Stereochemistry at Phosphorus of the Reaction Catalyzed by *myo*-Inositol Monophosphatase

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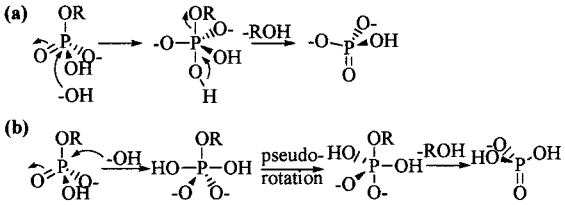
myo-Inositol monophosphatase (IMPase), the proposed target for lithium therapy for manic depression, is an important enzyme in the biosynthesis of second messengers. Earlier studies have shown that the IMPase-catalyzed hydrolysis of *myo*-inositol monophosphates to inorganic phosphate and *myo*-inositol proceeds by direct attack of water at phosphorus. However, research groups have independently proposed either an in-line displacement (with inversion of stereochemistry at phosphorus) or an adjacent attack with a pseudorotation (with retention of stereochemistry at phosphorus). Here, the elucidation of the stereochemical pathway is presented. The IMPase-catalyzed hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate in the presence of $H_2{}^{18}O$ gave inorganic R_p-[^{16}O,^{17}O,^{18}O]-thiophosphate, with inversion of configuration at phosphorus. This is only consistent with an in-line displacement, and it rules out the controversial adjacent/pseudorotation mechanism. This result will assist in the design of alternative inhibitors of IMPase.

Introduction

myo-Inositol monophosphatase (IMPase 3.1.3.25) is an important enzyme for mechanistic investigation due to its critical role in the phosphatidyl inositol signaling pathway and its inhibition by lithium used for the treatment of manic depression.[1,2] This enzyme, which has been purified from bovine[3] and human brain,[4] is responsible for supplying free *myo*-inositol by catalyzing the hydrolysis of all *myo*-inositol monophosphates, except the 2-isomer,[5] into inorganic phosphate and *myo*-inositol. The X-ray crystal structures of both the bovine and the human enzymes (2.1−2.6 Å resolution)[6−8] are known. The enzyme is a dimer with identical 30 kDa subunits (277 residues), with the active sites located in large hydrophilic caverns.[6,7] Two magnesium cations are present in the active site.[8,9]

Early mechanistic studies attempted to address whether the hydrolysis proceeded by direct nucleophilic attack by water at phosphorus or via a phospho-enzyme (P-E) intermediate[10,11] involving two in-line displacement reactions.[12] While a P-E intermediate is typical for nonspecific phosphatase enzymes such as alkaline phosphatase, experiments designed to trap such an intermediate in the case of IMPase failed.[10,12] X-ray crystallography of IMPase in the presence of D- or L-*myo*-inositol 1-phosphate also argued against the formation of a P-E intermediate, as there is no nucleophilic amino acid side chain close to the phosphoryl group.[8] In addition, kinetic and site-directed mutagenesis studies identified an activated water molecule rather than an amino acid as the nucleophile.[9,13] A dissociative mechanism via a metaphosphate intermediate has also been discounted; compounds bearing a hydroxyethyl arm are potent competitive enzyme inhibitors,[14] whereas transphosphorylation of the hydroxyethyl side chain may have been expected for a dissociative mechanism.

Scheme 1. Direct Attack by Water with Inversion (a)[9,17] or Retention (b)[15,17] of Stereochemistry at Phosphorus (R = *myo*-Inositol)



However, two different associative mechanisms (Scheme 1) with differing stereochemical outcomes have been suggested for IMPase[9,15,16] depending on the location of water in the active site. Pollack and co-workers[9] proposed an in-line displacement mechanism in which the nucleophile attacks opposite the leaving group, which would proceed with inversion of stereochemistry at phosphorus (Scheme 1a). From structural and mutagenesis studies, they proposed that the water nucleophile is activated by Glu-70, Mg^{2+}-1, and possibly Thr-95.[9] While the first metal ion activates water for nucleophilic attack, the second metal ion, coordinated by three aspartate residues, appears to act as a Lewis acid, stabilizing the leaving inositol oxyanion.

In contrast, Gani and co-workers[15,17] proposed a nonin-line associative mechanism where the nucleophile attacks the phosphorus at the same face as the leaving group (Scheme 1b). Pseudorotation is required to place the leaving group in an apical position, and the reaction would proceed with retention of stereochemistry at

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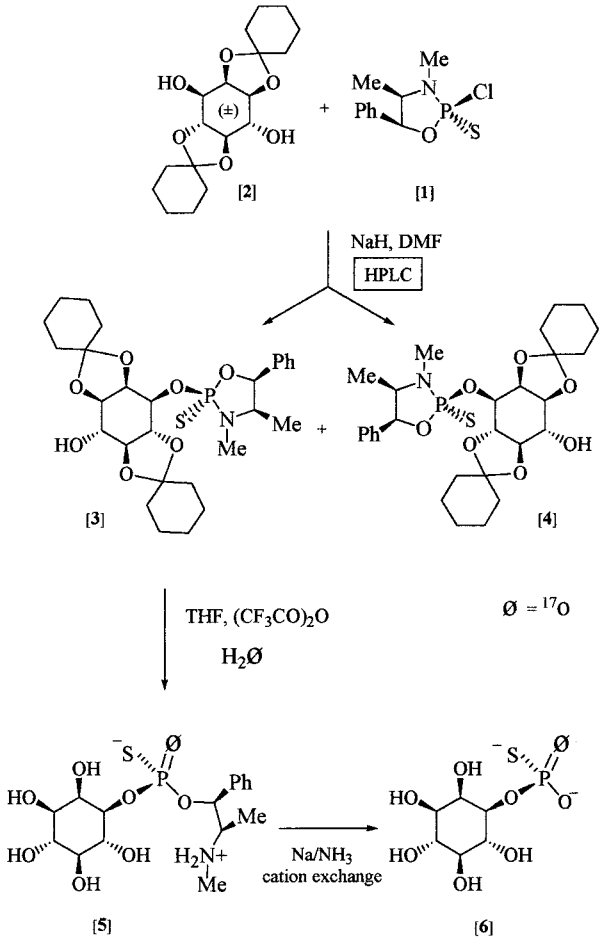
Scheme 2. Synthesis of D-1-S_p-*myo*-Inositol [^{17}O]-thiophosphate (**6**) Using (**1**) as a Chiral Phosphorylating Agent



phosphorus. The pseudorotation mechanism is unusual and, as yet, has not been proven for any enzymatic system.[18] They suggest that the 6-OH of *myo*-inositol forms a hydrogen bond with the water nucleophile that completes the primary coordination sphere of Mg^{2+}-2.

One of these two associative mechanisms can be ruled out by establishing the stereochemical course of the hydrolysis reaction catalyzed by IMPase. This can be achieved by analyzing the chiral product inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate formed from the hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate in the presence of $H_2{}^{18}O$.[19] In this study, we provide a firm stereochemical basis from which inhibitors and potential lead drugs can be securely designed.

Results and Discussion

Chemistry. The chiral phosphorylating agent (2*R*,4*S*,5*R*)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidin-2-one has been used by our group to prepare D-(+)-*myo*-inositol 3-phosphate.[20] The thio analogue (**1**) has been utilized to introduce ^{17}O and ^{18}O labels into organic thiophosphate analogues for stereochemical studies at phosphorus,[21−23] and here, **1** has been used to prepare D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate (**6**) (Scheme 2).

Phosphorylation of the anion from diol (**2**)[24] gave (D/L)-1,2:4,5-di-*O*-cyclohexylidene-3-[(2*S*,4*R*,5*S*)-3,4-dimethyl-5-phenyl-2-sulfide-1,3,2-oxazaphospholidinyl]-*myo*-inositol **3** and **4** with retention of configuration at phosphorus.[25] A small amount of phosphorylation at the 4/6-position was also observed. Diastereomers **3** and **4** coeluted by flash chromatography but could be separated by preparative high-performance liquid chromatography (HPLC), to give pure samples of the first (δ_P 82.4 ppm) and second (δ_P 81.0 ppm) eluted diastereomers, which were fully characterized by ^{31}P, ^{1}H, and ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

To establish which diastereomer was which, diol **2** was resolved via its biscamphanate using the method of Vacca and co-workers.[26] In their original paper, the compounds were misassigned, but a correction confirmed that the less polar diastereomer was D-1,2:4,5-di-*O*-cyclohexylidene 3,6-biscamphanate. Deprotection gave D-(−)-1,2:4,5-di-*O*-cyclohexylidene *myo*-inositol, which on phosphorylation with **1** gave diastereomer **4**. By comparison with the spectral data of the two diastereomers isolated by HPLC, it was established that the first eluted diastereomer was **4** and the second eluted **3**.

Treatment of **3** with a solution of trifluoroacetic anhydride in $H_2{}^{17}O$ (52.8% ^{17}O, 46.0% ^{16}O, and 1.2% ^{18}O) cleaved the P−N bond with inversion of configuration and removed the cyclohexylidene protecting groups to form zwitterion **5** (Scheme 2). Compounds containing the ^{17}O label attached to phosphorus cannot readily be detected by ^{31}P NMR spectroscopy; however, because of incomplete enrichment, other isotopes were also incorporated into this site, which allowed **5** to be observed at δ_P 57.0 ppm. Some phospho group migration (13.5 and 10.5%, respectively) to positions 6 (δ_P 58.6 ppm) and 4 (δ_P 58.9 ppm) was also observed. It is proposed that after acetal deprotection in a minor pathway, the adjacent equatorial hydroxy group forms a cyclic pentacoordinate intermediate at phosphorus, which on degradation cleaves to give these phospho migration products.

