Biologically Active Oligodeoxyribonucleotides. 5.[1] 5′-End-Substituted d(TGGGAG) Possesses Anti-Human Immunodeficiency Virus Type 1 Activity by Forming a G-Quadruplex Structure

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Received September 29, 1997

A series of hexadeoxyribonucleotides (6-mers), d(TGGGAG), substituted with a variety of aromatic groups at the 5′-end were synthesized and tested for anti-human immunodeficiency virus type 1 (HIV-1) activity. While unmodified d(TGGGAG) (**31**) had no anti-HIV-1 activity, compound **23** with a 3,4-di(benzyloxy)benzyl (DBB) group at the 5′-end potently inhibited the HIV-1_{IIIB}-induced cytopathicity of MT-4 cells in vitro (IC_{50} = 0.37 μM) without cytotoxicity up to 40 μM. A thermal denaturation study on the 5′-end-substituted 6-mers by means of the circular dichroism (CD) spectra demonstrated that the aromatic substituent attached at the 5′-end of the 6-mer strongly enhanced the formation of a parallel helical structure consisting of four strands (quadruplex). On the contrary, compound **36**, in which one of the guanosines of **23** was replaced by a thymidine, did not form a quadruplex, thus exhibiting no anti-HIV-1 activity. Moreover, both compound **15**, with a *tert*-butyldiphenylsilyl group solely at its 3′-end, and compound **21**, with a relatively small substituent, a benzyl group, at the 5′-end, formed quadruplexes but had no anti-HIV-1 activity. These findings led us to the conclusion that both the quadruplex structure and the aromatic substituent with adequate size at the 5′-end are crucial for the interaction of the 5′-end-substituted 6-mers with the V3 loop as well as the CD4 binding site on viral gp120, resulting in anti-HIV-1 activity.

Introduction

Since antisense oligodeoxyribonucleotides (AS-ODNs) can interfere with transcription, mRNA processing, or translation by the formation of a specific duplex with the cognate mRNA, the development of these compounds as potential therapeutic agents has received increasing attention in recent years.[2] There are a number of reports regarding the biological activities of both the AS-ODNs having natural-type oligodeoxyribonucleotide (ODN) structures[3] and the backbone-modified AS-ODNs that are designed to be resistant to enzymatic degradation[4,5] or more effectively transported into living cells than the natural-type counterpart.[6,7]

While the AS-ODN binds to the cognate mRNA, there are some other ODNs that bind to specific proteins in place of mRNAs. Bock et al.[8] reported that a pentadecadeoxyribonucleotide (15-mer) with a 5′-GGTTGGTGTGGTTGG-3′ sequence bound to thrombin to inhibit thrombin-catalyzed fibrin-clot formation in vitro. Ojwang et al.[9] reported that a heptadecadeoxyribonucleotide (5′-GTGGTGGGTGGGTGGGT-3′), which had single phosphorothioate internucleoside linkages at its 5′- and 3′-ends (T30177, also known as AR177), inhibited HIV integrase activity in HIV-infected cells. T30177 proved to favor the formation of a compact, intramolecularly folded structure dominated by two stacked guanosine-quartets (G-quartets), resulting in resistance to nucleases.[9−11] Wyatt et al.[12,13] reported that a phosphorothioate-type octadeoxyribonucleotide with a 5′-TTGGGGTT-3′ sequence (ISIS5320, Chart 1) formed a tetrameric structure arranged in a parallel helix stabilized by G-quartets and bound to the cationic V3 loop region of the viral envelope glycoprotein gp120. As a result, both cell-to-cell transmission and virus-to-cell transmission of HIV-1 were inhibited by submicromolar concentrations of ISIS5320.

For the last several years, we have studied the anti-HIV-1 activity of ODNs with natural-type phosphodiester backbone. First, the 15-mers complementary to six sites in HIV-1 RNA were tested for anti-HIV-1 activity, and all 15-mers were found to be inactive in our assay system. Unexpectedly, a synthetic precursor of one of the six 15-mers, with a 4,4′-dimethoxytrityl (DMTr) group at the 5′-end, complementary to the *tat* second splicing acceptor region of HIV-1 (5′-TGGGAGGTGGGTCTG-3′) (**1**), was found to be the only active

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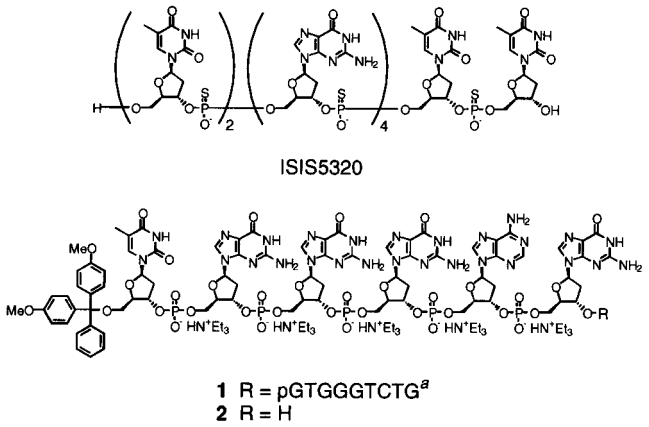
S0022-2623(97)00658-4 CCC: $15.00
Published on Web 08/15/1998

Chart 1



[a] This stands for 5′-phosphorylated nonanucleotide with a 5′-GTGGGTCTG-3′ sequence bound to the 3′-end of **2** via a phosphodiester linkage.

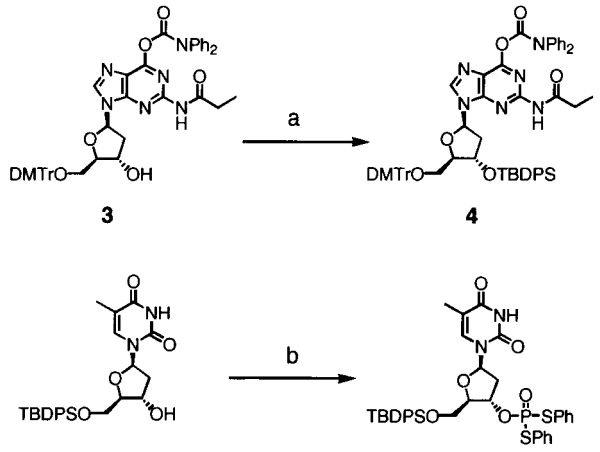
compound of all the compounds tested.[14] The following study on the structure−activity relationship (SAR) demonstrated that an aromatic group attached at the 5′-end strongly enhanced the anti-HIV-1 activity of the 15-mer.[15] Recently, the 15-mer with a DMTr group at its 5′-end proved to interfere with both viral adsorption to the cell membrane and cell fusion (syncytium formation) by interacting with both the V3 loop and the CD4 binding site on the HIV envelope glycoprotein gp120, instead of the so-called "antisense" fashion.[16] A recent study has also revealed that the 5′-region of the 5′-end-substituted 15-mer containing hexadeoxyribonucleotide (6-mer), 5′-TGGGAG-3′ (**2**), is important for its anti-HIV-1 activity.[17,18] ODN **2** also proved to exhibit anti-HIV-1 activity by the same mechanism of action as the 5′-end-substituted 15-mer **1**.[18] This paper describes the SAR of the 5′-end-substituted 6-mers and their conformations in solution.

