Studies of Transition-State Structures in Phosphoryl Transfer Reactions of Phosphodiesters of *p*-Nitrophenol

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Abstract: Heavy-atom kinetic isotope effects have been used to study the transition states for a number of phosphoryl transfer reactions of the phosphodiesters *p-tert*-butylphenyl *p*-nitrophenyl phosphate (**1**) and 3,3-dimethylbutyl *p*-nitrophenyl phosphate (**2**). The alkaline and acid hydrolysis reactions and the reaction with phosphodiesterase I from snake venom were studied with each substrate. In addition, the hydrolysis reactions of **1** catalyzed by bis-(imidazolyl)- and by mono(imidazolyl)-β-cyclodextrins were studied. The isotope effects measured were the primary ^{18}O isotope effect in the *p*-nitrophenyl leaving group, the secondary ^{18}O isotope effect in the nonbridge oxygen atoms, and the ^{15}N isotope effect in the leaving group. The data indicate similar early transition-state structures for the aqueous hydrolysis reactions of the two compounds with little bond cleavage to the leaving group. In contrast, significant differences in transition-state structure between the substrates are seen in their reactions with phosphodiesterase I. Compound **1** is a substrate for the imidazolyl-β-cyclodextrin catalysts, which operate as simple general base catalysts for this substrate. Transition-state bond cleavage to the leaving group is much further advanced in these reactions than in the uncatalyzed aqueous reactions.

Introduction

Phosphodiesters are the most stable phosphate esters, and their chemistry has great relevance to biological systems. Their hydrolysis reactions are bimolecular, with activation entropies of −25 eu, and Brønsted studies with diaryl phosphates give a value of $\beta_{lg} = -0.97$.[1] The question of whether these reactions proceed in two steps by way of a stable pentacovalent phosphorane intermediate, described in the IUPAC system[2] as $A_N + D_N$, or via a concerted A_ND_N (or $S_N2(P)$) process with a transition state resembling a phosphorane, is unsettled. Previous studies indicate that the nucleophilic displacement of aryl leaving groups from phosphate diesters and triesters is a concerted A_ND_N process,[3] although pentacoordinate phosphoranes have been shown to exist as intermediates in the transfer reactions of phosphoryl groups between strong nucleophiles, particularly with cyclic phosphates, and in acid-catalyzed phosphoryl transfer reactions.[4]

Heavy-atom isotope effects have been used to study the transition-state structures for a number of reactions of phosphate esters.[3b,c,5] The secondary oxygen-18 isotope effects in the nonbridge oxygen atoms, $^{18}k_{nonbridge}$, reflect changes in bond order between these atoms and the phosphorus atom. If the transition state resembles a phosphorane where these are single bonds, then the loss of bond order relative to the starting material, where the nonbridge phosphorus−oxygen bonds are intermediate between single and double bonds, should give rise to a normal $^{18}k_{nonbridge}$ isotope effect. The primary oxygen-18 isotope effect in the leaving group oxygen atom, $^{18}k_{lg}$, depends on the extent of bond cleavage to the leaving group in the transition state. With the *p*-nitrophenyl leaving group, bond fission is accompanied by a nitrogen-15 isotope effect, ^{15}k, in the nitrogen atom of the nitro group. We have previously shown that this effect gives an indication of the degree of bond cleavage to the leaving group by measuring charge delocalization into the aromatic ring.[6]

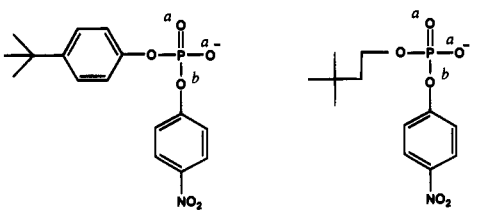

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Figure 1. Phosphodiesters studied in this work, showing the positions of oxygen isotope effect measurements. (a) Nonbridge oxygen atoms; site of $^{18}k_{nonbridge}$ isotope effects. (b) Leaving group oxygen atoms; site of $^{18}k_{lg}$ isotope effects. Isotope effects were also measured at the nitrogen atoms of both compounds.

This paper reports the ^{18}O and ^{15}N isotope effects on several phosphoryl transfer reactions of *p-tert*-butylphenyl *p*-nitrophenyl phosphate (**1**) (Figure 1). The described set of isotope effects has been measured for the alkaline and the acid hydrolysis reactions of **1**, for its hydrolysis by the enzyme phosphodiesterase I, and for the hydrolysis reactions catalyzed by three imidazolyl-β-cyclodextrin species. This particular diester was chosen for study because it is accepted as a substrate by the

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Table 1. Isotope Effect Data for Phosphodiesters **1** and **2**

reaction (t, °C)	^{15}k	$^{18}k_{lg}$	$^{18}k_{nonbridge}$[a]
	Diester **1**		
alkaline hydrolysis (70)	1.0010 ± 0.0001	1.0046 ± 0.0008	1.0040 ± 0.0001
acid hydrolysis (95)	1.0007 ± 0.0001	1.0058 ± 0.0005	0.9926 ± 0.0002[b] 1.0088 ± 0.0002[c]
phosphodiesterase (25)	1.0016 ± 0.0002	1.0150 ± 0.0005	0.9972 ± 0.0005
mono(imidazolyl)-β-cyclodextrin(70)	1.0007 ± 0.0001	1.0284 ± 0.0004	1.0040 ± 0.0001
bis(imidazolyl-β-cyclodextrin A,B (70)	1.0016 ± 0.0001	1.0243 ± 0.0007	1.0028 ± 0.0001
bis(imidazolyl-β-cyclodextrin A,C (70)	1.0016 ± 0.0002	1.0343 ± 0.0007	1.0056 ± 0.0001
	Diester **2**[d]		
alkaline hydrolysis (95)	1.0016 ± 0.0002	1.0059 ± 0.0005	0.9949 ± 0.0006
t corrected to 70 °C[e]	1.0017 ± 0.0002	1.0063 ± 0.0005	0.9945 ± 0.0006
acid hydrolysis (95)	1.0009 ± 0.0002	1.0039 ± 0.0004	1.0139 ± 0.0004[c]
phosphodiesterase (37)	1.0017 ± 0.0002	1.0073 ± 0.0008	0.9842 ± 0.0006

[a] Measured effects with both nonbridge oxygen atoms labeled. [b] Observed effect. [c] Corrected for protonation; see text for details. [d] The ^{15}k and $^{18}k_{nonbridge}$ isotope effects for this compound are from ref 3b; the $^{18}k_{lg}$ isotope effects are from this work. [e] An approximation of the temperature effect on the isotope effect was made using the equation ln(IE at 70 °C) = (368 K/343 K) ln(IE at 95 °C).

β-cyclodextrin catalysts. Breslow has described the use of bis(imidazolyl)-β-cyclodextrin compounds as catalysts for the hydrolysis of 4-*tert*-butylcatechol cyclic phosphate.[7] The reactions with the latter substrate exhibited bell-shaped profiles of the rates of hydrolysis versus pH and have been discussed as models for the reaction of the enzyme ribonuclease.[7] We studied the reactions of **1** with mono(imidazolyl)-β-cyclodextrin and with the A,B and A,C isomers of bis(imidazolyl)-β-cyclodextrin. The letters refer to which of the seven glucose rings which form β-cyclodextrin, designated A–G, bear the catalytic imidazole groups.