Sodium in liquid ammonia[22] was used to remove the chiral scaffold by reductive cleavage of the substituted benzyl ester group in **5** by C−O cleavage to give optically pure D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate (**6**), δ_P 44.85 (Scheme 2). A peak at δ_P 46.6 (26%) was also present for *myo*-inositol 4/6-thiophosphate. Mass spectrometry (MS) of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate established that the actual ^{17}O incorporation was 30% with the remaining isotope composition being 69% ^{16}O and 1% ^{18}O.

Unlabeled Hydrolysis Studies with IMPase. Ultimately, the hydrolysis of **6** in $H_2{}^{18}O$ catalyzed by IMPase[4,27] is required; however, it was first necessary to optimize the conditions to monitor the hydrolysis of unlabeled substrate.

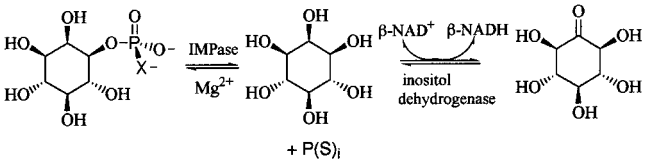
The activity of recombinant IMPase from bovine brain was confirmed by observing the rapid hydrolysis of D-*myo*-inositol 1/3-phosphate (δ_P 4.8 ppm) to inorganic phosphate (δ_P 3.4 ppm) by ^{31}P NMR spectroscopy. The corresponding hydrolysis of D-*myo*-inositol 1/3-thiophosphate was significantly slower, with 10% substrate (δ_P 44.8 ppm) remaining after 24 h at 37 °C. The major product was inorganic thiophosphate (PS_i, δ_P 32.2 ppm); however, inorganic phosphate (δ_P 5.1 ppm) and a desulfurization intermediate (δ_P 16.6 ppm, $^{2-}O_3P-S-S-PO_3{}^{2-}$) were also formed.

Table 1. Percentages of P_i (%) Observed with Time (h) from a Solution of PS_i (8.95 mM) in Tris-HCl, pH 9.00, at 25 °C with and without Different Concentrations of IMPase[a]

time (h)	without enzyme	0.119 mg mL^{-1} 3.97×10^{-6} M	0.235 mg mL^{-1} 7.82×10^{-6} M	0.343 mg mL^{-1} 11.43×10^{-6} M
24	4.3	4.7	8.7	14.7
48	11.4	8.1	17.9	26.9
72	15.3	16.4	19.6	34.6

[a] The reactions were monitored by ^{31}P NMR spectroscopy, and peak areas were used.

Scheme 3. Coupled Enzymatic Reaction with Inositol Dehydrogenase To Monitor Hydrolysis of *myo*-Inositol (Thio)phosphate (X = O or S) Catalyzed by IMPase



The rate of desulfurization of PS_i is critical for the labeled study as loss of sulfur leads to loss of the stereochemical information. Therefore, the chemical stability of PS_i was monitored at pD 11−12 and 8.2 by ^{31}P NMR spectroscopy at 22 and 37 °C. In agreement with Dittmer and Ramsay,[28] desulfurization was slower at pD 11−12 than at pD 8.2, and as anticipated, the rate increased with increasing temperature. Therefore, to minimize chemical desulfurization of PS_i, reactions catalyzed by IMPase were carried out at pH 9 and 25 °C. In addition to chemical instability, it is of importance to understand the possible role of IMPase in the desulfurization of PS_i. Solutions of PS_i (8.95 mM) were incubated at 25 °C in Tris-HCl buffer (pH 9.00) in the presence and absence of IMPase at various concentrations. The reactions were monitored by ^{31}P NMR spectroscopy over 72 h; the percentages of Pi formed are given in Table 1. The data, fitted with the Grafit program[29] to the Michaelis−Menten equation, showed that IMPase catalyzes desulfurization of PS_i. Therefore, the labeled reaction had to be designed to maximize the recovery of PS_i. This result is consistent with other enzymes,[30] for example, the desulfurization of PS_i is also catalyzed by acid and alkaline phosphatases.[31]

It was difficult using ^{31}P NMR spectroscopy to obtain good kinetic data for the hydrolysis of *myo*-inositol 1/3-thiophosphate; therefore, a UV assay was developed. The reaction catalyzed by IMPase can be monitored spectrophotometrically at 340 nm by coupling to inositol dehydrogenase, which catalyzes the oxidation of *myo*-inositol to *scyllo*-inosose (Scheme 3).[32−34] The k_{cat}, K_m, and k_{cat}/K_m values for the oxidation of *myo*-inositol catalyzed by inositol dehydrogenase at pH 9.13 were found to be 13.3 ± 2.0 s^{-1}, 0.58 ± 0.17 mM, and 2.3×10^4 M^{-1} sec^{-1}, respectively. The coupled enzyme assay required that the IMPase step was rate-determining. This was readily achieved by using inositol dehydrogenase in excess (0.14 units mL^{-1}), such that the oxidation of *myo*-inositol to *scyllo*-inosose was very fast. The observed velocity was shown to be independent of inositol dehydrogenase concentration but proportional to IMPase concentration. The kinetic constants for the hydrolysis of D-*myo*-inositol 3-phosphate catalyzed by IMPase at pH 9.00 at 25 °C were $K_m = 0.11 \pm 0.01$ mM (similar to literature values for the bovine recombinant IMPase),[4] $k_{cat} = 0.48 \pm 0.01$ s^{-1}, and $k_{cat}/K_m = 4.42 \times 10^3$ M^{-1} sec^{-1}.

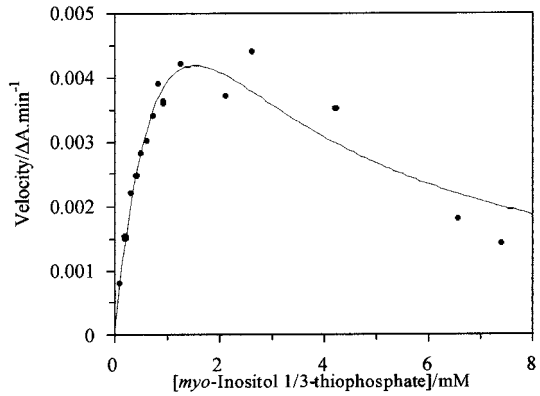


Figure 1. Kinetics of the hydrolysis of *myo*-inositol 1/3-thiophosphate [S] catalyzed by IMPase (6.7 μg ml^{-1}) in 50.4 mM Tris-HCl buffer containing 102 mM NaCl and 3 mM $MgCl_2 \cdot 6H_2O$, pH 9.00, at 25 °C with 5.0 mM β-NAD$^+$ and 0.19 units mL^{-1} inositol dehydrogenase. Plot of initial velocity vs [S].

The coupled assay was used to estimate the kinetic constants for the IMPase-catalyzed hydrolysis of *myo*-inositol 1/3-thiophosphate (Figure 1). At pH 9.00 at 25 °C, the K_m value was 2.07 ± 0.54 mM (by fitting the substrate inhibition equation using force estimates and simple weighting; see Figure 1), the k_{cat} value was 0.185 ± 0.037 s^{-1}, the K_{si} value was 1.14 ± 0.35 mM, and the k_{cat}/K_m value was 89.4 M^{-1} sec^{-1}. These are in the range of those published by Baker et al.[35] and Cole and Gani[15] ($K_m = K_{si} = 1$ mM). At high concentrations of *myo*-inositol 1/3-thiophosphate (>1 mM), inhibition of hydrolysis was observed. The turnover (k_{cat}) of *myo*-inositol 1/3-thiophosphate was 3 times slower than that observed for D-*myo*-inositol 3-phosphate. The specificity constant (k_{cat}/K_m) for D-*myo*-inositol 3-phosphate was 49 times higher than that for *myo*-inositol 1/3-thiophosphate confirming that the phosphate ester is a better substrate of IMPase than the thiophosphate ester. Substrate inhibition was confirmed when a high concentration of *myo*-inositol 1/3-thiophosphate (24 mM, δ_P (D_2O) 44.8 ppm) in the presence of IMPase (0.71 mg mL^{-1}) at pH 8.85 and 37 °C was shown to be stable toward hydrolysis by ^{1}H and ^{31}P NMR spectroscopy. The presence of the P−S group reduces the susceptibility of phosphorus to nucleophilic attack via an associative mechanism.

It was considered that substrate inhibition might arise through a covalent interaction between IMPase and *myo*-inositol 1/3-thiophosphate. The crystal structure of IMPase with D- or L-*myo*-inositol 1-phosphate bound[7,8] shows a disulfide bond between Cys-24 and Cys-125, although in the absence of substrate this disulfide bond was not observed. Electrospray mass spectrometry (ESI-MS) was used to probe the possibility of a covalent interaction between the cystine disulfide bond of IMPase and the *myo*-inositol 1/3-thiophosphate. The experimental mass of native, recombinant IMPase was 29 924.4 Da, which corresponds to 276 amino acids with loss of the N-terminal methionine. The recombinant enzyme, which had been in a 20.3 mM solution of *myo*-inositol 1/3-thiophosphate for 90 min, gave a molecular mass of 29 922.6 ± 3 Da, which confirms that

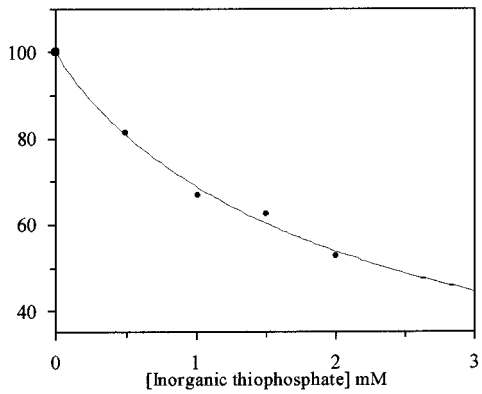


Figure 2. Plot of percentage activity in the hydrolysis of *myo*-inositol 1/3-phosphate vs concentration of PS_i as an inhibitor of IMPase in 50 mM Tris-HCl buffer containing 100 mM KCl and 2.7 mM $MgCl_2 \cdot 6H_2O$, pH 9.00, at 25 °C with 4.82 mM β-NAD^+, 0.19 units mL^{-1} inositol dehydrogenase, 1.42 $\mu g\ mL^{-1}$ IMPase, and 0.5 mM *myo*-inositol 1/3-phosphate. Points are experimental: line is theoretical assuming linear competitive inhibition with $K_i = 0.48$ mM.

myo-inositol 1/3-thiophosphate does not form a covalent complex with IMPase.

