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Oligonucleotides Containing Aminated 2'-Amino-LNA Nucleotides: Synthesis and Strong Binding to Complementary DNA and RNA

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Abstract: Mono- and diaminated 2'-amino-LNA monomers were synthesized and introduced into oligonucleotides. Each modification imparts significant stabilization of nucleic acid duplexes and triplexes, excellent sequence selectivity and significant nuclease resistance. Molecular modelling suggested that structural stabilization occurs via intrastrand electrostatic attraction between the protonated amino groups of the aminated 2'-amino-LNA monomers and the host oligonucleotide backbone.

INTRODUCTION

Considerable efforts are currently invested in the application of artificial nucleic acids within biochemistry, biology, nanotechnology and medical science.[1-6] Importantly, applications like oligonucleotide(ON)-based diagnostics and therapy usually requires chemically modified ONs to orchestrate improved binding affinity, high selectivity and increased biostability, all relative to what is offered by unmodified DNA and RNA ONs.[7-9] This demand has inspired many modifications such as nucleoside surrogates and phosphate backbone derivatives to achieve those favorable properties.[10-13] In this context, the locked nucleic acids (LNA and 2'-amino-LNA, Fig. 1) represent notable examples in which the ribofuranose moieties are constrained in North-type (C3'-*endo*) conformation via an oxymethylene or azamethylene bridge between the C2' and C4' atoms.[14-16]

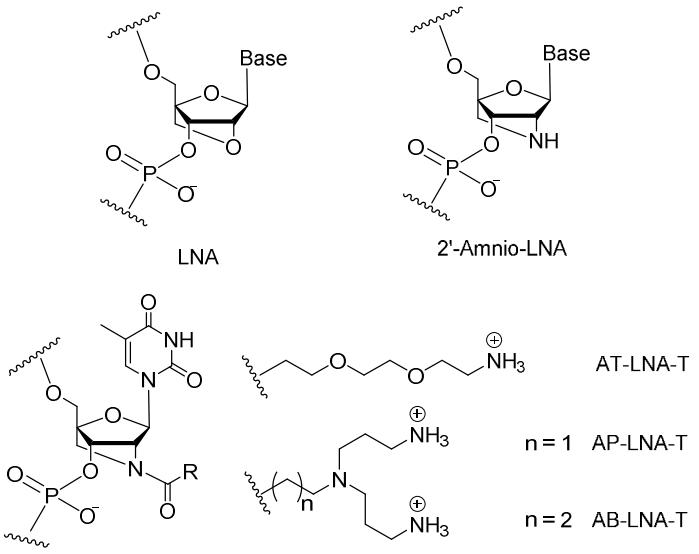


Figure 1. Structures of LNA, 2'-amino-LNA, AT-LNA-T, AP-LNA-T and AB-LNA-T nucleotide monomers.

Owing at least in part to their polyanionic nature, a main challenge when applying ONs for diagnostics and therapy is their limited cellular uptake.[4,11] One strategy to alleviate this problem has been to introduce positively charged groups.[17-24] For example, conjugation of an oligospermine to the 5'-end of an antisense ON or of one strand of an siRNA has been reported to induce the desired gene silencing activities.[25,26] In addition, introduction of piperazino-modified and glysino-modified amino-LNA-T nucleosides into bisLNAs enabled *in vitro* double strand invasion to an equal or even more significant level as to LNA-T.[27]

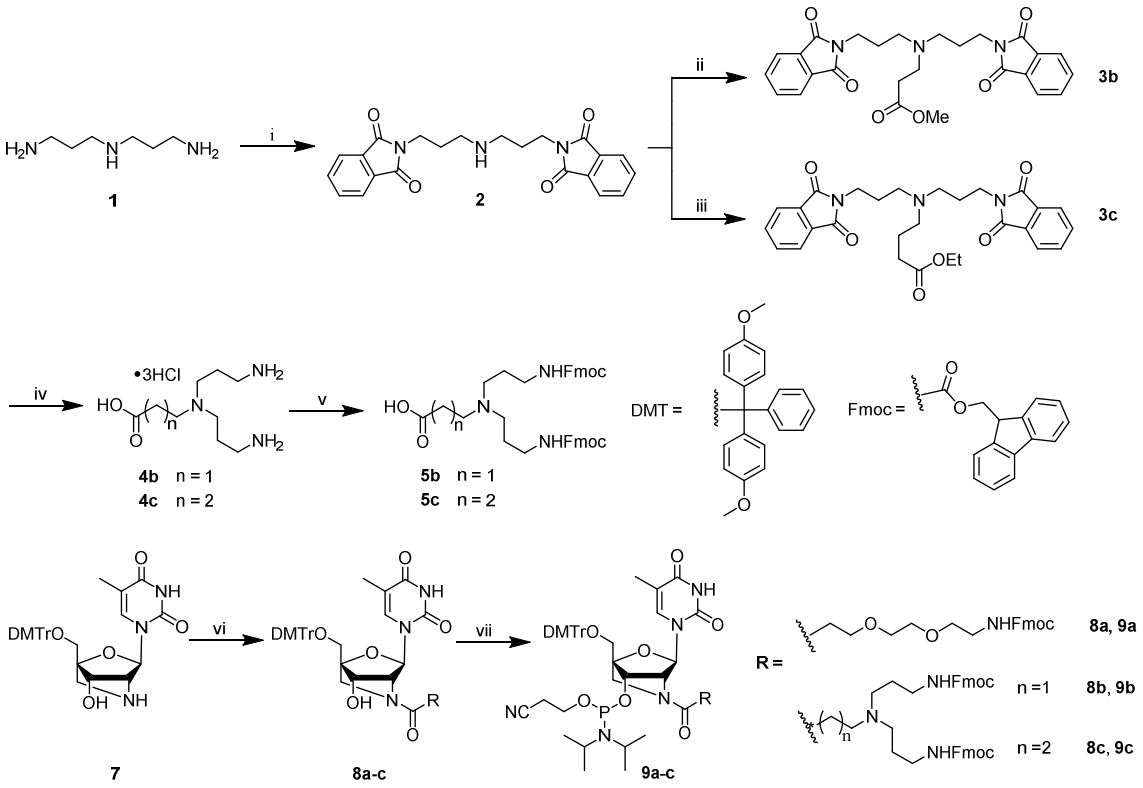
In order to combine high-affinity targeting and charge alleviation, we have attached norspermidine (pKa ~11)[28] via 2'-N-alkanoyl linkers to the 2'-amino-LNA-T monomer (monomers AP-LNA-T and AB-LNA-T, Fig. 1). We show below that both of these novel amino-LNA monomers induce high duplex and triplex stability, favourable mismatch discrimination and enhanced nuclease resistance. Molecular modelling demonstrated recurring electrostatic interactions between the protonated norspermidine moiety and the phosphate backbone, suggesting that backbone charge shielding is a key mechanism of structure stabilization. In parallel, a monoaminated analogue was also synthesized (AT-LNA-

