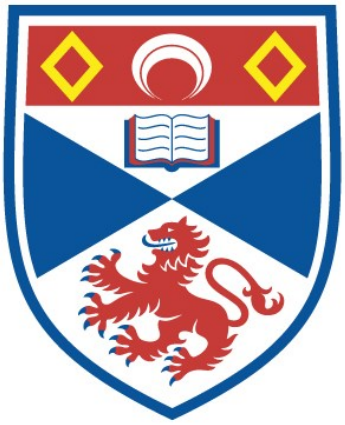
MECHANISTIC STUDIES ON MYO-INOSITOL MONOPHOSPHATASE

Andrew Graham Cole

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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MECHANISTIC STUDIES ON *myo*-INOSITOL MONOPHOSPHATASE

a thesis presented by

Andrew Graham Cole

to the

University of St. Andrews

submitted for the degree of

DOCTOR OF PHILOSOPHY

St. Andrews January 1994

DECLARATION

I, Andrew Graham Cole, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfiment of any other degree or professional qualification.

Signed ..... Date 18/2/94

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on 1/10/90 and as a candidate for the degree of Ph.D. on 1/10/90

Signed ..... Date 18/2/94.

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

Signature of supervisor .....

Date 21/2/94

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To all the people I have associated with in Southampton and St. Andrews, I wish them every success in their futures, and hope they achieve their goals in life.

Finally, but most importantly, I would like to thank my parents, my sisters and Jo for their support throughout my University career.

ABSTRACT

Enzymic phosphate monoester hydrolysis by inositol monophosphatase from bovine brain (EC 3.1.3.25) occurs via the direct displacement of phosphate by water rather than by a two step mechanism involving a phosphorylated enzyme intermediate. The catalytic process is believed to involve two Mg^{2+} ions, one of which is buried and acts as a Lewis acid and phosphate coordination site. The second metal ion appears to coordinate to the alkyl phosphate bridging oxygen, the 'catalytic' hydroxyl group (C-6OH of D-Ins 1-P) and to the nucleophile, water or hydroxide. Mechanistic differences have been identified between the hydrolysis of inositol phosphate and nucleoside 2'-monophosphate substrates in that although phosphate-oxygen ligand exchange with the solvent is facile in the presence of inositol, no such exchange occurs in the presence of adenosine. The minimum structural requirements of a substrate have been demonstrated via synthesis of ethane 1,2-diol monophosphate which shows enzyme activity (Vmax ca. 12% that of Vmax for Ins 1-P, Km = 0.7 mM and Ki = 1.0 mM). Elaboration of the free hydroxyl group to produce (S,R)-, (S)- and (R)-pentane 1,2,5-triol 2-phosphate gave inhibitors of contrasting potency ((S)- pentane 1,2,5-triol 2-phosphate Ki = 0.12 mM, (R)-pentane 1,2,5-triol 2-phosphate, Ki = 3.8 mM) as expected from the structural requirements for hydrolysis to occur. The proposed mechanism of adenosine 2'-monophosphate hydrolysis involving the N3-atom of the adenine moiety has been discounted through spectroscopic analysis of enzymic incubations of new nucleoside substrates (Uridine 2'-monophosphate; Vmax 230% that of Vmax for 2'-AMP, Km = 4.0 mM and 5,6-dihydrouridine 2'-monophosphate; Vmax 70% that of Vmax for 2'-AMP, Km = 1.4 mM), which showed no intermediate phosphates, with only the substrate and final enzymic product (uridine) detected. Ethane 1,2-diol phosphate has been further elaborated to produce 2-methoxyethanol phosphate, diethylene glycol phosphate, pentane 1,5-diol phosphate, diethylene glycol cyclic phosphate and pentane 1,5-diol cyclic phosphate (Ki values range between 3 & 8 mM). Inhibition is attributed to interaction of the substrate with the second Mg^{2+} ion, and displacement of the catalytic water (hydroxide) molecule. The fact that the cyclic phosphate diesters are not hydrolysed to phosphate monoesters by the enzyme demonstrates that the attacking nucleophile is not positioned on the first (buried) Mg^{2+} ion. The mechanistic difference of nucleoside 2'-monophosphate hydrolyses is attributeded to the ribofuranosyl oxygen acting as a surrogate for the catalytic hydroxyl group of inositol 1-phosphate. Modelling studies have shown that this results in 2'-AMP adopting an unfavourable conformation which is stabilised by the second (catalytic) Mg^{2+} ion. The absence of the phosphate moiety in adenosine prevents this conformation being achieved at the active site, accounting for the lack of inhibitory activity of adenosine, and the absence of phosphate-oxygen ligand exchange in the presence of adenosine. The proposed mechanism is consistent with all published kinetic data, and the substrate dependency of lithium inhibition.

