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Published on Web 01/03/2003

Solid-Phase Chemical Synthesis of Phosphonoacetate and Thiophosphonoacetate Oligodeoxynucleotides

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Abstract: Phosphonoacetate and thiophosphonoacetate oligodeoxynucleotides were prepared via a solid-phase synthesis strategy. Under Reformatsky reaction conditions, novel esterified acetic acid phosphinodiamidites were synthesized and condensed with appropriately protected 5′-*O*-(4, 4′-dimethoxytrityl)-2′-deoxynucleosides to yield 3′-*O*-phosphinoamidite reactive monomers. These synthons when activated with tetrazole were used with an automated DNA synthesizer to prepare phosphonoacetic acid modified internucleotide linkages on controlled pore glass. The phosphinoacetate coupling products were quantitatively oxidized at each step with (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine or 3*H*-1,2-benzodithiol-3-one-1,1-dioxide to produce mixed sequence phosphonoacetate and thiophosphonoacetate oligodeoxynucleotides with an average per cycle coupling efficiency of greater than 97%. Completely deprotected, modified oligodeoxynucleotides were purified by reverse-phase HPLC and characterized by ion exchange HPLC, ^{31}P NMR, and MALDI/TOF mass spectroscopy. Both analogues were stable toward hydrolysis with snake venom phosphodiesterase and stimulated RNase H1 activity.

Introduction

The search for viable antisense and diagnostic DNA analogues has led to a wide range of candidates.[2] However among these various derivatives, only a limited number possess the properties most commonly identified as necessary for antisense research: activation of RNase H, ability to form sequence-specific duplexes with complimentary oligoribonucleotides, and resistance toward nucleases.[3,4] Two analogues, phosphorothioate and phosphorodithioate DNAs, are especially attractive, as they possess all of these properties and can be prepared from the readily available 2′-deoxynucleosides. Although the antisense properties for both have been demonstrated,[3−6] phosphorothioate DNA is clearly the most advanced, as one sequence-defined derivative has entered phase III clinical trials for nonsmall cell lung cancer.[7] Because these analogues have certain undesirable biological properties,[3,8] there is clearly room for the development of new derivatives. One is an analogue whereby borane replaces a nonlinking oxygen in an internucleotide phosphate.[9−11] Although this derivative activates RNase H and is nuclease resistant, it so far has only been successfully prepared as oligodeoxythymidine presumably because of difficulties related to the use of borane with the remaining deoxynucleoside bases. Recently two new derivatives, cyclohexene nucleic acids[12] and 2′-fluoro-2′-deoxyarabinofuranosyl nucleic acids,[13] have been reported to possess the same favorable antisense properties, including RNase H activation. Although cellular uptake and in vivo toxicity studies have not been completed, these analogues represent the first sugar-modified nucleosides that show promise as antisense reagents. One serious disadvantage of these two analogues is that the natural, readily available 2′-deoxynucleo-

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(1) Abbreviations: B, appropriately protected purine or pyrimidine base; CPG, controlled pore glass; CSO, (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine; BDT, 3*H*-1,2-benzodithiol-3-one-1,1-dioxide; DMCE, 1,1-dimethyl-2-cyanoethyl; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene; TCA, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; ^{i}Pr, isopropyl; DMT, 4,4′-dimethoxytrityl; tetrazole, 1*H*-tetrazole; SVP, snake venom phosphodiesterase; ODN, oligodeoxynucleotide.

(2) Bennett, C. F.; Cowsert, L. M. *Biochim. Biophys. Acta* **1999**, *1489*, 19−30.
(3) Agrawal, S.; Zhao, Q. Y. *Curr. Opin. Chem. Biol.* **1998**, *2*, 519−528.
(4) Zhang, H.; Cook, J.; Nickel, J.; Yu, R.; Stecker, K.; Myers, K.; Dean, N. M. *Nat. Biotechnol.* **2000**, *18*, 862−867.
(5) Cummins, L.; Graff, D.; Beaton, G.; Marshall, W. S.; Caruthers, M. H. *Biochemistry* **1996**, *35*, 8734−8741.
(6) Vaughn, J. P.; Stekler, J.; Demirdji, S.; Mills, J. K.; Caruthers, M. H.; Iglehart, J. D.; Marks, J. R. *Nucleic Acids Res.* **1996**, *24*, 4558−4564.
(7) Yuen, A.; Halsey, J.; Fisher, G.; Advani, R.; Moore, M.; Saleh, M.; Ritch, P.; Harker, G.; Ahmed, F.; Jones, C.; Polikoff, P.; Keiser, W.; Kwoh, J. Holmlund, J.; Dorr, A.; Sikic, B. Phase I/II Trial of ISIS 3521, an Antisense Inhibitor of PKC-Alpha, with Carboplatin and Paclitaxel in Non-Small Cell Lung Cancer. American Society of Clinical Oncology's 37th Annual Meeting, San Francisco, CA, May 12, 2001.
(8) Stein, C. A.; Cheng, Y. C. *Science* **1993**, *261*, 1004−1012.
(9) Tomasz, J.; Shaw, B. R.; Porter, K.; Spielvogel, B. F.; Sood, A. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1373−1375.
(10) Higson, A. P.; Sierzchala, A.; Brummel, H.; Zhao, Z. Y.; Caruthers, M. H. *Tetrahedron Lett.* **1998**, *39*, 3899−3902.
(11) Sergueev, D. S.; Shaw, B. R. *J. Am. Chem. Soc.* **1998**, *120*, 9417−9427.
(12) Wang, J.; Verbeure, B.; Luyten, I.; Froeyen, M.; Hendrix, C.; Rosemeyer, H.; Seela, F.; Van Aerschot, A.; Herdewijn, P. *Nucleosides Nucleotides* **2001**, *20*, 785−788.
(13) Damha, M. J.; Wilds, C. J.; Noronha, A.; Brukner, I.; Borkow, G.; Arion, D.; Parniak, M. A. *J. Am. Chem. Soc.* **1998**, *120*, 13545−13545.

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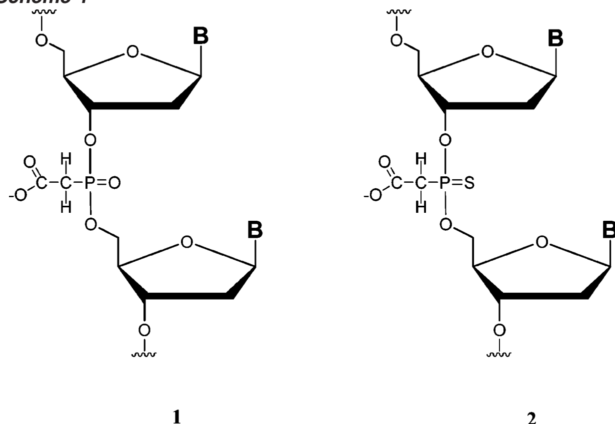
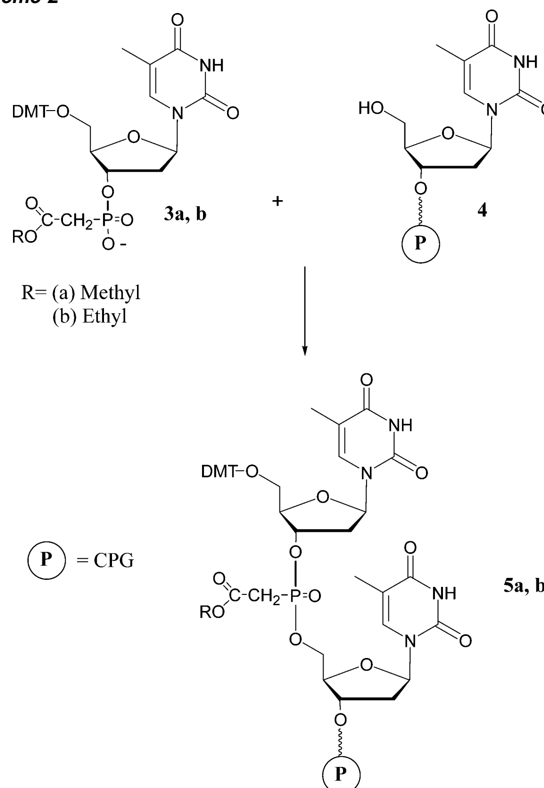
Scheme 1



Scheme 2



sides cannot be used to prepare the oligomers. Instead, entirely new synthons are needed which complicates their use because the synthetic methods are generally complex, low yielding, and costly.

In the diagnostic area, additional analogues such as peptide nucleic acids (PNA)[14] and locked nucleic acids (LNA),[15] among others, may prove useful, as water-solubility, cellular uptake, and RNase H activation are not always necessary. Unfortunately for these analogues as well, their synthesis requires the preparation of new monomer synthons, which suffer from complex and low yielding methodologies.

Because of these various limitations, we have continued to focus on the development of biochemically useful oligodeoxynucleotide analogues that are based upon the use of natural 2′-deoxynucleosides as starting materials. One set of candidates possesses either phosphonoacetate or thiophosphonoacetate internucleotide linkages (Scheme 1). Because of the presence of a phosphorus−carbon bond, these derivatives were expected to be nuclease resistant in a manner analogous to methylphosphonate modified DNA.[16] Because these analogues were also anionic, we speculated as well that they would be water soluble, perhaps hybridize to RNA, and thus activate RNase H. From a synthesis perspective, our main challenge was to develop an appropriate phosphitylating reagent that would lead to high repetitive coupling yields of **1** and **2** free from side products. In this manuscript, we report the chemical synthesis of these analogues, and initial biochemical results.

