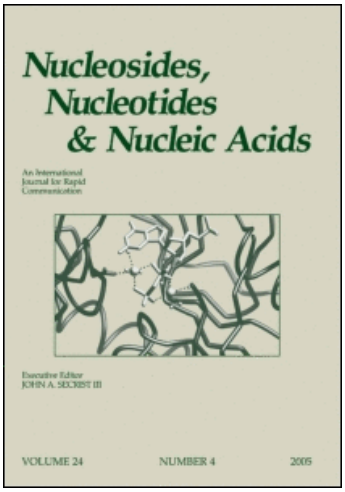

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Biologically Active Oligodeoxyribonucleotides - II[1]: Structure Activity Relationships of Anti-HIV-1 Pentadecadeoxyribonucleotides Bearing 5'-End-Modifications

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BIOLOGICALLY ACTIVE OLIGODEOXYRIBONUCLEOTIDES - II[1] : STRUCTURE ACTIVITY RELATIONSHIPS OF ANTI-HIV-1 PENTADECADEOXYRIBONUCLEOTIDES BEARING 5'-END-MODIFICATIONS[§]

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Abstract: 5'-End-modified pentadecadeoxyribonucleotides (15mers) with a sequence complementary to the *tat* 2nd splicing acceptor region of human immunodeficiency virus type 1 (HIV-1) were prepared and evaluated for anti-HIV-1 activity. The structures of modified 15mers were confirmed by negative ion LSI mass spectroscopy, and the anti-HIV-1 activities were evaluated *in vitro* by MTT assay using MT-4 cells. While the unmodified 15mer had no activity in our assay system, the 15mers bearing modifications with trityl-type substituents at the 5'-end showed potent anti-HIV-1 activities.

INTRODUCTION

Antisense oligodeoxyribonucleotides (AS-ODNs) have the ability to selectively inhibit the expression of deleterious genes, such as those involved in infectious diseases and cancer.[2] In particular, human immunodeficiency virus type 1 (HIV-1) has been one of the most frequently studied targets of AS-ODNs.[3] Recently, AS-ODNs bearing phosphorothioate-type internucleotidic phosphodiester linkages have been extensively studied[4~7] because of their resistance to degradation by nucleases.[8,9] On the other hand, modifications at the 5'-end and/or the 3'-end of AS-ODNs, which have natural-type

[§]This paper is dedicated to Dr. Morio Ikehara, an Emeritus Professor of Osaka University, on the occasion of his 70th birthday.
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internucleotidic phosphodiester linkages, have also been reported to be effective.[10~13] In our previous study,[14] it was found that the addition of a 4,4'-dimethoxytrityl (DMTr) group at the 5'-end of a 15mer AS-ODN (5'-TGGGAGGTGGGTCTG-3'), which is complementary to the *tat* 2nd splicing acceptor region of HIV-1 (Scheme 1), significantly enhanced the anti-HIV-1 activity of this AS-ODN (ODN-1 in Scheme 2). However, the DMTr group is known to be labile under weakly acidic conditions. We therefore planned to search a novel substituent for use in place of the DMTr group. In this paper, we compared the anti-HIV-1 activity of ODN-1 and those of the same 15mers substituted with a variety of hydrophobic groups instead of the DMTr group.

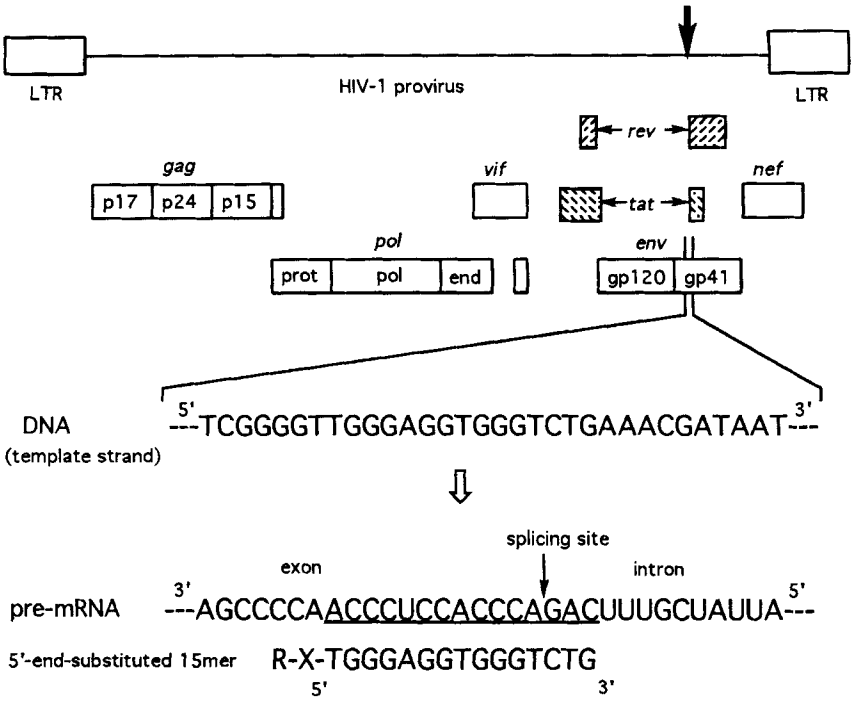
RESULTS AND DISCUSSION

Syntheses of 5'-end-substituted AS-ODNs---A variety of 15mers modified at their 5'-ends were synthesized by means of phosphoramidite chemistry on an automatic DNA synthesizer using 5'-substituted thymidine 3'-phosphoramidite derivatives as the 5'-terminal residues (Scheme 2). Trityl derivatives, substituted benzyl derivatives, silyl derivatives, and phosphoryl derivatives were employed as 5'-substituents.

First, 5'-substituted thymidine derivatives were prepared. In the cases of ODN-2 and ODN-3, thymidine was directly alkylated by monomethoxytrityl (MMT) chloride[15] and trityl (Tr) chloride,[16] respectively. In the cases of ODN-5, -7, -9, -10, and -11, 3'-*O*-(*t*-butyldimethylsilyl)thymidine was alkylated by the corresponding alkyl bromide in the presence of NaH and NaI, followed by desilylation using tetrabutylammonium fluoride (TBAF), to give the corresponding 5'-*O*-substituted thymidine. In the cases of ODN-4, -6, -8, -12, and -13, 5'-deoxy-5'-mercapto thymidine[17] was directly alkylated by the corresponding alkyl halide. To obtain the 5'-terminal residues of ODN-14, -15, and -16, thymidine was directly silylated by the corresponding silyl chloride.