In a previous study, we measured the $^{18}k_{nonbridge}$ and ^{15}k isotope effects in the compound 3,3-dimethyl *p*-nitrophenyl phosphate (**2**) (Figure 1) for a variety of phosphoryl transfer reactions.[3b] In conjunction with the present study, we have measured the $^{18}k_{lg}$ isotope effects in this compound for the alkaline and acid hydrolysis reactions and for the reaction with snake venom phosphodiesterase to allow comparisons of the full set of isotope effects between the two phosphodiesters for these reactions.

Results

The isotope effect data for *p-tert*-butylphenyl *p*-nitrophenyl phosphate (**1**) are given in the upper part of Table 1, and the data for 3,3-dimethylbutyl *p*-nitrophenyl phosphate (**2**) are in the lower portion of the table. The data for the imidazolyl-β-cyclodextrin-catalyzed reactions are all for reactions with compound **1**.

For hydrolysis reactions of diesters under acidic conditions, pH studies indicate that the protonated, neutral diester is the reactive species.[9] Because of the low pK_a of diester **1**, the acid hydrolysis reaction could not be run under conditions where the diester was completely protonated. Therefore, the observed $^{18}k_{nonbridge}$ isotope effect will have a component from the isotope effect for protonation to form the reactive neutral species. In order to extract the isotope effect on the phosphoryl transfer step, the observed effect must be corrected for protonation as previously described in a similar study of diester **2**.[3b] This correction requires knowledge of the value of the pK_a of **1**. The inductive effect of the second aryl group leads to the expectation that the pK_a of **1** should be even lower than the −0.35 value determined previously for the 3,3-dimethylbutyl compound **2**.[3b] Measurements of the change in the ^{31}P chemical shift of **1** as it was titrated with acid as described in the Experimental Section indicate the pK_a of **1** to be less than −1.2. Measurements of the isotope effects were performed in 2 M sulfuric acid; under those conditions, at least 90% of the diester will remain as the anion. Therefore, essentially the full equilibrium isotope effect for protonation will be expressed, and division of the observed $^{18}k_{nonbridge}$ isotope effect by this value, 0.984,[10] yields $^{18}k_{nonbridge}$ for the hydrolysis reaction. Both the observed and the corrected values are shown in Table 1.

In our previous studies with compound **2**, experiments in [^{18}O]water determined that no exchange occurs between the phosphoryl oxygen atoms and solvent during alkaline or acid hydrolysis. In addition, it was shown that both reactions proceed exclusively by P–O bond cleavage.[3b] These studies were not repeated with diester **1** since, given the close similarity between phosphodiesters **1** and **2**, we feel it is safe to assume that the same mechanisms will be followed.

In our search for viable substrates for the bis(imidazolyl)-β-cyclodextrins, a series of phosphodiesters having *p*-nitrophenol as the leaving group were tried as substrates. The second ester groups were methyl, 2,2-dimethylpropyl, 3,3-dimethylbutyl, 4-methylpentyl, phenyl, *p-tert*-butylphenyl, and *m-tert*-butylphenyl. Only the *p-tert*-butylphenyl compound **1** was hydrolyzed by the bis(imidazolyl)-β-cyclodextrins. The pH–rate profiles of this substrate measured with the A,C and A,B bis(imidazolyl)-β-cyclodextrins) and with mono(imidazolyl)-β-cyclodextrin are shown in Figure 2. In order to test the bis(imidazolyl)-β-cyclodextrin catalysts with a phosphodiester having a less labile leaving group, the compound *p-tert*-butylphenyl phenyl phosphate was tried as a substrate at several pH values between 5 and 9. No measurable reaction above the slow background hydrolysis was observed.

The cyclodextrin-catalyzed reactions and the alkaline hydrolysis of substrate **1** were carried out at 70 °C, and the other solution hydrolyses of **1** and **2** were carried out at 90 °C. The effect of this temperature difference on the isotope effects is negligible. An estimate of the correction to the isotope effects for the alkaline hydrolysis reaction of **2** was made in Table 1, using the equation ln(IE at 70 °C) = (368 K/343 K) ln(IE at 95 °C). This probably overestimates the magnitude of the corrections, but they are nonetheless smaller than the experimental errors in the measurements. Thus the isotope effects for the

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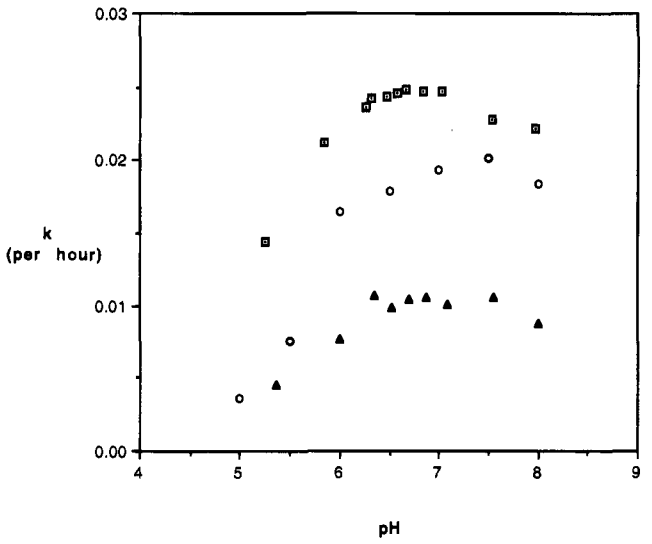


Figure 2. Rate constants at 70 °C for the hydrolysis of **1** (1 mM) in the presence of 5 mM A,C (□) and A,B (▲) bis(imidazolyl)-β-cyclodextrins and mono(imidazolyl)-β-cyclodextrin (○) with 100 mM buffer (acetate or phosphate) and 200 mM KCl.

nonenzymatic reactions can be directly compared with one another, ignoring the temperature differences.

Discussion

Kinetic isotope effects can give detailed quantitative information about transition-state structure. Measurements of $^{18}k_{lg}$, $^{18}k_{nonbridge}$, and ^{15}k have been reported for several phosphoryl transfer reactions involving phosphate esters of *p*-nitrophenol.[3b,c,5,6] These data can give information about the transition-state bonding to the leaving group and within the central phosphoryl group which is not obtainable by other methods.

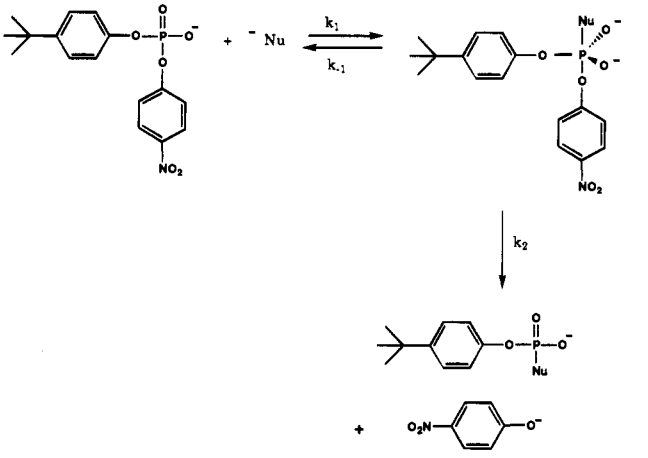
The correct interpretation of isotope effects in terms of bonding changes upon going from starting material to transition state depends on the proper choice of yardstick for comparison with the measured isotope effects. Changes in bonding to the *p*-nitrophenyl leaving group are measured by $^{18}k_{lg}$ and ^{15}k. These isotope effects are complicated by the delocalization of charge in the transition state into the aromatic ring, which gives rise to the ^{15}k isotope effect in the nitro group and lowers the magnitude of the $^{18}k_{lg}$ isotope effect.[5d,11] Relevant isotope effects for comparison are the corresponding equilibrium isotope effects on the deprotonation of *p*-nitrophenol, $^{18}K_{eq} = 1.0153 \pm 0.0002$[11] and $^{15}K_{eq} = 1.0023 \pm 0.0001$.[6] However, deprotonation involves breaking a bond to a proton, not to a phosphoryl group. One indication that these are not equivalent is the fact that an aryl group in a phosphodiester has an effective charge relative to the neutral phenol of +0.73 as measured from Bønsted correlations, reflecting the greater electron withdrawing character of the phosphoryl group relative to that of a proton.[12] This value is nearly identical to that of an aryl group in an acetate ester, +0.7.[13] These positive values reflect electron donation from the leaving group oxygen atom toward the phosphoryl (or carboxyl) group, which increases the P–O or C–O bond order in the ester ground states.

