Functionalization of 2′-amino-LNA with additional nucleobases†

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Thymin-1-yl-acetic acid and adenin-9-yl-acetic acid have been coupled to the N2′-atom of a 2′-amino-LNA thymine nucleoside, and these "double-headed" LNA monomers have been incorporated into oligodeoxyribonucleotides *via* their corresponding phosphoramidite derivatives. Oligonucleotides containing these modified nucleotides show in most cases increased thermal stability when forming duplexes with complementary DNA, even allowing multiple incorporations. Incorporation of "double-headed" LNA monomers in both strands also led to stable duplexes, however, no indication of Watson–Crick base pairing between the extra nucleobases could be found.

Introduction

One of the most important biological recognition events is hydrogen bond formation between nucleobases leading to Watson–Crick, Hoogsteen or wobble pairings. Such base pairings are pivotal for central processes like replication and transcription, and for formation of higher order structures of DNA and RNA. Modified nucleotides containing an additional nucleobase[1–5] are of interest as the base pairing or stacking involving the additional nucleobases could be exploited to create novel types of secondary structures and possibly enhance affinity towards a target. Herein we report the synthesis and properties of novel N2′-functionalized 2′-amino-LNA nucleotides possessing an additional adenine or thymine nucleobase. The corresponding phenyl derivative was included in this study as a planar hydrocarbon counterpart to the natural nucleobases (Fig. 1). Functionalization of the N2′-atom of the locked bicyclic 2-oxa-5-azabicyclo[2.2.1]heptane skeleton of 2′-amino-LNA (2′-amino-Locked Nucleic Acid)[6] monomers has previously been shown to present functionalities towards the minor groove of duplexes formed with complementary strands.[7–11]

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† Electronic supplementary information (ESI) available: General experimental; MALDI-MS of synthesized ONs (Table S1); full NMR assigment of **3a–3c**; NMR spectra of **3a–3c** and **4a–4c**. See DOI: 10.1039/b901028a

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Results and discussion

O5′-dimethoxytritylated 2′-amino-LNA thymine nucleoside **1**[12] was used as starting material for the synthesis of phosphoramidites **4a–c**. Acetic acid derivatives **2a** (thymin-1-ylacetic acid), **2b** ((6-*N*-benzoyladenin-9-yl)acetic acid),[13] and **2c** (phenylacetic acid) were reacted with nucleoside **1** using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) as the condensation reagent to give amides **3a–c** in excellent yields. The 3′-hydroxy functionality of amides **3a–c** was subsequently phosphitylated to give phosphoramidite building blocks **4a–c** in acceptable yields (Scheme 1), thus establishing a reliable and straightforward synthetic route towards "double headed" nucleoside derivatives.

Preparation of the oligonucleotides containing "double-headed" LNA monomers $\mathbf{T^L_T}$, $\mathbf{T^L_A}$ or $\mathbf{T^L_{Ph}}$ (Fig. 1) was carried out by conventional automated DNA synthesis. Functionalized phosphoramidites **4a–c** were coupled in high yield (>95%) using 1*H*-tetrazole as activator and a coupling time of 20 min. The synthesized oligonucleotides were purified by RP-HPLC, and their constitution and purity (>80%) was verified by MALDI-MS analysis and ion-exchange-HPLC, respectively. The sequences of the "double-headed" LNA oligonucleotides are depicted in Tables 1 and 2.

The influence of "double-headed" LNA monomers on the thermal stability of DNA duplexes was studied with unmodified complementary DNA strands (Table 1). No significant stability difference was observed between the three different modifications, though it seems that the contribution to duplex stability follows the trend of $\mathbf{T^L_A} < \mathbf{T^L_{Ph}} < \mathbf{T^L_T}$, as judged from the

