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New Strategies for Cyclization and Bicyclization of Oligonucleotides by Click Chemistry Assisted by Microwaves

Jory Lietard, Albert Meyer, Jean-Jacques Vasseur, and François Morvan*

Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS, Université Montpellier 1, Université Montpellier 2, Place Eugène Bataillon, CC1704, 34095 Montpellier Cedex 5, France

morvan@univ-montp2.fr

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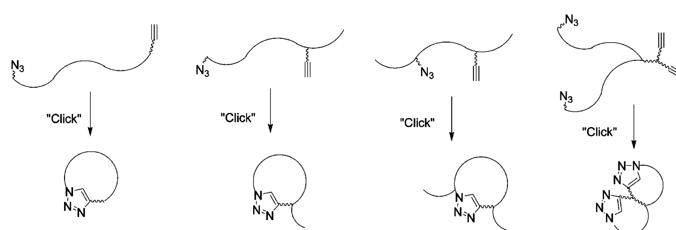
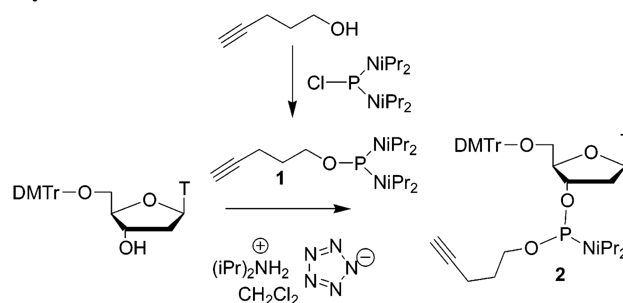


The synthesis of cyclic, branched, and bicyclic oligonucleotides was performed by copper-catalyzed azide−alkyne cycloaddition assisted by microwaves in solution and on solid support. For that purpose, new phosphoramidite building blocks and new solid supports were designed to introduce alkyne and bromo functions into the same oligonucleotide by solid-phase synthesis on a DNA synthesizer. The bromine atom was then substituted by sodium azide to yield azide oligonucleotides. Cyclizations were found to be more efficient in solution than on solid support. This method allowed the efficient preparation of cyclic (6- to 20-mers), branched (with one or two dangling sequences), and bicyclic (2 × 10-mers) oligonucleotides.

Introduction

DNA is a versatile material for the construction of nanostructures (for recent review see refs 1 and 2). There is great interest in constructs of oligonucleotides with unusual topologies. Among them, cyclic DNA and RNA were synthesized in the late 1980s,[3−7] and there appears to be a growing interest for them in several recent works.[8−14] Cyclic DNA and RNA present unusual chemical and biological properties in comparison with linear DNA and have been evaluated for several biological applications including antisense, triplex, and diag-

SCHEME 1. Synthesis of the Pentenyl Phosphoramidite of Thymidine



* Corresponding author. Tel: +33/467 144 961. Fax: +33/467 042 029.

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nostic applications.[3,15−17] Several methods have been developed for the synthesis of cyclic oligonucleotides using enzymatic or chemical protocols leading to cyclic oligonucleotides with phosphodiester,[3,18−21] phosphorothiolate diester,[13,22−24] disulfide,[25] or oxime linkages.[13,26]

Herein, we present a method for the circularization of short- to medium-size oligonucleotides (6- to 20-mers) that involves a 1,3-dipolar cycloaddition between an alkyne and an azide function, one borne on one end and the other on the other end of an oligonucleotide, leading to a triazole linkage. This method was then extended to the synthesis of branched and bicyclic oligonucleotides. The copper-catalyzed azide−alkyne cycloaddition (CuAAC) reaction[27,28] applied to DNA appeared as a useful reaction for anchored DNA on a solid support,[29] for preparation of DNA conjugates with carbohydrates[30−33] and recently for DNA ligation and DNA cyclization.[14]

Results and Discussion

We report an efficient method to prepare cyclic, bicyclic, and branched oligonucleotides based on CuAAC reaction. Initially, to set the conditions of cyclization the first reactions were performed on hexa- and nonathymidylate to form the corresponding cyclic oligonucleotides cT_6 and cT_9. In order to carry out the reaction of cyclization on solid support as well as in solution, both functionalities (i.e., akyne and azide) were introduced into the oligonucleotide still on the solid support. As a rule, it is more powerful to do so because excess reagents can be used to push the reaction to completion, and excess is removed by simple washings. The workups are easier and the resulting compounds generally have better purity.

Previously, we designed a protocol to introduce an alkyne function through an amidative oxidation of a *H*-phosphonate diester linkage by means of carbon tetrachloride in the presence of excess of propargylamine.[32−34] This method requires the use of *H*-phosphonate chemistry with pivaloyl chloride as a coupling reagent to introduce *H*-phosphonate diester linkages and a manual amidative oxidation. The oligonucleotide moiety is then synthesized by phosphoramidite chemistry. This protocol is efficient but requires the use of two chemistries on a DNA synthesizer, consequently necessitating multiple manipulations. In the present work, we developed a strategy using only the phosphoramidite chemistry. Thus, an alkyne function was introduced on the phosphorus atom of the 3′-end nucleoside by means of the incorporation of a new thymidine phosphoramidite **2** bearing a pentenyl chain. To do so, 4-pentenyl tetraisopropylphosphorodiamidite **1** was prepared from 4-penten-1-ol and bis(diisopropylamino)chlorophosphine in dichloromethane in the presence of triethylamine and was then reacted with commercially available 5′-dimethoxytritylthymidine under activation with diisopropylammonium tetrazolide affording **2** (Scheme 1).

DNA elongation was performed on an universal solid support constituted of a disulfide linker[35−37] that yields after its reduction to an intramolecular elimination of episulfide with the formation of a phosphodiester linkage. The solid-supported hexa- and nonathymidines bearing a 3′-end alkyne function **4ab** were synthesized on this solid support with standard phosphoramidite elongation cycle using the thymidine pentenyl phosphoramidite **2** and the commercially available cyanoethyl thymidine phosphoramidite (Scheme 2). A further, two-step 5′-azidation was performed according to Kool et al.[38] using first methyltriphenoxyphosphonium iodide[39,40] and second sodium azide yielding 5′-azido solid-supported oligonucleotides **5ab** (Scheme 2).

The corresponding solid-supported oligonucleotides were then split in two parts. One part was left on the solid support and the other part was treated with ammonia for 2 h at room temperature to afford the 5′-azido-3′-alkyne homothymidylate in solution. The HPLC analysis showed two major peaks that were characterized by MALDI-TOF mass spectrometry as the oligonucleotides with (**6ab**) and without (**7ab**) the disulfide linker, due to some cleavage of the disulfide bond followed by the elimination of episulfide yielding the phosphodiester linkage (Figure 1a, Scheme 3).

The 1,3-dipolar cycloaddition "click reaction" was performed with the oligonucleotide in solution or still on the solid support. Since this cycloaddition is catalyzed by Cu(I), we used $CuSO_4$, sodium ascorbate in water/methanol (1:1 v/v) to generate in situ the Cu(I) species and the reaction was assisted by microwave at 60 °C for 60 min.

