Structure–Activity Relationships of Cytotoxic Cholesterol-Modified DNA Duplexes

Michael W. Reed,*,† Eugeny A. Lukhtanov,† Vladimir V. Gorn,† Deborah D. Lucas,† James H. Zhou,‡ S. Balakrishna Pai,‡ Yung-chi Cheng,‡ and Rich B. Meyer, Jr.†

MicroProbe Corporation, 1725 220th Street SE, #104, Bothell, Washington 98021, and Department of Pharmacology, School of Medicine, Yale University, Sterling Hall of Medicine, New Haven, Connecticut 06510

Received July 25, 1995®

Short DNA duplexes with cholesterol linked at the 3′-terminus of each strand have unique, selective cytotoxic properties. The structural requirements for biological activity were explored through chemical synthesis of analogs and testing in cultured hepatoma cells. Effects of modifications to the sequence, backbone, 3′-sterol, 3′-linker, and 5′-terminus were evaluated. Self-complementary 3′-modified oligodeoxynucleotide (ODN) 10-mers were prepared from solid supports bearing the modification and linker of interest. Any changes to the normal phosphodiester backbone were poorly tolerated. The presence of cholesterol or a closely related sterol was an absolute requirement for activity. The length and position of attachment of the linker to cholesterol was important, with longer linkers showing reduced activity. Large, lipophilic groups at the 5′-terminus gave reduced cytotoxicity and poor solubility properties. The short length and unique structure of these ODNs allowed efficient automated synthesis on a 400 μmol scale and simplified purification.

Introduction

A new class of cytotoxic cholesterol-modified oligodeoxynucleotides (ODNs) with unique cell line specificity was recently reported.[1] These compounds were discovered in the course of screening cholesterol-modified ODNs designed as antisense agents for hepatitis B virus.[1] Closer examination of a particularly effective 14-mer showed cytotoxic effects in the host hepatoma cells. Synthesis and screening of related ODN sequences eventually led to a perfectly self-complementary 10-mer with a short, rigid linker between the 3′-phosphate residue and the cholesterol modification. The bioactive form of these compounds are short DNA duplexes (10–12 base pairs in length) as shown in Figure 1. Earlier studies determined the effects of ODN base composition and length in duplexes formed from equimolar concentrations of complementary ODN strands. Since a single self-complementary sequence is more practical for drug development, we describe here synthetic analogs designed to explore the effects of sequence, backbone modifications, sterol structure, linker length, and 5′-terminal groups.

Conjugation of a cholesterol group to ODNs has been shown to increase cellular uptake,[2] improve antisense efficacy,[3] alter tissue distribution,[4] and improve nuclease resistance.[5] The lipophilic cholesterol modification increases affinity of ODNs for cell membranes and low-density lipoproteins (LDL). Although lipophilic modifications have been shown to improve antiviral activity of ODNs, these groups can overwhelm the desired sequence specificity of mRNA-directed antisense agents.[6,7] For example, the mode of action of anti-human immunodeficiency virus (HIV) cholesterol-modified ODNs is a result of binding to key viral and cell surface proteins.[8] Recently, combinatorial methods have been developed to find unique ODN sequences that bind to specific proteins. Certain G-rich ODN sequences can form tetrameric structures that have been shown to have anti-HIV,[9] antithrombosis,[10] and antiproliferative[11] activity. The cholesterol-modified DNA duplexes described here are another example of specific nucleic acid structures that have unique biological properties.

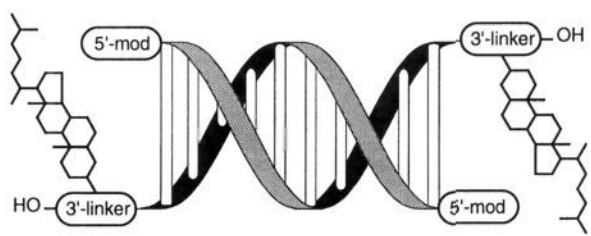


Figure 1. Schematic structure of the 3′-cholesterol-modified DNA duplexes.

We examined the toxicity of the cholesterol-modified DNA duplexes in a variety of culture systems and found that many other cell lines are unaffected. Screening in the tissue specific 60-cell line panel developed by the National Cancer Institute showed toxicity toward most colon carcinoma cell lines and two breast carcinoma cell lines (hepatoma cells were not tested).[12] Although the title compounds exhibit selective toxicity toward certain cultured cells, development as potential anticancer agents requires gram quantities for efficacy and toxicity testing in animals. These more advanced studies also require synthetic material of reproducible and specified purity. The phosphodiester 10-mer ODNs reported here are relatively easy to synthesize and purify on a 400 μmol scale. Synthetic details and physical properties are provided for this unique class of cytotoxic compounds.

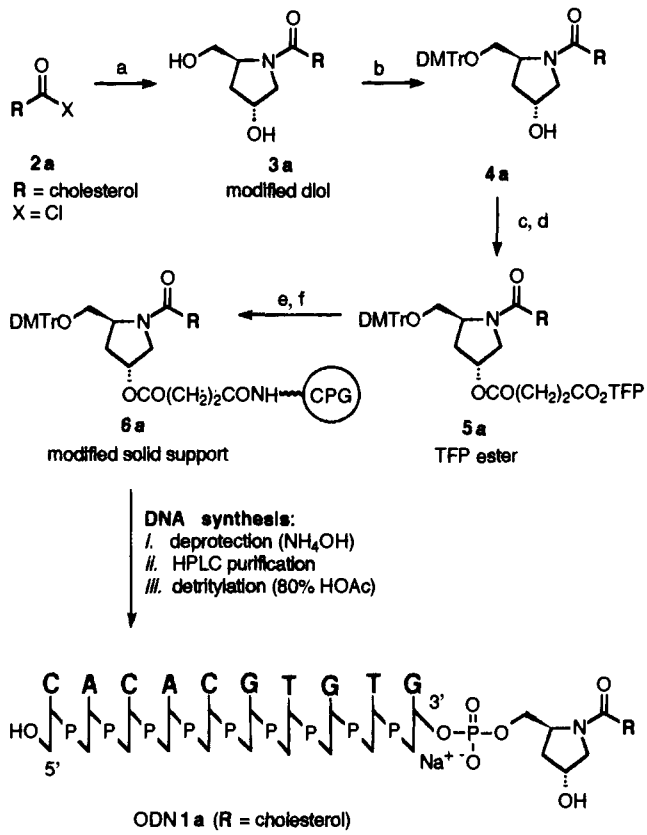
Chemistry

The chemistry used for preparation of the lead 3′-cholesterol-modified ODN **1a** and most other 3′-modified ODNs described here is shown in Scheme 1. Synthesis

* Author to whom correspondence should be addressed.
† MicroProbe Corp. Tel: 206-485-8566. Fax: 206-486-8336.
‡ Yale University. Tel: 203-785-7119. Fax: 203-785-7129.
® Abstract published in *Advance ACS Abstracts,* October 1, 1995.

0022-2623/95/1838-4587$09.00/0

Scheme 1. Synthesis of 3′-Modified Oligonucleotides[a]



[a] (a) *trans*-L-Hydroxyprolinol, Et_3N, CH_2Cl_2; (b) DMTCl, pyridine; (c) succinic anhydride, Et_3N, *N*-methylimidazole, CH_2Cl_2; (d) 2,3,5,6-tetrafluorophenyl trifluoroacetate, Et_3N, CH_2Cl_2; (e) LCAA-CPG, DMF, EtN(*i*-Pr)$_2$; (f) acetic anhydride, pyridine.

of the amino diol linker (*trans*-4-hydroxy-L-prolinol) and small scale preparation of 3′-cholesterol-modified ODNs via controlled pore glass (CPG) supports have been described earlier.[5,13] Modifications to the oligonucleotide sequence and backbone of lead ODN **1a** were examined first since these were easily accomplished on the DNA synthesizer using standard β-cyanoethyl phosphoramidite-coupling chemistry.[14] Phosphorothioate modifications were incorporated into the phosphate backbone through use of a sulfurizing agent in place of aqueous iodine in the appropriate coupling cycle.[15] Unless otherwise specified, all ODNs were >90% pure by C18 HPLC and one major band by polyacrylamide gel electrophoresis.

The synthetic approach to ODN **1a** is modular in that various 3′-modifications (R) are easily immobilized onto solid supports via 2,3,5,6-tetrafluorophenyl (TFP) ester intermediates. After removal of the dimethoxytrityl (DMTr) protecting group from the linker, the desired ODN sequence is synthesized directly from the exposed hydroxy group. After synthesis, ammonia hydrolysis cleaves the succinate ester to give the desired 3′-modified ODN. Amino diols other than *trans*-hydroxy-L-prolinol can also be incorporated into the solid supports to examine the effects of linkers between the ODN and cholesterol. As described below, the chemistry shown in Scheme 1 was used to prepare 10 different CPG supports (**6b**–**k**). The corresponding ODNs (**1b**–**k**) were prepared from these supports, and the cytotoxicity was examined relative to **1a**. Two series of analogs addressed the critical structural components at the 3′-terminus: The first series of 3′-modifications retained the hydroxyprolinol linker shown in Scheme 1 but varied the modifier (R), and the second series retained R = cholesterol and varied the amino diol linker.

Table 1. Effect of Sequence on Cytotoxicity of 3′-Cholesterol-Modified Self-Complementary 10-mer ODNs

ODN[a] 5′ → 3′	G–C content	activity[b]
CACACGTGTG	6/10	+
CTGGATCCAG	6/10	+
ACCACGTGGT	6/10	+
TCCACGTGGA	6/10	+
TCGACGTCGA	6/10	+
CACCCGGGTG	8/10	+
GTGGGCCCAC	8/10	+
TCGCGCGCGA	8/10	+
TGGCCGGCCA	8/10	+
GGGGGCCCCC	10/10	−
GCGCGCGCGC	10/10	±
GGGCCGGCCC	10/10	±

[a] All ODNs had the 3′-cholesterol modification shown in Scheme 1. [b] Cytotoxic activity was measured at an ODN concentration of 1 μM in Hep G2 cells using the cell morphology assay. Results are tabulated as either active (+), less active (±), or inactive (−) in comparison to ODN 1a and untreated cells.

