-Supporting Information-

Copper-Free Dual Labeling of DNA by Triazines and Cyclopropenes as Minimal Orthogonal *and* Bioorthogonal Functions

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Materials and Methods

Reactions and air or water sensitive reactants were dried and handled under argon atmosphere in oven-dried round-bottomed flasks. All compounds and solvents used were handled and stored according to supplier's procedures without any subsequent purification. The control of the reaction progress was conducted by thin layer chromatography (TLC) using silica gel coated aluminum plates (MERCK, silica gel 60, thickness 0.2 mm, F_{254}). Analysis of TLC plates was done by irradiation with UV light (λ_{exc} = 254 nm, emission quenching) and staining with 5% H_2SO_4 (in EtOH). Crude reaction products were purified by flash column chromatography on silica gel 60 (MERCK, 230 – 400 mesh). ^{1}H (500 MHz), ^{13}C (126 MHz) and ^{31}P NMR (202 MHz) measurements were performed in deuterated DMSO-d_6 or D_2O at a BRUKER Avance 500 and the resulting spectra were calibrated to the solvent signal at δ = 2.50 ppm (^{1}H) and δ = 39.52 ppm (^{13}C) for DMSO-d_6 or δ = 4.79 (^{1}H) for D_2O. Multiplicity of the signals was abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad singlet. MALDI (*matrix assisted laser desorption ionization*) spectra were recorded on a SHIMADZU AXIMA Confidence using THAP (2,4,6-trihydroxyacetophenon, 0.3 M in ethanol) as matrix in the linear negative mode. High-resolution mass spectra were obtained by *fast atom bombardment* (FAB) on a FINNIGAN MAT 95 mass spectrometer with FAB probe head or by *electrospray ionization* (ESI) on a THERMO FISHER SCIENTIFIC Q Exactive Orbitrap.

Synthetic Procedures

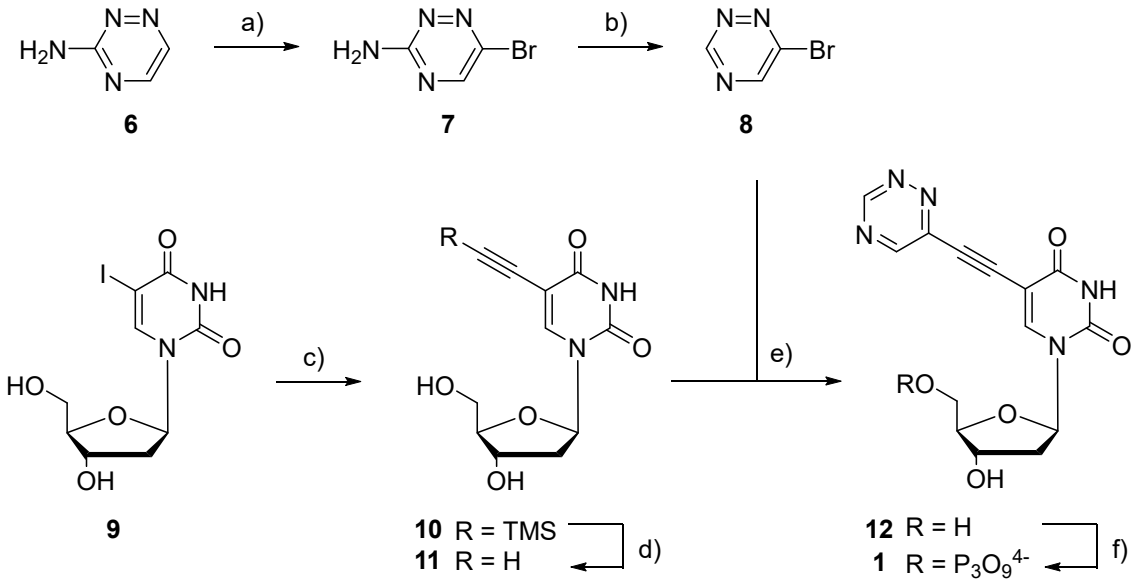
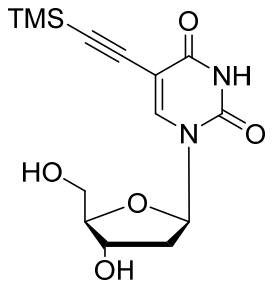


Figure S1: Synthesis of nucleoside triphosphate **1**: a) NBS, MeCN/H_2O, 1 h, 0 °C – r.t., 74% [1], b) isopentylnitrite, THF, 5 h, 65 °C [2], c) CuI, $Pd(PPh_3)_2Cl_2$, TMS-Acetylene, NEt_3, MeCN, o.n., r.t., 76%, d) TBAF (1 M in THF), THF, 30 min, r.t., quant. e) CuI, $Pd(PPh_3)_2Cl_2$, NEt_3, DMF, o.n., r.t., 66%, f) 1. proton sponge, TMP, 4 h, - 15 °C; 2. $(nBu_3NH)_2(P_2O_7)$, nBu_3N, DMF, 15 min, r.t.; 3. TEAB buffer (0.1 M), 4 h, r.t., 18%.

Compound 10



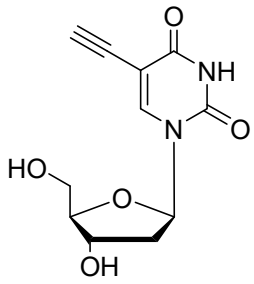
Chemical Formula: $C_{14}H_{20}N_2O_5Si$
Molecular Weight: 324,41

To a dry, argon-flushed Schlenk flask charged with 1.00 g 5-Iodo-2'-deoxyuridine (2.83 mmol, 1.00 equiv.) 25 mL dry NEt_3 and 25 mL dry acetonitrile were added. To this solution 34.9 mg CuI (0.18 mmol, 0.07 equiv.), 129 mg $Pd(PPh_3)_2Cl_2$ (0.18 mmol, 0.07 equiv.) and 1.60 mL TMS-acetylene (1.11 g, 11.3 mmol, 4.00 equiv.) were added. The resulting reaction mixture was stirred at room temperature overnight. The solvent was removed and the crude product purified by column chromatography (DCM; 10% MeOH). The product was obtained as light brown foam in 76% yield (700 mg, 2.16 mmol).

TLC (DCM; 10 % MeOH): R_f = 0.37.

The spectroscopic data were consistent with those reported in the literature.[3]

Compound 11



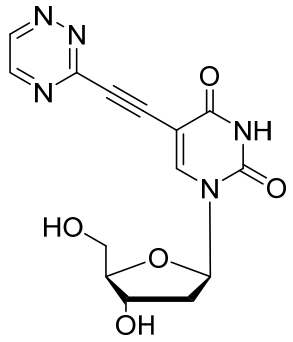
Chemical Formula: $C_{11}H_{12}N_2O_5$
Molecular Weight: 252,23

To a solution of 700 mg **10** (2.16 mmol, 1.00 equiv.) in 4 mL dry THF 5.61 mL TBAF (1.0 M in THF, 1.47 g, 5.61 mmol, 2.00 equiv.) was added. The reaction mixture was stirred at room temperature for 30 min. Afterwards silica was added to the mixture and the solvent was removed. After column chromatography (DCM; 0 – 10% MeOH) the desired product was obtained as white solid in quantitative yield (544 mg, 2.16 mmol).

TLC (DCM; 10 % MeOH): R_f =0.18.

The spectroscopic data were consistent with those reported in the literature.[3]

Compound 12



Chemical Formula: $C_{14}H_{13}N_5O_5$
Molecular Weight: 331,29

In a dry, argon-flushed Schlenk flask 310 mg **11** (1.23 mmol, 1.00 equiv.) were dissolved in 20 mL abs. DMF. To this solution 681 µL NEt_3 (497 mg, 4.92 mmol, 4.00 equiv.), 46.7 mg CuI (0.25 mmol, 0.20 equiv.) and 86.0 mg $Pd(PPh_3)_2Cl_2$ (0.12 mmol, 0.10 equiv.) were added and the mixture was degassed with argon for 5 min. An excess of freshly prepared **8**[1,2] was added and this mixture was stirred overnight at room temperature. The solvent was removed whereby the crude product was directly adsorbed to silica and purified by column chromatography (DCM; 0 - 10% MeOH). The product was obtained as a brown solid in 66% yield (269 mg, 0.81 mmol).

TLC (DCM; 10 % MeOH): R_f = 0.28.

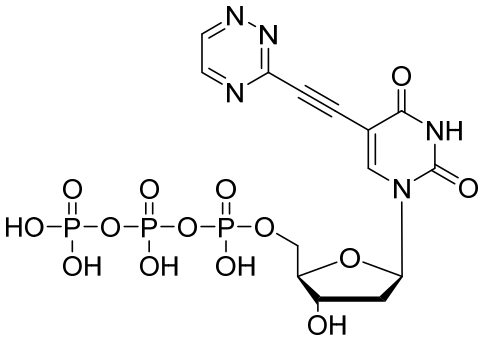
UV-Vis: $\varepsilon_{260\,nm}$ = 6083 L/(mol·cm).

^{1}H-NMR (500MHz, DMSO-d_6): δ (ppm) = 11.87 (s, 1H, NH), 9.75 (s, 1H, Ar-H), 8.96 (s, 1H, Ar-H), 8.66 (s, 1H, Ar-H), 6.11 (t, *J* = 6.3 Hz, 1H, 1‘-H), 5.32 (d, *J* = 4.3 Hz, 1H, 3‘-OH), 5.27 (t, *J* = 4.8 Hz, 1H, 5‘-OH), 4.27 (t, *J* = 4.4 Hz, 1H, 3‘-H), 3.86 – 3.80 (m, 1H, 4‘-H), 3.71 – 3.57 (m, 2H, 5‘-H), 2.20 (q, *J* = 4.9, 3.8 Hz, 2H, 2‘-H).

^{13}C-NMR (126 MHz, DMSO-d_6): δ (ppm) = 161.2 (C_q), 154.8 (Aryl-CH), 151.9 (Aryl-CH), 149.4 (C_q), 147.7 (C_q), 146.7 (Aryl-CH), 96.1 (C_q), 91.8 (C_q), 87.7 (4‘-C), 86.1 (C_q), 85.4 (1‘-C), 69.7 (3‘-C), 60.6 (5‘-C), 40.4 (2‘-C).

HR-MS (ESI): calc. 354.0814 [MNa^+], found 354.0815.

