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Phthalimide-oxy derivatives for 3'- or 5'-Conjugation of Oligonucleotides by Oxime Ligation and Circularization of DNAs by "Bis- or Tris-Click" Oxime Ligation

Albert Meyer, Jean-Jacques Vasseur, Pascal Dumy and François Morvan*

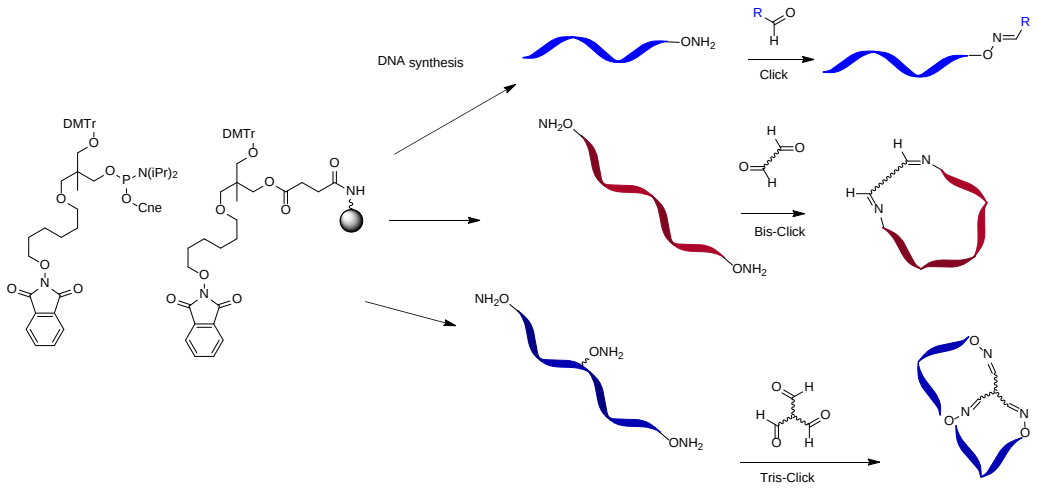
Institut des Biomolécules Max Mousseron, IBMM, UMR 5247, Université Montpellier, CNRS, ENSCM, 34095 Montpellier Cedex 5, France. Francois.morvan@umontpellier.fr

ABSTRACT

A solid support and two phosphoramidites exhibiting a phthalimide-oxy group were synthesized. First, after treatment with hydrazine, the resulting 5'- or 3'-oxyamine oligonucleotides were conjugated with aldehyde derivatives by oxime ligation. Second, oligonucleotides exhibiting at each end an oxyamine were circularized by means of different dialdehydes. The cyclic oligonucleotides of different lengths (9 to 31-mer) were rapidly obtained without the need of a template. Finally, a bis-cyclic oligonucleotide was synthesized starting form an oligonucleotide bearing three oxyamines which reacted with a trialdehyde forming three oxime ligations.

Modified oligonucleotides

Graphical abstract



Novel phthalimide-oxy phosphoramidites and a solid support were used to obtain oligonucleotides bearing one, two or three oxyamines for their subsequent conjugation, mono- and di-circularization by oxime ligation with mono-, di- or trialdehydes.

INTRODUCTION

Cyclic DNA and RNA exhibit unusual chemical and biological properties in comparison with linear ones, and have been evaluated for several biological applications such as antisense, triplex, and diagnostic applications.[1-5] First cyclic DNAs have been synthesized in the late 1980s[1,6-9] and new synthesis of cyclic DNAs and RNAs have been are reported along the years. Their synthesis was performed using enzymatic or chemical methods affording them with phosphodiester,[1,10-13] phosphorothiolatediester,[14-17] disulfide[18-20] oxime[17,21] and more recently with a triazole linker[22-29] As a rule cyclic oligonucleotides are synthesized thanks to complementary chemical functions borne on both ends of the sequence. Alternatively, the same chemical function could be introduced at each end and then the circularization performed with a linker bearing twice the complementary function. This strategy was recently applied to form cyclic oligonucleotides by Copper catalyzed Azide Alkyne Cycloaddition (CuAAC)[30-31] starting from a linear strand exhibiting an alkyne function at each end, and a linker bearing two azides.[26,28-29,32]

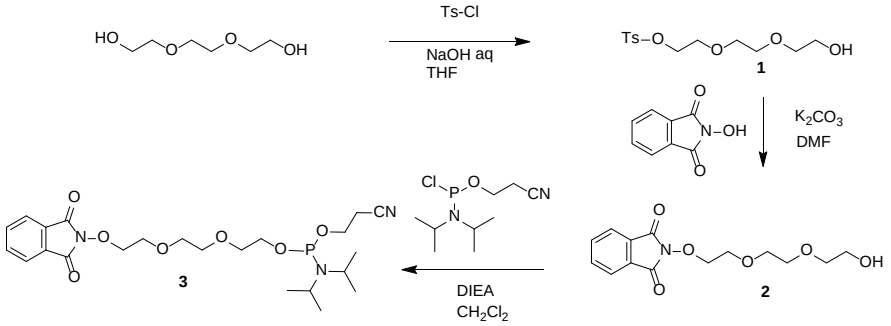
Herein, we report a method for the preparation of cyclic of oligonucleotides (9 to 31-mers) by two oxime ligations. To this purpose, a new solid support and phosphoramidites with a phthalimide-oxy group were prepared. Firstly, the solid support was used for the 3'-conjugation of oligonucleotides. Secondly in combination with the phthalimide-oxy phosphoramidites,

oligonucleotides with aminooxy function at both ends were synthesized. Their circularization was performed by different dialdehydes leading to cyclic oligonucleotides with two oxime linkages. As an extension of the scope of this strategy, a dumbbell structure was prepared bearing an alkyne function in a loop for derivatization with an azide dansyl through a CuAAC.

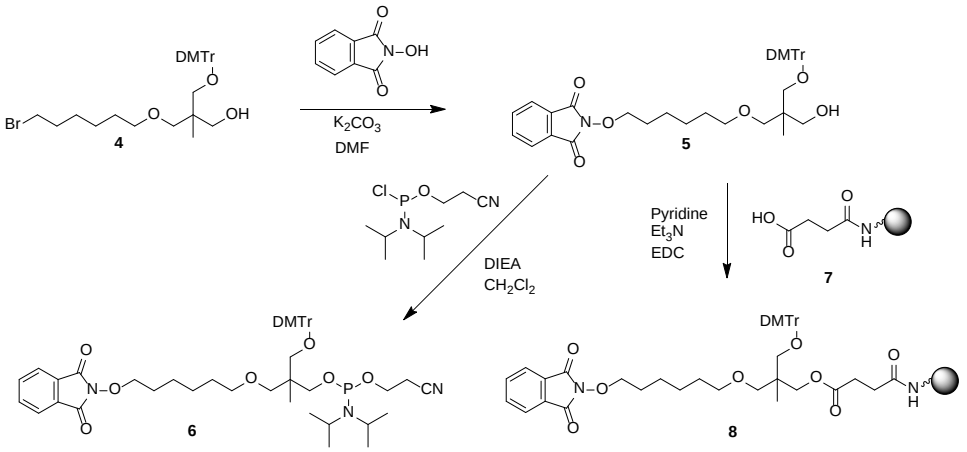
RESULTS AND DISCUSSION

Conjugation of oligonucleotides through oxime mainly occurred by the introduction of an aldehyde function into an oligonucleotide, either at the 3' or 5'-end, reacting then with the aminooxy derivatives.[33] There are only few examples of phosphoramidite derivatives allowing the introduction of the aminooxy in the oligonucleotides[21,34-36] Surprisingly, none solid support has been reported, so far, to introduce an aminooxy function at the 3'-end of an oligonucleotide for subsequent conjugation with an aldehyde derivative.

