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α-LNA, locked nucleic acid with α-D-configuration

Poul Nielsen* and Jakob Kragh Dalskov

Department of Chemistry, University of Southern Denmark, Odense University, 5230 Odense M, Denmark. E-mail: pon@chem.sdu.dk

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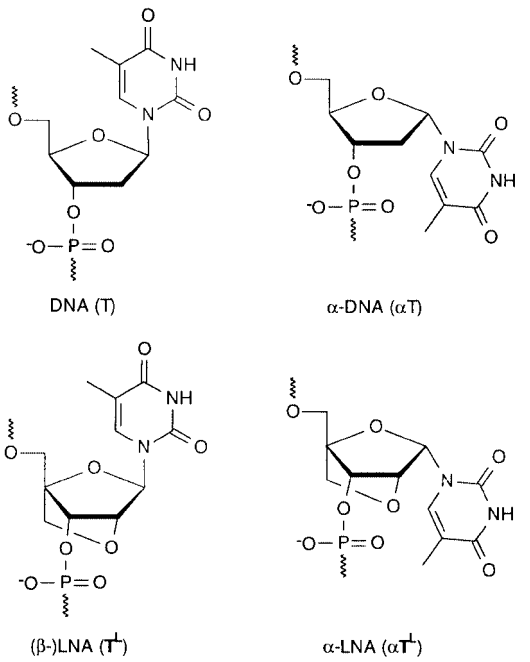
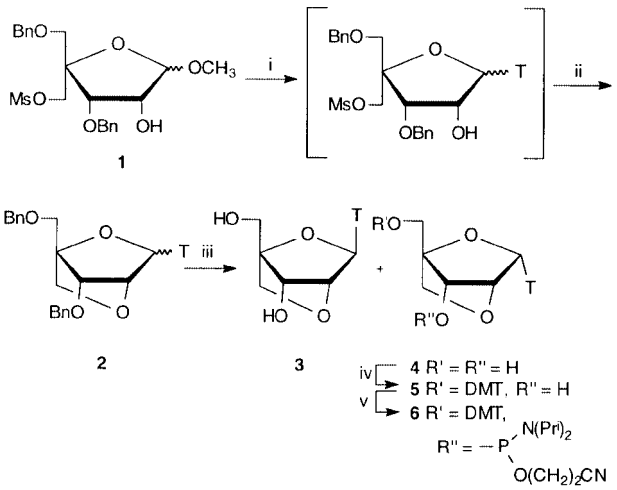
The bicyclic thymine monomer of α-LNA (αT^L) was efficiently synthesised and used in the synthesis of α-LNA sequences: incorporation of single αT^L-monomers in α-configured oligothymidylates destabilises the affinity towards both complementary DNA and RNA, whereas a fully modified α-LNA sequence displays a very efficient recognition of complementary RNA.

Conformationally restricted oligonucleotide analogues have been intensively investigated for their abilities in high affinity nucleic acid recognition.[1] As a prime example, LNA (locked nucleic acid)† has recently been introduced as a nucleic acid analogue displaying unprecedented affinity towards DNA and RNA.[2] The anomeric inverted analogue of DNA (α-DNA) has been demonstrated to hybridise efficiently with complementary DNA and RNA with a parallel strand orientation, and to be highly resistant towards degradation by nucleases.[3] Chemically modified analogues of α-DNA have also been investigated,[4] including the introduction of α-configured bicyclic nucleoside monomers.[5]

The α-2′-deoxynucleoside monomers in α-DNA as well as the β-2′-deoxynucleoside monomers in DNA exist in an equilibrium between the two low-energy N- and S-type conformational ranges.[6,7] In none of the α-DNA analogues, investigated so far,[4,5] have the monomers been efficiently restricted towards N-type conformations. Hoping to obtain an unprecedented parallel nucleic acid recognition and, thereby, a new tool in the development of diagnostic probes and antisense therapeutics, we therefore decided to examine the incorporation into oligonucleotides of an α-nucleoside analogue which is conformationally locked in an N-type conformation, *i.e.* α-LNA.‡



