SUPPLEMENTARY INFORMATION

A Traceless Aryl-Triazene Linker for DNA-Directed Chemistry

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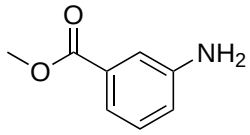
General Methods

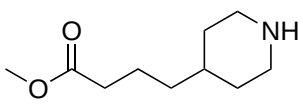
All purchased chemicals were used as received without further purification. Solvents were bought as HPLC-grade. All DNA oligonucleotides were synthesized on site with a MerMade-12 oligonucleotide synthesizer and HPLC purified, or supplied pure by Vipergen. Organic reactions were monitored by thin-layer chromatography (TLC) analysis whenever feasible and flash chromatography was carried out on Merck silica gel 60 (230-400 mesh). NMR spectra were recorded on a Varian AF 400 spectrometer (^{1}NMR at 400 MHz, ^{13}C NMR at 101 MHz). The chemical shifts are reported in ppm relative to the solvent residual peak.[1] The water used for the DNA experiments was purified on a MilliQ system. Mass spectra were recorded using a Bruker Daltonics Autoflex MALDI TOF MS spectrometer using AnchorChip as target plates or by using a Shimadzu LCMS-2020EV connected to a Shimadzu Prominence RP-HPLC system equipped with Phenomenex Gemini column and running gradient of MeOH in 1,1,1,3,3,3-hexafluoroisopropanol/triethylamine buffer (HFIP/TEA). Some masses of small molecules were measured with a Micromass LC-TOF instrument by using electrospray ionization (ESI). The matrix used for MALDI TOF analysis of oligonucleotides was 90% 3-hydroxypicolinic acid (50 mg/ml) in 1:1 H_2O MeCN and 10% diammonium citrate (50 mg/ml) in water. No calibration was used. RP-HPLC was done on a Hewlett Packard Agilent 1100 Series using Phenomenex Clarity 3u Oligo-RP 50x4.6mm columns. All DNA concentrations and yields of oligonucleotide reactions was determined using a ND-1000 NanoDrop spectrophotometer.



1a

Methyl 3-aminobenzoate (1a).[2]

To a round-bottomed flask containing MeOH (150 mL) and concentrated H_2SO_4 (3 mL, 56.282 mmol) was added 3-aminobenzoic acid (6.036 g, 44.016 mmol). The reaction mixture was refluxed overnight, followed by cooling to room temperature. The pH was adjusted to approximately 9 with a saturated solution of Na_2CO_3. The solution was concentrated *in vacuo*. Water (20 mL) was added and the mixture was extracted with EtOAc (3 x 30 mL). The organic phase was dried over Na_2SO_4, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography on silica gel using 100:100:3 pentane/Et_2O/Et_3N. This provided **1a** as a beige solid (6.319 g, 95%). M.p. 38.8-40.8 °C, literature 38 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.42 (m, 1H) 7.35 (m, 1H), 7.21 (dd, J = 7.84, 7.84, 1H), 6.86 (m, 1H), 3.89 (s, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 167.4, 146.6, 131.2, 129.3, 119.7, 119.5, 115.8, 52.1. HRMS (ESI): [M+Na]; Calc.: 174.0525 found: 174.0526.

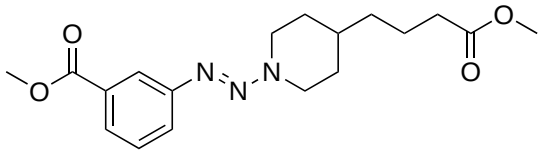


3

Methyl 4-(piperidin-4-yl)butanoate (3). [3]

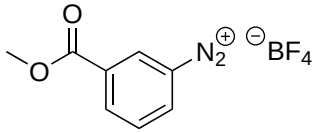
The compound 4-(piperidin-4-yl)butyric acid hydrochloride (0.796 g, 3.831 mmol) was added to a round-bottomed flask containing MeOH (20 mL) and concentrated H_2SO_4 (0.2 mL, 3.752

mmol) and the reaction was refluxed overnight. The reaction mixture was cooled to room temperature, and quenched with saturated $NaHCO_3$. Then the mixture was extracted with EtOAc (3 x 20 mL). Subsequently the organic phase was dried over Na_2SO_4, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography on silica gel using 100:10:3 toluene/MeOH/Et_3N.This provided **3** as a pale yellow oil (0.161 g, 23%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.67 (s, 3H), 3.15 (ddd, J = 12.3, 2.8, 2.8, 2H), 2.63 (ddd, J = 12.2, 2.6, 2.6, 2H), 2.29 (t, J = 7.5, 2H), 1.72 (d, J = 13.1, 2H), 1.64 (m, 2H), 1.37 (m, 1H), 1.29-1.16 (m, 4H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 173.7, 51.5, 44.0, 35.2, 33.9, 33.8, 28.7, 21.8. HRMS (ESI): [M+H]; Calc.: 186.1489 found: 186.1492.



