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Synthesis and biophysical characterization of *R*-6′-Me-α-L-LNA modified oligonucleotides

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ABSTRACT

The synthesis and biophysical properties of *R*-6′-Me-α-L-LNA, which has a methyl group in the (*R*) configuration on the 2′,4′-bridging substituent of α-L-LNA, is reported. The synthesis of the uracil nucleobase phosphoramidite was efficiently accomplished in 14 steps and 8 chromatographic purifications starting from a known sugar intermediate. Biophysical evaluation revealed that substitution along the edge of the major groove does not impair the high affinity duplex forming ability of α-L-LNA modified oligonucleotides.

2′,4′-Bridged nucleic acids (BNA),[1] of which locked nucleic acid (LNA or β-D-LNA **1**) is a representative member,[2] show unprecedented increases in the thermal stability of modified oligonucleotide duplexes.[3] The alpha anomer of enantio-LNA (α-L-LNA **3**) also exhibits LNA-like high affinity recognition of complementary nucleic acids despite the structural differences between the nucleoside monomers (Fig. 1).[4,5] However, while LNA forms highly A-type duplexes versus RNA,[6,7] the structure of α-L-LNA duplexes with RNA have an intermediate character between A- and B-type helical geometries.[8–10] Structural studies have also shown that the 2′,4′-bridge in LNA duplexes lies in the minor groove while this bridge is located inside the major groove for α-L-LNA modified duplexes. Consequently, the overall steric and hydration requirements for efficient hybridization are likely to be different in the β-D-series versus the α-L-series. As a manifestation of these differences, introducing alkyl substituents on the 2′-amino group in α-L-amino-LNA **7**[11,12] or replacing the 2′-oxygen atom in α-L-LNA with a substituted carbon atom (α-L-carba LNA **8**)[13,14] results in a reduction of duplex thermostability. In contrast, replacing the 2′-oxygen atom in β-D-LNA with a substituted amine (β-D-amino LNA **4**)[15] or carbon atom (β-D-carba LNA **5**)[16,17] is tolerated and does not impair duplex stability (Fig. 1).

We recently showed that introducing alkyl (methyl or methoxymethyl) substitution along the 2′,4′-bridge of LNA was tolerated and provided analogs (*S*-cEt **6**) that showed similar thermal stability as LNA but greatly improved resistance to exo-nuclease mediated digestion.[18] In addition, we also observed an improvement in the therapeutic profile of antisense oligonucleotides (ASO) containing these modifications.[19] As an extension of that program, we wanted to investigate the effect of introducing steric bulk at strategic locations on the α-L-LNA sugar moiety and examine the effect of these substitutions on the biological profile of α-L-LNA containing ASOs. As a prelude to that exercise, we first evaluated the effect of introducing steric bulk along the 2′,4′-bridge of α-L-LNA on the hybridization properties of the modified

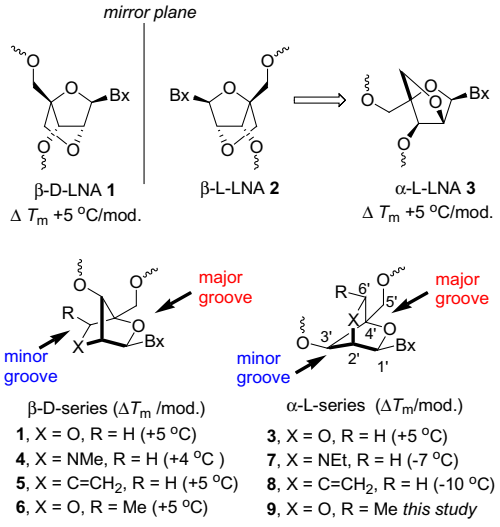

Figure 1. Structures of β-D-LNA, α-L-LNA and related analogs.

