baseline in the case of fits to unfiltered data where the baseline can also be refined.

Conclusions

Information regarding the structure of the Ni site in *Thiocapsa roseopersicina* hydrogenase poised in form C has been obtained from the analysis of features in the Ni K-edge EXAFS spectrum and is supported by data from structurally characterized model compounds and the analysis of X-ray absorption edge structure. The Ni ligands include 2 ± 1 S,Cl donors at 2.21 (2) Å and 3 ± 1 N,O donors at 2.06 (2) Å. Features arising from scattering atoms in the second and third coordination sphere can be accommodated by a model involving the association of the Ni center with an Fe_4S_4 cluster. The second coordination sphere Ni-Fe distance (4.3 Å) is too large for the Ni to be incorporated into an Fe,S cluster, and the absence of a 2.6-2.7 Å Ni-Fe vector suggests that a novel Ni,Fe,S cluster exists in Ni,Fe H_2ases. The observation of scattering from the Fe,S cluster implies the existence of a ligand that bridges between the cluster and the Ni site. The involvement of the Fe,S cluster is further corroborated by the observation of features that can be fit by Fe atoms in the third coordination sphere at a distance of 6.2 Å.

The structure that emerges from the EXAFS analysis has several features reminiscent of the structure of the metal cluster in *E. coli* sulfite reductase.[61] This enzyme contains a siroheme Fe atom linked to an Fe_4S_4 cluster via an Fe-S-Fe bridge. The closest Fe_{heme}-$Fe_{cluster}$ distance is 4.4 Å, with an average Fe_{heme}-$Fe_{cluster}$ distance of 4.7 Å. The closest S atom in the cluster lies at 4.3 Å. The distal Fe atoms in the cluster lie at 6.4 and 6.7 Å. Assuming a similar linkage involving a sulfido or thiolato bridge between the Ni center and an Fe_4S_4 cluster, several structures involving one or two Fe_4S_4 clusters can be envisioned that accommodate the Ni-Fe,S distances found in the second and third coordination spheres of the Ni. These results are not inconsistent with speculation that the H cluster[15] associated with H_2 activation in Fe-only hydrogenases may have a similar structure to the Ni,Fe,S cluster in Fe,Ni,S H_2ases, where a nonheme Fe is substituted for the Ni atom.[62]

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Supplementary Material Available: Table of fits generated to Fourier-filtered EXAFS spectra from 18 model compounds (24 pages). Ordering information is given on any current masthead page.

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Synthesis of Deoxydinucleoside Phosphorodithioates[1]

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Abstract: The synthesis of a new class of DNA analogues called phosphorodithioate DNA is described. This analogue, which has a deoxynucleoside-OPS_2O-deoxynucleoside internucleotide linkage, is isosteric and isopolar with the normal phosphodiester, inert toward nucleases, and potentially useful for a large number of biochemical and biological applications. Two methods are described for synthesizing this derivative. One route begins by condensing a deoxynucleoside phosphorodiamidite with a second appropriately protected deoxynucleoside to yield a deoxydinucleoside phosphoramidite. Sulfhydrolysis with H_2S generates the H-phosphonothioate, which upon oxidation with sulfur yields the deoxydinucleoside phosphorodithioate. Alternatively, sequential treatment of the deoxydinucleoside phosphoramidite with a mercaptan and sulfur yields the deoxydinucleoside phosphorodithioate triester. These deoxydinucleotides in protected form can then be used to introduce the dithioate internucleotide linkage into DNA. The second route for generating dithioate DNA uses deoxynucleoside phosphorothioamidites. Two derivatives, the deoxynucleoside 3'-*N*,*N*-dimethyl- or 3'-(*N*,*N*-tetramethylenephosphorothioamidite), were found to be especially attractive synthons as they could be prepared in stable form via a one-flask synthesis procedure and used to form the deoxydinucleoside thiophosphite rapidly (1-2 min with tetrazole as activator) in high yield. Subsequent oxidation with sulfur generates the completely protected phosphorodithioate linkage.

During the past decade, polynucleotides modified at phosphorus have found many new and potentially important applications in biochemistry and molecular biology. These include their use for studying enzyme mechanisms,[2] the interaction of proteins with nucleic acids,[3] and potentially as therapeutic drugs.[4] Although a large number of analogues have been synthesized and tested for biochemical reactivity, only a few are currently of interest. These include the polynucleotide methylphosphonates,[5] phos-

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phorothioates,[6-8] phosphoramidates,[7,9-12] and phosphate triesters.[13-16] These various analogues are all phosphorus chiral, which inevitably leads to a large number of nonresolvable diastereomeric oligomers having variable biophysical, biochemical, and biological properties.

Recently alternative analogues with achiral phosphorus internucleotide linkages have been developed and appear quite promising for many biochemical and biological applications. One series has either sulfur[17,18] or nitrogen[19] joining the nucleoside to phosphorus. Generally, however, these compounds are less stable than natural DNA toward basic or acidic workup conditions and have found limited use in the polynucleotide field. A particularly attractive achiral analogue is the deoxyoligonucleotide phosphorodithioate, which has deoxynucleoside–OPS_2O–deoxynucleoside internucleotide linkages.[20-31] It is isostructural and isopolar with the natural phosphate diester linkage, is stable toward enzymatic and chemical hydrolysis,[20-22,27] and has other biochemical properties similar to unmodified DNA. Here we describe in detail our initial investigations on the synthesis of deoxydinucleoside phosphorodithioates. A major objective of this work, which has now been realized,[32] was to develop an approach for synthesizing deoxyoligonucleotides with phosphorodithioate and natural DNA linkages in any predetermined order.

Experimental Section

General Procedures. Proton (^{1}H NMR) and phosphorus (^{31}P NMR) nuclear magnetic resonance spectra were recorded on a Brucker WM-250, Varian Gemini 300, or a JOEL 90XQ in deuterated chloroform with tetramethylsilane as internal standard (^{1}H NMR) and 85% phosphoric acid as external standard (^{31}P NMR). FAB mass spectrum analysis was completed with magic bullet (dithioerythritol–dithiothreitol, 3:2, v/v) unless otherwise specified. Thin-layer chromatography (TLC) was on aluminum-backed sheets (silica gel 60 F, 0.2 mm, E. Merck). Preparative chromatography was by flash column chromatography on silica gel 60, 230–400 mesh (Macherey Nagel, Dueren, FRG). Concentrations of solutions were carried out in vacuo at 40 °C or lower using an aspirator or an oil vacuum pump. Solids were dried at room temperature in a desiccator over phosphorus pentoxide and potassium hydroxide unless otherwise specified. All reactions were carried out at room temperature unless reported differently.

Pyridine and dichloromethane were freshly distilled over calcium hydride. Triethylamine was distilled over toluene–sulfonyl chloride and then calcium hydride. *N*-Methylaniline was purchased from Aldrich and passed over basic alumina (Woelm) before use. Anhydrous diethyl ether (ether, Merck or Mallinckrodt) was used directly. Acetonitrile was distilled over phosphorus pentoxide and then calcium hydride. Tetrahydrofuran (THF) was distilled over sodium metal in the presence of benzophenone.

Deoxyribonucleosides were obtained from Aldrich. 5′-*O*-4,4′-Dimethoxytritylated and N-protected deoxynucleosides,[33-35] 3′-*O*-acetylthymidine,[36] 3′-*O*-(4-chlorophenoxyacetyl)thymidine,[2] 5-(4-nitrophenyl)tetrazole,[37] and bis(*N*,*N*-dimethylamino)chlorophosphine[38] were prepared according to published procedures. 5-(4-Nitrophenyl)tetrazole was crystallized from anhydrous acetonitrile. (Trifluoromethyl)acetonitrile was prepared according to published procedures[39,40] and used directly after distillation. 1*H*-Tetrazole (tetrazole, Aldrich) was sublimed before use.

Synthesis of *N*-Methylanilinium Salts. Each appropriate acid (10 mmol) was dissolved in dry ether (950 mL) and added slowly with stirring to *N*-methylaniline (10 mmol) in dry ether (100 mL). Precipitation was completed by addition of *n*-pentane (50 mL) and the salt isolated by filtration, washing with ether and *n*-pentane, and drying in vacuo. This procedure was used to prepare *N*-methylanilinium trifluoroacetate[41] and *N*-methylanilinium (trifluoromethyl)tetrazolide. *N*-Methylimidazolinium trifluoroacetate was prepared analogously by substituting *N*-methylimidazole for *N*-methylaniline. *N*-Methylimidazolinium trifluoroacetate was very hygroscopic and volatile. For *N*-methylanilinium tosylate, *p*-toluenesulfonic acid monohydrate was dissolved in dry dichloromethane (50 mL) instead of ether prior to addition of *N*-methylaniline.

Synthesis of Onium Tetrafluoroborates. Tetrafluoroboric acid (Aldrich) as its etherate (85%, HBF_4 by volume) was used without further purification. A solution of the aromatic amine (20 mmol) in dry ether (100 mL) was prepared and HBF_4 (20 mmol, 3.8 g of a diethyl etherate) in dichloromethane (50 mL) was added with stirring. Precipitation was completed by addition of ether and the salt isolated by filtration, washing with ether, and drying in vacuo. This procedure was used to prepare pyridinium tetrafluoroborate and 4-(*N*,*N*-dimethylamino)pyridinium tetrafluoroborate as white, nonvolatile, and nonhygroscopic solids.

Kinetics of Phosphorothioite Synthesis. Generally a deoxynucleoside thioamidite (**14a**, **14b**, or **14c**, 0.2 mmol) was dissolved in acetonitrile. 3′-*O*-Acetylthymidine (1 equiv) and an appropriate activator in acetonitrile (2 mL) were added via cannulation. After 30 s, aliquots (0.02 mL) were removed periodically via a syringe and quenched by addition to a solution of elemental sulfur (0.02 g) in pyridine (0.1 mL). After analysis by TLC and exposure to HCl vapors, 4,4′-dimethoxytrityl-containing products were scrapped from the plate, eluted, and analyzed spectrophotometrically. Typical results are shown in Figure 5.

