Incorporation of 2′-Deoxy-2′-mercaptocytidine into Oligonucleotides via Phosphoramidite Chemistry

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Modified oligonucleotides that contain a sulfur atom in place of an oxygen atom have proven enormously valuable in studies of nucleic acid structure and function,[1−3] protein−nucleic acid interactions,[4−6] and antisense gene therapy.[7] Virtually every oxygen atom on the bases,[1,4] the sugar,[2,5,7] and the phosphoryl group[3,6] has been replaced by sulfur. Some of these nucleotide analogues have provided insight into the most intricate details of biological function.[8]

Substitution of the 2′-hydroxyl group in RNA by a mercapto (SH) group has received relatively little attention despite the potential use of 2′-deoxy-2′-mercaptonucleosides as probes for the role of the 2′-hydroxyl in RNA structure and function. Although the first 2′-deoxy-2′-mercaptonucleosides were synthesized more than two decades ago,[9] only recently has the synthesis of an RNA dinucleotide containing 2′-deoxy-2′-mercaptouridine been reported.[10] The synthesis employed phosphate triester methodology in which 5′-*O*-(9-phenylxanth-9-yl)-2′-deoxy-2′-(9-(*p*-anisyl)xanthen-9-ylthio)uridine-3′-*O*-(2-chlorophenyl)phosphate was coupled to 2′,3′-di-*O*-acetyluridine. This approach used relatively strong acidic conditions (0.1 M HCl) to remove the 2′-[9-(*p*-anisyl)xanthen-9-yl] sulfur protecting group and may therefore be problematic in the synthesis of longer oligonucleotides due to the tendency of adenosine and guanosine to depurinate under acidic conditions.[11]

Although phosphate triester methodology is useful for large scale solution synthesis of oligonucleotides, solid phase phosphoramidite methodology is generally the method of choice for most biochemical investigations because it enjoys near quantitative coupling yields and fewer side products.[12] Thus, the development of chemistry for the incorporation of 2′-mercaptonucleosides into RNA and DNA via the phosphoramidite approach would facilitate further investigation of nucleic acid structure and function. Herein, we report the synthesis of appropriately protected phosphoramidites that are suitable for the incorporation of 2′-deoxy-2′-mercaptocytidine and 2′-deoxy-2′-mercaptouridine into oligonucleotides. Methods for the postsynthetic protection and deprotection of the mercapto group via disulfide exchange chemistry are also described.

Standard preparation of nucleoside synthons used in solid phase synthesis of oligonucleotides includes protection of the 2′- and 5′-hydroxyls as *tert*-butyldimethylsilyl and dimethoxytrityl ethers, respectively, protection of the heterocyclic amines as amides, and phosphitylation of the 3′-oxygen to the β-cyanoethyl *N*,*N*-diisopropylphosphoramidite.[13] Protection of a 2′-mercapto group during solid phase synthesis is necessary to prevent side reactions due to the redox and nucleophilic properties of sulfur. Furthermore, because of the potential intramolecular S_N reactions at either the 1′-carbon[14] or 3′-phosphorus[15] under basic conditions, it is important that the sulfur protecting group remain stable during removal of the base labile groups. The most commonly used 2′-hydroxyl protecting group, *tert*-butyldimethylsilyl, is not suitable for sulfur protection.[16] However, protection of the mer-

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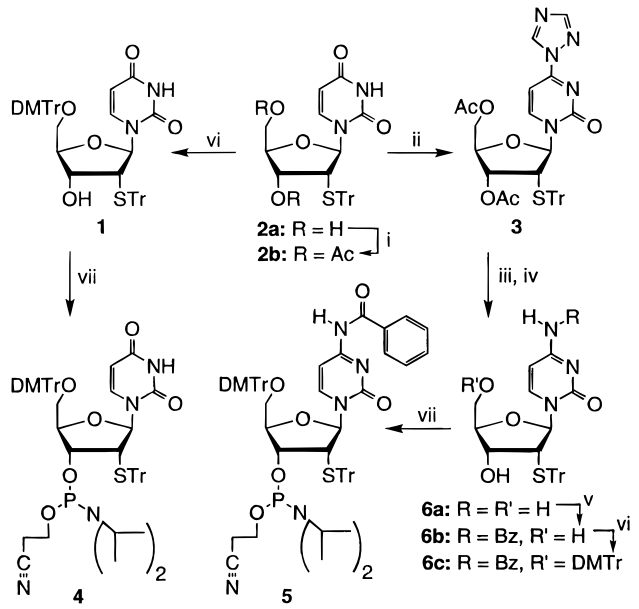
Scheme 1[a]



[a] Conditions: (i) Ac_2O/pyridine; (ii) NEt_3/PCl_3/1,2,4-triazole/CH_3CN; (iii) CH_2Cl_2/NH_3; (iv) MeOH/NH_3; (v) Bz_2O/DMF/pyridine; (vi) DMTrCl/DMAP/pyridine; (vii) $NCCH_2CH_2OP(Cl)N$*i*-Pr_2/1-Me-imidazole/*i*-Pr_2NEt/CH_2Cl_2.

