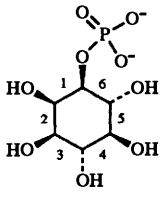
Probes for the position and mechanistic role of the second 'catalytic' magnesium ion in the inositol monophosphatase reaction[1]

Andrew G. Cole, John Wilkie and David Gani*
School of Chemistry, The Purdie Building, The University, St. Andrews, Fife KY16 9ST, UK

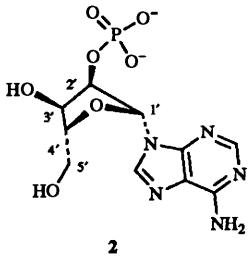
Two magnesium ions are required for the enzymic hydrolysis of phosphate monoester substrates of inositol monophosphatase. It has been suggested that one (buried) Mg^{2+} ion binds to the enzyme and the phosphate dianion moiety of the substrate through one or more of its negatively charged O-atoms while the second Mg^{2+} ion binds to the substrate bridging phosphate ester O-atom and one other substrate-derived O-atom. This second Mg^{2+} ion may also position and activate the attacking nucleophilic water molecule (A. G. Cole and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 1995, previous article). To determine the minimum structural requirements for a substrate, as deduced from the proposed interactions for natural and synthetic substrates with both Mg^{2+} ions, ethane-1,2-diol 1-phosphate was prepared and was found to be a substrate. The design and preparation of a range of minimal structure synthetic probes based on this new substrate including propyl, 2-methoxyethyl, 2-(2-hydroxyethoxy)ethyl and 5-hydroxypentyl monophosphate ester and both antipodes of 1,5-dihydroxypentan-2-yl phosphate allowed the specific interactions between the substrate and the enzyme and/or the second Mg^{2+} ion to be assessed. The results support the proposed roles for the metal ions and provide information on the position of the second Mg^{2+} ion. This information rationalises the properties of known organophosphate substrates and inhibitors for the enzyme and, furthermore, facilitates the construction of a 3-D catalytic mechanism for the inositol monophosphatase reaction which is described. This new catalytic mechanism explains why Li^{+} behaves as an inhibitor and accounts for its unusual inhibitory properties.

Introduction

Inositol monophosphatase plays a pivotal role in providing free inositol for the synthesis of secondary messenger precursors in mammalian brain cells and is able to hydrolyse both enantiomers of Ins 1-*P* and both enantiomers of Ins 4-*P*, as well as 2'-AMP.† The enzyme is the likely target for the action of Li^{+} salts in the treatment of manic depression[2] and, hence, there has been much interest in the mechanism of the enzyme and its mode of inhibition by Li^{+}.



1



2

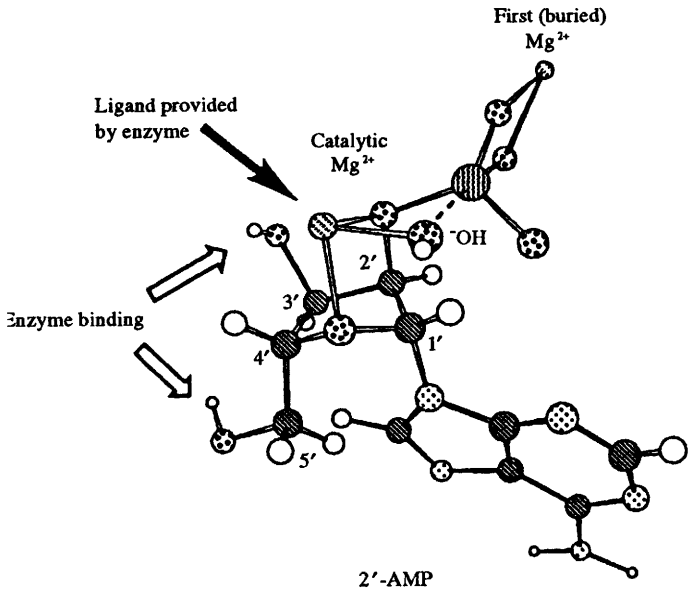
Since 1987, work from a number of laboratories has provided; (i) details of the roles of specific O-atoms on the inositol ring[3] in D-Ins 1-*P* **1** in binding to the enzyme and in conferring activity as a substrate; (ii) the X-ray crystal coordinates for a Gd^{3+}–sulfate form of the enzyme[4] and, (iii) information on the hydrolytic mechanism[5] and on the binding and debinding sequence for several species including the substrate, Mg^{2+} cofactor, both products (inositol and phosphate) and Li^{+}.[6] Building upon this information with the results of [^{18}O] water phosphate ligand-exchange experiments and alternative substrate and inhibition studies with the adenosine 2'-phosphate system **2**, we were recently able to put forward a new mechanism[1,7] in which the substrate binds to two Mg^{2+} ions at the active site, Fig. 1. This suggestion is completely consistent with the recent findings of the Merck, Sharp and Dohme group who reported on X-ray crystal structures for inositol monophosphatase complexes containing either two divalent Mn^{2+} ions and phosphate or one Gd^{3+} ion and Ins 1-*P*.[8-10] (A detailed comparison of the results of the Merck group and those of our own is presented in the Discussion section of this and the following article.)

The consensus structure of the proposed active complex is able to explain many features of the inositol system that had been hitherto difficult to rationalise, in particular with regard to Mg^{2+} binding.[6] Here we describe the results of experiments designed to refine our understanding of the catalytic role of the second Mg^{2+} ion, provide details of its coordination sphere and determine how Li^{+} cation inhibits the enzyme.

Results

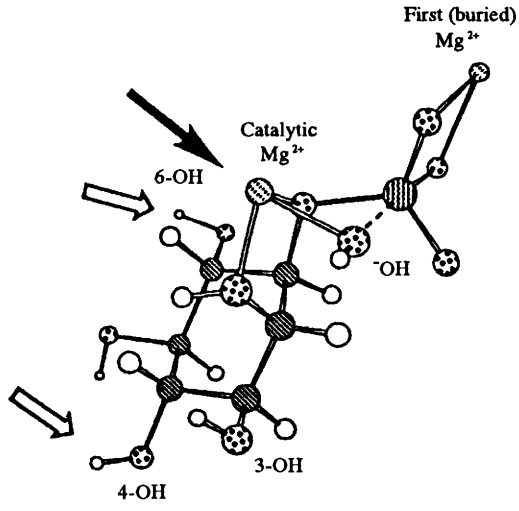
The structure of the proposed catalytic complex for 2'-AMP (Fig. 1) shows that only the adjacent 2'- and 1'-O-atoms of the ribofuranosyl ring make contact with the second Mg^{2+} ion.[7] The equivalent catalytic complex for D-Ins 1-*P* (Fig. 2) shows that only the adjacent transoid 1- and 6-O-atoms of the inositol ring make contact with the second Mg^{2+} ion. In the catalytic complex for L-Ins 1-*P* [*i.e.*, D-Ins 3-*P*] (Fig. 3) there are only interactions between the adjacent cisoid 1- and 2-O-atoms and the second Mg^{2+} ion (Mg^{2+}2). Note that the equivalent interactions with Mg^{2+}2 for both enantiomers of Ins 4-*P*, where the 4-O-atom and the adjacent catalytic O-atom are transoid, should resemble those for D-Ins 1-*P*, Fig. 2. For all five substrates there are, in fact, only two different conformational arrangements for the five-membered dioxametallocycles containing Mg^{2+}2. These differ only in which lone-pair of the catalytically essential O-atom adjacent to the phosphate ester is used in coordinating to Mg^{2+}2. For one group, that containing 2'-AMP and L-Ins 1-*P*, the phosphate ester is *endo* to the

† For abbreviations, see preceding paper.[7]



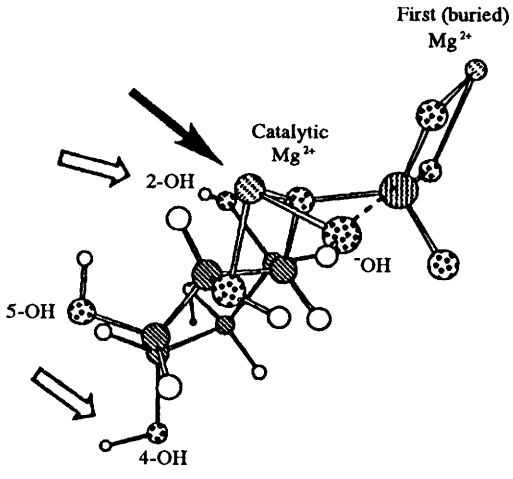
2'-AMP

Fig. 1



L-Ins 1-*P*
(D-Ins 3-*P*)

Fig. 3



D-Ins 1-*P*

Fig. 2

metallocycle and C-1′ of 2′-AMP is out of plane (Figs. 1 and 3) whereas, for D-Ins 1-*P* (Fig. 2) and for both enantiomers of Ins 4-*P* (not shown), the phosphate moiety is *exo* to the metallocycle and C-6 of D-Ins 1-*P* is out of plane. Note that the environment of the substrate-bound $Mg^{2+}2$ ion is hydrophilic and that for each of the substrates shown (Figs. 1–3) there is at least one non-chelating hydroxy group proximal to the $Mg^{2+}2$ ion. We should also be mindful of the fact that the 'catalytically essential' O-atom in each substrate might not interact *directly* with the $Mg^{2+}2$ ion but might, instead, form a hydrogen bond with a $Mg^{2+}2$-chelated water molecule.[7] Such a water molecule is well placed to serve as the nucleophile and H-bonding to the 'catalytically essential' O-atom in substrates could reasonably serve to position the nucleophile optimally for attack on the P-atom. This arrangement would give a seven-member trioxametallocycle containing an internal H-bond, and also fits all of the available experimental data.[3,6,7] Thus, regardless of how many atoms are involved in forming the metallocycle, the rudimentary requirements for a substrate seem to be associated with the ability of the molecule to adopt a conformation in which the C–OP bond and a lone-pair of the catalytically essential O-atom are parallel.

For all of the substrates discussed above only the phosphate moiety appears to make contact with the first (buried) Mg^{2+} ion.[4,7] Other interactions between the enzyme and the buried divalent metal ion ($M^{2+}1$), as deduced from X-ray crystal data for an enzyme–Gd^{3+} sulfate complex, have been considered by others previously and involve a β-carboxylate O-atom of Asp-90, the amide O-atom of Ile-92, the β-OH group of Thr-95, two of the O-atoms of bound sulfate (*in lieu* of phosphate), a γ-carboxylate O-atom of Glu-70 and two water molecules respectively, a total of eight ligands.[4,11] In the recently published crystal structures for the bis-Mn^{2+}–phosphate–enzyme complex and for the Gd^{3+}–Ins 1-*P*–enzyme complex, the side-chain hydroxy group of Thr-95 is not coordinated to the metal ion in site 1, and in the bis-Mn^{2+}–phosphate–enzyme complex there is only one water molecule associated with the Mn^{2+} ion that resides in site 1.[9,10] Thus, it is likely that Mg^{2+} is surrounded by six or seven ligands. With regard to a hydrolytic mechanism, the arrangement[7] would suggest that the first Mg^{2+} ion should act as a Lewis acid, by coordinating to the phosphate O-atoms to enhance the electrophilicity of the P-atom, while the second Mg^{2+} ion should stabilise the formation of hydroxide, from a chelated water molecule, which would then be activated and disposed correctly to attack the P-atom.[12] The associative process may be aided through further stabilisation of the developing negative charge by the positive dipole of the Thr-195–Thr-205 α-helix which points directly at the sulfate anion in the published crystal structure,[4] and the Thr-95–His-100 α-helix which points towards the leaving 1-O-atom of inositol in modelled enzyme–substrate complexes; see the following article.[13] The second Mg^{2+} ion also requires a second counter anion, in addition to hydroxide (from water), and must interact with the enzyme. Modelling studies indicate that the carboxylate group of Asp 220 could fulfil this role; see following article.[13] Alternative mechanisms involving the activation of a hydrolytic water chelated to $Mg^{2+}1$ are considered in the Discussion section below.

In view of the fact that only two adjacent O-atoms appear to interact with the second Mg^{2+} ion, while the phosphate moiety interacts with the first Mg^{2+} ion (Figs. 1 and 2), it seemed reasonable to expect that the minimum structure capable of acting as a substrate for the enzyme should be ethane-1,2-diol 1-phosphate **3**. This molecule could, in principle, bind in either or both of the two conformations proposed to be adopted by 2′-AMP (Fig. 4A; *cf.* Fig. 1) and D-Ins 1-*P* (Fig. 4B; *cf.* Fig. 2).