Chemistry

The 6-mers with a 5′-TGGGAG-3′ sequence and attaching a *tert*-butyldiphenylsilyl (TBDPS) group at the 3′-end were synthesized by the liquid-phase phosphotriester method. First, both 3′-*O*-TBDPS-guanosine derivative **4** and 5′-*O*-TBDPS-thymidine derivative **6** were synthesized as shown in Scheme 1. Next, the dimer unit of guanylyl-(3′→5′)-adenosine **9** was synthesized according to Hata's procedure as follows (Scheme 2).[19,20] One of two P−S bonds of **7** was cleaved by selective hydroxylation using Et_3N-H_2O−pyridine, followed by condensation with the compound obtained by the acidic treatment of **8** to give the dimer **9**.

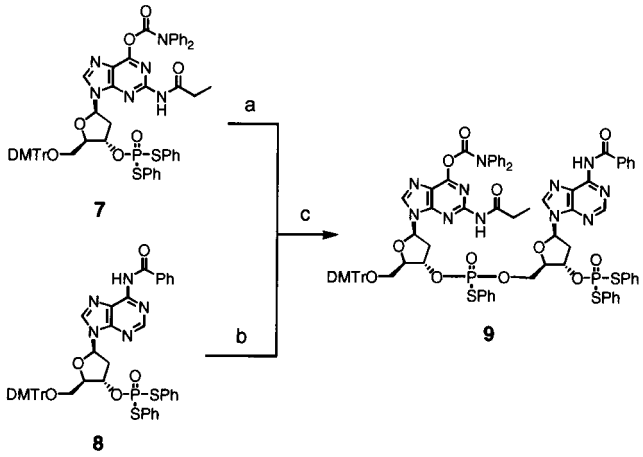
Scheme 3 illustrates the block condensation procedure used to obtain the 6-mers with TBDPS groups. One of two PhS groups at the 3′-end of **9** was selectively removed using triethylammonium hypophosphate, followed by condensation with the compound obtained by the acidic treatment of **4** to give the fully protected trimer **10**. The above procedure was repeated using the dimer unit **11** and thymidine monomers **13** or **6** to give two types of protected hexamers (**14a**,**b**). Deprotection of **14a** containing one TBDPS group was carried out as follows. Selective hydroxylation of the P−S bonds of **14a** using aqueous NaOH and the ammonolysis of the protecting groups bound to the nucleoside bases followed

Scheme 1[a]



[a] Reagents: (a) TBDPSCl, imidazole, DMF; (b) $(PhS)_2P(O)O^-H_3N^+C_6H_{11}$, 2,4,6-triisopropylbenzenesulfonyl chloride, 1*H*-tetrazole, pyridine.

Scheme 2[a]



[a] Reagents: (a) Et_3N, H_2O, pyridine; (b) TFA, $CHCl_3$; (c) 2,4,6-triisopropylbenzenesulfonyl chloride, 3-nitro-1,2,4-triazole, pyridine.

by the removal of the DMTr group attached at the 5′-end using aqueous acetic acid led to the desired compound **15** with a TBDPS group solely at its 3′-end. Deprotection of **14b** was carried out the same way as deprotection of **14a**, excluding treatment with aqueous acetic acid, to give the desired compound **16** with two TBDPS groups, one at each end. Compounds **36** and **37** were purchased from GENSET.

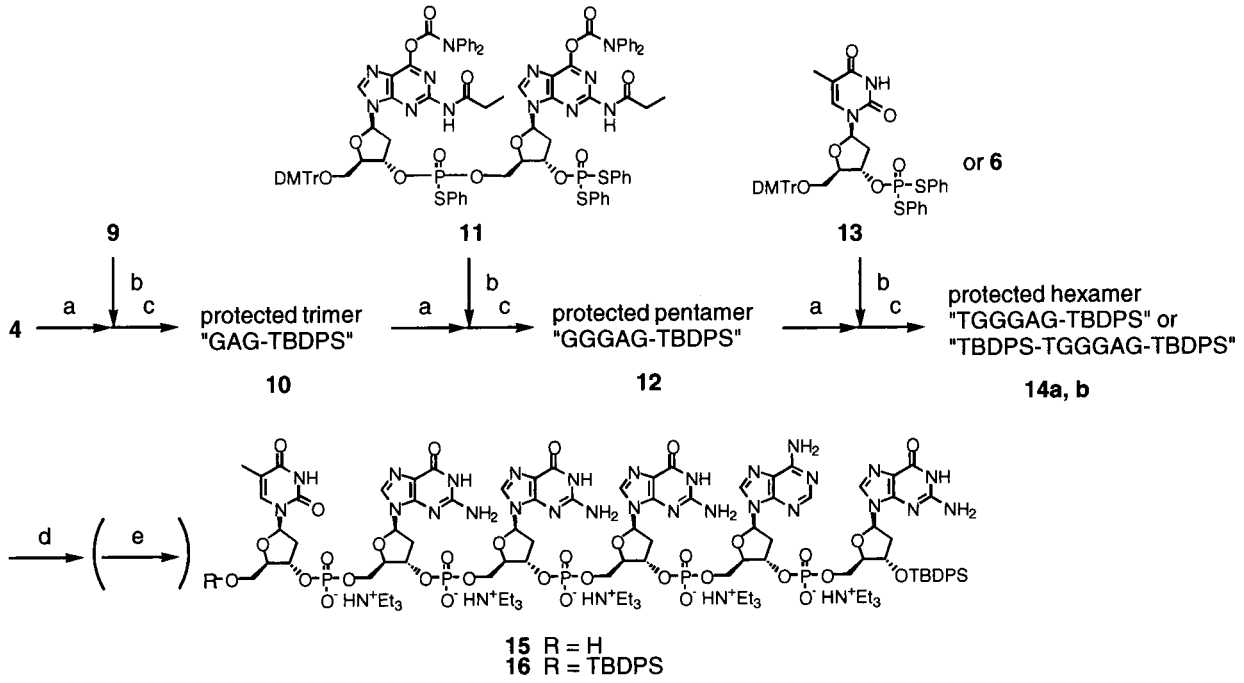
The rest of the 5′-end-substituted 6-mers were synthesized according to the solid-phase phosphoramidite method using an automatic DNA synthesizer by simple modification of the nucleotide sequence described in a previous paper.[15]

All compounds tested were purified using preparative reverse-phase HPLC before being used in biological assay. Negative ion LSI mass spectroscopy[21] has proved to be a good method for rapid confirmation of the structures (see Supporting Information).