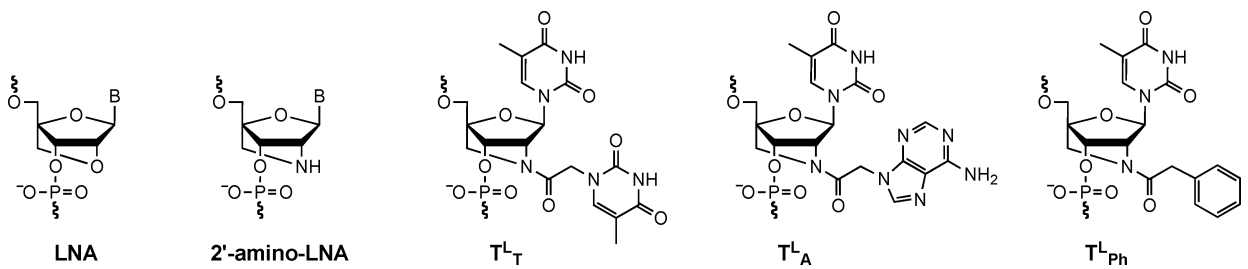
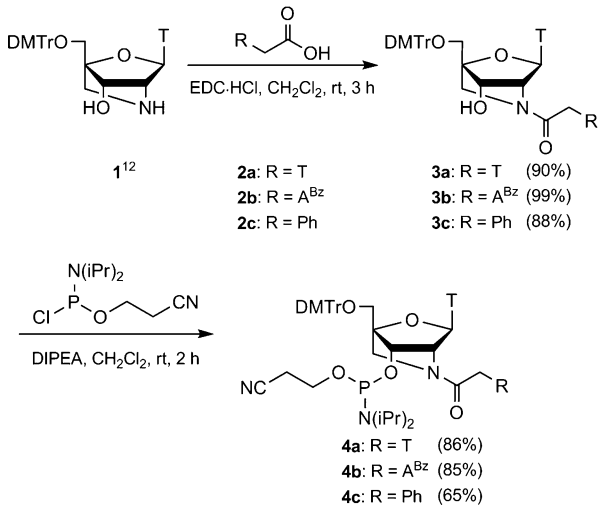


Fig. 1 LNA, 2′-amino-LNA and "double-headed" 2′-amino-LNAs.



Scheme 1 Synthesis of phosphoramidites for "double-headed" LNA. DMTr = 4,4′-dimethoxytrityl, T = thymin-1-yl, A^{Bz} = 6-*N*-benzoyl-adenin-9-yl.

Table 1 Thermal denaturation studies of duplexes with a single incorporation of a "double-headed" LNA monomer[a]

	T_m (ΔT_m)/°C		
	B = $\mathbf{T^L_T}$	**B** = $\mathbf{T^L_A}$	**B** = $\mathbf{T^L_{Ph}}$
5′-GTG AA**B** AGC C 3′-CAC TTA TCG G	36.5 (+1.5)	35.0 (±0.0)	37.0 (+2.0)
5′-GTG AAT AGC C 3′-CAC T**B**A TCG G	34.0 (−1.0)	32.0 (−3.0)	33.0 (−2.0)
5′-GTG AAT AGC C 3′-CAC TTA **B**CG G	38.0 (+3.0)	35.5 (+0.5)	37.0 (+2.0)

[a] Thermal denaturation temperatures (T_m values) of duplexes [T_m values/°C (ΔT_m = T_m of modified duplex − T_m of unmodified DNA:DNA duplex)] measured as the maximum of the first derivative of the melting curve (A_{260} vs. temperature) recorded in 10 mM phosphate buffer ([Na^+] = 110 mM, [Cl^-] = 100 mM, [EDTA] = 0.1 mM, pH 7.0) using 1.0 μM concentrations of the two complementary strands. For reference duplex 5′-GTG AAT AGC C:3′-CAC TTA TCG G, T_m = 35.0 °C.

thermal denaturation temperatures of the singly modified duplexes (Table 1). However, the specific positioning of the modification within the sequence context evidently exhibits a significant influence on duplex stability (*e.g.* ΔT_m of $\mathbf{T^L_T}$ modified duplexes range from −1.0 °C to +3.0 °C).

