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Kristensen, Kim Vejlegaard; Paul, Sibasish; Kosbar, Tamer; Wengel, Jesper; Caruthers, Marvin H

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Oligodeoxynucleotides containing 2'-amino-LNA nucleotides as constrained morpholino phosphorodiamidate and phosphorodiamidate monomers

Kim Vejlegaard a, Sibasish Paul b, Tamer Kosbar a,c, Jesper Wengel a,* and Marvin H. Caruthers b

a Biomolecular Nanoscale Engineering Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark
b Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80303, USA

Abstract

Incorporation in a 2'→5' direction of a phosphorodiamidate 2'-amino-LNA-T nucleotide as the morpholino phosphorodiamidate and N,N-dimethylamino phosphorodiamidate monomers into six oligonucleotides is reported. Thermal denaturation studies showed that the novel 2'-amino-LNA-based morpholino monomers exert a destabilizing effects on duplexes formed with complementary DNA and RNA.

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Phosphorodiamidate morpholino oligonucleotides (PMOs) (1, Fig. 1) are used as antisense tools in gene knockdown experiments. They act as steric blockers, do not degrade their target RNA, and are enzymatically stable. Current investigations of PMOs and conjugates thereof as therapeutics for amending splicing defects ex vivo and in vivo are underway. Recently the first PMO-drug Eteplirsen/Exondys 51 was approved by the FDA for treatment of Duchenne muscular dystrophy.

Methods to synthesize PMOs in a 5'→3' direction (reverse-direction compared to standard oligonucleotide synthesis) utilize either an N-trityl-5'-chlorophosphorodimethylamidate or an N-trityl-5'-H-phosphonate morpholino building block (2 and 3, Fig. 1). Recently, we have developed a method for synthesizing PMOs and PMO-DNA chimeras in 3'→5' direction. This procedure uses silyl-protected nucleobase morpholino building blocks and oxidative substitution of a borane phosphonate oligonucleotide intermediate.

Because of the current interest in PMOs, we decided to explore other morpholino-based monomers including the 2'-amino-LNA nucleotide (5, Fig. 1), a derivative of LNA (locked nucleic acid) (4, Fig. 1) having a 2'-N-C methylene bridge. LNA and 2'-amino-LNA are conformationally locked nucleotides of which the latter can be considered a bicyclic morpholino-based ring system (5, Fig. 1). When incorporated into oligonucleotides, the 2'-amino functionality faces the minor groove of nucleic acid duplexes. This site in oligonucleotides therefore provides a convenient handle when N-acylated and N-alkylated for appending amino acids residues, fluorescence probes, nucleobases and a piperizino group while preserving the LNA-type high-affinity hybridization with complementary DNA and RNA strands. A new synthons, 3'-O-benzyl-2'-amino-LNA-T phosphorodiamidite, was used to prepare an alternative morpholino analogue having a 2'-5' linkage (6 and 7, Fig. 1) and the 3'-hydroxyl protected through a benzyl group.

Molecular modelling studies were performed in order to assess the structure of 3'-O-benzyl-2'-amino-LNA-T phosphorodiamidate monomer 6 in a DNA:DNA duplex. In order to complete this study, a 9-mer duplex consisting of DNA:RNA [5'-d(CCTGATATGC);5'-r(GCAUAUCAG)] was downloaded from the protein data bank (PDB entry pdb 1HG9). The RNA strand was converted to DNA and 3'-O-benzyl-2'-amino-LNA phosphorodiamidate monomer 6 was inserted in d(CTGAXATGC) as monomer X. An AMBER force field in Macro Model 9.1 was used to generate representative low energy structures.

This modelling study indicated that the 3'-O-benzyl-2'-amino-LNA-T is locked into a 3'-endo (N-type) conformation (Fig. 2a) in a manner similar to 2'-amino-LNA-T monomer 5 inserted into the same duplex (Fig. 2b). Moreover both monomers 5 and 6 exhibited excellent stacking interactions with the neighboring
The phosphorodiamidite 9 (Scheme 1) was obtained in 87% yield from the 3′-O-benzyl derivative 8 using 2-cyanoethyl-N,N,N′,N′-tetraisopropylphosphorodiamidite as phosphitylating reagent and 4,5-dicyanoimidazole (DCI) as activator.