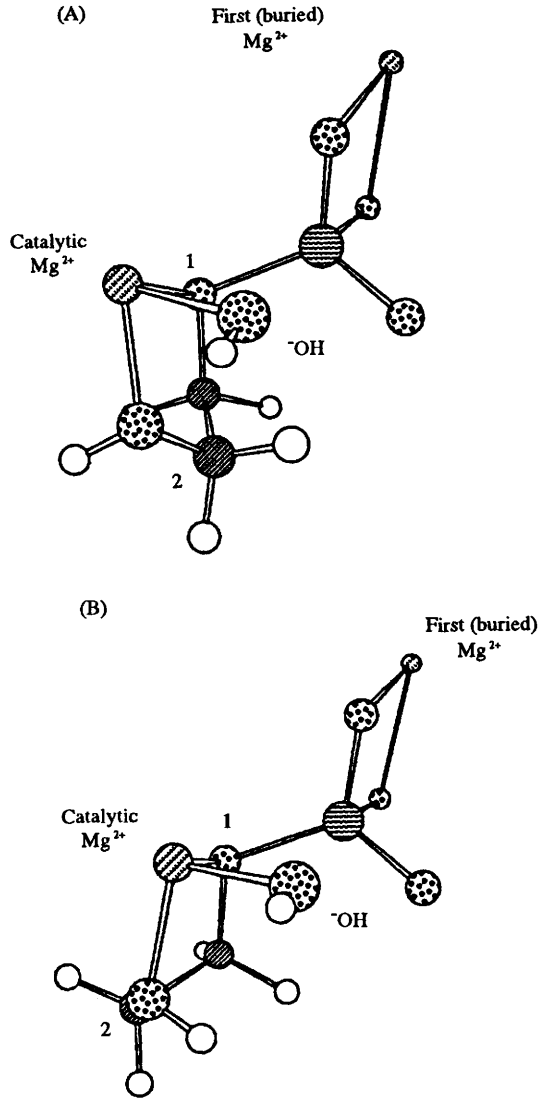
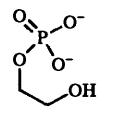
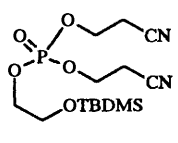


Fig. 4

Since this compound had not been used to probe the structure of the enzyme previously, it was prepared with a view to assessing its activity as a substrate and as an inhibitor. Accordingly, one hydroxy group of ethane-1,2-diol was protected with a *tert*-butyldimethylsilyl group (TBDMS) and the other was phosphitylated with bis(2-cyanoethyl) *NN*-diisopropyl phosphoramidate to give the phosphite triester. Oxidation of the phosphite to the phosphate triester **4** with MCPBA followed by removal of the cyanoethyl and silyl protecting groups gave the monoester **3** which was converted into, and stored as, its bis(cyclohexylammonium) salt.



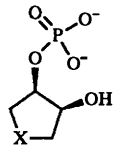
3



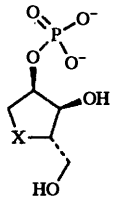
4

When tested as a substrate for inositol monophosphatase in the presence of 2 mmol dm^{-3} Mg^{2+} using a colorimetric inorganic phosphate assay, ethane-1,2-diol 1-phosphate **3** displayed a maximum velocity (V_{max}) of 12% of the V_{max}-value for Ins 1-*P* and a K_m-value of 0.7 mmol dm^{-3}. Note that the K_m-values for Ins 1-*P* and 2′-AMP are 0.1 and 0.9 mmol dm^{-3}, respectively, and that the values of k_{cat} for both Ins 1-*P* and 2′-AMP are approximately 45 s^{-1}.[6] In keeping with reports that the optimum concentration of Mg^{2+} for hydrolysis varies markedly with the structure of the substrate,[6,14] V_{max} for ethane-1,2-diol 1-phosphate **3** increased 1.5-fold upon increasing the Mg^{2+} ion concentration to 5 mmol dm^{-3}. At this high Mg^{2+} ion concentration, Mg^{2+} serves as an inhibitor when Ins 1-*P* is the substrate.[14]

When tested as an inhibitor using a radiochemical assay and [U-^{14}C]Ins 1-*P* as the substrate, ethane-1,2-diol 1-phosphate **3** behaved as a competitive inhibitor, as expected on the basis of its properties as a substrate, and displayed a K_i-value of 1.0 mmol dm^{-3}. Note that the K_i-value for inorganic phosphate under these conditions (pH 8.0; 2 mmol dm^{-3} Mg^{2+}) is 0.5 mmol dm^{-3}.[6] Thus, the small substrate showed moderate activity and would be suitable for elaboration in order to probe the binding interactions between the substrate and the second Mg^{2+} ion.



5



6

Before progressing further, it is useful to consider the results of reported studies of 1,2-diol phosphates within the context of the new two-Mg^{2+} ion mechanism.[7] Leeson *et al.* found that *cis*-cyclopentane-1,2-diol 1-phosphate **5** (X = CH_2) and, indeed, the 3-hydroxymethyl homologue **6** (X = CH_2) did not serve as substrates and displayed very weak properties as inhibitors (IC_{50} = 5 mmol dm^{-3} and 2 mmol dm^{-3} respectively).[15] The corresponding *cis*-cyclohexane-1,2-diol 1-phosphate **5** (X = CH_2CH_2), however, displayed weak substrate activity.[16] While these reports may seem difficult to understand, the above analysis of the active-site complex where the C–OP bond and a lone-pair of the adjacent catalytically essential O-atom must be aligned in a parallel manner [as for 2′-AMP (Fig. 1) and for D-Ins 1-*P* (Fig. 2)] can rationalise these findings. The latter compound, *cis*-cyclohexane-1,2-diol 1-phosphate, can adopt a conformation similar to L-Ins 1-*P* (Fig. 3) and, therefore, might be expected to serve as a substrate. Its low activity can be ascribed to the fact that the key enzyme-binding groups, equivalent to the 4-OH and 6-OH groups of L-Ins 1-*P*, are missing. On the other hand compound **5** (X = CH_2) can adopt neither of the allowed conformations available to compound **3** (Figs. 4A and 4B) because the C-2 atom is confined to a plane containing C-1 and both O-atoms by the cyclopentane ring, Fig. 5. Thus, the $Mg^{2+}2$ ion would be forced out of plane so that the trajectory for the attack of the $Mg^{2+}2$-chelated nucleophile on the phosphorus atom would be moved away from the optimum in both of the possible enzyme-bound conformations. This possibility is shown in Figs. 5A and 5B where a dashed line represents the optimum trajectory for attack.

This analysis assumes that $Mg^{2+}2$ can actually bind to the enzyme–$Mg^{2+}1$–potential substrate complex and there are ample data in support of this notion. While it is known that the affinity of the enzyme–$Mg^{2+}1$–substrate complex for $Mg^{2+}2$ is acutely dependent on the position of the 'catalytic O-atom' in substrates (*e.g.*, Ins 1-*P* **1**, 2′-AMP **2**, Ins 4-*P* and glycerol 2-phosphate),[6,14] one known substrate, 4-nitrophenyl phosphate,

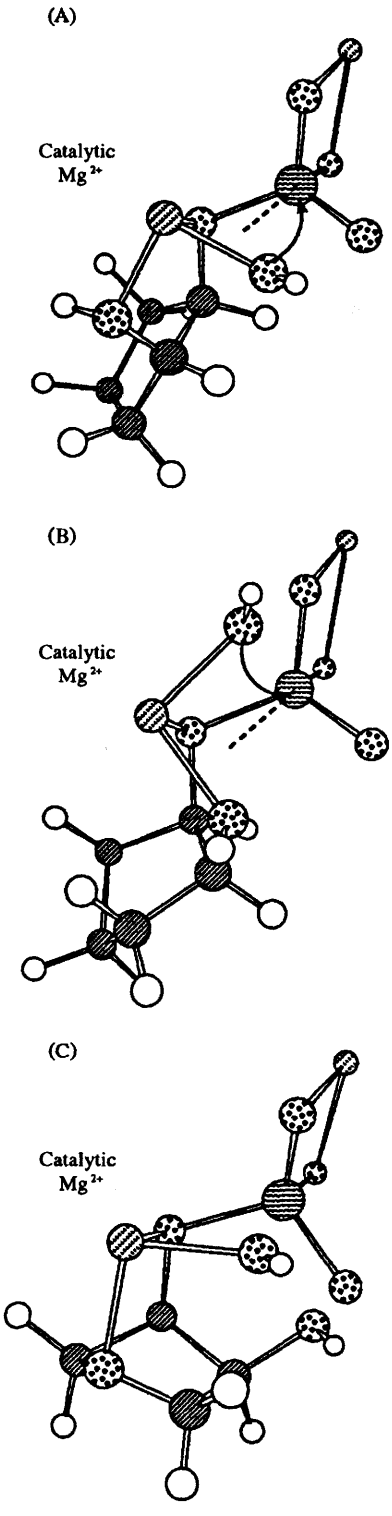
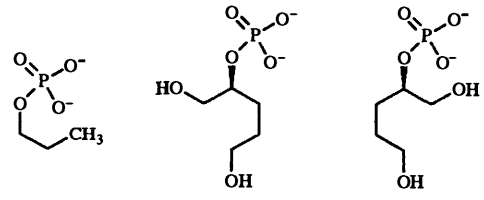


Fig. 5

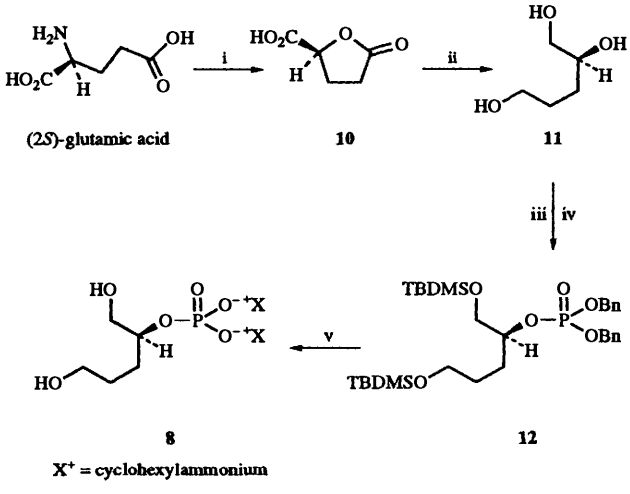
lacks the catalytically essential O-atom.[14] Although V_{max} for the compound is very low (5% of that for Ins 1-*P*), in spite of the fact that the leaving 4-nitrophenolate group (pK_a = 8) is a much better nucleofuge than alkoxide (pK_a = 16), that the compound does serve as a substrate indicates that $Mg^{2+}2$ binds. In accord with our own analysis, that the catalytic O-atom is important for $Mg^{2+}2$ binding,[1,7] the concentration of Mg^{2+} ions required to give a half-maximal rate of hydrolysis was found to be 1.4 mmol dm^{-3}, seven times higher than that for Ins 1-*P*.[11] Presumably, a water molecule can take the place of the catalytically essential O-atom in this instance. Thus, there is every reason to believe that $Mg^{2+}2$ does bind to the enzyme even in certain enzyme–$Mg^{2+}1$–inhibitor complexes where there is no group equivalent to the 6-OH group.[16] In accord with these arguments the phosphorylated tetrahydrofurandiol **6** (X = O) and cyclopentanediol **6** (X = CH_2) act as equally good inhibitors (IC_{50} = 2 mmol dm^{-3}),[15] but only the tetrahydrofuran **6** (X = O) is a substrate (of comparable activity to 2′-AMP **2**). In essence, it appears that $Mg^{2+}2$ does not bind productively to the enzyme–$Mg^{2+}1$–**6** (X = CH_2) complex, but does to the analogous complex derived from compound **6** (X = O) where the ether O-atom is able to chelate to $Mg^{2+}2$, as it does in 2′-AMP. This arrangement is shown in Fig. 5C (hydroxymethyl group omitted) and it is remarkable how closely the structure resembles one of the allowed conformations for ethane-1,2-diol 1-*P* **3**, Fig. 4B.

Proceeding with the functional analysis of the minimum substrate **3**, the importance of the 2-OH group was verified through its replacement by a methyl group, to give propyl phosphate **7**. The compound was completely inactive as a substrate, even at elevated Mg^{2+} concentrations (10 mmol dm^{-3}), and the compound showed no inhibitory activity at concentrations of up to 50 mmol dm^{-3}.



7 **8** **9**

In order to probe further the environment of the second Mg^{2+} ion, compound **3** was elaborated by incorporating extra oxygen-containing functionalities into specific positions. Thus, (2*S*)- and (2*R*)-pentane-1,2,5-triol 2-phosphate (**8** and **9**, respectively) were prepared from (2*S*)- and (2*R*)-glutamic acid, respectively, as outlined in Scheme 1 and as described in the Experimental section.