The effects of 5′-modifications on cytotoxicity of 3′-cholesterol-modified ODNs were also explored. These modifications were introduced into ODNs **1l**–**t** on the DNA synthesizer using suitably protected β-cyanoethyl phosphoramidites. The phosphoramidites were either obtained from commercial sources or prepared from the appropriate precursor alcohols. 5′-Cholesterol-modified ODNs (**1u**–**w**) were also prepared using cholesterol phosphoramidites and tested for cytotoxicity.

Structure–Activity Relationships

Cultured Hep G2 hepatoma cells form large vacuoles 24 h after treatment with ODN **1a** that are easily detected in affected cells using a microscopic assay.[1] This sensitive cell morphology assay was used for primary screening of the ODN analogs described here. Results were tabulated as either active (+), slightly less active (±), or inactive (−) in comparison to ODN **1a**. Since affected cells do not recover, a more quantitative assay based on the ability of transplanted cells to form colonies could be used. Concentrations of 0.1, 1, and 3 μM were tested, and colonies were counted after 14 days to determine IC_{50} values. The cytotoxic effect of **1a** was dose dependent with IC_{50} values of ca. 0.4 μM (1.6 μg/mL). Since we showed earlier[1] that there was excellent correlation between the morphology assay and the colony-forming assay, IC_{50} was not routinely determined for each ODN.

Sequence Modifications. Earlier studies showed that 3′-cholesterol ODNs which formed a duplex of 10–12 base pairs were most potent. Complementary 10-mer ODNs with at least four G–C base pairs showed similar potency. Thermal denaturation studies (UV) showed that as the melting temperature (T_m) of the duplexes dropped below 37 °C, potency dropped. ODNs with lower G–C content presumably do not form stable duplexes under physiological conditions. Twelve different 10-mer ODNs with G–C contents of 6/10, 8/10, and 10/10 were prepared with the same 3′-modification as **1a** and tested for potency. As shown in Table 1, additional duplex stability provided by increased G–C content did not translate into improved activity. The potency of the all G–C ODNs was less than **1a**, perhaps due to unusual complex formation. For example, de-

Table 2. Effect of 5′- and 3′-Nucleotide Overhangs on Cytotoxicity of 3′-Cholesterol-Modified Self-Complementary ODNs

ODN[a] (duplex structure)	overhang length (nt)	activity[b]
CACACGTGTC·Chol Chol·GTGTGCACAC	0	+
ACACACGTGTG·Chol Chol·GTGTGCACACA	1	+
TACACACGTGTG·Chol Chol·GTGTGCACACAT	2	+
TATACACACGTGTG·Chol Chol·GTGTGCACACATAT	4	+
CACACGTGTGC·Chol Chol·CGTGTGCACAC	1	+
CACACGTGTGCT·Chol Chol·TCGTGTGCACAC	2	±
CACACGTGTGCTAC·Chol Chol·CATCGTGTGCACAC	4	−

[a] All ODNs had the 3′-cholesterol modification shown in Scheme 1 and the same 10-mer duplex core as **1a**. [b] Cytotoxic activity was measured at an ODN concentration of 1 μM in Hep G2 cells using the cell morphology assay.

naturing gel electrophoresis showed one band for most ODNs, but the sequence GGGGGCCCCC appeared as a lower mobility smear. GC-rich DNA has also been shown to form Z helices, and this may be another cause for reduced potency. Since there was no apparent advantage to switching sequences, further modifications were explored using the sequence in ODN **1a.**

It is not yet clear why 3′-cholesterol DNA duplexes longer that 12 base pairs show reduced potency. In order to more fully explore ODN length requirements, a series of ODNs were prepared that retained the 10 base pair duplex "core" of ODN **1a** but had additional nucleotide "overhangs" added at either the 5′- or 3′-terminus. As shown in Table 2, 5′-nucleotide overhangs were well tolerated, but additional nucleotides between the duplex core and the 3′-cholesterol modification reduced potency. As the distance between the 3′-cholesterol group and the duplex core increases, activity decreases. The nature of the interactions of cholesterol with the duplex region is not clear. We[13] and others[16,17] have shown that lipophilic ODN modifications such as cholesterol can increase T_m with single-stranded DNA targets, perhaps through hydrophobic interactions with the heterocyclic bases in the interior of the duplex.

Backbone Modifications. Since the lead ODN **1a** had an unmodified phosphodiester backbone, synthetic efforts were focused here in efforts to improve drug stability and potency. Although 3′-modifications provide resistance to exonuclease degradation,[5] modifications to the internucleoside phosphates (i.e., phosphorothioates, methyl phosphonates) have been shown to give additional stability to single-stranded ODNs.[18] Phosphorothioates (S-ODNs) are especially useful as antisense agents since they are resistant to nucleases, yet their hybrids with RNA are still recognized by RNase H. As shown in Table 3, multiple phosphorothioate modifications in ODN **1** (especially at internal positions) significantly decreased potency.

The S-ODNs with multiple P–S modifications showed decreased T_m, presumably due to adverse effects in the population of diastereomers.[19] This decrease in duplex stability correlates well with decreased cytotoxicity. However, the ODN with a single P–S modification at the 5′-internucleoside phosphate gave no decrease in T_m but decreased activity in the clonogenic assay (data not shown). Two equal-sized product peaks were obtained from synthesis of this ODN which we believe are the Rp and Sp isomers. These isomers were completely resolved by C18 HPLC after synthesis on a 15 μmol scale. The isomers had similar biological activity in the morphology assay. Likewise, two distinct stereoisomers were observed when a single P–S modification was introduced at the 3′-terminal phosphate. It is possible that S-ODN analogs lose cytotoxic potency due to altered conformation of the active duplex structure or interactions with other macromolecular targets in the cell. It has been shown that S-ODNs have strong affinity for certain cellular proteins in comparison to phosphodiester ODNs.[20]

Table 3. Effect of Phosphorothioate Backbone Modifications on Cytotoxicity of ODN 1a

[a]position of modification (s)	activity[b]	T_m (°C)[c]
CACACGTGTG	+	46.8
CsACACGTGTG	+	46.8
CsAsCsACGTGTG	±	41.5
CsAsCsAsCsGTGTG	−	
CACsAsCsGTGTG	−	
CACACsGTGTG	±	
CACACTGTGTGs	+	
CACACGTGsTsGs	±	
CACACBGsTsGsTsGs	−	
CsAsCsAsCsGsTsGsTsGs	−	35.5

[a] All ODNs had the 3′-cholesterol modification shown in Scheme 1; s represents an internucleoside phosphorothioate linkage. [b] Cytotoxic activity was measured at an ODN concentration of 1 μM in Hep G2 cells using the cell morphology assay. [c] T_m for selected ODNs were determined at an ODN concentration of 4 μM as described in the Experimental Section.

3′-Modifications (R). Since early studies showed that the 3′-cholesterol modification was critical for biological activity, a series of 3′-modifications were prepared to explore the effects of different lipophilic groups. The structures of the 3′-modifications (R) and the relative cytotoxic activity of the corresponding ODNs **1a–f** are shown in Figure 2. Each of the solid supports (**6a–f**) were prepared from *trans*-L-hydroxyprolinol as shown in Scheme 1. Most of the starting electrophilic modifiers (**2**) were obtained by activation of a commercially available starting material. β-Estradiol-3-benzoate was used for preparation of **2e** (the benzoate protecting group is removed during ammonia hydrolysis). The sterols (ROH) were converted to chloroformates by treatment with phosgene. The TFP ester of methyllithocholic acid (**2f**) was prepared as shown in Scheme 2.

It is clear that the nature of the lipophilic group dramatically affects biological activity. ODNs **1a–c** were linked to closely related sterols at the 2-hydroxy position of the sterol nucleus. The cytotoxicity and lipophilicity (as evidenced by C18 HPLC retention time) of these analogs were similar. On the other hand, sterol-modified ODNs **1e,f** showed no cytotoxicity. These ODNs were linked to the D-ring of the sterol nucleus and somewhat less lipophilic than the active ODNs. The di-*O*-hexadecylglycerol derivative **1d** was more lipophilic than **1a** but lacked cytotoxicity. T_m studies of 15-mer DNA duplexes bearing this lipid structure at each 5′-terminus showed evidence of unusual complex formation.[21]

The exact structural requirements for the 3′-sterol modifier are not yet known, but this initial survey indicates that biological activity of the ODNs may be

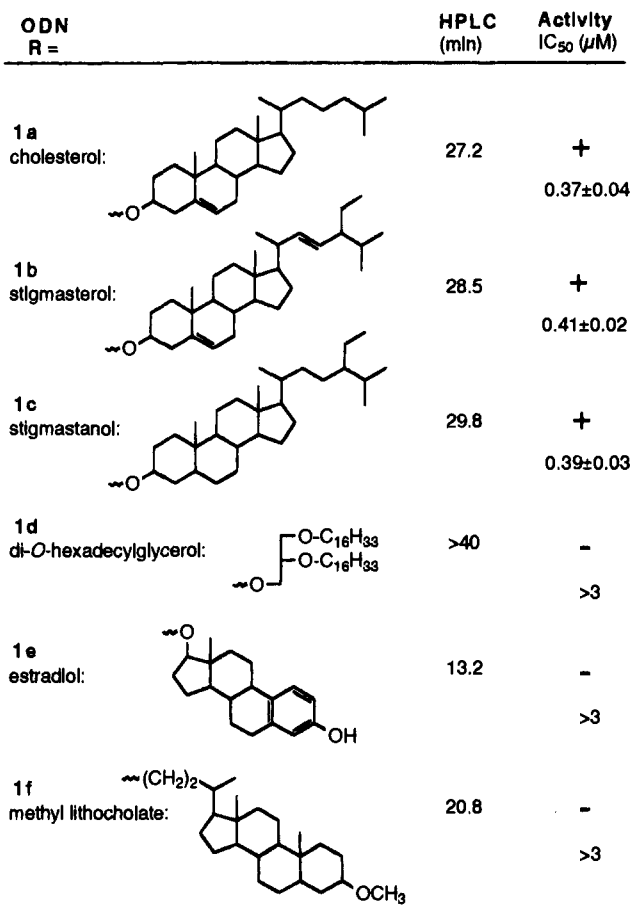
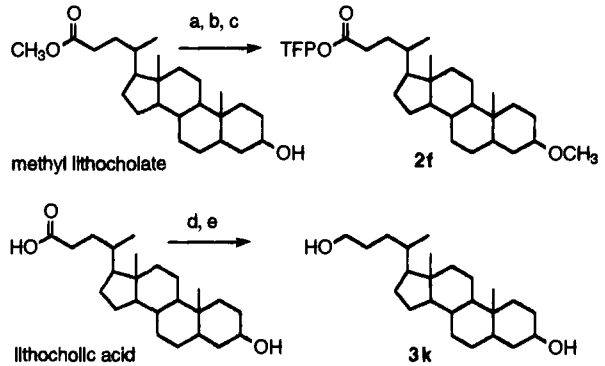


