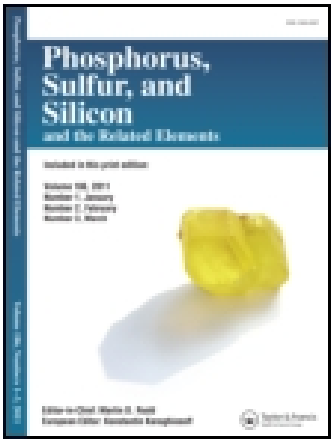

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Synthesis And Biological Activity of Borane Phosphonate DNA

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF BORANE PHOSPHONATE DNA

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Abstract *Borane phosphonate oligodeoxyribonucleotides are synthesized from 5′-O-[benzhydroxybis(trimethylsilyloxy)silyl]-2′-deoxyribonucleoside-3′-O-methylphosphoramidites. The exocyclic amine functions of adenine, guanine, and cytosine are protected with trimethoxytrityl, and thymine is unprotected. Using these synthons and under standard conditions via activation with S-ethylthiotetrazole, condensations on a highly crosslinked polystyrene support are in excess of 99%. Following the complete synthesis of the oligodeoxyribonucleotide phosphite triester, oxidation with THF·BH_3 yields the oligodeoxyribonucleotide borane phosphonate. Further treatment with 80% aqueous acetic acid followed by disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate removes trimethoxytrityl from the 2′-deoxyribonucleoside bases and the methyl protecting group from the internucleotide phosphate triester, respectively. Cleavage from the support with ammonium hydroxide and purification by reverse phase HPLC affords the pure oligodeoxyribonucleotide borane phosphonate. These oligomers are taken up by cells in the absence of cationic lipids and transport biologically active interfering RNA into cells.*

Keywords Borane; DNA; phosphonate

INTRODUCTION

The search for DNA and RNA analogs that may potentially fulfill the necessary requirements for classification as a therapeutic drug has led to a wide range of backbone oligonucleotide modifications. These criteria include stability toward nucleases, low toxicity, ease of transport across cell membranes, and biological activity. A promising member of this class, in which a nonbridging oxygen has been replaced by a borane group in DNA, is shown in Figure 1. The synthesis of borane phosphonate DNA has been reported by Shaw et al.[1] and the RNA analog by Chen et al.[2] This group is closely related to natural phosphodiesters and methylphosphonates, as it is isoelectronic and isostructural with methylphosphonates and carries a negative charge. Moreover, this linkage is nuclease resistant,[1,3] and as DNA containing borane phosphonate internucleotide linkages, it forms

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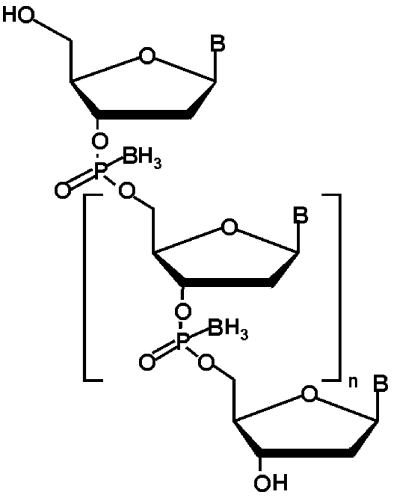

Figure 1 Chemical structure of borane phosphonate DNA. B = adenine, cytosine, guanine, and thymine (A,C,G,T, respectively); n = 0–20.

duplexes with complementary natural RNA. The resulting heteroduplex is recognized by RNase H, which catalyzes degradation of the unmodified RNA strand.[3] In related research Shaw and co-workers[4] have demonstrated that α-borane 2′-deoxynucleoside triphosphates are recognized by the polymerases that catalyze the formation of borane phosphonate internucleotide linkages. These results have encouraged us to continue to develop the chemistry associated with synthesizing borane phosphonate oligodeoxyribonucleotides (ODNs) and to study their biochemistry. In this article we report a new method for synthesis of these ODNs and demonstrate that the resulting oligomers are taken up by cells in culture. We also show that these oligomers, in complex with natural, interfering RNA, stimulate degradation of mRNA presumably through interacting with the RISC complex.

RESULTS

The challenge with this analog is to develop an orthogonal synthesis strategy. There are several key considerations. Of utmost importance, all base and phosphorus protecting groups must be stable toward boranation under conditions where the 2′-deoxynucleoside bases are not reduced or form irreversible complexes with borane. A second is the design of a transient 2′-deoxyribonucleoside hydroxyl protecting group, preferably 5′, which can be removed following each condensation cycle under conditions that do not modify the growing, support-bound borane phosphonate oligodeoxyribonucleotide.

