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5′-Bis-conjugation of Oligonucleotides by Amidative Oxidation and Click Chemistry

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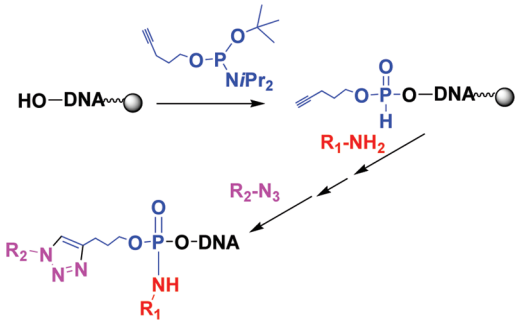

A pent-4-ynyl *tert*-butyl *N,N*-diisopropyl phosphoramidite was coupled at the 5′-end of oligonucleotides to give a phosphite triester linkage, which forms an *H*-phosphonate diester linkage during treatment with dichloroacetic acid. Then an amidative oxidation with CCl_4 in the presence of an amine and a 1,3-dipolar cycloaddition with an azide under copper(I) catalysis afforded the bis-conjugated oligonucleotides with high efficiency. The introduction of a bromoalkyl group as a precursor of azidoalkyl by amidative oxidation allowed the performance of two selective 1,3-dipolar cycloadditions.

Oligonucleotide conjugates are widely used for various applications in biology, biotechnology, and medicine.[1,2] This interest leads to the development of many strategies using specific reagents to introduce reporters into an oligonucleotide.[3] Since 2002, the well-known copper(I)-catalyzed[4,5] Huisgen 1,3-cycloaddition of azides and alkynes (CuAAC) has emerged as a reaction of choice for oligonucleotide conjugation with various reporters[6] such as peptides,[7,8] proteins,[9,10] carbohydrates,[6,8,11−20] or fluorescent dyes.[6,21] Multiconjugation of oligonucleotides by CuAAC has also been performed using sequential conjugation/modification or deprotection reactions.[15,18]

The oxime tethering procedure is another efficient click strategy for oligonucleotide conjugation,[22,23] and we recently reported the combination of CuAAC and formation of oxime ether to introduce two different molecules into an oligonucleotide at both ends.[8] New Cu-free click reactions have recently emerged with nitrile oxide-norbornene,[24] nitrile oxide-alkyne,[25−27] and nitrile oxide-styrene[28] 1,3-dipolar cycloadditions. Alternatively, amidative oxidation[29] is an easy way to introduce modifications into an oligonucleotide, forming a phosphoramidate linkage. Indeed introduction of a *H*-phosphonate diester linkage could be automated starting from rapidly accessible *H*-phosphonate monoester building blocks according to *H*-phosphonate chemistry.[30,31] Introduction of modifications by formation of a phosphoramidate linkage has been reported to bring nuclease resistance

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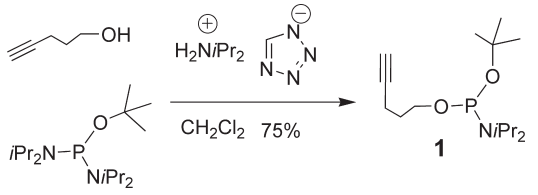
SCHEME 1. Synthesis of Pent-4-ynyl *tert*-Butyl *N,N*-Diisopropyl Phosphoramidite 1



to oligonucleotides[32] and to improve their cellular uptake.[33] Furthermore, the introduction of more than one label is sometimes required, such as oligonucleotides with a fluorescent dye and carbohydrate for cell targeting and fluorescent microscopy or with two carbohydrates for multivalency.

Herein, we report a new strategy combining amidative oxidation and CuAAC to introduce two labels at the 5′-end of oligonucleotides in close proximity. To this end, we designed a special phosphoramidite reagent, namely, pent-4-ynyl *tert*-butyl *N,N*-diisopropyl phosphoramidite, for bis-conjugation at the 5′-end of oligonucleotides. This reagent was introduced by phosphoramidite chemistry without oxidation and led to the formation of a *H*-phosphonate diester linkage bearing the alkyne function during the detritylation step according to an Arbusov-like reaction.[34,35] Thus, the use of such *tert*-butyl phosphoramidite building block avoids mixing phosphoramidite and *H*-phosphonate chemistries on the same synthesizer.

