[Supplementary Material]

Systematic characterization of 2′-deoxynucleoside-5′-triphosphate analogs as substrates for DNA polymerases by polymerase chain reaction and kinetic studies on enzymatic production of modified DNA

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General methods:

Mass spectral analysis for nucleoside analogues was performed on an ABI MDS-Sciex API-100 spectrometer under atmospheric pressure ionization conditions. Ultraviolet (UV) analyses were performed on a Shimadzu UV-1200 spectrometer. ^{1}H- and ^{31}P-nuclear magnetic resonance (NMR) Spectra were recorded on a JEOL JNM-AL300 or JNM-LA500 Fourier-transform-NMR spectrometer. Tetramethylsilane and 85% phosphoric acid were used as the internal standards for ^{1}H- and ^{31}P-NMR, respectively. The sodium salt of the dUTP analogue, generated from the corresponding triethylammonium salt using Dowex 50WX8 (Na^+ form), was used for NMR measurements. Thin-layer chromatography was performed using silica gel 60 F_{254} plates (Merck). Reversed-phase high-performance liquid chromatography (HPLC) was performed using a JASCO Gulliver system with UV detection at 260 nm and a packed Wakosil 5C18 (ϕ4.6 × 250 mm; Wako) or TSKgel ODS-80Ts (ϕ20 × 250 mm; Tosoh) column. Silica gel 60 (Kanto Chemical; 40–50 μm) was used for normal-phase column chromatography, and Wakosil 40C18 (Wako) was used for reversed-phase column chromatography. Reversed-phase medium-pressure liquid chromatography (MPLC) was performed using an YFLC-Wprep system (Yamazen) with a glass column (ϕ33× 250 mm) filled with Wakosil 40C18 (Wako). Ion exchange column chromatography was performed using an ECONO system (Bio-Rad) with a glass column (ϕ25× 500 mm) filled with diethylaminoethyl (DEAE) A-25-Sephadex (Amershambiosciences). Molecular absorption coefficients were 7330 $M^{-1}cm^{-1}$ at 290 nm for $\mathbf{T^{AL}}$, $\mathbf{T^{AC}}$, and $\mathbf{T^{AF}}$; 11050 $M^{-1}cm^{-1}$ at 291 nm for $\mathbf{T^{PA}}$, $\mathbf{T^{PN}}$, and $\mathbf{T^{PR}}$; 9300 $M^{-1}cm^{-1}$ at 260 nm for $\mathbf{T^{A2}}$, $\mathbf{T^{A4}}$, $\mathbf{T^{A6}}$, $\mathbf{T^{G6}}$, $\mathbf{T^{ME}}$, $\mathbf{T^{CN}}$, and $\mathbf{T^{DH}}$; 5040 $M^{-1}cm^{-1}$ at 290 nm for $\mathbf{C^{AL}}$, $\mathbf{C^{AC}}$, and $\mathbf{C^{AF}}$; 9450 $M^{-1}cm^{-1}$ at 295 nm for $\mathbf{C^{PA}}$, $\mathbf{C^{PN}}$, and $\mathbf{C^{PR}}$; and 7400 $M^{-1}cm^{-1}$ at 260 nm for $\mathbf{C^{A2}}$, $\mathbf{C^{A4}}$, $\mathbf{C^{A6}}$, $\mathbf{C^{G6}}$, $\mathbf{C^{ME}}$, $\mathbf{C^{CN}}$, and $\mathbf{C^{DH}}$.

Synthesis of modified nucleoside triphosphate analogs and their intermediates

The synthetic routes of the modified nucleoside triphosphate and their intermediates are shown in Scheme S1 and S2.

5-(2-(4-Trifluoroacetamidobutylamino)-2-oxoethyl)-2′-deoxyuridine (**2b**). Diaminobutane (275 mg; 3.12 mmol; 6.0 eq) was added to a stirred solution of nucleoside **1** (200 mg; 0.520 mmol) in methanol (3 mL). The reaction mixture was stirred at 50ºC for 3 h and then more diaminobutane (137 mg; 1.55 mmol; 3.0 eq) was added, and the mixture was stirred at 50ºC overnight. Ethyl trifluoroacetate (2.42 mL; 20.3 mmol; 39 eq) and triethylamine (181 μL; 1.30 mmol; 2.5 eq) were added, and the mixture was stirred at room temperature for 2 h, after which more ethyl trifluoroacetate (2.42 mL; 20.3 mmol; 39 eq) and triethylamine (181 μL; 1.30 mmol; 2.5 eq) were added, and the mixture was stirred at room temperature for 1.5 h. After evaporation, the residue was dissolved in water and washed with diethyl ether. The aqueous layer was evaporated, and the residue was dried well *in vacuo* and then purified by reversed-phase column chromatography (0% to 30% acetonitrile in water) to give nucleoside **2b** (118 mg; 0.261 mmol) as a white powder in 50% yield: *Rf* = 0.29 [1:4 methanol/chloroform]; ^{1}H NMR (300 MHz, CD_3OD) δ 7.90 (1H, s, H-6), 6.27 (1H, t, *J* = 6.8 Hz, H-1′), 4.38 (1H, m, H-4′), 3.90 (1H, q, *J* = 3.5 Hz, H-3′), 3.79 (1H, dd, *J* = 3.3 Hz, *J* = 12.0 Hz, H-5′), 3.71 (1H, dd, *J* = 3.8 Hz, *J* = 12.2 Hz, H-5"), 3.34 (2H, s, C5-C$\underline{H_2}$CONH-), 3.20 (4H, m, -NHC$\underline{H_2}$(CH$_2$)$_2$C$\underline{H_2}$NH-), 2.26 (2H, m, H-2′), 1.56 (4H, m, -NHCH$_2$(C$\underline{H_2}$)$_2$CH$_2$NH-); electrospray ionization-mass spectroscopy (ESI-MS; positive ion mode) *m/z*, found = 475.0, calculated for [(M+Na)$^+$] = 475.1.

5-(2-(4-Aminobutylamino)-2-oxoethyl)-2′-deoxyuridine-5′-triphosphate (**T^{A4}**). Nucleoside **2b** (100 mg; 0.221 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 71 mg; 0.331 mmol; 1.5 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (1.52 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (25 μL; 0.268 mmol; 1.2 eq) was then added dropwise with a micro syringe, and the reaction mixture was stirred at 0°C. After 45 min, *n*-tributylamine (196 μL; 0.823 mmol; 3.7 eq) and *n*-tributylamine pyrophosphate (2.1 mL of a 0.5 M solution in dimethylformamide [DMF]; 1.05 mmol; 4.8 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). The solvents were removed *in vacuo*, and the remaining crude mixture was dissolved in water. The product was purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05-1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing 5-(2-(4-trifluoroacetamidobutylamino)-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate were combined and evaporated under reduced pressure. To remove the excess pyrophosphate, the residue was purified by reversed-phase MPLC with a linear gradient of 0% to 20% acetonitrile in 10 mM triethylammonium acetate buffer (pH 7) to give the *N*-protected nucleotide: ESI-MS (negative ion mode) *m/z* , found = 691.0, calculated for [(M-H)$^-$] = 691.0. The *N*-protected nucleotide was treated with 4 N aqueous ammonia (4 mL), and the reaction mixture was stirred at room temperature for 1.5 h and then evaporated to dryness. The residue was purified by reversed-phase

HPLC (ϕ20 × 250 mm) to give the triphosphate $\mathbf{T}^{\mathbf{A4}}$ (14 μmol) in 6.3% yield starting from **2b**: ^{1}H NMR (500 MHz, D_2O) δ 7.78 (1H, s, H-6), 6.14 (1H, t, *J* = 6.8 Hz, H-1′), 4.49 (1H, m, H-4′), 3.95 (3H, m, H-3′ and H-5′), 3.20 (2H, s, C5-C$\underline{H}_2$CONH-), 3.04 (2H, t, *J* = 6.3 Hz, -HNC$\underline{H}_2$$(CH_2)_3NH_2$), 2.79 (2H, t, *J* = 7.5 Hz, -HN$(CH_2)_3$C$\underline{H}_2$$NH_2$), 2.20 (2H, m, H-2′), 1.48 (2H, m, -HNCH_2C$\underline{H}_2$$(CH_2)_2NH_2$), 1.40 (2H, m, -HN$(CH_2)_2C\underline{H}_2$$CH_2NH_2$); ^{31}P NMR (500 MHz, D_2O) δ -7.10 (d), -10.76 (d), -21.76 (t); ESI-MS (negative ion mode) *m/z*, found = 595.1, calculated for $[(M-H)^-]$ = 595.1.

