Stable Oligonucleotide-Directed Triplex Formation at Target Sites with CG Interruptions: Strong Sequence-Specific Recognition by 2′,4′-Bridged Nucleic-Acid-Containing 2-Pyridones under Physiological Conditions

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Abstract: A sequence of double-stranded DNA (dsDNA) which can be recognized by a triplex-forming oligonucleotide (TFO) is limited to a homopurine–homopyrimidine sequence. To develop novel nucleoside analogues which recognize CG interruption in homopurine–homopyrimidine dsDNA, we synthesized a novel 2′-*O*,4′-*C*-methyleneribonucleic acid (2′-*O*,4′-*C*-methylene *b*ridged *n*ucleic *a*cid; 2′,4′-BNA) that bears the unnatural nucleobases, 2-pyridone (P^B) or its 5-methyl congener ($^mP^B$); these analogues were introduced into pyrimidine TFOs using a DNA synthesizer. A TFO with a 2′-deoxy-β-D-ribofuranosyl-2-pyridone (P) or 2′,4′-BNA abasic monomer (H^B) was also synthesized. The triplex-forming ability of various synthesized 15-mer TFOs and the corresponding homopurine–homopyrimidine dsDNA, which contained a single pyrimidine–purine (PyPu) interruption, was examined in UV melting experiments. It was found that P^B and $^mP^B$ in the TFOs successfully recognized CG interruption under physiological conditions (7 mM sodium phosphate, 140 mM KCl, 5 mM spermine, pH 7.0). Furthermore, triplex formation between the dsDNA target which contained three CG interruptions and the TFO with three P^B units was also confirmed. Additional four-point 2′,4′-BNA modifications of the TFO containing three P^B units significantly enhanced its triplex-forming ability towards the dsDNA and had a T_m value of 43 °C under physiological conditions. These results indicate that a critical inherent problem of TFOs, namely, the sequence limitation of the dsDNA target, may be overcome to a large extent and this should promote antigene applications of TFOs in vitro and in vivo.

Keywords: molecular recognition · nucleic acids · nucleobases · oligonucleotides

Introduction

Triplex-forming oligonucleotides (TFOs) can interact with double-stranded DNA (dsDNA) in a sequence-specific manner. These TFOs have been largely studied as novel reagents for the control of gene expression in vitro and in vivo (antigene strategy).[1–4] Recognition of dsDNA by TFO follows two patterns that is a pyrimidine or purine. In a pyrimidine motif triplex, homopyrimidine TFO binds to the homopurine tract of the dsDNA target by the formation of Hoogsteen hydrogen bonds to form T·AT and C^+·GC triads (Figure 1a). In a purine motif triplex, homopurine TFO forms A·AT (or T·AT) and G·GC triads with the homopurine tract of the dsDNA target through reverse-Hoogsteen hydrogen bonds. In other words, the dsDNA target in both motifs is restricted to a homopurine–homopyrimidine duplex. Therefore, the development of nucleoside analogues is a prerequisite for effective recognition of a pyrimidine–purine (PyPu) base pair (a CG or TA base pair) in dsDNA. In order to overcome the restriction of the sequence of a dsDNA target, a large number of synthetic homopyrimidine TFOs containing unnatural nucleobases have been reported.[5] However, development of a modified TFO that has selective and strong binding affinity with a PyPu base pair has not yet been achieved. Although T or C bases have been known to bind to CG interruptions through a single hydrogen bond,[6, 7] these nucleobases recognize either AT or GC base pairs much more strongly. Results from a molecular modeling study suggested that the 2-carbonyl group in T or C play a crucial role in the recognition of a CG base pair (Figure 1b).[8] Therefore, we assumed that 2-pyridone, which lacks suitable functional groups for AT or GC base pair recognition, could succeed as a nucleobase for selective interaction with CG interruptions (Figure 1c).

We recently achieved the first synthesis of the novel nucleosides, 2′-*O*,4′-*C*-methyleneribonucleic acid (2′-*O*,4′-*C*-methylene *b*ridged *n*ucleic *a*cid, 2′,4′-BNA)[9, 10], which has a fixed N-type conformation, and 3′-*O*,4′-*C*-methyleneribonu-

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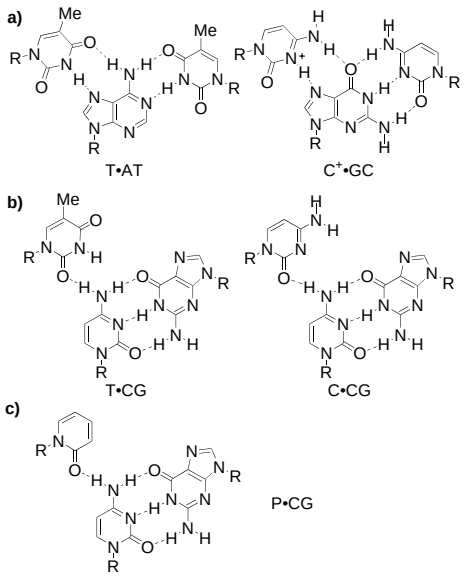


Figure 1. a) Canonical T•AT and C+•GC triads in a pyrimidine motif triplex; b) structure of T•CG and C•CG triads; c) proposed structure of a P•CG triad.

cleic acid (3′,4′-BNA)[11, 12] which has a restricted S-type conformation. Moreover, we have found that oligonucleotides with a 2′,4′-BNA modification leads to a marked increase in their triplex formation with dsDNA at neutral pH as well as in their duplex formation with single-stranded RNA (ssRNA).[10, 13–18] We report here the synthesis of the novel 2′,4′-BNA monomers, 2-pyridone derivative and its 5-methyl-2-pyridone congener (Figure 2), and the effective recognition of one or more CG base pairs in homopurine–homopyrimidine dsDNA by pyrimidine TFOs containing these 2′,4′-BNA monomers.[19]