capto group as a disulfide[17] or triphenylmethyl (trityl) ether[18] has proven suitable in the past; both are stable under basic conditions but can be removed under mild conditions. For example, 2′-deoxy-2′-(tritylthio)uridine (**2a**) is stable under strongly basic or mildly acidic conditions, but the trityl protecting group can be readily removed by treatment with silver nitrate.[9d]

Phosphoramidites for the incorporation of 2′-deoxy-2′-mercaptocytidine and 2′-deoxy-2′-mercaptouridine into oligonucleotides were prepared from the corresponding 2′-deoxy- 2′-(tritylthio)nucleosides (Scheme 1). 2′-Deoxy-2′-(tritylthio)cytidine (**6a**) was synthesized directly from 2′-deoxy-2′-(tritylthio)uridine (**2a**), analogous to previous work with 2′-deoxy-2′-(9-phenylxanthen-9-ylthio)uridine.[9d] The uridine derivative **2a** was first reacted with acetic anhydride to protect the 3′- and 5′-hydroxyl groups and then with the highly labile intermediate tris(1,2,4-triazol-1-yl)phosphine[19] to afford the 4-triazolyl pyrimidine derivative **3**. Nucleoside **3** was converted to the corresponding cytidine derivative by treatment with ammonia-saturated CH_2Cl_2. Subsequent treatment with half-saturated methanolic ammonia removed the acetyl protecting groups. This four-step sequence gave the cytosine nucleoside **6a** in 66% overall yield. The 4-amino and 5′-hydroxy groups were protected as the benzoyl amide[20] and the dimethoxytrityl ether,[13b] respectively, to afford **6c** in 65% yield. Phosphitylation of the 3′-hydroxyl of **6c** with β-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite[13a] produced phosphoramidite **5** which was characterized by HRMS and ^{31}P NMR. Analogous 5′ protection and 3′ activation reactions with starting material

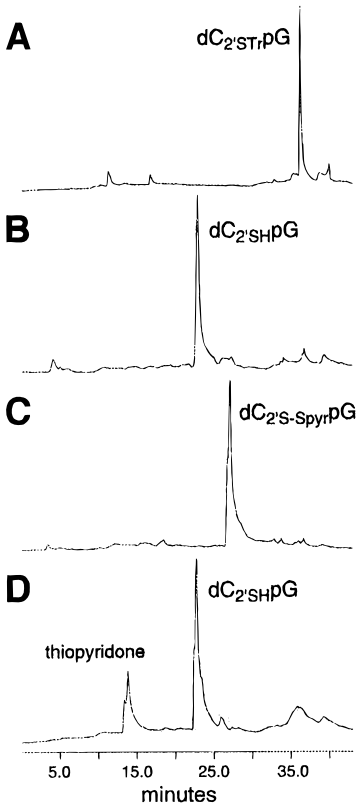


Figure 1. Reverse phase HPLC data for the dinucleotides $dC_{2'SX}pG$. HPLC-purified trityl-protected dimer **7a** (A) was treated with silver nitrate plus DTT or 2,2′-dipyridine disulfide to yield the 2′-mercapto derivative **10a** (B) or the 2′-(2-pyridyldithio) derivative **9a** (C), respectively. An HPLC, purified sample of **9a** was treated with DTT and gave the chromatogram shown in D.

2a gave the 2′-deoxy-2′-(tritylthio)uridine phosphoramidite **4** in 92% yield over the two steps.

To demonstrate the suitability of these phosphoramidites as building blocks for the incorporation of 2′-mercaptopyrimidines into oligonucleotides, we synthesized the dinucleotide 2′-deoxy- 2′-(tritylthio)cytidylyl (3′→5′) 2′-deoxyguanosine ($^{5'}dC_{2'STr}pG^{3'}$, **7a**) and characterized it by reverse phase HPLC (Figure 1A) and mass spectrometry (Figure 2A).[21] The trityl-protected dinucleotide **7a** was deprotected by treatment with aqueous silver nitrate followed by addition of dithiothreitol (DTT) which precipitates Ag^+ as the salt of DTT (Scheme 2).[18b] The resulting species had shorter retention time by HPLC relative to **7a** (Figure 1A and B), and its mass spectrum (Figure 2B) gave a molecular ion mass consistent with the desired 2′-mercapto dinucleotide $^{5'}dC_{2'SH}pG^{3'}$ (**10a**). The Ag^+/dimer complex **8a** can also be readily reacted to a mixed disulfide by disulfide exchange chemistry. Thus, treatment of the 2′-tritylthio derivative **7a** with silver nitrate and 2,2′-dipyridine disulfide resulted in quantitative conversion to a new product that had intermediate mobility on HPLC relative to **7a** and the 2′-mercapto derivative **10a** (Figure 1C), and a mass spectrum consistent with the 2′-(2-pyridyldithio) derivative $^{5'}dC_{2'S-Spyr}pG^{3'}$ (**9a**; Figure 2C). Treatment of **9a** with DTT[22] quantitatively reduced the disulfide to thiopyridone and **10a** (Figure 1D). This methodology can also

