Supporting Information: [4+2] and [2+4] cycloaddition reactions on single- and double-stranded DNA: a dual-reactive nucleoside

Reagents and solvents: Unless otherwise stated, all reagents were purchased from commercial sources (Carbosynth, Sigma-Aldrich, TCI) and used without further purification. Dry solvents were obtained from INERT dry solvent system and dry DIPEA was freshly distilled prior to use. Pure water was obtained from ELGA PURELAB Ultra water system. Tetrazine dyes were purchased from Jena Bioscience, stored at -20 °C in the dark and dissolved in DMSO to a proper concentration prior to use. SYBR Gold was provided by Invitrogen and stored as 10 mM solution in DMSO at -20 °C in the dark, 1:10000 dilution was prepared in TBE buffer directly before use. VdU (7) phosphoramidite was synthesized according to the literature procedure.1

Buffers: All buffers were prepared from electrophoresis or molecular biology grade reagents in pure water and filtered prior to use.

PBS buffer (155 mM NaCl, 3 mM Na2HPO4, 1.1 mM KH2PO4, pH 7.4)

Sodium cacodylate buffer (10 mM cacodylic acid, 100 mM NaOH, ( +/- 100 mM NaCl), pH 7.4)

TBE buffer 1X (89 mM Tris, 89 mM boric acid, 2mM EDTA, pH 8.3)

TEAA buffer (50 mM Et3N, 50 mM AcOH, pH 7.4)

Chromatography: Analytical thin-layer chromatography was performed on Merck 250 μm thick silica gel 60 aluminum plates, coated with fluorescent indicator F254, visualized with ultraviolet light (254 nm or 366 nm) and/or 5% solution of concentrated H2SO4 in MeOH. Flash column chromatography was carried out on silica gel (size 60, 230-400 mesh particle size) using compressed air. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on Varian Pro Star HPLC system coupled to a Varian Pro Star UV/VIS detector using semi preparative (semiprep) C8 or C18 reversed-phase columns (Reprospher 100 C8-Aqua μm, 150 x 10 mm (Maisch); Reprospher 100 C18-Aqua, 5 μm, 100 x 10 mm) with a flow rateof 3 mL/min or analytical C8 reversed-phase columns (Waters XBridge™ C8, 5 μm, 4.6 x 150 mm) with a flow rate of 0.4 mL/min. 100 mM TEAA buffer and MeCN were used as eluents.

Nuclear magnetic resonance spectroscopy: 1H NMR spectra were recorded on Bruker 400 MHz or Bruker 500 MHz spectrometers; residual solvent peaks were used as internal standards: CDCl3 (s, δH = 7.26 ppm), CD3OD (quint, δH = 3.31 ppm), (CD3)2SO (quint, δH = 2.50 ppm). 13C NMR spectra were recorded on 100 MHz or 125 MHz spectrometers; residual solvent peaks were used as internal standards: (CD3)2CO (sept, δC = 29.8 ppm), CDCl3 (s, δC = 77.2 ppm), (CD3)2SO (sept, δC = 39.5 ppm). CD3OD (sept, δH = 49.1 ppm). 31P NMR spectra were recorded without proton-coupling on Bruker 162 MHz spectrometers. Coupling
constants are reported in Hz, chemical shifts in ppm. The following abbreviations were used to describe multiplicities: s – singlet, d – doublet, t – triplet, q – quartet, quint – quintet, sept – septet, m – multiplet, dd – doublet-doublet, ddd – doublet-doublet-doublet dt – doublet-triplet, br – broad.

**Mass spectrometry:** Measurements were carried out at the Mass Spectrometry Facility of the Organic Chemistry Institute at the University of Zurich. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded on Bruker maXis QTOF-MS or high-resolution Orbitrap HPLC-MS/(MS) instruments. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on Bruker AutoFlex Speed with a 3-HPA matrix. Masses are given as m/z.

