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

Kim Vejlegaard [a], Sibasish Paul [b], Tamer Kosbar [a,c], Jesper Wengel [a,*], 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
[c] Department of Chemistry, Faculty of Science, Damietta University, New Damietta 34517, Egypt

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ABSTRACT

Incorporation in a 2′ → 5′ direction of a phosphorodiamidite 2′-amino-LNA-T nucleotide as the morpholino phosphoramidate 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.

Phosphorodiamidate morpholino oligonucleotides (PMOs) (**1**, Fig. 1) are used as antisense tools in gene knockdown experiments.[1] They act as steric blockers, do not degrade their target RNA,[2] and are enzymatically stable.[3] Current investigations of PMOs and conjugates thereof as therapeutics for amending splicing defects *ex vivo* and *in vivo* are underway.[4–14] Recently the first PMO-drug Eteplirsen/Exondys 51 was approved by the FDA for treatment of Duchenne muscular dystrophy.[15]

Methods to synthesize PMOs in a 5′ → 3′ direction (reverse-direction compared to standard oligonucleotide synthesis) utilize either an *N*-trityl-5′-chlorophosphorodimethylamidate[16,17] or an *N*-trityl-5′-H-phosphonate[18] 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.[19]

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*-4′*C* methylene bridge. LNA and 2′-amino-LNA are conformationally locked nucleotides[20] 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.[21] This site in oligonucleotides therefore provides a convenient handle when *N*-acylated and *N*-alkylated[21] for appending amino acids residues,[22] fluorescence probes,[23–26] nucleobases[27] and a piperizino group[28] while preserving the LNA-type high-affinity hybridization with complementary DNA and RNA strands. A new synthon, 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 phosphoramidate monomer **6** in a DNA:DNA duplex. In order to complete this study, a 9-mer duplex consisting of DNA:RNA [5′-d(CTGATATGC):5′-r(GCAUAUCAG)] was downloaded from the protein data bank (PDB entry pdb 1HG9),[29] the RNA strand was converted to DNA and 3′-*O*-benzyl-2′-amino-LNA phosphoramidate monomer **6** was inserted in d(CTGA**X**ATGC) 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

* Corresponding author.
E-mail address: jwe@sdu.dk (J. Wengel).

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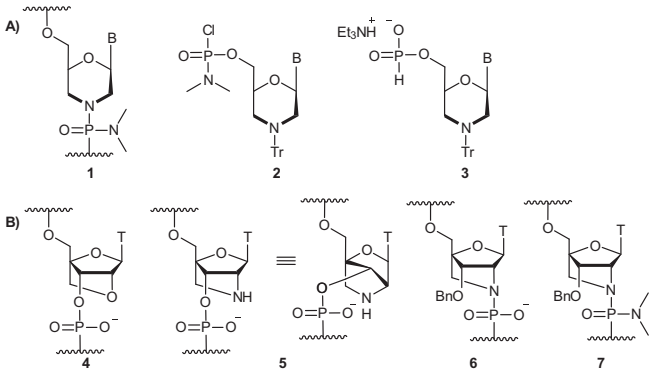


Fig. 1. A) Chemical structures of phosphorodiamidate morpholino oligonucleotides (PMO, **1**) and the morpholino building blocks (**2** and **3**) used to synthesize PMO in a 6′ → 3′ direction. B) Chemical structures of phosphordiester monomers of LNA, **4** and 2′-amino LNA, **5**. Structure **6** shows the 2′-amino-LNA-T phosphoramidate monomer and structure **7** the 2′-amino-LNA-T dimethylamino phosphorodiamidate monomer. B = nucleobases and T = thymin-1-yl.

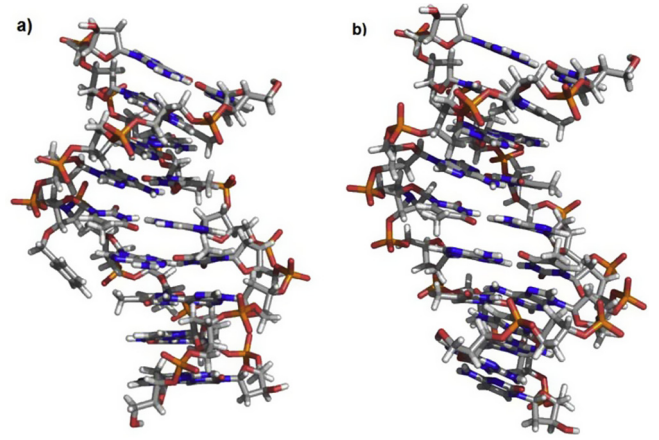
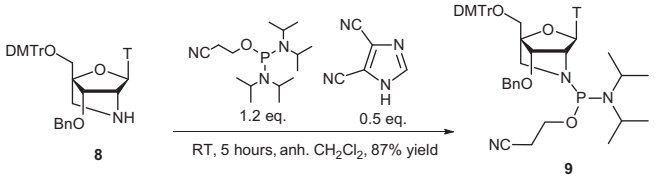


