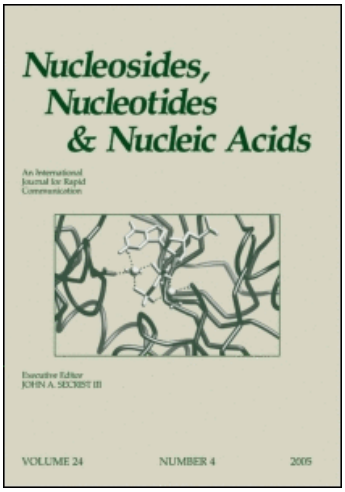

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SYNTHESIS AND PROPERTIES OF OLIGONUCLEOTIDES CONTAINING 8-BROMO-2'-DEOXYGUANOSINE

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SYNTHESIS AND PROPERTIES OF OLIGONUCLEOTIDES CONTAINING 8-BROMO-2′-DEOXYGUANOSINE

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ABSTRACT

The preparation of oligonucleotides containing 8-bromo-2′-deoxyguanosine is described. Substitution of G by 8-bromoguanine on an alternating CG decamer stabilizes the Z-form in such a way that the B-form was not observed. Melting temperatures showed that duplexes in which 8-bromo-2′-deoxyguanosine paired with natural bases were much less stable.

INTRODUCTION

Chemically modified oligonucleotides are widely used in the study of nucleic acid structure and protein-nucleic acid interactions (1,2). Specifically, the introduction of brominated and iodinated nucleosides in defined positions is applied in the following areas: (a) photo-cross-linking with nucleic acid-binding proteins, in which the halogen is excised selectively to generate the nucleobase radicals (3), (b) in X-ray diffraction experiments, in which the higher electron density of the bromine and iodine is used to solve the phase problem (4), (c) mutagenesis studies, in which changes in pKa and/or tautomerism induced by the halogen alter base pairing properties and polymerase fidelity (5).

In the recent years interest has focused on purine nucleosides carrying bulky substituents at the 8-position to favor the *syn* conformation at the N-glycoside bond

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(6–8). These oligonucleotides may be used as model compounds to study DNA fidelity and DNA repair. One of the most significant oxidative damage products in DNA is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG) (9). This lesion is repaired by several enzymes, and it has been suggested that the higher population of the *syn* conformation on 8-oxoG is the structural feature recognized by the repair enzymes to distinguish 8-oxoG and dG (10). Bulky substituents on purine nucleosides at the 8-position shift the equilibrium to the *syn* conformation (11). On the basis of these data, 8-bromo-2′-deoxyguanosine (**B**) was successfully used to probe *syn-anti* conformational preferences on G-quartet structures (6). Moreover, bromination of poly (d(GC)) stabilizes the Z-DNA form (12). A modification of 38% 8-bromoguanine and 18% 5-bromocytosine is enough to render a stable Z-DNA helix in physiological conditions.

Oligodeoxynucleotides containing 8-bromoguanine were prepared using the isobutyryl group for the protection of the amino group at position 2 (6). In this communication we report the synthesis of an 8-bromoguanine phosphoramidite derivative protected with the dimethylformamidine (dmf) (13,14) group and its use in the preparation of oligodeoxynucleotides carrying 8-bromoguanine. Moreover, base-pairing properties of oligodeoxynucleotides containing 8-bromoguanine and their ability to form Z-DNA are described.

RESULTS AND DISCUSSION

Preparation of the Protected Phosphoramidite

The synthesis of the 8-bromoguanine phosphoramidite building block is illustrated in Scheme 1. 8-Bromo-2′-deoxyguanosine (**1**) was prepared from dG as described elsewhere (15). Reaction of 8-bromo-2′-deoxyguanosine with *N,N*-dimethylformamide dimethyl acetal in dimethylformamide (16) gave compound **2** which was used without purification in the following step. Compound **2** was treated with dimethoxytrityl (DMT) chloride in pyridine for 5 hours at room temperature yielding compound **3** in good yield (73% from **1**). Reaction of compound **3** with 2-cyanoethyl-*N,N*-diisopropyl chlorophosphine and *N,N*-diisopropylethylamine gave the desired phosphoramidite **4** in 90% yield. Moreover, the DMT-nucleoside **3** was converted to the 3′-O-hemisuccinate derivative **5**, which was attached to amino-controlled pore glass (CPG, 500 Å) supports following previously described protocols (data not shown) (17,18).