**Photophysical experiments:** All photophysical measurements were performed using a Molecular Device Spectra spectrometer in a quartz cuvette (pathlength = 1 cm), 96-well UV-Star microplate (clear, flat bottom, purchased from Greiner) or 384-well (opaque, flat bottom, non-binding surface, purchased from Corning) and analyzed using SoftMax Pro 5.4.5 software. All measurements were corrected for background.

**Kinetic measurements:** Reaction rates between BDdU (2) and VdU (7) with tetrazine 5 were determined by absorbance measurements on a Molecular Device Spectra Spectrophotometer (pathlength = 1 cm) in 1:1 (v/v) mixture of water and methanol at 25 °C monitoring the exponential decay in the absorbance of tetrazine 5 at 530 nm. Stock solutions of VdU (7) and BDdU (2) (100 mM) were prepared in 1:1 (v/v) water/methanol mixture, containing 0.25% of DMSO. Tetrazine 5 (2 mM) was prepared in 1:1 (v/v) water/methanol mixture. The final concentrations (1 mM of tetrazine 5 and 5 mM to 15 mM of BDdU (2) and VdU (7)) were achieved upon dilution with water/methanol mixture. Reaction rates between BDdU (2) with maleimide 9 were determined by fluorescence measurements on a Molecular Device Spectra Spectrophotometer (pathlength = 1 cm) in 1:1 (v/v) mixture of water and DMSO at 25 °C monitoring the logarithmic growth of the emission of maleimide 9 at 365 nm (excitation wavelength: \( \lambda_{ex} = 310 \) nm). Stock solutions of BDdU (2) (20 mM) and maleimide 9 (1 mM) were prepared in 1:1 (v/v) water/DMSO mixture and DMSO, respectively, and diluted to final the concentrations (10 \( \mu \)M of maleimide 9 and 2.5 mM to 10 mM of BDdU (2)).

**Oligonucleotide synthesis and purification:** The wild-type complementary sequences were purchased from Sigma-Aldrich as HPLC grade products. Standard DNA phosphoramidites, solid supports and all necessary DNA synthesizer reagents were purchased from LinkTech. ETT activator was recrystallized from dry toluene, dried under high vacuum, and prepared to a 0.25 M solution in dry MeCN prior to use. Modified oligonucleotides were synthesized on a 1.0 \( \mu \)mol scale using a Bioautomation Co. Mermade 4 DNA synthesizer according to the standard DMTr-off procedure. The modified phosphoramidites were prepared in dry acetonitrile (concentration > 10 mg/mL) directly before use. Three coupling reactions were performed for the introduction of the modified nucleoside. Oligonucleotides were cleaved from the solid support and deprotected with 1.0 mL of 33% aqueous ammonium hydroxide at 55 °C for 16 hours in a 1.5 mL screw-top cap tube. The resin was filtered off through a cellulose
acetate membrane (0.22 μm pore size, purchased from Waters), the filtrates were lyophilized and purified by analytical RP-HPLC (Waters XBridge™ C8 5 μm, 4.6 x 150 mm) with the 2-25% gradient of MeCN over 35 min and eluent flow of 0.4 mL/min. Collected peaks were lyophilized four times and, if necessary, desalted on Bond Elut C18 cartridges (500 mg bed mass, purchased from Agilent) or Illustra NA P-10 columns, prepacked with Sephadex G-25 DNA grade (purchased from GE Healthcare).

**DNA analysis:** DNA analysis was carried out by polyacrylamide gel electrophoresis (PAGE) using Bio-Rad Powerpac 300 (160 V) in TBE running buffer. Reaction progress was analyzed by 22.5% denaturing gel, containing 4.5% of bis-acrylamide and 7 M urea at 60 °C. DNA folding were analyzed using 20% native gel at room temperature. Unless otherwise stated, DNA was stained with 0.01% solution of SYBR Gold in TBE buffer for 15 minutes at room temperature. Gels were scanned using a Typhoon FLA 9500 (λ<sub>Ex</sub> = 473 nm, λ<sub>Em</sub> = 520 nm) and were analyzed using ImageJ.