In order to synthesise the α-LNA thymine monomer (αT^L, **4**, Scheme 1) we first investigated the coupling of thymine to appropriate bicyclic carbohydrate precursors.[9] However, this approach has never been optimised to give the target compound in a satisfactory yield, and we hereby introduce an alternative synthetic strategy. The starting methyl furanoside **1** was obtained, as described previously,[9] and used in a modified Vorbrüggen nucleobase coupling reaction (Scheme 1). After varying the reaction conditions, the best result in terms of yield and ratio of products was obtained by using *in situ* TMS-protection of both the 2′- hydroxy functionality in **1** and the thymine, followed by coupling by using TMS-triflate as the Lewis acid in refluxing acetonitrile for seven days. After desilylation, the mixture of nucleosides was reacted with sodium hydride to give the anomeric mixture **2** (α:β ≈ 1.3:1 according to ^{1}H NMR) in 57% overall yield. After removal of the benzyl groups by hydrogenation, the β- and α-LNA monomers (**3**[2] and **4**,[9] respectively) were obtained in 98% yield and separated. The configuration of **4** was confirmed by comparison of NMR data with its exact enantiomer[8d] and by NOE-difference spectra as mutual contacts were observed between H-1′ and H-3′ and between H-5″ and H-6, respectively. The αT^L monomer **4** was prepared for incorporation into oligonucleotide sequences by protection, by using the dimethoxytrityl (DMT) group to give **5**, followed by phosphitylation to give the phosphoramidite synthon **6**.§ This compound was used in automated solid phase synthesis of oligonucleotides by using the phosphoramidite approach.[10] In connection with the α-thymidine (αT) phosphoramidite, the α-LNA sequences were obtained (Table 1) by using tetrazole activation and 10 min coupling times giving > 98% stepwise coupling yields. The modified oligomers **8**–**10** and **12**, **13** were synthesised by using the DMT-*ON* mode on universal CPG-support (Biogenex), which allowed the synthesis of fully modified sequences after cleavage from the solid support by using LiCl in aqueous



Scheme 1 *Reagents and conditions*: i, (a) TMS-Cl, *N*,*O*-bistrimethylsilylacetamide, thymine, MeCN, then TMS-OTf, (b) TBAF, THF; ii, NaH, DMF (57%, three steps); iii, H_2, $Pd(OH)_2$/C, EtOH (98%); iv, DMT-Cl, $AgNO_3$, Py, THF, DMF (80%); v, $EtN(Pr^i)_2$, $NC(CH_2)_2OP(Cl)N(Pr^i)_2$, CH_2Cl_2 (79%). T = thymine-l-yl.

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Table 1 Hybridisation data for α-LNA sequences and reference strands

	Sequence	dA_{14} complement		rA_{14} complement		rA_6CA_7 complement
		T_m/°C[a]	ΔT_m/°C[b]	T_m/°C[a]	ΔT_m/°C[b]	T_m/°C[a]
7	5′-T_{14}	33.0		30.0		n.d.[c]
8	5′-αT_{14}	32.0		43.0		n.d.[c]
9	5′-αT_7**T**$^{\mathbf{L}}$$T_6$	25.5	−6.5	35.0	−8.0	n.d.[c]
10	5′-αT_5**T**$^{\mathbf{L}}$$_4T_5$	26.0	−1.5	24.5	−4.6	n.d.[c]
11	5′-T_{10}	22.0		20.0		n.d.[c]
12	5′-αT_{10}	18.0		33.5		22.0
13	5′-α**T**$^{\mathbf{L}}$$_{10}$	no T_M[d]		45.0	+1.2[e]; +2.5[f]	37.0

[a] Melting temperatures (T_m) obtained from the maxima of the first derivatives of the melting curve (A_{260} *vs.* temperature) recorded in a buffer containing 10 mM Na_2HPO_4, 100 mM NaCl, 0.1 mM EDTA, pH 7.0 using 1.5 μM concentrations of the two complementary sequences, assuming identical extinction coefficients for all thymine nucleotides. [b] The change in T_m value per modification compared with the reference strand **8**. [c] Not determined. [d] No clear cooperative transition was seen. [e] Compared with **12**. [f] Compared with **11**.