Figure 2. Effect of 3′-modification (R) on cytotoxic activity of ODN 1. Each ODN was prepared from the corresponding CPG **6** via intermediates **2**–**5** as shown in Scheme 1. HPLC used a 250 × 4.6 mm C18 column and a gradient of 5–85% solvent B over 40 min (flow rate = 1 mL/min), where solvent A = 0.1 M triethylammonium acetate (pH 7.5) and solvent B = acetonitrile. UV detection was at 260 nm. Activity (+ or −) was determined in Hep G2 (hepatoma) cells using the cell morphology assay at an ODN concentration of 1 μM, and 50% inhibition concentration (IC_{50}) was determined using the clonogenic assay.

Scheme 2. Synthesis of Lithocholic Acid Intermediates[a]



[a] (a) CH_3I, Ag_2O; (b) NaOH, aqueous EtOH; (c) tetrafluorophenyl trifluoroacetate, Et_3N, CH_2Cl_2; (d) BH_3·THF; (e) MeOH.

related to their lipophilicity. Retention on C18 HPLC may be representative of affinity of the ODNs for the phospholipid bilayer of cell membranes. As described above, interactions of the 3′-terminal modifier with the double-stranded DNA region are important for activity. The size and shape of the active sterols may result in key contacts with certain hydrophobic residues in the duplex region. Since there was no increase in potency

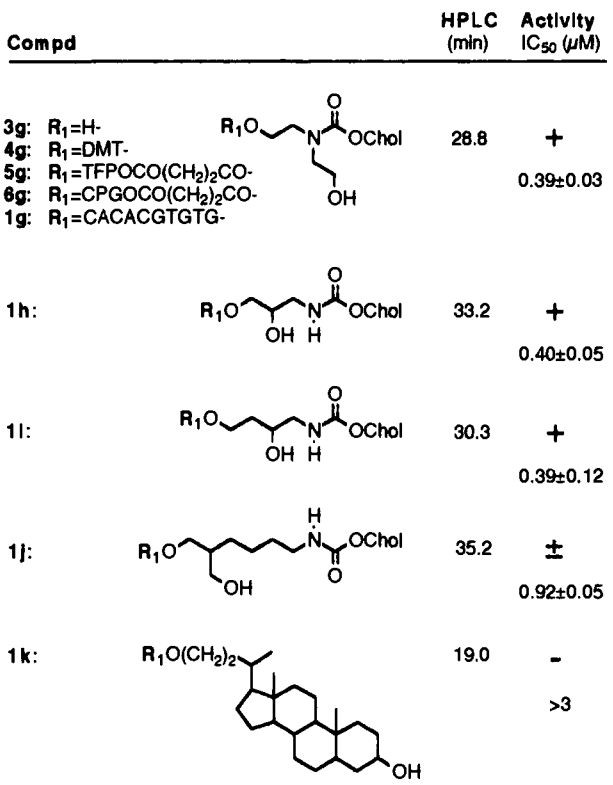


Figure 3. Effect of linker structure on cytotoxic activity of ODN 1. Each ODN was prepared from the corresponding CPG **6** via intermediates **3**–**5**. HPLC retention time and cytotoxic activity were determined as described in Figure 2.

with other sterols, further development of structure–activity relationships retained R = cholesterol.

Linker Structure. The nature of the linker between cholesterol and the 3′-terminal nucleotide is another structural variable that was examined. As shown in Table 2, a single nucleotide "linker" at the 3′-terminus was tolerated, but insertion of additional nucleotide linkers compromised cytotoxicity. The *trans*-hydroxyprolinol linker arm structure in ODN **1a** was chosen for its rigidity and its fixed stereochemistry (ODN **1a** is a single stereoisomer). However, this linker is not commercially available and therefore less attractive for future scaleup. To determine how linker arm length and structure affected activity, 3′-cholesterol-modified ODNs **1g**–**j** were prepared from the corresponding CPG supports **6g**–**j** and the potency was compared to the lead ODN **1a**. The structures of the 3′-linkers and the relative cytotoxic activity of the corresponding ODNs **1g**–**j** are shown in Figure 3.

Cholesterol was attached to the ODNs via the appropriate amino diol linker using the chemistry shown in Scheme 1. The 2-carbon long linker in ODN **1g** was especially attractive since there are no chiral carbon atoms. The other amino diol linkers were used as racemic mixtures. The 3-carbon linker in ODN **1h** has been used for the preparation of 3′-cholesterol-modified ODNs, but intermediates were not characterized and the loading level on the CPG was not determined.[16] We prepared this CPG from the corresponding TFP ester intermediate as usual. There was evidence for diastereomers in the HPLC purification of **1h** (two closely eluting peaks of equal intensity). The 4-carbon amino diol linker in ODN **1i** was prepared from CBZ-protected γ-amino-β-hydroxybutyric acid using a two-step reduction procedure similar to that used for synthesis of

trans-hydroxyprolinol.[22] ODN **1j** was prepared from a commercially available cholesterol CPG support. Cytotoxicity assays showed no significant increase in potency with other short linkers. Longer aliphatic linkers such as the 6-carbon linker in **1j** presumably lower potency by decreasing interactions between the attached steroid ring and the DNA duplex core. The increase in C18 HPLC retention time observed for **1j** may be predictive of these decreased interactions. CPG support **6k** was prepared from lithocholic acid as illustrated in Scheme 2. ODN **1k** was inactive, presumably due to its lower lipophilicity (19 min retention time by C18 HPLC), but perhaps due to the position of attachment at the D-ring of the steroid nucleus.

5′-Modifications. The foregoing structure–activity relationships show that interactions between the 3′-sterol and the duplex DNA region are important for activity. Since the 5′-terminus of the complementary strand is in close proximity to this region, a series of 3′-cholesterol-modified ODN **1a** analogs were prepared with 5′-modifications as shown in Figure 4. The ODNs containing 3-, 6-, and 12-carbon long aliphatic amines were prepared from commercially available monomethoxytrityl (MMTr)-protected phosphoramidites. The 5′-phosphate and thiophosphate residues were introduced into **1l,m** with a commercially available "chemical phosphorylation" reagent. The phosphoramidite for preparation of **1n** was synthesized from DMTr-protected propanediol.[23] The corresponding phosphoramidite for preparation of **1o** was synthesized from DMTr-protected hexanediol.[24] A bis-MMTr-protected diamine (**7**) was prepared and converted to phosphoramidite **8** for preparation of **1q**. An acridine alcohol (**9**) was synthesized and converted to phosphoramidite **10** for preparation of **1t**. Surprisingly, the C18 HPLC retention time of 3′-cholesterol-modified ODNs is not significantly altered by the addition of lipophilic 5′-groups. Unlike the 3′-modified ODNs shown in Figure 2, C18 HPLC retention time of the 5′-modified ODNs is not predictive of cytotoxicity.

It can be seen in Figure 4 that small, hydrophilic groups at the 5′-terminus are tolerated, but more lipophilic groups reduce cytotoxic activity. For example, short aliphatic amines or alcohols are as potent as ODN **1a**, but the 12-carbon long amine modification in **1s** destroyed activity. The 5′-acridine group in **1t** rendered the ODN inactive. Lipophilic 5′-groups clearly affect the physical properties of the ODN and the ability to form an active duplex structure.

Solubility problems were especially apparent with the 3′,5′-cholesterol-modified ODN **1u**. This ODN was prepared using a previously reported phosphoramidite containing a 6-carbon long linker.[25] Although **1u** was readily synthesized and isolated as the triethylammonium salt by reverse phase HPLC, the sodium salt was virtually insoluble in water. Since the bis-cholesterol duplexes were intractable, ODN **1v** was prepared with a 3′-hexylamine group from the appropriate CPG.[26] Likewise, ODN **1w** was prepared with a 3′-hexylamine group but using a phosphoramidite (**11**) derived from the hydroxyprolinol intermediate **4a**. The 5′-cholesterol-modified ODNs **1v,w** were inactive in the clonogenic assay (IC_{50} > 3 μM) and showed only minor morphology changes in Hep G2 cells (no vacuole formation, some cell floating). The same 3′- and 5′-modifications are present in active ODN **1r** and inactive ODN **1x**, but the position of attachment is switched. It is unclear why the 5′-cholesterol-modified ODNs are inactive. The T_m of **1w** (53 °C) is 6 °C higher than that of ODN **1a**, yet the resulting duplex is inactive. The 5′-cholesterol ODNs are somewhat more lipophilic than the 3′-cholesterol ODNs as evidenced by C18 HPLC.