**DNA folding:** Buffers were prepared as indicated in *Buffers* section. Stock solutions of modified oligonucleotides were prepared in water and concentrations were calculated using molar extinction coefficients at 260 nm. Molar extinction coefficients of the functionalized sequences were assumed to be equal to the wild-type oligonucleotides: ε = 255400 M⁻¹cm⁻¹ for BDdU and VdU modified DNA. Duplex DNA samples were prepared by mixing 1 eq. of the modified oligonucleotide with 1.1 eq. of the complementary strand in the indicated buffer and diluted to a final concentration of 6 μM, heated for 5 minutes at 95 °C, and slowly cooled to room temperature overnight before use. Duplex formation was analyzed by 20% native PAGE.

**Reaction conditions and analysis:** Single-stranded and duplex DNA were treated with the appropriate amount of the corresponding tetrazine or maleimide in DMSO, diluted with the indicated buffer to the final DNA concentration of 2 μM and incubated for the indicated period of time at 37 °C. All samples contained 20% of DMSO. In case of the time dependent experiment, reaction samples containing maleimide were quenched with glutathione (1 μL of 10 mM solution in water) at the appropriate time point. The samples were analyzed by 22.5% denaturing PAGE.

**Bodipy-Tz:**

![Bodipy-Tz](image)

Purchased from Jena Bioscience.
5-(1,3-butadienyl)-2'-deoxyuridine (2):

5-Bromovinyl-2'-deoxyuridine, 1 (Carbosynth, 530.0 mg, 1.59 mmol) and Pd(dppf)Cl₂ (0.05 eq., 58.2 mg, 0.08 mmol) were evacuated 3 times and dissolved in dry, degassed THF. Thereafter, vinyltributylstannane (1.5 eq, 0.70 ml, 2.40 mmol) was added in one portion. The reaction mixture was stirred at 65 °C for 16 h in a sealed tube, then filtered through a celite plug, evaporated, and subjected to silica purification. Purification by flash column chromatography (0 – 10% MeOH in DCM) to give 242 mg of BDdU (2) as a yellowish solid (54%).

1H NMR (400 MHz, CH₃OH) δ 8.23 (s, 1H), 7.10 (dd, J = 15.7, 10.6 Hz, 1H), 6.43 (dt, J = 17.0, 10.4 Hz, 1H), 6.33 – 6.27 (m, 1H), 5.27 (d, J = 16.7 Hz, 1H), 5.11 (d, J = 10.1 Hz, 1H), 4.49 – 4.41 (m, 1H), 3.96 (q, J = 3.3 Hz, 1H), 3.87 (dd, J = 17.0, 3.0 Hz, 1H), 3.78 (dd, J = 12.0, 3.4 Hz, 1H), 2.38 – 2.22 (m, 2H).

13C NMR (125 MHz, (CD₃)₂SO/CD₃OD) δ: 150.87, 138.89, 138.64, 130.97, 125.54, 117.41, 112.35, 111.09, 88.73, 86.09, 71.52, 62.24, 41.32. HR-ESI-MS (m/z): [M - H]- calc. for C₁₃H₁₅N₂O₅: 279.0986, found: 279.0985.

5'-O-(4,4-dimethoxytrityl)-5-(1,4-butadienyl)-2'-deoxyuridine (3):

BDdU (2) (250 mg, 0.892 mmol) was co-evaporated with dry pyridine (2x), then dissolved in 3 mL pyridine and treated with DMTr-Cl (1.2 eq, 360 mg, 1.070 mmol). The mixture was stirred at room temperature for 20 hours, subsequently quenched with sat. NaHCO₃ and extracted with DCM. Organic layers were collected, dried over anhydrous Na₂SO₄, filtered and evaporated under reduced pressure. The residue was purified on silica gel (DCM/MeOH/Et₃N 97:2:1) to give 3 as a white foam (348 mg, 67%).