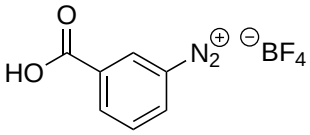
4

Methyl 3-((4-(4-methoxy-4-oxobutyl)piperidin-1-yl)diazenyl)benzoate (4).
BF_4NO (85 mg, 0.728 mmol) was weighed in open atmosphere and immediately transferred to a dry Schlenk tube, which subsequently was evacuated and filled with argon. MeCN (4 mL) was added and stirred until all solids were dissolved. The solution was transferred to a MeCN/CO_2 bath (-42 °C). To a dry Schlenk tube containing MeCN (1 mL), **1a** (65 mg, 0.430 mmol) was added and the mixture was stirred until **1a** had dissolved. This solution was added dropwise to the cooled BF_4NO solution. The mixed solution turned into a vague blue colour, and was left stirring for 30 min at room temperature. In a dry Schlenk tube **3** (80 mg, 0.432 mmol) and DIPEA (78 µL, 0.448 mmol) was mixed with MeCN (3 mL) and placed in the MeCN/CO_2 bath. The solution of diazotized **1a** (**2a**) was added dropwise to the cooled solution of **3**. After complete addition, the solution was left to stir for a 5 min on the MeCN/CO_2 bath and 5 without the MeCN/CO_2 bath. Solvent was removed *in vacuo*. The crude product was purified by flash chromatography on silica gel using 100:100:3 pentane/Et_2O/Et_3N. This provided **4** as a pale yellow oil (85 mg, 57%). ^{1}H-NMR (400 MHz, MeCN-d3) δ 7.96 (dd, J = 1.7, 1.7, 1H), 7.77 (ddd, J = 7.7, 1.6, 1.2, 1H), 7.59 (ddd, J = 8.0, 2.1, 1.1, 1H), 7.44 (dd, J = 8.0, 8.0, 1H), 3.87 (s, 3H), 3.61 (s, 3H), 2.30 (t, J = 7.5, 2H), 2.16 (wide s, 3H), 1.85 (wide d, J = 13.1, 2H), 1.67-1.55 (m, 3H), 1.33-1.19 (m, 5H). ^{13}C-NMR (101 MHz, MeCN-d3) δ 174.7, 167.7, 152.2, 132.1, 130.2, 127.0, 126.0, 121.5, 52.7, 51.9, 36.2, 36.2, 34.6, 22.9, with two signals not able to be resolved. HRMS (ESI): [M+Na]; Calc.: 370.1737 found: 370.1741.



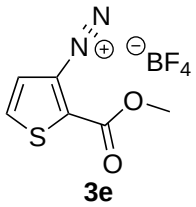
3a

3-(Methoxycarbonyl)benzenediazonium tetrafluoroborate (3a).[4]
BF_4NO (0.411 g, 3.515 mmol) was dissolved in MeCN (2 mL) in a dry Schlenk tube at 0 °C. To another dry Schlenk tube containing MeCN (2 mL) **1a** (0.393 g, 2.598 mmol) was dissolved. This solution of **1a** was added dropwise to the solution of BF_4NO. The mixture was stirred for 30 min at 0 °C, after which it was slowly left to heat to room temperature. The solvent was removed *in vacuo*. The crude product was recrystallized from MeCN by slow addition of Et_2O. This provided **3a** as a white cotton-like solid (0.558 g, 86%). M.p. 145.5 °C decomposes. ^{1}H-NMR (400 MHz, MeCN-d3) δ 9.00 (dd, J = 1.91, 1.91, 1H), 8.75 (ddd, J = 1.20, 1.60, 8.00, 1H), 8.64 (ddd, J = 1.13, 2.20, 8.39, 1H), 8.06 (dd, J = 7.98, 7.98, 1H), 3.98 (s, 3H). ^{13}C-NMR (101 MHz, MeCN-d3) δ 164.0, 142.7, 136.7, 134.6, 134.0, 133.5, 117.1, 54.2. HRMS (ESI): [M-BF_4]; Calc.: 163.0502 found: 163.0503.



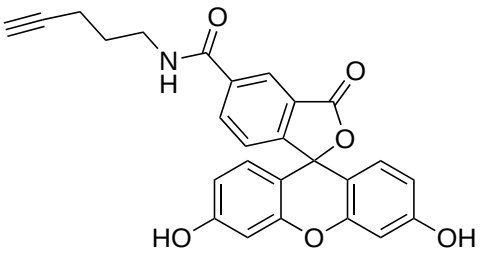
3b

3-Carboxybenzenediazonium tetrafluoroborate (3b).[5]
To a round-bottomed flask containing 96 % EtOH (10 mL) and HBF_4 (1.5 mL, 23.915 mmol) 3-aminobenzoic acid (0.509 g, 3.710 mmol) was added. The solution was stirred at room temperature until most solids were dissolved, followed by cooling to 0 °C. Isobutyl nitrite (1.5 mL, 12.655 mmol) was added dropwise over 30 min. The mixture was stirred for additional 10 min, followed by the addition of Et_2O (60 mL). Continued stirring for another 30 min lead to the precipitation of a white solid. The solution was filtered and the solid was washed with Et_2O. The product **3b** was used without further purification (0.832 g, 95 %). M.p. 153.4 °C decomposes. ^{1}H-NMR (400 MHz, MeCN-d3) δ 8.92 (s, 1H), 8.56 (d, J = 9.50, 1H), 8.36 (d, J = 7.83, 1H), 7.71 (dd, J = 8.26, 8.26, 1H). ^{13}C-NMR (101 MHz, MeCN-d3) δ 165.4, 142.9, 136.6, 135.2, 134.2, 133.4, 116.8. HRMS (ESI): [M-BF_4]; Calc.: 149.0346 found: 149.0345.



