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The Adenine Derivative of α-L-LNA (α-L-*ribo* Configured Locked Nucleic Acid): Synthesis and High-Affinity Hybridization towards DNA, RNA, LNA and α-L-LNA Complementary Sequences

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Abstract—Synthesis of a 9-mer α-L-LNA (α-L-*ribo* configured locked nucleic acid) containing three 9-(2-*O*,4-*C*-methylene-α-L-ribofuranosyl)adenine nucleotide monomer(s) has been accomplished. The work involved synthesis of the bicyclic adenine nucleoside via a condensation reaction between L-*threo*-pentofuranose derivative **1** and 6-*N*-benzoyladenine followed by C2′-epimerization. Hybridization studies demonstrated very strong duplex formation with 9-mer complementary DNA, RNA, LNA and α-L-LNA target sequences.

Synthesis of novel oligonucleotide analogues has been conducted with the aim of developing nucleic acid mimics with superior properties, for example, increased stability towards nucleolytic degradation and increased binding affinity and specificity towards complementary nucleic acid targets.[1] Following the discovery of LNA (locked nucleic acid, β-D-*ribo* isomer, Fig. 1),[2,3] we have recently introduced the stereoisomeric analogue termed α-L-LNA (α-L-*ribo* configured locked nucleic acid, α-L-*ribo* isomer, Fig. 1).[3,4] By virtue of their dioxabicyclo[2.2.1]heptane skeletons, the conformations of the furanose rings of an LNA monomer and an α-L-LNA monomer are efficiently locked in an *N*-type and an *S*-type (or *N*-type, C3′-*endo*)[5] conformation, respectively (Fig. 1). So far, only the properties of α-L-LNA containing the thymine α-L-LNA monomer in homo-thymine or in mixed sequence 9-mer contexts have been studied indicating helical thermostability of α-L-LNA/DNA and α-L-LNA/RNA duplexes approaching those of the corresponding LNA/DNA and LNA/RNA duplexes.[2,4]

A key step in the synthesis of the thymine α-L-LNA monomer was a cascade reaction on tri-*O*-mesyl nucleoside **A** involving C2′-epimerization, hydrolysis of the resulting anhydro-nucleoside intermediate, cyclization, and introduction of a hydroxy group at C5′ affording the bicyclic nucleoside **B** (Fig. 2).[6]

A similar reaction sequence is impossible for the corresponding purine derivatives because of the inability of these to form anhydro-nucleoside intermediates.

In this letter, a synthetic scheme for the purine α-L-LNA monomers is disclosed, as exemplified by the synthesis of the adenine derivative (Scheme 1). In addition, the hybridization properties of a 9-mer α-L-LNA (**α-L-LNA-2**, Table 1) containing three α-L-LNA adenine monomers ($^{\alpha L}\mathbf{A}^{\mathbf{L}}$, Scheme 1) towards complementary single-stranded 9-mer DNA, RNA, LNA and α-L-LNA target sequences are evaluated.

The starting material for the synthesis of the target bicyclic nucleoside **12** and the corresponding phosphoramidite derivative **15** was the known tetra-*O*-acyl-L-*threo*-pentofuranose **1**.[7] Coupling between furanose **1** and 6-*N*-benzoyladenine using $SnCl_4$ as Lewis acid under thermodynamic control afforded the desired N9-regioisomeric nucleoside **2** [9-(2-*O*-acetyl-5-*O*-benzoyl-4-*C*-benzoyloxymethyl-3-*O*-benzoyl-α-L-*threo*-pentofuranosyl)-6-*N*-benzoyladenine] in 52% yield.[8] As anticipated by the presence of the 2′-acetoxy group suggesting anchimeric assistance during the coupling reaction, the β-anomeric nucleoside product was neither detected by analytical TLC nor isolated. It should be noted that the assignment of the synthesized nucleoside derivatives **2**–**15** as the N9-regioisomers was confirmed by comparison of the obtained ^{13}C chemical shift values for derivatives **12** and **13** (vide infra) with the published values for N7- and N9-regioisomeric β-D-ribofuranosyladenines.[9] A

