Synthesis of 2′-Amino-LNA: A Novel Conformationally Restricted High-Affinity Oligonucleotide Analogue with a Handle

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Introduction

The potential of chemically modified oligonucleotide (ON) analogues as therapeutic agents or diagnostic molecules has stimulated great interest in nucleic acid mimics during recent years.[1] Conformationally restricted analogues containing bicyclic or tricyclic carbohydrate moieties have been synthesized, and interesting properties, e.g., enhanced affinity toward complementary DNA and/or RNA strands, have been obtained.[2] We have recently introduced LNA (locked nucleic acids) as a novel class of conformationally restricted oligonucleotide analogues containing 2′-*O*,4′-*C*-methylene ribonucleoside LNA monomers.[3−5] Among the intriguing properties of LNA are unprecedented affinity toward complementary DNA/RNA, excellent base-pairing selectivity obeying the Watson−Crick hydrogen bonding rules, and the structural similarity with parent DNA/RNA allowing efficient automated oligomerization without additional protecting groups.[3] The excellent hybridization characteristics of LNA are expected to be related to the fixed 3′-endo (^{3}E) conformation of the monomeric LNA nucleosides[6] (e.g., see monomer **U**) possibly resulting in entropically favored duplex formation. However, as hydration is known to be very important for the thermodynamic stability of duplexes,[7] the thermodynamic parameters could be very different in the case of, e.g., LNA/DNA and LNA/RNA duplexes.[8]

2′-Amino-LNA analogues appealed to us for several reasons. First, the 2′-amino functionality should be a structurally well-defined conjugation site[9] in a conformationally restricted LNA-type ON. Second, amine-derivatized ONs have displayed increased thermal affinities toward complementary ONs possibly because of the introduction of positively charged moieties (and thus partial neutralization of the otherwise negatively charged strands in a duplex) at physiological pH.[10] In this report, we describe the syntheses of the 2′-amino-LNA and 2′-methylamino-LNA monomeric nucleosides **4** and **10**, the efficient oligomerization of the corresponding phosphoramidite building blocks **7** and **12**, and the excellent hybridization properties of 9-mer 2′-amino-LNAs (monomers **V** and **X**).

Results and Discussion

A key step in our synthesis of the parent LNA monomeric nucleosides was base-induced ring closure of 4′-*C*-tosyloxymethyl derivatives by intramolecular nucleophilic attack from the 2′-OH group positioned at the α-face of the pentofuranose ring.[3a−c] We envisioned that synthesis of the 2′-benzylamino nucleoside **3** should be possible via a double nucleophilic substitution reaction on di-*O*-tosyl derivative **2**.[11] 4′-*C*-Hydroxymethyl nucleoside diol **1**[3b] was transformed into nucleoside **2** in 80% yield by treatment with *p*-toluenesulfonyl chloride and 4-*N*,*N*-(dimethylamino)pyridine (DMAP) in dichloromethane. Reaction of di-*O*-tosyl derivative **2** in neat benzylamine at 130 °C afforded in 52% yield the 2′-benzylamino-2′-deoxynucleoside **3** with the desired 2,5-oxazabicyclo[2.2.1]heptane skeleton. In a small scale

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(4) We have defined LNA as ONs containing one or more 2′-*O*,4′-*C*-methylene bicyclic ribonucleoside LNA monomers. 2′-Amino-LNA is defined as an ON containing one or more 2′-*N*,4′-*C*-methylene bicyclic 2′-amino-LNA monomers (possibly *N*-derivatized, e.g., *N*-methylated to give 2′-methylamino-LNA).

(5) The LNA nucleosides containing uracil and cytosine nucleobases have been synthesized independently by a linear approach: Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735. Oligomers containing these pyrimidine bases have recently been reported: Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Morio, K.; Doi, T.; Imanishi, T. *Tetrahedron Lett.* **1998**, *39*, 5401.

(6) Preorganization of LNA nucleosides into a 3′-endo type conformation has been shown by X-ray crystallography (ref 5) and by NMR studies (ref 3).

(7) The importance of hydration on duplex stability has been reported for conformationally restricted N3′-P5′-phosphoramidate DNA: Egli, M. *Antisense Nucleic Acids Drug Dev.* **1998**, *8*, 123.

(8) Preliminary thermodynamic analysis of binding between a fully modified LNA and complementary DNA has indicated a significant enthalpic advantage compared to the corresponding unmodified control: Koshkin, A. A.; Nielsen, P.; Meldgaard, M.; Rajwanshi, V. K.; Singh, S. K.; Wengel, J. *J. Am. Chem. Soc.*, in press. A favorable entropy term has been reported for a duplex between a partly modified LNA and RNA (ref 5).

(9) A 4′-*C*-alkylamino group has been used as a conjugation site: Maag, H.; Schmidt, B.; Rose, S. J. *Tetrahedron Lett.* **1994**, *35*, 6449.

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(11) In a communication, we have reported synthesis of the unprotected 2′-amino-LNA and 2′-thio-LNA nucleosides: Singh, S. K.; Kumar, R.; Wengel, J. *J. Org. Chem.* **1998**, 63, 6078.