Although several approaches have been proposed,[4] a viable methodology, which can be used to prepare heterologous constructs, has only recently been developed.[3,5] This approach yields oligomers 10–12 monomers in length with a combination of phosphate and borane phosphonate internucleotide linkages. In order to extend this approach to the synthesis of heterologous ODNs having at least 20 monomers and exclusively borane phosphonate internucleotide linkages, the synthesis strategy outlined in Figures 2 and 3 was developed.

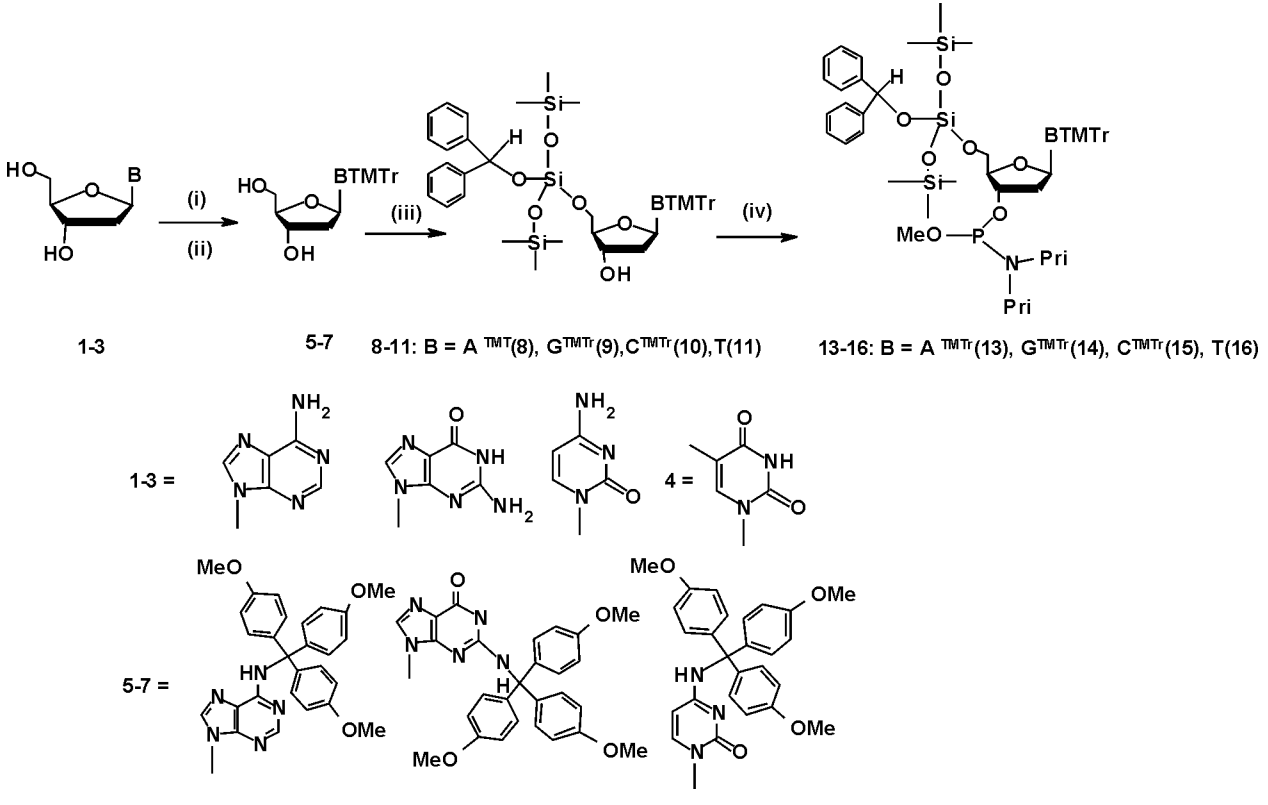

Figure 2 Schematic for the synthesis of the chemical synthons (compounds **13–16**) used to prepare borane phosphonate DNA. (i) Chlorotrimethylsilane; (ii) trimethoxytrityl chloride; (iii) benzhydroxy*bis*(trimethylsilyloxy)silyl chloride; (iv) methyl tetraisopropylphosphordiamidite/tetrazole.

