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Versatile Introduction of Azido Moiety into Oligonucleotides through Diazo Transfer Reaction

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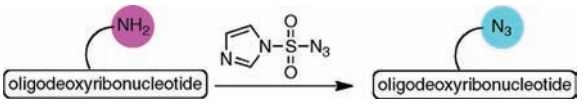
ABSTRACT



The use of a diazo transfer (DZT) reagent enables clean and efficient conversion of aminated oligodeoxyribonucleotides (ODNs) into their azido counterparts under mild conditions. ODNs bearing an amino tether at the 3′, 5′, or any internal position could be modified in this manner thus demonstrating the versatility of this reaction. Easy access to such azido-modified ODNs is of great interest for conjugation in particular through copper catalyzed 1,3-dipolar cycloaddition (CuAAC reaction).

Azides are versatile and bio-orthogonal chemical moieties for labeling many classes of biomolecules in any biological context. Indeed they are poorly electrophilic and thus do not react with amines or other nucleophiles found in many biological systems. In particular azides are involved in bioconjugation by either Staudinger ligation[1] or 1,3-dipolar cycloaddition (Huisgen reaction). The latter has undergone a considerable renewal of interest thanks to the use of Cu^{I} salts as a catalyst, which considerably enhances reaction yields and regioselectivity.[2] Thereby peptides,[3] sugars,[4] and oligonucleotides (ODNs),[5] have been efficiently linked to reporter groups using the copper catalyzed 1,3-dipolar cycloaddition (CuAAC reaction).

In the case of synthetic ODNs, the incorporation of a suitably functionalized tether is generally performed during on-support strand elongation *via* the introduction of a modified phosphoramidite synthon. However, the introduction of an azido moiety by this method is impossible in the case of phosphoramidite based ODN synthesis because of the interfering Staudinger reaction that takes place between azides and P^{III} derivatives.[6] Azide introduction during on-support synthesis is thus restricted to H-phosphonate[7] or phosphotriester[8] strategies.

As a consequence, many authors used a postsynthetic introduction of the azide function.[9] For example, the 5′ introduction of an azido group was performed by iodination of the 5′-alcohol of the supported oligonucleotidic strand, followed by substitution with an azide salt with a

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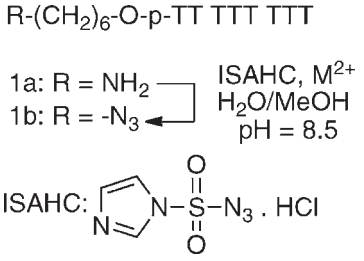
moderate yield ranging from 55 to 83% depending on the nature of the last nucleobase.[10] Several other halogenated reagents have been designed;[11] however their multistep preparation restricts their use as bioconjugation reaction precursors. Other strategies rely on acylation of an alkylaminated ODN by a presynthesized activated ester such as NHS bearing an azido group.[12] However in solution phase, the reaction of the NHS ester with the primary amine is competing against the hydrolysis of the NHS ester since the acylation is typically performed in aqueous buffer at pH 9. As a result it is recommended to perform the acylation on a solid support.

Because of the growing interest in CuAAC reactions for ODN conjugation a straightforward preparation of azido containing ODNs should represent an attractive alternative to the existing methods and would be of interest. Obviously, this method must be versatile enough to enable azidation in various positions (3′, 5′, and internal positions).

Our attention has focused on the use of a diazo transfer reagent, which enables the direct transformation of an amino function to an azide. Since ODNs bearing alkylaminated pendant groups can be easily purchased (or be routinely synthesized) the use of a diazo transfer reagent to convert a NH_2 group to N_3 could be a versatile one-step way to access azido functionalized ODNs. The initially developed triflyl azide was of poor synthetic interest due to explosive thermal decomposition.[13] However, Goddard-Borger and Stick recently developed imidazole-1-sulfonyl azide hydrochloride (**ISAHC**, Scheme 1), which circumvents these drawbacks.[14,15] We therefore decided to further investigate the synthetic usefulness of **ISAHC** for the postsynthetic modification of DNA.