Fig. 2. Snapshots from molecular dynamics simulations of a 9-mer duplex [(5′-d(CTGA**X**ATGC):5′-d(GCATATCAG)] modified at the central position (**X**) with a) 3′-*O*-benzyl-2-amino-LNA phosphoramidate monomer **6,** and b) 2′-amino-LNA-T monomer **5**. See ESI† Fig. S7 for a larger display including a snapshot of the dinucleotide structure showing more clearly the sugar pucker of the modified monomers **5** and **6**.

nucleobases and nucleobase positioning suitable for engaging into Watson–Crick base pairs (Fig. 2a and b). It is also evident from the molecular modelling that the 3′-*O*-benzyl group is protruding from the duplex into the minor groove and covers part of the phosphorous backbone. Based upon this modelling study, the 3′-*O*-benzyl and the locked morpholino scaffold seemed not to impede Watson-Crick base pairing thus giving us an incentive to begin an experimental evaluation of novel constrained morpholino monomers **6** and **7** in the context of DNA and RNA oligomers. In this manuscript, we describe the first synthesis procedures for incorporation of these monomers into oligonucleotides and present an evaluation of their duplex stability.



Scheme 1. Synthesis of the phosphorodiamidite **9**. T = Thymin-1-yl.

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 synthon **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. Synthon **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 synthon **9** or the standard 2′-deoxynucleoside 3′-phosphoramidites. The synthesis of **7** required modification of this cycle. The detritylation step had to be carried out with trimethylphosphite borane (TMPB) in the presence of acid. TMPB reacts with the generated dimethoxytrityl cation and therefore prevents formation of an internucleotide tritylphosphonate 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,[20] to the *N*,*N*-dimethylamino phosphorodiamidate (see Fig. S2, ESI†). This post-synthetic transformation consisted of 1) detritylation, 2) removal of the cyanoethyl substituent with MeCN:NEt_3 (1:1; v/v) (thereby also oxidizing P(III) to P(V)), 3) oxidative substitution by reaction with I_2 and dimethylamine, and 4) cleavage from the solid support using sat. aq·NH_3. The reaction steps 1–2 were performed on the column whereas steps 3–4 were completed on the polystyrene-resin in glass-vials.

Using synthon **9** and the standard 2′-deoxynucleoside 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 T_m 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 phosphoramidate monomer **6** with the *N*,*N*-dimethylamino phosphorodiamidate monomer **7** induced a significant but less pronounced decrease in stability as one incorporation reduced the T_m 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 T_m 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 T_m value was even more pronounced. Additionally, T_m-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

