Supporting information for **"Comparisons of Phosphorothioate with Phosphate Transfer Reactions for a Monoester, Diester and Triester: Isotope Effect Studies"**

Determinations of equilibrium isotope effects for deprotonation and metal ion complexation of *p*NPPT. For these experiments a 1:1 mixture of natural abundance *p*NPPT and [^{15}N, nonbridge-$^{18}O_2$]-*p*NPPT was used.[1] The ^{31}P NMR chemical shift data for the labeled and unlabeled compounds were recorded using a Bruker ARX 400 MHz spectrometer operating at 162 MHz. 65% H_3PO_4 was used as an external standard in a coaxial NMR tube.

*Deprotonation of pNPPT***.** The chemical shifts of the nonbridge ^{18}O-labeled and unlabeled pNPPT were recorded between pH 2 and pH 11. The buffers used for these experiments were as follows: pH 2, 0.25 M glycine; pH 3 and 3.72, 0.25 M formate; pH 4-5, 0.25 M acetate; pH 6, 0.25 M MES; pH 7-8, 0.25 M HEPES; pH 9, 0.25 M CHES; pH 10-11, 0.25 M CAPS. The substrate concentration was 20 mM and ionic strength was maintained using KCl (μ=1). Due to rapid hydrolysis at low pH samples at pH < 5 were prepared just before use.

The changes in chemical shifts with pH for unlabeled *p*NPPT were used to calculate the second pK_a of the *p*NPPT monoanion using equation 1.

$$\delta = \frac{\left(\delta_L + \delta_H \cdot K_a / [H^+]\right)}{1 + K_a / [H^+]} \qquad (1)$$

where:

δ	= observed chemical shift of pNPPT
δ_L, δ_H	= chemical shifts for the protonated (monoanion) and nonprotonated (dianion) substrate
K_a	= acidity constant
$[H^+]$	= hydronium ion concentration

[1] The ^{15}N label in this experiment is superfluous but since this compound had been prepared for use in KIE experiments, it was used in lieu of separately synthesizing [nonbridge-$^{18}O_2$]-*p*NPPT. The ^{15}N label does not result in a detectable isotope shift on the ^{31}P NMR signal.

The differences in ^{31}P chemical shift between ^{16}O and ^{18}O *p*NPPT were used to calculate the isotope effect for deprotonation using equation 2.[1]

$$\Delta\delta_{(^{16}O-^{18}O)} = d_H - (\delta_H - \delta_L)\cdot x + \frac{^{18}K\cdot x\cdot((\delta_H-\delta_L)+d_L-d_H)}{^{18}K\cdot x+1-x} \qquad (2)$$

where:

d_L = the difference in chemical shifts between dianionic ^{16}O and ^{18}O *p*NPPT

d_H = the difference in chemical shifts between monoanionic ^{16}O and ^{18}O *p*NPPT

^{18}K = the equilibrium isotope effect

x = the fraction of deprotonated pNPPT present, from equation 3

$$x = \frac{1}{1+\frac{1}{10^{pK_a-pH}}} \qquad (3)$$

Metal Complexation of pNPPT. Chemical shifts of nonbridge ^{18}O-labeled and unlabeled *p*NPPT were recorded over a range of concentrations of Zn^{2+} and of Cd^{2+}. In both sets of experiments the pH was such that *p*NPPT was present as the dianion. For the Zn^{2+} complexation experiments, the conditions were 3 mM *p*NPPT, 1 M TRIS (pH 6.5), and 0.1 M NH_4Cl (the presence of NH_4^+ was required to prevent the precipitation of zinc hydroxide). Data were recorded at Zn^{2+} concentrations of 0, 0.5 and 1.5 M. For the Cd^{2+} experiments, concentrations were 3 mM *p*NPPT; 0.25 M HEPES (pH 7.5), and ionic strength was maintained with NaCl (μ=1). The Cd^{2+} was added using a stock solution of 1 M $Cd(NO_3)_2$ to give a concentration range of 0 - 150 mM; this range was chosen based on the previously determined association constant of Cd^{2+} with *p*$NPPT^{2-}$.[2] By contrast, with zinc a ratio of 1.5:0.003 Zn^{2+}:*p*$NPPT^{2-}$ was found necessary to achieve significant complexation.

