Synthesis and Characterization of Oligonucleotides Containing 2′-*S*,3′-*O*-Cyclic Phosphorothiolate Termini

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Sulfur substitution of the oxygen atoms in nucleic acids has been widely used to study nucleic acid structure and function and the mechanisms of catalysis employed by ribozymes and enzymes that modify nucleic acids. In the case of metalloenzymes that catalyze phosphoryl transfer reactions, sulfur substitution of the oxygen atoms of the substrates and products is an especially powerful tool for mapping ground state and transition state interactions at the active site and for determining whether these interactions are mediated by metal ions.[1−8] A specific oxygen atom is identified as a ligand for a metal ion when sulfur substitution of that atom shifts the metal ion specificity of the metalloenzyme to a more thiophilic metal ion (for example, from Mg^{2+} to Mn^{2+}).[1] Accordingly, for those enzymes and ribozymes that catalyze site specific cleavage of RNA by facilitating attack of the 2′-hydroxyl on its neighboring phosphodiester (e.g., RNases and the hammerhead and HDV ribozymes), analogues of the substrate and product, in which the 2′-oxygen at the cleavage site is replaced by sulfur, should provide valuable probes for studying metal interactions with the nucleophile. Toward this goal, we recently described a method to install 2′-mercaptonucleosides into oligonucleotides, allowing the synthesis of substrate analogues.[9] To complement these studies, we now report methods for the synthesis of the corresponding product analogues—oligonucleotides containing 2′-*S*,3′-*O*-cyclic phosphorothiolate termini.

There are two examples of mononucleoside 2′-*S*,3′-*O*-cyclic phosphorothiolates reported in the literature. Patel et al. prepared the cytidine analogue by treating 2,2′-anhydro-1-*β*-D-arabinocytosine with P_2S_5 followed by incubation in pyridine/water and treatment of the resulting product with iodine.[10,11] Dantzman and Kiessling prepared the uridine analogue by transphosphorylation of 2′-deoxy-2′-mercaptouridine 3′-(*p*-nitrophenyl phosphate), whereby the 2′-sulfur attacks the activated neighboring phosphodiester and displaces *p*-nitrophenol.[12] Unfortunately, neither of these approaches is readily adaptable to the installation of a 2′-*S*,3′-*O*-cyclic phosphorothiolate at the 3′ terminus of oligonucleotides. Although oligonucleotides containing a 2′-mercaptonucleoside are synthetically accessible,[9] they cannot serve as precursors for cyclic phosphorothiolate formation via the transphosphorylation pathway because of the weak nucleophilicity of sulfur at nonactivated phosphate centers[12] and the tendency of the 2′-sulfur to attack the 1′-carbon and displace the heterocyclic base.[9] However, Liu et al. and Weinstein et al. showed that 3′-*S*-phosphorothiolate linkages are 2−3 orders of magnitude more susceptible to nucleophilic attack by the adjacent 2′-hydroxyl than the 3′-*O*-phosphate linkage in normal RNA, presumably due to geometric factors.[13,14] If a 2′-*S*-phosphorothiolate linkage is similarly susceptible to nucleophilic attack by its neighboring 3′-hydroxyl group, then an oligonucleotide containing this kind of linkage might be an effective precursor to a 2′-*S*,3′-*O*-cyclic phosphorothiolate. Here we describe a general method for the synthesis of oligonucleotides containing a 2′-*S*-phosphorothiolate linkage and show that this linkage reacts under alkaline conditions to form an oligonucleotide 2′-*S*,3′-*O*-cyclic phosphorothiolate.

