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Oligonucleotide Sequential Bis-Conjugation via Click−Oxime and Click−Huisgen Procedures

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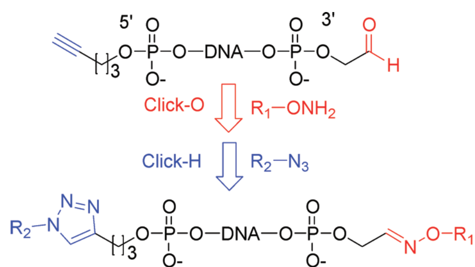

A novel procedure has been developed for the bis-conjugation of oligonucleotides using CuAAC (click-H) and oxime (click-O) tethering strategies. Oligonucleotides bearing a 5′-alkyne function and a 3′-aldehyde precursor were synthesized and were bis-conjugated with various reporters including azido carbohydrate or fluorescent dye and aminooxy peptide or carbohydrate. Versatility of the method was demonstrated by performing click-O prior to click-H and vice versa. Interestingly, when click-O is achieved prior to click-H, no purification is required in between, allowing a sequential one-pot protocol.

Conjugation of oligonucleotides (ODNs) to other molecules (i.e., reporters) has attracted significant research interest as it provides an easy way to modulate their biological properties (such as stability to degradation, cellular uptake, targeting, etc.).[1−3] Most of the reporters are linked either at the 5′ or the 3′ extremity of oligonucleotides, but conjugation at both 5′ and 3′-ends of ODNs is advantageous for both converging purposes, i.e., protection against nucleases[4,5] and combination of reporter properties (e.g., cell penetrating peptide and fluorescent probe). Two types of strategies have been developed to reach this challenging goal: solid-phase synthesis[6] and solution-phase fragment coupling.[7,8] This latter remains the most common approach to prepare those conjugates, the major advantages being that molecules with even incompatible chemistries can be conjugated to the ODNs. The solution-phase coupling approaches involve the separate synthesis and purification of ODNs and the reporter molecules incorporating mutually reactive functional moieties, followed by their solution-phase coupling leading to the formation of reversible or irreversible covalent bonds. Ideally, coupling reactions must respond to "click chemistry" criteria:[9] among other, they must be chemoselective, highly efficient and they must obviously be carried out in aqueous media. In the last years, the well-known copper(I) catalyzed[10,11] Huisgen 1,3-cycloaddition of azides and alkynes (CuAAC) emerged as a reaction of choice for ODN conjugation with various reporters[12] such as peptides,[13] carbohydrates,[6,14−20] or fluorescent dyes.[18] Multiconjugation of ODNs by CuAAC has also been performed using sequential conjugation/modification or deprotection reactions.[18,19] The oxime tethering procedure also fulfilled "click chemistry"

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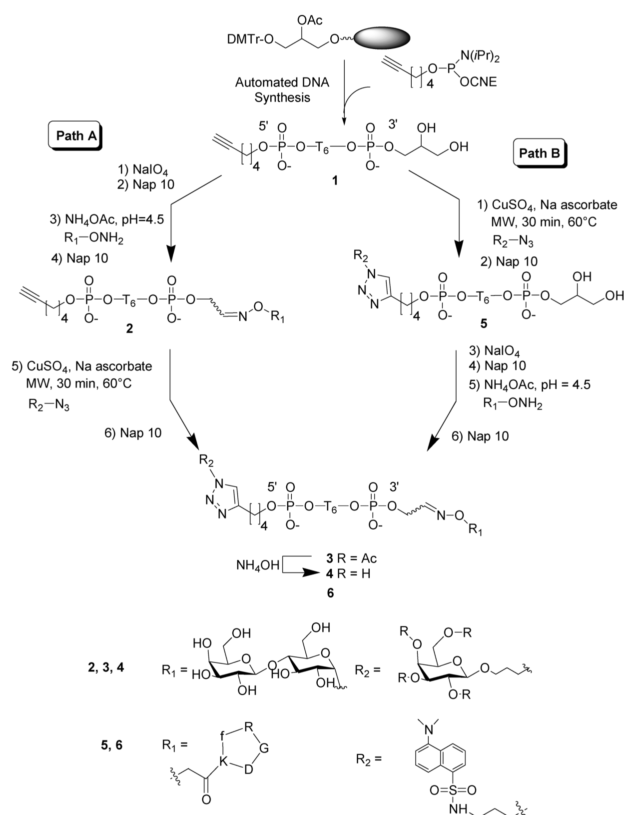
SCHEME 1. Synthesis of ODN 1 and Preparation of Bis-conjugates 4 and 6 According to Click-O Click-H Procedure (Path A) and to Click-H Click-O Procedure (Path B)