The equilibrium isotope effect for Cd^{2+} complexation was obtained from equation 2, after the appropriate substitutions (d_L= the difference in chemical shifts between fully complexed ^{16}O and ^{18}O *p*NPPT, d_H= the difference in chemical shifts between uncomplexed ^{16}O and ^{18}O *p*NPPT, x = the fraction of *p*NPPT complexed, obtained from $x = K_d \bullet M_t$ where K_d = dissociation constant obtained from previous experiments[2] and M_t= metal ion concentration.)

Kinetic Isotope Effect Determinations.

pNPPT Monoanion hydrolysis: Isotope effect experiments were carried out at pH 2 in 0.1 M Glycine buffer at 30 ˚C; under these conditions the *p*NPPT monoanion's half life is about 30 min. Typical isotope effect experiments were run with a 20 mL reaction volume, at 5 mM *p*NPPT concentration and were run in triplicate. After partial hydrolysis adding 8.3 mL of 0.5 M MES buffer at pH 6, which raised the pH to 5, and cooling on ice stopped the reactions. From this solution two small aliquots were taken; one was added to a 0.1 M TRIS solution (pH 9) where the reaction is very slow. The second aliquot was added to a 0.2 M glycine solution (pH 2), and heated at 50 ˚C for 10 h to hydrolyze the remaining substrate, in order to determine the total initial concentration of the substrate. The concentrations of *p*-nitrophenol in both aliquots were determined

spectrophotometrically at 400 nm in 0.1 N NaOH solution, and the ratio of the results gives the fraction of the reaction at which the experiments were stopped.

The reaction solutions at pH 5 were extracted three times with an equal volume of diethyl ether to quantitatively remove the *p*-nitrophenol product. The ether layers were dried over $MgSO_4$, filtered and evaporated to dryness. The aqueous layer was then titrated to pH 2 and left standing overnight at 30 ˚C to completely hydrolyze the remaining *p*NPPT. The *p*-nitrophenol formed in this second part of the reaction was then extracted using the same procedure described above. The *p*-nitrophenol samples were sublimed under vacuum at 90 ˚C, and 1-1.5 mg samples were prepared for isotopic analysis using an ANCA-NT combustion system coupled with a Europa 20-20 isotope ratio mass spectrometer.

pNPPT Dianion hydrolysis: These isotope effect experiments were performed at 50 ˚C in 0.1 M CHES at pH 10 and 0.2 M carbonate buffer at pH 11. The half-life of pNPPT at 50 ˚C is 5 days, and the rate is pH-independent in this range. The substrate concentrations were 10 mM in 10 mL volume for the reactions run in CHES buffer and 20 mM in 5 ml solution for the reactions run in carbonate buffer. At partial hydrolysis placing them on ice effectively stopped the reactions and the fraction of reaction was determined as described for the monoanion hydrolysis. The reaction solutions were carefully titrated to pH 5 using HCl and then extracted three times with an equal volume of diethyl ether to quantitatively remove *p*-nitrophenol. The aqueous layers were titrated to pH 2 and left overnight at 30 ˚C to completely hydrolyze the residual *p*NPPT. The *p*-nitrophenol formed in these reactions was isolated and prepared for isotopic analysis as described above.

Ethyl p-nitrophenyl phosphorothioate alkaline hydrolysis. Isotope effect experiments were run using 100 micromoles of the reactant in 5 to 10 mL volumes that were 1N in NaOH, at 95 ˚C. After partial hydrolysis, cooling on ice effectively stopped the reactions. The *p*-nitrophenol concentration at partial hydrolysis was determined by measuring the absorbance at 400 nm of an aliquot from the reaction solution diluted in 0.1 N NaOH. The total substrate concentration was determined from *p*-nitrophenol concentration present in a second aliquot in 1 N NaOH solution after complete hydrolysis at 95 ˚C (>12 h). The reaction solutions were then titrated to pH 4-5 with 1 N HCl, and *p*-nitrophenol was extracted with diethyl ether, as described for the monoester reactions. The aqueous layer was then brought back to 1 N NaOH with solid NaOH, then heated to 95 ˚C for > 12 h. The *p*-nitrophenol released was isolated by the usual method. The samples of *p*-nitrophenol were prepared for isotopic analysis as described for the monoester KIE experiments.

