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Microwave Assisted "Click" Chemistry for the Synthesis of Multiple Labeled-Carbohydrate Oligonucleotides on Solid Support†

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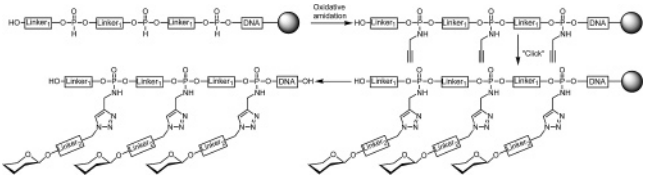
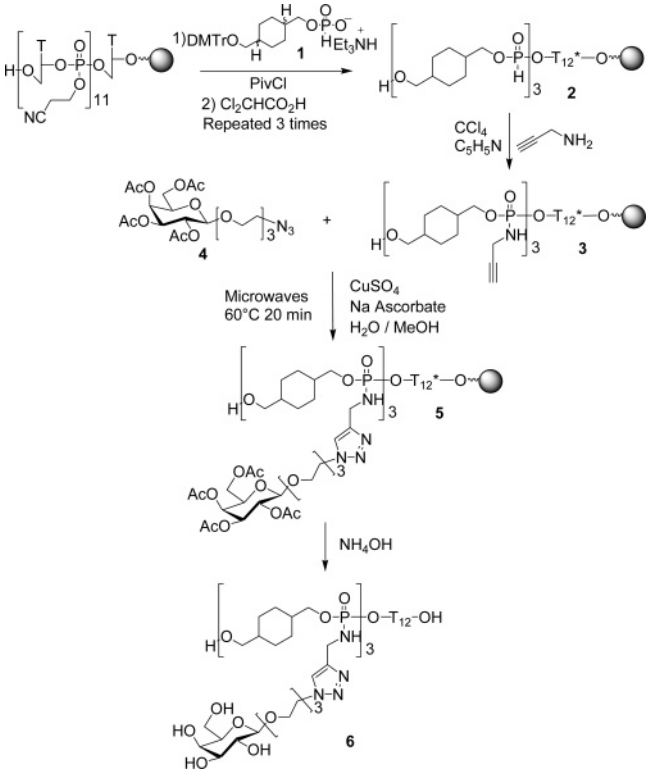


A versatile approach has been developed for the multiple labeling of oligonucleotides. First, three linkers as a *H*-phosphonate monoester derivative were condensed on a solid-supported T_{12} to introduce *H*-phosphonate diester linkages which were oxidized in the presence of propargylamine. Second, three galactosyl azide derivatives were conjugated to the solid-supported three-alkyne-modified T_{12} by a 1,3-cycloaddition so-called "click chemistry" in the presence of Cu(I) assisted by microwaves.

Oligonucleotides are important molecular tools for genomic research and biotechnology.[1] Most applications require labeling with dyes or other biomolecules such as peptides[2] or carbohydrates.[3] Oligonucleotides are typically synthesized on solid support using phosphoramidite chemistry.[4] Their conjugation with carbohydrates has been performed on one hand on solid support using either solid-supported carbohydrates[5,6] or carbohydrate phosphoramidites[5−13] and on the other hand in solution using reactive carbohydrate derivatives.[14−16] Nevertheless, these strategies require multistep synthesis and are time-consuming. In this paper, we report a general, simple and robust strategy for anchoring one or several carbohydrate derivative(s) to a solid-supported oligonucleotide (Scheme 1). The 1,3-dipolar cycloaddition between alkynes and azides, so-called "click" chemistry,[17,18] was applied to attach carbohydrate residues to

SCHEME 1. Solid-Supported Synthesis of a T_{12} Trispropargyl Phosphoramidate Oligonucleotide 3 and Its Conjugation to Galactosyl Azide 4 by Microwave-Assisted Click Chemistry



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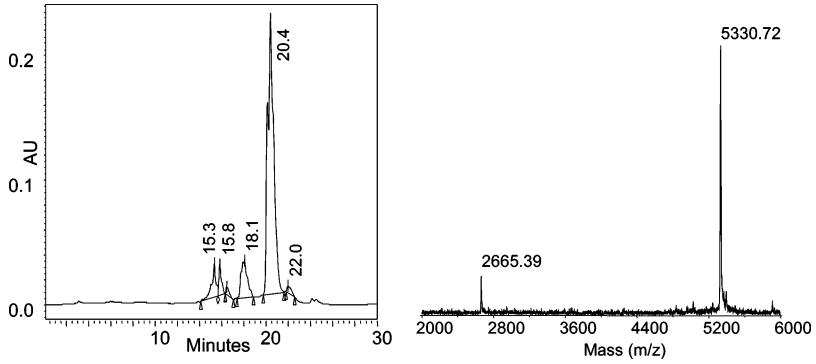

FIGURE 1. HPLC and MALDI-TOF MS of crude trigalactosylated T_{12} **6**: obtained by MW activation 100 W, 60 °C, 20 min on solid support after deprotection by ammonia.

the oligonucleotide backbone. We demonstrated that microwave activation significantly improved the reaction kinetic compared to standard conditions. Solid-supported reactions provided better results in terms of purity when compared to similar solution-phase conditions where some phosphoramidate hydrolysis was observed.