Synthesis of building blocks exhibiting a phthalimide-oxy group. Two phosphoramidites **3** and **6** and a solid support **8** bearing a phthalimide-oxy group were synthesized in order to introduce aminooxy functions at the 5'- or/and 3'-end of an oligonucleotide. Compound **3** was prepared in three steps (Scheme 1). Firstly, triethyleneglycol was monotosylated[37] and secondly, the tosyl was substituted by hydroxyphthalimide in presence of K_2CO_3[38] under microwaves assistance giving **2**. Thirdly, compound **2** was phosphitylated with 2-cyanoethyl-*N*,*N*-diisopropylchloro phosphoroamidite in presence of diisopropylethylamine affording the phosphoramidite derivative **3**. Similarly 1-*O*-(4,4′-dimethoxytrityl)-2-(6-bromohexyloxymethyl)-2-methyl-1,3-propanediol **4**[39] was substituted with hydroxyphthalimide giving **5** and then converted into its phosphoramidite derivative **6** or anchored on a succinyl LCAA-CPG solid support **7** using *N*-(3-dimethl aminopropyl)-*N*′-ethylcarbodiimide, as coupling agent, affording the solid support **8** (Scheme 2). A loading of 35 μmol/g was calculated by trityl assay.



Scheme 1. Synthesis of phthalimideoxy-triethyleneglycol phosphoramidite 3.



Scheme 2. Synthesis of phosphoramidite **6** and solid support **8** exhibiting a phthalimide-oxy-hexyl group.

The phosphoramidites **3** and **6** and the solid support **8** were validated on a hexathymidylate. On one hand, a T_6 was elongated starting from solid support **8** by standard phosphoramidite chemistry (Scheme 3) and on the other hand a T_6 was elongated from commercially available thymidine solid support and **3** or **6** was coupled using 20 eq and a 60 sec coupling step (Scheme 4).

A two-step deprotection protocol was applied with first a 30 min treatment with hydrazinium acetate in pyridine and second a 2 h aqueous ammonia treatment both at room temperature. [36] After ammonia evaporation, 3'- and 5'-aminooxyhexyl-THME-T_6 (**11** and **23** respectively) were analyzed by C_{18} reverse phase HPLC and characterized by MALDI-TOF mass spectrometry. While MS showed only the formation of the expected aminooxy-T_6 ($[M-H]^-$, m/z = 2059.50), the HPLC profile of **11** and **23** showed two main peaks and also small peaks before and after them (Fig. 1). This unexpected behavior was investigated. As an aminooxy function reacts rapidly and easily with any traces of aldehyde or ketone present in air or HPLC buffer, the aminooxy oligonucleotides are usually treated with acetone before their HPLC analysis.[35] So, aminooxy-T_6 were treated with acetone to form the corresponding adducts **12** and **24**. Both crude HPLC profiles showed only one peak which was characterized by MS as the expected oxime ($[M-H]^-$ = 2099.56) (Fig. 2). To explain this phenomenon, we can hypothesize that the

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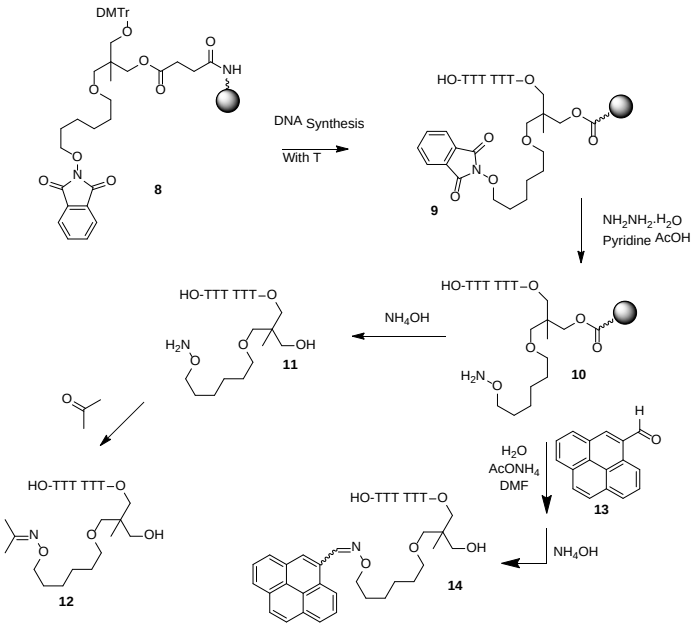
aminooxy function forms some interaction with the oligonucleotide leading to a constraint structure. Since the THME motif exhibits a chiral carbon, the two diastereoisomers are eluted differently. In contrast, when the oxime function is formed with the acetone, there is no more interaction and the difference between both diastereoisomers is no more visible by HPLC. We have already observed such phenomenon with cyclic and linear oligonucleotides built with this THME linkage.[25] For the aminooxy-EG_3-T_6 **18** and the corresponding acetone capped oligonucleotide **19**, there is no chiral center and so a single peak was visualized (Fig. 1-2). According to this fact, the HPLC profiles of aminooxy oligonucleotides, especially when a THME motif is present, do not give a realistic view of their purity while MALDI-TOS MS does even if this technique is not quantitative.



Scheme 3. 3'-Modified hexathymidylates

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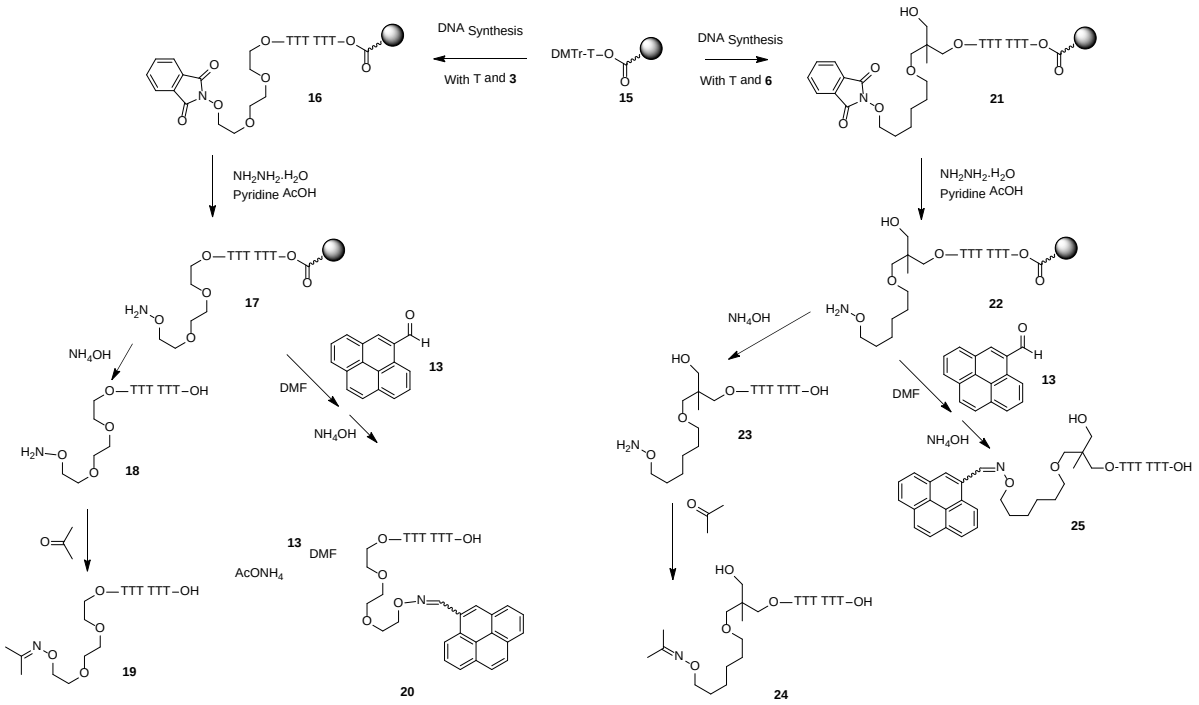