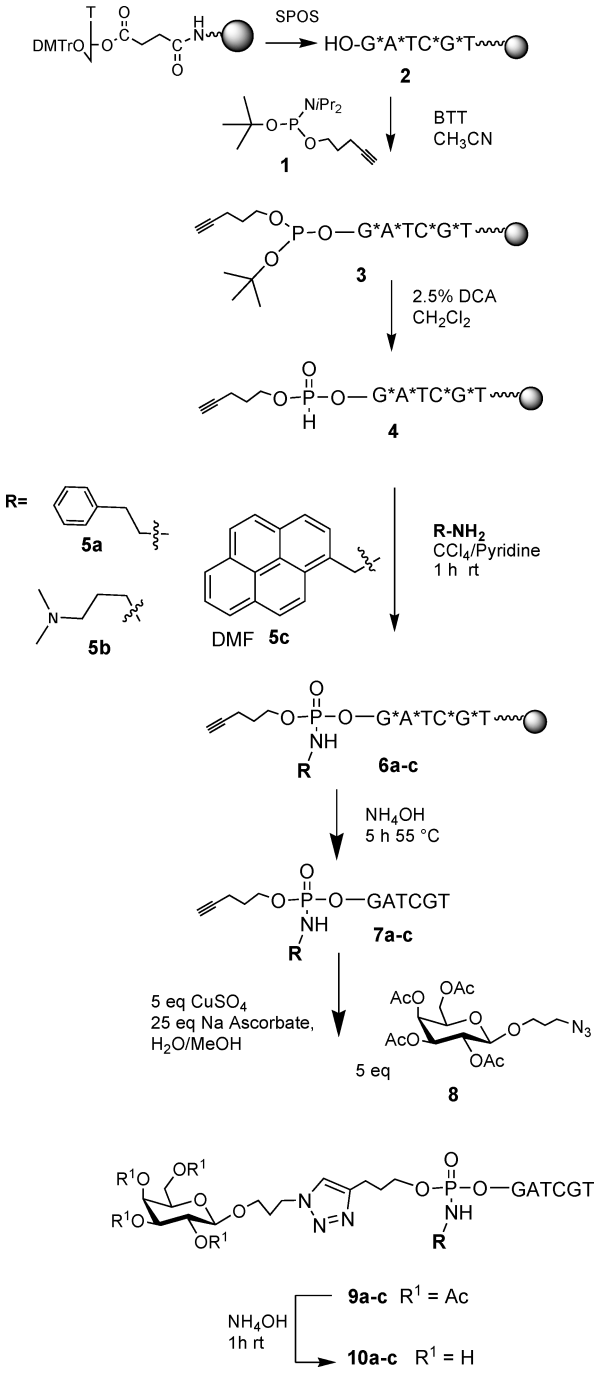
A first conjugation was performed by amidative oxidation of the *H*-phosphonate diester linkage of the resulting solid-supported oligonucleotide, while the second conjugation was done by CuAAC in solution (Scheme 2).

The pent-4-ynyl *tert*-butyl *N,N*-diisopropyl phosphoramidite **1** was rapidly and easily synthesized starting from commercially available pent-4-yn-1-ol and *tert*-butyl tetraisopropylphosphorodiamidite in dry dichloromethane in the presence of diisopropylammonium tetrazolide as activating agent with a fair yield of 75% (Scheme 1).

Hexanucleotide **2** was synthesized on solid support by phosphoramidite chemistry[36] on a DNA synthesizer. At the end of the sequence elongation, phosphoramidite **1** was coupled using an elongation cycle with an extended coupling time (120 s) and without performing the standard oxidizing step. During the acidic treatment used for the conventional detritylation step, the oligonucleotide with a *tert*-butyl phosphite triester linkage (**3**) led spontaneously to the formation of a *H*-phosphonate diester linkage affording **4**[34,35] (Scheme 2).

The amidative oxidation was performed manually on solid support using CCl_4 in the presence of three different amines (phenethylamine **5a**, 3-dimethylaminopropylamine **5b**, or pyren-1-ylmethylamine **5c**)[37] in pyridine for 1 h to form **6a**−**c** (Scheme 2).

SCHEME 2. Synthesis of 5′-Bis-conjugated Oligonucleotides 10a−c[a]



[a]SPOS: solid phase oligonucleotide synthesis. Conditions: (1) 2.5% dichloroacetic acid (DCA) CH_2Cl_2; (2) phosphoramidite derivative + benzylthiotetrazole (BTT); (3) Ac_2O, *N*-Me imidazole, 2,6-lutidine; (4) 0.1 M I_2 THF/H_2O/pyridine. Asterisk (*) represents protecting group on nucleobase (benzoyl or isobutyryl).