IMPase could also be affected by product inhibition; therefore, its activity in the presence of PS_i was monitored. P_i is a known competitive inhibitor of IMPase (K_i values 0.32−0.52 mM at pH 8.0 and 8 mM at pH 6.5).[3,10] The inhibition of IMPase by PS_i, evaluated at 0.5 mM *myo*-inositol 1/3-phosphate (Figure 2, data for pH 9.0), was similar to that for P_i: assuming linear competitive inhibition gave $K_i = 0.5$ mM at either pH 6.5 or pH 9.0. This result is consistent with PS_i being dianionic at both pH 6.5 and pH 9.0 (pK_{a1} 2.05, pK_{a2} 5.6, and pK_{a3} 10.2).[28] As product inhibition was shown not to be sensitive to pH, the labeled experiment was conducted at pH 9.00 because PS_i is chemically more stable at basic pH.

In summary, the following factors had to be considered in the experimental design for the IMPase-catalyzed hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate in $H_2{}^{18}O$. Hydrolysis of *myo*-inositol 1/3-thiophosphate competes with desulfurization of PS_i; therefore, the reaction should not be left longer than necessary. Product inhibition by PS_i occurs, which prevents the reaction from proceeding to completion. Substrate inhibition by *myo*-inositol 1/3-thiophosphate occurred, requiring that its concentration should not exceed 1−2 mM. For 25 mg of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate, this would require 100 mL of $H_2{}^{18}O$, which was prohibitively expensive. To minimize substrate inhibition, a method was developed in which *myo*-inositol 1/3-thiophosphate was added in several aliquots to IMPase in 5 mL of $H_2{}^{18}O$. This protocol, which maintained a low substrate concentration, was then used for the labeled experiment.

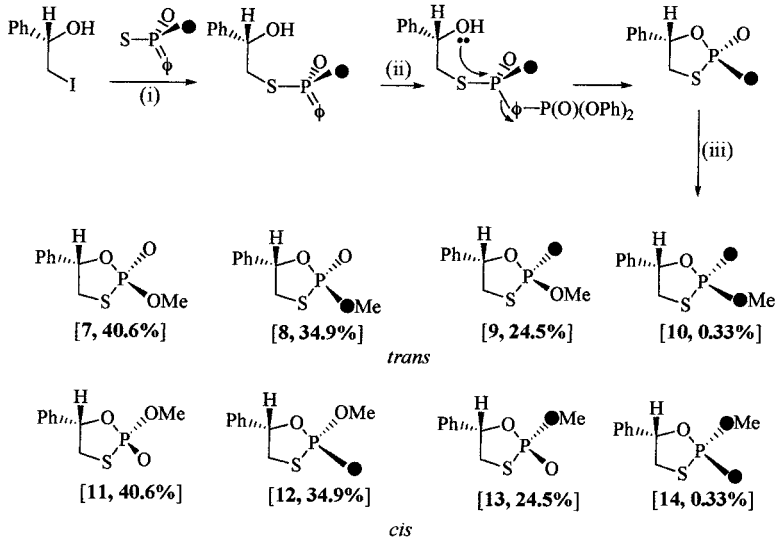
Stereochemical Study with IMPase. The hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate with $H_2{}^{18}O$ catalyzed by IMPase was completed in two experiments. In the first experiment, an aliquot of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate in $H_2{}^{18}O$ (25 μL, initial concentration 2.14 mM) was added every 2 h (8 additions) to a solution of IMPase in 97% $H_2{}^{18}O$ at 25 °C. The reaction was monitored 1 h after the addition of the first aliquot and shown to contain 1.14 mM of *myo*-inositol and 1.00 mM of substrate. To minimize substrate inhibition, aliquots of substrate were added every 2 h to allow the hydrolysis to proceed to near completion between additions. After 8 aliquots, the ^{31}P NMR (D_2O) spectrum gave δ 32.5 (29.4%, PS_i), 45.0 (58.0%, D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate), and 46.7 ppm (12.6%, *myo*-inositol 4/6-thiophosphate). The hydrolysis was not complete as some accumulation of substrate caused inhibition. In the second experiment, the concentration of substrate was halved; aliquots of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate (12 μL, initial concentration 1.07 mM) were added every 2 h (14 additions) to the IMPase solution in $H_2{}^{18}O$. The enzymatic reaction was monitored 90 min after the addition of the first aliquot and shown to contain 1.0 mM *myo*-inositol, with only 0.1 mM starting material remaining. After 14 aliquots, 12.15 mM of *myo*-inositol was formed with 3.25 mM D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate remaining. After complete addition, the ^{31}P NMR (D_2O) spectrum gave δ 5.5 (14.9%, P_i), 32.2 (39.7%, PS_i), 44.8 (35.4%, D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate), and 46.5 ppm (10.0%, *myo*-inositol 4/6-thiophosphate). Hydrolysis was more complete in the second experiment than in the first; however, 15% P_i was formed in the longer second experiment, arising from desulfurization of PS_i.

For each experiment, inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate was separated from D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate by anion exchange chromatography. By MS, the experimental ^{18}O incorporation during enzymatic hydrolysis was estimated to be 83%, with the remaining isotope composition being 1% ^{17}O and 16% ^{16}O. The reactions gave a total of 23 μmol of inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate (based on PS_i having an ϵ of 4200 $M^{-1}\ cm^{-1}$ at 225 nm, pH 7),[36] which was subjected to stereochemical analysis.

Arnold, Bethell, and Lowe developed a method for the stereochemical analysis of labeled inorganic thiophosphate, which is shown in Scheme 4 for inorganic R_p-[^{16}O,^{17}O,^{18}O]-thiophosphate.[37] Each ^{16}O, ^{17}O, or ^{18}O oxygen of the thiophosphate has an equal chance of reacting with diphenyl phosphorochloridate and therefore has an equal chance of being eliminated in the cyclization reaction. The isotopomeric cyclic phosphothiolate triesters derived from the chiral inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate were analyzed by high-field ^{31}P NMR spectroscopy (Figure 3). Any products containing an ^{17}O atom bonded to phosphorus are silent in the ^{31}P NMR spectrum. In addition, the incorporation of ^{17}O and ^{18}O into chiral inorganic thiophosphate is not 100% because both of the commercial isotopically labeled waters contain some of the other isotopes. A total of 12 triesters are formed, but only 8 are observable by ^{31}P NMR, the percentages of which are given in Scheme 4. In this experiment, only 6 species were detected because **10** and **14** did not appear in the ^{31}P NMR spectrum as there was only 1.2% ^{18}O in $H_2{}^{17}O$. Compounds with only ^{16}O, **7**, and **11** (Scheme 4) give rise to the most downfield peaks in the ^{31}P NMR spectrum.

From the chiral inorganic R_p-thiophosphate, compounds **8** and **12**, which contain ^{18}O−P and ^{18}O=P, respectively, are the major products. Comparison of the ratios (**8**/**9** and **12**/**13**) of the peak heights determines if the inorganic thiophosphate is of the *R* or *S* configuration at phosphorus.

Scheme 4. Stereochemical Analysis of Chiral Inorganic [^{16}O,^{17}O,^{18}O]-Thiophosphate, Showing the Cyclization Reaction that Eliminates ^{17}O[a,b]



[a] Shown are the eight triester products, which are observed by ^{31}P NMR spectroscopy. The percent of each species given is from mass spectral data with ^{17}O incorporation being 69% ^{16}O, 30% ^{17}O, and 1% ^{18}O and ^{18}O incorporation being 16% ^{16}O, 1% ^{17}O, and 83% ^{18}O. [b] Reagents: (i) 2[HN^{+}(*n*-octyl)$_3$], DMF. (ii) (PhO)$_2$POCl, NBu$^n_3$. (iii) CH_2N_2.