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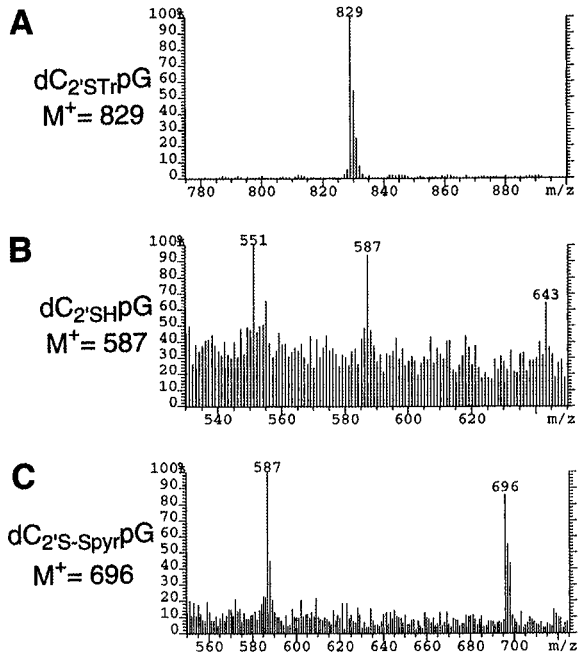
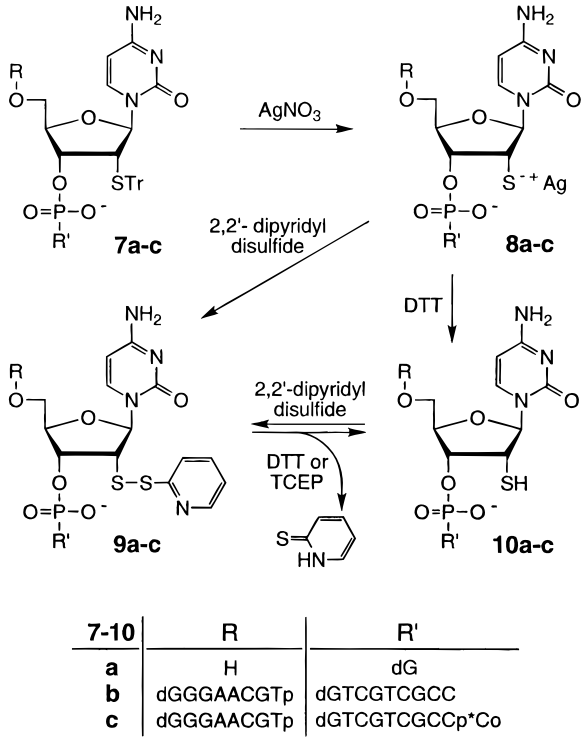


Figure 2. Mass spectral data of the dinucleotides $dC_{2'SX}pG$. Trityl-protected dimer **7a** (A), 2′-mercapto **10a** (B), and 2′-disulfide **9a** (C) all display appropriate M^+ *m/z* peaks. In B, the peaks at 551 and 643 arise from the matrix used for analysis.

Scheme 2



7-10	R	R'
a	H	dG
b	dGGGAACGTp	dGTCGTCGCC
c	dGGGAACGTp	dGTCGTCGCCp*Co

be applied to longer oligonucleotides as described in the Experimental Section.

We next examined the feasibility of 2′-mercaptocytidyl oligonucleotides for biochemical investigations by incorporation of phosphoramidite **5** into an internal position of an 18mer oligonucleotide (**7b**). Although the Ag^+/DTT deprotection scheme described above could be used to obtain 2′-mercaptocytidyl oligonucleotides, precipitation of the Ag^+/DTT complex always resulted in significant loss of oligonucleotide from the supernatant. For example, when 3′-radiolabeled oligonucleotide **7c** was treated with silver nitrate followed by DTT, a significant portion of radioactivity remained in the precipitate and could not be recovered by washing with 0.1 M triethylammonium acetate (TEAAC). We found it more convenient to convert the 2′-tritylthio oligonucleotide directly to the 2′-(2-pyridyldithio) derivative by treatment with silver nitrate and 2,2′-dipyridine disulfide followed by HPLC purification, thereby circumventing the precipitation step. The oligonucleotide could then be stored as the 2′-disulfide and deprotected with DTT[22] or tris(carboxyethyl)phosphine (TCEP)[23] to generate the 2′-mercapto derivative just prior to use. Figure 3 shows the electrophoretic mobilities and stabilities of **7c** (X = STr), **9c** (X = S−Spyr), and **10c** (X = SH; $^{5'}$dGGGAACGTC$_{2'X}$GTCGTCGCCp*Co$^{3'}$)[24] relative to the corresponding oligonucleotide containing ribocytidine at the internal position, $^{5'}$dGGGAACGTC$_{2'OH}$GTCGTCGCCp*Co$^{3'}$ (**11**). The relative mobilities of **7c**, **9c**, and **10c** parallel the relative HPLC retention times for the corresponding dinucleotide derivatives (**7a**, **9a**, and **10a**). Thus, the

