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**Synthesis of Oligonucleotides Carrying 5’-5’ Linkages Using Copper-Catalyzed
Cycloaddition Reactions**

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ABSTRACT

There is considerable interest in coupling oligonucleotides to molecules and surfaces. Although amino- and thiol-containing oligonucleotides are being successfully used for this purpose, cycloaddition reactions may offer greater advantages due to their higher chemoselectivity and speed. In this study, copper-catalyzed 1,3-dipolar cycloaddition reactions between oligonucleotides carrying azido and alkyne groups are examined. For this purpose several protocols for the preparation of oligonucleotides carrying these two groups are described. The non-templated chemical ligation of two oligonucleotides via copper-catalyzed [3+2] cycloaddition is described. Using solid-phase methodology oligonucleotides carrying 5'-5' linkages can be obtained in good yields.

Introduction.- Recent years have seen an increasing demand for oligonucleotide conjugates, while the Human Genome Project has triggered a demand for oligonucleotide chips. Oligonucleotides carrying amino and thiol groups are the most commonly used intermediates in the preparation of oligonucleotide conjugates and DNA chips. This is due to the special reactivity of thiol and amino groups, which allows formation of specific covalent bonds - thiols react with maleimido and bromoacetamido groups while aliphatic amino groups are reactive to active esters and isothiocyanates. Although these reactions are widely used, they are not completely chemoselective in aqueous solvents and hydrolysis

occurs together with the desired coupling reaction, thereby lowering the efficiency of these reactions.

This drawback has triggered the search for new chemoselective coupling reactions that may be used for the coupling of biomolecules in aqueous solvents. For example the Diels-Alder reaction has been described for the preparation of oligonucleotide conjugates [1, 2] and for the immobilization of oligonucleotides [3, 4].

A further cycloaddition reaction of interest is the [3+2] copper-mediated cycloaddition [5] or “Click Chemistry” [6]. This particular reaction has had a major impact on biomolecular research, especially in peptide [7, 8] and protein chemistry [9]. In oligonucleotide research the development of applications using Click Chemistry has been slower. This probably reflects the difficulties involved in preparing oligonucleotides modified with azido and alkynyl groups and the possible role of Cu(I) in producing hydroxyl radicals that may damage DNA [10-12]. Nevertheless, Seo *et al.* have shown that this reaction is useful for the immobilization of oligonucleotides on a chip as a first step for DNA sequencing [13, 14]. In addition, the preparation of oligonucleotide-carbohydrate conjugates [15, 16] and the synthesis of bis-nucleosides [17] using Click Chemistry have been described. Recently, the azide-alkyne cycloaddition reaction has been used for the template-mediated chemical ligation of two oligonucleotides and for the intramolecular circularization of a single oligonucleotide [18]

In this paper we seek to develop efficient protocols for the preparation of azido and alkyne groups. In addition we examine the use of the copper-catalyzed cycloaddition reaction for the preparation of oligonucleotide derivatives carrying a non-natural 5'-5' phosphate bond in the middle of the molecule. If the two halves of the oligonucleotide are complementary, oligonucleotide derivatives of this type may form parallel-stranded

duplexes [19]. If half of the hairpin is a polypurine sequence and the other half is a polypyrimidine sequence, the resulting parallel-stranded duplex will bind a polypyrimidine sequence by triple helix formation [20-22]. Our group has been especially interested in this type of oligonucleotides but until now the standard method for the preparation of parallel-stranded duplexes has required the use of reversed phosphoramidites which are less efficient and more expensive [20-22]. The present study seeks to identify the optimal conditions for preparing parallel-stranded clamps.

Results and Discussion.- 1. *Synthesis of oligonucleotides carrying alkynyl groups.* A general method for introducing functional groups at the 5'-end of oligonucleotides involves the reaction of 5'-amino-oligonucleotides with a compound carrying the desired functional group linked to a carboxylic acid. Using this approach, Seo *et al.* described the synthesis of oligonucleotides carrying a propargyl group at the 5'-end [13]. First, we used a variant of this method for the preparation of propargyl-oligonucleotides. Oligonucleotide **1** (T8-5'NH₂) was synthesized on a 1 μ mol scale. The phosphoramidite of 6-aminohexanol protected with the monomethoxytrityl [(MeO)Tr] group was used for introducing the amino group at the 5'-end. After removing the (MeO)Tr group, the resulting amino-oligonucleotide-support was treated with succinimidyl N-propargyl glutarimide [13], followed by cleavage and ammonia deprotection. Using this method, the propargyl oligonucleotide **2** was obtained in good yield and the mass spectrum of the purified compound was in agreement with the expected mass.