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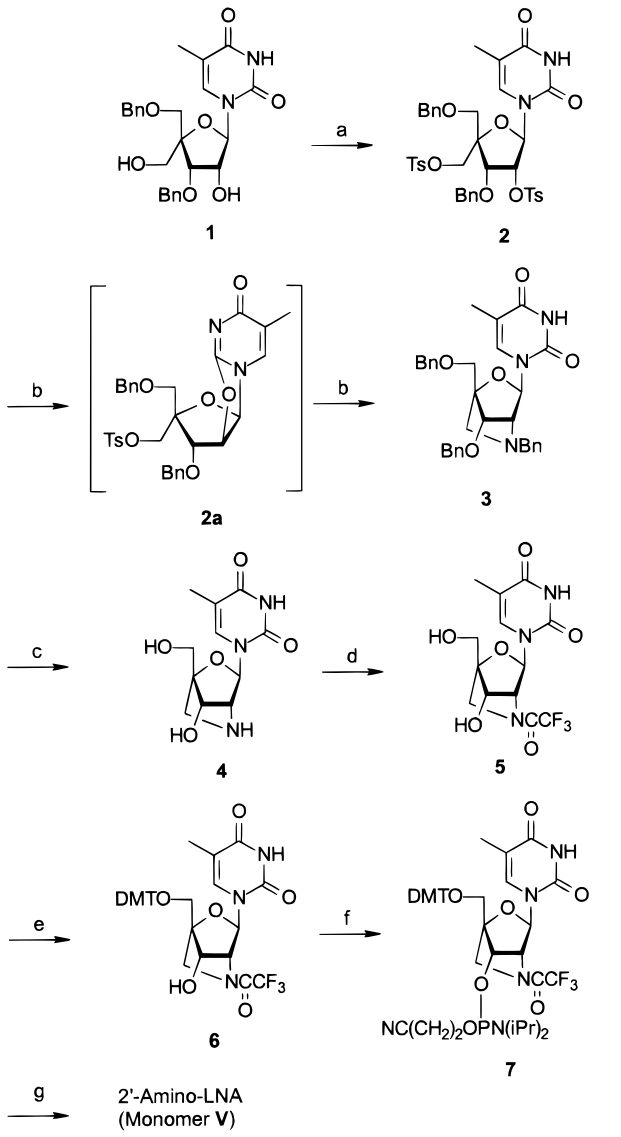
reaction conducted at only 80 °C, another product was isolated in trace amounts, which on the basis of NMR data (only one tosyl CH_3 signal, changed chemical shift values of the thymine moiety) and MS data was identified as the 2,2′-anhydro nucleoside derivative **2a**.[12] Thus, in analogy with earlier reports on double inversion at the 2′-position of nucleosides when introducing 2′-substituents,[13] we postulate the intermediacy of 2,2′-anhydro nucleoside **2a** in the transformation of nucleoside **2** into bicyclonucleoside **3**. However, as intramolecular displacement by an O-2 carbonyl is required for the suggested mechanism, it can be anticipated that an alternative synthetic strategy has to be developed for preparation of the corresponding purine bicyclonucleosides. Debenzylation of **3** to give the unprotected 2′-amino-LNA nucleoside **4** was accomplished in 68% yield using ammonium formate and 10% palladium on carbon (Scheme 1). This method proved superior to catalytic hydrogenation (20% $Pd(OH)_2/C$, H_2) furnishing a monobenzylated intermediate which eventually was transformed to product **4** using ammonium formate and 10% palladium on carbon.[11] For automated oligonucleotide synthesis by the phosphoramidite approach,[14] the 5′-*O*-dimethoxytrityl phosphoramidite **7** was synthesized. To avoid *N*-branching during oligomerization, the *N*-trifluoroacetyl-protected derivative was prepared. Thus, using essentially the method described by Ozaki et al.,[10b] nucleoside **5** was obtained in 81% yield from **4** by reaction with ethyl trifluoroacetate and DMAP. Subsequent 4,4′-dimethoxytritylation (to give nucleoside **6** in 93% yield) and 3′-*O*-phosphitylation (57% yield) afforded the nucleoside phosphoramidite **7** after precipitation from cold petroleum ether (Scheme 1). It is evident from the NMR data that the *N*-trifluoroacetyl derivatives **5**−**7** exist as a mixture of rotamers.

As mentioned above, the 2′-amino group of a 2′-amino-LNA nucleoside incorporated into an ON offers the possibility of attachment of, e.g., reporter groups, lipophilic carriers, or an aminoalkyl chain. Although some derivatizations of amino groups can be performed after oligomerization,[9] the use of *N*-alkylated 2′-amino-LNA monomers is a more appealing alternative expected to yield more well-defined oligomers. As the first step in this direction, we have developed a synthetic route to the 2′-methylamino-LNA nucleoside **10** and its phosphoramidite derivative **12** (Scheme 2). Direct alkylation of the unprotected 2′-amino-LNA nucleoside **4** using methyl iodide and NaH in DMF afforded a number of products according to analytical TLC. For simplicity, we therefore decided to block the hydroxyl groups by reaction with the bidentate reagent 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine, affording 3,5-di-*O*-silyl tricyclonucleoside **8** in 97% yield. By treatment with methyl iodide and 1,8-diazabicyclo[5.4.0]undec-7-ene in a mixture of THF and dichloromethane, alkylation proceeded efficiently to give 2′-methylamino derivative **9** in 74% yield and subsequent desilylation furnished 2′-methylamino-LNA nucleoside **10** in 79% yield. One significant advantage of introducing *N*-substituents before oligomerization is that, depending in the particular substituent, the need of introducing an additional *N*-protecting group is obliterated. To allow evaluation of *N*-methylated 2′-amino-LNA (monomer **X**), the nucleoside phsophoramidite **12** (Scheme 2) was synthesized by 5′-*O*-4,4′-dimethoxytritylation (to give nucleoside **11** in 72% yield) and subsequent 3′-*O*-phosphitylation (52% yield after precipitation from cold petroleum ether).

