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Supporting Information

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Supporting Information

for

Steric Constraints Dependent on Nucleobase Pair Orientation Vary in Different DNA Polymerase Active Sites

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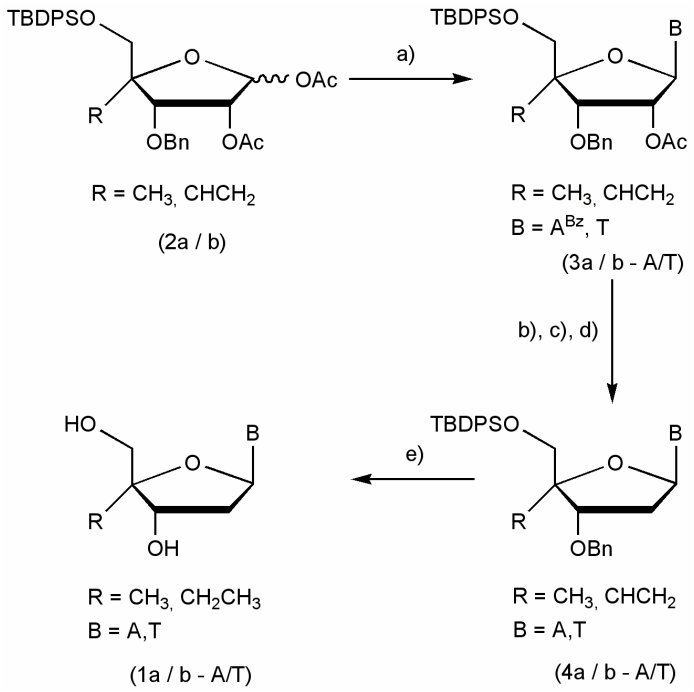
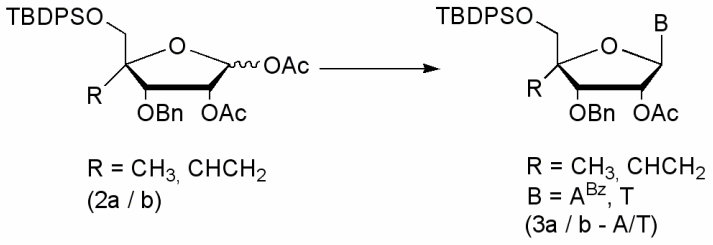
Synthesis of 2'-Deoxy-4'-Alkyl-Nucleosides

General: All temperatures quoted are uncorrected. All reagents are commercially available and were used without further purification. Solvents were purchased over molecular sieves (Fluka) and used directly without further purification unless otherwise noted. All reactions were conducted under rigorous exclusion of air and moisture. Petroleum ether used had a boiling point range of 35–80°C. TLC: Merck precoated plates (silica gel 60 F254). NMR spectroscopy: Bruker AC250, DPX400, DRX 600 and Varian INOVA400 MHz with the solvent peak as internal standard. Chemical shifts (δ) are given in parts per million, and tetramethyl silane was used as the external standard. ESI-MS: Bruker Daltonics esquire 3000+. High resolution electrospray ionization-Fourier transform ion cyclotron mass spectrometry (ESI-FTICR) was recorded on a Bruker Daltonics Apex II in positive mode.

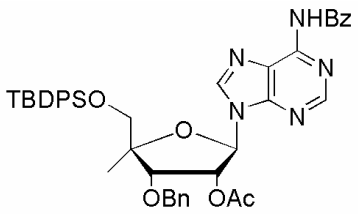
Synthetic strategy: Starting from the intermediates **2a/b** that are readily available in multigram scale, we successfully introduced the respective nucleobase by Vorbrüggen glycosylation to yield **3a/b-A/T**. Next, saponification, deoxygenation of the 2'-hydroxyl function and cleavage of the protection groups yielded **1a/b-A/T**. Noteworthy, for the successful hydrogenolysis of the benzyl ethers in **4a/b-A** basic conditions are required in order to suppress side reactions that occur to a significant extent in the absence of NaOH.



Scheme S1. Synthesis of modified nucleotides. a) Thymine, *N,O*-bis(trimethylsilyl)acetamide, TMSOTf, CH_3CN, reflux, 2h, 62% (Me) / 61% (Et); N^6-Benzoyladenine, TMSOTf, CH_3CN, 0℃, 1h, 55% (Me) / 48% (Et); b) NaOMe, MeOH, RT 1- 12h; c) PhOCSCl, DMAP, CH_3CN, RT, 1h; d) *n*Bu_3SnH, AIBN, toluene, reflux, 1h, 72% (T^{Me}) / 56% (T^{Et}) / 73% (A^{Me}) / 78% (A^{Et}) after 3 steps; e) 10% Pd/C, H_2, THF-EtOH, reflux, 24 h followed by TBAF, THF, RT, 7 h, 61% (T^{Me}) / 63% (T^{Et}); 10% Pd/C, H_2, THF-EtOH, 1N NaOH, reflux, 5-10 days, 94% (A^{Me}) / 98% (A^{Et}); f) 1. $POCl_3$ in $PO(OMe)_3$, (proton sponge for T) 0℃ -> RT, 2 - 4h, 2. Bu_3N, $(Bu_3NH)_2$-$H_2P_2O_7$ in DMF, 0℃, 15min, 3. TEAB buffer, 40 min, 25% (T^{Me}TP) / 41% (T^{Et}TP) / 9% (dA^{Me}TP) / 11% (dA^{Et}TP)

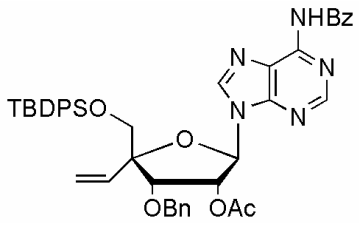


2'-*O*-Acetyl-6-*N*-benzoyl-3'-*O*-benzyl-5'-*O*-*tert*-butyldiphenylsilyl-4'-*C*-methyl-adenosine (3a-A)



N^6-Benzoyladenine (3.20 g, 13.38 mmol) was suspended in a solution of 1,2-di-*O*-acetyl-3-*O*-benzyl-5-*O*-*tert*-butyldiphenylsilyl-4-*C*-methyl-α,β-D-ribofuranose[1] **2a** (7.02 g, 12.18 mmol) in dry acetonitrile (100 mL) at 0 ℃. [2] A solution of Me_3SiOTf (4.40 mL, 24.77 mmol) in dry acetonitrile (10 mL) was added and the mixture was stirred at the same temperature. After 1 h, sodium bicarbonate (3 g) was added to the reaction mixture and filtered. The filtrate was evaporated and the residue was dissolved in CH_2Cl_2 (200 mL) and washed with H_2O (2 × 100 mL), and then dried ($MgSO_4$), concentrated, and purified by silica gel column chromatography (EtOAc/ petroleum, 2:3) to yield **3a-A** (5.06 g, 55%) as a pale yellow syrup; R_f 0.63 (EtOAc/ petroleum ether 3:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.04 (s, 9H; SiC(CH_3)$_3$), 1.34 (s, 3H; 4'-CH_3), 2.05 (s, 3H; 2'-OCOCH_3), 3.56 (d, ^2J = 11.0 Hz, 1H; H-5'a), 3.85 (d, ^2J = 11.0 Hz, 1H; H-5'b), 4.54 (d, ^2J = 11.4 Hz, 1H; CH_2Ph), 4.63 (m, 2H; CH_2Ph & H-3'), 5.94 (t, *J* = 5.4 Hz, 1H; H-2'), 6.28 (d, *J* = 5.1 Hz, 1H; H-1'), 7.20-7.67 (m, 17H; Ar*H*), 8.00 (m, 2H; Ar*H*), 8.18 (s, 1H; H-2), 8.64 (s, 1H; H-8), 9.51 (brs, 1H; N*H*-6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 18.4, 19.0, 20.6, 26.8, 67.9, 74.1, 74.8, 85.9, 87.4, 123.3, 127.7, 127.9, 128.3, 128.6, 129.7, 129.8, 132.4, 132.5, 133.5, 135.3, 135.5, 137.3, 141.7, 149.5, 151.4, 169.7. HRMS (MALDI) (*m*/*z*) calcd for $C_{43}H_{45}N_5O_6Si$ [*M*+H]$^+$: 756.3217; found: 756.3215.