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Vorbrüggen-type[10] coupling reaction with TMS-triflate as Lewis acid and *N*,*O*-bis(trimethylsilyl)acetamide as silylating agent furnished nucleoside **2** in only 21% yield. Next, epimerization at C2′ via intermediates **3** and **4** was performed. Selective deacylation of the 2′-*O*-acetyl group to give intermediate **3** was accomplished using half-saturated methanolic ammonia,[11] whereupon reaction with triflic anhydride in a mixture of dichloromethane and pyridine afforded intermediate **4** according to analytical TLC. No purification of this intermediate was necessary as direct reaction with potassium acetate and 18-crown-6 in toluene at 80 °C afforded the desired C2′-epimeric nucleoside **5** in excellent 84% yield from **2**.[12] Complete deacylation of **5** to give nucleoside **6** was accomplished using a mixture of saturated methanolic and aqueous ammonia, and via a transient protection protocol (*O*-trimethylsilylation, 6-*N*-benzoylation and desilylation) the corresponding 6-*N*-benzoyl derivative **7** was obtained in 91% from **5**. To prepare for cyclization by intramolecular attack from the 2′-hydroxy group, the di-*O*-mesyl derivative **8** was prepared in 79% yield by reaction with 2.2 equiv of mesyl chloride in pyridine at 0 °C. No mesylation of the secondary hydroxy group was observed. Cyclization of nucleoside **8** with concomitant demesylation and debenzoylation was performed in only 13% yield by reaction in a mixture of aqueous sodium hydroxide and 1,4-dioxane (reflux, 72 h). Due to the low yield and the need for repeated benzoylation of the adenine moiety (required for automated oligonucleotide synthesis), another approach was used. Thus, nucleoside **8** was treated with sodium hydride in THF which afforded derivative **9** with a bicyclic furanose moiety which was subsequently converted into the desired base-protected α-L-LNA adenine dihydroxy nucleoside **12**. Thus, substitution of the remaining mesyloxy group of **9** with an acetate group to give **10**, selective deacetylation (half-saturated methanolic ammonia) to give nucleoside **11** and subsequent debenzylation (Pd/C, ammonium formate) afforded the target nucleoside **12**[13] in 32% yield from **8** (Scheme 1). In addition, the fully deprotected nucleoside **13** was obtained in 17% yield. The assigned α-L-*ribo* configuration of nucleoside **12** was confirmed by ^{1}H NOE experiments, that is, mutual NOE effects between H2/H8 of the adenine moiety and the protons of the endocyclic methylene group (10% and 3% when irradiating H2/H8 and the protons of the endocyclic methylene group, respectively) and between H1′ and H2′/H3′ (5%/3% and 11% when irradiating H1′ and H2′/H3′, respectively).[14]

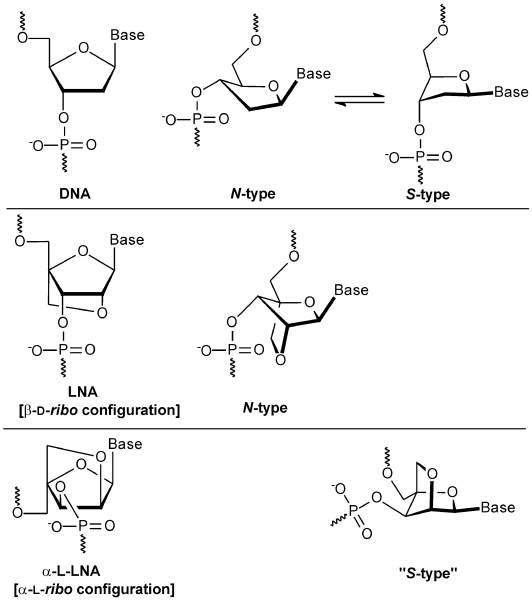


Figure 1. The structures of nucleotide monomers of DNA, LNA and α-L-LNA. The conformational equilibrium between *N*-type and *S*-type conformers (e.g., the C3′-*endo*/^3E *north*(*N*)-type and C3′-*exo*/$_3E$ *south*(*S*)-type conformations shown) of an unmodified DNA monomer is indicated. Also shown are the locked *N*-type (C3′-*endo*/^3E) and '*S*-type' (C3′-*exo*/$_3E$; *N*-type, C3′-*endo*, ^3E)[5] furanose conformations of an LNA and an α-L-LNA monomer, respectively.

