5′-Dithiophosphoryl Deoxyoligonucleotides: Synthesis and Biological Studies[1]

Peter H. Seeberger,[‡] Pia N. Jorgensen,[§] Danute M. Bankaitis-Davis,[⊥] Graham Beaton,[⊥] and Marvin H. Caruthers*[,†]

Contribution from the Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215, and Amgen, Inc., Boulder, Colorado 80301

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Abstract: The first synthesis of 5′-dithiophosphoryl deoxyoligonucleotides is reported. These oligomers are prepared by reacting *O*-(9-fluorenemethyl) *H*-phosphonothioate with an appropriately protected deoxyoligonucleotide and then oxidizing phosphorus with elementary sulfur. By using ^{35}S-sulfur, a long-lived radioactive isotope that is useful for monitoring biological and biochemical experiments can be introduced into deoxyoligonucleotides. 5′-Dithiophosphoryl deoxyoligonucleotides are stable toward alkaline phosphatase and do not serve as substrates for T4-polynucleotide ligase. These oligomers undergo oxidative phosphoryl disulfide formation to yield dimers via an intermolecular reaction or with cysteine to generate an amino acid-deoxyoligonucleotide joined through the 5′-dithiophosphoryl moiety and the cysteine sulfhydryl function.

Over the past several years, nucleoside and polynucleotide thiophosphates have proven to be valuable analogs for studying many biological and biochemical processes. Most significantly perhaps was the use of nucleoside thiophosphates to elucidate the mechanisms whereby several enzymes catalyze phosphoryl transfer reactions.[2] Similarly, oligonucleotide and deoxyoligonucleotide thiophosphates have proven to be very useful for examining the biochemistry and structure of ribozymes[3] and for controlling gene expression via antisense mechanisms.[4] Recently, research in this field has been further extended by developing methods for synthesizing deoxyoligonucleotides and oligonucleotides having internucleotide linkages where two nonbridging oxygens are replaced by sulfur.[5,6] Although biochemical studies with this class of compounds are only beginning, many interesting and potentially useful results have already been reported. These include observations that deoxyoligonucleotide dithiophosphates activate RNase H *in vitro*,[7] strongly inhibit human immunodeficiency virus (HIV) reverse transcriptase,[8] alter the binding of proteins to polynucleotide duplexes,[9] and are completely resistant to hydrolysis by various nucleases.[6,7,10−12]

As part of a study on the synthesis of biomolecules containing phosphate analogs, we recently reported the solution phase synthesis and initial biochemical studies of the deoxynucleoside 5′- and 3′-dithiophosphates.[13] In these studies, 9-fluorenemethanol was developed as an oxygen protecting group for the synthesis of this new class of monoesters. While these analogs showed moderate inhibition of avian myeloblastosis virus reverse transcriptase, deoxynucleoside dithiophosphates failed to inhibit HIV reverse transcriptase, T4-polynucleotide kinase, bacterial alkaline phosphatase, and the Klenow fragment of *E. coli* DNA polymerase. The dithiophosphate deoxynucleosides were also completely stable to phosphorolysis by bacterial alkaline phosphatase, and the 3′-derivatives were not substrates for T4 polynucleotide kinase.

Here we describe our initial investigations on the synthesis and potential biochemical utility of 5′-dithiophosphoryl deoxyoligonucleotides. In addition, we report the synthesis, via oxidation of the 5′-dithiophosphoryl moiety, of deoxyoligonucleotide dimers and of cysteine linked to deoxyoligonucleotides.

Results and Discussion

Synthesis of *O*-(9-Fluorenemethyl)-*H*-phosphonothioate. The successful synthesis of deoxynucleoside 3′- and 5′-phosphorodithioates[13,14] is based upon the synthesis scheme summarized in Figure 1. Thus, the method involves first condensation of tris-pyrrolidinophosphine with the appropriately protected deoxynucleoside followed by conversion to the H-phosphonodithioate with hydrogen sulfide. Oxidative coupling of 9-fluorenemethanol to **3** with iodine and deprotection

* To whom correspondence should be addressed.
† University of Colorado.
‡ Current address: Sloan-Kettering Institute for Cancer Research, Box 106, 1275 York Avenue, New York, NY 10021.
§ Current address: Department of Chemistry, University of Odense, Campusvej 55, DK-5230 Odense M, Denmark.
⊥ Current address: Amgen Inc., Boulder, CO 80301.
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(1) This is paper 46 in a series on nucleotide chemistry. Paper 45 is Kofoed, T.; Caruthers, M. H., *Tetrahedron Lett.* **1996** in press. This research was supported by the National Institutes of Health (Grant GM25680) and Amgen Inc. Abbreviations: T, thymine; TLC, thin layer chromatography; PAGE, polyacrylamide gel electrophoresis; TEAB, triethylammonium bicarbonate; HPLC, high performance liquid chromatography; DTT, dithiothreitol; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF, *N*,*N*-dimethylformamide; Fmol, 9-fluorenemethanol, DPCP, diphenylchlorophosphate; DMT, dimethoxytrityl; CPG, controlled pore glass.