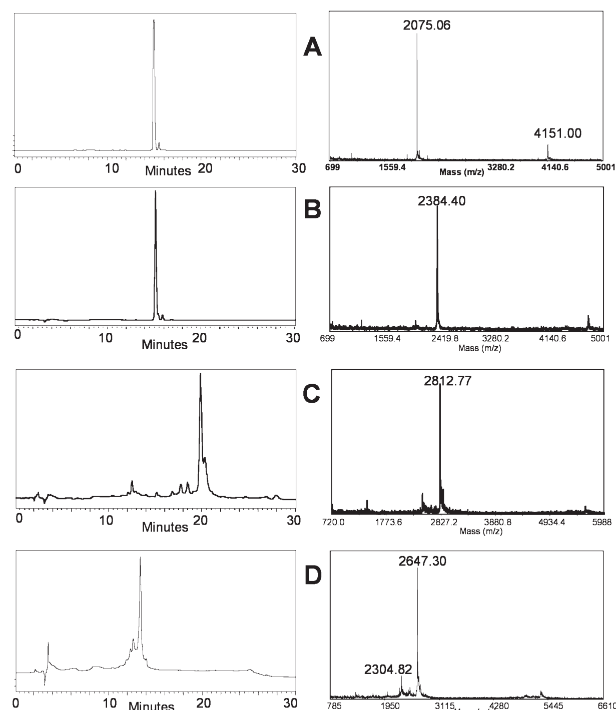


FIGURE 1. RP-HPLC (260 nm) crude profiles and MALDI-TOF MS spectra of **1** (A), monoconjugate **2** (B), and bis-conjugates **3** (C) and **4** (D).

criteria and has, more formerly, emerged as another efficient reaction for ODN conjugation.[21−26] It consists of the coupling reaction of an aminooxy moiety on an aldehyde group. It has also been used for bis-conjugation of oligonucleotide using sequential conjugation/deprotection reactions.[27] As those two reactions, i.e., click-O and click-H, are orthogonal, they can be used in the field of bis-conjugation of ODN avoiding intermediary deprotection or modification steps. This concept has been recently demonstrated in peptide conjugation chemistry,[28] and we report herein its validation in the field of ODN chemistry. During this work, Lonnberg et al. reported the use of both click-H and click-O reactions where click-H was used to build a trisaccharide derivative bearing an aldehyde function which was eventually conjugated to an aminoxy-ODN by click-O.[29]

We synthesized 5′-alkyne 3′-diol ODN **1** using commercially available glyceryl LCAA-CPG support and hexyn-5-yl phosphoramidite (Scheme 1). These oligonucleotides contain a 3′-diol function which is the precursor of the 3′-aldehyde[30] for a further reaction with aminooxy derivatives and a 5′-alkyne function for coupling with azido derivatives under Cu(I) catalysis.[10,11]

First, according to path A (Scheme 1), **1** was oxidized by sodium *m*-periodate to afford the corresponding 3′-aldehyde ODN which was, after desalting by size-exclusion chromatography (SEC), further conjugated with aminooxy lactose[31] at pH = 4.5 to give **2** within 2 h. Reactions were monitored by MALDI-TOF MS since **1**, aldehyde intermediate, and **2** exhibited same HPLC retention time (Figure 1A,B). Then, **2** was reacted with tetraacetylgalactopropyl azide[32] using $CuSO_4$ and sodium ascorbate under microwave assistance (MW) to speed up the reaction[14] for 30 min at 60 °C affording bis-conjugate ODN **3**. However, the HPLC profile of the crude showed the presence of impurities with a peak of longer retention time (Figure 1C) when acetyl groups were present and with lower retention time after ammonia treatment (Figure 1D), suggesting that the impurities have a galactose residue. The MS analysis of crude deprotected bis-conjugate **4** showed the expected ion at 2647.30 (calcd 2646.86) plus an ion at 2304.82 assigned to the 5′-galactose-T_6-3′-monophosphate as potassium salt (calculated 2303.61). The stability of oxime linkage under microwaves in the presence of $CuSO_4$/Na ascorbate for 20 min at 60 °C was studied, using an 11-mer−3′-oxime−c[RGD] conjugate. HPLC and MS analyses of the resulting mixture confirmed the negative effect of MW and copper on oxime linkages with a HPLC profile showing a large shoulder before the main peak of the oxime RGD−ODN conjugate and species of lower mass (data not shown).