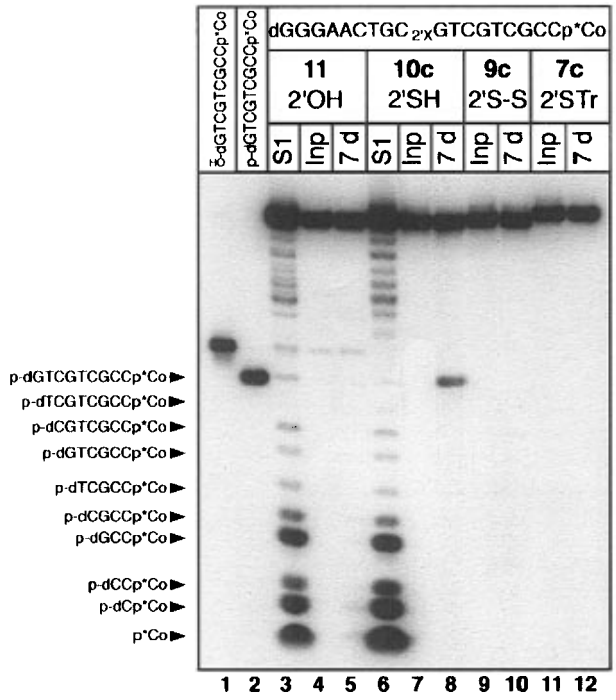


Figure 3. Analysis of the stability of 18mer oligonucleotides via electrophoresis. Input (Inp) oligonucleotides **11** (lane 4), **10c** (lane 7), **9c** (lane 9), and **7c** (lane 11) were incubated for 7 days at 25 °C, pH 7.5 (lanes 5, 8, 10, and 12, respectively). S1 nuclease digestions of **11** and **10c** are shown in lanes 3 and 6, respectively. Products from S1 nuclease-catalyzed cleavage to the 3′ side of $C_{2'X}$ in **11** (X = OH) and **10c** (X = SH) show identical mobilities as expected and are labeled at the left of the gel. The identity of the fragmentation product from **10c** (lane 8) was established as p-dGTCGTCGCCp*Co by comparison to an independently synthesized standard (lane 2). Both the **10c**-derived fragment and the independently synthesized oligonucleotide have identical electrophoretic mobilities and S1 nuclease digestion patterns (data not shown). In lane 6 the band that comigrates with p-dGTCGTCGCCp*Co (lane 2) may be due to decomposition of **10c** (lane 8) and not S1 nuclease activity.

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(24) p* indicates the presence of a radioactive isotope (^{32}P) in the phosphate moiety; Co = 3′-deoxyadenosine (cordycepin).

trityl derivative **7c** has reduced electrophoretic mobility (Figure 3, lane 11) compared to the 2′-mercapto derivative **10c** (lane 7) which has similar mobility to its 2′-hydroxyl counterpart **11** (lane 4). The disulfide derivative **9c** has intermediate mobility (lane 9).

Early work reported that 2′-mercaptocytidine is quite labile ($t_{1/2}$ = 29.5 min at pH 7.0, 37 °C) due to intramolecular S_N attack by sulfur on the adjacent 1′-carbon of the ribose, displacing the heterocyclic base.[9c] However, a more recent investigation reported that 2′-mercaptocytidine does not undergo glycosidic cleavage very readily, even at high pH.[14] The stability of 2′-mercaptocytidine as part of an internucleotide linkage has not yet been investigated. However, 2′-mercaptouridylyl (3′→5′) uridine and 2′-mercaptouridine 3′-(*p*-nitrophenyl)phosphate undergo fragmentation at high pH to give uracil and uridine 5′-phosphate[10] or *p*-nitrophenyl phosphate,[15] respectively. The latter products presumably result from further decomposition of the initial abasic product that results from glycosidic bond cleavage via the intramolecular S_N reaction. Incubation of the 2′-mercapto derivative **10c** at pH 7.5 and 25 °C for seven days (in the presence of TCEP to prevent oxidation) resulted in similar fragmentation to give the product $^{5'}$p-dGT-CCGTCGCCp*Co$^{3'}$ (Figure 3, lane 8); the calculated half-life was 3000 h.[25] In contrast, the blocked mercapto derivatives **7c** and **9c** and the 2′-hydroxyl derivative **11** showed no tendency to fragment (Figure 3, lanes 12, 10, and 5, respectively) under similar conditions and times.[26] These observations are consistent with a mechanism of decomposition that involves nucleophilic attack by the 2′-sulfur.

In conclusion, we have demonstrated the incorporation of 2′-deoxy-2′-mercaptocytidine into DNA oligonucleotides via the phosphoramidite approach. Protection of the sulfur as a trityl ether allows solid phase synthesis using standard reagents and protocols and requires no special precautions. Following oligonucleotide synthesis and deprotection, the tritylthio moiety is readily cleaved with silver nitrate followed by DTT, or is converted to the 2′-(2-pyridyldithio) derivative via treatment with silver nitrate and 2,2′-dipyridine disulfide. Synthesis of RNA oligonucleotides containing the modified nucleoside is also feasible (data not shown). Under mildly reducing conditions, oligonucleotides containing 2′-mercaptocytidine are stable at physiological pH on the time scale of most biochemical experiments and are therefore suitable as probes to explore the biological structure and function of nucleic acids.