The reactions were monitored by HPLC on a reversed-phase column. HPLC profiles showed peaks with different retention times for the starting linear oligonucleotides and the final cyclic oligonucleotides. In each case, the cyclic oligonucleotide displayed a peak at a lower retention time than the linear oligonucleotide. Since there is no change of mass during the

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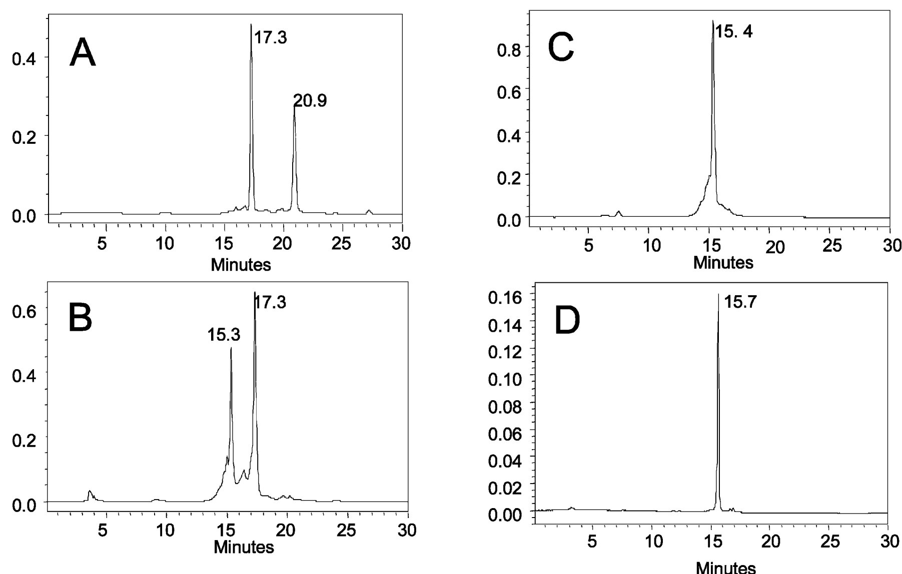
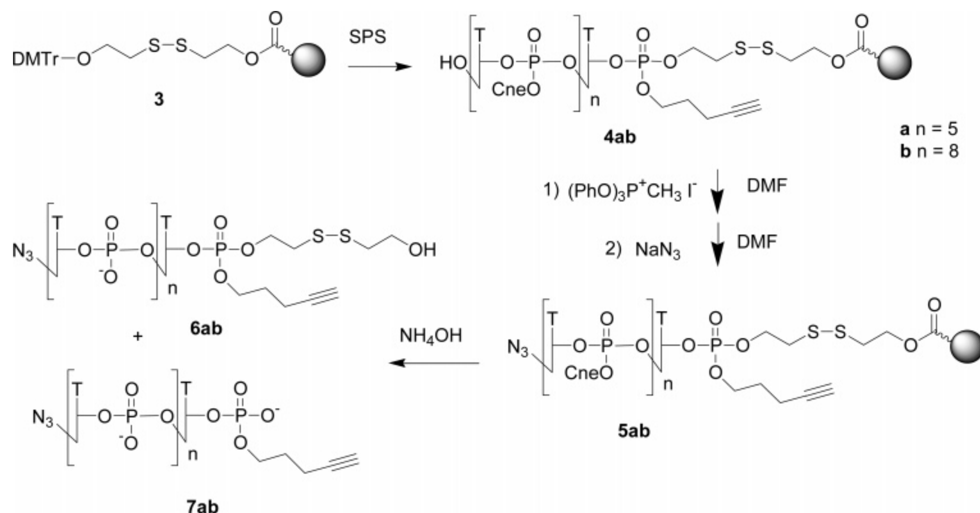


FIGURE 1. HPLC monitoring of click cyclization of 5′-azido-3′alkyne-T_9 affording cT_9: (A) crude linear T_9 **6b**, **7b**; (B) crude cT_9 **8b**, **9b**; (C) crude cT_9 **9b** after TCEP treatment; (D) pure cT_9 **9b**.

SCHEME 2. Synthesis of Solid-Supported 5′-Azido-3′-alkyne Homothymidylate[a]



[a] Key: (a) 2.5% dichloroacetic acid CH_2Cl_2; (b) **2** or DMTr-thymidine phosphoramidite + benzylthiotetrazole; (c) Ac_2O, *N*-Me imidazole, 2,6-lutidine; (d) 0.1 M I_2 THF/H_2O/pyridine. SPS = solid-phase synthesis.

cyclization, MALDI-TOF mass spectrometry is not the method of choice to monitor it, but is useful to confirm that the peak at lower retention time hypothesized to be the cyclic one has the same mass as the starting oligonucleotide.

Furthermore, to confirm cyclization, a second reaction of 1,3-dipolar cycloaddition was engaged with 5 molar equiv of propargyl alcohol under the same conditions. In all cases, no change of retention time in HPLC and no ion with +56 Da, corresponding to the click reaction of propargyl alcohol, was observed, suggesting that no azide function was still present. In contrast, when a competitive 1,3-dipolar cycloaddition was performed in solution with 5′-azido-3′-alkyne homothymidylate in presence of 5 molar equiv of propargyl alcohol, two compounds were obtained. The major one was the cyclic oligonucleotide and the minor one the linear oligonucleotide was reacted with propargyl alcohol (M + 56). Thus, although the propargyl alcohol was in excess, the cycloaddition proceeded preferentially through an intramolecular reaction.

Cyclization in Solution. Before cyclization, the HPLC chromatogram showed two peaks corresponding to the linear oligonucleotide without (**7b**, 17.3 min) and with the disulfide linker (**6b**, 20.9 min) (Figure 1a). The cyclization was performed on the mixture, since a treatment with triscarboxylethylphosphine (TCEP) to reduce the disulfide would also reduce the azide function to an amine. Click cyclization was carried out with $CuSO_4$, sodium ascorbate in water/methanol for 1 h at 60 °C assisted by MW. After reaction, two main peaks were visualized corresponding to the cyclic form without (**9b**, 15.3 min) and with (**8b**, 17.3 min) the disulfide linker (Figure 1b). A treatment with TCEP reduced the disulfide bound and afforded the expected cyclic T_9 (cT_9) **9b** (Figure 1c), which was finally purified by HPLC (Figure 1d).

Similar profiles were obtained for the cyclization of T_6 (**6a** and **7a**) affording cT_6 **9a** (see the Supporting Information).

Cyclization on Solid Support. The solid-supported 5′-azido-3′-alkyne homothymidylate was treated with $CuSO_4$ and sodium