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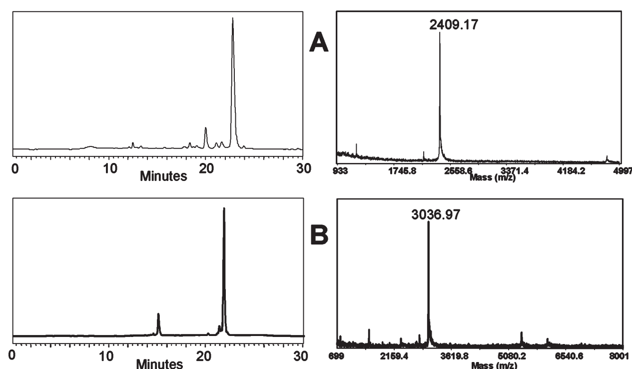


FIGURE 2. RP-HPLC (260 nm) crude profiles and MALDI-TOF MS spectra of monoconjugate **5** (A) and bis-conjugate **6** (B).

To avoid this degradation due to MW, we proceeded according to way B by performing the click-H under MW and subsequent click-O coupling reaction (Scheme 1). ODN **1** was first conjugated with 5-(dimethylamino)-*N*-(3-azidopropyl)-1-naphthalenesulfonamide[33] (dansyl azide dye) in the presence of $CuSO_4$ and sodium ascorbate for 30 min at 60 °C under microwave assistance to obtain **5** after SEC (Figure 2A). The crude was oxidized with sodium *m*-periodate to afford the expected 5′-dansyl-3′-aldehyde ODN which was desalted by SEC and directly engaged for the click-O with aminooxy c[RGD] peptide[34] affording the bis-conjugate **6** with a good purity (Figure 2B).

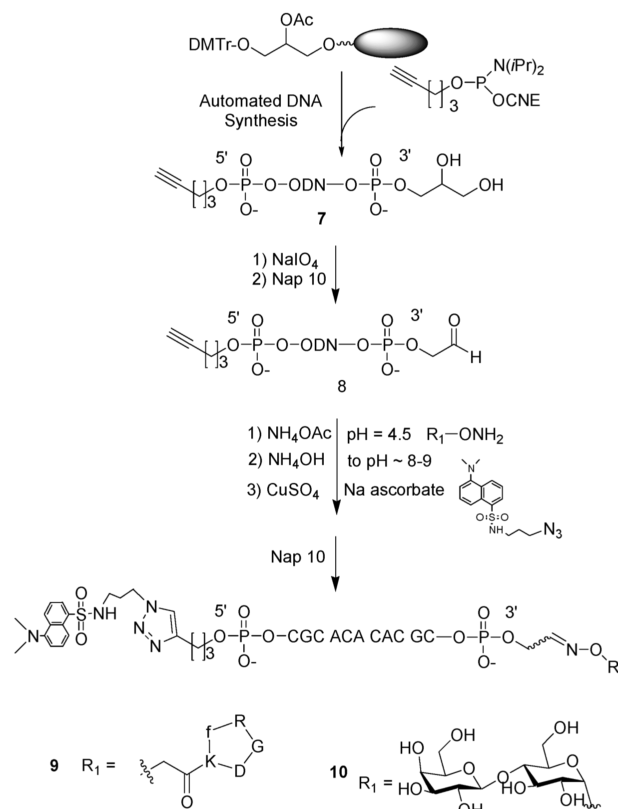
From these data it appeared that both strategies (i.e., paths A and B) allowed the synthesis of bis-3′,5′-ODN conjugate with fair efficacy.

However, to improve the protocol by reducing the number of manipulations (i.e., size-exclusion chromatography), and hence the time, we decided to work with a sequential protocol. As already reported, both clicks could not occur simultaneously since oxime ether is generally formed at pH = 4.5 and CuAAC must be performed at pH = 7−8.[35]

If we start from 5′-alkynyl-3′-glyceryl-ODN according to path B, since the click-H occurred in the presence of reducing agent, it will be difficult to oxidize the diol for the following click-O reaction. The alternative manner would be to start from a 5′-alkynyl-3′-aldehyde-ODN. However, since this aldehyde is not very stable, we observed some degradations with the formation of 3′-phosphate ODN species occurring during the click-H reaction. Hence, path B is not doable.

