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Biologically Active Oligodeoxyribonucleotides—IX.[1] Synthesis and Anti-HIV-1 Activity of Hexadeoxyribonucleotides, TGGGAG, Bearing 3′- and 5′-End-modification

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Abstract—We have determined that hexadeoxyribonucleotides (5′TGGGAG3′), with modified aromatic groups such as a trityl group at the 5′-end, have anti-HIV-1 activity in vitro. The 6-mer bearing a 3,4-dibenzyloxybenzyl (3,4-DBB) group at the 5′-end had the most potent activity and the least cytotoxicity. When the 3′-end of the 5′-(3,4-DBB)-modified 6-mer was substituted with a 2-hydroxyethylphosphate, a 2-hydroxyethylthiophosphate, or a methylphosphate group at the 3′-end, anti-HIV-1 activity increased. Moreover, among various 3′- and 5′-end-modified 6-mers that were tested, the 6-mer (R-95288) bearing a 3,4-DBB group at the 5′-end and a 2-hydroxyethylphosphate group at the 3′-end was the most stable, when incubated with mouse, rat, or human plasma. Therefore, R-95288 was chosen as the best candidate for possible use in therapy on the basis of its anti-HIV-1 activity. © 1997 Elsevier Science Ltd.

Introduction

Oligonucleotides are applicable in therapy as antisense nucleic acids that act against disease-related mRNA, aptamers that bind some proteins such as thrombin, and ribozymes that cleave RNAs.[2,3] At present, almost all oligoribonucleotides for phase study have phosphorothioate bonds, which are resistant to nuclease, substituted for their phosphodiester bonds.

We previously found that a pentadecadeoxyribonucleotide bearing a 4,4′-dimethoxytrityl (DMTr) group at the 5′-end had high anti-HIV activity.[4,5] Furthermore, the chain length of the oligodeoxyribonucleotide (ODN) was shortened[6] and the acid-labile DMTr group was substituted with a variety of aromatic groups, resulting in hexadeoxyribonucleotide **1a**, bearing a 3,4-dibenzyloxybenzyl (3,4-DBB) group at the 5′-end, which had high anti-HIV-1 activity and low cytotoxicity (Fig. 1).[7] The mechanism of action of such ODNs appears to be the inhibition of the adsorption and the entry of HIV-1 to the $CD4^+$ cell.[8] It is thought that these 6-mers act as aptamers.

It was previously reported that guanine-rich ODNs possess anti-HIV activity.[9–11] Wyatt et al. found an anti-HIV-active 8-mer (TTGGGGTT, ISIS-5320) with phosphorothioate modification by means of combinatorial chemistry, and the ternary structure responsible for its activity was a quadruplex that formed with the guanine-quartet (G-quartet).[9] The homoguanylate, whose chain length was four or five, with or without phosphorothioate modification, also had the anti-HIV-1 activity.[10] Ojwang et al. reported that a 17-mer (AR-177), which was composed of thymidine and deoxyguanosine with phosphorothioate bonds at the 3′- and 5′-ends, inhibited HIV integrase activity in HIV-infected cells.[11] These ODNs formed the quadruplex.

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Key words: nuclease-resistance, aptamer, 3,4-dibenzyloxybenzyl, 2-hydroxyethylphosphate, quadruplex.

Abbreviations: CD, circular dichroism; CPG, controlled pore glass; DBB, dibenzyloxybenzyl; DCC, *N,N′*-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMF, *N,N*-dimethylformamide; DMTr, 4,4′-dimethoxytrityl; HIV, human immunodeficiency virus; ib, isobutyryl; MSNT, mesitylenesulfonyl 4-nitrotriazolide; ODN, oligodeoxyribonucleotide; PBS, phosphate buffered saline; TEAA, triethylammonium acetate; TETD, tetraethylthiuram disulfide; THF, tetrahydrofuran; Tr, trityl.

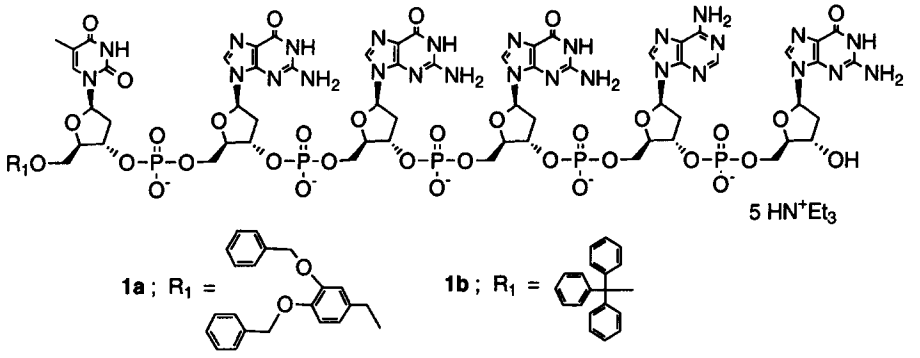


Figure 1. Structures of compounds **1a** and **1b** with anti-HIV-1 activity.

In the research reported here, various modified phosphate groups were introduced into the 5′-end-modified 6-mer (5′TGGGAG3′) at the 3′-end to promote resistance to 3′-exonuclease. These compounds had higher anti-HIV-1 activity and more resistance in plasma than 6-mers without 3′-end-modification. The 6-mer, **14d**, bearing a 3,4-DBB group at the 5′-end and a 2-hydroxyethylphosphate group at the 3′-end (R-95288) was chosen as the best candidate from among the 6-mers tested for its anti-HIV-1 activity.