Scheme 1. Synthesis of 2′-Amino-LNA Nucleoside 4 and Amidite Derivative 7[a]



[a] Key: (a) TsCl, DMAP, anhydrous CH_2Cl_2; (b) $BnNH_2$; (c) 10% palladium on carbon, ammonium formate, CH_3OH; (d) ethyl trifluoroacetate, DMAP, CH_3OH; (e) 4,4′-dimethoxytrityl chloride, anhydrous pyridine, anhydrous dichloromethane; (f) *N*,*N*-diisopropylethylamine, 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, anhydrous dichloromethane; (g) DNA synthesizer. DMT = 4,4′-dimethoxytrityl.

2′-Amino-LNAs (monomers **V** and **X**) were efficiently synthesized using the phosphoramidite approach[14] on an automated DNA synthesizer. The stepwise coupling efficiencies as evaluated spectrophotometrically by the release of the 4,4′-dimethoxytrityl cation after each coupling step were >99% for all amidites, with coupling times of 12 min for amidites **7** and **12** and 2 min for unmodified 2′-deoxynucleoside phosphoramidites. Due to the satisfactory stepwise coupling yields rendering reversed-phase purification unnecessary, the 5′-*O*-4,4′-

(12) Derivative **2a**: FAB-MS *m*/*z* 605 $[M + H]^+$; ^{13}C NMR ($CDCl_3$, 62.9 MHz, selected signals) 172.0, 159.1, 21.7. No trace of bicyclonucleoside **3** was detected.

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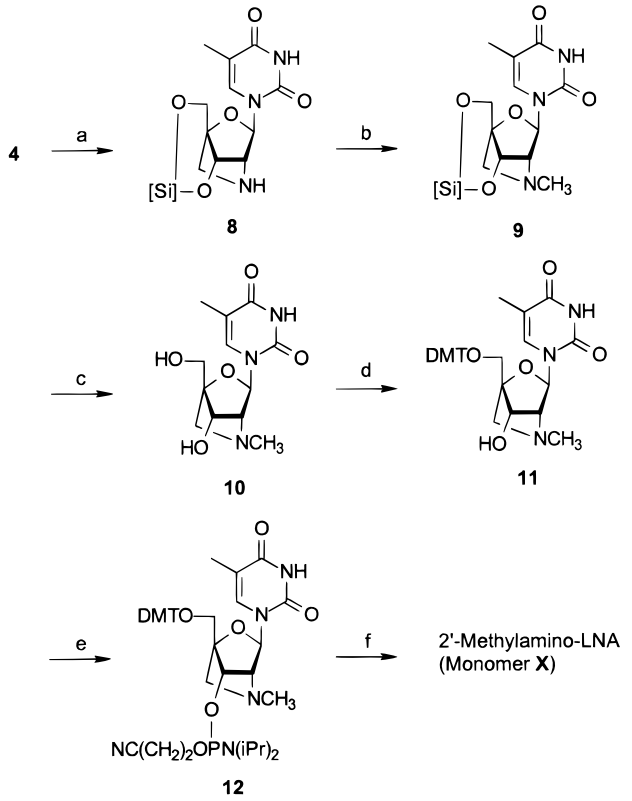
Scheme 2. Synthesis of 2′-Methylamino-LNA Nucleoside 10 and Amidite Derivative 12[a]

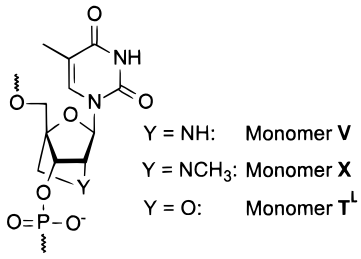


[a] Key: (a) 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, anhydrous pyridine; (b) methyl iodide, 1,8-diazabicyclo[5.4.0]undec-7-ene, anhydrous THF, anhydrous dichloromethane; (c) tetrabutylammonium fluoride, THF; (d) 4,4′-dimethoxytrityl chloride, anhydrous pyridine, anhydrous dichloromethane; (e) *N*,*N*-diisopropylethylamine, 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, anhydrous dichloromethane; (f) DNA-synthesizer. [Si] = 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl. DMT = 4,4′-dimethoxytrityl.

dimethoxytrityl group was removed after completion of the oligomerizations. After deprotection and cleavage from the solid support using concentrated ammonia, evaporation, and precipitation from ethanol, the 2′-amino-LNA 9-mer mixed-base sequences depicted in Table 1 (entries 5−9) were obtained. The purity was in all cases analyzed by capillary gel electrophoresis to be >90%, and the composition was verified by MALDI-MS. As expected, the *N*-trifluoroacetyl groups were efficiently removed during treatment with concentrated ammonia.

