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Double-Coding Nucleic Acids: Introduction of a Nucleobase Sequence in the Major Groove of the DNA Duplex Using Double-Headed Nucleotides

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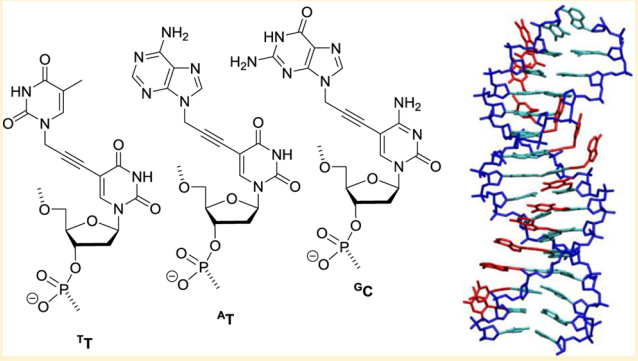
Supporting Information

ABSTRACT: A series of double-headed nucleosides were synthesized using the Sonogashira cross-coupling reaction. In the reactions, additional nucleobases (thymine, cytosine, adenine, or guanine) were attached to the 5-position of 2′-deoxyuridine or 2′-deoxycytidine through a propyne linker. The modified nucleosides were incorporated into oligonucleotides, and these were combined in different duplexes that were analyzed by thermal denaturation studies. All of the monomers were well tolerated in the DNA duplexes and induced only small changes in the thermal stability. Consecutive incorporations of the monomers led to increases in duplex stability owing to increased stacking interactions. The modified nucleotide monomers maintained the Watson−Crick base pair fidelity. Stable duplexes were observed with heavily modified oligonucleotides featuring 14 consecutive incorporations of different double-headed nucleotide monomers. Thus, modified duplexes with an array of nucleobases on the exterior of the duplex were designed. Molecular dynamics simulations demonstrated that the additional nucleobases could expose their Watson−Crick and/or Hoogsteen faces for recognition in the major groove. This presentation of nucleobases may find applications in providing molecular information without unwinding the duplex.



INTRODUCTION

The DNA double helix consists of two right-handed polynucleotide chains that are coiled about the same axis. The heterocyclic nucleobases project inward toward the center so that the base of one strand interacts or pairs with a base of the other strand by means of adenine:thymine and guanine:cytosine hydrogen bonding known as Watson−Crick base pairing.[1] In combination, the base pairing and the stacking of the bases hold the DNA double helix together, and the DNA carries the genetic information in the form of the sequence of bases. On this basis, DNA is an excellent scaffold for the design of artificial supramolecular systems, and the DNA duplex has been decorated and modified in numerous ways, finding applications in the development of therapeutics as well as in DNA nanotechnology.[2−6] For instance, the duplex has been used to organize chromophores in specific ways on the surface of the duplex.[7,8] In the present study, we introduce a range of additional nucleobases along the duplex. Nature has designed DNA in such a way that the genetic information is stored in the core of the double helix, and any access to this information requires the unwinding of the duplex. We envision that an array of nucleobases and thus chemical information on the exterior of the duplex could be used to transfer a code without unwinding the duplex. Furthermore, such a system could transfer one code to another based on one sequence of nucleobases in the core of the duplex and another sequence of nucleobases on the exterior of the duplex. Therefore, we aim to introduce a new series of so-called double-headed nucleotides with the additional nucleobases attached so to be organized in the major groove of the DNA double helix.

In recent years, we have studied a series of double-headed nucleotides as building blocks for artificial nucleic acids.[9−18] We define double-headed nucleotides as nucleotide monomers with two nucleobases attached on the same sugar, and several of these have been presented by us[9−18] and others[19−21] with the aim of using the base pairing and stacking potential of the additional nucleobases in various constructs. For instance, we have recently introduced 2′-deoxyuridine analogues with an additional thymine or adenine in the 2′-position attached through a methylene linker ($\mathbf{U}_\mathbf{T}$ and $\mathbf{U}_\mathbf{A}$, Figure 1).[10,11] Here, the additional nucleobases are positioned in the core of the

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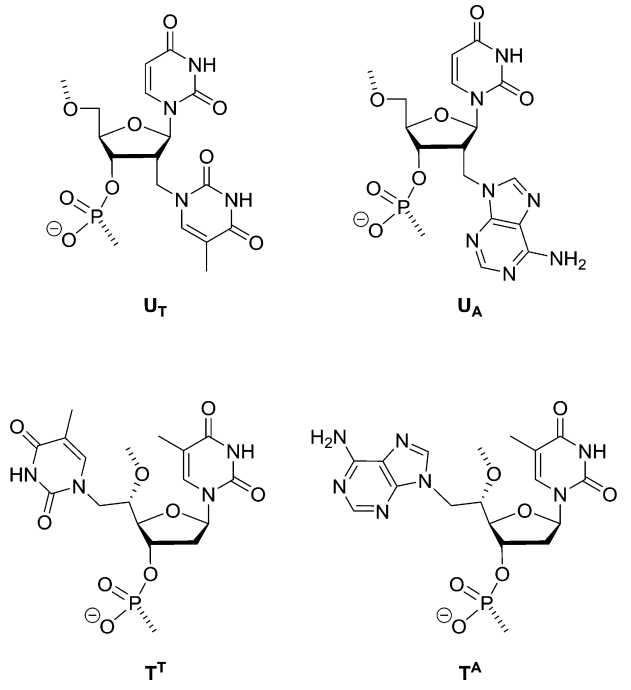

Figure 1. Two designs of double-headed nucleotides.

DNA duplex, and monomers $\mathbf{U_T}$ and $\mathbf{U_A}$ behave as compressed dinucleotides as the additional nucleobases participate in the Watson–Crick base pairing with complementary bases from the opposite strand.[11] The formation of a Watson–Crick A–T base pair between the additional bases of $\mathbf{U_T}$ and $\mathbf{U_A}$ led to the formation of a 5′-$\mathbf{U_T}$A:3′A$\mathbf{U_A}$ motif with high stability, and in this way, the duplex could carry an additional base pair based on a native backbone. In an earlier study, we showed that a homologue of $\mathbf{U_T}$ with an ethylene linker between the T and C2′ induced some stabilization of a three-way junction as the additional thymine perform stacking interactions in the core of the junction.[9,12] In addition, other double-headed analogues with the additional nucleobases in the 2′-position have been studied.[17,19]

Another example of double-headed nucleotides is the thymidine analogues with additional nucleobases in the 5′-position ($\mathbf{T^T}$ and $\mathbf{T^A}$, Figure 1).[13,15,16] When introduced into DNA duplexes, the additional thymine of $\mathbf{T^T}$ is positioned in the minor groove, where it can be recognized by another thymine introduced in the appropriate position of the opposite strand. Specific stacking interactions were observed but no base-pairing with the adenine of $\mathbf{T^A}$. Nevertheless, each incorporation of $\mathbf{T^T}$ or $\mathbf{T^A}$ leads to a decrease in thermal stability of the duplex by around 5 °C.[15] Herdewijn and co-workers have presented another pair of double-headed thymidine analogues with either thymine or adenine placed in the 4′-position through a methylene linker.[21] This design also positions the additional nucleobases in the minor groove, but no significant interactions between the bases in the minor groove were observed. Again, each modified nucleotide decreased the thermal stability of the duplex.[21] Simple acyclic double-headed nucleotides have also been studied, but without the natural (2′-deoxy)ribonucleoside skeleton, the thermal stability of the duplexes was decreased significantly.[18,20]

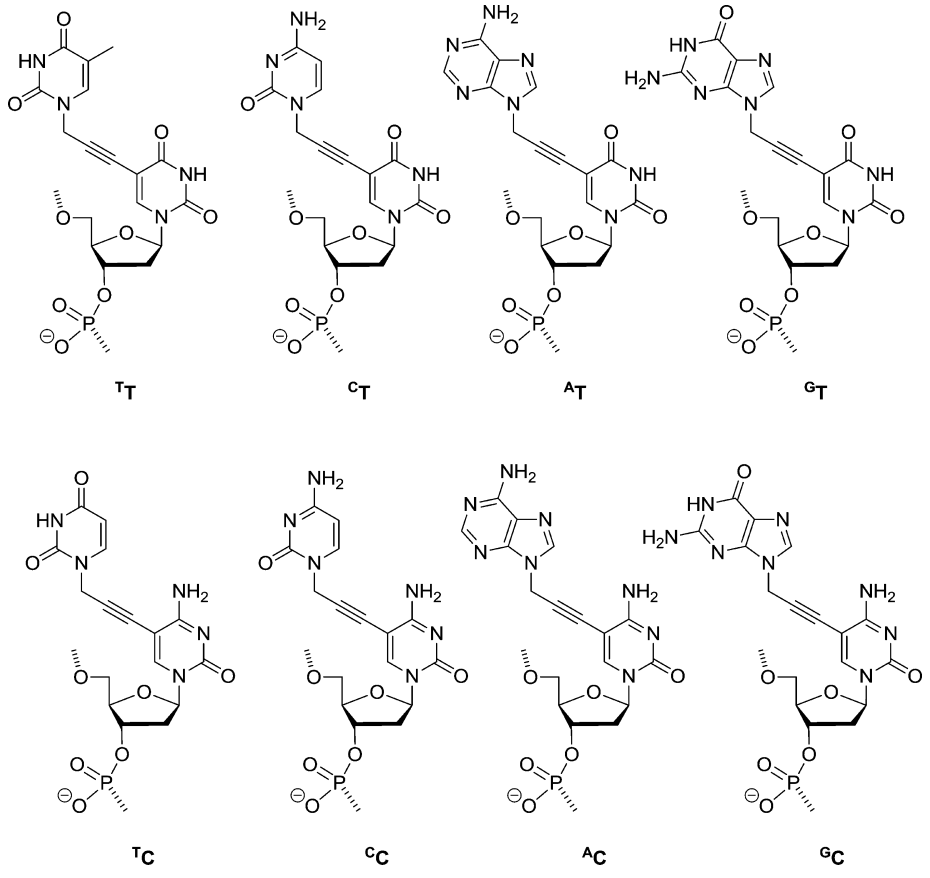

Figure 2. Double-headed nucleotide monomers $^{T}\mathbf{T}$, $^{C}\mathbf{T}$, $^{A}\mathbf{T}$, $^{G}\mathbf{T}$, $^{T}\mathbf{C}$, $^{C}\mathbf{C}$, $^{A}\mathbf{C}$, and $^{G}\mathbf{C}$.

