Reactivity of a 2‘-Thio Nucleotide Analog

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Reactivity of a 2′-Thio Nucleotide Analog

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Abstract: The chemical reactivity of ribonucleotide analog 2′-deoxy-2′-thiouridine 3′-(*p*-nitrophenyl phosphate) (**1**), in which the 2′ hydroxyl is replaced with a 2′-thiol group, has been characterized. The major reaction pathway for **1**, as monitored by ^{31}P NMR spectroscopy, is transphosphorylation to afford 2′,3′-cyclic phosphorothioate **3**, followed by hydrolysis of **3** to produce 2′-deoxy-2′-thiouridine 2′-phosphorothioate (**4**). Thus, the reaction pathway of **1** is similar to that of the hydrolysis of ribonucleotides, yet there are significant differences. The pH−rate profile for transphosphorylation of **1** was determined by monitoring the formation of *p*-nitrophenol or *p*-nitrophenolate by UV−visible spectroscopy. Analysis of the profile reveals the attacking nucleophile to be thiolate, and the pK_a of the 2′-thiol was determined to be 8.3 ± 0.1. At pH 7.4, the thiol-containing ribonucleotide analog **1** is hydrolyzed at an observed rate 27-fold slower than its 2′-hydroxyl counterpart. These results indicate that the rate of thiolate attack on the adjacent phosphodiester bond is 10^7-fold slower than that of the corresponding alkoxide. Thiolate nucleophiles, therefore, are remarkably reticent toward attack at electrophilic phosphate centers. In addition to providing new information about the reactivity of phosphodiester bonds, our studies highlight the potential of 2′-thiol-containing nucleotides for the study of an array of RNA processes, especially those in which the 2′-substituent plays a critical role.

Introduction

Impetus for studying RNA analogs has arisen from many directions. Investigations of the reactivities of these analogs have been propelled by a desire to understand the mechanisms by which enzymes and ribozymes facilitate RNA cleavage.[1] The possibility of using RNA derivatives as therapeutic agents has further fueled investigations into their properties.[2] Because of the critical role of the 2′-hydroxyl group in RNA degradation, nucleoside analogs with modifications at this site have been used to provide insight into the mechanism of hydrolysis and to increase the stability of nucleic acid-derived therapeutics.[1,2] One functional group substitution that could be used to investigate RNA chemistry and biology is the replacement of the 2′-hydroxyl with a 2′-thiol.[3] Because of the unique reactivity and metal binding properties of thiols, this functional group could be used to endow nucleic acids with tailored properties.

2′-Thiol groups in oligonucleotides could play significant roles in determining structure and promoting catalysis of nucleic acids. In analogy to cysteine residues in proteins, nucleotides containing thiols can participate in disulfide bond formation. This property may be exploited to study RNA tertiary structure[4] and RNA−protein interactions through the formation of covalent cross-links. In addition, the introduction of such thiol groups into a ribozyme could increase the diversity of functionality available for catalysis.[5] Another feature of sulfur substituents, their ability to coordinate metal ions, could be exploited to illuminate the roles of metals in nucleic acid catalysis[6] or to engineer new metal-dependent catalysts. For instance, the double-metal-ion mechanism, proposed for some enzyme- and ribozyme-mediated RNA hydrolysis reactions, can be explored using sulfur substituents to localize metals at specific sites.[7]

The utility of 2′-thio-substituted nucleotides for such RNA structure−function studies is contingent on their chemical reactivity, about which little is known. To provide insight into the reactivity of phosphodiesters adjacent to 2′-thiol groups, we synthesized ribonucleotide **1**[8] (Figure 1) and determined the rates of its transphosphorylation over a broad pH range. Previous reports suggested that thiols in the 2′-position of nucleotide analogs would react not through a transphosphorylation pathway

