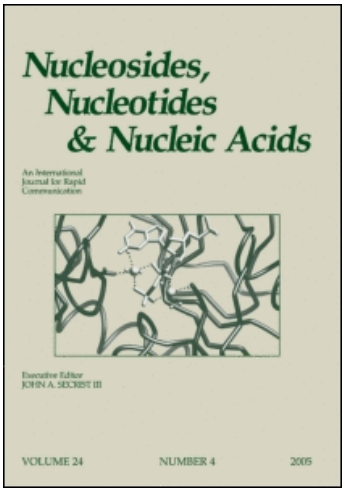


Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Lna (Locked Nucleic Acid)

Jesper Wengel[a]; Alexei Koshkin[ab]; Sanjay K. Singh[a]; Poul Nielsen[c]; Michael Meldgaard[a]; Vivek K. Rajwanshi[a]; Ravindra Kumar[a]; Jan Skouv[d]; Christina B. Nielsen[c]; Jens Peter Jacobsen[c]; Nana Jacobsen[b]; Carl E. Olsen[e]

[a] Center For Synthetic Bioorganic Chemistry, Department of Chemistry, University of Copenhagen, Copenhagen, Denmark [b] Exiqon A/S, Vedbæk, Denmark [c] Department of Chemistry, Odense University, Odense M, Denmark [d] Institute of Cancer Biology, Danish Cancer Society, Copenhagen [e] Department of Chemistry, Royal Veterinary and Agricultural University, Frederiksberg C, Denmark

To cite this Article Wengel, Jesper , Koshkin, Alexei , Singh, Sanjay K. , Nielsen, Poul , Meldgaard, Michael , Rajwanshi, Vivek K. , Kumar, Ravindra , Skouv, Jan , Nielsen, Christina B. , Jacobsen, Jens Peter , Jacobsen, Nana and Olsen, Carl E.(1999) 'Lna (Locked Nucleic Acid)', Nucleosides, Nucleotides and Nucleic Acids, 18: 6, 1365 — 1370

To link to this Article: DOI: 10.1080/07328319908044718

URL: http://dx.doi.org/10.1080/07328319908044718

NUCLEOSIDES & NUCLEOTIDES, 18(6&7), 1365–1370 (1999)

LNA (LOCKED NUCLEIC ACID)

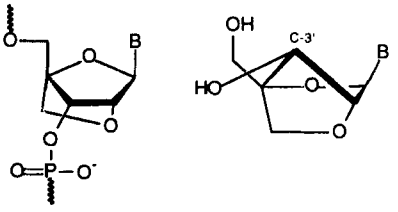
Jesper Wengel[a,*], Alexei Koshkin[a,b], Sanjay K. Singh[a], Poul Nielsen[c], Michael Meldgaard[a], Vivek K. Rajwanshi[a], Ravindra Kumar[a], Jan Skouv[d] Christina B. Nielsen[c], Jens Peter Jacobsen[c], Nana Jacobsen[b], and Carl E. Olsen[e]

[a]Center For Synthetic Bioorganic Chemistry, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark; [b]Exiqon A/S, Bygstubben 9, DK-2950 Vedbæk, Denmark; [c]Department of Chemistry, Odense University, DK-5230 Odense M, Denmark; [d]Institute of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen; [e]Department of Chemistry, Royal Veterinary and Agricultural University, DK-1871 Frederiksberg C, Denmark;

Abstract: LNA (Locked Nucleic Acid) forms duplexes with complementary DNA, RNA or LNA with unprecedented thermal affinities. CD spectra show that duplexes involving fully modified LNA (especially LNA:RNA) structurally resemble an A-form RNA:RNA duplex. NMR examination of an LNA:DNA duplex confirm the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA is demonstrated suggesting strand invasion by LNA. Lipofectin-mediated efficient delivery of LNA into living human breast cancer cells has been accomplished.

INTRODUCTION

Synthesis of a variety of conformationally restricted oligonucleotide analogues has been accomplished,[1,2] and we have recently introduced LNA (Locked Nucleic Acid; Figure 1)[3-7] containing 2'-*O*,4'-*C*-methylene linked bicyclic ribofuranosyl nucleosides locked in an N-type [3'-endo/^{3}E] conformation (Figure 1; B = nucleobase) as shown by X-ray crystallography[8] and NMR studies.[4,5] Convergent as well as linear syntheses of monomeric LNA nucleosides have been reported by us[4-6,9] and by Imanishi and coworkers.[8] Importantly, oligomerization



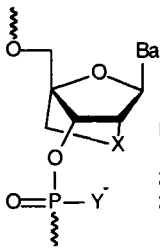
LNA **N-type conformation**

Figure 1.