Synthesis of *S*-(4-Chlorobenzyl) *N*,*N*,*N*′,*N*′-Tetraisopropylphosphorothiodiamidite (13a). 4-Chlorobenzyl mercaptan (50 mmol, 7.93 g) in ether (300 mL) and NaH (50 mmol, 2.4 g of a 50% suspension in oil) were stirred at room temperature under argon until evolution of hydrogen ceased (4 h). Bis(*N*,*N*-diisopropylamino)chlorophosphine (50 mmol, 13.34 g) was added to the white suspension and the reaction mixture stirred for an additional 2 h. After removal of the resulting salt by filtration under anhydrous conditions, the clear solution was concentrated in vacuo to a white, amorphous solid. Recrystallization from acetonitrile gave 27.23 g of product (70%): ^{31}P NMR δ 93.5; ^{1}H NMR

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δ 7.35 (aromatic), 3.4–3.9 (CH_2 (benzyl), α-CH (iPr)), 1.22 (CH_3 of iPr); FAB^+ mass spectrum, 389 (M^+); FAB^- mass spectrum, 387 ($(M - H)^-$).

Synthesis of Bis(*N*,*N*-pyrrolidino)chlorophosphine (15b). Trimethylchlorosilane (1 mol, 108.6 g, 126.9 mL) was added dropwise over a period of 3 h at 0 °C to a stirred solution of anhydrous pyrrolidine (2 mol, 148.24 g, 166.9 mL). The precipitate of pyrrolidine hydrochloride was removed by filtration through a Schlenk frit and washed extensively with ether. After removal of solvent at ambient temperature, the product was isolated by fractional distillation (40 °C, 16 mmHg) to yield 71.6 g (50%): ^1H NMR δ 2.85 (m, 4 H, α-CH_2 (pyrrolidinyl)), 1.66 (m, 4 H, β-CH_2 (pyrrolidinyl)), −0.03 (s, 9 H, CH_3 of trimethylsilyl); EI mass spectrum, 142 ($(M - H)^+$), 128 ($(M - CH_3)^+$), 100 ($(M - 3CH_3 + 2 H)^+$), 73 ($C(CH_3)_3Si)^+$), 59 ($((CH_3)_2SiH)^+$), 45 ($(CH_3SiH_2)^+$).

(Trimethylsilyl)pyrrolidine (0.5 mol, 71.5 g, 87.5 mL) was added dropwise over 20 min to a stirred solution of phosphorus trichloride (0.25 mol, 34.35 g, 21.8 mL) maintained at 0 °C in ether (200 mL). Immediately a white precipitate formed, which disappeared within 30 min. After 45 min, the reaction mixture was freed of a few insoluble drops of a yellow oil by cannulation into a distillation flask. Ether was removed at ambient temperature and the product obtained by distillation (90–92 °C, 16 mmHg) to yield 34.2 g (66.7%): ^1H NMR δ 3.24 (m, α-CH_2 (pyrrolidinyl)), 1.95 (m, β-CH_2 (pyrrolidinyl)); ^{31}P NMR δ 163.0 (s, br); FAB^- mass spectrum, 205 (M^+), 70 ($(pyrrolidinyl)^+$).

Synthesis of 2′-Deoxynucleosid-3-yl *S*-(4-Chlorobenzyl) *N*,*N*-Diisopropylphosphorothioamidite (14a). 5′-*O*-(4,4′-Dimethoxytrityl)thymidine (1 mmol, 0.545 g) and tetrazole (1 mmol, 0.070 g) were dissolved in acetonitrile–dichloromethane (10 mL, 4:1, v/v). *S*-(4-Chlorobenzyl) *N*,*N*,*N*′,*N*′-tetraisopropylphosphorothioamidite (1.2 mmol, 0.470 g) was added and the reaction carried out with stirring under argon at room temperature. Within 5 min, a precipitate of diisopropylammonium tetrazolide appeared. After 2 h, tetrazole (1 mmol, 0.070 g) was added and the reaction allowed to proceed for an additional 6 h. Analysis by TLC indicated almost complete conversion to product (longer reaction times lead to product decomposition). The reaction was quenched with triethylamine (1 mL), diluted with deacidified ethyl acetate (70 mL), and extracted successively with saturated aqueous bicarbonate (20 mL, twice) and brine. The aqueous layers were combined and back-extracted with deacidified ethyl acetate. The organic layers were combined, dried over sodium sulfate, and reisolated free of salt by filtration. The product mixture was fractionated over a silica gel column by use of chloroform–ethyl acetate–triethylamine (45:45:10, v/v/v). The product was collected, concentrated to a glass, dissolved in ethyl acetate–triethylamine (99:1, v/v), precipitated into *n*-pentane (300 mL), and isolated after drying in vacuo. The yield was 0.458 g (55%). This same procedure was used for synthesis of all four 2′-deoxynucleoside 3′-(*N*,*N*-diisopropylphosphorothioamidites) with yields of 50–60%.

Synthesis of 2′-Deoxynucleosid-3-yl *S*-(4-Chlorobenzyl) *N*,*N*-Tetramethylenephosphorothioamidite (14b) and 2′-Deoxynucleosid-3′-yl *S*-(4-Chlorobenzyl) *N*,*N*-Dimethylphosphorothioamidite (14c).[42] 5′-*O*-(4,4′-Dimethoxytrityl)4-*N*-benzoyl-2′-deoxycytidine (0.5 mmol, 0.317 g) was dissolved in acetonitrile (2 mL) and triethylamine (1 mL). Bis(*N*,*N*-pyrrolidinyl)chlorophosphine (0.6 mmol, 0.124 g) was added under argon with stirring. A precipitate formed immediately and the ^{31}P NMR of the reaction mixture exhibited only one signal (133.8 ppm). Presumably the excess of phosphitylating agent formed a compound insoluble in the reaction mixture and not observable in the NMR. After 5 min of stirring at room temperature, 4-chlorobenzyl mercaptan (1 mmol, 0.154 g) was added to the reaction mixture. A partial dissolution of the precipitate was observed. The reaction mixture was then evaporated to a glass and the resulting solid resuspended in acetonitrile (2 mL). The ^{31}P NMR of the solution indicated that the major products were the two diastereomers of the thioamidite (161.5 and 159.7 ppm). Hydrolysis products (12.4 ppm) and a minor product tentatively identified as an adduct of excess phosphitylating agent and mercaptan (107 ppm) were also present. The reaction mixture was quenched with triethylamine (1 mL), diluted with deacidified ethyl acetate (50 mL), and extracted successively with aqueous bicarbonate and brine. Aqueous layers were back-extracted with deacidified ethyl acetate and the combined organic layers were dried (30 min) over sodium sulfate in the presence of 10% triethylamine (based on the total volume of liquid). After the salt was removed by filtration, the product was concentrated to a white foam. The solid was dissolved in minimal toluene and precipitated into *n*-pentane (300 mL) containing 0.1% triethylamine (v/v). The precipitate was collected by filtration and dried in vacuo to yield 0.742 g of product (83.1%). In order to minimize oxidation to the phosphorothioamidates, this workup should be carried out rapidly and under an inert gas when possible. Otherwise variable amounts (10–30%) of the oxidized product will form. The same procedure was used to synthesize the four 2′-deoxynucleosid-3′-yl *S*-(4-chlorobenzyl) *N*,*N*-tetramethylenephosphorothioamidites and 2′-deoxynucleosid-3′-yl *S*-(4-chlorobenzyl) *N*,*N*-dimethylphosphorothioamidites (75–85% yields).

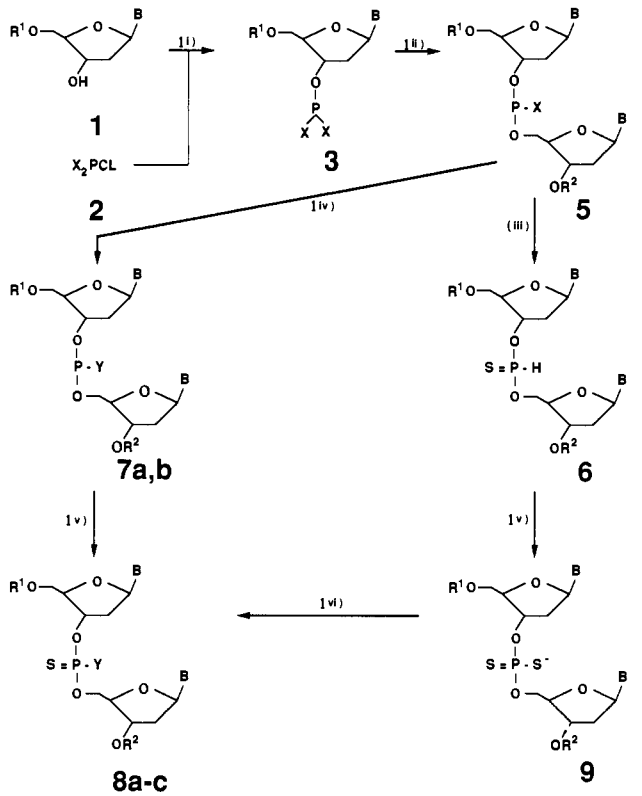

Figure 1. Synthesis of deoxydinucleoside phosphorodithioates via deoxynucleoside phosphordiamidites. Reaction conditions: (i) triethylamine; (ii) tetrazole + 3′-*O*-acetyl-2′-deoxynucleoside **4**; (iii) H_2S + tetrazole; (iv) mercaptan + tetrazole; (v) sulfur; (vi) α,2,4-trichlorotoluene. Abbreviations: R^1, 4,4′-dimethoxytrityl; R^2, acetyl; X, diisopropylamino; Y, (4-chlorobenzyl)thio (**7a**, **8a**); Y, (2-chlorobenzyl)thio (**7b**, **8b**); Y, (2,4-dichlorobenzyl)thio (**8c**).