Scheme 1 *Reagents, conditions and yields:* i, $NaNO_2$, aq.HCl, −10 °C, 76%; ii, $LiAlH_4$, THF, 45%; iii, TBDMSCl, imidazole, DMF, 68%; iv, (a) $Pr^i_2NP(OBn)_2$, 1*H*-tetrazole, MeCN; (b) MCPBA, CH_2Cl_2, 78%; v, (a) H_2, Pd/C; (b) TBAF, THF; (c) Amberlite 118(H^+); (d) cyclohexylamine, water, 69%

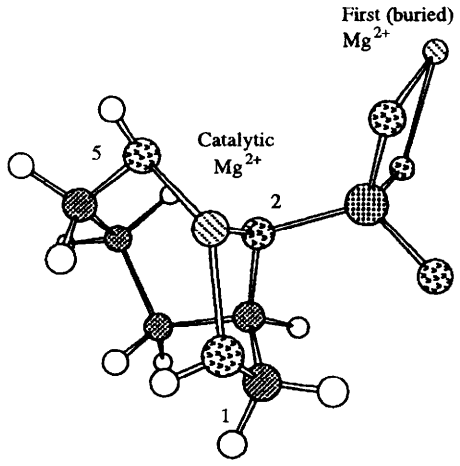


Fig. 6

(2*S*)-Pentane-1,2,5-triol 2-phosphate **8** served as a competitive inhibitor for the enzyme (K_i = 0.12 mmol dm^{-3}), as expected on the basis of the behaviour of the constrained cyclic analogue (1*S*,2*R*,4*S*)-cyclohexane-1,2,4-triol 1-phosphate **13** which is a competitive inhibitor.[16] Presumably, the interaction of the 1-OH and 5-OH groups of compound **8** with the sites for the 2-OH and 4-OH groups of Ins 1-*P* (Fig. 2) accounts for the 9-fold reduction in K_i over that for the diol phosphate **3** even though C-1 and C-5 in compound **8** cannot approach as closely as C-1 and C-4 in the constrained cyclic analogue **13**. For both compounds **8** and **13** it is possible that $Mg^{2+}2$ does not bind to the enzyme–inhibitor complex because a key metal ion-binding group is absent. On the other hand, by analogy with (1*R*,2*S*,4*R*)-cyclohexane-1,2,4-triol 1-phosphate **14**,[16] the (2*R*)-enantiomer of pentane-1,2,5-triol 2-phosphate, compound **9**, might have been expected to serve as a substrate since it possesses both of the putative Mg^{2+} ligands of the minimum substrate **3** as well as a hydroxy group capable of binding to the site for the 4-OH group of Ins 1-*P*. However, compound **9** did not serve as a substrate and, furthermore, was a weak inhibitor, K_i = 3.8 mmol dm^{-3}. A reasonable explanation is that the flexible 5-OH group binds to a site on the second Mg^{2+} ion, possibly normally occupied by the enzyme, and disrupts the geometry of the complex so that hydrolysis is prevented, Fig. 6. [A full analysis of these interactions within the environment of the protein is provided in the following article.][13] It is worthy of note that this compound is the only non-constrained compound known to us that possesses all of the features required for a substrate [*i.e.*, the minimal structure **3** plus a hydroxyalkyl side-arm of suitable length (to bind in the site for the 4-OH group of Ins 1-*P*)] that does not hydrolyse.

In another series of compounds, the free hydroxy group of ethane-1,2-diol 1-phosphate **3** was elaborated to provide molecules that might displace the nucleophilic water molecule from its site on the second Mg^{2+} ion; see Fig. 1. Accordingly, diethylene glycol phosphate **15** (X = O), diethylene glycol cyclic phosphate diester **16** (X = O), pentane-1,5-diol 1-phosphate **15** (X = CH_2) and pentane-1,5-diol cyclic phosphate diester **16** (X = CH_2) were prepared; see Experimental section. For comparison, 2-methoxyethyl-phosphate **17** was also prepared.

Each of the compounds was tested as a potential substrate and inhibitor for the enzyme, and none was a substrate, as expected. However, their competitive modes of inhibition and the values of the inhibition constants are informative and are consistent with structures depicted in Figs. 1–4.

For example, the 2-OH group of compound **3** was replaced by a methoxy group, to give compound **17**. 2-Methoxyethyl phosphate **17** showed no activity whatsoever as a substrate and displayed a massive 25-fold reduction in binding affinity as an inhibitor ($K_i \geqslant 25$ mmol dm^{-3}) compared with compound **3**. Indeed, the K_i-value is similar to that for propyl phosphate **7**, suggesting that the ether O-atom is virtually redundant. Interestingly, 6-*O*-methylinositol 1-phosphate (6-*O*-MeIns 1-*P*) has also been reported to be inactive as a substrate.[15] Like compound **17**, 6-*O*-MeIns 1-*P* behaves as an inhibitor which demonstrates that the ability of the O-atoms in the substrate (O-6 in D-Ins 1-P **1** and O-2 in compound **3**) to interact with $Mg^{2+}2$ or its chelated hydrolytic water molecule is severely compromised by alkylation to give the methyl ether derivatives. These observations indicate that steric crowding or adverse hydrophobic effects within the active site can prevent the lone-pairs of these O-atoms from assuming positions which support catalysis. Indeed, only in the case of highly ordered cyclic tetrahydrofuranol phosphate substrates [*e.g.*, 2′-AMP and compound **6** (X = O), where the lone-pair of the 1′-O-atom and 2′-C–O bond are aligned parallel, see Fig. 1,[7] and Fig. 5C] can an ether O-atom support catalysis.[7,15] Note that although, in principle, 2-methoxyethanyl phosphate **17** could adopt a similar conformation to cyclic ribofuranose 2-phosphate portion of the substrate 2′-AMP where the methyl group resides in the position occupied by C-4′ in 2′- AMP (Fig. 1), this arrangement is not one of the stable binding modes for the acyclic compound **17**; see following article.[13]

Replacement of the methyl group in compound **17** by a 2-hydroxyethyl group to give compound **15** (X = O), led to a ⩾8-fold increase in binding affinity (K_i = 3.5 mmol dm^{-3}) relative to compound **17**. This increase in affinity is ascribed to the ability of the inhibitor to provide an extra or alternative ligand for the second Mg^{2+} ion possibly by relocating or displacing the nucleophilic hydroxide ligand; see Fig. 7. The replacement of the ether O-atom to give compound **15** (X = CH_2) did not affect the K_i-value, as would be expected if the ω-hydroxy O-atom rather than the ether O-atom of compound **15** (X = O) is responsible for the major interaction with the second Mg^{2+} ion. The fact that compound **15** (X = O) did not serve as a substrate suggests that the ω-hydroxy group does not bind in the site for the 5′-OH group of 2′-AMP, Fig. 1. This is in accord with our analysis of the high free energy that such acyclic conformations would require in order to make the ether O-atom available for chelation to $Mg^{2+}2$ (see following article).[13]

The cyclic ether diester **16** (X = O) was prepared in order to determine whether the compound could serve as a substrate. Unfortunately, the structure reduces the negative charge on the phosphate moiety from −2 to −1. This change in charge was expected to have two effects, first to decrease binding affinity for the cyclic ether diester **16** (X = O) relative to the phosphate monoester **15** (X = O), due to the decreased charge available

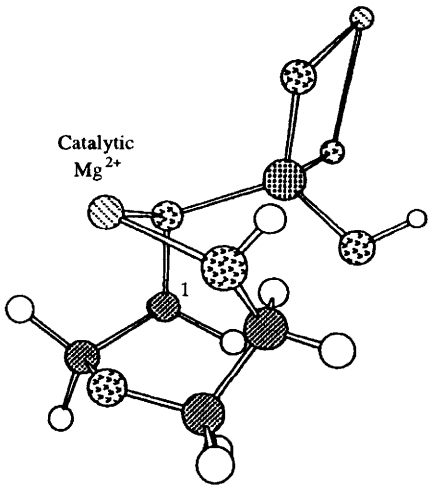


Fig. 7

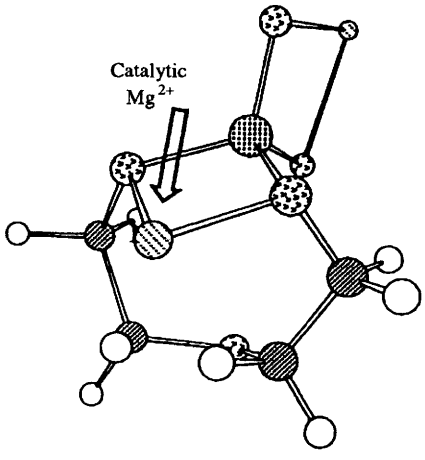


Fig. 8

for interaction with $Mg^{2+}1$, and; second, to decrease the affinity of the enzyme-bound species for $Mg^{2+}2$. In accord with these expectations, the diester **16** (X = O) was a weak inhibitor (K_i = 8 mmol dm^{-3}). Nevertheless, this result is remarkable given that the compound is a monoanion. Note that the value of K_i for sulfate dianion[17] is 20 mmol dm^{-3} and that the value of K_i for propyl phosphate dianion is > 50 mmol dm^{-3}; see above. The compound cannot bind to the enzyme in the same manner as the acyclic analogue **15** (X = O) because the surrogate for the nucleophile is now actually bonded to the P-atom. This will cause some reorganisation of the position of the phosphate O-atoms which might account for the low K_i-value. Fig. 8 shows one possible, highly symmetric binding mode which assumes that both bridging O-atoms interact equally well with $Mg^{2+}2$. That the compound was not a substrate for inositol monophosphatase is entirely consistent with a mechanism in which $Mg^{2+}2$ rather than $Mg^{2+}1$ provides the nucleophile, see the Discussion section below.

The removal of the ether O-atom in the monoanion **16** (X = O) to give compound **16** (X = CH_2) exerted little effect on the K_i-value, which was 7.5 mmol dm^{-3}. This result is in keeping with the earlier finding that the ether O-atoms in compounds **15** (X = O) and **17** do not interact strongly, if at all, with $Mg^{2+}2$. Taken together with the knowledge that ethane-1,2-diol phosphate is a substrate, these observations indicate that the catalytically essential O-atom in substrates does not interact with $Mg^{2+}2$ directly but, rather, provides a lone-pair to position the $Mg^{2+}2$-chelated nucleophile, water, properly and hold it in place on the metal ion. When the nucleophile is replaced by the terminal hydroxy group of compound **15** (X = O) to give an intramolecular complex, there may no longer be a need to stabilise the position of the coordinated O-atom such that the K_i-value of the compound is not affected by the substitution of the ether O-atom for a methylene group.

As alluded to above, none of the compounds **15–17** showed any activity as substrates. This was determined using the colorimetric enzyme assay and also by incubating each of the compounds with inositol monophosphatase for extended periods whilst monitoring the reaction solutions directly by ^1H, ^{13}C and/or ^{31}P NMR spectroscopy in experiments similar to those described previously.[7] The cyclic diesters were notably not cleaved to give monoesters.

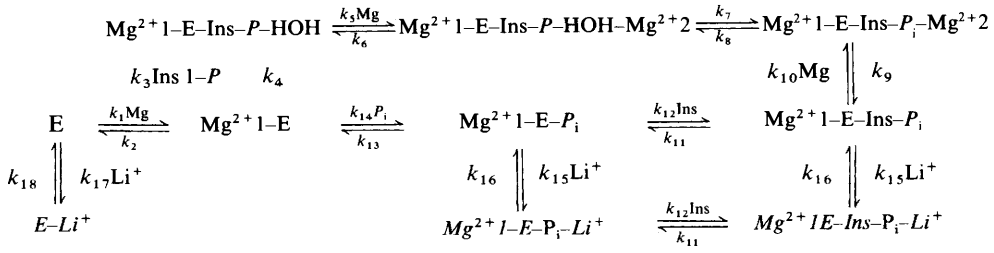
Discussion

Further consideration reveals that the results obtained from the study of the monoesters gives clues to the location of the active-site nucleophile. All of the monoesters [**9**,**15** (X = O, CH_2), **17**] described here as well as 6-*O*-methylinositol 1-*P* and the *cis*-cyclopentane-1,2-diol 1-phosphates[15] (**5** and **6**, X = CH_2) do contain the two requisite oxygen functionalities for binding to the second Mg^{2+} ion. For these compounds, those which serve as competitive inhibitors should also serve as substrates, if the first (buried) Mg^{2+} ion chelates and activates the nucleophilic water molecule. In these circumstances the role of the second metal ion would be merely to stabilise the leaving alkoxide group. Such a mechanism would predict an in-line displacement at phosphorus with inversion of configuration.[18]

Conversely, if the second Mg^{2+} ion provides the site for the nucleophile none of the compounds [**9**, **15** (X = O, CH_2), **17**], 6-*O*-methylinositol 1-*P* or *cis*-cyclopentane-1,2-diol 1-phosphates[15] (**5** and **6**, X = CH_2) which can provide a ligand to displace the nucleophile, or cause the reorganisation of the coordination geometry about $Mg^{2+}2$, would be hydrolysed. Indeed as is observed, the reaction would be extremely sensitive to small changes in the position of the metal-bound nucleophile. The results of this study and others[15] are therefore in accord with a mechanism involving two Mg^{2+} ions in which the *second* Mg^{2+} ion chelates and activates the nucleophile. Such a mechanism predicts adjacent displacement at phosphorus with pseudorotation and retention of configuration.[18] While adjacent displacement with pseudorotation at phosphorus has not yet been demonstrated for an enzymic system,[19] there is irrefutable chemical precedence for the mechanism[20] and other evidence to suggest that inositol monophosphatase utilises $Mg^{2+}2$ in activating the nucleophilic water molecule.