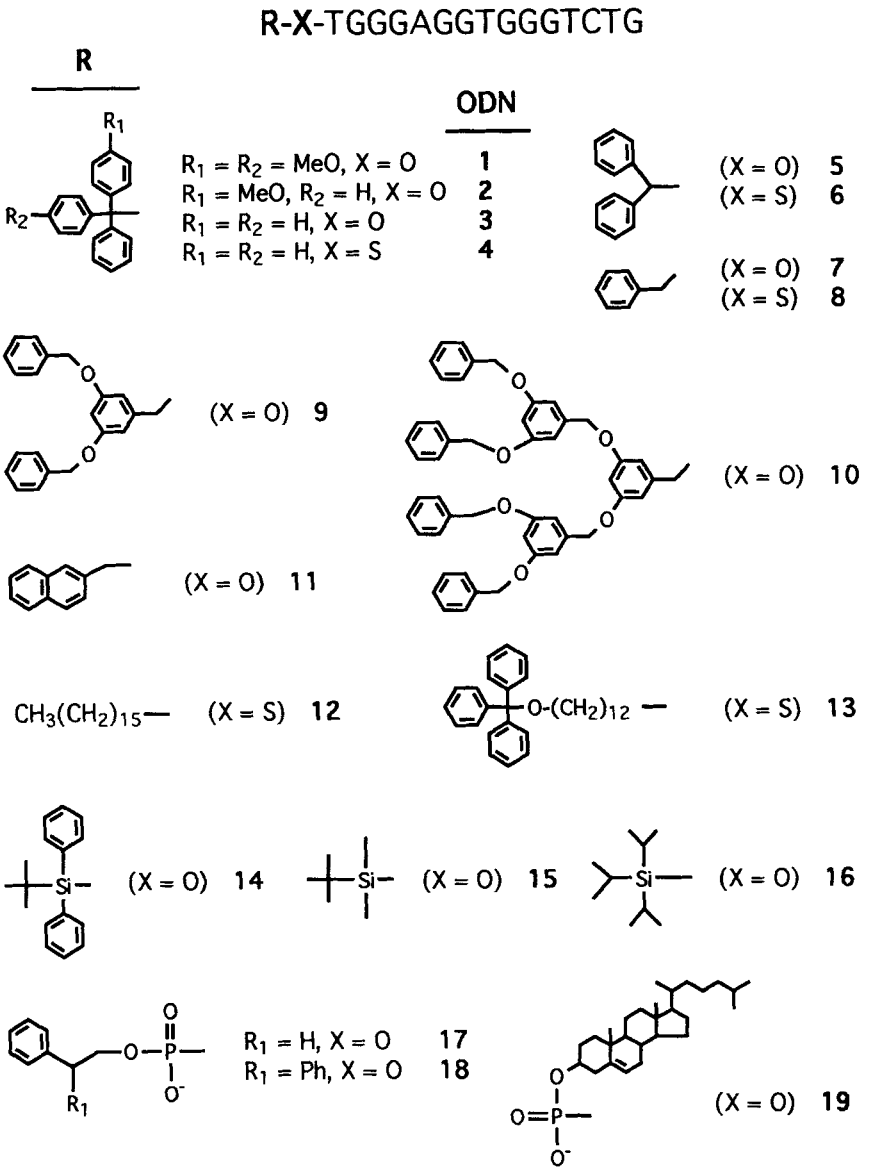
5'-End-substituted thymidine derivatives thus obtained were then phosphitylated by using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in the presence of diisopropylethylamine, to give the desired 5'-substituted thymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)phosphoramidite derivatives as 5'-terminal residues of ODN-2~16. Finally, these phosphoramidite derivatives were applied to an automatic DNA synthesizer, followed by deprotection using NH_4OH and purification using reversed-phase HPLC, to give ODN-2~16.

In the cases of ODN-17, -18, and -19, 2-phenylethanol, 2,2-diphenylethanol, and cholesterol were phosphitylated as described above, to give the 2-cyanoethyl *N,N*-diisopropylphosphoramidite derivatives. These derivatives were also applied to an automatic DNA synthesizer as extra-reagents. Thus, ODN-17, -18, and -19 were obtained in the same manner described above.



Scheme 1. *Tat* 2nd splicing acceptor region on the pre-mRNA of HIV-1

Negative ion LSI mass spectra of the AS-ODNs---To verify the sequence of these AS-ODNs, the typical sequencing technique using ^{32}P-labeling at the 5'-end was not applicable. Grotjahn et al. reported that negative ion mass spectroscopy was effective for analyzing the structure of natural-type ODN.[18, 19] It was also reported that some fragment ion peaks resulted from the fission of C-O bonds at the 5'-side or the 3'-side of internucleotidic phosphodiester linkages could be observed. These fragment ion peaks allow bi-directional sequence analysis[18] from both the 5'-end and the 3'-end of ODN. Thus, the negative ion LSI mass spectra were observed to confirm the molecular formulas and a few nucleotide sequences from both ends of AS-ODNs. As shown in Table 1, quasi-molecular ion peaks involving some sodium or potassium salts were detected under the low resolution conditions. Under the unit resolution conditions, some fragment ion peaks were observed, as shown in Table 2 and 3. In the case of ODN-10, the quasi-molecular ion peak could not be detected (Table 1). However, the 5'-end structure of ODN-10 was supported by the fragment ion peaks from the 5'-end of ODN-10 (Table 2).



Scheme 2. Structure of 5'-end-substituted 15mers

Table 1. Quasi-molecular ion peaks of AS-ODNs

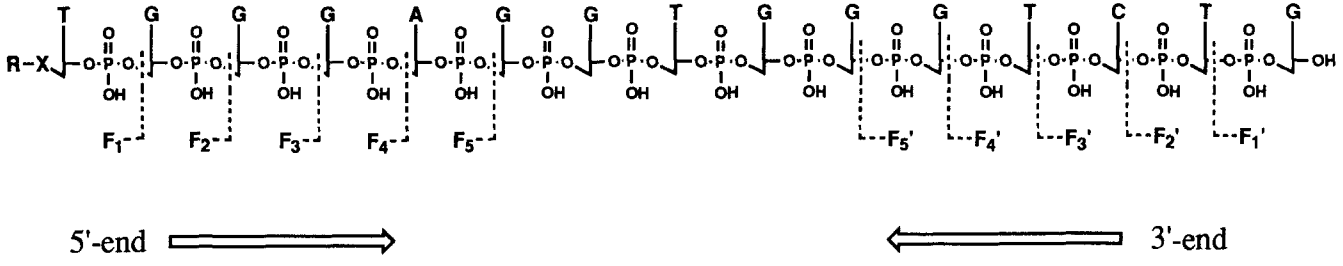
ODN	$[M-H]^-$	$[M+Na-2H]^-$	$[M+K-2H]^-$	$[M+2Na-3H]^-$	$[M+Na+K-3H]^-$
2	4989.6	5010.8	5027.7	N.D.	N.D.
3	4958.9	4980.9	4996.9	5002.9	N.D.
5	4882.73	4904.73	4920.74	N.D.	N.D.
6	N.D.	4922.3	4938.6	4943.0	N.D.
7	N.D.	4828.9	4846.4	N.D.	N.D.
8	N.D.	N.D.	N.D.	4867.4	4885.0
9	5018.77	5040.79	5056.82	N.D.	N.D.
11	4856.67	4878.72	4894.71	N.D.	N.D.
12	N.D.	N.D.	N.D.	5003.8	5019.3
14	4954.71	4976.74	4992.71	N.D.	N.D.
15	4830.80	4852.81	4868.85	N.D.	N.D.
16	4872.77	4894.77	4910.78	N.D.	N.D.
17	4900.70	4922.73	4938.71	N.D.	N.D.
18	4976.77	4998.79	5014.76	N.D.	N.D.

N.D. stands for "not detected".

Anti-HIV-1 activity of the AS-ODNs---In order to evaluate the anti-HIV-1 activities of 5'-end-substituted 15mers, the 50% inhibitory concentration (IC_{50}) for the cytopathic effect (CPE) induced by HIV-1_{IIIB} and the 50% cytotoxic concentration (CC_{50}) were determined by MTT assay using MT-4 cells *in vitro* according to the procedure reported previously.[20]

As shown in Table 4, the IC_{50} value for ODN-1 bearing DMTr group was 3.6 μg/ml, and the CC_{50} value for ODN-1 was greater than 100 μg/ml. The cell viability for ODN-1 increased in proportion to the concentration of ODN-1, as shown in Figure 1. In the previous study,[14] we reported that the natural-type AS-ODN, d(TGGGAGGTGGGTCTG), without any substituent exhibited no anti-HIV-1 activity in our assay system. Thus, the advantage of the introduction of a hydrophobic DMTr group to the natural-type AS-ODN was clearly revealed in the potent anti-HIV-1 activity of ODN-1.

