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Incorporation of α- and β-LNA (Locked Nucleic Acid) Monomers in Oligodeoxynucleotides with Polarity Reversals

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Abstract—The thymidine monomers of LNA with both α- and β-configuration are incorporated with polarity reversals (i.e., with 3′–3′ and 5′–5′ junctions) in oligodeoxynucleotides with β- and α-configuration, respectively. A 5′-*O*-phosphoramidite of the β-LNA monomer is synthesised. Large destabilisations of duplexes with both complementary DNA and RNA are observed for oligodeoxynucleotides containing the α-LNA monomer, whereas a duplex with complementary RNA of an α-oligodeoxynucleotide containing the β-LNA monomer is not destabilised.

Conformationally restricted nucleosides and corresponding oligodeoxynucleotides (ODNs) have received a lot of attention as potential candidates for antisense and diagnostic purposes.[1,2] For example, the use of bicyclic nucleosides has significantly improved the binding affinity of the corresponding ODNs towards complementary nucleic acid sequences.[2] As a prime example, a bicyclic nucleoside containing a 2′-*O*-4′-*C*-methylene bridge is known to be locked in a north (N) conformation[3] (Fig. 1) and ODNs containing this modification are known as LNA (locked nucleic acid).[4–6] LNA displays an unprecedented affinity towards complementary DNA and RNA (increases in thermal stability of duplexes ΔT_m from +3 to +9 °C per modification).[4–6]

ODNs containing exclusively α-configured nucleosides (α-ODNs) recognise complementary nucleic acids in a parallel manner.[7–9] α-ODNs are resistant towards degradation by nucleases and the affinity towards complementary DNA and RNA is comparable to the antiparallel recognition by β-ODNs.[7,8] However, the binding does not induce RNaseH mediated degradation of the RNA complement.[9] We have recently introduced α-LNA as α-ODNs containing α-D-configured bicyclic nucleosides which are locked in an N-conformation (the α-LNA monomers, Fig. 1).[10] In a fully modified sequence (T_{10}), also α-LNA displayed a high affinity towards complementary RNA but no recognition of DNA, and mixed sequences containing α-LNA monomers in combination with unmodified α-deoxynucleosides displayed poor affinity towards both DNA and RNA.[10]

Intensive investigation has been carried out on β-ODNs containing one or more α-nucleosides incorporated with reversed polarity allowing simultaneously for antiparallel recognition by the β-nucleosides and parallel recognition by the α-nucleosides.[11–16] Thus, chimeric α/β ODNs with stretches of 4 to 12 α-nucleosides give only minor decreases in affinity compared to pure β-ODNs.[11,12] They show increased stability towards nucleases[11] as well as activation of RNaseH in a duplex with complementary RNA.[12] The incorporation of one single α-configured thymidine with polarity reversals in the middle of an otherwise β-configured mixed nonamer ODN gives a decrease in affinity of 2.8 °C towards

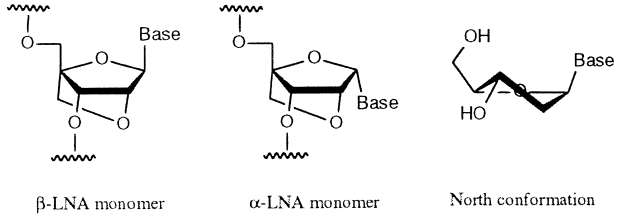

Figure 1. α- and β-LNA monomers and a nucleoside in a north conformation.

