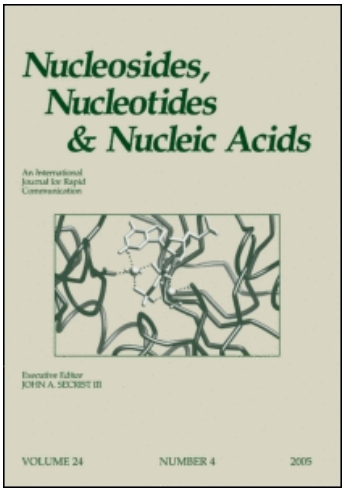

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Biologically Active Oligodeoxyribonucleotides. X. Anti-HIV-1 Activity and Stability of Modified Hexanucleotides Containing Glycerol-Skeleton

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BIOLOGICALLY ACTIVE OLIGODEOXYRIBONUCLEOTIDES. 10[1]: ANTI-HIV-1 ACTIVITY AND STABILITY OF MODIFIED HEXANUCLEOTIDES CONTAINING GLYCEROL-SKELETON§

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ABSTRACT: Deoxyribose-moieties of modified hexadeoxyribonucleotide **1**, which exhibits anti-HIV-1 activity, were partially replaced with glycerol-moieties. Compound **7** with two glycerylguanines at its 3'-end showed more potent anti-HIV-1 activity and more stability against digestion by nucleases than the parent compound **1**.

INTRODUCTION

Acyclic nucleosides are a type of sugar-modified nucleoside analogue. Oligonucleotides consisting of acyclic nucleotides[2] or carbocyclic nucleotides[3] are known to be resistant to digestion by nucleases.

We previously reported that the hexadeoxyribonucleotide, d(TGGGAG), substituted with a 3,4-di(benzyloxy)benzyl group at its 5'-end (**1**) interfered with both viral adsorption to the cell membrane and cell fusion (syncytium formation) by inhibiting the interaction between CD4 and the V3 domain of viral gp120, thus exhibiting potent anti-HIV-1 activity *in vitro*.[4-6] It was found that **1** formed a parallel

§This paper is dedicated to the memory of the late Professor Tsujiaki Hata, a former Professor Emeritus of the Tokyo Institute of Technology.

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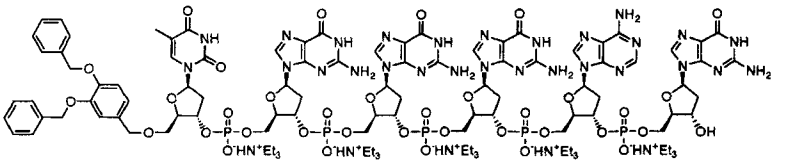
quadruplex and this might be the active species.[5] To obtain nuclease-resistant analogues, deoxyribose-moieties of **1** were partially replaced with acyclic glycerol-moieties. This paper describes the synthesis, anti-HIV-1 activity and stability of these modified oligonucleotides containing glycerol-skeleton.



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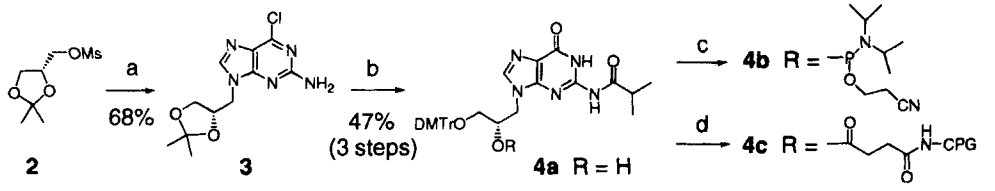
RESULTS AND DISCUSSION