T, Fig. 1) for comparison.

RESULTS and DISCUSSION

Synthesis of AT-LNA-T, AP-LNA-T and AB-LNA-T phosphoramidite monomers (**9a-9c**) was accomplished in two steps upon direct condensation between DMTr-protected 2'-amino-LNA-T (**7**)[29] and carboxy-functionalised precursors **5b**, **5c** and Fmoc-9-amino-4,7-dioxanonanoic acid (**6**) (Scheme 1). The latter is commercially available, while synthesis of precursors **5b** and **5c** followed a route similar to that described by Hujakka *et al.* with appropriate alterations in steps iii and v (Scheme 1).[30] Thus, the two primary amino groups of **1** were selectively protected using phthalic anhydride in glacial acetic acid to give **2**, which was further reacted with methyl acrylate in a Michael addition reaction or with ethyl 4-bromobutyrate in a nucleophilic substitution reaction to provide **3b** and **5c**, respectively. Full deprotection on **3b** and **3c** was performed with conc. hydrochloric acid to afford **4b** and **4c**, respectively, with two free primary amino groups and with the desired carboxy function. After neutralization using sodium hydroxide, subsequent attempts on Fmoc protection of the primary amino groups of **4b** and **4c** were unsuccessful following the published synthesis protocol.[30] Inspired by the well-developed NHS ester chemistry on NH_2-functionalized oligonucleotides,[31-33] Fmoc protection on **4b** and **4c** was efficiently achieved using Fmoc-OSu in a solvent system of acentonitrile and carbonate buffer (7:3) to produce **5b** and **5c**, respectively. The intermdiates **3c**, **4c** and **5c** are new compounds, whereas **2**, **3b**, **4b** and **5b** are known compounds.[30] 2'-Amino-LNA-T carboxylic groups of precursors **6**, **5b** and **5c** in the presence of 1-[bis(dimethylamino)methylene]-*1H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIPEA) to provide **8a**, **8b** and **8c**, respectively. These compounds were finally phosphitylated by reaction with 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphine to give the three desired phosphoramidite building blocks **9a**, **9b** and **9c**.

Scheme 1. **Synthesis of AT-LNA-T, AP-LNA-T and AB-LNA-T phosphoramidites 9a-c**[a]



[a]Reagents and conditions: (i) phthalic anhydride, glacial acetic acid, reflux, 1 h or 2 h, n.d. or 98%; (ii) methyl acrylate, RT 4 h, reflux, 4 h, 77% for two steps from **1**; (iii) ethyl 4-bromobutyrate, DIPEA, DMF, 70 °C, 168 h, 70%; (iv) conc. HCl, reflux, 24 h, n.d.; (v) 10% NaOH, carbonate buffer/MeCN, Fmoc-OSu, RT, 1 h or 2 h; 94% for two steps from **3b** to **5b**; 71% for two steps from **3c** to **5c**; (vi) Fmoc-9-amino-4,7-dioxanonanoic acid (**6**), HATU, DIPEA, DMF, RT, 1 h, 78%; **5b**, HATU, DIPEA, DMF, RT, 1 h, 75%; **5c**, HATU, DIPEA, DMF, RT, 2 h, 68%; (vii) 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphine, DIPEA, DCM, 1 h or 2 h, RT, **9a** 61%, **9b** 60%, **9c** 92%. (n.d. means that the yield was not determined).