The thermal stabilities (T_m values) of duplexes involving 2′-amino-LNAs were determined toward complementary DNA and RNA (Table 1, entries 5−9) and compared to the thermal stabilities of the unmodified reference duplexes and LNA reference duplexes (Table 1, entries 1−4, ΔT_m = change in T_m per 2′-amino-LNA monomer incorporated). Generally, as observed for LNA monomer $\mathbf{T^L}$,[3] significantly increased thermal affinities were obtained toward both DNA and RNA, however, most convincingly toward the latter (ΔT_m = +6.0 to +8.0 °C). Whereas the effect toward DNA was identical for monomers **V** and **X** (ΔT_m = +3.0 °C), it appears that toward RNA the stabilizing effect of 2′-methylamino-LNA monomer **X** is slightly more pronounced than that of 2′-amino-LNA monomer **V**. It is noteworthy that the 2′-amino-LNA shown in entry 9 containing both monomers **V** and **X** displayed enhanced thermal stabilities (ΔT_m = +3.0 °C toward DNA and ΔT_m = +6.6 °C toward RNA) corresponding to an approximately additive effect of the monomers. The lack of coupling constants between H-1′ and H-2′ in the ^{1}H NMR spectra of **4** and **10** indicate that these 2′-amino-LNA nucleosides, like the parent LNA nucleosides, exist in a 3′-endo type of conformation.[15] This is furthermore shown by the strong NOEs observed between H-3′ and H-6 (6% NOE (for **4**) and 8% NOE (for **10**) in H-3′ by irradiation of H-6). Thus, the comparable thermal stability of duplexes involving 2′-amino-LNA and parent LNA[3] probably reflects the similar monomeric pentofuranose conformation and similar extent of hydration, and no indication of an additional stabilizing effect by the introduction of the basic 2′-amino functionality was observed. Therefore, the locked *ribo*-configured bicyclo[2.2.1]pentofuranose skeleton appears in general to be a convenient structural element in high-affinity nucleic acid mimics designed to contain monomeric nucleosides in a conformationally restricted 3′-endo type conformation.

Table 1. Sequences Synthesized and Melting Temperatures (T_m Values) Measured



		DNA complement		RNA complement	
entry	sequence[a]	T_m, °C[b]	ΔT_m, °C[c]	T_m, °C[b]	$-\Delta T_m$, °C[c]
	Reference Sequences				
1	5′-d(GTGATATGC)	30		28	
2	5′-d(GTGTTTTGC)	32		30	
3	5′-(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	44		50	
4	5′-d(G$\mathbf{T^L}$G$\mathbf{T^L}\mathbf{T^L}\mathbf{T^L}\mathbf{T^L}$GC)	52		67	
	2′-Amino-LNA Sequences				
5	5′-d(GTGA**V**ATGC)	33	+3.0	34	+6.0
6	5′-d(G**V**GA**V**A**V**GC)	39	+3.0	47	+6.3
	2′-Methylamino-LNA Sequences				
7	5′-d(GTGA**X**ATGC)	33	+3.0	36	+8.0
8	5′-d(G**X**GA**X**A**X**GC)	39	+3.0	49	+7.0
9	5′-(G**X**G**V****X****V****X**GC)	47	+3.0	63	+6.6

[a] A = 2′-deoxyadenosine monomer, C = 2′-deoxycytidine monomer, G = 2′-deoxyguanosine monomer, T = thymidine monomer, **V** = 2′-amino-LNA monomer (see below), **X** = 2′-methylamino-LNA monomer (see below). [b] T_m = melting temperature (measured in medium salt buffer: 10 mM Na_2HPO_4, pH 7.0, 100 mM NaCl, 0.1 mM EDTA). [c] ΔT_m = change in T_m per 2′-amino-LNA monomer **V** and/or **X**.

Conclusion

A synthetic route to unprotected 2′-amino-LNA nucleosides has been developed embarking on double nucleophilic substitution of di-*O*-tosyl nucleoside **1** using benzylamine. The overall retention of configuration at the 2′-position is suggested to be a result of the intermediacy of 2,2′-anhydro nucleoside **2a**, which apparently limits the applicability of the described strategy to pyrimidine nucleosides. Alkylation of the 2′-amino group to give 2′-methylamino-LNA nucleoside **10** has been accomplished

efficiently, furnishing a convenient route for future derivatizations of 2′-amino-LNA nucleosides. High-yielding oligomerizations afforded the first 2′-amino-LNA oligomers (containing monomers **V** and/or **X**), which displayed significantly increased thermal stability toward complementary DNA ($\Delta T_m = +3.0$ °C) and RNA ($\Delta T_m = +6.0$ to $+8.0$ °C). The applicability of the 2′-amino group as a handle for conjugation of high-affinity LNAs has been indicated by the properties of 2′-methylamino-LNA.

Experimental Section

General. Reactions were conducted under an atmosphere of nitrogen when anhydrous solvents were used. Column chromatography was carried out on glass columns using Silica gel 60 (0.040−0.063 mm). Values for δ are in ppm relative to tetramethylsilane as internal standard (^{1}H and ^{13}C NMR) and relative to 85% H_3PO_4 as external standard (^{31}P NMR). ^{1}H NOE spectra were recorded for compounds **4** and **10**. Fast-atom bombardment mass spectra (FAB-MS) were recorded in positive ion mode. Microanalyses were performed at The Microanalytical Laboratory, Department of Chemistry, University of Copenhagen.