These similar Brønsted β values are indicative of similar electronic ground states of the leaving group in the two types of compounds. Therefore, the leaving group isotope effects for *p*-nitrophenyl acetate provide a reasonable standard for comparison with those of *p*-nitrophenyl phosphodiesters **1** and **2**.

Isotope effects have been reported for a series of reactions involving nucleophilic displacement of *p*-nitrophenol from *p*-nitrophenyl acetate and for the equilibrium between *p*-nitrophenolate and *p*-nitrophenyl acetate.[11] The equilibrium isotope effects in the *p*-nitrophenyl group were found to be $^{18}K_{eq} = 1.0277 \pm 0.0007$ and $^{15}K_{eq} = 1.0016 \pm 0.0006$. The largest kinetic isotope effects found in the leaving group were $^{18}k = 1.0330$ and $^{15}k = 1.0011$, for the reaction with methoxyethylamine at 25 °C.[11] Since the ^{18}k kinetic isotope effect is a primary one, it will have contributions from reaction coordinate motion and thus can be expected to exceed the equilibrium value if this bond is significantly broken in the transition state.

On these grounds, we expect the $^{18}k_{lg}$ isotope effect to have a maximum of about 1.03 for reactions of **1** and **2** if the bond to the leaving group is nearly completely broken in the transition state, and an upper limit for ^{15}k in the range 1.0011–1.0016.

The nucleophiles in the reactions of phosphodiesters **1** and **2** examined here each have pK_a values sufficiently greater than that of the *p*-nitrophenol leaving group so that nucleophilic attack should be rate-limiting, whether or not it is coupled with departure of the leaving group. This is because if the two-step $A_N + D_N$ mechanism is followed, the phosphorane intermediate that will result (eq 1, below) will partition essentially completely forward ($k_2 \gg k_{-1}$). In this case, only the first step of the



mechanism will exhibit isotope effects, and the largest isotope effects should then be observed in the nonbridge oxygen atoms. If instead a concerted A_ND_N mechanism is followed, then significant bond cleavage to the leaving group should be present in the transition state and will be reflected in significant isotope effects in the leaving group and small or negligible values for $^{18}k_{nonbridge}$ depending on the degree of associative character of the transition state. The postulated phosphorane intermediate depicted in eq 1 shows the *p*-nitrophenol leaving group in the apical position needed for departure. This need not be the case, and if a phosphorane intermediate is formed with the leaving group equatorial, pseudorotation must occur to place the *p*-nitrophenol group in the apical position before it can depart. In the unlikely event that such a pseudorotation step were sufficiently slow to be rate-limiting, then the isotope effects expressed would be those on the equilibrium formation of the phosphorane intermediate. This would be reflected in near unity values for the leaving group isotope effects ^{15}k and $^{18}k_{lg}$ and a significant normal value for $^{18}k_{nonbridge}$.

The phosphodiesters **1** and **2** possess the same leaving group but differ in the nature of the second ester group. The inductive effect of the second aryl group in compound **1** makes it the

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more reactive of the two diesters; at pH 13, the hydrolysis of **1** is nearly 4 times faster than that of **2**.

Solution Reactions. For the alkaline hydrolysis reactions, the isotope effects in the leaving group, $^{18}k_{lg}$ and ^{15}k, are somewhat larger for diester **2** than for **1**, indicating that at the transition state, bond cleavage to the leaving group is more advanced for **2**. The $^{18}k_{nonbridge}$ isotope effect is slightly inverse for compound **2** and is small but normal for the reaction with **1**. This indicates that the bond orders of the nonbridge oxygen atoms to phosphorus are slightly increased on going from the ground state to the transition state for the alkaline hydrolysis of **2** but are slightly decreased in the reaction with the diester **1**. This is indicative of a more associative transition-state structure for attack of hydroxide on diester **1**, with the bond order to the nonbridge oxygen atoms slightly weakened and bond cleavage to the leaving group less advanced. Interestingly, this picture is reversed in the acid hydrolysis reaction. Though the ^{15}k effects are the same within experimental error, $^{18}k_{lg}$ is somewhat larger for diester **1**. The $^{18}k_{nonbridge}$ isotope effect is normal for both compounds but is largest for **2**, indicating a significant reduction in bond order between phosphorus and the nonbridge oxygen atoms in the transition state. The data indicate a more associative transition-state structure of **2** in the acid hydrolysis reaction.

The magnitudes of $^{18}k_{lg}$ are small for the solution reactions of both compounds **1** and **2**, indicating that although differences between them are apparent, bond cleavage to the leaving group in the transition state is not very far advanced in any of these reactions.

Phosphodiesterase Reactions. Phosphodiesterase I hydrolyzes phosphodiesters by a ping-pong mechanism with a phosphorylated threonine intermediate.[14a] The active site contains a zinc atom which is necessary for catalysis, and the enzyme is activated by magnesium.[14b] Both diesters **1** and **2** are substrates for the enzyme, and the K_m for **1** was found to be 12 mM at the pH optimum of 8.0, close to that previously found for **2** of 10.5 mM.[3b] The pH−rate profile of V/K with phosphodiester **2** is bell-shaped with a broad optimum, with pK_a values of 6.3 and 9.6 indicated for the active site general base and acid, respectively.[3b]

A previous study of the enzymatic reaction with diester **2** measured only ^{15}k and $^{18}k_{nonbridge}$. The magnitude of ^{15}k indicated that bond cleavage to the leaving group was occurring in the transition state.[3b] The value for $^{18}k_{lg}$ measured in this study supports that conclusion. The large inverse value for $^{18}k_{nonbridge}$, close to the value of the 1.6% inverse equilibrium ^{18}O isotope effect for protonation of a phosphate ester, suggests that the phosphoryl group of **2** is activated by protonation in the transition state for the reaction.[3b]

There are significant differences between the isotope effect data for the enzymatic reaction of diester **1** compared with that for **2**. Both of the leaving group isotope effects are approximately twice as large with substrate **1**, and the value for $^{18}k_{nonbridge}$, while inverse, is considerably closer to unity for **1** than for substrate **2**. This unexpected result led us to perform several experiments to confirm that we were observing the same enzymatic reaction with the two substrates. As described in detail in the Experimental Section, an activity stain run on a native gel electrophoresis using both substrates showed that although the enzyme was contaminated with lower molecular weight proteins, the same protein band was responsible for hydrolyzing both substrates. Inhibition by EDTA[14b] abolished the turnover of both substrates, ruling out any non-active site reaction of either substrate.

