The 6-OH Group of D-Inositol 1-Phosphate Serves as an H-Bond Donor in the Catalytic Hydrolysis of the Phosphate Ester by Inositol Monophosphatase

David J. Miller,[a] Martin W. Beaton,[b] John Wilkie,[a] and David Gani*[a]

Inositol monophosphatase plays a pivotal role in the biosynthesis of secondary messengers and is believed to be a target for lithium therapy. It is established how a lithium ion works in inhibiting the enzyme but details of the mechanism for the direct magnesium ion activated hydrolysis of the substrate have been elusive. It is known that substrates require a minimal 1,2-diol phosphate structural motif, which in D-myo-inositol 1-phosphate relates to the fragment comprising the 1-phosphate ester and the 6-hydroxy group. Here it is shown that inhibitors that are D-myo-inositol 1-phosphate substrate analogues possessing 6-substituents larger than the 6-hydroxy group of the substrate, for example, the 6-O-methyl analogue, are able to bind to the enzyme in a congruous manner to the substrate. It is demonstrated, however, that such compounds show no substrate activity whatsoever. It is also shown that a 6-amino group is able to fulfil the role of the 6-hydroxy group of the substrate in conferring substrate activity and that a 6-methylamino group is similarly able to support catalysis. The results indicate that a 6-substituent capable of serving as a hydrogen-bond donor is required in the catalytic mechanism for hydrolysis. It has recently been shown that inositol is displaced from phosphorus with inversion of stereochemistry and we expect that the nucleophilic species is associated with Mg^{2+}-1. It is proposed here that the role of the 6-hydroxy group of the substrate is to H-bond with a water molecule or hydroxide ion located on Mg^{2+}-2. From this analysis, it appears that the water molecule bound to Mg^{2+}-2 serves as a proton donor for the inositolate leaving group in a process that stabilises the alkoxide product and retards the back-reaction.

KEYWORDS:

enzyme catalysis · hydrogen bonds · inositol phosphates · phosphatases · reaction mechanisms

Introduction

Mammalian brain inositol monophosphatase (IMPase, EC 3.1.3.25) provides inositol for the biosynthesis of the key secondary messenger precursor, phosphatidylinositol 4,5-bisphosphate. Phosphatidylinositol 4,5-bisphosphate is hydrolysed by phosphatidylinositidase C in response to receptor occupation to give diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3), both of which mediate signal transduction through specific interactions with their own targets.[1–4] DAG activates protein kinase C[5, 6] which modulates the activity of many enzymes through phosphorylation,[7] while Ins 1,4,5-P_3 causes the release of calcium ions from an intracellular store.[8] Brain cells vary in their ability to take up inositol[3, 9] and a series of phosphatases exist to sequentially hydrolyse Ins 1,4,5-P_3 and other inositol polyphosphates via the bisphosphates to give inositol 1- and 4-monophosphates, substrates for IMPase. The effect of blocking IMPase with inhibitor Li^+ cations is depletion of free inositol in brain cells[10, 11] and, thus, several groups have suggested that IMPase might be the target for the lithium ions in manic depression therapy. More recently the kinetics of inhibition by Li^+ ions have been probed[12–18] and there is now substantial evidence to show that the activity of IMPase would be very low at therapeutic concentrations of Li^+ ions ($\approx$1 mM). Importantly, the sensitivity of IMPase to Li^+ has been shown to be acutely dependent upon phosphate dianion concentration. This reaction product is present in brain cells at high concentrations, indicating that the efficacy of Li^+ is greater in cells than was originally thought.[18] Subsequent studies have defined how Li^+ ions interact with the enzyme (see below).

IMPase catalyses the hydrolysis of both enantiomers of *myo*-inositol 1-phosphate (Ins 1-P, **1**) and *myo*-inositol 4-phosphate (Ins 4-P),[14] ethane-1,2-diol phosphate[19] and 2′-ribonucleoside[13, 19] and 2′-ribofuranoside phosphates.[20] IMPase shows an absolute requirement for divalent metal ions,[13] such as Mg^{2+}, and two Mg^{2+} ions bind at each active site of the homodi-

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mer.[19, 21–25] Kinetic data[18, 23, 24] indicate that one metal ion (Mg^{2+}-1) binds to the enzyme before the substrate and the second (Mg^{2+}-2) binds after the substrate.[25] The lithium ion replaces Mg^{2+}-2 in phosphate-product complexes during its uncompetitive inhibition[17] of the enzyme.[25]

The 3-OH and 5-OH groups of the substrate **1** are not necessary for binding or catalysis[26–28] and 3,5-dideoxyinositol 1-P (**2**) is a good substrate. However, the 2-OH and 4-OH groups and the 1-O atom are important for binding and the 6-OH group is essential for catalysis (Figure 1). Deletion[26, 27] or alkylation[28] of

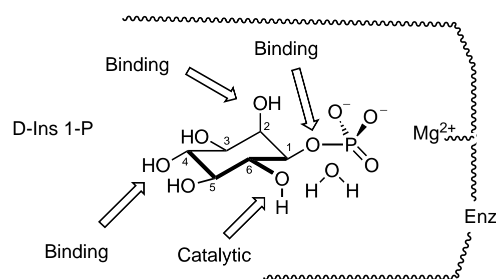
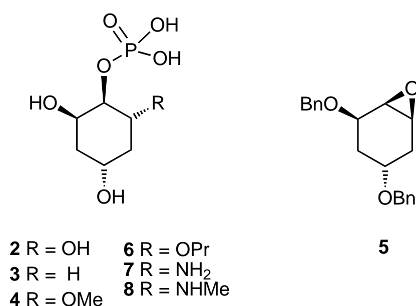


Figure 1. Schematic representation of enzyme–substrate interactions for inositol monophosphatase.



the 6-OH group in 3,5-dideoxyinositol 1-P (**2**) or Ins 1-P (**1**) lead to tightly binding competitive inhibitors of IMPase, for example, compounds **3** and 6-O-methyl Ins 1-P. Phosphate ^{18}O-ligand exchange studies established that water attacks the phosphate ester group directly,[18, 29] rather than by a substituted enzyme mechanism, but it was only recently that proposals emerged on how this might be achieved.

On the basis of kinetic data for the hydrolysis of different substrates, data for ^{18}O-phosphate ligand exchange and data for inhibition,[18, 30, 31] together with the X-ray crystal coordinates of a Gd^{3+} sulfate complex of the enzyme[32] and the results of extensive modelling studies, we proposed a three-dimensional (3-D) structure for the active complex in which the second ion to bind, Mg^{2+}-2, coordinates to a water molecule. It was further suggested that the interaction with Mg^{2+}-2 might activate the water molecule, or hydroxide ion, for nucleophilic attack on phosphorus (Figure 2 A).[19, 25] According to this model, the role of the catalytic 6-OH group of Ins 1-P (**1**) is to H-bond to the nucleophile so that it is properly positioned to attack the phosphate P-atom with adjacent displacement of the inositol moiety.[19, 25] Such a mechanism requires pseudorotation at the P-atom. This arrangement was able to account for the catalytic specificity of the enzyme in hydrolysing D-*myo*-Ins 3-P (L-*myo*-Ins 1-P) and both enantiomers of *myo*-Ins 4-P and for the inactivity of the 2- or 5-*myo*-inositol phosphates as substrates. Further support for the idea that the 6-OH group was important for binding to a water molecule associated with Mg^{2+}-2 was obtained from a consideration of the structural arrangement required for the hydrolysis of 2′-adenosine monophosphate (2′-AMP) and its analogues.[25] These compounds are good substrates but do not possess a group adjacent to the phosphate ester which is capable of forming the required hydrogen bond. Here it was shown that an additional "bridging" water molecule, linking the ribofuranosyl ring O atom to the water molecule associated with Mg^{2+}-2 through an H-bonding network, would occupy the same position in space as the catalytically essential hydroxy group of the natural inositol phosphate substrates in the enzyme–substrate complexes.

