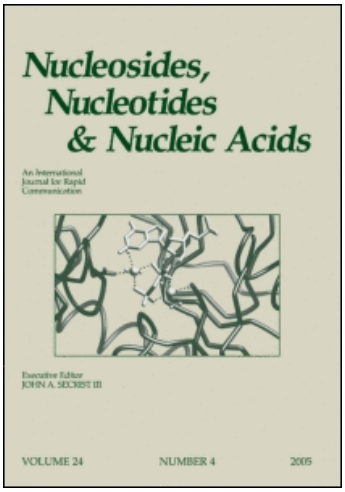

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How a Modification (8-Aza-3-deaza-2′-deoxyguanosine) Influences the Quadruplex Structure of Hotoda's 6-Mer TGGGAG with 5′- and 3′-End Modifications†

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ABSTRACT

We have synthesized a modified 6-mer using Hotoda's 6-mer TGGGAG with 5′- and 3′-end modifications as a template. We have replaced from one to all four 2′-deoxyguanosines by 8-aza-3-deaza-2′-deoxyguanosine (c^3z^8dG, 1) in order to investigate the anti-HIV structure activity relationship (SAR). ODN 4 (TGGG*AG) is the only one that exhibits a moderate anti-HIV-1 activity.

Key Words: 8-aza-3-deaza-2′-deoxyguanosine; 6-mer TGGGAG; Structure activity relationship.

†In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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INTRODUCTION

In recent years *antisense* oligonucleotides (ODNs) and triplex-forming ODNs were designed to be anti-HIV agents.[1,2] HIV-1 has been one of the most frequently studied targets of native and chemically modified ODNs.[3] These ODNs act at several steps, including HIV-binding to target cells. They also target to the viral RNA and prevent synthesis of proviral DNA through inhibition of reverse transcription. However, ODNs which aggregate into higher-order inter- or intramolecular structures due to repeating G-rich sequences that form tetramers stabilized by G-quartets, have been discovered as potent anti-HIV therapeutic drugs, acting through different mechanisms. Among these, AR 177 is an 17-mer ODN composed only of deoxyguanosine and thymidine with single phosphorothioate internucleoside linkages at its 5′- and 3′-end which is folded into guanosine-quartet structure.[4] This structure seems to result in a pronounced resistance to nucleases. Both, the G-quartet structure and a phosphorothioate backbone, were shown to be required for antiviral activity.[5] On the contrary, Furukawa et al. reported the anti-HIV-1 activity of ODNs with a natural type phosphodiester backbone, e. g. a 15-mer d(TGGGAGGTGGGTCTG) which is complementary to the '*Tat*' 2 nd splicing acceptor region of HIV-1, and possessed a 4, 4′-dimethoxytrityl group at the 5′-end.[6] This feature demonstrated the imperative protection with aromatic substituent at the 5′-end.[6] It was also revealed that adequate protection of the 5′-region is essential and responsible for the anti-HIV activity of the rationalized 6-mer (TGGGAG; R95288) as well.[7] This new lead *non-antisense* compound retains the structure of a parallel-stranded tetramer like in ISIS 5320 ($T_2G_4T_2$) possessing a thioate backbone structure.[5,8] Both compounds act by interaction with the V3 loop of the envelope glycoprotein as does the AR 177 (17-mer) which folds into an antiparallel intramolecular G-quartet structure.[9] The intrinsic structural properties define two classes of interesting lead compounds composed of G-quartets imperative for the inhibition of HIV replication in cell culture. The in vivo existence of such a tertiary structure motif was intensively studied, and both parallel and antiparallel motifs were recently disclosed with compelling evidence.[10] That means that specific proteins certainly preferentially bind to tetraplex structures and such an interaction was reported for human DNA topoisomerase I which controls the topological state of DNA.[11]

In order to ascertain the role of the guanine stretch, we reckoned, that replacement of an individual base would likely affect the overall conformation. Just to mimic the whole conformation and to assess the effect of more subtle differences, we introduced the non-natural guanosine residue (e. g. 8-aza-3-deaza-2′-deoxyguanosine 1, c3z8dG)[12,13] into Hotoda's ultimate ODN R95288[8] (Figure 1).