Some of this evidence stems from the results of a kinetic study where the rates of inositol-mediated ^{18}O-exchange from [^{18}O]water into inorganic phosphate were measured. The kinetic data gave a value for V_{Ex} (the maximum velocity of the ^{18}O-exchange reaction in the presence of saturating inositol and P_i) of ~70% of V_{max} for the hydrolysis reaction for Ins 1-*P*.[5,6] To appreciate why this fast exchange rate allows comment on which of the Mg^{2+} ions supplies the nucleophile we must first elaborate a published kinetic scheme[6] to allow for the fact that two Mg^{2+} ions are required for catalysis (Scheme 2) and recall that inositol monophosphatase does not form phosphorylated enzyme intermediates.[6]

In order to exchange ^{18}O-label from [^{18}O]water into P_i without forming phosphorylated enzyme intermediates, a substrate molecule must be first synthesized by the enzyme from inositol and P_i, along with an unlabelled water molecule, and then subsequently be hydrolysed with ^{18}O-water.[6] Two phosphoryl-transfer steps are therefore required. It is known that in the forward hydrolysis reaction direction the catalytic



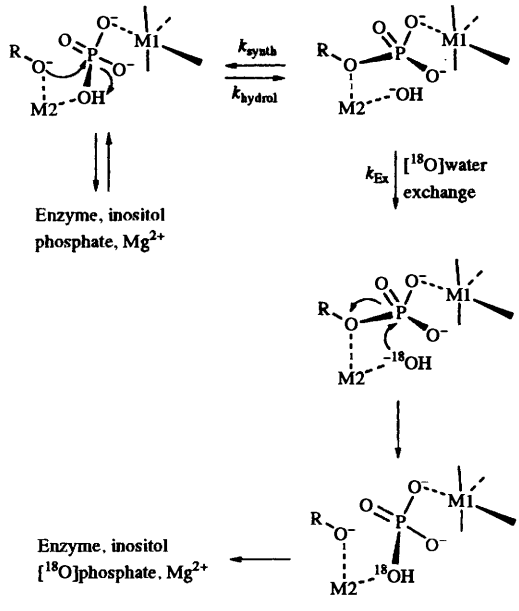
Scheme 2 Full kinetic scheme for catalysis and for Li^+ inhibition. The concentration of the bold complex increases at high Mg^{2+} concentration and causes inhibition. Inositol can probably dissociate from this complex to give $Mg^{2+}1$–E–P_i–$Mg^{2+}2$. Italicised complexes form only in the presence of Li^+ where Li^+ binds in the site for $Mg^{2+}2$. The complex E–Li^+ forms only at very high Li^+ concentration ($\geqslant 5$ mmol dm^{-3}) where Li^+ binds in the site for Mg^{2+} 1.

hydrolysis step, in which the phosphoryl group is transferred from inositol to water (k_7 in Scheme 2), is partially rate limiting.[14] Since the two phosphoryl-transfer steps involve similar nucleophiles and nucleofuges (*i.e.*, the steps are highly symmetric) it is reasonable to expect that the rate of phosphoryl transfer to inositol will not be faster than the partially rate-limiting catalytic hydrolysis step. Hence, *a priori*, it would be expected that the maximum observable ^{18}O-exchange rate (V_{Ex}) would be significantly lower than the maximum observable rate for hydrolysis (V_{max}). Put simply this analysis indicates that a value for V_{Ex} of 70% of the magnitude of V_{max} is very close to the theoretical maximum and that the on-off rate for (^{18}O-labelled) water must be very fast compared with k_7 and k_8 in Scheme 2.

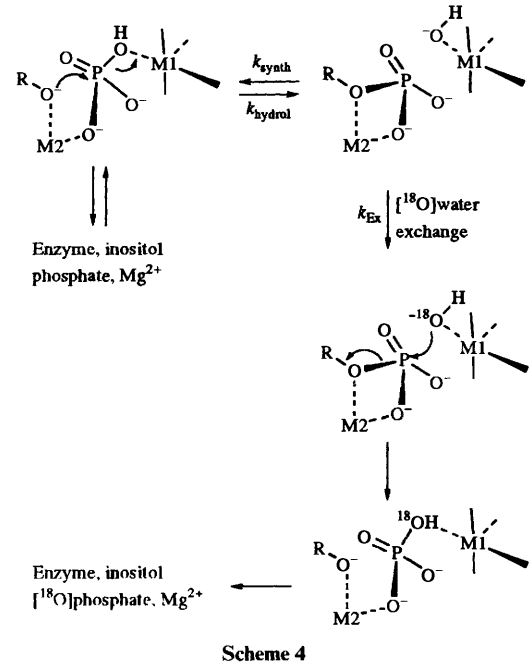
Mechanistically, this fast rate of phosphate-ligand exchange is much easier to accommodate if the nucleophilic water molecule is associated with the second Mg^{2+} ion (*i.e.*, the second Mg^{2+} ion on and the first one off). This is because the sequence for species binding is known for the hydrolysis reaction and occurs in the order $Mg^{2+}1$ on first, then substrate (or phosphate) and then $Mg^{2+}2$ (see Scheme 2).[6] Indeed, the first Mg^{2+} ion does not even need to vacate the active site between catalytic events and is almost certainly trapped in the active site by the phosphate moiety of substrates or inorganic phosphate at high concentrations of these species. Conversely, during the ^{18}O-exchange reaction, the *second* Mg^{2+} ion on must debind from the enzyme with its associated hydration ligands, between every observable catalytic event, in order to allow phosphate to debind from the active site.

The principle of microscopic reversibility requires that if $Mg^{2+}2$ provides the nucleophile for the hydrolysis of Ins 1-*P*, it must also accept the hydroxy group nucleofuge during the synthesis of Ins 1-*P* from inositol and P_i in the reverse reaction direction (Scheme 3). Therefore, most simply, the exchange reaction in [^{18}O]water needs only to reflect the partition ratio between, on the one hand, the hydrolysis (k_{Hydrol}) of the nascent Ins 1-*P* with [^{16}O]water newly derived from the unlabelled P_i molecule (to give unlabelled P_i) and, on the other hand, the off-rate for [^{16}O]water (with or without its coordinating $Mg^{2+}2$ ion) and its replacement with an [^{18}O]water molecule from the bulk solvent (k_{Ex}). This will lead to re-hydrolysis with [^{18}O]water (Scheme 3). Recall that the hydrolytic step is partially rate limiting in the overall physiological hydrolysis[14] where k_{cat} for each of the two subunits of the enzyme is 25–38 s^{-1} at 37 °C (depending on substrate).[6] Therefore, k_{Hydrol} has an estimated value of ~ 100 s^{-1}. Note that k_{Hydrol} (Schemes 3 and 4) is k_7 in Scheme 2.

Whilst we believe that this latter mechanism is the correct one, very recent work derived from X-ray crystallographic studies places the nucleophilic water molecule on $Mg^{2+}1$.[8–10] Essentially, a water molecule (W2) is located near the position required for the in-line displacement of O-1 of Ins 1-*P* from the phosphate ester moiety (3.5–3.6 Å from the P-atom) in an enzyme–Gd^{3+}–Ins 1-*P* complex that does not contain a second



Scheme 3



Scheme 4

metal ion.[9] Modelling studies by the same group on an annealed construct of a bis-Mn^{2+}–Ins 1-*P*–enzyme complex show that the position of W2 is stable during molecular dynamics simulation and that the O-atom is 3.3 Å from both $Mn^{2+}1$ and the phosphate P-atom such that the W2 O-atom, the P-atom

and the 1-O-atom of the inositol nucleofuge form an angle of 141°.[8] The W2 O-atom also forms hydrogen bonds with the side-chains of Glu-70 (2.89 Å) and Thr-95 (2.85–3.55 Å), which suggests that W2 is rather tightly bound (and, maybe, unsuitable as a nucleophile; see Discussion section in the following article).

Additional support for this conventional in-line displacement in which the nucleophile is associated with $M^{2+}1$ is also drawn from the fact that in the crystal structure of the bis-Mn^{2+}–phosphate–enzyme complex, the phosphate group is approximately inverted with respect to the situation for the substrate complex such that the water molecule (W2) is displaced (or at least, relocated) from the metal ion by a phosphate O-atom.[10] Thus, in simple terms the enzyme appears to be able to bind both halves of a distorted trigonal bypyramid where the apical O-atoms correspond to the positions occupied by O-1 of Ins 1-*P* and the O-atom of W2. Assuming that there are no gross changes in the motion of the phosphate moieties induced by changes in the nature of the metal ion occupancy, which may be reasonable, the observations are consistent with an in-line displacement. However, a pseudorotation mechanism would also give rise to an apparently inverted phosphate as the mechanism also generates a pentacoordinate intermediate in which one of the phosphate O-atoms in the product must be diametrically opposite to the position occupied by the departing ester O-atom.[18,19]

For an in-line displacement, the most simple mechanism that could support ^{18}O-label exchange into P_i under these circumstances, where the [^{16}O]water molecule newly derived from unlabelled P_i is coordinated to $Mg^{2+}1$, requires the very rapid exchange of this ($Mg^{2+}1$-coordinated) [^{16}O]water molecule with [^{18}O]water in the bulk solvent; while, at the same time, all the other interactions of $Mg^{2+}1$ with its coordinating ligands, derived from the substrate and the protein, remain intact (Scheme 4). Recall from above that $Mg^{2+}1$ is surrounded by at least four substrate and protein-derived ligands (see the following article[13]) and note that the rate of inner-ligand exchange for neutral water molecules on *unimpeded* Mg^{2+} ions is $\sim 3 \times 10^5$ s^{-1} at 37 °C.[21] Also note that the [^{16}O]water molecule newly derived from unlabelled P_i is, actually, W2 in the crystal structures referred to above and that W2 forms hydrogen bonds with protein-derived residues, which stabilise its position.[8]

The rapid exchange of water at the W2 site on $Mg^{2+}1$ would only be observable if it occurred in the higher order substrate-bound complexes (E–$Mg^{2+}1$–Ins-*P*–$Mg^{2+}2$ and E–$Mg^{2+}1$–Ins-*P*; see Schemes 2 and 4) since water that exchanges on the metal ion prior to formation of the bound substrate will not be suitably disposed for attacking the P-atom. This is because, prior to the formation of the substrate, the W2 site is blocked (by the inorganic phosphate O-atom which becomes the new W2 molecule) and the principle of microscopic reversibility requires that only W2 can re-attack before exchange takes place. Thus, ligand exchanges on the metal (k_{Ex}) must occur before, and, therefore, in competition with, the hydrolysis (k_{Hydrol}) of the newly formed substrate molecule by coordinated [^{16}O] water (W2) (Scheme 4), at a rate of at least 500–1000 s^{-1} (*i.e.*, significantly faster than k_{Hydrol}). Although it must be conceded that such a mechanism is not impossible, it requires the coordination sphere of the most deeply buried Mg^{2+} ion ($Mg^{2+}1$) to be easily accessible to bulk water in an enzyme conformation which is poised to catalyse its physiological reaction. At this time one would expect the structure of the protein to be most constrained, in order to stabilise the transition state for the hydrolytic process, and the location of key species, including the nucleophile and the activating metal ion, to be highly controlled by the enzyme and not free to participate in side-reactions. (Further evidence against the location of the nucleophile on $Mg^{2+}1$ is provided by analysis of the protein environment for substrate-bound structures, see the following article.)[13]