The columns containing **6a**−**c** were washed to remove reagents and treated with concentrated aqueous ammonia for deprotection and release, affording the monoconjugated oligonucleotides **7a**−**c** in solution. HPLC and MALDI-TOF MS analyses confirmed their expected structures (Figure 1 and Supporting Information).

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(37) Amines **5a** and **5b** were used at 1000 equiv to ensure the full reaction since they are inexpensive, while **5c** was used at 260 equiv and DMF was added to dissolve it. The same efficiency was observed for all of them.

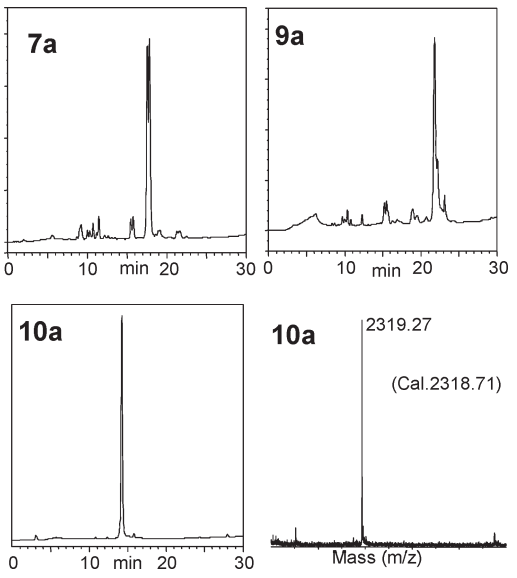

FIGURE 1. RP-HPLC (260 nm) profiles of crude **7a** and **9a** and pure **10a** and MALDI-TOF MS spectra of pure **10a**.

Each monoconjugated oligonucleotide **7a**−**c** was engaged in a CuAAC reaction with 3-azidopropyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **8** (5 equiv)[16] performed in solution under copper(I) catalysis generated in situ by reduction of $CuSO_4$ by sodium ascorbate. The reaction was finished within 2 h at room temperature, and the mixture was desalted by size-exclusion chromatography. The bis-conjugated oligonucleotides **9a**−**c** were purified by C_{18} HPLC. The acetyl groups on the galactose moiety were kept to facilitate the purification. The last ammonia treatment for 1 h at room temperature gave the fully deprotected 5′-bis-conjugated oligonucleotides **10a**−**c** with high purity without additional purification (see Supporting Information). For **10a** (Figure 1) and **10b** only one peak was observed by HPLC, while for more bulky conjugate **10c** two peaks, corresponding to Rp and Sp diastereoisomers of the phosphoramidate, were observed. MALDI-TOF MS confirmed the nature of the bis-conjugated oligonucleotides **10a**−**c** (see Supporting Information).

In another example, we introduced by amidative oxidation a bromoalkyl chain as a precursor of an azide function,[16,38,39] allowing two sequential CuAAC reactions to conjugate a galactose and a mannose residue at the 5′-end of an oligonucleotide within close vicinity. Starting from **4**, the amidative oxidation was performed with 3-bromopropylamine, affording the solid-supported oligonucleotide **12**, exhibiting bromopropyl and pentynyl groups. A first CuAAC reaction occurred with galactose azide derivative **8** on solid support with microwave assistance (MW)[11] to speed up the reaction, giving after 30 min at 60 °C the galactose-conjugated oligonucleotide **13**. It was converted into the azide derivative oligonucleotide **14** by treatment with *N*,*N*,*N*′*N*′-tetramethylguanidine azide (TMG N_3) and NaI in DMF for 40 min at 40 °C.

One half of **14** was directly engaged for a second CuAAC reaction on solid support with 1-*O*-propargyl-2,3,4,6-*O*-tetra-acetyl-α-D-mannopyranoside **15** under Cu(I) catalysis and

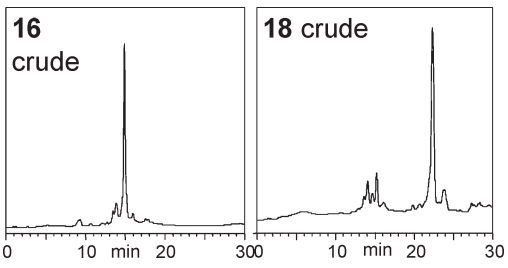

FIGURE 2. RP-HPLC (260 nm) profiles of crude **16** obtained by solid phase protocol and crude **18**.

