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Base−Base Interactions in the Minor Groove of Double-Stranded DNA

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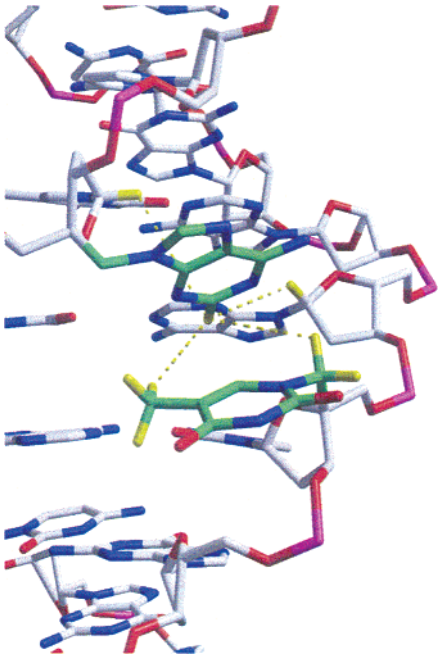

A method has been developed for the synthesis of bisheaded nucleosides with thymine and adenine base moieties. We have demonstrated that, when incorporated in oligonucleotides, extrahelical A−T base interactions are possible when the bisheaded nucleosides are positioned in opposite strands of the duplex and are separated from each other by one regular base pair.

Introduction

Two strands of complementary DNA form a helical duplex in which the base pairs form the core of the duplex, and these pairs are accessible from the outside via the major and minor grooves. These grooves can be used for sequence-specific recognition of double-stranded DNA with heterocyclic polyamides[1] or with triple helix forming oligonucleotides.[2] Presenting bases outside the helix has its precedent in biochemistry (base flipping for nucleic acid methylation),[3] in structural biology

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(1) Dervan, P. B.; Edelson, B. S. *Curr. Opin. Struct. Biol.* **2003**, *13*, 284−299.

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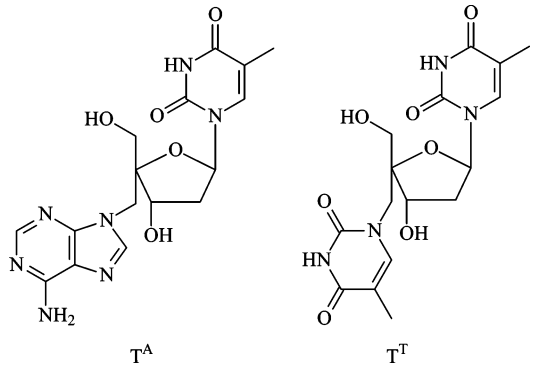

FIGURE 1. Nucleosides with two base moieties of the T^A and T^T motif.

(such as the formation of bulge loops),[4] as a spontaneous event (the flipped-out base can be trapped using β-cyclodextrin),[5] and in the field of synthetic nucleic acid chemistry.[6] In the latter example, two base moieties on the same backbone motif are involved in the formation of a stable duplex (i.e., one base is involved in base pairing within the double helix, while the second base is sticking out in the minor groove).

The structure repertoire of nucleic acids could be expanded, and the specificity of recognition between two oligonucleotides could be increased, when base pairing outside the double helix could be realized. Extending the genetic information system in regular DNA is usually done by increasing the number of specific base pairs[7] and not by increasing the number of canonical base pairs involved in the recognition process. Additional base pair recognition might be realized by introduction of bisheaded nucleotides in complementary DNA strands which can interact with each other in the grooves of the duplex.

To evaluate the potential of this recognition system, we synthesized an oligonucleotide based on a T^A and T^T motif (X^Y; X−base involved in base pairing within the double helix; Y−base presented at the outside of the helix) (Figure 1). The second base is connected at a 4′-*C*-methyl group of the nucleoside. A model shows that base pairing within the minor groove might be possible between a 4′-*C*-purine and a 4′-*C*-pyrimidine base, provided that the modified nucleotides are introduced within opposite strands of the double-stranded DNA and separated from each other by one regular base pair (Figure 2).

Results and Discussion

Synthesis. Synthetic chemistry for the introduction of a functionalized alkyl group in the 4′-position of a nucleoside has been described previously,[8,9] and our slightly modified procedure is based on this chemistry.

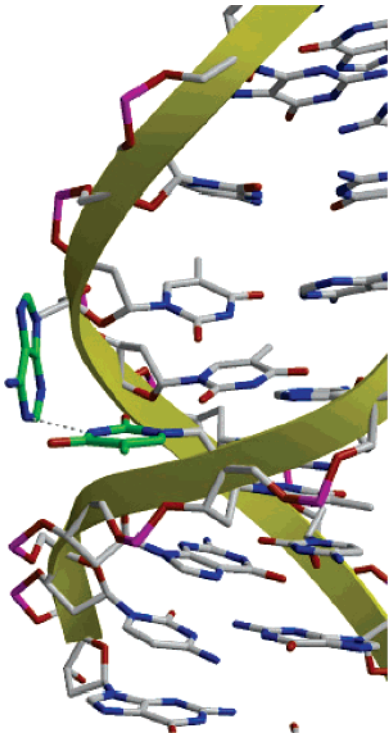

FIGURE 2. Potential for base pairing within the minor groove of double-stranded DNA using complementary bisheaded nucleosides.

The synthetic route to 4′-*C*-[(N^6-benzoyladenin-9-yl)methyl]-3′-*O*-[2-cyanoethoxy-(diisopropyl) aminophosphinyl]-5′-*O*-monomethoxytritylthymidine is outlined in Scheme 1.

The 3′-*O*-(*tert*-butyldimethylsilyl)-4′-(hydroxymethyl)-thymidine **1** was synthesized according to a previously described procedure.[10] Both primary hydroxyl groups of **1** were converted into their triflate by using trifluoromethanesulfonyl chloride in pyridine, giving compound **2** in 85% yield. The adenine base was introduced at the 4′-position using NaH and adenine in dry DMF at 50 °C in 64% yield. 2D NMR (gHMBC) was used to confirm the right connection between sugar and nucleobase moieties. The coupling between the 5′-H of sugar and the 2-C of thymine proved the formation of a 5′,O^2-anhydrobond. The protons of the 4′-methylene group were coupled with both the C-4 and C-8 of the adenine base.

Hydrolysis of the anhydride using 2 N NaOH in dioxane yielded compound **4** in 46% yield. The 5′-OH of compound **4** was protected with a TBDMS group using TBDMSCl and imidazole in acetonitrile in 58% yield. Protection of the exocyclic amino group of the adenine base was achieved using an excess of benzoyl chloride in pyridine and subsequent treatment with sat. NH_3/MeOH at 0 °C for half an hour. The desired monobenzoyl-protected amine **6** was obtained in 94% yield. Both TBDMS groups were removed using TBAF, giving compound **7** in 57% yield. Reaction of compound **7** with MMTrCl in anhydrous pyridine afforded **8** in 81% yield. Finally, the secondary hydroxyl group of **8** was phosphitylated to obtain the desired phosphoramidite **9**, which was then used as a building block for oligonucleotide synthesis.