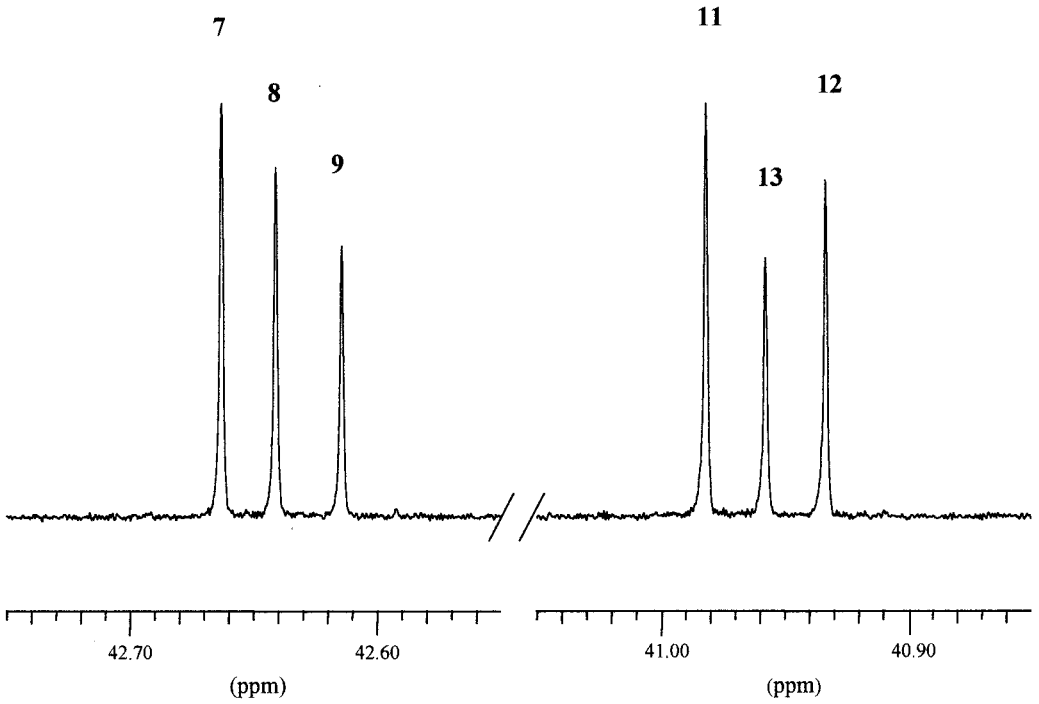


Figure 3. ^{31}P NMR spectrum (Bruker ARX400; 162 MHz) of the sample from the configurational analysis of inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate derived from the hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate in $H_2{}^{18}O$ catalyzed by recombinant bovine IMPase. (The spectrum was recorded at a digital resolution of 0.038 Hz per point and processed without resolution enhancement, with natural line widths of 0.27 Hz.)

From the mass spectral data of both the labeled substrate and the product, the predicted experimental ratios for the resonances from the trans ester (**7**:**8**:**9**) for inversion of configuration are 40.6:34.9:24.5, whereas for retention of configuration the ratios would be 40.6:24.5:34.9. The experimental ratios **7**:**8**:**9** of 40.5:33.5:26 (obtained from the peak heights in the ^{31}P NMR spectrum) are in excellent agreement with a complete inversion of configuration at phosphorus for the hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate to PS_i catalyzed by IMPase.

This result is entirely consistent with an in-line nucleophilic displacement reaction (Scheme 1a). It rules out a mechanism involving a P-E intermediate[10] and also the adjacent displacement reaction accompanied by pseudorotation (Scheme 1b).[17,38] It also argues against a mechanism involving a free metaphosphate intermediate (providing that this intermediate was able to rotate in the active site) since this would be accompanied by racemization of the stereochemistry at phosphorus. These observations are consistent with the "Merck" model for the hydrolysis mechanism, which is

based on their X-ray structural work.[6−9] In this mechanism, the nucleophilic water is coordinated to Mg^{2+}-1 and is activated by Glu-70 and Thr-95, allowing approach in-line with the inositol leaving group.

The stereochemical result has already led Gani and co-workers to revise their proposal regarding the crucial 6-hydroxy group in D-*myo*-inositol 1-phosphate: there is reduced catalytic activity or inhibition with substrate analogues that lack the 6-OH (e.g., D-6-*O*-methylinositol 1-phosphate and 6-deoxyinositol 1-phosphate).[39,40] They have now proposed that the 6-OH group hydrogen bonds with a water molecule bound to Mg^{2+}-2, while the nucleophilic water is associated with Mg^{2+}-1.

Conclusion

The stereochemical course of the reaction at phosphorus catalyzed by IMPase has been elucidated by the hydrolysis of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate in $H_2{}^{18}O$. Analysis of the chiral inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate product showed that the reaction proceeded with inversion of configuration (Scheme 1a), which is only consistent with the in-line displacement mechanism. It is interesting to note that fructose 1,6-bisphosphatase utilizes two magnesium cations, is sensitive to lithium, and also proceeds with inversion of stereochemistry at phosphorus.[39,41]

IMPase has been implicated in manic depression, and lithium inhibition is used currently in the treatment of this disease. Lithium concentrations are difficult to control, and new inhibitors for the treatment of manic depression are required.[2] The results of the present study provide the solid platform of structure/mechanism required in the design of novel inhibitors for IMPase, especially if mechanism-based inhibitors with advantages in potency and selectivity are to be contemplated.

Experimental Section

Labeled ^{17}O water (^{16}O: 46.0%, ^{17}O: 52.8%, and ^{18}O: 1.2%) was obtained from Cambridge Isotope Laboratories. Labeled ^{18}O water (O: 1.78%, ^{17}O: 0.96%, and ^{18}O: 97.26%) was obtained from Goss Scientific Instruments Ltd. Cyclohexylammonium D-*myo*-inositol 1/3-phosphate (∼75%) containing the 2-phosphate isomer (25%), malachite green carbinol, trisodium thiophosphate dodecahydrate (97%), *myo*-inositol, labeled ^{18}O water (^{18}O: 95%), and anhydrous solvents (dimethylformamide (DMF), diethyl ether, and ethanol) were obtained from Aldrich Chemical Co. Ammonium molybdate [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$], sodium chloride, sodium hydroxide pellets, magnesium chloride hexahydrate, disodium hydrogen orthophosphate dodecahydrate, potassium chloride, sodium hydrogen carbonate, tris(hydroxymethyl)methylamine, and standard pH buffers were obtained from BDH Chemicals Ltd. Low molecular weight protein standards, Phast gel sample applicator, Phast gel sodium dodecyl sulfate (SDS) buffer strips, Phast system separation, and control machine unit gels were obtained from Pharmacia, U.K. *myo*-Inositol dehydrogenase from *Enterobacter aerogenes* and bovine serum albumin (BSA, crystallized and lyophilised) were obtained from Sigma Chemical Company.

Routine NMR spectra were recorded on a JEOL NMR-EX270 spectrometer for ^{1}H (270.0 MHz), ^{31}P (109.2 MHz), and ^{13}C (67.8 MHz) data. The ^{31}P NMR (162 MHz) spectrum of the sample from the stereochemical analysis was recorded on a Bruker ARX400 spectrometer. ^{1}H spectra were referenced to tetramethylsilane for samples run in $CDCl_3$ and relative to benzene (at 7.44 pm) for samples run in D_2O. ^{31}P NMR spectra were referenced to 85% phosphoric acid (upfield chemical shifts are negative). ^{13}C NMR spectra were referenced to $CDCl_3$ (δ_C 77.0 ppm) or CH_3CN (δ_C 1.6 ppm) and benzene (δ_C 128.0 ppm) in D_2O. All ^{13}C and ^{31}P NMR spectra were ^{1}H-decoupled. Infrared spectra were recorded on a Philips PU 9516 infrared spectrophotometer. MS data were recorded in the Department of Chemistry, Manchester University. Fast atom bombardment (FAB) and chemical ionization (CI) mass spectra were recorded on a Fisons VG Trio 2000 mass spectrometer. FAB mass spectra were recorded with a mNBA matrix, while CI mass spectra were recorded using NH_3 as carrier gas. ESI mass spectra were recorded on a Micromass Platform spectrometer using acetonitrile−water (1:1) as the mobile phase. Melting points were determined using a Gallenkamp melting point apparatus. Optical rotations were measured on an AA-100 polarimeter (sensitivity 1 millidegree).

The solvents used were dried by heating under reflux over the appropriate drying agent for 1 h, followed by distillation with tetrahydrofuran (THF) (sodium wire, benzophenone) and triethylamine (KOH).

Flash column chromatography[42] was performed using chromatography grade silica gel 60 (35−75 μm, Prolabo). Thin-layer chromatography (TLC) was carried out using Merck 60 F_{254} silica gel TLC plates, and the components were revealed with phosphomolybdic acid reagent (PMA) in MeOH and molybdic acid reagent (1 g of molybdic acid in 30 mL concentrated H_2SO_4, diluted with 50 mL water) or observed by 254 nm UV lamp. TLC of phosphorus compounds was performed using Whatman K6 Silica gel on glass TLC plates. Spots were visualized using malachite green spray reagent.[43] Preparation of the Dowex-50 (H^+ form, length = 10 cm, diameter = 3 cm) and the Dowex-50 (Na^+ form, length = 20 cm, diameter = 2 cm) resin cation exchange columns was as follows: Two columns were prepared approximately two-thirds full of Dowex-50 (Dowex 50-X8, 20−50 Mesh). These were washed with H_2O (500 mL), 6 M HCl (500 mL), and then H_2O (500 mL) to give both columns in the H^+ form. One of the columns was washed with 6 M NaOH (500 mL) and then H_2O (500 mL) to obtain Dowex-50 in the Na^+ form. Anion exchange chromatography was performed using a fraction collector (Pharmacia LKB 2211-010 SuperRac). A conductivity meter (conductance bridge, Griffin and George Ltd.) with conductivity cell Griffin (PJK-320-518Q, platinum black) was used to measure the conductance (from 0.1 to 1 Ω^{1-}) of the triethylammonium bicarbonate (TEAB) buffer at different strengths and of the anion exchange loading sample. Anion exchange was performed with an analytical grade anion exchange resin (AG 1-X8, 200−400 mesh, formate form, BIO-RAD, total capacity of 2.9 mequiv mL^{-1} resin bed).

To determine concentrations of (organic) phosphate or thiophosphate solutions, samples were oxidized with perchloric acid to give inorganic phosphate, which was assayed at 660 nm as a complex with ascorbic acid and ammonium molybdate.

