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A traceless aryl-triazene linker for DNA-directed chemistry†

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DNA-directed synthesis of encoded combinatorial libraries of small organic compounds most often involves transfer of organic building blocks from one DNA strand to another. This requires cleavable linkers to enable cleavage of the link to the original DNA strand from which the building block is transferred. Relatively few cleavable linkers are available for DNA-directed synthesis and most often they leave an amino group at the organic molecule. Here we have extended the application of aryltriazenes as traceless linkers for DNA-directed synthesis. After reaction of one building block with a building block at another DNA strand the triazene linker is cleaved and reduced with hypophosphorous acid in high yield to leave the aryl group with a hydrogen in place of the triazene *i.e.* without a functional group trace. It was also demonstrated that alternatively the triazene could be converted to an azide, which was used in a cycloaddition reaction. The linker is generally stable at pH > 7 and could be stored for several months in a freezer without significant degradation.

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Introduction

The field of DNA-directed chemistry has its origin in chemical ligation of DNA strands,[1] and during the past decade it has been expanded to a broader range of chemical reactions and structural designs by Liu *et al.*[2] and others.[1,3–5]

A variety of covalent linkers are used to attach chemical building blocks to DNA oligonucleotides, and in studies where the building block must be transferred from one DNA strand to another, cleavable linkers are required. The transfer of building blocks in DNA-directed chemistry has been used to build DNA encoded combinatorial libraries of small peptides and other molecules. Liu *et al.* have reported on programmable synthesis of small molecules using DNA-directed chemistry.[6–9] In these studies a cleavable BSOCOES (bis[2-(succinimidooxycarbonyloxy)-ethyl]sulfone) linker was used. This is a homobifunctional linker that can link two amino residues and it is cleavable at elevated pH levels releasing the two formerly tethered amines.

In 2009 we reported on formation of an encoded library of short peptides in the yoctoreactor, which utilizes DNA junction structures, *e.g.* a 3-way junction. In this work, the yoctoreactor is used for sequential DNA-directed synthesis for small-molecule evolution.[10] In these studies we used a succinimidyl 2-(vinylsulfonyl)-ethyl carbonate linker, which is a heterobifunctional linker containing a sulfone conjugated alkene in one end and an NHS group in the other end.[11] It can selectively couple a thiol modified DNA strand with a building block containing an amino group. This linker is also cleavable at elevated pH values releasing the amino moiety of the building block.

The purpose of the yoctoreactor technology is to prepare and screen large combinatorial molecular libraries for compounds of pharmaceutical relevance in a quick and easy manner in a single tube format.[10] For both the above-mentioned linkers, the chemical building block is left with an amino group after cleavage of the linker. This is very useful for reactions with another building block; however, for the final building block it is inappropriate to terminate all products with an amino group. To increase the diversity of such libraries and to expand the linker chemistry in DNA-directed synthesis we have pursued the development of a cleavable linker that leaves the final building block in a synthesis without a functional group (a traceless linker). Here we present such a linker and its fundamental reaction pathway is shown in Fig. 1, where the coupled product is released with a hydrogen substituent instead of an amino moiety.

The traceless linker studied here is based on triazene chemistry. Aromatic triazenes are fairly common in classical organic chemistry[12,13] and Bräse *et al.* have pioneered the use of triazene linkers in solid phase synthesis.[14,15] The advantage of using triazenes is that the products can be cleaved from the solid support in a traceless manner. Triazenes are furthermore

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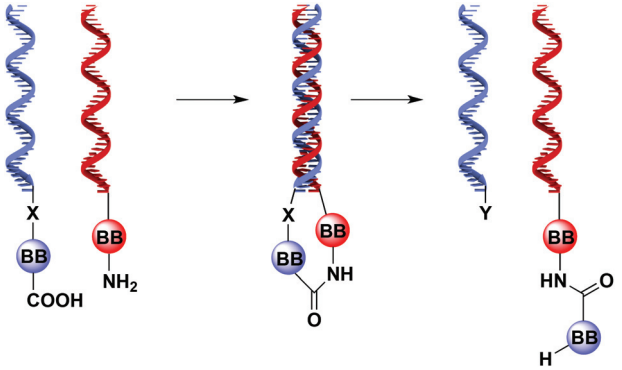