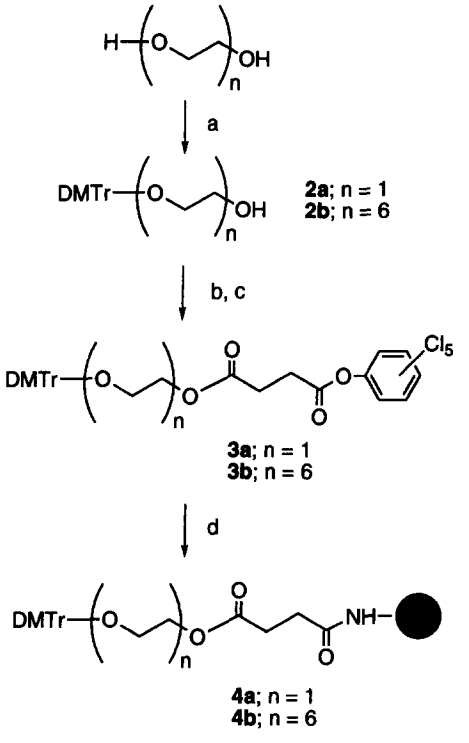
Results and Discussion

Chemistry

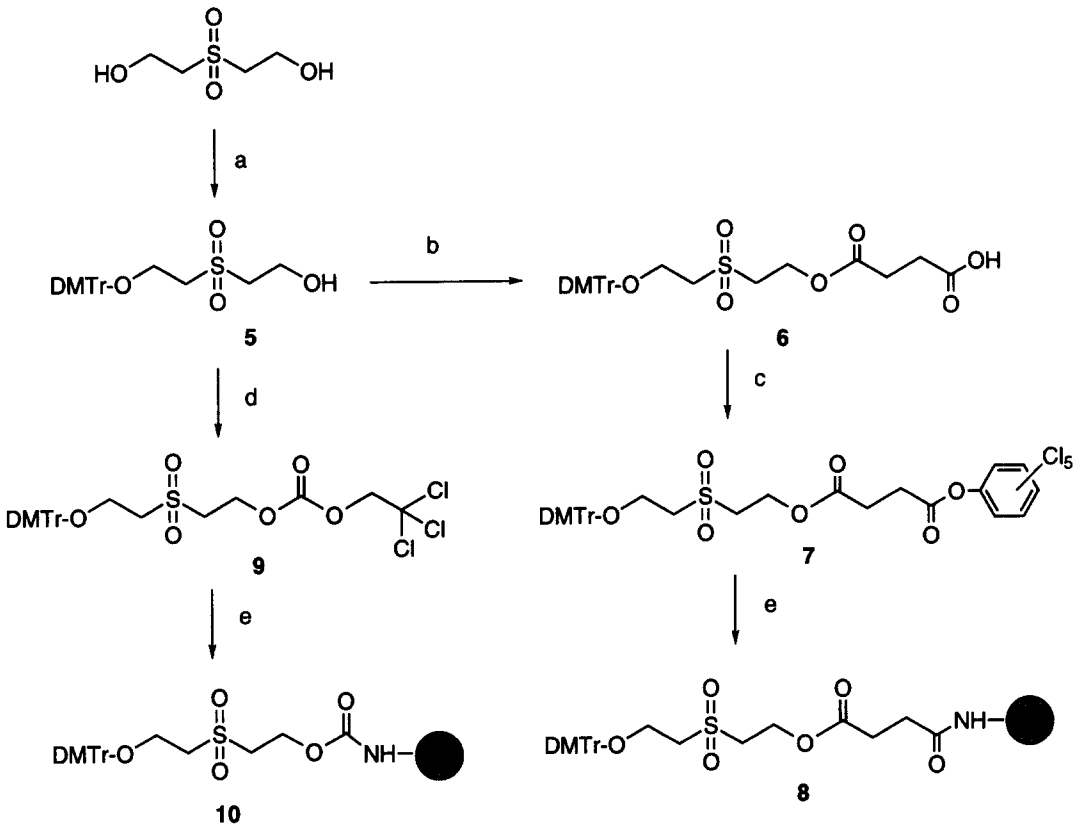
Various 3′- and 5″-end-modified ODNs were synthesized using the solid-phase method. Two types of controlled pore glass (CPG) support were prepared to introduce modified phosphate groups into ODNs at the 3′-end. For one type of support, CPGs **4a** and **4b** had diols via a succinate linker that could be cleaved from the support under alkaline conditions, such as aqueous ammonia (Scheme 1). In the synthesis of CPGs **4a** and **4b**, one alcohol in the diol, such as ethylene glycol or hexa(ethylene glycol), was protected with a DMTr group, to give DMTr-derivatives, **2a** and **2b**,[12] respectively. These compounds were introduced into the succinate linker, then esterification with 2,3,4,5,6-pentachlorophenol and *N,N′*-dicyclohexylcarbodiimide (DCC) was performed, and they were immobilized onto aminopropyl CPG to give CPGs **4a** and **4b**. The other type of support had a hydroxyethylsulfonyl group in which the hydroxy group was protected with a DMTr group (Scheme 2). *O*-(4,4′-Dimethoxytrityl) ethylsulfonylethanol **5**[13] was also immobilized onto the CPG by a procedure similar to synthesis of CPGs **4a** and **4b** to obtain CPG **8**. Compound **5** was reacted with 2,2,2-trichloroethoxycarbonylchloride to give compound **9**, and then compound **9** was directly incorporated into the aminopropyl CPG in the presence of triethylamine to give CPG **10** with a carbamate bond.

Modified nucleotide-bound CPGs **11a** and **11b**, prepared for the synthesis of ODNs having a 2-hydroxyethylphosphate group or a hexa(ethylene glyceryl)-phosphate group at the 3′-end, were coupled with 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine 3′-*O*-β -cyanoethylphosphoramidite[14] in the presence of 1-*H* tetrazole on the detritylated CPGs **4b** and **4a**, respectively, and then oxidized by iodine in H_2O–pyridine (Scheme 3). CPG **11c** was prepared for providing a 2-hydroxyethylthiophosphate group using a similar procedure to the preparation of CPG **11a** except for the sulfurization step with tetraethylthiuram disulfide (TETD).[15]

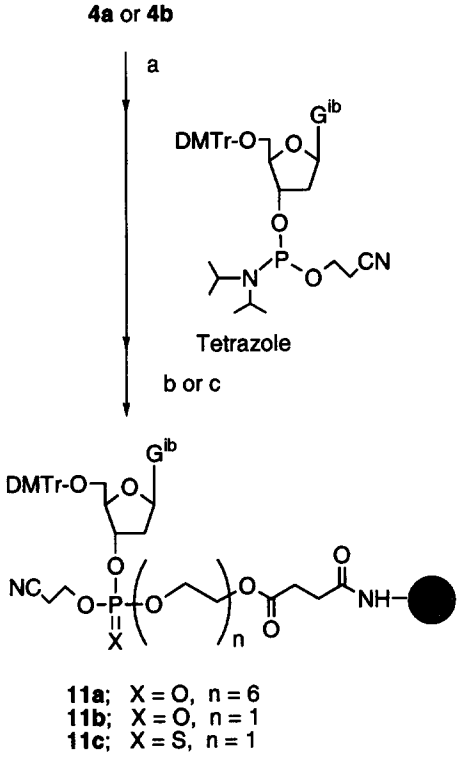
The CPGs **12a–i** for other 3′-modified phosphate groups were prepared from CPG **10** using phosphor-



Scheme 1. Preparation of CPGs with *O*-DMTr-protected diols. (a) DMTrCl, pyridine; (b) succinyl anhydride, DMAP, CH_2Cl_2; (c) 2,3,4,5,6-pentachlorophenol, DCC, DMF; (d) aminopropyl CPG, triethylamine, DMF.