The isotope effect data indicate the presence of significant bond cleavage to the leaving group in the transition states of both reactions, although to a greater extent with **1**, and with much less complete proton transfer to the nonbridge oxygen in the case of substrate **1**. The reason for this difference in transition-state structure probably arises from the difference in pK_a values of the nonbridge oxygen atoms of the two diesters. For diester **2**, this oxygen has a p$K_a = -0.35 \pm 0.05$.[3b] The pK_a of diester **1** was too low to be determined (see Experimental Section) but is more negative than −1.2. This difference arises from the change of the alkyl ester group in **2** to an aryl one in **1**. The pK_a values of phenyl phosphate (0.48 and 5.70)[12] are each depressed by about 1.2 units relative to those of *n*-butyl phosphate (1.80 and 6.84), and if similar effects hold for the diesters, then the pK_a of **1** can be estimated to lie in the neighborhood of −1.5. The transition state for nucleophilic attack by the active site threonine will resemble a phosphorane. The pK_a values of the nonbridge oxygen atoms will be significantly raised in the transition state to the extent that phosphorane geometry is achieved, as phosphoranes have much higher pK_a values than their corresponding diesters.[15] Theoretical estimates of the first pK_a of a trialkyl phosphorane range from 6.5[15a] to 9,[15b] but recent experimental evidence indicates a higher value of 11.4.[15c] Using the highest and lowest of these figures as extremes, if the inductive effects of a *p*-nitrophenyl and a *p-tert*-butylphenyl group in a phosphorane are similar to those observed in phosphates, then the phosphorane arising from diester **1** should have a pK_a between 3.6 and 8.5, and the phosphorane arising from **2** between 4.8 and 9.7.[16] Thus the transition-state structures will have pK_a values somewhere between these values and those of −1.5 and −0.3 for the respective diester substrates. Although this is a large range of uncertainty, the phosphorane transition-state pK_a values will be in the range where protonation by an enzymatic general acid is feasible. Whether the transition state bears more resemblance to a phosphorane or to the ground-state phosphodiester will depend upon whether the transition state is early or late. The difference between the pK_a values for the two structures may explain the $^{18}k_{nonbridge}$ isotope effect data. The large inverse value for **2** indicates essentially full proton transfer to the nonbridge oxygen in the transition state, whereas the much smaller inverse value for the less basic **1** suggests only partial protonation.

The magnitudes of ^{15}k and $^{18}k_{lg}$ indicate that the bond to the leaving group is being broken in the transition states of both reactions, with a similar degree of charge arising from bond cleavage already delocalized into the nitrophenyl groups. The overall picture is that of a concerted reaction with an associative transition state resembling a phosphorane.[17]

Imidazolylcyclodextrin Reactions. Anslyn and Breslow have described the use of bis(imidazolyl)-β-cyclodextrin compounds as catalysts for the hydrolysis of 4-*tert*-butylcatechol cyclic phosphate.[7b] The isomers of these catalytic compounds are designated by lettering A−G the seven glucose rings that constitute β-cyclodextrin. The isomer bearing the catalytic imidazole groups on adjacent glucose rings is designate as A,B.

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(16) The pK_a values for *p*-nitrophenyl phosphate are depressed an average of 1.7 units, and those of phenyl phosphate 1.2 units, relative to those of butyl phosphate. The estimated values for the phosphorane arising from **1** were obtained by subtracting 2.9, and for **2** by subtracting 1.7, from the high and low values of 11.4 and 6.5 for a trialkyl phosphorane.

The imidazole groups are successively farther apart around the ring of the β-cyclodextrin cavity in the A,C and A,D isomers.

The A,B, A,C, and A,D isomers show a bell-shaped pH–rate profile with 4-*tert*-butylcatechol cyclic phosphate, indicating bifunctional acid–base catalysis.[7b] The A,B isomer was the best catalyst of the three, and geometrical considerations led to the proposal that it functions by a mechanism involving protonation of the phosphoryl anion and general base-assisted attack of water to form an intermediate phosphorane monoanion.[7b] In contrast, the A,D isomer has its catalytic groups positioned nearly on opposite sides of the cavity, and thus positioned in a manner to facilitate protonation of the leaving group as nucleophilic attack occurs, suggesting a concerted mechanism. The A,C isomer could fit either mechanism. Using the same 4-*tert*-butylcatechol cyclic phosphate substrate with the A,B and A,C isomers of bis(imidazolyl)-β-cyclodextrin, we obtained kinetic results similar to those of Anslyn and Breslow.

We were unable to devise a cyclic phosphate substrate which could be remote labeled for our isotope effect studies, so we tested a variety of acyclic diesters bearing the *p*-nitrophenyl leaving group. Of those tested, only the *p*-*tert*-butylphenyl derivative **1** showed activity with the bis(imidazolyl)-β-cyclodextrin catalysts, presumably because only this substrate correctly positions the phosphoryl group with respect to the catalytic imidazole moieties. The hydrolysis of five-membered cyclic phosphate esters is millions of times more rapid than that of corresponding acyclic esters,[18] and as expected the catalyzed hydrolyses of **1** proceed much more slowly than those of the cyclic catechol substrate in spite of the better leaving group in **1**.

Reactions of the A,B and A,C isomers with **1** were run at 70 °C in order to shorten reaction times, but both isomers were quite effective catalysts. Control reactions without the catalyst, or containing β-cyclodextrin instead of the catalysts, showed negligible hydrolysis. The pH–rate profiles (Figure 2) are not bell-shaped, however, and indicate that no appreciable general acid catalysis occurs with this substrate and that each catalyst acts only as a general base.[19] The A,C isomer is about 2.5 times as effective as the A,B one, the reverse of the selectivity seen with the cyclic substrate, where the A,B isomer was about 5 times more active. For comparison, the mono(imidazolyl)-β-cyclodextrin compound was synthesized, and the pH–rate profile for the reaction catalyzed by it and **1** was found to be similar to that with the bis(imidazole) catalysts and of intermediate activity. This catalyst is by necessity monofunctional, and the similarity of the pH–rate profiles confirms that all three species are functioning solely as general base catalysts for nucleophilic attack by water on diester **1**. The slightly better catalytic activity of the A,C isomer may arise from simple geometrical considerations: since its two potential general bases are farther apart, there are probably more productive binding complex orientations possible than with the A,B isomer, which has the two imidazoles close together on one side of the cavity.

Possible reasons for the different catalytic mechanism with **1** are that the *p*-nitrophenyl leaving group is sufficiently labile so as not to require protonation (assuming leaving group protonation), or that the inductive effect of the *p*-nitrophenyl group in the diester substrate lowers the pK_a of the nonbridge oxygen atom to the point that its protonation by the conjugate acid of imidazole is unfavorable. To eliminate this difference, the diester substrate *p*-*tert*-butylphenyl phenyl phosphate was tried as a substrate, since the leaving group pK_a in this compound is very similar to that of catechol. Catalyzed hydrolysis of this substrate by both the A,B and A,C catalysts was negligible at 70 °C, and at higher temperatures decomposition of the catalyst occurred. These results indicate that the difference in mechanism followed by the cyclic substrate versus the acyclic one is not due to the difference in leaving group, but instead may arise from a difference in the reactivity of cyclic versus acyclic phosphodiesters.