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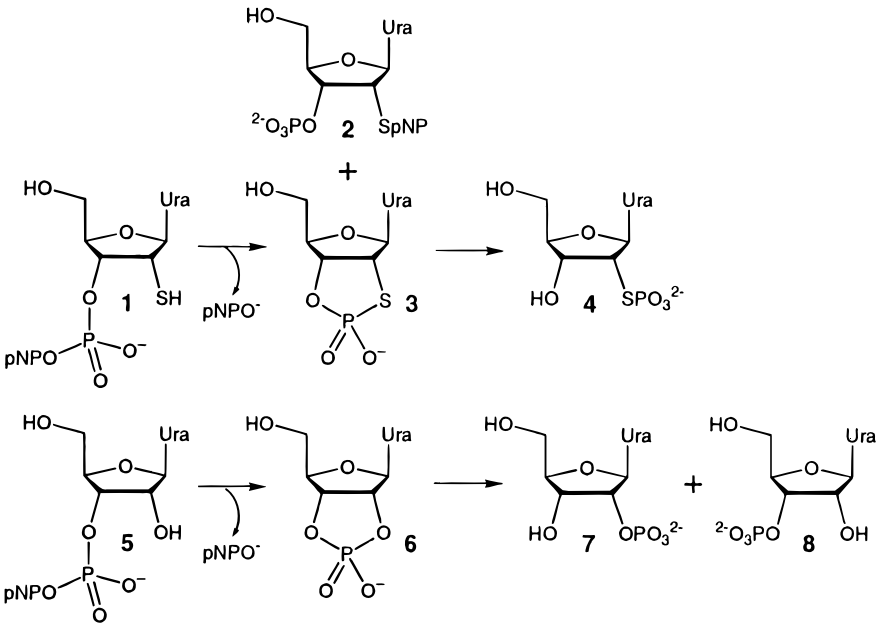

Figure 1. Scheme showing pathway for the hydrolysis of RNA analogs **1** and **5**. Abbreviations are as follows: pNP = *p*-nitrophenyl; Ura = uracil.

but by thiol attack at the glycosidic linkage to displace the nucleotide base.[9] With a goal of examining the transphosphorylation reaction, we equipped our 2′-thiol ribonucleotide analog with a *p*-nitrophenolate phosphodiester. The *p*-nitrophenol group activates the phosphate center toward nucleophiles and provides a convenient spectroscopic handle with which to follow the transphosphorylation reaction. A comparison of the rates of transphosphorylation of **1** with those obtained for the corresponding 2′-hydroxyl ribonucleotide analog **5**[10] provides insight into the propensity of nucleophilic thiols to attack electrophilic phosphodiesters.

Results and Discussion

Reactivity of 1 As Determined by ^{31}P NMR. To ascertain the course of the hydrolysis of **1**, the products of its reaction in aqueous solution were monitored at high and low pH by ^{31}P NMR spectroscopy.[11] Under strongly basic conditions (pH = 13), thiol **1** reacts to generate a mixture of 2′-deoxy-2′-thiouridine 2′-phosphorothioate (**4**) (65% by ^{31}P NMR), which is presumably derived from P−O scission of intermediate **3**,[12] and 2′-deoxy-2′-(*p*-nitrophenylthio)uridine 3′-phosphate, **2** (35% by ^{31}P NMR), the product of nucleophilic aromatic substitution.[13] The reaction of thiol **1** at pH values between 7 and 10 progressed to afford the 2′,3′-cyclic phosphorothioate **3**[14] as the major product, with some **2** generated by the competing reaction. The formation of product **2** reflects the high nucleophilicity of thiolates relative to alkoxides toward carbon centers and foreshadows the reactivity differences of these nucleophiles toward phosphodiesters. Under acidic conditions (pH = 2.3) the 2′-thiol derivative **1** is stable (no reaction was observed over 48 h), which contrasts with the reactivity of the 2′-hydroxyl analog **5** at low pH.

Cyclic phosphorothioate **3** is relatively stable at pH 7, but its hydrolysis to thiophosphate **4** under basic conditions could be monitored by NMR spectroscopy. The selective formation of the product **4** from the cyclic intermediate **3** contrasts with that observed for the breakdown of **6**, which yields both phosphate **7** and **8**. The exclusive P−O bond cleavage observed in the breakdown of intermediate **3** is precedented in studies of the alcoholysis of other 5-membered ring containing phosphorothioates. Unless reversible conditions are applied, only P−O bond scission occurs in such systems.[15] The susceptibility of the P−O over the P−S bond toward cleavage can be ascribed to the stereoelectronic features of the trigonal bipyramidal phosphoranes, which are formed during the course of the hydrolysis reactions (Figure 2).[16] The more apicophilic oxygen atom prefers an apical position in the transition state (or intermediate), as shown in structure **I.** Under these conditions, species **I** breaks down to generate only **4**; none of the 3′-phosphomonoester product that would arise through the higher energy species **II** is detected. Hydrolysis of **6** affords a mixture of 2′- and 3′-uridine monophosphates (**7** and **8**) since the trigonal bipyramidal phosphorane transition states formed are of similar energy.

pH−Rate Profile for the Reactions of 1 and 5. The ^{31}P NMR studies reveal that the predominant pathway for reaction of **1** is through transphosphorylation followed by hydrolysis of the cyclic phosphorothioate **3** (Figure 1) and not through attack

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(10) Davis, A. M.; Hall, A. D.; Williams, A. *J. Am. Chem. Soc.* **1988**, *110*, 5105−5108.

