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Replacing the 2′-oxygen with an exocyclic methylene group reverses the stabilization effects of α-L-LNA

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ABSTRACT

The synthesis and hybridization properties of an α-L-LNA analog where the 2′-oxygen atom is replaced with an exocyclic methylene group is reported. Contrary to the β-D series where the exocyclic methylene group is extremely well tolerated, this group was very poorly tolerated in the α-L-series and lead to duplex destabilization. Modeling studies showed that the exocyclic methylene group results in a steric clash with the nucleobase 3′ to the modified residue. Based on this structural model one can anticipate that replacing the 2′-oxygen atom of α-L-LNA with larger groups is likely to be detrimental to duplex stability. The model also provides insights into what type of 2′,4′-bridges are most likely to be tolerated in α-L-LNA modified oligonucleotide duplexes.

2′,4′-Bridged nucleic acid (BNA) analogs, of which locked nucleic acid (LNA) is a representative member, show unprecedented increases in the thermal stability of modified oligonucleotide duplexes.[1–3] Carba-locked nucleic acids (cLNA), where the 2′-oxygen atom in LNA **1** is replaced with a carbon atom, show interesting biological properties[4–6] and have been the subject of considerable interest lately (Fig. 1).[7–13] In general, cLNA analogs **4**–**6** reduce thermal stability of oligonucleotide duplexes relative to LNA **1** and related 'oxa' analogs **2** and **3**.[14,15] Nielsen proposed that the diminished binding affinity of cLNA analogs was a consequence of reduced hydration inside the minor groove.[11,12] This hypothesis was supported by T_m data which showed that introducing hydrophilic functionality along the 2′,4′-bridging group in cLNA analogs partially restores binding affinity for complementary nucleic acids.[11] cLNA modified nucleic acids also show modest increases in recognition of RNA relative to DNA as the more hydrophilic RNA is better able to compensate for the reduced duplex hydration as compared to DNA.

We showed for the first time that it was possible to obtain LNA-like binding affinity with cLNA analogs by replacing the 2′-oxygen atom in LNA with an exocyclic methylene group.[16] Examination of crystal structures of a self-complementary DNA decamers containing methylene-cLNA **7**, *R*-Me-cLNA **5**, and *S*-Me-cLNA **6** showed that the methylene group participates in a CH. . .O type interaction with the 4′-oxygen of a deoxyribose sugar residue of a neighboring strand in the crystal lattice. This indicated that the methylene-cLNA modification retains a net negative polarization at the edge of the minor groove and most likely does not disrupt the water of hydration around the sugar-phosphate backbone.

As a continuation of that exercise, we wanted to evaluate the antisense properties of α-L-methylene-cLNA **8**, where the 2′-oxygen atom in α-L-LNA is replaced with an exocyclic methylene group. α-L-LNA **10** is a class of nucleic acids that was first introduced by Wengel and co-workers and represents the alpha anomer of

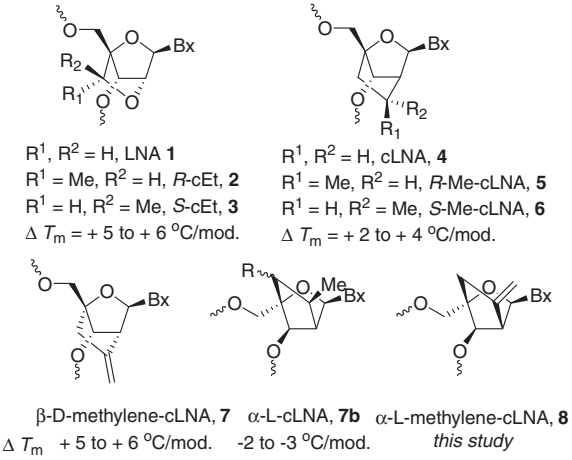

Figure 1. Structures of LNA and carba LNA analogs.