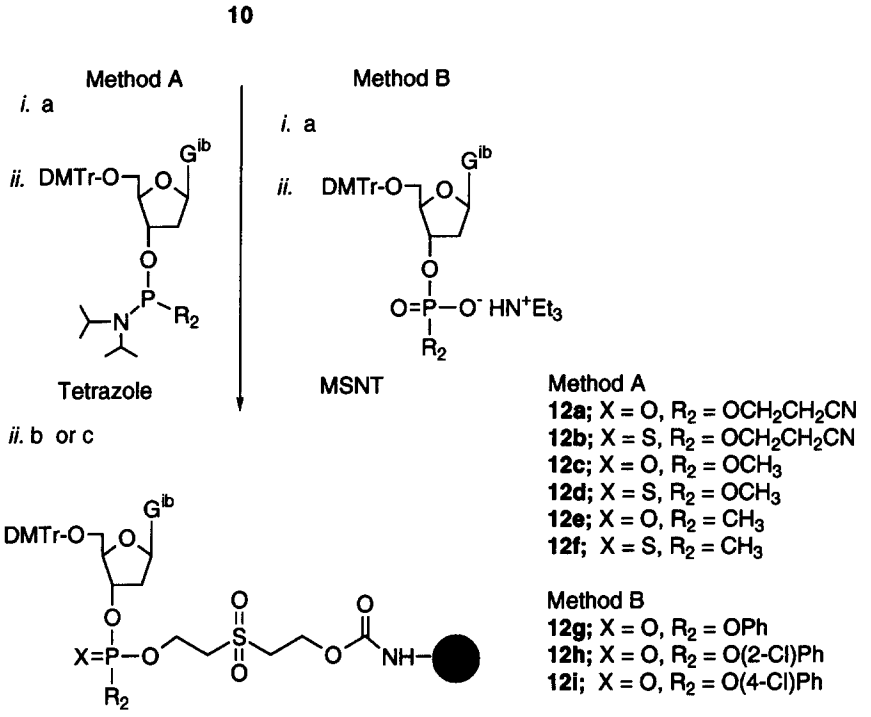
Scheme 2. Preparation of CPGs with *O*-DMTr-protected hydroxyethylsulfonyl groups. (a) DMTrCl, pyridine; (b) succinyl anhydride, DMAP, CH_2Cl_2; (c) 2,3,4,5,6-pentachlorophenol, DCC, DMF; (d) 2,2,2-trichloroethoxycarbonylchloride, pyridine; (e) aminopropyl CPG, triethylamine, DMF.



Scheme 3. Preparation of nucleotide-bound CPGs with diols. (a) dichloroacetic acid, CH_2Cl_2; (b) I_2–H_2O; (c) TETD, CH_3CN.

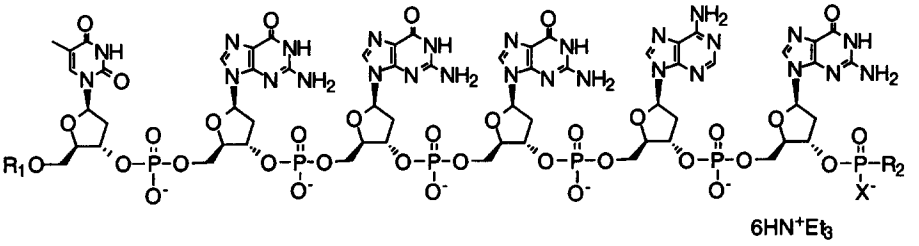
amidite chemistry[14] or phosphotriester chemistry[16] (Scheme 4). Detritylated CPG **10** was reacted with 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine-3′-*O*-β-cyanoethylphosphoramidite,[14] 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine-3′-*O*-methylphosphoramidite[17] or 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine-3′-methylphosphonamidite[18] and then was oxidized or sulfurized to give the CPGs **12a–f** bearing a protected 2′-deoxyguanosine 3′-phosphate, 3′-thiophosphate, 3′-methylphosphate, 3′-methylthiophosphate, 3′-methylphosphonate, or 3′-methylthiophosphonate group (Scheme 4, Method A). 3′-Phenylphosphodiester derivatives of the protected 2′-deoxyguanosine were coupled with detritylated CPG **10** using mesitylenesulfonyl 4-nitrotriazolide (MSNT),[16] to obtain CPGs **12g–i** for the 3′-phenylphosphate analogues (Scheme 4, method B).

The chain elongation of 3′- and 5′-end-modified ODNs was accomplished using CPGs **11a–c** or **12a–i** by the phosphoramidite method[14] (Table 1). 5′-*O*-Trityl or 5′-*O*-(3,4-dibenzyloxybenzyl) thymidine 3′-*O*-β-cyanoethylphosphoramidite[4] was finally coupled to the ODN-linked CPG for 5′-end modification. The CPG was treated with 28% aqueous ammonia solution for cleavage of oligonucleotides from the CPG support and the deprotection of acyl and 2-cyanoethyl groups to give crude ODNs bearing various 3′-end-modified phosphates. The purification of the ODNs was performed by



Scheme 4. Preparation of nucleotide-bound CPGs with sulfonylethoxy groups. (a) Dichloroacetic acid, CH_2Cl_2; (b) I_2–H_2O; (c) TETD, CH_3CN.

