'Active' conformation of the inositol monophosphatase substrate, adenosine 2'-phosphate: role of the ribofuranosyl O-atoms in chelating a second Mg^{2+} ion [1]

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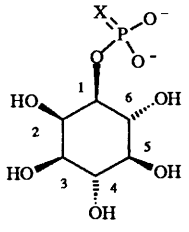
In the presence of Mg^{2+} ions, inositol monophosphatase from bovine brain catalyses the hydrolysis of the phosphate ester of a range of purine- and pyrimidine-containing nucleoside 2'-phosphates including adenosine 2'-phosphate (2'-AMP) but not adenosine 2'-phosphorothioate (2'-AMPS). 2'-AMPS also fails to serve as an inhibitor under these conditions. In contrast to the situation for the alcohol hydrolysis product, inositol, adenosine does not serve as a product inhibitor for the enzyme or mediate the enzyme-catalysed exchange of ^{18}O-label from [^{18}O]water into inorganic phosphate. However, in the presence of Mn^{2+} ions 2'-AMPS is a substrate for the enzyme. These findings indicate that the product adenosine does not bind to the enzyme in its ground-state conformations and that a strong phosphate group–holoenzyme interaction is required to stabilise a high-energy arrangement in the enzyme–substrate complexes of 2'-AMPS and, probably, 2'-AMP. On the basis of these results and those from previous kinetic and substrate modification studies it is proposed that a second Mg^{2+} ion might stabilise a conformation in which the adenine moiety of bound 2'-AMP occupies a C-1'-axial ribofuranosyl position through the direct chelation of the second Mg^{2+} ion to the bridging phosphate ester 2'-O-atom and the ribofuranose ring O-atom. An alternative high-energy arrangement in which the interaction of the second Mg^{2+} ion with the ribofuranose ring O-atom is mediated *via* water, such that the conformational strain in the furanose ring is relaxed, but where the entropy of the water is decreased, is also a possibility.

Introduction

In mammalian brain cells, inositol monophosphatase is responsible for supplying free inositol for the biosynthesis of phosphatidylinositol 1,4-bisphosphate, a precursor for the secondary messengers, inositol 1,4,5-trisphosphate and diacylglycerol.[2] The enzyme has a broad substrate specificity and is able to process both enantiomers of *myo*-inositol 1-phosphate Ins 1-*P* **1** (X = O) and *myo*-inositol 4-phosphate Ins 4-*P* **2** as well as 2'-AMP **3** (X = O). Recent work from a number of laboratories has probed the structure and mechanism of the enzyme and several key features are now documented.[2-7] For example, it is established that when Ins 1-*P* is the substrate the enzyme operates *via* a direct displacement ternary complex mechanism in which water is the attacking nucleophile,[3,4] rather than *via* the more common substituted enzyme mechanism.[8] It is also evident that the phosphate dianion moiety is the primary binding functionality for substrates and inhibitors and that the 1-O-atom and the 2- and 4-OH groups of Ins 1-*P* substrates [*e.g.*, D-Ins 1-*P* **1** (X = O)] are important in binding whilst the 6-OH group is important in catalysis, Fig. 1.[6,7] It is also known that Mg^{2+} is required for enzymic catalysis and is liganded by the phosphate group of substrates[4-9] and that Li^+ binds to enzyme–product complexes in place of Mg^{2+} in the inhibition of the enzyme.[4]



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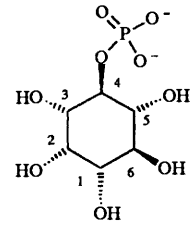

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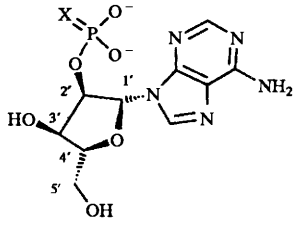


3 X = O, S

Since we communicated the preliminary results [1] of the studies described in this paper and the following paper in this series,[10] a number of detailed X-ray crystallographic studies have been reported by the Merck, Sharp and Dohme group.[11-13] These provide information on the structures for enzyme–metal ion–Ins 1-*P* complexes and for an enzyme–metal ion–phosphate product complex and are, at least qualitatively, consistent with the findings of our own studies. An important point is that the results of the two complementary approaches indicate that two Mg^{2+} ions are required for catalysis, and there is good agreement on the location of the two metal ions within the protein. A very detailed discussion of the similarity and differences in the conclusions of the Merck studies[11-13] and those of our own is provided in the third paper in this series[14] which considers the binding modes of several different substrates and inhibitors including D- and L-Ins 1-*P*.

Prior to these most recent studies, four important features of the system were difficult to rationalise and our objectives were to understand the mechanistic basis of each feature. The first was the ability of the enzyme to process ribonucleoside 2'-phosphates (*e.g.*, compound **3**) which lack the catalytically essential hydroxy group.[6,7] The second was that the position of

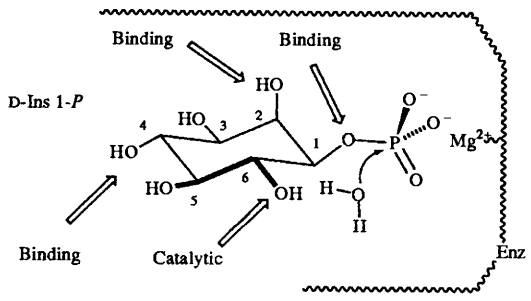


Fig. 1 Proposed roles of the flanking 2- and 6-hydroxy groups in binding to the enzyme and in facilitating catalysis. The active-site nucleophile is a water molecule.

the metal ion, Gd^{3+} (a competitive inhibitor for Mg^{2+}) in the X-ray crystal structure of a Gd^{3+} sulfate complex of the enzyme[9] was completely inconsistent with the published sequence for Mg^{2+} and substrate and product binding and debinding.[4] The Gd^{3+} ion appeared to be buried in such a way that it could not bind *after* the substrate (or debind before the product), contrary to expectations derived from kinetic studies.[4] The third was that the optimum concentration of Mg^{2+} required for catalysis varied with the structure of the substrate[4,15] and that Mg^{2+} binding was cooperative for some substrates but not for others.[4] The fourth was that the mode of inhibition of the enzyme by Li^+ changed from uncompetitive to noncompetitive with increasing concentration[4] and that K_i-values for uncompetitive inhibition by Li^+ depended acutely on the structure of the substrate.[2]

Here we provide structural and mechanistic information on the ability of the enzyme to recognise and process ribonucleoside 2'-phosphates and provide alternative evidence to show that two Mg^{2+} ions are required to support enzymic activity.

Results and discussion

2'-AMP **3** (X = O) is a good substrate for inositol monophosphatase from bovine brain (V_{max} is 90% of V_{max} for Ins 1-*P*; K_m = 0.86 mmol dm^{-3} [K_m for Ins 1-*P* = 0.1 mmol dm^{-3}, K_i for Ins 1-*P* = 0.1 mmol dm^{-3}, k_{cat} = 50 s^{-1}])[4] and is hydrolysed to give adenosine and inorganic phosphate (P_i). The value of K_i for 2'-AMP is 1.0 mmol dm^{-3} and, therefore, the binding affinity of 2'-AMP is ten-fold lower than that for Ins 1-*P*. The value of K_i for inorganic phosphate under similar conditions[4] is 0.3 mmol dm^{-3} and, therefore, interactions between the adenosine moiety and the active site do not quite compensate for the structural reorganisation required to accommodate the extra mass. 2'-AMP, like Ins 1-*P*, shows cooperativity for Mg^{2+} binding[4] and, like other non-physiological substrates for the enzyme, shows a steeper decrease in activity with decreasing Mg^{2+} concentration than Ins 1-*P* and Ins 4-*P*.[4,15]

It is generally accepted[16] that the 2'-, 3'- and 5'-oxygen atoms of the ribofuranosyl moiety in 2'-AMP **3** (X = O) serve the same functions as the 1-, 2- and 4-oxygen atoms in D-Ins 1-*P* **1** (X = O), respectively, in binding to the enzyme, Fig. 1. For Ins 1-*P*, the substrate-binding groups are now very well defined owing to elegant functional group-deletion studies by the Merck, Sharp and Dohme group.[6,7] These same studies have indicated that the 6-OH group of the substrate D-Ins 1-*P* is essential for catalysis and that its replacement by -OMe, -OR or by -H affords inhibitors. Since 2'-AMP does not possess an equivalent catalytic group but is, nevertheless, a good substrate, the ability of the enzyme to process ribonucleoside 2'-phosphates was further examined.

^{18}O-Label exchange

We had previously shown that the product alcohol, inositol, was able to mediate the incorporation of ^{18}O-label from [^{18}O]water into inorganic phosphate and that this exchange reaction displayed a similar pH-dependence and metal ion requirement to the forward inositol 1-phosphate hydrolysis reaction.[3,4] Furthermore, it was shown that inositol behaved as a noncompetitive product inhibitor (K_i = 400 mmol dm^{-3}) for the hydrolysis reaction and displayed a K_m-value of 190 mmol dm^{-3} for the exchange reaction.[3,4] It was therefore of interest to ascertain whether adenosine behaved similarly.

The rate for the enzyme-catalysed exchange of ^{18}O-label from [^{18}O]water (containing 45 atom % ^{18}O-label) into inorganic phosphate was accordingly measured in the presence of adenosine (30 mmol dm^{-3}) and Mg^{2+} ions, using a mass spectrometry-based isotope content analysis assay similar to that previously described for measuring inositol-dependent ^{18}O-label incorporation.[3,4] Control experiments in which adenosine was replaced by inositol showed the expected rates of label incorporation,[3,4] but those containing adenosine showed no incorporation whatsoever, even after prolonged periods. Furthermore, the addition of adenosine (30 mmol dm^{-3}) to standard enzyme activity assays performed using either [U-^{14}C]Ins 1-*P* or 2'-AMP (at low substrate concentration where the effects of competitive and noncompetitive inhibitors are pronounced) caused no detectable reduction in rate. Thus, adenosine does not serve as a product inhibitor. Together, these results indicate that adenosine is not recognised as a product and that the lower limit of the K_i-value for adenosine is 4 mol dm^{-3}.