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List of figures.

ABBREVIATIONS.

2'AMP	Adenosine 2'-monophosphate
5'AMP	Adenosine 5'-monophosphate
cAMP	cyclic Adenosine 2',3'-monophosphate
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
BSE	Bovine Spongiform encephalophathy
DABCO	1,4-Diazabicyclo[2,2,2]-octane
DEAE	Diethylaminoethyl
DG	Diacylglycerol
DMAP	4-dimethylaminopyridine
DMF	N'N-dimethylformamide
DMSO	Dimethyl sulfoxide
E. Coli	Escherichia coli
2'GMP	Guanosine 2'-monophosphate
Gly-1-P	Glycerol 1-phosphate
Gly-2-P	Glycerol 2-phosphate
Imp'ase	Inositol monophosphatase
InsOH	Inositol
K_i	Inhibition constant
K_m	Michaelis-Menten constant
m-CPBA	*m*-Chloroperbenzoic acid
NMR	Nuclear magnetic resonance
OD	Optical density
PCC	Phosphatidyl inositol lipase C
Pi	Inorganic phosphate
PLP	Pyridoxal 5'-phosphate
PPi	Inorganic pyrophosphate
PtdIns	Phosphatidylinositol
PtdIns x,y-P_n	Phosphatidylinositol x,y-polyphosphate
TBAB	Tetra-*n*-butyl ammonium bromide
TBAF	Tetra-*n*-butyl ammonium fluoride
TBDMS	*t*-Butyl dimethylsilyl

TLC	Thin layer chromatography
TMS	Tetramethylsilane
TRIS	Tris(hydroxymethyl)aminomethane
*p*TsCl	*p*-Toluene sulfonyl chloride
*p*TsOH	*p*-Toluene sulfonic acid
THF	Tetrahydrofuran
2'UMP	Uridine 2'-monophosphate
UV	Ultraviolet

DEDICATED TO MY
PARENTS AND MY SISTERS

CHAPTER ONE
INTRODUCTION

1. INTRODUCTION:

In brain cells, *myo*-inositol 1,4,5-trisphosphate, a hydrolysis product of phosphatidylinositol 4,5-bisphosphate, acts as a second messenger by stimulating calcium release from intracellular stores. The enzymic hydrolysis of phosphatidylinositol 4,5-bisphosphate (by phosphatidylinositol lipase C) is initiated on activation of an extracellular receptor by various agents such as hormones and neurotransmitters (first messengers). *myo*-Inositol second messenger metabolism occurs by stepwise enzymic dephosphorylations catalysed by specific phosphatase enzymes. The final dephosphorylation step involved in the metabolic pathway is mediated by the lithium sensitive enzyme *myo*-inositol monophosphatase. Since brain cells can not take up inositol, inhibition of *myo*-inositol monophosphatase by lithium depletes levels of free inositol, and prevents phosphoinositide biosynthesis. It is thought that this mechanism explains how lithium exerts its effect in the treatment of manic depression.

1.1 The inositols.

Inositol is a cyclohexanehexol, of which there are nine isomeric forms; *allo* -(**1**), (+)*chiro* -(**2**), (-)*chiro* -(**3**), *cis* -(**4**), *epi* -(**5**), *muco* -(**6**), *myo* -(**7**), *neo* -(**8**) and *schyllo* -(**9**).[1] (Figure 1.1.1) *myo*-Inositol (**7**), the only isomer of great importance with respect to receptor signalling, is an achiral molecule, possessing a plane of symmetry through C-2 and C-5[2] with chirality being introduced into the molecule as a result of unsymmetrical substitution (e.g. inositol 1-phosphate). It also has a single axial hydroxyl group at C2. The natural existence of its phosphoric esters has been known for many years, with the hexakisphosphate being a major constituent of plant seed (in the form of phytic acid), its function generally assigned the role of a phosphorus/ energy store.[3] The primary function of the inositol phosphates however (most importantly inositol 1,4,5-trisphosphate), is to act as intracellular messengers. The majority of human inositol intake is dietary (mainly from plants), however *myo*-inositol is biosynthesised in plants and mammals. The biosynthesis of inositol occurs *via* the *myo*-inositol 1-phosphate synthase catalysed isomerisation of D-glucose 6-phosphate (**10**) to L-*myo*-inositol 1-phosphate (**15**), and subsequent phosphate ester hydrolysis by *myo*-inositol

monophosphatase. *myo*-Inositol 1-phosphate synthase has been purified from bovine[4] and rat[5,6] testis, yeast[7,8] and a number of plant species[9,10] and the mechanism of isomerisation has been studied in depth.

