J|A|C|S
ARTICLES
Published on Web 03/25/2006

Synthesis and Biochemical Evaluation of Phosphonoformate Oligodeoxyribonucleotides

Christina M. Yamada, Douglas J. Dellinger,‡ and Marvin H. Caruthers*

Contribution from the Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received January 6, 2006; E-mail: marvin.caruthers@colorado.edu

Abstract: Phosphonoformate oligodeoxyribonucleotides were prepared via a solid phase synthesis strategy. The first step in the preparation of appropriate synthons was condensation of bis(*N*,*N*-diisopropylamino)-phosphine and diphenylmethylsilylethyl chloroformate in the presence of sodium metal to yield formic acid, [bis(*N*,*N*-diisopropylamino)phosphino]-β-(diphenylmethylsilylethyl) ester. The product of this reaction was then condensed with appropriately protected 2′-deoxynucleosides using 4,5-dicyanoimidazole to yield the 3′-*O*-phosphinoamidite reactive monomers. The exocyclic amines of cytosine, adenine, and guanine were protected with 9-fluorenylmethyloxycarbonyl, and oligodeoxyribonucleotides were synthesized on controlled pore glass using the hydroquinone-*O*,*O*′-diacetic acid linker. Synthons were sequentially added to this support using tetrazole as an activator, oxidized to phosphonoformate, and the transient 5′-protecting group was removed with acid. Following total synthesis of an oligomer, protecting groups were removed with TEMED·HF and products purified by HPLC. These analogues were resistant to nucleases, formed duplexes with complementary RNA (A-form), and, as chimeric oligomers containing phosphate at selected sites, stimulated RNase H1 activity.

Introduction

Readily available, rapidly synthesized oligodeoxyribonucleotides (ODNs) and modified ODNs have become indispensable tools for modern research in biology and biochemistry. Access to a wide repertoire of modified ODNs has specifically enabled many of the routine techniques used in molecular and cellular biology. They include sequencing, PCR applications, directed mutagenesis, study of gene expression, identification of single nucleotide polymorphisms, and modulation or control of gene expression. The latter application has broad implications from basic research on gene functions to the use of ODNs as therapeutic agents. Specifically, there have been two valuable techniques developed for the silencing of genes through the destruction of mRNA. These methods, which use antisense DNA or RNA,[2−7] require specific hybridization of oligonucleotides 18−22 nucleotides in length to mRNA before gene inhibition takes place by either RNase H or the RISC complex.[8] The utility of these oligonucleotides is greatly enhanced by incorporating modified phosphates or sugars, such as phosphorothioate,[9] or locked nucleic acids[10] among others[11] which render the internucleotide linkage more resistant to cellular nucleases and thus increases the longevity of the gene silencing effect. Recently, we have become interested in the potential advantages of the phosphonocarboxylate modification for increasing both nuclease resistance and cellular uptake. Phosphonocarboxylate, specifically phosphonoformic acids and phosphonoacetic acids, are well-known as good chemical and structural mimics of phosphoric acid and its biological derivatives. However, the synthesis chemistries historically used for synthesizing phosphonocarboxylate derivatives were too low yielding to be useful for the preparation of oligodeoxyribonucleotides. The construction of oligodeoxyribonucleotides with these modifications required the development of new, significantly higher yielding chemical methods.

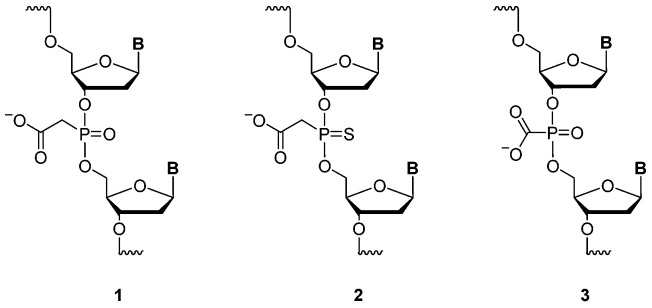
We recently developed a high yielding P(III) strategy that is useful for the construction of phosphonoacetate (**1**) and thiophosphonoacetate (**2**) oligodeoxyribonucleotides (Scheme 1).[12] This method relies upon the formation of a carbon−phosphorus bond using a zinc metal initiated Reformatsky reaction on a P(III) phosphorus electrophile (chlorophosphine) to produce an acetic acid phosphinodiamidite. The acetic acid phosphinodi-

‡ Current address: Agilent Laboratories, 3500 Deer Creek Road, Palo Alto, California 94304 USA.

(1) Abbreviations: ODN, oligodeoxyribonucleotide; PFA, phosphonoformic acid; CPG, controlled pore glass; CSO, (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; TCA, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; DMT, 4,4′-dimethoxytrityl; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; TEA, triethylamine; DPSE, diphenylmethylsilylethyl; LAH, lithium aluminum hydride; DCM, dichloromethane; TEAB, triethylammonium bicarbonate; THF, tetrahydrofuran; DCI, 4,5-dicyanoimidazole; T_m, melting temperature; SVP, snake venom phosphodiesterase; Fmoc, 9-fluorenylmethyloxycarbonyl.

(2) Chen, X.; Dudgeon, N.; Shen, L.; Wang, J. H. *Drug Discovery Today* **2005**, *10*, 587−593.
(3) Dorsett, Y.; Tuschl, T. *Nat. Rev. Drug Discovery* **2004**, *3*, 318−329.
(4) Campbell, T. N.; Choy, F. Y. *Curr. Issues Mol. Biol.* **2005**, *7*, 1−6.
(5) Dias, N.; Stein, C. A. *Mol. Cancer Ther.* **2002**, *1*, 347−355.
(6) Da Ros, T.; Spalluto, G.; Prato, M.; Sason-Behmoaras, T.; Boutorine, A.; Cacciari, B. *Curr. Med. Chem.* **2005**, *12*, 71−88.
(7) Wadhwa, R.; Kaul, S. C.; Miyagishi, M.; Taira, K. *Mutat. Res.* **2004**, *567*, 71−84.
(8) Nesterova, M.; Cho-Chung, Y. S. *Curr. Drug Targets* **2004**, *5*, 683−689.
(9) Kurreck, J. *Eur. J. Biochem.* **2003**, *270*, 1628−1644.
(10) Jepsen, J. S.; Wengel, J. *Curr. Opin. Drug Discovery Dev.* **2004**, *7*, 188−194.
(11) Micklefield, J. *Curr. Med. Chem.* **2001**, *8*, 1157−1179.
(12) Dellinger, D. J.; Sheehan, D. M.; Christensen, N. K.; Lindberg, J. G.; Caruthers, M. H. *J. Am. Chem. Soc.* **2003**, *125*, 940−950.

Scheme 1



B = thymine, adenine, cytosine, guanine

amidites are then used to produce highly reactive, protected 2′-deoxynucleotide monomers that can in turn be used in the standard phosphoramidite DNA synthesis protocols to produce the desired phosphonoacetate oligodeoxyribonucleotide analogues. These analogues, which possess an acetic acid functional group instead of a nonbridging oxygen atom at the internucleotide linkage, are P-chiral and isoelectronic with natural DNA at neutral pH. They have been shown to possess several attractive properties relative to their use as gene-silencing ODNs.[13] For example, both phosphonoacetate and thiophosphonoacetate form stable duplexes with complementary RNA to generate an A-form configuration similar to natural DNA:RNA duplexes. They also are completely resistant to snake venom phosphodiesterase, DNase I, and the nucleases present in HeLa cell nuclear extract. Most importantly, these analogues have the ability to direct and enhance RNase H1 activity, greatly enhance cellular uptake,[13] and can be used to produce highly active siRNA duplexes.[14]

These potentially useful biochemical properties led us to consider other phosphonocarboxylate modifications, such as the phosphonoformate internucleotide linkage (**3**), which structurally corresponds to replacement of a nonbridging oxygen atom with formate (Scheme 1). Although this modification as the internucleotide bond for DNA or RNA is unknown in the literature, phosphonoformic acid (PFA) or "Foscarnet" and its many derivatives and conjugates have been widely synthesized and shown to be highly effective as a DNA polymerase inhibitor useful for the treatment of viral diseases, such as herpes[15,16] or human cytomegalovirus retinitis.[17] PFA is also effective against HIV replication by blocking the pyrophosphate binding site of reverse transcriptase.[18,19] Although the standard chemistry for making PFA derivatives is widely known, it relies upon formation of the phosphorus−carbonyl bond through the oxidative process of an Arbuzov reaction.[20] The resulting P(V) molecules are chemically very stable and require extreme conditions for coupling in high yields to molecules, such as protected 2′-deoxynucleosides. Although these P(V) coupling reactions are satisfactory for the preparation of many nucleoside or 2′-deoxynucleoside antiviral agents, they are not effective under the mild conditions that are required for the synthesis of biologically relevant lengths of DNA and RNA. From our initial attempts at P(V) coupling of PFA to protected 2′-deoxynucleosides, we concluded that the synthesis of phosphonoformic acid modified DNA would require the development of phosphinoformic acid reagents that gave quantitative or near quantitative couplings under mild conditions. Development of these P(III) phosphonoformic acid reagents would also have broad applicability to the field of antiviral research.

Our success at developing similar reagents for the synthesis of phosphonoacetic acid modified DNA[12] required using a carbon nucleophile on any number of readily available chlorophosphines. This approach was not applicable to the synthesis of the corresponding formic acid derivative wherein the carbonyl is directly attached to the phosphorus atom. The formation of the carbonyl−phosphorus bond required the use of a P(III) phosphorus nucleophile on a carbon electrophile to produce formic acid phosphinodiamidites. The resulting formic acid phosphinodiamidites were then demonstrated to react in high yield with protected 2′-deoxynucleosides under acid-catalyzed conditions to produce the synthons used for preparing phosphonoformic acid modified ODNs (**3**). The synthesis chemistries and initial biochemical properties of phosphonoformate ODNs are outlined in this paper.