Table 1. Structures of 3′- and 5′-end-modified ODNs



Compound	R_1	X	R_2	CPG used[a]	HPLC[b] (min)	MS [M-H]$^-$ [c] Calcd	Found
13a	Tr	O	-$(OCH_2CH_2)_6OH$	**11a**	18.50	2456.58	2456.63
13b	Tr	O	-OPh	**12g**	18.22	2268.46	2268.58
13c	Tr	O	-O(2-Cl)Ph	**12h**	18.38	2302.42	2302.45
13d	Tr	O	-O(4-Cl)Ph	**12i**	18.55	2302.42	2302.50
13e	Tr	O	-OCH_2CH_2OH	**11b**	18.76	2236.45	2236.45
13f	Tr	S	-OCH_2CH_2OH	**11c**	18.92	2252.43	2252.55
13g	Tr	O	-OCH_3	**12c**	17.52	2206.44	2206.39
13h	Tr	S	-OCH_3	**12d**	17.72	2222.42	2222.53
13i	Tr	O	-CH_3	**12e**	18.03	2190.45	2190.34
13j	Tr	S	-CH_3	**12f**	18.07	2206.42	nd
14a	3,4-DBB	O	-$(OCH_2CH_2)_6OH$	**11a**	19.26	2516.60	2516.48
14b	3,4-DBB	O	-OPh	**12g**	19.51	2328.48	2328.43
14c	3,4-DBB	O	-O(2-Cl)Ph	**12h**	19.80	2362.44	2362.50
14d	3,4-DBB	O	-OCH_2CH_2OH	**11b**	19.16	2296.47	2296.30
14e	3,4-DBB	S	-OCH_2CH_2OH	**11c**	19.20	2312.45	2312.41
14f	3,4-DBB	O	-OCH_3	**12c**	19.15	2266.46	2266.28
14g	3,4-DBB	S	-OCH_3	**12d**	19.46	2282.44	2282.27
14h	3,4-DBB	O	-OH	**12a**	19.12	2252.45	2252.42
14i	3,4-DBB	S	-OH	**12b**	19.11	2268.42	2268.35

[a]Structures of the CPGs used are shown in Schemes 3 and 4.
[b]Retention time of the peak observed in HPLC (see conditions in the Experimental section)
[c]Measured mass using negative ion LSI mass spectroscopy.
nd: not determined.

C18 reversed phase column chromatography. Almost all ODNs were purified using a general procedure. However, because purity of ODNs **13g**, **13h**, **14f**, and **14g** bearing a methylphosphate group or a methylthiophosphate group was relatively low, due to demethylation in the ammonia treatment, these ODNs were purified by reverse phase HPLC. The structures of the 3′- and 5′-end-modified ODNs were determined by negative ion LSI mass spectroscopy (Table 1).

Anti-HIV-1 activity of 3′- and 5′-end-modified ODNs

The 50% inhibitory concentration (IC_{50}) for the cytopathic effect of MT-4 cells induced by HIV-1_{IIIB} and the 50% cytotoxic concentration (CC_{50}) of 3′- and 5′-end-modified ODNs were determined by MTT assay in vitro according to a procedure reported previously.[5] The 6-mer having a trityl (Tr) group at the 5′-end without 3′-modification was used as a control (IC_{50} = 2.1 μg/mL and CC_{50} = 61 μg/mL for ODN **1b**).[7] The anti-HIV-1 activity of 5′-*O*-Tr ODNs with a modified phosphate at the 3′-end is shown in Table 2. Though the anti-HIV-1 activity of ODN **13a**, having a hexa(ethylene glyceryl)phosphate group at the 3′-end, was about two-fold lower than that of ODN **1a**, ODN **13a** had no cytotoxicity up to 100 μg/mL. ODNs **13b–d** having phenylphosphate groups had high anti-HIV activity. As for smaller modified groups, when a 2-hydroxyethylphosphate group or a 2-hydroxyethylthiophosphate group were substituted at the 3′-end of ODNs **13e** and **13f**, respectively, they showed higher anti-HIV-1 activity than ODN **1b**. The 6-mers **13g–j** bearing other small modified groups, a methylphosphate, a methylthiophosphate, a methylphosphonate, or a methlythiophosphonate groups, respectively, also had high activity. Unfortunately, the ODNs bearing a Tr group at the 5′-end also had slight cytotoxicity in the range of 50–100 μg/mL.

Table 2. Anti-HIV-1 activity of 3′- and 5′-end-modified ODNs

Compound	IC_{50} (μg/mL)	CC_{50} (μg/mL)
1a	0.30	>100
1b	2.1	61
13a	4.7	>100
13b	0.55	50
13c	0.80	75
13d	0.45	75
13e	0.40	100
13f	0.45	70
13g	0.35	100
13h	0.55	>50
13i	0.50	>50
13j	0.80	60
14a	0.35	>100
14b	0.29	>100
14c	0.50	>100
14d	0.19	>100
14e	0.15	>100
14f	0.15	>100
14g	0.15	>100
14h	0.15	>100
14i	0.25	>100

When the Tr group of the 6-mer at the 5′-end was substituted with a 3,4-DBB group, we reported that this modified group exhibited less cytotoxicity (no cytotoxicity was observed up to 100 μg/mL).[7] Moreover, the IC_{50} value of 5′-*O*-(3,4-DBB)-modified ODN **1a** without 3′-modification, was 0.3 μg/mL.[7] The anti-HIV-1 activity of ODNs **14a–c** having a hexa(ethylene glyceryl)phosphate, 2-chlorophenylphosphate, or phenylphosphate group was not greater than that of ODN **1a**. However, ODNs **14d–i** having other small modifications exhibited about two-fold higher anti-HIV-1 activity than ODN **1a**.

These results suggest that the relationship between the anti-HIV-1 activity of ODNs and the structure of the 3′-end-modification corresponds with the size of the group substituted at the 3′-end. It was found that the relatively small substituted groups (a 2-hydroxyethylphosphate (**14d**), a 2-hydroxyethylthiophosphate (**14e**), a methylphosphate (**14f**), a methylthiophosphate (**14g**), a phosphate (**14h**), and a thiophosphate (**14i**)) were effective as highly active modifications. However, since the methylphosphate group of ODN **14f** and the methylthiophosphate group of ODN **14g** were demethylated under alkaline conditions for deprotection, they might be not suitable for large-scale synthesis for in vivo testing. Furthermore, the phosphate group of ODN **14h** and the thiophosphate group of ODN **14i** may be easily dephosphorylated by phosphatases in vivo. Consequently, we chose the ODNs with a 2-hydroxyethylphosphate or a 2-hydroxyethylthiophosphate modified group for next investigation of their enzymatic stability.

