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Solid-Phase Synthesis, Thermal Denaturation Studies, Nuclease Resistance, and Cellular Uptake of (Oligodeoxyribonucleoside)methylborane Phosphine−DNA Chimeras

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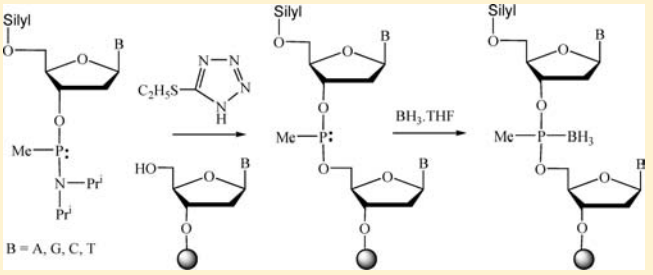
Supporting Information

ABSTRACT: The major hurdle associated with utilizing oligodeoxyribonucleotides for therapeutic purposes is their poor delivery into cells coupled with high nuclease susceptibility. In an attempt to combine the nonionic nature and high nuclease stability of the P−C bond of methylphosphonates with the high membrane permeability, low toxicity, and improved gene silencing ability of borane phosphonates, we have focused our research on the relatively unexplored methylborane phosphine (Me−P−BH_3) modification. This Article describes the automated solid-phase synthesis of mixed-backbone oligodeoxynucleotides (ODNs) consisting of methylborane phosphine and phosphate or thiophosphate linkages (16-mers). Nuclease stability assays show that methylborane phosphine ODNs are highly resistant to 5′ and 3′ exonucleases. When hybridized to a complementary strand, the ODN:RNA duplex was more stable than its corresponding ODN:DNA duplex. The binding affinity of ODN:RNA duplex increased at lower salt concentration and approached that of a native DNA:RNA duplex under conditions close to physiological saline, indicating that the Me−P−BH_3 linkage is positively charged. Cellular uptake measurements indicate that these ODNs are efficiently taken up by cells even when the strand is 13% modified. Treatment of HeLa cells and WM-239A cells with fluorescently labeled ODNs shows significant cytoplasmic fluorescence when viewed under a microscope. Our results suggest that methylborane phosphine ODNs may prove very valuable as potential candidates in antisense research and RNAi.



INTRODUCTION

The ideal oligodeoxynucleotide drug molecule would be one that possesses enhanced intracellular delivery, high target-binding affinity, and biological activity, while maintaining low nuclease susceptibility, nonspecific binding, and toxicity. The most prevalent approach toward this goal has been the construction of chimeric oligodeoxynucleotides (ODNs) containing segments of different chemistries, each of which confers a certain advantage to the oligomer.[1,2] Taking a step further, we have now optimized the chemistry behind the automated synthesis of a relatively unexplored[3] "hybrid" modification, which has overlapping structural motifs of two well-known antisense molecules, the methylphosphonate[4] and borane phosphonate.[5] Methylphosphonate oligodeoxynucleotides contain nonionic internucleotide linkages that are highly lipophilic and resist enzymatic degradation. However, due to their lipophilicity, they have low water solubility and are often bound to the cell membrane and not available for biological activity in cells.[6] Borane phosphonate oligodeoxynucleotides, on the other hand, have high water solubility, possess favorable cell uptake properties, and show siRNA activity even as single strands.[7] In this Article, we report our research on combining the nonionic nature and high nuclease stability of the P−C bond with the high water solubility, low toxicity, and improved gene silencing ability of borane phosphonates to generate the methylborane phosphine internucleotide linkage.

In terms of size, the substitution of a nonbridging oxygen of the phosphate with a methyl group to generate a methylphosphonate represents the smallest possible structural change that eliminates the negative charge on the backbone. A borane phosphonate modification is isosteric with a methylphosphonate and also isoelectronic with the native phosphate. It also imparts higher lipophilicity to the molecule as compared to a regular phosphate while also retaining its negative charge. In the methylborane phosphine linkage described here, the nonbridging oxygens of a phosphodiester are replaced by a borane and a methyl group, giving rise to a P(IV) center. In the absence of nonbridging oxygens, the electronegativities of the substituents follow the order: C (2.5) > P (2.2) > B (2.0). As a result, the biophysical properties of the resultant methylborane phosphine (Me−P−BH_3) linkage are expected to be different from those of

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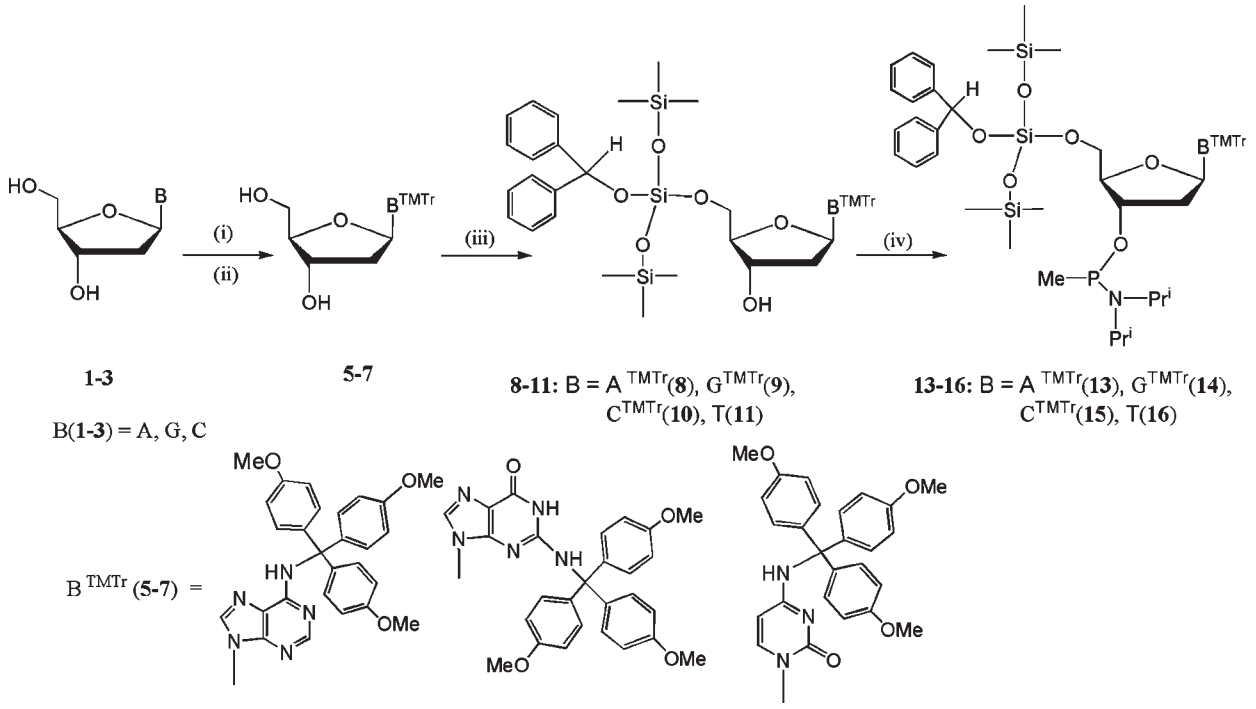
Scheme 1. Synthesis of 5′-*O*-Silyl-*N*-trimethoxytrityl-2′-deoxynucleoside-3′-*O*-Methylphosphinoamidites (A, G, C) and 5′-*O*-Silyl-2′-deoxythymidine-3′-*O*-methylphosphinoamidite[a]



[a] Reagents and conditions: (i) 4 equiv of Me_3SiCl, pyridine, 2 h; (ii) 1.2 equiv of 4,4′,4″-trimethoxytritylchloride, pyridine, 16 h; (iii) 1.1 equiv of [benzhydryloxy-bis(trimethylsilyloxy)silyl]chloride, 3 equiv of imidazole, DMF, 6 h; (iv) 1.2 equiv of $MeP[N(i\text{-}C_3H_7)_2]_2$, CH_2Cl_2, 4,5-dicyano imidazole, 40 min.

the methylphosphonate and the borane phosphonate analogues. We hypothesize that the resultant new class of compounds might embody the best characteristics of both precursor molecules, the high nuclease stability of the P−C bond of methylphosphonates combined with the high aqueous solubility, membrane permeability, low toxicity, and improved gene silencing ability of borane phosphonates.