The preparation of ribonucleoside phosphoramidite building blocks for standard oligonucleotide synthesis requires protection of the 5′- and 2′-oxygens as 4,4′-dimethoxytrityl (DMTr) and *tert*-butyldimethylsilyl (TBDMS) ethers, respectively, and phosphitylation of the 3′-oxygen. Using the same 5′ and 2′ protecting groups, Sun et al. prepared 3′-thioribonulceoside 3′-*S*-phosphoramidites and used them to install 3′-*S*-phosphothiolate linkages into oligonucleotides.[15] Analogously, we approached the installation of 2′-*S*-phosphorothiolate linkages into oligonucleotides using 2′-mercaptonucleoside 2′-*S*-phosphoramidites, in which the 5′- and 3′-hydroxyl groups were protected as DMTr and TBDMS ethers, respectively. Attempts to protect the 3′-oxygen of 4-*N*-benzoyl-5′-*O*-(dimethoxytrityl)- 2′-deoxy-2′-(tritylthio)cytidine as a TBDMS ether failed even in the presence of strong base catalysts presumably due to steric hindrance from the large trityl group. Instead, we first converted the 2′-tritythio moiety to a 2′-pyridyl disulfide moiety and synthesized 4-*N*-benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxy-2′-thiocytidin-2′-yl *β*-cyanoethyl *N*,*N*-diisopropylphosphoramidite **6** according to Scheme 1. Treatment of 4-*N*-benzoyl-2′-deoxy-2′-(tritylthio)cytidine (**1**)[9] with $AgNO_3$ followed by Aldrithiol-2 gave the disulfide **2** in 83% yield (Scheme 1). Reaction of **2** with DMTrCl in pyridine using DMAP as a catalyst gave **3** in 85% yield. Treatment of **3** with a large excess of

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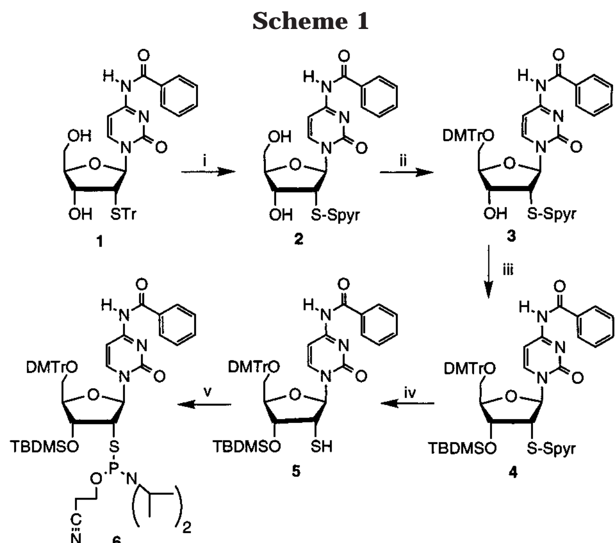
Scheme 1



i) $CHCl_3$:MeOH (1:2), $AgNO_3$, aldrithiol-2. ii) DMTrCl, DMAP, pyridine. iii) TBDMSCl, imidazole, pyridine. iv) DTT, TEA, THF. v) 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite, methylimidazole, *i*-Pr_2NEt, CH_2Cl_2.

imidazole and TBDMSCl in a small volume of pyridine afforded **4** in 86% yield. The disulfide was reduced with dithiothreitol (DTT) to generate the 2′-mercapto derivative **5** in 95% yield, which was phosphitylated to the phosphoramidite **6** in 90% yield (Scheme 1).

The thiophosphoramidite was then used to install a 2′-*S*-phosphorothiolate linkage within a DNA tetramer ($^{5'}TC_{2'SP}TU^{3'}$). We chose to incorporate 2′-deoxyribose residues at positions flanking the modified linkage to prevent cleavage at these sites during exposure of the oligonucleotide to acid or base. As described previously for the installation of 3′-thionucleosides into oligonucleotides, the thiophosphoramidite **6** was coupled manually using *p*-nitrophenyltetrazole as an activator.[15] As a control oligonucleotide, we synthesized a tetramer containing a 2′-*O*-phosphodiester linkage ($^{5'}TC_{2'OP}TU^{3'}$) using 4-*N*-benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)cytidin-2′-yl β-cyanoethyl *N*,*N*-diisopropylphosphoramidite. Both oligonucleotides were deprotected using standard conditions: treatment with NH_4OH:EtOH (3:1) for 16 h at 55 °C to remove the phosphate and heterocyclic protecting groups and treatment with tetrabutylammonium fluoride (TBAF; 1 M in THF) for 36 h at room temperature to remove the 3′-silyl protecting group.

The tetramers were incubated with polynucleotide kinase and [γ-^{32}P]-ATP to generate the corresponding 5′-[^{32}P]-oligonucleotides **7a** and **7b**, which were purified by polyacrylamide gel electrophoresis (PAGE). Because the 2′-*S*-phosphorothiolate linkage is expected to be susceptible to nucleophilic attack by the adjacent 3′-hydroxyl group, we eluted **7a** and **7b** from the electrophoresis gel into a low pH buffer (pH 5.5) at 4 °C and carried out subsequent workup procedures at pH 5.5.