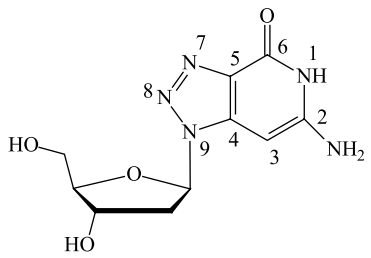

Figure 1. 8-Aza-3-deaza-2′-deoxyguanosine 1 (c^3z^8dG), purine numbering.

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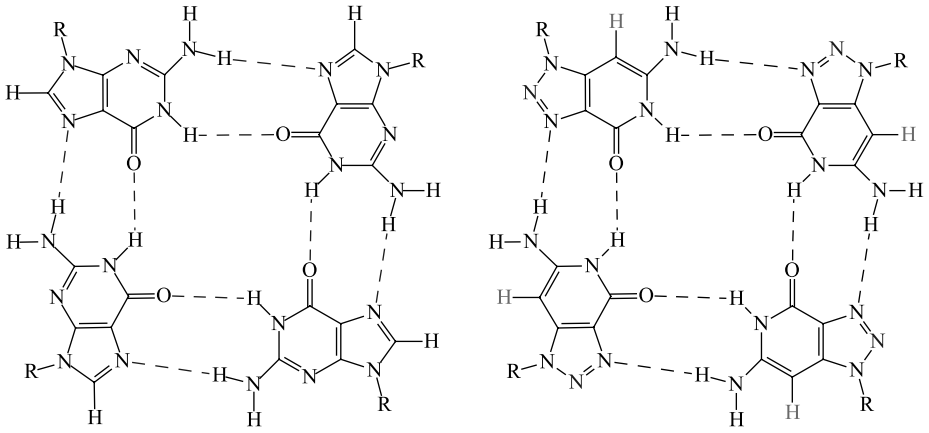

Figure 2. Schematic presentation of deoxyguanosine tetrad and theoretical 8-aza-3-deaza-2′-deoxyguanosine tetrad. (*View this art in color at www.dekker.com.*)

RESULTS AND DISCUSSION

In a previous communication we have reported on chemical and hybridization properties of duplexes containing 1.[14] In a 16-mer with a sequence devoid of quadruplex formation, 1 caused minimal destabilization *per* base. In the comparison of the hydrogen-bonding network of G-tetrads in the Hoogsteen manner (Figure 2) with N^7, O^6, $NH^{[1]}$ and one proton of amino group involved, no disruption is expected with

Table 1. Position(s) of modified guanosine in sequence (3,4-DBB)-TGGGAG-2hEtP.

ODN	Position of modified G (G*) in sequence (3,4-DBB)-TGGGAG-2hEtP	
ODN 1		TGG GAG
ODN 2	1	TG*G GAG
ODN 3	2	TGG* GAG
ODN 4	3	TGG G*AG
ODN 5	4	TGG GAG*
ODN 6	12	TG*G* GAG
ODN 7	13	TG*G G*AG
ODN 8	14	TG*G GAG*
ODN 9	23	TGG* G*AG
ODN 10	24	TGG* GAG*
ODN 11	34	TGG G*AG*
ODN 12	123	TG*G* G*AG
ODN 13	124	TG*G* GAG*
ODN 14	134	TG*G G*AG*
ODN 15	234	TGG* G*AG*
ODN 16	1234	TG*G* G*AG*

the insertion of c^3z^8dG. This prompted us to incorporate 1 into an ODN possessing a sequence that has the tendency toward formation of a quadruplex structure.

For SAR reasons, it was reasonable to initiate this investigation adopting the most biologically reliable ODN, possessing a 3,4-dibenzyloxybenzyl group (3,4-DBB) at 5′- and a 2-hydroxyethylphosphate group (2hEtP) at the 3′-end,[8] and we have synthesized all possible combinations of ODNs that are shown in Table 1, using the phosphoramidite method on an automated DNA synthesizer.