Some years ago, our group developed a phosphoramidite-based method for the high yielding synthesis of mixed-sequence borane phosphonate DNA.[8] These and further research efforts have led us to replace the conventional 5′-*O*-dimethoxytrityl protecting group of the phosphoramidite synthons with a fluoride labile 5′-*O*-silyl ether (5′-*O*-[benzhydryloxy-*bis*(trimethylsilyloxy)-silyl], Bzh). The exocyclic amines of adenine, guanine, and cytosine are protected with the mild acid-labile trimethoxytrityl group. Because phosphoramidite chemistry is being used, thymine is not protected.[9] This new base protection strategy had to be employed because the borane reagents reduce the commonly used amide protecting groups to *N*-alkyl or aryl exocyclic amines or form stable, irreversible borane adducts with these bases.[10] Apart from conferring complete orthogonality to the fluoride labile 5′-*O*-silyl chemistry, the trimethoxytrityl protecting group cannot be reduced by borane and, perhaps because of its large size, prevents the formation of irreversible borane adducts. It should also be noted that the trimethoxytrityl cation generated during the conventional detritylation step (3% trichloroacetic acid in CH_2Cl_2) of DNA synthesis is incompatible with the borane phosphonate linkage.[11,12] We observed that the methylborane phosphine linkage was also prone to decomposition under similar conditions. However, in agreement with a previous literature report,[13] decomposition of the P−BH_3 linkage was negligible when 80% aqueous acetic acid was used for detritylation. Drawing upon these studies, we have now developed an efficient synthetic route to methylborane phosphine ODNs. In this Article, we describe the automated solid-phase synthesis, hybridization experiments (T_m), nuclease susceptibilities, and preliminary in vivo studies on a series of mixed-sequence methylborane phosphine 2′-deoxyoligonucleotide analogues.

RESULTS AND DISCUSSION

Chemical Synthesis. Our primary objective was to develop the methylphosphinoamidite synthons for all four bases that are needed to generate the methylborane phosphine linkages (Scheme 1). The phosphitylating reagent, bis(*N*,*N*-diisopropylamino)methylphosphine, was synthesized in high yield (90%) via the Grignard reaction, starting from bis(*N*,*N*-diisopropylamino)chlorophosphine and methylmagnesium bromide.[14] This diamidite was allowed to react with appropriately N-protected 5′-*O*-silyl-2′-deoxynucleosides to yield the 5′-*O*-silyl-2′-deoxynucleoside-3′-*O*-methylphosphinoamidites (amidites **13**, **14**, and **15**). The thymidine synthon (**16**) does not require base protection. A detailed procedure for the synthesis of amidites **13**−**16** can be found in the Experimental Section. To synthesize chimeric ODNs having the methylborane phosphine and phosphate (or thiophosphate) linkages, *N*-trimethoxytrityl-5′-*O*-silyl-2′-deoxynucleoside-3′-*O*-methylphosphoramidite synthons (**17**−**19**) and 5′-*O*-silyl-2′-deoxythymidine-3′-*O*-methylphosphoramidite (**20**) were prepared from compounds (**8**−**11**) and bis(*N*,*N*-diisopropylamino)methoxyphosphine [Supporting Information, S8−S9].

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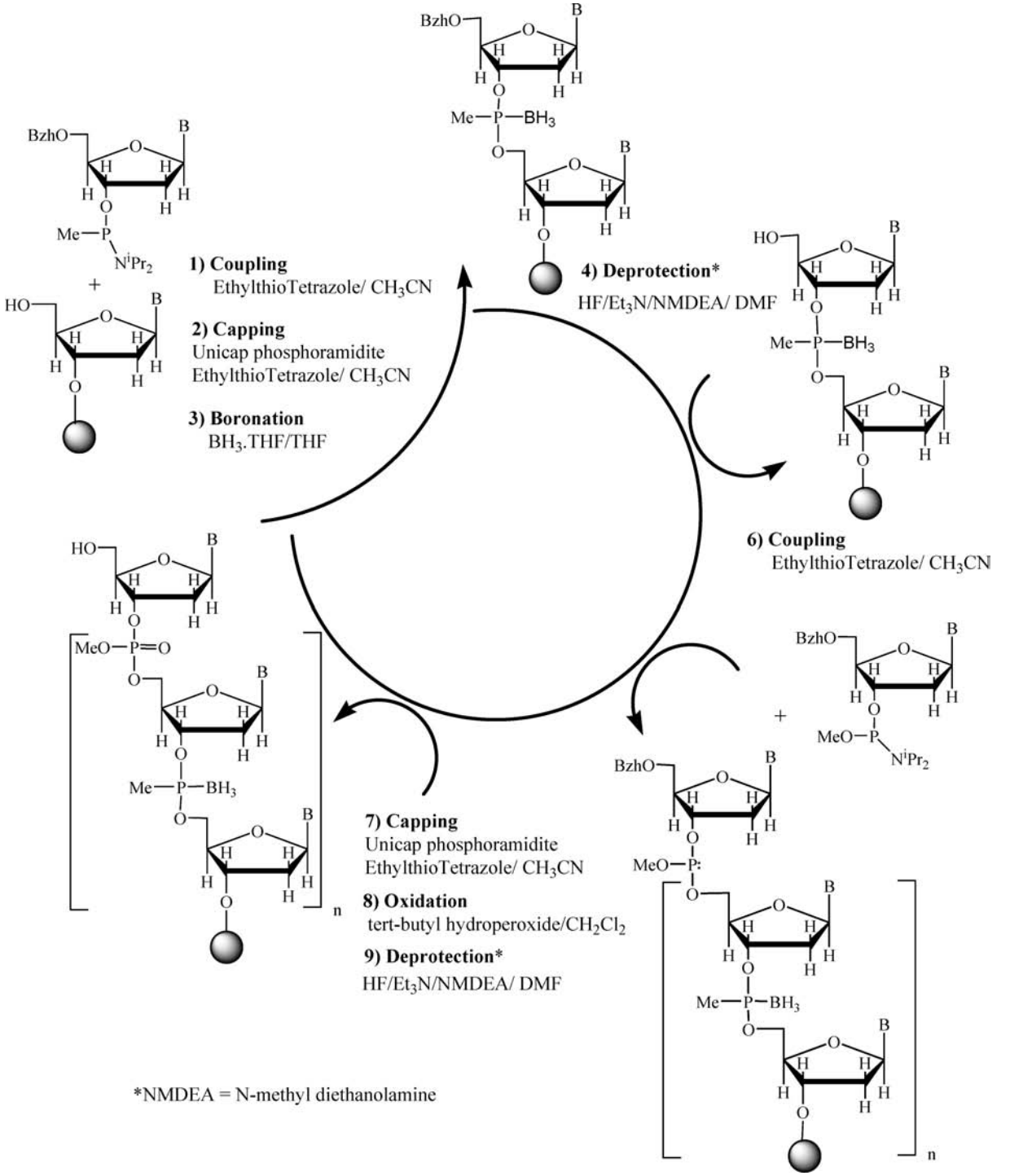
Scheme 2. Solid-Phase Synthesis of Methylborane Phosphine−DNA Chimeras



All amidite synthons (**13**−**20**) have been characterized by NMR and mass spectral analysis. The compounds were obtained in high purity after column chromatography with average yields ranging from 40% to 60%.