Interestingly, multiple incorporations in the same strand were well tolerated and let to thermally stable duplexes (Table 2). In particular, only a minor difference between the thermal denaturation temperatures of a duplex containing eight contiguous LNA thymine monomers ($\mathbf{T^L}$) and a duplex containing eight contiguous "double-headed" LNA monomers is notable. The obtained results demonstrate that the N2′-atom of 2′-amino-LNA monomers is a highly suitable site for attachment, allowing one or several additional nucleobases to be oriented towards the minor groove of thermally stable duplexes. Thus, "double-headed" LNA compares favorably with previously reported "double-headed" nucleosides, where single incorporations typically result in considerable destabilization of the duplexes formed and multiple incorporations in the same strand result in much less stable duplexes.[1–5]

Table 2 Thermal denaturation studies of duplexes with multiple incorporations of "double headed" LNA monomers[a]

	T_m (ΔT_m)/°C
5′-GTG AA$\mathbf{T^L_T}$ $\mathbf{T^L_T}$GC C 3′-CAC TTA ACG G	43.0 (+4.5)
5′-GAC G$\mathbf{T^L}\mathbf{T^L}$ $\mathbf{T^L}\mathbf{T^L}\mathbf{T^L}$ $\mathbf{T^L}\mathbf{T^L}\mathbf{T^L}$ GCA C 3′-CTG CAA AAA AAA CGT G	67.5 (+16.0)
5′-GAC G$\mathbf{T^L_T}\mathbf{T^L_A}$ $\mathbf{T^L_A}\mathbf{T^L_T}\mathbf{T^L_A}$ $\mathbf{T^L_T}\mathbf{T^L_T}\mathbf{T^L_A}$ GCA C 3′-CTG CAA AAA AAA CGT G	62.0 (+10.5)

[a] For conditions of thermal denaturation experiments see Table 1. $\mathbf{T^L}$ = LNA thymine monomer. For reference duplexes 5′-GTG AAT TGC C:3′-CAC TTA ACG G, T_m = 38.5 °C and 5′-GAC GTT TTT TTT GCA C:3′-CTG CAA AAA AAA CGT G, T_m = 51.5 °C.

The effect of incorporation of "double-headed" LNA monomers in both strands of a duplex was evaluated by thermal denaturation studies using duplexes containing the different "double-headed" LNA monomers in either downstream or upstream constitutions (Table 3, entries 1–3 and 4–6, respectively). In both constructs, stable duplexes were formed, but the upstream arrangement invariably led to thermally more stable duplexes than the downstream arrangement, regardless of N2′-functionalities. Furthermore, incorporation of two modifications led to more than additive increases in the thermal stability of the duplex (*e.g.* compare T_m's of the singly modified duplexes 5′-GTG AA $\mathbf{T^L_T}$ AGC C:3′-CAC TTA TCG G and 5′-GTG AA TAGC C:3′-CAC TTA $\mathbf{T^L_T}$CG G in Table 1 with T_m of the upstream arrangement of doubly modified duplex 5′-GTG AA $\mathbf{T^L_T}$ AGC C:3′-CAC TTA $\mathbf{T^L_T}$CG G in Table 3). The differences in thermal stability of the doubly modified duplexes seem to follow the trend of the singly modified duplexes, with $\mathbf{T^L_A}$ being the least stabilizing and $\mathbf{T^L_T}$ being the most stabilizing. Accordingly, the duplex with an upstream arrangement of two $\mathbf{T^L_A}$ monomers is noted as the least stable duplex with an upstream constitution. This is most likely explained by steric hindrance between the

Table 3 Thermal denaturation studies of duplexes with incorporation of a "double-headed" LNA monomer in each strand[a]

	T_m (ΔT_m)/°C		
	B = $\mathbf{T^L_T}$	**B** = $\mathbf{T^L_A}$	**B** = $\mathbf{T^L_{Ph}}$
5′-GTG AA**B** AGC C 3′-CAC TT$\mathbf{T^L_T}$A TCG G	38.0 (+3.0)	37.0 (+2.0)	38.0 (+3.0)
5′-GTG AA**B** AGC C 3′-CAC TT$\mathbf{T^L_A}$A TCG G	39.0 (+4.0)	39.0 (+4.0)	38.0 (+3.0)
5′-GTG AA**B** AGC C 3′-CAC TT$\mathbf{T^L_{Ph}}$A TCG G	39.5 (+4.5)	37.0 (+2.0)	36.0 (+1.0)
5′-GTG AA**B** AGC C 3′-CAC TTA $\mathbf{T^L_T}$CG G	45.0 (+10.0)	40.5 (+5.5)	43.5 (+8.5)
5′-GTG AA**B** AGC C 3′-CAC TTA $\mathbf{T^L_A}$CG G	42.0 (+7.0)	39.5 (+4.5)	40.5 (+5.5)
5′-GTG AA**B** AGC C 3′-CAC TTA $\mathbf{T^L_{Ph}}$CG G	42.0 (+7.0)	40.5 (+5.5)	41.5 (+6.5)