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(12) The ^{31}P NMR chemical shift of **4** is 15 ppm, which is similar to previously reported values for *S*-alkyl phosphorothioates, see: (a) Meade, T. J.; Iyengar, R.; Frey, P. A. *J. Org. Chem.* **1985**, *50*, 936−940. (b) Müller, C. E.; Roth, H. J. *Tetrahedron Lett.* **1990**, *31*, 501−502.

(13) The ^{31}P NMR chemical shift of **2** is 4 ppm at high pH. For additional spectroscopic data on this compound, see the Experimental Section.

(14) The ^{31}P NMR chemical shift of **3** is 37 ppm, which is characteristic of oxathiaphospholanes; see: Nuretdinova, O. N.; Guseva, F. F.; Arbuzov, B. A. *Izv. Akad. Nauk. SSSR, Ser. Khim* **1976**, 2625−2627.

(15) (a) It was also observed that hydrolysis of *O*,*S*-diethyl phosphorothioate occurs 25 times faster than ethylene cyclic phosphate. Gay, D. C.; Hamer, N. K. *J. Chem. Soc. B* **1970**, 1123−1127. (b) Kudelska, W.; Michalska, M. *Tetrahedron* **1986**, *42*, 629−636.

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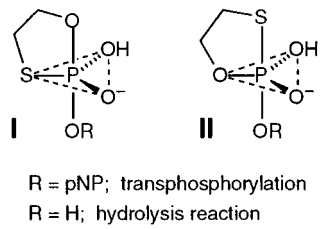

Figure 2. Possible trigonal bipyramidal phosphorane transition states formed during the transphosphorylation and hydrolysis reactions.

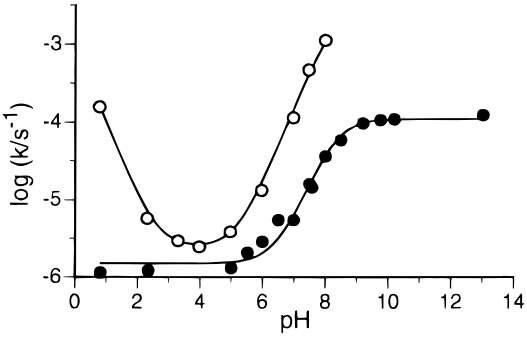

Figure 3. pH−rate profile for the hydrolysis of 2′-thiol $UpOC_6H_4$-*p*-NO_2, **1** (●), and $UpOC_6H_4$-*p*-NO_2, **5** (○), at 25 °C with [buffer] = 0.10 M and $I = 0.20$ M was obtained by monitoring the formation of *p*-nitrophenol or *p*-nitrophenolate (330 nm, pH < 7; 400 nm, pH ≥ 7).

at the glycosidic linkage.[17] Thus, the transphosphorylation of **1** is similar to that of a ribonucleotide, although there are intriguing differences. To further characterize the reactions of **1**, the pH−rate profile for its transphosphorylation was determined (Figure 3). By monitoring formation of *p*-nitrophenolate, the rate for the formation of **3** could be dissected from other processes. For comparison, the rate of transphosphorylation for hydroxyl analog **5** under the same conditions was also determined.

From the pH−rate profile, parallels between the reaction of **1** and that of **5** under basic conditions are apparent. Specifically, the transesterification of both nucleotides are first order with respect to hydroxide ion concentration from pH 6 to 8.[18] Moreover, the reaction rate for **1** becomes pH-independent above pH 9; the rate of transphosphorylation of 2′-hydroxyl ribonucleotide analogs also reaches a maximum when the basicity of the solution exceeds the pK_a of the alkoxide nucleophile.[19] Analysis of the pH−rate profile for **1** affords a value of 8.3 ± 0.1 for the pK_a of 2′-thiol analog **1**. An independent determination of the pK_a of the related compound 2′-deoxy-2′-thiouridylyl-(3′→5′)adenosine, obtained by monitoring the chemical shift of the H2′ uridine proton by ^{1}H NMR as a function of pD, afforded a similar pK_a of 8.2 ± 0.1.[20] These data indicate that the transphosphorylation reactions of **1** and **5** occur by related mechanisms, and they are consistent with thiolate serving as the nucleophile in the transphosphorylation of **1**.