Alternatively we used the phosphoramidite of 10-hydroxydecanoic acid N-hydroxysuccinimide ester for the introduction of the N-hydroxysuccinimide ester group at

the 5'-end. Oligonucleotide sequences **4** (A8-5'COOH) and **6** (T8-5'COOH) were synthesized on a 1 μ mol scale. The resulting 5'-carboxy-oligonucleotide-supports were treated with propargylamine followed by ammonia deprotection. Propargyl-oligonucleotides **5** and **7** were obtained in good yields and they were characterized by mass spectrometry. This method was simpler than that described above as the use of propargylamine avoided the need to prepare succinimidyl N-propargyl glutarimide.

As a further step in the simplification process, we prepare the phosphoramidite derivative of an alcohol carrying a terminal alkyne. Commercially available 5-hexyn-1-ol was reacted with chloro-N,N-diisopropylamino-O-(2-cyanoethoxy) phosphine yielding the desired phosphoramidite. This phosphoramidite was used to introduce an alkynyl group at the 5'-end of oligonucleotides **11** (GA-5'alkynyl) and **12** (CT-5'alkynyl). Alkynyl-oligonucleotides **11** and **12** were obtained in excellent yields and they were characterized by mass spectrometry. No oxidation products resulting from the interaction of the iodine solution used in the DNA synthesizer and the alkyne function were observed.

2. *Synthesis of oligonucleotides carrying azido groups.* The azido group is not compatible with the phosphoramidite group because azido groups react with phosphites yielding phosphoramidates (Staudinger reaction [23]). For this reason azido groups need to be introduced in the oligonucleotide after the completion of the sequence. The preparation of oligonucleotides carrying azidonucleosides has also shown that azido groups attached to the nucleobases are stable to ammonia solutions only at room temperature, but not at higher temperature [24-26].

First we used the method described by Seo *et al* [13] for the preparation of oligonucleotides carrying 5-azido groups introducing some modifications. Oligonucleotide **1** (T8-5'NH₂) was synthesized as described above. After the removal of the (MeO)Tr group the resulting amino-oligonucleotide-support was treated with 5-azidopentanoic acid N-hydroxysuccinimide ester [13]. The resulting support was treated with concentrated ammonia at room temperature to avoid azide decomposition [24-26]. Oligonucleotide **3** carrying an azido group at the 5'-end was obtained in good yields as determined by HPLC analysis. The purified product had the expected mass.

A second protocol based on the iodination of the 5'-end followed by azide displacement was studied. Oligonucleotide **8** (CT) was synthesized on a 1 µmol scale and the last dimethoxytrityl [(MeO)₂Tr] group was removed. The resulting support was treated with triphenoxymethylphosphonium iodide as described by Miller and Kool [27] to yield the iodo-oligonucleotide **9** (CT_I) and the resulting support was treated with sodium azide [11]. Finally, the support was treated with ammonia to yield the 5'-azido-oligonucleotide **10** (CT_N3) in excellent yields as determined by HPLC analysis. The purified oligonucleotide was characterized by mass spectrometry and enzymatic digestion using snake venom phosphodiesterase and alkaline phosphatase followed by HPLC analysis [25] showing the presence of 5-azido-2',5'-dideoxycytosine.

The success of the previous method suggested the need to prepare of a phosphoramidite to introduce the haloethyl group at the 5'-end of the oligonucleotide. We decided to study the potential use of the bromoethyl group in DNA as an intermediate group in the introduction of the azido group. In addition the ethyl linker would provide less

steric hindrance to the azido group than the previous 5'-azido-2'-deoxynucleoside derivative.

Starting from commercially available 6-bromohexanol, the phosphoramidite derivative was prepared. This phosphoramidite was introduced in the DNA synthesizer and incorporated into the CT oligonucleotide sequence **13**. The support carrying the 5'-bromo oligonucleotide was treated with sodium azide and the resulting support was treated with concentrated ammonia at room temperature, giving the desired 5'-azido-oligonucleotide **14** (CT_N3hexyl) in good yields. The purified oligonucleotide had the expected mass.