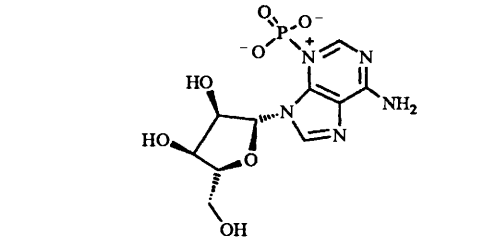
Adenosine-mediated ^{18}O-exchange into inorganic phosphate (P_i) is expected on the basis of microscopic reversibility, and since inositol-mediated exchange occurs readily, the results indicate that, for the adenosine system, the reverse reaction possesses at least one very slow step, *i.e.* a step in the hydrolysis reaction direction is essentially irreversible. This conclusion is informative as only two explanations can account for the differences in the kinetics of the two systems; either (a) 2'-AMP is hydrolysed by an alternative mechanism such that adenosine is not the true enzymic product; for example, the AMP is first transphosphorylated to a new phosphoadenosine which then hydrolyses non-enzymically; or (b) the product-binding complex for adenosine exists in a high-energy state. This high-energy state could be caused by unfavourable steric and/or electronic interactions in the nascent bound adenosine molecule (*i.e.*, a high internal energy) or by the loss of the primary (phosphate moiety) and/or secondary binding interactions with the enzyme or its hydration sphere or by any combination of these. The result in each case would be that free adenosine would be unable to bind in its product site.

The possibility of transphosphorylation could not be excluded by inspection of the structure of 2'-AMP. We had previously proposed that the catalytic 6-OH group of Ins 1-*P* **1** (X = O) might provide an anchor to orientate the nucleophilic water molecule to attack the phosphate P-atom *via* a seven-membered-ring transition state,[2] Fig. 1. Although 2'-AMP **3** (X = O) lacks an equivalent hydroxy group it could also form a seven-membered-ring transition state leading to N^3-phosphoadenosine **4** *via* intramolecular transphosphorylation, if its pyrimidine ring N^3-atom could serve as a nucleophile. Spontaneous non-enzymic rapid hydrolysis of the reactive intermediate, N^3-phosphoadenosine **4**, would give the observed products, adenosine and P_i, and would account for the inability of the enzyme to recognise adenosine.

In order to test this first possibility, the operation of an alternative mechanism involving the intermediacy of N^3-phosphoadenosine, the rate of hydrolysis of the putative N^3-phosphopurine intermediate was considered. In accord with

Table 1 Relative rates of hydrolysis of ribonucleoside 2'-phosphates by bovine inositol monophosphatase

Substrate	Relative v_{max}	Metal ion cofactor	K_m (mmol dm^{-3})
Adenosine 2'-phosphate	100	Mg^{2+}	0.5
Adenosine 2'-phosphate	100	Mn^{2+}	n.d.
8-Bromoadenosine 2'-phosphate	100	Mg^{2+}	n.d.
Uridine 2'-phosphate	230	Mg^{2+}	4.0
5,6-Dihydrouridine 2'-phosphate	70	Mg^{2+}	1.4
Adenosine 2'-phosphorothioate	< 0.2	Mg^{2+}	n.d.
Adenosine 2'-phosphorothioate	2.0	Co^{2+}	n.d.
Adenosine 2'-phosphorothioate	20	Mn^{2+}	n.d.



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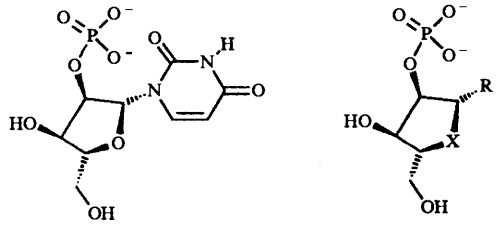

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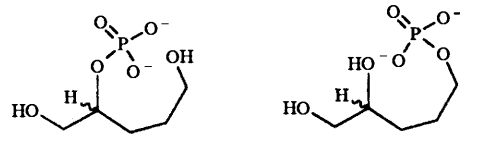

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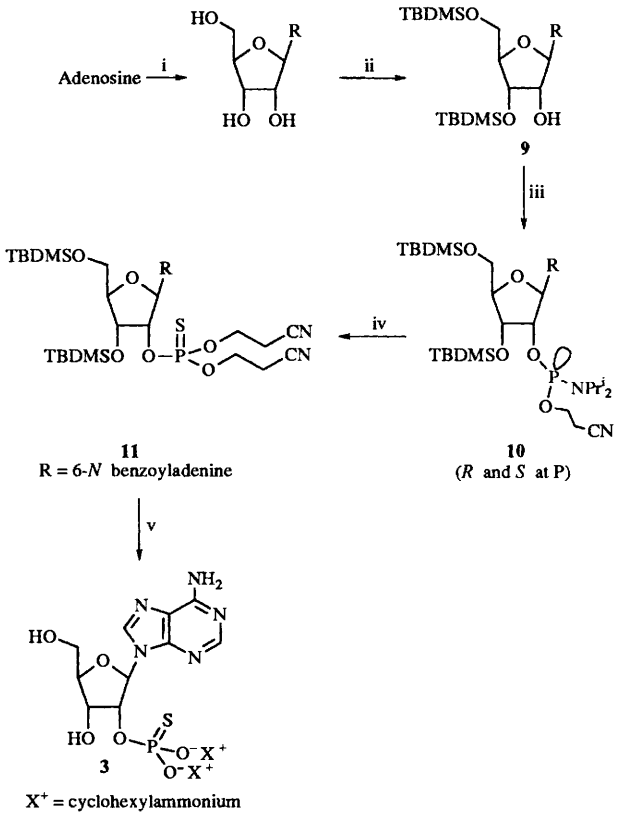
expectations, examination of the course for the complete enzymic hydrolysis of 2'-AMP over a period of several minutes by ^{1}H NMR spectroscopy did not reveal any signals due to the accumulation of N^3-phosphoadenosine. Phosphopyridinium monoanions[17] hydrolyse quite rapidly under the conditions employed for the enzyme incubations (k = 0.02–5 × 10^{-4} s^{-1}), and it seemed likely that the more electron-deficient pyrimidine ring of the putative intermediate would be a better leaving group than a pyridine. This could give rise to faster rates. Therefore, the potential substrates, compounds **5**–**7** containing nucleophilic O-atoms in place of the N^3-atom of 2'-AMP, were examined. It was reasoned that if the alternative mechanism operated, phosphoryl transfer *via* seven-membered transition states would give transphosphorylated products that would be stable to non-enzymic hydrolysis and would accumulate. In considering the hydrolytic stability of the possible imino phosphoester intermediates that might form in the reaction of uridine 2'-phosphate **5** and the 5,6-dihydrouridine analogue **6** (R = 5,6-dihydrouracil, X = O) with the enzyme, it was noted that 2,4-dinitrophenyl phosphate[18] (which contains an excellent leaving group for mechanisms involving P–O bond cleavage) hydrolyses at a low rate (k = 1.43 × 10^{-4} s^{-1} in water at pH 12, 43.2 °C) and would be stable to non-enzymic hydrolysis over the time scale and under the standard conditions employed for our NMR spectroscopic experiments, pH 8.0 and at 25–37 °C.[4]

When tested as substrates uridine 2'-phosphate **5** and the 5,6-dihydrouridine analogue **6** (R = 5,6-dihydrouracil, X = O) readily underwent enzymic hydrolyses at rates similar to 2'-AMP, see Table 1. No evidence was obtained for the intermediacy of C-2 phosphorylated uridine or dihydrouridine moieties by examination of the incubation solutions by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy during the hydrolyses.

To determine if an imino phosphoester intermediate was being formed but was being hydrolysed *via* an alternative mechanism involving non-enzymic nucleophilic attack by water at C-2 (this would result in C–O bond cleavage), the phosphatase catalysed hydrolysis of uridine 2'-phosphate **5** was repeated in [^{18}O]water. No ^{18}O-label was detected in the uridine product as determined by mass spectrometry, which together which the NMR spectroscopic data indicates that no transphosphorylated intermediates are formed. Furthermore, (2*RS*)-pentane-1,2,5-triol 2-phosphate **7** did not undergo transphosphorylation to give compound **8** and was not hydrolysed by the enzyme as judged by ^{1}H NMR spectroscopy. Compound **8** was prepared to provide a reference standard for this NMR experiment. These results are not in accord with the involvement of the N^3-atom of 2'-AMP in an alternative mechanism for 2'-AMP hydrolysis by the enzyme. Note that in agreement with this analysis, Leeson *et al.*[19] have reported that ribofurans which lack the heterocyclic base (*e.g.*, **6**; R = H, X = O) serve as substrates for the enzyme. Thus, to explain the fact that adenosine is not recognised by the enzyme as a product inhibitor or as a cofactor for ^{18}O-label exchange between the solvent and P_i, it appears that the enzyme-bound form must demand a high-energy arrangement which is not significantly populated in aqueous buffer solution. Any such high-energy structure would need to account for why the removal of the ribofuranosyl ring O-atom to give carbocyclic ribofuran analogues (*e.g.*, **6**; R = H, X = CH_2) results in compounds that are not substrates,[19] and whether the ribofuranosyl ring O-atom could serve as the surrogate catalytic site for the 6-OH group in Ins 1-*P* substrates.