(2) Eckstein, F. *Ann. Rev. Biochem.* **1985** , *54*, 367.
(3) (a) Herschlag, D.; Piccirilli, J. A.; Cech, T. R. *Biochemistry* **1991** *30*, 4844; (b) Piccirilli, J. A., Vyle, J. S.; Caruthers, M. H.; Cech, T. R. *Nature* **1993**, *361*, 85.
(4) Stein, C. A.; Cohen, J. S. Phosphorothioate Oligonucleotide Analogues in Oligonucleotides: Antisense Inhibitors of Gene Expression; Cohen, J. S., Ed.; Macmillan Press: London, 1989, p 97.
(5) Dahl, O. *Sulfur Reports* **1991**, *11*, 167.
(6) Okruszek, A.; Sierzchala, A.; Fearon, K. L.; Stec, W. J. *J. Org. Chem.* **1995**, *60*, 6998.
(7) Cummins, L.; Graff, D.; Beaton, G.; Marshall, W. S.; Caruthers, M. H. *Biochemistry* **1996,** *35*, 8734.
(8) Marshall, W. S.; Caruthers, M. H. *Science* **1993**, *259*, 1564.
(9) Caruthers, M. H.; Beaton, G.; Graff, D. A.; Hall, M. J.; Marshall, W. S.; Wiesler, W. T. *Structural Biology: The State of the Art*; Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: 1994; pp 191−198.
(10) Nielsen, J.; Brill, W. K.-D.; Caruthers, M. H. *Tetrahedron Lett.***1988**, *29*, 2911.
(11) Grandas, A.; Marshall, W. S.; Nielsen, J.; Caruthers, M. H. *Tetrahedron Lett* **1989**, *30*, 543.
(12) Porritt, G. M.; Reese, C. B. *Tetrahedron Lett.* ***1989***, *30*, 4713.
(13) Seeberger, P. H.; Yau, E.; Caruthers, M. H. *J. Am. Chem. Soc.* **1995**, 117, 1472.
(14) Seeberger, P. H.; Caruthers, M. H. *Tetrahedron Lett.* **1995**, *36*, 695.

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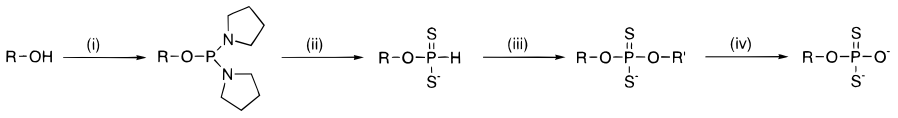

Figure 1. Oxidative formation of deoxynucleoside phosphorodithioates: (i) trispyrrolidinophosphine; (ii) H_2S + tetrazole; (iii) 1 eq 0.1 M iodine/pyridine + R′-OH; and (iv) NH_4OH. R-OH, appropriately protected deoxynucleoside; R′-OH, 9-fluorenemethanol.

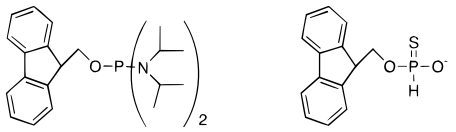

Figure 2. Synthons for the solid phase synthesis of 5′-dithiophosphoryl deoxyoligonucleotides.

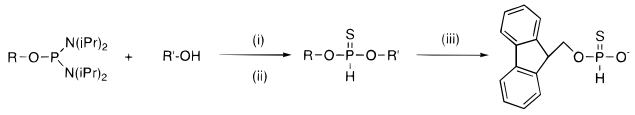

Figure 3. Synthesis of *O*-(9-fluorenemethyl) H-phosphonothioate: (i) tetrazole; (ii) H_2S + tetrazole; and (iii) 2-carbamoyl-2-cyanoethylene-1,1-dithiolate. R, CH_3; R′-OH, Fmol.

using ammonium hydroxide generates the final product (**5**). This method cannot easily be applied to the synthesis of 5′-dithiophosphoryl deoxyoligonucleotides as the yields of intermediates (5′-H-phosphonodithioate, **3**, and protected 5′-phosphorodithoate, **4**) are low, and side products including the 5′-phosphorothioate and 5′-phosphate are produced in considerable amounts. Thus on solid supports, this method fails because of the low yields for each of the synthetic steps shown in Scheme 1 and the generation of side products that cannot easily be separated from the desired deoxyoligonucleotide.