This proposal, which invokes the interaction of a water molecule with Mg^{2+}-2, differed only in detail from ideas put forward by the Merck, Sharp and Dohme (MSD) group at the same time. These were derived largely from X-ray crystal data for different enzyme–metal-ion complexes (see Figure 2 B).[22, 24, 33] Here, it was proposed that the more deeply buried metal ion (Mg^{2+}-1) coordinates to and activates the attacking nucleophile for an inline displacement of inositol with inversion of config-

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was born in 1957 in Beckenham, England, and worked at the Wellcome Research Laboratories before moving to Sussex University where he obtained a BSc degree by thesis in 1981. He completed his DPhil with Douglas Young at the same university before moving to Southampton in 1983 as a Royal Society University Fellow to pursue independent interests in enzyme mechanisms. In 1990 he moved to the University of St. Andrews to the Chair of Organic Chemistry and later became Purdie Professor of Chemistry and the first Director for the Centre of Biomolecular Sciences. Since 1998 he has held the Chair of Organic Chemistry at the University of Birmingham. He is a passionate believer in applying biomolecular understanding to the design and preparation of effect molecules and is pursuing several programmes at the interface of chemistry, biology and molecular medicine. These include studies into the mechanism and inhibition of enzymes PP1, 2A, inositol monophosphatase and methylaspartase, the synthesis of protein structural motifs for use in drug design and proteomics, ribosomal polyprotein processing and solid/gel phase synthesis and catalysis.

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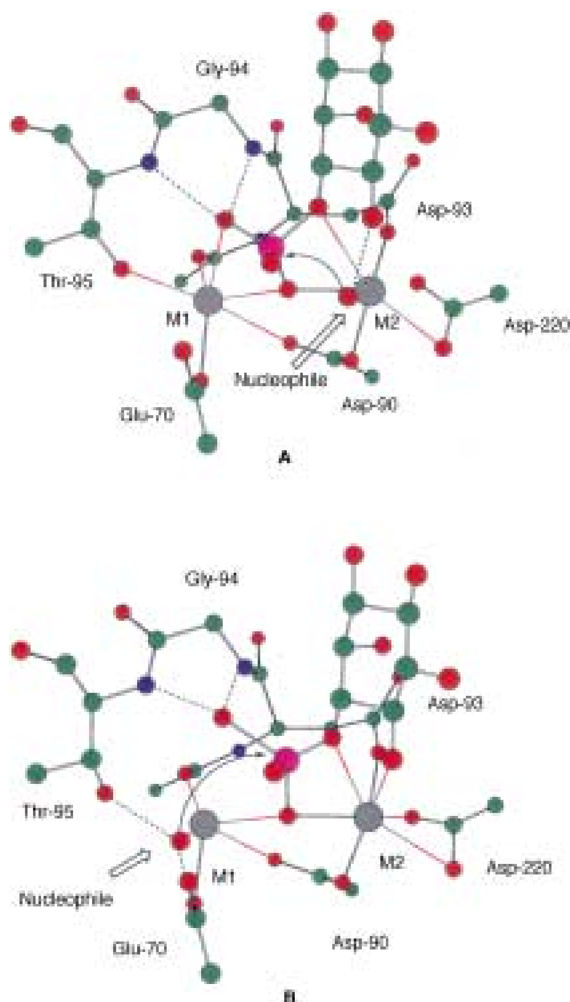


Figure 2. 3-D Structures showing A) adjacent displacement and B) inline displacement mechanisms for phosphate hydrolysis catalysed by inositol monophosphatase. Colour scheme: pink = phosphorous, grey = magnesium, blue = nitrogen, red = oxygen, green = carbon.

uration at the P atom.[22] A significant problem with this mechanism is that it did not explain why 2′-AMP should serve as a substrate or why the "nonbinding" 6-OH group in the substrate Ins 1-P was essential for catalysis. The crystal data obtained by the MSD group indicated that the 6-OH group might interact directly with Mg^{2+}-2 or with an associated water molecule. Therefore, it could be argued that the binding affinity of the second magnesium ion, Mg^{2+}-2, to bind would be compromised by the alteration of the 6-OH group to a bulkier 6-OMe group. However, at the time it was difficult to extrapolate these ideas to rationalise the substrate activity of 2′-AMP and the substrate inactivity of the tight-binding 6-deoxyinositol 1-P.

In spite of the differences in the two suggested mechanisms at a structural level, the positions of the substrate binding groups and the metal ions within the proposed active complex were virtually identical. Indeed the differences concern only the locations of, and charges on, bound solvent molecules. Theoretical studies on the mechanism of phosphoryl transfer indicate that the transition state energy differences for adjacent displacement, with retention of configuration, and inline displacement, with inversion, are small.[34] As there was chemical precedent for both mechanisms,[35] it was not possible, a priori, to favour one over the other. Here we describe experiments designed to further probe the position and role of the bound solvent molecules.

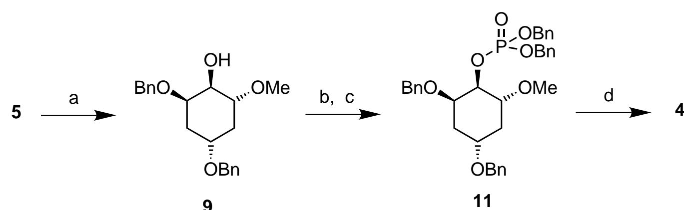
Results

Stereochemical course of phosphoryl transfer

Determination of the stereochemical course of phosphoryl transfer from the substrate to water would have distinguished between the two mechanisms. Such a strategy requires the synthesis of samples of the substrate analogues, inositol phosphorothioates, stereospecifically labelled with two isotopes of oxygen and enzymatic hydrolysis with water derived from a third isotope of oxygen to give samples of chiral [^{18}O,^{17}O]-thiophosphate. Inositol phosphorothioates are known to be substrates[36] and appropriately chirally labelled samples have been prepared in our laboratory and assigned absolute configurations.[37] Full details will be reported elsewhere. However, the rate of hydrolysis of the chiral phosphorothioates was very low and we were unable to isolate sufficient quantities of the chiral [^{18}O,^{17}O]-thiophosphates for determination of the stereochemical course.[38] Other groups experienced similar problems[39] but a breakthrough was achieved by Cullis and co-workers very recently; it was shown that hydrolysis occurs with inversion at phosphorus.[40] Within the context of the active site, the result indicates that the nucleophile is associated with Mg^{2+}-1. Since the result excludes the proposed role of the 6-OH group in positioning the nucleophilic water molecule on Mg^{2+}-2, it was important to understand why the presence of such a group and its interaction with a Mg^{2+}-2-bound water molecule was essential for catalysis.