As expected, Fmoc protection of the primary amino groups was compatible with standard solid-phase oligonucleotide synthesis,[33,34] and phosphoramidite monomers **9a**, **9b** and **9c** were efficiently incorporated into ONs by so-called hand-couplings[35] in stepwise coupling yields 99%, 97% and 95%, respectively. Diethylamine treatment is indispensable before cleavage of the ONs from solid support to ensure initial removal of the cyanoethyl group thus eliminating the risk of attack from deprotected free amino groups on acrylonitrile upon treatment with aqueous ammonia. Interestingly, the standard deprotection and cleavage protocol (28% aqueous ammonia, 55 °C, 16 h) was not suitable for AP-LNA-T-containing ONs, as the

desired ONs were found not to be the major product components after such treatment. For example, MALDI-TOF MS after attempted synthesis of single-modified **ON4** showed a dominant peak which was ~115 Daltons lower than the calculated molecular weight of **ON4**. It is hypothesized that the bis(3-aminopropyl)amino moiety of the AP-LNA-T monomer was replaced by a primary amine via a reverse-Michael addition reaction followed by re-addition of ammonia. This issue was resolved using a milder deprotection condition (aqueous ammonia, room temperature, 14 h), leading to the desired ONs as major products. Reasonably, when an extra methylene unit was introduced to incorporate monomer AB-LNA-T, ON synthesis was fully compatible with standard deprotection and cleavage condition strongly supporting the proposed reverse-Michael addition pathway as described above. After DMTr-ON reversed-phase HPLC and/or ion-exchange HPLC purification, the composition and purity (>85%) of modified ONs were confirmed by MALDI-MS and ion-exchange HPLC analysis, respectively.

Each monomer (AP-LNA-T, AB-LNA-T and AT-LNA-T) was incorporated into a 9-mer sequence and their binding affinity was evaluated towards complementary DNA and RNA strands (Table 1). Both single (**ON3**, **ON4** and **ON5**) and multiple modifications (**ON7**, **ON8** and **ON10**) were investigated using ONs containing the corresponding DNA-T (**ON1**) and 2'-amino-LNA-T monomers (**ON2**, **ON6** and **ON9**) as controls. The endeavour to synthesize ONs containing three poly-aminated monomers (AP-LNA-T or AB-LNA-T) failed, but incorporation of two modifications (**ON7** and **ON8**) was successful. When the central DNA-T was substituted by one of the three aminated monomers (**ON3**, **ON4** and **ON5**), remarkably increased duplex stabilities were observed in DNA/DNA contexts. Compared to DNA-T and 2'-amino-LNA-T controls, up to 8.5 °C and 4.5 °C increase in melting temperature was observed respectively, with the bisaminated monomers AP-LNA-T and AB-LNA-T displaying the most significant increases. Except for **ON4** and **ON5**, the most significant

increases in thermal denaturation temperatures were observed against complementary RNA which is consistent with previous results for other 2'-amino-LNA-T monomers.[14]

Incorporation of three AT-LNA-T nucleosides (**ON10**) enhanced duplex stability significantly more than single incorporation (**ON3**) regardless of the target strand being DNA or RNA. For AP-LNA-T and AB-LNA-T it is evident that the position of the monomer plays a vital role on DNA:DNA duplex stability, with singly-modified ONs (central, **ON4** and **ON5**) conferring almost equal duplex stability to that of the corresponding doubly-modified ones (**ON7** and **ON8**). This latter result, however, represent an exception and after changing to low salt experimental condition, higher stabilization for two inclusions than for one was observed. These binding data are consistent with a favorable electrostatic interaction that becomes less shielded at lower ionic strength (see modeling section below).

Table 1. Thermal Denaturation Temperatures of Duplexes[a]

Sequence	X		Complementary DNA		Complementary RNA	
			Medium Salt	Low salt	Medium Salt	Low salt
5'-GTGATATGC	-	ON1	32.0	17.5	29.5	15.5
5'-GTGA**X**ATGC	2'-amino-LNA-T	ON2	36.0 (+4.0)	22.5 (+5.0)	38.0 (+8.5)	23.5 (+8.0)
	AT-LNA-T	ON3	39.5 (+7.5)	26.5 (+9.0)	40.0 (+10.5)	26.5 (+11.0)
	AP-LNA-T	ON4	40.5 (+8.5)	30.0 (+12.5)	38.5 (+9.0)	26.5 (+11.0)
	AB-LNA-T	ON5	40.5 (+8.5)	30.5 (+13.0)	38.0 (+8.5)	27.0 (+11.5)
5'-G**X**GATA**X**GC	2'-amino-LNA-T	ON6	36.0 (+4.0)	22.0 (+ 4.5)	42.5 (+13.0)	27.5 (+12.0)
	AP-LNA-T	ON7	41.5 (+9.5)	34.0 (+16.5)	42.0 (+12.5)	33.0 (+17.5)
	AB-LNA-T	ON8	41.0 (+9.0)	34.5 (+17.0)	42.5 (+13.0)	35.0 (+19.5)
5'-G**X**GA**X**A**X**GC	2'-amino-LNA-T	ON9	41.0 (+9.0)	27.0 (+9.5)	49.5 (+20.0)	35.0 (+19.5)
	AT-LNA-T	ON10	49.0 (+17.0)	39.0 (+21.5)	55.5 (+26.0)	44.0 (+28.5)

[a] T_m values (°C) of unmodified and modified (**X** = 2'-amino-LNA-T, AT-LNA-T, AP-LNA-T or AB-LNA-T) DNA/DNA and DNA/RNA duplexes measured as an average of two independent melting temperature determinations with a deviation ≤ 0.5 °C. Values in brackets are ΔT_m values measured as the difference in T_m values between modified and unmodified duplexes. The experiments were carried out at pH 7.0 in medium salt buffer (5.8 mM NaH_2PO_4/Na_2HPO_4 buffer, containing 100 mM NaCl and 0.10 mM EDTA) and low salt buffer (6.7 mM NaH_2PO_4/Na_2HPO_4 buffer, containing 0.10 mM EDTA). The increase in melting temperature of 2'-amino-LNA-T is in general agreement with previous results reported for **ON2** and **ON9**.[14]