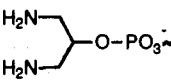
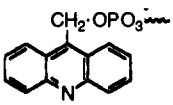
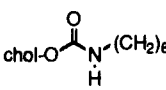
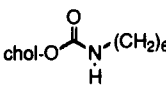
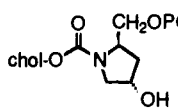
5′-Modification	ODN	HPLC (min)	Activity IC_{50} (μM)
HO~	**1a**	27.3	+ (0.37±0.04)
	**1l**	31.3	+ (0.34±0.04)
	**1m**	29.6	+ (0.53±0.08)
$HO(CH_2)_3O-PO_3^-$~	**1n**	27.8	+
$HO(CH_2)_6O-PO_3^-$~	**1o**	27.6	+ (0.52±0.04)
$H_2N(CH_2)_3O-PO_3^-$~	**1p**	27.4	+
	**1q**	27.2	+
$H_2N(CH_2)_6O-PO_3^-$~	**1r**	29.3	+ (0.30±0.03)
$H_2N(CH_2)_{12}O-PO_3^-$~	**1s**	26.2	- (>3)
	**1t**	29.2	- (>3)
	**1u**	>40	Insoluble
 [3′-$(CH_2)_6NH_2$]	**1v**	35.7	± (>3)
 [3′-$(CH_2)_6NH_2$]	**1w**	33.3	± (>3)

Figure 4. Effect of 5′-modifications on cytotoxic activity of ODN 1. Except for 1v,w, each ODN had the 3′-cholesterol modification shown in Scheme 1. HPLC retention time and cytotoxic activity were determined as described in Figure 2.

Summary

Structure–activity relationships of 3′-cholesterol-modified, self-complementary 10-mer ODNs showed that these novel cytotoxic compounds have strict structural requirements. Although none of the analogs was more potent than the lead ODN **1a**, much was learned about the limits of modification. Stability of the DNA duplex structures under physiological conditions was required for activity. Phosphorothioate modifications

destabilized the duplexes and therefore decreased potency. Hydrophobic interactions between the 3'-terminal cholesterol modification and the duplex core were also critical. Long nucleotide or aliphatic linkers between the 3'-terminal phosphate and the cholesterol modification reduced or eliminated potency. 3'-Modifications more or less lipophilic than cholesterol eliminated activity, but this may have been due to changes in the structure of the appended sterol. Large lipophilic 5'-terminal groups were poorly tolerated and could give rise to insoluble complexes.

An active ODN analog (**1g**) with a short, achiral linker was efficiently prepared on a large scale using standard solid phase chemistry and fully characterized. The availability of gram quantities of compound will aid the future development of these compounds as potential anticancer agents. Molecular modeling and NMR studies may help determine the conformation of the active duplex structures. The biophysical properties of these compounds, and their interaction with cellular membranes and membrane proteins are currently being explored. The precise mechanism of action by which these compounds act is a subject of further investigation.

Experimental Section

Chemistry. Anhydrous solvents were obtained from Aldrich (Milwaukee, WI). Proton magnetic resonance spectra were recorded using either a Varian Gemini 200 or 300 MHz spectrometer. All chemical shifts are presented in parts per million (ppm) downfield from tetramethylsilane. Where elemental analysis are indicated by symbols of the elements, results were within ±0.4% and performed by Quantitative Technologies, Inc. (Bound Brook, NJ), or Robertson Microlit Labs (Madison, NJ). Flash chromatography was performed with J.T. Baker Inc. silica gel (40 μm, 60 Å). Analytical thin-layer chromatography was carried out on EM Science F254 aluminum-backed, fluorescent indicator plates. Compounds were visualized with UV light or by charring after acid spray. Chemical yields refer to isolated compounds of >95% purity, as indicated by NMR spectroscopy.

General Procedure for Synthesis of Chloroformate Derivatives 2b–e. The lipophilic modifications (R) listed in Figure 2 were converted to the corresponding chloroformate derivatives by treatment with phosgene. Stigmasterol, stigmastanol, and 1,2-di-*O*-hexadecyl-*rac*-glycerol were purchased from Sigma Chemical Co. (St. Louis, MO). β-Estradiol-3-benzoate was used for preparation of **2e**. The appropriate alcohol (10 mmol) was stirred in 30 mL of 20% phosgene in toluene. As reaction progressed, the starting material dissolved completely. The reactions were monitored by TLC (hexanes–ethyl acetate). After 1–5 h at room temperature, solvent and excess phosgene were removed by evaporation on a rotary evaporator. Residual phosgene was removed by coevaporation with toluene. The desired chloroformates were dried *in vacuo*, and the resulting white solids were used in the acylation reactions without further purification.

2,3,5,6-Tetrafluorophenyl 3-*O*-Methyllithocholate (2f). Methyl lithocholate (Sigma) was converted to **2f** as shown in Scheme 2. The alcohol (2.5 g, 6.4 mmol) was first converted to the methyl ether by treatment with silver(I) oxide (2.0 g, 8.6 mmol) and methyl iodide (3.0 g, 20.8 mmol) in 10 mL of DMF. After stirring for 4 days at room temperature, the mixture was filtered, diluted with 200 mL of water, and extracted with 2 × 100 mL of hexanes. The organic phase was evaporated to give 2.0 g of an oil. The product was purified by flash chromatography (4 × 20 cm, silica gel) using a mixture of hexanes and ethyl acetate (8:1) to give 1.3 g (50%) of methyl 3-*O*-methyllithocholate as a white solid: ^{1}H NMR ($CDCl_3$) δ 3.64 (s, 3 H), 3.32 (s, 3 H), 3.13 (m, 1 H), 2.4–2.1 (m, 2 H), 2.0–0.8 (m, 32 H), 0.61 (s, 3 H). Anal. ($C_{26}H_{44}O_3$) C, H, N.

A solution of methyl 3-*O*-methyllithocholate (1.2 g, 3.0 mmol) in 15 mL of ethanol was treated with 2 mL of 5 M NaOH. The progress of the saponification was monitored by TLC (CH_2Cl_2/CH_3OH/Et_3N, 18:1:1). After 2 h, the mixture was acidified with concentrated HCl and evaporated to dryness. The residue was extracted with CH_2Cl_2 (100 mL), washed with water, dried over Na_2SO_4, and concentrated to an oil. This crude product was dissolved in 10 mL of CH_2Cl_2 and treated with Et_3N (680 μL, 4.9 mmol) and 2,3,5,6-tetrafluorophenyl trifluoroacetate[5] (800 μL, 4.6 mmol). After 30 min, the reaction mixture was evaporated to dryness and resuspended in 20 mL of hexanes. The suspension was purified by flash chromatography (4 × 20 cm, silica gel). The product was eluted with hexanes–ethyl acetate (10:1). The fractions containing the desired product were combined and evaporated on a rotary evaporator to give 1.0 g (62%) of TFP ester **2f** as a white solid: ^{1}H NMR ($CDCl_3$) δ 6.96 (m, 1 H), 3.33 (s, 3 H), 3.14 (m, 1 H), 2.8–2.5 (m, 2 H), 2.0–0.8 (m, 32 H), 0.64 (s, 3 H). Anal. ($C_{31}H_{42}O_3F_4$) C, H, F.

General Procedure for Synthesis of Modified Diols 3. For compounds **3a–f**, a solution of 4 mmol of the appropriate electrophile **2** in 10 mL of anhydrous CH_2Cl_2 was added to an ethanolic solution of *trans*-hydroxy-L-prolinol[13] (5 mmol) and Et_3N (5 mmol). For the cholesterol-modified diols **3g–i**, cholesterol chloroformate (Sigma) was added to the appropriate amino diol: **3g**, diethanolamine; **3h**, (±)-1-amino-2,3-propanediol; **3k**, (±)-1-amino-2,4-butanediol.[22] After stirring for 1–2 h, the solution was evaporated to dryness. The crude mixtures were separated by flash chromatography (4 × 20 cm, silica gel, 5–10% CH_3OH/CH_2Cl_2) to provide the desired products **3**.

3b: white solid (53%); ^{1}H NMR ($CDCl_3$) δ 5.39 (d, 1 H, J = 5 Hz), 5.15 (dd, 1 H, J_1 = 8.5 Hz, J_2 = 7.0 Hz), 5.02 (dd, 1 H, J_1 = 8.5 Hz, J_2 = 7.0 Hz), 4.9–4.0 (m, 3 H), 3.9–3.4 (m, 4 H), 2.5–2.2 (m, 2 H), 2.1–0.75 (m, 40 H), 0.70 (s, 3 H). Anal. ($C_{35}H_{57}NO_4$) C, H, N.

3c: white solid (40%); ^{1}H NMR ($CDCl_3$) δ 5.0–4.0 (m, 3 H), 3.9–3.4 (m, 4 H), 2.2–0.75 (m, 47 H), 0.65 (s, 3 H). Anal. ($C_{35}H_{61}NO_4$) H, N; C: calcd, 75.09; found, 74.65.

3d: white solid (75%); ^{1}H NMR ($CDCl_3$) δ 4.7–4.0 (m, 4 H), 3.75 (m, 1 H), 3.7–3.4 (m, 10 H), 2.06 (m, 1 H), 1.8–1.5 (m, 5 H), 1.26 (s, 28 H), 0.88 (t, 6 H, J = 7.0 Hz). Anal. ($C_{41}H_{81}NO_6$) C, N; H: calcd, 11.94; found: 11.34.

3e: white solid (74%); ^{1}H NMR ($CDCl_3$) δ 8.21 (d, 2 H, J = 7.0 Hz), 7.64 (t, 1 H, J = 7.0 Hz), 7.51 (t, 2 H, J = 8.0 Hz), 7.35 (d, 1 H, J = 8.0 Hz), 7.0–6.9 (m, 2 H), 4.8–3.4 (m, 7 H), 2.90 (m, 2 H), 2.4–1.2 (m, 15 H), 0.86 (s, 3 H). Anal. ($C_{31}H_{37}NO_6$) C, H, N.

3f: white solid (82%); ^{1}H NMR ($CDCl_3$) δ 4.5–4.25 (m, 2 H), 3.7–3.5 (m, 4 H), 3.32 (s, 3 H), 3.13 (m, 1 H), 2.4–0.8 (m, 37 H), 0.62 (s, 3 H). Anal. ($C_{30}H_{51}NO_4$) C, H, N.