Compound 1



Chemical Formula: $C_{14}H_{16}N_5O_{14}P_3$
Molecular Weight: 571,22

In a dry argon-flushed flask **12** (30.0 mg, 90.6 µmol, 1.00 equiv.) and proton sponge (29.1 mg, 0.14 mmol, 1.50 equiv.) were dried overnight *in vacuo*. Trimethylphosphate and nBu$_3$N were dried under argon atmosphere overnight with 4 Å molecular sieve.

The solids were dissolved under argon atmosphere in 0.95 mL trimethylphosphate and cooled to - 15 °C. $POCl_3$ (8.86 µL, 14.6 mg, 0.10 mmol, 1.05 equiv.) was added and after 4 h at – 15 °C another portion $POCl_3$ (3.37 µL, 5.55 mg, 0.04 mmol, 0.40 equiv.) was added and stirred for an additional hour. Separately $(nBu_3NH)_2H_2P_2O_7$ (298 mg, 0.54 mmol, 6.00 equiv.) was dired *in vacuo* for 1 h, dissolved in abs. DMF (0.4 M) and mixed with nBu$_3$N (94.9 µL, 73.9 mg, 0.40 mmol, 4.40 equiv.). This solution was then added at once to the reactin mixture and the cooling bath was removed. After 15 min the reaction mixture was added dropwise into 0.1 M TEAB-buffer (3 mL, pH = 7.3) over 40 min and stirred for 4 h at room temperature. Afterwards the solution was frozen and lyophilized. The crude product was first purified by reversed phase HPLC (0 – 35 % MeCN in TEAB buffer (50 mM, pH = 7.3) over 60 min) and then purified by SAX-HPLC (0 – 100 % MeCN/H_2O (15:85) in TEAB buffer (0.85 M, pH = 7.3) over 60 min). The fractions containing product were combined, freeze-dried and the yield was determined by absorption at 260 nm ($\varepsilon_{260\,nm}$ = 6083 L/(mol·cm)). The product was obtained as yellowish solid in 18% yield (16.5 µmol).

^{1}H-NMR (500MHz, D_2O): δ (ppm) = 9.58 (s, 1H, Ar-H), 9.10 (s, 1H, Ar-H), 8.50 (s, 1H, Ar-H), 6.29 (t, *J* = 6.7 Hz, 1H, 1‘-H), 4.66 (dt, *J* = 6.8, 3.3 Hz, 1H, 3‘-H), 4.27 – 4.19 (m, 3H, 4‘-H, 5‘-H), 2.55 – 2.35 (m, 2H, 2‘-H).

^{31}P-NMR (202 MHz, D_2O): δ (ppm) = - 10.54 (d, J = 20.1 Hz, 1P), - 11.65 (d, J = 20.5 Hz, 1P), - 23.33 (t, J = 20.3 Hz, 1P).

MALDI-MS: m/z: 566.78 $[M-4H]^-$

Primer Extension (PEX) and Labeling

Primer extension experiments were conducted in a thermocycler TGradient 96 (BIOMETRA/ANALYTIK JENA) using micro tubes (THERMO FISHER SCIENTIFIC). Reaction mixtures (9.5 µL) containing primer (750 nM), template (900 nM) and reaction buffer (2 µL of *ThermoPol* for *Vent(exo-)* and *Deep Vent (exo-)* or 4 µL *Hemo KlenTaq* buffer, respectively) were hybridized by heating to 90 °C for 10 min and subsequent cooling to 25 °C (1.2 °C/ min). Afterwards commercially available DNA-polymerase (1 U; NEW ENGLAND BIOLABS) and the appropriate dNTP-mixtures (400 µM each) were added and dissolved to a final sample volume of 20 µL. The elongation was performed at 37 °C with different elongation times as described in the figure captions of the corresponding PAGE analysis. Afterwards, the product mixture was cooled to room temperature and diluted with 20 µL of loading buffer (20 mM EDTA, 89 mM TRIS, 89 mM boric acid, 4% Ficoll, 0.1% bromophenol, 0.02% xylene cyanol FF, 7.0 M urea, pH 8.0) to inhibit further elongation.

For bioorthogonal labeling the enzymatic primer extension was conducted as described above and the primer extension product was subsequently desalinated without the addition of loading buffer using *illustra MicroSpin G-25* columns (GE HEALTHCARE). The resulting mixture was lyophilized and the residue was resolved in *Millipore-water* to the original concentration and afterwards incubated with 1000 equiv. (compared to the used primer) of the respective dye for a defined period of time (as stated in the figure captions) at room temperature. Labeling reactions were stopped by the addition of loading buffer and PAGE analysis was performed. For PAGE analysis of samples containing cy5-modified primer **P2** loading buffer excluding xylene cyanol FF was used. For dual labeling experiments primer extension was carried out on a 500 µl scale with an elongation time of 30 min at 37 °C. Afterwards the mixture was desalinated which led to a total volume of 1 mL. This dilution was used for labeling with the respective dye. After incubation with BCN-modified rhodamine **3** another desalination step was performed, the resulting solution was lyophilized and the residue was resolved in *Millipore-water* before conjugation with the respective tetrazine-dye was carried out.

For the incorporation of conjugated triphosphates, the KOD XL polymerase was used. Therefore, reaction mixtures (9.6 µL) containing primer (740 nM), template (900 nM) and KOD XL DNA reaction buffer (2 µL) were hybridized as described above. Afterwards KOD XL DNA-

polymerase (1 U, MERCK NOVAGEN) was added. Triphosphate mixtures were prepared directly before use. Therefore, **1** or **2** was mixed with 1 equivalent of the respective dye and incubated at room temperature (**1** + **3** (30 min); **2** + **4** (30 min); **2** + **4** (15 min) + **3** (30 min); **2** + **5** (15 min)). Afterwards the residual natural triphosphates were added. Those dNTP-mixtures (800 µM of the labeled triphosphate; 400 µM natural triphosphates) were added to the reaction mixtures and dissolved to a final sample volume of 20 µL. The elongation was performed at 72 °C for 30 min. Afterwards the product mixtures were cooled to room temperature, diluted with 20 µL loading buffer and analsed by denaturing polyacrylamide gel electrophoresis.

Denaturing Polyacrylamide Gel Electrophoresis

The product mixtures from PEX and iEDDA experiments were analysed by denaturing (12.5%) polyacrylamide gel electrophoresis (PAGE). Therefore, aqueous acrylamide/bisacrylamide (19:1) gel mixtures containing urea (8.3 M), TEMED (2.65 mM), EDTA (2 mM, pH 8.0), TRIS base (89 mM) and boric acid (89 mM) were polymerized (30 min) by the addition of ammonium persulfate (4 mM) as radical source in a *Sequi-Gen GT* Sequencing Cell (38 cm × 50 cm) with a *PowerPac HV* (BIO-RAD) at constant 50 W (max 3 kV) and a temperature of 50 °C for ~ 60 min. As electrolyte a freshly prepared TBE buffer (89 mM TRIS base, 89 mM boric acid, and 2 mM EDTA, pH 8.0) was used. The subsequent analysis of the resulting PAGE gel was carried out using a *Stella 8300* fluorescence imager (RAYTEST) by emission read out of the used dyes. These were irradiated from above with different LED-lamps with the following excitation wavelengths: λ_{exc} = 470 ± 20 nm for fluorescein and BODIPY FL, λ_{exc} = 540 ± 10 nm for rhodamine B and tetramethylrhodamine (TAMRA) and λ_{exc} = 630 ± 10 nm for cy5. The resulting fluorescence signals were detected by a CCD camera (cooled to −20 °C) by the use of different emission filters (535 ± 20, 605 ± 10 and 700 ± 17.5 nm). An *AIDA Image Analyzer* software (RAYTEST) was used for the analysis of the acquired pictures. Labeling yields were quantified in the *1D Multi Labeling mode*.

Images of polyacrylamide gel electrophoresis (PAGE) analysis

Polymerase screening for the primer extension with 1

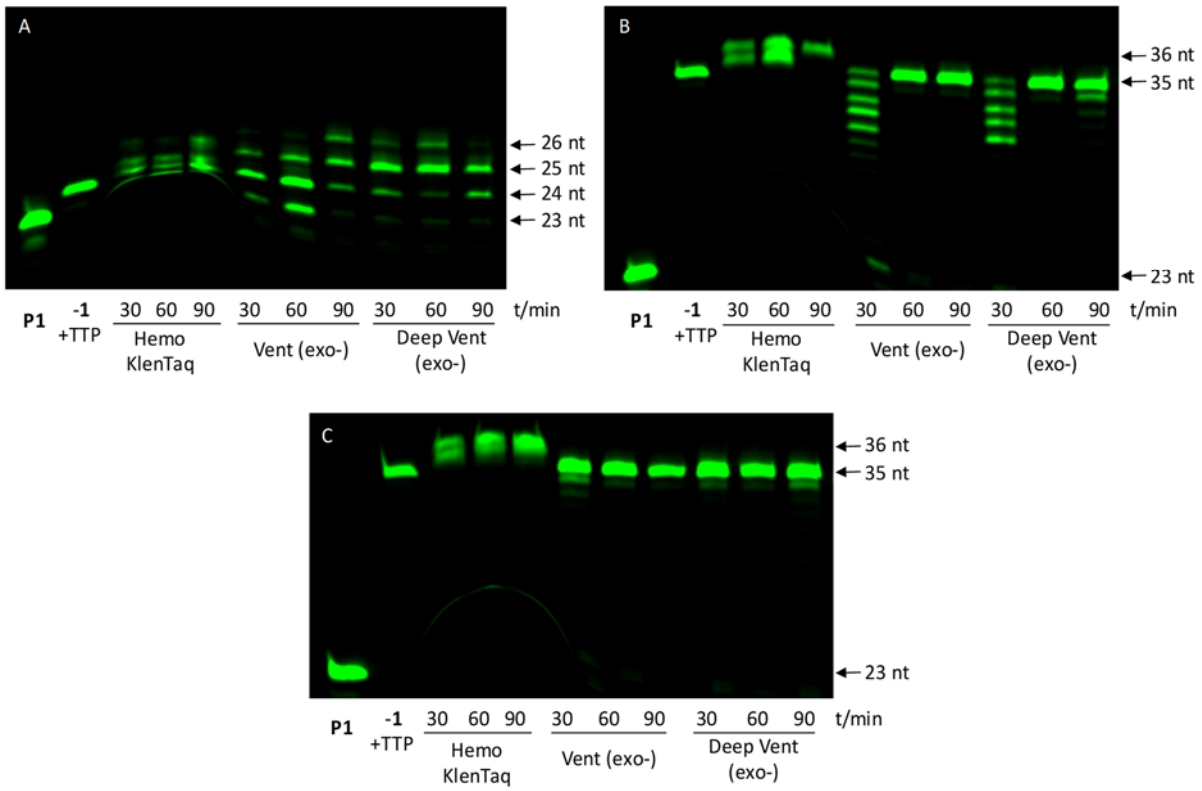


Figure S2: PAGE analysis of the polymerase screening for the incorporation of **1** at 37 °C using different elongation times (30, 60, 90 min); fluorescein fluorescence: λ_{exc} = 470 nm, λ_{em} = 535 nm. (A) standing start experiment with **P1** (23nt), **T1** and **1**. "-**1**/+TTP" refers to **P1** elongated by only 1 TTP (24 nt). (B) standing start experiment with **P1** (23 nt), **T1**, **1**, dATP, dCTP and dGTP. "-**1**/+TTP" refers to **P1** fully elongated with natural nucleotides (35 nt). (C) running start experiment with **P1** (23 nt) **T2**, **1**, dATP, dCTP and dGTP. "-**1**/+TTP" refers to **P1** fully elongated with natural nucleotides (35 nt).