5-Cyanomethyl-2′-deoxyuridine (**4**). Nucleoside **3** (200 mg, 0.569 mmol) was treated with 7 N aqueous ammonia (10 mL) and the reaction mixture was stirred at room temperature for 1 h and then evaporated to dryness. The residue was purified by reversed-phase column chromatography (0% to 30% acetonitrile in water) to give nucleoside **4** (132 mg, 0.494 mmol) as a yellow powder in 87% yield: *Rf* 0.34 [1:4 methanol/chloroform]; ^{1}H NMR (300 MHz, D_2O) δ 7.83 (1H, s, H-6), 6.14 (1H, t, *J* = 6.8 Hz, H-1′), 4.31 (1H, m, H-4′), 3.91 (1H, q, *J* = 3.7 Hz, H-3′), 3.71 (1H, dd, *J* = 3.5 Hz, *J* = 12.5 Hz, H-5′), 3.62 (1H, dd, *J* = 4.8 Hz, *J* = 12.6 Hz, H-5"), 3.43 (2H, s, C5-C$\underline{H}_2$CN), 2.26 (2H, m, H-2′); ESI-MS (positive ion mode) *m/z*: found = 290.3, calculated for $[(M+Na)^+]$ = 290.1.

5-Cyanomethyl-2′-deoxyuridine-5′-triphosphate ($\mathbf{T}^{\mathbf{CN}}$). Nucleoside **4** (122 mg; 0.457 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 0.147 g; 0.686 mmol; 1.5 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (3.0 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (60 μL; 0.644 mmol; 1.4 eq) was then added dropwise using a micro syringe, and the reaction mixture was stirred at 0°C. After 45 min, *n*-tributylamine (404 μL; 1.70 mmol; 3.7 eq) and *n*-tributylamine pyrophosphate (4.7 mL of a 0.5 M solution in DMF, 2.35 mmol, 5.1 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). The solvents were removed *in vacuo*, and the remaining crude mixture was dissolved in water. The product was purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing $\mathbf{T}^{\mathbf{CN}}$ were combined and evaporated under reduced pressure. To remove the excess pyrophosphate, the residue was purified by reversed-phase MPLC with a linear gradient of 0% to 20% acetonitrile in 10 mM triethylammonium acetate buffer (pH 7). Further purification was performed by reversed-phase HPLC (ϕ20 × 250 mm) with a linear gradient of 0% to 4.9% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7) to give the triphosphate $\mathbf{T}^{\mathbf{CN}}$ (101 μmol) in 22% yield starting from **4**: ESI-MS (negative ion mode) *m/z*, found = 506.0, calculated for $[(M-H)^-]$ = 506.0.

5-Cyanomethyl-5′,3′-O-diacetyl-2′-deoxycytidine (**5**). Phosphorus oxychloride (2 mL; 21.5 mmol; 2.2 eq) was added dropwise to a stirred solution of nucleoside **3** (3.50 g, 9.96 mmol) in dry pyridine (142 mL), and the solution was stirred at room temperature. After 4 h, ice-cold water was added to the reaction mixture, and after 0.5 h, concentrated aqueous ammonia (19 mL) was added, and the solution was heated at 50°C for 2 h. The solvents were removed *in vacuo*, and the residue was purified by flash chromatography on silica gel and elution with 1% to 10% methanol in chloroform

to yield nucleoside **5** (1.43 g; 4.08 mmol) as a brown powder in 41% yield: R_f 0.25 [1:9 methanol/chloroform]; ^{1}H NMR (300 MHz, D_2O) δ 7.82 (1H, s, H-6), 6.16 (1H, dd, *J* = 5.7 Hz, *J* = 6.0 Hz, H-1′), 5.20 (1H, d, *J* = 6.6 Hz, H-4′), 4.28 (3H, m, H-3′ and H-5′), 3.59 (2H, m, C5-C$\underline{H_2}$CN), 2.59 - 2.52 (1H, 2dd, *J* = 1.8 Hz, H-2′), 2.18 (1H, m, H-2"), 2.05 (6H, m, 3′-OC(=O)C$\underline{H_3}$ and 5′-OC(=O)C$\underline{H_3}$); ESI-MS (positive ion mode) *m/z*, found = 351.0, calculated for $[(M+H)^+]$ = 351.1.

5-Cyanomethyl-2′-deoxycytidine (**6**). Nucleotide **5** (117 mg; 0.334 mmol) was treated with 7 N aqueous ammonia (10 mL), and the reaction mixture was stirred at room temperature for 1 h and then evaporated to dryness. The residue was purified by reversed-phase column chromatography (0% to 30% acetonitrile in water) to give nucleoside **6** (65 mg, 0.244 mmol) as a yellow powder in 73% yield: *Rf* 0.20 [1:4 methanol/chloroform]; ^{1}H NMR (300 MHz, D_2O) δ 7.84 (1H, s, H-6), 6.10 (1H, t, *J* = 6.5 Hz, H-1′), 4.32 (1H, m, H-4′), 3.91 (1H, m, H-3′), 3.65 (2H, m, H-5′), 3.54 (2H, s, C5-C$\underline{H_2}$CN), 2.11 (2H, m, H-2′); ESI-MS (positive ion mode) *m/z* , found = 289.2, calculated for $[(M+Na)^+]$ = 289.1.

5-Cyanomethyl-2′-deoxycytidine-5′-triphosphate (**C**$^{\mathbf{CN}}$). Nucleoside **6** (60 mg, 0.225 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 72.4 mg; 0.338 mmol; 1.5 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (1.7 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (44 μL; 0.472 mmol; 2.1 eq) was then added dropwise using a micro syringe, and the reaction mixture was stirred at 0°C. After 45 min, *n*-tributylamine (200 μL; 0.839 mmol; 3.7 eq) and *n*-tributylamine pyrophosphate (2.26 mL of a 0.5 M solution in DMF; 1.13 mmol; 5 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). The solvents were removed *in vacuo*, and the remaining crude mixture was dissolved in water. The product was purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing **C**$^{\mathbf{CN}}$ were combined and evaporated under reduced pressure. To remove the excess of pyrophosphate, the residue was purified by reversed-phase MPLC with a linear gradient of 0% to 20% acetonitrile in 10 mM triethylammonium acetate buffer (pH 7). Further purification was performed on reversed-phase HPLC (ϕ20 × 250 mm) with a linear gradient of 0% to 4.9% acetonitrile in 50 mM triethylammonium acetate buffer to give the triphosphate **C**$^{\mathbf{CN}}$ (11.1 μmol) in 5% yield starting from **6**: ESI-MS (negative ion mode) *m/z* , found = 505.1, calculated for $[(M-H)^-]$ = 505.0.

5-(2-Methoxy-2-oxoethyl)-2′-deoxycytidine (**7**). Nucleotide **5** (1.30 g; 3.71 mmol) was treated with 1 N aqueous KOH (15.6 mL; 15.6 mmol; 4.2 eq) and the reaction mixture was refluxed at 86°C for 1 h, cooled to room temperature, neutralized with 1 N aqueous HCl, and evaporated to dryness. The residue was purified by reversed-phase column chromatography (0% to 16% acetonitrile in water) to give 5-(2-hydroxy-2-oxoethyl)-2′-deoxycytidine (690 mg; 2.42 mmol) as a pale yellow powder in 65% yield: *Rf* 0.27 [5:4:1 water/1-butanol/chloroform]; ^{1}H NMR (300 MHz, D_2O) δ 7.82 (1H, s, H-6), 6.12 (1H, t, *J* = 6.5 Hz, H-1′), 4.31 (1H, m, H-4′), 3.94 (1H, q, *J* = 4.1 Hz, H-3′),

3.73 (1H, dd, *J* = 3.5 Hz, *J* = 12.5 Hz, H-5′), 3.63 (1H, dd, *J* = 4.8 Hz, *J* = 12.3 Hz, H-5"), 3.29 (2H, s, C5-C$\underline{H_2}$CO-) 2.26 (2H, m, H-2′); ESI-MS (negative ion mode) *m/z* , found = 284.1, calculated for [(M-H)$^-$] = 284.1. To the obtained nucleoside (344 mg; 1.21 mmol) in dry methanol (50 mL), chlorotrimethysilane (0.57 mL; 4.46 mmol; 3.7 eq) was added, and the mixture was stirred at 31°C for 1.5 h and then evaporated to dryness. The residue was suspended in methanol and the insoluble material was removed by vacuum filtration. The filtrate was evaporated to dryness and the residue was purified by flash chromatography on silica gel and elution with 10% to 20% methanol in chloroform to yield nucleoside **7** (295 mg, 0.986 mmol) as a pale yellow powder in 81% yield: R_f 0.24 [1:5 methanol/chloroform]; ^{1}H NMR (300MHz, D_2O) δ 7.68 (1H, s, H-6), 6.10 (1H, t, *J* = 6.5 Hz, H-1′), 4.27 (1H, m, H-4′), 3.90 (1H, q, *J* = 4.2 Hz, H-3′), 3.70 (1H, dd, *J* = 3.5 Hz, *J* = 12.5 Hz, H-5′), 3.63 - 3.54 (3H, m, H-5" and -$CH_2COOC\underline{H_3}$), 3.42 (2H, s, C5-C$\underline{H_2}$CO-), 2.29 (1H, m, H-2′), 2.13 (1H, m, H-2′); ESI-MS (positive ion mode) *m/z* , found = 300.0, calculated for [(M+H)$^+$] = 300.1.

