Supporting Information

1. Chemical syntheses of Cy3- and Cy5-dPxTPs.

2. $^1$H NMR spectrum of Cy3-dPxTP.

3. $^{31}$P NMR spectrum of Cy3-dPxTP.

4. Mass spectrum of Cy3-dPxTP.

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8. Real-time qPCR using a Ds-containing primer and fluorophor-dPxTPs (Figures S1-S3).
1. Chemical syntheses of Cy3- and Cy5-dPxTPs.

Conditions: (a) NH₂-dPxTP in 100 mM NaHCO₃, Na₂CO₃ buffer (pH 8.5), Cy3 or Cy5 Mono NHS ester in DMF, r.t., 12h.

General methods and materials

Reagents and solvents were purchased from standard suppliers and used without further purification. ¹H NMR and ³¹P NMR spectra were recorded on a Bruker (300-AVM) magnetic resonance spectrometer. The triphosphate derivatives were purified with a DEAE-Sephadex A-25 column (300 × 15 mm) and a C18 column (Shiseido). Electrospray ionization mass spectra (ESI-MS) and ESI-TOF MS spectra were recorded on a Waters Micromass ZMD 4000 system equipped with a Waters 2690 LC system and a Micromass (Waters) Q-Tof Ultima global mass spectrometer, respectively. Cy3™ and Cy5™ Mono NHS esters were purchased from GE Healthcare.

1-(2-Deoxy-β-D-ribofuranosyl)-4-[3-(Cy3-carboxamidohexanamido)-1-propynyl]-2-nitropyrrrole 5′-triphosphoric acid (Cy3-dPxTP).

A 0.1 M NaHCO₃-Na₂CO₃ buffer solution (pH 8.6, 500 μl) of 1-(2-deoxy-β-D-ribofuranosyl)-4-[3-(6-aminohexanamido)-1-propynyl]-2-nitropyrrrole
5′-triphosphoric acid (NH$_2$-dPxTP) (8.4 μmol) was reacted with Cy3-Mono NHS ester (6.0 mg, 7.6 μmol) in DMF (300 μl) in the dark at room temperature. After 12 h, 50 mM TEAB (3.0 ml) was added to the reaction mixture. The product (2.7 μmol, 35%) was purified by DEAE Sephadex A-25 column chromatography (1.5 cm x 30 cm, eluted by a linear gradient from 50 mM to 1 M TEAB) and C18 HPLC (eluted by a linear gradient of CH$_3$CN in 100 mM TEAA, pH 7.0).

$^1$H NMR (300 MHz, D$_2$O) δ 8.55 (t, 1H, $J = 13.6$ Hz), 7.90 (t, 2H, $J = 1.7$ Hz), 7.85 (dd, 2H, $J = 2.1$ Hz), 7.39 (dd, 2H, $J = 1.9$, 8.5 Hz), 7.19 (d, 1H, $J = 2.1$ Hz), 6.64 (t, 1H, $J = 5.9$ Hz), 6.39 (dd, 2H, $J = 2.8$, 13.5 Hz), 4.59 (m, 1H), 4.22-4.08 (m, 9H), 3.20 (q, 32H, $J = 7.3$ Hz), 3.07 (t, 2H, $J = 6.5$ Hz), 2.59 (dt, 1H, $J = 1.1$, 13.3 Hz), 2.38 (dt, 1H, $J = 6.2$, 13.8 Hz), 2.27-2.17 (m, 4H), 1.86 (m, 2H), 1.77 (s, 12H), 1.67-1.54 (m, 4H), 1.42-1.25 (m, 56H). $^{31}$P NMR (121 MHz, D$_2$O) δ -8.65 (bs, 1P), -10.72 (d, 1P, $J = 19.7$ Hz), -22.32 (t, 1P, $J = 20.4$ Hz). ESI-MS for C$_{49}$H$_{63}$N$_6$O$_{22}$P$_3$S$_2$ Calcd. 1247.28 (M+H)$^+$, Found 1247.28 (M+H)$^+$, Calcd. 1348.40 (M+H/TEA)$^+$, Found 1348.55 (M+H/TEA)$^+$, Calcd. 1449.52 (M+H/2TEA)$^+$, Found 1449.39 (M+H/2TEA)$^+$, Calcd. 1245.28 (M-H)$^-$, Found 1244.91 (M-H)$^-$.

UV (10 mM sodium phosphate buffer, pH 7.0) $\varepsilon$ 293 nm = 18,400, $\varepsilon$ 366 nm = 11,600, $\varepsilon$ 550 nm = 180,000.

1-(2-Deoxy-β-D-ribofuranosyl)-4-[3-(Cy5-carboxamidohexanamido)-1-propynyl]-2-nitropyrrrole 5′-triphosphoric acid (Cy5-dPxTP).

