Electronic Supporting Information

Synthesis of γ-Labeled Nucleoside 5'-Triphosphates using Click Chemistry

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1 Materials and Methods

Nucleoside triphosphates were purchased from JENA BIOSCIENCE as 100 mM solutions (for the general procedure 1, p. 3) or as the sodium salts from SIGMA-ALDRICH (for the general procedure 2, p. 4) in the case of dTTP and ATP. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) and 1-aminobut-3-yne were purchased from SIGMA-ALDRICH. DMF and THF used for the synthesis were of at least 99.5% purity from ACROS ORGANICS. $CuSO_4$ (≥ 99% purity) was ordered in an anhydrous form from FLUKA and sodium ascorbate (99% purity) was bought from ABCR. 5-Carboxytetramethylrhodamine azide **c** (5-TAMRA azide) was purchased from BASECLICK. All other chemicals that were needed for the synthesis of the fluorophore azides were purchased from SIGMA-ALDRICH, FLUKA, ABCR or ACROS ORGANICS and used without further purification.

Sensitive compounds like triphosphate derivatives were freeze-dried using a lyophylizer (CHRIST alpha 2-4 LD). All other solutions were concentrated *in vacuo* on a HEIDOLPH rotary evaporator with a Vario PC2001 diaphragm pump by VACUUBRAND. Chromatographic purification of products was accomplished by reversed-phase high-performance liquid chromatography (RP-HPLC, p. 3) or flash column chromatography on MERCK Geduran Si 60 (40–63 μm) silica gel (normal phase). Thin layer chromatography (TLC) was performed on MERCK 60 (silica gel F_{254}) plates.

^{1}H, $^{13}C\{^{1}H\}$ and ^{31}P NMR spectra were recorded in deuterated solvents on BRUKER ARX 300, VARIAN VXR400S, VARIAN Inova 400 and BRUKER AMX 600 spectrometers and calibrated to the residual solvent peak if possible. As an external reference tetramethylsilane was applied for ^{13}C NMR measurements in D_2O and H_3PO_4 was used for ^{31}P NMR spectra. The chemical shifts (δ) are given in ppm, the coupling constants (J) in Hz. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad and combinations of these. For assignement of the structures additional 2D NMR spectra (COSY, HSQC, HMBC) were measured. Please note that the numbering in the assignements does not follow IUPAC rules and neither residual solvent signals (D_2O 4.79 ppm) nor triethylammonium salt signals (buffer from HPLC purification: quartet around 3.3 ppm, singlet around 2.0 ppm and triplet around 1.3 ppm) have been assigned for clarity. Note that signals labeled with *, **, *** or **** are interchangeable.

High resolution electronspray ionization mass spectra (HRESIMS) were recorded on a THERMO FINNIGAN LTQ-FT (ESI-FTICR), high resolution electron impact ionization mass spectra (HREIMS) were recorded on a THERMO FINNIGAN MAT 95. IR measurements were performed with neat compounds on a PERKIN ELMER Spectrum BX FT-IR spectrometer

equipped with a diamond-ATR (Attenuated Total Reflection) setup. The pH-values were adjusted using an MP 220 pH-meter (METTLER TOLEDO). UV-Vis measurements were either carried out on a JASCO V-650 (1 mL sample volume), a VARIAN Cary 5000 (1.4 mL sample volume) or a NanoDrop ND-1000 spectrophotometer from THERMO SCIENTIFIC (1.5 µL sample volume). For fluorescence measurements a JASCO FP-750 or a VARIAN Cary Eclipse spectrofluorometer was used.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Analytical and semipreparative RP-HPLC was performed on an analytical HPLC WATERS Alliance (2695 Separation Module, 2996 Photodiode Array Detector) equipped with the column Nucleosil 120-3 C18 from MACHEREY NAGEL. Using a flow of 0.5 mL/min a gradient of 0 → 20% B in 45 min was applied for the reaction control of γ-alkyne labeled NTPs, a gradient of 0 → 40% B in 25 min and 40 → 60% from 25-45 min was used for the click reactions. Preparative RP-HPLC was performed on a HPLC WATERS Breeze (2487 Dual λ Array Detector, 1525 Binary HPLC Pump) equipped with the columns Nucleosil 100-7 C18, VP 250/10 C18 or VP 250/32 C18 from MACHEREY NAGEL. Using a flow of 5 mL/min a gradient of 0 → 10% B was applied for γ-alkyne labeled nucleotides of cytidine (**2**, **6**), 0 → 15% B for all other γ-alkyne labeled nucleotides (**1**, **3**-**5**, **7**-**8)** and 0 → 20% B was applied for coumarin labeled NTPs (**4a**, **5a**). As an eluent, mixtures of 0.1 M triethylammonium acetate in H_2O (buffer A) and in 80% (w/w) MeCN (buffer B) were used.

2 Synthesis of γ-Alkyne Labeled Nucleoside Triphosphates

The synthesis of γ-alkyne labeled nucleoside triphosphates was generally applicable for all nucleotides **1**-**8** (only one minor adjustment was necessary in the case of dGTP) and can be performed in two different ways. The first protocol allows to carry out the synthesis in a biochemistry lab while the second protocol is suitable for standard chemical synthesis with common equipment.

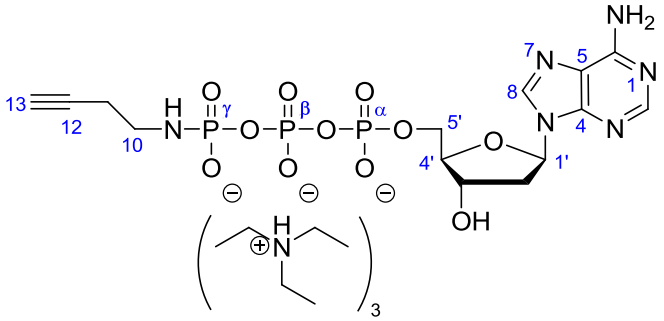
General Procedure 1: In a 1.5 mL reaction tube 300 µL of the nucleoside triphosphate solution (100 mM, 30 µmol, 1.0 eq) were mixed with 300 µL EDC·HCl solution (500 mM in ddH_2O, pH = 7.5, 150 µmol, 5.0 eq) and incubated at 25 °C, 1200 rpm (in a thermomixer from EPPENDORF) for 7 min. Then, 600 µL of 1-aminobut-3-yne solution (55 mM in DMF, 33 µmol, 1.1 eq) were added and incubated for 4.5 h at 25 °C, 1200 rpm. DMF was removed by extraction with $CHCl_3$ (3 x 600 µL) and the aqueous layer (aprox. 600 µL) was transferred to a 15 mL reaction tube. Precipitation was achieved by addition of 180 µL NaCl (3 M in

ddH_2O) and 4 mL of abs. EtOH. After vortexing, the mixture was cooled to -80 °C for 1 h (alternatively -20 °C overnight). After centrifugation (4000 x g, 10 min) the supernatant was removed, the colorless solid was redissolved in H_2O (500 µL) and then dried by lyophylization. Purification by preparative RP-HPLC yielded γ-alkyne labeled nucleotides with >95% purity (according to analytical RP-HPLC).

General Procedure 2: All reactions were performed with magnetic stirring. EDC·HCl (5.0 eq) was dissolved in ddH_2O, the nucleoside triphosphate (1.0 eq) was added and the pH was adjusted to 7.5 with aqueous NaOH (0.1 M) if necessary. After stirring at rt for 5 min 1-aminobut-3-yne (55 mM in DMF, 1.1 eq) was added. After complete consumption of the starting material (reactions were monitored by RP-HPLC), the solvent was removed *in vacuo* and the colorless crude product was purified by preparative RP-HPLC to yield the γ-alkyne labeled nucleotides.

Synthesis of γ-Alkyne Labeled dNTPs

Synthesis of γ-*N*-(But-3-yn-1-ylamido)-2'-deoxyadenosine-5'-triphosphate (1)



The synthesis was performed with a 100 mM dATP solution (300 µL, 30.0 µmol) as described in the general procedure 1 (p. 3). RP-HPLC purification afforded the tris-triethylammonium salt of γ-alkyne labeled dATP **1** (19.9 mg, 23.5 µmol, 78%) as a colorless solid after lyophylization.

^{1}H NMR (400 MHz, D_2O): δ (ppm) = 8.56 (s, 1H, 2-H), 8.31 (s, 1H, 8-H), 6.55 (t, ^{3}J = 4.0 Hz, 1H, 1'-H), 4.84–4.82 (m, 1H, 3'-H), 4.33–4.25 (m, 1H, 4'-H), 4.33–4.10 (m, 2H, 5'-H), 3.10–2.99 (m, 2H, 10-H), 2.89–2.83 (m, 1H, 2'-H), 2.67–2.60 (m, 1H, 2'-H), 2.36 (td, ^{3}J = 6.4 Hz, ^{4}J = 2.6 Hz, 2H, 11-H), 2.31 (t, ^{4}J = 2.4 Hz, 1H, 13-H).

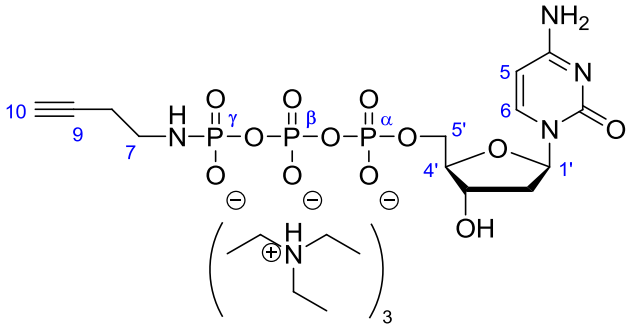
^{13}C NMR (102 MHz, D_2O): δ (ppm) = 155.3 (6-C), 152.5 (2-C), 148.7 (4-C), 140.0 (8-C), 118.5 (5-C), 85.7 (d, $^{3}J_{PC}$ = 9.2 Hz, 4'-C), 83.6 (1'-C), 83.2 (12-C), 72.3 (3'-C), 71.0 (13-C), 65.4 (d, $^{2}J_{PC}$ = 7.1 Hz, 5'-C), 40.4 (10-C), 39.0 (2'-C), 20.8 (d, $^{3}J_{PC}$ = 8.2, 11-C).

^{31}P NMR (162 MHz, D_2O): δ (ppm) = -2.00 (d, ^{2}J = 19.4 Hz, γ-P), -11.58 (d, ^{2}J = 19.6 Hz, α-P), -23.15 (t, ^{2}J = 21.6 Hz, β-P).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3177, 2982, 2884, 2610, 2471, 1650, 1597, 1570, 1473, 1397, 1330, 1294, 1212, 1083, 991, 901, 836, 799, 723.

HRESIMS: calculated for $C_{14}H_{20}N_6O_{11}P_3^-$ [M-H]$^-$: 541.0408, observed: 541.0399.

Synthesis of γ-*N*-(But-3-yn-1-ylamido)-2'-deoxycytidine-5'-triphosphate (2)



The synthesis was performed with a 100 mM dCTP solution (300 µL, 30.0 µmol) as described in the general procedure 1 (p. 3). RP-HPLC purification afforded the tris-triethylammonium salt of γ-alkyne labeled dCTP **2** (18.9 mg, 23.0 µmol, 76%) as a colorless solid after lyophylization.

^{1}H NMR (400 MHz, D_2O): δ = 8.04 (d, ^{3}J = 7.53 Hz, 1H, 6-H), 6.37 (t, ^{3}J = 6.92 Hz, 1H, 1'-H), 6.19 (d, ^{3}J = 7.57 Hz, 1H, 5-H), 4.67–4.64 (m, 1H, 3'-H), 4.25–4.24 (m, 3H, 4'-H, 5'-H_2), 3.12–3.06 (m, 2H, 7-H), 2.69–2.65 (m, 1H, 2'-H), 2.47–2.46 (m, 1H, 2'-H), 2.45–2.40 (m, 2H, 8-H), 2.38–2.36 (m, 1H, 10-H).

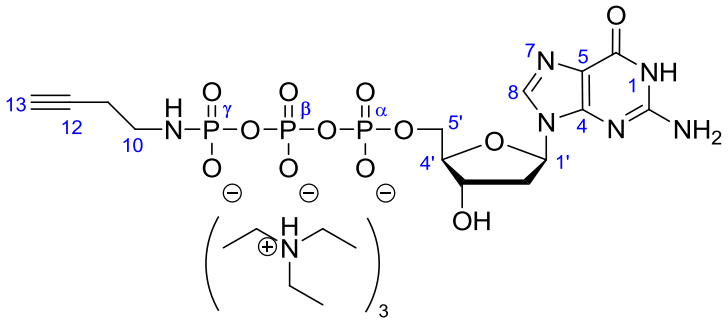
^{13}C NMR (102 MHz, D_2O): δ = 166.0 (4-C), 157.3 (2-C), 141.7 (6-C), 96.5 (1'-C), 85.8 (5-C), 85.4 (d, $^{3}J_{PC}$ = 9.6 Hz, 4'-C), 83.3 (9-C), 70.4 (3'-C), 70.1 (10-C), 65.0 (d, $^{2}J_{PC}$ = 5.6 Hz, 5'-C), 40.4 (7-C), 39.4 (2'-C), 20.7 ($^{3}J_{PC}$ = 8.7 Hz, 8-C).

^{31}P NMR (162 MHz, D_2O): δ = -1.97 (d, ^{2}J = 20.7 Hz, γ-P), -11.63 (d, ^{2}J = 19.5 Hz, α-P), -23.10 (t, ^{2}J = 20.6 Hz, β-P).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3276, 2973, 2937, 2877, 2437, 1692, 1647, 1524, 1488, 1385, 1223, 1057, 995, 902, 840, 809, 787, 767.

HRESIMS: calculated for $C_{13}H_{20}N_4O_{12}P_3^-$ $[M-H]^-$: 517.0296, observed: 517.0296.

Synthesis of γ-*N*-(But-3-yn-1-ylamido)-2'-deoxyguanosine-5'-triphosphate (3)



The synthesis was performed with a 100 mM dGTP solution (300 µL, 30.0 µmol) as described in the general procedure 1 (p. 3) except that after EDC·HCl incubation 200 µL of extra H_2O were added to prevent precipitation upon addition of the DMF solution. RP-HPLC purification afforded the tris-triethylammonium salt of γ-alkyne labeled dGTP **3** (20.3 mg, 23.6 µmol, 78%) as a colorless solid after lyophylization.

^{1}H NMR (400 MHz, D_2O): δ (ppm) = 8.11 (s, 1H, 8-H), 6.33 (t, ^3J = 4.6 Hz, 1H, 1'-H), 4.81–4.78 (m, 1H, 3'-H), 4.28–4.25 (m, 1H, 4'-H), 4.20 (t, ^3J = 3.6 Hz, 2H, 5'-H), 3.10–3.01 (m, 2H, 10-H), 2.89–2.83 (m, 1H, 2'-H), 2.53–2.48 (m, 1H, 2'-H), 2.37–2.34 (m, 2H, 11-H), 2.31–2.29 (m, 1H, 13-H).

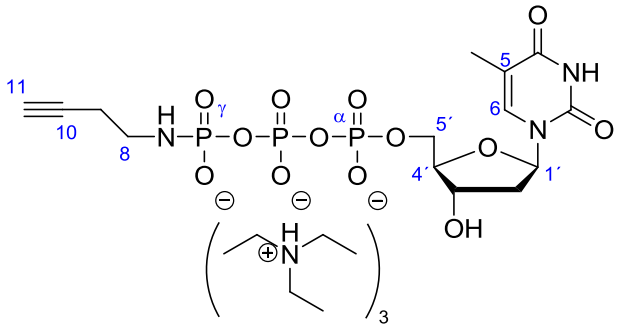
^{13}C NMR (150.6 MHz, D_2O): δ (ppm) = 158.9 (6-C), 153.8 (2-C), 151.4 (4-C), 137.8 (8-C), 116.3 (5-C), 85.6 (d, $^3J_{PC}$ = 9.2 Hz, 4'-C), 83.6 (1'-C), 83.3 (12-C), 71.2 (3'-C), 70.0 (13-C), 65.3 (d, $^2J_{PC}$ = 5.7 Hz, 5'-C), 40.4 (10-C), 38.2 (2'-C), 20.7 (d, $^3J_{PC}$ = 8.4 Hz, 11-C).

^{31}P NMR (162 MHz, D_2O): δ (ppm) = -1.94 (d, ^2J = 21.6 Hz, γ-P), -11.51 (d, ^2J = 19.8 Hz, α-P), -23.08 (t, ^2J = 20.3 Hz, β-P).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3212, 2986, 2945, 2694, 2497, 1678, 1635, 1602, 1568, 1531, 1478, 1454, 1397, 1358, 1320, 1225, 1085, 1060, 996, 911, 837, 783, 734, 677.

HRESIMS: calculated for $C_{14}H_{20}N_6O_{13}P_3^-$ $[M-H]^-$: 557.0358, observed: 557.0346.

Synthesis of γ-*N*-(But-3-yn-1-ylamido)-2'-deoxythymidine-5'-triphosphate (4)



The synthesis was performed with dTTP sodium salt (13.8 mg, 25.0 µmol, 1.0 eq), EDC·HCl (24.0 mg, 125.0 µmol, 5.0 eq) and 1-aminobut-3-yne (1.9 mg, 28.0 µmol, 1.1 eq) in ddH_2O (0.5 mL) within 4.5 h as described in the general procedure 2 (p. 4). RP-HPLC purification afforded the tris-triethylammonium salt of γ-alkyne labeled dTTP **4** (17.5 mg, 21.0 µmol, 84%) as a colorless solid after lyophylization.

^{1}H NMR (400 MHz, D_2O): δ (ppm) = 7.80 (d, ^3J = 7.7 Hz, 1H, 6-H), 6.37 (t, ^3J = 6.9 Hz, 1H, 1'-H), 4.70–4.66 (m, 1H, 3'-H), 4.26–4.18 (m, 3H, 4'-H, 5'-H), 3.11–3.05 (m, 2H, 8-H), 2.44–2.33 (m, 5H, 2'-H, 9-H, 11-H), 1.96 (s, 3H, 7-H).

^{13}C NMR (102 MHz, D_2O): δ (ppm) = 166.5 (4-C), 151.7 (2-C), 137.3 (6-C), 111.7 (5-C), 85.4 (d, $^3J_{PC}$ = 9.1 Hz, 4'-C), 84.8 (1'-C), 83.4 (10-C), 70.7 (3'-C), 70.1 (11-C), 65.3 (d, $^2J_{PC}$ = 5.7 Hz, 5'-C), 40.4 (8-C), 38.5 (2'-C), 20.8 (d, $^3J_{PC}$ = 8.6 Hz, 9-C), 11.6 (7-C).

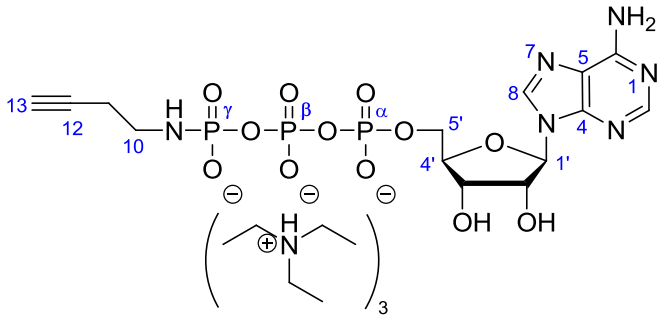
^{31}P NMR (162 MHz, D_2O): δ (ppm) = -1.94 (d, ^2J = 20.6 Hz, γ-P), -11.85 (dd, ^2J = 19.9 Hz, ^4J = 3.8 Hz, α-P), -23.15 (t, ^2J = 20.2 Hz, β-P).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3250, 2914, 2847, 2692, 2493, 1660, 1463, 1399, 1223, 1060, 991, 906, 814, 764, 717.