Other observations which support the notion that the nucleophile is located on $Mg^{2+}2$ come from studies of phosphorothioate substrates. It is known from kinetic studies that $Mg^{2+}2$ binds to the enzyme after the substrate binds[6] and that the affinity of the ternary E–$Mg^{2+}1$–substrate complex for $Mg^{2+}2$ varies markedly with the structure of the substrate.[6,14] Therefore, a ternary complex must exist and must provide the correct chelation environment for $Mg^{2+}2$. These conclusions are borne out by a significant amount of structural data provided both here (in this series of papers) and by X-ray crystallographic data from the Merck, Sharp and Dohme group, which supply the structures of two ternary E–Gd^{3+}–Ins 1-*P* complexes.[9] It is also known that 2′-AMP is a good substrate for the magnesium form of the enzyme[6] and that adenosine 2′-phosphorothioate fails to react or show any activity as an inhibitor.[7] These observations indicate that 2′-AMPS does not bind to the Mg^{2+} form of the enzyme, as was discussed in the previous paper in this series.[7] Careful analysis indicates that the result implies that even the ternary complex (E–$Mg^{2+}1$–2′-AMPS) does not form, since if it did, inhibition would be observed. $Mg^{2+}1$ is, therefore, clearly associated with binding to the phosphate group and when 2′-AMPS is the substrate it is necessary to replace $Mg^{2+}1$ with a thiophilic Mn^{2+} ion in order to ensure that the substrate can bind and undergo hydrolysis. This is because 2′-AMPS exists in a high-energy bound arrangement that requires the stabilisation provided by a strong primary interaction between the buried metal ion and the sulfur atom of the phosphorothioate group.[7]

The rate of 2′-AMPS hydrolysis by the Mn^{2+} enzyme is 20% of the rate for 2′-AMP hydrolysis by the Mg^{2+} or Mn^{2+} forms of the enzyme.[7] Moreover, the phosphorothioates of D-Ins 1-*P* and L-Ins 1-*P* are hydrolysed by the Mg^{2+} form of the enzyme at 10% of the rate for the analogous phosphates.[22] These high rates of hydrolysis are extremely difficult to accommodate into a mechanism in which $M^{2+}1$ provides the nucleophile given that $M^{2+}1$ is involved in binding to the sulfur atom of the phosphorothioate group. Owing to the larger size of sulfur, such an interaction would separate the P-atom from $M^{2+}1$ by 0.2–0.3 Å compared with the situation for oxygen and increase the steric hindrance for an in-line attack by the nucleophile on phosphorus. Each of these factors should significantly reduce the reaction rate. These problems need not be encountered for an adjacent displacement mechanism[20a] where the nucleophile can attack either in-line from opposite the sulfur atom *via* an associative mechanism, or from the same face as the departing nucleofuge in a dissociative 'metaphosphate-type' mechanism,[20b] as could be the case if the nucleophile were to be provided by $M^{2+}2$.

Of relevance to the present analysis, fructose 1,6-bisphosphatase shows significant primary structural identity to inositol monophosphatase for the metal ion-binding region and some similar mechanistic properties.[19] This enzyme hydrolyses the 1-phosphate ester group of the α-anomer of D-fructose 1,6-bisphosphate to give fructose 6-phosphate and P_i and also employs two divalent metal ions. The enzyme catalyses ^{18}O-exchange between solvent and P_i but, as for inositol monophosphatase, only in the presence of the product, in this case fructose 6-phosphate.[23] The hydrolysis occurs *via* an in-line displacement, with inversion of configuration.[24] However, the binding affinity of the two metal ions is reversed compared with inositol monophosphatase and it is the second Mg^{2+} ion to bind that supplies the activated water molecule in the hydrolysis of the substrate.[19] This scenario is analogous to the one that we are proposing for inositol monophosphatase and, hence, it appears that the mechanism (but not the

stereochemical course) for exchanging ^{18}O-label from the solvent into P_i for the two enzymes might be the same.

Given that there is now strong evidence to show that two Mg^{2+} ions are required for catalysis[6,7,8–10,25] and because it is known that Li^+ and Mg^{2+} (at high Mg^{2+} concentration) serve as mutually exclusive uncompetitive inhibitors[14] and bind to the E–P_i product complex,[6] it appears that the proposal that K_i for inhibition by Li^+ is different for different substrates because the steady-state concentration of E–P_i varies for different substrates[6] is correct. However, with the emergence of the new catalytic Mg^{2+} ion site it is necessary to point out that Li^+ must occupy the second site for Mg^{2+} in the Mg^{2+}–E–P_i product complex (see Scheme 2). At higher concentration Li^+ is able to bind into the first Mg^{2+} (buried) site (occupied by Gd^{3+} in an X-ray crystal structure[4]) as is demonstrated by the almost unique change in the mode of inhibition by Li^+ from uncompetitive to noncompetitive (with respect to substrate) with increasing Li^+ concentration.[6] A full kinetic mechanism, consistent with all known properties for the enzyme and which is modified from our earlier analysis[6] to take account of the fact that two Mg^{2+} ions are required for catalysis, is shown in Scheme 2.

The results and analyses described in this paper provide a rationale for the mechanism and substrate specificity of inositol monophosphatase. The involvement of two Mg^{2+} ions in the catalytic mechanism is now certain and the most plausible location for the hydrolytic water molecule is on the second Mg^{2+} ion. All of the conformational analyses performed in this paper consider only the position and spatial organisation of the second Mg^{2+} ion and the hydrolytic water molecule with respect to the substrate (and its putative metal-binding groups) and the position of the first Mg^{2+} ion, for a range of substrates and inhibitors. A more refined and detailed analysis which considers the structural and mechanistic implications within the environment of the protein is presented in the following article.[13] Since we started this work and reporting our preliminary findings,[1] several articles have appeared that support a two-metal-ion mechanism.[8–10] There is a high degree of agreement in the findings of these recently reported studies, which are derived largely from X-ray crystal data, and those of our own. However, there are notable differences, in particular with regard to the position of the metal bound nucleophile. These details are also discussed in the following article.

Experimental

NMR Spectra were recorded on a Bruker AM-300 spectrometer (^1H, 300 MHz; ^{13}C, 75 MHz; ^{31}P, 121.5 MHz) and a Varian Gemini spectrometer (^1H, 200 MHz; ^{13}C, 50 MHz). High-field NMR spectra were obtained on an SERC service basis at the University of Warwick (^1H, 400 MHz) and the University of Edinburgh (^1H, 600 MHz; ^{13}C, 150 MHz; ^{31}P, 242 MHz). ^1H NMR spectra were referenced internally on ^2HOH (δ 4.68), $CHCl_3$ (δ 7.27) or DMSO (δ 2.47). ^{13}C NMR spectra were referenced on MeOH (δ_C 49.9), C^2HCl_3 (δ_C 77.5), or DMSO (δ_C 39.70) and ^{31}P spectra on external H_3PO_4 (δ_P 0). *J*-Values are given in Hz. IR spectra were recorded using a Perkin-Elmer 1420 ratio recording spectrometer and a Perkin-Elmer 1710 FT IR spectrometer. The samples were prepared as Nujol mulls or thin films between sodium chloride discs. Absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. Mps were measured using an electrothermal mp apparatus and are uncorrected. Optical rotations were measured on an Optical Activity Ltd. AA-100 polarimeter using 10 cm path length cells at room temperature; $[\alpha]_D$-values are given in 10^{-1} deg cm^2 g^{-1}. Mass spectra were recorded on a Kratos MS50, and were obtained on an SERC service basis at the University of Swansea using a VG ZAB E. Major fragments are given as percentages of the base-peak intensity. GLC/MS spectra were recorded on a Hewlett Packard 5890A 6C. UV–visible optical densities were measured on a Cam Spec M302 spectrophotometer. Solvents and common reagents were purified according to the method of Perrin and Armarego.[26] Flash chromatography was performed according to the procedure of Still[27] using Sorbisil C60 (40–60 μm) silica gel. Analytical TLC was carried out on 0.25 mm precoated silica gel plates (MN SIL G/UV_{254}) or on 0.1 mm precoated cellulose plates (CEL MN 300–10/UV_{254}), and compounds were visualised by UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid, aq. potassium permanganate, acidic palladium(II) chloride or ninhydrin. Light petroleum refers to the fraction boiling at 40–60 °C. Scintillation counting for ^{14}C-compounds was performed using a Packard Tri-Carb 4000 scintillation counter.

Buffers, salts and deuterium oxide were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Inositol 1-phosphates were prepared as described previously,[6,28,29] other substrates were prepared as described below. Amberlite IR 118 (H^+) and Dowex 1 × 8(OH^-) ion-exchange resins were obtained from British Drug Houses (Poole, Dorset, UK) and [U-^{14}C]inositol 1-phosphate was obtained from Amersham International (Amersham, Bucks, UK). Water-miscible scintillant (ES-199) was obtained from Canberra Packard (Pangbourne, Berks, UK). Phosphitylating agents were prepared from phosphorus trichloride using literature procedures and were stored under argon at −20 °C prior to use. All other chemicals were of analytical grade or were recrystallised or redistilled before use.

Enzyme

Bovine brain inositol monophosphatase was purified from a recombinant strain of *E. coli* as described previously.[6] Enzyme-activity assays were performed using a colorimetric assay developed by Itaya and Ui[30] (employing molybdic acid and Malachite Green) or using a radiochemical assay.[6] Rate determinations were performed at 37 °C in triplicate under conditions identical with those described in the previous article.[7] All of the compounds described here were also assessed as potential substrates by using NMR spectroscopy. The experimental protocols were similar to those described in the previous article.[7]

2-(*tert*-Butyldimethylsiloxy)ethyl bis-2-cyanoethyl phosphate 4

tert-Butyldimethylsilyl chloride (1.5 g, 10 mmol) was added to a solution of ethylene glycol (2.5 g, 50 mmol) and imidazole (1.85 g, 20 mmol) in dry DMF (30 cm^3) under dinitrogen and the solution was stirred at 20 °C. After 3 h aq. sodium hydrogen carbonate (30 cm^3; 5%) was added and the solution was extracted with diethyl ether (3 × 30 cm^3). The pooled organic fractions were washed successively with water (20 cm^3) and saturated brine (30 cm^3) and then dried (Na_2SO_4). The solvent was removed under reduced pressure to yield a liquid which displayed the expected NMR spectral data. The silylated glycol was redissolved in dry acetonitrile (30 cm^3), and 1*H*-tetrazole (0.7 g, 10 mmol) and freshly prepared bis(2-cyanoethyl) *N*,*N*′-diisopropylphosphoramidite[30] (3.50 g, 13 mmol) were added. The reaction mixture was stirred at 20 °C under dinitrogen for 1 h and was then cooled to −10 °C. A solution of MCPBA (50–60%; 5.3 g, 15 mmol) in dry dichloromethane (50 cm^3) was added and stirring was continued for a further 1 h. The reaction mixture was diluted with dichloromethane (30 cm^3), washed successively with aq. sodium sulfite (3 × 50 cm^3; 10%) and saturated brine (30 cm^3) and dried (Na_2SO_4). The solvent was removed to yield a pale yellow oil, which was purified by chromatography on triethylamine-treated silica (elution with ethyl acetate) to give *phosphate* **4** as a liquid (1.94 g, 54%), *m/z* (Found: $[M + H]^+$, 363.1505. $C_{14}H_{28}N_2O_5PSi$ requires *m/z*,

363.1505); ν_{max}(Nujol)/cm^{-1} 3000s (C–H), 1285s (P=O), 1050s and 820s; δ_H(200 MHz; C^2HCl_3) 0.08 (6 H, s, SiMe), 0.90 (9 H, s, Bu^t), 2.85 (4 H, t, *J* 6.2, CH_2CN), 3.82 (2 H, t, *J* 4.7, 1-H_2), 4.20 (2 H, m, 2-H_2) and 4.28 (4 H, m, cyanoethyl $POCH_2$); δ_C(50.5 MHz; C^2HCl_3) −4.9 (SiMe), 10.6 (*C*Me$_3$), 20.1 (*C*H_2CN), 26.3 (C*Me*$_3$), 62.4 (C-1), 62.6 (*J* 5.5, cyanoethyl $POCH_2$), 70.2 (*J* 6.2, C-2) and 116.8 (CN); δ_P(121.5 MHz; C^2HCl_3) −1.95; *m/z* (CI) 380 (45%, $[M + NH_4]^+$), 363 (100, $[M + H]^+$), 310 (10, $[M + NH_4 - \text{cyanoethyl}]^+$), 266 (10, $[M + NH_4 - \text{TBDMS}]^+$) and 249 (15, $[M + H - \text{TBDMS}]^+$).