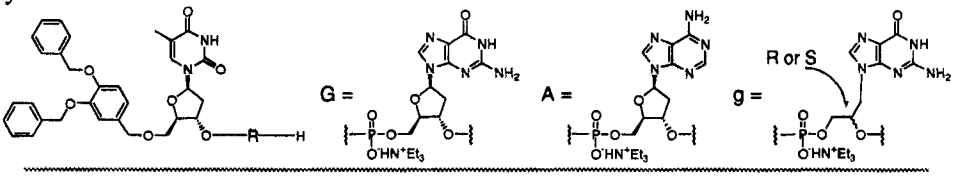
Glycerylguanine was previously synthesized by Holy's group,[7] however, the yield was not sufficient. Thus, the (S)-glycerylguanine unit (**4b**) was synthesized as shown in SCHEME 1. Mesylate **2** was condensed with 2-amino-6-chloropurine to give **3**.[8] Acidic hydroxylation of **3** followed by the protection of the amino group and the primary hydroxyl group led to the partially protected glycerylguanine **4a**. Compound **4a** was phosphitylated to give the amidite unit **4b**. Compound **4a** was also loaded onto controlled pore glass (CPG) via a succinate linker by the conventional procedure[9] to give **4c**. The antipodes (R-isomers) of **4b** and **4c** were prepared from the antipode of **2** using the same procedure shown in SCHEME 1. Compounds **4b**, **4c** and their antipodes were applied to an automatic DNA synthesizer to obtain the modified oligonucleotides with a glycerol-skeleton listed in TABLE 1. The structures of all compounds listed in TABLE 1 were confirmed by means of negative ion LSI mass spectra.

The 3'-end-substitution of **1** with a 2-hydroxyethylphosphoryl group has already been found to be effective in terms of both stability and activity.[1] Since natural-type oligonucleotides are digested mostly from the 3'-end,[10] the replacement of the sugar-moieties of **1** from the 3'-end towards the 5'-direction would be a logical route of investigation to pursue.



SCHEME 1. Reagents and conditions: (a) 2-amino-6-chloropurine, K_2CO_3, DMF, 90 °C; (b) (i) 2N aq.HCl, reflux, (ii) TMSCl, pyridine, (iii) $(^iPrCO)_2O$, (iv) NH_4OH, (v) DMTrCl; (c) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite, diisopropylamine-tetrazole, CH_2Cl_2; (d) (i) succinic anhydride, DMAP, pyridine, 73%, (ii) pentachlorophenol, DCC, pyridine, THF, (iii) aminopropyl CPG, Et_3N, DMF.

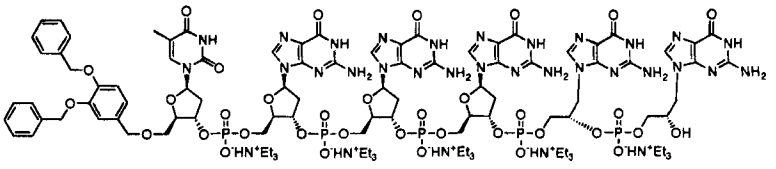
TABLE 1. *In vitro* anti-HIV-1 activity for modified oligonucleotides containing glycerol-skeleton.[a]



compd.	sequence of R	configuration of g	IC_{50} (μg/ml)[b]	IC_{90} (μg/ml)[b]	CC_{50} (μg/ml)[c]
1	GGGAG	--	1.6	4.7	>50
5	GGGAGg	S	5.3	10	>50
6	GGGAg	S	1.2	3.1	>50
7	GGGgg	S	1.0	1.9	>50
8	GGggg	S	37.5	50	>50
9	Ggggg	S	9.1	12	>50
10	ggggg	S	36	50	>50
11	GGGAGg	R	3.8	6.0	>50
12	GGGAg	R	2.0	2.8	>50
13	GGGgg	R	4.3	10	>50
14	GGggg	R	13.5	24	>50
15	Ggggg	R	4.3	14	>50
16	ggggg	R	49	>50	>50

[a]All entries are the average of two determinants. [b]Both the IC_{50} and the IC_{90} values for the inhibition of HIV-1_{IIIB}-induced cytopathicities were determined *in vitro* by MTT assay using MT-4 cells. [c]The CC_{50} values were also determined by MTT assay using MT-4 cells.