HRESIMS: calculated for $C_{14}H_{21}N_3O_{13}P_3^-$ $[M-H]^-$: 532.0293, observed: 532.0291.

Synthesis of γ-Alkyne Labeled NTPs

Synthesis of γ-*N*-(But-3-yn-1-ylamido)adenosine-5'-triphosphate (5)



The synthesis was performed with ATP disodium salt (27.6 mg, 50.0 µmol, 1.0 eq), EDC·HCl (48.0 mg, 250.0 µmol, 1.0 eq) and 1-aminobut-3-yne (3.8 mg, 55.0 µmol, 1.1 eq) in ddH_2O (1.0 mL) within 3 h as described in the general procedure 2 (p. 4). RP-HPLC purification afforded the tris-triethylammonium salt of γ-alkyne labeled ATP **5** (36.7 mg, 43.0 µmol, 85%) as a colorless solid after lyophylization.

^{1}H NMR (400 MHz, D_2O): δ (ppm) = 8.60 (s, 1H, 2-H), 8.31 (s, 1H, 8-H), 6.18 (d, ^3J = 6.0 Hz, 1H, 1'-H), 4.84–4.80 (m, 1H, 2'-H), 4.62–4.60 (m, 1H, 3'-H), 4.44–4.43 (m, 1H, 4'-H), 4.30–4.26 (m, 2H, 5'-H), 3.07–3.03 (m, 2H, 10-H), 2.38–2.26 (m, 3H, 11-H, 13-H).

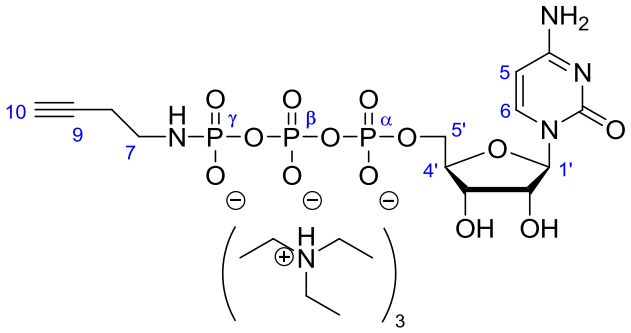
^{13}C NMR (102 MHz, D_2O): δ (ppm) = 155.4 (6-C), 152.7 (2-C), 149.3 (4-C), 140.0 (8-C), 118.4 (5-C), 86.6 (1'-C), 84.1 (d, $^3J_{PC}$ = 9.3 Hz, 4'-C), 83.3 (12-C), 74.2 (2'-C), 70.4 (3'-C), 70.3 (13-C), 65.2 (d, $^2J_{PC}$ = 6.1 Hz, 5'-C), 40.4 (10-C), 20.8 (d, $^3J_{PC}$ = 8.5 Hz, 11-C).

^{31}P NMR (162 MHz, D_2O): δ (ppm) = -1.94 (d, ^2J = 20.7 Hz, γ-P), -11.58 (d, ^2J = 19.6 Hz, α-P), -23.03 (t, ^2J = 20.2 Hz, β-P).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3189, 2982, 2680, 2489, 1645, 1600, 1571, 1475, 1399, 1331, 1297, 1215, 1060, 990, 897, 799.

HRESIMS: calculated for $C_{14}H_{20}N_6O_{12}P_3^-$ $[M-H]^-$: 557.0358, observed: 557.0355.

Synthesis of γ-*N*-(But-3-yn-1-ylamido)cytidine-5'-triphosphate (6)



The synthesis was performed with a 100 mM CTP solution (300 µL, 30.0 µmol) as described in the general procedure 1 (p. 3). RP-HPLC purification afforded the tris-triethylammonium salt of γ-alkyne labeled CTP **6** (19.2 mg, 23.0 µmol, 76%) as a colorless solid after lyophylization.

^{1}H NMR (400 MHz, D_2O): δ (ppm) = 8.08 (d, ^{3}J = 8.0 Hz, 1H, 6-H), 6.22 (d, ^{3}J = 8.0 Hz, 1H, 5-H), 6.03 (d, ^{3}J = 4.4 Hz, 1H, 1'-H), 4.43–4.41 (m, 1H, 3'-H), 4.37–4.34 (m, 1H, 2'-H), 4.32-4.25 (m, 3H, 4'-H, 5'-H), 3.08 (dt, $^{2}J_{HP}$ = 10.4 Hz, ^{3}J = 6.8 Hz, 2H, 7-H), 2.41 (td, ^{3}J = 6.8 Hz, ^{4}J = 2.6 Hz, 2H, 8-H), 2.37 (t, ^{4}J = 2.6 Hz, 1H, 10-H).

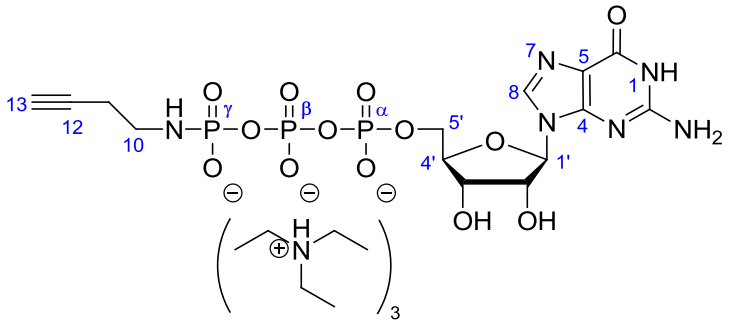
^{13}C NMR (102 MHz, D_2O): δ (ppm) = 165.6 (4-C), 157.2 (2-C), 141.6 (6-C), 96.6 (5-C), 89.0 (1'-C), 83.3 (9-C), 82.7 (d, $^{3}J_{PC}$ = 9.6 Hz, 4'-C), 74.2 (2'-C), 70.0 (10-C), 69.0 (3'-C), 64.4 (d, $^{2}J_{PC}$ = 5.6 Hz, 5'-C), 40.4 (7-C), 20.8 (d, $^{3}J_{PC}$ = 8.7 Hz, 8-C).

^{31}P NMR (162 MHz, D_2O): δ (ppm) = -1.98 (d, ^{2}J = 20.7 Hz, γ-P), -11.63 (d, ^{2}J = 19.5 Hz, α-P), -23.09 (t, ^{2}J = 20.5 Hz, β-P).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3248, 2994, 2502, 1645, 1489, 1398, 1285, 1220, 1109, 1061, 1011, 903, 902, 787.

HRESIMS: calculated for $C_{13}H_{20}N_4O_{13}P_3^-$ $[M-H]^-$: 533.0245, observed: 533.0233.

Synthesis of γ-*N*-(But-3-yn-1-ylamido)guanosine-5'-triphosphate (7)



The synthesis was performed with a 100 mM GTP solution (300 µL, 30.0 µmol) as described in the general procedure 1 (p. 3). RP-HPLC purification afforded the tris-triethylammonium salt of γ-alkyne labeled GTP **7** (22.8 mg, 26.0 µmol, 86%) as a colorless solid after lyophylization.

^{1}H NMR (400 MHz, D_2O): δ (ppm) = 8.15 (s, 1H, 8-H), 5.94 (d, ^{3}J = 6.4 Hz, 1H, 1'-H), 4.87–4.82 (m, 1H, 2'-H), 4.61–4.57 (m, 1H, 3'-H), 4.39–4.35 (m, 1H, 4'-H), 4.30–4.20 (m, 2H, 5'-H), 3.09–3.01 (m, 2H, 10-H), 2.40–2.34 (m, 2H, 11-H), 2.33–2.31(m, 1H, 13-H).

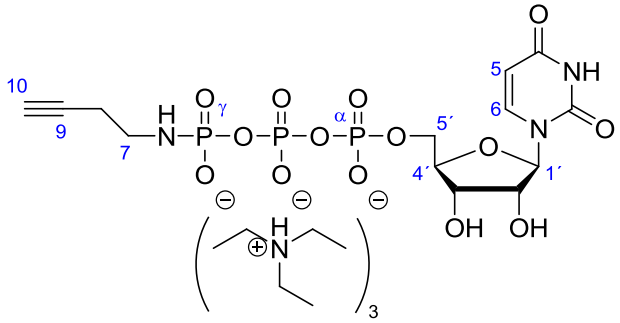
^{13}C NMR (D_2O, 102 MHz): δ (ppm) = 159.0 (6-C), 154.0 (2-C), 151.8 (4-C), 137.7 (8-C), 116.3 (5-C), 86.7 (1'-C), 83.9 (d, $^{3}J_{PC}$ = 9.2 Hz, 4'-C), 82.9 (12-C), 73.4 (2'-C), 70.3 (3'-C and 13-C), 65.2 (d, $^{2}J_{PC}$ = 5.6 Hz, 5'-C), 40.3 (10-C), 20.7 (d, $^{2}J_{PC}$ = 8.5 Hz, 11-C).

^{31}P NMR (162 MHz, D_2O): δ (ppm) = -1.97 (d, ^{2}J = 20.6 Hz, γ-P), -11.64 (d, ^{2}J = 19.6 Hz, α-P), -23.12 (t, ^{2}J = 20.2 Hz, β-P).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3301, 2986, 2948, 2632, 2491, 1678, 1567, 1532, 1477, 1454, 1398, 1223, 1116, 1061, 1011, 912, 837, 810, 783.

HRESIMS: calculated for $C_{14}H_{20}N_6O_{13}P_3^-$ $[M-H]^-$: 573.0307, observed: 573.0307.

Synthesis of γ-*N*-(But-3-yn-1-ylamido)uridine-5'-triphosphate (8)



The synthesis was performed with a 100 mM UTP solution (300 µL, 30.0 µmol) as described in the general procedure 1 (p. 3). RP-HPLC purification afforded the tris-triethylammonium salt of γ-alkyne labeled UTP **8** (17.6 mg, 21.0 µmol, 70%) as a colorless solid after lyophylization.

^{1}H NMR (400 MHz, D_2O): δ (ppm) = 8.02 (d, ^{3}J = 7.7 Hz, 1H, 6-H), 6.02 (d, ^{3}J = 7.6 Hz, 1H, 5-H), 6.02–5.99 (m, 1H, 1'-H), 4.46–4.41 (m, 2H, 3'-H, 2'-H), 4.33–4.29 (m, 1H, 4'-H), 4.28–4.25 (m, 2H, 5'-H), 3.12–3.05 (m, 2H, 7-H), 2.44–2.40 (m, 2H, 8-H), 2.38–2.37 (m, 1H, 10-H).

^{13}C NMR (102 MHz, D_2O): δ (ppm) = 166.1 (4-C), 151.8 (2-C), 141.7 (6-C), 102.7 (5-C), 88.1 (1'-C), 83.3 (d, $^{3}J_{PC}$ = 9.8 Hz, 4'-C), 83.2 (9-C), 73.7 (2'-C), 70.1 (10-C), 69.6 (3'-C), 64.8 (d, $^{2}J_{PC}$ = 5.6 Hz, 5'-C), 40.4 (7-C), 20.8 (d, $^{3}J_{PC}$ = 8.2 Hz, 8-C).

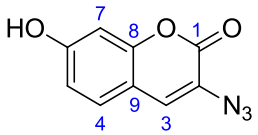
^{31}P NMR (162 MHz, D_2O): δ (ppm) = -1.95 (d, ^{2}J = 21.0 Hz, γ-P), -11.65 (dd, ^{2}J = 19.4 Hz, ^{4}J = 3.8 Hz, α-P), -23.10 (t, ^{2}J = 21.0 Hz, β-P).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3259, 2988, 2690, 2504, 1680, 1463, 1390, 1220, 1009, 902, 813, 764.

HRESIMS: calculated for $C_{13}H_{20}N_3O_{14}P_3^-$ [M-H]$^-$: 534.0085, observed: 534.0074.

3 Synthesis of Fluorophore Azides

Synthesis of 3-Azido-7-hydroxy-2*H*-chromen-2-one (a)



The synthesis was performed according to the procedure of *Sivakumar et al.*.[1]

^{1}H NMR (400 MHz, DMSO): δ (ppm) = 10.52 (s, 1H, OH), 7.60 (s, 1H, 3-H), 7.48 (d, ^{3}J = 8.5 Hz, 1H, 4-H), 6.81 (dd, ^{3}J = 8.5 Hz, ^{4}J = 2.3 Hz, 1H, 5-H), 6.76 (d, ^{4}J = 2.3 Hz, 1H, 7-H).

^{13}C NMR (102 MHz, DMSO): δ (ppm) = 160.2 (1-C), 157.3 (6-C), 152.7 (8-C), 129.1 (3-C), 127.8 (4-C), 121.1 (2-C), 113.8 (9-C), 111.3 (5-C), 102.0 (7-C).

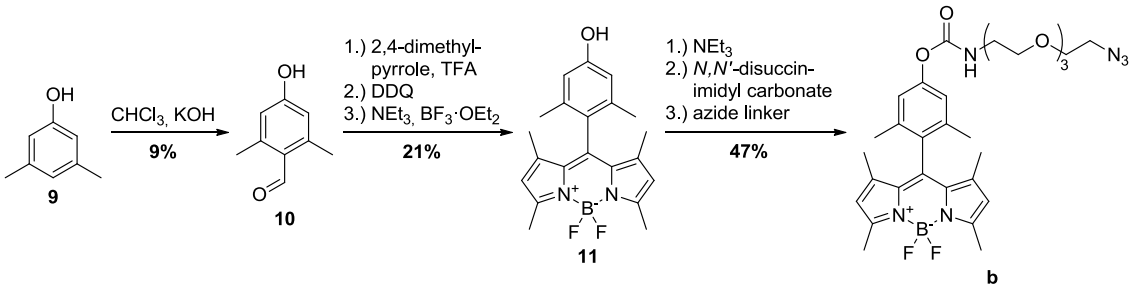
IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3204, 2920, 2100, 1739, 1608, 1544, 1505, 1447, 1307, 1243, 1175, 1108, 993, 940, 837, 761, 661.

HREIMS: calculated for $C_9H_5N_3O_3^+$ $[M]^+$: 203.0331, observed: 203.0321.

UV-Vis (DMSO): λ_{Abs} (nm) = 345.

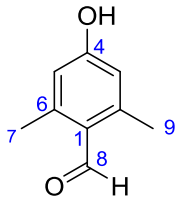
Synthesis Overview of BODIPY Azide b

The synthetic route for the BODIPY azide **b** is shown in scheme 1.



Scheme 1. Non-optimized synthesis of BODIPY azide **b**.

Synthesis of 4-Hydroxy-2,6-dimethylbenzaldehyde (10)

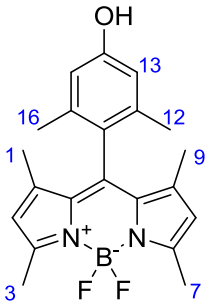


To a stirring solution of 3,5-dimethylphenol (**9**) (86.9 g, 0.71 mol, 1.0 eq) in H_2O (280 mL), KOH (73.0 g, 1.30 mol, 1.8 eq) was added and the reaction mixture was heated to 60 °C. Then, $CHCl_3$ (112 mL, 1.39 mol, 2.0 eq) was added slowly within 6 h and stirring was continued at 60 °C for 14 h. After cooling to rt the mixture was poured into aqueous H_2SO_4 (1% v/v, 400 mL). The resulting yellow precipitate was filtered off, washed with cold $CHCl_3$ (300 mL) and dried *in vacuo*. The desired aldehyde **10** (10.2 g, 0.07 mol, 9%) was obtained as a white-off solid.

^{1}H NMR (300 MHz, CD_3OD): δ (ppm) = 10.35 (s, 1H, 8-H), 6.50 (s, 2H, 3-H and 5-H), 2.52 (s, 6H, 7-H and 9-H).

HRESIMS: calculated for $C_9H_9O_2^-$ $[M-H]^-$: 149.0608, observed: 149.0603.

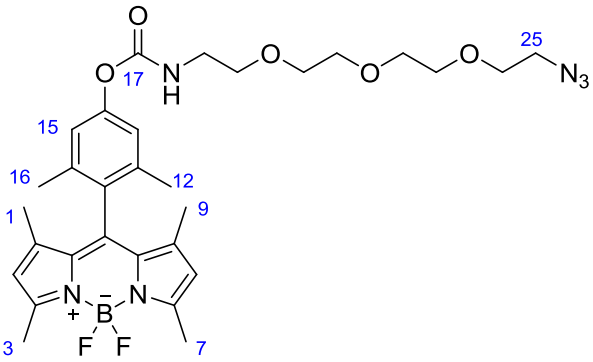
Synthesis of 5,5-Difluoro-10-(4-hydroxy-2,6-dimethylphenyl)-1,3,7,9-tetramethyl-5*H*-dipyrrolo[1,2-c:1',2'-f][1,3,2]diazaborinin-4-ium-5-uide (11)



The synthesis was performed according to the procedure of *Liu et al.* using **10** (300 mg, 2.0 mmol, 1.0 eq) instead of 4-hydroxybenzaldehyde as starting material.[2] After flash column chromatography (DCM/*iso*hexane = 5:1 → 10:1) **11** (155 mg, 0.4 mmol, 21%) was obtained as an orange-brown solid.

^{1}H NMR (300 MHz, CD_3OD): δ (ppm) = 6.64 (s, 2H, 13-H and 15-H), 6.04 (s, 2H, 2-H and 8-H), 2.48 (s, 6H, 1-H and 9-H), 2.03 (s, 6H, 12-H and 16-H), 1.46 (s, 6H, 3-H and 7-H).
HRESIMS: calculated for $C_{21}H_{24}BF_2N_2O^+$ $[M+H]^+$: 369.1945, observed: 369.1946.

Synthesis of 10-(4-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethylcarbamoyloxy)-2,6-dimethylphenyl)-5,5-difluoro-1,3,7,9-tetramethyl-5*H*-dipyrrolo[1,2-c:1',2'-f][1,3,2]diazaborinin-4-ium-5-uide (b)



To a stirring solution of BODIPY phenol **11** (200 mg, 0.54 mmol, 1.0 eq) in THF (10 mL), NEt_3 (302 µL, 220 mg, 2.17 mmol, 4.0 eq) was added, followed by the addition of *N,N'*-disuccinimidyl carbonate (278 mg, 1.09 mmol, 2.0 eq). After stirring at rt for 16 h, TLC monitoring indicated quantitative conversion. 11-Azido-3,6,9-trioxaundecan-1-amine (480 µL, 514 mg, 2.44 mmol, 4.5 eq) was added and stirring at rt was continued for 1 h. H_2O (150 mL) was added and the mixture was extracted with EtOAc (4 x 100 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*. Flash

column chromatography (*iso*hexane/EtOAc = 3:1 → 0:1) afforded the desired BODIPY azide **b** (156 mg, 0.26 mmol, 47%) as a deep red oil with green shine.

^{1}H NMR (400 MHz, CD_3OD): δ (ppm) = 7.00 (s, 2H, 13-H and 15-H), 6.07 (s, 2H, 2-H and 8-H), 3.71–3.64 (m, 10H, linker-CH_2), 3.62 (t, ^{3}J = 5.4 Hz, 2H, linker-CH_2), 3.38 (q, ^{3}J = 5.2 Hz, 4H, linker-CH_2), 2.50 (s, 6H, 1-H and 9-H), 2.13 (s, 6H, 12-H and 16-H), 1.44 (s, 6H, 3-H and 7-H).