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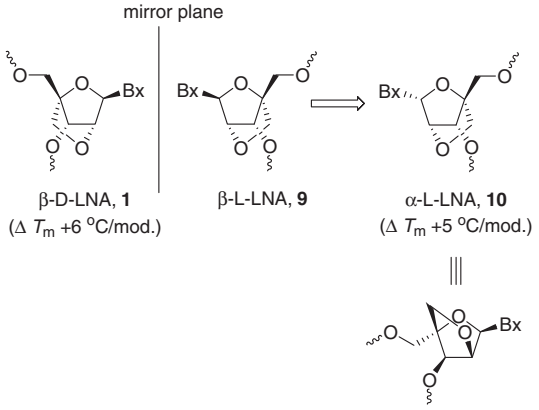


Figure 2. Structures of β-D-LNA, β-L-LNA, α-L-LNA.

enantio-LNA (L-LNA) **9** (Fig. 2).[17] Wengel evaluated all eight stereoisomers of LNA in thermal denaturation studies and showed that α-L-LNA modified oligonucleotides possess almost identical hybridization properties as LNA.[18,19] Petersen and co-workers reported the NMR structure of α-L-LNA modified DNA/RNA duplex.[20,21] They observed that α-L-LNA fits seamlessly into the duplex structure, despite the structural differences between the LNA and α-L-LNA nucleoside monomers. The structural data also showed that the 2′,4′-bridge in α-L-LNA modified duplexes resides inside the major groove. In contrast, the 2′,4′-bridging group in LNA and other β-D-configured analogs resides in the minor groove. Thus it was possible that the steric and hydration requirements of α-L-cLNA modified oligonucleotides were likely to be very different from that of β-D-cLNA containing oligonucleotides. Chattopadhyaya recently reported the synthesis and properties of α-L-configured carbocyclic LNA analogs **7b** where the 2′-oxygen atom in α-L-LNA was replaced with a substituted carbon atom. All those analogs were found to be destabilzing versus complementary RNA and DNA.[22] In this report, we present the synthesis and hybridization properties of α-L-methylene-cLNA modified oligonucleotides and further elucidate the structure thermal stability relationships of this interesting class of nucleic acid analogs.

Synthesis of the α-L-methylene-cLNA uracil nucleobase phosphoramidite **23** commenced from the known sugar intermediate **11**[14] and is similar to the synthesis of the methylene-cLNA **7** reported by us recently (Scheme 1).[16,23] Oxidation of the primary alcohol followed by a Wittig reaction provided olefin **12**. Hydroboration of the olefin in **7** using 9-BBN provided primary alcohol **13**. Oxidation of the primary alcohol followed by a Corey-Fuchs olefination provided the dibromo-olefin **14**, which was converted to the alkyne **15** by treatment with *n*-butyl lithium. Interestingly, during the preparation of β-D-methylene-cLNA **7**, we were unable to convert the dibromo-olefin to the alkyne in the presence of the 3′-*O*-naphthyl group. In that case, both, the dibromo-olefin functionality and the 3′-*O*-naphthyl group were on the alpha face of the sugar ring and treatment with *n*BuLi resulted in the formation of unidentified products arising from single electron transfer into the naphthyl ring.

Acetolysis of the 1,2-*O*-acetonide in **15**, followed by a Vorbruggen reaction with per-silylated uracil and deprotection of the 2′-*O*-acetyl protecting group provided nucleoside **16**. The 2′-hydroxyl group was converted to a thiono-carbonate by reaction with tolyl-chlorothionoformate to provide **17**. Treatment of **17** with nBu$_3$SnH/AIBN in refluxing toluene provided the cyclized nucleoside **18**. The cyclization occurs by generation of a carbon based radical at the 2′-position, followed by an intra-molecular cyclization into pendant alkyne functionality. The next step involved removal of the 3′-*O*-naphthyl group with DDQ to provide **19**. Inversion of the 3′-hydroxyl group was accomplished by an oxidation/reduction sequence. Oxidation of the hydroxyl group in **19** to the bridge-head ketone followed by reduction with sodium borohydride provided the inverted alcohol **20** (2.5:1 mixture) as the major product. Presumably, hydride delivery occurs from the less hindered *si* face of the bridge-head ketone. The 5′-*O*-TBDPS group in **20** was removed using buffered triethylamine trihydrofluoride ($Et_3N{\cdot}3HF$) to provide nucleoside **21**. $Et_3N{\cdot}3HF$ was preferred over TBAF to avoid contamination of the nucleoside product with tetrabutylammonium fluoride salts during purification using silica gel chromatography. Reprotection of the 5′-hydroxyl group as the dimethoxytrityl ether provided **22**, which was converted to phosphoramidite **23** by means of a phosphitylation reaction.

