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Synthesis and Characterization of an RNA Dinucleotide Containing a 3′-*S*-Phosphorothiolate Linkage

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Abstract: The synthesis of an RNA dinucleotide (IspU) containing a 3′-*S*-phosphorothiolate linkage is described. The compound is prepared from 9-(3-deoxy-3-iodo-β-D-xylofuranosyl)hypoxanthine with installation of the phosphorothiolate group via an Arbusov reaction and protection of the ribose 2′-hydroxyl as a silyl ether. IspU is found to be a substrate for several enzymes including T4 polynucleotide kinase, snake venom phosphodiesterase, and ribonuclease T_2. Base-catalyzed cleavage of the dinucleotide is accelerated (∼2000-fold) relative to that of the phosphate-linked compound IpU. Product characterization and kinetic analysis show that IspU is cleaved through the same mechanism as IpU. The observed rate acceleration is argued to reflect stabilization of the anionic transition state by the polarizable sulfur atom.

Introduction

Nucleic acids lack the diversity of functional groups which protein enzymes employ to achieve structural organization and catalytic efficiency. Nevertheless, RNA and DNA molecules, like proteins, are capable of folding into stable tertiary structures and catalyzing chemical reactions.[1−5] Whereas many functional group changes in protein enzymes can be achieved using site-directed mutagenesis, related approaches to nucleic acid studies merely result in changing hydrogen-bonding patterns. Thus, synthetic methods have been developed for introducing non-native functional groups into nucleic acids.[6] Some of the most informative substitutions have involved replacing the phosphoryl oxygens with sulfur.

Phosphorothioate diastereoisomers (R_p, S_p), resulting from substitution of one nonbridging phosphoryl oxygen with sulfur, have become common tools in nucleic acid biochemistry. Selective incorporation of one or the other diastereoisomer has been used to investigate the stereochemistry of reaction mechanisms,[7−12] to locate protein−nucleic acid interactions,[13] and to probe for metal ion−phosphate interactions.[14−16]

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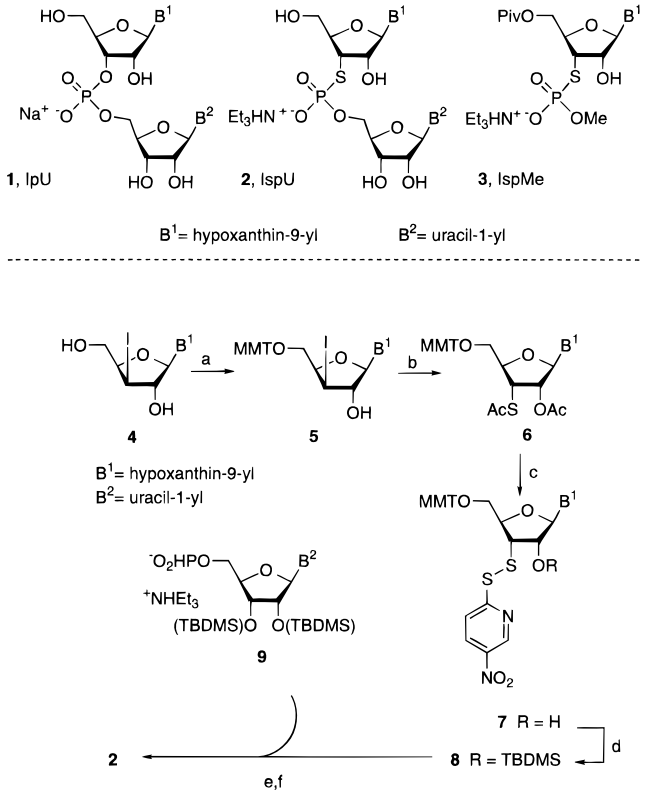
Sulfur substitution of the bridging atoms has received less attention. DNA oligonucleotides containing a 5′-bridging sulfur atom have been synthesized.[17−21] Recently, reports of incorporation of a 2′,5′-dideoxy-5′-thionucleotide within an otherwise all-RNA hammerhead ribozyme and of a single 5′-deoxy-5′-thioribonucleotide in a DNA oligonucleotide have appeared.[22,23] Synthesis of an RNA dinucleotide containing a 5′-deoxy-5′-thio-substituted phosphate linkage has also been reported.[24] Replacement of the 3′-bridging oxygen with sulfur in a deoxyribodinucleotide was first reported by Cosstick and coworkers in 1988.[25] Since the initial synthesis of these DNA 3′-*S*-phosphorothiolates, the methods have been improved[26−29] and extended to both a purine−pyrimidine ribodinucleotide precursor[30] and a pyrimidine−pyrimidine ribodinucleotide, UspU.[31] The DNA analog has been investigated for enzymatic and chemical cleavage properties in the context of large DNAs.[32,33]

In a slightly different approach, a DNA 3′-*S*-phosphorothiolate was used to investigate the active site of the *Tetrahymena* ribozyme. Analysis of cleavage of a DNA substrate with a 3′-*S*-phosphorothiolate at the scissile phosphate, d(CCCUCUspA), identified a specific metal ion interaction that contributes to ribozyme catalysis.[34] Use of a DNA substrate complicated the study because the 2′-OH at the cleavage site enhances reactivity.[35] Because 2′-hydroxyls contribute to the structure and activity of many ribozymes, it is advantageous to be able to make functional group substitutions in a ribose context.

Investigation of both RNA and protein enzymes could be extended by the availability of RNA 3′-*S*-phosphorothiolates. Before these molecules can be used as specific mechanistic probes, they need to be evaluated for their suitability as RNA analogs. Herein, we report the synthesis of a purine−pyrimidine dinucleotide containing a ribo-3′-*S* -phosphorothiolate linkage (IspU) and characterize its reactivity with phosphodiesterases, RNA-specific nucleases, and chemical cleavage reagents. IspU was selected as a target because it is a potential substrate for the *Tetrahymena* ribozyme.[36]

Scheme 1[a]



[a] Reagents and conditions: (a) MMTCl, pyridine; (b) potassium thioacetate, DMF, 37 °C, 24−36 h; (c) NaOH/MeOH, 1 h, then acetic acid, 2,2′-dithiobis(5-nitropyridine) in CH_2Cl_2; (d) *tert*-butyldimethylsilyl triflate, pyridine, 4 h; (e) **9**, bis(trimethylsilyl)trifluoroacetamide, THF; (f) acetic acid−H_2O (8:2), then NEt_3·3HF, 6 h.

Results

Synthesis of 3′-(Thioinosylyl)-(3′→5′)-uridine (IspU). The synthesis of IspU (**2**) was based on that reported for a 9-[3-deoxy-3-*S*-(5-nitropyridyl-2-disulfanyl)-β-D-ribofuranosyl]hypoxanthine derivative which had previously been shown to react readily with trialkyl phosphites.[30] Although a protected version of the dinucleotide has been reported, the stability of the compound under conditions for deprotection of the 2′-silyl group was uncertain.[30] The suitability of the TBDMS group for the protection of the 2′-hydroxyl function vicinal to the sensitive phosphorothiolate group was established in a model study on a 2′-*O*-TBDMS-protected inosine phosphorothiolate, IspMe (**3**). NEt_3·3HF has been reported to be superior to tetrabutylammonium fluoride (TBAF) for the deprotection of silylated oligoribonucleotides.[37] Indeed, treatment of silylated precursor with a large excess of neat NEt_3·3HF cleanly removed the TBDMS group to give **3** as the triethylammonium salt in 85% yield. The crude reaction mixture showed a single peak in the ^{31}P NMR spectrum ($\delta = 20.69$ ppm) with no upfield resonances indicative of cleavage of the P−S bond.