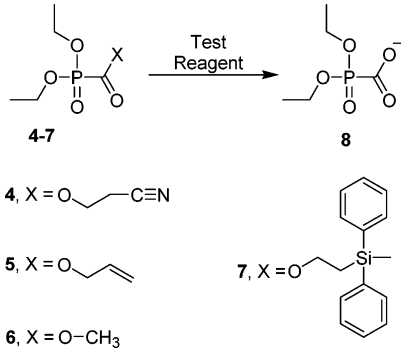
Results

Protection and Stability of Phosphonoformate. Our major focus was to maintain carbon−phosphorus bond stability under DNA synthesis conditions, including those used to remove formate and base protecting groups and to cleave the final product, phosphonoformate DNA, from the support. The cleavage of this bond from alkylformate phosphonates has been studied under nucleophilic hydrolysis conditions and found to proceed by attack of the nucleophile on the carbonyl followed by cleavage of the phosphorus−carbon bond. The facile nucleophilic cleavage of this bond is potentially useful as an acylating agent[21] and also as a protecting group for H-phosphonate internucleotide linkages.[22] For our purposes, it was important to find conditions for solid phase synthesis that did not lead to carbon−phosphorus bond cleavage.

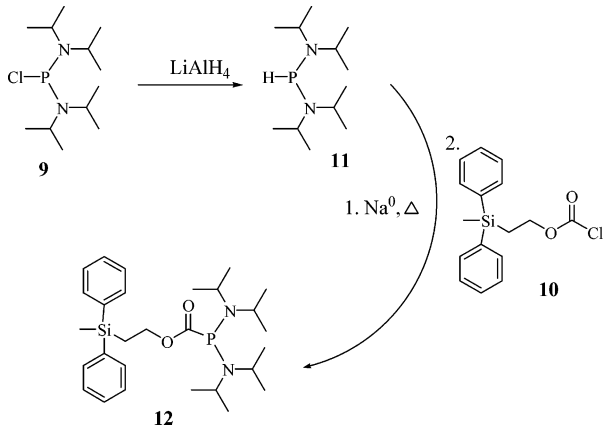
To address this problem, a series of model compounds were synthesized from triethyl phosphite and the appropriate chloroformate using Michaelis−Arbuzov conditions.[20] These compounds (**4**−**7**) were then used to study the stability of phosphonoformates with solid phase DNA synthesis reagents and to establish satisfactory conditions for formate ester hydrolysis (Scheme 2). Phosphorus NMR was used to assess the stability of this linkage toward various reagents. From previous research with acyloxyalkyl esters of phosphonoformates,[23] the tri-, di-, and monoesters in this series all had chemical shifts upfield between −3 and −11 ppm. Similarly, we expected that the model compounds **4**−**7** as well as the desired diester **8** would have similar chemical shifts. Downfield chemical shifts of −3 to 10 ppm, perhaps with proton decoupling, would indicate carbon−phosphorus bond cleavage with the generation of a

(13) Sheehan, D.; Lunstad, B.; Yamada, C. M.; Stell, B. G.; Caruthers, M. H.; Dellinger, D. J. *Nucleic Acids Res.* **2003**, *31*, 4109−4118.
(14) Yamada, C. M.; Dellinger, D. J.; Caruthers, M. H. *Enhanced Cellular Uptake of siRNA Active Duplexes using Phosphonocarboxylate Modified Oligonucleotides*. Poster presented at Keystone Symposia: Diverse Roles of RNA in Gene Regulation; Breckenridge, CO, January, 2005.
(15) Lambert, R. W.; Martin, J. A.; Thomas, G. J.; Duncan, I. B.; Hall, M. J.; Heimer, E. P. *J. Med. Chem.* **1989**, *32*, 367−374.
(16) De Clercq, E. *J. Clin. Virol.* **2004**, *30*, 115−133.
(17) Chrisp, P.; Clissold, S. F. *Drugs* **1991**, *41*, 104−129.
(18) Wagstaff, A. J.; Bryson, H. M. *Drugs* **1994**, *48*, 199−226.
(19) Noormohamed, F. H.; Youls, M. S.; Higgs, C. J.; Martin, M.; Gazzard, B. G.; Lant, A. F. *Antimicrob. Agents Chemother*. **1998**, *42*, 293−297.
(20) Bhattacharya, A.; Thyagarajan, G. *Chem. Rev.* **1981**, *81*, 415−430.
(21) Sekine, M.; Kume, A.; Hata, T. *Tetrahedron Lett.* **1981**, *22*, 3617−3620.
(22) Sekine, M.; Mori, H.; Hata, T. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 239−242.
(23) Iyer, R. P.; Boal, J. H.; Phillips, L. R.; Thacker, D. R.; Egan, W. *J. Pharm. Sci.* **1994**, *83*, 1269−1273.

Scheme 2



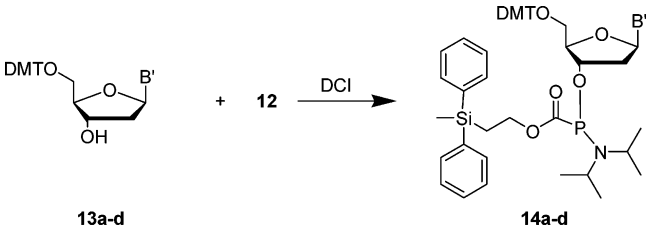
Scheme 3



phosphinic acid or H-phosphonate. Of these four model compounds, only **7** (previously used for phosphate protection[24,25]) proved satisfactory. It was stable toward all DNA synthesis reagents, and the formate ester could be removed without formate−phosphorus cleavage to yield **8** with an aqueous TEMED·HF solution in acetonitrile. In contrast, compound **4** generated either the H-phosphonate or phosphinic acid under all conditions tested for removal of the β-cyanoethyl ester (ammonium hydroxide, methylamine, TEA, and DBU). Similarly, the allylester **5** was either inert toward various cleavage conditions (Pd(0), triphenylphosphine, aniline; Pd(0), triphenylphosphine, ethylhexanoic acid) or degraded to the H-phosphonate (Pd(0), triphenylphosphine, aniline at 65 °C; butylamine, morpholine). With compound **6**, conditions could be found (thiophenol, TEA) that generated **8** (75%) contaminated with the H-phosphonate (20%) and unreacted starting material (5%). On the basis of these results, further research continued using the diphenylmethylsilylethyl (DPSE) ester to protect the phosphonoformate linkage.

Synthesis of Phosphinylformic Acid Ester. Synthesis of formic acid [bis(diisopropylamino)phosphino]-β-(diphenylmethylsilyl)ethyl ester (**12**) from bis(*N*,*N*-diisopropylamino)chlorophosphine (**9**) and the appropriate chloroformate (**10**) is outlined in Scheme 3. The first step was reduction of **9** with LAH in THF to yield bis(diisopropylamino)phosphine (**11**) in quantitative yield (^{31}P NMR, δ = 4l.4 ppm). This reaction has been hypothesized to be efficient due to the inability of three bulky diisopropyl groups to bond with a single phosphorus which,

Scheme 4



a, B' = thymine
b, B' = N4-(9-fluorenylmethyloxycarbonyl) cytosine
c, B' = N6-(9-fluorenylmethyloxycarbonyl) adenine
d, B' = N2-(9-fluorenylmethyloxycarbonyl) guanine

consequently, inhibits redistribution reactions leading to reversal of product formation.[26] When **10** was reacted with **11** using procedures that had been successful in similar systems,[26,27] no reaction product was found. Consequently, **11** was refluxed in THF with sodium for 2 h in an attempt to activate the phosphine. There was no apparent shift in the phosphorus NMR after this step, and hydrogen was still bonded to phosphorus as shown by decoupling the phosphorus−proton signal. However, when this phosphine was cannulated into a solution of **10** and stirred for 30 min, analysis of the reaction mixture showed a major ^{31}P NMR product peak with a chemical shift of 51.9 ppm. Following purification by trituration in hexanes to yield a clear, yellow oil, analysis by ^{1}H NMR and high-resolution mass spectrometry confirmed the synthesis of **12**.

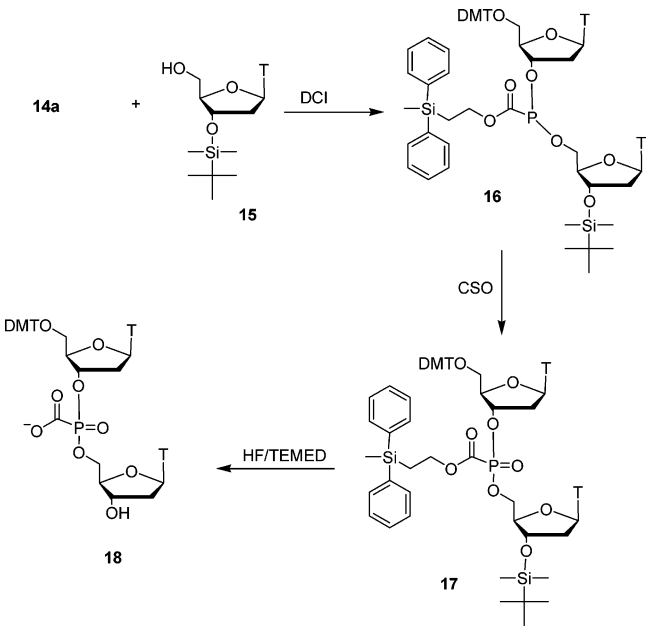
Synthesis of Phosphonoformate Linked Dinucleotides. Compound **12** reacted quantitatively with **13a** in the presence of 4,5-dicyanoimidazole (DCI) as an acid catalyst (4−16 h) to yield **14a** (Scheme 4). This synthesis was first attempted with tetrazole as an activator, but yields were low. The products (**14a**, diastereomers) were purified by silica gel column chromatography and characterized by ^{31}P NMR and ESI mass spectroscopy (see Experimental Section).

To evaluate possible chemical steps leading to the synthesis of phosphonoformate DNA, a thymidine dinucleotide (**16**) was first prepared (Scheme 5) from **14a** and 3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxythymidine (**15**) using DCI in anhydrous acetonitrile. When monitored by ^{31}P NMR, all of **14a** was converted after 5 min to the dinucleotide formic acid phosphonite (**16**, δ = 138.9, 138.0 ppm). After oxidation in situ with (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine (CSO), compound **17** was isolated by silica gel column chromatography (89% yield) and characterized by ^{31}P NMR (δ = −4.8, −5.0 ppm). Removal of the DPSE protecting group from phosphonoformate was found to proceed to completion using a solution of 20% TEMED and 10% HF (aq) in acetonitrile (TEMED·HF) at pH 8.6 (30 min). This solution also removed the 3′-*O*-silyl protecting group. Once these protecting group cleavages were complete, the dimer (**18**) was purified by reverse phase HPLC and characterized by ^{31}P NMR (δ = −6, −6.5 ppm) and ESI mass spectrometry (Figure 1).