As outlined in Fig. 3, two generalized pathways were used with synthyn 9 in order to prepare oligonucleotides containing the monomers 6 or 7. For the preparation of 6, the first step was treatment with acid to remove the dimethoxytrityl group. Synthyn 9 was then activated and coupled with the growing oligonucleotide in order to generate the phosphoramidite internucleotide linkage. Following oxidation and capping, the cycle can be repeated using synthyn 9 or the standard 2′-deoxyxynucleoside 3′-phosphoramidites. The synthesis of 7 required modification of this cycle. The detritylation step had to be carried out with trimethylphosphate borane (TMPB) in the presence of acid. TMPB reacts with the generated dimethoxytrityl cation and therefore prevents formation of an internucleotide triplyphosphonate linkage. Following coupling with 9, boronation and capping completed the synthesis steps for one cycle. In order to evaluate the overall yield and the extent of degradation, various coupling conditions were explored and the results are shown in Fig. S3 and Table S1 (ESI). Based upon these results, we conclude that the optimal conditions were 0.10 M tetrazole for each of two coupling rounds of 900 s each giving ~80% total stepwise coupling yield.

For the synthesis of PMO-DNA chimeras containing monomer 7, the borane phosphonate intermediate was converted, through oxidative substitution, to the N,N-dimethylamino phosphoramidate (see Fig. S2, ESI). This post-synthetic transformation consisted of 1) detritylation, 2) removal of the cyanethyl substituent with MeCN:NEt3 (1:1; v/v) (thereby also oxidizing P(III) to P(V)), 3) oxidative substitution by reaction with I2 and dimethylamine, and 4) cleavage from the solid support using sat. aq. NH3. The reaction steps 1–2 were performed on the column whereas steps 3–4 were completed on the polystyrene-resin in glass-vials.

Using synthyn 9 and the standard 2′-deoxyxynucleoside 3′-phosphoramidites, oligonucleotides ON2-ON5 and ON7-ON8 were synthesized, characterized by LC-MS, and used to evaluate the effect of the novel constrained morpholino monomers on duplex stability. ON1-ON10 were hybridized to complementary DNA or RNA and thermal denaturation experiments were conducted in a medium salt buffer (Table 1). When ON2-ON5 were hybridized to DNA, one incorporation of monomer 6 induced a drastic drop in Tm, value of −14.5 °C for ON2 while no detectable transition above 5 °C was observed for ON3 with four incorporations of monomer 6. Replacing the phosphoramidite monomer 6 with the N,N-dimethylamino phosphoramidate monomer 7 induced a significant but less pronounced decrease in stability as one incorporation reduced the Tm value by −10.5 °C for ON4. This difference is most likely due to less electrostatic charge repulsion, since monomer 7 does not have a negative charge. With two incorporations of monomer 7 into ON5 no transition was observed. With complementary RNA, no transition above 5 °C was observed for ON2-ON5.

The mixed-base sequences ON7 and ON8 show a similar pattern with a significant drop in Tm of −10 °C towards complementary DNA and of −7 °C towards complementary RNA for one incorporation of monomer 6, and with three incorporations the drop in Tm value was even more pronounced. Additionally, Tm-measurements were performed on two reference oligonucleotides containing 2′-amino-LNA-T nucleotides incorporated in the 3′ → 5′ (standard manner) with a phosphodiester backbone as monomer 5 (Fig. 1) (ON9 and ON10). These measurements showed the expected large increases in thermal stability in sharp contrast to the effects of the two morpholino-based incorporation motifs introduced herein. The decrease in thermal stability might in part be due to the 2′ → 5′ incorporation pattern of monomer 6 and 7 as strong destabilization has previously been reported upon incorporation of one

**Scheme 1.** Synthesis of the phosphorodiamidite 9. T = Thymin-1-yl.
Temperatures were reported only for the latter which makes direct comparison with our data (Table 1) impossible. In conclusion the 3'-O-benzyl-2'-amino-LNA-T phosphorodiamidate 9 was successfully synthesized and incorporated in a 2' → 5' direction into six oligonucleotides as either one or three phosphorodiamidate monomer(s) 6 (ON2, ON3, ON7, and ON8) or, through a borane phosphonate intermediate, as one or two N,N-dimethylamino phosphorodiamidate monomer(s) 7 (ON4 and ON5) giving chimeric PMO-DNA oligomers. Thermal denaturation experiments revealed a significant decreasing effect on duplex stability of these monomers which may at least in part be explained by interference of 3'-O-benzyl group on the hydration of the phosphorus backbone. A further development could be to select a removable protecting group for the 3'-hydroxy function or to use a 3'-O-methyl substituent as a sterically less demanding group.

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A. Supplementary data

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