Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2219–2222

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

THE FIRST ANALOGUES OF LNA (LOCKED NUCLEIC ACIDS): PHOSPHOROTHIOATE-LNA AND 2'-THIO-LNA

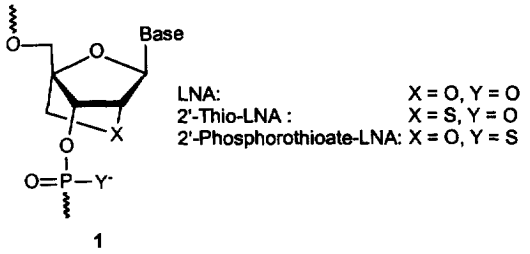
Ravindra Kumar, Sanjay K. Singh, Alexei A. Koshkin, Vivek K. Rajwanshi, Michael Meldgaard, and Jesper Wengel*

Center for Synthetic Bioorganic Chemistry, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark

Received 27 May 1998; accepted 14 July 1998

Abstract: LNA (Locked Nucleic Acids, **1**, X = O, Y = O) is a novel oligonucleotide analogue capable of recognizing complementary DNA and RNA with unprecedented thermal affinities. Synthesis of the first chemically modified LNA analogues is reported. A 9-mer phosphorothioate- LNA containing three LNA thymine monomers (**1**, X = O, Y = S, Base = thymin-1-yl) and 9-mer LNAs containing one, three or five 2'-thio-LNA monomers (**1**, X = S, Y = O, Base = uracil-1-yl) were able to recognize both complementary DNA and RNA with thermal affinities comparable to those of parent LNA.. © 1998 Elsevier Science Ltd. All rights reserved.

Conformationally restricted oligonucleotide (ON) analogues containing bicyclic monomers have attracted considerable attention due to their potential for formation of entropically favourable duplexes.[1-5] We have recently introduced LNA (Locked Nucleic Acids, **1**, X = O, Y = O)[6,7] as a novel ON analogue containing one or more 2'-*O*,4'-*C*-linked bicyclo[2.2.1]nucleoside LNA monomers locked in a 3'-endo conformation.[6-8] The highly interesting properties and characteristics of LNA are summarized below:[6,7]



1

LNA: X = O, Y = O
2'-Thio-LNA : X = S, Y = O
2'-Phosphorothioate-LNA: X = O, Y = S

Characteristics of LNA:[6,7]

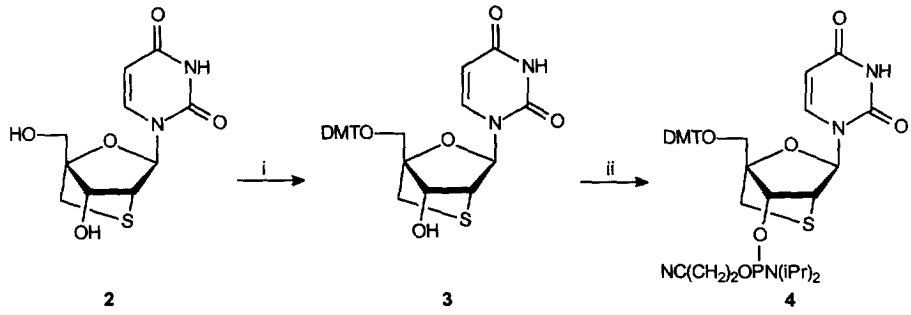
- Unprecedented thermal stabilities of duplexes towards complementary DNA and RNA (ΔT_m = +3 to +8 °C)
- Stability towards 3'-exonucleolytic degradation
- Efficient automated oligomerization
- Good aqueous solubility

We are interested in evaluating chemical variants of LNA and in this report we introduce the sulphur analogues phosphorothioate-LNA (**1**, X = O, Y = S) and 2'-thio-LNA (**1**, X = S, Y = O). Though a 14-mer oligothymidylate LNA displayed stability towards the 3'-exonuclease snake venom phosphordiesterase,[6] degradation of LNA by other nucleases cannot at this moment be ruled out. As the introduction of phosphorothioate internucleoside linkages has proved to be a solution to this problem for unmodified ONs,[9] we decided to synthesize an all-phosphorothioate-LNA (Table, entry 6).[10] 4'-Thio analogues (with a sulphur atom replacing the oxygen atom in the furanose ring) of natural ONs have shown satisfactory affinities towards complementary ONs.[11-13] This fact, and molecular modeling suggesting the compatibility of a similar modification in the additional ring of LNA monomers,[14] have prompted

