

C5-Amino acid functionalized LNA: positively poised for antisense applications†

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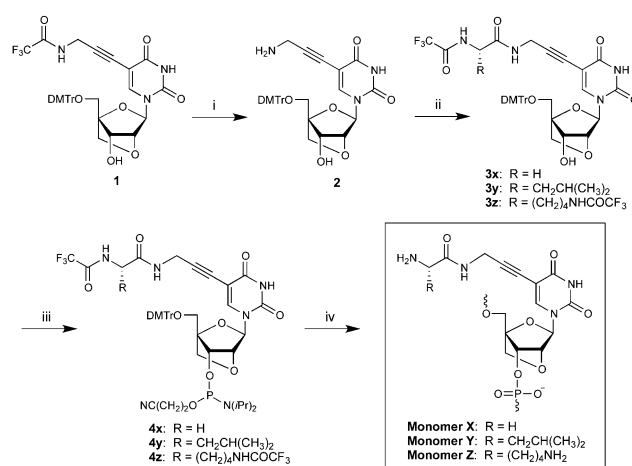
Incorporation of positively charged C5-amino acid functionalized LNA uridines into oligodeoxyribonucleotides (ONs) results in extraordinary RNA affinity, binding specificity and stability towards 3'-exonucleases.

Modulation of gene expression by antisense-based approaches continues to stimulate the development of novel chemically modified nucleotides in search of oligonucleotides with improved RNA affinity, binding specificity, and pharmacokinetic profiles.¹ Of the many modifications studied for antisense applications, conformationally restricted nucleotides,² and locked nucleic acid (LNA) in particular,³ have emerged as particularly promising chemistries toward this end. Over the past 15 years, major efforts have been devoted to increase the chemical diversity of LNA nucleotides through additional modification of the sugar ring, with the aim of improving properties for antisense applications.^{3,4} Modification of the nucleobase of LNA, on the other hand, is an underexplored strategy for optimization of pharmacodynamic and pharmacokinetic properties.

Modification of the C5-position of pyrimidines is particularly interesting due to synthetic feasibility and functional group tolerance, as attached moieties are directed into the major groove with minimal perturbation of the duplex structure.^{5,6} We have previously shown that attachment of small hydrophilic moieties to the C5-position of LNA uridine (U) such as 3-aminopropyn-1-yl promotes even greater target affinity than canonical LNA, while attachment of large hydrophobic substituents such as cholesterol confers complete stability against exonucleases, albeit at the expense of decreased target affinity.⁷ At the onset of the present study, we hypothesized that hydrophilic moieties of intermediate size would contribute to both additionally improved affinity and nuclease stability. Furthermore, positively charged moieties often

improve cellular uptake through electrostatic interactions.^{8,9} Amino acids are a class of molecules that fit the size, hydrophilicity and charge criteria.¹⁰ Driven by the desire to exploit the properties of both LNA and basic amino acid residues, we report the synthesis of three C5-amino acid functionalized LNA-U phosphoramidites and the biophysical characterization of ONs modified with these units.

Synthesis of phosphoramidites **4x/y/z** initiates from known nucleoside **1** (Scheme 1).⁷ Deprotection of the trifluoroacetamide (TFA) group using saturated methanolic ammonia reveals the aminopropynyl group at the C5-position of **2** in 97% yield.‡ TSTU-mediated coupling of TFA-protected glycine, leucine or lysine gives **3x/y/z** in moderate yields (48–58%).§ Subsequent O3'-phosphitylation affords phosphoramidites **4x/y/z**, which were used for incorporation of monomers **X/Y/Z** into ONs by automated DNA solid-phase synthesis (15 min, 4,5-dicyanoimidazole as activator, average coupling yield > 90%). The composition and



Scheme 1 Reagents and conditions: (i) sat. NH₃/MeOH (97%); (ii) TFA-protected amino acid, *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU), *N,N*-diisopropylethylamine (DIPEA), DMF (48–58%); (iii) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, DIPEA, CH₂Cl₂ (37–64%); (iv) DNA synthesis. DMTr = 4,4'-dimethoxytrityl.

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† Electronic supplementary information (ESI) available: Synthetic protocols, NMR spectra and MS data for new nucleosides and ONs; thermal denaturation curves; additional *T_m* and thermodynamic data; sequences of AONs. See DOI: 10.1039/c4cc03623a



Table 1 T_m 's of duplexes between **B1–B4**-series and complementary RNA^a

ON	Sequence	$\Delta T_m/\text{mod}$				
		B = L^b	B = N^b	B = X	B = Y	B = Z
B1	5'-GTG ABA TGC	+8.5	+13.0	+10.5	+9.5	+12.5
B2	3'-CAC BAT ACG	+5.5	+10.0	+10.5	+10.5	+11.0
B3	3'-CAC TAB ACG	+8.5	+12.5	+10.0	+7.0	+14.0
B4	3'-CAC BAB ACG	+7.5	+11.0	+10.8	+9.3	+13.0

^a $\Delta T_m/\text{mod}$ = change in T_m per modification relative to unmodified reference duplex (**R1:D2** or **D1:R2** both $T_m = 28.0$ °C); **R1**: 5'-GUG AUA UGC; **R2**: 3'-CAC UAU ACG; T_m 's determined as the maximum of the first derivative of the melting curve (A_{260} vs. T) recorded in medium salt phosphate buffer ($[\text{Na}^+] = 110$ mM, $[\text{Cl}^-] = 100$ mM, pH 7.0 ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$)), using 1.0 μM of each strand. Monomer **L** = LNA-T. Monomer **N** = C5-(3-aminopropyn-1-yl)-LNA-U. ^b Previously reported.⁷

purity of all modified ONs was ascertained by MALDI-MS analysis (Table S1, ESI[†]) and ion-pair reversed-phase HPLC, respectively.