In the key step for the synthesis of IspU (Scheme 1), the disulfide **7** was reacted with 2′,3′-bis(*O*-*tert*-butyldimethylsilyl)-uridin-5′-yl *O*,*O*-bis(trimethylsilyl) phosphite, generated *in situ* from **9**. After partial purification by flash column chromatography, the protected dinucleotide was deblocked in two steps using aqueous acetic acid followed by NEt_3·3HF. IspU (**2**) was purified by flash chromatography on C_{18} reversed phase silica. The spectroscopic data obtained for the dinucleotide were

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Table 1. Identified Products of Enzymatic and Chemical Cleavage of p*IspU and p*IpU[a]

	p*IspU		p*IpU	
reagent	product(s)[b]	methods	product(s)	methods
SVPD	pIsH	GE/D, TLC,[d] IM	pIoH	GE, TLC
nuclease P1		GE	pIoH	GE
RNase T_2	pIsp	GE, TLC	pIp	GE, TLC
RNase T_1	pIsp	GE, TLC	pIp	GE, TLC
$AgNO_3$	pIsH	GE, TLC, IM		GE
−OH	pIs>p	GE, TLC, AC, IM	pIp	GE, TLC
	pIsp	GE, TLC, AC		
	pIsH	GE, TLC, IM		
H_3O^+	pI(2′-p,3′-SH)[d]	GE, IM	nd	

[a] Reactions were performed and analyzed as described in the Experimental Section. [b] Abbreviations: pIsH, 5′-phosphoryl-3′-deoxy-3′-thioinosine; pIsp, 3′-*S*,5′-diphosphorylinosine; pIs>p, 5′-phosphoryl(3′-*S*,2′-*O*-cyclic phosphoryl)inosine; pI(2′-p,3′-SH), 2′,5′-diphosphoryl-3′-thioinosine; GE, mobility on polyacrylamide gels compared to appropriate markers; GE/D, polyacrylamide gel electrophoresis ± DTT; TLC, thin layer chromatography; IM, iodoacetamide modification; AC, $AgNO_3$ cleavage. [c] TLC data not shown. [d] Initial product formed at pH 1, 50 °C, 90 min.

consistent with the proposed structure (see the Experimental Section). The ^{13}C NMR spectrum of IspU showed only one significant difference from that of an authentic sample of IpU, with the C3′-inosyl resonance of IspU showing an upfield shift to 44.05 ppm (*cf.* 71.65 ppm in IpU). In the ^1H spectrum, a small $^3J_{1'2'}$ coupling constant was observed for both **2** (essentially a singlet) and **3** (1.2 Hz), indicating that the thiosugar exists predominantly in the 3′-*endo* pucker, and indeed exceeds the 3′-*endo* distribution found in natural ribonucleotides (*cf.* $^3J_{1'2'}$ = 5.2 Hz in IpU). This conformational effect, which results from replacing the 3′-oxygen atom by the larger, more electropositive sulfur, is predicted by the gauche effect and should make these analogs very good mimics of RNA.[38−40]

Enzymatic Studies with IspU. (See Table 1 for a summary of the methods used for product comparison and identification.) In order to facilitate analysis of the dinucleotides, IpA, IpU, and IspU were phosphorylated with T4 polynucleotide kinase and [γ-^{32}P]ATP. A direct comparison of IspU and IpU indicated that while IspU was a substrate for this kinase, the relative rate of reaction (ratio of k_{cat}/K_M values) was slowed by approximately 100-fold (data not shown). Reduced reactivity is consistent with this kinase's known interaction with the 3′-phosphate[41−43] and with the sulfur substitution perturbing the linkage.

p*IspU was investigated as a substrate for snake venom phosphodiesterase (SVPD) and nuclease P1. Both enzymes cleave RNA and DNA to leave 3′-hydroxyls and 5′-monophosphates (p*NpMoH → p*NoH + pMoH), so they would need to cleave the S−P bond of IspU.[44,45] As shown in Figure 1, p*IspU was cleaved by SVPD; resolution of a distinct product band required addition of 10 mM DTT to both the gel matrix and running buffer. As observed previously with a DNA *S*-phosphorothiolate,[34] without the reducing agent, the radioactivity smears uniformly along the lane. The unexpectedly high electrophoretic mobility of p*IsH relative to p*IoH (compare the two SVPD lanes in Figure 1) can be attributed to deprotonation of the 3′-sulfhydryl, resulting in an additional negative charge on the molecule.[46] Thiol-specific modification reagents ICH_3 and ICH_2CONH_2 were used to demonstrate that SVPD cleaves to give the anticipated product p*IsH (Figure 2).[51,52]

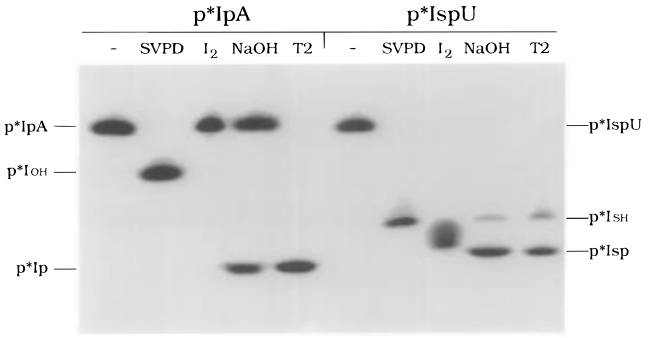

Figure 1. Enzymatic and nonenzymatic cleavage of dinucleotides containing 3′-*S*-phosphorothiolate and normal phosphodiester linkages. The 5′-^{32}P-labeled dinucleotides were analyzed without additional treatment (−) and after treatment with the following reagents: 0.02 mg/mL SVPD at 37 °C, 50 mM I_2/aqueous pyridine at room temperature, 0.1 N NaOH at 50 °C, and 0.2 unit/μL RNase T_2 (T2) at 50 °C. All reactions were incubated at room temperature for 30 min. Because the dinucleotides are 5′-end-labeled, their different 3′-nucleotides do not affect the labeled cleavage products.

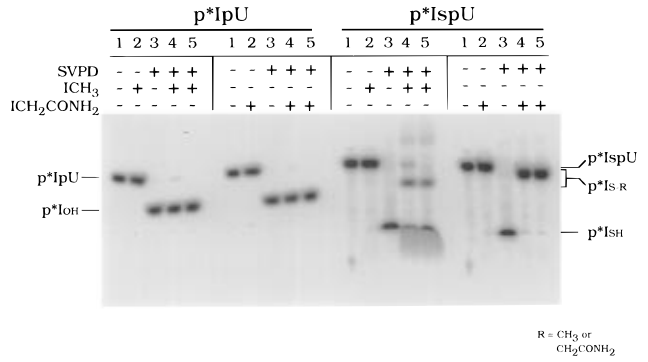

Figure 2. Selective modification of a reaction product containing a free thiol. 5′-^{32}P-labeled dinucleotides were incubated in 50 mM HEPES (pH 8) and 10 mM Mg^{2+} with thiol-specific modifying reagents ICH_3 and ICH_2CONH_2, and with (+) or without (−) concomitant treatment with SVPD. SVPD reactions were conducted with 2×10^{-4} mg/mL SVPD. Incubation conditions: lane 1, 0 °C, 1 h; lane 2, 25 °C, 10 min, then room temperature, 50 min; lane 3, 25 °C, 10 min; lane 4, 25 °C, 10 min, and lane 5, 25 °C, 10 min, then room temperature, 50 min.

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(46) The 3′-sulfhydryl should have a significantly lower pK_a than the corresponding 3′-hydroxyl. The pK_a values for two 2′-deoxy-2′-thionucleosides have been reported: 2′-thio-U, 7.3 (ref 47); 2′-thio-C = 7.5 (ref 48). These values reflect a shift of 5 pK_a units from the unmodified nucleosides: 12.59 and 12.24−12.5 for U and C, respectively (refs 49 and 50). Christensen et al. also determined the pK_a for the secondary ribose hydroxyl on inosine to be 12.36 (ref 50). Assuming the same magnitude of shift as seen with the pyrimidines, the 3′-sulfhydryl on the SVPD reaction product (p*IsH) should have a pK_a in the range of 7.4, be substantially deprotonated within the gel (pH 8.3−8.5), and migrate with an additional negative charge.
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After treatment with the alkyl iodides, the modified cleavage products showed shifts in mobility consistent with the addition of R groups having different molecular weights (CH_3 vs CH_2CONH_2). Nuclease P1 did not cleave p*IspU under conditions that gave total cleavage of p*IpU (data not shown).

RNA 3′-*S*-phosphorothiolates are also potential substrates for ribonucleases. These protein enzymes cleave RNA using the ribose 2′-hydroxyl as the nucleophile in a transphosphorylation reaction to generate cyclic phosphates which they then hydrolyze to generate 3′-monophosphates (p*NpM → p*N>p + ^{HO}M → p*Np + ^{HO}M).[53] p*IspU was digested with either RNase T_2 (Figure 1) or RNase T_1 (data not shown). In both cases, the product formed migrated approximately with p*Ip which had been generated by cleavage of either p*IpA or p*IpU with RNase T_2. The product was tentatively identified as p*Isp (see below for further characterization).

Specific Cleavage of the *S*-Phosphorothiolate Linkage. In work with DNA 3′-*S*-phosphorothiolates, it has been demonstrated that iodine as well as silver ions specifically cleave the S−P bond without apparent damage to native phosphodiester linkages [d(p*NspM) → p*(dNsH) + p(dM)].[26,27,32] Each reagent was tested with p*IspU, and cleavage occurred as anticipated (Figures 1 and 3A). $AgNO_3$ cleavage followed by quenching into a stop solution containing DTT (20−100 mM) produced p*IsH as identified by comigration with the SVPD product in both TLC (data not shown) and PAGE analysis. Iodine cleavage resulted in formation of several products, all of which had similar mobility on a high percentage polyacrylamide gel. The species presumably represent oxidation states of the terminal sulfur. In previous work, the major product of iodine cleavage of a DNA analog did not co-elute with an authentic standard on HPLC.[27] Unlike iodine cleavage of RNA and DNA phosphorothioates, cleavage of the 3′-*S*-phosphorothiolates with either $AgNO_3$ or I_2/pyr proceeds quantitatively.