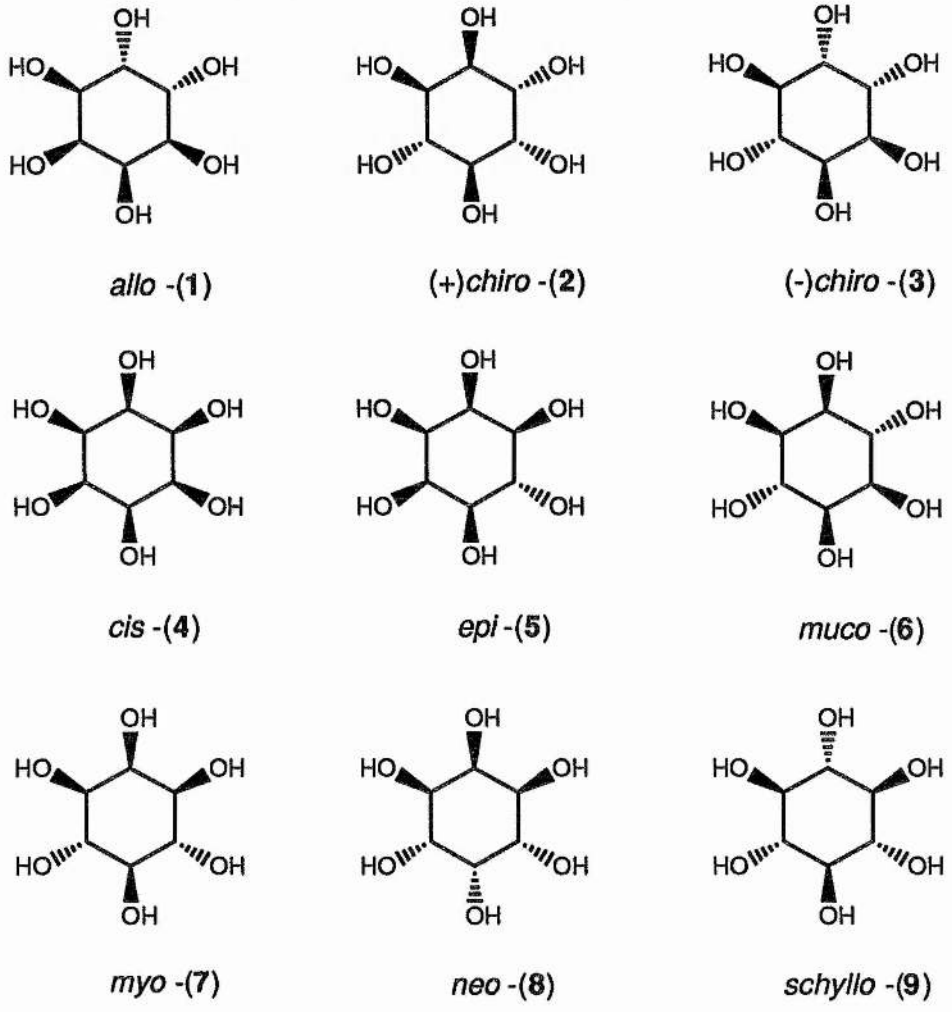
