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α-L-RNA (α-L-*ribo* Configured RNA): Synthesis and RNA-Selective Hybridization of α-L-RNA/α-L-LNA Chimera

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Abstract—Synthesis of the novel α-L-ribofuranosyl phosphoramidite derivative **7** was accomplished via the α-L-ribofuranosyl thymine nucleoside **4**. Amidite **7** was used in automated syntheses of chimeric oligonucleotides composed of mixtures of the novel α-L-RNA nucleotide monomer ($^{\alpha L}$**T**, α-L-*ribo* configured RNA), and DNA, LNA (**T**$^{\mathbf{L}}$, locked nucleic acid) or α-L-LNA ($^{\alpha L}$**T**$^{\mathbf{L}}$, α-L-*ribo* configured locked nucleic acid) nucleotide monomers. For α-L-RNA/DNA and α-L-RNA/α-L-LNA chimeras, RNA-selective hybridization was obtained, for α-L-RNA/α-L-LNA chimera we found increased binding affinity compared to the corresponding DNA:RNA reference duplex. In addition, α-L-RNA/α-L-LNA chimera displayed significant stabilization towards 3′-exonucleolytic degradation. These results indicate that α-L-RNA/α-L-LNA chimeras deserve further evaluation as antisense molecules.

In the antisense therapeutic strategy, chemically modified oligonucleotides are administered in order to specifically inhibit the translation of disease-related mRNA sequences by duplex formation. Essential requirements for therapeutic efficiency of antisense oligonucleotides include good aqueous solubility, resistance toward enzymatic degradation, and high binding affinity and specificity towards the RNA target strand. Accordingly, synthesis of a large number of chemically modified oligonucleotides has been accomplished.[1] The unprecedented thermal stability of duplexes involving LNA (locked nucleic acid, β-D-*ribo* isomer, Fig. 1)[1b,2,3] has prompted us to study the properties of various LNA stereoisomers, including α-L-LNA (α-L-*ribo* configured LNA, Fig. 1).[1b,3–5] Very efficient recognition of single-stranded DNA and RNA has been demonstrated not only for LNA but also for α-L-LNA.[2–5]

Although significantly less stabilizing than LNA or α-L-LNA, 2′-*O*-alkyl-RNAs[6–8] are presently among the preferred nucleotide modifications in antisense oligonucleotides. The fact that 2′-*O*-alkyl-RNA/RNA duplexes[9] and LNA/RNA duplexes[2f] are not substrates for the RNA-cleaving enzyme RNase H limits the use of fully modified 2′-*O*-alkyl-RNA and fully modified LNA to RNase H independent antisense applications, for example, as steric blockers, as agents interfering with double-stranded RNA targets, or as end-gaps in gapmer[9] antisense oligonucleotides. However, high binding affinity and efficient mis-match discrimination are of utmost importance for any antisense application, as is

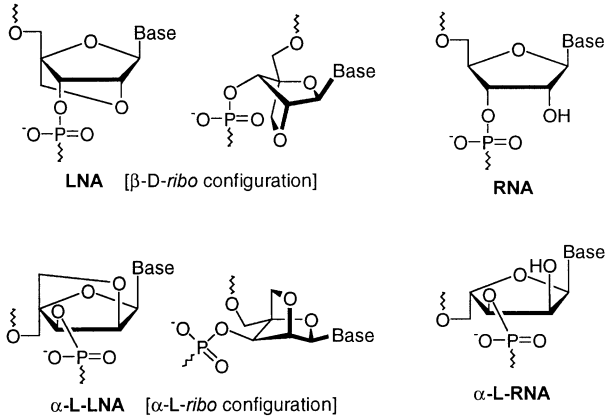

Figure 1. Structures of LNA and α-L-LNA nucleotide monomers and sketches of their locked *N*-type (C3′-*endo*, ^{3}E) furanose conformations.[5] Also shown are structures of RNA and α-L-RNA nucleotide monomers. In Table 1, the notations $^{\alpha L}$**T** (α-L-*ribo* configured RNA), **T**$^{\mathbf{L}}$ (LNA) and $^{\alpha L}$**T**$^{\mathbf{L}}$ (α-L-LNA) are used for the monomers shown above (base = thymin-1-yl).