Fig. 1 Principle of the transfer of building blocks from one strand to another followed by cleavage of the linker showing the possible cleavage residues from this work. (X: cleavable linker, Y: residue from the cleavable linker.)

stable towards bases, nucleophiles, reductants and a variety of metal-catalysts, whereas they are labile towards acids and electrophiles. Triazenes are typically formed by the reaction of an aryldiazonium salt with a secondary amine. When subjected to acids or electrophiles, the triazene cleaves and regenerates the diazonium salt, which can be reduced to the naked arene (hydrogen) with a mild reductant.[16]

For the triazene linker described in this work the key issues addressed are: (a) the triazene linkers must be installed on the DNA conjugates, (b) the linker must be sufficiently stable under aqueous conditions at various pH values to function in DNA-directed chemical reactions efficiently, and (c) the linker must be cleavable under conditions that are compatible with DNA and aqueous conditions.

Results and discussion

A model linker **4** was first synthesized by addition of the piperidine derivative **3** to the aryl diazonium salt **2a** made *in situ* from **1a** as illustrated in Fig. 2 in 57% yield. Subsequently we have screened for useful reagents to cleave the linker **4** into **5** and **3** under conditions that are not harmful to DNA.

The two steps involved in the cleavage and reduction reaction of **4** can be followed by ^{1}H NMR as shown in Fig. 3. Upon treatment with acid, **4** is cleaved back into the diazonium salt **2a** resulting in a downfield shift of the proton signals, because of the decrease in electron density on the aromatic ring causing decreased shielding. Subsequent addition of a reductant led to formation of **5** as verified by the upfield shift.

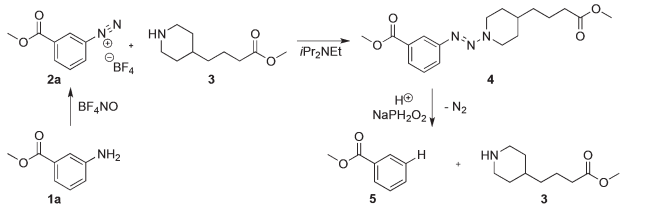

Fig. 2 Synthesis and reductive cleavage of the prototype triazene linker **4** from the secondary amine **3** and the *in situ* formed diazonium salt **2a**. The linker **4** is cleaved into the compounds **5** and **3** by the addition of a reductant and an acid.

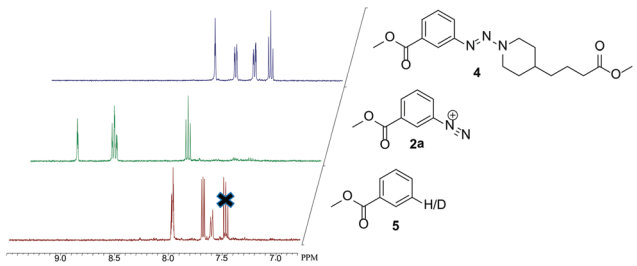

Fig. 3 Showing the resulting states of the triazene **4** prior to acidic cleavage and **2a** and **5** post cleavage of the triazene with their respective ^{1}H NMR spectra in blue, green and red. The "X" marks a signal from H_3PO_2 which was used as a reagent in this case. The disparity between signal intensities in **5** is caused by partial deuteration at the 3-position.

Several conditions were tested for the cleavage and all solvents and reactants used for the NMR study were deuterated to minimize interference with the signals of interest in ^{1}H NMR (Table 1). The cleavage with $HSiCl_3$ is well described as a highly efficient cleavage and reductive agent for triazenes.[16] It worked very well in neat MeCN, but unfortunately trichlorosilane is incompatible with water, and thus not applicable for DNA (entries 1 and 2).