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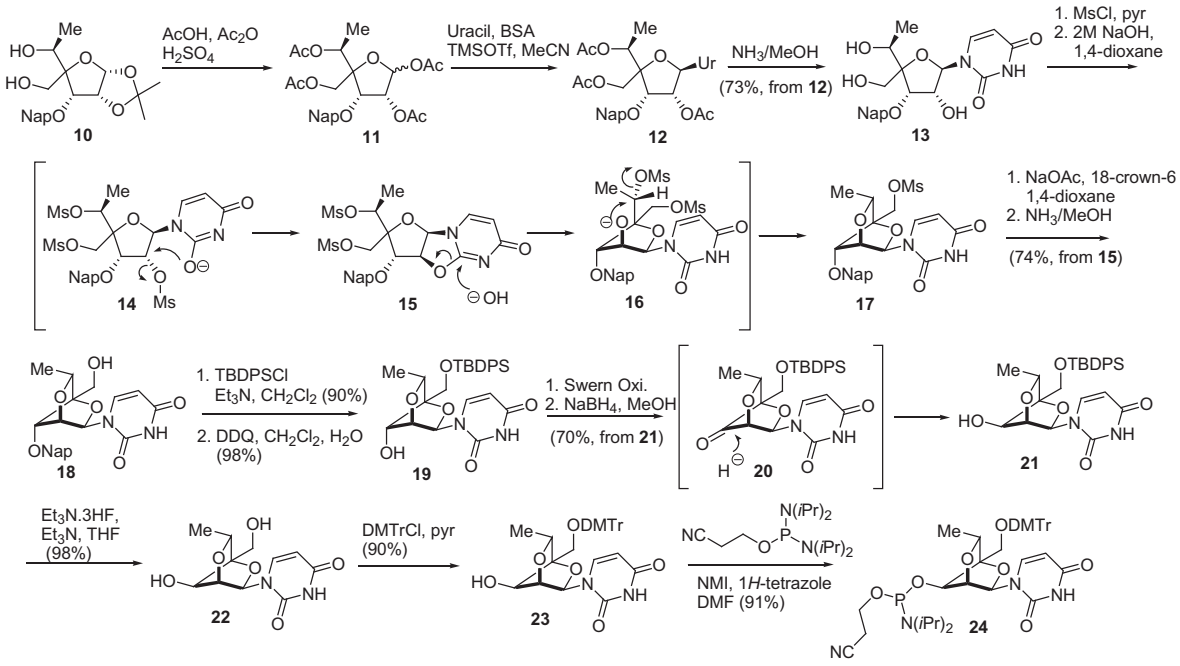


Scheme 1. Synthesis of *R*-6′-Me-α-L-LNA nucleoside phosphoramidite.

duplexes. In this report, we present the synthesis of *R*-6′-Me-α-L-LNA modified oligonucleotides and show that substitution is tolerated at the edge of the major groove of α-L-LNA modified duplexes.