Synthesis of *O*-[5′-*O*-(4,4′-Dimethoxytrityl)nucleosid-3′-yl] *S*-(4-Chlorobenzyl) *O*-(3′-*O*-Acetylnucleosid-5′-yl) Phosphorodithioate as Well as the *S*-(2-Chlorobenzyl) and *S*-(2,4-Dichlorobenzyl) Derivatives (8a–c) via 2′-Deoxynucleosid-3′-yl Phosphorodiamidites (Figure 1). Two methods were developed. One method involved (1) preparation of 2′-deoxynucleosid-3′-yl phosphorodiamidites, (2) condensation of the 2′-deoxynucleosid-3′-yl phosphorodiamidite with a 2′-deoxynucleoside to yield a 2′-deoxydinucleoside phosphoramidite, (3) sulfhydrolysis with H_2S, (4) oxidation with sulfur, and (5) protection of the resulting dithioate via alkylation. For the second method, the 2′-deoxydinucleoside phosphoramidite was condensed directly with a mercaptan and oxidized with sulfur to yield the product.[43] Descriptions of both methods in sequential order are presented in this section.

The first method has the following five steps.

(1) Synthesis of 5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (**3**). 5′-*O*-(4,4′-Dimethoxytrityl)-thymidine (2.21 mmol, 1.20 g) was dried by coevaporation with THF and then dissolved in THF (10 mL) containing triethylamine (3.3 mmol, 0.46 mL). Bis(diisopropylamino)chlorophosphine (2.44 mmol, 0.650 g) was added with stirring. After 35 min, triethylammonium hydrochloride was removed by filtration from the soluble reaction product and washed with THF (1 mL). The filtrates were combined and concentrated to dryness. The product was redissolved in acetonitrile (5 mL) and used without further purification.

(2) Synthesis of *O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *O*-(3′-*O*-acetylthymidin-5′-yl) *N*,*N*-diisopropylphosphoramidite (**5**). 3′-*O*-Acetylthymidine (**4**; 2.25 mmol, 0.639 g) and tetrazole (2.0 mmol, 0.142 g) were dried by coevaporation with anhydrous THF (10 mL) and dissolved in acetonitrile (5 mL). The resulting solution was mixed with **3** (prepared as described above and added in an amount equimolar with

(42) We thank Dr. J.-Y. Tang and Dr. Y.-X. Ma for applying these procedures to deoxyoligonucleotide synthesis on silica supports (see ref 32).

(43) We thank Dr. A. Grandas for elaborating this synthesis strategy (see ref 22).

3′-*O*-acetylthymidine), and the reaction mixture stirred for 45 min. The products were diluted with dichloromethane (75 mL), extracted with saturated sodium bicarbonate and brine, and dried over sodium sulfate. After removal of salt by filtration, the product was purified by silica gel column chromatography using ethyl acetate–chloroform–triethylamine (45:45:10, v/v/v) and obtained in 75% yield (1.59 g): ^{31}P NMR (C-H_3CN) δ 148.5, 148.1.

(3) Synthesis of *O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *O*-[3′-*O*-acetylthymidin-5′-yl] hydrogenphosphonothioate (**6**). Compound **5** (0.5 mmol, 0.470 g) and tetrazole (0.5 mmol, 0.035 g) were dissolved in acetonitrile (1 mL) and H_2S was bubbled through the solution for 1 min. The reaction flask was sealed and stored with stirring at room temperature for 16 h. Evaporation of the solvent (*danger*: H_2S) afforded a gum, which was redissolved in ethyl acetate and extracted twice (20 mL each) with 2 M triethylammonium bicarbonate (pH 7.4). The product was dried over sodium sulfate, concentrated to a gum, redissolved in dichloromethane (5 mL), and precipitated into *n*-pentane (250 mL). After filtration and desiccation over molecular sieves, the desired product was obtained in 90% yield (0.400 g): ^{1}H NMR δ 7.81, 7.80 (2 d, J_{PH} = 671.4, 676.7 Hz, 1 H, PH (hydrogenphosphonothioate, 2 diastereomers)), 7.55, 7.53 (2 s, 2 H, H_6 (thymine)), 7.20–7.37 (m, 9 H, H_2 and H_6 (anisyl), phenyl), 6.82 (d, J_{HH} = 8.8 Hz, 4 H, H_3 and H_5 (anisyl)), 6.49, 6.26 (2 m, 2 H, $H_{1'}$), 5.49, 5.25 (2 m, 2 H, $H_{3'}$), 4.35 (m, 1 H, $H_{4'}$), 4.19 (m, 2 H, H_5, (near hydrogenphosphonothioate)), 4.07 (m, 1 H, $H_{4'}$), 3.76 (s, 6 H, CH_3 (anisyl)), 3.42 (m, 2 H, H_5, (near DMTr)), 2.32–2.54 (m, 4 H, $H_{2'}$), 2.08, 2.07 (2 s, 3 H, CH_3 of acetyl, 2 diastereomers), 1.90 (s, 3 H, CH_3 of thymine, 3′-end), 1.43 (s, 3 H, CH_3 of thymine, 5′-end); ^{31}P NMR δ 70.7, 71.7; FAB$^+$ mass spectrum, 527 (5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*^{2}-anhydrothymidine); FAB$^-$ mass spectrum, 890 (M^-), 623 (*O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] hydrogenphosphonate)$^-$ (desulfurized product)), 363 ((*O*-(3′-*O*-acetylthymidin-5′-yl) hydrogenphosphonate)$^-$ (desulfurized product)).

(4) Synthesis of triethylammonium *O*-[5′-*O*-(4,4′-dimethoxytrityl)-thymidin-3′-yl] *O*-(3′-*O*-acetylthymidin-5′-yl) phosphorodithioate (**9**). Compound **6** (0.1 mmol, 0.104 g) in dichloromethane (1 mL) was added to elemental sulfur (0.125 mmol elementary sulfur in toluene–2,6-lutidine (19:1, v/v)). After 0.5 h the reaction products were concentrated to a yellow gum, dissolved in dichloromethane, and fractionated by silica gel column chromatography (0–12% methanol in dichloromethane–triethylamine, 995:5, v/v). The product was isolated in 70% yield by concentration of appropriate fractions to a white solid: ^{1}H NMR δ 8.12 (br s, 2 H, H_3 (thymine)), 7.90, 7.60 (2 s, 2 H, H_6 (thymine)), 7.24–7.40 (m, 9 H, H_2 and H_6 (anisyl), phenyl), 6.80 (d, J_{HH} = 8.8 Hz, 4 H, H_3 and H_5 (anisyl)), 6.43 (m, 2 H, $H_{1'}$), 5.36–5.46 (m, 2 H, $H_{3'}$), 4.40 (m, 2 H, $H_{4'}$), 4.16 (m, 2 H, $H_{5'}$ (near phosphorodithioate)), 3.76 (s, 6 H, CH_3 (anisyl)), 3.52 (m, 2 H, $H_{5'}$ (near DMTr)), 2.28 (m, 4 H, $H_{2'}$), 2.05 (s, 3 H, CH_3 (acetyl)), 1.97 (s, 3H, CH_3 of thymine, 3′-end), 1.58 (s, 3 H, CH_3 of thymine, 5′-end); ^{31}P NMR δ 112.7; FAB$^+$ mass spectrum, 303 (DMTr); FAB$^-$ mass spectrum, 921 ((O-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *O*-(3′-*O*-acetylthymidin-5′-yl) phosphorodithioate)$^-$ (M), 395 (*O*-(3′-*O*-acetylthymidin-5′-yl) phosphorodithioate)$^-$).

(5) Alkylation of **9** with α,2,4-trichlorotoluene. *O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *O*-(3′-*O*-acetylthymidin-5′-yl) phosphorodithioate (0.057 g, 0.06 mmol) and α,2,4-trichlorotoluene (0.05 mL) were dissolved in acetonitrile (2 mL). After 1 h at 55 °C, TLC analysis indicated quantitative conversion to *O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] S-(2,4-dichlorobenzyl) *O*-(3′-*O*-acetylthymidin-3′-yl) phosphorodithioate (**8c**). The reaction product was concentrated to an oil, dissolved in dichloromethane, precipitated into *n*-pentane, and dried in vacuo (90% yield): ^{1}H NMR δ 7.52, 7.55 (2 s, 1 H, H_6 (thymine, 2 diastereomers)), 7.23–7.37 (m, 13 H, H_2 and H_6 (anisyl), aromatic (2,4-dichlorobenzyl), phenyl, H_6 (thymine)), 6.82 (d, J_{HH} = 8.6 Hz, 4 H, H_3 and H_5 (anisyl), 6.34, 6.28 (2 m, 2 H, $H_{1'}$), 5.38, 5.01 (2 m, 2 H, $H_{3'}$), 4.08–4.24 (m, 6 H, CH_2 (2,4-dichlorobenzyl), $H_{4'}$ and $H_{5'}$ (3′-end), 3.76 (s, 6 H, CH_3 (anisyl)), 3.42 (m, 2 H, $H_{5'}$ (5′-end)), 2.39 (m, 4 H, $H_{2'}$), 2.08 (s, 3 H, CH_3 (acetyl)), 1.89, 1.87 (2 s, 3 H, CH_3 of thymine, 3′-end, 2 diastereomers), 1.43, 1.42 (2 s, 3 H, CH_3 of thymine, 5′-end, 2 diastereomers); ^{31}P NMR δ 93.7, 94.4; FAB$^+$ mass spectrum, 527 (5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*^{2}-anhydrothymidine); FAB$^-$ mass spectrum, 922 (M + H − (2,4-dichlorobenzyl)$^-$), 813 (*O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *S*-(2,4-dichlorobenzyl) phosphorodithioate)$^-$), 553 ((*O*-(3′-*O*-acetylthymidin-5′-yl) *S*-(2,4-dichlorobenzyl) phosphorodithioate)$^-$).