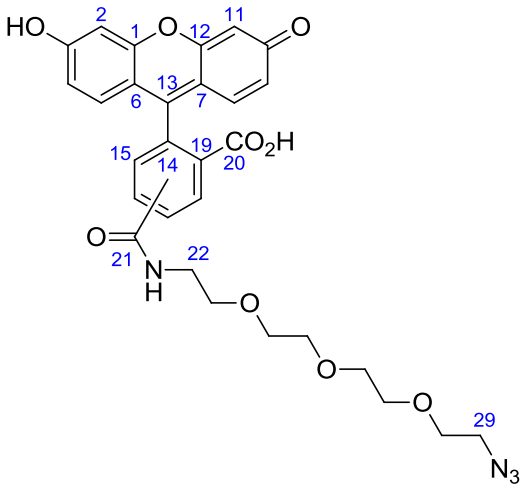
^{13}C NMR (102 MHz, CD_3OD): δ (ppm) = 156.8 (ipso-C), 153.2 (17-C), 143.8 (ipso-C), 141.9 (ipso-C), 137.9 (ipso-C), 132.1 (ipso-C), 131.7 (ipso-C), 122.6 (13-C and 15-C), 122.1 (2-C and 8-C), 71.69 (linker-C), 71.67 (linker-C), 71.6 (linker-C), 71.5 (linker-C), 71.3 (linker-C), 71.14 (linker-C), 71.10 (linker-C), 70.8 (linker-C), 62.2 (linker-C), 51.8 (linker-C), 42.0 (linker-C), 19.6 (12-C and 16-C), 14.6 (1-C and 9-C), 13.7 (3-C and 7-C).

HRESIMS: calculated for $C_{30}H_{39}BF_2N_6NaO_5^+$ $[M+H]^+$: 635.2935, observed: 635.2934.

UV-Vis (H_2O): λ_{Abs} (nm) = 498.

Fluorescence (H_2O): λ_{Em} (nm) = 508.

Synthesis of 4(5)-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)carbamoyl)-2-(6-hydroxy-oxo-3*H*-xanthen-9-yl)benzoic acid (5(6)-d)



To a stirring solution of 5(6)-carboxyfluorescein (188.2 mg, 0.50 mmol, 1.0 eq) in DMF (1.5 mL), *N*,*N*-diisopropylethylamine (0.26 mL, 1.50 mmol, 3.0 eq), 4-dimethylamino-pyridine (6.1 mg, 0.05 mmol, 0.1 eq), 1-(Bis(dimethylamino)methylene)-1*H*-1,2,3-triazolo-[4,5-b]pyridinium-3-oxid hexafluorophosphate (228.1 mg, 0.60 mmol, 1.2 eq) and 11-azido-3,6,9-trioxaundecan-1-amine (120.0 mg, 0.55 mmol, 0.11 mL, 1.1 eq) were added and stirred at rt for 24 h. The solvent was removed *in vacuo* and the crude product was purified by flash column chromatography (DCM/MeOH = 9:1 → 5:1) to yield 5(6)-**d** (278.4 mg, 0.48 mmol, 97%) as an orange solid.

5 mg of the regioisomers 5(6)-**d** were separated by preparative RP-HPLC (0 → 50% B in 45 min) and the 5-regioisomer **d** was used for click reactions.

^{1}H NMR (400 MHz, CD_3OD): δ (ppm) = 8.44 (dd, ^{4}J = 1.6 Hz, J = 0.7 Hz, 0.5H, 18-H), 8.25 (dd, ^{3}J = 8.0 Hz, ^{4}J = 1.6 Hz, 0.5H, 16-H), 8.19 (dd, ^{3}J = 8.0 Hz, ^{4}J = 1.4 Hz, 0.5H, 17-H), 8.12 (dd, ^{3}J = 8.0 Hz, J = 0.7 Hz, 0.5H, 18-H), 7.69 (dd, ^{4}J = 1.4 Hz, J = 0.8 Hz, 0.5H, 15-H), 7.34 (dd, ^{3}J = 8.0 Hz, J = 0.7 Hz, 0.5H, 15-H), 6.75–6.71 (m, 2H, 5-H and 8-H), 6.64–6.58 (m, 2H, 2-H and 11-H), 6.54 (ddd, ^{3}J = 8.7 Hz, ^{3}J = 5.2 Hz, ^{4}J = 2.4 Hz, 2H, 4-H and 9-H), 3.77–3.48 (m, 16 H, linker-CH_2), 2.15 (s, 1 H, OH).

Note: Long-range couplings can occure.

^{13}C NMR (102 MHz, CD_3OD): δ (ppm) = 170.7 (21-C), 168.6 (20-C), 168.4 (20-C), 161.8 (3-C, 10-C), 154.3 (1-C, 12-C), 142.4 (14-C), 138.0 (13-C), 135.6 (19-C), 130.5 (5-C, 8-C), 130.3 (5-C, 8-C), 126.4 (16-C)*, 125.9 (17-C)*, 125.1 (18-C)*, 124.2 (15-C)*, 113.9 (4-C, 9-C), 111.1 (6-C, 7-C), 103.77 (2-C, 11-C), 71.8 (25-C)**, 71.8 (26-C)**, 71.8 (25-C)**, 71.7 (23-C, 26-C)**,***, 71.6 (23-C)***, 71.5 (24-C)****, 71.3 (24-C)****,

71.2 (27-C)****, 71.2 (27-C)****, 70.6 (28-C), 70.4 (28-C), 51.9 (29-C), 51.9 (29-C), 41.4 (22-C), 41.3 (22-C).

IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3302, 3051, 2117, 1678, 1621, 1516, 1458, 1373, 1343, 1319, 1260, 1226, 1158, 1121, 1070, 982, 953, 926, 860, 837, 816, 756, 745, 720.

HRESIMS: calculated for $C_{29}H_{27}N_4O_9^-$ $[M]^-$: 575.1784, observed: 575.1784.

UV-Vis (H_2O): λ_{Abs} (nm) = 495.

Fluorescence (H_2O): λ_{Em} (nm) = 520.

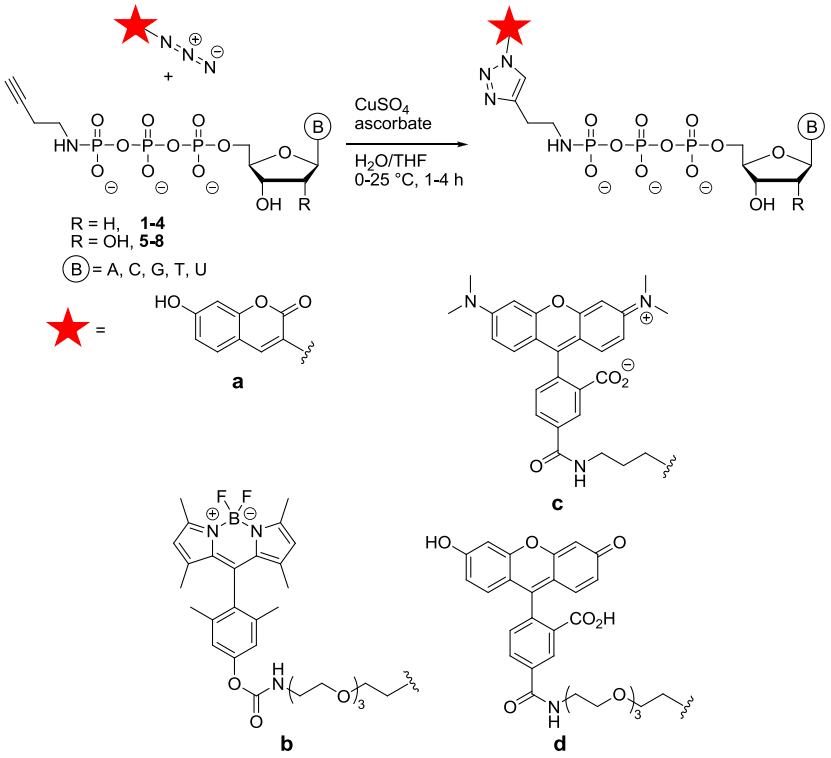
4 Synthesis of γ-Fluorophore Labeled Nucleoside Triphosphates

General Procedure 3: In a 0.2 mL PCR tube 8.0 µL of the γ-alkyne labeled nucleoside triphosphate solution (50 mM in ddH_2O, 400 nmol, 1.0 eq) was mixed with 10.4 µL of the fluorophore azide solution (58 mM, 600 nmol, 1.5 eq) (THF solution for the coumarin **a**, BODIPY **b** and carboxyfluorescein **d**, THF/H_2O = 1:1 mixture for TAMRA **c**). 0.64-1.92 µL $CuSO_4$ solution (10 mg/mL in ddH_2O, 40 nmol, 0.1-0.3 eq) and 1.60-4.80 µL of a freshly prepared sodium ascorbate solution (100 mg/mL in H_2O, 800 nmol, 2.0-6.0 eq) were added, mixed and incubated at 0 °C to 25 °C for 1-4 h (exact conditions: Table S1, p. 20). Purification by semipreparative RP-HPLC yielded γ-fluorophore labeled nucleotides.

General Procedure 4: All reactions were performed in glassware under an argon atmosphere and with magnetic stirring. This procedure was only applied for the synthesis of **4a** and **5a**.

The γ-alkyne labeled nucleoside triphosphate (1.0 eq) was dissolved in ddH_2O, the fluorophore azide **a** (1.5 eq) in THF was added at rt and the brownish solution was degassed (5x). Sodium ascorbate (2.0 eq) and $CuSO_4$ (10 mol%) were added and after complete consumption of the starting material (reactions were monitored by RP-HPLC; exact conditions: Table S1, p. 20 or procedures, p. 21-23) the solvent was removed and the pale brown crude product was purified by preparative RP-HPLC to yield the γ-fluorophore labeled nucleotides.

5 Click Reactions Overview (Table S1)



Scheme 2. Cu(I)-catalyzed click reactions using different γ-alkyne labeled nucleotides (**1-8**) and fluorophore azides (**a-d**).

entry	γ-NTP	azide	product	conditions	yield (%)[1)]	chemical formula [M-H]⁻	MS calc. m/z	MS found m/z
1	dATP	d	1d	A	≥90	$C_{43}H_{48}N_{10}O_{20}P_3^-$	1117.2265	1117.2221
2	dCTP	d	2d	A	≥90	$C_{42}H_{48}N_8O_{21}P_3^-$	1093.2152	1093.2110
3	dGTP	d	3d	A	≥90	$C_{43}H_{48}N_{10}O_{21}P_3^-$	1133.2214	1133.2168
4	dTTP	d	4d	A	≥90	$C_{43}H_{49}N_7O_{22}P_3^-$	1108.2149	1108.2113
5	dTTP	a	4a	A	≥90, 70[2)]	$C_{23}H_{26}N_6O_{16}P_3^-$	735.0624	735.0620
6	dTTP	b	4b	C	≥90	$C_{44}H_{60}BF_2N_9O_{18}P_3^-$	1144.3336	1144.3344
7	dTTP	c	4c	B	≥90	$C_{42}H_{49}N_9O_{17}P_3^-$	1044.2465	1044.2438
8	ATP	a	5a	A	≥90,77[2)]	$C_{23}H_{25}N_9O_{15}P_3^-$	760.0688	760.0687
9	ATP	d	5d	A	≥90	$C_{43}H_{48}N_{10}O_{21}P_3^-$	1133.2214	1133.2224
10	CTP	d	6d	A	≥90	$C_{42}H_{48}N_8O_{22}P_3^-$	1109.2101	1109.2122
11	GTP	d	7d	A	≥90	$C_{43}H_{48}N_{10}O_{22}P_3^-$	1149.2122	1149.2163
12	UTP	d	8d	A	≥90	$C_{42}H_{47}N_7O_{23}P_3^-$	1110.1942	1110.1913

1) yield determined by analytical RP-HPLC; 2) isolated yield after RP-HPLC purification.

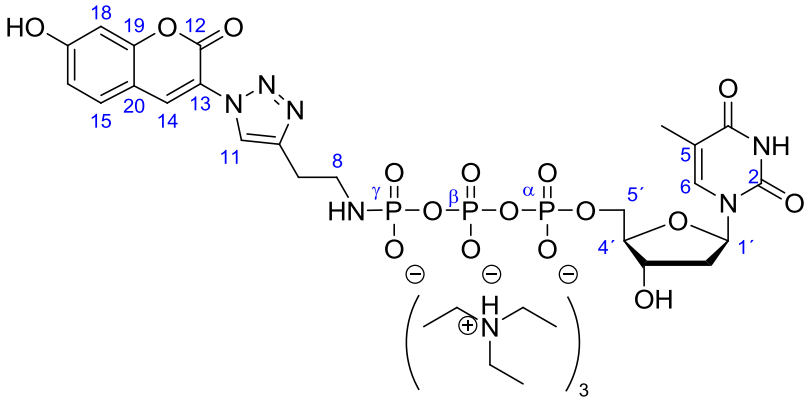
Reaction conditions:

A) 1.5 eq fluorophore azide, 0.1 eq Cu_2SO_4, 2.0 eq sodium ascorbate, 0 °C to 25 °C, 1 h, THF/H_2O = 1:1.

B) 1.5 eq fluorophore azide, 0.1 eq Cu_2SO_4, 2.0 eq sodium ascorbate, 0 °C to 25 °C, 2 h, THF/H_2O = 1:3.

C) 1.5 eq fluorophore azide, 0.3 eq Cu_2SO_4, 6.0 eq sodium ascorbate, 25 °C, 3 h, THF/H_2O = 3:1.

Synthesis of γ-*N*-(2-(1-(7-Hydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-1,2,3-triazol-4-yl)ethyl-amido)-2´-deoxythymidine-5´-triphosphate (4a)



The synthesis was performed with **4** (16.9 mg, 20.0 µmol, 1.0 eq), **a** (6.2 mg, 30.3 µmol, 1.5 eq), sodium ascorbate (7.9 mg, 40.0 µmol, 2.0 eq) and $CuSO_4$ (0.3 mg, 10 mol%) in ddH_2O/THF = 1:1 (1.0 mL) at 0 °C within 1 h as described in the general procedure 4 (p. 19). Purification by RP-HPLC afforded the tris-triethylammonium salt of γ-fluorophore labeled dTTP **4a** (14.6 mg, 14.0 µmol, 70%) as a bright yellow solid after lyophylization.

One pot synthesis of 4a from dTTP

The synthesis was performed with a 100 mM dTTP solution (200 µL, 20 µmol, 1.0 eq) as described in the general procedure 1 (p. 3) within 4.5 h, using adjusted amounts of EDC·HCl and 1-aminobut-3-yne in ddH_2O/DMF = 1:1 (0.8 mL). After precipitation, the crude γ-alkyne labeled dTTP **4** was directly used for the click reaction with fluorophore azide **a** as described above in the general procedure 3 using the reagent amounts as described on p. 21. Purification by RP-HPLC afforded the tris-triethylammonium salt of the γ-fluorophore labeled dTTP **4a** (12.6 mg, 12.1 µmol, 60%) as a bright yellow solid after lyophylization.

^{1}H NMR (600 MHz, D_2O): δ (ppm) = 8.38 (s, 1H, 11-H), 8.36 (s, 1H, 14-H), 7.63 (d, ^3J = 8.6 Hz, 1H, 15-H), 7.58 (d, ^4J = 1.2 Hz, 1H, 6-H), 6.95 (dd, ^3J = 8.8 Hz, ^4J = 2.4 Hz, 1H, 16-H), 6.87 (d, ^4J = 1.9 Hz, 1H, 18-H), 6.15 (t, ^3J = 6.9 Hz, 1H, 1'-H), 4.58–4.52 (m, 1H, 3'-H), 4.18–4.05 (m, 3H, 4'-H, 5'-H), 3.36–3.25 (m, 2H, 8-H), 3.03 (t, ^3J = 7.3 Hz, 2H, 9-H), 2.29–2.24 (m, 1H, 2'-H), 2.22–2.16 (m, 1H, 2'H), 1.85 (s, 3H, 7-H).

^{13}C NMR (151 MHz, D_2O): δ (ppm) = 166.0 (4-C), 161.9 (12-C), 158.5 (17-C), 154.6 (19-C), 151.1 (2-C), 145.9 (13-C), 138.1 (14-C), 136.9 (6-C), 131.0 (15-C), 124.3 (11-C), 118.9 (10-C), 114.8 (16-C), 111.3 (5-C), 110.8 (20-C), 102.6 (18-C), 85.4 (d, $^3J_{PC}$ = 9.0 Hz, 4´-C), 84.8 (1'-C), 70.7 (3'-C), 65.2 (d, $^2J_{PC}$ = 6.0 Hz, 5'-C), 41.2 (8-C), 38.6 (2'-C), 27.2 (d, $^3J_{PC}$ = 8.9 Hz, 9-C), 11.5 (7-C).

^{31}P NMR (162 MHz, D_2O): δ (ppm) = -1.78 (d, ^2J = 19.0 Hz, γ-P), -11.81 (d, ^2J = 19.2 Hz, α-P), -23.04 (t, ^2J = 20.5 Hz, β-P).

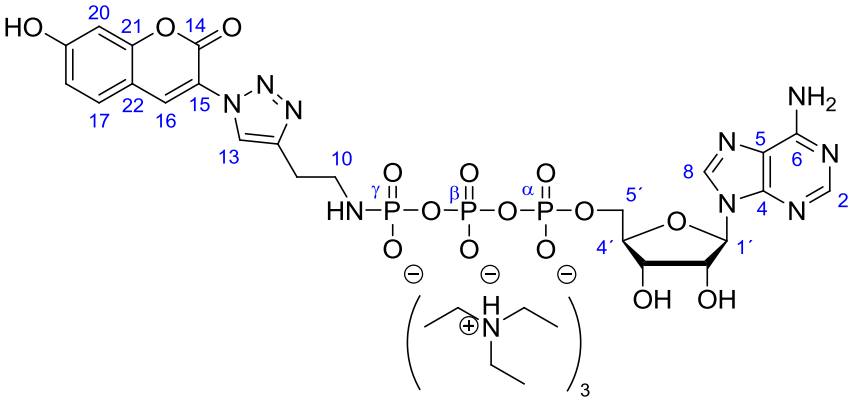
IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3331, 2987, 2692, 2500, 1728, 1695, 1651, 1605, 1475, 1419, 1398, 1327, 1222, 1116, 1082, 1052, 989, 902, 812, 798, 760, 720.

HRESIMS: calculated for $C_{23}H_{26}N_6O_{16}P_3^-$ $[M-H]^-$: 735.0624, observed: 735.0620.

UV-Vis (H_2O): λ_{Abs} (nm) = 393, 262.

Fluorescence (H_2O): λ_{Em} (nm) = 476.

Synthesis of γ-*N*-(2-(1-(7-Hydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-1,2,3-triazol-4-yl)ethyl-amido)adenosine-5´-triphosphate (5a)



The synthesis was performed with **5** (8.6 mg, 10.0 µmol, 1.0 eq), **a** (3.0 mg, 15.0 µmol, 1.5 eq), sodium ascorbate (4.0 mg, 20.0 µmol, 2.0 eq) and $CuSO_4$ (0.2 mg, 10 mol%) in ddH_2O/THF = 1:1 (0.5 mL) at 0 °C within 1 h as described in the general procedure 4 (p. 19). Purification by RP-HPLC afforded the tris-triethylammonium salt of γ-fluorophore labeled ATP **5a** (8.2 mg, 7.7 µmol, 77%) as a bright yellow solid after lyophylization.

^{1}H NMR (600 MHz, D_2O): δ (ppm) = 8.28 (s, 1H, 2-H), 8.24 (s, 1H, 13-H), 8.07 (s, 1H, 16-H), 7.90 (s, 1H, 8-H), 7.40 (d, ^3J = 8.6 Hz, 1H, 17-H), 6.80 (dd, ^3J = 8.6 Hz, ^4J = 2.2 Hz, 1H, 18-H), 6.65 (d, ^4J = 2.2 Hz, 1H, 20-H), 5.91 (d, ^3J = 5.4 Hz, 1H, 1'-H), 4.52 (t, ^3J = 5.2 Hz, 1H, 2'-H), 4.46 (t, ^3J = 4.8 Hz, 1H, 3'-H), 4.32–4.28 (m, 1H, 4'-H), 4.27–4.15 (m, 2H, 5'-H), 3.33–3.25 (m, 2H, 10-H), 2.99 (t, ^3J = 7.4, 2H, 11-H).