Protonation of one or more of the primary amino groups seems to induce a crucial effect for duplex stabilization, and its importance was further emphasized by studying thermal denaturation temperatures at reduced ionic strength (Table 1). Thus, compared to DNA-T and 2'-amino-LNA-T, all modified ONs containing monomer(s) AT-LNA-T, AP-LNA-T and AB-LNA-T showed even stronger affinity towards DNA and RNA complements at the low salt buffer condition than at medium salt. It should be noted that it is not surprising that 2'-amino-LNA-T is unable to alleviate the negative influence from the reduced ionic strength, as the pKa value of the protonated 2'-amino group is only 6.17.[36]

The binding specificity was assessed for the three singly-modified ONs (**ON3**, **ON4** and **ON5**) with the nucleobase varied in the complementary strand opposite to the site of modification (ESI). Relative to the all-DNA control **ON1**, all modified ONs generally exhibited better mismatch discriminating ability, except against the RNA target with a central RNA-C. The mismatch studies were also extended to **ON10** that includes three AT-LNA-T monomers, and this study confirmed the satisfactory base-pairing selectivity of this monomer as **ON10** showed similar discriminative ability as **ON1** (ESI).

We used molecular modelling (for details, see ESI S7) to investigate the mechanisms of stabilization of single AT-LNA-T, AP-LNA-T and AB-LNA-T monomers inserted into a DNA:RNA hybrid duplex (i.e. **ON3**, **ON4**, **ON5** with complementary RNA, see Table 1). The possible interaction partners of the amine moiety were explored by performing a conformational search on the 2'-*N*-alkanoyl linkers with amine moieties while keeping the atoms of the DNA:RNA duplex frozen to the positions in the most representative structure of the NMR ensemble (PDB-ID: 1HHW).[37] The most favoured conformations found are shown in Fig 2. Energies and additional parameters are listed for the 10 lowest conformations in Table S4. Intrastrand hydrogen bonds to phosphate oxygens were found in most conformations, and the low energy conformations shown in Fig 2 are representative of the

binding modes found in the 40 kJ/mol energy window searched. The conformational search did not find interstrand interaction of the type where the protonated amino moieties of the LNA-T modification form hydrogen bonds with opposite strand phosphates. This reflects the inability of the linkers to bridge the minor groove of the DNA:RNA duplex. However, another type of interstrand interaction was found as the second-lowest energy conformer (~0.6 kJ/mol higher than the lowest energy conformer) for AP-LNA-T (Fig 2c), with one protonated primary amine binding to a neighbouring phosphate oxygen and the other towards the O2'-atom on the opposite strand.

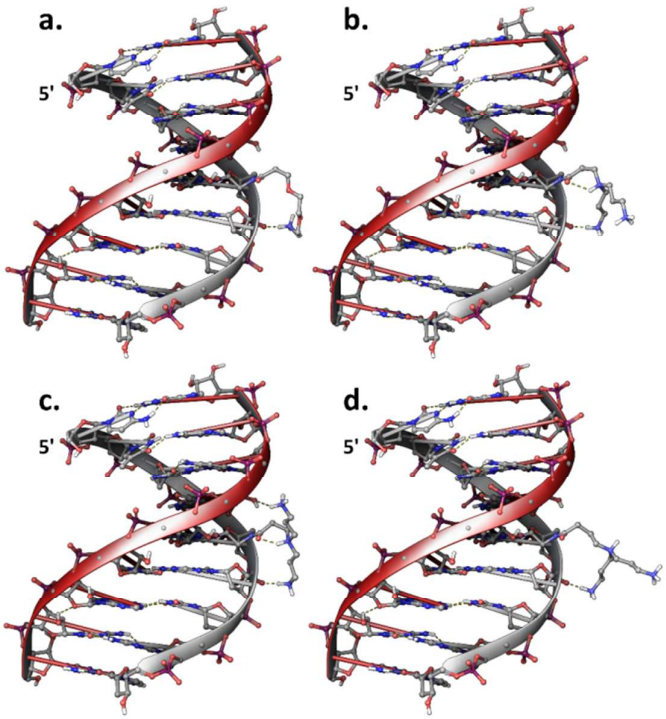


Figure 2. Low energy binding modes found in conformational searches on single AT-LNA-T, AP-LNA-T and AB-LNA-T monomers inserted into the DNA:RNA duplex (PDB-ID: 1HHW).[37] **a**. lowest energy conformer for the AT-LNA-T monomer; **b**. lowest energy conformer for the AP-LNA-T monomer; **c**. second lowest energy conformer (ΔE = 0.6 kJ/mol) for the AP-LNA-T monomer; **d**. lowest energy conformer for the AB-LNA-T monomer. For clarity, only polar hydrogens are displayed.

This binding mode was also found for AB-LNA-T (ESI, Figure S28b), but at a higher relative energy (~15 kJ/mol higher than the lowest energy conformer). Despite the substantial

predicted difference in energy barrier to populating the interstrand interaction mode for AB-LNA-T and AP-LNA-T, the two modifications yield very similar experimental stabilizations of the singly modified DNA:RNA duplexes (Table 1). This suggests that the primary mode of duplex stabilization by AT-LNA-T, AP-LNA-T and AB-LNA-T is the interaction between the protonated primary amines and the neighbouring negatively charged phosphates on the same strand (i.e. Fig 2a, b, d), as recurrently found in the conformational search. This electrostatic/hydrogen bond interaction may shield the interstrand electrostatic backbone repulsion, resulting in increased thermal denaturation temperatures.