ammonia. The oligomers were purified by using disposable reverse phase chromatography cartridges (Cruachem), which yielded products with > 90% purity, as judged from capillary gel electrophoresis. The compositions of α-LNA sequences were verified from MALDI-MS spectra.¶

The α-LNA sequences **9**, **10** and **13**, as well as their α-DNA counterparts **8** and **12**, were mixed with their DNA and RNA complements and the resulting hybridisation data are shown in Table 1. Compared to the unmodified sequence **7**, the affinity of the unmodified α-sequence **8** towards dA_{14} is, as expected,[3] similar. However, the introduction of one or four α-LNA monomers (**9** or **10**, respectively) results in strongly decreased affinities towards dA_{14}. Towards complementary RNA, rA_{14}, **8** has a higher affinity than **7** and the destabilising effect of one or four α-LNA monomers is even more pronounced. However, in both cases, the introduction of a block of α-LNA monomers diminishes the combined destabilising effect (comparing ΔT_m for **9** and **10**). This suggests that the N-type conformation α-LNA monomers do not have the ability to alter neighbouring nucleosides and, thereby, change the overall single strand conformation towards a form which is more preferable for duplex formation. As judged from NMR studies, this is an important feature of the original (β-)LNA.[11]

The fully modified α-LNA sequence **13** displays strong recognition of the complementary RNA-strand (ΔT_m = +1.2 °C per monomer compared to α-DNA **12** and +2.5 °C compared to the unmodified oligodeoxynucleotide sequence **11**). On the other hand, no clear cooperative transition was seen when **13** was mixed with dA_{14}. This indicates either that α-LNA is unable to recognise DNA and is thereby extraordinarily RNA-selective or, alternatively, that the two sequences might form a secondary structure not detectable by UV-spectroscopy at 260 nm. Thus, a broad non-cooperative transition at 40–45 °C is seen in the mixture, but a similar transition is also observed for **13** alone. Even though the possibility of self-melting has been described earlier for longer α-oligothymidylate sequences,[12] the broad transition and low hyperchromicity observed for **13** alone does not indicate the melting process of a duplex structure, but rather a transition between secondary forms of single strands. The presence of a duplex between **13** and rA_{14} was confirmed by the fact that a clear melting transition of a duplex between **13** and a mis-matching complementary sequence was observed with T_m decreased by 8 °C (Table 1). Furthermore, thermal stabilities measured at higher ionic concentrations (data not shown) were increased, as expected, for all the duplexes involving **11**–**13** and RNA-complements as well as **11** and **12** with dA_{14}, whereas no clear cooperative transitions at increased temperature were observed with **13**:dA_{14} or with **13** alone.

In conclusion, α-LNA is able to form a duplex with complementary RNA with a high thermal stability comparable to other stereoisomers of LNA,[8] even though the original (β)LNA still displays the highest affinity towards complementary nucleic acids. Nevertheless, α-LNA in the present oligothymidylate sequence displays the highest affinity towards RNA of any α-D-configured oligonucleotide analogue. However, from the sequences presented here we are not able to determine whether α-LNA prefers a parallel strand orientation upon hybridisation. This subject is under investigation in our laboratories *via* the synthesis of α-LNA sequences with mixed nucleobase compositions, in addition to further examination of the properties and applications of α-LNA.

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Notes and references

† LNA is defined as an oligonucleotide containing one or more LNA monomers which are bicyclic nucleosides preorganized in N-type conformations. α-LNA is therefore defined as an oligonucleotide containing one or more monomeric α-D-LNA nucleosides in connection to unmodified α-D-nucleosides.

‡ Three other stereoisomers of LNA have been recently introduced[8] and their affinities towards both complementary RNA and the enantiomeric L-RNA have been investigated.[8c] In that sense, an α-LNA sequence has been examined in form of the duplex between its enantiomer α-L-LNA and L-RNA, but only as an (almost) fully modified sequence and only against RNA.[8c]

§ Selected data for **6**: δ_P($CDCl_3$, 121.5 MHz with 85% H_3PO_4 as external standard) 150.9, 151.1.

¶ MALDI-MS: *m/z* ([M − H]$^-$ (found/calc.): **9** (4227.1/4223.8); **10** (4309.2/4307.8); **13** (3261.6/3260.1).

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