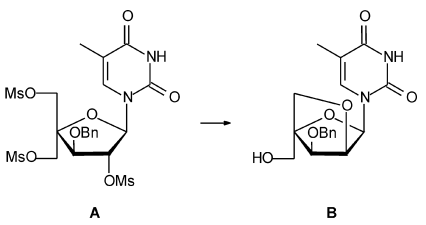
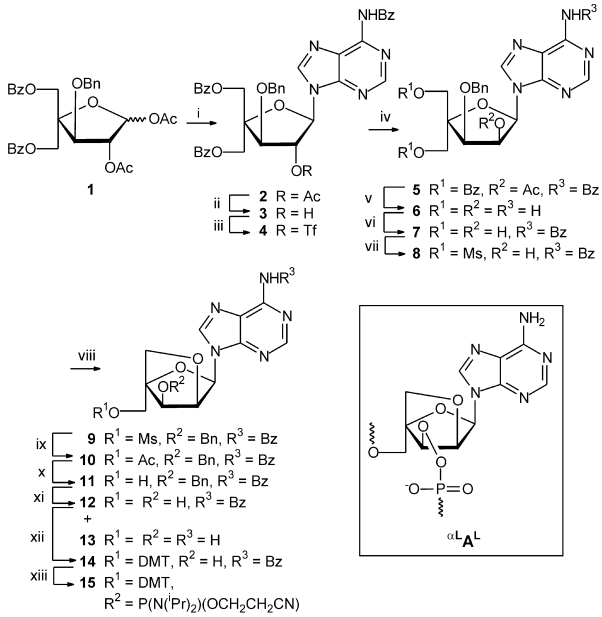


Figure 2. The key cascade reaction during the synthesis of the thymine α-L-LNA monomer. The yield for the conversion shown was 58% and the reagents used were 6 M NaOH and EtOH (1:1, reflux, 43 h; ref 6).



Scheme 1. Synthesis of the α-L-LNA adenine nucleosides **12** and **13** and the phosphoramidite monomer **15** suitable for incorporation of the α-L-LNA adenine monomer $^{\alpha L}\mathbf{A}^{\mathbf{L}}$ into oligonucleotides. Reagents, conditions and yields: (i) 6-*N*-benzoyladenine (2 equiv), $SnCl_4$ (3 equiv), acetonitrile, rt (52%); (ii) half-saturated NH_3 in methanol, 0 °C; (iii) Tf_2O (3 equiv), pyridine, dichloromethane, −30 to 0 °C; (iv) KOAc (5 equiv), 18-crown-6 (2 equiv), toluene, 80 °C (84%, 3 steps); (v) saturated NH_3 in methanol/32% aq NH_3 (4:1), rt; (vi) (a) TMSCl (15 equiv), pyridine, rt; (b) BzCl (5 equiv), pyridine, rt; (c) 32% aq NH_3/H_2O/methanol (6:3:4), rt (91%, two steps); (vii) MsCl (2.2 equiv), pyridine, 0 °C (79%); (viii) NaH (2 equiv), THF, rt; (ix) KOAc (5 equiv), 18-crown-6 (2 equiv), 1,4-dioxane, 100 °C (87%, two steps); (x) half-saturated NH_3 in methanol, 0 °C (83%); (xi) $HCOONH_4$ (3 equiv), 10% Pd/C, methanol, reflux (**12**: 44%, **13**: 24%); (xii) DMTCl (2 equiv), pyridine, rt (82%); (xiii) 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite (2 equiv), *N*,*N*-diisopropylethylamine (4 equiv), dichloromethane, rt (63%).