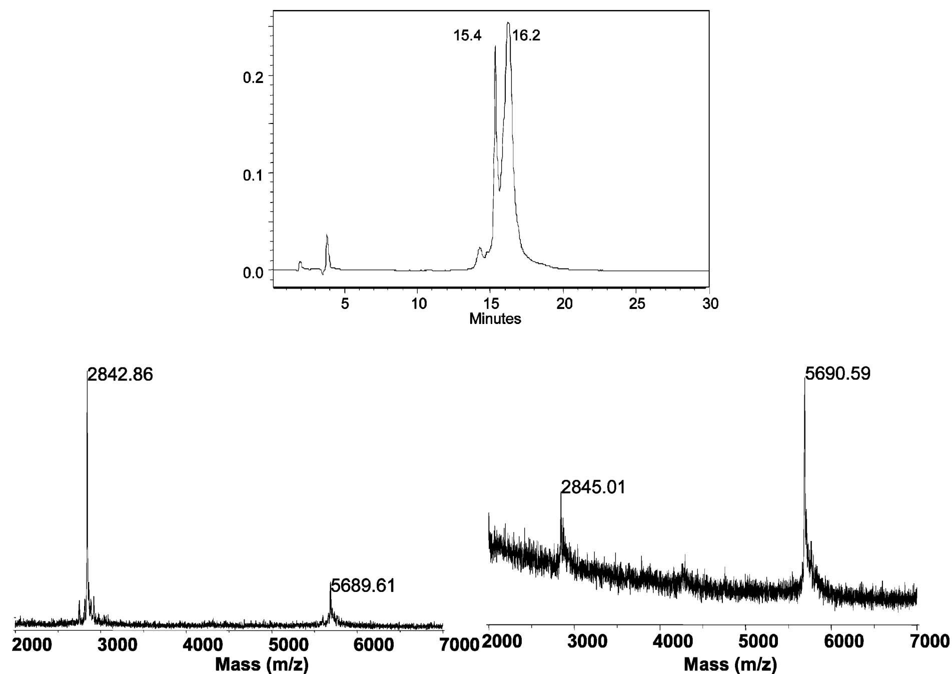
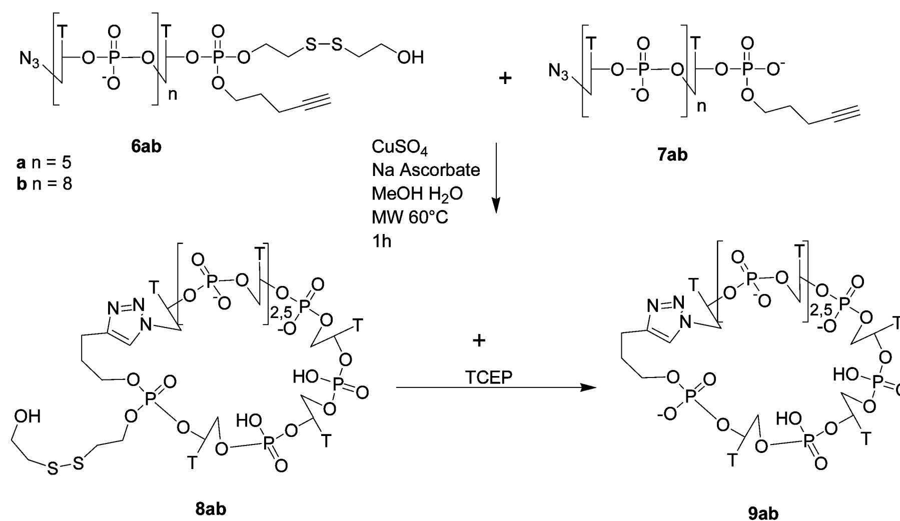


FIGURE 2. HPLC profile of click cyclization performed on solid support affording **9b** (t_R = 15.3 min) and **12b** (t_R = 16.2 min) and MALDI-TOF MS spectra of the peak at 15.3 min (**9b**, left) and the peak at 16.2 min (**12b**, right).

SCHEME 3. Click Cyclization of T6 and T_9 Performed in Solution



ascorbate in water/methanol (1:1 v/v) by microwave for 1 h at 60 °C. After filtration, the solid support was treated with ammonia in the presence of TCEP to release the cyclic oligonucleotide without the disulfide linker. HPLC profiles showed a different pattern in comparison with the cyclization done in solution, with a narrow peak corresponding to the expected cT_6 or cT_9 cyclic oligo (**9ab**) and a broad peak at higher retention time (Figure 2). The amount of the latter increased with the length of the homothymidylate (41% for T_6 and 71% for T_9). Both peaks were isolated by HPLC and characterized by MS. For the click reaction performed with T_9, the first peak at 15.3 min showed an ion at m/z = 2842.86 corresponding to the cyclic cT_9 and the second peak at 16.2 min showed an ion at m/z = 5690.59 corresponding to a dimerization of the oligonucleotide. Two possibilities were either the production of a cyclic dimer with two triazole rings or a linear dimer from a single cycloaddition and still one azide and one alkyne functions (Scheme 4). To distinguish the two species, a second "click" reaction was performed with propargyl alcohol, and no extra ion was visualized by MS, confirming that only cyclic dimer (**12b**) was present. Similar results were previously reported for the cyclization of solid-supported peptides.[41] It was suggested that the click reaction on solid support could be considered a reaction in a concentrated medium while the click reaction in solution is performed in a diluted media. Hence, intermolecular reaction is favored on solid support while only the intramolecular reaction occurred in solution. This first result showed that cyclization in solution was more efficient than on solid support with only the formation of an intramolecular cyclic oligonucleotide.

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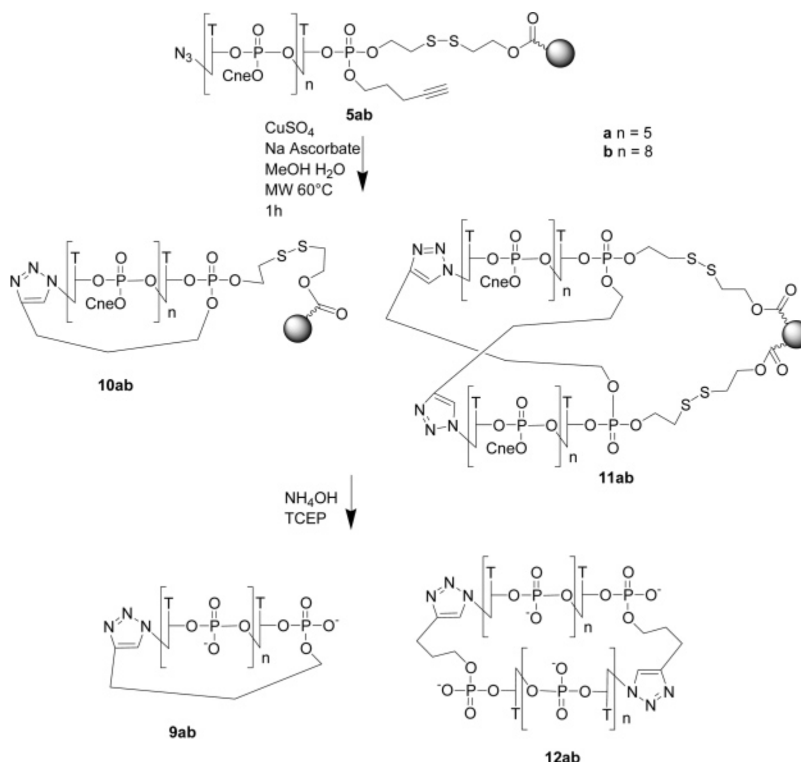
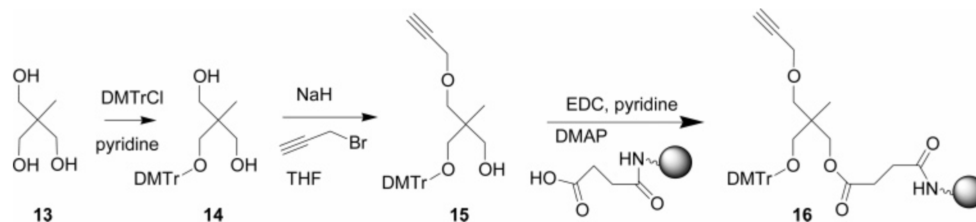
SCHEME 4. Click Reaction Performed on Solid-Supported Oligonucleotide 5ab Bearing One Azide and One Alkyne Functions



SCHEME 5. Synthesis of Monoalkyne Universal Solid Support 16



SCHEME 6. Synthesis of Bromohexyl Phosphoramidite 17

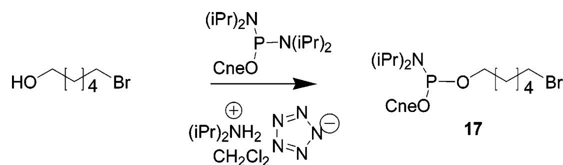


TABLE 1. T_m Values of cT_{20}:A_n and T_n:A_n:Phosphate Buffer 10 mM, NaCl 100 mM pH 7, 1 μM of Each Oligonucleotide

	A_6	A_7	A_8	A_9	A_{10}	A_{20}
cT_{20}	25.2	25.9	27.9	30.6	33.6	32.9
T_n[a]	0	<3	7.2	16.8	23.0	53.0

[a] *n* corresponds to the length of the homoadenylate.

