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Investigation of restricted backbone conformations as an explanation for the exceptional thermal stabilities of duplexes involving LNA (Locked Nucleic Acid):† synthesis and evaluation of abasic LNA

Lisbet Kværnø and Jesper Wengel*

Center for Synthetic Bioorganic Chemistry, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark. E-mail: wengel@kiku.dk

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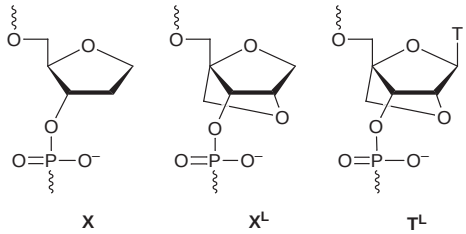
In order to investigate the structural basis of the unique hybridization properties of LNA (Locked Nucleic Acid), an abasic LNA monomer (a 1-deoxy-2-*O*,4-*C*-methylene-D-ribofuranose derivative) was synthesized and evaluated with respect to influence on duplex stability, showing that effects mediated *via* the nucleobase are pivotal for the properties of LNA.

In recent years much attention in the search for effective antisense oligonucleotides has been focused on the synthesis of conformationally restricted analogues, stimulated by the promise of entropically advantageous duplex formation.[1,2] So far,



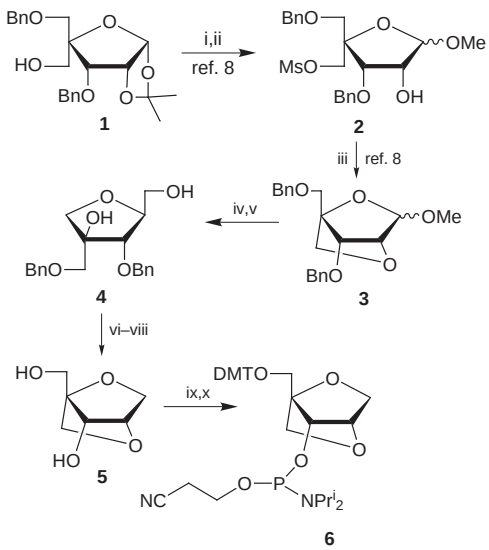
X **X^{L}** **T^{L}**

LNA (Locked Nucleic Acid, thymine monomer **T^{L}**) is showing the most promising properties,[3–7,†] *e.g.* unprecedented thermal stabilities towards complementary DNA and RNA (ΔT_m/modification = +3 to +9 °C), stability towards 3′-exonucleolytic degradation, efficient automated oligomerization and good aqueous solubility. As an attempt to investigate the structural elements of LNA responsible for the remarkably enhanced duplex stabilities we have synthesized and evaluated the hydridization properties of oligonucleotides containing the novel abasic LNA monomer **X^{L}** in comparison with unmodified reference sequences, sequences containing the abasic DNA monomer **X**, and sequences containing thymine LNA monomer **T^{L}**.

The 4-*C*-hydroxymethylfuranose **1** was converted in two steps to the anomeric methyl furanosides **2** (Scheme 1).[8] As previously reported, treatment of **2** with NaH induced cyclization to give furanosides **3** which after hydrolysis afforded the monocyclic aldehyde.[8] Reduction of this aldehyde with $NaBH_4$ proceeded smoothly to give the novel diol **4** in 84% yield (from **2**). Selective tosylation of the primary hydroxy group followed by treatment with NaH afforded the bicyclic skeleton of abasic LNA whereafter hydrogenolysis to give diol **5**,‡ dimethoxytritylation and phosphitylation yielded the phosphoramidite building block **6** suitable for oligonucleotide synthesis. Except for prolonged coupling times (8 min compared with standard 1 min), normal coupling conditions were used for amidite **6** on an automated DNA synthesizer. The stepwise coupling yield was approximately 99% as was also obtained for both the unmodified DNA and RNA phosphoramidites as well as for the commercially available amidite leading to the abasic DNA monomer **X**.