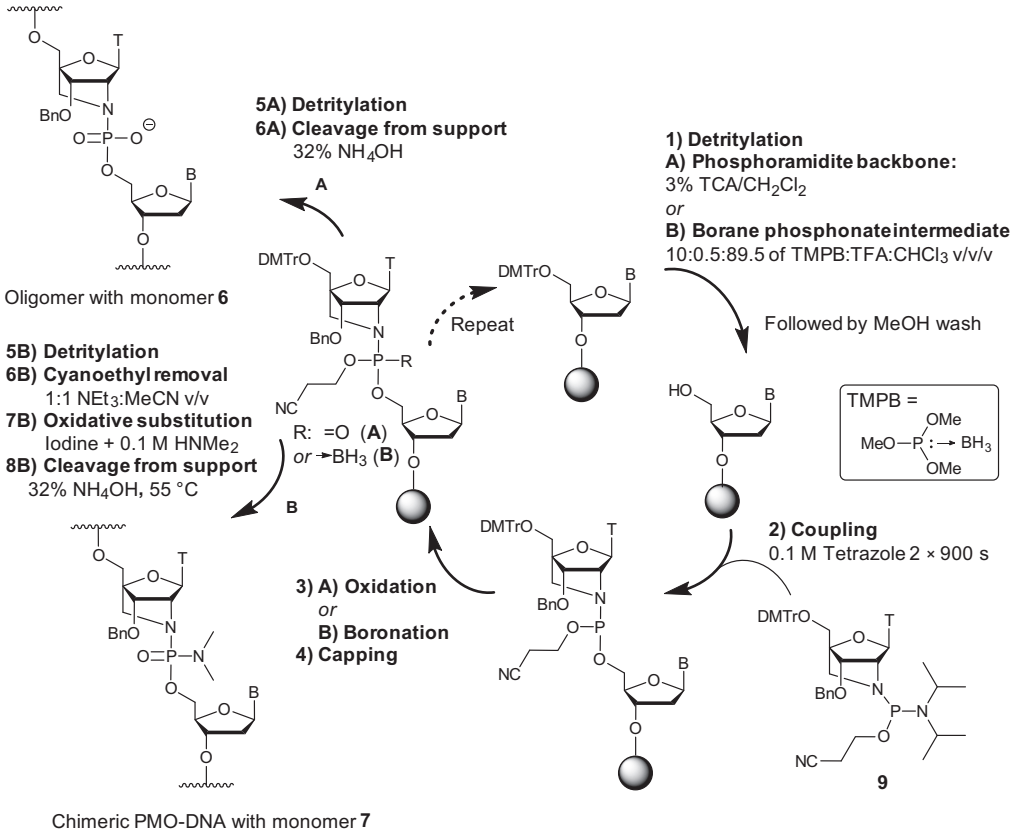


Fig. 3. Synthetic cycle for incorporation of the phosphorodiamidite **9** with the optimal coupling condition of 0.1 M tetrazole for two rounds of 900 s. Route A enables the formation of phosphoramidate monomer **6** using standard detritylation, oxidation with aqueous iodine and cleavage from resin using sat. aq·NH_3. Route B enables the formation of chimeric PMO-DNA oligomers containing *N,N*-dimethylamino phosphorodiamidate monomer **7** using the trityl-scavenger mixture TMPB:CF_3COOH:$CHCl_3$ 10:0.5:89.5 v/v/v, boronation using 0.05 M BH_3 in THF and the post-synthetic transformation followed by cleavage from resin using sat. aq·NH_3. B = Nucleobases and T = Thymin-1-yl monomer.

Table 1
Thermal denaturation studies for synthesized oligonucleotides[a]

Sequence		T_m-values in medium salt buffer	
		Complementary DNA	Complementary RNA
5′-TTT-TTT-TTT-T-3′	**ON1**	19.5 °C	13.5 °C
5′-TTT-TT**6**-TTT-T-3′	**ON2**	5.0 °C (−14.5 °C)	<5.0 °C
5′-TTT-**666-6**TT-T-3′	**ON3**	<5.0 °C	<5.0 °C
5′-TTT-TT**7**-TTT-T-3	**ON4**	9.0 °C (−10.5 °C)	<5.0 °C
5′-TTT-T**77**-TTT-T-3′	**ON5**	<5.0 °C	<5.0 °C
5′-GTG-ATA-TGC-3′	**ON6**	31 °C	27.5 °C
5′-GTG-A**6**A-TGC-3′	**ON7**	20.5 °C (−10 °C)	20.5 °C (−7 °C)
5′-G**6**G-A**6**A-**6**GC-3′	**ON8**	5.0 °C (−26 °C)	6.0 °C (−21.5 °C)
5′-GTG-A**5**A-TGC-3′	**ON9**	34.5 °C (+3.5 °C)	35.5 °C (+8 °C)
5′-G**5**G-A**5**A-**5**GC-3′	**ON10**	40.0 °C (+9 °C)	48.5 °C (+21 °C)

[a] Thermal denaturation temperatures of DNA-DNA and DNA-RNA duplexes measured as the average of the maxima of the first derivatives of the melting curves (A_{260} versus temperature) from two independent melting temperature determination with a deviation <0.5 °C. Numbers in brackets are ΔT_m values measured in degrees Celsius as the difference in T_m-values between modified and unmodified duplexes. The experiments were recorded in medium salt buffer (6.1 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0 using 1.5 M concentrations of the two complementary strands (assuming identical extinction coefficients for all modified and unmodified oligonucleotides); **5** = 2′-amino-LNA-T phosphodiester monomer, **6** = 2′-amino-LNA-T phosphoramidate, **7** = 2′-amino-LNA-T *N,N*-dimethylamino phosphorodiamidate, T = thymin-1-yl monomer, C = cytosine-1-yl monomer, A = adenine-9-yl monomer and G = guanine-9-yl monomer.

or few 2′ → 5′ linked 3′-deoxy monomers into DNA duplexes.[30] Notably, a 3′-deoxy derivative of monomer **6** has been incorporated into DNA and morpholino oligomers, but melting temperatures were reported only for the latter which makes direct comparison with our data (Table 1) impossible.[31]

In conclusion the 3′-*O*-benzyl-2′-amino-LNA-T phosphorodiamide **9** was successfully synthesized and incorporated in a 2′ → 5′ direction into six oligonucleotides as either one or three phosphoramidate 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

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.05.023.

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