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Methylphosphonate LNA: A Locked Nucleic Acid with a Methylphosphonate Linkage

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Abstract—Synthesis of an oligonucleotide containing one methylphosphonate locked nucleic acid (LNA) thymine monomer using the phosphoramidite approach is described. The binding affinity of this 9-mer methylphosphonate LNA towards complementary DNA and RNA oligonucleotides was increased compared to the reference DNA, but decreased compared to the reference LNA. In the 9-mer sequence context studied, introduction of a single methylphosphonate LNA monomer, contrary to a single LNA monomer, efficiently inhibits 3′-exonucleolytic degradation.

LNA (locked nucleic acid, Fig. 1) is a conformationally restricted RNA mimic displaying unprecedented binding affinities towards complementary DNA/RNA.[1–6] We have recently reported synthesis and high-affinity hybridization of oligonucleotides (ONs) containing amide-linked LNA-type dinucleotides.[7] Methylphosphonate-linked ONs[8–14] (Fig. 1) is another class of modified ONs containing non-ionic internucleoside linkages which has been studied for antisense applications.[15–17] Compared to the corresponding unmodified ONs, methylphosphonate DNA displays stabilization towards nucleolytic degradation but also reduced binding affinity towards DNA/RNA target strands.[12–14] Most studies have been performed on methylphosphonate ONs containing diastereoisomeric (Rp/Sp) linkages, but introduction of chirally pure methylphosphonate linkages (Rp isomer) increased the binding.[16] Especially a 2′-*O*-methyl-ON with alternating phosphate and chirally pure methylphosphonate linkages proved promising as not only very efficient protection against exonucleolytic degradation, but also increased thermal stability of the duplex formed with complementary RNA, were obtained, even relative to the corresponding DNA/RNA reference duplex.[14] Based on these promising data, and the fact that the furanose rings of both 2′-*O*-methyl-RNA and LNA are conformationally restricted or locked, respectively, in *N*-type conformations (Fig. 1),[17] we decided to study methylphosphonate-linked LNA (Fig. 1).

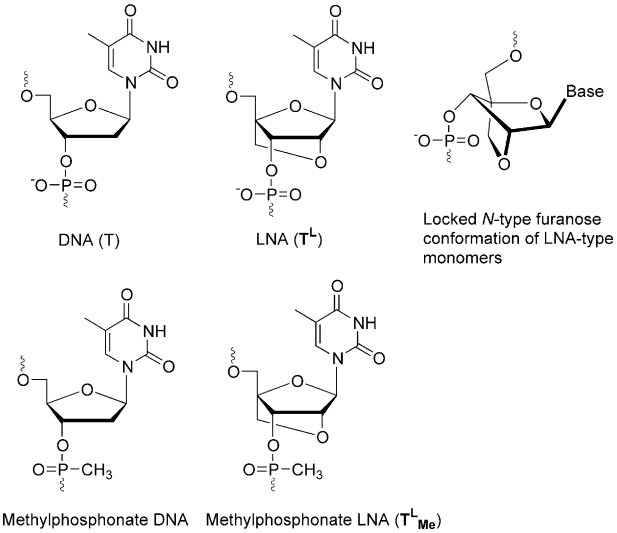

Figure 1. Structures of the thymine nucleotide monomers of DNA, LNA (T^L), methylphosphonate DNA, and methylphosphonate LNA (T^L_{Me}).

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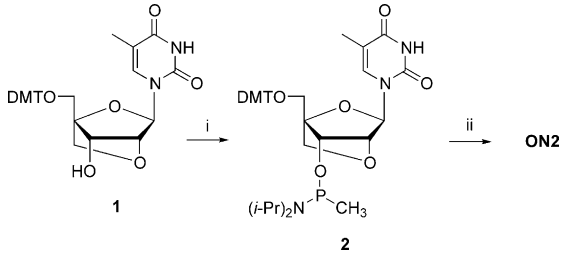
The LNA-type methylphosphonamidite **2** needed for the automated synthesis of methylphosphonate LNA, that is **ON2** containing eight DNA monomers and one methylphosponate LNA monomer (Table 1), was obtained as shown in Scheme 1. Thus, reaction of nucleoside **1**[2,18] with bis(diisopropylamino)methylphosphine[19] and 1*H*-tetrazole in dichloromethane afforded methylphosphonamidite **2** as a mixture of two diastereoisomers in 44% yield after column chromatographic purification.[20] Whereas the peaks in the ^{31}P NMR spectrum for the two diastereoisomers of the corresponding thymidine phosphonamidite appeared closely together (121.0 and 120.7 ppm),[10] two clearly separated peaks were observed for the two diastereoisomers of **2** (129.4 and 122.3 ppm). Steric constraints imposed by the C2′-oxymethylene-C4′ linker on the O3′-substituent are likely to contribute to this difference.