Probing the role of the 6-hydroxy group

There are two possible ways in which the 6-OH group in the substrate can interact with an Mg^{2+}-2-bound water molecule. In the first, the 6-OH group could serve as an H-bond donor, and in the second, as an H-bond acceptor. To distinguish between these possibilities the homochiral inhibitor (1*R*,2*R*,4*R*,6*R*)-3,5-dideoxy-6-*O*-methylinositol 1-phosphate (**4**) was prepared from (1*R*,2*R*,4*R*,6*S*)-2,4-bis-(benzyloxy1)-1,6-epoxycyclohexane (**5**)[41] using methods described previously for the racemic material[42] (see Scheme 1; compound **10**, described in the Experimental Section, is formed after step (b)). The chiral 6-methyl ether **4** served as a potent competitive inhibitor ($K_i = 0.9$ μM) and was more active than the racemate ($K_i = 2.5$ μM),[42] thereby confirming the stereochemical requirements for inhibition. Incubation of compound **4** with vast excesses of IMPase resulted in the formation of no inorganic phosphate whatsoever, as determined by ^{31}P NMR spectroscopy. Even after several days, no hydrolysis was detected and the result was verified using a highly sensitive malachite-green spectrophotometric assay.[18] Ins 1-P or 2′-AMP



Scheme 1. Synthesis of (−)-(1*R*,2*R*,4*S*,6*R*)-6-O-methyl-3,5-dideoxyinositol 1-phosphate **4**: a) $Yb^{III}(OTf)_3$ (0.2 equiv), MeOH, CH_2Cl_2, 65 °C, 3 h, 97 %; b) diphenylchlorophosphate, TEA, DMAP, CH_2Cl_2, 20 °C, 12 h, 37 %; c) BnONa, THF, −70 °C, 2 h, 55 %; d) Pd/C (10 %), H_2 (gaseous), MeOH, 20 °C, 24 h, 90 %. OTf = triflate = trifluoromethanesulfonate, TEA = triethylamine, DMAP = 4-dimethylaminopyridine, Bn = benzyl.

that were added to the incubation mixture containing IMPase, magnesium ions and inhibitor **4** were hydrolysed at the expected rate. These findings indicate that the active isomer of methyl ether **4** is not a slow, tight-binding substrate for the enzyme but is, indeed, a reversible competitive inhibitor.[42, 43] The results also suggest that the 6-OH group in the substrate **1** does not serve as a hydrogen-bond acceptor to the Mg^{2+}-2-bound water molecule. However, it could not be ruled out that the methyl ether group was either too large to allow the cyclitol ring to bind in a productive fashion, or, that the lone-pairs on the 6-O atom were displaced from their normal position in the substrate through a rotation about the C-6–O bond.

Modelling active site interactions with 6-ethers and 6-amines

Modelling studies on the protein–**4** complex, with the coordinates generated in fitting the original mechanistic data to the X-ray crystal structure,[25] indicated that small 6-*O*-alkyl ether groups such as those present in compounds **4** and **6** would not disturb any interaction between the cyclitol and the protein (Figure 3). Indeed, it was evident from modelling that the 6-OMe-inhibitor **4** could bind in two different conformations, either with the methyl group buried in an active site pocket (Figure 3 A), or exposed to solution (Figure 3 B). In both cases, a water molecule (or hydroxide ion) is able to access the remaining coordination site on Mg^{2+}-2. In the optimised structures, the bound water molecule showed strong interactions with a nonbridging phosphate oxygen atom and the sides chain of either Glu-70 or Asp-220 rather than with the 6-O atom of the inositol analogue, but only a slight perturbation in the structure was required to change this. In the case where the methyl group was exposed to solvent, the distance between the O atoms of the water and the ether was 2.93 Å, so that only a reorientation of the water hydrogen atoms is required to form a good hydrogen bond. As the pocket, bounded by Gly-215, Ile-216, His-217, Trp-219, Asp-220 and Arg-248 is quite small and is unable to accommodate larger 6-*O*-alkyl ethers, such as the propyl ether in **6**, which is also a good, submicromolar inhibitor, it is reasonable to suppose that the ethers bind with the alkyl group exposed as in Figure 3 B.

Optimisation of the bound complexes with the 6-methylamino analogue **8** showed two similar binding conformations. In addition, due to the potential for inversion at the nitrogen atom

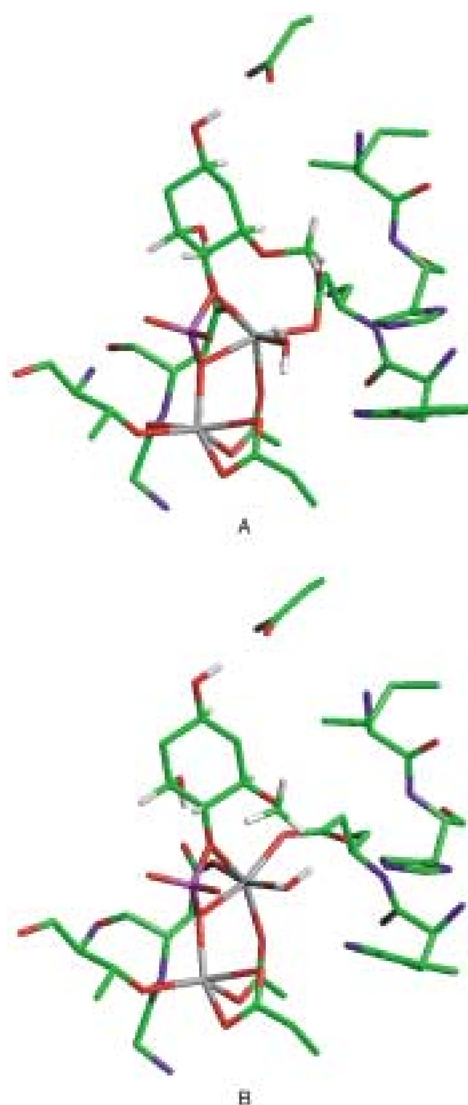


Figure 3. 6-OMe-inhibitor **4** complex showing favourable interactions between the cyclitol group and the protein where: A) the methyl is buried in a pocket in the active site; B) the methyl is exposed to the solution. Colour scheme: white = hydrogen, other colours are as in Figure 2.

and the presence of both a lone pair and hydrogen atom on the amine, both the "buried" and "open" complexes could adopt two low energy structural arrangements in which there was a hydrogen bond between the Mg^{2+}-2-bound water molecule and the N^6 atom. These differed primarily in whether the bound water molecule served as a hydrogen-bond donor to the N^6 atom or as a hydrogen-bond acceptor (Figure 4).

Such interactions appeared to be able to fulfil the perceived requirements for catalysis in the natural inositol phosphate substrates, for example, Ins 1-P (**1**), and thus it appeared that 6-amino-3,5,6-trideoxyinositol 1-phosphates **7** and **8** should serve as substrates. No previous work had investigated the properties of 2-aminocyclitol 1-phosphates and so it was necessary to prepare and test the parent 6-amino-3,5,6-trideoxyinositol 1-phosphate **7** as a substrate first, before addressing the question of the steric influence of the N^6-alkyl group.