Although the successful synthesis of a dinucleotide suggested that phosphonoformate oligomers could be prepared, the lability of the phosphorus−carbon bond to nucleophilic attack precluded the use of standard amide protection on the exocyclic amines of the other bases. Initially, these 2′-deoxynucleosides were prepared as the diphenylmethylsilylethylamino derivatives and

(24) Ravikumar, V.; Wyrzkiewicq, T.; Cole, D. *Tetrahedron* **1994**, *50*, 9255−9266.
(25) Krotz, A.; Cole, D.; Ravikumar, V. *Tetrahedron Lett.* **1996**, *37*, 1999−2002.
(26) King, R. B.; Sundaram, P. M. *J. Org. Chem.* **1984**, *49*, 1784−1789.
(27) King, R. B.; Fu, W. K. *Inorg. Chem.* **1986**, *25*, 2384−2389.

Scheme 5



assessed for their lability to TEMED·HF solutions. Unfortunately, and even after overnight treatment, the DPSE group was not completely removed from these bases. Our attention then turned to the 9-fluorenylmethyloxycarbonyl (Fmoc) group. When attached to the exocyclic amino groups of 2′-deoxycytidine (**13b**), 2′-deoxyadenosine (**13c**), and 2′-deoxyguanosine (**13d**), Fmoc could be removed completely in 2 h with the TEMED·HF solution used to cleave the DPSE ester from phosphonoformate. On the basis of these results, compounds **14b**, **14c**, and **14d** were synthesized using the same conditions as described for the preparation of **14a**. Typically, during purification by silica gel column chromatography, a small amount of triethylamine (0.1%) is added to column chromatography solvents in order to prevent loss of the 5′-*O*-dimethoxytrityl group from various synthons (due to the acidity of silica gel). However, the lability of Fmoc toward base led us to modify the column chromatography procedure. First a solvent containing 0.1% triethylamine was pre-run through the silica gel column to neutralize acids. This step was followed by several column volumes of hexanes to remove excess triethylamine before introduction of the synthon for purification.

Synthesis of Phosphonoformate DNA on Polymer Supports. The standard linkage joining synthetic DNA to controlled pore glass is a succinate ester which is hydrolyzed under basic conditions. Unfortunately, and because these conditions lead to cleavage of the phosphorus−carbon bond in phosphonoformate DNA, a new linker was needed. These studies led us to a hydroquinone-*O*,*O*′-diacetic acid support joined 3′- to a 2′-deoxynucleoside (Scheme 6, Q-linker).[28,29] The ester joining the Q-linker to DNA is hydrolyzed by several mild reagents, including NH_3/methanol, K_2CO_3/methanol, *tert*-butylamine, and fluoride ion, while remaining stable to standard DNA synthesis conditions. When we tested the Q-linker with the TEMED·HF solution, complete cleavage was observed within 2 h. These are the same conditions developed to remove Fmoc from the exocyclic amines of 2′-deoxynucleotide bases and also to eliminate the DPSE group from the phosphonoformate ester. As a result, a single reagent was found that completely removes all protecting groups within 2 h and also cleaves phosphonoformate DNA from the support.

For the preparation of DNA containing this analogue, a synthesis cycle was developed that led to phosphonoformate oligodeoxyribonucleotides or to chimeric oligomers having both phosphonoformate and phosphate internucleotide linkages (Scheme 6). As an initial test of this cycle, dimers were prepared from the Q-linker, tetrazole, and compounds **14a**−**d**. Although the condensation of **14a** to the support was complete (>99%) within 30 min, the same reaction with **14b**−**d** was 95% complete only after introducing **14b**−**d** twice to the support followed by 2 h wait steps (double coupling, 4 h total reaction time). There was no improvement when DCI was substituted for tetrazole. Perhaps the slower coupling rates for **14b**−**d** were due to the increased steric hindrance of Fmoc-protected bases during reaction on polymeric supports. Following treatment with TEMED·HF, these dimers were isolated by reverse phase HPLC and characterized. With ^{31}P NMR, all four dimers displayed two peaks having chemical shifts between −5 to −6 ppm, as expected from a P-chiral phosphonoformate linkage (Figure 2). The structures of these dimers were confirmed by ESI mass spectrometry (see Experimental Section).

This cycle was then used to synthesize 14mers—initially from **14a** and then **14a**−**d** (Table 1). Reverse phase HPLC of the total reaction mixture from the synthesis of an oligodeoxyribonucleotide having exclusively phosphonoformate internucleotide linkages (dT_{14} formate, compound **20**) yields a broad peak (31−35 min) with only minor contaminants (Figure 3A). ^{31}P NMR of this pooled fraction generates multiple peaks with chemical shifts between −5 and −6 ppm as expected for an oligomer having many stereoisomers. No other chemical shifts could be detected between 10 and −10 ppm (Figure 3B). When this pooled fraction was analyzed by gel electrophoresis, compound **20** was observed to have the same mobility as unmodified dT_{14} (inset to Figure 3A). This was expected from similar results with oligodeoxyribonucleotides having phosphonoacetate internucleotide linkages.[12,13] Unfortunately, fully modified phosphonoformate oligomers with length larger than tetramers could not be detected by MALDI-TOF mass spectroscopy. Matrices including hydroxypicolinic acid and α-cyano-4-hydroxycinnamic acid under different pH conditions were tested but without success. Similar results (broad major peak by reverse phase HPLC, multiple chemical shifts between −4.8

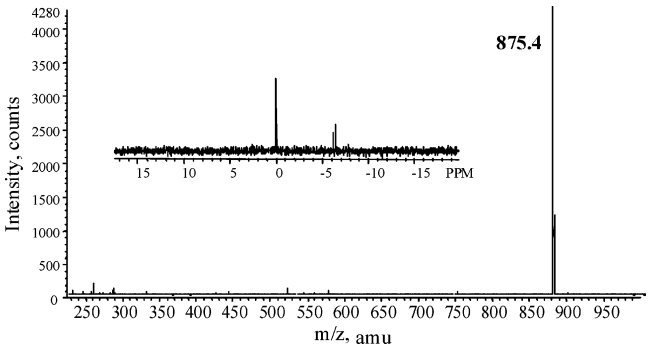
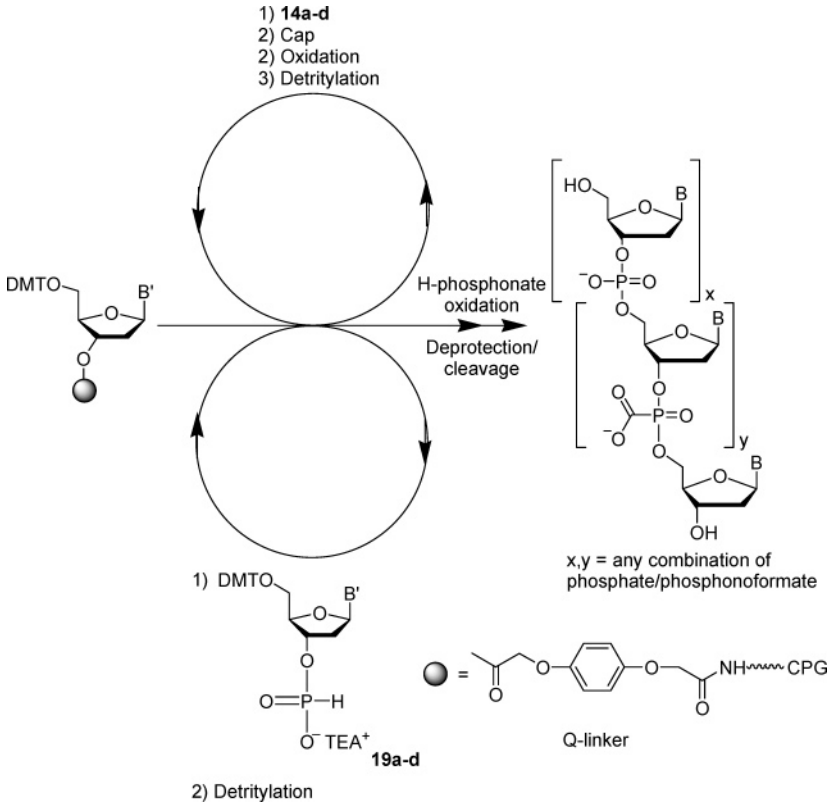


Figure 1. Characterization of **18**. ESI mass spectra (calcd mass = 875.8). Inset is ^{31}P NMR showing two stereoisomers at −6 and −6.5 ppm. The peak at 0.0 ppm is a phosphoric acid reference.

(28) Pon, R. T.; Yu, S. *Tetrahedron Lett.* **2001**, *42*, 8943−8946.
(29) Pon, R. T.; Yu, S. *Nucleic Acids Res.* **1997**, *25*, 3629−3635.

Scheme 6



and −5.6 ppm in the expected region of the ^{31}P NMR, and an inability to observe any results by MALDI-TOF mass spectrometry) were obtained with a fully modified 14mer having all four 2′-deoxynucleotides (Table 1, compound **29**).

For the synthesis of chimeric oligodeoxyribonucleotides having both phosphate and phosphonoformate internucleotide linkages, 2′-deoxynucleoside-3′-*H*-phosphonates (**19a**−**d**) and compounds **14a**−**d** were used interchangeably. The choice of H-phosphonate chemistry to introduce the phosphate linkage was predicated on observations that standard 2′-deoxynucleoside phosphoramidites were not compatible with phosphonoformate

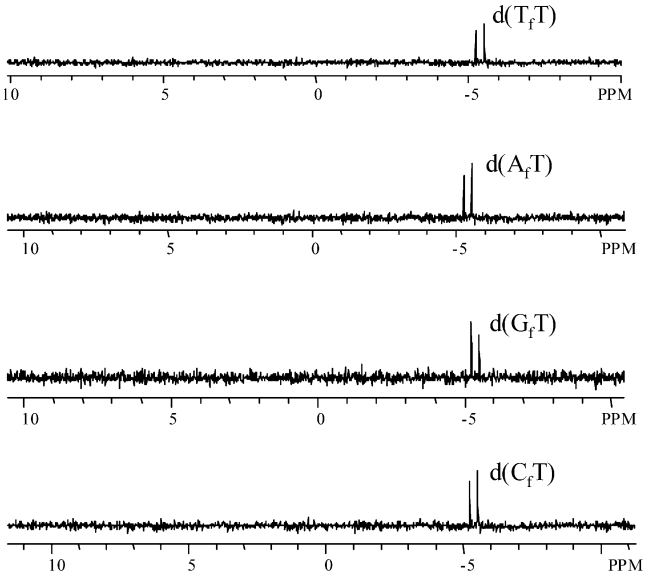


Figure 2. ^{31}P NMR spectra of phosphonoformate dimers; f = phosphonoformate internucleotide linkage.

