Fast and Efficient Postsynthetic DNA Labeling in Cells by Means of Strain-Promoted Sydnone-Alkyne Cycloadditions

Katja Krell, Bastian Pfeuffer, Franziska Rönicke, Zoeisha S. Chinoy, Camille Favre, Frédéric Friscourt,* and Hans-Achim Wagenknecht*
Table of Content

1. Materials and methods ................................................................. 2

2. Kinetics measurements with isolated sydrones............................... 3

3. Synthetic Procedures .................................................................... 7

4. Oligonucleotide Synthesis ............................................................ 13

5. SPSAC Labeling Experiments ......................................................... 14

6. Cell experiments ........................................................................... 20

7. Images of NMR spectra and MS analyses ..................................... 21

8. Kinetic measurements of nucleosides and DNA ............................. 39

9. References .................................................................................... 44
1. Materials and methods

All reactions were carried out under a dry argon environment. All solvents were of reagent grade and used as received. Chemicals and reagents were used as commercially supplied without any further purification unless otherwise stated. Room temperature refers to ambient temperature (20-22 °C). Reactions were monitored by Thin Layer Chromatography (TLC) using aluminum backed silica gel 60 (F254) plates, visualized using UV254 nm and 365 nm and potassium permanganate dips as appropriate. Column chromatography was carried out using silica gel G60 (Fluka analytical, 230-400 mesh, 40-63 μm particle size, 60 Å) as the stationary phase.

PhSydH,i PhSydCl,i MeO-DIBO,ii MeO-DIBAC,iii BCNiv, benzoic acid sydnones 3i and 4i, deprotected nucleoside 7 were synthesized as reported.

NMR Spectroscopy

$^1$H and $^{13}$C-NMR spectra were recorded on a Bruker 300 MHz and Bruker Ascend 400 MHz spectrometer and kinetics measurements were recorded on a Bruker 400 MHz. Chemical shifts are reported in δ units, parts per million (ppm) downfield from TMS. Coupling constants ($J$) are reported in Hertz (Hz) without adjustments; therefore, due to limits in digital resolution, in some cases there are small differences (<1 Hz) in the measured J value of the same coupling constant determined from different signals. Splitting patterns are designed as follows: s – singlet, d – doublet, t – triplet, dd – doublet of doublets, dt – doublet of triplets, td – triplet of doublets, ddd – doublet of doublet of doublets, tt – triplet of triplets, sp – septet, hept – heptet, m – multiplet, br – broad. Various 2D techniques experiments were used to establish the structures and to assign the signals.

Mass Analysis

High-resolution mass spectra were obtained with an electrospray ionization Thermo Exactive orbitrap mass spectrometer. Some of mass spectra were also measured on a Shimadzu Axima Confidence using 3-hydroxypicolinic acid as matrix substance.

Optical Spectroscopy

Spectroscopic measurements were carried out on a Cary 100 Scan UV/vis spectrometer by Varian and a Fluoromax-4 spectrofluorometer by Horiba Jobin-Yvon.

Oligonucleotide Synthesis

Oligonucleotide synthesis was performed on a H-6 synthesizer by K&A Laborgeräte. After cleavage, the oligonucleotides were purified on a semi-preparative reversed-phase HPLC ThermoFisher system (RP-C18 column, A = NH$_4$HCO$_3$ buffer, B = acetonitrile). The purified oligonucleotide strands were quantified photometrically using a NanoDrop ND-1000 spectrometer.

HeLa cells

HeLa cells were bought ATCC (Manassas Virginia).
2. Kinetics measurements with isolated sydnone

The rate measurements of cycloaddition of various cyclooctynes (MeO-DIBO, MeO-DIBAC, BCN) with either 3-phenylsydnone (PhSydH) or 4-chloro-3-phenylsydnone (PhSydCl) were conducted by using ¹H-NMR spectroscopy (Bruker 400 MHz) at 25 °C. A 20 mM solution of sydnone (0.2 mL) in CD₃OD was added to a thermally equilibrated solution of cyclooctynes (20 mM, 0.2 mL) in CD₃OD, leading to a mixture of both reactants in 1:1 ratio with a respective concentration of 6.66 mM. Reactions were monitored by following the decay of characteristic peaks of the cyclooctynes and the sydnone as well as the formation of characteristic pyrazole peaks. Consumption of starting materials followed a second-order equation and the second-order rate constants were obtained by least squares fitting of the data to a linear equation (Figures S1-S6).