3e

2-(Methoxycarbonyl)thiophene-3-diazonium tetrafluoroborate (3e).[6]
To a round-bottomed flask containing 96 % EtOH (20 mL) and HBF_4 (1.5 mL, 23.915 mmol) methyl 3-amino-2-thiophenecarboxylate (1.060 g, 63.47 mmol) was added. The solution was stirred at room temperature until most solids were dissolved, followed by cooling to 0 °C. Isobutyl nitrite (1.5 mL, 12.655 mmol) was added dropwise over 30 min. The mixture was stirred for an additional 10 min, followed by the addition of Et_2O (80 mL). Continued stirring for another 30 min lead to the precipitation of a white solid. The solution was filtered and the solid was washed with Et_2O. The product **3e** was used without further purification (1.58 g, 92 %). M.p. 152.6 °C decomposes. ^{1}H-NMR (400 MHz, MeCN-d3) δ 8.14 (s, 2H), 4.04 (s, 3H). ^{13}C-NMR (101 MHz, DMSO-d6) δ 157.4, 147.4, 135.7, 129.8, 109.2, 54.4. HRMS (ESI): [M-BF_4]; Calc.: 169.0066 found: 169.0067.



7

5-Carboxamide-*N*-(pent-4-yn-1-yl)-fluorescein (7).
To a round-bottomed flask 5-carboxyfluorescein (12.1 mg, 32.2 µmol), HOBt (10.5 mg, 77.7 µmol) and HBTU (17.7 mg, 52.0 µmol) were added. The solids were dissolved by adding 2 mL dry DMF. To the reaction DIPEA (100 µL, 574.1 µmol) and pent-4-yn-1-amine (7 µL, 72.3 µmol) was added. The mixture was stirred at room temperature overnight in the dark and under a dry argon atmosphere. The DMF was removed *in vacuo* and the solid was redissolved in 1:1 MeCN:MeOH and evaporated on silica. The compound were purified with flash column chromatography in 1:10 EtOH:toluene (R_f=0.28) with slowly increasing polarity with EtOH

towards 1:1 EtOH:toluene (R_f=0.77). The fractions of relevance were concentrated *in vacuo*, yielding the product **7** as an orange solid (11.7 mg, 82 %). ^{1}H-NMR (400 MHz, MeCN-d3) δ 8.34 (dd, J = 1.6, 0.7, 1H), 8.14 (dd, J = 8.0, 1.6, 1H), 7.52 (bs, 2H), 7.32 (bt, 1H), 7.26 (dd, J = 8.0, 0.7, 1H), 6.72 (d, J = 2.4, 2H), 6.65 (d, J = 8.7, 2H), 6.56 (dd, J = 8.7, 2.4, 2H), 3.48 (td, J = 6.8, 6.0, 2H), 2.30 (td, J = 7.1, 2.7, 2H), 2.21 (t, J = 2.7, 1H), 1.82 (p, J = 7.0, 2H). HRMS (ESI): [M+H]; Calc.: 442.1285 found: 442.1287.

General procedure for DMTMM coupling of *N*-Fmoc-piperidine-4 –carboxylic acid (6) with amino-modified oligonucleotides followed by alkaline cleavage (O2).
All the listed concentrations are final concentrations of the mixture. The compound **6** (50 mM) was dissolved in DMF and added to an Eppendorf tube, which contained the oligonucleotide of interest (**O1**, 10 nmol) in a HEPBS pH 9 buffer (100 mM). To this, DMTMM (50 mM) was added. Final solvent mixture was 1:1 H_2O/DMF (300 µL). The mixture was agitated overnight at room temperature. If the solution had precipitates, DMF was added to make it homogeneous. The mixture was added NaOAc pH 5.2 (300 mM), glycogen (1 µL) and vortexed briefly, followed by addition of 2.5-3 volumes of EtOH. The sample was put in dry ice for 15 min and subsequently centrifuged at 20000 g at 4 °C for 30 min. The supernatant was decanted, the pellet air dried for 10 min, followed by being redissolved in TEAA 0.1 M, 100 µL) and purified by RP-HPLC (260 nm signal trace shown in figure S1, starting material at ~8.0 min and product at ~14.4 min). The retention time should be expected to increase by 6-8 minutes. The purified fractions were lyophilized. The pellet was redissolved in LiOH (200 mM, 200 µL) and agitated for 2 hours at room temperature. The solution was EtOH precipitated using the procedure above followed by an additional RP-HPLC purification (260 nm signal trace shown in figure S2, starting material at ~14.7 min and product at ~8.2 min), which – after lyophilisation – yielded the product **O2** (7.54 nmol, 75%).

