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Probing Potential Medium Effects on Phosphate Ester Bonds Using ^{18}O Isotope Shifts on ^{31}P NMR

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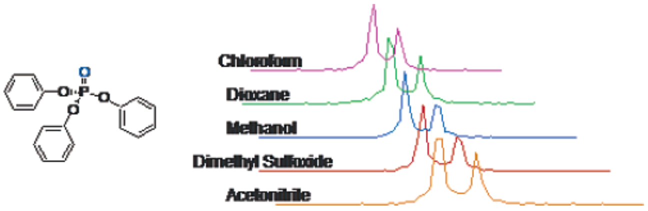

Dipolar aprotic cosolvents, such as DMSO and acetonitrile, accelerate the rates of hydrolysis of phosphate monoester dianions. It has been speculated that the rate acceleration arises from the disruption of hydrogen bonding to the phosphoryl group. An aqueous solvation shell can stabilize the dianionic phosphoryl group by forming hydrogen bonds to the phosphoryl oxygens, whereas solvents such as DMSO are incapable of forming such bonds. It has been proposed that the loss of stabilization could result in a weakened P−OR ester bond, contributing to the observed faster rate of hydrolysis. Computational results support this notion. We have used the ^{18}O-induced perturbation to the ^{31}P chemical shift to ascertain whether solvent changes result in alterations to the P−O(R) bond. We have studied $^{16}O^{18}O$-labeled methyl, ethyl, phenyl, *p*-nitrophenyl, diethyl *p*-nitrophenyl, triphenyl, and di-*tert*-butyl ethyl phosphate in the solvents water, methanol, chloroform, acetonitrile, dioxane, and DMSO. The results suggest no significant solvent-induced weakening of the phosphate ester bonds in any of the solvents tested, and this is unlikely to be a significant source for the acceleration of hydrolysis in mixed solvents.

Introduction

Phosphoryl transfer reactions are ever-present in biochemistry. The hydrolysis of phosphate monoesters, most commonly tyrosine, serine, and threonine phosphates, by phosphatases is involved in cell signaling and regulation. The substrates for most phosphatases are believed to be the dianion forms, which have very slow rates of uncatalyzed hydrolysis; the estimated half-life for attack by water on alkyl phosphate dianions is 10^{12} years at 25 °C.[1]

The rate of hydrolysis reactions of phosphomonoester dianions is strongly affected by the medium. The rate of hydrolysis of the *p*-nitrophenyl phosphate (pNPP) dianion is accelerated by up to 10^6 in 95% DMSO[2] and an increase of over 10^6-fold has also been reported in acetonitrile containing 0.02 M water as compared to that of water alone. It has been speculated[2] that this increase in rate is due to the disruption of hydrogen bonding to the phosphoryl group, resulting in a weakening of the P−O ester bond. One way to rationalize this effect is that the loss of stabilizing hydrogen bonds results in a greater contribution from a resonance form consisting of metaphosphate and the ester group oxyanion. If it occurs, such an effect would make hydrolysis of the phosphate ester easier in the presence of dipolar aprotic cosolvents. The enthalpy of activation for the aqueous hydrolysis of the (pNPP dianion, $\Delta H^\ddagger = 30.6$ kcal/mol, is reduced to $\Delta H^\ddagger = 20.7 \pm 0.6$ kcal/mol in 95:5 DMSO/H_2O.[3] This observation is consistent with the hypothesis that, relative to the aqueous reaction, in the mixed solvent the dianionic reactant is destabilized more than the transition state, as illustrated in Figure 1. Other conclusions are also possible; for example, the reactant energies could be similar, but the transition state more stable in the mixed solvent, relative to pure water.