The isotope effects for both bis(imidazolyl)- and the mono(imidazolyl)-β-cyclodextrins with **1** were measured at 70 °C at pH 6.3, where the bis(imidazole) catalysts should be monoprotonated, in order to look for any indications of participation of the protonated imidazole in the transition states for the reactions. If the nonbridge oxygen atom were to be protonated in the transition state, this would result in a significant inverse isotope effect for $^{18}k_{nonbridge}$. If the leaving group oxygen were being protonated, the secondary ^{15}k isotope effect should be abolished and the primary $^{18}k_{lg}$ effect diminished, depending on the relative progress in the transition state of proton transfer compared with the extent of bond cleavage. As the data in Table 1 show, none of these predicted events occur, thus there is no indication of proton transfer to either the leaving group or the nonbridge oxygen atom, consistent with the kinetic results. The $^{18}k_{nonbridge}$ isotope effects are all very similar to that from the alkaline hydrolysis reaction of **1**. The large magnitudes of the isotope effects in the leaving group are indicative of a transition state in which the bond to the leaving group is largely broken in a concerted mechanism with no phosphorane intermediate.

Conclusions. The solution alkaline and acidic hydrolysis reactions of phosphodiesters **1** and **2** proceed by concerted mechanisms with no evidence of a phosphorane intermediate. While bond cleavage to the leaving group is occurring at the transition state, it is not far advanced. The transition states are similar for the two compounds but exhibit small differences in involvement of the nonbridge oxygen atoms. The two diesters

(17) A reviewer raised the question of whether the differences in isotope effects for the enzymatic reactions with substrates **1** and **2** could be caused by differences in commitment factors. For this to be the case, the mechanism of cleavage would have to be stepwise, instead of the concerted mechanism indicated by the data. But for the sake of argument, if a stepwise mechanism as shown in eq 1 is operating, then the observed isotope effects are given by the equation $^{18}(V/K) = [^{18}K_{eq}{}^{18}k_2 + {}^{18}k_1(cf)]/(1 + cf)$, where $^{18}K_{eq}$ is the isotope effect on the equilibrium formation of the protonated monoanionic intermediate, $^{18}k_1$ and $^{18}k_2$ are respectively the kinetic isotope effects on the formation and breakdown of the intermediate, and cf is the commitment factor, k_2/k_{-1}. In the case of the leaving group isotope effect $^{18}k_{lg}$, since there should be no significant isotope effects at this atom for the formation of the phosphorane, the equation reduces to $^{18}k_{lg} = (^{18}k_2 + cf)/(1 + cf)$. The observation that $^{18}k_{lg}$ for diester **2** is half as large as that for **1** could be explained by a commitment factor of near unity for diester **2** (that is, $k_2 = k_{-1}$) and a commitment of zero for diester **1** (that is, $k_2 \ll k_{-1}$). Applying the same analysis to ^{15}k leads to the prediction of a similar reduction in magnitude for this isotope effect with **2** compared to **1**, but this is not observed. The values of ^{15}k for the two substrates are identical within experimental error. Considering the effect of the proposed commitment on the value for $^{18}k_{nonbridge}$ with diester **2**, this will then be equal to the expression $(^{18}K_{eq}{}^{18}k_2 + {}^{18}k_1)/2$, while that with diester **1** will be $(^{18}K_{eq}{}^{18}k_2)/1$. The values for $^{18}K_{eq}$ and $^{18}k_1$ at the nonbridge oxygen atoms will be inverse; the equilibrium effect for formation of the phosphorane $^{18}K_{eq}$ will probably be greater than the kinetic effect $^{18}k_1$. The effect on the breakdown step, $^{18}k_2$, will be normal. Assuming similar transition states for formation and breakdown of the phosphorane intermediate, the magnitudes of $^{18}k_1$ and $^{18}k_2$ will be similar, though in opposite directions. The inverse $^{18}K_{eq}$ will be reduced in magnitude by multiplication by the normal $^{18}k_2$. The magnitudes of the kinetic effects will depend upon whether the transition state is early or late, but in general the equations lead to the expectation that the commitment factor should result in $^{18}k_{nonbridge}$ effects that are smaller for substrate **2** than for **1**, which is the opposite of what is observed.

(18) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70–78.

(19) We do not attribute any kinetic significance to the small decrease in rates above pH 7. Since this occurs with the monoimidazole catalyst as well as the A,B and A,C species, it cannot be attributed to the occurrence of bifunctional catalysis. The pH–rate profiles for the A,C and A,D catalysts with the 4-*tert*-butylcatechol cyclic phosphate substrate (see ref 7b) also do not drop completely on the alkaline side, indicating that acid catalysis is less important than base catalysis in these reactions as well.

are accepted as substrates by phosphodiesterase I, but the isotope effects reveal significant differences in the transition-state structures. The large inverse value for $^{18}k_{nonbridge}$ for the more basic diester **2** strongly indictes protonation of the nonbridge oxygen atom in the transition state. Bond cleavage to the leaving group is similar to those for the solution hydrolysis reactions. The data for the less basic substrate **1** suggest only partial proton transfer to the nonbridge oxygen atom and about twice the degree of bond cleavage to the leaving group in the transition state. Phosphodiester **1** is a substrate for imidazolyl-β-cyclodextrin catalysts, but, unlike the bifunctional catalysis observed with a cyclic diester substrate, **1** undergoes catalysis by a simple general base mechanism. A concerted mechanism is indicated with no involvement of the nonbridge oxygen atoms different than that observed in the solution hydrolytic reactions. The degree of bond cleavage to the leaving group in these general base-catalyzed reactions is significantly more advanced than that in the uncatalyzed aqueous reactions.

Experimental Section

Materials. Tetrahydrofuran was distilled under nitrogen from sodium and benzophenone ketyl just before use. Pyridine, collidine, triethylamine, and acetonitrile were distilled under nitrogen from calcium hydride just before use. Anhydrous dioxane was purchased from Aldrich and used as received. Acetone was freshly distilled from calcium sulfate. Tetrazole was purified by sublimation and dried over P_2O_5. $H_2{}^{18}O$ (96% ^{18}O) was purchased from Isotec. Thin-layer chromatography (TLC) was performed on polyester-backed plates of silica gel with a fluorescent indicator; compounds were detected under ultraviolet light.

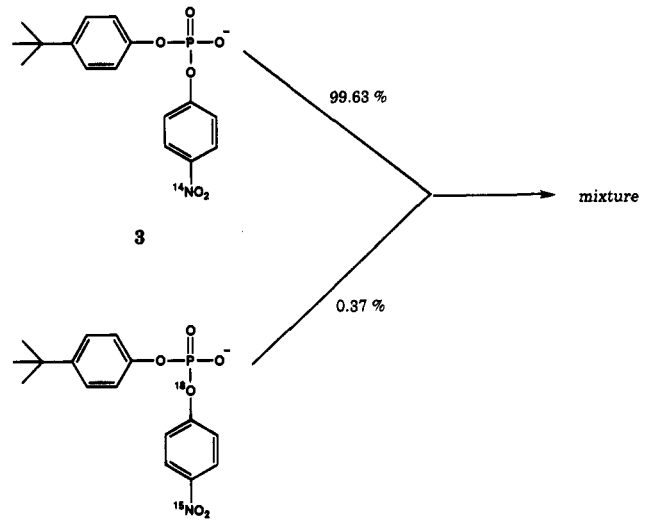
[^{14}N]-*p*-Nitrophenol, [^{18}O,^{15}N]-*p*-nitrophenol, and [^{14}N]-*p*-nitrophenyl phosphorodichloridate were synthesized as previously reported.[3b] All natural abundance diesters were synthesized by reacting *p*-nitrophenyl phosphorodichloridate with the respective phenol or alcohol and purified using methods previously described.[3b] A,B and A,C bis(imidazolyl)-β-cyclodextrins were synthesized according to procedures of Breslow.[7b] The mono(imidazolyl)-β-cyclodextrin was prepared by similar methods from the 6-*O*-β-cyclodextrin monotosylate.[20]