Table 1. Characterization of Fully Modified Phosphonoformate and Chimeric Oligodeoxynucleotides

oligomer	structure[a]	calcd mass	obsd mass	NMR [$^{31}P_f$]/[$^{31}P_f$ + $^{31}P_p$]
20	d($T_fT_fT_fT_fT_fT_fT_fT_fT_fT_fT_fT_fT_fT$)	4559.0		1.0
21	d($TTT_fTTT_fTTT_fTTT_fTT$)	4308.8	4309.2	0.3
22	d($T_fT_fT_fT_fT_fTTTT_fT_fT_fT_fT_fT$)	4476.8		0.8
23	d($T_fT_fT_fT_fTTTTTT_fT_fT_fT_fT$)	4420.8		0.6
24	d($T_fT_fT_fTTTTTTTT_fT_fT_fT$)	4364.8		0.5
25	d($T_fT_fTTTTTTTTTT_fT_fT$)	4308.8	4309.0	0.3
26	d($T_fTTTTTTTTTTTT_fT$)	4252.8	4254.6	0.1
27	d($TTC_fTTA_fTTG_fTTC_fTT$)	4360.6	4363.7	0.3
28	d($A_fTGTCAACTCGTC_fT$)	4317.6	4319.4	0.1
29	d($T_fT_fC_fT_fT_fA_fT_fT_fG_fT_fT_fC_fT_fT$)	4563.0		1.0

[a] Nomenclature: f, phosphonoformate; the absence of a symbol between 2′-deoxynucleoside letters corresponds to a normal phosphate linkage (i.e., CT).

internucleotide linkage. For example, as discussed previously (Scheme 2), the conditions used to remove β-cyanoethyl and methyl lead to degradation of phosphonoformate. The cytosine, adenine, and guanine exocyclic amines of 2′-deoxynucleoside-3′-*H*-phosphonates were protected with a 9-fluorenylmethyloxycarbonyl group. For both series, the remaining steps in each cycle were similar (Scheme 6). Chimeric oligomers were purified by reverse phase HPLC after H-phosphonate oxidation and oligomer cleavage with the TEMED·HF solution. Analysis of these chimeras by ^{31}P NMR generates a set of chemical shifts between −4.5 and −6 ppm (phosphonoformate) and a peak at −0.3 ppm (phosphate diester). When these peaks were integrated, the correct signal ratio of phosphate/phosphonate was observed (Table 1). For chimeric 14mers having up to four phosphonoformate internucleotide linkages, analysis by MALDI-TOF mass spectrometry was possible, and the observed mass corresponds to the calculated value (Table 1). For all phospho-

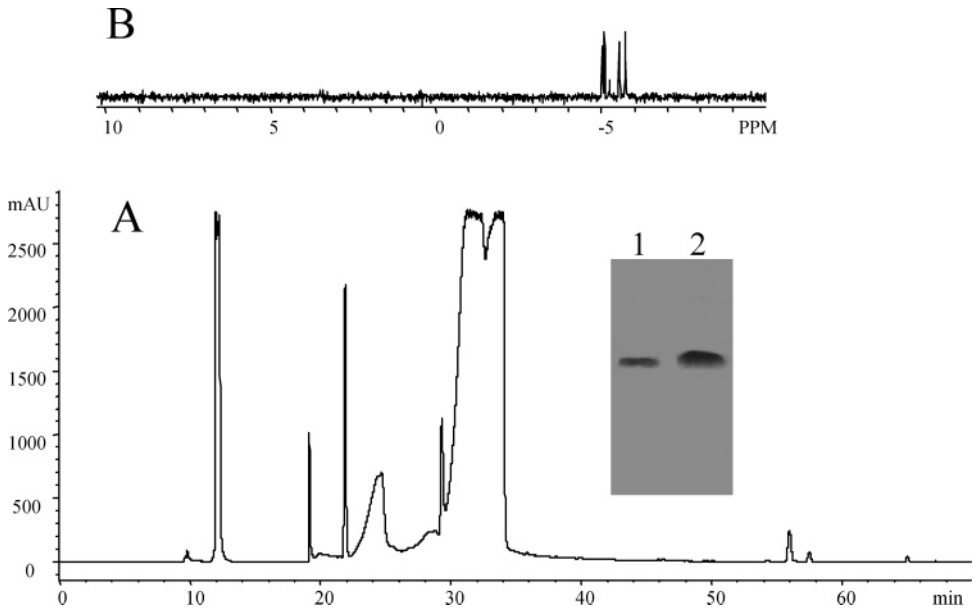


Figure 3. Characterization of oligodeoxythymidine having phosphonoformate linkages (compound **20**, Table 1). (A) Reverse phase HPLC of the total reaction mixture isolated from a polymer support. Inset shows gel electrophoresis of fractions 31−35 min (lane 1) visualized by UV light. Lane 2: $d(TpTp)_6$-(TpT). (B) ^{31}P NMR of the pooled fractions 31−35 min.

Table 2. Melting Temperatures (T_m)[a] and Observed RNase H1 Catalytic Rates[b] for Phosphonoformate DNA:RNA Heteroduplexes

duplex[c]	DNA oligomer	T_m (°C)	ΔT_m (°C)	K_{obs} (min^{-1})	k_{rel}
I	d(TTTTTTTTTTTTTT)	36.2		1.067	1
II	d($T_fT_fT_fT_fT_fT_fT_fT_fT_fT_fT_fT_fT$)	47.6	+11.4	NR	0
III	d($T_fT_fT_fT_fT_fTTTT_fT_fT_fT_fT_fT$)	45.0	+8.8	0.130	0.12
IV	d($T_fT_fT_fT_fTTTTTT_fT_fT_fT_fT$)	43.8	+7.6	0.346	0.32
V	d($T_fT_fT_fTTTTTTTT_fT_fT_fT$)	42.0	+5.8	2.42	2.30
VI	d($T_fT_fTTTTTTTTT_fT_fT$)	39.0	+2.8	2.72	2.50
VII	d($T_fTTTTTTTTTTT_fT$)	36.1	−0.1	6.46	6.00
VIII	d($TTT_fTTT_fTTT_fTTT_fTT$)	35.2	−1.0	0.00512	0.005
IX	d(TTCTTATTGTTCTT)	38.2		1.048	1
X	d($T_fT_fC_fT_fT_fA_fT_fT_fG_fT_fT_fC_fT_fT$)	48.0	+9.8	NR	0
XI	d($TTC_fTTA_fTTG_fTTC_fTT$)	38.1	−0.1	0.0171	0.02
XII	d(ATGTCAACTCGTCT)	40.1		1.54	1
XIII	d($A_fTGTCAACTCGTC_fT$)	40.3	+0.2	6.095	4.00

[a] Melting temperatures and initial rates were determined in RNase H1 buffer (see Experimental Section). [b] Initial rate under enzyme saturating conditions at 25 °C. [c] Duplexes are formed from the DNA oligomer in complex with complementary, unmodified RNA of the same length.

noformate containing ODNs (compounds **20**−**29**, Table 1), isolated yields of fully deprotected oligomers were 45−50%.

Biochemical and Biophysical Properties. Initial biophysical studies were carried out to determine the charge state of the phosphonoformate internucleotide linkage and to assess the ability of this analogue to form heteroduplexes with unmodified RNA. In a manner similar to previous research with the phosphonoacetate derivative, the charge state of phosphonoformate DNA was determined by comparing retention times on reverse phase HPLC columns under different pH conditions (data not shown). The observed pK_a for phosphonoformate was 3.0, while the value for phosphonoacetate was 3.8.[13] These results were expected as they mirror the differences in pK_a for phosphonoacetic acid (5.2) and phosphonoformic acid (4.3).[30]

Of particular interest were results on the stability of phosphonoformate DNA:RNA heteroduplexes (Table 2). The T_m values for oligomers having only phosphonoformate linkages when in complex with natural RNA are considerably higher than the corresponding unmodified DNA:RNA heteroduplexes. For example, the homopolymer of deoxythymidine as phosphonoformate forms a duplex with unmodified RNA (duplex **II**) that has a T_m 11.4 °C higher than that of the unmodified DNA:RNA. With a phosphonoformate oligomer containing all four 2′-deoxynucleotides, a similar result was observed (compare duplexes **IX** and **X**, ΔT_m = 9.8 °C). As the number of phosphonoformate linkages decreases in an oligodeoxyribonucleotide, the ΔT_m also decreases (relative to the comparable oligomer having all phosphonoformate linkages). The increased stability of phosphonoformate DNA containing duplexes was unexpected since oligodeoxyribonucleotides having phosphonoacetate internucleotide linkages, when formed as a duplex with natural RNA, had decreased melting temperatures when compared to those of unmodified DNA:RNA heteroduplexes.[13] An additional observation, which is perhaps useful relative to understanding these results, is that the placement of the phosphonoformate linkages may be important relative to stability. When four phosphonoformate linkages are located at the extremities of an oligomer (duplex **VI**), the ΔT_m with natural RNA is +2.8 °C (relative to unmodified DNA), whereas when these four linkages are equally distributed (duplex **VIII**), there is a small negative effect on heteroduplex T_m. Perhaps the adjacent phosphonoformate internucleotide linkages stabilize duplexes through metal chelation or hydrogen bonding with complementary RNA. Adjacent, multiple interactions of this type would suppress the duplex ends from locally denaturing and initiating the melting process.

To further characterize the phosphonoformate duplexes listed in Table 2, CD spectra were recorded at 25 °C and compared with duplex **I** (Figure 4). The CD spectra of duplex **I** exhibited the characteristics of A-form as expected for an RNA−DNA hybrid.[31] For example, the positive band at 266 nm and the negative band at 211 nm are characteristic of the A-form pattern. Similarly, duplexes **II** and **X**, which contain only phosphono-

(30) Kyuji, O. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 7065.

