JOC Article

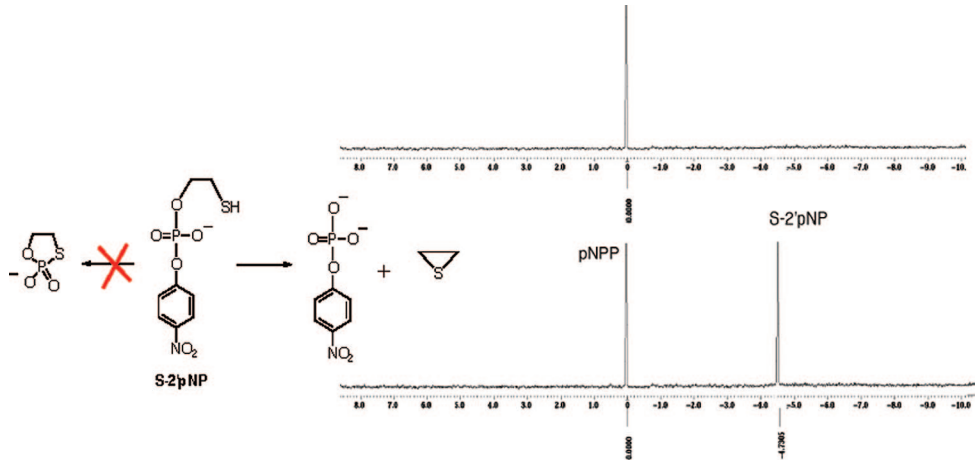
The Effects of Sulfur Substitution for the Nucleophile and Bridging Oxygen Atoms in Reactions of Hydroxyalkyl Phosphate Esters

Subashree Iyer and Alvan C. Hengge*

Utah State University, Department of Chemistry and Biochemistry, Logan, Utah 84322-0300

hengge@cc.usu.edu

Received January 28, 2008



The effects of sulfur substitution on the reactions of hydroxyalkyl phosphate esters are examined. These compounds are models for the intramolecular phosphoryl transfer reaction involved in the cleavage of the internucleotide bond in RNA. The models studied here lack the ribose ring and their conformational flexibility results in greater stability and the availability of different reaction pathways. Sulfur in the nucleophilic position shows no nucleophilic reaction at phosphorus, instead rapidly attacking at the beta carbon atom, forming thiirane with departure of a phosphomonoester. Sulfur substitution at either of the two bridging positions leads to cleavage of the diester via formation of a cyclic intermediate, but with significant rate acceleration when compared to the oxygen analogues. The bridge-substituted models react substantially slower than the analogous ribose compounds with sulfur substitution at comparable positions. Kinetic isotope effects reveal significant differences in the transition state depending on which bridging position sulfur occupies. When sulfur is in the scissile bridging position, a highly associative transition state is indicated, with a largely formed bond to the nucleophile and the scissile P−S bond is little changed. When sulfur occupies the other bridging position, the isotope effects imply a very early transition state in a concerted reaction.

Introduction

The chemistry of the phosphodiester linkage in RNA is of great interest due to its critical role in biology. Numerous studies have been devoted to the reactions of dinucleotides and related model systems in order to understand details of the most common reaction of this system, which is intramolecular attack by the 2′ hydroxyl group on the phosphodiester bond. One of the strategies utilized in mechanistic studies has been to examine the effect on mechanism and rate imparted by the substitution of sulfur for oxygen at various positions. Despite differences in electronegativity and polarizability, sulfur is typically construed as a conservative substitution for oxygen. As a result, oligonucleotide analogues incorporating such a substitution are expected to mimic the conformation of natural DNA or RNA and, therefore, are more likely to exhibit biochemical properties similar to those of their parent compounds. Studies of compounds in which sulfur replaces nonbridging phosphate oxygen atoms in DNA and RNA analogues have led to important insights into enzymatic and RNA-catalyzed cleavage of phosphodiesters.[1–3] Sulfur replacement for oxygen has also been carried out at the 2′ position of RNA and at the 3′ and 5′

10.1021/jo8002198 CCC: $40.75 © 2008 American Chemical Society
Published on Web 06/06/2008

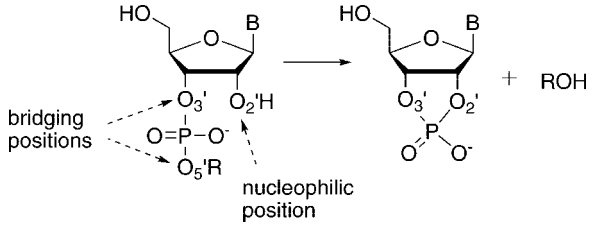


FIGURE 1. Cyclization reaction typical of RNA and analogous model systems, designating the oxygen atoms located in what are referred to as bridging positions and the nucleophilic position.

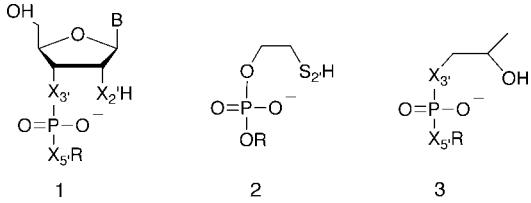


FIGURE 2. Ribonucleotide diester **1** and the model systems studied for this report, which were **2** and **3**. X represents either O or S. The designations 2′, 3′, and 5′ follow the conventions commonly used in nucleotide chemistry. The simplified model system 2 with sulfur in the nucleophilic position was modified by addition of a methyl group in 3 to permit the detection of isomerization, or phosphate migration, competitive with hydrolysis.

positions of RNA and DNA. The 3′ and 5′ positions are commonly referred to as the "bridging" positions. The atom at the 2′ position is the nucleophile in a typical reaction sequence, leading to the hydrolysis of the phosphodiester linkage in RNA (Figure 1).

The substitution of sulfur in either of the bridging positions or in the nucleophilic position of RNA models has been shown to affect both reactivity and mechanism in ways that are summarized below.[4–6] Computational studies of the reverse reaction, the methanolysis of ethylene phosphate phosphodiester resulting in ring-opening, concluded that differences in the free energy profiles result from sulfur substitution, particularly when sulfur is in either of the bridging positions, or if sulfur is the nucleophilic atom.[7]

In this study, we report thio-substitutional effects on simplified ribonucleoside phosphodiester models (Figure 2). The thio-substituted systems studied in this work differ from those previously reported in conformational flexibility, lacking the ribose ring and maintaining only the atoms involved in the cleavage process. In the native system, the ribose ring positions the 2′ nucleophile in a position facilitating attack at phosphorus. In the absence of the ring, greater conformational flexibility and increased hydrolytic stability results, and other possible reaction pathways are available.

We have investigated the effects of substitution of sulfur for oxygen at the 2′, 3′, and 5′ positions on the reactivity and on the reaction mechanism in the conformationally flexible models **2** and **3**. The leaving groups were either *p*-nitrophenyl (pK_a 7.1) or *m*-nitrobenzyl (pK_a 14.9). In order to obtain further mechanistic information about how sulfur substitutions affects the transition states of these reactions, we measured heavy-atom kinetic isotope effects (KIEs) on the cyclization reactions of the sulfur-bearing analogues **3** with the *m*-nitrobenzyl (mNB) R group. Heavy atom KIEs are very useful for investigating mechanisms of chemical and enzymatic reactions. Measurements of ^{18}O KIEs have been used to analyze a number of nonenzymatic[8–12] and enzymatic[13,14] reactions of phosphodiesters. These data present a useful framework for the interpretation of KIE data in the present study. Such isotope effects provide information about changes in the bonding of isotopically labeled atoms in the transition state of the rate-determining step. KIEs have been measured in the nucleophilic and in the leaving group positions for the cyclization reactions of the sulfur analogues where R = *m*-nitrobenzyl (mNB), **S-3′mNB**, and **S-5′mNB** depicted in Figure 3, in order to ascertain the extent of bond formation to the nucleophile and bond fission to the leaving group.

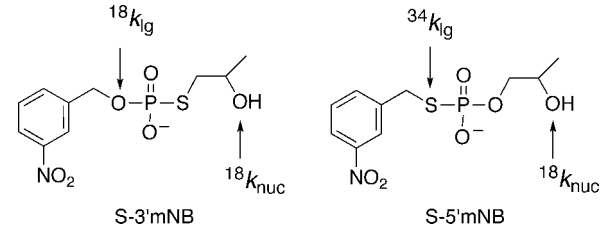


FIGURE 3. KIEs measured at the nucleophilic and leaving group positions in **S-3′mNB** and **S-5′mNB**, indicated by arrows.