As a consequence of these considerations, we chose to investigate synthons **6** and **7** for the preparation of 5′-dithiophosphoryl deoxyoligonucleotides (Figure 2). Our expectation was that these synthons would generate higher yields and fewer side products. The synthesis of **6** proved to be especially difficult. Although ^{31}P-NMR analysis of the reaction mixture generated by condensing bis(diisopropylamino)chlorophosphine with 9-fluorenemethanol indicated a very high yield of **6**, all isolation attempts failed due to its instability during aqueous workup and column chromatography. Because nucleoside H-phosphonothioates have been used to successfully synthesize dithioate DNA on solid supports,[5] we next turned to **7**, where Fmol substitutes for the deoxynucleoside in this structure, as a potential synthon. The choice of Fmol as the transient blocking group was based upon our earlier work where Fmol protected deoxynucleoside 5′- or 3′-phosphorodithioates were shown to be readily converted via ammonium hydroxide treatment to the dithiophosphoryl deoxynucleosides.[13] Thus the overall plan, as outlined in Figures 3 and 4, was to prepare **7** and use it as a synthon for generating 5′-dithiophosphoryl deoxyoligonucleotides.

Synthesis of **7** begins by condensing bis(diisopropylamino)-methoxyphosphine with 1 equiv of 9-fluorenemethanol using 0.5 equiv tetrazole as activator (Figure 3). Further treatment of the reaction mixture with hydrogen sulfide followed by flash silica column chromatography yields the H-phosphonothioate diester **10**. The choice of R was limited because removal of Fmol is very sensitive to basic conditions, and the H-phosphonothioate diester is labile to acid. Of the various R groups examined, methyl proved to be most useful as it can be removed from **10** using the sodium salt of 2-carbamoyl-2-cyanoethylene-1,1-dithiolate.[15] Even with this reagent, the reaction is not entirely selective as 20% cleavage of Fmol occurs. Purification of **7** involves several steps: two sequential silica gel columns followed by either reverse phase HPLC or crystallization from water. This extensive purification scheme is necessary in order to completely remove the dithiolate salt.

Synthesis of 5′-*O*-(dithiophosphoryl)deoxyoligonucleotides. After assembly of a deoxyoligonucleotide (modified or unmodified) on a solid support using standard procedures[16] and removal of the 5′-dimethoxytrityl group with acid, **7** is joined to the 5′-hydroxyl of the deoxyoligonucleotide using DPCP as an activator (Figure 4). Following this coupling step, the thiophosphite is sulfurized with elemental sulfur in carbon disulfide/pyridine containing 5% triethylamine. Inclusion of 5% triethylamine in the sulfurization mixture reduces the reaction time significantly and does not cause cleavage of Fmol. Because thiophosphate DNA having ^{35}S-sulfur at internucleotide positions has proven to be extremely useful for many biological and pharmacological experiments,[17] we investigated the labeling of 5′-dithiophosphate deoxyoligonucleotides with this isotope. This was accomplished successfully using ^{35}S-sulfur as the oxidant (see Experimental Section). The specific activity was determined to be 4.76×10^8 cpm/μmol which is comparable (4.9×10^8 cpm/μmol) to results obtained through labeling the backbone of phosphorothioate deoxyoligonucleotides with ^{35}S-sulfur at multiple sites.[17]

Following oxidation, the Fmol protecting group is removed with DBU (0.1 M in acetonitrile) rather than 20% piperidine or concentrated ammonium hydroxide[13,14] as fewer side products and far less desulfurization is observed with this reagent (^{31}P-NMR analysis). A convenient method for measuring the progress of Fmol cleavage is to monitor the liberated dibenzofulvene by UV absorption at 294 and 304 nm. Under these basic conditions with DBU, Fmol cleavage, as monitored by production of dibenzofulvene, has a half-life of under 1 min but in practice dithiophosphorylated deoxyoligonucleotides are treated for 10 min to assure complete deprotection. When the amount of dibenzofulvene released is compared to the deoxyoligonucleotide present on the solid support (measured by the generation of dimethoxytrityl cation), the coupling efficiency for dithiophosphorylation is greater than 95%. 5′-Dithiophosphoryl deoxyoligonucleotides are removed from the support, freed of protecting groups by standard treatment with concentrated ammonium hydroxide, and then purified by reverse-phase HPLC. Purification of radioactively labeled DNA is by denaturing, preparative PAGE followed by extraction and desalting on an appropriate column. Precipitation of the gel purified product in 0.3 M sodium acetate (pH 5.3) is not possible as desulfurization and loss of radioactive label occurs under these acidic conditions.