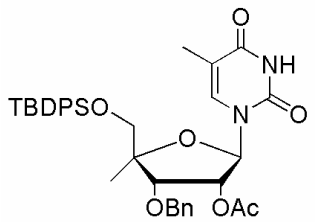
2'-*O*-Acetyl-6-*N*-benzoyl-3'-*O*-benzyl-5'-*O*-*tert*-butyldiphenylsilyl-4'-*C*-vinyl-adenosine (3b-A)



N^6-Benzoyladenine (4.40 g, 18.41 mmol) was suspended in a solution of 1,2-di-*O*-acetyl-3-*O*-benzyl-5-*O*-*tert*-butyldiphenylsilyl-4-*C*-vinyl-α,β-D-ribofuranose[1] **2b** (9.85 g, 16.69 mmol) in dry acetonitrile (100 mL) at 0 ℃. A solution of Me_3SiOTf (6.05 mL, 34.02 mmol) in dry acetonitrile (10 mL) was added and the mixture was stirred at the same temperature. After 1 h, sodium bicarbonate (5 g) was added to the reaction mixture and filtered. The filtrate was evaporated and the residue was dissolved in CH_2Cl_2 (200 mL) and washed with H_2O (2 × 100 mL), and then dried ($MgSO_4$), con-

centrated, and purified by silica gel column chromatography (EtOAc/petroleum ether, 2:3) to yield **3b-A** (6.19 g, 48%) as a pale yellow syrup; R_f 0.70 (EtOAc/petroleum ether 3:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.04 (s, 9H; SiC(C*H*$_3$)$_3$), 2.03 (s, 3H; 2'-OCOC*H*$_3$), 3.67 (d, ^2J = 11.4 Hz, 1H; H-5'a), 3.78 (d, ^2J = 11.4 Hz, 1H; H-5'b), 4.58 (d, ^2J = 11.3 Hz, 1H; C*H*$_2$Ph), 4.65 (d, ^2J = 11.3 Hz, 1H; C*H*$_2$Ph), 4.95 (d, J = 5.8 Hz, 1H; H-3'), 5.28 (d, J = 11.0 Hz, 1H; 4'-CH=C*H*$_2$), 5.52 (d, J = 17.3 Hz, 1H; 4'-CH=C*H*$_2$), 5.88 (dd, ^3J = 4.2 Hz, ^3J = 5.7 Hz, 1H; H-2'), 6.03 (dd, ^3J = 11.0 Hz, ^3J = 17.3 Hz, 1H; 4'-C*H*=CH$_2$), 6.30 (d, J = 4.0 Hz, 1H; H-1'), 7.20-7.70 (m, 17H, Ar*H*), 8.00 (m, 2H; Ar*H*), 8.26 (s, 1H; H-2), 8.67 (s, 1H; H-8), 9.58 (brs, 1H; N*H*-6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 19.0, 20.5, 26.7, 30.7, 65.9, 73.8, 74.2, 86.2, 88.5, 116.0, 123.2, 127.5, 127.6, 127.7, 127.8, 128.2, 128.5, 129.7, 129.8, 132.3, 132.4, 132.5, 133.5, 134.1, 135.3, 135.4, 137.2, 141.6, 151.4, 169.7. HRMS (MALDI) (*m*/*z*) calcd for $C_{44}H_{45}N_5O_6Si$: 768.3217 [*M*+H]$^+$; found: 768.3206.

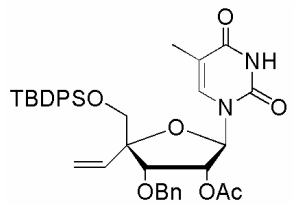
2'-*O*-Acetyl-3'-*O*-benzyl-5'-*O*-*tert*-butyldiphenylsilyl-4'-*C*-methyl-thymidine (3a-T)



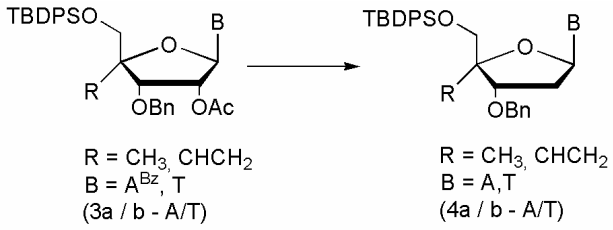
To a solution of compound **2a** (4.62 g, 8.02 mmol) and thymine (2.02 g, 16.03 mmol) in dry acetonitrile (100 mL) was added *N*,*O*-bis(trimethylsilyl)acetamide (11.76 mL, 47.57 mmol) and refluxed for 1 h. After cooling to RT, Me_3SiOTf (1.88 ml, 10.57 mmol) was added, and the mixture was refluxed again for 1 h. The mixture was quenched with sat. $NaHCO_3$ solution (30 mL). The organic solvent was removed under reduced pressure and extracted with CH_2Cl_2 (3 × 100 mL). The organic layer was dried ($MgSO_4$), concentrated, and purified by silica gel column chromatography (EtOAc-petroleum ether, 3:7) to give **3a-T** (3.21 g, 62%) as a white foam; R_f 0.20 (EtOAc-petroleum ether 1:3). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.97 (s, 9H; SiC(C*H*$_3$)$_3$), 1.05 (s, 3H; 4'-C*H*$_3$), 1.41 (s, 3H; 5-C*H*$_3$), 1.96 (s, 3H; 2'-OCOC*H*$_3$), 3.42 (d, ^2J = 11.2 Hz, 1H; H-5'a), 3.67 (d, ^2J = 11.2 Hz, 1H; H-5'b), 4.25 (d, J = 6.2 Hz, 1H; H-3'), 4.31 (d, ^2J = 12.5 Hz, 1H; C*H*$_2$Ph), 4.47 (d, ^2J = 12.5 Hz, 1H; C*H*$_2$Ph), 5.25 (t, J = 5.9 Hz, 1H; H-2'), 6.12 (d, J = 5.9 Hz, 1H; H-1'), 7.10-7.56 (m, 16H; Ar*H* & H-6), 9.82 (brs, 1H; N*H*-3). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 11.8, 18.0, 19.2, 20.6, 26.9, 68.0, 74.0,

74.7, 85.3, 86.4, 111.5, 127.6, 127.7, 127.8, 129.9, 130.0, 132.1, 132.7, 135.2, 135.4, 137.3, 150.6, 164.0, 170.1. HRMS (MALDI) (*m*/*z*) calcd for $C_{36}H_{42}N_2O_7Si$ [*M*+Na]$^+$: 665.2569; found: 665.2642.