Monomers

The 5′-*O*-(3,4-DBB)thymidine-3′-*O*-phosphoramidite building block was prepared as described earlier.[8] At this point it should be noted that the general procedure led to two products, 3′- and 5′-(3,4-dibenzyloxybenzxyl) derivatives (in a control experiment we observed partial deprotection of the 3′-*O*-*tert*-butyldimethylsilyl group). After desilylation we have first separated 3′- from 5′-(3,4-DBB)-T. In the ^{1}H NMR spectrum the chemical shifts of the sugar proton H_3' and of the methyl group on the base were siutable to differentiate between 5′-(3,4-DBB)-T and 3′-(3,4-DBB)-T by performing decoupling experiments (decoupling of H_3' has an influence on 3′-OH and also on H_2', H_2'' and H_4'). Then, we used 5′-(3,4)-DBB-T for the preparation of the appropriate phosphoramidite building block.

Hotoda et al.[8] prepared CPG with the first dG and then continued the synthesis on a synthesizer. In spite of the fact that we started the ODN synthesis on a synthesizer with a ‘naked’ ethylene glycol modified CPG, we have observed good coupling efficiency using trityl monitoring. We started with detritylation, which was followed by coupling of the first nucleoside (dG or 1 in modified ODNs). The overall yields in all cases were adequate and comparable to the synthesis of Hotoda’s 6-mer (R95288). The benefit of our alternative approach is a considerably more rapid synthesis.

Oligonucleotides

ODNs were synthesized on an Expedite synthesizer (model 8909) and purified on RP-HPLC using a column oven (T = 55°C) avoiding problems with higher structures. The 3,4-dibenzyloxybenzyl group is as hydrophobic as DMT, and the retention times of (3,4-DBB)-ODNs are comparable with that of DMT-ODNs.

Quadruplexes were formed by dissolving ODNs in the appropriate buffer and by heating the solutions at 95°C for 5 min. The particular solution was cooled and then equilibrated for at least 1 day at 4°C. Before further experiments, the samples were equilibrated at room temperature or at the initial temperature of the experiment.

Amongst different techniques proposed to monitor G-quartet formation,[15] gel electrophoresis,[16–18] CD[19] and ESI MS[20] were used in the present study. In vitro anti-HIV activity tests were performed for evaluation of the structure-activity relationship. The quadruplex structure of R95288 was first determined by CD spectroscopy[8] and was used as a control. It is generally considered that parallel-type G quartets (quadruplex formed from four independent short strands) have a positive ellipticity maximum at 264 nm and a negative ellipticity minimum at 240 nm (Figure 3).

For SAR reasons, anti-HIV assays were performed on ODNs containing c^3z^8dG in all feasible positions. The in vitro inhibitory effect on the HIV-induced cytophathic

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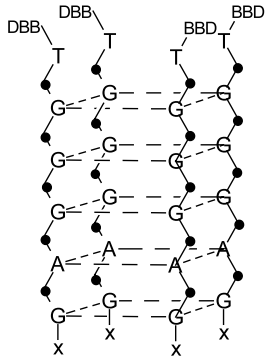

Figure 3. Schematic presentation of parallel quadruplex.

effect and the cytotoxcity of the ODNs in human lymphocyte MT-4 cells were determined by the MTT assay.[21] Only ODN 4 among all modified ODNs exhibited sufficient inhibitory activity against the replication of HIV-1 strain III_B in MT-4 cells to determine IC_{50}, while ODN 1 demonstrated comparable activity as reported.[8] ODN 4 did not inhibit the replication of HIV-2 strain ROD in cell culture. Interestingly, ODN 5 gave a maximal protection against the cytopathogenic effect of HIV-1 strain III_B in MT-4 cells of 21 to 23%. Other modified ODNs did not demonstrate any anti-HIV 1 activity. Anti-HIV-1 data are presented in Table 2. They were the basis for further SAR corellations.