^{13}C NMR (151 MHz, D_2O): δ (ppm) = 161.6 (14-C), 157.7 (19-C), 154.8 (21-C), 152.1 (2-C), 148.2 (4-C), 145.6 (15-C), 139.1 (8-C), 136.7 (16-C), 130.6 (17-C), 123.8 (13-C), 118.1 (5-C)*, 118.0 (12-C)*, 114.6 (18-C), 110.3 (22-C), 102.2 (20-C), 86.9 (1'-C), 83.6 (d, $^3J_{PC}$ = 8.5 Hz, 4'-C), 74.6 (2'-C), 70.0 (3'-C), 65.0 (d, $^2J_{PC}$ = 5.6 Hz, 5'-C), 41.2 (10-C), 27.1 (d, $^3J_{PC}$ = 8.3 Hz, 11-C).

^{31}P NMR (162 MHz, D_2O): δ (ppm) = -1.77 (d, ^2J = 19.9 Hz, γ-P), -11.51 (d, ^2J = 18.3 Hz, α-P), -22.95 (t, ^2J = 17.6 Hz, β-P).

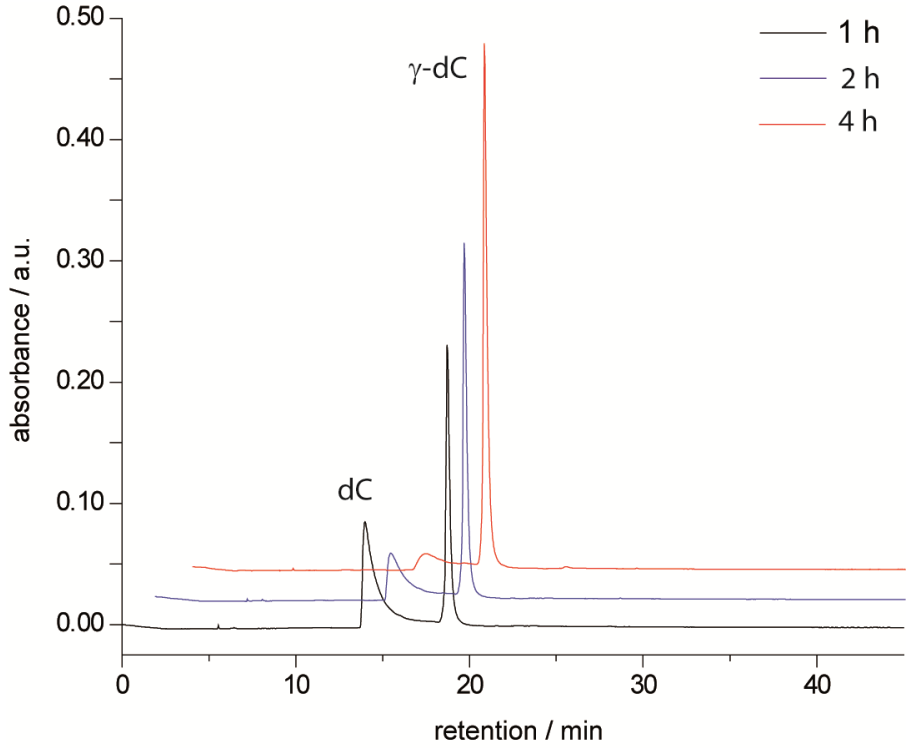
IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3327, 3172, 2982, 2878, 2733, 2503, 1728, 1700, 1645, 1605, 1513, 1475, 1418, 1397, 1327, 1218, 1113, 1083, 1060, 988, 899, 846, 811, 798, 758, 718.

HRESIMS: calculated for $C_{23}H_{25}N_9O_{15}P_3^-$ [M-H]$^-$: 760.0688, observed: 760.0687.

UV-Vis (H_2O): λ_{Abs} (nm) = 354, 258.

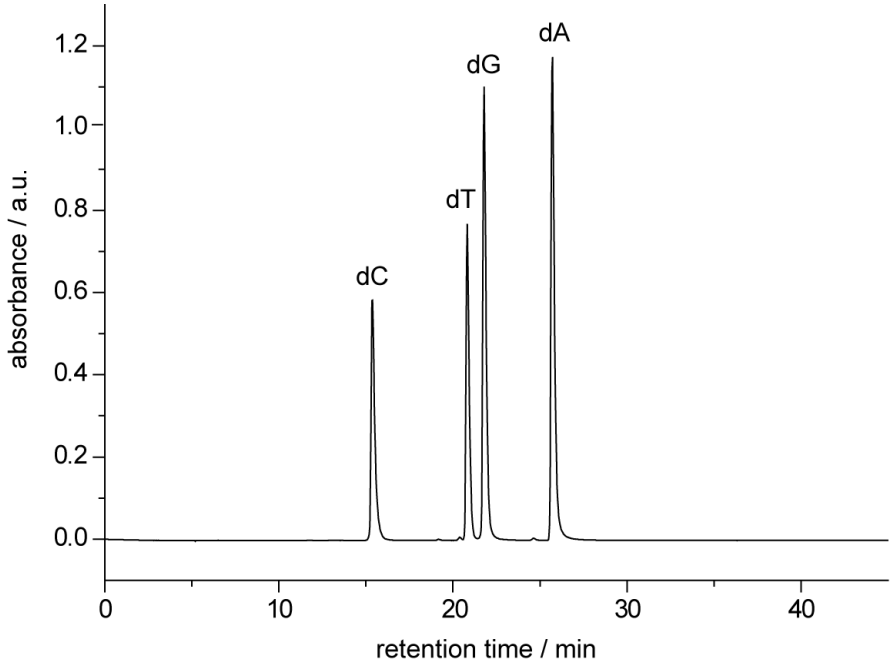
Fluorescence (H_2O): λ_{Em} (nm) = 478.

6 RP-HPLC Profiles of the Reaction to γ-Alkyne Labeled dCTP 2 (Figure S1)

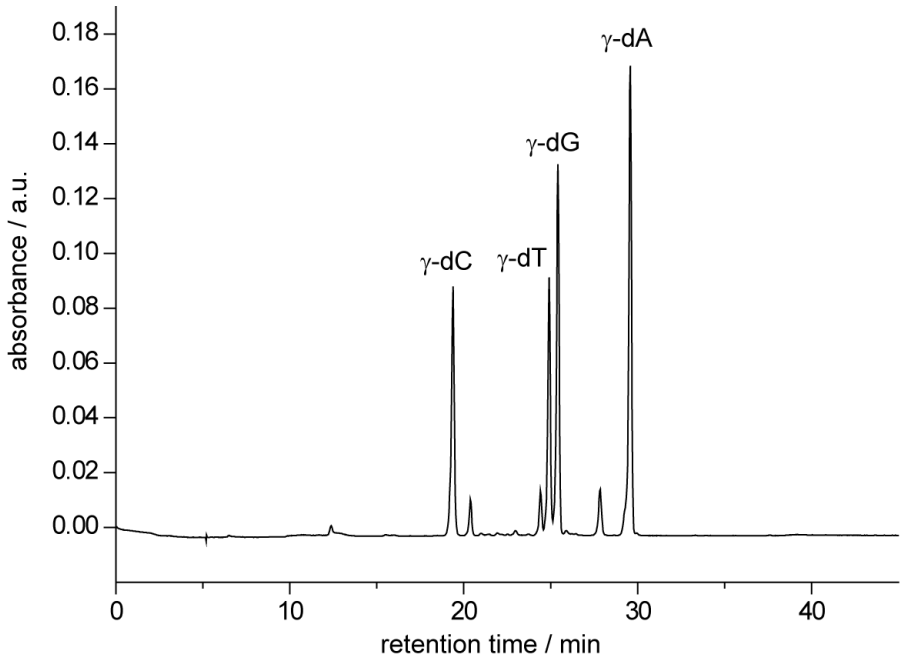


HPL-chromatograms of the reaction from dCTP to γ-alkyne labeled dCTP **2** after 1 h, 2 h and 4 h reaction time using EDC and our described method. Please note that this is the crude mix and time-point 0 h (pure dCTP) was omitted for clarity.

7 RP-HPLC Profiles of dNTPs and Crude γ-Alkyne Labeled dNTPs (Figure S2)

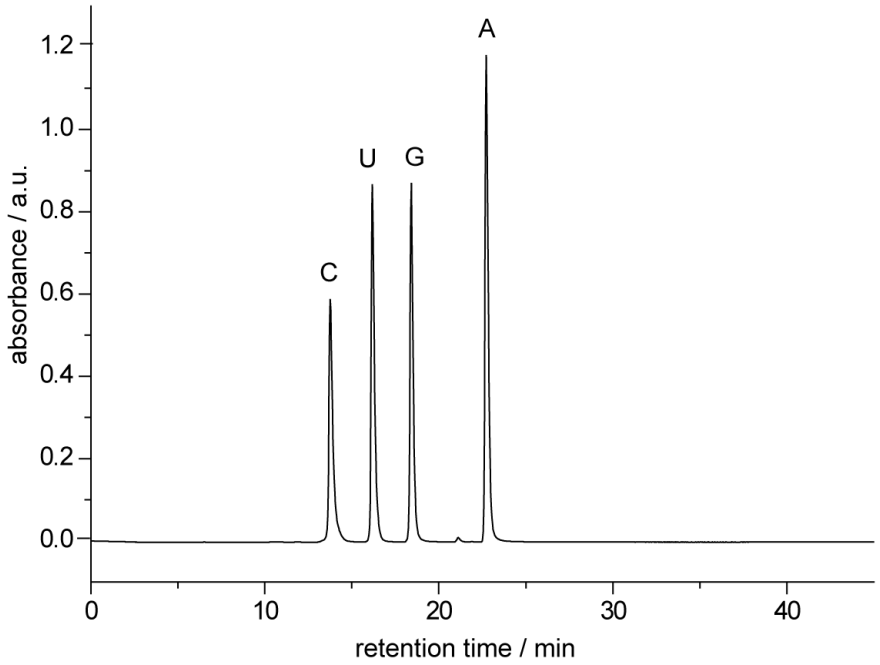


A. HPL-chromatogram of an authentic dNTP standard mix at 260 nm (0 → 20% B in 45 min). The R_t for the respective nucleotides are 15.4 min, 20.8 min, 21.8 min and 25.7 min. Note that although the mix contains almost equal concentrations of each nucleotide, the peak size area varies according to the different extinction coefficients.

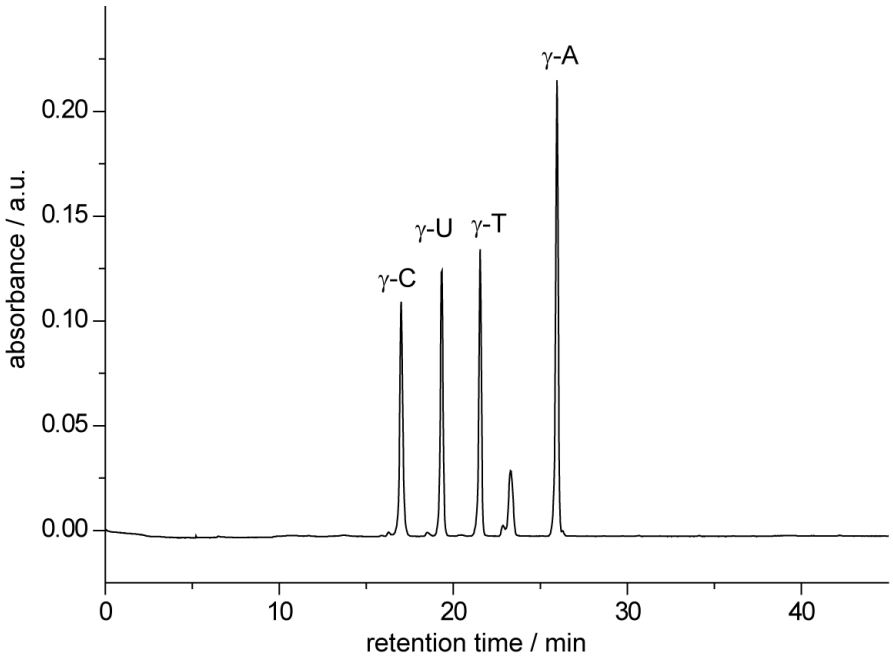


B. HPL-chromatogram of a crude reaction mix containing γ-alkyne labeled dNTPs after 4 h reaction time. The R_t for the respective nucleotides are 19.4 min, 24.9 min, 25.4 min and 29.6 min.

8 RP-HPLC Profiles of NTPs and Crude γ-Alkyne Labeled NTPs (Figure S3)



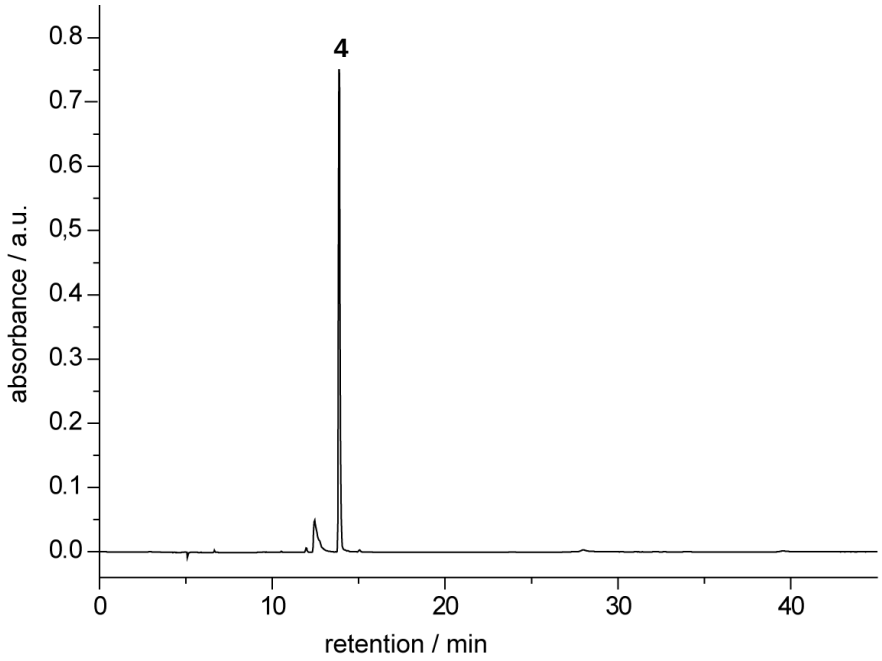
A. HPL-chromatogram of an authentic NTP standard mix at 260 nm (0 → 20% B in 45 min). The R_t for the respective nucleotides are 13.8 min, 16.2 min, 18.4 min and 22.7 min.



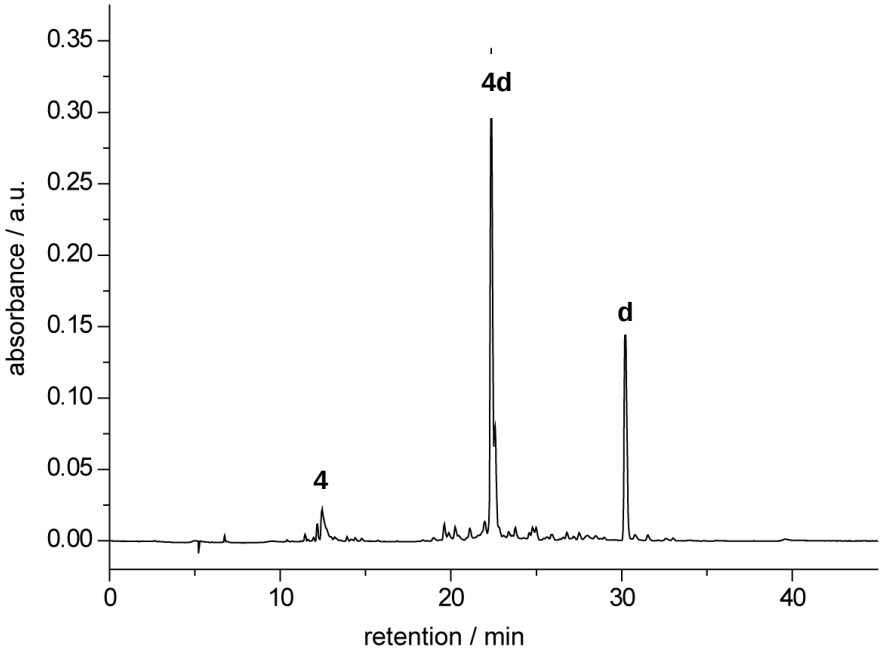
B. HPL-chromatogram of a crude reaction mix containing γ-alkyne labeled NTPs after 4 h reaction time. The R_t for the respective nucleotides are 17.0 min, 19.3 min, 21.5 min, 26.0 min.