One of the main differences between this and the previous preparation of isobutyryl-protected 8-bromoguanine derivative (6), is that, bromination is performed before the introduction of the DMT and the amino protecting group. In this way some possible side reactions such as bromination of the DMT group and premature cleavage of the acid labile DMT group are avoided, resulting in a much higher yield. Also, the use of the more labile dmf group for the protection of the amino group results in a more versatile building block.

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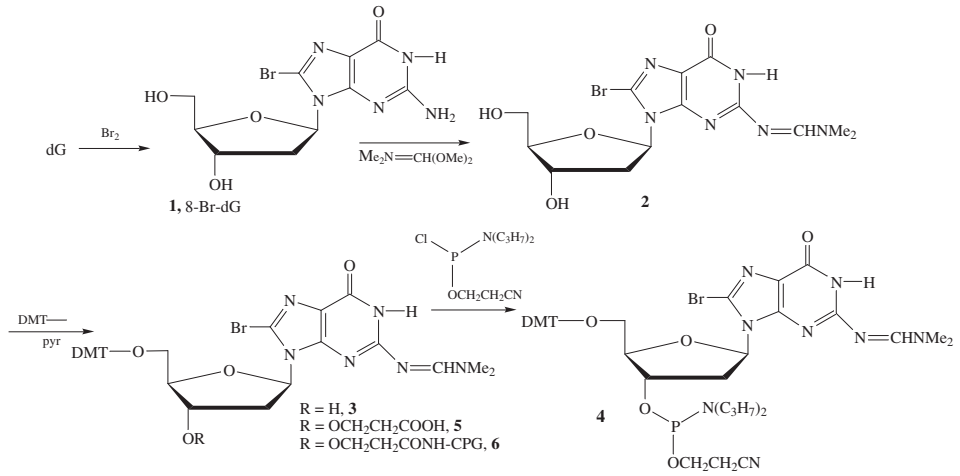

Scheme 1. Preparation of the phosphoramidite derivative of N-protected-8-bromo-2′-deoxyguanine.

Preparation of Oligonucleotides

Oligonucleotides A (5′ T**B** 3′), B (5′ CACA**B** 3′), C (5′ GCAATGGA**B**CCTC TA 3′), D (5′ C**B**C**B**CGCGCG 3′), E (5′ **5B5B**CGCGCG 3′) and F (5′ **5G5**GCGCG CG 3′) where **B** = 8-bromoguanine and 5 = 5-bromocytosine were prepared on a DNA synthesizer using both standard (benzoyl-dA and dC and isobutyryl-dG) and *t*-butylphenoxyacetyl (TBPA)-protected (19) 2-cyanoethyl phosphoramidites together with phosphoramidite **4** and the commercially available phosphoramidite of 5-bromo-2′-deoxycytidine (protected with the benzoyl group) and the appropriate succinyl-CPG supports. During the syntheses, 0.02 M iodine solution (tetrahydrofurane: water: pyridine 7:1:2) was used to prevent the formation of N-cyano nucleosides (20). *t*-Butylphenoxyacetyl anhydride was used instead of acetic anhydride in the syntheses with TBPA-protected phosphoramidites to prevent acetylation of TBPA-protected bases (19). After the assembly of the sequences, oligonucleotide-supports were treated with concentrated (30%) aqueous ammonia solution. The time needed for the removal of the dmf group on the dimer A was found to be less than 1 hour at room temperature, because a single peak was observed on reverse phase HPLC that had the expected molecular weight and the expected nucleoside composition after enzymatic digestion. Sequences carrying 5-bromocytosine (E and F) were prepared using *t*-butylphenoxyacetyl (TBPA)-protected phosphoramidites and deprotected with concentrated ammonia at room temperature to avoid the formation of 5-aminocytosine (21). Sequences B, C and D prepared with standard phosphoramidites were treated with concentrated ammonia overnight at 50°C. Special attention was paid to detect the decomposition of 8-bromoguanine to 8-aminoguanine or 8-oxoguanine as it has been described for 5-bromo-pyrimidines (21) and 8-bromo-dA (22) when using hot ammonia solutions for the removal of protecting groups. In all cases, a major peak was observed that had the correct