Base-Catalyzed Cleavage of p*IspU. Product Analysis. Base-catalyzed (or buffer-catalyzed) cleavage of RNA is often examined as a model for the mechanism of action of ribonucleases.[54−58] The reactions differ in that (1) the nucleophile is deprotonated by hydroxide in solution as opposed to by an amino acid side chain within the enzyme active site and (2) opening of the 2′,3′-cyclic phosphate intermediate loses regiospecificity in solution, producing the 2′-monophosphate as well as the 3′-monophosphate. In both the enzyme- and base-catalyzed reactions, the leaving group is the 5′-hydroxyl. The accurate production of this leaving group was confirmed with base cleavage of the 3′-end-labeled molecule IspUp*A_H (where A_H is 3′-deoxyadenosine). As expected, partial cleavage produced Up*A_H which comigrated with the authentic dinucleotide (data not shown).

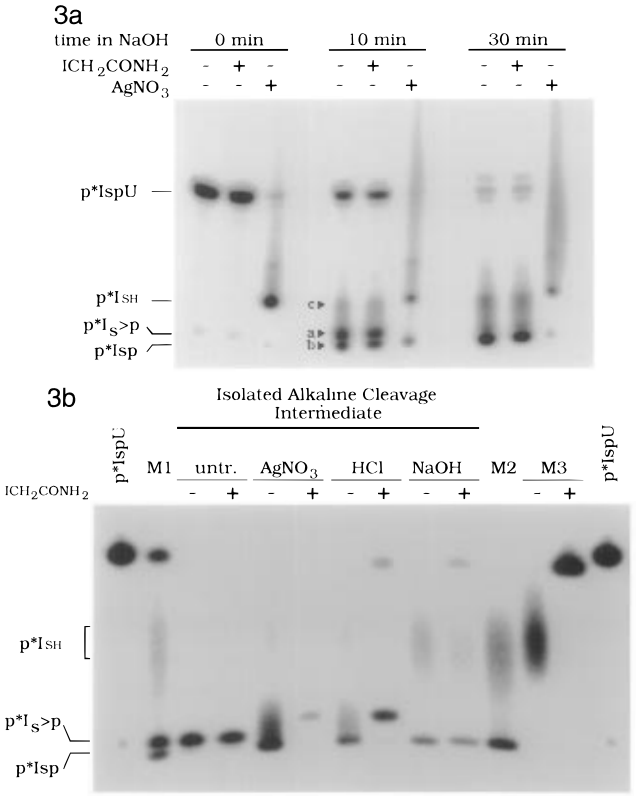

Figure 3. Identification of the products of alkaline cleavage of p*IspU: p*IspU → p*Is>p → p*Isp. (A) Reaction of alkaline cleavage products with ICH_2CONH_2 and $AgNO_3$. p*IspU was cleaved with 0.01 N NaOH at 30 °C. Aliquots were removed and neutralized with HCl at the indicated times. Each sample was then divided into thirds: one-third was diluted directly into DTT−stop solution, one-third was treated with 50 mM aqueous ICH_2CONH_2 at 30 °C for 10 min, and one-third was reacted with 50 mM $AgNO_3$ at room temperature for 10 min. Silver nitrate treatment led to some smearing in the lane as the samples were not filtered to remove any precipitates before analysis. (B) Characterization of the intermediate in alkaline cleavage. The intermediate species **a** was isolated and then either left untreated (untr.) or reacted for 30 min with 50 mM $AgNO_3$ at room temperature, 0.1 N HCl at 50 °C, or 0.1 N NaOH at 50 °C; it was then either quenched (−) or treated (+) with 40 mM ICH_2CONH_2 (DMF stock) in 50 mM HEPES (pH 8) at room temperature for 15 min. Comparative markers were generated from p*IspU: M1, partial alkaline cleavage; M2, RNase T_2 digest; M3, SVPD cleavage.

Alkaline cleavage of p*IspU resulted in the appearance of three 5′-radiolabeled products (Figure 3A). Characterization of the reaction products relied on two methods: modification of free thiols with ICH_2CONH_2 and specific cleavage of the sulfur−phosphorus bond with $AgNO_3$. The starting dinucleotide (i.e., intact S−P bond) was not modified with ICH_2CONH_2 but was cleaved with $AgNO_3$, providing a basis of comparison (Figure 3A, left). Alkaline cleavage of p*IspU first generated species **a**, which was converted directly to one of faster mobility (**b**); **b** comigrated with the product of RNase T_2 cleavage. After this conversion had begun, a minor product (**c**), migrating slower than the intermediate **a**, appeared (Figure 3A).

Although the RNase cleavage product and the alkaline hydrolysis product **b** were presumed to be p*Isp on the basis of the mechanism of ribonuclease cleavage, it was necessary to rule out formation of the 2′-phosphate, 3′-sulfhydryl [p*I(2′-p,3′-SH)] via phosphoryl migration. To distinguish between the two potential monophosphorylated products (*S*-linked and *O*-linked), the reaction products were probed for both accessible

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thiols and intact S−P bonds. The *S*-linked monophosphate would be expected to be inert to thiol modification, but to be sensitive to S−P bond cleavage with $AgNO_3$. Conversely, *O*-linked monophosphate should be sensitive to ICH_2CONH_2 modification and inert to $AgNO_3$.

As shown in Figure 3A, an alkaline cleavage reaction was sampled at the indicated times; the aliquots were neutralized and either modified with ICH_2CONH_2 or cleaved with $AgNO_3$. After 10 min, the two major species **a** and **b** were in evidence; neither had altered mobility after ICH_2CONH_2 treatment, suggesting that both lacked accessible thiols. After 30 min, essentially complete conversion to the fastest moving product **b** had occurred. Iodoacetamide modification again produced no change, but $AgNO_3$ converted the final alkaline product to one comigrating with cleavage product (p*Ish) from the intact dinucleotide. On the basis of this reactivity, the ultimate product of alkaline transphosphorylation and hydrolysis **b** was identified as p*Isp. This conclusion correlates well with model studies on *S*-phosphorylated derivatives of β-mercaptoethanol[59] which demonstrated exclusive formation of an *S*-linked phosphate when a 2′-*O*,3′-*S* cyclic phosphate derivative, sodium *O*,*S*-ethylenephosphorothioate, was incubated under alkaline conditions (0.1 N NaOH, 25 °C).

The identity of the intermediate **a** could not be ascertained by analysis of the partial digest. However, on the basis of the expected mechanism for base-catalyzed cleavage of RNA, the intermediate was postulated to be the 2′-*O*,3′-*S*-cyclic phosphate p*Is>p. On the basis of work with model compounds and 2′-sulfur-substituted nucleotides, it was anticipated that the putative cyclic phosphorothiolate would open upon treatment with either acid or base.[59−61] However, pseudorotation under acidic conditions should favor cleavage of the S−P bond to produce p*I(2′-p,3′-SH), while basic conditions should prohibit pseudorotation, limiting the mechanism to direct displacement of the O−P bond, leading to p*Isp. To better characterize the intermediate **a**, it was generated by partial cleavage, and then isolated from a denaturing gel. Treatment of the isolated intermediate with either $AgNO_3$ or HCl resulted in a product that did react with ICH_2CONH_2 (Figure 3B). The resulting iodoacetamide conjugate had unique electrophoretic mobility relative to those of other observed species, and was concluded to be p*I(2′-p,3′-SR), R = CH_2CONH_2. NaOH hydrolyzed intermediate **a**, but the resulting product was not reactive with the modifying reagent as seen previously with alkaline cleavage of p*IspU (Figure 3). Both cleavage products, p*Isp from alkaline cleavage and p*I(2′-p, 3′-SH) from either acid or $AgNO_3$ treatment, had similar mobility although the $AgNO_3$/HCl product displayed a slight tendency to smear, also consistent with the presence of a sulfhydryl group. The differential reactivity confirmed the identity of the intermediate as the 2′-*O*,3′-*S*-cyclic phosphate p*Is>p.

Kinetics of Base-Catalyzed Cleavage. Over the full range of pH 10−14, the modified dinucleotide p*IspU was cleaved 2000-fold more rapidly than the control dinucleotide p*IpU (10 °C, I = 1.0 M, Figure 4). Thus, while the products of the reaction suggested identical cleavage mechanisms for the *S*-phosphorothiolate and the native phosphodiester, comparative rate analysis indicated a substantial difference between the two dinucleotides. One possible source of the rate acceleration could be perturbation of the pK_a of the 2′-hydroxyl nucleophile. The pH curves in Figure 4 were fit to derive an apparent pK_a for that nucleophile (Table 2).[62,63] The apparent pK_a values were essentially identical for the two dinucleotides.