Scheme 4. 5'-Modified hexathymidylates

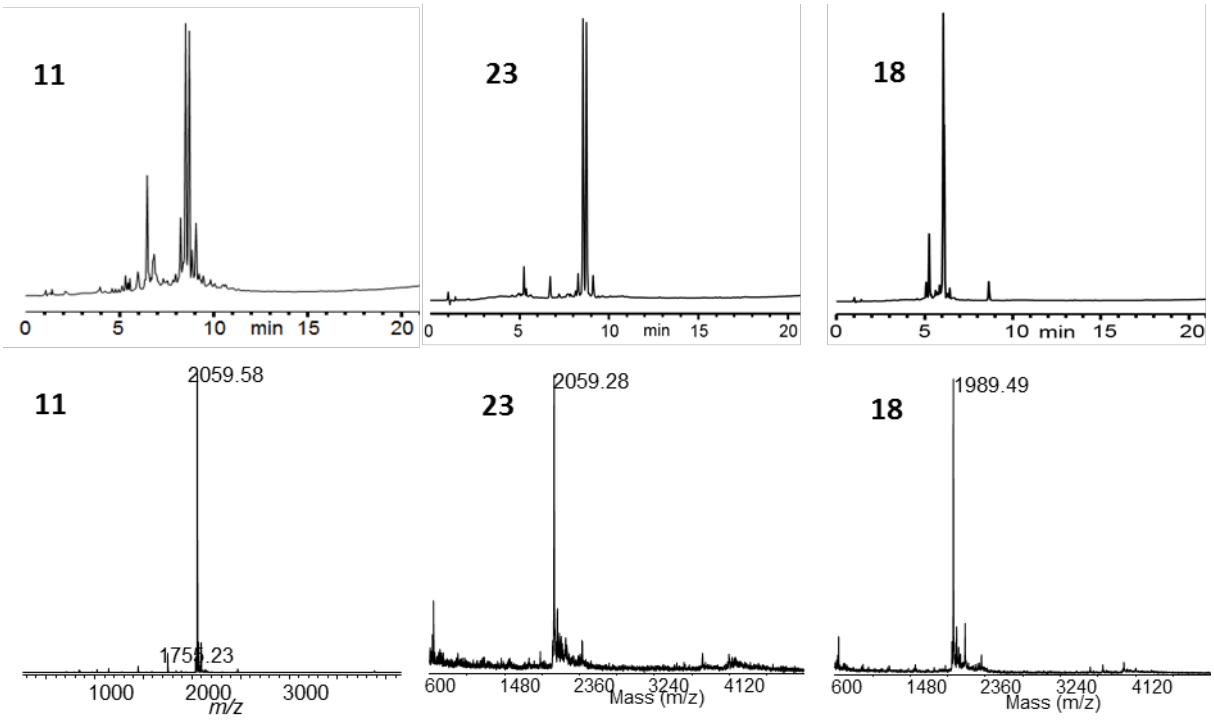

Figure 1. HPLC profiles (top) and MALDI-TOF MS (bottom) of crude aminooxy hexathymidylates. Calculated $[M-H]^-$, m/z = 2059.50 for **11** and **23** and 1989.36 for **18**.

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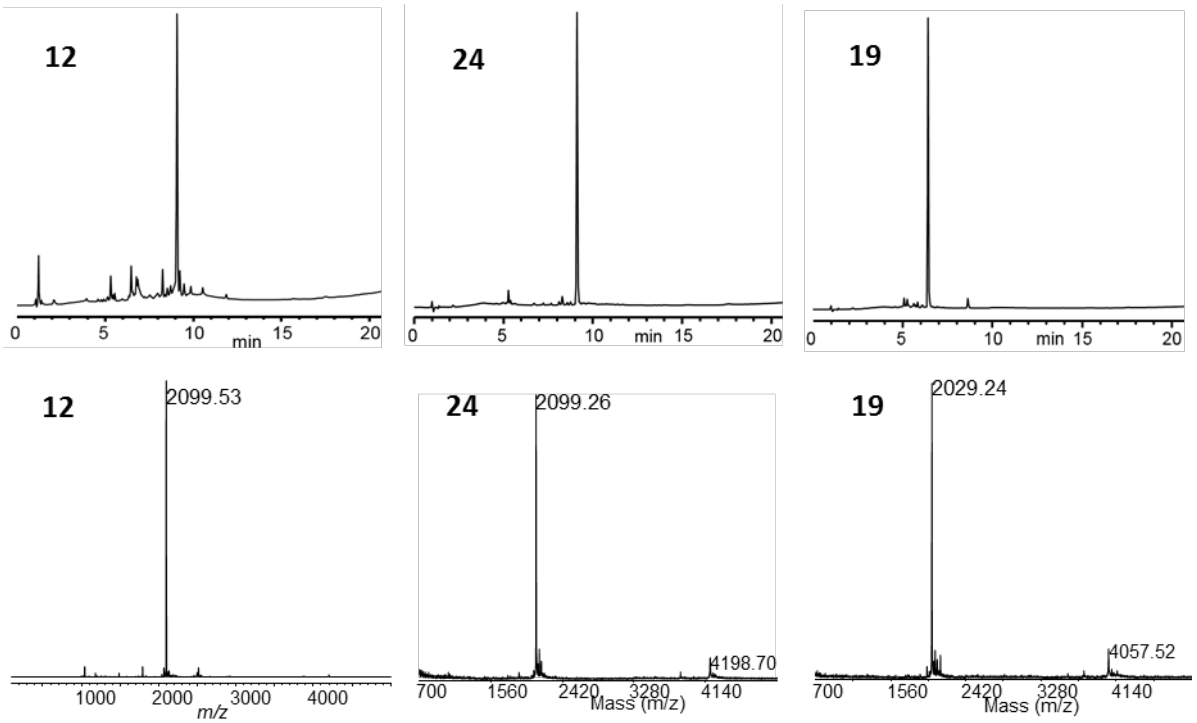


Figure 2. HPLC profiles (top) and MALDI-TOF MS (bottom) of crude acetone-capped oxime hexathymidylates. Calculated $[M-H]^-$, m/z = 2099.56 for **12** and **24** and 2029.43 for **19**.

The three supported 3'- and 5'-aminooxy T_6 models were conjugated with pyrene aldehyde affording, after déprotection, the expected 3'-conjugate **14** and 5'-conjugates **25** and **20** (schemes 3 and 4). They were eluted as two peaks in C_{18} reverse phase HPLC corresponding to the E and Z isomers of the oxime as already reported (Fig. 3).[34] Each conjugate was characterized by MALDI-TOF MS and showed a characteristic UV band at 363 nm corresponding to the pyrene. We visualized the expected ion plus that corresponding to a fragmentation at the nitrogen-oxygen bound of the oxime linkage [M - 227 Da] during the MS analysis.[40]