*E-mail: wengel@kiku.dk; fax: +45 35 32 02 12; tel: +45 35 32 01 70

0960-894X/98/$ - see front matter © 1998 Elsevier Science Ltd. All rights reserved.
PII: S0960-894X(98)00366-7

us to synthesize the 2'-thio-LNA nucleoside **2** shown in the Scheme.[15] For incorporation of monomer **1** (X = S, Y = O, Base = uracil-1-yl) into ONs, the phosphoramidite derivative **4**[16] was synthesized from **2** in 41% yield by 4,4'-dimethoxytritylation affording the 5'-*O*-protected nucleoside **3** followed by phosphitylation (Scheme). Amidite **4** was used to synthesize 9-mer 2'-thio-LNAs containing one, three or five 2'-thio-LNA monomers (Table, entries 7-9).[17]



Scheme. (i) 4,4'-Dimethoxytrityl chloride (DMTCl), pyridine (83%); (ii) $NC(CH_2)_2OP(Cl)N(iPr)_2$, diisopropylethylamine, dichloromethane (49%). The experimental procedures were equivalent to those reported for the corresponding parent LNA nucleosides.[7]

The thermal affinity studies towards complementary DNA and RNA are depicted in the Table as T_m values (melting temperatures) and ΔT_m values (change in T_m *per* modification). The remarkable effect of introducing three LNA thymine monomers $\mathbf{T^L}$ (**1**, X = O, Y = O, Base = thymin-1-yl) appears clearly (entries 1 and 2).[6,7] The results shown in entries 3 and 4 are in line with the earlier published data (ΔT_m = +5.0 °C / $\mathbf{T^L}$). It is well known that the thermal affinity of phosphorothioate ONs is decreased by approximately -1 °C per phosphorothioate linkage.[18] However, comparison of the thermal affinities of the phosphorothioate-LNA (**1**, X = O, Y = S, Base = thymin-1-yl) of entry 6 with the corresponding phosphordiester reference of entry 2 (both containing three $\mathbf{T^L}$ monomers) reveals a significantly less pronounced decrease (ΔT_m = -0.4/-0.4 °C per phosphorothioate linkage) than that observed between the corresponding oligodeoxynucleotides (entries 1 and 5, ΔT_m = -0.9/-1.4 °C per phosphorothioate linkage). The reason for this effect is presently not clear, but it could possibly originate from the dominating effects of the rigid bicyclo[2.2.1]nucleoside system partially compensating the unfavourable effect from diastereoisomerism at the phosphorus atoms. An interesting effect can be extracted when comparing the results for the two all-phosphorothioate sequences of entries 5 and 6. Thus, impressively increased thermal affinities towards both DNA (ΔT_m = +6.7 °C / $\mathbf{T^L}$) and RNA (ΔT_m = +10.0 °C / $\mathbf{T^L}$) were obtained by introducing the three LNA thymine monomers. Comparison with the results of entries 1 and 2 reveals a possible conclusion with important implications for antisense therapeuticals, namely that the stabilizing effect of LNA monomers is even more pronounced for phosphorothioate ONs than for unmodified phosphordiester ONs. Thus, *e.g.*, if parent LNA is degraded *in vivo*, or if a stretch of unmodified phosphorothioate nucleotides is necessary to effect RNase H activity, phosphorothioate-LNA appears to be a very attracting molecule.

