Reactions of phosphate and phosphorothiolate diesters with nucleophiles: comparison of transition state structures

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A series of methyl aryl phosphorothiolate esters (SP) were synthesized and their reactions with pyridine derivatives were compared to those for methyl aryl phosphate esters (OP). Results show that SP esters react with pyridine nucleophiles *via* a concerted S_N2(P) mechanism. Brønsted analysis suggests that reactions of both SP and OP esters proceed *via* transition states with dissociative character. The overall similarity of the transition state structures supports the use of phosphorothiolates as substrate analogues to probe mechanisms of enzyme-catalyzed phosphoryl transfer reactions.

Introduction

Biological systems rely heavily on phosphoester transfer reactions, devoting significant fractions of their genomes to encode protein and RNA enzymes that catalyze these reactions.[1–3] Phosphoester transferases serve as integral components of pathways involved in cellular replication and differentiation, playing central roles in gene replication, recombination, and expression. Understanding these important cellular processes at a chemical level requires detailed knowledge of the catalytic mechanisms exploited by the enzymes that mediate these processes. We now know that enzymes frequently employ metal ions[4–14] and/or general acids and bases to catalyze phosphoester transfer reactions.[15–21] Nevertheless, defining these mechanisms within specific protein and RNA enzymes remains challenging.

Phosphorothiolates, in which a sulfur atom replaces a bridging oxygen, have provided effective probes for investigating the mechanistic basis of phosphoryl transferases. This modification is frequently used to study RNA enzymes including splicing machineries (Group I, Group II, and the spliceosome),[4–6,22–25] the hepatitis delta virus (HDV)[17] and hammerhead ribozymes,[26–28] RNase P,[29] as well as protein enzymes.[30] These probes have unveiled intricate and fundamental features of catalysis, including metal ion coordination, hydrogen bond donation, and proton transfer to the leaving group. These studies have relied on the underlying assumption that phosphorothiolate and phosphate diesters react similarly with nucleophiles and share a common pathway and transition state. However, this assumption has never been tested. Here, we synthesize a series of methyl aryl phosphorothiolate esters and compare their non-enzymatic reactions directly with those of an analogous series of methyl aryl phosphate esters. We obtain an initial physical organic description of the transition state structure.

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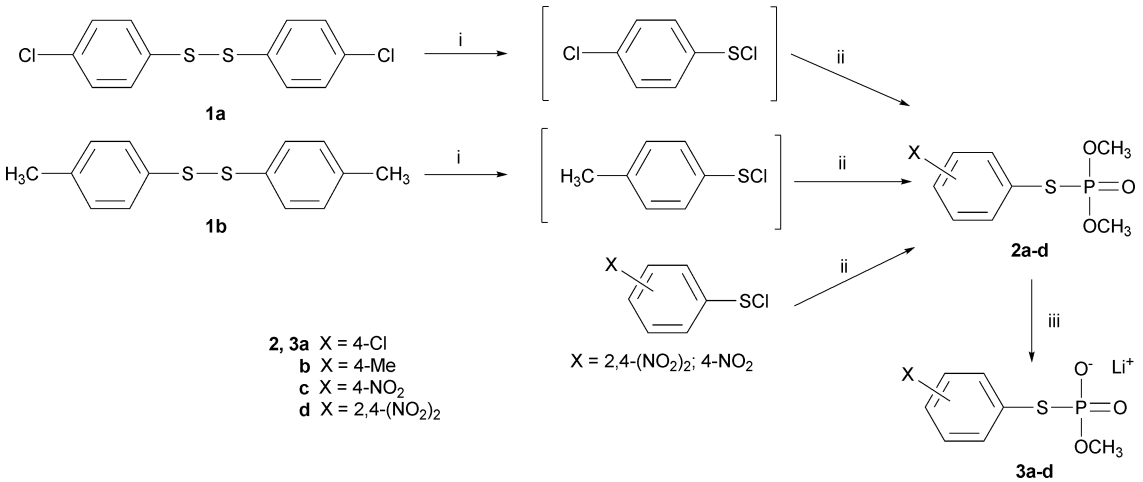
Results and discussion

Synthesis of phosphates and phosphothiolate diesters

We chose to investigate reactions of methyl aryl phosphorothiolate diester monoanions with pyridine nucleophiles for two reasons: (1) aryl groups allow systematic perturbation of the nucleophile and leaving group pK_a values with minimal steric perturbation, and (2) Kirby and Younas previously investigated reactions of OP diesters with pyridine nucleophiles.[31] We synthesized a series of *S*-aryl methyl phosphorothiolate (SP) diesters (Scheme 1, **3a–d**) and aryl methyl phosphate (OP) diesters containing substituted thiophenol and phenol leaving groups, respectively (Scheme 1). OP diesters were synthesized following the procedure of Ba-Saif *et al.*[32] To synthesize SP diesters, we first prepared *S*-aryl *O*,*O*-dimethyl phosphorothiolates **2a–d** using a modified version of the method described by Murdock and Hopkins for the preparation of *S*-(4-chlorophenyl) *O*,*O*-dimethyl phosphorthioic acid ester **2a**.[33] Rather than using bromotrichloromethane to facilitate the radical-chain-transfer reaction, we reacted aryl sulfenyl chlorides (generated *in situ* for 4-methylbenzenesulfenyl chloride and 4-chlorobenzenesulfenyl chloride by reducing the corresponding disulfides with chlorine gas) directly with trimethyl phosphite to generate **2a–d**. Dimethyl phosphorothiolates **2a–d** were demethylated with LiCl in dry acetone to give *S*-aryl methyl phosphorothiolates **3a–d**.[31]