The most suitable reactant for application under aqueous conditions was hypophosphorous acid, which has previously been used as a reducing agent for diazonium salts (entry 4).[17] An acid, such as 2,2,2-trifluoroacetic acid (TFA), is only able to cleave the linker, but not reduce it (entry 3). To optimize the conditions, sodium hypophosphite was used instead, to enable separate addition of the acid and thereby making it easier to control the pH value and avoid the risk of depurination of DNA (entry 5). It was also attempted to replace the acid with an electrophile. We speculated that chloral (2,2,2-trichloroacetaldehyde) could react with the terminal nitrogen

Table 1 Examples of various cleavage conditions of the triazene linker in the ^{1}H-NMR study

Entry	Solvent (deuterated)	Cleavage agent	Cleavage conversion	Reductant	Reduction conversion
1	MeCN	$HSiCl_3$	99%	$HSiCl_3$	95%
2	MeCN + 12% D_2O	$HSiCl_3$	100%	$HSiCl_3$	0%
3	MeCN–D_2O 1 : 1	4% TFA	100%	—	0%
4	MeCN–D_2O 4 : 1	D_3PO_2	100%	D_3PO_2	100%
5	MeCN–D_2O 1 : 1	4% TFA	100%	NaH_2PO_2	100%
6	MeCN–D_2O 1 : 1	Chloral	15%	NaH_2PO_2	0%

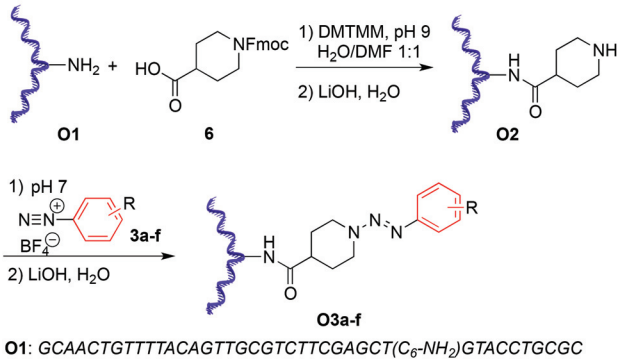

Fig. 4 General reaction pathway for coupling an aromatic diazonium salt to a DNA strand *via* a triazene.

atom of the triazene and cause cleavage. Unfortunately the cleavage efficiency of chloral was very poor (entry 6), curiously, however, it improved in the presence of water.

For the introduction of the triazene linker directly onto DNA we have synthesized a 40-mer oligonucleotide carrying an internal dT(C6-amino) modifier (**O1**). Our first approach in construction of triazene-linked DNA conjugates was to attach a building block already containing the triazene linker to amino-functionalized DNA. However, due to problems related to the presence of two carboxylic acid groups in these derivatives this approach was abandoned. Instead, a strategy in which the triazene is formed directly on the DNA was implemented. First, the amino modified DNA **O1** was linked to the *N*-Fmoc-piperidine-4-carboxylic acid (**6**) (Fig. 4). The deprotected secondary amine conjugate **O2** was subsequently subjected to the reaction with the aryldiazonium salts **3a–f** at pH 7 in a 1 : 1 H_2O–MeCN mixture. The triazenes **O3a–f** were formed on DNA without any side reactions and in good to average yields.

Six different diazonium salts were linked to the DNA *via* the triazene linker as shown in Table 2. In all cases the coupling reactions were very clean leading to the expected conjugates. In entry 2 it is demonstrated that the method tolerates the presence of a free acid group as in **3b**. If the ester **3a** is applied it can subsequently be converted into **O3b** by hydrolysis of the ester. The coupling to various triazenes shows overall good yield and purity, although coupling with the free acid **3b** shows lower conversion.