Kinetic Isotope Effect Determinations. All isotope effects were measured by the competitive method, using an isotope ratio mass spectrometer. The measurement of heavy-atom isotope effects using an isotope ratio mass spectrometer is much more accurate and reliable than direct spectrophotometric comparisons of rates, due to the small magnitudes of the observed effects. The ^{18}O isotope effects were measured by the remote label method.[8] In this method, substrate is synthesized with labels at two positions, one at the site of chemical interest and the other at a position that lends itself to isolation and isotopic measurement, to function as a marker. In this work, the nitrogen atom of the substrate served as the remote label. For measurement of $^{18}k_{lg}$, **1** was synthesized either with oxygen-18 in the bridge oxygen atom and nitrogen-15 in the nitro group or with nitrogen-14 in the nitro group and only the natural abundance of oxygen-18 in the various oxygen atoms (Scheme 1). These were then mixed to reconstitute the natural abundance of 0.37% of nitrogen-15, as the isotope ratio measurements are most accurate when samples approximate natural abundance. The $^{18}k_{nonbridge}$ effects were measured similarly, using the corresponding compound with the two nonbridge oxygen atoms labeled with oxygen-18 and with nitrogen-15 in the nitro group. The synthetic routes for the multiply labeled compounds are summarized in Schemes 2 and 3. The general methodology has been previously described in other studies of phosphoryl transfer mechanisms using isotope effects.[3b,c,5]

General Methods. Reactions were run at diester concentrations from about 2 to 4 mM, under conditions described in detail below for each reaction. When the reactions were partially complete, they were stopped by cooling and titrated to pH 3, and an aliquot was removed for assay and added to cold 1 N NaOH solution. The amount of *p*-nitrophenol present in the assay solution was found by measuring

(20) Melton, L.; Slessor, K. N. *Carbohydr. Res.* **1971**, *18*, 29.

Scheme 1. Diagrammatic Representation of the Preparation of the Mixture Used To Measure the Leaving Group Oxygen-18 Isotope Effects by the Remote Label Method[a]



[a] The isotopic isomers are mixed to approximately reconstitute the natural abundance of nitrogen-15.

the absorbance at 400 nM of a portion added to dilute NaOH. The assay solutions were then heated to 100 °C for >8 half-lives to completely hydrolyze remaining substrate, and the amount of *p*-nitrophenol present was measured again. The fraction of reaction, *f*, used in calculating isotope effects was the ratio of the initial and final assays.

Molecular nitrogen was produced by combustion of *p*-nitrophenol and analyzed for isotopic composition by methods previously described.[5d] The isotope effects were calculated using the isotopic ratios of nitrogen in the *p*-nitrophenol product at partial reaction (R_p) and in the starting material (R_0) at known fractions of reaction using eq 2. Equation 3

$$\text{isotope effect} = \log(1-f)/\log[1-f(R_p/R_0)] \quad (2)$$

$$\text{isotope effect} = \log(1-f)/\log[(1-f)(R_s/R_0)] \quad (3)$$

was used to calculate the observed isotope effect from the isotopic ratios of residual substrate (R_s) and R_0. Isotope effects with compound **2** were calculated using both equations. Because of difficulties in isolating residual substrate in pure form from reactions involving **1**, isotope effects were calculated from R_p and R_0 only.

The isotopic ratio of the starting material was determined from nitrogen obtained from combustion of samples of the substrates and, as a control, by completely hydrolyzing samples of the substrates and analyzing the nitrogen obtained from the *p*-nitrophenol product. The isotopic ratios obtained from both methods were the same within experimental error, showing that no isotopic fractionation occurs during procedures used to recover *p*-nitrophenol.

The experiments using the double-labeled substrate mixtures yield an observed isotope effect which is the product of the effect due to nitrogen-15 substitution and that due to the oxygen-18 substitution. The observed isotope effects from these experiments were corrected for the ^{15}k effect and for incomplete levels of isotopic incorporation in the starting material as previously described.[3c]

Conditions for Isotope Effect Reactions. The solution reactions to determine $^{18}k_{lg}$ of compound **2** were run at 95 °C for consistency with the prior measurements of ^{15}k and $^{18}k_{nonbridge}$ with this compound. The alkaline hydrolysis reactions of compound **1** were run at 70 °C to facilitate comparison with the data obtained from the imidazolylcyclodextrin reactions run at this temperature. The acid hydrolysis reactions of **1** were run at 90 °C to shorten the reaction times. All reactions were run to a variety of fractional reaction values ranging from about 11% to about 50%. The isotope effects calculated at the various values gave excellent agreement in all cases and were averaged

to give the values in Table 1. At least six determinations were made of each isotope effect.

Alkaline Hydrolysis. Reactions with diester **1** were run at 70 °C and those with **2** at 95 °C at substrate concentrations from 2 to 6 mM in 1 N NaOH. The half-life for reaction of **1** was about 2.3 h, and that for **2** was about 2.5 h.

Acid Hydrolysis. Reactions were run at 95 °C at substrate concentrations from 5 to 7 mM in 2 N H_2SO_4. The half-life for reaction of **1** was about 8.5 h, and that for **2** was about 5 h.

Imidazolyl-β-cyclodextrin-Catalyzed Reactions. Reactions of compound **1** with all three catalysts were run at substrate concentrations from 2.5 to 4 mM and catlyst concentration 6 mM in 0.5 M phosphate buffer at pH 6.1. Reactions were allowed to proceed for ~11−130 h to obtain a variety of fractional reaction values. Reactions were run at 70 °C to shorten reaction times; at higher temperatures, decomposition of the catalyst occurred.

As a control experiment, solutions of each of the three catalysts were heated with 15 mg of *p*-nitrophenol under conditions identical to those used in the isotope effect experiments. The *p*-nitrophenol was isolated and analyzed for isotopic composition, which was found to be unchanged. This verifies that no nitrogen-containing contaminants or isotopic fractionation interferes with the data from these experiments.

The catalysts were quantitatively recovered unchanged after reaction, demonstrating that their function was indeed catalytic and ruling out nucleophilic participation by the secondary hydroxyl groups of the cyclodextrins.

Phosphodiesterase Reactions. Reactions were run at pH 8 in TAPS buffer with substrate **2** at 37 °C as previously reported.[3b] Reactions with **1** were performed similarly but at 25 °C.