Results

Synthesis of 2′,4′-BNA containing 2-pyridone and 5-methyl-2-pyridone: The synthetic route to the phosphoramidites **6a** and **6b** is shown in Scheme 1. According to method of Vorbrüggen,[20] the starting material **1**,[21] which was easily prepared from D-glucose, was treated with 2-pyridone, *N*,*O*-bis(trimethylsilyl)acetamide and trimethylsilyl trifluoromethanesulfonate (TMSOTf) in 1,2-dichloroethane to give **2a** as the sole β-anomer in 74% yield. By using 5-methyl-2-pyridone, **2b** was produced in 87% yield in a similar manner. The exposure of **2** to potassium carbonate in methanol gave the bicyclic nucleosides **3** in yields of 96–100%. Subsequent hydrogenolysis of **3** afforded the diols **4** in 91–95% yields. ^{1}H NMR analysis of **4a** and **4b** showed that

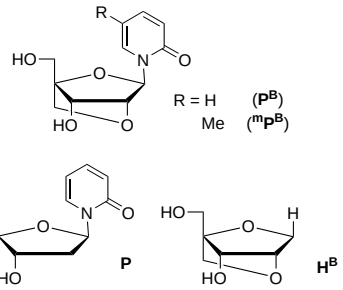
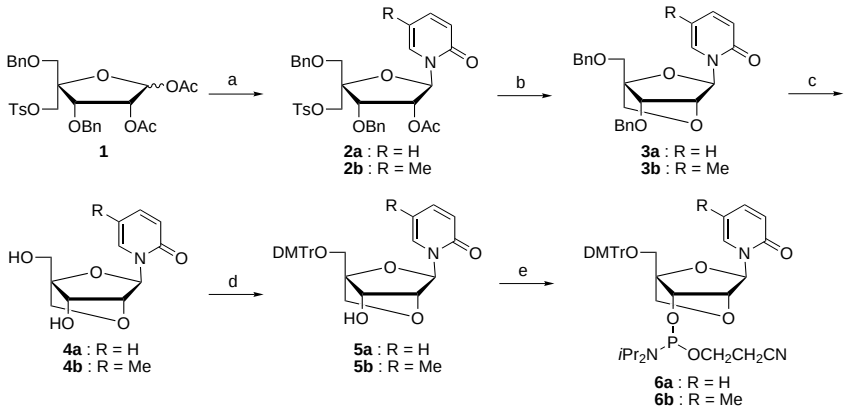


Figure 2. Structure of 2′,4′-BNA monomers and a nucleoside bearing 2-pyridone.

the 2′,4′-BNA monomers exhibited singlet signals for the C1′-, C2′- and C3′-hydrogens of the sugar moiety. This indicated that the sugar in their 2′,4′-BNA monomers was locked in N-type conformation.[22] Furthermore, X-ray crystallographic analysis[19, 23] of the structure of **4a** confirmed that the conformation of the sugar moiety was in a 3′-*endo* (N-type) conformation, in which the sugar pseudorotation phase angle (*P*) was 18.0°.[24] Protection of the primary alcohol of **4** with 4,4′-dimethoxytrityl chloride (DMTrCl) in pyridine gave compound **5** in 96–97% yields. The phosphoramidite **6a**, a suitable building block for DNA synthesis, was obtained in 98% yield by phosphitylation of **5a**. The yield of phosphoramidite **6b** was 91%. Then the phosphoramidites **6a** and **6b** were incorporated into TFOs by standard phosphoramidite protocol in a DNA synthesizer (Figure 3). TFOs that contained P[25, 26] or H^B[16, 27] were also synthesized. The purity of the modified TFOs was verified using reversed-phase high performance liquid chromatography (HPLC) and the compositions were determined by matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) (see Experimental Section).

The triplex-forming ability of P^B and ^{m}P^B-containing TFO with dsDNA (single interruption): Initially, UV-melting experiments were used to investigate whether the nucleoside analogue P, which bears 2-pyridone, could recognize a CG interruption in dsDNA when incorporated into TFOs. The



Scheme 1. a) 2-Pyridone or 5-methyl-2-pyridone, *N*,*O*-bis(trimethylsilyl)acetamide, 1,2-dichloroethane, reflux, then TMSOTf, reflux, 74% (**2a**) or 87% (**2b**); b) potassium carbonate, methanol, RT, 100% (**3a**), 96% (**3b**); c) 20% palladium hydroxide on carbon powder, cyclohexene, ethanol, reflux, 95% (**4a**), 91% (**4b**); d) 4,4′-dimethoxytrityl chloride, pyridine, RT, 96% (**5a**), 97% (**5b**); e) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, CH_3CN/THF, RT, 95% (**6a**), 91% (**6b**).

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TFOs sequence

7 5'-TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT-3'
8 5'-TTTTT^{m}CT^{m}**C**T^{m}CT^{m}CT^{m}CT-3'
9 5'-TTTTT^{m}CT**P**T^{m}CT^{m}CT^{m}CT-3'
10 5'-TTTTT^{m}CT**P^{B}**T^{m}CT^{m}CT^{m}CT-3'
11 5'-TTTTT^{m}CT^{m}**P^{B}**T^{m}CT^{m}CT^{m}CT-3'
12 5'-TTTTT^{m}CT**H^{B}**T^{m}CT^{m}CT^{m}CT-3'
13 5'-TTTTT^{m}CT**T**^{m}C^{m}CT^{m}CT^{m}CT-3'
14 5'-TTTTT^{m}CT**P^{Bm}**C^{m}CT^{m}CT^{m}CT-3'
15 5'-TTTTT^{m}C^{m}CT**T**T^{m}CT^{m}CT^{m}CT-3'
16 5'-TTTTT^{m}C^{m}C**P^{B}**T^{m}CT^{m}CT^{m}CT-3'
17 5'-TTTTT^{m}C^{m}CT^{m}C^{m}CT^{m}CT^{m}CT-3'
18 5'-TTTTT^{m}C^{m}C**P^{Bm}**C^{m}CT^{m}CT^{m}CT-3'
19 5'-TTTTT**T**T**T**T**T**T^{m}CT^{m}CT-3'
20 5'-TTTTT**P^{B}**T**P^{B}**T**P^{B}**T^{m}CT^{m}CT-3'
21 5'-TT^{B}TT^{B}T**P^{B}**T**P^{B}**T**P^{B}**T^{m}C^{B}T^{m}C^{B}T-3'
22 5'-TT^{B}TT^{B}T^{m}**P^{B}**T^{m}**P^{B}**T^{m}**P^{B}**T^{m}C^{B}T^{m}C^{B}T-3'

target dsDNA

23 5'-GCTAAAAAGA**X**AGAGAGATCG-3'
3'-CGATTTTTCT**Y**TCTCTCTAGC-5'