Kinetic measurements

We used ^{1}H and ^{31}P NMR to monitor the reactions of the monoanions of the OP and SP diesters with pyridine nucleophiles (23 °C, 1.7 M ionic strength, 30 mM K_2CO_3, pH 10) (Fig. 1A and 1B). In these reactions, the initial products, pyridine methyl phosphoramidates, undergo rapid hydrolysis to pyridine and methyl phosphate, the only phosphorus-containing product observed.[31] Pseudo-first order rate constants (k_{obs}, Fig. 1C) were obtained by single exponential fits of the reactions with nucleophiles present in large excess (10–50 eq.). Least-squares analysis of the plots of k_{obs} *versus* nucleophile concentration gave good linear fits (Fig. 1D). Second order rate constants (k_2) were obtained from the slopes of these plots. For reactions of pyridines with OP diesters at 39 °C investigated previously, k_2 plots showed curvature due to



Scheme 1 *Reagents and conditions*: (i) Cl_2, CH_2Cl_2, rt, 0.5 ~ 1 h; (ii) trimethyl phosphite, 0 ~ 40 °C, 0.5 ~ 2 h, 65 ~ 97%; (iii) LiCl, acetone, rt, 4 ~ 24 h, 64 ~ 90%.

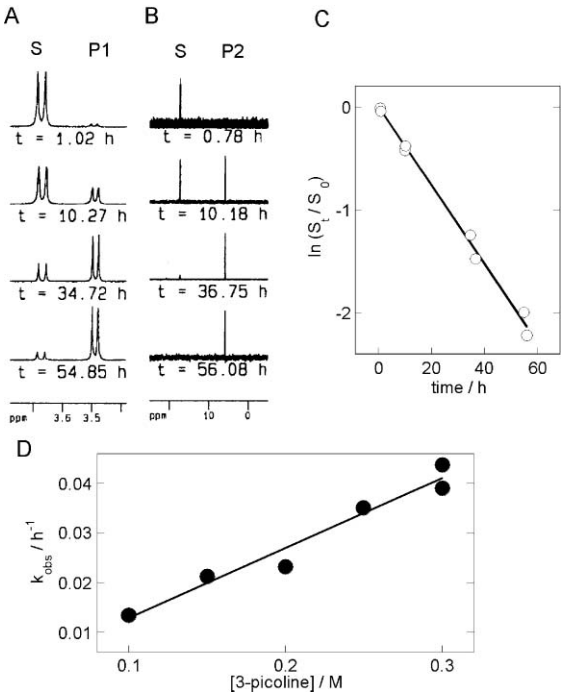


Fig. 1 Monitoring the reaction of *S*-(4-nitrophenyl) *O*-methyl phosphorothiolate with 3-picoline. ^{1}H (A) and ^{31}P (B) NMR spectra of the reaction mixture at different times. The ^{1}H NMR spectrum shows the methyl peak as a doublet in the starting ester (S) and the methyl phosphate product (P1). The ^{31}P NMR spectrum shows the phosphorus peak in the starting ester (S) and the phosphate monoester product (P2). (C) Data from A and B are plotted against time and the observed rate constant (k_{obs}) is obtained from the slope of the plot. (D) Dependence of observed rate constant (k_{obs}) on the concentration of 3-picoline. The slope of this linear dependence gives the second order rate constant k_2.

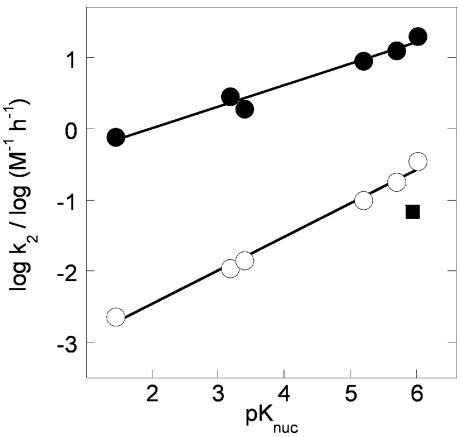


Fig. 2 Plot of k_2 *versus* pK_a of the nucleophile for the reactions of *S*-(2,4-dinitrophenyl) *O*-methyl phosphorothiolate (closed circles) and 2,4-dinitrophenyl methyl phosphate (open circles) with pyridine nucleophiles. Second order rate constants (k_2) were obtained as described in Fig. 1. The pK_a values[43] of the pyridine nucleophiles are: 3-CN, 1.45; 3-$COCH_3$, 3.18; 3-$CONH_2$, 3.40; 3-H, 5.20; 3-methyl, 5.70; and 4-methyl, 6.02. The solid square symbol represents k_2 for the reaction of *S*-(2,4-dinitrophenyl) *O*-methyl phosphorothiolate with 2-picoline.

pyridine self-association.[31,34] The reactions studied here showed no curvature, possibly due to the lower temperature (23 °C) and lower concentration of pyridines used.