In nucleic acids the conformation of the furanose ring is decisive for the type of duplex formed. The pentofuranose moieties in RNA normally exist in an N-type conformation leading to A-type duplexes, while an S-type conformation as generally seen in DNA leads to B-type duplexes. Also the dihedral phosphate backbone angle C4′–C3′–O3′–P (denoted ε^{t} and ε^{-} for *trans* and *gauche*, respectively[9]) and the furanose conformation have been shown to be interdependent for monomeric nucleotides or single stranded oligonucleotides.[9–12] Thus, for dinucleotides an N-type conformation strongly favours the ε^{t} dihedral angle while a mixture of ε^{t} and ε^{-} is seen for S-type conformations.[9] Studies of ethyl phosphate mononucleotides which mimic dinucleotides with total elimination of base–base stacking interactions likewise show an interdependency between the N/S and $\varepsilon^{t}/\varepsilon^{-}$ equilibria for ethyl ribonucleotides while these two equilibria seem to be independent for ethyl 2′-deoxyribonucleotides.[10–12] The rigid structure of an LNA monomer which restricts the furanose ring to the N-type conformation[3,4,13] is therefore expected to influence the local phosphate backbone fluctuations by quenching the $\varepsilon^{t}/\varepsilon^{-}$ equilibrium.

The question under investigation here is whether the abasic LNA monomer **X^{L}**, which is anticipated to display the same fixed furanose and restricted backbone conformations as the parent LNA monomers (*e.g.* monomer **T^{L}**) but with the exclusion of base–base stacking interactions, induces an increased duplex stability relative to the abasic DNA monomer **X** comparable to the one obtained for the parent LNA monomers relative to the DNA monomers. In this respect, the modified 9- and 13-mer oligodeoxyribonucleotides and oligo-



Scheme 1 *Reagents and conditions:* i, MsCl, pyridine; ii, 20% HCl, MeOH–H_2O (7 : 1), iii, NaH, DMF; iv, 80% AcOH; v, $NaBH_4$, MeOH, 84% for 3 steps; vi, TsCl, pyridine, 71%; vii, NaH, DMF, 82%; viii, H_2, Pd/C, 80%; ix, DMTCl, pyridine, 73%; x, $NC(CH_2)_2OP(Cl)NPr^{i}_2$, $EtNPr^{i}_2$, CH_2Cl_2, 62%.

Table 1 Sequences synthesized and T_m values towards fully complementary DNA sequences[a]

Entry	Sequence	Y	T_m/°C
1	5′-d(GTGA-**Y**-ATGC)	T	27
2		**T**^{L}	35
3		**X**	[b]
4		**X**^{L}	[b]
5	5′-r(GUGA-**Y**-AUGC)	U	27
6		**T**^{L}	38
7		**X**^{L}	~3
8	5′-d(CAGTGA-**Y**-ATGCGA)	T	48
9		**T**^{L}	53
10		**X**	27
11		**X**^{L}	27
12	5′-r(CAGUGA-**Y**-AUGCGA)	U	46
13		**T**^{L}	53
14		**X**	29
15		**X**^{L}	29

[a] T_m values measured as the maximum of the first derivative of the melting curve (A_{260} *vs.* temperature) recorded in medium salt buffer (10 mM $NaHPO_4$, 100 mM NaCl, 0.1 mM EDTA, pH 7.0) using 1.5 mM concentrations of the two complementary strands (assuming identical extinction coefficients for T and **T**^{L} and for the different monomeric nucleotides whether present in LNA, the unmodified references or the strands containing an abasic monomer). A = adenosine monomer, C = cytidine monomer, G = guanosine monomer, U = uridine monomer, T = thymidine monomer, **X** = abasic DNA monomer, **X**^{L} = abasic LNA monomer, **T**^{L} = LNA thymine monomer. Oligo-2′-deoxynucleotide sequences are depicted as d(sequence) and oligoribonucleotide sequences as r(sequence). [b] No T_m detected.