3. Cu-catalyzed cycloaddition of azido-oligonucleotides and alkynyl-oligonucleotides.

Next, the use of copper-catalyzed cycloaddition reactions to chemically ligate two oligonucleotides was studied. In order to find the optimal conditions for the coupling reaction, a small excess of T8-5'propargyl (**2**) was mixed with T8-5'azide (**3**) in the presence of either CuSO₄/ascorbic acid or CuI as catalyst. Best results were obtained when a large excess (10-25 times excess) of copper catalyst was used (*Figure 1*). When the copper catalyst was only 0.1 equivalents, yields were between 10-15%. The length of the product of cycloaddition was confirmed by gel electrophoresis. Mass spectrometry gave a higher mass than expected probably due to the presence of copper ions that were not completely eliminated by HPLC purification.

In order to facilitate the purification and removal of the copper ions we studied the Cu-catalyzed cycloaddition reactions on the solid support. The solid support carrying oligonucleotide sequence T8-5'azide (**3**) was treated with 2 equivalents of T8-5'propargyl (**2**) using a large excess of CuI as catalyst. The resulting support was treated with

concentrated ammonia. Analysis of the reaction by HPLC showed the formation of the expected product **15** as the major component (data not shown).

Likewise the solid support carrying oligonucleotide sequence CT-N3hexyl (**14**) was reacted with T8-5'propargyl (**7**) and CuI to yield oligonucleotide **17** (*Figure 2*). This was also characterized by mass spectrometry and gel electrophoresis.

Tris(benzyltriazolylmethyl)amine (TBTA) has been recommended as a copper ligand to enhance speed and prevent damage to the oligonucleotides [11, 12]. We compared the results using TBTA and CuI as catalysts in the reaction between CT-N3hexyl (**14**) and T8-5'propargyl (**7**) to yield oligonucleotide **16**. Although the resulting chromatogram was slightly better when using TBTA, no great differences in the yield were observed. It is important to notice that the use of Click Chemistry in the oligonucleotide field is strongly influenced from the negative results described by Kanan et al [12]. These authors treated 17 pmols of an oligonucleotide with a large excess of copper sulphate/ ascorbic acid (more than 2000 time molar excess) and found approximately 50% degradation of the oligonucleotide after 10 min at room temperature [12]. These conditions are far away from the preparative work such as the study described here (150-300 nmols of oligonucleotide in the presence of 10-20 molar excess of copper). In our conditions we found only a slight degradation after 2-3 days of treatment as seen by the fast eluting peaks in front of the desired compound (*Figure 2*).

Next, the solid support carrying oligonucleotide sequence T8-5'azide (**3**) was treated with A8-5'propargyl (**5**) and CuI (3 mg) as described above. After the reaction, the support was extensively washed to eliminate the copper ions. The desired oligonucleotide **17** was obtained in good yield. The purified oligonucleotide **17** was characterized by UV, mass

spectrometry, gel electrophoresis and enzymatic digestion. CD spectra of the purified **17** show the presence of a parallel duplex structure as expected (*Figure 3*).

When cycloaddition reactions were performed using oligonucleotides **11** and **12** carrying alkynyl groups the cycloaddition reaction resulted in low yields and the final product could not be isolated (data not shown). Nevertheless, reaction of 5-hexyn-1-ol with 5'-azidothymidine and oligonucleotides **11** and **12** with benzylazide gave the expected cycloaddition products (*Figure 4*). Most probably the 5-hexynyl group is not reactive enough to link two large molecules such as the oligonucleotides, but it may be used for linking small organic molecules to oligonucleotides. This result is in agreement with Click reactions involving alkynes without a neighbouring electron-withdrawing group [28, 29].

Conclusions. The synthesis of oligonucleotides carrying alkynyl and the synthesis of oligonucleotides carrying azido groups were carried out using three methods. We have demonstrated that the 5'-ends of two oligonucleotides can be chemically linked using a Cu-catalyzed cycloaddition reaction with the following observations: A) An excess of copper ions is required. B) CuI is a more efficient catalyst than CuSO₄/ascorbic acid. C) The use of the azido-oligonucleotide anchored still in the solid phase allows the efficient removal of the excess of copper ions. D) 5-Hexynyl groups are not reactive enough to produce the cycloaddition products between oligonucleotides.

Oligonucleotides with a parallel duplex structure with 5'-5' linkages are of interest for their triplex-forming properties [19-22]. The synthesis of these compounds by the linking of two parts is a considerable challenge. Previously we sought to link these two parts using thiol and maleimido groups but our attempts were unsuccessful. Using Click Chemistry,

however, this synthesis has been possible. We believe that the power of this reaction will enable a large number of oligonucleotide conjugates to be synthesized.