Enzymatic stability of ODNs bearing 3′-end-modified group

Zendegui et al.[19] and Temsamani et al.[20] showed that phosphodiester ODNs bearing a aminopropylphosphate group at the 3′-end were stable in mice. To evaluate the stability of our synthesized ODNs against 3′-exonuclease, ODNs **13e** and **13f** bearing a 2-hydroxyethylphosphate or a 2-hydroxyethylthiophosphate at the 3′-end, respectively, were incubated with snake venom phosphodiesterase that acts as a 3′-exonuclease, and amounts of these ODNs that remained in this reaction were detected by reverse phase HPLC. No short chain length products that were predicted, such as a 5-mer or a 4-mer, were detected (data not shown), perhaps due to rapid digestion of these predicted products by 3′-exonuclease to the mononucleotide. Compared to ODN **1b** without 3′-end-modification, ODNs **13e** and **13f** were very stable against snake venom phosphodiesterase as shown in Figure 2. The results suggest that these modified groups at the 3′-end were resistant to 3′-exonuclease. These modifications may be useful for the designation of antisense oligonucleotides.

Stability of ODNs bearing 3′- and 5′-end-modification in plasma

Besides the stability of the 3′- and 5′-end-modified ODNs against 3′-exonuclease, we investigated their stability in plasma, because a high concentration needs to be maintained in the body for a long time for the anti-HIV therapy. ODNs **14d** and **14e** bearing a 2-hydroxyethylphosphate or a 2-hydroxyethylthiophosphate group at the 3′-end, respectively, were tested for their stability in mouse, rat, and human plasma. ODNs **14d** and **14e** were incubated with plasma for 4 h, then these reaction mixtures were analyzed by reverse phase HPLC to quantify the amount of ODNs remaining (Fig. 3). The 5′-end-modified ODN **1a** without 3′-end-modification was used as a control. ODNs **14d** and **14e** were more stable in the plasma of all species compared to ODN **1a**. Further, we found that ODN **14d** was more stable than ODN **14e**. Therefore, ODN **14d** (which is called R-95288) bearing a 3,4-DBB group at the 5′-end and a 2-hydroxyethylphosphate group at the 3′-end was chosen as the best candidate from among the 6-mers tested for its anti-HIV-1 activity.

Prediction of ternary structure of ODN 14d

The CD spectrum of ODN **14d** (R-95288) is shown in Figure 4a. The CD spectrum of ODN **14d** showed a single maximum at 264 nm that was consistent with the data for the parallel quadruplex.[21] The CD spectrum of ODN **1a** bearing a 3,4-DBB group at the 5′-end was similar to that of ODN **14d** (data not shown). On the other hand, the molecular ellipticity of ODN **14d** at about 260 nm was larger than that of the 6-mer (5′TGGGAG3′) without 3′- and 5′-end-modification having no anti-HIV-1 activity (IC_{50} >100μg/mL) at 20 °C. The CD spectrum of the 6-mer without 3′- and 5′-end-modification measured at 20 °C was similar to those of ODN **14d** at 60, 70, and 80 °C (Fig. 4a, lines e, f, and g, respectively). Moreover, the maxium wave-

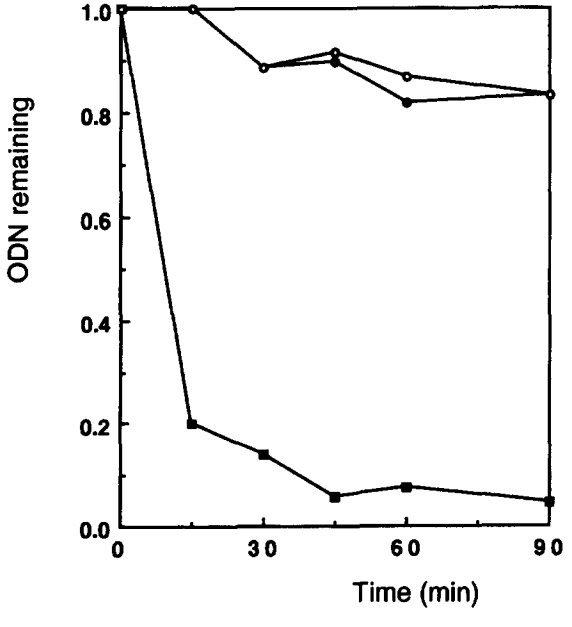


Figure 2. Measurement of the stability of ODNs **1b**, **13e**, and **13f** against snake venom phosphodiesterase. Percentages of ODNs **1b**, **13e** and **13f** remaining are shown as solid squares, open circles, and solid circles, respectively.