(31) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry*; W. H. Freeman: San Francisco, CA; pp 118−124.

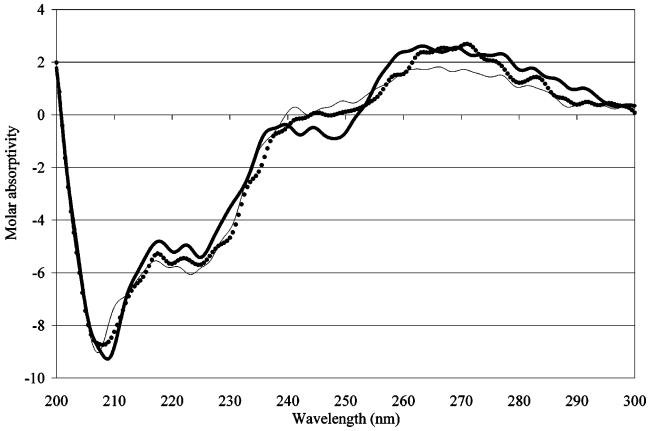


Figure 4. CD spectra of duplexes **I** (—), **II** (—), and **X** (- - -) at 25 °C in 25 mM Na_2HPO_4, 100 mM NaCl (pH 7.4), and 10 μM duplex. Each spectra are the average of three experiments.

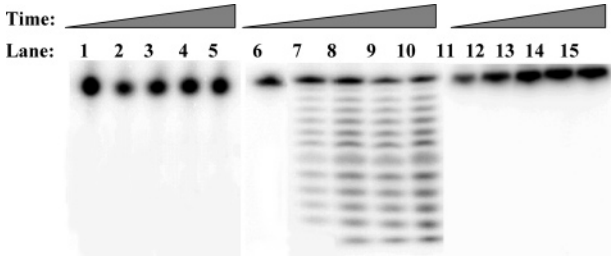


Figure 5. DNase I analysis of phosphonoformate DNA. 5′-^{32}P-end-labeled oligomers were treated with DNase I followed by analysis using gel electrophoresis and an autoradiograph of the gels. Time points were taken at 0, 0.5, 1, 2, and 3 h. Lanes 1−5, oligomer **20**; lanes 6−10, d(TpTp)$_6$TpT; lanes 11−15, oligomer **21**.

formate internucleotide linkages in the DNA strand, also form A-like conformations. The remaining duplexes listed in Table 2 also exhibit A-form CD spectra (data not shown). This A-form configuration appears to be important for the recognition and cleavage of substrates by RNase H.[32]

Because these analogues contain a phosphorus−carbon bond, we expected that the phosphonoformate linkage would be resistant to degradation by nucleases. To test this possibility, nuclease studies were completed using DNase I, SVP, and HeLa cell nuclear extract. Monitoring degradation by DNase I and SVP was conveniently possible by radiolabeling the 5′-end using γ-^{32}P ATP and T4-kinase. Unlike phosphonoacetate DNA,[13] fully modified and chimeric phosphonoformate oligomers could be successfully labeled using T4-kinase. Results from DNase I degradation of the fully modified phosphonoformate analogue of a thymidine 14mer (oligomer **20**) and a similar compound having phosphonoformate linkages every third position (oligomer **21**) are presented in Figure 5. Both phosphonoformate containing oligomers were completely resistant to degradation by DNase I under conditions where normal DNA is being degraded. Furthermore, fully modified phosphonoformate DNA did not inhibit enzymic degradation of normal ODNs. Similar results were observed with SVP and HeLa cell nuclear extracts (data not shown; degradation with HeLa cell extract was monitored by reverse phase HPLC).

Since phosphonoformate DNAs are anionic at physiological pH, nuclease stable, and form A-like duplexes with complementary RNA, their ability to stimulate *E. coli* RNase H1 activity was examined. 5′-Radiolabeled ^{32}P phosphate RNA was annealed to complementary phosphonoformate containing DNA and tested for cleavage with RNase H1 under enzyme saturating conditions. Results are summarized in Table 2. With fully modified phosphonoformate DNA, there was no RNase H1 activity for either the oligodeoxythymidine or mixed sequence analogues. This was the result at any of three temperatures (4, 25, and 37 °C) and when a 100-fold excess of RNase H1 (relative to our standard assay) was added. There also was no inhibition of RNase H1 activity when these fully modified phosphonoformate DNAs were added to a normal DNA:RNA duplex undergoing degradation. In contrast, chimeric oligomers containing phosphonoformate and phosphate internucleotide linkages activated RNase H1. Those having phosphonoformate interspersed with phosphate (duplexes **VIII** and **XI**) had much slower observed rates (0.00512 and 0.0171 min^{-1}) than the controls (1.067 and 1.048 min^{-1}), respectively. In contrast many oligomers having phosphonoformate internucleotide linkages at the ends (one to three at each end), and phosphate in the center had faster rates than the control duplexes. Those with four and five phosphonoformates at each end activated RNase H1 but at a reduced rate relative to controls.

Discussion

When we first considered the formation of a carbonyl−phosphorus bond by the use of a P(III) phosphorus nucleophile on a carbon electrophile to produce phosphinoformic acid diamidites, our initial review of the literature was discouraging. It had been previously reported by King et al. that bis-(dialkylamino)phosphines were ineffective nucleophiles for the formation of phosphorus−carbon bonds using carbon electrophiles, such as methyl iodide, and that conversion to the sodium metal salts had no effect at increasing the nucleophilicity of the phosphorus.[33] However, we were surprised that the same reagents that were unreactive with methyl iodide gave very high yield reactions with a variety of chloroformates.

Once we were able to successfully synthesize the formic acid phosphinodiamidites and evaluate their reactivity, it was clear that they should have broad utility beyond the synthesis of ODNs. As a reagent, the phosphinoformic acid diamidites can be used to make any variety of esterified versions of PFA conjugates in high yield and under mild conditions. These reagents should be especially useful in screening novel PFA conjugates for enhanced bioavailability.[19,34,35]

We show that phosphinoformic acid diamidites can react with the secondary hydroxyl of a protected 2′-deoxynucleoside in quantitative yield to produce extremely active phosphinoformate amidites. The reactivity of these monomers was somewhat surprising due to the deactivating nature of a carbonyl directly attached to a phosphorus atom. Since these monomers did couple in near quantitative yields on solid support, they made possible the synthesis of phosphonoformate oligodeoxyribonucleotides.

These results demonstrate that phosphonoformate oligodeoxyribonucleotides can be successfully synthesized and are

(32) Lima, W. F.; Mohan, V.; Crooke, S. T. *J. Biol. Chem.* **1997**, *272*, 18191−18199.

(33) King, R. B.; Sadanani, N. D.; Sundaram, P. M. *Phosphorus Sulfur Relat. Elem.* **1983**, *18*, 125−128.

(34) Velazquez, S.; Lobaton, E.; De Clercq, E.; Koontz, D. L.; Mellors, J. W.; Balzarini, J.; Camarasa, M.-J. *J. Med. Chem.* **2004**, *47*, 3418−3426.

(35) Marma, M. S.; Kashemirov, B. A.; McKenna, C. E. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1787−1790.

potentially useful in various biochemical systems. They form specific duplexes with complementary RNA, are stable toward exo- and endonucleases, and activate RNase H1. Further research is needed to test the value of these compounds toward activation of RNase H1 in cells and to determine if phosphonoformate DNA, in a manner similar to thiophosphonoacetate DNA,[13] transports into cells in the absence of cationic lipids.

Experimental Section

General Procedures. ^{1}H NMR spectra were recorded on Varian 500 MHz and Varian 400 MHz spectrometers with tetramethylsilane as an internal reference. ^{31}P NMR spectra were recorded on a Varian 400 MHz spectrometer using an external capillary containing 85% H_3PO_4 in D_2O as a reference. Downfield chemical shifts were recorded as positive values for ^{31}P NMR. The University of Colorado Central Analytical Laboratories performed ESI, EI, FAB, and accurate mass spectroscopy analysis. Reverse phase (Zorbax 300SB C-18 column, Agilent Technologies, Palo Alto, CA) chromatography was performed on an Agilent Technologies Model 1100 HPLC. Solid phase DNA synthesis was accomplished using an ABI model 394 automated DNA synthesizer (Applied Biosystems, Foster City, CA) modified for the synthesis cycle shown in Scheme 6. All reagents, columns, standard 2′-deoxynucleotide-3′-phosphoramidites, and 2′-deoxythymidine-3′-*H*-phosphonate monomer (**19a**) were purchased from Glen Research (Sterling, VA).

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Anhydrous solvents were purchased from Sigma-Aldrich Co. (Milwaukee, WI). Protected 2′-deoxynucleosides (compounds **13a**−**d**) were purchased from ChemGenes Corporation (Wilmington, MA). Medium-pressure, preparative, column chromatography was performed using 230−450 mesh silica gel from Sorbent Technologies (Atlanta, GA). Thin layer chromatography was performed on aluminum-backed silica gel 60 F_{254} plates from EM Sciences (Gibbstown, NJ).

Synthesis of Diphenylmethylsilylethyl Chloroformate. In a fume hood, 500 mL of a 20% solution of phosgene (0.94 mol, 1 equiv) in toluene was added into a 2 L round-bottom flask with a stir bar.[36] The flask was placed into an ice bath and cooled to 0 °C. In a flat-bottomed 500 mL flask, 214 mL of 2-(methyldiphenylsilyl)ethanol (0.94, 1 equiv) was diluted into toluene, slowly cannulated into the phosgene solution, and allowed to stir overnight, resulting in a greenish/brown clear solution. Using a Teflon high vacuum pump, the reaction was evaporated to produce a greenish/brown oil. The exit hose of the pump was placed in a large flask of water to neutralize the excess phosgene. No further purification was necessary. Typical yields were 95−97%. ^{1}H NMR ($CDCl_3$): δ 7.16−7.49 (m, aromatic, 10 H), δ 4.40 (t, 2 H), δ 1.68 (t, 2 H), δ 0.61 (s, 1H). Electron impact mass spectrometry gave a molecular ion of 304 *m*/*e*.