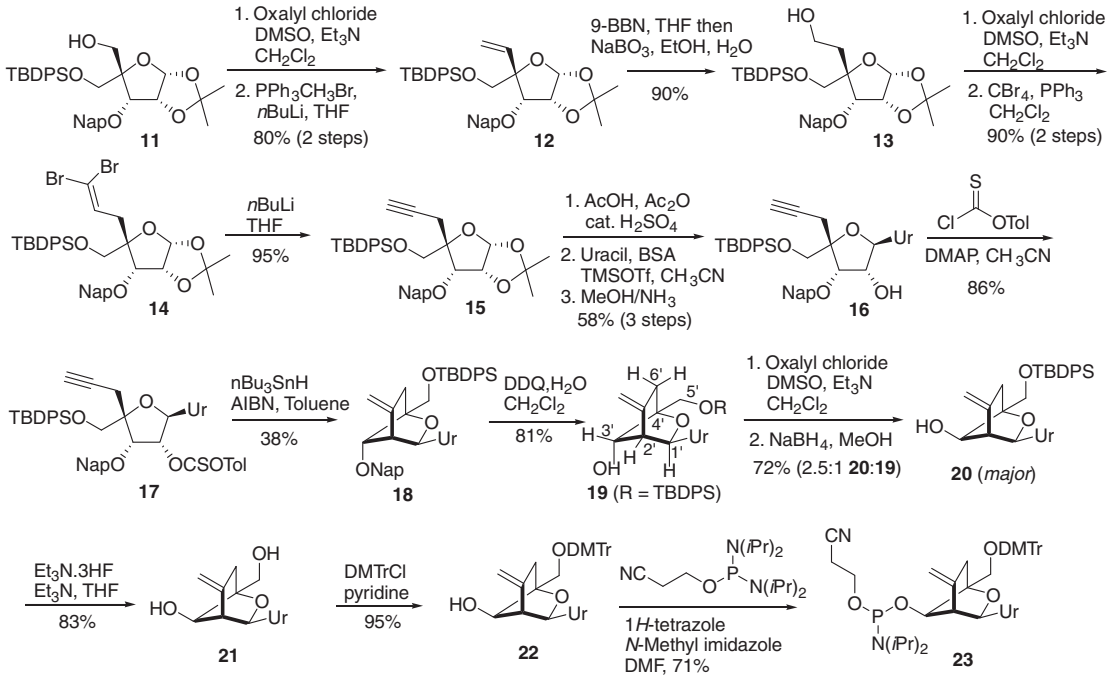
The identity of the cyclized nucleoside **20** was established by NMR spectroscopy. Consistent with the formation of a 2.2.1 bicyclo ring system, the 1′-H and 2′-H appear as singlets in nucleosides **19** and **20**. Further evidence of inversion of configuration of the 3′-hydroxyl group was obtained by examination of the chemical shifts of the 1′H. This proton appears more downfield in **19** (6.3 ppm) as compared to **20** (5.9 ppm) as a result of deshielding effect of the axial 3′-hydroxyl in **19**. Conversely, the chemical shift of the one of the 6′H is more downfield (2.7 ppm) in **20** as compared to **19** (2.4 ppm), presumably because of proximity to the 3′-hydroxyl group after the inversion. Lastly, NOESY crosspeaks were observed between the 1′-H and the 3′-H in nucleoside **20** but not in nucleoside **19**.

The α-L-methylene-cLNA phosphoramidite **23** was incorporated into oligonucleotides using standard phosphoramidite chemistry. The syntheses were carried out at 2 μm scale using deoxythymidine loaded CPG support and 10 equiv of the modified amidite (5 min coupling cycle; ~75% yield coupling yield for the modified amidite). Two oligonucleotide sequences were prepared with one (**A2**) and two tandem incorporations (**A3**) of the modified nucleoside and evaluated in thermal denaturation experiments (Table 1). The α-L-methylene-cLNA modification was found to be

Table 1
Thermal stability measurements of α-L-methylene-cLNA, methylene-cLNA, and α-L-LNA modified oligonucleotides versus RNA complements

Oligomer	Mass calcd	Mass observed	% UV purity	Modification	Sequence[a] (5′–3′)	T_m[b] (°C) versus RNA	ΔT_m (°C)/mod.
A1	3633.4	3632.9		DNA[c]	d(GCGTTTTTTGCT)	45.6	0
A2	3657.4	3656.7	98.3	α-L-Methylene-cLNA	d(GCGTT**U**TTTGCT)	35.5	−10.1
A3	3681.4	3681.4	97.7	α-L-Methylene-cLNA	d(GCGTT**UU**TTGCT)	32.9	−6.4
A4	3647.4	3646.5	97.8	α-L-LNA	d(GCGTT**U**TTTGCT)	50.2	+4.6
A5	3661.3	3660.8	97.9	α-L-LNA	d(GCGTT**UU**TTGCT)	54.7	+4.6
A6	3657.4	3656.7	98.0	Methylene-cLNA	d(GCGTT**U**TTTGCT)	50.4	+4.9
A7	3647.4	3646.8	98.1	LNA	d(GCGTT**U**TTTGCT)	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] DNA olignucleotides were purchased from commercial vendors and used as supplied.