The three new monomers were further incorporated into triplex-forming oligonucleotides (TFOs) to evaluate their triplex-stabilizing properties towards the wild-type HIV polypurine tract[38] in a 29-mer dsDNA sequence. The studies were performed on singly- or doubly-modified TFOs using **TFO1**, **TFO2**, **TFO6** and **TFO10** containing DNA-T and/or LNA-T monomers as references (Table 2). Two substitution patterns were studied with a two-base or five-base gap between the two monomers to discern a spatial effect. At pH 6, the TFOs containing monomers AT-LNA-T, AP-LNA-T and AB-LNA-T all exhibited significantly higher triplex stability than **TFO1** with a ΔT_m value of up to 12.5 ˚C for a single incorporation. Incorporation of an additional of the amidated monomers led to even greater increases in thermal denaturation temperature (up to 17.5 ˚C for the two-base gap and 20.0 ˚C for the five-base gap). It is noteworthy that only one methylene insertion in the linkage imparts ~2 ˚C increase in triplex melting temperature (from AP-LNA-T to AB-LNA-T), which underlines that seemingly minor changes in molecular design can lead to significant differences in biophysical properties. In general, the thermal stability of the triplexes decreased drastically when the pH was increased from 6 to 7, probably due to deprotonation of N3 of cytosine (pKa ≈ 5.5)[39-41] compromising the formation of the stable C^+.GC triplet under neutral pH.[42] However, the amidated monomers induced remarkable increases in triplex

stability also at pH 7 with the order of stability being AB-LNA-T > AP-LNA-T > AT-LNA-T ≥ LNA-T.

Table 2. Parallel Triplex Melting Analysis[a]

		5'-TT**X**TC**Y**TTT**Z**CCCCCCT 5'-CCACTTTTTAAAAGAAAAGGGGGGACTGG 3'-GGTGAAAAATTTTCTTTTCCCCCCTGACC		TFO Duplex	
Sequence	**X**	**Y**	**Z**	**pH 6.0**	**pH 7.0**
TFO1	DNA-T	DNA-T	DNA-T	23.0	n.d.
TFO2	DNA-T	DNA-T	LNA-T	31.5 (+8.5)	12.0
TFO3	DNA-T	DNA-T	AT-LNA-T	31.0 (+8.0)	12.0
TFO4	DNA-T	DNA-T	AP-LNA-T	33.0 (+10.0)	15.0
TFO5	DNA-T	DNA-T	AB-LNA-T	35.5 (+12.5)	15.5
TFO6	DNA-T	LNA-T	LNA-T	36.0 (+13.0)	17.0
TFO7	DNA-T	AT-LNA-T	AT-LNA-T	33.5 (+10.5)	16.0
TFO8	DNA-T	AP-LNA-T	AP-LNA-T	36.0 (+13.0)	17.0
TFO9	DNA-T	AB-LNA-T	AB-LNA-T	40.5 (+17.5)	20.0
TFO10	LNA-T	DNA-T	LNA-T	38.5 (+15.5)	22.0
TFO11	AT-LNA-T	DNA-T	AT-LNA-T	37.0 (+14.0)	23.5
TFO12	AP-LNA-T	DNA-T	AP-LNA-T	41.0 (+18.0)	26.0
TFO13	AB-LNA-T	DNA-T	AB-LNA-T	43.5 (+20.0)	28.0

[a] T_m values (˚C) are an average of two independent melting temperatures with deviation no more than 0.5 ˚C. Values in brackets are ΔT_m = T_m (LNA-T, AT-LNA-T, AP-LNA-T or AB-LNA-T) - T_m (DNA-T) at pH 6.0. The experiments were performed in 10 mM sodium cacodylate, 150 mM NaCl and 10 mM $MgCl_2$ under pH 6.0 and pH 7.0. The concentration of TFO: target DNA was 1.5 μM:1.0 μM. n.d. = not detected.

A 3'-exonuclease assay was used to evaluate the resistance of ^{32}P-labelled **ON1-ON5** towards nucleolytic degradation upon incubation with snake venom phosphordiesterase (SVPDE). As depicted in Fig. 3, both the all-DNA sequence (**ON1**) and 2'-amino-LNA-T sequence (**ON2**) were digested rapidly as no trace of corresponding full-length ONs remained after 5 min incubation. For **ON1**, the digestion was completed in 20 min, whereas **ON2** indicated some resistance but still being fully degraded during the 60-min time course. Retarded mobility was observed when DNA-T was replaced with AT-LNA-T, AP-LNA-T or AB-LNA-T, probably due to a combined effect of additional bulkiness and increased mass/charge ratio derived from protonation of the primary amines (considering charge as a sum of negative and positive charges). In stark contrast to **ON1** and **ON2**, **ON3-ON5** all showed steadfast resistance towards 3'-exonuclytic digestion with no or only little full-degradation after 60 min incubation, each displaying a neat major band which persisted over

the time course of the experiment. Intriguingly, the final products for **ON4** and **ON5** showed even slower gel mobility than the corresponding intact 9-mer sequence. That was not the case for **ON1-ON3.** To decipher the composition of these products, a parallel 3'-exonuclease assay was performed on **ON3-ON5** (without 5'-^{32}P-labelling) and each sample was desalted using NAP-Micro columns. The ensuing MALDI-TOF MS analysis showed that the molecular weights of three final products (ON3*, ON4* and ON5*) were consistent with the corresponding 6-mer fragments (5'-GTGA**X**A; see ESI). Apparently, the relative bulkiness and protonation of the primary amines in **ON4**-**ON5** influence so heavily that the full length GTGA**X**ATGC 9-mers runs even faster than the GTGA**X**A 6-mers digest products.