24 5'-GCTAAAAAGA**X**GGAGAGATCG-3'
3'-CGATTTTTCT**Y**CCTCTCTAGC-5'

25 5'-GCTAAAAAGG**X**AGAGAGATCG-3'
3'-CGATTTTTCC**Y**TCTCTCTAGC-5'

26 5'-GCTAAAAAGG**X**GGAGAGATCG-3'
3'-CGATTTTTCC**Y**CCTCTCTAGC-5'

27 5'-GCTAAAAA**C**A**C**A**C**AGAGATCG-3'
3'-CGATTTTT**G**T**G**T**G**TCTCTAGC-5'

XY = CG, GC, TA or AT (**23-26**)

Figure 3. The TFOs sequence and the sequence of the DNA targets.

melting temperature (T_m) was measured in 7 mM sodium phosphate buffer (pH 7.0) that also contained 140 mM KCl and 5 mM spermine to approach physiological conditions in the nucleus.[28] The T_m value of the triplex **9·23** (XY = CG) was 45 °C, while those of the triplexes **9·23** (XY = GC, TA and AT), which contained a P·GC, P·TA and P·AT triad, were much lower (33, 34 and 34 °C, respectively). This indicated that the nucleoside analogue, P, effectively recognized the CG interruption in dsDNA (Table 1, Figure 4). The thermal stabilization effect of a T·CG and ^{m}C·CG triad (^{m}C: 5-methylcytosine) was comparable to that of a P·CG triad, while T and ^{m}C formed stable triads, T·AT and ^{m}C^{+}·GC with T_m values of 59 and 58 °C, respectively (Table 1). This result suggests that the 2-carbonyl oxygen in P (or T and ^{m}C) plays an important role in the binding with a CG base pair and that the absense of a functional group at the 3- and 4-position in a pyrimidine nucleobase effectively prevents hybridization with PyPu base pairs.

Next, the effect of the 2′,4′-BNA modification of P was examined. The triplex formation of P^{B}-containing TFO **10** was also found to be sequence-selective, and the T_m value of the triplex **10·23** (XY = CG) was 10–13 °C higher than those of the triplexes **7·23** (XY = CG), **8·23** (XY = CG), **9·23** (XY = CG) or **12·23** (XY = CG) (Table 1, Figure 5). Moreover, the thermal stability of the triplex **10·23** (XY = CG) was almost comparable to that of the canonical triplexes **7·23** (XY = AT) and **8·23** (XY = GC), which contained a T·AT and ^{m}C^{+}·GC

Table 1. T_m values [°C] of TFOs **7**–**18** with their corresponding duplexes.[a]

TFO	Target duplex	XY			
		CG	GC	TA	AT
7 (T)	**23**	44	35	33	59
8 (^{m}C^{B})	**23**	42	58	35	35
9 (P)	**23**	45	33	34	34
10 (P^{B})	**23**	55	37	36	44
11 (^{m}P^{B})	**23**	56	42	35	44
12 (H^{B})	**23**	44	35	44	35
13 (T)	**24**	39	33	29	53
14 (P^{B})	**24**	49	33	29	37
15 (T)	**25**	34	33	33	55
16 (P^{B})	**25**	43	33	35	34
17 (T)	**26**	15[c]	17[c]	nd[b,c]	15[c]
18 (P^{B})	**26**	34	21	nd[b]	23

[a] Conditions: 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl and 5 mM spermine, concentration of triplex = 1.5 μM. [b] A typical transition in the UV-melting curve was not observed. [c] All the T_m values were extremely low, and that might mean incomplete formation of the corresponding triplexes, perhaps due to sequence-peculiarity of the TFO used though the details in this respect are not clear.

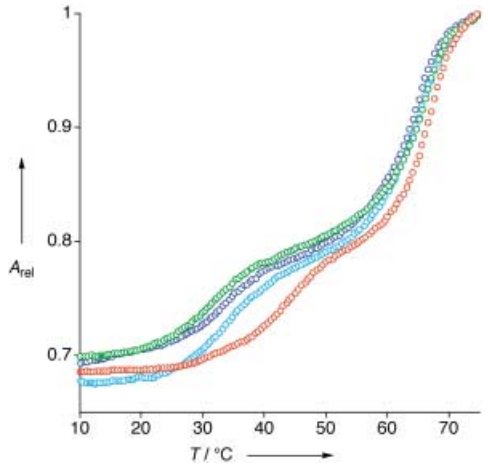

Figure 4. UV-melting profiles (260 nm) for the triplexes **9·23** (XY = CG, red; GC, green; TA, blue; AT, cyan). The presented melting curves were normalized.

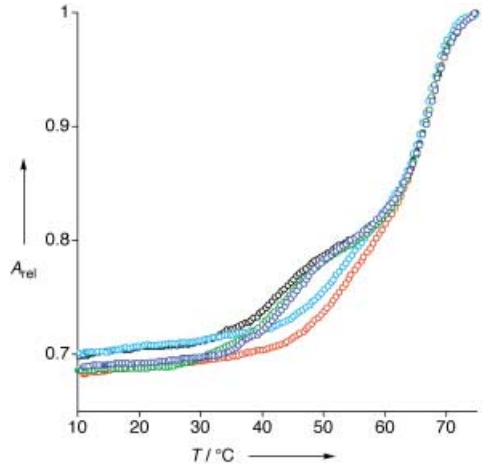

Figure 5. UV-melting profiles (260 nm) for the triplexes which were formed with the duplex **23** (XY = CG) by TFOs **7** (blue), **9** (green), **10** (red), **11** (cyan) and **12** (black) triads. These melting curves have been normalized.

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triad, respectively. The 5-methyl substitution of U or C in TFO is known to cause relatively large enhancement of triplex stability,[29] but notable improvement in recognition of a CG interruption was not observed in the $^{m}P^{B}$-containing TFO (Table 1). Possibly the difference in the spatial position of the 5-methyl group between T·AT (^{m}C·GC) and $^{m}P^{B}$·CG triads may explain why the 5-methyl group in $^{m}P^{B}$ did not give the desired effect.