Results and Discussion

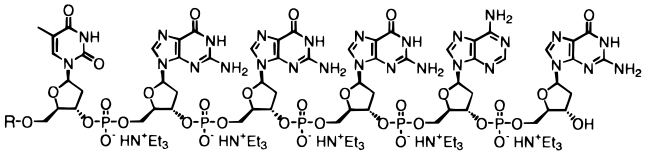
To evaluate the anti-HIV-1 activity of the 5′-end-substituted 6-mers along with an unmodified 6-mer,

Scheme 3[a]



[a] Reagents: (a) TFA, $CHCl_3$; (b) 3.3 M H_3PO_4, Et_3N, pyridine; (c) 2,4,6-triisopropylbenzenesulfonyl chloride, 3-nitro-1,2,4-triazole, pyridine; (d) (i) 0.1 N NaOH, H_2O, pyridine, (ii) 28% aqueous NH_4OH; (e) AcOH, H_2O.

Table 1. In Vitro Anti-HIV-1 Activity of 5′-End-Modified d(TGGGAG)[a]



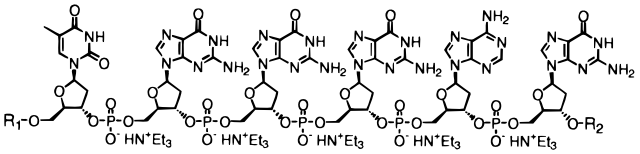
compd	R	IC_{50} (μM)[b]	CC_{50} (μM)[c]
2	$(4\text{-MeO-}C_6H_4)_2PhC$	1.0 ± 0.21	38
17	Ph_3C	0.80 ± 0.10	23 ± 2.4
18	9-Ph-xanthen-9-yl	0.95 ± 0.18	>40
19	9-Ph-fluoren-9-yl	1.3 ± 0.16	>40
20	Ph_2CH	4.3 ± 0.71	>40
21	$PhCH_2$	>40	>40
22	$4\text{-}(PhCH_2O)\text{-}C_6H_4CH_2$	1.9 ± 0.35	>40
23	$3,4\text{-}(PhCH_2O)_2\text{-}C_6H_3CH_2$	0.37 ± 0.060	>40
24	$3,5\text{-}(PhCH_2O)_2\text{-}C_6H_3CH_2$	0.49 ± 0.064	>40
25	$2\text{-Ph-}C_6H_4CH_2$	1.2 ± 0.19	>40
26	$4\text{-Ph-}C_6H_4CH_2$	2.8 ± 0.66	>40
27	2-naphthyl-CH_2	2.0 ± 0.44	>40
28	1-pyrenyl-CH_2	1.8 ± 0.24	>40
29	$^t\text{Bu(Ph)}_2\text{Si}$	0.88 ± 0.070	>40
30	$^t\text{Bu(Me)}_2\text{Si}$	>40	1.2 ± 0.82

[a] Values represent the mean of duplicate or quadruplicate ± SD. [b] The 50% inhibitory doses for the cytopathicities induced by $HIV\text{-}1_{IIIB}$ were determined in vitro by MTT assay using MT-4 cells. [c] The 50% cytotoxic doses were determined by MTT assay using MT-4 cells.

d(TGGGAG), the 50% inhibitory concentrations (IC_{50}) for inhibiting the cytopathicity induced by $HIV\text{-}1_{IIIB}$ were determined in vitro by MTT assay using MT-4 cells.[22] The 50% cytotoxic concentrations (CC_{50}) were also determined using MT-4 cells. The 6-mer d(TGGGAG) with a DMTr group at its 5′-end (**2**) has been found to possess potent anti-HIV-1 activity with moderate cytotoxicity (Table 1).[17,18] Consequently, 6-mers with a variety of aromatic substituents were examined initially to clarify the SAR for the 5′-end-substituted 6-mers. As shown in Table 1, the 6-mer substituted with a trityl group (**17**) in place of a DMTr group had relatively potent anti-HIV-1 activity along with moderate cytotoxicity like **2**. Compounds **18** and **19**, in which two of three aromatic moieties of the tertiary alkyl substituents were linked covalently, showed no cytotoxicity up to 40 μM but potent anti-HIV-1 activity. Compound **20** with a secondary alkyl (benzhydryl) group showed moderate anti-HIV-1 activity without cytotoxicity, and compound **21** with a primary alkyl (benzyl) group showed no anti-HIV-1 activity and no cytotoxicity. These results demonstrate that reduction in the number of phenyl groups of **17** resulted in a reduction of both anti-HIV-1 activity and cytotoxicity. Next, the phenyl group of **21** was further substituted with some aromatic groups. The anti-HIV-1 activity of **22** with a benzyloxy group as an extra substituent of **21** was moderate. Addition of another benzyloxy group to **22** gave **23** and **24**, both of which showed very potent anti-HIV-1 activity. The anti-HIV-1 activity of 6-mers containing biphenyl moieties (**25** and **26**) or fused aromatics (**27** and **28**) as substituents was moderate. A TBDPS group was also found to be as effective as the 5′-substituent of the 6-mer (**29**). However, the 6-mer with a *tert*-butyldimethylsilyl (TBDMS) group (**30**) showed no anti-HIV-1 activity and potent cytotoxicity. As a result, compound **23** with a 3,4-di(benzyloxy)benzyl (DBB) group was the most potent of all candidates listed in Table 1.

Next, the effect of 5′-end substitution was assessed by comparing it with 3′-end substitution. A TBDPS group was used as the substituent because it was relatively easy to introduce at the 3′-end of the 6-mer. As shown in Table 2, it is obvious that 5′-end substitution is crucial for anti-HIV-1 activity. While compound **29** with a TBDPS group at its 5′-end possessed potent anti-HIV-1 activity, the unmodified 6-mer (**31**) showed

Table 2. Modification Effect of a TBDPS Group on the in Vitro Anti-HIV-1 Activity of d(TGGGAG)[a]



compd	R_1	R_2	IC_{50} (μM)[b]	CC_{50} (μM)[c]
31	H	H	>40	>40
15	H	^{t}Bu(Ph)$_2$Si	>40	7.7
29	^{t}Bu(Ph)$_2$Si	H	0.88 ± 0.070	>40
16	^{t}Bu(Ph)$_2$Si	^{t}Bu(Ph)$_2$Si	6.0	>40

[a−c] See legends of Table 1.