A critical feature of thiol derivative **1** is its stability over the entire pH range studied, relative to **5**. Near physiological pH (7.4) the 2′-thiol nucleotide undergoes transphosphorylation at an observed rate 27-fold slower than that of **5**. Given the increased concentration of the reactive nucleophilic thiolate in **1** relative to that of the corresponding alkoxide in **5** under these conditions, this 27-fold observed difference indicates the rate of thiolate attack on the adjacent phosphodiester bond is a striking 10^7-fold slower than that of the corresponding alkoxide. Thus, thiolate groups are remarkably reticent nucleophiles when the target is an electrophilic phosphate center.[9,21]

The decreased propensity of thiolate relative to alkoxide attack on the phosphodiester bond may be attributed to stereoelectronic features of the trigonal bipyramidal phosphoranes, which are formed during the course of the transphosphorylation reactions. For **1** to undergo transphosphorylation, the incoming thiolate nucleophile must attack the adjacent phosphodiester from an apical position (structure **II**, Figure 2). Sulfur, being less apicophilic than oxygen, prefers the equatorial position (structure **I**, Figure 2). The necessity for sulfur to occupy an apical position during attack destabilizes the transition state for the transphosphorylation of **1**.[15,22] In addition, the incipient P−O bond formed in the transition state for reaction of **5** is expected to be stronger than the corresponding P−S bond generated by reaction of **1**.[23]

Another aspect of the reactivity of the 2′-thiol nucleotide **1** is its transesterification under acidic conditions, which deviates from the behavior of a hydroxyl nucleotide, such as **5**. Below pH 5 compound **1** undergoes hydrolysis at a basal rate, which is pH-independent.[24] By analogy to mechanisms proposed for 2′-hydroxyl nucleotides, we hypothesize that the pH-independent region under acidic conditions for **1** is due to the ionized thiol attacking a neutral phosphodiester.[25] Below pH 2, the rate of transphosphorylation of hydroxyl derivative **5** exhibits a first-order dependence on hydronium ion concentration attributed to hydroxyl attack on the neutral phosphodiester group.[18b] In this pH range, which is approaching the pK_a of the phosphodiester (*ca.* −0.36),[26] the *p*-nitrophenyl group is most likely departing as its anion from the neutral pentacoordinated phosphorane intermediate. The increase in rate for **5** contrasts with that for thiol analog **1**, a result that suggests an equivalent mechanism does not occur in the acidic region (pH <2). Our data are consistent with the transphosphorylation occurring via thiolate attacks; the sulfhydryl group has little tendency to attack either an anionic or a neutral phosphodiester.

Our quantification of the modest reactivity of thiolate nucleophiles toward phosphodiesters illuminates previous find-

(17) Only under very basic conditions (pH = 14) did a significant amount (30%) of glycosidic bond cleavage occur to afford uracil and *p*-nitrophenyl phosphate.

(18) Others have also demostrated this first order dependence on hydroxide for hydrolysis of 2′-hydroxyl compounds similar to **5**; see: (a) Usher, D. A.; Richardson, D. I., Jr.; Oakenfull, D. G. *J. Am. Chem. Soc.* **1970**, *92*, 4699−4712. (b) Oivanen, M.; Lönnberg, H. *Acta Chem. Scand.* **1991**, *45*, 968−971.

(19) For an estimate of the pH-independent rate for transphosphorylation of **5**, see the Experimental Section and ref 18a.

(20) (a) The pK_a of the 2′-hydroxyl of a ribonucleotide derivative is similarly depressed (pK_a of 13.9 obtained by stopped-flow kinetics)[18a] relative to that of 2-propanol. A pK_a value of 8.0 was obtained for the thiol in 2′-deoxy-2′-thiocytidine 3′-phosphate by ultraviolet spectroscopy.[3d]

(21) For thiols reacting with phosphodiester monochloridates, see: (a) Merckling, F. A.; Rüedi, P. *Tetrahedron Lett.* **1996**, *37*, 2217−2220. For thiols reacting with phosphorochloridothioates, see: (b) Miller, B. *J. Am. Chem. Soc.* **1962**, *84*, 403−409.