Synthesis of the *R*-6′-Me-α-L-LNA nucleoside phosphoramidite **24** started from the known diol **10** (Scheme 1).[20] Treatment with AcOH/Ac_2O/catalytic sulfuric acid resulted in cleavage of the 1,2-*O*-acetonide and peracetylation of the sugar intermediate to provide **11**. No formation of the pyranose product was observed indicating that acetylation of the free alcohols or trapping of the intermediate furanose oxonium ion with acetate occurs faster than rearrangement to the pyranose anomer. A Vorbruggen reaction with persilylated uracil and TMSOTf provided the nucleoside **12** with exclusive formation of the beta-isomer being directed by anchimeric assistance from the 2′-*O*-acetyl group. Removal of all the acetyl protecting groups in **12** was accomplished by treatment with methanolic ammonia to provide nucleoside **13** in excellent yield (73% over three steps). Treatment of **13** with methanesulfonyl chloride in pyridine provided the trimesyl nucleoside **14**, which was treated with excess sodium hydroxide to provide the cyclized nucleoside **17**.[21,22] The formation of **17** is explained as follows. Treatment of **14** with sodium hydroxide results in the formation of the cyclic anhydro nucleoside **15** by displacement of the 2′-*O*-mesyl group by the 2-oxygen atom on the pyrimidine ring. Under basic conditions, **15** is converted to the 'ara' nucleoside **16** by hydrolytic opening of the anhydro nucleoside resulting in net inversion of configuration of the 2′-hydroxyl group. Cyclization to the [2.2.1] bicyclic α-L-LNA scaffold occurs by a S_N2 displacement of the secondary 6′-*O*-mesylate by the 2′-hydroxyl anion to provide **17**. The 5′-*O*-mesyl group in crude **17** was converted to the corresponding acetate by displacement with potassium acetate and 18-crown-6 in 1,4-dioxane followed by hydrolysis of the acetate group using methanolic ammonia to provide **18** in excellent yield (74%, five steps from **13**). Next, the 5′-hydroxyl was reprotected as the TBDPS ether to facilitate aqueous workup during removal of the 3′-*O*-Nap protecting group with DDQ as described by us[18] and others[23] previously to provide **19**. The 3′-hydroxyl group in **19** was inverted by oxidation to the ketone **20**, followed by reduction with sodium borohydride to give **21** exclusively. The stereoselectivity during the reduction process is controlled by the 6′-methyl group which completely blocks the *Re* face of the ketone in **20** and directs attack of the nucleophile from the less hindered *Si* face. The TBDPS group in **21** was then removed using triethylamine trihydrofluoride to provide the nucleoside **22** in essentially quantitative yield. $Et_3N{\cdot}3HF/Et_3N$ was preferred over TBAF for silyl group removal to avoid contamination of the water soluble nucleoside product with tetrabutylammonium salts during chromatography on silica gel. Protection of the 5′-hydroxyl group as the dimethoxytrityl ether to give **23**, followed by a phosphitilation reaction provided the desired nucleoside phosphoramidite **24** in good yield.

Structural elucidation of the cyclized nucleosides was carried out by NMR spectroscopy (Fig. 2).[24] Consistent with the formation of a 2.2.1 bicyclic ring system, the 1′, 2′ and 3′ protons in nucleoside **17** and **18** and the 1′ and 2′ protons in **19** and **21** appear as singlets (H3′ in **19** and **21** appears as a doublet due to coupling with the 3′-OH). In addition, NOESY crosspeaks were observed between the H3′ and the 6′-Me in **19** but not in **21**. In contrast, NOESY crosspeaks were observed between the H3′ and the H1′ in nucleoside **21** but not in **19**. NOESY crosspeaks were also visible between the 3′-OH and H1′ in **19** and between the 6′-Me and the 3′-OH in **21**. The chemical shift of the methyl group is more downfield in **21** (1.4 ppm) as compared to **19** (1.2 ppm), while the chemical shift of the 1′H is more downfield in **19** (6.2 ppm) as compared to **21** (5.8 ppm). Presumably, the axial 3′-hydroxyl group deshields the 1′H in **19** but the equatorial 3′-hydroxyl deshields the methyl group in **21**.

We evaluated the *R*-6′-Me-α-L-LNA modification in an oligonucleotide sequence previously reported by Imanishi[1] and us[17,18] to

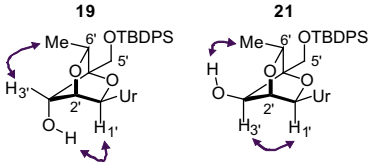
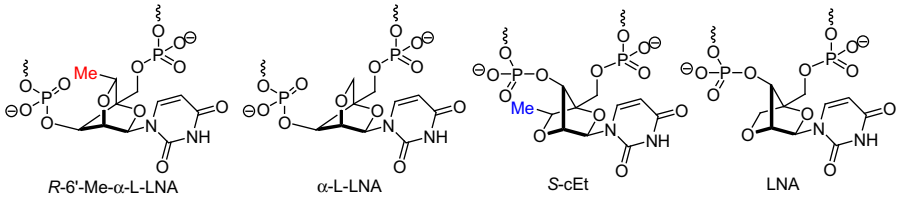


Figure 2. Structural elucidation of *R*-6′-Me-α-L-LNA nucleosides.