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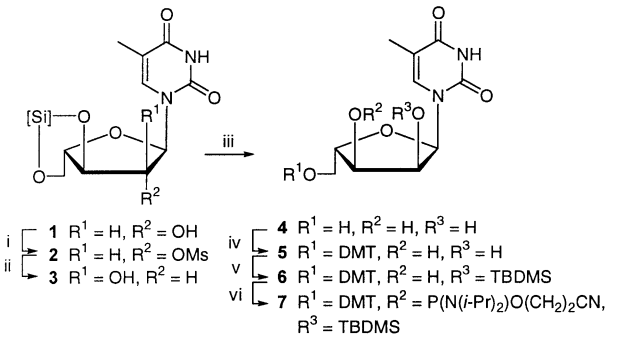

Scheme 1. Synthesis of the α-L-ribofuranosyl thymine nucleoside **4** and the phosphoramidite derivative **7:** (i) MsCl, pyridine (84%); (ii) aq NaOH, ethanol; (iii) aq NaOH, ethanol, 65 °C (59%, two steps); (iv) DMTCl, pyridine (93%); (v) TBDMSCl, imidazole, pyridine (45%); (vi) $NC(CH_2)_2OP(Cl)N(i\text{-}Pr)_2$, $EtN(i\text{-}Pr)_2$, CH_2Cl_2 (73%). [Si] = 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl. DMT = 4,4′-dimethoxytrityl.

the opportunity to fine-tune these parameters, for example, by combining nucleotide monomers of different chemical structures. This report is focused on the potential of modulating RNA-recognition by use of the novel α-L-RNA (α-L-*ribo* configured RNA, Fig. 1) with special focus on α-L-RNA/DNA and α-L-RNA/α-L-LNA chimeras.

Starting from L-arabinose, the known nucleoside **1**[10] was synthesized in five steps. Mesylation of compound **1** in pyridine gave compound **2** in an acceptable yield (84%). Subsequent treatment with aq NaOH in ethanol afforded the *ribo*-configured nucleoside **3**, presumably via an O2′,C2-anhydronucleoside intermediate. Subsequent heating of the reaction mixture for 18 h effected removal of the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl protecting group and 1-(α-L-ribofuranosyl)thymine (**4**) was isolated in 59% yield (from **2**). The NMR data of **4** were identical to those of its enantiomer, 1-(α-D-ribofuranosyl)thymine.[11] Nucleoside triol **4** was selectively DMT-protected in 93% yield at the primary hydroxy group by reaction with 1.5 equiv DMTCl in pyridine to give derivative **5**, which after reaction with 3.0 equiv TBDMSCl in pyridine in the presence of imidazole afforded a mixture of the 2′-*O*- and 3′-*O*-silylated products. Separation of these by column chromatography (2.0–6.0% acetone/0.5% pyridine/97.5–93.5% dichloromethane; v/v/v) furnished nucleoside **6** in 45% yield. Nucleoside **6** was dissolved in anhydrous dichloromethane in the presence of *N*,*N*-diisopropylethylamine and 2-cyanoethyl *N*,*N*-diisopropylphophoramidochloridite to give phosphoramidite **7** in 73% yield (Scheme 1).

The oligomers (Table 1) used in this study were synthesized on an automated DNA synthesizer using the phosphoramidite approach.[12] The α-L-ribofuranosyl phosphoramidite building block **7** was used for the synthesis of the α-L-RNA oligomers **11**, **12** and **16**–**20**. The stepwise coupling yield for amidite **7** was approximately 98% (20 min coupling time; 1*H*-tetrazole as activator) using procedures described previously.[4a] After detritylation, cleavage from the solid support and deacylation were effected using 40% aqueous methylamine (10 min, 55 °C). After cooling to −18 °C, the solid support was removed (centrifugation), washed [2 × 0.25 cm^3; $EtOH/CH_3CN/H_2O$ (3:1:1, v/v/v)], and the combined liquid phase evaporated to dryness under reduced pressure. The residue was desilylated using a method described previously[13] during 20 h at 55 °C. Desilylation of the oligomers was incomplete when using milder desilylation conditions as revealed by MALDI-MS analysis. Standard conditions of the synthesizer were used for incorporation of DNA monomers whereas the incorporation of LNA or α-L-LNA monomers followed procedures described previously.[2a,2b,4a] Analysis by capillary gel electrophoresis verified the purity of the novel α-L-RNA oligomers **11**, **12** and **16**–**20** as being >90%, whereas MALDI-MS analysis confirmed their compositions.[14] The reference DNA oligomers **8** and **13**,[2a,4b] the LNA oligomers **9** and **14**,[2a,2b] and the α-L-LNA oligomers **10** and **15**[4b,4c] have been prepared and studied previously.

