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Azide Solid Support for 3′-Conjugation of Oligonucleotides and Their Circularization by Click Chemistry

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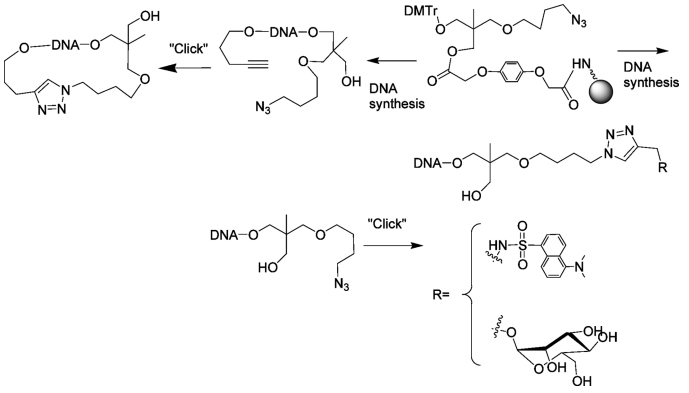

A solid support bearing an azido linker was used to synthesize a 3′-azido-alkyl-oligonucleotide by phosphoramidite chemistry. The resulting oligonucleotide was either conjugated by 1,3-dipolar cycloaddition on solid support or in solution with mannose-propargyl derivative and in solution with dansyl propargyl. Besides, after introduction of an alkyne function at the 5′-end, the resulting oligonucleotide bearing both 3′-azide and 5′-alkyne functions was circularized.

Introduction

Oligonucleotide (ODN) conjugates are widely used for various applications in biology, biotechnology, and medicine. Many methods of conjugation are reported in the literature.[1−4] ODN conjugates with fluorescent dyes and biomolecules such as peptides[5−9]or carbohydrates[7,10−16] are commonly described. Most of the conjugation methods require the introduction of functional groups to synthetic ODNs by a DNA synthesizer. Amine or thiol functions are from the most popular groups for the conjugation to the ends of oligonucleotides via commercially available phosphoramidite reagents or solid supports. Recently an alternative approach using the azide/alkyne system arose as an efficient and powerful method for conjugation. Both functions react

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together through a Huisgen 1,3-dipolar cycloaddition[17] called "click chemistry".[18] This reaction was found to be catalyzed by the Cu(I) ion,[19,20] leading to fast reactions. Hence, a wide range of applications for bioconjugation have been reported (for reviews see refs 21−27). It is generally considered that both alkyne and azide functions are mainly orthogonal with other functionalities so that the click reaction is chemoselective and can be performed in water and organic solvents. Many publications reported the introduction of the alkyne function into ODN using phosphoramidite derivatives of modified nucleosides on the nucleobases,[28−31] on the sugar at the 2′ position[32,33] or on the phosphorus atom,[34] or using non-nucleosidic derivatives.[34−45] Methods to introduce propargyl function through a phosphoramidate linkage were also described.[15,16,46]

In contrast only a few publications showed the introduction of the azide function into an oligonucleotide still on the solid support. The main reason is that it was shown that a nucleoside with an azide function reacts with the phosphoramidite derivative according to the Staudinger reaction when both compounds are in solution.[47] Likewise, van der Marel et al.[33] showed more recently that a nucleoside exhibiting both phosphoramidite and an azide function rapidly decomposed in solution. Thus to avoid this side reaction the azide function was mainly introduced by a time-consuming post-elongation protocol in solution.[33,38,48] As an alternative Kool et al. reported the 5′-iodination and subsequent treatment with sodium azide to gain 5′-azido-oligonucleotide[49] but this approach was restricted since deoxyadenosine led to a side reaction during the iodination.[50] Along this line, the use of building blocks with a bromine-alkyl group was developed and introduced automatically by a DNA synthesizer into a sequence[34,51] or at the 5′-end.[36,52] The bromo function was then converted to an azido upon treatment with sodium azide leading to azide oligonucleotides. However, very recently Lonnberg et al. showed that the azide function can be introduced by a 4′-azidomethyl-thymidine-3′-*H*-phosphonate derivative and then the oligonucleotide can be elongated with either *H*-phosphonate or phosphoramidite chemistry without observing the Staudinger reaction.[53]