Solid-Phase Synthesis. Once these synthons were available, our next goal was to optimize the solid-phase synthesis cycle for preparing methylborane phosphine-modified ODNs. A schematic of this cycle is depicted in Scheme 2. Prior to synthesis, the 5′-DMT group on the 2′-deoxythymidine bound to the polystyrene support[15] was removed with 3% trichloroacetic acid in dichloromethane. The unprotected 2′-deoxynucleoside was allowed to react with the appropriate 5′-*O*-silyl-2′-deoxynucleoside-3′-*O*-methylphosphinoamidite in anhydrous acetonitrile in the presence of 5-ethylthio-1*H*-tetrazole to generate a family of dimers having a methylphosphite diester internucleotide linkage. The coupling wait time was 500 s for the methylphosphinoamidites **13**−**16**; it was decreased to 180 s for the phosphoramidites **17**−**20**. This condensation step was followed by capping with

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Table 1. Synthesis Cycle for Methylborane Phosphine ODNs

reaction	wash/reagents/solvents	time (s)
coupling	0.1 M phosphinoamidites (**13**−**16**) and 0.25 M ethylthio tetrazole in acetonitrile (1:1)	wait: 500 s
	or	
	0.1 M phosphoramidites (**17**−**20**) and 0.25 M ethylthio tetrazole in acetonitrile (1:1)	wait: 180 s
wash	acetonitrile	40 s
capping	0.1 M Unicap Phosphoramidite and 0.25 M ethylthio tetrazole in acetonitrile (1:1) at port 5[a]	wait: 40 s
boronation	25 mM BH_3·THF in THF at port 12	12 to column, 60 s; wait, 120 s
or		
oxidation/sulfurization	1 M anhydrous *tert*-butyl hydroperoxide or Beaucage reagent in acetonitrile at port 15	15 to column, 15 s; wait, 45 s
wash	acetonitrile,	40 s
	dichloromethane	45 s
	dimethylformamide	45 s
5′-deprotection	1.1 M HF/1.1 triethylamine/0.2 M NMDEA[b] in dimethylformamide (pH 9.2)	10 to column, 25 s; wait, 45 s
wash	dimethylformamide,	45 s
	acetonitrile	55 s

[a] The amidite capping was performed as per the manufacturer's instructions.[23] [b] NMDEA = *N*-methyl diethanolamine.

Unicap Phosphoramidite[16] to block failure sequences. The methylphosphite diester was boronated using a freshly prepared solution of 25 mM BH_3·THF in THF to generate a methylborane phosphine (P−IV) linkage. Boronation wait time of 120 s was essential to prevent incomplete boronation, which leads to the generation of methylphosphonate linkages during a subsequent oxidation step. After boronation, the support was washed successively with acetonitrile (40 s), dichloromethane (45 s), and dimethylformamide (45 s) to complete one synthesis cycle. After each condensation step followed by capping and boronation, 5′-*O*-silyl deprotection was carried out using a freshly prepared solution of 1.1 M HF/1.1 M Et_3N/0.2 M *N*-methyl diethanolamine in DMF (pH = 9.2) to generate a family of dinucleotides having a combination of any of the four bases. These dimers could then be extended using the same repetitive cycle to generate multiple methylborane phosphine linkages. To introduce a phosphate or a thiophosphate linkage, the growing 2′-deoxyoligonucleotide chain was allowed to react with the appropriate *N*-trimethoxytrityl protected 5′-*O*-silyl-2′-deoxynucleoside-3′-*O*-methylphosphoramidite using 5-ethylthio-1*H*-tetrazole as activator, followed by capping with Unicap Phosphoramidite. The resultant phosphite triester was treated with anhydrous *tert*-butyl hydroperoxide[17,18] or Beaucage reagent[19] to generate the phosphate or the thiophosphate triester linkage, respectively. The 5′-*O*-silyl deprotection using triethylammonium−hydrogen fluoride mixture as described above completed the cycle. In this manner, either synthesis cycle was repeated until the ODN of appropriate length, sequence, and internucleotide linkage was generated. The protecting groups were removed sequentially as reported for the synthesis of borane phosphonate DNA[8] (see Experimental Section). After deprotection, the ODN was cleaved from the polystyrene support using ethylenediamine in methanol−water−acetonitrile.[20] Table 1 summarizes the synthetic cycle.

Numerous mixed backbone ODNs (16-mers) bearing methylborane phosphine and phosphate or thiophosphate internucleotide linkages have been generated using this synthesis strategy. For all oligomers, similar profiles were obtained during RP-HPLC purification, with each having one major, relatively broad product peak and capped failure sequences, which appeared as earlier eluting products. Broad product peaks were anticipated due to the presence of multiple chiral phosphorus centers. The average yields (95% purity) for a 0.2 μM synthesis ranged from 50 to 80 OD.[21] Aliquots of these ODNs were characterized by analytical RP-HPLC. Figures 8−11 in the Supporting Information show analytical RP-HPLC profiles for the ODNs **1**, **4**, **6**, and **7**, respectively. The mass data for ODNs **1**−**7** having combinations of methylborane phosphine modified segments and internucleotide phosphate or thiophosphate linkages are listed in Table 2. The observed masses for all ODNs correspond to those as calculated. The ^{31}P NMR analyses of the oligomers display a broad signal at 146−150 ppm (methylborane phosphine) and a sharp peak at −1.2 ppm when phosphate is part of the backbone (Figure 1 and Supporting Information, S9, Figure 6), or at 57 ppm for a thiophosphate (Supporting Information, S10, Figure 7). Very similar chemical shifts have been reported for small molecules containing Me−P−BH_3 linkages.[22]

Table 2. MALDI-TOF Analysis of Mixed-Backbone ODNs

		mol. wt[b]	
no.	ODN[a]	calcd	obs
1	$T_{\underline{b}}A_{\underline{b}}A_pC_pA_pC_pG_pA_pT_pA_pC_pG_pC_pG_pA_{\underline{b}}T$	4862.01	4863.11
2	$T_{\underline{b}}A_{\underline{b}}A_pC_pA_pC_pG_pA_pT_pA_pC_pG_pC_pG_{\underline{b}}A_{\underline{b}}T$	4856.10	4853.44
3	$T_{\underline{b}}A_{\underline{b}}A_{\underline{b}}C_pA_pC_pG_pA_pT_pA_pC_pG_pC_{\underline{b}}G_{\underline{b}}A_{\underline{b}}T$	4848.22	4845.92
4	$T_{\underline{b}}A_sA_sC_sA_sC_sG_sA_sT_sA_sC_sG_sC_sG_sA_{\underline{b}}T$	5071.69	5068.33
5	$T_{\underline{b}}A_{\underline{b}}A_sC_sAC_sG_sA_sT_sA_sC_sG_sC_sG_sA_{\underline{b}}T$	5051.77	5052.62
6	$T_{\underline{b}}A_{\underline{b}}A_sC_sA_sC_sG_sA_sT_sA_sC_sG_sC_sG_{\underline{b}}A_{\underline{b}}T$	5031.85	5030.92
7	$T_{\underline{b}}A_sA_sC_sA_{\underline{b}}C_sG_{\underline{b}}A_sT_sA_sC_sG_sC_sG_sA_{\underline{b}}T$	5031.85	5028.73

[a] p, phosphate; b, methylborane phosphine; s, thiophosphate. [b] Observed masses represent MALDI-TOF data acquired on a Voyager DE-STR instrument.

Polyacrylamide-gel electrophoresis (14% gel, 8 M urea) of chimeric methylborane phosphine−phosphate ODNs was carried out to determine their electrophoretic mobility relative to an unmodified phosphate control (oligo **8**) and the tracking dye bromophenol blue. The electrophoretic mobility followed the order: oligo **8** > ODN **2** > bromophenol blue ≈ ODN **3**. When the ODN was 53% modified (ODN **10**), the mobility decreased further (Supporting Information, Table 1, S18). Thus, the electrophoretic mobility of the methylborane phosphine modified ODNs

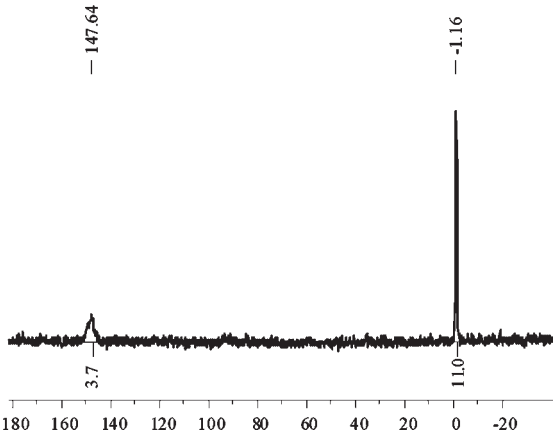

Figure 1. ^{31}P NMR spectrum (300 MHz, D_2O, 13 064 scans) of ODN **2** (5′- $T_bA_bA_pC_pA_pC_pG_pA_pT_pA_pC_pG_pC_pG_bA_bT$-3′).

decreased with increasing number of modifications. A similar reduction in gel mobility has been reported for oligodeoxynucleotides containing neutral methylphosphonate linkages.[4] Such an effect was also found in zwitterionic oligomers bearing positively charged ω-aminohexyl groups[24] and oligoarginine–oligonucleotide conjugates.[25]