The presence of a bridging sulfur in tetramer **7b** was confirmed by treatment with Ag^+, which specifically cleaves bridging phosphorothiolates but leaves normal phosphodiester linkages intact.[16] As expected, silver treatment did not affect **7a** (Figure 1, lane 1) but quantitatively converted **7b** to a faster migrating species, presumably the 2′-mercapto derivative **11** (Figure 1, lane 10; Scheme 2). To determine the feasibility of using 2′-*S*-phosphorothiolate-linked oligonucleotides to generate 2′-*S*,3′-*O*-cyclic phosphorothiolate termini, we examined the alkaline cleavage reactions of **7a** and **7b** (Figure 1). The reactivity of 2′-5′-linked RNA **7a** under basic conditions (Figure 1, lanes 3−8) is expected to be similar to the well-documented pathway by which natural RNA reacts: the neighboring hydroxyl group attacks the phosphodiester to form a 2′,3′-*O*-cyclic phosphate **8a** (the slower migrating band) that then hydrolyzes to a mixture of 2′- and 3′-monophosphates, **9a** and **10a**, respectively (the faster migrating band).[17−19] Consistent with these assignments, the band corresponding to **8a** decreases in intensity relative to that for **9a**/**10a** upon longer exposure to alkaline conditions, suggesting that the former is converted to the latter. As observed previously for the reaction of 3′-*S*-phosphorothiolate linkages under basic conditions,[13−15] the 2′-*S*-phosphorothiolate linkage was much more susceptible to alkaline cleavage than the corresponding phosphate linkage. In 0.01 M NaOH, **7a** required heating at 90 °C (Figure 1, lanes 3−8) to show a rate of cleavage comparable to that of **7b** at 30 °C (lanes 12−17). Under identical conditions (0.1 M NaOH and 50 °C), **7b** reacts completely within 10 min (Figure 1, lane 18) whereas **7a** remains mostly unreacted after 10 min (lane 9). The initial stages of the reaction of **7b** at 30 °C give rise predominately to a product that migrates in a manner similar to that of the cyclic phosphate product **8a** (derived from **7a**) and is presumably the 2′-*S*,3′-*O*-cyclic phosphorothiolate **8b**. As the reaction proceeds, two additional radioactive bands form: one that migrates faster than **8b** and in a manner similar to that of **9a**/**10a** and one that migrates slower than **8b** and in a manner similar to that of **11**. The former product presumably arises from hydrolysis of the 2′-*S*,3′-*O*-cyclic phosphorothiolate, which is expected to yield exclusively the 2′-*S*-phosphorothiolate monoester **10b**.[20] The latter

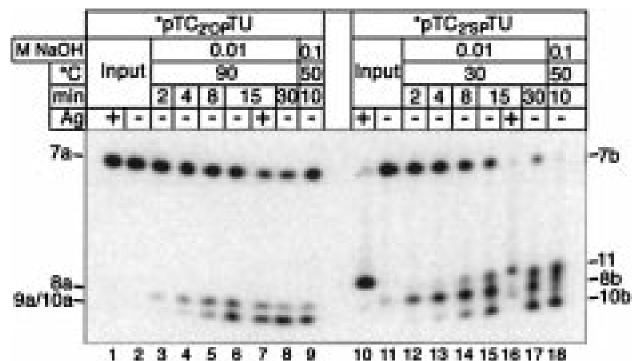


Figure 1. The alkaline cleavage reactions of oligonucleotides containing 2′-*O*- (**7a**; lane 2) and 2′-*S*-linked (**7b**; lane 11) phosphodiesters. **7a** was treated with 0.01 M NaOH for 2, 4, 8, 15, and 30 min at 90 °C (lanes 3−8), and **7b** was treated at 30 °C (lanes 12−17). To compare reactivity under the same conditions, both oligonucleotides were incubated with 0.1 M NaOH at 50 °C for 10 min (lanes 9 and 18). Oligonucleotides were tested for the presence of a phosphorothiolate linkage by treatment with 20 mM $AgNO_3$ for 20 min in the dark (lanes 1,7, 10, and 16).