Since the disulfide bond was partially cleaved during the ammonia treatment leading to complicated HPLC profiles, we then performed the syntheses on another universal solid support with a propanediol linker (pro).[42] Three new oligonucleotides were synthesized on it: a T_6, a T_{20}, and a heterooligonucleotide (15-mer), according to the same procedure as described above. Click cyclization was done in solution, and similar results were obtained (see the Supporting Information). Only the intramolecular cyclization occurred to afford cT_6pro, cT_{20}pro, and cTTACACCCAATTCTTpro (c15-mer-pro). For the two shorter oligonucleotides, HPLC profiles showed two peaks corresponding to the two diastereoisomers due to the phosphotriester linkage, while for cT_{20}pro the two diastereoisomers were eluted together. For the T_{20}, click cyclization was also performed on solid support. In that case, after 1 h at 60 °C under MW the cyclization achieved only 50% and did not go further even after an extra treatment of 2 h at 60 °C under MW. This result could be explained by the fact that 3′-alkyne functions are poorly accessible due to the steric hindrance of the long oligonucleotide. Then, cyclic and linear T_{20} were cleaved from the solid support by ammonia treatment and finally click cyclization was performed in solution affording the expected cT_{20}pro.

Click without the MW. As previously observed in our hands, click reaction on solid support was not efficient at all without

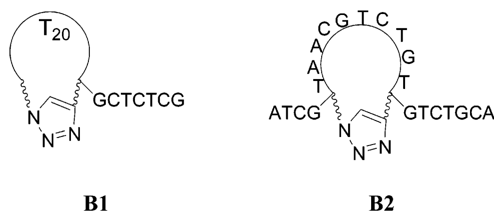


FIGURE 3. Schematic representation of cyclic branched oligonucleotides with one (**B1**) and two dangling sequences (**B2**).

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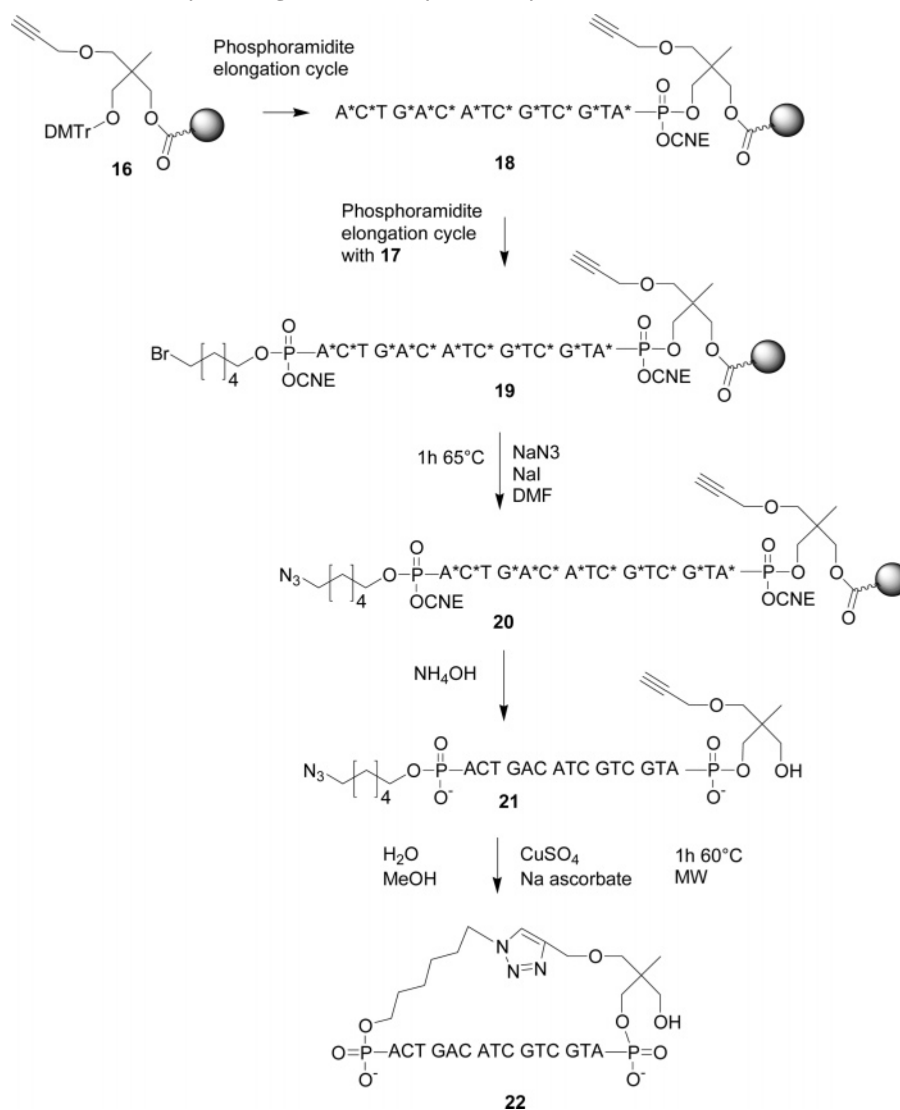
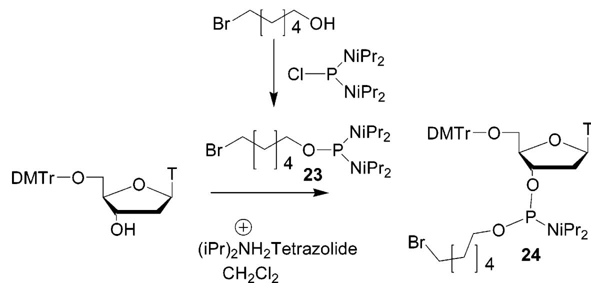
SCHEME 7. Universal Method for Cyclic Oligonucleotide Synthesis by MW-CuAAC Reaction[a]



[a] * corresponds to nucleobase protecting group: benzoyl for A and C and isobutyryl for G.