ONs modified with C5-amino acid functionalized LNA-U monomers **X/Y/Z** display higher affinity toward complementary RNA than ONs modified with canonical LNA thymidine (monomer **L**) (Table 1). Incorporation of glycine-conjugated monomer **X** consistently increases the thermal denaturation temperatures (T_m 's) of 9-mer mixed-sequence model duplexes by 10–11 °C, while leucine-conjugated monomer **Y** results in slightly less thermostable duplexes ($\Delta T_m = +7.0$ to +10.5 °C). ONs containing lysine-conjugated monomer **Z** display the highest RNA affinity in this series ($\Delta T_m = +11.0$ to +14.0 °C). In fact, **Z** is as affinity-enhancing as C5-aminopropynyl LNA-U (monomer **N**).⁷ Similar trends are observed towards DNA targets, although slightly less pronounced stabilization is observed (Table S2, ESI[†]).

Thermodynamic parameters for duplex formation were determined from denaturation curves through curve fitting (Table S3, ESI[†]).¹¹ Changes in free energy (ΔG) follow similar trends as T_m 's. Thus, ONs containing monomers **X/Y/Z** show greater affinity toward RNA than DNA targets (RNA: $\Delta G^{298} = -8$ to -23 kJ mol⁻¹; DNA: $\Delta G^{298} = -5$ to -17 kJ mol⁻¹), and greater target affinity than ONs modified with LNA-T. The additional stabilization provided by the C5-amino acid functionalized LNA monomers is a result of more favorable enthalpy. This is likely due to extended π -conjugation of the nucleobase and improved stacking within the duplex,^{6b} as well as electrostatic screening of the negatively charged duplex by protonated amino acid residues.^{6c–g} The latter is a reasonable assumption considering that the pK_a for α -amino groups of free amino acids and the ε -amino group of lysine is >9.16 and 10.67 , respectively.¹²

To study in greater detail if protonation of the amino acid residues of monomers **X/Y/Z** contributes to duplex stabilization, thermal denaturation profiles were recorded in buffers of decreasing ionic strength (110, 40, and 10 mM sodium phosphate buffer).^{6a} As expected, lower absolute T_m 's are observed at low ionic strengths due to decreased electrostatic shielding of the two negatively charged duplex strands (Tables S4 and S5, ESI[†]). Interestingly, ONs modified with monomers **X/Y/Z** show greater relative affinity increases toward RNA and DNA targets at lower ionic strengths as evidenced by the trend in ΔT_m 's (Fig. 1 and Fig. S2, ESI[†]). For example, the ΔT_m for the duplex

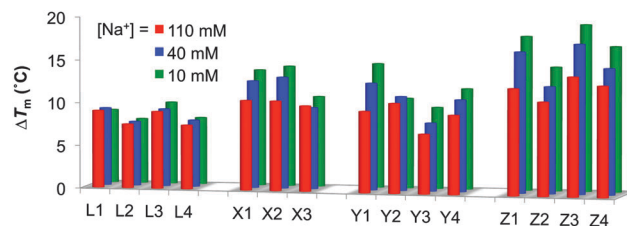


Fig. 1 Thermostabilities of duplexes between **B1–B4**-series and RNA at different ionic strengths. ΔT_m = change in T_m 's relative to unmodified reference duplex **D1:R2** ($T_{m,110\text{mM}} = 28.0$ °C, $T_{m,40\text{mM}} = 21.0$ °C, $T_{m,10\text{mM}} = 11.5$ °C); **R1:D2** ($T_{m,110\text{mM}} = 28.0$ °C, $T_{m,40\text{mM}} = 22.0$ °C, $T_{m,10\text{mM}} = 12.0$ °C). Buffer conditions: $[\text{Na}^+] = 110$ mM and $[\text{Cl}^-] = 100$ mM; $[\text{Na}^+] = 40$ mM and $[\text{Cl}^-] = 30$ mM; $[\text{Na}^+] = 10$ mM, pH 7.0 ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) in all cases. See also Table S4 (ESI[†]).

between lysine-modified **Z1** and complementary RNA is 12.5 °C and 18.0 °C at $[\text{Na}^+] = 110$ mM and 10 mM, respectively (Table S4, ESI[†]). These effects are much weaker for LNA-modified ONs (**L1–L4**), which strongly suggests that the C5-amino acid moieties of monomers **X/Y/Z** indeed are protonated and contribute to duplex stability.^{6a}