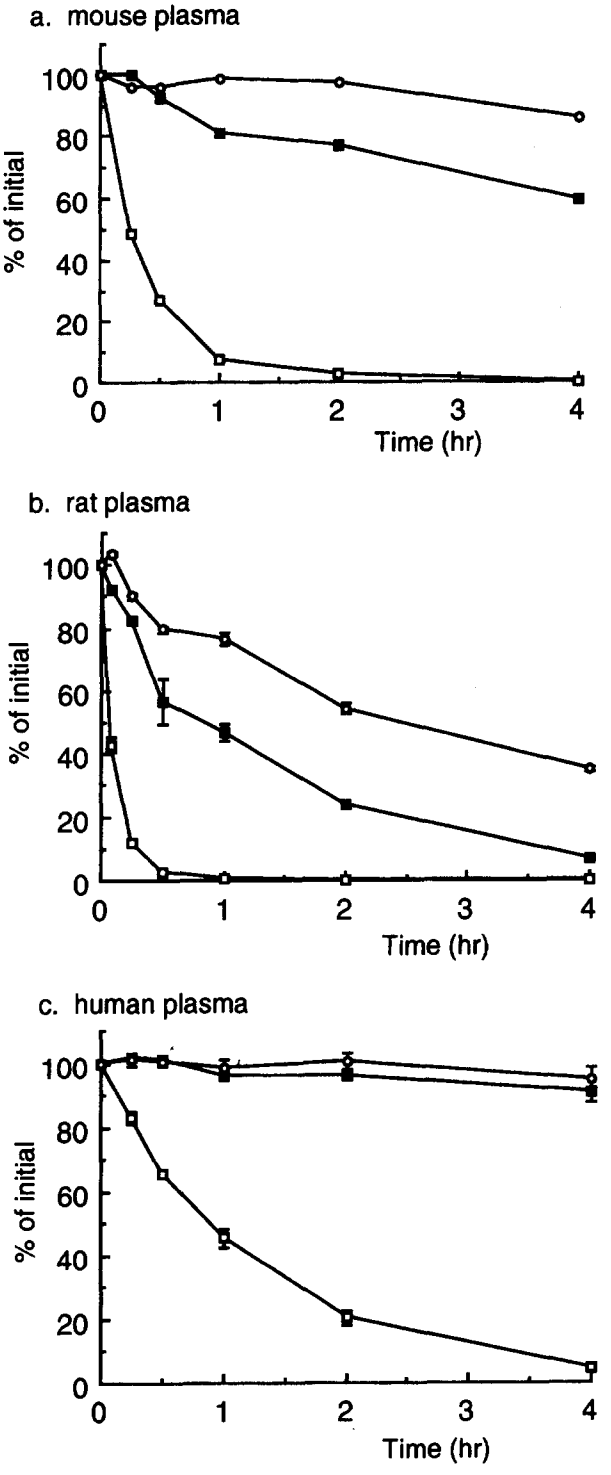


Figure 3. Measurement of the stability of ODNs **1a**, **14d**, and **14e** in (a) mouse, (b) rat, and (c) human plasma. Percentages of ODNs **1a**, **14d**, and **14e** remaining are shown as open squares, open circles, and solid squares, respectively.

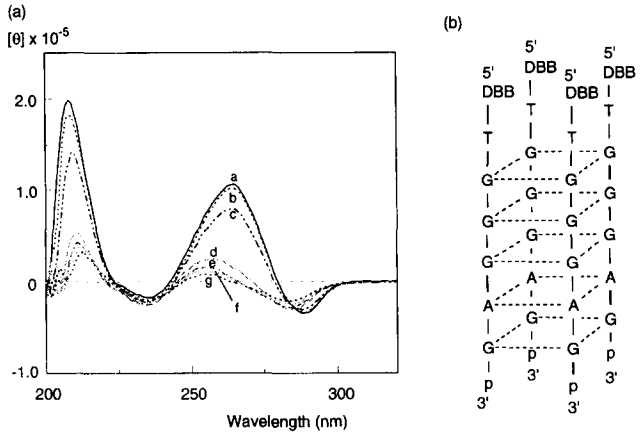


Figure 4. (a) CD spectra of ODN **14d**. The measurement conditions are described in the Experimental section. a: 20 °C, b: 30 °C, c: 40 °C, d: 50 °C, e: 60 °C, f: 70 °C, g: 80 °C. (b) Proposed quadruplex structure of ODN **14d**. DBB: 3,4-dibenzyloxybenzyl group, p: 2-hydroxyethylphosphate group.

length of these CD spectra was at about 255 nm and was blue-shifted about 9 nm compared to that of ODN **14d** at 20 °C. These results show that the quadruplex structure of ODN **14d** is disrupted at high temperatures over 60 °C, and the 3,4-DBB group is important for formation of the ternary structure. It was shown that ODN **14d** might form a quadruplex structure as shown in Figure 4b. Recently, Wolfe and Goodchild showed that covalent attachment of a cholesteryl group to ODNs promoted quadruplex formation.[22] The hydrophobic 3,4-DBB group of ODN **14d** may induce a similar effect on the formation of the quadruplex. A detailed structural analysis of ODN **14d** (R-95288) using NMR spectroscopy will be reported in the near future.

Conclusion

The structure-activity relationship of 3′- and 5′-end-modified 6-mers with a 5′TGGGAG3′ sequence showed that the size of the substituent at the 3′-end of affects the anti-HIV-1 activity. Among these ODNs, the 6-mer (R-95288) bearing a 3,4-DBB group at the 5′-end and a 2-hydroxyethylphosphate group at the 3′-end had the highest anti-HIV-1 activity and the highest stability in mouse, rat and human plasma.

Experimental

General methods

The protected nucleoside 3′-*O*-β-cyanoethylphosphoramidites were obtained from Millipore. Proton magnetic resonance spectra were recorded using a JEOL JNM-EX 270 spectrometer (270 MHz). All chemical shifts are presented in parts per million (ppm) downfield from tetramethylsilane. UV absorption spectra were recorded on a Hitachi U-3210 spectrophotometer. Analytical thin layer chromatography was carried out using Merck Kieselgel 60 F_{254} precoated plates. Column chromatography was performed with Merck Kieselgel 60 (70-230 mesh). HPLC was performed on a Hitachi 655A-11 Liquid Chromatography pump equipped with an L-5000 LC controller, an L-3000 photo diode array detector, and a D-2500 chromato-integrator.

***O*-4,4′-dimethoxytrityl ethylene glycol (2a).** To a stirred solution of 50 mmol of ethylene glycol in 40 mL of dry pyridine was added 3.38 g (10 mmol) of 4,4′-dimethoxytrityl chloride. After stirring for 2 h, the reaction mixture was quenched by addition of 5 mL of methanol and then the mixture was concentrated on a rotary evaporator. The mixture was diluted with CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, and concentrated in vacuo. The crude mixture was separated by silica gel column chromatography (100 g) in 1% methanol/CH_2Cl_2 to give the desired product **2a** (1.97 g, 54%): FAB-MS M^+; 364, ^{1}H-NMR ($CDCl_3$) δ 7.45–6.82 (m, 13H, Ph), 3.79 (s, 6H, CH_3-), 3.75 (t, 2H, -C$\underline{H}_2$OH, J = 4.62Hz), 3.25 (t, 2H, DMTr-O-C$\underline{H}_2$-, J = 4.62Hz), 1.95(t, 1H, OH).