Side Reaction of the Bioorthogonal Dual Labeling (P1/T2)

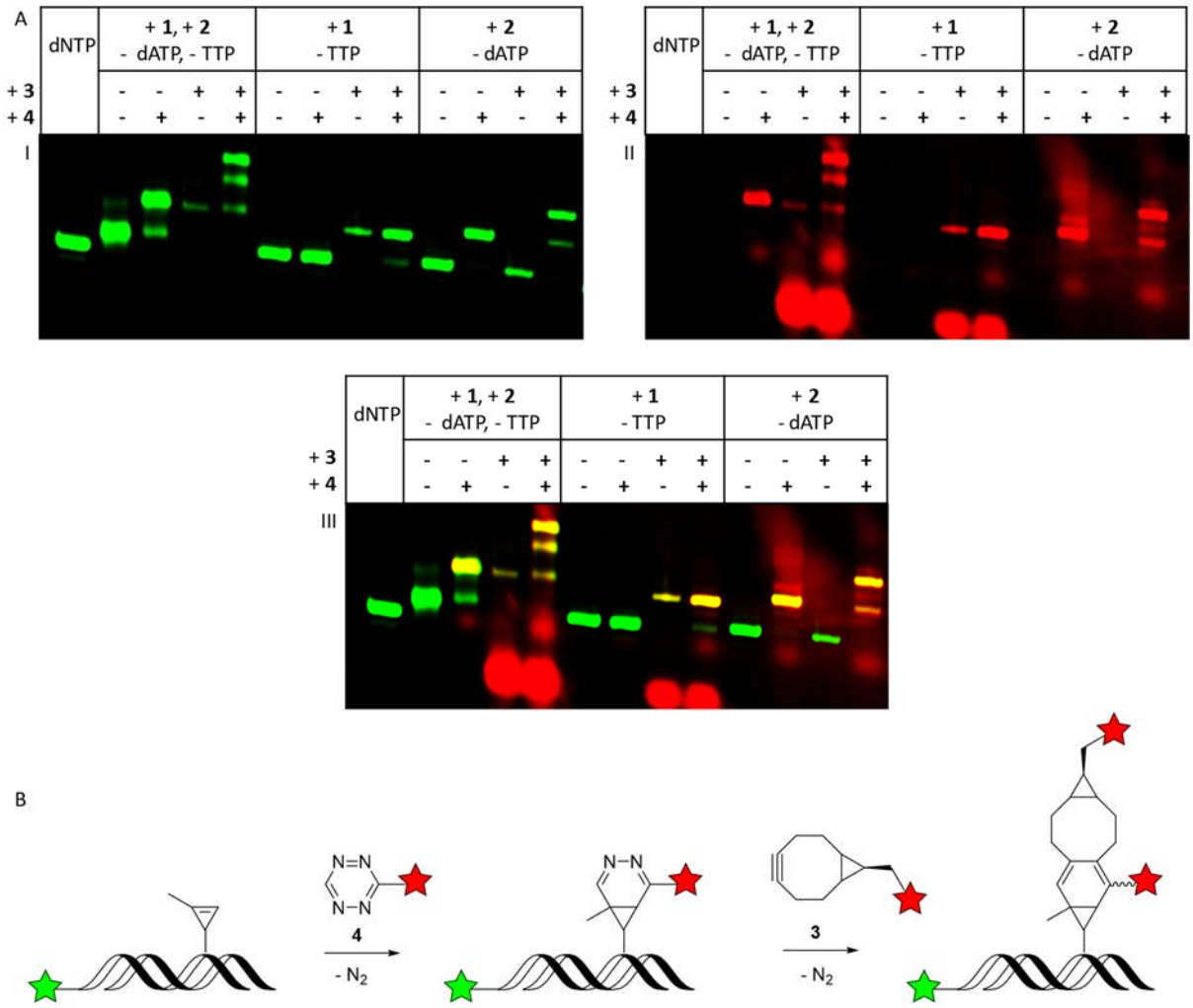


Figure S3: (A) PAGE analysis of labeling reactions using primer extension products from **P1**/**T2** elongated by *Vent (exo-)* at 37 °C for 30 min, yielded by different triphosphate mixtures including **1** and/or **2** in combination with the other unmodified dNTPs, and the fluorescent dyes **3** and **4** (60 min incubation). If both dyes are added the sample was first incubated with **4** and afterwards without any purification with **3** leading to an additional click-product in the event that **1** is present in the oligonucleotide. The lanes "dNTP" refer to the fully extended **P1** using only unmodified triphosphates and serve as references. I) fluorescein fluorescence: λ_{exc} = 470 nm, λ_{em} = 535 nm; II) rhodamine/TAMRA fluorescence: λ_{exc} = 540 nm, λ_{em} = 605 nm, III) overlay of I and II. (B) proposed mechanism for the formation of an additional conjugation product when the cyclopropene moiety is allowed to react with a tetrazine- and a BCN-modified dye.

Control Experiments for the Dual Labeling using P2/T2

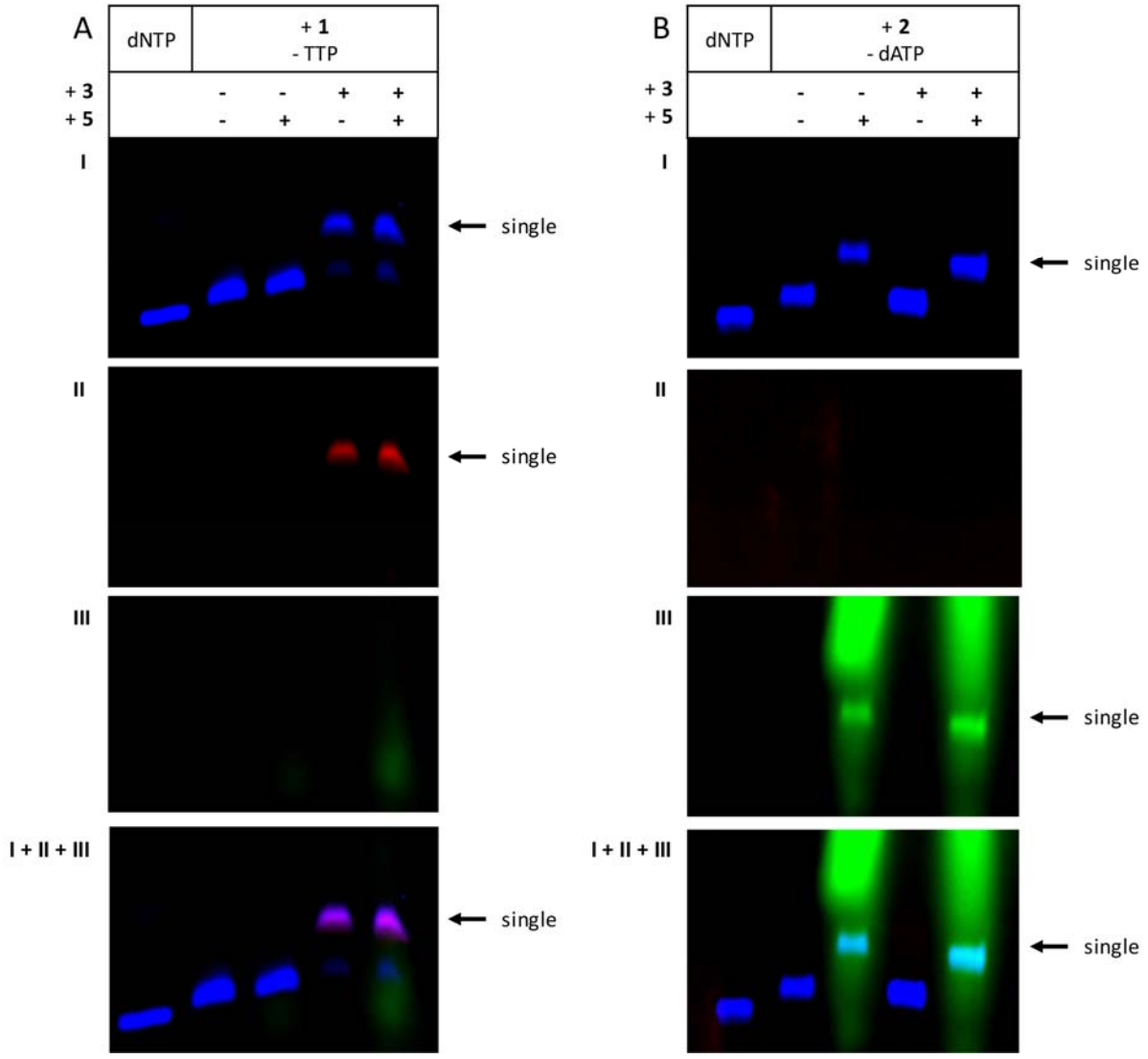


Figure S4: PAGE analysis of control experiments for labeling reactions using elongated **P2** (*Vent (exo-)*, 37 °C with a triphosphate mixture containing either **1** in combination with dATP, dCTP and dGTP (A) or **2** in combination with TTP, dCTP and dGTP (B) for 30 min) and the fluorescent dyes **3** (30 min) and **5** (15 min). The lanes "dNTP" refer to the fully extended **P2** using only unmodified triphosphates and serve as references. I) cy5 λ_{exc} = 630 nm, λ_{em} = 700 nm; II) rhodamine λ_{exc} = 540 nm, λ_{em} = 605 nm; III) BODIPY λ_{exc} = 470 nm, λ_{em} = 535 nm; IV) overlay of I, II and III, whereby a mixed color occurs whenever different signals are present: magenta for cy5 (blue) + rhodamine (red); cyan for cy5 (blue) + BODIPY (green).