The linear dependence of k_{obs} on the nucleophile concentration and of k_2 on the pK_a of the nucleophile (Fig. 2) suggest that the SP diesters react with pyridines by an S_N2 mechanism. Moreover, even though 2-picoline and 3-picoline have similar pK_a values, the sterically hindered 2-picoline reacts ($k_2 = 0.069$ M^{-1} h^{-1}) with *S*-(2,4-dinitrophenyl) methyl phosphorothiolate more than 100-fold slower than does 3-picoline ($k_2 = 13$ M^{-1} h^{-1}). Reactions of *S*-(4-nitrophenyl) methyl phosphorothiolate with peroxide exhibit an α effect:[35] peroxide reacts more than 100-fold faster than hydroxide, even though peroxide has a pK_a value almost four pH units lower than hydroxide. This sensitivity of the reaction to steric and electronic features of the nucleophile further suggests that the pyridines act directly as nucleophiles rather than as general acid/base catalysts. We conclude that SP diesters, like OP diesters, react with nucleophiles through an S_N2(P) mechanism.[31]

Reactions of 3-picoline with a series of SP and OP diesters show that k_2 for these esters strongly depends on the leaving group pK_a (Fig. 3), exhibiting Brønsted β_{lg} values of −1.1 and −1.3 for SP and OP diesters, respectively. β_{lg} values provide the change of the effective charge that occurs on the leaving group as the

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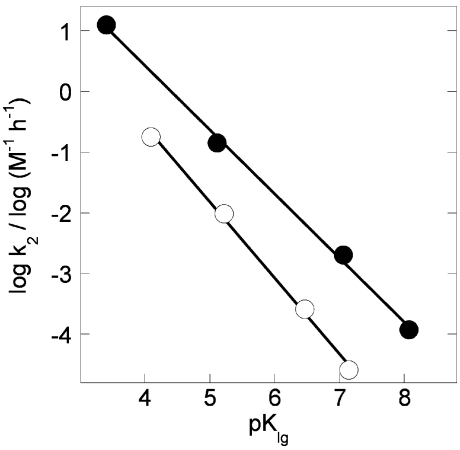


Fig. 3 Plot of k_2 *versus* pK_a of the leaving group for the reactions of *S*-aryl *O*-methyl phosphorothiolates (closed circles) and aryl methyl phosphates (open circles) with 3-picoline. The pK_a values[36,37] of the leaving groups for *S*-aryl *O*-methyl phosphorothiolates are: 2,4-dinitro, 3.2; 4-nitro, 5.11; 4-chloride, 7.06; and 4-methyl, 8.07. The pK_a values[32] of the leaving groups for aryl methyl phosphates are: 2,4-dinitro, 4.1; 2,5-dinitro, 5.22; 4-Cl-2-nitro, 6.46; and 4-nitro, 7.14.

reaction progresses from ground state to transition state, relative to the effective charge change defined by the ionization equilibrium: $RXH \rightleftharpoons RX^- + H^+$ (X = O for OP and S for SP diesters). The effective charges on X for RXH and RX^- are defined as 0 and −1, respectively.

To obtain the effective charge and bonding extent for the leaving group in the transition state, β_{lg} values require calibration against β_{eq}, the substituent effect on the reaction equilibrium.[38–40] β_{eq} defines the effective charge change on the leaving group for the overall reaction equilibrium, K_{eq}. For reactions of aryl phosphate monoanion, $\beta_{eq} = -1.74$,[41] meaning that the oxygen leaving group bears an effective charge of +0.74 in the starting ground state and an effective charge of −1 (by definition) in the phenolate product state. The value of $\beta_{lg} = -1.3$ for OP diesters suggests that the phenolate oxygen bears an effective charge of −0.6 (+0.74 − 1.3) in the transition state. β_{eq} for cleavage of *S*-aryl phosphorothiolate esters has not been determined and is challenging to measure. However, acyl and -PO_3H^- groups exhibit similar electropositive character in phenol transfer equilibria (with $\beta_{eq} = 1.70$ and 1.74, respectively[42]). Assuming that the acyl and -PO_3H^- groups exhibit similar electropositivity in thiophenol transfer, we may use $\beta_{eq} = 1.38$ for thiophenol-acyl transfer equilibria[42] as a crude estimate of β_{eq} for thiophenol-PO_3H^- and thiophenol-$PO_2(OCH_3)^-$ transfer equilibria. The value of $\beta_{lg} = -1.1$ for SP diesters suggests that the sulfur leaving group has an effective charge of −0.7 (+0.38 − 1.1) in the transition state. OP and SP diesters therefore appear to undergo similar extents of bond breaking (α) in their respective reactions with pyridine: $\alpha_S = \beta^S_{lg}/\beta^S_{eq} = 0.79$; $\alpha_O = \beta^O_{lg}/\beta^O_{eq} = 0.75$.

To probe the degree of bond making between the nucleophile and OP and SP diesters, we studied reactions of OP and SP diesters with a series of pyridine derivatives. The second order rate constants for reaction of pyridine nucleophiles with *S*-(2,4-dinitrophenyl) methyl phosphorothiolate and 2,4-dinitrophenyl methyl phosphate show linear dependencies on nucleophile pK_a (Fig. 2). The slopes give the Brønsted coefficient, β_{nuc}, as 0.3 and 0.47 for SP and OP diesters respectively. The β_{nuc} of 0.47 for OP diesters is comparable to the published[31] value of 0.44 measured at 39 °C and 1 M ionic strength.‡ These apparent β_{nuc} values for the phosphoryl transfer to pyridine likely deviate from the intrinsic β_{nuc} by the well-documented solvation effect.[44,45] Using the equation $\beta^{corr}_{nuc} = (\beta_{nuc} - \beta_d)/1 - \beta_d)$ with $\beta_d = -0.2$,[46] we obtained corrected β^{corr}_{nuc} values of 0.42 and 0.56 for SP and OP diesters, respectively.