Preparation of modified CPG 4. To a stirred solution of 0.5 mmol of the DMTr derivative **2a** and 4-(dimethylamino)pyridine (92 mg, 0.75 mmol) in 2 mL of CH_2Cl_2 was added succinic anhydride (75 mg, 0.75 mmol). After stirring for 1 h, the mixture was diluted with CH_2Cl_2, washed with 0.5 M KH_2PO_4 (pH 5.0) and water, dried over Na_2SO_4, and concentrated in vacuo to give the monosuccinate. 2,3,4,5,6-Pentachlorophenol (0.16 g, 0.75 mmol) and DCC (0.16 g, 0.75 mmol) were added to the solution of the monosuccinate in 3 mL of DMF. After stirring for 42 h, the insoluble material was removed by filtration. The filtrate was concentrated in vacuo, and benzene was added to the residue. Insoluble material was repeatedly removed to give the desired product **3a**. Compound **3b** was prepared according to a similar procedure to **3a** using **2b**[12] as a starting material.

Aminopropyl CPG (CPG Inc., 129 μmol NH_2 group/gram) was suspended in 4 mL of DMF containing 60 μL of triethylamine, and pentachlorophenyl ester **3a** was added. After standing for 36 h, the CPG support was filtered, washed with pyridine and CH_2Cl_2, and dried in vacuo to give the modified CPG **4a**. DMTr loading levels were determined by acid hydrolysis and measurement of the trityl cation (A_{500}; ε =71700). The loading level was 40 μmol/g. The residual amino groups were capped with acetic anhydride in the presence of 1-methylimidazole in pyridine. **4b**: Loading level was 59.1 μmol/g.

2-[2-*O*-(4,4′-dimethoxytrityloxy)ethylsulfonyl]ethyl monosuccinate (6). To a stirred solution of 3.0 mmol of the DMTr derivative **5**[13] and DMAP (384 mg, 3.15 mmol) in 12 mL of CH_2Cl_2 was added succinic anhydride (315 mg, 3.15 mmol). After stirring for 30 min, the mixture was diluted with CH_2Cl_2, washed with 0.5 M KH_2PO_4 (pH 5.0) and water, dried over Na_2SO_4, and concentrated in vacuo to give the monosuccinate **6** (1.6 g, 96%): ^{1}H-NMR ($CDCl_3$) δ

7.40–6.83 (m, 13H, Ph), 4.58–4.53 (t, 2H, $-SO_2CH_2\underline{CH_2}OCO-$, J = 5.94 Hz), 3.79(s, 6H, CH_3-), 3.68–3.64 (t, 2H, DMTr-O-$CH_2\underline{CH_2}$-, J = 5.61 Hz), 3.50–3.45 (t, 2H, $-SO_2\underline{CH_2}CH_2OCO-$, J = 5.94 Hz), 3.18–3.14 (t, 2H, DMTr-O-$\underline{CH_2}CH_2$, J = 5.61 Hz) 2.71–2.61 (m, 4H, $-OCH_2CH_2O-$).

Preparation of modified CPG 8. Pentachlorophenyl ester **7** was prepared according to a similar procedure to ester **3** using compound **6** as a starting material, and then modified CPG **8** was obtained from ester **7** as described in the procedure for CPG **4**. Loading level was 53.1 μmol/g.

2-[2-*O*-(4,4′-dimethoxytrityloxy)ethylsulfonyl]ethyl 2,2,2-trichloroethoxycarbonate (9). To a stirred solution of 2.4 mmol of DMTr derivative **5**[13] in 12 mL of CH_2Cl_2 was added 2,2,2-trichloroethoxycarbonylchloride (0.35 mL, 2.6 mmol). After stirring for 2 h, the mixture was extracted with 100 mL of CH_2Cl_2 and 100 mL of 5% aqueous $NaHCO_3$. The organic phase was collected, washed with 5% aqueous $NaHCO_3$, and dried over Na_2SO_4. The crude product was purified by reversed phase silica gel column chromatography (Preparative C18, Waters, 2.2 × 7 cm) in CH_3CN–H_2O to give the desired product as a white foam (1.35 g, 89%): FAB-MS M^+; 630, ^{1}H-NMR ($CDCl_3$) δ 7.41–6.83 (m, 13H, Ph), 4.75 (s, 2H, $-CH_2CCl_3$), 4.70–4.66 (t, 2H, $CH_2\underline{CH_2}OCO-$, J = 5.94Hz), 3.80 (s, 6H, CH_3-), 3.68–3.64 (t, 2H, DMTr-O-$CH_2\underline{CH_2}$-, J = 5.28 Hz), 3.61–3.56 (t, 2H, $\underline{CH_2}CH_2OCO-$, J = 5.94 Hz), 3.23–3.19 (t, 2H, DMTr-O-$\underline{CH_2}CH_2$-, J = 5.28 Hz).

Preparation of modified CPG 10. Aminopropyl CPG (CPG Inc., 85.7 μmol NH_2 group/g) was suspended in 5 mL of DMF containing 15 μL of triethylamine, and the trichloroethoxycarbonate **9** was added. After standing for 4 days, CPG support was filtered, washed with pyridine and CH_2Cl_2, and dried in vacuo to give modified CPG **10**. DMTr loading levels were determined by acid hydrolysis and measurement of the trityl cation (A_{500}; ε =71700). The loading level was 24 μmol/g. The residual amino groups were capped with acetic anhydride in the presence of 1-methylimidazole in pyridine.

Preparation of nucleotide-bound CPGs 11a–c. Nucleotide-bound CPG **11a** was synthesized from 5 μmol of CPG **4b** on a Cyclon DNA synthesizer (Milligen). 5′-*O*-(4,4′-Dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine 3′-*O*-β-cyanoethylphosphoramidite (Milligen) in dry CH_3CN (20 mL) was used for coupling in the presence of 1-*H* tetrazole on the detritylated CPG **4b**. The CPG was oxidized by iodine in H_2O-pyridine. CPG **11b** was obtained using a similar procedure. CPG **11c** was also prepared using a similar procedure except for the sulfurization step with TETD (Perkin–Elmer).[15] The coupling yields for these reactions were quantified.

Preparation of nucleotide-bound CPGs 12a–f (method A in Scheme 4). Nucleotide-bound CPGs **12a–f** were also prepared from 5 μmol of CPG **10** on a Cyclon DNA synthesizer using 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine 3′-*O*-β-cyanoethylphosphoramidite in dry CH_3CN, 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine-3′-*O*-methylphosphoramidite (Sigma) in dry CH_3CN or 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine-3′-methylphosphonamidite (American Bionetic Inc.) in dry THF. The coupling yields for these reactions were quantified.