To confirm the general apllicability of this result, we also studied the ability of P^{B} to recognize CG interruption in other dsDNA targets (Table 1). Due to the low stability of the $^{m}C^{+}$·GC triad under physiological conditions,[30] the replacement of an AT base pair by a GC base pair at the neighboring sites of the target CG interruption caused a decrease in the T_m values. However, the TFOs **14**, **16** or **18** maintained reasonable triplex-forming ability towards the corresponding target duplexes **24**, **25** or **26** (XY = CG) with almost no effect on a variety of base pairs at the neighboring sites (Table 1). The increase in T_m values of TFOs **14**, **16** and **18** for a CG interruption ranged from +9–19 °C with respect to those of TFOs **13**, **15** and **17**.

Triplex-forming ability of TFO containing P^{B} and $^{m}P^{B}$ with dsDNA including three CG interruptions: It is of considerable interest as to whether the TFO that bears P^{B} or $^{m}P^{B}$ can form a stable triplex with dsDNA that includes multiple CG interruptions. Therefore, the triplex-forming ability of TFOs **19**–**22**, which have three-point P^{B} or $^{m}P^{B}$ modifications, with the target duplex **27** was evaluated (Figure 6).[31] In the case of

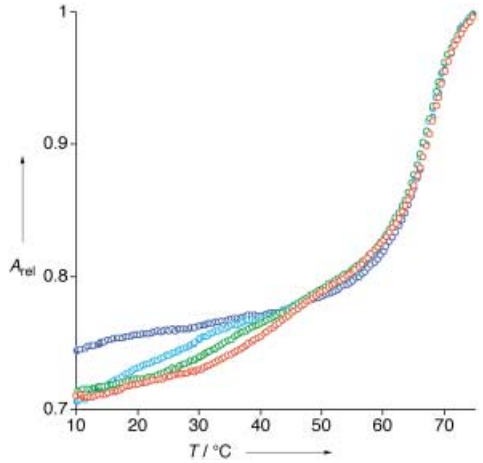

Figure 6. UV-melting profiles (260 nm) for the triplexes **19**·**27** (blue), **20**·**27** (cyan), **21**·**27** (red) and **22**·**27** (green). Their T_m values were 22 °C (**20**·**27**), 43 °C (**21**·**27**) and 35 °C (**22**·**27**). Typical hyperchromicity of triplex **19**·**27** was not observed. These melting curves have been normalized.

natural TFO **19**, the formation of a triplex with three T·CG triads was not observed. However, under physiological conditions, the TFO **20** was found to form a triplex containing three P^{B}·CG triads with a T_m value of 22 °C. As we previously reported, 2′,4′-BNA modification of T or ^{m}C in homopyrimidine TFO significantly promoted the triplex formation.[16–18] Therefore, to enhance the triplex stability, an additional four 2′,4′-BNA modifications were introduced into TFO **20**. This new TFO, **21**, exhibited a potent triplex-forming ability with T_m value of 43 °C, even though the target duplex **27** had three CG interruptions in its 15-bp TFO-binding region. The TFO **22**, the 5-methyl congener of TFO **21**, was also found to form a stable triplex with the target duplex **27** with T_m value of 35 °C.

Discussion

Extensive research on TFOs, which involve nucleoside analogues for the recognition of CG interruption in dsDNA, has been reported to date.[8, 32–35] Most of the nucleoside analogues have complicated structures that consist of highly fused-aromatic ring and many functional groups in order to form hydrogen bonds with the 4-amino hydrogen in C and the 6-carbonyl oxygen and 7-nitrogen atoms in G. However, their mode of recognition of a CG base pair remained vague, and the development of an ideal nucleoside analogue to interact with a CG interruption was not been achieved. However, the simple structure of 2-pyridone, which we selected as a nucleobase, has only one functional group that can form a hydrogen bond in a triplex. By using the 2-pyridone as a nucleobase, successful recognition of CG interruption in dsDNA was demonstrated (Table 1, Figure 5), and this indicates the importance of the hydrogen bond between the 2-carbonyl oxygen in P and the 4-amino hydrogen in C (Figure 1c). Moreover, the stability of a triplex that contained a P·CG triad was comparable to that of triplexes containing a T·CG or C·CG triad (Table 1). Again this demonstrates the requirement of the 2-carbonyl oxygen not only in P but also in T and C.[8]

On the other hand, Jayaraman and co-workers investigated triplex-forming ability of TFOs that contained 2-pyridone or 4-pyridone nucleobases in order to confirm the hydrogen bond arrangement of an antiparallel T·CG triad.[26] Consequently, the 4-carbonyl oxygen in T was shown to play an important role in an antiparallel triplex in the recognition of the 4-amino hydrogen in C. Their results also support the requirement of the 2-carbonyl oxygen in P, C or T for recognition of CG interruption in a parallel triplex formation since the 4-carbonyl oxygen at T in an antiparallel motif is considered to be spatially identical with the 2-carbonyl oxygen at T in a parallel motif. A 2′,4′-BNA modification of the P nucleoside in TFO retained the sequence-selectivity and promoted a significant increase in the binding affinity with CG interruption (Table 1). However, 2′,4′-BNA abasic analogue (H^{B}) did not show particular specific recognition of a CG base pair (Table 1). The excellent binding properties of P^{B} is likely to arise from a combination of the sequence-selectivity of the 2-pyridone nucleobase moiety and of the potent triplex-forming ability by the 2′,4′-methylene bridged sugar moiety. As described by Dervan et al.,[30a, 32] their nucleoside analogue D3 achieved a shape-selective recognition of PyPu base pairs in dsDNA when the D3·PyPu triad was flanked by two T·AT triads but its binding affinity was drastically decreased when the T·AT triad in the adjacent 3′ direction was replaced by a C^{+}·GC triad. In the case of the interaction of a TA base pair with G,[36–38] the thermal stability of TFOs containing a G·TA triad was deeply influenced by a variety of neighboring base triads.[30a] Moreover, in the case of

