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Examination of P−OR Bridging Bond Orders in Phosphate Monoesters using ^{18}O Isotope Shifts in ^{31}P NMR

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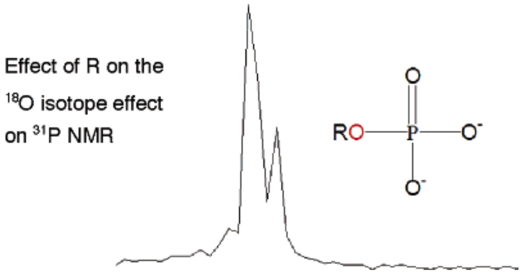

Evidence indicates that phosphate monoesters undergo hydrolysis by a loose transition state with extensive bond fission to the leaving group. It has been proposed that part of the high dependence of the rate on the leaving group pK_a ($\beta_{lg} = -1.23$) arises from weaker ester bonds in the reactants as the pK_a of the leaving group decreases, on the basis of X-ray structures and calculations. In contrast, IR and Raman studies suggest that the leaving group has little effect on the length of the P−OR bridging bond in solution. To gather additional data on this issue, we have used ^{18}O isotopic shifts in ^{31}P NMR to monitor the bond order of P−O bonds in a range of phosphate esters with different leaving groups. Using this technique, we have been able to evaluate whether significant changes are observed in the P−O bond orders for the bridging and nonbridging positions of methyl, ethyl, phenethyl, propargyl, phenyl, and *p*-nitrophenyl phosphate using [^{16}O^{18}O] labeled species in deuterium oxide. The results indicate that the bridging and nonbridging bond orders to phosphorus in phosphate monoesters are not significantly altered by differences in the pK_a of the leaving group or by the counterion of the phosphate ester dianion.

Introduction

Phosphoryl transfer reactions are ubiquitous in biochemistry. The hydrolysis of phosphate monoesters, most commonly tyrosine, serine, and threonine phosphates, by phosphatases is involved in cell signaling and regulation. Except for the acid phosphatases, 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 rates of hydrolysis reactions of phosphomonoester dianions are strongly affected by the pK_a of the leaving group, as reflected by the significant, negative Brønsted $\beta_{lg} = -1.23$.[2] The origin of this large leaving group dependence has been debated in the literature. The β_{eq} for this dephosphorylation of substituted phenyl monoesters is −1.36, as compared to $\beta_{eq} = -1.0$ for the deprotonation of phenols.[3] The larger substituent effect for the conversion of RO−PO$_3^{2-}$ (as well as for the analogous conversions of acyl, sulfuryl, and related esters) to RO$^-$ than for the conversion of RO−H to RO$^-$ is attributed to the greater electron-attracting character of the phosphoryl and related groups, which results in a partial positive charge on the ester oxygen atom not present in ROH.[3,4] It has been proposed that the basicity of the leaving group may also have an effect on the ground-state strength of the scissile P−OR bond, perhaps contributing to the large substituent effect on the reaction rate. A study based on the X-ray structures of several phosphomonoester dianions (Table 1) suggested that substituents that reduce the pK_a of the ester group do, in fact, result in a longer, weaker P−OR ester bonds.[5]

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TABLE 1. P-OR Bridging Bond Lengths from Crystal Structures of a Series of Phosphomonoester Dianions

ester group	pK_a of HOR	P−O(R) bond (Å)
p-nitrophenyl[a]	7.14	1.664[6]
phenyl[b]	9.95	1.64[7]
propargyl[a]	13.6	1.626[8]
methyl[c]	15.5	1.597[9]

[a] Bis(cyclohexylammonium) salt. [b] Dipotassium salt. [c] Diammonium salt.