SCHEME 8. Synthesis of Bromohexyl Phosphoramidite of Thymidine

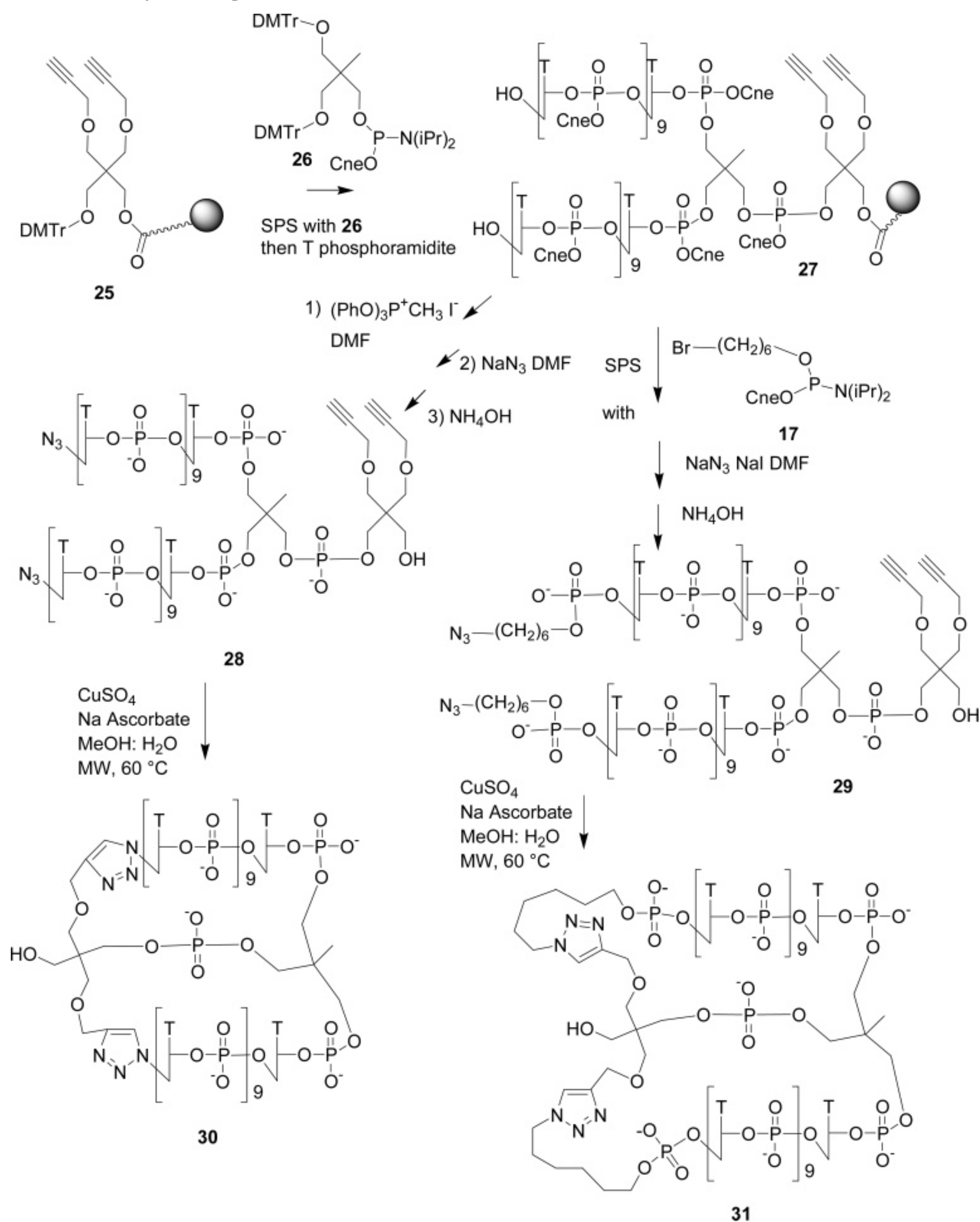


MW. In contrast, in solution, CuAAC click reaction carried out at 65 °C without MW gave a 1,3-dipolar cycloaddition. However, the results were less robust. Indeed, under the same conditions (i.e., 65 °C with $CuSO_4$ sodium ascorbate under argon), the cyclization of T_6 was incomplete after 24 h while that of T_9 and T_{20} was complete within 1 h 20 min to 2 h.

Universal Method for Preparation of Cyclic Oligonucleotides. The strategy described above if applied to the synthesis of any sequence would require the synthesis of the four pentenyl phosphoramidites corresponding to each nucleoside (A, C, G, and T) to introduce the alkyne function on the 3′-end. In addition, the 5′-azidation is not efficient for all the nucleosides, Kool et al. reported that the formation of 5′-iodide deoxyadenosine, the precursor of the 5′-azido deoxyadenosine, proceeded with only a moderate yield (56%).[39] In order to circumvent these limitations, we designed a universal strategy using a new solid support and a new phosphoramidite that could be used for any sequence. Thus, we synthesized a new universal solid support **16** bearing an alkyne function (Scheme 5), and we prepared a new 6-bromo-1-hexyl-phosphoramidite **17** for introduction of an azide function on the 5′-end of any oligonucleotide (Scheme 6). After its coupling on the oligonucleotide, the bromine atom was substituted by sodium azide in presence of sodium iodide affording the 5′-azidohexyl oligonucleotide.

Alkyne universal solid support was elaborated with a three-step protocol from tris(hydroxymethyl)ethane **13** which was monoprotected with DMTr group (**14**, 56%), monoalkylated with propargyl bromide and sodium hydride in THF (**15**, 74%), and was finally loaded on a succinyl LCAA CPG using *N*-(3-

SCHEME 9. Synthesis of Bicyclic Oligonucleotides 30 and 31



dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) as coupling reagent affording **16** (32.2 μmol/g) (Scheme 5).

Bromohexyl phosphoramidite **17** was obtained in one step from 6-bromo-1-hexanol and cyanoethyl tetraisopropylphosphorodiamidite activated with diisopropylammonium tetrazolide in dichloromethane (60%) (Scheme 6).

To evaluate the efficiency of both these building blocks, a 15-mer (ACT GAC ATC GTC GTA) was synthesized on the alkyne universal solid support **16** applying a standard phosphoramidite elongation cycle, and likewise, the bromohexyl phosphoramidite **17** was introduced at the 5′-end of the oligonucleotide (Scheme 7). The 5′-azido-15mer was obtained by treatment with a mixture of NaN_3 and NaI in DMF for 1 h at 65 °C. After ammonia treatment, the 5′-azidohexyl 3′-alkyne oligonucleotide was obtained in solution and characterized by HPLC and MALDI-TOF analyses (see the Supporting Information).

Cyclization of the oligonucleotide by CuAAC reaction assisted by microwave at 60 °C was complete within 1 h 30 min. HPLC profile after reaction showed the disappearance of the starting linear oligonucleotide and a major peak of lower retention time having the expected mass. Finally, cyclic oligonucleotide **22** was obtained as pure material after HPLC purification (see the Supporting Information).

Branched Cyclic Oligonucleotides. Then, we extended our strategy to the synthesis of branched oligonucleotides corresponding to cyclic oligonucleotides with a single dangling strand **B1** and with two single dangling strands **B2** (Figure 3). The **B1** structure could be considered mimics of a lariat structure.[43,44]

The first branched oligonucleotide **B1,** corresponding to a cyclic T_{20} with a dangling 3′-GCTCTCG sequence, was synthesized on solid support using the pentyne phosphoramidite **2** introduced into the sequence after the synthesis of the GCTCTCG sequence and followed by the elongation of 19 thymidines and finally the bromohexyl phosphoramidite **17** at

