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Synthesis of Monoconjugated and Multiply Conjugated Oligonucleotides by "Click Thiol" Thiol-Michael-Type Additions and by Combination with CuAAC "Click Huisgen"

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Monoconjugated and multiply conjugated oligonucleotides were efficiently synthesized by starting from monothiohexyl- or tetra-thiohexyl-oligonucleotides and treatment with acrylamide derivatives (carbohydrates, ferrocene, biotin, fluorescent dyes, deoxycholic acid). The thiol Michael-type additions (TMTAs) occurred during the deprotection and release of the oligonucleotides from the solid support, so no additional time for conjugation was needed. Sequential combination either of Copper-catalyzed Azide–Alkyne Cycloaddition (CuAAC) on solid support and then TMTA or of TMTA followed by CuAAC in solution allowed the synthesis of several oligonucleotides decorated with two different biomolecules.

Introduction

Hydrothiolation of alkenes occurs either by radical or by nucleophilic mechanisms, corresponding either to thiol radical additions to electron-rich enes (termed thiol-ene reactions) or to thiol Michael-type additions (TMTAs) to electron-deficient enes, respectively. Both reactions are also termed "click thiol" reactions. Thiol-ene reactions have been applied for the synthesis of multiple structures, especially for polymer synthesis,[1–4] but also for the preparation of glycoclusters.[5] The reactions usually proceed between thiols and enes in the presence of radical initiators under UV irradiation conditions. Although the reactions work well, the conditions are not very convenient because specific equipment is required. In contrast, the use of activated enes bearing electron-withdrawing substituents (such as ester, amide or cyano) leads to TMTA under basic catalysis conditions without the need for an activator or special equipment.[3]

With regard to oligonucleotide applications, the literature shows this strategy to be undeveloped, because reactions under UV irradiation conditions can lead to photodamage with the formation of a large variety of photoproducts, more specifically dimeric pyrimidine units.[6,7] TMTA is therefore used, but is mostly reserved for reactions between thiol-modified oligonucleotides and maleimide derivatives[8–10] or more recently between maleimide oligonucleotides and thiol derivatives.[11] In contrast, these reactions have not been widely applied to conjugation of oligonucleotides with acrylamide derivatives.[12,13] However, acrylamide derivatives are easily and rapidly obtained from virtually any amine or carboxylic acid derivative under mild conditions. A key advantage of acrylamide derivatives over maleimide ones is their better stability in basic media.

Results and Discussion

Here we report the application of TMTA to monoconjugation of a 5′-thiol oligonucleotide and different acrylamide derivatives (i.e., phenyl, mannose, ferrocene, dansyl, biotin or deoxycholic acid) and also for multiple conjugation of a tetrathiol-based oligonucleotide and an acrylamide mannose derivative to afford a glycocluster. Finally, the orthogonality of TMTA with copper-catalyzed azide–alkyne cycloaddition (CuAAC)[14,15] in application to oligonucleotides was studied. It was demonstrated that the two reactions could not be performed together, due to reaction between thiol and copper, but could be performed sequentially to allow the preparation of bis-conjugated oligonucleotides (mannose-galactose, biotin-galactose, biotin-mannose or dansyl-mannose).

The acrylamide derivatives **4a**–**f** (Scheme 1) were synthesized in a few steps with yields between 34 to 90%. Phenethylamine was treated with acryloyl chloride in the presence of triethylamine to afford phenethylacrylamide (**4a**). The 2,3,4,6-tetraacetyl-mannosyl-propyl-acrylamide **4b** was prepared from 3-azidopropyl 2,3,4,6-tetraacetylmannoside,[16] which was firstly reduced to the amine by treatment