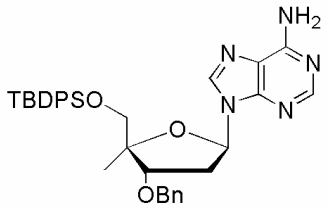
2'-*O*-Acetyl-3'-*O*-benzyl-5'-*O*-*tert*-butyldiphenylsilyl-4'-*C*-vinyl-thymidine (3b-T)



To a solution of compound **2b** (5.37 g, 9.10 mmol) and thymine (2.30 g, 18.25 mmol) in dry acetonitrile (100 mL) was added *N*,*O*-bis(trimethylsilyl)acetamide (13.39 mL, 54.17 mmol) and refluxed for 1 h. After cooling to RT, Me_3SiOTf (2.14 mL, 12.03 mmol) was added and the mixture was refluxed again for 1 h. The mixture was quenched with sat. $NaHCO_3$ solution (30 mL). The organic solvent was removed under reduced pressure and extracted with CH_2Cl_2 (3 × 100 mL). The organic layer was dried ($MgSO_4$), concentrated, and purified by silica gel column chromatography (EtOAc-petroleum ether, 3:7) to give **3b-T** (3.65 g, 61%) as a white foam; R_f 0.26 (EtOAc-petroleum ether 1:3). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.97 (s, 9H; SiC(C*H*$_3$)$_3$), 1.43 (s, 3H; 5-C*H*$_3$), 1.93 (s, 3H; 2'-OCOC*H*$_3$), 3.52 (d, ^2J = 11.6 Hz, 1H; H-5'a), 3.58 (d, ^2J = 11.6 Hz, 1H; H-5'b), 4.35 (d, ^2J = 11.3Hz, 1H; C*H*$_2$Ph), 4.48 (m, 2H; C*H*$_2$Ph & H-3'), 5.10 (d, ^2J = 11.0 Hz, 1H; 4'-CH=C*H*$_2$), 5.23 (t, *J* = 5.0 Hz, 1H; H-2'), 5.33 (d, ^2J = 17.3 Hz, 1H; 4'-CH=C*H*$_2$), 5.71 (dd, ^3J = 11.0 Hz, ^3J = 17.3 Hz, 1H; 4'-C*H*=CH$_2$), 6.11 (d, *J* = 5.0 Hz, 1H; H-1'), 7.10-7.54 (m, 16H; Ar*H* & H-6), 9.80 (brs, 1H; N*H*-3). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 11.8, 19.3, 20.6, 26.9, 66.0, 73.9, 74.2, 76.7, 85.8, 87.9, 111.6, 127.4, 127.6, 127.7, 127.8, 128.3, 128.4, 129.9, 130.0, 132.1, 133.4, 135.1, 135.4, 137.2, 150.5, 164.0, 170.1, 175.2. HRMS (MALDI) (*m*/*z*) calcd for $C_{37}H_{42}N_2O_7Si$ [*M*+Na]$^+$: 677.2659; found: 677.2632.

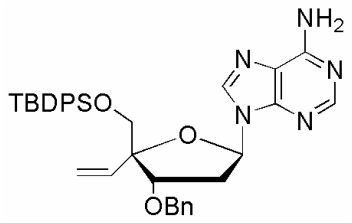


3'-*O*-Benzyl-5'-*O*-*tert*-butyldiphenylsilyl-2'-deoxy-4'-*C*-methyl-adenosine (4a-A)



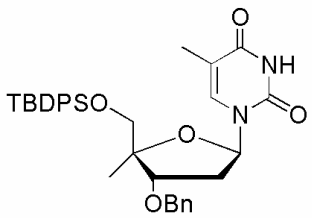
To a solution of compound **3a-A** (4.92 g, 6.51 mmol) in MeOH (100 mL), was added NaOMe (1.05 g, 19.43 mmol) and the mixture was stirred at RT for 12 h. After completion of the reaction, the reaction mixture was treated with ion exchanger Amberlite IR-120 (H^+ form) to neutralize the basic condition. The reaction mixture was filtered through sintered funnel, evaporated the solvent and dried under vacuum. The resulting compound was dissolved in dry CH_2Cl_2 (50 mL) and to this solution were added DMAP (4.12 g, 33.72 mmol) and phenyl chlorothionoformate (0.98 mL, 7.08 mmol), and the mixture was stirred at RT for 1 h. After completion of the reaction, the reaction mixture was washed with aq. 5% citric acid (50 mL) and H_2O (2×50 mL). The extracts were dried ($MgSO_4$) and evaporated to give a crude yellowish foam. To a solution of this crude compound in anhyd. toluene (100 mL) were added *n*Bu_3SnH (9.96 mL, 37.02 mmol) and AIBN (101 mg, 0.61 mmol). The mixture was refluxed for 1 h, and the solvents were evaporated. The residue was purified by silica gel column chromatography (EtOAc-petroleum ether, 3:2) to give **4a-A** (2.78 g, 72%) as a white foam; R_f 0.33 (EtOAc-petroleum ether 3:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.97 (s, 9H; SiC(C*H*$_3$)$_3$), 1.27 (s, 3H; 4'-C*H*$_3$), 2.50-2.75 (m, 2H; H-2'), 3.53 (d, ^2J = 10.8 Hz, 1H; H-5'a), 3.75 (d, ^2J = 10.8 Hz, 1H; H-5'b), 4.38 (t, J = 5.4 Hz, 1H; H-3'), 4.44 (d, ^2J = 12.0 Hz, 1H; C*H*$_2$Ph), 4.57 (d, ^2J = 12.0 Hz, 1H; C*H*$_2$Ph), 6.32 (t, J = 6.2 Hz, 1H, H-1'), 6.47 (brs, 2H; N*H*$_2$-6), 7.15-7.60 (m, 15H; Ar*H*), 7.94 (s, 1H; H-2), 8.19 (s, 1H; H-8). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 18.1, 19.1, 26.8, 37.2, 68.0, 72.0, 78.4, 83.0, 87.5, 119.9, 127.3, 128.3, 129.7, 132.6, 132.8, 135.4, 135.5, 137.7, 138.6, 149.2, 152.7, 155.7. HRMS (MALDI) (*m*/*z*) calcd for $C_{34}H_{39}N_5O_3Si$ [*M*+Na]$^+$: 616.2720; found: 616.2699.