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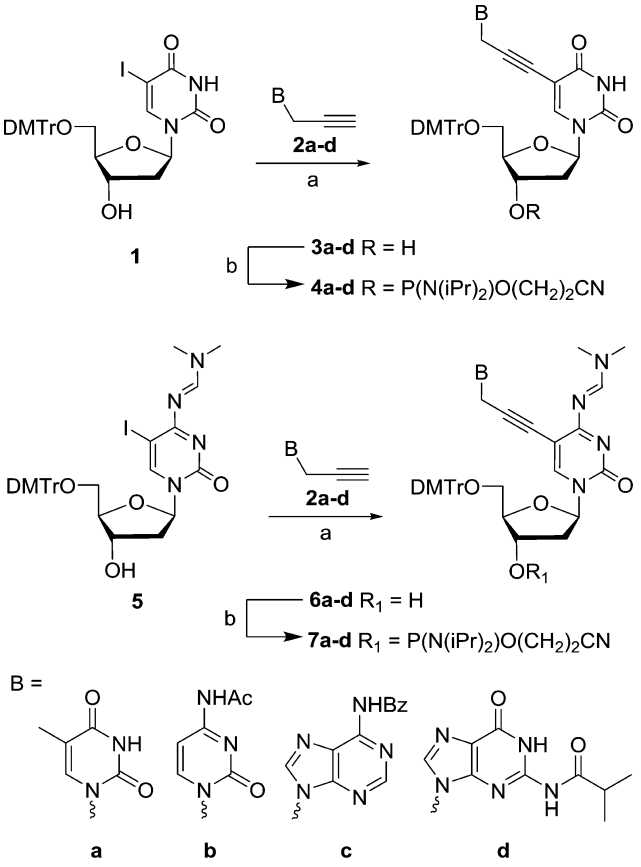
The double-headed nucleotides studied so far place the additional nucleobases either in the core of the duplex forming Watson–Crick base pairs ($\mathbf{U}_T$ and $\mathbf{U}_A$)[10,11] or in the minor groove ($\mathbf{T}^T$ and $\mathbf{T}^A$).[16] In the B-type duplex, the minor groove is probably too narrow to contain a full array of nucleobases, and our examination of the 5′-*C*-position ($\mathbf{T}^T$, $\mathbf{T}^A$ and other analogues) has demonstrated that a decrease in duplex stability generally follows this series of double-headed nucleotides.[15,16] We therefore turned our focus toward investigating the recognition potential of additional nucleobases in the major groove of the B-type DNA duplex, and the 5-position of pyrimidine nucleotides offers an attractive site for introducing substituents that point into the major groove away from the Watson–Crick binding face. Therefore, substitution at this position is expected neither to interfere with base pairing nor to influence the general structure of the double helix. The presence of alkyne groups such as propynyl at the 5-position of pyrimidines is known to increase the thermal stability of the duplex.[22,23] In light of this, we decided to attach additional nucleobases at the 5-position of pyrimidines through a propyne linker. With this strategy, we synthesized and incorporated the eight double-headed nucleotide monomers ($^T\mathbf{T}$, $^C\mathbf{T}$, $^A\mathbf{T}$, $^G\mathbf{T}$, $^T\mathbf{C}$, $^C\mathbf{C}$, $^A\mathbf{C}$, and $^G\mathbf{C}$, Figure 2) into a DNA duplex to present a sequence of additional bases outside the duplex core. By making the full series of eight monomers, the full variety of outside sequences can be made based on a polypyrimidine core sequence.[24,25]

RESULTS AND DISCUSSION

Chemical Synthesis. The two series of four double-headed nucleosides **3a–d** and **6a–d** were prepared in good yields using the Sonogashira cross-coupling reaction between the propargylated nucleobases (**2a–d**) and the 5′-ODMTr-protected 5-iodo-2′-deoxyuridine **1**[26,27] or the corresponding 5-iodo-2′-deoxycytidine derivative **5**,[28] respectively (Scheme 1). The appropriately protected N1- or N9-propargylated nucleobases **2a–c** were made from literature methods,[17,29−31] and the guanine derivative **2d** was made by a similar method[32] and a subsequent protection. The Sonogashira couplings proceeded in generally high yields (65–81%). Subsequent phosphitylation afforded the phosphoramidites **4a–d** and **7a–d**, respectively, as appropriate building blocks for automated oligonucleotide synthesis. The identity of all the new compounds was fully ascertained by NMR spectroscopy (1H, ^{13}C, ^{31}P, COSY, HSQC, and HMBC) and HRMS. Thus, the phosphoramidites **4a–d** and **7a–d** were all obtained from commercially available 5-iodo-2′-deoxyuridine or 5-iodo-2′-deoxycytidine in only three high-yielding steps and then introduced into oligonucleotides (ONs) to give the modified nucleotide monomers ($^T\mathbf{T}$, $^C\mathbf{T}$, $^A\mathbf{T}$, $^G\mathbf{T}$, $^T\mathbf{C}$, $^C\mathbf{C}$, $^A\mathbf{C}$, and $^G\mathbf{C}$), by using standard solid-phase DNA synthesis and 1*H*-tetrazole as the activator. Extended coupling time of 15 min was applied for the modified amidites affording >90% coupling yields. In the standard deprotection step, where ONs were treated with concentrated ammonia at 55 °C, some degree of substitution of the additional base for ammonia was observed in MALDI-MS. However, with prolonged ammonia treatment at room temperature, this was not observed, and the ONs were obtained in high yields and purity.

Hybridization Studies. At first, two monomers, $^T\mathbf{T}$ and $^A\mathbf{T}$, were incorporated once or three times into a 9-mer oligonucleotide sequence (Table 1). The modified oligonucleotides (ONs) were mixed with the complementary DNA and

Scheme 1[a]



[a]Reagents and conditions: (a) $Pd(PPh_3)_4$, CuI, Et_3N, DMF (**3a** 69%, **3b** 79%, **3c** 65%, **3d** 71%, **6a** 81%, **6b** 65%, **6c** 72%, **6d** 69%); (b) $NC(CH_2)_2OP(N(i\text{-}Pr)_2)Cl$, $(i\text{-}Pr)_2NEt$, CH_2Cl_2 (**4a** 74%, **4c** 78%, **4d** 70%, **7a** 75%, **7c** 78%) or $NC(CH_2)_2OP(N(i\text{-}Pr)_2)_2$, $(i\text{-}Pr)_2NH_2^+CHN_4^-$, CH_2Cl_2 (**4b** 71%, **7b** 71%, **7d** 63%). DMTr = 4,4′-dimethoxytrityl.

Table 1. Hybridization Data for Modified Duplexes

		T_m (ΔT_m/mod)[a] (°C)	
	sequence	cDNA 3′-CACAAAACG	cRNA 3′-CACAAAACG
ON1	5′-GTGTTTTGC	33.5	30.0
ON2	5′-GTGT^{T}TTTGC	36.0 (+2.5)	32.0 (+2.0)
ON3	5′-GTGT^{A}TTTGC	30.0 (−3.5)	26.5 (−3.5)
ON4	5′-GTG^{T}T^{T}T^{T}TTGC	37.0 (+1.2)	37.0 (+2.3)
ON5	5′-GTG^{A}T^{A}T^{A}TTGC	34.0 (+0.2)	36.5 (+2.2)

[a]Melting temperatures obtained from the maxima of the first derivatives of the melting curves (A_{260} vs temperature) recorded in a buffer containing 2.5 mM Na_2HPO_4, 5.0 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, pH 7.0 using 1.5 μM concentrations of each strand.

RNA sequences in medium salt buffer, and the melting temperatures (T_m) of the resulting duplexes were determined from the UV melting curves at neutral pH 7. A single incorporation of monomer $^T\mathbf{T}$ (ON2) is well tolerated in both DNA:DNA and DNA:RNA duplexes and induced an increase in the duplex stability compared to the unmodified duplex (ON1) by ~2.0 °C in both cases when placed in the center of the duplex. However, a single incorporation of monomer $^A\mathbf{T}$ (ON3) led to a destabilization of the corresponding modified

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Table 2. Hybridization Data for Modified Duplexes

		T_m[a] (°C)			
		cDNA1 3′-AAAAGXAAAGGGGGA, X =			
	sequence	A	C	T	G
ON6	5′-TTTTCTTTTCCCCCCT	50.5	40.5	43.0	44.0
ON7	5′-TTTTC^{T}TTTTCCCCCCT	51.5	41.5	42.5	43.0
ON8	5′-TTTTC^{C}TTTTCCCCCCT	50.5	41.5	43.0	43.0
ON9	5′-TTTTC^{A}TTTTCCCCCCT	50.0	40.5	42.5	43.0
ON10	5′-TTTTC^{G}TTTTCCCCCCT	50.5	41.0	42.5	42.5
ON11	5′-TTTT^{A}C^{A}T^{T}TTTCCCCCCT	54.0	44.0	45.0	45.5
ON17	5′-T^{T}T^{T}T^{A}T^{A}C^{A}T^{T}T^{T}T^{A}T^{T}C^{A}C^{T}C^{T}C^{T}CT	>75.0	62.5	61.0	66.0
		T_m[a] (°C)			
		cDNA1 3′-AAAAGAAAAXGGGGGA, X =			
	sequence	G	C	T	A
ON6	5′-TTTTCTTTTCCCCCCT	50.5	37.0	38.0	39.0
ON12	5′-TTTTCTTTT^{T}CCCCCCT	51.0	37.5	40.0	40.0
ON13	5′-TTTTCTTTT^{C}CCCCCCT	52.0	38.0	40.0	40.0
ON14	5′-TTTTCTTTT^{A}CCCCCCT	50.0	38.0	39.0	38.5
ON15	5′-TTTTCTTTT^{G}CCCCCCT	50.5	38.5	40.5	40.5
ON16	5′-TTTTCTTT^{A}T^{C}C^{A}CCCCCT	54.0	40.0	41.0	42.0
ON17	5′-T^{T}T^{T}T^{A}T^{A}C^{A}T^{T}T^{T}T^{A}T^{T}C^{A}C^{T}C^{T}C^{T}CT	>75.0	57.0	59.0	60.5
ON18	5′-^{C}T^{G}T^{G}T ^{C}T^{A}C^{G}T ^{C}T^{C}T^{G}T ^{T}C^{A}C^{T}C^{T}C^{T}CT	>75.0	n.d.	n.d.	n.d.
		T_m[a] (°C)			
	sequence	cDNA7 3′-GGCC GAA AGA AAA GAA GAG CGCG			
ON19	5′-CCGG CTT TCT TTT CTT CTC GCGC-3′	66.0			
ON20	5′-CCGG^{G}C^{C}T^{T}T^{G}T^{A}C^{C}T^{G}T^{A}T^{T}T^{A}C^{T}T^{G}T^{C}C^{C}T^{G}C GCGC-3′	>75.0			