Anti-HIV-1 activity was estimated *in vitro* using HIV-1_{IIIB} and MT-4 cells. As shown in TABLE 1, there were no significant differences between modified oligonucleotides containing (S)-glycerylguanines (**5~10**) and those containing (R)-glycerylguanines (**11~16**). No candidates showed any cytotoxicity up to 50 μg/ml. Among these, compound **7** containing two (S)-glycerylguanines possessed the most potent anti-HIV-1 activity in terms of both IC_{50} and IC_{90} values. Modified



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oligonucleotides containing more than three glycerylguanines were less potent than the rest of the candidates.

The stability of modified oligonucleotides in 93% serum of rat, mouse and human was estimated by means of HPLC (FIG. 1). The half-lives ($T_{1/2}$) of **1** in 93% serum of rat, mouse and human were ca. 5 min, 15 min and 1 h, respectively. The replacement of sugar-moieties by glycerol-moieties strongly stabilized the oligonucleotides in the serum. In the case of human serum, oligonucleotides with more than one glycerylguanine (**6**, **7**, **10**, **12**, **13** and **16**) remained more than 90% intact after 4 hours (FIG. 1E, F). There was no notable difference in stability between the S-series (**6**, **7** and **10**) and the R-series (**12**, **13** and **16**).

The circular dichroism (CD) spectra for compounds **6**~**10** and **12**~**16** were measured at ca. 1 A_{260} units/ml in 10 mM PBS buffer (pH 7) at ambient temperature (FIG. 2). In the previous study, we found that compound **1** formed a G-quadruplex structure, that is, a helical structure consisting of four strands, stabilized by the formation of G-quartets.[5] As shown in FIG. 2A, compounds **6** and **12** have a positive Cotton effect at 262 and 264 nm, which is typical of CD spectra of parallel quadruplexes with guanine-rich sequences.[11] Therefore, compounds **6** and **12** formed G-quadruplexes like **1**. In the case of other oligonucleotides with more than two glycerylguanines, the CD spectra were far different from those of typical G-quadruplexes with a natural-type deoxyribose-backbone (FIG. 2B~E). The CD spectra of compounds **10** and **16**, in which five of the six deoxyribose-moieties were replaced by glycerol-moieties, were almost symmetric to each other (FIG. 2E).

CONCLUSION

Twelve new oligonucleotides containing a partial glycerol-skeleton were prepared and tested for anti-HIV-1 activity *in vitro*. Compound **7** with two (S)-glycerylguanines was found to be the most potent of all tested. Recently, Wilson-Lingardo *et al.* used a phosphodiester-linked glycerol backbone for combinatorial libraries for drug discovery.[12] The glycerol-skeleton used in our present study proved to be effective for resistance against digestion by nucleases. In conclusion, compound **7** was found to be more potent and more stable against digestion by nucleases than the parent compound **1**.

EXPERIMENTAL

^{1}H-NMR spectra were recorded on a JEOL JNM-EX 270 Spectrometer (270 MHz) with tetramethylsilane as an internal standard. UV absorption spectra were recorded on a HITACHI U-3210 Spectrophotometer. CD spectra were recorded on a JASCO J-500C Spectrometer. Negative ion LSI mass spectra were recorded on a VG 70-4SE using 3-nitrobenzyl alcohol as a matrix. TLC was done on Merck Kieselgel $60F_{254}$ precoated plates. Column chromatography was performed with Merck Kieselgel 60 (70-230 mesh). HPLC was performed on a HITACHI 655A-11 Liquid Chromatograph equipped with L-5000 LC Controller, L-3000 Photo Diode Array Detector, and D-2500 Chromato-Integrator.