triplexes that contain a T·CG triad, UV-melting experiments showed that the triplex **17·26** (XY = CG), which has a $^{m}C^{+}$·GC triad at the adjacent 5′ direction, was the most destabilized although the destabilization was not large. The triplexes with a P^{B}·CG triad also displayed similar tendencies. These results suggest that the nearest 5′-sided, rather than the nearest 3′-sided, GC base pair of CG interruption in a dsDNA target causes greater destabilization of a formed triplex. In any event, it was clearly shown that P^{B} interacts strongly with CG interruption in a sequence-specific manner, regardless of the triads adjacent to the P^{B}·CG triad (Table 1). In a triplex between TFO **20** and dsDNA **27**, which included three CG interruptions (Figure 6), the T_m value was 22 °C and the triplex was showed to decrease in ΔT_m per P^{B} modification by approximately 9 °C with respect to the fully matched triplex **7·23** (XY = AT), whereas the triplex **10·23** (XY = CG) with a P^{B}·CG triad was 4 °C less stable in T_m value than the full-matched triplex. As we have previously reported,[14, 16–18] in the case of 2′,4′-BNA (T^{B} or $^{m}C^{B}$), which has natural nucleobases, similar results were also obtained. For example, a triplex, which contained a T^{B}·AT triad, dramatically increased in T_m value by 13 °C with respect to the corresponding natural triplex, while the T_m values of triplexes containing multiple T^{B}·AT triads showed an increase by 5–6 °C per 2′,4′-BNA modification. These results are probably due to a slight warp of the sugar moiety by 2′,4′-BNA modification. However, it was shown that **20**, which contains three P^{B} modifications, successfully recognized the dsDNA target **27** with three CG interruptions and formed a triplex that included three P^{B}·CG triads.

An additional four 2′,4′-BNA modifications of the TFO **21** further stabilized the triplex, which included three P^{B}·CG triads, and its T_m value was 43 °C. On the other hand, a triplex consisting of the TFO (5′-TT^{B}TT^{B}TTTTTTT^{m}C^{B}T^{m}C^{B}T-3′), with T instead of the P^{B} in **21**, and a duplex **27** had a T_m value of 18 °C. These results showed that the excellent recognition of CG interruption shown by P^{B} was maintained even if natural-type 2′,4′-BNA such as T^{B} and $^{m}C^{B}$ were introduced into the TFO with P^{B} to enhance the stability of a triplex.

To the best of our knowledge, this is the first example of a modified TFO to have achieved effective recognition of the dsDNA which contained three CG interruptions.[39] The use of P^{B} should substantially overcome one of the critical problems in pyrimidine motif triplex formation, that is, a limitation of the target sequence, and these results should afford a vital clue to the development of practical antigene strategy.

Conclusion

The results presented here demonstrate that the 2-carbonyl oxygen of pyridone or pyrimidine nucleobases plays an important role in the recognition of a CG base pair in a pyrimidine motif triplex. The 2-pyridone derivative (P) has sufficient CG selectivity due to the lack of a 3-nitrogen atom and a 4-carbonyl group which are crucial for the formation of hydrogen bonds with AT base pairs. The combination of 2-pyridone or 5-methyl-2-pyridone and the 2′-*O*,4′-*C*-methylene bridged sugar moiety significantly enhances the binding affinity with a CG base pair without loss of selectivity. The triplexes were formed between the TFO, which contained P^{B} or $^{m}P^{B}$, and the target duplex, which included three CG interruptions. Moreover, the additional four-point 2′,4′-BNA modifications of the TFO to involve three P^{B} units led to dramatic enhancement of the triplex stability. We believe that the novel 2′,4′-bridged nucleic acid analogue, P^{B}, should eventually lead to the advancement of practical applications of the antigene strategy.

Experimental Section

1-[2-*O*-Acetyl-3,5-di-*O*-benzyl-4-(*p*-toluenesulfonyloxymethyl)-β-D-ribofuranosyl]-2-pyridone (2a): Under a nitrogen atmosphere at room temperature, 2-pyridone (52 mg, 0.52 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (0.16 mL, 0.57 mmol) were added to a solution of compound **1** (154 mg, 0.26 mmol) in anhydrous 1,2-dichloroethane (4 mL) and the mixture was heated under reflux for 2 h. Trimethylsilyl trifluoromethanesulfonate (51 μL, 0.28 mmol) was added to the reaction mixture at 0 °C. The mixture was then heated under reflux for 4 h. After the addition of a saturated aqueous solution of sodium hydrogen carbonate, the organic phase was extracted with ethyl acetate, washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 1:1) to give compound **2a** (169 mg, 74 %) as a colorless oil. $[\alpha]_D^{26} = +55.9$ (c = 1.00 in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.75 (d, J = 8 Hz, 2H), 7.65 (dd, J = 7, 2 Hz, 1H), 7.38–7.18 (m, 13H), 6.43 (d, J = 9 Hz, 1H), 6.00 (d, J = 3 Hz, 1H), 5.83 (m, 1H), 5.37 (dd, J = 6, 3 Hz, 1H), 4.53, 4.34 (AB, J = 12 Hz, 2H), 4.45 (d, J = 6 Hz, 1H), 4.45, 4.38 (AB, J = 11 Hz, 2H), 4.27, 4.17 (AB, J = 11 Hz, 2H), 3.83, 3.54 (AB, J = 10 Hz, 2H), 2.42 (s, 3H), 2.03 (s, 3H); ^{13}C NMR (68 MHz, $CDCl_3$, 25 °C): δ = 169.26, 161.78, 144.94, 137.05, 136.93, 133.31, 129.67, 128.35, 128.32, 128.00, 127.95, 127.86, 120.74, 120.41, 105.67, 88.63, 85.48, 76.64, 75.03, 74.15, 73.53, 69.74, 69.42, 21.72, 20.77; IR (KBr): $\tilde{\nu}_{max}$ = 1748, 1667, 1591, 1363, 1229, 1180, 1111 cm^{-1}; MS (EI): m/z (%): 633 (4) $[M]^+$, 91 (100); elemental analysis calcd (%) for $C_{34}H_{35}NO_9S$ (633.7): C 64.44, H 5.57, N 2.21, S 5.06; found: C 64.25, H 5.56, N 2.18, S 4.94.

