Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Fast and efficient DNA crosslinking and multiple orthogonal labelling by copper-free click chemistry

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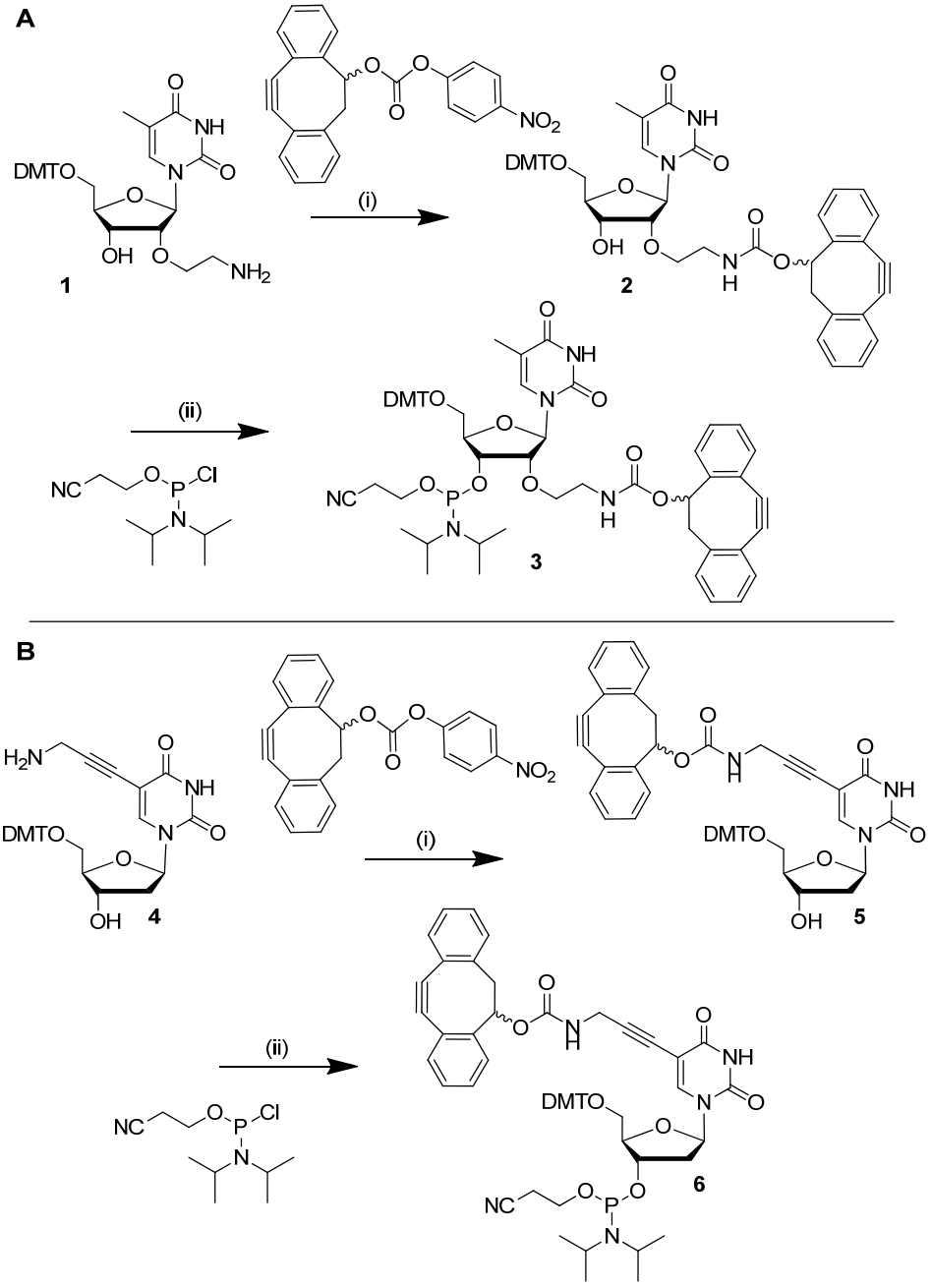
Contents

Organic synthesis: general Information

All reagents were purchased from Aldrich, Fluka, Avocado, or Acros Organics and used without purification with the exception of the following solvents, which were purified by distillation: THF (over sodium wire), dichloromethane, di-isopropylethylamine, triethylamine and pyridine (over calcium hydride). Chemical transformations were carried out under an atmosphere of argon using oven-dried glassware. Thin layer chromatography (TLC) was performed using Merck Kieselgel 60 F24 silica gel plates (0.22 mm thickness, aluminium backed) and the compounds were visualised by irradiation at 254 nm or by staining with anisaldehyde, ceric sulfate or ninhydrin solution. Column chromatography was carried out under argon pressure using Fisher Scientific DAVISIL 60Å (35-70 micron) silica.

^{1}H NMR spectra were measured at 300 MHz on a Bruker AC300 spectrometer or at 400 MHz on a Bruker DPX400 spectrometer. The ^{13}C NMR spectra were measured at 75 MHz and 100 MHz respectively on the same spectrometers. The ^{31}P NMR spectrum was recorded on a Bruker AC300 spectrometer at 121 MHz. Chemical shifts are given in ppm relative to tetramethylsilane, and *J* values are quoted in Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal, assignment of the compounds was aided by COSY (^{1}H-^{1}H), HMQC and HMBC (^{1}H-^{13}C) experiments.

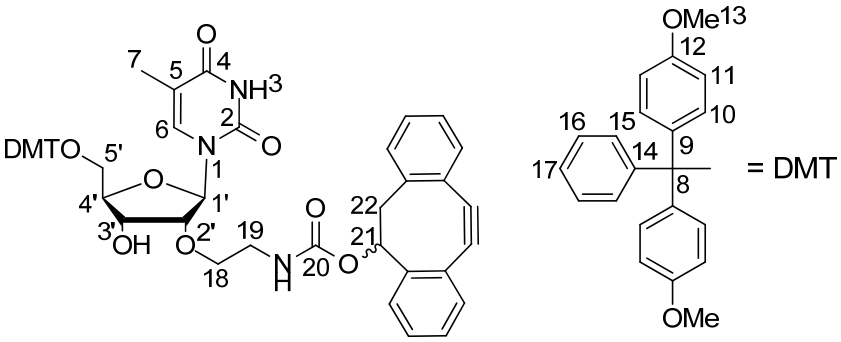
All the low-resolution mass spectra were recorded using electrospray ionisation on a Fisons VG platform instrument or a Waters ZMD quadrupole mass spectrometer in HPLC grade acetonitrile or methanol. High-resolution mass spectra were recorded in HPLC grade acetonitrile or methanol using electrospray ionisation on a Bruker APEX III FT-ICR mass spectrometer. Electrospray mass spectrometry of oligonucleotides was recorded in water using a Bruker micrOTOF^{TM} II focus ESI-TOF MS instrument in ES^{-} mode.



A. Synthesis of $^{2'\text{-DIBO-}}$K phosphoramidite monomer. (i) DCM, Et_3N, room temperature, 5 h, 88%, (ii) DIPEA, DCM, room temperature, 3 h, 59%.

B. Synthesis of $^{5\text{-DIBO-}}$K phosphoramidite monomer. (i) DCM, Et_3N, 4 h, 55 °C, 73% (ii) DIPEA, DCM, 1 h, room temperature, 72%.

2′-*O*-{2-[3-(7,8-Didehydro-1,2:5,6-dibenzocyclooctynyl)oxyamido]ethyl}-5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine (2)



To a solution of DIBO p-nitrophenyl carbonate[1] (0.75 g, 1.93 mmol) in anhydrous dichloromethane (14 mL) was added anhydrous triethylamine (1.5 mL) followed by 5′-DMT 2′-aminoethoxy thymidine (compound 1 in scheme 1, 1.4 g, 2.32 mmol) under an argon atmosphere at room temperature. The reaction mixture was stirred for 5 h at room temperature, then diluted with dichloromethane (10 mL) and the resulting mixture was extracted with saturated brine (2 x 150 mL). The combined extracts were dried (Na_2SO_4) and the solvent was removed *in vacuo*. The crude product was purified by column chromatography (60-100% EtOAc/Hexane) to give compound **2** as a white foam (1.43 g, 1.69 mmol, 88%). R_f 0.45 (10% MeOH/dichloromethane).

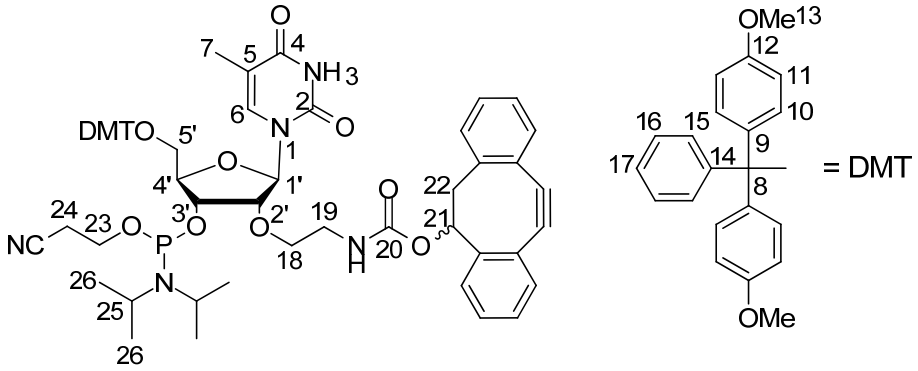
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.16 (s, 1H, N**H**), 7.66 (s, 1H, **H^6**), 7.49 - 7.44 (m, 1H, **H^{Ar}**), 7.39 (app. d, 2H, *J* = 7.6 Hz, **H^{Ar}**), 7.36 - 7.17 (m, 14H, **H^{Ar}**), 6.82 (d, 4H, *J* = 8.6 Hz, **H^{11}**), 5.95 (d, 1H, *J* = 2.0 Hz, **H$^{1'}$**) 5.62 (s, 1H, N**H**), 5.47 (app. s., 1H, **H^{21}**), 4.45 - 4.39 (m, 1H, **H$^{3'}$**), 4.06 - 3.98 (m, 2H, **H$^{2'}$** and **H$^{4'}$**), 3.94 (app. s., 1H, **H^{18}** or **^{19}**), 3.81 (app. s., 1H, **H^{18}** or **^{19}**), 3.76 (s, 6H, 2×OC**H$_3$**), 3.57 - 3.46 (m, 2H, **H$^{5'}$** and **H^{18}** or **^{19}**), 3.44 - 3.34 (m, 2H, **H$^{5'}$** and **H^{18}** or **^{19}**), 3.14 (app. d, 1H, *J* = 14.7 Hz, **H^{22}**), 2.87 (dd, 1H, *J* = 16.1, 2.5 Hz, **H^{22}**), 1.37 (s, 3H, **H^7**)

^{13}C NMR (100 MHz, $CDCl_3$) δ 164.2 (**C^4**), 159.0 (**C^{12}**), 156.2 (**C^{20}**), 151.2 (**C^{Ar}**), 151.0 (**C^2**), 144.6 (**C^{Ar}**), 135.7 (**C^9**), 135.6 (**C^{Ar}**), 135.4 (**C^6**), 130.4 (**CH^{Ar}**), 130.2 (**CH^{Ar}**), 128.4 (**CH^{Ar}**), 128.3 (**CH^{Ar}**), 128.2 (**CH^{Ar}**), 127.4 (**CH^{Ar}**), 127.3 (CH^{Ar}), 126.5 (**CH^{Ar}**), 126.2 (**CH^{Ar}**), 124.0 (**CH^{Ar}**), 121.6 (**C^{Ar}**), 116.0 (**CH^{Ar}**), 113.6 (**C^{11}**), 113.2 (**C^{Ar}**), 111.5 (**C^{Ar}**), 110.2 (**C^{Ar}**), 88.1 (**C$^{1'}$**), 87.1 (**C^8**), 83.6 (**C$^{2'}$**), 83.1 (**C$^{4'}$**), 70.7 (**C^{18}** or **^{19}**), 69.2 (**C$^{3'}$**), 62.1 (**C$^{5'}$**), 55.5 (2×O**C**H$_3$), 46.5 (**C^{22}**), 41.2 (**C^{18}** or **^{19}**), 12.2 (**C^7**)

LRMS [ESI$^+$, MeOH] *m/z* (%): 872.5 ([M+Na]$^+$, 75%).