Previous Studies of Thio Effects in RNA Models

Sulfur at the 2′ Nucleophilic Position. Two previous studies [5,15] have investigated ribonucleotide models with sulfur at the 2′ position. When 2′-thiouridylyl-(3′→5′)-uridine[15] is reacted under acidic conditions, only disulfide bond formation was observed, with no reactivity of the phosphodiester bond toward either cyclization or migration. Under alkaline conditions (pH = 9) the cleavage of the glycosidic linkage by attack of sulfur and loss of uracil is the predominant reaction. In contrast, the 2′-OH group in uridylyl-(3′→5′)-uridine (3′,5′-UpU) is a very competent nucleophile toward phosphorus; at pH < 6, isomerization of 3′,5′-UpU to 2′,5′-UpU is the dominant reaction, while at pH > 6, cyclization is the major pathway.[6]

The 2′-SH is capable of attacking the phosphorus in the more activated *p*-nitrophenyl ester 2′-thiouridylyl-3′-*p*-nitrophenylphosphate.[5] This compound reacts predominantly by a cyclization pathway with elimination of *p*-nitrophenol, though a competing pathway resulting in nucleophilic aromatic substitution by sulfur also occurs. The rate of thiolate attack on the adjacent phosphodiester bond is 10^7-fold slower than attack of the corresponding alkoxide.[5] The subsequent hydrolysis of the cyclic

(1) Frey, P. A. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1989**, *62*, 119–201.
(2) Stivers, J. T.; Nagarajan, R. *Chem. Rev.* **2006**, *106*, 3443–67.
(3) Lonnberg, T.; Ora, M.; Virtanen, S.; Lonnberg, H. *Chem. Eur. J.* **2007**, *13*, 4614–27.
(4) Almer, H.; Stromberg, R. *J. Am. Chem. Soc.* **1996**, *118*, 7921–7928.
(5) Dantzman, C. L.; Kiessling, L. L. *J. Am. Chem. Soc.* **1996**, *118*, 11715–11719.
(6) Oivanen, M.; Kuusela, S.; Lonnberg, H. *Chem. Rev.* **1998**, *98*, 961–990.
(7) Liu, Y.; Gregersen, B. A.; Hengge, A.; York, D. M. *Biochemistry* **2006**, *45*, 10043–53.
(8) Cassano, A. G.; Anderson, V. E.; Harris, M. E. *J. Am. Chem. Soc.* **2002**, *124*, 10964–10965.
(9) Catrina, I. E.; Hengge, A. C. *J. Am. Chem. Soc.* **2003**, *125*, 7546–52.
(10) Hengge, A. C. *Acc. Chem. Res.* **2002**, *35*, 105–12.
(11) Hengge, A. C.; Tobin, A. E.; Cleland, W. W. *J. Am. Chem. Soc.* **1995**, *117*, 5919–5926.
(12) Hengge, A. C.; Bruzik, K. S.; Tobin, A. E.; Cleland, W. W.; Tsai, M. D. *Bioorg. Chem.* **2000**, *28*, 119–133.
(13) Cassano, A. G.; Anderson, V. E.; Harris, M. E. *Biochemistry* **2004**, *43*, 10547–10559.
(14) Sowa, G. A.; Hengge, A. C.; Cleland, W. W. *J. Am. Chem. Soc.* **1997**, *119*, 2319–2320.
(15) Reese, C. B.; Simons, C.; Zhang, P. Z. *J. Chem. Soc., Chem. Commun.* **1994**, 1809–1810.

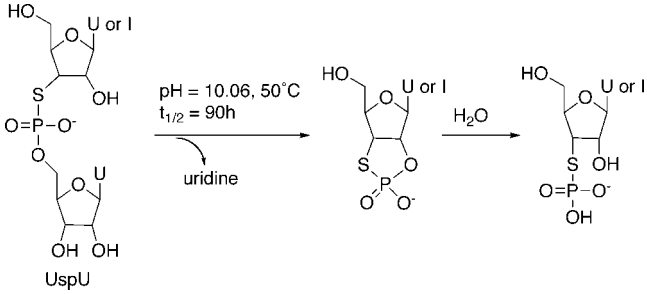


FIGURE 4. Reaction pathway followed by UspU and IspU under alkaline conditions. U = uracil, I = inosine.

diester proceeds exclusively by fission of the P−O bond to form the 2′-*S*-phosphorothioate. Precedent for the selective breakdown of such intermediates has also been reported in the alcoholysis of 5-membered phosphorothioates, in which only P−O bond fission occurs unless reversible conditions are employed, discussed below.[16,17]

Sulfur at the 3′-Bridging Position. 3′-*S*,5′-*O*-Phosphorothiolate internucleotide linkages, depicted in Figure 4, are cleaved considerably more readily under alkaline conditions than the native phosphodiester bond. The "3′-bridging thio-effect" for the hydroxide ion catalyzed cleavage of 3′,5′-UpU and 3′,5′-IpU and its thio analogues, 3′,5′-UspU and 3′,5′-IspU (henceforth referred to as UspU and IspU) ranges from 200 to 2000.[18–20] Under acidic conditions, the thio effect is close to unity and isomerization occurs competitively with hydrolysis.[21]

UspU undergoes rapid cyclization in pH 10.06 buffer at 50 °C, with a half-life of 90 min. The primary hydrolysis products of this reaction are uridine and 3′-thiouridine 2′,3′-cyclic phosphorothioate (Figure 4).[18,20] Under these conditions, the cyclic phosphorothioate undergoes further hydrolytic cleavage to give 3′-thiouridine 3′-phosphorothioate as the only product. In glacial acetic acid, the cyclic product from UspU is reported to give the same product from P−O fission as shown in Figure 4.[19] In contrast, the analogous product from IspU cyclization at pH 1 is reported to give the initial product of P−S fission, which subsequently reacted to give products that were not identified.[20] The reason for this difference is unclear.

Two previous examinations of simplified models analogous to those employed in this study have been reported with sulfur in the 3′ bridging position. The phosphorothioate diester **4** (Figure 5, top) undergoes cyclization followed by nucleophilic attack and P−O bond fission to yield the phosphorothioate after ring opening.[16] With sodium methoxide at reflux, the product undergoes slow equilibration to the product resulting from P−S fission of the cyclic diester, which subsequently forms thiirane and methyl phosphate. The phosphorothioate triester **5** (Figure 5, bottom) generated in situ under neutral conditions in toluene undergoes cyclization competitively with migration of the phosphoryl group from sulfur to oxygen. The latter compound undergoes intramolecular sulfur attack at carbon to form the thiirane with elimination of diphenyl hydrogen phosphate.[17]

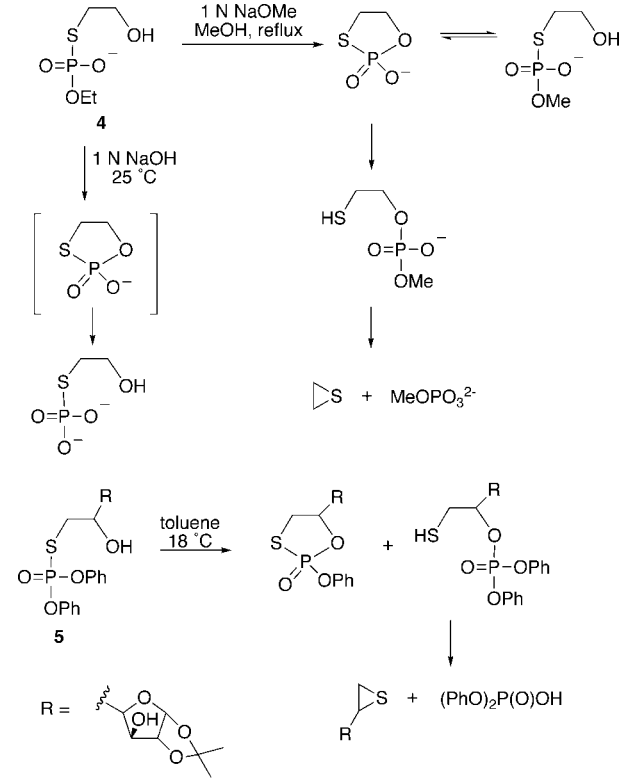


FIGURE 5. Reaction pathways followed by previously studied models **4** and **5** with sulfur in the 3′ bridging position.