[a]See Table 1.

duplexes by 3.5 °C in both cases. This indicates an unfavorable interaction of the A as compared to the T in the major groove, either steric or by disturbance of the groove hydration. Three incorporations of the monomer ^{T}T (ON4) gave a higher T_m with complementary DNA than with one incorporation (37 °C as compared to 36 °C for ON2). However, this corresponds to a lower increase of 1.2 °C for each incorporation. The increase for each incorporation against complementary RNA is still around 2.0 °C. Three incorporations of monomer ^{A}T (ON5) induced a positive effect on the thermal stability of modified duplexes in both cases although the effect was slightly more pronounced with the RNA target as compared to the DNA target. With DNA, the T_m was identical to the unmodified duplex, i.e., an increase of 4.0 °C as compared to a single incorporation of ^{A}T. With RNA, a decrease of 3.5 °C was converted to an increase of 2.2 °C for each incorporation. These results indicate favorable stacking interactions from the alkynyl groups in the modified monomers. Furthermore, this stacking effect seems to compensate for the slight negative influence that a single introduction of an additional adenine nucleobase has in the major groove.

Next, we studied the incorporation of each of the eight monomers (Figure 2) in 16-mer oligonucleotide sequences, ON7−10 and ON12−15 (Table 2), and the hybridization of these ONs to complementary DNA. As evident from Table 2, introduction of any of the monomers in the DNA duplex does not produce any major change in the thermal stability of the duplex as compared to the unmodified duplex (ON6:cDNA1). Monomers ^{T}T, ^{T}C and ^{C}C (ON7, ON12 and ON13) induced small increases in the duplex stability (0.5−1.5 °C), monomers ^{C}T, ^{G}T and ^{G}C (ON8, ON10 and ON15) had no net effect on the thermal stability, whereas monomers ^{A}T, and ^{A}C (ON9 and ON14) induced a slight decrease in the duplex stability (0.5 °C). Hence, both the positive effect of ^{T}T and the negative effect of ^{A}T were much smaller in this longer duplex as compared to the 9-mer duplex in Table 1.

Hereafter, we investigated the core Watson−Crick fidelity of the modified ONs (Table 2). The data revealed that the mismatch discrimination ability of the modified ONs is comparable to that of the unmodified ON6. Thus, a C opposite the modified T′s in ON7−10 is discriminated with 9−10 °C as compared to 10 °C for the unmodified T in ON6, whereas a T is discriminated with 7.5−9 °C (7.5 °C for ON6) and a G with 7−8.5 °C (6.5 °C for ON6). Opposite the modified C's in ON12−15, a C is discriminated with 12−14 °C as compared to 13.5 °C for ON6, a T is discriminated with 10−12 °C (12.5 °C for ON6), and an A is discriminated with 10−12 °C (11.5 °C for ON6). Hence, the studied double-headed monomers maintain both the affinity and specificity in Watson−Crick base pairing, which strongly suggests that the presence of an additional nucleobase at the 5-position does not interfere with the Watson−Crick base-pairing properties of the core pyrimidine nucleobases of the modified monomers.

Next, ONs with three consecutive incorporations of different monomers (ON11 and ON16) were synthesized to test the synergy between different monomers from the potential stacking effects. Modifications were introduced either in the thymidine or cytosine rich region of the unmodified ON6. Both ON11 and ON16 displayed a T_m of 54 °C corresponding to an increase in the thermal stability by 3.5 °C as compared to the unmodified duplex, which further supports our earlier observation from Table 1 of favorable stacking interactions from the modified monomers (Table 1). Importantly, ON11 and ON16 also maintained the fidelity of Watson−Crick base

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pairing. Thus, single mismatches were introduced opposite the central of the three modifications, and all mismatched nucleobases were discriminated with at least the same decrease in T_m as observed for ON6 (8.5−14.0 °C as compared to 6.5−13.5 °C).

With these indications that neither affinity for complementary DNA nor the fidelity in the recognition were hampered by the introduction of the double-headed nucleotides into the oligonucleotides, we decided to synthesize more heavily modified sequences. The 16-mer ON17 contains 14 modifications and was composed in such a way that the outer sequence is an oligo A−T sequence. Four of the eight modified nucleotides (^{T}**T**, ^{A}**T**, ^{T}**C**, and ^{A}**C**, Figure 2) were used to synthesize this sequence. ON17 was mixed with the complementary DNA-strand (cDNA1) to form a duplex on which a sequence of nucleobases is pointing outward into the major groove. This duplex was very stable with a T_m of >75 °C corresponding to an increase in T_m of more than 25 °C compared to the unmodified duplex. The ability of ON17 to discriminate between matched and mismatched targets was tested with the mismatches in either the T-rich or in the C-rich region. Successful discrimination was seen in all cases with decreases in T_m that were slightly higher as compared to the unmodified duplex formed by ON6. The formation of a duplex between ON17 and the matched cDNA1 was also confirmed by using nondenaturating gel electrophoresis (Figure 3). Both

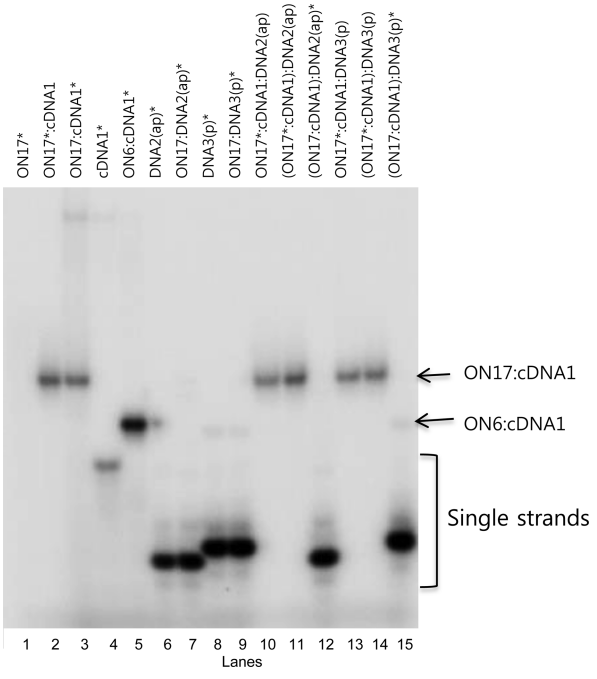

Figure 3. Nondenaturing PAGE to investigate oligonucleotide hybridization. *Indicates the labeled ON. For sequences of ON6, ON17, and cDNA1, see Table 2. DNA2(ap): 5′-AAAATATAATTTAA. DNA3(p): 5′-AATTTAATATAAAA.

ON17 and cDNA1 were ^{32}P-labeled in the experiment. In lane 2 and 3, the duplex is clearly observed with the label positioned on either of the two strands. The single stranded cDNA1 is seen in lane 4, but the labeled single stranded ON17 is for unknown reasons not observable in lane 1 (or in any other repeated experiments). Nevertheless, the presence of a duplex between ON17 and cDNA1 is clearly established.

In order to explore the recognition potential of the outer sequence of ON17, we used nondenaturating gels to search for complexes between ON17 and DNA-strands matching the outer oligo T-A sequence. Both an antiparallel and a parallel DNA were used (DNA2(ap) and DNA3(p), respectively), together with ON17 as a single strand or as the preformed ON17:cDNA1 duplex (Figure 3). Only bands corresponding to the single strands were observed in all cases without cDNA1 (Figure 3, lanes 6−9). In a mixture of ON17 with both cDNA1 and DNA2(ap), only the ON17:cDNA1 duplex was observed (lane 10), and this is also the case when ON17 and cDNA1 were premixed before addition of DNA2(ap) (lane 11). When only DNA2(ap) was labeled, it is clear that none of this sequence is complexed with other strands (lane 12). Exactly the same picture was observed with DNA3(p) instead of DNA2(ap) (lanes 13−15). In addition, UV−melting experiments showed no UV-meltings with mixtures of the unpaired ON17 and either DNA2(ap) or DNA3(p). Hereby, it is clear that the outer sequence of our double-coding oligonucleotide ON17 cannot be recognized by standard parallel or antiparallel DNA strands. This is not a surprising result, as the natural DNA backbone is not optimized for this sort of recognition. However, the fidelity of the inner duplex is confirmed by the fact that no non-Watson−Crick complexes are formed between ON17 and either DNA2(ap) or DNA3(p). As the recognition potential in an outer oligo T−A sequence as exposed by ON17 is indeed very low (the melting temperature of the duplex formed between DNA2(ap) and its natural complement was determined to be just 23 °C), we decided to test the potential in another sequence context. We therefore synthesized ON18 (Table 2) based on the same 16-mer oligopyrimidine sequence but with a mixed outer sequence of 15 nucleobases. Four of the eight modified building blocks (^{C}**T**, ^{G}**T**, ^{A}**C**, and ^{T}**C**) were used in this preparation. The melting temperature of the inner duplex again exceeded 75 °C confirming that stable duplexes are formed with these double-headed oligonucleotides despite of the sequence, and the duplex band was observed in a nondenaturating gel (see the Supporting Information). Nevertheless, the analysis of the recognition potential to outer parallel or antiparallel DNA-sequences again failed to indicate recognition of the outer sequence. Furthermore, the analysis was hampered by the unexpected formation of some secondary structures in the unmodified DNA-sequences (see the Supporting Information). For that reason, also a third heavily modified ON was made, ON20 (Table 2). Herein another core sequence was used, and 15 modified double-headed nucleotides were flanked by four CG-pairs in each end. Seven of our eight different monomers (Figure 2) were used for this 23-mer sequence. As expected, also ON20 formed a very stable duplex with its inner complement, cDNA7 (Table 2), which was confirmed in a nondenaturating gel (see the Supporting Information). Nevertheless, no recognition of outer DNA-complements was observed neither of the single stranded ON20 nor of the ON20:cDNA7 duplex.

