LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition

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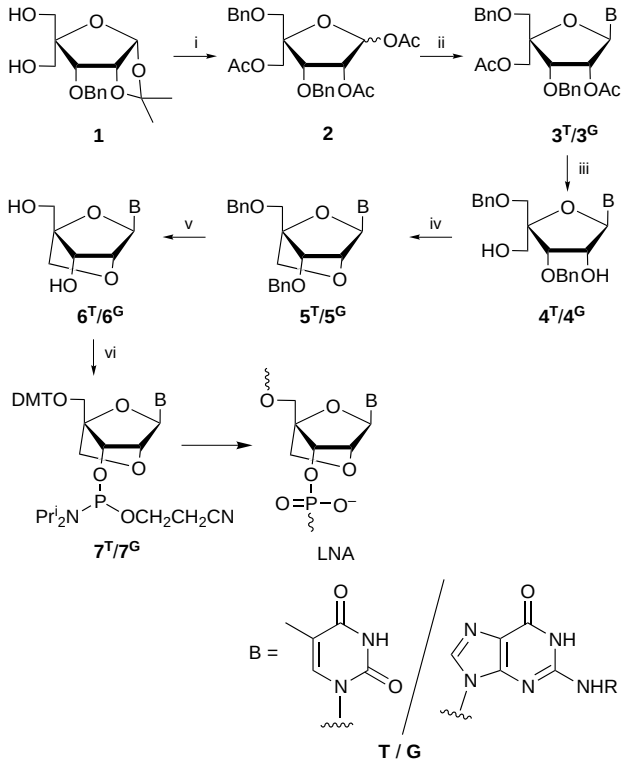
A novel class of nucleic acid analogues, termed LNA (locked nucleic acids), is introduced. Following the Watson–Crick base pairing rules, LNA forms duplexes with complementary DNA and RNA with remarkably increased thermal stabilities and generally improved selectivities.

During the last decade a plethora of DNA and RNA analogues have been chemically synthesized,[1–6] *e.g.* with the aim of improving nucleic acid recognition. We[7] and others[8] have reported promising, but not satisfactory, properties of oligonucleotides containing conformationally restricted monomers. We report here the synthesis of a novel conformationally restricted nucleic acid mimic, LNA (see Scheme 1). Molecular modelling† and simple model building suggested to us that the LNA monomers would be favourably preorganized in an N-type conformation thus enabling the formation of entropically favoured duplexes with complementary DNA and RNA. As an attractive feature, the structural change from DNA (or RNA) to LNA is limited from a chemical perspective, namely the introduction of an additional 2′-*C*,4′-*C*-oxymethylene link.



Scheme 1 *Reagents and conditions* (for **7T**): i, (*a*) NaH, BnBr, DMF, (*b*) Ac_2O, Py (64%, two steps), (*c*) 80% AcOH, (*d*) Ac_2O, Py (86%, two steps); ii, thymine, *N*,*O*-bis(trimethylsilyl)acetamide, TMS triflate, acetonitrile (76%) [for **3G**: 2-*N*-isobutyrylguanine, *N*,*O*-bis(trimethylsilyl)acetamide, TMS triflate, dichloroethane]; iii, NaOMe, MeOH (97%); iv, (*a*) TsCl, Py (47%), (*b*) NaH, DMF (89%); v, 20% $Pd(OH)_2$/C, EtOH, H_2 (98%); vi, (*a*) DMTCI, Py (93%), (*b*) *N*,*N*-diisopropylethylamine, 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, CH_2Cl_2 (70%)

For synthesis of the LNA monomers we chose a strategy starting from the known 4′-*C*-hydroxymethyl pentofuranose derivative **1**[9] (Scheme 1). Regioselective 5-*O*-benzylation, acetylation and acetolysis, followed by another acetylation, afforded furanose **2**, a key intermediate for coupling with silylated nucleobases. Stereoselective reaction with silylated thymine[10] yielded nucleoside **3T** which was deacetylated to give nucleoside diol **4T**. Tosylation followed by base-induced ring closure afforded the 2′-*O*,4′-*C*-linked bicyclic nucleoside derivative **5T**. Debenzylation yielded the unprotected analogue **6T** as the first example of a nucleoside diol with the (1*S*,3*R*,4*R*,7*S*)-7-hydroxy-1-hydroxymethyl-2,5-dioxabicyclo-[2.2.1]heptane structure. The assigned structure of **6T** was verified by NMR spectroscopy, including NOE experiments.‡ The absence of a coupling constant between 1′-H and 2′-H, and the unusual and strong mutual NOE effects (9%/8%) between 3′-H and 6-H (thymine base), strongly indicate structural preorganization of the pentofuranose ring of the LNA monomer into an N-type conformation.[5,11] Similar synthetic procedures were applied to synthesize the guanine derivatives **3G**–**6G** *via* coupling of **2** and 2-*N*-isobutyrylguanine. Transformation of nucleosides **6** into the 5′-*O*-4,4′-dimethoxytrityl (5′-*O*-DMT) protected analogues and subsequently into the phosphoramidite derivatives **7**§ yielded the desired monomeric building blocks for automated oligonucleotide synthesis.