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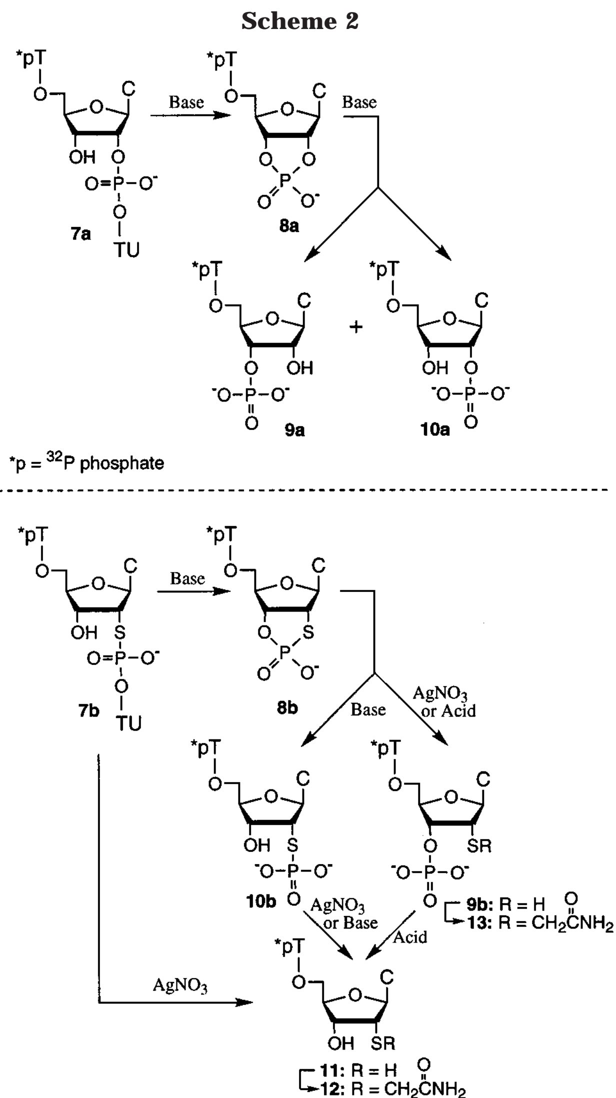
(19) The 2′- and 3′-monophosphates commonly migrate together in PAGE.

Scheme 2



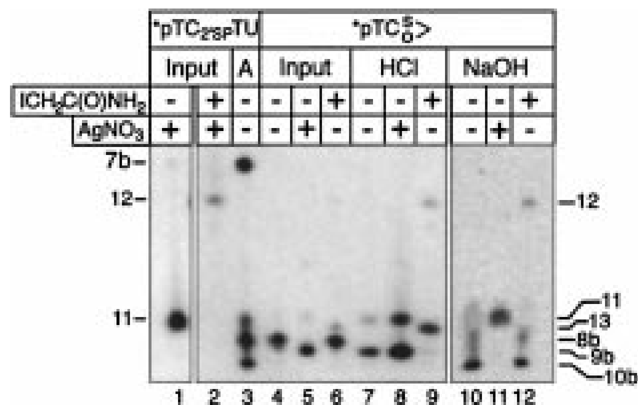


Figure 2. Biochemical characterization of the 2′-*S*,3′-*O*-cyclic phosphorothiolate **8b**. Standards were generated either by incubation of **7b** with 20 mM $AgNO_3$ in the dark for >20 min (lane 1) followed by treatment with 20 mM iodoacetamide for >20 min at 30 °C (lane 2), or by alkaline treatment (A) with 0.01 M NaOH at 30 °C for 15 min (lane 3). **8b** was incubated with either 0.1 M HCl (lanes 7–9) or 0.1 M NaOH (lanes 10–12) at 50 °C for 15 min before neutralization with NaOH or HCl, respectively. These reactions, as well as untreated **8b** (lanes 4–6), were divided into thirds. One-third was loaded directly on the gel (lanes 4, 7 and 9), one-third was reacted with $AgNO_3$ (lanes 5, 8, and 11), and one-third was reacted with iodoacetamide (lanes 6, 8, and 12) as described above.

product may arise from dephosphorylation of **10b** to give **11**. Consistent with these assignments, when 15 min aliquots of the cleavage reactions (Figure 1, lanes 6 and 15) were treated with Ag^+ (lanes 7 and 16), only those species that are expected to contain a sulfur–phosphorus (S–P) bond (**7b**, **8b**, **10b**; Scheme 2) disappear, whereas those species lacking an S–P bond (**7a**, **8a**, **9a**/**10a**, **11**) are unaffected.