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complementary RNA.[13] With four single reverse incorporations of different α-deoxynucleosides each separated by three or four β-nucleosides smaller decreases ($\Delta T_m = 1.5\,^{\circ}C$ per α-monomer) have been observed, and furthermore, these sequences allowed RNaseH mediated degradation of the RNA-strand and exhibited in vivo antitumor activity.[14] ODNs with alternating α- and β-nucleosides (and hence, alternating 3′–3′ and 5′–5′ junctions) do not activate RNaseH,[15] and the decreases in affinity towards complementary RNA and DNA strands are more pronounced (ΔT_m from -1 to $-5\,^{\circ}C$ per α-monomer).[11,15] In self-complementary DNA decamers, single incorporations of different α-deoxynucleosides with polarity reversals afforded decreases in duplex stability between 2.5 and 5.0 °C per modification.[16]

In order to perform a preliminary investigation on the effects of conformational restriction in this context, we decided to incorporate the α-LNA monomer with polarity reversals into normal β-ODNs (Fig. 2A). Hereby, the effect of an α-nucleoside with locked north conformation concerning the affinity towards DNA and RNA complements is investigated. For a further examination of strong conformational restriction in this type of nucleic acid analogues, we also decided to incorporate the β-LNA monomer with polarity reversals into α-ODNs (Fig. 2B).

The building blocks needed for the automated synthesis of ODNs using phosphoramidite chemistry[17] are shown in Figure 3. For synthetic simplicity, only the LNA thymidine monomers and α-pyrimidine nucleosides were used for this study. The synthesis of α-ODNs containing reversely incorporated β-LNA monomers demanded unmodified α-nucleoside phosphoramidites **1** in combination with the LNA 5′-*O*-phosphoramidite **2** (Fig. 3) and was performed in the usual 3′ to 5′ direction. On the other hand, the synthesis of β-ODNs containing reversely incorporated α-LNA monomers was performed in the 5′ to 3′ direction using unmodified 5′-*O*-phosphoramidites **3** as well as an α-LNA phosphoramidite **4** (Fig. 3). The α-thymidine and benzoyl protected α-5-methylcytidine amidites **1** were both synthesised from α-thymidine using published procedures[18] and any required 5′-*O*-phosphoramidites **3** were purchased from Chemgenes. The synthesis of the α-LNA phosphoramidite **4** has been reported earlier,[10] whereas a synthesis of the 3′-*O*-dimethoxytrityl protected LNA 5′-*O*-phosphoramidite **2** was developed (Scheme 1). Thus, the LNA nucleoside monomer **5**[4,5] was protected on the primary hydroxyl group using a silyl ether to give **6** followed by tritylation of the 3′-*O* position. The conventional method using 4,4′-dimethoxytrityl chloride in pyridine failed in this case, and several combinations of bases including DMAP as well as $AgNO_3$ as an additive did not bring the yield of **7** over 20%. However, the use of 4,4′-dimethoxytrityl triflate[19] which has been used before for tritylation of hindered secondary alcohols on bicyclic nucleosides[19] improved the yield of **7** to 66%. Simple desilylation to give **8**[20] followed by conventional phosphorylation gave the phosphoramidite building block **2** in 77% yield over the two steps.[21]

All modified ODNs were synthesised in the 0.2 μmol scale using phosphoramidite chemistry[17] and the DMT-*ON* mode on universal CPG-supports purchased from Biogenex. All couplings were performed with tetrazole activation. For **4**, prolonged coupling time and, for **2**

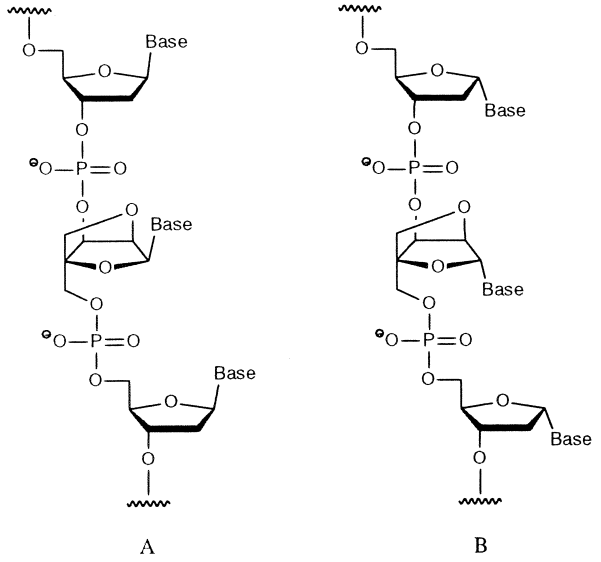


Figure 2. (A) Segment of a β-ODN containing α-LNA with polarity reversals; (B) segment of an α-ODN containing β-LNA with polarity reversals.