After correction of the observed β_{nuc} values, the difference between OP and SP diesters decreases modestly but remains significant. The interaction coefficient p_{xy} could account for part of this difference. p_{xy} defines the dependence of β_{nuc} on the pK_a of the leaving group or the dependence of β_{lg} on the pK_a of the nucleophile ($p_{xy} = \partial\beta_{nuc}/\partial pK^{lg}_a = \partial\beta_{lg}/\partial pK^{nuc}_a$).[47] The p$K_a$ values of 2,4-dinitrothiophenol[36] and 2,4-dinitrophenol[32] are 3.2 and 4.1, respectively. Using an interaction coefficient p_{xy} of 0.013 for pyridine attack on OP diesters,[31,47] we obtain $\beta_{nuc} = 0.56 - 0.013 \times (4.1 - 3.2) = 0.55$ for pyridine nucleophiles reacting with a hypothetical aryl methyl phosphate diester containing a leaving group with pK_a = 3.2. Thus, the pK_a difference of 2,4-dinitrothiophenol and 2,4-dinitrophenol is not sufficient to explain the difference in β_{nuc} between the OP and SP diesters.

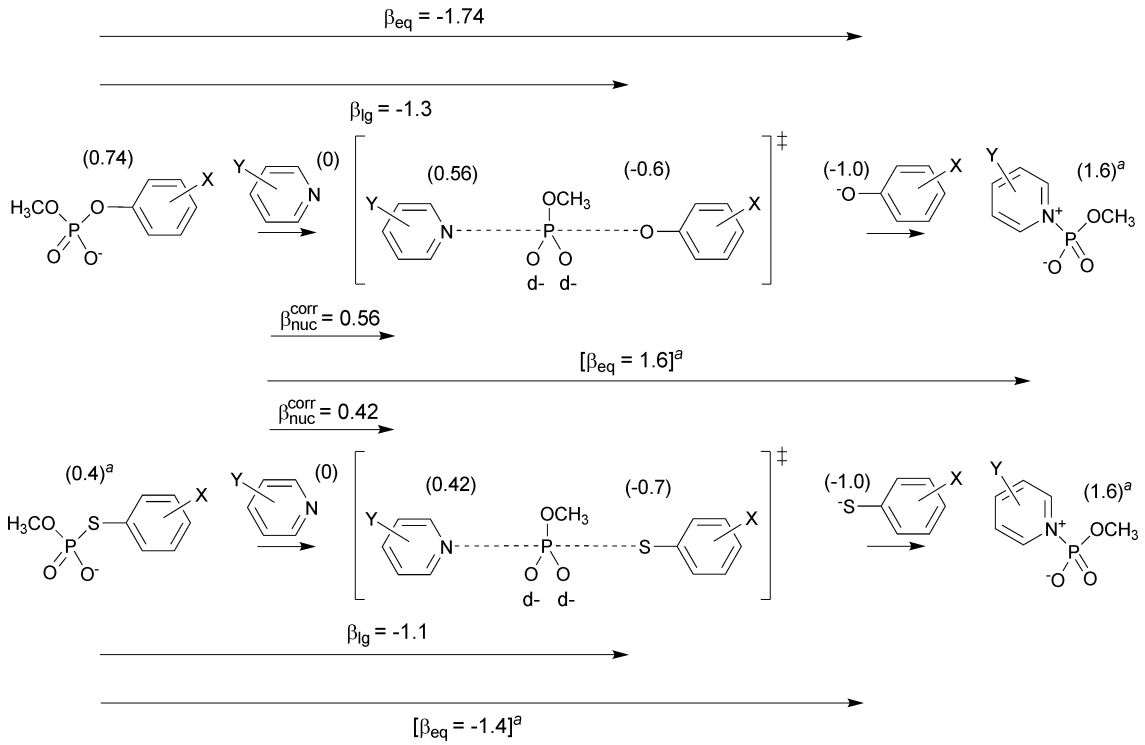
The β_{eq} for phosphoryl group transfer to pyridines is independent of the leaving group, allowing direct comparison of β^{corr}_{nuc} values. The β^{corr}_{nuc} values indicate that SP diesters have less N-P bonding in the transition state than do OP diesters. Given that the P–O and P–S bonds undergo similar extents of cleavage in the transition state, these results suggest that SP diesters require less bonding to the nitrogen nucleophile than do OP diesters to achieve the same degree of bond cleavage to the leaving group. The Hammond effect may account for this difference,[35,48] reflecting the weaker thermodynamic stability of the P–S bond compared to the P–O bond.[49,50]

To facilitate comparison of the OP and SP diester reactions, we may obtain a crude estimate of the β_{eq} for pyridine-$PO_2(OCH_3)^-$ transfer equilibria from the pyridine-acyl transfer data (where $\beta_{eq} = +1.6$),[42] as described above for thiophenol-$PO_2(OCH_3)^-$ transfer equilibria. This enables construction of the effective charge map (Scheme 2) and representation of the transition state in a More O'Ferrall–Jencks diagram (Fig. 4).[51,52] The data suggest that OP and SP diesters react with nucleophiles *via* a concerted mechanism§ involving a dissociative transition state.[53]¶ Previous studies on chemical models of phosphate diester monoanions,[32,54] *ab initio* calculations of phosphorothiolate diesters,[55] and Kirby's β_{nuc} values[31]‡corroborate the OP diester transition state model that

‡ The text of the published report[31] states that $\beta_{nuc} = 0.31$; the plot in Fig. 2 in that report has a slope of 0.44. We plotted the data from Table 2 in that report and obtained $\beta_{nuc} = 0.44$, in excellent agreement with our measurement.