Ethane-1,2-diol 1-phosphate bis(cyclohexylammonium) salt 3

The TBDMS phosphate **4** (0.5 g, 1.38 mmol) was dissolved in dry methanol (5 cm^3), and a solution of sodium methoxide in methanol (5.0 cm^3; 1.0 mol dm^{-3}) added. The reaction mixture was stirred at room temperature for 4 h and the solvent was removed under reduced pressure. The residue was dissolved in dry THF (1 cm^3) and a solution of tetrabutylammonium fluoride (TBAF) in THF (1.4 cm^3; 1.0 mol dm^{-3}) was added. The solution was stirred overnight at room temperature and the solvent was removed. The residue was redissolved in water (5 cm^3) and was subjected to chromatography on Amberlite IR 118 $(H)^+$ ion-exchange resin, eluted with water. The acidic fractions were combined and concentrated to 10 cm^3, and an excess of cyclohexylamine (4 cm^3) was added. The mixture was stirred at 20 °C for 4 h, and was then extracted with diethyl ether (3 × 30 cm^3) to remove the excess of cyclohexylamine. The aqueous phase was lyophilised and the residue was recrystallised from aq. acetone to yield the *phosphate salt* **3** as needles (0.31 g, 60%) (Found: C, 44.95; H, 9.95; N, 7.55. Calc. for $C_{14}H_{33}N_2O_5P{\cdot}2H_2O$: C, 44.7; H, 9.9; N, 7.45%); mp 134–136 °C; ν_{max}(Nujol)/cm^{-1} 3400–2400br s (O–H and N–H), 1270 (P=O), 1100s, 970s and 750s; δ_H(200 MHz; ^2H_2O) 1.00–2.00 (20 H, m, cyclohexyl CH_2), 3.05 (2 H, m, cyclohexyl CH), 3.65 (2 H, t, *J* 4.5, 2-H_2), 3.80 (2 H, m, 1-H_2); δ_C(50.5 MHz; ^2H_2O) 26.6, 27.1 and 33.1 (cyclohexyl CH_2), 53.0 (cyclohexyl CH), 64.7 (C-2) and 67.8 (*J* 5.8, C-1); δ_P(121.5 MHz; ^2H_2O) 3.98; *m/z* (FAB) 341 (3%, $[M + H]^+$), 242 (100, $[M - C_6H_{14}N + H]^+$ 165 (45, $[M - 2(C_6H_{14}N) + Na]^+$), 143 (7, $[M - 2(C_6H_{14}N) + H]^+$) and 101 (20, [cyclohexylamine + $H]^+$).

Diphenyl propyl phosphate

Using a modification of the method of Billington *et al.*)[28]

Propan-1-ol (0.5 g, 8.33 mmol), 4-(dimethylamino)pyridine (DMAP) (50 mg) and triethylamine (2.1 cm^3, 1.55 g, 15.4 mmol) were dissolved in dry THF under argon and the reaction mixture was cooled to −10 °C. Diphenyl phosphorochloridate (1.88 g, 145 cm^3, 7.0 mmol) was added dropwise and the mixture was stirred at room temperature for 2 h. The precipitated salts were removed by filtration and the solvent was removed under reduced pressure. The residue was purified by silica column chromatography (elution with 30% ethyl acetate in light petroleum) to yield the *diphenyl ester* as a liquid (1.58 g, 84%) (Found: M^+, 292.0864. $C_{15}H_{17}O_4P$ requires *m/z*, 292.0864); ν_{max}(Nujol)/cm^{-1} 2900s (C–H), 1600s (Ar), 1490s, 1300s, 1200s (P=O), 1100s, 1050s, 790s and 595s; δ_H(200 MHz; C^2HCl_3) 0.95 (3 H, t, $J_{2,3}$ 7.4, Me), 3.55 (2 H, m, CH_2Me), 4.25 (2 H, m, CH_2OP) and 7.20 (10 H, m, ArH); δ_C(50.5 MHz; C^2HCl_3) 9.9 (Me), 23.5 (*C*H_2Me), 70.8 ($J_{P,C}$ 6.5, CH_2OP), 120.0, 125.3 and 129.8 (Ar-C) and 136.4 ($J_{P,C}$ 5.9, Ar-C quaternary); δ_P(121.5 MHz; C^2HCl_3) −11.4; *m/z* (CI) 310 (75%, $[M + NH_4]^+$), 293 (100, $[M + H]^+$) and 93 (20, PhO^+).

Propyl phosphate bis(cyclohexylammonium) salt 7

This was prepared from diphenyl propyl phosphate by using similar protocols to those described by Billington *et al.*[28] in which the phenyl esters are transesterified to give benzyl esters which are then subsequently removed by catalytic hydrogenolysis. Recrystallisation from aqueous acetone afforded the required *bis(cyclohexylammonium) salt* **7** (0.42 g, 59%), mp 96–99 °C (Found: [M (derivatised as the dimethyl ester) + $H]^+$, 169.0630. $C_5H_{14}O_4P$ requires *m/z* 169.0630); ν_{max}(Nujol)/cm^{-1} 2850s (N–H and C–H), 1210m (P=O), 1050s (C–O), 790w and 700w; δ_H(200 MHz; ^2H_2O) 0.95 (2 H, t, $J_{2,3}$ 7.5, Me), 1.10–2.00 (22 H, m, cyclohexyl CH_2 and CH_2Me), 3.10 (2 H, m, cyclohexyl CH) and 3.70 (2 H, m, CH_2OP); δ_C(50.5 MHz; ^2H_2O) 12.5 (Me) 26.6, 27.1, 33.1 (cyclohexyl CH_2 and CH_2 Me), 53.1 (cyclohexyl CH), 68.9 ($J_{P,C}$ 4.4, CH_2OP); δ_P(121.5 MHz; ^2H_2O) 4.10; *m/z* (derivatised as the dimethyl ester) (CI) 186 (40%, $[M + NH_4]^+$) and 169 (100, $[M + H]^+$).

(2*S*)-5-Oxotetrahydrofuran-2-carboxylic acid 10

By use of the method of Ravid *et al.*,[32] (*S*)-glutamic acid (10.0 g, 70.0 mmol) was dissolved in hydrochloric acid (100 cm^3; 1 mol dm^{-3}) and the solution was cooled to −10 °C. Sodium nitrite (6.21 g, 90 mmol) was added portionwise over a period of 1 h, and the reaction mixture was stirred at 20 °C for 16 h. The solvents were removed under reduced pressure and the residue was stirred with hot acetone (200 cm^3). The inorganic salts were removed by filtration, the solvent was removed, and the residue was stirred with hot ethyl acetate (200 cm^3) for 1 h. The solution was decanted and the solvent was removed under reduced pressure to give a residue, which was recrystallised from hot ethyl acetate to afford white crystals (7.78 g, 76%), mp 70–71 °C (lit.,[32] 71–73 °C) (Found: $[M + NH_4]^+$, 148.0610. Calc. for $C_5H_{10}NO_4$: *m/z*, 148.0610); ν_{max}(Nujol)/cm^{-1} 3000br s (O–H), 1725s, 1750s (C=O lactone and acid), 1470s and 1490s; $[\alpha]_D$ +16.0 (*c* 6, MeOH) {lit.,[32] $[\alpha]_D$ +15.6 (*c* 2, EtOH)}; δ_H(200 MHz; ^2H_2O) 2.05–2.35 (1 H, m, 4-H), 2.40–2.65 (3 H, m, 4-H and 3-H_2) and 4.90–5.05 (1 H, q, $J_{A,X}$ 1.6, $J_{B,X}$ 7.0, 5-H); δ_C(50 MHz; ^2H_2O) 26.5 and 28.4 (C-3 & -4), 78.0 (C-2), 175.1 (C-5) and 181.8 (CO_2H); *m/z* (CI) 148 (100%, $M + NH_4]^+$), 102 (6, $[M + H - CO_2H_2]^+$) and 85 (4, $[M - CO_2H]^+$).

(2*R*)-5-Oxotetrahydrofuran-2-carboxylic acid 10A

This was prepared in an identical manner to compound **10** starting from (*R*)-glutamic acid; $[\alpha]_D$ −14.0 (*c* 6, MeOH) {lit.,[32] $[\alpha]_D$ −15.7 (*c* 2.4, EtOH)}; all spectral data were identical with those obtained for the (*S*)-antipode **10** described above.

(2*S*)-Pentane-1,2,5-triol 11

Following the general method of Brunner and Lautenschlager,[33] (2*S*)-5-oxotetrahydrofuran-2-carboxylic acid **10** (2.0 g, 15.4 mmol) in dry THF (30 cm^3) was added dropwise under dinitrogen to a stirred suspension of lithium aluminium hydride (1.17 g, 30.8 mmol) in THF (50 cm^3) over a period of 30 min. The reaction mixture was stirred for 1 h, and then a solution of water (3 cm^3) in THF (15 cm^3) was added with extreme caution. The resulting suspension was filtered and the solvent was removed under reduced pressure. The residue was redissolved in acetone (50 cm^3) and the solution was dried (Na_2SO_4). The solvent was removed under reduced pressure to give the *triol* **11** as a liquid (0.84 g, 45%), $[\alpha]_D$ −8. °C (*c* 1.7, MeOH), −14.7 (*c* 2.1, EtOH) {lit.,[33] $[\alpha]_D$ −15.1 (*c* 4.8, EtOH); lit.,[34] $[\alpha]_D$ −19.6 (*c* 5.0 EtOH)}. All other spectral data were identical with those obtained for the racemic compound.[7]

(2*R*)-Pentane-1,2,5-triol 11A

This was prepared in an identical manner to compound **9** starting from (2*R*)-5-oxotetrahydrofuran-2-carboxylic acid **10A**; $[\alpha]_D$ +7.5 (*c* 2.05, MeOH). All spectral data were identical with those obtained for the (2*S*)-antipode **11**.

(2*S*)-1,5-Bis(*tert*-butyldimethylsiloxy)pentan-2-ol 12
This compound was prepared in a manner identical with that described in the previous article for the racemic material[7] starting from (2*S*)-pentane-1,2,5-triol **11** compound **12**. $[\alpha]_D$ −7.9 (*c* 5, Et_2O). All other spectral data were identical with those obtained for the racemic compound.[7]

(2*R*)-1,5-Bis(*tert*-butyldimethylsiloxy)pentan-2-ol 12A
This compound was prepared in a manner identical with that described in the previous article for the racemic material[7] starting from (2*R*)-pentane-1,2,5-triol **11A**; $[\alpha]_D$ +8.5 (*c* 4.2, Et_2O). All other spectral data were identical with those obtained for the racemic compound.[7]

Dibenzyl (2*S*)-1,5-bis(*tert*-butyldimethylsiloxy)pentan-2-yl phosphate
This compound was prepared in a manner identical with that described in the previous article for the racemic material[7] starting from (2*S*)-1,5-bis-(*tert*-butyldimethylsiloxy)pentan-2-ol **12**; $[\alpha]_D$ −10.2 (*c* 2.5, Et_2O). All spectral data were identical with those obtained for the racemic compound.[7]

Dibenzyl (2*R*)-1,5-bis(*tert*-butyldimethylsiloxy)pentan-2-yl phosphate
This compound was prepared in a manner identical with that described in the previous article for the racemic material[7] starting from (2*R*)-1,5-bis-(*tert*-butyldimethylsiloxy)pentan-2-ol **12A**; $[\alpha]_D$ +10.3 (*c* 2.5, Et_2O). All spectral data were identical with those obtained for the racemic compound.[7]

(2*S*)-Pentane-1,2,5-triol 2-phosphate bis(cyclohexylammonium) salt 8
This compound was prepared in a manner identical with that described in the previous article for the racemic material[7] starting from dibenzyl (2*S*)-1,5-bis(*tert*-butyldimethylsilyloxy)pentan-2-yl phosphate; $[\alpha]_D$ −2.1 (*c* 1.4, water). All spectral data were identical with those obtained for the racemic compound.[7]

(2*R*)-Pentane-1,2,5-triol 2-phosphate bis(cyclohexylammonium) salt 9
This compound was prepared in a manner identical with that described in the previous article for the racemic material[7] starting from dibenzyl (2*R*)-1,5-bis-(*tert*-butyldimethylsilyloxy)pentan-2-yl phosphate; $[\alpha]_D$ +1.7 (*c* 1.4, water). All other spectral data were identical with those obtained for the racemic compound.[7]