Incorporation of labeled triphosphates into oligonucleotides

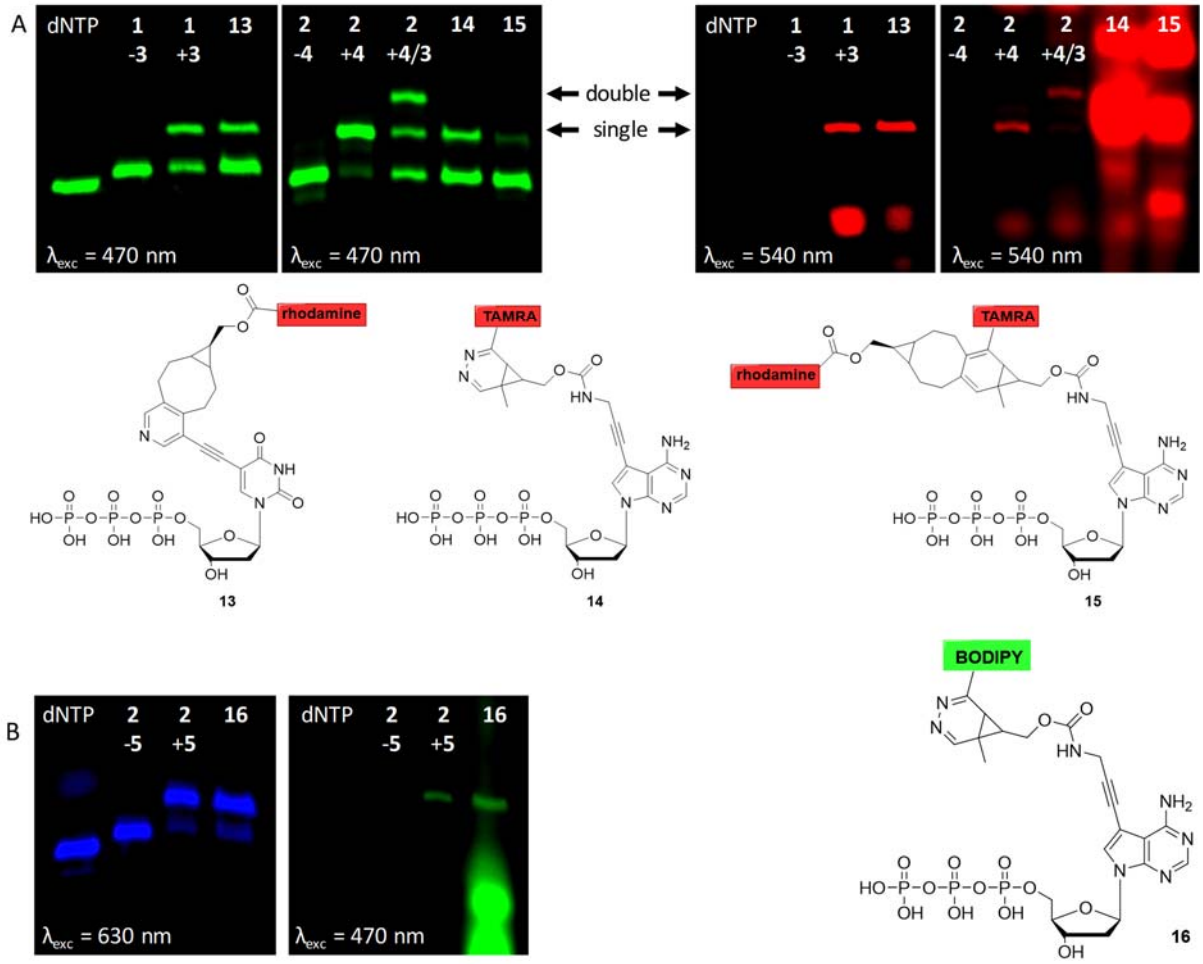


Figure S5: PAGE analysis of primer extension experiment with either the unlabeled triphosphates **1** and **2** or the labeled triphosphates **13**, **14**, **15** or **16** in combination with the other unnatural dNTPs. Unlabeled triphosphates are incorporated using *Vent (exo)* and an elongation time of 30 min at 37 °C. After subsequent desalination the primer extension products are labeled with the respective dyes. Labeled triphosphates are incorporated using KOD XL and an elongation time of 30 min at 72 °C. Fluorescein/BODIPY: λ_{exc} = 470 nm, λ_{em} = 535 nm; rhodamine/TAMRA: λ_{exc} = 540 nm, λ_{em} = 605 nm; cy5: λ_{exc} = 605 nm, λ_{em} = 700 nm. (A) primer extension using **P1**/**T2**. Incorporation of **13** results in the same bands in the fluorescein and the rhodamine channel as incorporation of **1** and subsequent labeling with **3**. Incorporation of **14** shows the same two bands in the fluorescein channel as incorporation of **2** and subsequent labeling with **4**. Evaluation of the TAMRA fluorescence is difficult due to residual dye overlapping with the bands. Whenever **2** is incorporated and labeled with **3** and **4** another side product is visible. In comparison to this, there is no successful incorporation of the double-labeled dATP **15**, which might be due to the large steric hinderence. (B) primer extension using **P2**/**T2**. When **16** is used for primer extension, mostly the BODIPY-dATP-conjugate is incorporated yielding the same bands with fluorescence in the cy5 as well as the BODIPY channel, showing the same product as incorporation of **2** and subsequent labeling with **5**.

Images of NMR spectra and Mass spectra

Compound 12

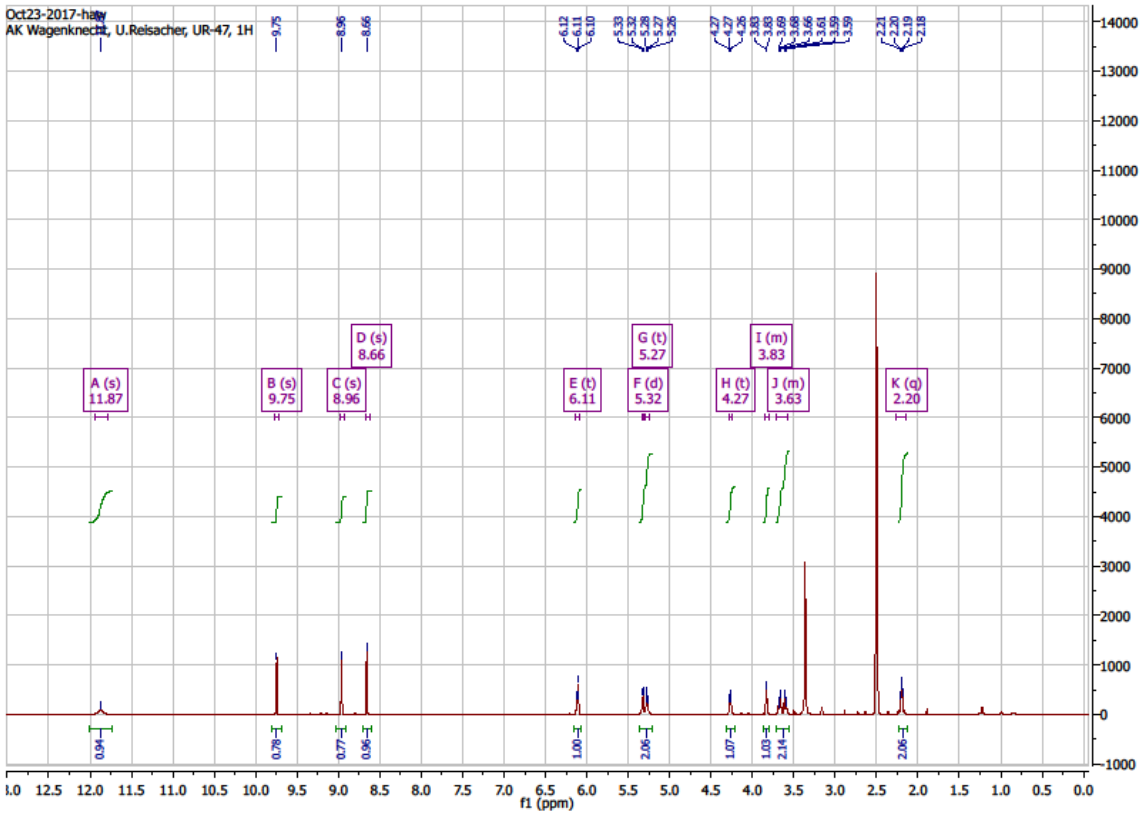


Figure S6: ^{1}H-NMR spectrum of **12** (500 MHz, DMSO-d_6).

