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High-affinity nucleic acid recognition using 'LNA' (locked nucleic acid, β-D-*ribo* configured LNA), '*xylo*-LNA' (β-D-*xylo* configured LNA) or 'α-L-LNA' (α-L-*ribo* configured LNA)

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Remarkably strong binding affinities towards complementary single stranded DNA and RNA were obtained for 10- and 14-mer LNAs (locked nucleic acids) containing diastereoisomeric β-D-*ribo*, β-D-*xylo* or α-L-ribo configured LNA thymine monomers; a possible relevance of these results in relation to nucleic acid evolution is discussed.

As recently summarized, Eschenmoser and co-workers have intensively studied the basic chemical properties of a number of potential natural nucleic acid alternatives containing various hexopyranosyl or pentopyranosyl monomeric units.[1] Many of these alternatives have displayed efficient and strong Watson–Crick base-paring, indicating that this property has not been a decisive driving force during the evolution of RNA (ribofuranosyl nucleic acids),[1b] at least not with respect to the carbohydrate part of RNA. The possibilities exist that RNA was selected for synthetic reasons favouring the formation of RNA, or for functional reasons inherent in the β-D-ribofuranosyl structure of RNA. In the latter scenario it is conceivable that the RNA structure resulted from selection and optimization among irregular nucleic acids consisting of mixtures of, *e.g.* different pentofuranosyl and/or pentopyranosyl monomeric nucleotide units. Although several pentofuranosyl configurational isomers of RNA have been synthesized, *e.g.* the β-L-ribofuranosyl[2] and β-D-arabinofuranosyl[3] isomers, their binding affinities, especially in stereoirregular oligonucletides, have not been much studied.

We and others have recently reported unprecedented thermal affinities of duplexes involving the oligonucleotide analogue 'LNA'[4] (locked nucleic acid, $\mathbf{T^L}$, β-D-*ribo* isomer, Fig. 1).† The furanose ring of LNA, being part of a dioxabicyclo[2.2.1]heptane skeleton, is efficiently locked in a C3'-*endo* (N-type) conformation. We have initiated a program focusing on the synthesis and properties of conformationally locked configurational isomers of LNA. Very recently, we have published the syntheses of the first two diastereoisomeric forms of LNA, namely '*xylo*-LNA' ($\mathbf{xT^L}$, β-D-*xylo* isomer,† Fig. 1) containing one or more 2'-*O*,4'-*C*-methylene-β-D-xylofuranosyl nucleotide monomer(s), and 'α-L-LNA' ($^{\alpha L}\mathbf{T^L}$, α-L-*ribo* isomer,† Fig. 1) containing one or more 2'-*O*,4'-*C*-methylene-α-L-ribofuranosyl nucleotide monomer(s).[5]

The LNA oligomers **5** and **7**–**9** (Table 1) were synthesized on an automated DNA synthesizer by use of the phosphoramidite approach[6] using conditions (pyridine hydrochloride; 10 min coupling time; > 98% step-wise coupling yields) optimized earlier for synthesis of *xylo*-LNA and α-L-LNA.[5] Importantly, the use of polystyrene supports, loaded with the 3'-end penultimate thymidine monomer, allowed synthesis of the (almost)‡ fully modified *xylo*-LNA **7** and α-L-LNA **8**. The oligomers **5** and **7**–**9** were synthesized in the DMT-*ON* mode and, after cleavage from the solid support, purified using disposable reversed phase chromatography cartridges (Cruachem) yielding products with > 80% purity as judged from capillary gel electrophoresis. The compositions of LNAs **5**, **7** and **9** were verified by MALDI-MS analysis.§

In Table 1, hybridization data for 10- and 14-mer thymine LNA derivatives are shown. Sharp and monophasic transitions were obtained in all experiments (except with *xylo*-LNA **5**) towards complementary DNA (dA_{14}) or complementary RNA (rA_{14}). Control experiments without complementary strands showed no indication of the formation of well-defined and stable homocomplexes (no cooperative self-melting). The results obtained for references **1** and **2** and for the different LNA derivatives **3**, **4** and **6** have been reported earlier.[4a,5] Especially noteworthy is the unprecedented binding affinity of the fully modified parent LNA **6**[4a] and the apparent and significant