ODN-2~4 bearing trityl-type substituents showed moderate anti-HIV-1 activities (3.8~8.0 μg/ml) and no cytotoxicity (Table 4). ODN-2~4 showed dose-dependent anti-HIV-1 activities almost as same as those of ODN-1 (Figure 1). The CC_{50} value for ODN-3 bearing a trityl group was 3.8 μg/ml. The cytotoxicity of ODN-3 was particularly examined up to 400 μg/ml and the CC_{50} value for ODN-3 was greater than 400 μg/ml. Thus, the selectivity index ($SI = CC_{50} / IC_{50}$) was greater than 105. In comparison of ODN-3, -5, and -7, it was found that reducing the number of phenyl groups of ODN-3 resulted in a reduction of the anti-HIV-1 activity and an increase in cytotoxicity. ODN-6



Bi-directional sequence analysis

Table 2. Fragment ion peaks from the 5'-end of modified ODNs

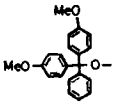
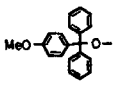
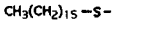
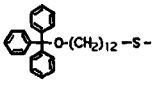
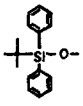
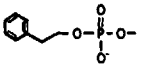
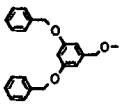
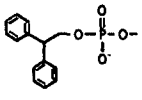
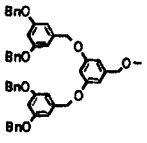
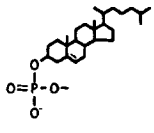
ODN	F_1	F_2	F_3	F_4	F_5
2	----	----	----	----	----
3	563	892	1221	1550	----
5	487	816	1145	1474	----
9	623	952	1281	----	----
10	1047	1376	1705	----	----
11	461	790	1119	1448	1761
12	561	890	1219	----	----
14	559	888	1217	1546	----
15	435	764	1093	1422	1735
16	477	806	1135	1464	----
17	505	834	1163	1492	----
18	581	910	1239	1568	1881

Table 3. Fragment ion peaks from the 3'-end of modified ODNs

ODN	F_1'	F_2'	F_3'	F_4'	F_5'
2	----	650	939	1243	----
3	346	650	939	1243	1572
5	346	650	939	1243	----
9	346	650	939	1243	1572
10	----	----	----	----	----
11	346	650	939	1243	1572
12	----	----	----	----	----
14	----	----	----	----	----
15	346	650	----	----	----
16	346	650	939	----	----
17	346	650	939	1243	1572
18	346	650	939	1243	1572

Table 4. Anti-HIV-1 activities and cytotoxicities of AS-ODNs.

R-X-TGGGAGGTGGGTCTG

ODN	R-X-	(μg/ml) IC_{50}	(μg/ml) CC_{50}	ODN	R-X-	(μg/ml) IC_{50}	(μg/ml) CC_{50}
1		3.6	>100	11		7.4	>100
2		8.0	>100	12		12.5	>100
3		3.8	>400[ξ]	13		37	>100
4		7.2	>100	14		5.3	>100
5		6.0	>100	15		2.5	25
6		2.5	>100	16		2.5	35
7		>100	50				
8		20	100	17		>100	>100
9		4.3	90	18		6.5	>100
10		22.5	>100	19		16.7	>100

ξ : Only ODN-3 was examined up to 400 μg/ml.

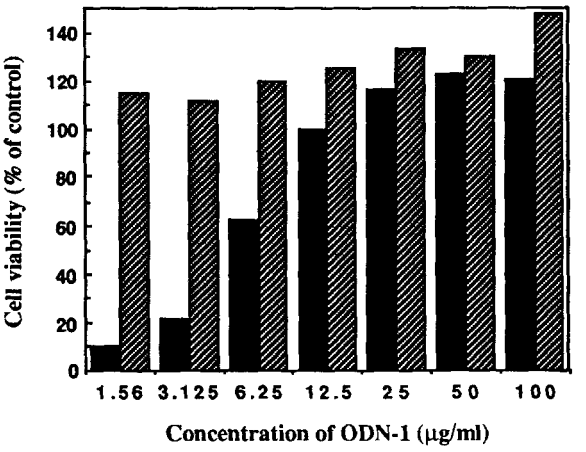


Figure 1. Dose-dependent anti-HIV-1 activity and cytotoxicity of ODN-1

was very potent from the viewpoint of IC_{50} value. However, the cell viability for ODN-6 at a concentration greater than 50 μg/ml was reduced in proportion to the concentration of ODN-6, regardless of the cytotoxicity (CC_{50} >100 μg/ml).

Figure 2 shows the anti-HIV-1 activities and cytotoxicities of ODN-7, -9, and -10. ODN-7 exhibited very weak anti-HIV-1 activity at 100 μg/ml, and the CC_{50} value was 50 μg/ml (Figure 2.(A)). In the case of ODN-9, the benzyl group of ODN-7 was substituted with two benzyloxy groups. ODN-9 exhibited dose-dependent anti-HIV-1 activity up to 25 μg/ml (Figure 2.(B)). The cytotoxicity of ODN-9, though reduced slightly as compared with that of ODN-7, still existed (CC_{50} = 90 μg/ml). In the case of ODN-10, two benzyloxy groups of ODN-9 were further substituted with four benzyloxy groups. While cytotoxicity of ODN-10 was not observed up to 100 μg/ml (Figure 2.(C)), the anti-HIV-1 activity of ODN-10 was reduced as compared with that of ODN-9 (IC_{50} = 22.5 μg/ml). Considering these results, there was a marked tendency for the cytotoxicity to be reduced when the AS-ODN was substituted with larger hydrophobic moiety than a benzhydryl group. It was also found that substituents involving three benzene rings (e.g. Tr or 3,5-dibenzyloxybenzyl) were suitable for potent anti-HIV-1 activity. In comparison of ODN-1~4 and ODN-7~10, it was apparent that the trityl-type moieties had better property than the substituted benzyl-type moieties from the viewpoint of both anti-HIV-1 activity and cytotoxicity.

ODN-12 bearing a long alkyl chain as an aliphatic substituent at the 5'-end of ODN also showed anti-HIV-1 activity. However, the IC_{50} value for ODN-12 was greater than 12