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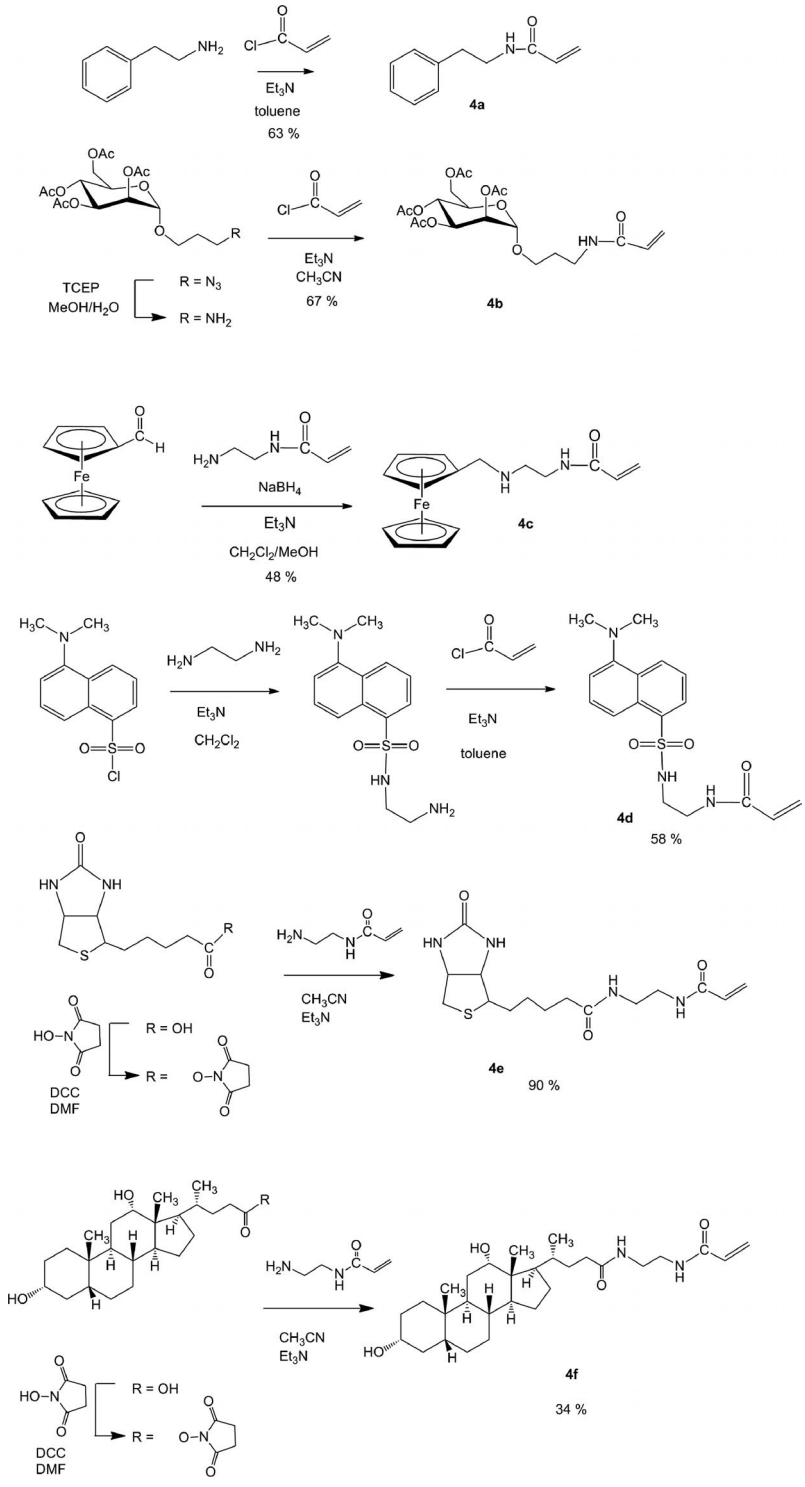
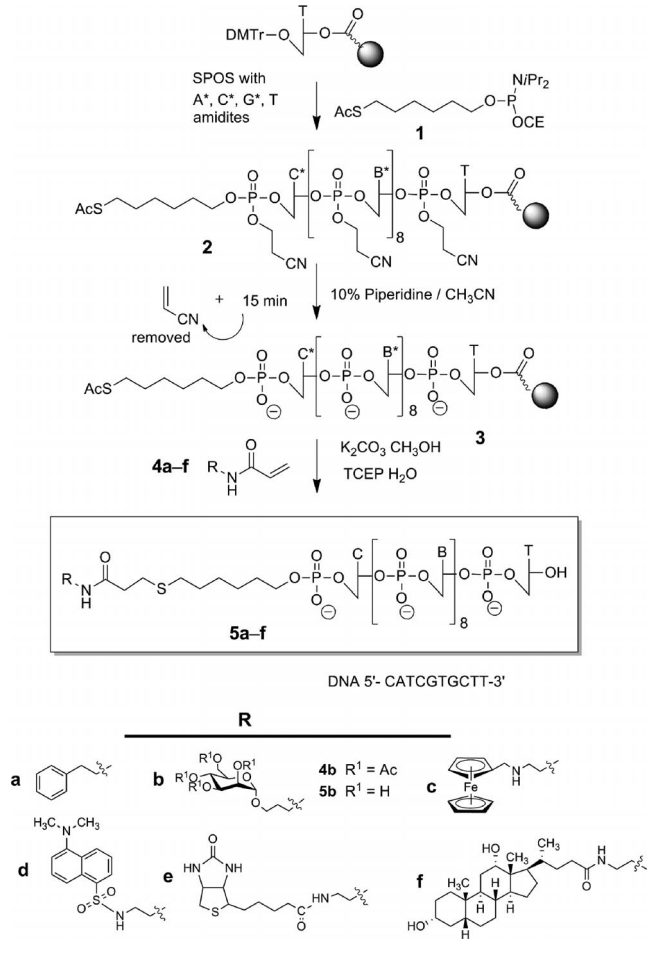
with tris(2-carboxyethyl)phosphane (TCEP) and secondly, after workup, further transformed by direct treatment of the crude product with acryloyl chloride. The reaction between ferrocenaldehyde and *N*-acryloyl-ethylenediamine[17] gave the Schiff base, which was reduced in situ with sodium borohydride to afford the ferrocene acrylamide derivative **4c**. Dansyl chloride was treated with 8 equiv. of ethylenediamine to form the corresponding monosulfonamide ethylamine compound. After workup, the crude product was treated with acryloyl chloride to give the dansylacrylamide derivative **4d**. The biotin acrylamide derivative **4e** was prepared as previously reported[17] by starting from biotin, which was converted into its *N*-hydroxysuccinimide derivative. Treatment with *N*-acryloyl-ethylenediamine then gave **4e**. Finally, the same protocol was applied to deoxycholic acid, leading to deoxycholic acid acrylamide derivative **4f**.



Scheme 1. Synthesis of acrylamide derivatives **4a**–**f**.

The fully protected solid-supported 5′-*S*-acetylthiohexyl-decanucleotide **2** (Scheme 2) was straightforwardly synthesized from commercially available building blocks (nucleosides and *S*-acetylthiohexyl phosphoramidites) by standard phosphoramidite chemistry[18]. After oligonucleotide elongation, the cyanoethyl protecting groups of the phosphates are removed by β-elimination during the ammonia treatment, leading to the formation of acrylonitrile. Acrylonitrile reacts rapidly with thiols, so it is essential to remove it before the deprotection of the thiol function. For this purpose, we applied a two-step deprotection procedure. Firstly, the solid-supported decamer was treated for 15 min with 10% piperidine/acetonitrile solution, allowing the β-elimination of cyanoethyl groups without the hydrolysis of the acetyl protecting group of the thiol. Secondly, after washing with acetonitrile to remove acrylonitrile, the solid-supported phosphodiester decamer containing the S-acetylthiohexyl group was treated with ammonia to afford the 5′-thiohexyl decamer. After concentration, HPLC and mass spectrometry analyses showed it to exist mainly in its dimeric form, due to rapid oxidation of the thiol function into the disulfide by air. Treatment with tris-(2-carboxylethyl)-phosphane (TCEP) gave the expected 5′-thiol decamer with good purity.



Scheme 2. Synthesis of 5′-conjugate oligonucleotides **5a**–**f**. SPOS: solid-phase oligonucleotide synthesis; 1) phosphoramidite derivative + benzylthiotetrazole; 2) phenoxyacetyl anhydride, *N*-Me-imidazole and 2,6-lutidine; 3) 0.1 M I_2, THF/H_2O/pyridine; 4) 2.5% dichloroacetic acid, CH_2Cl_2. B* asterisk represents A or C with a benzoyl protecting group and G with the isopropyl-phenoxyacetyl protecting group, and B represents A, C, G or T.

