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Carbohydrate-Functionalized Locked Nucleic Acids: Oligonucleotides with Extraordinary Binding Affinity, Target Specificity, and Enzymatic Stability

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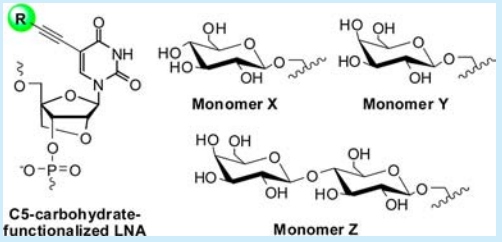
Supporting Information

ABSTRACT: Three different C5-carbohydrate-functionalized LNA uridine phosphoramidites were synthesized and incorporated into oligodeoxyribonucleotides. C5-Carbohydrate-functionalized LNA display higher affinity toward complementary DNA/RNA targets (ΔT_m/modification up to +11.0 °C), more efficient discrimination of mismatched targets, and superior resistance against 3′-exonucleases compared to conventional LNA. These properties render C5-carbohydrate-functionalized LNAs as promising modifications in antisense technology and other nucleic acid targeting applications.



Chemically modified oligonucleotides are widely used in molecular biology, biotechnology, and biomedical sciences to alter gene expression and detect specific nucleic acid targets.[1] Incorporation of conformationally restricted nucleotides is a particularly popular strategy to increase the binding affinity of oligonucleotides.[2,3] LNA[4,5] (locked nucleic acid, Figure 1), also known as bridged nucleic acid (BNA),[6] is one of the most promising members of this compound class, as it results in some of the largest increases in thermal denaturation temperatures (T_m's) of duplexes reported thus far. Moreover, LNA-modified oligonucleotides display moderately improved target specificity and enzymatic stability[7] and have accordingly been used extensively in RNA-targeting applications.[8] The promising characteristics of LNA has fuelled substantial efforts to develop next-generation LNA monomers with even more desirable biophysical characteristics.[2,3,9]

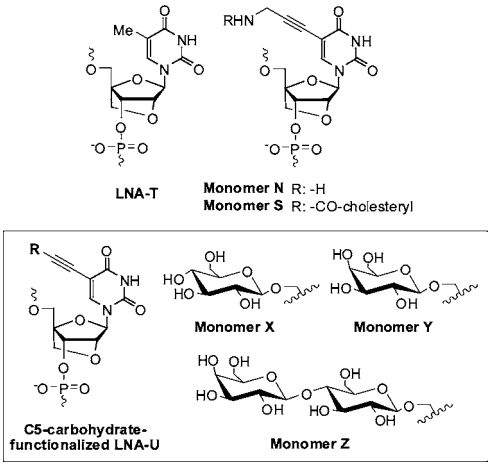


Figure 1. Chemical structures of LNA-T and C5-alkynyl-functionalized LNA-U monomers discussed herein.

As part of our ongoing interest in LNA chemistry,[2e,10] we recently set out to synthesize and study oligodeoxyribonucleotides (ONs) modified with various C5-alkynyl-functionalized LNA uridine (U) monomers.[11] The thermostabilities of the corresponding duplexes with complementary DNA/RNA strongly depend on the nature of the C5-alkynyl group. For example, ONs that are modified with C5-aminopropynyl-functionalized LNA-U monomer **N** (Figure 1) display significantly higher affinity toward DNA/RNA targets and moderately improved protection against snake venom phosphordiesterase (SVPDE, a 3′-exonuclease) compared to unmodified ONs or conventional LNA (duplex T_m's are increased by 6.5−13.0 °C and 2.5−3.5 °C per modification, relative to unmodified ONs or conventional LNA, respectively).[11] In contrast, ONs modified with C5-cholesterol-functionalized LNA-U monomer **S** (Figure 1) display much lower affinity toward DNA/RNA targets but are essentially inert toward SVPDE-mediated degradation.[11]