Table 1
Thermal stability measurements of *R*-6′-Me-α-L-LNA, α-L-LNA, LNA and *S*-cEt modified oligonucleotides versus RNA and DNA complements



Oligomer	Modification	Sequence[a] (5′–3′)	Mass calcd	Mass found	% UV purity	T_m[b] (°C) versus RNA	ΔT_m (°C)/mod.
A1	DNA	d(GCGTTTTTTGCT)	3633.4	3632.9		45.6	0
A2	*R*-6′-Me-α-L-LNA	d(GCGTT**U**TTTGCT)	3661.4	3660.9	98.6	51.3	+5.5
A3	*R*-6′-Me-α-L-LNA	d(GCGTT**UU**TTGCT)	3689.4	3688.9	97.9	55.2	+4.8
A4	α-L-LNA[c]	d(GCGTT**U**TTTGCT)	3647.4	3646.5	97.8	50.2	+4.6
A5	α-L-LNA[c]	d(GCGTT**UU**TTGCT)	3661.3	3660.8	97.9	54.7	+4.6
A6	*S*-cEt[d]	d(GCGTT**U**TTTGCT)	3661.4	3660.6	95.8	50.1	+4.6
A7	LNA[d]	d(GCGTT**U**TTTGCT)	3647.4	3646.8	98.1	50.1	+4.6

[a] Bold letter indicates modified residue.
[b] T_m values were measured in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 0.1 mM EDTA. Sequence of RNA complement 5′-r(AGCAAAAAACGC)-3′.
[c] Ref. 14.
[d] Ref. 18.

characterize the hybridization properties of LNA oligonucleotides using a single and two tandem incorporation of the modified nucleoside (Table 1). Oligonucleotides were synthesized at 2 μmol scale using T-CPG support, 0.l M solutions of all phosphoramidites in acetonitrile, 0.5 M 1*H*-tetrazole as the activator and standard oxidizing and capping reagents. An extended coupling time of 8 min was used for incorporation of the modified nucleosides and the efficiency of incorporation was ~85% per incorporation. In this sequence, *R*-6′-Me-α-L-LNA showed excellent hybridization properties (ΔT_m +4.8 to +5.5 °C/mod.), which were slightly better than LNA (ΔT_m +4.6 °C/mod.), α-L-LNA (ΔT_m +4.6 °C/mod.) and *S*-cEt (ΔT_m +4.6 °C/mod.) modified oligonucleotides. This result was gratifying given the poor hybridization properties exhibited by a number of α-L-LNA analogs, which have steric bulk along the 2′,4′-bridging substituent, reported by Hrdlicka,[11] Chattopadhyaya[13] and us[14] recently.

To better understand the position of the methyl group relative to the 2′,4′-bridge in the major groove of the modified duplex, we created a structural model using a published structure[10] of an α-L-LNA duplex as described previously (Fig. 3).[14] The model indicated that the methyl group in *R*-6′-Me-α-L-LNA is pointed towards the 3′-phosphodiester linkage at the edge of the major groove and does not make steric contacts with any of the adjacent nucleotides. Moreover, the model indicates that larger substituents such as intercalators and fluorescent tags could be incorporated into the major groove of α-L-LNA modified duplexes using flexible linkers tethered off the 2′,4′-bridge of the nucleoside monomer. This is especially relevant given the recent report by Hrdlicka which showed that tethering pyrene off the 2′-nitrogen in α-L-amino-LNA could be utilized to detect abasic sites and for other oligonucleotide based diagnostic applications.[11,25,26]

In conclusion, we report the synthesis and biophysical characterization of *R*-6′-Me-α-L-LNA modified oligonucleotides. Synthesis of the uracil phosphoramidite was accomplished in 14 steps and 8 chromatography purifications starting from a known sugar precursor intermediate. The synthetic sequence was efficient and could be conveniently carried out on a multi-gram scale to provide enough pyrimidine amidites to support the biophysical and biological