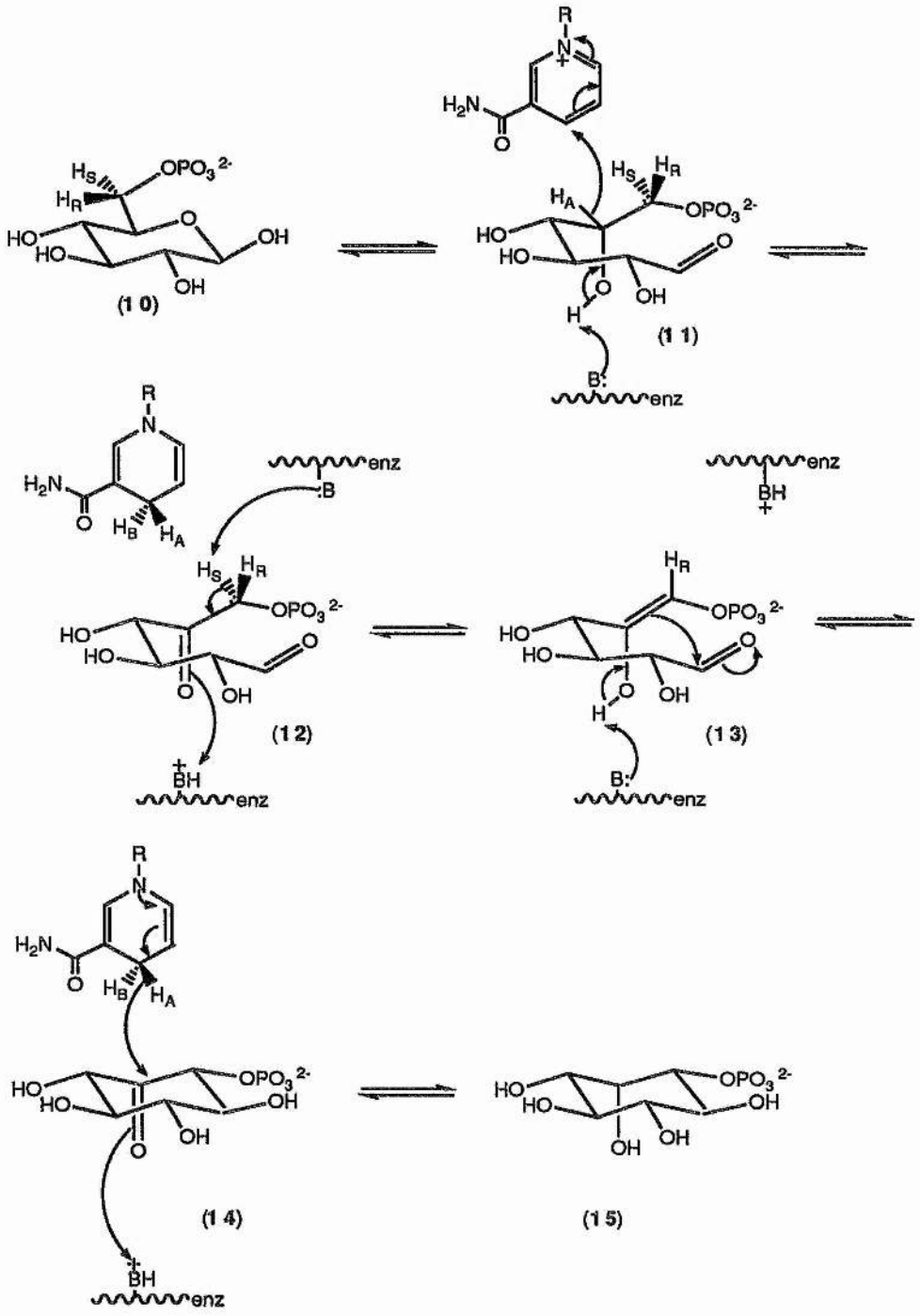