In contrast, according to path A, it is possible to perform a sequential "one pot" bi-click without any purification between the two steps. Obviously, reactions were performed without MW assistance to avoid the degradation of the oxime linkage. Thus, starting from a 5′-alkynyl-3′-aldehyde-ODN, the click-O reaction was performed at pH 4.5 with an aminooxy derivative, the pH was increased to 8−9 by addition of a drop of aqueous ammonia, and finally, the click-H reaction occurred by addition of an azide derivative, $CuSO_4$, and sodium ascorbate. This protocol was applied for the synthesis of two hetero undecamers bis-conjugated on one hand with 3′-lactose and 5′-dansyl and on the other hand with 3′-c[RGD] and 5′-dansyl (Scheme 2).

SCHEME 2. Synthesis of ODN 7 and Preparation of Bis-conjugates 9 and 10 According to Sequential Click-O Click-H One-Pot Procedure



Starting from glyceryl solid support (1 μmol), undecamer (CGCACACACGC) was assembled on a DNA synthesizer and the pent-4-yn-1-yl phosphoramidite[20] was coupled at the last step to afford **7** (0.7 μmol) after ammonia treatment. One part of **7** (0.2 μmol) was oxidized with sodium *m*-periodate providing 5′-pent-4-yn-1-yl-3′-aldehyde 11-mer **8** which was desalted by using SEC and directly engaged for a click-O reaction at pH 4.5 by using 10 molar equiv of aminooxy-c[RGD] over 24 h. The reaction was monitored by HPLC and MS. After completion, the pH was increased by addition of one drop of ammonia, and dansyl propyl azide (5.5 mol equiv), $CuSO_4$, and Na ascorbate were added. The click-H reaction was done within 30 min at room temperature without MW assistance, and the crude mixture was desalted by SEC. The pure bis-conjugate **9** was obtained with a 50% isolated yield after purification by reversed-phase HPLC (0.1 μmol) (Figure 3).

The synthesis of 3′-lactose 5′-dansyl 11-mer **10** was achieved similarly. To speed up the click-O reaction we started with 6 molar equiv of aminooxy lactose, and 1.5 molar equiv was added after 2 h. The reaction was complete within 3 h. After addition of ammonia (to increase the pH ~7−8), dansyl propyl azide (5.5 molar equiv), $CuSO_4$, and Na ascorbate were added. Since the reaction was incomplete after 1.5 h, an additional 3.5 equiv was added. The click-H reaction was finished within 3.5 h, and the mixture was treated as described above to afford 50% (0.1 μmol) of pure **10**.

Bis-conjugates **9** and **10** were thereby obtained by a sequential procedure demonstrating that no purification is

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(35) When the formation of oxime ether was performed at pH 7, the reaction occurred very slowly leading to some degradation of the 3′-aldehyde ODN to the corresponding 3′-monophosphate ODN.

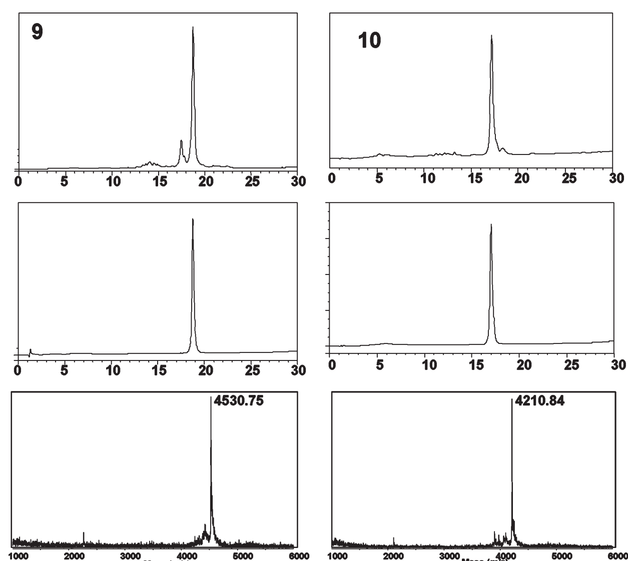

FIGURE 3. RP-HPLC (260 nm) profiles of crude and pure and MALDI-Tof MS spectra (top from bottom) of bis-conjugate **9** (left) and bis-conjugate **10** (right) obtained via the sequential "one pot" procedure.

necessary between each click reaction if click-O is applied prior to click-H.