With the DNA–triazene conjugates at hand, we next wanted to show that building block **3b** can undergo a DNA-directed coupling with a functional group at another DNA strand and subsequently be fully transferred to the other strand by traceless cleavage of the triazene linker. The reactions were performed in the yoctoreactor setup as shown in Fig. 5a, where conjugates **O3b** (40 nt), the amino modified **O4** (51 nt) and a DNA helper strand **O6** (21 nt) are annealed to form a DNA 3-way junction in which the reactions occur.[10] Both the sequences **O3b** and **O4** contain a hairpin loop, which principally enables ligation of the complete 3-way junction structure to one strand, as previously shown for the yoctoreactor. The amide coupling reaction is

Table 2 Coupling of various other diazonium compounds to the piperidine modified oligonucleotide

Entry	Diazonium compound	Product	Yield[a]	Calculated mass	Observed mass[b]
1	**3a**	**O3a**	52%	12 671.92	12 671.4
2	**3b**	**O3b**	10%	12 657.90	12 657.6
3	**3c**	**O3c**	33%	12 631.90	12 631.3
4	**3d**	**O3d**	28%	12 691.82	12 692.4
5	**3e**	**O3e**	47%	12 677.88	12 677.9
6	**3f**	**O3f**	3%	12 672.91	N.D.

[a] Measured by absorbance following lyophilisation of HPLC purified samples. [b] Obtained with ESI-MS.

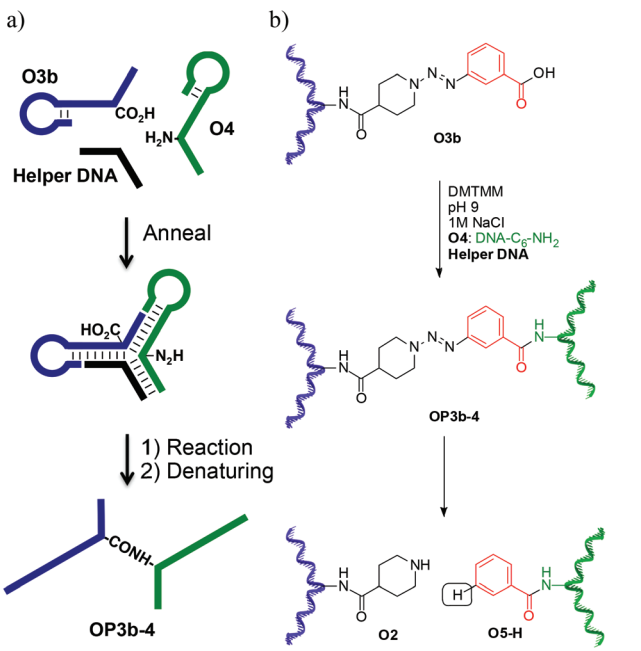

Fig. 5 (a) DNA-directed coupling carried out in a yoctoreactor type setup using a third helper DNA strand (brown) to form the 3-way junction. (b) A detailed illustration of the reaction between the triazene-linked benzoic acid moiety **O3b** and the amino-modified DNA **O4**, followed by cleavage of the triazene linker resulting in transfer of the benzoic acid from one strand to the other.

mediated by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) to form the oligonucleotide coupling product **OP3b-4** (Fig. 5b).[18] The reaction is analyzed by

polyacrylamide gel electrophoresis (PAGE) as shown in Fig. 6. The starting oligos are shown in lanes 1–3 while the product of the reaction before purification is shown in lane 4 and in lane 5 after purification. The yield of the amide bond formation was estimated to be 38% based on the analysis of the gel band intensities (densitometry), by comparing the band intensity of the cross-linked product with the unreacted DNA.

The final cleavage and reduction of the purified **OP3b-4** were performed in one step to give **O5-H** (and **O2**) and this was affected by treatment with 450 mM NaOAc, pH 5 and 50 mM NaH_2PO_2. The outcome of the reaction is shown in Fig. 6, lane 6. The yield was approximated to 78% and LCMS analysis resulted in a mass of 28 382.3, corresponding well to the calculated mass of 28 383.3. A sample of the non-cleaved residue band was isolated and identification by LCMS (ESI) was attempted. This was, however, not successful indicating that residual material is not simply residual cross-linked material with the expected structure, but more likely several species in small amounts. However, the amount of residual material was very low.