Figure 1.1.1. The stereochemistry of the inositols.

The enzyme is a complex one in that it combines the activities of an NAD^+ dependent oxidoreductase with an aldolase.[11] Initial opening of the hemiacetal ring (**10**) followed by NAD^+ mediated oxidation of the 5-hydroxyl group (via abstraction of the 5 hydrogen) gives the putative 5-keto-glucose 6-phosphate (**12**). Stereospecific deprotonation followed by stereospecific ring closure forms L-*myo*-inosose 2,1-phosphate (**14**) which is reduced (NADH) to L- *myo*-inositol 1-phosphate (**15**). Evidence for the formation of *myo*-inosose

2,1-phosphate (**14**) has been found in the reduction of a testis synthase catalysed reaction mixture with [^{3}H]-$NaBH_4$, which allows both *myo*- and *scyllo*- tritiated inositol 1-phosphate to be isolated. All of the intermediates in



Scheme 1.1.1. Mechanism of inositol 1-phosphate synthase.

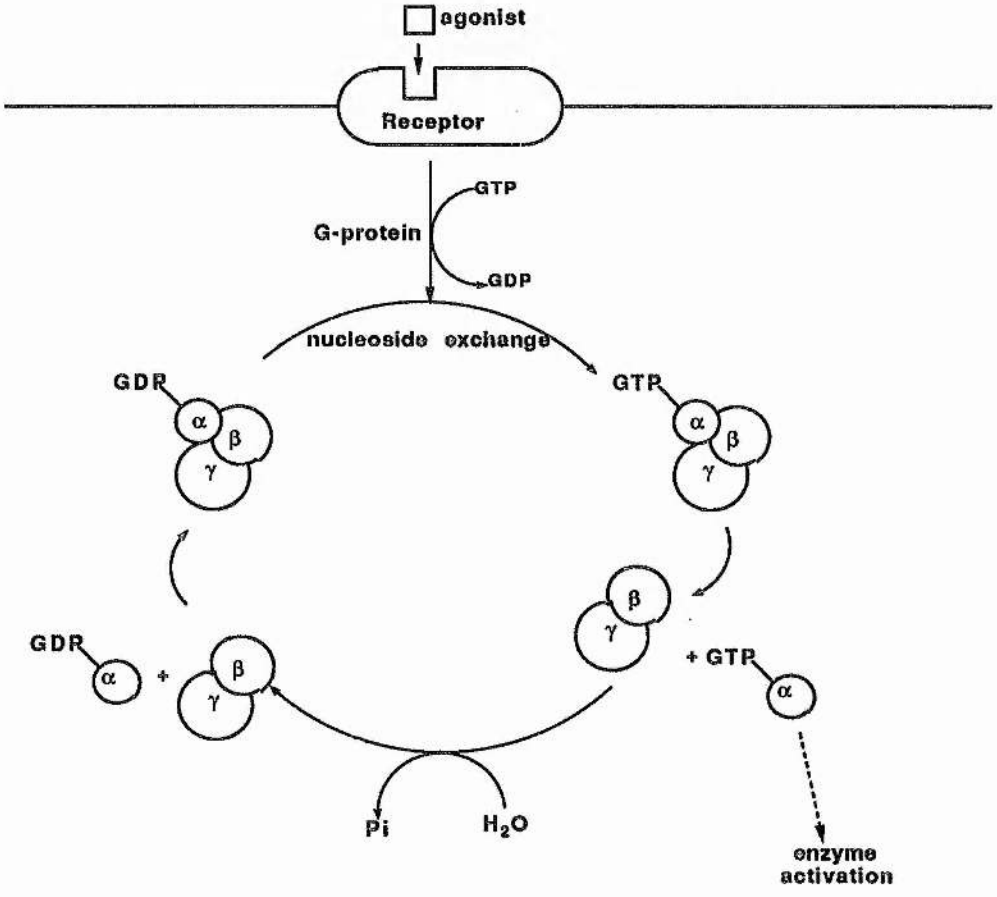
the catalytic pathway are reported to be tightly bound,[10] however the first three steps of the pathway were shown to be reversible, by demonstrating the incorporation of deuterium (from the $[^2H]$-H_2O incubation medium) into the 6-position of glucose 6-phosphate (**10**). No overall reversibility of the isomerisation has been observed,[4] suggesting that the cyclisation step is irreversible. The hydrolysis of inositol 1-phosphate by inositol monophosphatase will be discussed in more detail elsewhere. The mechanism of catalysis of inositol phosphate synthase is shown in scheme 1.1.1.

1.2 Intercellular signalling.

Cells respond when a variety of extracellular ligands (hormones, growth factors or neurotransmitters) interact with receptors on the outer surface of the plasma membrane. Such interactions result in a cascade of biochemical processes causing a change in the cell behaviour, for example, the secretion of a specific enzyme or in the case of muscle cells, physical contraction. The signalling mechanisms known to be coupled to the activation of plasma membrane receptors fall into three main categories.[16] The first class of receptors either contain, or are closely linked to an ion channel which spans the plasma membrane. Stimulation of these receptors leads to a change in the ability of the ion channel to allow ions for which it is specific to pass across the cell membrane, resulting in a change in the intracellular concentration of that ion. The second class of signalling mechanism involves a receptor that is actually a membrane spanning protein (a tyrosine kinase). The tyrosine kinase receptors have sites on the outer surface which recognise agonists, and active sites on the inner surface within the cell. The binding of an agonist to these enzymes results in the phosphorylation of tyrosine residues on target proteins within the cell. This type of signalling mechanism is used by many growth factors and hormones such as insulin. The third class of receptors are coupled to a class of glycoproteins called 'G-proteins', by which agonist binding causes a response within the cell.[16,17] Receptor activation causes the associated trimeric G-protein (consisting of an α-, a β- and a γ- subunit) to release guanosine 5'-diphosphate and bind guanosine 5'-triphosphate. The GTP-G-protein complex then dissociates into two separate units, an α-GTP unit and a β,γ- unit, and it is the α-GTP subunit which is involved in the activation of the