Following a similar synthetic strategy for obtaining compound **9**, 4′-*C*-(thymin-1-yl)methyl-5′-monomethoxytrityl-3′-*O*-[2-cya-

(3) Allan, B. W.; Beechem, J. M.; Lindstrom, W. M.; Reich, N. O. *J. Biol. Chem.* **1998**, *273*, 2368−2373.
(4) Shen, L. X.; Cai, Z.; Tinoco, I., Jr. *FASEB J.* **1995**, *9*, 1023−1033.
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(6) Wu, T.; Froeyen, M.; Schepers, G.; Mullens, K.; Rozenski, J.; Busson, R.; Van Aerschot, A.; Herdewijn, P. *Org. Lett.* **2004**, *6*, 51−54.
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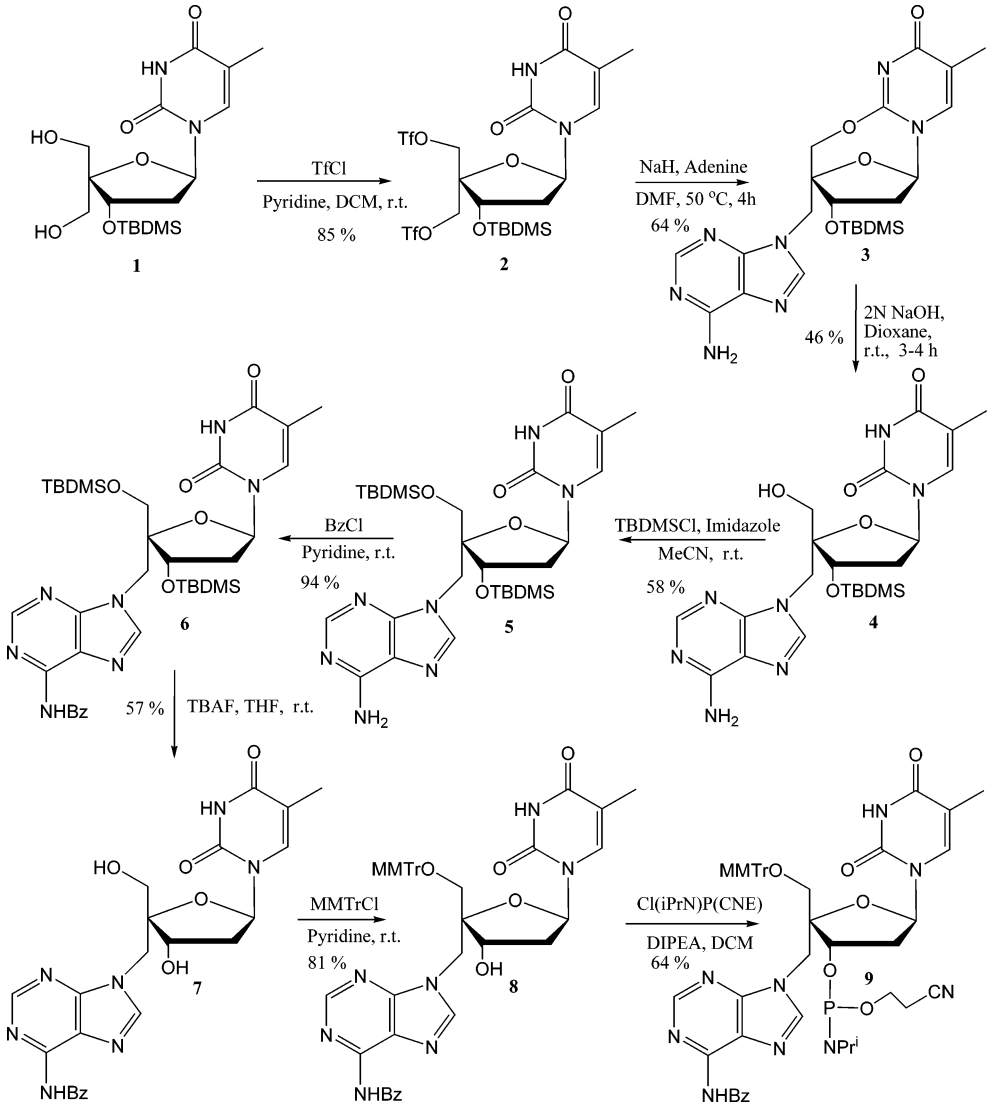
SCHEME 1. Synthesis of the T^A Building Block



noethoxy diisopropylaminophosphinyl]-thymidine was obtained as outlined in Scheme 2.

Substitution of **2** using NaH and thymine in dry DMF at 50 °C gave compound **10**, which was treated with 2 N NaOH in dioxane. This compound was difficult to purify using column chromatography due to comigration of an impurity. Therefore, preparative TLC was used to obtain a pure sample. The 2D NMR spectrum (gHMBC) showed that the protons on the extra methylene group at 4′-C of **11** are coupled with C-2 and C-6 of the newly introduced thymine base of compound **11**, proving the desired connection between thymine and methylene group on 4′-C. The right connection between N^1 of the newly introduced thymine and carbon 4′-C is characterized by the peak at 46.92 ppm in the ^{13}C NMR spectrum. NMR (^1H and ^{13}C) can be used to distinguish between a C−N and C−O bond in the alkylation reaction of pyrimidine bases (for example, see ref 11).

The TBDMS group of compound **11** was removed using tetrabutylammonium fluoride. Compound **12** was obtained in 12% overall yield from compound **2**. Tritylation of **12** with MMTrCl in anhydrous pyridine afforded **13** in 83% yield. Finally, phosphitylation of compound **13** gave the desired phosphoramidite **14**.

With the phosphoramidites **9** and **14** at hand, several oligonucleotides were synthesized comprising single or triple incorporations of the modified thymidine nucleoside analogues. The assembly was performed using the phosphoramidite approach. The nucleoside analogues were used at 0.07 M, and coupling was allowed to proceed for 12 min. Ethylthiotetrazole (ETT) was used as activator, resulting mostly in good coupling yields (>95%). The synthesized oligomers were isolated following ion exchange chromatography at pH 12 and desalting by gel filtration. The alkaline pH ensures denaturing conditions and allows straightforward isolation of the desired peak in >90% purity. All synthesized oligonucleotides were analyzed by mass spectrometry (LC/MS, see the Experimental Section) to prove the correct incorporation of the nucleotide analogues, and the obtained data are shown in Table 1. After submission of this manuscript, an article was published describing the incorporation of a 2′-*C*-(2-(thymine-1-yl)ethyl nucleoside in an LNA-modified oligonucleotide and studying its influence on the stability of three-way junctions.[12]

(11) Hossain, N.; Rozenski, J.; De Clercq, E.; Herdewijn, P. *J. Org. Chem.* **1997**, *62*, 2442−2447.

SCHEME 2. Synthesis of the TT Building Block

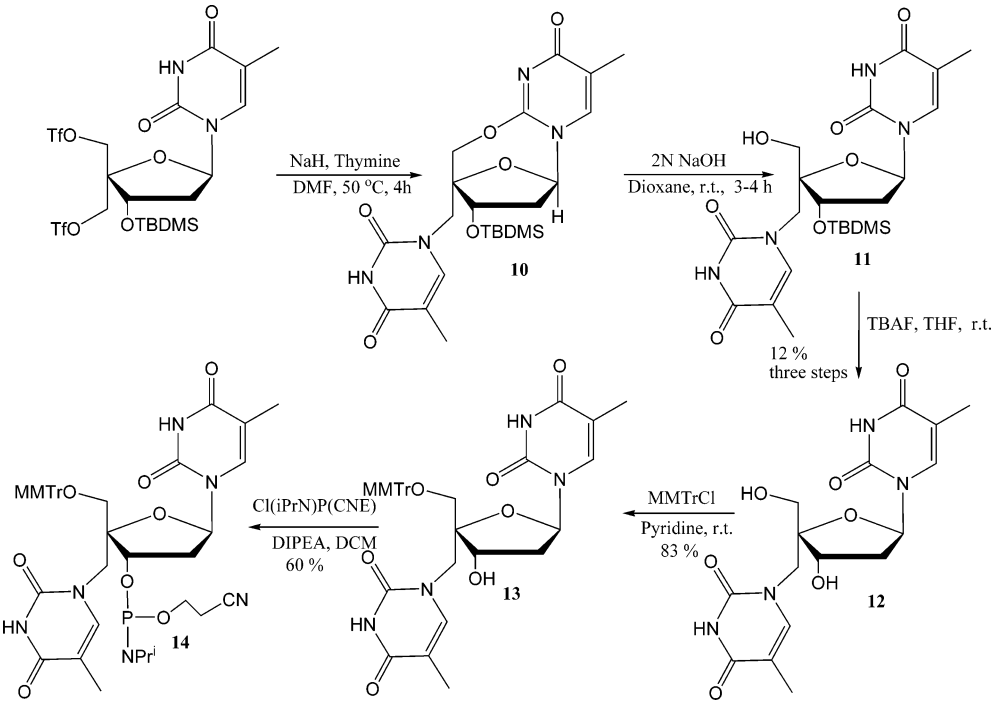


TABLE 1. ESI-MS Monoisotopic Data of Oligonucleotides Containing Modified Monomeric Units

DNA sequences	mass calcd[a]	ESI-MS
d(3′-CGC $T^AT^AT^AT^ACGC$-5′)	3556.7	3556.7
d(3′-GCGT^{T}AAGCG-5′)	2900.6	2900.6
d(3′-CGCT^{A}TACGC-5′)	2820.5	2820.5
d(3′-CGCT^{T}TACGC-5′)	2811.5	2811.5
d(3′-GCGT^{A}AAGCG-5′)	2909.6	2909.5

[a] Calculated value on average mass.