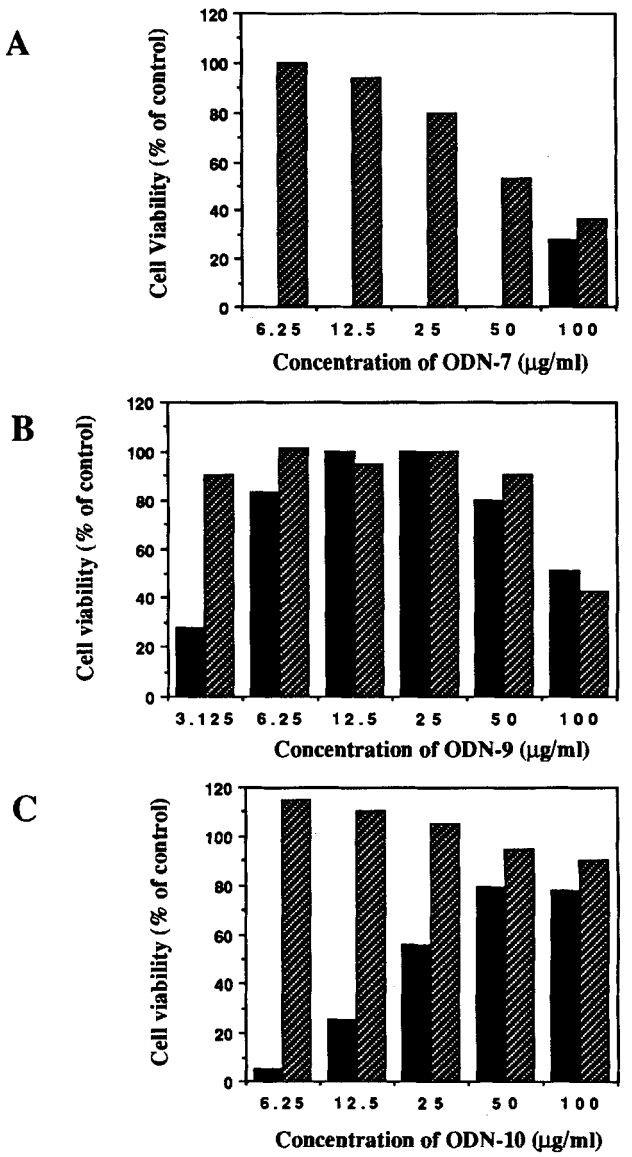


Figure 2. Anti-HIV-1 activity and cytotoxicity of (A) ODN-7, (B) ODN-9, and (C) ODN-10. ■: Viability of cells which were exposed to HIV-1; ▨: Viability of cells which were not exposed to HIV-1.

μg/ml. ODN-13 bearing a long alkyl linker between the trityl group and the 5'-end of ODN exhibited low anti-HIV-1 activity (IC_{50} = 37 μg/ml). Consequently, it was evident that the aromatic moiety should be attached in the vicinity of the 5'-end of 15mer.

ODN-14 bearing a *t*-butyldiphenylsilyl (TBDPS) group exhibited potent anti-HIV-1 activity that was almost as same as those of AS-ODNs bearing trityl-type substituents. ODN-15 and ODN-16 bearing a *t*-butyldimethylsilyl (TBDMS) and a triisopropylsilyl group (TPS), respectively, seemed to be potent from the viewpoint of IC_{50} values. However, the CC_{50} values for ODN-15 and ODN-16 were 25 and 35 μg/ml, and hence the SI values for ODN-15 and ODN-16 were 10 and 14, respectively. The cytotoxicities of ODN-15 and ODN-16 can be attributable to the small size of the substituents, TBDMS and TPS, because this tendency was consistent with the results of ODN-7 and ODN-8.

ODN-17 linking a 2-phenylethyl group via a phosphodiester bond exhibited no anti-HIV-1 activity and no cytotoxicity up to 100 μg/ml. On the other hand, ODN-18 bearing a 2,2-diphenylethyl group in the same fashion as ODN-17 exhibited moderate anti-HIV-1 activity (IC_{50} = 6.5 μg/ml). Letsinger et al. reported that a cholesteryl group attached to the internucleotidic phosphodiester bond enhanced the antisense activity.[12] Thus, in the case of ODN-19, a cholesteryl group was attached to the 5'-end of 15mer via a phosphodiester bond. However, the anti-HIV-1 activity of ODN-19 was very low (IC_{50} = 16.7 μg/ml).

CONCLUSION

Nineteen AS-ODNs bearing a variety of 5'-end-substituents were synthesized in the phosphoramidite method using an automatic DNA synthesizer. The structures of these AS-ODNs were successfully confirmed by negative ion LSI mass spectroscopy. The study on structure activity relationships of the 5'-end-substituted AS-ODNs led us to the following conclusions: (1) aromatic substituents were superior to aliphatic substituents; (2) trityl-type substituents were superior to the others involving substituted benzyl-, silyl-, and phosphoryl-type substituents, except for a TBDPS group; (3) AS-ODNs bearing small-size substituents (e.g. a benzyl group) exhibited cytotoxicities; (4) Substituents involving three benzene rings (e.g. Tr or 3,5-dibenzyloxybenzyl) were suitable for potent anti-HIV-1 activity, and in general, substituents involving more, or less, than three benzene rings led to low anti-HIV-1 activities.

We could not find a nobel modified 15mer which was apparently more potent than ODN-1 bearing a DMTr group. ODN-3 bearing a Tr group exhibited almost the same anti-HIV-1 activity as that of ODN-1. Since a Tr ether bond is known to be almost 200-fold more stable under weakly acidic conditions (80% AcOH, 20°C) than a DMTr ether bond,[21] ODN-3 seems to be more suitable for manipulation in large scale than ODN-1. In

addition, the CC_{50} value for ODN-3 was greater than 400 μg/ml. Thus, ODN-3 is recommended at present.

The natural-type 15mer, d(TGGGAGGTGGGTCTG), without substituents exhibited no anti-HIV-1 activity in our assay system. On the other hand, 15mers bearing a variety of substituents at the 5'-end of ODNs showed anti-HIV-1 activities. If the mechanism for the anti-HIV-1 action of these modified ODNs was antisense fashion, a possible explanation would be that the substituent enhanced the permeability of ODN, such that it could be taken up into the target cells. Mechanistic study of the 5'-end-substituted 15mers is now in progress.

Experimental Section

^{1}H-NMR spectra were recorded on a JEOL JNM-EX 270 spectrometer (270 MHz) or a Varian EM360L NMR spectrometer with tetramethylsilane as an internal standard. UV absorption spectra were recorded on a HITACHI Model 200-20 Spectrophotometer or a HITACHI U-3210 Spectrophotometer. Negative ion LSI mass spectra were recorded on VG 70-4SE using 3-nitrobenzyl alcohol as a matrix. TLC was done on 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 Chromatograph pump equipped with an L-5000 LC Controller, an L-3000 Photo Diode Array Detector, and a D-2500 Chromato-Integrator.

General methods for the preparation of 5'-substituted thymidine.

Method A---To a solution of 713 mg (2 mmol) of 3'-*O*-(*t*-butyldimethylsilyl)thymidine in 4 mL of tetrahydrofuran (THF) was added 175 mg (4 mmol) of 55% NaH under an Ar atmosphere, and the total mixture was stirred at 60 °C. After 2 h, a solution of 2 mmol of appropriate alkyl halide in 1 mL of THF and 150 mg (1 mmol) of NaI were added, and the total mixture was stirred overnight at room temperature. The solvent was removed by evaporation and the residue was dissolved in 50 mL of ethyl acetate (EtOAc). The solution was washed twice with 50 mL of 0.1 N HCl and dried over anhydrous $MgSO_4$. The solvent was removed by evaporation and the residue was re-dissolved in 2 mL of THF. Then 2 mL of the 1 M solution of TBAF in THF was added and the total mixture was stirred at room temperature for 2 h. The solvent was removed by evaporation and the residue was re-dissolved in 50 mL of EtOAc. The solution was washed twice with 50 mL of sat. aq. NaCl and the organic layer was dried over anhydrous $MgSO_4$. The solvent was removed by evaporation, the residue was applied on a silica gel column (50 g), and the desired product was eluted with 1~3% MeOH-CH_2Cl_2.

Method B---To a solution of 516 mg (2 mmol) of 5'-deoxy-5'-mercaptothymidine[17] in 30 mL of acetone were added 3 mmol of appropriate alkyl bromide and 1 g (9.4 mmol) of

Na_2CO_3, and the total mixture was stirred under reflux. After 5 h, the mixture was filtered and the filtrate was evaporated. The residue was applied on a silica gel column and the desired product was eluted with 5% MeOH-CH_2Cl_2.