IMPase, purified from bovine brain,[44] was stored in 60% $(NH_4)_2SO_4$ containing 10 mM ethylenediaminetetraacetic acid (EDTA). Assay conditions of pH 8.0, 37 °C, 50 mM Tris/HCl containing KCl (100 mM), $MgCl_2$ (7 mM), and *myo*-inositol 1-monophosphate (1 mM) gave a specific activity of 13 μmol min^{-1} mg^{-1}. Electrophoresis was carried out using the Pharmacia Phast system with manual gel staining using precast Phastgel Gradient 10−15 gels and PhastGel SDS buffer strips, which contained 0.2 M Tricine, 0.2 M Tris, and 0.55% SDS, pH 8.1, in a 3% agarose isoelectric focusing support. Gels were stained with Coomassie blue stain. Protein concentration was determined by the method of Bradford (1976)[45] using BSA as standard.

Ultrafiltration (10 YM 10 (25 mm) 136 12 DIAFLO membrane, Amicon Corporation) of the enzyme was used to dilute the storage EDTA concentration (10 mM) to 10 μM against 3 times 10 mL of Tris/HCl buffer (10 mM, pH ∼8.1) in the presence of the antioxidant dithioerythritol (DTE) (0.5 mM) as required. The diluted solution of protein was concentrated to a volume of 1 mL and stored in a sterilized Eppendorf tube in the freezer (∼ −30 °C). All metal ion solutions were prepared in 50 mM Tris/HCl, pH 8.0. A stock solution of antioxidant DTE (5mM) was prepared in 50 mM Tris/HCl, pH ∼8.1. The

pH required was obtained by addition of HCl or NaOH. The pD value was calculated from the measured pH value.[46]

Kinetic parameters were determined by fitting the data to the appropriate equation by nonlinear least squares regression analysis using Grafit[29] (Erithacus Software Ltd., distributed by Sigma Chemical Co.).

Measurements of pH values were taken with a Corning digital 240 pH meter and BDH Colorkey buffer standards were used as reference solutions. Values of pH of small volumes of buffer were determined using a Kent EIL 7005 pH meter calibrated with standard buffers at 20 °C. Enzyme assays, spectral scans, and absorbance readings at fixed wavelengths were performed using either a Perkin-Elmer λ3 spectrophotometer thermostated to 37 °C with a Haake GH water bath or a Cary UV/vis spectrophotometer thermostated to 25 °C using a Cary temperature controller. Poly(methyl methacrylate) (PMMA) plastic cuvettes or quartz cuvettes were used for readings in the UV region at 280 and 340 nm; otherwise, disposable polystyrene (PS) plastic cuvettes were used for readings in the visible region. All buffer solutions were prepared using analytical grade reagents and water, which had been distilled, deionized, and then treated using a Milli-Q purification system. All stock solutions (except buffer) were stored over ice during kinetic experiments.

(D)-2,3:5,6-Di-*O*-cyclohexylidene 1-[(2*S*,4*R*,5*S*)-3,4-Dimethyl-5-phenyl-2-sulfide-1,3,2-oxazaphospholidinyl]*myo*-inositol (3) and (D)-1,2:4,5-Di-*O*-cyclohexylidene 3-[(2*S*,4*R*,5*S*)-3,4-Dimethyl-5-phenyl-2-sulfide-1,3,2-oxazaphospholidinyl]*myo*-inositol (4). (D/L)-1,2:4,5-Di-*O*-cyclohexylidene-*myo*-inositol[24] (2.57 g, 7.5 mmol) and 60% NaH (1.6 equiv, 0.297 g, 12.4 mmol) in dry DMF (25 mL) was stirred for 1 h at 0 °C under argon. To this reaction mixture was added dropwise (2*R*,4*R*,5*S*)-(+)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidin-2-sulfide (1.974 g, 7.5 mmol) in dry DMF (7.5 mL), and the mixture was stirred for 2 h allowing the temperature to rise to room temperature. Flash chromatography on silica gel eluting with EtOAc−hexane (1:5, 1:3, and then 1:2) gave one pair of diastereomers as the major product, 42.7% (1.286 g): R_f = 0.36 (EtOAc−hexane, 1:2). ^{31}P NMR ($CDCl_3$) δ_P (ppm): 81.4 (s, 39%, **4**) and 83.1 (s, 61%, **3**). ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.83 (d, J_{HH} = 6.6 Hz, **3**) and 0.84 (d, J_{HH} = 6.6 Hz, **4**), (total 3H, CHCH_3), 2.75 (d, J_{PH} = 12.9 Hz, **3**) and 2.76 (d, J_{PH} = 12.5 Hz, **4**), (total 3H, NCH_3), 4.56 (t, J_{HH} = 4.6 Hz, **3**) and 4.69 (t, J_{HH} = 4.6 Hz, **4**), (total 1H, H-2). IR (Nujol): ν_{max} 3410 (OH) cm^{-1}.

Diastereomers **3** and **4** were separated by HPLC on a preparative C_{18} reverse phase column, eluting with CH_3CN−H_2O (60:40). The diastereomer that eluted first (160 mg) with a retention time of 34.2 min was **4**. ^{31}P NMR (d_4-MeOH) δ_P (ppm): 81.0 (s). ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.84 (3H, d, J_{HH} = 6.6 Hz, CHCH_3), 1.2−1.8 (20H, m, 10 × CH_2 of cyclohexylidene), 2.53 (1H, d, J_{HH} = 3.0 Hz, 6-OH), 2.76 (3H, d, J_{PH} = 12.5 Hz, NCH_3), 3.42 (1H, dd, J_{HH} = 10.6 Hz, J_{HH} = 9.6 Hz, H-5), 3.6−3.8 (1H, m, C*H*CH_3), 3.8−3.95 (1H, m, H-6), 4.0−4.1 (2H, m, H-1/H-4), 4.69 (1H, t, J_{HH} = 4.6 Hz, H-2), 4.8−5.0 (1H, m, H-3), 5.68 (1H, dd, J_{HH} = 6.3 Hz, J_{HH} = 3.0 Hz, C*H*Ph), 7.3−7.4 (5H, m, Ph). ^{13}C NMR ($CDCl_3$) δ_C (ppm): 13.8 (s, CH*C*H_3), 23.6, 23.7, 23.8, 24.1, 24.9, 25.1, 34.9, 36.3, 36.5, 37.9 (10 × *C*H_2 of cyclohexylidene), 29.3 (d, J_{PC} = 6.1 Hz, N*C*H_3), 59.8 (d, J_{PC} = 9.7 Hz, *C*HCH_3), 75.0 (d, J_{PC} = 6.1 Hz, inositol-CH), 75.2 (inositol-CH), 75.3 (d, J_{PC} = 6.1 Hz, inositol-CH), 76.0 (inositol-CH), 77.7 (inositol-CH), 81.3 (inositol-CH), 82.3 (*C*HPh), 110.7, 113.3 (C-1 of cyclohexylidene), 126.1, 128.1, 128.2 (CH of Ph), 136.1 (d, J_{PC} = 6.1 Hz, C-1 of Ph).

The diastereomer that eluted second (360 mg) with a retention time of 35.7 min was **3**. ^{31}P NMR (d_4-MeOH) δ_P (ppm): 82.4 (s). ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.84 (3H, d, J_{HH} = 6.6 Hz, CHCH_3), 1.2−1.75 (20H, m, 10 × CH_2 of cyclohexylidene), 2.59 (1H, d, J_{HH} = 3.0 Hz, 4-OH), 2.75 (3H, d, J_{PH} = 12.5 Hz, NCH_3), 3.42 (1H, dd ~ appears at t, J_{HH} ~ 10.1 Hz, H-5), 3.6−3.8 (2H, m, C*H*CH_3), 3.8−3.95 (1H, m, H-4), 4.0−4.1 (2H, m, H-3/H-6), 4.56 (1H, t, J_{HH} = 4.6 Hz, H-2), 4.9−5.05 (1H, m, H-1), 5.63 (1H, dd, appears as t, J_{HH} ~J_{PH} ~ 5.8 Hz, C*H*Ph), 7.3−7.4 (5H, m, Ph). ^{13}C NMR data ($CDCl_3$, assignments made with the aid of a DEPT spectrum) δ_C (ppm): 14.4 (d, J_{PC} = 2.5 Hz, CH*C*H_3), 23.5, 23.6, 23.9, 24.0, 24.9, 29.7, 34.8, 36.3, 36.5, 38.1 (10 × *C*H_2 of cyclohexylidene), 29.5 (d, J_{PC} = 7.3 Hz, NCH_3), 59.5 (d, J_{PC} = 11 Hz, *C*HCH_3), 75.2 (d, J_{PC} = 6.1 Hz, inositol-CH), 75.6 (inositol-CH), 75.7 (inositol-CH), 76.5 (inositol-CH), 77.8 (inositol-CH), 81.4 (inositol-CH), 82.4 (*C*HPh), 110.9, 113.3 (C-1 of cyclohexylidene), 126.5, 128.2 (*C*H of Ph, 1 overlapping), 136.2 (d, J_{PC} = 4.9 Hz, C-1 of Ph). IR (Nujol): ν_{max} 3405 (OH) cm^{-1}; *m/z* (CI) 566 (M + H$^+$, [^{12}C] 12.3%), 567 (M + H$^+$, [^{13}C] 4%), 148 (Ph$^+$CH−CHMe−NMeH, 100%).

To identify each diastereomer, 1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol 3,6-bis-camphanate and 2,3:5,6-di-*O*-cyclohexylidene-*myo*-inositol 1,4-bis-camphanate were prepared[26] from (D/L)-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol and *S*-(−)-camphanic chloride. The less polar diastereomer was 1,2:4,5-di-*O*-cyclohexylidene 3,6-bis-camphanate, which was deprotected using KOH in absolute ethanol to give D-(−)-1,2:4,5-di-*O*-cyclohexylidene *myo*-inositol in 99% yield. This diol was phosphorylated using (2*R*,4*R*,5*S*)-(+)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidin-2-sulfide to give **4** with all of the spectroscopic data in agreement with those reported above.