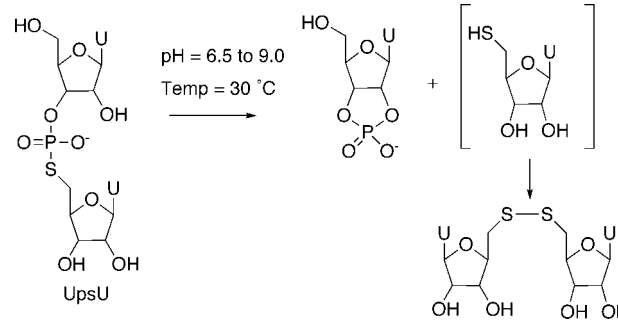


FIGURE 6. Reaction pathway followed by UpsU under neutral to alkaline conditions.[22,23]

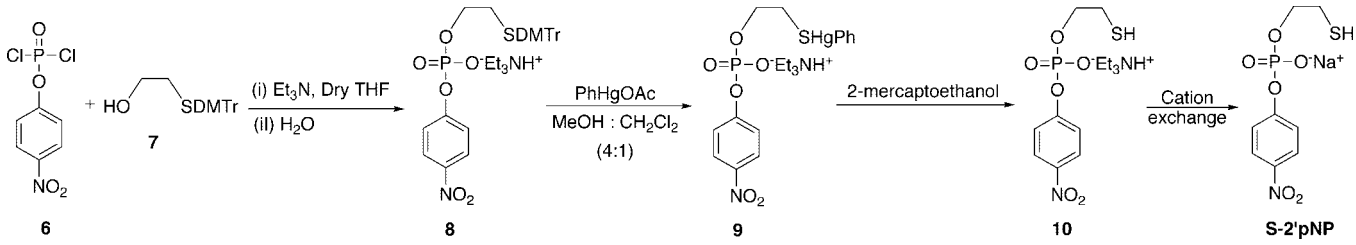
Sulfur at the 5′ Bridging Position. The replacement of this key oxygen atom by sulfur to form the uridylyl (3′,5′)-5′-deoxy-5′-thiouridine (UpsU) results in a nearly 10^5-fold rate acceleration on the cyclization reaction (Figure 6).[22,23] This rate enhancement is attributable to the reduced pK_a of thiols, which are 10^5-10^6 times more acidic than alcohols. At 30 °C, the $t_{1/2}$ for hydrolysis at pH 6.5, 7.0, 8.0, and 9.0 are 49, 13, 2.2, and 0.48 h, respectively. The departed thiolate immediately oxidizes into the symmetrical disulfide.

Results and Discussion

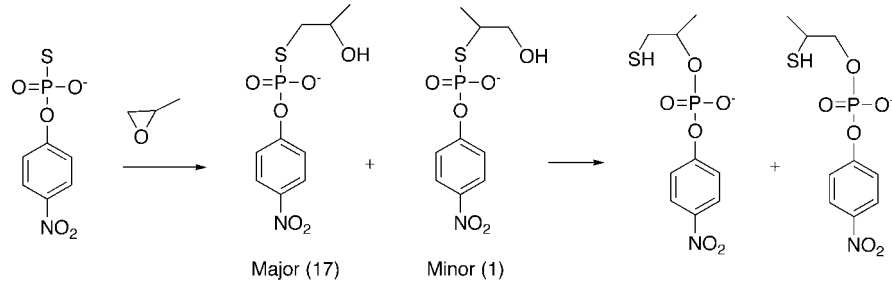
a. Kinetic Results. Sulfur at the 2′ Position. Since previous results indicate that a sulfur nucleophile is capable of phosphodiester cleavage only when a good leaving group is present, the *p*-nitrophenyl analogue of compound **2**, **S-2′pNP** (Figure

(16) Gay, D. C.; Hamer, N. K. *J. Chem. Soc. B* **1970**, 1123–1127.
(17) Kudelska, W.; Michalska, M. *Tetrahedron* **1986**, *42*, 629–636.
(18) Liu, X.; Reese, C. B. *Tetrahedron Lett.* **1996**, *37*, 925–928.
(19) Liu, X. H.; Reese, C. B. *J. Chem. Soc., Perkin Trans. 1* **2000**, 2227–2236.
(20) Weinstein, L. B.; Earnshaw, D. J.; Cosstick, R.; Cech, T. R. *J. Am. Chem. Soc.* **1996**, *118*, 10341–10350.
(21) Elzagheid, M. I.; Oivanen, M.; Klika, K. D.; Jones, B. C. N. M.; Cosstick, R.; Lonnberg, H. *Nucleosides Nucleotides* **1999**, *18*, 2093–2108.
(22) Liu, X.; Reese, C. B. *Tetrahedron Lett.* **1995**, *36*, 3413–3416.
(23) Thomson, J. B.; Patel, B. K.; Jimenez, V.; Eckart, K.; Eckstein, F. *J. Org. Chem.* **1996**, *61*, 6273–6281.

SCHEME 1. Synthetic Route for S-2′pNP



SCHEME 2. Synthesis and Subsequent Isomerization of S-3′pNP



7) was synthesized. An analogous compound with oxygen in the nucleophilic position, 2-hydroxypropyl-*p*-nitrophenyl phosphate (HPpNP)[24] undergoes facile cyclization and elimination of *p*-nitrophenol. The synthetic route for **S-2′pNP** is outlined in Scheme 1.

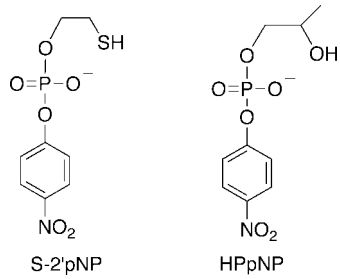


FIGURE 7. *p*-Nitrophenyl analogue of compounds **2**, **S-2′pNP**, and its oxygen analogue **HP*p*NP**.

In contrast to the reactivity of 2′-thiouridylyl-*p*-nitrophenyl phosphate, **S-2′pNP** does not undergo cyclization or nucleophilic substitution in the pH range 7.0 to 10.5, instead undergoing rapid and quantitative thiirane formation via expulsion of *p*-nitrophenyl phosphate (*p*NPP) (Figure 8). Monitoring of this reaction by ^{31}P NMR revealed no other phosphorus containing products (the hydrolysis of *p*NPP is extremely slow under these conditions, and no inorganic phosphate was observed). By ^{1}H NMR only the loss of reactant and appearance of *p*NPP were observed. Thiirane was not detected, presumably due to its volatility, but its formation was inferred by the formation of the *p*NPP product, and from the precedents for facile thiirane formation cited above. The ^{31}P NMR spectra of the reaction mixture at pH 7.0, 25 °C after 15 min, and after completion of reaction, are shown in Figure 9. A $t_{1/2}$ of 15 min was found for the conversion of **S-2′pNP** to *p*NPP under these conditions. This reaction pathway echoes the propensity noted in previous models for sulfur to perform an intramolecular S_N2 displacement of a phosphate monoester or diester accompanying the formation of thiirane. [16,17]

In the reaction of **S-2′pNP** this pathway is followed exclusively, despite the presence of the labile *p*-nitrophenyl leaving group. This pathway is not available in the ribose ring analogue, uridine-3′-*p*-nitrophenylphosphate, in which the nucleophile and leaving group are fixed in a syn conformation. Thus, the conformational restriction imposed by the ribose ring enables sulfur to perform nucleophilic attack at phosphorus, by preventing the preferred attack at carbon.

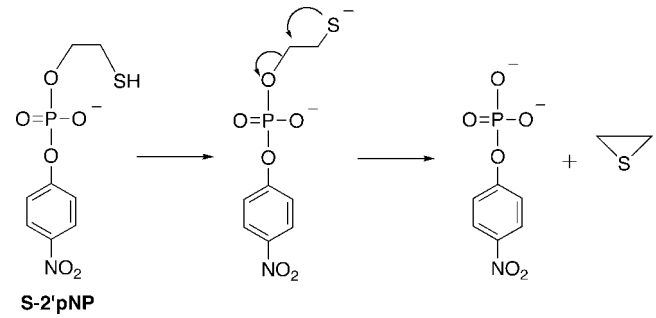


FIGURE 8. Intramolecular S_N2 reaction observed for **S-2′pNP**.

Sulfur at the 3′-position. The synthesis of the conformationally flexible analogue with a *p*-nitrophenyl leaving group **S-3′pNP** described in Scheme 2 was accomplished by coupling *p*-nitrophenyl phosphorothioate with excess propylene oxide, conditions used for preparation of the similar compound HPpNP (Figure 7).[24] The reaction proceeded smoothly to give the two possible isomers in a ratio of 17:1 (as determined by ^{31}P NMR; data not shown). The diester bond in both isomers of **S-3′pNP** was found to be highly susceptible to migration, and upon attempted purification or ion exchange, ^{31}P NMR indicated that migration of the phosphoryl group from sulfur to oxygen had occurred. Because this isomerization could not be avoided, we proceeded to the synthesis of the alkyl analogue **S-3′mNB**.

The *O*-alkyl ester model **14** and the *S*-alkyl ester **S-3′mNB** were less prone to isomerization, and could be synthesized and isolated in a pure form. The oxygen and sulfur analogues were synthesized using *H*-phosphonate chemistry as shown in Schemes 3 and 4.