General method for the preparation of 5'-substituted thymidine 3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite.

Method C---5'-*O*-Substituted thymidine (0.5 mmol) was dried by repeated coevaporation with pyridine and was dissolved in 2.5 mL of THF. To the solution were added 348 μL (2 mmol) of diisopropylethylamine and 223 μL (1 mmol) of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, and the total mixture was stirred at room temperature under an Ar atmosphere. After 30 min, the mixture was filtered and the filtrate was evaporated to dryness. The residue was dissolved in 50 mL of EtOAc and the solution was washed twice with ice-cold 10% aq. Na_2CO_3, followed by drying over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was applied on a silica gel column (30 g). The desired product was eluted with CH_2Cl_2-EtOAc-Et_3N (45 : 45 : 10, v/v/v).

General method for the synthesis of 5'-substituted AS-ODNs.

Method D---An appropriate 5'-substituted thymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)phosphoramidite (0.1 g) was dissolved in 4 mL of CH_3CN and the solution was applied to the Cyclone™ Plus DNA Synthesizer (MilliGen/Biosearch) as an extra amidite reagent. Synthesis of 15mer was performed by the standard conditions starting from 5 μmol scale. Deprotection was performed using 29% aq. NH_4OH at 60 °C for 5 h. The crude product was purified by HPLC under one of the conditions described below: Inertsil PREP-ODS, 20 × 250 mm; 0.1M TEAA (pH 7.3); 254 nm; 9mL/min; A: 20-50% CH_3CN, 30 min; B: 0-40% CH_3CN, 40 min; C: 10-40% CH_3CN, 30 min; D: 10-70% CH_3CN, 60 min; E: 20-70% CH3CN, 50 min; F: YMC SH-243-7 (C8); 25mM TEAP (pH 6.8); 5-75% CH_3CN, 100 min; 254 nm; 9mL/min.

Synthesis of ODN-2---5'-*O*-Monomethoxytritylthymidine[15] (257 mg, 0.5 mmol) was phosphitylated by Method C, to give 261 mg (73%) of 5'-*O*-monomethoxytritylthymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.64, 7.59 (2s, 1 H, H6); 7.46-7.20, 6.88-6.82 (m, 14 H, Ph); 6.46-6.39 (m, 1 H, H1'); 4.73-4.62 (m, 1 H, H3'); 4.19, 4.15 (2br s, 1 H, H4'); 3.90-3.30 (m, 6 H, H5', POC*H*$_2$ and PNC*H*); 3.78 (s, 3 H, C*H*$_3$O); 2.66-2.29 (m, 4 H, H2', C*H*$_2$CN); 1.46 (s, 3 H, C*H*$_3$); 1.17 (d, 12 H, J = 7.3 Hz, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition A) and the fraction eluted at 14.4 min was collected, to give 91 A_{260} units of ODN-2.

Synthesis of ODN-3---5'-*O*-Tritylthymidine[16] (969 mg, 2 mmol) was phosphitylated by Method C, to give 1.35 g (98%) of 5'-*O*-tritylthymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.62, 7.57 (2s, 1 H, H6); 7.46-7.20 (m, 15 H, Ph); 6.46-6.37 (m, 1 H, H1'); 4.68 (br s, 1 H, H3'); 4.19, 4.15 (2br s, 1 H, H4'); 3.90-3.30 (m, 6 H, H5', $POCH_2$ and PNC*H*); 2.63-2.28 (m, 4 H, H2', CH_2CN); 1.47 (s, 3 H, CH_3); 1.23-1.00 (m, 12 H, $(CH_3)_2CH$).

The above compound was applied to the DNA synthesizer according to Method D in 15 μmol scale. Purification was performed by HPLC (condition A) and the fraction eluted at 12.6 min was collected, to give 464 A_{260} units of ODN-3.

Synthesis of ODN-4---5'-Tritylthio-5'-deoxythymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite[17] was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition A) and the fraction eluted at 13.1 min was collected, to give 38 A_{260} units of ODN-4.

Synthesis of ODN-5---377 mg (23%) of 5'-*O*-Benzhydrylthymidine was obtained by Method A starting from 1.426 g (4 mmol) of 3'-*O*-(*t*-butyldimethylsilyl)thymidine.

^{1}H-NMR ($CDCl_3$) δ : 9.85 (br s, 1 H, N*H*); 7.56 (s, 1 H, H6); 7.38-7.20 (m, 10 H, Ph); 6.45 (t, 1 H, J = 6.9 Hz, H1'); 5.40 (s, 1 H, Ph_2CH); 4.62-4.58 (m, 1 H, H3'); 4.17-4.15 (m, 1 H, H4'); 3.75-3.58 (m, 2 H, H5'); 2.47-2.22 (m, 2 H, H2'); 1.36 (s, 3 H, CH_3).

The above compound (204 mg, 0.5 mmol) was phosphitylated by Method C, to give 213 mg (70%) of 5'-*O*-benzhydrylthymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)-phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.55, 7.51 (2s, 1 H, H6); 7.43-7.22 (m, 10 H, Ph); 6.48-6.42 (m, 1 H, H1'); 5.44, 5.42 (2s, 1 H, Ph_2CH); 4.73-4.64 (m, 1 H, H3'); 4.25, 4.19 (2br s, 1 H, H4'); 3.90-3.55 (m, 6 H, ,H5', $POCH_2$, PNC*H*); 2.68-2.24 (m, 4 H, H2', CH_2CN); 1.39 (s, 3 H, CH_3); 1.30-1.10 (m, 12 H, $(CH_3)_2CH$).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition B) and the fraction eluted at 29.2 min was collected, to give 130 A_{260} units of ODN-5.

Synthesis of ODN-6---334 mg of 5'-benzhydrylthio-5'-deoxythymidine was obtained by Method B.

^{1}H-NMR (DMSO-d_6) δ : 11.28 (s, 1 H, N*H*); 7.47-7.20 (m, 11 H, H6, Ph); 6.15 (t, 1 H, J = 6.8 Hz, H1'); 5.37-5.32 (m, 2 H, O*H*, Ph_2CH); 4.16-4.12 (m, 1 H, H3'); 3.85-3.79 (m, 1 H, H4'); 2.71-2.50 (m, 2 H, H5'); 2.21-2.00 (m, 2 H, H2'), 1.79 (s, 3 H, CH_3).

The above compound (212 mg, 0.5 mmol) was phosphitylated by Method C, to give 253 mg (81%) of 5'-benzhydrylthio-5'-deoxythymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.47-7.17 (m, 11 H, Ph, H6); 6.35-6.27 (m, 1 H, H1'); 5.29, 5.27 (2s, 1 H, Ph_2CH); 4.50-4.40 (m, 1 H, H3'); 4.19-4.10 (m, 1 H, H4'); 3.92-3.50 (m, 4 H, $POCH_2$, PNCH); 2.80-2.10 (m, 6 H, H2', H5', CH_2CN); 1.77 (s, 3 H, CH_3); 1.20-1.15 (m, 12 H, $(CH_3)_2CH$).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition B) and the fraction eluted at 30.3 min was collected, to give 73 A_{260} units of ODN-6.

Synthesis of ODN-7---392 mg of 5'-*O*-benzylthymidine was obtained by Method A.

^{1}H-NMR ($CDCl_3$) δ : 7.59 (s, 1 H, H6); 7.35-7.22 (m, 5 H, Ph); 6.42 (t, 1 H, J = 6.6 Hz, H1'); 4.56 (s, 2 H, $PhCH_2$); 4.51 (br s, 1 H, H3'); 4.13 (br s, 1 H, H4'); 3.80-3.65 (m, 2 H, H5'); 2.42-2.12 (m, 2 H, H2'); 1.58 (s, 3 H, CH_3).