(1*S*,2*R*)-(2-Methylamino-1-phenylpropyl) 1-*myo*-Inositol-(*R*$_p$)-[^{16}O,^{17}O]-thiophosphate (5). A solution of diastereomer **4** (0.167 g, 0.295 mmol) in THF (0.5 mL) was added to a solution of trifluoroacetic anhydride (0.38 mL, 2.83 mmol) in the presence of labeled ^{17}O water (0.5 mL, 52.8% ^{17}O) under argon at ~5 °C. The mixture was stirred for 30 min, and then, the solution was concentrated under vacuum. Water (20 mL) and diethyl ether (30 mL) were added, and the aqueous layer was separated and concentrated under vacuum. The reaction mixture gave optically pure ^{17}O-labeled zwitterion (**5**) (120 mg, 96%) in the presence of ^{16}O- and ^{18}O-labeled zwitterions, which were observed by ^{31}P NMR. ^{31}P NMR (D_2O) δ (ppm): 56.51 (s, 1.7%, [^{16}O,^{16}O]-zwitterion phosphorylated at position 3), 56.98 (s, 70.54%, [^{16}O,^{16}O]-zwitterion phosphorylated at position 1), 57.4 (s, 3.4%, impurities), 58.6 (s, 13.5%, [^{16}O,^{16}O]-zwitterion phosphorylated at position 6), 58.9 (s, 10.5%, [^{16}O,^{16}O]-zwitterion phosphorylated at position 4). ^{1}H NMR (D_2O, referenced to water at 4.7 ppm) δ (ppm): 0.87 (3H, d, J_{HH} = 6.93 Hz, CHCH_3), 2.52 (3H, s, NCH_3), 3.06 (1H, t, J_{HH} = 9.24 Hz, H-5), 3.25−3.45 (2H, m, H-1/H-3), 3.53 (1H, dd appears as a br t, J_{HH} = 9.9 Hz, J_{HH} = 9.57 Hz, H-2), 3.96−4.02 (2H, m, H-4/H-6), 5.4−5.5 (1H, m, C*H*Ph), 7.15 (5H, m, Ph). *m/z* (ES$^-$): 422 (M − H$^+$ [^{16}O,^{16}O], 100), 423 (M − H$^+$ [^{16}O,^{17}O] or M − H$^+$ [^{16}O,^{16}O,1^{13}C], 65.24), 424 (M − H$^+$ [^{16}O,^{18}O] or M − H$^+$ [^{16}O,^{17}O,1^{13}C], 10.97). Considering the natural ^{13}C content, this mass spectral data corresponds to 54% [^{16}O,^{16}O], 36% [^{16}O,^{17}O].

D-1-*S*$_p$-*myo*-Inositol [^{17}O]-Thiophosphate (6). A small piece of sodium was added to distilled liquid NH_3 (5 mL) to give a blue solution, and the labeled zwitterion (**5**) (122 mg, 0.29 mmol) was added. The reaction mixture was stirred for 30 min at − 78 °C, the reaction was quenched by the addition of EtOH (5 mL), and the reaction mixture was allowed to warm to room temperature. Water (20 mL) was added and then Dowex-50 (H$^+$ form) resin to adjust the pH to 5. This solution was applied to a Dowex-50 (Na$^+$ form) resin column and eluted with H_2O (100 mL). The eluant was concentrated on a high vacuum rotary evaporator to give disodium D-1-*S*$_p$-*myo*-inositol [^{17}O]-thiophosphate (80 mg, 86%), which was stored under argon in the freezer. ^{31}P NMR (D_2O) δ (ppm): 44.85 (s, 74.3%, *myo*-inositol 1-[^{16}O,^{16}O]-thiophosphate), 46.6 (s, 25.7%, *myo*-inositol 4-[^{16}O,^{16}O]-thiophosphate and *myo*-inositol 6-[^{16}O,^{16}O]-thiophosphate). Compound **6** is not observed by ^{31}P NMR spectroscopy. ^{1}H NMR (270.0 MHz, D_2O, referenced to water at 4.7 ppm) δ (ppm): 1.79 (1H, s, impurity not assigned), 3.24 (1H, t, J_{HH} = 8.91 Hz, H-5), 3.42−3.57 (2H, m, H-3/H-4), 3.65 (1H, dd appears as a br t, J_{HH} = 9.6 Hz, J_{HH} = 9.9 Hz, H-6), 3.99 (1H, dd appears as br t, J_{PH} ~ $J_{1/6}$ ~ 10.4 Hz, H-3), 4.18 (1H, br s, H-2). *m/z* (ES$^-$): 275 (M-2Na$^+$ + H$^+$ [^{16}O,^{16}O], 59.3), 276 (M-2Na$^+$ + H$^+$ [^{16}O,^{17}O] or M-2Na$^+$ + H$^+$ [^{16}O,^{16}O,1^{13}C] 27.6), 277 (M-2Na$^+$ + H$^+$ [^{16}O,^{18}O] or M-2Na$^+$ + H$^+$ [^{16}O,^{17}O, 1^{13}C] 6.1). Considering the natural ^{13}C content, this mass

spectral data corresponds to 69% [^{16}O,^{16}O], 30% [^{16}O,^{17}O], and 1% [^{16}O,^{18}O].

Disodium (D)-*myo*-Inositol 1/3-Thiophosphate. This compound was prepared for kinetic studies using a method similar to that described for the labeled material except that a mixture of diastereomers **3** and **4** were used. ^{31}P NMR (D_2O) δ (ppm): 44.8 (s, 95%, 1/3-phosphorylated compounds), 46.5 (s, 5%, 4/6-phosphorylated compounds). ^{1}H NMR (D_2O): δ 3.37 (1H, t, $J_{HH} \sim 9.1$ Hz, H-5), 3.55−3.7 (2H, m, H-1 and H-6), 3.78 (1H, t, $J_{HH} = 9.7$ Hz, H-4), 4.11 (1H, br t, $J_{PH} = 10.4$ Hz, H-3), 4.31 (1H, br s, H-2). ^{13}C NMR (D_2O, referenced to acetonitrile at 1.6 ppm): δ 70.3 (s, inositol-CH), 71.0 (s, inositol-CH), 71.6 (d, $J_{PC} \sim 3.7$ Hz, inositol-CH), 71.8 (s, CH-inositol), 73.9 (s, CH-inositol), 74.2 (d, $J_{PC} \sim 4.9$ Hz, CH-inositol). IR (Nujol): ν_{max} 3250 (OH) cm^{-1}. *m/z* (ES$^-$): 275 (M − H, [^{12}C], 100%), 276 (M − H, [^{13}C], 8.1%).

Hydrolysis of D-*myo*-Inositol 1/3-Monophosphate with IMPase Monitored by ^{31}P NMR Spectroscopy. A solution of cyclohexylammonium D/L-*myo*-inositol 1-monophosphate (75% approximately, 1.3 mg, 4 mM) in 50 mM Tris/HCl buffer in D_2O (0.8 mL, pD ∼ 8.5) containing 100 mM KCl and 7 mM $MgCl_2 \cdot 6H_2O$ in the presence or absence of 5mM DTE (0.1 mL) gave ^{31}P NMR (D_2O) δ 4.8 (s, ∼77%, *myo*-inositol 1/3-monophosphate) and 5.5 (s, ∼23%, *myo*-inositol 2-monophosphate). IMPase (0.2 mL, concentration 0.54 mg mL^{-1}, after ultrafiltration) was added, and the reaction was monitored by ^{31}P NMR spectroscopy over 40 min at 37 °C, which showed the hydrolysis of *myo*-inositol 1/3-monophosphate to inorganic phosphate: δ 3.4 (s, P_i), 5.5 (d, $J_{PH} = 7.8$ Hz, *myo*-inositol 2-monophosphate). In agreement with the literature, *myo*-inositol 2-monophosphate is not a substrate for IMPase.[5]

Hydrolysis of D-*myo*-Inositol 1/3-Thiophosphate with IMPase Monitored by ^{31}P NMR Spectroscopy. The ^{31}P NMR spectrum of *myo*-inositol 1/3-thiophosphate (2 mg, 4.8 mM) in Tris/HCl buffer in D_2O (0.8 mL, pD ∼ 8.5), KCl (100 mM), $MgCl_2 \cdot 6H_2O$ (7 mM), and DTE (0.2 mL, 5mM) gave δ (D_2O) 3.4 (s, 6%, P_i), 45.1 (s, 94%). After IMPase (0.3 mL, 0.54 mg mL^{-1}, after ultrafiltration to remove EDTA) was added, the reaction was followed by ^{31}P NMR spectroscopy at 37 °C, which after 24 h, showed δ 3.4 (sharp s, P_i), 36−38 (br s, PS_i), 45.1 (sharp s, *myo*-inositol 1/3-thiophosphate). To sharpen the broad peak for PS_i to estimate the percentage of each component, the pD of the solution was adjusted to 11−12 with NaOH (1 M, 3 drops), and the ^{31}P NMR spectrum was recorded as follows: δ 5.1 (s, 20%, P_i), 16.6 (s, 2.2%, intermediate in the oxidative desulfurization of PS_i), 32.2 (sharp, s, 66.7%, PS_i), 44.8 (s, 11.1%, *myo*-inositol 1/3-thiophosphate).

Desulfurization of Inorganic Thiophosphate Monitored by ^{31}P NMR Spectroscopy. A solution of PS_i (4.6 mg, 11.6 mM) in Tris/HCl buffer (1 mL) at pD 11−12 (adjusted with 1 M NaOH) was left for 6 days at 22 °C after which reaction was assessed by ^{31}P NMR δ 5.7 (P_i, 6%), 16.6 ($^{2-}O_3P-S-S-PO_3^{2-}$, 5%), 32.2 (PS_i, 89%).