The oxygen analogue **14** of **S-3′mNB** is extremely robust and does not undergo cleavage or isomerization between pH 8 to 11, up to a temperature of 80 °C. Previous studies have found

(24) Brown, D. M.; Usher, D. A. *J. Chem. Soc.* **1965**, *87*, 6558–6564.

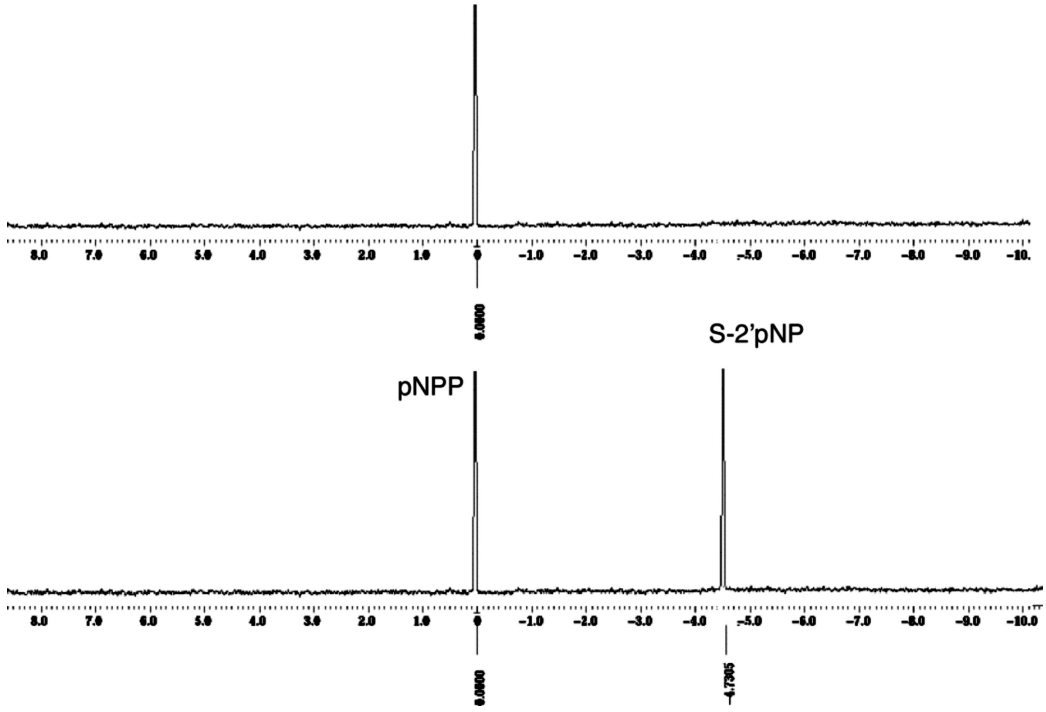
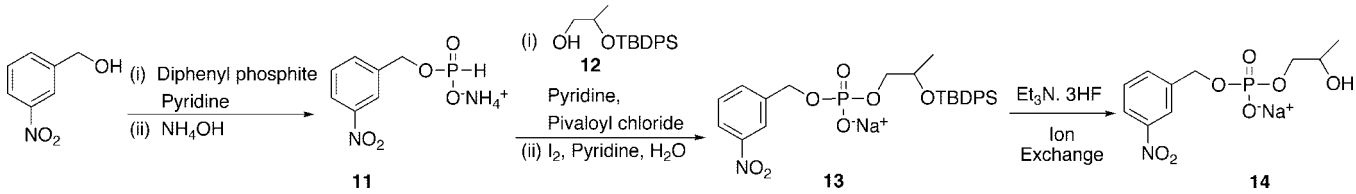
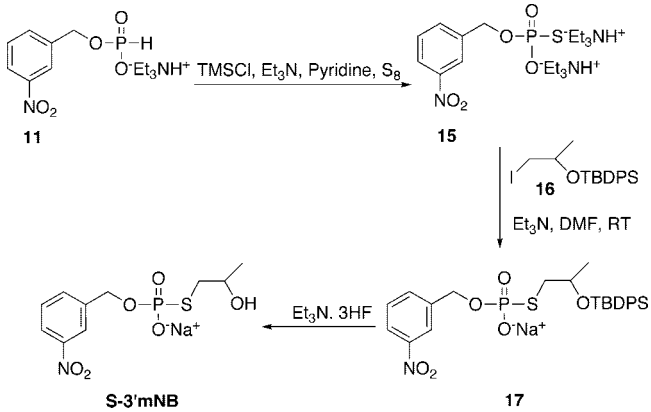


FIGURE 9. Reaction progress monitored by ^{31}P NMR for **S-2′pNP** at pH = 7.0, 25 °C. Bottom: ^{31}P NMR of the reaction mixture after 15 min. Top: ^{31}P NMR of the reaction mixture after completion of reaction and after spiking with *p*NPP. A larger image of these spectra appears in the Supporting Information.

SCHEME 3. Synthesis of 14, the Oxygen Analogue of S-3′mNB



SCHEME 4. Synthesis of S-3′mNB



that compounds of this type with alkyl ester groups react slowly under alkaline conditions, primarily by a pathway to form the epoxide and the alkyl phosphate monoester.[24] For example, the isopropyl ester reacts in 1N hydroxide at 80 °C with a half-life of 64 h; 81% of the reaction proceeds via a pathway to form isopropyl phosphate and propylene oxide, and 19% via the competing pathway of hydroxide attack at phosphorus to yield isopropyl alcohol.[24] In contrast, the sulfur analogue **S-3′mNB** undergoes reaction exclusively by cyclization. Analysis of the reaction progress by ^{31}P NMR between pH 8.0 to 13.0 showed no signal corresponding to *m*-nitrobenzyl phosphate, the product expected from isomerization followed by thiirane formation, ruling out this pathway. The formation of the cyclic phosphorothiolate intermediate was detected by its characteristic ^{31}P chemical shift (δ = 37.03). The rate of disappearance of **S-3′mNB** is first order in hydroxide concentration, consistent with the deprotonated hydroxyl group as the nucleophile (see Supporting Information). The cyclic intermediate undergoes hydrolysis and ring opening with an outcome that is influenced by pH. Below pH 11.0 no direct products of hydrolytic ring opening were observed by NMR (Figure 10). The only phosphorus-containing final product is inorganic phosphate, implying the intermediacy of the phosphoric acid ester product with a free thiol, which rapidly forms a thiirane and inorganic phosphate analogous to reactions described earlier. At higher pH, the thiaphosphoric acid ester product expected from P−O ring-opening is observed (δ = 17.08) along with inorganic phosphate (δ = 2.89) (Figure 11). This thiaphosphoric acid ester gradually disappears and is eventually replaced by inorganic phosphate, the final product under all conditions. The reaction pathway implied by the ^{31}P NMR data is summarized in Figure 12.

The failure to observe the thiaphosphoric acid ester product below pH 11 deserves comment. Inorganic phosphate is the final product across the pH range; the difference in the observation of intermediates implies that these have different, pH-dependent lifetimes. It is unlikely that the mode of ring-opening is different

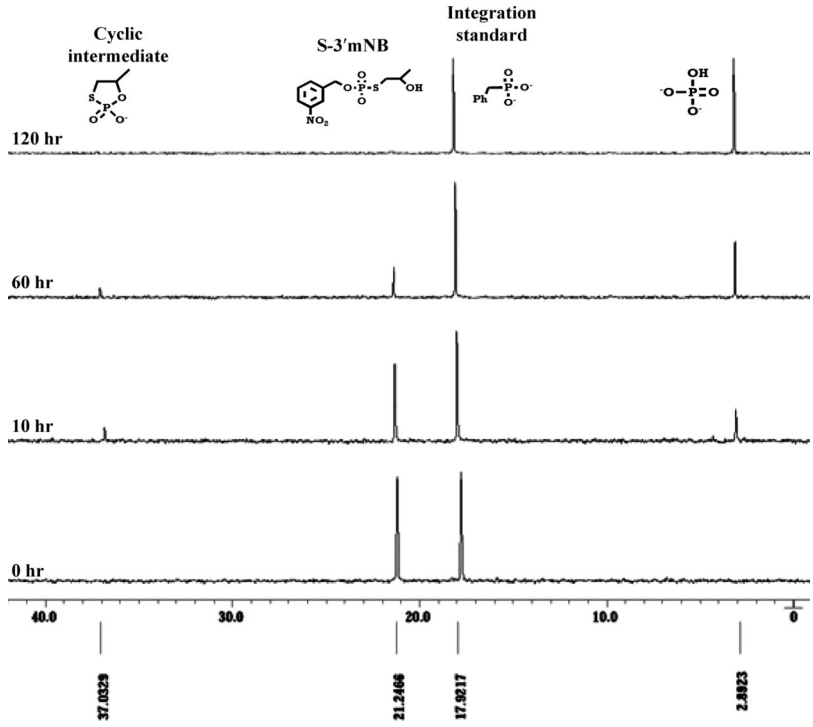


FIGURE 10. NMR time course of the reaction of compound **S-3′mNB** at pH = 10.5, 80 °C. Similar results were obtained at pH 8.0, 9.0, and 10.0.