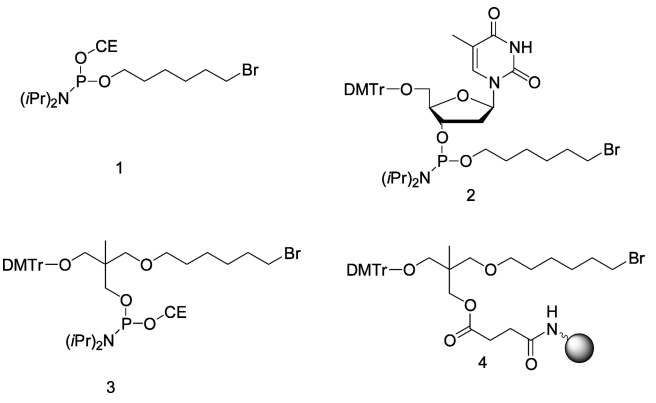

FIGURE 1. Schematic structure of phosphoramidite derivative **1**−**3** and solid support **4** bearing a bromohexyl group (CE: 2-cyanoethyl).

Results and Discussion

This recent result prompted us to design a solid support allowing the introduction of an azide function for subsequent 3′-conjugation by Cu(I) alkyne/azide cycloaddition (CuAAC) and also the cyclization of an oligonucleotide bearing both 3′-azide and 5′-alkyne functions.

To this end, two strategies were explored. The first one was based on the use of a solid support bearing a bromohexyl group **4** (Figure 1) as an extension of our previous work where phosphoramidite derivatives **1**−**3** were used and the bromine atom was easily substituted after elongation by sodium azide affording the azido-oligonucleotides.[34,51,52] However, with the solid support **4**, prepared from CPG with 500 Å pore size, we were unable to substitute the bromine atom using either sodium azide or the more reactive

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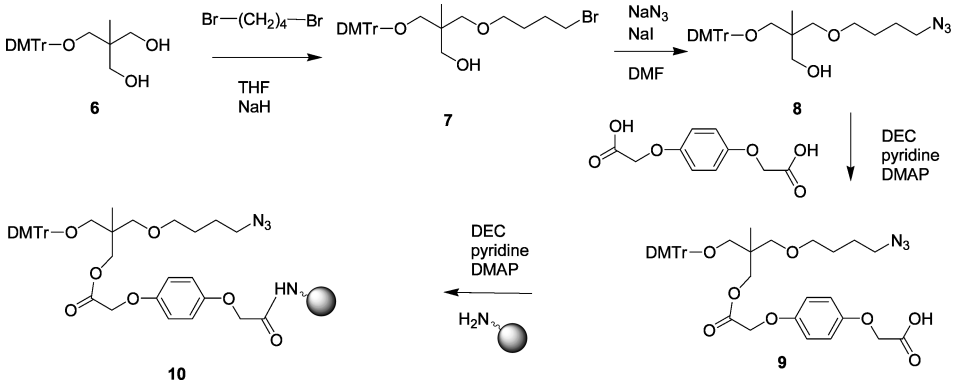
SCHEME 1. Synthesis of Azide Solid Support 10



tetramethylguanidine azide reagent. As the steric hindrance due to the presence of the oligonucleotide was suspected, we also tried to perform the substitution before the oligonucleotide elongation directly on the solid support **4** but here again without success. A last attempt with less loaded CPG with 1000 Å pore size was also unsuccessful. These results are surprising since we have already substituted bromide atoms on solid supported oligonucleotides many times.[34,51,52] However, in these cases the alkyl bromide chain was always borne on a phosphotriester linkage[34,52] or between two phosphotriester linkages.[51] We suspect a detrimental long distance effect of the ester function linking the bromine compound to the solid support, but we were unable to find any information in the literature to support this hypothesis.

The second strategy uses a solid support **10** bearing an azide alkyl chain that was successfully applied to synthesized 3′-azide alkyl-oligonucleotide according to the phosphoramidite chemistry.