HRMS [ESI$^+$] calcd. for $C_{50}H_{47}N_3O_{10}$ (M) 849.3261, [M+Na]$^+$ = 872.3154, found 872.3155.

2′-*O*-{2-[3-(7,8-Didehydro-1,2:5,6-dibenzocyclooctynyl)oxyamido]ethyl}-5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (3)



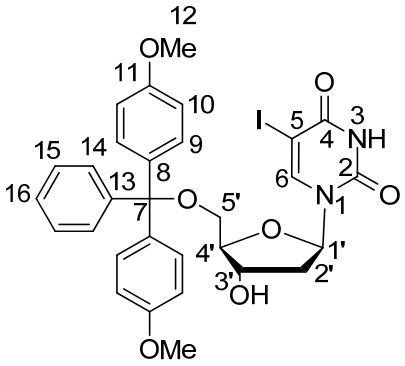
To a solution of nucleoside **2** (0.35 g, 0.42 mmol) in distilled dichloromethane (5 mL) and distilled di-isopropylethylamine (0.15 mL, 0.83 mmol), under an argon atmosphere excluding moisture, was added 2-cyanoethyl-*N,N*-diisopropyl-chlorophosphoramidite (0.13 mL, 0.58 mmol) dropwise. The reaction was stirred at room temperature for 3 h then was transferred to a separating funnel containing distilled dichloromethane (25 mL), and washed with saturated aq KCl (25 mL). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. Purification by column chromatography (60% EtOAc/hexane, 0.5% pyridine) under argon pressure, gave the desired product **3** as a diastereomeric mixture (*ca. 1:1:1:1*), as an air-sensitive white foam (0.26 g, 0.25 mmol, 59.4%). R_f 0.26 (55% EtOAc/Hexane)

^{31}P NMR (121 MHz, CD_3CN) δ 150.86, 150.79, 149.98, 149.82 (isomers)

^{1}H NMR (400 MHz, CD_3CN) δ 9.20 (s, 1H, N**H**) 7.60 - 7.53 (m, 1H, $\mathbf{H^{Ar}}$), 7.51 - 7.22 (m, 17H, $\mathbf{H^{Ar}}$), 6.87 (dd, 4H, J = 8.3, 5.3 Hz, $\mathbf{H^{11}}$), 6.09 (br. s., 1H, N**H**), 5.94 (d, 1H, J = 9.6 Hz, $\mathbf{H^{1'}}$), 5.36 (app. s., 1H, $\mathbf{H^{21}}$), 4.59 - 4.41 (m, 1H, $\mathbf{H^{3'}}$), 4.25 - 4.13 (m, 2H, $\mathbf{H^{2'}}$ and $\mathbf{H^{4'}}$), 3.91 - 3.78 (2 × m, 1H, $\mathbf{H^{23}}$), 3.77 - 3.71 (m, 8H, 2×OC**H$_3$** and $\mathbf{H^{18}}$ or $\mathbf{^{19}}$), 3.70 - 3.59 (2 × m, 1H, $\mathbf{H^{23}}$), 3.58 - 3.54 (m, 2H, $\mathbf{H^{25}}$), 3.51 - 3.41 (m, 1H, $\mathbf{H^{18}}$ or $\mathbf{^{19}}$), 3.39 - 3.26 (m, 3H, $\mathbf{H^{5'}}$ and $\mathbf{H^{18}}$ or $\mathbf{^{19}}$), 3.17 (d, 1H, J = 15.2 Hz, $\mathbf{H^{22}}$), 2.82 (d, 1H, J = 14.7 Hz, $\mathbf{H^{22}}$), 2.64 (br. t, 1H, J = 5.8 Hz, $\mathbf{H^{24}}$), 2.49 (t, 1H, J = 5.8 Hz, $\mathbf{H^{24}}$), 1.41, 1.39, 1.38 and 1.37 (s, 3H, C**H$_3$**), 1.18 - 1.11 (m, 9H, $\mathbf{H^{26}}$), 1.03 (dd, 3H, J = 6.6, 3.5 Hz, $\mathbf{H^{26}}$)

LRMS [ESI$^+$, MeCN] *m/z* (%) 1072.9 ([M+Na]$^+$, 100%)

5′-*O*-(4,4′-Dimethoxytrityl)-5-iodo-2′-deoxyuridine[2]



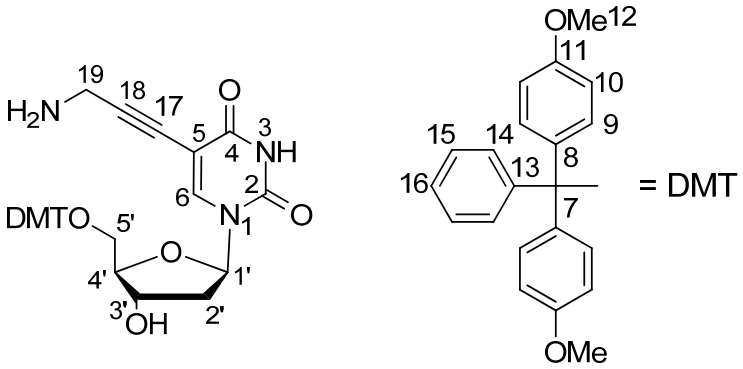
5-Iodo-2′-deoxyuridine (5.5 g, 15.5 mmol) was co-evaporated with distilled pyridine (3 x 10 mL) and suspended in distilled pyridine (40 mL). To this was added drop-wise a solution of 4,4′-dimethoxytrityl chloride (6.31 g, 18.6 mmol) in distilled pyridine (35 mL) over a period of 40 min and the reaction was stirred at room temperature for 4 h 15 min. The reaction was quenched by the addition of MeOH (40 mL) and then stirred for 20 min. The reaction volume was reduced by two thirds *in vacuo*, diluted with dichloromethane (200 mL) and extracted with distilled water (200 mL) and saturated aq. $NaHCO_3$ (200 mL). The organic layers were combined, dried (Na_2SO_4) and the solvent was removed *in vacuo*. Following purification by column chromatography (0-4% MeOH/dichloromethane with 0.5 % pyridine) the title compound was afforded as a white foam (8.35 g, 12.7 mmol, 82 %). R_f 0.44 (10% MeOH/dichloromethane).

^{1}H NMR (300 MHz, DMSO-d_6) δ 11.74 (s, 1H, N**H**), 8.02 (s, 1H, **H^6**), 7.44 - 7.11 (m, 9H, **H^{Ar}**), 6.90 (d, 4H, J = 8.8 Hz, **H^{10}**), 6.11 (t, 1H, J = 6.8 Hz, **H$^{1'}$**), 5.31 (d, 1H, J = 4.4 Hz, O**H$^{3'}$**), 4.27 - 4.19 (m, 1H, **H$^{3'}$**), 3.90 (q, 1H, J = 3.8 Hz, **H$^{4'}$**), 3.74 (s, 6H, **H^{12}**), 3.24 - 3.13 (m, 2H, **H$^{5'}$**), 2.28 - 2.11 (m, 2H, **H$^{2'}$**)
^{13}C NMR (75 MHz, DMSO-d_6) δ 160.5 (**C^2**), 158.1 (**C^4**), 150.0 (**C**H^6), 149.6 (**C^{Ar}**), 144.7 (**C**H^{Ar}), 144.2 (**C^{Ar}**), 136.1 (**C**H^{Ar}), 135.4 (**C^{Ar}**), 135.4 (**C^{Ar}**), 129.7 (**C**H^{Ar}), 127.9 (**C**H^{Ar}), 127.6 (**C**H^{Ar}), 126.7 (**C**H^{Ar}), 123.9 (**C**H^{Ar}), 113.3 (**C^{10}**), 85.8 (**C$^{4'}$**), 84.9 (**C$^{1'}$**), 70.5 (**C$^{3'}$**), 69.8 (**C^5**), 63.7 (**C$^{5'}$**), 55.0 (**C^{12}**), 39.9 (**C$^{2'}$**)

LRMS [ESI$^+$, MeOH] *m/z* (%): 679.2 ([M+Na]$^+$, 100%).

Data matches literature source[2]

5-(3-Aminopropynyl)-5´-*O*-(4,4´-dimethoxytrityl)-2´-deoxyuridine (4)[3]



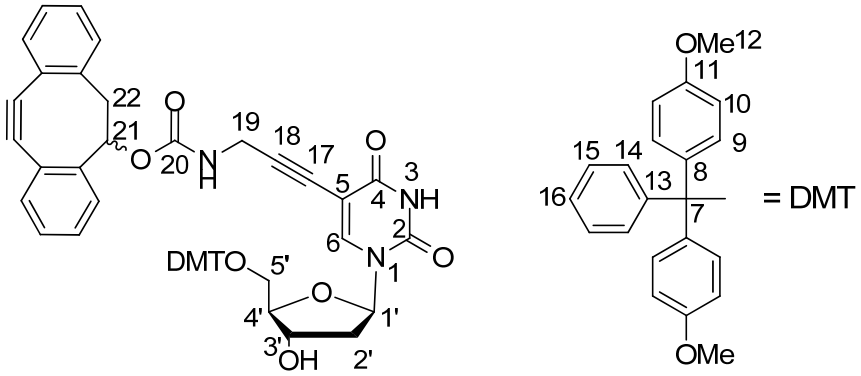
To a mixture of 5´-*O*-(4,4´-dimethoxytrityl)-5-iodo-2´-deoxyuridine[2] (3.0 g, 4.57 mmol) and CuI (0.17 g, 0.91 mmol) in anhydrous DMF (23 mL) under an argon atmosphere, was added propargylamine (0.6 mL, 9.14 mmol) and triethylamine (14 mL). The reaction was stirred for 10 min at room temperature before $Pd(PPh_3)_4$ (0.53 g, 0.46 mmol) was added, followed by stirring at room temperature for a further 3 h. The solvent was removed *in vacuo* and purification by column chromatography (2-4% MeOH/dichloromethane with 1 % pyridine) afforded the product **4** as a pale yellow foam (2.11 g, 3.61 mmol, 79 %). R_f 0.31 (10% MeOH/dichloromethane).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (1H, s, **H^6**), 7.38 (2H, d, *J* = 8 Hz, **H^{Ar}**), 7.31 - 7.14 (7H, m, **H^{Ar}**), 6.79 (4H, d, *J*=8 Hz, **H^{10}**), 6.28 (1H, t, *J* = 6 Hz, **H$^{1´}$**), 4.54 - 4.44 (1H, m, **H$^{3´}$**), 4.03 (1H, d, *J* = 2 Hz, **H$^{4´}$**), 3.73 (6H, s, 2×OC**H$_3$**), 3.40 (1H, dd, *J* = 11 and 2 Hz, **H$^{5´}$**), 3.23 (1H, dd, *J* = 11 and 3 Hz, **H$^{5´}$**), 3.21 - 3.03 (2H, br. s, C**H$_2$**), 2.50 - 2.39 (1H, m, **H$^{2´}$**), 2.32 - 2.20 (1H, m, **H$^{2´}$**)

^{13}C NMR (100 MHz, $CDCl_3$) δ 161.6 (**C^4**), 158.7 (**C^{11}**), 149.2 (**C^2**), 144.6 (**C^{Ar}**), 142.5 (**C^6**), 135.6 (**C^8**), 135.4 (**C^8**), 130.1 (**CH^{Ar}**), 130.0 (**CH^{Ar}**), 128.1 (**CH^{Ar}**), 127.9 (**CH^{Ar}**), 127.0 (**CH^{Ar}**), 113.4 (**C^{10}**), 100.2 (**C^5**), 87.0 (**C^7**), 86.6 (**C$^{4´}$** and **C^{18}**), 85.6 (**C$^{1´}$**), 73.1 (**C^{17}**) 72.1 (**C$^{3´}$**), 63.5 (**C$^{5´}$**), 55.3 (**C^{12}**), 41.6 (**C$^{2´}$**), 31.7 (**C^{19}**)

LRMS [ESI$^+$, MeOH] *m/z* (%): 606.4 ([M+Na]$^+$, 100%).
HRMS [ESI$^+$]: calcd. for $C_{33}H_{33}N_3O_7$ (M) 853.6310, [M+H]$^+$ = 854.2391, found 854.2383.