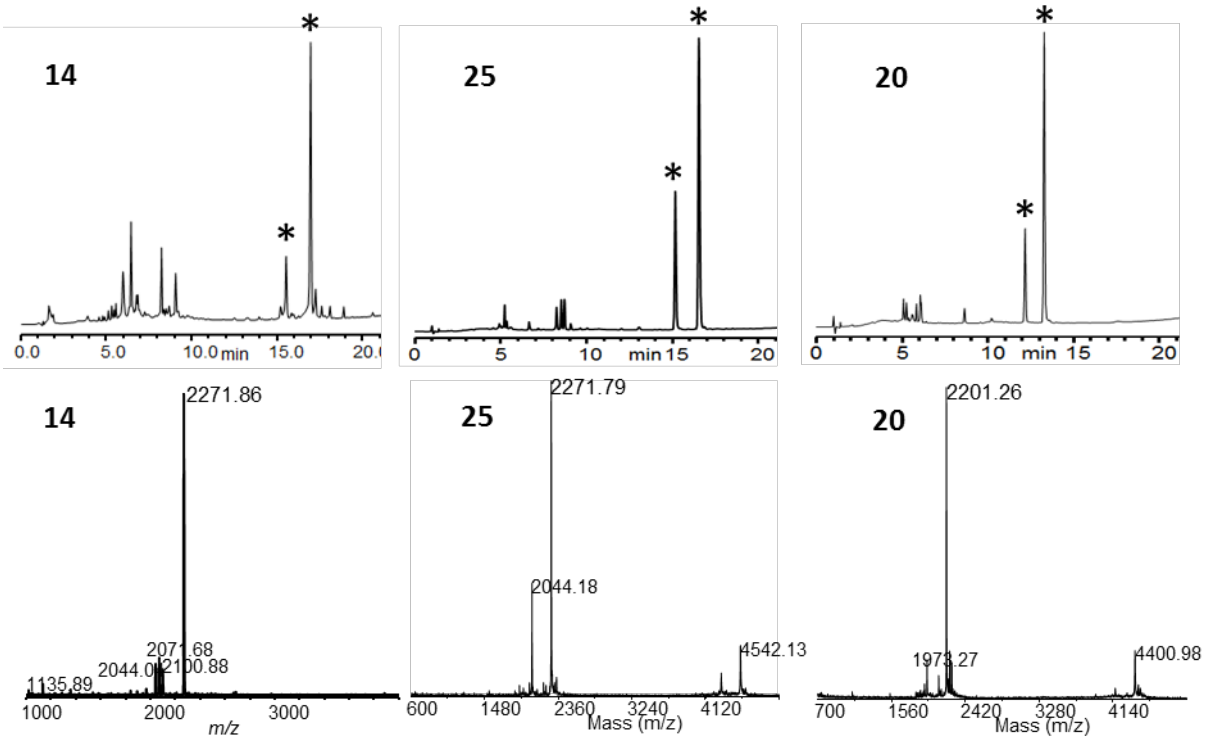


Figure 3. HPLC profiles (top) and MALDI-TOF MS (bottom) of crude pyrenyl oxime hexathymidylates. * shows peaks of E/Z of pyrenyl conjugates. Calculated $[M-H]^-$, m/z = 2271.75 for **14** and **25** and 2201.61 for **20**.

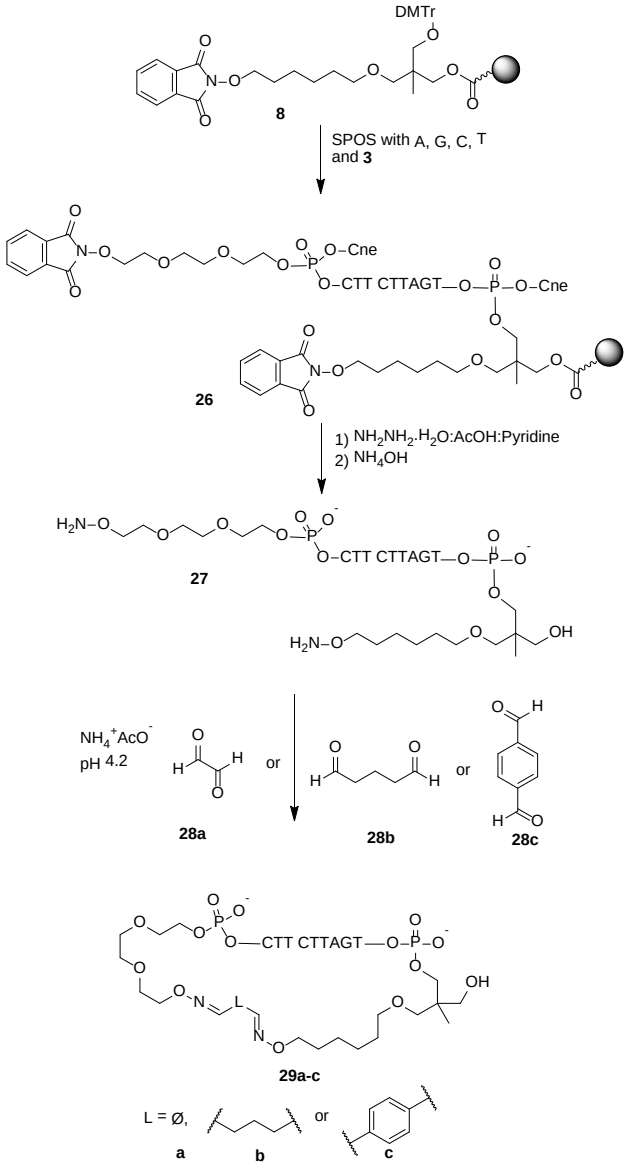
These results showed that it is possible to introduce an aminooxy function at the 3' or 5' ends of an oligonucleotide. The next step was to introduce an aminooxy function at both ends of oligonucleotides to circularize them by means of a dialdehyde.

Oligonucleotide elongation and circularization

A first 9-mer oligonucleotide (CTT CTT AGT) was synthesis starting from solid support **8** by standard phosphoramidite chemistry, with a 30 sec coupling time for regular nucleoside phosphoramidites and 60 sec for phosphoramidite **3** to ensure a high coupling (Scheme 5). The deprotection of the aminooxy functions was performed on the solid support using the standard treatment with aqueous hydrazine in acetic acid pyridine for 30 min. Then an ammonia treatment was applied to release and deprotect the linear 9-mer **27** exhibiting an aminooxy function at each end. It was characterized by MALDI-TOF mass spectrometry and analyzed by

C_{18} reverse phase HPLC. MALDI-TOF MS confirmed the synthesis of **27** with the presence of shortmers and the HPLC profile displayed two mains peaks (Fig. S1).

The cyclization was performed with three different dialdehydes: glyoxal **28a**, glutaraldehyde **28b** and terephthalaldehyde **28c**. The linear bis-aminooxy oligonucleotide **27** (100 nmol) was dissolved in sodium acetate buffer pH 4.2 (10 µM) and 1.2 molar equivalent of dialdehyde was added. After 30 min, the solution was purified by size exclusion chromatography (SEC) to remove the small excess of dialdehyde and buffer. The efficiency of the cyclization affording oligonucleotides **29a-c** was monitored by MALDI-TOF MS and HPLC. (Fig. S2-S4) Whatever the dialdehyde, the reaction gave only the expected cyclic compound. The three dialdehydes exhibited the same reactivity.



Scheme 5. Circularization of oligonucleotides by bis-oxime ligation

Table 1. Sequences and MALDI-TOF mass spectrometry data of the linear and circular oligonucleotides

oligonucleotides	Sequences 5'—3'	$[M-H]^-$ Calcd	$[M-H]^-$ Found
27	NH_2O-EG_3 CTT CTT AGT ~C_6ONH_2[a]	3203.24	3203.35
29a	Circular **27** with glyoxal	3225.25	3225.80
29b	Circular **27** with glutaraldehyde	3267.33	3266.66
29c	Circular **27** with terephthalaldehyde	3301.35	3301.89
21-mer linear $S31_{21mer}$	NH_2O-EG_3 TTT CCG CGT TGG ATT AGC TTT ~C_6ONH_2	6932.64	6932.33
21-mer circular $S33_{21mer}$	Circularized with terephthalaldehyde	7030.75	7029.92
31-mer linear $S31_{31mer}$	NH_2O-EG_3 TTT GAT TAC AGC CGG TGT ACG ACC CTT TTT ~C_6ONH_2	9981.63	9982.11
31-mer circular $S33_{31mer}$	Circularized with terephthalaldehyde	10079.73	10080.56
24-mer linear 31	NH_2O-EG_3 GAC GCT AAT C-TTTT -G ATT AGC GTC ~C_6ONH_2	7857.26	7858.04
[b]cap24-mer linear 32	Me_2C=NO-EG_3 GAC GCT AAT C-TTTT -G ATT AGC GTC ~C_6ON=CMe_2	7937.39	7938.86
24-mer circular dumbbell 33	Circularized with terephthalaldehyde	7955.36	7957.36
Dansyl 23-mer linear 39	NH_2O-EG_3 GAC GCT AAT C-TXTT -G ATT AGC GTC ~C_6ONH_2 with X = linker with dansyl	8106.64	No hit
Cap Dansyl 23-mer linear 40	Me_2C=NO-EG_3 GAC GCT AAT C-TXTT -G ATT AGC GTC ~C_6ON=CMe_2 with X = linker with dansyl	8186.77	8186.17
Dansyl 23-mer circular dumbbell 41	Circularized with terephthalaldehyde	8204.74	8206.00

30-mer linear 43	NH_2OC_6~ GAC GCT AAT CTT CTA NH_2OC_6~ GCT GAA CAT TAG TCC ~C_6ONH_2	10031.87	10037.30
Cap30-mer linear 44	$Me_2C=NOC_6$~ GAC GCT AAT CTT CTA $Me_2C=NOC_6$ GCT GAA CAT TAG TCC ~$C_6ON=CMe_2$	10152.06	10152.49
Bi-cyclic 30-mer 45	Circularized with Benzene-1,3,5-tricarboxaldehyde	10139.97	10139.82

[a]~C_6ONH_2 or NH_2OC_6~ correspond to $HOCH_2C(CH_2OH)(CH_3)$-$CH_2O(CH_2)_6$-ONH_2 conjugated to the oligonucleotide through a phosphodiester linkage at the 3'- and 5'-end respectively, [b]Cap means that aminooxy functions were capped with acetone.