1-[2-*O*-Acetyl-3,5-di-*O*-benzyl-4-(*p*-toluenesulfonyloxymethyl)-β-D-ribofuranosyl]-5-methyl-2-pyridone (2b): Under a nitrogen atmosphere, 5-methy-2-pyridone (161 mg, 1.47 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (0.44 mL, 1.78 mmol) were added to a solution of compound **1** (590 mg, 0.99 mmol) in anhydrous 1,2-dichloroethane (10 mL) at room temperature and the mixture was heated under reflux for 0.5 h. Trimethylsilyl trifluoromethanesulfonate (0.14 mL, 0.77 mmol) was at room temperature added to the reaction mixture, which was then heated under reflux for 3 h. After the addition of a saturated aqueous solution of sodium hydrogen carbonate, the organic phase was extracted with ethyl acetate. The residue was obtained as in the synthesis of **2a** and further purified by flash silica gel column chromatography (*n*-hexane/ethyl acetate 2:3) afforded compound **2b** (553 mg, 87 %) as a white powder. M.p. 42–45 °C; $[\alpha]_D^{27} = +32.9$ (c = 0.64 in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS):δ = 7.74 (d, J = 8 Hz, 1H), 7.36–7.19 (m, 13H), 7.09 (dd, J = 9, 2 Hz, 1H), 6.39 (d, J = 9 Hz, 1H), 6.03 (d, J = 4 Hz, 1H), 5.40 (dd, J = 6, 4 Hz, 1H), 4.53, 4.38 (AB, J = 12 Hz, 2H), 4.52–4.44 (m, 2H), 4.50 (d, J = 6 Hz, 1H), 4.24, 4.19 (AB, J = 11 Hz, 2H), 3.82, 3.58 (AB, J = 10 Hz, 2H), 2.42 (s, 3H), 2.03 (s, 3H), 1.71 (s, 3H); ^{13}C NMR (68 MHz, $CDCl_3$, 25 °C): δ = 169.46, 161.17, 144.93, 142.444, 137.14, 137.04, 132.22, 130.43, 129.70, 128.41, 128.33, 127.99, 127.95, 127.87, 127.84, 127.54, 120.13, 115.00, 88.40, 85.41, 77.13, 75.12, 74.30, 73.58, 70.17, 69.36, 21.74, 20.79, 16.96; IR (KBr): $\tilde{\nu}_{max}$ = 1748, 1674, 1602, 1363, 1229, 1180, 1105 cm^{-1}; MS (EI): m/z (%): 647 (2) $[M]^+$, 91 (100); elemental analysis calcd (%) for $C_{35}H_{37}NO_9S \cdot \frac{1}{3}H_2O$ ($C_{35}H_{37}NO_9S$, 647.7): C 64.30, H 5.81, N 2.14, S 4.90; found: C 64.23, H 5.75, N 2.18, S 4.86.

1-(3,5-Di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)-2-pyridone (3a): Potassium carbonate (72 mg, 0.52 mmol) was added to a solution of compound **2a** (110 mg, 0.17 mmol) in methanol (3 mL) at room temperature and the mixture was stirred for 17 h. The solvent was concentrated under reduced pressure. Ethyl acetate and water were added to the residue

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and the organic phase was extracted with ethyl acetate. The residue was obtained as in the synthesis of **2a** and further purified by silica gel column chromatography (*n*-hexane/ethyl acetate 3:2 to 1:2) to give compound **3a** (73 mg, 100%) as a white powder. M.p. 117–118 °C; $[\alpha]_D^{26} = +202.9$ ($c =$ 1.00 in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS):$\delta = 7.79$ (dd, $J = 7$, 2 Hz, 1H), 7.39–7.20 (m, 11H), 6.51 (d, $J = 9$ Hz, 1H), 6.10 (m, 1H), 5.90 (s, 1H), 4.67 (s, 1H), 4.65, 4.63 (AB, $J = 12$ Hz, 2H), 4.59, 4.44 (AB, $J = 12$ Hz, 2H), 4.05, 3.90 (AB, $J = 8$ Hz, 2H), 3.97 (s, 1H), 3.86, 3.84 (AB, $J = 11$ Hz, 2H); ^{13}C NMR (68 MHz, $CDCl_3$, 25 °C): $\delta = 161.78$, 139.50, 137.53, 136.84, 132.03, 128.35, 128.35, 128.27, 127.83, 127.73, 127.53, 127.31, 120.30, 105.64, 88.00, 87.18, 76.29, 75.60, 73.60, 72.15, 72.04, 64.49; IR (KBr): $\tilde{\nu}_{max} = 1661$, 1584 cm^{-1}; MS (EI): *m*/*z* (%): 419 (10) $[M]^+$, 91 (100); elemental analysis calcd (%) for $C_{25}H_{25}NO_5$ (419.5): C 71.58, H 6.01, N 3.34; found: C 71.53, H 6.03, N 3.37.

1-(3,5-Di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)-5-methyl-2-pyridone (3b): Potassium carbonate (399 mg, 2.45 mmol) was added to a solution of compound **2b** (530 mg, 0.82 mmol) in methanol (15 mL) at room temperature and the mixture was stirred for 11 h. The solvent was concentrated under reduced pressure. Ethyl acetate and water were added to the residue and the organic phase extracted with ethyl acetate. The residue was obtained as in the synthesis of **2a** and further purified by silica gel column chromatography (*n*-hexane/ethyl acetate 4:7) to give compound **3b** (340 mg, 96%) as a colorless oil. $[\alpha]_D^{23} = +159.9$ ($c = 0.70$ in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 7.54$ (m, 1H), 7.37–7.16 (m, 11H), 6.46 (d, $J = 9$ Hz, 1H), 5.89 (s, 1H), 4.66 (s, 1H), 4.64, 4.63 (AB, $J =$ 12 Hz, 2H), 4.60, 4.46 (AB, $J = 12$ Hz, 2H), 4.06, 3.89 (AB, $J = 8$ Hz, 2H), 4.00 (s, 1H), 3.88, 3.86 (AB, $J = 11$ Hz, 2H), 1.86 (s, 3H); ^{13}C NMR (68 MHz, $CDCl_3$, 25 °C): $\delta = 161.03$, 142.15, 137.51, 136.90, 129.18, 128.34, 128.23, 127.77, 127.73, 127.54, 127.43, 119.82, 114.67, 87.91, 87.04, 76.30, 75.44, 73.67, 72.11, 71.96, 64.77, 17.33; IR (KBr): $\tilde{\nu}_{max} = 1668$, 1596, 1094, 1051 cm^{-1}; MS (EI): *m*/*z* (%): 433 (12) $[M]^+$, 91 (100); elemental analysis calcd (%) for $C_{26}H_{27}NO_5 \cdot 1/4H_2O$ ($C_{26}H_{27}NO_5$, 433.5): C 71.30, H 6.33, N 3.20; found: C 71.39, H 6.41, N 3.19.