The Huisgen's 1,3-dipolar cycloaddition between alkyne and azide is very attractive since it is nearly quantitative, can be performed in water with an organic cosolvent, and multiple cycloadditions can be performed on multivalent scaffolds.[19,20] Furthermore, this reaction is orthogonal to most typical organic transformations and therefore highly chemoselective. Nevertheless, even though the 1,4-disubstituted triazoles are preferred to their 1,5-regioisomers, complete regioselectivity is sometimes difficult to achieve, in particular when high temperature and long reaction times are required. The regioselectivity of the cycloaddition reaction was not determined in this study since no analytical tool was available for that purpose. We can assume that the triazoles formed in the 1,3-dipolar cycloaddition are predominantly the 1,4-disubstituted regioisomers as already reported.[21,22] Both regioisomers would lead to an analogous glycoconjugate which should display similar biochemical and biophysical properties. Click chemistry has been successfully applied for fluorescent labeling of oligonucleotides in solution[23] and recently for the attachment of an oligonucleotide on a monolayer[24] and for DNA metallization.[25]

We first prepared a cyanoethyl-protected dodecathymidine (T_{12}^{*}) on solid support using well-established phosphoramidite chemistry.[4] Then three *H*-phosphonate diester linkages were introduced using *H*-phosphonate monoester **1** to yield the modified supported oligonucleotide **2** (Scheme 1). An amidative oxidation with CCl_4 in the presence of propargylamine afforded the alkyne-functionalized oligonucleotide **3** with three propargyl phosphoramidate linkages. An aliquot was treated with aqueous ammonia and analyzed by HPLC and MS to determine the efficiency of these synthetic steps (i.e., three *H*-phosphonate couplings and amidative oxidation). The first *H*-phosphonate coupling was not complete (86−94%), while the two subsequent couplings were higher yielding. The resulting mixture was composed of **3** (∼75%), unreacted T_{12} (∼14%), and intermediates with one (∼3%) and two propargyl groups (∼4%) as determined by MALDI-TOF MS analyses (see the Supporting Information).

TABLE 1. Microwave-Assisted 1,3-Dipolar Cycloaddition on Solid Support of Alkyne-Functionalized Oligonucleotide 3 (0.5 μmol) and Galactosyl Azide Derivative 4 (5 μmol, 3.3 molar equiv/Alkyne) with $CuSO_4$ (0.2 μmol) and Sodium Ascorbate (1 μmol) in MeOH/H_2O (200 μL, 1:1, v/v) under MW Activation (100 W)

entry	*T* (°C)	time (min)	conversion[a] (%)
1	100	20	100
2	75	20	100
3	60	20	100
4	60	15	84[b]
5	20[c]	420	73

[a] Conversion to the triply functionalized oligonucleotide **3**. [b] Contaminated with one unreacted alkyne residue. [c] Without microwave activation.

Oligonucleotide **3** was then used to optimize the 1,3-dipolar cycloaddition with azide derivative **4**. The reaction proceeds slowly at room temperature[18] and was therefore performed under microwave (MW) conditions to shorten reaction times.[21] Several reaction conditions under microwave activation were investigated with temperatures ranging from 60 to 100 °C and reaction times from 15 to 60 min (Table 1). The cycloaddition was performed between the trivalent alkyne oligonucleotide **3** and the monovalent azide **4** with 3.3 molar equiv of azide per alkyne residue in the presence of $CuSO_4$/sodium ascorbate[22] in water/methanol to yield the solid-supported protected trigalactosylated oligonucleotide **5**. Subsequent treatment of **5** with aqueous ammonia afforded the fully deprotected trigalactosylated T_{12} **6** in solution. The percentage of cycloaddition was determined by HPLC and MS analysis of the crude material (Figure 1). The main peak (t_R ∼20 min) corresponds to the expected trigalactosylated T_{12} **6**, the broad peak at ∼18 min corresponds to the digalactosylated T_{12} formed from the dialkyne T_{12}, the two peaks at ∼16 min correspond to the monogalactosylated T_{12} formed from the monoalkyne T_{12}, and the peak at ∼15 min is the T_{12}. The splitting of the HPLC peak is due to the presence

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of diastereoisomers since the phosphorus atom of the phosphoramidate linkage is chiral.