The circularization of longer oligonucleotides (21, 24 and 31-mer) was performed with the terephthalaldehyde (Schemes 6 and S1). The 24-mer **31** is able to form a hairpin with a T_4 loop while the two others ($S31_{\_21mer}$, $S31_{\_31mer}$) are not complementary. The synthesis of the linear oligonucleotides was performed as described above. The circularization was checked after 60 min by MALDI-TOF MS showing the full cyclization affording 33 $S33_{21mer}$ and $S33_{31mer}$ (Table 1, Fig. S5-S8). Even for long oligonucleotides it was not necessary to use a template for their circularization. Each of them was purified by C_{18} reverse phase HPLC.

After the elongation and deprotection of the 24-mer forming an hairpin structure **31**, one part was capped with acetone to further study the melting temperature of the linear hairpin **32** versus the circular hairpin or dumbbell structure **33** (Scheme 6, Fig. S9-S11).

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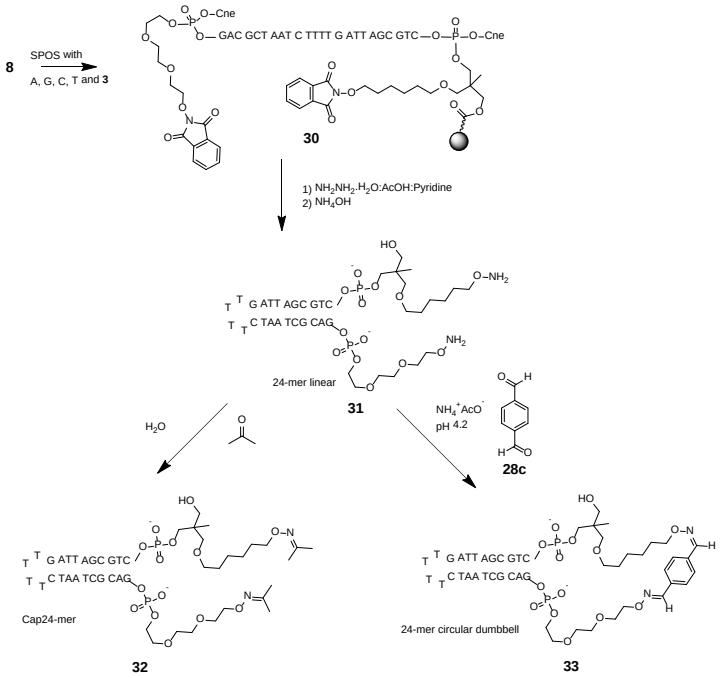

Scheme 6. Synthesis of capped 24-mer and 24-mer circular dumbbell.

Then, a dumbbell structure with a fluorescent dye was circularized. To this end, we combined two click reactions corresponding to the oxime ligation for circularization and copper catalyzed azide alkyne cycloaddition (CuAAC) for the conjugation with dansyl propylazide **37**. We previously showed that both reactions are orthogonal under certain conditions.[41-42] The oligonucleotide was synthesized from solid support **8** and the phosphoramidite compound **34** exhibiting a propargyl function was introduced in the loop. The sequence was finished with the phosphoramidite **3** (Scheme 7). The CuAAC was performed on solid-supported oligonucleotide **36**, with dansyl propylazide **37** using $CuSO_4$ and sodium ascorbate under microwaves assistance[43] affording **38**. The phthalimide groups were removed by hydrazine treatment and the dansyl linear oligonucleotide **39** was obtained after ammonia treatment. One part of it was treated with acetone to form the capped linear entity **40**. On another part, terephathalaldehyde was added in ammonium acetate buffer leading to the circularized oligonucleotide **41** as confirmed by MALDI-TOF MS and HPLC analyses. Pure **41** was isolated by HPLC (Fig. S12-S14).

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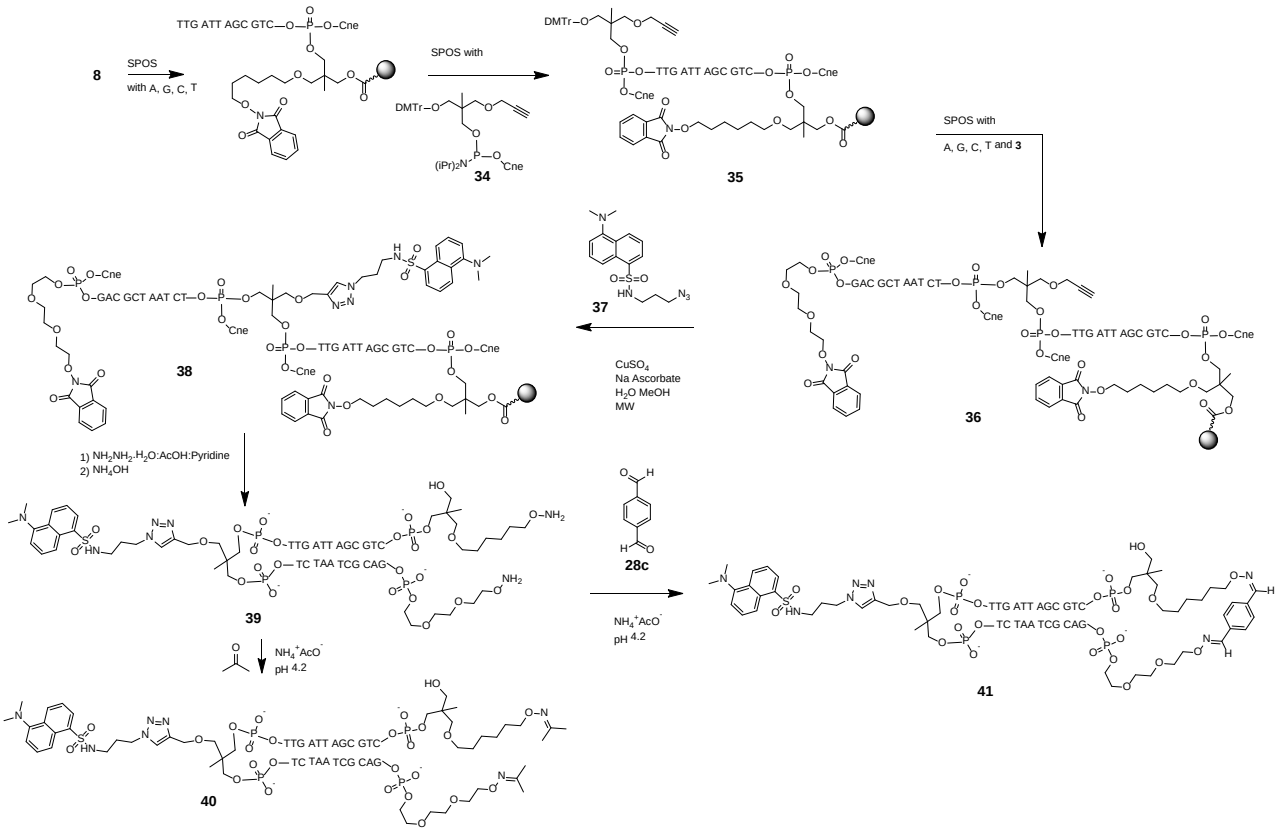