The second method involved condensation of a dinucleoside phosphoramidite (**5**) with a mercaptan followed by sulfur oxidation. The method is illustrated by the synthesis of *O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *S*-(4-chlorobenzyl) *O*-(3′-*O*-acetylthymidin-5′-yl) phosphorodithioate (**8a**). Compound **5** (1.66 mmol, 1.59 g), tetrazole (4.01 mmol, 0.281 g), and 4-chlorobenzyl mercaptan (7.6 mmol, 1 mL, 1.2 g) were added to acetonitrile (7 mL) and the reaction mixture stirred for 30 min at room temperature. After being quenched (10 min) by addition of elemental sulfur (10 mL of a 0.05 M solution in toluene–2,6-lutidine, 19:1, v/v), the reaction products were diluted with ethyl acetate (75 mL), extracted sequentially with aqueous saturated sodium bicarbonate and brine, dried over sodium sulfate, and filtered from the salt. The resulting product mixture was concentrated to an oil, diluted with ethyl acetate (40 mL), and triturated with hexanes (200 mL) to give a white powder. Purification by silica gel column chromatography (2–12% methanol in dichloromethane) yielded the completely protected product in 91% yield. Compound **8b** was synthesized by the same procedure. Other derivatives containing the 2,4-dichlorobenzyl (**8c**) or 2,4-dinitrobenzyl[29] phosphorus-protecting groups could presumably be synthesized by this procedure as well.

Synthesis of *O*-[5′-*O*-(4,4′-Dimethoxytrityl)nucleosid-3′-yl] *S*-(4-chlorobenzyl) *O*-(3′-*O*-Acetylnucleosid-5′-yl) Phosphorodithioate (8a) via 2′-Deoxynucleosid-3′-yl Phosphorothioamidites (Figure 4). This method involved condensation of a 2′-deoxynucleosid-3′-yl phosphorothioamidite with a 2′-deoxynucleoside followed by sulfur oxidation. The following general procedure was used to synthesize deoxydinucleoside phosphorodithioates from 2′-deoxynucleosid-3′-yl *S*-(4-chlorobenzyl) *N*,*N*-diisopropylphosphorothioamidites (**14a**) and pyridinium tetrafluoroborate as activator. *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *S*-(4-chlorobenzyl) *N*,*N*-diisopropylphosphorothioamidite (1 mmol, 0.832 g) and 3′-*O*-acetylthymidine (0.5 mmol, 0.136 g) were dissolved under argon with stirring in acetonitrile (1 mL). Pyridinium tetrafluoroborate (2 mmol, 0.158 g) in acetonitrile (2 mL) was added and the reaction allowed to proceed for 15 min. Sulfur (20 atomic equiv, 0.640 g) suspended in pyridine (2 mL) was added to quench the reaction, and after 5 min, the reaction mixture was concentrated to a gum in vacuo. The reaction products were dissolved in ethyl acetate (50 mL), filtered through glass wool to remove the excess sulfur, extracted consecutively with saturated aqueous bicarbonate and brine, and dried over sodium sulfate for 4 h. After the salt was removed by filtration, the reaction products were concentrated to a glass, dissolved in 1,1,1-trichloroethane–methanol (93:7, v/v), and fractionated by flash column chromatography on silica gel. The product fractions were combined and evaporated to a white foam. The product was dissolved in ethyl acetate (2 mL), isolated by precipitation from *n*-pentane, and dried in vacuo to yield 0.629 g (60%). Following this procedure, isolated yields were generally 50–60% for various deoxynucleoside phosphorodithioates.

Deoxynucleoside *N*,*N*-tetramethylenephosphorothioamidites **14b** and deoxynucleoside *N*,*N*-dimethylphosphorothioamidites **14c** showed much greater reactivity than the deoxynucleoside *N*,*N*-diisopropylphosphorothioamidites **14a**. Thus, a standard coupling reaction involving 0.1 M solutions of either **14b** or **14c** and 3′-*O*-acetylthymidine (1:1 molar equiv for **14b** or **14c** to 3′-*O*-acetylthymidine rather than 2:1 as reported above for **14a**) using the conditions described above was complete in less than 30 s with tetrazole as catalyst. Oxidations and workups with **14b** or **14c** were identical with those described for **14a**.

Arbuzov Reaction with *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *O*-(β-Cyanoethyl) *S*-(4-Chlorobenzyl) Phosphorothioite (18a). 5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl β-cyanoethyl *N*,*N*-diisopropylphosphoramidite (**10**; 0.5 mmol, 0.372 g) and tetrazole (2.5 mmol, 0.175 g) were dissolved in acetonitrile (4 mL) and 4-chlorobenzyl mercaptan (5 mmol, 0.793 g) was added. The progress of the reaction was monitored by ^{31}P NMR. Initially, the phosphorothioite forms (**18a**; 191.1, 190.5 ppm) and then rearranges to a major product (**19a**; 93.5 ppm, 83% yield based on ^{31}P NMR). The reaction mixture was poured into ethyl acetate (50 mL), extracted twice with saturated aqueous sodium bicarbonate and once with brine, and dried over sodium sulfate. After removal of the salt, the reaction products were concentrated to an oil and fractionated by silica gel column chromatography using 1,1,1-trichloroethane–methanol (95:5, v/v) to elute the main product. The minor product (**20a**) was then eluted with 1,1,1-trichloroethane–methanol (9:1, v/v). Fractions containing these compounds were concentrated to dryness, redissolved in ethyl acetate (5 mL), and isolated by precipitation from *n*-pentane (200 mL of solvent each).

Main product, *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *O*-(β-cyanoethyl) (4-chlorobenzyl)phosphonothioate (**19a**): ^{1}H NMR δ 8.4 (br s, 1 H, H_3 (thymine)), 7.50 (2 d, 1 H, H_6 (thymine, 2 diastereomers)), 7.18–7.34 (m, 13 H, H_2 and H_6 (anisyl), aromatic (4-chlorobenzyl), phenyl), 6.79 (2 d, J_{HH} = 8.7 Hz, 4 H, H_3 and H_5 (anisyl)), 6.35 (m, 1 H, $H_{1'}$), 5.30 (m, 1 H, $H_{3'}$), 4.17 (m, 1 H, $H_{4'}$), 4.06 (d, J_{PH} = 17.5 Hz), 3.99 (d, J_{PH} = 17.3 Hz, 2 H, CH_2 (4-chlorobenzyl)), 4.04 (m, 2 H, α-CH_2 (β-cyanoethyl)), 3.74, 3.73 (2 s, 6 H, CH_3 (anisyl)), 3.33 (m, 2 H, $H_{5'}$), 2.51–2.64 (m, 2 H, β-CH_2 (β-cyanoethyl)), 2.20–2.34 (m, 2 H, $H_{2'}$), 1.40 (s, 3 H, CH_3 of thymine); ^{31}P NMR δ 95.3, 95.9 (2 diastereomers); FAB$^-$ mass spectrum, 800 ((M-2H)$^-$).

Minor product, *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *O*-(β-cyanoethyl) *S*-(4-chlorobenzyl)phosphorothioate (**20a**): ^{1}H NMR δ 8.52,

8.48 (2 s, H_3 (thymine, 2 diastereomers)), 7.50, 7.49 (2 s, H_6 (thymine, 2 diastereomer)), 7.12–7.35 (m, 13 H, H_2 and H_6 (anisyl), aromatic (4-chlorobenzyl), phenyl), 6.80 (2 d, J_{HH} = 9 Hz, 4 H, H_3 and H_5 (anisyl)), 6.36 (m, 1 H, $H_{1'}$), 5.11 (m, 1 H, $H_{3'}$), 4.16 (m, 1 H, $H_{4'}$), 4.00 (2 d, J_{PH} = 16 Hz, 2 H, CH_2 (4-chlorobenzyl, 2 diastereomers)), 4.05 (m, 2 H, α-CH_2 (β-cyanoethyl)), 3.75, 3.74 (2 s, 6 H, CH_3 (anisyl)), 3.20–3.64 (m, 2 H, $H_{5'}$), 2.56 (2 t, β-CH_2 (β-cyanoethyl)), 2.27–2.40 (m, 2 H, $H_{2'}$), 1.37 (2 s, 3 H, CH_3 of thymine); ^{31}P NMR δ 27.46; FAB$^+$ mass spectrum, 856 ((M + K)$^+$), 840 ((M + Na)$^+$), 817 (M$^+$); FAB$^-$ mass spectrum, 764 ((M − (β-cyanoethyl)$^-$).