Isolation of *p*-Nitrophenol for Analysis. After reaction mixtures were cooled to room temperature, they were titrated to pH 3 and extracted three times with ethyl ether. After removal of the ether by rotary evaporation, the residue was dissolved in 40 mL of water and 1 mL of 5 N NaOH. The basic solution was washed three times with ether, and the ether layer were discarded. The aqueous solution was neutralized with 1 mL of 5 N H_2SO_4, and *p*-nitrophenol was extracted with ether (3 × 50 mL). The ether layers were combined, dried over magnesium sulfate, and evaporated to dryness. The *p*-nitrophenol was further purified by vacuum sublimation at 90 °C and prepared for combustion.[5d]

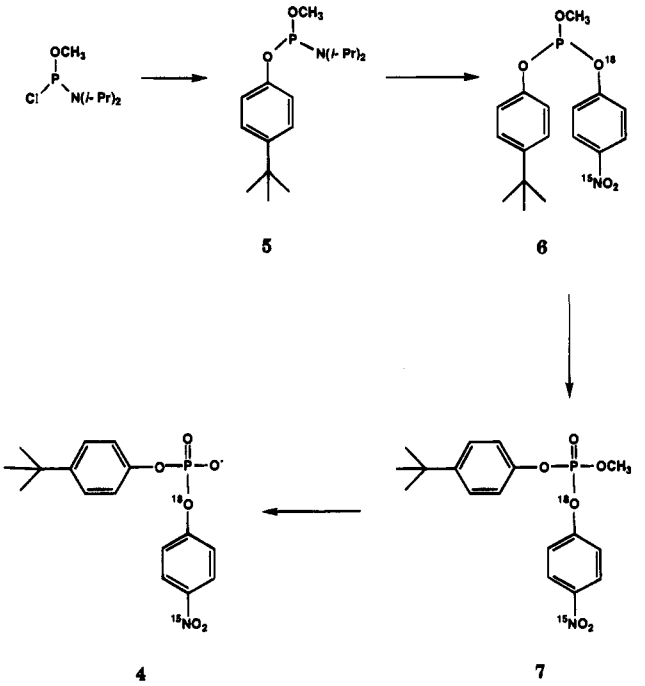
pH−Rate Profiles of Diester 1 with Imidazolylcyclodextrin Catalysts. These reactions were run at 70 °C using acetate and phosphate buffers. Concentrations were 100 mM buffer, 5 mM catalyst, 1 mM substrate, and 200 mM KCl. Reactions were followed by periodically assaying for *p*-nitrophenol at 400 nm. Pseudo-first-order rate constants were obtained by fitting the kinetic data to the equation $y = A(1 - \exp(-kt))$ using the program EXPFIT of Cleland.

Determination of the pK_a of Diester 1. The pK_a values of phosphate esters can be found from measurements of the pH dependence of the ^{31}P NMR chemical shift. Phosphodiester **1** was titrated with perchloric acid, but ^{31}P NMR data indicated that the endpoint had not yet been reached in 11.6 M acid, indicating that the pK_a was less than −1.2.

Inactivation Experiments with Phosphodiesterase. A solution of the enzyme in 1 mL of 5 mM TAPS buffer, pH 8, was inactivated by addition of 50 μL of a 20 mM solution of EDTA.[14b] After 15 min, assays with both substrates **1** and **2** showed no reaction, indicating that complete inactivation had occurred.

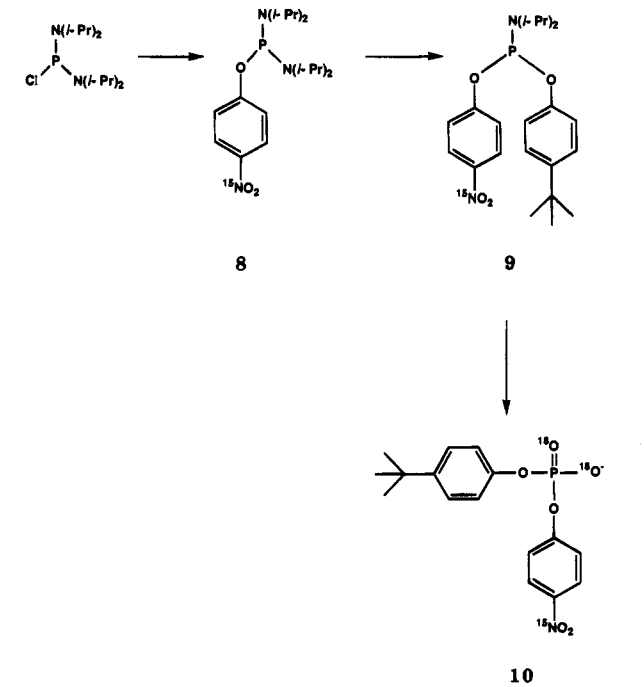
Samples of the enzyme were run on a 10% SDS−PAGE gel and stained with Coomassie brillant blue, revealing the presence of several low-molecular-weight impurities in the enzyme preparation. Samples of the enzyme were then run in several lanes on a 10% native PAGE gel which was then cut into thirds. One of these was stained with Coomassie brillant blue. Activity stains were run on each of the remaining two gels with the substrates **1** and **2**. The gel was soaked in a Petri dish containing a 5 mM concentration of one of the diesters in TAPS buffer, pH 8. A yellow band resulting from release of *p*-nitrophenol appeared with both substrates within 20 min at the same place on the gel, at the band expected to correspond to the diesterase protein on the basis of its known molecular weight. No indications of substrate turnover by lower molecular weight proteins was observed.

Scheme 2. Outline of the Synthetic Route Used To Prepare [^{15}N,phenolic-^{18}O]-*p*-*tert*-Butylphenyl *p*-Nitrophenyl Phosphate[a]



[a] See Experimental Section for details.

Scheme 3. Outline of the Synthetic Route Used To Prepare [^{15}N,nonbridge-$^{18}O_2$]-*p*-*tert*-Butylphenyl *p*-Nitrophenyl Phosphate[a]



[a] See Experimental Section for details.

Synthesis of Compounds. The synthetic routes to the labeled versions of compound **1** are outlined in Schemes 2 and 3, and are given in detail below.

Preparation of [^{14}N]-*p*-Nitrophenyl *p*-*tert*-Butylphenyl Phosphate (3). [^{14}N]-*p*-Nitrophenyl phosphorodichloridate (3.89 g, 15.4 mmol) was placed in a flame-dried flask, and under a nitrogen atmosphere 6 mL of anhydrous dioxane was added, followed by pyridine (2.5 mL, 31 mmol). *p*-*tert*-Butylphenol (1.55 g, 10.3 mmol) was separately dissolved in 6 mL of anhydrous dioxane, and this solution was added slowly to the reaction mixture. The reaction was stirred at room

temperature for 2 h until *p-tert*-butylphenol was not detected by TLC. The reaction mixture was then cooled in an ice bath, and a solution consisting of 7 mL of water and 0.7 mL of pyridine was added. The reaction mixture was concentrated to an oil by rotary evaporation, 30 mL of water was added, and the product was extracted with chloroform (3 × 75 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated by rotary evaporation. The pyridinium salt of *p*-nitrophenyl *p-tert*-butylphenyl phosphate was purified by column chromatography using silica gel, eluting with 5:1 ethyl acetate/methanol. The sodium form of the diester was obtained using ion-exchange chromatography with Sephadex SP-C25-20. A 10-fold excess of resin was swelled in 0.5 N sodium acetate buffer at pH 5.5 and then washed with 10 column volumes of deionized water, followed by 1 column volume of 25% ethanol in water. The pyridinium salt of diester **3** was dissolved in 750 mL of 25% ethanol in water, and this solution was passed through the column, followed by 1 column volume of water. The diester product contained a small amount of *p*-nitrophenol, which was removed using reverse phase chromatography. Reverse phase silica gel[21] (25 g) was loaded into a column with methanol, followed by 2 column volumes of degassed water. The crude product was loaded onto the column as an aqueous solution, and then the column was washed with solvents of decreasing polarity from 10 to 50% methanol in water. The product was found to elute with 40−50% methanol in water. The product-containing fractions were combined, and the solvent was removed by rotary evaporation, followed by lyophilization to give a white powder (3.15 g, 82% yield): ^{1}H NMR (D_2O) δ 1.28 (s, 9H), 7.14 (d, 2H, J = 8 Hz), 7.35 (d, 2H, J = 9 Hz), 7.44 (d, 2H, J = 9 Hz).