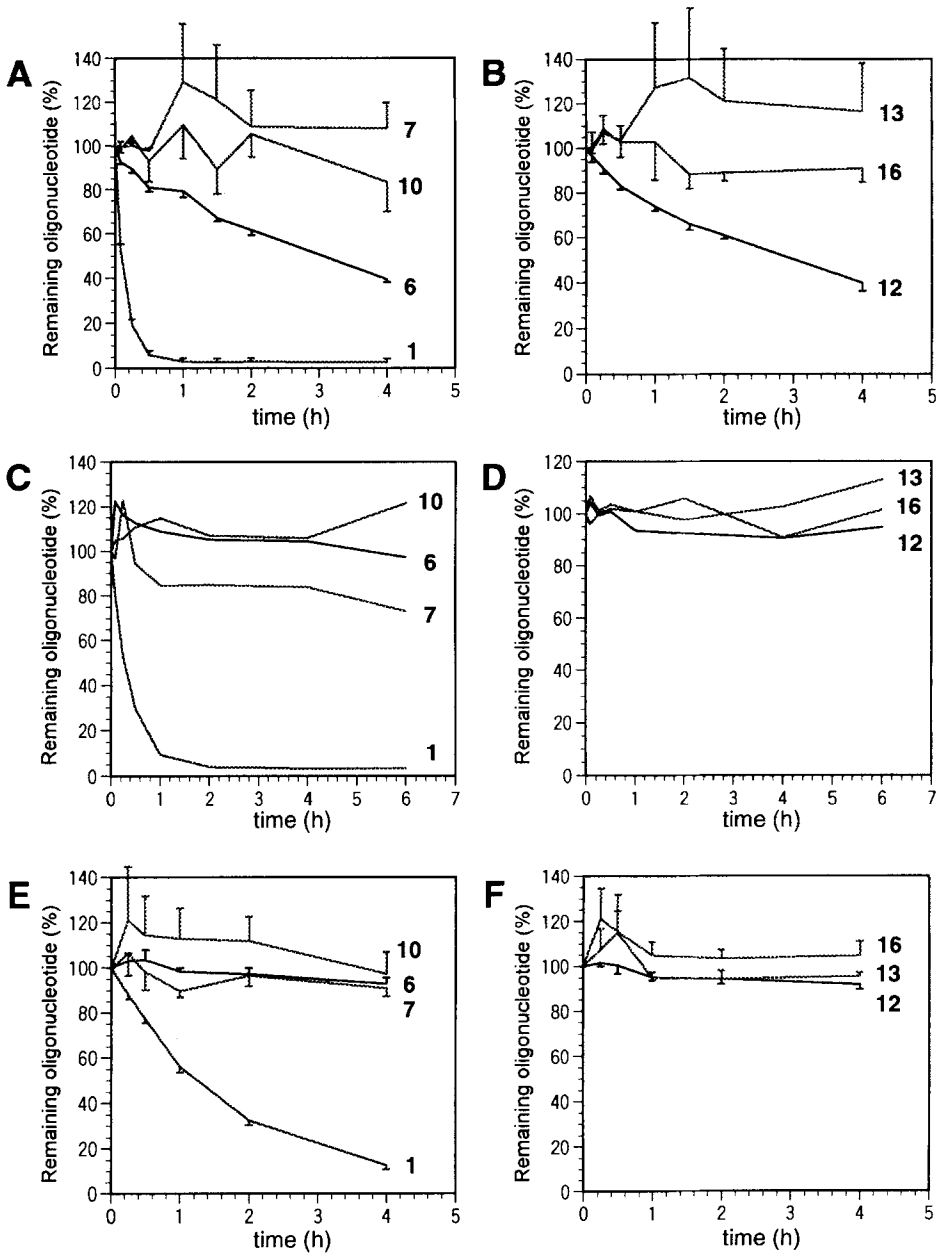

FIG. 1. The stability of modified oligonucleotides in 93% serum of rat (**A**, **B**; n = 3), mouse (**C**, **D**; n = 1) and human (**E**, **F**; n = 3). These values determined by means of HPLC indicate the percentages of remaining samples after incubation of 20 μg/ml of each sample in the presence of 93% serum.