The solid support **10** was easily prepared by a 4-step synthesis starting from dimethoxytrityl-1,1,1-tris(hydroxymethyl)ethane **6**[34] that was alkylated with 1,4-dibromobutane in the presence of NaH affording **7** as a racemic mixture and then treated with sodium azide and sodium iodide to give the azido derivative **8**. This compound was converted into its *O*-hydroquinone-*O*,*O*′-diacetyl hemiester[54] derivative **9** and finally anchored on LCAA-CPG by using EDC (500 Å loading of 65 μmol/g) (Scheme 1).

A first heptathymidylate was synthesized on solid support **10** by using standard phosphoramidite chemistry. Undeniably, after deprotection upon ammonia treatment, the expected 3′-azide-linker-T_7 was obtained (data not shown). We then synthesized on solid support **10** an oligonucleotide exhibiting the four nucleobases (Scheme 2). One-third of the resulting solid-supported dodecamer (CAT CGG GCT TGG) bearing a 3′-azide-linker group **11** was directly used for a CuAAC with 1-*O*-propargyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranose **12** (5 equiv) on solid support under microwave (MW) activation[15] at 60 °C for 30 min (2 equiv of $CuSO_4$, 10 equiv of Na ascorbate, methanol−water 1:1) then deprotected with aqueous ammonia to provide the 3′-mannosyl 12-mer **13** (29 $OD^{260\ nm}$ 240 nmol, Figure 2). The remaining two-thirds were treated with ammonia to release in solution the fully deprotected 3′-azide-linker oligonucleotide **14** (440 nmol, 54 $OD^{260\ nm}$). After evaporation, the crude (18 $OD^{260\ nm}$ for each) was used for CuAAC reaction with dansyl-propargyl **15**[55] (5 molar equiv) or 1-*O*-propargyl-2,3,4,6-*O*-tetraacetyl-α-mannose **12** (5 molar equiv). The reactions were performed at room temperature, using $CuSO_4$ (2 molar equiv) and sodium ascorbate (10 molar equiv) in methanol−water (1:1, v/v), and were complete within 2 h affording the 3′-dansyl oligonucleotide **16** (140 nmol, 17 $OD^{260\ nm}$) and the 3′-tetraacetyl-mannose oligonucleotide, respectively. The acetyl protections of the 3′-tetraacetyl-mannose oligonucleotide were removed under ammonia to give **13** (115 nmol, 14 $OD^{260\ nm}$).

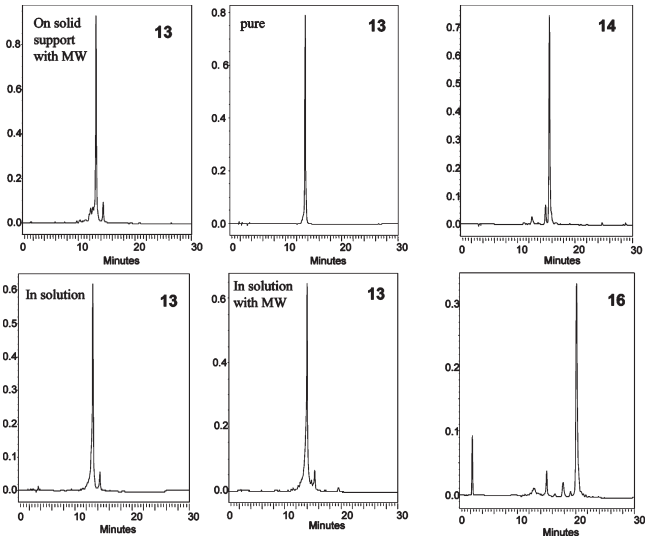


FIGURE 2. HPLC profiles of 3′-mannosylated CAT CGG GCT TGG **13** obtained by CuAAC on solid support with MW assistance or in solution with and without MW assistance and after purification. HPLC profiles of the crude 3′-azide 9-mer **14** and its 3′-conjugate **16** with dansyl obtained by CuAAC in solution without MW.