Thermal Stability Study. The phosphoramidites **9** and **14** were used to incorporate the 4′-branched thymidines T^A and T^T into several oligonucleotides. In the first sequence (3′-CGCT^{A}T^{A}T^{A}T^{A}CGC-5′), we incorporated four consecutive T^A moieties. This allowed us to evaluate the influence of the sugar moiety versus an acyclic backbone on dsDNA stability. When using four consecutive acyclic monomers as described before,[6] the ΔT_m/mod was as high as −4.0 °C. However, when using four T^A analogues, the ΔT_m/mod amounted to only −0.4 °C (Table 2 entries 1 and 2). A considerable increase in duplex stability therefore is observed when replacing the acyclic backbone by a 4′-*C*-branched furanose backbone. Recently, Wengel et al. reported that introduction of 4′-*C*-(*N*-methylpiperazino)methyl-substituted nucleosides into DNA significantly increased the thermal stability of a DNA duplex,[13] due to the possibility of salt formation of the piperazine moiety. In contrast to this 4′-*C*-(*N*-methylpiperazino)methyl moiety, the base (A) connected at the 4′-*C*-methyl group is not expected to be protonated at physiological pH. With a single incorporation, however, there was a detrimental loss in affinity, albeit still smaller than with the acyclic monomers (entries 4 and 6). With the thymine base at the branching point (T^T), the destabilization

TABLE 2. Influence of the Incorporation of Several Modifications (T^T and T^A) on the Stability of DNA Duplexes[a]

entry	sequences	T_m	ΔT_m	ΔT_m/mod
1	d(3′-CGC TTTTCGC-5′) d(5′-GCG AAAAGCG-3′)	46.4		
2	d(3′-CGC $T^AT^AT^AT^A$CGC-5′) d(5′-GCG A A A A GCG-3′)	45.2	−1.5	−0.4
3	d(3′-GCGTAAGCG-5′) d(5′-CGCATT CGC-3′)	40.7		
4	d(3′-GCGT^{A}AAGCG-5′) d(5′-CGCA TT CGC-3′)	30.0	−10.7	−10.7
5	d(3′-GCGT^{T}AAGCG-5′) d(5′-CGCA TT CGC-3′)	39.0	−1.7	−1.7
6	d(3′-GCGTAA GCG-5′) d(5′-CGCATT^A CGC-3′)	34.7	−6.0	−6.0
7	d(3′-GCGTAA GCG-5′) d(5′-CGCATT^T CGC-3′)	38.5	−2.2	−2.2
8	d(3′-GCGT^{T}AAGCG-5′) d(5′-CGCATT^A CGC-3′)	35.3	−5.4	−2.7
9	d(3′-GCGT^{T}AAGCG-5′) d(5′-CGCATT^T CGC-3′)	36.3	−4.4	−2.2
10	d(3′-GCGT^{A}AAGCG-5′) d(5′-CGCATT^A CGC-3′)	37.5	−3.2	−1.6

[a] T_m was determined at UV 260 nm in NaCl (0.1 M), KH_2PO_4 (20 mM, pH 7.5), EDTA (0.1 mM). Entries are in °C.

effect remained small (entries 5 and 7). This difference between a purine and pyrimidine base could not been explained.

To try to prove the hypothesis that base pair interaction might occur within the minor groove of dsDNA, T^A and T^T were incorporated into both strands of the dsDNA sequence d(3′-GCGTAAGCG-5′)(5′-CGCATTCGC-3′). In each case, as required by the model, the modified nucleotides were separated by a regular A−T base pair (Table 2, entries 8−10). The drop in T_m varied between −3.2 and −5.4 °C (or −1.6 and −2.7 °C/mod) and thus is lower than the destabilization caused by a single T^A moiety (but higher than a single T^T moiety), indicating a stabilizing effect by introduction of the T^T nucleotide in the opposite strand of the T^A nucleotide. It is rewarding to notice that the T_m rises 0.6 °C when a second modification is introduced

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(13) Raunkjaer, M.; Bryld, T.; Wengel, J. *Chem. Commun.* **2003**, *13*, 1604−1605.

d(5′-C_1 G_2 C_3 A_4 T_5 T_6^A C_7 G_8 C_9-3′)
d(3′-G_{18} C_{17} G_{16} T_{15}^T A_{14} A_{13} G_{12} C_{11} G_{10}-5′)

FIGURE 3. Numbering of duplex **8**.

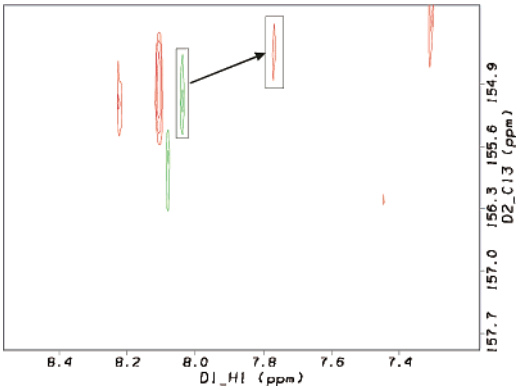

FIGURE 4. Overlay of the aromatic gHSQC spectra of single-stranded d(5′-CGCATT^A CGC-3′) (drawn in green) and the aromatic gHSQC spectrum of construct **8** (drawn in red). The boxed resonance signals originate from the H2 proton of the extra adenine base moiety.

in the opposing strand, hinting to a T−A interaction in the minor groove (entry 6 versus 8). However, a minor groove A−A interaction looks even much stronger in the studied sequence context, as the T_m increases from 30.0 and 34.7 °C, respectively, to 37.5 °C when a T^A block is incorporated in both opposing strands (entries 4 and 6 versus 10). The analogous T^T incorporation has no positive effect on duplex stabilities, as here the T_m is lowered further compared to a single T^T incorporation (entries 5 and 7 versus 9). It is interesting to observe that introduction of identical bisheaded bases (entries 9 and 10) gives less destabilization than introduction of different bisheaded bases (entry 8) in the opposite strands, and hydration of the minor groove might play a role in this phenomenon.

It can be concluded that the studied interaction of T^T with T^A or T^A with T^A in opposite strands separated by one regular base pair leads to a duplex stabilization when compared to incorporation of a single T^A unit. The reason for the stabilization effect is not clear. The 4′-*C*-methylthymine or 4′-*C*-methyladenine substituent can be situated outside the helix or within the minor groove. Interactions in the grooves are possible with the information present in the floor of the groove or by base pairing or stacking interactions of the extra nucleobases.

Therefore, we started an NMR investigation on two representative examples (i.e., the T−A interaction of the duplex of entry 8 and the position of the T base in the duplex of entry 5). Duplex **8** was selected (instead of the more stable duplex **10**) for structural studies because of the potential for Watson−Crick base pairing within the minor groove.

NMR Analysis and Molecular Modeling of Duplexes 5 and 8: Localization of the Extrahelical Bases. To shed light on the structural mechanisms that stabilize the above duplexes in which T^A and T^T building blocks are present in opposite strands, a preliminary NMR study was undertaken. As a model to study these interactions, duplex **8** (Figure 3) was selected because the extra bases (A and T) could interact as well by base pairing as by stacking. As discussed above, it is worth noting that the introduction of a T^T building block in construct **6** increases the stability of the resulting duplex (construct **8**) by 0.6 °C.