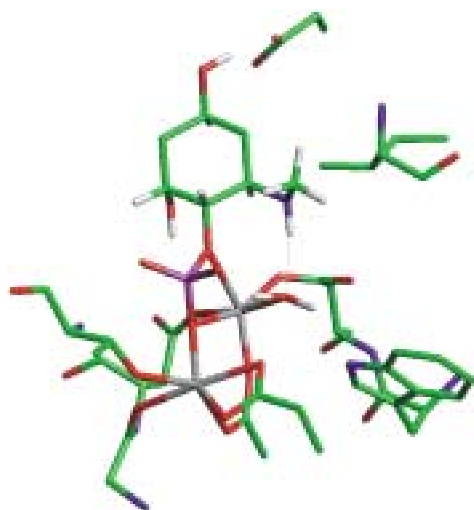


*Figure 4. 6-Methylamino-inhibitor **8** complex showing that the secondary NH proton served as an H-bond donor to the Mg^{2+}-2-coordinated hydroxide ion rather than the Mg^{2+}-2-bound water molecule serving as an H-bond donor to the N^6-atom. Colour scheme: as in Figures 2 and 3.*

Synthesis of 6-amino 3,5-dideoxyinositol 1-phosphates

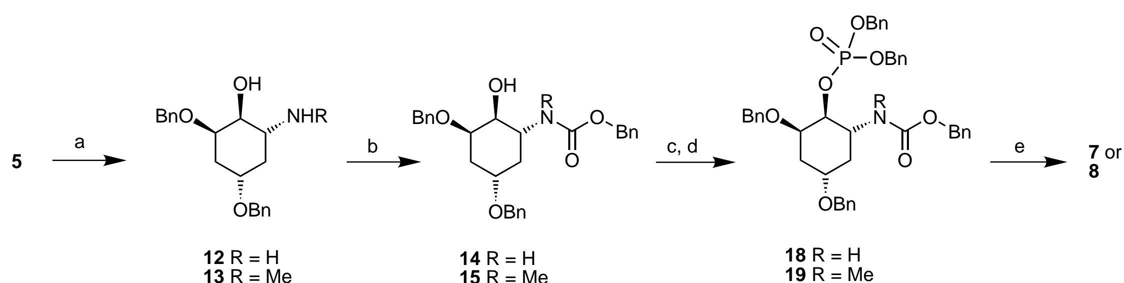
Recently, we reported the preparation of (1*R*,2*R*,4*R*,6*S*)-2,4-bis(benzyloxy1)-1,6-epoxycyclohexane (**5**) from (−)-quinic acid.[41] The absolute stereochemistry of this intermediate facilitated its conversion, with inversion of configuration at C-6, into 6-alkyloxy derivatives; these could be subsequently converted into 1-phosphate ester derivatives, for example, compound **6**, that were known to be the most active stereoisomers for inositol monophosphatase inhibition.[41, 42] Therefore, it seemed expedient to employ the epoxide **5** in the synthesis of the amine derivatives **7** and **8**. Accordingly, using a modification of the Crotti's procedure[44] for epoxide aminolysis, epoxide **5** was heated with either aqueous ammonia or methylamine in a sealed tube in the presence of 0.2 equivalents of ytterbium(III) triflate to afford each of the amino alcohols **12** and **13** with near quantitative conversion.[45] The compounds displayed the expected analytical and spectroscopic properties and analysis of the ^{1}H NMR spectra showed that the reaction had proceeded with inversion of configuration at C-6 and that no or very little reaction had occurred through attack at C-1.

The 6-amino cyclohexanols **12** and **13** were treated with benzyl chloroformate (Scheme 2) to give the benzyl urethanes **14** and **15** in yields of 57 and 65 %, respectively, and the products were phosphorylated with diphenyl chlorophosphate to give the diphenyl phosphate triesters, **16** and **17**. Transesterification with sodium benzyloxide to give the dibenzyl phosphate esters **18** and **19** and catalytic hydrogenolytic removal of all five benzyl protecting groups to give the required amines **7** and **8** was achieved using procedures previously optimised for the 6-alkyloxy derivatives (such as **4**).[42] The amines **7** and **8** were obtained in yields of 88 % and 89 %, respectively, from the dibenzyl esters **18** and **19**, and were stored as their cyclohexylammonium salts.[45] The salts and all of their synthetic precursors displayed the expected analytical and spectral data (full details will be reported elsewhere) and were ready to be tested for biological activity.

Determination of biological activity

Buffered deuterium oxide solutions of each of the amino phosphate esters (15 mM) containing magnesium chloride and IMPase were monitored by ^{1}H NMR spectroscopy and spectra were acquired at 15 minute intervals. The 6-amino phosphate **7** was hydrolysed completely within six hours to give the expected product, indicating that it is a substrate. Note that the physiological substrate **1** was hydrolysed almost completely within 15 minutes under these conditions. The result indicates that a 6-amino group is able to fully support catalysis and, indeed, is the first example of a compound possessing any substituent other than a hydroxy group at C-6 to have been shown to display substrate activity.

The 6-methylamino phosphate **8** took ten hours before an obvious time-dependent change could be observed. The result indicates that the 6-methylamino phosphate **8** is a substrate for IMPase. Albeit encouraging, the low rate of reaction precluded a firm conclusion on the basis of the observation alone because the presence of trace levels of contaminating nonspecific phosphatases, which can act upon the 6-methylamino phosphate **8**, could not be excluded.[45] Furthermore, it is known that inorganic phosphate is a good competitive product inhibitor ($K_i = 0.3$ mM).[18] Thus, a very slow observed rate of reaction for the substrate could have been indicative of a high substrate K_m value and/or a low value of k_{cat}.



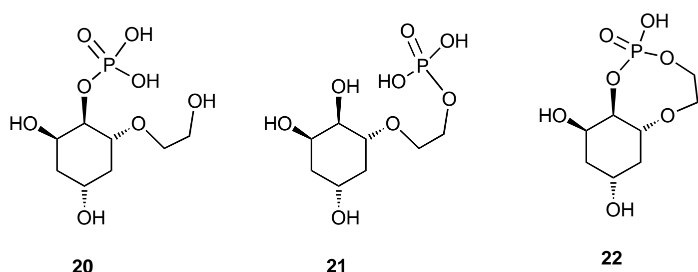
*Scheme 2. Synthesis of (−)-(1S,2R,4S,6R)-6-amino-3,5,6-trideoxyinositol 1-phosphate **7** and (−)-(1S,2R,4S,6R)-6-aminomethyl-3,5,6-trideoxyinositol 1-phosphate **8**: a) aqueous amine or methylamine, $Yb^{III}(OTf)_3$ (0.2 equiv), sealed tube, 65 °C, 48 h, 51 – 99 %; b) 2m NaOH, benzyl chloroformate, 0 °C, 2 h, 57 – 83 %; c) diphenylchlorophosphate, TEA, DMAP, CH_2Cl_2, 20 °C, 12 h, 99 %; d) BnONa, THF, −70 °C, 2 h; e) Pd/C (10 %), H_2 (gaseous), MeOH, 20 °C, 24 h, 88 %.*

In order to resolve the ambiguities the V_{max} and K_m values of both 6-aminocyclitol 1-phosphates **7** and **8** were determined using standard assay conditions[18] except that a solution of Mg^{2+} ions at a concentration of 6 mM was used. Fresh batches of each substrate were prepared and all traces of inorganic phosphate were removed by recrystallisation. The primary amine **7** displayed a V_{max} value of 8.00 μM min^{-1} using 15 μg of enzyme per assay and a K_m value of 300 μM, which is nine times the V_{max} value for 2′-AMP under standard conditions. The methyl homologue **8** gave values of 4.00 μM min^{-1} for V_{max} and 140 μM for K_m, respectively. In these experiments it was demonstrated that all of the phosphatase activity was sensitive to inhibition by Li^+, which indicates that nonspecific phosphatases were not present. Thus, it is established that both primary and secondary amino groups can provide the necessary interactions with the Mg^{2+}-2-bound water molecule to support catalysis. It is also evident that the lack of substrate activity displayed by methyl ether **4** is not due to an inability to bind in the appropriate manner, in accord with the theoretical predictions.

Discussion

In the absence of information on the stereochemical course of the IMPase reaction, until very recently,[40] we focused on the design and evaluation of structural probes for the coordination sphere of Mg^{2+}-2. The mechanism proposed in Figure 2 A,[25] is now known to be incorrect as the nucleophile is delivered from Mg^{2+}-1. However, the proposed interaction of Mg^{2+}-2 with a water molecule that also binds to the 6-OH group of the substrate[25] does appear to exist and seems to correctly account for the substrate specificity of the enzyme. Before attempting to provide a unified mechanism for the action of IMPase it is, therefore, instructive to summarize the results of experiments designed to probe the coordination sphere of Mg^{2+}-2.