Table 1. Melting temperatures (T_m values) obtained from the maxima of the first derivatives of the melting curves (A_{260} vs temperature) recorded in a medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 1.5 mM concentrations of the two complementary strands assuming identical extinction coefficients for modified and unmodified oligonucleotides. The complementary 9-mer DNA, RNA, LNA and α-L-LNA sequences are also shown. **α-L-LNA-2** represents the first α-L-LNA containing α-L-LNA purine monomers. References are given in the table for published T_m values. A, C, G, T and U denotes standard DNA/RNA monomers. $\mathbf{T^L}$ and $\mathbf{A^L}$ denotes LNA monomers. $\mathbf{^{\alpha L}T^L}$ and $\mathbf{^{\alpha L}A^L}$ denote α-L-LNA monomers

DNA-1	5′-(GTGATATGC)
RNA-1	5′-r(GUGAUAUGC)
LNA-1	5′-(G$\mathbf{T^L}$G$\mathbf{AT^L}$A$\mathbf{T^L}$GC)
α-L-LNA-1	5′-(G($\mathbf{^{\alpha L}T^L}$)GA($\mathbf{^{\alpha L}T^L}$)A($\mathbf{^{\alpha L}T^L}$)GC)
DNA-2	5′-(GCATATCAC)
RNA-2	5′-r(GCAUAUCAC)
LNA-2	5′-(GC$\mathbf{A^L}$T$\mathbf{A^L}$TC$\mathbf{A^L}$C)
α-L-LNA-2	5′-(GC($\mathbf{^{\alpha L}A^L}$)T($\mathbf{^{\alpha L}A^L}$)TC($\mathbf{^{\alpha L}A^L}$)C)

T_m values/°C	DNA-2	RNA-2	LNA-2	α-L-LNA-2
DNA-1	29	28[2b]	40[2d]	37
RNA-1	27[2e]	38[2e]	46	42
LNA-1	44[2b]	50[2b]	74[2d]	60
α-L-LNA-1	37[4c]	45[4c]	56	56

Following standard procedures, the phosphoramidite building block **15**[15] was obtained from diol **12** by selective 4,4′-dimethoxytritylation to give derivative **14** in 82% yield and subsequent phosphitylation (63% yield). Phosphoramidite **15** was used on an automated synthesizer to give a 9-mer α-L-LNA [**α-L-LNA-2**, 5′-(GC($\mathbf{^{\alpha L}A^L}$)T($\mathbf{^{\alpha L}A^L}$)TC($\mathbf{^{\alpha L}A^L}$)C)][16] containing three α-L-LNA adenine monomers ($\mathbf{^{\alpha L}A^L}$, Scheme 1). The synthesis was performed as described earlier[4] with a stepwise coupling yield of >90% for amidite **15** (with coupling times of 10, 15 or 30 min) using 1*H*-tetrazole as activator compared with >99% for unmodified 2′-deoxynucleoside phosphoramidites. It is noteworthy that another sample of amidite **15** afforded stepwise coupling yields of >99% using similar conditions[8] which shows that there is no significant sterical hindrance at the α-face of the furanose ring of **15** during the coupling reactions on the DNA synthesizer. Instead, variations in purity in between the phosphoramidite samples may explain the different stepwise coupling yields obtained.

The hybridization properties of **α-L-LNA-2** containing three α-L-LNA adenine monomers and six DNA monomers towards complementary 9-mer single-stranded DNA, RNA, LNA and α-L-LNA targets were evaluated and are shown in Table 1. In addition, a number of already published T_m values are included for comparison. The data obtained for **α-L-LNA-2** show that the affinity- enhancing effect of introducing α-L-LNA thymine monomers[4] can be extended to α-L-LNA adenine monomers. Thus, the T_m values for **α-L-LNA-2** are increased by 8 and 15 °C towards complementary DNA and RNA, respectively, compared with the corresponding unmodified references (DNA:DNA and DNA:RNA duplexes). It is furthermore revealed that **α-L-LNA-2** binds very strongly indeed towards the LNA (**LNA-1**) and α-L-LNA (**α-L-LNA-1**) complements as shown by T_m values of 60 and 56°C, respectively. Only the corresponding LNA:LNA duplex is thermally more stable ($T_m = 74$ °C). As LNA has been established as an RNA mimic[2d,17] and as α-L-LNA, as shown herein, hybridizes very efficiently with RNA targets, the formation of very stable LNA:α-L-LNA duplexes was expected.[18]

Conclusion

A viable synthetic route for the first α-L-LNA purine monomer has been developed. The required C2′-epimerization was efficiently accomplished by substituting a 2′-triflyloxy group with an acetate group. Subsequent cyclization and preparation of the phosphoramidite building block allowed synthesis of a 9-mer α-L-LNA containing three α-L-LNA adenine monomers. In hybridization studies towards complementary DNA and RNA, high-affinity recognition was demonstrated thus indicating the general affinity-enhancing character of α-L-LNA compared with the corresponding unmodified references (**DNA-2:DNA-1** and **DNA-2:RNA-1**). It has furthermore been demonstrated that α-L-LNA:α-L-LNA and α-L-LNA:LNA, like LNA:LNA, constitute exceptionally stable duplex structures.