Clearly, it would be attractive to develop antisense building blocks that display very high target affinity *and* impart complete resistance against nucleases upon incorporation into ONs. At the onset of the present study, we hypothesized that conjugation of bulky yet polar groups such as mono- or disaccharides to the C5-position of LNA-U monomers would yield building blocks with such characteristics. Our hypothesis was based on the assumption that the steric bulk of carbohydrates would impart protection against nucleases, while their polar nature would allow them to be positioned in the major groove without exhibiting the same detrimental effects on duplex stability as large hydrophobic C5-substituents. Conjugation of carbohydrate moieties on LNA

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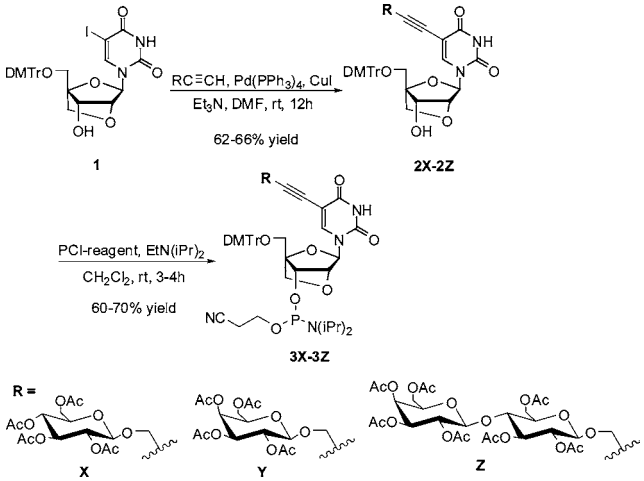
nucleotides would also be interesting from a pharmacokinetic perspective, as antisense ONs linked to glycoclusters are known to display improved cellular uptake through receptor-mediated endocytosis.[12]

Here, we describe the synthesis and biophysical characterization of ONs modified with C5-carbohydrate-functionalized LNA-U monomers **X**-**Z** (Figure 1). These monomers were chosen to study the influence of steric bulk (mono- vs disaccharides, monomers **X**/**Y** vs **Z**) and stereochemical configuration (glucose vs galactose configuration, monomers **X** vs **Y**).

Phosphoramidites **3X**−**3Z** were synthesized in a similar manner as other C5-alkynyl-functionalized LNA-U building blocks (Scheme 1).[11] Thus, glucose/galactose/lactose-functionalized alkynes[13] were coupled to known C5-iodo LNA-U nucleoside **1**[11] using Sonogashira conditions[14] to furnish **2X**−**2Y** in 62−66% yield, which upon treatment with 2-cyanoethyl-*N*,*N*′-diisopropylchlorophosporamidite and *N*,*N*′-diisopropylethylamine provided **3X**−**3Z** in 60−70% yield.

Scheme 1. Synthesis of C5-Carbohydrate-Functionalized LNA Phosphoramidites[a]



[a]DMTr: 4,4′-dimethoxytrityl chloride. PCl-reagent: 2-cyanoethyl-*N*,*N*′-diisopropylchlorophosporamidite.

Phosphoramidites **3X**−**3Z** were incorporated into ONs via machine-assisted solid-phase DNA synthesis using extended hand-coupling (20 min, 5-(ethylthio)-1*H*-tetrazole as activator)), which resulted in stepwise coupling yields of ∼95%. ONs were purified by ion-pair reversed-phased HPLC, and their composition was verified by MALDI MS analysis (Table S1, Supporting Information). The hybridization characteristics of ONs modified with one or two **X**−**Z** monomers were studied in 9-mer mixed-sequence contexts (Table 1). The T_m's of duplexes between C5-carbohydrate-functionalized LNA and complementary DNA/RNA (cDNA/cRNA) were determined by thermal denaturation experiments using medium salt phosphate buffers.