Arbuzov Reaction of *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *O*-(β-Cyanoethyl) *S*-(2,4-Dichlorobenzyl) Phosphorothioite (18b). *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *O*-(β-cyanoethyl) *N*,*N*-diisopropylphosphoramidite (**10**; 0.5 mmol, 0.372 g) and tetrazole (2.5 mmol, 0.175 g) were dissolved in acetonitrile (4 mL) and 2,4-dichlorobenzyl mercaptan (5 mmol, 0.965 g) was added. The progress of the reaction was monitored by ^{31}P NMR. Initially the phosphorothioite forms (**18b**, 190.9, 190.3 ppm), which then rearranges at a slower rate than the (4-chlorobenzyl)thio derivative to the corresponding 2,4-dichlorobenzylphosphonothioate ($t_{1/2}$ = 20 and 150 min, respectively) as the major product. The phosphonothioate was isolated and characterized; **19b**: ^{1}H NMR δ 8.68, 8.65 (2 s, 1 H, H_6 (thymine)), 7.15–7.42 (m, 12 H, H_2 and H_6 (anisyl), aromatic (2,4-dichlorobenzyl), phenyl), 6.83 (d, J_{HH} = 7.52, 4 H, H_3 and H_5 (anisyl)), 6.34 (m, 1 H, $H_{1'}$), 5.38 (m, 1 H, $H_{3'}$), 4.26 (m, 1 H, $H_{4'}$), 4.22 (d, J_{PH} = 16.2), 4.12 (d, J_{PH} = 16.4 Hz, 2 H, CH_2 (2,4-dichlorobenzyl, 2 diastereomers)), 4.16 (m, 2 H, α-CH_2 (β-cyanoethyl)), 3.77, 3.76 (2 s, 6 H, CH_3 (anisyl, 2 diastereomers)), 3.33–3.42 (m, 2 H, $H_{5'}$), 2.69, 2.61 (2 t, J_{HH} = 6.2 Hz, 2 H, β-CH_2 (β-cyanoethyl)), 2.34–2.44 (m, 2 H, $H_{2'}$), 1.44 (s, 3 H, CH_3 of thymine); ^{31}P NMR δ 95.1, 94.8 (2 diastereomers); FAB$^-$ mass spectrum, 835 ((M − 2H)$^-$). The minor product (**20b**) with a ^{31}P NMR signal at 27.5 ppm was not characterized.

Reaction of Excess 3′-*O*-Acetylthymidine with *O*-[5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-benzoyl-2′-deoxycytidin-3′-yl] *S*-(4-Chlorobenzyl) *O*-(3′-*O*-Acetylthymidin-5′-yl) Phosphorothioite. *O*-[5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-benzoyl-2′-deoxycytidin-3′-yl] *S*-(4-chlorobenzyl) *N*,*N*-tetramethylenephosphorothioamidite (0.5 mmol, 0.370 g), tetrazole (2.5 mmol, 0.175 g), and 3′-*O*-acetylthymidine (0.5 mmol, 0.143 g) were dissolved in acetonitrile (5 mL). After formation of the phosphorothioite triester (1 min; ^{31}P NMR δ 192.6 and 192.2 (2 diastereomers), excess 3′-*O*-acetylthymidine (2 mmol, 0.569 g) was added. Over the next 20 h, the phosphorothioite was converted to a trialkylphosphite triester (^{31}P NMR δ 139.5, reaction mixture). After the reaction was quenched with sulfur (5 min, 10 atomic equiv, 0.160 g) in order to convert the phosphite to the phosphorothioate triester, the reaction products were poured into ethyl acetate and the excess sulfur was removed by filtration through glass wool. The clear solution containing reaction products was then extracted sequentially with aqueous saturated sodium bicarbonate (twice) and brine, dried over sodium sulfate, and concentrated to a foam. Flash column chromatography (CH_3CCl_3–CH_3OH, 85:15, v/v) yielded a major product, *O*-[5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-benzoyl-2′-deoxycytidin-3′-yl] *O*,*O*-di-3′-*O*-acetylthymidin-5′-yl phosphorothioate, which was dissolved in chloroform, precipitated from *n*-pentane (250 mL), and dried in vacuo to yield 0.390 g (57%): ^{1}H NMR δ 9.62 (br s, 2 H, H_3 (thymine)), 9.22 (br s, 1 H, 4-NH (cytosine)), 8.09 (d, J_{HH} = 7.4 Hz, 2 H, H_2 and H_6 (benzoyl)), 7.41–7.56 (m, 3 H, H_3, H_4 and H_5 (benzoyl)), 7.15–7.36 (m, 9 H, H_2 and H_6 (anisyl), phenyl), 6.8 (d, J_{HH} = 7.8 Hz, 4 H, H_3 and H_5 (anisyl)), 6.15–6.24 (m, 3 H, $H_{1'}$), 5.11–5.25 (m, 3 H, $H_{3'}$), 4.39 (m, 2 H, $H_{5'}$ (thymidine)), 4.11 (m, 2 H, $H_{4'}$ (thymidine)), 4.06 (m, 1 H, $H_{4'}$ (cytidine)), 3.73, 3.72 (2 s, 6 H, CH_3 (anisyl, rotational hindrence)), 3.40 (m, 2 H, $H_{5'}$), 2.87 (m, 1 H, $H_{2'}$ (cytidine)), 2.05–2.24 (m, 5 H, $H_{2'}$ (4 of thymidine, 1 of cytidine), 2.02 (s, 6 H, CH_3 (acetyl)), 1.83 (s, 6 H, CH_3 of thymine); ^{31}P NMR δ 67.4; FAB$^+$ mass spectrum, 1285 ((M − H + Na)$^+$), 1261 ((M − H)$^+$), 994 ((M − 3′-*O*-acetylthymidine)$^+$), 960 ((M − DMTr)$^+$).

Reactivity toward Electrophiles—Alkylation and Dealkylation of Phosphorodithioates. Alkylation with ethyliodide. *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *O*-(3′-*O*-acetylthymidin-5′-yl) phosphorodithioate (**9**) was dissolved in D_2O (0.5 mL) and ethyl iodide (0.1 mL) in ethanol (0.5 mL) was added. No reaction was observed at room temperature. Upon heating to 55 °C for 1 h, alkylation to the phosphorodithioate triester occurred: ^{31}P NMR (D_2O) δ 97.4 ppm, >95% yield based upon NMR; R_f = 0.69 (triethylamine–methanol–chloroform, 1:14:85, v/v/v). The phosphorodithioate triester was stable under these reaction conditions for 18 h and therefore used to examine reactivity toward hard nucleophiles.

Reactivity of Dinucleoside Phosphorodithioate Triesters toward Hard Nucleophiles. *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *S*-ethyl *O*-thymidin-5′-yl phosphorodithioate was dissolved in water (0.4 mL) and 1.0 M sodium hydroxide (0.1 mL) added. After 30 min at room temperature, the reaction mixture was neutralized with 80% acetic acid (0.11 mL) and evaporated in vacuo. ^{31}P NMR analysis indicated complete hydrolysis to a phosphorothioate diester (54.8 and 54.6 ppm, two diastereomers).

Reactivity of Dinucleoside Phosphorodithioate Triesters toward Soft Nucleophiles. *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *S*-(4-chlorobenzyl) *O*-(3′-*O*-acetylthymidin-5′-yl) phosphorodithioate (**8a**) and *O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *S*-(2,4-dichlorobenzyl) *O*-(3′-*O*-acetylthymidin-4′-yl) phosphorodithioate (**8c**) were treated with a freshly prepared solution consisting of thiophenol–triethylamine–dioxane (1:1:2, v/v/v). Aliquots were removed at various times (1, 2, 4, 6, 10, 20, 60, 90, and 120 min) and analyzed by TLC in CH_3CCl_3–CH_3OH (4:1, v/v) as described under Kinetics of Phosphorothioite Triester Formation. The results gave half-times ($t_{1/2}$) of 12 and 3 min for the removal of the 4-chlorobenzyl and 2,4-dichlorobenzyl groups, respectively. There were no detectable cleavages (95% sensitivity) of internucleotidic bonds within 120 min.

Stability of S-Protected Phosphorodithioates to DNA Synthesis Conditions.[44] (1) Detritylation. Compound **8c** (1.3 mg) was treated with 1% trifluoroacetic acid in dichloromethane (1 mL) for 90 min. The dimer was stable when analyzed by TLC (R_f = 0.51 in triethylamine–methanol–chloroform, 1:14:85, v/v/v) and ^{31}P NMR (94.6, 93.7 ppm in D_2O). (2) Oxidation. Compound **8c** (2.2 mg) was treated with 0.1 M I_2 in water–2,6-lutidine–tetrahydrofuran (2:1:2, v/v/v) for 90 min. Decomposition was not observed by TLC. (3) Carbon disulfide. Because carbon disulfide is a commonly used solvent for sulfurization of trialkylphosphites and trialkylphosphorothioites, it was tested toward reactivity with phosphorodithioate triesters. Compound **8c** (2 mg) was treated with carbon disulfide (0.2 mL) and acetonitrile (0.35 mL) for 60 min. It was stable in this solvent when analyzed by TLC and ^{31}P NMR. (4) Pyridinium tetrafluoroborate. Compound **8a** (2 mg) was treated with a 0.2 M solution (0.35 mL) of pyridinium tetrafluoroborate in acetonitrile. No changes were observed when the reaction mixture was analyzed by ^{31}P NMR. (5) Capping. Compound **8a** (3 mg) was treated with 0.5 mL of 4-(*N*,*N*-dimethylamino)pyridine–THF (3.25:46.75, v/v) and 0.1 mL of acetic anhydride–2,6-lutidine (1:1, v/v). No changes were observed (^{31}P NMR).