Molecular Modeling. To ascertain the structural impact of the double-headed nucleotides in a duplex, we conducted molecular dynamics simulations of the unmodified dsDNA duplex (ON6:cDNA1) and two duplexes with an (almost) fully modified strand, ON17:cDNA1 and ON18:cDNA1. The simulations were stable and converged, thus showing that there is no steric hindrance for duplex formation with the double-headed nucleotides as already shown experimentally by melting analysis. The integrity of the Watson−Crick duplex is

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fully maintained with only slight structural perturbation by the extra nucleobases that are located in the major groove. To accommodate the additional array of nucleobases, the Watson–Crick core is only slightly perturbed. The width of the minor groove is changed slightly, broadened in the TA region of the duplex and narrowed in the CG region (see the Supporting Information, Table S2), and the Watson–Crick nucleobases are displaced a few angstroms from the helix axis to form a slightly unwound and ladder-like duplex (Figure 4). Overall, the minor groove width is more uniform in the modified duplexes than in the native duplex where a variation of ∼2 Å is observed between the TA and CG regions.

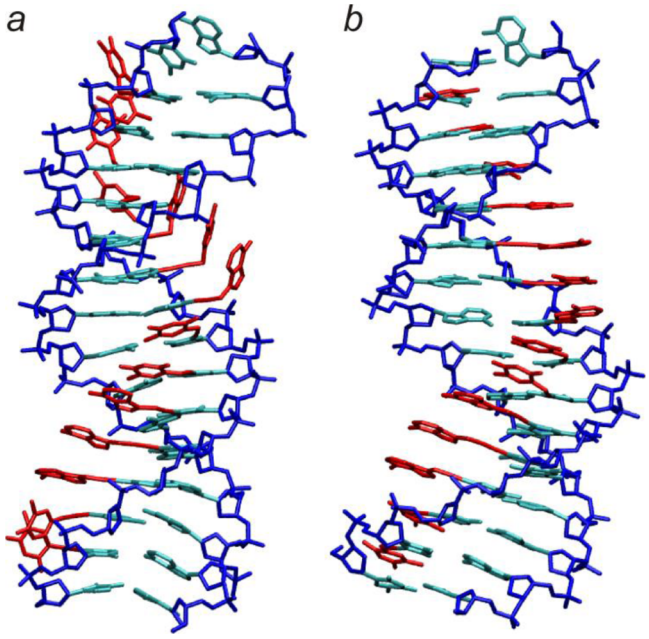

Figure 4. Snapshots from molecular dynamics simulations; the sugar–phosphate backbone is colored blue, Watson–Crick nucleobases cyan and major groove nucleobases red. (a) Free MD simulation of ON17:cDNA1: the two representative major groove conformations are both observed, type 1 with nucleobases almost planar with the Watson–Crick stack and type 2 with nucleobases tilted. (b) Restrained MD simulation of ON17:cDNA1 with all major groove nucleobases restrained in type 1 conformation.

To form a platform for recognition by a third nucleotide strand of some sort, the additional nucleobases in the major groove need to present either their Watson–Crick or Hoogsteen faces in a regular and continuous array. First we note that the hydrophilic edges of nucleobases are indeed exposed to the solvent and not buried in the major groove. Second, the nucleobases in the major groove are flexible and adopt a range of different conformations over the course of the simulations presenting mainly the Watson–Crick edge but also the Hoogsteen edge in accessible positions, and third, the nucleobases never present themselves in a continuous array.

The additional nucleobases adopt two major ranges of conformations (Figure 4 and Figure S4, Supporting Information) and we tested if it was possible to form a regular and continuous array of nucleobases by restraining the nucleobases in each of these geometries. In each case, only weak restraints, 1 kcal/(mol rad^2), were needed to enforce the desired geometry, but a regular array of nucleobases over time was formed only in one case (Type 1 conformation) (Figure 4). The continuous array of nucleobases in the major groove was achieved without any notable structural rearrangement of the Watson–Crick duplex. We gauged the regularity of the stack of nucleobases by measuring the interbase N1 (purines)/N3 (pyrimidines) distance between juxtaposed nucleobases. Even in the case shown in Figure 4b where a regular stack of nucleobases was formed in the major groove, the interbase N1/N3 distance was on average approximately 1 Å longer than in a Watson–Crick stack over the course of the trajectory (Table S3, Supporting Information). This does indicate that a native DNA backbone might not be the optimum design for recognition of the major groove-based nucleobases.

Finally, we observed in our simulations that the alkyne linkers are prone to stack with adjacent nucleobases rather than with adjacent alkynes (Figure S5, Supporting Information). This is to some extent contrary to the observation that an increase in melting temperature mainly follows upon consecutive incorporations of the double-headed nucleotides. Nevertheless, the alkyne clearly extends the size of the conjugated system and thereby also increase the stacking and the reason why a single incorporation is generally not increasing duplex stability is elusive but might be due to hydration effects.

DISCUSSION

The eight double-headed nucleotide monomers (^{T}**T**, ^{C}**T**, ^{A}**T**, ^{G}**T**, ^{T}**C**, ^{C}**C**, ^{A}**C**, and ^{G}**C**) were all very conveniently synthesized via the phopsphoramidites **4a–d** and **7a–d**. The present synthesis of phosphoramidites involves a very simple three-step procedure from commercially available 5-iodo-2′-deoxyuridine and 5-iodo-2′-deoxycytidine in combination with simple N1- or N9-propargylated nucleobases prepared in one to three steps from the commercial nucleobases. The incorporation into ONs is straightforward, and heavily modified sequences can easily be formed. Very stable duplexes are formed, and the thermal stability is in general increased with at least 1.5 °C for each modification owing to stacking of the alkyne linkers with adjacent nucleobases. Importantly, all the modified nucleotide monomers maintained the Watson–Crick base pairing specificity in the duplex formation. Thus, the outer nucleobases are not interfering with the base-pairing of the core pyrimidines. Thereby we have shown that stable duplexes with an array of additional nucleobases in the major groove can easily be produced and that any sequence compositions of the outer sequences and any oligopyrimidine composition of the inner sequence might be produced. This might in the future be extended to inner purine nucleobases, as Sonogashira couplings to N7-iodo-N7-deazapurines is well-known chemistry, known to position various substituents into the major groove of DNA duplexes.[25,33,34]

Simulations show how the array of additional nucleobases is placed in the major groove where they are rather flexible. However, weak restraints are sufficient to order these nucleobases in a regular array where they present the Watson–Crick face for recognition. The simulations corroborate the experiments and indicate that a native DNA backbone is not optimum for recognition as the distance between adjoining Watson–Crick faces is larger than in a Watson–Crick base-paired duplex.

The open question is how the array of nucleobases in the major groove can be recognized by other nucleic acids. In other words, can these double-headed oligonucleotides form the basis of a true double-coding DNA system? So far, we have explored

the possibility of recognizing complementary DNA-strands in a parallel or antiparallel orientation, but as evident from the PAGE analyses and rationalized from the modeling, base-pairing in this way is not possible. This can be explained by incompatible geometry in combination with the rigidity of the duplex already formed, but repulsion between negatively charged phosphate backbones might also play a role. Overall, the backbone constitution of the third strand is not right for this complexation, and therefore, we plan to study other nucleic acid systems for this recognition. For instance, more flexible and/or neutral backbones might be able to recognize the outside sequence.

CONCLUSION

We have described an efficient synthetic access to double-headed nucleotides and oligonucleotides with additional nucleobases attached in the 5-position of the pyrimidines. We have shown the formation of very stable duplexes with a full array of additional nucleobases protruding outward into the major groove. Native DNA oligonucleotides could not recognize and bind to the major groove bases but we continue to search for an appropriately modified nucleic acid system that might do so. This would then generate a double coding DNA system, where part of the information encoded is accessible without unwinding the duplex, the information content is doubled and where information can be translated from one code to another. This might be a new paradigm in nucleic acid nanotechnology.

EXPERIMENTAL SECTION

General Methods. All commercial reagents were used as supplied, except CH_2Cl_2, which was distilled prior to use. Anhydrous solvents were dried over 4 Å activated molecular sieves (CH_2Cl_2, pyridine, and DCE) or 3 Å activated molecular sieves (DMF). All reactions were carried out under an atmosphere of argon or nitrogen when anhydrous solvents were used. Reactions were monitored using TLC analysis with Merck silica gel plates (60 F_{254}). To visualize the plates, they were exposed to UV light (254 nm) and/or immersed in a solution of 5% H_2SO_4 in methanol followed by charring. Standard column chromatography was performed using silica gel 60 (0.040 0.063 mm). Silica gel was pretreated with 1% pyridine in CH_2Cl_2 (v/v) for the purification of 4,4′-dimethoxytrityl-protected nucleosides. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 400, 101, and 162 MHz, respectively. Chemical shift values (δ) are reported in ppm relative to either tetramethylsilane (^{1}H NMR) or the deuterated solvents as internal standard for ^{13}C NMR (δ $CDCl_3$ 77.160 ppm, DMSO-d_6 39.52 ppm), and relative to 85% H_3PO_4 as external standard for ^{31}P NMR. 2D spectra (^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and HMBC) have been used in assigning ^{1}H and ^{13}C NMR signals. High-resolution ESI (quadrupole) mass spectra were recorded in positive mode.