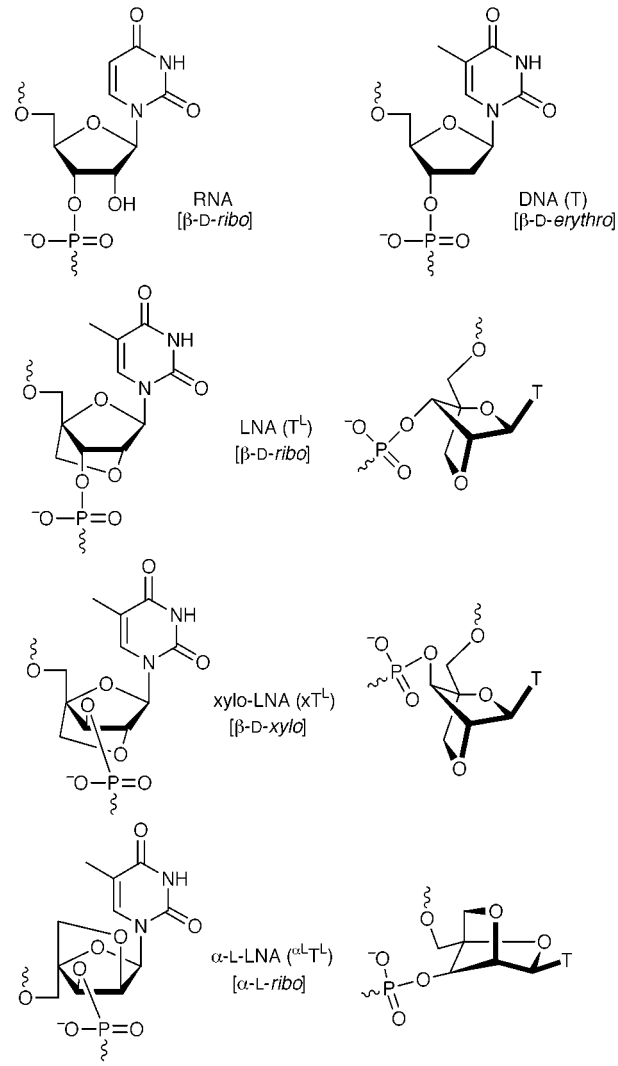

Fig. 1 Structure of the monomeric nucleotide units of RNA, DNA (T), LNA ($\mathbf{T^L}$), *xylo*-LNA ($\mathbf{xT^L}$) and α-L-LNA ($^{\alpha L}\mathbf{T^L}$); the configurations are shown in the square brackets; thymine derivatives are shown except in the case of RNA (uracil derivative shown). Also indicated are the locked furanose conformations of the three differently configured LNA monomers as obtained from molecular modelling ($\mathbf{T^L}$: C3'-*endo* conformation; $\mathbf{xT^L}$: C3'-*endo* conformation: $^{\alpha L}\mathbf{T^L}$: C3'-*exo* conformation).

affinity enhancing properties of LNA monomers as well as α-L-LNA monomers when incorporated into otherwise unmodified DNA strands (LNA **3**[4a] and α-L-LNA **4**;[5] ΔT_m = +5.0 and +4.5 °C towards complementary RNA, respectively; ΔT_m = increase in T_m value per modification; T_m = melting temperature, see text below Table 1).

A detrimental effect caused by the introduction of *xylo*-LNA monomers (*xylo*-LNA **5**) on the thermal stability can be seen from Table 1, confirming earlier obtained results for a singly substituted *xylo*-LNA.[5] In contrast, the fully-modified *xylo*-LNA **7** displayed very strong binding affinity towards DNA and RNA (ΔT_m = +3.1 and +4.3 °C, respectively). Likewise, remarkable thermal affinities were obtained for the fully-modified α-L-LNA **8** (ΔT_m = +4.8 °C towards DNA and +5.3 °C towards RNA). Modeling studies on α-L-LNA monomer $^{\alpha L}\mathbf{T}^{L}$ and LNA monomer $\mathbf{T}^{L}$ indicate a three-dimensional proximity of the thymine moieties and of the 5'- and 3'-oxygen atoms. This apparent structural similarity, despite the inverted configurations at the 2'-, 3'- and 4'-carbon atoms, offers an explanation of the observed very strong binding of the 14-mer 'stereoirregular LNA' **9** (containing four consecutive $\mathbf{T}^{L}$ monomers and four consecutive $^{\alpha L}\mathbf{T}^{L}$ monomers) towards both DNA and RNA (ΔT_m = +4.0 and +4.4 °C, respectively).

The results presented herein demonstrate that conformational restriction of fully modified diastereoisomeric LNA derivatives with β-D-*xylo* and α-L-*ribo* configurations (*xylo*-LNA **7** and α-L-LNA **8**) leads to high-affinity recognition of complementary DNA and RNA.¶ Thus, whereas LNA[4] is the nucleic acid analogue synthesized so far displaying the strongest binding affinity towards complementary DNA and RNA, the T_m values obtained herein likewise rank *xylo*-LNA and α-L-LNA, together with *e.g.* 2'-fluoro N3'-P5' phosphoramidates[7] and hexitol nucleic acids,[8] among the strongest DNA and RNA binders known. This is an important point with respect to the possibility of using LNA derivatives as antisense oligonucleotides or diagnostic probes.