At this stage we compared the structures of ODN 4 and ODN 5 with ODN 1 using CD. The CD spectra of ODN4 and ODN 5 are very similar in shape and ellipticity to the spectrum of ODN 1. However, the intensity of ODN 4 at the same concentration was weaker. ODN 4 displayed a shoulder in the range of 210 nm–230 nm which changed into a peak with increasing substitutions of G^*. Obviously, modified guanosine c^3z^8dG has a very strong impact on the shape of the particular CD spectrum. This may be atributed to a lack of quadruplex formation or to a significant bathochromic shift in the UV spectra of monomer 1 (Figure 4).

Table 2. Anti-HIV activity.

Compds[a]		EC_{50} (μg/ml)[b]	CC_{50} (μg/ml)[c]	SI[d]	Max. Prot.
ODN 1	III_B	0.87	>50	>58	
	ROD	16.1	>50	>3	
ODN 4	III_B	78	>100	>1	57–62%
	ROD	>100	>100	>1	0–1%
ODN 5	III_B	>100	>100	1	21–23%
	ROD	>100	>100	1	0%

[a]For position of modification (c^3z^8dG) in sequence, see Table 1.
[b]50% effective concentration, required to inhibit HIV-1 replication in MT-4 cells by 50%.
[c]50% cytotoxic concentration, required to inhibit cell viability by 50%.
[d]Selectivity index, or ratio of CC_{50} to EC_{50}.

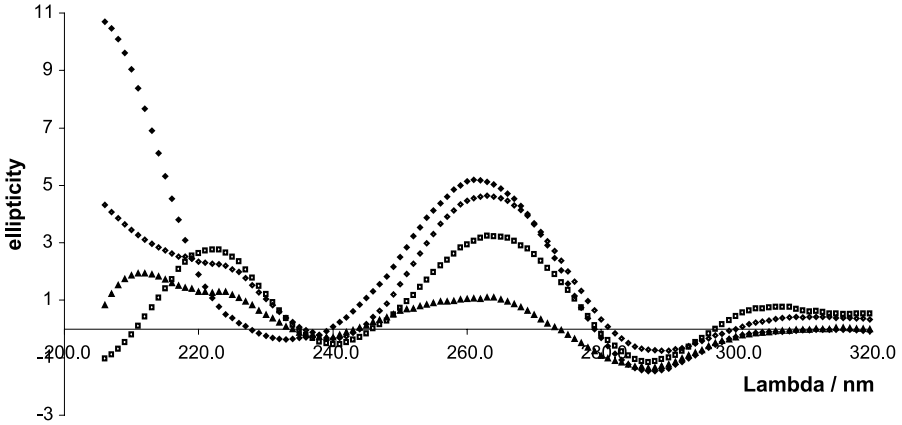

Figure 4. CD spectra of ODN 1, ODN 5, ODN 4, ODN 8, ODN 11 from top to bottom respectively (at 210 nm).

Electrospray ionization mass spectrometry (ESI-MS) is the method that is definitely not influenced by modification of the base. Goodlett et al.[20] studied the ESI-MS stability of a noncovalent, four-stranded oligonucleotide, $d(CGCG_4GCG)_4$ in a 10 mM phosphate buffer containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). Four-stranded complexed strucuture could be observed only in the presence of monoatomic cations like K^+, Na^+, Li^+, Ca^{2+}. In our case the very best results were obtained with 2.5 mM potassium acetate and 2.5 mM ammonium acetate. More significant results are presented in Table 3. The figure below shows three examples of MS spectra of ODN 1, ODN 5, ODN 8 from top to bottom, respectively. Solely monomer (M) and quadruplex (Q, 85%) peaks were detected with the basic ODN, while all modified analogs showed quite lower percentage of $[M^+]_4$ signals that correspond to quadruplex structures and additional signals demonstrating triplex (T) and duplex (D) entities in different ratios. The results are nicely comparable with CD results but unfortunately do not correlate with anti-HIV-1 activity data.