Experimental Section

General. All reactions were preformed at rt unless otherwise noted. All reagents and anhydrous solvents were from Aldrich and all drying reagents and other solvents were from Fisher unless otherwise noted. All nucleotide modifying enzymes were from U.S. Biochemicals. [α-^{32}P]-3′-Deoxyadenosine triphosphate ([α-^{32}P]cordycepin triphosphate or [α-^{32}P]CoTP) was obtained from New England Nuclear. Solutions of $AgNO_3$ and TCEP (Strem) were prepared fresh daily and stored in the dark. Solutions of DTT and 2,2′-dipyridine disulfide were stored at −20 °C and discarded after two months of use. Merck silica gel, 8983 grade, 230−400 mesh, 60 Å (Aldrich) was used for column chromatography. Silica gel on glass with fluorescent indicator (Sigma) was used for TLC. Reverse phase HPLC was performed using a Beckman Ultrasphere ODS C_{18} column (10 × 250 mm). HPLC solvent gradients were performed with 0.1 M TEAAC (pH 6.5; solvent A) and CH_3CN (solvent B). Two gradient programs were used: for dinucleotides **7a**−**10a**, 0−12% B from 0−24 min and 12−50% B from 24−42 min, and for oligonucleotides **7b**−**10b**, 100% A from 0−5 min, 0−12% B from 5−20 min, and 12−30% B from 20−35 min. ^{1}H, ^{31}P, and ^{13}C spectra were obtained on a GE 500 MHz NMR spectrometer. ^{31}P spectra were referenced to aqueous phosphoric acid, and ^{1}H and ^{13}C spectra were referenced to TMS, CH_2Cl_2, or DMSO. Mass spectral data were obtained from the Mass Spectral Analysis Lab at the University of California, Riverside, on a VG ZAB high resolution mass spectrometer. Elemental analyses were obtained from Atlantic Microlabs Inc. Polyacrylamide gel electrophoresis (PAGE) was performed using 20% polyacrylamide (Fisher; acrylamide:bis-acrylamide, 29:1) with 7 M urea. Gel loading buffer contained 8 M urea (Fisher), 50 mM EDTA (pH 8.0, Fisher), 0.02% bromophenol blue (EM Science), and 0.02% xylene cyanol FF (Kodak).

1-[3′,5′-Di-*O*-acetyl-2′-deoxy-2′-(tritylthio)-β-D-ribofuranosyl]-4-(1,2,4-triazol-1-yl)pyrimidin-2-(1*H*)-one (3). The uridine nucleoside **2a**[9d] (2.7 g, 5.4 mmol) was dried by azeotropic distillation with pyridine (3 × 5 mL) and redissolved in anhydrous pyridine (10 mL). Acetic anhydride (2.0 mL, 21.5 mmol) was added, and the solution was stirred for 4 h under Ar. The reaction mixture was concentrated and purified by column chromatography (1−3% MeOH in $CHCl_3$) to afford a white foam, presumably 3′,5′-di-*O*-acetyl-2′-deoxy-2′-(tritylthio)-uridine, **2b** (3.06 g, 97%), which was used directly in the next reaction. **2b** (2.7 g, 4.6 mmol) was dissolved in anhydrous CH_3CN (10 mL) under Ar. In a separate flask, triazole (2.6 g, 37.6 mmol), NEt_3 (5 mL), and PCl_3 (1.0 mL, 11.5 mmol) were added to anhydrous CH_3CN (10 mL) and stirred under Ar. Within 5 min a white precipitate formed. This suspension was filtered directly into the solution containing **2b**, and the remaining solid was washed with CH_3CN (5 mL). After the reaction was stirred under Ar for 12 h, NEt_3 (18 mL), water (3 mL), and saturated aqueous $NaHCO_3$ (100 mL) were added, and the mixture was extracted with $CHCl_3$ (2 × 100 mL). The organic phases were combined, dried with $MgSO_4$, and evaporated to yield **3** (2.49 g, 85%) as a white foam. The compound was analyzed spectroscopically and used in the next reaction without further purification. ^{1}H NMR ($CDCl_3$) δ: 9.36 (s, 1H), 8.21 (s, 1H), 7.76 (d, J = 7.4, 1H), 7.37 (m, 6H), 7.17 (m, 9H), 7.04 (d, J = 7.4, 1H), 6.49 (d, J = 10.1, 1H), 4.15 (dd, J = 11.9, 1H), 4.08 (s, 1H), 3.96 (dd, J = 11.9, 1H), 3.60 (d, J = 5.0, 1H), 3.02 (dd, J = 5.0, 10.1, 1H), 2.13 (s, 3H), 1.92 (s, 3H). ^{13}C NMR ($CDCl_3$) δ: 167.0, 169.7, 159.2, 154.5, 154.3, 146.1, 143.7, 143.4, 129.0, 128.2, 127.2, 95.8, 87.7, 82.4, 73.8, 67.8, 63.7, 51.0, 20.8, 20.6. HRMS (MNa^+) calcd for $C_{34}H_{31}N_5O_7S_1Na_1$ 660.1893, found 660.1905.