Phosphothioate substrates

In order to probe further the enzyme's ability to recognise 2'-AMP but not adenosine, adenosine 2-phosphorothioate (2'-AMPS, **3**; X = S) was prepared starting from adenosine. Benzoylation at N^6 followed by protection of the 5'-hydroxy group and either of the 2'- or 3'-hydroxy groups gave a mixture of protected adenosines containing either a free 3'- or 2'-hydroxy group, Scheme 1. The isomers were separated by column chromatography and the required 3',5'-bis-*tert*-butyldimethylsilyl (TBDMS)-protected adenosine **9** was treated with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite to give the separable diastereoisomeric phosphoramidite diesters **10A** and **10B**; see Experimental section. These were treated with 2-



Scheme 1 *Reagents and conditions:* i (a) BzCl, pyridine, 0 °C; (b) NaOH, pyridine; 71%; ii (a) TBDMSCl, imidazole, DMF; (b) separate isomers; 36%; iii, 2-cyanoethyl *N,N*- diisopropylchlorophosphoramidochloridite, DMAP, Pr^i_2NEt, THF; 62%; iv, (a) 1*H*-tetrazole, 3-hydroxypropionitrile, MeCN; (b) S_8, pyridine; 48%; v (a) NH_4OH–EtOH (3:1), 60 °C; (b) TBAF, THF; (c) Amberlite 118 $(cyclohexylammonium)^+$; 53%

cyanoethanol to give the phosphite triester **11**. Sulfurisation in pyridine followed by removal of the protecting groups with ethanolic ammonia and then treatment with cyclohexylamine gave 2'-AMPS **3** (X = S) as its dicyclohexylammonium salt in 4.3% overall yield from adenosine. 2'-AMPS **3** (X = S) was tested as a substrate and as an inhibitor for inositol monophosphatase.

It was already known that, in the presence of Mg^{2+} ions, both L- and D-inositol phosphorothioate **1** (X = S) were substrates for the enzyme and possessed K_m- and K_i-values of ~ 1.0 mmol dm^{-3}. These values are of the order of 10-fold higher than the values for the corresponding inositol phosphates.[20] Conversely, the phosphorothioate, 2'-AMPS **3** (X = S) did not serve as a substrate as judged by ^1H NMR spectroscopic examination of the incubation mixture over a long period (up to 48 h) against control incubations containing 2'-AMP **3** (X = O). The maximum rate of hydrolysis was 0.2% of the rate of 2'-AMP **1** (X = O) hydrolysis in the presence of Mg^{2+} ion under otherwise identical conditions. The compound **3** (X = S) also failed to act as an inhibitor as assessed in standard enzyme assays. Together these results indicate that the enzyme cannot recognise the molecule. However, when the compound was tested as a substrate in enzyme assays which contained thiophilic Mn^{2+} ions in place of Mg^{2+} ions, the expected hydrolysis reaction occurred at ~ 20% of the rate of 2'-AMP **1** (X = O) in the presence of Mg^{2+} ions. Co^{2+} ions also supported the catalytic hydrolysis of 2'-AMP **3** (X = O) at lower levels but, of the order of 10-fold better than Mg^{2+} ions. (Note that formation of a precipitate greatly hampered attempts to obtain precise rates for the reactions containing thiophilic metal ions.)

These findings indicate that a strong binding interaction between the enzyme-bound metal ion and the phosphate or phosphorothioate group is required to confer activity as a substrate. In the light of the fact that neither adenosine nor 2'-AMPS serve as inhibitors for the Mg^{2+} form of the enzyme or show any detectable interaction with the enzyme, it is reasonable to propose that the primary binding interaction of the phosphate group with the metal ion must be strong in order to off-set the energy required to reorganise the adenosine moiety into its active conformation.

Comparison of 2'-AMP to D-Ins 1-*P* binding

Given that inositol is recognised as a product, the enzyme-bound form must exist in a low-energy conformation and can thus serve as a template for deducing the high-energy arrangement of the adenosine system.

In order to occupy the same position as the 6-OH group in D-Ins 1-*P*, relative to all other binding and catalytically important groups, the furanosyl ring O-atom in 2'-AMP must move toward the 2'-O atom, so that their respective lone-pairs point towards each other, and so bring the adenine moiety into an unfavourable axial position. This conformation also suffers from an adverse 1,3-interaction between the adenine moiety and the 4'-hydroxymethyl group and from a 1,2-interaction between the 2'-O and 3'-O atoms. Calculations predict that the structure may be as high as 100–105 kJ mol^{-1} less stable than the unconstrained form ($K \sim 10^{-17}$) which would certainly explain why adenosine is not recognised by the enzyme. [See the third paper in this series for full details of the calculated internal energies for unbound and bound 2'-AMP and adenosine. Structures are given in that paper's Fig. 7.][14]

By analogy to the D-Ins 1-*P* system (and with particular reference to the 1-O and 6-O atoms, Fig. 1) which this strained conformation now accurately mimics, the 2'-O atom and ribofuranosyl O-atom are key binding[6] and catalytic functionalities[7] respectively and must, therefore, interact with other species on the enzyme. Neither of the O-atoms possess a hydrogen atom so that H-bonding cannot stabilise the system. However, chelation to a second Mg^{2+} ion to produce a five-membered metallocycle would substantially stabilise the system and off-set some of the high-energy penalty associated with the strained conformation. The remaining binding energy would need to be derived from the interaction of the phosphate group with its binding site and from the peripheral interactions of the 3'-OH and 5'-OH groups of 2'-AMP with the binding sites for the 2-OH and 4-OH groups of D-Ins 1-*P*. The second Mg^{2+} ion could also provide a possible site for a hydroxide ion suitably disposed for attacking the P-atom: see Fig. 2(*a*).

Another possible arrangement that would fit the available data would be to relax some of the internal strain of the ribofuranosyl ring by removing the direct interaction of furanosyl O-atom with the second Mg^{2+} and replacing it with an indirect water-mediated interaction. This latter arrangement is discussed in detail in the third paper in this series within the context of the protein environment.[14]

Transposition of the interactions of the second Mg^{2+} ion with 2'-AMP [Fig. 2(*a*)] onto the structure of D-Ins 1-*P* gives a possible 3-D structure for the key binding interactions of both metal ions with the substrate [Fig. 2(*b*)].

The involvement of a second Mg^{2+} ion is completely consistent with all of the observed properties of all published substrates and inhibitors of the enzyme.[2] Moreover, since substrate O-atoms provide (at least) two ligands for the second Mg^{2+} ion, its involvement accounts for the variance of observed K_{Mg}-values for different substrates[15] as well as the observed cooperativity effects that are displayed for Mg^{2+} with some substrates (highlighted above) but not others.[4] Moreover, the new findings explain the inconsistencies in the deduced

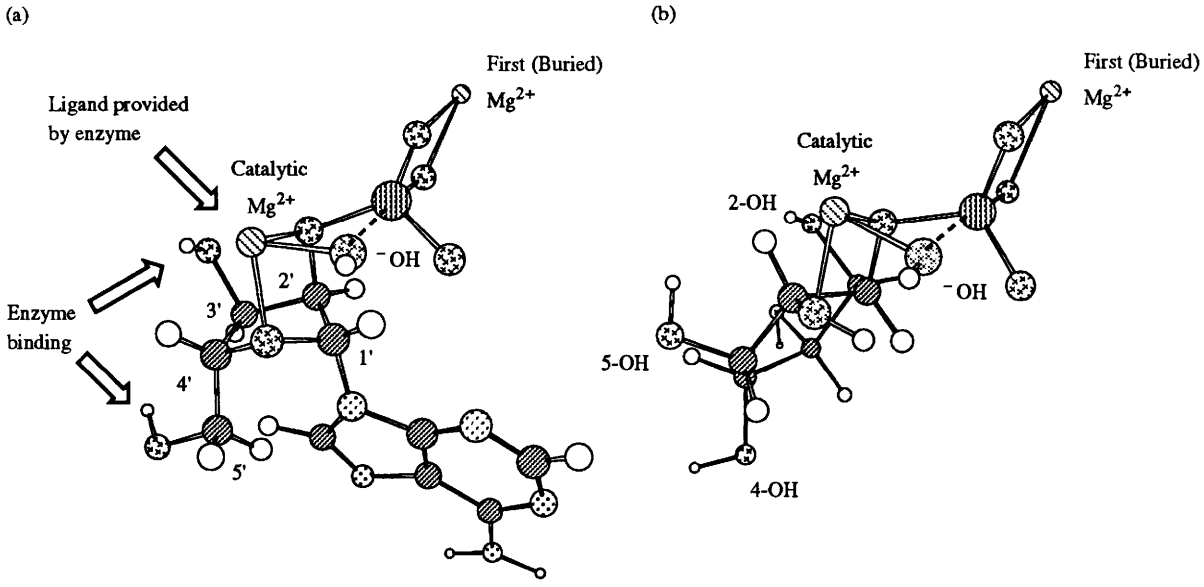


Fig. 2 (*a*) Model showing the role of the second (catalytic) Mg^{2+} ion in stabilising the conformation of the active form of 2'-AMP through chelation by the 2'- and ribofuranosyl O-atoms. Other ligands for the second Mg^{2+} ion are possibly the nucleophilic water molecule (hydroxide) and Asp-220. (*b*) Showing the conformation of D-Ins 1-*P* and the important catalytic and binding interactions with the two Mg^{2+} ions.

binding sequences for species derived from the crystal data for the Gd^{3+} sulfate–enzyme complex, which indicated that the metal was buried deeper in the active-site cleft than sulfate (as a surrogate for phosphate) and, therefore, should bind first,[9] and our own kinetic studies which indicated that Mg^{2+} binds after the substrate and debinds before the product.[4] Put simply, the two experimental approaches were each detecting one of two different metal ions. As noted above, since performing the work described here,[1] other research groups have provided data to support a catalytic mechanism involving two divalent metal cations.[11-13,21] In one of these studies two Mn^{2+} ions were observed in a Mn^{2+} phosphate complex of the enzyme.[13] The positions of the two metal ions in this structure and in modelled substrate bound active complexes[11] are extremely similar to those we report here.