3g: white needles from methanol (78%); ^{1}H NMR ($CDCl_3$) δ 5.38 (d, 1 H, J = 4.6 Hz), 4.52 (m, 1 H), 3.80 (m, 4 H), 3.48 (apparent br s, 4 H), 2.4–0.8 (m, 40 H), 0.66 (s, 3 H). Anal. ($C_{32}H_{55}NO_4$) C, H, N.

3h: white solid (74%); ^{1}H NMR ($CDCl_3$) δ 5.39 (d, 1 H, J = 4.6 Hz), 5.05 (t, 1 H, J = 6 Hz), 4.50 (m, 1 H), 3.77 (m, 1 H), 3.60 (m, 2 H), 3.33 (m, 2 H), 2.86 (d, 2 H, J = 5 Hz), 2.78 (br t, 1 H), 2.4–0.8 (m, 40 H), 0.68 (s, 3 H). Anal. ($C_{31}H_{53}NO_4$) C, H, N.

3i: white solid (79%); ^{1}H NMR ($CDCl_3$) δ 5.38 (d, 1 H), 5.14 (t, 1 H), 4.50 (m, 1 H), 4.00–3.70 (m, 3 H), 3.60–3.00 (m, 2 H), 2.40–2.20 (m, 2 H), 2.10–0.60 (m, 41 H).

Compound 3k. Lithocholic acid (0.5 g, 1.33 mmol) was treated with 5 mL of 1 M BH_3·THF with stirring to give a gellike product. After 30 min, 5 mL of CH_3OH was added to quench the reaction and the mixture was evaporated to dryness. The residue was dissolved in hot CH_3OH/water and allowed to crystallize at −10 °C overnight. The crystals were collected by filtration, washed with cold CH_3OH/water, and dried *in vacuo* affording 0.41 g (85%) of **3k** as a white solid: ^{1}H NMR ($CDCl_3$) δ 3.59 (m, 3 H), 2.0–1.8 (m, 34 H), 0.61 (s, 3 H). Anal. ($C_{24}H_{42}O_2$) C, H.

General Procedure for Synthesis of DMTr-Protected Diols 4. To a stirred solution of 2 mmol of the appropriate diol **3** in 10 mL of dry pyridine was added 0.85 g (2.5 mmol) of

dimethoxytrityl chloride. After stirring for 5 h, the reactions were quenched by addition of 500 μL of water and then the mixtures concentrated on a rotary evaporator. The crude mixtures were separated by flash chromatography (4 × 20 cm, silica gel, ethyl acetate–hexanes) to provide the desired products **4**. A drop of deuterated pyridine was added to $CDCl_3$ before NMR analysis to prevent hydrolysis of the DMTr group.

4b: white foam (60%); ^{1}H NMR ($CDCl_3$) δ 7.5–7.1 (m, 9 H), 6.83 (d, 4 H, J = 8.5 Hz), 5.3 (m, 1 H), 5.15 (dd, 1 H, J_1 = 8.5 Hz, J_2 = 6.0 Hz), 5.03 (dd, 1 H, J_1 = 8.5 Hz, J_2 = 7.0 Hz), 4.6–4.0 (m, 3 H), 3.76 (s, 6 H), 3.7–3.4 (m, 4 H), 2.5–0.75 (m, 42 H), 0.70 (s, 3 H). Anal. ($C_{56}H_{75}NO_6$) C, H, N.

4c: pale yellow foam (76%); ^{1}H NMR ($CDCl_3$) δ 7.5–7.1 (m, 9 H), 6.83 (d, 4 H, J = 8.0 Hz), 4.53 (m, 2 H), 4.13 (m, 1 H), 3.79 (s, 6 H), 3.7–3.1 (m, 4 H), 2.3–0.75 (m, 47 H), 0.65 (s, 3 H). Anal. ($C_{56}H_{79}NO_6$) C, H, N.

4d: pale yellow gum (80%); ^{1}H NMR ($CDCl_3$) δ 7.4–7.1 (m, 9 H), 6.82 (d, 4 H, J = 9.0 Hz), 4.57 (m, 1 H), 4.3–3.9 (m, 3 H), 3.78 (s, 6 H), 3.7–3.0 (m, 11 H), 2.24 (m, 1 H), 2.04 (m, 1 H), 1.6–1.4 (m, 4 H), 1.25 (s, 28 H), 0.88 (t, 6 H, J = 7.0 Hz). Anal. ($C_{62}H_{99}NO_8$) C, H, N.

4e: white foam (82%); ^{1}H NMR ($CDCl_3$) δ 8.21 (d, 2 H, J = 7.0 Hz), 7.64 (t, 1 H, J = 7.0 Hz), 7.51 (t, 2 H, J = 8.0 Hz), 7.4–7.2 (m, 10 H), 7.0–6.9 (m, 2 H), 6.84 (d, 4H, J = 9.0 Hz), 4.7–4.4 (m, 2 H), 4.20 (m, 1 H), 3.79 (s, 6 H), 3.7–3.0 (m, 4 H), 2.89 (m, 2 H), 2.4–1.0 (m, 15 H), 0.88 (s, 1 H), 0.56 (s, 2 H). Anal. ($C_{52}H_{55}NO_8$·0.3EtOAc) C, H, N.

4f: pale yellow foam (71%); ^{1}H NMR ($CDCl_3$) δ 7.3–7.1 (m, 9 H), 6.83 (d, 4 H, J = 9.0 Hz), 4.5–4.25 (m, 2 H), 3.77 (s, 6 H), 3.75–3.45 (m, 4 H), 3.33 (s, 3 H), 3.13 (m, 1 H), 2.5–0.8 (m, 37 H), 0.62 (s, 3 H). Anal. ($C_{51}H_{69}NO_6$) H, N; C: calcd, 77.31; found, 76.31.

4g: prepared using 0.9 equiv of dimethoxytrityl chloride; pale yellow foam (44%); ^{1}H NMR ($CDCl_3$) δ 7.4–7.2 (m, 9 H), 6.84 (d, 4 H, J = 9.0 Hz), 5.34 (m, 1 H), 4.46 (m, 1 H), 3.78 (s, 6 H), 3.75 (m, 2 H partially obscured by OCH_3), 3.48 (m, 4 H), 3.24 (m, 2 H), 2.4–0.8 (m, 40 H), 0.68 (s, 3 H). Anal. ($C_{53}H_{73}NO_6$) C, H, N.

4h: pale yellow foam (75%); ^{1}H NMR ($CDCl_3$) δ 7.4–7.2 (m, 9 H), 6.83 (d, 4 H, J = 9.0 Hz), 5.36 (d, 1 H, J = 6 Hz), 4.98 (br t, 1 H), 4.45 (m, 1 H), 3.88 (m, 1 H), 3.78 (s, 6 H), 3.39 (m, 1 H), 3.17 (m, 3 H), 2.4–0.8 (m, 40 H), 0.67 (s, 3 H). Anal. ($C_{52}H_{71}NO_6$) C, H, N.

4i: pale yellow foam (73%); ^{1}H NMR ($CDCl_3$) δ 7.45–7.10 (m, 9 H), 6.80 (d, 4 H, J = 8.8 Hz), 5.38 (d, 1 H), 5.05 (br t, 1 H), 4.50 (m, 1 H), 3.90–3.80 (m, 2 H), 3.80 (s, 6 H), 3.50–2.95 (m, 4 H), 2.30 (m, 2 H), 2.10–0.60 (m, 43 H). Anal. ($C_{33}H_{73}NO_6$) C, H; N: calcd, 1.17; found, 1.60.

4k: white solid foam (75%); ^{1}H NMR ($CDCl_3$) δ 7.4–7.1 (m, 9 H), 6.83 (d, 4 H, J = 8.0 Hz), 3.77 (s, 6 H), 3.59 (m, 3 H), 2.0–0.8 (m, 34 H), 0.62 (s, 3 H). Anal. ($C_{45}H_{60}O_4$) C, H.

General Procedure for Synthesis of 2,3,5,6-Tetrafluorophenyl Esters 5. To a stirred solution of 1.5 mmol of the appropriate DMTr derivative **4** and Et_3N (275 μL, 2 mmol) in 10 mL of CH_2Cl_2 were added succinic anhydride (0.2 g, 2 mmol) and *N*-methylimidazole (10 μL). The mixture was stirred overnight. TLC ($CHCl_3$/MeOH, 9:1) showed no residual starting material and appearance of a product with lower R_f. Tetrafluorophenyl trifluoroacetate (437 μL, 2.5 mmol) was added dropwise to the vigorously stirred mixture. After 1 h, 100 mL of CH_2Cl_2 was added. The solutions were washed with water (2 × 100 mL) and brine (100 mL) and dried over Na_2SO_4. The desired TFP ester **5** was isolated by flash chromatography (4 × 25 cm, silica gel) using a gradient of ethyl acetate in hexanes. This two-step procedure provided 50–95% yield starting from the DMTr derivative.

5b: white foam (51%); ^{1}H NMR ($CDCl_3$) δ 7.5–7.1 (m, 9 H), 7.00 (m, 1 H), 6.83 (d, 4 H, J = 8.0 Hz), 5.42 (apparent br s, 1 H), 5.3 (m, 1 H), 5.15 (dd, 1 H, J_1 = 8.5 Hz, J_2 = 7.0 Hz), 5.03 (dd, 1 H, J_1 = 8.5 Hz, J_2 = 7.0 Hz), 4.45 (m, 1 H), 4.3–4.0 (m, 1 H), 3.78 (s, 6 H), 3.6–3.6 (m, 2 H, partially obscured by OCH_3), 3.5–3.1 (m, 2 H), 3.01 (m, 2 H), 2.77 (t, 2 H, J = 7.0 Hz), 2.5–0.75 (m, 42 H), 0.70 (s, 3 H). Anal. ($C_{66}H_{79}NO_9F_4$·0.5EtOAc) C, H, N.