Our first attempted conjugation was performed with the solid-supported thiohexyl phosphodiester decamer and *N*-phenethylacrylamide (15 equiv.) in methanol and aqueous ammonia (1:4, v/v) in the presence of TCEP at 55 °C for 4 h and showed full conversion of the starting compound into the expected conjugate. We envisioned that because both deprotection of the oligonucleotide and TMTA occur in ammonia it should be possible to proceed by a deprotection/reaction cascade by adding all of the reagents together (i.e., *N*-phenethylacrylamide, TCEP and ammonia) to the solid-supported thiohexyl phosphodiester decamer. By this strategy, the expected conjugate was obtained easily with the same efficiency. It is noteworthy that the presence of TCEP was necessary, because oxidation to the disulfide was more rapid here than TMTA.

When the reaction was performed with dansylacrylamide derivative **4d** we observed some instability in ammonia, leading to some degradation of **4d**. This result prompted us to look for a milder basic treatment that would allow deprotection and activation without degradation of the acrylamide derivatives.

We therefore used a methanolic solution of potassium carbonate, which is known to deprotect oligonucleotides bearing easily labile protecting groups on their nucleobases.[19] To test this approach, we synthesized the 5′-*S*-acetyl-thiohexyl decamer **2** (Scheme 2) with benzoyl protecting groups for A and C and isopropylphenoxyacetyl groups for G. Decamer **2** was treated with 10% piperidine solution in acetonitrile to remove the cyanoethyl groups and washed to afford **3**.[20] Compound **3** was engaged in TMTA with *N*-phenethylacrylamide (15 equiv.) either in the presence of TCEP (8 equiv.) in concentrated aqueous ammonia (1.0 mL) or in methanolic K_2CO_3 solution (50 mM, 0.5 mL) at 55 °C for 4 h. After concentration, HPLC analyses showed the formation of the expected conjugate with a slightly better purity for the reaction carried out with K_2CO_3 (80% vs. 70%). For further conjugation we therefore used a methanolic K_2CO_3 solution.

The reaction was attempted with only 4 equiv. of acrylamide derivative and 8 equiv. of TCEP. After 3 h at 55 °C, 57% conversion had been achieved. After one night, only a 63% yield of conjugate had been produced and we observed the presence of some dimerisation (ca. 10%) through disulfide bond formation. Addition of further TCEP (4 equiv.) and acrylamide derivative (4 equiv.) allowed full conversion. Thus, to obtain rapid and full conversion of the thiol oligonucleotide into its conjugate it is more efficient to use 10 to 15 equiv. of acrylamide derivative.

To extend the scope of this reaction, we applied it to conjugations of oligonucleotides with different acrylamide derivatives bearing mannose (compound **4b**),[21] ferrocene (compound **4c**), dansyl (compound **4d**), biotin (compound **4e**) or deoxycholic acid (compound **4f**) moieties (Scheme 2). To allow fast and complete reactions, TMTA was per-

formed under the following conditions: the solid-supported 5′-thiol decamer **3** (ca. 0.25 μmol) was treated with 8 equiv. of TCEP and 15 equiv. of **4b**–**f** in 500 μL of 50 mM K_2CO_3 in methanol for 4 h at 55 °C. The completion of the reaction was checked by MALDI-TOF MS. After filtration and washing of the CPG beads with water (500 μL) the solution was concentrated to half its original volume and subjected to size exclusion chromatography (SEC) to remove excess acrylamide derivative **4a**–**f**, TCEP and K_2CO_3. HPLC analyses of the crude products showed that the reactions were clean, with purities from 75% to 95%, and the main peaks were characterized by MS as the expected conjugates (see Table S1 in the Supporting Information). Figure 1 shows MALDI-TOF and HPLC profiles of **5b** (crude and pure) as an example (see the Supporting Information for **5a** and **5c**–**f**). It can be seen that the reaction is very clean, effectively with the formation of only one product. The non-conjugated oligonucleotide (*m*/*z* = 3189.2) was not observed, showing that the TMTA was quantitative. The conjugates **5a**–**f** were easily purified by reversed-phase HPLC and characterized by MALDI-TOF MS (Table S1 in the Supporting Information).

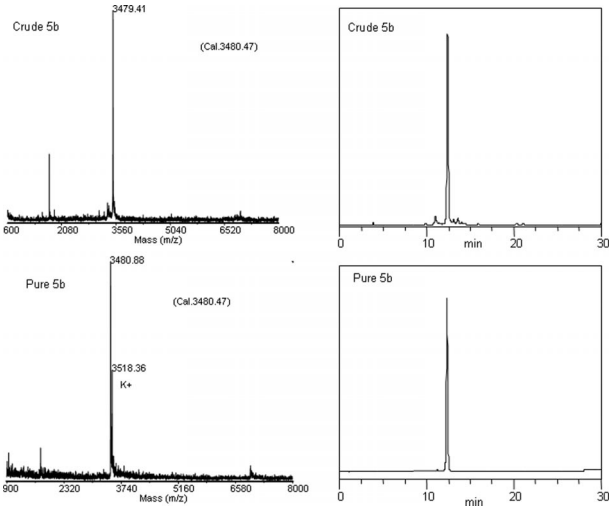
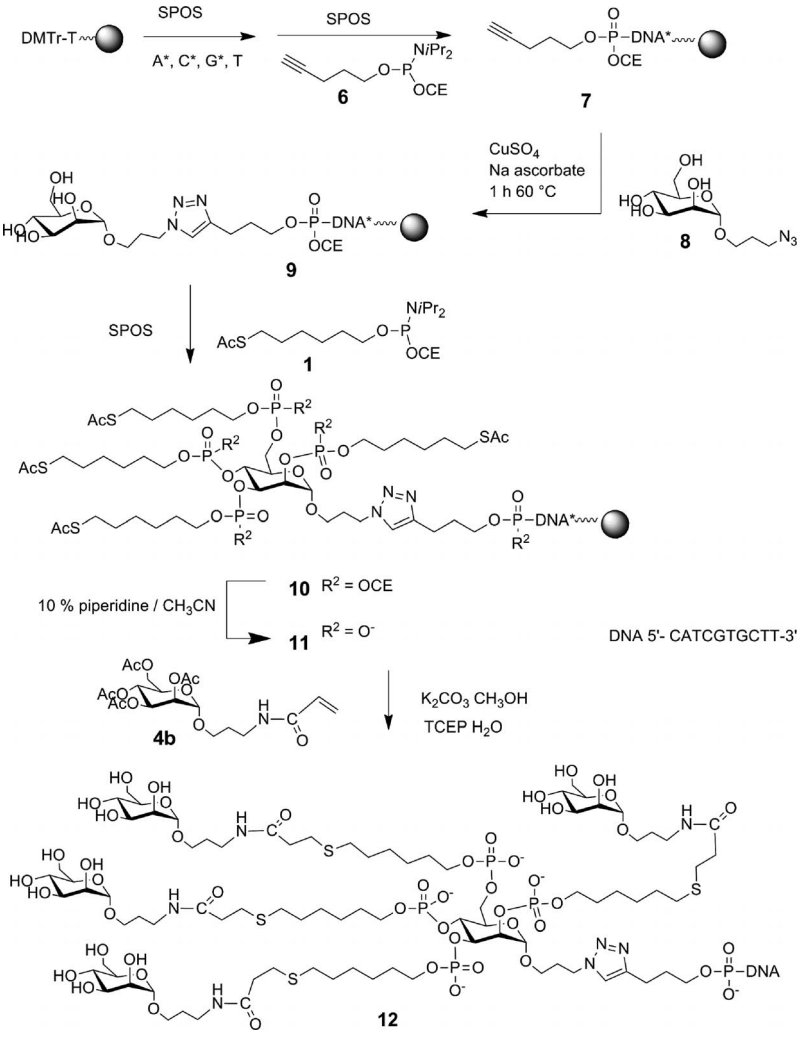