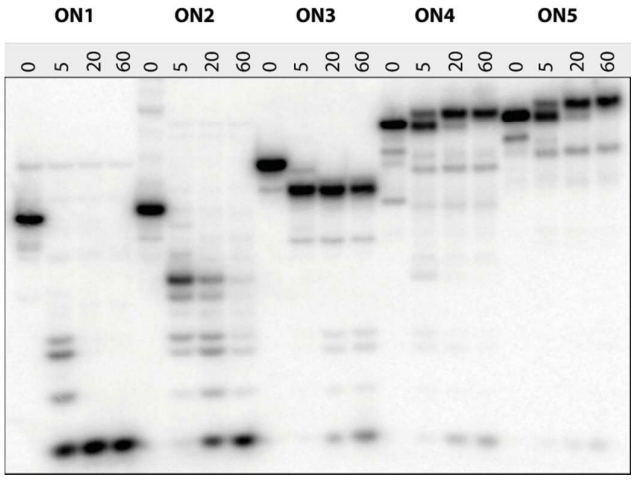


Figure 3. 20% PAGE denaturing gel showing the time-course of SVPDE-mediated degradation of 5′-^{32}P-GTGA**X**ATGC with **ON1** (**X** = T), **ON2** (**X** = 2′-amino-LNA-T), **ON3** (**X** = AT-LNA-T), **ON4** (**X** = AP-LNA-T) and **ON5** (**X** = AB-LNA-T) at pH 8.5 and at 21 ˚C (ESI). Samples were incubated for 5, 20 and 60 min. The negative control (0 min) was taken before the enzyme was added. The gel was visualized by autoradiography.

Strong stability was independently reported for ONs containing various amino-LNA-T and LNA-T derivatives.[21,43-46] Our recent results with a piperazino-modified amino-LNA-T monomer likewise demonstrated strong resistance to 3'-nucleolytic digestion.[22] Such extraordinary nuclease resistance for ONs containing a single AT-LNA-T, AP-LNA-T or AB-

LNA-T is in a sharp contrast to LNA-T for which a single modification only induce limited resistance towards exonucleolytic degradation[43-46] wherefore LNA-containing antisense ONs typically are constructed as phosphorothioate oligomers. Thus, the aminated 2'-amino-LNA-T derivatives may enable the use of antisense and antigene oligomers based in full or in part on phosphodiester linkages.

CONCLUSION

In conclusion, three novel aminated 2'-amino-LNA monomers (AT-LNA-T, AP-LNA-T and AB-LNA-T) nucleosides have been synthesized. The incorporation of these monomers into oligonucleotides in substitute for DNA or LNA monomers induced significantly increased duplex and triplex stabilities, enhanced mismatch discriminating ability and robust stability against 3'-exonucleolytic digestion, with the bis-amidated monomers AB-LNA-T and AP-LNA-T generally outperforming the mono-aminated monomer AT-LNA-T. Molecular modelling indicated that the duplex stabilization occurs via intrastrand electrostatic interactions between the phosphate group of the oligonucleotide backbone and the protonated amino-functionalities of the novel amino-LNA-T analogues. The resulting charge screening is proposed to contribute to increased denaturation temperatures by reducing oligo backbone-backbone repulsion. Those favourable properties render the three monomers promising candidates for the further development of oligonucleotide-based tools for biotechnology and therapeutic applications. Furthermore, these novel monomers will in future experiments be explored to answer the important question of relevance of overall net charge on cell membrane permeability of oligonucleotides.

EXPERIMENTAL PROCEDURES

Experimental procedures and synthetic data can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACA Publications website at DOI:

Details on synthesis of substituted 2'-amino-LNA-T monomers, oligonucleotide synthesis, purification and analysis (IE-HPLC and MAIDI-TOF MS), ultraviolet duplex melting studies, ultraviolet triplex melting studies, nuclease resistance assay, molecular modelling; copies of representative IE-HPLC curves, MAIDI-TOF mass spectra, and UV-melting curves.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu. Rev. Biochem. 73*, 39-85.
(2) Sessler, J. L., Lawrence, C. M., and Jayawickramarajah, J. (2007) Molecular recognition via base-pairing. *Chem. Soc. Rev. 36*, 314-325.
(3) Seeman, N. C. (2010) Nanomaterials based on DNA. *Annu. Rev. Biochem. 79*, 65-87.
(4) Guo, P. X., Coban, O., Snead, N. M., Trebley, J., Hoeprich, S., Guo, S. C., and Shu, Y. (2010) Engineering RNA for targeted siRNA delivery and medical application. *Adv. Drug Deliv. Rev. 62*, 650-666.
(5) Boersma, A. J., Megens, R. P., Feringa, B. L., and Roelfes, G. (2010) DNA-based asymmetric catalysis. *Chem. Soc. Rev. 39*, 2083-2092.
(6) Martin, A. R., Vasseur, J. J., and Smietana, M. (2013) Boron and nucleic acid chemistries: merging the best of both worlds. *Chem. Soc. Rev. 42*, 5684-5713.
(7) Davidson, B. L., and McCray, P. B. (2011) Current prospects for RNA interference-based therapies. *Nat. Rev. Genet. 12*, 329-340.
(8) Li, Z. H., and Rana, T. M. (2014) Therapeutic targeting of microRNAs: current status and future challenges. *Nat. Rev. Drug Discov. 13*, 622-638.
(9) Lundin, K. E., Gissberg, O., and Smith, C. I. E. (2015) Oligonucleotide therapies: the past and the present.