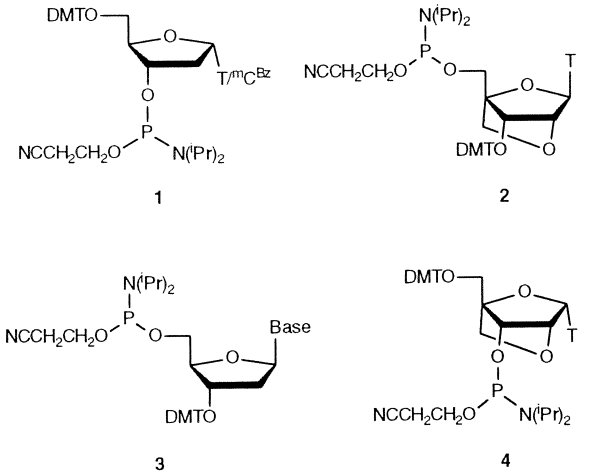
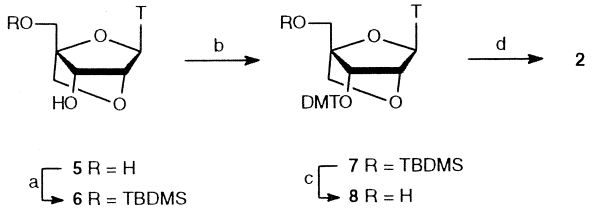


Figure 3. Building blocks used for ODN synthesis. T = thymin-1-yl. $^{m}C^{Bz}$ = 3-*N*-benzoyl-5-methylcytosin-1-yl. DMT = 4,4′-dimethoxytrityl.



Scheme 1. (a) TBDMS-Cl, pyridine, 91%; (b) DMT-triflate, pyridine, 66%; (c) TBAF, THF, 88%; (d) $NC(CH_2)_2OP(Cl)N(^{i}Pr)_2$, CH_2Cl_2, $Et_2N^{i}Pr$, 88%. T = thymin-1-yl.

and **3,** prolonged detritylation times were used giving >98% stepwise coupling yields. The modified oligomers were cleaved from the solid support using LiCl in aqueous ammonia and purified on disposable reverse phase chromatography cartridges (Cruachem), yielding products with >90% purity according to capillary gel electrophoresis.[22] The composition of the sequences (Table 1) were verified from MALDI-MS spectra.[23]

The modified ODNs as well as their unmodified reference sequences were mixed with complementary single stranded DNA or RNA sequences and the thermal stabilities of the resulting duplexes were determined (Table 1). When normal α-thymidine is incorporated with polarity reversals into β-ODNs (via **1** and **3**), we observe a decrease in thermal stability compared to unmodified references that is comparable or slightly more pronounced (ΔT_m from −3.5 to −5.0 °C) than what has previously been reported[13–16] (Table 1, ODNs **10** and **13** compared to **9** and **12**). However, the decrease is much more significant when the α-LNA nucleoside (via **4**) is similarly incorporated (ODNs **11** and **14**). Thus, with the mixed sequence **14** no stable duplexes are formed. This indicates that the locked north conformation of the α-monomer is very unfavourable for this type of duplex formation. When β-thymidine was incorporated reversely into α-ODNs (via **1** and **3**) comparable destabilisations (ODNs **16** and **19** compared to **15** and **18**) were observed (ΔT_m from −3 to −7 °C) most significantly of duplexes with complementary RNA. However, when the β-LNA nucleoside (via **2**) was incorporated, completely different results were obtained. Thus, for an oligothymidylate sequence **17**, a comparable decrease in affinity towards DNA (−5 °C) but a very large decrease in affinity towards RNA (−13 °C) was observed. On the other hand, the mixed pyrimidine sequence (ODN **20**) displayed a larger decrease in affinity towards DNA (−7 °C) but almost no decrease in affinity towards RNA (−0.5 °C).[24] Thus, in the latter case the locked N-conformation of the β-monomer neutralises the destabilising effect of the polarity reversal incorporation.