§ Formally, the reactions may occur *via* a stepwise addition-elimination (associative) mechanism in which the nucleophile first adds to the phosphorous center to form a dianionic phosphorane intermediate followed by expulsion of the leaving group. Our results argue against the associative mechanism. If the addition step limited the reaction rate, we would expect small values of β_{nuc} and β_{lg} (relative to the corresponding β_{eq}). If the elimination step limited the reaction rate, we would expect more substantial values for both β_{nuc} and β_{lg}. However, we observe relatively large β_{lg} values and relatively small β_{nuc} values, consistent with neither rate-limiting addition nor rate-limiting elimination.

¶ A dissociative transition state, defined by $\beta_{nuc}/\beta_{eq} + (1 - \beta_{lg}/\beta_{eq}) < 1$, indicates that bond fission exceeds bond formation. An associative transition state, defined by $\beta_{nuc}/\beta_{eq} + (1 - \beta_{lg}/\beta_{eq}) > 1$, indicates that bond formation exceeds bond fission).



Scheme 2 Effective charges of the nucleophiles and leaving groups in the reactions of pyridine derivatives with OP and SP esters. [a]Values are estimated.

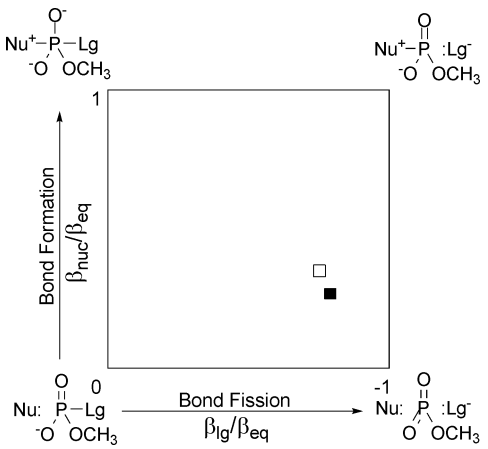


Fig. 4 Reaction maps for transfer of the methyl phosphoryl group from phenols and thiophenols to pyridine nucleophiles. The horizontal and vertical scales are calibrated in terms of the overall effective charge change on the leaving and entering atoms, respectively using values reported in Scheme 2. The position of the transition state of the reaction of *S*-aryl *O*-methyl phosphorothiolates with pyridines is shown as a closed square and that of the reaction of aryl methyl phosphates with pyridines is shown as an open square.

emerges from the current work. Comparison of our data for SP diesters to these OP data suggests that nucleophiles react with the SP diesters *via* a similar mechanism and transition state structure.

The analysis above provides a foundation for studying the phosphoester transfer in biological systems. In the case of nucleic acid substrates, in which the leaving oxygen atoms are bonded to alkyl groups rather than to aryl groups like the model compounds used here, the pK_a differences between the sulfur and oxygen leaving groups become more pronounced. For example, the pK_as of 2,4-dinitrophenol and 2,4-dinitrothiophenol differ by 0.9 units, whereas the pK_as of ethanol and ethanethiol differ by ~5 units. For OP esters, the p_{xy} value predicts that a 5-unit pK_a decrease in the leaving group would decrease β_{nuc} by more than 10%, thereby increasing the dissociative character of the transition state. Therefore the pK_a difference between S and O leaving groups in nucleic acids would be expected to induce an even greater increase in the dissociative character of the transition state than observed here for sulfur substitution in methyl aryl phosphodiesters. Together with the increases in volume and bond lengths that accompany sulfur substitution, the more open transition state structure may explain in part why enzymes catalyze reactions of phosphorothiolates less efficiently than those of their natural substrates.

Conclusions

In summary, we have obtained an initial model for the transition state structure of nonenzymatic reactions of phosphodiester and phosphorothiolate diesters with pyridine nucleophiles. Both oxygen and sulfur leaving groups bear similar effective charges and undergo similar degrees of bond cleavage in the transition state. Although we can only estimate bonding extents due to lack of knowledge about β_{eq}, this work provides a useful direct comparison of the reactions of SP and OP diesters with pyridine nucleophiles. Both types of esters react with pyridine nucleophiles *via* a concerted, dissociative mechanism, but the SP diesters react *via* a slightly more open transition state than do the OP diesters. We conclude that sulfur substitution of the leaving group does not grossly perturb the reaction pathway, supporting the use of

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phosphorothiolates as substrate analogues to probe mechanisms of enzyme-catalyzed phosphoryl transfer reactions.