The results of experiments which further substantiate and probe the role of the second Mg^{2+} ion[10] together with full details of the structural and mechanistic consequences for catalysis and for inhibition by Li^+, in the form of 3-D protein–ligand structures,[14] are described in the following two articles.

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 a 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 dimethyl sulfoxide (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 in Hz. IR spectra were recorded using a Perkin-Elmer 1420 ratio recording spectrometer and a Perkin-Elmer 1710 f.t. 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 melting point 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 obtained on a 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–VIS optical densities were measured on a Cam Spec M302 spectrophotometer. Solvents and common reagents were purified according to the method of Perrin and Armarego.[22] Flash chromatography was performed according to the procedure of Still[23] 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, deuterium oxide, 2'-AMP and uridine 2'-phosphate were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Inositol 1-phosphates were prepared as described previously,[4] 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 by 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 *E. coli* strain[24] as described previously in a routine yield of 20%.[4] Only homogeneous enzyme was used for the experiments described here. Purity was assessed using polyacrylamide gel electrophoresis (PAGE) as described previously.[4] Enzyme-activity assays were performed using a colorimetric assay developed by Itaya and Ui[25] (employing molybdic acid and Malachite Green) or using a radiochemical

assay.[4] Rate determinations were performed at 37 °C in triplicate in assay buffer A containing KCl (300 mmol dm^{-3}), $MgCl_2$ (2 mmol dm^{-3}) and Tris·HCl at pH 7.8 (50 mmol dm^{-3}). Background phosphatase activity was assessed in each experiment by performing parallel assays in the presence of Li^+ ion in buffer B (buffer B is buffer A plus 150 mmol dm^{-3} LiCl). Rate data were analysed and processed graphically and by using non-linear regression analysis as described previously.[4]

Colorimetric assay. *Colorimetric assay reagent.*—Malachite Green (1.5 g) was dissolved in hydrochloric acid (5 mol dm^{-3}; 25 cm^3) and diluted with water (750 cm^3). To this solution was added a solution of ammonium molybdate (10.5 g) in hydrochloric acid (5 mol dm^{-3}; 225 cm^3) and the solution was stirred at room temperature for 10 min. The solution was filtered by gravity, and stored in the dark for periods of up to 1 month.

Incubation samples contained the following: assay buffer A (210 mm^3), substrate (Ins 1-*P* or 2′-AMP) at various concentrations in assay buffer (30 mm^3), inhibitor at various concentrations in assay buffer (30 mm^3) [in the absence of an inhibitor, this addition was substituted by plain assay buffer (30 mm^3)] and enzyme solution (activity pre-determined for the requirements of individual experiments) (30 mm^3).

The assay solutions were incubated at 37 °C and the reaction was quenched by the addition of colorimetric assay reagent (2.0 cm^3) at the required time (relative to the addition of the enzyme solution). 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 value to a preconstructed standard curve prepared using known phosphate concentrations.

Radiochemical assay. The standard conditions for the radiochemical assay were identical with those for the colorimetric assay. Enzyme solutions (70 mm^3) were added to [U-^{14}C]Ins 1-*P* (10 mmol dm^{-3}, 139 dpm $nmol^{-1}$; 30 mm^3) and assay buffer (200 mm^3) in several identical reaction vessels. The reactions were incubated at 37 °C and were quenched at zero time and at various time intervals thereafter with aq. NaOH (1 mol dm^{-3}; 30 mm^3). The resulting solutions were diluted with water (1 cm^3) and applied to columns of Dowex-1 formate 1 × 8, 400 mesh (20 × 6 mm). The [^{14}C]inositol was washed off the columns with water (2 × 1 cm^3), the eluates (3.3 cm^3) were emulsified with scintillation fluid (10 cm^3) and the radioactivity in each sample was determined by scintillation counting. The use of both assay procedures has been described in detail previously.[4]

One unit of enzyme is defined as the amount of activity which catalyses the hydrolysis of 1 μmol min^{-1} of *myo*-inositol 1-phosphate under the assay conditions.[4]

The activity of all substrates (other than Ins 1-*P*) described in this work were determined using the colorimetric assay. The activity of adenosine 2′-phosphorothioate was determined by NMR spectroscopy, as indicated below.

The activity of inhibitors was assessed using the colorimetric or radiochemical assays with Ins 1-*P* as the substrate over a range of inhibitor concentrations.

^{18}O-Label exchange experiments[3,4]

(a) Inositol-mediated exchange. Solutions which contained 50 mm^3 of buffered substrate (150 mmol dm^{-3} Tris·HCl at pH 8, 100 mmol dm^{-3} KH_2PO_4), 30 mmol dm^{-3} aq. inositol solution (25 mm^3) [for control, distilled water (25 mm^3) was used], 20 mmol dm^{-3} $MgCl_2$ (25 mm^3) and enzyme solution {[12 units cm^{-3} inositol monophosphatase, 1 mg cm^{-3} bovine serum albumin (BSA)], 100 mm^3} were prepared. The solutions were incubated at 37 °C for 30 min and then [^{18}O]water (60 atom%; 50 mm^3) was added to each. The samples were quenched in liquid nitrogen at t = 0, 40 and 80 min, and the control (containing no inositol) was quenched at t = 80 min. The water was removed by lyophilisation, and the residues were extracted with HCl in methanol (100 mm^3; 0.2 mol dm^{-3}) by vortexing for 30 s. Excess of ethereal diazomethane was added to each sample until the yellow colour persisted. Excess of diazomethane was removed by bubbling nitrogen gas through the solutions, and the supernatant liquid was separated from the residue by decantation after centrifugation. The derivatised trimethyl phosphate samples were analysed by GLC–MS which showed a time-dependent increase in the incorporation of solvent-derived label into the phosphate sample. The sample quenched after 80 min showed a 140:142 amu ratio of 3:2 indicating that 40% of the trimethyl phosphate sample contained one ^{18}O-atom. The control experiment showed no ^{18}O-label exchange.

(b) Adenosine-mediated exchange. The above experiment was repeated using adenosine (30 mmol dm^{-3}; 25 mm^3) in place of inositol. The isolated derivatised phosphate sample showed no ^{18}O-label incorporation.

The experiment was repeated using a concentration of adenosine of 30 mmol dm^{-3} in the incubation and a greater overall concentration of [^{18}O]water (45 atom% in the incubation solution) and longer incubation periods (t_{max} = 24 h). The isolated derivatised phosphate sample showed no ^{18}O-label incorporation whatsoever.

Reaction courses for synthetic substrates as assessed by NMR spectroscopy

The substrate to be tested was dissolved in assay buffer C [20 mmol dm^{-3} NH_4HCO_3, 2 mmol dm^{-3} $MgCl_2$ (or Co^{2+} or Mn^{2+}) at pH 8.0 in ^2H_2O (500 mm^3)] to a concentration of 60 mmol dm^{-3} in a 5 mm NMR tube. The ^{1}H NMR spectrum was recorded, and enzyme solution [(7 units cm^{-3} inositol monophosphatase, 0.5 mg cm^{-3} BSA in buffer C), 100 mm^3] was added. [Note that this is sufficient enzyme to hydrolyse all of the substrate (where it is Ins 1-*P* or 2′-AMP) within 90 min.] The ^{1}H NMR spectrum was recorded immediately to account for any signals arising from the enzyme buffer system. The samples were incubated at 37 °C for periods of up to 72 h, and the ^{1}H and ^{31}P NMR spectra were recorded at regular time intervals to assess the extent of hydrolysis and/or transphosphorylation.

Using these protocols it was established that 8-bromoadenosine 2′-phosphate, uridine 2′-phosphate and 5,6-dihydrouridine 2′-phosphate were excellent substrates for the enzyme and displayed no intermediates during the conversion into the dephosphorylated products. Under identical conditions (2*RS*)-pentane-1,2,5-triol 2-phosphate **7** failed to react, as was monitored by examination of the chemical shift for 2-H in the proton NMR spectrum. This is well separated from other signals in the 2-phosphate at δ 4.10 but is partially obscured and occurs at δ ~3.7 in both the putative transphosphorylation product **8** and the hydrolysis product. Both of these compounds were synthesized and were fully characterised as part of this study, see below. Similarly, pentane-1,2,5-triol 5-phosphate **8** failed to transphosphorylate to give compound **7** and did not hydrolyse to the triol.

Experiments in which the rate of hydrolysis was assessed for adenosine phosphorothioate in the presence of Co^{2+} and Mn^{2+} gave precipitates of metal ion thiophosphate salts. These were removed by centrifugation prior to recording NMR spectra. The approximate relative rates for the hydrolysis of 2′-AMPS in the presence of Mg^{2+}, Co^{2+} and Mn^{2+} compared with the rate of 2′-AMP hydrolysis in the presence of Mg^{2+} were <0.2%, 2% and 20% as judged by integration of the well separated signals for the C-1′ hydrogens in the substrate and product at δ 6.10 and 5.95, respectively. Note that Mg^{2+} and Mn^{2+} supported

similar hydrolysis rates for 2'-AMP as determined using the colorimetric assay, see Table 1.

Experiment to measure possible ^{18}O-incorporation at C-2 of uridine 2'-monophosphate during enzymic hydrolysis

Uridine 2'-monophosphate was dissolved in assay buffer C (300 mm^3) and [^{18}O]H_2O (200 mm^3) was added. The reaction was incubated at 37 °C for 24 h, after which time the reaction mixture was lyophilised, and the uridine hydrolysis product was analysed by chemical ionisation (CI) mass spectroscopy. The CI mass spectrum was identical with that for an authentic sample of uridine, indicating that no ^{18}O-label had become incorporated into the molecule.