Enzymatic Studies. It is known that both the methylphosphonate and the borane phosphonate modifications are highly resistant to exonuclease activity.[26−28] To evaluate the relative exonuclease susceptibility of the methylborane phosphine-modified ODNs, we synthesized a series of 16-mers having the random sequence 5′-TAA CAC GAT ACG CGA T-3′ with an increasing number of methylborane phosphine caps and phosphate or phosphorothioate gaps. Unmodified phosphate and phosphorothioate oligomers having the same sequence were used as controls. The oligomers were tested for stability against snake venom phosphodiesterase (SVPDE, 3′-exonuclease) and calf spleen phosphodiesterase (CSPDE, 5′-exonuclease). For the SVPDE assay, a sample of the oligomer (1 OD) was incubated at 37 °C with 5 μg of the enzyme in Tris-HCl buffer (pH 9) containing 10 mM $MgCl_2$. The CSPDE assay was carried out using 1 OD of the oligomer with 0.1 U enzyme in ammonium acetate buffer (pH 6.8) containing 2.5 mM EDTA at 37 °C. Aliquots were removed at different time points, denatured, and analyzed by RP-HPLC as described in the Supporting Information. The half-lives were determined by calculating the time taken for 50% degradation of the starting oligodeoxynucleotide as observed by RP-HPLC. ODN **3**, with three methylborane phosphine "caps" on each end and a phosphate "gap", was found to have a half-life of ∼2.5 h in the presence of SVPDE. A rapid degradation of the internal phosphodiester linkages was observed as soon as the methylborane phosphine cap at the 3′ end was degraded by the enzyme. The all-phosphate DNA control was fully degraded in less than 5 min under the same experimental conditions. ODN **2** and ODN **1** with two and one methylborane phosphine caps on each end respectively were degraded at a relatively faster rate (**2**, $t_{1/2}$ = ∼90 min; **1**, $t_{1/2}$ = ∼ 60 min) by the enzyme (Figure 2). The stability of oligomers increased at least 6-fold when the internal phosphate linkages were substituted by thiophosphates. Only a slight increase in the rate of degradation of ODNs **4** and **6** was observed even when the enzyme concentration was increased 10-fold. With CSPDE, even a single modification at each end of the oligomer (ODN **1**) prevented the

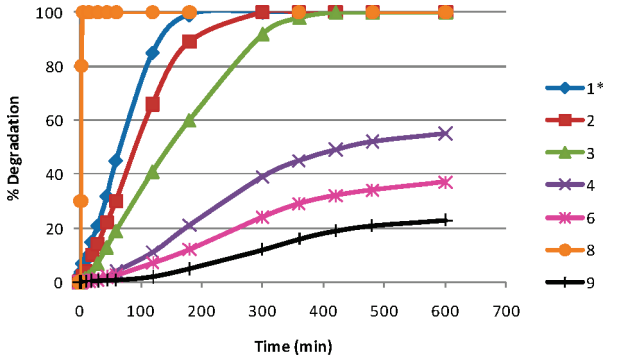

Figure 2. Nuclease resistance of ODNs against SVPDE. Half-lives are defined as time taken for 50% degradation of the starting oligodeoxynucleotide; **8** = phosphate DNA; **9** = thiophosphate DNA. *Legends 1−9 correspond to ODNs **1**−**9** respectively, as listed in Tables 2 and 3.

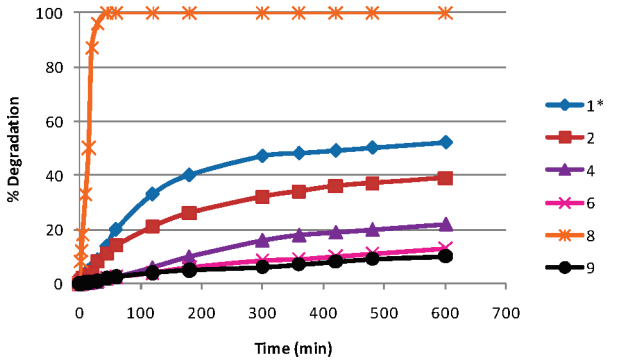

Figure 3. Nuclease resistance of ODNs against CSPDE. Half-lives are defined as time taken for 50% degradation of the starting oligodeoxynucleotide; **8** = phosphate DNA; **9** = thiophosphate DNA. *Legends 1−9 correspond to ODNs **1**−**9**, respectively, as listed in Tables 2 and 3.

degradation of the vicinal phosphodiester linkages for a longer time ($t_{1/2}$ = 6 h; Figure 3). ODN **2** with two modifications at each end was almost twice as stable ($t_{1/2}$ > 12 h). Under the same conditions, the all-phosphate control was completely degraded in less than 20 min. As anticipated, when the phosphate gap was substituted with phosphorothioate linkages (ODNs **4**, **6**), the nuclease resistance increased considerably ($t_{1/2}$ >20 h).

Early reports on methylphosphonate dimers suggest that one diastereoisomer is slowly hydrolyzed by SVPDE.[29] It has also been demonstrated that only the (*Sp*)-stereoisomer of the deoxyribonucleoside borane phosphonate dimer is digestible by SVPDE.[27] It was interesting to see whether SVPDE can specifically degrade one stereoisomer (*Rp* or *Sp*) of a methylborane phosphine linkage. A pentameric oligonucleotide (5′-TTT_bTT-3′) bearing a single methylborane phosphine linkage flanked by phosphates was subjected to SVPDE assay under the above experimental conditions. Analytical RP-HPLC data indicate that there is no enzyme specificity toward either isomer; both isomers were degraded at a similar rate (Supporting Information, S19, Figure 15).

Hybridization Experiments. The ability of an oligonucleotide to bind to its target is an important parameter for its biological activity. To assess the target binding ability of the methylborane phosphine modified ODNs, duplex hybridization studies were performed. ODNs **1**−**7** were mixed with the cDNA

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Table 3. T_m Data for ODN/DNA Duplexes

	no. of modifications				
duplex	$MePBH_3$	thio	T_m[a] (°C)	ΔT_m (°C)	$\Delta T_m'$[d] (°C)
1/**8**	2	0	53.5	1	0.5
2/**8**	4	0	48.0	6.5	1.6
3/**8**	6	0	42.5	12.0	2.0
4/**8**	2	13	45.9	1.6	0.8
5/**8**	3	12	45.0	2.5	0.8
6/**8**	4	11	41.9	5.6	1.4
7/**8**	4	11	40.3	7.2	1.8
8[b]/**8**	0	0	54.5		
9[c]/**8**	0	15	47.5		

[a] All T_m's represent an average of at least two experiments. [b] **8** = phosphate DNA. [c] **9** = thiophosphate DNA. [d] $\Delta T_m'$ represents the ΔT_m per modification.

Table 4. T_m Data for ODN/RNA Duplexes

	no. of modifications				
duplex	$MePBH_3$	thio	T_m[a] (°C)	ΔT_m (°C)	$\Delta T_m'$[d] (°C)
1/**8**″[a]	2	0	55.5	1	0.5
2/**8**″	4	0	53.5	3.0	0.8
3/**8**″	6	0	48.5	8.0	1.3
4/**8**″	2	13	47.1	1	0.5
6/**8**″	4	11	44.2	3.8	0.9
8[b]/**8**″	0	0	56.5		
9[c]/**8**″	0	15	48.0		

[a] All T_m's represent an average of at least two experiments. [b] **8** = phosphate DNA. [c] **9** = thiophosphate DNA. [d] $\Delta T_m'$ represents the ΔT_m per modification; **8**″ = phosphate RNA.

strand in a 1:1 ratio in citrate buffer (150 mM NaCl, 15 mM sodium citrate, pH 7.0) to obtain an overall concentration of 0.2 A_{260} units of the duplex. The samples were denatured at 80 °C and cooled to 15 °C. They were then heated at a rate of 1 °C/min, and A_{260} versus time was recorded. Melting temperatures were taken as the temperature of half dissociation and were obtained from the first derivative plots. T_m values for unmodified phosphate and thiophosphate DNA of the same sequence with cDNA were determined for comparison.