(22) Cleavage of 3′-thiouridylyl(3′→5′)uridine (pH 10, 50 °C) occurs at a rate 200-fold faster than that of UpU. In the transition state for the former substrate, the sulfur substituent can occupy an equatorial position; see: Liu, X.; Reese, C. B. *Tetrahedron Lett.* **1996**, *37*, 925−928.

(23) For bond enthalpies for P−O and P−S, see: (a) Pedley, J. B.; Marshall, E. M. *J. Phys. Chem. Ref. Data* **1983**, *12*, 967−1026. (b) Drowart, J.; Myers, C. E.; Szwarc, R.; Auwera-Mahieu, A. V.; Uy, O. M. *High Temp. Sci.* **1973**, *5*, 482−488.

(24) Alternatively, the hydrolysis of **1** between pH 0.8 and 5 may only be seemingly pH-independent. Specifically, the observed rate of hydrolysis under increasingly acidic conditions may be due to intermolecular attack by water rather than by thiol- or thiolate-facilitated transphosphorylation, since the rate of hydrolysis of ethyl *p*-nitrophenyl phosphodiester at pH = 0.8 at 25 °C with $I = 0.20$ M was determined to be 6.3×10^{-7} s^{-1}.

(25) The pH-independent region observed for 2′-hydroxyl nucleotides similar to **5** has been postulated to be the result of an ionized 2′-hydroxyl attacking a neutral phosphodiester backbone; see: Kosonen, M.; Oivanen, M.; Lönnberg, H. *J. Org. Chem.* **1994**, *59*, 3704−3708.

(26) This pK_a was obtained for 3,3-dimethylbutyl *p*-nitrophenyl phosphate. Hengge, A. C.; Cleland, W. W. *J. Am. Chem. Soc.* **1991**, *113*, 5835−5841.

ings in which the reactions of 2′-thiol-substituted nucleotides were examined. For example, Reese and co-workers did not detect any products of transphosphorylation when 2′-deoxy-2′-thiouridylyl(3′→5′)uridine was treated with acid or base; rather, they found that the major products were the symmetrical disulfide dimer and uracil.[3g] The nucleobase uracil is presumably derived from reaction of the thiolate at the anomeric position, which forms an episulfide that undergoes further degradation to multiple products. The origin of the differences in the observed products between this study and ours lies in the structures of the phosphodiester substituents employed. With the unactivated phosphodiester investigated previously, the rate of transphosphorylation by a thiolate is expected to be even more diminished than that of **1**. *p*-Nitrophenylate serves as a better leaving group than a primary alkoxide, and this substituent renders the phosphodiester in **1** more electrophilic. Therefore, different reaction pathways for 2′-thiol nucleotide substrates can be promoted by changes in the electronic properties of the phosphodiester group. For instance, compounds such as 2′-deoxy-2′-thiouridylyl(3′→5′)uridine may undergo transphosphorylation in the presence of catalysts that can increase the electrophilicity of the phosphodiester or stabilize the leaving group.

Despite our findings that thiolates exhibit limited nucleophilicity toward phosphodiesters, these species are used by biological catalysts that hydrolyze phosphomonoesters. Protein tyrosine phosphatases (PTPases),[27] a family of enzymes that participates in signal transduction, use a cysteine-derived thiolate as a nucleophile to attack the tyrosine phosphate moiety. In these reactions, which proceed through thiophosphate intermediates, the catalytic cysteine residue is essential; it cannot be replaced by serine. A distinguishing feature of the reactions of the PTPases that require thiolate nucleophiles is that these processes occur through dissociative mechanisms in which there are metaphosphate-like transition states.[28] In contrast, transesterification of phosphodiesters generally occurs through an associative pathway.[16g] Interestingly, in the examples of biological catalysts that use a thiolate to attack phosphorus, the phosphorus species are electron deficient. Similarly, the increased propensity for thiolate attack at an electrophilic phosphorus center is reflected in the different results of our studies and previous investigations.

