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Functionalized LNA (locked nucleic acid): high-affinity hybridization of oligonucleotides containing N-acylated and N-alkylated 2′-amino-LNA monomers†

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A molecular dynamics (MD) simulation, synthesis and very efficient hybridization for a series of N-acylated and N-alkylated derivatives of 2′-amino-LNA are reported.

Studies on the attachment of hydrophobic and hydrophilic moieties at the furanose ring of internally positioned ribonucleotides of oligonucleotides (ONs) have been intensified over recent years for the purpose of improved detection systems for molecular diagnostics or enhanced antisense activity of ONs.[1–6] Based on the high-affinity hybridization reported for LNA (locked nucleic acid)[7–9] and 2′-amino-LNA,[10] § we decided to evaluate N-acylated and N-alkylated derivatives of 2′-amino-LNA monomers as novel building blocks for functionalized ONs.

An MD simulation (1 ns duration) of a hybrid between a fully modified nonamer *N*-benzoyl 2′-amino-LNA and its complementary RNA was performed using standard methods.[11] The benzoyl moieties are located at the brim of the minor groove, and they occupy roughly half of the space in the groove (Fig. 1). The carbonyl oxygen is directed toward the solvent, and the distance to one of the non-bridging phosphate oxygens is ~5.3 Å. The benzoyl moieties do not stack with one another, but rather partake in van der Waals interactions with the sugar moieties of the 3′-flanking sugars. A control simulation of the corresponding LNA:RNA hybrid shows that the attachment of the benzoyl moieties at the 2′-nitrogen does not introduce significant changes in the duplex geometry.

Based on this simulation we synthesized phosphoramidite derivatives **4** as building blocks for ONs that contain the functionalized 2′-amino-LNA monomers **V–Z** (Scheme 1; see ESI for structure drawings). The bicyclic nucleoside **1** was synthesized according to the published procedure[12] and was selectively N-benzoylated giving N-benzoyl derivative **2a** and N-alkylated by reductive alkylation furnishing the *N*-benzyl, *N*-(pyren-1-yl)methyl and *N*-(2-phthalimido)ethyl derivatives **2b–d**. Subsequent protection of the 5′-hydroxyl groups afforded the 5′-*O*-4,4′-dimethoxytrityl (5′-*O*-DMT) derivatives **3a–d**. DMT-protection after acylation failed for the pyren-1-ylcarbonyl derivative. Instead, the known 5′-*O*-DMT protected *N*-trifluoroacetyl nucleoside **3e**[10] was converted into **3f** by deacylation followed by reaction with pyrenyl-1-carbonyl chloride. The phosphoramidite building blocks **4a–d** and **4f** were obtained by standard phosphitylation of nucleosides **3a–d** and **3f**.

Synthesis of the novel 2′-amino-LNAs (Tables 1 and 2) containing monomers **V–Z** was performed in 0.2 μmol scale on an automated DNA synthesizer. Standard procedures were used with modifications[13] for amidites **4**. For synthesis of ONs that contain **V** and **X**, *tert*-butylphenoxyacetyl protected DNA amidites were applied to allow mild deprotection. The coupling yields were *ca*. 99% for all modified (10 min coupling time) and unmodified phosphoramidites. After deprotection and cleavage from the solid support (32% aq. ammonia)¶ and desalting, satisfactory purities (>80%) of all 2′-amino-LNAs were verified by capillary gel electrophoresis‖ and their composition by MALDI-MS analysis.

The influence on thermal denaturation of the functionalized 2′-amino-LNA monomers **V–Z** was studied toward DNA and RNA complements (Table 1). All novel mixed sequence 9-mers that contain two or three modified 2′-amino-LNA monomers (**ON4–ON8**) hybridize efficiently and in general with very high thermal stabilities comparable to those obtained for the LNA (**ON2** and **ON10**[7,8]) and *N*-methyl 2′-amino-LNA (**ON3**)[10]

