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# Head-to-Tail Oxime Cyclization of Oligodeoxynucleotides for the Efficient Synthesis of Circular DNA Analogues

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**Abstract:** A convenient strategy for the synthesis of the analogue of cyclic oligodeoxyribonucleotides is presented. The cyclization of the oligonucleotide was accomplished through intramolecular oxime bond formation between a 5′-oxyamine moiety and a 3′-aldehydic group.

Circular oligonucleotides possess unusual chemical and biological properties with respect to the standard linear oligonucleotides that have spurred their use as molecular tools during the past decade.[1] Since no extremity is left for the digestion process to begin, they are much more resistant to exonuclease activity than their corresponding linear oligonucleotides.[2] In addition, they appear to have excellent DNA- and RNA-binding affinity and high sequence selectivity.[3] The circular DNA have also excellent strand displacement activity which would, in theory, help them to hybridize in the areas of folded RNA. Promising biological activities have thus been reported from large to small circular oligonucleotides.[4] Moreover, conformational studies of cyclic oligonucleotides by using NMR have taken much interest as the occurrence of such structures in solution is of primary importance to assess their possible biological relevance.[5]

Considering their wide potential applications, several efforts have been dedicated so far for the development of chemical methods for the synthesis of cyclic oligonucleotides. Cyclization has been achieved using intramolecular chemical ligation of partially unprotected oligonucleotide in diluted solution, by template-directed cyclization for larger cycles up to 24-mer or by solid-phase synthesis.[6−8] Pedroso et al. have thus developed a solid support which allows the preparation of cyclic DNA with moderate (4% for a 32 mer) to good 50% yield for the smallest cycles.[8b] More recently, Kool et al. have reported a solid-phase synthesis using a cyclization step by $S_N2$ displacement of a 5′-iodo by a 3′-sulfur moiety.[8a]

In the context to design new procedures for the conjugation of oligonucleotide with a reporter group, our investigations have focused on the utility of the oxime linkage. This linkage has been used efficiently for the chemical ligation of peptides,[9] conjugation of peptides with carbohydrates,[10] and more recently for the conjugation of peptides with oligonucleotides.[11] The major advantage of this ligation technique is that it requires neither a coupling reagent nor chemical manipulations except mixing of the two components, namely the oxyamine and the aldehyde moieties.

We thus envisage exploiting the favorable characteristics of the oxime bond formation for the preparation of analogues of circular oligonucleotides. We envisioned the formation of these analogues by using a bifunctionalized synthetic oligonucleotide bearing a trityl-protected aminooxy nucleophile and a masked aldehyde at the 5′- and 3′-end, respectively. The oligonucleotide should be "armed" by mild generation of the aldehyde and deprotection of the oxyamine. Intramolecular head to tail reaction between these two reactive moieties should result in the cyclization of the oligonucleotide (Figure 1).

In this study, we describe the preparation of synthetic oligonucleotides sustaining the two reactive moieties (i.e., the oxyamine and the aldehyde) at each extremity and the cyclization reaction using sequences of various lengths from 3-mer to 11-mer. We show that the intramolecular oxime bond formation is very efficient for affording cyclic oligonucleotides analogues in good yield.

The envisioned strategy necessitated first the preparation of 3′,5′-bifunctionalized oligonucleotide. For the introduction of the masked aldehyde at the 3′-extremity, the solid support 3′-glyceryl CPG **1** bearing a 1,2-diol was chosen as starting material (Scheme 1). This support was preferred to the previously reported 1,2-amino alcohol containing support.[12] In fact, the efficiency of the latter