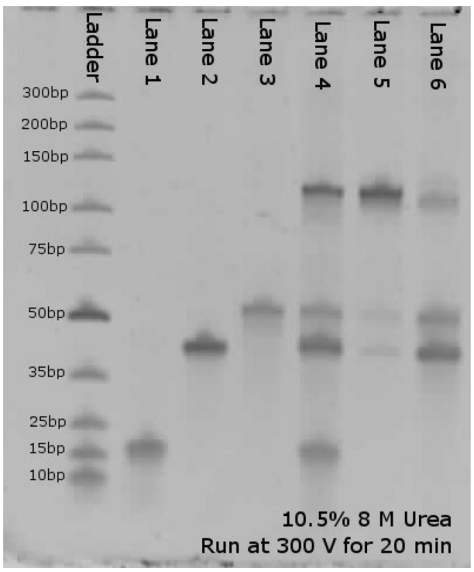

Fig. 6 A 10.5% denaturing PAGE stained with SYBR® Gold. Lane (1) 21mer DNA strand (**O6**). Lane (2) 40mer DNA strand (**O3**). Lane (3) 51mer DNA strand (**O4**). Lane (4) crude reaction mixture from a DNA cross-linking reaction (**OP3b-4**). Lane (5) purified cross-linked 40 + 51mer DNA. Lane (6) cleavage of the cross-linked DNA with AcOH and NaH_2PO_2.

The potential usefulness of the linker in DNA-directed chemistry is highly dependent on the storage stability of the triazene linked conjugates. Therefore, the stability of the linker was tested by incubating the cross-linked product at different temperatures and different pH values. The samples were assayed by PAGE (ESI†) and after analysis of the relative intensities of the bands in the gels; the results of these investigations are collected in Fig. 7. The three graphs show the percentage of non-degraded **OP3b-4** over time at three different temperatures (−20 °C; room temperature (19–25 °C); and 50 °C), and each graph contains data from 5 different pH values (values 5, 6, 7, 8 and 9).

The linker is generally most stable at high pH values and in particular at −20 °C. At a low pH value the linker degrades rapidly at room temperature and with heating, which was expected. From these results it is evident that the linker should preferably be stored at −20 °C and at pH > 7. In other experiments we have stored the cross-linked product for 8 months at −20 °C at pH 7 without seeing any significant degradation. We therefore believe that the minor degradation observed at −20 °C in Fig. 7a occurs during the gel loading or elution.

In addition to the traceless cleavage of the triazene linker we also explored the opportunity to introduce an azide group. This was achieved by replacing the sodium hypophosphate with sodium azide and thus converting the diazonium salt into an azide (**O5-N_3**). The resulting azide product was obtained in 72% conversion by densitometry and was identified by LCMS. It was also attempted to use the classic Sandmeyer conditions for introduction of halides onto the aromatic moiety in place of the diazo group, but so far without success.

The mechanism behind the conversion of the diazo group in the intermediate to the azide is probably due to its unique mechanism which is not a nucleophilic aromatic substitution. Rather it is the formation of an azidodiazene or a pentazole by attack of the azide on the diazonium group. This azidodiazene or pentazole collapses to form N_2 and the azide substituent.[19,20] To further test the presence of the introduced azide, a Huisgen–Sharpless–Meldal cycloaddition reaction was performed with an alkyne modified fluorophore (7) (Fig. 8).

The product **O5-7** was identified by LCMS and denaturing PAGE as shown in Fig. 9. The green bands are an overlay from a fluorescence scan prior to staining the gel, and the black bands