Biological Studies. Previous research demonstrated that deoxynucleoside dithiophosphates are stable toward alkaline

(15) Dahl, B.; Bjergårde, K.; Henriksen, L.; Dahl, O. *Acta. Chem. Scand.* **1990**, 44, 639.
(16) Caruthers, M. H. *Science* **1985**, *230*, 281.
(17) Stein, C. A.; Iversen, P. L.; Subasinghe, S.; Cohen, J. S.; Stec, W. J.; Zon, G. *Analytical Biochemistry* **1990**, *188*, 11.

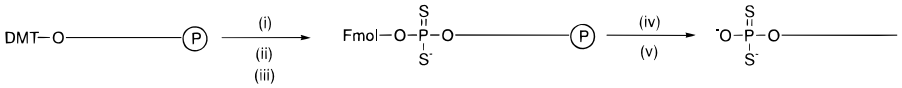

Figure 4. Synthesis of 5′-dithiophosphoryl deoxyoligonucleotide: (i) dichloroacetic acid; (ii) compound **7** + DPCP; (iii) sulfur; (iv) DBU; and (v) NH_4OH. Abbreviations: an encircled P, solid support; —, deoxyoligonucleotide.

phosphatase.[13] In agreement with this work, alkaline phosphatase also fails to cleave a dithiophosphate moiety on the 5′-terminus of a deoxyoligonucleotide. As expected, calf spleen phosphodiesterase (a 5′−3′ exonuclease requiring a 5′-hydroxyl group) does not recognize 5′-dithiophosphoryl deoxyoligonucleotides as substrates. Snake venom phosphodiesterase (3′−5′ phosphodiesterase), however, completely degrades a 5′-dithiophosphorylated oligomer to 5′-mononucleotides at a rate comparable to that observed with natural DNA. T4-DNA ligase fails to link a 5′-dithiophosphoryl deoxythymidine decanucleotide to the 3′-hydroxyl of a deoxythymidine decanucleotide when dA_{20} is present as a template. These results are consistent with our earlier observations that enzymes fail to process 5′- or 3′-dithiophosphate deoxynucleosides[13] and suggest that ^{35}S-sulfur labeled 5′-dithiophosphoryl deoxyoligonucleotides may prove to be very useful for many biochemical studies not only as a result of their enzymic stability but also because oligomers labeled with ^{35}S-sulfur generate data that can be readily analyzed and interpreted by modern phosphorimager technology. ^{35}S-Sulfur tagged dithiophosphate oligomers could also prove useful for many *in vivo* biological experiments because this phosphoryl group should be stable to cellular phosphatases which is not the case for 5′-phosphate and 5′-thiophosphate containing oligomers. Because this is a chemical labeling method, it does not require any enzymes and thus can be used to tag a large number of polynucleotide analogs—even those that are not recognized by T4-kinase and ligase.

Dimerization and Oxidative Coupling. After storage of ^{35}S-labeled 5′-dithiophosphoryl deoxythymidine decanucleotide as a frozen solution in distilled water, analytical PAGE reveals two products. One corresponds to the decanucleotide, whereas the second has the mobility expected of an oligomer twice this size. This slower migrating product increases in relative intensity to 30% yield with prolonged storage (120 h) and disappears upon addition of DTT (Figure 5). No second, slower migrating product is observed when solutions are stored as liquids. These decamers must presumably aggregate in frozen solution and undergo coupling through the dithiophosphoryl moieties to form 20mers possessing a -OP(O)(S^-)SSP(O)(S^-)-*O*-bridge. Since 5′-thiophosphoryl deoxyoligonucleotides and nucleosides have been shown to undergo a similar coupling reaction in aqueous solution with oxidants such as KI_3 or $K_3Fe(CN)_6$,[18−20] various mild oxidizing agents were explored in attempts to enhance both the rate and yield of dimers from 5′-dithiophosphoryl deoxyoligonucleotides. These included aqueous solutions of 20% and 50% dimethyl sulfoxide[21] (10 mM Tris, pH 7.5, 24 h) and 0.7% hydrogen peroxide but none led to any detectable dimerization. However upon treatment of 5′-dithiophosphoryl deoxythymidine octanucleotide with 10 eq $K_3Fe(CN)_6$ in aqueous 10 mM Tris, pH 7.5, complete formation of dimer was observed after 10 min (HPLC and ^{31}P NMR analysis). These results compare favorably to the oxidation of terminal thiophosphoryl deoxyoligonucleotides under the same conditions where three hours were necessary in order to obtain 90% dimers.[20]