The specificity of ONs modified with C5-amino acid functionalized LNA-U monomers **X/Y/Z** was evaluated by measuring the T_m 's of duplexes between modified ONs and RNA or DNA containing a mismatched nucleotide either directly across from the central modification (**B1**-series) or 2'-deoxyadenosine (**B4**-series). Just like LNA-T (**L1**) or C5-aminopropynyl modified (**N1**), **X1/Y1/Z1** show comparable or improved discrimination of mismatched RNA and DNA relative to **D1** (Table 2 and Table S6, ESI[†]). Discrimination of the challenging T/U:G mismatch is particularly effective. Doubly modified **X4/Y4/Z4** also discriminate mismatched RNA targets well, whereas discrimination of mismatched DNA targets is far less efficient (Table S7, ESI[†]). The latter may be a result of stabilizing non-specific electrostatic interactions occurring in DNA duplexes.

ONs with C5-amino acid functionalized LNA-U monomers positioned close to the 3'-terminus (**X2/Y2/Z2**) were evaluated for nuclease stability using snake venom phosphodiesterase (SVPDE), a 3'-exonuclease (Fig. 2). As expected, unmodified DNA (**D2**) is completely cleaved within 40 min. Incorporation of a single LNA-T (**L2**) still results in complete digestion, although at a significantly slower rate. Satisfyingly, **X2/Y2/Z2** were completely stable to SVPDE once degradation of the 2–3 terminal 2'-deoxyribonucleotides

Table 2 Thermal discrimination of mismatched RNA targets by **B1**-series^a

ON	Sequence	RNA: 3'-CAC UMU ACG			
		T_m (°C)	ΔT_m (°C)		
		M = A	M = C	M = G	M = U
D1	5'-GTG ATA TGC	28.0	< -18.0	-5.5	< -18.0
L1^b	5'-GTG ALA TGC	36.5	-20.0	-9.0	-19.5
N1^b	5'-GTG ANA TGC	41.0	-18.5	-11.5	-22.5
X1	5'-GTG AXA TGC	38.5	-17.0	-11.5	-19.0
Y1	5'-GTG AYA TGC	37.5	-16.0	-9.0	-19.0
Z1	5'-GTG AZA TGC	40.5	-17.0	-11.5	-19.0

^a ΔT_m = change in T_m relative to fully matched **B1:R2** duplex (**M = A**) shown in bold. ^b Previously reported.⁷



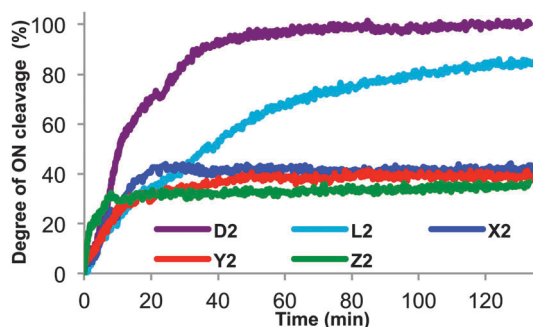


Fig. 2 Enzymatic degradation of **B2**-series by 3'-exonuclease (SVPDE) performed in magnesium buffer (50 mM Tris-HCl, 10 mM Mg^{2+} , pH 9.0) by using 3.3 μ M ONs and 0.52 μ g (0.03 U) of SVPDE at 37 °C. Degree of ON cleavage calculated as the change in absorbance at 260 nm with respect to unmodified DNA $[(A_{260}(\mathbf{B2}) - A_{260,initial}(\mathbf{B2})) / (A_{260,final}(\mathbf{D2}) - A_{260,initial}(\mathbf{D2}))]$.

had ceased, as evidenced by the plateau in their degradation profiles.

Encouraged by these results, we designed a fully phosphorothioated 3-14-3 gapmer antisense ON (ASO) targeting firefly luciferase mRNA (Table S8, ESI[†]). Very preliminary experiments using murine 3T3-L1 cells indicate that ASOs modified with two C5-lysine LNA-U Z monomers, one in each LNA wing, display similar knockdown efficiency (~50% at 80 nM), with no observable cell death, as the corresponding ASO with LNA-T when cationic transfection agents are used (results not shown). Additional studies are necessary to assess the full potential of C5-amino acid functionalized LNA-U monomers as ASO modifications, including their promise for enhanced and/or gymnotic ASO delivery.

In summary, C5-amino acid functionalized LNA uridine phosphoramidites represent a novel approach to chemical diversification of LNA. ONs containing monomers X/Y/Z display significantly higher affinity toward complementary RNA than canonical LNA ONs due to extended conjugation of the nucleobase, as well as stabilizing electrostatic interactions. The excellent specificity of LNA modified ONs towards singly mismatched RNA is maintained. Additionally, monomers X/Y/Z are inert towards enzymatic digestion by 3'-endonuclease (SVPDE), which sets them apart from other high-affinity C5-LNA-U monomers.⁷ The promising biophysical properties and cationic character render C5-amino acid functionalized LNA positively poised for antisense applications.

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Notes and references

† For an alternative synthesis of compound **2**, see ESI[†].

§ Coupling between Fmoc-protected amino acids and **2** was unsuccessful.

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