Reaction of 2′-Deoxynucleosid-3′-yl Phosphoramidites with Thiols and Alcohols. *O*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidin-3′-yl] *O*-(β-cyanoethyl) *N*,*N*-diisopropylphosphoroamidite (**10**; 0.13 mmol, 0.099 g) was dissolved in acetonitrile (0.4 mL) and the ^{31}P NMR recorded (147.5 ppm). 2-Mercaptoethanol (0.16 mmol, 0.011 mL, ca. 12.3 mg) was added without observing a reaction (^{31}P NMR). Addition of tetrazole (0.15 mmol, 0.3 mL of a 0.5 M solution in acetonitrile, ca. 10.65 mg) catalyzed a reaction leading to phosphite **11** (>95%) within 20 min (^{31}P NMR δ 138.4 and 138.1). Addition of sulfur (0.5 mL of a 0.5 M elemental sulfur in toluene–2,6-lutidine, 19:1, v/v) converted the phosphite to the phosphorothioate triester (^{31}P NMR δ 66.1). Purification by silica gel column chromatography (0–10% methanol in dichloromethane) yields 0.075 g: ^{1}H NMR δ 7.54 (s, 1 H, H_6), 7.38–7.24 (m, 9 H, H_2 and H_6 of anisyl, phenyl), 6.82 (d, J_{HH} = 8.4 Hz, 4 H, H_3 and H_5 anisyl), 6.40 (m, 1 H, $H_{1'}$), 5.29 (m, 1 H, $H_{3'}$), 4.25–4.09 (m, 3 H, $H_{4'}$ + α-CH_2 (β-cyanoethyl)), 3.83 (s, 6 H, CH_3 (anisyl)), 3.42 (s, 2 H, $H_{5'}$), 2.72–2.41 (m, 3 H, $H_{2'}$ + β-CH_2 (β-cyanoethyl)), 1.43 (s, 3 H, CH_3 of thymine); ^{31}P NMR δ 66.8. With the same reaction procedure with a 1:1 mixture (molar equivalents of ethanol and ethanethiol) and tetrazole as catalyst, the phosphite triester was observed and characterized after oxidation to the phosphorothioate.

Results and Discussion

Synthesis of Deoxydinucleoside Phosphorodithioates via Deoxynucleoside Diamidites. Phosphordiamidites have been used in the polynucleotide field for the preparation of deoxynucleoside phosphoramidites[45,46] and, as deoxynucleoside phosphordiamidites, for the synthesis of deoxydinucleoside phosphoramidites.[7,47,48] Here procedures are described for using deoxynucleoside phosphordiamidites for the synthesis of deoxydinucleoside phosphordithioates (Figure 1). The first step is synthesis of the deoxynucleoside phosphordiamidite **3** from an appropriately protected deoxynucleoside **1** and bis(diisopropylamino)chlorophosphine (**2**).

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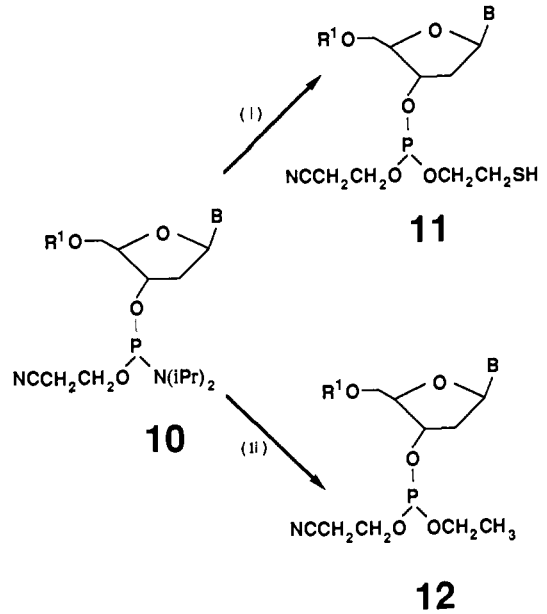

Figure 2. Reactions of deoxynucleoside phosphoramidites with alcohols and mercaptans. Reaction conditions: (i) 2-mercaptoethanol and tetrazole; (ii) ethanol, ethanethiol, and tetrazole. Abbreviations: B, thymine; iPr, isopropyl.

The resulting deoxynucleoside phosphordiamidite reacts without isolation with a 3′-protected deoxynucleoside **4** to yield the deoxydinucleoside phosphoramidite **5**. Compound **5** as the diisopropylphosphoramidite is then activated with tetrazole and reacted with H_2S or a mercaptan to yield, respectively, the hydrogen phosphonothioate **6** or the phosphorothioite triester **7a,b**. Further oxidation of **7a** or **7b** with sulfur yields the completely protected deoxydinucleoside phosphorodithioate triester **8a,b**. The 4-chlorobenzyl mercaptan derivative proved to be superior to the 2-chlorobenzyl compound. Based upon ^{31}P NMR, the yield of **8a** was higher and it could be more easily purified from side products by column chromatography. Of the two routes shown in Figure 1, this pathway is preferred as iv and v can be combined without an intermediate isolation step. Synthesis of **6** proceeds to completion with 1 h in the presence of 2 equiv of tetrazole but the reaction must be carefully monitored as the resulting H-phosphonothioate is unstable toward prolonged exposure to these conditions. For example, in model experiments with *O*-[5′-*O*-(4,4′-dimethoxytrityl)4-*N*-benzoyl-2′-deoxycytidin-3′-yl] *O*-(β-cyanoethyl) *N,N*-diisopropylphosphoramidite, tetrazole, and H_2S, the H-phosphonothioate initially forms in high yield but then degrades slowly (ca. 60% remains after 16 h at room temperature as measured by ^{31}P NMR) to various unidentified compounds. This instability also appears to be base dependent as the 4-*N*-benzoylcytosine derivative decomposes more rapidly than the thymine compound. Generation of the fully protected deoxydinucleoside phosphorodithioate from **6** proceeds in two steps. First oxidation with sulfur yields **9** which, after isolation, is then alkylated with α,2,4-trichlorotoluene to yield **8c**. Although this is the more lengthy route, proceeding through **6** has more versatility as deoxydinucleoside phosphorothioamidates, phosphorothioate triesters, and phosphorothioate diesters can be synthesized in addition to deoxydinucleoside phosphorodithioates merely by oxidation of **6** with iodine and an amine, alcohol, or water, respectively.[20]

In order to investigate the relative reactivity of deoxynucleoside phosphoramidites toward alcohols and thiols, two competition experiments were performed (Figure 2). The reaction of *O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *O*-(β-cyanoethyl) *N,N*-diisopropylphosphoramidite (**10**) with 2-mercaptoethanol in the presence of tetrazole shows only attack from the oxygen nucleophile. Thus, the O-phosphite **11** is the end product of the thermodynamically controlled reaction. Similarly, when the same model deoxynucleoside phosphoramidite reacts with a 1:1 (molar equivalents) mixture of ethanol and ethanethiol in the presence of tetrazole, only the O-phosphite **12** is present. This result indicates that attack from the oxygen nucleophile is also favored in the kinetically controlled reaction. These observations demonstrate a need for anhydrous conditions and carefully controlled handling of **5** so as to prevent inadvertent hydrolysis or oxidation during synthesis of **7a**, **7b**, or **6**.

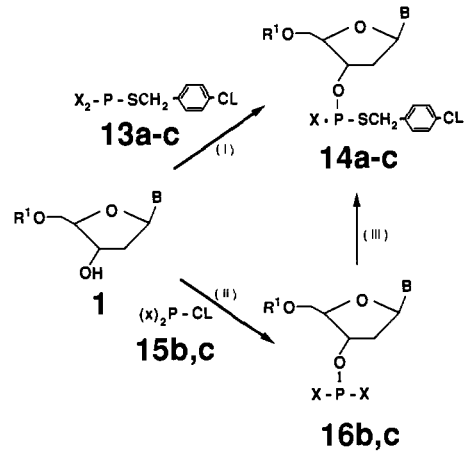

Figure 3. Synthesis of deoxynucleosid-3′-yl *S*-(4-chlorobenzyl) phosphorothioamidites. Reaction conditions: (i) tetrazole; (ii) triethylamine; (iii) 4-chlorobenzyl mercaptan. Abbreviations: X, diisopropylamino (**13a**, **14a**); X, pyrrolidino (**13b**, **14b**, **15b**, **16b**); X, dimethylamino (**13c**, **14c**, **15c**, **16c**).

Synthesis of Deoxydinucleoside Phosphorodithioates via Deoxynucleoside 3′-Phosphorothioamidites. Although deoxydinucleoside phosphorodithioates can be synthesized from deoxynucleoside phosphordiamidites as outlined in Figure 1, the procedure has certain critical limitations relative to its use in synthesizing DNA. This is primarily because the highly reactive deoxynucleoside phosphordiamidites, which are most useful for DNA synthesis, cannot be stored or purified, and must be used immediately after synthesis. As a consequence, it is difficult to develop a DNA synthesis procedure using these compounds as mononucleotide synthons. One could, however, prepare all 16 deoxydinucleoside phosphorodithioates (**8a** or **8c**) and use these synthons for introducing the phosphorodithioate internucleotide linkage at any defined site in DNA. The procedure, however, requires considerable preparation of precursors and lacks the flexibility needed for a general synthesis method. For these reasons and because of success using deoxynucleoside 3′-phosphoramidites to synthesize unmodified DNA,[49] deoxynucleoside 3′-phosphorothioamidites were studied as intermediates for preparing phosphorodithioate DNA. The objective was to develop a stable, highly reactive deoxynucleoside 3′-phosphorothioamidite that could be used to introduce the phosphorodithioate linkage rapidly and quantitatively into any predetermined position in a deoxyoligonucleotide.

Because of earlier success in DNA synthesis using deoxynucleoside 3′-diisopropylphosphoramidites,[50] the corresponding phosphorothioamidites were initially examined as synthons (Figure 3). These compounds, the deoxynucleoside 3′-(diisopropylphosphorothioamidites) **14a** were available by condensation of a suitably protected deoxynucleoside (**1**) with *S*-(4-chlorobenzyl) *N,N,N′,N′*-tetraisopropylphosphorothiodiamidite (**13a**) in the presence of 2 equiv of tetrazole. The products were remarkably stable toward acid hydrolysis and air oxidation. For example they could be purified by silica gel column chromatography and then stored dry indefinitely without degradation or oxidation. This route was also attractive because **13a** could be synthesized as an air-stable, crystalline compound by condensation of bis(diisopropylamino)chlorophosphine and sodium (4-chlorobenzyl)-mercaptide.