Dibenzyl 2-methoxyethyl phosphate
2-Methoxyethanol (0.38 g, 0.5 mmol) and 1*H*-tetrazole (0.08 g, 1.15 mmol) were dissolved in dry acetonitrile (5 cm^3) under argon and a solution of dibenzyl *N*,*N*-diisopropylphosphoramidite[35] (0.21 g, 0.6 mmol) in dry acetonitrile (5 cm^3) was added. The reaction mixture was stirred at room temperature for 2 h and was then cooled to −10 °C. A solution of MCPBA (55–60% purity; 0.22 g, 0.7 mmol) in dichloromethane (10 cm^3) was added dropwise, and the resulting solution was stirred at 0 °C for 1 h. The reaction mixture was diluted with dichloromethane (15 cm^3), washed successively with aq. sodium sulfite (3 × 10 cm^3; 10%), aq. sodium hydrogen carbonate (2 × 5 cm^3; 5%) and saturated brine (10 cm^3), and then dried (Na_2SO_4). The solvent was removed under reduced pressure to give a pale yellow oil, which was purified by silica column chromatography (elution with 50% ethyl acetate in light petroleum) to give the desired *triester* as an oil (1.31 g, 78%) (Found: $[M + H]^+$, 337.1205. $C_{17}H_{22}O_5P$ requires *m*/*z*, 337.1205); ν_{max}(neat)/cm^{-1} 2900s (C–H), 1485s, 1260s (P=O), 1050s (C–O), 760s and 700s; δ_H(200 MHz; C^2HCl_3) 3.38 (3 H, s, OMe), 3.55 (2 H, m, CH_2OMe), 4.15 (2 H, m, CH_2OP), 5.05 (4 H, d, *J* 7.9, $PhCH_2$) and 7.35 (10 H, m, ArH); δ_C(50.5 MHz; C^2HCl_3) 59.5 (OMe), 67.1 (CH_2OMe), 69.8 ($J_{P,C}$ 5.7, Ph_2-CH_2), 71.7 ($J_{P,C}$ 7.2, CH_2OP), 128.3, 128.5, 129.0 and 129.1 (Ar-C) and 136.4 (Ar-C quaternary); δ_P(121.5 MHz; C^2HCl_3) −0.50; *m*/*z* (CI) 354 (3%, $[M + NH_4]^+$) and 337 (100, $[M + H]^+$).

2-Methoxyethyl phosphate bis(cyclohexylammonium) salt 17
Dibenzyl 2-methoxyethyl phosphate (1.00 g, 2.99 mmol) was dissolved in absolute methanol (50 cm^3) and palladium on activated charcoal (400 mg, 10%) was added with stirring of the solution. The reaction vessel was flushed with hydrogen gas (~500 cm^3) and the reaction mixture was stirred under hydrogen at 20 °C for 16 h. The mixture was filtered through Celite and the solvent was removed under reduced pressure. The residue was dissolved in water (10 cm^3) and was subjected to chromatography on Amberlite 118(H^+) ion-exchange resin eluted with water. The acidic fractions were combined, treated with freshly distilled cyclohexylamine (6 cm^3, 53 mmol) and the reaction mixture was stirred at room temperature for 4 h. The aqueous solution was extracted with diethyl ether (3 × 50 cm^3) to remove the excess of cyclohexylamine, and the sample was lyophilised. The residue was recrystallised from aq. acetone to give the *bis(cyclohexylammonium) salt* as crystals (0.72 g, 68%), mp 191–195 °C (Found: $[M (free acid) + H]^+$, 157.0266. $C_3H_{10}O_5P$ requires *m*/*z*, 157.0266); ν_{max}(Nujol)/cm^{-1} 2850s (N–H and H-bonding), 1200m (P–O), 1050s (C–O), 770w and 720w; δ_H(200 MHz; ^2H_2O) 1.10–2.00 (20 H, m, cyclohexyl CH_2), 3.0 (2 H, m, cyclohexyl CH), 3.27 (3 H, s, OMe), 3.50 (2 H, t, $J_{1H,2H}$ 4.7, CH_2OMe) and 3.50 (2 H, dt, $J_{1H,2H}$ 4.6, $J_{P,C}$ 4.9, CH_2OP); δ_C(50.5 MHz; ^2H_2O) 26.6, 27.1, 33.1 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 60.8 (OMe), 65.6 (CH_2OMe) and 75.0 ($J_{P,C}$ 8.1, $PhCH_2$); δ_P(121.5 MHz; ^2H_2O) 3.52; *m*/*z* (EI) 157 (5%, $[M + H]^+$), 99 (5, [phosphate + $H]^+$) and 45 (100, $CH_3OCH_2^+$).

Dibenzyl 5-benzyloxy-3-oxapentyl phosphate
Diethylene glycol (2.0 g, 18.9 mmol) and benzyl bromide (0.64 g, 3.78 mmol) were dissolved in dry DMF (30 cm^3) under dinitrogen and sodium hydride [0.36 g, 7.5 mmol (50% dispersion in oil)] was added portionwise over a period of 30 min. The reaction mixture was stirred at room temperature for 2 h and was then quenched by the addition of water (30 cm^3). The benzylated product was extracted into diethyl ether (3 × 50 cm^3), and the combined extracts were washed successively with aq. sodium hydrogen carbonate (3 × 30 cm^3; 5%) and saturated brine (30 cm^3), and then dried ($MgSO_4$). The solvents were removed under reduced pressure and the residue was dissolved in dry acetonitrile (20 cm^3). 1*H*-Tetrazole (0.53 g, 7.5 mmol) and a solution of dibenzyl *N*,*N*-diisopropylphosphoramidite[35] (1.68 g, 4.9 mmol) in dry acetonitrile (10 cm^3) were added and the reaction mixture was stirred at room temperature under dinitrogen for 30 min. The reaction mixture was cooled to −10 °C and a solution of MCPBA (1.72 g, 4.9 mmol; 50–60%) in dichloromethane (30 cm^3) was added dropwise over a period of 10 min. After 30 min, the reaction mixture was diluted with dichloromethane (100 cm^3), washed successively with 10% aq. sodium sulfite (3 × 30 cm^3), sodium hydrogen carbonate (30 cm^3; 5%) and saturated brine (30 cm^3) and then dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue was purified by silica column chromatography on triethylamine-basified silica (60% ethyl acetate–light petroleum) to give the *triester* as an oil (0.76 g, 44% from diethylene glycol) (Found: $[M + H]^+$, 457.1780. $C_{25}H_{30}O_6P$ requires *m*/*z*, 475.1780); ν_{max}(neat)/cm^{-1} 2900–3100s (C–H), 1280s (P=O), 1050s (C–O), 750s and 700s; δ_H(200 MHz; C^2HCl_3) 3.60 (6 H, m, diethylene glycol CH_2), 4.15 (2 H, m,

CH_2OP), 4.50 (2 H, s, benzyl ether CH_2), 5.05 (4 H, d, J 8, benzyl ester CH_2) and 7.30 (15 H, m, Ar H); δ_C(50.5 MHz; ^2H_2O) 67.2, 67.3, 69.7, 69.9, 70.3, 70.5 and 71.2 (CH_2), 128.1, 128.2, 128.5, 128.9 and 129.0 (Ar–C) and 136.3, 138.8 (Ar–C quaternary); δ_P(121.5 MHz; C^2HCl_3) −0.55; m/z (CI) 457 (100%, $[M + H]^+$), 277 (10, [M(free acid) + H]$^+$), 259 (5, $[M + H - 2\ BnO]^+$), 108 (85, BnOH) and 91 (55, Bn^+).

Diethylene glycol monophosphate bis(cyclohexylammonium) salt 15 (X = O)

This compound was prepared from dibenzyl 5-benzyloxy-3-oxapentyl phosphate through catalytic hydrogenolytic cleavage of the benzyl groups followed by treatment of the deprotected material with cyclohexylamine essentially as described for compound **17** above. The required product was obtained as crystals from aq. acetone (0.28 g, 78%); mp 135–145 °C (phase transition); m/z (of the dimethyl ester derivative) (Found: $[M + H]^+$, 215.0685. $C_6H_{16}O_6P$ requires m/z, 215.0685); ν_{max}(Nujol)/cm^{-1} 3300br s (O–H), 2850br s (N–H and C–H), 1210s (P=O), 1070s (C–O), 770w and 700w; δ_H(200 MHz; ^2H_2O) 1.00–2.00 (20 H, m, cyclohexyl CH_2), 3.00 (2 H, m, cyclohexyl CH_2), 3.65 (6 H, m, diethylene glycol CH_2) and (4.15 (2 H, m, CH_2OP); δ_C(50.5 MHz; ^2H_2O) 26.6, 27.1 and 33.2 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 63.1 (C-3, 65.9 (C-2), 73.4 ($J_{P,C}$ 7.7, C-1) and 74.5 (C-4); δ_P(121.5 MHz; ^2H_2O) 2.96; m/z (CI) (of the dimethyl ester derivative) 232 (65%, $[M + NH_4]^+$), 215 (100, $[M + H]^+$), 183 (5, $[M - OCH_3]^+$) and 153 (7, $[M - 2\ OCH_3]^+$).

Dibenzyl 5-benzyloxypentyl phosphate

This compound was prepared in an identical manner to dibenzyl 5-benzyloxy-3-oxapentyl phosphate starting from pentane-1,5-diol (0.62 g, 28%) (Found: $[M + H]^+$, 455.1990. $C_{26}H_{32}O_5P$ requires m/z, 455.1987); ν_{max}(neat)/cm^{-1} 2900s (C–H), 1270s (P=O), 1050s (C–O), 750s and 700s; δ_H(200 MHz; C^2HCl_3) 1.30–1.65 (6 H, m, 2-, 3- and 4-H_2), 3.45 (2 H, t, $J_{4,5}$ 6.2, 5-H_2), 4.00 (2 H, dt, $J_{1,2}$ 6.3, $J_{1,P}$ 6.6, 5-H_2), 4.50 (2 H, s, benzyl ether CH_2), 5.05 [4 H, d, J(benzyl H_2P) 8.2, benzyl ester CH_2] and 7.30 (15 H, m, ArH); δ_C(50.5 MHz; C^2HCl_3) 22.7 (C-3), 29.7 (C-4), 30.5 (C-2), 68.3, 69.6, 70.5 and 73.4 (C-1, -5 and PhCH_2), 128.0, 128.1, 128.4, 128.9 and 129.1 (Ar-C) and 136.4 and 138.5 (Ar-C quaternary); δ_P(121.5 MHz; C^2HCl_3) −1.90; m/z (CI) 472 (5%, $[M + NH_4]^+$), 455 (100, $[M + H]^+$), 275 {5, [M (free acid) + H]$^+$}, 257 (20, $[M + H - 2\ BnO]^+$), 195 {5, $[M + H - PO(OBn)_2)]^+$}, 108 (85, BnOH) and 91 (55, Bn^+).

Pentane-1,5-diol monophosphate bis(cyclohexylammonium) salt 15 (X = CH_2)

This compound was prepared in an identical manner to diethylene glycol monophosphate bis(cyclohexylammonium) salt **15** (X = O) starting from dibenzyl 5-benzyloxypentyl phosphate. The title compound was obtained as crystals from aq. acetone (0.31 g, 83%), mp 149–151 °C; m/z (of the dimethyl ester derivative) (Found: $[M + H]^+$, 213.0890. $C_7H_{18}O_5P$ requires m/z, 213.0892); ν_{max}(Nujol)/cm^{-1} 3300br s (O–H), 2850 br s (N–H), 1200m (P=O), 1050s (C–O), 770m and 700m; δ_H(200 MHz; ^2H_2O) 1.00–2.00 (26 H, m, cyclohexyl CH_2, 2-, 3- and 4-H_2), 3.00 (2 H, m, cyclohexyl CH_2), 3.45 (2 H, m, 5-H_2) and 3.65 (2 H, m, 1-H_2); δ_C(50.5 MHz; ^2H_2O) 24.3 (C-5), 26.6 and 33.1 (cyclohexyl CH_2), 27.0, 32.6 (C-2 and -4), 27.1 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 64.5 (C-5) and 67.3 ($J_{P,C}$ 5.0, C-1); δ_P(121.5 MHz; ^2H_2O) 3.57; m/z (CI) (of the dimethyl ester derivative) 230 (35% $[M + NH_4]$), 213 (100, $[M + H]^+$) and 181 (3, $[M - OCH_3]^+$).