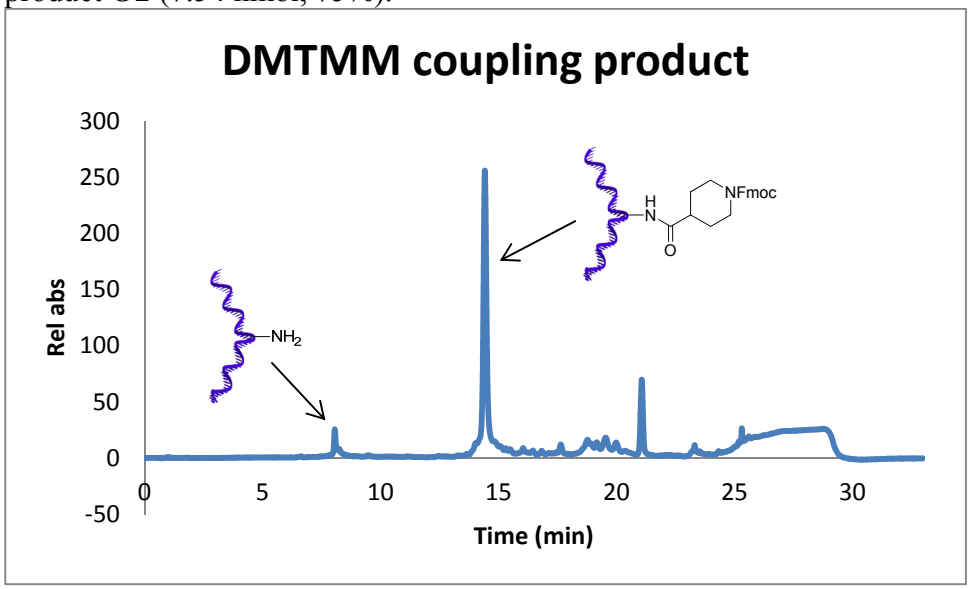


Figure S1: HPLC trace of coupling of Fmoc protected piperidine moiety to amino modified DNA.

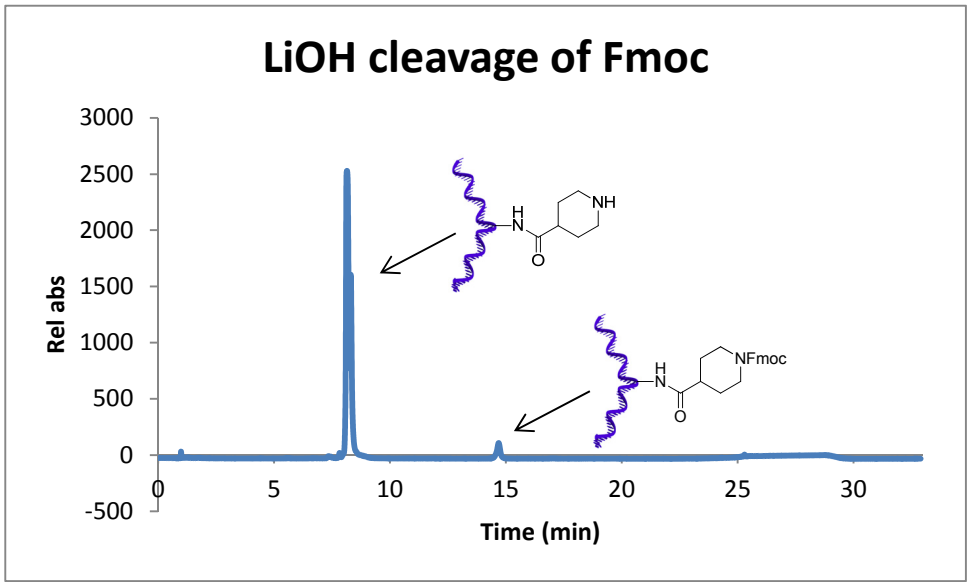


Figure S2: HPLC trace of cleavage of Fmoc group on piperidine modified DNA

Coupling 3-(methoxycarbonyl)benzenediazonium tetrafluoroborate (3a) with piperidine modified oligonucleotides (O3b).

All the listed concentrations are final concentrations of the mixture. The oligonucleotide **O2** (2.12 nmol) was dissolved in a MOPS pH 7 buffer (100 mM), to this aqueous **3a** (10 mM) was added together with 1 volume of MeCN so final mixture was 1:1 H_2O/MeCN. The mixture was agitated for 30 min, followed by EtOH precipitation as above but with LiCl (500 mM) replacing the NaOAc. The sample was purified by RP-HPLC at pH 9 (260 nm signal trace shown in figure S3, starting material at ~7.4 min and product at ~12.2 min), followed by lyophilisation of the purified fractions. The pellet was redissolved in LiOH (100 mM) and was agitated for 2 hours, followed by EtOH precipitation with LiCl (500 mM) and RP-HPLC purification at pH 9 (260 nm signal trace shown in figure S4, starting material at ~11.8 min and product at 8.5 min). The purified fractions were lyophilized. This yielded a 3-((4-carbamoylpiperidin-1-yl)diazenyl)benzoic acid modified oligonucleotide, which corresponded to the product **O3b** (1.16 nmol, 55 %).