2′-Deoxy-2′-(tritylthio)cytidine (6a). The triazole derivative **3** (200 mg, 0.3 mmol) was dissolved in ammonia saturated CH_2Cl_2 (5 mL) and stirred for 36 h. The solution was evaporated, and the residue was redissolved in half-saturated methanolic ammonia (5 mL). After 48 h the solution was evaporated to dryness, and the residue was recrystallized from methanol to afford compound **6a** (126 mg, 80%) as white crystals. ^{1}H NMR (DMSO-d_6) δ: 7.32 (d, 1H), 7.27 (m, 7H), 7.17 (m, 9H), 6.07 (d, J = 10.1, 1H), 5.61 (d, 1H), 4.98 (d, J = 4.3, 1H), 4.72 (t, J = 4.3, 1H), 3.67 (s, 1H), 3.18 (m, 2H), 2.79 (dd, J = 3.7, 10.1, 1H), 2.71 (m, J = 3.7, 4.3, 1H). ^{13}C NMR (DMSO-d_6) δ: 165.7, 155.7, 144.6, 141.2, 129.3, 128.2, 126.9, 95.4, 86.6, 96.1, 71.7, 67.1, 62.1, 53.0. Anal. Calcd for $C_{28}H_{27}N_3O_4S_1$: C, 67.0; H, 5.4; N, 8.4; S, 6.3. Found: C, 66.9; H, 5.5; N, 8.4; S, 6.3. HRMS (MNa^+) calcd 524.1620, found 524.1639.

4-*N*-Benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxy-2′-(tritylthio)cytidine (6c). The cytidine derivative **6a** (675 mg, 1.4 mmol) was coevaporated with pyridine (3 × 10 mL) and redissolved in a mixture of anhydrous DMF (4.5 mL) and anhydrous pyridine (10.5 mL). Benzoic anhydride (379 mg, 1.7 mmol) was added under Ar, and the solution stirred at 100 °C for 4 h. After evaporation of the solvent, the residue was

(25) The half-life ($t_{1/2}$) was determined as follows: Aliquots (1 μL) were removed from the reaction at 0, 3, 10, 26, 63, 109, and 168 h, quenched with gel loading buffer (3 μL), and stored at −20 °C before PAGE. The degree of decomposition was visualized and quantitated with a Molecular Dynamics Phosphorimager. The data were plotted as log [S_t/S_o] vs time, where [S_t/S_o] is the fraction of **10c** remaining at time t. The observed rate constant for decomposition (k_{obs}) was determined from the slope (m) of a linear fit to the data as $m = -k/2.303$. The half life was calculated as $t_{1/2} = 0.693/k_{obs}$.

(26) TCEP was absent from incubations containing **9c**.

redissolved in $CHCl_3$ (40 mL) and extracted first with saturated aqueous $NaHCO_3$ (2 × 20 mL) and then with water (20 mL). The organic layer was dried with $MgSO_4$ and concentrated to afford a white foam, presumably 4-*N*-benzoyl-2′-deoxy-2′-(tritylthio)cytidine (**6b**) (630 mg, 77%), which was converted without further purification to the dimethoxytrityl derivative as follows. **6b** (353 mg, 0.6 mmol) and DMAP (36 mg, 0.3 mmol) were coevaporated with pyridine (3 × 5 mL) and redissolved in anhydrous pyridine (10 mL). Dimethoxytrityl chloride (396 mg, 1.2 mmol) was added, and the reaction stirred for 30 h under Ar. The solution was concentrated and purified by column chromatography (0−2% MeOH in $CHCl_3$) to yield **6c** (452 mg, 85% yield) as a yellow foam. ^{1}H NMR ($CDCl_3$) δ: 8.85 (s, 1H), 8.21 (m, 1H), 7.99 (m, 2H), 7.64 (t, J = 7.3, 1H), 7.55 (t, J = 7.3, 2H), 7.40 (m, 6H), 7.20 (m, 20H), 6.67 (dd, 4H), 6.47 (d, 1H), 3.99 (s, 1H), 3.78 (s, 6H), 3.41 (dd, J = 4.4, 1H), 3.24 (m, 2H), 2.51 (d, J = 4.4, 1H). ^{13}C NMR ($CDCl_3$) δ: 158.7, 158.7, 143.9, 143.1, 135.4, 135.1, 133.3, 130.0, 129.9, 129.2, 128.6, 128.2, 127.9, 127.1, 113.2, 87.0, 86.3, 85.4, 70.7, 67.2, 63.9, 56.8, 56.8, 55.3, 55.3. HRMS (MNa^+) calcd for $C_{56}H_{49}N_3O_7S_1Na_1$ 930.3189, found 930.3176.

4-*N*-Benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxy-2′-(tritylthio)cytidin-3′-yl β-Cyanoethyl *N*,*N*-Diisopropylphosphoramidite (5). Fully protected nucleoside **6c** (300 mg, 0.3 mmol) was coevaporated with pyridine (3 × 8 mL) and redissolved in CH_2Cl_2 (10 mL) that had been freshly distilled over CaH_2. Diisopropylethylamine (115 μL, 0.7 mmol), β-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (150 μL, 0.7 mmol) and redistilled 1-methylimidazole (13 μL, 0.2 mmol) were then added, and the reaction was stirred for 2 h under Ar. The solution was concentrated and purified by column chromatography (0.2% NEt_3 and 2% acetone in CH_2Cl_2; both the acetone and CH_2Cl_2 were filtered through basic alumina before mixing) to afford **5** (335 mg, 92%) as a white foam. ^{31}P NMR ($CDCl_3$) δ: 153.1, 150.0. HRMS (MNa^+) calcd for $C_{65}H_{66}N_5O_8S_1P_1Na_1$ 1130.4267, found 1130.4333.