Oxidative formation of dithiophosphoryl dimers through the synthesis of a disulfide bridge suggests the possibility of using this reaction to conjugate cysteine to a dithiophosphate. When 10 equivalents of cysteine were added to a solution of 5′-[^{35}S]-dithiophosphoryl deoxythymidine decanucleotide, 15% cross-linking was observed after 72 h (HPLC and PAGE), and the reaction proceeds linearly with respect to time (Figure 6). Of particular interest was the observation that this reaction proceeds without added oxidant in aqueous solution at room temperature. This model study provides the basis for further research currently underway on the attachment of 5′-dithiophosphoryl deoxyoligonucleotides to cysteine containing peptides and proteins.

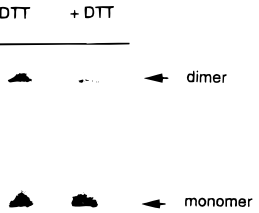

Figure 5. PAGE analysis of 5′-[^{35}S]dithiophosphoryl dT_{10} dimers. Samples were stored frozen as described in the experimental section. Aliquots were then analyzed in the presence (+ DTT) and absence (−DTT) of dithiothreitol.

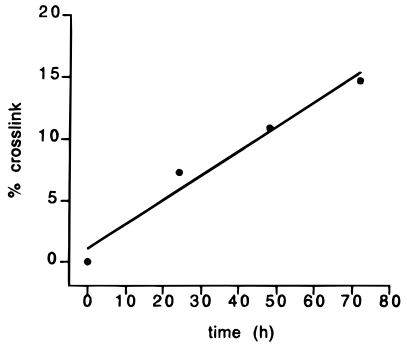

Figure 6. Time course for the reaction of cysteine with 5′-[^{35}S]-dithiophosphoryl dT_{10}.

Conclusion. This study describes the preparation of a synthon that facilitates the synthesis on solid supports of deoxyoligonucleotides having a 5′-dithiophosphoryl moiety. Because the synthetic scheme also can be used to incorporate radioactive sulfur into the dithiophosphate, the method provides synthetic access to oligomers containing a phosphatase stable, radioactive label. Therefore dithiophosphate monoesters joined to the 5′-end of DNA have great potential as phosphatase stable markers for monitoring the fate of deoxyoligonucleotides in nuclease rich environments such as cell culture media, cell extracts, and within cells or tissues. These methods should prove useful for the synthesis on solid supports of a large variety of dithiophosphorylated bioorganic molecules. These include deoxyoligonucleotides, oligonucleotides, sugars, and peptides that are potential inhibitors of protein phosphatases and protein kinases. It was found that deoxyoligonucleotide dithiophosphate monoesters undergo coupling to cysteine and dimerization by oxidative formation of a disulfide linkage. These observations suggest several additional potential applications including their use as sequence specific suicide inhibitors of DNA processing enzymes and as a new approach for delivering antisense DNA to cells when these oligomers are covalently joined via a reversible disulfide linkage to carrier proteins.

(18) Eckstein, F. *J. Am. Chem. Soc.* **1966**, *88*, 4292.
(19) Wu, T.; Orgel, L. E. *J. Mol. Evol.* **1991**, *32*, 274.
(20) Gryaznov, S. M.; Letsinger, R. L. *Nucleic Acids Res.* **1993**, *21*, 1403.

Experimental Section

Materials and Methods. 9-Fluorenemethanol, hydrogen sulfide, DBU, DPCP, sulfur, carbon disulfide and triethylamine were purchased from Aldrich Chemicals. Dry pyridine, dichloromethane, and acetonitrile (containing less than 0.005% water) were also purchased from Aldrich and used as supplied. ^{35}S-sulfur was obtained from Amersham Inc. (Arlington Heights, IL). Reagents for deoxyoligonucleotide synthesis such as appropriately protected deoxynucleoside phosphoramidites, tetrazole, and oxidation solutions were purchased from Glen Research (Sterling, VA). Ammonium hydroxide, acetic acid, methanol and *N*,*N*-dimethylformamide were obtained from Mallinckrodt. Dithiothreitol and L-cysteine were purchased from Sigma. A 0.5 M solution of 1*H*-tetrazole in dry acetonitrile was purchased from Applied Biosystems, Inc. Solid support syntheses were carried out on an Applied Biosystems, Inc. 380A or 394 automated DNA synthesizer.

^{1}H NMR spectra were recorded on a Varian VXR-300S and a General Electric GE 300 in the solvent indicated. ^{31}P NMR spectra were recorded on a Varian VXR-300S spectrometer operating at 121.4 Hz, a Bruker AM-400 spectrometer operating at 162.0 Hz, and a General Electric GE 300 spectrometer with broad band decoupling referenced to 85% H_3PO_4 as an external standard.