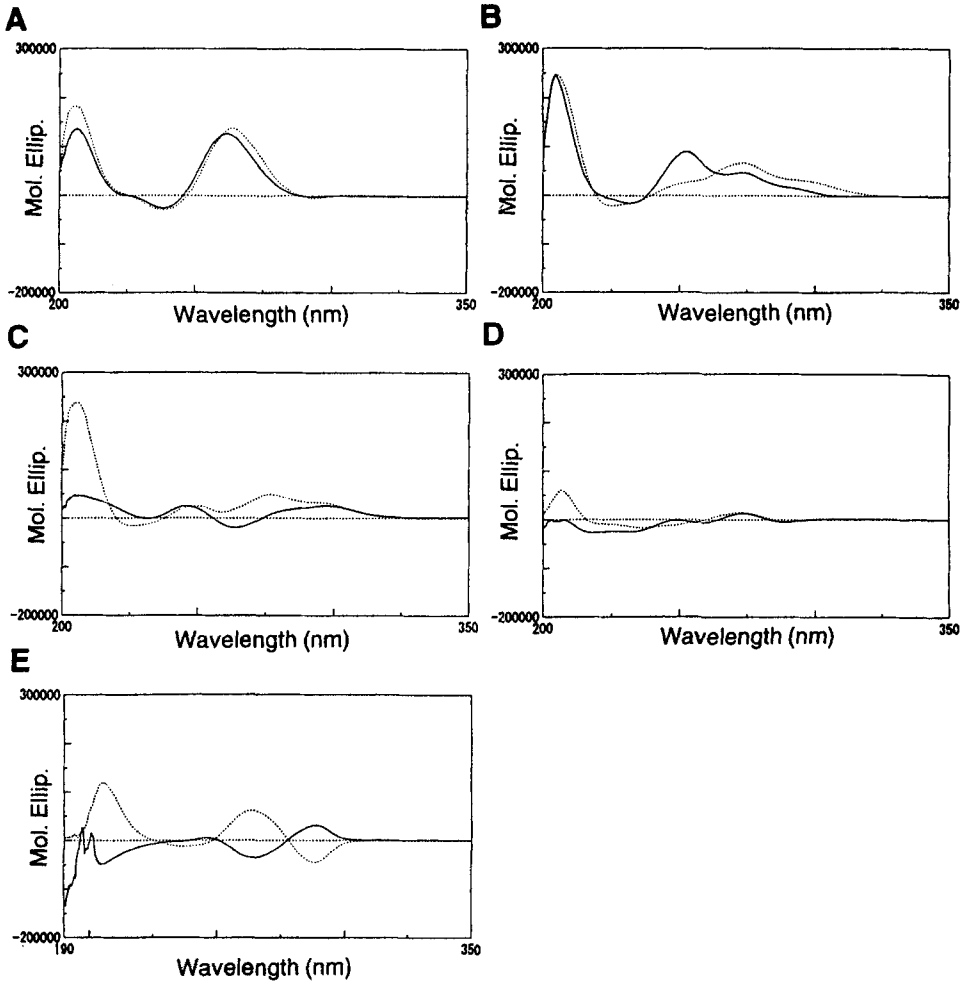

FIG. 2. CD spectra for oligonucleotides having 3,4-di(benzyloxy)benzyl groups at their 5'-ends with sequences, TGGGAg (**A**; *6* and **12**), TGGGgg (**B**; *7* and **13**), TGGggg (**C**; *8* and **14**), TGgggg (**D**; **9** and **15**) and Tggggg (**E**; **10** and **16**). g: See structure of TABLE 1. Solid line: S-isomer; dotted line: R-isomer. Sample ca. 1 A_{260} units/ml in 10 mM PBS buffer (pH 7) at ambient temperature.