1H NMR (500 MHz, CDCl₃) δ: 7.73 (s, 1H), 7.41 (d, J = 7.36 Hz, 2H), 7.32 – 7.27 (m, 6H), 7.26 – 7.23 (m, 1H), 7.02 (dd, J = 15.62, 10.70 Hz, 1H), 6.83 (d, J = 8.88 Hz, 4H), 6.39 (dd, J = 7.37, 6.06 Hz, 1H), 6.00 (dt, J = 16.91, 10.40 Hz, 1H), 5.54 (d, J = 15.62 Hz, 1H), 5.01 (d, J = 16.08 Hz, 1H), 4.98 (d, J = 9.01 Hz, 1H), 4.57 (dt, J = 6.07, 3.02 Hz, 1H), 4.07 (q, J = 3.02 Hz, 1H), 3.78 (s, 6H), 3.49 (dd, J = 10.58, 3.21 Hz, 1H), 3.37 (dd, J = 10.58, 3.21 Hz, 1H), 2.46 (dd, J = 13.58, 5.87, 3.07 Hz, 1H), 2.33 (dd, J = 13.61, 7.24, 6.38 Hz, 1H). 13C NMR (125 MHz, CDCl₃) δ: 161.94, 159.34, 149.67, 144.84, 138.02, 136.97, 136.01, 135.95, 132.36, 130.62, 128.70, 127.82, 124.13, 118.47, 113.98, 113.01, 118.47, 113.87, 87.54, 86.77, 85.59, 72.84, 63.97, 55.85, 41.87. HR-ESI-MS (m/z): [M + Na]+ calc. for C₃₄H₃₄N₂O₇Na: 605.2264, found: 605.2246.
5′-O-(4,4′-dimethoxytrityl)-3′-O-[2-cyanoethoxy-(N,N-diisopropylamino)-phosphino]-5-(1,4-butadienyl)-2′-deoxyuridine (4):

Freshly distilled DIPEA (5.0 eq., 180 μL, 1.03 mmol) was added to a cooled down to 0 °C solution of 3 (120 mg, 0.206 mmol) in dry DCM (2 mL). After 5 min the mixture was treated with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (2.05 eq., 94 μL, 0.422 mmol) and stirred at room temperature for 3 h. Reaction mixture was loaded directly on a short silica gel column and purified (EtOAc/Hex/Et3N 60:40:1) to give 4 (diastereomeric mixture) as a white foam (68 mg, 42%).

\[ \text{\textsuperscript{1}H NMR (400 MHz, CDCl}_3\text{)} \delta: 7.79 (s, 1H), 7.75 (s, 1H), 7.41 (d, \textit{J} = 9.75 \text{ Hz}, 4H), 7.34 – 7.27 (m, 14H), 7.01 (dd, \textit{J} = 15.55, 10.19 \text{ Hz}, 2H), 6.83 (dd, \textit{J} = 8.83, 4.02 \text{ Hz}, 8H), 6.39 (dd, \textit{J} = 13.82, 7.87 \text{ Hz}, 2H), 6.02 – 5.89 (m, 2H), 5.47 (dd, \textit{J} = 15.74, 2.90 \text{ Hz}, 2H), 5.01 – 4.92 (m, 4H), 4.68 – 4.61 (m, 2H), 4.21 – 4.07 (m, 4H), 3.87 – 3.72 (m, 14H), 3.66 – 3.45 (m, 10H), 3.36 – 3.28 (m, 2H), 2.62 (t, \textit{J} = 6.2 \text{ Hz}, 2H), 2.42 (t, \textit{J} = 5.2 \text{ Hz}, 2H), 1.18 (s, 3H), 1.18 (s, 6H), 1.16 (s, 3H), 1.15 (s, 6H), 1.06 (s, 3H), 1.04 (s, 3H). \]

\[ \text{\textsuperscript{31}P NMR (162 MHz, CDCl}_3\text{)} \delta: 150.34, 149.88. \]

5-((E)-2-(3,6-di(pyridin-2-yl)pyridazin-4-yl)vinyl)-2′-deoxyuridine (6):