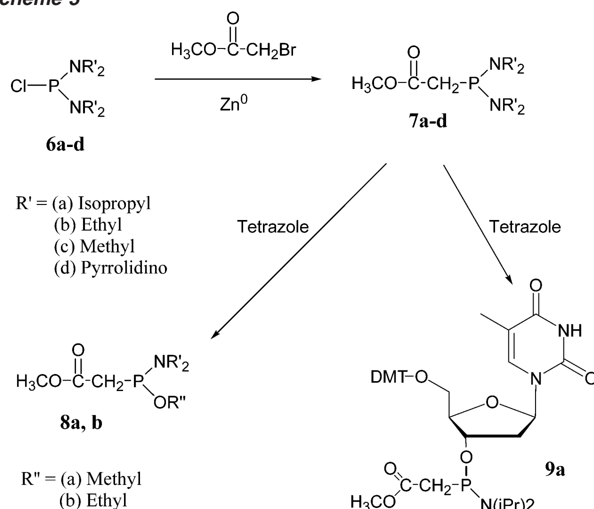
Results

P(V) Chemistry. Our initial investigations directed toward the synthesis of **1** focused on using the P(V) synthons introduced by others in preliminary research carried out in this area.[17−19] These experiments were completed by coupling either the 3′-*O*-phosphonoacetate methyl (**3a**) or ethyl (**3b**) ester of 5′-DMT-thymidine to thymidine attached to controlled pore glass (**4**)[20] using a series of arylsulfonyl chlorides and 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole as activating agents[21] (Scheme 2). Coupling yields to generate **5a** and **5b** were monitored from the absorbance at 498 nm of the dimethoxytrityl carbocation released from the product in anhydrous acid. For these experiments, with reaction times ranging from 1 to 24 h, yields never exceeded 5%, which is consistent with previous results.[19] Several attempts were also made to hydrolyze the ethyl or methyl ester of **5a** and **5b** as well as cleave the dimers from the support. These included treatment with concentrated ammonium hydroxide, dilute aqueous sodium hydroxide, and aqueous triethylamine. The products from these reactions, when analyzed by reverse-phase HPLC, were only present in trace amounts never exceeding 1%. By synthesizing the dimer as a methyl ester in solution (5% yield) and converting it to the 3′-*O*-phosphoramidite, Rudolph and co-workers reported the support synthesis of a 13mer oligodeoxythymidine having alternating phosphate and phosphonoacetate methyl ester linkages (dimer couplings through the 3′-*O*-phosphoramidite were 88%).[19] On the basis of these unacceptable results, we concluded that the successful synthesis of phosphonoacetate oligodeoxynucleotides would require the development of P(III) methods.

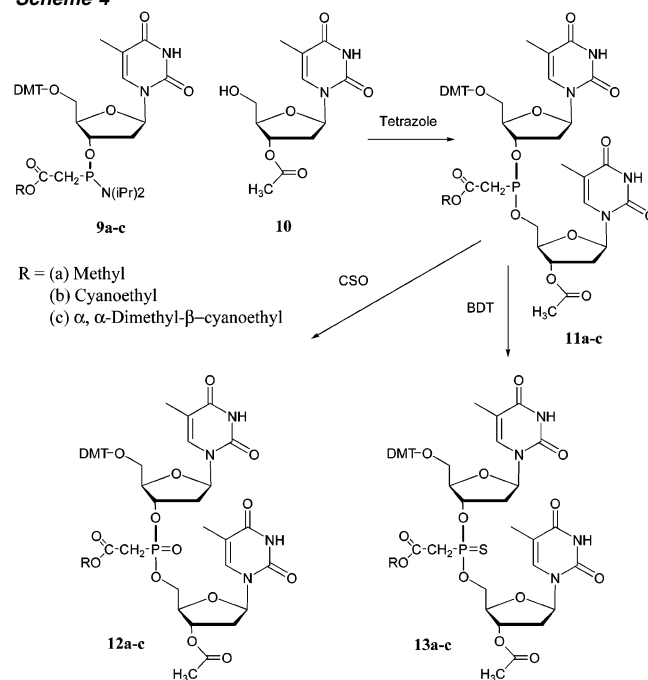
P(III) Chemistry. Although a few reports have appeared on the chemical and physical properties of phosphinylacetic acid, these compounds have never been used as chemical synthons.[22−24] Three brief communications on acetic acid phosphinodiamidites have been published, but each contained very limited experimental descriptions and no product analysis.[25−27] The results

(14) Nielsen, P. E. *Curr. Opin. Biotechnol.* **2001**, *12*, 16−20.
(15) Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607−3630.
(16) Miller, P. S.; Tso, P. O. P. *Annu. Rep. Med. Chem.* **1988**, *23*, 295−304.
(17) Griengl, H.; Hayden, W.; Penn, G.; Declercq, E.; Rosenwirth, B. *J. Med. Chem.* **1988**, *31*, 1831−1839.
(18) 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.
(19) Rudolph, M. J.; Reitman, M. S.; MacMillan, E. W.; Cook, A. F. *Nucleosides Nucleotides* **1996**, *15*, 1725−1739.
(20) Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* **1981**, *103*, 3186−3191.
(21) Efimov, V. A.; Reverdatto, S. V.; Chakmakhcheva, O. G. *Nucleic Acids Res.* **1982**, *10*, 6675−6694.
(22) VanDoorn, J. A.; Meijboon, N. *Phosphorus, Sulfur Silicon Relat. Elem.* **1989**, *42*, 211−222.
(23) Matrosov, E. I.; Tsvetkov, E. N.; Malevannaya, R. A.; Kabachnik, M. I. *Zh. Obshch. Khim.* **1972**, *42*, 1695−1700.
(24) Podlahova, J. *Collect. Czech. Chem. Commun.* **1978**, *43*, 57−72.

Scheme 3



Scheme 4



from this literature were incorporated into our initial studies, as outlined in Scheme 3.

Phosphinylacetic acid derivatives (compounds **7a−d**) useful for oligodeoxynucleotide phosphonate syntheses were prepared from bis(*N*,*N*-dialkylamino)chlorophosphines (**6a−d**) and methylbromoacetate. Compounds **6a−d** were first synthesized by the addition of the corresponding dialkylamine to phosphorus trichloride in anhydrous acetonitrile,[28] and the products were purified by distillation or recrystallization.[29] In our initial attempt at the formation of the carbon−phosphorus bond using the Reformatsky derivative of methylbromoacetate,[30] the organometallic reagent was preformed by the reaction of methyl bromoacetate with granular zinc metal in THF prior to the addition of the chlorophosphine (**6a−d**). The ^{31}P NMR spectrum of these reaction mixtures showed a complex series of products. In a previous communication by Novikova,[25] acetic acid [bis-(*N*,*N*-diethylamino)phosphino]methylester (**7b**) was reported to have a ^{31}P NMR chemical shift of δ 82 ppm. In our hands, neither the reaction mixtures nor the fractionally distilled products exhibited this chemical shift. The Reformatsky reactions were repeated by the in situ formation of the organometallic reactant as described by Bayles.[31] Under these conditions, compounds **6a−d** were converted to a major or single product with a ^{31}P NMR chemical shift of 49−52 ppm. Attempts to purify these products by vacuum distillation (40 microns) resulted in decomposition, whereas the removal of solvent in vacuo followed by trituration into hexanes or pentanes generated **7a−d**, as characterized by mass spectroscopy and ^{1}H NMR. When compound **7b** was distilled under high vacuum, an uncharacterized decompostion product appeared in the ^{31}P NMR spectrum near the previously reported 82 ppm. Compounds **7b−d** were significantly less stable than **7a** to storage and manipulation.

The reactivity of these bis-amidites toward the formation of phosphite esters was initially tested by condensing **7a** with methanol or ethanol in the presence of an acid catalyst such as 0.8 molar equiv of tetrazole to yield **8a** and **8b**. As judged by ^{31}P NMR, both **8a** and **8b** formed quantitatively (δ = 124.4 and 124.2 ppm). Further isolation of these products by silica gel column chromatography and analysis using ^{1}H NMR and EI mass spectroscopy confirmed their synthesis. These results encouraged us to test the use of **7a** in the formation of **9a**, a potential synthon for the preparation of **1** and **2**. Compound **7a** was treated with 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxythymidine using 0.8 molar equiv of tetrazole to yield **9a**. Isolation via silica gel column chromatography (84% yield) and characterization by ^{31}P NMR and FAB mass spectroscopy confirmed the identification of **9a**. To complete these initial model studies, **9a** was reacted with 3′-*O*-acetyl-2′-deoxythymidine (**10**) using an excess of tetrazole in anhydrous acetonitrile to generate the phosphinodiester **11a** (Scheme 4). Oxidation of the unpurified reaction mixture containing **11a** to the P(V) derivative (**12a**) using aqueous iodine generated both the protected phosphonoacetate and a small amount (3−5%) of the phosphate, as assayed by ^{31}P NMR and reverse-phase HPLC. Elimination of the phosphate product was achieved with either a low-water oxidation procedure[32] or oxidation under anhydrous conditions using (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine.[33] Conversion of **11a** to the thiophosphonodiester (**13a**) in near quantitative yield was accomplished with 3-*H*-1,2-benzodithiol-3-one-1,1-dioxide[34] without detectable decarboxylation.

Although these initial studies were quite encouraging, as ester protected phosphonoacetate dinucleotides could be obtained rapidly and in high yield, an additional serious problem was deprotection of the acetic acid methyl ester. Using the depro-

(25) Novikova, Z. S.; Zdorova, S. N.; Kirzner, V. N.; Lutsenko, I. F. *Zh. Obshch. Khim.* **1976**, *46*, 575−578.
(26) Novikova, Z. S.; Skorobogatova, Y. S.; Lutsenko, I. F. *Zh. Obshch. Khim.* **1976**, *46*, 2213−2217.
(27) Stepanov, I. A.; Platonov, A. Y.; Chistokletov, V. N. *Zh. Obshch. Khim.* **1979**, *49*, 2389−2390.
(28) King, R. B.; Sadanani, N. D.; Sundaram, P. M. *Phosphorus Sulfur Relat. Elem.* **1983**, *18*, 125−128.
(29) Compound **6a** was purified by recrystallization, whereas **6b−d** were distilled under high vacuum.
(30) Malenko, D. M.; Gololobov, Yu. G. *Zh. Obshch. Khim.* **1976**, *46*, 2391−2392.
(31) Bayles, P. L.; Hauser, C. R. *J. Am. Chem. Soc.* **1954**, *76*, 2306−2308.
(32) Hogrefe, R. I.; Vaghefi, M. M.; Reynolds, M. A.; Young, K. M.; Arnold, L. J. *Nucleic Acids Res.* **1993**, *21*, 2031−2038.
(33) Ugi, I.; Jacob, P.; Landgraf, B.; Rupp, C.; Lemmen, P.; Verferth, U. *Nucleosides Nucleotides* **1988**, *7*, 605−608.
(34) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693−4699.