A solution of PS_i (10 mg, 25.2 mM) in Tris/HCl buffer (1 mL) at pD ∼ 8.24 was left for 6 days at 22 °C after which reaction was assessed by ^{31}P NMR δ 5.7 (Pi, 21%) and 32.2 (PS_i, 79%).

A solution of PS_i (10.7 mg, 27 mM) in Tris/HCl buffer (1 mL) at pD ∼ 8.24 was left for 3 days at 37 °C after which reaction was assessed by ^{31}P NMR δ 5.7 (Pi, 25%) and 32.2 (PS_i, 75%).

Inositol Dehydrogenase Assay. The rate of oxidation of *myo*-inositol was shown to be proportional to the concentration of inositol dehydrogenase, which was varied at constant concentrations of *myo*-inositol (0.39 M) and β-NAD$^+$ (4.75 mM). The observed reaction velocities (5.95×10^{-2} and 12.6×10^{-2} min^{-1}) were proportional to the inositol dehydrogenase concentration (1.39 and 2.78 μg mL^{-1}).

To determine the effect of the concentration of *myo*-inositol in the coupled assay at a constant concentration of inositol dehydrogenase at pH 9.13, the assay cuvettes contained 0.1 mL of β-NAD$^+$ (4.75 mM final concentration), *myo*-inositol (0.039−0.784 mM from 0.01 to 0.2 mL of a stock solution of 3.92 M *myo*-inositol in 50 mM Tris-HCl buffer, pH 9.13), and Tris-HCl buffer containing 100 mM KCl and 3.3 mM $MgCl_2 \cdot 6H_2O$, pH 9.13 (0.695−0.885 mL), to give a total volume of 1.00 mL. The mixture was incubated at 25 °C for 5 min, after which inositol dehydrogenase (5 μL, final concentration 0.0486 units mL^{-1}, 1.39 μg mL^{-1}) was added. The absorbance at 340 nm was monitored with time.

Stability of IMPase at Various pH Values. The stability of IMPase was evaluated under the conditions required for the continuous coupled enzymatic assay in the presence of a small amount of 1,10-phenanthroline as a chelating agent. The incubation media consisted of 50 mM Tris-HCl at different pH values (7.06, 8.06, or 9.00), 100 mM KCl, 3 mM $MgCl_2 \cdot 6H_2O$, 20 mM β-NAD$^+$, 0.55 mM cyclohexylammonium *myo*-inositol 1/3-phosphate, and IMPase (0.129 mg mL^{-1}). These solutions were incubated at 25 °C for 5 min, and the reaction was initiated by addition of IMPase (30 μL, final concentration 3.81 μg mL^{-1}). The absorbance at 340 nm was monitored for several minutes after intervals of 30 and 75 min incubation. The activity of IMPase was not detectably changed by incubation at pH 7.06, 8.06, or 9.00.

Validation of the Coupled Enzyme Assay. The coupled enzyme assay (Scheme 3) was arranged so that the reaction catalyzed by IMPase was rate-determining, with inositol dehydrogenase in excess; the observed velocity was independent of inositol dehydrogenase and proportional to IMPase under the optimized coupled assay conditions. To establish the concentration of inositol dehydrogenase required that its concentration was varied, with constant concentrations of *myo*-inositol 1/3-phosphate, IMPase, and β-NAD$^+$, until the rate of the overall enzymatic coupled assay was constant. Assays were completed at concentrations of *myo*-inositol 1/3-phosphate both around the K_m value (0.1 mM) and less than the K_m value (0.038 mM). The minimum quantity of inositol dehydrogenase required was 0.14 units mL^{-1}. When the concentration of inositol dehydrogenase was doubled (0.284 units mL^{-1}), the rate of the enzymatic reaction remained the same for 0.1 and 0.038 mM *myo*-inositol 1/3-phosphate.

The rate of reaction was shown to be proportional to the concentration of IMPase, which was varied at constant concentrations of *myo*-inositol 1/3-phosphate (0.1 mM), β-NAD$^+$ (1.99 mM), and inositol dehydrogenase (0.14 units mL^{-1}). The observed velocities of the reaction (6.12×10^{-3}, 1.1×10^{-2}, 1.3×10^{-2}, and 1.77×10^{-2} ΔA min^{-1}) were proportional to the concentrations of IMPase (7.36, 13.9, 20.78, and 27.6 μg mL^{-1}), respectively. Under similar conditions, the velocities (3.71×10^{-3}, 5.49×10^{-3}, and 7.95×10^{-3} ΔA min^{-1}) of the reaction were also proportional to the concentration of IMPase (6.3, 12.55, and 18.73 μg mL^{-1}) at a *myo*-inositol monophosphate concentration of 0.038 mM. Assays with D-*myo*-inositol 1/3-thiophosphate also needed a concentration of inositol dehydrogenase of 0.14 units mL^{-1}.

Kinetic Study of D-*myo*-Inositol 3-Phosphate Using the Inositol Dehydrogenase Coupled Assay. The cuvettes contained 0.1 mL of β-NAD$^+$ (5.22 mM), 20 μL of inositol dehydrogenase (0.19 units mL^{-1}), sodium D-*myo*-inositol 3-phosphate (final concentrations: 14.83, 11.92, 6.82, 3.41, 2.22, 1.02, 0.68, 0.34, 0.17, and 0.085 mM), and 50.4 mM Tris-HCl buffer containing 102 mM NaCl and 3.37 mM $MgCl_2 \cdot 6H_2O$ at pH 9.10. This mixture was incubated at 25 °C for 5 min, and the reaction was initiated by addition of IMPase (5 μL, final concentration 2.9 μg mL^{-1}). The change in absorbance at 340 nm was monitored over several minutes.

Kinetic Study of D-*myo*-Inositol 1/3-Thiophosphate Using the Inositol Dehydrogenase Coupled Assay. The cuvettes contained 0.1 mL of β-NAD$^+$ (5 mM), 20 μL of inositol dehydrogenase (0.19 units mL^{-1}), *myo*-inositol 1/3-thiophosphate (final concentrations: 7.4, 6.56, 4.23, 2.63, 2.12, 1.27, 0.95, 0.85, 0.74, 0.64, 0.53, 0.42, 0.32, 0.21, or 0.11 mM), and 50.4 mM Tris-HCl buffer containing 102 mM NaCl and 3.4 mM $MgCl_2 \cdot 6H_2O$ at pH 9.00. This mixture was incubated at 25 °C for 5 min, and then, the reaction was initiated by the addition of IMPase (10 μL, final concentration 6.7 μg mL^{-1}). The change in absorbance at 340 nm was monitored over several minutes.

Standard Curve of *myo*-Inositol. After *myo*-inositol 1/3-thiophosphate was hydrolyzed, the *myo*-inositol formed was quantitated by means of a standard curve. The cuvettes contained 0.1 mL of β-NAD$^+$ (final concentration: 4.42 mM), *myo*-inositol (0.00349−0.0349 mM from 0.01 to 0.1 mL of a stock solution of 0.349 mM *myo*-inositol in Tris-HCl buffer (pH 8.90)), and 0.78−0.87 mL) Tris-HCl buffer (52.8 mM, pH 8.90) containing 99.8 mM NaCl and 3.6 mM $MgCl_2 \cdot 6H_2O$ to give a total volume of 1.00 mL. This mixture was incubated at 25 °C for 5 min after which time the reaction was initiated by the addition of inositol dehydrogenase (20 μL, final concentration 0.107 92 units mL^{-1}, 1.08×10^{-2} mg mL^{-1}). The absorbance at 340 nm was monitored with time until a steady state was reached. The absorbance (0.006 23−0.0693) was proportional to the *myo*-inositol concentration (0.003 49−0.0349 mM).

Studies of Putative Interactions of IMPase with High Concentrations of *myo*-Inositol 1/3-Thiophosphate by ESI-MS. IMPase (50 μL of 0.436 mg mL^{-1}) was added to 0.95 mL of 50 mM Tris-HCl containing 100 mM NaCl and 3.44 mM $MgCl_2 \cdot 6H_2O$ at pH 8.80. To half of this mixture (0.5 mL) was added *myo*-inositol 1/3-thiophosphate (7.36 mg, 20.3 mM), and the reaction was incubated at 37 °C for 90 min. The other half of the mixture, containing only IMPase, was also incubated at 37 °C for 90 min as a control. The samples were kept at 4 °C overnight, and the Tris-HCl buffer was exchanged with ammonium acetate (5 mM) by centrifugation through an Ultrafree-MC filter (Millipore). An aliquot (250 μL) of each sample was kept at 4 °C, diluted with ammonium acetate (250 μL, 5 mM), and centrifuged until 50 μL remained. Ammonium acetate was added (4 times) to return the volume to 250 μL. The solutions were concentrated to 50 μL to give final concentrations of IMPase of ∼0.46 mg mL^{-1}. The two samples were analyzed by ESI-MS.

Desulfurization of Inorganic Thiophosphate Catalyzed by IMPase. Sodium thiophosphate tribasic dodecahydrate (0.020 63 g) was dissolved in Tris-HCl buffer (5 mL, 50.3 mM, pH 9.00, containing $MgCl_2 \cdot 6H_2O$ (3 mM) and NaCl (100.5 mM)) to give a 10.44 mM stock solution. Into three NMR tubes were added 0.6 mL of the above stock solution of PS_i and D_2O (0.1 mL). The reactions were initiated by the addition of IMPase to give final enzyme concentrations of 0.119, 0.235, and 0.343 mg mL^{-1} corresponding to 3.97×10^{-6}, 7.82×10^{-6}, and 11.43×10^{-6} M. The samples were incubated at 25 °C, and the ^{31}P NMR spectra were recorded after 24, 48, and 72 h. The area of the peaks of PS_i remaining and P_i formed were analyzed by weighing the paper cut-outs of the peaks. Desulfurization with time (h) is shown in Table 1.