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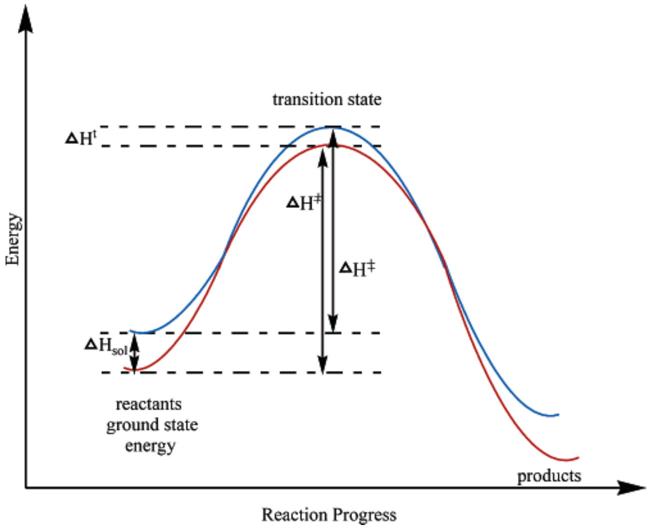

FIGURE 1. Diagram of reaction coordinate for phosphate ester hydrolysis showing a previously hypothesized origin of the different $\Delta H^{\ddagger}$ values in water (red) and 95:5 DMSO/water (blue).

TABLE 1. P−O Ester Bond Lengths for Methyl, Phenyl, and *p*-Nitrophenyl Phosphate Dianions from Previously Reported[3] Calculations at the HF/6-31++G Level[a]**

	leaving group/pK_a		
	OMe/ 15.5	OPh/ 9.95	OpNP/ 7.14
P−O in water, HF/6-31++G**, PCM	1.663 Å	1.699 Å	1.729 Å
P−O in DMSO, HF/6-31++G**, PCM	1.680 Å	1.725 Å	1.866 Å
P−O in bond order in DMSO relative to that in H_2O	0.96	0.91	0.60

[a] The bond orders were calculated using the bond length of each respective phosphate ester in water as D(1) in the Pauling equation.

KIE data[3] indicate that a similar transition state occurs in the reaction in both solvent systems, indicating that the rate acceleration does not result from a mechanistic change. The transition state is very loose, resembling the anionic leaving group and metaphosphate, with little nucleophilic participation. With respect to charge distribution, the charge of −2 on the phosphoryl group in the reactant is much more dispersed in the transition state; the phosphoryl group retains about a unit of negative charge, with the remainder on the leaving group.

Computational results,[3] reproduced in Table 1, suggest that upon going from water to DMSO, the P−O ester bond becomes significantly weaker. In these computations, solvent molecules were not explicitly added, and solvation was modeled using polarizable continuum models (PCM) for DMSO and water. The Pauling equation[4] (eq 1, where $D(n)$ is the bond length for a bond order of n, and $D(1)$ is the bond length for a bond order of unity) can be used to estimate the degree of bond order loss implied by the calculations. If the Pauling equation is rearranged to the form of eq 2, and the computed P−O distance for each ester in water is assigned to a bond order of unity, the resulting relative bond order (n) in DMSO calculated for each ester is shown in the bottom row of Table 1.

TABLE 2. Bond Lengths from Analysis of the P−O Ester Bond of pNPP in DMSO/Water Mixtures by Raman Spectroscopy[5]

%DMSO	$R_{bridging}$ (Å)	Pauling bond order
0	1.623	1.00
25	1.624	1.00
50	1.626	0.99
60	1.627	0.98
70	1.628	0.98
80	1.630	0.97

$$D(n) = D(1) - 0.6 \log n \qquad (1)$$

$$D(\text{DMSO}) - D(\text{H}_2\text{O}) = -0.6 \log n \qquad (2)$$

The calculations predict significant medium-induced effects on the ester bond for pNPP, and smaller effects for phosphates with more basic ester groups. It is noted that the calculations also predict an effect of the ester group on the P−O bond length. Subsequent experimental investigations have not found such an effect.[5,6]

An investigation[5] using Raman spectroscopy of pNPP in water/DMSO mixtures determined that the bridging P−OR bond weakens by a small extent as the amount of DMSO increases (Table 2). The Pauling bond orders in Table 2 were calculated using the bond length of 1.623 Å found in pure water as D(1). In 80% DMSO, the highest DMSO content for which measurements could be obtained, a change in bond order of 0.03 is implied by these data. This is much smaller than the computationally predicted effect for pNPP in pure DMSO. DMSO at 80% still contains 11 M concentration of water, leaving open the question of whether a more complete change of medium might result in significant ester bond weakening effects.