[a] For conditions of thermal denaturation experiments see Table 1.

two purine nucleobases, though changes in hydration pattern in and around the minor groove might also play a role in the effects observed. The observed changes in thermal affinity contrast with previous results where any observed stabilizing effect was attributed to stacking between the additional nucleobases.[2,4] However, the variation of attachment site of the additional nucleobases combined with the conformationally restricted LNA-skeleton render direct comparison difficult.

Incorporation of both an additional thymine and adenine nucleobase opens the possibility of additional hydrogen bonding. The potential of additional hydrogen bond formation between the two strands of a doubly modified duplex was evaluated by simple molecular modeling. A model structure of 5′-GTG AA**T**$^{L}_{T}$ AGC C:3′-CAC TTA **T**$^{L}_{A}$CG G, containing a thymine and adenine modified "double-headed" 2′-amino-LNA monomer in the two strands, respectively, was constructed in MacroModel V9.1.[14] The duplex structure was subjected to Monte Carlo conformational analysis, and the generated structures were energy minimized using the AMBER force field[15,16] as implemented in MacroModel V9.1. Two protocols were employed, either allowing the N2′-functionalities to move freely in space or forcing the two extra nucleobases to be positioned at a distance favoring hydrogen bonding. Subsequently, the lowest energy structure obtained by each of the protocols was subjected to a stochastic dynamics simulation followed by energy minimization of structures taken evenly distributed along the simulation trajectory. In both simulation protocols the global duplex conformation is found to be an intermediate A/B-type duplex. This is in agreement with previous studies for LNA-modified DNA-type duplexes, which have shown that the LNA-skeleton is conformationally driving the local duplex geometry from a B-type towards an intermediate A/B-type duplex.[17] The lowest energy structure obtained with non-restricted N2′-functionalities, indicates, in agreement with previous results, that N2′-functionalities are positioned in the minor groove of the duplex. Furthermore, the functionalities are found to be directed towards the 3′-end of the appended strand, without any interaction with each other (Fig. 2). However, forcing hydrogen bonding between the additional nucleobases indicates that the two functionalities potentially could reach each other across the minor groove of the duplex (Fig. 2). Inspection of the measured T_m values, however, does not give any clear-cut indication of such inter-strand communication *via* base-pairing. Thus, the energy gain associated with base pairing is most likely to be too small to facilitate the necessary rearrangement of the N2′-functionalities.

Conclusion

The efficient synthesis of novel "double-headed" LNA nucleosides and oligonucleotides thereof has been achieved. Incorporation of one or more of these novel monomers into DNA strands generally resulted in thermally very stable duplexes with unmodified DNA complements. This demonstrates the suitability of the N2′-atom of 2′-amino-LNA monomers as an attachment site for additional nucleobases oriented towards the minor groove. These "double-headed" LNA monomers are thus one step forward towards increasing the information content of synthetic oligonucleotides, and further structural studies and design optimization is warranted.