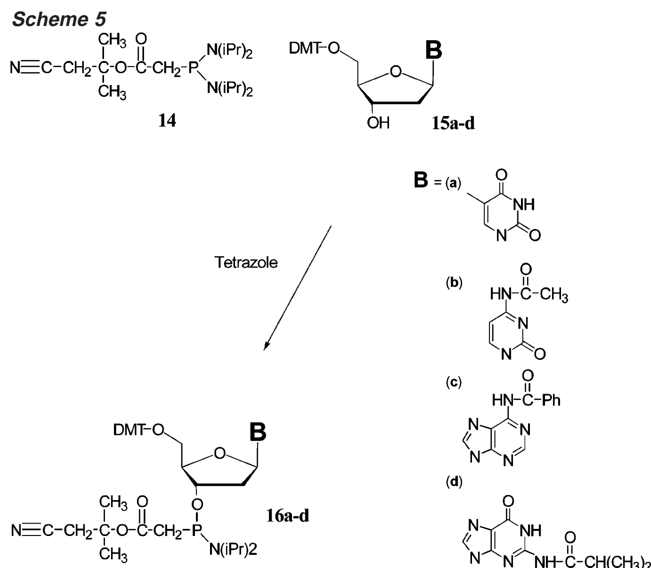
tection conditions published previously for the hydrolysis of the methyl ester,[17,18,35] cleavage of the internucleotide linkage and decarboxylation were the major products, as assayed by reverse-phase HPLC and ^{31}P NMR. When oligodeoxythymidine phosphonoacetate methyl ester (10mer) was prepared on CPG and the reaction mixture treated with a deprotection reagent that had been reported to give the least cleavage (0.1 M piperidine dissolved in 1:1 methanol−water, 88 h, 25 °C),[19] degradation still predominated (HPLC and PAGE analysis) with only approximately 15% of the product being isolated. These results suggested that an attack by base on the ester carbonyl was probably responsible for the generation of intermediates leading to decarboxylation or cleavage via a Perkow-type rearrangement.[36] This was previously shown to be the case, where facile cleavage of the phosphorus−carbon bond for simple formic acid ester phosphonate derivatives occurred under conditions of nucleophilic hydrolysis.[37−39]

As a consequence of these observations, carboxyl esters that undergo β-elimination by a non-nucleophilic base were investigated. Although these types of protecting groups are generally less useful with carboxylic acid esters when compared to phosphate esters, the presence of an electron-withdrawing phosphonodiester joined to acetate was expected to enhance β-elimination. Initially, the β-cyanoethyl[40] derivative (Scheme 4, compound **12b**) was tested, but results indicated that approximately 4−8% of this internucleotide linkage was converted to phosphate (^{31}P NMR) with anhydrous solutions of DBU, presumably because of trace amounts of water leading to a hydroxide attack on the ester carbonyl. We therefore reasoned that the placement of methyl groups on the ester would further shield the carbonyl from nucleophilic attack. Experimentally, this proved to be the case as the α,α-dimethyl-β-cyanoethyl ester analogue (Scheme 4, compound **12c**) yielded only trace amounts of the phosphate hydrolysis product after deprotection using anhydrous DBU in acetonitrile (1−2%, ^{31}P NMR). This derivative proved to be satisfactory for further work on oligodeoxynucleotide synthesis.

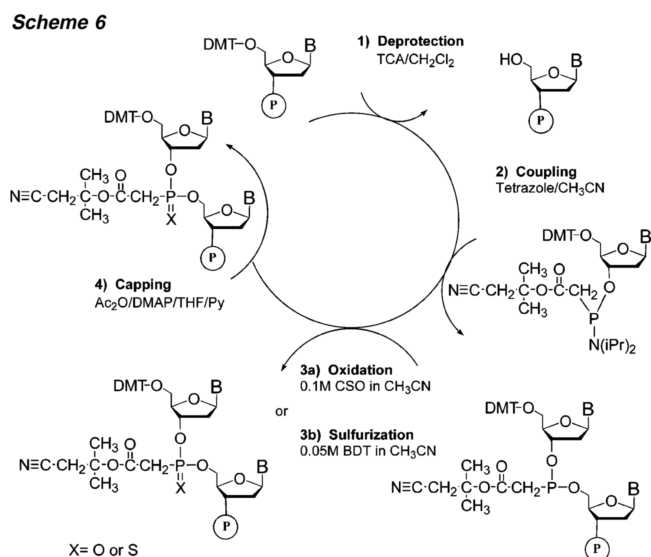
Introduction of the dimethylcyanoethyl group into the appropriate 2′-deoxynucleoside synthons proceeded via the reaction sequence summarized in Scheme 5. Initially, the bromoacetate used in the Reformatsky reaction was synthesized by condensing bromoacetyl bromide with 3-hydroxy-3-methylbutyronitrile in toluene under reflux for 12 h. Compound **14** was then prepared via the procedure of Bayles[31] as described previously for the synthesis of **7a**. Further condensation of **14** with appropriately protected 2′-deoxynucleosides **15a−d** using tetrazole led to synthons **16a−d**. Because of the electron-withdrawing properties of the dimethylcyanoethyl group, there were initial concerns that **14** and **16a−d** would be generated in lower amounts than the corresponding methyl ester. However, for both series, the purified yields were comparable (80−90%).

These encouraging results were then extended to the development of a solid-phase synthesis cycle for preparing phosphonoacetate-modified oligodeoxynucleotides. Starting with the standard phosphoramidite synthesis cycle for the preparation of DNA,[41] we required several modifications to successfully generate this analogue (Scheme 6). These included changing the condensation time, modifying the oxidation step, and altering the capping solution. On the basis of previous results with other analogues, including the methylphosphonate derivative, a slower condensation rate was expected, but we were surprised to discover that 33 min was necessary in order to obtain 97% coupling efficiency per cycle. Presumably, this slower rate was due to the presence of an electron-withdrawing acetyl ester linked to phosphorus through a carbon−phosphorus bond. For the capping and oxidation steps, the research of others with the methylphosphonate analogue proved to be quite useful.[32] The standard iodine oxidation solution[20] was altered to contain less iodine and water[32] or substituted with (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) in anhydrous acetonitrile.[42] Similarly, when the original capping solution was exchanged with

Scheme 5



Scheme 6



(35) Cook, A. F. Published International Patent Application Number WO 93/10140.
(36) Emsley, J.; Hall, D. *The Chemistry of Phosphorus*; Harper and Row: London, 1976; pp 133−139.
(37) Sekine, M.; Satoh, M.; Yamagata, H.; Hata, T. *J. Org. Chem.* **1980**, *45*, 4162−4167.
(38) Narayanan, K. S.; Berlin, K. D. *J. Am. Chem. Soc.* **1979**, *101*, 109−116.
(39) Kluger, R.; Chin, J. *J. Am. Chem. Soc.* **1978**, *100*, 7382−7388.
(40) Tener, G. *J. Am. Chem. Soc.* **1961**, *83*, 159−168.
(41) Caruthers, M. H.; Barone, A. D.; Beaucage, S. L.; Dodds, D. R.; Fisher, E. F.; McBride, L. J.; Matteucci, M.; Stabinsky, Z.; Tang, J.-Y. *Methods Enzymol.* (Wu, R., Grossman, L., Eds.) **1987**, *154*, 287−313.
(42) The Glen Report, *Glen Research Corporation Technical Report*, Vol. 9; Glen Research Sterling, VA, 1996; pp 8−9.

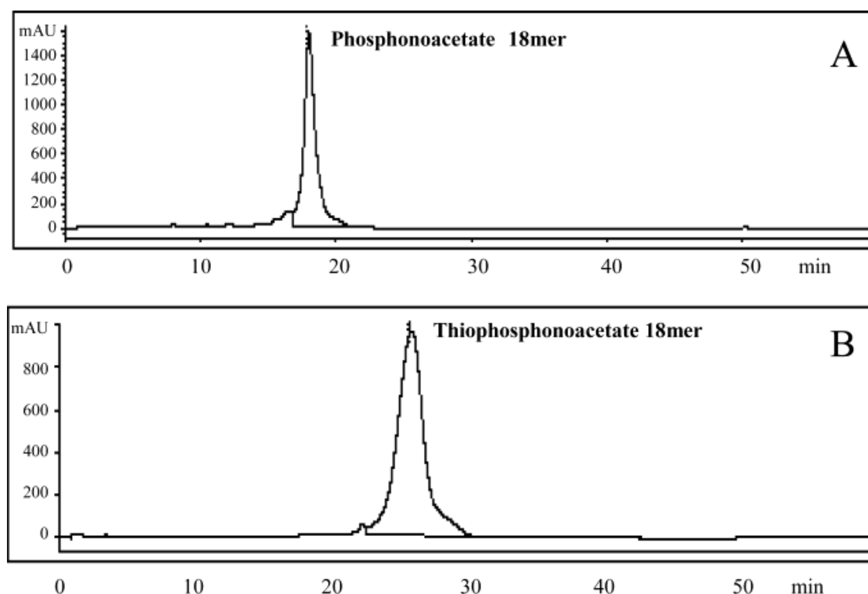


Figure 1. Ion-exchange HPLC of the phosphonoacetate (panel A) and thiophosphonoacetate (panel B) analogues of 5′-d(CTCAAGTGGGCTGGTGAC).

the reagent recommended by Arnold and Hogrefe[32] for the synthesis of methylphosphonate containing oligomers, reaction mixtures contained fewer uncharacterized minor side products, as monitored by HPLC. Sulfurization of the acetic acid phosphinyl ester with BDT[34] to generate the thiophosphonoacetate analogue proved quite successful, and very few side products were observed.

After synthesis, the phosphonoacetate and thiophosphonoacetate modified oligomers were cleaved from the support and protecting groups removed. On the basis of our initial studies with dimers, the deprotection strategy involved first the removal of the DMCE group by treatment of the CPG with an anhydrous solution of DBU in acetonitrile. The resulting oligomers were then freed of base-protecting groups and cleaved from the support using conditions similar to those previously published for the rapid deprotection of oligodeoxynucleotides[43] (40% methylamine in water at 55 °C for 15 min). Although these conditions are considerably milder than the standard procedure with concentrated ammonium hydroxide overnight at 55°C, our experience with methyl ester hydrolysis gave us concerns regarding the stability of the phosphonate linkage. To investigate this potential problem, oligothymidylate 15mers were synthesized in which the eighth internucleotide linkage from the 5′-end was either phosphonoacetate or thiophosphonoacetate (the remainder were natural phosphodiester linkages). The resulting oligodeoxynucleotides were characterized by HPLC, ^{31}P NMR, and MALDI-TOF mass spectroscopy. Following 5′-[^{32}P]-phosphate labeling of each oligomer, we incubated them at 55 °C with 40% methylamine in water, with aliquots removed at various times and products separated by PAGE. Autoradiographic analysis demonstrated that 98.6% of the full-length phosphonoacetate containing oligomer remained after 15 min and 95.4% after 60 min. Comparable numbers for the thiophosphonoacetate containing oligomer were 98.8% (15 min) and 96.5% (60 min). For 20mers having all phosphonoacetate or thiophosphonoacetate internucleotide linkages, these observations suggested that 75−80% of products (calcd) would arrive intact. These results encouraged us to continue using 40% methylamine at 55 °C (15 min) to remove base-protecting groups and cleave the oligomers from supports.