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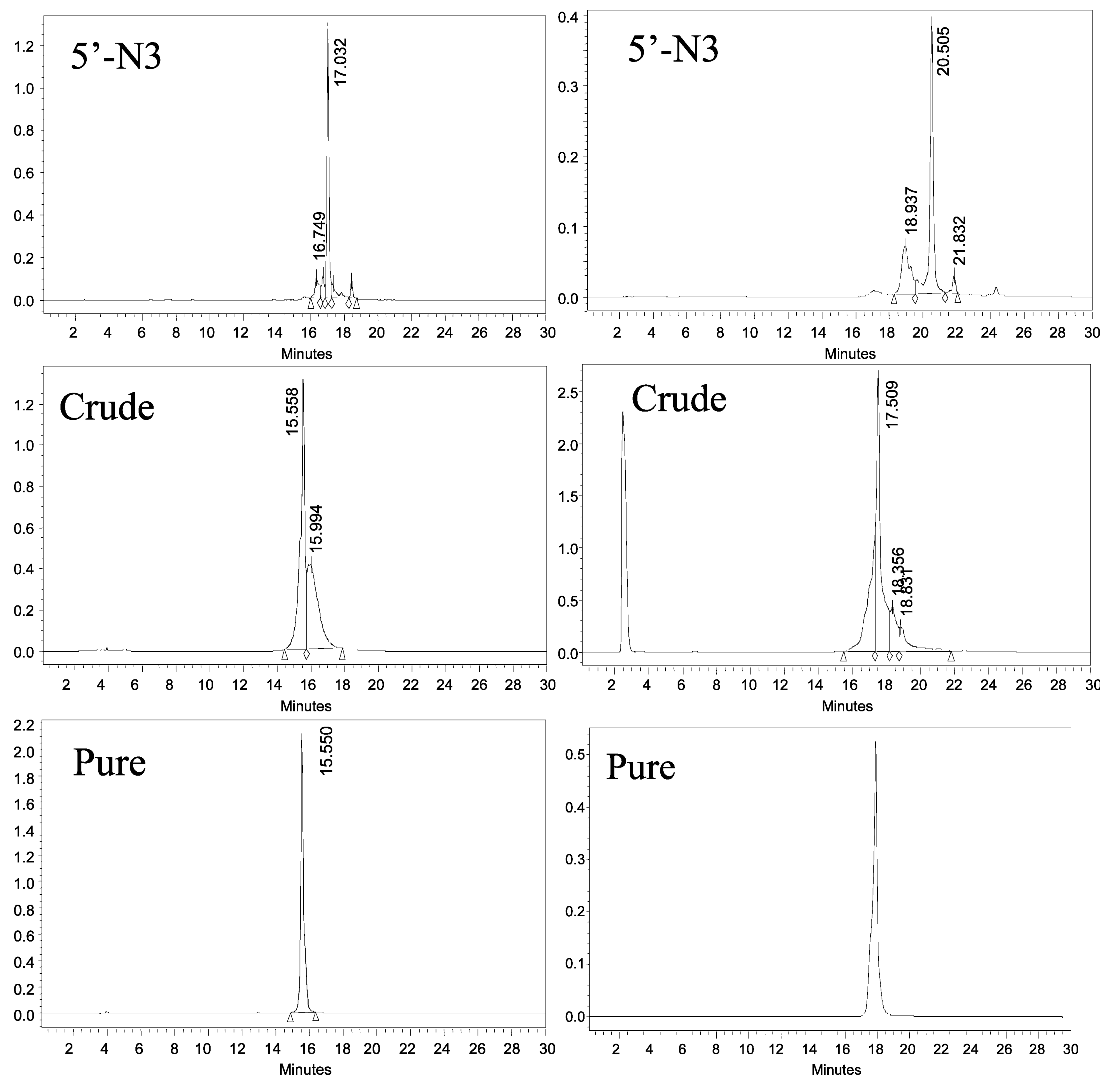

FIGURE 4. HPLC profiles before and after cyclization affording the crude bicyclic oligonucleotides **30** (left) and **31** (right) and after purification.

the 5′-end. A subsequent treatment with NaN_3 and NaI led to its 5′-azidation. For the double-branched oligonucleotide **B2**, we first synthesized a new 6-bromohexyl diisopropyl thymidine phosphoramidite **24** in order to introduce an azide function into the sequence. This compound was synthesized following the same strategy used for the preparation of **2** starting from 6-bromo-1-hexanol by reaction with bis(diisopropylamino) chlorophosphine to form the diamidite derivative **23**, which reacted with 5′-DMTr-thymidine under diisopropylammonium tetrazolide catalysis (Scheme 8).

Thus, according to the phosphoramidite method on commercial solid support, we synthesized the GTCTGCA sequence, introduced phosphoramidite **2** with an alkyne function, followed the synthesis with the AACGTCTG sequence, introduced the phosphoramidite **24** with a bromo linker, and finished the elongation with the ATCG sequence. A post-elongation treatment with NaN_3 and NaI led to an azidation on the hexyl linker into the sequence.

Both oligonucleotides bearing azide and alkyne functions were deprotected and cleaved from the solid support by an ammonia treatment at room temperature for 18 h. This mild treatment avoided strand cleavage due to nucleophilic attack on the phosphotriester linkages (i.e., phosphorus bearing the pentyne or the azidohexyl chain). Both $N_3(CH_2)_6T_{19}T$(pentyne)-GCTCTCG and ATCGT(azidohexyl)-AACGTCTGT(pentyne)-GTCTGCA linear constructions were obtained in solution.

A CuAAC cyclization was performed in solution assisted by MW for 1 h 30 min at 60 °C. Cyclization was monitored by HPLC (see the Supporting information). Total disappearance of starting linear oligonucleotide was always observed. Clear monitoring was sometimes difficult since **B1** and **B2** (as well as their precursors) are constituted of two and four diastereoisomers, respectively, due to the chiral phosphorus atom of the phosphotriester linkages providing broad peaks. The HPLC profiles of the crude showed some impurities that were assumed to come from nucleophilic attack on the phosphotriester linkages yielding oligonucleotides without the pentyne or the hexylazide arm. This fact was emphasized when CuAAC cyclization to obtain **B1** was carried out without MW at 60 °C for several hours. Indeed, the cyclization only achieved 49% after 3 h, and during the time of consumption of starting compound (18 h) several other compounds were formed, broadening the HPLC peak of the cyclic compound. It is likely that the phosphotriester functions were hydrolyzed affording a mixture of different linear and cyclic oligonucleotides according to the cleavage of the PO bond. Furthermore, it was reported that some degradations

occur due to the copper, even with degassed solvents.[45] Nevertheless, both pure cyclic oligonucleotides **B1** and **B2** were obtained after purification by HPLC and were characterized by mass spectrometry. This is a clear advantage of the MW-assisted click over the non-assisted reaction.

Bicyclic Oligonucleotides. To our knowledge, there is only one example of synthesis of bicyclic oligonucleotides reported in the literature. They were prepared through the formation of a disulfide bridge between two thymidine bases modified at the C-5 position carrying a thiol at the end of an alkyl chain.[46] In our case, we developed another method based on a double CuAAC cyclization from a construction bearing two alkyne and two azide functions. For that purpose, a new solid support **25** bearing two alkyne functions was designed. It was prepared from pentaerythritol according to the same procedure as above for preparation of **16**. Pentaerythritol was first monoprotected with a dimethoxytrityl group (30%), alkylated twice by means of propargyl bromide and sodium hydride in THF (89%),[47] and finally loaded on succinyl LCAA-CPG (40.0 μmol/g). Starting from **25**, a phosphoramidite derivative with two masked hydroxyl functions **26**[48] was incorporated (Scheme 9). Then two T_{10} were synthesized in parallel affording **27**, and each 5′-end was converted into azido. For comparison, the two azidation protocols described above were applied leading to one construction with an azido function on the 5′-carbon of the oligonucleotide and the other one with an azido function on a hexyl linker (Scheme 9). After release from the solid support by ammonia treatment, HPLC and MS analysis of **28** and **29** showed that azidation was almost complete (Figure 4). Then a click cyclization assisted by MW was applied to both constructions for 1 h 30 min at 60 °C affording the bicyclic oligonucleotides **30** and **31**.

HPLC profiles of the each crude showed complete disappearance of the diazide starting oligonucleotide and a major peak corresponding to the bicyclic oligonucleotide with some impurities. These side products were attributed to the monocyclic species after MALDI-TOF analysis with a 5′-OH function, a product of incomplete azidation and the dimerization of two monocyclic species. Each bicycle was finally purified by HPLC and characterized by mass spectrometry.

Hybridization Properties of Cyclic T_{20} (cT_{20}). The cyclic T_{20} was hybridized with hexa- to eicosa-adenylate (A_6, A_7, A_8, A_9, A_{10}, and A_{20}), and the melting temperature (T_m) of each complex was determined (Table 1). For comparison, the T_m of T_n:A_n duplexes were also determined.