1-(3,5-Di-*O*-benzyl-2-*O*-*p*-toluenesulfonyl-4-*C*-(*p*-toluenesulfonyloxymethyl)-*β*-D-ribofuranosyl)thymine (2). A solution of 1-(3,5-di-*O*-benzyl-4-*C*-(hydroxymethyl)-*β*-D-ribofuranosyl)-thymine[3b] (**1**, 1.48 g, 3.16 mmol), *N*,*N*-(dimethylamino)pyridine (1.34 g, 10.5 mmol), and *p*-toluenesulfonyl chloride (1.45 g, 7.6 mmol) in dichloromethane (20 mL) was stirred for 3 h at room temperature. The reaction mixture was diluted with dichloromethane (30 mL), washed with saturated aqueous solutions of sodium hydrogencarbonate (3 × 20 mL) and sodium chloride (2 × 25 mL), dried (Na_2SO_4), and filtered. After evaporation to dryness under reduced pressure, the residue was subjected to column chromatography on silica gel using methanol/dichloromethane (1:99, v/v) as eluent to give nucleoside **2** as a white solid material after evaporation of the solvents under reduced pressure (1.95 g, 80%): FAB-MS *m*/*z* 776 [M$^+$]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 8.50 (bs, 1H), 7.70−6.79 (m, 19H), 6.03 (d, 1H, *J* 8.0 Hz), 5.00−4.93 (m, 2H), 4.59 (m, 2H), 4.48 (d, 1H, *J* 10.8 Hz), 4.37 (d, 1H, *J* 4.9 Hz), 4.10 (d, 1H, *J* 10.4 Hz), 4.02 (d, 1H, *J* 10.4), 3.75 (d, 1H, *J* 10.2 Hz), 3.66 (d, 1H, *J* 10.2 Hz), 2.41 (s, 3H), 2.32 (s, 3H), 1.37 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 162.9, 149.8, 145.8, 145.2, 136.9, 136.8, 134.3, 132.1, 132.0, 130.0, 129.9, 129.0 128.9, 128.4, 128.3, 128.2, 128.0, 127.7, 111.2, 85.3, 84.0, 78.9, 78.3, 75.2, 74.3, 72.7, 69.1, 21.7, 11.9. Anal. Calcd for $C_{39}H_{40}N_2S_2O_{11}$: C, 60.3; H, 5.2; N, 3.6. Found: C, 60.0; H, 5.1; N 3.8.

1-(2-Amino-2-*N*,4-*C*-methylene-2-*N*,3-*O*,5-*O*-tribenzyl-*β*-D-ribofuranosyl)thymine (3). A solution of nucleoside **2** (8.0 g, 10.0 mmol) in benzylamine (5 mL) was stirred at 130 °C for 20 h. The reaction mixture was subsequently directly subjected to column chromatography on silica gel using methanol/dichloromethane (1:99, v/v) as eluent to give nucleoside **3** as a white solid material after evaporation of the solvents under reduced pressure (2.9 g, 52%): FAB-MS *m*/*z* 540 [M + H]$^+$; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.58 (d, 1H, *J* 1.1 Hz), 7.40−7.23 (m, 15H), 5.79 (s, 1H), 4.68−4.48 (m, 4H), 4.08 (s, 2H), 3.95 (s, 1H), 3.78 (m, 2H), 3.66 (s, 1H, 2′-H), 3.04 (d, 1H, *J* 9.5 Hz), 2.73 (d, 1H, *J* 9.5 Hz), 1.57 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 163.9, 149.8, 139.2, 137.6, 137.3, 135.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.7, 127.0, 109.6, 88.2, 86.3, 76.7, 73.8, 72.0, 66.0, 63.8, 57.9, 57.8, 12.2. Anal. Calcd for $C_{32}H_{33}N_3O_5 \cdot 0.5H_2O$: C, 70.1; H, 6.3; N, 7.7. Found: C, 70.0; H, 6.1; N, 7.5.

1-(2-Amino-2-*N*,4-*C*-methylene-*β*-D-ribofuranosyl)thymine (4). To a solution of nucleoside **3** (2.80 g, 5.20 mol) in methanol (120 mL) was added ammonium formate (1.70 g, 0.027 mol) and 10% palladium on carbon (4 g), and the resulting suspension was heated under reflux for 4 h. The catalyst was filtered off (silica gel, washed with methanol, 15 mL), the filtrate was concentrated to dryness under reduced pressure, and the residue was subjected to column chromatography on silica gel using methanol/dichloromethane (1:9, v/v) as eluent to give nucleoside **4** as a white solid material after evaporation of the solvents under reduced pressure (0.95 g, 68%): FAB-MS *m*/*z* 270 [M + H]$^+$; ^{1}H NMR ($(CD_3)_2SO$, 250 MHz) δ 11.29 (bs, 1H, NH), 7.73 (d, 1H, *J* 1.1 Hz, 6-H), 5.31 (s, 1H, 1′-H), 5.29 (bd, 1H, *J* 3.7 Hz, 3′-OH), 5.13 (t, 1H, *J* 5.3 Hz, 5′-OH), 3.81 (s, 1H, 3′-H), 3.69 (d, 2H, J 5.1 Hz, 5′-H), 3.23 (s, 1H, 2′-H), 2.88 (d, 1H, *J* 9.8 Hz, 1″-H_a), 2.55 (d, 1H, *J* 9.8 Hz, 1″-H_b), 1.77 (d, 3H, *J* 0.8 Hz, CH_3); ^{13}C NMR ($(CD_3)_2SO$, 62.9 MHz) δ 164.0, 150.1, 135.6, 107.8, 89.5, 87.9, 68.7, 61.9, 57.1, 49.4, 12.4. Anal. Calcd for $C_{11}H_{15}N_3O_5 \cdot 0.5H_2O$: C, 47.5; H, 5.8; N, 15.1. Found: C, 47.6; H, 5.3; N, 14.8.