![Figure S1.](image)

**Figure S1.** Kinetics measurement plots of MeO-DIBO and PhSydH. Decay of MeO-DIBO: 2.22-2.39 (m, CH₂) and sydnone: 7.86-7.92 (d, 2 × CHar) and formation of the pyrazole product: 3.75 (s, 2×OCH₃), 8.21 (s, CHpyr).
Figure S2. Kinetics measurement plots of MeO-DIBO and PhSydCl. Decay of MeO-DIBO: 2.22-2.39 (m, CH₂), 6.92 (d, 2×CHar) and formation of the pyrazole product: 3.75 (s, 2×OCH₃), 6.87 (d, CHar).

Figure S3. Kinetics measurement plots of MeO-DIBAC and PhSydH. Decay of MeO-DIBAC: 1.76-1.82 (m, CH₃) and formation of the pyrazole product: 1.58-1.62 (2×s, CH₃), 4.41-4.52 (2×d, CH₂), 8.29-8.36 (2×s, CHpyr).
Figure S4. Kinetics measurement plots of MeO-DIBAC and PhSydCl. Decay of MeO-DIBAC: 1.765-1.825 (m, CH$_3$) and formation of the pyrazole product: 1.58-1.65 (2×s, CH$_3$), 4.36-4.52 (2×d, CH$_2$), 5.86-6.08 (2×d, CH$_2$).

Figure S5. Kinetics measurement plots of BCN and PhSydH. Decay of BCN: 0.82-0.96 (m, CH$_2$) and sydnone: 7.87-7.92 (m, 2×CHar) and formation of the pyrazole product: 2.51-2.62 (m, CH), 7.20-7.27 (m, CHar).
Figure S6. Kinetics measurement plots of BCN and PhSydCl. Decay of BCN: 0.82-0.96 (m, CH$_2$) and sydnone: 7.65-7.82 (m, 5×CHar) and formation of the pyrazole product: 2.47-2.59 (m, CH), 7.39-7.56 (m, 5×CHar).
3. Synthetic Procedures

**Compound 7**

Sydnone 5 (525 mg, 2.55 mmol, 1.00 equiv.) was dissolved in anhydrous DMF (20 mL). Subsequently, *N*-hydroxysuccinimide (439 mg, 3.82 mmol, 1.50 equiv.) and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (732 mg, 3.82 mmol, 1.50 equiv.) were added and the reaction mixture was stirred overnight at room temperature. The crude mixture was diluted with ethyl acetate (50 mL), washed with water (3 x 150 mL) and brine (1x 150 mL). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to obtain the desired product as a powder (600 mg, 78%).

R<sub>f</sub> (DCM/MeOH 50:1) = 0.19.

$^1$H-NMR (400 MHz, DMSO-<em>d</em>$_6$): $\delta$ 8.40 (d, $J = 9.0$ Hz, 2H, CH$_{ar}$), 8.23 (d, $J = 8.9$ Hz, 2H, CH$_{ar}$), 7.98 (s, 1H, CH), 2.92 (s, 4H, CH$_2$).

$^{13}$C-NMR (101 MHz, DMSO-<em>d</em>$_6$): $\delta$ 170.1 (Cq), 168.4 (Cq), 160.7 (Cq), 139.1 (Cq), 132.0 (CH$_{ar}$), 127.7 (Cq), 122.7 (CH$_{ar}$), 95.5 (CH), 25.6 (CH$_2$).

HR-ESI-MS (m/z): [M+H]$^+$ calcd. for C$_{14}$H$_9$O$_5$N$_3$, 304.0564; found, 304.0554.
Compound 8

Sydnone 6 (103 mg, 0.429 mmol) was added to a cooled (0 °C) solution of N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (98.6 mg, 0.514 mmol, 1.20 eq) in anhydrous DMF (1.6 mL), and the mixture was stirred for 10 min. N-Hydroxysuccinimide (52.8 mg, 0.459, 1.07 eq) was added to the mixture, and the reaction was stirred at 0 °C for 30 min, and then at room temperature for 2.5 h. The reaction was diluted with ethyl acetate (30 mL), washed with saturated NH₄Cl (3 x 40 mL), dried over MgSO₄, filtered and the filtrate was concentrated under reduced pressure to give the desired NHS-sydnone 8 (122 mg, 84%) as an orange solid.

$^1$H-NMR (300 MHz, CDCl₃): δ 8.45 (d, $J = 8.6$ Hz, 2H, 2 × C₆H₄), 7.87 (d, $J = 8.6$ Hz, 2H, 2 × C₆H₄), 2.95 (s, 4H, 2 × NHS-C₂H₂).