5-{3-[3-(7,8-Didehydro-1,2:5,6-dibenzocyclooctynyl)oxyamido]propynyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (5)



To a solution of DIBO p-nitrophenyl carbonate[1] (1.42 g, 3.68 mmol) in anhydrous dichloromethane (25 mL) was added anhydrous triethylamine (2.4 mL) followed by compound **4** (1.79 g, 3.07 mmol) under an argon atmosphere. The reaction mixture was stirred for 4 h at 55 °C, then diluted with dichloromethane (250 mL) and extracted with saturated brine (2 x 250 mL). The combined extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo*. The crude product was purified by column chromatography (60-100% EtOAc/Hexane, 0.5% Pyridine) to give compound **5** as a white foam (1.85 g, 2.23 mmol, 73%). R_f 0.47 (10% MeOH/dichloromethane).

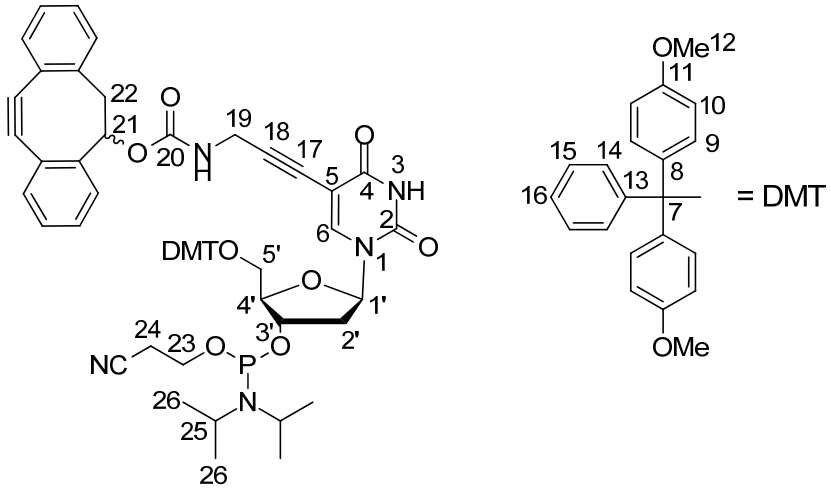
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.96 (s, 1H, N**H**), 8.15 (s, 1H, **H^6**), 7.38 - 7.45 (m, 3H, **H^{Ar}**), 7.17 - 7.35 (m, 14H, **H^{Ar}**), 6.83 (d, 4H, *J* = 9.1 Hz, **H^{10}**), 6.32 (t, 1H, *J* = 6.6 Hz, **H$^{1'}$**), 5.52 (br. s., 1H, N**H**), 5.44 (app. s., 1H, **H^{21}**), 4.56 (app. s., 1H, **H$^{3'}$**), 4.14 (d, 1H, *J* = 2.5 Hz, **H$^{4'}$**), 3.84 (d, 2H, *J* = 4.5 Hz, **H^{19}**), 3.71 (s, 6H, 2×OC**H$_3$**), 3.34 (app. s., 2H, **H$^{5'}$**), 3.10 (d, 1H, *J* = 14.7 Hz, **H^{22}**), 2.85 (d, 1H, *J* = 13.1 Hz, **H^{22}**), 2.49 - 2.58 (m, 1H, **H$^{2'}$**), 2.24 - 2.32 (m, 1H, **H$^{2'}$**).

^{13}C NMR (100 MHz, $CDCl_3$) δ 162.3 (**C^4**), 158.5 (**C^{11}**), 154.8 (**C^{20}**), 151.8 (**C^{Ar}**), 150.8 (**C^{Ar}**), 149.4 (**C^2**), 144.5 (**C^{Ar}**), 143.1 (**C^6**), 135.4 (**C^8**), 135.4 (**C^8**), 129.9 (**CH^{Ar}**), 129.9 (**CH^{Ar}**), 128.0 (**CH^{Ar}**), 127.9 (**CH^{Ar}**), 127.8 (**CH^{Ar}**), 127.0 (**CH^{Ar}**), 126.9 (**CH^{Ar}**), 126.9 (**CH^{Ar}**), 126.1 (**CH^{Ar}**), 125.8 (**CH^{Ar}**), 123.7 (**CH^{Ar}**), 123.7 (**CH^{Ar}**), 121.1 (**C^{Ar}**), 113.3 (**C^{10}**), 112.8 (**C^{Ar}**), 109.9 (**C^{Ar}**), 99.4 (**C^5**), 89.5 (**C^{18}**), 86.9 (**C^7**), 86.7 (**C$^{4'}$**), 85.9 (**C$^{1'}$**), 74.2 (**C^{17}**), 72.1 (**C$^{3'}$**), 63.5 (**C$^{5'}$**), 55.2 (**C^{12}**), 46.0 (**C^{22}**), 41.5 (**C$^{2'}$**), 31.6 (**C^{19}**)

LRMS [ESI$^+$, MeCN]: *m/z* (%): 852.6 ([M+Na]$^+$, 100%)

HRMS [ESI$^+$]: calcd. for $C_{50}H_{43}N_3O_9$ (M) 829.2999, [M+Na]$^+$ = 852.2892, found 852.2884.

5-{3-[3-(7,8-Didehydro-1,2:5,6-dibenzocyclooctynyl)oxyamido]propynyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (6)



To a solution of nucleoside **5** (1.75 g, 2.11 mmol) in distilled dichloromethane (20 mL) and distilled di-isopropylethylamine (0.73 mL, 4.21 mmol), under an argon atmosphere excluding moisture, was added 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.66 mL, 2.95 mmol) dropwise. The reaction was stirred at room temperature for 1 h then transferred to a separating funnel containing distilled dichloromethane (60 mL), and washed with saturated aq KCl (70 mL). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. Purification by column chromatography (65% EtOAc/hexane, 0.5% pyridine) under argon pressure, gave the desired product **6** (diastereomeric mixture) as an air-sensitive, powdery white foam (1.57 g, 1.52 mmol, 72%). R_f 0.32 (60% EtOAc/Hexane).

^{31}P NMR (121 MHz, CD_3CN) δ 149.26

^{1}H NMR (400 MHz, CD_3CN) δ 9.17 (s, 1H, N**H**), 7.95 (d, 1H, *J* = 11.6 Hz, **H^{Ar}**), 7. 56 - 7.43 (m, 3H, **H^{Ar}**), 7.41 - 7.19 (m, 14H, **H^{Ar}**), 6.91 - 6.83 (m, 4H, **H^{10}**), 6.17 - 6. 08 (m, 1H, **H$^{1'}$**), 5.94 (br. s., 1H, N**H**), 5.36 (app. s., 1H, **H^{21}**), 4. 72 - 4.58 (m, 1H, **H$^{3'}$**), 4.16 and 4.12 (dd, 1H, *J* = 6.6, 3.0 Hz and *J* = 6.1, 3.5 Hz, **H$^{4'}$**), 3. 87 - 3.80 (m, 2H, **H^{19}**), 3. 79 - 3.65 (m, 8H, 2×OC**H$_3$** and **H^{23}**), 3.65 - 3.52 (m, 2H, **H^{25}**), 3.41 - 3.31 (m, 1H, **H$^{5'}$**), 3.24 and 3.21 (dd, 1H, *J* = 11.1, 2.5 Hz and *J* = 10.6, 2.5, **H$^{5'}$**), 3.17 - 3.11 (m, 1H, **H^{22}**), 2.82 (dt, 1H, J=15.0, 3.9 Hz, **H^{22}**), 2.64 (t, 1H, *J* = 6.1 Hz, **H^{24}**), 2.54 (t, 1H, *J* = 6.1 Hz, **H^{24}**), 2.51 - 2.32 (m, 2H, **H$^{2'}$**), 1.18 - 1.14 (m, 9H, **H^{26}**), 1.08 (d, 3H, *J* = 6.6 Hz, **H^{26}**)

LRMS [ESI$^+$, MeCN] *m/z* (%): 1052.9 ([M+Na]$^+$, 100%).

Oligonucleotide synthesis and purification

Standard DNA phosphoramidites, solid supports, and additional reagents were purchased from Link Technologies and Applied Biosystems Ltd. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/ RNA synthesizer using a standard 0.2 or 1.0 μmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 35 s, and the coupling time for the modified phosphoramidite monomers was extended to 360 s. Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C. The oligonucleotides were purified by reversed-phase HPLC on a Gilson system using an XBridge^{TM} BEH300 Prep C18 10μM 10x250 mm column (Waters) with a gradient of acetonitrile in ammonium acetate (0% to 50% buffer B over 30 min, flow rate 4 mL/min), buffer A: 0.1 M ammonium acetate, pH 7.0, buffer B: 0.1 M ammonium acetate, pH 7.0, with 50% acetonitrile. Elution was monitored by UV absorption at 300 nm. After HPLC purification, oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare) and analysed by gel electrophoresis. All oligonucleotides were characterised by electrospray mass spectrometry and capillary electrophoresis (CE). Mass spectrometry of oligonucleotides was recorded in water using a Bruker micrOTOF^{TM} II focus ESI-TOF MS instrument in ES^{-} mode.

General method for azide labelling

6-Azidohexanoic acid NHS ester[4] (2 mg) in DMSO (80 μL) was added to the freeze-dried amino-modified oligonucleotides (1.0 μmole synthesis with one addition of the amino modified monomer) in 80 μL of 0.5 M Na_2CO_3/$NaHCO_3$ buffer at pH 8.75. The reaction was left for 4 h, room temperature to give the azide-labelled oligonucleotide. The reaction mixture was then desalted using a NAP-10 Sephadex column and purified by reversed-phase HPLC as described above. Following the same method, oligonucleotides with two additions of the aminoC_6dT monomer were labelled using 4 mg of 6-azidohexanoic acid NHS ester[4].

Labelling of oligonucleotide with bicyclo[6.1.0]nonyne (BCN) alkyne active carbonate

The BCN active carbonate (Berry & Associates, cat. No: LK 4320**,** 2 mg) in DMF (80 µL) was added post-synthetically to the freeze dried 2′-aminoethoxythymidine-modified oligonucleotide[5] (1.0 µmole) in 0.5 M Na_2CO_3/$NaHCO_3$ buffer (80 µL) at pH 8.75 and left to react for 4 h, room temperature. The resultant BCN-labelled oligonucleotide was desalted by using a NAP-10 Sephadex column and purified by reversed-phase HPLC.