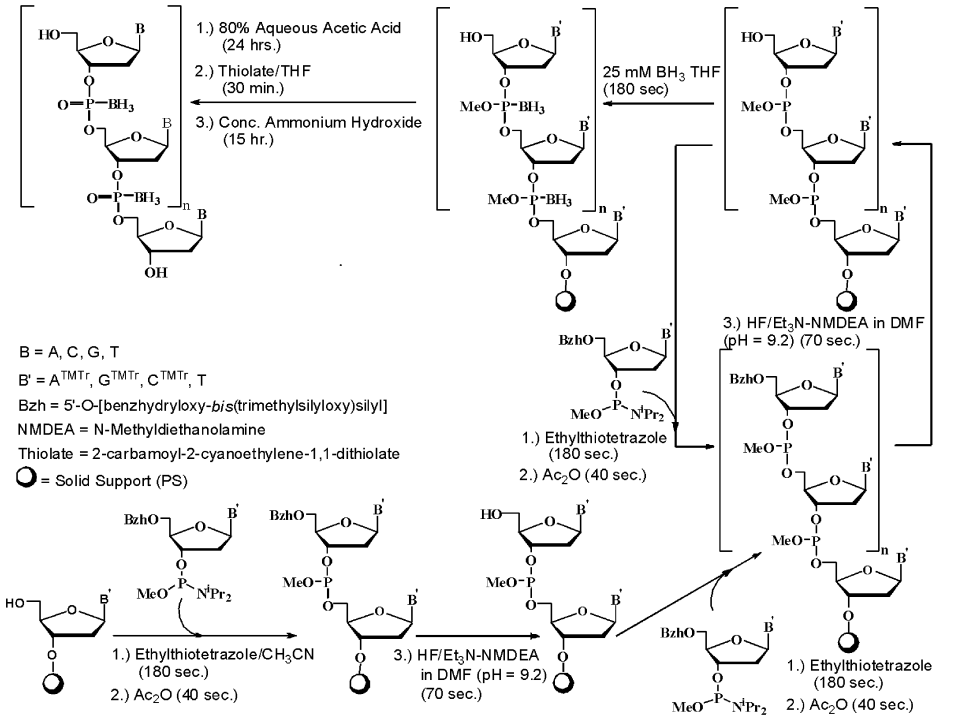

Figure 3 Schematic for the synthesis of borane phosphonate DNA.

The serious challenge that must be overcome in order to synthesize these ODNs is to develop protecting groups that are stable toward boranation. In the previous approach,[3,5] we used a combination of fluorenylmethoxycarbonyl, dimethoxytrityl, and trimethoxytrityl on the amino groups of guanine, adenine, and cytosine, respectively. However, the guanine and adenine protection strategies proved unsatisfactory, as both the N2-(9-fluorenylmethoxycarbonyl) and N6-dimethoxytrityl groups proved to be difficult to remove completely. We therefore turned to the strategy shown in Figure 2, where the amino groups on all three bases are protected with trimethoxytrityl. Because we planned to use phosphoramidites as synthons, thymine does not require protection.[1] We retained the benzhydroxy*bis(*trimethylsilyoxy)silyl for transient 5′-hydroxyl protection, as it could be completely removed in 70 s. Additionally, we chose to continue the use of the 3′-O-methylphosphoramidites, because condensation rates are rapid and lead to very high yielding product (98–99% per nucleotide addition). Removal of the methyl protecting group from the final product with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate is also rapid.[8]

Synthesis of these ODNs is carried out using the approach outlined in Figure 3 and Table 1. The first step is to condense an appropriately base-protected 5′-[benzhy droxy*bis*(trimethylsilyloxy)silyl]-3′-O-methylphosphoramidite-2′-deoxynucleoside and a polystyrene-linked and base-protected 2′-deoxynucleoside using S-ethylthiotetrazole to

[1]Previous research has shown that the use of 2′-deoxynucleoside 3′-H-phosphonates as synthons requires that N3 of thymine be protected preferably with N3-anisoyl. Otherwise, the combination of silylation to activate an internucleotide H-phosphonate linkage toward boranation, followed by boranation, causes reduction of the thymine base.[6,7]

Table 1 Synthesis cycle for borane phosphonate DNA

Reaction	Wash/reagents/solvents	Time (s)
Coupling	0.1M phosphoramidites (13–16) and 0.25 M S-ethylthiotetrazole in acetonitrile (1:1)	Wait: 180 s
Wash	Acetonitrile	40 s
	Dichloromethane	30 s
Capping	Cap Mix A (Tetrahydofuran/pyridine/acetic anhydride) and Cap Mix B (16% 1-methylimidazole in tetrahydrofuran, Equal volumes were mixed at port 14 just prior to synthesis	14 to column: 10 s Wait: 30 s
Wash	Acetonitrile	40 s
	Dichloromethane	45 s
	Dimethylformamide	45 s
5′- Deprotection	1.1M HF/ 1.1 Triethylamine/0.2 M NMDEA[a] in Dimethylformamide (pH 9.2)	10 to column: 25 s Wait: 45 s
Wash	Dimethylformamide,	45 s
	Acetonitrile	55 s