$^{13}$C-NMR (75 MHz, CDCl₃): δ 168.8 (3 × C), 160.3 (C), 137.6 (C), 132.5 (2 × CH), 129.6 (C), 125.2 (2 × CH), 25.8 (2 × CH₂), missing C–Cl signal due to relaxation issue.

HR-ESI-MS (m/z): [M+H]$^+$ calcd for C₁₃H₉ClN₃O₆⁺, 338.0174; found, 338.0175.
Deprotected nucleoside 9 (450 mg, 1.58 mmol, 1.00 equiv.) and triethylamine (879 µL, 6.31 mmol, 4.00 equiv.) were dissolved in anhydrous DMF (10 mL). Sydnone 7 (717 mg, 2.37 mmol, 1.50 equiv.) was added and the reaction was stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the crude product was redissolved in methanol (50 mL). The solution was treated with Amberlite IRA-402 bicarbonate form for 30 minutes. After filtration, the solvent was evaporated. The crude mixture was purified via column chromatography on silica gel (dichloromethane/methanol 10:1). The product was obtained as a pale yellow powder (217 mg, 29%).

R f (DCM/MeOH 9:1) = 0.17.

1H-NMR (400 MHz, DMSO-d6): δ 11.29 (s, 1H, 3-NH), 8.75 (t, J = 5.7 Hz, 1H, NH), 8.13 – 8.09 (m, 2H, CHar), 8.07 – 8.02 (m, 2H, CHar), 7.86 (s, 1H, CH), 7.73 (s, 1H, CH), 6.17 (dd, J = 7.6, 6.2 Hz, 1H, 1'-CH), 5.23 (d, J = 4.3 Hz, 1H, 3'-OH), 5.02 (t, J = 5.2 Hz, 1H, 5'-OH), 4.25 (tt, J = 5.8, 3.3 Hz, 1H, 3'-CH), 3.77 (q, J = 3.7 Hz, 1H, 4'-CH), 3.57 (ddd, J = 11.8, 8.6, 5.2 Hz, 2H, 5'-CH2), 3.29 (q, J = 6.8 Hz, 2H, CH2), 2.26 (m, 2H, CH2), 2.17 – 2.03 (m, 2H, 2'-CH2), 1.71 (p, J = 7.2 Hz, 2H, CH2).

13C-NMR (101 MHz, DMSO-d6): δ 168.4 (Cq), 164.6 (Cq), 163.4 (Cq), 150.3 (Cq), 137.9 (Cq), 136.4 (Cq), 136.1 (CH), 128.9 (CHar), 121.5 (CHar), 112.9 (Cq), 95.1 (CH), 87.3 (4'-CH), 83.9 (1'-CH), 70.4 (3'-CH), 61.3 (5'-CH2), 39.1 (2'-CH2), 38.8 (CH2)27.8 (CH2), 24.0 (CH2).

HR-ESI-MS: m/z calcd. for C_{21}H_{24}O_{8}N_{5} [MH]+: 474.1619, found: 474.1617.
Compound 2

Deprotected nucleoside 9 (70.0 mg, 245 µmol, 1.00 equiv.) and triethylamine (136 µL, 981 µmol, 4.00 equiv.) were dissolved in anhydrous DMF (5 mL). Sydnone 7 (100 mg, 294 µmol, 1.20 equiv.) was added and the reaction was stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the crude product was redissolved in methanol (50 mL). The solution was treated with Amberlite IRA-402 bicarbonate form for 30 minutes. After filtration, the solvent was evaporated. The crude mixture was purified via column chromatography on silica gel (dichloromethane/methanol 10:1). The product was obtained as a pale yellow foam (66 mg, 54%).

R<sub>f</sub> (DCM/MeOH 9:1) = 0.15.

<sup>1</sup>H-NMR (400 MHz, DMSO-<sup>d6</sup>) δ = 11.26 (s, 1H, 3'-NH), 8.76 (t, J = 5.7 Hz, 1H, NH), 8.12 (d, J = 8.6 Hz, 2H, CH<sub>ar</sub>), 7.94 (d, J = 8.7 Hz, 2H, CH<sub>ar</sub>), 7.73 (s, 1H, 6-CH), 6.18 (dd, J = 7.5, 6.1 Hz, 1H, 1'-CH), 5.23 (d, J = 4.2 Hz, 1H, 3'-OH), 5.02 (d, J = 5.5 Hz, 1H, 5'-OH), 4.29 – 4.21 (m, 1H, 3'-CH), 3.77 (q, J = 3.6 Hz, 1H, 4'-CH), 3.58 (qd, J = 11.8, 5.9 Hz, 2H, 5'-CH<sub>2</sub>), 3.29 (dd, J = 6.9 Hz, 2H, CH<sub>2</sub>), 2.34 – 2.20 (m, 2H, CH<sub>2</sub>), 2.16 – 2.03 (m, 2H, 2'-CH<sub>2</sub>), 1.71 (p, J = 7.2 Hz, 2H, CH<sub>2</sub>).