The LNA methylphosphonate oligonucleotide **ON2** (Table 1) was synthesized in 0.2 μmol scale on an automated DNA synthesizer using amidite **2** and commercial DNA phosphoramidites (*tert*-butylphenoxyacetyl protected G and phenoxyacetyl protected A and C). The use of base-labile protecting groups on the base moieties was necessary because of the known instability of methylphosphonate-linked ONs under the strongly basic conditions otherwise needed for deprotection.[21,22] Standard conditions were applied during ON synthesis except for extended coupling time for the amidite **2** (15 min coupling using manual coupling conditions,[23] ~83% coupling yield; 2 min coupling time for DNA phosphoramidites, >99% coupling yields). **ON2**, as the 5′-DMT-ON derivative, was deprotected and simultaneously cleaved off the solid support using saturated methanolic ammonia (55 °C, 2.5 h). Purification (which includes detritylation) was performed on a disposable reversed phase purification cartridge furnishing the fully deprotected **ON2**.[24]



Scheme 1. Reagents and conditions: (i) $CH_3P(N(i\text{-}Pr)_2)_2$, 1*H*-tetrazole, CH_2Cl_2 (44%); (ii) DNA synthesizer.

The hybridization properties of the methylphosphonate LNA/DNA mixmer **ON2** towards complementary single-stranded DNA and RNA strands were evaluated by thermal denaturation studies (Table 1). Reference duplex melting temperatures (T_m values) were obtained for the unmodified DNA oligomer **ON1** and the corresponding LNA oligomer **ON3**[25] containing a single LNA thymine monomer ($\mathbf{T^L}$, Fig. 1, Table 1) as the central nucleotide. Relative to the fully matched reference DNA/DNA and DNA/RNA duplexes (**Y** = A), incorporation of the methylphosphonate LNA monomer $\mathbf{T^L_{Me}}$ induced a significant increase in T_m values towards both complementary DNA ($\Delta T_m = +3\,^\circ C$) and complementary RNA ($\Delta T_m = +6\,^\circ C$). A similar, but even more pronounced, affinity enhancing effect was obtained for the LNA **ON3** ($T_m = 35\,^\circ C$ towards complementary DNA,[25] $T_m = 37\,^\circ C$ towards complementary RNA[26]). Also shown in Table 1 are T_m values for **ON1** and **ON2** towards singly mis-matched complements. These data demonstrate that the methylphosphonate LNA **ON2** discriminates towards mis-matches at least as efficiently as the DNA reference **ON1** (evaluated for mis-matches positioned directly opposite the $\mathbf{T^L_{Me}}$ monomer).

Our interest in methylphosphonate LNA as antisense molecules was in part stimulated by the promise of obtaining efficient protection towards nucleolytic degradation as both methylphosphonate linkages[10,14] and LNA monomers[1,27] confer some stabilization towards 3′-exonucleolytic degradation. Thus, whereas LNA oligomers containing three LNA monomers at each end display a 10-fold stabilization in human serum compared to unmodified DNA,[27] introduction of a single LNA monomer in the end of a DNA oligomer only leads to a 2–3-fold stabilization.[27,28] These results are confirmed in Figure 2 which depicts degradation of [^{32}P]-labelled **ON1**, **ON2** and **ON3** by snake venom phosphordiesterase (3′-exonuclease).[29,30] Thus, whereas the unmodified DNA **ON1** was completely degradated after 10 min of incubation, the centrally positioned thymine LNA monomer clearly retarded digestion of the central segment of **ON3**. However, almost complete degradation of **ON3** was seen after 10 min of incubation. On the contrary, after initial rapid digestion of a

Table 1. Melting temperatures (T_m values) towards complementary single-stranded DNA [d(sequence)] and RNA [r(sequence)] targets obtained as the maximum of the first derivative of the melting curve (A_{260} vs temperature) in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 1.5 μM concentrations of the two complements[a]

Sequence		T_m/°C [3′-d(CACT**Y**TACG)]				T_m/°C [3′-r(CACU**Y**UACG)]	
	Y =	A	C	G	T	A	C
5′-d(GTGATATGC) (**ON1**)		29	n.d.[b]	19	<10[c]	27	<10[c]
5′-d(GTGAT$\mathbf{T^L_{Me}}$ATGC) (**ON2**)		32	<10[c]	15	<10[c]	33	<10[c]
5′-d(GTGAT$\mathbf{T^L}$ATGC) (**ON3**)		35[d]	n.d.[b]	n.d.[b]	n.d.[b]	37	n.d.[b]

[a]A, C, G, T and U denote unmodified DNA/RNA monomers; $\mathbf{T^L}$ denotes a thymin-1-yl LNA monomer; $\mathbf{T^L_{Me}}$ denotes a thymin-1-yl methylphosphonate LNA monomer.
[b]Not determined.
[c]No cooperative transition above 10 °C was observed.
[d]Ref 25.