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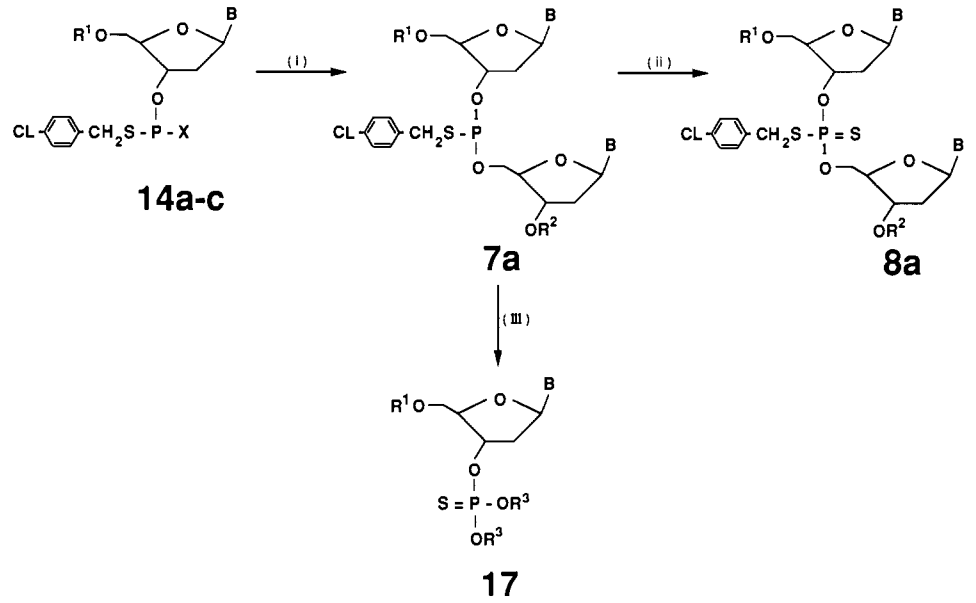

Figure 4. Synthesis of deoxydinucleoside phosphorodithioates and deoxytrinucleoside phosphorothioates via deoxynucleoside 3′-phosphorothioamidites. Reaction conditions: (i) pyridinium tetrafluoroborate (**14a**) or tetrazole (**14b**, **14c**); (ii) sulfur; (iii) tetrazole + 3′-*O*-acetylthymidine followed by sulfur. Abbreviations: R^3, 3′-*O*-acetylthymidin-5′-yl.

Table I. Acid Activation Studies with Deoxynucleoside 3′-Diisopropylphosphorothioamidites **14a**[a]

activator	equiv	time, min	results
N-methylanilinium trifluoroacetate	2	12	stable intermediate, detritylation during coupling
N-methylanilinium trifluorotetrazolide, 2 trifluoroacetate, and tosylate	2	12	stable intermediate, detritylation during coupling
N-methylimidazolinium trifluoroacetate	5	20	detritylation during coupling, trace of product
tetrazole	5	20	incomplete reaction after 20 min
5-(4-nitrophenyl)tetrazole	2	10	low solubility in acetonitrile, incomplete reaction
pyridinium tetrafluoroborate	2	10	high yield of product in 20 min with minimum detritylation and side-product formation
4-(*N*,*N*-dimethylamino)pyridinium trifluoroacetate	5	20	trace of product

[a] All coupling reactions were performed in a 0.1 M acetonitrile solution of deoxynucleoside phosphorothioamidite and 3′-*O*-acetylthymidine. The amount of activator is listed in the table.

Synthesis of deoxydinucleoside phosphorodithioates from **14a** (Figure 4), however, proved to be less than satisfactory. This was because **14a** could not be rapidly activated with tetrazole (Table I and Figure 5) under conditions where the corresponding deoxynucleoside 3′-(diisopropylphosphoramidite) was very reactive (complete conversion to the dinucleotide in 1 min or less).

In an attempt to identify an appropriate activator for this condensation, several acids were screened (Table I). Most, however, were unsatisfactory as conversion to product was slow or incomplete (Figure 5) and the prolonged reaction times promoted formation of several side products via oxidation and hydrolysis. Activators such as *N*-methylanilinium trifluoroacetate and *N*-methylanilinium(trifluoromethyl)tetrazolide caused excessive detritylation whereas 4-(*N*,*N*-dimethylamino)pyridinium trifluoroacetate and *N*-methylimidazolinium trifluoroacetate were not acidic enough to be useful. The latter was also volatile and hygroscopic. 5-(4-Nitrophenyl)tetrazole was unattractive due to its low solubility in acetonitrile, a solvent commonly used in internucleotide coupling, and tetrazole, because of insufficient acidity, routinely failed to catalyze complete conversion to product. Pyridinium tetrafluoroborate was the most satisfactory of these activators as internucleotide thiophosphite triester formed within 10 min in satisfactory yield.

During these attempts to activate **14a** with various acids, a large number of side reactions were observed. For example, although **14a** is an air-stable solid, it is very susceptible to air oxidation in acidic solutions. The resulting oxidized products do not interfere with coupling reactions but the amount of **14a** available for synthesis is reduced. This leads to a lower yield of **7a**. Similarly, the product of this reaction, a deoxydinucleoside phosphorothioite triester (**7a**), is equally sensitive to air oxidation and hydrolysis

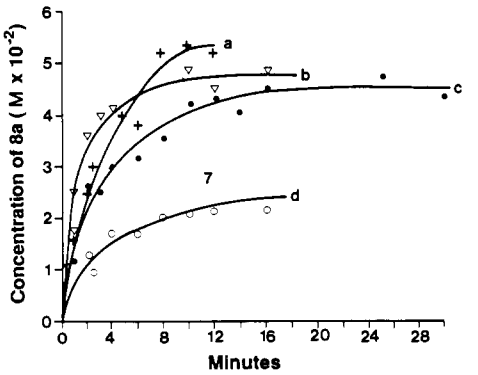

Figure 5. Activation of *O*-[5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl] *S*-(4-chlorobenzyl) *N*,*N*-diisopropylphosphorothioamidite (**14a**) in the presence of 3′-*O*-acetylthymidine. The deoxynucleosides were present in 0.1 M concentrations. The resulting deoxydinucleotide was analyzed as its phosphorodithioate after quenching of an aliquot of the reaction mixture with pyridine and elementary sulfur at the appropriate time points (see Experimental Section for details). Several activation conditions were used: (a) pyridinium tetrafluoroborate (2 equiv), (b) 5-(4-nitrophenyl)tetrazole (2 equiv, slurry in acetonitrile), (c) tetrazole (5 equiv), (d) *N*-methylimidazolidinium trifluoroacetate (5 equiv).

in the presence of acid activators. For these reasons, several of the more acidic activators could not be used. Even tetrazole is sufficiently acidic to yield these side products if reaction mixtures are not maintained free of oxygen and are allowed to proceed for long time periods (hours).

These various side reactions and the extreme precautions required to eliminate molecular oxygen from reaction mixtures

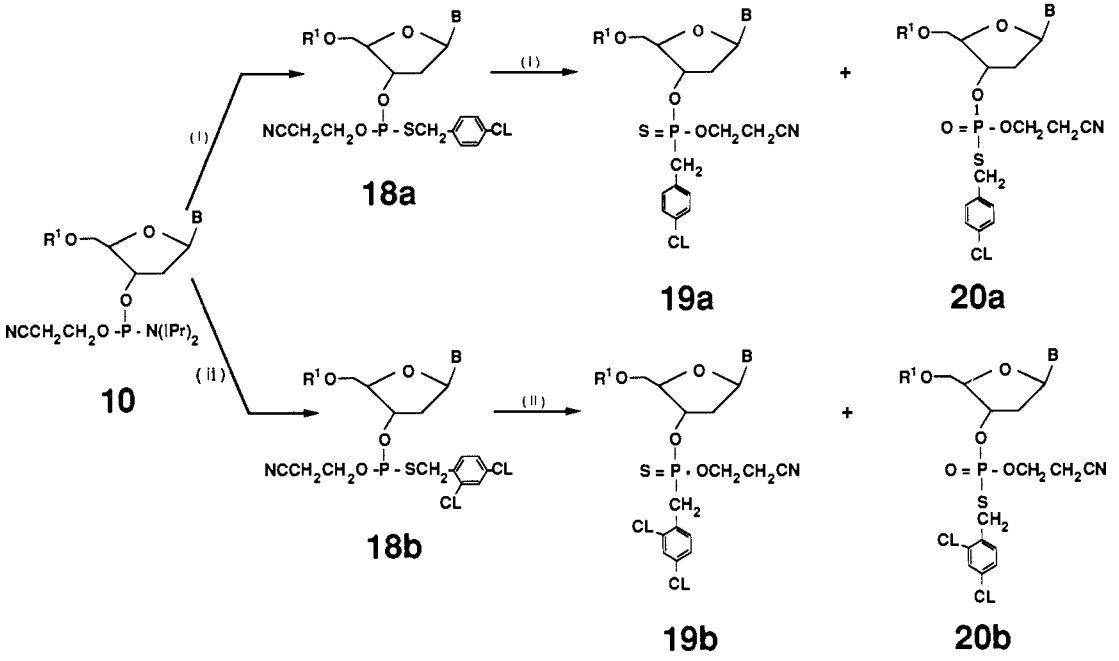

Figure 6. Arbuzov reactions with deoxynucleoside derivatives. reaction conditions: (i) tetrazole and 4-chlorobenzyl mercaptan; (ii) tetrazole and 2,4-dichlorobenzyl mercaptan.

suggested that even pyridinium tetrafluoroborate was only marginally useful as an activator. This conclusion was reinforced by the small but detectable amount of detritylation that always occurred with this activator (approximately 5% as estimated by TLC analysis). Detritylation would limit this activator to nonpolymer support synthesis procedures where side products can be removed by column chromatography.