Synthesis of 2-Cyanoethyl Chloroformate. In a fume hood, 500 mL of a 20% solution of phosgene (0.94 mol, 1 equiv) in toluene was added into a 2 L round-bottom flask with a stir bar. The flask was placed into an ice bath and cooled to 0 °C. In a flat-bottomed 500 mL flask, 23.4 g of 3-hydroxypropionitrile (0.33 equiv) was diluted into toluene, slowly cannulated into the phosgene solution, and allowed to stir overnight. The reaction was evaporated to an oil as described above. ^{1}H NMR ($CDCl_3$): δ 4.44 (t, 2 H), δ 2.8 (t, 2 H). Electron impact mass spectrometry gave a molecular ion of 133 *m*/*e*.

General Procedure for Arbuzov Reactions. Model compounds were synthesized using the following general procedures. All chloroformates were commercially available except the 2-cyanoethyl chloroformate and the methyldiphenylsilylethyl chloroformate (syntheses described above). Triethyl phosphite (1 equiv) and chloroformate (1 equiv) were added neat to a 50 mL round-bottom flask. The reaction was allowed to stir for 10 min and then checked by ^{31}P NMR for completion. Triethyl phosphite has a chemical shift of δ 138 ppm, whereas the products had chemical shifts of approximately δ −4 ppm. There was no need for purification as all reactions went to completion and no side products were detected. The model compounds were analyzed by ^{31}P NMR, ^{13}C NMR, and mass spectroscopy (see below).

Diethyl 2-Cyanoethyl Phosphonoformate (4). 6.7 g (0.05 mol, 1 equiv) of 2-cyanoethyl chloroformate was reacted with 8.6 mL (1 equiv) of triethyl phosphite in a 50 mL round-bottom flask. Because the density of the 2-cyanoethyl chloroformate was not known, the liquid was simply weighed into a tared flask. The reaction was very rapid at room temperature, creating heat and emitting bubbles of chloroethane. After 10 min, the reaction was checked by ^{31}P NMR, and no starting material was present. Yield: 95%, 11.2 g. ^{13}C NMR ($CDCl_3$): δ 164.8, 116.6, 64.5, 59.8, 17.4, 15.8. ^{31}P NMR: δ −5.0. Electron impact mass spectrometry gave a molecular ion of 236.0 *m*/*e*.

Diethyl Allyl Phosphonoformate (5). 10 mL (0.09 mol, 1 equiv) of allyl chloroformate was reacted with 16 mL (1 equiv) of triethyl phosphite in a 50 mL round-bottom flask. The reaction was very rapid at room temperature, creating heat and emitting bubbles of chloroethane. After 10 min, the reaction was checked by ^{31}P NMR, and no starting material was present. Yield: 95%, 18.9 g. ^{13}C NMR ($CDCl_3$): δ 165.3, 130.9, 119.8, 66.3, 64.5, 15.3. ^{31}P NMR: δ −4.2. FAB mass spectrometry gave a molecular ion of 223.0 *m*/*e*.

Diethyl Methyl Phosphonoformate (6). 4 mL (0.05 mol, 1 equiv) of methyl chloroformate was reacted with 8.9 mL (1 equiv) of triethyl phosphite in a 50 mL round-bottom flask. The reaction was very rapid at room temperature, creating heat and emitting bubbles of chloroethane. After 10 min, the reaction was checked by ^{31}P NMR, and no starting material was present. Yield: 95%, 9.3 g. ^{13}C NMR ($CDCl_3$): δ 165.7, 64.2, 52.1, 15.9. ^{31}P NMR: δ −4.1. FAB mass spectrometry gave a molecular ion of 197.0 *m*/*e*.

Diethyl *β*-(Diphenylmethylsilyl)ethyl Phosphonoformate (7). 8.9 g (0.03 mol, 1 equiv) of methyldiphenylsilylethyl chloroformate was reacted with 5 mL (1 equiv) of triethyl phosphite in a 50 mL round-bottom flask. Because the density of the DPSE chloroformate was not known, the liquid was simply weighed into a tared flask. The reaction was very rapid at room temperature, creating heat and emitting bubbles of chloroethane. After 10 min, the reaction was checked by ^{31}P NMR, and no starting material was present. Yield: 95% 11.6 g. ^{13}C NMR ($CDCl_3$): δ 165.7, 135.4, 134.4, 129.7, 128.2, 64.5, 64.4, 16.4, 15.5, −4.0. ^{31}P NMR: δ −4.3. Electron impact mass spectrometry gave a molecular ion of 406 *m*/*e*.

Synthesis of Formic Acid, [B*is*(*N*,*N*-diisopropylamino)phosphino]-*β*-(diphenylmethylsilyl)ethyl ester (12). The synthesis of the phosphine was a three-step reaction. First, bis(*N*,*N*-diisopropylamino)-chlorophosphine (26 g, 0.1 mol) was dissolved in dry THF in a 1 L round-bottom flask. The solution was placed in an ice bath and cooled to 0 °C. A 100 mL bottle of 1 M lithium aluminum hydride (0.1 mol) in tetrahydrofuran (Sigma-Aldrich, Co) was transferred directly by cannula into the flask containing the bis(*N*,*N*-diisopropylamino)-chlorophosphine. The reaction was stirred for 10 min and then checked by ^{31}P NMR (δ = 41.44 ppm). The ice bath was removed, and a heating mantle along with a Friederich's condenser was placed on the flask. Sodium pieces (4.6 g, 0.2 mol) were added carefully to the flask. The solution was allowed to reflux in tetrahydrofuran with stirring. After 2 h, the reaction was removed from the heating mantle and cooled to room temperature. *β*-(Diphenylmethylsilyl)ethyl chloroformate (121.6 g, 0.4 mol) was dissolved in tetrahydrofuran in a 1 L round-bottom flask. The phosphine reaction was cannulated into the chloroformate solution, leaving behind unreacted sodium. (Unreacted sodium was neutralized with 2-propanol.) The reaction was stirred for 1 h at room temperature and then checked for completeness by ^{31}P NMR. If complete, the tetrahydrofuran was removed by rotary evaporation. The resulting viscous oil was extracted three times with anhydrous hexanes.

(36) Phosgene is a toxic and flammable gas and should be used with appropriate safety precautions (see the Sigma-Aldrich Material Safety Data Sheet for appropriate handling and safety instructions).

All hexane fractions were combined, filtered, and evaporated under vacuum to a slightly yellow oil to yield 39.9 g (80%). ^{1}H NMR ($CDCl_3$): δ 7.34−7.54 (m, aromatic,10H), 4.23 (t, 2H), 3.6 (m, 4H), 1.62 (t, 2H), 1.3 (d, 24H), 0.61 (s, 1H). ^{31}P NMR: δ 51.89. Electron impact mass spectrometry gave a molecular ion of 500.3 *m*/*e*. Accurate mass measurements for $C_{28}H_{45}N_2O_2PSi$: calculated, 500.2988; found, 500.2886.

General Procedure for Synthesis of Protected 2′-Deoxynucleoside Formic Acid Phosphinoamidites (14a−d). The synthesis of protected 2′-deoxynucleoside formic acid phosphinoamidites was accomplished by the following general procedure. Protected 2′-deoxynucleoside (**13a**−**d**, 1 equiv)[37] was dissolved in anhydrous dichloromethane at a concentration of 100 mM, and 1.2 molar equiv of the formic acid ester phosphinodiamidite (**12**) was added with stirring. 4,5-Dicyanoimidazole (1.1 equiv) was then added. The reaction was allowed to stir for 4−16 h and analyzed for completeness by ^{31}P NMR and TLC (hexanes:ethyl acetate, 1:4). The reaction was monitored by TLC (protected 2′-deoxynucleoside R_f < product R_f) and by loss of the formic acid phosphinodiamidite starting material (^{31}P NMR). If incomplete, another 0.5 equiv of compound **12** was added and the reaction allowed to stir for an additional 2 h. The reaction was quenched by addition of 1 equiv of anhydrous triethylamine (relative to 4,5-dicyanoimidazole). The mixture was stirred briefly, the solvent removed by evaporation on a rotary evaporator, and the resulting viscous oil purified by silica gel column chromatography (hexanes:ethyl acetate, 0.1% triethylamine, 6 × 25 cm). The viscous oil was redissolved in a minimum volume of ethyl acetate and added to the top of the column. The column was eluted using a gradient of 0−80% ethyl acetate with the product typically eluting in 40−60% ethyl acetate. Fractions containing UV-active material were collected and analyzed by TLC (hexanes:ethyl acetate, 1:4). Fractions containing the product were combined and concentrated on a rotary evaporator to a foam. The resulting white solids were analyzed by ^{31}P NMR and ESI mass spectroscopy.

Synthesis of 3′-*O*-(*N*,*N*-Diisopropylamino)phosphinoformic acid-*β*-(diphenylmethylsilyl)ethyl ester-5′-*O*-di-*p*-anisylphenylmethyl-2′-deoxythymidine (14a). 5′-DMT-2′-deoxythymidine (**13a**) (5 g, 9.2 mmol) was reacted with formic acid [bis(*N*,*N*-diisopropylamino)-phosphino]-*β*-(diphenylmethylsilyl)ethyl ester (4.60 g, 1 equiv) in the presence of 4,5-dicyanoimidazole (1.2 g, 1.1 equiv) for 4 h at room temperature. The product eluted from the column in 40% ethyl acetate giving 4.3 g (50%). All correct fractions were collected and evaporated to a foam. The resulting purified phosphinoamidite was analyzed by ^{31}P NMR giving diastereomers (δ = 108.7 and 109.3 ppm) and by ESI mass spectroscopy 944 $[M + H]^+$. Accurate mass measurement for $C_{53}H_{62}N_3O_9PSi$, M + H: calculated, 944.4093; found, 944.4061.