[a]NMDEA = N-methyl diethanolamine.

activate the condensation. This reaction is followed by capping with a solution of acetic anhydride and removal of the 5′-silyl group with fluoride at pH 9.2. This cycle (condensation with an appropriately protected 2′-deoxynucleoside-3′-phosphoramidite, capping with acetic anhydride, and removal of the 5′-silyl group with fluoride) can be repeated until the synthesis of the desired oligodeoxyribonucleotide phosphite triester is complete.

Further steps involving boranation and removal of protecting groups generate the final product. Boranation with BH_3·THF converts the internucleotide linkage to a PIV analog. Relative to the total synthetic scheme, this P(IV) compound is extremely important, as it is stable to aqueous acetic acid, which is used as the next step to remove the *N*-trimethoxytrityl groups from cytosine, adenine, and guanine. Moreover, unlike the borane phosphonate internucleotide linkage,[6] the P(IV) adduct does not react with the trimethoxytrityl cation (present during the aqueous acetic acid step). Thus, in order to complete deprotection of the oligodeoxyribonucleotide analog, the P(IV) borane adduct is treated with aqueous acetic acid to remove the trimethoxytrityl group from the 2′-deoxynucleoside bases. This is followed first with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate to remove the methyl group[8] and convert the internucleotide linkage to borane phosphonate and finally conc. ammonium hydroxide to remove the completely deprotected ODN from the polystyrene support. Purification is by reverse phase HPLC.[3,5]

The products of this chemistry have been fully characterized by NMR and mass spectral analyses. Phosphorus NMR analyses display a broad signal at 94.4–94.8 ppm (borane phosphonate) and a sharp peak at –2 ppm, indicating that a small fraction (3–4%) of the internucleotide linkages are phosphate (Figure 4A and 4B). B^{11} NMR spectra display a broad signal at –40 ppm, which is characteristic of the borane phosphonate linkage. As found for all ODNs synthesized by this procedure, the observed mass corresponds to those as calculated. The mass spectral analyses for two oligomers are included in the legend to Figure 4. These results demonstrate that oligomers as prepared by this method have the predicted structures and are free of any bases or sugars complexed with borane.

These ODNs form duplexes with complementary RNA but, when compared to the unmodified oligomers, have a reduced melting temperature (Tm). For example the duplex

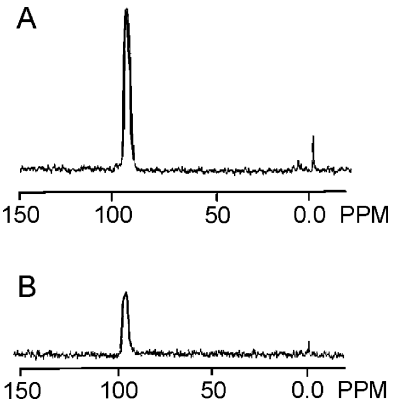

Figure 4 Phosphorus NMR of two ODNs having borane phosphonate internucleotide linkages. (A) d ($G_bA_bT_bT_bA_bT_bG_bT_bC_bC_bG_bG_bT_bT_bA_bT_bG_bT_bA_bT_bT$); (B) d($A_bA_bC_bC_bA_bT_bA_bC_bA_bA_bC_bC_bT_bA_bC_bT_bA_bC_bC_bT_bC_bT$). The major peak corresponding to borane phosphonate is 94.4–94.8; the minor peak is phosphate. Compound A, calc mass 6414.69, observed mass 6415.7; Compound B, calc mass 6522.78, observed mass 6526.8.

formed between the oligomer whose sequence is shown in the legend to Figure 4A and a complementary unmodified RNA with two uridine nucleotides at the 3′-end (i.e., both strands in the duplex have two unpaired nucleotides at the 3′-ends) has a Tm of 55°C, whereas the Tm of the natural RNA/RNA duplex (both oligomers have two unpaired uridine nucleotides at the 3′-ends) is 79°C. (T_m studies were completed in siRNA buffer × 1, as this buffer was used for studying cell uptake of ODNs.) This difference corresponds to about 1.18°C drop in the Tm per borane phosphonate internucleotide linkage. The corresponding Tm reduction for oligomers having phosphorothioate is 0.6°C per linkage.