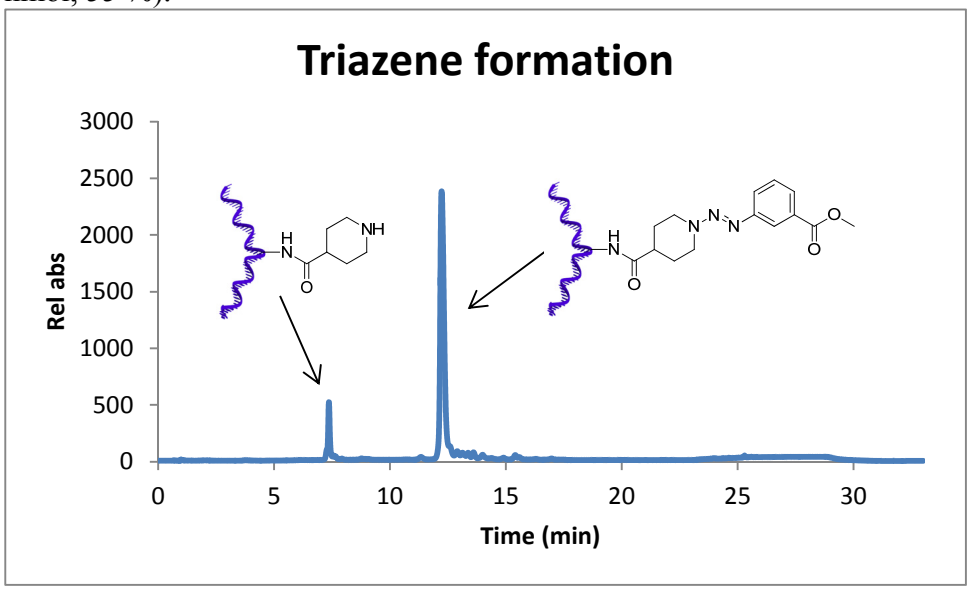


Figure S3: HPLC trace of the formation on a triazene on a piperidine modified DNA strand.

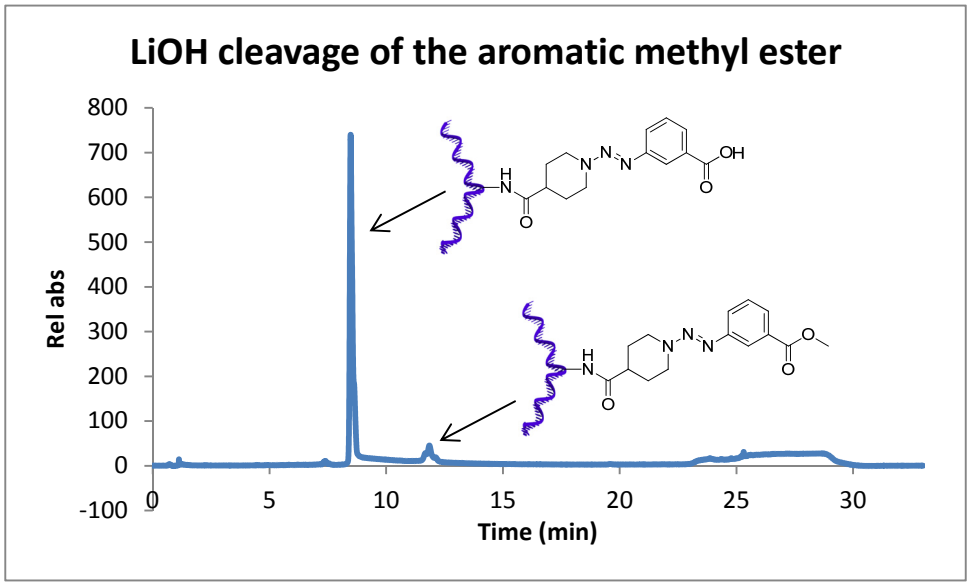


Figure S4: HPLC trace of the cleavage of a methyl ester on a DNA conjugated triazene.

Triazene formation test with small organic molecules analyzed by LCMS.
All the concentrations are final concentrations of the mixture. Twelve solutions of 4-(hydroxymethyl)piperidine (10 mM) in H_2O or H_2O – MeCN (1:1) were prepared: Two solutions were buffered with NaOAc pH 5.2, two solutions buffered with MOPS pH 5, two solutions were buffered with MOPS pH 6 and so forth with MOPS pH 7 and 8 and HEPBS pH 9 and 10. One set of the solutions were adjusted with H_2O to 90 µL while the other set were adjusted with H_2O (40 µL) and MeCN (50 µL) to a total volume of 90 µL. Each set comprised each of the 6 buffer combinations. To all 12 solutions, aqueous **3a** (10 mM) was added to a total volume of 100 µL in each mixture. After reacting for 30 min, the samples were injected directly into a LCMS for analysis. The product peak integrals from UV detection at 215 nm are listed below.

H_2O	Abs x 10^{-4}
NaOAc pH 5.2	132
MOPS pH 5	26
MOPS pH 6	39
MOPS pH 7	29
MOPS pH 8	36
HEPBS pH 9	27
HEPBS pH 10	41

H_2O/MeCN	Abs x 10^{-4}
NaOAc pH 5.2	311
MOPS pH 5	175
MOPS pH 6	231
MOPS pH 7	281
MOPS pH 8	304
HEPBS pH 9	305
HEPBS pH 10	272

Surprisingly, this showed efficient triazene formation at low pH values, but only when using NaOAc as the buffer salt. Using amino-sulfonic acid buffers resulted in higher conversions at increasing pH values as would be expected. The reasoning behind the expectations is that the triazene is more stable at higher pH values, as the protonation of the formed tertiary amine is no longer possible. Also, the starting secondary amine is more nucleophilic at higher pH.