5-(2-Methoxy-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate (**C^{ME}**). Nucleoside **7** (101 mg, 0.337 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1, 8-naphthalenediamine (Proton Sponge®; 108 mg; 0.504 mmol; 1.5 eq) were dried together in a flask under vacuum overnight. Trimethylphosphate (3.5 mL) was added to the flask under argon, and the solution was cooled to 0°C for 30 min. Distilled phosphorus oxychloride (50 μL; 0.536 mmol; 1.6 eq) was then added dropwise using a micro syringe, and the reaction mixture was allowed to stir at 0°C. After 45 min, *n*-tributylamine (298 μL; 1.25 mmol; 3.7 eq) and *n*-tributylamine pyrophosphate (3.5 mL of a 0.5 M solution in DMF; 1.75 mmol; 5.2 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). The solvents were removed *in vacuo*, and the remaining crude mixture was dissolved in water. The product was purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing **C^{ME}** were combined and evaporated under reduced pressure. To remove the excess pyrophosphate, the residue was purified by reversed-phase MPLC with a linear gradient of 0% to 20% acetonitrile in 10 mM triethylammonium acetate buffer (pH 7). Further purification was performed by reversed-phase HPLC (ϕ20 × 250 mm) with a linear gradient of 0% to 4.9% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7) to give the triphosphate **C^{ME}** (3.24 μmol) in 1% yield starting from **7**: ESI-MS (negative ion mode) *m/z* , found = 538.0, calculated for [(M-H)$^-$] = 538.0.

5-(2-(6-Trifluoroacetamidohexylamino)-2-oxoethyl)-2′-deoxycytidine (**8a**). Diaminohexane (510 mg; 4.39 mmol; 5.0 eq) was added to a stirred solution of nucleoside **7** (265 mg, 0.885 mmmol) in methanol (3 mL). The reaction mixture was stirred at 50ºC for 2 h. Ethyl trifluoroacetate (3.3 mL; 27.7 mmol; 31 eq) and triethylamine (339 μL; 2.43 mmol; 2.7 eq) were added, and the mixture was stirred at room temperature for 1 h, after which more ethyl trifluoroacetate (3.3 mL; 27.7 mmol; 31 eq) and triethylamine (339 μL; 2.43 mmol; 2.7 eq) were added and stirred at room temperature for 2 h. After evaporation, the residue was purified by reversed-phase column

chromatography (0% to 30% acetonitrile in water) to give nucleoside **8a** (294 mg; 0.613 mmol) as a white powder in 69% yield: *Rf* 0.35 [1:4 methanol/chloroform]; ^{1}H NMR (300 MHz, CD_3OD) δ 7.68 (1H, s, H-6), 6.13 (1H, t, *J* = 6.6 Hz, H-1′), 4.32 (1H, m, H-4′), 3.94 (1H, m, H-3′), 3.72 (1H, dd, *J* = 3.0 Hz, *J* = 12.3 Hz, H-5′), 3.63 (1H, dd, *J* = 5.1 Hz, *J* = 12.3 Hz, H-5"), 3.26 (2H, s, C5-C$\underline{H}_2$CONH-), 3.18 (2H, t, *J* = 6.9 Hz, -NHC$\underline{H}_2$(CH$_2$)$_4$CH$_2$NH-), 3.08 (2H, t, *J* = 6.9 Hz, -NHCH$_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 2.31 (1H, m, H-2′), 2.19 (1H, m, H-2′), 1.41 (4H, m, -NHCH$_2$C$\underline{H}_2$(CH$_2$)$_2$C$\underline{H}_2$CH$_2$NH-), 1.19 (4H, br, -NH(CH$_2$)$_2$(C$\underline{H}_2$)$_2$(CH$_2$)$_2$NH-); ESI-MS (positive ion mode) *m/z* , found = 502.3, calculated for [(M+Na)$^+$] = 502.2.

5-(2-(6-Aminohexylamino)-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate (**C^{A6}**). Nucleoside **8a** (243 mg; 0.507 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 163 mg; 0.761 mmol; 1.5 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (3 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (100 μL; 1.07 mmol; 2.1 eq) was then added dropwise using a micro syringe, and the reaction mixture was allowed to stir at 0°C. After 50 min, *n*-tributylamine (411 μL; 1.73 mmol; 3.4 eq) and *n*-tributylamine pyrophosphate (5.33 mL of a 0.5 M solution in DMF; 2.67 mmol; 5.3 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). The solvents were removed *in vacuo*, and the remaining crude mixture was dissolved in water. The product was purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing 5-(2-(6-trifluoroacetamidohexylamino)-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate were combined and evaporated under reduced pressure. To remove the excess pyrophosphate, the residue was purified by reversed-phase MPLC with a linear gradient of 0% to 35% acetonitrile in 10 mM triethylammonium acetate buffer (pH 7) to give the *N*-protected nucleotide (43 mg; 59.8 μmol) in 12% yield: ESI-MS (negative ion mode) *m/z* , found = 718.5, calculated for [(M-H)$^-$] = 718.1. The *N*-protected nucleotide (32 mg; 44.5 μmol) was treated with 2 N aqueous ammonia (4 mL), and the reaction mixture was stirred at room temperature for 1 h and then evaporated to dryness. The residue was purified by reversed-phase HPLC (*ϕ*20 × 250 mm) with 13% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7) containing 0.1% tetrabutylammonium hydroxide. Further purification was performed by reversed-phase MPLC with a linear gradient of 0% to 35% acetonitrile in 10 mM triethylammonium acetate buffer (pH 7) to give the triphosphate **C^{A6}** (36.3 μmol) in 9.6% yield starting from **8a**: ESI-MS (negative ion mode) *m/z* , found = 622.2, calculated for [(M-H)$^-$] = 622.1.

5-(2-(6-Guanidinohexylamino)-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate (**C^{G6}**). The triethylammonium salt of **C^{A6}** (16.2 μmol) was dissolved in 1.0 M *S*-ethylthiourea hydrobromide in DMF (3.57 mL; 3.57 mmol; 220 eq). Triethylamine (994 μL; 7.13 mmol, 440 eq) was added, and the mixture was stirred at room temperature for 4 h. After evaporation, the residue was purified by reversed-phase HPLC (*ϕ*20 × 250 mm) with a linear gradient of 3.5% to 8.4% acetonitrile in 50 mM

triethyl ammonium acetate buffer to give the triphosphate **C**$^{\mathbf{G6}}$ (8.85 μmol) in 55% yield: ESI-MS (negative ion mode) *m/z* , found = 664.1, calculated for [(M-H)$^-$] = 664.1.