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Experimental Part

General. Phosphoramidites and ancillary reagents used during oligonucleotide synthesis were from *Applied Biosystems* (*PE Biosystems Hispania S.A.*, Spain), *Link technologies* (*Link Technologies Ltd*, Scotland) and *Glen Research* (*Glen Research Inc.*, USA). The rest of the chemicals were purchased from *Aldrich*, *Sigma* or *Fluka* (*Sigma-Aldrich Química S.A.*, Spain). Solvents were from *S.D.S.* (*S.D.S.*, France).

Instrumentation. ^1H -NMR spectra were measured at 300 MHz on a Varian spectrometer and ^{13}C NMR spectra were measured at 75 MHz. ^{31}P NMR spectra were recorded at 121 MHz and were externally referenced to 85% phosphoric acid. UV spectra were recorded on an UV-2301PC *Shimadzu* spectrophotometers. Mass spectra (electrospray or matrix-assisted laser desorption ionization time-of-flight, MALDI-TOF) were done at the Mass

spectrometry service at the University of Barcelona. CD spectra were recorded on a Jasco J-810 spectropolarimeter.

2-Cyanoethyl hex-5-ynyl- N,N-Diisopropylphosphoramidite. 5-hexyn-1-ol (0.54 ml, 5 mmol) was dissolved in dry acetonitrile (6 ml) under argon and N,N-diisopropylethylamine (2.6 ml, 15 mmol) was added with exclusion of moisture. The solution was cooled on ice and 2-cyanoethoxy-N,N- diisopropylaminochlorophosphine (1.7 ml, 7.5 mmol) was added dropwise. The solution was stirred at room temperature for 2 hours. The solvent was then removed *in vacuo* and the residue dissolved in dichloromethane with 1 % triethylamine. The solution was washed with H₂O and brine solution, dried over Na₂SO₄, and evaporated. The crude product was purified by silica gel column chromatography (1:9 ethyl acetate/hexane with 4 % triethylamine) to give the desired phosphoramidite (670 mg) as a pale yellow oil in 46 % yield. ¹H NMR (CDCl₃) δ_H: 3.90-3.55 (m, 6H), 2.64 (t, J = 5.4 Hz, 2H), 2.23 (t, J = 6.9 Hz, 2H), 1.95 (t, J = 2.7 Hz, 1H), 1.78-1.57 (m, 4H), 1.18 (d, J = 6.9 Hz, 12H); ¹³C NMR (CDCl₃) δ_C (two diastereoisomers): 117.6, 84.2, 68.5, 63.2 and 63.0, 58.4 and 58.2, 43.1 and 42.9, 30.2 and 30.1, 24.7 and 24.6, 20.4 and 20.3, 18.1; ³¹P NMR (CDCl₃) δ_P: 147.76; MS(CI) Found 299.5 [M + H⁺] (expected for C₁₅H₂₇N₂O₂P 298.4).

2-Cyanoethyl-6-bromohexyl-N,N-Diisopropylphosphoramidite. 6-bromohexanol (0.26 ml, 2 mmol) was dissolved in dry acetonitrile (4 ml) under argon and N,N-diisopropylethylamine (1 ml, 6 mmol) was added with exclusion of moisture. The solution was cooled on ice and 2-cyanoethoxy-N,N- diisopropylaminochlorophosphine (0.7 ml, 3 mmol) was added dropwise. The solution was stirred at room temperature for 2.5 hours.

The solvent was then removed *in vacuo* and the residue dissolved in dichloromethane with 1 % triethylamine. The solution was washed with H₂O and brine solution, dried over Na₂SO₄, and evaporated. The crude product was purified by silica gel column chromatography (1:9 ethyl acetate/hexane with 4 % triethylamine) to give the desired phosphoramidite (360 mg) as a pale yellow oil in 47 % yield. ¹H NMR (CDCl₃) δ_H: 3.9-3.76 (m, 2H), 3.7-3.52 (m, 4H), 3.41 (t, J = 6.9 Hz, 2H, -CH₂Br), 2.64 (t, J = 6.6 Hz, 2H), 1.92-1.82 (m, 2H), 1.67-1.59 (m, 2H), 1.50-1.36 (m, 4H), 2.35 (d, J = 6.6 Hz, 12H); ¹³C NMR (CDCl₃) δ_C (two diastereoisomers): 117.6, 63.6 and 63.4, 58.4 and 58.2, 43.1 and 42.9, 33.8, 32.7, 31.0 and 30.9, 27.8, 25.2, 24.7 and 24.6 and 24.5 (4 -CH₃), 20.4 and 20.3; ³¹P NMR (CDCl₃) δ_P: 147.73; MS(ES) Found 381.3 [M + H⁺] (expected for C₁₅H₃₀BrN₂O₂P 381.3).