3'-*O*-Benzyl-5'-*O*-*tert*-butyldiphenylsilyl-2'-deoxy-4'-*C*-vinyl-adenosine (4b-A)



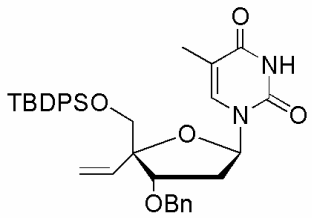
To a solution of compound **3b-A** (6.01 g, 7.83 mmol) in MeOH (100 mL), was added NaOMe (1.27 g, 23.50 mmol) and the mixture was stirred at RT for 12 h. After completion of the reaction, the reaction mixture was treated with ion exchanger Amberlite IR-120 (H^+ form) to neutralize the basic condition. The reaction mixture was filtered through sintered funnel, evaporated the solvent and dried under vacuum. The resulting compound was dissolved in dry CH_2Cl_2 (50 mL) and to this solution were added DMAP (4.86 g, 39.78 mmol) and phenyl chlorothionoformate (1.16 mL, 8.38 mmol), and the mixture was stirred at RT for 1 h. After completion of the reaction, the reaction mixture was washed with aq. 5% citric acid (50 mL) and H_2O (2×50 mL). The extracts were dried ($MgSO_4$) and evaporated to give a crude yellowish foam. To a solution of this crude compound in anhyd. toluene (100 mL) were added *n*-Bu_3SnH (12.59 mL, 46.80 mmol) and AIBN (128 mg, 0.77 mmol). The mixture was refluxed for 1 h and the solvents were evaporated. The residue was purified by silica gel column chromatography (EtOAc-petroleum ether, 3:2) to give **4b-A** (2.68g, 56%) as a white foam; R_f 0.36 (EtOAc-petroleum ether 3:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.06 (s, 9H; SiC(C*H*$_3$)$_3$), 2.61 (m, 2H; H-2'), 3.74 (d, ^2J = 11.4 Hz, 1H; H-5'a), 3.80 (d, ^2J = 11.4 Hz, 1H; H-5'b), 4.55 (d, ^2J = 11.9 Hz, 1H; C*H*$_2$Ph), 4.62 (d, ^2J = 11.9 Hz, 1H; C*H*$_2$Ph), 4.81 (t, *J* = 7.2 H, 1H; H-3'), 5.31 (d, *J* = 10.8 Hz, 1H; 4'-CH=C*H*$_2$), 5.57 (d, *J* = 17.2 Hz, 1H; 4'-CH=C*H*$_2$), 6.03 (dd, ^3J = 10.8 Hz, ^3J = 17.2 Hz, 1H; 4'-C*H*=CH$_2$), 6.48 (brm, 3H; H-1' & N*H*-6), 7.23-7.67 (m, 15 H; Ar*H*), 8.21 (s, 1H; H-2), 8.30 (s, 1H; H-8). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 19.1, 26.8, 36.9, 65.7, 72.4, 82.4, 88.7, 116.3, 119.8, 127.4, 127.7, 127.8, 127.9, 128.4, 129.7, 129.8, 132.5, 132.6, 134.6, 135.4, 135.5, 137.6, 138.7, 149.1, 152.8, 155.7. HRMS (MALDI) (*m*/*z*) calcd for $C_{35}H_{39}N_5O_3Si$ [*M*+Na]$^+$: 628.2720; found: 628.2694.

3'-*O*-Benzyl -5'-*O*-*tert*-butyldiphenylsilyl-2'-deoxy-4'-*C*-methyl-thymidine (4a-T)



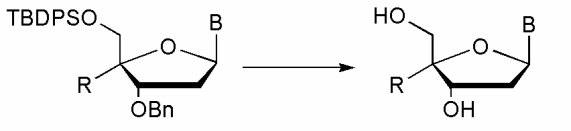
To a solution of compound **3a-T** (3.53 g, 5.49 mmol) in MeOH (100 mL), was added NaOMe (445 mg, 8.23 mmol) and the mixture was stirred at RT for 1 h. After completion of the reaction, the reaction mixture was treated with ion exchanger Amberlite IR-120 (H^+ form) to neutralize the basic condition. The reaction mixture was filtered through sintered funnel, evaporated the solvent and dried under vacuum. The resulting compound was dissolved in dry CH_3CN (100 mL) and to this solution were added DMAP (1.88 g, 15.38 mmol) and phenyl chlorothionoformate (0.83 mL, 6.00 mmol), and the mixture was stirred at RT for 1 h. After completion of the reaction, the solvent was removed and the residue was dissolved in CH_2Cl_2 (200 mL). The CH_2Cl_2 layer was washed with aq. 5% citric acid (50 ml) and H_2O (2×50 mL). The extracts were dried ($MgSO_4$) and evaporated to give a crude yellowish foam. To a solution of this crude compound in anhyd. toluene (100 mL) were added *n*-Bu_3SnH (8.83 mL, 32.82 mmol) and AIBN (90 mg, 0.54 mmol). The mixture was refluxed for 1 h and the solvents were evaporated. The residue was purified by silica gel column chromatography (EtOAc-petroleum ether, 3:7) to give **4a-T** (2.35g, 73%) as a white foam; R_f 0.36 (EtOAc-petroleum ether 1:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.07 (s, 9H; SiC-(CH_3)$_3$), 1.22 (s, 3H; 4'-CH_3), 1.58 (s, 3H; 5-CH_3), 2.19 (m, 1H; H-2'a), 2.54 (m, 1H; H-2'b), 3.62 (d, ^2J = 11.0 Hz, 1H; H-5'a), 3.79 (d, ^2J = 11.0Hz, 1H; H-5'b), 4.28 (d, ^3J = 4.1 Hz, ^3J = 6.8 Hz, 1H; H-3'), 4.45 (d, ^2J = 12.0 Hz, 1H; CH_2Ph), 4.63 (d, ^2J = 12.0 Hz, 1H; CH_2Ph), 6.30 (d, J = 6.6 Hz, 1H; H-1'), 7.28-7.70 (m, 16H; Ar*H* & H-6), 9.04 (brs, 1H; N*H*-3). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 12.0, 18.0, 27.0, 37.9, 68.5, 72.0, 83.3, 87.1, 110.9, 127.4, 127.8, 127.9, 128.4, 130.0, 132.4, 132.9, 135.3, 135.5, 137.7, 150.3, 163.8. HRMS (MALDI) (*m*/*z*) calcd for $C_{34}H_{40}N_2O_5Si$ [*M*+Na]$^+$: 607.2604; found: 607.2582.

3'-*O*-Benzyl-5'-*O*-*tert*-butyldiphenylsilyl-2'-deoxy-4'-*C*-vinyl-thymidine (4b-T)



To a solution of compound **3b-T** (3.09 g, 4.72 mmol) in MeOH (100 mL), was added NaOMe (383 mg, 7.08 mmol) and the mixture was stirred at RT for 1 h. After completion of the reaction, the reaction mixture was treated with ion exchanger Amberlite