Our previous research has demonstrated that esterified phosphonoacetate analogs of DNA are taken up by cells in culture.[9] Most importantly, and unlike previous research with other analogs such as phosphorothioate DNA, transfection with these oligomers takes place in the absence of cationic lipids. This is a major advantage, as cationic lipids are toxic to cells and their uptake must be carefully controlled. The toxicity also varies considerably for various cell lines. This earlier work has encouraged us to investigate the possibility that borane phosphonate DNA, either as single strands or as a duplex, could be taken up by cells in the absence of cationic lipids. Our first experiment involved synthesizing the ODN whose sequence is shown in Figure 4A and attaching a 5′-fluoroscein label in order to monitor results. This ODN is then added to cells in culture at a concentration of 1μM. After 16 h, the cells are rinsed and uptake examined with fluorescence microscopy and fluorescence assisted cell sorting (FACS) as a quantitative method. Figure 5 shows representative data with HeLa cells as acquired from FACS analysis. The left panel shows results with untreated cells where a small amount of background fluorescence is observed primarily from flavins and other small molecules found in these cells. The right panel shows that all HeLa cells, in the absence of cationic lipid, are transfected by this 5′-fluorescein labeled ODN. Controls such as 5′-fluorescein labeled phosphorothioate DNA are not taken up by HeLa cells in the absence of cationic lipid. When examined by fluorescence microscopy, the 5′-fluorescein labeled ODN is primarily localized throughout the cytoplasm (data not shown).

We then wanted to assess if a complementary, unmodified RNA strand hybridized to the ODN could also be transfected into cells without cationic lipids. This experiment

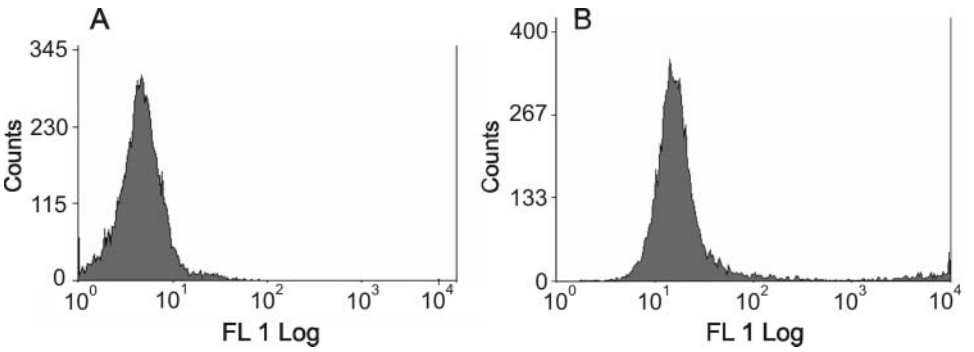

Figure 5 FACS analysis of 5′-fluorescein labeled d($G_bA_bT_bT_bA_bT_bG_bT_bC_bC_bG_bG_bT_bT_bA_bT_bG_bT_bA_bT_bT$). (A) HeLa cells; (B) HeLa cells tranfected for 16 h with ODN at 1 μM.

was carried out using 5′-fluorescein labeled RNA (no internucleotide modifications) that is complementary to the ODN whose sequence is shown in Figure 4A, as illustrated in the legend to Figure 6. This duplex has two unpaired 2′-deoxythymidine nucleotides at the 3′-ends of both the ODN and the unmodified RNA strands. The duplex that is formed is then added to HeLa cells at 5 μM (as presented previously, this duplex would be stable at 37°C in cell culture as the Tm is 55°C). Results by FACS analysis are shown in Figure 6. The left panel displays only the low level of background fluorescence as observed in HeLa cells. When the duplex is added in the absence of cationic lipid (results shown in the right panel), approximately 85% of the cells show an uptake of the complementary RNA strand. When a fluorescein-labeled, noncomplementary RNA is added with the borane phosphonate oligomers, there is no RNA uptake. Thus a duplex must form in order for transfection to occur. When uptake is examined by fluorescence microscopy (data not shown), the fluorescein-labeled RNA is localized in the cytoplasm.