Oligonucleotide synthesis. Oligonucleotides sequences were prepared using solid-phase methodology and 2-cyanoethyl phosphoramidites as monomers. The syntheses were performed on an Applied Biosystems Model 3400 DNA synthesizer using 0.2 and 1 μmol scales. After the assembly of sequences, ammonia deprotection was performed overnight at 55 °C. Oligonucleotides were purified by reverse-phase HPLC. HPLC solutions are as follows. Solvent A: 5% ACN in 100 mM triethylammonium acetate (pH 6.5) and solvent B: 70% ACN in 100 mM triethylammonium acetate pH 6.5. Columns: Nucleosil 120C18 (10 μm), 200 x 10 mm. Flow rate: 3 ml/min. Conditions A: 20 min linear gradient from 15-80% B. Conditions B: 20 min linear gradient from 0-50% B. Conditions C: 20 min linear gradient from 5-35% B.

Synthesis of oligonucleotides carrying alkynyl groups.

Method 1. Synthesis of oligonucleotides carrying a propargyl group at the 5'-end using the 5'-amino-oligonucleotides. Oligonucleotide **1** (T8-5'NH₂, *Table 1*) was synthesized on 1 μ mol scale. The phosphoramidite of 6-(4-monomethoxytrityl [(MeO)Tr])-aminohexanol was used for the introduction of the amino group at the 5'-end. After the removal of the (MeO)Tr group the resulting amino-oligonucleotide-support was treated with 20 times excess of succinimidyl N-propargyl glutarimide [13] in dioxane for 1 hr at room temperature. The resulting support was washed and treated with concentrated ammonia at room temperature for 2 hours. Oligonucleotide **2** was purified by reverse-phase HPLC (Conditions B). The desired oligonucleotide **2** eluted at 12.5 min (starting T8-5'NH₂ eluted at 11 min). Mass spectrometry (electrospray): Found 2760 (M+ 3Na⁺); expected for C₉₄H₁₂₈N₁₈O₅₉P₈, 2701.6.

Method 2. Synthesis of oligonucleotides carrying a propargyl group at the 5'-end using the 5'-carboxy-oligonucleotides. Oligonucleotides **4** (A8-5'COOH) and **6** (T8-5'COOH) were synthesized on 1 μ mol scale. The phosphoramidite of 10-hydroxydecanoic acid N-hydroxysuccinimide ester (5'carboxy modifier C10, Glen Research) was used for the introduction of the N-hydroxysuccinimide ester group at the 5'-end. The resulting 5'-carboxy-oligonucleotide-supports were treated with 10 times excess of propargylamine in dichloromethane carrying 10% triethylamine for 4 hours at room temperature. The resulting supports were washed and treated with concentrated ammonia at 55 °C for 3 hours. Oligonucleotides **5** and **7** were purified by reverse-phase HPLC. Oligonucleotide **5** eluted at 10.8 min (conditions B). Mass spectrometry found 2728 (M-H); expected for

$C_{93}H_{119}N_{41}O_{42}P_8$, 2730.6. Oligonucleotide **7** eluted at 14.8 min (conditions C). Mass spectrometry: Found 2657.4 (M-H); expected for $C_{93}H_{127}N_{17}O_{58}P_8$, 2658.4.

Method 3. Synthesis of oligonucleotides carrying an alkynyl group at the 5'-end using the phosphoramidite derivative of 5-hexyn-1-ol. The 5-hexyn-1-ol phosphoramidite was used for the introduction of an alkynyl group at the 5'-end of oligonucleotides **11** (GA-5'alkynyl) and **12** (CT-5'alkynyl). After ammonia deprotection, the resulting oligonucleotides were purified by reverse-phase HPLC. Oligonucleotide **11** eluted at 10.3 min (conditions B). Mass spectrometry (MALDI): Found 3636.3 (M-H); expected for $C_{116}H_{142}N_{55}O_{62}P_{11}$, 3638.9. Oligonucleotide **12** eluted at 9.7 min (conditions B). Mass spectrometry (MALDI): Found 3351 (M-H); expected for $C_{110}H_{147}N_{28}O_{72}P_{11}$, 3353.8.