Synthesis of *N*2-(Isobutyryl)-*N*9-propargylguanine (2d). *N*9-Propargylguanine[32] (0.40 g, 2.11 mmol) and isobutyric anhydride (3.5 mL, 21.1 mmol) were mixed with DMA (30 mL), and the suspension was heated to 145 °C to obtain a clear solution and then stirred at this temperature for another 4 h. The mixture was concentrated under reduced pressure, and the residue was mixed with 80 mL of EtOH/H_2O (1:1, v/v). The mixture was heated to 100 °C to obtain a clear solution and then concentrated under reduced pressure until a solid appeared. The mixture was cooled to room temperature and then kept at 5 °C for 2 h. The precipitated solid was filtered, washed with cold EtOH/H_2O (15 mL, 1:1, v/v), and dried under reduced pressure to obtain **2d** (0.38 g, 69%) as a brown powder: R_f 0.3 (6% MeOH in CH_2Cl_2); ^{1}H NMR (DMSO-d_6) δ 12.12 (s, 1H, NH), 11.76 (s, 1H, NH), 8.05 (s, 1H, H8), 4.96 (s, 2H, CH_2), 3.51 (s, 1H, C≡CH), 2.83−2.76 (m, 1H, C*H*(CH_3)$_2$), 1.12 (d, J = 6.8 Hz, 6H, CH(CH_3)$_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.1 (COCH(CH_3)$_2$), 154.7 (C4), 148.3, 148.1 (C2, C6), 139.0 (C8), 119.9 (C5), 77.9 (C≡CH), 76.2 (*C*≡CH), 34.6 (CH(CH_3)$_2$), 32.6 (CH_2), 18.8 (CH(CH_3)$_2$); HR-ESI MS *m*/*z* 282.0975 ([M + Na]$^+$, $C_{12}H_{13}N_5O_2Na^+$ calcd 282.0961).

General Procedure for the Synthesis of Double-Headed Nucleosides 3a−d and 6a−d. Nucleoside **1** or **5** (0.50−1.0 mmol), propargylated nucleobase **2a−d** (0.75−1.50 mmol), CuI (0.10−0.20 mmol), and $Pd(PPh_3)_4$ (0.05−0.10 mmol) were suspended in DMF (5−10 mL), and the content was degassed using argon. To this was added Et_3N (0.3−0.6 mL), and the resulting mixture was stirred under an argon atmosphere in the dark 12−16 h at room temperature. When the reaction was completed according to TLC, the reaction mixture was diluted with EtOAc (50−100 mL) and a saturated aqueous solution of $NaHCO_3$ (50−100 mL) was added. The phases were separated, and the organic phase was washed with brine (30−50 mL). The combined aqueous phase was extracted with EtOAc (30−50 mL), and the combined organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (0−5% MeOH in CH_2Cl_2) to afford the desired double-headed nucleoside.

*5′-O-(4,4′-Dimethoxytrityl)-5-(3-(thymin-1-yl)propyn-1-yl)-2′-deoxyuridine (**3a**).* The general procedure was applied with **1** (0.32 g, 0.50 mmol), 1-propargylthymine **2a** (164 mg, 1.00 mmol), CuI (20 mg, 0.1 mmol), $Pd(PPh_3)_4$ (60 mg, 0.05 mmol), and Et_3N (0.3 mL): yield 240 mg (69%); R_f 0.4 (6% MeOH in CH_2Cl_2); ^{1}H (DMSO-d_6) δ 11.72 (bs, 1H, NH), 11.37 (bs, 1H, NH), 7.99 (s, 1H, H6(T)), 7.41−7.39 (m, 2H, DMTr), 7.36 (s, 1H, H6(U)), 7.32−7.27 (m, 6H, DMTr), 7.23−7.19 (m, 1H, DMTr), 6.89 (d, 4H, *J* = 8.4 Hz, DMTr), 6.09 (t, 1H, *J* = 6.4 Hz, H1′), 5.33 (d, 1H, *J* = 3.2 Hz, 3′−OH), 4.50 (s, 2H, CH_2), 4.27 (bs, 1H, H3′), 3.91 (bs, 1H, H4′), 3.73 (s, 6H, 2 × OCH_3), 3.24 (dd, 1H, *J* = 10.1 Hz, 4.9 Hz, H5′), 3.10 (d, 1H, *J* = 10.1 Hz, H5′), 2.32−2.25 (m, 1H, H2′), 2.21−2.16 (m, 1H, H2′), 1.70 (s, 3H, CH_3); ^{13}C (DMSO-d_6) δ 164.1 (C4(T)), 161.6 (C4(U)), 158.1 (DMTr), 150.3 (C2(T)), 149.3 (C2(U)), 144.8 (C6(U)), 143.9 (DMTr), 139.6 (C6(T), 135.6, 135.3, 129.7, 129.7, 127.9, 127.5, 126.7, 113.3, 113.2 (DMTr), 109.2 (C5(T)), 97.6 (C5(U)), 86.6 (C4′), 85.9 (DMTr, C≡C), 85.2 (C1′), 77.0 (C≡C), 70.4 (C3′), 63.7 (C5′), 55.0 (OCH_3), 39.9 (C2′), 36.9 (CH_2), 11.9 (CH_3); HR-ESI MS *m*/*z* 715.2367 ([M + Na]$^+$, $C_{38}H_{36}N_4O_9Na^+$ calcd 715.2374).

*5-(3-(4-N-Acetylcytosin-1-yl)propyn-1-yl)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**3b**).* The general procedure was applied with **1** (0.32 g, 0.50 mmol), 4-*N*-acetyl-1-propargylcytosine **2b** (120 mg, 0.63 mmol), CuI (20 mg, 0.10 mmol), $Pd(PPh_3)_4$ (60 mg, 0.05 mmol), and Et_3N (0.3 mL): yield 0.28 g (79%); R_f 0.5 (6% MeOH in CH_2Cl_2); ^{1}H (DMSO-d_6) δ 11.74 (s, 1H, NH(U)), 10.88 (s, 1H, NH(C)), 8.03 (s, 1H, H6(U)), 7.88 (d, 1H, *J* = 7.6 Hz, H6(C)), 7.40−7.38 (m, 2H, Ar), 7.31−7.26 (m, 6H, DMTr), 7.22−7.18 (m, 1H, DMTr), 7.12 (d, 1H, *J* = 7.6 Hz, H5(C)), 6.88 (d, 4H, *J* = 8.4 Hz, DMTr), 6.11 (t, 1H, *J* = 6.4 Hz, H1′), 5.33 (d, *J* = 4.4 Hz, 3′−OH), 4.63 (s, 2H, CH_2), 4.30−4.25 (m, 1H, H3′), 3.93−3.90 (m, 1H, H4′), 3.73, (s, 6H, 2 × OCH_3), 3.23 (dd, 1H, *J* = 10.4 Hz, 5.2 Hz, H5′), 3.11 (dd, 1H, *J* = 10.4 Hz, 2.7 Hz, H5′), 2.33−2.26 (m, 1H, H2′), 2.23−2.17 (m, 1H, H2′), 2.10 (s, 3H, CH_3); ^{13}C (DMSO-d_6) δ 170.8 (CO), 162.4 (C4(C)), 161.4 (C4(U), 158.0 (DMTr), 154.4 (C2(C)), 149.1 (C2(U)), 148.1 (C6(C)), 144.7 (DMTr), 143.9 (C6(U)), 135.5, 135.1, 129.6, 129.6, 127.8, 127.4, 126.5, 113.0 (DMTr), 97.3 (C5(U)), 95.5 (C5(C)), 85.9, 85.8, 85.7 (DMTr, C4′, C≡C), 85.1 (C1′), 78.0 (C≡C), 70.2 (C3′), 63.6 (C5′), 54.9 (OCH_3), 40.3 (C2′), 38.5 (CH_2), 24.3 (CH_3); HR-ESI MS *m*/*z* 742.2481 ([M + Na]$^+$, $C_{39}H_{37}N_5O_9Na^+$ calcd 742.2483).

*5-(3-(6-N-Benzoyladenin-9-yl)propyn-1-yl)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**3c**).* The general procedure was applied with **1** (0.32 g, 0.50 mmol), 6-*N*-benzoyl-9-propargyladenine **2c** (0.28 mg, 1.01 mmol), CuI (20 mg, 0.10 mmol), $Pd(PPh_3)_4$ (60 mg, 0.05 mmol), and Et_3N (0.3 mL): yield 0.25 g (65%); R_f 0.4 (6% MeOH in CH_2Cl_2); ^{1}H (DMSO-d_6) δ 11.73 (bs, 1H, NH), 11.20 (bs, 1H, NH), 8.76 (s, 1H, H2(A)), 8.40 (s, 1H, H8(A)), 8.06 (d, 2H, *J* = 7.6 Hz, Bz), 8.01 (s, 1H, H6 (U)), 7.65 (t, 1H, *J* = 7.6 Hz, Bz), 7.55 (t, 1H, *J* = 7.6 Hz, Bz), 7.40−7.38 (m, 2H, DMTr), 7.31−7.27 (m, 6H, DMTr), 7.19−7.16 (m, 1H, DMTr), 6.88 (d, 4H, *J* = 8.0 Hz, DMTr), 6.10 (t, *J* = 6.4 Hz, 1H, H1′), 5.33 (d, 1H, *J* = 4.4 Hz, 3′−OH), 5.19 (s, 2H,

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CH_2), 4.28−4.24 (m, 1H, H3′), 3.94−3.90 (m, 1H, H4′), 3.71 (s, 6H, 2 × OCH_3), 3.25 (dd, 1H, *J* = 10.0 Hz, 5.4 Hz, H5′), 3.08 (d, 1H, *J* = 10.0 Hz, H5′), 2.33−2.26 (m, 1H, H2′), 2.23−2.16 (m, 1H, H2′); ^{13}C (DMSO-d_6) δ 165.6 (CO), 161.5 (C4(U)), 158.1 (DMTr), 151.8 (C4(A)), 151.7 (C2(A)), 150.3 (C6(A)), 149.3 (C2(U)), 144.8 (DMTr), 144.1 (C8(A)), 143.6 (C6 (U)), 135.6, 135.3, 133.3, 132.4 (Bz), 129.7, 129.7, 128.5 (Bz), 127.9, 127.5, 126.6, 125.2 (C5(A)), 113.2, 113.2, (DMTr) 97.4 (C5(U), 85.8, 85.8 (C4′, C≡C, DMTr), 85.2 (C1′), 77.4 (C≡C), 70.3 (C3′), 63.8 (C5′), 55.0 (OCH_3), 39.8 (C2′), 33.5 (CH_2); HR-ESI MS *m*/*z* 828.2747 ([M + Na]$^+$, $C_{45}H_{39}N_7O_8Na^+$ calcd 828.2752).