LNAs¶ and unmodified reference strands (Tables 1 and 2) were effectively synthesized using the phosphoramidite approach.[12] Stability against 3′-exonucleolytic degradation is a prerequisite for most *in vivo* applications of oligonucleotide analogues The LNA 5′-$\mathbf{T^L}_{13}$T displayed complete 3′-exonucleolytic stability when measured by a procedure described earlier using snake venom phosphodiesterase.[13] The thermal stabilities (melting temperatures, T_m) of duplexes involving LNA oligonucleotides towards both DNA and RNA complements were determined and compared to their unmodified references (Tables 1 and 2). In all experiments, sharp monophasic transitions were observed with hyperchromicities of 1.2–1.4. No transitions were observed when running LNAs without complements in control experiments.

Table 1 Hybridization data of oligothymidylates

		DNA Complement dA_{14}		RNA Complement A_{14}	
Entry	Sequence[a] 5′ → 3′	T_m/°C	($\Delta T_m/\mathbf{T^L}$)/°C	T_m/°C	($\Delta T_m/\mathbf{T^L}$)/°C
1	T_{14}	35.5	Ref.	32.0	Ref.
2	$T_7\mathbf{T^L}T_6$	35.5	0.0	36.0	+4.0
3	$T_3(\mathbf{T^L}T)_4T_3$	47.0	+2.9	52.5	+5.1
4	$T_5\mathbf{T^L}_4T_5$	42.0	+1.6	51.5	+4.9
5	$\mathbf{T^L}_{13}$T	> 90.0	> +4.2	87.5	+4.3
6	T_{10}	24.0	Ref.	18.0	Ref.
7	$\mathbf{T^L}_9$T	80.0	+4.9	70.5	+4.3
8	T_6	< 10.0	Ref.	< 10.0	Ref.
9	$\mathbf{T^L}_5$T	32.0	> +4.4	40.0	> +6.0

[a] See footnote to Table 2.

Table 2 Hybridization data of mixed sequences

Entry	Sequence[a] 5′ → 3′		T_m/°C	[ΔT_m/ $\mathbf{T^L}$($\mathbf{G^L}$)]/ °C
1	d(GTGATATGC)	d(GCATATCAC)	28.0	Ref.
2	d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	d(GCATATCAC)	44.0	+5.3
3	d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	d(——— T ———)	27.0	—
4	d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	d(——— G ———)	30.0	—
5	d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	d(——— C ———)	23.0	—
6	d(GTGAGATGC)	d(GCATCTCAC)	33.0	Ref.
7	d(G$\mathbf{T^L}$GA$\mathbf{G^L}$A$\mathbf{T^L}$GC)	d(GCATCTCAC)	49.0	+5.3
8	d(GTGATATGC)	GCAUAUCAC	28.0	Ref.
9	d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	GCAUAUCAC	50.0	+7.3
10	d(GTGAGATGC)	GCAUCUCAC	33.0	Ref.
11	d(G$\mathbf{T^L}$GA$\mathbf{G^L}$A$\mathbf{T^L}$GC)	GCAUCUCAC	58.0	+8.3

[a] A = adenosine monomer, C = cytidine monomer, G = guanosine monomer, U = uridine monomer, T = thymidine monomer, $\mathbf{T^L}$/$\mathbf{G^L}$ = LNA monomers. Oligodeoxynucleotide sequences are depicted as d(sequence). 'Ref' indicates reference duplex. Hybridization mixtures of 1 ml were prepared using a buffer solution (10 mM Na_2HPO_4, pH 7.0, 100 mM NaCl, 0.1 mM EDTA) and equimolar (1.0 or 1.5 μM) amounts of the oligonucleotides. The absorbance at 260 nm was recorded while the temperature was raised linearly from 10 to 93 °C (1 °C min^{-1}). The melting temperatures (T_m values) were obtained as the maxima of the first derivatives of the melting curves.

The results for oligothymidylates are depicted in Table 1. Generally, significant increases in T_m values were obtained. Against complementary DNA (dA_{14}), the results ranged from no effect (incorporation of one LNA monomer, entry 2) to an increase in T_m of 4.9 °C per $\mathbf{T^L}$ incorporated for the LNA $\mathbf{T^L}_9$T (entry 7). The effect on the thermal stabilities of duplexes towards RNA (A_{14}) was significant (and additive) in all experiments (entries 2–9, increases in T_m of 4.0–6.0 °C per $\mathbf{T^L}$ incorporated).