Table 1. Melting temperatures (T_m values) towards complementary single-stranded DNA and RNA targets obtained as the maximum of the first derivative of the melting curve (A_{260} vs temperature) in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 1.5 μM concentrations of the two complements[a]

Entry	Description of oligomers	Sequence of oligomers	DNA complement T_m (°C)	RNA complement T_m (°C)
1	DNA reference	**8**: GTGATATGC	30	28
2	LNA/DNA	**9**: $GT^LGAT^LAT^LGC$	44	50
3	α-L-LNA/DNA	**10**: $G(^{\alpha L}T^L)GA(^{\alpha L}T^L)A(^{\alpha L}T^L)GC$	37	45
4	**α-L-RNA/DNA**	**11**: $GTGA(^{\alpha L}T)ATGC$	26	28
5	**α-L-RNA/DNA**	**12**: $G(^{\alpha L}T)GA(^{\alpha L}T)A(^{\alpha L}T)GC$	<5	12
6	DNA reference	**13**: $\mathbf{T_{10}}$	20	19
7	LNA	**14**: $(T^L)_9T$	80	71
8	α-L-LNA	**15**: $(^{\alpha L}T^L)_9T$	63	66
9	**α-L-RNA**	**16**: $(^{\alpha L}T)_9T$	<5	<5
10	**α-L-RNA/LNA**	**17**: $(^{\alpha L}T)_4(T^L)_4(^{\alpha L}T)T$	<5	27
11	**α-L-RNA/LNA**	**18**: $[(^{\alpha L}T)(T^L)]_4(^{\alpha L}T)T$	<5	<5
12	**α-L-RNA/α-L-LNA**	**19**: $(^{\alpha L}T)_4(^{\alpha L}T^L)_4(^{\alpha L}T)T$	<5	29
13	**α-L-RNA/α-L-LNA**	**20**: $[(^{\alpha L}T)(^{\alpha L}T^L)]_4(^{\alpha L}T)T$	<5	27

[a]All oligomers are depicted with the 5′-end positioned to the left. A = adenine monomer, C = cytosine monomer, G = guanine monomer, T = thymine monomer; A, C, G and T are DNA monomers, that is, 2′-deoxy-β-D-ribofuranosyl derivatives. See Fig. 1 for structures of the T^L, $^{\alpha L}T^L$, and $^{\alpha L}T$ monomers (Base = thymin-1-yl).

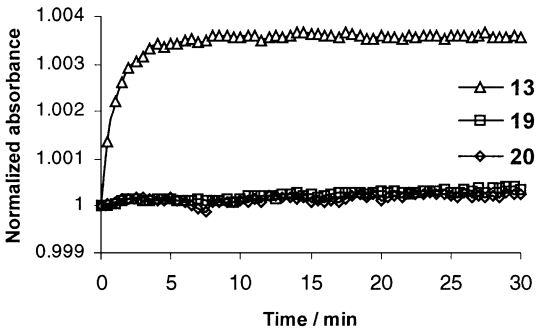

Figure 2. Time course of snake venom phosphodiesterase digestion of the DNA reference **13** and the α-L-RNA/α-L-LNA chimeras **19** and **20**. A solution of the oligomers (~0.2 OD) in 2 mL of a buffer (0.1 M Tris–HCl; pH = 8.6; 0.1 M NaCl; 14 mM $MgCl_2$) was digested with 1.2 U snake venom phosphodiesterase [30 μL of a solution in the following buffer: 5 mM Tris–HCl; pH = 7.5; 50% glycerol (v/v)] at 25 °C.