6-*N*-Benzoyladenosine

6-*N*-Benzoyladenosine was prepared from adenosine according to the method of Schaller *et al.*[26] Recrystallisation from methanol afforded crystals (6.60 g, 71%), mp 152 °C (decomp.) (lit.,[26] mp not given) (Found: C, 52.45; H, 4.95; N, 18.0. Calc. for $C_{17}H_{17}N_5O_5{\cdot}H_2O$: C, 52.45; H, 4.65; N, 17.8%); *m/z* (Found: $[M + H]^+$, 372.1308. $C_{17}H_{18}N_5O_5$ requires *m/z* 372.1308); $[\alpha]_D$ −49.5 (*c* 0.052, MeOH); ν_{max}(Nujol)/cm^{-1} 3500w, 3420w, 3340w, 3100w (OH and NH), 1700s (amide C=O), 1300m, 1220m, 1050m and 700m; δ_H(200 MHz; [$^2H_6$]-DMSO) 3.70 (2 H, ABX, $J_{5'A,5'B}$ 12, $J_{4',5'A}$ 4, $J_{4',5'B}$ 3, 5'-H_2), 4.05 (1 H, q, $J_{4',3'}$ 4, $J_{4',5'}$ 4, 4'-H), 4.24 (1 H, t, $J_{3',4'}$ 4, $J_{2',3'}$ 4, 3'-H), 4.65 (1 H, t, $J_{2',3'}$ 4, $J_{1',2'}$ 5, 2'-H), 6.13 (1 H, d, $J_{1',2'}$ 5, 1'-H), 7.60 (3 H, m, ArH *meta* and *para*), 8.08–8.12 (2 H, m, ArH *ortho*), 8.87 (1 H, s, adenine CH) and 9.11 (1 H, s, adenine CH); δ_C(50.3 MHz; [$^2H_6$]DMSO) 61.6 (C-5'), 70.7 (C-3'), 74.0 (C-2'), 86.0 (C-4'), 87.9 (C-1'), 126.1 (C-5), 128.8 (Ar-C *meta* and *para*), 132.7 (Ar-C *ortho*), 133.7 (Ar-C quaternary), 143.4 (C-2), 150.8, 151.9 and 152.6 (C-8, -4 and -6) and 166.1 (amide C=O); *m/z* (EI) 371 (5%, M^+), 238 (8, benzoyladenine), 135 (100, [adenine − H]$^+$), 105 (95, $PhCO^+$) and 77 (85, Ph^+).

6-*N*-Benzoyl-3',5'-bis-*O*-*tert*-butyldimethylsilyladenosine 9

Following the method of Ogilvie *et al.*[27] 6-*N*-benzoyladenosine (4.0 g, 10.8 mmol) was azeotropically dried with dry dimethylformamide (DMF) (3 × 20 cm^3) and the residue and imidazole (4.40 g, 64 mmol) were dissolved in dry DMF (15 cm^3) under argon and *tert*-butyldimethylsilyl chloride (TBDMSCl) (4.54 g, 32.3 mmol) was added. The reaction mixture was stirred at room temperature for 2 h and aq. ammonia solution (2 mol dm^{-3}; 40 cm^3) was added. The silylated adenosine derivatives were extracted into ethyl acetate (3 × 50 cm^3) and the extracts were dried ($MgSO_4$). The solvents were removed under reduced pressure to give a mixture of four compounds (which were well separated on TLC, see below) as a yellow oil. These were separated by short silica column chromatography, eluting with a stepped gradient of 0–35% ethyl acetate in light petroleum, in 5% steps, to give the desired product as a foam (3.89 g, 36%), mp 55–65 °C (lit.,[27] 66–70 °C) (Found: C, 57.6; H, 7.95; N, 11.4. Calc. for $C_{29}H_{45}N_5O_5Si_2$: C, 58.05; H, 7.55; N, 11.65%); *m/z* (Found: $[M + H]^+$, 600.3040. $C_{29}H_{46}N_5O_5Si_2$ requires *m/z*, 600.3037); $[\alpha]_D$ −21 (*c* 0.33, Et_2O) (lit.,[27] value not given); ν_{max}(Nujol)/cm^{-1} 3300m (O–H), 2900s (C–H), 1710s (amide C=O), 1450s, 1240m, 1050m and 850m; δ_H(200 MHz; C^2HCl_3) 0.03 (3 H, s), 0.05 (3 H, s), 0.13 (3 H, s) and 0.14 (3 H, s) (SiMe), 0.87 (9 H, s) and 0.93 (9 H, s) (SiBu^t), 3.85 (2 H, ABX, $J_{5'A,5'B}$ 12, $J_{4',5'A}$ 3, $J_{4',5'B}$ 3, 5'-H_2), 4.11 (1 H, q, $J_{4',3'}$ 4, $J_{4',5'}$ 3, 4'-H), 4.57 (2 H, m, 2'- and 3'-H), 6.07–6.09 (1 H, d, $J_{1',2'}$ 4, 1'-H), 7.53 (3 H, m, ArH *meta* and *para*), 8.02 (2 H, m, ArH *ortho*), 8.21 (1 H, s, adenine H), 8.76 (1 H, s, adenine H) and 9.24 (1 H, br s, amide NH); δ_C(75 MHz; C^2HCl_3) −10.2 and −10.3 (SiMe), 18.6 and 18.9 (SiBu^t, quaternary C), 26.4 and 26.9 (SiC*Me*$_3$), 64.7 (C-5'), 72.1, 75.6, 86.0 and 89.4 (ribose ring C), 123.8 (C-5), 128.4 and 129.3 (Ar-C *meta* and *para*), 133.2 (Ar-C *ortho*), 134.2 (Ar-C quaternary), 142.1 (C-8), 150.1, 152.1 and 153.1 (C-2, -4 and -6) and 165.3 (amide C=O); *m/z* (Cl) 600 (70%, $[M + H]^+$), 343 (5, [TBDMSO$_2$ ribose + H]$^+$), 240 (100, [AdBz + H]$^+$) and 121 (10, $BzNH_2^+$); TLC: 2',3',5'-tris-*O*-TBDMS-6-*N*-BzAd, R_f = 0.93; 6-*N*-benzoyl-2',5'-bis-*O*-TBDMS-Ad, R_f = 0.67; 6-*N*-benzoyl-3',5'-bis-*O*-TBDMS-Ad, R_f = 0.58 and 6-*N*-benzoyl-5'-*O*-TBDMS-Ad, R_f = 0.48 on silica (50% ethyl acetate–diethyl ether).

6-*N*-Benzoyl-3',5'-bis-*O*-*tert*-butyldimethylsilyladenosin-2'-yl-2-cyanoethyl *N*,*N*-diisopropylphosphoramidite 10A and 10B