Scheme 1. Diazo Transfer (DZT) Reaction



The diazo transfer reaction was first explored with 5′-aminated model ODN **1a**. The reaction was carried out in mild basic media ($NaHCO_3$ buffer, pH = 8.5) at 55 °C in the presence of $CuSO_4$. The course of the reaction was followed by reversed-phase HPLC, and the reaction proceeded essentially to completion within 1 h to yield exclusively azido ODN **1b** (see the HPLC profile in the Supporting Information). When a similar reaction was performed at room temperature, a poor 4% yield was obtained. Even after overnight reaction, only 44% conversion was obtained. We investigated the influence of the cation, since other divalent cations have been reported to accelerate DZT reactions. Addition of Cu^{2+}, Co^{2+}, and Zn^{2+} salts (2.8 equiv) lead to complete conversion in less than 20 min. On the other hand incomplete conversions were observed when using Mg^{2+} and Ni^{2+} salts even after prolonged reaction times (see Figure 1). For longer ODNs **2a** and **3a** (Table 1), near quantitative reaction yields were observed affording the azido containing ODNs **2b** and **3b**,

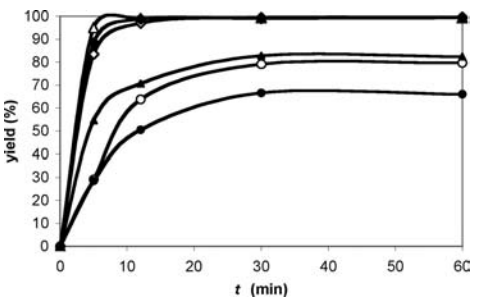


Figure 1. Conversion of **1a** in presence of divalent cations: control without addition (●), Ni^{2+} (▲), Mg^{2+} (○), Zn^{2+} (◆), Co^{2+} (◇), and Cu^{2+} (Δ) cations. The course of the reactions was monitored by HPLC (see the Supporting Information).

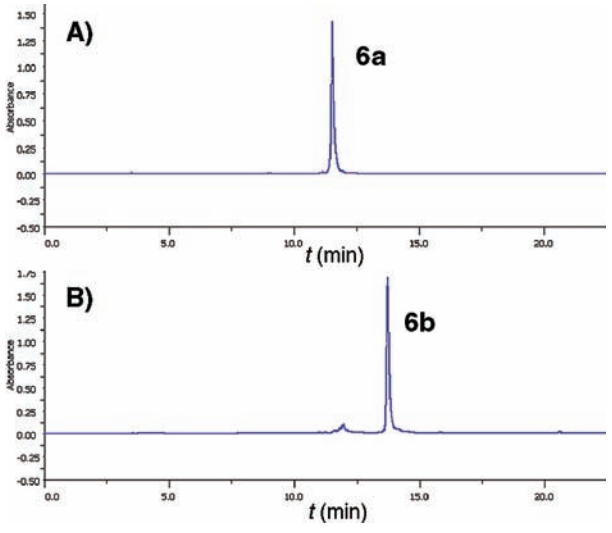


Figure 2. HPLC profiles of (A) starting material 3′-amino ODN **6a** (nontritylated ODN) and (B) crude reaction mixture after DZT reaction. Detection at 260 nm, gradient A was used (see Supporting Information).