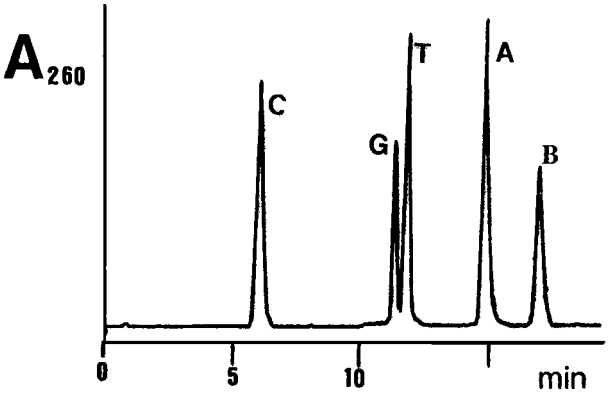

Figure 1. Reversed phase chromatography analysis of the mixture obtained after phosphodiesterase and alkaline phosphatase degradation of purified pentadecamer C (5′ GCA ATG GAB CCT CTA 3′).

nucleoside composition (see Fig. 1) and the expected molecular weight. The higher stability of 8-bromoguanine to nucleophiles was also evident from the treatment of dimer A with lithium azide. This treatment was performed in order to obtain a dimer containing the photoreactive 8-azido-dG (data not shown). When support carrying sequence A was treated with 0.16 M lithium azide in DMF overnight at 50°C, followed by ammonia deprotection (1 h, R.T.) the dimer containing unaltered 8-bromo-G was isolated. The same experiment with a similar dimer but containing 8-bromo-A instead of 8-bromo-G gave total conversion of 8-bromo-A dimer to the dimer containing 8-azido-A (23). Therefore, 8-bromoguanine oligonucleotides can be prepared without detectable degradation using the standard ammonia deprotection conditions (60°C, overnight).

Melting Experiments

Duplexes having 8-bromoguanine base pairs with the four natural bases were analyzed. Melting temperatures (T_m) are shown in Table 1. Guanine and

Table 1. Melting Temperatures (T_m,°C) of 8-bromoguanine (B) and 8-oxo-G Duplexes (0.1 M sodium phosphate pH 7.0)

	5′GCA ATG GAX GCC TCT A 3′ 3′CGT TAC CTY CGG AGA T 5′		
	X = G	X = **B**	X = 8-oxo-G
Y = C	62	56	60
Y = A	55	49	55
Y = G	55	n.c.#	54
Y = T	54	56	50

#Curve with low hyperchromicity and not cooperative

8-oxoguanine base pairs were included as reference. The oligonucleotide containing 8-oxoguanine were prepared as previously described (16). As expected in all cases the most stable base pair is formed between the guanine derivative and cytosine. The relative stability of the different guanine derivatives is G•C>8-oxo-G•C> B•C. A similar trend is observed with the G•A mispairs. Duplex containing B•G basepair did not exhibit the cooperative helix-to-coil transition and for this reason T_m was not calculated. In contrast, G•T and 8-oxo-d G•T mispairs were less stable than B•T mispair. In general, melting temperatures of duplexes containing **B** were lower than 8-oxo-G and guanine duplexes. The degree of destabilization is similar to the destabilization found in duplexes containing 8-methoxy-dA (7). This destabilization has been assigned to a major content of the *syn* conformer that prevents the normal Watson-Crick hydrogenbonding (7).

B to Z Transition Studies

Previously it was shown that bromination of poly (d(GC)) stabilizes the Z form (12) but no data have appeared on the role of each individual reaction product: 5-bromocytosine and 8-bromoguanine. We addressed this question by preparing decamers containing alternating CG sequences in which two of the five residues were replaced by the corresponding bromo derivative. The contribution of each bromo derivative to the stabilization was estimated from the mid-point NaCl concentration for the B-Z transition of different self-complementary decamers measured by circular dichroism. As seen in Table 2, the presence of 5-bromocytosine induced only moderate stabilization of the Z-form (mid-point 1.2 M NaCl compared to a mid-point of 2.2 M NaCl for $d(CGCGCGCGCG)_2$. On the other hand, 8-bromoguanine induces a large stabilization of the Z-form in such a way that even without NaCl added the CD spectra of 8-bromoguanine decamers correspond to the Z-form (see Fig. 2). The strong stabilization of the Z-form caused by 8-bromoguanine is in agreement with the strong stabilization observed for 8-methylguanine (24) and 7-deaza-8-methylguanine (25,26). Incorporation of 8-bromoguanine, 8-methylguanine or 7-deaza-8-methylguanine into oligonucleotides may be useful for the