Using modifications of the methods of Usman *et al.*[28] and Scaringe *et al.*[29] for the preparation of similar compounds, diisopropylethylamine (2.97 cm^3, 16.9 mmol), 4-(dimethylamino)pyridine (DMAP) (106 mg, 1.69 mmol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (1.31 g, 0.97 cm^3, 5.5 mmol) were dissolved in tetrahydrofuran (THF) (40 cm^3) under argon and the mixture was stirred at room temperature for 10 min. A solution of 6-*N*-benzoyl-3',5'-bis-*O*-*tert*-butyldimethylsilyladenosine **9** (2.5 g, 4.24 mmol) in THF (30 cm^3) was added dropwise, and the reaction mixture was stirred at room temperature for 3 h. The solvents were removed under reduced pressure, and the resulting oil was partitioned between ethyl acetate (50 cm^3) and saturated aq. sodium hydrogen carbonate solution (50 cm^3). The organic layer was washed with saturated aq. sodium hydrogen carbonate solution (3 × 30 cm^3) and dried ($MgSO_4$). The solvents were removed under reduced pressure, and the residue was purified on a triethylamine-basified silica column eluted with 35% ethyl acetate in light petroleum, to give the required product as two separate diastereoisomers (2.11 g, 62%); lower R_f diastereoisomer; *m/z* (Found: $[M + Na]^+$, 822.3935. $C_{38}H_{62}N_7NaO_6Si_2$ requires *m/z*, 822.3935); $[\alpha]_D$ −62.2 (*c* 0.8, MeOH); ν_{max}(Nujol)/cm^{-1} 3200br s, 1700s (amide C=O), 1611s, 1460s, 1250s and 1073s; δ_H(200 MHz; C^2HCl_3) 0.09 (3 H, s, SiMe), 0.11 (3 H, s, SiMe), 0.13 (3 H, s, SiMe) and 0.14 (3 H, s, SiMe), 0.92 (9 H, s, SiBu^t) and 0.94 (9 H, s, SiBu^t), 1.13 [12 H, dd, J(CH*Me*$_2$, P) 14.6, J(CH*Me*$_2$, C*H*Me$_2$) 6.6, CH*Me*$_2$], 3.58 (4 H, m, CH_2CN), 3.90 (2 H, ABX, $J_{5'A,5'B}$ 11.4, $J_{4',5'A}$ 3.6, $J_{4',5'B}$ 3.4, 5'-H_2), 4.15 (1 H, q, $J_{4',3'}$, 3.6, $J_{4',5'}$ 4, 4'-H), 4.55 (1 H, t, $J_{4',3'}$ 4, $J_{2',3'}$ 4.4, 3'-H), 4.70 (1 H, dt, $J_{2',3'}$ 4.5, $J_{1',2'}$ 5.2, $J_{2',P}$ 10, 2'-H), 6.35 (1 H, d, $J_{1',2'}$ 4.9, 1'-H), 7.58 (3 H, m, ArH *meta* and *para*), 8.05 (2 H, m, ArH *ortho*), 8.39 (1 H, s, adenine-H) and 8.80 (1 H, s, adenine-H) and 9.14 (1 H, s, amide NH); δ_C(75 MHz; C^2HCl_3) −4.9 to −3.9 (SiMe groups), 18.6 (SiBu^t, quaternary C), 18.9 (SiBu^t, quaternary C), 23.1 (CH*Me*$_2$), 23.2 (CH*Me*$_2$) 24.9 (SiC*Me*$_3$), 43.5 (*C*HMe$_2$), 43.9 (*C*HMe$_2$), 58.4 ($J_{P,C}$ 18, $POCH_2$), 62.8 (C-5'), 70.8 (C-4'), 77.0 and 77.2 (C-2', $J_{P,C}$ 15), 86.0 and 87.9 (C-1' and C-4'), 116.9 (CN), 123.7 (C-5), 128.4 and 129.3 (ArC *meta* and *para*), 133.2 (Ar-C *ortho*), 134.3 (Ar-C quaternary), 142.6 (C-8), 149.9, 152.3 and 152.9 (C-2, -4 and -6) and 165.3 (amide C=O); δ_P(121.5 MHz; C^2HCl_3) 150.4; *m/z* (FAB) 823 (45%, $[M + Na]^+$), 801 (10, $[M + H]^+$), 699 (25, [M − Pr^i_2NH]$^+$), 343 (45, [(TBDMSO)$_2$ ribose + H]$^+$), 262 (15, [AdBz + Na]$^+$) and 240 (45, [AdBz + Na]$^+$); R_f = 0.32 (50% ethyl acetate–light petroleum). Higher R_f diastereoisomer; *m/z* (Found: $[M + Na]^+$, 822.3935. $C_{38}H_{62}N_7NaO_6Si_2$ requires *m/z*, 822.3935); $[\alpha]_D$ −54.4 (*c* 0.8, MeOH); ν_{max}(Nujol)/cm^{-1} 3200br s, 1700s (amide C=O), 1611s, 1456s, 1255s, 1073s and 839s; δ_H(200 MHz; C^2HCl_3) 0.10 (3 H, s, SiMe), 0.11 (3 H, s, SiMe), 0.15 (3 H, s, SiMe) and 0.20 (3 H, s, SiMe), 0.92 (9 H, s, SiBu^t), 0.95 (9 H, s, SiBu^t), 1.15 [12 H, dd, J(CH*Me*$_2$, P) 14.6, J(CH*Me*$_2$, C*H*Me$_2$) 6.6, CH*Me*$_2$], 3.60 (4 H, m, CH_2CN), 4.00 (3 H, m, 5'-H_2 and 4'-H), 4.55 (1 H, t, $J_{4',3'}$ 4, $J_{2',3'}$ 4.4, 3'-H), 4.86 (1 H, dt, $J_{2',3'}$ 4.5, $J_{1',2'}$ 5.2, $J_{2',P}$ 10, 2'-H), 6.25 (1 H, d, $J_{1',2'}$ 4.9, 1'-H), 7.58 (3 H, m, ArH *meta* and *para*), 8.05 (2 H, m, ArH *ortho*), 8.39 (1 H, s, adenine CH), 8.80 (1 H, s adenine CH) and 9.12 (1 H, br s, amide NH); δ_C(75

MHz; C^2HCl_3) −4.9 to −3.9 ($SiMe_2$), 17.0 and 17.3 ($SiCMe_3$, quaternary C), 23.1 and 23.2 ($CHMe_2$), 24.7 and 25.0 ($SiCMe_3$), 41.9 and 42.1 ($CHMe_3$), 57.1 and 57.3 ($J_{P,C}$ 18, $POCH_2$), 61.0 (C-5'), 69.9 (C-4'), 74.9 and 75.1 (C-2', $J_{P,C}$ 15), 84.3 and 86.4 (C-1' and -4'), 115.6 (CN), 122.1 (C-5), 126.6 and 127.7 (Ar-C *meta* and *para*), 131.6 (Ar-C *ortho*), 132.7 (Ar-C quaternary), 140.8 (C-8), 148.3, 150.4 and 151.5 (C-2, -4 and -6) and 165.4 (amide C=O); δ_P(121.5 MHz; C^2HCl_3) 150.6; *m/z* (FAB) 823 (90%, $[M + Na]^+$), 801 (5, $[M + H]^+$), 700 (40, $[M + H - {}^iPr_2NH]^+$), 343 {100, $[(TBDMSO)_2$ ribose + $H]^+$}, 262 (45, $[AdBz + Na]^+$) and 240 (55, $[AdBz + Na]^+$); R_f = 0.39 (50% ethyl acetate–light petroleum).

6-*N*-Benzoyl-3',5'-bis-*O*-(*tert*-butyldimethysilyl)adenos-2'ylbis-2-cyanoethylphosphorothioate 11

To a solution of 1 *H*-tetrazole (0.3 g, 4.2 mmol) in dry acetonitrile (10 cm^3) under argon was added 3-hydroxypropionitrile (0.41 cm^3, 6.0 mmol). A solution of the protected phosphoramidite nucleoside **10A** + **10B** (1.58 g, 2.0 mmol) in dry acetonitrile (10 cm^3) was added, and the reaction mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the resulting oil was redissolved in dry pyridine (20 cm^3). Sulfur (0.64 g, 20 mmol) was added and the reaction mixture was stirred at room temperature for 15 min. Excess of sulfur was removed by filtration and the pad was washed with ethyl acetate. The required adenosine phosphorothioate product **11** was purified by silica column chromatography on triethylamine-basified silica gel eluted with 65% ethyl acetate–light petroleum to give a foam (0.75 g, 48%); *m/z* (Found: $[M + NH_4]^+$, 802.3003. $C_{35}H_{53}N_7O_7PSSi_2$ requires *m/z*, 802.3003); $[\alpha]_D$ −50.6 (*c* 0.6, MeOH); ν_{max}(Nujol)/cm^{-1} 2520w, 1700s, 1580s, 1550s, 1300s, 1150s, 1000s, 950w, 930w, 890w, 830m, 790m and 725s; δ_H(200 MHz; C^2HCl_3) 0.12 (3 H, s, SiMe), 0.13 (3 H, s, SiMe), 0.17 (3 H, s, SiMe), 0.19 (3 H, s, SiMe), 0.93 (9 H, s, SiBu^t), 0.96 (9 H, s, SiBu^t), 2.62 (2 H, t, CH_2CN), 2.69 (2 H, t, CH_2CN), 3.90 (2 H, ABX, $J_{A,B}$ 11.6, $J_{A,X}$ 2.9, $J_{B,X}$ 2.3, 5'-H), 4.20 (5 H, m, 4'-H and $POCH_2$), 4.60 (1 H, t, $J_{4',3'}$ 3.1, $J_{2',3'}$ 4.3, 3'-H), 5.40 (1 H, dq, $J_{1',2'}$ 6.2, $J_{2',3'}$ 4.7, $J_{2',P}$ 10.6, 2'-H), 6.40 (1 H, d, $J_{1',2'}$ 6.2, 1'-H), 7.55 (3 H, m, ArH *meta* and *para*), 8.05 (2 H, d, *J* 6.8, ArH *ortho*), 8.81 (1 H, s, adenine-H) and 8.82 (1 H, s, adenine-H); δ_C(75.5 MHz; C^2HCl_3) −4.8, −4.4 and −4.0 (SiMe groups), 18.9 and 19.3 (SiBu^t quaternary C), 20.0, 20.1 and 20.2 (CH_2CN), 26.5, 26.7 and 26.8 ($SiCMe_3$), 60.3, 62.2, 62.4 and 62.8 (C-5' and $POCH_2$), 72.0, 80.4, 86.3 and 87.2 (ribose C-H), 117.0 and 117.1 (CN), 123.9 (C-5), 128.7 and 129.6 (Ar-C *meta* and *para*), 133.6 (Ar-C *ortho*), 134.3 (Ar-C quaternary), 142.5 (C-8), 150.4, 152.7 and 153.3 (C-2, -4 and -6) and 165.6 (amide C=O); δ_P(121.5 MHz; C^2HCl_3) 66.5; *m/z* (FAB) 824 (100%, $[M + Na]^+$), 802 (55, $[M + H]^+$) and 600 (5, $[M + H - PS(OCH_2CH_2CN)_2]^+$).

Adenosine 2'-phosphorothioate biscyclohexylamine salt 3 (X = S)

The phosphorothioate triester **11** (300 mg, 0.375 mmol) was dissolved in a 3:1 water–ethanolic conc. ammonia solution (20 mol dm^{-3}; 5 cm^3) in a sealed glass vial and the solution was stirred at 60 °C for 20 h. The solvents were removed under reduced pressure and the residue was partitioned between 5% aq. ammonia (5 cm^3) and diethyl ether (10 cm^3). The organic phase was washed with water (3 × 5 cm^3) and the combined aqueous phases were concentrated under reduced pressure. The resulting oil was dissolved in dry THF (2.0 cm^3) and a solution of tetrabutylammonium fluoride (TBAF) (1.0 mol dm^{-3} in THF; 0.8 cm^3) was added. The reaction mixture was stirred for 10 h and the solvents were removed under reduced pressure. The residue was redissolved in water (2 cm^3) and the product was subjected to ion-exchange chromatography on Amberlite IR 118 (cyclohexylammonium)$^+$. The UV-active fractions were combined and lyophilised to give a solid (35 mg, 53%); mp > 250 °C (Found: C, 47.65; H, 7.25; N, 17.1. $C_{22}H_{40}N_7O_6PS$ requires C, 47.1; H, 7.2; N, 17.45%); $[\alpha]_D$ −41.2 (*c* 1.4, MeOH); ν_{max}(Nujol)/cm^{-1} 3400br s, 2200w, 1700s, 1300s, 1150s and 700s; δ_H(200 MHz; ^2H_2O) 0.9–2.1 (cyclohexyl CH_2), 3.10 (cyclohexyl CH), 3.85 (2 H, m, 5'-H_2), 4.28 (1 H, m, 4'-H), 4.38 (1 H, m, 3'-H), 5.20 (1 H, m, 2'-H), 6.00 (1 H, d, $J_{1',2'}$ 6.0, 1'-H), 8.2 (1 H, s, adenine-H) and 8.4 (1 H, s, adenine-H); δ_C(75.5 MHz; ^2H_2O) 26.7, 27.3 and 33.3 (cyclohexyl CH_2), 53.2 (cyclohexyl CH), 64.7 (C-5'), 73.6 (C-4'), 79.1 (C-3'), 88.5 (C-1'), 90.5 (C-2'), 121.8 (C-5), 144.0 (C-8), 151.5, 155.2 and 158.2 (C-2, -4 and -6); δ_P(121.5 MHz; ^2H_2O) 45.2; *m/z* (FAB) 463 (2%, $[M - C_6H_{14}N + H]^+$), 430 {4, [M(disodium salt) + $Na]^+$}, 408 {10, [M (monosodium salt) + $Na]^+$}, 386 {15, [M (monosodium salt) + $H]^+$}, 364 {15, [M (free acid) + $H]^+$} and 272 (25, [M − thiophosphate + $Na]^+$).