To assess a broad range of organic solvents for potential solvation-induced changes in bonding in phosphate esters, we have measured the ^{18}O-induced perturbation to the ^{31}P chemical shift ($\Delta\delta_p$) in a series of phosphate esters, using the solvents water, DMSO, dioxane, acetonitrile, chloroform, and methanol, to ascertain whether changes in solvent result in a significant alteration to either the P−OR ester bond or the nonbridging P−O phosphoryl bonds.

In this study, the phosphate esters were synthesized with a mixture of ^{16}O and ^{18}O at the position(s) of interest, shown in Figure 2. The ^{31}P NMR spectra of ^{18}O-labeled phosphate esters show from two to four resonances, depending on the number of ^{18}O atoms present. The ^{18}O-labeled analogue always gives a more upfield signal than that with ^{16}O. Published data show that the magnitude of this isotope-induced shift ($\Delta\delta_p = \delta^{16}\text{O} - \delta^{18}\text{O}$) is proportional to the P−O bond order; $\Delta\delta_p$ increases from ~0.016 ppm for single bonds up to ~0.041 ppm for double bonds.[7,8]

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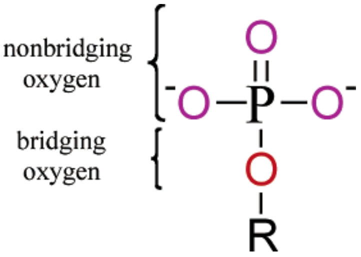

FIGURE 2. Positions of ^{18}O labeling in the phosphate esters studied by NMR. Red indicates the bridging oxygen, and purple indicates the nonbridging oxygens.

TABLE 3. Isotope Shifts for a Series of Phosphate Esters and Inorganic Phosphate[6−8]

compound, position of ^{18}O	formal P−O bond order	$\Delta\delta_P = \delta^{16}O - \delta^{18}O$ (ppb)
ATP β-γ bridging	1	16.5
ADP α-β bridging	1	16.6
inorganic phosphate trianion	1.25	20.5
ADP β-nonbridging	1.33	21.5
ATP γ-nonbridging	1.33	22.0
ATP β-nonbridging	1.5	28.5
diethyl pNPP	2	40.0

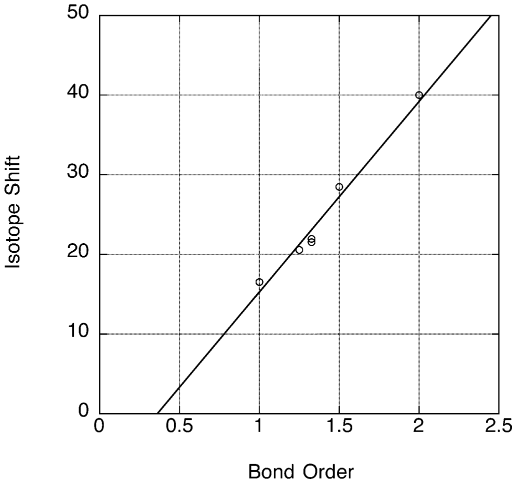

FIGURE 3. Graph of the isotope shift data in Table 3. The line represents a least-squares fit to the data, and obeys the relation $\Delta\delta_P = (23.8) \times (\text{bond order}) - 8.4$, with $r^2 = 0.97$.

The magnitudes of isotope shifts result from a combination of two factors: the first derivative of nuclear shielding with respect to bond length (the magnitude of which increases with increasing bond order) and anharmonicity, the manifestation of an altered bond length arising from substitution by the heavier isotope.[9,10] The degree of anharmonicity has been shown to vary depending on molecular structure, making the relative contributions from these two factors variable and difficult to correlate with any one property. However, the cumulative data have led to the observation that, within the same or similar functional groups, the magnitude of isotope shift is proportional to bond order.[9−11]