Diethylene glycol cyclic phosphate benzyl ester

To *N,N*-diisopropyldichlorophosphoramidite (1.27 g, 6.3 mmol) in dry dichloromethane (10 cm^3) at −10 °C under dinitrogen was added a solution of diethylene glycol (0.85 g, 9 mmol) and anhydrous triethylamine (1.32 g, 13 mmol) in dry dichloromethane (10 cm^3). The reaction mixture was stirred for 30 min at −10 °C and then at 20 °C for a further 5 h. The reaction mixture was diluted with dichloromethane (10 cm^3), washed successively with aq. sodium hydrogen carbonate (10 cm^3; 5%) and saturated brine (10 cm^3), and dried ($MgSO_4$), and the solvent was removed under reduced pressure to give diethylene glycol 1,5-cyclic *N,N*-diisopropyl phosphoramidite as an oil.

Without further purification the compound was dissolved in dry acetonitrile (20 cm^3), benzyl alcohol (1 g, 9.3 mmol) and 1-*H*-tetrazole (0.53 g, 7.5 mmol) in dry acetonitrile (10 cm^3) were added, and the reaction mixture was stirred at room temperature under dinitrogen for 30 min. The reaction mixture was cooled to −10 °C and a solution of MCPBA (1.72 g, 4.9 mmol; 50–60%) in dichloromethane (30 cm^3) was added dropwise over a period of 10 min. After 30 min, the reaction mixture was diluted with dichloromethane (100 cm^3) and was washed successively with 10% aq. sodium sulfite (3 × 30 cm^3), aq. sodium hydrogen carbonate (30 cm^3; 5%) and saturated brine (30 cm^3), and dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue was purified by silica column chromatography on triethylamine-basified silica (60% ethyl acetate – light petroleum) to give the *cyclic triester* as an oil (0.75 g, 46%) (Found: $[M + H]^+$, 259.0735. $C_{11}H_{16}O_5P$ requires m/z, 259.0735); ν_{max}(Nujol)/cm^{-1} 2900br s (C–H) 1485s and 1250s (P=O), 1100s, 1050s, 900s and 720s; δ_H(200 MHz; C^2HCl_3) 3.60 (2 H, m, 2- and 4-H), 4.00 (4 H, m, 1-, 2-, 3- and 4-H), 4.40 (2 H, m, 1- and 4-H), 5.10 (2 H, d, $J_{P,H}$ 7.2, PhCH_2) and 7.35 (5 H, m, ArH); δ_C(50.5 MHz; C^2HCl_3) 69.5 and 69.7 (C-1, -5 and PhCH_2), 73.1 (C-2 and -4), 128.2 and 128.5 (Ar-C) and 139.5 (Ar-C quaternary); δ_P(121.5 MHz; C^2HCl_3) −0.79; m/z (CI) 517 (5%, $[2 M + H]^+$), 276 (3, $[M + NH_4]^+$), 259 (100, $[M + H]^+$), 108 (4, BnOH) and 91 (5, $PhCH_2^+$).

Diethylene glycol cyclic phosphate diester cyclohexylammonium salt 16 (X = O)

This compound was prepared in an identical manner to diethylene glycol monophosphate bis(cyclohexylammonium) salt **15** (X = O) starting from diethylene glycol cyclic phosphate benzyl ester to give the required *salt* as a solid (0.52 g, 86%), mp 190–193 °C {Found: [M (free acid) + H]$^+$, 169.0266. $C_4H_{10}O_5P$ requires m/z, 169.0266}; ν_{max}(Nujol)/cm^{-1} 2900s (C–H), 1270s (P=O), 1100s, 1050s and 750s; δ_H(200 MHz; ^2H_2O) 1.00–2.00 (10 H, m, cyclohexyl CH_2), 3.00 (1 H, m, cyclohexyl CH_2), 3.75 (4 H, m, 2- and 4-H_2) and 3.95 (4 H, m, 1- and 5-H_2); δ_C(50.5 MHz; ^2H_2O) 26.6, 27.1 and 33.1 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 69.5 ($J_{P,C}$ 6.1, CH_2OP) and 73.7 (CH_2OCH_2); δ_P(121.5 MHz; ^2H_2O) −0.25; m/z (CI) 337 (5%, $[2 M + H]^+$), 186 (100, $[M + NH_4]^+$) and 169 (100, $[M + H]^+$).

Pentane-1,5-diol cyclic phosphate benzyl ester

This compound was prepared in an identical manner to diethylene glycol cyclic phosphate benzyl ester from pentane-1,5-diol to afford the required *triester* as an oil (0.35 g, 34%) (Found: M^+, 256.0864. $C_{12}H_{17}O_4P$ requires m/z, 256.0864); ν_{max}(neat)/cm^{-1} 2860br s (N–H and H-bonding), 1220s (P=O), 1050s, 900s, 760m and 750m; δ_H(200 MHz; C^2HCl_3) 1.60–2.10 (6 H, m, 2-, 3- and 4-H_2), 4.20 (4 H, m, CH_2OP) and 5.15 (2 H, d, $J_{P,C}$ 8.5, PhCH_2); δ_C(50.5 MHz; C^2HCl_3) 24.2 and 29.6 (C-2, -3 and -4), 69.4 and 69.6 (C-1, -5 and PhCH_2), 128.4 and 129.0 (C-Ar) and 137.5 (Ar-C quaternary); δ_P(121.5 MHz; C^2HCl_3) −0.80; m/z (CI) 274 (15%, $[M + NH_4]^+$), 257 (100, $[M + H]^+$), 184 (5, $[M - PhCH_2 + H]^+$), 167 (5, $[M - PhCH_2 + NH_4]^+$), 108 (10, BnOH) and 91 (5, $PhCH_2^+$).

Pentane-1,5-diol cyclic phosphate cyclohexylammonium salt 16 (X = CH_2)

This compound was prepared in an identical manner to diethylene glycol cyclic phosphate diester cyclohexylammonium salt **16** (X = O) starting from pentane-1,5-diol cyclic phosphate benzyl ester to afford the required salt as a solid (0.41 g, 85%), mp 160–162 °C (Found: [M (free acid) + NH_4]$^+$, 184.0739. $C_{15}H_{15}NO_4P$ requires *m/z*, 184.0738); ν_{max}(Nujol)/cm^{-1} 2850br s (N–H and H-bonding), 1210s (P=O), 1050s, 950s and 790s; δ_H(200 MHz; ^2H_2O) 1.0–2.0 (16 H, m, cyclohexyl CH_2, 2-, 3- and 4-H_2), 3.00 (1 H, m, cyclohexyl CH) and 3.85 (4 H, m, CH_2OP); δ_C(50.5 MHz; ^2H_2O) 26.1, 26.6, 27.0, 31.4 and 33.1 (C-2, -3, -4 and cyclohexyl CH_2), 53.1 (cyclohexyl CH_2) and 70.3 ($J_{P,C}$ 6.1, $POCH_2$); δ_P(121.5 MHz; ^2H_2O) 0.70; *m/z*(CI) 333 (15%, [2 M + H]$^+$), (100, [M + NH_4]$^+$) and 167 (30, [M + H]$^+$).

Acknowledgements

We thank the SERC for grant GR/J29893 and for a studentship to A. G. C., and Drs M. Akhtar and J. Schulz for useful comments.

Abbreviations

2′-AMP, Adenosine 2′-phosphate; Ins 1-*P*, *myo*-inositol 1-phosphate; Ins 3-*P*, *myo*-inositol 3-phosphate; Ins 4-*P*, *myo*-inositol 4-phosphate; P_i, inorganic phosphate; MCPA, *m*-chloroperbenzoic acid; $Mg^{2+}1$, buried Mg^{2+} ion (first Mg^{2+} ion to bind to the enzyme); $Mg^{2+}2$, catalytic Mg^{2+} ion (second Mg^{2+} ion to bind to the enzyme); TBDMS, *tert*-butyldimethylsilyl; THF, tetrahydrofuran; TBAF, tetrabutylammonium fluoride.

References

1 A. G. Cole and D. Gani, *J. Chem. Soc., Chem. Commun.*, 1994, 1139.
2 D. Gani, C. P. Downes, I. Batty and J. Bramham, *Biochim. Biophys. Acta*, 1993, **1177**, 253.
3 R. Baker, J. J. Kulagowski, D. C. Billington, P. D. Leeson, I. C. Lennon and N. Liverton, *J. Chem. Soc., Chem. Commun.*, 1989, 1383; J. J. Kulagowski, R. Baker and S. R. Fletcher, *J. Chem. Soc., Chem. Commun.*, 1991, 1649.
4 R. Bone, J. P. Springer and J. R. Atack, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 10031.
5 G. R. Baker and D. Gani, *BioMed. Chem. Lett.*, 1991, **1**, 193.
6 A. P. Leech, G. R. Baker, J. K. Shute, M. A. Cohen and D. Gani, *Eur. J. Biochem.*, 1993, **212**, 693.
7 A. G. Cole and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 1995, preceding article.
8 S. J. Pollack, J. R. Atack, M. R. Knowles, G. McAllister, C. I. Ragan, R. Baker, S. R. Fletcher, L. L. Iversen and H. B. Broughton, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 5766.
9 R. Bone, L. Frank, J. P. Springer, S. J. Pollack, S. Osborne, J. R. Atack, M. R. Knowles, G. McAllister, C. I. Ragan, H. B. Broughton, R. Baker and S. J. Fletcher, *Biochemistry*, 1994, **33**, 9460.
10 R. Bone, L. Frank, J. P. Springer and J. R. Atack, *Biochemistry*, 1994, **33**, 9468.
11 S. J. Pollock, M. R. Knowles, J. R. Atack, H. B. Broughton, C. I. Ragan, S.-A. Osborne and G. McAllister, *Eur. J. Biochem.*, 1993, **217**, 281.
12 D. Herschlag and W. P. Jencks, *Biochemistry*, 1990, **29**, 5172.
13 J. Wilkie, A. Cole and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 1995, following article.
14 A. J. Ganzhorn and M.-C. Chanal, *Biochemistry*, 1990, **29**, 6065.
15 P. D. Leeson, K. James, I. C. Lennon, N. J. Liverton, S. Aspley and R. G. Jackson, *Biomed. Chem. Lett.*, 1993, **3**, 1925.
16 R. Baker, P. D. Leeson, N. J. Liverton and J. J. Kulagowski, *J. Chem. Soc., Chem. Commun.*, 1990, 462.
17 L. M. Hallcher and W. R. Sherman, *J. Biol. Chem.*, 1980, **255**, 10896.
18 P. A. Frey, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1989, **62**, 119.
19 D. Gani and J. Wilkie, *Chem. Soc. Rev.*, 1995, **24**, 55.
20 (a) S. L. Buchwald, D. H. Pliura and J. R. Knowles, *J. Am. Chem. Soc.*, 1984, **106**, 4916; (b) P. M. Cullis and R. Misra, *J. Am. Chem. Soc.*, 1991, **113**, 9679.
21 C. M. Frey and J. Stuehr in *Metal Ions in Biological Systems*, ed. H. Sigel, Marcel Dekker, New York, 1974, vol. 1, p. 51.
22 G. R. Baker, D. C. Billington and D. Gani, *BioMed. Chem. Lett.*, 1991, **1**, 17.
23 T. R. Sharp and S. J. Benkovic, *Biochemistry*, 1979, **18**, 2910.
24 P. L. Domanico, J. F. Rahil and S. J. Benkovic, *Biochemistry*, 1985, **24**, 1623.
25 P. J. Greasley and M. G. Gore, *FEBS Lett.*, 1993, **331**, 114; P. J. Greasley, L. G. Hunt and M. G. Gore, *Eur. J. Biochem.*, 1994, **222**, 453.
26 D. D. Perrin and W. F. L. Armarego, *Purification of Laboratory Reagents*, Pergamon Press, Oxford, 3rd edn., 1988.
27 W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.
28 D. C. Billington, R. Baker, J. J. Kulagowski and I. M. Mawer, *J. Chem. Soc., Chem. Commun.*, 1987, 314.
29 G. R. Baker, D. C. Billington and D. Gani, *Tetrahedron*, 1991, **47**, 3895.
30 K. Itaya and M. Ui, *Clin. Chim. Acta*, 1966, **14**, 361.
31 N. D. Sinha, J. Biernat, J. McManus and H. Koester, *Nucleic Acids Res.*, 1984, **12**, 4539.
32 U. Ravid, R. M. Silverstein and L. R. Smith, *Tetrahedron*, 1978, **34**, 1449.
33 H. Brunner and H.-J. Lautenschlager, *Synthesis*, 1989, 706.
34 F. C. Hartman and R. Barker, *J. Org. Chem.*, 1964, **29**, 873.
35 K.-L. Yu and B. Fraser Reid, *Tetrahedron Lett.*, 1988, **29**, 979.

Paper 5/01469G
Received 9th *March* 1995
Accepted 6th *June* 1995