ribonucleotides depicted in Table 1 were synthesized§ and their hybridization to fully complementary oligodeoxyribonucleotide sequences [5′-d(GCATATCAC) and 5′-d(TCGCATATCACTG)] was studied. For each oligonucleotide sequence, the melting temperature (T_m value) for the unmodified reference was compared with the T_m values for the analogues containing either one LNA thymine monomer (**T**^{L}), one abasic DNA monomer (**X**) or one abasic LNA monomer (**X**^{L}). The effect of a single LNA monomer on the thermal stability, which hitherto has not been reported, is profound, with ΔT_m values of +8 and +5 °C for deoxy-LNA† (entries 2 and 9, **T**^{L} compared to a thymidine monomer) and +11 and +7 °C for ribo-LNA† (entries 6 and 13, **T**^{L} compared to a uridine monomer¶). In analogy with previously reported data,[14] we observed a detrimental effect of the abasic DNA monomer **X** on the thermal stability (entries 3, 10 and 14, ΔT_m values < -17 °C). The T_m values obtained for the abasic LNA monomer **X**^{L} (entries 4, 7, 11 and 15) were identical to those obtained for **X**. Thus, despite the very large stabilizing effect induced by incorporation of one LNA monomer **T**^{L} instead of a thymidine monomer, no effect resulted from the exchange of the abasic DNA monomer **X** with the abasic LNA monomer **X**^{L}.

NMR investigations[13] on single stranded LNA and LNA:DNA duplexes have shown that LNA monomers induce a shift towars an N-type conformation in neighboring unmodified monomers. Quenched backbone torsions and/or improved nucleobase stacking are possible explanations for this conformational effect. Since the structural difference for both pairs examined herein (**T**^{L} *vs.* T and **X**^{L} *vs.* **X**) is an oxymethylene bridge linking the 2(′)- and the 4(′)-carbon atoms the thermal denaturation studies have shown that conformational restrictions of the pentofuranose and backbone alone are not sufficient to induce an effect. We therefore conclude that the nucleobase is essential as a mediator of the conformational changes.

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Notes and references

† We have defined LNA as an oligonucleotide containing one or more 2′-*O*,4′-*C* methylene linked bicyclic ribonucleoside LNA monomers. Deoxy-LNA consists of 2′-deoxynucleotide and LNA monomers, while ribo-LNA consists of ribonucleotide and LNA monomers (see ref. 3–5).

‡ *Selected data* for **5**: δ_H (CD_3OD) 4.74 (2 H, br s, OH), 4.01 and 4.04 (2 H, 2 s, H-2, H-3), 3.85 (1 H, d, *J* 8.0, H-4'a), 3.85 (1 H, d, *J* 8.0, H-1a), 3.75 (1 H, d, *J* 7.9, H-4'b), 3.74 (1 H, d, *J* 8.2, H-1b), 3.68 (2 H, d, *J* 2.7, H-5), assignment of H-1 and H-4′ signals may be interchanged; δ_C (CD_3OD) 86.28 (C-4), 78.72, 72.54, 72.38 and 71.21 (C-1, C-2, C-3, C-4′), 57.68 (C-5); *m/z* (FAB^+) 147 $[M + H]^+$.

§ MALDI mass data $[M\text{-}H^-]^-$: 5′-d(GTGA-**X**-ATGC) 2630, calc. 2628; 5'-d(GTGA-**X**^{L}-ATGC) 2657, calc. 2656; 5′-r(GUGA-**X**^{L}-AUGC) 2759, calc. 2755; 5′-d(CAGTGA-**X**-ATGCGA) 3874, calc. 3873; 5′-d(CAGTGA-**X**^{L}-ATGCGA) 3901, calc. 3901; 5′-r(CAGUGA-**X**-AUGCGA) 4037, calc. 4036; 5′-r(CAGUGA-**X**^{L}-AUGCGA) 4068, calc. 4064.

¶ We have previously shown for the identical 9-mer sequence that a 'U-LNA monomer' and a 'T-LNA monomer' induce identical T_m values (see ref. 4).

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