Conclusions

The unique reactivity of 2′-thiol-substituted ribonucleotides is an attribute that highlights their potential as important tools for the study of nucleic acid chemistry and biology. Our finding that ribonucleotide analog **1** reacts through transphosphorylation suggests that oligonucleotides bearing 2′-sulfhydryl groups can be used to provide mechanistic insight into enzyme- and ribozyme-promoted RNA cleavage. Catalysts that adequately activate the leaving 5′-hydroxyl and/or polarize the scissile phosphodiester bond may be expected to facilitate the cleavage of 2′-thiol-containing oligonucleotides by a similar mechanism. Moreover, nucleoside analogs endowed with a 2′-thiol can be used to equip ribozymes with a functional group that has widespread use in reactions catalyzed by enzymes. Finally, 2′-thiol substituents can be used to engineer metal binding sites into RNA molecules. Such modifications would provide the means to dissect the contributions of specific metal ions to RNA structure or catalysis. Chemical investigations into the intrinsic reactivity of ribonucleotide derivatives containing 2′-thiol groups are essential prerequisites for the multiple applications of these compounds.

Experimental Section

General Methods. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker AM-300 spectrometer at 300, 75, and 121 MHz, respectively. Chemical shifts are reported relative to external references of sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$, 1,4-dioxane, or 85% H_3PO_4 for ^{1}H, ^{13}C, and ^{31}P, respectively, unless otherwise stated. ^{1}H NMR data are assumed to be first order with apparent doublets and triplets reported as d and t, respectively. A Corning 320 pH meter equipped with a standard calomel electrode was used for pH measurements. Ultraviolet absorbance measurements were made on a Cary Model 3 spectrophotometer equipped with a Cary temperature controller. To prevent reaction due to contaminating ribonucleases, water used in the preparation of buffers for ^{31}P NMR and UV−visible kinetics measurements was treated with diethyl pyrocarbonate then sterilized with an autoclave. Buffers used were glycine (pH = 2.3, 9.2−10.2), formate (pH = 3.3−4.0), acetate (pH = 5.0−5.5), MES (pH = 6.0−6.5), HEPES (pH = 7.0−7.6), Tris (pH = 8.5), and triethanolamine (pH = 7.5−8.0), and the buffer pH was adjusted with hydrogen chloride or sodium hydroxide. Reactions carried out at pH's above 13 and below 1 were done in NaOH or HCl solutions with the pH of the solution measured by a pH meter. Buffers were filtered through a 0.1 μm filter. To prevent oxidation of thiol containing compounds to the disulfide, solutions were deoxygenated by three successive freeze−pump−thaw cycles, and hydrolysis reactions monitored by NMR were performed under argon.

Spectral Data for 1, 2, and 2′-Deoxy-2′-Thiouridylyl (3′→5′) Adenosine.[8] **2′-Deoxy-2′-thiouridine 3′-(*p*-nitrophenyl phosphate) (1):** ^{1}H NMR (300 MHz, D_2O) δ 8.29 (d, J = 9.2 Hz, 2 H), 7.82 (d, J = 8.1 Hz, 1 H), 7.41 (d, J = 8.8 Hz, 2 H), 6.03 (d, J = 9.2 Hz, 1 H), 5.92 (d, J = 8.1 Hz, 1 H), 4.78−4.76 (m, 1 H), 4.36−4.34 (m, 1 H), 3.78 (d, J = 3.3 Hz, 2 H), 3.75−3.72 (m, 1H); ^{13}C NMR (75 MHz, D_2O) δ 165.8, 157.1 (d, J_{CP} = 7.0 Hz), 151.9, 143.5, 141.4, 125.8, 120.5 (d, J_{CP} = 5.1 Hz), 103.0, 89.8, 85.1, 77.5 (d, J_{CP} = 5.1 Hz), 61.1, 44.0 (d, J_{CP} = 7.0 Hz); ^{31}P NMR (121 MHz, D_2O) δ −5.57 (d, J = 6.9 Hz); ultraviolet absorption λ_{max} 266 nm (ϵ 13 000) at pH = 4.9; mass spectrum (LSIMS, 3-NBA, negative ion mode) *m*/*z* 460.1 [M$^-$, calcd for $C_{15}H_{15}N_3O_{10}PS$ 460.1].