(*S*)-2-Amino-6-chloro-9-(2,2-dimethyl-1,3-dioxolan-4-ylmethyl)purine (3). A mixture of (R)-4-methylsulfonyloxymethyl-2,2-dimethyl-1,3-dioxolane (**2**, 1.05 g, 5 mmol), 2-amino-6-chloropurine (1.06 g, 6.25 mmol), K_2CO_3 (888 mg, 6.43 mmol), and DMF (90 ml) was stirred at 90°C. After 17 h, the solvent was removed by evaporation. The residue was dissolved in EtOAc (100 ml), washed with 5% aq. $NaHCO_3$, and the washing was extracted with EtOAc (2 x 100 ml). The organic layers were combined, dried over $MgSO_4$ and evaporated to dryness. The residue was eluted with 4% MeOH-

CH_2Cl_2 on a silica gel column (100 g) to give **3** (962 mg, 68%) as a white amorphous solid. $[\alpha]_D$ -8.95° (c 1.17, $CHCl_3$). UV (MeOH): 223 (22300), 248 (4800), 310 (6000). ^{1}H-NMR (DMSO-d_6) δ: 8.08 (s, 1H, H-8), 6.92 (s, 2H, NH_2), 4.52-4.43 (m, 1H, H-2'), 4.20-4.10 (m, 2H, H-1'), 4.01 (dd, J = 6.6 and 8.6 Hz, 1H, H-3'a), 3.77 (dd, J = 5.3 and 8.6 Hz, 1H, H-3'b), 1.29 (s, 3H, CH_3), 1.24 (s, 3H, CH_3). IR (KBr): 3430, 3312, 3186, 2984, 2941, 2860, 1642, 1616, 1560, 1517, 1429. FAB-MS: m/z 283 $(M+H)^+$.

(*S*)-N^2-Isobutyryl-N^9-(2-hydroxy-3-(4,4'-dimethoxytrityloxy)propyl) guanine (4a). Compound **3** (925 mg, 3.26 mmol) was dissolved in 2N aq. HCl (3.3 ml) and the solution was stirred under reflux. After 75 min, the mixture was cooled in an ice-water bath, neutralized with 2.5N aq. NaOH (ca. 2.6 ml), then evaporated. The residue was dried by repeated coevaporation with pyridine, then suspended in pyridine (33 ml). TMSCl (2.1 ml, 16.3 mmol) was added to the mixture, which was then stirred in an ice-water bath under N_2 atmosphere. After 30 min, isobutyryl anhydride (2.8 ml, 16.3 mmol) was added and the total mixture was stirred at rt. After 140 min, the mixture was cooled in an ice-water bath, then H_2O (6.5 ml) was added. After 15 min, 28% aq. NH_4OH (6.5 ml) was added and stirred for 1 h. The solvent was removed by evaporation and the residue was dried by repeated coevaporation with pyridine. The residue was dissolved in pyridine (33 ml), DMTrCl (1.47 g, 3.77 mmol) was added, and the mixture was stirred at rt for 5 h. The mixture was diluted with CH_2Cl_2 (200 ml), washed with 5% aq. $NaHCO_3$, then dried over $MgSO_4$. The solvent was removed by evaporation and the residue was eluted with 4% MeOH-CH_2Cl_2 on a silica gel column (100 g) to give **4a** (925 mg, 47%) as a colorless foam. ^{1}H-NMR ($CDCl_3$) δ: 11.81 (s, 1H, N*H*), 8.55 (s, 1H, N*H*), 7.54 (s, 1H, H-8), 7.47-6.81 (m, 13H, Ar-*H*), 4.90 (br s, 1H, O*H*), 4.40-3.98 (m, 3H, H-1' and H-2'), 3.79 (s, 6H, CH_3O), 3.35-3.20 (m, 2H, H-3'), 2.70-2.55 (m, 1H, C*H*$(CH_3)_2$), 1.27-1.22 (m, 6H, CH(CH_3)$_2$).