Experimental

Synthesis

***S*-(4-Chlorophenyl) *O*,*O*-dimethyl phosphorothiolate (2a).** An ice cooled solution of bis(4-chlorophenyl) disulfide **1a** (20 g, 0.068 mol) in dichloromethane (100 ml) was saturated with chlorine gas by bubbling it through the solution for 1 hour then excess gas and solvent were removed by vacuum evaporation. Trimethyl phosphite (18 ml, 0.15 mol) was added dropwise under argon over 15 minutes to the stirred solution of 4-chlorobenzenesulfenyl chloride intermediate. The solution was stirred for an additional 30 minutes, solvent was then removed by evaporation under vacuum, and excess trimethyl phosphite was distilled off at 70 °C/0.01 mmHg to give **2a** as a colorless oil (27 g, 79%); ν_{max}(film)/cm^{-1} 3116, 3087, 2966, 2864, 2362, 2345, 1603, 1546, 1459, 1444, 1345, 1263, 1186, 1032, 905, 843, 799, 733, 559 (lit.,[33] 3070, 1263, 1180); δ_H(acetone-d$_6$) 3.83 (6 H, d, *J* 12.7), 7.40–7.44 (2 H, m), 7.58–7.62 (2 H, m); δ_C 54.57 (d, *J* 6.6), 126.07 (d, *J* 7.1), 130.28 (d, *J* 2.2), 135.78 (d, *J* 3.3), 136.89 (d, *J* 5.6); δ_P 24.53.

***S*-(4-methylphenyl) *O*,*O*-dimethyl phosphorothiolate (2b).** Compound **2b** was prepared from *p*-tolyldisulfide (15 g, 0.061 mol) using the procedure described for the synthesis of **2a** with the following modifications: chlorine gas was bubbled for 30 minutes, trimethyl phosphite (16 ml, 0.14 mol) was added dropwise over 10 minutes under argon at 0 °C. The resulting solution of *p*-tolylsulfenyl chloride was stirred for 10 minutes. Vacuum distillation yielded **2b** as a yellow oil (28 g, 97%); ν_{max}/cm^{-1} (KBr) 3022, 2953, 2850, 2358, 1902, 1800, 1596, 1492, 1457, 1261, 1182, 1017, 811, 788, 765, 600, 567; δ_H(acetone-d6) 2.32 (3 H, d, *J* 1.7), 3.77 (6 H, d, *J* 12.5), 7.19–7.23 (2 H, m), 7.46–7.50 (2 H, m); δ_C 21.04, 54.35 (d, *J* 6.0), 123.53 (d, *J* 7.1), 130.99 (d, *J* 2.2), 135.45 (d, *J* 4.9), 140.21 (d, *J* 3.3); δ_P 25.56.

***S*-(4-nitrophenyl) *O*,*O*-dimethyl phosphorothiolate (2c).** Under argon, trimethyl phosphite (8.8 ml, 0.074 mol) was added dropwise over 10 minutes to a solution of 4-nitrobenzenesulfenyl chloride (13 g, 0.068 mol) in dichloromethane (55 ml); the solution was brought to reflux and then stirred for an additional hour at room temperature. Dichloromethane was evaporated, and the residue was recrystallized in 15 ml methanol to yield **2c** (10 g, 65%); mp 56–58 °C; ν_{max}/cm^{-1} (KBr) 3104, 2955, 2852, 2366, 2345, 1578, 1513, 1444, 1349, 1265, 1179, 1012, 854, 835, 789, 765, 746, 555; δ_H (acetone-d6) 3.85 (6 H, d, *J* 12.7), 7.86–7.90 (2 H, m), 8.22–8.26; δ_C 54.97 (d, *J* 6.0), 124.99 (d, *J* 1.6), 135.51 (d, *J* 5.5), 136.54 (d, *J* 6.6), 148.96; δ_P 14.32.

***S*-(2,4-dinitrophenyl) *O*,*O*-dimethyl phosphorothiolate (2d).** The general procedure as described for the synthesis of **2b** was followed. The reaction mixture containing 2,4-dinitrobezenesulfenyl chloride starting material was stirred for 2 hours at 20 °C after addition of trimethyl phosphite. Recrystallization in methanol gave **2d** (15 g, 73%); mp 69–70 °C; ν_{max}/cm^{-1} (KBr) 3116, 3087, 2966, 2864, 2362, 2345, 1603, 1546, 1345, 1263, 1186, 1023, 905, 843, 799, 734, 559; δ_H 3.87 (6 H, d, *J* 12.9), 8.33 (1 H, dd, *J* 1.7 and 8.8), 8.56 (1 H, dd, *J* 2.7 and 8.8), 8.84 (1 H, d, *J* 2.4); δ_C 55.60 (d, *J* 6.6), 121.43, 127.88, 131.89 (d, *J* 4.9), 148.43, 151.87 (d, *J* 4.4); δ_P 21.14.