Figure 1. MALDI-TOF and HPLC profiles of conjugate **5b**.

We then applied this efficient thiol click reaction protocol, developed for monoconjugation, to multiple conjugation to produce conjugate **12** (Scheme 3), a tetrathiol oligo-



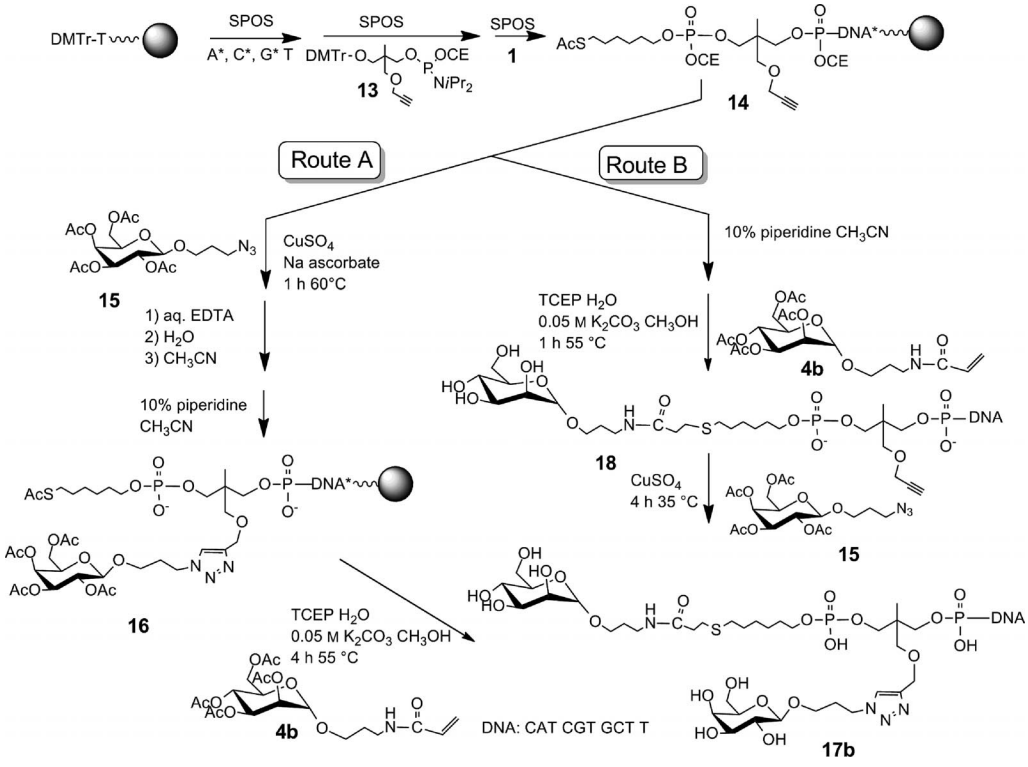
Scheme 3. Synthesis of mannosyl-centered tetramannosyl-substituted oligonucleotide conjugate **12**.

nucleotide containing a mannosyl-centred tetramannosyl-substituted motif. To this end, firstly 5′-pent-4-ynyl decamer **7** was synthesized on solid support from pent-4-ynyl phosphoramidite **6**.[22] Mannosylpropyl azide **8**[23] was then conjugated by CuAAC in the presence of sodium ascorbate and $CuSO_4$ for 1 h at 60 °C, which allowed the introduction of a mannose core with four free hydroxy functions (compound **9**).[23] Thiol phosphoramidite **1** was introduced onto each hydroxy group by use of double coupling (180 s). The resulting tetra-thiol-modified oligonucleotide **10** was treated with piperidine to remove the cyanoethyl groups. Finally, solid-supported phosphodiester oligonucleotide **11** (ca. 0.25 μmol) was engaged in a deprotection TMTA cascade with **4b** (20 equiv.) as described above to afford exclusively the neoglyco-oligonucleotide conjugate **12** containing the mannosyl-centred tetramannosyl motif. This result demonstrated that multiple conjugation of oligonucleotides by TMTA is also very efficient with use only of 5 equiv. of **4b** per thiol function.