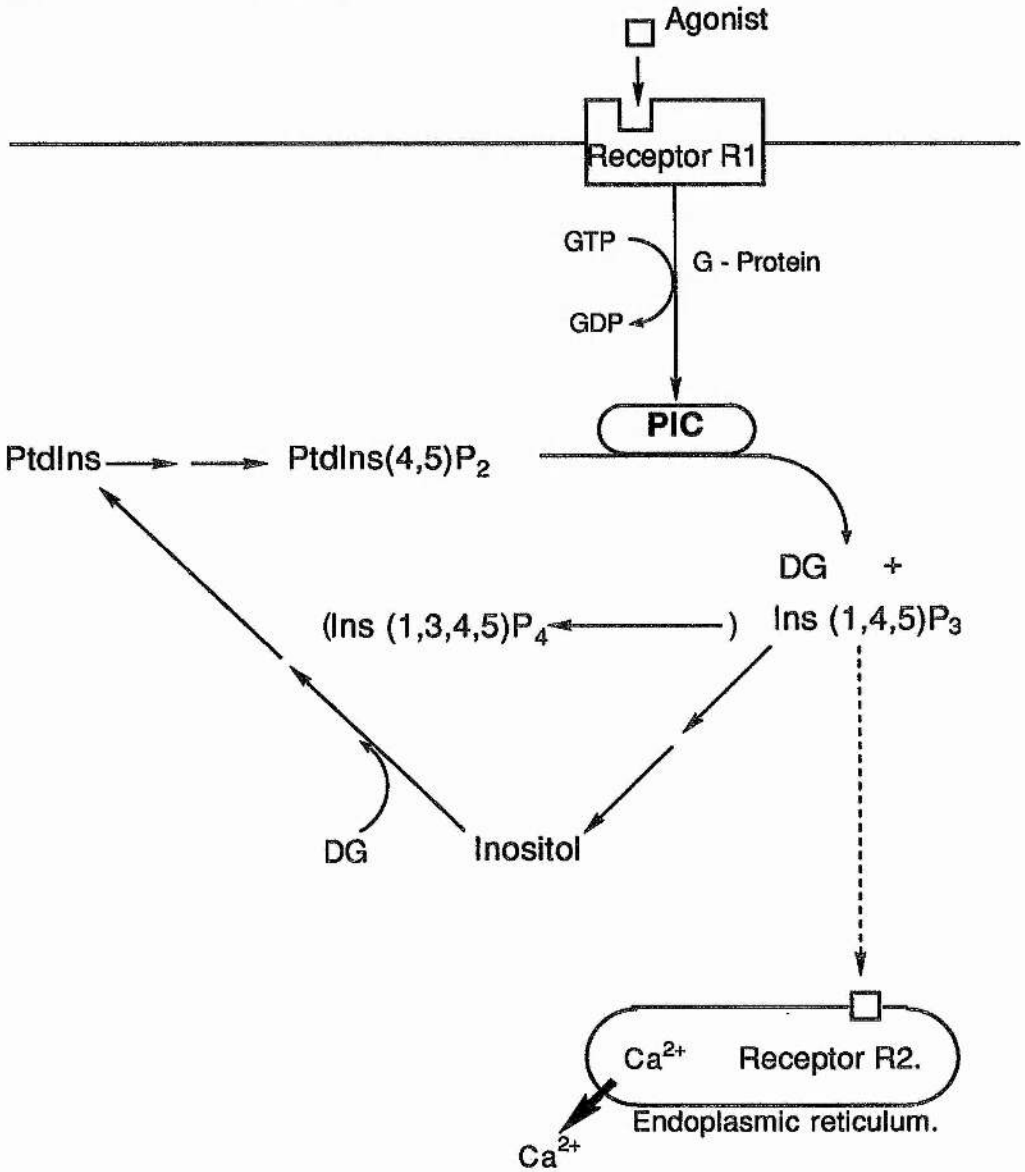
enzyme to which the receptor is coupled. The bound GTP is then hydrolysed by the GTP'ase activity of the α-subunit, and the resulting α-GDP complex recombines with the β,γ- unit, allowing the cycle to continue again on agonist binding (Scheme 1.2.1).

The two main examples of G-protein linked receptors are the activation of adenylate cyclase and the activation of phosphatidylinositol lipase C. In both cases, the products of enzyme activation are known as second messengers, ultimately leading to a response within the cell. In the first example, (yielding adenosine 3',5'-cyclic monophosphate from adenosine 5'-triphosphate[18,19]) cAMP acts by stimulating a specific protein kinase which in turn phosphorylates specific target proteins within the cell leading to overall response.[18,19] This system has been the subject of much research, and unlike the second example, has been very well understood for many years.



Scheme 1.2.1. G-protein signal transduction.

Hokin and Hokin's discovery in 1953 that acetylcholine and carbamoylcholine stimulated the turnover of phosphate into the total phospholipid of slices of pancreas[20] (shown by the ^{32}P-incorporation from [^{32}P]-inorganic phosphate into the phospholipid) was the first indication that phosphatidyl inositol was metabolised on stimulation of the muscarinic cholinergic receptors of cells.