Extension of the 6-OH group of Ins 1-P by an ethylene bridge, as in compound **20**, places the 2′-OH group of the "pendant arm" into the position of the Mg^{2+}-2-bound water molecule. The activity of both enantiomers of **20** and some related monophosphate analogues (compounds **4** and **6**), including the 2′-phosphate **21** and the 1,2′-cyclic phosphate **22**, have been assessed.[42, 43] The racemic 6-methyl ether **4** is a competitive



inhibitor (K_i = 2.5 μM) of almost identical efficacy to the 6-deoxy analogue.[26] The racemic 6-propyl ether analogue **6** is slightly more potent (K_i = 1.2 μM),[44] as was expected on the basis of earlier results which established that a long lipophilic side-chain appended to C-6 enhanced inhibitor binding.[28, 41] In the light of this analysis it might have been expected that the potency of the racemate of the 6-hydroxyethyl ether **20** with its hydrophilic side-chain should be very much lower than the isosteric hydrophobic propyl ether **6**. However, it was known that groups attached to the cyclitol 6-position can access either of two distinct regions of the active site.[25] The lipophilic pocket formed by Val-40 and Leu-42 and, to a lesser extent, Trp-219 and Ile-216 (space occupied by the adenine moiety in the substrate 2′-AMP (and used by MSD in the design of inhibitors[28])) and the hydrophilic site near the Mg^{2+}-2 ion are normally occupied by a water molecule or hydroxide ion (Figure 2 A).

When tested, the racemic hydroxyethyl ether **20** was a good competitive inhibitor (K_i = 1.8 μM)[43] and showed no tendency to serve as a substrate or to undergo transesterification to give compound **21**. These results were consistent with the idea that the hydroxyethyl arm can access the coordination sphere of the Mg^{2+}-2 ion and, on the basis of modelling work,[25] it was predicted that the (1*R*,2*R*,4*R*,6*R*)-enantiomer should be the better inhibitor. In the event the (−)-(1*R*,2*R*,4*R*,6*R*)-hydroxyethyl ether **20** was 120-fold more potent as a competitive inhibitor (K_i = 0.5 μM) than its enantiomer.[43] It was noted that the (−)-(1*S*,2*R*,4*S*)-antipode of the 6-deoxy analogue **3**, which possesses the same spatial configuration as (−)-**20**, was found to be more potent than its (+)-antipode.[26] It was further reasoned that compound **3** might behave as an inhibitor because there is no 6-hydroxy group available to H-bond to the water molecule (which at that time was believed to be the possible nucleophile). Also in accord with this finding was the observation that the (−)-(1*R*,2*R*,4*R*,6*R*)-antipode of the propyl ether **6**, in which the absolute stereochemistry is defined by the stereochemistry of the starting material, (−)-quinic acid, is more active as an inhibitor than the racemate with a K_i value of 0.87 μM.[41]

The hypothesis that the hydroxyethyl ether **20** could displace the Mg^{2+}-2-bound water molecule was further tested with the theoretical intramolecular transesterification product, inhibitor **21**. It was argued that maintenance of all of the interactions of the cyclitol OH groups simultaneously with the interactions of the phosphate group with the enzyme-bound metal ions would only be possible if the bridging O atom of the ester in the phosphoethyl group in **21** could interact with Mg^{2+}-2. Supporting evidence was obtained when compound **21** was shown to be a good competitive inhibitor. The K_i value of 8.5 μM was only fivefold higher than for the racemic isomeric 6-hydroxyethyl ether **20** and the lowest, by far, for any primary alkyl phosphate inhibitor for IMPase.[42, 43]

In spite of the weight of evidence that suggested that the hydrophilic cyclitol C-6-appended side chains could interact with Mg^{2+}-2 by forcing the water molecule from the coordination sphere, the catalytic role of the 6-OH group in Ins 1-P remained obscure. The properties of the 6-methyl ether as an inhibitor rather than a substrate could not be easily rationalised, particularly in the light of the finding that Mg^{2+}-1 delivers the nucleophile.[40] If water is indeed bound to Mg^{2+}-2, why should it not act as a hydrogen-bond donor in the complex of the enzyme with 6-methyl ether **4**? Since it is established here that the compound shows no substrate activity whatsoever, there are

only two likely explanations. First, it could be because the electron density on the 6-O atom is not optimally located for H-bond formation due to steric interactions caused by the methyl group. This explanation is not in accord with the submicromolar K_i value for the active enantiomer of **4** reported here or the modelling studies presented here. These indicate that there is space for an extra methyl group to exist in the correct conformation for H-bond formation between the 6-O atom and the water molecule. Second, it could be that the Mg^{2+}-2-bound water needs to serve as an H-bond acceptor and requires the functionality at the 6-position of the substrate to be an H-bond donor. The findings presented here, that both the 6-amino cyclitol 1-P **7** and 6-aminomethyl cyclitol 1-P **8** serve as good substrates while the isosteric 6-methyl ether **4** does not, clearly demonstrate that a C-6 H-bond donor is absolutely essential for catalysis.

A unified mechanism

The finding that the Mg^{2+}-2-bound water donates a lone pair of electrons to an acidic hydrogen attached to the cyclitol at C-6 or, indeed, that similar H-bond donors exist in acyclic substrates[30, 31] suggests that the water should function as an acid. Alternatively or additionally, the H-bond might stabilise the formation of a hydroxide ion on Mg^{2+}-2. Indeed, if the water does serve as an acid, then later in the reaction, after deprotonation, the species must exist as an H-bonded, Mg^{2+}-2-coordinated hydroxide ion. Given that the nucleophilic species is derived from water associated with Mg^{2+}-1 (see above), it is useful to consider the protonation states of the water molecules in the overall phosphoryl transfer mechanism as a whole.

Hydroxide ion associated with Mg^{2+}-1 is likely to be the nucleophile and many properties of the system are consistent with this notion. First, *V/K* for Ins 1-P increases with increasing pH values and does not plateau below pH 9.2, the upper limit of the study, although V_{max} peaks at pH 8.5.[18] This indicates that a deprotonation step with an apparent pK_a value of greater than 9.2 is required for catalysis in either the *free* enzyme – Mg^{2+}-1 ion complex, or any other *unbound* species, the cofactor (Mg^{2+}-2), the substrate (Ins 1-P) or the other substrate (water).[46] Note that the cyclitol phosphate substrate **1** is known to be "sticky",[17] to bind to the enzyme before the Mg^{2+}-2 ion and to ionise as the dianion with a pK_a value of 6.2.[18] The unbound species Mg^{2+}-OH_2 possesses a pK_a value of 12.6[47] and would be quite easy to deprotonate when bound in the Mg^{2+}-1 site. The side-chains of residues Glu-70 and Thr-95 are well positioned to remove a proton from the associated hydrate which may be rendered even more acidic by its position close to the positive pole of an α-helix.

