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 (1H NMR at 400 MHz, 13C 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 H2O 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 were determined using a ND-1000 NanoDrop spectrophotometer.

Methyl 3-aminobenzoate (1a).2
To a round-bottomed flask containing MeOH (150 mL) and concentrated H2SO4 (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 Na2CO3. 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 Na2SO4, and the solvent was removed in vacuo. The crude product was purified by flash chromatography on silica gel using 100:100:3 pentane/Et2O/Et3N. This provided 1a as a beige solid (6.319 g, 95%). M.p. 38.8-40.8 °C, literature 38 °C. 1H-NMR (400 MHz, CDCl3) 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). 13C-NMR (101 MHz, CDCl3) δ 167.4, 146.6, 131.2, 129.3, 119.7, 119.5, 115.8, 52.1.


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 H2SO4 (0.2 mL, 3.752
mmol) and the reaction was refluxed overnight. The reaction mixture was cooled to room
temperature, and quenched with saturated NaHCO₃. Then the mixture was extracted with EtOAc
(3 x 20 mL). Subsequently the organic phase was dried over Na₂SO₄, and the solvent was
removed in vacuo. The crude product was purified by flash chromatography on silica gel using
100:10:3 toluene/MeOH/Et₃N. This provided 3 as a pale yellow oil (0.161 g, 23%). ¹H-NMR
(400 MHz, CDCl₃) 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).
¹³C-NMR (101 MHz, CDCl₃) 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.

Methyl 3-((4-(4-methoxy-4-oxobutyl)piperidin-1-yl)diazenyl)benzoate (4).
BF₄NO (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/CO₂
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 BF₄NO 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/CO₂ 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 5 min on the MeCN/CO₂ bath and 5 without
the MeCN/CO₂ bath. Solvent was removed in vacuo. The crude product was purified by flash
chromatography on silica gel using 100:100:3 pentane/Et₂O/Et₃N. This provided 4 as a pale
yellow oil (85 mg, 57%). ¹H-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). ¹³C-NMR (101 MHz, MeCN-d₃) δ 174.7, 167.7, 152.2, 132.1, 130.2,
127.0, 126.0, 121.5, 51.9, 36.2, 34.6, 22.9, with two signals not able to be resolved.

3-(Methoxycarbonyl)benzenediazonium tetrafluoroborate (3a).⁴
BF₄NO (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 BF₄NO. 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 Et₂O. This
provided 3a as a white cotton-like solid (0.558 g, 86%). M.p. 145.5 °C decomposes. ¹H-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). ¹³C-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-BF₄]; Calc.: 163.0502 found: 163.0503.
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 an additional 10 min, followed by the addition of Et\(_2\)O (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\(_2\)O. The product 3b was used without further purification (0.832 g, 95 %). M.p. 153.4 °C decomposes. 1H-NMR (400 MHz, MeCN-d3) \(\delta\) 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). 13C-NMR (101 MHz, MeCN-d3) \(\delta\) 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.

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\(_2\)O (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\(_2\)O. The product 3e was used without further purification (1.58 g, 92 %). M.p. 152.6 °C decomposes. 1H-NMR (400 MHz, MeCN-d3) \(\delta\) 8.14 (s, 2H), 4.04 (s, 3H). 13C-NMR (101 MHz, DMSO-d6) \(\delta\) 157.4, 147.4, 135.7, 129.8, 109.2, 54.4. HRMS (ESI): [M-BF\(_4\)]; Calc.: 169.0066 found: 169.0067.

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 \textit{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 (Rf=0.77). The fractions of relevance were concentrated in vacuo, yielding the product 7 as an orange solid (11.7 mg, 82 %). ¹H-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 H2O/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.](image-url)
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 H2O/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 S2: HPLC trace of cleavage of Fmoc group on piperidine modified DNA

**Triazene formation**

Figure S3: HPLC trace of the formation on a triazene on a piperidine modified DNA strand.
**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$_2$O or H$_2$O – 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$_2$O to 90 µL while the other set were adjusted with H$_2$O (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.

<table>
<thead>
<tr>
<th></th>
<th>H$_2$O Abs x 10$^{-4}$</th>
<th>H$_2$O/MeCN Abs x 10$^{-4}$</th>
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<tr>
<td>NaOAc pH 5.2</td>
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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
<table>
<thead>
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<td>NaOAc pH 5.2</td>
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<tr>
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<td>MOPS pH 7</td>
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<tr>
<td>MOPS pH 8</td>
<td>85</td>
</tr>
<tr>
<td>HEPBS pH 9</td>
<td>42</td>
</tr>
<tr>
<td>HEPBS pH 10</td>
<td>61</td>
</tr>
</tbody>
</table>

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 H2O. 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 NaPH2O2 (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: 

\[ \% = 100 \times \left(1 - \frac{10^{B1+ B2}}{10^{TP + B1 + B2}}\right) \]

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.
Cleaving the triazene-linked DNA with NaPH$_2$O$_2$ or NaN$_3$ (O5-N3).

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$_2$O$_2$ (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. NaN₃ cleaved sample: [M]; Calc: 15991.4 found: 15991.0. NaN₃ 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-N₃ to yield O5-7.
The crude lyophilized mixture – from the formation of O5-N₃ – was redissolved in 65 µL H₂O, 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), CuSO₄ (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**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' → 3')</th>
</tr>
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<tbody>
<tr>
<td>O7 20mer</td>
<td>GTAAGTCCTCGTAAAGTCTTC-PO₄⁻-C₆H₁₂O-NH₂</td>
</tr>
<tr>
<td>O1 40mer</td>
<td>GCAACGTGTGGCTGTCCTGCAGCT-(C₆H₁₂-NH₂)GTAACCTGCGC</td>
</tr>
<tr>
<td>O4 51mer</td>
<td>AGAAAGGTGTCCTGGCAGTCTCCCTTCTGACCAGTCTCCT-(C₆H₁₂-NH₂)GCTCGAGAC</td>
</tr>
<tr>
<td>O6 21mer</td>
<td>GCCGAGGTACTGGGAGTCGTC</td>
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