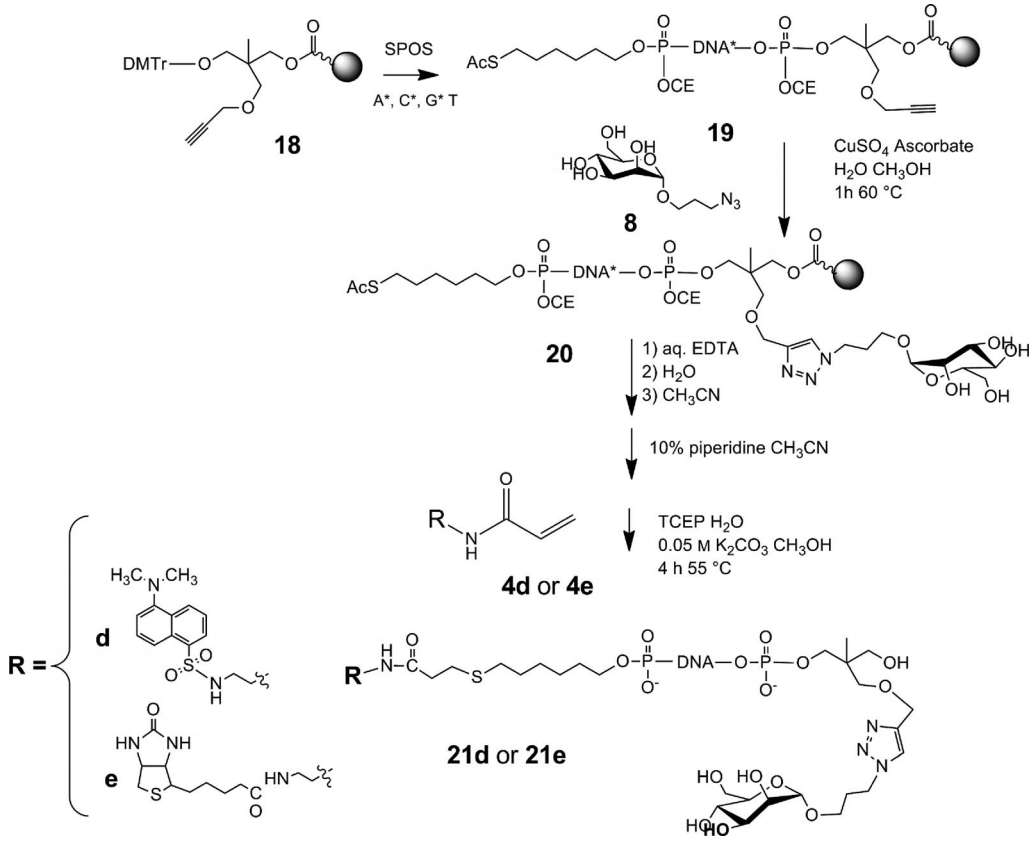
The combination of TMTA and CuAAC reactions has previously been reported in the polymer field,[24,25] but to the best of our knowledge this combination has never been applied for bis-conjugation of oligonucleotides. We had previously shown that CuAAC could be combined with oxime formation[26] or with amidative oxidation[27] to prepared bis-conjugated oligonucleotides. Here, we studied conditions for combining CuAAC and TMTA in the synthesis of an oligonucleotide containing mannose and galactose residues. TMTA and CuAAC are not fully orthogonal, due to the possibility of reaction between a thiol moiety and copper,[28] so it is necessary to proceed sequentially. The conjugate **17b** (Scheme 4), containing both mannose and galactose residues, was synthesized in two ways. By Route A, CuAAC on the solid support[29] was applied first, and after washes TMTA was carried out. By Route B, TMTA was performed first and CuAAC was then carried out in solution. To this end, a solid-supported oligonucleotide was successively elongated with monoalkyne phosphoramidite **13**[30] and thiol phosphoramidite **1** to afford **14**, containing an alkyne function and a *S*-acetylthiohexyl group (Scheme 4).

A portion of compound **14** was conjugated with tetraacetyl-galacto-propyl azide **15** by CuAAC in the presence of $CuSO_4$ and sodium ascorbate (1 h 60 °C). The CPG beads were then thoroughly washed with a saturated aqueous solution of EDTA to remove any trace of copper. Simple washing with water and organic solvents was not efficient, so traces of copper were still present and quenched the TMTA. After removal of cyanoethyl groups with piperidine, yielding **16**, the second conjugation with **4b** was carried out by TMTA as described above to afford **17b**. Excesses of reagents were mainly removed by SEC and the final bis-conjugate was isolated by HPLC and characterized by MS (see the Supporting Information).

In parallel, another portion of **14** was treated with piperidine and the first conjugation was performed by TMTA to afford **18**, within 1 h. Compound **18** was directly treated with tetraacetyl-galactose azide **15**[31] and $CuSO_4$ for the second conjugation by CuAAC. Because TCEP was present, there was no need to add sodium ascorbate to form Cu^I in situ. Furthermore, because of the presence of K_2CO_3/methanol, the acetyl groups of galactose were hydrolysed



Scheme 4. Synthesis of bis-conjugated oligonucleotide **17b** by TMTA and CuAAC.



Scheme 5. Synthesis of oligonucleotides bearing 5′-dansyl or 5′-biotin and 3-mannose groups by CuAAC and then TMTA.

during the reaction to afford **17b** directly; it was treated as described above.

Both strategies led to the formation of the bis-*glyco*-conjugated oligonucleotide **17b** with the same efficacy. It should be noted, however, that sequential CuAAC and TMTA could not be performed in solution because the presence of copper quenches the latter reaction.

Likewise, the 5′-biotin-galactose **17e** oligonucleotide was prepared from **16** by TMTA conjugation with biotin acrylamide **4e** (see Scheme S1 in the Supporting Information).

Finally, oligonucleotides decorated at both ends with 5′-dansyl and 3′-mannose (compound **21d**, Scheme 5) or with 5′-biotin and 3′-mannose (compound **21e**) components were prepared by Route A with CuAAC on solid support first, to introduce a mannose residue from mannose propyl azide **8** at the 3′-end, and then by divergent TMTA, with either **4d** or **4e**, to introduce a dansyl or a biotin motif, respectively, at the 5′-end (Scheme 5). An oligonucleotide was thus elongated on monoalkyne solid support **18**[32] and *S*-acetyl-thiohexyl phosphoramidite **1** was coupled at the 5′-end to afford **19**. CuAAC was carried out on solid support with mannose propyl azide **8**, $CuSO_4$ and sodium ascorbate for 1 h at 60 °C to afford **20**. After washing with saturated aqueous EDTA solution, water and acetonitrile, cyanoethyl groups were removed by piperidine treatment. One portion of the 3′-mannnose solid-supported oligonucleotide was treated with dansylacrylamide **4d** and another with biotin acrylamide **4e** in the presence of TCEP, K_2CO_3 and methanol for 4 h at 55 °C to afford the bis-conjugated oligonucleotides **21d** and **21e**, respectively, with dansyl or biotin groups at their 5′-ends and mannose at their 3′-ends.