Preparation of nucleotide-bound CPG 12g–i (method B in Scheme 4). CPGs **12g–i** were prepared from 4 μmol of CPG **10** using phosphotriester chemistry.[16] Triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine 3′-*O*-phenylphosphate (20 mg, 22.3 μmol) in dry pyridine (1 mL) was coupled in the presence of MSNT (40 mg, 135 μmol) on detritylated CPG **10** for 30 min at 40 °C to give CPG **12g**. Unreacted hydroxy groups on the CPG were capped with acetic anhydride in the presence of 1-methylimidazole in THF. CPGs **12h** and **12i** were also obtained using triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine 3′-*O*-(2-chlorophenyl)phosphate or triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine 3′-*O*-(4-chlorophenyl)phosphate, respectively.

Oligodeoxyribonucleotide synthesis of 3′- and 5′-end-modified ODNs. 3′- And 5′-end-modified ODNs **13a–j** and **14a–i** were prepared using CPGs **11a–c** or **12a–i** on a Cyclon DNA synthesizer. 5′-*O*-Trityl thymidine 3′-*O*-β-cyanoethylphosphoramidite[4] or 5′-*O*-(3,4-dibenzyloxybenzyl) thymidine 3′-*O*-β-cyanoethylphosphoramidite[4] was finally coupled with ODN-linked CPGs for 5′-end modification. After synthesis, the CPGs were treated with 29% aqueous ammonia at 60°C for 5 hr. The crude products were purified by C18 column chromatography (Preparative C18, Waters, 1.5 × 15 cm, 50 mM triethylammonium bicarbonate (pH 7.5), 20–50% CH_3CN; linear gradient). The purity of the ODNs was analyzed by reverse phase HPLC (Inertsil ODS, GL Science Inc., 6 × 150 mm, 0.1 M triethylammonium acetate (TEAA, pH 7.5), 10–60% CH_3CN (20 min), 1 mL/min, 260 nm).The retention times for the ODNs are shown in Table 1.

Measurement of anti-HIV-1 activity in MT-4 cells. The inhibition of the cytopathic effect (CPE) of HIV-1 by ODNs was assayed using MT-4 cells as described previously.[4] Briefly, MT-4 cells were suspended at 1×10^5 cells/mL and infected with HIV-1 at a multiplicity of infection of 0.01. After exposure to HIV-1 for 1 h at 37 °C, the MT-4 cells were resuspended at 4×10^5 cells/mL in RPMI-1640 medium containing 10% FCS, and then 4×10^4 cells in 0.1 mL were put into each well of a flat-bottomed 96-well culture plate containing 0.1 mL serial two-fold dilutions of each of the 3′- and 5′-end-modified

ODNs. After a 5-day incubation at 37°C, the CPE of HIV-1 was measured using a method based on the conversion of MTT to a reduced form in viable cell. Cytotoxicity of ODNs in MT-4 cells was also assessed by the MTT method. The 50% inhibitory concentrations (IC_{50}) and cytotoxic concentrations (CC_{50}) were defined as the compound concentrations required to reduce by 50% the number of viable cells in the virus- and mock-infected cell cultures, respectively.

Measurement of stability of ODNs 13e and 13f against snake venom phosphodiesterase. To each solution of 10 μg of ODNs **1a**, **13e**, and **13f** in 750 μL of the reaction buffer (50 mM Tris–HCl (pH 8.0) and 10 mM $MgCl_2$) was added 0.1 μg of snake venom phosphodiesterase (Boehringer Mannheim) in 25 μL of H_2O at 37 °C. An amount of 150 μL of the reaction mixture was taken into 50 μL of 50 mM EDTA (pH 8.0) at 15, 30, 45, 60, and 90 min after the initial mixing, washed with 200 μL of $CHCl_3$:phenol (1:1 v/v) twice and with ether three times. The reaction mixture was analyzed by reverse phase HPLC (YMC A-312, YMC Co., Ltd, Japan, 6 × 150 mm, solution A; 0.1 M TEAA (pH 7.5), solution B; 0.1 M TEAA (pH 7.5), 60% CH_3CN, B%; 0–5% (10 min), 5–100% (20 min), 1 mL/min, 260 nm). 5′-dGMP and 5′-dAMP were detected as cleaved products at 8.9 and 17.6 min, respectively. The ratio of the remaining 6-mer to the initial was determined from the peak areas of the remaining 6-mer and the initial.

Measurement of stability of ODNs 14d and 14e in plasma. To each solution of 30 μg of ODNs **1a**, **14d**, and **14e** in 100 μL of PBS buffer was added 1.4 mL of mouse, rat, or human plasma (final concentration; 93.3% plasma) at 37°C. 100 mL of the reaction mixture was taken into 100 μL of lysis buffer (Perkin–Elmer) at 0, 0.25, 0.5, 1, 2, and 4 h after the initial mixing, and in the case of rat plasma, an additional sampling was performed at 5 min after mixing. To the sampling mixture was added 70 μL of PBS buffer (pH 7.4), 10 μL of Tris–HCl (pH 8), 10 μL of 25 mg/mL proteinase K (Perkin–Elmer), and ODN **14c** as an internal standard at 37°C. After 30 min, the reaction mixture was washed with 300 mL of phenol:$CHCl_3$: isoamylalcohol (25:24:1 v/v/v) and with 300 μL of $CHCl_3$. The reaction mixture was analyzed by reverse phase HPLC (Wakopak WS-DNA, Wako Co. Ltd, Japan, 4.6 × 150 mm, 0.1 M TEAA (pH 7.0): CH_3CN=74.5:25.5 (v/v), 1 mL/min, 260 nm). The amount of remaining 6-mer (retention time of ODN **1a**; 12.1 min, ODN **14d**; 11.6 min, ODN **14e**; 12.2 min) was calculated from the peak area of the internal standard (retention time of ODN **14c**; 15.1 min).

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Measurement of CD spectra of ODN 14d. CD spectra were recorded with a JASCO J-500C Spectropolarimeter. ODN **14d** (1 A_{260} unit/mL) was dissolved in PBS buffer.

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