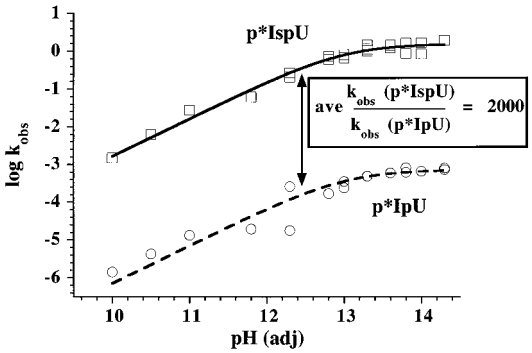

Figure 4. Kinetically determined pK_a for the 2′-OH in alkaline cleavage of p*IspU (squares) and p*IpU (circles). Reactions were performed at constant ionic strength (I = 1.0) as described in the Experimental Section. Measured pH values were adjusted for temperature and salt effects as described. Each point represents log k_{obs} derived from the first-order loss of substrate at the indicated pH. Values determined from the best fit line for a single ionization event are listed in Table 2.

Second-order rate constants for transphosphorylation were determined for p*IspU and p*IpU under several conditions. As seen in Table 2, a 10 °C increase in temperature increased the rate by 2−3-fold, and increasing the ionic strength to I = 1.0 provided a rate enhancement of 6-fold. This latter result is consistent with charge screening of the interaction between the anionic nucleophile and the negatively charged phosphate.

Acid-Catalyzed Cleavage of p*IspU. As described previously, acid-catalyzed cleavage of RNA proceeds via a more complex set of reaction pathways than does the alkaline reaction.[63] Below pH 3, changing protonation states lead to shifts in the predominant cleavage pathways (e.g., phosphoryl migration vs transphosphorylation and hydrolysis). With a purine−pyrimidine dinucleotide, depurination can also contribute to loss of substrate.[63]

Although several products resulting from cleavage of p*IspU at pH 2 were observed via PAGE analysis, we chose to characterize only the initial reaction product (Figure 5). Unlike alkaline cleavage of the substituted dinucleotide, acid cleavage afforded an initial product which was sensitive to ICH_2CONH_2 modification. The product was identified as p*I(2′-p,3′-SH) on the basis of its comigration with p*Isp and the presence of a free thiol.

Initial kinetic studies revealed similar rates of acid-catalyzed cleavage for p*IspU and p*IpU (data not shown). No attempt was made to deconvolute the contributing reaction pathways and their respective rates.

Discussion

Although sulfur is typically considered to be a conservative substitution for oxygen, the two differ substantially. Oxygen is more electronegative and is considered to be a hard atom.[64] Sulfur is considered to be soft, and very polarizable.[64] Despite these basic chemical differences, IspU was determined to be a suitable analog for RNA. The enzymatic and chemical characterization is summarized in Figure 6. The dinucleotide was found to be a substrate for two enzymes commonly used to introduce radiolabels into nucleic acids, polynucleotide kinase and polyA polymerase. As previously demonstrated for DNA analogs, SVPD and two chemical reagents, $AgNO_3$ and I_2/pyr, were found to cleave the S−P bond. Additionally, ribonucleases T_1 and T_2 cleaved p*IspU to form the expected products.

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Table 2. Apparent pK_a Values and Second-Order Rate Constants for Cleavage of p*IspU and p*IpU

	apparent pK_a[a]		k_{OH}[b] (M^{-1} min^{-1})			
	adjusted	unadjusted	10 °C, I = 1.0	20 °C, I = 1.0	20 °C, I = var	30 °C, I = var
p*IspU	12.99 ± 0.08	12.86 ± 0.08	22.7	62.0	10.9	20.3
p*IpU	13.0 ± 0.2	12.9± 0.2	5.7×10^{-3}	nd	nd	7.8×10^{-3}

[a] Adjusted pK_a values reflect corrections in the calculated pH for temperature and ionic strength as described in the Experimental Section. [b] Second-order rate constants were determined from the slopes of plots of k_{obs} vs hydroxide concentration. Correlation factors were greater than 0.97 in all cases. var = variable monovalent cation concentration based on buffer used or NaOH added; nd = not determined.

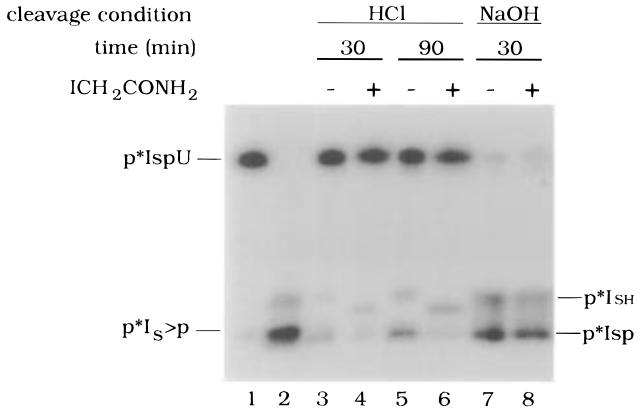

Figure 5. Modification of the initial product of acid cleavage of p*IspU. p*IspU was incubated with 0.1 N HCl at 50 °C. At the indicated times, aliquots were removed and neutralized with NaOH. The samples were divided and either quenched (−) or reacted (+) with 40 mM ICH_2CONH_2 (DMF stock) at room temperature for 15 min. p*IspU (lane 1) and p*Is>p (lane 2) were run as markers. Alkaline cleavage of p*IspU (0.01 N NaOH, 50 °C, 15 min) before (lane 7) and after (lane 8) modification is provided for comparison. In lanes 4 and 6, the species migrating between p*Isp and p*Ish is the iodoacetamide derivative of p(I(2′-p, 3′-SH).

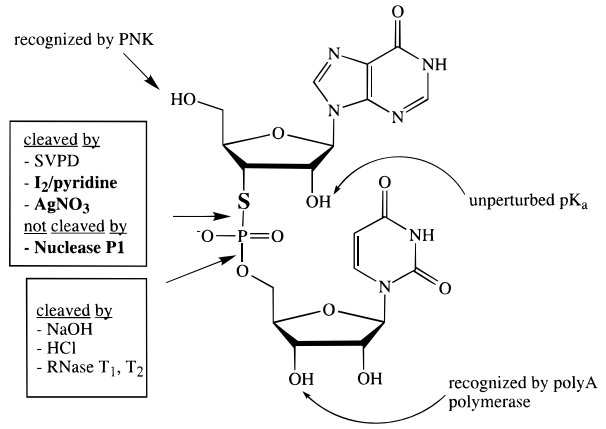

Figure 6. Summary of IspU reactivity. Reagents that react differently with IspU and IpU are shown in bold type. Not illustrated is the predominant 3′-*endo* sugar pucker induced by the sulfur substitution. PNK, polynucleotide kinase; SVPD, snake venom phosphodiesterase.

Although some reactions were essentially unaffected by the sulfur substitution, differences between IspU and IpU were detected and characterized more fully.

The mechanism of base-catalyzed cleavage of RNA is analogous to that of some RNA and protein enzymes which have active sites that facilitate in-line nucleophilic attack by the 2′-hydroxyl on the proximal phosphate with liberation of the 5′-oxygen-linked portion of the chain.[53,65,66] Regardless of the catalyst, the reaction proceeds via a trigonal bipyramidal transition state, with the nucleophile and leaving group positioned at opposing apices. While the ribonucleases appeared to be relatively insensitive to the sulfur substitution in p*IspU (Figure 1), it was clear that base-catalyzed cleavage was strongly affected as p*IspU decomposed 2000-fold faster than p*IpU under alkaline conditions (Figure 4). An accelerated rate of cleavage has also been reported for UspU under different conditions (50 mM sodium glycinate buffer, pH 10.06, 50 °C).[31]

For analysis of the source of the enhanced reactivity, the reaction can be considered in terms of its components: the leaving group, the nucleophile, and the reaction center. The leaving group, the 5′-oxyanion of uridine, is the same for both molecules studied and consequently should affect both cleavage rates equally. The nucleophile could be altered in two ways. First, another moiety could replace the 2′-hydroxyl in that role, effectively changing the cleavage mechanism. This alternative is unlikely because of the entropic advantage conferred by intramolecular attack versus intermolecular attack. Moreover, identification of the 2′-*O*, 3′-*S*-cyclic phosphate intermediate confirmed participation of the intramolecular nucleophile. Second, enhancement of the nucleophilicity of the 2′-hydroxyl could produce an increase in the rate of cleavage, since attack of the 2′-hydroxyl is considered to be the rate-limiting step of RNA scission under alkaline conditions.[67] However, determination of the reaction rate as a function of pH showed that the apparent pK_a of the nucleophile is identical for p*IpU and p*IspU, thereby eliminating nucleophilic enhancement as the source of rate acceleration.