In agreement with the trend[5] shown in Table 1, a computational study[10] comparing methyl, phenyl, and *p*-nitrophenyl phosphates found a significant lengthening of the bridging P−OR bond lengths as the pK_a of the ester decreased. In contrast to the solid state and computational results, a study using vibrational spectroscopy in solution found only a very small trend toward a longer ester bond with ester group pK_a.[11] That study used the nonbridging bond orders on the phosphoryl group to indirectly assess the bridging P−OR ester bond order; the sum of the P−O nonbridging bond lengths was inferred from their IR vibrations, and this sum was subtracted from 5.0 to yield the ester bond length.[11] Relevant to the assumption of the formal total bond order to phosphorus, computational studies have indicated that P−O pi bonds in phosphate esters have ionic character.[12−15]

We sought a different technique to assess whether P−OR ester bonds vary significantly with ester group pK_a. Toward this end, we have measured the magnitude of the ^{18}O-induced perturbation to the ^{31}P NMR chemical shift ($\Delta\delta_P$) in a series of phosphomonoesters to ascertain whether significant alterations to the nonbridging P−OR bond result from a change to the ester group.

Results and Discussion

In this study, phosphate esters were synthesized as a mixture of ^{16}O and ^{18}O isotopomers at the position of interest, either the bridging (ester) position or the nonbridging positions (See Figure 1). The ^{31}P NMR shows two (for labeling in the bridging position) to four (for labeling in the nonbridging positions) resonances, with the most highly ^{18}O substituted isotopic isomer giving rise to the most upfield signal. Figure 2 shows a typical ^{31}P spectrum for an ester with a mixture of ^{16}O and ^{18}O in the bridging position. The ^{18}O species gives rise to the more upfield resonance.

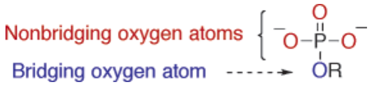

FIGURE 1. Diagram of the ^{16}O/^{18}O labeled positions of the phosphate monoesters studied.

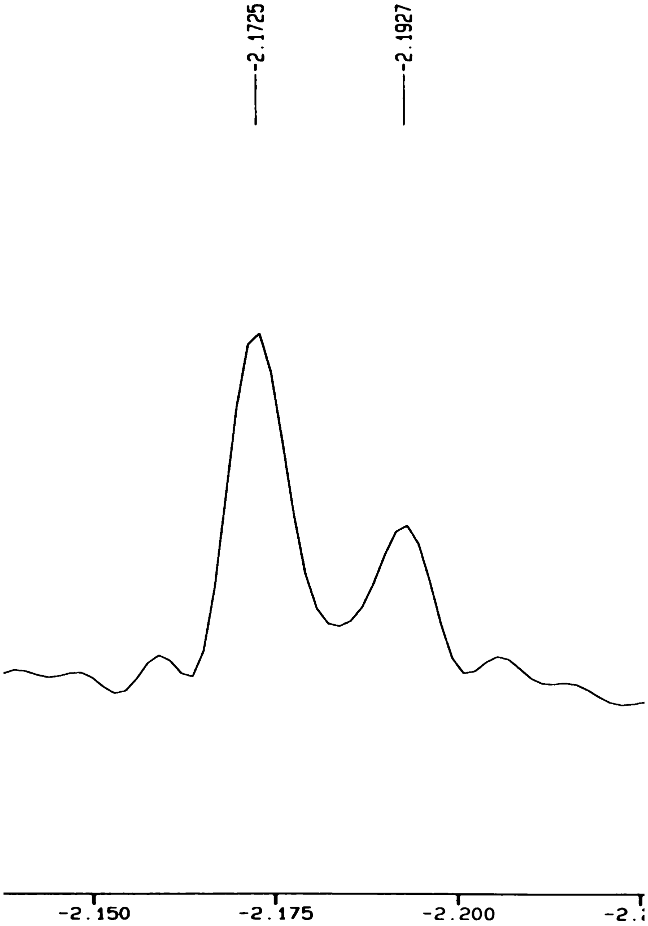

FIGURE 2. Sample ^{31}P NMR spectrum showing the resonances from the ^{16}O and ^{18}O isotopic isomers of a phosphate monoester labeled in the bridging position.