For comparison **11** (66 nmol) was used for CuAAC reaction with **12** under MW assistance for 30 min at 60 °C, using the same conditions as above. A complete cycloaddition was observed with a similar HPLC profile. A final ammonia treatment gave conjugate **13** with a similar purity to **13** obtained without MW assistance (Figure 2).

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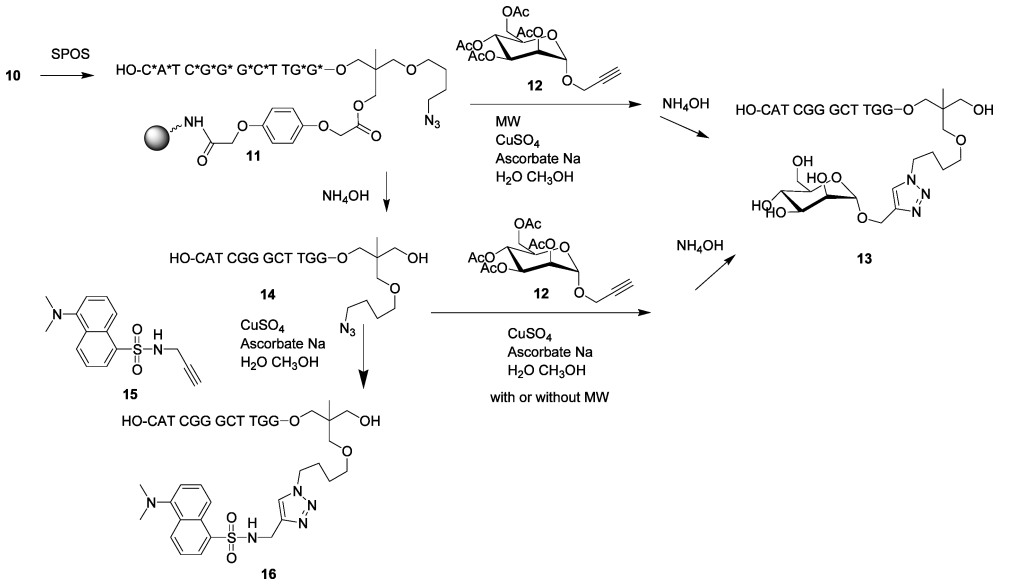
SCHEME 2. Synthesis of 3′-Mannose and 3′-Dansyl Oligonucleotide Conjugates[a]



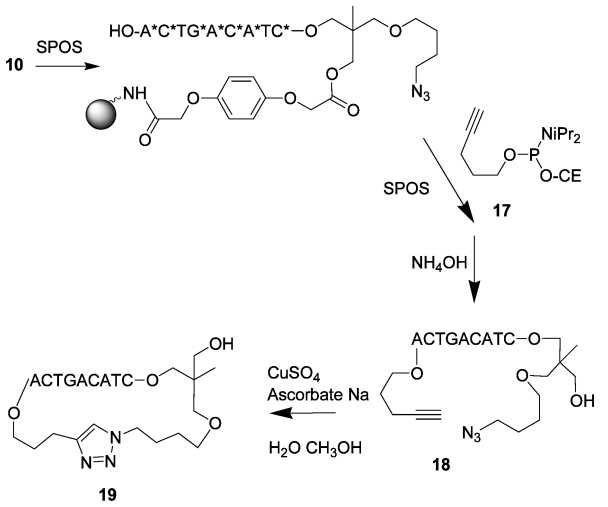
[a]SPOS: solid-phase oligonucleotide synthesis: (1) 2.5% dichloroacetic acid CH_2Cl_2; (2) phosphoramidite derivative + benzylthiotetrazole; (3) Ac_2O, *N*-Me imidazole, 2,6-lutidine; (4) 0.1 M I_2 THF/H_2O/pyridine. The asterisk represents protecting groups: benzoyl for A and C and isobutyryl for G.

We observed that it is quite compulsory to use MW assistance when CuAAC reactions are carried out on solid support to rapidly obtain (less than 30 min) a complete reaction.[15] In contrast, when CuAAC reactions were carried out in solution, the MW assistance could be omitted and the reaction is usually done within 2 h at rt but obviously CuAAC reactions under MW assistance are more rapid since the temperature is set at 60 °C.