5c: white foam (76%); ^{1}H NMR ($CDCl_3$) δ 7.5–7.1 (m, 9 H), 7.00 (m, 1 H), 6.83 (d, 4 H, J = 8.0 Hz), 5.41 (apparent br s, 1 H), 4.53 (m, 1 H), 4.2–4.0 (m, 1 H), 3.79 (s, 6 H), 3.75–3.1 (m, 2 H, partially obscured by OCH_3), 3.5–3.1 (m, 2 H), 3.00 (t, 2 H, J = 6.0 Hz), 2.76 (t, 2 H, J = 6.0 Hz), 2.4–0.7 (m, 47 H), 0.65 (s, 3 H). Anal. ($C_{66}H_{83}NO_9F_4$·0.5EtOAc) C, H, N.

5d: colorless syrup (77%); ^{1}H NMR ($CDCl_3$) δ 7.4–7.1 (m, 9 H), 7.00 (m, 1 H), 6.83 (d, 4 H, J = 9.0 Hz), 5.44 (m, 1 H), 4.3–3.9 (m, 3 H), 3.78 (s, 6 H), 3.7–3.0 (m, 11 H), 3.00 (t, 2 H, J = 7.0 Hz), 2.76 (t, 2 H, J = 7.0 Hz), 2.36 (m, 1 H), 2.15 (m, 1 H), 1.6–1.4 (m, 4 H), 1.25 (s, 28 H), 0.88 (t, 6 H, J = 7.0 Hz). Anal. ($C_{72}H_{103}NO_{11}F_4$) C, H, N.

5e: white foam (95%); ^{1}H NMR ($CDCl_3$) δ 8.21 (d, 2 H, J = 7.0 Hz), 7.64 (t, 1 H, J = 7.0 Hz), 7.51 (t, 2 H, J = 8.0 Hz), 7.4–7.2 (m, 10 H), 7.0–6.9 (m, 3 H), 6.84 (d, 4H, J = 9.0 Hz), 5.44 (apparent br s, 1 H), 4.67 (t, $^2/_5$ H, J = 8.0 Hz), 4.52 (t, $^3/_5$ H, J = 7.0 Hz), 4.18 (m, 1 H), 3.79 (s, 6 H), 3.7–2.7 (m, 10 H), 2.5–1.0 (m, 15 H), 0.85 (s, $^6/_5$ H), 0.57 (s, $^9/_5$ H). Anal. ($C_{62}H_{59}NO_{11}F_4$·0.5EtOAc) C, H, N.

5f: white foam (85%); ^{1}H NMR ($CDCl_3$) δ 7.3–7.1 (m, 9 H), 7.00 (m, 1 H), 6.83 (d, 4 H, J = 9.0 Hz), 5.28 (m, 1 H), 4.30 (m, 1 H), 3.78 (s, 6 H), 3.75–3.45 (m, 4 H), 3.33 (s, 3 H), 3.13 (m, 1 H), 3.00 (t, 2 H, J = 7.0 Hz), 2.74 (t, 2 H, J = 7 Hz), 2.4–2.0 (m, 5 H), 1.9–0.8 (m, 32 H), 0.62 (s, 3 H). Anal. ($C_{61}H_{73}NO_9F_4$) C, H, N, F.

5g: white foam (75%); ^{1}H NMR ($CDCl_3$) δ 7.5–7.1 (m, 9 H), 6.99 (m, 1 H), 6.83 (d, 4 H, J = 8.0 Hz), 5.36 (m, 1 H), 4.46 (m, 1 H), 4.24 (m, 2 H), 3.78 (s, 6 H), 3.63 (m, 2 H), 3.46 (m, 2 H), 3.22 (m, 2 H), 2.98 (t, 2 H, J = 6.8 Hz), 2.71 (m, 2 H), 2.4–0.8 (m, 40 H), 0.68 (s, 3 H). Anal. ($C_{63}H_{77}NO_9F_4$) C, H, N.

5h: white foam (75%); ^{1}H NMR ($CDCl_3$) δ 7.4–7.2 (m, 9 H), 6.99 (m, 1 H), 6.83 (d, 4 H, J = 9.0 Hz), 5.35 (d, 1 H, J = 4 Hz), 5.12 (m, 1 H), 4.81 (br t, 1 H), 4.43 (m, 1 H), 3.77 (s, 6 H), 3.53 (m, 1 H), 3.37 (m, 1 H), 3.24 (m, 2 H), 3.01 (t, 2 H, J = 7 Hz), 2.79 (t, 2 H, J = 7 Hz), 2.4–0.8 (m, 40 H), 0.67 (s, 3 H).

5k: white foam (93%); ^{1}H NMR ($CDCl_3$) δ 7.4–7.1 (m, 9 H), 6.98 (m, 1 H), 6.83 (d, 4 H, J = 8.0 Hz), 4.76 (m, 1 H), 3.77 (s, 6 H), 3.59 (t, 2 H, J = 6.0 Hz), 2.98 (t, 2 H, J = 7.0 Hz), 2.71 (t, 2 H, J = 7.0 Hz), 2.0–0.8 (m, 34 H), 0.62 (s, 3 H). Anal. ($C_{55}H_{64}O_7F_4$) C, H, F.

Preparation of Modified CPG Supports 6. Long chain alkylamine CPG (Sigma; 80–120 mesh, 500 Å) was derivatized with the appropriate TFP ester **5**, and residual amino groups were capped with acetic anhydride as previously described for preparation of **6a**.[5] Unless otherwise noted, 100 μmol of TFP ester was offered per gram of CPG. DMTr loading levels were determined by acid hydrolysis and measurement of the trityl cation (A_{498}). Loading levels were generally 35–45 μmol/g. CPG support **6i** was prepared directly from the corresponding *p*-nitrophenyl ester according to the literature method to give a loading level of 24 μmol/g.[14] A 1 μmol column containing CPG support **6j** was purchased from Clontech Laboratories Inc. (Palo Alto, CA).

General Procedure for Synthesis of β-Cyanoethyl Phosphoramidites. Three new phosphoramidites were prepared for synthesis of the 5′-modified ODNs described in Figure 4. The diamine amidite **8** used for preparation of **1q** was obtained from the bis-monomethoxytrityl-protected compound **7** as described in the procedure below. The acridine amidite **10** used for synthesis of **1t** was prepared from the corresponding alcohol **9** using a similar procedure. The cholesterol amidite 11 used for synthesis of **1w** was prepared from compound **4a**.[13]

***N,N′*-Bis(4-monomethoxytrityl)-1,3-diamino-2-hydroxypropane (7).** A solution of monomethoxytrityl chloride (10.3 g, 33.3 mmol) in 10 mL of anhydrous CH_2Cl_2 was added dropwise to a solution of 1,3-diamino-2-hydroxypropane (3.0 g, 33.3 mmol) in 50 mL of CH_2Cl_2. After stirring for 30 min, the mixture was diluted with the same solvent to 100 mL, washed twice with 100 mL of water, dried over Na_2SO_4, and concentrated *in vacuo* to give a pale yellow foam. The crude product was purified by flash chromatography (4 × 30 cm, silica gel) in ethyl acetate/hexanes (3:1) to give the desired product as a colorless foam (7.4 g, 35%): ^{1}H NMR ($CDCl_3$) δ 7.5–7.1 (m, 24 H), 6.80 (d, 4 H, J = 9 Hz), 3.9–3.7 (m, 1 H, overlapping with OCH_3), 3.78 (s, 6 H), 2.30 (m, 4 H). Anal. ($C_{43}H_{42}N_2O_3$·0.25EtOAc) C, H, N.

2-Cyanoethyl [*N,N'*-Bis(4-monomethoxytrityl)-1,3-diamino-2-propyl]-*N,N*-diisopropylphosphoramidite (8). To a solution of **7** (2.54 g, 4.0 mmol) in 10 mL of anhydrous CH_2Cl_2 was added diisopropylethylamine (4 mL) followed by 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.3 mL, 5.8 mmol). After 30 min, 0.1 mL of CH_3OH was added to quench the excess phosphitylating reagent. After being diluted with ethyl acetate to 50 mL, the reaction mixture was washed twice with 50 mL of saturated $NaHCO_3$ and 50 mL of brine and dried over Na_2SO_4. The solution was concentrated to 10 mL and applied onto a silica gel column (4 × 20 cm). The product was eluted with hexanes/ethyl acetate/Et_3N (8:2:1) to give a colorless foam after evaporation of the solvent: yield 2.6 g (78%); ^1H NMR ($CDCl_3$) δ 7.5–7.1 (m, 24 H), 6.80 (m, 4 H), 4.14 (1 H), 3.79 (s, 3 H), 3.77 (s, 3 H), 3.7–3.3 (m, 4 H), 2.5–2.2 (m, 6 H), 1.12 (d, 6 H, J = 7 Hz), 1.04 (d, 6 H, J = 7 Hz). Anal. ($C_{52}H_{59}N_4O_4P$) C, H, N.

5-(9-Acridinyl)-1-pentanol (9). To an ice-cooled slurry of 3.84 g (13.8 mmol) of 5-(9-acridinyl)pentanoic acid[13] in 150 mL of dry THF was added 21 mL of 1 M BH_3·THF (dropwise with stirring). After 1 h at room temperature, the reaction was quenched with 25 mL of 10% acetic acid in CH_3OH. The mixture was dried *in vacuo*, and the residue was stirred at 100 °C with 100 mL of 1.5 M HCl and 65 mL of 1 M ferric chloride. After 1 h, the heterogeneous mixture was filtered and the solid was washed with 100 mL of hot water. The filtrate was made basic with concentrated ammonia, and the heterogeneous mixture was extracted with ethyl acetate, dried over Na_2SO_4, and dried *in vacuo*. The residue was purified by flash chromatography (silica gel, 4 × 16 cm) using 5% CH_3OH/CH_2Cl_2 to give 2.1 g of yellow solid. Recrystallization from ethyl acetate gave 1.8 g (50%) of **9**: mp 152–154 °C; ^1H NMR ($CDCl_3$) δ 8.25 (m, 4 H), 7.77 (t, 2 H, J = 7.3 Hz), 7.56 (t, 2 H, J = 7.3 Hz), 3.67 (m, 4 H), 1.86 (m, 2 H), 1.7–1.5 (m, 7 H). Anal. ($C_{18}H_{19}NO$) C, H, N.