The remarkable nucleic acid recognition potential of oligothymidylate LNAs was extended to mixed sequences containing the pyrimidine LNA monomer $\mathbf{T^L}$ and/or the purine LNA monomer $\mathbf{G^L}$ (Table 2). Thus, convincing stabilizing effects were observed when hybridizing nonamer LNAs towards DNA (ΔT_m = +5.3 °C per LNA monomer incorporated), whereas unprecedented increases in thermal stability (ΔT_m = +7.3 and +8.3 °C per LNA monomer incorporated) of the corresponding LNA:RNA duplexes were observed. The selectivity of the mixed sequence LNAs is, if anything, improved compared to the corresponding DNA references. This can be extracted from experiments where mis-matched nucleotides were in turn introduced in the position opposite to the middle $\mathbf{T^L}$/$\mathbf{G^L}$ monomers (and T/G monomers). The decreases in thermal stabilities obtained were slightly more pronounced for the LNAs compared to the reference DNAs (representative results are shown in entries 3–5; similar results were obtained when evaluating the binding selectivity of $\mathbf{G^L}$ and the binding selectivity against complementary RNA). These results indicate that LNA obeys the standard Watson–Crick pairing rules and that the complexes are bimolecular duplexes.**

In this report it has been demonstrated that preorganized LNAs display 3′-exonucleolytic stability and excellent ability to recognize complementary DNA and RNA. These results should make LNA a prime candidate for development of oligonucleotide-based therapeutics and diagnostic probes, and LNA-mediated nucleic acid recognition a novel concept of general applicability.

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Footnotes and References

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† Molecular modelling was performed using HYPERCHEM™ version 4.0; MM+ force field; Polak-Ribiere conjugate gradient geometry optimization.

‡ *NMR data* for $\mathbf{6^T}$ (conventional nucleoside numbering is used): δ_H [$(CD_3)_2SO$] 11.33 (1 H, br s, NH), 7.62 (1 H, d, *J* 1.1, 6-H), 5.65 (1 H, d, *J* 4.4, 3′-OH), 5.41 (1 H, s, 1′-H), 5.19 (1 H, t, *J* 5.6 Hz, 5′-OH), 4.11 (1 H, s, 2′-H), 3.91 (1 H, d, *J* 4.2, 3′-H), 3.82 (1 H, d, *J* 7.7, 5″-H_a), 3.76 (2 H, d, *J* 5.7, 5′-H), 3.63 (1 H, d, *J* 7.7, 5″-H_b), 1.78 (3 H, d, *J* 0.7, CH_3); key NOE contacts, signifying close interatom distances, were identified between: 5″-H_b and 1′-H, 6-H and 3′-H, 5′-OH and 5′-H, 5′-OH and 3′-H, and 5′-OH and 6-H.

§ *NMR data* for: $\mathbf{7^T}$ δ_P($CDCl_3$) 149.06, 148.74. For $\mathbf{7^G}$ δ_P ($CDCl_3$) 148.17, 146.07.

¶ The term LNA describes oligonucleotides containing one or more LNA monomer(s). For synthetic convenience, the LNAs of this first study were synthesized (0.2 μmol scale) on commercial supports carrying a natural 2′-deoxynucleoside. Step-wise coupling yields: $\mathbf{7^T}$ > 98% (12 min coupling), $\mathbf{7^G}$ > 95% (12 min coupling), deoxynucleoside phosphoramidites > 98% (2 min coupling). After standard cleavage and deprotection, capillary gel electrophoresis or reversed-phase HPLC was used to verify the purity (> 90%) of the synthesized oligonucleotides. Selected MALDI-MS experiments: 5′-$T_5\mathbf{T^L}_4T_5$ [M − H]$^-$ 4307.0 (calc. 4307.8); 5′-$\mathbf{T^L}_{13}$T [M − H]$^-$ 4557.8 (calc. 4559.7); 5′-d(G$\mathbf{T^L}$GA$\mathbf{G^L}$A$\mathbf{T^L}$GC) [M − H]$^-$ 2862.2 (calc. 2861.9).

** At pH 7.0, cytosine nucleobases are non-protonated and thus unable to form triple-helical structures. Triple-helical complexation for the oligothymidylate LNAs of Table 1 is theoretically possible, but no biphasic transitions were detected, even for the lower melting complexes. As further evidence, strikingly similar CD curves for LNA complexes and the corresponding reference duplexes were obtained.

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