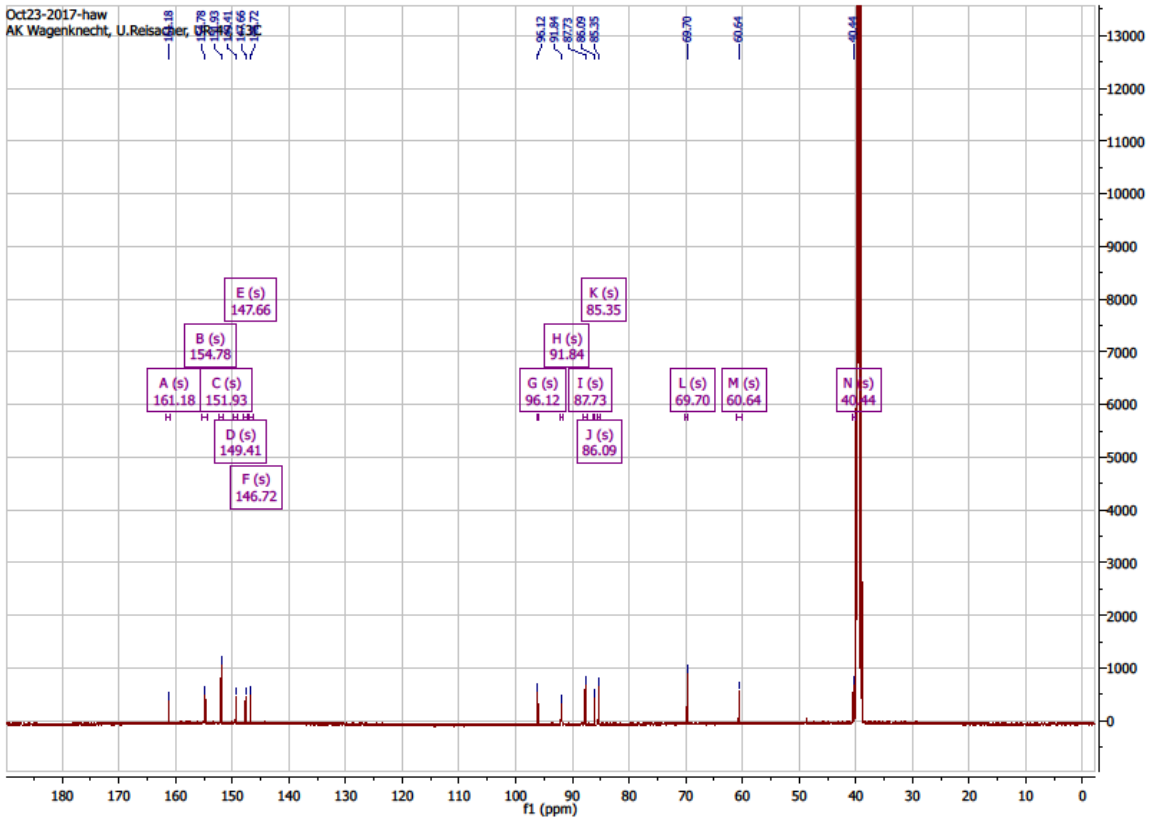


Figure S7: ^{13}C-NMR spectrum of **12** (126 MHz, DMSO-d_6)

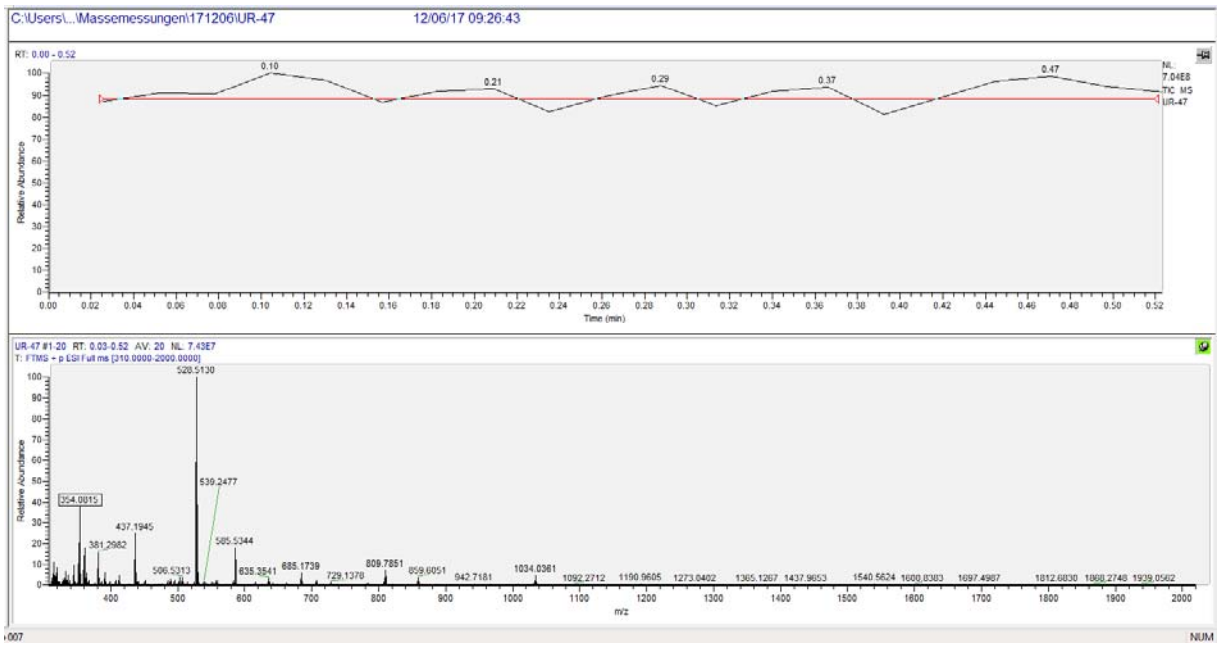


Figure S8: MS-ESI spectrum of **12**.

Compound 1

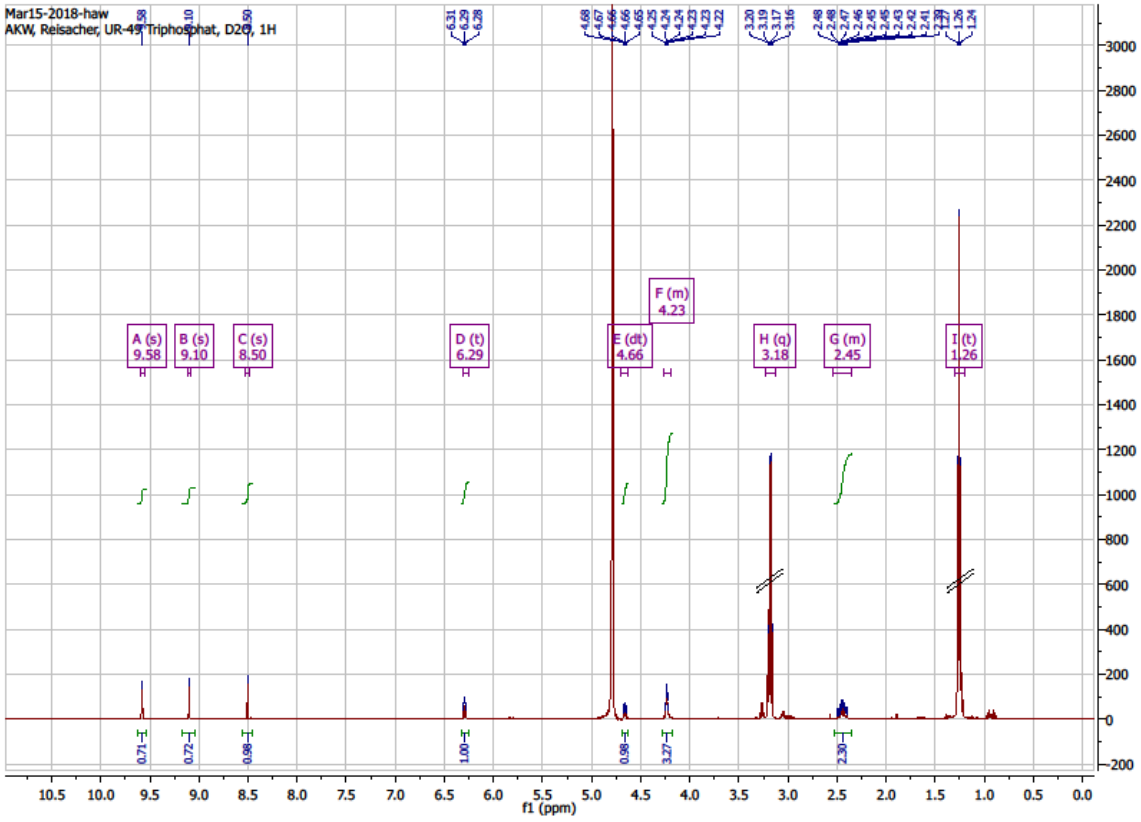


Figure S9: ^{1}H-NMR spectrum of **1** (500 MHz, D_2O). The signals at 3.18 ppm (q) and 1.26 ppm (t) correspond to the triethylammonium ion.

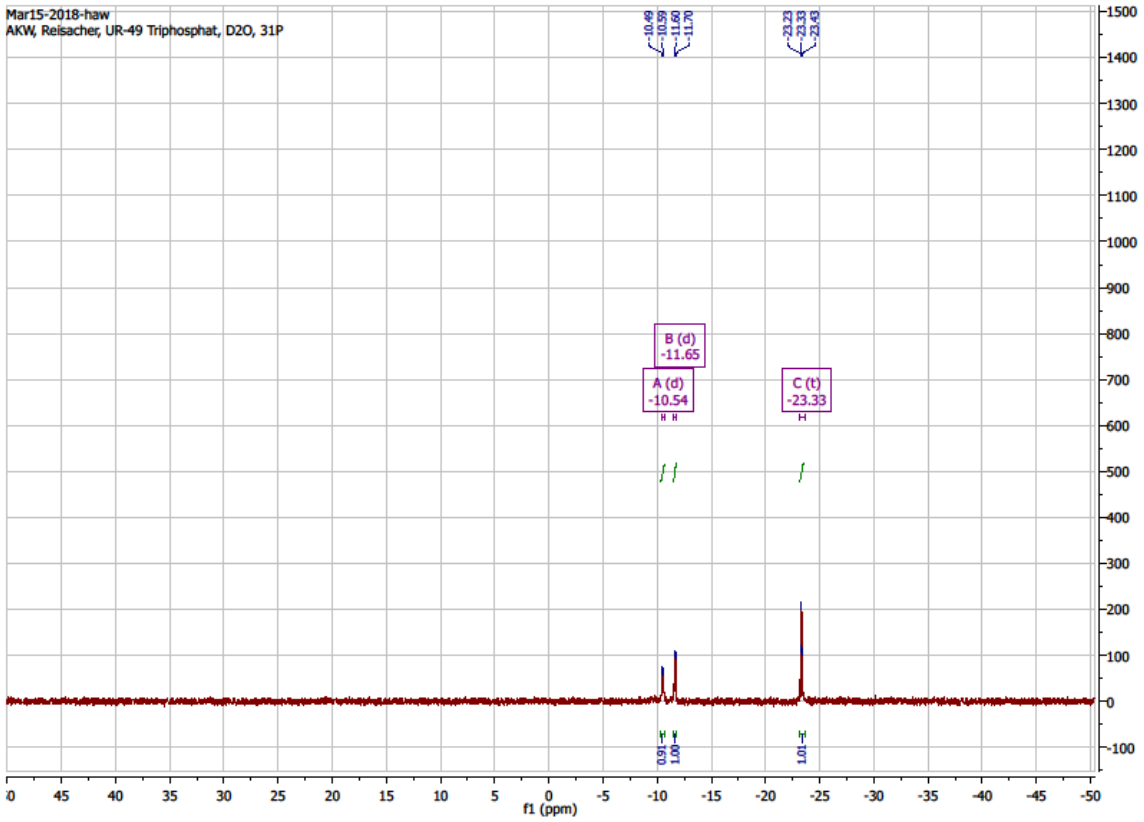


Figure S10: ^{31}P-NMR spectrum of **1** (202 MHz, D_2O).