There are few examples in the literature of determination of quadruplex structure on the basis of electrophoretic mobility. In native PAGE, the secondary structures are not disrupted, and the mobility depends on the ratio mass/charge, the presence of cations and the concentration etc.[16–18] When a quadruplex is build up from four intermolecular strands, a delayed retention time is expected for the quadruplex. Positive results are obtained only with K^+, other ions proved ineffective in formations of a quadruplex at salt concentration of 200 mM. Among all ODNs, only ODN 1 showed almost 100% retained mobility. ODN 4 showed two peaks that were highly resolved, one for a single strand and the second for the quadruplex structure. Two exchanging Gs in positions 1 and 4 (ODN 8) also furnished two peaks, though the retained peak belonging to higher molecular weight is very weak. Other ODNs displayed only one peak, namely that of a single strand. These results correlate quite well with the anti-HIV-1 activity (Figure 5).

Slight disagreement of native PAGE data with the results of the CD spectra could be assigned to the strong dependence of the latter method on stacking interactions. The

Table 3. Characterization of ODNs by ESI MS (quadruplex determination) and results from native electrophoresis.

Compds[a]	M (3−)	M (2−)	D (3−)	T (4−)	Q (5−)	Native page
ODN 1				min	1853 (Q-7H + 2K)$^{5-}$ 85%	Q (> 95%)
ODN 4			1532 (D-3H)$^{3-}$ 18%	1724 (T-4H)$^{4-}$ 4%	1847 (Q-6H + K)$^{5-}$ 5%	Q (∼ 30%)
ODN 5	765 (M-3H)$^{3-}$ 18%	1148 (M-2H)$^{2-}$ 100%	1531 (D-3H)$^{3-}$ 15%	1732 (T-5H + K)$^{4-}$ 3%	1853 (Q-7H + 2K)$^{5-}$ 16%	Q (∼ 5%)
ODN 8			1531 (D-3H)$^{3-}$ 30%	1724 (T-4H)$^{4-}$ 10%	1838 (Q-5H)$^{5-}$ 8%	Q min
ODN 16			1544 (D-4H + 4K)$^{3-}$ 15%	1760 (T-8H + 4K)$^{4-}$ 5%	1890 (Q-4H + 6K)$^{5-}$ 3%	M (100%)

[a]For position of modification (c^3z^8dG) in sequence, see Table 1.

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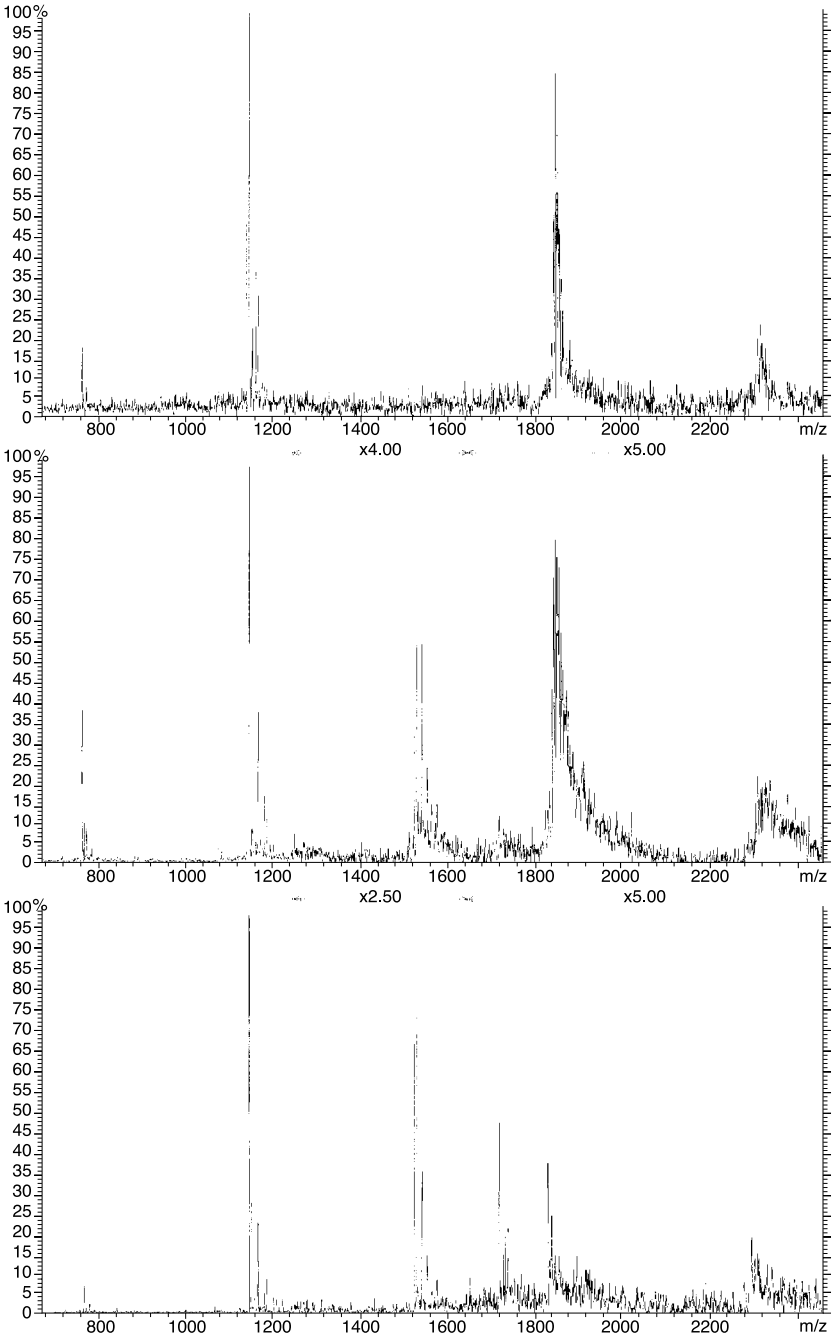