5-(2-(4-Trifluoroacetamidobutylamino)-2-oxoethyl)-2′-deoxycytidine (**8b**). Diaminobutane (177 mg; 2.01 mmol; 5 eq) was added to a stirred solution of nucleoside **7** (120 mg; 0.401 mmmol) in methanol (3 mL). The reaction mixture was stirred at 50ºC for 3.5 h. Ethyl trifluoroacetate (1.8 mL; 15.1 mmol; 38 eq) and triethylamine (101 μL; 0.72 mmol; 1.8 eq) were added, and the mixture was stirred at room temperature for 1.5 h and then more ethyl trifluoroacetate (1.8 mL; 15.1 mmol; 38 eq) and triethylamine (101 μL; 0.72 mmol; 1.8 eq) were added and stirred at room temperature for 1 h. After evaporation, the residue was purified by reversed-phase column chromatography (0% to 30% acetonitrile in water) to give nucleoside **8b** (100 mg; 0.222 mmol) as a yellow powder in 55% yield: *Rf* 0.28 [1:4 methanol/chloroform]; ^{1}H NMR (300 MHz, D_2O) δ 7.67 (1H, s, H-6), 6.13 (1H, t, *J* = 6.6 Hz, H-1′), 4.32 (1H, m, H-4′), 3.94 (1H, m, H-3′), 3.72 (1H, dd, *J* = 3.2 Hz, *J* = 12.5 Hz, H-5′), 3.63 (1H, dd, *J* = 5.3 Hz, *J* = 12.5 Hz, H-5"), 3.27 (2H, s, C5-C$\underline{H_2}$CONH-), 3.20 (2H, t, *J* = 5.9 Hz, -NHC$\underline{H_2}$(CH$_2$)$_2$CH$_2$NH-), 3.10 (2H, t, *J* = 6.0 Hz, -NHCH$_2$(CH$_2$)$_2$C$\underline{H_2}$NH-), 2.31 (1H, m, H-2′), 2.19 (1H, m, H-2′), 1.43 (4H, br, -NHCH$_2$(C$\underline{H_2}$)$_2$CH$_2$NH-); ESI-MS (positive ion mode) *m/z*, found = 474.1, calculated for [(M+Na)$^+$] = 474.2.

5-(2-(4-Aminobutylamino)-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate (**C**$^{\mathbf{A4}}$). Nucleoside **8b** (95 mg; 0.210 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 61.7 mg; 0.288 mmol; 1.4 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (1.5 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (37.6 μL; 0.403 mmol; 1.9 eq) was then added dropwise using a micro syringe, and the reaction mixture was allowed to stir at 0°C. After 45 min, *n*-tributylamine (170 μL; 0.714 mmol; 3.4 eq) and *n*-tributylamine pyrophosphate (2.0 mL of a 0.5 M solution in DMF; 1.0 mmol; 4.8 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). The solvents were removed *in vacuo*, and the remaining crude mixture was dissolved in water. The product was purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing 5-(2-(4-trifluoroacetamidobutylamino)-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate were combined and evaporated under reduced pressure. To remove the excess pyrophosphate, the residue was purified by reversed-phase MPLC with a linear gradient of 0% to 35% acetonitrile in 10 mM triethylammonium acetate buffer to give the *N*-protected nucleotide (8.9 mg; 12.9 μmol) in 6.1% yield: ESI-MS (negative ion mode) *m/z*, found = 690.1, calculated for [(M-H)$^-$] = 690.1. The *N*-protected nucleotide (9.0 mg; 13.0 μmol) was treated with 4 N aqueous ammonia (4 mL), and the reaction mixture was stirred at room temperature for 1 h and then evaporated to dryness. The residue was purified by reversed-phase HPLC (ϕ20 × 250 mm) with a linear gradient of 0% to 4.9% acetonitrile in 50 mM triethylammonium acetate buffer to give the triphosphate **C**$^{\mathbf{A4}}$ (11.8 μmol) in 5.6% yield starting from **8b**: ESI-MS (negative ion mode) *m/z*, found = 594.2, calculated for [(M-H)$^-$] = 594.1.

5-(2-(2-Trifluoroacetamidoethylamino)-2-oxoethyl)-2′-deoxycytidine (**8c**). Diaminoethane (120 mg; 2.00 mmol; 5 eq) was added to a stirred solution of nucleoside **7** (120 mg; 0.401 mmol) in methanol (3 mL). The reaction mixture was stirred at 50ºC for 3.5 h. Ethyl trifluoroacetate (1.8 mL; 15.1 mmol; 38 eq) and triethylamine (101 μL; 0.72 mmol; 1.8 eq) were added, and the mixture was stirred at room temperature for 1.5 h, after which more ethyl trifluoroacetate (1.8 mL; 15.1 mmol; 38 eq) and triethylamine (101 μL; 0.72 mmol; 1.8 eq) were added and stirred at room temperature for 1 h. After evaporation, the residue was purified by reversed-phase column chromatography (0% to 30% acetonitrile in water) to give nucleoside **8c** (81 mg; 0.191 mmol) as a white powder in 48% yield: *Rf* 0.14 [1:4 methanol/chloroform]; ^{1}H NMR (300 MHz, D_2O) δ 7.65 (1H, s, H-6), 6.11 (1H, t, *J* = 6.6 Hz, H-1′), 4.29 (1H, m, H-4′), 3.91 (1H, m, H-3′), 3.71 (1H, dd, *J* = 1.7 Hz, *J* = 12.8 Hz, H-5′), 3.62 (1H, dd, *J* = 4.7 Hz, *J* = 12.5 Hz, H-5"), 3.29 (4H, m, -NH(C$\underline{H_2}$)$_2$NH-), 3.25 (2H, s, C5-C$\underline{H_2}$CONH-), 2.28 (1H, m, H-2′), 2.19 (1H, m, H-2′); ESI-MS (negative ion mode) *m/z*, found = 421.9, calculated for [(M-H)$^-$] = 422.1.

5-(2-(4-Aminobutylamino)-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate (**C^{A2}**). Nucleoside **8c** (100 mg; 0.236 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 76 mg; 0.355 mmol; 1.5 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (1.1 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (46.8 μL; 0.502 mmol; 2.1 eq) was then added dropwise using a micro syringe, and the reaction mixture was allowed to stir at 0°C. After 45 min, *n*-tributylamine (208 μL; 0.873 mmol; 3.7 eq) and *n*-tributylamine pyrophosphate (2.36 mL of a 0.5 M solution in DMF; 1.18 mmol; 5 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). The solvents were removed *in vacuo*, and the remaining crude mixture was dissolved in water. The product was purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing 5-(2-(4-trifluoroacetamidoethylamino)-2-oxoethyl)-2′-deoxycytidine-5′-triphosphate were combined and evaporated under reduced pressure. To remove the excess of pyrophosphate, the residue was purified by reversed phase MPLC with a linear gradient of 0% to 35% acetonitrile in 10 mM triethylammonium acetate buffer to give the *N*-protected nucleotide: ESI-MS (negative ion mode) *m/z*, found = 662.0, calculated for [(M-H)$^-$] = 662.0. The *N*-protected nucleotide was treated with 4 N aqueous ammonia (4 mL), and the reaction mixture was stirred at room temperature for 1 h and then evaporated to dryness. The residue was purified by reversed-phase HPLC (ϕ20 × 250 mm) with a linear gradient of 2.1% to 2.8% acetonitrile in 50 mM triethylammonium acetate buffer to give the triphosphate **C^{A2}** (16.3 μmol) in 6.9% yield starting from **8c**: ESI-MS (negative ion mode) *m/z*, found = 566.0, calculated for [(M-H)$^-$] = 566.1.

5-Iodo-5′,3′-O-di-tert-butyldimethylsilyl-2′-deoxyuridine (**10**). A solution of 5-iodo-2′-deoxyuridine (**9**; 2.0 g; 5.65 mmol) and imidazole (1.69 g; 24.8 mmol; 4.4 eq) in dry DMF (22.6 mL) was stirred at room temperature and mixed with *tert*-butyldimethylsilyl chloride (2.23 g; 14.8 mmol; 2.6 eq). After 3 h, the reaction mixture was evaporated to dryness, and the

residue was dissolved in 1:1 (v/v) ethyl acetate/diethyl ether and then successively washed with aqueous saturated $NaHCO_3$ and brine. After being dried over $MgSO_4$, the solvent was evaporated, and the remaining residue was purified by flash chromatography on silica gel with elution using 0% to 1% methanol in dichloromethane to yield nucleoside **10** (3.19 g; 5.48 mmol) as a white foam in 97% yield: R_f 0.19 [1:99 methanol/dichloromethane]; ^{1}H NMR (300 MHz,$CDCl_3$) δ 8.00 (1H, s, H-6), 6.18 (1H, dd, *J* = 5.4 Hz, *J* = 5.7 Hz, H-1′), 4.32 (1H, m, H-4′), 3.90 (1H, q, *J* = 2.2 Hz, H-3′), 3.80 (1H, dd, *J* = 2.3 Hz, *J* = 11.3 Hz, H-5′), 3.68 (1H, dd, *J* = 2.3 Hz, *J* = 11.3 Hz, H-5"), 2.26 - 2.19 (1H, 2dd, *J* = 2.1 Hz, *J* = 2.4 Hz, H-2′), 1.91 (1H, m, H-2′), 0.86, 0.81 (18H, 2s, tBu-3′ and tBu-5′), 0.074, 0.063, 0,000, -0.008 (12H, 4s, $(C\underline{H}_3)_2$Si-3′ and $(C\underline{H}_3)_2$Si-5′); ESI-MS (positive ion mode) *m/z*, found = 583.2, calculated for $[(M+H)^+]$ = 583.2; and found = 604.9, calculated for $[(M+Na)^+]$ = 605.1.