SPAAC click DNA crosslinking ligation reactions

To a solution of azide oligonucleotide (2 nmoles) in 0.2 M NaCl (90 µL) was added the alkyne oligonucleotide (2 nmoles in 10 µL 0.2 M NaCl). The concentration of each oligonucleotide was 20 µM. The reaction mixture was left at room temperature for a specific period of time before adding formamide (50 µL) and loading directly onto a 20% polyacrylamide/7 M urea gel which was electrophoresed at a constant power of 20W for 3 h in 0.09 M Tris-borate-EDTA buffer. The gel-purified product was analysed and characterised by mass spectrometry. The reaction was repeated on a 20 nmole scale under the same conditions. The reaction mixture was left at room temperature for 1 h before desalting using NAP-10 column and purifying by HPLC as described above. The purified product was analysed and characterised by mass spectrometry and capillary electrophoresis.

A) Gel electrophoresis for A_pT steps (5′- ACAGAATTCATATT – 3′)

Major to Major groove ligation:

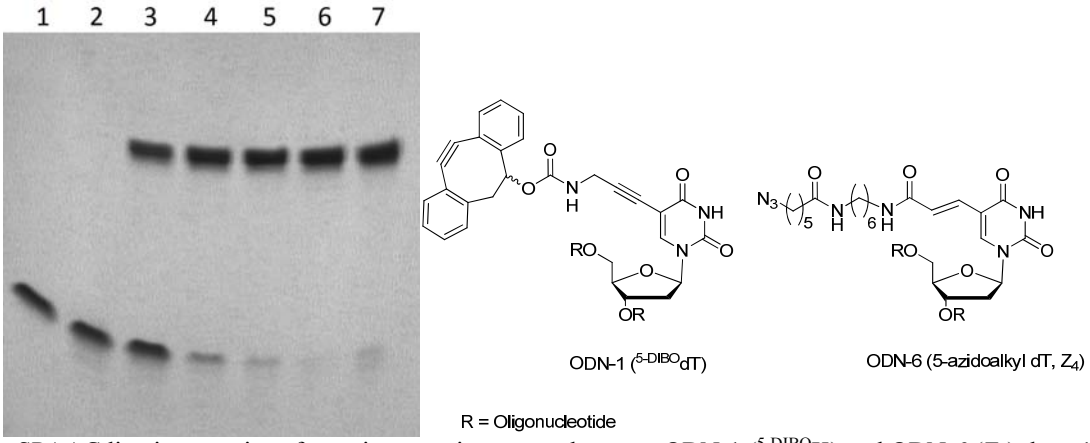


Figure S1: SPAAC ligation reactions for major to major groove between ODN-1 ($^{5\text{-DIBO}}$K) and ODN-6 (Z_4), lane **1-2**: control starting oligonucleotides ODN-6 and ODN-1 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

Minor to Minor groove ligation:

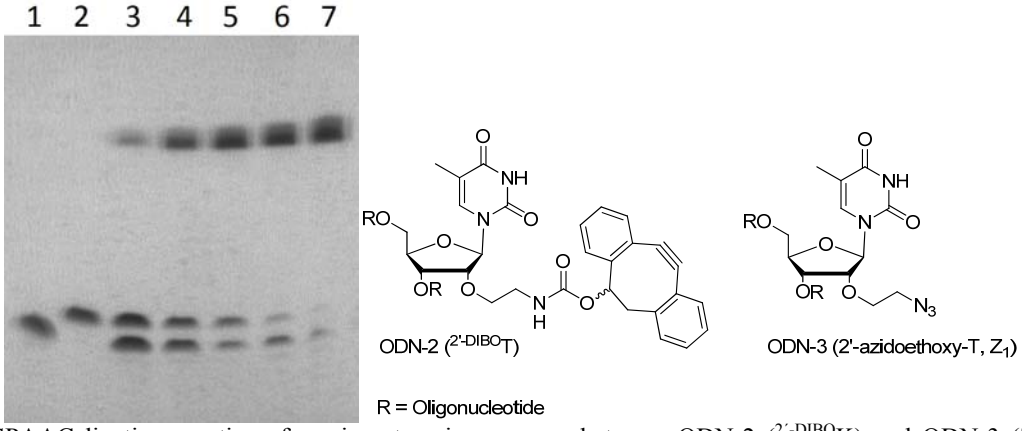


Figure S2: SPAAC ligation reactions for minor to minor groove between ODN-2 ($^{2'\text{-DIBO}}$K) and ODN-3 (Z_1), lane **1-2**: control starting oligonucleotides ODN-3 and ODN-2 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

Major to Minor groove ligation:

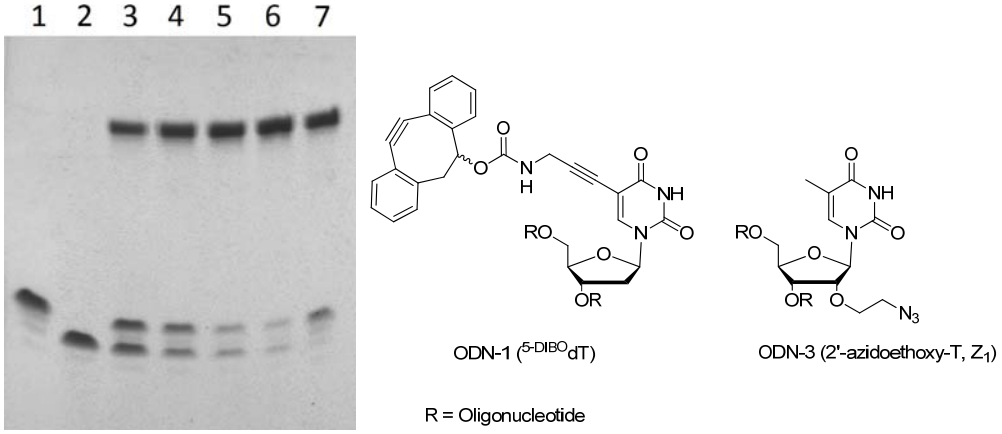


Figure S3: SPAAC ligation reactions for major to minor groove between ODN-1 ($^{5\text{-DIBO}}$K) and ODN-3 (Z_1), lane **1-2**: control starting oligonucleotides ODN-1 and ODN-3 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

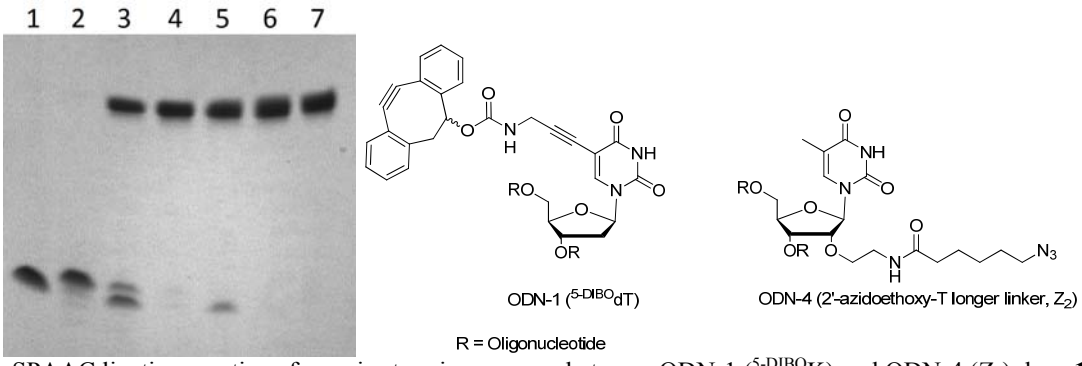


Figure S4: SPAAC ligation reactions for major to minor groove between ODN-1 ($^{5\text{-DIBO}}$K) and ODN-4 (Z_2), lane **1-2**: control starting oligonucleotides ODN-4 and ODN-1 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

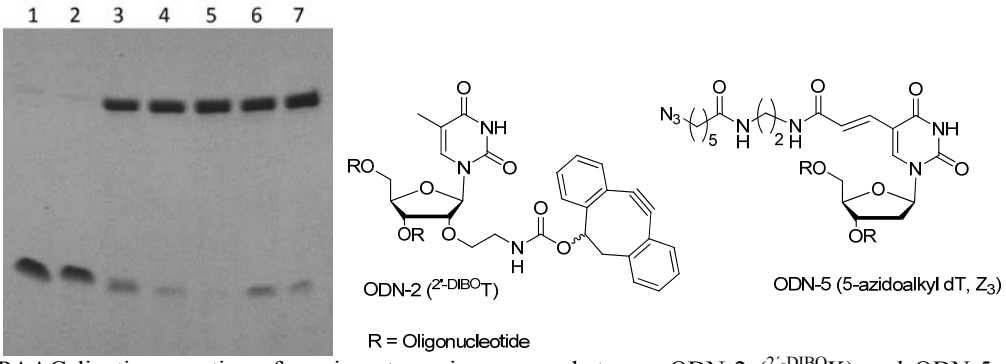


Figure S5: SPAAC ligation reactions for minor to major groove between ODN-2 ($^{2'\text{-DIBO}}$K) and ODN-5 (Z_3), lane **1-2**: control starting oligonucleotides ODN-5 and ODN-2 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

B) Gel electrophoresis for T_pA steps (5′- ACAGATATCATATT – 3′)

Major to Major groove ligation:

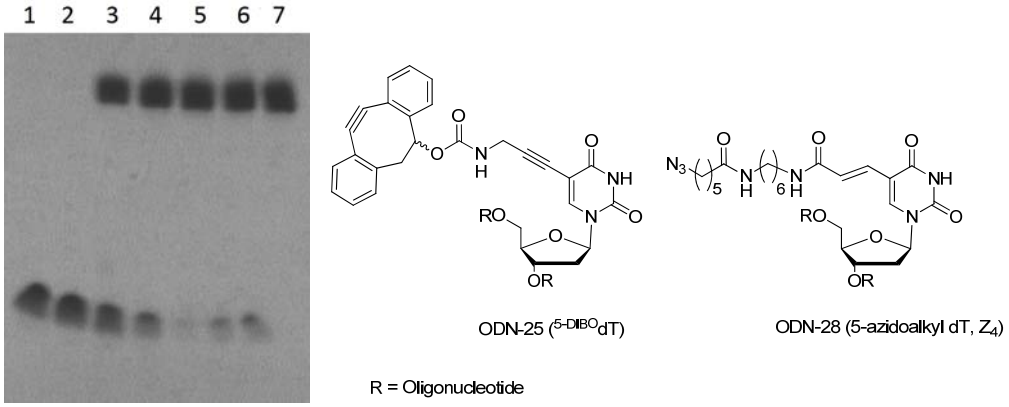


Figure S6: SPAAC ligation reactions for major to major groove between ODN-25 ($^{5\text{-DIBO}}$K) and ODN-28 (Z_4), lane **1-2**: control starting oligonucleotides ODN-28 and ODN-25 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

Minor to Minor groove ligation:

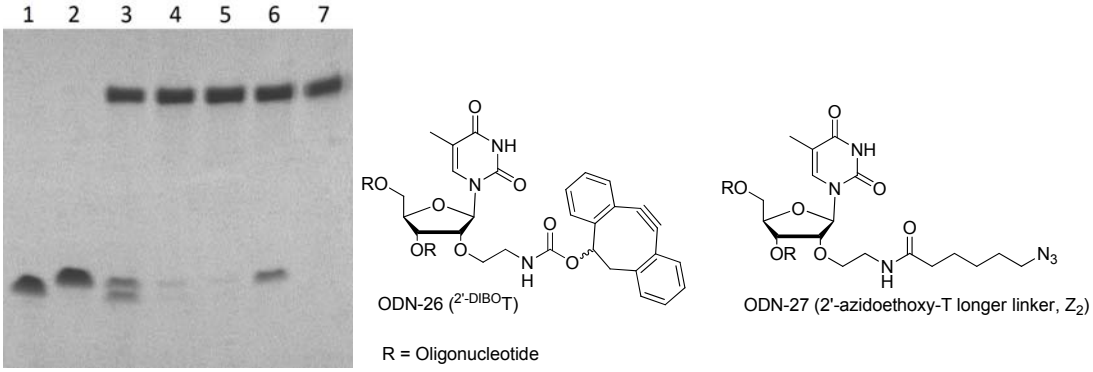


Figure S7: SPAAC ligation reactions for minor to minor groove between ODN-26 ($^{2'\text{-DIBO}}$K) and ODN-27 (Z_2), lane **1-2**: control starting oligonucleotides ODN-27 and ODN-26 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

Major to Minor groove ligation:

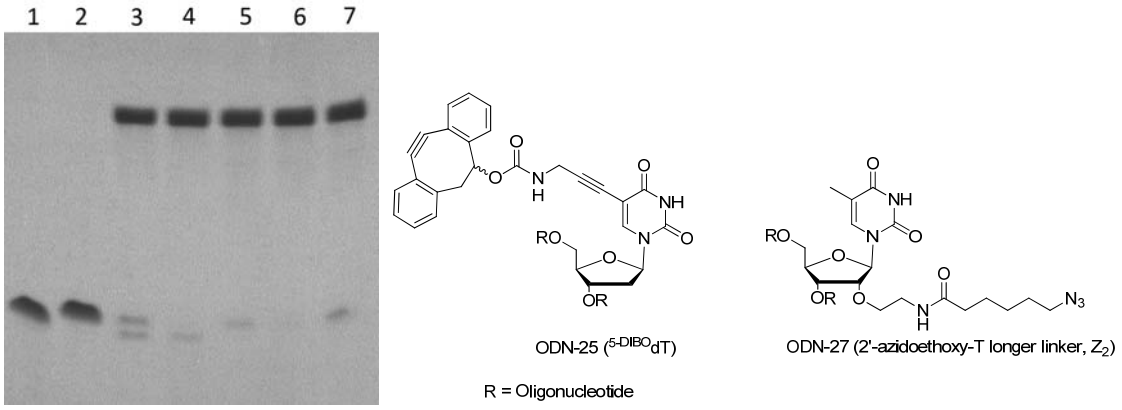


Figure S8: SPAAC ligation reactions for major to minor groove between ODN-25 ($^{5\text{-DIBO}}$K) and ODN-27 (Z_2), lane **1-2**: control starting oligonucleotides ODN-27 and ODN-25 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

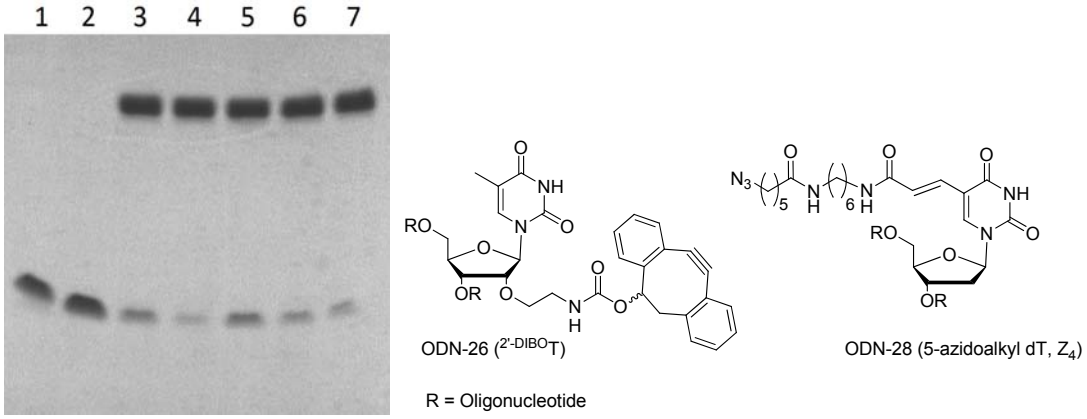


Figure S9: SPAAC ligation reactions for minor to major groove between ODN-26 ($^{2'\text{-DIBO}}$K) and ODN-28 (Z_4), lane **1-2**: control starting oligonucleotides ODN-28 and ODN-26 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

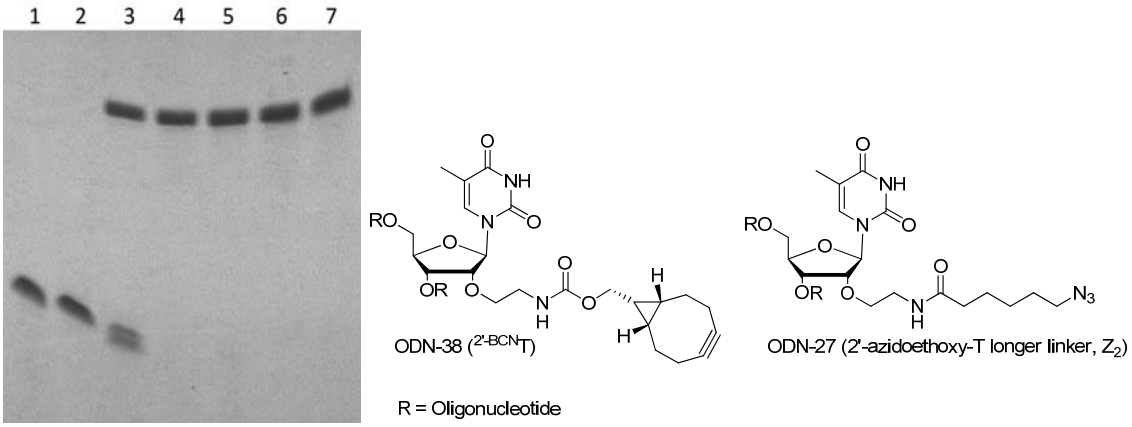


Figure S10: SPAAC ligation reactions for minor to minor groove between ODN-38 ($^{2'\text{-BCN}}$K) and ODN-27 (Z_2), lane **1-2**: control starting oligonucleotides ODN-27 and ODN-38 respectively, lanes **3-7**: crude reaction mixtures; 0 min, 5 min, 15 min, 30 min and 1 h.

Orthogonal labelling of ODN-7 and ODN-8 with carboxyfluorescein-5-amidohexylazide and Texas red NHs-ester to form HyBeacons ODN-9 and ODN-10

Carboxyfluorescein-5-amidohexylazide[6] (50 nmol in 20 µL DMSO) and Texas red active ester (100 nmol in 20 µL DMSO) (single isomer, Life Technologies, cat. No: T-20175, Figure S12) were added to the alkyne-amino modified oligonucleotide (ODN-7, 5 nmol) in 40 µL 0.5 M Na_2CO_3/$NaHCO_3$ buffer at pH 8.75. The reaction mixture was heated for overnight at 55 °C then desalted by using a NAP-10 Sephadex column, purified by reverse phase HPLC and analysed by capillary electrophoresis and mass spectrometry.

The same method, using 100 nmole of the fluorescein azide and 200 nmole of Texas red active ester, was used to synthesis the HyBeacon ODN-10 with 4 additions of the dyes.

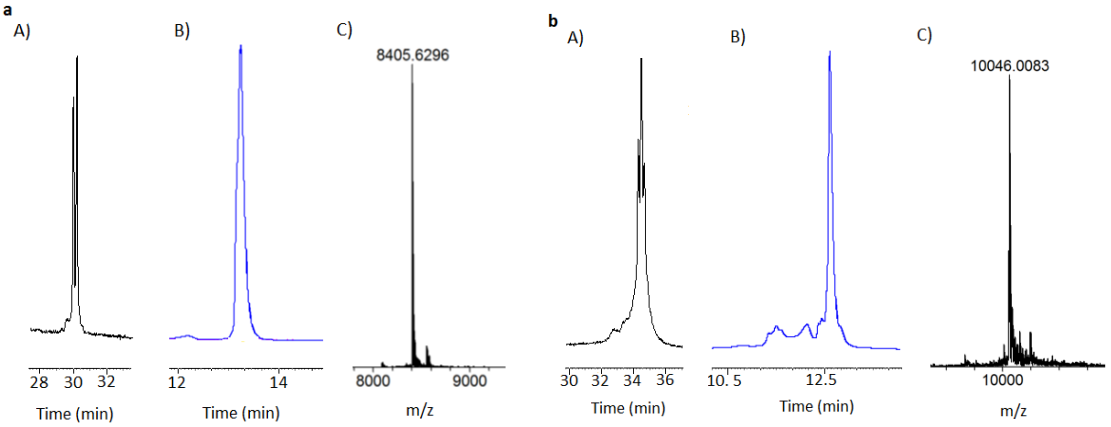


Figure S11: Characterisation of labelled HyBeacon ODN-9 with 2 additions of dyes (a) and HyBeacon ODN-10 with 4 additions of dyes (b). A) CE electropheregrams show more than one peak due to the stereoisomers of the DIBO triazoles. B) HPLC chromatograms monitoring at 295 nm and C) Electrospray mass spectra (ES^-)**.** Capillary Electrophoresis (CE) analysis of oligonucleotides was performed by injection of 0.4 OD/100 µL. ssDNA 100-R Gel, Tris-Borate-7 M Urea (Kit No 477480) on a Beckman Coulter P/ACETM MDQ Capillary Electrophoresis system using 32 Karat software. UV-254 nm, inject-voltage 10.0 kv and separation-voltage 9.0 kv (45.0 min duration). X-axis is time (min), Y-axis is UV absorbance at 254 nm. See oligonucleotide synthesis and purification for conditions of HPLC and mass spectrometry.