Values of $\Delta\delta_p$ from published data of ^{18}O-isotopomers of ATP and ADP,[7,8] inorganic phosphate, and a phosphotriester[6] (Table 3) have been plotted as a function of formal P−O bond order in Figure 3. The observation that this fit does not pass through the origin suggests that the correlation may not be linear at lower bond orders. Alternatively, this may reflect the fact that the P−O bond order for each ester is only a formalism, and may not reflect the true bond order. A fit of the same data forced through the origin yields a slightly poorer correlation, with $r^2 = 0.91$.[6] While the exact mathematical relationship between $\Delta\delta_p$ and bond order is uncertain, it is clear that there is a systematic relationship. The correlation between $\Delta\delta_p$ and P−O bond order in phosphate esters bond order has been further demonstrated by the linear relationship between the magnitude of $\Delta\delta_p$ and the P−O stretching frequencies in methyl, dimethyl, and trimethyl phosphates.[12] The correlation was also found in our previous work in water with different counterions for various phosphate monoesters.[6] ^{18}O-isotope shifts in ^{31}P NMR have been used in a number of other studies of bond order for phosphate[7,8,12,13] and phosphorothioate[14−17] esters. Thus, a significant difference in bond order will be accompanied by a difference in isotope shift well within the range of detectability.

Measurements of ^{18}O-isotope shifts on ^{13}C NMR of carbons in C−O bonds for a number of compounds in various solvents have been reported. The results have shown an independence of significant solvent effects for formal bond orders ranging from 1.0 to 2.0. For *n*-propyl [*carbonyl*-^{18}O]benzoate, the same isotope shift of 33 ± 1 ppb is observed in $CDCl_3$, DMSO-d_6, pyridine-d_5, benzene-d_6, and methanol-d_4.[18] Similar isotope shifts are also found for 1-phenyl-ethanol-^{18}O in several solvents: 23 ± 1 ppb in $CDCl_3$, 21 ± 1 ppb in benzene-d_6, and 20 ± ppb in pyridine-d_5.[18] The results suggest the isotope shift for C−O bonds is independent of the solvent and solely dependent upon the C−O bond order. A similar trend is seen in the isotope shifts for acetone[*carbonyl*-^{18}O] (50 ppb) and benzoic acid[*carbonyl*-^{18}O] (31 ppb) in both $CDCl_3$ and D_2O.[19] A very small (up to 4.2 ppb) dependence of the isotope shift on solvent is reported for 1-methylcyclohexanol, 1-cyclohexylethanol, and 1-octanol, which was attributed to variations in the ability of solvents to hydrogen bond to the alcohol, indirectly affecting the C−O bond.[20] No dependence of the isotope shift for *carbonyl*-^{18}O compounds was found.[20] These results show that the neutral compounds in these studies do not

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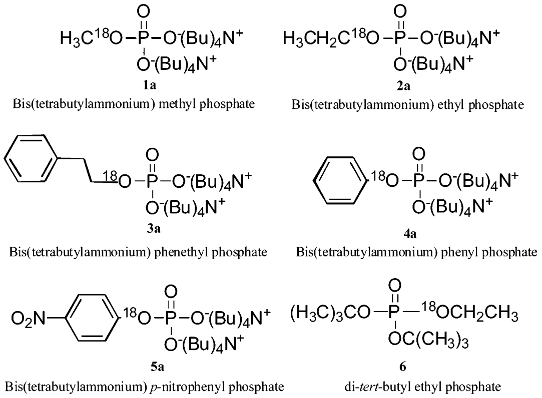

FIGURE 4. Bridge-labeled phosphate esters used in this study. All monoesters were used as their bis(tetrabutylammonium) salts.

undergo significant changes in bonding in the solvents studied. However, the phosphate esters in the present study are dianionic, with resonance-stabilized charges that could be affected by solvation more than the less polarized bonds of the carbon compounds.

Results and Discussion

We previously synthesized several alkyl (methyl, ethyl, and phenethyl) and aryl (phenyl and *p*-nitrophenyl) phosphate monoesters with a mixture of ^{16}O and ^{18}O at the ester position and measured the $\Delta\delta_p$ values for the bis(tetrabutylammonium) salt forms in water.[6] In this work, we have also included the triester di-*tert*-butyl ethyl phosphate with a mixture of ^{16}O and ^{18}O in the ethyl ester position and obtained $\Delta\delta_p$ values for all of the esters in the solvents water, DMSO, dioxane, acetonitrile, chloroform, and methanol. This solvent set covers a range of polarities, and includes protic and aprotic members. Figure 4 identifies each phosphate ester studied. The data for the bridge-labeled phosphate esters in the different solvents are shown in Table 4.