Conclusions

TMTA was efficiently applied to the synthesis of mono-conjugated oligonucleotides and also, for the first time, for the synthesis of a multiply conjugated oligonucleotide, under very convenient and simple conditions. A great advantage of TMTA with acrylamide derivatives for the synthesis of oligonucleotide conjugates is that the reactions take place during the deprotection and release from the solid support, so no additional time is required for the conjugation. Such a strategy could not be applied with maleimide derivatives because they, unlike acrylamide derivatives, are not very stable under basic conditions. Finally, sequential combination of CuAAC and TMTA allowed the synthesis of bis-conjugated oligonucleotides. This result opens the way to the synthesis of oligonucleotides decorated with different labels.

Experimental Section

***N*-Phenethylacrylamide (4a):** Acryloyl chloride (80 μL, 1 mmol) was added to a solution of phenethylamine (126 μL, 1 mmol) and anhydrous triethylamine (280 μL, 2 mmol) in dry toluene (5 mL). After

2 h stirring at room temp., the mixture was diluted with toluene and poured into a saturated aqueous $NaHCO_3$ solution (50 mL). The compound was extracted with CH_2Cl_2 (3× 15 mL). The organic layer was then collected and dried with Na_2SO_4, and the solvents were evaporated under vacuum. Purification by flash chromatography on silica gel (AcOEt in cyclohexane 25% to 50%) afforded **4a** as a colourless oil (110.3 mg, 63%). TLC R_f = 0.6 (cyclohexane/AcOEt 1:4 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.79 (t, J = 6.9 Hz, 2 H, CH_2), 3.54 (q, J = 6.9 Hz, 2 H, CH_2NH), 5.48 (br. s, 1 H, NH), 5.55 (dd, J = 1.4, 10.4 Hz, 1 H, CH=CH_2,*cis*), 5.95 (dd, J = 10.2, 17 Hz, 1 H, CH=CH_2), 6.2 (dd, J = 1.5, 17 Hz, 1 H, CH=CH_2,*trans*), 7.12–7.28 (m, 5 H, ar) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 35.6, 40.7, 126.3, 126.5, 128.7, 128.8, 130.9, 138.8, 165.6 ppm. HRMS (ESI+): calcd. for $C_{11}H_{14}NO$ $[M + H]^+$ 176.1075; found 176.1051.

2,3,4,6-*O*-Tetraacetyl-1-*O*-[3-(acryloylamino)propyl]-α-D-mannose (4b): Tris(2-carboxyethyl)phosphane (TCEP, 330 mg, 1.15 mmol) was added to a solution of 2,3,4,6-*O*-tetraacetyl-1-*O*-azidopropyl-α-D-mannose[16] (100 mg, 0.23 mmol) in MeOH/H_2O (8:2, v/v, 6 mL). The reaction was monitored by TLC. After 1.5 h stirring at 65 °C, the mixture was concentrated and filtered to remove the white precipitate. The filtrate was evaporated to dryness to afford a clear oil, which was diluted with anhydrous CH_3CN (10 mL). Anhydrous Et_3N (64 μL, 2 mmol) and acryloyl chloride (19 μL, 0.23 mmol) were then added successively. The cloudy mixture was stirred for 2 h at room temp. The reaction was then quenched with saturated $NaHCO_3$ solution (50 mL) and the mixture was extracted with CH_2Cl_2 (2× 50 mL). The organic layers were concentrated and dried with Na_2SO_4, and the residue was purified by flash column silica gel (MeOH in CH_2Cl_2 0 to 5%) to afford **4b** as a clear oil (70 mg, 67%). TLC R_f = 0.45 (cyclohexane/AcOEt 1:1, v/v). ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 1.86–199 (m, 2 H, CH_2CH$_2$NH), 1.97, 2.05, 2.14 and 2.20 (4×s, each 3 H, 4×$COCH_3$), 3.37–4.38 (m, 7 H, OCH$_2$CH$_2$CH_2N$_3$, OCHHCH$_2$CH$_2$N$_3$, 5-H and OCHHCH$_2$CH$_2$N$_3$, 6-Ha and 6-Hb), 4.85 (d, J = 1.4 Hz, 1 H, 1-H), 5.28–5.35 (m, 3 H, 3-H, 2-H, 4-H), 5.69 (dd, J = 2.3, 9.5 Hz, 1 H, CH_2=CH,*cis*), 6.14–6.31 (m, 3 H, CH_2=CH, NH) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 20.8, 20.9, 21.0, 29.2, 37.4, 62.77, 66.3, 66.8, 68.7, 69.2, 69.5, 97.7, 126.6, 130.9, 165.8, 169.8, 170.1, 170.2, 170.7 ppm. HRMS (ESI+): calcd. for $C_{20}H_{30}NO_{11}$ $[M + H]^+$ 460.1817; found 460.1819.

***N*-[2-(Ferrocenylmethylamino)ethyl]acrylamide (4c):** Ferrocenecarbaldehyde (214 mg, 1 mmol) was added to a solution of *N*-acryloylethylenediamine[17] (114 mg, 1 mmol) and triethylamine (140 μL, 1 mmol) in CH_2Cl_2/MeOH (5 mL). After the system had been stirred for 1 h at room temp., $NaBH_4$ (70 mg, 2 mmol) was added. After 1 h, the mixture was diluted with CH_2Cl_2 and poured into water (50 mL). After washing, the organic layer was collected and dried with Na_2SO_4, and the solvents were evaporated under vacuum. The residue was purified by column chromatography (silica gel, 2 to 10% MeOH in CH_2Cl_2) to afford **4c** as a yellow powder (150 mg, 48%). TLC R_f = 0.55 (CH_2Cl_2/MeOH 8:2 v/v). ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 2.94 (br. s, 2 H, CH_2NH), 3.49 (br. s, 2 H, $NHCH_2$), 3.88 (s, 2 H, $FeCH_2NH$), 4.11 (s, 5 H, cyclopent), 4.16 (s, 2 H, cyclopent), 4.4.37 (s, 2 H, cyclopent), 5.58 (t, J = 5.4 Hz, 1 H, CH_2=CH,*cis*), 6.19 (d, J = 5.8 Hz, 2 H, CH_2=CH), 8.15 (br. s, 1 H, NH) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 18.4, 30.9, 36, 46.8, 47.8, 69, 69.7, 70.7, 75.4, 126.8, 130.8, 166.7 ppm. HRMS (ESI+): calcd. for $C_{16}H_{21}N_2OFe$ $[M + H]^+$ 313.0987; found 313.1003.