TLC was performed on Kieselgel 60 Platten (Merck Darmstadt) eluted with the solvent system indicated and examined by UV light. Reverse phase HPLC was performed with a Waters dual-pump 6000A system in combination with a Maxima 820 gradient control system and a Model 440 UV detector operating at 254 nm. Columns were packed with PRP-1 (5 μm, Hamilton) and eluted with a linear gradient of acetonitrile containing 50 mM triethylammonium bicarbonate (TEAB), pH 7.5. NAP 25 DEAE-Sephadex desalting columns were purchased from Pharmacia Biotech (Upsalla, Sweden).

Alkaline phosphatase from bovine brain, calf spleen phosphodiesterase, snake venom phosphodiesterase, and T4 ligase were obtained from Boehringer Mannheim. Quantitation of radioactivity analyzed by PAGE was achieved with a Molecular Dynamics PhosphorImager.

Synthesis of *O*-(9-Fluorenemethyl) *O*-(Methyl) *H*-Phosphonothioate (10). A 0.5 M solution (2.0 mL, 0.5 eq) of 1*H*-tetrazole in anhydrous acetonitrile was added over 5 min to a solution of 386 μL (2 mmol) bis(diisopropylamino)methoxyphosphine[22] and 392 mg (2 mmol) of 9-fluorenemethanol in 150 mL of anhhydrous dichloromethane. The solution was stirred for 1 h. A steady stream of H_2S was bubbled through the solution for 1 min and 20 mL (5 equiv) of 0.5 M tetrazole in acetonitrile was added after which H_2S addition continued for another 4 min. After stirring the solution under argon for 1 h, extraction with 5% $NaHCO_3$ (2 × 15 mL) was followed by drying over Na_2SO_4. The crude product was purified by flash silica column chromatography using dichloromethane as eluant. Yield: 420 mg (72.3%); TLC (CH_2Cl_2): R_f 0.92; ^{31}P NMR ($CDCL_3$): δ 73.32 ppm; ^{1}H NMR ($CDCl_3$): δ 3.65 (d, J = 14.4 Hz, 3H, *O*-CH_3), 4.44 (t, J = 12.2, 6.4 Hz, 1H, Fmol), 4.47 (q, J = 10.0, 6.2, 6.1, 4.8 Hz, 2H, Fmol), 7.36−7.82 (m, 8H, Fmol), 7.73 (d, J = 656.8 Hz, 1H, P-H); ^{13}C NMR ($CDCl_3$): δ 48.4 (Fmol), 52.5 (OCH_3), 68.2 (Fmol), 120.5 (Fmol), 125.5 (Fmol), 127.5 (Fmol), 128.4 (Fmol), 141.8 (Fmol), 143.5 (Fmol); MS (FAB$^+$; NOBA): 340 (15), 291 (M^+, 42), 179 (100); MS (FAB$^-$; NOBA): 289 (M^-, 20), 199 (40), 168 (30), 153 (100).

Synthesis of *O*-(9-Fluorenemethyl) *H*-phosphonothioate Sodium Salt (7). 10 (145 mg, 0.5 mmol) was dissolved in 1.0 mL of a 1 M solution of 2-carbamoyl-2-cyanoethylene-1,1-dithiolate in DMF (2 equiv). The mixture was stirred for 20 min, and DMF was removed by rotary evaporation yielding a yellow gum, which was purified by silica column flash chromatography [CH_2Cl_2, 1 L; CH_2Cl_2/MeOH (95/5), 2 L; CH_2Cl_2/MeOH (90/10), 2 L]. The product containing fractions were combined, the solvent was removed, and the mixture was further purified by rechromatography on a silica gel column as described above. The crude product was crystallized from water (50 mg/1 mL H_2O) to remove residual dithiolate salt. Yield: 94.4 mg (61.4%); TLC (CH_2Cl_2): R_f 0.11; ^{31}P NMR (D_2O): δ 57.70 ppm; ^{1}H NMR (acetone-d_6): δ, 4.15−4.29 (m, 3H, Fmol), 7.20−7.75 (m, 8H, Fmol), 7.91 (d, J = 585 Hz, 1H, P-H); ^{13}C NMR (acetone-d_6): δ, 48.8 (CH(Fmol)), 66.7 (CH_2(Fmol)), 120.3 (C4), 126.2 (C1), 127.5 (C2), 128.0 (C3), 141.7 (C12, C13), 145.1 (C10, C11); MS (FAB$^+$; NOBA): 273 (M^+, 10), 154 (100); MS (FAB$^-$; NOBA): 275 (M^-, 10), 199 (40), 153 (100).