5-(3-Hydoxypropyn-1-yl)-5′,3′-O-di-tert-butyldimethylsilyl-2′-deoxyuridine (**11**). Tetrakis(triphenylphosphine)palladium(0) (632 mg; 0.547 mmol; 0.1 eq), triethylamine (1.53 mL; 11.0 mmol; 2.0 eq), propargyl alcohol (0.96 mL; 16.5 mmol; 3.0 eq), and copper iodide (208 mg; 1.09 mmol; 0.2 eq) were added to a solution of nucleoside **10** (3.19 g; 5.48 mmol) in dry DMF. The reaction was stirred for 6 h under argon at room temperature and evaporated to dryness. The residue was dissolved with ethyl acetate and then successively washed with aqueous saturated $NaHCO_3$ and brine. After being dried over $MgSO_4$, the solvent was evaporated, and the remaining residue was purified by flash chromatography on silica gel with elution using 1:3:2:0.2 (v/v/v/v) diethyl ether/hexane/ethyl acetate/methanol to yield nucleoside **11** (2.34 g, 4.58 mmol) as a yellow foam in 84% yield: R_f 0.27 [1:4:2:0.2 diethyl ether/hexane/ethyl acetate/methanol]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7,96 (1H, s, H-6), 6.20 (1H, dd, *J* = 6.0 Hz, H-1′), 4.33 (3H, m, H-4′ and -C≡C-C$\underline{H}_2$-), 3.89 (1H, q, *J* = 2.2 Hz, H-3′), 3.82 (1H, dd, *J* = 2.4 Hz, *J* = 11.7 Hz, H-5′), 3.68 (1H, dd, *J* = 2.3 Hz, *J* = 11.6 Hz, H-5"), 2.28 - 2.21 (1H, 2dd, *J* = 3.0 Hz, *J* = 2.7 Hz, H-2′), 1.96 (1H, m, H-2′), 0.851, 0.809 (18H, 2s, tBu-3′ and tBu-5′), 0.067, 0.051, 0.000, -0.007 (12H, 4s, $(C\underline{H}_3)_2$Si-3′ and $(C\underline{H}_3)_2$Si-5′); ESI-MS(positive ion mode) *m/z*, found = 511.4, calculated for $[(M+H)^+]$ = 511.3; and found = 533.3, calculated for $[(M+Na)^+]$ = 533.3.

5-(3-Acetoxypropyn-1-yl)-5′,3′-O-di-tert-butyldimethylsilyl-2′-deoxyuridine (**12**). Acetic anhydride (4.2 mL; 44.4 mmol; 10 eq) was added slowly to a solution of nucleoside **11** (2.24 g; 4.39 mmol) in dry pyridine. The reaction was stirred for 1 h at room temperature and evaporated to dryness. The residue was purified by flash chromatography on silica gel with elution using 2:1 (v/v) hexane/ethyl acetate to yield nucleoside **12** (1.99 g; 3.60 mmol) as a yellow foam in 82% yield: R_f 0.31 [2:1 hexane/ethyl acetate]; ^{1}H NMR (300 MHz,$CDCl_3$) δ 7,98 (1H, s, H-6), 6.20 (1H, t, *J* = 6.6 Hz, H-1′), 4.78 (2H, s, -C≡C-C$\underline{H}_2$-), 4.33 (1H, m, H-4′), 3.91 (1H, d, *J* = 2.4 Hz, H-3′), 3.83 (1H, dd, *J* = 2.4 Hz, *J* = 11.4 Hz, H-5′), 3.69 (1H, dd, *J* = 2.1 Hz, *J* = 11.4 Hz, H-5"), 2.29 - 2.22 (1H, 2dd, *J* = 2.7 Hz, *J* = 3.0 Hz, H-2′), 2.01 (4H, m, H-2′ and -OC(=O)C$\underline{H}_3$), 0.856, 0.817 (18H, 2s, tBu-3′ and tBu-5′), 0.068, 0.057, 0.008, 0.000 (12H, 4s, $(C\underline{H}_3)_2$Si-3′ and $(C\underline{H}_3)_2$Si-5′); ESI-MS (positive ion mode) *m/z*, found = 553.4, calculated for $[(M+H)^+]$ = 553.3; and found = 575.2, calculated for $[(M+Na)^+]$ = 575.3.

5-(3-Acetoxypropyn-1-yl)-2′-deoxyuridine (**13**). Triethylamine trihydrofluoride (3.45 mL; 21.2 mmol; 6.0 eq) was added slowly to a solution of nucleoside **12** (1.94 g; 3.51 mmol) in tetrahydrofuran (THF; 22 mL). The reaction was stirred for 2 h at room temperature and evaporated to dryness. The residue was purified by reversed-phase column chromatography (5% to 30% acetonitrile in water) to give nucleoside **13** (846 mg; 2.61 mmol) as a white powder in 74% yield: *Rf* 0.26 [1:9 methanol/ chloroform]; ^{1}H NMR (300 MHz, CD_3OD) δ 8.35 (1H, s, H-6), 6.23 (1H, t, *J* = 6.5 Hz, H-1′), 4.87 (2H, s, -C≡C-C$\underline{H}_2$-), 4.40 (1H, m, H-4′), 3.93 (1H, q, *J* = 3.3 Hz, H-3′), 3.81 (1H, dd, *J* = 3.0 Hz, *J* = 12.0 Hz, H-5′), 3.73 (1H, dd, *J* = 3.5 Hz, *J* = 11.9 Hz, H-5"), 2.24 (2H, m, H-2′), 2.07 (3H, s, -OC(=O)C$\underline{H}_3$); ESI-MS (negative ion mode) *m/z*, found = 323.0, calculated for [(M-H)$^-$] = 323.1.

5-(3-Hydoxypropyn-1-yl)-2′-deoxyuridine-5′-triphosphate (**T^{PN}**). Nucleoside **13** (240 mg; 0.740 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 238 mg; 1.11 mmol; 1.5 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (4.28 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (82.9 μL; 0.889 mmol; 1.2 eq) was then added dropwise using a micro syringe, and the reaction mixture was allowed to stir at 0°C. After 45 min, *n*-tributylamine (596 μL; 2.50 mmol; 3.4 eq) and *n*-tributylamine pyrophosphate (7.55 mL of a 0.5 M solution in DMF; 3.78 mmol; 5.1 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). After evaporation, the residue was dissolved in water and washed with diethyl ether. The aqueous layer was evaporated, and the residue was dried well *in vacuo* and then purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing 5-(3-acetoxypropyn-1-yl)-2′-deoxyuridine-5′-triphosphate were combined and evaporated under reduced pressure. The residue was purified by reversed-phase MPLC with a linear gradient of 0% to 8% acetonitrile in 10 mM triethylammonium acetate buffer (pH 7) to give the *O*-acetyl nucleotide: ESI-MS (negative ion mode) *m/z*, found = 563.0, calculated for [(M-H)$^-$] = 563.0. The *O*-acetyl nucleotide was treated with 2 N aqueous ammonia (20 mL), and the reaction mixture was stirred at room temperature for 1 h and then evaporated to dryness. The residue was purified by reversed-phase MPLC with a linear gradient of 0% to 8% acetonitrile in 10 mM triethylammonium acetate buffer to give the triphosphate **T^{PN}** (189 μmol) in 26% yield starting from **13**: ESI-MS (negative ion mode) *m/z*, found = 520.7, calculated for [(M-H)$^-$] = 521.0.