The duplexes comprising ODNs **1**−**7** (Table 3) with complementary DNA showed increase in A_{260} upon increasing temperature and gave the characteristic sigmoidal melting curve. As the number of modifications increased from zero to two, the T_m dropped from 54.5 to 53.5 °C. This modest depression, however, was followed by a steep decline to 48.5° when four modifications were introduced. Two additional modifications depressed it further to 42.5 °C. Thus, the depression in T_m for the ODN:DNA duplexes was 0.5 °C/mod for the first two modifications and by 1.6−2 °C/mod for successive modifications (Table 3). In the case of the oligomers **4**−**7** with thiophosphate gaps, a similar trend was observed, although the ΔT_m was −(0.8−1.8) °C/mod. In comparison, a fully modified stereorandom borane phosphonate DNA with all four bases depressed the T_m of a DNA:DNA duplex by 1.3 °C/mod,[30] whereas a fully modified stereorandom methyl phosphonate DNA:DNA duplex was found to have a T_m that was almost equivalent to the corresponding unmodified phosphodiester duplex.[31]

Because the intended intracellular antisense targets are mRNAs, it was of interest to explore the binding affinity of methylborane phosphine ODNs toward complementary all-phosphate RNA. Hybridization studies on ODNs **1**−**4** and **6** with RNA showed that for a given ODN, the ODN:RNA duplex (Table 4) is more stable relative to its ODN:DNA duplex (Table 3). As observed for the ODN**1**:DNA duplex, the ΔT_m for the ODN:RNA duplex was −0.5 °C/mod for the first two modifications introduced. Four modifications depressed the T_m by −0.8 °C/mod as opposed to −1.6 °C/mod for the ODN**2**:DNA duplex, and six modifications depressed the T_m by −1.3 °C/mod as opposed to −2.0 °C/mod for the ODN**3**:DNA duplex. Following the same trend, the depression in T_m was 0.5 and 0.9 °C/mod in a DNA:RNA duplex for ODNs **4** and **6** with thiophosphate gaps, as opposed to 0.8 and 1.4 °C/mod, respectively, for their DNA:DNA duplexes. Thus, a stereorandom methylborane phosphine linkage is more helix stabilizing in a DNA:RNA duplex.

A comparable trend has been observed in the case of borane phosphonate DNA duplexes. Shimizu et al. have reported that a DNA:RNA duplex of stereorandom borane phosphonate DNA with its complementary phosphate RNA [$d(C_bA_bG_bT_b)_3$:r-$(ACUG)_3$, (T_m = 45.0 °C)] is more stable as compared to a DNA:DNA duplex of stereorandom borane phosphonate with complementary phosphate DNA[30] [$d(C_bA_bG_bT_b)_3$:$d(ACTG)_3$] (T_m = 39.7 °C). The control all-phosphate DNA:RNA duplex has a T_m of 52.4 °C under the same conditions. In contrast, the DNA:RNA duplex of the stereorandom methylphosphonate oligodeoxynucleotide $(C_mT_m)_7$A:RNA has a highly depressed T_m (ΔT_m = −26.5 °C) relative to the all-phosphate DNA:RNA control.[32] In the case of borane phosphonates as well as methylborane phosphine ODNs, the reasons for the higher binding affinity for a complementary RNA strand as opposed to a DNA strand could arise from the different conformations of the resultant DNA:DNA and DNA:RNA duplexes.[33] However, more in-depth thermodynamic and structural studies need to be undertaken to precisely determine the reasons behind this effect.

Barawkar et al. have reported that DNA:DNA and DNA:RNA duplexes of oligonucleotides containing internucleoside guanidinium linkages are stabilized when the ionic strength is lowered.[2] This salt-effect is opposite of that observed in the case of phosphate DNA:DNA and DNA:RNA duplexes where increasing the salt concentration improves the stability of the duplex by reducing electrostatic repulsion between the negatively charged phosphate backbones. The reverse salt effect arises due to the strong interactions of the positively charged guanidinium moiety with the phosphodiester linkage of the complementary strand, which becomes the predominant electrostatic interaction under low salt concentrations.

If the methylborane phosphine linkage were positively charged, we expected to see such an effect during the hybridization of chimeric ODNs with cDNA and RNA. To test this hypothesis, the hybridization experiments of ODNs **1**−**3** with RNA were carried out at low salt concentrations (10 mM NaCl, 10 mM Na_2HPO_4, pH 7.1). Indeed, under these conditions, the binding affinity of ODNs **1**−**3** was found to be higher. ODN **1** with two methylborane phosphine linkages exhibited a T_m of 39.5 °C. As expected for an increasing number of positive charges, the T_m for the ODN**2**:RNA duplex was 41.5 °C, and that for the ODN**3**:RNA duplex was 41.9 °C. The T_m for the

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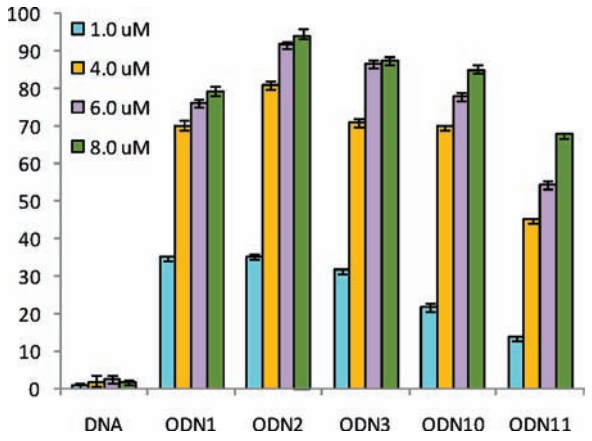
Chart 1. Bar Diagram Showing the Percentage of HeLa Cells Showing Uptake at Various Concentrations of ODNs 1−3, 8, 10, 11 after 16 h of Transfection[a]



[a]The data represent the means ± SEM of three independent experiments.

all-phosphate DNA:RNA control duplex was 41.9 °C. Thus, the binding affinity of ODN**3** with complementary RNA approached that for the unmodified DNA:RNA duplex at 10 mM NaCl (close to physiological conditions), suggesting that the methylborane phosphine moiety might be positively charged. These results are encouraging relative to the use of methylborane phosphine containing ODNs in various biological experiments carried out under physiological salt conditions.

Flow Cytometric Experiments. Encouraged by the results obtained from preliminary biochemical experiments, we next explored the cell-uptake properties of single-stranded methylborane phosphine ODNs as a function of an increasing number of modifications. For this purpose, 5′-fluorescein labeled ODNs **1**−**3** (Table 2) were synthesized. In an attempt to compare the efficiency of a cap-gap ODN versus an ODN having alternating methylborane phosphine and phosphate internucleotide linkages, fluorescein labeled ODN **10** ($T_bA_bA_bC_bA_pC_pG_pA_pT_pA_p$-$C_pG_bC_bG_bA_bT$) and **11** ($T_bA_pA_bC_pA_bC_pG_bA_pT_bA_pC_bG_pC_bG_p$-$A_bT$) were synthesized. The fluorescein tags on ODNs **1**−**3**, **8**, **10**, and **11** were introduced via borane phosphonate linkages to reduce the rate of enzymatic hydrolysis resulting in the release of fluorescein. The fluorescence detected by FACS was expected to arise from fluorescein attached to intact ODNs because Fisher et al. have shown that fluorescein, if detached from ODNs, is quickly discarded from cells.[34]

We first studied the uptake of methylborane phosphine ODNs **1**−**3**, **10**, and **11** in HeLa cells. Exponentially growing HeLa cells maintained as subconfluent monolayers in Dulbecco's Modified Eagle Medium (DMEM) were seeded on 12-well culture plates 24 h before transfection. During transfection, the medium was replaced with DMEM premixed with the ODNs at various concentrations (1.0, 4.0, 6.0, and 8.0 μM), and the cells were further incubated for 16 h. The cells were then rinsed three times with D-PBS (Dulbecco's phosphate buffer saline), trypsinized, and resuspended in D-PBS. Fluorescence intensity was analyzed for cells presenting higher fluorescence than the background (Chart 1). The background was defined as the autofluorescence of cells incubated only with DMEM during transfection; single-stranded 5′-fluorescein tagged phosphate DNA (**8**) was used as the control.