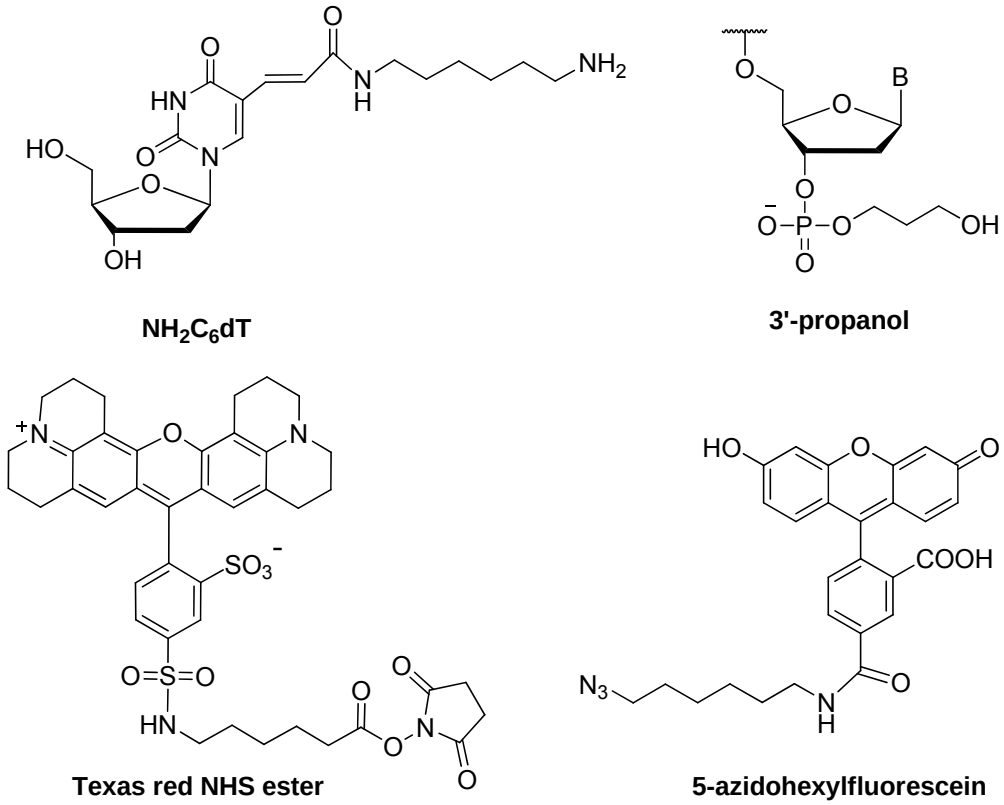


Figure S12: Modifications used in HyBeacon probes

Fluorescence melting analysis

Measurements were made on a Perkin Elmer LS50B fluorimeter equipped with a Perkin Elmer PTP-1 Peltier system. FLWinlab TempScan software was used with optimum excitation/emission slit widths, excitation wavelength and emission wavelength settings for each sample, the slit width (ext. slit: 7 and emi. Slit: 8) and excitation wavelength (490 nm) settings were kept constant for comparison of samples. Samples were analysed in a 200 µL cuvette (Hellma quartz SUPRASIL QS; 200 µL volume, 10 mm path-length) with a collection angle of 90°. A total sample volume of 200 µL was used, with labelled oligonucleotide concentration of 0.3 µM. Samples were prepared in 10 mM phosphate buffer with a total of 200 mM NaCl at pH 7.0. The samples were heated from 25°C to 85°C with steps of 1°C, ramp speed 0.6°C/min. A fluorescence scan between 490 nm and 800 nm was performed at each step.

UV Melting analysis

UV melting experiments were performed on a Varian Cary 4000 UV-VIS spectrophotometer with Cary temperature controller, Cary Win UV Thermal software and an absorption wavelength of 260 nm. Samples were made to 1.7 µM oligonucleotide concentration in 10 mM sodium phosphate with 200 mM NaCl buffer at pH 7.0. The samples were initially denatured by heating to 85 °C at 10 °C/min then cooled to 20 °C at 1 °C/min and heated to 85 °C at 1 °C/min. Three successive melting and annealing curves were measured, and the average melting temperatures (T_m) were calculated with Cary Win UV Thermal application software.

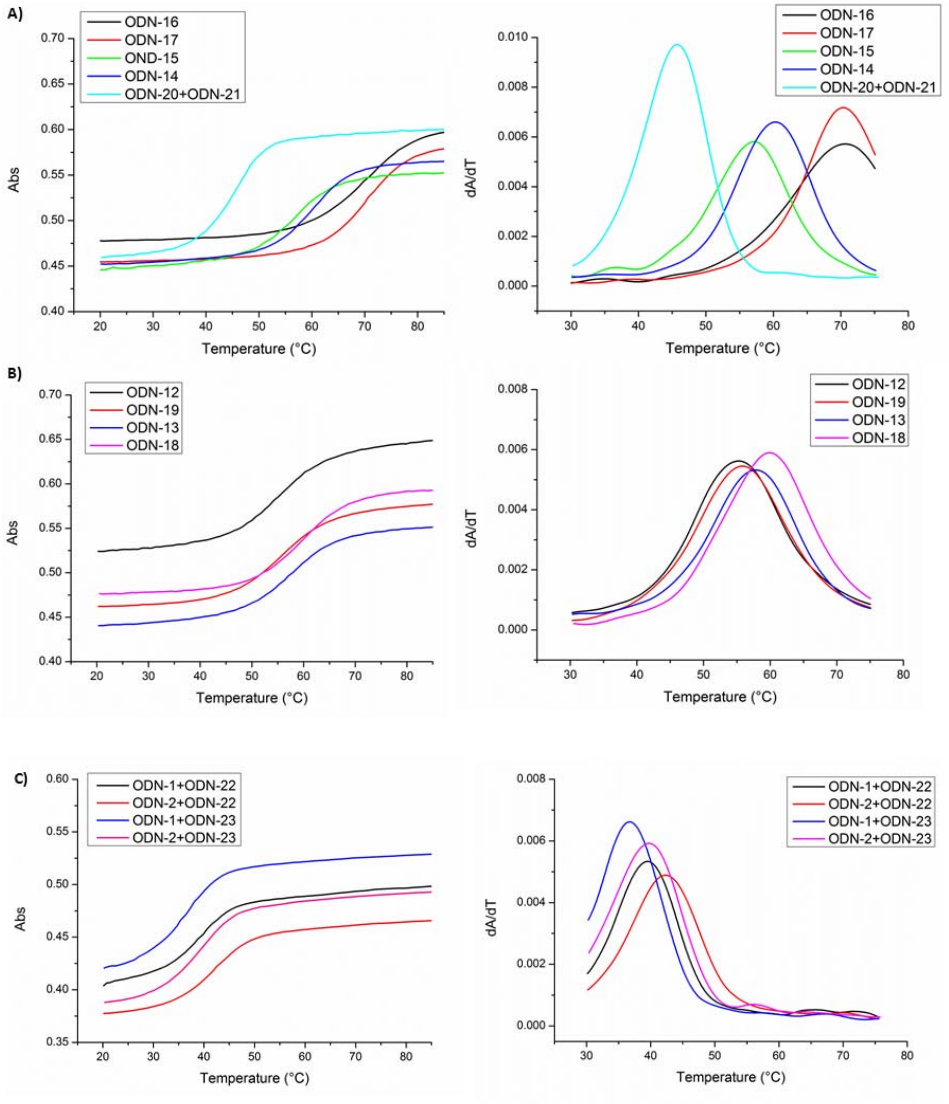


Figure S13. Examples of UV melting curves and derivatives for the A_pT step; A) and B) Melting curves and derivatives of the crosslinked duplexes and control unmodified non-crosslinked duplex. C) Melting curves and derivatives of the modified non-crosslinked duplexes.

Modelling studies on duplexes crosslinked with DIBO at A_pT step

The idealised structure of the double-stranded B-form DNA was generated from its sequence, using the program Coot.[7] For each linkage (and for each of four diastereoisomers) 1000 random conformers were generated with the terminal atoms attached to the modified DNA bases/sugars. Energy minimization was carried out on each structure, firstly with the positions of the nucleic acid atoms constrained, and then with the constraints removed. Linkage conformer generation and energy minimization (molecular mechanics) were carried out using the Open Babel chemical toolbox.[8] The lowest energy structures for each linkage were analysed. The modelling studies confirmed that each linker can stretch between the attachment points with reasonable geometry.

Circular dichroism analysis on duplexes modified at A_pT step

Circular dichroism spectra were recorded on a Jasco J-720 spectropolarimeter. Samples were made to 1.7 μM oligonucleotide concentration in 10 mM sodium phosphate with 200 mM NaCl buffer at pH 7.0. Spectra were recorded between 200-320 nm at a rate of 100 nm/min, with a bandwith of 1.0 nm, step resolution of 0.2 nm and sensitivity of 20 mdeg. 16 successive spectra were recorded and an average was taken. A blank (buffer) baseline was subtracted from each spectrum.

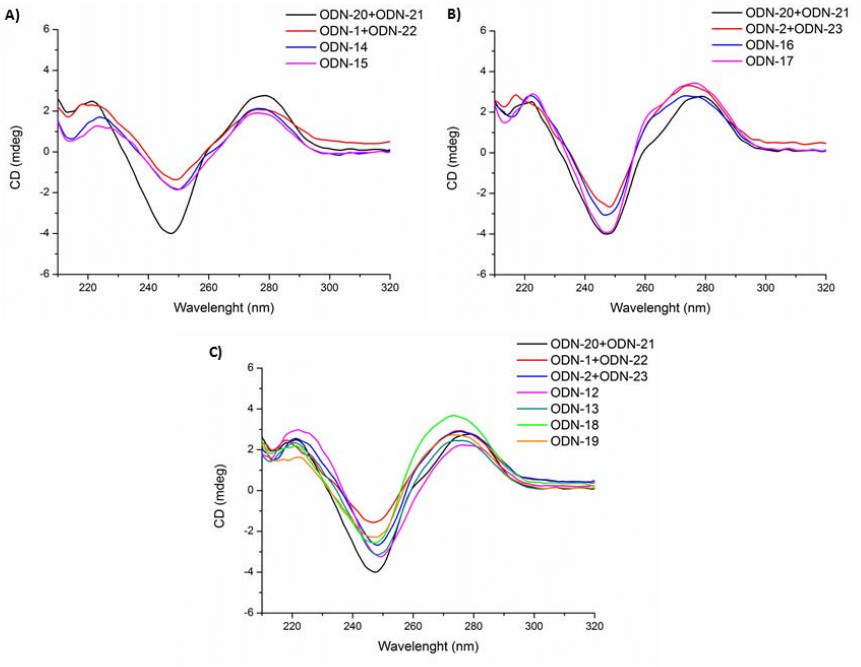


Figure S14: Circular dichroism spectra of unmodified normal and crosslinked duplexes A) major to major groove, B) minor to minor groove and C) major to minor groove.

DIBO crosslinking at A_pT step

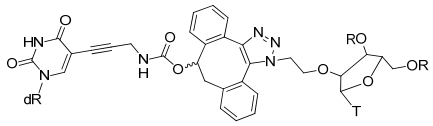
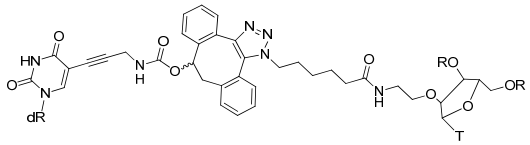
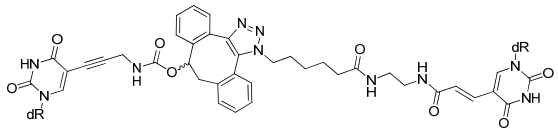
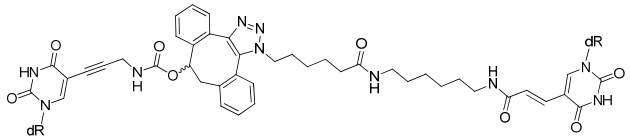
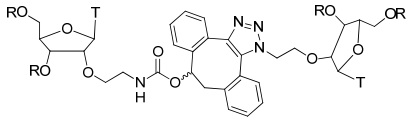
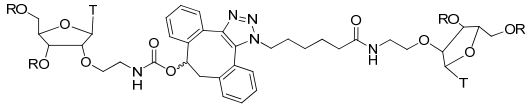
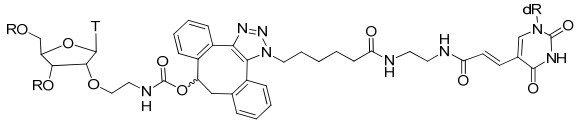
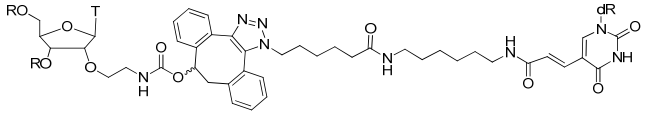
Crosslink (type)	T_m °C
ODN-12 (ODN-1 + ODN-3) = $^{5\text{-DIBO-}}K + Z_1$ (Mm) 	56.0
ODN-13 (ODN-1 + ODN-4) = $^{5\text{-DIBO-}}K + Z_2$ (Mm) 	57.5
ODN-14 (ODN-1 + ODN-5) = $^{5\text{-DIBO-}}K + Z_3$ (MM) 	60.5
ODN-15 = ODN-1 + ODN-6 = $^{5\text{-DIBO-}}K + Z_4$ (MM) 	57.0
ODN-16 (ODN-2 + ODN-3) = $^{2'\text{-DIBO-}}K + Z_1$ (mm) 	70.5
ODN-17 (ODN-2 + ODN-4) = $^{2'\text{-DIBO-}}K + Z_2$ (mm) 	70.0
ODN-18 (ODN-2 + ODN-5) = $^{2'\text{-DIBO-}}K + Z_3$ (mM) 	60.0
ODN-19 (ODN-2 + ODN-6) = $^{2'\text{-DIBO-}}K + Z_4$ (mM) 	55.5
ODN-20/21 5′-AATATGAATTCTGT unmodified uncrosslinked 3′-TTATACTTAAGACA	46.0

Table S1. Double strand crosslinking by the SPAAC reaction, one regioisomer of the DIBO triazole is shown. M = major groove, m = minor groove. Oligonucleotide sequences in Table S7. The crosslinks are at an A_pT step. T_m values are the average of 3 separate melting and annealing curves.