PtdIns = Phosphatidyl inositol.
PIC = Phosphatidyl inositol lipase C.
DG = Diacylglycerol.

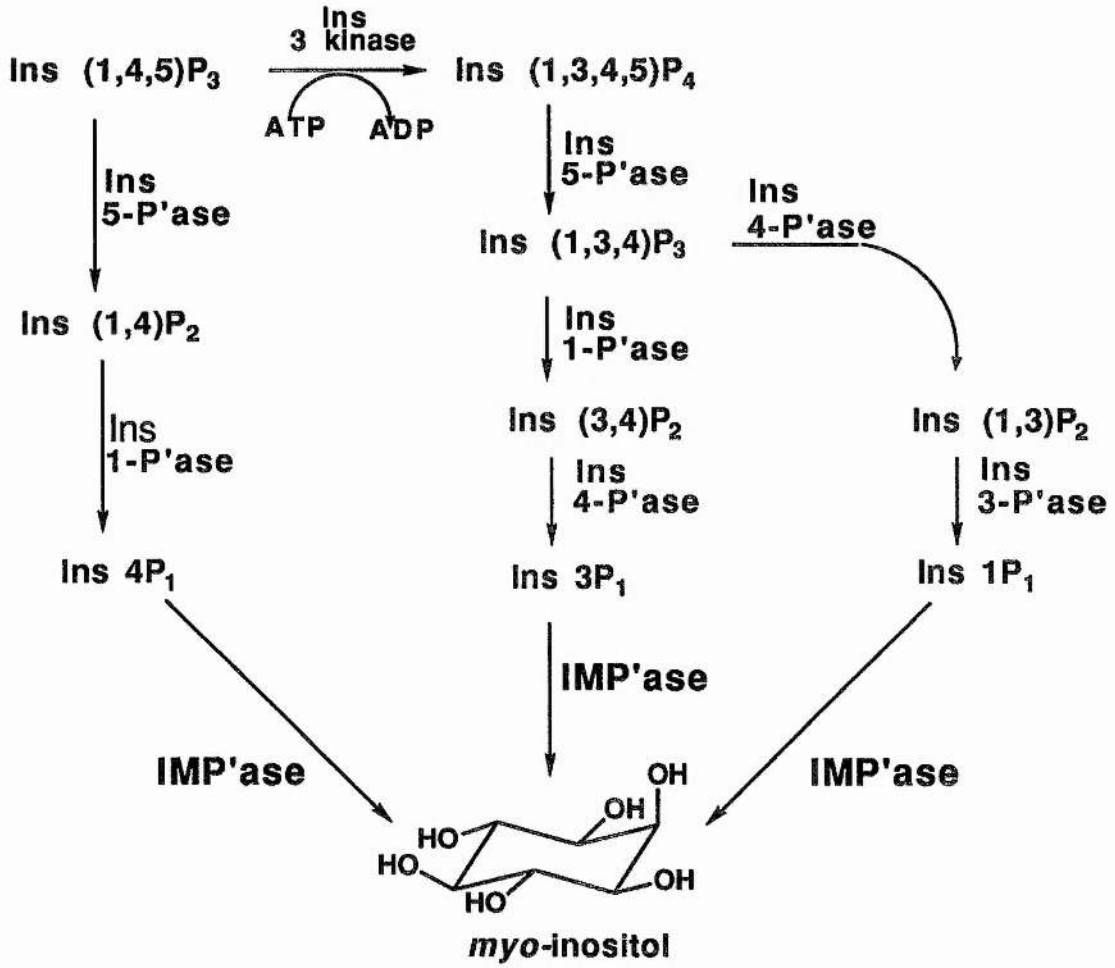
Scheme 1.2.2. The inositol cycle.

Since this discovery, it has become apparent that a large number of agonists can stimulate an increase in the metabolism of membrane phosphoinositides, for example, noradrenaline,[20] histamine,[21] vasopressin[22] and angiotensin.[23] Although the change in phosphoinositide metabolism is always specific for one particular receptor class such as the α-adrenergic receptor, the H_1-histaminergic receptor or the V_1-vasopressin receptor.[25] In 1975, Michell noted that receptors which stimulated phosphoinositide turnover also activated calcium dependent processes within the cell.[26] It is now known that the G-protein controlled hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate releases the two second messengers inositol 1,4,5-trisphosphate and diacyl glycerol.[27] Inositol 1,4,5-trisphosphate diffuses into the cytosol and releases calcium from an intracellular storage site *via* a second receptor on the endoplasmic reticulum.[25,28] Diacyl glycerol activates a calcium and phospholipid requiring kinase enzyme (protein kinase C)[29,30] which is responsible for the phosphorylation of certain regulatory proteins. It can also be hydrolysed by diacyl glycerol lipase[31] resulting in the release arachidonic acid[32,33] which is involved in the biosynthesis of prostaglandins. The formation of these second messengers is summarised in scheme 1.2.2.