Table 3. Sequence Effect on in Vitro Anti-HIV-1 Activity of ODNs Having a 3,4-Di(benzyloxy)benzyl Group[a]

compd	sequence	IC_{50} (μM)[b]	CC_{50} (μM)[c]
23	TGGGAG	0.37 ± 0.060	>40
32	TGGGA	1.9 ± 0.24	>40
33	TGGG	4.6 ± 0.56	>40
34	TGG	>70	>70
35	TG	>100	>100
36	TGTGAG	>24	>24
37	TTTTTT	>24	>24
38	TGGGG	0.66 ± 0.036	>40
39	TGGGGG	0.26 ± 0.032	>40
40	TTGGGG	0.56 ± 0.15	>40
41	TTGGG	>40	40
42	TTGG	>50	33 ± 2.4

[a−c] See legends of Table 1.

no anti-HIV-1 activity and no cytotoxicity up to 40 μM. Compound **15** with a TBDPS group solely at its 3′-end showed no anti-HIV-1 activity with moderate cytotoxicity. The additional substitution of another TBDPS group at the 3′-end of **29** reduced the anti-HIV-1 activity (**16**).

The results obtained so far indicate that the 5′-end substitution of the 6-mer is essential for anti-HIV-1 activity and the DBB group is the most effective substituent of all tested. The remaining part of the SAR study was to examine the sequence effect of the 6-mer. Thus, the 5′-end substituent was fixed with a DBB group, and the sequence of the ODN was changed. First, the nucleotide sequence of **23** was truncated gradually from the 3′-end toward the 5′-direction (**32**−**35**). As shown in Table 3, it is obvious that the anti-HIV-1 activity becomes less potent as the nucleotide chain is decreased. Compound **33**, with a TGGG sequence, was found to contain the minimum structural requirement for anti-HIV-1 activity in our assay system. While the 4,4′-dimethoxytritylated ODNs with sequences TGGGAG, TGGGA, TGGG, and TGG possessed moderate cytotoxicity,[17,18] compounds **23** and **32**−**35** containing a DBB group in place of a DMTr group showed no cytotoxicity up to 40 μM. Both compounds **36**, in which one of the guanosines of **23** was replaced by a thymidine, and **37**, with a TTTTTT sequence, were inactive. Compound **39**, in which the adenosine of **23** was replaced by a guanosine, was found to be more potent than the parent compound **23**. The ODN containing a TT sequence at its 5′-end was not appropriate because of its tendency to exert moderate cytotoxicity (**41**, **42**). It is well-known that an ODN with a high content of guanosine forms a G-quadruplex structure, that is, a helical structure consisting of four ODN strands, stabilized by the formation of G-quartets.[23,24] Accordingly, compound **39** with a sequence containing five contiguous guanosines could form a G-quadruplex.[25] In fact, the purification of **39** by preparative reverse-phase HPLC was somewhat troublesome because a major peak with a longer retention time than the peak of monomeric **39** appeared. This additional peak was thought to be attributable to the G-quadruplex formed by self-assembly of **39** under HPLC conditions because the peak was reproduced by HPLC analysis of the fraction of monomeric **39** isolated by preparative HPLC.

To clarify the presence of the G-quadruplex structures of the 6-mers in solution, the CD spectra of compounds **15**, **21**, **23**, **29**, **31**−**37**, and **39** were measured. Figure 1A−G shows the CD spectra of these ODNs in 10 mM PBS buffer (pH 7) at about 1 A_{260} unit/mL concentrations (ca. 20 μM). As shown in Figure 1A, the spectrum of **31**, which was not substituted and had no anti-HIV-1 activity, had a positive Cotton effect at 253 nm. The presence of the 253-nm band is consistent with the CD spectrum expected by calculation using the nearest-neighbor method.[26] The molecular ellipticity for this area decreased with the increase in temperature. However, the temperature dependency of the spectrum of **31** was not so drastic. The clear isosbestic points at 262 and 238 nm demonstrate a two-state transition probably between disordered monomer and the monomer strand ordered by intramolecular stacking interactions. On the contrary, the spectra of **23** having a DBB group at the 5′-end was drastically changed as a function of temperature (Figure 1B). While the spectra of **23** at above 50 °C were almost the same as those of **31**, the spectra at below 40 °C had a strong positive Cotton effect at about 264 nm, which is typical of CD spectra of parallel quadruplexes with guanine-rich sequences.[27] The shift of the CD band as a function of temperature demonstrates that **23** forms a parallel G-quadruplex stabilized by G-quartets (Chart 2) at ambient temperature and about 20 μM concentration, and the helix dissociates gradually at above 50 °C. Compound **29** with a TBDPS group at the 5′-end gave almost the same result as **23** (Figure 1C). Compound **15** having a TBDPS group at the 3′-end without anti-HIV-1 activity also forms a G-quadruplex; however, the G-quadruplex of **15** is less stable than that of **29** (Figure 1D). It is presumed by comparison of compounds **29** and **15** that the formation of a G-quadruplex is not enough for anti-HIV-1 activity and the aromatic substituent must be attached not at the 3′-end but at the 5′-end. Another example is **21** having a relatively small substituent, a benzyl group, at the 5′-end. Compound **21** having no anti-HIV-1 activity also forms a G-quadruplex (Figure 1E). This result demonstrates that the 5′-substituents of the G-quadruplex-forming ODNs must have enough size (or suitable number of phenyl groups) as mentioned above. Next, the 5′-substituent was fixed with the most suitable DBB group, and the sequence effect was examined. Figure 1F shows the CD spectra for the ODNs truncated gradually from the 3′-end of **23**. While compounds **23**, **32**, and **33** having anti-HIV-1 activity proved to form G-quadruplexes, compounds **34** and **35** without anti-HIV-1 activity were too short to form G-quadruplexes. Moreover, compound **36**, in which one of the guanosines