DNA Cyclization

Several chemical methods are described in literature to circularize DNA or RNA.[31,34,38,45,56−62] Among them, recent examples of DNA circularization by CuAAC reaction have been reported but in each case the protocol is not straightforward and requires an extra postelongation step.[31,34,38,45,62] Along this line, we previously reported a universal method to synthesize cyclic oligonucleotides starting from a monoalkyne solid support. After the oligonucleotide elongation the introduction of phosphoramidite bromohexyl **1** was also done with a DNA synthesizer. Then a postelongation treatment on solid supported oligonucleotide with a NaN_3 NaI solution leads to the substitution of the bromide affording the 5′-azidooligonucleotide.[34] After the ammonia treatment to deprotect and release the 3′-alkyne 5′-azide oligonucleotide from the solid support, this compound was finally circularized in solution by CuAAC reaction. Here, we proceeded in the reverse way, starting from the azide solid support **10**, synthesizing the oligonucleotide (ACTGACATC) and introducing the alkyne function by means of pentyn-4-oyl phosphoramidite (Scheme 3). After ammonia deprotection the linear 5-alkyne 3′-azide nonamer was afforded in solution as a single peak. The CuAAC reaction was efficiently performed with $CuSO_4$ and sodium ascorbate at room temperature for 3.5 h to give the cyclic nonamer as a double peak due to the chiral carbon of the trishydroxymethylethane moiety. A similar behavior was already observed on other cyclic oligonucleotides where a phosphorus atom was the chiral center.[34] The reaction was monitored by reverse phase HPLC (Figure 3) and not by MALDI-TOF MS since the mass of both linear and cyclic oligonucleotide is the same. As observed before, the cyclic ODN exhibited a lower retention time than the linear ODN due to the high lipophilicity of the azide function. Both linear and cyclic compounds were characterized by MALDI-TOF MS.

SCHEME 3. Synthesis of Cyclic 9-Mer ACTGACATC 19



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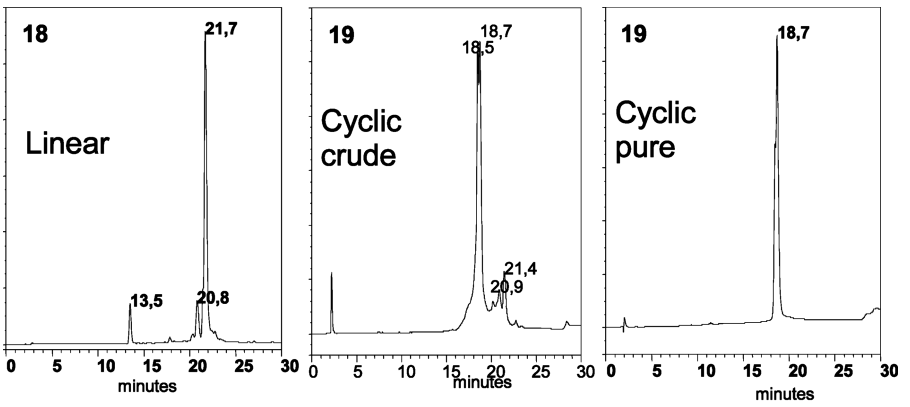

FIGURE 3. HPLC profiles of linear and cyclic 9-mer ACTGACATC **19**.

The present method is more straightforward than all of the previous ones since all the steps to obtain the linear oligonucleotide bearing both alkyne and azide functions are performed on a DNA synthesizer without any postelongation step.

Conclusion

In conclusion, an azide solid support was efficiently prepared. This solid support could be used with phosphoramidite chemistry allowing 3′-end conjugation of oligonucleotide either on solid support or in solution through CuAAC reaction. Its use in combination with an alkyne phosphoramidite derivative introduced at the 5′-end afforded an oligonucleotide with both alkyne and azide functions allowing a straightforward cyclization.