*5′-O-(4,4′-Dimethoxytrityl)-5-(3-(2-N-isobutyrylguanin-9-yl)-propyn-1-yl)-2′-deoxyuridine (**3d**).* The general procedure was applied with **1** (0.32 g, 0.50 mmol), 2-*N*-isobutryl-9-propargylguanine **2d** (195 mg, 0.75 mmol), CuI (20 mg, 0.10 mmol), $Pd(PPh_3)_4$ (60 mg, 0.05 mmol) and Et_3N (0.3 mL): yield 0.28 g (71%); R_f 0.3 (6% MeOH in CH_2Cl_2); ^{1}H NMR (DMSO-d_6) δ 12.12 (s, 1H, NH), 11.76, (s, 1H, NH), 11.74 (s, 1H, NH), 7.98 (s, 1H, H6(U)), 7.86 (s, 1H, H8(G)), 7.39−7.37 (m, 2H, DMTr), 7.29−7.24 (m, 6H, DMTr), 7.18−7.16 (m, 1H, DMTr), 6.87−6.84 (m, 4H, DMTr), 6.08 (t, 1H, *J* = 6.4 Hz, H1′), 5.32 (d, 1H, *J* = 4.8 Hz, 3′−OH), 5.03−4.93 (m, 2H, CH_2), 4.26−4.22 (m, 1H, H3′), 3.92−3.89 (m, 1H, H4′), 3.72, (s, 6H, 2 × OCH_3), 3.21 (dd, 1H, *J* = 10.5 Hz, 5.5 Hz, H5′), 3.09 (dd, 1H, *J* = 10.5 Hz, 2.9 Hz, H5′), 2.83−2.75 (m, 1H, C*H*(CH_3)$_2$), 2.33−2.26 (m, 1H, H2′), 2.23−2.17 (m, 1H, H2′), 1.11 (d, J = 6.8 Hz, 6H, CH(C*H*$_3$)$_2$); ^{13}C (DMSO-d_6) δ 180.0 (CO), 161.4 (C4 (U), 157.9 (DMTr), 154.7 (C6(G)), 149.2 (C2(U)), 148.0 (C2(G), C4(G)), 144.7 (H6(U)), 144.1, 135.4, 135.1, 129.6, 129.6, 127.8, 127.4, 126.5 (DMTr), 120.0 (C5(G)), 112.9 (DMTr), 97.0 (C5(U)), 85.7, 85.7 (C4′, C≡C, DMTr), 85.3 (C1′), 77.5 (C≡C), 70.2 (C3′), 63.7 (C5′), 54.9 (OCH_3), 39.3 (C2′), 34.5 (CH(CH_3)$_2$), 33.3 (CH_2), 18.6 (CH(CH_3)$_2$). HR-ESI MS *m*/*z* 810.2817 ([M + Na]$^+$, $C_{42}H_{41}N_7O_9Na^+$ calcd 810.2858).

*5′-O-(4,4′-Dimethoxytrityl)-4-N-(dimethylaminomethylene)-5-(3-(thymin-1-yl)propyn-1-yl)-2′-deoxycytidine (**6a**).* The general procedure was applied with **5** (0.30 g, 0.42 mmol), 1-propargylthymine **2a** (140 mg, 0.85 mmol), CuI (20 mg, 0.10 mmol), $Pd(PPh_3)_4$ (60 mg, 0.05 mmol), and Et_3N (0.3 mL): yield 0.32 g (81%); R_f 0.4 (6% MeOH in CH_2Cl_2); ^{1}H NMR (DMSO-d_6) δ 11.37 (s, 1H, NH), 8.62 (s, 1H, CH=N), 8.10 (s, 1H, H6(C)), 7.44−7.40 (m, 3H H6(T), DMTr), 7.33−7.28 (m, 6H, DMTr), 7.24−7.21 (m, 1H, DMTr), 6.90 (d, 4H, *J* = 8.0 Hz, DMTr), 6.12 (t, 1H, *J* = 6.6 Hz, H1′), 5.33 (d, 1H, *J* = 4.4 Hz, 3′−OH), 4.54−4.44 (m, 2H, CH_2), 4.30−4.26 (m, 1H, H3′), 3.99−3.94 (m, 1H, H4′), 3.73 (s, 6H, 2 × OCH_3), 3.27 (dd, 1H, *J* = 10.3 Hz, 5.0 Hz, H5′), 3.19 (s, 3H, CH_3), 3.12 (dd, 1H, *J* = 10.3 Hz, 2.0 Hz, H5′), 3.04 (s, 3H, CH_3), 2.33−2.27 (m, 1H, H2′), 2.19−2.12 (m, 1H, H2′), 1.70 (s, 3H, CH_3); ^{13}C (DMSO-d_6) δ 169.7 (C4(C)), 164.0 (C4(T)), 158.0, 157.8 (CH=N, DMTr), 153.2 (C2 (C)), 150.2 (C2(T)), 144.7 (C6 (C)), 139.6 (C6(T)), 135.54, 135.2, 129.6, 129.6, 127.8, 127.4, 126.6, 113.2 (DMTr), 109.0 (C5(T)), 96.7 (C5(C)) 86.0, 85.9, 85.9, 85.8 (C4′, C1′, DMTr, C≡C), 78.8 (C≡C), 70.4 (C3′), 63.5 (C5′), 54.9 (OCH_3), 40.9 (CH_3), 40.8 (C2′), 36.8 (CH_2), 34.5 (CH_3), 11.6 (CH_3); HR-ESI MS *m*/*z* 747.3172 ([M + H]$^+$, $C_{41}H_{43}N_6O_8^+$ calcd 747.3137).

*5-(3-(4-N-Acetylcytosin-1-yl)propyn-1-yl)-5′-O-(4,4′-dimethoxytrityl)-4-N-(dimethylaminomethylene)-2′-deoxycytidine (**6b**).* The general procedure was applied with **5** (710 mg, 1.00 mmol), 4-*N*-acetyl-1-propargylcytosine **2b** (270 mg, 1.41 mmol), CuI (40 mg, 0.20 mmol), $Pd(PPh_3)_4$ (120 mg, 0.10 mmol), and Et_3N (0.6 mL): yield 0.50 g (65%); R_f 0.4 (6% MeOH in CH_2Cl_2); ^{1}H NMR (DMSO-d_6) δ 10.89 (s, 1H, NH), 8.64 (s, 1H, CH=N), 8.15 (s, 1H, H6(C_1)), 8.05 (d, 1H, *J* = 7.6 Hz, H6(C_2), 7.43−7.41 (m, 2H, DMTr), 7.33−7.29 (m, 6H, DMTr), 7.23−7.19 (m, 1H, DMTr), 7.13 (d, 1H, *J* = 7.6 Hz, H5(C_2), 6.91−6.88 (m, 4H, DMTr), 6.11 (t, 1H, *J* = 6.4 Hz, H1′), 5.34 (d, 1H, *J* = 4.4 Hz, 3′−OH), 4.67−4.57 (m, 2H, CH_2), 4.32−4.27 (m, 1H, H3′), 3.99−3.96 (m, 1H, H4′), 3.73, (s, 6H, 2 × OCH_3), 3.23 (dd, 1H, *J* = 10.4 Hz, 5.0 Hz, H5′), 3.21 (s, 3H, CH_3), 3.14 (dd, 1H, *J* = 10.4 Hz, 2.4 Hz, H5′), 3.05 (s, 3H, CH_3), 2.34−2.28 (m, 1H, H2′), 2.22−2.14 (m, 1H, H2′), 2.11 (s, 3H, CH_3); ^{13}C (DMSO-d_6) δ 170.9 (CO), 169.8 (C4(C_1)), 162.4 (C4(C_2)), 158.1 158.0 (DMTr, CH=N), 154.6 (C2(C_2)), 153.3 (C2(C_1)), 148.0 (C6(C_2)), 145.0 (C6(C_1)), 144.8, 135.6, 135.2, 129.7, 129.7, 127.9, 127.5, 126.6, 113.2, 113.2, (DMTr) 96.7 (C5(C_1)), 95.4 (C5(C_2)), 85.9, 85.9, 85.3, (C4′, C1′, DMTr), 80.8 (C≡C) 70.4 (C3′), 63.6 (C5′), 55.0 (OCH_3), 41.0 (C2′), 40.9 (CH_3), 39.0 (CH_2), 34.8 (CH_3), 24.3 (CH_3); HR-ESI MS *m*/*z* 774.3263 ([M + H]$^+$, $C_{42}H_{43}N_7O_8H^+$ calcd 774.3246).