The above compound (166 mg, 0.5 mmol) was phosphitylated by Method C, to give 271 mg of 5'-*O*-benzhydrylthymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)-phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.59-7.56 (2s, 1 H, H6); 7.38-7.26 (m, 5 H, Ph); 6.40 (t, 1 H, J = 7.3 Hz, H1'); 4.68-4.55 (m, 1 H, H3'); 4.60, 4.59 (2s, 2 H, $PhCH_2$); 4.24-4.18 (m, 1 H, H4'); 3.90-3.55 (m, 6 H, H5', $POCH_2$, PNCH); 2.65, 2.58 (2t, 2 H, J = 6.4, 6.4 Hz, CH_2CN); 2.52-2.15 (m, 2 H, H2'); 1.63 (s, 3 H, CH_3); 1.18 (m, 12 H, $(CH_3)_2CH$).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition C) and the fraction eluted at 14.1 min was collected, to give 126 A_{260} units of ODN-7.

Synthesis of ODN-8---To a solution of 775 mg (3 mmol) of 5'-deoxy-5'-mercapto-thymidine[17] were added 564 mg (3.3 mmol) of benzyl bromide and 660 mg (6.6 mmol) of Na_2CO_3, and the total mixture was stirred at room temperature for 17 h. The mixture was filtered and the filtrate was evaporated. The residue was dissolved in 40 mL of CH_2Cl_2 and washed twice with H_2O. The organic layer was dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure. The residue was recrystallized from 10 mL of ethanol, to give 451 mg of 5'-benzylthio-5'-deoxythymidine.

^{1}H-NMR (DMSO-d_6) δ : 11.28 (s, 1 H, NH); 7.46 (s, 1 H, H6); 7.46-21 (m, 5 H, Ph); 6.16 (t, 1 H, J = 6.7 Hz, H1'); 5.32 (d, 1 H, J = 4.4 Hz, OH); 4.15 (m, 1 H, H3'); 3.83 (m, 1 H, H4'); 3.78 (s, 2 H, $PhCH_2$); 2.78-2.59 (m, 2 H, H5'); 2.25-2.00 (m, 2 H, H2'); 1.77 (s, 3 H, CH_3).

The above compound (174 mg, 0.5 mmol) was phosphitylated by Method C, to give 262 mg (95%) of 5'-benzylthio-5'-deoxythymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)-phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.37 (s, 1 H, H6); 7.34-7.20 (m, 5 H, Ph); 6.32-6.25 (m, 1 H, H1'); 4.49-4.38 (m, 1 H, H3'); 4.18-4.08 (m, 1 H, H4'); 3.90-3.50 (m, 6 H, PhC*H*$_2$, POC*H*$_2$, PNC*H*); 2.87-2.68 (m, 2 H, H5'); 2.67-2.56 (m, 2 H, C*H*$_2$CN); 2.55-2.12 (m, 2 H, H2'); 1.91 (s, 3 H, C*H*$_3$); 1.20-1.15 (m, 12 H, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition B) and the fraction eluted at 25.8 min was collected, to give 46 A_{260} units of ODN-8.

Synthesis of ODN-9---258 mg (23%) of 5'-*O*-(3,5-dibenzyloxybenzyl)thymidine was obtained by Method A using 3,5-dibenzyloxybenzyl bromide.[22]

^{1}H-NMR ($CDCl_3$) δ : 7.87 (s, 1 H, N*H*); 7.52 (s, 1 H, H6); 7.43-7.27, 6.59-6.52 (m, 13 H, Ph), 6.37 (t, 1 H, J = 6.8 Hz, H1'); 5.03 (s, 4 H, PhC*H*$_2$); 4.51 (d, 2 H, J = 3.3 Hz, PhC*H*$_2$OCH$_2$); 4.50-4.44 (m, 1 H, H3'); 4.06-4.03 (m, 1 H, H4'); 3.77-3.63 (m, 2 H, H5'); 2.32-2.12 (m, 2 H, H2'); 1.67 (s, 3 H, C*H*$_3$).

The above compound (258 mg, 0.47 mmol) was phosphitylated by Method C, to give 307 mg (87%) of 5'-*O*-(3,5-dibenzyloxybenzyl)thymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.56, 7.53 (2s, 1 H, H6); 7.42-7.28, 6.56 (m, 13 H, Ph); 6.40 (t, 1 H, J = 6.6 Hz, H1'); 5.02 (s, 4 H, PhC*H*$_2$); 4.67-4.58 (m, 1 H, H3'); 4.53, 4.51 (2s, 2 H, PhC*H*$_2$OCH$_2$); 4.23, 4.17 (2br s, 1 H, H4'); 3.90-3.52 (m, 6 H, H5', POC*H*2, PNC*H*); 2.68-2.53 (m, 2 H, C*H*$_2$CN); 2.49-2.12 (m, 2 H, H2'); 1.65 (s, 3 H, C*H*$_3$); 1.18 (d, 12 H, J = 5.9 Hz, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition A) and the fraction eluted at 15.7 min was collected, to give 85 A_{260} units of ODN-9.

Synthesis of ODN-10---381 mg (20%) of 5'-*O*-[3,5-bis{3,5-(dibenzyloxy)benzyl-oxy}benzyl]thymidine was obtained by Method A using 3,5-bis{3,5-(dibenzyloxy)benzyl-oxy}benzyl bromide.[22]

^{1}H-NMR ($CDCl_3$) δ : 7.86 (s, 1 H, N*H*); 7.52 (s, 1 H, H6); 7.45-7.28, 6.68-6.50 (m, 29 H, Ph), 6.35 (t, 1 H, J = 8.1 Hz, H1'); 5.03 (s, 8 H, PhC*H*$_2$); 4.98 (s, 4 H, PhC*H*$_2$); 4.50 (m, 2 H, PhC*H*$_2$OCH$_2$); 4.42-4.38 (m, 1 H, H3'); 4.03-3.98 (m, 1 H, H4'); 3.73-3.59 (m, 2 H, H5'); 2.30-2.09 (m, 2 H, H2'); 1.70 (s, 3 H, C*H*$_3$).

The above compound (242 mg, 0.25 mmol) was phosphitylated by Method C, to give 146 mg (50%) of 5'-*O*-[3,5-bis{3,5-(dibenzyloxy)benzyloxy}benzyl]thymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.56, 7.53 (2s, 1 H, H6); 7.45-7.28, 6.66, 6.58-6.52 (m, 29 H, Ph); 6.39 (t, 1 H, J = 6.8 Hz, H1'); 5.01 (s, 8 H, PhC*H*$_2$); 4.95 (s, 4 H, PhC*H*$_2$); 4.66-4.57 (m, 1 H, H3'); 4.52, 4.51 (2s, 2 H, PhC*H*$_2$OCH$_2$); 4.22, 4.16 (2br s, 1 H, H4'); 3.83-3.34 (m, 6 H, H5', POC*H*2, PNC*H*); 2.69-1.77 (m, 4 H, H2', C*H*$_2$CN); 1.66 (s, 3 H, C*H*$_3$); 1.30-1.08 (m, 12 H, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition D) and the fraction eluted at 36.6 min was collected, to give 59 A_{260} units of ODN-10.

Synthesis of ODN-11---179 mg (35%) of 5'-*O*-(2-Naphtylmethyl)thymidine was obtained by Method A.