From the correlation shown in Figure 3, a solvent-induced change in bond order of 0.1 should result in a difference in $\Delta\delta_p$ of ~2.4 ppb; for a bond order change of 0.05, a $\Delta\delta_p$ difference of ~1.2ppb is expected. If the P−O bond is affected by solvation as much as the calculations predict, then compound **5a** should give a $\Delta\delta_p$ value in water that is about 9.5 ppb greater than that in DMSO. The magnitude of $\Delta\delta_p$ for **5a** in pure DMSO could not be obtained, but the values in water and 50% DMSO are very similar: in water $\Delta\delta_p = 19.8 \pm 0.4$ ppb and in 50% DMSO $\Delta\delta_p = 20.7 \pm 0.2$ ppb. The calculations lead to the expectation of a greater $\Delta\delta_p$ by about 2.2 ppb for phenyl phosphate (**4a**) in water than DMSO, but a difference of 1.2 in the opposite direction is observed. For methyl phosphate (**1a**) a $\Delta\delta_p$ in water that is ~1.0 ppb higher than DMSO is expected, but the small difference of 0.7 ppb that is observed is in the opposite direction and the values for ethyl phosphate are indistinguishable in water and DMSO. These data indicate that the calculations significantly overestimated the degree of bond order effects caused by the solvent change from water to DMSO.

In only a few cases are significant differences in isotope shifts observed from solvent to solvent. The $\Delta\delta_p$ values for ethyl phosphate (**2a**) in dioxane, acetonitrile, and chloroform range from 2.0 to 2.5 ppb higher than in water, DMSO, and methanol. No such differences in these solvents are noted for any of the other esters, including the triester (**6**), where the labeled ester group is also ethyl. A reasonable rationalization for the larger magnitude of $\Delta\delta_p$ for ethyl phosphate in these solvents is not evident. If the difference arose from a difference in the degree of anharmonicity in these solvents, then a similar trend should be seen in all of the esters, but this is not present in the data.

It is also noted that in chloroform, $\Delta\delta_p$ is reduced for the two aryl phosphates relative to other solvents. Phenyl phosphate (**4a**) has a $\Delta\delta_p$ value of 15.7 ppb in chloroform, about 3.5 ppb lower than the values in the other solvents; for *p*-nitrophenyl phosphate (**5a**), the reduction is smaller, about 2 ppb. This observation is consistent with a small weakening of the ester bond. These aryl phosphates might be more susceptible to this effect due to the more polarizable nature of the aryl oxide moiety compared to an alkoxide.

It is noted that the $\Delta\delta_p$ values for the aryl phosphates are consistently higher than those for the alkyl phosphates. The reason for this slight increase in $\Delta\delta_p$ is uncertain, but it has been speculated that it may be related to the conjugation of the oxygen with the delocalized phenyl ring, resulting in greater nuclear shielding by the heavier aromatic group.[9] Another possibility is a difference in the relative contribution of anharmonicity to the isotope shift in the phenyl esters compared to the alkyl esters. Since this difference is consistent across all of the solvents studied, it implies that isotope shifts are most reliably compared within similar groups such as within alkyl esters and aryl esters. The results suggest that there are no significant differences in the ester bond in the solvents examined in this study, with the exception of the aryl esters in chloroform, which are small.