Figure 5. MS spectra of ODN 1, ODN 5, ODN 8.

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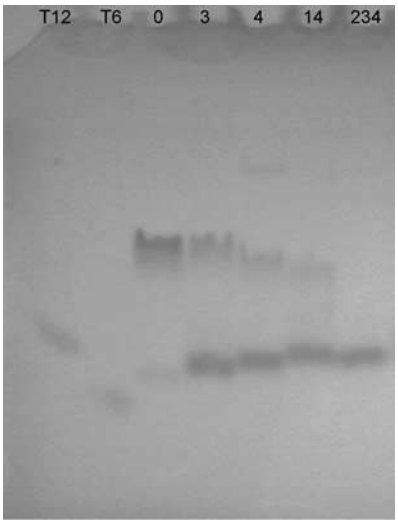

Figure 6. Native electrophoresis of standards T12, T6, ODN 1, ODN 4, ODN 5, ODN 8, ODN 15.

interactions between A and G* may have a slighter effect on the CD spectra of ODN 5, because of the flanking of the terminal base.

Results described above disclose that the replacement of 2′-deoxyguanosine by 8-aza-3-deaza-2′-deoxyguanosine[1] disrupted the anticipated parallel quadruplex structure more efficiently that expected (Figure 6).

EXPERIMENTAL PART

General. NMR spectra were obtained on a Varian (299.97 MHz). ^{1}H NMR spectra were referenced to TMS. 85% Phosphoric acid was used as an external standard for ^{31}P. MS spectra were recorded on a Pneumatic Assistant ESI MS instrument (AutoSpecE). The UV spectra were recorded on BioLambda 40 Perkin Elmer spectrophotometer. Precoated Merck Silica gel 60F 254 plates were used for TLC. Anhydrous solvents were freshly distilled from the appropriate drying agents. All other chemicals were of reagent grade or better quality and were used as received.

Monomers. 5′-O-(3,4-Dibenzyloxybenzyl)thymidine was prepared according to the general procedure from the literature[8] with an additional step needed for separation of 5′-O-(3,4-dibenzyloxybenzyl)-thymidine and 3′-O-(3,4-dibenzyloxybenzyl)-thymidine. Flash chromatography (60 × 130 mm; CH_2Cl_2 : MeOH, 100 : 1 to 40 : 1) afforded 5′-O-(3,4-dibenzyloxybenzyl)-thymidine and 3′-O-(3,4-dibenzyloxybenzyl)-thymidine in a ratio of 2 : 1.