<sup>13</sup>C-NMR (101 MHz, DMSO) δ = 164.8 (C<sub>q</sub>), 163.4 (C<sub>q</sub>), 150.3 (C<sub>q</sub>), 138.6 (C<sub>q</sub>), 136.4 (6-CH<sub>2</sub>), 134.4 (C<sub>q</sub>), 128.9 (CH<sub>ar</sub>), 125.4 (CH<sub>ar</sub>), 112.9 (C<sub>q</sub>), 99.4 (C<sub>q</sub>), 87.3 (4'-CH), 83.9 (1'-CH), 70.4 (3'-CH), 61.3 (4'-CH, 5'-CH<sub>2</sub>), 39.4 (2'-CH<sub>2</sub>), 38.8 (CH<sub>2</sub>), 27.8 (CH<sub>2</sub>), 23.9 (CH<sub>2</sub>).

HR-ESI-MS: m/z calcd. for C<sub>21</sub>H<sub>23</sub>O<sub>8</sub>N<sub>5</sub>Cl [MH]<sup>+</sup>: 508.1230, found: 508.1224.
Deprotected nucleoside 10 (256 mg, 0.832 mmol, 1.00 equiv.) and triethylamine (464 µL, 3.33 mmol, 4.00 equiv.) were dissolved in anhydrous DMF (10 mL). Sydnone 7 (378 mg, 1.25 mmol, 1.50 equiv.) was added and the reaction was stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the crude product was redissolved in methanol (50 mL). The solution was treated with Amberlite IRA-402 bicarbonate form for 30 minutes. After filtration, the solvent was evaporated. The crude mixture was purified via column chromatography on silica gel (dichloromethane/methanol 9:1). The product was obtained as a pale yellow powder (119 mg, 28%).

Rf (DCM:MeOH 9:1): = 0.11

$^1$H-NMR (400 MHz, DMSO) δ (ppm) = 8.87 (s, 1H, NH), 8.16-8.13 (m, 2H, CH$_{2}$), 8.06-8.04 (m, 2H, CH$_{2}$), 8.02 (s, 1H, H-2), 7.88 (s, 1H, CH), 7.16 (s, 1H, H-8), 6.58 (s, 2H, NH$_{2}$), 6.47 (dd, J = 8.3, 5.9 Hz, 1H, H-1'), 5.25 (s, 1H, 3'-OH), 5.09 (s, 1H, 5'-OH), 4.33-4.31 (m, 3'-H), 3.80-3.77 (m, 1H, 4'-H), 3.57-3.45 (m, 2H, H-5'), 3.38 (q, J = 6.7 Hz, 2H, CH$_{2}$-NH), 2.82 (t, J = 7.6 Hz, 2H, CH$_{2}$), 2.47 (s, 1H, H-2'a'), 2.10 (ddd, J = 13.0, 6.0, 2.5 Hz, 1H, H-2'a'), 1.83 (p, J = 7.4 Hz, 2H, CH$_{2}$).

$^{13}$C-NMR (400 MHz, DMSO) δ (ppm) = 168.96 (Cq), 165.08 (Cq), 158.08 (Cq), 158.08 (C-6), 151.81 (C-2), 150.91 (C-4), 138.43 (Cq), 136.52 (Cq), 129.60 (C$_{Ar}$), 121.90 (C$_{Ar}$), 119.20 (C-8), 115.18 (Cq), 102.56 (C-5), 95.60 (CH), 87.59 (Cq), 83.17 (C-1'), 71.59 (C-3'), 62.62 (C-5'), 40.20 (NH$_{2}$-CH$_{2}$), 39.78 (C-2'), 30.39 (CH$_{2}$), 23.97 (CH$_{2}$).