5-Iodo-5′,3′-O-di-tert-butyldimethylsilyl-2′-deoxycytidine (**15**). A solution of 5-iodo-2′-deoxycytidine **14** (400 mg; 1.13 mmol) and imidazole (339 mg; 4.98 mmol; 4.4 eq) in dry DMF (4.5 mL) was stirred at room temperature and mixed with *tert*-butyldimethylsilyl chloride (443 mg; 2.94 mmol; 2.6 eq). After 3 h, the reaction mixture was evaporated to dryness, and the residue was dissolved in 1:1 (v/v) ethyl acetate/diethyl ether and then successively washed with saturated aqueous $NaHCO_3$ and brine. After being dried over $MgSO_4$, the solvent was evaporated, and the remaining residue was purified by flash chromatography on silica gel with elution using 1%

methanol in dichloromethane to yield nucleoside **15** (601 mg; 1.03 mmol) as a white foam in 91% yield: R_f 0.49 [1:99 methanol/dichloromethane]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (1H, s, H-6), 6.24 (1H, t, *J* = 6.6 Hz, H-1′), 4.35 (1H, m, H-4′), 3.98 (1H, q, *J* = 2.7 Hz, H-3′), 3.89 (1H, dd, *J* = 2.6 Hz, *J* = 11.6 Hz, H-5′), 3.75 (1H, dd, *J* = 2.6 Hz, *J* = 11.3 Hz, H-5"), 2.51 - 2.44 (1H, 2dd, *J* = 3.0 Hz, *J* = 2.7 Hz, H-2′), 1.96 (1H, m, H-2′), 0.93, 0.88 (18H, 2s, tBu-3′ and tBu-5′), 0.14, 0.13, 0.070, 0.062 (12H, 4s, $(C\underline{H}_3)_2$Si-3′ and $(C\underline{H}_3)_2$Si-5′); ESI-MS (positive ion mode) *m/z*, found = 604.0, calculated for $[(M+Na)^+]$ = 604.2.

5-(3-Hydoxypropyn-1-yl)-5′,3′-O-di-tert-butyldimethylsilyl-2′-deoxycytidine (**16**). Tetrakis(triphenylphosphine)palladium(0) (38 mg; 32.9 μmol; 0.1 eq), triethylamine (92 μL; 0.660 mmol; 2.0 eq), propargyl alcohol (57.3 μL; 0.984 mmol; 3.0 eq) and copper iodide (12.5 mg; 65.6 μmol; 0.2 eq) were added to a solution of nucleoside **15** (191 mg; 0.328 mmol) in dry DMF. The reaction was stirred for 6 h under argon at room temperature and evaporated to dryness. The residue was dissolved in ethyl acetate and then successively washed with saturated aqueous $NaHCO_3$ and brine. After being dried over $MgSO_4$, the solvent was evaporated, and the remaining residue was purified by flash chromatography on silica gel with elution using 5:2:0.4 (v/v/v) diethyl ether/ethyl acetate/methanol to yield nucleoside **16** (157 mg; 0.308 mmol) as a white powder in 94% yield: R_f 0.57 [5:2:0.2 diethyl ether/ethyl acetate/methanol]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16 (1H, s, H-6), 6.25 (1H, t, *J* = 6.0 Hz, H-1′), 4.46 (2H, s, -C≡C-C$\underline{H}_2$-), 4.37 (1H, m, H-4′), 3.95 (2H, m, H-5′), 3.78 (1H, m, H-3′), 2.47 (1H, m, H-2′), 2.04 (1H, m, H-2′), 0.93, 0.88 (18H, 2s, tBu-3′ and tBu-5′), 0.14, 0.13, 0.065, 0.059 (12H, 4s, $(C\underline{H}_3)_2$Si-3′ and $(C\underline{H}_3)_2$Si-5′); ESI-MS (negative ion mode) *m/z*, found = 508.4, calculated for $[(M-H)^-]$ = 508.3.

5-(3-Acetoxypropyn-1-yl)-N4-acetyl-5′,3′-O-di-tert-butyldimethylsilyl-2′-deoxycytidine (**17**). Acetic anhydride (1.2 mL; 12.7 mmol; 27 eq) was added slowly to a solution of nucleoside **16** (242 mg; 0.475 mmol) in dry pyridine. The reaction was stirred for 1.5 h at room temperature and evaporated to dryness. The residue was purified by flash chromatography on silica gel with elution using 2:1 (v/v) hexane/ethyl acetate to yield the nucleoside **17** (160 mg; 0.269 mmol) as a white powder in 57% yield: R_f 0.60 [1 / 2, hexane / ethyl acetate]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.38 (1H, s, H-6), 6.21 (1H, t, *J* = 5.9 Hz, H-1′), 4.86 (2H, s, -C≡C-C$\underline{H}_2$-), 4.35 (1H, m, H-4′), 3.98 (2H, m, H-5′ and H-3′), 3.78 (1H, dd, *J* = 2.3 Hz, *J* = 11.6 Hz, H-5"), 2.69 (3H, s, N^4-C(=O)C$\underline{H}_3$), 2.59 - 2.51 (1H, 2dd, *J* = 4.5 Hz, *J* = 4.8 Hz, H-2′), 2.12 (3H, s, -OC(=O)C$\underline{H}_3$), 2.09 (1H, m, H-2′), 0.92, 0.89 (18H, 2s, tBu-3′ and tBu-5′), 0.13, 0.12, 0.076, 0.067 (12H, 4s, $(C\underline{H}_3)_2$Si-3′ and $(C\underline{H}_3)_2$Si-5′); ESI-MS (positive ion mode) *m/z*: found = 594.4, calculated for $[(M+H)^+]$ = 594.3.

5-(3-Acetoxypropyn-1-yl)-N4-acetyl-2′-deoxycytidine (**18**). Triethylamine trihydrofluoride (255 μL, 1.56 mmol, 6.0 eq) was added slowly to a solution of nucleoside **17** (155 mg, 0.261 mmol) in THF (1.0 mL). The reaction was stirred for 9 h at room temperature and evaporated to dryness. The residue was purified by flash chromatography on silica gel with elution using 2% to 8% methanol in chloroform to yield to yield the nucleoside **18** (70 mg, 0.192 mmol) as a white powder in 74% yield: R_f 0.34 [1:9 methanol/chloroform]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (1H, s, H-6),

6.15 (1H, t, *J* = 5.9 Hz, H-1′), 4.87 (2H, s, -C≡C-C$\underline{H}_2$-), 4.56 (1H, m, H-4′), 4.10 (1H, m, H-3′), 4.02 (1H, dd, *J* = 2.7 Hz, *J* = 11.7 Hz, H-5′), 3.90 (1H, dd, *J* = 2.7 Hz, *J* = 11.7 Hz, H-5"), 2.67 (3H, s, N^4-C(=O)C$\underline{H}_3$), 2.62 - 2.54 (1H, 2dd, *J* = 4.2 Hz, *J* = 5.1 Hz, *J* = 4.5 Hz, H-2′), 2.37 (1H, m, H-2′), 2.14 (3H, s, -OC(=O)C$\underline{H}_3$); ESI-MS (negative ion mode) *m/z*, found = 364.0, calculated for $[(M-H)^-]$ = 364.1.

5-(3-Hydoxypropyn-1-yl)-2-deoxycytidine-5′-triphosphate (**C**$^{\mathbf{PN}}$). Nucleoside **18** (63 mg; 0.172 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 55.4 mg; 0.259 mmol; 1.5 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (1.0 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (33.8 μL; 0.363 mmol; 2.1 eq) was then added dropwise using a micro syringe, and the reaction mixture was stirred at 0°C. After 45 min, *n*-tributylamine (153 μL; 0.642 mmol; 3.7 eq) and *n*-tributylamine pyrophosphate (1.72 mL of a 0.5 M solution in DMF; 0.860 mmol; 5 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). After evaporation, the residue was dissolved in water and washed with diethyl ether. The aqueous layer was evaporated, and the residue was dried well *in vacuo* and then purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The fractions containing 5-(3-acetoxypropyn-1-yl)-2′-deoxycytidine-5′-triphosphate were combined and evaporated under reduced pressure. The residue was purified by reversed-phase MPLC with a linear gradient of 0% to 20% acetonitrile in 10 mM triethylammonium acetate buffer to give the *O*-acetyl nucleotide: ESI-MS (negative ion mode) *m/z*, found = 604.1, calculated for $[(M-H)^-]$ = 604.0. The *O*-acetyl nucleotide was treated with 2 N aqueous ammonia (20 mL), and the reaction mixture was stirred at room temperature for 1 h and then evaporated to dryness. The residue was purified by reversed-phase HPLC (*ϕ*20 × 250 mm) with a linear gradient of 2.1% to 3.5% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7) to give the triphosphate **C**$^{\mathbf{PN}}$ (1.95 μmol) in 1.1% yield starting from **19**: ESI-MS (negative ion mode) *m/z*, found = 520.2, calculated for $[(M-H)^-]$ = 520.0.