BDdU (2) (20 mg, 0.0714 mmol) was dissolved in dioxane/H₂O/EtOH 1:2:2 (1 mL) and tetrazine 5 (1.5 eq., 25 mg, 0.107 mmol) was added in one portion. Reaction mixture was stirred for 3 hours at room temperature, subsequently evaporated under reduced pressure, and purified on the silica gel (DCM/MeOH 9:1) to give 6 as a yellow solid (31 mg, 60%).

\[ \text{\textsuperscript{1}H NMR (500 MHz, (CD}_3\text{)_2SO)} \delta: 8.83 (d, \textit{J} = 4.51 \text{ Hz}, 1H), 8.73 (d, \textit{J} = 4.63 \text{ Hz}, 1H), 8.64 (d, \textit{J} = 7.93 \text{ Hz}, 1H), 8.23 (s, 1H), 8.07 (dd, \textit{J} = 9.17, 7.69, 1.57 \text{ Hz}, 2H), 8.00 (d, \textit{J} = 7.65 \text{ Hz}, 2H), 7.98 (d, \textit{J} = 16.10 \text{ Hz}, 2H), 7.58 (ddd, \textit{J} = 25.71, 6.78, 4.87 \text{ Hz}, 2H), 7.34 (d, \textit{J} = 16.13 \text{ Hz}, 1H), 6.17 (t, \textit{J} = 6.67 \text{ Hz}, 1H), 5.30 (d, \textit{J} = 3.52 \text{ Hz}, 1H), 5.06 (t, \textit{J} = 4.62 \text{ Hz}, 1H), 4.32 – 4.21 (m, 1H), 3.80 (q, \textit{J} = 3.87 \text{ Hz}, 1H), 3.65 – 3.53 (m, 2H), 2.24 – 2.11 (m, 2H). \]

\[ \text{\textsuperscript{13}C NMR (125 MHz, (CD}_3\text{)_2SO)} \delta: 161.77, 157.36, 155.37, 152.94, 149.80, 149.42, 148.85, 141.70, 137.84, 137.33, 136.24, 129.48, 125.35, 124.78, 123.97, 122.38, 121.27, 118.59, 110.31, 109.59, 87.64, 84.56, 70.22, 61.27, 45.73. \]

\[ \text{HR-ESI-MS (m/z): [M + H]+ calc. for C}_{25}\text{H}_{23}\text{N}_6\text{O}_5: 487.1730, \text{ found: 487.1729.} \]
5-((Z)-2-(3,6-di(pyridin-2-yl)pyridazin-4-yl)vinyl)-2'-deoxyuridine (6):

*Trans*- (6) was dissolved in a DMSO/MeOH mixture and irradiated under the UV lamp (irradiation wavelength: 365 nm) for 30 minutes at room temperature. After irradiation, the mixture was purified on RP-HPLC (3-25% of MeCN over 30 min, flow: 3 mL/min). Reported yield determined by integration of ¹H-NMR peaks from crude reaction.

**¹H NMR** (500 MHz, CD₃OD) δ: 8.74 (d, J = 5.39 Hz, 1H), 8.67 (d, J = 4.88 Hz, 1H), 8.63 – 8.58 (m, 2H), 8.06 – 7.98 (m, 2H), 7.95 – 7.88 (m, 2H), 7.57 – 7.50 (m, 2H), 6.71 (d, J = 12.05 Hz, 1H), 6.49 (d, J = 12.04 Hz, 1H), 6.15 (dd, J = 7.43, 6.10 Hz, 1H), 4.01 (dt, J = 5.89, 2.89 Hz, 1H), 3.70 (q, J = 3.14 Hz, 1H), 2.08 (ddd, J = 13.55, 7.47, 6.12 Hz, 1H). ¹³C NMR (125 MHz, (CD₃)₂SO) δ: 161.78, 157.36, 157.10, 155.37, 152.94, 149.79, 149.42, 148.85, 141.69, 137.83, 137.32, 136.24, 129.46, 125.34, 124.78, 123.96, 122.38, 121.27, 118.59, 87.63, 84.55, 70.22, 61.27.