1-(2-*O*,4-*C*-Methylene-β-D-ribofuranosyl)-2-pyridone (4a): Compound **3a** (68 mg, 0.16 mmol), 20% palladium hydroxide on carbon powder (60 mg), cyclohexene (0.82 mL, 8.10 mmol) and ethanol (2 mL) were heated under reflux for 1.5 h. The reaction mixture was filtered and silica gel (0.2 g) was added to the solution. The mixture was concentrated under reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$/methanol 20:1) to give compound **4a** (37 mg, 95%). Crystallization from acetone gave the analytical specimen as plate crystals. M.p. 206–207 °C; $[\alpha]_D^{26} = +162.8$ ($c = 1.00$ in CH_3OH); ^{1}H NMR (270 MHz, CD_3OD, 25 °C, TMS):$\delta = 8.01$ (dd, $J = 7$, 1 Hz, 1H), 7.59–7.53 (m, 1H), 6.53–6.45 (m, 2H), 5.79 (s, 1H), 4.33 (s, 1H), 4.04 (s, 1H), 3.99, 3.82 (AB, $J = 8$ Hz, 2H), 3.94 (s, 2H); ^{13}C NMR (68 MHz, CD_3OD, 25 °C): $\delta = 163.83$, 142.15, 133.68, 120.24, 108.10, 90.42, 89.09, 80.59, 72.49, 69.92, 57.76; IR (KBr): $\tilde{\nu}_{max} = 3293$, 2952, 1655, 1567, 1051 cm^{-1}; MS (EI): *m*/*z* (%): 239 (30) $[M]^+$, 96 (100); elemental analysis calcd (%) for $C_{11}H_{13}NO_5$ (239.2): C, 55.23, H 5.48, N 5.86; found: C 55.05, H 5.44, N 5.82.

5-Methyl-1-(2-*O*,4-*C*-methylene-β-D-ribofuranosyl)-2-pyridone (4b): A mixture of compound **3b** (265 mg, 0.61 mmol), 20% palladium hydroxide on carbon powder (140 mg), cyclohexene (3.10 mL, 30.6 mmol), and ethanol (6 mL) was heated under reflux for 2 h, then filtered. The filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$/methanol 13:1) to give compound **4b** (141 mg, 91%) as a white powder. M.p. 209–211 °C; $[\alpha]_D^{28} =$ $+114.8$ ($c = 0.93$ in CH_3OH); ^{1}H NMR (270 MHz, CD_3OD, 25 °C, TMS): $\delta = 7.82$ (d, $J = 2$ Hz, 1H), 7.45 (dd, $J = 9$, 2 Hz, 1H), 6.46 (d, $J = 9$ Hz, 1H), 5.76 (s, 1H), 4.31 (s, 1H), 4.05 (s, 1H), 3.79, 3.97 (AB, $J = 8$ Hz, 2H), 3.93 (s, 2H), 2.15 (s, 3H); ^{13}C NMR (68 MHz, CD_3OD, 25 °C): $\delta = 162.93$, 144.59, 131.01, 119.79, 117.77, 90.38, 89.04, 80.58, 72.46, 69.83, 57.66, 17.49; IR (KBr): $\tilde{\nu}_{max} = 3406$, 2925, 1658, 1567 cm^{-1}; MS (EI): *m*/*z* (%): 253 (38) $[M]^+$, 109 (100); elemental analysis calcd (%) for $C_{12}H_{15}NO_5 \cdot 1/3H_2O$ ($C_{12}H_{15}NO_5$, 253.3): C 56.11, H 6.04, N 5.45; found: C 56.15, H 5.95, N 5.44.

1-[5-*O*-(4,4′-Dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]-2-pyridone (5a): Under a nitrogen atmosphere, 4,4′-dimethoxytrityl chloride (113 mg, 0.33 mmol) was added to a solution of compound **4a** (50 mg, 0.21 mmol) in anhydrous pyridine (1 mL) and the mixture was stirred at room temperature for 1 h. After the addition of saturated aqueous solution of sodium hydrogen carbonate, the organic phase was extracted with ethyl acetate. The residue was obtained as in the synthesis of **2a** and further purified by silica gel column chromatography (*n*-hexane/ethyl acetate/triethylamine 20:100:1) to afford compound **5a** (109 mg, 96%). Reprecipitation from *n*-hexane/ethyl acetate gave the analytical specimen as a white powder. M.p. 114–115 °C; $[\alpha]_D^{25} = +56.5$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (270 MHz, CD_3COCD_3, 25 °C, TMS): $\delta = 8.07$ (dd, $J = 7$, 2 Hz, 1H), 7.56–7.26 (m, 10H), 6.92 (d, $J = 8$ Hz, 4H), 6.38 (d, $J = 9$ Hz, 1H), 6.23 (s, 1H), 5.77 (s, 1H), 4.78 (d, $J = 5$ Hz, 1H), 4.38 (d, $J = 5$ Hz, 1H), 4.34 (s, 1H), 3.94 (d, $J = 8$ Hz, 1H), 3.82–3.80 (m, 7H), 3.59, 3.52 (AB, $J = 11$ Hz, 2H); ^{13}C NMR (68 MHz, CD_3COCD_3, 25 °C): $\delta = 161.85$, 159.40, 145.71, 140.38, 140.36, 136.39, 136.19, 130.77, 130.75, 128.74, 128.46, 127.48, 120.54, 113.76, 105.72, 105.70, 88.56, 88.48, 87.06, 79.78, 72.30, 70.15, 59.44, 55.40; IR (KBr): $\tilde{\nu}_{max} = 3279$, 2952, 1657, 2574, 1508, 1252 cm^{-1}; MS (EI): *m*/*z* (%): 303 (100) [$C_{21}H_{19}^+$ (DMTr$^+$)]; elemental analysis calcd (%) for $C_{32}H_{31}NO_7 \cdot$ $1/2H_2O$ ($C_{32}H_{31}NO_7$, 541.6): C 69.81, H 5.86, N 2.54; found: C 69.90, H 5.78, N 2.55.