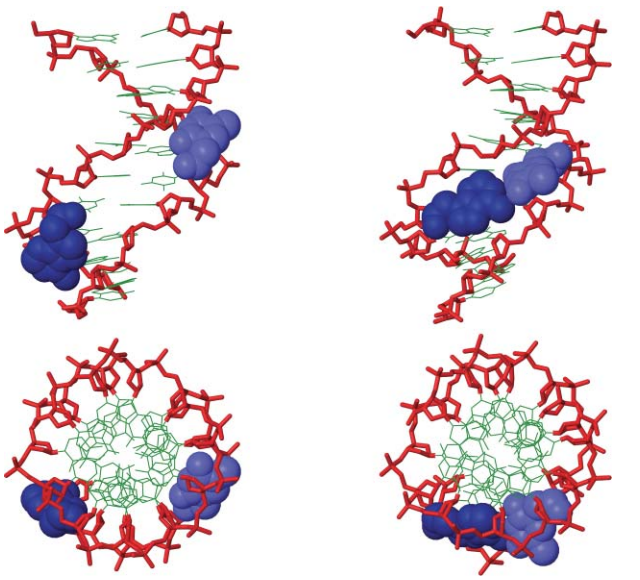


Fig. 2 Illustrations of the lowest energy structures of doubly modified duplex 5′-GTG AA **T**$^{L}_{T}$ AGC C:3′-CAC TTA **T**$^{L}_{A}$CG G, without (to the left) and with forced hydrogen bonding of the extra nucleobases, respectively. Coloring scheme: nucleobases: *green*; sugar–phosphate backbone: *red*; N2′-thymin-1-ylacetyl: *light blue*; N2′-adenin-9-ylacetyl: *blue.*

Experimental

Preparation of "double-headed" LNA phosphoramidites

General procedure for synthesis of 3.

*1-(2-Amino-2-deoxy-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-2-*N*-(thymin-1-ylacetyl)-β-*D*-ribofuranosyl)thymine (**3a**).* A mixture of 1-(2-amino-2-deoxy-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-β-D-ribo-furanosyl)thymine[12] (**1**, 286 mg, 0.50 mmol) and thymin-1-ylacetic acid (**2a**, 110 mg, 0.60 mmol) was dried by co-evaporation with 1,2-dichloroethane (2 × 2 ml), and suspended in dry dichloromethane (5 ml). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 115 mg, 0.60 mmol) was added to the suspension at 0 °C. After stirring the reaction mixture for 3 hours water (5 ml) was added. The reaction mixture was diluted with dichloromethane (50 ml) and washed with water (2 × 50 ml). The aqueous phase was back extracted with dichloromethane (50 ml). The combined organic layer was dried with Na_2SO_4, then evaporated and the residue was purified by silica-gel column chromatography (0–8% MeOH in CH_2Cl_2 (v/v)) to afford a rotameric mixture (~1:1.5 by ^{1}H NMR) of nucleoside **3a** as a colorless foam (334 mg, 90%). Physical data for rotameric mixture: R_f = 0.4 (10% MeOH in CH_2Cl_2, (v/v)); Selected signals ^{1}H NMR (400 MHz, DMSO-d_6):[18,19] δ 11.50 (br s, 1.5H, ex, N3-H$_A$), 11.39 (br s, 1H, ex, N3-H$_B$), 11.31 (br s, 2.5H, ex, N3‴-H$_{A+B}$), 7.54 (s, 1H, H6$_B$), 7.53 (s, 1.5H, H6$_A$), 7.22–7.47 (m, 25H, H6‴$_{A+B}$, Ar$_{A+B}$), 6.09 (d, J = 4.2 Hz, 1.5H, ex, 3′-OH$_A$), 6.04 (d, J = 4.3 Hz, 1H, ex, 3′-OH$_B$), 5.63 (s, 1.5H, H1′$_A$), 5.44 (s, 1H, H1′$_B$), 4.76 (d, J = 16.7, 1.5H, COCH$_{2,A,a}$), 4.57 (d, J = 16.7, 1.5H, COCH$_{2,A,b}$), 4.49 (s, 2H, COCH$_{2,B}$), 3.75 (s, 15H, OCH$_3$), 1.76 (s, 7.5H, 5‴-CH$_{3,A+B}$), 1.51 (s, 4.5H, 5-CH$_{3,A}$), 1.49 (s, 3H, 5-CH$_{3,B}$); Selected signals ^{13}C NMR (101 MHz, DMSO-d_6): δ 165.9 (*C*OCH$_{2,A}$), 165.6 (*C*OCH$_{2,B}$), 142.1 (C6‴$_A$), 142.0 (C6‴$_B$), 134.3 (C6$_B$), 134.0 (C6$_A$), 86.2 (C1′$_A$), 86.0 (C1′$_B$), 55.0 (OCH$_{3,A+B}$), 48.3

(CO$CH_{2,A}$), 47.9 (CO$CH_{2,B}$), 12.21 (5-$CH_{3,A}$), 12.18 (5-$CH_{3,B}$), 11.81 (5‴-$CH_{3,B}$), 11.77 (5‴-$CH_{3,A}$); HRMS MALDI FT-MS *m/z* 760.2556 ([M + Na]$^+$, calcd $C_{39}H_{39}N_5O_{10}\cdot Na^+$ 760.2589).