Experimental Section

1-*O*-(4,4′-Dimethoxytrityl)-2-(4-bromobutoxymethyl)-2-methyl-1,3-propanediol, 7. 2-[(4,4′-Dimethoxytrityl)oxymethyl]-2-methylpropane-1,3-diol[34] (1.7 g, 4 mmol), sodium iodide (62 mg, 0.1 mmol), and dibromobutane (1.54 mL, 10 mmol) were added to a solution of sodium hydride (60% in oil, 645 mg, 4 mmol) in anhydrous THF (16 mL) at 0 °C. After 5 h under magnetic stirring at rt, the reaction was quenched by adding 50 mL of CH_2Cl_2 and 2 mL of water. The organic layer (300 mL) was washed with brine (2 × 150 mL), dried over Na_2SO_4, and evaporated. The crude product was purified by silica gel column chromatography (0 to 30% acetone in cyclohexane containing 1% triethylamine) to afford a racemic mixture of **7** (1.4 g, 64%) as a clear oil. R_f 0.5 (cyclohexane/acetone 1:1 v/v); H NMR ($CDCl_3$, 300 MHz) δ 0.89 (s, 3H), 1.68−1.92 (m, 4H), 2.70 (t, 1H, J = 5.9 Hz), 3.06−3.14 (m, 2H), 3.40−3.80 (m, 8H), 3.80 (s, 6H), 6.84−7.45 (m, 13H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 16.9, 27.2, 28.6, 32.8, 39.8, 54.2, 65.4, 68.4, 69.7, 74.8, 84.9, 112.1, 125.9, 126.8, 127.1, 129.3, 135.1, 138.5, 144.0, 157.5. Mass spectrometry characterization was unsuccessful.

1-*O*-(4,4′-Dimethoxytrityl)-2-(4-azidobutoxymethyl)-2-methyl-1,3-propanediol, 8. 1-*O*-(4,4′-Dimethoxytrityl)-2-(4-bromobutoxymethyl)-2-methyl-1,3-propanediol (**7**; 0.36 mmol, 200 mg) was dried three times by coevaporation with anhydrous acetonitrile and then dissolved in anhydrous acetonitrile (3 mL) before addition of tetramethylguanidinium azide (0.72 mmol, 114 mg). The reaction was stirred at rt for 5 h, and then diluted with 25 mL of CH_2Cl_2. The organic layer was washed with brine (2 × 70 mL), dried over Na_2SO_4, and evaporated. The crude product was purified by silica gel column chromatography (0 to 3% acetone in CH_2Cl_2 containing 0.5% triethylamine) to afford **8** (140 mg, 74%) as a clear oil. R_f 0.5 (CH_2Cl_2/acetone 7.5:2.5 v/v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.92 (s, 3H), 1.62−1.65 (m, 4H), 2.72 (m, 1H), 3.08−3.15 (m, 2H), 3.27−3.31 (m, 2H), 3.44−3.58 (m, 6H), 3.82 (s, 6H), 6.84−7.46 (m, 13H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.9, 25.8, 26.9, 40.8, 51.3, 55.2, 66.5, 69.4, 71.0, 75.9, 85.9, 113.1, 126.8, 127.8, 128.2, 130.1, 136.1, 145.0, 158.4; HRMS ESI (positive mode) m/z calcd for $C_{30}H_{37}O_5N_3Na$ $[M + Na]^+$ 542.2631, found 542.2646.

(4-CPG-long chain alkyl-carbamoylmethoxy-phenoxy)acetic Acid 2-(4-Azido-butoxymethyl)-3-(4,4′-dimethoxytrityloxymethyl)-2-methylpropyl Ester 10. Compound **8** (250 mg, 0.48 mmol) was dissolved in dry pyridine with hydroquinone-*O*,*O*′-diacetic acid (130 mg, 0.58 mmol), DMAP (12 mg, 0.1 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide DEC (92 mg, 0.4 mmol) then the mixture was stirred at rt for 3 h. Pyridine was evaporated and the residue was redissolved in CH_2Cl_2 and washed with water (three times). After evaporation the crude hemiester was directly anchored on CPG by using DMAP (10 mg), DEC (120 mg), and dry Et_3N (80 μL) in dry pyridine and shaken overnight. After filtration and drying the solid support was capped by using Cap A and Cap B solution for 1 h. Loading was determined by trityl assay: 65 μmol/g