HR-MS (FAB) m/z: calc. for C$_{23}$H$_{26}$O$_{6}$N$_{7}$ [M+H]: 496.1939, found: 496.1937.
Compound 4

Deprotected nucleoside 10 (150 mg, 0.488 mmol, 1.00 equiv.) and triethylamine (283 µL, 2.03 mmol, 4.00 equiv.) were dissolved in anhydrous DMF (5 mL). Sydnone 8 (198 mg, 0.586 mmol, 1.20 equiv.) was added and the reaction was stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the crude product was redissolved in methanol (50 mL). The solution was treated with Amberlite IRA-402 bicarbonate form for 30 minutes. After filtration, the solvent was evaporated. The crude mixture was purified via column chromatography on silica gel (dichloromethane/methanol 9:1). The product was obtained as a pale yellow powder (175 mg, 36%).

Rf (DCM:MeOH 9:1): = 0.41

$^1$H-NMR (400 MHz, DMSO): δ (ppm) = 8.79 (s, 1H, NH), 8.15-8.13 (m, 2H, CH$_{Ar}$), 8.02 (s, 1H, H-2), 7.95-7.93 (m, 2H, CH$_{Ar}$), 7.16 (s, 1H, H-8), 6.59 (s, 2H, NH$_2$), 6.49 (dd, J = 8.3, 6.0 Hz, H-1'), 5.23 (d, J = 4.1 Hz, 3'-OH), 5.06 (t, 5.7 Hz, 1H), 4.33-4.31 (m, 1H, 3'-H), 3.80-3.78 (m, 1H, 4'-H), 2.46 (s, 1H, H-2'), 2.10 (ddd, J = 13.0, 6.0, 2.6 Hz, 1H, H-2'$_b$), 1.85 (p, J = 7.0 Hz, 2H, CH$_2$).

$^{13}$C-NMR (400 MHz, DMSO) δ (ppm) = 166.30 (Cq), 163.91 (Cq), 162.80 (Cq), 158.09 (C-6), 151.81 (C-2), 150.93 (C-4), 139.11 (Cq), 134.81 (Cq), 129.45 (CAr), 125.84 (CAr), 119.25 (C-8), 115.15 (Cq), 102.57 (C-5), 99.89 (CH), 87.60 (Cq), 83.20 (C-1'), 71.60 (C-3'), 62.64 (C-5'), 40.21 (NH2-CH2), 39.79f (C-2'), 30.46 (CH2), 23.91 (CH2).

HR-MS (FAB) m/z: calc. for C$_{23}$H$_{25}$ClN$_7$O$_6$Cl$^+$ [M+H]: 530.1549, found: 530.1548.
4. Oligonucleotide Synthesis

DNA1 and DNA2 were synthesized by using standard solid-phase phosphoramidite synthesis protocol on a H-6 DNA/RNA synthesizer by K&A Laborgeräte. Natural phosphoramidites (2’-deoxyadenosine, 2’-deoxyguanosine, 2’-deoxycytidine, thymidine, Sigma Aldrich) were used as a 67 mM solution in acetonitrile. Artificial phosphoramidite 11 was used as a 100 mM solution in dichloromethane. As solid phase, CPG columns (1 µmol, Sigma Aldrich) were used. After synthesis, the CPG columns were dried in high vacuum. The CPG was removed from the columns and transferred to an Eppendorf reaction vial. 700 µL of 25% ammonium hydroxide was added and the suspension incubated over night at 55 °C, followed by removal of the solvents by vacuum centrifugation (35 min, 35 °C, 100 mbar (ammonium hydroxide) followed by 4 h, 25 °C, 0.100 mbar). The crude product was used without any further purification for synthesis of DNA3-DNA6.

Approximately 1 µmol of DNA1 (or DNA2) was dissolved in anhydrous DMSO (300 µL). NHS-ester 7 or 8 (2 mg) was dissolved in anhydrous DMSO (100 µL) and added to the DNA1 solution. DIPEA (5 µL) was added and the vials shook on a laboratory shaker for 16 hours. After completion of the reaction, the solvent was removed in the vacuum centrifuge (8 h, 25 °C, 0.100 mbar).

The DNA pellet was dissolved in water (600 µL) and purified via semi-preparative HPLC VDSpher OptiBio PUR 300 S18-SE column (250 x 10 mm, 5 µm, 300 µL injection, 0-20% acetonitrile, 0.1 M ammonium acetate, 40 °C, 30 minutes). The detection wavelength was set to 260 and 280 nm (oligonucleotide absorbance) and 320 nm (sydnone absorbance). The success of the synthesis was determined via MALDI-TOF with 3-HPA as a matrix substance.