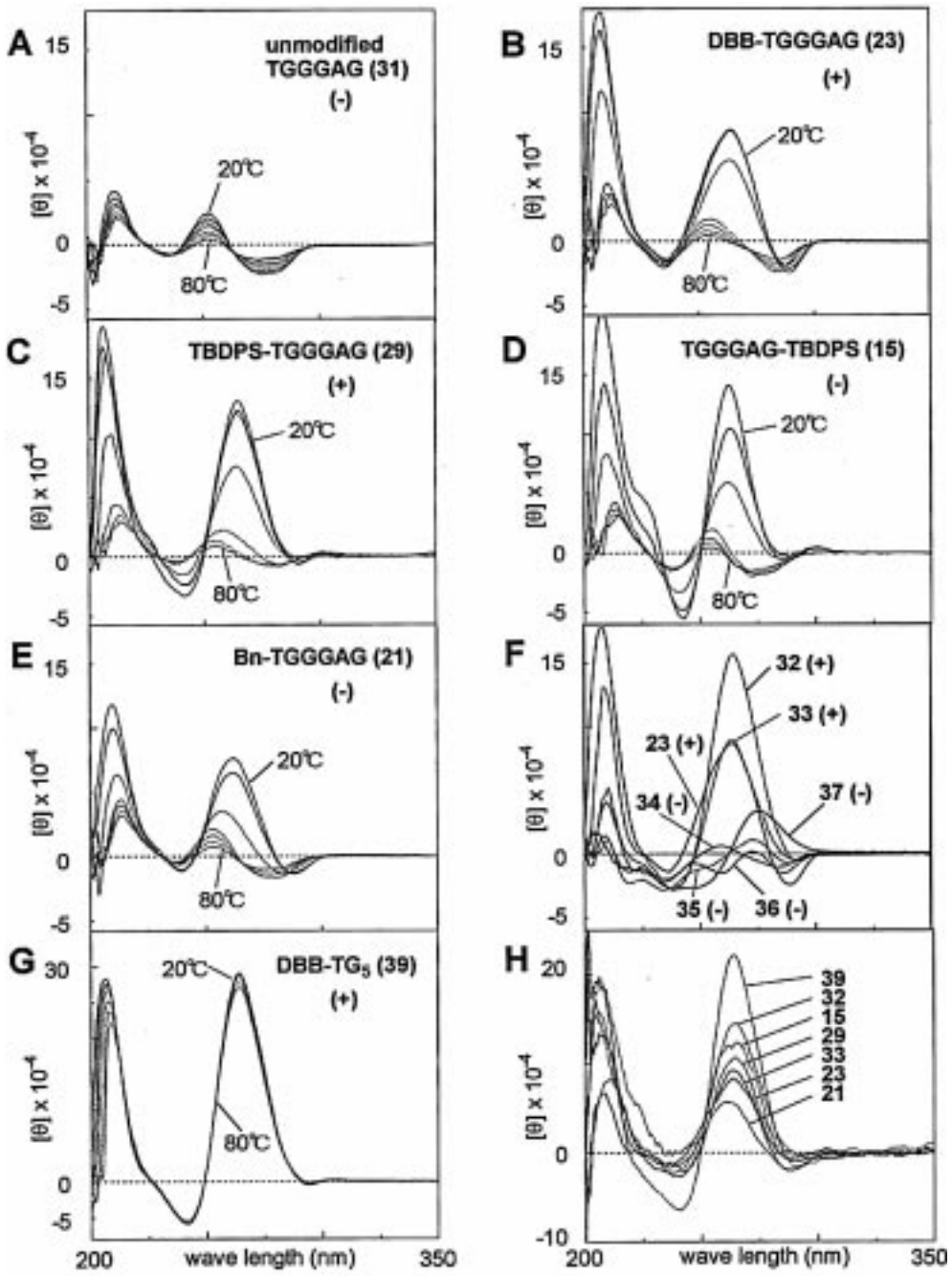


Figure 1. CD spectra for the 5′-end-modified 6-mers. A−E, G: CD spectra for ca. 1 A_{260} unit/mL concentrations (ca. 20 μM) of **31** (A), **23** (B), **29** (C), **15** (D), **21** (E), and **39** (G) in 10 mM PBS buffer (pH 7) at 20 °C (black), 30 °C (blue), 40 °C (red), 50 °C (green), 60 °C (pink), 70 °C (light blue), and 80 °C (light brown). F: CD spectra for ca. 1 A_{260} concentrations of **23** and **32**−**37** in 10 mM PBS buffer (pH 7) at ambient temperature. H: CD spectra for ca. 0.5 μM concentrations of G-quadruplex-forming ODNs; (+), anti-HIV-1 active; (−), anti-HIV-1 inactive.

of **23** was replaced by a thymidine, did not form a G-quadruplex, thus exhibiting no anti-HIV-1 activity. Inactive compound **37** with a TTTTTT sequence did not form a G-quadruplex, as was to be expected because of the absence of guanosine. The above results suggest the apparent relationship between G-quadruplex formation and anti-HIV-1 activity. The spectrum of **39** with a 5′-TGGGGG-3′ sequence indicates that **39** shows a very thermodynamically stable G-quadruplex (Figure 1G). This is attributable to the presence of five contiguous guanosines as discussed above. The G-quadruplex of **39** hardly dissociated even at 80 °C. Figure 1H shows the CD spectra of G-quadruplex-forming ODNs at about 0.5 μM concentrations in PBS buffer at ambient temperature. All the CD spectra still had a positive Cotton effect at around 264 nm, demonstrating that these ODNs form G-quadruplexes at the minimum concentrations (about IC_{50} for **23**) required for anti-HIV-1 activity.

Finally, the stability of **23** and **31** in human plasma was assessed in vitro. As shown in Figure 2, an apparent stabilization effect by the modification at the 5′-end of **31** was observed. The half-lives of **23** and **31** were roughly 1 h and 15 min, respectively. The nuclease resistance of **23** may be partially attributable to the G-quadruplex structure containing G-quartet motifs.[11]

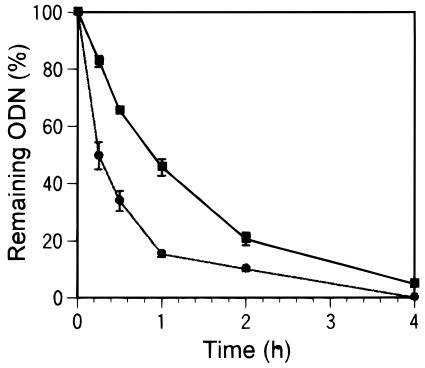
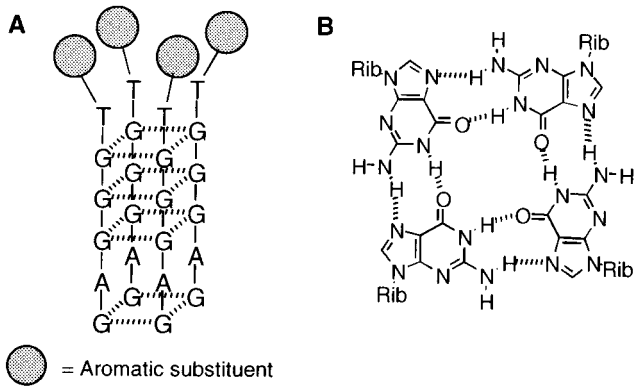


Figure 2. Stability of **23** (■) and **31** (●) in 93% human plasma. Initial concentration of ODN: 20 μg/mL; $n = 3$; mean ± SD. ODNs **23** and **31** were incubated with human plasma, and these reaction mixtures were analyzed by reverse-phase HPLC at 0, 0.25, 0.5, 1, 2, and 4 h after initial mixing to quantify the amount of ODNs remaining.