5′-*O*-(Dimethoxytrityl)-2′-deoxy-2′-(tritylthio)uridine (1). Nucleoside **2a**[9d] (1.47 g, 2.9 mmol) and DMAP (178 mg, 1.6 mmol) were coevaporated with pyridine (3 × 15 mL) and redissolved in anhydrous pyridine (15 mL). Dimethoxytrityl chloride (2.03 g, 6.0 mmol) was added, and the reaction was stirred for 4 h under Ar. The reaction mixture was concentrated and purified by column chromatography ($CHCl_3$) to afford **1** (2.25 g, 96%) as a yellow foam. ^{1}H NMR ($CDCl_3$) δ: 7.92 (d, J = 8.1, 1H), 7.48 (d, 6H), 7.27 (m, 9H), 7.14 (m, 6H), 7.05 (dd, J = 3.9, 8.9, 4H), 6.68 (d, J = 8.9, 2H), 6.63 (d, J = 8.9, 2H), 6.25 (d, J = 9.7, 1H), 6.32 (d, J = 8.1, 1H), 3.97 (s, 1H), 3.78 (s, 3H), 3.77 (s, 3H), 3.43 (dd, J = 4.7, 9.7, 1H), 3.18 (d, J = 10.9, 1H), 3.10 (d, J = 10.9, 1H), 2.52 (d, J = 4.7, 1H). ^{13}C NMR ($CDCl_3$) δ: 163.0, 158.7, 158.6, 150.5, 143.8, 143.7, 140.9, 135.2, 134.8, 130.0, 129.8, 129.0, 128.6, 128.0, 127.8, 127.2, 127.1, 113.1, 102.9, 87.1, 85.8, 85.2, 70.6, 67.3, 63.9, 55.7, 55.3. HRMS (MNa^+) calcd for $C_{49}H_{44}N_2O_7S_1Na_1$ 827.2767, found 827.2746.

5′-*O*-(Dimethoxytrityl)-2′-deoxy-2′-(tritylthio)uridin-3′-yl β-Cyanoethyl *N*,*N*-Diisopropylphosphoramidite (4). Protected nucleoside **1** (100 mg, 0.1 mmol) was coevaporated with pyridine (3 × 3 mL) and redissolved in CH_2Cl_2 (5 mL) that had been freshly distilled over CaH_2. Diisopropylethylamine (53 μL, 0.3 mmol), β-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (100 μL, 0.5 mmol), and redistilled 1-methylimidazole (6 μL, 0.1 mmol) were then added, and the reaction was stirred for 6 h under Ar. The solution was concentrated and purified by column chromatography (1% NEt_3 in CH_2Cl_2 that had been filtered through basic alumina) to afford **4** (125 mg, 96%) as a yellow foam. ^{31}P NMR ($CDCl_3$) δ: 152.6, 149.6. HRMS (MNa^+) calcd for $C_{58}H_{61}N_4O_8P_1S_1Na_1$ 1027.3845, found 1027.3852.

Incorporation of 2′-Deoxy-2′-(tritylthio)cytidine into Oligonucleotides by Solid Phase Synthesis (7). Oligonucleotides were synthesized on a 1 μmol scale with standard phosphoramidites (Perseptive Biosystems) using a Millipore Expidite Nucleic Acid Synthesis System and standard DNA and RNA protocols. Phosphoramidite **5** was coupled according to the standard RNA synthesis protocol and gave yields comparable to those of the standard phosphoramidites. Synthesized oligonucleotides were deprotected at 55 °C for 14−18 h with concentrated aqueous NH_3 and evaporated to dryness. Oligonucleotides that contained ribonucleotides were desilylated by treatment with 1.0 M tetrabutylammonium fluoride (TBAF)/THF solution (0.5 mL), gently shaken in the dark for 24 h, neutralized with 0.1 M TEAAC (pH 6.9), and purified by elution through a NAP-10 gel-filtration column (Pharmacia Biotech). Deprotected oligonucleotides were purified by reverse phase HPLC and stored at −20 °C in unbuffered water. Overall yields of oligonucleotides containing the 2′-modification were comparable to those for oligonucleotides lacking the modification.

General Procedure for Deprotection to 2′-Deoxy-2′-mercaptocytidyl Oligonucleotides (10). (a) From 2′-Deoxy-2′-(tritylthio)cytidyl Oligonucleotides. Dinucleotide **7a** or oligonucleotide **7b** (5 nmol) was treated with a solution of $AgNO_3$ (150 nmol) in aqueous 0.1 M TEAAC (pH 6.5; 48 μL) for 30 min. Aqueous DTT (0.1 M; 2 μL) was added, and the mixture was incubated for 5 min before centrifugation for 5 min. The supernatant was removed from the Ag^+/DTT precipitate and analyzed by HPLC. Conversion appeared quantitative by UV/HPLC. Dinucleotide **10a** had a retention time of 22 min (Figure 1B).