Several consequences of sulfur substitution on the reactive phosphorus can be considered. Replacing the 3′-oxygen with sulfur is expected to increase the separation between C3′ and the phosphorus by ∼1 Å due to changes in bond lengths: C−S = 1.82 Å, C−O = 1.43Å, S−P = 1.95 Å, O−P = 1.57 Å.[68] Additionally, calculations with model phosphoranes predict that sulfur-centered bond angles will be more acute than the oxygen-centered counterparts: $P(H_5)XH$ has ∠P−X−H = 112.0° when X = O and 99.5° when X = S.[69] In the dinucleotide, the longer bonds and sharper ∠C3′−S−P could reduce the distance between the nucleophile and the reactive phosphate, facilitating the first step of the reaction. The intermediate 2′,3′-cyclic phosphorothiolate could also be stabilized by those same factors. In fact, under conditions of partial cleavage we observed a greater buildup of the cyclic phosphate intermediate with p*IspU than with p*IpU. The increased bond length and smaller bond angle preferences may combine to alleviate some of the strain energy in the five-membered ring. Liu et al. suggested that decreased ring strain contributed to lowering the energy of the transition state in cleavage of UspU.[31]

However, acid-catalyzed cleavage of p*IspU was determined to be accelerated by only 3-fold over p*IpU (data not shown). Since acid-catalyzed transesterification and hydrolysis presum-

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ably proceed via transition states with geometries similar to those of the base-catalyzed reactions, the geometric perturbation by the sulfur substitution should be comparable in each case. It seems unlikely then that the large increase in rate of base-catalyzed cleavage can be attributed exclusively to geometric factors. It should be noted that changes in phosphate protonation under acidic conditions result in increased use of pathways involving pseudorotation[63] and that, until each step is examined independently, the full impact of the sulfur substitution on acid-catalyzed hydrolysis cannot be assessed.

Oxygen and sulfur have been shown to bond differently with phosphorus when placed in a nonbridging position. Oxygen forms a supplementary bonding interaction ($p_\Pi - d_\Pi$) which appears as an O−P bond order intermediate between 1 and 2. In contrast, sulfur loses the added bonding interaction and localizes a full negative charge.[70,71] In spite of this electronic rearrangement, studies with phosphorothioates showed little or no alteration in the rates of alkaline hydrolysis.[15,72−74]

Sulfur in the 3′-bridging position may contribute to stabilization of the transition state through the added polarizability of that atom. In model studies on acyclic phosphates, Kirby and Younas found that phosphate diesters were more refractory to attack by anionic nucleophiles than to neutral ones presumably due to electrostatic repulsion between the phosphate and the incoming anion.[75] In carbanion chemistry, the stabilizing effect of α-sulfur atoms is well documented, and is largely attributed to increased polarizability of the sulfur.[76] Viewing the phosphate as a center of negative charge, which increases in anionic character on moving to the transition state, the adjacent sulfur could facilitate distribution of the charge thereby stabilizing the transition state. This type of effect coupled with geometric factors would be consistent with the observed rate enhancement for base-catalyzed hydrolysis which utilizes an anionic nucleophile, and also with the modest enhancement seen with acid-catalyzed cleavage where the nucleophile is a neutral hydroxyl.

Enzymatic cleavage of the 3′-sulfur−phosphorus bond was first characterized with TspT, a DNA dinucleotide containing a 3′-*S*-phosphorothiolate.[27] Compared with TpT, TspT was an equivalent substrate for SVPD, but was a much poorer substrate for nuclease P1. IspU showed the same differential reactivity as TspT. Although SVPD and nuclease P1 sever the same bond in native phosphodiester linkages, their mechanisms of action are dramatically different. SVPD forms a covalent enzyme−substrate intermediate by in-line attack of the hydroxyl of a threonine side chain followed by hydrolysis of the P−O bond, resulting in retention of configuration at phosphorus.[72,77,78] SVPD has no binding requirement for the atoms preceding the scissile bond, indicating that the 3′-uridine should be sufficient to identify p*IspU as a substrate.[44,79,80] In contrast, nuclease P1 is argued to utilize a zinc-activated water molecule as the nucleophile for attack on the diester substrate, and cleaves with inversion of the phosphorus configuration.[45,81] In an X-ray crystallographic study, an arginine side chain (R48) was modeled to form interactions with the 3′-oxygen and one of the nonbridging phosphate oxygens, thereby positioning the phosphate for cleavage.[45] As discussed above, sulfur substitution leads to geometric changes in the backbone resulting from elongated bonds and sharpened angles. These variations could shift the trajectory of nucleophilic attack. Thus, the reduced rate of *S*-phosphorothiolate cleavage by nuclease P1 might result from disruption of in-line attack by the proposed H_2O nucleophile or loss of one or both of the putative R48 interactions.

Implications for Use in Biochemical Studies. These enzymatic and chemical studies have several implications for future biochemical applications of ribo-3′-*S*-phosphorothiolates. Reported here is the synthesis of a dinucleoside 3′-*S*-phosphorothiolate, IspU (**2**), which has been prepared using an Arbusov reaction as the key step and TBDMS protection of the 2′-hydroxyl group. This methodology should prove readily adaptable to the synthesis of a fully protected derivative that could be incorporated into oligoribonucleotides by a standard dimer-block strategy. Enzymatic ligation methods should then facilitate selective incorporation of this analog into large RNA molecules of interest.[82] Furthermore, the IspU sequence was chosen because it can be used directly as a substrate for the reverse reaction of the *Tetrahymena* ribozyme. Recently, Liu and Reese published a report of the synthesis of 3-(thiouridylyl)-(3′→5′)-uridine (UspU).[31] Their synthesis, which relied upon the 1-(2-fluorophenyl)-4-methoxypiperidinyl group to protect both 2′- and 5′- hydroxy functions of the thiouridine moiety, departs from TBDMS protection of the 2′-position which is compatible with the incorporation of the dimer in RNA oligonucleotides.

In characterizing the susceptibility of IspU to base-catalyzed cleavage, we determined that the 3′-*S*-phosphorothiolate linkage is substantially more labile than an unsubstituted dinucleotide at pH > 10. However, most biochemical studies are performed between pH 5 and pH 9. Within this range, the 3′-*S*-phosphorothiolate linkage is stable. For example, at pH 8 and 30 °C, IspU has a calculated half-life of 575 h (Table 2), whereas UpsU (5′-bridging substitution) has a reported half-life of 2.2 h.[24] Even though the native phosphodiester has far greater stability,[83] the 3′-sulfur substitution would clearly be an acceptable replacement.

Experimental Section

Materials. Anhydrous DMF, THF, pyridine, and iodomethane were from Aldrich. Iodoacetamide, silver nitrate, and inosylyl-(3′→5′)-uridine (IpU) were from Sigma. Acrylamide solutions (40%) (29:1 acrylamide/bisacrylamide) were purchased from Fisher Biotech. T4 polynucleotide kinase was from New England Biolabs. Yeast polyA polymerase and 5X polyA polymerase buffer were from USB. Snake venom phosphodiesterase was from Boehringer Mannheim. Nuclease P1, RNase T_1, and RNase T_2 were from CALBIOCHEM. Radionucleotides [γ-^{32}P]ATP and [α-^{32}P]cordycepin (cordycepin = 3′-deoxyadenosine triphosphate) were from New England Nuclear. PEI cellulose

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plates (F-254) were from Selecto Scientific. 5′-DMT-riboinosine-2′-TBDMS-3′-CNEt amidite was purchased from ChemGenes Corp. Disposable reversed phase C_{18} columns were purchased from Waters.

Spectroscopy. FAB mass spectra were recorded on a VG Analytical 7070E mass spectrometer operating with a PDP 11/250 data system and an Ion Tech FAB ion gun working at 8 kV. 3-Nitrobenzyl alcohol was used as a matrix unless stated otherwise. High-resolution FAB mass spectra were obtained on a VG ZAB/E spectrometer at the EPSRC Mass Spectrometry Service Centre (Swansea, U.K.). NMR spectra were measured on a Bruker AMX400, a Bruker AC200, or a Varian VXR-300S spectrometer, and chemical shifts are given in parts per million downfield from an internal standard of tetramethylsilane, unless stated otherwise. ^{31}P NMR spectra are referenced to 85% phosphoric acid.

Chromatography. Analytical thin-layer chromatography was performed on silica gel coated aluminum plates impregnated with a fluorescent indicator (254 nm). Nucleosides were visualized either as black spots by spraying with a solution of 5% (v/v) sulfuric acid and 3% (w/v) phenol in ethanol and charring at 120 °C or by quenched fluorescence under illumination with short wavelength UV light. Flash column chromatography was performed using either silica gel 60 (230−400 mesh) or octadecyl-functionalized silica gel (Aldrich). HPLC was conducted using a Varian 500 Star liquid chromatograph equipped with a Varian UV50 detector recording at 260 nm, and analysis performed on a Nucleosil C_{18} reversed phase column, using a gradient of 30% acetonitrile in 50 mM triethylammonium acetate (pH 6.5) over 20 min, with a flow rate of 1 mL/min.