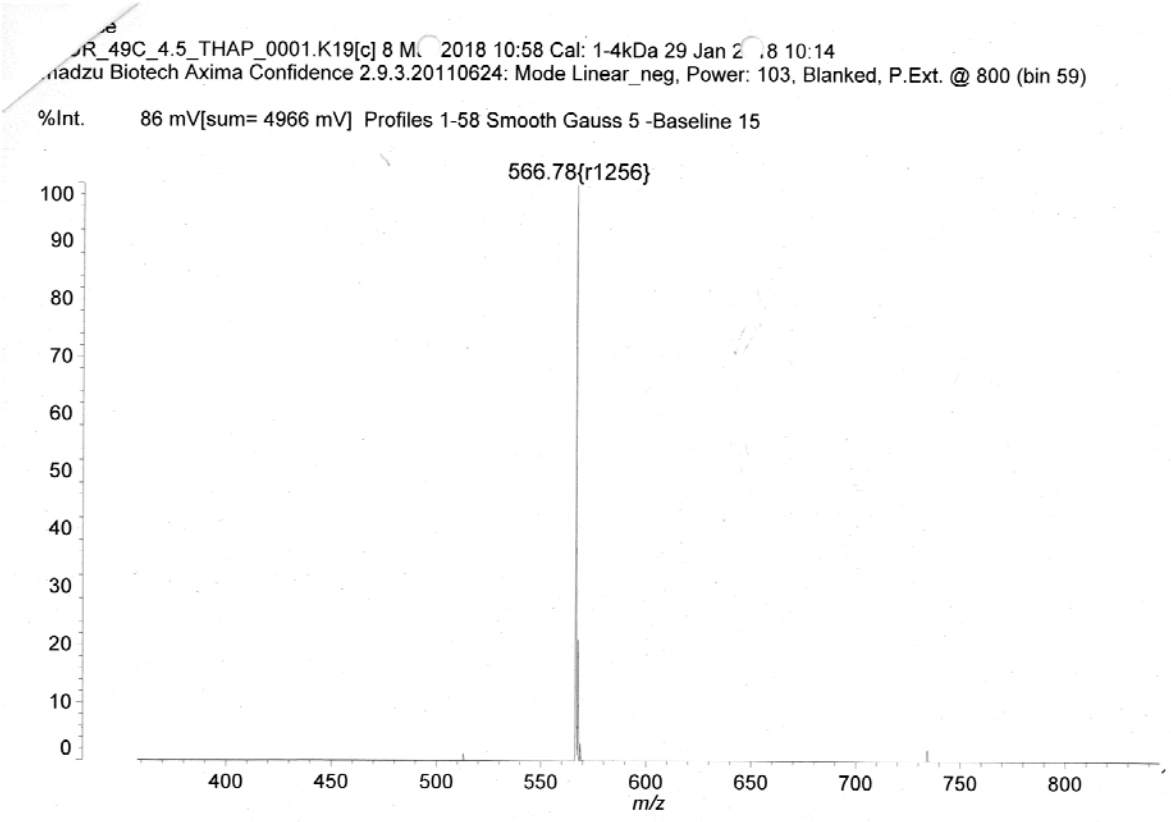
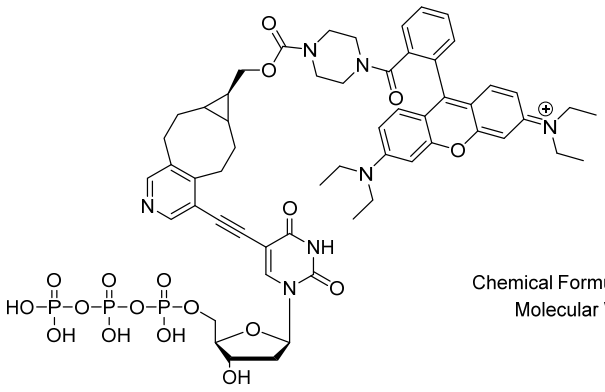


Figure S11: MALDI-TOF MS spectrum of **1.**

Triphosphate Conjugate 13



Chemical Formula: $C_{57}H_{67}N_7O_{18}P_3^+$
Molecular Weight:1231,11

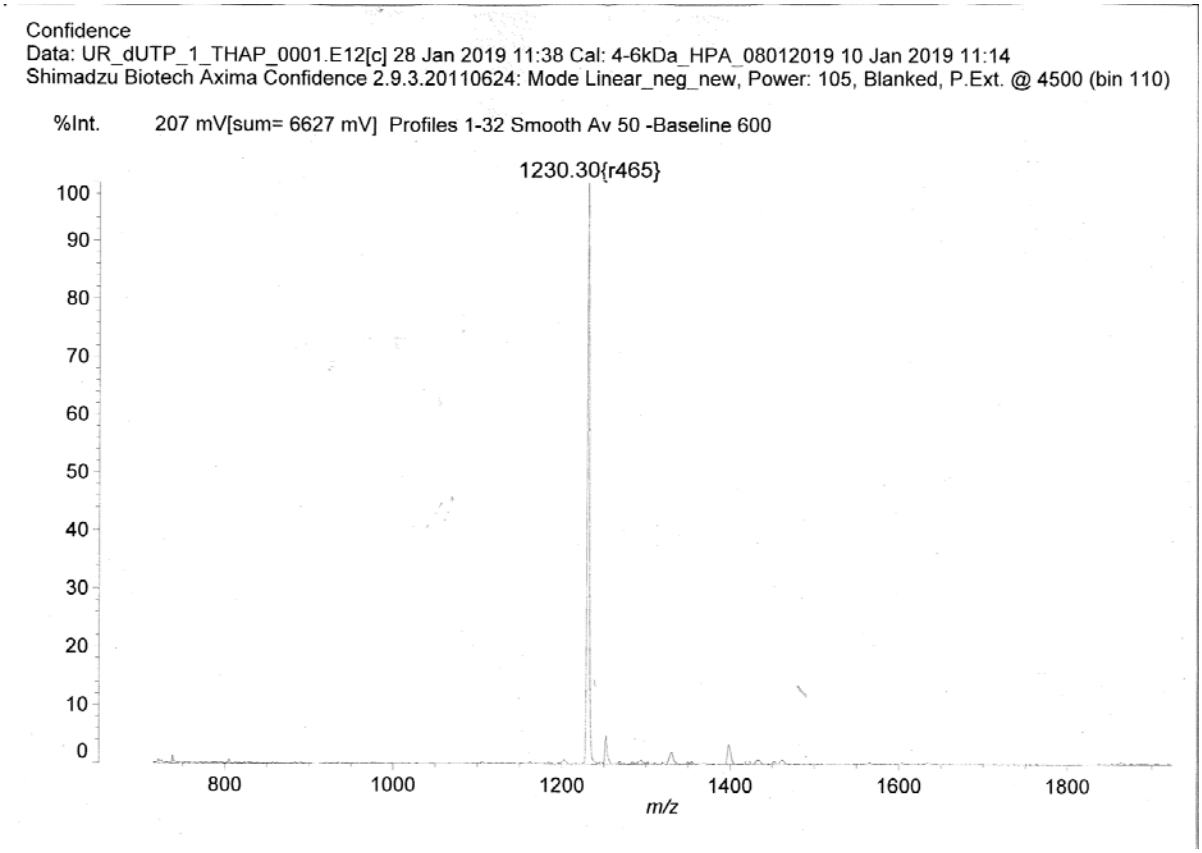
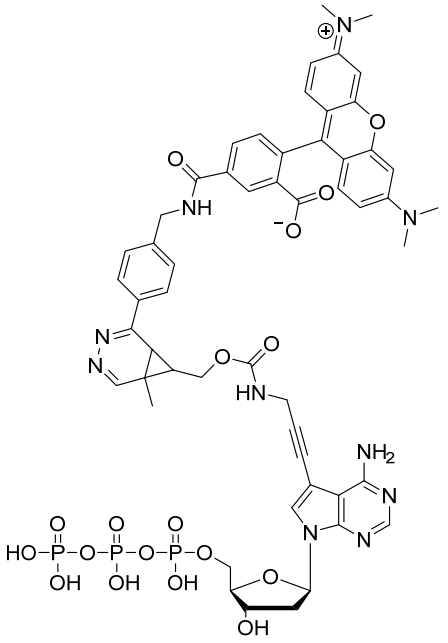


Figure S12: Maldi-TOF MS Spectrum of **1** labeled with BCN-rhodamine: *m/z* = 1230.30 $[M-H]^-$.