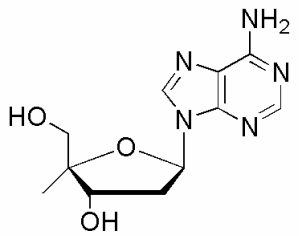
IR-120 (H^+ form) to neutralize the basic condition. The reaction mixture was filtered through sintered funnel, evaporated the solvent and dried under vacuum. The resulting compound was dissolved in dry CH_3CN (100 mL) and to this solution were added DMAP (1.64 g, 13.42 mmol) and phenyl chlorothionoformate (0.72 mL, 5.20 mmol), and the mixture was stirred at RT for 1 h. After completion of the reaction, the solvent was removed and the residue was dissolved in CH_2Cl_2. The CH_2Cl_2 layer was washed with aq. 5% citric acid (50 mL) and H_2O (2 × 50 mL). The extracts were dried ($MgSO_4$) and evaporated to give a crude yellowish foam. To a solution of this crude compound in anhyd. toluene (100 mL) were added *n*-Bu_3SnH (7.24 mL, 26.91 mmol) and AIBN (82 mg, 0.49 mmol). The mixture was refluxed for 1 h and the solvents were evaporated. The residue was purified by silica gel column chromatography (EtOAc/petroleum ether, 3:7) to give **4b-T** (2.19g, 78%) as a white foam; R_f 0.23 (EtOAc/petroleum ether 1:3). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.92 (s, 9H; SiC-(C*H*$_3$)$_3$), 1.38 (s, 3H; 5-C*H*$_3$), 2.05 (m, 1H; H-2'a), 2.30 (m, 1H; H-2'b), 3.60 (brs, 2H; H-5'), 4.33 (d, ^2J = 11.9 Hz, 1H; C*H*$_2$Ph), 4.44 (m, 2H; C*H*$_2$Ph & H-3'), 5.10 (dd, ^2J = 1.4 Hz, ^3J = 10.7 Hz, 1H; 4'-CH=C*H*$_2$), 5.34 (dd, ^2J = 1.5 Hz, ^3J = 17.2 Hz, 1H; 4'-CH=C*H*$_2$), 5. 77 (dd, ^3J = 10.8 Hz, 17.2 Hz, 1H; 4'-C*H*=CH$_2$), 6.16 (dd, ^3J = 4.8 Hz, ^3J = 7.0 Hz, 1H; H-1'), 7.10-7.50 (m, 16H; Ar*H* & H-6), 9.56 (brs, 1H; N*H*-3). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 11.9, 19.4, 27.0, 37.3, 65.9, 72.2, 82.7, 88.6, 111.0, 116.5, 127.4, 127.8, 128.4, 129.9, 130.0, 132.3, 132.9, 135.1, 135.2, 135.3, 137.5, 150.5, 164.0. HRMS (MALDI) (*m*/*z*) calcd for $C_{35}H_{40}N_2O_5Si$ [*M*+Na]$^+$: 619.2604; found: 619.2598.



R = CH_3, $CHCH_2$
B = A,T
(4a / b - A/T)

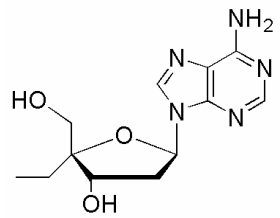
R = CH_3, CH_2CH_3
B = A,T
(1a / b - A/T)

2'-Deoxy-4'-*C*-methyl-adenosine (1a-A)



To a solution of compound **4a-A** (1.36 g, 2.29 mmol) in EtOH/THF/1 N NaOH (200: 200:27.5 mL) was added an equivalent weight amount of 10% Pd/C. The mixture was subsequently filled with H_2 (balloon) and refluxed for 4 days. After 4 days, again another 0.5 equivalent weight amount of 10% Pd/C was added and again refluxed for 1 more day. After completion of the reaction, the mixture was filtered through Celite on a sintered funnel and washed thoroughly. The solvent was removed under reduced pressure, and purified by silica gel column chromatography (EtOAc/EtOH/ water, 8:1:1(used the organic layer of the mixture)) to give **1a-A** (597 mg, 98%) as a pale yellow solid; R_f 0.36 (EtOAc-1-PrOH-H_2O 4:1:1). ^{1}H NMR (250 MHz, CD_3OD): δ = 1.22 (s, 3H; 4'-CH_3), 2.43 (m, 1H; H-2'a), 2.93 (m, 1H; H-2'b), 3.57 (d, ^2J = 12.0 Hz, 1H; H-5'a), 3.70 (d, ^2J = 12.0 Hz, 1H; H-5'b), 4.54 (dd, ^3J = 3.4 Hz, ^3J = 6.0 Hz, 1H; H-3'), 6.36 (t, J = 6.2 Hz, 1H; H-1'), 8.17 (s, 1H; H-2), 8.26 (s, 1H; H-8). ^{13}C NMR (62.9 MHz, CD_3OD): δ = 18.2, 41.8, 68.5, 73.2, 78.2, 78.7, 79.2, 85.8, 85.9, 90.1, 120.7, 149.41, 157.0. HRMS (MALDI) (*m*/*z*) calcd for $C_{11}H_{15}N_5O_3$ [*M*+Na]$^+$: 288.1073; found: 288.1087.

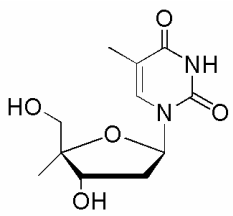
2'-Deoxy-4'-*C*-ethyl-adenosine (1b-A)



To a solution of compound **4b-A** (1.25 g, 2.06 mmol) in EtOH/THF/1 N NaOH (150: 150:25 mL) was added an equivalent weight amount of 10% Pd/C. The mixture was subsequently filled with H_2 (balloon) and refluxed for 4 days. After 4 days, again another 0.5 equivalent weight amount of 10% Pd/C was added and again refluxed for 2 more days. The reaction mixture was filtered through sintered funnel, and again another equivalent weight amount of 10% Pd/C was added and refluxed for another 3 days. After completion of the reaction, the mixture was filtered through Celite on a sintered funnel and washed thoroughly. The solvent was removed under reduced pressure, and purified by silica gel column chromatography (EtOAc/EtOH/water, 8:1: 1 (used the organic layer of the mixture)) to give **1b-A** (559 mg, 94%) as a pale yellow; R_f 0.46 (EtOAc-1-PrOH-H_2O 4:1:1). ^{1}H NMR (250 MHz, CD_3OD): δ = 0.97 (t, 3H; 4'-$CH_2C$$H_3$), 1.58-1.86 (m, 2H; 4'-CH_2CH_3), 2.40 (m, 1H; H-2'a), 2.95 (m, 1H; H-

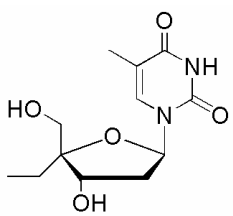
2'b), 3.62 (d, ^2J = 12.0 Hz, 1H; H-5'a), 3.75 (d, ^2J = 12.0 Hz, 1H; H-5'b), 4.60 (dd, ^3J = 3.0 Hz, ^3J = 6.0 Hz, 1H; H-3'), 6.34 (dd, ^3J = 6.2 Hz, ^3J = 7.7Hz, 1H; H-1'), 8.17 (s, 1H; H-2), 8.24 (s, 1H; H-8). ^{13}C NMR (62.9 MHz, CD_3OD): δ = 8.8, 25.5, 42.2, 66.3, 73.4, 78.1, 78.6, 79.1, 86.1, 91.9, 120.8, 141.2, 149.4, 153.0, 157.0. HRMS (MALDI) (*m*/*z*) calcd for $C_{12}H_{17}N_5O_3$ [*M*+H]$^+$: 280.1410; found: 280.1421.