2′-Deoxy-2′-(*p*-nitrophenylthio)uridine 3′-phosphate (2): ^{1}H NMR (300 MHz, D_2O) δ 8.00 (d, J = 8.6 Hz, 2H), 7.53 (d, J = 8.5 Hz, 2H), 7.50 (d, J = 7.7 Hz, 1H), 6.19 (d, J = 9.0 Hz, 1H), 5.57 (d, J = 8.1 Hz, 1H), 4.78−4.83 (m, 1H), 4.29−4.31 (m, 1H), 4.04 (dd, J = 8.6, 5.7 Hz, 1H), 3.57 (d, J = 3.5 Hz, 2H); ^{13}C NMR (126 MHz, D_2O) δ 165.2, 150.7, 146.3, 141.3, 141.1, 132.4, 123.9, 103.1, 89.7, 86.1, 75.5 (d, J_{CP} = 5.4 Hz), 61.3, 53.3 (d, J_{CP} = 5.4 Hz); ^{31}P NMR (121 MHz, D_2O) δ 1.13 (d, J = 5.4 Hz); ultraviolet absorption λ_{max} 260 nm (ϵ 7400), λ 332 nm (ϵ 3900), at pH = 5.8; mass spectrum (LSIMS, 3-NBA, negative ion mode) *m*/*z* 460.1 [M$^-$, calcd for $C_{15}H_{15}N_3O_{10}PS$ 460.1].

2′-Deoxy-2′-thiouridylyl (3′→5′) adenosine: ^{1}H NMR (300 MHz, D_2O) δ 8.45 (s, 1 H), 8.26 (s, 1 H), 7.73 (d, J = 8.3 Hz, 1 H), 6.12 (d, J = 5.0 Hz, 1 H), 5.89 (d, J = 8.1 Hz, 1 H), 5.82 (d, J = 8.8 Hz, 1 H), 4.87−4.85 (m, 1 H), 4.57−4.51 (m, 2 H), 4.38−4.37 (m, 1 H), 4.26−4.11 (m, 3 H), 3.72−3.60 (m, 2 H), 3.54 (dd, J = 7.0, 5.2 Hz, 1 H); ^{13}C NMR (75 MHz, D_2O) δ 165.8, 155.4, 152.7, 151.8, 148.9, 141.2, 139.6, 118.6, 102.8, 89.8, 87.2, 84.9, 83.4 (d, J_{CP} = 9.5 Hz), 76.6 (d, J_{CP} = 5.1 Hz), 73.7, 70.2, 65.2 (d, J_{CP} = 5.1 Hz), 61.0, 44.2 (d, J_{CP} = 7.0 Hz); ^{31}P NMR (121 MHz, D_2O) δ −0.50; ultraviolet absorption λ_{max} 260 nm (ϵ 14 000) at pH = 5.6; mass spectrum (LSIMS, 3-NBA, negative ion mode) *m*/*z* 588.1 [M$^-$, calcd for $C_{19}H_{24}N_7O_{11}PS$ 588.1].

Experimental Procedures. ^{31}P NMR Spectroscopy Studies of the Hydrolysis of 1. A deoxygenated solution of **1** (3.6 mg, 0.0074 mmol) in water (0.205 mL) was added to a deoxygenated solution of buffer. The final solution was 15 mM in **1** at a buffer concentration of 150 mM with the ionic strength of the solution adjusted to 250 mM by the addition of NaCl. Reactions were carried out at room temperature in a 5 mm NMR tube equipped with a D_2O insert. The resulting solutions were periodically monitored by ^{31}P NMR. For the reaction at pH 13 (0.49 mM NaOH), the solution concentration of **1** was 15 mM.

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UV−Visible Studies of the Hydrolysis of 1 and 5. The transphosphorylation reactions of 2′-thiol-UpOC$_6$H$_4$-*p*-NO$_2$, **1**, and UpOC$_6$H$_4$-*p*-NO$_2$, **5**, were monitored at 330 nm for pH <7 and at 400 nm for pH ≥7. Kinetic measurements were carried out at 25 °C with substrate concentrations at 0.15 mM and buffer concentrations at 100 mM. The ionic strength of each reaction was adjusted to 200 mM using NaCl. Conditions for the kinetic studies were chosen to minimize buffer-induced catalysis. These precautions include the specific choice of buffers used (listed under General Methods) and the reaction conditions, which were selected so that each was carried out at a pH near the pK_a for each buffer. At pH 7.5, the rates of transphosphorylation of **1** in two different buffers, HEPES and triethanolamine, were found to be the same.