MW for 30 min at 60 °C. A last ammonia treatment gave the 5′-(galactose-mannose)-conjugated oligonucleotide **16**, which was purified by HPLC. The second half of **14** was deprotected by ammonia, and the corresponding 5′-galactose-azidopropyl oligonucleotide **17** was conjugated in solution with the mannose propargyl derivative **15** under Cu(I) catalysis for 90 min at room temperature to afford **18**, which was purified by reverse phase HPLC and finally treated with ammonia to give the 5′-(galactose-mannose)-conjugated oligonucleotide **16**. Both on solid support and in solution protocols afforded **16** with high efficiency. However, fully solid phase synthesis is more straightforward since less manipulation and time are required. Furthermore, HPLC profiles of crude **16** obtained from solid phase synthesis and of crude **18** show that fully solid phase synthesis yielded less impurities (Figure 2).

In conclusion, we have demonstrated that a combination of amidative oxidation and 1,3-dipolar cycloaddition can be performed sequentially on an oligonucleotide for bis-conjugation. All the reactions could be performed on solid support, allowing straightforward and easy workups. Using the new phosphoramidite **1**, we obtained 5′-bis-conjugation of an oligonucleotide with close proximity of both labels. Alternatively, this strategy could be applied with an alkyne and a *H*-phosphonate diester linkage at different positions in the sequence of an oligonucleotide (e.g., 5′- and 3′-end). To this end, *tert*-butyl hydroperoxide must be used as oxidizer of phosphite triesters to keep the *H*-phosphonate diesters unchanged.[40] Furthermore, several alkynes and *H*-phosphonate diester linkages could be introduced into oligonucleotides, allowing the synthesis of multilabeled oligonucleotides with different reporters.

Experimental Section

Synthesis of Pent-4-ynyl *tert*-Butyl *N*,*N*-Diisopropyl Phosphoramidite 1. To a solution of pent-4-yn-1-ol (168 mg 2.0 mmol) and *tert*-butyl tetraisopropyl phosphorodiamidite (731 mg 2.4 mmol) in anhydrous dichloromethane (10 mL) was added diisopropylammonium tetrazolide (172 mg, 1.0 mmol). The resulting mixture was stirred overnight at room temperature, diluted with ethyl acetate (50 mL), and washed with brine (2 × 100 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by flash column chromatography (silica gel; isocratic cyclohexane containing 5% Et_3N) to afford the resulting phosphoramidite **1** (430 mg, 75% yield) as a colorless oil. TLC (cyclohexane/Et_3N, 9:1; v/v) R_f: 0.49. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 136.77 ppm. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.15−1.19 (2d, 12H, *J* 6.8 Hz); 1.35 (s, 9H); 1.79−1.84 (m, 2H);

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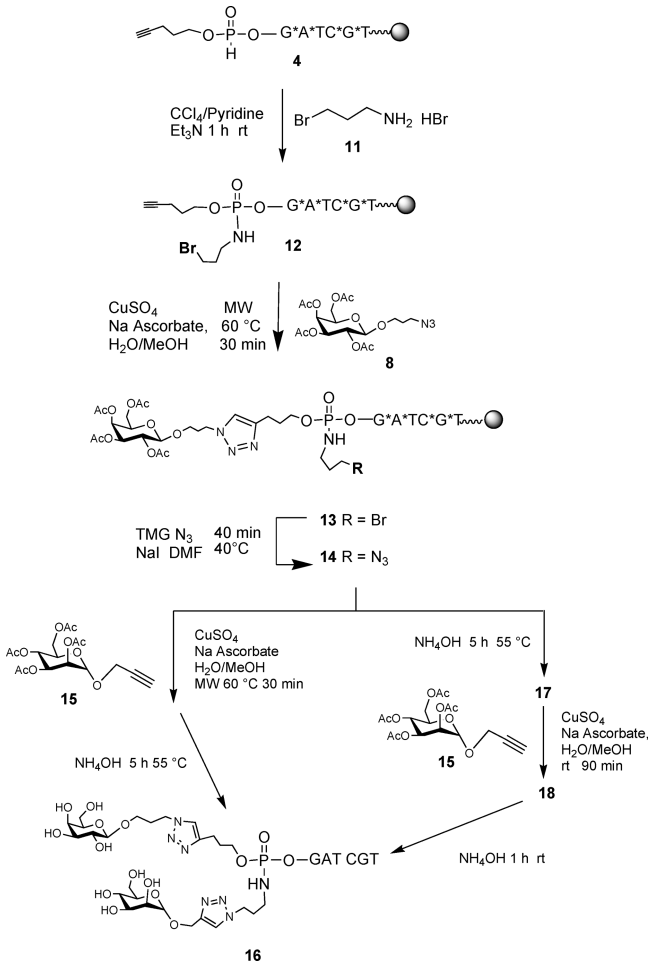
SCHEME 3. Synthesis of 5′-(Galactose-mannose)-Conjugated Oligonucleotide 16