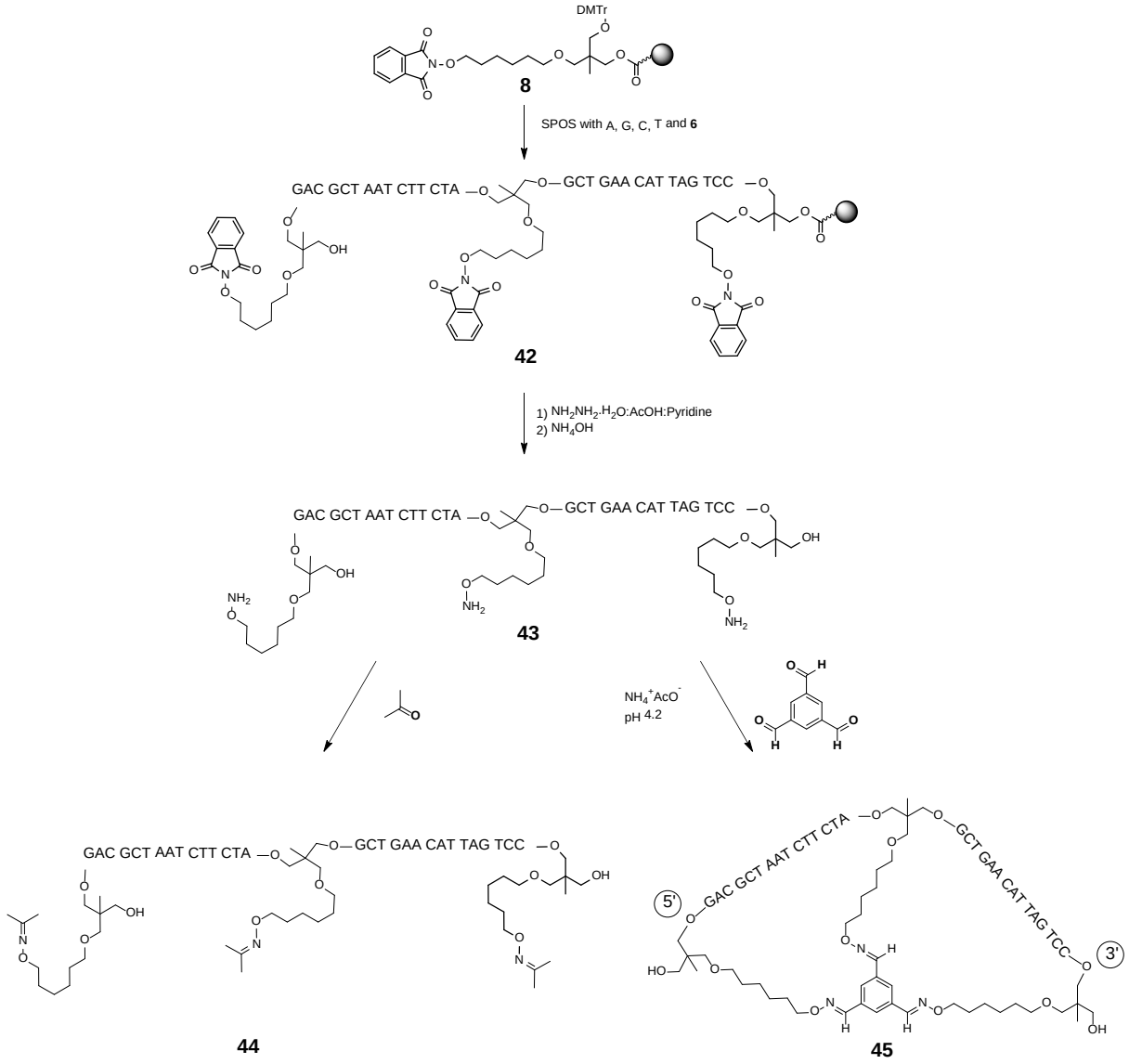
Scheme 7. Synthesis of dansyl-23-mer dumbbell.

Melting temperatures (Tm) of capped linear 24-mer **32** and 24-mer circular dumbbell **33** as well as the dansyl conjugates as linear **40** and cyclic **41** oligonucleotides were measured. For both linear structures a Tm of 77 °C was found while the circular structures displayed a Tm superior to 95°C. Thus the cyclization increases the Tm by more than 18°C.

Bicyclic DNA

Finally, to extend the scope of the cyclization by oxime ligation, a bicyclic oligonucleotide was synthesized applying a triple oxime ligation. Starting from solid support **8** a first 15-mer oligonucleotide sequence was elongated and then phosphoramidite **6** was coupled and a second 15-mer oligonucleotide was elongated and phosphoramidite **6** was coupled affording construction **42** (Scheme 8). A two-step deprotection was performed leading to oligonucleotide **43** with three aminooxy functions (Fig S15). One part was treated with acetone and another part was bi-cyclized using benzene-1,3,5-tricarboxaldehyde affording the tri-capped sequence **44** and the bi-cyclic oligonucleotide **45**, respectively (Fig. 4). Both were purified by C_{18} HPLC and characterized by MALDI-TOF MS (Fig. 4). For the linear structure **44** a single peak was

obtained while for the bi-cyclic structure **45**, HPLC profile shows three main peaks due to the presence of the E and Z isomers for each oxime motif. For both, only the ions corresponding to the expected linear and bi-cyclic oligonucleotides were observed by MS.



Scheme 8. Synthesis of a bicyclic oligonucleotide

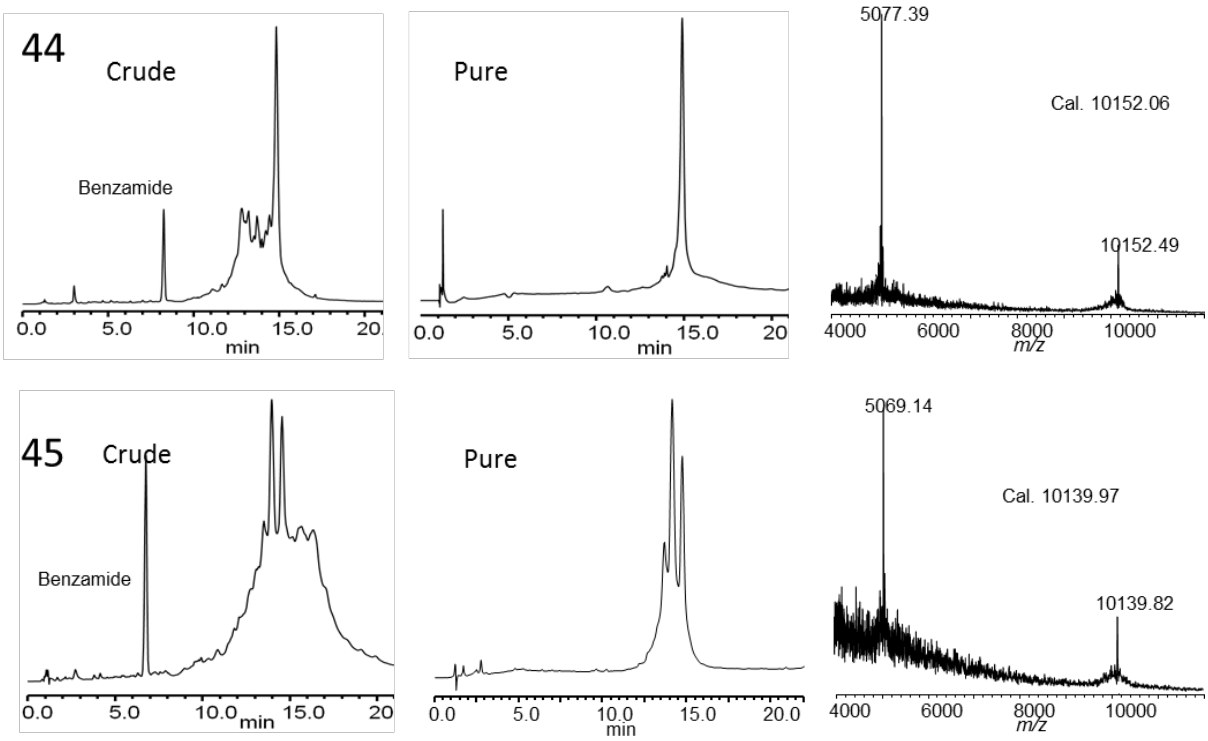


Figure 4. HPLC profiles and MALDI-TOF MS of (top) acetone-capped linear oligonucleotide **44** and (bottom) bicyclic oligonucleotide **45**.