**Table S1.** MS analysis of DNA1-DNA6.

| sequence | calc. mass | meas. mass |
|----------|------------|------------|
| DNA1     | 5191.9     | 5193.8     |
| DNA2     | 5214.0     | 5214.1     |
| DNA3     | 5379.9     | 5374.4     |
| DNA4     | 5402.0     | 5408.7     |
| DNA5     | 5413.9     | 5414.9     |
| DNA6     | 5436.0     | 5440.4     |
5. SPSAC Labeling Experiments

Nucleosides

For the labeling of sydnone-modified nucleosides (Figure 3): to a 25 µM solution of nucleosides 1-4 in methanol in a quartz glass cuvette, the respective amount of BCN (stock solutions in methanol) was added (final concentration 250 µM, 10.0 eq. relative to nucleoside). Absorbance and emission spectra were recorded at exactly defined times using the automatic settings of each spectrometer (UV/Vis: Varian Cary Bio 100, average time 0.1 s, data interval 1.0 nm, scanning speed 600 nm/min, source changeover at 350 nm. The spectra were corrected against the UV/Vis absorbance of the solvent. Emission: Jobin-Yvon Fluoromax-3, increment time 0.2 s, integration time 0.1 s, slit width 3 nm. The spectra were corrected against the Raman scattering of the solvent.)

Oligonucleotides

For the labeling of sydnone-modified oligonucleotides (Figure S7-S13), to a 2.5 µM solution of DNA3 (or DNA4, DNA5, DNA6) in water in a 1 mL reaction tube, the respective amount of Cy3-DIBAC (stock solution in DMSO) was added and separated in 100 µL portions for monitoring via HPLC chromatography (ThermoFisher Scientific Ultimate 3000, VDSpher OptiBio PUR 300 S18-SE column, reversed phase, 0-80% acetonitrile in 40 minutes at 40 °C, detection wavelengths 260, 280, 315 and 555 nm).

The success of the reaction was verified by MALDI-TOF MS.

Table S2. MALDI-TOF MS analysis of DNA3-Cy3-DIBAC–DNA6-Cy3-DIBAC

| adduct              | calculated mass [Da] | measured mass [Da] |
|---------------------|----------------------|--------------------|
| DNA3-Cy3-DIBAC      | 6093.4               | 6093.8             |
| DNA4-Cy3-DIBAC      | 6115.0               | 6115.3             |
| DNA5-Cy3-DIBAC      | 6127.4               | 6129.5             |
| DNA6-Cy3-DIBAC      | 6149.0               | 6148.6             |
Figure S7. Analytical HPLC (260 nm detection wavelength) of the reaction of DNA3 (2.5 µM) and Cy3-DIBAC (25 µM) in water (+5% DMSO) at 20 °C. The spectrum contains impurities (marked with an asterisk) from either the oligonucleotide synthesis (9 min retention time) or the dye (37 and 41 min retention time).

Figure S8. Analytic HPLC (260 nm detection wavelength) of the reaction between 2.5 µM DNA3 and 3.75 µM Cy3 DIBAC (1.50 equiv.). The signal of DNA3 (9.5 min) and Cy3 DIBAC (40 min) are decreasing, whereas the product signal (22 min) is increasing.
Figure S9. Analytical HPLC (260 nm detection wavelength) of the reaction between 2.5 µM DNA5 and 3.75 µM Cy3 DIBAC (1.50 equiv.). The signal of DNA5 (9.5 min) and Cy3 DIBAC (40 min) are decreasing, whereas the product signal (23 min) is increasing. Additionally, the chromatogram shows impurities (marked with an asterisk) at 9 min, 37 min and 41 min retention time.

Figure S10. Analytical HPLC (260 nm detection wavelength) of the reaction of DNA4 (2.5 µM) and Cy3-DIBAC (3.75 µM, 1.50 eq) in water (+5% DMSO) at 20 °C. The spectrum contains impurities (marked with an asterisk) from either the oligonucleotide synthesis (9 min retention time) or the dye.
**Figure S11.** Analytical HPLC (260 nm detection wavelength) of the reaction of DNA4 (2.5 µM) and Cy3-DIBAC (12.5 µM, 5.00 eq) in water (+5% DMSO) at 20 °C. The spectrum contains impurities (marked with an asterisk) from either the oligonucleotide synthesis (9 min retention time) or the dye (40 min retention time).