1.93 (t, 1H, *J* 2.7 Hz); 2.27−2.33 (m, 2H); 3.56−3.66 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 15.2, 24.1, 24.2, 24.5, 24.6, 30.2, 30.3, 30.8, 30.9, 42.9, 43.0, 61.0, 61.2, 68.2, 74.6, 74.8, 76.6, 77.0, 77.4, 84.2. HRMS ESI+: *m*/*z* calcd for $C_{15}H_{31}NO_2P$ $(M + H)^+$ 288.2092, found 288.2095.

5′-(Pent-4-ynyl-*H*-phosphonate diester) Solid-Supported Hexamer 4. The 6-mer GATCGT was synthesized starting from commercially available solid support (1 μmol) according to a standard phosphoramidite method[36] on a DNA synthesizer (ABI 394). The elongation cycle was (1) 2.5% dichloroacetic acid, 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; and (4) 0.1 M I_2 THF/H_2O/pyridine for 15 s. Then, the phosphoramidite **1** (0.2 M) was coupled for 120 s with BTT and directly treated with 2.5% dichloroacetic acid, CH_2Cl_2, for 35 s, affording **4**.

Procedure for Amidative Oxidation Affording 6a−c and 11. Amines **5a**,**b** (63 μL, 500 μmol) in CCl_4/pyridine (940 μL, 1:1, v/v) were applied to a column containing 0.5 μmol of solid-supported oligonucleotide **4** for 1 h at room temperature with frequent shaking and finally washed with pyridine, water, methanol, and acetonitrile (1 mL each).

Hydrochloride **5c** (70 mg, 260 μmol) and Et_3N (80 μL) in DMF/pyridine (3.0 mL, 1:1, v/v) were centrifuged, and the supernatant was added to CCl_4 (1.0 mL). The resulting oxidizing solution was applied to 1 μmol of **4** for 1 h as described above.

Hydrobromide **11** (218 mg, 1.0 mmol) and Et_3N (139 μL) in CCl_4/pyridine (940 μL, 1:1, v/v) were centrifuged, and the supernatant was applied to 1 μmol of **4** for 1 h as described above.

Procedure for Azidation. The solid-supported bromopropyl derivative **13** (1 μmol) was treated with a solution of TMG N_3 (160 mg) and NaI (8 mg) in DMF (1.0 mL) for 40 min at 40 °C. Then, the CPG beads were washed with DMF (2 × 1 mL) and CH_3CN (3 × 1 mL) and dried under vacuum.

Procedure for 1,3-Dipolar Cycloadditions. Solutions of $CuSO_4$ (80 mM) in water, sodium ascorbate (200 mM) in freshly degassed water, and sugars (**8** or **15**) (100 mM) in methanol were prepared.

CuAAC in Solution. To **7a**−**c** (500 nmol) and **8** (5 equiv, 25 μL) or to **17** (500 nmol) and **15** (5 equiv, 25 μL) in 90 μL of water/methanol (1:1 v/v) was added a premixed solution of $CuSO_4$ (2 equiv, 12 μL) and sodium ascorbate (20 equiv, 50 μL). After the reaction the salts were removed on Nap10.

CuAAC on Solid Support. To the solid-supported oligonucleotide **12** (1 μmol) were added carbohydrate derivative (**8**, 50 μL), water/methanol (100 μL 1:1, v/v), and CuSO4 (25 μL and Na ascorbate 100 μL). The resulting solution was placed in a sealed tube and introduced into a microwave synthesizer (Initiator from Biotage) set at 60 °C, 100 W for 30 min under magnetic stirring. Finally, the beads were filtered off and washed with water and methanol. The same protocol was applied for **14** on a 0.5 μmol scale using carbohydrate derivative **15**.

Deprotection of Oligonucleotides. This was performed by treatment with concentrated ammonia for 5 h at 55 °C, while hydrolysis of acetyl groups was carried out for 1 h at rt.

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Supporting Information Available: HPLC profiles and MALDI-TOF spectra of all compounds, fluorescence spectrum of **7c**, and ^{1}H, ^{13}C, and ^{31}P NMR data of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.