Triphosphate Conjugate 14



Chemical Formula: $C_{54}H_{55}N_{10}O_{18}P_3$
Molecular Weight:1225,01

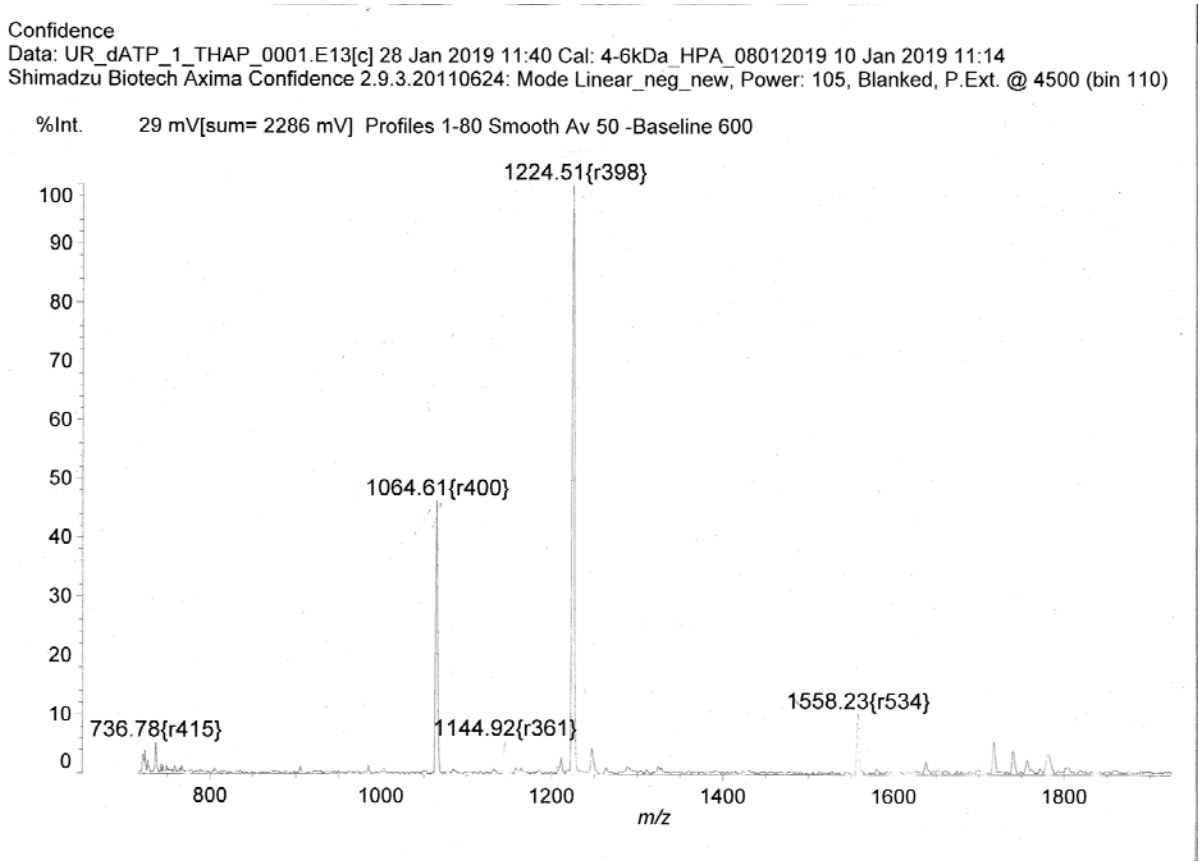
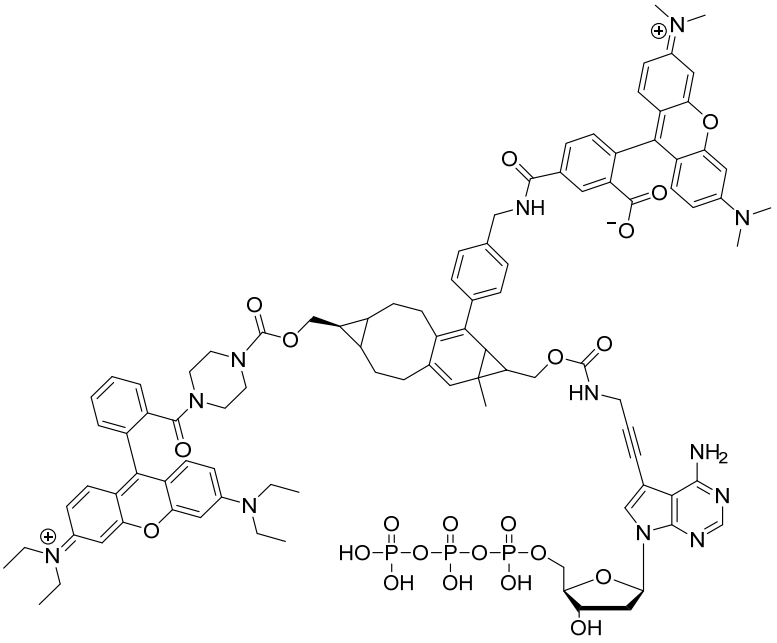


Figure S13: MADLI-TOF MS of **2** labeled with tetrazine-TAMRA **4**: *m/z* = 1224.51 $[M-H]^-$. *m/z* = 1064.61 corresponds to $[M-H]^-$ of the conjugate between **4** and the cyclopropenylated deazaadenosine-monophosphate (TAMRA-dAMP).

Triphosphate Conjugate 15



Chemical Formula: $C_{97}H_{106}N_{12}O_{22}P_3^+$
Molecular Weight:1884,90

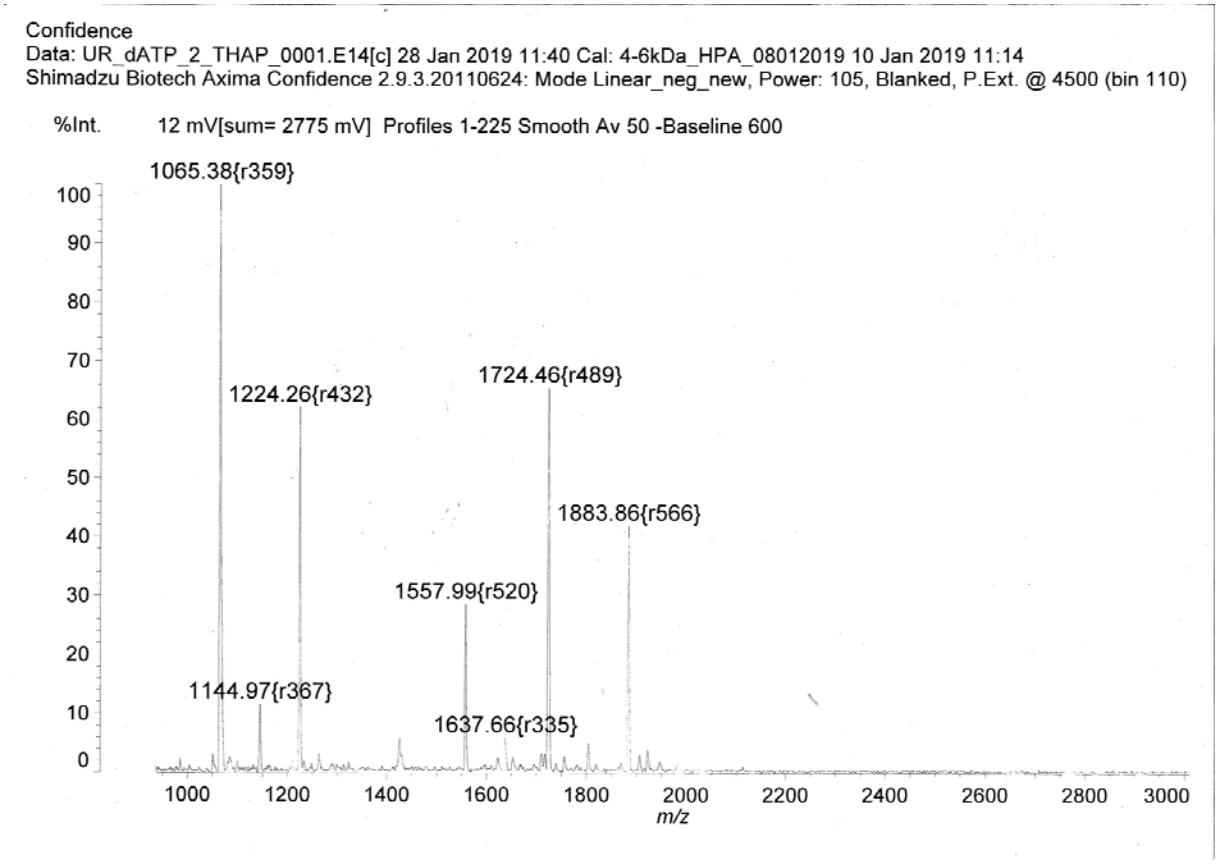
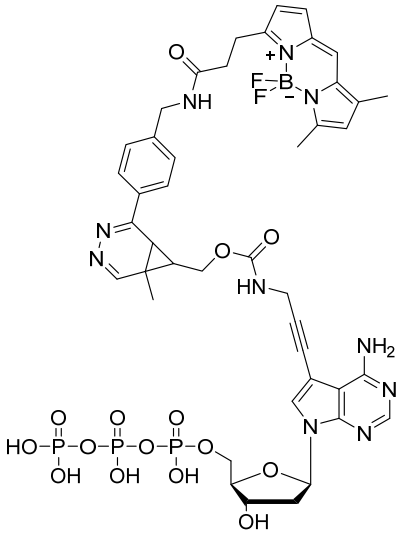


Figure S14: MALDI-TOF MS spectrum of the side product of the dual bioorthogonal labeling when **2** is allowed to react with **3** and **4** (TAMRA-BCN-dATP): *m/z* = 1883.86 $[M-H]^-$. *m/z* = 1065.38: TAMRA-dAMP $[M-H]^-$, *m/z* = 1224.26: TAMRA-dATP $[M-H]^-$, *m/z* = 1724.46: TAMRA-BCN-dAMP $[M-H]^-$.