**Figure S12.** Analytical HPLC (260 nm detection wavelength) of the reaction of DNA6 (2.5 µM) and Cy3-DIBAC (3.75 µM, 1.50 eq) in water (+5% DMSO) at 20 °C. The spectrum contains impurities (marked with an asterisk) from either the oligonucleotide synthesis (9 min retention time) or the dye.
Figure S13. Analytical HPLC (260 nm detection wavelength) of the reaction of DNA6 (2.5 µM) and Cy3-DIBAC (12.5 µM, 5.00 eq) in water (+5% DMSO) at 20 °C. The spectrum contains impurities (marked with an asterisk) from either the oligonucleotide synthesis (9 min retention time) or the dye (40 min retention time).
Figure S14. UV/vis absorbance (left) and fluorescence (right, $\lambda_{\text{exc}}$=315 nm) changes recorded during SPSAC labeling experiments with 25 µM nucleoside 3 and 250 µM BCN 11 (10.0 equiv.) in MeOH at 20 °C over 4 h.

Figure S15. UV/vis absorbance (left) and fluorescence (right, $\lambda_{\text{exc}}$=315 nm) changes recorded during SPSAC labeling experiments with 25 µM nucleoside 4 and 250 µM BCN 11 (10.0 equiv.) in MeOH at 20 °C over 4 h.
6. Cell experiments

Human cervix carcinoma cells (HeLa) were cultured in Dulbecco’s modified Eagle Medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin (100 µg/mL) at 37°C/5% CO₂. For sub-culturing, cells were detached with 0.25% trypsin-EDTA solution. Unless stated otherwise, 4×10⁴ cells per well were seeded into Ibidi 8-well µ-Slides with ibiTreat surface.

For the SPSAC labeling experiments, cells were transfected for 16 hours with 75 ng per well DNA3 or DNA5 using ScreenFect A according to the protocol supplied by the manufacturer: Screenfect A (1.4 µL) was diluted with dilution buffer (38.6 µL). DNA (0.55 µL, 100 µM solution in water) was diluted with dilution buffer (39.5 µL). Both solutions were combined and incubated for 30 minutes allow lipoplex formation. Subsequently, lipoplexes were diluted with DMEM (720 µL), and 200 µL of the solution was added to each well. Cells were fixed using 4% paraformaldehyde in PBS for 15 minutes, 50 mM glycine/50 mM ammonium chloride in PBS for 5 minutes and treated with 7 nM sulfo-Cy3 in PBS for 3 hours, followed by washing with 2 × PBS.

Visualization of sulfo-Cy3 was performed with a Leica DMi8, TCS SP8 confocal microscopy with a 40x water objective. Image acquisition was conducted at a resolution of 1024 × 1024 pixels and 8 bit depth using LAS X 3.5.7.23225 sofware. Excitation was set to 488 nm (OPSL 488 laser, 15% laser power) and emission detected at 550-650 nm (HyD detector, 100% smart gain). Fluorescence settings were complemented with a transmission channel, detected with a PMT detector.
7. Images of NMR spectra and MS analyses

Figure S16. $^1$H-NMR (400 MHz, DMSO-$d_6$) spectrum of 7.
Figure S17. $^{13}$C-NMR (101 MHz, DMSO) spectrum of 7.

Figure S18. ESI-MS analysis of 7.
Figure S19. $^1$H-NMR (400 MHz, DMSO-d$_6$) spectrum of 1.

Figure S20. $^{13}$C-NMR (101 MHz, DMSO) spectrum of 1.
Figure S21. ESI-MS analysis of 1.

Figure S22. $^1$H-NMR (400 MHz, DMSO-d$_6$) spectrum of 2.
Figure S23. $^{13}$C-NMR (101 MHz, DMSO) spectrum of 2.

Figure S24. ESI-MS analysis of 2.
Figure S25. $^1$H-NMR (400 MHz, DMSO-d$_6$) spectrum of 3.

Figure S26. $^{13}$C-NMR (101 MHz, DMSO) spectrum of 3.
Figure S27. HR-MS (FAB) m/z calculation for 3.

Figure S28 ¹H-NMR (400 MHz, DMSO-d₆) spectrum of 4.
Figure S29. $^{13}$C-NMR (101 MHz, DMSO) spectrum of 4.

Figure S30. HR-MS (FAB) m/z calculation for 4.
Figure S31. MALDI-TOF MS analysis of DNA1.

Figure S32. MALDI-TOF MS analysis of DNA2.
Figure S33. MALDI-TOF MS analysis of DNA3.