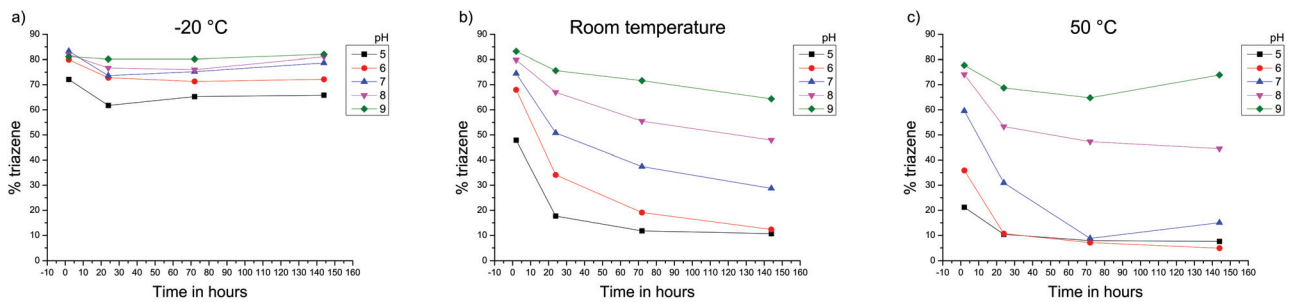

Fig. 7 Graphs showing stability of the triazene linker with varying pH values at (a) −20 °C, at (b) room temperature, and at (c) 50 °C.

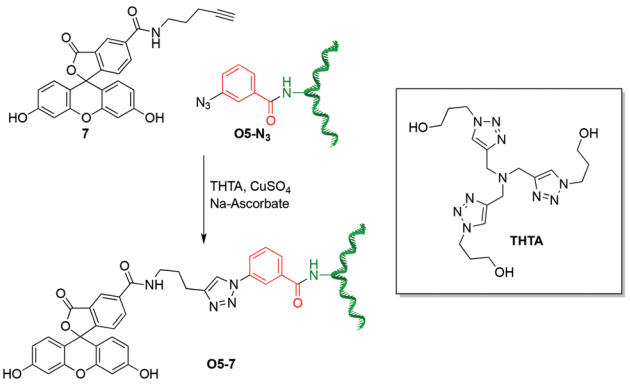

Fig. 8 Copper catalysed click reaction between the modified oligonucleotide **O5-N$_3$** and an alkyne modified 5-carboxyfluorescein (**7**), which result in the product **O5-7**.

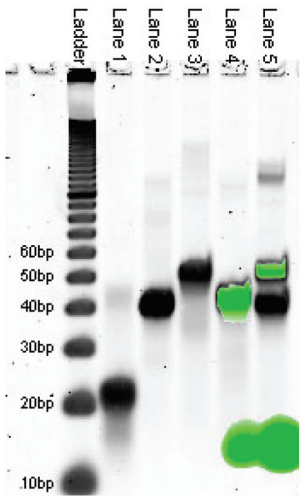

Fig. 9 A 10% denaturing PAGE stained with SYBR® Gold with a fluorescence overlay coloured green. Lane (1) 21mer DNA strand (**O6**). Lane (2) 40mer DNA strand (**O3**). Lane (3) 51mer DNA strand (**O4**). Lane (4) reference 40mer DNA strand with fluorescein (**O**). Lane (5) click reaction performed on the azide cleaved **OP3b-4** mixture resulting in **O5-7**.

are from the SYBR® Gold stain. It is shown that the upper right-most band corresponds to the correct product **O5-7**. To the left of the product band is a reference band, which is an azide modified **O3** subjected to the same click conditions as **O5-N$_3$**.

With the results from this gel, it was shown that the azide functionality was indeed incorporated and fully functional for chemical modification, and thus vastly increasing the possibility for linker diversity.

Conclusion

We have shown that triazene linkers can be applied to link aromatic building blocks to DNA oligonucleotides containing secondary amines. If the aryl groups contain another functional group such as a carboxylic acid it can react with a building block at another DNA strand in a DNA-directed reaction. Subsequently, the triazene linker could efficiently be cleaved and reduced by hypophosphorous acid, leaving the aryl group with a hydrogen in place of the triazene. The overall process is a DNA-directed and traceless transfer of an aromatic building block from one DNA strand to another. We believe this will find application in DNA-directed synthesis of encoded chemical libraries. Furthermore, it was shown that the triazene could also be substituted with an azide, and since the Huisgen–Sharpless–Meldal 1,3-dipolar cycloaddition reaction of azides with alkynes is compatible with DNA, the azide approach provides further opportunities for increasing the diversity in such libraries.

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