In order to complete this research, we wanted to test whether this RNA/borane phosphonate DNA duplex is biologically active (Figure 7). The RNA of this duplex corresponds in sequence to a small interfering RNA (siRNA) that, when taken up by the RISC complex, degrades luciferase RNA.[10] Therefore, we added this duplex at 5 μM to HeLa cells and allowed transfection to proceed for 24 h. The media containing the duplex was removed,

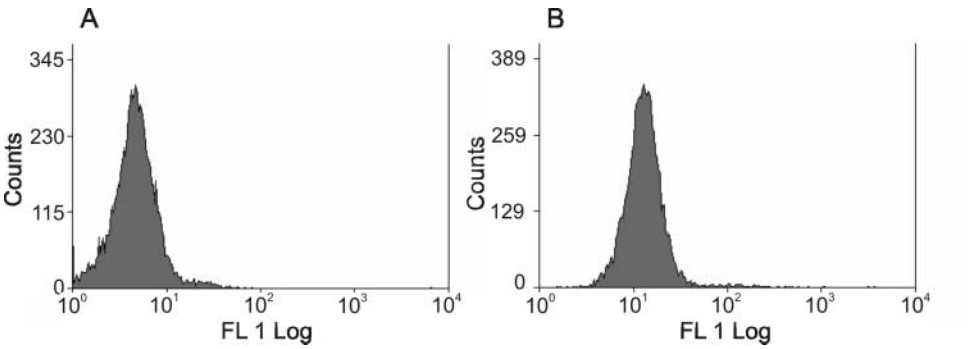

Figure 6 FACS analysis of 5′-fluorescein labeled natural RNA as a duplex with d($G_bA_bT_bT_bA_bT_b$ $G_bT_bC_bC_bG_bG_bT_bT_bA_bT_bG_bT_bA_bT_bT$). The RNA sequence was 5′-(Fl-U-A-C-A-U-A-A-C-C-G-G-A-C-A-U-A-A-U-C-U-U). (A) HeLa cells; (B) HeLa cells transfected for 24 h with the duplex at 5 μM.

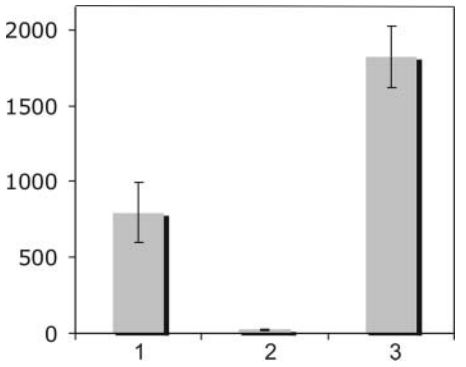

Figure 7 Luciferase activity of HeLa cells following transfection. Lane 1, siRNA/borane phosphonate DNA duplex at 5 μM. Lane 2, siRNA/RNA duplex at 5 μM plus lipofectin. Lane 3, pGL 3 firefly luciferase plasmid plus lipofectin (no duplex). For all experiments, firefly luciferase activity was measured 24 h after transfection with plasmid. siRNA/borane phosphonate duplex: 5′-(U-A-C-A-U-A-A-C-C-G-G-A-C-A-U-A-A-U-C-U-U)/5′-d($G_bA_bT_bT_bA_bT_bG_bT_bC_bC_bG_bG_bT_bT_bA_bT_bG_bT_bA_bT_bT$); siRNA/RNA duplex: 5′-(G-A-U-U-A-U-G-U-C-C-G-G-U-U-A-U-G-U-A-U-U)/ 5′-(U-A-C-A-U-A-A-C-C-G-G-A-C-A-U-A-A-U-C-U-U).

and the cells were washed three times with fresh media to ensure removal of the duplex from the cell culture plate. These cells were then transfected in fresh media with pGL 3 plasmid and lipofectin (Dharmafect 1, Thermofisher was essential for this step in order for cells to take up plasmid DNA). After 24 h, analysis for luciferase activity clearly shows that the siRNA/borane phosphonate DNA duplex reduces luciferase activity by approximately 60% relative to controls having no duplex (100% active luciferase) or the natural siRNA/RNA duplex (no luciferase activity).

DISCUSSION

These results demonstrate that a new high-yielding procedure has been developed for the synthesis of borane phosphonate DNA. The method is based on the 3′-*O*-methylphosphoramidites in combination with a series of base protecting groups (trimethoxytrityl) that are completely stable to borane but are rapidly removed by mild acid. The oligomers are obtained in high yield with a minimum (3–4%) contamination with phosphate internucleotide linkages. Previous results have shown that borane phosphonate ODNs are stable toward nuclease and stimulate RNase H activity.[3,5] Here we demonstrate that these analogs are readily taken up by cells and, in complex with complementary siRNA, show biological activity. Because borane phosphonate DNA stimulates RNase H activity, the possibility exists that these analogs degrade the siRNA in situ in the presence of endogenous RNase H. This RNase H activity would, as a consequence perhaps, partially counter the expected reduction in luciferase activity. Such a possibility might be responsible for incomplete inhibition of luciferase activity by this duplex. Of interest would be to prepare 2′-*O*-methyl RNA as the borane phosphonate oligomer and test its ability, in complex with siRNA, to reduce luciferase activity. Because 2′-*O*-methyl RNA does not stimulate RNase H activity, a 2′-*O*-methyl RNA borane phosphonate oligomer should deliver siRNA without endogenous degradation by RNase H to cells and thus exhibit enhanced activity.