First NOESY, TOCSY, and H^1−C^{13} gHSQC spectra were recorded for both single-stranded molecules. Following titration

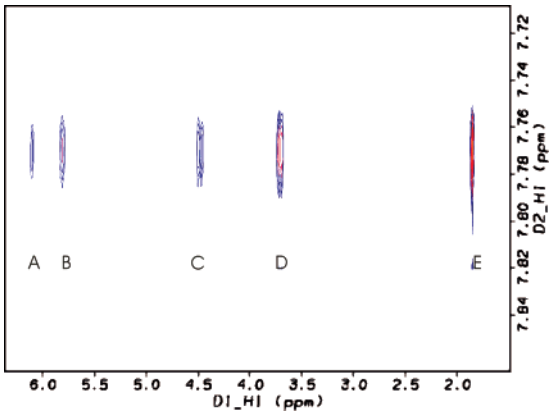

FIGURE 5. Part of the NOESY spectrum (mixing time 150 ms) of duplex **8**. Indicated are some interesting cross-peaks between H2(T_6^A) and proton signals of the opposite strand. (A) H1′(T_6^T), (B) H1′(A_{14}), (C,D) H6′/H6″(T_{15}^T), (E) CH3(T_{15}^T extra base moiety).

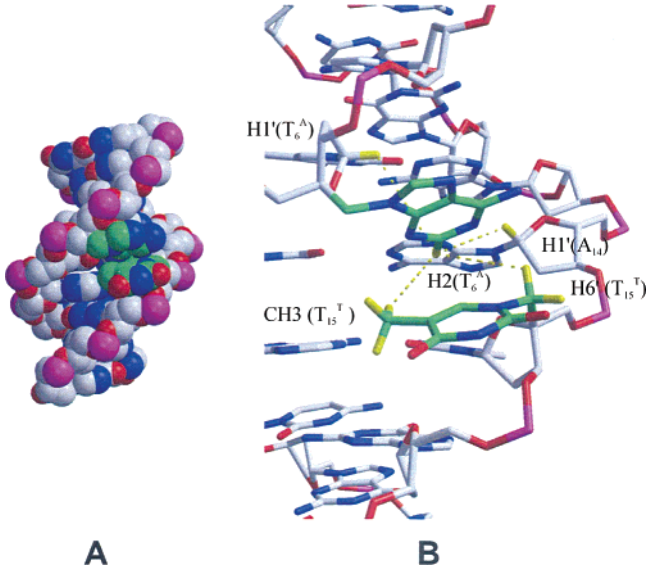

FIGURE 6. Averaged (A) and detailed (B) structure of the molecular modeling experiment showing the interaction of the T and A bases in the minor groove of the duplex. Figure B also shows the observed NOE contacts in the modified region.

of both strands to obtain an equimolar solution, spectra of the double-stranded molecule were recorded. It was encouraging to notice that the resonance signals for the additional bases did shift significantly following the formation of the duplex structure, indicating structural changes within the modified region. The proton and carbon signal of the H2 proton in the extra adenine base moiety was shifted from 8.04 ppm (H^1) and 155.12 ppm (C^{13}) in the single strand to 7.77 ppm (H^1) and 154.85 ppm (C^{13}) in the duplex (Figure 4). The methylene signal of the extra thymine base moiety also shifted from 1.82 ppm (H^1) to 1.73 ppm (H^1).

Considering these promising initial results, partial assignment of the resonance signals in the modified region was started using the gHSQC, TOCSY, and NOESY spectra of the duplex. Some interesting NOE contacts could be observed between the adenine proton H2(T_6^A) and proton signals of the opposite strand [H1′-(A14), H6′/H6″(T15T), and CH3(T15T extra base moiety)] as is indicated in Figures 5 and 6B.

This pattern of NOE cross-peaks is not corresponding to the expected pattern in a canonical AT base pair where no cross-

peak is expected between the methyl protons of the thymine base and the H2 proton of adenine. The observed pattern better fits a situation in which the two bases are stacked upon each other in the minor groove. In this case, an NOE contact cross-peak is expected as well between H2 and the H6′/H6″ protons (cross-peaks C and D, Figure 5) as between H2 and the methyl group (cross-peak E, Figure 5). This positioning of the bases also explains the NOE contacts with the H1′ protons of residues A_{13} and A_{14}.

On the basis of the available NMR restraints, a model was built using the Amber software and the energy was minimized in Sander. The duplex was solvated in a water box, and counterions were added followed by molecular dynamics simulation with all restraints removed (500 ps at 300 K). The results of the modeling experiments are in agreement with the observed NOE effects. This model demonstrates that the extrahelical bases (A and T) are situated in the minor groove. The interaction, however, is not specific (no Watson−Crick TA base pairing), and the stabilization of the interaction is mainly of hydrophobic nature.

To evaluate if the extrahelical T base is occupying a minor groove position when no A base is present in the opposite strand (preorganization for base−base interactions), we analyzed the structure of duplex **5** (Table 2) by NMR. The signals in the NMR spectra of the extra thymine base, however, do not really shift when the spectra of the single-strand T^T-oligo is compared with the spectra of the double-stranded oligo. No NOE effects are observed between the extrahelical thymine base and the protons of the regular DNA duplex (except for NOE contact between protons of the extrahelical thymine base and its own sugar moiety). These signals are broader than these of the NOE signals of bases involved in base pairing in the center of the helix. Therefore, we may conclude that the extrahelical thymine base has a high degree of conformational freedom when no adenine base is present in the opposite strand.

Conclusion

Oligonucleotides have been synthesized containing bisheaded furanose nucleosides in opposite strands and separated from each other by one regular base pair. Oligonucleotides with a bisheaded nucleotide may, likewise, be considered as a mimic for base flipping, although the orientation of the base is different. NMR analysis and molecular modeling have demonstrated that the supplementary bases attached to a 4′-*C*-methyl group are situated in the minor groove and may communicate with each other mainly by hydrophobic interactions. Future research activities will focus on the properties of the spacer between the groove-oriented base and the sugar moiety to allow W−C pairing of the extra nucleobases in the minor (or major) groove of dsDNA.

Experimental Section

Chemical Synthesis. 5′-*O*-[(Trifluoromethyl)sulfonyl]-4′-*C*-[(trifluoromethylsulfonyl]-oxymethyl]-3′-*O*-[(1,1-dimethylethyl)-dimethylsilyl]-thymidine (2). To a solution of compound **1** (2.15 g, 5.56 mmol), which was prepared according to the reported procedure,[8] in 30 mL of anhydrous DCM was added 3.3 mL of dried pyridine and trifluoromethanesulfonyl chloride (2.34 mL, 13.8 mmol) at 0 °C. The reaction mixture was stirred for 1 h and partitioned between 20 mL of H_2O and 200 mL of EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by column chromatography on a silica gel column (*n*-hexane/EtOAc, 4:1, 2:1) to afford compound **2** (3.08 g, 4.73 mmol, 85%) as a yellow amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 0.12 (s, 3H, $SiCH_3$), 0.13 (s, 3H, $SiCH_3$), 0.91 (s, 9H, CH_3), 1.93 (s, 3H, T-CH_3), 2.40−2.50 (m, 1H, 2′-H_a), 2.68−2.73 (m, 1H, 2′-H_b), 4.55 (d, J = 11.1 Hz, 1H, 5′-H_a), 4.59 (d, J = 10.6 Hz, 1H, 6′-H_a), 4.68 (d, J = 10.6 Hz, 1H, 6′-H_b), 4.74 (d, J = 11.1 Hz, 1H, 5′-H_b), 4.83 (t, J = 6.2 Hz, 1H, 3′-H), 5.91 (t, J = 6.2 Hz, 1H, 1′-H), 7.04 (s, 1H, 6-H), 8.81 (br s, 1H, NH); ^{13}C NMR (500 MHz, $CDCl_3$) δ_C −5.3, −4.7 ($Si(CH_3)_2$), 12.2 (T-CH_3), 17.8 ($C(CH_3)_3$), 25.5 ($C(CH_3)_3$), 40.1 (C-2′), 73.6 (C-5′ or C-6′), 73.7 (C-3′), 73.9 (C-6′ or C-5′), 84.5 (C-4′), 89.2 (C-1′), 111.6 (C-5), 118.5 (q, J = 318 Hz, CF_3), 126.7 (q, J = 366 Hz, CF_3), 137.7 (C-6), 149.7 (C-2), 163.4 (C-4); ESI HRMS calcd for $C_{19}H_{29}F_6N_2O_{10}S_2Si$ $[M + H]^+$ 651.0937; found 651.0922.