2-Cyanoethyl [5-(9-acridinyl)-1-pentyl]-*N,N*-diisopropylphosphoramidite (10): pale yellow syrup (73%); ^1H NMR ($CDCl_3$) δ 8.24 (m, 4 H), 7.77 (t, 2 H, J = 7.6 Hz), 7.56 (t, 2 H, J = 7.6 Hz), 3.8–3.4 (m, 8 H), 2.61 (t, 2 H, J = 6.5 Hz), 2.0–1.6 (m, 6 H), 1.2–1.0 (m, 12 H). Anal. ($C_{27}H_{36}N_3O_2P·0.3H_2O$) C, H, N.

2-Cyanoethyl [[(cholesteryloxy)carbonyl]-5-[[bis(4-methoxyphenyl)phenylmethoxy]methyl]-(3*R*)-*trans*-3-pyrrolidinyl]-*N,N*-diisopropylphosphoramidite (11): colorless foam (69%); ^1H NMR ($CDCl_3$) δ 7.5–7.1 (m, 9 H), 6.83 (d, 4 H, J = 8 Hz), 5.4–5.2 (m, 1 H), 4.6–4.3 (m, 2 H), 4.2–4.0 (m, 1 H), 3.78 (s, 6 H), 3.78–3.0 (m, 8 H, partially overlapped with OCH_3), 2.60 (t, 2 H, J = 5 Hz), 2.4–0.7 (m, 55 H), 0.66 (s, 3 H). Anal. ($C_{63}H_{90}N_3P$) C, H, N, P.

Small Scale Oligonucleotide Synthesis. 3′-Modified ODNs for determining SAR were synthesized from 2 μmol (~50 mg) of the appropriate modified CPG support **6** on an Applied Biosystems Model 394 DNA synthesizer using the 1 μmol coupling program supplied by the manufacturer. 3′-Hexylamine-modified ODNs **1t,w** were prepared using hexylamine CPG.[26] ODN **1l** was prepared on a 1 μmol scale. Standard reagents for the β-cyanoethyl phosphoramidite-coupling chemistry were used. Phosphorothioate modifications were introduced at the desired internucleoside position using 3*H*-1,2-benzodithiol-3-one 1,1-dioxide as the sulfurizing agent.[15] 5′-Modifications were introduced into ODNs **1l–w** on the DNA synthesizer using the appropriate phosphoramidite reagent. Phosphoramidites were either prepared as described above or purchased from Glen Research (Sterling, VA). After ammonia deprotection, the ODNs were HPLC purified, detritylated, and precipitated from butanol as described previously.[13] Unless otherwise mentioned, the ODN products gave one major band by gel electrophoresis (20% polyacrylamide–7 M urea gel, silver staining). Analytical C18 HPLC was used to determine product purity as described in Figure 2. If ODNs were less than 90% pure, they were repurified on a 10 × 250 mm C18 column (flow rate = 4.7 mL/min). To ensure complete removal of (volatile) triethylammonium salts, the ODNs were dissolved in 1 mL of water and dried with 3 equiv of $NaHCO_3$. The ODNs were filtered through 0.45 μm syringe filters as a final purification step before cell culture assay. The ODNs were redissolved in 1 mL of water, and ODN concentrations of 1:100 dilutions were determined in 0.1 M Tris buffer (pH 7.5) using extinction coefficients calculated for single-stranded ODNs.[27] A_{260} values were obtained on a Beckman DU-40 spectrophotometer using 0.1 mL quartz cuvettes.

Thermal Denaturation Studies. Selected ODNs were prepared in the specified concentrations as solutions in pH 7.2 1× PBS (9.2 mM disodium phosphate, 0.8 mM monosodium phosphate, 0.131 M sodium chloride). A Perkin Elmer Lambda 2S UV–vis spectrophotometer equipped with a PTP6 thermal programmer was used. Thermal dissociation curves were obtained by heating samples from 10 to 90 °C with a temperature increase of 0.5 °C/min. A_{260} vs time values and the first derivative data were determined automatically. The T_m was determined from the derivative maximum. Data from one representative run are given in Table 3. An average from three synthetic runs (in triplicate) is given in Table 4.

Synthesis of Cholesterol-Modified Poly(styrene) Support 6g. Amino-derivatized poly(styrene) support with a loading level of ~50 μmol/g was purchased from Pharmacia (Uppsala, Sweden). A mixture of 100 g of the poly(styrene) support, 30 mL of diisopropylethylamine, and 7.0 g (6.6 mmol) of **5g** in 300 mL of dry DMF were swirled in a 500 mL round-bottom flask (orbital shaker, 150 rpm). After 24 h, the solid support was filtered on a sintered glass funnel and washed with 1.5 L portions of DMF, acetone, and ether. The solid support was dried under vacuum and treated with with a mixture of pyridine (300 mL), *N*-methylimidazole (30 mL), and acetic anhydride (30 mL). After swirling for 30 min, the support was filtered and washed as described above and then dried under vacuum for 24 h. This procedure provided a loading of 28 μmol/g as determined by DMTr content.

Large Scale Oligonucleotide Synthesis (1g). ODN **1g** was synthesized from 14.8 g (414 μmol) of the cholesterol-modified poly(styrene) support **6g** described above. The desired 10-mer was prepared on a Pharmacia "OligoPilot" DNA synthesizer using the 400 μmol program supplied by the manufacturer. Reagent consumption and specifications are provided in Table 4. Final detritylation was executed on the instrument. After the synthesis was complete (5.5 h), the column reactor was dried on a vacuum flask (15 min), and the support was transferred to a 250 mL glass bottle. After wetting the support with 40 mL of EtOH, 160 mL of 30% NH_4OH was added. The bottle was capped, shaken, and incubated at 50 °C for 16 h. After cooling to room temperature, the solution was carefully (foaming) filtered into a 500 mL round-bottom flask. The poly(styrene) support was washed with water, and the combined filtrates were concentrated to dryness on a Rotavap at 35 °C. The amber syrup residue was dissolved in 30 mL of water, and the crude product was analyzed by capillary gel electrophoresis (CGE). CGE was performed on a Beckman P/ACE 2100 system using an eCAP U100P gel column (40 cm) and showed 70–80% full length product. Samples were analyzed at 14.1 kV using Tris borate buffer containing 7 M urea.

$NaClO_4$ (2 M, 2 mL) and 5 mL of CH_3CN were added to the crude sample of **1g**, and the solution was purified in a single HPLC run. Preparative HPLC was carried out using a Rainin dual-pump system equipped with a Rainin UV-1 detector. Pump control and data processing used Rainin Dynamax software on a Macintosh computer. Purification was accomplished on a 250 × 41.4 mm Rainin Dynamax C18 column (5 μm, 300 Å). The product was eluted with a linear gradient of 20–80% CH_3CN in 0.1 M $NaClO_4$ (pH 7) over 45 min (flow rate = 50 mL/min). The product eluted at ~25 min in ~250 mL of solvent. The smaller (cholesterol-bearing) "failure" peaks eluted after the desired product peak. The solvent was removed on a Rotavap, and the residual white gel was triturated wtih 80 mL of acetone. The white solid gum was dissolved in 35 mL of water (with heating) and added in 2 mL portions to a vigorously stirred solution of 400 mL of 1% $NaClO_4$ in acetone. After 5 min, the white precipitate was allowed to settle and the majority of the acetone was decanted. The remaining suspension was centrifuged for 10 min at 2000 rpm in a 150 mL poly(propylene) tube. The precipitated **1g** was washed with 120 mL of acetone to remove excess sodium

Table 4. Summary of Results from Large Scale Synthesis of ODN 1g[a]

synthesis scale (from solid support)[b]	414 μmol
phosphoramidite consumption[c]	dA: 1.54 g (1.8 mmol) dC: 2.25 g (2.7 mmol) dG: 2.27 g (2.7 mmol) T: 1.34 g (1.8 mmol)
yield of lyophilized product (Na^+ salt)	633 mg (36% yield)[b]
molecular formula[d]	$C_{129}H_{167}N_{39}O_{64}P_{10}Na_{10}\cdot 25H_2O$
molecular weight	4277
combustion analysis (calcd/found)	C = 36.22/36.34 H = 5.11/5.03 N = 12.77/12.57 P = 7.24/7.09
UV absorbance[e] (λ_{max} = 258 nm)	1 au = 57.9 μg/mL (±0.9)
T_m (50 μg/mL in 1 × PBS, pH 7.2)[f]	50.0 °C (±0.9)
purity specifications	C18 HPLC (>98%)[g] CGE (>98%)[h]

[a] Yield and analysis results are from a single production run. UV properties are averages from three runs. [b] Cholesterol-modified poly(styrene) support **6g** was the limiting reagent. [c] Does not include holdup of reagent in synthesizer tubing. [d] The degree of hydration was confirmed experimentally by Karl–Fisher titration. [e] UV absorbance was determined for a 50 μg/mL solution in 1× PBS (pH 7.2) at 10 °C. [f] T_m was determined for a 50 μg/mL solution as described in the Experimental Section. [g] HPLC conditions are described in Figure 2. [h] Capillary gel electrophoresis conditions are described in the Experimental Section.

perchlorate, recentrifuged, and dried *in vacuo* for 16 h. Finally, the solid product was dissolved in 20 mL of sterile water, filtered through a 0.45 μm syringe filter, and lyophilized to constant weight to give 633 mg (36% yield based on starting immobilized cholesterol) of **1g** as a white solid foam. The product was further characterized by C18 HPLC, CGE, UV, and elemental analysis. Results are presented in Table 4.

Knowledge of the degree of hydration allowed the molecular weight and molar extinction coefficient to be determined for ODN **1g**. The UV absorbance was determined at 10 °C at the λ_{max} (258 nm) since the T_m curve was flattest at this temperature. T_m was routinely determined since this property is intimately linked to the biological activity of the product; 1 A_{258} unit was determined to be ~58 μg/mL. The earlier calculated value for **1g** used for determining ODN concentration was 38 μg/mL (determined from extinction coefficients derived from single-stranded structures[27]). Therefore the corrected IC_{50} for ODN **1g** (shown in Figure 2) is ~0.6 μM (2.5 μg/mL).