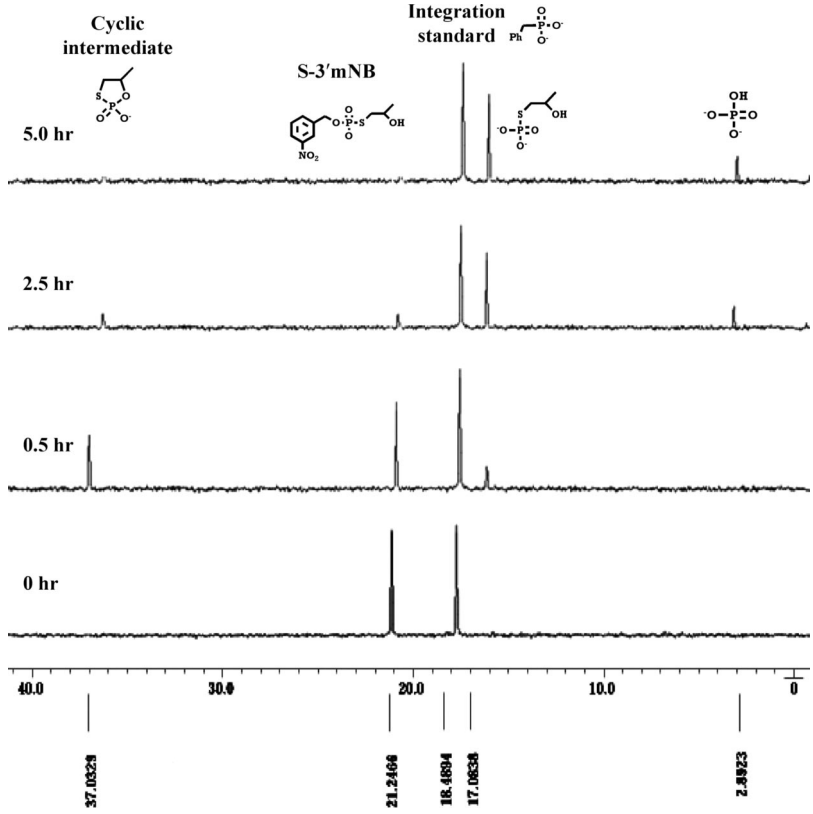


FIGURE 11. NMR time course of the reaction of compound **S-3′mNB** at pH 11.0, 80 °C. Similar results were obtained at pH 11.5, 12.0, and 13.0. At longer times, the phosphorothioate ester signal is replaced by inorganic phosphate, the only final product.

above and below pH 11, and analogous ribose systems discussed above react at alkaline pH by P−O fission to yield the thiaphosphoric acid ester (B in Figure 12). This suggests that intermediate B is on the reaction pathway even though it is only observed at pH 11 and above. The failure to observe B below pH 11 suggests that it undergoes migration to C more rapidly than it is formed under these conditions.

Sulfur at the 5′ Position. The synthesis of the aryl analogue of **S-5′pNP** is outlined in Scheme 5. This required the synthesis of *p*-nitrothiophenyl phosphorodichloridate.[25] This was synthe-

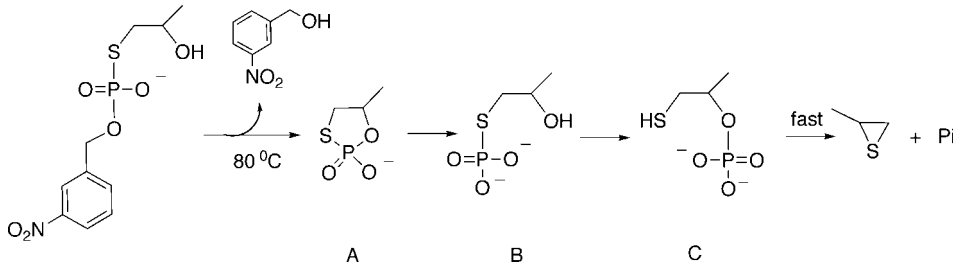
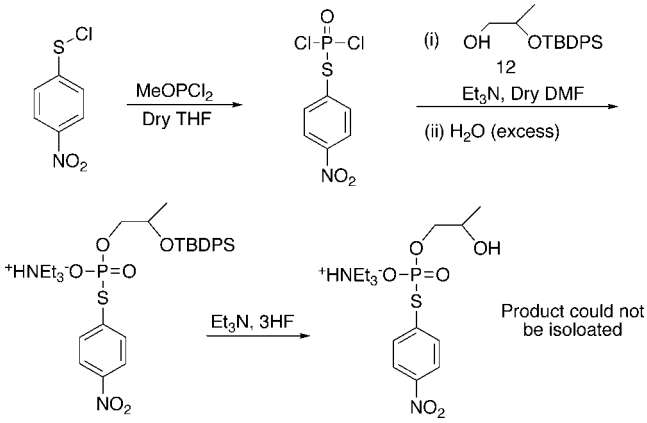


FIGURE 12. The hydrolytic pathway of **S-3′mNB** as inferred from ^{31}P NMR data. Under all conditions intermediate A was observed, and inorganic phosphate was the final product. At pH 11 and above, intermediate B and inorganic phosphate were observed, but not C. At pH 10.5 and under, neither B nor C was observed.

SCHEME 5. Synthetic Route for S-5′pNP



sized by reacting the commercially available *p*-nitrobenzenesulfenyl chloride with freshly distilled dichloromethyl phosphite in stoichiometric amounts followed by Kugelrohr vacuum distillation. The chlorines on *p*-nitrothiophenyl phosphorodichloridate were substituted consecutively by the alcohol **12** and water. The resulting product was fairly pure as determined by ^{31}P NMR, so it was directly subjected to desilylation. During deprotection the reaction mixture turned dark orange, and the ^{31}P NMR indicated that the diester had undergone spontaneous hydrolysis. The lability of this compound can be attributed to the low pK_a (4.2) of *p*-nitrothiophenol. Difficulties associated with isolation of the product precluded us from subjecting **S-5′pNP** to further kinetic studies. The nitrobenzylmercapto ester **S-5′mNB**, the alkyl analogue of **S-5′mNB**, was less labile and synthesized as shown in Scheme 6.

The hydrolysis of **S-5′mNB** over the pH range 8.0−12.0 was followed by ^{31}P NMR, and a compilation of spectra at pH = 10.5 over time appears in Figure 13. To maintain uniformity kinetic measurements were performed under conditions similar to those used for **S-3′mNB**. The flexible model **S-5′mNB** reacts in a manner similar to UpsU (Figure 6) but more slowly. The hydrolytic pathway for **S-5′mNB** implied by the NMR data is presented in Figure 14. The cyclic intermediate undergoes hydrolytic opening to give a mixture of the two possible products. The thiolate product was not observed, and rapidly oxidized to the disulfide, as was found in the earlier study of UpsU.[22,23] Like **S-3′mNB**, The rate of disappearance of **S-5′mNB** is first order in hydroxide concentration, consistent with the deprotonated hydroxyl group as the nucleophile (see the Supporting Information). The first-order dependence on [$^-$OH] found is consistent with nucleophilic attack by the deprotonated alcohol, with a pK_a >12.

SCHEME 6. Synthetic Route for S-5′mNB

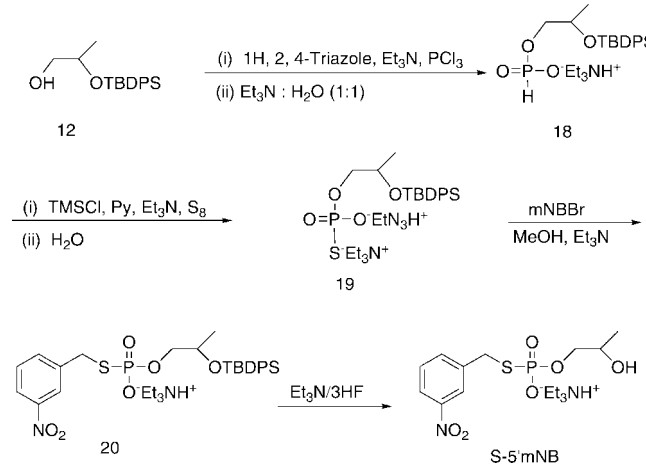


TABLE 1. Nucleophile and Leaving Group Isotope Effects Measured for S-3′mNB and S-5′mNB[a]

compd	$^{18}k_{nuc}$ (obsd)	leaving group KIE
S-3′mNB	1.1188 ± 0.0055	$^{18}k_{lg}$ = 1.0118 ± 0.0003
S-5′mNB	1.0245 ± 0.0047	$^{34}k_{lg}$ = 1.0009 ± 0.0001

[a] The $^{18}k_{nuc}$ (observed) values include contributions from the EIE for deprotonation and from the kinetic effect for nucleophilic attack.

b. Kinetic Isotope Effects. Calculations of the methanolysis of ethylene phosphate predict that sulfur substitution into either of the bridging positions should affect the free energy profile, and thus the transition state.[7] It is logical to assume that sulfur substitution will also affect the transition states of the reactions studied here. While the oxygen analogue with the *m*-nitrobenzyl leaving group was too unreactive for isotope effect measurements, the KIEs in the nucleophile and leaving group positions in **S-3′mNB** and **S-5′mNB** permit an evaluation of the effect of changing the position of sulfur on the transition state of the cyclization reaction.