Synthesis of oligonucleotides carrying azido groups

Method 1. Synthesis of oligonucleotides carrying an azido group at the 5'-end using the 5'-amino-oligonucleotides. Oligonucleotide **1** (T8-5'NH₂) was synthesized on 1 μ mol scale as described above. After the removal of the (MeO)Tr group the resulting amino-oligonucleotide-support was treated with 20 times excess of succinimidyl 5-azido valerate [12] in dioxane for 1 hr at room temperature. The resulting support was washed and treated with concentrated ammonia at room temperature for 2 hours. Oligonucleotide **3** was purified by reverse-phase HPLC as described above (Conditions B). The desired oligonucleotide **3** eluted at 13.2 min (starting T8-5'NH₂ eluted at 11 min). Mass spectrometry (electrospray): Found 2716 (M+ 2Na⁺); expected for $C_{91}H_{126}N_{20}O_{58}P_8$, 2675.5.

Method 2. Synthesis of oligonucleotides carrying a propargyl group at the 5'-end using the iodination followed by azide displacement. Oligonucleotide **8** (CT) was synthesized on 1 μ mol scale and the last DMT group was removed. An aliquot of this support was treated with concentrated ammonia to yield oligonucleotide **8** (CT) which was purified by HPLC (retention time, conditions B, 9.7 min) and characterized by mass spectrometry (electrospray, found 3194.7 expected for $C_{104}H_{138}N_{28}O_{69}P_{10}$, 3193.7). The resulting support was treated with triphenoxymethylphosphonium iodide (68 mg, 1 mmol) [24] in DMF (1 ml) to yield the iodo-oligonucleotide **9** (CT_I). An aliquot of this support was treated with concentrated ammonia to yield oligonucleotide **9** (CT_I) which was purified by HPLC (retention time, conditions B, 10.9 min) and characterized by mass spectrometry (MALDI, found 3302.2 (M-H) expected for $C_{104}H_{137}N_{28}O_{68}P_{10}I$, 3303.6). Finally the support carrying the 5'-iodo oligonucleotide was treated with sodium azide in DMF overnight at room temperature. The resulting support was treated with concentrated ammonia at room temperature for 3 hours yielding the desired 5'-azido-oligonucleotide **10** (CT_N3). Oligonucleotide **10** was purified by reverse-phase HPLC (Conditions B) eluting at 10.4 min. Mass spectrometry (MALDI) found 3216.9 (M-H) expected for $C_{104}H_{137}N_{31}O_{68}P_{10}$, 3218.7). The presence of 5-azido-2',5'-dideoxycytosine at the 5'-end was also confirmed by enzymatic digestion of the purified 5'-azido-oligonucleotide using snake venom phosphodiesterase and alkaline phosphatase followed by HPLC analysis of the resulting nucleosides [25].

Method 3. Synthesis of oligonucleotides carrying a propargyl group at the 5'-end using the phosphoramidite derivative of 6-bromohexanol. The phosphoramidite of 6-bromohexanol was introduced in the DNA synthesizer and incorporated into the CT oligonucleotide sequence **13**. The support carrying the 5'-bromo oligonucleotide was treated with sodium azide in DMF overnight at room temperature. The resulting support was treated with concentrated ammonia at room temperature for 3 hours yielding the desired 5'-azido-oligonucleotide **14** (CT_N3hexyl). Oligonucleotide **14** was purified by reverse-phase HPLC (conditions C) eluting at 12 min. Mass spectrometry (MALDI): Found 3397.8 (M-H) expected for $C_{110}H_{150}N_{31}O_{72}P_{11}$, 3398.8.

Cu-catalyzed cycloaddition of azido-oligonucleotides and alkynyl-oligonucleotides.

T8-5' propargyl (**2**) and T8-5' azide (**3**) to yield oligonucleotide **15** (solution phase). In order to find the optimal conditions for the coupling reaction a small excess of T8-5' propargyl (**2**) was mixed with T8-5' azide (**3**) in the presence of either $CuSO_4$ / ascorbic acid or CuI as catalyst. In all cases we used T8-5' propargyl (**2**, 19.5 nmol), and T8-5' azide (**3**, 15 nmol). Four conditions were tested:

- 1) $CuSO_4$ / ascorbic in low amounts: 2 nmol (0.1 eq) of $CuSO_4$ and 10 nmol (0.5 eq) of ascorbic acid in 0.35 ml of water / 0.15 ml *tert*-butanol, room temperature under argon atmosphere, 48 hours.
- 2) $CuSO_4$ / ascorbic in low amounts, longer time: 2 nmol (0.1 eq) of $CuSO_4$ and 10 nmol (0.5 eq) of ascorbic acid in 0.05 ml of water / 0.025 ml *tert*-butanol, room temperature under argon atmosphere, 72 hours.