(*S*)-1-(N^2-Isobutyrylguanin-9-yl)-3-*O*-(4,4'-dimethoxytrityl)-propanediol-2-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (4b). Compound **4a** (690 mg, 1.15 mmol) was dried by repeated coevaporation with pyridine, then dissolved in CH_2Cl_2 (6 ml). Diisopropylamine-tetrazole (99 mg, 0.557 mmol) and 2-cyanoethyl *N*,*N*,*N*',*N*'-tetraisopropylphosphorodiamidite (404 μl, 1.27 mmol) were added and the mixture was stirred at rt. After 7.5 h, 2-cyanoethyl *N*,*N*,*N*',*N*'-tetraisopropylphosphorodiamidite (100 μl, 0.314 mmol) was added and stirring was continued for 14 h. The solvent was removed by evaporation, the residue was dissolved in EtOAc (50 ml), washed with ice-cold 10% aq. Na_2CO_3 (2 x 50 ml), washed with sat. aq. NaCl (50 ml), then dried over Na_2SO_4. The solvent was removed by evaporation and the residue was eluted with EtOAc on a silica gel column (40 g) to give **4b** (806 mg, 88%) as an oil. This material was a mixture of diastereomers. ^{1}H-NMR ($CDCl_3$) δ: 11.90, 11.85 (2s, 1H, N*H*), 8.65, 8.31 (2s, 1H, N*H*), 7.54, 7.49 (2s, 1H, H-8), 7.45-6.79 (m, 13H, Ar-*H*), 4.50 (br s, 1H, H-2'), 4.33 (d, J = 5.9 Hz, 2H, H-1'), 3.79 (s, 6H, CH_3O), 3.70-3.40 (m, 4H, POCH_2 and PNC*H*), 3.20-3.00 (m, 2H, H-3'), 2.65-2.55 (m, 1H, COC*H*), 2.53-2.45 (m, 2H, CH_2CN), 1.30-1.10 (m, 18H, COCH(CH_3)$_2$ and NCH(CH_3)$_2$).

(*S*)-1-(N^2-Isobutyrylguanin-9-yl)-3-*O*-(4,4'-dimethoxytrityl)-propanediol-2-*O*-succinate. Compound **4a** (179 mg, 0.3 mmol) was dried by evaporation with pyridine, then dissolved in pyridine (4.5 ml). To the solution were added succinic anhydride (180 mg, 1.8 mmol), DMAP (24 mg, 0.2 mmol) and Et_3N (41 µl, 0.3 mmol), and the total mixture was stirred at rt. After 42 h, the solvent was removed by evaporation and the residue was dissolved in EtOAc (20 ml), washed with 10% aq. citrate (2 x 20 ml), washed with H_2O (20 ml), then dried over $MgSO_4$. The solvent was removed by evaporation and the residue was recrystallized from CH_3CN to give the desired succinate derivative (152 mg, 73%). ^{1}H-NMR ($CDCl_3$) δ: 9.49 (s, 1H, N*H*), 7.56 (s, 1H, H-8), 5.22 (br s, 1H, H-2'), 4.52-4.30 (m, 2H, H-1'), 3.79 (s, 6H, CH_3O), 3.27-2.40 (m, 7H, H-3' and succinate and CH(CH_3)$_2$), 1.17 (d, 6H, J = 7.3 Hz, CH(CH_3)$_2$).

(*S*)-1-(N^2-Isobutyrylguanin-9-yl)-3-*O*-(4,4'-dimethoxytrityl)-propanediol-2-*O*-pentachlorophenylsuccinate. The above succinate derivative (152 mg, 0.217 mmol) was dried by coevaporation with pyridine, then dissolved in THF (2 ml). To the solution were added pentachlorophenol (58 mg, 0.217 mmol), DCC (113 mg, 0.55 mmol) and pyridine (50 µl), and the total mixture was stirred in an ice-water bath for 1 h, then at rt for 16 h. The precipitate was filtered off and the filtrate was evaporated to dryness. The residue was eluted with 3% MeOH-CH_2Cl_2 on a silica gel column (10 g) to give the desired pentachlorophenyl ester (239 mg). This material containing a small amount of dicyclohexyl urea was used without further purification.