T_m values of cT_{20}:A_n increased from A_6 to A_{10} and then reached a plateau. These results suggest that cT_{20} is able to hybridize up to 10 nucleobases. It is known that such a cyclic thymidylate forms a triplex helix with the cyclic oligo in a dumbbell form with two loops of 3- to 5-nucleotide length.[18] According to this structure, the cT_{20} could form a triplex with five to seven TAT triplets. To explain the present result we could hypothesize that extra nucleobases form base pairs with the nucleobases of the loops. The T_m values of T_n:A_n duplexes were always lower than those of cT_{20}:A_n except for T_{20}:A_{20} indicating a benefit of the cyclic partner able to form Hoogsteen hydrogen bounds plus a lower entropic parameter.

Conclusion

We applied the CuAAC reaction to the synthesis of cyclic oligonucleotides, branched (lariat mimic) and bisbranched cyclic oligonucleotides, and also bicyclic ones. For that purpose, we designed different building blocks (i.e., phosphoramidites and solid supports) allowing introduction of one or two alkyne and azide functions into the same oligonucleotide. The CuAAC reaction was found more efficient in solution than on solid support. Furthermore, microwave assistance allowed the formation of the cyclic oligonucleotide within 1 to 1 h 30 min with only little degradation. The evaluation of their biophysical properties is in progress.

Experimental Section

General Remarks. All commercial chemicals were reagent grade and were used without further purification. DNA synthesis reagents, phosphoramidites, and benzylmercaptotetrazole (BMT) are commercially available. Long-chain alkylamine controlled pore glass (LCAA-CPG) 500 Å, 80−120 mesh, amino group 80−90 μmol/g was from Sigma, and amino-SynBase CPG 1000, amino group 68 μmol/g was from Link Technologies.

4-Propyn-1-yl Tetraisopropylphosphorodiamidite (1). To a solution of 4-propyn-1-ol (95 μL, 1.0 mmol) and Et_3N (278 mL, 2 mmol) in dry diethyl ether (2.5 mL) was added bis(diisopropylamino)chlorophosphine (267 mg, 1 mmol) and the mixture stirred for 2 h at rt. The solution was diluted with diethyl ether/triethylamine (9:1, v/v, 10 mL), and the salts were removed by filtration and washed. The solution was evaporated to half, and cyclohexane was added. Diethyl ether was removed by evaporation keeping cyclohexane in solution. The solution was applied to a silica gel column (25 g), and the compound was purified using cyclohexane containing 6% Et_3N: 280 mg, 89%, R_f 0.60 (cyclohexane/CH_2Cl_2/Et_3N, 6:3:1, v/v/v). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.20 (24H, dd, J = 10.4 and 13.0 Hz), 1.83 (2H, quint, J = 6.6 Hz,), 1.95, (1H, t, J = 2.6 Hz), 2.32 (2H, td, J = 2.6 and 7.1 Hz,), 3.44−3.77 (6H, m). ^{13}C NMR (CD_3CN,400 MHz): δ 14.9, 23.4, 24.1, 30.5, 44.2, 62.4, 68.7, 83.9, 117.1. ^{31}P NMR (CD_3CN,80 MHz): δ 125.0.

5′-*O*-Dimethoxytrityl-3′-*O*-[(4-propynyl)-*N*,*N*-diisopropylphosphoramidite]thymidine (2). Dry 5′-*O*-Dimethoxytritylthymidine (544.5, 1 mmol) and dry diisopropylammonium tetrazolide (86 mg, 0.5 mmol) were dissolved in dry CH_2Cl_2 (8 mL), and 4-propyn-1-ol tetraisopropylphosphorodiamidite **1** (377 mg, 1.2 mmol) was added. After 3 h of stirring, the solution was diluted with ethyl acetate (80 mL), and the solution was washed with brine (2 × 150 mL). The organic layer was dried over Na_2SO_4 and evaporated. The compound was purified by flash chromatography on silica gel using an increasing amount of CH_2Cl_2 (14 to 44%) in cyclohexane containing 6% of Et_3N: 650 mg, 85%, R_f 0.50 (cyclohexane/CH_2Cl_2/Et_3N, 5:4:1, v/v/v). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.07 (3H, d, J = 6.7 Hz), 1.18 (9H, d, J = 6.8 Hz), 1.43 (3H, bs), 1.70−2.03 (3H, m), 2.20−2.36 (4H, m), 3.50−3.71 (6H, m), 3.81 (3H, s), 4.17−4.22 (1H, m) 4.65−4.68 (1H, m), 6.43−645 (1H, m), 6.83−7.45 (13H, m), 7.68 (1H, d), 8.50 (1H, br s). ^{13}C NMR (CD_3CN, 400 MHz): δ 11.7, 15.1, 23.0, 24.5, 26.9, 30.0, 40.3, 43.1, 55.3, 61.9, 63.2, 68.7, 83.7, 84.8, 85.5, 85.9, 86.9, 111.2, 113.3, 127.2, 128.0, 128.2, 130.2, 135.3, 135.5, 135.7, 144.3, 150.4, 158.7, 163.9. ^{31}P NMR (CD_3CN, 80 MHz): δ 148.3 and 148.7.

2-[(4,4′-Dimethoxytrityl)oxymethyl]-2-methylpropane-1,3-diol (14). To 1,1,1-tris(hydroxymethyl)ethane (1.2 g, 10 mmol) dissolved in dry pyridine (50 mL) was added dimethoxytrityl chloride (2.4 g, 8 mmol) in two parts over 30 min. After 3 h of

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stirring at room temperature, the reaction was quenched with methanol (2 mL). The mixture was treated with aqueous $NaHCO_3$ (300 mL), and the title compound was extracted with CH_2Cl_2 (3 × 150 mL). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The oily residue was purified by silica gel chromatography using an increasing amount of methanol (0−2%) in CH_2Cl_2 containing 1% of Et_3N affording **14** as a colorless oil: 1.9 g, 56% R_f = 0.28 (CH_2Cl_2/MeOH, 19:1, v/v). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.87 (3H, s), 2.42 (2H, br s), 3.16 (2H, s), 3.69 (2H, d, J = 25.2 Hz) 3.64 (2H, d, J = 25.2 Hz), 3.82 (6H, s), 6.79−7.49 (13H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.4, 41.1, 55.2, 67.2, 68.2, 86.3, 113.3, 126.9, 128.0, 130.0, 135.8, 144.7, 158.5. HRFABMS (positive mode, nitrobenzylic alcohol) m/z: calcd for $C_{26}H_{30}O_5$ [M + H]$^+$ 422.2093, found 422.2098.

1-Propargyl-2-[(4,4′-dimethoxytrityl)oxymethyl]-2-methylpropane-1,3-diol (15). Compound **14** (620 mg, 1.47 mmol) was dissolved in anhydrous THF (6 mL), and sodium hydride (60% in oil, 590 mg, 14.7 mmol) was added. After 10 min of stirring, propargyl bromide (80% in toluene, 650 mL, 5.9 mmol) was added, and the mixture was stirred at rt for 1 h. Then CH_2Cl_2 (100 mL) and 2 mL of water were added. The organic layer was washed with water (2 × 100 mL) and dried over Na_2SO_4. After evaporation, the residue was purified by flash chromatography using an increasing amount of ethyl acetate (10% to 50%) in cyclohexane containing 1% of triethylamine: 500 mg 74%. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.95 (3H, s), 2.58 (1H, t), 2.60 (1H, br s), 3.03 (1H, d, J = 20.7 Hz) 3.13 (1H, d, J = 20.7 Hz) 3.58−3.6 (2H, m), 3.65 (2H, s), 3.82 (6H, s), 4.18 (2H, dd, J = 0.9 and 1.4 Hz), 6.85−7.54 (13H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.8, 40.8, 55.2, 58.7, 66.5, 68.9, 74.3, 74.5, 79.7, 86.0, 113.2, 126.7, 127.8, 128.1, 129.1, 130.1, 136.0, 145.0, 158.4. HRFABMS (positive mode, nitrobenzylic alcohol) m/z: calcd for $C_{29}H_{32}O_5$ [M + H]$^+$ 460.2250, found 460.2248.