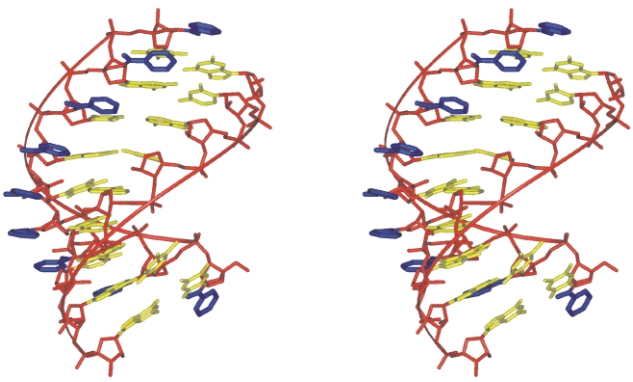
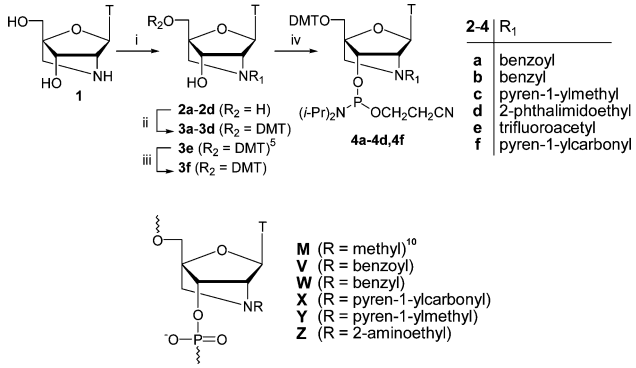


Fig. 1 Average structure from molecular dynamics simulation. Stereo view of a fully modified nonamer *N*-benzoyl 2′-amino-LNA [5′-(^{Me}CTGATATG^{Me}C)^{L}:RNA] hybrid viewed into the minor groove. For clarity, no hydrogens are shown. The colouring scheme is: nucleobases, yellow; sugar–phosphate backbone, red; benzoyl moieties, blue.



Scheme 1 *Reagents and conditions*: i) (**2a**): benzoyl chloride, Na_2CO_3, MeOH, 0 °C, 93%; (**2b**): benzaldehyde, AcOH, $NaCNBH_3$, MeOH, 85%; (**3c**) pyrene-1-carbaldehyde, AcOH, $NaCNBH_3$, MeOH, 94%; (**3d**) phthalimidoacetaldehyde, AcOH, $NaCNBH_3$, MeOH, 55%; ii) DMTCl, pyridine (**3a**: 88%, **3b**: 92%, **3c**: 77%, **3d**: 72%); iii) a) **3e**,[10] sat. NH_3 in MeOH, 92%, b) pyren-1-ylcarbonyl chloride, Na_2CO_3, MeOH, 0 °C, 67%; iv) $NC(CH_2)_2OP(Cl)N(i\text{-}Pr)_2$, $(i\text{-}Pr)_2NEt$, CH_2Cl_2 (**4a**: 82%, **4b**: 70%, **4c**: 76%, **4d**: 58%, **4f**: 57%). T = thymin-1-yl.

† Electronic supplementary information (ESI) available: structures of 2′-amino-LNA monomers **V–Z**. See http://www.rsc.org/suppdata/cc/b3/b307026c/

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Table 1 Thermal denaturation studies toward DNA/RNA

	Complementary DNA		Complementary RNA	
Oligonucleotide (5′–3′)	T_m/°C	ΔT_m/°C	T_m/°C	ΔT_m/°C
ON1:d(GTG ATA TGC)	27	Ref.	28	Ref.
ON2:d(G**T^L**G A**T^L**A **T^L**GC)	44[7,8]	—	50[7,8]	—
ON3:d(G**M**G A**M**A **M**GC)	39[10]	—	49[10]	—
ON4:d(G**V**G A**V**A **V**GC)	47	+6.7	56	+9.3
ON5:d(G**W**G A**W**A **W**GC)	37	+3.3	50	+7.3
ON6:d(GTG A**X**A **X**GC)	38	+5.5	41	+6.5
ON7:d(GTG A**Y**A **Y**GC)	26	−0.5	34	+3
ON8:d(G**Z**G A**Z**A **Z**GC)	41	+4.7	49	+7
ON9:T_{10}	18	Ref.	18	Ref.
ON10: (**T^L**)$_9$T	80[7]	—	71[7]	—
ON11: **V**$_9$T	75	+6.3	73	+6.1
ON12: **X**$_9$T	n.t.	—	n.t.	—
ON13: **Y**$_9$T	65	+5.2	n.t.	—
ON14: **Z**$_9$T	86	+7.8	77	+6.6