Table 2. Midpoint NaCl Concentration in B->Z Transitions for 8-bromoguanine (**B**) and 5-bromocytosine (**5**) oligodeoxynucleotides (50 mM tris HCl pH 7.5)

Oligonucleotide	NaCl (M)[a]
$d(CGCGCGCGCG)_2$	2.2
$d(\mathbf{5}G\mathbf{5}GCGCGCG)_2$	1.2
$d(C\mathbf{B}C\mathbf{B}CGCGCG)_2$	no transition, Z-form even at 0 M NaCl
$d(\mathbf{5B5B}CGCGCG)_2$	no transition, Z-form even at 0 M NaCl

[a]In all cases the solutions were 50 mM Tris HCl buffer, pH 7.5

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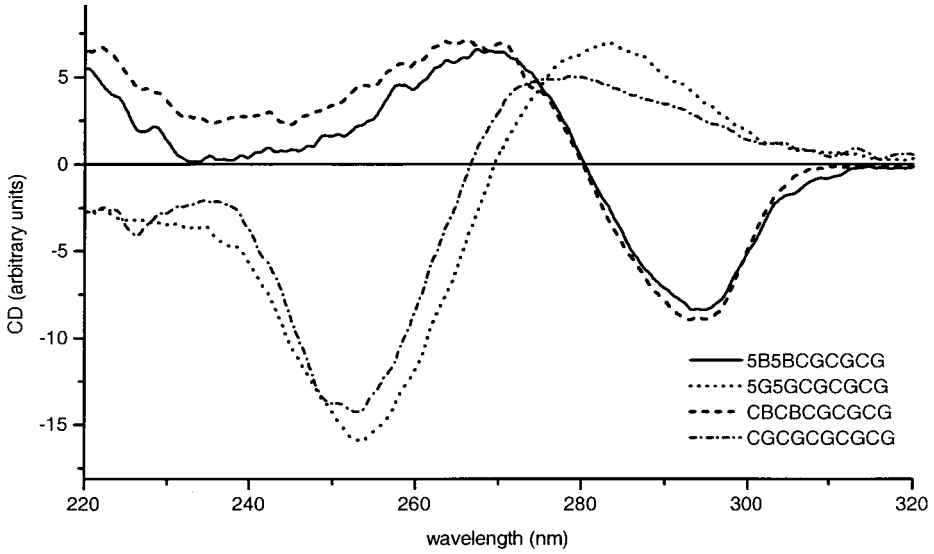

Figure 2. CD spectra of decamers containing 5-bromocytosine and 8-bromoguanine in 50 mM Tris HCl buffer, pH 7.5, (without NaCl added) at 20°C.

preparation of DNA substrates to study Z-DNA binding proteins, (27,28) although we believe 8-bromoguanine oligonucleotides are easier to prepare.

Conclusion

In conclusion, a new 8-bromo-dG phosphoramidite derivative is described. The new derivative is easily prepared and can be removed in mild conditions. Contrary to other brominated nucleosides (21,22), 8-bromo-dG was found to be very stable to ammonia deprotection. Therefore, the use of more labile groups for the natural bases is not required. Substitution of G by 8-bromo-G destabilizes the duplex structure as expected from the higher content of the *syn* conformer, but it also strongly stabilizes the Z-form. Thus, 8-bromo-dG may be a useful probe for locating subtle differences in DNA polymerases (29) and DNA repair glycosylases due to differences in conformational properties, and for producing substrates for the study of Z-DNA binding proteins.