Biology. Addition of ODNs to Cell Culture and Cell Morphology Examination. Hep G2 hepatoma cells were obtained from the American Type Culture Collection and grown in minimal essential medium with Earl's salts and 10% fetal bovine serum in 5% CO_2 conditions at 37 °C. During ODN treatment, the regular fetal bovine serum in the medium was substituted with heat-inactivated fetal bovine serum. Cells (10 000/well) were plated in 24-well plates and grown in 0.5 mL of indicated medium for 24 h. The cultures were then exposed to ODN at indicated concentrations. Cultures were examined for cell morphology changes after 24 h under a phase contrast invertoscope (Reichert-Jung). The examination was extended to the subsequent 3 days. Cultures not treated with any ODN were also run in parallel as controls. In each case, duplicate treatments were conducted. Careful examination of the cultures were made to detect any cellular secretion, cell floating, etc. Cell morphology changes (CMC) were scored in comparison to active ODN **1a** as follows: equally active (+), less active (±), or inactive (−).

Clonogenic Assay. Hep G2 cells (500–700) were plated in triplicate in a 6-well plate. After 24 h, the cultures were treated with varying concentrations of ODN. Control cultures received media change without any ODN. The cultures were incubated for 24 h, at which point the medium was aspirated and fresh medium was added. After 14 days, the cultures were fixed and stained with methylene blue. Macroscopic colonies were counted. IC_{50} values were determined graphically as the concentration that inhibits colony formation by 50%.

Acknowledgment. We thank David Adams and Enjia Yang for technical assistance and Drs. Howard Gamper and Mikhail Podyminogin for helpful discussions during the course of this work. This work was supported by grants from the National Institutes of Health (AI29277, CA44358, and CA09085) and a gift from MicroProbe Corp.

References

(1) Zhou, J. H.; Pai, B. S.; Reed, M. W.; Gamper, H. B.; Lukhtanov, E.; Podyminogin, M.; Meyer, R. B., Jr.; Cheng, Y.-c. Discovery of Short, 3′-Cholesterol-Modified DNA Duplexes with Unique Antitumor Cell Activity. *Cancer Res.* **1994**, *54*, 5783–5787.
(2) Boutorin, A. S.; Gus'kova, L. V.; Ivanova, E. M.; Kobetz, N. D.; Zarytova, V. F.; Ryte, A. S.; Yurchenko, L. V.; Vlassov, V. V. Synthesis of Alkylating Oligonucleotide Derivatives Containing Cholesterol or Phenazinium Residues at their 3′-Terminus and their Interaction with DNA within Mammalian Cells. *FEBS Lett.* **1989**, *254*, 129–132.
(3) Kreig, A. M.; Tonkinson, J.; Matson, S.; Zhao, Q.; Saxon, M.; Zhang, L.-M.; Bhanja, U.; Yakubov, L.; Stein, C. A. Modification of Antisense Phosphodiester Oligodeoxynucleotides by a 5′ Cholesterol Moiety Increases Cellular Association and Improves Efficacy. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1048–1052.
(4) de Smidt, P. C.; Le Doan, T.; de Falco, S.; van Berkel, T. J. C. Association of Antisense Oligonucleotides with Lipoproteins Prolongs the Plasma Half-life and Modifies the Tissue Distribution. *Nucleic Acids Res.* **1991**, *17*, 4695–4700.
(5) Gamper, H. B.; Reed, M. W.; Cox, T.; Virosco, J. S.; Adams, A. D.; Gall, A. A.; Scholler, J. K.; Meyer, R. B., Jr. Facile Preparation of Nuclease Resistant 3′-Modified Oligodeoxynucleotides. *Nucleic Acids Res.* **1993**, *21*, 145–150.
(6) Letsinger, R. L.; Zhang, G.; Sun, S.; Ikeuchi, T.; Sarin, P. S. Cholesterol-Conjugated Oligonucleotides: Synthesis, Properties, and Activity as Inhibitors of Replication of Human Immunodeficiency Virus in Cell Culture. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6553–6556.
(7) Shea, R. G.; Marsters, J. C.; Bischofberger, N. Synthesis, Hybridization Properties and Antiviral Activity of Lipid-Oligodeoxynucleotide Conjugates. *Nucleic Acids Res.* **1990**, *18*, 3777–3783.
(8) Stein, C. A.; Pal, R.; DeVico, A. L.; Hoke, G.; Mumbauer, S.; Kinstler, O.; Sarngadharan, G.; Letsinger, R. L. Mode of Action of 5′-Linked Cholesterol Phosphorothioate Oligodeoxynucleotides in Inhibiting Syncytia Formation and Infection by HIV-1 and HIV-2 In Vitro. *Biochemistry* **1991**, *30*, 2439–2444.
(9) Wyatt, J. R.; Vickers, T. A.; Roberson, J. L.; Buckheit, R. W., Jr.; Klimkait, T.; Debaets, E.; Davis, P. W.; Rayner, B.; Imbach, J. L.; Ecker, D. J. Combinatorially Selected Guanosine-Quartet Structure is a Potent Inhibitor of Human Immunodeficiency Virus Envelope-Mediated Cell Fusion. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 1356–1360.
(10) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. Selection of Single-Stranded DNA Molecules that Bind and Inhibit Human Thrombin. *Nature* **1992**, *355*, 564–566.
(11) Burgess, T. L.; Fisher, E. L.; Ross, S. L.; Bready, J. V.; Qian, Y.-x.; Bayewitch, L. A.; Cohen, A. M.; Herrera, C. J.; Hu, S. S.-F.; Kramer, T. B.; Lott, F. D.; Martin, F. H.; Pierce, G. F.; Simonet, L.; Farrell, C. L. The Antiproliferative Activity of c-myb and c-myc Antisense Oligonucleotides in Smooth Muscle Cells is Caused by a Nonantisense Mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4051–4055.
(12) Grever, M. R.; Schepartz, S. A.; Chabner, B. A. The National Cancer Institute: Cancer Discovery and Development Program. *Semin. Oncol.* **1992**, *19*, 622–638.
(13) Reed, M. W.; Adams, A. D.; Nelson, J. S.; Meyer, R. B., Jr. Acridine- and Cholesterol-Derivatized Solid Supports for Improved Synthesis of 3′-Modified Oligonucleotides. *Bioconjugate Chem.* **1991**, *2*, 217–225.
(14) Atkinson, T.; Smith, M. Solid-Phase Synthesis of Oligodeoxyribonucleotides by the Phosphite-Triester Method. In *Oligonucleotide Synthesis, A Practical Approach*; Gait, M., Ed.; IRL Press: Washington, DC, 1984; pp 35–81.
(15) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. The Automated Synthesis of Sulfur-Containing Oligodeoxynucleotides using 3*H*-1,2-Benzodithiole-3-one 1,1-dioxide as a Sulfur-Transfer Reagent. *J. Org. Chem.* **1990**, *55*, 4693–4699.
(16) Gryaznov, S. M.; Lloyd, D. H. Modulation of Oligonucleotide Duplex and Triplex Stability via Hydrophobic Interactions. *Nucleic Acids Res.* **1993**, *21*, 5909–5915.
(17) Jager, A.; Levy, M. J.; Hecht, S. M. Oligonucleotide *N*-Alkylphosphoramidates: Synthesis and Binding to Polynucleotides. *Biochemistry* **1988**, *27*, 7237–7246.

(18) Milligan, J. F.; Matteucci, M. D.; Martin, J. C. Current Concepts in Antisense Drug Design. *J. Med. Chem.* **1993**, *36*, 1923–1937.
(19) Stein, C. A.; Subasinghe, C.; Shinozuka, K.; Cohen, J. S. Physicochemical Properties of Phosphorothioate Oligodeoxynucleotides. *Nucleic Acids Res.* **1988**, *16*, 3209–3221.
(20) Brown, D. A.; Kang, S.-H.; Gryaznov, S. M.; Dedionisio, L.; Heidenreich, O.; Sullivan, S.; Xu, X.; Nerenberg, M. I. Effect of Phosphorothioate Modification of Oligodeoxynucleotides on Specific Protein Binding. *J. Biol. Chem.* **1994**, *43*, 26801–26805.
(21) Shea, R. G.; Marsters, J. C.; Bischofberger, N. Synthesis, Hybridization Properties and Antiviral Activity of Lipid-Oligodeoxynucleotide Conjugates. *Nucleic Acids Res.* **1990**, *18*, 3777–3783.
(22) Reed, M. W. Synthesis of a Host Specific Toxin from *Alternaria alternata* f. sp. *lycopersici*. Dissertation, University of California, Davis, CA, 1986; pp 83–86.
(23) Seela, F.; Kaiser, K. Oligodeoxyribonucleotides Containing 1,3-Propanediol as Nucleoside Substitute. *Nucleic Acids Res.* **1987**, *15*, 3113–3129.
(24) Hinrichsen, R. D.; Fraga, D.; Reed, M. W. 3′-Modified Antisense Oligodeoxyribonucleotides Complementary to Calmodulin mRNA Alter Behavioral Responses in *Paramecium*. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8601–8605.
(25) MacKellar, C.; Graham, D.; Will, D. W.; Burgess, S.; Brown, T. Synthesis and Physical Properties of *anti*-HIV Antisense Oligonucleotides Bearing Terminal Lipophilic Groups. *Nucleic Acids Res.* **1992**, *20*, 3411–3417.
(26) Petrie, C. R.; Reed, M. W.; Adams, A. D.; Meyer, R. B., Jr. An Improved CPG Support for the Synthesis of 3′-Amine-Tailed Oligonucleotides. *Bioconjugate Chem.* **1992**, *3*, 85–87.
(27) Cantor, C. R.; Warshaw, M. M.; Shapiro, H. Oligonucleotide Interactions. III. Circular Dichroism Studies of the Conformation of Deoxyoligonucleotides. *Biopolymers* **1970**, *9*, 1059–1077.

JM950388M