The structures of duplexes containing ODNs with single α-nucleosides incorporated with polarity reversals have been studied by Germann and co-workers using NMR techniques.[13,16,25,26] It was demonstrated that the presence of these unnatural moieties leads to structural variations in the section containing the modification compared to an unmodified control. Thus, the α-nucleoside is found to be in a south (S) conformation[3] in both DNA–DNA and DNA–RNA hybrids,[13,25] whereas the nucleotide downstream of the 5′–5′ junction in a DNA–DNA duplex is shifted in conformational equilibrium towards an S to N ratio of approximately 1:1 compared to higher than 80% S-conformation in the unmodified control.[16,25] In a DNA–RNA hybrid, where the nucleotides of the DNA strand display a higher propensity for adopting an N-conformation, the two nucleotides upstream of the 3′–3′ junction turn out to be shifted towards a higher percentage of S.[13] In neither case, however, does the overall structure of the duplex alter decisively.[13,25] Our results with reversely incorporated α-LNA are in line with these NMR analyses, as the locked N-conformation of the α-nucleosides has been found to be very unfavourable for duplex stabilities. Hence, the high preference of α-nucleosides for adopting S-conformations seems to be essential in duplexes with polarity reversals.

On the other hand, the LNA monomer incorporated with polarity reversals into α-ODNs improves the affinity towards complementary RNA, at least in a mixed pyr-

Table 1. Hybridisation data of duplexes containing unmodified and modified ODNs with polarity reversals

ODN	Sequence	Complementary ssDNA		Complementary ssRNA	
		T_m (°C)[a]	ΔT_m (°C)	T_m (°C)[a]	ΔT_m (°C)
	β-ODNs containing α-nucleosides[b]				
9	5′-β-T_{14}	33.0	ref.	30.0	ref.
10	5′-β-$T_7{}^{3'-3'}\alpha T^{5'-5'}T_6$	29.5	−3.5[d]	25.0	−5.0[d]
11	5′-β-$T_7{}^{3'-3'}\alpha \mathbf{T}^{\mathbf{L}5'-5'}T_6$	21.5	−11.5[d]	22.0	−8.0[d]
12	5′-β-CTGATATGC	29.0	ref.	26.5	ref.
13	5′-β-CTGA$^{3'-3'}\alpha T^{5'-5'}$ATGC	25.0	−4.0[e]	22.5	−4.0[e]
14	5′-β-CTGA$^{3'-3'}\alpha \mathbf{T}^{\mathbf{L}5'-5'}$ATGC	<10	<−19[e]	<10	<−16[e]
	α-ODNs containing β-nucleosides[c]				
15	5′-α-T_{14}	31.5	ref.	43.0	ref.
16	5′-α-$T_7{}^{3'-3'}\beta T^{5'-5'}T_6$	28.5	−3.0[f]	36.0	−7.0[f]
17	5′-α-$T_7{}^{3'-3'}\beta \mathbf{T}^{\mathbf{L}5'-5'}T_6$	26.5	−5.0[f]	29.0	−13.0[f]
18	5′-α-^{m}CT^{m}C^{m}CTT^{m}CTTT	42.5	ref.	32.0	ref.
19	5′-α-^{m}CT^{m}C^{m}CT$^{3'-3'}\beta T^{5'-5'm}$CTTT	39.5	−3.0[g]	28.5	−3.5[g]
20	5′-α-^{m}CT^{m}C^{m}CT$^{3'-3'}\beta \mathbf{T}^{\mathbf{L}5'-5'm}$CTTT	35.5	−7.0[g]	31.5	−0.5[g]