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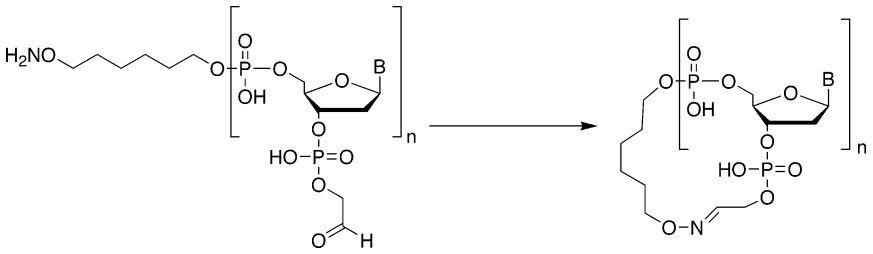
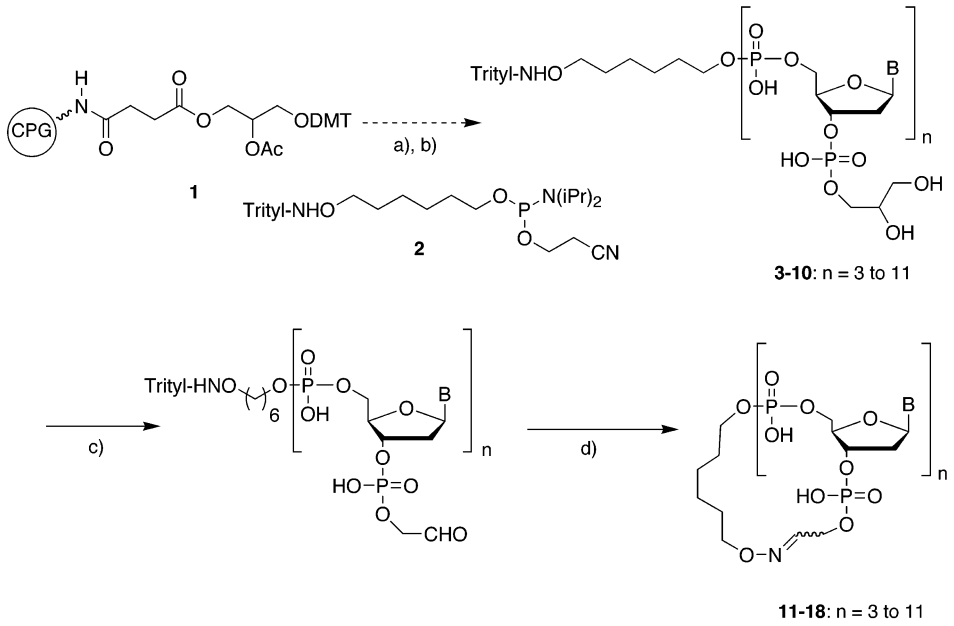


**FIGURE 1.** Oxime strategy for the synthesis of analogues of cyclic oligonucleotides.

**SCHEME 1**[a]



[a] Conditions: (a) automated DNA synthesis with incorporation of phosphoramidite **2** at the last stage; (b) 28% aq. $NH_4OH$, 55 °C, 16 h; (c) $NaIO_4/H_2O$; (d) AcOH 80%, rt, 2−5 h.

**TABLE 1. Sequences and Analytical Data for Linear and Cyclic Oligonucleotides**

| | [M − H]$^-$ [b] | | $t_R$[c] | | reaction | yield[d] | $t_R$[c] | [M−H]$^-$ [b] | |
|---|---|---|---|---|---|---|---|---|---|
| linear sequence[a] | calcd | found | (min) | cyclic sequence | time (h) | (%) | (min) | calcd | found |
| XTTTY **3** | 1440.63 | 1440.85 | 26.6 | c(TTT) **11** | 2 | 75 | 15.6 | 1149.35 | 1148.20 |
| XGGGY **4** | 1515.67 | 1515.46 | 27.8 | c(GGG) **12** | 2 | 48 | 14.6 | 1223.39 | 1223.16 |
| XACGTY **5** | 1763.84 | 1763.49 | 26.4 | c(ACGT) **13** | 2.5 | 68 | 15.1 | 1471.56 | 1471.12 |
| XTTTTTTY **6** | 2353.22 | 2352.86 | 25.4 | c(TTTTTT) **14** | 3 | 70 | 14.3 | 2060.94 | 2060.45 |
| XTGCTCGCTY **7** | 2966.61 | 2966.56 | 23.2 | c(TGCTCGCT) **15** | 4 | 58 | 13.2 | 2674.61 | 2674.82 |
| XCATTCATTY **8** | 2949.62 | 2949.51 | 23.1 | c(CATTCATT) **16** | 4 | 60 | 13.7 | 2657.62 | 2657.62 |
| XGCGTGTGTGCGY **9** | 3994.27 | 3994.26 | 19.9 | c(GCGTGTGTGCG) **17** | 4 | 64 | 13.1 | 3702.27 | 3701.91 |
| XCGCACACACGCY **10** | 3861.21 | 3860.88 | 21.2 | c(CGCACACACGC) **18** | 5 | 43 | 14.4 | 3569.21 | 3568.91 |