In a 0.1 M NaHCO$_3$-Na$_2$CO$_3$ buffer solution (pH 8.6, 500 μl), 1-(2-deoxy-β-D-ribofuranosyl)-4-[3-(6-aminohexanamido)-1-propynyl]-2-nitropyrrrole 5′-triphosphoric acid (NH$_2$-dPxTP) (7.6 μmol) was reacted with Cy5-Mono NHS ester (5.0 mg, 6.3 μmol) in DMF (300 μl) in the dark at room temperature. After 12 h, 50 mM TEAB (3.0 ml) was added to the reaction mixture. The product (3.3 μmol, 52%) was purified by DEAE Sephadex A-25 column chromatography (1.5 cm x 30 cm, eluted by a linear gradient from 50
mM to 1 M TEAB) and C18 HPLC (eluted by a linear gradient of CH₃CN in 100 mM TEAA, pH 7.0).

¹H NMR (300 MHz, D₂O) δ 8.05 (dt, 2H, J = 6.1, 13.0 Hz), 7.86-7.76 (m, 5H), 7.33 (dd, 2H, J = 4.9, 8.4 Hz), 7.20 (d, 1H, J = 1.9 Hz), 6.64 (t, 1H, J = 6.0 Hz), 6.56 (t, 1H, J = 12.4 Hz), 6.30 (dd, 2H, J = 13.9, 16.5 Hz), 4.57 (m, 1H), 4.21-4.05 (m, 9H), 3.20 (q, 23H, J = 7.3 Hz), 3.07 (t, 2H, J = 6.5 Hz), 2.59 (dt, 1H, J = 6.1, 13.6 Hz), 2.39 (dt, 1H, J = 6.2, 13.6 Hz), 2.22 (dt, 4H, J = 7.4, 16.4 Hz), 1.84 (m, 2H), 1.70 (s, 12H), 1.59 (m, 4H), 1.41-1.25 (m, 45H). ³¹P NMR (121 MHz, D₂O) δ -10.23 (d, 1P, J = 19 Hz), -10.79 (d, 1P, J = 17.2 Hz), -22.60 (t, 1P, J = 18.3 Hz).

ESI-TOF MS for C₅₁H₆₇N₆O₂₂P₃S₂ Calcd. 1273.30 (M+H)⁺, Found 1273.32 (M+H)⁺, Calcd. 1374.42 (M+H/TEA)⁺, Found 1374.44 (M+H/TEA)⁺, Calcd. 1475.54 (M+H/2TEA)⁺, Found 1475.55 (M+H/2TEA)⁺. UV (10 mM sodium phosphate buffer, pH 7.0) ε 648 nm = 280,000.
2. $^1$H NMR spectrum of Cy3-dPxTP.

$^1$H NMR (300 MHz, D$_2$O) spectrum of Cy3-dPxTP.

3. $^{31}$P NMR spectrum of Cy3-dPxTP.

$^{31}$P NMR (121 MHz, D$_2$O) spectrum of Cy3-dPxTP.
4. Mass spectrum of Cy3-dPxTP.

Calcd.: 1247.28 (M+H)+
Found : 1247.28 (M+H)+
Calcd.: 1348.40 (M+H/TEA)+
Found : 1348.55 (M+H/TEA)+
Calcd.: 1449.52 (M+H/2TEA)+
Found : 1449.39 (M+H/2TEA)+

5. $^1$H NMR spectrum of Cy5-dPxTP.

$^1$H NMR (300 MHz, D$_2$O) spectrum of Cy5-dPxTP.
6. $^{31}$P NMR spectrum of Cy5-dPxTP.

$^{31}$P NMR (121 MHz, D$_2$O) spectrum of Cy5-dPxTP.

7. Mass spectrum of Cy5-dPxTP.

Calcd.: 1273.30 (M+H)$^+$
Found : 1273.32 (M+H)$^+$
Calcd.: 1374.42 (M+H/TEA)$^+$
Found : 1374.44 (M+H/TEA)$^+$
Calcd.: 1475.54 (M+H/2TEA)$^+$
Found : 1475.55 (M+H/2TEA)$^+$

ESI-TOF MS (positive) spectra of Cy5-dPxTP.
8. Real-time qPCR using a Ds-containing primer and fluorophor-dPxTPs (Figures S1-S3)

Figure S1. Amplification plots (A), dissociation curves (B), and linear standard curve analysis (C) of a 10-fold dilution series from 3 to $3 \times 10^6$ copies of a 98-bp double-stranded DNA, using a Ds-containing primer and Cy3-dPxTP. The cycle threshold values (Ct) obtained from panel B were plotted against the log of the template copies.

Figure S2. Amplification plots (A), dissociation curves (B), and linear standard curve analysis (C) of a 10-fold dilution series from 3 to $3 \times 10^6$ copies of a 98-bp double-stranded DNA, using a Ds-containing primer and Cy5-dPxTP. The cycle threshold values (Ct) obtained from panel B were plotted against the log of the template copies.
Figure S3. Amplification plots (A), dissociation curves (B), and linear standard curve analysis (C) of a 10-fold dilution series from 3 to $3 \times 10^6$ copies of a 98-bp double-stranded DNA, using a Ds-containing primer and FAM-dPxTP. The cycle threshold values (Ct) obtained from panel B were plotted against the log of the template copies.