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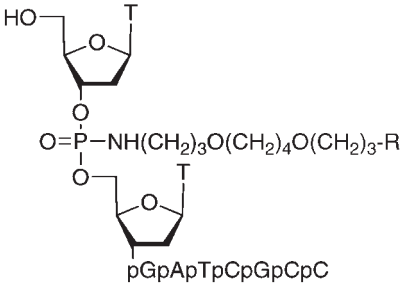
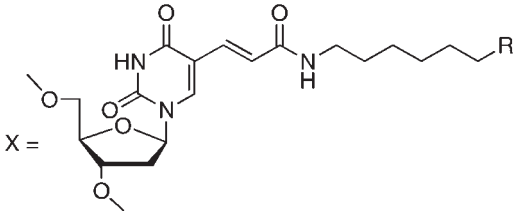
Table 1. Structure of Studied ODNs (p = Phosphodiester Linkage) and ESMS Analyses of Azido Compounds

Structure of starting materials[a] and products[b]	Azido ODNs numbering[c]	Calcd MW	Measured[d] MW
R-$(CH_2)_6$-O-p-TTT TTT TT	**1b**	2575.6	2575.9
R-$(CH_2)_6$-O-p-TTT TTT TTT TTT TTT T	**2b**	5007.9	5008.8
R-$(CH_2)_6$-O-p-TTT TTT TTT TTT TTT TTT TTT TTT	**3b**	7441.5	7441.3
R-$(CH_2)_6$-O-p-TAA TTA AT	**4b**	2611.6	2611.3
R-$(CH_2)_6$-O-p-CCC TTC CT	**5b**	2500.6	2500.6
R-$(CH_2)_6$-O-p-CTA CTG CTA CGA GTC AGG TCG CAT	**6b**	7546.4	7546.5
R-$(CH_2)_6$-O-p-CGC ATA TAC GC	**7b**	3504.7	3504.8
GGA TCG ATC-p-O-$(CH_2)_6$-R	**8b**	2942.6	2942.7
TGC ATC GAT CGG ATC G-p-O-$(CH_2)_6$-R	**9b**	5100.0	5100.8
R-$(CH_2)_6$-O-p-TGC CAT CGT-p-O-$(CH_2)_6$-R	**10b**	3098.8	3098.8
R-$(CH_2)_6$-O-p*-G*C*A*T*G*C*A*G*A*C*A*T*T*[e]	**11b**	4256.4	4256.4[f]
 pGpApTpCpGpCpC	**12b**	2900.8	2900.8[g]
TAC GCA AG**X** TTG C 	**13b**	4128.4	4128.1[f]

[a] R = NH_2. [b] R = N_3. [c] Corresponding amino-ODNs are numbered as **1a**–**13a**. [d] Deconvoluted mass measured from multicharged ions. [e] * = phosphorothioate linkage. [f] Measured from observed Na^+ adducts. [g] Both diastereoisomers due to phosphoramidate linkage were isolated and subjected to reaction conditions.

respectively, although slightly longer reaction times were needed. For convenience purposes, we fixed the reaction times at 2 h (see Supporting Information).

Before extending our reaction conditions to more complex ODNs, we decided to investigate the chemospecificity of the DZT reaction for the alkyl-amine moiety over the pendant amino groups of the nucleobases. Thus, four natural tetramers ($^{5'}$TNTT$^{3'}$; N = A, T, G, or C) were subjected to harsher reaction conditions (reagent concentrations doubled; increased *T* to 65 °C). Even after 2 days under such harsh conditions, no unspecific reaction was observed by RP-HPLC and ES-MS analysis.

The 5′-introduction of the azido moiety was then applied to 5′-amino ODNs **4a**–**7a** containing the four nucleobases. A quantitative conversion into azido derivatives **4b**–**7b** was observed in each case (Figure 2 shows a representative example of an HPLC profile of such a transformation).