(b) From 2′-Deoxy-2′-(2-pyridyldithio)cytidyl Oligonucleotides. Dinucleotide **9a** or oligonucleotide **9b** (5 nmol) was treated for 15 min with DTT (200 nmol) in aqueous 50 mM Tris-HCl (pH 8.0; 50 μL). The solution was centrifuged for 5 min and analyzed by HPLC. Conversion appeared quantitative by UV/HPLC. Thiopyridone and dinucleotide **10a** had retention times of 13 min and 22 min, respectively (Figure 1D).

General Procedure for Preparation of 2′-Deoxy-2′-(2-pyridyldithio)cytidyl Oligonucleotides (9). (a) From 2′-Deoxy-2′-(tritylthio)cytidyl Oligonucleotides. Dinucleotide **7a** or oligonucleotide **7b** (5 nmol) was treated for 30 min with $AgNO_3$ (150 nmol) in unbuffered water (43 μL). Treatment of this mixture with methanolic 2,2′-dipyridine disulfide (0.1 M; 2 μL) and aqueous 0.5 M Tris-HCl (pH 8.0; 5 μL) resulted in the formation of a yellow precipitate. After incubation for 1 h, the suspension was centrifuged for 5 min, and the supernatant was analyzed by HPLC. Conversion appeared quantitative by UV/HPLC. Dinucleotide **9a** had a retention time of 27 min (Figure 1C).

(b) From 2′-Deoxy-2′-mercaptocytidyl Oligonucleotides. Dinucleotide **10a** (<1 nmol), methanolic 2,2′-dipyridine disulfide (0.1 M; 2 μL), aqueous 0.5 M Tris-HCl (pH 8.0; 5 μL), and unbuffered water were combined to a final volume of 50 μL. After incubation for 1 h, the solution was centrifuged for 5 min and analyzed by HPLC. Conversion appeared quantitative by UV/HPLC.

Radiolabeling of Oligonucleotides. Oligonucleotides were 3′-end labeled with [α-^{32}P]CoTP and terminal deoxynucleotidyl transferase (TDT). Reactions included [α-^{32}P]CoTP (30 μCi), TDT (17 units), oligonucleotide (20 pmol), and cobalt(II) chloride (20 nmol) in a 10 μL volume. After 1 h incubation at 37 °C, the reactions were quenched with gel loading buffer (8 μL), and the components were separated by PAGE. Products were visualized by autoradiography, excised from the gel, and eluted at 4 °C with water. The eluent was applied to a Sep-Pac C_{18} cartridge (Waters) which was then washed with water (2 mL), followed by 50% aqueous CH_3CN (2 mL). Fractions containing oligonucleotide were evaporated to dryness and brought up in water to a concentration of 20 nM as determined by specific radioactivity.

To obtain p-dGTCGTCGCCp*Co (Figure 3, lane 2) as a marker for the fragmentation product from **10c**, dGTCGTCGCC was 3′ end labeled as described above (lane 1) and then 5′-phosphorylated with T4 Polynucleotide Kinase (PNK) and adenosine triphosphate (ATP) by addition of the following ingredients directly to the TDT reaction: 0.5 M Tris-HCl (pH 7.5, 1.5 μL), 0.1 M $MgCl_2$ (1.5 μL), 50 mM ATP (1.7 μL), and 30 units/μL PNK (0.3 μL). The reaction mixture was incubated at 37 °C for 30 min before quenching with gel loading buffer. The product was purified by PAGE/Sep-Pac C_{18} as described above.

Digestion of Oligonucleotides with S1 Nuclease. Radiolabeled oligonucleotide **9c** or **11** (20 fmol) was incubated for 5 min with S1 nuclease (25 units) in aqueous buffer (10 μL) containing 50 mM sodium acetate (pH 5.2), 1 mM $ZnCl_2$, 0.25 M NaCl, and 1 mM TCEP. The reaction was stopped by the addition of gel loading buffer (10 μL) followed by cooling on dry ice.

Stability of Oligonucleotides. Radiolabeled oligonucleotides **7c**, **9c**, **10c**, and **11** (20 fmol) were incubated for 7 days

at 25 °C in aqueous 50 mM Tris-HCl (pH 7.5, 10 μL). The 2′-mercapto derivative **10c** was obtained by reduction of the 2′-(2-pyridyldithio) derivative **9c** with TCEP. For consistency, all reaction mixtures except **9c** initially contained 1mM TCEP. Mildly reducing conditions were maintained throughout the 7 day incubation by adding fresh TCEP (0.1 μL of a solution containing 0.1 M TCEP and 50 mM Tris, pH 7.5) after 2, 4, and 6 days. After 7 days, the reaction mixtures were diluted with gel loading buffer (10 μL) and analyzed by PAGE (Figure 3).

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Supporting Information Available: Copies of ^{1}H, ^{13}C, and ^{32}P NMR spectra (10 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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