1-(2-Amino-2-*N*,4-*C*-methylene-2-*N*-trifluoroacetyl-*β*-D-ribofuranosyl)thymine (5). To a suspension of nucleoside **4** (0.050 g, 0.186 mmol) in methanol (2 mL) was added *N*,*N*-(dimethylamino)pyridine (0.013 mg, 0.106 mmol) and ethyl trifluoroacetate (0.029 mL, 0.242 mmol), and the mixture was stirred at room temperature for 2.5 h. The solvent was removed under reduced pressure, and the residue was subjected to column chromatography on silica gel using methanol/dichloromethane (2.5:97.5, v/v) as eluent to give nucleoside **5** as a white solid material after evaporation of the solvents under reduced pressure (0.055 g, 81%): FAB-MS *m*/*z* 366 [M + H]$^+$; ^{13}C NMR (CD_3OD, 62.9 MHz) δ 166.5, 157.7 (q, $^2J_{C,F}$ 37.5 Hz, $COCF_3$), 157.6 (q, $^2J_{C,F}$ 37.2 Hz, $COCF_3$), 151.8, 136.8, 136.8, 117.6 (d, $^1J_{C,F}$ 287.5 Hz, CF_3), 117.5 (d, $^1J_{C,F}$ 286.5 Hz, CF_3), 110.8, 110.8, 90.7, 89.3, 87.7, 87.3, 70.1, 68.6, 66.2, 66.2, 64.5, 57.9, 53.3, 12.7. Anal. Calcd for $C_{13}H_{14}N_3O_6F_3$: C, 42.8; H, 3.9; N, 11.5. Found: C, 42.5; H, 4.0; N, 11.2.

1-(2-Amino-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-2-*N*-trifluoroacetyl-*β*-D-ribofuranosyl)thymine (6). To a solution of nucleoside **5** (0.030 g, 0.082 mmol) in anhydrous pyridine (0.6 mL) at 0 °C was added dropwise (during 20 min) 4,4′-dimethoxytrityl chloride (0.054 g, 0.159 mmol) dissolved in anhydrous pyridine/dichloromethane (0.6 mL, 1:1, v/v), and the mixture was stirred for 10 h at room temperature. A mixture of ice and water was added (5 mL), and the resulting mixture was extracted with dichloromethane (3 × 5 mL). The combined organic phase was washed with a saturated aqueous solution of sodium hydrogencarbonate (3 × 2 mL), dried (Na_2SO_4), and filtered. The filtrate was evaporated to dryness under reduced pressure, and the residue was subjected to column chromatography on silica gel using methanol/dichloromethane/pyridine (1.5:98.0:0.5, v/v/v) as eluent to give nucleoside **6** as a white solid material after evaporation of the solvents under reduced pressure (0.051 g, 93%): FAB-MS *m*/*z* 667 [M]$^+$, 668 [M + H]$^+$; FAB-HRMS calcd for $C_{34}H_{32}N_3O_8F_3$+ 667.2142, found 667.2146; ^{13}C NMR (C_5D_5N, 100.6 MHz) δ 165.1, 165.0, 159.5, 159.5, 151.4, 145.7, 136.3, 136.1, 134.8, 134.6, 130.9, 130.9, 130.9, 128.9, 128.9, 128.7, 128.7, 128.4, 127.7, 123.2, 114.1, 114.1, 114.0, 110.4, 89.4, 87.9, 87.5, 87.4, 87.2, 70.8, 69.0, 66.0, 64.4, 60.5, 60.2, 55.5, 53.6, 53.4, 49.9, 13.2, 13.1.

1-(2-Amino-3-*O*-(2-cyanoethoxy(diisopropylamino)-phosphinoxy)-5-*O*-4,4′-dimethoxytrityl-2-*N*,4-*C*-methylene-2-*N*-trifluoroacetyl-*β*-D-ribofuranosyl)thymine (7). To a solution of nucleoside **6** (0.121 g, 0.181 mmol) in anhydrous dichloromethane (2 mL) were added *N*,*N*-diisopropylethylamine (0.093 mL, 0.54 mmol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (0.057 mL, 0.26 mmol) at 0 °C, and the mixture was stirred for 10 h at room temperature. The mixture was diluted with dichloromethane (20 mL), extracted with a saturated aqueous solution of sodium hydrogencarbonate (3 × 10 mL), dried (Na_2SO_4), and filtered. The filtrate was evaporated to dryness under reduced pressure, and the residue was subjected to column chromatography on silica gel using methanol/dichloromethane/pyridine (1.5:98.0:0.5, v/v/v) as eluent to give crude **7** (0.107 g) after evaporation of the solvents under reduced pressure. The residue was dissolved in anhydrous dichloromethane (1 mL), and by dropwise addition to vigorously stirred petroleum ether (60−80 °C, 30 mL) at −30 °C, nucleotide **7** precipitated to give a white solid material after filtration (0.090 g, 57%): FAB-MS *m*/*z* 868 [M + H]$^+$, 890 [M + Na]$^+$; ^{31}P NMR (CD_3CN, 121.5 MHz) δ 150.4, 150.2, 148.8, 149.1.

1-(2-Amino-2-*N*,4-*C*-methylene-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-*β*-D-ribofuranosyl)thymine (8). To a solution of nucleoside **4** (0.20 g, 0.74 mmol) in anhydrous pyridine (3 mL) at −15 °C was added dropwise (during 3 h) 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.305 mL, 0.0011 mol), and the mixture was stirred for 10 h at room temperature. MeOH (3 mL) was added, and the mixture was evaporated to dryness under reduced pressure. The residue was subjected to