ODNs **8a** and **9a** bearing an aminated tether at the 3′ end (prepared from a commercially available 3′-hexylamino support) were also efficiently converted into azido containing ODNs **8b** and **9b**, respectively. Since 3′-amino ODNs are routinely obtained by automated synthesis, the present strategy represents a straightforward route toward 3′-azido ODNs compared to previously published methods based on sophisticated modified supports.[11b,12a,16] ODN **10b** bearing azido tethers at both 3′ and 5′ ends could also be obtained in a single step from 3′, 5′-*bis*-amino modified ODN **10a** prepared from the aforementioned 5′-amino linker and 3′-amino modified solid support. Phosphorothioate ODNs are far more resistant to exo- and endonuclease activity as compared to natural ODNs and have been extensively studied as new therapeutic tools.[17] Quantitative conversion of phosphorothioate ODN **11a** to azido **11b** was achieved by using the same protocol.

Introduction of an azido function at an internal position has also been explored in both internucleotidic positions and at modified nucleobases. Introduction of an amino tether at the internucleotidic junction in ODN **12a** was accomplished by standard protocol, using H-phosphonate

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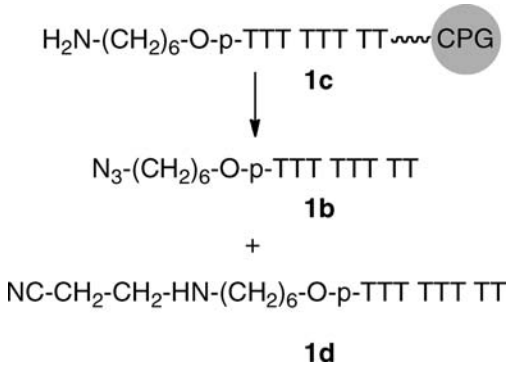
chemistry and subsequent oxidation.[18] ODN **13a** bearing amino pendant groups was obtained by automated synthesis using commercially available C-5 modified thymine. Both ODNs **12a** and **13a** were cleanly converted into the corresponding azido ODNs **12b** and **13b**, respectively.

Scheme 2. On-Support DZT Reaction



The DZT reaction was also investigated for supported ODNs. For this purpose, model ODN **1c** bearing a 5′-amino moiety was kept on the support (controlled pore-glass solid support) for the subsequent DZT reaction (Scheme 2). One major problem can arise from the base sensitivity of the succinyl ester bond linking the protected ODNs on the CPG support. As anticipated, the DZT reaction performed in 50 mM K_2CO_3 methanolic solution at RT leads to complete release of the azido-ODN **1b** into the supernatant as monitored by UV and trityl cation assay. On the other hand, when performed under milder basic conditions (0.5 mM K_2CO_3 concentration), we did not observe any formation of the expected azido product. Instead 34% of a later eluting byproduct was isolated by reversed-phase HPLC, with a +53 a.m.u. compared to the starting material. When treated under similar conditions, the unmodified supported T_8 ODN did not lead to such a byproduct. This byproduct was assigned to 5′-*N*-cyanoethyl adduct **1d**, potassium carbonate not being concentrated enough to efficiently quench acrylonitrile.[19] When performed at 55 °C, only the acrylonitrile adduct was observed. We finally found that K_2CO_3 concentrations between 2 and 5 mM lead to a 76% yield of the azido ODN after ammonolysis with only 8% of the acrylonitrile byproduct. Moreover, no significant release of ODN into the liquid phase was noticed during the DZT reaction.

In conclusion the use of diazo tranfer reagent **ISAHC** allows a clean and efficient preparation of azido functionalized ODNs.[20] Azido moieties can be introduced at various positions within the sequence (3′, 5′, or any internal positions) from readily accessible amino containing ODNs. Phosphorothioate ODNs could also be modified to contain azido moieties. In all cases quantitative conversion of amino containing ODNs was achieved. This represents a powerful tool for the preparation of azido-containing ODNs for subsequent conjugation using the well-described CuAAC and Staudinger reactions.

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Supporting Information Available. Experimental procedures, RP-HPLC profiles, and ESI-MS spectra for compounds **1b**–**13b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) Unfortunately the use of diazo transfer reagent **ISAHC** was found inefficient for introducing the azido moiety in ribo-oligonucleotides due to their instability under the basic conditions required for the DZT reaction.