Preparation of (*N,N*-Diisopropylamino)methoxy-*p-tert*-butylphenoxyphosphine (5). Chloro(*N,N*-diisopropylamino)methoxyphosphine (238 mg, 1.21 mmol) and collidine (0.32 mL, 2.4 mmol) were dissolved in 2 mL of freshly distilled THF under nitrogen atmosphere in a flame-dried flask. A solution of p-*tert*-butylphenol (91 mg, 0.6 mmol, recrystallized from EtOAc and dried under vacuum) in 1 mL of anhydrous THF was added dropwise. The reaction mixture was stirred for 20 min at room temperature until the phenol was undetectable according to TLC analysis. Solvent was removed by rotary evaporation, and the resulting oily residue was purified by flash chromatography on silica gel using 20:1 hexane/ethyl ether (contining 0.5% triethylamine). Fractions containing product were pooled and then concentrated to a colorless oil that solidified in the freezer (173 mg, 82% yield): ^{1}H NMR ($CDCl_3$) δ 1.17 (d, 6H, $CH(CH_3)_2$), 1.22 (d, 6H, $CH(CH_3)_2$), 1.27 (s, 9H, $C(CH_3)_3$), 3.49 (d, 3H, OCH_3), 3.71 (m, 2H, arom H), 6.97 (d, 2H), 7.27 (d, 2H, arom H).

Preparation of ([^{15}N,^{18}O]-*p*-Nitrophenoxy)methoxy-*p-tert*-butylphenoxyphosphine (6). **5** (173 mg, 0.56 mmol) was dissolved in 2 mL of acetonitrile and kept under nitrogen atmosphere. Tetrazole (52 mg, 0.74 mmol) and [^{15}N,^{18}O]-*p*-nitrophenol (73.9 mg, 0.52 mmol) were added, and the reaction mixture was stirred at room temperature for 2 h until phosphine **5** could not be detected by TLC. Ethyl acetate (2 mL) was added, and the resulting white precipitate of tetrazolium salts was removed by filtration. The filtrate was concentrated by rotary evaporation, and the residual oil was purified by column chromatography on silica gel, eluting with petroleum ether/diethyl ether (20:1) containing 0.5% triethylamine. Fractions containing product were combined, and the solvent was removed to give **6** as a colorless oil (131 mg, 72% yield): ^{1}H NMR ($CDCl_3$) δ 1.30 (s, 9H), 3.81 (d, 3H), 7.01 (d, 2H), 7.35 (d, 2H), 8.22 (d, 2H).

Preparation of Methyl [^{15}N,^{18}O]-*p*-Nitrophenyl *p-tert*-Butylphenyl Phosphate (7). To the solution of phosphite **6** (131 mg, 0.37 mmol) in 3 mL of dichloromethane was added *tert*-butyl hydroperoxide (149 μL of 3 M solution in 2,2,4-trimethylpentane, 0.45 mmol). The reaction mixture was stirred at room temperature for 1 h. After this time, TLC did not show the presence of starting material (R_f of product, 0.15 in 4:1 petroleum ether/ether). An aqueous solution of sodium thiosulfate (0.1 N, 4 mL) was added, and the product was then extracted with ether (3 × 20 mL). Combined ether extracts were dried over magnesium sulfate and concentrated by rotary evaporation. The crude product was purified by column chromatography on silica gel eluting with 4:1 petroleum ether/diethyl ether. Fractions containing product were combined and concentrated to give triester **7** as a white solid (154 mg, 94% yield): ^{1}H NMR ($CDCl_3$) δ 1.30 (s, 9H), 3.99 (d, 3H), 7.12 (d, 2H), 7.36 (d, 2H), 7.39 (d, 2H), 8.24 (d, 2H).

Preparation of [^{15}N,^{18}O]-*p*-Nitrophenyl *p-tert*-Butylphenyl Phosphate Sodium Salt (4). Phosphate triester **7** (154 mg, 0.42 mmol) was dissolved in 3 mL of anhydrous acetone. Sodium iodide (180 mg, 1.2 mmol) was added, and the reaction mixture was refluxed for 1 h. Solvent was removed by rotary evaporation, and the residual white solid was purified by column chromatography on silica gel using 10:1 ethyl acetate/methanol. The product had an R_f of 0.4 on TLC. The ^{1}H NMR was identical with that of the natural abundance compound. ^{15}N and ^{18}O incorporations in the product were determined by mass spectroscopy to be 99% and 86%, respectively.

Preparation of Bis(*N,N*-diisopropylamino)[^{15}N]-*p*-nitrophenoxyphosphate (8). Bis(diisopropylamino)chlorophosphine (362 mg, 1.32 mmol) was placed in a flame-dried flask under nitrogen atmosphere. To this was added 10 mL of anhydrous THF, followed by triethylamine (730 μL, 5.44 mmol). A solution of [^{15}N]-*p*-nitrophenol (190 mg, 1.36 mmol) in 5 mL of anhydrous THF was added dropwise to the reaction mixture. After 10 min, TLC showed no remaining *p*-nitrophenol. Solvent was removed by rotary evaporation, and the crude reaction mixture was purified by column chromatography on silica gel eluting with 20:1 hexane/ether with 0.5% triethylamine. Fractions containing product were combined and concentrated to dryness to yield a slightly yellow oil that solidified upon cooling in the freezer (426 mg, 85% yield): ^{1}H NMR ($CDCl_3$) δ 1.13 (d, 6H), 1.20 (d, 6H), 3.61 (m, 2H), 7.08 (d, 2H), 8.15 (d, 2H).

Preparation of ([^{15}N]-*p*-Nitrophenoxy)-*p-tert*-butylphenoxyphosphine (9). Phosphine **8** (426 mg, 1.15 mmol) was dissolved in 8 mL of anhydrous acetonitrile. Tetrazole (121 mg, 1.73 mmol) was added, followed by *p-tert*-butylphenol (173 mg, 1.15 mmol). The reaction mixture was stirred at room temperature for 10 min. The solvent was removed by rotary evaporation, and the reaction mixture was purified by column chromatography on silica gel, eluting with 40:1 petroleum ether/ether. The product has an R_f of 0.3 on TLC with a solvent ratio of 20:1. Fractions containing product were pooled and concentrated to a yellow oil (216 mg, 45% yield): ^{1}H NMR ($CDCl_3$) δ 1.24 (d, 6H), 1.27 (s, 9H), 3.59 (m, 2H), 7.14 (d, 2H), 7.30 (d, 2H), 7.39 (d, 2H), 8.19 (d, 2H).

Preparation of [^{15}N,nonbridge-$^{18}O_2$]-*p*-Nitrophenyl *p-tert*-Butylphenyl Phosphate Sodium Salt (10). To a solution of **9** (216 mg, 0.52 mmol) in 5 mL of anhydrous THF was added $H_2{}^{18}O$ (200 μL). Iodine (141 mg, 0.55 mmol) in 2 mL of anhydrous THF was added dropwise to the reaction mixture until the solution remained yellow. Sodium hydroxide (22 mg, 0.55 mmol) was added, followed by an aqueous solution of sodium thiosulfate until the reaction mixture became colorless. The solvent was removed by rotary evaporation, and then 10 mL of ether was added. The white crystalline precipitate that formed was collected by Buchner filtration and was washed with ether. The product was further purified by column chromatography on silica gel and then by reverse phase chromatography, as described for product **4**. The mass spectroscopy analysis indicated 90% ^{18}O incorporation in product.

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