Melting temperatures (T_m values) measured as the maximum of the first derivative of the melting curve (A_{260} *vs.* temperature; 10 to 70 °C or 10 to 95 °C; increase 1 °C min^{-1}) recorded in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 1.5 μM concentrations of the two complementary strands. Each T_m value was determined in two independent experiments, and ΔT_m values, calculated per monomer **V–Z** relative to the T_m value for **ON1** and **ON9**, were within ±0.5 °C consistent for the two experiments. "n.t.": No cooperative melting transition. "**T^L**" denotes an LNA thymine monomer.[7,8]

Table 2 Thermal denaturation studies toward *N*-(pyren-1-yl)carbonyl 2′-amino-LNA (**ON18**) and *N*-(pyren-1-yl)methyl 2′-amino-LNA (**ON19**). See the footnote in Table 1 for details and conditions

	ON18 d(GCA **X**A**X** CAC)	**ON19**: d(GCA **Y**A**Y** CAC)
Oligonucleotide (5′–3′)	T_m/°C	T_m/°C
ON15: d(GTG A**T^L**A TGC)	49	36
ON16: d(GTG A**X**A TGC)	48	38
ON17: d(GTG A**Y**A TGC)	47	41
ON6: d(GTG A**X**A **X**GC)	55	50
ON7: d(GTG A**Y**A **Y**GC)	51	50

references. Favourable hydration of the carbonyl oxygen atoms compared to the methylene groups is indicated by comparison of the influence on thermal stabilities of **V** *vs.* **W** and **X** *vs.* **Y** [**ON4–ON7** and the corresponding singly modified ONs (not shown)]. In agreement with the MD simulation (Fig. 1), the (almost) fully modified *N*-benzoyl 2′-amino-LNA **ON11** shows remarkably efficient binding toward DNA and RNA complements. However, with the corresponding *N*-(pyren-1-yl)carbonyl 2′-amino-LNA **ON12** no duplex is formed. Possibly, unfavourable steric interactions impede duplex formation in this case. In contrast, the more flexible **ON13** containing *N*-(pyren-1-yl)methyl moieties is able to hybridize with DNA. The *N*-(2-amino)ethyl 2′-amino-LNA **ON14** is able to efficiently target both DNA and RNA, and it displays increased stability relative to reference **ON9** at lower salt conditions due to the presence of the basic amino groups (data not shown).

The possibility of interstrand stacking of the pyrenyl moieties of monomers **X** and **Y** was evaluated (Table 2). With **ON18** [containing two *N*-(pyren-1-yl)carbonyl 2′-amino-LNA monomers **X**] as target strand, **ON15–ON17**, **ON6** and **ON7** containing monomers **T^L** (purely conformational effect), **X** or **Y** (once or twice) all displayed near to additive affinity increases following the trend in Table 1. With **ON19** [containing two *N*-(pyren-1-yl)methyl 2′-amino-LNA monomers **Y**] as target strand, significantly lower thermal affinities were obtained, except for **ON7**, *i.e.* duplex **ON7**:**ON19** that includes a "2 + 2 pyrene unit". This indicates an interstrand stabilizing effect, possibly stacking of the pyrenyl moieties, for duplexes that contain the rather flexible *N*-(pyren-1-yl)methyl 2′-amino-LNA monomer **Y** in the two strands. Furthermore, juxtapositioning and interstrand interaction between the pyrene units of duplex **ON7**:**ON19** was indicated by steady-state fluorescence experiments that show a strong pyrene–pyrene excimer band at 430–530 nm not observed for the duplex between **ON7** and complementary DNA or for single-stranded **ON7**.[14]

In conclusion, the 2′-nitrogen atom of 2′-amino-LNA monomers is very suitable for functionalization of high-affinity ONs, and we are currently expanding the structural diversity for exploratory applications within biology and nanobiotechnology.

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Notes and references

§ We have defined a 2′-amino-LNA as an ON containing one or more 2′-amino-2′-deoxy-2′-*N*,4′-*C*-methylene-*β*-D-ribofuranosyl monomer(s).[10]

¶ *Conditions*: 2 h at room temperature for **ON4**, **ON6**, **ON11**, **ON12**, **ON16** and **ON18**; 48 h at 55 °C for **ON8**; 48 h at 55 °C followed by 48 h at 70 °C for **ON14**; 16 h at 55 °C for the remaining ONs.

‖ Analysis by standard capillary gel electrophoresis of the almost fully modified **ON12** and **ON14** could not be performed.

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