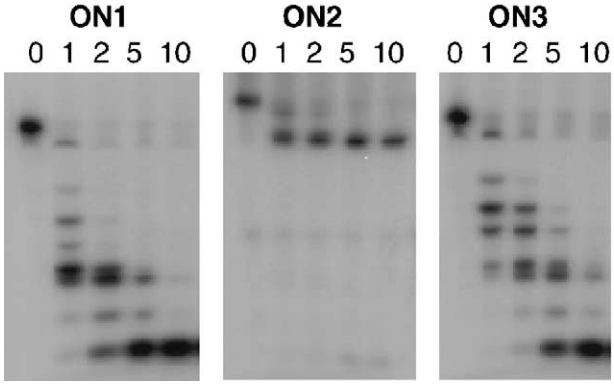


Figure 2. Snake venom phosphordiesterase degradation of [^{32}P]-labelled DNA oligo **ON1**, methylphosphonate LNA oligo **ON2** and LNA oligo **ON3**. The numbers above the lanes indicate the time of incubation (in minutes).[30]

few of the unmodified DNA nucleotides from the 3′-end of the methylphosphonate LNA **ON2**, the presence of the methylphosphonate LNA monomer $\mathbf{T^{L}_{Me}}$ induced complete resistance towards further degradation. Digestion using 25 times as much enzyme as above[30] and up to 120 min of incubation time did not induce significant further degradation of **ON2** (data not shown).

Incorporation of a methylphosphonate LNA monomer into a DNA strand significantly increases the binding affinity, especially towards RNA. Thus, a locked *N*-type furanose conformation efficiently improves the binding affinity of oligonucleotides containing not only natural phosphordiester linkages but also neutral methylphosphonate linkages. In addition, the general compatibility and affinity-enhancing effect of LNA monomers[1–7] in various ON contexts has been underlined, and it appears likely that it should be possible to synthesize a methylphosphonate LNA displaying high overall binding affinity towards a given RNA target. Very encouraging for antisense applications is the obtained result that introduction of a single methylphosphonate LNA monomer efficiently inhibits 3′-exonucleolytic degradation. Future studies should be directed towards a comprehensive evaluation of the chemical and structural characteristics and the biological properties of methylphosphonate LNA, including the stereopure variants.