EXPERIMENTAL

Chemical Synthesis

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Dichloromethane was distilled over P_2O_5. Commercially available DNA synthesis reagents and 5′-fluorescein phosphoramidite were purchased from Glen Research (Sterling, VA, USA). Medium-pressure, preparative column chromatography was performed using 60 Å standard grade silica gel from Sorbent Technologies. The silica gel was dried overnight in the oven before being used for phosphoramidite purification. Thin-layer chromatography was performed on aluminum-backed silica 60 F254 plates from EMD Chemicals, USA. ^{1}H and ^{31}P NMR spectra were recorded on Bruker Avance-III 300 NMR and Varian INOVA 400 NMR spectrometers using a $CDCl_3$ lock, and TMS was used as an internal reference.

HPLC Methods

Analytical HPLC injections were carried out using an Agilent Hypersil ODS 5 μm column, 4.0 mm i.d. × 250 mm, eluting at 1.5 mL/min with a gradient of acetonitrile/50 mM triethylammonium bicarbonate buffer, pH 8.5. Preparative HPLC (Agilent Zorbax SB-C18, 5 μm column 9.4 mm i.d. × 250 mm) was performed on an Agilent Technologies Model HPLC 1100, eluting at 1.5 mL/min with a gradient of acetonitrile/50 mM triethylammonium bicarbonate buffer, pH 8.5. The eluent was monitored for absorption at 280 nm. Mass spectral and analytical data were obtained via the PE SCIEX/ABI API QSTAR Pulsar i Hybrid LC/MS/MS at the University of Colorado Central Analytical Laboratories.

General Procedure for the Synthesis of N-Trimethoxytrityl Protected 2′-Deoxyribonucleosides [2′-Deoxyriboadenosine (5), 2′-Deoxyriboguanosine (6), 2′-Deoxyribocytidine (7)]

The 2′-deoxyribonucleoside (0.02 mol) was co-evaporated three times with pyridine and dried under vacuum for 12 h. The 2′-deoxyribonnucleoside was transferred to a round-bottomed flask equipped with a magnetic stir-bar, and anhydrous pyridine (350 mL) was added with stirring. To this suspension, chlorotrimethylsilane (0.1 mol, 5 equiv.) was added. After the mixture had been stirred at room temperature for 2 h, trimethoxytrityl chloride (0.03 mol, 1.5 equiv.) was added. The reaction mixture was stirred for 16 h at room temperature. The precipitated salts were removed by filtration using Whatman filter paper. To the filtrate, 60 mL of water and aqueous ammonium hydroxide (10 mL, 28–30%) were added, and the reaction mixture was stirred until complete deprotection of silyl groups from the 5′ and 3′ positions of the N-trimethoxytrityl protected 2′-deoxyribonucleoside was observed by TLC [$CHCl_3$/MeOH (9:1)]. The above mixture was concentrated on a rotary evaporator to approximately 30–40 mL. The crude mixture was dissolved in ethyl acetate, and this solution was washed first with a 5% aqueous solution of sodium bicarbonate. The organic phase was separated and extracted from ethyl acetate/water. The crude product in ethyl acetate was dried over anhydrous magnesium sulfate. The product was further purified by column chromatography using hexane/chloroform/pyridine (48:48:4) and a gradient of chloroform/methanol (100:0–6%). Average yields: 85–90%.

General Procedure for the Synthesis of 5′-O-[Benzhydroxy*bis*(trimethylsilyloxy)silyl]-2′-deoxyribonucleosides (8–11)

The 5′-O-silyl-N-trimethoxytrityl-2′-deoxyribonucleosides (**8–10**) and 5′-O-silyl-2′-deoxythymidine (**11**) were prepared according to published procedures.[5] The N-protected 2′-deoxyribonucleoside (0.02 mol) was dried in vacuo for 16 h and then dissolved in anhydrous N,N-dimethylformamide (600 mL). Imidazole (0.06 mol, 3 equiv.) was added to the mixture, and the flask was placed on ice and stirred. Benzhydroxy*bis*(trimethylsilyloxy)silyl chloride (0.022 mol, 1.1 equiv.) was added slowly over 1 h via syringe. The flask was allowed to stir at room temperature for ~16 h. Distilled water (60 mL) was added, and the solvent was removed in vacuo to a final volume of 50 mL. This solution was dissolved in dichloromethane and rinsed with an aqueous solution of 5% sodium bicarbonate saturated with sodium chloride. The organic layer was dried over anhydrous sodium sulfate. The product was filtered and purified by column chromatography. Elution initially was with $CHCl_3$/hexane (1:1) followed by a gradient of 2–10% methanol in chloroform. (For N-trityl analogs, 2% pyridine was added to the eluting system.) The product eluted in 5–10% methanol. Average yields: 55–65%.