Following inline attack of Mg^{2+}-1-bound hydroxide ion on the P atom of the phosphate ester, negative charge would begin to build up on the inositol 1-O atom and some of this charge would be dissipated to Mg^{2+}-2 through its direct interaction with the *pro-R* lone pair (Figure 5). However, the product ground state would contain inverted phosphoric acid dianion, with the non-ionised O atom associated with Mg^{2+}-1 and 1-inositolate anion coordinated to Mg^{2+}-2. This complex would appear to be very likely to react through reverse steps to give the substrate ground state unless the inositolate moiety could be protonated. Two potential acidic protons exist near the alkoxide ion, the substrate 6-OH proton, which is involved in H-bonding to the Mg^{2+}-2-associated water molecule, and a proton of water molecule itself. An important role of the Mg^{2+}-2 ion would appear to be to locate its water molecule and reduce the pK_a value of this hydrate such that it can serve as an acid. The donation of a lone pair of electrons from the hydrate to the 6-OH proton in the substrate would further reduce its pK_a value such that it can directly protonate the 1-inositolate anion in the modified inline displacement mechanism (Figure 5). This action would generate an H-bonded Mg^{2+}-2-bound hydroxide ion as a product. Of course, it is possible that the 6-OH proton serves as the acid that initially quenches the charge on the 1-inositolate leaving group and that the Mg^{2+}-2-bound water molecule reprotonates the 6-alkoxide. It should be noted that the 6-OH proton cannot interact directly with the 1-O atom until the P–O bond is cleaved because the only accessible lone pair on the 1-O atom is ligated to Mg^{2+}-2.

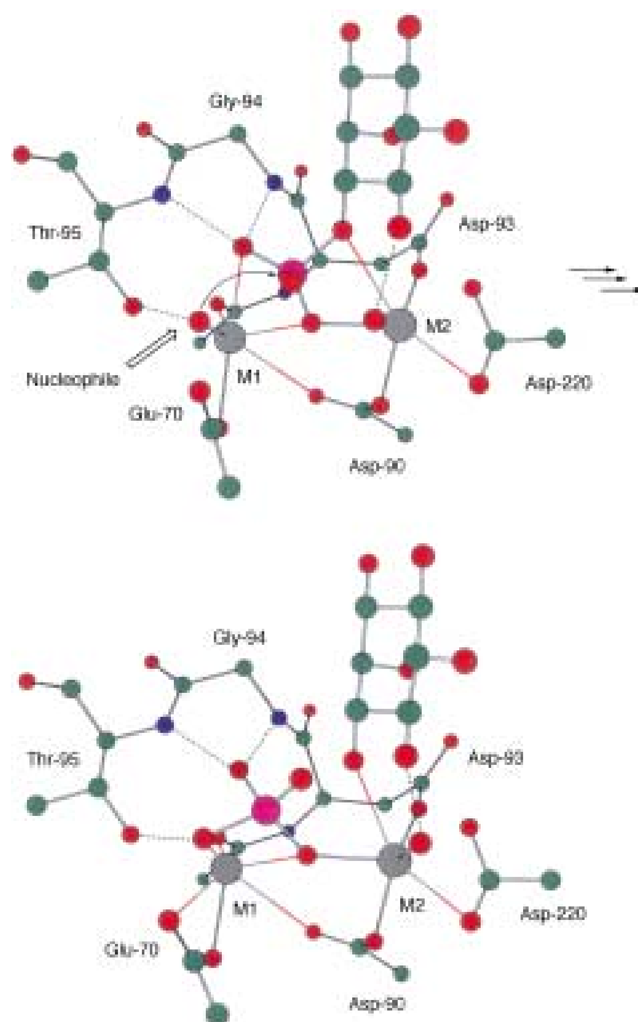


Figure 5. Modified inline displacement mechanism for phosphate hydrolysis catalysed by inositol monophosphatase. Colour scheme: as in Figures 2 and 3.

In accord with these ideas, it is known that Zn^{2+} forms of IMPase (previously referred to as zinc-dependent acid phospha-

tase)[48] operate best at much lower hydroxide ion concentrations (approximately pH 5.0 – 6.5) than the Mg^{2+} forms (approximately pH 6.7 – > 9.0) for the substrates Ins 1-P and 4-nitrophenyl phosphate (4-NPP). At low pH values with the Zn^{2+} form of the enzyme, 4-NPP is a much better substrate than Ins 1-P. Obviously 4-NPP does not possess a group equivalent to that of the 6-OH group of Ins 1-P **1**. The fact that 4-NPP is a substrate at all and, indeed, is the only substrate type known for which a substrate-derived H-bond donor is not absolutely essential, can be ascribed to the enhanced electrophilicity of the P atom and the low pK_a value of the nitrophenol leaving group. It is interesting to note that, with the Mg^{2+} form of the enzyme, the activity of 4-NPP is 30-times lower than that of Ins 1-P, and its optimal activity is shifted to higher pH values. Moreover, the system is barely sensitive to lithium ion inhibition,[16–18] which is a good test for rate-limiting inorganic phosphate release as displayed by several natural substrates.[18] These observations are consistent with a change in rate-limiting step to one where Mg^{2+}-1-bound water deprotonation and attack on phosphorus becomes slow relative to the release of the products. In the hydrolysis of 4-NPP it would seem to be unnecessary to protonate the phenolate since its pK_a value, when associated with Mg^{2+}-2, would be below the pH value of the incubation solutions. The effect of changing ions from Mg^{2+} to Zn^{2+} seems to reduce the requirement for hydroxide ions by a factor of approximately 10^3-fold for 4-NPP, which is consistent with the proposed roles of an Mg^{2+} ion in activating the nucleophile and consistent with the differences in the pK_a values of 9.0[49] and 12.6[47] for water molecules in the complexes $[Zn(H_2O)_6]^{2+}$ and $[Mg(H_2O)_6]^{2+}$, respectively.

Significance

The finding that the 6-amino phosphate ester **7** serves as a substrate was expected and is in accord with the predictions of earlier molecular modelling work.[25, 50] The complete lack of substrate activity for the potent competitive inhibitor, 6-methyl ether **4**, together with the substrate activity of the 6-methylamine **8**, clearly indicates that the C-6 substituent needs to be an H-bond donor or an acid. The results provide support for the structural detail of the previously proposed active complex (Figure 2 A), in which a water molecule or hydroxide ion is chelated to Mg^{2+}-2 and H-bonded to the C-6 heteroatom. In the light of the recent finding that the phosphate ester hydrolysis reaction occurs with inversion of configuration at phosphorus, it is possible to conclude that the nucleophile is delivered from Mg^{2+}-1 and not from Mg^{2+}-2. In rationalising all of this information, a new unified mechanism is proposed in which the Mg^{2+}-2-bound water molecule serves as a proton donor for the inositolate leaving group. This new mechanism accommodates all known properties of the reactions catalysed by the enzyme and, importantly, is able to explain why the replacement of the substrate 6-OH group by an alkoxy group or a hydrogen atom should give a tight-binding non-hydrolysable compound. This new mechanistic insight will aid in the rational design of inhibitors of reduced polarity in the quest for safer alternatives to lithium therapy in the treatment of manic depression.