9 RP-HPLC Profiles of the Click Reaction to Compound 4d (Figures S4)

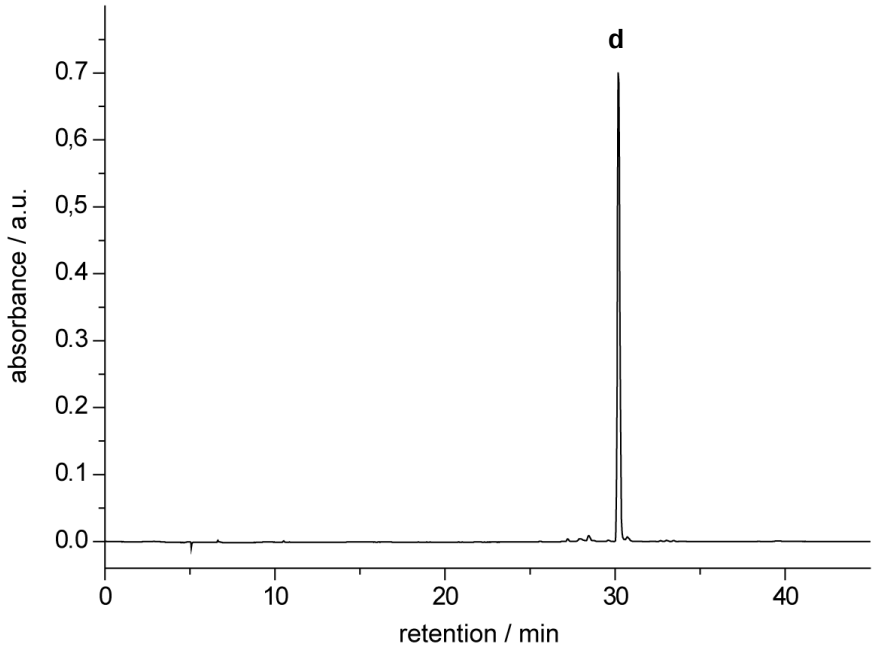
4



A. HPL-chromatogram of γ-alkyne labeled dTTP **4** at 260 nm (0 → 20 → 60% B in 45 min).



B. HPL-chromatogram of the crude click reaction to compound **4d** at 260 nm after 1 h reaction time.

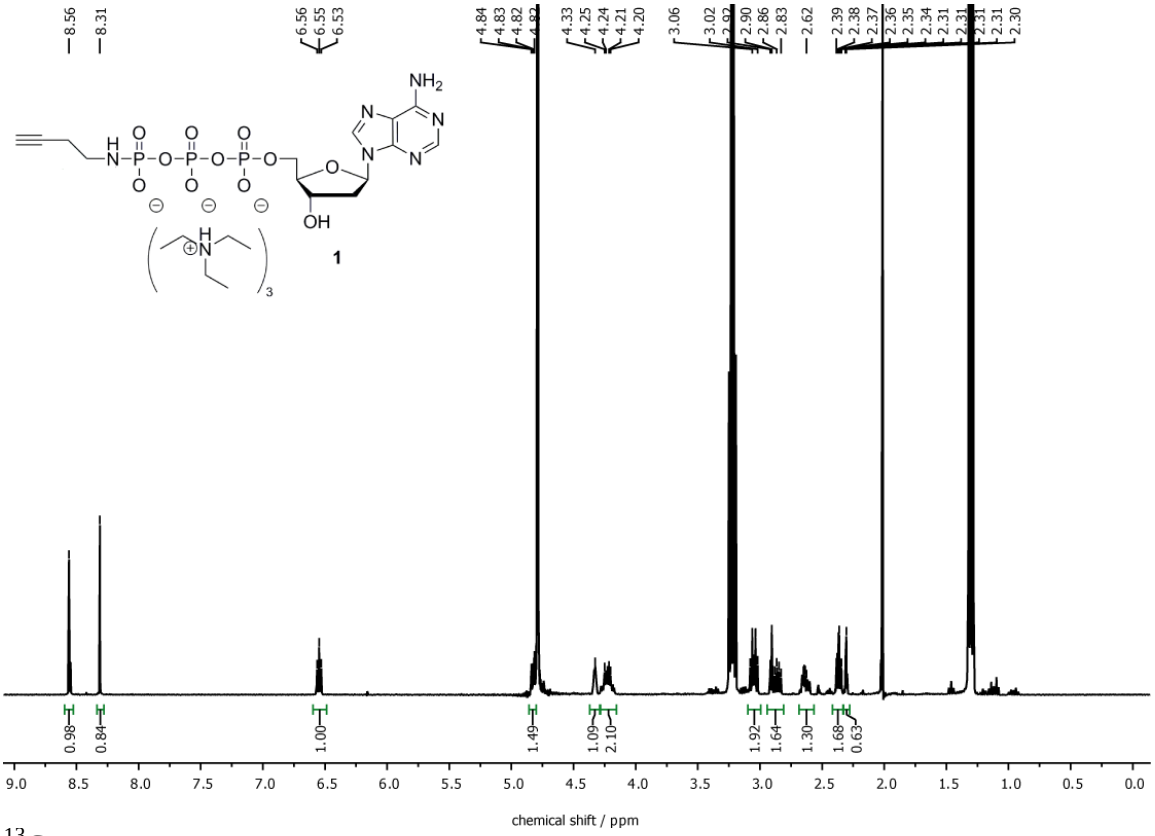


C. HPL-chromatogram of carboxyfluorescein azide **d** at 260 nm.

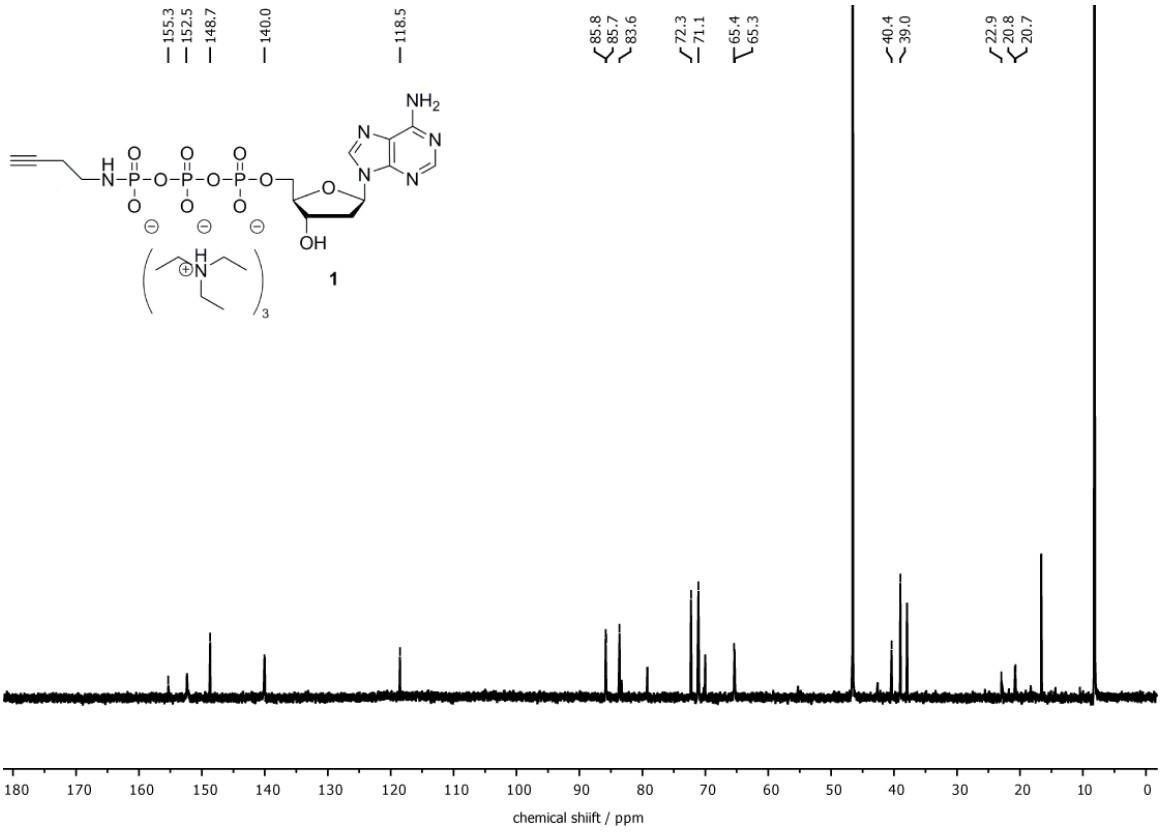
10 ^{1}H and ^{13}C NMR of γ-Alkyne Labeled NTPs (1-8) (Figure S5)

γ-*N*-(But-3-yn-1-ylamido)-2'-deoxyadenosine-5'-triphosphate (1)

^{1}H NMR:

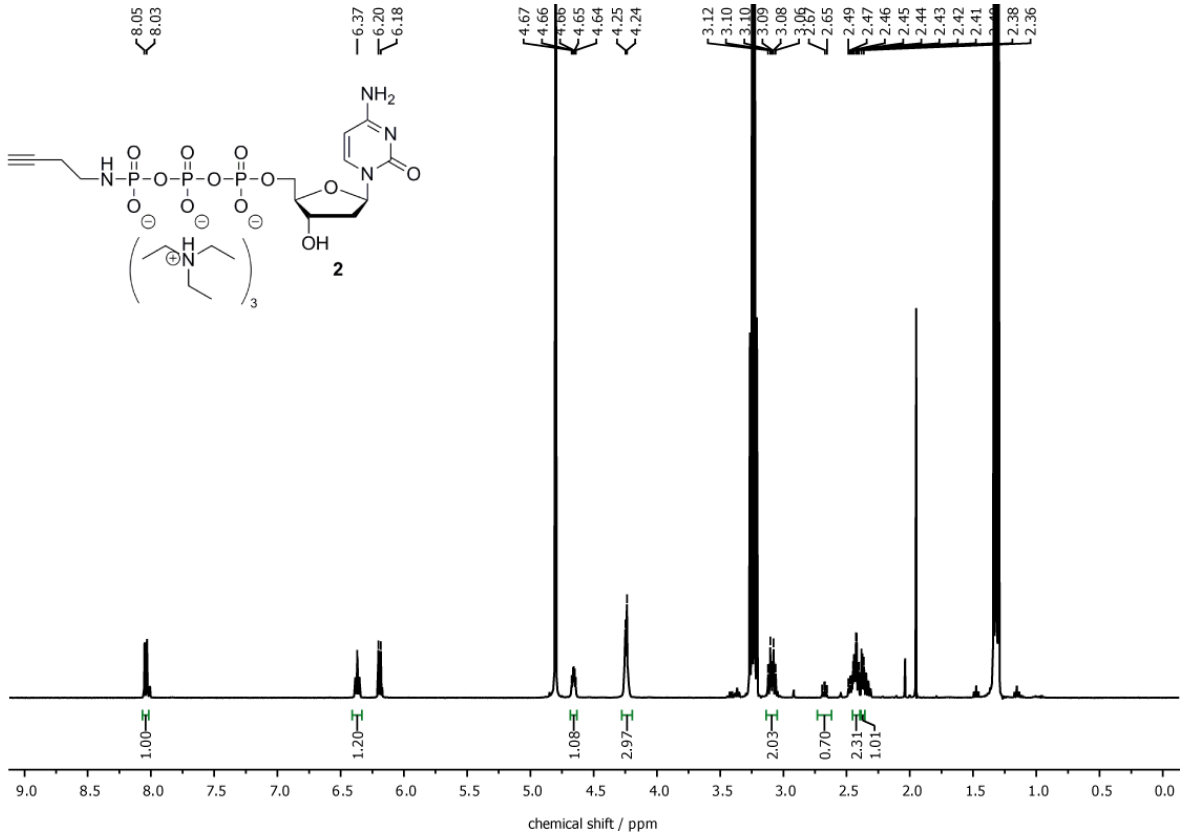


^{13}C NMR:

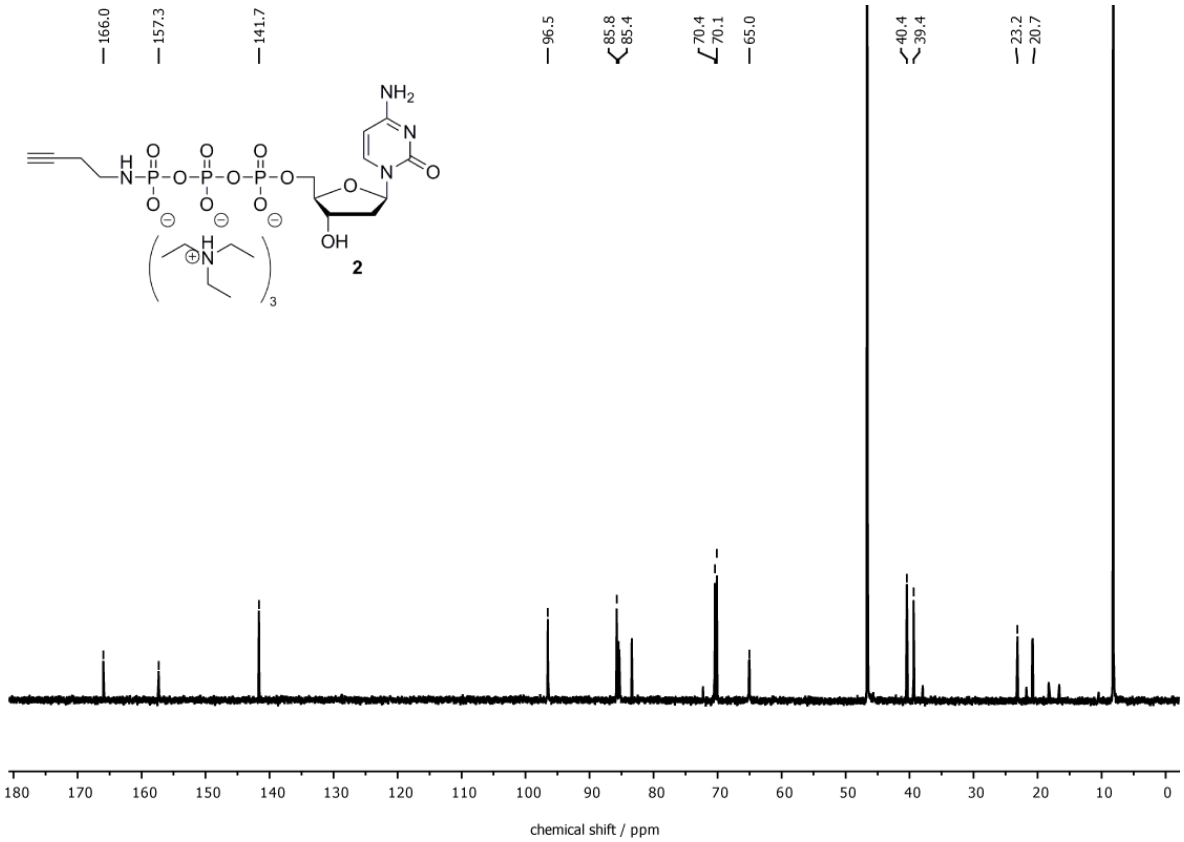


γ-*N*-(But-3-yn-1-ylamido)-2'-deoxycytidine-5'-triphosphate (2)

^{1}H NMR:

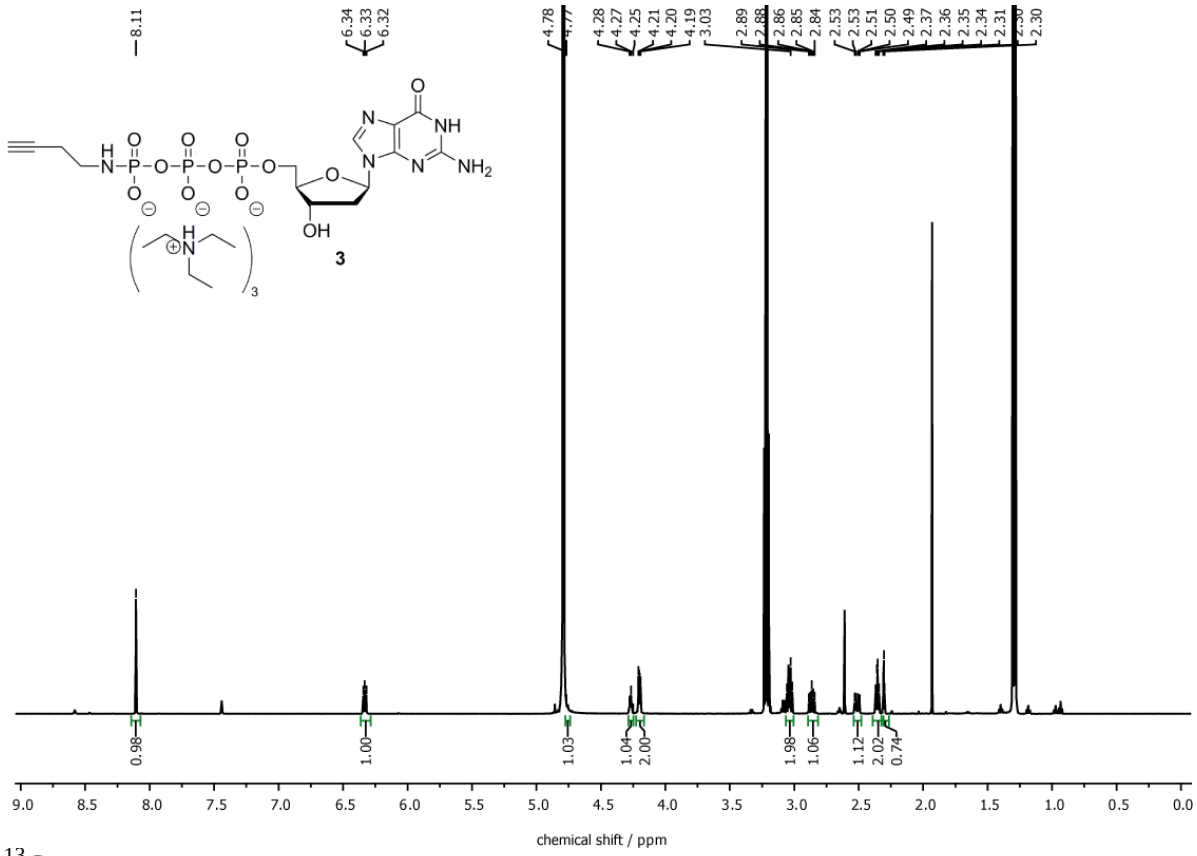


^{13}C NMR:

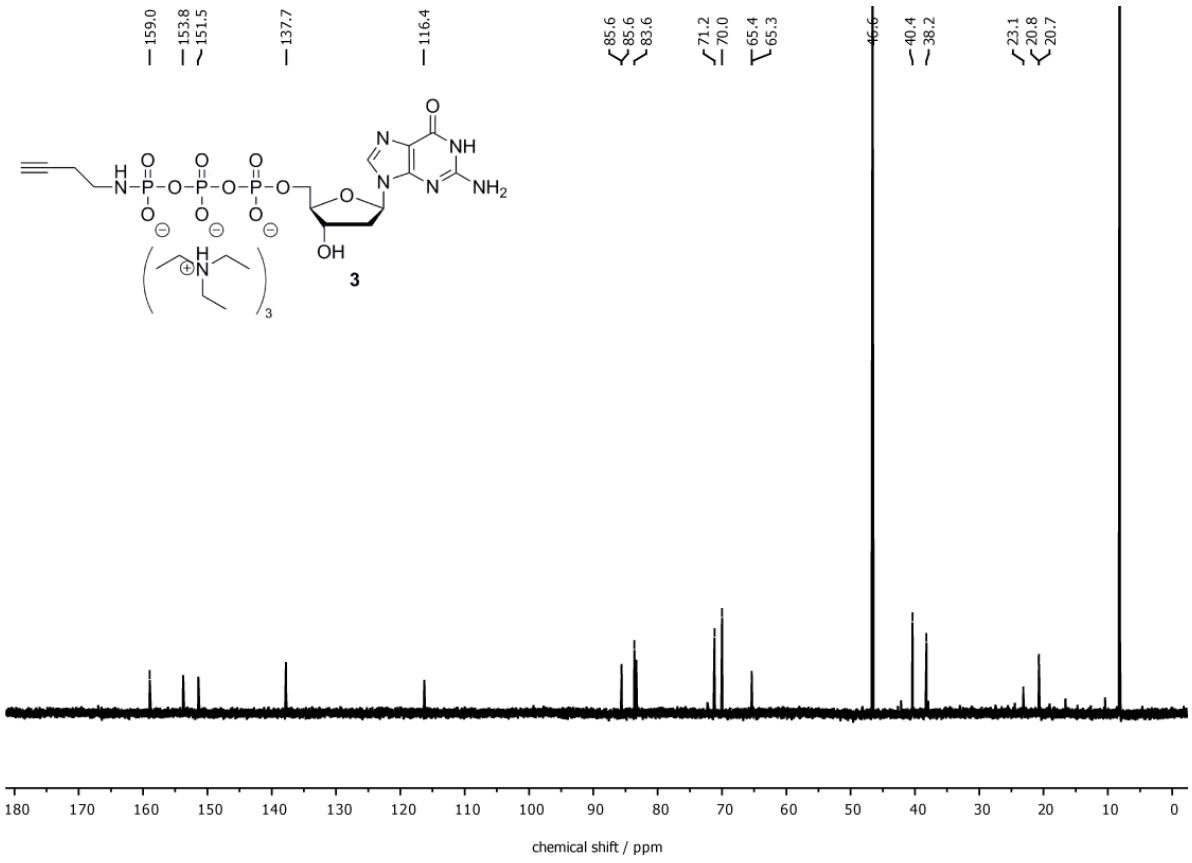


γ-*N*-(But-3-yn-1-ylamido)-2'-deoxyguanosine-5'-triphosphate (3)

^{1}H NMR:

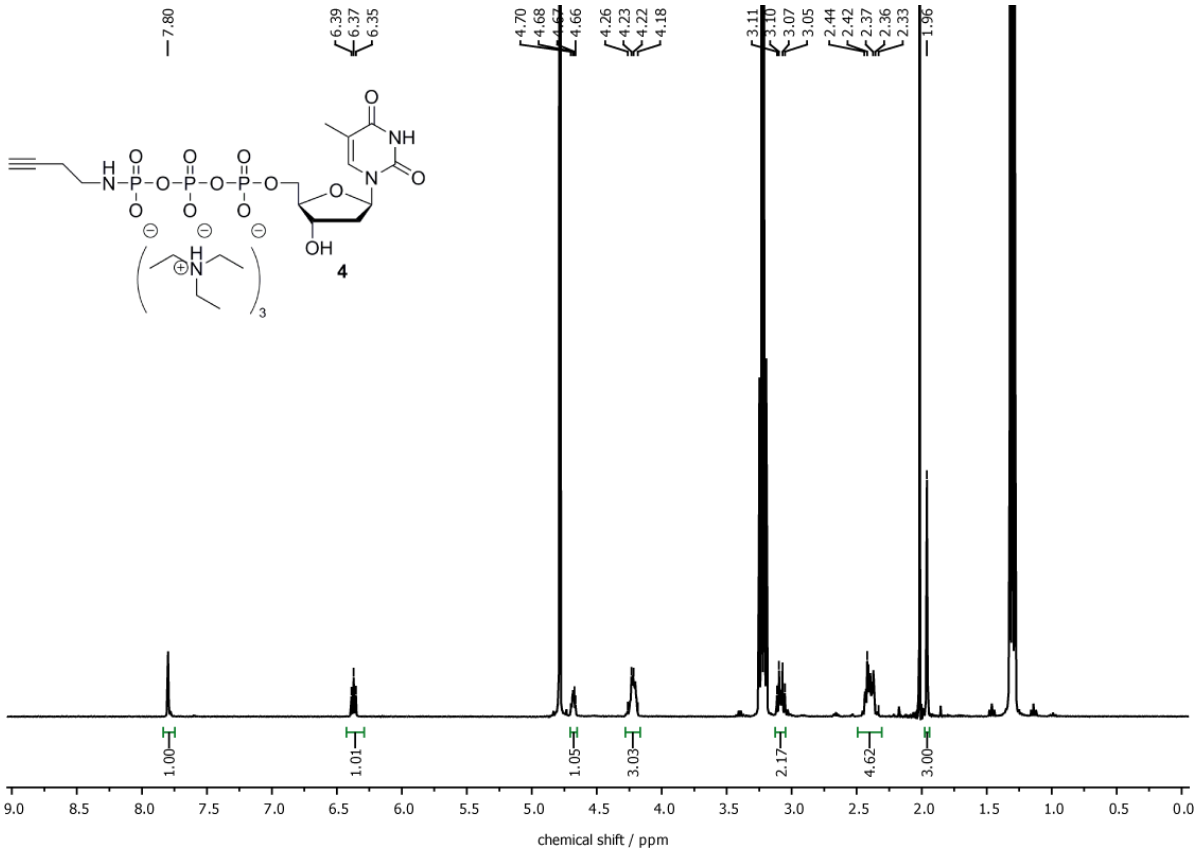


^{13}C NMR:

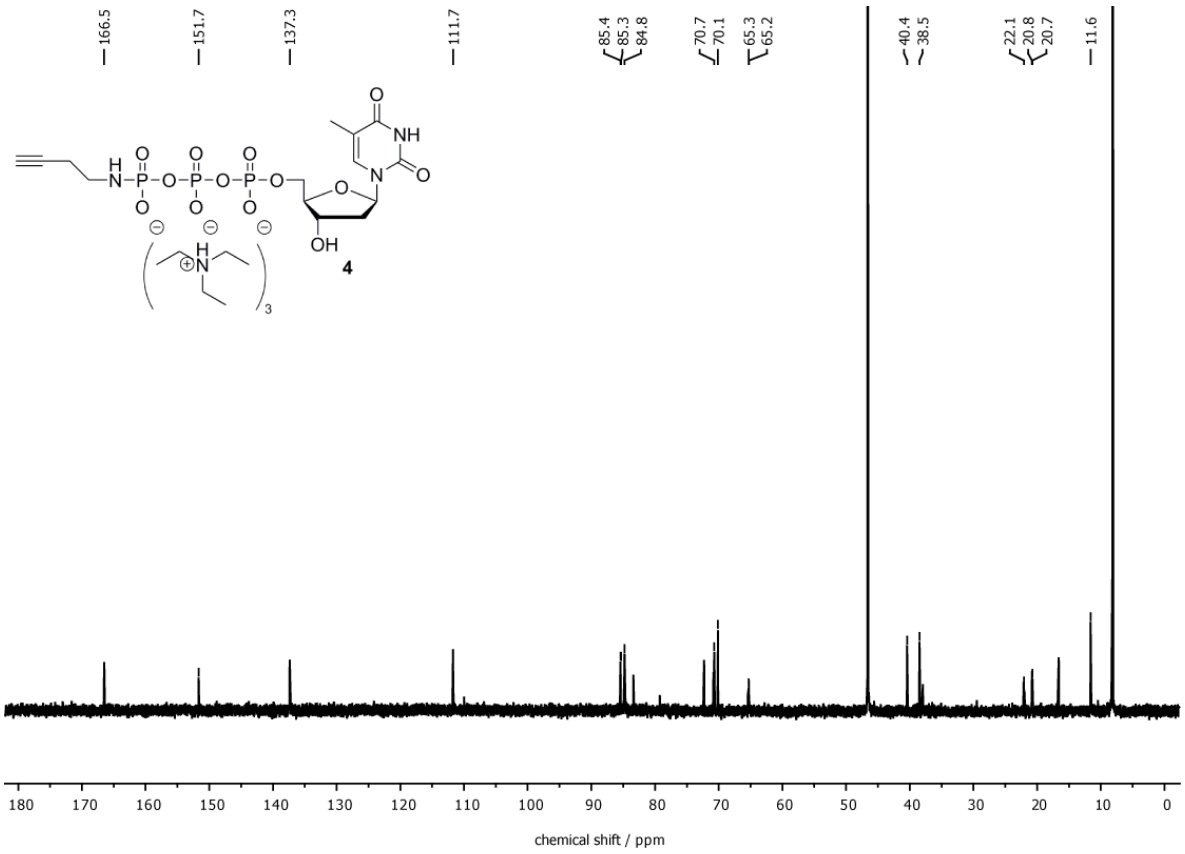


γ-*N*-(But-3-yn-1-ylamido)-2'-deoxythymidine-5'-triphosphate (4)

^{1}H NMR:

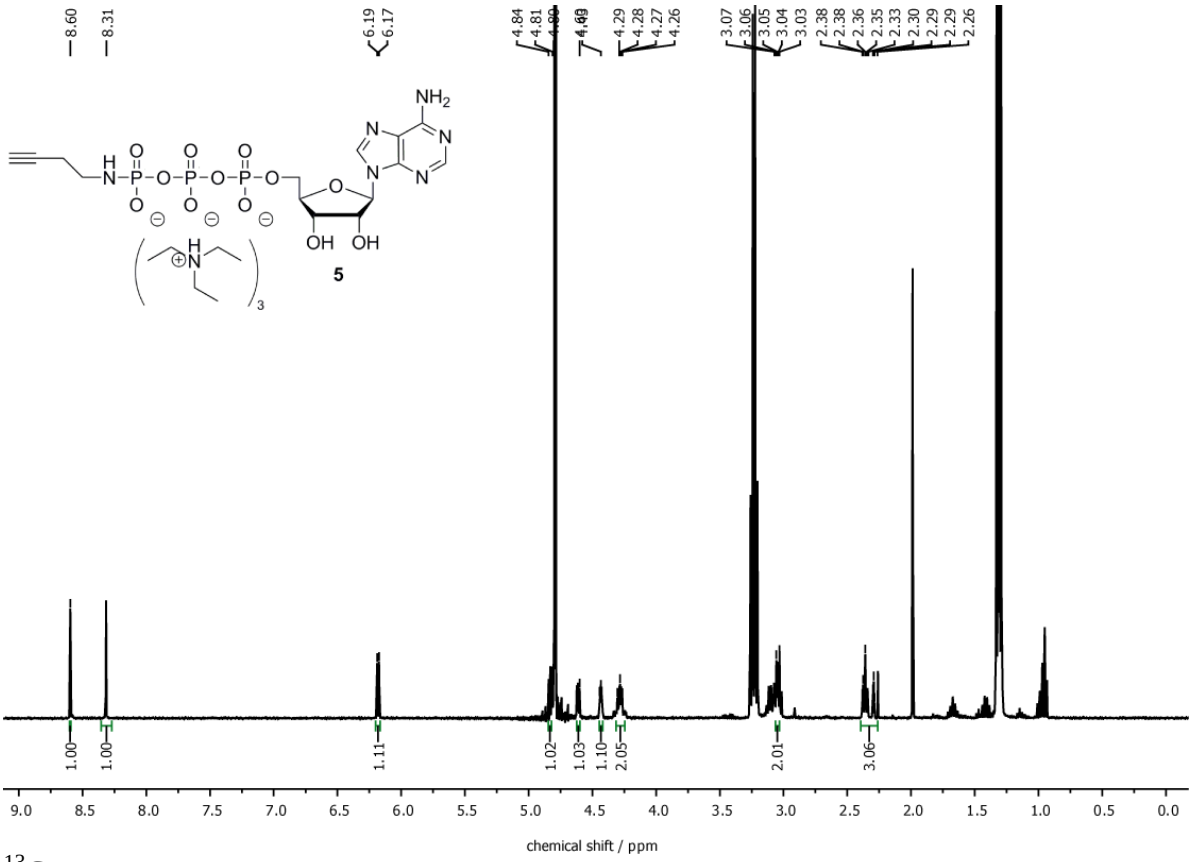


^{13}C NMR:

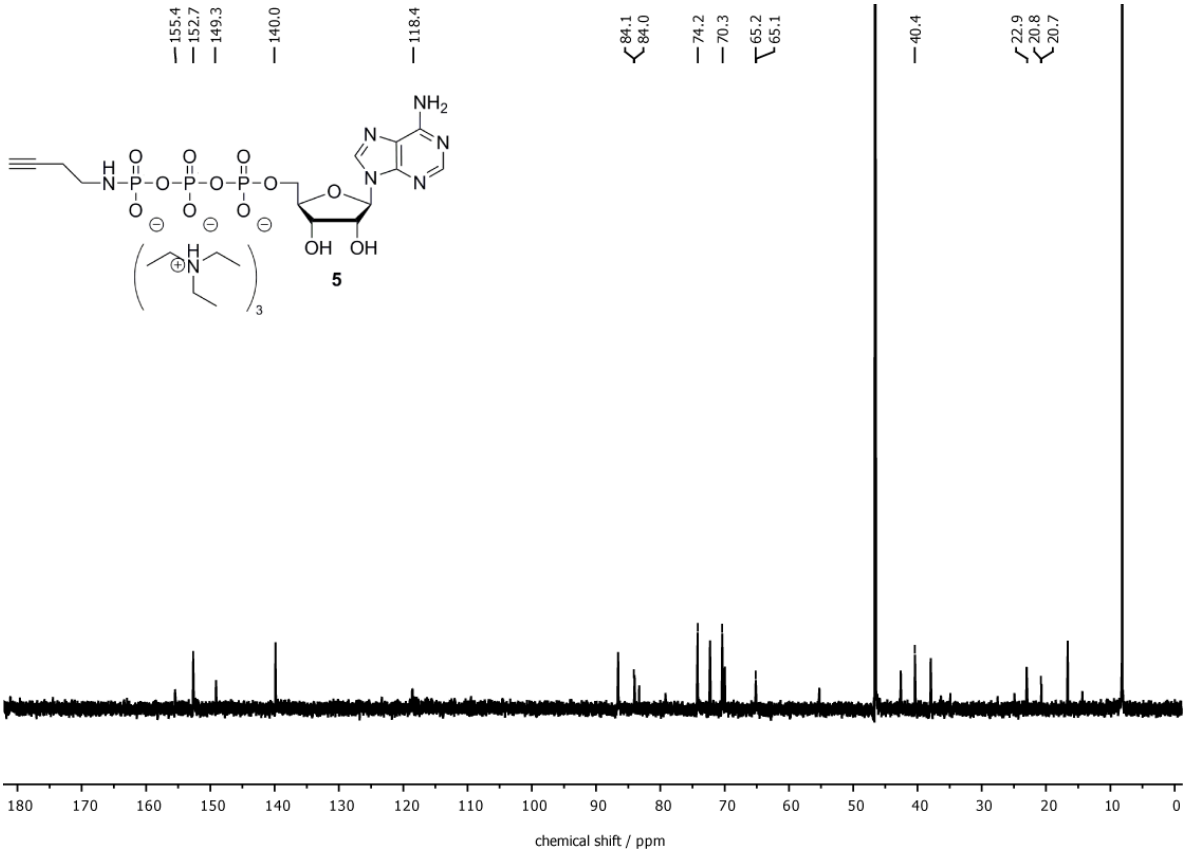


γ-*N*-(But-3-yn-1-ylamido)adenosine-5'-triphosphate (5)

^{1}H NMR:

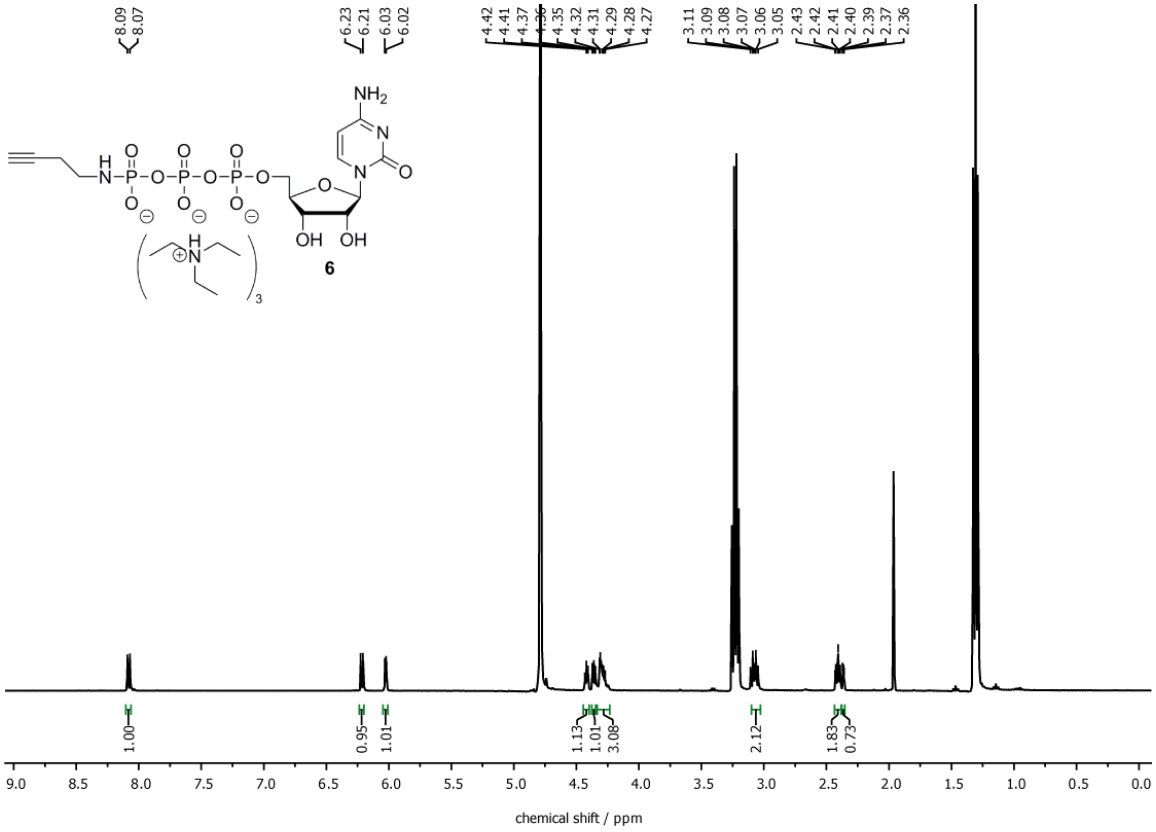


^{13}C NMR:

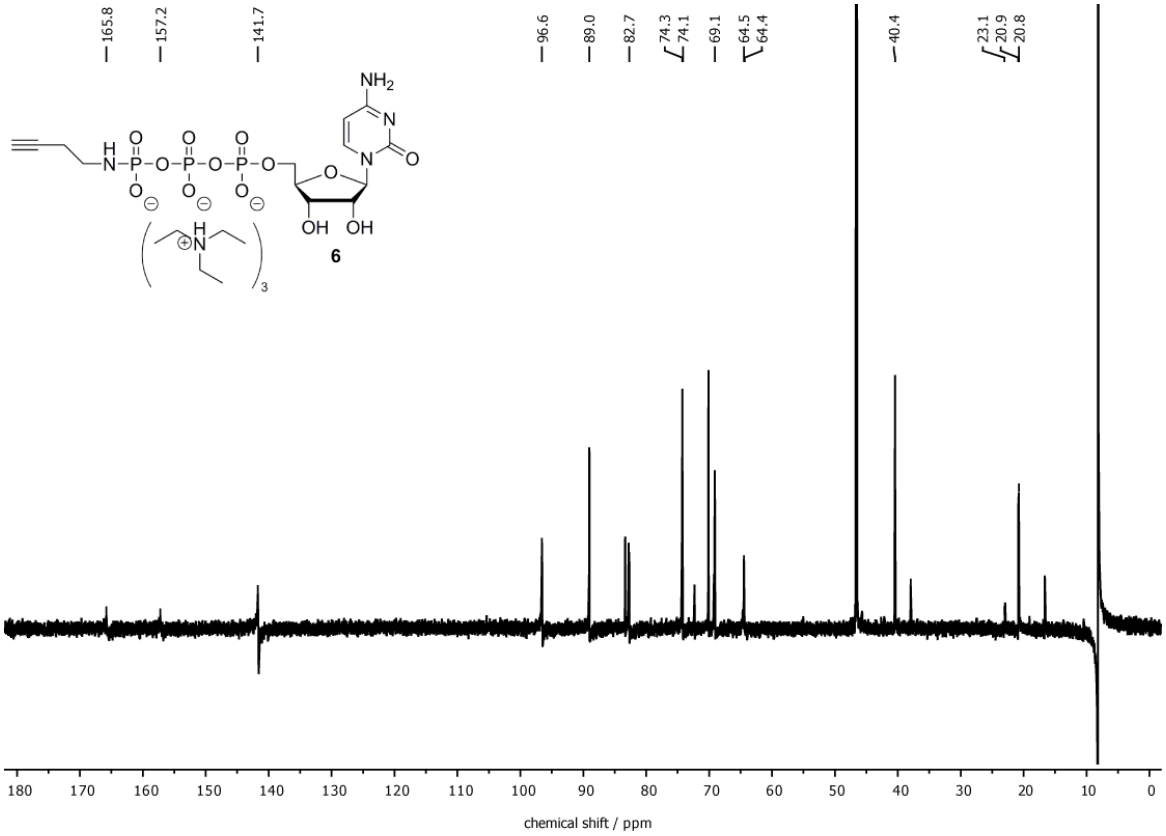


γ-*N*-(But-3-yn-1-ylamido)cytidine-5'-triphosphate (6)

^{1}H NMR:

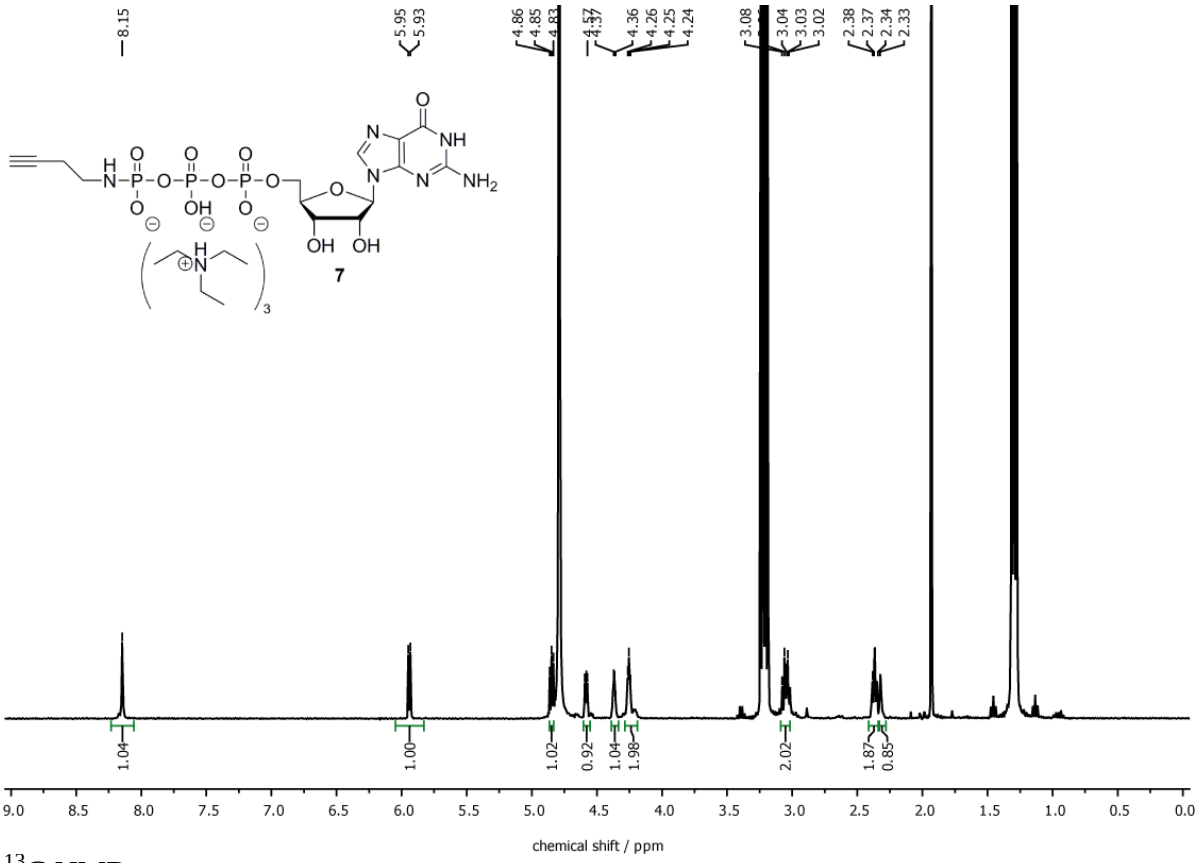


^{13}C NMR:

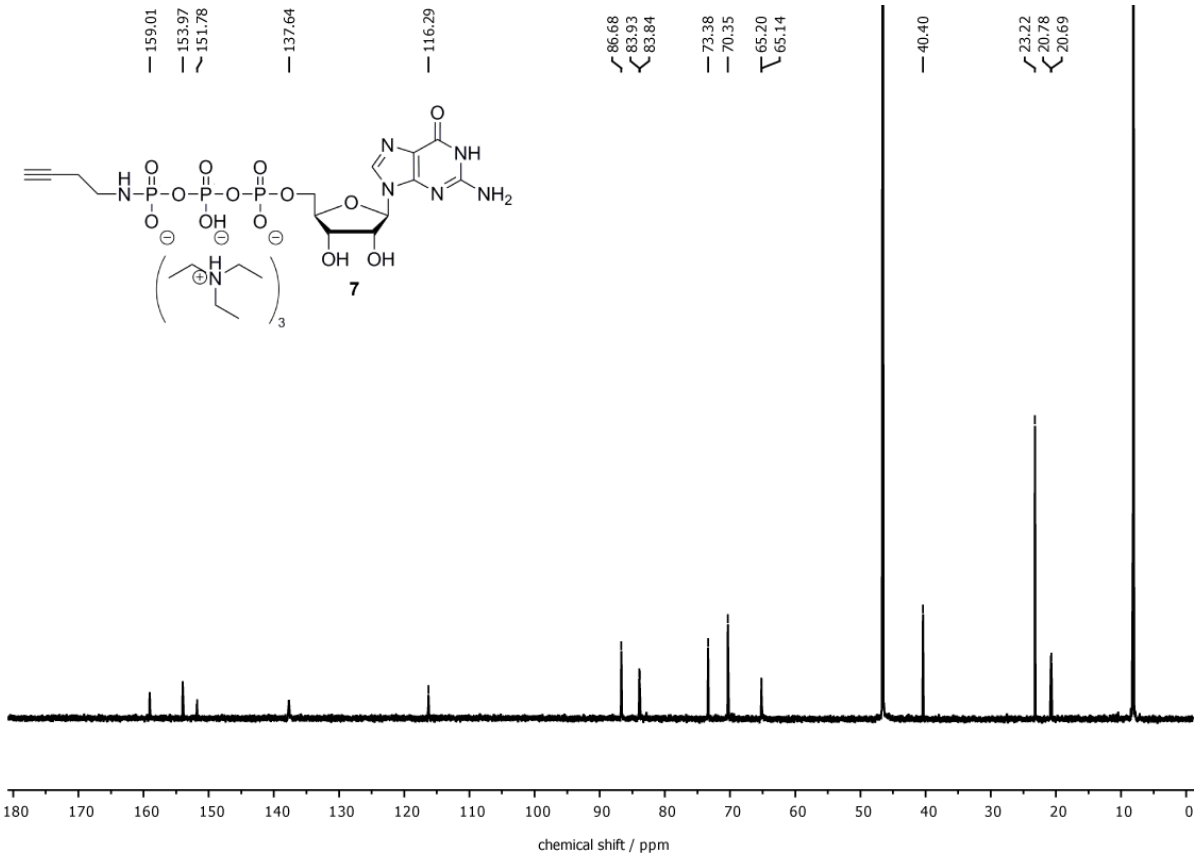


γ-*N*-(But-3-yn-1-ylamido)guanosine-5'-triphosphate (7)

^{1}H NMR:

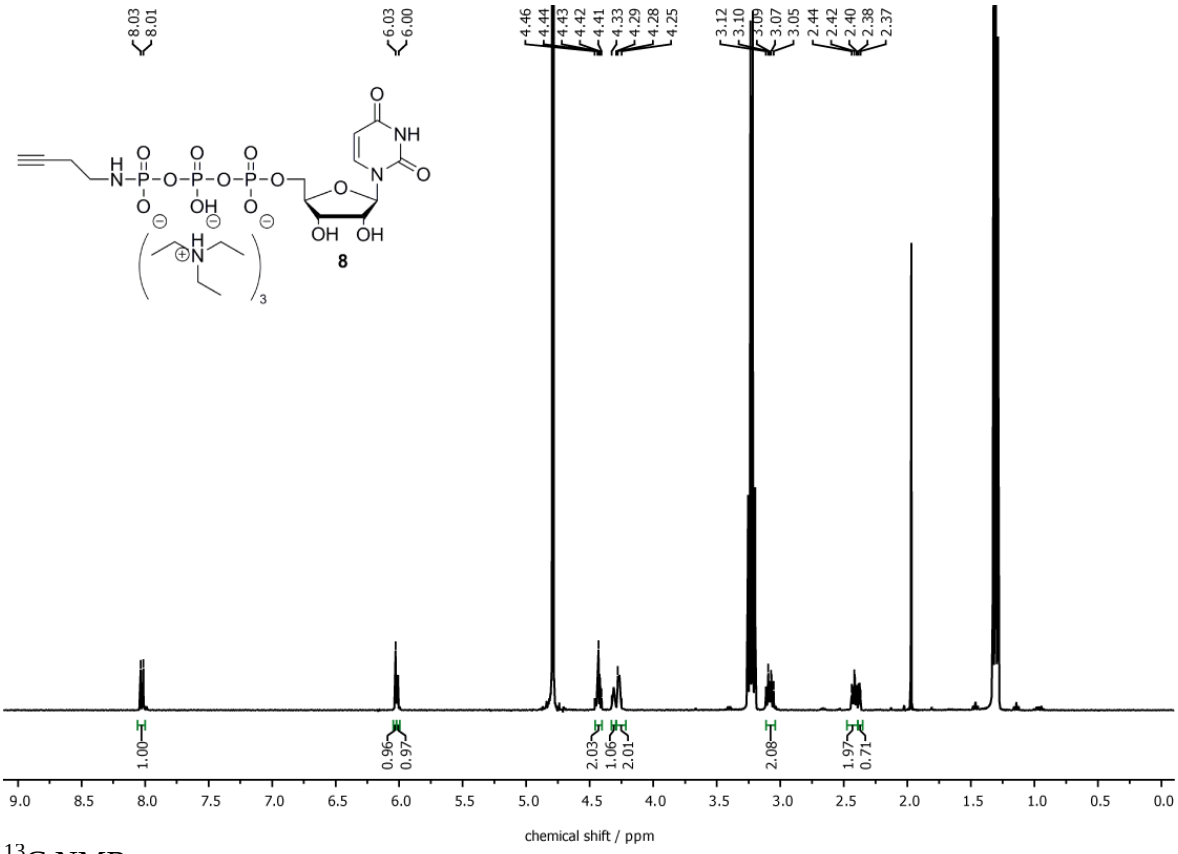


^{13}C NMR:

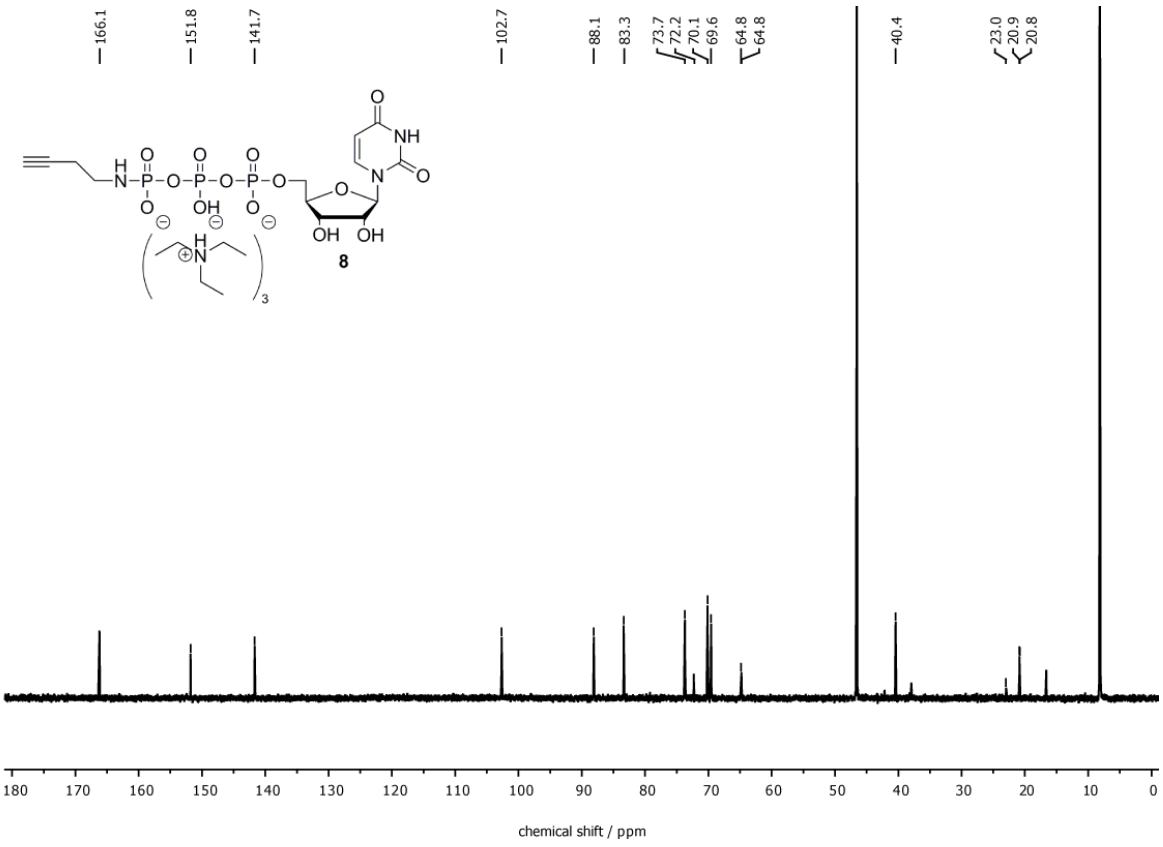


γ-*N*-(But-3-yn-1-ylamido)uridine-5'-triphosphate (8)

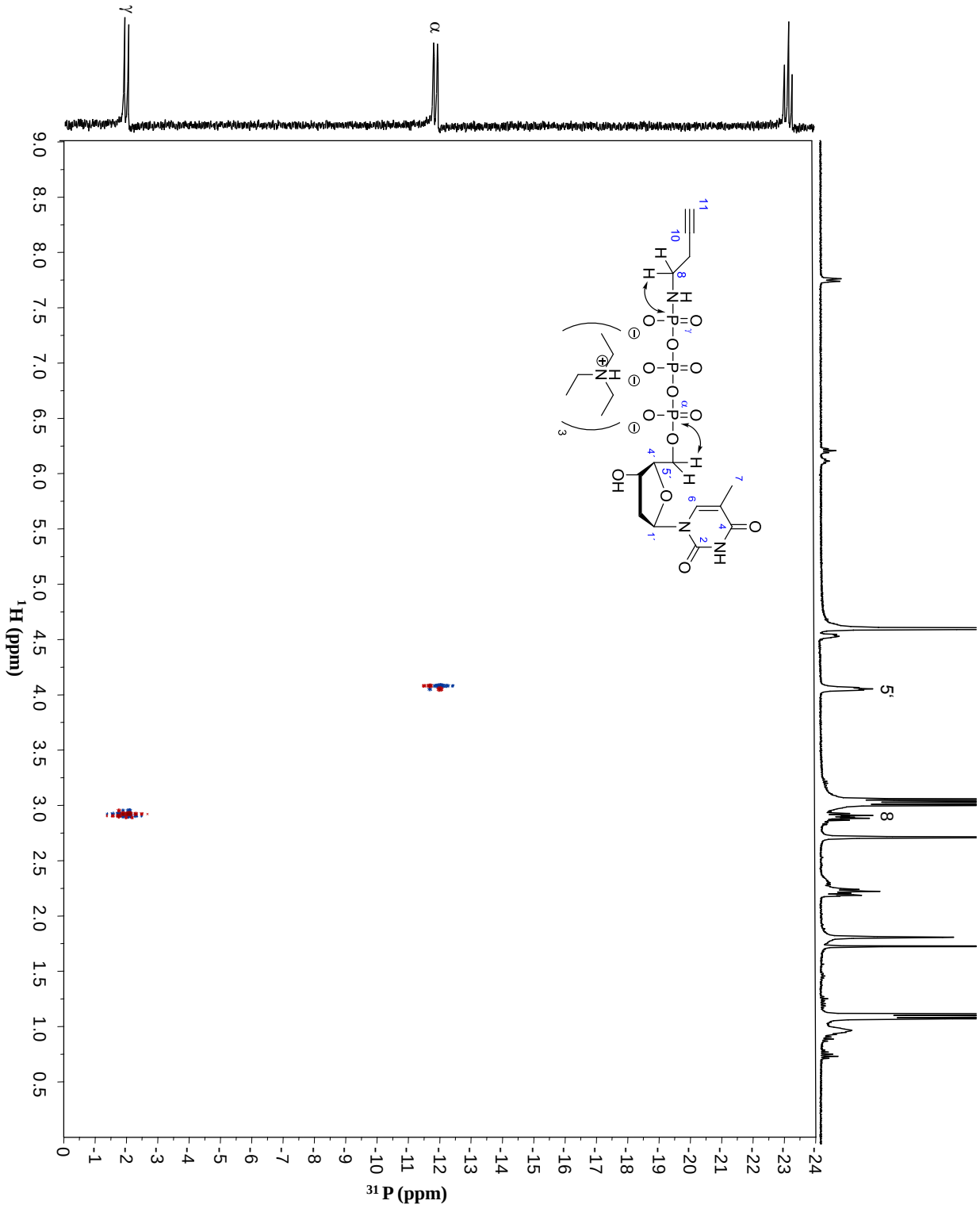
^{1}H NMR:



^{13}C NMR:



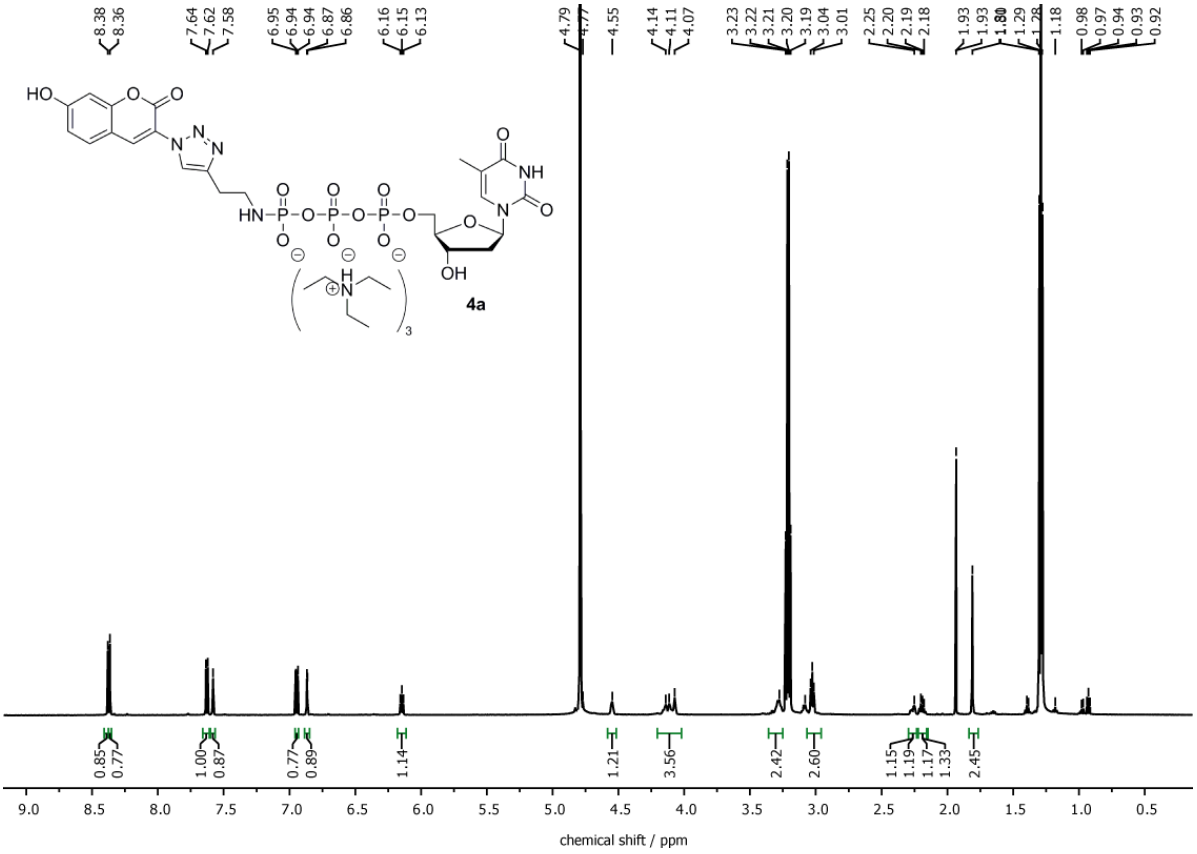
11 ^{31}P-^{1}H HMBC NMR Spectrum of γ-Alkyne Labeled dTTP 4 (Figure S6)