The results of cycloaddition are summarized in Table 1. Impurities present in the solid-supported starting material with only one and two alkyne residues leading to the mono- and bis-cycloadducts were not considered, but cycloaddition reactions proceeded with the same efficiency. It is worth pointing out that cycloaddition to the cyanoethyl protecting groups was not observed under these conditions.[26,27]

The first attempt was performed at 100 °C with 20 min MW activation (entry 1), and complete conversion of all three alkyne residues into the desired triazoles was observed. Decreasing the temperature to 75 and then 60 °C also gave a complete reaction within 20 min (entries 2 and 3). Nevertheless, conversion was not complete when the temperature was kept at 60 °C, and the time was reduced to 15 min where 16% of digalactosylated T_{12} with one remaining alkyne residue was detected by HPLC and MS (entry 4). Finally, the reaction did not reach completion (73%) even after 7 h without MW activation (entry 5).

Similarly, the cycloaddition reaction was performed in solution on the crude T_{12} phosphodiester with three alkyne phosphoramidate linkages and was complete either within 20 min at 75 °C under MW or 18 h without MW (see the Supporting Information). Nevertheless, a loss of 375.3 Da was observed by mass spectrometry corresponding to a phosphodiester linkage probably resulting from hydrolysis of one phosphoramidate P−N bond (13% under MW and 20% without MW). No decomposition of the solid-supported oligonucleotide **5** was observed when the reaction was performed even at 100 °C for 60 min under MW activation.

In conclusion, we observed a very efficient "click" coupling of alkyne-bearing oligonucleotide **3** with azide-functionalized galactoside **4** under MW activation at 60 °C for 20 min. The reaction could be performed on solid support or in solution under similar conditions. The main advantages of the solid-supported microwave assisted click chemistry are as follows: (1) introduction of several alkynes anywhere within the oligonucleotide backbone (i.e., at the 3′- or 5′-end or in the oligonucleotidic sequence);[28] (2) modulation of the distance between each alkyne using different diol-type linkers between each *H*-phosphonate diester function; (3) cycloadditions can be easily performed with an oligonucleotide on solid support; (4) use and recovery of excess azide derivatives; (5) conjugation of various azides to an oligonucleotide using this approach; (6) rapid and high-yielding multiple 1,3-dipolar cycloadditions and lack of hydrolysis of the phosphoramidate bonds.

We are currently studying the conjugation of various azide-labeled biomolecules to oligonucleotides containing the four natural nucleobases for potential biomedical, therapeutic, and diagnostic applications.

Experimental Section

Synthesis of Solid-Supported T_{12} (2). The solid-supported T_{12} was synthesized using a DNA synthesizer (ABI 381A) using standard phosphoramidite chemistry on a commercially available thymidine succinyl CPG solid support (500A). Then **1** (60 mM in C_5H_5N/CH_3CN, 1:1, v/v) was coupled using a *H*-phosphonate chemistry cycle with pivaloyl chloride as activator (200 mM in C_5H_5N/CH_3CN, 1:1, v/v). Each solution was passed through the column alternatively for 5 s, 12 times (60 molar excess) for the first coupling and 6 times (30 molar excess) for the next couplings. The detritylation step was performed with 3% dichloroacetic acid in CH_2Cl_2 for 60 s (3.0 mL/min flow).

General Procedure for Amidative Oxidation. The solid-supported oligonucleotide (1 μmol) was treated, back and forth using two syringes, with a solution of 10% propargylamine (100 μL) in CCl_4/C_5H_5N (900 μL, 1:1, v/v) for 30 min. The CPG beads were washed with C_5H_5N (1 mL) and MeCN (3 × 2 mL) and then dried by flushing with nitrogen.

General Procedure for Cu(I)-Catalyzed 1,3-Dipolar Cycloaddition. To a solid-supported oligonucleotide (0.5 μmol) were added protected galactosyl azide **4** (10 equiv, 5 μmol, 100 μL of a 50 mM solution in MeOH), $CuSO_4$ (0.4 equiv, 0.2 μmol, 5 μL of a 40 mM solution in H_2O), freshly prepared sodium ascorbate (2 equiv, 1 μmol, 20 μL of a 50 mM solution in H_2O), and water (75 μL). The resulting preparation in a sealed tube was treated with a microwave synthesizer Initiator from Biotage set at 100 W with a 30 s premixing time. The temperature was monitored with an internal infrared probe. The solution was removed, and the CPG beads were washed with H_2O/MeOH (2 mL, 1:1, v/v) and MeOH (1 mL) and then dried.

General Procedure for Deprotection. The beads were placed into a sealed vial and treated with concentrated aqueous ammonia (1 mL) for 4 h at room temperature. The beads were filtered off, and the solution was evaporated. The residue was dissolved in water for subsequent analyses.

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Supporting Information Available: Detailed experimental procedures for **1** and **4** with NMR characterization data, HPLC, and MALDI-TOF spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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