Synthesis of 5′-Dithiophosphoryl Deoxyoligonucleotides. Deoxyoligonucleotides were assembled on CPG supports using commercially available reagents for phosphoramidite chemistry and standard synthesis cycles. The terminal 5′-DMT protecting group was removed in the usual manner following deoxyoligonucleotide synthesis, and the column was washed with dichloromethane and dried. After washing the column with acetonitrile/pyridine (1/1, v/v) for 30 s, a 0.1 M solution of **7** in acetonitrile/pyridine (95/5, v/v) and the activator solution (0.1 M DPCP in acetonitrile/pyridine (95/5, v/v)) were delivered simultaneously to the column and remained in contact with the CPG support for 5 min after which the column was washed with dichloromethane/pyridine (1/1, v/v). Delivery, wait, and wash were repeated. Sulfurization with 1.56 M sulfur in carbon disulfide/pyridine/triethylamine (95/95/10; v/v/v) for 1 h was followed by extensive washes with carbon disulfide and dichloromethane. The 9-fluorenemethanol protecting group was removed by suspending the solid support in 1 mL of a 0.1 M solution of DBU in acetonitrile for 10 min. In order to quantitate coupling efficiency, 100 μL of the deprotection solution was diluted with 900 μL acetonitrile, and the UV absorbance at 304 nm was determined. After washing with diethyl ether, the deoxyoligonucleotide was removed from the support by concentrated ammonium hydroxide for 30 min. Nonradioactive samples were purified by reverse phase HPLC with a linear gradient of 0−40% acetonitrile in 50 mM TEAB buffer.

Synthesis of ^{35}S-Labeled 5′-Dithiophosphoryl Decathymidine. The decathymidine deoxyoligonucleotide was assembled using commercially available reagents for phosphoramidite chemistry and standard synthesis cycles on a CPG support containing 1 μmol deoxynucleoside. The terminal 5′-DMT protecting group was removed, and the synthesis column was washed with dichloromethane and dried over argon. After washing the column with acetonitrile/pyridine (1/1,v/v) for 30 s, a 0.1 M solution of **7** in acetonitrile/pyridine (95/5, v/v) and the activator solution (0.1 M DPCP in acetonitrile/pyridine (95/5, v/v)) were delivered simultaneously to the column and remained in contact with the CPG support for 5 min after which the column was washed with dichloromethane/pyridine (1/1, v/v). For preparation of ^{35}S-sulfur labeled 5′-dithiophosphoryl deoxyoligonucleotides, sulfurization using ^{35}S-sulfur was carried out manually. Elemental ^{35}S-sulfur (1 mCu) (1.14 mg) was dissolved in 23 μL of carbon disulfide/pyridine (1/1) to give a 5% solution of sulfur. This solution was added to a small vial containing the support bound deoxyoligonucleotide. Carbon disulfide/pyridine (230 μL) (1/1) was added in order to cover the support completely, and the reaction was allowed to proceed for 1 h. Nonradioactive elemental sulfur (11.4 mg) was added to the reaction mixture to generate a 5% solution of sulfur, and the sulfurization allowed to proceed for an additional hour. The solvents were removed under vacuum, and a 0.1 M solution of DBU in acetonitrile was added to the vial. After 3 h, solvents were removed under vacuum. Residual solvents were then removed from CPG by coevaporation with diethyl ether. Cleavage from the support was affected by reaction with 1 mL of concentrated ammonium hydroxide for 30 min. Following fractionation by polyacrylamide gel electrophoresis, ^{35}S-labeled 5′-dithiophosphoryl deoxyoligonucleotides were extracted from the gel and desalted by passage through a NAP 25 column using 50 mM TEAB buffer as eluant. The product concentration was determined by measuring absorbance at 260 nm. The specific activity of the radioactive deoxyoligonucleotide was determined from the OD_{260} values and the counts per minute. The specific activity of 5′-[^{35}S]dithiophosphoryl dT_{10} was 4.76 × 10^8 cpm/μmole.

Enzyme Studies. General Procedure. Solutions specified below containing substrates, reaction buffers, enzyme, and 5′-[^{35}S]-dithiophosphoryl dT_{10} were incubated at 37 °C for 30 min. Aliquots were removed at specified time intervals, quenched with formaldehyde loading buffer, and analyzed by PAGE. After soaking the gel for 30 min in 5% acetic acid/5% methanol/90% H_2O solution to remove urea, the radioactivity was analyzed by placing the gel in a Molecular Dynamics PhosphorImager, recording radioactivity, and quantitating the results.