*1-(2-Amino-2-*N*-(6-*N*-benzoyladenin-9-ylacetyl)-2-deoxy-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-β-*D*-ribofuranosyl)thymine (**3b**).* A rotameric mixture (~1:1.3 by ^{1}H NMR) of adenine derivative **3b** was obtained from nucleoside **1**[12] (286 mg, 0.50 mmol) and *N*-6-benzoyl(adenin-9-yl)acetic acid[13] (**2b**, 178 mg, 0.60 mmol) as a colorless foam (421 mg, 99%) in a similar manner as thymine derivative **3a** except for the column chromatography (0–7% MeOH in CH_2Cl_2 (v/v)). Physical data for rotameric mixture: $R_f = 0.5$ (10% MeOH in CH_2Cl_2, (v/v)); ^{1}H NMR (DMSO-d_6):[18,19] δ 11.56 (br s, 1.3H, ex, NH_A/6‴-NH_A), 11.39 (br s, 1H, ex, NH_B/6‴-NH_B), 11.17 (br s, 2.3H, ex, NH_{A+B}/6‴-NH_{A+B}), 8.72 (s, 2.3H, H2‴$_{A+B}$), 8.47 (s, 1.3H, H8‴$_A$), 8.38 (s, 1H, H8‴$_B$), 7.53–7.59 (m, 6.9H, $H6_{A+B}$, Ar_{A+B}), 6.18 (d, $J = 4.3$ Hz, 1.3H, ex, 3′-OH_A), 6.08 (d, $J = 4.3$ Hz, 1H, ex, 3′-OH_B), 5.75 (s, 1.3H, H1′$_A$), 5.53 (s, 1H, H1′$_B$), 5.48 (d, $J = 17.0$ Hz, 1.3H, $COCH_{2,A,a}$), 5.30 (d, $J = 17.0$ Hz, 1.3H, $COCH_{2,A,b}$), 5.28 (d, $J = 17.2$ Hz, 1H, $COCH_{2,B,a}$), 5.20 (d, $J = 17.2$ Hz, 1H, $COCH_{2,B,b}$), 3.74–3.77 (m, 15.8H, $OCH_{3,A+B}$, H5″$_B$), 1.54 (s, 3.9H, 5-$CH_{3,A}$), 1.51 (s, 3H, 5-$CH_{3,B}$); ^{13}C NMR (DMSO-d_6): δ 165.5 (*C*OPh_{A+B}), 165.2 (*C*O$CH_{2,A}$), 165.1 (*C*O$CH_{2,B}$), 151.5 (C2‴$_{A/B}$), 151.4 (C2‴$_{A/B}$), 145.6 (C8‴$_A$), 145.5 (C8‴$_B$), 134.3 ($C6_B$), 134.1 ($C6_A$), 86.4 (C1′$_A$), 86.0 (C1′$_B$), 55.1 ($OCH_{3,A+B}$), 44.6 (CO*C*$H_{2,A}$), 44.3 (CO*C*$H_{2,B}$), 12.29 (5-$CH_{3,A}$), 12.26 (5-$CH_{3,B}$); HRMS MALDI FT-MS *m/z* 873.2943 ([M + Na]$^+$, calcd $C_{46}H_{42}N_8O_9\cdot Na^+$ 873.2967).