Lithium *S*-(4-chlorophenyl) *O*-methyl phosphorothiolate (3a). A solution of **2a** (23 g, 90 mmol) in 50 ml acetone was stirred with LiCl (3.8 g, 90 mmol) for 8 hours at 20 °C; filtration yielded a white precipitate **3a** (15 g, 69%); mp >250 °C; (Found: C, 34.11; H, 2.95; P, 12.73; S, 13.99. $C_7H_7ClLiO_3PS$ requires C, 34.38; H, 2.89; P, 12.66; S, 13.11%); λ_{max} (MeOH)/nm 226 (ε/dm^3 mol^{-1} cm^{-1} 81 000); ν_{max}(KBr)/cm^{-1} 3429, 2988, 2946, 2844, 2359, 2343, 1637, 1476, 1390, 1209, 1084, 1013, 820, 783, 744; δ_H 3.56 (3 H, d, *J* 12.9), 7.29–7.32 (2 H, m), 7.44–7.47 (2 H, m); δ_C 54.12 (d, *J* 6.6), 128.77 (d, *J* 6.6), 130.06 (d, *J* 1.6), 134.87 (d, *J* 3.3), 136.36 (d, *J* 4.9); δ_P 19.26; HRMS (FAB$^-$) calculated for $C_7H_7ClO_3PS$: 236.9542, found: 236.9536.

Lithium *S*-(4-methylphenyl) *O*-methyl phosphorothiolate (3b). The general synthetic procedure described for the synthesis of **3a** was followed. After 24 hours of stirring the reaction mixture containing **2b**, a precipitate of **3b** was isolated by filtration (15 g, 64%); mp >250 °C; (Found: C, 42.08; H, 4.69; P, 14.00; S, 13.92. $C_8H_{10}LiO_3PS$ requires C, 42.87; H, 4.50; P, 13.82; S, 14.30%); λ_{max} (MeOH)/nm 226 (ε/dm^3 mol^{-1} cm^{-1} 67 000); ν_{max}/cm^{-1} 3422, 3020, 2980, 2946, 2844, 2360, 2343, 1636, 1493, 1224, 1082, 810, 782; δ_H 2.22 (3 H, s), 3.54 (3 H, d, *J* 12.7), 7.09–7.13 (2 H, m), 7.34–7.38 (2 H, m); δ_C 21.06, 54.12 (d, *J* 6.0), 126.29 (d, *J* 6.6), 130.87 (d, *J* 1.6), 135.10 (d, *J* 4.4), 139.90 (d, *J* 3.0); δ_P 20.06; HRMS (FAB$^-$) calculated for $C_8H_{10}O_3PS$: 217.0137, found: 217.0077.

Lithium *S*-(4-nitrophenyl) *O*-methyl phosphorothiolate (3c). The general procedure described for the synthesis of **3a** was followed except that the reaction mixture containing **2c** was stirred for 15 hours. Crystals were isolated by filtration, and dried under vacuum to yield 6.2 g (90%) of **3c** containing 0.77 equivalents of acetone (by ^{1}H NMR). Removal of solvent from crystal lithium salt proved difficult, recrystallization in chloroform–methanol (12 : 1) gave **3c** with 0.87 equivalents of crystal methanol, mp >250 °C; (Found: C, 30.84; H, 3.22; N, 5.13; P, 10.79; S, 11.93. $C_7H_7LiNO_5PS{\cdot}0.87\ CH_3OH$ requires C, 33.40; H, 3.73; N, 4.95; P, 10.94; S, 11.33%.); λ_{max} (MeOH)/nm 306 (ε/dm^3 mol^{-1} cm^{-1} 68 000); ν_{max}/cm^{-1} (KBr) 3380, 3102, 2984, 2845, 2366, 2345, 1638, 1598, 1578, 1514, 1344, 1236, 1082, 1045, 854, 787, 744, 685, 580; δ_H 3.61 (3 H, d, J 12.9), 7.69–7.72 (2 H, m), 8.11–8.14 (2 H, m); δ_C 54.21, 124.82, 134.21 (d, *J* 5.5), 140.86 (d, *J* 6.0); δ_P 17.23; HRMS (FAB$^-$) calculated for $C_7H_7NO_5PS$: 247.9783, found: 247.9796.

Lithium *S*-(2,4-dinitrophenyl) *O*-methyl phosphorothiolate (3d). The general procedure described for the synthesis of **3a** was followed. A reaction mixture containing starting material **2d** became yellow, and crystals formed after 4 hours, crystals were isolated and dried under vacuum. The yield of **3d** (16 g) was quantitative, however acetone was present. Recrystallization in chloroform–methanol (4 : 1) was accompanied by decomposition, and yellow crystals of **3d** were recovered containing 0.7 equivalents of methanol, mp >250 °C; Found: C, 30.84; H, 3.22; N, 5.13; P, 10.79; S, 11.93. $C_7H_7LiNO_5PS{\cdot}0.7\ CH_3OH$ requires C, 33.40; H, 3.73; N, 4.95; P, 10.94; S, 11.33%.); λ_{max} (MeOH)/nm 312 (ε/dm^3 mol^{-1} cm^{-1} 67 000); ν_{max}/cm^{-1} (KBr) 3418, 3110, 2952, 2848, 2364, 2344, 1595, 1529, 1458, 1344, 1267, 1093, 1044, 917, 843, 786, 746, 735, 669; δ_H 3.59 (3 H, d, *J* 12.9), 8.14 (1 H, d, *J* 9.0), 8.31 (1 H, dd, *J* 9.0), 8.72 (1 H, d, *J* 2.2); δ_C 54.45 (d, *J* 6.6),

121.64, 127.75, 135.78 (d, J 4.4), 138.02 (d, J 4.4), 146.61, 150.00 (d, J 2.7); δ_P 14.74; HRMS (FAB$^-$) calculated for $C_7H_7N_2O_7PS$: 292.9633, found: 292.9622.