4′-*C*-[(Adenin-9-yl)methyl]-3′-*O*-[(1,1-dimethylethyl)dimethylsilyl]-O^2,5′-anhydrothymidine (3). To a solution of adenine (0.61 g, 4.62 mmol) in 45 mL of dried DMF was added NaH (80%, 345 mg, 11.6 mmol) at room temperature. The mixture was heated to 70 °C and kept stirring for 10 min. The reaction mixture was cooled to 50 °C, and a solution of compound **2** (3.0 g, 4.62 mmol) and a catalytic amount of 18-crown-6 in 10 mL of DMF were added. After being stirred for 4 h, the solvent was removed in vacuo. The residue was partitioned between 50 mL of sat. $NaHCO_3$ and EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography to afford compound **3** (1.43 g, 2.9 mmol) as a yellow amorphous solid in 64% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 0.17 (s, 3H, $SiCH_3$), 0.20 (s, 3H, $SiCH_3$), 0.92 (s, 9H, CH_3), 1.72 (d, J = 1.0 Hz, 3H, T-CH_3), 2.39 (ddd, J = 1.4, 8.0 and 15.1 Hz, 1H, 2′-H_a), 2.75 (ddd, J = 1.7, 7.1 and 15.1 Hz, 1H, 2′-H_b), 3.52 (d, J = 12.6 Hz, 1H, 5′-H_a), 4.42 (d, J = 15.3 Hz, 1H, 6′-H_a), 4.54 (d, J = 15.3 Hz, 1H, 6′-H_b), 4.78 (d, J = 12.5 Hz, 1H, 5′-H_b), 4.83 (dd, J = 6.8 and 1.5 Hz, 1H, 3′-H), 6.09 (dd, J = 8.3 and 1.7 Hz, 1H, 1′-H), 7.20 (br s, 2H, NH_2), 7.67 (d, J = 1.1 Hz, 1H, T-6-H), 8.02 (s, 1H, A-2-H), 8.16 (s, 1H, A-8-H); ^{13}C NMR (500 MHz, DMSO-d_6) δ_C −5.1, −4.8 ($Si(CH_3)_2$), 12.8 (T-CH_3), 17.7 ($C(CH_3)_3$), 25.6 ($C(CH_3)_3$), 43.3 (C-6′), 44.8 (C-2′), 74.0 (C-3′), 76.4 (C-5′), 88.2 (C-4′), 91.6 (C-1′), 117.1 (T−C-5), 118.2 (A−C-5), 138.7 (T−C-6), 141.3 (A−C-8), 149.8 (A−C-4); 152.5 (A−C-2), 155.9 (A−C-6), 156.2 (T−C-2), 170.9 (T−C-4); ESI HRMS calcd for $C_{22}H_{32}N_7O_4Si$ $[M + H]^+$ 486.2285; found 486.2268.

4′-*C*-[(Adenin-9-yl)methyl]-3′-*O*-[(1,1-dimethylethyl)dimethylsilyl]-thymidine (4). To a solution of compound **3** (1.40 g, 2.88 mmol) in 45 mL of dioxane was added aqueous NaOH (2 N, 8.64 mL, 17.3 mmol) at room temperature, and the mixture was stirred for 4 h. Then 8 mL of saturated $NaHCO_3$ was added, and the neutralized reaction mixture was concentrated in vacuo and partitioned between 20 mL of H_2O and 150 mL of EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography to afford compound **4** (0.67 g, 1.33 mmol) as a yellow amorphous solid in 46% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ_H 0.14 (s, 3H, $SiCH_3$), 0.16 (s, 3H, $SiCH_3$), 0.92 (s, 9H, CH_3), 1.71 (s, 3H, T-CH_3), 2.33−2.41 (m, 2H, 2′-H), 4.21 (d, J = 16.0 Hz, 1H, 6′-H_a), 4.41 (d, J = 16.0 Hz, 1H, 6′-H_b), 4.74 (t, J = 6.0 Hz, 1H, 3′-H), 5.45 (br s, 1H, OH), 6.40 (t, J = 6.1 Hz, 1H, 1′-H), 7.27 (br s, 2H, NH_2), 7.63 (s, 1H, T-6-H), 8.09 (s, 1H, A-8-H), 8.15 (s, 1H, A-2-H), 11.32 (s, 1H, NH), 5′-H was obscured in a water peak; ^{13}C NMR (500 MHz, DMSO-d_6) δ_C −4.8, −4.5 ($Si(CH_3)_2$), 12.5 (T-CH_3), 18.0 ($C(CH_3)_3$), 26.0 ($C(CH_3)_3$), 43.9 (C-6′), 61.6 (C-5′), 72.3 (C-3′), 83.5 (C-4′), 87.1 (C-1′), 109.9 (T−C-5), 118.4 (A−C-5), 136.7 (T−C-6), 142.8 (A−C-8), 150.6 (A−C-4); 150.9 (T−C-2), 153.1 (A−C-2), 156.2 (A−C-6), 164.4 (T−C-4), C-2′ was hidden by solvent peaks; ESI HRMS calcd for $C_{22}H_{34}N_7O_5Si$ $[M + H]^+$ 504.2391; found 504.2391.

3′,5′-*O*-Di[(1,1-dimethylethyl)dimethylsilyl]-4′-*C*-[(adenin-9-yl)-methyl]-thymidine (5). To a solution of compound **4** (0.46 g,

0.92 mmol) in 25 mL of pyridine were added TBDMSCl (0.28 g, 1.84 mmol) and imidazole (0.16 g, 2.30 mmol) at room temperature. The reaction mixture was stirred overnight. The solvent was removed in vacuo and coevaporated twice with toluene. The residue was partitioned between water and EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, and then concentrated in vacuo. The residues were purified by column chromatography. Compound **5** (0.33 g, 0.53 mmol) was obtained as a colorless amorphous solid in 58% yield. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H 0.02 (s, 3H, $SiCH_3$), 0.05 (s, 3H, $SiCH_3$), 0.18 (s, 3H, $SiCH_3$), 0.19 (s, 3H, $SiCH_3$), 0.90 (s, 9H, CH_3), 0.97 (s, 9H, CH_3), 1.88 (s, 3H, T-CH_3), 2.19−2.27 (m, 1H, 2′-H_a), 2.32−2.48 (m, 1H, 2′-H_b), 3.29 (d, J = 11 Hz, 1H, 5′-H_a), 3.82 (d, J = 11 Hz, 1H, 5′-H_b), 4.32 (d, J = 15 Hz, 1H, 6′-H_a), 4.50 (d, J = 15 Hz, 1H, 6′-H_b), 4.74 (br d, J = 8.2 Hz, 1H, 3′-H), 6.79 (dd, J = 8.8 and 5.8 Hz, 1H, 1′-H), 7.23 (d, J = 1.1 Hz, T-6-H), 8.23 (s, 1H, A-8-H), 8.36 (s, 1H, A-2-H); ^{13}C NMR (200 MHz, $CDCl_3$) δ_C −5.6, −5.5, −5.1, −4.7 ($Si(CH_3)_2$), 12.3 (T-CH_3), 18.0, 18.3 ($C(CH_3)_3$), 25.7, 25.8 ($C(CH_3)_3$), 40.5 (C-2′), 44.2 (C-6′), 65.4 (C-5′), 73.1 (C-3′), 81.8 (C-4′), 86.8 (C-1′), 111.3 (T−C-5), 118.0 (A−C-5), 135.3 (T−C-6), 143.0 (A−C-8), 150.4 (A−C-4), 151.3 (T−C-2), 153.2 (A−C-2), 155.6 (A−C-6), 165.6 (T−C-4); ESI HRMS calcd for $C_{28}H_{48}N_7O_5Si_2$ $[M + H]^+$ 618.3255; found 618.3247.