The results for the 2'-thio-LNAs (Table, entries 7-9) clearly indicate a positive effect on the thermal stability of duplexes towards both DNA and RNA by the introduction of 2'-thio-LNA monomer **1** (X = S, Y = O). This effect (ΔT_m ~ +5 °C / modification towards DNA; ΔT_m ~ +8 °C / modification towards RNA) is comparable with that

Entry	Sequence	DNA complement		RNA complement	
		T_m/°C	ΔT_m/°C	T_m/°C	ΔT_m/°C
	Reference sequences				
1[6,7]	5'-d(GTGATATGC)	28		28	
2[6,7]	5'-d(GT^{L}GAT^{L}AT^{L}GC)	44	+5.3[a]	50	+7.3[a]
3	5'-d(GTGTTTTGC)	32		n.d.	
4	5'-d(GT^{L}GT^{L}T^{L}T^{L}T^{L}GC)	52	+5.0[b]	n.d.	
	Phosphorothioate sequences				
5	5-d($G_ST_SG_SA_ST_SA_ST_SG_SC$)	21	−0.9[a]	17	−1.4[a]
6	5'-d($G_ST^L{}_SG_SA_ST^L{}_SA_ST^L{}_SG_SC$)	41	−0.4[c]/+6.7[d]	47	−0.4[c]/+10.0[d]
	2'-Thio-LNA sequences				
7	5'-d(GTGAU^{LS}ATGC)	34	+6.0[a]	36	+8.0[a]
8	5'-d(GU^{LS}GAU^{LS}AU^{LS}GC)	42	+4.7[a]	52	+8.0[a]
9	5'-d(GU^{LS}GU^{LS}U^{LS}U^{LS}U^{LS}GC)	51	+3.8[b]	n.d.	

Table. LNAs synthesized and melting temperatures towards complementary DNA and RNA measured in 100 mM NaCl, 10 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.0.[7] A = 2'-deoxyadenosine monomer, C = 2'-deoxycytidine monomer, G = 2'-deoxyguanosine monomer, T = thymidine monomer, T^L = LNA thymine monomer (**1**, X = O, Y = O, Base = thymin-1-yl), U^{LS} = 2'-thio-LNA uracil monomer (**1**, X = S, Y = O, Base = uracil-1-yl). The subscript "s" in the sequences depicted in entries 5 and 6 denotes a phosphorothioate internucleoside linkage. T_m = melting temperature, ΔT_m = change in T_m *per* modification, n.d. = not determined. [a]The results of entry 1 used as reference T_m values. [b]The result of entry 3 used as reference T_m value. [c]The results of entry 2 used as reference T_m values. [d]The results of entry 5 used as reference T_m values.

observed for parent LNA (entries 1-4). The picture is complicated by the simultaneous introduction of two modifications (the 2'-thio functionality and uracil instead of thymine). However, as we have earlier observed identical melting temperatures for the LNA thymine and uracil monomers,[7] and as the references containing 2'-deoxyuridine instead of thymidine, if anything, would be expected to display lower T_m values than those found in entries 1 and 3,[18] the comparison seems relevant. The results obtained for 2'-thio-LNAs are in accordance with the molecular modeling results which indicated that the additional 2'-*S*,4'-*C*-linked ring is positioned at the rim of the duplexes with no apparent unfavourable intermolecular steric interactions.[14] Consequently, *e.g.*, the corresponding 2'-amino-LNA monomer (**1**, X = NH, Y = O) appears to be very interesting as a possible conjugation site in an LNA-type ON.

In conclusion, the first analogues of LNA, namely phosphorothioate-LNA and 2'-thio-LNA, have been synthesized. Preliminary studies of their binding behavior towards complementary DNA and RNA revealed very interesting results. Thus, hitherto unprecedented thermal stabilities of phosphorothioate ONs were obtained, and the 2'-thio substitution did not interfere with the superior nucleic acid recognition properties of LNA. With these results, the importance of the conformationally restricted LNA structure, and its compatibility with much of the chemistry

already developed for natural ONs, have been stressed. Further studies on these and other chemically modified LNAs are underway.

Acknowledgments

Ms Britta Dahl is thanked for LNA synthesis. Dr Carl Erik Olsen, Department of Chemistry, The Royal Danish Veterinary and Agricultural University is thanked for performing MALDI-MS. The Danish Natural Science Research Council, The Danish Technical Research Council, The Danish International Development Agency and Exiqon A/S, Denmark are thanked for financial support.