Synthesis of 3′-*O*-(*N*,*N*-Diisopropylamino)phosphinoformic acid-*β*-(diphenylmethylsilyl)ethyl ester-5′-*O*-di-*p*-anisylphenylmethyl-N4-(9-fluorenylmethyloxycarbonyl)-2′-deoxycytidine (14b). 5′-*O*-DMT-N4-(9-Fmoc)-2′-deoxycytidine (**13b**) (5 g, 6.7 mmol) was reacted with formic acid [bis(*N*,*N*-diisopropylamino)phosphino]-*β*-(diphenylmethylsilyl)ethyl ester (3.66 g, 1 equiv) in the presence of 4,5-dicyanoimidazole (0.86 g, 1.1 equiv) for 16 h. To neutralize the silica gel, one volume of 0.1% triethylamine in hexanes was eluted from the column followed by 2 column volumes of hexanes to remove triethylamine before the product was charged on the column. The compound eluted from the column in 40% ethyl acetate. Fractions containing product were collected and evaporated to a foam (3.9 g, 51%). The resulting purified phosphinoamidite was analyzed by ^{31}P NMR giving diastereomers (δ = 108.5 and 110.1 ppm) and by ESI mass spectroscopy 1151.4 $[M + H]^+$; 1173.4 $[M + Na]^+$; 1189.4 $[M + K]^+$. Accurate mass measurement for $C_{67}H_{71}N_4O_{10}PSi$, M + H: calculated, 1151.4755; found 1151.4771.

Synthesis of 3′-*O*-(*N*,*N*-Diisopropylamino)phosphinoformic acid-*β*-(diphenylmethylsilyl)ethyl ester-5′-*O*-di-*p*-anisylphenylmethyl-N6-(9-fluorenylmethyloxycarbonyl)-2′-deoxyadenosine (14c). 5′-*O*-DMT-N6-(9-Fmoc)-2′-deoxyadenosine (**13c**) (5 g, 6.4 mmol) was reacted with formic acid [bis(*N*,*N*-diisopropylamino)phosphino]-*β*-(diphenylmethylsilyl)ethyl ester (3.9 g, 1.2 equiv) in the presence of 4,5-dicyanoimidazole (0.84 g, 1.1 equiv) for 4 h. To neutralize the silica gel, one column volume of 0.1% triethylamine in hexanes was eluted from the column followed by 2 column volumes of hexanes to remove triethylamine before the product was charged on the column. The compound eluted from the column in 40% ethyl acetate. Fractions containing product were collected and evaporated to a foam (3.7 g, 50%). The resulting purified phosphinoamidite was analyzed by ^{31}P NMR giving diastereomers (δ = 108.9 and 109.9 ppm) and by ESI mass spectroscopy 1176 $[M + H]^+$; 1197 $[M + Na]^+$. Accurate mass measurement for $C_{68}H_{71}N_6O_9PSi$, M + H: calculated, 1175.4868; found 1175.4879.

Synthesis of 3′-*O*-(*N*,*N*-Diisopropylamino)phosphinoformic acid-*β*-(diphenylmethylsilyl)ethyl ester-5′-*O*-di-*p*-anisylphenylmethyl-N2-(9-fluorenylmethyloxycarbonyl)-2′-deoxyguanosine (14d). 5′-*O*-DMT-N2-(9-Fmoc)-2′-deoxyguanosine (**13d**) (5 g, 6.3 mmol) was reacted with formic acid [bis(*N*,*N*-diisopropylamino)phosphino]-*β*-(diphenylmethylsilyl)ethyl ester (3.8 g, 1.2 equiv) in the presence of 4,5-dicyanoimidazole (0.82 g, 1.1 equiv) for 4 h. To neutralize the silica gel, one column volume of 0.1% triethylamine in hexanes was eluted from the column followed by 2 column volumes of hexanes to remove triethylamine before the product was charged on the column. The compound eluted from the column in 60% ethyl acetate. Fractions containing product were collected and evaporated to a foam (3.8 g, 50%). The resulting purified phosphinoamidite was analyzed by ^{31}P NMR giving diastereomers (δ = 109.0 and 109.6 ppm) and by ESI mass spectroscopy 1192 $[M + H]^+$; 1214 $[M + Na]^+$; 1229 $[M + K]^+$. Accurate mass measurement for $C_{68}H_{71}N_6O_{10}PSi$, M + H: calculated, 1191.4817; found, 1191.4826.

General Procedure for Synthesis of Fmoc-Protected 2′-Deoxynucleoside 3′-*H*-Phosphonate Monomers (19b−d). The synthesis of Fmoc-protected 2′-deoxynucleoside H-phosphonate monomers was accomplished by the following general procedures. Anhydrous pyridine was added to protected 2′-deoxynucleosides (**13b**−**d**,[37] 1 equiv) in a 500 mL flask. The pyridine was removed by evaporation on a rotary evaporator. This step was repeated three times, and the flask was left on high vacuum overnight to remove any water from the 2′-deoxynucleoside. The next day, the 2′-deoxynucleoside was dissolved in anhydrous pyridine. Once completely dissolved, diphenyl phosphite (5 equiv) was added to each reaction flask, the reaction was allowed to stir for 30 min and then analyzed for extent of reaction by silica gel TLC (9:1 $CHCl_3$:MeOH, protected 2′-deoxynucleoside R_f < P(III) intermediate R_f). If complete, 1 M TEAB (aq) was added and the reaction stirred for another 30 min. The reaction was monitored by TLC (9:1 $CHCl_3$:MeOH) (P(III) intermediate R_f > product R_f). When complete, DCM was added and the reaction extracted three times in a separatory funnel. The organic layer was dried with sodium sulfate, the solvent removed on a rotary evaporator, and the product purified by column chromatography on silica gel (DCM:MeOH). Before the product was added to the column, the column was eluted with one volume of DCM containing 1% 1M TEAB; 1% 1 M TEAB was added to all solvents applied to the column to prevent removal of the trityl group on the 5′-position of the 2′-deoxynucleoside. The 1 M TEAB is an aqueous solution and forms a layer on top of the DCM in the column. It is important to not flow this water layer into the column because it will effect the silica gel and hence product elution. The crude product was redissolved in a minimum volume of DCM and added to the top of the column. To remove byproducts with a high mobility on TLC, DCM:benzene (1:1) was passed through the column. Once all byproducts were removed, a gradient of MeOH (0−7%) with DCM was begun in order to elute the product. Fractions containing UV-active material were collected and analyzed by silica gel TLC (9:1 $CHCl_3$:MeOH). Fractions containing the product were combined and evaporated to a

(37) Heikkilä, J.; Chattopadhyaya, J. *Acta Chem. Scand.* **1983**, *B37*, 263−265.

foam on a rotary evaporator. The white solid was analyzed by ^{31}P NMR and ESI mass spectroscopy.

Synthesis of 5′-*O*-Di-*p*-anisylphenylmethyl-N4-(9-fluorenylmethyloxycarbonyl)-2′-deoxycytidine 3′-*H*-Phosphonate Triethylammonium Salt (19b). 5′-DMT-N4-Fmoc-2′-deoxycytidine (**13b**) (1.58 g, 2 mmol) was reacted with diphenyl phosphite (1.9 mL, 10 mmol) in anhydrous pyridine (10 mL) for 30 min; 1 M TEAB (aq) (50 mL) was added and the reaction allowed to stir for another 30 min. The product was extracted and purified on silica gel as described in the general procedure for H-phosphonoate monomer synthesis. The compound eluted from the column in 7% methanol giving 1.77 g (96% yield). The resulting purified monomer was analyzed by ^{31}P NMR (δ = 2.5 ppm) and by ESI mass spectroscopy 916 $[M + H]^+$.

Synthesis of 5′-*O*-Di-*p*-anisylphenylmethyl-N6-(9-fluorenylmethyloxycarbonyl)-2′-deoxyadenosine 3′-*H*-Phosphonate Triethylammonium Salt (19c). 5′-DMT-N9-Fmoc-2′-deoxyadenosine (**13c**) (1.55 g, 2 mmol) was reacted with diphenyl phosphite (1.9 mL, 10 mmol) in anhydrous pyridine (10 mL) for 30 min; 1 M TEAB (aq) (50 mL) was added and the reaction allowed to stir for another 30 min. The product was extracted and purified on silica gel as described in the general procedure for H-phosphonoate monomer synthesis. The compound eluted from the column in 7% methanol giving 1.66 g (88% yield). The resulting purified monomer was analyzed by ^{31}P NMR (δ = 2.7 ppm) and by ESI mass spectroscopy 941 $[M + H]^+$.

Synthesis of 5′-*O*-Di-*p*-anisylphenylmethyl-N2-(9-fluorenylmethyloxycarbonyl)-2′-deoxyguanosine 3′-*H*-Phosphonate Triethylammonium Salt (19d). 5′-DMT-N2-Fmoc-2′-deoxyguanosine (**13d**) (1.5 g, 2 mmol) was reacted with diphenyl phosphite (1.9 mL, 10 mmol) in anhydrous pyridine (10 mL) for 30 min; 1 M TEAB (aq) (50 mL) was added and the reaction allowed to stir for another 30 min. The product was extracted and purified on silica gel as described in the general procedure for H-phosphonoate monomer synthesis. The compound eluted from the column in 7% methanol giving 1.08 g (56% yield). The resulting purified monomer was analyzed by ^{31}P NMR (δ = 2.6 ppm) and by ESI mass spectroscopy 957 $[M + H]^+$.

Synthesis of Thymidylyl-(3′-5′)-Thymidine Phosphonoformic Acid-*β*-(diphenylmethylsilyl)ethyl ester (17). Synthesis of **17** involved condensation of 5′-*O*-DMT-2′-deoxythymidinephosphinoamidite (**14a**) and 3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxythymidine (**15**).[38] 3′-*O*-(*N*,*N*-diisopropylamino)phosphinoformic acid-*β*-(diphenylmethylsilyl)ethyl ester-5′-*O*-di-*p*-anisylphenylmethyl-2′-deoxythymidine (100 mg, 0.1 mmol) and 3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxythymidine (41.5 mg, 0.12 mmol) were dissolved in anhydrous acetonitrile (5 mL). Once dissolved, 4,5-dicyanoimidazole (0.59 g, 5 mmol) was added to the solution. The reaction was allowed to stir for 10 min and then analyzed by ^{31}P NMR (δ 138.9 and 138.0 ppm). Oxidation of the dinucleotide formic acid phosphonite to the corresponding dinucleotide phosphonoformate was completed by adding (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine (23 mg, 1 mmol)[39] to the crude coupling reaction. This solution was allowed to stir for 5 min and then analyzed by ^{31}P NMR (δ −4.8 and −5.0 ppm). The product was purified by silica gel chromatography (DCM:MeOH, 0.1% TEA, 3 × 20 cm). A step gradient of 0−2% methanol was used and the product eluted in 2% methanol. Fractions containing **17** were collected, and solvent was evaporated on a rotary evaporator. The purified white foam (152.9 mg, 89%) was analyzed by ^{31}P NMR: δ −4.5 and −4.8 ppm.