Because of these difficulties, more reactive synthons that could potentially be activated with weaker acids were examined. Both the deoxynucleoside 3′-(tetramethylenephosphorothioamidite) **14b** and 3′-(*N*,*N*-dimethylphosphorothioamidite) **14c** were found to be satisfactory as they were rapidly activated with tetrazole to yield the deoxydinucleoside phosphorothioite triester (Figure 4, **7a**) in high yield.

The synthesis of **14b** or **14c** could not be completed by the same pathway developed for **14a** (Figure 3). This was because the bis(pyrrolidino)-*S*-(4-chlorobenzyl)thiophosphine (**13b**) or bis-(*N*,*N*-dimethylamino)-*S*-(4-chlorobenzyl)thiophosphine (**13c**) could not be crystallized or obtained pure by fractional distillation. Consequently, impure **13b** or **13c** and **1** yielded complex mixtures of reaction products, which, because **14b** and **14c** were unstable toward silica gel, could not be separated by column chromatography. A second, equally unsuccessful route, involved using dichloro(pyrrolidino)phosphine or dichloro-(*N*,*N*-dimethylamino)-phosphine as the phosphitylating reagent. In this pathway, the dichlorophosphine was first condensed with deoxynucleoside and then mercaptan. This route was of little value because the symmetrical deoxydinucleoside was the predominant product irrespective of temperature (room temperature to −40 °C), the relative mole ratios of mercaptan and deoxynucleoside, or whether the deoxynucleoside and mercaptan were added sequentially to the dichlorophosphine. Attempts to purify these complex product mixtures also failed primarily because of decomposition on silica gel columns.

A third, much more successful procedure was based upon the use of deoxynucleosid-3′-yl *N*,*N*-tetramethylenephosphorodiamidites **16b** or dimethylphosphorodiamidites **16c** as intermediates. This route has several novel features as it leads to the desired products **14b** or **14c**) in high isolated yield (75–85%) and purity via a one-flask procedure that does not require silica gel column chromatography. The synthesis begins by forming the deoxynucleosid-3′-yl phosphorodiamidite (**16b** or **16c**) from the protected deoxynucleoside and **15b** or **15c** in the presence of triethylamine. The products react without isolation with 4-chlorobenzyl mercaptan to yield the deoxynucleoside thioamidites (**14b** or **14c**).[51] The acid catalyst driving the second step is the amine hydrochloride derived from synthesis of the diamidite. The major side product (**13b** or **13c**), which is formed from excess **15b** or **15c** and mercaptan, can be removed during the aqueous workup and precipitation of the product.

Deoxydinucleoside phosphorodithioates can then be synthesized from **14b** or **14c**, 3′-*O*-acetylthymidine, and tetrazole as activator. After 1-5 min, the reaction is quenched with base to yield **7a** (Figure 4) and the product further converted via oxidation with elemental sulfur to yield the dinucleoside phosphorodithioate triester **8a**. These results show that both **14b** and **14c** are much more reactive than **14a** under the same conditions. Assuming that the phosphorus–nitrogen bonding is the same for **14a–c**, enhanced reactivity would appear to be due to the reduced steric bulk surrounding phosphorus in **14b** and **14c**. This is because diisopropylamine is more basic than dimethylamine but **14a** reacts more slowly than **14c**. Furthermore, the enhanced basicity of pyrrolidine as compared to dimethylamine does not appear to have a detectable influence on the reactivity of **14b** and **14c**. Both have similar reactivities under the conditions tested.

Tetrazole activation of **14b** or **14c** appeared to generate very few side products. For example, during the time required for complete reaction to form **7a**, tetrazole did not catalyze oxidation of **14b**, **14c**, or **7a** or the hydrolysis of **7a** (analyzed by ^{31}P NMR). Two potential additional side reactions, nucleophilic substitution on phosphorothioite triesters and Arbuzov rearrangements, were carefully investigated. When **7a** was treated with 1 equiv of 3′-*O*-acetylthymidine and tetrazole, a trinucleoside phosphite triester formed (characterized after oxidation with sulfur to the phosphorothioate triester **17**. However the half-life for tetrazole-catalyzed displacement of the mercaptan is 12 h at room temperature. As a consequence, this reaction has little significance as coupling reactions are complete in 5 min or less and the thiophosphite is immediately oxidized to the phosphorodithioate **8a**. In order to test the significance of potential tetrazole-catalyzed Arbuzov rearrangements, the *S*-(4-chlorobenzyl) and *S*-(2,4-di-

(51) The (2,4-dichlorobenzyl)thio derivatives corresponding to **14b** and **14c** have been prepared analogously by Y.-X. Ma and J.-Y. Tang and used to synthesize oligonucleotides containing the phosphorodithioate linakge (see ref 32). This sulfur protecting group is preferred over the 4-chlorobenzyl because it is removed rapidly by thiophenolate ($t_{1/2}$ = 3 min; see Experimental Section) without detectable cleavage at the 5′-carbon.

chlorobenzyl) derivatives of O-[5'-O-(4,4'-methoxytrityl)thymidin-3'-yl], O-(β-cyanoethyl) thiophosphite were prepared and kept at room temperature in a 1 M solution of tetrazole for several hours (Figure 6). When monitored by ^{31}P NMR, Arbuzov rearrangement of the 4-chlorobenzyl group of **18a** to yield **19a** occurred with a $t_{1/2}$ = 20 min whereas the 2,4-dichlorobenzyl derivative rearranged with $t_{1/2}$ = 150 min. During these prolonged exposures to tetrazole, a small fraction (see Experimental Section) of **18a** or **18b** was oxidized to the deoxynucleoside phosphorothioate (**20a** and **20b**, respectively). The amount of oxidation was dependent upon the oxygen content of solvents and the atmosphere over the reaction mixture. Surprisingly, when **7a** was tested under these same conditions, it did not rearrange detectably to the (4-chlorobenzyl) thiophosphonate in 3 h. These results demonstrate that Arbuzov rearrangements are not significant side reactions during the time (5 min) required to synthesize a thiophosphite linkage. They also are in agreement with earlier observations that dinucleotide methylphosphites do not undergo Arbuzov reactions with iodine or iodomethane[3,9,44] even though phosphites bearing simple alkyl groups readily rearrange with these reagents.

Conclusions

Two methods were developed for synthesizing deoxydinucleoside phosphorodithioates. With deoxynucleoside 3'-phosphordiamidites, the intermediate H-phosphonothioate could be used to synthesize deoxydinucleoside phosphorothioamidates, phosphorodithioates, and phosphorothioate triesters in addition to phosphorodithioate triesters. Thus, this approach can be used for introducing several unique analogues bearing specific reporter groups into DNA. This may well be the long-term utility of the method. The second approach, whereby deoxynucleoside 3'-(*N*,*N*-dimethylphosphorothioamidites) or deoxynucleoside 3'-(*N*,*N*-tetramethylenephosphorothioamidites) are used as synthons, appears to be more useful for the synthesis of dithioate oligonucleotides as the intermediates are readily activated with tetrazole, are stable toward storage, and yield deoxydinucleoside phosphorodithioates rapidly without forming detectable side products. Deoxydinucleoside phosphorodithioate triesters were found to be stable to all the current conditions used for the repetitive synthesis of DNA on polymeric supports (acid removal of the dimethoxytrityl group, acylation with acetic anhydride and (dimethylamino)-pyridine, and iodine oxidation) and could be freed of protecting groups by using reagents (triethylammoniumthiophenolate and concentrated ammonium hydroxide) compatible with the synthesis of natural internucleotide linkages.

Recently deoxynucleoside 3'-phosphorothioamidites have been used to synthesize several oligonucleotides with both phosphorodithioate and natural internucleotide linkages.[32] Biochemical and biological reports, which are now beginning to appear in abbreviated form,[52,53] demonstrate that DNA containing phosphorodithioate linkages mimic natural DNA in certain important properties such as forming duplexes with complementary deoxyoligonucleotides, stimulating RNase H activity in Hela cell nuclear extracts and the binding of *lac* repressor and *cro* repressor to their respective operators. Phosphorodithioate DNA has certain unique and very useful properties as well. These include complete resistance to cellular nucleases, ease of derivatization with various reporter groups such as fluoresceine and biotin, and as an extremely potent inhibitor of HIV reverse transcriptase. This combination of observations suggests a large number of potential applications for phosphorodithioate-linked DNA in biology and biochemistry. Undoubtedly the value of these compounds as research tools will continue to grow in the years to come.

Acknowledgment. We thank Mr. Ron Sadecky and Dr. Robert Barkley for recording the FAB mass spectra.

Supplementary Material Available: Spectral data for the synthesis of **14a-c** and **8a** (10 pages). Ordering information is given on any current masthead page.

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(+)-CC-1065 DNA Alkylation: Key Studies Demonstrating a Noncovalent Binding Selectivity Contribution to the Alkylation Selectivity

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Abstract: A comparative study of the selectivity and relative intensity of DNA alkylation with a series of (+)-CC-1065 analogues is detailed. The results of the study (1) reveal a nonselectivity of the simple alkylation event in the absence of noncovalent binding selectivity, (2) highlight the enhanced selectivity of the alkylation in the event of noncovalent binding selectivity, (3) demonstrate that a DNA autocatalytic phosphate activation of the alkylation reaction (Lewis acid complexation/protonation) may not be uniquely responsible for the nonselective or selective alkylations, and (4) address the assumption that stereoelectronic effects associated with the (+)-CC-1065 cyclopropane alkylation contribute uniquely to the alkylation selectivity.

(+)-CC-1065 (**1**), a potent antitumor antibiotic and the subject of extensive investigations,[2-4] has been shown to exert its effects through the sequence selective alkylation of DNA. In initial and continued investigations, the Upjohn group in collaboration with

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