Results from hybridization experiments (T_m values) toward single-stranded DNA and RNA complements are depicted in Table 1. In entries 1–5, different variations of a 9-mer mixed-base sequence are studied. Introduction of three thymine LNA monomers (**9**)[2a,2b] or three α-L-LNA monomers (**10**)[4c] significantly improves the thermal stability towards both DNA and RNA when compared to the results obtained with the corresponding DNA reference **8**. Both **9** and **10** display a weak RNA selectivity as witnessed by the slightly lower thermal stabilities of the duplexes involving the DNA complement. Results for the two α-L-RNA/DNA chimeras **11** and **12** are despicted in entries 4 and 5. Incorporation of one α-L-RNA nucleotide leads to unchanged (toward RNA) or reduced (toward DNA, $\Delta T_m = -4$ °C) thermal stability when compared to the DNA reference **8**. When three α-L-RNA monomers are incorporated (oligomer **12**), hybridization towards both DNA and RNA is adversely influenced, most so, however, toward DNA.

Various combinations of the different monomers in a homothymine 10-mer context are evaluated in the second series of oligomers (Table 1, entries 6–13). As reported earlier, the (almost) fully modified LNA and α-L-LNA oligomers **14** and **15**, respectively, indeed display very efficient hybridization towards both DNA and RNA.[2a,3b,3c] In contrast, a melting point was not obtained for the corresponding α-L-RNA **16** neither toward DNA nor RNA. Exchange of four α-L-RNA monomers of **16** with LNA monomers gave the α-L-RNA/LNA chimeras **17** and **18**. With four consecutive LNA monomers (**17**), no hybridization toward the DNA complement was detected. However, a T_m value of 27 °C toward the RNA complement was observed. This strong RNA selectivity is thought to be caused by hybridization of the LNA segment (-$\mathbf{T^L_4}$-) with the RNA but not with the DNA complement, as supported by hybridization results reported earlier for a $\mathbf{T^L_5}$T oligomer.[2a] A comparable RNA selectivity has not been observed neither for longer homothymine sequences (e.g., **14**) nor for partly or fully modified LNAs with mixed base compositions.[2b,15] The α-L-RNA/LNA chimera **18** with alternating α-L-RNA and LNA monomers hybridizes neither with the DNA nor the RNA complement. The two α-L-RNA/α-L-LNA chimeras **19** and **20** containing four consecutive α-L-LNA monomers and alternating α-L-RNA and α-L-LNA monomers, respectively, display very similar binding properties. Thus, T_m values of 29 °C and 27 °C for **19** and **20**, respectively, were observed toward the RNA target but no T_m values toward the DNA target. Of utmost importance for antisense applications are not only binding affinity but also base-pairing selectivity. It is therefore encouraging that oligomers **19** and **20** both display satisfactory discriminatory behavior towards an RNA target with one mis-matched base [r(A_7CA_6) target: $T_m < 5$ °C]. However, more experiments are needed in order to fully understand the binding interactions between RNA and α-L-RNA/α-L-LNA chimera.

The stability of α-L-RNA/α-L-LNA chimera toward 3′-exonucleolytic degradation in vitro was evaluated using snake venom phosphodiesterase (SVPDE).[16] During SVPDE digestion of unmodified oligonucleotides, for example the DNA reference **13** (see Fig. 2), the absorbance at 260 nm rapidly increases due to conversion into the nucleoside constituents.[16] In contrast, the α-L-RNA/α-L-LNA chimeras **19** and **20** are both very significantly stabilized toward degradation by SVPDE (Fig. 2; no significant hyperchromicity observed). These qualitative experiments indicate that α-L-RNA/α-L-LNA chimera, like α-L-DNA,[17] α-L-LNA,[18] and DNA/α-L-LNA chimera,[18] are significantly protected toward 3′-exonucleolytic degradation.