Removal of DPSE Group from Ester-Protected 2′-Deoxydinucleotide Phosphonoformate 17 to Yield 18. In a one-dram screw cap vial, DPSE-protected 2′-deoxydinucleotide phosphonoformate (**17**) (20 mg, 20.5 μmol) was dissolved in 1 mL of acetonitrile. To this solution was added *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (250 μL) and 48% hydrofluoric acid (aq) (125 μL) (pH 8.6). The pH of this solution was determined by adding 10 mL of H_2O to a 1 mL aliquot of a TEMED·HF solution (prepared using the 2:1 volume ratios of TEMED and HF) and measuring the pH. The reaction was allowed to stir for 30 min at room temperature or until quantitative ester deprotection was achieved as assessed by ^{31}P NMR. Purification was accomplished by reverse phase HPLC utilizing a 25 cm Zorbax 300SB-C18 column with 9.4 mm inside diameter. Gradient eluents were (A) water and (B) acetonitrile. The following gradient conditions were used to elute the trityl-on 2′-deoxydinucleotide dimers: 0−2 min, 10% B, then 2−52 min, 10−92% B at a flow rate of 1.2 mL/min. The desired fractions were collected, concentrated under vacuum, and the purified product was dissolved in water. The deprotected dinucleotide phosphonoformate was characterized by ^{31}P NMR (diastereomers, δ −6.1 and −6.4 ppm) and by ESI mass spectroscopy 875.4 $[M - H]^-$.

Synthesis of Phosphonoformate Oligodeoxyribonucleotides. The solid phase synthesis of phosphonoformate oligodeoxyribonucleotides was accomplished using an ABI model 394 automated DNA synthesizer from Applied Biosystems (Foster City, CA). The synthesis cycle was adapted from a standard 1 μM 2-cyanoethyl phosphoramidite DNA synthesis cycle.[40] Instead of a standard succinate linker, a 1.0 μmol Q-support (Glen Research) containing hydroquinone-*O*,*O*′-diacetic acid linker on controlled pore glass (CPG) was used.[28] The 3′-*O*-(*N*,*N*-diisopropylamino)phosphinoformic acid-*β*-(diphenylmethylsilyl)ethyl ester-5′-*O*-di-*p*-anisylphenylmethyl-protected 2′-deoxynucleosides (**14a**−**d**) were freshly dissolved in anhydrous acetonitrile (100 mM) prior to each synthesis. 2′-Deoxythymidine-3′-phosphonoformate amidite (**14a**) had a coupling time of 30 min, while the corresponding 2′-deoxyadenosine-3′-phosphonoformate amidite (**14c**), 2′-deoxyguanosine-3′-phosphonoformate amidite (**14d**), and 2′-deoxycytidine-3′-phosphonoformate amidite (**14b**) all had double couplings with a 2 h wait per coupling. Tetrazole dissolved in anhydrous acetonitrile at a concentration of 0.45 M was used as an activator. Trichloroacetic acid (3% w/v) dissolved in anhydrous dichloromethane was used to deprotect the 5′-*O*-di-*p*-anisylphenylmethyl groups prior to each round of coupling. Capping was accomplished using the standard two-part capping solution following the coupling step where cap A was 10% acetic anhydride in anhydrous THF and pyridine and cap B was 10% 1-methylimidazole in anhydrous THF. Oxidation of the nascent internucleotide formic acid phosphonite to the phosphonate was accomplished using the standard 0.1 M I_2 in a solution of THF/pyridine/H_2O.

Postsynthesis, the CPG was washed with anhydrous acetonitrile for 60 s and then flushed with a stream of argon until dry. The CPG was poured into a one-dram vial, the TEMED·HF solution (20% TEMED, 10% HF (aq) in acetonitrile at pH 8.6) was added and the vial sealed with a Teflon cap. The CPG was left at room temperature for 2 h with occasional agitation. The CPG was removed by filtration using a 0.45 μm filter in an Eppendorf tube (Fisher Scientific). The supernatant containing the cleaved and deprotected oligodeoxyribonucleotide was then purified by reverse phase HPLC.

Reverse Phase HPLC Purification of Phosphonoformate Oligodeoxyribonucleotides. Fully modified phosphonoformate and chimeric oligomers were purified by reverse phase HPLC. Preparative HPLC utilized a 25 cm Zorbax 300SB-C18 column with 9.4 mm i.d. (Agilent Technologies). Gradient eluents were (A) 0.22 μm filtered H_2O and (B) acetonitrile. All oligomers were purified DMT off and only one reverse phase purification was performed with a gradient of 0−100% B over 60 min and a flow rate of 1.2 mL/min. The product fractions were collected, concentrated under vacuum, and dissolved in 0.22 μm filtered water.

Mass Spectrometry of Oligodeoxyribonucleotides. Two types of mass spectrometry were performed with the choice dependent upon the length of the oligomer. For dimers and trimers, all analyses were performed on an Applied Biosystems/MDS SCIEX Pulsar Q-Star using direct infusion ESI in negative ion mode. Oligomers were dissolved in

(38) Ogilvie, K. K.; Iwacha, D. J. *Tetrahedron Lett.* **1973**, *14*, 317−319.
(39) Ugi, I.; Jacob, P.; Landgraf, B.; Rupp, C.; Lemmen, P.; Verfürth, U. *Nucleosides Nucleotides* **1988**, *7*, 605−608.
(40) Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* **1981**, *103*, 3185−3191.

acetonitrile and water (50:50) at a concentration of 10−100 μM and infused via a 50 μL syringe. Dimer masses: T_fT, expected 573.4, observed 573.2; C_fT, expected 558.4, observed 558.1; A_fT, expected 582.4, observed 582.2; G_fT, expected 598.4, observed 598.2.

For longer oligomers, MALDI-TOF was performed on a PerSeptive Biosystems Voyager-DE STR Biospectrometry Workstation. Modified oligomers were dissolved in water at a concentration of 100 μM. The matrix was 2,4,6-trihydroxyacetophenone monohydrate (THAP).[41,42] It was prepared by adding 0.2 mmol of THAP (45 mg) and 8.2 μmol of ammonium citrate (2 mg) to 500 μL of acetonitrile/water (1:1), which forms a supersaturated solution (a cloudy suspension). The THAP matrix suspension (1 μL) was pipetted onto a gold-plated, 100-well plate. The oligomer solution (1 μL) was pipetted onto the same location. There was no need for a desalting step if the oligodeoxyribonucleotide was purified by reverse phase HPLC utilizing volatile salts. After the spot was dry, the plate was inserted into the Voyager Biospectrometry Workstation. All measurements were observed in the negative ion mode for increased sensitivity and resolution.

Melting Point Measurements. Melting points (T_ms) for the DNA:RNA heteroduplexes were determined on a Varian Cary 1E UV−visible spectrometer. The absorbance at 260 nm was measured, while the temperature of the sample was increased at a rate of 1.0 °C/min. All phosphonoformate and control oligomers were separately mixed with target RNA in a 1 mL cuvette, and the T_m was determined as the maximum of the first derivative of the melting curve. Concentrations were 1 μM in each strand. The buffer was 20 mM HEPES−KOH (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT. Prior to analysis, each sample was incubated briefly at 95 °C, equilibrated to room temperature, chilled on ice for 30 min, and then re-equilibrated to room temperature (25 °C).

Circular Dichroism Spectra. CD spectra were recorded using a JASCO J-720 spectrophotometer. All CD experiments were performed at 25 °C in 1 mm path-length cuvettes with a buffer adjusted to pH 7.4 containing 25 mM NaH_2PO_4 and 100 mM NaCl. The concentrations were 10 μM for each strand in a total volume of 350 μL. Prior to CD analysis, hybridization of the duplexes was performed as described for the melting curve analysis.

Stability toward DNase I. Endonuclease digestion experiments were carried out using DNase I from bovine pancrease lyophilisate (Roche Applied Science). The assays were performed using a mixture of 5′-^{32}P-labeled oligomer (100 000 cpm) and unlabeled oligomer (50 pmol) in a buffer containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$. Enzyme was added to a final concentration of 2 U/μL (20 μL total reaction volume), and the reaction was incubated at 37 °C. Aliquots (3.5 μL) were removed and quenched by adding 7 M urea in TBE buffer (6.5 μL) and heating to 95 °C for 2 min. Time points were taken for 3 h after the addition of the enzyme. Samples were stored on ice until analysis by PAGE (20%, 7 M urea). Autoradiographic imaging was performed using an Amersham Biosciences Phosphorimager (Typhoon 9400). Gel bands were quantitated using ImageQuant software (version 5.1).

Hydrolysis of RNA Heteroduplexes with *E. coli* RNase H1. A mixture of 5′-^{32}P-labeled RNA (100 000 cpm/reaction), unlabeled RNA (100 pmol), and complementary oligodeoxyribonucleotide (100 pmol) was added to a buffer (pH 7.8) containing 20 mM HEPES−KOH, 50 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT. The duplexes were hybridized by heating briefly to 95 °C and then incubated at 4 °C for 30 min. *E. coli* RNase H1 (Promega) was added (8 units), and the reactions were allowed to proceed for various times at 25 °C (40 μL total reaction volume). Aliquots of the reaction mixture (3.5 μL) were quenched with 6.5 μL of 7 M urea and 20 mM EDTA and stored on ice until analysis by gel electrophoresis (20%, 19:1 cross-linked). All reactions were performed in triplicate. Data were analyzed by KaleidaGraph.

Acknowledgment. This work was supported by the University of Colorado. We thank David Sheehan and Agnieszka Sierzchala for scientific discussion, and Robert Barkley and Richard Shoemaker for technical assistance in the Central Analytical Laboratory. This paper is dedicated to Professor Wojciech J. Stec on the occasion of his 65th birthday.

Supporting Information Available: Table containing further information on the protocol for synthesizing phosphonoformate ODNs. This material is available free of charge via the Internet at http://pubs.acs.org.

JA060112B

(41) Koomen, J. M.; Russell, W. K.; Hettick, J. M.; Russell, D. H. *Anal. Chem* **2000**, *72*, 3860−3866.
(42) Pieles, U.; Zurcher, W.; Schar, M.; Moser, H. E. *Nucleic Acids Res.* **1993**, *21*, 3191−3196.