2'-Deoxy-4'-*C*-methyl-thymidine (1a-T)



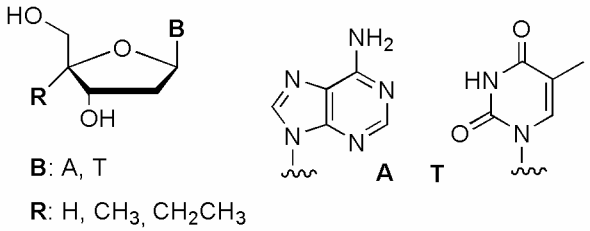
To a solution of compound **4a-T** (2.35 g, 4.02 mmol) in EtOH (100 mL) was added an equivalent weight amount of 10% Pd/C. The mixture was subsequently filled with H_2 balloon and refluxed for 24 h. After completion of the reaction, the mixture was filtered through Celite on a sintered funnel and washed thoroughly. The solvent was removed under reduced pressure and the resulting white foam was dissolved in anhyd. THF (50 mL). A 1 M solution of TBAF in THF (4.11 mL) was added and the mixture was allowed to stir for over night. After completion of the reaction, the mixture was concentrated and purified by silica gel column chromatography (MeOH/EtOAc, 1:4) to give **1a-T** (651 mg, 63%) as a white solid; R_f 0.66 (MeOH-EtOAc 1:3). ^{1}H NMR (250 MHz, CD_3OD): δ = 1.04 (s, 3H; 4'-CH_3), 1.76 (s, 3H; 5-CH_3), 2.13-2.32 (m, 2H; H-2'), 3.43 (d, ^2J = 11.7 Hz, 1H; H-5'a), 3.51 (d, ^2J = 11.7Hz, 1H; H-5'b), 4.30 (t, *J* = 6.7 Hz, 1H; H-3'), 6.07 (t, *J* = 6.1 Hz, 1H; H-1'), 7.63 (s, 1H; H-6). ^{13}C NMR (62.9 MHz, CD_3OD): δ = 11.9, 17.0, 39.6, 65.9, 70.3, 83.6, 87.4, 110.3, 141.6, 150.6, 164.6. HRMS (MALDI) (*m*/*z*) calcd for $C_{11}H_{16}N_2O_5$ [*M*+Na]$^+$: 279.0957; found: 279.1002.

2'-Deoxy-4'-*C*-ethyl-thymidine (1b-T)



To a solution of compound **4b-T** (2.19 g, 3.67 mol) in EtOH (100 mL) was added an equivalent weight amount of 10% Pd/C. The mixture was subsequently filled with H_2 balloon and refluxed for 24 h. After completion of the reaction, the mixture was filtered through Celite on a sintered funnel and washed thoroughly. The solvent was removed under reduced pressure and the resulting white foam was dissolved in anhyd. THF (50 mL). A 1 M solution of TBAF in THF (3.20 mL) was added and the mixture was allowed to stir for over night. After completion of the reaction, the mixture was concentrated and purified by silica gel column chromatography (MeOH/EtOAc 1:2) to give **1b-T** (600 mg, 61%) as a white solid; R_f 0.7 (MeOH-EtOAc 1:3). ^{1}H NMR (250 MHz, CD_3OD): δ = 0.81 (t, *J* = 7.4 Hz, 3H; 4'-CH_2CH_3), 1.35-1.63 (m, 2H, 4'-CH_2CH_3), 1.75 (s, 3H; 5-CH_3), 2.20 (m, 2H; H-2'), 3.44 (d, ^2J = 11.8 Hz, 1H; H-5'a), 3.56 (d, ^2J = 11.8 Hz, 1H; H-5'b), 4.35 (t, *J* = 6.5 Hz, 1H; H-3'), 6.01 (t, *J* = 6.2 Hz, 1H; H-1'), 7.62 (s, 1H; H-6). ^{13}C NMR (62.9 MHz, CD_3OD): δ = 8.6, 12.5, 25.0, 64.9, 72.6, 90.7, 111.3, 152.3, 166.4. HRMS (MALDI) (*m*/*z*) calcd for $C_{12}H_{18}N_2O_5$ [*M*+Na]$^+$: 293.1113; found: 293.1136.

Conformational Analysis



Nucleosides were dissolved in D_2O at concentrations of ca. 10 mM. NMR spectra were recorded at 300 K on a Bruker DRX 600 MHz spectrometer equipped with an inverse triple resonance probe head with actively shielded *z*-gradient coil. Spectra were processed and analyzed with Bruker's TOPSPIN 1.3 software. Chemical shifts and coupling constants were extracted from 1-dimensional proton spectra recorded with a spectral width of 16 ppm and 32k complex data points. After processing with a squared sine bell window function shifted by π/10 data were zero-filled to 128k points resulting in a digital resolution of 0.07 Hz/point. In most cases, chemical shifts and scalar couplings could directly be determined by first-order analysis. In case of 4'-methyl/ethyl-thymidine (**1a/b-T**) the chemical shift difference between the signals of H2' and H2'' was smaller than 15 Hz. The exact shifts and couplings were thus deter-

mined by computer simulation with the program gNMR 4.1.2 (Adept Scientific plc, Amor Way, Letchworth Garden City, Herts, SG6 1ZA, UK, kindly provided by Prof. Dr. U. Groth, Universität Konstanz). In case of unmodified thymidine the H2'-H2'' chemical shift difference was smaller than 4 Hz. Here, sums of couplings were used to determine the conformational equilibrium as described.[3]

Conformational analysis was carried out with the program PSEUROT 6.3 (van Wijk, J., Westra Hoekzema, A.J.A, Altona, C., Leiden Institute of Chemistry, Leiden University, The Netherlands). A standard Donders type generalized Karplus equation with PSEUROT default parameters suitable for deoxyribonucleosides was chosen. The lack of a $^{3}J_{HH}$-coupling describing the 3'-4'-torsion angle in 4'-substituted nucleosides leads to insufficient parameters for a full description of pseudorotation. For that reason, the ring puckering amplitude theta was constrained to 35 deg for both conformers, northern and southern, respectively, leaving three parameters to be optimized by PSEUROT, specifically the phase angle of pseudorotation of each conformer, phiN and phiS, and the molar ratio between both conformers. In most cases, four coupling constants, $^{3}J_{H1'H2'}$, $^{3}J_{H1'H2''}$, $^{3}J_{H2'H3'}$, $^{3}J_{H2''H3'}$ were provided to PSEUROT. Only in the case of unmodified thymidine, H2' and H2'' have almost degenerate resonances which made it more convenient to use sums of couplings as input to the software. For dA all five homonuclear ^{3}J-couplings were available. However, in order to ensure consistency, the same three-parameter fitting procedure as for the modified nucleosides was applied.

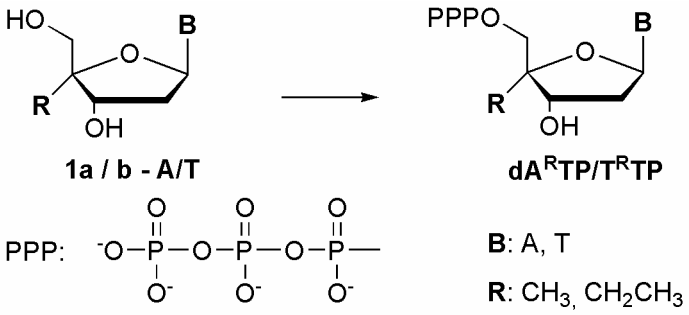
Table S1. Analysis of deoxyribofuranose ring conformation.