CONCLUSION

The use of the phosphoramidites **3** and **6** and of the solid support **8** bearing a phthalimide-oxy group allowed the conjugation of oligonucleotide at the 3' and 5'-extremities with aldehyde derivatives through the formation of an oxime. Furthermore, we demonstrated that oligonucleotides exhibiting aminooxy at both ends were efficiency circularized by means of either aliphatic or aromatic dialdehyde by a bis-oxime ligation. Finally, a bis-cyclization can be performed starting from an oligonucleotide exhibiting three aminooxy functions one at both ends and one in the middle of the sequence. This bis-circularized was carried out by reaction of a tri-aldehyde leading to a tris-oxime ligation. According to this strategy the multiple oxime ligation is an effective method to make DNA-based objects.

EXPERIMENTAL PROCEDURE

All commercial chemicals were reagent grade and were used without further purification. DNA synthesis reagents and phosphoramidites are commercially available. Flash column chromatography was performed on silica gel 60 (40-63 μm). ^{1}H NMR (300-400 MHz), ^{13}C NMR spectra (100 MHz), and ^{31}P NMR (121 MHz) were recorded in the stated solvent at room temperature unless otherwise specified. The compounds **1**[37] and **4**[39] were prepared according to literature protocols.

8-phthalimidooxy-3,6-dioxaoctanol 2. To a solution of *N*-hydroxyphthalimide (700 mg, 4 mmol) and 8-tosyloxy-3,6-dioxaoctanol **1**[37] (610 mg, 2 mmol) in DMF (20 ml) K_2CO_3 (550 mg, 4 mmol) was added. The red mixture was heated under microwaves at 60 °C for 2 h. After filtration, the DMF solution was evaporated. The dark residue was diluted with CH_2Cl_2 (100 ml) and washed by water (2 x 100 ml). The organic layer was dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by silica gel chromatography (0 to 40% acetone in CH_2Cl_2), affording compound **2** (379 mg, 64 %) as a red oil. TLC (2.5 % MeOH in CH_2Cl_2, twice elutions) R_f: 0.40, ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 2.72 (sb, 1H, OH), 3.48-3.65 (m, 8H, -O-CH_2CH_2-O-, -CH_2-O-, -O-CH_2-), 3.81-3.84 (m, 2H, CH_2-OH), 4.32-4.35 (m, 2H, N-O-CH_2-), 7.70-7.80 (m, 4H, phth). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 61.8 (-CH_2-OH), 69.2, 70.3, 70.8 (-O-CH_2-CH_2-O-), 72.5 (-CH_2O-Phth), 123.5 (CAr), 128.9 (CAr, Cq), 134.5 (CAr), 163.5 (C=O). HRMS (ESI/Q-TOF) *m/z* calcd for $C_{14}H_{18}NO_6$ $[M+H]^+$, 296.1134 found = 296.1132.

8-phthalimidooxy-3,6-dioxaoctanol-[(2-cyanoethyl)-**(*N*,*N*-diisopropyl)phosphoramidite] 3.** To the solution of **2** (650 mg, 2.2 mmol) in dry CH_2Cl_2 (20 ml) were added successively DIEA (453 µl, 2.6 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (511 µl, 2.2 mmol). The mixture was stirred under magnetic stirring for 1 hour at room temperature. Then, the reaction was quenched by addition of 500 µl of water and diluted with CH_2Cl_2 (150 ml). The organic layer was washed with $NaHCO_3$ (100 mL), dried over Na_2SO_4, and evaporated. The crude product was purified by silica gel chromatography (0 to 60% AcOEt in cyclohexane containing 4% Et_3N) to afford compound **3** (823 mg, 76%) as a clear oil. TLC (AcOEt/Et_3N, 9:1, v/v) R_f : 0.40, ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.16 (t, 12H, J=6.4Hz, Isopropyl), 2.64 (t, 2H, J=6.5Hz, -C$\underline{H}_2$CN), 3.55-3.88 (m, 14H, -C$\underline{H}$-,-C$\underline{H}_2$-OP, O-C$\underline{H}_2$-C$\underline{H}_2$-O, -O-C$\underline{H}_2$-), 4.35-4.37 (m, 2H, phthN-O-C$\underline{H}_2$-), 7.72-7.84 (m, 4H, phth).^{13}C-NMR ($CDCl_3$, 100 MHz): δ 20.3 (-$\underline{C}H_2$-CN), 24.6, 24.6, 24.6, 24.6, (CH_3.), 43.1, 43.1, (-$\underline{C}$H-, iPr), 58.5, 62.5, 69.3, 70.5, 70.8, 71.2 (-$\underline{C}H_2$-O-), 77.2 (PhthN-O-$\underline{C}H_2$-), 117.8 (-$\underline{C}$N), 123.5, 123.5 (CAr), 129.0, 129.0 (CAr),

134.4, 134.4 (CAr.), 163.4, 163.4 (-C=O). ^{31}P-NMR ($CDCl_3$, 162 MHz): δ 148.57ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd for $C_{23}H_{37}N_3O_8P$ $[M+H_2O+H]^+$, 514.2318 found = 514.2321.

2-(4,4'-Dimethoxytrityloxymethyl)-2-methyl-3-(6-phthalimidooxyhexyloxy)-propanol 5. To a solution of 2-(4,4'-dimethoxytrityloxymethyl)-2-methyl-3-(6-bromohexyloxy)-propanol **4**[39] (700 mg, 1.2 mmol) in DMF anhydrous (15 ml) was added *N*-hydroxyphthalimide (340 mg, 2 mmol) and K_2CO_3 (550 mg, 4 mmol). The red mixture was heated under microwaves for 60 min at 60°C. After filtration and concentration, the residue was diluted with CH_2Cl_2 (100 ml), washed with brine (2 × 70 mL), dried over Na_2SO_4, and evaporated. The crude product was purified by silica gel column chromatography (10 to 30% AcOEt in cyclohexane) to afford title compound **5** (460 mg, 57%) as a clear oil. TLC (cyclohexane/AcOEt 7:3 v/v) *Rf* : 0.30, ^{1}H NMR ($CDCl_3$, 300 MHz): 0.89 (s, 3 H, CH_3); 1.36-1.63 (m, 6 H, -CH_2-), 1.73-1.83 (m, 2 H, -CH_2-), 2.87 (bs, 1H, OH), 3.09 (s, 2 H, -CH_2-ODmtr), 3.40-3.56 (m, 6 H, -O-CH_2-, -CH_2-O-CH_2-), 3.77 (s, 6 H, OCH_3), 4.19 (2H, -CH2-O-phtha, J = 6.6 Hz), 6.80-6.83 (m, 4 H, Dmtr), 7.17-7.45 (m, 9 H, Dmtr), 7.69-7.83 (m, 4 H, phth). ^{13}C NMR ($CDCl_3$, 100 MHz): *δ* 18.0 (CH_3), 25.5, 25.9, 28.1, 29.5 (-CH_2-), 40.8 (Cq), 55.2, 55.2 (-OCH_3), 66.4, 69.6, 71.7, 76.1 (-CH_2-O-CH_2-, HO-CH_2-, -CH_2-O-Dmtr), 78.5 (-CH_2-O-phth), 85.9 (Cq), 113.0, 113.0, 113.0, 113.0 (CAr.Dmtr), 123.5, 123.5, 126.7, 126.7, 127.8, 127.8, 128.2, 128.2, 129.0, 130.2, 130.2, 130.2, 130.2 (CAr), 134.5, 134.5 (CAr.Phth), 136.2, 136.2, 145.1, 158.4, 158.4 (Cq, Dmtr), 163.7, 163.7 (CO). HRMS (ESI/Q-TOF) *m*/*z* calcd for $C_{40}H_{45}N_1O_8Na$ $[M+Na]^+$, 690.3034 found 690.3040.