[a] X = 5′-protected aminooxy linker, Y = 3′-glyceryl linker. [b] Electrospray MS. [c] Retention time ($t_R$) by analytical C18 reversed-phase HPLC using a gradient of 0−30% of acetonitrile over 20 min, at a flow rate of 1 mL min$^{-1}$. [d] Yields are calculated by UV at $\lambda_{max}$ = 260 nm

support for the introduction of the 1,2-amino alcohol moiety at the 3′-end was found to dramatically decreased after a period of 3−4 months of storage at 4 °C. The 5′-aminooxy-containing linker was introduced as described according to standard $\beta$-cyanoethylphosphoramidite chemistry and by incorporating the phosphoramidite **2** at the final step of the automated DNA synthesis.[11a] The 3′,5′-bifunctionalized oligonucleotides were thus prepared using the aforementionned support **1** and incorporation of the phosphoramidite **2** at the 5′-end. After cleavage from the support and deprotection of bases using standard protocol, the bifunctionalized 5′-protected aminooxy oligonucleotides **3**−**10** were purified by reversed-phase HPLC (Figure 2A shows the crude protected aminooxy mixture in case of **6** as a representative example) and characterized by ES-MS analysis (Table 1). The oxidative cleavage of the diol at the 3′-end was then performed using sodium periodate leading to the 3′-aldehyde containing oligonucleotides in quantitative yield (Figure 2C depicted the HPLC profile of the crude oxidation mixture

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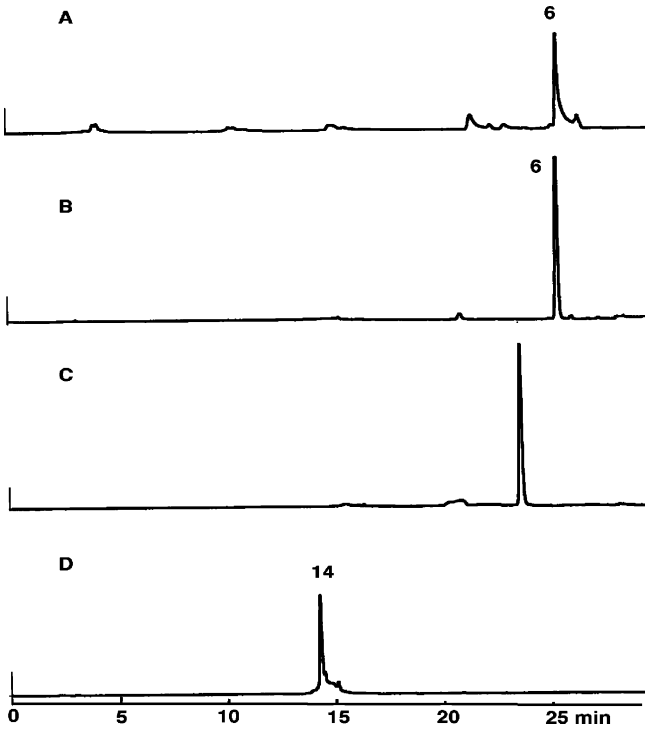


**FIGURE 2.** Reversed-phase HPLC chromatograms (detection at 260 nm) of (A) the crude mixture of the 5′-protected aminooxy oligonucleotide **6**, (B) the purified 5′-protected aminooxy oligonucleotide **6**, (C) the crude mixture of oxidation of **6**, and (D) the crude mixture of cyclization of **6** leading to **14**.

of **6**, which revealed a single major product). The cleavage of the trityl protection on the aminooxy moiety at the 5′-end was then performed in 80% aqueous acetic acid. The liberation of the oxyamine moiety resulted in the immediate cyclization of the linear oligonucleotide through the formation of the oxime bond. The linear deprotected oligonucleotide intermediate could never be observed by HPLC. The circularization was carried out at different concentrations ranging from $10^{-5}$ to $10^{-3}$ M with comparable efficiency. No product of dimerization was evidenced by HPLC. The cyclic oligonucleotides **11**−**18** were purified by reversed-phase HPLC. Due to the presence of the hydrophobic trityl group, the retention time of the starting material is largely different from the cyclic oligonucleotide that facilitated greatly the purification. The cyclic oligonucleotides **11**−**18** were then characterized by ES-MS analysis (Table 1).