Such isotope shifts result from the combination of two major factors. One is greater nuclear shielding by the heavier nucleus, which is manifested to a greater degree for shorter, higher order bonds. The other major contributor to isotope shifts is anharmonicity, manifested in a shortened bond length arising from substitution by the heavier isotope.[16−18] The degree of anharmonicity can vary depending on molecular structure, making the relative contributions from these two factors variable, complicating the interpretation of comparative isotope shifts among different types of compounds. Isotope shift data have been compiled for a large number of compounds. The variability of the relative contribution of

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TABLE 2. Isotope Shifts for a Series of Phosphate Esters and Inorganic Phosphate[a]

compound, position of ^{18}O	formal P−O bond order	$\Delta\delta_P = \delta^{18}O - \delta^{16}O$ (ppb)
ATP, $\beta-\gamma$, bridging	1	16.5
ADP, $\alpha-\beta$, 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, nonbridging	2	40.0

[a] ADP and ATP isomers are from previously published data;[19,20] isotope shifts for inorganic phosphate and diethyl *p*-nitrophenyl phosphate were measured as part of this paper.

TABLE 3. $\Delta\delta_P$ Values from ^{31}P NMR of Mixtures of $^{16}O/^{18}O$ Bridge-Labeled Phosphate Monoester Dianions in D_2O[a]

ester	salt	ester pK_a	$\Delta\delta_P$ (ppb)
methyl-$^{18}O-PO_3^{2-}$	Na_2	15.5	14.1 ± 0.2
methyl-$^{18}O-PO_3^{2-}$	TBA_2	15.5	13.6 ± 0.2
ethyl-$^{18}O-PO_3^{2-}$	CHA_2	15.9	13.4
ethyl-$^{18}O-PO_3^{2-}$	Na_2	15.9	14.2 ± 0.1
ethyl-$^{18}O-PO_3^{2-}$	TBA_2	15.9	14.4 ± 0.1
phenethyl-$^{18}O-PO_3^{2-}$	Na_2	15.5	14.4 ± 0.1
phenethyl-$^{18}O-PO_3^{2-}$	CHA_2	15.5	14.5 ± 0.2
phenethyl-$^{18}O-PO_3^{2-}$	TBA_2	15.5	13.3 ± 0.4
phenyl-$^{18}O-PO_3^{2-}$	CHA_2	9.95	19.4 ± 1.4
phenyl-$^{18}O-PO_3^{2-}$	Na_2	9.95	16.7 ± 1.1
phenyl-$^{18}O-PO_3^{2-}$	TBA_2	9.95	19.1 ± 0.9
p-nitrophenyl-$^{18}O-PO_3^{2-}$	CHA_2	7.14	19.5 ± 0.7
p-nitrophenyl-$^{18}O-PO_3^{2-}$	Na_2	7.14	19.0 ± 0.5
p-nitrophenyl-$^{18}O-PO_3^{2-}$	TBA_2	7.14	19.8 ± 0.4

[a] TBA = tetrabutylammonium and CHA = cyclohexylammonium.

nuclear shielding and anharmonicity is one reason isotope shifts are difficult to correlate with any one molecular property across ranges of compounds. However, the cumulative data has led to the observation that, within the same or similar functional groups, the magnitude of the isotope shift is proportional to the bond order.[17,18] Published data for phosphate esters and anhydrides confirm that the magnitude of the ^{18}O-induced shift ($\Delta\delta_P$) is proportional to the P−O bond order; $\Delta\delta_P$ increases from ~0.016 ppm for P−O single bonds to ~0.041 ppm for double bonds (Table 2).[19,20] Figure 3 shows the correlation between $\Delta\delta_P$ (in ppb) and formal P−O bond order for a series of compounds. The data are plotted in two ways. At left (plot A) is the least-squares fit to the data; at right (panel B) is a fit that is forced to go through the origin. Plot A is a better fit of the data, although the failure to pass through the origin suggests that at very low bond orders, the relationship becomes nonlinear. Plot B meets the intuitive expectation that the magnitude of the isotope shift should become zero at zero bond order but gives an inferior fit to the experimental data. The two plots would yield slightly different numbers for the conversion of isotope shift to bond order; however, we do not propose to use isotope shift values to derive bond orders. Rather, we look for differences in isotope shift, as a function of the ester group, as an indicator as to whether significant differences in bond order are present. Either way the data are fitted in Figure 3, the empirical relationship between the two parameters is clear. The correlation between $\Delta\delta_P$ and the P−O bond order in phosphate esters 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.[21] ^{18}O-Isotope shifts have been used in a number of other studies as indicators of bond orders in phosphates[19,20,22] and phosphorothioates.[23−26]