Triazene formation test on DNA followed by LCMS analysis.
Six Eppendorf tubes were charged with an aliquot of the conjugate **O2** (50 pmol), buffer (either NaOAc pH 5.2; MOPS pH 5, 6, 7 and 8; or HEPBS pH 9 and 10, final 100 mM), MeCN (50 µL) and water was added to a total volume of 90 µL. Finally, aqueous **3a** (10 µL, final 10mM) was added to all seven solutions. After reacting for 30 min, the samples were precipitated as previously described. The pellets were redissolved, and 5 % of each sample was analyzed by RP-HPLC. The product peak integrals (260 nm) are listed below. The product from the reaction with MOPS pH 7 were analyzed with LCMS: [M]; Calc: 6551.12 found: 6550.58

H_2O	Abs x 10^{-4}
NaOAc pH 5.2	53
MOPS pH 5	0
MOPS pH 6	45
MOPS pH 7	124
MOPS pH 8	85
HEPBS pH 9	42
HEPBS pH 10	61

Clearly, pH 7 provides an optimum for the reaction. This describes an experimental window between a low pH value that would cleave the triazene, and a high pH value, which would lead to decomposition of the diazonium salt before it could react with the secondary amine on DNA. In figure S5 is shown three representative HPLC chromatograms, from which the above table was generated.

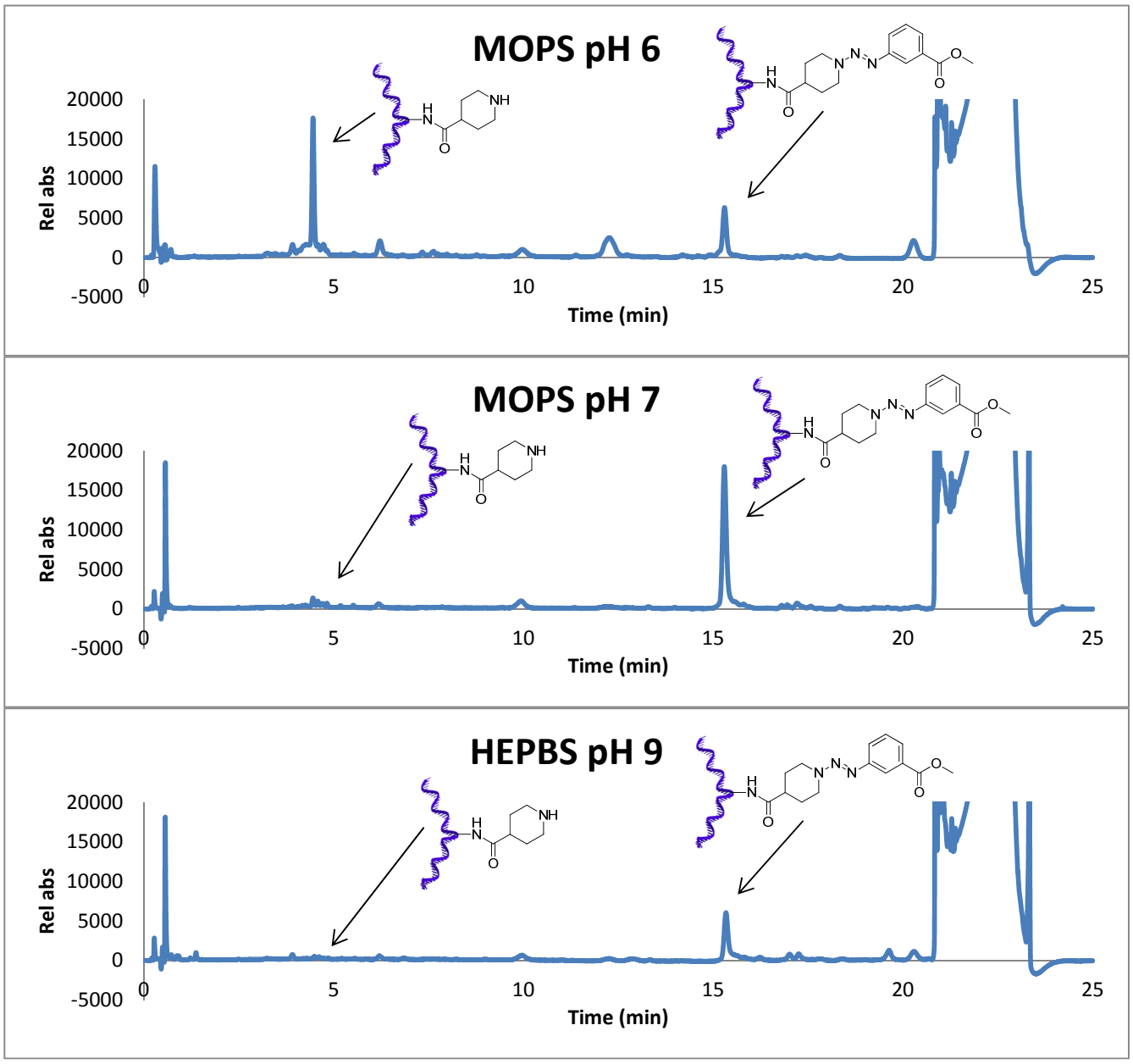


Figure S5: Three HPLC traces of testing the direct formation of a triazene on piperidine modified DNA.