The kinetic isotope effects measured for analogues **S-3′mNB** and **S-5′mNB** are shown in Table 1. The observed nucleophile KIEs reflect both the fractionation of the oxygen isotopes for equilibria before formation of the transition state and the kinetic isotope effect on the rate-limiting step. The KIE, in turn, is composed of two factors, the temperature-independent factor (TIF) and the temperature-dependent factor (TDF).[26] The TIF, or the imaginary frequency factor, reflects the extent to which the isotopically labeled atom participates in reaction coordinate motion. This factor always favors the lighter isotope since the

(25) Bull, E. O.; Naidu, M. S. R. *Phosphorus, Sulfur Silicon Relat. Elem.* **2000**, *162*, 231–243.

(26) Melander, L.; Saunders, W. H. *Reaction Rates of Isotopic Molecules*; Robert E. Krieger: Malabar, FL, 1987.

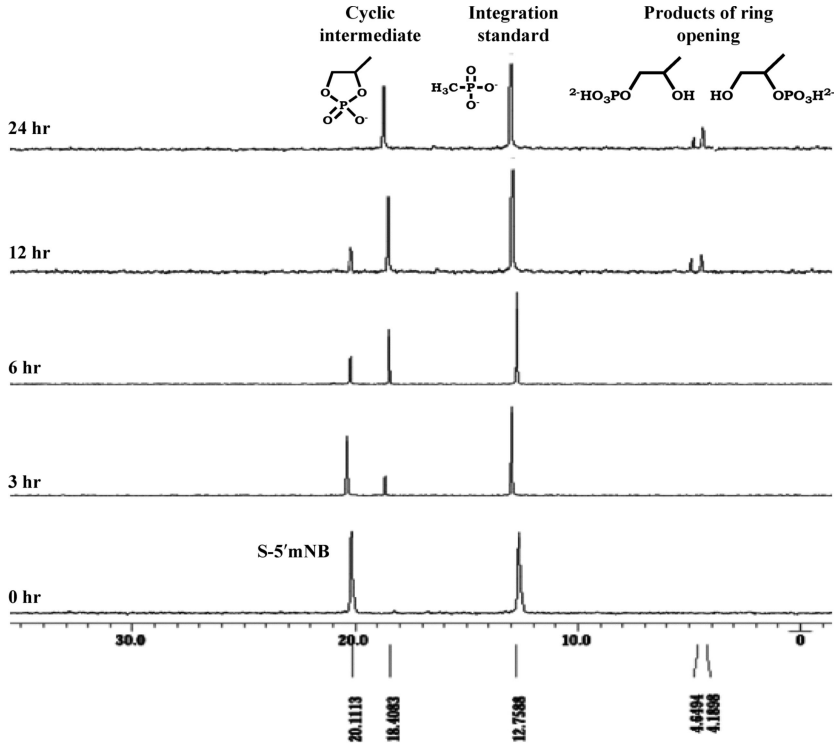


FIGURE 13. NMR time course of the reaction of compound **S-5′mNB** at pH 10.5, 80 °C. This is representative of the reaction pathway observed in the pH range 7.0−12.

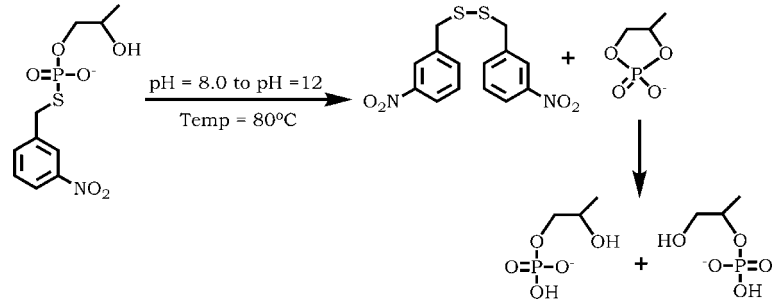


FIGURE 14. Hydrolytic pathway for **S-5′mNB** as determined by ^{31}P NMR.

imaginary frequency is larger for the lighter isotope, producing a normal contribution to the observed isotope effect. The TDF reflects differences in bonding of the labeled atom in the ground-state compared to the transition state. Tighter or stiffer bonding to the labeled atom favors the heavier isotope and results in an inverse isotope effect, while looser bonding yields a normal isotope effect.

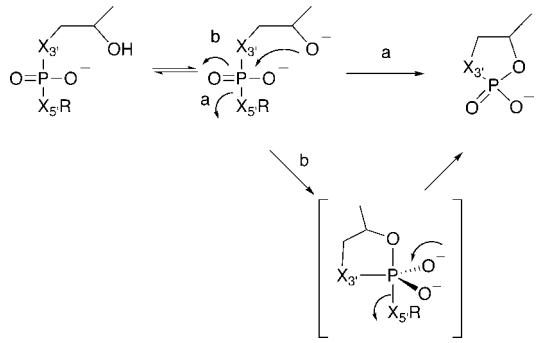
Nucleophile KIEs are generally normal, because the normal contribution from the TIF usually outweighs the inverse contribution from bond formation.[8,13,27–30] Inverse nucleophile KIEs are possible in late transition states if bond formation is sufficiently far advanced and the inverse contribution from the TDF outweighs the TIF.[29,31] An observed nucleophile isotope effect may also be inverse in a stepwise mechanism in which nucleophilic attack forms an intermediate when a subsequent step is rate-determining.[32] In such a mechanism, the observed nucleophile KIE represents the equilibrium isotope effect on formation of the intermediate. There is no TIF since there is no imaginary frequency involving the labeled atom in the transition state of the rate-determining step.

The nucleophile KIEs for **S-3′mNB** and **S-5′mNB** were determined under specific base conditions, as determined by the absence of an effect on the reaction rate as buffer concentration was varied. This implies that the nucleophile is deprotonated in a pre-equilibrium step, which allows for an estimation to be made for the individual contributions to the observed KIE from the separate steps of deprotonation and nucleophilic attack.

The equilibrium isotope effect (EIE) for deprotonation of an alcohol has not been reported; however, the deprotonation of water results in a normal ^{18}O EIE of 1.04 (in other words, a 4% enrichment of ^{16}O).[33] This fractionation is for the ensemble of the solvated hydroxide molecule, and includes secondary

(27) Marlier, J. F. *J. Am. Chem. Soc.* **1993**, *115*, 5953–5956.
(28) Marlier, J. F.; Dopke, N. C.; Johnstone, K. R.; Wirdzig, T. J. *J. Am. Chem. Soc.* **1999**, *121*, 4356–4363.
(29) Paneth, P.; O'Leary, M. H. *J. Am. Chem. Soc.* **1991**, *113*, 1691–1693.
(30) Westaway, K. C.; Fang, Y.; Persson, J.; Matsson, O. *J. Am. Chem. Soc.* **1998**, *120*, 3340–3344.
(31) Hogg, J. L.; Rodgers, J.; Kovach, I.; Schowen, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 79–85.
(32) Humphry, T.; Forconi, M.; Williams, N. H.; Hengge, A. C. *J. Am. Chem. Soc.* **2002**, *124*, 14860–1.
(33) Green, H.; Taube, H. *J. Phys. Chem.* **1963**, *67*, 1565–1566.