TLC analysis of enzymatic and chemical reactions was done on PEI cellulose.[86] Solvent systems were (a) 0.9 M NH_4OAc (pH 7)/100 mM DTT, (b) 0.9 M NH_4OAc (pH 8)/100 mM DTT, and (c) 0.5 M LiCl/100 mM DTT. In all cases, resolution was improved by developing the plate in H_2O followed by complete air-drying before applying the samples.

9-(3-Deoxy-3-iodo-β-D-xylofuranosyl)hypoxanthine (4). Compound **4** was synthesized using modifications of methods reported for adenosine.[87,88] 2′,3′-anhydroinosine[89] (5.88 g, 23.5 mmol) and sodium iodide (15.8 g, 105 mmol, 4.5 equiv) were suspended in dry acetonitrile (120 mL). To this stirred solution was added dropwise boron trifluoride-etherate (63 mL) at room temperature. After 1 h, the reaction mixture was quenched with 400 mL of a saturated aqueous $NaHCO_3$ solution, containing NaS_2O_3 (3 g). The resultant solution was evaporated *in vacuo* to give a white solid. Nucleosidic material was extracted into methanol (100 mL). The insoluble inorganic salts were removed by filtration, and the methanolic solution was evaporated *in vacuo*. The crude product was recrystallized from hot water, and a white solid was collected by filtration. The mother liquor was concentrated to yield a further crop of material (5.33 g, 60%): ^{1}H NMR (200 MHz, DMSO-d_6) δ 9.05 (1H, br s), 8.38 (1H, s), 8.17 (1H, s), 6.38 (1H, d, J = 5.5 Hz), 5.86 (1H, d, J = 4.96 Hz, 5.30 (1H, t, J = 4.94 Hz), 4.99 (1H, dd, J = 5.48, 6.77 Hz), 4.58 (1H, t, J = 6.6 Hz), 4.26 (1H, m, H4′), 3.86 (2H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 156.83, 148.32, 146.23, 138.72, 124.50, 88.32, 81.04, 80.08, 65.88, 28.56; MS *m/z* (FAB$^+$) 401 (M + Na$^+$, 6.5), 379 (M + H$^+$, 40). FAB HRMS found *m/z* (M + Na)$^+$ 400.9725, $C_{10}H_{11}N_4O_4INa$ requires *m/z* (M + Na)$^+$ 400.9722.

9-[5-*O*-(Monomethoxytrityl)-3-deoxy-3-iodo-β-D-xylofuranosyl]-hypoxanthine (5). 9-(3-Deoxy-3-iodo-β-D-xylofuranosyl)hypoxanthine (5.7 g, 15.1 mmol) was dried by coevaporation with dry pyridine (3 × 20 mL) and finally suspended in dry pyridine (80 mL). Monomethoxytrityl chloride (MMTCl) (9.32 g, 30.2 mmol, 2 equiv) was added, and the reaction was stirred overnight. The solution was partitioned between CH_2Cl_2 (250 mL) and saturated $NaHCO_3$/NaCl (1:1) (250 mL), and the organic phase was washed with water. The organic phase was dried (Na_2SO_4), filtered, evaporated *in vacuo*, and then coevaporated with toluene. The resulting residue was purified by silica gel column chromatography eluting with MeOH (0−6%) in CH_2Cl_2 to give **5** as a pale yellow foam (6.4 g, 65%): ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.26 (1H, br s), 8.06 (1H, br s), 7.47−7.19 (12H, m), 6.81 (2H, d, J = 8.8 Hz), 6.45 (1H, br s), 5.97 (1H, s), 5.29 (1H, br s), 4.45 (1H, m), 4.13 (1H, m), 3.71 (3H, s), 3.6−3.3 (2H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.79, 158.12, 148.01, 146.34, 144.11, 143.92, 139.55, 135.14, 130.49, 128.56, 127.93, 127.15, 123.76, 113.24, 91.11, 86.98, 83.44, 79.87, 68.51, 55.12, 29.52; MS *m/z* (FAB$^+$) 673 (M + Na$^+$, 6.7), 651 (M + H$^+$, 9.5), 273 (trityl, 100), 137 (hypoxanthine + 2H, 62.8); FAB HRMS found *m/z* (M + H)$^+$ 651.1119, $C_{30}H_{28}N_4O_5I$ requires *m/z* (M + H)$^+$ 651.1104.

9-[5-*O*-(Monomethoxytrityl)-3-deoxy-3-thio-3-*S*,2-*O*-diacetyl-β-D-ribofuranosyl]hypoxanthine (6). 9-[5-*O*-(Monomethoxytrityl)-3-deoxy-3-iodo-β-D-xylofuranosyl]hypoxanthine (**5**) (3.85 g, 5.92 mmol) was dissolved in DMF (40 mL). Potassium thioacetate (2 g, 17.7 mmol, 3 equiv) was added, and the solution stirred for 36−48 h at 40 °C. The solution was partitioned between CH_2Cl_2 (100 mL) and saturated $NaHCO_3$/NaCl (1:1) (100 mL), and the organic phase was washed with water (3 × 50 mL). The organic phase was dried ($MgSO_4$), filtered, evaporated *in vacuo*, and coevaporated with toluene. The resulting products were purified by silica gel column chromatography eluting with MeOH (0−3%) in CH_2Cl_2. The fractions collected which contained the products were combined and evaporated *in vacuo* to give **6** as a white amorphous solid (1.9 g, 50%): ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.19 (1H, br s), 8.12 (1H, br s), 7.43−7.19 (12H, m), 6.82 (2H, d, J = 8.8 Hz), 6.15 (1H, d, J = 1.64 Hz), 5.85 (1H, dd, J = 1.64, 5.5 Hz), 4.82 (1H, dd, J = 5.5, 9.9 Hz), 4.32 (1H, m), 3.77 (3H, s), 3.45 (2H, m); MS *m/z* (FAB$^+$) 641 (M + H, 3), 137 (hypoxanthine + 2H, 25.4). Anal. Calcd for $C_{34}H_{32}O_7N_4S$: C, 63.74; H, 5.03; N, 8.74. Found: C, 63.44; H, 5.01; N, 8.69.

9-[5-*O*-(Monomethoxytrityl)-3-deoxy-3-*S*-(5-nitropyridyl-2-disulfanyl)-β-D-ribofuranosyl]hypoxanthine (7). The mixed disulfide was prepared essentially as described.[30] To a stirred solution of 5′-*O*-(monomethoxytrityl)-3′-*S*,2′-*O*-diacetyl-3′-thioinosine (1.28 g, 2 mmol) dissolved in the minimum amount of methanol was added a solution of methanolic sodium hydroxide (10 M, 0.6 mL). After stirring for 1−2 h under an atmosphere of nitrogen, the reaction was complete [TLC, MeOH/CH_2Cl_2 (9:1) as eluent] and the solution was neutralized with dilute acetic acid. The solution was partitioned between CH_2Cl_2 (25 mL) and H_2O (25 mL), the CH_2Cl_2 layer was separated, and 2,2′-dithiobis(5-nitropyridine) (1.86 g, 6 mmol, 3 equiv) was added. After stirring for 30 min, saturated aqueous $NaHCO_3$ solution (20 mL) was added. The organic phase was separated, washed with water (40 mL), dried ($MgSO_4$), filtered, and evaporated *in vacuo*. The crude product was purified by silica gel column chromatography eluting with a gradient of MeOH (0−5%) in CH_2Cl_2. Appropriate fractions were pooled and evaporated *in vacuo* to give a yellow foam (1.1 g, 74%): ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.20 (1H, d, J = 2.2 Hz,), 8.24 (1H, d, J = 9.4 Hz), 8.12 (1H, s), 8.06 (1H, s), 7.57 (1H, d, J = 9.4 Hz), 7.39−7.14 (12H, m), 6.77 (2H, d, J = 8.1 Hz), 6.27 (1H, br s), 6.11 (1H, s), 4.72 (1H, m), 4.33 (1H, m), 4.12 (1H, m), 3.77 (3H, s), 3.62(1H, m), 3.48 (1H, m); MS *m/z* (FAB$^+$) 711 (M + H$^+$, 3). It was noted that **7** slowly decomposed to give the symmetrical dinucleoside disulfide; it was therefore converted to **8** immediately after isolation.