After synthesis and on the basis of these observations, the DMCE group was first removed with 1.5% DBU in anhydrous acetonitrile and 40% methylamine in water was then used to cleave the products from the supports and eliminate base-protecting groups. These reaction mixtures were purified by reverse-phase HPLC to generate a major peak of 5′-DMT containing oligomers. This material was then treated with 80% acetic acid to remove the DMT group, and the products were repurified by reverse-phase HPLC to yield the fully deprotected 18mers. Aliquots of the phosphonoacetate and thiophosphonoacetate modified oligomers were then analyzed by ion-exchange HPLC (Figure 1). For both oligomers, similar profiles were obtained with each having one major, broad peak and several minor and uncharacterized earlier eluting products. Broad product peaks were expected, as these oligomers each contained 17 P-chiral centers. On the basis of the amount of deoxynucleoside attached to the support, isolated yields of these 18mers following the complete removal of protecting groups and two sequential reverse-phase purifications were 33% (phosphonoacetate) and 32% (thiophosphonoacetate). As expected for oligomers with many P-chiral centers, the ^{31}P NMR spectra generated multiple peaks for the phosphonoacetate (27.5−28.5 ppm) and the thiophosphonoacetate (93−94 ppm) oligomers (Figure 2). In both spectra, the product peaks contained greater than 98% of the total signal with only trace amounts of ^{31}P contaminates. When these oligomers were analyzed by MALDI-TOF mass spectroscopy (Figure 3), each oligomer's observed mass (phosphonoacetate 18mer, 6270 *m*/*e*; thiophosphonoacetate 18mer, 6540 *m*/*e*) was as calculated (6271 and 6542, respectively).

Because of the presence of a carbon−phosphorus bond, the phosphonoacetate and thiophosphonoacetate ODNs were expected to be nuclease resistant. To test this possibility, 5′-fluorescein labeled phosphonoacetate and thiophosphonoacetate ODNs were prepared and tested for susceptibility to degradation by snake venom phosphodiesterase. Similar phosphate and thiophosphate oligomers were used as controls (Figure 4). Under

(43) Reddy, M. P.; Hanna, N. B.; Farooqui, F. *Tetrahedron Lett.* **1994**, *35*, 4311−4314.

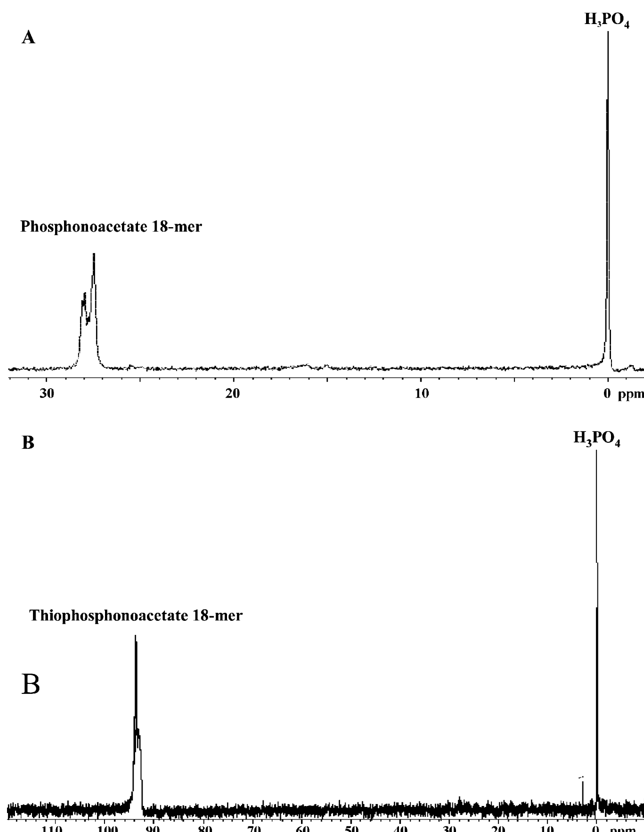


Figure 2. ^{31}P NMR analysis of the phosphonoacetate (panel A) and thiophosphonoacetate (panel B) analogues of 5′-d(CTCAAGTGGGCTG-GTGAC).

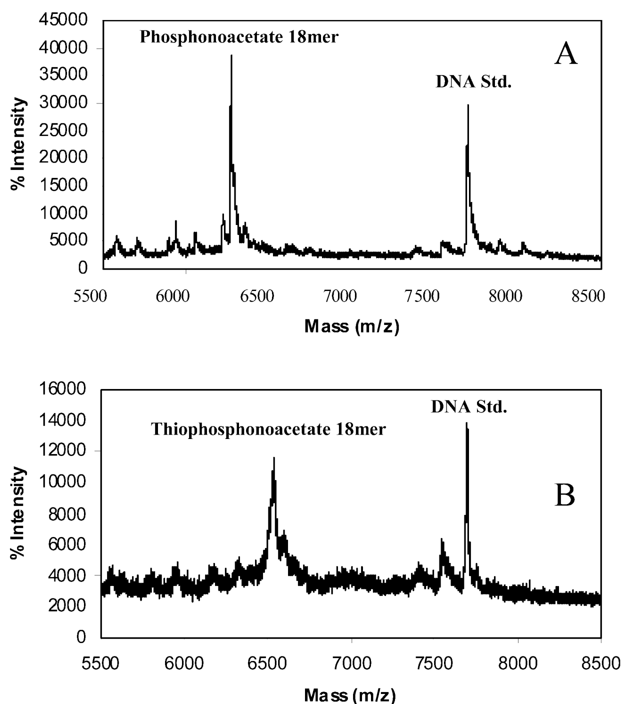


Figure 3. MALDI-TOF analysis of the phosphonoacetate (panel A) and thiophosphonoacetate (panel B) analogues of 5′-d(CTCAAGTGGGCTG-GTGAC). The peak at 7697 *m/e* corresponds to an internal DNA standard.

conditions where the ODN having phosphodiester linkages was completely degraded (lanes 22−28), both the thiophosphonoacetate (lanes 1−7) and phosphonoacetate (lanes 8−14) ODNs were stable. Furthermore, both analogues were more stable

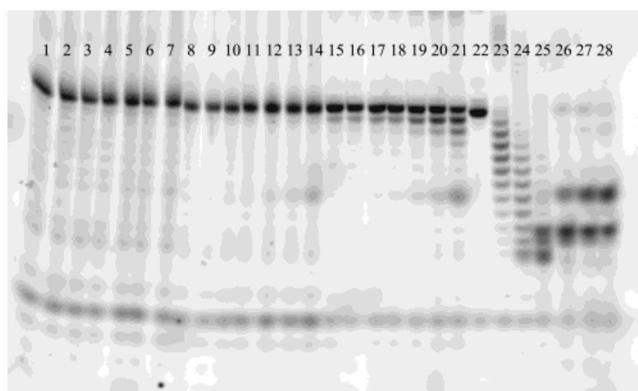


Figure 4. Exonuclease degradation of ODNs with snake venom phosphodiesterase. 5′-Fluorescein labeled ODNs were treated with SVP for various times and analyzed by PAGE. Digestion times: (0 min) lanes 1, 8, 15, 22; (15 min) lanes 2, 9, 16, 23; (30 min) lanes 3, 10, 17, 24; (60 min) lanes 4, 11, 18, 25; (180 min) lanes 5, 12, 19, 26; (360 min) lanes 6, 13, 20, 27; (1080 min) lanes 7, 14, 21, 28. Thiophosphonoacetate ODN: lanes 1−7. Phosphonoacetate ODN: lanes 8−14. Phosphorothioate ODN: lanes 15−21. Phosphate ODN: lanes 22−28.

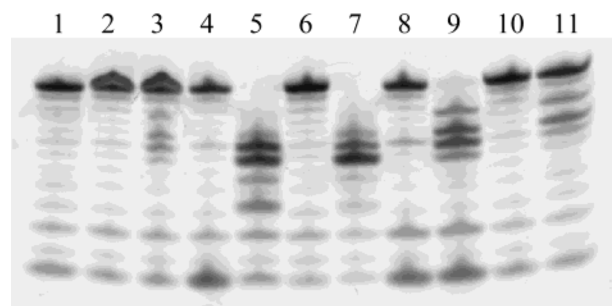


Figure 5. *E. coli* RNase H1 degradation of RNA. 5′-Fluorescein labeled RNA was allowed to form duplexes with complementary ODNs, which were then treated with *E. coli* RNase H1 for 12 h and analyzed by PAGE. Lane 1: RNA, enzyme. Lane 2: 2′-*O*-methyl RNA, RNA. Lane 3: 2′-*O*-methyl RNA, RNA, enzyme. Lane 4: phosphate ODN, RNA. Lane 5: phosphate ODN, RNA, enzyme. Lane 6: phosphorothioate ODN, RNA. Lane 7: phosphorothioate ODN, RNA, enzyme. Lane 8: phosphonoacetate ODN, RNA. Lane 9: phosphonoacetate ODN, RNA, enzyme. Lane 10: thiophosphonoacetate ODN, RNA. Lane 11: thiophosphonoacetate ODN, RNA, enzyme.

toward this exonuclease than the phosphorothioate ODN (lanes 15−21).