The generality of the method was emphasized using a set of sequences of increasing length. In each case, the synthesis was conducted in triplicate. In all trials, we observed the HPLC profiles of crude cyclized products with a single major peak along with minor impurities (Figure 2D shows the crude mixture of cyclization in case of **14** as an example). The cyclic oligonucleotides **11**−**18** were thus obtained in good yield (40−50%) after purification by HPLC even for the longest sequences **17** and **18** (Table 1). The time of reaction for the complete disappearance of the starting material was less than 5 h. During the course of the reaction, careful HPLC analysis revealed the formation of the expected two isomers especially for shorter sequences. $^1$H NMR analysis of the sequence c(TTT) **11** indicated the presence of two set of signals for the oximic proton (at $\delta = 7.02$ and $\delta = 7.59$ ppm, respectively (two isomers in ratio 60:40)) characteristic of two diastereoisomers in slow exchange around the oxime bond.

The chemical stability of these compounds was also studied by incubating the cyclic oligonucleotide **14** (as an example) in aqueous buffer at various pH from 4 to 9. No significant hydrolysis, degradation, or ring-opening products were observed even after 48 h of incubation at 37 °C. It was confirmed by ESMS analysis of the final incubated sample, which revealed only the presence of the cyclic starting material.

In conclusion, the oxime bond formation strategy has been successfully applied for the ring closure of ODN by using standard DNA synthesizer and phosphoramidite chemistry. The ring closure of 3- to 11-mer oligonucleotides was thus achieved with high yield. Moreover, the use of commercial CPG support and standard DNA synthesis combined with an easy purification from the linear precursor make this method very attractive. Others sequences (i.e., dumbbell, hairpin, and longer oligonucleotides) are currently in preparation to study the binding properties of these analogues with RNA and DNA target. The high efficiency in DNA series also convinced us to apply the oxime strategy for the synthesis of cyclic RNA, which appear more challenging to prepare.[13]

## Experimental Section

Automated DNA synthesis was carried out using standard $\beta$-cyanoethyl nucleoside phosphoramidites chemistry and the commercial 3′-glyceryl-CPG solid support **1** (loading = 71 $\mu$mol g$^{-1}$) on a 1 $\mu$M scale. The modified phosphoramidite **2** was prepared according to the reported procedure.[11a] It was incorporated at the last stage of the automated DNA synthesis using the protocol for modified bases described by the constructor. After cleavage from the solid support and deprotection by treatment with concentrated ammonia (28%) for 16 h at 55 °C, the oligonucleotides **3**−**10** were purified by reversed-phase HPLC on a $\mu$-Bondapak C-18 column (10 × 250 mm, 7 $\mu$m). The following system of solvent was used: solvent A, 20 mM ammonium acetate/$CH_3CN$, 95:5 (v/v); solvent B ($CH_3CN$), flow rate, 4 mL/min; a linear gradient from 0 to 30% of B in 20 min was applied. Peroxidation of the oligonucleotides **3**−**10** ($C = 10^{-3}$ M) was carried out with $NaIO_4$ in excess (50 equiv) in water (350 $\mu$L) at room temperature for 1 h. The resulting aldehyde-containing oligonucleotides were then purified by simple filtration on reversed-phase C18 silica gel. Cyclization was achieved by cleavage of the trityl protection with 80% aq AcOH (500 $\mu$L) for 2−5 h (concentration ranging from $10^{-5}$ to $10^{-3}$ M). After evaporation of the solvent by lyophilization, the oligonucleotides **11**−**18** were purified by reversed-phase HPLC under the same conditions as above.

All the oligonucleotides **3**−**18** were characterized by ESMS. The ESMS analysis was performed in the negative mode. The eluent was 50% aqueous acetonitrile and the flow rate was 8 $\mu$L/min. The oligonucleotides were dissolved in 50% aqueous acetonitrile, and 1% of $NEt_3$ was added.

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