Following base-catalyzed cleavage of **7b** (0.01 M NaOH at 30 °C; Figure 1, lane 13) for 4 min, we purified the 2′-*S*,3′-*O*-cyclic phosphorothiolate **8b** by DTT-PAGE, and in a manner similar to that of **7b**, maintained the pH at 5.5 during elution from the gel and subsequent workup steps. To obtain further evidence for our structural assignments and the aforementioned reaction pathway, we analyzed the reactivity of **8b** under acidic and basic conditions and probed the susceptibility of the resulting products and **8b** itself to modification by Ag^+ and iodoacetamide. The latter reagent converts mercaptans to acetamide thioethers but does not modify alcohols.[21] Figure 2 and Scheme 2 show the results of this biochemical analysis. The electrophoretic mobilities of the products were compared to standards generated by Ag^+ cleavage (**11**; Figure 2, lane 1) and base cleavage (**8b**, **10b**, **11**; lane 3) of tetramer **7b**. **11** can be further reacted with iodoacetamide to a slower migrating species (**12**; Figure 2, lane 2). The much faster mobility of **11** relative to **12** (compare Figure 2, lanes 1 and 2) may be because **11** harbors an additional negative charge due to ionization of the mercapto group under the electrophoresis conditions.[14]

To test for the presence of a phosphorothiolate linkage in the putative 2′-*S*,3′-*O*-cyclic phosphorothiolate **8b** (Figure 2, lane 4), we treated it with Ag^+. As expected, Ag^+ converted **8b** completely to a faster migrating product (Figure 2, lane 5). This product migrates more slowly than the 2′-*S*-monophosphorothiolate **10b** (Figure 2, lane 3) and is converted to a slower migrating species by iodoacetamide (see below; lane 9), consistent with the structure **9b**. Treatment of **8b** with acid (0.1 M HCl, 50 °C, 15 min) results in two new products (Figure 2, lane 7), one of which comigrates with **9b** from silver cleavage. Neither of these two products is affected by Ag^+ (Figure 2, lane 8), but both are converted to slower migrating species by iodoacetamide (lane 9), indicating the presence of a free mercapto group and suggesting that ring opening at the cyclic phosphorothiolate under acidic conditions occurs preferentially through scission of the S–P bond.[22] One of the iodoacetamide-modified products comigrates with **12**, suggesting that acid treatment also gives rise to the dephosphorylated species **11**.

Finally, we studied the reactivity of **8b** under basic conditions (Figure 2, lane 10). Upon exposure to 0.1 M

(20) An NMR study showed that 2′-*S*,3′-*O*-uridine cyclic phosphorothiolate yields exclusively the 2′-*S*-phosphorothiolate under basic conditions.[12]

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(22) It is known that hydrolytic ring opening of inosine 2′-*O*,3′-*S*-cyclic phosphorothiolate under acidic conditions also cleaves the 3′-S–P bond.[14] The observation that one of the acid cleavage products of **8b** (Figure 2, lane 7) comigrates with the silver cleavage product (lane 5) suggests that acidic conditions also cleave the 2′-S–P bond of the 2′-*S*,3′-*O*-cyclic phosphorothiolate. The reasons for the different regioselectivities resulting from base and acid cleavage are discussed in refs 12 and 14 and references therein.

NaOH for 15 min, **8b** disappeared completely, forming products that comigrate with the 2′-*S*-phosphorothiolate **10b** and the dephosphorylated product **11**. The response of the products to Ag^+ (Figure 2, lane 11) or iodoacetamide (lane 12) treatment is consistent with these assignments: following Ag^+ treatment **10b** disappears, presumably being converted to **11**, which consequently increases in relative intensity compared to that of the untreated lanes. Iodoacetamide treatment provides a new band that comigrates with **12**, presumably arising from modification of **11**, while **10b** remains unaffected. These data strongly argue that **8b** contains a 2′-*S*,3′-*O*-cyclic phosphorothiolate terminus, imply that no modifications to the sulfur occurs during synthesis, isolation, or purification of the oligonucleotide, and provide a biochemical "signature" for a cyclic phosphorothiolate terminus.