*5-(3-(6-N-Benzoyladenin-9-yl)propyn-1-yl)-5′-O-(4,4′-dimethoxytrityl)-4-N-(dimethylaminomethylene)-2′-deoxycytidine (**6c**).* The general procedure was applied with **5** (0.71 g, 1.00 mmol), 6-*N*-benzoyl-9-propargyladenine **2c** (0.35 g, 1.26 mmol), CuI (40 mg, 0.20 mmol), $Pd(PPh_3)_4$ (120 mg, 0.10 mmol), and Et_3N (0.6 mL): yield 0.62 g (72%); R_f 0.4 (7% MeOH in CH_2Cl_2); ^{1}H NMR (DMSO-d_6) δ 11.21 (s, 1H, NH), 8.76 (s, 1H, H2(A)), 8.59 (s, 1H, CH=N), 8.46 (s, 1H, H8(A)), 8.12 (s, 1H, H6(C)), 8.02 (d, 2H, *J* = 7.6 Hz, Bz), 7.65 (t, 1H, *J* = 7.6 Hz, Bz), 7.58−7.54 (m, 2H, Bz), 7.43−7.41 (m, 2H, DMTr), 7.32−7.30 (m, 6H, DMTr), 7.23−7.19 (m, 1H, DMTr), 6.90 (d, 4H, *J* = 7.8 Hz, DMTr), 6.12 (t, 1H, *J* = 6.6 Hz, H1′), 5.33 (d, 1H, *J* = 4.4 Hz, 3′−OH), 5.20−5.10 (m, 2H, CH_2), 4.29−4.26 (m, 1H, H3′), 3.98−3.95 (m, 1H, H4′), 3.72 (s, 6H, 2 × OCH_3), 3.28 (dd, 1H, *J* = 10.5 Hz, 5.2 Hz, H5′), 3.17 (s, 3H, CH_3), 3.12 (dd, 1H, *J* = 10.5 Hz, 2.4 Hz, H5′), 2.94 (s, 3H, CH_3), 2.32−2.26 (m, 1H, H2′), 2.18−2.13 (m, 1H, H2′). ^{13}C (DMSO-d_6) δ 169.7 (C4(C)), 165.5 (CO), 158.0, 157.9 (DMTr, CH=N), 153.2 (C2(C), 151.8 (C4(A)), 151.6 (C2(A)), 150.1 (C6(A)), 144.9, 144.7 (DMTr, C6(C)), 143.6 (C8(A)), 135.5, 135.2, 133.3, 132.3, 129.6, 129.6, 128.4, 127.8, 127.5, 126.6 (Bz, DMTr), 125.2 (C5(A)), 113.1 (DMTr), 96.5 (C5(C)), 85.9, 85.8, 85.87 (C4′, DMTr, C≡C), 85.2 (C1′), 79.5, 70.3 (C3′), 63.6 (C5′), 54.9 (OCH_3), 40.8 (CH_3), 40.7 (C2′), 34.6 (CH_3), 33.5 (CH_2); HR-ESI MS *m*/*z* 882.3350 ([M + Na]$^+$, $C_{48}H_{45}N_9O_7Na^+$ calcd 882.3334).

*5′-O-(4,4′-Dimethoxytrityl)-4-N-(dimethylaminomethylene)-5-(3-(2-N-isobutyrylguanin-9-yl)propyn-1-yl)-2′-deoxycytidine (**6d**).* The general procedure was applied with **5** (0.71 g, 1.00 mmol), 2-*N*-isobutyryl-9-propargylguanine **2d** (0.39 g, 1.50 mmol), CuI (40 mg, 0.1 mmol), $Pd(PPh_3)_4$ (120 mg, 0.05 mmol), and Et_3N (0.3 mL): yield 0.58 g (69%); R_f 0.4 (7% MeOH in CH_2Cl_2); ^{1}H NMR (DMSO-d_6) δ 12.11 (s, 1H, NH), 11.73 (s, 1H, NH), 8.62 (s, 1H, CH=N), 8.11 (s, 1H, H6(C)), 8.02 (s, 1H, H8(G)), 7.43−7.41 (m, 2H, DMTr), 7.33−7.28 (m, 6H, DMTr), 7.23−7.19 (m, 1H, DMTr), 6.89 (d, 4H, *J* = 8.0 Hz, DMTr), 6.12 (t, 1H, *J* = 6.4 Hz, H1′), 5.33 (d, 1H, *J* = 4.4 Hz, 3′−OH), 4.99−4.88 (m, 2H, CH_2), 4.29−4.24 (m, 1H, H3′), 3.98−3.95 (m, 1H, H4′), 3.73, (s, 6H, 2 × OCH_3), 3.26 (dd, 1H, *J* = 10.4 Hz, 5.0 Hz, H5′), 3.18 (s, 3H, N−CH_3), 3.12 (dd, 1H, *J* = 10.4 Hz, 2.4 Hz, H5′), 2.97 (s, 3H, N−CH_3), 2.79 (septet, 1H, *J* = 6.8 Hz, C*H*(CH_3)$_2$), 2.34−2.28 (m, 1H, H2′), 2.21−2.14 (m, 1H, H2′), 1.13 (d, 6H, *J* = 6.8 Hz, CH(C*H*$_3$)$_2$). ^{13}C (DMSO-d_6) δ 180.2 (CO), 169.8 (C4(C)), 161.7, 158.1, 157.4 (DMTr, CH=N), 154.8 (C6(G)), 153.2 (C2(C)), 148.3, 148.1 (C2(G), C4(G)), 145.0, 144.8 (DMTr, C6(C)), 138.7 (C8(G)), 135.5, 135.3, 129.7, 127.9, 127.5, 126.6, 125.8 (DMTr), 120.1 (C5(G)), 113.2 (DMTr), 96.5 (C5(C)), 86.0, 85.9, 85.8, 85.0 (DMTr, C4′, C1′, C≡C), 79.9 (C≡C), 70.4 (C3′), 63.7 (C5′), 55.0 (OCH_3), 40.9 (N−CH_3), 40.7 (C2′), 34.7 (N−CH_3), 34.6 (CH(CH_3)$_2$), 33.8 (CH_2), 18.8 (CH(CH_3)$_2$)); HR-ESI MS *m*/*z* 842.3617 ([M + H]$^+$, $C_{45}H_{47}N_9O_8H^+$ calcd 842.3620).

General Procedure for Preparation of Phosphoramidites 4a−d and 7a−d. Procedure A: Nucleoside **3** or **6** (0.20 mmol) was coevaporated with anhydrous 1,2-dichloroethane (2 × 5 mL) and dissolved in anhydrous CH_2Cl_2 (5 mL). DIPEA (1.00 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.60 mmol) were added, and the reaction mixture was stirred at room temperature for 2 h and then quenched by the addition of 2−3 drops of anhydrous ethanol. The residue was concentrated under reduced pressure and purified by column chromatography (0−3% MeOH in CH_2Cl_2) to afford the desired phosphoramidite.

Procedure B: Nucleoside **3** or **6** (0.23−0.28 mmol) was coevaporated with anhydrous 1,2-dichloroethane (2 × 5 mL) and dissolved in anhydrous CH_2Cl_2 (5 mL). Diisopropylammonium tetrazolide salt (0.47−0.56 mmol) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite (0.46−0.56 mmol) were added, and the reaction mixture was stirred at room temperature for 16h, and then

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quenched by the addition of 2−3 drops of anhydrous ethanol. The residue was concentrated under reduced pressure and purified by column chromatography (0−3% MeOH in CH_2Cl_2) to afford the desired phosphoramidite.

*3′-O-(P-2-Cyanoethyl-N,N-diisopropylaminophosphinyl)-5′-O-(4,4′-dimethoxytrityl)-5-(3-(thymin-1-yl)propyn-1-yl)-2′-deoxyuridine (**4a**).* The general procedure A was applied with nucleoside **3a** (220 mg, 0.31 mmol), *N,N*-diisopropylchlorophosphoramidite (280 μL, 1.19 mmol), and DIPEA (354 μL, 2.03 mmol): yield 210 mg (74%); R_f 0.4 (4% MeOH in CH_2Cl_2); ^{31}P ($CDCl_3$) δ 149.07, 148.77; HR-ESI MS *m/z* 915.3467 ([M + Na]$^+$, $C_{47}H_{53}N_6O_{10}PNa^+$ calcd 915.3453).

*5-(3-(4-N-Acetylcytosin-1-yl)propyn-1-yl)-3′-O-(P-2-cyanoethyl-N,N-diisopropylaminophosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**4b**).* The general procedure B was applied with nucleoside **3b** (200 mg, 0.27 mmol), 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphoramidite (176 μL, 0.55 mmol), and diisopropylammonium tetrazolide salt (110 mg, 0.55 mmol): yield 180 mg (71%); R_f 0.4 (4% MeOH in CH_2Cl_2); ^{31}P ($CDCl_3$) δ 149.07, 148.70; HR-ESI MS *m/z* 942.3563 ([M + Na]$^+$, $C_{48}H_{54}N_7O_{10}PNa^+$ calcd 942.3562).

*5-(6-N-Benzoyladenin-9-yl)propyn-1-yl)-3′-O-(P-2-cyanoethyl-N,N-diisopropylaminophosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**4c**).* The general procedure A was applied with nucleoside **3c** (225 mg, 0.28 mmol), *N,N*-diisopropylchlorophosphoramidite (200 μL, 0.85 mmol), and DIPEA (260 μL, 1.49 mmol): yield 228 mg (78%); R_f 0.4 (4% MeOH in CH_2Cl_2); ^{31}P ($CDCl_3$) δ 149.06, 148.77; HR-ESI MS *m/z* 1006.4033 ([M + H]$^+$, $C_{54}H_{56}N_9O_9PH^+$ calcd 1006.4011).

*3′-O-(P-2-Cyanoethyl-N,N-diisopropylaminophosphinyl)-5′-O-(4,4′-dimethoxytrityl)-5-(3-(2-N-isobuturylguanin-9-yl)propyn-1-yl)-2′-deoxyuridine (**4d**).* The general procedure A was applied with nucleoside **3d** (200 mg, 0.25 mmol), *N,N*-diisopropylchlorophosphoramidite (180 μL, 0.77 mmol), and DIPEA (220 μL, 1.26 mmol): yield 175 mg (70%); R_f 0.4 (4% MeOH in CH_2Cl_2); ^{31}P ($CDCl_3$) δ 149.04, 148.76; HR-ESI MS *m/z* 1010.3924 ([M + Na]$^+$, $C_{51}H_{58}N_9O_{10}PNa^+$ calcd 1010.3936).

*3′-O-(P-2-Cyanoethyl-N,N-diisopropylaminophosphinyl)-5′-O-(4,4′-dimethoxytrityl)-4-N-(dimethylaminomethylene)-5-(3-(thymin-1-yl)propyn-1-yl)-2′-deoxycytidine (**7a**).* The general procedure A was applied with nucleoside **6a** (220 mg, 0.29 mmol), *N,N*-diisopropylchlorophosphoramidite (200 μL, 0.85 mmol), and DIPEA (260 μL, 1.49 mmol): yield 210 mg (75%); R_f 0.4 (4% MeOH in CH_2Cl_2); ^{31}P ($CDCl_3$) δ 149.02, 148.45; HR-ESI MS *m/z* 969.4039 ([M + Na]$^+$, $C_{50}H_{59}N_8O_9PNa^+$ calcd 969.4035).