^{1}H-NMR ($CDCl_3$) δ : 7.88-7.77, 7.53-7.43 (m, 7 H, naphtyl); 7.61 (s, 1 H, H6); 6.35 (t, 1 H, J = 6.6 Hz, H1'); 4.76 (s, 2 H, naphtyl-C*H*$_2$); 4.52-4.47 (m, 1 H, H3'); 4.11-4.09 (m, 1 H, H4'); 3.89-3.71 (m, 2 H, H5'); 2.38-2.14 (m, 2 H, H2'); 1.54 (s, 3 H, C*H*$_3$).

The above compound (179 mg, 0.47 mmol) was phosphitylated by Method C, to give 165 mg (60%) of 5'-*O*-(2-naphtylmethyl)thymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)-phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.88-7.77, 7.52-7.42 (m, 7 H, naphtyl); 7.60, 7.57 (2s, 1 H, H6); 6.40 (t, 1 H, J = 5.9 Hz, H1'); 4.81-4.71 (m, 2 H, naphtyl-C*H*$_2$); 4.67-4.58 (m, 1 H, H3'); 4.24, 4.18 (2br s, 1 H, H4'); 3.90-3.50 (m, 6 H, H5', POC*H*$_2$, PNC*H*); 2.68-2.15 (m, 4 H, H2', C*H*$_2$CN); 1.58, 1.56 (2s, 3 H, C*H*$_3$); 1.17 (d, 12 H, J = 6.6 Hz, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition C) and the fraction eluted at 17.6 min was collected, to give 141 A_{260} units of ODN-11.

Synthesis of ODN-12---To a solution of 258 mg (1 mmol) of 5'-deoxy-5'-mercaptothymidine[17] in 20 mL of acetone were added 457 mg (1.5 mmol) of hexadecyl bromide and 0.5 g (4.7 mmol) of Na_2CO_3, and the total mixture was stirred under reflux for 3 h. After the same workup as in Method B, 303 mg of 5'-hexadecylthio-5'-deoxythymidine was obtained.

^{1}H-NMR (DMSO-d_6) δ : 11.28 (s, 1 H, N*H*); 6.16 (dd, 1 H, J = 6.4, 7.8 Hz, H1'); 5.31 (d, 1 H, J = 4.4 Hz, O*H*); 4.19-4.12 (m, 1 H, H3'); 3.84-3.79 (m, 1 H, H4'); 2.83-2.68 (m, 2 H, H5'); 2.58-2.47 (m, 2 H, C*H*$_2$S); 2.25-2.00 (m, 2 H, H2'); 1.79 (s, 3 H, C*H*$_3$); 1.57-1.45 (m, 2 H, C*H*$_2$); 1.30-1.23 (s, 26 H, C*H*$_2$); 0.85 (t, 3 H, J = 6.8 Hz, C*H*$_3$).

The above compound (241 mg, 0.5 mmol) was phosphitylated by Method C, to give 193 mg (56%) of 5'-hexadecylthio-5'-deoxythymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.46, 7.41 (2s, 1 H, H6); 6.29 (t, 1 H, J = 7.3 Hz, H1'); 4.55-4.45 (m, 1 H, H3'); 4.22-4.12 (m, 1 H, H4'); 3.92-3.54 (m, 4 H, POC*H*$_2$, PNC*H*); 2.93-2.17 (m, 6 H, H2', H5', C*H*$_2$CN); 1.93 (s, 3 H, C*H*$_3$); 1.60 (br s, 2 H, C*H*$_2$S); 1.26 (s, 30 H, C*H*$_2$); 1.20 (d, 12 H, J = 6.6 Hz, (C*H*$_3$)$_2$CH); 0.88 (t, 3 H, J = 7.0 Hz, C*H*$_3$).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition D) and the fraction eluted at 42.3 min was collected, to give 81 A_{260} units of ODN-12.

Synthesis of ODN-13---712 mg of 5'-(12-Hydroxydodecylthio)-5'-deoxythymidine was obtained by Method B using 12-hydroxydodecyl bromide. 598 mg (1.3 mmol) of the above compound was dissolved in 30 mL of pyridine. To the solution was added 541 mg (1.95 mmol) of trityl chloride and the total mixture was refluxed for 4 h. The solvent was removed under reduced pressure and the residue was re-dissolved in 30 mL of CH_2Cl_2. The solution was washed with 30 mL each of 0.2 N HCl, sat. aq. NaCl, and sat. aq. $NaHCO_3$. The organic layer was dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure. The residue was applied to a silica gel column, and 757 mg of the desired compound, 5'-(12-trityloxydodecylthio)-5'-deoxythymidine, was obtained.

^{1}H-NMR (DMSO-*d*$_6$) δ : 11.28 (s, 1 H, N*H*); 7.50 (s, 1 H, H6); 7.38-7.21 (m, 15 H, Ph); 6.16 (t, 1 H, J = 6.3 Hz, H1'); 5.31 (d, 1 H, J = 4.4 Hz, O*H*); 4.17-4.14 (m, 1 H, H3'); 3.83-3.80 (m, 1 H, H4'); 2.95 (t, 2H, J = 6.4 Hz, C*H*$_2$S); 2.77-2.73 (m, 2 H, H5'); 2.20-2.05 (m, 2 H, H2'); 1.78 (s, 3 H, C*H*$_3$); 1.10-1.06 (m, 20 H, C*H*$_2$).

The above compound (342 mg, 0.5 mmol) was phosphitylated by Method C, to give 423 mg (96%) of 5'-(12-trityloxydodecylthio)-5'-deoxythymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.55-7.22 (m, 16 H, H6, Ph); 6.36 (t, 1 H, J = 6.6 Hz, H1'); 4.61-4.51 (m, 1 H, H3'); 4.30-4.12 (m, 1 H, H4'); 3.97-3.55 (m, 4 H, POC*H*$_2$, PNC*H*); 3.10 (t, 2 H, J = 6.4 Hz, C*H*$_2$O); 2.99-2.20 (m, 8 H, H2', H5', C*H*$_2$CN, C*H*$_2$S); 1.97 (s, 3 H, C*H*$_3$); 1.72-1.25 (m, 10 H, C*H*$_2$); 1.25 (d, 12 H, J = 7.3 Hz, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method E. Purification was performed by HPLC (condition D) and the fraction eluted at 36.0 min was collected, to give 48 A_{260} units of ODN-13.

Synthesis of ODN-14---1.21 g (5 mmol) of thymidine and 749 mg (11 mmol) of imidazole were dissolved in 10 mL of DMF. To the solution was added 1.43 mL (5.5 mmol) of TBDPSCl, and the total mixture was stirred at room temperature under an Ar atmosphere. After 140 min, the solvent was removed under reduced pressure and the residue was re-dissolved in 100 mL of CH_2Cl_2. The solution was washed twice with 100 mL of sat. aq. NaCl and the organic layer was dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure and the residue was applied on a silica gel column (100 g). Elution was performed with 0.5~3% MeOH-CH_2Cl_2, and 2.4 g (62%) of 5'-*O*-(*t*-butyl-diphenylsilyl)thymidine was obtained.

^{1}H-NMR ($CDCl_3$) δ : 10.51 (br s, 1 H, N*H*); 7.72-7.64, 7.40-7.32 (m, 10 H, Ph); 7.56 (s, 1 H, H6); 6.51 (t, 1 H, J = 6.6 Hz, H1'); 4.63 (br s, 1 H, H3'); 4.47 (br s, 1 H, O*H*); 4.12 (br s, 1 H, H4'); 4.05-3.85 (m, 2 H, H5'); 2.58-2.47, 2.30-2.17 (m, 2 H, H2'); 1.58 (s, 3 H, C*H*$_3$); 1.10 (s, 9 H, *t*Bu).