column chromatography on silica gel using methanol/dichloromethane (1:99, v/v) to give nucleoside **8** as a white solid material after evaporation of the solvents under reduced pressure (0.370 mg, 97%): FAB-MS *m*/*z* 512 $[M + H]^+$; ^{1}H NMR ($(CD_3)_2SO$, 400 MHz) δ 11.37 (bs, 1H), 7.48 (s, 1H), 5.32 (s, 1H), 4.06 (d, 1H, *J* 13.5 Hz), 4.00 (s, 1H), 3.84 (d, 1H, *J* 13.5 Hz), 3.41 (s, 1H), 2.92 (d, 1H, *J* 10.2 Hz), 2.64 (d, 1H, *J* 10.2 Hz), 1.74 (s, 3H), 1.10−0.92 (m, 28 H); ^{13}C NMR ($(CD)_3SO_2$, 62.9 MHz) δ 163.8, 149.8, 134.1, 107.9, 89.5, 87.9, 70.1, 61.1, 57.9, 49.3, 17.2, 17.2, 17.0, 16.9, 16.8, 16.7, 12.6, 12.2, 11.7. Anal. Calcd for $C_{23}H_{41}N_3O_6Si_2$: C, 54.0; H, 8.1; N, 8.2. Found: C, 54.0; H, 8.3; N, 7.8.

1-(2-Methylamino-2-*N*,4-*C*-methylene-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl)thymine (9). To a solution of nucleoside **8** (0.33 g, 0.64 mmol) in anhydrous THF/dichloromethane (4:1, v/v) at −10 °C was added dropwise (during 30 min) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.125 mL, 0.836 mmol) and methyl iodide (0.05 mL, 0.79 mmol), and the mixture was stirred for 48 h at 10 °C. Additional DBU (0.05 mL, 0.33 mmol) and methyl iodide (0.020 mL, 0.32 mmol) was added dropwise (during 15 min) to the reaction mixture, and stirring at 10 °C was continued for 24 h. The mixture was evaporated to dryness under reduced pressure, and the residue was subjected to column chromatography on silica gel using methanol/dichloromethane (1:99, v/v) as eluent to give nucleoside **9** as a white solid material after evaporation of the solvents (0.25 g, 74%): FAB-MS *m*/*z* 526 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.19 (bs, 1H), 7.65 (d, 1H, *J* 1.3 Hz), 5.59 (s, 1H), 4.11 (s, 1H), 4.05 (d, 1H, *J* 13.2 Hz), 3.87 (d, 1H, *J* 13.2 Hz), 3.44 (s, 1H), 2.98 (d, 1H, *J* 9.5 Hz), 2.71 (d, 1H, *J* 9.5 Hz), 2.72 (s, 3H), 1.91 (d, 1H, *J* 1.1 Hz), 1.12−0.96 (m, 28H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 163.7, 149.6, 135.2, 109.7, 90.9, 85.7, 71.4, 67.3, 58.6, 58.2, 41.2, 17.5, 17.4, 17.3, 17.2, 17.1, 16.9, 13.3, 13.1, 13.0, 12.6, 12.1. Anal. Calcd for $C_{24}H_{44}N_3O_6Si_2 \cdot 0.25H_2O$: C, 54.4; H, 8.3; N, 7.9. Found: C, 54.4; H, 8.1; N, 7.7.

1-(2-Methylamino-2-*N*,4-*C*-methylene-β-D-ribofuranosyl)-thymine (10). To a solution of nucleoside **9** (0.40 g, 0.76 mmol) in THF at room temperature was added a solution of tetrabutylammonium fluoride in THF (1.0 M, 2.2 mL, 2.2 mmol), and the reaction mixture was stirred for 20 min whereupon pyridine/water/methanol (6 mL, 3:1:1, v/v/v) was added. The mixture was added to Dowex 50 × 200 resin (2.2 g, H^+ (pyridinium) form, 100−200 mesh) suspended in pyridine/water/methanol (6 mL, 3:1:1, v/v/v), and the resulting mixture was stirred for 20 min. After filtration, the residue was washed with pyridine/water/methanol (3 × 3 mL, 3:1:1, v/v/v), and the combined filtrate was evaporated to dryness under reduced pressure to give an oily residue after coevaporation with methanol (2 × 5 mL). Column chromatography on silica gel using methanol/dichloromethane (1:49, v/v) as eluent gave nucleoside **10** as a white solid material after evaporation of the solvents under reduced pressure (0.17 g, 79%): FAB-MS *m*/*z* 284 $[M + H]^+$; FAB-HRMS calcd for $C_{12}H_{18}N_3O_5+$ 284.12465, found 284.12402; ^{1}H NMR ($(CD_3)_2SO$, 400 MHz) δ 11.3 (bs, 1H, NH), 7.70 (d, 1H, *J* 1.1 Hz, 6-H), 5.50 (s, 1H, 1′-H), 5.26 (d, 1H, *J* 4.9 Hz, 3′-OH), 5.12 (t, 1H, *J* 5.7 Hz, 5′-OH), 3.87 (d, 1H, *J* 4.8 Hz, 3′-H), 3.67 (d, 2H, *J* 5.5 Hz, 5′-H), 3.12 (s, 1H, 2′-H), 2.87 (d, 1H, *J* 9.3 Hz, 5″-H_a), 2.56 (s, 3H, NCH_3), 2.52−2.49 (1H, m, 5″-H_b), 1.77 (s, 3H, CH_3); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.80 (d, 1H, *J* 1.3 Hz, 6-H), 5.71 (s, 1H, 1′-H), 4.07 (s, 1H, 3′-H), 3.83 (s, 2H, 5′-H), 3.36 (s, 1H, 2′-H), 3.08 (d, 1H, *J* 9.9 Hz, 5″-H_a), 2.68 (s, 3H, NCH_3), 2.57 (d, 1H, *J* 9.9 Hz, 5″-H_b), 1.88 (d, 3H, *J* 1.1 Hz, CH_3); ^{13}C NMR (CD_3OD, 62.9 MHz) δ 166.6, 151.9, 137.4, 110.4, 91.3, 85.2, 71.4, 69.1, 59.4, 58.7, 40.2, 12.2.