Taken together, our results show that the 2′-*S*-phosphorothiolate linkage is correctly installed into oligonucleotides via phosphoramidite chemistry and is a suitable precursor for generating oligonucleotides containing 2′-*S*,3′-*O*-cyclic phosphorothiolate termini. Although this study establishes the method using a short DNA oligonucleotide, the approach is readily adapted to longer oligonucleotides (data not shown). In cases where PAGE does not readily separate the cyclic phosphorothiolate from its ring-opened hydrolysis product, exposure of the 2′-*S*-phosphorothiolate precursor to base-catalyzed cleavage conditions (0.01 M NaOH, 30 °C) must be limited to 4 min so as to minimize hydrolysis. Because standard ribonucleotide linkages undergo minimal base-catalyzed cleavage under these conditions (data not shown), it should be possible to generate RNA oligonucleotides containing 2′-*S*,3′-*O*-cyclic phosphorothiolate termini. The methods described herein provide access to new probes with which to explore the biological structure and function of macromolecules that interact with 2′,3′-cyclic phosphates.

Experimental Section

General Methods. All reactions were preformed at room temperature unless otherwise indicated. All reagents were from Aldrich, and all solvents were from Fisher unless otherwise indicated. Solutions of DTT (IDL) were stored in the dark and discarded after 2 months of use. Merck silica gel, 8983 grade, 230−400 mesh, 60 Å, was used for column chromatography. Oligonucleotides were synthesized on a 1 μmol scale using a Millipore Expidite Nucleic Acid Synthesis System and standard DNA and RNA protocols and phosphoramidites (Glenn) unless otherwise indicated. Reverse phase high-pressure liquid chromatography (HPLC) was performed using a C_{18} column (10 × 250 mm). ^1H, ^{31}P, and ^{13}C spectra were obtained on a 500 MHz NMR spectrometer. Mass spectral data were obtained from the Mass Spectral Analysis Lab at the University of California, Riverside, on a high-resolution mass spectrometer. PAGE was performed using 20% polyacrylamide (Fisher; acrylamide: bisacrylamide 29:1) with 7 M urea. Gel loading solution contained 8 M urea (VWR), 50 mM EDTA (Fisher), 0.02% bromophenol blue (EM Science), and 0.02% xylene cyanol FF (Kodak). DTT-PAGE included 10 mM DTT in both the gel and the gel running buffer. Gels were pre-electrophoresed for at least 5 h before use.

4-*N*-Benzoyl-2′-deoxy-2′-(2-pyridyldithio)cytidine (2). The benzoyl-protected cytidine derivative **1**[9] (167 mg; 0.28 mmol) was dissolved in chloroform (6 mL) and MeOH (3 mL). $AgNO_3$ (51 mg; 0.3 mmol) and Aldrithiol-2 (132 mg; 0.6 mmol) were then added, and after a few minutes of stirring, a white precipitate formed. Stirring was continued for 2 h, and the reaction mixture was concentrated and purified by column chromatography (eluting with a gradient of 0−2% MeOH in $CHCl_3$) to afford **2** as a white powder (110 mg; 83% yield). ^1H NMR (DMSO-d_6) δ: 8.37 (d, 1H), 8.26 (d, 1H), 8.02 (d, 2H), 8.02 (d, 2H), 7.74 (t, 1H), 7.64 (t, 2H), 7.54 (t, 2H), 7.23 (d, 1H), 7.18 (t, 1H), 6.50 (d, 1H), 6.23 (d, 1H), 5.15 (s, 1H), 4.40 (s, 1H), 4.11 (s, 1H), 3.85 (m, 1H), 3.58 (m, 2H). ^{13}C NMR (DMSO-d_6) δ: 168.1, 164.0, 159.5, 155.8, 150.3, 146.2, 138.6, 133.9, 133.7, 129.4, 122.1, 120.1, 97.6, 89.5, 87.7, 73.0, 62.0, 61.8. HRMS (MH^+) calcd 473.0953, found 473.0936.