All of the modified ONs form duplexes that are significantly more thermostable than unmodified reference duplexes, suggesting that the bulky polar carbohydrate units are well-tolerated in the major groove of nucleic acid duplexes. Invariably, greater relative increases in T_m's are observed with RNA targets (ΔT_m = 4.5−8.5 °C and 8.0−11.0 °C for **B1**−**B3** series with cDNA and cRNA, respectively, Table 1). Duplexes modified with monosaccharide-functionalized monomers **X**/**Y** are up to 4.0 °C more thermostable than duplexes modified with lactose-conjugated monomer **Z** (compare ΔT_m's for **X**/**Y**- vs **Z**-series, Table 1). In most cases, **Y**-modified ONs display slightly higher target affinity than **X**-modified ONs, suggesting that the stereochemical configuration of the C4-position in the carbohydrate plays a small role in determining duplex thermostability. Incorporation of a second **X**−**Z** monomer as a next-nearest neighbor results in additional stabilization of the duplexes although the increases in T_m per modification are slightly lower than for singly modified duplexes (compare ΔT_m's for **B2**/**B3**- vs **B4**-series, Table 1). Interestingly, ONs modified with C5-carbohydrate-functionalized LNA-U monomers display similar

Table 1. Thermal Denaturation Temperatures for Duplexes between C5-Carbohydrate-Functionalized LNA and cDNA/cRNA[a]

			ΔT_m (°C)				
ON	sequence	**B** =	**L**[b]	**N**[b]	**X**	**Y**	**Z**
B1	5′-GTG A**B**A TGC		+5.0	+8.0	+6.0	+7.0	+5.5
D2	3′-CAC TAT ACG						
D1	5′-GTG ATA TGC		+4.0	+6.5	+5.5	+8.5	+4.5
B2	3′-CAC **B**AT ACG						
D1	5′-GTG ATA TGC		+6.5	+9.5	+7.0	+5.5	+4.5
B3	3′-CAC TA**B** ACG						
D1	5′-GTG ATA TGC		+5.5	+8.0	+5.8	+5.8	+3.8
B4	3′-CAC **B**A**B** ACG						
B1	5′-GTG A**B**A TGC		+9.5	+13.0	+10.0	+11.0	+8.5
R2	3′-CAC UAU ACG						
R1	5′-GUG AUA UGC		+6.5	+10.0	+9.0	+11.0	+9.0
B2	3′-CAC **B**AT ACG						
R1	5′-GUG AUA UGC		+9.5	+12.5	+9.0	+9.0	+8.0
B3	3′-CAC TA**B** ACG						
R1	5′-GUG AUA UGC		+8.0	+11.0	+8.3	+8.5	+7.0
B4	3′-CAC **B**A**B** ACG						

[a]ΔT_m = change in T_m's relative to unmodified reference duplexes **D1**:**D2** ($T_m \equiv$ 29.5 °C), **D1**:**R2** ($T_m \equiv$ 27.0 °C) and **D2**:**R1** ($T_m \equiv$ 27.0 °C); T_m's determined as the first derivative maximum of denaturation curves (A_{260} vs T) recorded in medium salt phosphate buffer ($[Na^+]$ = 110 mM, $[Cl^-]$ = 100 mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4)), using 1.0 μM of each strand. T_m values are averages of at least two measurements within 1.0 °C. [b]Data from ref 11.

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Table 2. Discrimination of Singly Mismatched DNA/RNA Targets by C5-Carbohydrate-Functionalized LNAs and Reference Strands[a]

		DNA: 3′-CAC T**B**T ACG				RNA: 3′-CAC U**B**U ACG			
		T_m (°C)	ΔT_m (°C)			T_m (°C)	ΔT_m (°C)		
ON	**B** =	A	C	G	T	A	C	G	U
D1		**29.5**	−16.5	−8.0	−15.5	**27.0**	−17.0	−4.5	−17.0
L1[b]		**34.5**	−18.0	−11.0	−16.0	**36.5**	−19.0	−8.0	−18.5
N1[b]		**37.5**	−19.0	−12.0	−17.5	**40.0**	−18.5	−11.5	−22.5
X1		**35.5**	−19.5	−13.0	−20.0	**37.0**	−19.0	−12.5	−21.5
Y1		**36.5**	−20.5	−15.0	−20.0	**38.0**	−20.0	−11.0	−22.0
Z1		**35.0**	−20.5	−25.0	−25.0	**35.5**	−25.5	−25.5	−25.5