These collective data indicate that, within this functional group, the relative contributions from anharmonicity and the distance dependence of nuclear shielding remain sufficiently constant that the magnitude of the isotope shift gives an indication of P−O bond order. Thus, a significant difference in bond order will be revealed by a change in isotope shift. Using the bridging P−OR bond length of 1.60 Å from the X-ray structure of diammonium methyl phosphate as an arbitrary starting point for a bond order of unity, Pauling's rule[27] gives a reduced bond order of 0.72 for the longer bond of 1.66 Å for bis-(cyclohexylammonium) *p*-nitrophenyl phosphate (Table 1). This should give rise to a reduction of ~6.7 ppb in the magnitude of $\Delta\delta_P$ between the two compounds. Thus, if the bridging bond orders of the esters in Table 1 vary in aqueous solution as much as is implied by their X-ray structures, then differences in $\Delta\delta_P$ should be easily observable.

We synthesized several alkyl (methyl, ethyl, and phenethyl) and two aryl (phenyl and *p*-nitrophenyl) phosphate monoesters with a mixture of ^{16}O and ^{18}O in the ester position and measured their $\Delta\delta_P$ values, with different counterions, in aqueous solution. These data are shown in Table 3.

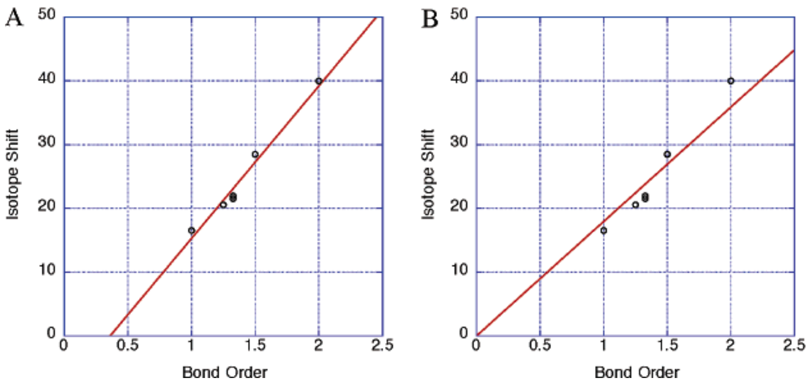

FIGURE 3. Graph of the isotope shift data in Table 2. The line in plot A represents a least-squares fit to the data and obeys the relation $\Delta\delta_P = (23.8)(\text{bond order}) - 8.4$, with $r^2 = 0.97$. The fit in plot B is forced through the origin and yields the equation $\Delta\delta_P = (17.9)(\text{bond order})$ with $r^2 = 0.91$.

It is emphasized that the present study is not an attempt to calculate bond orders from isotope shifts. Rather, the empirical relationship between bond order and $\Delta\delta_{\mathrm{P}}$ is used to examine whether the R moiety imparts significant differences in P−OR bond order in aqueous solution, as are implied by the computational results and seen in X-ray structures. The comparative values (bond order differences implied by differences in $\Delta\delta_{\mathrm{P}}$, or their absence) are the focus of this investigation. Using bond orders calculated from Pauling's rule and the X-ray data in Table 1, the magnitudes of $\Delta\delta_{\mathrm{P}}$ for phenyl and p-nitrophenyl phosphates should decrease by ~3.6 and ~5.4 ppb, respectively, relative to $\Delta\delta_{\mathrm{P}}$ for methyl phosphate.

The difference in $\Delta\delta_{\mathrm{P}}$ between the alkyl and the aryl esters is in the opposite direction expected if the bridging bonds of the aryl esters are longer and weaker than those of the alkyl esters. The larger $\Delta\delta_{\mathrm{P}}$ values for phenyl and p-nitrophenyl phosphate may be due to greater anharmonicity in the aryl esters relative to the alkyl esters. However, the magnitude of $\Delta\delta_{\mathrm{P}}$ is the same, within experimental error, for the cyclohexylammonium and the tetrabutylammonium salts of phenyl phosphate and p-nitrophenyl phosphate. This indicates that the P−OR ester bond is essentially unchanged in these two esters, despite the ~3 unit difference in pK_a between these two aryl ester groups, evidence that the basicity of the ester group does not significantly affect the bridging bond order in aqueous solution. The $\Delta\delta_{\mathrm{P}}$ for disodium phenyl phosphate of 16.7 ± 1.1 ppb lies just outside the experimental uncertainty of the values for other salts of phenyl phosphate and those of p-nitrophenyl phosphate.