(21) Tam, J. P.; Wu, C.-R.; Liu, W.; Zhang, J.-W. *J. Am. Chem. Soc.* **1991**, *113*, 6657.

(22) Barone, A. D.; Tang, J. Y.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 4051.

(a) Alkaline Phosphatase. Reaction mixtures contained 5′-[^{35}S]-dithiophosphoryl dT_{10} (2.5 μM), DTT (500 μM), buffer (Tris-HCl 50 mM, EDTA, 0.1 mM, pH 8.5), and alkaline phosphatase (2 units/μL, 0.2 units/μL or 0.02 units/μL).

(b) Calf Spleen Phosphodiesterase. Reaction mixtures contained 5′-[^{35}S]dithiophosphoryl dT_{10} (2.5 μM), DTT (500 μL), buffer (0.01 M citric acid, pH 6), and calf spleen phosphodiesterase (1.6×10^{-4} U/μL, 1.6×10^{-5} U/μL, or 1.6×10^{-6} U/μL).

Snake Venom Phosphodiesterase Studies. Reaction mixtures contained 5′-[^{35}S]dithiophosphoryl dT_{10} (2.5 μM), buffer (25 mM Tris, 0.006% Triton X-100), and snake venom phosphodiesterase (1.5×10^{-6} U/μL, 1.87×10^{-6} U μL, or 2.25×10^{-6} U/μL).

T4 DNA Ligase Studies. Reaction mixtures contained 5′-[^{35}S]-dithiophosphoryl dT_{10} (1 μM), dT_{10} (1 μM), dA_{20} (1 μM), buffer (Tris-HCl 66 mM, $MgCl_2$ 5 mM, DTT 1 mM, ATP 1 mM, pH 7.5), and T4 DNA ligase (1 unit/μL).

Dimerization Studies. Solutions containing 5′-[^{35}S]dithiophosphoryl dT_{10} (30 μM) were incubated at −70 °C for 6 days in distilled water (pH 6.5). Aliquots were removed at specified time intervals, quenched with formaldehyde loading buffer, and analyzed by PAGE. After soaking the gel for 30 min in 5% acetic acid/5% methanol/90% H_2O solution to remove urea, radioactivity was analyzed using a Molecular Dynamics PhosphorImager. Under these conditions, dimerization was 30% complete after 120 h. No dimerization was observed after 6 days of incubation at room temperature

Similar results were obtained by analysis on HPLC. Solutions containing 5′-[^{35}S]dithiophosphoryl dT_{10} (4 μM) in distilled water were incubated for 8 days at −70 °C. Aliquots were removed at specified time intervals and injected onto a reverse-phase HPLC column. Retention times on the HPLC: 5′-dithiophosphoryl deoxyoligonucleotide, 32.4 min; dimer of dithiophosphoryl deoxyoligonucleotide, 37.2 min.

To a 10 mM Tris, pH 7.5, solution of 5′-dithiophosphoryl-dT_8 (4.4 mM, 200 μl) was added $K_3Fe(CN)_6$ (10 eq), and the reaction incubated for 10 min. The stereoisomeric mixture of dimers (100% yield) were purified by reverse phase HPLC. ^{31}P NMR (D_2O) δ 0.0 ppm(14P, $ROP(O)_2OR$), 67.42 ppm (P, $J_{pp} = 8.14$ Hz, $(ROP(S)(O)S-)_2$), 68.47 ppm (P, $J_{pp} = 14.2$ Hz, $(ROP(S)(O)S-)_2$); ^{31}P NMR (10 mM Tris, pH 7.8) δ 0.0 ppm (14P, $ROP(O_2)OR$), 67.39 (P, $J_{pp} = 16.28$ Hz $(ROP(S)(O)S-)_2$), 68.42 ppm (P, $J_{pp} = 24.42$ Hz, $(ROP(S)(O)S-)_2$); HPLC retention times, 37.8 and 38.3 min.

Oxidative Coupling of Cysteine and 5′-Dithiophosphoryl Deoxyoligonucleotides. Solutions containing 5′-[^{35}S]dithiophosphoryl dT_{10} (400 μM) and cysteine (4 mM, 10 equiv) were incubated for 3 days at room temperature. Aliquots were removed at specified time intervals, injected onto a PRP-1 reverse-phase HPLC column, and analyzed over 60 min using a gradient of 0−40% acetonitrile in 50 mM TEAB. Retention times on the HPLC: 5′-dithiophosphoryl deoxyoligonucleotide, 32.4 min; dimer of 5′-dithiophosphoryl deoxyoligonucleotide, 37.2 min; cysteine conjugate, 34.2 min.

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