Dihydrouridine 2'-phosphate disodium salt 6 (R = 5,6-dihydrouracil, X = O)

A modification of the method of Kondo and Witkop[30] and Gani and Young[31] for the reduction of thymidine and uracil was used. Uridine 2'-phosphate dilithium salt (200 mg, 0.59 mmol) was dissolved in hydrochloric acid (20 cm^3; 1 mmol dm^{-3}) and 5% rhodium on alumina (20 mg) was added. The reaction vessel was flushed with hydrogen gas (~500 cm^3), and the mixture was stirred at room temperature under hydrogen. After 12 h the reaction mixture was filtered through Celite and the solvent was removed under reduced pressure. The residue was redissolved in water (5 cm^3) and was subjected to ion-exchange chromatography on Amberlite 118 $(Na)^+$ and eluted with water. The eluent was lyophilised to give an amorphous solid (170 mg, 88%); mp 145–155 °C (decomp.); $[\alpha]_D$ −32.3 (*c* 1.0, water); ν_{max}(Nujol)/cm^{-1} 3400–2400br s (O–H and N–H), 1650 and 1670 (C=O), 1270 (P=O), 1100s, 970s and 750s; δ_H(200 MHz; ^2H_2O) 2.70 (2 H, t, *J* 6.5, CH_2CO), 3.50 (2 H, t, *J* 6.4, CH_2NR), 3.65 (2 H, m, 5'-H_2), 3.95 (1 H, m, 4'-H), 4.15 (1 H, m, 3'-H), 4.45 (1 H, m, 2'-H) and 5.80 (1 H, d, *J* 6.2, 1'-H); δ_C(50.5 MHz; ^2H_2O) 32.9 (CH_2CO), 39.5 (CH_2NR_2), 64.1, (C-5'), 72.9 (C-4'), 75.5 (C-3'), 86.0 (C-1'), 89.8 ($J_{P,C}$ 9.7, C-2'), 157.5 [NC(O)N] and 176.8 [$CH_2C(O)N$]; δ_P(121.5 MHz; ^2H_2O) 3.90; *m/z* (FAB) 393 (5%, $[M + Na]^+$), 371 (5, $[M + H]^+$), 137 (15, [dihydrouracil + $Na]^+$) and 115 (10, [dihydrouracil + $H]^+$).

The compound served as a good substrate for inositol monophosphatase, V_{max} = 70% of V_{max} for 2'-AMP, K_m = 1.4 mmol dm^{-3}.

(2*RS*)-Pentane-1,2,5-triol

A solution of 2-oxoglutaric acid (2.5 g, 17.1 mmol) in dry THF (50 cm^3) was added over a period of 30 min to a stirred suspension of lithium aluminium hydride (1.63 g, 42.9 mmol) in dry THF (75 cm^3) under nitrogen. The reaction mixture was stirred for a further 1 h. Water (2.5 cm^3) in THF (10 cm^3) was added with extreme caution, and the resulting suspension was filtered. The precipitate was washed with acetone (100 cm^3) and the solvents were removed under reduced pressure. The residue was redissolved in acetone, and the solution was dried ($MgSO_4$). The solvent was removed under reduced pressure to give the triol as a liquid (0.93 g, 45%) similar to that prepared from α-hydroxyglutarolactone;[32] *m/z* (Found: $[M + NH_4]^+$, 138.1130. $C_5H_{16}NO_3$ requires *m/z*, 138.1130); ν_{max}(Nujol)/cm^{-1} 3350br s (O–H), 2900s (C–H) and 1075s (C–O); δ_H(200 MHz; ^2H_2O), 1.45 (4 H, m, 3- and 4-H_2), 3.50 (4 H, m, 1- and 5-H_2) and 3.60 (1 H, m, 2-H); δ_C(50.5 MHz; ^2H_2O) 30.3 and 31.6 (C-3 and -4), 64.4 (C-5), 68.2 (C-1) and 74.4 (C-2); *m/z* (CI) 138 (100%, $[M + NH_4]^+$), 121 (100, $[M + H]^+$), 103 (5, $[M + H - H_2O]^+$) and 85 (10, $[M + H - 2H_2O]^+$).

(2*RS*)-1,5-Bis-(*tert*-butyldimethylsiloxy)pentan-2-ol

To a solution of pentane-1,2,5-triol (0.72 g, 6.0 mmol) and imidazole (0.9 g, 13.4 mmol) in dry DMF (20 cm^3) was added TBDMSCl (2.0 g, 13.4 mmol). The reaction mixture was stirred at room temperature under nitrogen for 24 h. The solution was added to aq. sodium hydrogen carbonate (30 cm^3; 5%), and was extracted into diethyl ether (3 × 50 cm^3). The organic phase was washed with saturated brine (30 cm^3) and was dried (Na_2SO_4). The solvent was removed under reduced pressure to yield a pale yellow liquid, which was purified by silica column chromatography and elution with 10% ethyl acetate in light petroleum to give a viscous liquid (1.43 g, 68%) (Found: C, 58.7; H, 11.7. $C_{17}H_{40}O_3Si_2$ requires C, 58.6; H, 11.6%); *m/z* (Found: $[M + H]^+$, 349.2594. $C_{17}H_{41}O_3Si_2$ requires *m/z*, 349.2594); ν_{max}(Nujol)/cm^{-1} 3400br s (O–H), 2950s (C–H), 1100br s (Si–O), 840s and 790s; δ_H(200 MHz; C^2HCl_3) 0.05 and 0.06 (12 H, 2 × s, SiMe), 0.89 and 0.90 (18 H, 2 × s, Bu^t), 1.30–1.75 (4 H, m, 3- and 4-H_2), 2.70 (1 H, br s, OH), 3.45 (1 H, dt, 2-H) and 3.60 (4 H, m, 1- and 5-H_2); δ_C(50.5 MHz; C^2HCl_3) −4.9 (SiMe), 18.8 (*C*Me$_3$), 26.4 (C*Me*$_3$), 28.4 (C-4), 30.2, (C-3), 63.7 (C-5), 67.7 (C-1) and 77.2 (C-2); *m/z* (CI) 349 (100%, $[M + H]^+$), 331 (10, $[M + H - H_2O]^+$), 235 (5, $[M + NH_4 - TBDMSO]^+$), 217 (15, $[M + H - TBDMSOH]^+$), 132 (15, $TBDMSOH^+$) and 58 (3, $[Bu^t + H]^+$).

Dibenzyl (2*RS*)-1,5-Bis-(*tert*-butyldimethylsiloxy)pentan-2-yl phosphate

(*RS*)-1,5-Bis-*O*-(*tert*-butyldimethylsilyl)pentane-1,2,5-triol (0.80 g, 2.29 mmol) and 1*H*-tetrazole (0.32 g, 4.59 mmol) were dissolved in dry acetonitrile (10 cm^3) under argon and a solution of dibenzyl *N*,*N*-diisopropylphosphoramidite[33] (1.03 g, 2.98 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 (*m*-chloroperbenzoic acid (MCPBA) (55–60% purity; 1.08 g, 3.44 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 (60 cm^3), washed successively with 10% aq. sodium sulfite (3 × 30 cm^3), aq. sodium hydrogen carbonate (2 × 20 cm^3; 5%) and saturated brine (30 cm^3) and then was dried over (Na_2SO_4). The solvent was removed under reduced pressure to give a pale yellow oil, which was purified by silica column chromatography on triethylamine-basified silica and eluted with 30% ethyl acetate in light petroleum to give the title compound as a liquid (1.09 g, 78%) (Found: C, 61.0; H, 9.2. $C_{37}H_{56}O_6PSi_2$ requires C, 61.15; H, 8.8%); *m/z* (Found: $[M + H]^+$, 609.3200. $C_{31}H_{57}O_6PSi_2$ requires *m/z*, 609.3196); ν_{max}(Nujol)/cm^{-1} 2800s (C–H), 1500s, 1275s (P=O), 1100s and 1000s; δ_H(200 MHz; C^2HCl_3) 0.08 (12 H, s, SiMe), 0.88 (18 H, s, Bu^t), 3.60 (2 H, t, *J* 5.9, 5-H_2), 3.70 (2 H, ABX, J_{AB} 10.6, J_{AX} 4.8, 1-H_2), 4.40 (1 H, m, 2-H), 5.05 (4 H, d, $J_{P,H}$ 7.7, PhCH_2) and 7.35 (10 H, m, ArH); δ_C(50 MHz; C^2HCl_3) −4.9 and −4.8 (SiMe), 18.8 (*C*Me$_3$), 26.3 and 26.4 (C*Me*$_3$), 28.6 and 29.0 (C-3 and -4), 63.2 (C-5), 65.2 (C-1), 69.6 ($J_{P,C}$ 5.7, PhCH_2), 80.1 ($J_{P,C}$ 6.4, C-2), 128.3, 128.8 and 128.9 (Ar–C) and 136.5 (Ar–C quaternary); δ_P(121.5 MHz; C^2HCl_3) −1.1; *m/z* (CI) 609 (10%, $[M + H]^+$), 132 (5, $[TBDMSO + H]^+$) and 58 (60, $[Bu^t + H]^+$).