Acknowledgements

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References and Notes

1. Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun*. **1998**, 455. LNA was herein defined as an oligonucleotide containing one or more conformationally locked 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide(s) ['LNA monomer(s)'].
2. Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607.
3. Singh, S. K.; Wengel, J. *Chem. Commun*. **1998**, 1247.
4. Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Morio, K.; Doi, T.; Imanishi, T. *Tetrahedron Lett*. **1998**, *39*, 5401.
5. Wengel, J. *Acc. Chem. Res*. **1999**, *32*, 301.
6. Braasch, D. A.; Corey, D. R. *Chem. Biol*. **2000**, *55*, 1.
7. Lauritsen, A.; Wengel, J. *Chem. Commun*. **2002**, 530.
8. Miller, P. S.; McParland, K. B.; Jayaraman, K.; Ts'o, P. O. P. *Biochemistry* **1981**, *20*, 1874.
9. Smith, C. C.; Aurelian, L.; Reddy, M. P.; Miller, P. S.; Ts'o, P. O. P. *Proc. Natl. Acad. Sci. U.S.A*. **1986**, *83*, 2787.
10. Agrawal, S.; Goodchild, J. *Tetrahedron Lett*. **1987**, *28*, 3539.
11. Löshner, T.; Engels, J. W. *Nucleosides Nucleotides* **1988**, *7*, 729.
12. Sarin, P. S.; Agrawal, S.; Civeira, M. P.; Goodchild, J.; Ikeuchi, T.; Zamecnik, P. C. *Proc. Natl. Acad. Sci. U.S.A*. **1988**, *85*, 7448.
13. Tidd, D. M.; Hawley, P.; Warenius, H. M.; Gibson, I. *Anticancer Drug Des*. **1988**, *3*, 117.
14. Reynolds, M. A.; Hogrefe, R. I.; Jaeger, J. A.; Schwartz, D. A.; Riley, T. A.; Marvin, W. B.; Daily, W. J.; Vaghefi, M. M.; Beck, T. A.; Knowles, S. K.; Klem, R. E.; Lyle, J. A., Jr. *Nucleic Acids Res*. **1996**, *24*, 4584.
15. De Mesmaeker, A.; Häner, R.; Martin, P.; Moser, H. E. *Acc. Chem. Res*. **1995**, *28*, 366.
16. Freier, S. M.; Altmann, K.-H. *Nucleic Acids Res*. **1997**, *25*, 4429.
17. Kværnø, L.; Wengel, J. *Chem. Commun*. **2001**, 1419.
18. Koshkin, A. A.; Fensholdt, J.; Pfundheller, H. M.; Lomholt, C. *J. Org. Chem*. **2001**, *66*, 8504.
19. Moriarty, R. M.; Hiratake, J.; Liu, K. *J. Am. Chem. Soc*. **1990**, *112*, 8575.
20. Experimental procedure for preparation of (1*R*,3*R*,4*R*,7*S*)-1-(4,4′-dimethoxytrityloxymethyl)-7-((diisopropylamino)(methyl)phosphinoxy)-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (**2**). To a stirred solution of nucleoside **1** (0.78 g, 1.40 mmol)[2,18] in anhydrous CH_2Cl_2 (10 mL) at room temperature was added 1*H*-tetrazole (0.10 g, 1.50 mmol) and bis(diisopropylamino)methylphosphine[19] (0.50 mL, 2.00 mmol). After stirring for 24 h at room temperature, additional 1*H*-tetrazole (0.02 g, 0.30 mmol) and bis(diisopropylamino)-methylphosphine (0.15 mL, 0.60 mmol) were added and the mixture was stirred for another 24 h. Dichloromethane (20 mL) was added and washing was performed using saturated aqueous sodium hydrogen carbonate (3 × 20 mL). The separated organic phase was dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using EtOAc/petroleum ether/triethylamine (50/50/0.5) as eluent. The fractions containing product were pooled and evaporated to dryness under reduced pressure affording amidite **2** (0.43 g, 44%) as a white solid material. ^{31}P NMR (CH_3CN) δ 129.4, 122.3; MS-FAB (*m*/*z*) 718 $[M]^+$.
21. Miller, P. S.; Yano, J.; Yano, E.; Carrol, C.; Jayaraman, K.; Ts'o, P. O. P. *Biochemistry* **1979**, *18*, 5134.
22. Sinha, N. D.; Michaud, D. P.; Roy, S. K.; Casale, R. A. *Nucleic Acids Res*. **1994**, *22*, 3119.
23. Rajwanshi, V. K.; Håkansson, A. E.; Dahl, B. M.; Wengel, J. *Chem. Commun*. **1999**, 1395.
24. MALDI-MS (*m*/*z*) 2778.0 (calcd 2779.9) $[M-H]^-$; Analysis by capillary gel electrophoresis verified the purity as >80%.
25. Kværnø, L.; Kumar, R.; Dahl, B. M.; Olsen, C. E.; Wengel, J. *J. Org. Chem*. **2000**, *65*, 5167.
26. Lauritsen, A.; Wengel, J. Unpublished results.
27. Kurreck, J.; Wyszko, E.; Gillen, C.; Erdmann, V. A. *Nucleic Acids Res*. **2002**, *30*, 1911.
28. Morita, K.; Hasegawa, C.; Kaneko, M.; Tsutsumi, S.;

Sone, J.; Ishikawa, T.; Imanishi, T.; Koizumi, M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 73.
29. Likely due to the presence of the uncharged methylphosphonate linkage, the methylphosphonate LNA **ON2** displays a slightly different gel mobility than **ON1** and **ON3**.
30. Oligoes were 5′-^{32}P-labelled with polynucleotide kinase and mixed with unlabelled oligo prior to digest reactions. The reactions were performed at 25 °C in 8 μL of the following buffer: 0.1 M Tris–HCl pH 8.9, 0.1 M NaCl, 14 mM $MgCl_2$. In each reaction, 5 pmol of oligo was digested with 0.002 U of snake venom phosphordiesterase (*Crotalus adamanteus*). The reactions were stopped by addition of formamide with EDTA. Samples were analyzed on 20% acrylamide/urea gels.