Scheme 1. Synthesis of α-L-methylene-cLNA uridine phosphoramidite.

extremely destabilizing (ΔT_m −10.1 °C/mod. for **A2** and −6.4 °C/mod. for **A3**) in both the sequences evaluated even relative to DNA. This was surprising considering that methylene-cLNA **A6** (ΔT_m +4.9 °C/mod.) actually improves binding affinity slightly relative to LNA **A7** (ΔT_m +4.6 °C/mod.).

To understand this divergent behavior in the α-L-series relative to the β-D series, we modeled the exocyclic methylene group into the published structure of a α-L-LNA modified DNA/RNA duplex (Fig. 3).[20] The structural model indicated that, because the exocyclic methylene group in the α-L-series lies in the major groove, it creates a steric clash with the nucleobase of the 3′-flanking nucleotide. These types of steric interactions are missing in the β-D series where the 2′,4′-bridging substituent is located in the minor groove. Based on this structural model, one could anticipate that introducing larger groups in place of the 2′-oxygen atom of α-L-LNA will result in destabilization of oligonucleotide duplexes and most likely explains the poor hybridization behavior of *N*-alkyl α-L-2′-amino-LNA[24,25] and the α-L-cLNA[22] analogs reported recently. However, the model also indicates that introducing substitutents in the *R*-configuration on the bridging methylene unit of the α-L-LNA scaffold is likely to be tolerated.

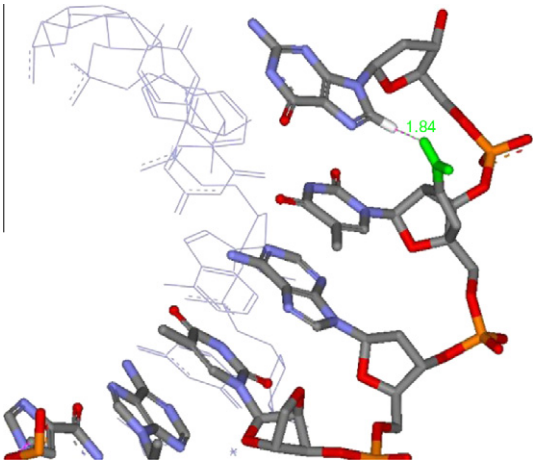


Figure 3. Structure of an α-L-LNA modified DNA/RNA duplex with an exocyclic methylene group (green) modeled into the structure. One of the methylene hydrogen is within 2 Å of H-8 of a deoxyadenine nucleotide 3′ to the modified residue. Complementary strand is shown in blue.

It should be noted that in the T_m sequence used for this study, the α-L-LNA modified nucleotide has a uracil nucleobase and the 3′-nucleotide is deoxythymidine. In contrast, the α-L-LNA modified nucleotide has a thymine nucleobase and the 3′-nucleotide is deoxyguanosine in the published structure used for constructing the model. However, since the overall topology of the duplex structure is determined primarily by the sugar-phosphate backbone, the changes in base composition should not affect the conclusions derived from the structural model. In fact, one can expect that the steric interaction of the exocyclic methylene group is likely to be worse when the 3′-nucleotide has a pyrimidine base since the six-membered ring will position the H-6 proton even closer to the exocyclic methylene group of the modified nucleotide.

In conclusion, we show that replacing the 2′-oxygen with an exocyclic methylene group in α-L-LNA disrupts hybridization. This behavior is opposite to that observed in the β-D series, where an exocyclic methylene group was well tolerated. A structural model based on the published structure of an α-L-LNA/RNA duplex indicated that the exocyclic methylene group results in a steric clash with the nucleobase 3′ to the modified residue. This most likely prevents the oligonucleotide from assuming the desired conformation required for hybridization. Based on this structural model one could anticipate that replacing the 2′-oxygen atom of α-L-LNA with larger groups will be detrimental to duplex stability. The model also provides insights into what type of 2′,4′-bridges are most likely to be tolerated in α-L-LNA modified oligonucleotide duplexes. These hypotheses are currently being investigated experimentally and the results will be reported in due course.

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