Hum. Gene Ther. 26, 475-485.
(10) Lennox, K. A., and Behlke, M. A. (2011) Chemical modification and design of anti-miRNA oligonucleotides. *Gene Ther. 18*, 1111-1120.
(11) Deleavey, G. F., and Damha, M. J. (2012) Designing chemically modified oligonucleotides for targeted gene silencing. *Chem. Biol. 19*, 937-954.
(12) Zheng, J., Yang, R. H., Shi, M. L., Wu, C. C., Fang, X. H., Li, Y. H., Li, J. H., and Tan, W. H. (2015) Rationally designed molecular beacons for bioanalytical and biomedical applications. *Chem. Soc. Rev. 44*, 3036-3055.
(13) Ozcan, G., Ozpolat, B., Coleman, R. L., Sood, A. K., and Lopez-Berestein, G. (2015) Preclinical and clinical development of siRNA-based therapeutics. *Adv. Drug Deliv. Rev. 87*, 108-119.
(14) Singh, S. K., Kumar, R., and Wengel, J. (1998) Synthesis of 2 '-amino-LNA: A novel conformationally restricted high-affinity oligonucleotide analogue with a handle. *J. Org. Chem. 63*, 10035-10039.
(15) Campbell, M. A., and Wengel, J. (2011) Locked vs. unlocked nucleic acids (LNAvs.UNA): contrasting structures work towards common therapeutic goals. *Chem. Soc. Rev. 40*, 5680-5689.
(16) Astakhova, I. K., and Wengel, J. (2014) Scaffolding along nucleic acid duplexes using 2'-amino-locked nucleic acids. *Acc. Chem. Res. 47*, 1768-1777.
(17) Hojland, T., Kumar, S., Babu, B. R., Umemoto, T., Albaek, N., Sharma, P. K., Nielsena, P., and Wengel, J. (2007) LNA (locked nucleic acid) and analogs as triplex-forming oligonucleotides. *Org. Biomol. Chem. 5*, 2375-2379.
(18) Noir, R., Kotera, M., Pons, B., Remy, J. S., and Behr, J. P. (2008) Oligonucleotide-oligospermine conjugates (zip nucleic acids): a convenient means of finely tuning hybridization temperatures. *J. Am. Chem. Soc. 130*, 13500-13505.
(19) Winkler, J., Saadat, K., Diaz-Gavilan, M., Urban, E., and Noe, C. R. (2009) Oligonucleotide-polyamine conjugates: influence of length and position of 2'-attached polyamines on duplex stability and antisense effect. *Eur. J. Med. Chem. 44*, 670-677.
(20) Astakhova, I. K., Hansen, L. H., Vester, B., and Wengel, J. (2013) Peptide-LNA oligonucleotide conjugates. *Org. Biomol. Chem. 11*, 4240-4249.
(21) Shrestha, A. R., Kotobuki, Y., Hari, Y., and Obika, S. (2014) Guanidine bridged nucleic acid (GuNA): an effect of a cationic bridged nucleic acid on DNA binding affinity. *Chem. Commun. 50*, 575-577.
(22) Lou, C., Vester, B., and Wengel, J. (2015) Oligonucleotides containing a piperazino-modified 2'-amino-LNA monomer exhibit very high duplex stability and remarkable nuclease resistance. *Chem. Commun. 51*, 4024-4027.
(23) Boisguerin, P., Deshayes, S., Gait, M. J., O'Donovan, L., Godfrey, C., Betts, C. A., Wood, M. J. A., and Lebleu, B. (2015) Delivery of therapeutic oligonucleotides with cell penetrating peptides. *Adv. Drug Deliv. Rev. 87*, 52-67.
(24) Menzi, M., Lightfoot, H. L., and Hall, J. (2015) Polyamine-oligonucleotide conjugates: a promising direction for nucleic acid tools and therapeutics. *Future Med. Chem. 7*, 1733-1749.
(25) Gagnon, K. T., Watts, J. K., Pendergraff, H. M., Montaillier, C., Thai, D., Potier, P., and Corey, D. R. (2011) Antisense and antigene inhibition of gene expression by cell-permeable oligonucleotide-oligospermine conjugates. *J. Am. Chem. Soc. 133*, 8404-8407.
(26) Paris, C., Moreau, V., Deglane, G., Karim, L., Couturier, B., Bonnet, M. E., Kedinger, V., Messmer, M., Bolcato-Bellemin, A. L., Behr, J. P., Erbacher, P., and Lenne-Samuel, N. (2012) Conjugating phosphospermines to siRNAs for improved stability in serum, intracellular delivery and RNAi-mediated gene silencing. *Mol. Pharm. 9*, 3464-3475.
(27) Geny, S., Moreno, Pedro M. D., Krzywkowski, T., Gissberg, O., Andersen, N. K., Isse, A. J., El-Madani, A. M., Lou, C., Pabon, Y. V., Anderson, B. A., Zaghloul, E. M., Zain, R., Hrdlicka, P. J., Jørgensen, P. T., Nilsson, M., Lundin, K. E., Pedersen, E. B., Wengel, J., and Smith, C. I. E. (2016) Next-generation bis-locked nucleic acids with stacking linker and 2′ -glycylamino-LNA show enhanced DNA invasion into supercoiled duplexes. *Nucleic Acids Res. 44*, 2007-2019.
(28) Bottcher, T., Kolodkin-Gal, I., Kolter, R., Losick, R., and Clardy, J. (2013) Synthesis and activity of biomimetic biofilm disruptors. *J. Am. Chem. Soc. 135*, 2927-2930.
(29) Madsen, A. S., Jorgensen, A. S., Jensen, T. B., and Wengel, J. (2012) Large scale synthesis of 2'-amino-LNA thymine and 5-methylcytosine nucleosides. *J. Org. Chem. 77*, 10718-10728.
(30) Hujakka, H., Ratilainen, J., Korjamo, T., Lankinen, H., Kuusela, P., Santa, H., Laatikainen, R., and Narvanen, A. (2001) Synthesis and antimicrobial activity of the symmetric dimeric form of temporin a based on 3-*N*,*N*-di(3-aminopropyl)amino propanoic acid as the branching unit. *Bioorg. Med. Chem. 9*, 1601-1607.
(31) Paredes, E., Evans, M., and Das, S. R. (2011) RNA labeling, conjugation and ligation. *Methods 54*, 251-259.
(32) Shelbourne, M., El-Sagheer, A. H., and Brown, T. (2012) Fast and efficient DNA crosslinking and multiple orthogonal labelling by copper-free click chemistry. *Chem. Commun. 48*, 11184-11186.