On the basis of these encouraging results, the phosphonoacetate and thiophosphonoacetate analogues were also tested for their ability to stimulate RNase H1 activity. The test systems were composed of a 5′-fluorescein labeled RNA and complementary phosphonoacetate, thiophosphonoacetate, or phosphorothioate ODNs. Controls were natural, complementary DNA (which activates RNA degradation) and 2′-*O*-methyl RNA (which does not stimulate RNase H1 activity). As can be seen from the results presented in Figure 5, under conditions where DNA (lanes 4,5) and phosphorothioate DNA (lanes 6,7) stimulated RNA degradation, both phosphonoacetate (lanes 8,9) and thiophosphonoacetate (lanes 10,11) ODNs also activated RNase H1. However, there were differences. For example, the phosphonoacetate analogue appeared to be more active than thiophosphonoacetate (compare lanes 9,11). Perhaps the reduced activity with the thiophosphonoacetate derivative was due to a combination of steric and hydrophobic effects in the enzyme active site. Alternatively and because of sulfur steric effects, the thiophosphonoacetate ODN/RNA duplex could be less stable which would lead to a slower RNA hydrolysis rate. Another

interesting observation was the RNA degradation profile (compare lanes 5,7 to 9,11). Clearly, some cleavage sites were different when phosphonoacetate and thiophosphonoacetate derivatives were used to stimulate RNase H1 degradation. Perhaps these profile differences relate to sequence dependent instabilities of ODN/RNA duplexes, but these observations require further analysis.

These results demonstrate that phosphonoacetate and thiophosphonoacetate oligodeoxynucleotides having sufficient length for biochemical experiments can be synthesized by the procedures outlined in this manuscript. On the basis of preliminary biochemical results, these analogues may prove quite useful for numerous applications, as they are stable toward nucleases, activate RNase H, and can be used as primers with DNA polymerases.[44]

Experimental Section

General Procedures. ^{1}H NMR spectra were recorded on Varian VXR-300 and Bruker 400 MHz spectrometers with tetramethylsilane as an internal reference. ^{31}P NMR spectra were recorded on a Bruker 400 MHz spectrometer using an external capillary containing 85% H_3PO_4 as a reference. Downfield chemical shifts were recorded as positive values for ^{31}P NMR. The University of Colorado Central Analytical Laboratories performed ESI, FAB, and absolute mass spectroscopy analysis. Reverse-phase (Zorbax 300SB-C-18 column, Agilent Technologies, Palo Alto, CA) and ion-exchange (1-mL Resource-Q column, Amersham Biosciences, Piscataway, NJ) HPLC were performed on an Agilent Technologies Model 1100 HPLC. Autoradiography and fluorescent imaging were performed on a Molecular Dynamics Typhoon model 8600 (Amersham Biosciences). DNA synthesis was carried out on an Applied Biosystems model 394 automated DNA synthesizer (Applied Biosystems, Foster City, CA) modified for the synthesis cycle shown in Scheme 6. 5′-Fluorescein phosphoramidite was purchased from Glen Research (Sterling, VA).

Dichloromethane and pyridine were distilled over calcium hydride. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Commercially available DNA synthesis reagents were obtained from Glen Research Corp. Medium-pressure, preparative column chromatography was performed using 230−400 mesh silica gel from EM Sciences. Thin-layer chromatography was performed on glass-backed silica 60 F_{254} plates from EM Sciences. 3*H*-1,2-benzodithiol-3-one-1,1-dioxide was purchased from Glen Research, and (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Phosphodiesterase I from *Crotalus Adamanteus* was obtained from US Biochemicals (Cleveland, OH). *E. coli* RNase H1 was purchased from Promega (Madison, WI).

Synthesis of Bis(*N*,*N*-diisopropylamino)chlorophosphine (6a). A 5 L three neck round-bottom flask was equipped with a Friedrich's condenser, a ground glass stirrer bearing, and a silicon rubber septum and placed under dry argon. Anhydrous diisopropylamine (2 L, 1.6 kg, 15.9 mol) was added to the flask and diluted by the addition of anhydrous acetonitrile (2 L). The solution was mixed with a mechanical stirrer attached to a glass rod and a Teflon blade. An ice−water bath was placed under the flask, and the solution was allowed to cool with stirring for 30 min. Phosphorus trichloride (313 g, 2.3 mol) was placed in a dry 2 L flask, and anhydrous acetonitrile (1 L) was added. This phosphorus trichloride solution was then added slowly by cannulation to the vigorously stirred solution of diisopropylamine. Once addition was complete, the ice bath was removed and the reaction mixture was stirred overnight. Complete conversion of the phosphorus trichloride (δ 201 ppm) to product (δ 134 ppm) was monitored by ^{31}P NMR. The reaction mixture was filtered to remove diisopropylamine hydrochloride, and the precipitate was washed with anhydrous ether. The combined filtrates were concentrated in vacuo to a semicrystalline solid. Anhydrous hexanes (1.5 L) were added to the flask, and the mixture was heated. The hot liquid was filtered through a Schlenk filter funnel to remove residual amine hydrochloride, and the resulting clear liquid was concentrated in vacuo to half the original volume. The product was then isolated by crystallization, filtration, and drying in vacuo to yield 447 g (74% yield). ^{31}P NMR (CD_3CN) δ 134.4 (s); electron impact mass spectrometry gave a molecular ion of 267 *m*/*e*.

Synthesis of Bis(*N*,*N*-diethylamino)chlorophosphine (6b). *N*,*N*-Diethyltrimethylsilylamine (100 g, 688 mmol) was dissolved in 1 L of anhydrous ether and placed under dry argon in a 2 L three neck round-bottom flask equipped with a Friedrich's condenser, a ground glass stirrer bearing, and a silicon rubber septum. The solution was mixed with a mechanical stirrer attached to a glass rod and a Teflon blade. An ice−water bath was placed under the flask, and the solution was allowed to cool with stirring for 30 min. Phosphorus trichloride (23.7 g, 172 mmol) was placed in a dry 500 mL flask, and anhydrous ether (250 mL) added. This phosphorus trichloride solution was then added slowly by cannulation to the vigorously stirred solution of *N*,*N*-diethyltrimethylsilylamine. Once addition was complete, the ice bath was removed and the reaction mixture was stirred overnight. Complete conversion of the phosphorus trichloride (δ 201 ppm) to the tris(*N*,*N*-diethylamino)phosphine product (δ 102 ppm) was monitored by ^{31}P NMR. The reaction mixture was filtered through a Schlenk filter funnel to remove amine hydrochloride, and the filtrate was concentrated to an oil in vacuo. The product was distilled using a two-stage oil pump at 0.04 mmHg to yield 34.8 g (82%): bp 67−69 °C; ^{31}P NMR (CD_3CN) δ 102.6 (s); electron impact mass spectrometry gave a molecular ion of 247 *m*/*e*. The resulting tris(*N*,*N*-diethylamino)phosphine was then converted to the bis(*N*,*N*-diethylamino)chlorophosphine by disproportionation with phosphorus trichloride. Tris(*N*,*N*-diethylamino)phosphine (34.8 g, 141 mmol) and anhydrous acetonitrile were added to a dry 500 mL round-bottom flask containing a Teflon stir bar. The flask was fitted with a silicon rubber stopper and placed under argon. An ice−water bath was placed under the flask, the solution was allowed to cool with stirring for 30 min, and phosphorus trichloride (9.69 g, 70.5 mmol) was added slowly by syringe to the stirred solution. The solution was allowed to warm to room temperature, and the reaction was stirred overnight. Complete conversion of the tris(*N*,*N*-diethylamino)phosphine (δ 102 ppm) to the bis(*N*,*N*-diethylamino)chlorophosphine product (δ 159 ppm) was monitored by ^{31}P NMR. The product was distilled using a two-stage oil pump at 0.04 mmHg to yield 42.5 g (95%): bp 56−59 °C; ^{31}P NMR (CD_3CN) δ 159.2 (s); electron impact mass spectrometry gave a molecular ion of 211 *m*/*e*.

Synthesis of Bis(*N*,*N*-dimethylamino)chlorophosphine (6c). Starting with *N*,*N*-dimethyltrimethylsilylamine (100 g, 853 mmol) and phosphorus trichloride (29.3 g, 213 mmol), we synthesized the product using the procedure outlined for **6b**, distilled using a two-stage oil pump at 0.04 mmHg, and yielded 32.9 g (98%) of **6c**: bp 50−56 °C; ^{31}P NMR (CD_3CN) δ 158.9 (s); electron impact mass spectrometry gave a molecular ion of 155 *m*/*e*.

Synthesis of Bis(pyrrolidino)chlorophosphine (6d). A 3 L three neck round-bottom flask was equipped with a Friedrich's condenser, a ground glass stirrer bearing, and a silicon rubber septum and placed under dry argon. Pyrrolidine (85.2 g, 1.2 mol) was added to the flask and diluted by the addition of 1.5 L of anhydrous acetonitrile. The solution was mixed with a mechanical stirrer attached to a glass rod and a Teflon blade. An ice−water bath was placed under the flask, and the solution was allowed to cool with stirring for 30 min. Phosphorus trichloride (18.3 g, 133 mmol) was placed in a dry 2 L flask, and anhydrous acetonitrile (1 L) was added. This phosphorus trichloride solution was then added slowly by cannulation to the vigorously stirred solution of pyrrolidine. Once addition was complete, the ice bath was removed and the reaction mixture was stirred overnight.

(44) Sheehan, D.; Dellinger, D. J.; Lindberg, J. G.; Caruthers, M. H. Manuscript in preparation.

Complete conversion of the phosphorus trichloride (δ 201 ppm) to the tris(pyrrolidino)phosphine (δ 103 ppm) was monitored by ^{31}P NMR. The reaction mixture was filtered to remove pyrrolidine hydrochloride, and the precipitate was washed with anhydrous ether. The filtrate was evaporated in vacuo, and the crude tris(pyrrolidino)phosphine was isolated as an oil. The oil was distilled using a two-stage oil pump at 0.04 mmHg to yield 28.2 g (88%): bp 80−82 °C; ^{31}P NMR (CD_3CN) δ 103.4 (s); electron impact mass spectrometry gave a molecular ion of 241 *m*/*e*. The resulting tris(pyrrolidino)phosphine was then converted to the bis(pyrrolidino)chlorophosphine by disproportionation with phosphorus trichloride. Tris(pyrrolidino)phosphine (28.2 g, 117 mmol) and anhydrous acetonitrile (150 mL) were placed in a dry 500 mL round-bottom flask containing a Teflon stir bar. The flask was fitted with a silicon rubber stopper and placed under argon. The flask was immersed in an ice−water bath, and the solution was allowed to cool with stirring for 30 min. Phosphorus trichloride (8.05 g, 58.5 mmol) was added slowly by syringe to the stirred solution. The solution was allowed to warm to room temperature, and the reaction was stirred overnight. Complete conversion of the tris(pyrrolidino)phosphine (δ 103 ppm) to the bis(pyrrolidino)chlorophosphine (δ 161 ppm) was monitored by ^{31}P NMR. The product was distilled using a two-stage oil pump at 0.04 mmHg to yield 36.2 g (92%): bp 59−62 °C; ^{31}P NMR (CD_3CN) δ 161.3 (s); electron impact mass spectrometry gave a molecular ion of 206 *m*/*e*.