of 3'-*O*-phosphoramidite LNA monomers proceeds efficiently on an automated DNA synthesizer using standard procedures.[4,5,7]

RESULTS AND DISCUSSION

Representative results from thermal denaturation studies of duplexes formed between 9-mer LNAs and complementary DNA and RNA are summarized in Table 1.[4,5,7,9,10] From entries 4, 5 and 6 it can be extracted that LNA ("deoxy-LNA", "ribo-LNA" and "homo-LNA")[3,11] recognizes both complementary DNA and RNA with remarkable thermal affinities. Thus, significantly increased melting temperatures (T_m values) were observed for the duplexes involving LNA compared with the corresponding unmodified reference duplexes (ΔT_m = +4.0 to +9.3 °C towards DNA; ΔT_m = +4.0 to +8.3 °C towards RNA).[4,5,7] Incorporation of LNA monomers into an oligoribonucleotide (entry 5; ribo-LNA) has a pronounced stabilizing effect possibly originating from the expected conformational fit between the ribonucleotide and the LNA monomers (N-type conformations).[7] Stimulated by the fact that the majority of first generation antisense drugs are nuclease resistant phosphorothioate oligodeoxynucleotide analogues, we synthesized the phosphorothioate-LNA depicted in entry 7.[9] A major draw-back of phosphorothioates is decreased affinity (ΔT_m ~ -1 °C) towards complementary nucleic acids, an effect that is evident from the hybridization data of the phosphorothioate reference of entry 3 compared with the data of entry 1. Relative to the corresponding LNA (entry 4), the phosphorothioate-LNA (entry 7) displays only minor decreases in the thermal affinity (ΔT_m = -0.4 °C), and compared to the corresponding phosphorothioate reference (entry 3) or oligodeoxynucleotide reference (entry 1), the phosphorothioate-LNA display strongly increased thermal affinities.[9] Thus, it appears that the incorporation of LNA monomers into all-phosphorothioate oligodeoxynucleotides is an effective way of improving the hybridization properties of phosphorothioate oligos. Two other LNA analogues, namely 2'-thio-LNA[9,10] and 2'-amino-LNA[10,12] have been synthesized. Evaluation of their hybridization properties (entries 8 and 9)[9,12] suggests that the "ribo-configurated" LNA-type bicyclo[2.2.1]heptane skeleton is a generally applicable structural element in high-affinity oligonucleotides. Especially the possibility of utilizing the 2'-amino functionality of 2'-amino-LNA as a structurally well-defined conjugation site is appealing (see the description of the first NMR experiment on an LNA:DNA duplex in a latter paragraph).



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

Table 1. Hybridization data of 9-mer LNAs towards complementary DNA and RNA. A = adenosine monomer, C = cytidine monomer, G = guanosine monomer, U = uridine monomer, T = thymidine monomer, ^{Me}C = 5-methylcytidine monomer, $\underline{X}^{L}$ = LNA monomer. $\underline{U}^{LS}$ = 2'-thio-LNA monomer; $\underline{T}^{LNH}$ = 2'-amino-LNA monomer; subscript "s" denotes a phosphorothioate linkage. Oligodeoxynucleotide sequences are depicted as d(sequence) and oligoribonucleotide sequences as r(sequence). The melting temperatures (T_m values; medium salt buffer consisting of 10 mM sodium phosphate, pH 7.0, 100 mM sodium chloride) were obtained as the maxima of the first derivatives of the melting curves (A_{260} *vs.* temperature) using 1.5 μM concentrations of the two complementary strands (assuming identical extinction coefficients for all strands). All transitions were monophasic. ΔT_m values = the increases in the thermal stability *per LNA monomer incorporated* compared to the corresponding reference duplex. [a]Relative to entry 1. [b]Relative to entry 2. [c]Relative to entry 4. [d]Relative to entry 3. [e]Relative to entry 1 for which a T_m of 30 °C towards DNA was measured in this series of experiments.