**HR-ESI-MS** (m/z): [M + H]⁺ calc. for C₂₅H₂₃N₆O₅: 487.1730, found: 487.1728.

2-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-4-(2'-deoxyuridin-5-yl)-3a,4,7,7a-tetrahydro-1H-isoinodole-1,3(2H)-dione (10):

BDdU (2) (10 mg, 0.0357 mmol) was dissolved in 1:1 (v/v) DMSO/H₂O mixture (1 mL) and maleimide 9 (1.5 eq., 16 mg, 0.0535 mmol) was added in one portion. The reaction mixture was stirred for 12 h at room temperature, subsequently evaporated under reduced pressure and purified on preparative TLC (DCM/MeOH 95:5) to give 10 (8 mg, 37%) in 1:1 diastereomeric ratio.

**¹H NMR** (400 MHz, (CD₃)₂SO) 10a δ: 12.97 (s, 1H), 11.44 (s, 1H), 8.24 (d, J = 8.71 Hz, 2H), 7.90 (s, 1H), 7.68 (d, J = 7.16 Hz, 1H), 7.54 (d, J = 7.15 Hz, 1H), 7.35 (d, J = 8.70 Hz, 2H), 7.22 (dt, J = 10.47, 6.51 Hz, 2H), 6.25 (t, J = 6.72 Hz, 1H), 6.20 – 6.03 (m, 2H), 5.24 (d, J = 4.19 Hz, 1H), 5.17 (t, J = 4.45 Hz, 1H), 4.29 (tt, J = 5.41, 2.59 Hz, 1H), 3.83 (q, J = 3.12 Hz, 1H), 3.72 – 3.46 (m, 5H), 2.72 – 2.64 (m, 1H), 2.37 – 2.26 (m, 1H), 2.19 – 1.94 (m, 2H).
$^1$H NMR (400 MHz, (CD$_3$)$_2$SO) 10b $\delta$: 12.97 (s, 1H), 11.44 (s, 1H), 8.24 (d, $J = 8.71$ Hz, 2H), 7.90 (s, 1H), 7.68 (d, $J = 7.16$ Hz, 1H), 7.54 (d, $J = 7.15$ Hz, 1H), 7.35 (d, $J = 8.70$ Hz, 2H), 7.22 (dt, $J = 10.47$, 6.51 Hz, 2H), 6.25 (t, $J = 6.72$ Hz, 1H), 6.20 – 6.03 (m, 2H), 5.24 (d, $J = 4.19$ Hz, 1H), 5.17 (t, $J = 4.45$ Hz, 1H), 4.29 (tt, $J = 5.41$, 2.59 Hz, 1H), 3.83 (q, $J = 3.12$ Hz, 1H), 3.72 – 3.46 (m, 5H), 2.72-2.64 (m, 1H), 2.37 – 2.26 (m, 1H), 2.19 – 1.94 (m, 2H). HR-ESI-MS (m/z): [M + H]$^+$ calc. for C$_{30}$H$_{28}$N$_5$O$_7$: 570.1989, found: 570.1986.

Figure S1. Changes in absorbance of tetrazine 5 (530 nm) following addition of BDdU (2) or VdU (7). Pseudo-first-order reaction rates ($k'$) versus alkene concentration for the consumption of tetrazine 5 (1 mM) in a 1:1 (v/v) mixture of water and methanol. The slope of each linear regression provides the rate constant $k$.

Figure S2. Absorbance and emission spectra of trans-6 measured in water (black) and acetonitrile (red); excitation wavelength: $\lambda_{ex}= 333$ nm. Sample concentration: 40 $\mu$M.

Figure S3. Changes in absorbance and emission spectra upon trans-cis isomerization of 6 determined in water; excitation wavelength: $\lambda_{ex}= 333$ nm.
**Figure S4.** $^1$H NMR analysis of trans-to-cis isomerization according to changes in chemical shift (ppm) and coupling constants (Hz) of signals corresponding to alkene protons. A pure solution of trans-6 was irradiated with the 365 nm UV light at room temperature for 15 min. NMR spectra of the pure isomers are provided at the end of the SI document.