EXPERIMENTAL SECTION

General Methods

All reactions were carried out in oven-dried glassware, under a nitrogen or argon atmosphere, unless specified otherwise. Before use, starting materials were dried by evaporation with the dry solvent which was later used for the reaction. Reagents for oligonucleotide synthesis were from Glen Research and PE Applied

Biosystems. Dry solvents were from SDS and Romil. HPLC grade solvents were from Romil and E. Merck. Snake venom phosphodiesterase (from *Crotalus durissus*) and alkaline phosphatase were from Boehringer Mannheim. The rest of the reagents were from Aldrich and Fluka and were used without further purification. Analytical TLC was run on aluminum sheets coated with silica gel 60 F_{254} from Merck. Silica gel column chromatography was performed with Chromatogel 60 A C.C. (40–60 microns, 230–400 mesh, SDS).

Instrumental

^{1}H-NMR (250 MHz) ^{13}C-NMR (63 MHz) and ^{31}P-NMR (101 MHz) spectra were recorded on a Brüker AM-250. HPLC chromatography was performed on an HPLC System Gold (Beckman) and a Waters instrument. Mass spectra were obtained on a API III SCIEX-Perkin Elmer equipped with a triple quadrupole detector.

5′-O-(Dimethoxytrityl)-N^2-(*N,N*-dimethylaminomethyliden)-8-bromo-2′-deoxyguanosine (3)

8-Bromo-2′-deoxyguanosine (15) (1.5 g, 4.3 mmol) was reacted with *N,N*-dimethylformamide dimethyl acetal (1.72 ml, 15.9 mmol) in 20 cm^3 of *N,N*-dimethylformamide for 4 hours at room temperature giving quantitatively compound **2**, which was used without purification in the following step.

The residue of the previous reaction (compound **2**) was dissolved in 20 ml of pyridine and treated with dimethoxytrityl chloride (6.5 mmol) for 5 hours at room temperature. Methanol (1 ml) was added to stop the reaction and the mixture was concentrated to dryness. The residue was dissolved in dichloromethane and washed with 1M aq. $NaHCO_3$. The organic phase was dried and concentrated to dryness. The residue was purified on silica gel eluted with 0-10% methanol gradient in dichloromethane to afford compound **3** (2.2 g, 73% from **1**) (Found: C, 57.7; H, 5.3; N, 12.1. $C_{34}H_{35}N_6O_6$ requires C, 58.0; H, 5.0; N, 11.9), Rf 0.10 (5% methanol in chloroform); $\delta_H(CD_3OD)$ 2.29–2.42 (2H, m), 2.98 (3H, s), 3.03 (3H, 1s), 3.16–3.49 (2H, m), 3.69 (6H, s), 4.05 (1H, m), 4.81 (1H, m), 6.34 (1H, dd, J = 7.8 Hz, J = 4.4 Hz), 6.64 (4H, d, J = 5.8 Hz), 6.70 (4H, d, J = 5.8 Hz), 7.05-7.42 (5H, m), 8.34 (1H, s), 8.50 (1H, d, J = 4.2 Hz); $\delta_C(CD_3OD)$ 38.3, 35.4, 41.6, 55.6, 65.0, 72.4, 87.0, 87.1, 87.4, 113.9, 121.6, 127.6, 129.1, 129.8, 130.9, 131.1, 138.0, 138.1, 144.6, 152.5, 158.1, 159.0, 159.3, 150.9.

3′-*O*-[(2-Cyanoethoxy)-(*N,N*-diisopropylamino)phosphinyl]-5′-*O*-(dimethoxytrityl)-N^2-[(*N,N*-dimethylamino)methyliden]-8-bromo-2′-deoxyguanosine (4)

Compound **3** (0.50 g, 0.74 mmol) was dissolved in dry acetonitrile and reacted with 0.20 ml (0.88 mmol) of (2-cyanoethoxy)-(N,N-diisopropylamino)

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chlorophosphine and 0.40 ml (2.22 mmol) of ethyldiisopropylamine. After 1 hour of magnetic stirring, the reaction was stopped by adding 1 ml of MeOH. The solvent was evaporated and the residue was dissolved in DCM (20 ml) and washed with 10% aq. $NaHCO_3$ followed by saturated aq. NaCl. The organic phase was dried and concentrated to dryness. The residue was purified on silica gel eluted with dichloromethane/ ethyl acetate/triethylamine (45:45:10) to give compound **4** (0.55 g, 90%) (Found: C, 56.7; H, 5.7; N, 12.7. $C_{43}H_{52}N_8O_7BrP$ requires C, 57.1; H, 5.8; N, 12.4); Rf 0.8 (dichloromethane/ethyl acetate/triethylamine 45:45:10); δ_p (CH_3CN, external reference H_3PO_4) 149.2 and 149.3 (two diastereoisomers).