1-[5-*O*-(4,4′-Dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]-5-methyl-2-pyridone (5b): Under a nitrogen atmosphere, 4,4′-dimethoxytrityl chloride (151 mg, 0.45 mmol) was added to a solution of compound **4b** (87 mg, 0.34 mmol) in anhydrous pyridine (1 mL) at room temperature and the mixture was stirred for 1 h. After the addition of water, the organic phase was extracted with ethyl acetate. The residue was obtained as in the synthesis of **2a** and further purified by silica gel column chromatography (*n*-hexane/ethyl acetate 1:5) to give compound **5b** (186 mg, 97%). Reprecipitation from *n*-hexane/ethyl acetate gave the analytical sample as a white powder. M.p. 118–121 °C; $[\alpha]_D^{23} = +34.8$ ($c = 1.11$ in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 7.68$ (s, 1H), 7.50–7.19 (m, 10H), 6.85 (d, $J = 8$ Hz, 4H), 6.47 (d, $J = 9$ Hz, 1H), 5.85 (s, 1H), 4.50 (s, 1H), 4.28 (s, 1H), 3.87 (s, 2H), 3.79 (s, 6H), 3.48, 3.58 (AB, $J = 11$ Hz, 2H), 1.93 (s, 3H); ^{13}C NMR (68 MHz, $CDCl_3$, 25 °C): $\delta = 161.15$, 158.49, 144.47, 142.37, 135.44, 135.42, 129.99, 129.96, 129.04, 127.99, 127.90, 126.90, 119.93, 115.08, 113.23, 113.22, 87.83, 87.66, 86.59, 79.18, 71.73, 70.25, 58.63, 55.25, 17.60; IR (KBr): $\tilde{\nu}_{max} = 3327$, 2951, 1664, 1581, 1508, 1252, 1179, 1081, 1046 cm^{-1}; MS (FAB): *m*/*z*: 556 $[M+H]^+$; elemental analysis calcd (%) for $C_{33}H_{33}NO_7 \cdot 1/3H_2O$ ($C_{33}H_{33}NO_7$, 555.6): C 70.57, H 6.04, N 2.49; found: C 70.55, H 6.14, N 2.45.

1-[3-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5-*O*-(4,4′-dimethoxytrityl)-2-*O*-4-*C*-methylene-β-D-ribofuranosyl]-2-pyridone (6a): Under a nitrogen atmosphere, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (52 μL, 0.16 mmol) was added to compound **5a** (20 mg, 37 μmol) and diisopropylammonium tetrazolide (17 mg, 98 μmol) in anhydrous acetonitrile/tetrahydrofuran (3:1, 0.8 mL) at room temperature and the mixture was stirred for 9 h. The solvent was removed under reduced pressure and residue purified by flash silica gel column chromatography (*n*-hexane/ethyl acetate/triethylamine 50:50:1) to give compound **6a** (26 mg, 95%) as a white powder. M.p. 68–69 °C; ^{31}P NMR (81 MHz, $CDCl_3$, 25 °C): $\delta = 149.3$, 148.69; MS (FAB): *m*/*z*: 742 $[M+H]^+$; HRMS (FAB): calcd for $C_{41}H_{48}N_3O_8PNa$: 764.3077; found: 764.3077 $[M+Na]^+$.

1-[3-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5-*O*-(4,4′-dimethoxytrityl)-2-*O*-4-*C*-methylene-β-D-ribofuranosyl]-5-methyl-2-pyridone (6b): Under a nitrogen atmosphere, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (33 μL, 0.10 mmol) was added to compound **5b** (34 mg, 61 μmol) and diisopropylammonium tetrazolide (10 mg, 58 μmol) in anhydrous acetonitrile/tetrahydrofuran (3:1, 2 mL) at room temperature and the mixture was stirred for 10 h. The solvent was removed under reduced pressure and the residue purified by flash silica gel column chromatography (*n*-hexane/ethyl acetate 50:50) to give compound **6b** (42 mg, 91%) as a white powder. M.p. 72–75 °C; ^{31}P NMR (81 MHz, $CDCl_3$, 25 °C): $\delta = 149.09$, 148.77; MS (FAB): *m*/*z*: 756 $[M+H]^+$; HRMS (FAB): calcd for $C_{42}H_{50}N_3O_8PNa$: 778.3233; found: 778.3236 $[M+Na]^+$.

Syntheses of modified oligonucleotides: Oligonucleotides were synthesized on a 0.2 μmol scale on an Applied Biosystems DNA synthesizer by using standard phosphoramidite chemistry. The 5′-terminal DMTr group-protected oligonucleotides were treated with concentrated ammonia hydroxide for 18 h at 60 °C and the solvents were removed. The residue was purified with NENSORP PREP to give the purposed oligonucleotides. The purity of the modified oligonucleotides was verified using reversed-phase HPLC (ChemcoPak CHEMCOSORB 300—5C18, 4.6 mm × 250 mm), and the compositions determined by MALDI-TOF-MS (Voyager-DE).

MALDI-TOF-MS data: **9**, calcd for $[M-H]^-$: 4465.04; found: 4463.87; **10**, calcd for $[M-H]^-$: 4493.05; found: 4493.37; **11**, calcd for $[M-H]^-$: 4507.08;

found: 4507.76; **12**, calcd for $[M-H]^-$: 4399.97; found: 4400.41; **14**, calcd for $[M-H]^-$: 4492.07; found: 4491.60; **16**, calcd for $[M-H]^-$: 4492.07; found: 4492.83; **18**, calcd for $[M-H]^-$: 4491.08; found: 4490.48; **20**, calcd for $[M-H]^-$: 4489.01; found: 4489.89; **21**, calcd for $[M-H]^-$: 4601.06; found: 4600.91; **22**, calcd for $[M-H]^-$: 4643.14; found: 4642.37.

UV-melting experiments: UV-melting experiments were carried out on Beckman DU—650 spectrometer. The profiles were measured at a scan rate of 0.5 °C min^{-1} at 260 nm in sodium phosphate buffer (7 mM, pH 7.0) which also contained KCl (140 mM) and spermine (5 mM). The final concentration of each oligonucleotide used was 1.5 μM. The T_m value was designated as the maximum of the first derivative calculated from the UV-melting profile.

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