For substrates **1** and **5**, the observed rate of transphosphorylation was calculated from the plot of absorbance versus time using Beer's law. The change in molar absorptivity for both compounds at each pH was determined from the difference in absorbance of completely hydrolyzed compound compared to the initial absorbance of starting material at the same concentration, pH, and ionic strength. Each data point plotted in the pH−rate profile is an average of at least two runs.

The observed rate constant for the hydrolysis of **1** was fit to the following Henderson−Hasselbach-derived equation:

$$k_{obs} = k_{max}K_a/([H^+] + K_a) + k_{min}[H^+]/([H^+] + K_a)$$

where K_a is the acid dissociation constant for the 2′-thiol, k_{max} is the asymptotic maximum rate of thiolate hydrolysis, and k_{min} is the asymptotic minimum. A value to 8.3 ± 0.1 was obtained for the pK_a of the 2′-thiol in **1**.

The observed rate constant for the hydrolysis of **5** was expressed using the following equation:

$$k_{obs} = k_{max}K_a/([H^+] + K_a) + k_{max}'[H^+]/([H^+] + K_a') + k_{ind}$$

where K_a is the acid dissociation constant for the 2′-hydroxyl, K_a' is the acid dissociation constant for the phosphate group, k_{max} is the asymptotic maximum rate of alkoxide mediated hydrolysis, k_{max}' is the maximum rate of hydroxide attack on the neutral phosphate, and k_{ind} is the rate of pH-independent hydrolysis.

The asymptotic maximum rate of alkoxide-mediated transphosphorylation of **5** was obtained by extrapolation of the above fit past the pK_a of the 2′-hydroxyl using 1.2×10^{-14} as the acid dissociation constant for the 2′-hydroxyl.[18a] By comparing the asymptotic maximum observed rates of transphosphorylation of **1** and the extrapolated maximum of **5**, the ratio of intramolecular alkoxide versus thiolate attack on the phosphate was calculated to be 10^7.

Titration of the Thiol in 2′-deoxy-2′-thiouridylyl(3′→5′)adenosine As Monitored by 1H NMR. Spectra are reported relative to an internal reference of sodium 3-(trimethylsilyl)propionate-*2,2,3,3,-d*$_4$. The ionic strength of a solution of 2′-deoxy-2′-thiouridylyl(3′→5′)adenosine (11 mM in D_2O) was adjusted to 130 mM by the addition of NaCl. The pD of the solution was adjusted to 4.6 by addition of a solution of DCl in D_2O. After acquisition of a ^{1}H NMR spectrum, titration of 2′-deoxy-2′-thiouridylyl(3′→5′)adenosine with NaOD in D_2O was monitored by ^{1}H NMR and the pD of the solution was measured on a pH meter. Ten such measurements were made up to a pD of 11.5 (Figure 4). The pK_a was calculated from the fit of the chemical shift of the H2′ proton of uridine versus pD using the following equation

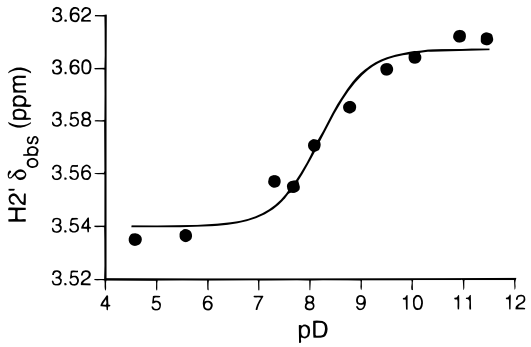

Figure 4. Graph of the chemical shift of the H2′proton in 2′-deoxy-2′- thiouridylyl(3′→5′)adenosine as a function of pD.

$$\delta_{obs} = \delta_{down}\delta_{up}\,[(10^{pK_a} + 10^{pD})/(\delta_{down}10^{pD} + \delta_{up}10^{pK_a})]$$

where δ_{obs} is the chemical shift of the H2′ in ppm, δ_{down} is the downfield limit for the chemical shift, and δ_{up} is the upfield limit. A value of 8.2 ± 0.1 was obtained for the pK_a of the 2′-thiol.

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Supporting Information Available: ^{1}H NMR spectra for **1**, **2**, and 2′-deoxy-2′- thiouridylyl(3′→5′)adenosine (4 pages). See any current masthead page for ordering and Internet access instructions.

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