These experiments revealed that the uptake of the methylborane phosphine ODNs was highly dependent on concentration as well as the percentage of modifications introduced. The cells treated with the DNA control exhibited a very weak fluorescence at all concentrations. For all ODNs studied, there was an increase in uptake efficiency when the concentration was increased from 1.0 to 4.0 μM. In the case of ODNs **1**−**3**, a plateau of maximum uptake was reached around 6−8.0 μM. The cellular uptake pathway appeared to be saturable at higher concentrations, suggesting the possible involvement of cell-surface receptors. In contrast, the increase in cellular accumulation of methylphosphonate oligonucleotides which predominantly occurs by endocytotic pathways was shown to be unsaturable even at 100 μM.[35] Another interesting observation was that the percentage of cells showing uptake was not directly proportional to the number of modifications on the ODNs. Thus, ODN **2** with two modifications at both the 5′ and 3′ ends seemed to be more efficiently taken up by cells as compared to ODNs **1**, **3**, and **10**; ODN **3** with three caps (six modifications) was comparable to ODN **1**, but slightly less efficient than ODN **2**. ODN **10** with eight modifications and the ODN **11** variant did not show improved cell uptake properties relative to ODN **3**. It was also noted that cap-gap ODN **10** was more efficient than ODN **11** at all concentrations studied even though both had 8 methylborane phosphine linkages. Although there are numerous reports in the literature wherein a single molecule has been conjugated to DNA or RNA to improve cell delivery,[36−39] there are no studies detailing the effect of increasing number of borane phosphonate modifications on uptake. Further cell uptake experiments and mechanistic studies dealing with single-stranded borane phosphonate oligonucleotides and methylborane phosphine ODNs will be needed to confirm whether this effect is brought about by the BH_3 moiety, or if it is specific to the methylborane phosphine linkage or the positive charge, which appears to be associated with this site.

Fluorescence Microscopy. It has been demonstrated that chemically modified oligonucleotides are generally taken up by cells through various endocytotic pathways[35,40] leading to entrapment in endocytotic vesicles, which results in poor biological activity. Subcellular distribution patterns of methylborane phosphine ODNs after 24 h of passive transfection were visualized by fluorescence microscopy. HeLa cells were incubated with 5.0 μM fluorescein-tagged ODN **12** ($T_bT_bT_pT_pT_pT_pT_pT_pT_pT_pT_pT_p$-$T_pT_bT_bT$, 16-mer) for 24 h. The cells were washed with D-PBS, trypsinized to remove membrane-bound fluorescence, pelleted, and fixed by formalin before analysis. The fluorescence intensity was found to be both vesicular and diffuse in the cytoplasm, but no intensity was detected in the nucleus (Figure 4).

Because cellular uptake efficiency is known to be highly dependent on cell type, we extended our studies to a human melanoma cell line, WM-239A. WM-239A cells were transfected with fluorescein tagged ODN **3** premixed in RPMI to give a final concentration of 8.0 μM. The cells were fixed with formalin before analysis, as described in the Experimental Section. After 20 h of incubation, fluorescence was found mainly within the cytoplasm and to a very low extent in the nucleus. The cytoplasmic fluorescence appeared to be distributed throughout the cytoplasm in a diffuse, not punctated manner (Figure 5).

These experiments imply that methylborane phosphine ODNs are not confined to vesicular structures inside cells and therefore might show potent biological activity. Even as the mechanism of uptake of these oligonucleotides remains largely unclear, further studies will address the temperature dependence of internalization,

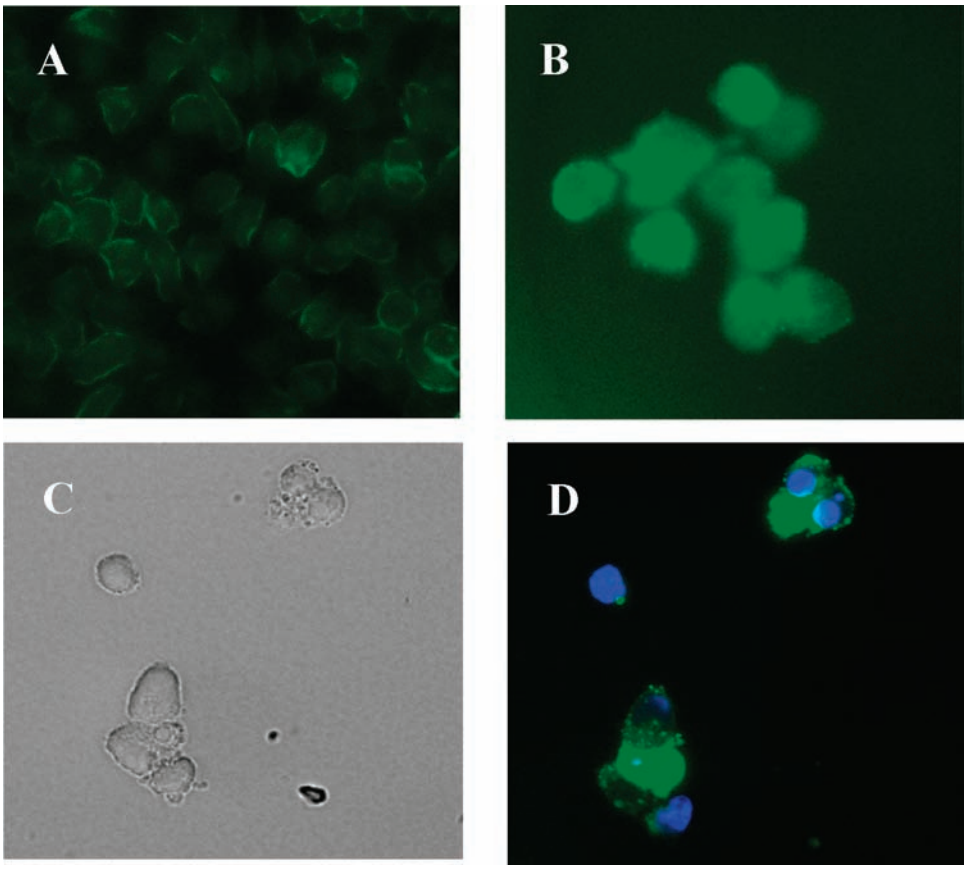


Figure 4. Uptake of fluorescein labeled ODN **12** into HeLa cells in the absence of transfecting agents. (A) Auto fluorescence of HeLa cells. (B) Incubation of cells with ODN **12** (5.0 μM, 24 h) followed by formalin fixation. (C,D) Incubation of cells with ODN **12** (5.0 μM, 24 h) followed by formalin fixation, nucleus stained with DAPI. (C) versus (D) corresponds to the cells photographed under phase-contrast versus the same cells under fluorescence microscopy.

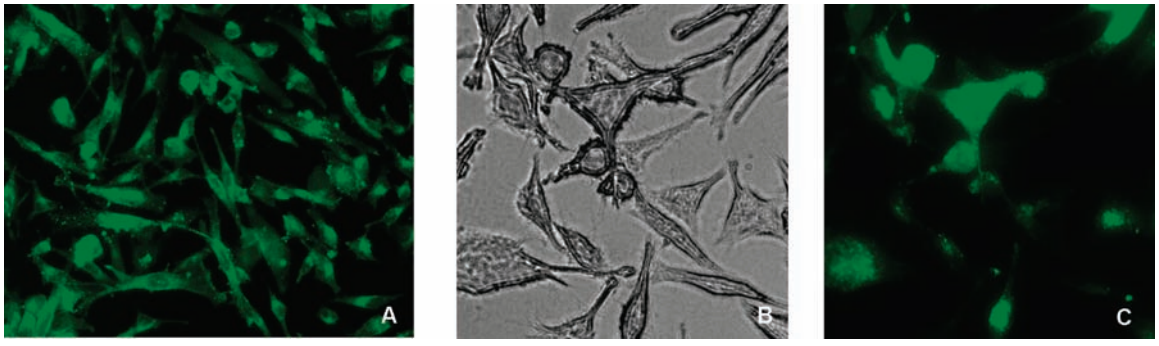


Figure 5. (A) Fluorescence microscopy image (20× magnification) of WM239A cells transfected with 8.0 μM ODN **2** for 20 h followed by formalin fixation. (B) versus (C) corresponds to WM239A cells photographed at 40× magnification under phase-contrast (B) versus the same cells under fluorescence microscopy using the same transfection conditions as for the cells shown in (A).

time-dependent efflux rates, and also how well these modifications improve the potency of ODNs when applied to various gene knock-down assays such as the Dual Luciferase Reporter assay[41] and the Let-7b assay (Applied Biosystems, assay ID 002619).