To assess whether any change to the P−O bond in the nonbridging oxygen atoms occurs, $\Delta\delta_p$ values for

TABLE 4. **$\Delta\delta_p$ Values from ^{31}P NMR of Mixtures of ^{16}O-^{18}O Bridge-Labeled Phosphate Esters**

	$\Delta\delta_p$ (ppb)					
ester	deuterium oxide	DMSO	dioxane	acetonitrile	chloroform	methanol
1a	13.6 ± 0.2	12.9 ± 0.1	14.2 ± 0.1	14.4 ± 0.2	14.3 ± 0.1	14.1 ± 0.5
2a	14.4 ± 0.1	14.5 ± 0.1	17.0 ± 0.4	17.0 ± 0.2	16.3 ± 0.2	14.9 ± 0.9
3a	13.3 ± 0.4	N/A[b]	N/A[b]	N/A[b]	N/A[b]	N/A[b]
4a	19.1 ± 0.9	20.3 ± 1.3	21.4 ± 0.4	19.9 ± 1.1	15.7 ± 0.6	19.1 ± 0.6
5a	19.8 ± 0.4	20.7 ± 0.2[a]	N/A[b]	N/A[b]	18.2 ± 1.0	20.2 ± 0.9
6	N/A[b]	14.8 ± 0.1	14.3 ± 0.1	14.8 ± 0.1	15.6 ± 0.4	15.6 ± 0.1

[a] Value was obtained in 50% DMSO/50% H_2O; peaks were not resolvable in higher fractions of DMSO. [b] N/A: Resolution of the peaks could not be achieved.

TABLE 5. $\Delta\delta_p$ Values (per ^{18}O Atom) from ^{31}P NMR of Mixtures of ^{16}O and ^{18}O Nonbridge-Labeled Phosphate Esters[a]

	$\Delta\delta_p$ (ppb)					
ester	deuterium oxide	DMSO	dioxane	acetonitrile	chloroform	methanol
1b	24.9 ± 0.8	28.9 ± 0.7	27.5 ± 0.1	28.9 ± 0.3	25.1 ± 0.6	26.7 ± 0.9
2b	23.7 ± 0.4	24.8 ± 0.0	24.0 ± 0.1	24.2 ± 0.1	24.0 ± 0.1	28.5 ± 0.1
3b	23.8 ± 0.4	N/A[c]	N/A[c]	N/A[c]	N/A[c]	27.3 ± 0.7
7	23.5 ± 0.3	N/A[c]	N/A[c]	N/A[c]	N/A[c]	N/A[c]
4b	22.8 ± 0.2	23.4 ± 1.2	28.9 ± 0.9	28.2 ± 1.0	24.1 ± 0.6	24.0 ± 0.6
5b	20.7 ± 0.4	23.3 ± 0.4[b]	N/A[c]	22.7 ± 0.2	21.1 ± 0.1	22.2 ± 0.6
8	N/A[c]	41.0 ± 0.0	41.2 ± 0.2	41.2 ± 0.2	40.6 ± 0.2	39.4 ± 0.2
9	N/A[c]	41.1 ± 0.1	41.1 ± 0.5	41.0 ± 0.1	40.3 ± 0.2	38.7 ± 0.4

[a] In the monoesters, the isotope shift between the $^{16}O_3$- and the $^{18}O_3$-labeled species was obtained and divided by 3, to give the per atom $\Delta\delta_p$. [b] Value was obtained in 50% DMSO/50% H_2O; peaks were not resolvable in higher fractions of DMSO. [c] N/A: Resolution of the isotopomers could not be achieved. First four entries are alkyl esters, followed by aryl esters.

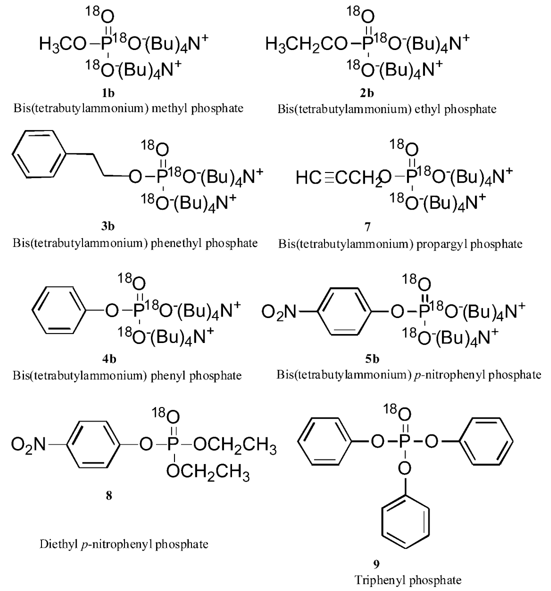

FIGURE 5. Nonbridge oxygen-labeled phosphate esters used in this study.