3′,5′-*O*-Di[(1,1-dimethylethyl)dimethylsilyl]-4′-*C*-[(*N*^{6}-benzoyladenin-9-yl)methyl]-thymidine (6). To a solution of compound B (320 mg, 0.52 mmol) in 20 mL of pyridine was added benzoyl chloride (150 μL, 1.30 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred overnight. The solvent was removed in vacuo and coevaporated twice with toluene. The residue was partitioned between water and EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, and then concentrated in vacuo. The oily residue was dissolved in MeOH (20 mL), saturated with ammonia, and allowed to stand at 0 °C for half an hour. The mixture was concentrated, and the residue was partitioned between water and EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by column chromatography, affording compound **6** (353 mg, 0.49 mmol) as a colorless amorphous solid in 94% yield. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H 0.02 (s, 3H, $SiCH_3$), 0.04 (s, 3H, $SiCH_3$), 0.19 (s, 3H, $SiCH_3$), 0.20 (s, 3H, $SiCH_3$), 0.89 (s, 9H, CH_3), 0.98 (s, 9H, CH_3), 1.83 (s, 3H, T-CH_3), 2.29−2.36 (m, 2H, 2′-H), 3.24 (d, J = 11 Hz, 1H, 5′-H_a), 3.77 (d, J = 11 Hz, 1H, 5′-H_b), 4.50 (br s, 2H, 6′-H), 4.69 (t, J = 4.0 Hz, 1H, 3′-H), 6.63 (t, J = 7.3 Hz, 1H, 1′-H), 7.45−7.59 (m, 4H, $Bz_{m,p}$-H, T-6-H), 8.07 (d, J = 6.6 Hz, 2H, Bz_o-H), 8.25 (s, 1H, A-8-H), 8.86 (s, 1H, A-2-H), 9.66 (br s, 1H, NH), 10.09 (br s, 1H, NH); ^{13}C NMR (200 MHz, $CDCl_3$) δ_C −5.6, −5.5, −5.1, −4.7 ($Si(CH_3)_2$), 12.2 (T-CH_3), 18.0, 18.2 ($C(CH_3)_3$), 25.7, 25.8 ($C(CH_3)_3$), 41.2 (C-2′), 45.0 (C-6′), 65.2 (C-5′), 73.8 (C-3′), 83.7 (C-4′), 87.6 (C-1′), 111.3 (T−C-5), 122.1 (A−C-5), 127.3, 128.3, 128.6, 132.6 (ar-C), 135.2 (T−C-6), 144.6 (A−C-8), 149.8 (A−C-4), 150.5 (T−C-2), 152.7 (A−C-2, A−C-6), 164.0 (Bz-CO), 165.3 (T−C-4); ESI HRMS calcd for $C_{35}H_{52}N_7O_6Si_2$ $[M + H]^+$ 722.3518; found 722.3526.

4′-*C*-[(*N*^{6}-Benzoyladenin-9-yl)methyl]-thymidine (7). To a solution of compound **6** (310 mg, 0.43 mmol) in THF was added 1 M TBAF (1.0 mL, 1.0 mmol) at room temperature. The reaction mixture was kept stirring for 3−4 h and concentrated in vacuo. The residue was purified by column chromatography to afford compound **7** (120 mg, 0.24 mmol) as a colorless amorphous solid in 57% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ_H 1.72 (s, 3H, T-CH_3), 2.34 (t, J = 6.0 Hz, 2H, 2′-H), 3.18−3.27 (m, 1H, 5′-H_a), 3.44−3.53 (m, 1H, 5′-H_b), 4.41 (d, J = 15 Hz 1H, 6′-H_a), 4.54 (d, J = 15 Hz, 1H, 6′-H_b), 4.61 (q, J = 5.1 Hz, 3′-H), 5.30 (t, J = 5.0 Hz, 1H, 5′-OH), 5.75 (d, J = 4.8 Hz, 1H, 3′-OH), 6.41 (t, J = 6.2 Hz, 1H, 1′-H), 7.52−7.65 (m, 4H, $Bz_{m,p}$-H), 7.69 (br s, 1H, T-6-H), 8.05 (d, J = 7.0 Hz, 2H, Bz_o-H), 8.42 (s, 1H, A-8-H), 8.74 (s, 1H, A-2-H), 11.15 (br s, 1H, NH), 11.27 (br s, 1H, NH); ^{13}C NMR (200 MHz, DMSO-d_6) δ_C 12.2 (T-CH_3), 43.8 (C-6′), 61.8 (C-5′), 70.3 (C-3′), 83.2 (C-4′), 86.9 (C-1′), 109.4 (T−C-5), 125.1 (A−C-5), 128.6 (Bz-C), 132.6 (Bz-C), 133.6 (Bz-C), 136.2 (T−C-6), 145.9 (A−C-8), 150.3 (A−C-4), 150.6 (T−C-2), 151.6 (A−C-2), 153.4 (A−C-6), 163.9 (BzCO), 165.8 (T−C-4), C-2′ was hidden by solvent peaks; ESI HRMS calcd for $C_{23}H_{24}N_7O_6$ $[M + H]^+$ 494.1788; found 494.1783.

5′-*O*-(Monomethoxytrityl)-4′-*C*-[(*N*^{6}-benzoyladenin-9-yl)methyl]-thymidine (8). To a solution of compound **7** (302 mg, 612 mmol) in 20 mL of dried pyridine was added MMTrCl (245 mg, 796 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred overnight. Then 1 mL of methanol was added to quench the reaction. The solvent was removed in vacuo and coevaporated twice with toluene. The residue was partitioned between water and EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by column chromatography to afford compound **8** (310 mg, 496 mmol) as a colorless amorphous solid in 81% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ_H 1.34 (s, 3H, T-CH_3), 3.70 (s, 3H, OCH_3), 4.49 (br s, 2H, 6′-H), 4.87 (br s, 1H, 3′-H), 5.90 (br s, 1H, OH), 6.42 (t, J = 6.4 Hz, 1H, 1′-H), 6.77 (d, J = 8.8 Hz, 2H, Ar H), 7.04 (d, J = 8.8 Hz, 2H, Ar H), 7.23−7.36(m, 10H, Ar H), 7.44 (s, 1H, T-6-H), 7.52−7.69 (m, 3H, Bz-H), 8.04 (d, J = 6.9 Hz, 2H, Bz-H), 8.22 (s, 1H, A-8-H), 8.59 (s, 1H, A-2-H), 11.13 (br s, 1H, NH), 11.33 (br s, 1H, NH), 2′-H was obscured in solvent peaks. 5′-H was obscured in water peak at 3.35 ppm; ^{13}C NMR (200 MHz, DMSO-d_6) δ_C 11.7 (T-CH_3), 44.4 (C-6′), 55.1 (OCH_3), 64.8 (C-5′), 70.8 (C-3′), 83.3 (C-4′), 86.2 (C-1′), 86.7 ($C(Ar)_3$), 109.6 (T−C-5), 113.3 (ar-C), 124.6 (A−C-5), 127.1, 128.0, 128.6, 130.1, 132.5, 133.7, 134.3 (Ar−C), 135.9 (T−C-6), 143.7, 144.0 (Ar−C), 145.5 (A−C-8), 150.1 (A−C-4), 150.5 (T−C-2), 151.3 (A−C-2), 153.0 (A−C-6), 158.4 (Ar−C), 163.9 (BzCO), 165.7 (T−C-4), C-2′ was hidden by solvent peaks; ESI HRMS calcd for $C_{43}H_{40}N_7O_7$ $[M + Na]^+$ 766.2989; found 766.2980.

5′-*O*-[(4-Methoxyphenyl)diphenylmethyl]-4′-*C*-[(*N*^{6}-benzoyladenin-9-yl)methyl]-3′-*O*-(*P*-*β*-cyanoethoxy-*N*,*N*-diisopropylaminophosphinyl)-thymidine (9). The monomethoxytritylated derivative **8** (300 mg, 0.39 mmol) was dissolved in 10 mL of DCM under argon, and diisopropylethylamine (DIEA, 204 μL, 1.17 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (CEPNCl, 175 μL, 0.78 mmol) were added. The mixture slowly became clear, and overall the solution was stirred for 2 h, when TLC indicated complete reaction. Water (2 mL) was added, and the solution was stirred for 10 min and partitioned between CH_2Cl_2 (50 mL) and aqueous $NaHCO_3$ (30 mL). The organic phase was washed with aqueous sodium chloride (2 × 30 mL), and the aqueous phases were extracted with DCM (30 mL). Evaporation of the organic solvent left a white foam that was purified twice on 40 g of silica gel (hexane/acetone/TEA, 40:59:1) to afford the product as a foam after several coevaporations with DCM. Dissolution in 2 mL of DCM and precipitation in 50 mL of cold (−70 °C) hexane afforded 242 mg (0.25 mmol, 64%) of the title product **9** as a white powder. R_f (hexane/acetone/TEA 40:59:1): 0.33. ESI-MS pos. calcd. for $C_{52}H_{57}N_9O_8P_1$ $[M + H]^+$ 966.4067; found 966.4073; ^{31}P NMR δ ppm, (external ref = H_3PO_4 capil.) 150.72, 151.02.