Chart 2. Parallel G-Quadruplex Structure (A) and G-Quartet (B)



Conclusion

Our previous efforts in both synthetic and biological studies on 5′-end-substituted ODNs with anti-HIV-1 activity have led to the identification of non-antisense lead compound **2** with a 5′-TGGGAG-3′ sequence.[17,18,28] The present study dealt with the optimization of both the 5′-end substituent and the sequence of the ODN. A 3,4-di(benzyloxy)benzyl (DBB) group was found to be the best substituent of all tested with respect to both potent anti-HIV-1 activity and low cytotoxicity. In terms of optimal sequence, compound **39** with a 5′-TGGGGG-3′ sequence was perhaps the best on the basis of the anti-HIV-1 activity. Fujihashi et al.[29] reported that oligodeoxyriboguanylic acids with a chain length of more than three nucleotides and their oligoriboguanylic acid counterparts both inhibited cytopathicity induced by HIV-1 in vitro. Since these oligonucleotides containing contiguous guanosines have a tendency to form tetrameric aggregates, the manipulation of these ODNs involving **39** on a large scale was expected to be troublesome. Therefore, we concluded that compound **23**, which showed a simple profile on HPLC analysis, was superior to **39** on the basis of both potent anti-HIV-1 activity and ease of manipulation.

In vitro anti-HIV-1 activity of **23** has proved to be almost the same as that of ISIS5320 ($IC_{50} = 0.3$ μM), a phosphorothioate (PS) octamer.[12] PS-ODNs have appeared to possess a number of biological effects, i.e., (i) nonspecific binding to protein, (ii) prolonged blood coagulation, (iii) complement activation, and (iv) liver and kidney toxicity, that are unrelated to their intended antisense activity.[30] Compound **23** with a natural-type phosphodiester backbone might not have the drawbacks of PS-ODNs.

Recently, Lacoste et al.[31] reported that an acridine moiety attached at the 5′-end of triplex-forming ODN stabilized the triplex by intercalation of the acridine ring at the triplex/duplex junction. Bates et al.[32] successfully used a psoralen moiety as a 5′-end substituent of triplex-forming ODN to form triplex-directed photoadduct. As to the stability of the quadruplex, Wolfe et al.[33] reported that a cholesteryl substituent attached at the 3′-terminal phosphodiester bond of the phosphorothioate 20-mer, 5′-TGGGGCTTACCTTGCGAACA-3′, stabilized the quadruplex structure. In our present study, thermal denaturation of the 5′-end-substituted 6-mers measured by CD spectra indicated that the aromatic substituents strongly enhanced the formation of G-quadruplex structures. In both cases, hydrophobic interactions of the substituents might have contributed to the stability of the structure.

All ODNs with anti-HIV-1 activity in the present study form G-quadruplexes. In addition, compound **36**, in which one of the guanosines of **23** was simply replaced by a thymidine, did not form a G-quadruplex, thus exhibiting no anti-HIV-1 activity. These results suggest that the G-quadruplex structures are the ultimate active species. On the other hand, both compound **15** with a 3′-end substituent and compound **21** with a relatively small 5′-end substituent, a benzyl group, form G-quadruplexes but have no anti-HIV-1 activity. These findings led us to the conclusion that both the G-quadruplex structure and the aromatic substituent with adequate size at the 5′-end were crucial for anti-HIV-1 activity. The mechanism for anti-HIV-1 activity of 5′-end-substituted 6-mers has been elucidated as the interference of both cell-to-cell transmission and virus-to-cell transmission of HIV-1 by interaction of the 6-mers with both the V3 loop and the CD4 binding site on viral gp120.[17,18] We presume that both the G-quadruplex-forming ODN backbone and the cluster of 5′-end substituents might have contributed to the interaction of the 6-mers with viral gp120. At present, the results of the CD experiment are the only clues for determining the fundamental conformation of **23** which possesses potent anti-HIV-1 activity. Further experiments involving NMR analysis are currently underway in an attempt to gain insight into the structural and conformational requirements of this series of compounds.

Experimental Section

Chemistry. ^{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 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 equipped with an L-5000 LC controller, an L-3000 photodiode array detector, and a D-2500 chromato-integrator.

Compounds **17**−**35** and **38**−**42** were synthesized using a Cyclone Plus DNA synthesizer equipped with additional

reagents, 5′-end-substituted thymidine derivatives, which were synthesized according to the previous method.[15] Compounds **36** and **37** were purchased from GENSET. Compounds **15** and **16** were synthesized on the basis of Hata's procedure.[19] Thus, intermediates **3**, **7**, **8**, **11**, and **13** were synthesized according to the literature.[20] Intermediate **5** was also synthesized according to the literature.[15] Syntheses of other intermediates (**4**, **6**, **9**, **10**, **12**, and **14a**,**b**) and desired compounds **15** and **16** were performed as follows.

3′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine (4). Alcohol **3** (164 mg, 0.2 mmol) and imidazole (27 mg, 0.4 mmol) were dissolved in DMF (1 mL). To the solution was added *tert*-butyldimethylsilyl chloride (78 μL, 0.3 mmol), and the total mixture was stirred at room temperature. After 42 h, 5% aqueous $NaHCO_3$ (50 mL) was added, and the resulting mixture was extracted with EtOAc (50 mL). The organic layer was washed with 5% aqueous $NaHCO_3$ (50 mL) and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was eluted on a silica gel column (20 g) with 1% MeOH−CH_2Cl_2 to give **4** (151 mg, 71%). Rotational isomers were observed: ^{1}H NMR ($CDCl_3$) δ 7.96 and 7.81 (2s, 1H), 7.63−6.68 (m, 33H), 4.52 (br s, 1H), 4.22 (br s, 1H), 3.79 and 3.72 (2s, 6H), 3.07 (m, 2H), 2.80 (m, 2H), 2.43 (m, 2H), 1.19 (t, J = 7.3 Hz, 3H), 1.07 (s, 9H); HRMS (FAB) m/z 1059.4476 (M + H)$^+$ Δ_{ppm} = 0.