$^{16}O-^{18}O$ nonbridge-labeled phosphate esters were also measured in the same solvents. Figure 5 identifies each phosphate ester by number. The data for the nonbridge-labeled phosphate esters in each solvent are shown in Table 5.

If a loss of hydrogen bonding results in an increase in P−O nonbridging bond orders as discussed earlier, then a larger $\Delta\delta_p$ should result in aprotic solvents. This is observed in a few cases, but there is no consistent trend. Phenyl phosphate (**4b**) shows an increased $\Delta\delta_p$ in dioxane and acetonitrile, but not *p*-nitrophenyl phosphate (**5b**) (though a value in dioxane could not be obtained). The data in Table 4 show no accompanying changes to the P−OR ester bond in these solvents. In the case of **4b** in chloroform, where an indication of P−OR ester bond weakening relative to water was noted, the nonbridging $\Delta\delta_p$ is increased somewhat (24.1 ± 0.6 ppb versus 22.8 ± 0.2 ppb). However, this increase in the nonbridging $\Delta\delta_p$ is smaller than in DMSO and dioxane, where no accompanying reduction in the ester bond $\Delta\delta_p$ occurs.

Among the alkyl esters, relative to its value in water, methyl phosphate (**1b**), but not ethyl (**2b**), shows a larger $\Delta\delta_p$ in DMSO, dioxane, and acetonitrile. As the smallest ester, **1b** would permit the most facile solvation of the charged phosphoryl group. Comparisons with the bridging P−OR data in Table 4 show that any changes in the nonbridging P−O bonds of **1b** in DMSO, dioxane, and acetonitrile do not result in an observable effect on the ester bond.

The data do not show any significant effects from solvation in the phosphate esters examined in this study, nor any general or predictable trends in the small differences in isotope shifts in the tested solvents. This suggests that the small changes that are observed and noted above most likely result from idiosyncratic differences in the solvation shells around specific esters. The data do not support the notion that specific solvent properties, such as solvent polarity or hydrogen bond acceptor or donor ability, will result in significant, or easily predictable, differences in bonding in phosphate esters.

Conclusions

In summary, the data do not support a significant weakening of the ester bond upon transfer from water to a dipolar aprotic solvent. Nor do the data suggest any correlation between P−OR bond order and pK_a of the leaving group of the ester bond in any of the solvents. Any solvent-induced weakening of the ester bond is below the threshold of detection of the isotope shifts, and is, thus, probably too small to be a major contribution to the enhanced rate of hydrolysis observed in DMSO/water and acetonitrile/water mixtures. The differences in the isotope shifts in ^{31}P NMR in various solvents are greater than those reported for alcohols, and carbonyl compounds using C−O bonds with ^{13}C NMR, but the differences observed are small and follow no systematic trend. The results suggest that the increased rate of hydrolysis observed in the mixed solvents arises from some feature of the reaction other than weakening of the ester bond, possibly a difference in the relative stabilization of the reactant state and transition states in the different solvent systems. Experiments to assess this possibility are being explored.

Experimental Section

Phosphorus nuclear magnetic resonance (^{31}P NMR) spectra were recorded with a 400 MHz instrument operating at 161.9 MHz. All samples were ~20 mM in total phosphate and consisted of ~30−40% of the ^{18}O-labeled analogue. Five spectra were obtained for each sample, the isotope shifts obtained were averaged, and the standard deviations are reported. All ^{31}P NMR spectra were obtained with the following parameters: spectral width 8196.722 Hz, acquisition time 0.61 s, total number of data points 10000, number of scans 64, pulse delay of 8.00 μs, and pulse width of 60°.

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Supporting Information Available: Procedures for the synthesis of the phosphate esters and a sample ^{31}P spectrum (of nonbridge-O-labeled phenethyl phosphate). This material is available free of charge via the Internet at http://www.pubs.acs.org.

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