In conclusion, we have demonstrated that a combination of click-O and click-H can be performed sequentially in oligonucleotide bis-conjugation field. According to this protocol, the resulting bis-conjugates were obtained with high efficiency and a fair 50% overall yield.

Experimental Section

Synthesis of 5′-Pent-4-yn-1-yl-3′-glyceryl ODN 7. The 11-mer CGCACACACGC was synthesized starting from commercially available glyceryl solid support (1 μmol) according to the standard phosphoramidite method[36] on a DNA synthesizer (ABI 394). The elongation cycle was as follows: (1) 2.5% dichloroacetic acid in CH_2Cl_2 for 35 s; (2) phosphoramidite derivative (0.09 M) + benzylthiotetrazole (0.3 M) in CH_3CN for 20 s; (3) Ac_2O, *N*-Me imidazole, 2,6-lutidine for 15 s; (4) 0.1 M I_2 THF/H_2O/pyridine for 15 s. The pent-4-yn-1-yl phosphoramidite[20] was then coupled (0.15 M for 30 s). The deprotection and release were performed with concentrated ammonia overnight at 30 °C to afford after evaporation **7** (0.7 μmol).

Synthesis of 5′-Pent-4-yn-1-yl-3′-aldehyde ODN 8. A solution of sodium *m*-periodate (200 mM, 163 μL, 33 μmol) in water was added to **7** (0.2 μmol, 200 μL) in water. After 10 min, the mixture was desalted on NAP 10 to afford **8** (0.15 μmol, 17 $OD_{260\ nm}$, 75%).

Sequential Click-O and Click-H Reactions with Aminoxy-c[RGD] and Dansyl Propyl Azide To Afford 9. 5′-Pent-4-yn-1-yl-3′-aldehyde-ODN **8** (0.15 μmol) in 120 μL of water was dissolved in 0.4 M $AcO^-NH_4^+$ buffer, pH = 4.5 (250 μL). A solution of aminoxy-c[RGD], TFA salt 30 mM in water was then added, starting with 3 molar equiv followed by additions at 2 h (3 molar equiv), 20 h (3 molar equiv), and 22 h (1 molar equiv). The reaction was monitored by HPLC and MS. After completion (24 h), the pH was increased until pH ~7−8 by addition of one drop of ammonia. Then, the main excess of ammonia was removed for 10 min in vacuo, and the resulting solution (400 μL) was used directly for the next step. A solution of 5-(dimethylamino)-*N*-(2-propyl azide)-1-naphthalenesulfonamide (70 mM, 5.5 molar equiv, 12 μL) in methanol and a freshly prepared solution of $CuSO_4$ (80 mM, 5 molar equiv, 11 μL) and sodium ascorbate (200 mM, 25 molar equiv, 20 μL) in water and methanol (50 μL) were added. The coupling was monitored by HPLC. After 1 h, the mixture was desalted on NAP 10 to afford the bis-oligonucleotide conjugate **9** (12 $OD_{260\ nm}$, 0.1 μmol, 66%).

Sequential Click-O and Click-H Reactions with Aminooxy Lactose and Dansyl Propyl Azide To Afford 10. 5′-Pent-4-yn-1-yl-3′-aldehyde-ODN **8** (0.12 μmol) in 50 μL of water was dissolved in 0.4 M $AcO^-NH_4^+$ buffer, pH = 4.5 (250 μL). Then, a solution of aminoxy lactose 70 mM in water was added starting with 6 molar equiv followed by another addition of 1.5 molar equiv at 2 h. The reaction was monitored by HPLC and MS. After completion (3 h), the pH was increased by addition of one drop of ammonia. The main excess of ammonia was then removed for 10 min in vacuo, and the resulting solution (300 μL) was used directly in the next step. A solution of 5-(dimethylamino)-*N*-(2-propyl azide)-1-naphthalenesulfonamide (70 mM, 9 molar equiv, 15 μL) in MeOH and a freshly prepared solution of $CuSO_4$ (80 mM, 10 molar equiv, 18 μL) and sodium ascorbate (200 mM, 50 molar equiv, 32 μL) in water and methanol (100 μL) were added. The coupling was monitored by HPLC. After 3.5 h, the mixture was desalted on NAP 10 to afford the bis-oligonucleotide conjugate **10** (12 $OD_{260\ nm}$, 0.1 μmol, 83%).

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Supporting Information Available: HPLC profiles and MALDI-TOF spectra of crude compounds from **7** to **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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