Oligonucleotide Synthesis

The following sequences have been synthesized: A (5′ T**B** 3′), B (5′ CACA**B** 3′), C (5′ GCAATGGA**B**CCTCTA 3′), D (5′ C**B**C**B**CGCGCG 3′), E(5′ **5B5B**CGCG CG 3′), and F (5′ **5G5**GCGCGCG 3′) being **B** = 8-bromo-G and **5** = 5-bromo-cytosine. Oligonucleotides were prepared on an automatic DNA synthesizer using commercially available 2-cyanoethyl phosphoramidites and the modified phosphoramidite. Oligonucleotide sequences B and C were prepared using using both standard (benzoyl-dA and dC and isobutyryl-dG) and *t*-butylphenoxyacetyl (TBPA)-protected (19) phosphoramidites. Oligonucleotide sequences E and F were prepared using using *t*-Butylphenoxyacetyl (TBPA)-protected phosphoramidites as decribed (23). During the syntheses, 0.02 M iodine solution (tetrahydrofurane: water: pyridine 7:1:2) was used to prevent the formation of N-cyano nucleosides (20). *t*-Butylphenoxyacetyl anhydride was used instead of acetic anhydride on the syntheses with TBPA-protected phosphoramidites to prevent acetylation of TBPA-protected bases (19).

Oligonuclcotide-supports were treated with 32% aqueous ammonia at 50 °C for 16 h (when benzoyl and isobutyryl groups are used) and at room temperature for 16 hr (when TBPA groups are used). Ammonia solutions were concentrated to dryness and the products were purified either by cartridge purification (COP), by reversed phase HPLC or by PAGE electrophoresis. All purified products presented a major peak that was collected and analyzed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis of the nucleosides (HPLC conditions B, see Fig. 2). HPLC solutions are as follows. Solvent A: 100 mM triethylammonium acetate (pH 7.8) and solvent B: 50% ACN in 100 mM triethylammonium acetate pH 7.8. For analytical runs the following conditions were used. Column: Nucleosil 120C18, 250 × 4 mm, flow rate: 1 ml min^{-1}. Conditions A) a 40 min linear gradient from 0 to 100% B. Conditions B) a 20 min linear gradient from 0 to 40% B. For semipreparative runs the following conditions were used: Columns: Nucleosil 120C_{18}, 250 × 10 mm. Flow rate: 3 ml min^{-1}. A 20 min linear gradient from 20-80% B (DMT-on), or a 30 min linear gradient from 0-60% B (DMT-off).

Mass spectra (electrospray) of dimer A: 648.9 and 650.9 (expected for $C_{20}H_{26}N_7O_{11}PBr$ 649.3, 651.3), pentamer B: 1550.7 (expected for $C_{48}H_{60}N_{21}O_{26}P_5$

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Br 1549.7, 1551.7), pentadecamer C: 4654.3 (expected for $C_{146}H_{183}N_{58}O_{87}P_{14}Br$ 4654.2, 4656.3), decamer D: 3186.3 (expected for $C_{95}H_{119}N_{40}O_{58}P_9Br_2$: 3187.6), decamer E: 3344.6 (expected for $C_{95}H_{117}N_{40}O_{58}P_9Br_4$: 3345.6).

Melting and Circular Dichroism Experiments

Melting experiments of pentadecamers duplexes were performed as previously described (17). Duplex concentration was 4 μM.

Circular dichroism (CD) spectra were recorded on a Jasco J-710 spectropolarimeter with a Peltier temperature controller. CD measurements were taken at 20°C with a 5 mm pathlength cell. Duplex concentration was 4 μM. UV melting curves were performed with the samples from CD measurements. Melting temperatures of dodecamers were between 60 and 85°C depending on the oligonucleotide and the salt concentration.

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