The results reported herein suggest that further studies should be performed in order to evaluate the full potential of α-L-RNA/α-L-LNA chimeras as antisense molecules. If the pronounced RNA selectivity obtained for **20** (composed of alternating α-L-RNA and α-L-LNA monomers) turns out to be a general feature of α-L-RNA/α-L-LNA chimeras, one may envision reduced toxicity and improved specificity compared to the current antisense molecules which are known to be able also to hybridize toward DNA targets. A similarily pronounced RNA selectivity has been reported for a few other oligonucleotide analogues, for example, β-L-DNA,[19] arabinonucleic acids,[20] 2′-*O*,3′-*C*-linked bicyclic oligonucleotides,[21] and α-D-LNA.[22] However, their usefulness as antisense molecules is hampered either by comparatively low binding affinity toward RNA[19,20] or the necessity of using fully modified oligomers in order to obtain efficient RNA binding.[21,22] It is therefore important that the α-L-RNA/DNA chimera **11** retains the ability to hybridize to RNA and that the α-L-RNA/α-L-LNA chimera **20**, with alternating α-L-RNA and α-L-LNA monomers, displays increased binding affinity towards RNA (ΔT_m value = +8 °C compared to the DNA reference **13**). It is striking that both DNA/α-L-LNA and α-L-RNA/α-L-LNA chimeras display increased binding affinity toward RNA which stresses the flexibility and power of these LNA-type chimeric oligonucleotides.