Preliminary cytotoxicity assays have been carried out using fluorescein labeled ODN **3** on HeLa cells and the WM-239A cell line. The cells were transfected with ODN **3** at 6 μM concentration for 24 h and stained with propidium iodide as described in the Supporting Information (S20). The number of dead cells was determined by flow cytometry. About 5% dead cells were found in the HeLa cell line (Supporting Information, S20, Figure 16) as opposed to 3% for the control with no ODN. In the WM-239A cell line, the apoptosis was 7% (Supporting Information, S20, Figure 17) as opposed to 5% in the control experiment.

We have successfully developed a method for synthesizing ODNs containing multiple methylborane phosphine internucleotide linkages. This strategy required the choice of suitable protecting groups and cleavage conditions that are compatible

with both the P−C internucleotide bond and borane chemistry for removing the ODNs from the support. Using this chemical approach, chimeric methylborane phosphine ODNs (16-mers) with phosphate and thiophosphate internucleotide linkages can be synthesized in reasonable yields. A single modification at each end generated an oligomer that was approximately 20 times more stable than native DNA toward SVPDE digestion; the nuclease resistance toward CSPDE was enhanced by at least 30 fold. Despite the methyl and borane groups being nearly isosteric with the phosphate, the polarity and charge distribution of the methylborane phosphine linkage are unlike those of either the methylphosphonate or the borane phosphonate, and are expected to give rise to interesting and unforeseen biological activity.

Hybridization experiments suggest that, like borane phosphonates, these linkages are more helix destabilizing in a DNA:DNA duplex than a DNA:RNA duplex. It should also be noted that stereorandom methylborane phosphine−DNA chimeras have much higher binding affinity to a complementary RNA strand relative to stereorandom methylphosphonate oligonucleotides in a DNA:RNA duplex, possibly due to the structural differences between the duplexes.

Observations on the slower electrophoretic mobility of chimeric ODNs (relative to its all-phosphate control) and the low salt effect on the stability of their duplexes with unmodified RNA lead to a discussion of the nature of charge distribution as well as the structure of the tetra-coordinated phosphorus in the methylborane phosphine linkage. It has been theorized, on the basis of Gaussian 94 calculations, that the reduction of atomic charge on phosphorus roughly correlates with chemical shifts observed for borane phosphonates and phosphodiester linkages.[42] Phosphorus that bears a theoretical atomic charge of +2.78 (phosphate, P(V)) resonates at −2.41 ppm, whereas the phosphorus of the borane phosphonate linkage (atomic charge: +2.36, P(V)) resonates at 95.0 ppm. In comparison, $(MeO)_3P$ (P(III)) with an atomic charge of +1.77 resonates at +140.0 ppm. Following this trend, a chemical shift of +148.0 ppm would suggest that the atomic charge on the Me−P−BH_3 phosphorus (P(IV) species) might be slightly lower than +1.77. Alternatively, it could also be hypothesized that the phosphorus (P−IV) in the Me−P−BH_3 linkage exists as a phosphonium species. Tetra-coordinated phosphonium-based ionic liquids are well-known in the literature and have been reported to be stable even at elevated temperatures.[43] X-ray crystallographic data on decyl(tricyclohexyl) phosphonium cation have shown that the phosphorus center adopts a near-symmetrical tetrahedral environment, although with consistently shorter P−C bond lengths (−0.038 Å) than its phosphine (P−III) counterpart.[44] Borane phosphonate and methyl phosphonate diesters are also known to adopt a tetrahedral geometry around the phosphorus.[42,45] Interestingly, in contrast to the bond shortening that was observed for the phosphonium species, the P−B bond (∼1.905 Å) in a borane phosphonate diester[42] and the P−C bond (∼1.77 Å) in a methylphosphonate diester[45] are longer than their respective P═O bonds (1.52, 1.48 Å). Thus, irrespective of the charge on the phosphorus center or the distortion in P−C and P−B bond lengths, we speculate that the Me−P−BH_3 internucleotide linkage might presumably adopt a tetrahedral geometry. Our current research has yielded no definitive proof to support either the existence of a positively charged phosphonium species or that a neutral species as has been postulated.[46] However, both the electrophoretic mobility data and the T_m measurements at low salt conditions suggest the possibility of the methylborane phosphine linkage being positively charged. Further research will include structural characterization of these molecules so as to gain a better understanding of their three-dimensional conformation.

Preliminary studies on the cell-uptake of chimeric ODNs with varying number of modifications indicate that they are very efficiently taken up by cells without the aid of transfecting agents even when the ODN is only 13% modified. Fluorescence microscopy shows that the ODN is localized in the cytoplasm and not bound to the cell membrane. These results indicate that methylborane phosphine chimeras could possibly show potent biological activity when applied to in vivo experiments.

■ CONCLUSION

In summary, we have demonstrated that methylborane phosphine linkages have increased lipophilicity, impart resistance to cellular nucleases, have duplex stabilities similar to natural DNA:RNA duplexes under physiological salt conditions, and are efficiently taken up by various cell lines without the aid of transfecting agents. These desirable qualities make the methylborane phosphine modification an ideal candidate for various biological applications such as antisense therapy, RNAi experiments, and as antagomirs against miRNAs.

■ EXPERIMENTAL SECTION

Chemical Synthesis. The general procedure for the synthesis of protected 2′-deoxynucleoside-3′-*O*-methyl phosphinoamidites (**13**−**16**) is as follows: Protected 2′-deoxynucleosides (**8**−**11**, Supporting Information, S2−S3) were dried in vacuo for 6 h. The protected 2′-deoxynucleosides (0.02 mol), 4,5-dicyano imidazole (0.018 mol, 0.9 equiv) (DCI), and anhydrous dichloromethane (100 mL) were taken up in a 250 mL round-bottom flask and stirred under nitrogen. Anhydrous CH_2Cl_2 (250 mL), bis(*N*,*N*-diisopropylamino)methylphosphine (0.02 mols, 1.0 equiv), and a magnetic stir bar were placed in a 500 mL round-bottom flask fitted with a 150 mL addition funnel. The freshly prepared deoxynucleoside−DCI solution was taken up in the addition funnel and added slowly over 30 min to the 500 mL round-bottom flask, under nitrogen. The solution was allowed to stir for an additional 30 min. A small amount of triethylamine (approximately 0.2 mL) was added to neutralize the solution, and the solvent was removed in vacuo. The crude product was isolated by chromatography using a 30−80% gradient of ethylacetate in hexane containing 1% triethylamine.

Compound ***13***. 5′-*O*-Silyl-*N*6-trimethoxytrityl-2′-deoxyadenosine-3′-*O*-methylphosphinoamidite.

Yield 47.7%. ^{31}P NMR ($CDCl_3$): δ 121.36, 119.95. ^{13}C NMR ($CDCl_3$): δ 158.2, 154.12, 152.19, 148.56, 144.11, 143.06, 144.03, 138.19, 130.02, 128.23, 128.21, 128.18, 127.14, 127.11, 126.36, 126.30, 126.24, 121.23, 121.19, 116.41, 113.08, 86.86, 86.62, 84.35, 82.21, 76.18, 76.07, 75.95, 75.85, 70.17, 63.47, 55.20, 44.26, 44.13, 39.32, 24.70, 24.02, 18.30, 18.21, 18.15, 18.05, 1.53, 1.51, 1.49.

Compound ***14***. 5′-*O*-Silyl-*N*2-trimethoxytrityl-2′-deoxyguanosine-3′-*O*-methylphosphinoamidite.

Yield 40.5%. ^{31}P NMR ($CDCl_3$): δ 119.65, 118.16. ^{13}C NMR ($CDCl_3$): δ 158.4, 158.28, 151.32, 151.19, 150.46, 150.28, 144.06, 144.00, 139.72, 134.86, 129.85, 128.24, 127.18, 126.35, 126.27, 117.39, 113.56, 113.36, 113.24, 113.11, 86.30, 83.92, 83.66, 75.51, 69.43, 63.21, 55.24, 55.15, 44.22, 44.09, 40.34, 24.71, 24.13, 24.03, 18.25, 18.18, 18.09, 18.02, 1.94, 1.85, 1.54, 1.52, 1.51, 1.15.

Compound ***15***. 5′-*O*-Silyl-*N*4-trimethoxytrityl-2′-deoxycytidine-3′-*O*-methylphosphinoamidite.