	Chemical shifts [ppm]					Scalar couplings (relevant for conformational analysis) [Hz]						Conformational analysis with PSEUROT [a]		
	1'	**2'**	**2''**	**3'**	**4'**	$J_{1'2'}$	$J_{1'2''}$	$J_{2'3'}$	$J_{2''3'}$	$J_{2'2''}$	$J_{3'4'}$	**% (N)-type**	**% (S)-type**	**Rmsd**
dA	6.460	2.828	2.541	4.627	4.164	7.59	6.18	6.13	3.31	14.07	3.00	30	70	0.096
1a-A	6.411	2.932	2.596	4.597	n.a.	6.55	6.66	6.49	4.7	14.13	n.a.	45	55	0.028
1b-A	6.420	2.942	2.577	4.664	n.a.	6.9	6.7	6.51	4.37	14.2	n.a.	43	57	0.014
						$J_{1'2'}+J_{1'2''}$	$J_{2'3'}+J_{2''3'}$	$J_{2'3'}+J_{2''3'}+J_{3'4'}$						
T	6.270	2.351	2.351	4.443	3.998	13.6	10.76	14.81				42	58	0.505
						$J_{1'2'}$	$J_{1'2''}$	$J_{2'3'}$	$J_{2''3'}$	$J_{2'2''}$	$J_{3'4'}$			
1a-T	6.220	2.438	2.463	4.449	n.a.	5.92	7.07	6.87	5.97	14.15	n.a.	58	42	0.249
1b-T	6.223	2.466	2.418	4.523	n.a.	6.15	6.98	6.98	5.51	14.28	n.a.	53	47	0.177

[a] During the fitting procedure, the ring puckering amplitude was constrained to 35deg for both conformers, (S)-type and (N)-type, respectively. The remaining three parameters, i.e. the phase angle of pseudorotation of both conformers and the molar ratio of both conformers, were optimized in order to fit the coupling constants provided. n.a.: not accessible

The ratio between (S)-type and (N)-type conformation was determined from $^3J_{HH}$ coupling constants using published procedures. Nucleosides were dissolved in D_2O to ca. 10 mM. 1D ^1H NMR spectra were acquired at 300K on a 600 MHz spectrometer. Chemical shifts were referenced to the residual HDO signal at 4.750 ppm. Coupling constants were mainly determined by first-order analysis. The higher-order spectra of **1a/b-T** were analyzed by computersimulation with the gNMR software. In case of unmodified thymidine, sums of *J*-coupling served as input for PSEUROT calculations as described.[3] Unmodified nucleosides were found to adopt about 60-70 % southern conformation [(S)-type], while for **1a/b-A/T** approximately 50% (S)-type conformations were observed, indicating that 4'-alkylation had only moderate impact on sugar puckering in solution in general. Noteworthy, the observed effects on the conformational equilibria are independent of the nature of the nucleobase as indicated by comparable fractions of (S)-type conformations for all nucleotides (**1a/b-A/T**).

2'-Deoxy-4'-*C*-Alkyl-Nucleoside Triphosphates



Nucleoside triphosphates were synthesized according to standard procedures[4] and purified by Sephadex®-DEAE A-25 (HCO_3^- form) anion exchange chromatography by using a linear gradient of $(Et_3NH)HCO_3$ (0.01 - 1 M) and by RP-18 MPLC with a linear gradient of acetonitrile (0 - 20%) in $(Et_3NH)OAc$ (0.05 M). Concentrations were determined using the extinction coefficient of unmodified nucleosides. The nucleoside triphosphates were analyzed by means of ^1H-, ^{31}P NMR and ESI-MS.

2'-Deoxy-4'-*C*-methyl-adenosine-5'-*O*-triphosphate (dA^{Me}TP) To a stirred ice-cooled solution of **1a-A** (65 mg, 0.24 mmol) in trimethylphosphate (0.5 mL), phosphorus oxychloride (27 µL, 0.29 mmol) was added dropwise and allowed to stir at 0 °C. After 2 h, further again 7 µL (0.07 mmol) of $POCl_3$ was added and allowed to stir for another 2 h. A solution of *n*-tributylamine (293 µL, 1.23 mmol) and 0.5 M tributylammonium pyrophosphate in dry DMF (2.45 mL, 1.23 mmol)[4e] was added to

the reaction mixture, and the solution was stirred for 15 min at 0 °C, then quenched with 20 mL of 0.1 M TEAB buffer (pH 7.5) and allowed to stir at 0 °C for 10 min and then the reaction mixture was allowed to stir at room temperature for 30 min. The reaction mixture was washed with methyl *tert*-butyl ether and the aqueous layer was lyophilized to dryness. Further purification was done by anion exchange chromatography and RP-18 MPLC to yield **dA^{Me}TP** (10.55 mg, 9%). ^{1}H NMR (400 MHz, D_2O): δ = 1.92 (brs, 3H; 4'-C*H*$_3$), 3.05 (m, 1H; H-2'a), 3.27 (m, 1H; H-2'b), 4.14 (m, 1H; H-5'a), 4.18 (d, 1H; H-5'b), 4.75 (brs, 1H; H-3'), 6.09 (m, 1H; H-1'), 7.55 (s, 1H; H-2), 7.77 (s, 1H; H-8). ^{31}P NMR (D_2O, 162 MHz): δ = -22.86 (t, $P_ß$), -11.1 (d, $P_γ$), -10.0 (d, $P_α$). ESI-MS (*m*/*z*) calcd for $C_{11}H_{18}N_5O_{12}P_3$ [*M*-H]$^+$: 504.0; found: 503.8.

2'-Deoxy-4'-*C*-ethyl-adenosine-5'-*O*-triphosphate (dA^{Et}TP) To a stirred ice cooled solution of **1b-A** (64 mg, 0.22 mmol) in trimethylphosphate (0.6 mL), phosphorus oxychloride (25 µL, 0.27 mmol) was added dropwise and allowed to stir at 0 °C. After 2 h, further again 7 µL (0.07 mmol) of $POCl_3$ was added and allowed to stir another 2 h. A solution of *n*-tributylamine (274 µL, 1.15 mmol) and 0.5 M tributylammonium pyrophosphate in dry DMF (2.29 mL, 1.15 mmol) was added to the reaction mixture, and the solution was stirred for 15 min at 0 °C, and then quenched with 20 mL of 0.1 M TEAB buffer (pH 7.5) and allowed to stir at 0 °C for 10 min and then the reaction mixture was allowed to stir at room temperature for 30 min. The reaction mixture was washed with methyl *tert*-butyl ether and the aqueous layer was lyophilized to dryness. Further purification was done by anion exchange chromatography and RP-18 MPLC to yield **dA^{Et}TP** (11.95mg, 11%). ^{1}H NMR (400 MHz, D_2O): δ = 0.94 (m, 3H; 4'-CH_2-C*H*$_3$), 1.67 (m, 2H; 4'-C*H*$_2$CH$_3$), 2.59 (m, 1H; H-2'a), 2.87 (m, 1H; H-2'b), 4.05 (m, 2H; H-5'), 4.90 (brs, 1H; H-3'), 6.41 (m, 1H; H-1'), 8.23 (s, 1H; H-2), 8.51 (s, 1H; H-8). ^{31}P NMR (D_2O, 162 MHz): δ = -22.88 (brs, $P_ß$), -10.9 (d, $P_γ$), -9.9 (d, $P_α$). ESI-MS (*m*/*z*) calcd for $C_{12}H_{20}N_5O_{12}P_3$ [*M*+H]$^+$: 520.0; found: 520.0.