***S,S′*-Diphenyl 5′-*O*-(*tert*-Butyldiphenylsilyl)thymidine-3′-*O*-phosphorodithioate (6).** Alcohol **5** (48 mg, 0.1 mmol), cyclohexylammonium *S,S′*-diphenylphosphorodithioate (76 mg, 0.2 mmol), and 1*H*-tetrazole (14 mg, 0.2 mmol) were coevaporated with pyridine, and the residue was dissolved in pyridine (1 mL). To the solution was added 2,4,6-triisopropylbenzenesulfonyl chloride (60 mg, 0.2 mmol), and the total mixture was stirred at room temperature. After 1 h, the reaction mixture was diluted with EtOAc (50 mL), and the solution was washed with 5% aqueous $NaHCO_3$ (2 × 50 mL) followed by drying over $MgSO_4$. The solvent was removed by evaporation, and the residue was eluted on a silica gel column (5 g) with 3% MeOH−CH_2Cl_2 to give **6** (62 mg, 91%): ^{1}H NMR ($CDCl_3$) δ 8.05 (br s, 1H), 7.67−7.30 (m, 21H), 6.33 (dd, J = 5.2, 9.3 Hz, 1H), 5.34 (dd, J = 5.8, 9.5 Hz, 1H), 4.02 (m, 1H), 3.90 (dd, J = 2.1, 11.7 Hz, 1H), 3.81 (dd, J = 2.1, 11.7 Hz, 1H), 2.39 (dd, J = 5.3, 13.9 Hz, 1H), 2.20 (m, 1H), 1.54 (s, 3H), 1.09 (s, 9H); HRMS (FAB) m/z 745.1972 (M + H)$^+$ Δ_{ppm} = −2.6. Anal. ($C_{38}H_{41}N_2O_6S_2PSi\cdot 0.5H_2O$) C, H, N, S, P.

[*S*-Phenyl 5′-*O*-(4,4′-dimethoxytrityl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S,S′*-diphenyl N^6-benzoyladenosine-3′-*O*-phosphorodithioate] (9). *S,S′*-Diphenyl 5′-*O*-(4,4′-dimethoxytrityl)-N^6-benzoyladenosine-3′-*O*-phosphorodithioate (**8**) (856 mg, 1 mmol) was dissolved in $CHCl_3$ (100 mL) and then stirred in an ice−water bath. To the solution was added trifluoroacetic acid (2 mL), and the mixture was stirred for 10 min. The mixture was neutralized by pyridine (3 mL), washed with 5% aqueous $NaHCO_3$ (2 × 50 mL), dried over $MgSO_4$, and then evaporated to give a residue containing a 5′-hydroxyl component. Separately, *S,S′*-diphenyl 5′-*O*-(4,4′-dimethoxytrityl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorodithioate (**7**) (1.225 g, 1.2 mmol) was treated with pyridine (12 mL), triethylamine (12 mL), and H_2O (6 mL) in an ice−water bath for 5 min. The temperature was raised to room temperature, and the reaction was continued for 1 h. The solution was repeatedly coevaporated with pyridine, combined with the above 5′-hydroxyl component and 3-nitro-1,2,4-triazole (342 mg, 3 mmol), coevaporated with pyridine to dryness, and dissolved in pyridine (10 mL). To the solution was added 2,4,6-triisopropylbenzenesulfonyl chloride (606 mg, 2 mmol), and the total mixture was stirred at room temperature for 1 h. The mixture was diluted with CH_2Cl_2 (100 mL) and then washed with 5% aqueous $NaHCO_3$ (2 × 100 mL), and the washings were extracted with CH_2Cl_2 (50 mL). The organic layers were combined, dried over $MgSO_4$, and then evaporated. Chromatography (70−230 mesh silica gel, MeOH−CH_2Cl_2, 2:98) provided **9** (1.227 g, 84%). Compound **9** is a mixture of diastereomers: ^{1}H NMR ($CDCl_3$) δ 9.02 and 9.00 (2s, 1H), 8.24 and 8.22 (2s, 1H), 8.14 (m, 1H), 8.05 (m, 1H), 7.65−7.15 (m, 39H), 6.81 (m, 4H), 6.40 (m, 1H), 6.18−6.04 (m, 1H), 5.57 and 5.43 (2m, 1H), 5.32 (m, 1H), 4.73−4.46 (m, 2H), 4.33−4.18 (m, 2H), 3.72 (s, 6H), 3.48−3.32 (m, 2H), 3.00−2.80 (m, 4H), 2.60−2.38 (m, 1H), 1.18 (m, 3H); HRMS (FAB) m/z 1616.3867 (M + Na)$^+$ Δ_{ppm} = −0.4.

[*S*-Phenyl 5′-*O*-(4,4′-dimethoxytrityl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenyl N^6-benzoyladenosine-3′-*O*-phosphorothioyl]-(3′→5′)-[3′-*O*-(*tert*-butyldimethylsilyl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine](10). Compound **9** (250 mg, 0.17 mmol) was dissolved in 5 M H_3PO_2−pyridine (10.2 mL) and triethylamine (5.1 mL), and the solution was stirred at 40 °C. After 1 h, the mixture was diluted with $CHCl_3$ (100 mL) and washed with 1 M triethylammonium bicarbonate (TEAB) (pH 8, 3 × 100 mL), and the washings were extracted with $CHCl_3$ (100 mL). The organic layers were combined, dried over $MgSO_4$, and then evaporated to give a residue containing a phosphodiester component. This residue and **4** (151 mg, 0.142 mmol) were subjected to the reactions described in the synthesis of **9** to give **10** (150 mg, 50%). Compound **10** is a mixture of diastereomers: ^{1}H NMR ($CDCl_3$) δ 8.97 (m, 1H), 8.72−8.53 (m, 1H), 8.23 (m, 1H), 8.16−7.98 (m, 4H), 7.70−7.04 (m, 54H), 6.80 (m, 4H), 6.40 (m, 2H), 6.13 (m, 1H), 5.41 (m, 1H), 5.25 (m, 1H), 4.68−3.92 (m, 8H), 3.74 (s, 3H), 3.72 (s, 3H), 3.47−3.27 (m, 2H), 3.05−2.12 (m, 10H), 1.28−0.70 (m, 15H).

[*S*-Phenyl 5′-*O*-(4,4′-dimethoxytrityl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenyl N^2-propionyl-O^6-(diphenylcarbamoyl)-guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenyl N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenyl N^6-benzoyladenosine-3′-*O*-phosphorothioyl]-(3′→5′)-[3′-*O*-(*tert*-butyldimethylsilyl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine] (12). Compounds **10** (159 mg, 0.071 mmol) and **11** (139 mg, 0.085 mmol) were subjected to the reactions described in the synthesis of **10** to give **12** (87.6 mg, 37%). Compound **12** is a mixture of diastereomers: ^{1}H NMR ($CDCl_3$) δ 8.92−8.57 (m, 2H), 8.20−7.95 (m, 6H), 7.70−6.90 (m, 87H), 6.74 (m, 4H), 6.45−6.07 (m, 5H), 5.29 (m, 4H), 4.60−3.90 (m, 14H), 3.68 (br s, 6H), 3.45−3.22 (m, 2H), 2.85−2.20 (m, 18H), 1.30−0.70 (m, 21H).