3) CuSO₄/ ascorbic in excess: 200 nmol (10 eq) of CuSO₄ and 1000 nmol (50 eq) of ascorbic acid in 0.05 ml of water / 0.025 ml *tert*-butanol, room temperature under argon atmosphere, 72 hours with stirring.

4) CuI in excess: 500 nmol (25 eq) of CuI and 6 μmols of DIPEA in 0.03 ml of water / 0.03 ml of acetonitrile, room temperature under argon atmosphere, 40 hours with stirring.

Best results were obtained in trials 3-4 where an excess of the copper catalysts was used (75% yield). When copper catalyst was only 0.1 equivalents (trials 1-2) yields were between 10-15%. The presence of the product of cycloaddition was confirmed by gel electrophoresis. Mass spectrometry gave a higher than expected mass (found 6157, expected for C₁₈₅H₂₅₄N₃₈O₁₁₇P₁₆, 5377.8) probably due to the presence of copper ions that were not completely eliminated from the reaction.

T8-5'propargyl (**7**) and CT-N3hexyl (**14**) to yield oligonucleotide **16** (solid phase). The solid support carrying oligonucleotide sequence CT-N3hexyl (**14**, 100 nmol) was treated with T8-5'propargyl (**7**, 500 nmols) in the presence of CuI (4 mg), 4 μl of DIPEA and 2 mg of ascorbic acid 0.2 ml of water / acetonitrile (1:1) at room temperature, stirring for 48 hours. After the reaction, the support was extensively washed with acetonitrile, a solution of ascorbic acid (0.02 g/ ml) in water, water, 0.1M EDTA, water and dichloromethane/methanol (1:1). The resulting support was treated with concentrated ammonia at 55 °C for 6 hours. Oligonucleotide **16** was purified by reverse-phase HPLC as described above (*Figure 2*). The desired compound was obtained in a 38% yield. The purified compound was further analyzed by gel electrophoresis. Mass spectrometry (MALDI): Found 6051.5 (M -H) expected for C₂₀₃H₂₇₇N₄₈O₁₃₀P₁₉, 6056). A similar

experiment was performed but adding 4 mg of TBTA in the mixture. The HPLC profile of the final product was similar to the experiment without TBTA. The desired compound was obtained in a 41% yield.

A8-5'propargyl (**5**) and T8-5'azide (**3**) to yield oligonucleotide **17** (solid phase). The solid support carrying oligonucleotide sequence T8-5'azide (**3**, 150 nmol) was treated with A8-5'propargyl (**5**, 300 nmols) in the presence of CuI (3 mg) and 5 μ l of DIPEA, as described above. After the reaction, the support was extensively washed with acetonitrile, dimethylformamide (DMF), a solution of ascorbic acid (0.02 g/ ml) in DMF/ pyridine 6:5, DMF, water 0.1M EDTA, water, DMF and dichloromethane/ methanol (1:1). The resulting support was treated with concentrated ammonia at 55 °C for 6 hours. Oligonucleotide **17** was purified by reverse-phase HPLC as described above (Conditions C). The desired oligonucleotide **17** eluted at 14.3 min and was obtained in a 40% yield. Mass spectrometry (MALDI) found 5501 ($M + Cu^{2+} + Na^{+}$) expected for $C_{184}H_{245}N_{61}O_{100}P_{16}$, 5415.9).

The desired compound was characterized by UV and enzymatic digestion. CD spectra show the presence of a parallel duplex structure as expected (*Figure 3*).

CT-5'alkynyl (**12**) and benzyl azide (solid phase). The solid support carrying oligonucleotide sequence CT-5'alkynyl (**12**, 1.6 mg, 50 nmols) was treated with benzyl azide (2 μ l) in the presence of CuI (4 mg), 4 μ l of DIPEA and 2 mg of ascorbic acid at room temperature, in 0.15 ml of water/acetonitrile (1:1) stirring for 17 hours. After the reaction, the support was extensively washed with acetonitrile, a solution of ascorbic acid (0.02 g/ ml) in water, water, 0.1M EDTA, water and dichloromethane/methanol (1:1). The

desired compound was obtained as a major product (*Figure 4*). Mass spectrometry (MALDI): Found 3489.5, expected 3484.2).