1-(5-*O*-4,4′-Dimethoxytrityl-2-methylamino-2-*N*,4-*C*-methylene-β-D-ribofuranosyl)thymine (11). To a solution of nucleoside **10** (0.135 g, 0.477 mmol) in anhydrous pyridine (1.5 mL) at 0 °C was added dropwise (during 20 min) a solution of 4,4′-dimethoxytrityl chloride (0.238 g, 0.702 mmol) in anhydrous pyridine/dichloromethane (1.0 mL, 1:1, v/v), and the resulting mixture was stirred for 10 h at room temperature. A mixture of ice and water was added (5 mL), and the mixture was extracted with dichloromethane (3 × 10 mL). The combined organic phase was washed with a saturated aqueous solution of sodium hydrogencarbonate (3 × 5 mL), dried (Na_2SO_4), and filtered. The filtrate was evaporated to dryness under reduced pressure, and the residue was subjected to column chromatography on silica gel using methanol/dichloromethane/pyridine (1:98:1, v/v/v) as eluent to give nucleoside **11** as a white solid material after evaporation of the solvents under reduced pressure (0.20 g, 72%): FAB-MS *m*/*z* 586 $[M + H]^+$; ^{1}H NMR (C_5D_5N, 400 MHz) δ 13.2 (bs, 1H), 7.98 (d, 1H, *J* 1.3 Hz), 7.98−7.00 (m, 13H), 6.12 (s, 1H), 4.78 (d, 1H, *J* 3.7 Hz), 3.88−3.79 (m, 4H), 3.71 (s, 3H), 3.71 (s, 3H), 3.29 (d, 1H, *J* 9.3 Hz), 2.84 (d, 1H, *J* 9.3 Hz), 2.81 (s, 3H), 1.85 (d, 3H, *J* 0.9 Hz); ^{13}C NMR (C_5D_5N, 62.9 MHz) δ 165.1, 159.2, 151.4, 145.9, 136.5, 136.4, 130.8, 130.7, 128.7, 128.4, 127.4, 113.8, 109.6, 89.8, 86.8, 85.1, 72.0, 68.7, 60.9, 59.4, 55.2, 40.1, 13.1. Anal. Calcd for $C_{33}H_{35}N_3O_7 \cdot 0.25H_2O$: C, 67.2; H, 6.1; N, 7.1. Found: C, 67.2; H, 6.2; N, 6.9.

1-(3-*O*-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-5-*O*-4,4′-dimethoxytrityl-2-methylamino-2-*N*,4-*C*-methylene-β-D-ribofuranosyl)thymine (12). To a solution of nucleoside **11** (0.130 g, 0.222 mmol) in anhydrous dichloromethane (2 mL) at 0 °C were added *N*,*N*-diisopropylethylamine (0.088 mL, 0.514 mmol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (0.065 mL, 0.291 mmol), and the mixture was stirred for 10 h at room temperature. Dichloromethane (30 mL) was added, and the mixture was extracted with a saturated aqueous solution of sodium hydrogencarbonate (3 × 10 mL), dried (Na_2SO_4), and filtered. The filtrate was evaporated to dryness under reduced pressure, and the residue was subjected to column chromatography on silica gel using methanol/dichloromethane/pyridine (0.5:98.5:1.0, v/v/v) as eluent to give crude **12** (0.120 g) after evaporation of the solvents under reduced pressure. The residue was dissolved in anhydrous dichloromethane (1 mL), and by dropwise addition to vigorously stirred petroleum ether (60−80 °C, 30 mL) at −30 °C, nucleotide **12** precipitated to give a white solid material after filtration (0.090 g, 52%): ^{31}P NMR (CD_3CN, 121.5 MHz) δ 147.7.

2′-Amino-LNA Synthesis and Analysis. The 2′-amino-LNA 9-mers (Table 1, entries 3−7) were synthesized on an automated DNA synthesizer using standard procedures and amidites **7** and **12** (12 min couplings) and commercial 2′-deoxynucleoside 2-cyanoethyl *N*,*N*-diisopropylphosphoramidites (2 min couplings). The stepwise coupling yield for all amidites was >99%. After completion of the syntheses of the 5′-*O*-4,4′-dimethoxytrityl-OFF 2′-amino-LNAs, deprotection and cleavage from the solid support was accomplished using concentrated ammonia (32% (w/w), 55 °C, 12 h). After evaporation under reduced pressure and precipitation from ethanol, the purity was analyzed by capillary gel electrophoresis to be >90% for all 2′-amino-LNAs. The composition of the 2′-amino-LNAs was verified by MALDI-MS $[M - H]^-$: calcd for 5′-d(GTGA**V**ATGC) 2779.9, found 2778.5; calcd for 5′-d(G**V**GA**V**A**V**GC) 2833.9, found 2832.3; calcd for 5′-d(GTGA**X**ATGC) 2793.9, found 2793.5; calcd for 5′-d(G**X**GA**X**A**X**GC) 2876.0, found 2875.5; calcd for 5′-d(G**X**G**V**X**V**X**V**GC) 2912.0, found 2910.8.

Melting Temperature Measurements. The thermal affinity studies were performed as described earlier[3b] using 1.5 μM concentrations of the two complementary strands, assuming identical extinction coefficients for 2′-amino-LNA and the corresponding unmodified oligonucleotides.

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