Synthesis of Acetic Acid, [Bis(*N*,*N*-diisopropylamino)phosphino]-methyl Ester (7a). Granular zinc metal (7.2 g, 110 mmol) and a magnetic stir bar were placed in a 1 L three neck round-bottom flask equipped with a Fredrich's condenser and two 500 mL addition funnels. Bis(*N*,*N*-diisopropylamino)chlorophosphine (20 g, 75 mmol) and methyl bromoacetate (11.6 g, 82.5 mmol) were each dissolved in 200 mL of anhydrous ether. These two solutions were placed in dropping funnels, and the reflux condenser was fitted with a dry argon line. Approximately one-third of each solution was added to the flask, and the mixture was heated with a heat gun until the Reformatsky reaction was initiated. Once initiated, the reaction was continued by the constant addition of the two solutions. After the addition was complete, the reaction was kept at reflux until the starting material was consumed, as monitored by ^{31}P NMR. The reaction mixture was decanted from the unreacted zinc, and the ether was removed in vacuo using a rotory evaporator. The product was isolated by triturating with pentanes to yield 11.6 g (54%). ^{1}H NMR ($CDCl_3$) δ 3.68 (s, 3H), 3.55 (m, 4H), 2.93 and 2.91 (d, 2H), 1.30 (m, 24H); ^{31}P NMR (CD_3CN) δ 49 (s); electron impact mass spectrometry gave a molecular ion of 304 *m*/*e* with a fragmentation loss of CH_2COOCH_3 at 231 *m*/*e*.

Synthesis of Acetic Acid, [Bis(*N*,*N*-diethylamino)phosphino]-methyl Ester (7b). Starting with granular zinc (13.1 g, 200 mmol), bis(*N*,*N*-diethylamino)chlorophosphine (21.1 g, 100 mmol), and methyl bromoacetate (14.1, 100 mmol), we synthesized the product using the procedure outlined for **7a** to yield 2.2 g (9%) of **7b**. ^{1}H NMR ($CDCl_3$) δ 3.66 (s, 3H), 2.93 and 2.91 (d, 2H), 2.72 (q, 8H), 1.26 (t, 12H); ^{31}P NMR (CD_3CN) δ 50.9 (s); electron impact mass spectrometry gave a molecular ion of 248 *m*/*e* with a fragmentation loss of CH_2COOCH_3 at 175 *m*/*e*.

Synthesis of Acetic Acid, [Bis(*N*,*N*-dimethylamino)phosphino]-methyl Ester (7c). Starting with granular zinc (13.1 g, 200 mmol), bis(*N*,*N*-dimethylamino)chlorophosphine (15.5 g, 100 mmol), and methyl bromoacetate (14.1, 100 mmol), we synthesized the product using the procedure outlined for **7a** to yield an unpurified product. The product was unstable to further purification by triturating with pentanes or hexanes. ^{31}P NMR (CD_3CN) δ 51.0 (s); electron impact mass spectrometry gave a molecular ion of 192 *m*/*e* with a fragmentation loss of CH_2COOCH_3 at 119 *m*/*e*.

Synthesis of Acetic Acid, [Bis(pyrrolidino)phosphino]methyl Ester (7d). Starting with granular zinc (13.1 g, 200 mmol), bis-(pyrrolidino)chlorophosphine (20.7 g, 100 mmol), and methyl bromoacetate (14.1, 100 mmol), we synthesized the product using the procedure outlined for **7a** to yield an unpurified product. The product was unstable to further purification by triturating with pentanes or hexanes. ^{31}P NMR (CD_3CN) δ 51.2 (s); electron impact mass spectrometry gave a molecular ion of 244 *m*/*e* with a fragmentation loss of CH_2COOCH_3 at 171 *m*/*e*.

Synthesis of 1,1-Dimethylcyanoethyl Bromoacetate. Bromoacetyl bromide (108 g, 600 mmol) was added to a 1 L round-bottom flask containing 500 mL of anhydrous toluene. 3-Hydroxy-3-methylbutyronitrile (50 g, 500 mmol) was added slowly with stirring. The round-bottom flask was fitted with a Friedrich's condenser and a drying tube vented to an acid trap. The reaction mixture was heated to reflux using a mantel and refluxed overnight. The reaction was allowed to cool to room temperature, and the mixture was concentrated in vacuo to an oil. The oil was purified by vacuum distillation at ~0.1 mmHg. The oil was distilled into two fractions: an initial minor fraction that gave a wide boiling range and a second major fraction that distilled at a temperature range of 120−122 °C. The initial fraction was discarded, and the constant boiling fraction gave 97.3 g of a clear, colorless liquid (88% yield). ^{1}H NMR ($CDCl_3$) δ 3.75 (s, 2H), 2.86 (2, 2H), 1.53 (s, 6H). Electron impact mass spectrometry gave molecular ions of 205 *m*/*e* and 207 *m*/*e* with isotopic abundances of 51% and 49%.

Synthesis of Acetic Acid, [Bis(*N*,*N*-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl Ester (14). Anhydrous THF (160 mL), bis-(*N*,*N*-diisopropylamino)chlorophosphine (20 g, 75 mmol), and a magnetic stir bar were added to a 500 mL round-bottom flask which was stoppered, and the solution was allowed to be stirred until the phosphine dissolved. After dissolution, anhydrous ether (100 mL) was added. 1,1-Dimethyl-2-cyanoethyl bromoacetate (18.2 g, 82.5 mmol) was placed in a 125 mL round-bottom flask, and anhydrous THF (75 mL) added. Granular zinc metal (7.2 g, 110 mmol), anhydrous ether (50 mL), and a magnetic stir bar were placed in a 1 L three necked round-bottom flask fitted with a Friedrich's condenser and two 500 mL addition funnels. The phosphine solution and the 1,1-dimethyl-2-cyanoethyl bromoacetate solution were added to the two addition funnels. Aliquots of the phosphine solution (85 mL) and the bromoacetate solution (25 mL) were added to the three necked round-bottom flask. The reaction mixture was then heated under reflux until an exothermic reaction was noticeable (the slightly cloudy, colorless reaction became clear and slightly yellow). The reaction was continued at reflux by the addition of the remainder of the phosphine and bromoacetate solutions. Once the addition was complete, the reaction was kept at reflux for 30 min by heating, allowed to cool to room temperature, and analyzed for completeness by ^{31}P NMR. The starting material at δ 135 ppm was converted to a single product at δ 48 ppm. The cooled reaction mixture was concentrated in vacuo to a viscous oil. The resulting viscous oil was extracted three times with anhydrous hexanes which converted the oil to a solid. The solid was then dissolved in acetonitrile, and this solution was extracted twice with anhydrous hexanes. The acetonitrile solution was analyzed by ^{31}P NMR for absence of the product at δ 48 ppm and discarded. All hexane fractions were combined and concentrated in vacuo to a slightly yellow oil, redissolved in anhydrous hexanes, and placed in a freezer overnight. The hexane solution was decanted into a clean, dry 500 mL round-bottom flask, and the hexanes were removed by evaporation to give 16.4 g (88% yield) of a slightly yellow oil. The product could be further purified by recystalization in anhydrous pentanes to yield a crystalline solid. ^{1}H NMR ($CDCl_3$) δ 3.48 (m, 4H), 2.99 (s, 2H), 2.80 and 2.78 (d, 2H), 1.30 (m, 24H). Electron impact mass spectrometry gave a molecular ion of 371 *m*/*e* with a fragmentation loss of $CH_2COOC(CH_3)_2CH_2CN$ at 231 *m*/*e*. ^{31}P NMR (CD_3CN) δ 48.1 ppm.

Synthesis of Acetic Acid, [*O*-Methyl(*N*,*N*-diisopropylamino)-phosphino]methyl Ester (8a). Compound **7a** (2.0 g, 6.5 mmol) was placed in a 100 mL round-bottom flask and dissolved in anhydrous acetonitrile (50 mL). Anhydrous methanol (210 mg, 6.5 mmol) and tetrazole (360 mg, 5.2 mmol) were added, and the flask was stirred for 24 h. The reaction mixture was neutralized by the addition of 5 mL of

triethylamine and concentrated to an oil using a rotary evaporator. The oil was applied to a silica gel column equilibrated with ethyl acetate/hexanes (3:1, v/v), and the column was eluted with the same solvent mixture. The product fractions were collected and concentrated using a rotary evaporator to generate 1.3 g of a clear liquid (85%). ^{31}P NMR (CD_3CN) δ 124.4 (s); electron impact mass spectroscopy gave a molecular ion of 235 *m*/*e* with fragmentation loss of CH_2COOCH_3 at 162 *m*/*e*.

Synthesis of Acetic Acid, [*O*-Ethyl(*N*,*N*-diisopropylamino)phosphino]methyl Ester (8b). Compound **7a** (2.0 g, 6.5 mmol) was placed in a 100 mL round-bottom flask and dissolved in anhydrous acetonitrile (50 mL). Anhydrous ethanol (300 mg, 6.5 mmol) and tetrazole (360 mg, 5.2 mmol) were added, and the flask was stirred for 24 h. The reaction mixture was neutralized by the addition of 5 mL of triethylamine and concentrated to an oil using a rotary evaporator. The product was isolated as a clear liquid (1.4 g, 86%) using the same procedure as that followed for **8a**. ^{31}P NMR (CD_3CN) δ 124.2 (s); electron impact mass spectroscopy gave a molecular ion of 249 *m*/*e* with a fragmentation loss of CH_2COOCH_3 at 176 *m*/*e*.