Triphosphate Conjugate 16



Chemical Formula: $C_{43}H_{48}BF_2N_{10}O_{15}P_3$
Molecular Weight:1086,64

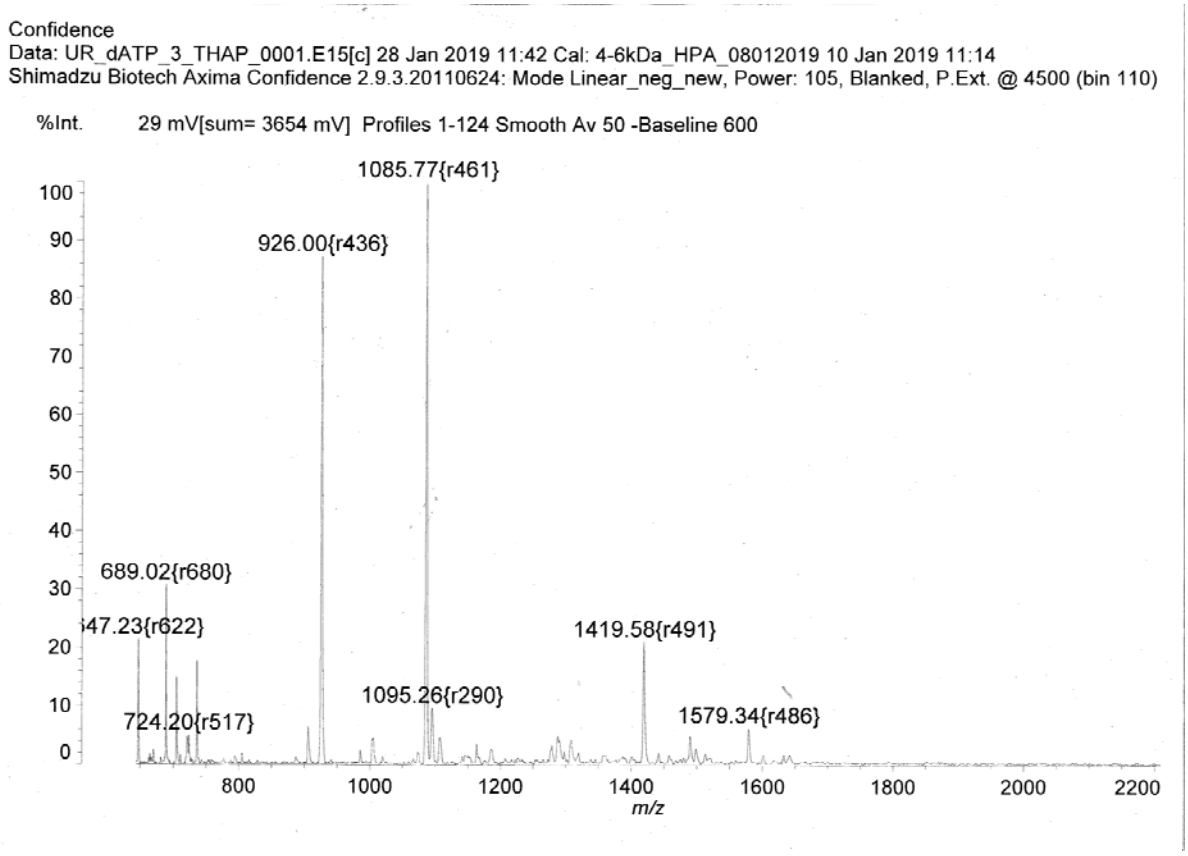


Figure S15: MALDI-TOF MS spectrum **2** labeled with tetrazine-BODIPY **5**: *m/z* = 1085.77 $[M-H]^-$. *m/z* = 926.00 corresponds to $[M-H]^-$ of BODIPY-dAMP.

Cell culture and transfection experiments

HeLa wt (Human cervix carcinoma cells) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose, gibco) supplemented with 10 % fetal calf serum (FCS, PAA) and 1 U/mL Penicillin/Streptomycin and incubated at 37 °C, 5 % CO_2 and 95 % humidity.

Transfection experiments were carried out according to the one-step transfection protocol provided by *ScreenFect®*. Therefore, 150 ng of the DNA primer extension product of **P2**/**T2** labelled with **3** and **5**, the DNA primer extension product of **P2**/**T2** labelled with **3** and the DNA primer extension product with **P2**/**T2** labelled with **5** were incubated with 0,85 µl *ScreenFect®A* and the corresponding *Dilution Buffer* to a total volume of 40 µl for 20 minutes at room temperature for lipoplex formation. Subsequently, 2 x 10^4 (per 200 µl) freshly detached and resuspended HeLa cells were added to the complex and transferred to 8 well slides (IBIDI® µ-Slide 8 well). After 24 h, transfection was stopped by washing the cells 3x with DMEM.

Visualization of the transfected DNA constructs was conducted by confocal microscopy using Leica TCS SPE (DMI4000) with a ACS APO 40x/1.15 OIL objective. BODIPY (**5**) was excited using a 488 nm laser line and BCN rhodamine (**3**) was excited at 532 nm. Fluorescence emission was measured at 502-522 nm for BODIPY and 560-580 nm for BCN rhodamine. Image acquisition was conducted at a lateral resolution of 1024 × 1024 pixels and 8 bit depth using Leica Application Suite X (LAS X).

Steady-state fluorescence in vitro

Fluorescecne and excitation spectra were recorded with a HORIBA JOBIN-YVON *Fluoromax-4* spectrofluorometer (equipped with *LFI-3751* Peltier-Element from WAVELENGTH ELECTRONICS) in semi-micro quartz glass cuvettes (width 3 mm, volume 0.14 mL) from STARNA. The spectrometer was calibrated against the raman peak of water (λ = 397 nm). All spectra were corrected against the raman scattering of the pure solvent. Following parameters were used: slits 7 nm (Figures S16 and S17) or slits 9 nm (Figure S18), increment 1.0 nm, integration time 0.1 s, acquisitions 3 (average scans).

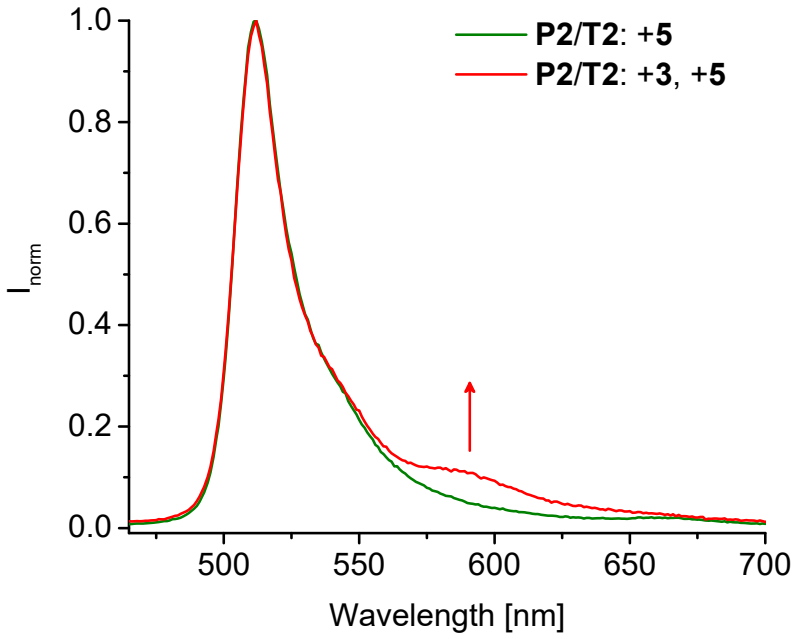


Figure S16. Normalized fluorescence of the primer extension product of **P2**/**T2** after labeling by BCN-rhodamine **3** and tetrazine-BODIPY **5** (red) in comparison with the corresponding primer extension product after labelling only by tetrazine-BODIPY **5** (green), in 50 mM Na-Pi buffer, pH 7, λ_{exc}=450 nm. Please not that there is remaining "unclicked" **5** in the samples which cannot completely by separated by the desalination process.

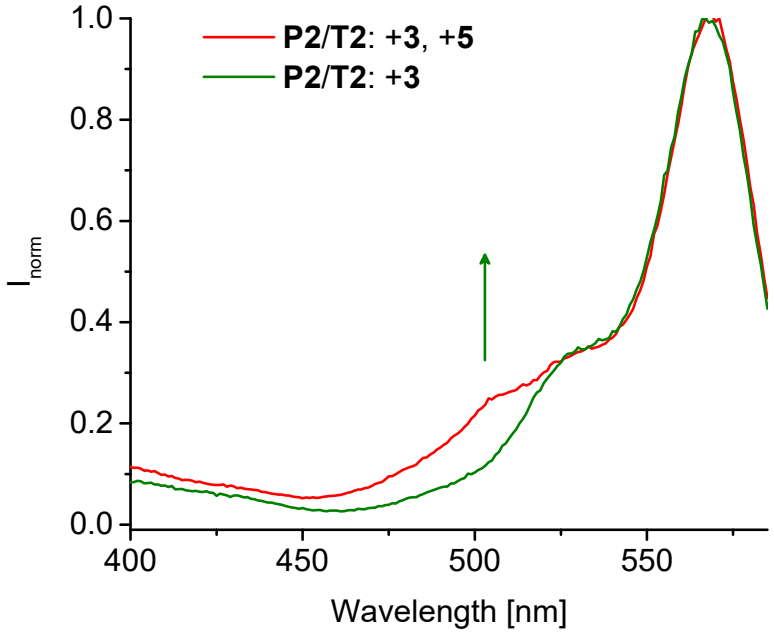


Figure S17. Normalized fluorescence excitation spectra of the primer extension product of **P2**/**T2** after labeling by BCN-rhodamine **3** and tetrazine-BODIPY **5** (red) in comparison with the corresponding primer extension product after labelling only by tetrazine-BODIPY **5** (green), in 50 mM Na-Pi buffer, pH 7, λ_{em}=600 nm. Please not that there is remaining "unclicked" **5** in the samples which cannot completely by separated by the desalination process.

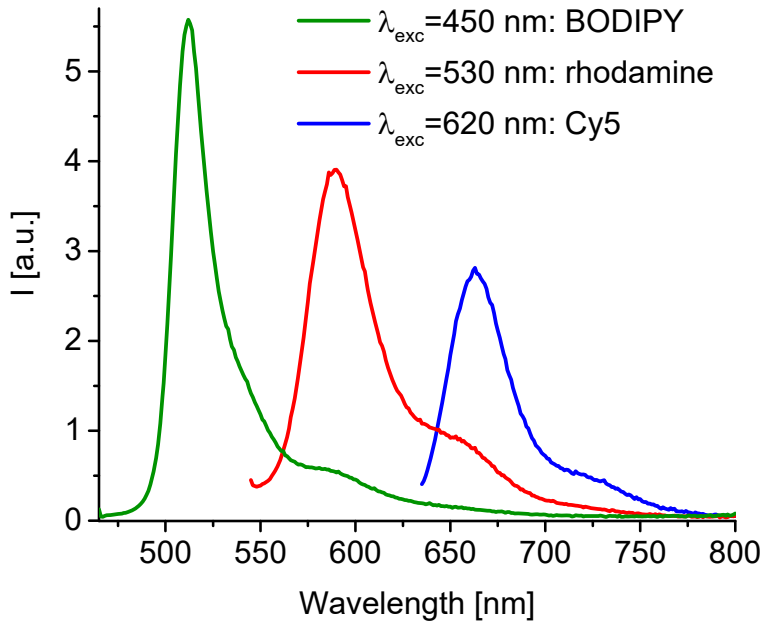


Figure S18. Fluorescence of the primer extension product of **P2**/**T2** after labeling by BCN-rhodamine **3** and tetrazine-BODIPY **5** in 50 mM Na-Pi buffer, pH 7. The different excitation wavelengths reveal the presence of all three dyes.

References

1. *US Pat.,* US2016251361, **2016**.
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