[a]For conditions of thermal denaturation experiments, see Table 1. T_m's of fully matched duplexes are shown in bold. ΔT_m = change in T_m relative to fully matched duplexes (**B** = A). [b]Data from ref 11.

affinity toward DNA/RNA targets as ONs modified with conventional LNA, and only slightly lower target affinities than **N**-modified ONs (Table 1).

The binding specificities of centrally modified C5-carbohydrate-functionalized LNAs were determined using DNA/RNA targets with mismatched nucleotides opposite of the modification (Table 2). Importantly, **X1**/**Y1**/**Z1** display significantly improved mismatch discrimination relative to unmodified reference strand **D1** and similar, if not improved, binding specificity relative to ONs modified with conventional LNA-T or C5-aminopropynyl LNA monomer **N** (Table 2).

Lastly, we studied the enzymatic stability of **D2**/**X2**/**Y2**/**Z2** in the presence of snake venom phosphordiesterase by recording the change in absorbance at 260 nm as a function of time (Figure 2). As expected, unmodified reference strand **D2** is quickly

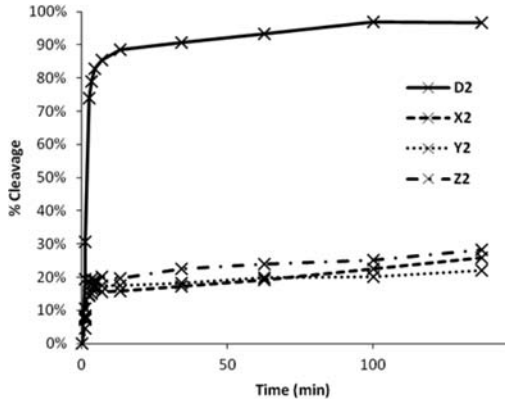

Figure 2. 3′-Exonuclease degradation of singly modified C5-carbohydrate-functionalized LNA and reference strands (**B2** series). Nuclease degradation studies were performed in magnesium buffer (50 mM Tris-HCl, 10 mM Mg^{2+}, pH 9.0) using 3.3 μM ONs and 0.03 U of snake venom phosphodiestaerase.

degraded (~90% degradation within ~10 min, Figure 2). Gratifyingly, C5-carbohydrate-functionalized LNA **X2**/**Y2**/**Z2** are essentially inert against SVPDE-mediated degradation, once an initial ~5 min period of cleavage, corresponding to degradation of the 3′-terminal deoxyribonucleotides, has elapsed (see degradation profiles for **X2**/**Y2**/**Z2**, Figure 2). Pseudo-first-order rate constants, determined for the first ~5 min of SVPDE-mediated degradation of ONs, are 6- to 8-fold lower for **X2**/**Y2**/**Z2** than for **D2** (Figure S3 and Table S2, Supporting Information). This strongly suggests that the SVPDE-inhibitory influence of the bulky carbohydrate moieties extends to 3′-flanking deoxyribonucleotides.

In conclusion, a series of C5-carbohydrate-functionalized LNA-U phosphoramidites have been synthesized and incorporated into ONs. The modified ONs display extraordinary affinity toward complementary DNA/RNA targets, binding specificity and resistance against 3′-exonucleases. These properties render C5-carbohydrate-functionalized LNAs as promising agents for antisense technology and other nucleic acid targeting applications. Their potential as mRNA knockdown agents and splice-switching ONs[15] will be evaluated in the near future.

■ ASSOCIATED CONTENT

Supporting Information

General experimental section; experimental protocols; MS data of modified ONs; representative thermal denaturation curves; additional enzymatic degradation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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