General Procedure for Synthesis of Protected Deoxynucleoside Acetic Acid Phosphinoamidites. Protected deoxynucleoside acetic acid phosphinoamidites were synthesized by the following general procedure. Protected deoxynucleosides were dissolved in anhydrous dichloromethane at 0.05−0.1 M, depending upon their solubility, and 1.2 molar equiv of the appropriate acetic acid phosphinodiamidite were added with stirring. Upon complete dissolution of the reaction components, 0.8 molar equiv of tetrazole was added to the reaction mixture as a 0.45 M solution in anhydrous acetonitrile. The reaction mixture was allowed to stir for 24 h at room temperature and then analyzed for the extent of the reaction by ^{31}P NMR and silica gel TLC (eluted with ethyl acetate). The reaction was determined to be complete by spot to spot conversion to a faster eluting product on TLC and by a complete loss of the acetic acid phosphinodiamidite ^{31}P NMR signal. If incomplete, the reaction was allowed to stir for another 24 h. Upon completion, the reaction was quenched by the addition of 0.8 molar equiv of anhydrous triethylamine. After 5 min, the reaction mixture was concentrated to a viscous oil in vacuo using a rotary evaporator. The viscous oil was redissolved in a minimum volume of ethyl acetate and was added to the top of a silica gel column preequilibrated with various mixtures of ethyl acetate/hexanes as outlined in each preparation. Isocratic elution of the column with appropriate solvents for each preparation (monitored by TLC) was used to collect the product. Fractions containing the product were combined and concentrated to a foam in vacuo on a rotary evaporator, redissolved in a minimal amount of anhydrous dichloromethane, and added dropwise to rapidly stirring anhydrous hexanes. The solid precipitate was isolated by filtration and dried overnight in vacuo. The resulting white solids were analyzed by ^{31}P NMR and FAB mass spectroscopy.

Synthesis of 3′-*O*-(Diisopropylamino)phosphinoacetic Acid Methyl Ester 5′-*O*-(4, 4′-Dimethoxytrityl)thymidine (9a). Compound **15a** (10 g, 18.4 mmol) was reacted with **7a** (5.6 g, 18.4 mmol) in the presence of tetrazole (1.03 g, 14.7 mmol) for 24 h. Following the workup as described in the general procedure, we isolated the product as a solid (11.55 g, 84% yield) from a silica gel column, which was eluted with a solvent mixture of ethyl acetate/hexanes (50:50, v/v). ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 120.6 and δ 120.8 ppm). FAB Mass Spectroscopy FAB$^+$ 748 *m*/*e* (m + 1), FAB$^-$ 746 *m*/*e* (m − 1).

Synthesis of 3′-*O*-(Diisopropylamino)phosphinoacetic Acid α,α-Dimethyl-*β*-cyanoethyl Ester 5′-*O*-(4, 4′-Dimethoxytrityl)thymidine (16a or 9c). Compound **15a** (10 g, 18.4 mmol) was reacted with **14** (6.8 g, 18.4 mmol) in the presence of tetrazole (1.03 g, 14.7 mmol) for 48 h. Following the workup described in the general procedure, we isolated the product as a solid (12.88 g, 86% yield) from a silica gel column eluted with ethyl acetate. ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 120.3 and δ 120.8 ppm). FAB mass spectroscopy FAB$^+$ 815 *m*/*e* (m + 1), FAB$^-$ 813 *m*/*e* (m − 1). Accurate mass measurement for $C_{44}H_{55}N_4O_9P$, M + H: calculated, 815.3785; found, 815.3775.

Synthesis of 3′-*O*-(Diisopropylamino)phosphinoacetic Acid α,α-Dimethyl-*β*-cyanoethyl Ester 5′-*O*-(4,4′-Dimethoxytrityl)-*N*-4-acetyl-2′-deoxycytidine (16b). Compound **15b** (10 g, 17.5 mmol) was reacted with **14** (6.5 g, 17.5 mmol) in the presence of tetrazole (0.98 g, 14.0 mmol) for 24 h. Following the workup described in the general procedure, we isolated the product as a solid (11.48 g, 78% yield) from a silica gel column eluted with ethyl acetate/hexanes (50:50). ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 121.3 and δ 121.8 ppm). FAB mass spectroscopy FAB$^+$ 842 *m*/*e* (m + 1), FAB$^-$ 840 *m*/*e* (m − 1). Accurate mass measurement for $C_{45}H_{56}N_5O_9P$, M + H: calculated, 842.3894; found, 842.3914.

Synthesis of 3′-*O*-(Diisopropylamino)phosphinoacetic Acid α,α-Dimethyl-*β*-cyanoethyl Ester 5′-*O*-(4,4′-Dimethoxytrityl)-*N*-6-benzoyl-2′-deoxyadenosine (16c). Compound **15c** (10 g, 15.3 mmol) was reacted with **14** (5.7 g, 15.3 mmol) in the presence of tetrazole (0.86 g, 12.24 mmol) for 24 h. Following the workup described in the general procedure, we isolated the product as a solid (10.64 g, 75% yield) from a silica gel column eluted with ethyl acetate. ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 120.8 and δ 121.6 ppm). FAB mass spectroscopy FAB$^+$ 928 *m*/*e* (m + 1), FAB$^-$ 926 *m*/*e* (m − 1). Accurate mass measurement for $C_{51}H_{58}N_7O_8P$, M + H: calculated, 928.4163; found, 928.4159.

Synthesis of 3′-*O*-(Diisopropylamino)phosphinoacetic Acid α,α-Dimethyl-*β*-cyanoethyl Ester 5′-*O*-(4,4′-Dimethoxytrityl)-*N*-2-isobutyryl-2′-deoxyguanosine (16d). Compound **15d** (10 g, 15.7 mmol) was reacted with **14** (5.8 g, 15.7 mmol) in the presence of tetrazole (0.88 g, 12.56 mmol) for 24 h. Following the workup as described in the general procedure, we isolated the product as a solid (12.70 g, 89% yield) from a silica gel column eluted with ethyl acetate. ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 121.7 and δ 122.0 ppm). FAB mass spectroscopy FAB$^+$ 910 *m*/*e* (m + 1), FAB$^-$ 908 *m*/*e* (m − 1). Accurate mass measurement for $C_{48}H_{60}N_7O_9P$, M + H: calculated, 910.4268; found, 910.4235.

Synthesis of Thymidylyl-(3′-5′)-Thymidine Phosphonoacetic Acid Methyl Ester (12a). Compounds **9a** (133 mg, 0.18 mmol) and **10** (120 mg, 0.28 mmol) were dissolved in anhydrous acetonitrile (0.4 mL). Tetrazole (53 mg, 0.72 mmol) in 1.6 mL of anhydrous acetonitrile was transferred via syringe to the reaction mixture. Completion of the synthesis of **11a** was marked by the quantitative conversion of **9a** to **11a**, as monitored by ^{31}P NMR and silica gel TLC (methanol/dichloromethane, 1:9). Following the completion of the internucleotide coupling reaction, 100 mM CSO in anhydrous acetonitrile (20 equiv) was added. The oxidation was allowed to proceed until complete conversion to the phosphonoacetate (**12a**) was observed by ^{31}P NMR and silica gel TLC (methanol/dichloromethane, 1:9). The reaction mixture was then concentrated to dryness in vacuo, dissolved in 0.5 mL of acetonitrile and 0.5 mL of water. The product was isolated by preparative HPLC, utilizing a 25 cm Zorbax 300SB, C-18 column with a 9.4 mm ID. Gradient eluents were 100 mM triethylammonium acetate at pH 8.0 (A) and acetonitrile (B). The following gradient conditions were used to elute the trityl-on dimers: 0−2 min, 40% B; 2−52 min, 40−80% B; and finally 52−58 min, 80−100% B at a flow rate of 1.2 mL/min. The product containing fractions were collected and concentrated in vacuo, giving 138 mg of purified product (81% yield). ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 22.1 and δ 21.3). FAB mass spectroscopy FAB$^+$ 948 *m*/*e* (m + 1).

Synthesis Thymidylyl-(3′-5′)-Thymidine Thiophosphonoacetic Acid Methyl Ester (13a). Compound **11a** was synthesized as described in the previous section. It was converted to **13a** using 50 mM BDT in anhydrous acetonitrile (20 equiv). The reaction was allowed to proceed until the complete conversion to **13a** was observed by ^{31}P NMR and silica gel TLC (methanol/dicholormethane, 1:9). Preparative HPLC was performed as summarized for the isolation of **12a**. The product

containing fractions were collected and concentrated in vacuo giving 149 mg of purified product (86% yield). ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 86.3 and δ 85.9). FAB mass spectroscopy FAB$^+$ 964 *m*/*e* (m + 1).

Synthesis of Thymidylyl-(3′-5′)-Thymidine Phosphonoacetic Acid α,α-Dimethyl-β-cyanoethyl Ester (12c). Compound **12c** was prepared in an 84% isolated yield using the procedure outlined for the synthesis of **12a**. ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 21.1 and δ 20.0). FAB mass spectroscopy FAB$^+$ 1015 *m*/*e* (m + 1).

Synthesis of Thymidylyl-(3′-5′)-Thymidine Thiophosphonoacetic Acid α,α-Dimethyl-β-cyanoethyl Ester (13c). Compound **13c** was prepared in an 87% isolated yield using the procedure outlined for the synthesis of **13a**. ^{31}P NMR (CD_3CN) gave a set of diastereomers (δ 85.4 and δ 85.1). FAB mass spectroscopy FAB$^+$ 1031 *m*/*e* (m + 1).

Synthesis of Phosphonoacetate Oligodeoxynucleotides. The chemical synthesis of phosphonoacetate oligodeoxynucleotides was accomplished using an ABI model 394 automated DNA synthesizer. The synthesis cycle was adapted from a standard procedure commonly used to prepare DNA according to the phosphoramidite method.[41] The oxidation step was accomplished prior to the capping step. Compounds **16a**−**d** were dissolved in anhydrous acetonitrile at 100 mM and placed on the appropriate ports of the synthesizer. The activator was freshly sublimed tetrazole dissolved in anhydrous acetonitrile at 0.45 M. The coupling wait time was increased to 33 min. Trichloroacetic acid (3%, w/v) was dissolved in anhydrous dichloromethane and used to deprotect the DMT group prior to each addition of **16a**−**d**. Blocking of unreacted oligomers after each synthesis step was accomplished with a two part capping solution (cap A and cap B). Cap A was 10% acetic anhydride in anhydrous tetrahydrofuran, and cap B was 0.625% (w/v) DMAP in anhydrous pyridine. Oxidation of the nascent internucleotide acetic acid phosphonite to the phosphonate was accomplished using CSO (100 mM) dissolved in anhydrous acetonitrile. The oxidation wait time was 3 min.