9-[5-*O*-(Monomethoxytrityl)-3-deoxy-3-*S*-(5-nitropyridyl-2-disulfanyl)-2-*O*-(*tert*-fbutyldimethylsilyl)-β-D-ribofuranosyl]hypoxanthine (8). Compound **8** was prepared as described.[30] The product was obtained as a pale yellow amorphous solid (88%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.13 (1H, d, J = 2.2 Hz), 8.24 (1H, dd, J = 2.6, 9.4 Hz), 8.19 (1H, s), 8.07 (1H, s), 7.67 (1H, d, J = 9.4 Hz), 7.34−7.20 (12H, m), 6.75 (2H, d, J = 8.2 Hz), 6.07 (1H, d, J = 2.5 Hz), 4.99 (1H, dd, J = 2.6, 5.2 Hz), 4.56 (1H, m), 4.05 (1H, dd, J = 5.4, 7.9 Hz), 3.75 (3H, s, OMe), 3.73 (1H, dd, J = 2.4, 10.8 Hz), 3.73 (1H, dd, J = 3.9, 10.8 Hz), 0.94 (9H, s), 0.20 (3H, s), 0.11 (3H, s, Me); MS *m/z* (FAB$^+$) 825 (M + H$^+$, 3.6), 767 (M − tBu$^+$, 3.4); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.36, 159.14, 158.71, 148.45, 145.18, 145.10, 143.83, 143.73, 142.18, 138.78, 134.80, 131.54, 130.40, 128.38, 127.89, 127.18, 125.35, 119.45, 113.14, 90.39, 87.01, 83.65, 77.53, 63.26, 55.21, 53.91, 25.69, 18.13, −4.60, −4.90. Anal. Calcd for $C_{41}H_{44}N_6O_7S_2Si$: C, 59.69; H, 5.38; N, 10.19. Found: C, 59.59 H, 5.37 N, 10.14.

2′,3′-Bis(*O*-*tert*-butyldimethylsilyl)uridin-5′-yl-*H*-phosphonate, Triethylammonium Salt (9). Freshly distilled phosphorus trichloride (1 mL, 11.45 mmol, 5 equiv) and *N*-methylmorpholine (12.64 mL, 114.5 mmol) were mixed with anhydrous CH_2Cl_2 (13 mL). To this solution

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was added 1,2,4-triazole (2.66 g, 38.6 mmol), and the reaction was stirred under a nitrogen atmosphere for 30 min at room temperature. After this time, the reaction mixture was cooled to 0 °C, and 2′,3′-bis(*O*-*tert*-butyldimethylsilyl)uridine (1.1 g, 2.29 mmol) in dry CH_2Cl_2 (30 mL) was added dropwise over 20 min. The mixture was stirred for a further 10 min. The reaction mixture was then poured into a 1 M aqueous triethylammonium bicarbonate solution (TEAB; 100 mL), and the organic layer was separated. The aqueous phase was further extracted with CH_2Cl_2 (2 × 100 mL). The combined organic extracts were dried (Na_2SO_4) and evaporated *in vacuo*. The crude product was purified by silica gel column chromatography eluting with a gradient of MeOH (0−7%) in CH_2Cl_2/Et_3N (98:2). Appropriate fractions were pooled and evaporated *in vacuo* to give a white foam (0.57 g, 68%): ^{31}P NMR (81 MHz, $CDCl_3$) δ 4.1; ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.44 (1H, br s), 8.29 (1H, d, J_{P-H} = 626 Hz), 7.93 (1H, d, J = 8.26 Hz), 5.76 (1H, d, J = 4.38 Hz), 5.56 (1H, d, J = 7.7 Hz), 4.00 (1H, t, J = 4.96 Hz), 3.95 (1H, m), 3.44 (2H, m), 2.61 (2H, q, J = 7.3 Hz), 0.85 (3H, t, J = 7.3 Hz), 0.88 (9H, s), 0.84 (9H, s), 0.1−0.05 (12H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.77, 150.57, 140.88, 101.95, 88.72, 83.27, 75.51, 71.17, 61.66, 45.47, 25.72, 25.65, 17.89, 17.81, 8.98, −4.68,-4.75, −4.89; MS *m/z* (FAB^-) 535 (M, 100).

9-[5-*O*-Pivaloyl-3-deoxy-3-*S*-(*O*-methyl phosphorothiolate)-β-D-ribofuranosyl]hypoxanthine, Triethylammonium Salt (IspMe, 3). The phosphorothiolate precursor 9-[5-*O*-Pivaloyl-3-deoxy-3-*S*-(*O*-methyl phosphorothiolate)-2-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-hypoxanthine[90] (70 mg, 0.103 mmol) was dissolved, with stirring, in NEt_3·3HF (0.5 mL). After 2 h the solution was diluted with H_2O (10 mL) and the pH adjusted to 7.0 with NEt_3. The solution was applied to a C_{18} reversed phase column and eluted with MeOH (0−30%) in H_2O to give **3** as a colorless glass (1075 OD_{248} units, 0.087 mmol, 85%): ^{1}H NMR (300 MHz, CD_3OD) δ 8.27 (1H, s), 8.07 (1H, s), 6.05 (1H, d, J = 1.2 Hz), 4.75 (1H, d, J = 4.8 Hz), 4.51 (1H, dd, J = 2.1, 12.6 Hz), 4.39 (1H, dd, J = 4.8, 12.6 Hz), 4.30 (1H, ddd, J = 2.1,4.8, 10.6 Hz), 3.86 (1H, ddd, J = 4.8, 10.5, 12.0 Hz), 3.58 (3H, d, J = 13.2 Hz), 3.19 (6H, q, J = 7.2 Hz), 1.29 (9H, t, J = 7.2 Hz), 1.18 (9H, s); ^{31}P NMR (162 MHz, CD_3OD) δ 20.69; ^{13}C NMR (100 MHz, CD_3OD) δ 180.02, 159.21, 149.78, 147.19, 140.38, 126.32, 92.81, 84.21 (d, J_{PC} = 7.2 Hz), 77.84, 64.78, 53.65 (d, J_{PC} = 5.8 Hz), 48.06, 47.84, 40.18, 27.92, 9.52; MS *m/z* (FAB^-) 461 (M^-); UV λ_{max} (MeOH) 248 nm.

3′-(Thioinosylyl)-(3′→5′)-uridine, Sodium Salt (IspU, 2). To a solution of the bis(trimethylsilyl) phosphite[30] derived from **9** (293 mg, 0.46 mmol) in anhydrous CH_2Cl_2 (2 mL) was added the disulfide **8** (379 mg, 0.46 mmol) in CH_2Cl_2 (1 mL). An intense red color was formed, and after 2 h the reaction was quenched with water (0.1 mL) and subsequently partitioned between CH_2Cl_2 and saturated $NaHCO_3$ solution. The organic phase was dried (anhydrous $MgSO_4$) and reduced to an oil. Silica gel chromatography [MeOH (0−7%) in CH_2Cl_2/Et_3N (98:2)] gave the protected dimer (0.32 g, 55%). The protected dinucleotide (200 mg, 0.18 mmol) was dissolved in 80% acetic acid and stirred at room temperature overnight. The acetic acid was evaporated *in vacuo* and the residual acetic acid removed by coevaporation with ethanol. The residue was triturated with 30 mL of petroleum ether (40−60) and dried by coevaporation with toluene. Half of the residue was treated with NEt_3·3HF (1.8 mL, 10.7 mmol) in THF (1 mL) and was stirred overnight. The reaction was quenched with distilled water, and aqueous TEA was added until neutrality was achieved. The solution was evaporated *in vacuo*. The crude product was purified by flash chromatography on C_{18} reversed phase silica eluting with a gradient of MeOH (0−6%) in distilled water. The fractions containing the product were evaporated *in vacuo* to give the triethylammonium salt of the title compound as a colorless amorphous solid. The dinucleotide was converted to its sodium salt by passage of its solution (5 mL of H_2O) through a column of Dowex 50W-X8 (Na^+) ion exchange resin (25 mg, 18%): ^{31}P NMR (81 MHz, D_2O) δ 20.54; ^{1}H NMR (250 MHz, D_2O, inversion recovery, referenced to HOD at 4.80 ppm) δ 8.2 (1H, br s), 8.13 (1H, s), 7.73 (1H, d, J = 8.2 Hz), 6.07 (1H, s), 5.58 (1H, d, J = 2.55 Hz), 5.45 (1H, d, J = 8.2 Hz), 4.71 (1H, d, J = 4.4 Hz), 4.38 (1H, m), 4.27−4.06 (6H, m), 3.93, (1H, dd, J = 3.4, 13.5), 3.62 (1H, ddd, J = 4.4, 10.6, 12.2 Hz); ^{13}C NMR (100 MHz, D_2O) δ 165.38, 158.08, 150.94, 145.85, 140.14, 101.61, 91.09, 89.39, 84.96 (d, J_{PC} = 5.9 Hz), 81.70 (d, J_{PC} = 10.1 Hz), 75.77, 74.00, 68.41, 63.62, 59.29, 44.05; MS *m/z* (FAB^-, glycerol) 589 (M^-, 100), 135 (hypoxanthine, 8.0); UV λ_{max} (MeOH) 252 nm; HPLC retention time 10.95 min.