4-*N*-Benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxy-2′-(2-pyridyldithio)cytidine (3). The disulfide **2** (140 mg; 0.3 mmol) was dissolved in dry pyridine (4 mL). Dimethoxytrityl chloride (203 mg; 0.6 mmol) and (dimethylamino)pyridine (18 mg; 0.15 mmol) were added, and the solution was stirred for 6 h. The solution was concentrated and purified by column chromatography (eluting with a gradient of 0−1% MeOH in $CHCl_3$) to afford **3** as a yellow foam (195 mg; 85% yield). ^1H NMR ($CDCl_3$) δ: 8.71 (s, 1H), 8.55 (d, 1H) 8.22 (s, 1H), 7.94 (s, 2H), 7.77 (t, 1H), 7.64 (t, 2H), 7.55 (t, 2H), 7.4 (m, 9H), 7.25 (d, 1H), 6.86 (dd, 4H), 6.42 (d, 1H), 4.47 (s, 1H), 4.34 (d, 1H), 3.83 (s, 6H), 3.78 (dd, 1H), 3.55 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 159.1, 158.1, 150.1, 144.5, 137.7, 135.8, 135.5, 133.6, 130.6, 130.5, 129.6, 129.5, 128.6, 128.5, 128.3, 128.2, 128.1, 127.6, 122.8, 122.3, 113.7, 113.6, 87.6, 85.3, 72.2, 64.0, 63.7, 55.7. HRMS (MNa^+) calcd 797.2080, found 797.2124.

4-*N*-Benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxy-2′-(2-pyridyldithio)cytidine (4). Nucleoside **3** (195 mg; 0.25 mmol) was dissolved in dry pyridine (3 mL) followed by addition of imidazole (858 mg; 12.6 mmol) and *tert*-butyldimethylsilyl chloride (1.5 g; 10 mmol). The solution turned clear immediately, and after a few minutes of stirring, a white solid appeared. After stirring for 24 h, the reaction mixture was concentrated and purified by column chromatography (eluting with a gradient of 0−1% MeOH in $CHCl_3$) to afford nucleoside **4** (190 mg; 86% yield) as a white foam. ^1H NMR ($CDCl_3$) δ: 8.45 (d, 1H), 7.64 (m, 6H), 7.39 (d, 2H), 7.3 (m, 10H), 7.21 (d, 2H), 7.02 (m, 1H), 6.85 (dd, 4H), 6.58 (d, 1H), 4.61 (d, 1H), 4.16 (s, 1H), 3.94 (dd, 1H), 3.84 (s, 6H), 3.52 (dd, 2H), 0.94 (s, 9H), 0.13 (s, 3H), 0.04 (s, 3H). ^{13}C NMR ($CDCl_3$) δ: 137.8, 135.5, 130.6, 130.3, 129.7, 128.5, 128.3, 128.1, 128.0, 127.4, 126.2, 124.8, 120.2, 116.8, 116.1, 113.6, 113.4, 55.8, 54.5, 50.3, 26.0, 0.3, −4.7. HRMS (MNa^+) calcd 911.2944, found 911.2924.

4-*N*-Benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxy-2′-mercaptocytidine (5). Nucleoside **4** (190 mg; 0.21 mmol) was dissolved in anhydrous, argon-saturated THF (6 mL); anhydrous DTT (129 mg; 0.84 mmol) and triethylamine (100 μL) were added. The reaction was stirred for 3 h, concentrated, and purified by column chromatography (eluting with a gradient of 1−2% MeOH in $CHCl_3$) to afford nucleoside **5** (155 mg; 95% yield) as a white foam. ^1H NMR ($CDCl_3$) δ: 8.43 (d, 1H), 8.07 (d, 1H), 7.89, (d, 2H), 7.61 (t, 1H), 7.53 (t, 2H), 7.42 (d, 2H), 7.36 (m, 7H), 6.90 (d, 4H), 6.27 (d, 1H), 4.27 (s, 1H), 3.81 (s, 6H), 3.71 (m, 2H), 3.60 (m, 1H), 3.41 (d, 1H), 0.86 (s, 9H), 0.02 (s, 3H), −0.03 (s, 3H). ^{13}C NMR ($CDCl_3$) δ: 159.2, 145.1, 144.3, 135.6, 135.5, 133.6, 130.6, 129.5, 128.7, 128.5, 127.9, 127.7, 113.8, 97.0, 93.1, 87.6, 85.0, 71.6, 62.1, 55.7, 49.2, 30.1, 26.1, 18.5, 1.4, 0.4, −4.3, −4.5. HRMS (MNa^+) calcd 802.2958, found 802.2977.