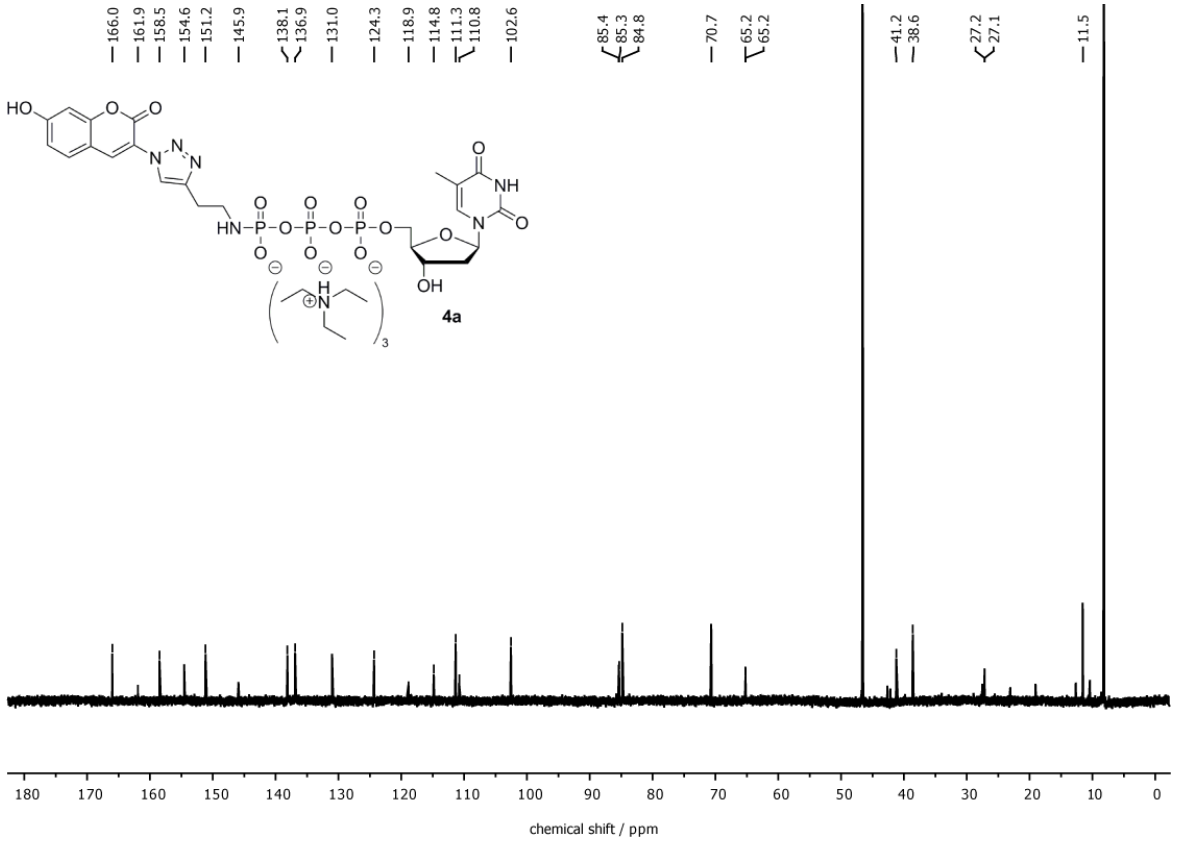
12 ^{1}H, ^{13}C and ^{31}P NMR of γ-Fluorophore Labeled Nucleoside Triphosphates 4a, 5a (Figure S7)

γ-*N*-(2-(1-(7-Hydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-1,2,3-triazol-4-yl)ethylamido)-2´-deoxythymidine-5´-triphosphate (4a)

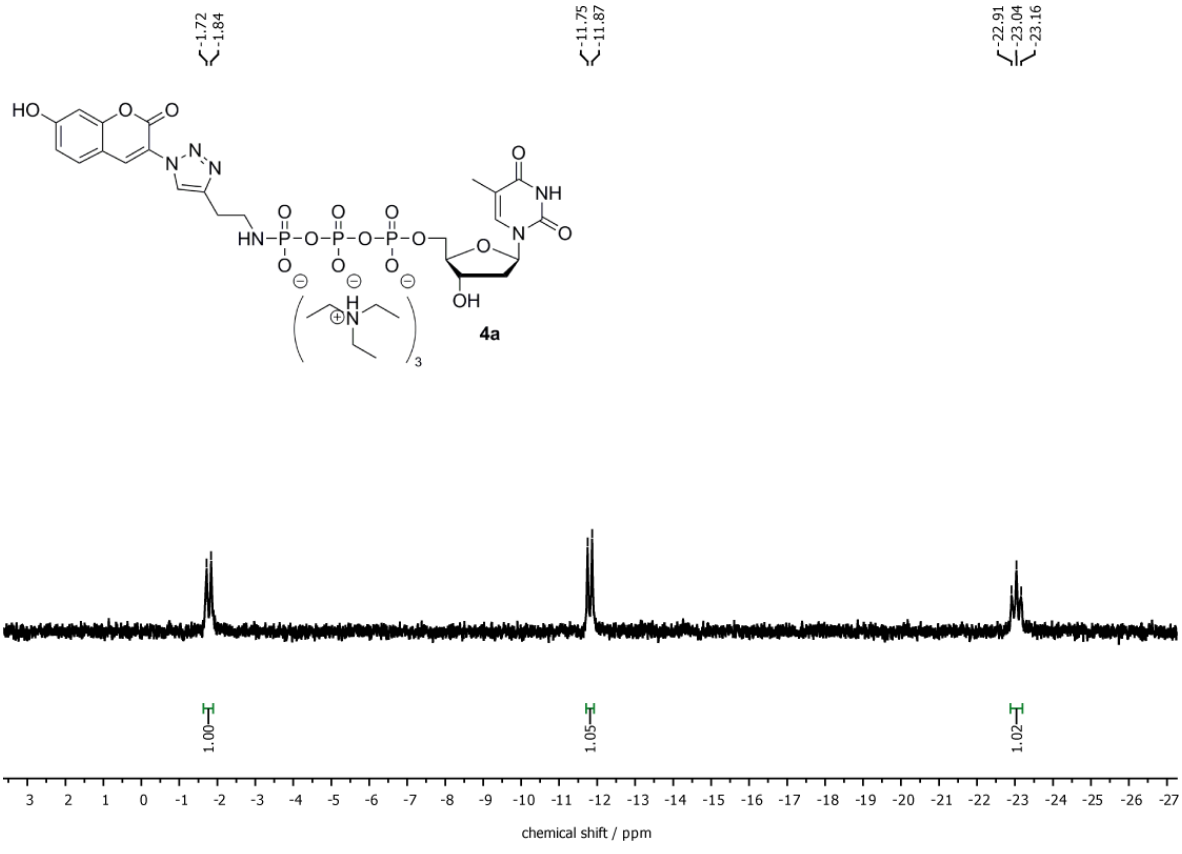
^{1}H NMR:



^{13}C NMR:

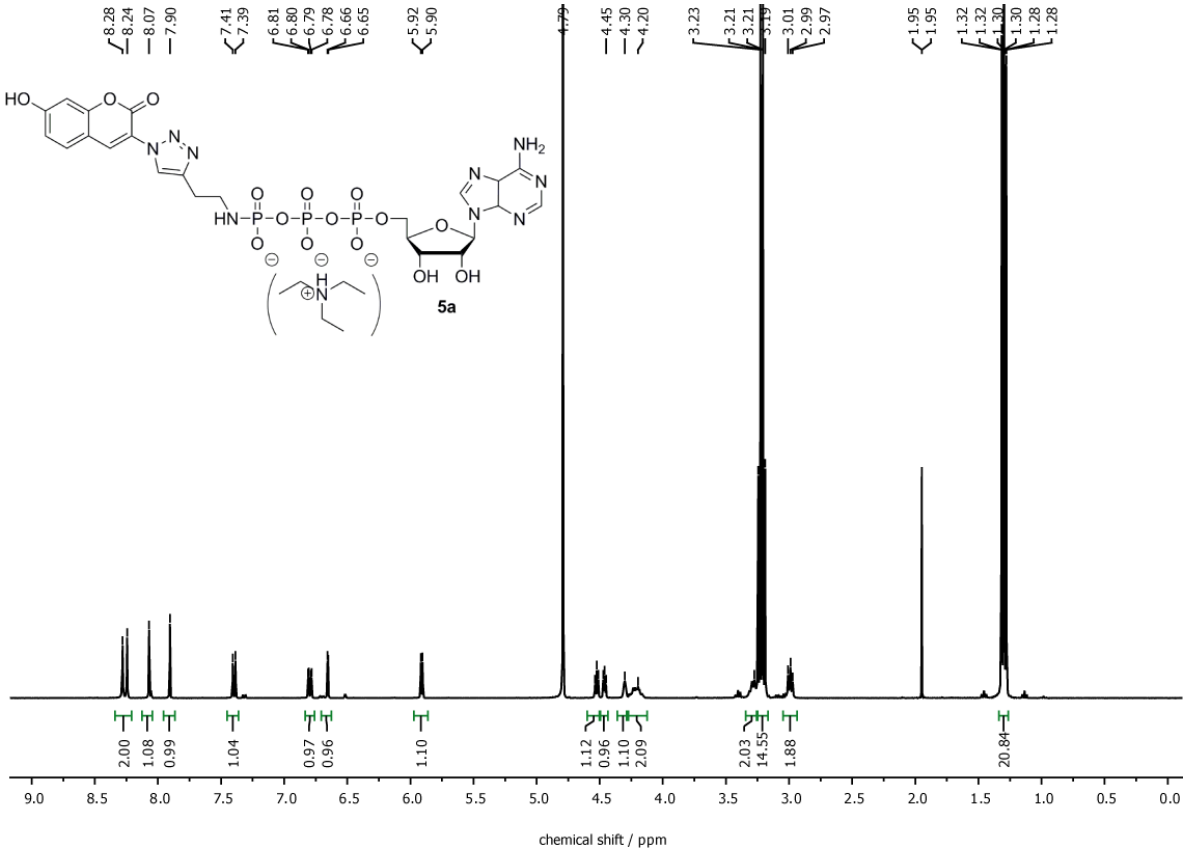


^{31}P NMR:

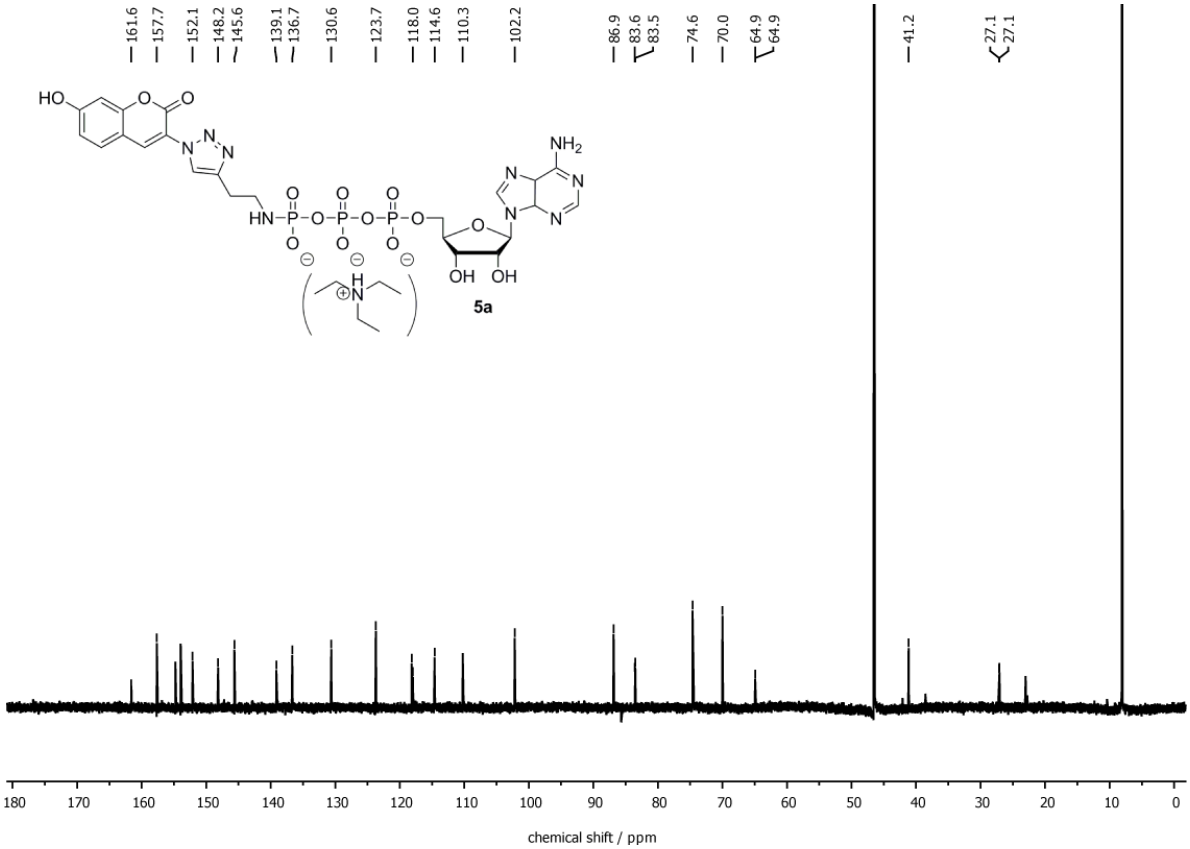


γ-*N*-(2-(1-(7-Hydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-1,2,3-triazol-4-yl)ethylamido)adenosine-5´-triphosphate (5a)

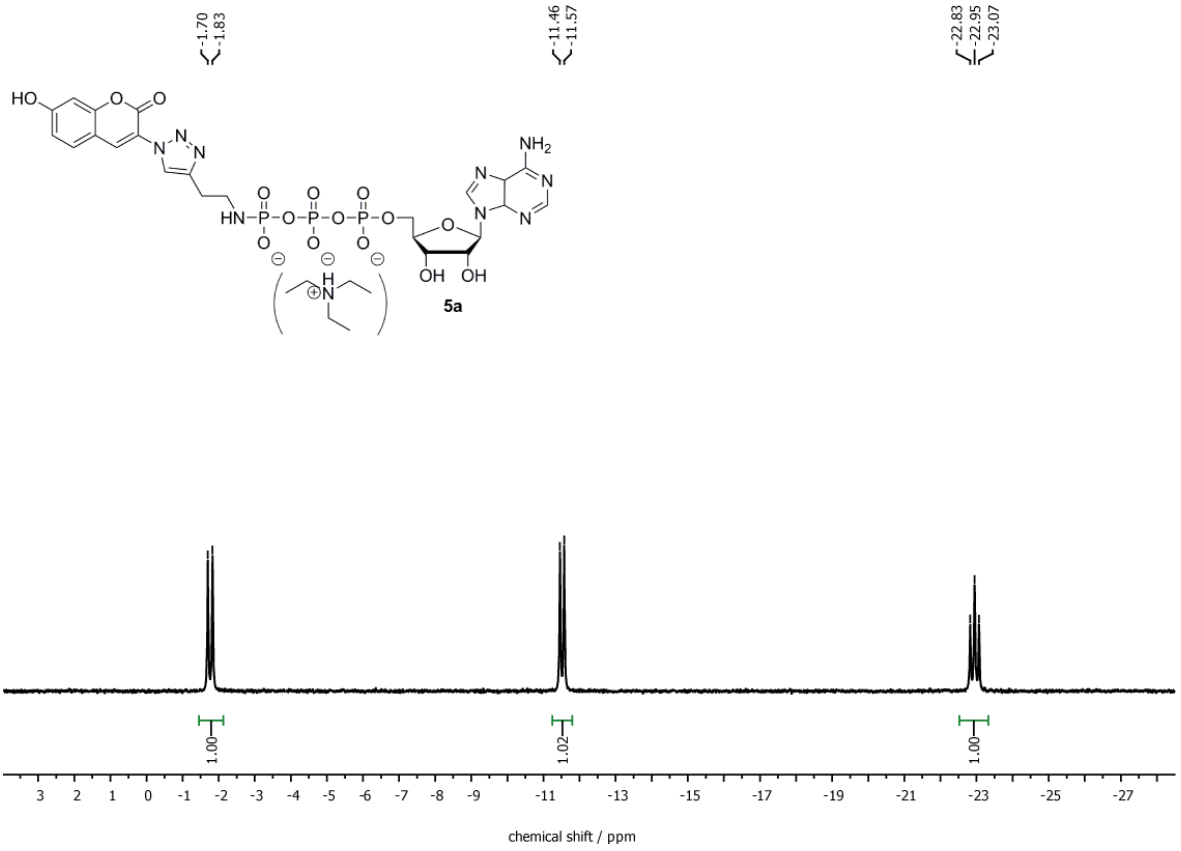
^{1}H NMR:



^{13}C NMR:



^{31}P NMR:



13 Primer Extension Experiments

The primer and template with the following sequences were obtained from METABION:

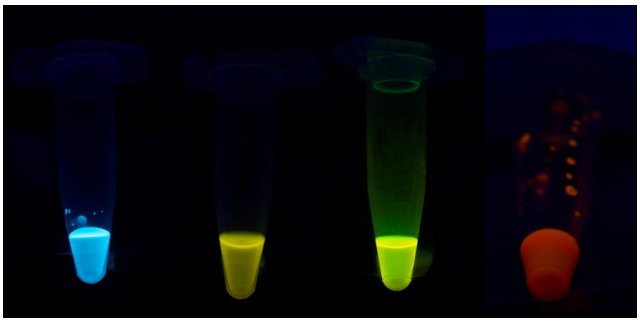
Primer: 5´-Fluo-dGCAGTCTCGCATGTCTCTC-3´

Template: 5´-dGACTGAGAGACATGCGAGACTGC-3´

Using a thermocycler (Mastercycler Personal from EPPENDORF) 20 µM of the 5'-fluoresceine labeled 19mer primer was annealed to a 50% excess of the 23mer template DNA strand in buffer (100 mM NaCl, 25 mM Tris-HCl, pH = 7.6 at 25 °C) prior to primer extension experiments. Therefore, the following temperature gradient was applied: 95 °C for 4 min followed by cooling with 2 °C/min to 4 °C.

Subsequently, primer extension experiments were performed with the exonuclease deficient DNA polymerase I from *E. coli* (Klenow exo$^-$, NEW ENGLAND BIOLABS). In a 10 µL setup 5 pmol of dsDNA was premixed in NEBuffer 2 (NEW ENGLAND BIOLABS; 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9). 200 µM of dNTPs or RP-HPLC purified γ-fluorophore labeled dNTPs were added, followed by the addition of one unit polymerase. The mixtures were incubated at 37 °C for 30 min and then stopped by addition of one volume of loading dye (7 M urea, 30% glycerine, 88 mM Tris-HCl, 88 mM NaH_2BO_4, 2 mM EDTA, 0.025% bromophenol blue). For analysis, the primer extension products were resolved on 20% denaturing polyacrylamide gels (7 M urea, 35 mA, 1000 V) and visualized using a LAS-3000 imaging system (RAYTEST).

14 Optical Appearance of the Fluorophore-Labeled dTTPs 4a-4d



Optical appearance of the fluorophore-labeled dTTP solutions **4a-4d** upon excitation with light at 366 nm. From left to right: Coumarin-dTTP **4a**, fluorescein-dTTP **4d**, BODIPY-dTTP **4b**, TAMRA-dTTP **4c** at concentrations from 4.2-5.0 mM.

15 References

[1] K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill, Q. Wang, *Org. Lett.*, 2004, **6**, 4603-6.

[2] J.-Y. Liu, H.-S. Yeung, W. Xu, X. Li, D. K. P. Ng, *Org. Lett.*, 2008, **10**, 5421-24.