References and Notes

1. Bolli, M.; Litten, J.C.; Schütz, R.; Leumann, C. J. *Chem. Biol.* **1996**, *3*, 197.
2. Marquez, V. E.; Siddiqui, M. A.; Ezzitouni, A.; Russ, P.; Wang, J.; Wagner, R. W.; Matteucci, M. D. *J. Med. Chem.* **1996**, *39*, 3739.
3. Herdewijn, P. *Liebigs Ann.* **1996**, 1337.
4. Nielsen, P.; Pfundheller, H. M.; Wengel, J. *Chem. Commun.* **1997**, 825.
5. Nielsen, P.; Pfundheller, H. M.; Olsen, C. E.; Wengel, J. *J. Chem. Soc. Perkin Trans. 1* **1997**, 3423.
6. Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun.* **1998**, 455.
7. Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607.
8. The uracil and cytosine LNA monomers have been prepared and analyzed by X-ray crystallography: Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735. No oligomers were reported.
9. Zon, G.; Stec, W. J. Phosphorothioate oligonucleotides, in *Oligonucleotides and Analogues* (Eckstein, F., ed.) IRL Press, Oxford **1991**, 87 and references cited therein.
10. The all-phosphorothioate LNA (Table, entry 6) and the corresponding all-phosphorothioate oligodeoxynucleotide reference (Table, entry 5) were synthesized on a Biosearch 8750 DNA synthesizer using standard conditions and Beaucages' reagent as sulphurizing agent. The stepwise coupling yields were >98%. After completion of the syntheses, deprotection and cleavage from the solid support was effected using concentrated ammonia (55 °C, 14 h). After precipitation from ethanol the oligos were analyzed using capillary gel electrophoresis (>90% purity).
11. Bellon, L.; Barascut, J.-L.; Maury, G.; Divita, G.; Goody, R.; Imbach, J.-L. *Nucleic Acids Res.* **1993**, *21*, 1587.
12. Hancox, E. L.; Connolly, B. A.; Walker, R. T. *Nucleic Acids Res.* **1993**, *21*, 3485.
13. Jones, G. D.; Lesnik, E. A.; Owens, S. R.; Risen, L. M.; Walker, R. T. *Nucleic Acids Res.* **1996**, *24*, 4117.
14. Molecular modeling was performed using Hyperchem™ program. The predicted structure of the 2'-thio-LNA nucleoside **2** was in accordance with a 3'-endo conformation (^{3}E, N-type conformation) equivalent to that reported for the parent LNA nucleosides.[6-8] The only difference between **2** and the corresponding LNA nucleoside was as expected a slight enlargement of the additional ring. Meldgaard, M.; Scheuer-Larsen, C. unpublished results.
15. Synthesis of 2'-thio-LNA nucleoside **2** [1-(2-deoxy-2-mercapto-2-*S*,4-*C*-methylene-β-D-ribofuranosyl)uracil] will be reported elsewere: Singh, S. K.; Kumar, R.;Wengel, J., submitted.
16. ^{31}P NMR data for **4**: δ($CDCl_3$) 148.9, 149.0.
17. The 2'-thio-LNAs were synthesized on a Pharmacia Gene Assembler Special DNA synthesizer using standard conditions. The step-wise coupling yield for amidite **4** was approximately 85% (12 min couplings) as compared to >99% for the commercial 2'-deoxynucleoside phosphoramidites. After completion of the syntheses, deprotection and cleavage from the solid support was effected using concentrated ammonia (55 °C, 8 h). The DMT-ON 2'-thio-LNAs were purified and analyzed (>90% purity) by reversed phase HPLC as described earlier.[7] The composition of the 2'-thio-LNAs was verified using MALDI-MS [M-H] observed/calculated: 5'-d(GTGAU^{LS}ATGC):2782.3/2783.7;5'-d(GU^{LS}GAU^{LS}AU^{LS}GC):2842.3/2844.0;5'-d(GU^{LS}G$U^{LS}$$U^{LS}$$U^{LS}$$U^{LS}$GC): 2886.4/2886.1.
18. Freier, S. M.; Altmann, K.-H. *Nucleic Acids Res.* **1997**, *25*, 4429.