Experimental Section

Chemical syntheses

(−)-(1*S*,2*R*,4*R*,6*R*)-6-Amino-3,5,6-trideoxyinositol 1-phosphate (7) and (−)-(1*S*,2*R*,4*R*,6*R*)-6-methylamino-3,5,6-trideoxyinositol 1-phosphate (8) were prepared as communicated recently[45] and gave the following analytical data: Compound **7**: mp > 200 °C (decomp); $[\alpha]_D = -97$ ($c = 0.37$ in methanol); ^{1}H NMR (300 MHz, D_2O): $\delta = 4.21$ (m, 1 H; 1-CH), 3.47 – 3.32 (m, 2 H; 2-CH and 4-CH), 3.42 – 3.31 (m, 1 H; 6-CH), 2.19 – 1.36 (m, 4 H; 3-CH_2 and 5-CH_2); ^{13}C NMR (75 MHz, D_2O): $\delta = 74.70$ (1-C), 67.80 (2-C), 63.18 (4-C), 48.88 (6-C), 37.59 (5-C), 36.38 (3-C); ^{31}P NMR (121.5 MHz, D_2O): $\delta = +3.44$; HR-MS (FAB): calcd for $C_6H_{15}NO_6P$ $[M+H]^+$: 228.0637, found: 228.0626. Compound **8**: mp > 200 °C (decomp); $[\alpha]_D = -24$ ($c = 0.20$ in methanol); ^{1}H NMR (300 MHz, D_2O): $\delta = 4.25$ (m, 1 H; 1-CH), 4.07 – 3.92 (m, 2 H; 2-CH and 4-CH), 3.49 – 3.38 (m, 1 H; 6-CH), 2.72 (s, 3 H; NMe), 2.81 – 1.39 (m, 4 H; 3-CH_2 and 5-CH_2); ^{13}C NMR (75 MHz, D_2O): δ 72.03 (1-C), 67.93 (2-C), 63.72 (4-C), 50.28 (6-C), 39.88 (5-C), 39.31 (3-C), 37.46 (NMe); ^{31}P NMR (121.5 MHz, D_2O): $\delta = +3.07$; MS (FAB): m/z (%): 242 (15, $[M+H]^+$); MS (ES$^+$ TOF): m/z (%): 264 (30, $[M+Na]^+$), 242 (100, $[M+H]^+$); HR-MS (ES$^+$ TOF): calcd for $C_7H_{17}NO_6P$ $[M+H]^+$: 242.0794, found: 242.0791.

(−)-(1S,2R,4S,6R)-2,4-Bis(benzyloxy)-6-methyloxy-cyclohexanol (9):[51] $Yb^{III}(OTf)_3$ (40 mg, 0.06 mmol) was added to a stirred solution of the epoxide **5** (100 mg, 0.32 mmol) in CH_2Cl_2 (10 mL), followed by methanol (1 mL). The resulting mixture was refluxed for 3 h, and then the solvents were removed under reduced pressure. The residue was washed with water (10 mL) and then extracted with ethyl acetate (2 × 10 mL). The pooled organic phases were dried (Na_2SO_4) and then concentrated under reduced pressure to give the alcohol (−)-**9** (107 mg, 97 %) as a light coloured oil. $[\alpha]_D = -47$ ($c = 0.19$ in EtOAc); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.25 – 7.4$ (m, 10 H; Ar-H), 4.6 (s, 2 H; OCH_2Ph), 4.5 (s, 2 H; OCH_2Ph), 4.0 – 3.9 (m, 1 H; tertiary H), 3.85 – 3.65 (m, 1 H; tertiary H), 3.6 – 3.3 (m, 2 H; 2 × tertiary H), 3.45 (s, 3 H; OCH_3), 2.6 – 2.45 (m, 1 H; secondary H), 2.45 (m, 1 H; secondary H), 1.7 (s, 1 H; OH), 1.45 – 1.2 (m, 2 H; 2 × secondary H); ^{13}C NMR (50.3 MHz, $CDCl_3$): $\delta = 138.9$ and 138.8 (Ar-C, quaternary), 130.2, 130.0, 128.8, 128.7, 128.2, 128.1, 128.0 (Ar-CH), 78.8 (1-C), 76.8 and 75.8 (2-C and 6-C), 72.4 (OCH_2Ph), 72.0 (4-C), 71.1 (OCH_2Ph), 57.5 (OCH_3), 35.0 and 34.3 (3-C and 5-C); IR (neat): $\tilde{\nu} = 3485$, 2927, 1453, 1364, 1093 cm^{-1}; MS (EI): m/z (%): 342 (8, M^+), 250 (37, $[M - C_7H_7 - H]^+$), 235 (11, $[M - C_7H_7O]^+$), 91 (100, $C_7H_7^+$); HR-MS (EI): calcd for $C_{21}H_{26}O_4$ (M^+): 342.1831, found: 342.1838; elemental analysis calcd (%) for $C_{21}H_{26}O_4$: C 73.65, H 7.65; found C 72.9, H 7.5.

(−)-(1S,2R,4S,6R)-1-(Diphenoxyphosphoryloxy)-2,4-bis(benzyloxy)-6-methyloxycyclohexane (10):[51] DMAP (10 mg, 0.09 mmol), triethylamine (40 mg, 0.44 mmol, 0.06 mL) and diphenylchlorophosphate (120 mg, 0.44 mmol, 0.10 mL) were added to a stirred solution of alcohol (−)-**9** (100 mg, 0.29 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred overnight and the solvent was removed under reduced pressure. The residue was then washed with water (10 mL) and extracted with ethyl acetate (2 × 10 mL). The pooled organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residual oil was purified by chromatography on silica (EtOAc/petroleum ether 1/10) to give the phosphate triester (−)-**10** (60 mg, 37 %) as a colourless oil. $[\alpha]_D = -37.5$ ($c = 0.2$ in EtOAc); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.25 – 7.4$ (m, 20 H; Ar-H), 4.5 – 4.6 (m, 1 H; 1-H), 4.5 (s, 2 H; OCH_2Ph), 4.4 (s, 2 H; OCH_2Ph), 4.0 – 4.1 (m, 1 H; tertiary H), 3.6 – 3.85 (m, 2 H; 2 × tertiary H), 3.3 (s, 3 H; OCH_3), 2.4 – 2.6 (m, 1 H; secondary H), 2.2 – 2.4 (m, 1 H; secondary H), 1.5 – 1.35 (m, 2 H; 2 × secondary H); ^{13}C NMR (50.3 MHz, $CDCl_3$): $\delta = 150.5$ and 150.4 (POC_6H_5 quaternary), 138.9 and 138.8 (Ar-C, quaternary), 130.2, 130.1, 128.9, 128.8, 128.2, 128.1, 128.0, 125.7, 125.6, 120.7, 120.6 and 120.5

(Ar-CH), 83.2 (^2J(C,P) = 6.3 Hz; 1-C), 76.5 (^3J(C,P) = 5.1 Hz; C tertiary), 75.8 (^3J(C,P) = 3 Hz; C tertiary), 72.7 (OCH_2Ph), 71.5 (4-C), 71.2 (OCH_2Ph), 57.7 (OCH_3), 34.5 and 35.3 (3-C and 5-C); ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = -12.0$; MS (EI): m/z (%): 575 (20, $[M+H]^+$), 251 (58, $[(PhO)_2PO_2H_2]^+$), 91 (100, $C_7H_7^+$); HR-MS (FAB): calcd for $C_{33}H_{36}O_7P$ $[M+H]^+$; 575.2199, found: 575.2204.