***N*-(2-Acrylamidoethyl)-5-(dimethylamino)naphthalene-1-sulfonamide (4d):** Dansyl chloride (136 mg, 0.25 mmol) was added at room temp. to a solution of ethylenediamine (134 μL, 2 mmol) and anhydrous triethylamine (70 μL, 0.5 mmol) in anhydrous CH_2Cl_2 (4 mL). The mixture was stirred for 1 h, diluted with CH_2Cl_2 (50 mL) and washed with cold water (2× 25 mL). The organic phase was dried with Na_2SO_4 and filtered, and the solvent was evaporated under vacuum to afford a pale green powder. The crude product (50 mg, 0.17 mmol) was dissolved in anhydrous triethylamine (47 μL, 0.34 mmol) in anhydrous CH_2Cl_2 (2 mL), and acryloyl chloride (14 μL, 0.17 mmol) was added. After 2 h stirring at room temp., the mixture was diluted with CH_2Cl_2 and poured into a saturated aqueous $NaHCO_3$ solution (50 mL). The organic layer was then collected and dried with Na_2SO_4, and the solvents were evaporated under vacuum. Purification by flash chromatography on silica gel (MeOH in CH_2Cl_2 0 to 5%) yielded **4d** as a pale green powder (50 mg, 58%). TLC R_f = 0.5 (CH_2Cl_2/MeOH 9:1, v/v). ^{1}H NMR ($CDCl_3$,300 MHz): δ = 2.94 (s, 6 H), 3.08–3.17 (m, 2 H, -CH_2NH-), 3.40–3.48 (m, 2 H, -CH_2NHSO_2-), 5.62 (d, J = 1.6, 8.6 Hz, 1 H, CH=CH_2,*cis*), 5.83 (t, J = 5.8 Hz, 1 H, NH), 5.96 (dd, J = 8.6, 16.8 Hz, 1 H, CH=CH_2), 6.24 (dd, J = 1.6, 16.8 Hz, 1 H, CH=CH_2,*trans*), 6.30 (m, 1 H, NH), 7.24 (d, J = 7.6 Hz, 1 H, Ar), 7.6 (q, J = 8.4 Hz, 2 H, Ar), 8.3 (t, J = 8.6 Hz, 2 H, Ar), 8.6 (d, J = 8.6 Hz, 1 H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 35.4, 38.8, 41.4, 46.5, 111.3, 114.8, 119.2, 122.6, 124.5, 125.5, 125.5, 126.00, 126.4, 126.6, 130.6, 148.1, 162.7 ppm. HRMS (ESI+): calcd. for $C_{17}H_{22}N_3O_3S$ $[M + H]^+$ 348.1379; found 348.1382.

***N*-[2-(Acrylamido)ethyl]biotinamide (4e)** was prepared as described in the literature.[17]

Deoxycholin Derivative 4f: Deoxycholic acid (392 mg, 1 mmol) and *N*-hydroxysuccinimide (130 mg, 1.1 mmol) were dissolved in DMF (10 mL), followed by addition of *N*,*N*′-dicyclohexylcarbodiimide at room temp. After overnight stirring, the precipitate (DCU) was filtered off and the filtrate was concentrated under vacuum. The crude oil was washed with Et_2O (3× 50 mL) to afford a white precipitate, which was diluted with anhydrous CH_3CN (10 mL). A solution of anhydrous Et_3N (240 μL, 1.7 mmol) and *N*-acryloylethylenediamine[17] (90 mg, 0.8 mmol) was then added. The cloudy mixture was stirred overnight at 50 °C and then concentrated under vacuum. The residue was purified by flash column chromatography (silica gel, MeOH in CH_2Cl_2 0 to 10%) to afford **4f** as a clear oil (166 mg, 34%). TLC R_f = 0.5 (CH_2Cl_2/MeOH 95:5 v/v). ^{1}H NMR (CD_3OD, 600 MHz): δ = 0.65 (s, 3 H, CH_3), 0.88 (s, 3 H, CH_3), 0.95 (d, J = 9 Hz, 3 H CH_3), 1.01–1.88 (m, 24 H, CH_2, CH), 2.01–2.24 (m, 2 H, C_{23}, CH_2), 3.25–3.27 (m, 4 H, CH_2), 3.44–3.51 (m, 1 H, C_3, CH), 3.90 (br, 1 H, C_{12}, CH), 5.61 (dd, J = 5.4, 12 Hz, 1 H, CH_2=CH,*cis*), 6.16–6.18 (m, 2 H, CH_2=CH) ppm. ^{13}C NMR (CD_3OD, 151 MHz): δ = 13.2, 17.7, 23.7, 24.8, 27.4, 28.4, 28.6, 29.9, 31.0, 33.2, 34.1, 34.8, 35.3, 36.4, 36.8, 37.2, 37.4, 39.9, 40.1, 43.6, 47.5, 48.0, 72.5, 73.9, 126.8, 132.0, 168.4, 177.2 ppm. HRMS (ESI+): calcd. for $C_{29}H_{49}N_2O_4$ $[M + H]^+$ 489.3692; found 489.3685.

General Procedure for the Synthesis of Oligonucleotides: Oligonucleotides were synthesized on a 1 μmol scale with a DNA synthesizer and use of standard phosphoramidite chemistry. For the coupling step, benzylmercaptotetrazole (BMT, 0.3 M in anhydrous CH_3CN) was used as the activator, commercially available nucleoside phosphoramidites (with benzoyl for A and C and isopropylphenoxyacetyl for G, 0.09 M in anhydrous CH_3CN) were introduced with 20 s coupling times, and *S*-acetyl-thiohexyl phosphoramidite **1** (0.15 M in anhydrous CH_3CN), pent-4-ynyl phosphoramidite **6**[22] and 1-*O*-(4,4′-dimethoxytrityl)-2-propargyloxymethyl-2-methyl-3-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite]propane-1,3-diol (**13**[30], 0.09 M in anhydrous CH_3CN) were introduced with 60 s coupling times. The capping steps were per-

formed with acetic anhydride in commercially available solutions (Cap A: phenoxyacetyl anhydride, pyridine, THF 10:10:80 and Cap B: 10% *N*-methylimidazole in THF) for 60 s. Oxidation was performed with a commercially available solution of iodide (I_2, 0.1 M, THF/pyridine/water 90:5:5, 13 s). Detritylation was performed with DCA in CH_2Cl_2 (2.5%, 35 s).