DIBO crosslinking at T_pA step

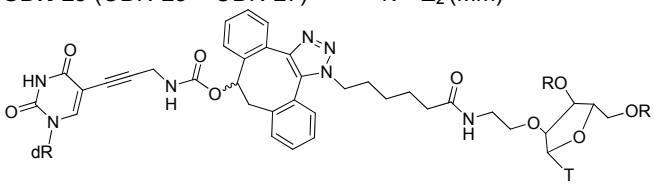
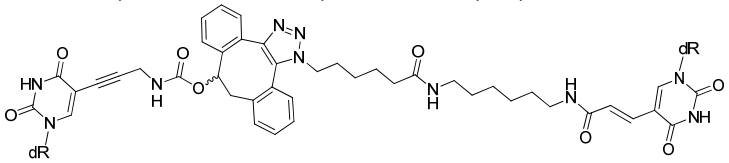
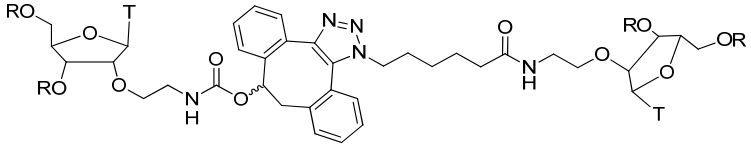
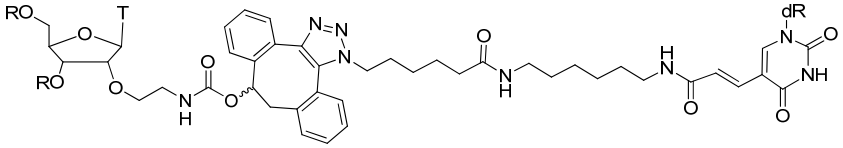
Crosslink (type)	T_m °C
ODN-29 (ODN-25 + ODN-27) = $^{5\text{-DIBO-}}K + Z_2$ (Mm) 	**65.0**
ODN-30 = (ODN-25 + ODN-28) = $^{5\text{-DIBO-}}K + Z_4$ (MM) 	**68.0**
ODN-31 (ODN-26 + ODN-27) = $^{2'\text{-DIBO-}}K + Z_2$ (mm) 	**68.5**
ODN-32 (ODN-26 + ODN-28) = $^{2'\text{-DIBO-}}K + Z_4$ (mM) 	**67.0**

Table S2. Double strand crosslinking by the SPAAC reaction, one regioisomer of DIBO triazole linkage is shown. M = major groove, m = minor groove. Oligonucleotide sequences in Table S9. The crosslinks are at a T_pA step. T_m values are the average of 3 separate melting and annealing curves. Melting temperatures accurate to ± 0.5 °C.

Uncrosslinked duplexes, modifications at T_pA step

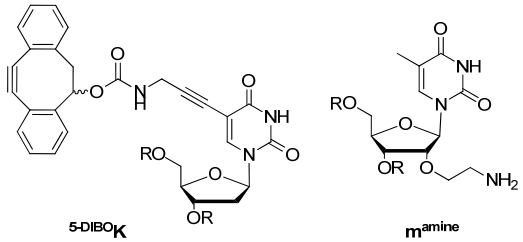
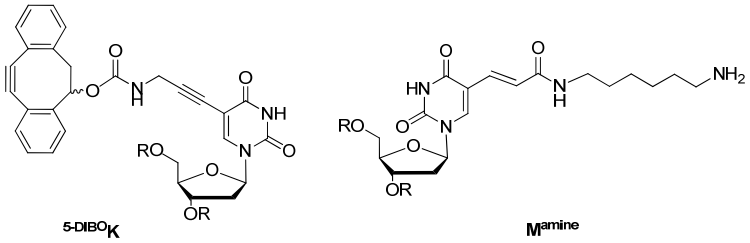
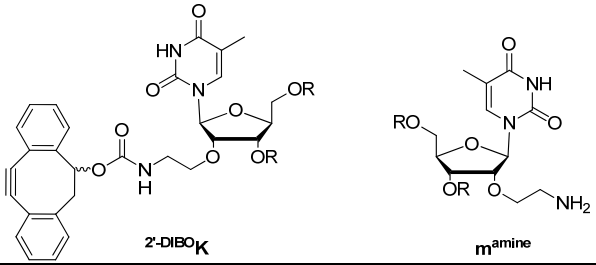
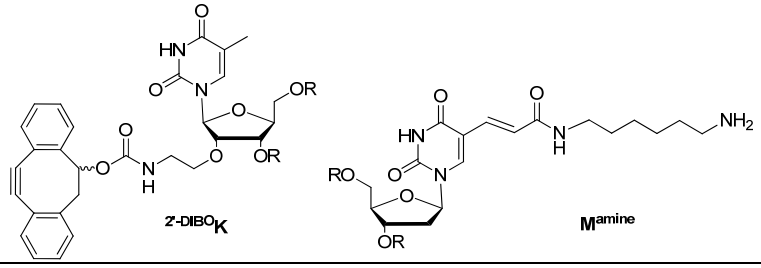
Modified thymidine nucleosides	Type	Duplex T_m °C
ODN-25 + ODN-33 	mM	41.0
ODN-25 + ODN-34 	MM	43.0
ODN-26 + ODN-33 	mm	46.0
ODN-26 + ODN-34 	Mm	46.0

Table S3. UV melting of uncrosslinked duplexes with one DIBO-T and one amino-T at T_pA step. Only one regioisomer of DIBO triazole linkage is shown. M = major groove, m = minor groove. D = DIBO, Z = azide. Average of 3 separate melting and annealing curves. Melting temperatures accurate to ± 0.5 °C. Oligonucleotide sequences in Table S9. The modifications are at a T_pA step. DNA sequence is:

5´-AATATGA**T**ATCTGT
3´-TTATACTA**T**AGACA

Where the bold underlined **T** in the top strand indicates the position of DIBO labelling in the modified strand. Similarly the bold underlined **T** in the bottom strand indicates the position of amine labelling in the modified strands

Uncrosslinked duplexes, modifications at A_pT step

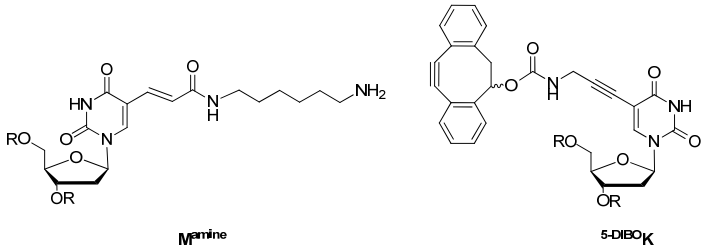
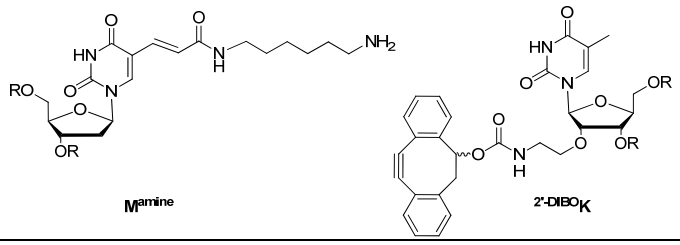
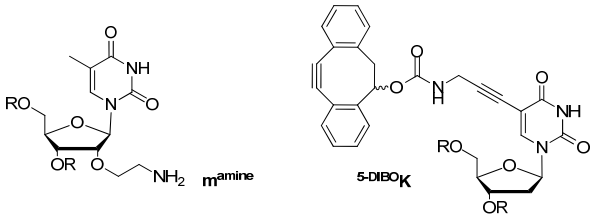
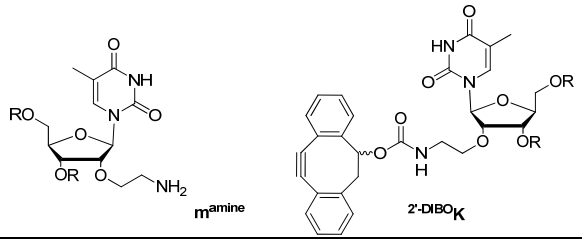
Modified thymidine nucleosides	Type	Duplex T_m °C
ODN-22 + ODN-1 	MM	39.0
ODN-22 + ODN-2 	Mm	42.0
ODN-23 + ODN-1 	mM	36.5
ODN-23 + ODN-2 	mm	39.5
ODN-20/21 control unmodified DNA duplex		46.0

Table S4. UV melting of uncrosslinked duplexes, A_pT step. Only one regioisomer of DIBO triazole linkage is shown. M = major groove, m = minor groove. Average of 3 separate melting and annealing curves. Melting temperatures accurate to ± 0.5 °C. Oligonucleotide sequences in Table S7. The modifications are at an A_pT step. DNA sequence is:

5´-AATATGAA**T**TCTGT
3´-TTATACT**T**AAGACA

Where the bold underlined **T** in the top strand indicates the position of DIBO labelling in the modified strand. Similarly the bold underlined **T** in the bottom strand indicates the position of amine labelling in the modified strand.

Uncrosslinked duplexes, modifications at A_pT step

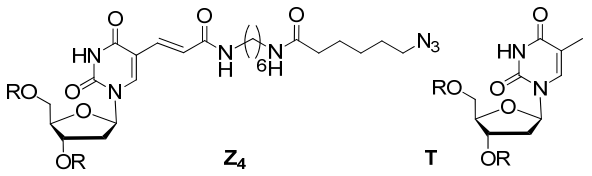
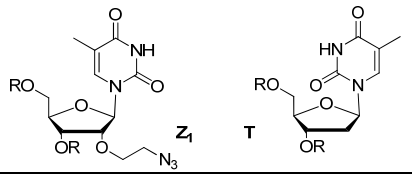
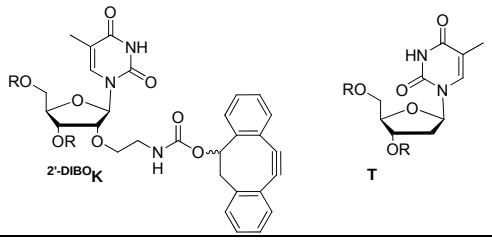
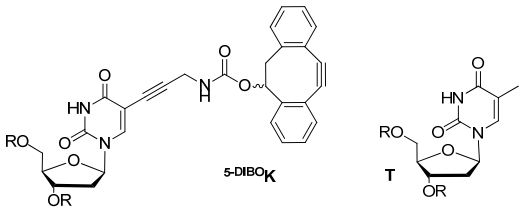
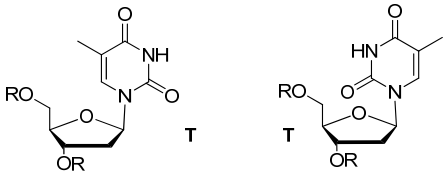
Modified thymidine nucleosides	Type	Duplex T_m °C
ODN-6 + ODN-21 	M^Z	42.5
ODN-3 + ODN-21 	m^Z	41.5
ODN-2 + ODN-20 	m^D	43.0
ODN-1 + ODN-20 	M^D	40.5
ODN-20 + ODN-21 control unmodified DNA duplex 	-	46.0

Table S5. UV melting of uncrosslinked duplexes with one modified and one unmodified T. Only one regioisomer of DIBO triazole linkage is shown. M = major groove, m = minor groove. D = DIBO, Z = azide. Average of 3 separate melting and annealing curves. Melting temperatures accurate to ± 0.5 °C. Oligonucleotide sequences in Table S7. The modifications are at an A_pT step. DNA sequence is:

5´-AATATGAA**T**TCTGT
3´-TTATACT**T**AAGACA

Where the bold underlined **T** in the top strand indicates the position of DIBO labelling in the modified strand. Similarly the bold underlined **T** in the unmodified bottom strand indicates the position of thymidine in the A_pT step under investigation.