Cross-linking the yoctoreactor with the triazene (OP3b-4) and testing its stability.
All concentrations are final. To oligonucleotide **O3b** (1.16 nmol) was added oligonucleotide **O4** (1.16 nmol), oligonucleotide **O6** (1.16 nmol), NaCl (1 M), HEPBS pH 9 (100 mM) and DMTMM (50 mM), and the volume was adjusted to 300 µL with H_2O. The mixture was agitated overnight at room temperature. The sample was EtOH precipitated with LiCl as before. The sample was PAGE purified on a 10.5%/8 M acrylamide/urea gel. The extracted sample was EtOH precipitated with LiCl resulting in the pure **OP3b-4** (320 pmol, 28 %). LCMS: [M]; Calc.: 28383.8 found: 28382.3. The sample was split into 15 PCR tubes – each with 20 pmol **OP3b-4** – containing $NaPH_2O_2$ (5 mM) and, in groups of three, one of the following buffers at 100 mM: NaOAc pH 5.2, MES pH 6, MOPS pH 7, HEPES pH 8 and HEPBS pH 9, resulting in a total volume of 30 µL. One group was furthermore divided into 5 µL aliquots. The samples were split in three groups of five samples, with all five pH values in each group. One group was put on a Thermomixer Compact at 50 °C, another group was left at room temperature and the third - which was the aliquoted sample - was stored at -20 °C. An 10.5%/8 M acrylamide/urea gel PAGE was performed after 2 hours (**a)** below), 24 hours (**b)** below), 3 days (**c)** below) and 6 days (**d)** below) with each gel containing each of the 15 different samples. The gels were compared using ImageJ (http://rsbweb.nih.gov/ij/) to measure the band intensities. Gel intensity calculations: The values were calculated as follows: the top band which is the cross-linked product is labeled TP, bottom bands are the cleaved products labeled B1 and B2 from top to bottom, and % is the amount of triazene left: $\% = 100 \times (1 - \frac{B1+B2}{TP+B1+B2})$. This formula assumes that if the percentage of triazene that is left equals 50%, the sum of the intensity of the bottom bands is equal to the top band. These calculated values are not absolute, as some margin of error is to be expected from extracting data in this manner. The graphs in figure 7 are made with cross gel results, but this should be valid as the percentages are standardized with itself in each lane. It must be emphasized that these values are not absolutes.