(33) Lou, C., Dallmann, A., Marafini, P., Gao, R., and Brown, T. (2014) Enhanced H-bonding and pi-stacking in DNA: a potent duplex-stabilizing and mismatch sensing nucleobase analogue. *Chem. Sci. 5*, 3836-3844.
(34) Lou, C., Shelbourne, M., Fox, K. R., and Brown, T. (2011) 2'-Aminoethoxy-2-amino-3-methylpyridine in triplex-forming oligonucleotides: high affinity, selectivity and resistance to enzymatic degradation. *Chem. Eur. J. 17*, 14851-14856.
(35) Rajwanshi, V. K., Hakansson, A. E., Dahl, B. M., and Wengel, J. (1999) LNA stereoisomers: xylo-LNA (beta-D-xylo configured locked nucleic acid) and alpha-L-LNA (alpha-L-ribo configured locked nucleic acid). *Chem. Commun.*, 1395-1396.
(36) Plashkevych, O., Chatterjee, S., Honcharenko, D., Pathmasiri, W., and Chattopadhyaya, J. (2007) Chemical and structural implications of 1',2'- versus 2',4'-conformational constraints in the sugar moiety of modified thymine nucleosides. *J. Org. Chem. 72*, 4716-4726.
(37) Petersen, M., Bondensgaard, K., Wengel, J., and Jacobsen, J. P. (2002) Locked nucleic acid (LNA) recognition of RNA: NMR solution structures of LNA : RNA hybrids. *J. Am. Chem. Soc. 124*, 5974-5982.
(38) Faria, M., Wood, C. D., Perrouault, L., Nelson, J. S., Winter, A., White, M. R. H., Helene, C., and Giovannangeli, C. (2000) Targeted inhibition of transcription elongation in cells mediated by triplex-forming oligonucleotides. *Proc. Natl. Acad. Sci. U. S. A. 97*, 3862-3867.
(39) Callahan, D. E., Trapane, T. L., Miller, P. S., Tso, P. O. P., and Kan, L. S. (1991) Comparative circular-dichroism and fluorescence studies of oligodeoxyribonucleotide and oligodeoxyribonucleoside methylphosphonate pyrimidine strands in duplex and triplex formation. *Biochemistry 30*, 1650-1655.
(40) Xodo, L. E., Manzini, G., Quadrifoglio, F., Vandermarel, G. A., and Vanboom, J. H. (1991) Effect of 5-methylcytosine on the stability of triple-stranded DNA - a thermodynamic study. *Nucleic Acids Res. 19*, 5625-5631.
(41) Singleton, S. F., and Dervan, P. B. (1992) Influence of pH on the equilibrium association constants for oligodeoxyribonucleotide-directed triple helix formation at single DNA sites. *Biochemistry 31*, 10995-11003.
(42) Frankkamenetskii, M. D., and Mirkin, S. M. (1995) Triplex DNA structures. *Annu. Rev. Biochem. 64*, 65-95.
(43) Ostergaard, M. E., Kumar, P., Baral, B., Raible, D. J., Kumar, T. S., Anderson, B. A., Guenther, D. C., Deobald, L., Paszczynski, A. J., Sharma, P. K., and Hrdlicka, P. J. (2009) C5-functionalized LNA: unparalleled hybridization properties and enzymatic stability. *Chembiochem 10*, 2740-2743.
(44) Hari, Y., Morikawa, T., Osawa, T., and Obika, S. (2013) Synthesis and properties of 2'-*O*,4'-*C*-ethyleneoxy bridged 5-methyluridine. *Org. Lett. 15*, 3702-3705.
(45) Kumar, P., Ostergaard, M. E., Baral, B., Anderson, B. A., Guenther, D. C., Kaura, M., Raible, D. J., Sharma, P. K., and Hrdlicka, P. J. (2014) Synthesis and Biophysical Properties of C5-Functionalized LNA (Locked Nucleic Acid). *J. Org. Chem. 79*, 5047-5061.
(46) Kaura, M., Guenther, D. C., and Hrdlicka, P. J. (2014) Carbohydrate-functionalized locked nucleic acids: oligonucleotides with extraordinary binding affinity, target specificity, and enzymatic stability. *Org. Lett. 16*, 3308-3311.

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