[*S*-Phenyl 5′-*O*-(4,4′-dimethoxytrityl)thymidine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenyl N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenylN^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenyl N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenylN^6-benzoyladenosine-3′-*O*-phosphorothioyl]-(3′→5′)-[3′-*O*-(*tert*-butyldimethylsilyl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine] (14a). Compounds **12** (43 mg, 0.013 mmol) and **13** (20 mg, 0.026 mmol) were subjected to the reactions described in the synthesis of **10** to give **14a** (19.4 mg, 40%). Compound **14a** is a mixture of diastereomers: ^{1}H NMR ($CDCl_3$) δ 9.15−8.62 (m, 3H), 8.20−7.90 (m, 6H), 7.73−6.90 (m, 92H), 6.82 (m, 4H), 6.45−6.05 (m, 6H), 5.40−5.12 (m, 5H), 4.60−3.90 (m, 17H), 3.74 (m, 6H), 3.47−3.12 (m, 2H), 2.85−2.20 (m, 20H), 1.45−0.70 (m, 24H).

[*S*-Phenyl 5′-*O*-(*tert*-butyldimethylsilyl)thymidine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenyl N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenylN^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenyl N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine-3′-*O*-phosphorothioyl]-(3′→5′)-[*S*-phenylN^6-benzoyladenosine-3′-*O*-phosphorothioyl]-(3′→5′)-[3′-*O*-(*tert*-butyldimethylsilyl)-N^2-propionyl-O^6-(diphenylcarbamoyl)guanosine] (14b). Compounds **12** (43 mg, 0.013 mmol) and **6** (20 mg, 0.03 mmol) were subjected to the reactions described in the synthesis of **10** to give **14b** (13.3 mg, 28%). Compound **14b** is a mixture of diastereomers: ^{1}H NMR ($CDCl_3$) δ 9.20−8.60 (m, 3H), 8.20−7.93 (m, 6H), 7.70−

6.90 (m, 93H), 6.45−6.05 (m, 6H), 5.40−5.12 (m, 5H), 4.60−3.54 (m, 19H), 2.90−1.93 (m, 20H), 1.45−0.70 (m, 33H).

Triethylammonium Salt of Thymidine-3′-*O*-phosphoryl-(3′→5′)-guanosine-3′-*O*-phosphoryl-(3′→5′)-guanosine-3′-*O*-phosphoryl-(3′→5′)-guanosine-3′-*O*-phosphoryl-(3′→5′)-adenosine-3′-*O*-phosphoryl-(3′→5′)-3′-*O*-(*tert*-butyldimethylsilyl)guanosine (15). Compound **14a** (10 mg), pyridine (1 mL) and 0.2 N aqueous NaOH (1 mL) were stirred in an ice−water bath for 30 min. The mixture was applied on a column of Dowex 50Wx2 (ϕ1 × 5 cm, pyridinium form), followed by elution with pyridine−H_2O (1:1, v/v, 20 mL). The eluent was evaporated, and the residue was dissolved in 28% aqueous NH_4OH (20 mL). The resulting solution was stirred at room temperature for 18 h and then at 50 °C for 3 h. The solution was evaporated to dryness, and the residue was dissolved in AcOH−H_2O (4:1, v/v, 10 mL), stirred at room temperature for 15 min, evaporated to dryness, and then dissolved in 0.1 M triethylammonium acetate (TEAA) (pH 7, 2 mL). The solution was subjected to preparative HPLC (Inertsil Prep-ODS, ϕ20 × 250 mm; 0.1 M TEAA, pH 7, 20→50% CH_3CN/30 min, 9 mL/min, 254 nm), and the fraction that eluted at 19.7 min was collected. The solvent was removed by evaporation, and the residue was dissolved in H_2O (20 mL) and then lyophilized to give the triethylammonium salt of **15** (46.7 A_{260} units).

Triethylammonium Salt of 5′-*O*-(*tert*-Butyldimethylsilyl)-thymidine-3′-*O*-phosphoryl-(3′→5′)-guanosine-3′-*O*-phosphoryl-(3′→5′)-guanosine-3′-*O*-phosphoryl-(3′→5′)-guanosine-3′-*O*-phosphoryl-(3′→5′)-adenosine-3′-*O*-phosphoryl-(3′→5′)-3′-*O*-(*tert*-butyldimethylsilyl)guanosine (16). Compound **14b** (13 mg) was subjected to the reaction described in the synthesis of **15** except for treatment with aqueous AcOH. The crude product was subjected to reversed-phase open column chromatography (Preparative C18 125, 55−105 μm, Waters; A: 50 mM TEAB, pH 7, 5% CH_3CN; B: 50 mM TEAB, pH 7, 60% CH_3CN, linear gradient; 150 drops/fraction, 254 nm), and the main fraction (fractions 30 and 31) was collected. The solvent was removed by evaporation, and the residue was dissolved in H_2O (20 mL) and then lyophilized to give the triethylammonium salt of **16** (48.1 A_{260} units).

The structures of all compounds tested (**15**−**42**) were confirmed by means of negative ion LSI mass spectroscopy.

Biology. Measurement of Stability in Human Plasma. To each solution of 30 μg of ODNs **23** and **31** in 100 μL of PBS buffer was added 1.4 mL of human plasma at 37 °C; 100 μL 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. To the sampling mixture were added 70 μL of PBS buffer (pH 7.4), 10 μL of Tris-HCl (pH 8), and 10 μL of 25 mg/mL proteinase K (Perkin-Elmer). After 30 min, the reaction mixture was washed with 300 μL of phenol−$CHCl_3$−isoamyl alcohol (25:24:1, v/v/v) and with 300 μL of $CHCl_3$. The resulting 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).

Inhibition of HIV-1_{IIIB}-Induced Cytopathicity. The measurement of anti-HIV-1 activity in MT-4 cells was performed as described previously.[15] Briefly, exponentially growing MT-4 cells were centrifuged for 5 min at 140*g*. The cell pellet 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 × 10^4 cells in 200 μL of medium. At 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.

Acknowledgment. We thank Drs. Hideyuki Haruyama and Takemichi Nakamura for critical reading of this manuscript.

Supporting Information Available: Table of negative ion LSI MS and retention times of HPLC for compounds **15**−**42** (Table 4), PAGE for compounds **17**, **20**, **21**, **23**, **28**−**30**, **32**−**35**, and **38** (Figure 3), and HPLC profile for **39** (Figure 4) (3 pages). Ordering information is given on any current masthead page.

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JM970658W