2'-Deoxy-4'-*C*-methyl-thymidine-5'-*O*-triphosphate (T^{Me}TP) To a stirred ice cooled solution of **1a-T** (99 mg, 0.38 mmol), proton sponge (125 mg, 0.58 mmol) in trimethylphosphate (1.0 mL), phosphorus oxychloride (43 µL, 0.46 mmol) was added dropwise and allowed to stir at 0 °C. After 2 h, further again 10 µL (0.10 mmol) of $POCl_3$ was added and allowed to stir for another 2 h. A solution of *n*-tributylamine (461 µL, 1.93 mmol) and 0.5 M tributylammonium pyrophosphate in dry DMF (3.86 mL, 1.93 mmol) was added to the reaction mixture, and the solution was stirred for 15 min at

0 ℃, and then quenched with 20 mL of 0.1 M TEAB buffer (pH 7.5) and allowed to stir at 0 ℃ for 10 min and then the reaction mixtu re was allowed to stir at room temperature for 30 min. The reaction mixture was washed with methyl *tert*-butyl ether and the aqueous layer was lyophilized to dryness. Further purification was done by anion exchange chromatography and RP-18 MPLC to yield **T^{Me}TP** (48.3 mg, 25%). ^{1}H NMR (400 MHz, D_2O): δ = 1.30 (s, 3H, 4'-CH_3), 1.96 (s, 3H; 5-CH_3), 2.49 (m, 2H, H-2'), 4.05 (m, 1H, H-5'a), 4.13 (m, 1H, H-5'b), 4.71 (t, *J* = 8 Hz, 1H; H-3'), 6.30 (t, *J* = 8 Hz, 1H; H-1'), 7.81 (s, 1H; H-6). ^{31}P NMR (D_2O, 162 MHz): δ = -22.54 (t, $P_ß$), -11.0 (d, $P_γ$), -10.1 (d, $P_α$). ESI-MS (*m*/*z*) calcd for $C_{11}H_{19}N_2O_{14}P_3$ [*M*-H]$^+$: 494.9; found: 495.0.

2'-Deoxy-4'-*C*-ethyl-thymidine-5'-*O*-triphosphate (T^{Et}TP) To a stirred ice cooled solution of **1b-T** (63 mg, 0.23 mmol), proton sponge (75 mg, 0.34 mmol) in trimethylphosphate (0.5 mL), phosphorus oxychloride (26 µL, 0.28 mmol) was added dropwise and allowed to stir at 0 ℃. After 2 h, furth er again 6 µL (0.06 mmol) of $POCl_3$ was added and allowed to stir for another 2 h. A solution of *n*-tributylamine (278 µL, 1.16 mmol) and 0.5 M tributylammonium pyrophosphate in dry DMF (2.33mL, 1.16 mmol) was added to the reaction mixture, and the solution was stirred for 15 min at 0 ℃, and then quenched with 20 mL of 0.1 M TEAB buffer (pH 7.5) and allowed to stir at 0 ℃ for 10 min and then the reaction mixtu re was allowed to stir at room temperature for 30 min. The reaction mixture was washed with methyl *tert*-butyl ether and the aqueous layer was lyophilized to dryness. Further purification was done by anion exchange chromatography and RP-18 MPLC to yield **T^{Et}TP** (48.75mg, 41%). ^{1}H NMR (400 MHz, D_2O): δ = 1.00 (brs, 3H; 4'-CH_2CH_3), 1.69 (m, 1H; 4'-C$H_2$$CH_3$), 1.74 (m, 1H; 4'-C$H_2$$CH_3$), 1.96 (s, 3H; 5-C$H_3$); 2.45 (m, 1H; H-2'), 2.51 (m, 1H; H-2'), 4.13 (m, 2H; H-5'); 4.80 (brs, 1H; H-3' merged with D_2O peak), 6.29 (t, *J* = 8 Hz, 1H; H-1'), 7.88 (s, 1H; H-6). ^{31}P NMR (D_2O, 162 MHz): δ = -22.51 (t, $P_ß$), -10.8 (d, $P_γ$), -10.1 (d, $P_α$). ESI-MS (*m*/*z*) calcd for $C_{12}H_{21}N_2O_{14}P_3$ [*M*-H]$^+$: 509.0; found: 509.0.

DNA substrates and kinetic analysis. DNA oligonucleotide synthesis was carried out by using an Applied-Biosystems 392 DNA/RNA synthesizer and purified by reversed-phase HPLC (DMT-ON) and afterwards by preparative PAGE on a 12% polyacrylamide gel containing 8M urea (DMT-OFF). DNA primer oligonucleotides were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Fermentas) according to the

procedure recommended by the manufacturer. Unmodified dNTPs are commercially available (Roche).

Purification of recombinant DNA polymerases. A 3′-5′-exonuclease-deficient variant of the Klenow Fragment of *E. coli* DNA polymerase I and Dpo4 of *S. solfataricus* were expressed and purified as described.[5] The purity of the proteins was >95% as controlled by SDS-PAGE. The concentrations were determined by the Bradford assay.

Single nucleotide insertion assays. Appropriate primer/template substrates (for sequence see Figure 2) were annealed by mixing 5'-^{32}P labeled primer with 1.5-fold the amount of template in the specific reaction buffer (see below). The mixture was heated to 95℃ for 5 min and subsequently allowed to co ol to room temperature over 1 h. After annealing, the appropriate DNA polymerase amount (see below) was added and the solution was incubated at 0℃ for 10 min. D NA/enzyme mixture and dN^{R}TP solution were incubated at 37℃ for 2 min. Then the reactions were initiated by addition of the DNA/enzyme mixture (10 µL) to an equal amount of dN^{R}TP solution in the reaction buffer and incubated at 37℃. Reactions pr omoted by the Klenow fragment of *E. coli* DNA polymerase I (*exo-* mutant) were performed in Tris-HCl (pH 7.3, 50 mM), $MgCl_2$ (10 mM), and DTT (1 mM). Reactions promoted by the DNA Polymerase IV of *Sulfolobus solfataricus* were performed in Tris-HCl (pH 8.0, 50 mM), $MgCl_2$ (10 mM), KCl (20 mM), DTT (2 mM), BSA (100 µg/ml) and Glycerol (10%). Assays included: 150 nM primer and 10 nM enzyme for nucleotide insertion. After incubation for the indicated time (15 min matched / 45 min mismatched) the reactions were quenched by addition of 35 µL of gel loading buffer (80% formamide, EDTA (20 mM)) and subsequently heated to 95℃ for 10 min. Reactions were analyzed by 12% polyacrylamide gel electrophoresis containing 8M urea, transferred to filter paper, dried under vacuum, and visualized by phosphor imaging.

Steady-state kinetics assays.[6] The steady-state kinetic data were obtained from single nucleotide insertion assays as described above except that concentration of nucleotides (at least eight different nucleotide concentrations were used in each investigation), enzyme concentration, and reaction time were adjusted for different reactions to allow 25% or less primer extension ensuring single completed hit conditions according to published procedures.[6] The reactions were fractionated by 12% denaturing polyacrylamide gel electrophoresis, and the data were quantified by phos-

phor imager analysis. Relative velocity *v* was measured as the ratio of the extended product (I_{ext}) to remaining primer (I_{prim}) as follows $v = I_{ext}/I_{prim}\ t$, where *t* represents the reaction time, and normalized for the enzyme and primer-template concentration used. The apparent K_M and k_{cat} values were obtained from Michaelis-Menten kinetics as described. [6] The depicted data derives from at 3-times repeated experiments.

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