4′-*C*-[(Thymin-1-yl)methyl]-3′-*O*-[(1,1-dimethylethyl)dimethylsilyl]-thymidine (11). To a solution of thymine (3.35 g, 26.7 mmol) in 100 mL of DMF was added NaH (80%, 0.96 g, 32.0 mmol) at room temperature. The mixture was warmed to 70 °C and kept stirring for 10 min. Once the reaction mixture was cooled to 50 °C, a solution of compound **2** (1.73 g, 2.67 mmol) and a catalytic amount of 18-crown-6 in 10 mL of DMF was added. After being stirred for 4 h, the solvent was removed in vacuo. The residue was partitioned between 50 mL of sat. $NaHCO_3$ and 100 mL of DCM. The organic layer was washed with water and brine, dried over Na_2SO_4, and then concentrated in vacuo. The residue was used directly in the next reaction, as compound **10** comigrated with thymine and proved difficult to purify.

To a solution of this residue in 45 mL of dioxane was added aqueous NaOH (2 N, 11 mL, 22 mmol) at room temperature, and the mixture was stirred for 4 h. Then 15 mL of saturated $NaHCO_3$ solution was added. The reaction mixture was concentrated in vacuo to a small volume and partitioned between 20 mL of H_2O and 100 mL of DCM. The organic layer was washed with water and brine, dried over Na_2SO_4, and then concentrated in vacuo. A pure sample as a colorless amorphous solid was obtained by preparative TLC. ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 0.10 (s, 3H, $SiCH_3$), 0.11 (s, 3H, $SiCH_3$), 0.89 (s, 9H, $C(CH_3)_3$), 1.74 (s, 6H, T-CH_3), 2.24−2.29 (m, 1H, 2′-H_a), 2.33−2.39 (m, 1H, 2′-H_b), 3.37−3.46 (m, 2H, 5′-H), 3.79 (d, J = 14.7 Hz, 1H, 6′-H_a), 3.92 (d, J = 14.7 Hz, 1H, 6′-H_b), 4.68 (t, J = 6.7 Hz, 1H, 3′-H), 5.23 (t, J = 4.9 Hz, 1H, OH), 6.26 (t, J = 6.0 Hz, 1H, 1′-H), 7.47 (s, 1H, T*-6-H), 7.76 (s, 1H, T-6-H), 11.25 (br s, 2H, NH); ^{13}C NMR (200 MHz, DMSO-d_6) δ_C −5.2, −4.8 ($Si(CH_3)_2$), 12.0*,12.3 (T-CH_3), 17.7 ($C(CH_3)_3$), 25.6 ($C(CH_3)_3$), 46.9 (C-6′), 61.61 (C-5′), 71.4 (C-3′), 82.9 (C-1′), 87.2 (C-4′), 108.1*, 109.1 (T−C-5), 136.1, 142.6* (T−C-6), 150.4, 151.7* (T−C-2), 163.8, 164.1* (T−C-4), C-2′ was hidden by the solvent peaks; ESI HRMS calcd for $C_{22}H_{34}N_4NaO_7Si$ $[M + Na]^+$ 517.2094; found 517.2096.

4′-*C*-[(Thymin-1-yl)methyl]-thymidine (12). To a solution of **11** in THF was added 1 M TBAF (3.0 mL, 3.0 mmol) at room temperature. The reaction mixture was stirred for 4 h and concentrated in vacuo. The residue was purified by column chromatography to afford compound **12** (121 mg, 0.32 mmol) as a colorless amorphous solid in 12% yield from **2**. ^{1}H NMR (200 MHz, DMSO-d_6) δ_H 1.74 (s, 6H, T-CH_3), 2.26 (t, J = 6.4 Hz, 2H, 2′-H), 3.30−3.50 (m, 2H, 5′-H), 3.76 (d, J = 15.0 Hz, 1H, 6′-H_a), 3.97 (d, J = 15.0 Hz, 1H, 6′-H_b), 4.46−4.60 (m, 1H, 3′-H), 5.18 (t, J = 5.3 Hz, 1H, 5′-OH), 5.53 (d, J = 4.4 Hz, 1H, 3′-OH), 6.27 (t, J = 6.0 Hz, 1H, 1′-H), 7.46 (app d, J = 1.1 Hz, 1H, T-6-H), 7.79 (app d, J = 1.1 Hz, 1H, T-6-H), 11.27 (br s, 2H, NH); ^{13}C NMR (200 MHz, DMSO-d_6) δ_C 12.0, 12.3 (T-CH_3), 47.1 (C-6′), 61.5 (C-5′), 70.1 (C-3′), 83.0 (C-1′), 87.5 (C-4′), 108.2, 109.2 (T−C-5), 136.3, 142.7 (T−C-6), 150.6, 152.0 (T−C-2), 163.9, 164.3 (T−C-4), C-2′ was hidden by solvent peaks; ESI HRMS calcd for $C_{16}H_{20}N_4NaO_7$ $[M + Na]^+$ 403.1230; found 403.1230.

5′-*O*-(Monomethoxytrityl)-4′-*C*-[(thymin-1-yl)methyl]-thymidine (13). To a solution of compound **12** (110 mg, 0.29 mmol) in 20 mL of dry pyridine was added MMTrCl (108 mg, 0.35 mmol) at 0 °C. The reaction mixture was allowed to reach room temperature and kept stirring overnight. Then 1 mL of methanol was added. The solvent was removed in vacuo and coevaporated twice with toluene. The residue was partitioned between water and DCM. The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography to afford compound **13** as a colorless amorphous solid (156 mg, 0.24 mmol) in 83% yield. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H 1.54, 1.73 (two s, 6H, T-CH_3), 2.29−2.53 (m, 2H, 2′-H), 3.06 (d, J = 10.4 Hz, 1H, 5′-H_a), 3.34 (d, J = 10.4 Hz, 1H, 5′-H_b), 3.78 (s 3H, OCH_3), 4.05 (br s, (d, 2H, 6′-H), 4.23 (d, J = 5.8 Hz, 1H, OH), 4.60 (br s, 1H, 3′-H), 6.34 (t, J = 6.6 Hz, 1H, 1′-H), 6.82 (d, J = 8.8 Hz, 2H, Ar H), 7.06 (s, 1H, T-6-H), 7.24−7.48 (m, 13H, Ar H, T-6-H), 9.07 (s, 1H, NH), 9.19 (s, 1H, NH); ^{13}C NMR (200 MHz, $CDCl_3$) δ_C 12.0 (T-CH_3), 39.4 (C-2′), 48.7 (C-6′), 55.2 (OCH_3), 66.0 (C-5′), 73.3 (C-3′), 84.5 (C-1′), 87.3 (C-4′ or C(Ar)$_3$), 87.7 (C(Ar)$_3$ or C-4′), 111.4, (T−C-5), 113.2 (T−C-5), 113.3 (Ar−C), 127.2, 127.5, 127.9, 128.1, 128.3, 129.2, 130.4, 134.2 (ar-C), 135.7 (T−C-6), 141.6 (Ar−C), 143.3; 143.6 (Ar−C), 150.2, 152.2 (T−C-2), 159.0, 163.6 (T−C-4); ESI HRMS calcd for $C_{36}H_{36}N_4NaO_8$ $[M + Na]^+$ 675.2431; found 675.2439.