1-Propargyl-2-[(4,4′-dimethoxytrityl)oxymethyl]-2-methyl-3-(succinic-LCAA CPG)-propane-1,3-diol (16). Same protocol as for **11**. Loading 32.2 μmol/g for LCAA CPG 500 Å and 30.1 μmol/g for LCAA CPG 1000 Å.

6-Bromohexyl 2-cyanoethyl diisopropylphosphoramidite (17). To a solution of anhydrous 6-bromo-1-hexanol (325 mg, 1.8 mmol) and diisopropylammonium tetrazolide (154 mg, 0.9 mmol) in anhydrous dichloromethane (5 mL) was added 2-cyanoethyl tetraisopropylphosphorodiamidite (570 μL, 1.8 mmol). The resulting mixture was stirred for 5 h at room temperature, diluted with ethyl acetate (30 mL), and washed with brine (2 × 100 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was purified by flash column chromatography (silica gel; cyclohexane with 3% Et_3N) affording phosphoramidite **1** (400 mg 60% yield) as a colorless oil. TLC (cyclohexane/CH_2Cl_2/Et_3N; 5/4/1; v/v/v) R_f: 0.5. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.17−1.21 (12H, d), 1.44−1.88 (8H, m), 2.62−2.69 (2H, m), 3.39−3.87 (8H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.9, 24.0, 24.1, 24.2, 24.7, 27.3, 29.7, 30.4, 30.5, 32.2, 33.3, 42.4, 42.6, 57.7, 57.9, 62.9, 63.1, 117.1 ^{31}P NMR ($CDCl_3$,80 MHz): δ 148.5 ppm.

6-Bromo-1-hexyl Tetraisopropylphosphorodiamidite (23). Using the same protocol as for **1** starting from 6-bromo-1-hexanol 271.6 mg 1.5 mmol gave 410 mg (90%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.16−1.20 (24H, dd), 1.42−1.47 (4H, m), 1.56−1.65 (2H, m), 1.85−1.92 (2H, m), 3.39−3.62 (8H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 23.6, 23.7, 24.6, 24.7, 27.9, 31.4, 31.5, 32.8, 33.7, 44.1, 44.4, 63.9, 64.1, ^{31}P NMR ($CDCl_3$,80 MHz): δ 125.3 ppm.

5′-*O*-Dimethoxytrityl-3′-*O*-[(6-bromohexyl)-*N*,*N*-diisopropylphosphoramidite]thymidine (24). Using the same protocol as for **2** starting from 5′-dimethoxytritylthymidine (300 mg, 0.54 mmol) and using 6-bromo-1-hexytetraisopropylphosphorodiamidite **(23)** afforded 377 mg 82% of **24**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.4−1.20 (12H, m), 1.36−1.88 (9H, m), 2.50−2.65 (2H, m), 3.35−3.7 (8H, m), 3.81 (6H, s), 4.17−4.22 (1H, m), 4.61−4.69 (1H, m), 6.40−6.46 (1H, m), 6.83−7.67 (14H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): 11.7, 24.5, 24.6, 25.1, 25.2, 27.8, 31.0, 32.7, 33.8, 339, 42.9, 43.0, 43.1, 55.3, 63.4, 84.8, 86.9, 111.2, 113.3, 127.1, 128.0, 128.2, 130.1, 135.3, 135.4, 135.5, 135.8, 144.4, 150.2, 158.7, 163.7. ^{31}P NMR ($CDCl_3$, 80 MHz): δ 147.8 and 148.3.

1-*O*-(4,4′-Dimethoxytrityloxymethyl)-2,2-bis-propargyloxymethyl-3-*O*-(succinic-LCAA CPG)-1,3-propanediol (26). LCAA-CPG (1.00 g), 1-*O*-(4,4′-dimethoxytrityl)-2,2-bis-propargyloxymethyl-1,3-propanediol **8**[47] (0.114 g, 0.2 mmol), EDC (0.191 g, 1 mmol), DMAP (0.012 g, 0.1 mmol), and Et_3N (0.1 mL) were shaken in anhydrous pyridine (5 mL) at room temperature for 48 h. Then pentachlorophenol (135 mg, 0.5 mmol) was added, and the mixture was shaken for 10 h. Piperidine (5 mL) was added, and after 5 min of shaking, the solid support was filtered off, washed with CH_2Cl_2, and dried. A capping step with standard Cap A and Cap B solutions was applied for 2 h, and the solid support was filtered off, washed with CH_2Cl_2, and dried. Trityl assay indicated a loading of 40 μmol/g.

Oligonucleotide Synthesis. Oligonucleotides (1 μmol scale) were synthesized on an ABI 381A or 394 DNA synthesizer using a cycle involving phosphoramidite chemistry. Detritylation was performed with 2.5% DCA in CH_2Cl_2 for 60 s. Coupling step: BMT (0.3 M in dry acetonitrile) was used as activator; propynyl and bromohexyl phosphoramidites (0.09 M in CH_3CN) were introduced with a 45 s coupling time; commercially available phosphoramidites (0.09M in CH_3CN) were introduced with a 30 s coupling time. The capping step was performed with acetic anhydride using commercial solution (Cap A: Ac_2O, pyridine, THF 10/10/80 and Cap B: 10% *N*-methylimidazole in THF) for 15 s. Oxidation was performed with commercial solution of iodide (0.1 M I_2, THF, pyridine/water 90/5/5) for 10 s.

General Procedure for Azidation. Azidation of 5′-hydroxyl oligonucleotides was performed according to the procedure described by Kool et al.[38]

Azidation from bromohexyl oligonucleotides was performed as follows: A solution of NaN_3 (13 mg) and NaI (30 mg) in dry DMF (1.5 mL) was applied on the solid-supported bromohexyl oligonucleotide for 1 h 15 min at 65 °C. Then the CPG beads bearing the oligonucleotide were washed with DMF (2 × 1 mL) and CH_2Cl_2 (5 mL) and dried in a desiccator under reduced pressure for 30 min.

General Procedure for Deprotection. The beads were placed into a sealed vial and treated with concentrated aqueous ammonia (1 mL) for 24 h at room temperature for oligonucleotides containing phosphotriester functions or 2 h at room temperature and then 5 h at 55 °C for the others. The beads were filtered off, and the solution was evaporated. The residue was dissolved in water for subsequent analyses.

General Procedure for Cu(I)-Catalyzed 1,3-Dipolar Cycloaddition. To azido-alkyne oligonucleotide (∼1 μmol) were added $CuSO_4$ (0.4 equiv, 0.4 μmol, 13.2 μL of a 20 mM solution in H_2O), freshly prepared (from degassed water) sodium ascorbate (2 equiv, 2 μmol, 13.2 μL of a 100 mM solution in H_2O), methanol (100 μL), and water (23.6 μL). The tube containing the resulting preparation was flushed with argon and sealed. The reaction was placed in a microwave synthesizer Initiator from Biotage set at 100 W with a 30 s premixing time for 1 to 1 h 30 min at 60 °C. Temperature was monitored with an internal infrared probe. The solution was then desalted on NAP10.

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Supporting Information Available: HPLC profiles and MALDI-TOF MS spectra of oligonucleotides synthesized. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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