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3. 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 assigned to LNA (and LNA monomers) as the positioning of the N1, O2′, O3′, and O5′ atoms are equivalent to the one found in RNA. Analogously, α-L-LNA has been defined as an oligonucleotide containing one or more 2′-*O*,4′-*C*-methylene-α-L-ribofuranosyl nucleotide monomer(s).

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13. Data for (1*R*,3*R*,4*S*,7*R*)-3-(6-*N*-benzoyladenin-9-yl)-7-hydroxy-1-(hydroxymethyl)-2,5-dioxabicyclo[2.2.1]heptane (**12**): R_f: 0.23 (13% MeOH in CH_2Cl_2); FAB-MS *m*/*z* 384 $[M+H]^+$; ^{1}H NMR ($(CD_3)SO$) δ ~11.1 (1H, br s, 6-NH), 8.72 (1H, s, 2-H), 8.65 (1H, s, 8-H), 8.04 (2H, d, $J=8.2$ Hz, Bz), 7.65–7.51 (3H, m, Bz), 6.51 (1H, s, 1′-H), 5.94 (1H, d, $J=4.4$ Hz, 3′-OH), 4.93 (1H, t, $J=5.6$ Hz, 5′-OH), 4.44–4.42 (2H, m), 4.09 (1H, d, $J=8.2$ Hz, 5″-H), 4.02 (1H, d, $J=8.2$ Hz, 5″-H), 3.73 (2H, d, $J=5.5$ Hz, 5′-H); ^{13}C NMR ($(CD_3)SO$) δ 152.2, 151.6, 150.3, 133.4, 132.5, 128.5, 125.2 (Bz, C-2, C-4, C-5, C-6), 142.5 (C-8), 90.7 (C-4′), 84.4 (C-1′), 79.4 (C-2′), 72.9 (C-3′), 72.6 (C-5″), 57.5 (C-5′).
14. Cyclization by intramolecular nucleophilic attack from the 5′-hydroxy group [2′-*O*-mesyl derivative of nucleoside **3**, NaOH (10 equiv) in H_2O/dioxane (1:1), reflux, 94 h; 2′-*O*-trifluoromethanesulfonyl derivative of nucleoside **3**, NaH (app. 6 equiv) in DMF, rt to 140 °C, 140 h] was unsuccessfully attempted. In contrast, Robins et al. have earlier reported successful cyclization of the structurally similar nucleoside 9-(3-azido-3-deoxy-2-*O*-mesyl-β-D-xylofuranosyl)adenine to the corresponding 2′,5′-anhydro nucleoside derivative: Robins, M. J.; Hawrelak, S. D.; Kanai, T.; Siefert, J.-M.; Mengel, R. *J. Org. Chem.* **1979**, *44*, 1317. It is therefore conceivable that the cyclization described above will be possible using the right conditions/reagents.
15. Data for (1*S*,3*R*,4*S*,7*R*)-3-(6-*N*-benzoyladenin-9-yl)-7-(2-cyanoethoxy(diisopropylamino)phosphinoxy-1-(4,4′-dimethoxytrityloxymethyl)-2,5-dioxabicyclo[2.2.1]heptane (**15**): R_f: 0.70, 0.77 (8% MeOH in CH_2Cl_2); ^{31}P NMR (CH_3CN) δ 150.2, 150.4.
16. The 9-mer **α-L-LNA-2** was purified (after standard deprotection and cleavage from the solid support using 32% aqueous ammonia) by DMT-ON reversed phase chromatography on disposable purification cartridges which includes detritylation. The composition of the α-L-LNA was confirmed by MALDI-MS analysis and the purity (>90%) by capillary gel electrophoresis.
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18. The formation of very stable LNA:α-L-LNA duplexes is confirmed by the T_m value of 56 °C obtained for the **α-L-LNA-1:LNA-2** duplex.