*5-(3-(4-N-Acetylcytosin-1-yl)propyn-1-yl)-3′-O-(P-2-cyanoethyl-N,N-diisopropylaminophosphinyl)-4-N-(dimethylaminomethylene)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxycytidine (**7b**).* The general procedure B was applied with nucleoside **6b** (200 mg, 0.25 mmol), 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphoramidite (163 μL, 0.51 mmol), and diisopropylammonium tetrazolide salt (102 mg, 0.51 mmol): yield 180 mg (71%); R_f 0.4 (4% MeOH in CH_2Cl_2); ^{31}P ($CDCl_3$) δ 149.05, 148.41; HR-ESI MS *m/z* 974.4371 ([M + H]$^+$, $C_{51}H_{60}N_9O_9PH^+$ calcd 974.4324).

*5-(3-(6-N-Benzoyladenin-9-yl)propyn-1-yl)-3′-O-(P-2-cyanoethyl-N,N-diisopropylaminophosphinyl)-5′-O-(4,4′-dimethoxytrityl)-4-N-(dimethylaminomethylene)-2′-deoxycytidine (**7c**).* The general procedure A was applied with nucleoside **6c** (175 mg, 0.20 mmol), *N,N*-diisopropylchlorophosphoramidite (150 μL, 0.64 mmol), and DIPEA (175 μL, 1.00 mmol): yield 170 mg (78%); R_f 0.4 (4% MeOH in CH_2Cl_2); ^{31}P ($CDCl_3$) δ 149.05, 148.48; HR-ESI MS *m/z* 1060.4595 ([M + H]$^+$, $C_{57}H_{62}N_{11}O_8PH^+$ calcd 1060.4593).

*3′-O-(P-2-Cyanoethyl-N,N-diisopropylaminophosphinyl)-5′-O-(4,4′-dimethoxytrityl)-4-N-(dimethylaminomethylene)-5-(3-(2-N-isobutyrylguanin-9-yl)propyn-1-yl)-2′-deoxycytidine (**7d**).* The general procedure B was applied with nucleoside **6d** (200 mg, 0.23 mmol), 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphoramidite (157 μL, 0.47 mmol), and diisopropylammonium tetrazolide salt (95 mg, 0.47 mmol): yield 155 mg (63%); R_f 0.4 (4% MeOH in CH_2Cl_2); ^{31}P ($CDCl_3$) δ 149.00, 148.42; HR-ESI MS *m/z* 1042.4712 ([M + H]$^+$, $C_{54}H_{64}N_{11}O_9PH^+$ calcd 1042.4699).

Oligonucleotide Synthesis. Oligonucleotide synthesis was carried out on an automated DNA synthesizer following the phosphoramidite approach. Synthesis of oligonucleotides **ON1**−**ON20** were performed on a 0.2 μmol scale (CPG support) by using the modified nucleoside phosphoramidites **4a**−**d** and **7a**−**d** to introduce monomers ^{T}**T**, ^{C}**T**, ^{A}**T**, ^{G}**T**, ^{T}**C**, ^{C}**C**, ^{A}**C**, and ^{G}**C** as well as the corresponding commercial 2-cyanoethyl phosphoramidites of the natural 2′-deoxynucleosides. The synthesis followed the regular protocol for the DNA synthesizer. For the modified phosphoramidites a prolonged coupling time of 15 min was used. 1*H*-Tetrazole was used as activator. In general coupling yields for all 2-cyanoethyl phosphoramidites were >90%. The 5′-*O*-DMT-ON oligonucleotides were removed from the solid support by treatment with concentrated aqueous ammonia at room temperature for 24 h. The oligonucleotides were purified by reversed-phase HPLC on a Waters 600 system using a XBridge OST C18 column, 19 × 100 mm, 5 μm + precolumn: XBridge 10 × 10 mm, 5 μm. Temperature 50 °C; Buffer A: 0.05 M triethylammonium acetate pH 7.4. Buffer B: MeCN/H_2O (3:1 v/v). Program used: 2 min 100% A, 100%−30%:0%−70% A:B over 17 min, 4 min 100% B, 6 min 100% A. Flow 5 mL/min. All oligonucleotides were detritylated by treatment with 80% aqueous acetic acid for 20 min, neutralized by addition of sodium acetate (3 M, 15 μL), then added sodium perchlorate (5 M, 15 μL) followed by acetone (1 mL). The pure oligonucleotides precipitated overnight at −20 °C. The mixture was then placed in a centrifuge and subjected to 12000 rpm, 10 min at 4 °C. The supernatant was removed and the pellet washed with cold acetone (2 × 1 mL). The pellet was then dried for 30 min under nitrogen flow at 50 °C, dissolved in pure water (1 mL) and the concentration measured as OD 260 nm. The extinction coefficients for the modified monomers were calculated based on the assumption that contribution from the additional nucleobase is equivalent to that of corresponding 2′-deoxynucleoside. For instance, extinction coefficient for ^{C}T was obtained as a sum of extinction coefficients of dC and dT. The purity and constitution of the ONs were confirmed by IC analysis and MALDI-TOF MS [M-H]$^+$, respectively (Table S1, Supporting Information).

Thermal Denaturation Experiments. Samples were dissolved in a medium salt buffer containing 2.5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 100 mM NaCl, and 0.1 mM EDTA at pH = 7.0 with 1.5 μM concentrations of the two complementary oligonucleotide sequences. The increase in UV absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 10 to 80 °C at a rate of 1.0 °C/min by means of a Peltier temperature programmer. The melting curves were found to be reversible.

Hybridization Analysis by Nondenaturing PAGE (Polyacrylamide Gel Electrophoresis). The oligonucleotides were ^{32}P-labeled at the 5′-end using T4 polynucleotide kinase (New England Biolab) according to standard methods. The labeled samples were mixed with appropriate amounts of unlabeled oligonucleotide. Hybridization using 2 pmol of each oligonucleotide was performed in 20 mM Tris-HCl pH 7,5, 100 mM NaCl, and 10 mM $MgCl_2$ by incubation at 80 °C 1 min, slow cooling to 20 °C, and then 15 min on ice. For samples with preannealing of two oligonucleotides, the third oligonucleotide was added at 20 °C followed by 30 min incubation and then 15 min on ice. The samples were run in a cold room in nondenaturing 13% polyacrylamide gels without urea in a buffer with 90 mM boronic acid, 90 mM Tris base pH 8.3, and 50 mM KCl. The bands in the gel were visualized using a Typhoon trio laser scanner.

Molecular Dynamics. *Parametrization of the Modified Nucleosides.* The modified nucleosides were built in *xleap*, and the geometry was optimized at the HF/6-31G* level using Gaussian03.[35] Atomic charges were calculated using the RESP methodology[36] keeping the native charges from the force field of Cornell et al. for backbone and sugar atoms except for C1′ and H1′.[37,38] In this manner, we were consistent with the charge derivation for the native atomic charges. The atoms in the triple bond were assigned the c1 atom type from the general AMBER force field (gaff),[39] and missing force field parameters were deduced by analogy between gaff and ff99-bsc0 parameters.[40]

MD Simulations. All MD simulations were carried out with AMBER12 program suite using the ff99-bsc0[40] and gaff[39] parameters

for nucleic acids and ion. The TIP3P water model was used.[41] Starting coordinates were generated using idealized B-DNA geometries. In *LEaP*, net-neutralizing Na^+ ions were added, and the whole system was surrounded by a truncated octahedron of TIP3P waters with a minimum distance of 10.0 Å from the helix to the edges of the box.

In the initial energy minimization harmonic positional restraints with a force constant of 500 kcal mol^{-1} $Å^{-2}$ were applied to the DNA molecule. The system was minimized for 500 steps of steepest descent and 500 steps of conjugate gradient minimization. Finally, a further 1000 steps of steepest descent and 1500 steps of conjugate gradient minimization was carried out with the entire system free to move. In the MD equilibration, the SHAKE algorithm was applied with a 1 fs time step. The nonbond cutoff was 9 Å and the particle mesh Ewald method with default parameters was used to calculate long-range electrostatic interactions. The temperature of the system was raised from 0 to 300 K over 20 ps at constant volume using the Berendsen thermostat with a 0.2 ps coupling parameter and applying harmonic positional restraints with a force constant of 10 kcal mol^{-1} $Å^{-2}$ to the DNA molecules. Centre of mass movement was removed every 1000th step. Subsequently, 10 ps MD was carried out at 300 K at constant volume and temperature with parameters as in the first round of equilibration. The final step of equilibration consisted of 200 ps unrestrained *NPT* MD at 300 K with a time step of 2 ps. The reference pressure was 1 atm, the barostat coupling parameter 2.0 ps, and the thermostat coupling parameter 1.0 ps. Production runs were carried out for 30 ns with parameters as for the last step of the equilibration except for the pressure and temperature coupling parameters, which both were 5.0 ps.

In an attempt to force the extra bases in the major groove into a continuous stack, we performed two sets of restrained simulations with varying force constants corresponding to two representative geometries from the free MD simulations. In the type 1 conformation, the θ_1 (C6−C5−C3L−N9*/N1*) torsion angle was restrained between 180° and 190° and θ_2 (C5−C3L−N9*/N1*−C8*/C6*) between −10° and 0°, while in the type 2 conformation, θ_1 was restrained between 75° and 85° and θ_2 between 50° and 70°. Force constants in simulations were 1, 2, or 5 kcal/(mol rad^2), respectively, and restrained simulations were run for 10 ns.

Analysis of Trajectories. Torsion angles, distances, helix parameters, and rmsd's were determined with *ptraj* from the AMBER program suite.

■ ASSOCIATED CONTENT

Supporting Information

MALDI-TOF data for oligonucleotides. Further non-denaturating gel analyses. Further modeling data. Selected NMR-spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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