*1-(2-Amino-2-deoxy-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-2-*N*-phenylacetyl-β-*D*-ribofuranosyl)thymine (**3c**).* A rotameric mixture (~1:1.6 by ^{1}H NMR) of phenyl derivative **3c** was obtained from nucleoside **1** (286 mg, 0.50 mmol) and phenylacetic acid (**2c**, 82 mg, 0.60 mmol) as a colorless foam (303 mg, 88%) in a similar manner as thymine derivative **3a** except for the column chromatography (0–5% MeOH in CH_2Cl_2 (v/v)). $R_f = 0.6$ (10% MeOH in CH_2Cl_2, (v/v)); ^{1}H NMR (DMSO-d_6):[18,19] δ 11.50 (br s, 1.5H, ex, NH_A), 11.39 (br s, 1H, ex, NH_B), 7.57 (s, 1H, $H6_B$), 7.54 (s, 1.5H, $H6_A$), 5.99 (d, $J = 4.2$ Hz, 1.5H, ex, 3′-OH_A), 5.96 (d, $J = 4.1$ Hz, 1H, ex, 3′-OH_B), 5.53 (s, 1.5H, H1′$_A$), 5.40 (s, 1H, H1′$_B$), 3.77 (s, 3H, $COCH_{2,A}$), 3.74 (s, 15H, $OCH_{3,A+B}$), 3.63 (d, $J = 15.8$ Hz, 1H, $COCH_{2,B,a}$), 3.59 (d, $J = 15.8$ Hz, 1H, $COCH_{2,B,b}$), 1.51 (s, 4.5H, 5-$CH_{3,A}$), 1.47 (s, 3H, 5-$CH_{3,B}$); ^{13}C NMR (DMSO-d_6): δ 169.3 (*C*O$CH_{2,A}$), 169.2 (*C*O$CH_{2,B}$), 134.4 ($C6_B$), 134.1 ($C6_A$), 86.8 (C1′$_A$), 86.2 (C1′$_B$), 55.0 ($OCH_{3,A+B}$), 39.9 (CO*C*$H_{2,A+B}$, overlap with DMSO-d_6), 12.3 (5-$CH_{3,A}$), 12.2 (5-$CH_{3,B}$); HRMS MALDI FT-MS *m/z* 712.2623 ([M + Na]$^+$, calcd $C_{40}H_{39}N_3O_8\cdot Na^+$ 712.2629).

General procedure for synthesis of 4.

*1-(2-Amino-3-*O*-(2-cyanoethoxy(diisopropylamino)phosphinoxy)-2-deoxy-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-2-*N*-(thymin-1-ylacetyl)-β-*D*-ribofuranosyl)thymine (**4a**).* Amide **3a** (275 mg, 0.37 mmol) was dried by co-evaporation with dry dichloroethane, then dissolved in dichloromethane (5 ml). *N,N′*-diisopropylethylamine (129 µl, 0.74 mmol) and *O*-(2-cyanoethyl)-*N,N′*-diisopropyl-aminophosphorochloridite (123 µl, 0.55 mmol) were added at 0 °C. After stirring at rt for 2 h, absolute EtOH was added (2 ml). The reaction mixture was diluted with dichloromethane (50 ml) and washed with phosphate buffer (5 × 50 ml, pH 7.0). Organic layer was dried with Na_2SO_4, then evaporated, and the resulting residue was purified by silica-gel column chromatograpy (25% acetone in petroleum ether (v/v)), affording a rotameric and diastereomeric mixture of phosphoramidite **4a** as a colorless foam (298 mg, 86%). ^{31}P NMR (121 MHz, $CDCl_3$): δ 150.9, 150.4, 150.2, 149.0 ppm. HRMS MALDI FT-MS m/z (M + Na$^+$) found 960.3646 calc. 960.3668.

*1-(2-Amino-2-*N*-(6-*N*-benzoyladenin-9-ylacetyl)-3-*O*-(2-cyanoethoxy(diisopropylamino)phosphinoxy)-2-deoxy-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-β-*D*-ribofuranosyl)thymine (**4b**).* Phosphoramidite **4b** was obtained from amide **3a** (327 mg, 0.38 mmol) as a colorless foam (339 mg, 85%) in a similar manner as phosphoramidite **4a** except for the column chromatography (50% acetone in petroleum ether (v/v)). ^{31}P NMR ($CDCl_3$): δ 151.2, 150.7, 150.5, 149.1 ppm. HRMS MALDI-FT MS m/z (M + Na$^+$) found 1073.4035 calc. 1073.4045.