Postsynthesis, the CPG was washed with anhydrous acetonitrile for 60 s and then flushed with a stream of argon until dry. The CPG was removed from the column and placed in a 1.5 mL screw-cap, conical glass reaction vial. A 1.5% DBU solution in anhydrous acetonitrile (15 μL in 1 mL of anhydrous acetonitrile) was added, and the vial was sealed using a Teflon-lined, silicon septum under the screw-cap. The vial was vortexed to stir the contents, and the reaction to remove the dimethylcyanoethyl groups was allowed to proceed for 60 min. The DBU solution was removed using a glass pipet, and the CPG was washed with three 1 mL aliquots of anhydrous acetonitrile. The phosphonoacetate oligodeoxynucleotide was cleaved from the CPG, and the exocyclic amine protecting groups were removed using 40% methylamine in water. Deprotection and cleavage were carried out at 55 °C for 15 min. The vial's contents were cooled to room temperature, and the solution was transferred to a 1.5 mL Eppendorf tube and evaporated to dryness in vacuo. The oligodeoxynucleotide product was redissolved in water and purified by HPLC.

Synthesis of Thiophosphonoacetate Oligodeoxynucleotides. The preparation of thiophosphonoacetate oligodeoxynucleotides was accomplished as described for the synthesis of phosphonoacetate oligodeoxynucleotides. Oxidation of the nascent internucleotide acetic acid phosphonite to the thiophosphonate was accomplished using 50 mM BDT in anhydrous acetonitrile. The oxidation wait time was 60 s. Postsynthesis, the CPG was washed with anhydrous acetonitrile for 60 s and then flushed with a stream of argon until dry. The CPG was removed from the column and placed in a 1.5 mL screw-cap, conical glass reaction vial. A 1.5% DBU solution in anhydrous acetonitrile (15 μL in 1 mL of anhydrous acetonitrile) was added, and the vial was sealed using a Teflon-lined, silicon septum under the screw-cap. The vial was vortexed to stir the contents, and the reaction to remove the dimethylcyanoethyl groups was allowed to proceed for 60 min. The DBU solution was removed using a glass pipet, and the CPG was washed with three 1 mL aliquots of anhydrous acetonitrile. The phosphonoacetate oligodeoxynucleotide was cleaved from the CPG, and the exocyclic amine protecting groups were removed using 40% methylamine in water. Deprotection and cleavage were carried out at 55 °C for 15 min. The vial's contents were cooled to room temperature, and the solution was transferred to a 1.5 mL Eppendorf tube and evaporated to dryness in vacuo. The oligodeoxynucleotide product was redissolved in water and purified by HPLC.

HPLC Purification of Phosphonoacetate and Thiophosphonoacetate Oligodeoxynucleotides. Phosphonoacetate and thiophosphonoacetate oligodeoxynucleotides were purified by reverse-phase HPLC using the DMT protecting group for hydrophobic affinity. The crude oligodeoxynucleotide mixture was dissolved in 0.5 mL of water for purification by HPLC. Preparative HPLC utilized a 25 cm Zorbax 300SB-C18 column with a 9.4 mm ID. Gradient eluents were 100 mM triethylammonium acetate pH 8.0 (A) and acetonitrile (B). The following gradient conditions were used to elute the trityl-on oligodeoxynucleotides: 0−2 min, 8% B; 2−27min, 8 to 20% B; and finally 27−52 min, 20 to 80% B at a flow rate of 1.2 mL/min. The desired fractions were collected, concentrated under vacuum, and dissolved in 100 μL of 10 mM Tris-HCl (pH 8.0). Phosphonoacetate oligodeoxynucleotide 18mer gave 417 nmol of DMT-on product (42% yield). Thiophosphonoacetate oligodeoxynucleotide 18mer gave 461 nmol of DMT-on product (46% yield). The trityl group was removed by treatment with 80% acetic acid (1 mL). After 1 h, the solution was concentrated to dryness, dissolved in 0.5 mL of 50mM triethylammonium acetate (pH 8), and reapplied to the 25 cm Zorbax 300SB-C18 column to isolate the DMT-off oligodeoxynucleotide product. Phosphonoacetate oligodeoxynucleotide 18mer gave 329 nmol of DMT-off products (33% yield). Thiophosphonoacetate oligodeoxynucleotide 18mer gave 322 nmol of DMT-off product (32% yield). The elution profile was as follows: 0% B for 30 min followed by a gradient of 0 to 80% B from 30 to 50 min at a flow rate of 1.2 mL/min.

5′-Fluorescein Oligodeoxynucleotides and Oligoribonucleotides. 5′-Fluorescein labeled oligodeoxynucleotides and phosphorothioate oligodeoxynucleotides were synthesized using standard procedures.[41] 5′-Fluorescein labeled phosphonoacetate and thiophosphonoacetate oligodeoxynucleotides were synthesized as described previously using the chemistry outlined in Scheme 6. The 5′-fluorescein was chemically added to these analogues using standard procedures.[41] These oligomers were purified by reverse-phase HPLC as described in the previous section. The deoxynucleotide sequence for these ODNs was 5′-GAGTGATCTATG-3′. The 5′-fluorescein oligoribonucleotide had the complementary sequence 5′-CAUAGAUCACUC-3′ and was obtained from Dharmacon Research, Inc. (Lafayette, CO).

Ion-Exchange HPLC Analysis of Phosphonacetate and Thiophosphonoacetate Oligodeoxynucleotides. Oligodeoxynucleotide mixtures were analyzed for the yield of full-length product by ion-exchange HPLC. The HPLC was performed using a 1 mL Resource-Q column (30 cm × 6.4 mm ID). Gradient eluents were (A) 10 mM NaOH/80 mM NaBr and (B) 10 mM NaOH/1.5 M NaBr. The gradient was 0% A to 100% B in 45 min at a flow rate of 1.5 mL/min.

MALDI-TOF Mass Spectroscopy. Analysis was performed on a Perseptive Biosystems Voyager Biospectrometry Work Station. The acetic acid modified oligodeoxynucleotides were concentrated to dryness and dissolved in 2-propanol/water (1:1) to a final concentration of 200 pmol/μL. Samples were prepared as described in the Sequazyme Oligonucleotide Sequencing Kit (Perseptive Biosystems) with the following modifications: 1μL of oligodeoxynucleotide, 1μL 25mer DNA standard, 1μL of ammonium citrate buffer, and 7μL of matrix were combined on a layer of Parafilm coated with ammonium cation exchange beads. After pipetting the solution over the beads for 60 s, we transferred 5μL to a gold-plated 100 well plate. The MALDI-TOF measurements were observed in the positive ion mode.

^{31}P NMR of Phosphonoacetate and Thiophosphonoacetate Oligodeoxynucleotides. Oligodeoxynucleotides obtained following the trityl-off, reverse-phase purification step were purified for ^{31}P NMR

characterization using preparative ion exchange HPLC on a 1 mL Resource-Q column with buffers and gradients as described for ion-exchange HPLC analysis. The desired fractions were collected, and the pH was adjusted with 80% acetic acid to approximately pH 7. Purified oligodeoxynucleotides were evaporated to dryness and redissolved in D_2O for ^{31}P NMR analysis of the phosphorus backbone using a Varian VXR-300 broadband NMR.

Stability Studies of Chimerical Oligodeoxynucleotides toward Methylamine. Chimerical dT_{15} oligodeoxynucleotides were synthesized in which the eighth internucleotide linkage from the 5′ end was either phosphonoacetate or thiophosphonoacetate. These oligomers were 5′ end labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase. The labeled oligodeoxynucleotides were gel purified and ethanol precipitated. Mixtures of ^{32}P 5′-labeled oligodeoxynucleotide (1 000 000 cpm/reaction) and unlabeled chimerical dT_{15} oligodeoxynucleotide (20 pmol) in 1 μL were diluted with 99 μL of 40% aqueous methylamine. The reaction mixtures were incubated at 55 °C. At each time point, an aliquot was removed from the reaction tube and quickly cooled in a dry ice bath and the methylamine was removed in vacuo. Each dried sample was resuspended in 10 μL of Tris-HCl (pH 8.03) and diluted with 20 μL of 80% formamide containing 50 mM EDTA. The reaction products were resolved by denaturing PAGE (20%, 19:1 cross-linking, 7 M urea) and quantitated by autoradiography.

Exonuclease Digestion. Exonuclease digestion experiments were carried out as described by Cummins[5] using phosphodiesterase I from *Crotalus Adamanteus* venom. The assays were performed using 1 μM Tris-HCl (pH 8.5) and 14 mM $MgCl_2$. The enzyme concentration was 0.05 units/mL. Aliquots for analysis were removed at 15, 30, 60, 180, 360, and 1080 min. These reactions were terminated by dilution with an equal volume of 80% formamide gel loading buffer containing tracking dyes and then placed immediately on dry ice. The reaction mixture aliquots were analyzed using polyacrylamide gel electrophoresis (20%, 19:1 cross-linking, 7 M urea), and the gels were analyzed using a Molecular Dynamics Typhoon Phosphorimager.

Hydrolysis of RNA Heteroduplexes with *E. coli* RNase H1. RNase H1 experiments were performed with *E. coli* RNase H1 using the conditions described by Hogrefe et al.[45] The reactions were carried out using an assay buffer of 50 mM tris-HCl (pH 8.0), 20 mM KCl, 9 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 250 μg/mL bovine serum albumin. An oligodeoxynucleotide or modified oligodeoxynucleotide (200 pmol) and 5′-fluorescein labeled complementary oligoribonucleotide were added to the assay buffer (35 μL). Following the addition of *E. coli* RNase H1 (3 units), reaction mixtures were placed in a circulating water bath at 18 °C over 12 h. The reaction mixtures were then diluted with an equal volume of 80% formamide gel loading buffer containing tracking dyes and analyzed by polyacrylamide gel electrophoresis (20%, 19:1 cross-linking, 7 M urea). The developed gels were analyzed using a Molecular Dynamics Typhoon Phosphorimager.

Acknowledgment. We thank Dr. R. Barkley for the mass spectral analysis and Brian Stell and Christina Tucker for technical assistance. This research was supported by the National Institutes of Health (Grant GM25680), the University of Colorado, and MetaSense Technologies.

JA027983F

(45) Hogrefe, H. H.; Hogrefe, R. I.; Walder, R. Y.; Walder, J. A. *J. Biol. Chem.* **1990**, *265*, 5561−5566.