Yield 45.3%. ^{31}P NMR ($CDCl_3$): δ 120.04, 119.47. ^{13}C NMR ($CDCl_3$): δ 158.55, 158.40, 158.28, 151.32, 151.19, 150.46, 50.28, 144.04, 143.95, 139.72, 134.86, 128.24, 127.18, 126.35, 126.27, 117.39,

113.56, 113.36, 113.24, 113.11, 86.30, 83.92, 83.66, 75.51, 69.81, 69.77, 63.43, 63.21, 55.24, 55.15, 44.22, 44.09, 40.34, 24.71, 24.13, 24.03, 18.25, 18.18, 18.09, 18.02, 1.94, 1.85, 1.54, 1.52, 1.51, 1.14.

Compound **16**. 5′-*O*-Silyl-2′-deoxythymidine-3′-*O*-methylphosphinoamidite. Yield 60%. ^{31}P NMR ($CDCl_3$): δ 121.18, 120.96. ^{13}C NMR ($CDCl_3$): δ 163.53, 150.15, 135.85, 126.11, 110.79, 110.73, 87.43, 86.47, 85.00, 87.46, 76.21, 75.95, 63.43, 63.33, 44.28, 44.15, 40.14, 39.61, 24.74, 24.16, 18.26, 18.11, 12.70, 1.54, 1.52, 1.49.

Soild-Phase Synthesis. The chemical synthesis of chimeric methylborane phosphine 2′-deoxyoligonucleotides was accomplished using an ABI model 392 automated DNA synthesizer. The synthesis cycle (Table 1) was a slightly modified version of the previously reported procedure.[47] The 5′-*O*-silyl-*N*-trimethoxytrityl-3′-*O*-methoxy-2′deoxynucleoside phosphoramidites of A (**17**), G (**18**), C (**19**) and the 5′-*O*-silyl-2′-deoxythymidine-3′-*O*-methoxyphosphoramidites (**20**) were prepared as previously reported.[47] Suitably protected 3′*O*-methylphosphinoamidites **13**−**16** as well as the 3′*O*-methoxyphosphoramidites **17**−**20** were dissolved in anhydrous acetonitrile at 100 mM and placed on the appropriate ports of the synthesizer. The coupling wait time was 500 s for the 3′-*O*-methylphosphinoamidites **13**−**16**; it was decreased to 180 s for the 3′-*O*-methoxyphosphoramidites **17**−**20**. The activator was 5-ethylthio-1*H*-tetrazole (0.25 M in anhydrous acetonitrile, Glen Research, VA). After each amidite condensation step, the support was washed with acetonitrile for 40 s, and Unicap Phosphoramidite (Glen Research, VA) was used as per the manufacturer's recommendations for capping the failure sequences. Anhydrous *tert*-butyl hydroperoxide[17] or Beaucage reagent[19] was used to generate the phosphate or the thiophosphate triester linkage, respectively. Boronation of the methylphosphonite internucleotide linkages was carried out using a freshly prepared solution of 25 mM BH_3·THF in THF.

Postsynthesis, the polystyrene (PS) was washed with anhydrous acetonitrile for 60 s and then flushed with a stream of argon until dry. The bases were detritylated using an 80% aqueous acetic acid solution for 24 h. The methyl groups were then removed from the phosphate backbone using 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (Acacia Lifesciences, IL) in DMF. The polystyrene beads were thoroughly washed with water followed by anhydrous acetonitrile and dichloromethane and flushed with argon until dry. The PS support was removed from the column and placed in a 1.5 mL screw-cap, conical glass reaction vial. A 50% solution of ethylenediamine in ethanol/acetonitrile/water (47:48:5 v/v) was added, and the vial was sealed. The vial was vortexed to stir the contents, and the cleavage was allowed to proceed for 120 min at room temperature. The vial's contents were transferred to a 1.5 mL Eppendorf tube and evaporated to dryness in a SpeedVac. The crude ODN was redissolved in 5% acetonitrile−water and purified by RP-HPLC.

Nuclease Stability Determination. Snake Venom Phosphodiesterase I (*Crotalus adamanteus*) and calf spleen phosphodiesterase II were purchased from Sigma (St. Louis, MO). While performing enzymatic hydrolysis experiments, the oligodeoxynucleotide (1 OD) was incubated at 37 °C in either 150 μL of 100 mM Tris-HCl buffer (pH 9.0), 10 mM $MgCl_2$ (5 μg), Snake Venom Phosphodiesterase I, and the total volume made up to 200 μL; or 160 μL of 125 mM ammonium acetate buffer (pH 6.8), 2.5 mM EDTA, and 0.1 U of CSPDE, made up to a total volume of 200 μL. Aliquots of the reaction mixtures were removed at the indicated time points, quenched by the addition of 1.0 M EDTA (SVPDE) or 2.0 M Urea (CSPDE), and stored in dry ice until analyzed by analytical RP-HPLC.

Preparation of Cells. HeLa cells were obtained from American Type Culture Collection (Rockville, MD) and serially maintained at monolayer cultures in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) in 12-well culture plates, and incubated for 24 h prior to 2′-deoxyoligonucleotide transfection. The cells were used at passages 12−18. WM-239A cell line was a gift from the Natalie Ahn laboratory, Department of Chemistry and Biochemistry, University of Colorado, Boulder. Cells were routinely maintained as monolayers in RPMI containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL), and used at passages 7−12.

Cell-Uptake Experiments. For uptake experiments, ~1 × 10^5 cells/well (12-well plates) were incubated in appropriate medium containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) for 24 h. The concentration (OD) of 5′-fluorescein labeled, chimeric methylborane phosphine ODN dissolved in siRNA buffer 1X (Thermoscientific) was measured by UV spectroscopy. The medium was removed, and the cells were transfected with the ODN in DMEM or RPMI to give the required final concentration. The cells were then incubated at 37 °C for 16 h. After the incubation, the medium was removed from the wells and cells washed three times with 0.5 mL of D-PBS. Cells were then treated for 3 min at 37 °C with a prewarmed solution of trypsin-EDTA (1X) until all cells became round and detached from the plates; the cells from each plate were taken up in 1 mL of D-PBS and centrifuged at 1000 rpm for 5 min to form cell pellets. The pellets were washed and resuspended in D-PBS and kept at 0 °C in the dark for flow cytometric analysis.

Flow Cytometry. Flow cytometric data on at least 10 000 cells per sample were acquired on a Moflow flow cytometer (Beckman-Coulter) equipped with a single 488 nm argon laser, 530/40 nm emission filter (Fluorescein). Raw flow cytometry data were manipulated and visualized using Summit 4.3 software (Beckman-Coulter). Fluorescence intensity of the 5′-fluorescein tag was analyzed for cells presenting higher fluorescence than the background. The background was defined as the auto fluorescence of cells incubated only with the vehicle (DMEM + siRNA buffer).

Fluorescence Microscopy. An inverted microscope (Olympus IX 81) equipped with a Hamamatsu C4742-95 CCD and CoolSNAP ES digital camera (Photometrics) was used for fluorescence microscopy. For microscopic analysis, ~1 × 10^5 cells were seeded onto sterile coverslips (18 mm diam, round, VWR) placed in 12-well plates and incubated in appropriate medium containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) for 24 h. The cells were transfected with the chimeric ODNs and incubated at 37 °C for 20 h. After the incubation, the medium was removed from the wells and cells washed four times with 0.5 mL of D-PBS. The cells were covered with 1 mL of 10% neutral, buffered formalin solution (Sigma-Aldrich) for 15 min. The formalin solution was removed, and the cells were washed with 2 mL of D-PBS for 10 min at room temperature. The coverslips were carefully removed from the wells and mounted upside-down on cover slides (25 × 75 mm, 1.0 mm thick) using Fluoromount-G (Southern Biotech) as the mounting medium, and were observed under the microscope.

■ ASSOCIATED CONTENT

Supporting Information. Procedures for the chemical synthesis of 5′-*O*-silyl-2′-deoxynucleotides-3′-*O*-methylphosphoroamidites, ^{31}P NMR data, analytical RP-HPLC profiles, and MALDI-TOF data for ODNs, data on PAGE, enzyme degradation experiments performed using SVPDE to study the relative stability of (*Rp*)- and (*Sp*)- enantiomers, and apoptosis assay protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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