*1-(2-Amino-3-*O*-(2-cyanoethoxy(diisopropylamino)phosphinoxy)-2-deoxy-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-2-*N*-phenylacetyl-β-*D*-ribofuranosyl)thymine (**4c**).* Phosphoramidite **4c** was obtained from amide **3c** (264 mg, 0.38 mmol) as a colorless foam (220 mg, 65%) in a similar manner as phosphoramidite **4a** except for the column chromatography (50% acetone in petroleum ether (v/v)). ^{31}P NMR ($CDCl_3$): δ 150.8, 150.3, 149.5 ppm. HRMS MALDI-FT MS m/z (M + Na$^+$) found 912.3680 calc. 912.3708.

Synthesis of oligonucleotides containing "double-headed" LNA

Syntheses of modified ONs were performed on 0.2 µmol scale using an automated DNA synthesizer, using standard conditions for unmodified phosphoramidites. Incorporation of "double-headed" 2′-amino-LNA thymine monomers was carried out by a manual coupling protocol, using extended coupling time (20 min) and 1*H*-tetrazole as activator. Stepwise coupling yields were >99% for unmodified phosphoramidites and approximately 95% for modified phosphoramidites. After deprotection and cleavage from the solid support (32% aq. NH_3, 12 h, 55 °C), the oligonucleotides were purified by RP-HPLC, and their purity (>80%) and composition verified by IE-HPLC and MALDI-MS analysis (Table S1, see ESI†), respectively.

Thermal denaturation studies

Melting temperatures (T_m values) were determined as the maximum of the first derivative of the melting curve (A_{260} vs. temperature; temperature ramp 1 °C/min) and were recorded in 10 mM sodium phosphate buffer (100 mM NaCl, 0.1 mM EDTA, adjusted to pH 7.0 by 10 mM NaH_2PO_4/5 mM Na_2HPO_4) using 1.0 µM concentrations of the two complementary strands. The reported thermal denaturation temperatures are an average of at least two measurements within ±0.5 °C.

Molecular modeling

Unmodified duplex 5′-d(GTG AAT AGC C):3′-d(CAC TTA TCG G) was built having standard B-type helical geometry and subsequently modified to duplex 5′-d(GTG AA$\mathbf{T^L_T}$ AGC C):3′-d(CAC TTA $\mathbf{T^L_A}$CG G) in MacroModel V9.1.[14] The charge of the phosphodiester backbone was neutralized with sodium ions, which were placed 3.0 Å from the non-bridging oxygen atoms. Monte Carlo conformational search was performed, with torsional sampling of the N2′-functionalities (for monomer $\mathbf{T^L_T}$,

N2′-C(O) through C_α-N1″ were sampled; for monomer $\mathbf{T^L}_A$, N2′-C(O) through C_α-N9″ were sampled). Where applicable, hydrogen bonding of additional nucleobases was ensured by restraining $N3_T$–$N1_A$ to 2.87 Å and 4-O_T–6-N_A to 2.83 Å by a force constant of 100 kJ/molÅ^2. Sodium–oxygen distances were restrained by a force constant of 100 kJ/molÅ^2, and all atoms except the sugar and N2′-functionality moiety of modified monomers $\mathbf{T^L}_T$ and $\mathbf{T^L}_A$ and the adjacent phosphodiester groups (including O3′ of the preceding nucleotide and O5′ of the following nucleotide) were frozen. 1000 structures were generated and subsequently minimized using the Polak-Ribiere conjugate gradient method, the all-atom AMBER force field[15,16] and GB/SA solvation model[20] as implemented in MacroModel V9.1. Non-bonded interactions were treated with extended cut-offs (van der Waals 8.0 Å and electrostatics 20.0 Å). The lowest energy structures obtained were then subjected to 3 ns of stochastic dynamics (simulation temperature 300 K, time step 2.2 fs, SHAKE all bonds to hydrogen), during which 300 structures were collected evenly and subsequently minimized. During dynamics and the following minimization all atoms were allowed to move freely, but the previously applied atom-to-atom restraints were kept intact.

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