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Table 1: Sequences of Oligonucleotides Prepared.

Number	Oligonucleotide	Sequence ^a
1	T8-5'NH ₂	5'-NH ₂ -(CH ₂) ₆ -TTTTTTTT-3'
2	T8-5'propargyl	5'-CHC-CH ₂ NHCO(CH ₂) ₃ CONH-(CH ₂) ₆ -TTTTTTTT-3'
3	T8-5'azido	5'-N ₃ -(CH ₂) ₄ CONH-(CH ₂) ₆ -TTTTTTTT-3'
4	A8-5'COOH	5'-SUOOC-(CH ₂) ₉ -AAAAAAAA-3'
5	A8-5'propargyl	5'-CHC-CH ₂ NHCO-(CH ₂) ₉ -AAAAAAAA-3'
6	T8-5'COOH	5'-SUOOC-(CH ₂) ₉ -TTTTTTTT-3'
7	T8-5'propargyl	5'-CHC-CH ₂ NHCO-(CH ₂) ₉ -TTTTTTTT-3'
8	CT	5'-CTTCCTCCTCT-3'
9	CT_I	5'-I-CTTCCTCCTCT-3'
10	CT_N3	5'-N ₃ -CTTCCTCCTCT-3'
11	GA-5'alkynyl	5'-CHC-(CH ₂) ₄ -GAAGGAGGAGA-3'
12	CT-5'alkynyl	5'-CHC-(CH ₂) ₄ -CTTCCTCCTCT-3'
13	CT_Br	5'-Br-(CH ₂) ₆ -CTTCCTCCTCT-3'
14	CT_N3hexyl	5'-N ₃ -(CH ₂) ₆ -CTTCCTCCTCT-3'
15	T8-5'-5'-T8	3'-TTTTTTTT-5'-(CH ₂) ₆ -NHCO(CH ₂) ₄ -tri-CH ₂ NHCO(CH ₂) ₃ CONH-(CH ₂) ₆ -TTTTTTTT-3'
16	CT-5'-5'-T8	3'-TCTCCTCCTTC-5'-(CH ₂) ₆ -NHCO(CH ₂) ₄ -tri-CH ₂ NHCO-(CH ₂) ₉ -TTTTTTTT-3'
17	T8-5'-5'-A8	3'-TTTTTTTT-5'-(CH ₂) ₆ -NHCO(CH ₂) ₄ -tri-CH ₂ NHCO-(CH ₂) ₉ -AAAAAAAA-3'

^a SU: N-hydroxysuccinimide ester, tri: 1,2,3-triazol.

LEGENDS

Scheme 1. Copper-Catalyzed [3+2] Cycloaddition or Click Chemistry Between Oligonucleotides Carrying Azido and Alkynyl Groups.

Scheme 2. Protocols for the Synthesis of Oligonucleotides Carrying Alkynyl Groups at the 5'-end: A) using 5'-amino-oligonucleotides, B) using 5'-carboxy-oligonucleotides and C) using the phosphoramidite derivative of 5-hexyn-1-ol.

Scheme 3. Protocols for the Synthesis of Oligonucleotides Carrying Azido Groups at the 5'-end: A) using 5'-amino-oligonucleotides, B) via 5'-iodo-oligonucleotides, and C) using the phosphoramidite derivative of 6-bromohexanol.

Figure 1. *HPLC profile of Cu-catalyzed cycloaddition between T8-5'propargyl (2) and T8-5'azido (3) to yield oligonucleotide 15 (solution phase) using 19 eq of CuSO₄/ascorbic acid (trial 3).*

Figure 2. *HPLC profile Cu-catalyzed cycloaddition between T8-5'propargyl (7) and CT-N3hexyl (14) to yield oligonucleotide 16 (solid phase) using CuI.*

Figure 3. *CD spectra of oligonucleotide 17 and T8-5'azido 3 (1M NaCl, 50 mM sodium phosphate/ citric acid pH 7.0). The CD spectrum of oligonucleotide 17 contains a strong*

maximum at 217 nm and a strong minimum at 248 nm as described for parallel reversed Watson-Crick [15].

Figure 4. *HPLC profile of Cu-catalyzed cycloaddition between CT-5'alkynyl (12) and benzyl azide.*

Scheme 1

Scheme 2

Scheme 3

Figure 1

Figure 2

Figure 3

Figure 4

Graphical illustration for the table of contents