We measured $\Delta\delta_{\mathrm{P}}$ values with three different counterions since X-ray structures of phosphate esters with different counterions have shown some variability in bridging P−OR bond lengths. X-ray structures reported after the original study[5] cited in Table 1 report an essentially identical P−OR bond length for pentakis(imidazole)copper(II) phenyl phosphate dianion[28] as for the potassium salt. However, a P−OR bond of 1.626 Å is found in disodium methyl phosphate,[29] in contrast to the earlier finding of 1.597 Å for the diammonium salt.[9] In our work, no significant differences were observed in $\Delta\delta_{\mathrm{P}}$ due to differences in the identity of the counterions. Clearly, then, the variations in bridging bond lengths as a function of ester group pK_a and counterions that occur in X-ray structures are not seen in the aqueous ^{31}P NMR studies reported here. The differences seen in the X-ray structures are likely due to effects manifested in the crystal packing environments, including differences in interactions of the phosphoryl group within the crystals of the solids, versus the solvated ions in solution.

TABLE 4. $\Delta\delta_{\mathrm{P}}$ Values from ^{31}P NMR of Mixtures of ^{16}O-^{18}O Nonbridge-Labeled Phosphate Monoester Dianions in D_2O[a]

ester	salt	$\Delta\delta_{\mathrm{P}}$ (ppb)
methyl-O-P^{18}O$_3^{2-}$	Na$_2$	23.6 ± 0.1
methyl-O-P^{18}O$_3^{2-}$	TBA$_2$	24.9 ± 0.8
ethyl-O-P^{18}O$_3^{2-}$	CHA$_2$	20.1 ± 1.2
ethyl-O-P^{18}O$_3^{2-}$	TBA$_2$	23.7 ± 0.4
phenethyl-O-P^{18}O$_3^{2-}$	CHA$_2$	23.2 ± 0.1
phenethyl-O-P^{18}O$_3^{2-}$	Na$_2$	23.8 ± 0.4
phenethyl-O-P^{18}O$_3^{2-}$	TBA$_2$	23.8 ± 0.4
propargyl-O-P^{18}O$_3^{2-}$	CHA$_2$	23.6 ± 0.1
propargyl-O-P^{18}O$_3^{2-}$	Na$_2$	23.5 ± 0.5
propargyl-O-P^{18}O$_3^{2-}$	TBA$_2$	23.5 ± 0.3
phenyl-O-P^{18}O$_3^{2-}$	CHA$_2$	23.3 ± 0.2
phenyl-O-P^{18}O$_3^{2-}$	Na$_2$	23.3 ± 0.4
phenyl-O-P^{18}O$_3^{2-}$	TBA$_2$	22.8 ± 0.2
p-nitrophenyl-O-P^{18}O$_3^{2-}$	CHA$_2$	23.5 ± 0.1
p-nitrophenyl-O-P^{18}O$_3^{2-}$	Na$_2$	23.5 ± 0.2
p-nitrophenyl-O-P^{18}O$_3^{2-}$	TBA$_2$	20.7 ± 0.4

[a] TBA=tetrabutylammonium, CHA=cyclohexylammonium.

We also measured the ^{13}C−^{31}P coupling constants for methyl, phenyl, and p-nitrophenyl phosphates. This two-bond coupling constant reflects the P−O−C interaction, not solely the P−O bond. Previously published data show that two-bond phosphorus−carbon coupling constants depend on the hybridization of the carbon atom, with sp^2 carbons exhibiting slightly higher coupling constants than sp^3 carbon atoms.[30,31] We obtained similar results; the P−O−C coupling constant is 4.8 Hz in methyl phosphate, 6.0 Hz in phenyl phosphate, and 5.8 Hz in p-nitrophenyl phosphate. The differences between these coupling constants for alkyl versus aryl phosphate esters may be explained by the aid of Jameson's s-character theory.[32] This theory holds that as a phosphorus atom is bound to more electronegative atoms, or groups of atoms, the coupling constant becomes increasingly more positive. Since hybridization makes the aryl ester group slightly more electronegative than the alkyl ester group, the coupling constants for aryl phosphate esters are predicted to be slightly larger. The close similarity of the two-bond coupling constants for the two aryl esters further suggests that no significant difference in the P−O bond order exists between these two esters in aqueous solution.