3′-O-(3,4-Dibenzyloxybenzyl)thymidine. ^{1}H NMR ($CDCl_3$) δ (ppm) 8.44 (1 H, s, N-H), H-6 in aromatic proton region, 7.47–7.26, 6.93–6.81 (13 H, m, Ar), 6.09 (1 H, t, J = 7.1 Hz, H_1'), 5.17 (4 H, 2 × s, 2 × CH_2Ph), 4.42 (2 H, 2 × d, J = 11.4 Hz,

$PhCH_2O$), 4.20 (1 H, m, H_3'), 4.07 (1 H, m, H_4'), 3.90–3.83, 3.69–3.62 (2 H, m, H_5', H_5''), 2.36–2.22 (2 H, m, H_1', H_1''), 1.91 (3 H, d, J = 1.2 Hz, CH_3).

5′-*O*-(3,4-Dibenzyloxybenzyl) Thymidine. ^{1}H NMR ($CDCl_3$) δ (ppm) 8.33 (1 H, s, N-H), 7.50 (1 H, d, J = 1.2 Hz, H-6), 7.47–7.28, 6.92–6.80 (13 H, m, Ar), 6.37 (1 H, t, J = 6.8 Hz, H_1'), 5.17 (4 H, s, 2 × CH_2Ph), 4.46 (2 H, 2 × d, J = 4.8 Hz, $PhCH_2O$), 4.38 (1 H, m, H_3'), 4.03 (1H, m, H_4'), 3.72–3.67, 3.62–3.57 (2 H, m, H_5', H_5''), 2.15–2.07 (2 H, m, H_1', H_1''), 1.62 (3 H, d, J = 1.2 Hz, CH_3).

Synthesis, Purification and Characterization of the ODNs 1–16. The synthesis was carried out on an automated Expedite synthesizer in a 1 μmol scale with 3′-phosphoramidites. Longer coupling times were used for incorporation of modified guanosine 1 and 5′-O-(3,4-dibenzyloxybenzyl) thymidine. After cleavage from the solid support, the ODNs were deprotected in concentrated aqueous ammonia at 55°C overnight. The purification of the 5′-O-(3,4-dibenzyloxybenzyl)-oligomers were performed by RP-HPLC at 55°C (pump A: 50 mM triethylammonium acetate + 5% acetonitrile; pump B: acetonitrile + 5% 50 mM treithylammonium acetate; gradient: 95–45% A in 37 min; flow rate 1 ml/min at analytical scale, flow rate 2.5 ml/min at preparative scale). ODNs were desalted, lyophilized on a Speed-Vac evaporator and stored in deep freezer.

Mass Spectrometry. MS spectra were recorded on a Pneumatic Assistant ESI–MS instrument (AutoSpecE). ODN samples were prepapred in 2.5 mM ammonium acetate, 2.5 mM potassium acetate.

CD Spectroscopy. CD spectra were measured on a 62A DS AVIV spectropolarimeter in a 1 mm path length cell. The samples were prepared in 10 mM cacodylate buffer containing 1 M NaCl at the equal ODN concentration. CD spectra were recorded at 25°C from 400 to 200 nm with an average time of 3 sec and were normalized by substraction of the background scan with buffer.

Gel Electrophoresis. Native electrophoresis experiments were run on 20% nondenaturing polyacrylamides gels in 0.6 × TBE buffer with 50 mM of added salt (the same as in ODN solution). ODN solutions were prepared in 50 mM Tris-HCl, 200 mM K^+ (Na^+, Cs^+, Li^+), annealed, mixed with 1 μl of 30% glycerol and kept in refrigerator for at least 24 hours. The gels were run at 4–6°C, at constant voltage (100 V) for 14 hours (until bromophenol blue migrated 14 cm). Gels were visualized at 254 nm and photographed.

Anti-HIV Activity. The in vitro inhibitory effect on HIV-induced cytophathic effect and the cytotoxicity of the ODNs in human lymphocyte MT-4 cells were determined by the MTT assay.[21]

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