Protocol for the Removal of Cyanoethyl Groups: After elongation, the solid-supported *S*-acetyl-thiolhexyl-oligonucleotides were treated with a solution of piperidine in dry CH_3CN (10%, 1.5 mL, back and forth between two syringes for 15 min), washed with dry acetonitrile and dried with nitrogen flushing.

General Protocol for TMTA: An acrylamide derivative (**4a**–**f**, 3.8 μmol, 15 equiv., 38 μL of a 100 mM solution in MeOH), TCEP (2 μmol, 8 equiv., 20 μL of a 100 mM solution in H_2O) and K_2CO_3 in MeOH (50 mM, 500 μL) were added to a solid-supported *S*-acetyl-thiohexyl-oligonucleotide **3** (≈0.25 μmol). The vial containing the mixture was heated for 4 h at 55 °C. The mixture was filtered off and desalted on NAP10.

Synthesis of the Mannosyl-Centred Tetramannosyl-Substituted Oligonucleotide 12: 1-*O*-Azidopropyl-α-mannose[16] (4 μmol, 40 μL of a 100 mM solution in MeOH) and freshly prepared solutions of $CuSO_4$ (1 μmol, 24 μL of a 40 mM in water) and of sodium ascorbate (5 μmol, 50 μL of a 100 mM in water) were added to a vial containing the solid-supported 5′-(pent-4-ynyl)-oligonucleotide **7** (1 μmol) in H_2O/MeOH (1:1, v/v, 200 μL). The vial was heated at 60 °C (oil bath) under magnetic stirring for 1 h. The solid-supported 5′-mannosyl-oligonucleotide **9** was then filtered off, washed (2 mL) with water, MeOH and CH_3CN, dried and loaded onto a DNA synthesis column. Thiohexyl phosphoramidite **1** was conjugated by application of a double coupling with a 180 s wait between each coupling. Compound **10** was treated over 15 min with a solution of piperidine in CH_3CN (10%, 1.5 mL) and washed with CH_3CN, and a quarter of the beads (ca. 0.25 μmol) were transferred into a vial. 1-*O*-Propylacrylamide-2,3,4,6-*O*-tetraacetyl-α-mannose (**4b**, 5 μmol, 5 equiv./thiol, 50 μL of a 100 mM solution in MeOH), TCEP (20 μL, 8 equiv., 2 μmol of a 100 mM solution in H_2O) and K_2CO_3 in MeOH (50 mM, 500 μL) were added into the vial, and the mixture was heated for 4 h at 55 °C. After completion of the reaction (HPLC monitoring), the mixture was filtered, diluted with water (500 μL) and concentrated to remove MeOH. The solution was applied onto a NAP 10 column for desalting, to afford **12**.

Synthesis of Bis-Conjugated Oligonucleotide 17b by CuAAC and then TMTA (Route A): Mannose-propyl-azide **15** (100 mM, 1 μmol, 10 μL of a 100 mM solution in MeOH), a freshly prepared solution of $CuSO_4$ (0.25 μmol, 6 μL of 40 mM solution in water) and sodium ascorbate (1.25 μmol, 18 μL of 100 mM solution in water) in H_2O/MeOH (1:1, v/v, 100 μL) were added to compound **14** (≈0.25 μmol). The vial containing the mixture was sealed and placed in an oil bath at 60 °C under magnetic stirring for 1 h. The CPG beads were then filtered off and washed with water (2 mL), MeOH (2 mL), water (2 mL), a saturated solution of EDTA (2 mL), water (2 mL) and CH_3CN (2 mL). The CPG beads were dried, treated for 15 min with a solution of piperidine in CH_3CN (10%, 2 mL) and washed with CH_3CN (2 mL). The CPG beads were transferred into a vial and 1-*O*-propylacrylamide-2,3,4,6-*O*-tetraacetyl-α-mannose (**4b**, 3.75 μmol, 37 μL of a 100 mM solution in MeOH), TCEP (2 μmol, 20 μL of a 100 mM solution in H_2O) and K_2CO_3 in MeOH (50 mM, 500 μL) were added. The vial containing the mixture was heated for 2 h at 55 °C. Finally, the mixture was filtered, diluted with water (500 μL) and concentrated to remove MeOH. Compound **17b** was obtained after desalting on NAP 10.

Synthesis of Bis-conjugated Oligonucleotide 17b by TMTA and then CuAAC (Route B): Compound **14** (≈0.25 μmol) was first treated with piperidine (see above). 1-*O*-Propylacrylamide-2,3,4,6-*O*-tetraacetyl-α-mannose (**4b**, 3.75 μmol, 37 μL of a 100 mM solution in MeOH), TCEP (2 μmol, 20 μL of a 100 mM solution in H_2O) and K_2CO_3 in MeOH (50 mM, 500 μL) were then added. The vial containing the mixture was heated for 1 h at 55 °C. After completion of the reaction (HPLC monitoring), 1-*O*-propyl-azide 2,3,4,6-tetraacetyl-α-galactose (**15**,[31] 100 mM, 1.0 μmol, 10 μL of a 100 mM solution in MeOH) and a freshly prepared solution of $CuSO_4$ (0.25 μmol, 6 μL of 40 mM solution in water) were added. The vial was heated for 4 h at 35 °C. The mixture was then filtered, diluted with water (500 μL) and concentrated to remove MeOH. Compound **17b** was obtained after desalting on NAP 10.

Bis-conjugated oligonucleotides **17e**, **21a** and **21e** were synthesized by Route A.

Supporting Information (see footnote on the first page of this article): ^1H and ^{13}C NMR spectra of **4a**–**d** and **4f**, HPLC profiles of conjugates **5a**–**f**, **12**, **17b**, **17e**, **21d** and **21e** with MS spectra.

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