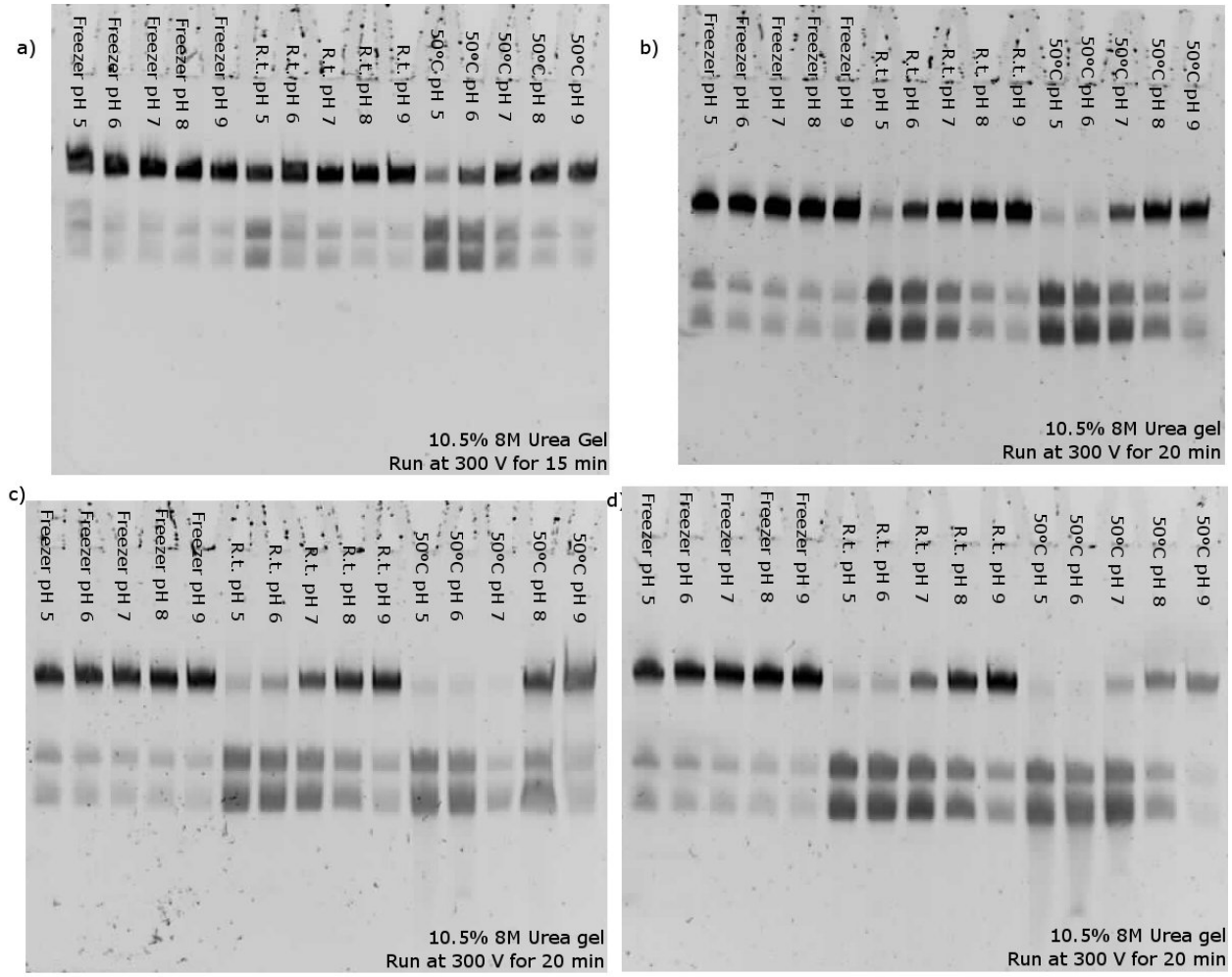


Figure S6: DE-PAGE of stability testing of a DNA conjugated triazene.

% triazene after 2 hours			
pH	-20 °C	r.t.	50 °C
5	72	48	21
6	80	68	36
7	83	74	60
8	82	80	74
9	81	83	78

% triazene after 24 hours			
pH	-20 °C	r.t.	50 °C
5	62	18	10
6	73	34	11
7	74	51	31
8	77	67	53
9	80	76	69

% triazene after 3 days			
pH	-20 °C	r.t.	50 °C
5	65	12	8
6	71	19	7
7	75	37	9
8	76	55	47
9	80	72	65

% triazene after 6 days			
pH	-20 °C	r.t.	50 °C
5	66	11	8
6	72	12	5
7	79	29	15
8	81	48	45
9	82	64	74

Cleaving the triazene-linked DNA with $NaPH_2O_2$ or NaN_3 (O5-N_3).
Starting with 4 nmol of oligonucleotide **O1** using the exact same conditions as in experiment for **OP3b-4** to couple oligonucleotide **O1** and oligonucleotide **O4** together with a triazene followed by PAGE purification. This lead to 80 pmol purified **OP3b-4**, which was split into two Eppendorf tubes. To both Eppendorf tubes was added NaOAc pH 5 (3 M, 15 µL). To one of the tubes was added $NaPH_2O_2$ (500 mM, 10 µL) and to the other NaN_3 (500 mM, 10 µL). The

volume was adjusted to 100 µL in each tube. The mixtures were agitated overnight at 37 °C. Both samples were EtOH precipitated with standard procedures and products were visualized using an 8 M urea/10.5% PAGE. Gel results are shown in figure S7. Furthermore both reactions were analyzed with LCMS. $NaPH_2O_2$ cleaved sample: [M]; Calc: 15991.4 found: 15991.0. NaN_3 cleaved sample: [M]; Calc: 16032.4 found: 16031.9.

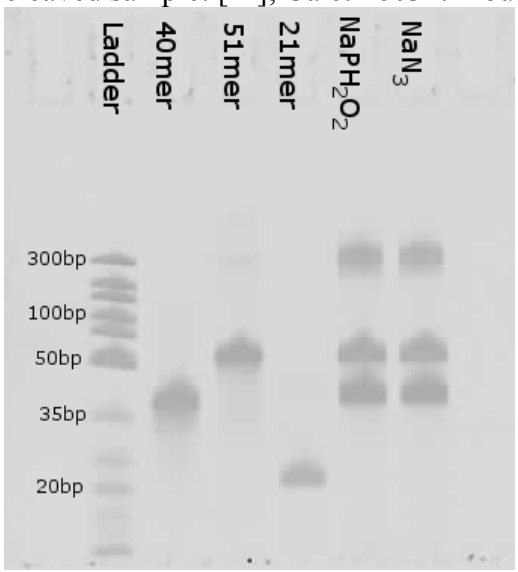


Figure S7: DE-PAGE of cleavage of the triazene with different conditions.

"Click" reaction of the fluorophore 7 and the oligo O5-N_3 to yield O5-7.

The crude lyophilized mixture – from the formation of **O5-N_3** – was redissolved in 65 µL H_2O, and was added the following reagents in order, concentrations are from stock solutions: HEPES pH 7 buffer (1 M, 20 µL), **7** (20 mM, 50 µL), THTA (*tris*-(1-[3-hydroxypropyl]triazolyl-4-methyl)amine)[7] (25 mM, 12.8 µL), $CuSO_4$ (2 mM, 20 µL), DMSO (24 µL), sodium L-ascorbate (500 mM, 8 µL). The mixture was agitated for 2 h at room temperature, which was followed by the addition of EDTA (50 mM, 5 µL) to quench the reaction. The reaction was EtOH precipitated as previously described yielding **O5-7**, which was visualized using an 8 M urea/10% PAGE. Gel results shown in figure 9.

DNA sequences

Name	**Sequence (5' → 3')**
O7 20mer	GTAAGTCCTCGTAAGTCCTC-PO_4^--$C_7H_{14}O$-NH_2
O1 40mer	GCAACTGTTTTACAGTTGCGTCTTCGAGCT-(C_6H_{12}-NH_2)GTACCTGCGC
O4 51mer	AGAAGGTGGTCCCTGGCAGTCTCCCCTTCTGACCGACTCCT-(C_6H_{12}-NH_2)GCTCGAAGAC
O6 21mer	GCGCAGGTACTGGAGTCGGTC

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