2-(4,4'-Dimethoxytrityloxymethyl)-2-methyl-3-(6-phthalimidooxyhexyloxy)-propanol-[(2-cyanoethyl)-*N*,*N*-diisopropyl-phosphoramidite] 6. To a solution of **5** (460 mg, 0.69 mmol) and diisopropylethylamine (180 µl, 1.0 mmol) in CH_2Cl_2, anhydrous 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (154 µl, 0.69 mmol) was added. The mixture was stirred for 3 hours under magnetic stirring at room temperature. Then, the reaction was quenched by addition of 500 µl of water and diluted with CH_2Cl_2 (100 ml). The organic layer was washed with $NaHCO_3$ (100 mL), dried over Na_2SO_4, and evaporated. The crude product was purified by silica gel column chromatography (10 to 30% AcOEt in cyclohexane containing 4% Et_3N) to afford a compound **6** (370 mg, 62%) as a clear oil. TLC (cyclohexane/AcOEt/Et_3N 7:2/1 v/v/v) Rf : 0.50. ^{1}H NMR ($CDCl_3$, 400 MHz) : 0.98 (d, 3 H, J = 9.5 Hz, CH_3); 1.11–1.18 (m, 12 H, 2 $CH(CH_3)_2$), 1.36-1.42 (m, 4 H, -CH_2-), 1.47-1.60 (m, 2 H, -CH_2-), 1.76-1.83 (m, 2 H, -CH_2-), 2.54 (dt, 2H, J_1 = 3.6 Hz, J_2 = 6.5 Hz, CH_2-CN), 2.98-3.05 (m, 2 H, -CH_2-ODmtr), 3.34-

3.75 (m, 10 H, -CH-, -O-CH_2-, -CH_2-O-CH_2-), 3.8 (s, 6 H, OCH_3), 4.21 (2H, -CH_2-O-phth, J = 6.7 Hz), 6.81-6.84 (m, 4 H, Dmtr), 7.18-7.45 (m, 9 H, Dmtr), 7.74-7.85 (m, 4 H, phtha). ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 17.8 (CH_3), 20.4 (-CH_2-CN), 24.6, 24.6, 24.6, 24.6 (CH_3,iPr), 25.5, 25.9, 28.1, 29.6 (-CH_2-), 41.3 (Cq), 43.1, 43.1, (-CH-, iPr), 55.2, 55.2 (-OCH_3), 58.3, 65.1, 66.5, 71.4, 73.4 (-O-CH_2-), 78.5 (-CH_2-O-pht), 85.4 (Cq), 112.9, 112.9, 112.9, 112.9, (CAr), 117.7 (-CN), 123.5, 123.5, 126.6, 126.6, 127.6, 127.6, 128.3, 128.3, 129.0, 130.2, 130.2, 130.2, 130.2, 134.4, 134.4, (CAr), 136.6, 136.6, 145.4, 158.3, 158.3 (Cq), 163.7, 163.7 (CO). ^{31}P-NMR ($CDCl_3$, 162 MHz): δ 147.42-147.54 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd for $C_{49}H_{63}N_3O_9P$ $[M+H]^+$, 868.4302 found 868.4296.

2-(4,4'-Dimethoxytrityloxymethyl)-2-methyl-3-(6-phthalimidooxyhexyloxy)-propyl (succinate-LCAA-CPG) 8. Succinate-LCAA CPG 7 (750 g), 2-(4,4'-dimethoxytrityloxymethyl)-2-methyl-3-(6-bromohexyloxy)-propanol **5** (350 mg, 0.52 mmol), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (460 mg, 2.4 mmol), DMAP (60 mg, 0.5 mmol), and Et_3N (220 µL, 1.5 mmol) were gently shaken in anhydrous pyridine (6 mL) for 48 h at room temperature. Then, the solid support was filtered off, washed with CH_2Cl_2, and dried. A capping step with standard Cap A and Cap B solutions (1:1, v/v) 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 35 µmol/g.

Synthesis of oligonucleotides. The oligonucleotides were synthesized at the 1µmol-scale on a DNA synthesizer (ABI 394) by standard phosphoramidite chemistry. For the coupling step, benzylmercaptotetrazole (BMT) was used as activator (0.3 M in anhydrous CH_3CN), with a coupling time of 30 s for standard nucleosides phosphoramidites (0.075 M in anhydrous CH_3CN) and of 60 s for phosphoramidites **3** and **6** (0.1 M in anhydrous CH_3CN). The capping step was performed with acetic anhydride using commercial solutions (Cap A: Ac_2O/pyridine/THF, 10:10:80, v/v/v and Cap B: 10% *N*-methylimidazole in THF) for 15 s. Oxidation was performed for 15 s using 0.1 M I_2, THF/pyridine/water 90:5:5. Detritylation was performed with 3% trichloroacetic acid (TCA) in CH_2Cl_2 for 65 s.

General procedure for deprotection of solid-supported oligonucleotides: The CPG beads bearing modified oligonucleotides were treated with a solution of $NH_2NH_2.H_2O$/Pyridine/acetic acid 0.124:4:1 v/v/v for 30 min at room temperature. The solution was withdrawn and the beads were washed with pyridine, methanol and acetonitrile. The beads were transferred to a 4 mL

screw top vial and treated with 2 mL of concentrated aqueous ammonia at 55°C for 5 h. For each compound, the supernatant was withdrawn and evaporated to dryness. The residue was dissolved in water for subsequent analysis and characterization.

Capping with acetone. To an aliquot of aminooxy oligonucleotide in water a drop of acetone was added. After 15 min, the solution was concentrated and the resulting oligonucleotide was analysed by HPLC and characterized by MS.

Protocol for circularization. Bis-aminooxy oligonucleotide (0.1 μmol) was dissolved in 1.0 mL of ammonium acetate buffer (400 mM pH 4.5) and terephthalate dialdehyde (0.12 μmol) was added. The resulting mixture was stirred for 1h at rt. The crude was applied on a NAP10 column for desalting.

Protocol for bis-circularization. tris-aminooxy oligonucleotide (0.1 μmol) was dissolved in 1.0 mL of ammonium acetate buffer (400 mM pH 4.5) and benzene-1,3,5-tricarboxaldehyde (0.12 μmol) was added. The resulting mixture was stirred for 1h at rt. The crude was applied on a NAP10 column for desalting.

Protocol for CuAAC. Solid-supported oligonucleotide 0.5 μmol with $CuSO_4$ 0.2 μmol (5 μL of freshly prepared 40 mM aqueous solution) sodium ascorbate 1 μmol (10 μL of 100 mM aqueous solution), dansyl propyl azide 1 μmol, 150 μL of water and 150 μL of methanol were added in a sealed tube and heated at 60 °C under microwaves assistance for 1 h. Beads were filtered off and washed with water and methanol and dried.

Melting temperature*:* Tm values of oligonucleotides were measured at a concentration of 0.5 μM in 10 mM sodium cacodylate, 100 mM NaCl, pH 7 buffer. A linear gradient of temperature of 0.5 °C/min was applied from 95°C to 60°C and 60°C to 95°C. UV absorbance was read at 260 nm.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the website at DOI:

NMR spectra for **2**, **3**, **5** and **6**. HPLC and MALDI-TOF MS profiles.

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