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**Figure S5.** Absorbance (A.) and emission (B.) spectra of 10a and 10b determined for each diastereomer. Sample concentrations: 20 μM. For solubility reasons, the measurements were performed in DMSO. Excitation wavelength: $\lambda_{ex}$: 310 nm. C. Increase in fluorescence of maleimide 9 upon addition of BDdU (2) illustrating the fluorogenic character of the cycloaddition reaction. The measurement was conducted in 1:1 (v/v) mixture of DMSO and water using the following concentrations of the reactants: Concentration (9) = 250 μM, Concentration (2) = 500 μM.
Figure S6. HPLC and MALDI-TOF-MS analyses of oligonucleotides modified with VdU (A), or BDdU (B). Oligonucleotides were analyzed by HPLC using a Waters XBridgeTM C8, 5 µm, 4.6 x 150 mm column with a 2-25% gradient of MeCN in 1X TEAA (0.4 mL/min). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on Bruker AutoFlex Speed using a 3-HPA matrix.
Table S1. MALDI-TOF-MS of oligonucleotides modified with VdU (7), and BDdU (2) as well as reactions products of modified oligonucleotides with Bodipy FL tetrazine and maleimide 9.

| Sequence 5'-3'               | Molecular formula   | Calculated m/z | Found m/z |
|------------------------------|---------------------|----------------|-----------|
| TTGGGTTAGGVT(VdU)AGGGTTAGGGA | C_{241}H_{297}N_{96}O_{146}P_{23} | 7583           | 7580      |
| TTGGGTTAGGVT(VdU-Bodipy)AGGGTTAGGGA | C_{264}H_{319}B_{1}F_{2}N_{101}O_{147}P_{23} | 8016           | 8019      |
| TTGGGTTAGGVT(BDdU)AGGGTTAGGGA | C_{243}H_{299}N_{96}O_{146}P_{23} | 7609           | 7620      |
| TTGGGTTAGGVT(BDdU-Bodipy)AGGGTTAGGGA | C_{266}H_{319}B_{1}F_{2}N_{101}O_{147}P_{23} | 8040           | 8044      |
| TTGGGTTAGGVT(BDdU-Maleimide (9))AGGGTTAGGGA | C_{260}H_{310}N_{99}O_{148}P_{23} | 7898           | 7904      |

Figure S7. Analysis of reactions between maleimide (9) and single- (A) and double-stranded DNA (B) modified with BDdU (22.5% denaturing PAGE). Oligonucleotides were incubated with a 10-, 100-, 250-, 500-, 1000- and 2000-fold excess of dienophile for 2 hours at 37 °C in 10 mM sodium cacodylate buffer (pH 7.4). The gel was stained with SYBR Gold solution in TBE buffer and scanned using Typhoon FLA 9500.
Figure S8. The stability of BDdU in the presence of 6-fold excess DL-Dithiothreitol (DTT) was monitored by $^1$H NMR in deuterated methanol at room temperature over the course of 24 hours ($^1$H NMR, 500 MHz). $^1$H NMR spectra were recorded at 0, 4, 16, and 24 hours. BDdU nucleoside (25.0 μmol, 1 eq) and DTT (150.0 μmol, 6 eq) were dissolved into 500 μL of methanol to give a final concentration of 50 mM for the nucleoside and 300 mM for DTT. No reaction between DTT and BDdU was observed.
NMR Spectra:

$^1$H NMR (CD$_3$OD, 400MHz)

$^{13}$C NMR ((CD$_3$)$_2$SO/CD$_3$OD, 125 MHz)
References:
1. Naik, A.; Alzeer, J.; Triemer, T.; Bujalska, A.; Luedtke, N. W., Angew. Chem. Int. Ed. 2017, 56 (36), 10850-10853.