4-*N*-Benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxy-2′-thiocytidin-2′-yl β-Cyanoethyl *N*,*N*-Diisopropylphosphoramidite (6). 2′-Mercapto nucleoside **5** (70 mg; 0.9 mmol) was added to dry CH_2Cl_2 (3 mL). Diisopropylethylamine (78 μL; 0.45 mmol), redistilled methylimidazole (3.5 μL; 0.045 mmol), and β-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (40 μL; 0.18 mmol) were added, and the reaction was stirred for 1 h under argon. The solution was concentrated and purified by column chromatography (eluting with a gradient of 2−6% acetone in CH_2Cl_2; both solvents were filtered through basic alumina before mixing) to afford **6** (80 mg; 90% yield) as a white foam. ^{31}P NMR ($CDCl_3$) δ: 166.17, 161.81. HRMS (MNa^+) calcd 1002.4037, found 1002.4065.

Synthesis of Oligonucleotides. The oligonucleotide containing the 2′-*S*-phosphodiester linkage ($^{5'}TC_{2'SP}TU^{3'}$) was synthesized using the 2′-*S*-phosphoramidite **6**, which was coupled in the presence of *p*-nitrophenyl tetrazole as described by Sun et al. for 3′-*S*-phosphoramidites.[15] The oligonucleotide containing the 2′-*O* phosphodiester linkage was synthesized using *N*-benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-cytidin-2′-yl β-cyanoethyl *N*,*N*-diisopropylphosphoramidite (Chem-

Genes). Following synthesis, the oligonucleotides were deprotected with concentrated NH_4OH:EtOH (3:1, 2 mL) at 55 °C for 16 h. Each solution was concentrated and then shaken in 0.1 M TBAF in THF (0.5 mL) in the dark for 36 h. Then 0.1 M TEAAC (0.4 mL) was added, and the solutions were spun under vacuum for 3 h. The oligonucleotides were passed through a Sephadex G-25 column, followed by purification by reverse phase HPLC. The HPLC column was heated at 40 °C to prevent blockage by the thick TBAF solution.

Radiolabeling of Oligonucleotides. Oligonucleotides (20 pmol) were 5′- radiolabeled with [α-^{32}P]ATP (NEN) and T4 Polynucleotide Kinase (USB) according to the manufacturer's (USB) protocol (10 μL scale). The reactions were quenched with gel loading buffer (10 μL), and the oligonucleotides were separated from failure sequences by PAGE and visualized by autoradiography. The radiolabeled tetramers were excised and eluted from the gel at 4 °C with triethylammoniumacetate (TEAAC) buffer (pH 5.5) for 8−12 h followed by desalting on a Sep-pak C_{18} cartridge (Waters). Before concentrating the fractions containing the oligonucleotides, TEAAC was added to a concentration of 1 mM to maintain a low pH. After drying, the oligonucleotides were dissolved in 1 mM TEAAC to a concentration of 20 nM.

Chemical Cleavage Reactions. Complete alkaline or acid hydrolysis of the oligonucleotides was carried out at 50 °C for 10 or 15 min with 0.1 M NaOH or 0.1 M HCl, respectively, before neutralizing with HCl or NaOH, respectively. Partial alkaline cleavage of **7a** and **7b** was achieved by incubation with 0.01 M NaOH at 90 °C or 30 °C, respectively, for various times (Figure 1), before quenching with HCl. Specific cleavage of the S−P bond was attained through incubation with 20 mM $AgNO_3$ in the dark for over 20 min. Treatment with 20 mM iodoacetamide, a well-known mercaptan modification reagent, at 30 °C for over 20 min was used to assay for free mercapto groups.

Isolation of the 2′-*S*,3′-*O*-Cyclic Phosphorothiolate (8b). **7b** (~5 pmol; essentially $^1/_2$ or $^1/_4$ of the amount obtained from the kinase reaction described above) was incubated with 0.01 M NaOH for 4 min at 30 °C in a 50 μL reaction. Then 0.1 M HCl (5 μL) was added to neutralize the base, and the mixture was dried and redissolved in water (10 μL). Gel loading buffer (10 μL) was added, and the mixture was purified by DTT-PAGE. The product was visualized by autoradiography and excised and eluted from the gel with TEAAC (pH 5.5). The oligonucleotide was purified by Sep-Pak C_{18} cartridge as described above. Before drying the fractions containing the oligonucleotide, TEAAC buffer (pH 5.5) was added to a final concentration of 1 mM. After drying, the oligonucleotide was dissolved in 1 mM TEAAC (pH 5.5) to a concentration of 20 nM.

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Supporting Information Available: ^{1}H NMR spectra of compounds **2**−**5** and ^{31}P NMR spectrum of compound **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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