Dimethyl p-nitrophenyl phosphorothioate alkaline hydrolysis. Reactions were initiated by addition of 25 mL of 0.2 N NaOH to a solution of 100 μmoles of reactant in 5.5 mL of dioxane. After partial hydrolysis the reactions were slowed dramatically by titration to pH 10 using HCl. An aliquot of the reaction mixture was added to a vial containing an equal volume of pH 7 phosphate buffer, and a second aliquot added to a vial containing an equal volume of 1N NaOH. The first vial was assayed for *p*-nitrophenol immediately, the second after 24 hours, the ratio of the *p*-nitrophenol assays giving the fraction of reaction. The pH 10 reaction mixture was twice extracted with an equal volume of methylene chloride, which control experiments showed removes the unreacted triester but leaves *p*-nitrophenol (as the phenolate) in the aqueous layer. The

methylene chloride layers were combined, added to 20 mL of 1N NaOH, and the organic solvent was gradually removed by rotary evaporation. The resulting aqueous solution was allowed to stand for 24 hours. Isolation of *p*-nitrophenol from both aqueous fractions was performed by acidification followed by ether extraction, as described for the monoester and diester reactions.

Kinetic Isotope Effect Data Analysis. For each isotope effect at least three reactions were run. The $^{15}N/^{14}N$ ratios were measured for the product (R_p) and of the remaining starting material (R_s) at partial reaction, as well as in the original mixture (R_o). The isotope effects were calculated using equations 1 and 2.[3]

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

$$\text{isotope effect} = \log (1 - f) / \log (1 - f\ (R_P / R_O)) \quad (2)$$

For each isotope effect the value calculated from R_o and R_p (equation 1) and from R_o and R_s (equation 2) agreed within experimental error and these were averaged to give the results reported. The ^{15}N KIE is given directly from these equations. In the ^{18}O isotope effect experiments the observed KIEs given by the above equations were corrected for the ^{15}N isotope effect and for incomplete levels of isotopic incorporation.

Calculation of corrected ^{18}O kinetic isotope effects

In the ^{18}O isotope effect experiments the observed KIEs were corrected for the ^{15}N isotope effect and for incomplete levels of isotopic incorporation. The derivations of the equations used for these corrections in experiments with one ^{18}O label ($^{18}O_{lg}$) and for experiments with two labels ($^{18}O_{nuc}$) have been described.[4] For one ^{18}O label the following equation is used:

$$^{18}k = \frac{^{15,18}ky}{^{15}k - [^{15,18}k - {}^{15}k][(1-b)z/(bx)] - {}^{15,18}k(1-y)}$$

where,

$^{15,18}k$ = the observed KIE due to both labels

^{15}k = the ^{15}N KIE

^{18}k = the corrected ^{18}O KIE

b = the fraction of doubly labeled compound in the remote labeled mixture

x = the fraction of ^{15}N in the remote label position of the doubly labeled (^{15}N, ^{18}O) compound used for mixing.

y = the fraction of ^{18}O in the doubly - labeled compound used for mixing

z = the fraction of ^{15}N in the remote label position of the ^{14}N - labeled compound used for mixing.

For the labeled substrates and the mixtures used for the $^{18}O_{lg}$ experiments, these values were as follows.
*p*NPPT: b=0.00368, x=0.99, y=0.87, z=0.0001.
Ethyl *p*-nitrophenyl phosphorothioate: b=0.00370, x=0.99, y=0.91, z=0.0002.
Dimethyl *p*-nitrophenyl phosphorothioate: b=0.00371, x=0.99, y=0.93, z=0.0002.

For two ^{18}O labels the following equation was used:

$$^{18}k = 1 + \left[\sqrt{\frac{^{15,18,18}k}{^{15}k - [(1-b)z/(bx)](^{15,18,18}k - ^{15}k)}} - 1 \right] \times [1 + (1-y)/2]$$

where,

$^{15,18,18}k$ = the observed KIE due to all three labels

^{15}k = the ^{15}N KIE

^{18}k = the corrected ^{18}O KIE per ^{18}O atom

b = the fraction of ^{15}N,^{18}O,^{18}O - labeled compound in the remote labeled mixture

x = the fraction of ^{15}N in the remote label position of the doubly labeled (^{15}N,^{18}O,^{18}O) compound used for mixing.

y = the fraction of doubly-^{18}O labeled species in the compound used for mixing

z = the fraction of ^{15}N in the remote label position of the ^{14}N - labeled compound used for mixing.

For the labeled substrates and the mixture used for the $^{18}O_{nonbridge}$ experiments, these values were:

*p*NPPT: b=0.00376, x=0.99, y=0.92, z=0.0001.
Ethyl *p*-nitrophenyl phosphorothioate: b=0.00367, x=0.99, y=0.87, z=0.0002.

References

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