5′-*O*-(Monomethoxytrityl)-4′-*C*-[(thymin-1-yl)methyl]-3′-*O*-(*P*-*β*-cyanoethoxy-*N*,*N*-diisopropylaminophosphinyl)-thymidine (14). The monomethoxytritylated derivative **13** (100 mg, 0.15 mmol) was dissolved in 10 mL of DCM under argon, DIEA (80 μL, 0.45 mmol) and CEPNCl (75 μL, 0.33 mmol) were added, and the solution was stirred for 2 h, when TLC indicated incomplete reaction. An additional quantity of 40 μL each of DIEA and CEPNCl were added, and after being stirred for 1 h more, water (2 mL) was added. The solution was stirred for 10 min and partitioned between DCM (50 mL) and aqueous $NaHCO_3$ (30 mL). The organic phase was washed with aqueous sodium chloride (2 × 30 mL), and the aqueous phases were back-extracted with CH_2Cl_2 (30 mL). Evaporation of the organics left a light yellow foam that was flash purified twice on 30 g of silica gel (hexane/acetone/TEA, 50:49:1) to afford the product as a foam after several coevaporations with DCM. Dissolution in 2 mL of DCM and precipitation in 50 mL of cold (−70 °C) hexane afforded 77 mg (0.09 mmol, 60%) of the title product **14** as a white powder. R_f (hexane/acetone/TEA, 40:59:1): 0.38; ESI-MS pos. calcd for $C_{45}H_{54}N_6O_9P_1$ $[M + H]^+$ 853.3690; found 853.3715; ^{31}P NMR δ ppm, (external ref = H_3PO_4 capil.) 150.47, 150.95.

Oligonucleotide Synthesis. Oligonucleotide assembly was performed on a DNA synthesizer using the phosphoramidite approach. Standard DNA assembly protocols were used, either at the 0.2-μmol scale or at the 10-μmol scale for the NMR oligonucleotides. Coupling time for the modified bases was increased to 12 min using 0.07 M of the newly synthesized unnatural amidites, with ETT as the activator substituting for tetrazole. The oligomers were deprotected and cleaved from the solid support by treatment with concentrated aqueous ammonia (55 °C, 16 h). After gel filtration with water as eluent, the crude was analyzed on an anion exchange column, after which purification was achieved on a HR 10/10 with the following gradient system (A = 10 mM NaOH, pH 12.0, 0.1 M NaCl; B = 10 mM NaOH, pH 12.0, 0.9 M NaCl; gradient used depending on the oligo; flow rate 2 mL/min). The low-pressure liquid chromatography system consisted of an intelligent pump, a UV detector, and a recorder. The product-containing fraction was desalted and lyophilized.

Oligonucleotides were characterized, and their purity was checked by HPLC/MS on a capillary chromatograph. Columns of 150 mm × 0.3 mm length were used. Oligonucleotides were eluted with a triethylammonium/1,1,1,3,3,3-hexafluoro-2-propanol/acetonitrile solvent system. Flow rate was 5 μL/min. Electrospray spectra were acquired on an orthogonal acceleration/time-of-flight mass spectrometer in negative ion mode. Scan time used was 2 s. The combined spectra from a chromatographic peak were deconvoluted using the MaxEnt algorithm of the software. Theoretical oligonucleotide masses were calculated using the monoisotopic element masses.

Melting Temperatures. Oligomers were dissolved in 0.1 M NaCl, 0.02 M potassium phosphate, pH 7.5, 0.1 mM EDTA. The concentration was determined by measuring the absorbance in water at 260 nm at 80 °C and assuming the cyclic nucleoside analogues to have the same extinction coefficients in the denatured state as the natural nucleosides with for T^A (ϵ = 23 500) and for T^T (ϵ = 17 000). The concentration in all experiments was 4 μM for each strand unless otherwise stated. Melting curves were determined with a UV spectrophotometer. Cuvettes were maintained at constant temperature by means of water circulation through the cuvette holder. The temperature of the solution was measured with a thermistor directly immersed in the cuvette. Temperature control and data acquisition were done automatically. The samples were heated at a rate of 0.2 °C/min starting at 10 °C up to 80 °C and cooling again at the same speed. Melting temperatures were determined by plotting the first derivative of the absorbance versus temperature curve and are the average of two runs. Up and down curves in general showed identical T_m values.

NMR Experiments. NMR Sample Preparation. The duplexes used for the NMR experiments [d(3′-GCGT^{T}AAGCG-5′)-(5′-CGCATT^A CGC-3′)] and [d(3′-GCGT^{T}AAGCG-5′)-(5′-CGCATTCGC-3′)] were obtained by titrating (i) a solution of d(3′-GCGT^{T}AAGCG-5′) with the complementary sequence (5′-CGCATT^A CGC-3′) and (ii) a solution of d(3′-GCGT^{T}AAGCG-5′) with the complementary sequence (5′-CGCATT^A CGC-3′). The degree of complex formation was monitored by one-dimensional NMR spectra of nonexchangeable base protons and anomeric protons. After

titration, the pD of the sample was adjusted to 7.2. The sample was lyophilized and redissolved in 0.25 mL of D_2O, resulting in a concentration of 1.9 mM of the [d(3′-GCGT^{T}AAGCG-5′)-(5′-CGCATT^A CGC-3′)] and 1.7 mM of [d(3′-GCGT^{T}AAGCG-5′)-(5′-CGCATTCGC-3′)]. The solutions were briefly heated to 80 °C and slowly cooled to room temperature to promote duplex formation.

NMR Spectroscopy. Spectra were recorded on a spectrometer, operating at 499.140 MHz. Unless stated otherwise, spectra were recorded at 22 °C. Spectra were processed using the FELIX 97.00 software package running on a Silicon Graphics O2 R10000 workstation (IRIX version 6.3).

The TOCSY[14] and NOESY[15] spectra in D2O were recorded with a sweep width of 4200 Hz in both dimensions. For the TOCSY experiments, a clean MLEV17[16] version was used, with a low power 90° pulse of 18.2 ms and the delay set to 47.3 ms. The total TOCSY mixing time was set to 50 ms. The spectrum was acquired with 32 scans, 2048 data points in t2 and 512 FIDs in t1. The data were apodized with a shifted sine bell square function in both dimensions and processed to a 2K × 1K matrix. The NOESY experiments were acquired with mixing times of 150 ms, 32 scans, 2048 data points in t2 and 512 increments in t1. The natural abundance ^{1}H, ^{13}C HSQC spectra were recorded with sensitivity enhancement and gradient coherence selection[17] using 72 scans and 256/512 complex data points and 10 000/12 500 Hz spectral widths in t1 and t2, respectively.

Molecular Modeling. A model of duplex **8** (Figure 6) was created using the Amber software.[18] Atomic electrostatic charges to be used in the Amber software package were calculated from the electrostatic potential at the 6-31G* level using the package games[19] and the two-stage RESP fitting procedure.[20] The charges of the atoms in the additional bases and atom C4′ were calculated by the RESP procedure. All other charges were fixed to the values as those of DNA in the Amber 94 topology files.[21] The parameters used in the Amber simulations are those from the parm99 dataset.[20] An initial model of a nine-residue-long double-stranded DNA was created using nucgen, which is part of the Amber suite of programs. Modified T nucleotides with an additional adenine or thymine base were constructed in a macromodel.[22] Their geometry was optimized in the Amber* force field.[23] Then quatfit was used to fit the modified nucleotides RTT on residue 15 and RTA on residue 6 of the double helix.[24]

The energy of the system was minimized in Sander (Amber 6) for 1000 steps, while maintaining NMR restraints (equilibrium H−H distances of 5.0 Å for proton pairs obtained from observed NOE contacts in the modified region, Figures 5 and 6). Examination of the model showed that the extra bases had to be oriented in the minor groove of the double helix.

The DNA was then solvated in a TIP3P water box.[25] 16 Na+ counterions were then added to get an electrostatic neutral system. The water molecules and counterions were then allowed to relax their positions while keeping the solute fixed. Molecular dynamics simulations were then initiated with all restraints removed, with periodic boundary conditions and using a cutoff distance of 9 Å for the nonbonded interactions and the particle mesh Ewald method for the summation of the Coulombic interactions.[26] Simulation temperature was 300 K and continued for 500 ps. During the last 50 ps, structures were collected. From those, one average structure was calculated (shown in Figure 6a, detail shown in Figure 6b). The NOE distances are marked in yellow dotted lines. Figures were created by bobscript, molscript, and Raster3D.[27−29]

The proton−proton distances in the model are between H2(T_6^A) and protons H1′(A_{14}) 3.34 Å, HB71 ($T1_5^T$) 3.99 Å, H6′ (T_{15}^T) 4.34 Å, H1′ (T_6^T) 4.28 Å, H5′ (T_{15}^T) 4.64 Å, confirming the NOE measurements.

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Supporting Information Available: General experimental section and ^{1}H NMR data of compounds **2**, **3**, **4**, **5**, **6**, **7**, **8**, **11**, **12**, **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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