Figure S34. MALDI-TOF MS analysis of DNA4.
Figure S35. MALDI-TOF MS analysis of DNA5.

Figure S36. MALDI-TOF MS analysis of DNA6.
Figure S37. MALDI-TOF MS analysis of 12.
Figure S38. MALDI-TOF MS analysis of 13.
Figure S39. MALDI-TOF MS analysis of the 1-DIBAC adduct.
Figure S40. MALDI-TOF MS analysis of 14.
Figure S41. MALDI-TOF MS analysis of 15.
Figure S42. MALDI-TOF MS analysis of DNA3-Cy3-DIBAC adduct.

Figure S43. MALDI-TOF MS analysis of DNA4-Cy3-DIBAC adduct.
**Figure S44.** MALDI-TOF MS analysis of DNA5-Cy3-DIBAC adduct.

**Figure S45.** MALDI-TOF MS analysis of DNA6-Cy3-DIBAC adduct.
8. Kinetic measurements of nucleosides and DNA

**Figure S46.** Analytical HPLC (260 nm detection wavelength) of the reaction between 2.5 µM DNA4 and 12.5 µM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference. The signal of DNA4 (10 min) is vanished, whereas the product signal (23 min) is formed. Additionally, the chromatogram shows the thymidine at 9 min, the dye at 42 min and impurities at 3 min and 54 min retention time.

**Figure S47.** Analytical HPLC (260 nm detection wavelength) of the reaction between 2.5 µM DNA4 and 12.5 µM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference.
Figure S48. Analytical HPLC (260 nm detection wavelength) of the reaction between 2.5 µM DNA6 and 12.5 µM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference. The signal of DNA6 (10 min) is vanished, whereas the product signal (23 min) is formed. Additionally, the chromatogram shows the thymidine at 9 min, the dye at 42 min and impurities at 3 min, 31 min and 54 min retention time.

Figure S49. Analytical HPLC (260 nm detection wavelength) of the reaction between 2.5 µM DNA6 and 12.5 µM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference.
Figure S50. Analytical HPLC (260 nm detection wavelength) of the reaction between 150 µM 3 and 750 µM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference. The signal of 3 (13 min) is decreasing, whereas the product signal (17 min) is formed. Additionally, the chromatogram shows the thymidine at 8 min and impurities at 59 min retention time.

Figure S51. Analytical HPLC (260 nm detection wavelength) of the reaction between 150 µM 3 and 750 µM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference.
**Figure S52.** Determination of the reaction rate constant between 150 µM 3 and 750 µM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference. Analytical HPLC runs were performed at different reaction times to determine the rate constant. $k_{i, \text{obs}} = 4.9464 \times 10^{-5} \pm 9.9191 \times 10^{-6}$ yields $k_{i, \text{cyc}} = 0.0659 \pm 0.0132 \text{ M}^{-1}\text{s}^{-1}$.

**Figure S53.** Analytical HPLC (260 nm detection wavelength) of the reaction between 150 µM 4 and 750 µM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference. The signal of 4 (14 min) is vanished, whereas the product signal (18 min) is formed. Additionally, the chromatogram shows the thymidine at 8 min and impurities at 59 min retention time.
Figure S54. Analytical HPLC (260 nm detection wavelength) of the reaction between 150 mM 4 and 750 mM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference.

Figure S55. Determination of the reaction rate constant between 150 mM 3 and 750 mM Cy3 DIBAC (5.00 equiv.) with thymidine as a reference. Analytical HPLC runs were performed at different reaction times to determine the rate constant. $k_{i,obs} = 0.00329 \pm 0.00112 \rightarrow k_{i,cyc} = 4.39 \pm 1.49 \text{ M}^{-1}\text{s}^{-1}$. 
9. References

i Favre, C.; de Cremoux, L.; Badaut, J.; Friscourt, F. J. Org. Chem. 2018, 83, 2058.
ii Friscourt, F.; Ledin, P. A.; Mbuia, N. E.; Flanagan-Steet, H. R.; Wolfert, M. A.; Steet, R.; Boons, G. J. J. Am. Chem. Soc. 2012, 134, 5381.
iii Debets, M. F.; Prins, J. S.; Merkx, D.; van Berkel, S. S.; van Delft, F. L.; van Hest, J. C. M.; Rutjes, F. Org. Biomol. Chem. 2014, 12, 5031.
iv Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F.; van Hest, J. C. M.; Lefeber, D. J.; Friedl, P.; van Delft, F. L. Angew. Chem. Int. Ed. 2010, 49, 9422.