$\mathbf{T}^{\mathbf{L}}$ refers to LNA thymidine monomers.
[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. All values are means of double determinations.
[b]Mixed with antiparallel complementary sequences dA_{14}, rA_{14}, 5′-dGCATATCAG or 5′-rGCAUAUCAG.
[c]Mixed with parallel complementary sequences dA_{14}, rA_{14}, 5′-dGAGGAAGAAA or 5′-rGAGGAAGAAA.
[d]The change in T_m value per modification compared with the reference strand **9**.
[e]Compared with **12**.
[f]Compared with **15**.
[g]Compared with **18**.

imidine sequence, suggesting that the N-conformation is preferable for the reverse β-nucleoside in this duplex type. This confirms the potential of conformationally locked nucleosides in ODNs also with polarity reversals and indicates that more improved thermal stabilities might be obtained in other sequences with chimeras of conformationally restricted α- and β-nucleosides. Taken together, our results and the NMR analyses (vide supra) indicate that other conformational restrictions can afford ODNs with higher binding affinities, for example by incorporating restricted monomers up- or downstream of the polarity reversals. Furthermore, the fact that α/β-chimeras with polarity reversals in some duplexes with RNA induce RNaseH mediated cleavage of the RNA strand[14] and the fact that one incorporation of (β-)LNA does not significantly destabilise duplex stability suggest, in combination, that chimeras of α/β-LNA and other modified or unmodified nucleosides might be useful in the construction of conformationally restricted high-affinity ODNs with the ability of inducing RNaseH mediated degradation of the target RNA.

In conclusion, we have demonstrated the efficient incorporation of conformationally locked α- and β-LNA monomers into ODNs with polarity reversals. In this context, we have efficiently synthesised a 5′-*O*-phosphoramidite of an LNA monomer. In accordance with the NMR examination by Germann and co-workers,[13,25] our results suggest that the binding affinity of ODNs containing α-nucleosides with polarity reversals might instead be significantly improved by α-nucleoside analogues that are conformationally restricted in S-type conformations. The development of other conformationally restricted α/β-chimeric ODNs with potential high-affinity nucleic acid recognition and, therefore, also antisense purposes is now in progress.

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20. Selected data for **8**: ^{1}H NMR; δ_H ($CDCl_3$, 300 MHz) 7.43–7.22 (9H, m, arom.), 7.18 (1H, s, H-6), 6.81–6.77 (4H, m, arom.), 5.46 (1H, s, H-1′), 4.30 (1H, d, *J* 7.6 Hz, H-1″), 4.02 (2H, m, H-5′), 3.89 (1H, d, 7.6 Hz, H-1″), 3.77 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.66 (1H, s, H-2′ or 3′), 3.34 (1H, s, H-2′ or 3′), 1.79 (3H, s, CH_3). HR-MALDI MS; *m*/*z* found 595.2062, calcd for $C_{32}H_{32}N_2O_8$ + Na 595.2051.
21. Selected data for **2**: ^{31}P NMR; δ_P ($CDCl_3$, 121.5 MHz with 85% H_3PO_4 as external standard) 150.68, 150.85.
22. ODN **13** was synthesised on a 3′-*O*-DMT-dC CPG-support from Chemgenes and cleaved using concentrated aqueous ammonia. After the usual chromatography (Cruachem), the sequence was >70% pure according to capillary gel electrophoresis.
23. MALDI MS data for oligonucleotide sequences: *m*/*z* (found/calcd); **10** (4195.3/4195.8 Da); **11** (4223.6/4223.8 Da); **13** (2712.2/2712.8 Da); **14** (2742.1/2740.8 Da); **16** (4194.8/4195.8 Da); **17** (4220.8/4223.8 Da); **19** (2976.7/2975.0 Da); **20** (3003.3/3003.0 Da).
24. All thermal stabilities of duplexes containing **15**–**20** were also determined in a high salt buffer (750 mM NaCl) and the expected increases were observed independent of the modifications.
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