(E)-5-(3-Acetamidoprop-1-enyl)-2′-deoxyuridine-5′-triphosphate (**T**$^{\mathbf{AC}}$). Acetic anhydride (10 μL; 106 μmol; 424 eq) and diethyl amine (10 μL; 97 μmol; 388 eq) was added to a solution of the triphosphate **T**$^{\mathbf{AL}}$ (250 nmol) in 50 mM triethylammonium acetate buffer (pH 7; 50 μL). The reaction was stirred for 1 h at room temperature and evaporated to dryness. The residue was purified by reversed-phase HPLC (*ϕ*4.6 × 250 mm) with a linear gradient of 0% to 7% acetonitrile in 50 mM triethylammonium acetate buffer to give the triphosphate **T**$^{\mathbf{AC}}$ (165 nmol) in 66% yield starting from **T**$^{\mathbf{AL}}$: ESI-MS (negative ion mode) *m/z*, found = 564.1, calculated for $[(M-H)^-]$ = 564.0.

(E)-5-(3-Trifluoroacetamidoprop-1-enyl)-2′-deoxyuridine-5′-triphosphate (**T**$^{\mathbf{AF}}$). Ethyl trifluoroacetate (36 μL; 303 μmol; 1010 eq) and triethyl amine (36 μL; 258 μmol; 860 eq) were added to a solution of the triphosphate **T**$^{\mathbf{AL}}$ (300 nmol) in methanol (300 μL). The reaction was stirred for 3 h at room temperature and evaporated to dryness. The residue was dissolved in water

and purified by reversed-phase HPLC (ϕ4.6 × 250 mm) with a linear gradient of 0% to 21% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7) to give the triphosphate $\mathbf{T^{AF}}$ (240 nmol) in 80% yield starting from $\mathbf{T^{AL}}$: ESI-MS (negative ion mode) *m/z*, found = 618.0, calculated for $[(M-H)^-]$ = 618.0.

(E)-5-(3-Acetamidoprop-1-enyl)-2′-deoxycytidine-5′-triphosphate ($\mathbf{C^{AC}}$). Acetic anhydride (10 μL; 106 μmol; 353 eq) and diethyl amine (10 μL; 97 μmol; 323 eq) were added to a solution of the triphosphate C^{AL} (300 nmol) in 50 mM triethylammonium acetate buffer, (pH 7; 47 μL). The reaction was stirred for 1 h at room temperature and evaporated to dryness. The residue was purified by reversed-phase HPLC (ϕ4.6 × 250 mm) with a linear gradient of 0% to 7% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7) to give the triphosphate C^{AC} (214 nmol) in 71% yield starting from $\mathbf{C^{AL}}$: ESI-MS (negative ion mode) *m/z*, found = 562.9, calculated for $[(M-H)^-]$ = 563.0.

(E)-5-(3-Trifluoroacetamidoprop-1-enyl)-2′-deoxycytidine-5′-triphosphate ($\mathbf{C^{AF}}$). Ethyl trifluoroacetate (84 μL; 706 μmol; 2017 eq) and triethyl amine (84 μL; 603 μmol; 1723 eq) were added to a solution of the triphosphate C^{AL} (350 nmol) in methanol (700 μL). The reaction was stirred for 4 h at room temperature and evaporated to dryness. The residue was purified by reversed-phase HPLC (ϕ4.6 × 250 mm) with a linear gradient of 0% to 21% acetonitrile in 50 mM triethylammonium acetate buffer to give the triphosphate $\mathbf{C^{AF}}$ (254 nmol) in 73% yield starting from $\mathbf{C^{AL}}$: ESI-MS (negative ion mode) *m/z*, found = 617.0, calculated for $[(M-H)^-]$ = 617.0.

PCR assays

The PAGE gel images by 6-FAM detection corresponding to Figure 3 were shown in Figure S1.

Kinetic study on polymerase reactions using modified primers/templates/triphosphates

Standing-start experiments were performed using *KOD(exo-)* and *Vent(exo-)* DNA polymerases. Kinetic parameters obtained from the experiment are summarized in Table S1.

Table S1. Kinetic parameters obtained from the standing-start experiments using *KOD(exo-)* DNA polymerase.

Primer/Template	dNTP	K_m (μM)	$(k_{cat})_{rel}$	$(k_{cat}/K_m)_{rel}$	Accuracy
P0/TA	TTP	126	1[a]	1[c]	1[c]
	$\mathbf{T}^{\mathbf{A6}}$	140	0.88[a]	0.79[c]	0.79[c]
P1/TA	TTP	414	0.22[a]	0.066[c]	1[e]
	$\mathbf{T}^{\mathbf{A6}}$	237	0.12[a]	0.058[c]	0.88[e]
P2/TA	TTP	*NA*	*NA*	*NA*	
	$\mathbf{T}^{\mathbf{A6}}$	823	0.016[a]	0.0024[c]	
PA/T0	dATP	12.3	1[b]	1[d]	
PA/T1	dATP	20.0	1.0[b]	0.63[d]	
PA/T2	dATP	38.7	1.3[b]	0.42[d]	
PA/T3	dATP	57.6	0.56[b]	0.12[d]	

dNTP, deoxynucleotide triphosphate; *NA*, not available.

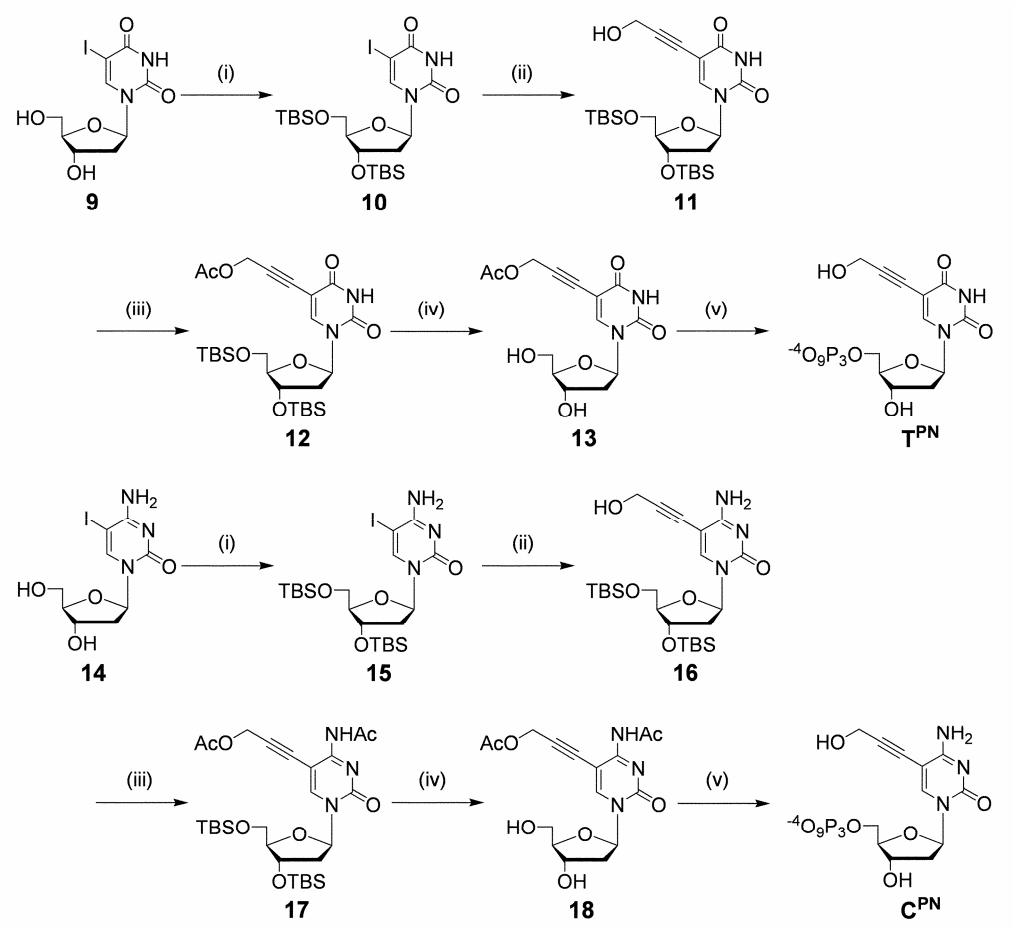
[a]Relative value of the apparent k_{cat} obtained from the experiment using P0, TA, and TTP.

[b]Relative value of the apparent k_{cat} obtained from the experiment using PA, T0, and dATP.