General Procedure for the Synthesis of Protected 2′-Deoxyribonucleoside 3′-O-Methyl Phosphoramidites

Protected 2′-deoxyribonucleosides (**8–11**; 3.6 mmol each) were dried in vacuo for 12 h and dissolved in anhydrous dichloromethane (30 mL). Methyl tetraisopropylphosphordiamidite (4 mmol, 1.1 equiv.) was added with stirring. Then 0.4 M solution of 1H-tetrazole in anhydrous acetonitrile (3.6 mmol, 1.0 equiv.) was added slowly over 2 h, and the reaction mixture was stirred for an additional 2 h. A small amount of triethylamine (approximately 0.4 mL) was added to neutralize the solution, and the solvent was removed in vacuo. The products were isolated by column chromatography using a 0–100% gradient of ethyl acetate in benzene, containing 1% triethylamine.

Solid-Phase Synthesis

Low volume (LV) polystyrene columns (0.2-μmol synthesis scale) were purchased from Glen Research (Sterling, VA). Prior to synthesis, the 5′-DMT group on the support-bound 2′-deoxyribonucleoside was removed with 3% dichloroacetic acid in dichloromethane. DNA synthesis was carried out on an Applied Biosystems Model 392 automated DNA synthesizer (Applied Biosystems, Foster City, CA, USA) optimized for fluoride-ion chemistry. Using appropriately protected 2′-deoxyribonucleoside 3′-O-methylphosphoramidites and the synthesis cycle outlined in Table 1, an oligodeoxyribonucleotide having phosphite triester internucleotide linkages was generated. This oligomer on the solid support was next treated twice (90 s each) with a 25 μM complex of $THF{\cdot}BH_3$ in THF followed by a wash with THF (30 s).

For oligomers having 5′-fluorescein, the 5′-fluorescein phosphoramidite was condensed with the oligomer using standard synthesis methods (Table 1) to generate the phosphite triester. These oligomers, including the fluorescein phosphite linkage, were then converted to borane phosphonate using 25 μM complex of $THF{\cdot}BH_3$ in THF (2×90 s) followed by THF wash (30 s).

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% 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 (30 min, Acacia Lifesciences, IL, USA) 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 conc. solution of ammonium hydroxide was added, and the vial was sealed. The vial was vortexed to stir the contents, and the cleavage was allowed to proceed for 15 h at 55°C. The vial's contents were transferred to a 1.5 mL Eppendorf tube and evaporated to dryness in a Speedvac. The ODN product was redissolved in 5% acetonitrile-water and purified by RP-HPLC.

Preparation of Cells

HeLa cells were obtained from American Type Culture Collection (Rockville, MD, USA) 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 oligonucleotide transfection. The cells were used at passages 12–18.

Incubation of Cells with ODNs

For uptake experiments, $\sim 1 \times 10^5$ cells/well (12 well plates) were incubated in DMEM containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) for 24 h. The concentration of 5′-fluorescein-labeled boranephosphonate ODN dissolved in siRNA buffer 1X (Thermoscientific) was measured by UV spectroscopy. The media was removed, and the cells were transfected with the ODN in DMEM to give a final concentration of 1 uM, 5 uM, or 10 uM. The cells were then incubated at 37°C for 24 h. After the incubation, the medium was removed from the wells, and cells were washed four times with 0.5 mL PBS. Cells were then treated for 3 min at 37°C with a pre-warmed 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 PBS and centrifuged at 1000 rpm for 5 min to form cell pellets. The pellets were washed and resuspended in PBS and kept at 0°C in the dark for flow cytometric analysis.

Cell Viability

Viability of the cells was determined before and after incubation with ODNs by trypan blue exclusion (conventional microscopy) or by propidium iodide exclusion (flow cytometric analysis). By both experiments, greater than 86–90% cells were found to be viable after 24 h of ODN transfection.

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), and 630/30 nm emission filter (propidium iodide). In two-color flow cytometric analyses (cell viability test), spectral overlap was corrected by compensation. 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).

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