To assess whether the identity of the ester group affects the nonbridge P−O bond orders, these isotope shifts were measured as well and are shown in Table 4. This table includes propargyl phosphate, which is absent from Table 3 because we were not able to synthesize bridge-^{18}O-propargyl phosphate.

The data in Table 4 demonstrate that none of the esters has significantly different nonbridge P−O bonds from one another, and none is significantly different from the formal P−O bond order of 1.33. Nor, at least in water, does the identity of the counterion result in a consequential difference in bond order. The magnitudes of the nonbridge P−O isotope shifts for phenyl phosphate and pNPP are the same as those for the other esters, despite

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the somewhat larger isotope shifts seen in the bridging P−O bond with these esters. The absence of any significant variation in $\Delta\delta_P$ among the three alkyl esters demonstrates that steric effects that might affect solvation of the ester group do not have an effect on the bond orders of the bridging or nonbridging P−O bonds.

Conclusions

In summary, the NMR data do not support a significant weakening of the bridging P−OR bond order in aqueous solution with decreasing leaving group pK_a, and this conclusion is consistent with the very small differences inferred by a previous vibrational spectroscopy study.[11] The identity of the counterion also makes no significant difference in the magnitudes of the isotope shifts and, by implication, of either the bridging or the nonbridging bond orders for the phosphate esters examined. The variations in the X-ray data of esters with different counterions, and with different ester groups, may be a result of local medium effects resulting from close contacts in the crystal environment, as opposed to the solvation shell in water. The differences present in the crystal structures do not reflect the situation in aqueous solution. The significant differences predicted by the computational study most likely reflect shortcomings in the modeling of the effects of solvation, and the failure to observe such differences in aqueous solution highlights the importance of this consideration when modeling phosphate esters.

The Brønsted dependency of the hydrolysis of phosphate monoester dianions arises from substituent-influenced changes to the relative energies of the ground state and transition state along the reaction coordinate. Differences between ground state and transition state electrostatic interactions with solvent will be a significant contribution to these energies. In the transition state, since a significant negative charge is present on the leaving group, less basic ester groups should favor greater charge dispersion. As a result, the solvation of the transition state, and hence its energy, could be affected. Electrostatic interactions with the solvent that affect the ground-state energy might also change with ester group basicity, but the results reported here, and the vibrational spectroscopy study,[11] indicate that the bond order of the ester bond is not affected. These results do not provide an explanation for the large leaving group dependency, but rule out a substituent-induced weakening of the scissile P−O bond.

Experimental Procedures

All of the phosphate esters used in this study are known compounds and were synthesized by standard methods. The synthetic procedures used to prepare the isotopic isomers of the phosphate esters, and the spectral characterizations, are reported in the Supporting Information. Phosphorus nuclear magnetic resonance (^{31}P NMR) spectra were recorded with a 400 MHz spectrometer equipped with a 52-mm bore magnet. The isotope effects on the chemical shifts in Tables 3 and 4 are reported in delta (δ) units, parts per billion (ppb), as the difference between the ^{16}O and ^{18}O isotopic isomers. All samples were brought up to pH 9−10 with the appropriate base. All were ~20 mM in the phosphate ester and had ~30−40% incorporation of the ^{18}O label. For each isotopically labeled ester, five independent spectra were obtained, the measured isotope shifts were averaged, and the standard errors were calculated. All ^{31}P NMR spectra were obtained with the following parameters: spectral width 8196.722 Hz, acquisition time 0.61 s, 10 000 data points, 64 scans, pulse delay of 8.00 μs, and a pulse width of 60°.

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Supporting Information Available: Synthetic procedures used for the preparation of bridge-^{18}O and nonbridge-^{18}O-labeled phosphate esters in Tables 3 and 4 and their spectral characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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