Polyacrylamide Gel Electrophoresis (PAGE). Dinucleotides were separated on 20−24% polyacrylamide gels for both isolation and analysis. Gels and running buffer contained 1X TBE (100 mM Tris base, 83 mM boric acid, 1 mM EDTA). Radiolabeled molecules were isolated from nondenaturing gels unless indicated. Denaturing gels contained 7 M urea. Denaturing analytical gels including those for product identification and kinetics also contained 10 mM DTT in the gel matrix and in the running buffer. DTT-containing gels were pre-electrophoresed at constant power (30−35 W) for 2−4 h. Analytical polyacrylamide gels were dried and then analyzed using a Molecular Dynamics PhosphorImager.

Oligonucleotide Synthesis. Inosylyl-(3′→5′)-adenosine (IpA) was synthesized on an Applied Biosystems 395 DNA/RNA synthesizer. The dinucleotide was deprotected using standard protocols,[91] and purified on 20% denaturing preparative gels. The appropriate band was visualized by UV shadowing, excised from the gel slab, frozen, crushed, and eluted into 8−10 mL of H_2O at 4 °C overnight. The oligonucleotide was concentrated with a disposable reversed phase cartridge and used directly.

End-Labeling Reactions. 5′-End-labeling was accomplished with T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP (e.g., IpU → p*IpU, where p* indicates the location of the radioactive phosphorus). A typical reaction included 1X kinase buffer (50 mM Tris−HCl (pH 7), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA), 20−100 pmol of dinucleotide, 10 units of PNK, and 0.15 mCi of [γ-^{32}P]-ATP in a 10 μL volume. The reaction was incubated at 37 °C for 30 min, quenched with stop solution (90% ultrapure formamide, 0.02X TBE, 0.007% bromophenol blue, 0.007% xylene cyanole, 2% sucrose, 20 mM EDTA), and loaded directly onto a 24% nondenaturing gel. The gel was exposed to X-ray film, and the appropriate bands were identified and excised from the gel. The radiolabeled RNA was eluted into 100−200 μL of sterile H_2O overnight at 4 °C. Yeast polyA polymerase was used to 3′-end-label RNA dinucleotides with [α-^{32}P]-cordycepin (e.g., IpU→IpUp*A_H).[92] A typical reaction included 10 mM Tris−HCl (pH 7), 25 mM KCl, 0.35 mM $MnCl_2$, 0.1 mM EDTA, 50 μg/mL acetylated bovine serum albumin (BSA), 5% glycerol, 30−500 pmol dinucleotide, 500 units of polyA polymerase, and 0.05 mCi of [α-^{32}P]cordycepin in a 10 μL reaction. After incubation at 30 °C for 0.5−2 h, products were isolated as above.

Enzymatic Cleavage Reactions. Total digests of the dinucleotides were accomplished by incubation with either 2 × 10^{-4} or 0.2 mg/mL SVPD (final concentration) at 25−37 °C for 10−30 min. At the lower enzyme concentration, the reaction required addition of Mg^{2+} (10 mM), and buffer [50 mM MES (pH 5.9) or 50 mM HEPES (pH 8)] was typically added as well. Nuclease P1 (0.1 mg/mL) reactions were incubated at 37 °C for 30 min. Complete cleavage of the dinucleotides with ribonuclease was achieved after incubation with either RNase T_2 (0.2 unit/μL) or RNase T_1 (0.2−2 units/μL) at 50 °C for 10−30 min. The enzymatic reactions were quenched with an equal volume of DTT−stop solution (stop solutions as above with DTT added to 20 mM) and analyzed on denaturing polyacrylamide gels containing 10 mM DTT.

Chemical Cleavage Reactions. Cleavage with I_2 in aqueous pyridine (I_2/pyr) and $AgNO_3$ was performed essentially as described.[27] I_2 was dissolved in pyridine to 100 mM. Equal volumes of the I_2/pyr stock solution and the dinucleotide sample were mixed (typically 5−10 μL total volume), and the reaction was left in the dark at room temperature for 15−30 min. An equal volume of DTT−stop solution was added to dilute the reaction. Dinucleotides or reaction aliquots were treated with 20 mM $AgNO_3$, and reactions were left in the dark at room temperature or at 30 °C for 10−30 min. Cleavage extents were unaltered by the time or temperature of the incubation. For PAGE analysis, the reactions were diluted to 10−20 μL with DTT−stop solution. Stop solutions contained between 20 and 100 mM DTT; the distribution of the reaction products was unaffected by the DTT

(90) Synthetic details for the precursors to IspMe (**3**) are contained in the Supporting Information.

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concentration. Stock solutions of both $AgNO_3$ and I_2 were stored at room temperature in the dark, used for 2−3 days, and then discarded.

Total alkaline cleavage was accomplished by incubating p*IspU with 0.01 M NaOH at 30 °C for ≥30 min and p*IpU with 0.1 M NaOH at 50−65 °C for 0.5−1 h. Partial acid cleavage was carried out with 0.1 M HCl at 50−65 °C for both p*IspU and p*IpU. The duration of the reaction varied and is specified in individual figure legends. Base- and acid-catalyzed cleavage reactions were neutralized before treatment with a secondary reagent (e.g., $AgNO_3$).

Modification of Free Thiols. Free thiols generated by cleavage of p*IspU were capped with thiol-specific modification reagents. In a typical reaction, an aliquot of a cleavage reaction mixture (e.g., p*IspU + SVPD) was treated with either iodomethane (ICH_3) or iodoacetamide (ICH_2CONH_2) in a 5−10 μL volume for 10−60 min. Reaction temperatures ranged from ambient to 30 °C. No variation in the extent of reaction was observed as a function of temperature; however, reactions done with additional HEPES (pH 8.0) (50 mM) gave more complete modification. ICH_3 was used neat to give a final concentration of 1.6 M. ICH_2CONH_2 was either dissolved in 500 mM HEPES pH 8.0 to make a 200 mM stock and used at a final concentration of 40 mM or dissolved in DMF to make a 500 mM stock which was used at 50 mM final concentration. Stock solutions were stored at −20 °C.

Kinetics of Base- and Acid-Catalyzed Cleavage Reactions. Kinetic determinations were made between 10 and 30 °C at pHs from 8 to 14. Samples were buffered with HEPES (pH 8.0), CHES (pH 9.0), and CAPS (pH 10−11). The buffer pH was adjusted at ambient temperature, and reported values are uncorrected. For pH 12−14, NaOH was used. Since high concentrations of NaOH provide high concentrations of monovalent counterions, we sought to saturate any charge screening effects by performing kinetic analyses at constant ionic strength. At both 10 and 20 °C, base-catalyzed cleavage reactions were performed with buffer solutions supplemented with NaCl to maintain a constant ionic strength, $I = 1.0$. In a typical kinetics experiment, seven 1−2 μL aliquots were removed at intervals from a reaction tube and quenched into 5 μL of DTT−stop solution. For pH > 12, the quench contained sufficient added HCl to neutralize the reaction (pH ∼7). Samples were placed directly on ice and, if needed, stored at −20 °C before PAGE analysis. Acid cleavage was investigated over a range of pH from 0.3 to 3. The ionic strength was adjusted as above. Time points were taken as described for alkaline conditions with the quench solutions containing NaOH to neutralize all of the acidic reactions. For both basic and acidic reactions, time points were taken over 3 half-lives except where the reactions were too slow, in which case initial rates were measured as indicated. First-order rate constants (k_{obs}) were determined from the fraction of substrate remaining versus time with data fit as single exponentials. Second-order rate constants were determined from the slope of linear plots of k_{obs} vs hydroxide concentration.

For determination of the apparent pK_a of the ribose 2′-hydroxyl, alkaline cleavage reactions were carried out from pH 10 to pH 14 at 10 °C, $I = 1.0$. The pH values were corrected for both temperature and salt effects by adjusting the ionization constant for water with known parameters.[63,93−95] The two correction factors were of opposite sign, and similar though not identical magnitude. Consequently, the value of each determined pK_a was changed only slightly by the adjustment. A plot of log k_{obs} vs pH (Figure 4) was fit to describe a single ionizing system since it has been demonstrated for hydrolysis of dinucleotides that hydroxide is the only contributing catalyst above pH 9.[62,63,74]

Isolated Alkaline Cleavage Intermediate. p*IspU (18 μL) was added to 0.1 N NaOH (2 μL) and incubated at 30 °C for 25 min. The reaction was neutralized with 0.1 N HCl (2 μL), diluted with stop solution (20 μL), and loaded onto a 20% denaturing gel. The intermediate and the uncleaved p*IspU were isolated from the gel and eluted, separately, into 200 μL of H_2O at 4 °C overnight.

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Supporting Information Available: Synthetic procedures for the precursors to IspMe (**3**) (5 pages). See any current masthead page for ordering and Internet access instructions.

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