(2*RS*)-Pentane-1,2,5-triol 2-phosphate biscyclohexylammonium salt 7

Dibenzyl (2*RS*)-1,5-bis-(*tert*-butyldimethylsiloxy)pentan-2-yl phosphate (0.80 g, 1.31 mmol) was dissolved in methanol (10 cm^3) and palladium on activated charcoal (80 mg, 10%) was added to the stirred 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 redissolved in THF (5 cm^3) and TBAF (1.0 mol dm^{-3} solution in THF; 1.31 cm^3) was added. The mixture was stirred for a further 4 h, and the solvent was removed under reduced pressure. The residue was redissolved in water (5 cm^3) and was subjected to chromatography on Amberlite 118 $(H)^+$ ion-exchange resin and eluted with water. The acidic fractions were combined, treated with freshly distilled cyclohexylamine (3 cm^3, 26.5 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 water–acetone to give compound **7** as crystals (0.36 g, 69%); mp 165–167 °C (Found: C, 46.7; H, 9.8; N, 6.1. $C_{17}H_{39}N_2O_6P{\cdot}2H_2O$ requires C, 47.0; H, 10.0; N, 6.45%); ν_{max}(Nujol)/cm^{-1} 3200–2400br s (OH and H-bonding), 1280m (P=O), 1055s and 880s; δ_H(300 MHz; ^2H_2O) 1.00–2.00 (24 H, m, cyclohexyl CH_2 and 3- and 4-H_2), 3.15 (2 H, m, cyclohexyl CH), 3.50–3.70 (4 H, m, 1- and 5-H_2) and 4.10 (1 H, m, 2-H); δ_C(75 MHz; ^2H_2O) 26.5 and 27.0 (cyclohexyl CH_2), 30.0 (C-4), 30.8 (C-3), 35.0 (cyclohexyl CH), 64.4 (C-5), 67.5 (C-1) and 78.0 ($J_{P,C}$ 5.4, C-2); δ_P(121.5 MHz; C^2HCl_3) 2.6; *m/z* (FAB) 300 (95%, $[M - C_6H_{14}N + H]^+$), 223 (50, $[M - 2(C_6H_{14}N) + Na]^+$), 143 (5, $[M - phosphate + Na]^+$) and 121 (100, $[M - phosphate + H]^+$).

This compound failed to undergo transphosphorylation (to give the 5-phosphate **8** described below) and failed to serve as a hydrolysis substrate upon incubation with the enzyme, as determined by NMR spectroscopy; see above. In standard activity assays the compound acted as a weak inhibitor, K_i = 0.5 mmol dm^{-3}.

Bis-(2-cyanoethyl)-4,5-epoxypentyl phosphate

Pent-4-en-1-ol (0.30 g, 3.48 mmol) was dissolved in dry acetonitrile (10 cm^3) under argon and 1*H*-tetrazole (0.24 g, 3.48 mmol) was added. A solution of bis(2-cyanoethyl) *N*,*N*-diisopropylphosphoramidite (1.18 g, 4.35 mmol) was added and the solution was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was redissolved in dichloromethane. The solution was cooled to −10 °C and a solution of MCPBA (50–60% purity; 4.2 g, 12.0 mmol) in dichloromethane (50 cm^3) was added dropwise, and the mixture was then stirred for 1 h. The reaction mixture was diluted with dichloromethane (60 cm^3), washed successively with aq. sodium sulfite (3 × 30 cm^3; 10%), aq. sodium hydrogen carbonate (2 × 20 cm^3; 5%) and saturated brine (30 cm^3) and was then dried (Na_2SO_4). The solvent was removed under reduced pressure to give a pale yellow oil. This was purified by silica column chromatography on triethylamine-basified silica, and eluted with ethyl acetate, to give a liquid (0.63 g, 63%); *m/z* (Found: $[M + H]^+$, 289.0953. $C_{11}H_{18}N_2O_5P$ requires *m/z*, 289.0953); ν_{max}(neat)/cm^{-1} 3000s (C–H), 1260s (P=O), 1050s and 950s; δ_H(200 MHz; C^2HCl_3) 1.3–1.9 (4 H, m, 2- and 3-H_2), 2.45 (1 H, q, 5-H), 2.75 (5 H, m, 5-H and cyanoethyl CH_2CN), 2.9 (1 H, m, 4-H) and 4.2 (6 H, m, 1-H_2 and cyanoethyl $POCH_2$); δ_C(50 MHz; C^2HCl_3) 20.2 (CH_2CN), 27.2 (C-2), 28.9 (C-3), 47.4 (C-5), 51.0 (C-4), 62.8 ($J_{P,C}$ 5.3, cyanoethyl $POCH_2$), 68.8 ($J_{P,C}$ 6.2, C-1) and 117.2 (CN); δ_P(121.5 MHz; C^2HCl_3) −1.98; *m/z* (CI) 306 (70%, $[M + NH_4]^+$), 289 (100, $[M + H]^+$), 253 (15, $[M - C_3H_4N + NH_4]^+$), 236 (17, $[M - C_3H_4N + H]^+$), 222 (25, [biscyanoethyl phosphate + $NH_4]^+$) and 222 (10, [biscyanoethyl phosphate + $H]^+$).

(2*RS*)-Pentane-1,2,5-triol 5-phosphate biscyclohexylammonium salt 8

The preceding 4,5-epoxide (85 mg, 0.3 mmol) was dissolved in acetone (0.5 cm^3) and 2 mol dm^{-3} sodium hydroxide (0.44 cm^3,

0.88 mmol) was added. The solution was stirred at room temperature for 2 h and then the solvent was removed under reduced pressure. Excess of sodium hydroxide (2.5 cm^3; 2 mol dm^{-3}) was added and the reaction mixture was stirred for a further 3 h. The solvent was removed under reduced pressure and the residue was subjected to chromatography on Amberlite 118 $(H)^+$ ion-exchange resin with water as eluent. The acidic fractions were combined, treated with freshly distilled cyclohexylamine (1 cm^3, 8.8 mmol) and 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 solution was lyophilised to give a pale yellow gum which resisted crystallisation (60 mg, 68%); *m/z* (Found: [M (derivatised as the dimethyl ester) + H]$^+$, 229.0841. $C_7H_{18}O_6P$ requires *m/z*, 229.0841); ν_{max}(Nujol)/cm^{-1} 3200–2400br s (OH and NH), 1270m (P=O), 955s and 900s; δ_H(200 MHz; ^2H_2O) 1.00–2.00 (24 H, m, cyclohexyl CH_2 and 3- and 4-H_2), 3.00 (2 H, m, cyclohexyl CH), 3.40 (2 H, m, 5-H_2) and 3.50–3.90 (3 H, m, 1-H_2 and 2-H); δ_C(75 MHz; ^2H_2O) 26.7 and 27.2 (cyclohexyl CH_2), 29.0 (C-4), 31.6 (C-3), 33.2 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 67.5 ($J_{P,C}$ 5.0, C-5), 68.2 (C-1) and 74.4 (C-2); δ_P(121.5 MHz; C^2HCl_3) 2.66; *m/z* (CI) (as the dimethyl ester) 243 (40%, [M + NH_4]$^+$), 229 (100, [M + H]$^+$), 214 (10, [M + H − Me]$^+$), 114 ([dimethyl phosphate + NH_4]$^+$) and 127 ([dimethyl phosphate + H]$^+$).

This compound failed to undergo transphosphorylation to give compound **7** upon incubation with the enzyme as determined by NMR spectroscopy; see above.

8-Bromoadenosine 2′-phosphate

By use of a modification of the procedure of Gani *et al.*,[34] adenosine monophosphate (100 mg, 0.29 mmol) was added to an acetate buffer solution at pH 4.5 prepared by dissolution of sodium acetate (0.82 g) and glacial acetic acid (0.3 g) in water (5 cm^3). When dissolution was complete, bromine (50 mm^3, 0.97 mmol) was added and the reaction mixture was stirred at room temperature. After 3 h, solid sodium metabisulfite ($Na_2S_2O_5$) was added, until the solution became colourless, when it was adjusted to pH 6.0. After storage at −5 °C for several hours, small crystals precipitated and these were filtered off and dried (86.7 mg, 70%); δ_H(300 MHz; ^2H_2O), 3.86 (2 H, m, 5′-H_2), 4.23 (1 H, m, 4′-H), 4.59 (1 H, m, 3′-H), 5.20 (1 H, m, 2′-H), 6.11 (1 H, d, $J_{1',2'}$ 6.0, 1′-H) and 8.07 (1 H, s, adenine 2-H); δ_C(75.5 MHz; ^2H_2O) 64.85 (C-5′), 73.66 (C-4′), 77.75 (C-3′), 88.79 (C-1′), 93.01 (C-2′), 131.3 (C-5), 143.6 (C-8) and 151.2, 155.0 and 157.1 (C-2, -4 and -6); δ_P(121.5 MHz; ^2H_2O) 4.07.

The sample contained a small amount of unchanged adenosine 2′-phosphate which could not be removed by repeated recrystallisation, and which was, therefore, used directly in NMR spectroscopic experiments to determine its activity as a substrate for inositol monophosphatase. The 8-bromoadenosine derivative was hydrolysed at a similar rate to 2′-AMP.

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