SCHEME 7. Two Potential Pathways for the Cyclization Reactions[a]



[a] The concerted reaction is shown in pathway a. If a phosphorane intermediate forms, as in pathway b, the leaving group may or may not initially be in the apical position required for departure as shown. If not, pseudorotation will be required before expulsion and product formation can occur.

contributions from strengthened hydrogen bonds between hydroxide and solvating waters. The EIE solely from deprotonation is uncertain but is likely to be smaller, and may more nearly resemble the 1.5% fractionation factor for deprotonation of acids and *p*-nitrophenol,[34] measurements that are not complicated by contributions from labeled solvent. The uncertainty of the fractionation factor of the 2′ hydroxyl group precludes an exact correction of the observed $^{18}k_{nuc}$ values to extract the portion due to nucleophilic attack, but this reduction will be in the neighborhood of from 1.02 to 1.04. Regardless of this uncertainty, the correction will be the same for **S-3′mNB** and **S-5′mNB**, and the significantly different $^{18}k_{nuc}$ values imply very different transition states for the cyclization reactions of these two analogues. The corrected $^{18}k_{nuc}$ for the reaction of **S-3′mNB** will be near the upper limit expected for an ^{18}O KIE. This implies that the normal reaction coordinate effect dominates over the inverse effect on bond formation, and therefore suggests low bond order to the nucleophile in an early transition state. In the case of **S-5′mNB**, a small normal or small inverse corrected $^{18}k_{nuc}$ will result. This suggests a transition state with significantly advanced bond formation to the nucleophile, or, a stepwise mechanism. Because the nucleophile in these reactions is intramolecular, as the new bond forms in the transition state, there may be more stiffening of bending and torsional modes involving the nucleophilic atom than typically occur during nucleophilic attack. To the extent that this occurs, the nucleophile isotope effect could be more inverse than in a bimolecular transition state with a similar degree of bond formation.

A dramatic difference on the nucleophile KIE results depending on whether sulfur is in the 3′ or 5′ bridging position. The leaving group pK_a will be significantly different when sulfur or oxygen is in the 5′ position. In the all-oxygen system, linear free energy data have led to the proposal that uridine 3′ phosphate esters cyclize by a phosphorane pathway in which the rate-determining step depends upon the leaving group (Scheme 7).[35] Sulfur substitution may also affect the geometry of the trigonal bipyramidal transition state, or intermediate, if the reaction is stepwise. In the transition state of the cyclization of **S-3′mNB**, sulfur must reside in an equatorial position because the nucleophile must be apical, and the five-membered ring mandates that only one of the ring atoms can be accommodated in an apical position in the new geometry. The leaving group is free to occupy the other apical position, facilitating a concerted reaction. In the reaction of **S-5′mNB**, the sulfur, since it is the incipient leaving group, may or may not initially occupy an apical position. Normally the more electronegative element oxygen will prefer the apical position, by way of one of the nonbridging oxygens. The larger steric requirement of the −SR moiety may outweigh this consideration, and favor its position in the apical site. However, if the −SR group is initially in an equatorial position, its departure would require the formation of an intermediate that must pseudorotate before its expulsion. Formation of such an intermediate would result in an inverse nucleophile KIE if its breakdown is rate limiting or a normal $^{18}k_{nuc}$ if formation is rate-limiting.

Consideration of the leaving group isotope effects gives additional information about transition state and mechanism in these reactions. The cyclization of **S-3′mNB** shows $^{18}k_{lg}$ = 1.0118. Estimates of the upper limit for this KIE with the alkyl *m*-nitrobenzyl leaving group are in the range of 1.06 to 1.07 from the following precedents. The departure of a methoxide leaving group in the hydrazinolysis of methyl formate has a leaving-group ^{18}O KIE of 1.06.[36] The C−O cleavage reactions catalyzed by fumarase and crotonase give leaving-group ^{18}O KIEs of 1.07 and 1.05, respectively.[37,38] Finally, estimated maximum KIE values for C−O and P−O cleavage reactions with alkyl leaving groups of 1.05 have been predicted on the basis of vibrational stretching frequencies.[36,39] The leaving-group ^{18}O KIE on the cyclization of **S-3′mNB** is only about 1/6th of the estimated maximum, and implies a small extent of P−O bond fission. Though small, the presence of a significant KIE in both the nucleophile and leaving group atoms implies that bond formation and bond fission in **S-3′mNB** occurs in the same step, in a concerted mechanism.

The scissile atom in the case of **S-5′mNB** is sulfur; few sulfur kinetic isotope effects have been reported in the chemical literature. Due to the proportionately smaller difference between the isotopes, the theoretical magnitude of a ^{34}S KIE is expected to be less than an ^{18}O KIE. ^{34}S KIEs in the range of 1.0108 to 1.0133 have been reported for the rearrangement of organic monothiopyrophosphates, a reaction involving fission of a P−S bond.[40] Sulfur KIEs of 1.0154 and 1.0172 have been measured for the sulfuryl transfer reactions of *p*-nitrophenyl sulfate and *p*-acetylphenyl sulfate, respectively.[41] A ^{34}S KIE of 1.0126 has been predicted for P−S bond fission in the attack by methoxide on a 5-membered cyclic phosphorothioate ester.[7] The $^{34}k_{lg}$ measured for the cyclization of **S-5′mNB** is very small compared to these reported values, implying a transition state in which the P−S bond remains largely intact. Together with the nucleophile KIE, a phosphorane-like transition state is indicated for this reaction.

Does a Phosphorane Intermediate Form in the Reaction of. S-5′mNB? If a phosphorane intermediate forms, its formation should be rate-limiting, as it should break down with a large preference for P−S fission based on the large difference between the pK_a values of the two potential leaving groups. This

(34) Rishavy, M. A.; Cleland, W. W. *Can. J. Chem.* **1999**, *77*, 967–977.
(35) Lonnberg, H.; Stromberg, R.; Williams, A. *Org. Biomol. Chem.* **2004**, *2*, 2165–2167.
(36) Sawyer, C. B.; Kirsch, J. F. *J. Am. Chem. Soc.* **1973**, *95*, 7375–7381.
(37) Bahnson, B. J.; Anderson, V. E. *Biochemistry* **1989**, *28*, 4173–81.
(38) Blanchard, J. S.; Cleland, W. W. *Biochemistry* **1980**, *19*, 4506–13.
(39) Gorenstein, D. G.; Lee, Y.-G.; Kar, D. *J. Am. Chem. Soc.* **1977**, *99*, 2264–2267.
(40) Paneth, P.; Reimschussel, W. *J. Am. Chem. Soc.* **1985**, *107*, 1407–1408.
(41) Burlingham, B. T.; Pratt, L. M.; Davidson, E. R.; Shiner, V. J. J.; Fong, J.; Widlanski, T. S. *J. Am. Chem. Soc.* **2003**, *125*, 13036–13037.

mechanism might result in a very small $^{34}k_{lg}$; since bonds to phosphorus in pentacoordinate phosphoranes are slightly longer than phosphate esters, modest weakening of the P−S bond is expected. If pseudorotation is needed to bring sulfur into an apical position this should not have a large effect on the leaving group KIE, since whether pseudorotation is required or not, the KIEs on the reaction in such a mechanism will be those on phosphorane formation as long as all subsequent steps are rapid. While the data point to a highly associative transition state, the data from this study are not sufficient to distinguish between a concerted reaction with a phosphorane-like transition state and a two-step mechanism for **S-5′mNB**.

Conclusions

Sulfur at the 2′ position in the conformationally flexible model **S-2′pNP** is completely dysfunctional as a nucleophile toward the adjacent phosphorus center. In spite of the presence of the labile *p*-nitrophenyl leaving group, the thiolate reacts exclusively with the neighboring carbon. Thus, the ribose ring is crucial to position a 2′ thiolate in a suitable geometry to force nucleophilic attack on the phosphorus center in 2′-thio nucleotides.

The combination of aryl leaving groups and sulfur at either bridging position proved unfavorable to the stability of these compounds. Aryl leaving groups activate the phosphorus center such that these compounds undergo either spontaneous isomerization or hydrolytic cleavage, depending on the location of the sulfur atom. Sulfur at the bridging positions in models with alkyl leaving groups exhibited much greater stability compared to their aryl analogues. The overall cleavage mechanisms of these model compounds are essentially similar to those of the ribose-containing systems. The conformationally flexible models in this study react substantially slower than ribose compounds with sulfur substitution at equivalent positions. Kinetic isotope effects reveal that when sulfur is in the 3′ position, the transition state is very early, with bond formation to the nucleophile and leaving group bond fission both only modestly advanced. With sulfur in the scissile 5′ position, KIEs indicate a highly associative transition state.

Experimental Section

Reactivity of S-2′pNP as Determined by ^{31}P NMR. Compound **S-2′pNP** was allowed to react over the pH range 7.0−10.5 buffered with MOPS (3-(*N*-morpholino)propanesulfonic acid) (pH 7.0, 7.5), TRIS (trishydroxymethylaminomethane)−HCl (pH 8.0, 8.5), CHES (3-cyclohexylaminoethylsulfonic acid) (pH 9.0, 9.5), and CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) (pH 10.0, 10.5). A 100 μmol portion of **S-2′pNP** was dissolved in 500 μL of buffer and allowed to react at rt. The reaction progress was monitored by ^{31}P NMR at 162 MHz. The reactant ($\delta = -4.7$) was converted cleanly into a single phosphorus-containing product that had a ^{31}P chemical shift identical to *p*NPP. The identity of the product was confirmed by spiking the NMR tube after reaction was complete with authentic *p*NPP. The externally added *p*NPP overlapped with the ^{31}P and the ^{1}H NMR signals of the reaction product.