Entry	Sequence	DNA complement		RNA complement	
		T_m/°C	ΔT_m/°C	T_m/°C	ΔT_m/°C
	References				
1	5'-d(GTGATATGC)	28		28	
2	5'-r(GUGAUAUGC)	27		38	
3	5'-d($G_sT_sG_sA_sT_sA_sT_sG_sC$)	21		17	
	Deoxy-LNA (X=Y=O)				
4	5'-d(G$\underline{T}^{L}$GA$\underline{T}^{L}$A$\underline{T}^{L}$GC)	44	+5.3[a]	50	+7.3[a]
	Ribo-LNA (X=Y=O)				
5	5'-r(G$\underline{T}^{L}$GA$\underline{T}^{L}$A$\underline{T}^{L}$GC)	55	+9.3[b]	63	+8.3[b]
	Homo-LNA (X=Y=O)				
6	5'-($\underline{G}^{L}\underline{T}^{L}\underline{G}^{L}\underline{A}^{L}\underline{T}^{L}\underline{A}^{L}\underline{T}^{L}\underline{G}^{L\,Me}\underline{C}^{L}$)	64	+4.0[a]/+4	74	+5.1[a]/+4.0[b]
	Phosphorothioate-LNA (X=O, Y=S)				
7	5'-($G_s\underline{T}^{L}{}_sG_sA_s\underline{T}^{L}{}_sA_s\underline{T}^{L}{}_sG_sC$)	41	-0.4[c]/+6	47	-0.4[c] /+10.0[d]
	2'-Thio-LNA (X=S, Y=O)				
8	5'-(G$\underline{U}^{LS}$GA$\underline{U}^{LS}$A$\underline{U}^{LS}$GC)	42	+4.7[a]	52	+8.0[a]
	2'-Amino-LNA (X=NH, Y=O)				
9	5'-(G$\underline{T}^{LNH}$GA$\underline{T}^{LNH}$A$\underline{T}^{LNH}$GC)	39	+3.0[e]	47	+6.3[a]

Table 2. LNA:LNA Base pairing. See caption of Table 1. [a] Medium salt buffer: 10 mM sodium phosphate, pH 7.0, 100 mM sodium chloride. [b] Low salt buffer: 1 mM sodium phosphate, pH 7.0.

Entry	Duplex	T_m (°C)
1	5'-d(G$\underline{T^L}$GA$\underline{T^L}$A$\underline{T^L}$GC) / 3'-d(C$\underline{A^L}$CT$\underline{A^L}$T$\underline{A^L}$CG)	63
2	5'-($\underline{G^LT^LG^LA^LT^LA^LT^LG^{L\,Me}C^L}$) / 3'-($\underline{^{Me}C^LA^{L\,Me}C^LT^LA^LT^LA^{L\,Me}C^LG^L}$)	>93[a]/93[b]
3	5'-($\underline{G^LT^LG^LA^LA^LA^LT^LG^{L\,Me}C^L}$) / 3'-($\underline{^{Me}C^LA^{L\,Me}C^LT^LA^LT^LA^{L\,Me}C^LG^L}$)	>93[a]/76[b]

In Table 2, results from LNA:LNA base-pairing experiments are displayed. An LNA:LNA duplex (entry 1; three $\mathbf{T^L}$:$\mathbf{A^L}$ base pairs) exhibited a T_m value of 63 °C which should be compared with 28 °C for the DNA:DNA reference. No duplex dissociation was detected for the two complementary homo-LNAs (entry 2) in the standard medium salt buffer (T_m estimated >93 °C). However, a transition (T_m ~93° C) was evident in a buffer with very low concentration of salt (1 mM sodium phosphate). The excellent ability of LNA to discriminate mismatched bases when hybridizing with unmodified oligonucleotides has been demonstrated earlier.[5] As judged from the decreased thermal stability (T_m = 76 °C; in the low salt buffer; entry 3) of a duplex between two homo-LNAs with a single $\mathbf{A^L}$:$\mathbf{A^L}$ mismatch, LNA:LNA base pairing selectivity appears to be very satisfactory. Based on these melting results, LNA:LNA hybridization constitutes the most stable nucleic acid type duplex system hitherto discovered[13] which underlines the importance of conformational restriction in molecular recognition.

Sequence selective recognition of double stranded nucleic acids continues to be a major goal at the chemistry and biology interface, and we have performed the first experiments towards evaluating LNA in this context.[14] Capture by hybridization of digoxygenin(DIG)-labeled double stranded PCR products (up to five hundred base pairs in length; deoxynucleotide monomers) were evaluated using a DNA capture probe [5'-biotin-d(GGTGGTTTGTTTG] or an LNA capture probe [5'-biotin-($\mathbf{G^LG^LT^LG^LG^LT^LT^LT^LG^L}$-$\mathbf{T^LT^LT^L}$dG)]. After immobilisation using streptavidin coated microtiter plates (immobilisation by biotin/strepavidin interaction), the amount of captured DIG-labeled PCR product was quantified by evaluating the peroxidase activity after application of peroxidase-conjugated anti-DIG antibodies. After initial denaturation (5 min at 95 °C; then 15 min at 25 °C), both the DNA and the LNA capture probe were able to capture a PCR product containing in one

of the stands a sequence complementary to the capture probes (the LNA-mediated capture was significantly more efficient). Without denaturation (15 min at 25 °C), capture was exclusively possible when using the LNA capture probe (and with a signal intensity comparable to the one after initial denaturation). Capture of a PCR product without a sequence complementary to the capture probes was negative in all control experiments. The results from these preliminary experiments, which have been confirmed using other capture probe sequences and other double stranded PCR products, suggest that LNA is able to recognize a mixed sequence segment of a double stranded nucleic acid duplex by strand-invasion.