Crosslinking studies on BCN cyclooctyne

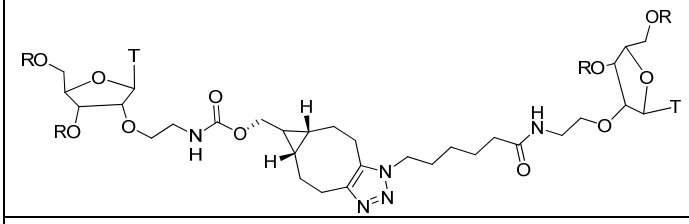
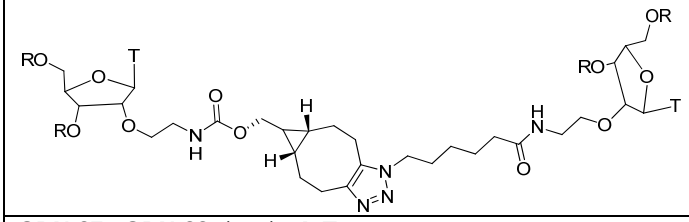
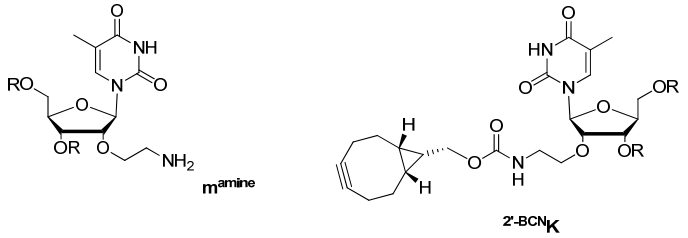
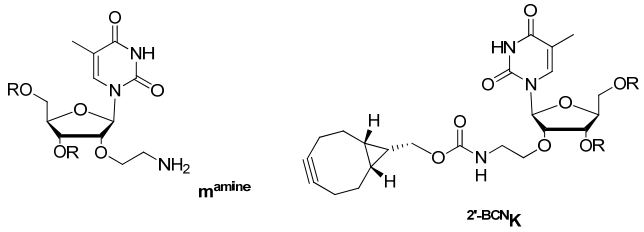
Crosslink and modified thymidine nucleosides	T_m °C
ODN-39 (ODN-37 + ODN-4) = $^{2\text{-BCN-}}$K + Z_2 (mm) **A_pT** 	**69.0**
ODN-40 = (ODN-38 + ODN-27) = $^{2'\text{-BCN-}}$K + Z_2 (mm) **T_pA** 	**71.0**
ODN-37+ ODN-23 (mm) **A_pT** 	**38.0**
ODN-38+ODN-33 (mm) **T_pA** 	**44.0**

Table S6. UV melting table of the double strand crosslinking by the copper free ligation of $^{2'\text{-BCN}}$K and of uncrosslinked duplexes with one modified and one unmodified T. M = major groove, m = minor groove. Average of 3 separate melting and annealing curves. Melting temperatures accurate to ± 0.5 °C. Oligonucleotide sequences in Tables S7, S9, S10. The crosslinks are at T_pA and A_pT steps.

Code	Oligonucleotide sequences (5´ to 3´)	Calc.	Found
ODN-1	AATATGAA$^{5\text{-DIBO}}$KTCTGT	4561	4560
ODN-2	AATATGAA$^{2'\text{-DIBO}}$KTCTGT	4582	4581
ODN-3	ACAGAAZ$_1$TCATATT	4330	4330
ODN-4	ACAGAAZ$_2$TCATATT	4443	4443
ODN-5	ACAGAAZ$_3$TCATATT	4483	4482
ODN-6	ACAGAAZ$_4$TCATATT	4539	4538
ODN-12	ODN-1 + ODN-3	8892	8892
ODN-13	ODN-1 + ODN-4	9006	9005
ODN-14	ODN-1 + ODN-5	9045	9044
ODN-15	ODN-1 + ODN-6	9101	9100
ODN-16	ODN-2 + ODN-3	8912	8912
ODN-17	ODN-2 + ODN-4	9026	9025
ODN-18	ODN-2 + ODN-5	9065	9064
ODN-19	ODN-2 + ODN-6	9121	9120
ODN-20	ACAGAATTCATATT	4245	4244
ODN-21	AATATGAATTCTGT	4276	4275
ODN-22	ACAGAAM^{A}TCATATT	4399	4399
ODN-23	ACAGAAm^{A}TCATATT	4304	4304
ODN-24	ACAGAAM$^{\underline{A}}$TCATATT	4343	4343

Table S7: Oligonucleotide sequences and mass spectrometry for the A_pT step. $^{5\text{-DIBO}}$K = 5- propargyl DIBO-dT, $^{2'\text{-DIBO}}$K = 2´-ethoxy DIBO-T, Z_1 = 2´-azidoethoxy-T labelled with 6-azidohexanoic, Z_2 = 2´-aminoethoxy-T labelled with 6-azidohexanoic acid, Z_3 = aminoC_2dT labelled with 6-azidohexanoic acid, Z_4 = aminoC_6dT labelled with 6-azidohexanoic acid, M^A = aminoC_6dT, m^A = 2´-aminoethoxy-T and $M^{\underline{A}}$ = aminoC_2dT. Electrospray Mass spectrometry of oligonucleotides was recorded in water using a Bruker micrOTOF^{TM} II focus ESI-TOF MS instrument in ES$^-$ mode.

ODN-7	CGCT$^{5\text{-DIBO}}$K CTGTAM^{A}CTATATTCATCP	7202	7203
ODN-8	CGC$^{5\text{-DIBO}}$KTCTGM^{A}ATCTA$^{5\text{-DIBO}}$KATTCAM^{A}CP	7641	7642
ODN-9	CGCT Fz^{DIBO}CTGTATr^{C6}CTATATTCATCP	8404	8405
ODN-10	CGCFz^{DIBO}TCTGTr^{C6}ATCTAFz^{DIBO}ATTCATr^{C6}CP	10045	10046
ODN-11	CTATGATGAATATAGATACAGAAGCGTCAT	9261	9261

Table S8: Oligonucleotide sequences and mass spectrometry for the Hybeacons and complementary template. $^{5\text{-DIBO}}$K = 5- propargyl DIBO-dT, M^A = aminoC_6dT, P = propanol, Fz^{DIBO} = 5-propargyl DIBO-dT labelled with fluorescein-5-amidohexyl azide, Tr^{C6} = aminoC_6dT labelled with Texas red NHs-ester. Electrospray Mass spectrometry of oligonucleotides was recorded in water using a Bruker micrOTOF^{TM} II focus ESI-TOF MS instrument in ES$^-$ mode.

Code	Oligonucleotide sequences (5′ to 3′)	Calc.	Found
ODN-25	AATATGA$^{5\text{-DIBO}}$KATCTGT	4561	4561
ODN-26	AATATGA$^{2'\text{-DIBO}}$KATCTGT	4582	4581
ODN-27	ACAGAZ$_2$ATCATATT	4443	4443
ODN-28	ACAGAZ$_4$ATCATATT	4539	4538
ODN-29	ODN-25+ODN-27	9005	9005
ODN-30	ODN-25+ODN-28	9101	9100
ODN-31	ODN-26+ODN-27	9026	9025
ODN-32	ODN-26+ODN-28	9121	9120
ODN-33	ACAGAm^{A}ATCATATT	4304	4304
ODN-34	ACAGAM^{A}ATCATATT	4399	4399

Table S9: Oligonucleotide sequences and mass spectrometry for the T$_p$A step. $^{5\text{-DIBO}}$K = 5- propargyl DIBO-dT, $^{2'\text{-DIBO}}$K = 2′-ethoxy DIBO-T, Z$_2$ = 2′-aminoethoxy-dT labelled with 6-azidohexanoic acid, Z$_4$ = aminoC$_6$dT labelled with 6-azidohexanoic acid, m^A = 2′- aminoethoxy-T and M^A = aminoC$_6$dT. Electrospray Mass spectrometry of oligonucleotides was recorded in water using a Bruker micrOTOF^{TM} II focus ESI-TOF MS instrument in ES$^-$ mode.

Code	Oligonucleotide sequences (5′ to 3′)	Calc.	Found
ODN-35	AATATGAAm^{A} TCTGT	4335	4334
ODN-36	AATATGAm^{A}ATCTGT	4335	4334
ODN-37	AATATGAA$^{2'\text{-BCN}}$KTCTGT	4511	4511
ODN-38	AATATGA$^{2'\text{-BCN}}$KATCTGT	4511	4510
ODN-39	ODN-37+ ODN-4	8956	8955
ODN-40	ODN-38+ODN-27	8956	8955

Table S10: Oligonucleotide sequences and mass spectrometry for the BCN alkyne in the A$_p$T and T$_p$A steps. $^{2'\text{-BCN}}$K = 2′-ethoxy BCN-T and m^A = 2′- aminoethoxy-T. Electrospray Mass spectrometry of oligonucleotides was recorded in water using a Bruker micrOTOF^{TM} II focus ESI-TOF MS instrument in ES$^-$ mode.

References:

1. M. Shelbourne, X. Chen, T. Brown and A. H. El-Sagheer, *Chem. Commun.*, 2011, **47**, 6257-6259.
2. G. Deglane, F. Morvan, F. Debart and J. J. Vasseur, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 951-954.
3. A. Brewer, G. Siligardi, C. Neylon and E. Stulz, *Org. Biomol. Chem.*, 2011, **9**, 777-782.
4. P. Kocalka, A. H. El-Sagheer and T. Brown, *Chembiochem*, 2008, **9**, 1280-1285.
5. J. A. Richardson, M. Gerowska, M. Shelbourne, D. French and T. Brown, *ChemBioChem*, 2010, **11**, 2530-2533.
6. N. Gale, P. Kocalka, C. Mardle and T. Brown, *Artificial DNA: PNA & XNA*, 2011, **2**, 79-89.
7. P. Emsley, B. Lohkamp, W. G. Scott and K. Cowtan, *Acta Crystallogr. Sect. D-Biol. Crystallogr.*, 2010, **66**, 486-501.
8. N. M. O′Boyle, M. Banck, C. A. James, C. Morley, T. Vandermeersch and G. R. Hutchison, *J Cheminformatics*, 2011, **3**.