Measurement of k_{obs} and k_2

All reaction solutions contained 10 mM arylmethylphosphate (OP) or phosphorothiolate esters (SP) in deuterium oxide and were maintained at 1.7 M ionic strength using potassium chloride. Reactions were carried out in NMR tubes at room temperature and kept in the dark. A Bruker Avance DMX 500 (500 MHz) and Avance DRX 400 (400 MHz) were used to record ^{1}H NMR and ^{31}P NMR, ^{1}H NMR was referenced to deuterium oxide (δ_H 4.63) and ^{31}P NMR was referenced externally to 85% H_3PO_4 (δ_P 0.00).

Generally, to measure k_{obs}, we monitored the conversion of substrate into methyl phosphate by ^{1}H NMR, reactions were followed for at least seven time points and, unless otherwise noted, rates were measured for more than 3.5 half lives. Values of k_{obs} were measured for five different nucleophile concentrations. A total of five to six runs were performed for each substrate–nucleophile combination. The second order rate constant, k_2, was obtained from the slopes of the linear plots of k_{obs} *versus* nucleophile concentration. We used ^{31}P NMR to confirm the assignment of the product peak as methyl phosphate for each substrate–nucleophile combination. Precipitate developed in some reactions with phosphorothiolate diesters but not with phosphate diesters. The formation of precipitate did not appear to affect measurements of k_{obs}.

Measurement of phosphorothiolate diester and methyl phosphate relaxation times

To acquire accurate integration values by ^{31}P NMR for phosphorothiolate diesters and methyl phosphate, we must allow the ^{31}P nuclei to relax fully following excitation. Therefore, we measured the relaxation times of the phosphorous nucleus for each compound by a standard inversion recovery test. Relaxation times (Table 1) were 5.303 s, 5.339 s, 4.906 s, and 3.390 s for phosphorothiolates **3a-d**, respectively. Variation of phosphorothiolate concentration does not appear to influence relaxation time measurements except for **3a**. Relaxation times of **3a** were measured to be 5.123 s at 50 mM and 5.483 s at 200 mM. The average value was used. Methyl phosphate, synthesized by hydrolysis of phosphorothiolate diesters with 1.0 M potassium hydroxide, had an average relaxation time of 11.003 s (literature,[56] 12 s).

Table 1 Relaxation times of the phosphorous nucleus for SP diesters

	Relaxation times/s[a]	Chemical shift (δ_P)
3a	5.123	17.54
	5.483 (50 mM)	17.77
3b	5.339	20.00
3c	4.906	18.98
3d	3.39	15.0
Methyl phosphate	10.823 (50 mM)	5.15
	11.183	5.30
	11.184	5.23

[a] Concentrations are at 200 mM unless otherwise noted.

Kinetics of reactions of oxyanion nucleophiles with *S*-(4-nitrophenyl) methyl phosphorothiolate

In measuring k_2 for the reaction of hydroxide nucleophile with *S*-(4-nitrophenyl) methyl phosphorothiolate, potassium hydroxide concentrations of 0.3 to 1.7 M were used. In addition, to determine the rate by following the formation of methyl phosphate, the k_{obs} value was calculated from conversion of substrate peak to the product thiophenolate peak. The latter measurement gave slightly lower k_2. This may be due to the formation of a by-product, as suggested by new resonances in the aryl region (according to the ^{1}H NMR spectra). We did not identify the structure of the by-product. The k_2 value was not corrected for carbon attack because we didn't detect any products arising from attack at carbon.

In reactions of peroxide nucleophile with *S*-(4-nitrophenyl) methyl phosphorothiolate, peroxide nucleophile stock solution was prepared fresh with one equivalent of potassium hydroxide. Nucleophile concentration ranged from 0.1 to 0.3 M. Values of k_{obs} were determined by following the reaction over the first two half lives.

Kinetics of reactions of phosphorothiolate and phosphate diesters with pyridine nucleophiles

Reactions of phosphorothiolate and phosphate diesters with pyridine nucleophiles were buffered with 30 mM potassium bicarbonate buffer (pH 10). The concentration ranged from 0.1 to 0.3 M for the picoline nucleophiles and from 0.1 to 0.5 M for all other pyridine nucleophiles. Concentrations of bases were not adjusted for association, as we observed, under our reaction conditions, no obvious curvature in the plots of k_{obs} *vs.* pyridine nucleophile concentration (pyridine nucleophile concentration $\leq$ 0.5 M, 23 °C; 1.7 M ionic strength).

In the reaction of 3-picoline with **4c** and **5c–d**, k_{obs} was determined by following reactions for more than 3.5 half lives. For **4a–b** and **5a–b**, k_{obs} was determined from initial rates. For phosphate diester **5a**, ten runs were performed and k_{obs} was averaged due to the slow rate of this reaction. NMR tubes were flame closed to prevent the evaporation of 3-picoline.

In the reaction of 3-cyanopyridine with phosphate diester **5d**, a substituted pyridine by-product was observed. Although this by-product does not appear to affect k_{obs} over the first 1.5 half lives, it does affect it increasingly thereafter. Consequently, k_{obs} was determined from the initial rate for this reaction (seven time points). The by-product was isolated by TLC (ethyl acetate–hexane, 6 : 1) and identified by ^{1}H NMR and mass spectra as nicotinamide.

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