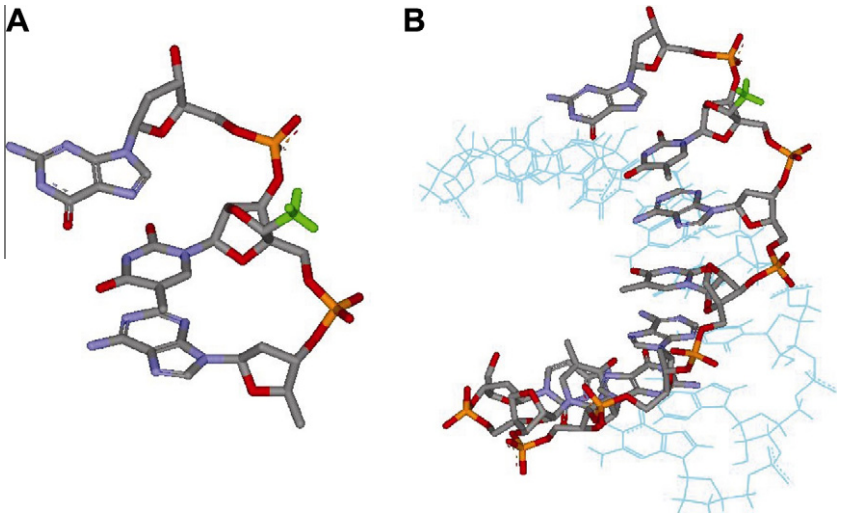


Figure 3. Hypothetical model of *R*-6′-Me-α-L-LNA modified DNA/RNA duplex showing (A) 6′-Me group in green pointed towards the 3′-phosphodister linkage and (B) along the edge of the major groove in a modified duplex. Complementary RNA strand shown in blue.

characterization of this modification. Biophysical evaluation showed that introduction of substitution at the edge of the major groove is tolerated in α-L-LNA modified duplexes without any loss in duplex thermal stability. This result is in contrast with the duplex destabilization properties reported for other closely related α-L-LNA analogs with alkyl substituents along the 2′,4′-bridging group.[11–14] Using a structural model, we show that the methyl group in *R*-6′-Me-α-L-LNA is pointed towards the 3′-phosphodiester linkage at the edge of the major groove and does not make steric contacts with any of the adjacent nucleotides, thus providing a rationale for the observed hybridization properties. Further characterization of the structural and biological properties of this modification is underway and will be reported in due course.

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24. Compound **19** ^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.18 (br s, 1H, NH), 7.84–7.62 (m, 5H, aryl), 7.53–7.32 (m, 6H, aryl), 6.23 (s, 1H, 1′H), 5.71 (d, J = 8.1 Hz, 1H, H5), 4.60 (br s, 1H, 3′H), 4.54 (s, 1H, 2′H), 4.16 (m, 1H, 6′H), 4.06 (m, 2H, 5′H), 3.69 (br s, 1H, 3′OH), 1.17 (d, J = 6.4 Hz, 3H, CH_3), 1.08 (s, 9H, *t*-Bu). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 163.5, 150.3, 140.4, 135.7, 135.6, 132.3, 132.1, 130.2, 130.1, 127.9, 101.2, 90.8, 90.3, 81.3, 77.8, 75.0, 61.5, 26.8, 19.6, 19.2. HRMS (QTOF) calcd for $C_{27}H_{33}N_2O_6Si$, 509.2108; found 509.2114. Compound **21** ^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.05 (br s, 1H, NH), 7.82–7.58 (m, 5H, aryl), 7.53–7.32 (m, 6H, aryl), 5.79–5.66 (m, 2H, 1′H and H5 overlapped), 4.73 (s, 1H, 2′H), 4.29 (d, J = 4.9 Hz, 1H, 3′H), 4.19 (d, J = 6.6 Hz, 1H, 6′H), 4.16–4.04 (m, 2H, 5′H), 2.63 (d, J = 5.1 Hz, 1H, 3′OH), 1.39 (d, J = 6.6 Hz, 3H, CH_3), 1.08 (s, 9H, *t*-Bu). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 163.3, 150.3, 139.4, 135.6, 132.8, 132.7, 130.1, 130.0, 127.9, 101.3, 91.9, 87.5, 81.4, 79.4, 74.7, 60.3, 26.8, 19.4, 17.2. HRMS (QTOF) calcd for $C_{27}H_{33}N_2O_6Si$, 509.2108; found 509.2111.
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