CD spectra of different complexes involving homo-LNA have been recorded. The CD spectra show that the LNA:RNA complex structurally resemble the A-form duplex of the RNA:RNA reference. The structures of the LNA:DNA and LNA:LNA complexes deviate to some extent from the structure of the RNA:RNA duplex and elements in the CD spectra characteristic of a B-form duplex are evident.

As the first step towards a deeper understanding of the structure of duplexes involving LNA, the results from the first NMR experiment are summarized below.[14] An LNA [5'-d(CCGC$\mathbf{T^L}$AGCG)] was synthesized and annealed to its complementary oligodeoxy-nucleotide. The overall structure of the duplex closely resembles the corresponding unmodified B-form duplex and the 2'-*C*,4'-*C*-oxymethylene linkage is situated at the rim of the duplex facing the minor groove. As expected, the LNA monomer is locked in a 3'-endo conformation. Minor conformational changes in the C-monomer preceeding the LNA monomer and in a number of other monomers (in both strands) are seen. It thus appears that an LNA monomer is able to induce a certain degree of conformational shifts in the neighbouring monomers. Ongoing NMR experiments on duplexes of LNA containing a larger number of LNA monomers are expected to allow more definite conclusions concerning this aspect to be drawn.

Delivery by transfection of fluorescein-labeled LNA [5'-fluorescein-($\mathbf{T^LG^LC^LC^LT^L}$-$\mathbf{G^LC^LA^LG^LG^LT^LC^LG^LA^LC^L}$dT)] into living MCF-7 breast cancer cells has been accomplished (250 nM LNA, 0.8 μg/ml lipofectin, 37 °C, 24 h).[14] The fluorescence was found in approximately 35% of the MCF-7 cells, and from analysis of fixed cells it can be concluded that the fluorescein-labeled LNA is localized in the cell nucleus.

CONCLUSION

The universality of LNA mediated high-affinity hybridization has been demonstrated. The RNA-mimicking character of LNA is reflected in the N-type conformational restriction

of the monomeric nucleotides, and in the secondary structure of an LNA:RNA duplex. Preliminary experiments suggest LNA to be a promising candidate for efficient recognition of a given mixed sequence in a nucleic acid duplex. These results, together with the demonstration that fluorescein-labeled LNA can be efficiently delivered into living MCF-7 breast cancer cells by Lipofectin-mediated transfection, should stimulate the evaluation of LNA as antisense or antigene molecules.

ACKNOWLEDGEMENTS

The Danish Natural Science Research Council and The Danish Technical Research Council are thanked for financial support.

REFERENCES AND NOTES

1. Herdewijn, P. *Liebigs Ann.* **1996**, 1337 and references cited therein.
2. Cool, E. T. *Chem. Rev.* **1997**, *97*, 1473 and references cited therein.
3. LNA is defined as an ON containing one or more 2'-*O*,4'-*C*-methylene bicyclic ribonucleoside LNA monomers.
4. Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun.* **1998**, 455.
5. Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607.
6. Koshkin, A. A.; Rajwanshi, V. K.; Wengel, J. *Tetrahedron Lett.* **1998**, *39*, 4381.
7. Singh, S. K.; Wengel, J. *Chem. Commun.* **1998**, 1247.
8. Uracil and cytosine derivatives: (a) Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735; (b) Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Morio, K.; Doi, T.; Imanishi, T. *Tetrahedron Lett.* **1998**, *39*, 5401.
9. Kumar, R.; Singh, S. K.; Koshkin, A. A.; Rajwanshi, V. K.; Meldgaard, M.; Wengel, J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2219.
10. Singh, S. K.; Kumar, R.; Wengel, J. *J. Org. Chem.*. In press.
11. Deoxy-LNA is defined as an LNA consisting of LNA monomers and 2'-deoxy-nucleotide monomers. Ribo-LNA is defined as an LNA consisting of LNA monomers and ribonucleotide monomers. Homo-LNA is defined as an all-modified LNA.
12. Singh, S. K.; Kumar, R.; Wengel, J. *J. Org. Chem.***1998**, *63*, 6078.
13. A pentameric guanidinium linked thymidylate has been reported to display an estimated T_m value of more than 100 °C towards poly(rA). However, it has been shown that this association involves a triple helical complex. (a) Brown, K. A.; Dempcy, R. O.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6097; (b) Linkletter, B. A.; Bruice, T. C. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1285.
14. Manuscript in preparation.