(−)-(1S,2R,4S,6R)-1-[Bis(benzyl)phosphoryloxy]-2,4-bis(benzyloxy)-6-methyloxycyclohexane (11):[51] Benzyl alcohol (30 mg, 0.27 mmol, 0.03 mL) was added to a stirred suspension of NaH (60% dispersion in oil, 11 mg, 0.45 mmol) in THF (15 mL). The mixture was cooled −70 °C and a solution of phosphate triester (−)-**10** (100 mg, 0.18 mmol) in THF (10 mL) was added. The mixture was allowed to warm to room temperature (over 4 h) and then water (10 mL) was added. The mixture was extracted with ethyl acetate (2 × 10 mL) and the combined organic layers were dried (Na_2SO_4) and then concentrated under reduced pressure. The residual oil was purified by chromatography on silica (EtOAc/petroleum ether 1/10) to give the benzyl ether (−)-**11** (60 mg, 55%) as a colourless oil. $[\alpha]_D = -52.2$ ($c = 0.06$ in EtOAc); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.5-7.25$ (m, 20 H; Ar-H), 5.1 – 4.95 (m, 4 H; 2 × POCH_2Ph), 4.5 – 4.4 (s, 4 H; 2 × OCH_2Ph), 4.32 – 4.28 (m, 1 H; 1-H), 4.03 – 3.98 (m, 1 H; tertiary H), 3.75 – 3.65 (m, 1 H; 4-H), 3.65 – 3.6 (m, 1 H; tertiary H), 3.35 (s, 3 H; OCH_3), 2.46 – 2.43 (m, 1 H; secondary H), 2.23 – 2.18 (m, 1 H; secondary H), 1.4 – 1.3 (m, 2 H; 2 × secondary H); ^{13}C NMR (50.3 MHz, $CDCl_3$): $\delta = 138.6$, 138.5, 136.1, 136.0 (Ar-C, quaternary), 128.5, 128.4, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 127.6 (Ar-CH), 81.8 (^2J(C,P) = 6.3 Hz; 1-C), 76.4 (^3J(C,P) = 6.0 Hz; C tertiary), 75.7 (C tertiary), 72.8 (OCH_2Ph), 71.6 (4-C), 71.2 (OCH_2Ph), 69.7 (^2J(C,P) = 5.5 Hz; POCH_2Ph), 69.5 (^2J(C,P) = 5.1 Hz; POCH_2Ph), 57.8 (OCH_3), 35.2 and 34.5 (C secondary); ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = -1.25$; IR (neat): $\tilde{\nu} = 1273$, 1106, 1023, 931, 740, 696 cm^{-1}; MS (EI): m/z (%): 603 (15, $[M+H]^+$) and 91 (100, $C_7H_7^+$); HR-MS (EI): calcd for $C_{35}H_{40}O_7P$ $[M+H]^+$: 603.2512, found: 603.2520.

(−)-(1R,2R,4R,6R)-6-Methoxy-2,4-dihydroxycyclohexyl bis-(cyclohexylammonium) phosphate (the bis(cyclohexylammonium) salt of **4**):[51] Palladium on carbon (10%; 5 mg) and a drop of acetic acid were added to a stirred solution of benzyl ether (−)-**11** (50 mg, 0.07 mmol) in methanol. The mixture was stirred vigorously under an atmosphere of hydrogen gas for 24 h. The mixture was then filtered through a prewashed Celite pad. The pad was washed with water and methanol and the combined filtrates were then lypholised. The resulting white solid was stirred with cylcohexylamine and then again lypholised to give the phosphate **4** (15 mg, 90%) as a white solid. mp > 200 °C (decomp); $[\alpha]_D = -40.7$ ($c = 0.03$ in H_2O); ^{1}H NMR (500 MHz, D_2O): $\delta = 4.33-4.3$ (m, 1 H; 2-H), 4.02 – 3.96 (m, 1 H; 1-H), 3.98 – 3.94 (m, 1 H; 4-H), 3.62 – 3.58 (m, 1 H; 6-H), 3.43 (s, 3 H; OCH_3), 3.15 – 3.1 (m, 2 H; 2 × 1-H of cyclohexylammonium (Cha)), 2.4 – 2.35 (m, 1 H; 5-H), 2.1 – 2.05 (m, 1 H; 3-H), 2.0 – 1.9 (m, 4 H; 4 × 2-H of Cha), 1.82 – 1.72 (m, 4 H; 4 × 3-H of Cha), 1.65 – 1.6 (m, 2 H; 2 × 4-H of Cha), 1.6 – 1.55 (m, 1 H; 3-H), 1.45 – 1.35 (m, 1 H; 5-H), 1.35 – 1.25 (m, 8 H; 4 × 2-H and 4 × 3-H of Cha), 1.2 – 1.1 (m, 2 H; 4-H of Cha); ^{13}C NMR (125.8 MHz, D_2O): $\delta = 77.5$ (6-C), 76.8 (^2J(C,P) = 5.5 Hz; 1-C), 67.6 (2-C), 64.8 (4-C), 57.0 (OCH_3), 50.7 (1-C of Cha), 37.4 (3-C), 36.1 (5-C), 30.7 (2-C of Cha), 24.6 (4-C of Cha), 24.1 (3-C of Cha); ^{31}P NMR (121.2 MHz, D_2O): $\delta = +3.0$; MS (FAB): m/z (%): 319 (1, $[M+2K+H]^+$), 281 (8, $[M+K+2H]^+$), 243 (7, $[M+3H]^+$) and 147 (100) where M is the molecular weight of the phosphate dianion; MS (CI): 145 (26, $[C_7H_{13}O_3]^+$) and 127 (100); HR-MS (CI): calcd for $C_7H_{13}O_3$ $[M-PO_4]^+$: 145.0865, found: 145.0869.

Enzyme: Bovine brain inositol monophosphatase was purified from a recombinant *E. coli* strain[52] as described previously and was routinely obtained in a yield of 20%. The purity was assessed using polyacrylamide gel electrophoresis.[18] Enzyme activity assays were performed using a colourimetric assay developed by Itaya and Ui[53] employing molybdic acid and malachite green. Rate determinations were performed at 37 °C in triplicate in assay buffer A, which contained KCl (300 mM), $MgCl_2$ (2 mM or 6 mM) and Tris · HCl at pH 7.8 (50 mM). Incubation samples contained the following: assay buffer A (240 µL), substrate (Ins 1-P or aminocyclitol 1-phosphates **7** or **8**) at various concentrations in assay buffer (30 µL), and enzyme solution (activity predetermined for the requirements of individual experiments; 30 µL). The reaction began with the addition of enzyme and were incubated at 37 °C. The reaction was quenched by the addition of an acidic colourimetric assay reagent (2.0 mL). The colour was allowed to develop over a period of 30 min, and the absorbance at 660 nm was measured in a 10 mm pathlength cuvette. Phosphate concentrations were determined by comparison of absorbance values to a preconstructed standard curve prepared using known phosphate concentrations. Background phosphatase activity was assessed in each experiment by performing parallel assays in the presence of Li^+ ions in buffer B (buffer B is buffer A plus 150 mM LiCl). One unit of IMPase hydrolyses 1 µmol of Ins 1-P per minute under these assay conditions. Rate data were analysed and processed graphically by nonlinear regression analysis methods as described previously.[18] The kinetic parameters for the 6-aminocyclitol 1-phosphates are presented in the Results Section above.

NMR spectroscopy: The experiments were performed in 5 mm tubes which contained IMPase (30 units) and substrate (Ins 1-P, 6-methyl ether **4** or aminocyclitol 1-phosphates **7** or **8**; 15 mM) in buffered deuterium oxide at pH 8.0 in a total volume of 0.5 mL. Reactions were started by the addition of enzyme and ^{1}H NMR spectra were recorded every 15 minutes. All of the samples showed a time-dependent phosphate ester hydrolysis reaction except for compound **4**. ^{31}P NMR spectral analysis of the reaction mixtures after several days verified that compound **4** was not a substrate and aliquots removed from the NMR spectroscopy tube and assayed with the colourimetric test confirmed that no inorganic phosphate had been formed.

Analysis of binding interactions: All calculations were performed using the AMBER all-atom molecular mechanics force field and the Discover program. A distance-dependant dielectric constant ($\varepsilon = 4r$) and nonbonded cut-off of 15 Å were used. Optimisation terminated with all gradients less than 4 kJ Å^{-1} mol^{-1}. Starting points for the calculation were produced by modification of the bound D-Inositol 1-phosphate described in our previous work. Resulting complexes were viewed using the Insight software from Biosym. Both the 6-OMe and the 6-NMe inositol derivatives **4** and **8**, respectively, had multiple binding conformations, all of which were optimised and are presented in the discussion. It is not possible to reliably compare binding energies for different conformations as the overall energy of the system is dominated by interactions within the enzyme but outside the active site.

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