The above compound (240 mg, 0.5 mmol) was phosphitylated by Method C, to give 254 mg (74%) of 5'-*O*-(*t*-butyldiphenylsilyl)thymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.70-7.63, 7.50-7.36 (m, 11 H, H6, Ph); 6.43-6.39 (m, 1 H, H1'); 4.70-4.62 (m, 1 H, H3'); 4.17-4.09 (m, 1 H, H4'); 4.05-3.53 (m, 6 H, H5', POC*H*$_2$, PNC*H*); 2.67-2.14 (m, 4 H, H2', C*H*$_2$CN); 1.61 (s, 3 H, C*H*$_3$); 1.23-1.05 (m, 12 H, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition A) and the fraction eluted at 20.4 min was collected, to give 72 A_{260} units of ODN-14.

Synthesis of ODN-15---356 mg (0.5 mmol) of 5'-*O*-(*t*-butyldimethylsilyl)thymidine was phosphitylated by Method C, to give 203 mg (73%) of 5'-*O*-(*t*-butyldimethylsilyl)-thymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.54, 7.50 (2s, 1 H, H6); 6.39-6.33 (m, 1 H, H1'); 4.56-4.48 (m, 1 H, H3'); 4.20, 4.11 (2br s, 1 H, H4'); 3.95-3.53 (m, 6 H, H5', POC*H*$_2$, PNC*H*); 2.67-2.62 (m, 2 H, C*H*$_2$CN); 2.52-2.00 (m, 2 H, H2'); 1.91 (s, 3 H, C*H*$_3$); 1.23-1.15 (m, 12 H, (C*H*$_3$)$_2$CH); 0.91 (s, 9 H, *t*Bu); 0.12 (s, 6 H, C*H*$_3$).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition C) and the fraction eluted at 19.2 min was collected, to give 71 A_{260} units of ODN-15.

Synthesis of ODN-16---To a solution of 1.21 g (5 mmol) of thymidine were added 1.17 mL (5.5 mmol) of triisopropylsilyl chloride and 374 mg (5.5 mmol) of imidazole, and the mixture was stirred at room temperature under an Ar atmosphere. After 5 d, the solvent was removed under reduced pressure and the residue was re-dissolved in 100 mL

of EtOAc. The solution was washed twice with 100 mL of sat. aq. $NaHCO_3$ and the organic layer was dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure and the residue was applied on a silica gel column (100 g). Elution was performed with 3% MeOH-CH_2Cl_2 and 1.07 g (53%) of 5'-O-(triisopropylsilyl)thymidine was obtained.

^{1}H-NMR ($CDCl_3$) δ : 8.57 (br s, 1 H, N*H*); 7.45 (s, 1 H, H6); 6.38-6.33 (m, 1 H, H1'); 4.59-4.53 (m, 1 H, H3'); 4.05-4.00 (m, 1 H, H4'); 3.97-3.94 (m, 2 H, H5'); 2.43-2.08 (m, 3 H, H2', O*H*); 1.91 (s, 3 H, C*H*$_3$); 1.20-1.05 (m, 21 H, *i*Pr).

The above compound (199 mg, 0.5 mmol) was phosphitylated by Method C, to give 252 mg (84%) of 5'-*O*-(triisopropylsilyl)thymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)-phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.48, 7.44 (2s, 1 H, H6); 6.38-6.31 (m, 1 H, H1'); 4.67-4.58 (m, 1 H, H3'); 4.17, 4.10 (2br s, 1 H, H4'); 4.05-3.53 (m, 6 H, H5', POC*H*$_2$, PNC*H*); 2.67-2.05 (m, 4 H, H2', C*H*$_2$CN); 1.92 (s, 3 H, C*H*$_3$); 1.33-1.02 (m, 33 H, (C*H*$_3$)$_2$CHN, *i*PrSi).

The above compound was applied to the DNA synthesizer according to Method D. Purification was performed by HPLC (condition A) and the fraction eluted at 15.1 min was collected, to give 107 A_{260} units of ODN-16.

Synthesis of ODN-17---220 μL (2 mmol) of 2-phenylethanol was phosphitylated by Method C, to give 397 mg (63%) of 2-cyanoethyl 2-phenylethyl *N,N*-diisopropyl phosphoramidite.

^{1}H-NMR (60 MHz, $CDCl_3$) δ : 7.28 (s, 5 H, Ph); 4.10-3.25 (m, 6 H, POC*H*$_2$, PNC*H*); 2.90 (t, 2 H, J = 7.2 Hz, PhC*H*$_2$); 2.50 (t, 2 H, J = 6.6 Hz, C*H*$_2$CN); 1.30-1.00 (m, 12 H, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method D using pyridine as a solvent of the final coupling reaction. Purification was performed by HPLC (condition B) and the fraction eluted at 22.2 min was collected, to give 195 A_{260} units of ODN-17.

Synthesis of ODN-18---99 mg (0.5 mmol) of 2,2-diphenylethanol was phosphitylated by Method C, to give 187 mg (94%) of 2-cyanoethyl 2,2-diphenylethyl *N,N*-diisopropyl phosphoramidite.

^{1}H-NMR ($CDCl_3$) δ : 7.32-7.13 (m, 10 H, Ph); 4.35-3.47 (m, 7 H, POC*H*$_2$, PNC*H*, Ph$_2$C*H*); 2.35-2.27 (m, 2 H, C*H*$_2$CN); 1.15-1.02 (m, 12 H, (C*H*$_3$)$_2$CH).

The above compound was applied to the DNA synthesizer according to Method D using pyridine as a solvent of the final coupling reaction. Purification was performed by HPLC (condition B) and the fraction eluted at 27.3 min was collected, to give 173 A_{260} units of ODN-18.

Synthesis of ODN-19---773 mg (2 mmol) of cholesterol was phosphitylated by Method C, to give 1.04 g (89%) of cholesteryl 2-cyanoethyl *N*,*N*-diisopropyl phosphoramidite.

^{1}H-NMR (60 MHz, $CDCl_3$) δ : 5.33 (br s, 1 H, vinyl-*H* of cholesterol); 4.05-3.20 (m, 5 H, POC*H*$_2$, POC*H*, PNC*H*); 2.75-0.50 (m, 57 H, C*H*$_2$CN, (C*H*$_3$)$_2$CH, cholesterol).

The above compound was applied to the DNA synthesizer according to Method D using pyridine as a solvent of the final coupling reaction. Purification was performed by HPLC (condition F) and the fraction eluted at 61.3 min was collected, to give 33 A_{260} units of ODN-19.

Measurement of anti-HIV-1 activity in MT-4 cells

Measurement of anti-HIV-1 activity in MT-4 cells was performed as described previously.[20] Exponentially growing MT-4 cells were centrifuged for 5 min at 140 x g. The MT-4 cell pellet was infected with HIV-1 stock solution at 100 $CCID_{50}$ in RPMI-1640 medium. After a 1-hr incubation at 37 °C, the MT-4 cells were resuspended at 4 x 10^5 cells/ml in RPMI-1640 medium containing 10% FCS, and then 4 x 10^4 cells in 0.1 ml were brought into each well of a flat-bottomed 96-well culture plate containing 0.1 ml each of serial two-fold dilutions of each of the 5'-end-substituted AS-ODNs. After a 6-day incubation at 37 °C, the cytopathic effect of HIV-1 was estimated from the viability of HIV-1-infected cells according to the MTT method.

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