***O*-4-Pentynyl-*O*′-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite, 17.** To a solution of anhydrous 4-pentyn-1-ol (1.8 mmol, 151 mg) and diisopropylammonium tetrazolide (0.9 mmol, 154 mg) in anhydrous CH_2Cl_2 (5 mL) was added 2-cyanoethyl diisopropylphosphorodiamidite (1.8 mmol, 570 μL). The resulting mixture was stirred for 5 h at room temperature, diluted with CH_2Cl_2 (30 mL), and washed with brine (2 × 100 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The crude product was purified by silica gel column chromatography (0 to 50% CH_2Cl_2 in cyclohexane containing 5% triethylamine) to afford **17** (350 mg, 68%) as a clear oil. R_f 0.65 (cyclohexane/CH_2Cl_2/Et_3N 5:4:1, v/v/v); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.18−1.21 (d, 12H, J = 6.4 Hz), 1.77−1.97 (m, 3H), 2.27−2.36 (m, 2H), 2.62−2.69 (m, 2H), 3.55−3.88 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.3, 20.4, 29.2, 42.1, 58.2, 58.4, 61.9, 68.9, 83.7, 117.6; ^{31}P NMR ($CDCl_3$ 81 MHz) δ 148.8 ppm; HRMS ESI (positive mode) m/z calcd for $C_{14}H_{26}O_2N_2P$ $[M + H]^+$ 285.1732, found 285.1732.

General Procedure for Cu(I)-Catalyzed 1,3-Dipolar Cycloaddition: a. In Solution. A solution of 5-(dimethylamino)-*N*-(2-propynyl)-1-naphthalene-sulfonamide **15**[55] (70 mM, 10 μL) or 1-*O*-propargyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranose **12**[51] (100 mM, 7.5 μL) in MeOH and freshly prepared solutions of $CuSO_4$ (40 mM 7.5 μL) and sodium ascorbate (100 mM, 15 μL) in water were added to oligonucleotide **14** (0.146 μmol, 123 μL of H_2O) in solution. The coupling was monitored by HPLC. After 2 h, the mixture was desalted on NAP 10, affording the oligonucleotide conjugate (**16**: 17 DO at 260 nm, 140 nmol,

97%; **13**: 14 DO at 260 nm, 115 nmol, 81% after ammonia treatment at 55 °C for 5 h).

b. On Solid Support. A solution of 1-*O*-propargyl-2,3,4,6-*O*-tetraacetyl-α-mannose **12** (100 mM, 7.5 μL) in MeOH and freshly prepared solutions of $CuSO_4$ (40 mM, 7.5 μL) and sodium ascorbate (100 mM, 15 μL) in water were added to the solid-supported oligonucleotide (∼1/3 μmol). The vial containing the resulting mixture was sealed and placed in a microwave synthesizer, with a 30 s premixing time, for 30 min at 60 °C. The temperature was monitored with an internal infrared probe. Then, the CPG beads were filtered and washed with water (2 mL) and MeOH (2 mL) and dried. A final ammonia treatment (55 °C for 5 h) removed all the protecting groups and released the oligonucleotide conjugate in solution (29 DO at 260 nm, 239 nmol, 80%).

DNA Circularization by Cu(I)-Catalyzed 1,3-Dipolar Cycloaddition Reaction. To the linear alkyne-azide oligonucleotide **18** (200 nmol) dissolved in 120 μL of water were added $CuSO_4$ (10 μL of a 40 mM solution in H_2O), freshly prepared sodium ascorbate (20 μL of a 100 mM solution in H_2O), and methanol (90 μL). After 3.5 h the solution was desalted on NAP 10.

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Supporting Information Available: Protocol to synthesize modified oligonucleotides, MALDI-TOF MS data of **13**, **14**, **16**, **18**, and **19**, and ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR of compounds **7**, **8**, and **17**. This material is available free of charge via the Internet at http://pubs.acs.org.