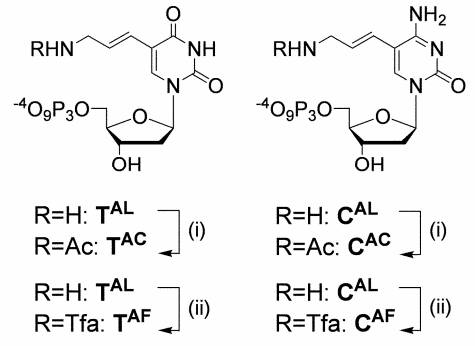
[c]Relative value of the apparent k_{cat}/K_m obtained from the experiment using P0, TA, and TTP.

[d]Relative value of the apparent k_{cat}/K_m obtained from the experiment using PA, T0, and dATP.

[e]Relative value of the apparent k_{cat}/K_m obtained from the experiment using P1, TA, and TTP.



Scheme S1. Synthesis of C5-(3-hydoxypropyn-1-yl) analogues of dUTP and dCTP: (i) *tert*-butyldimethylsilyl chloride, imidazole, DMF, room temperature, 3 h; (ii) propargyl alcohol, tetrakis(triphenylphosphine)palladium(0), triethylamine, CuI, DMF, room temperature, 6 h; (iii) acetic anhydride, pyridine, room temperature, 1 h; (iv) triethylamine trihydrofluoride, THF, room temperature, 2 h; (v) $POCl_3$, *N*,*N*,*N*′,*N*′-tetramethyl-1, 8-naphthalendiamine, trimethyl phosphate, 0ºC, 45 min, followed by *n*-tributylamine pyrophosphate, DMF, room temperature, 1 h.



Scheme S2. Synthesis of C5-(3-acylamidoprop-1-enyl) analogues of dUTP and dCTP: (i) acetic anhydride, triethylammonium acetate buffer/1,4-dioxane, room temperature, 1 h; (ii) ethyl trifluoroacetate, triethylamine, methanol, room temperature, 3–4 h.

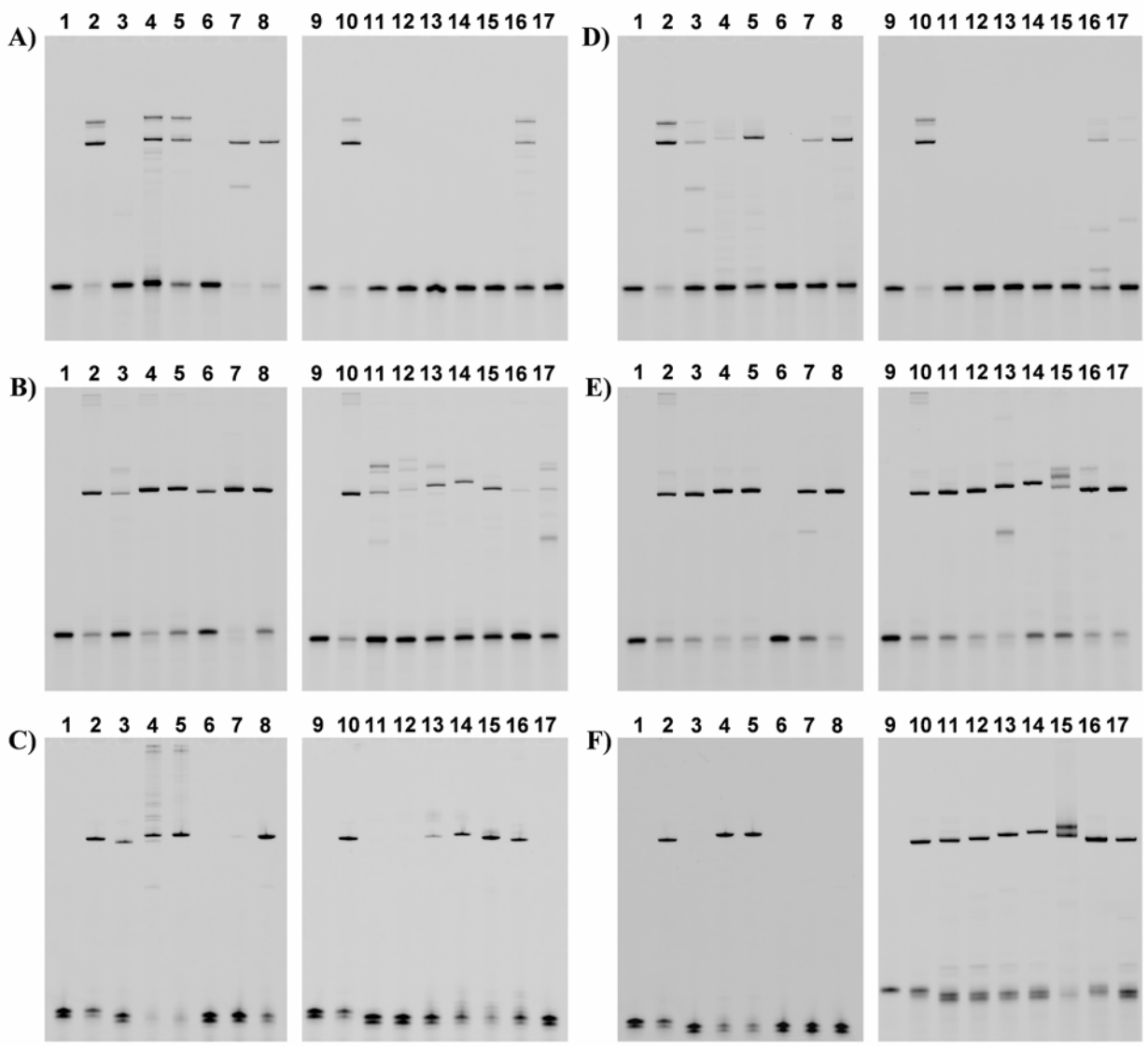


Figure S1. The PAGE gel images by 6-FAM detection corresponding to Figure 3. (A–C) The reaction mixtures containing dATP, dGTP, dCTP, and $\mathbf{T}^{\mathbf{AL}}$ (lane 3), $\mathbf{T}^{\mathbf{AC}}$ (lane 4), $\mathbf{T}^{\mathbf{AF}}$ (lane 5), $\mathbf{T}^{\mathbf{PA}}$ (lane 6), $\mathbf{T}^{\mathbf{PN}}$ (lane 7), $\mathbf{T}^{\mathbf{PR}}$ (lane 8), $\mathbf{T}^{\mathbf{A2}}$ (lane 11), $\mathbf{T}^{\mathbf{A4}}$ (lane 12), $\mathbf{T}^{\mathbf{A6}}$ (lane 13), $\mathbf{T}^{\mathbf{G6}}$ (lane 14), $\mathbf{T}^{\mathbf{ME}}$ (lane 15), $\mathbf{T}^{\mathbf{CN}}$ (lane 16), or $\mathbf{T}^{\mathbf{DH}}$ (lane 17) were separated by electrophoresis on denaturing PAGE. Except for the positive control, the reaction mixtures do not contain natural TTP. The PCR product was generated by the positive control (reaction mixture containing dATP, dGTP, dCTP, and TTP; lanes 2 and 10) but not by the negative control (reaction mixture containing dATP, dGTP, and dCTP; lanes 1 and 9). (D–F) Reaction mixtures containing dATP, dGTP, dCTP, and $\mathbf{C}^{\mathbf{AL}}$ (lane 3), $\mathbf{C}^{\mathbf{AC}}$ (lane 4), $\mathbf{C}^{\mathbf{AF}}$ (lane 5), $\mathbf{C}^{\mathbf{PA}}$ (lane 6), $\mathbf{C}^{\mathbf{PN}}$ (lane 7), $\mathbf{C}^{\mathbf{PR}}$ (lane 8), $\mathbf{C}^{\mathbf{A2}}$ (lane 11), $\mathbf{C}^{\mathbf{A4}}$ (lane 12), $\mathbf{C}^{\mathbf{A6}}$ (lane 13), $\mathbf{C}^{\mathbf{G6}}$ (lane 14), $\mathbf{C}^{\mathbf{ME}}$ (lane 15), $\mathbf{C}^{\mathbf{CN}}$ (lane 16) or $\mathbf{C}^{\mathbf{DH}}$ (lane 17) were separated by electrophoresis on denaturing PAGE. Except for the positive control, the reaction mixtures did not contain natural dCTP. The PCR product was generated by the positive control (reaction mixture containing dATP, dGTP, dCTP, and TTP; lanes 2 and 10) but not by the negative control (reaction mixture containing dATP, dGTP, and TTP; lanes 1 and 9). The thermostable DNA polymerases used were *Taq* (A, D), *Vent(exo-)* (B, E), and *KOD Dash* (C, F).