Interestingly, whereas Eschenmoser and co-workers have found efficient cross-pairing between the diastereoisomeric conformationally restricted *pentopyranosyl* family of nucleic acid analogues but no pairing of these towards RNA,[1] the three differently configured *pentofuranosyl* LNAs discussed herein are able to very efficiently bind to RNA (itself a pentofuranosyl system). These results, in combination with the RNA-binding reported for β-D-arabinofuranosyl nucleic acids,[3f] suggest that constitution (rather than configuration) could have been a decisive factor in an early-stage combinatorial chemical evolution of the nucleic acid structure. It can be imagined that eventual optimization based on functional aspects, *e.g.* base-pairing strength or potential for stereoregular non-enzymatic replication, caused by differences in configuration and thus conformational equilibria between the various pentofuranosyl (or pentopyranosyl) diastereoisomers, could have favoured the current β-D-ribofuranosyl structure of the nucleic acids.

We are continuing our examination of the LNA family of molecules. A fourth LNA diastereoisomer ('α-L-*xylo*-LNA') is currently being synthesized which, together with the three diastereoisomers discussed herein and an enantiomeric RNA target, will allow us to study the properties of all eight possible LNA stereoisomers (in stereoregular or stereoirregular oligomers).‖

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Table 1 Hybridization data for diastereosiomeric LNAs and reference strands

	Sequence	dA_{14} complement		rA_{14} complement	
		T_m/°C[a]	ΔT_m/°C[b]	T_m/°C[a]	ΔT_m/°C[b]
1[c]	T_{14}	36/32[d]	Ref.[e]	32/28[d]	Ref.[e]
2[c]	T_{10}	24/20[d]	Ref.[e]	18	Ref.[e]
3[c]	$T_5(\mathbf{T}^{L})_4T_5$	42	+1.5	52	+5.0
4[f]	$T_5(^{\alpha L}\mathbf{T}^{L})_4T_5$	36	+1.0	46	+4.5
5	$T_5(\mathbf{xT}^{L})_4T_5$	n.t.[g]		n.t.[g]	
6[c]	5'-$(\mathbf{T}^{L})_9$T	80	+6.2[h]	71	+5.9[h]
7	5'-$(\mathbf{xT}^{L})_9$T	48	+3.1	57	+4.3
8	5'-$(^{\alpha L}\mathbf{T}^{L})_9$T	63	+4.8	66	+5.3
9	5'-$T_3(\mathbf{T}^{L})_4(^{\alpha L}\mathbf{T}^{L})_4T_3$	64	+4.0[i]	63	+4.4[i]

[a] Melting temperatures (T_m values) obtained from the maxima of the first derivatives of the melting curves (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 all thymine nucleotides [ref. 4(*b*)]. [b] The change in T_m value per modification (ΔT_m) compared with the T_m value for the reference of similar length. [c] Ref. 4(*a*). [d] The T_m values obtained for the reference strands varied from one experimental series to another [ref. 4(*a*), 5]. [e] Reference duplex. [f] Ref. 5. [g] No transition was observed above 20 °C. [h] In the original publication [ref. 4(*a*)], these ΔT_m values were erroneously calculated as +4.9 and +4.3 °C. [i] These ΔT_m values are calculated average values for the different LNA monomers incorporated.

Notes and references

† We have defined LNA as an oligonucleotide containing one or more 2'-*O*,4'-*C*-methylene-β-D-ribofuranosyl nucleotide monomer(s). The natural β-D-ribo configuration is generally assigned to LNA (and LNA monomers) as the positioning of the 1-nitrogen and 2'-, 3'- and 5'-oxygen atoms are equivalent to the one found in RNA. Strictly following carbohydrate nomenclature, however, the configuration of LNA nucleosides depends on the priorities between the two 4'-*C*-substituents (β-D-*ribo* or α-L-*lyxo* configuration in the case of parent LNA). Analogously, all *xylo*-LNA derivatives and all α-L-LNA derivatives are considered as having β-D-*xylo* and α-L-*ribo* configuration, respectively.

‡ For synthetic ease, the LNAs **6**-**8** were synthesized on commercially available T-supports. However, these LNAs are described herein as 'fully modified'.

§ MALDI-MS ($[M-H]^-$; found/calc.): **5** (4305.4/4307.8); **7** (3229.8/3231.1); **9** (4421.4/4419.8).

¶ The generality (base-pairing selectivity, mixed sequence contexts, strand orientations *etc*.) of the hybridization of diastereoisomeric forms of LNA has yet to be proven as has already been done for the parent LNA (ref. 4).

‖ The indirect evaluation of the RNA binding affinity of the remaining four LNA stereoisomers will be undertaken in a collaborative project with Dr Stefan Pitsch (Institute of Organic Chemistry, ETH, Zürich), involving the use of enantiomeric RNA. It should be noted that the formation of LNA derivatives of *arabino* and *lyxo* configuration† is precluded because of the inherent *syn*-positioning of the 2'-oxygen and 4'-carbon atoms.

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