Large-Scale Enzymatic Reaction with Unlabeled *myo*-Inositol 1/3-Thiophosphate. IMPase (10.72 mg) was added to Tris-HCl buffer (1.42 mL, 49.8 mM, pH 9.00) containing $MgCl_2 \cdot 6H_2O$ (3 mM) and NaCl (100 mM). *myo*-Inositol 1/3-thiophosphate (12.19 mg, stock solution: 476.0 mM) was dissolved in Tris-HCl buffer (80 μL, pH 9.00). An aliquot of *myo*-inositol 1/3-thiophosphate solution (10 μL, final concentration: 3.33 mM) was added every 2 h (for 6 additions) to the solution containing IMPase (7.15 mg mL^{-1}) at 25 °C. After the first aliquot was added, the enzymatic reaction mixture (0.01 mL) was added to Tris-HCl buffer (0.87 mL, pH 9.00), β-NAD$^+$ (0.1 mL, final concentration 4.68 mM), and inositol dehydrogenase (0.02 mL). A slope of 0.0098 ΔA min^{-1} at 340 nm corresponded to a concentration of 2.5 mM of *myo*-inositol. The initial concentration of the substrate was 3.33 mM; therefore, 0.83 mM of starting material remained. After 11 h and 6 aliquots was added, the *myo*-inositol assay (0.002 mL of aliquot, 0.878 mL of Tris-HCl, 0.1 mL of β-NAD$^+$, and 0.02 mL of inositol dehydrogenase) gave a slope of 0.0096 ΔA min^{-1} corresponding to a concentration of 12.23 mM. The concentration of the sample was 19.98 mM *myo*-inositol, with 7.75 mM starting material remaining.

After 12 h, the reaction was stopped by the addition of NaOH solution (4 drops, 1 M), and ^{31}P NMR spectroscopy (109.2 MHz, D_2O) gave δ (ppm) 4.76 (s, 19.6%, P_i), 31.45 (s, 57.7%, PS_i), 44.02 (s, 19.9%, *myo*-inositol 1/3-thiophosphate), 45.74 (s, 2.8%, *myo*-inositol 4/6-thiophosphate). Separation of inorganic thiophosphate from *myo*-inositol, inorganic phosphate, and *myo*-inositol 1/3-thiophosphate was performed by anion exchange chromatography. Fractions 25−31 corresponded to *myo*-inositol 1/3-thiophosphate, and fractions 38−42 corresponded to inorganic thiophosphate.

Hydrolysis of D-1-S_p-*myo*-Inositol [^{17}O]-Thiophosphate (6) in $H_2{}^{18}O$ Catalyzed by IMPase. First reaction: Tris-HCl buffer (2 mL, 50.0 mM, pH 9.00) containing $MgCl_2 \cdot 6H_2O$ (3.0 mM) and NaCl (100.4 mM) was lyophilyzed overnight. To this was added $H_2{}^{18}O$ (400 μL), and lyophilyzation of the resulting solution was repeated. $H_2{}^{18}O$ (1.8 mL) was added under argon, followed by IMPase (16.34 mg previously lyophilyzed with 250 μL of $H_2{}^{18}O$), and the solution was incubated at 25 °C under argon for the duration of the experiment. D-1-S_p-*myo*-Inositol [^{17}O]-thiophosphate (10.5 mg, 0.033 mmol) was lyophilyzed with $H_2{}^{18}O$ (250 μL), and the resulting solid was dissolved in Tris-HCl buffer (pH 9.00) in $H_2{}^{18}O$ (210 μL) to give a 157 mM substrate stock solution.

An aliquot of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate stock solution (25 μL, 2.14 mM) was added every 2 h (8 additions) to the IMPase solution at 25 °C. One hour after the addition of the first aliquot, the formation of *myo*-inositol was measured. An aliquot of the enzymatic reaction mixture (0.01 mL) was added to Tris-HCl buffer (0.87 mL, pH 9.00), β-NAD$^+$ (0.1 mL, 4.89 mM), and inositol dehydrogenase (0.02 mL). A slope of 0.0067 ΔA min^{-1} at 340 nm corresponded to a concentration of 1.14 mM of *myo*-inositol. The initial concentration of the substrate was 2.14 mM; therefore, 1.00 mM starting material remained. Aliquots were added every 2 h. After the 8 additions, the reaction was put in the fridge to slow the hydrolysis and then quenched by the addition of aqueous NaOH (4 drops, 1 M, pH 13.0). ^{31}P NMR spectroscopy (109.2 MHz, D_2O) gave δ (ppm) 32.46 (s, 29.4%, inorganic thiophosphate), 45.00 (s, 58%, *myo*-inositol 1-thiophosphate), 46.74 (s, 12.6%, *myo*-inositol 6-thiophosphate. The mixture was subjected to anion exchange chromatography. Fractions 25−31 corresponded to D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate**,** and fractions 37−43 corresponded to inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate (observed by UV at 222 nm) and were analyzed by ^{31}P NMR spectroscopy. Fractions 37−43 were concentrated by evaporation and freeze-dried overnight, dissolved in distilled water (3 mL), and diluted 10-fold. An absorbance of 1.180 at 222 nm gave a concentration of 2.8 mM of inorganic thiophosphate, which corresponds to 8.4 μmol (based on PS_i having an ϵ of 4200 M^{-1} cm^{-1} at 225 nm, pH 7).[36]

Second reaction: This was identical to the first reaction except that the 160 mM stock solution of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate (10.8 mg, 0.034 mmol) in Tris-HCl buffer/$H_2{}^{18}O$ (pH 9.00, 210 μL) was added in 15 × 12 μL aliquots every 2 h to the buffer containing 14.4 mg of IMPase in $H_2{}^{18}O$ at 25 °C. Ninety minutes after the addition of the first aliquot, the inositol dehydrogenase assay showed the formation of 1.00 mM of *myo*-inositol. The initial concentration of the substrate was 1.1 mM; therefore, 0.1 mM starting material remained. Aliquots were added every 2 h to avoid accumulation of starting material. After 28 h (14 aliquots), another inositol dehydrogenase assay gave a *myo*-inositol concentration of 12.15 mM. The total concentration of added substrate was 15.4 mM; therefore, 3.25 mM of D-1-S_p-*myo*-inositol [^{17}O]-thiophosphate remained. After all of the additions, the reaction was put in the fridge to slow hydrolysis and stopped by the addition of aqueous NaOH (3 drops, 1 M, pH 12.0). ^{31}P NMR spectroscopy (109.2 MHz, D_2O) δ (ppm) gave 5.53 (s, 14.9%, inorganic phosphate), 32.23 (s, 39.7%, inorganic thiophosphate), 44.78 (s, 35.4%, *myo*-inositol 1-thiophosphate), 46.51 (s, 10.0%, *myo*-inositol 6-thiophosphate). Separation by anion exchange chromatography (as described for the first reaction) gave inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate (14.8 μmol).

Altogether, 23 μmol of inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate was isolated by anion exchange chromatography by combining both samples. This was analyzed using the method of Arnold, Bethell, and Lowe.[37]

(*S*)-1-Phenylethanol-2-S-[^{16}O,^{17}O,^{18}O]-thiophosphate. The inorganic [^{16}O,^{17}O,^{18}O]-thiophosphate (approximately 23 μmol)

isolated from the two enzymatic reactions was converted to its trioctylammonium salt and dried by evaporation of dry DMF. The residue was dissolved in DMF (0.3 mL) and treated with excess (*S*)-2-iodo-1-phenylethanol (14 mg, 56 μmol). After ca. 26 h, the reaction was shown to be complete by ^{31}P NMR, and the product was isolated by ion exchange chromatography on (diethylamino)ethyl (DEAE) Sephadex (A25) eluting with a gradient of TEAB buffer (25−250 mM; pH 7.8). (*S*)-1-Phenylethanol-2-S-[^{16}O,^{17}O,^{18}O]-thiophosphate (11 μmol based on integration of ^{1}H NMR signals with respect to dioxan added as an internal integration standard) was isolated and cyclized directly without further characterization.

***cis*- and *trans*-4-(*S*)-Phenyl-2-methoxy-2-oxo-1,3,2-thiaoxaphospholane.** The tributylammonium salt of (*S*)-1-Phenylethanol-2-S-[^{16}O,^{17}O, ^{18}O]-thiophosphate (11 μmol) was dried by evaporation of dry DMF and then reacted with diphenylphosphorochloridate (13.2 μmol) and tributylamine (11 μmol) in dry DMF (0.3 mL). After 15 min, the cyclization reaction was quenched by the addition of TEAB buffer (2 mL; 100 mM solution, pH 7.8), and the solution was evaporated to dryness and purified by ion exchange chromatography on DEAE Sephadex (A25) eluting with a gradient of triethylammonium biocarbonate buffer (5−120 mM; pH 7.8). The triethylammonium salt of 4-(*S*)-phenyl-2,2-dioxo-1,3,2-thiaoxaphospholane was converted directly to the pyridinium salt using Dowex 50W (pyridinium form), and the salt was dried by coevaporation of dry CH_3CN. The residue was dissolved in CD_3CN (0.5 mL) and treated with excess distilled diazomethane solution (dried over KOH pellets). The excess diazomethane was evaporated with a stream of dry nitrogen, and the solution was filtered and analyzed by high field ^{31}P NMR spectroscopy (Figure 3).

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