To obtain a quantitative estimate of **S-2′pNP** reactivity, the half-life for the conversion of **S-2′pNP** to *p*NPP was determined at pH 7.0 by ^{31}P NMR. A 100 μmol portion of **S-2′pNP** was dissolved in 500 μL of 0.5 M MOPS buffer along with an equivalent amount of integration standard (methylphosphonic acid) and allowed to react at rt. The ^{31}P NMR was recorded at an interval of 2.5 min until the peak for **S-2′pNP** had disappeared completely. A $t_{1/2}$ of approximately 15 min was obtained for the conversion of **S-2′pNP** to *p*NPP under these conditions.

Kinetics Studies of S-3′mNB. Kinetic studies were carried out at 80 °C from pH 9.0−13.0 buffered with CHES (pH 9.0) and CAPS (pH 10.0, 10.5, 11.0, 11.5). For pH 12.0 and 13.0, 0.01 and 0.1 N NaOH were used, respectively. Constant ionic strength (I = 1.0) was maintained using KCl. The reaction progress was monitored using ^{31}P NMR. In a typical experiment, 100 μmol of **S-3′mNB** and an equivalent amount of integration standard (benzyl phosphonic acid) were dissolved in 500 μL of buffer and allowed to react in an NMR tube immersed in an oil bath maintained at 80 °C. The NMR tube was cooled, and ^{31}P NMR was recorded at appropriate intervals. Reaction was allowed to progress until three half-lives. At pH 12 and 13, the experiment was carried out in the NMR with the probe temperature set to 80 °C and spectra automatically recorded at fixed time intervals. The rate constant (k) for the disappearance of **S-3′mNB** was determined at each pH from the first order loss of the reactant. A graph of log k as a function of pH appears in the Supporting Information.

Kinetic Studies of S-5′mNB. Compound **S-5′mNB** was allowed to react over the pH range 8.0−12.0 buffered with TRIS−HCl (pH 8.0), CHES (pH 9.0), and CAPS (pH 10.0, 10.5, 11.0, 11.5). For pH 12.0, 0.01N NaOH was used. Constant ionic strength (I = 1.0) was maintained using KCl. The reaction progress was monitored using ^{31}P NMR. In a typical experiment, 100 μmol of **S-5′mNB** and an equivalent amount of integration standard (methyl phosphonic acid) were dissolved in 500 μL of buffer and allowed to react in an NMR tube immersed in an oil bath maintained at 80 °C. The NMR tube was cooled, and ^{31}P NMR was recorded at appropriate intervals. Reaction was allowed to progress until 3 half-lives. At pH 11.0, 11.5, and 12.0, the experiment was carried out in the NMR with the probe temperature set to 80 °C and spectra automatically recorded at fixed time intervals. The rate constant (k) for the disappearance of **S-5′mNB** was determined at each pH from the first order loss of the reactant. A graph of log k as a function of pH appears in the Supporting Information.

Kinetic Isotope Effect Studies. The isotope effects were measured by the competitive method using an isotope ratio mass spectrometer (IRMS) to measure the change in isotopic composition over the course of the reaction. The ^{34}S isotope effect was measured using natural abundance compound. The ^{18}O KIEs were measured using the double label method,[42] in which a nitrogen atom in the departing ester group was used as a reporter for ^{18}O ratios at the positions of interest. This methodology has been used in numerous previous KIE studies involving phosphate esters.[10] A description of the isotopic isomers needed for the measurement of the ^{18}O KIEs and their synthesis can be found in the Supporting Information.

Measurement of Kinetic Isotope Effects. The isotope effects for **S-3′mNB** and **S-5′mNB** were measured under similar conditions at pH 10.5. The absence of an effect of buffer concentration on the rate demonstrated that the reactions proceed by specific base catalysis (data not shown). The leaving group in **S-3′mNB** is *m*NBA, and in the case of **S-5′mNB** it is the *m*-nitrobenzylthiolate anion, which subsequently oxidizes to the symmetrical dimer, *m*-nitrobenzyl disulfide, under the reaction conditions. Thus, for KIEs on reactions of **S-3′mNB**, isotopic analysis was carried out on isolated *m*NBA, while for **S-5′mNB** the disulfide product was isolated and analyzed for either sulfur isotopic analysis or nitrogen isotopic analysis.

KIE Measurements for S-3′mNB. S-3′mNB (100 μmol) was dissolved in 0.25 M CAPS buffer at pH = 10.5. The solution temperature was maintained at 80 °C in a block heater. Reactions were run in triplicate and were allowed to proceed to 50% completion. The reaction progress was monitored by ^{31}P NMR. Once the desired amount of hydrolysis was reached, the progress of reaction was stopped by cooling in an ice bath. The solution was extracted three times with diethyl ether (20 mL) to separate the *m*NBA from the remaining reaction mixture. The ether from all three extractions was pooled together and dried over $MgSO_4$.

The $MgSO_4$ was filtered off and the ether was removed by rotary evaporation. The *m*NBA was then purified by distillation on a coldfinger apparatus at 105 °C under reduced pressure. The unreacted substrate in the aqueous layer was again subjected to hydrolysis at 80 °C at pH = 12.5 to expedite the hydrolysis process. The reaction completion was confirmed by ^{31}P NMR. The solution was cooled to RT and titrated to pH = 10 to protonate the *m*NBA, which was then isolated and purified as described above. One milligram samples were prepared for combustion and isotopic ratio analysis in tin capsules containing chromosorb W. Isotope analysis was collected on the samples by IRMS using an ANCA-NT combustion system, working in tandem with a Europa 20-20 IRMS.

KIE Measurement for S-5′mNB. S-5′mNB (100 μmol) was dissolved in 0.25 M CAPS buffer at pH = 10.5. The solution temperature was maintained at 80 °C in a block heater. Reactions were run in triplicate and were allowed to proceed to 50% completion. The reaction progress was monitored by ^{31}P NMR. Once the desired amount of hydrolysis was reached, the reaction was brought to a standstill by cooling it in an ice bath. The solution was extracted three times with diethyl ether (20 mL) to separate the *m*NBA from the remaining reaction mixture. The ethers from all three extractions were pooled together and dried over $MgSO_4$. The $MgSO_4$ was filtered off, and the ether was removed by rotary evaporation. The bis(*m*-nitrobenzyl) disulfide was further purified to remove salts and other water soluble impurities by passing it through a short silica gel column. The product was eluted with ether. The ether was again removed under reduced pressure to obtain an off-white color solid. The solid was dried for an hour under vacuum. One mg samples were added to 5 × 9 mm tin capsules and compressed for combustion and IRMS analysis. The leaving group ^{34}S KIE was obtained using natural abundance compound and the leaving group ^{18}O KIE obtained using the remote-labeled mixture.

Three values are needed to calculate the observed isotope effects using eqs 1 and 2, shown below.[43]

$$\text{isotope effect} = \log(1 - f)/\log[(1 - f)(R_s/R_o)] \quad (1)$$

$$\text{isotope effect} = \log(1 - f)/\log[(1 - f(R_p/R_o))] \quad (2)$$

The isotope ratio for the unreacted starting material (R_o), the isotope ratio for either the residual starting material (R_s) or the product (R_p) at the point the reaction is halted, and the fraction of reaction (f) when the reaction was halted are included. Control experiments with natural abundance **S-2′mNB** and **S-3′mNB** gave no observable ^{15}N KIE, demonstrating that the procedures used for isolation and purification of *m*-nitrobenzyl alcohol for isotopic analysis did not introduce artifactual isotopic fractionation. The ^{18}O isotope effects were determined by correcting the observed isotope effect for incomplete levels of ^{18}O incorporation.[44]

Acknowledgment. We thank the NIH for financial support (GM47297). We thank Dr. Vernon Anderson for helpful discussions.

Supporting Information Available: A larger image of Figure 11, kinetic plots of **S-3′mNB** and **S-5′mNB** versus pH, and synthetic procedures for natural abundance and labeled compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

JO8002198

(42) O'Leary, M. H. In *Methods in Enzymology*; Purich, D. L., Ed.; Academic Press: New York, 1980; Vol. *64*.
(43) Bigeleisen, J.; Wolfsberg, M. *Adv. Chem. Phys.* **1958**, *1*, 15–76.
(44) Cleland, W. W. In *Isotope effects in chemistry and biology*; Kohen, A., Limbach, H.-H., Eds.; CRC Press: Boca Raton, FL, 2006; pp 915–930.