Compound 4a Loaded onto CPG (4c). The above pentachlorophenyl ester (239 mg) was dried by repeated coevaporation with pyridine, then dissolved in DMF (5 ml). To the solution were added aminopropyl CPG (CPG INC., AMP00500B, 521 Å, 120/200, 85.7 µmol/g; 1.2 g) and Et_3N (28 µl, 0.2 mmol), and the total mixture was allowed to stand with occasional shaking for 19 h. The solvent was removed by filtration and the CPG was washed adequately with DMF then CH_2Cl_2. The CPG thus obtained was mixed with pyridine (9 ml), acetic anhydride (1 ml) and DMAP (122 ml), and the total mixture was allowed to stand with occasional shaking for 1 h. The solvent was removed by filtration and the CPG was washed adequately with pyridine and CH_2Cl_2, then dried to give **4c** (46.7 µmol/g of **4a** was loaded onto CPG).

Synthesis of Oligonucleotides Containing Glycerol-skeleton. The antipodes (R-isomers) of **4b** and **4c** were also prepared in the same procedure described above starting from the antipode of **2**. Compounds **4b**, **4c** and their antipodes were applied to an automatic DNA synthesizer according to the previous method to give the oligonucleotides containing a glycerol-skeleton. The yields of compounds **5~16** are listed in TABLE 2. The structures were confirmed by means of negative ion LSI mass spectra. The purity was assessed by means of reverse phase HPLC.

Inhibition of HIV-1_{IIIB}-induced Cytopathicity. The measurement of anti-HIV-1 activity in MT-4 cells was performed as described previously.[13] Briefly, exponentially growing MT-4 cells were centrifuged for 5 min at 140 x g. The cell pellet

TABLE 2. Yields, negative ion LSI-MS and HPLC analyses for compounds **5~16**.

compds	Yield[a] A_{260} units	formula	Negative ion LSI-MS [M-H]⁻ calcd	found	HPLC[b] conditions[c]	Rt (min)
5	85.5	$C_{89}H_{102}N_{32}O_{41}P_6$	2459.523	2459.910	A	13.1
6	124.8	$C_{79}H_{90}N_{27}O_{35}P_5$	2130.470	2130.413	A	13.4
7	48.6	$C_{77}H_{88}N_{27}O_{35}P_5$	2104.455	2104.164	A	13.3
8	37.3	$C_{75}H_{86}N_{27}O_{34}P_5$	2062.444	2062.035	A	13.2
9	107.7	$C_{73}H_{84}N_{27}O_{33}P_5$	2020.433	2019.994	A	13.3
10	54.9	$C_{71}H_{82}N_{27}O_{32}P_5$	1978.423	1977.883	A	14.5
11	64.4	$C_{89}H_{102}N_{32}O_{41}P_6$	2459.523	2460.112	B	13.0
12	68.6	$C_{79}H_{90}N_{27}O_{35}P_5$	2130.470	2130.413	B	13.4
13	54.8	$C_{77}H_{88}N_{27}O_{35}P_5$	2104.455	2104.164	B	13.3
14	48.1	$C_{75}H_{86}N_{27}O_{34}P_5$	2062.444	2062.035	B	13.3
15	46.8	$C_{73}H_{84}N_{27}O_{33}P_5$	2020.433	2019.994	B	13.4
16	83.1	$C_{71}H_{82}N_{27}O_{32}P_5$	1978.423	1977.883	B	14.3

[a]Syntheses were performed on a 5 μmol scale. [b]All compounds proved to be more than 95% pure calculated by A_{260} area. [c]Conditions: 20→50% CH_3CN-0.1 M TEAA (pH 7) / 30 min (linear gradient), 2 ml/min, 60 °C; A: YMC-Pack A-312, S-5, 120 Å, ODS; B: Inertsil ODS-2.

was suspended in a small quantity of RPMI-1640 medium and infected with 100 $TCID_{50}$ HIV-1 for 1 h. The cells were then washed with RPMI-1640 medium, resuspended in the same medium, and distributed in 96-well plates containing serial dilutions of modified oligonucleotides. Each well contained 2.5 x 10^4 cells in 200 μl medium. After day 6, cell viability was assessed by the MTT method. The concentrations of the compounds giving 50% inhibition of HIV-induced cytopathic effect (IC_{50}) were determined from the dose-response curves.

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