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References and Notes

1. (a) Freier, S. M.; Altmann, K.-H. *Nucleic Acids Res*. **1997**, *25*, 4429. (b) Wengel, J. *Acc. Chem. Res*. **1999**, *32*, 301. (c) Herdewijn, P. *Biochim. Biophys. Acta* **1999**, *1489*, 167. (d) Uhlmann, E. *Curr. Opin. Drug Discov. Dev*. **2000**, *3*, 203. (e) Kværnø, L.; Wengel, J. *Chem. Commun*. **2001**, 1419.
2. (a) Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun*. **1998**, 455. (b) Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607. (c) Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Morio, K.; Doi, T.; Imanishi, T. *Tetrahedron Lett*. **1998**, *39*, 5401. (d) Koshkin, A. A.; Nielsen, P.; Meldgaard, M.; Rajwanshi, V. K.; Singh, S. K.; Wengel, J. *J. Am. Chem. Soc*. **1998**, *120*, 13252. (e) Singh, S. K.; Wengel, J. *Chem. Commun*. **1998**, 1247. (f) Wahlestedt, C.; Salmi, P.; Good, L.; Kela, J.; Johnsson, T.; Hökfelt, T.; Broberger, C.; Porreca, F.; Lai, J.; Ren, K.; Ossipov, M.; Koshkin, A.; Oerum, H.; Jakobsen, N.; Skouv, J.; Jacobsen, M. H.; Wengel, J. *Proc. Natl. Acad. Sci. U.S.A*. **2000**, *97*, 5633.
3. We have defined LNA as an oligonucleotide containing one or more 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide monomer(s) and α-L-LNA as an oligonucleotide containing one or more 2′-*O*,4′-*C*-methylene-α-L-ribofuranosyl monomer(s).
4. (a) Rajwanshi, V. K.; Håkansson, A. E.; Dahl, B. M.; Wengel, J. *Chem. Commun*. **1999**, 1395. (b) Rajwanshi, V. K.; Håkansson, A. E.; Kumar, R.; Wengel, J. *Chem. Commun*. **1999**, 2073. (c) Rajwanshi, V. K.; Håkansson, A. E.; Sørensen, M. D.; Pitsch, S.; Singh, S. K.; Kumar, R.; Nielsen, P.; Wengel, J. *Angew. Chem. Int. Ed*. **2000**, *39*, 1656. (d) Håkansson, A. E.; Wengel, J. *Bioorg. Med. Chem. Lett*. **2001**, *11*, 935.
5. Despite the apparent differences in the furanose conformations between an LNA monomer and an α-L-LNA monomer (Fig. 1), the furanose conformation of an α-L-LNA monomer is also of the *N*-type (C3′-*endo*, ^3E) because of its L-configuration. A similar furanose conformation, although more flexible, is likely to be preferred for an α-L-RNA monomer as indicated by calculations, see: Latha, Y. S.; Yathindra, N. *Biopolymers* **1992**, *32*, 249.
6. Inoue, H.; Hayase, Y.; Iwai, A.; Miura, K.; Ohtsuka, E. *Nucleic Acids Res*. **1987**, *15*, 6131.
7. Lesnik, E. A.; Guinosso, C. J.; Kawasaki, A. M.; Sasmor, H.; Zounes, M.; Cummins, L. L.; Ecker, D. J.; Cook, P. D.; Freier, S. M. *Biochemistry* **1993**, *32*, 7832.
8. Martin, P. *Helv. Chim. Acta* **1995**, *78*, 486.
9. Manoharan, M. *Biochim. Biophys. Acta* **1999**, *1489*, 117.
10. Czernecki, S.; le Diguarher, T. *Synthesis* **1991**, 683.
11. Sawai, H.; Nakamura, A.; Hayashi, H.; Shinozuka, K. *Nucleosides Nucleotides* **1994**, *13*, 1647.
12. Caruthers, M. H. *Acc. Chem. Res*. **1991**, *24*, 278.
13. Wincott, F.; DiRenzo, A.; Schaffer, C.; Grimm, S.; Tracz, D.; Workman, C.; Sweedler, D.; Gonzalez, C.; Scaringe, S.; Usman, N. *Nucleic Acids Res*. **1995**, *23*, 2677.
14. MALDI-MS ($[M-H]^-$ measured/$[M-H]^-$ calc): **11** (2770/2769), **12** (2804/2801), **16** (3121/3121), **19** (3170/3169), **20** (3172/3169).
15. Oerum, H.; Jacobsen, M. H.; Koch, T.; Vuust, J.; Borre, M. B. *Clin. Chem*. **1999**, *45*, 1898.
16. (a) Rosemeyer, H.; Seela, F. *Helv. Chim. Acta* **1991**, *74*, 748. (b) Svendsen, M. L.; Wengel, J.; Dahl, O.; Kirpekar, F.; Roepstorff, P. *Tetrahedron* **1993**, *49*, 11341.
17. Asseline, U.; Hau, J.-F.; Czernecki, S.; Le Diguarher, T.; Perlat, M.-C.; Valery, J.-M.; Thuong, N. T. *Nucleic Acids Res*. **1991**, *19*, 4067.
18. Sørensen, M. D.; Kværnø, L.; Bryld, T.; Håkansson, A. E.; Verbeure, B.; Gaubert, G.; Herdewijn, P.; Wengel, J. *J. Am. Chem. Soc*. in press.
19. (a) Fujimori, S.; Shudo, K. *J. Am. Chem. Soc*. **1990**, *112*, 7436. (b) Ashley, G. W. *J. Am. Chem. Soc*. **1992**, *114*, 9731.
20. (a) Damha, M. J.; Wilds, C. J.; Noronha, A.; Brukner, I.; Borkow, G.; Arion, D.; Parniak, M. A. *J. Am. Chem. Soc*. **1998**, *120*, 12976. (b) Noronha, A. M.; Wilds, C. J.; Lok, C.-N.; Viazovkina, K.; Arion, D.; Parniak, M. A.; Damha, M. J. *Biochemistry* **2000**, *39*, 7050. (c) Wilds, C. J.; Damha, M. J. *Nucleic Acids Res*. **2000**, *28*, 3625.
21. (a) Nielsen, P.; Pfundheller, H. M.; Olsen, C. E.; Wengel, J. *J. Chem. Soc., Perkin Trans. 1* **1997**, 3423. (b) Raunkjær, M.; Olsen, C. E.; Wengel, J. *J. Chem. Soc., Perkin Trans. 1* **1999**, 2543.
22. (a) Nielsen, P.; Dalskov, J. K. *Chem. Commun*. **2000**, 1179. (b) Christensen, N. K.; Dahl, B. M.; Nielsen, P. *Bioorg. Med. Chem. Lett*. **2001**, *11*, 1765.