1.3 The inositol cycle and its inhibition by lithium.

Chemical messengers acting within a cell must not only be synthesised rapidly, but also metabolised rapidly to ensure fast cellular response and fast return to the cell's prestimulated state.[34] The pathway involved in the synthesis and metabolism of diacyl glycerol and the inositol phosphate second messengers is known as the inositol cycle, from which the majority of enzymes have been isolated and characterised. Diacyl glycerol can be phosphorylated by diacyl glycerol kinase to form phosphatidic acid which in turn is converted into CMP-phosphatidate, and recycled into the phospholipid *via* recombination with inositol[35]. The breakdown of inositol 1,4,5-trisphosphate occurs through the sequential loss of phosphate groups by the action of certain phosphatase enzymes, initially by inositol 5-phosphatase to form the inactive inositol 1,4-bisphosphate.[36,37] The fact that inositol 1,4-bisphosphate is inactive suggests that this enzyme is responsible for the termination of the calcium mobilising

signal. The bisphosphate is further dephosphorylated by inositol 1-phosphatase and inositol monophosphatase to form free inositol, which reacts with the diacyl glycerol derivative CMP-phosphatidate. An additional metabolic pathway involves phosphorylation of the inositol 1,4,5-trisphosphate second messenger, to form inositol 1,3,4,5-tetrakisphosphate. The tetrakisphosphate is again sequentially dephosphorylated ultimately leading to *myo*-inositol (Scheme 1.3.1).



IMP'ase = Inositol monophosphatase

Scheme 1.3.1. The fate of the second messenger Ins 1,4,5-P_3.

Irvine *et al* first noticed that two different isomers of inositol trisphosphate and also inositol 1,3,4,5-tetrakisphosphate were present in stimulated rat

cortical slices.[38] The further phosphorylation step is known to be catalysed by the specific 3-kinase enzyme.[39] Although not fully understood, inositol 1,3,4,5-tetrakisphosphate has been shown to possess second messenger activity, but is active only in the presence of inositol 1,4,5-trisphosphate. This tetrakisphosphate is thought to be responsible for the activation of calcium mobilisation between different intracellular storage sites and also for the entry of calcium ions into the cell.[40] Inositol 1,3,4,5- tetrakisphosphate is also believed to be hydrolysed by the same enzyme (Ins(1,4,5)P_3-5-phosphatase) that causes Ins 1,4,5-P_3 deactivation through specific dephosphorylation.[41] Other higher phosphates that have been identified include inositol 1,3,4,6-tetrakisphosphate[42] and inositol 1,3,4,5,6-pentakisphosphate, but their functions in mammals remain unclear.

One important feature of the inositol cycle is that the enzyme inositol monophosphatase is the only enzyme capable of regenerating inositol. Inhibition of inositol monophosphatase within the cell should therefore result in the partial or complete failure of the inositol cycle. Lithium is one such agent which blocks inositol formation through inhibition the monophophatase (in an uncompetitive manner at low lithium concentration).[43] The inhibitory action of lithium was first discovered in 1971 when Allison *et al.* noted that there was a considerable reduction in the concentration of inositol in the brains of lithium treated rats[44] (this attributed to inositol monophosphatase inhibition). It has since been shown that the reduction in the level of inositol in the cytosol is accompanied by decreased levels of the inositol phosphate second messengers.[45] An increase in the levels of CMP-phosphatidate,[46] the species requiring inositol to reform phosphatidylinositol 4,5-bisphosphate has also been observed. The mode of action of lithium as an inhibitor has been of considerable academic and industrial interest over the last twenty years as lithium is widely used (usually as its citrate or carbonate salt) in the treatment of manic depression, a disease affecting approximately 1% of the world population. Although lithium exhibits properties as a therapeutic agent, a greater understanding of the mechanism of inhibition is essential to allow the design of new drugs, as a number of problems are associated with lithium therapy. To achieve the desired therapeutic effect, a plasma concentration of between 0.5 mM and 1.2 mM lithium is required, however serious side effects

occur at plasma concentrations of, or greater than, 2 mM. A plasma concentration of 3 mM can be fatal so strict monitoring of plasma lithium levels is necessary on initiation of lithium therapy. Also prolonged lithium therapy (periods in the region of 2-3 years) result in histological changes in the kidneys which may impair renal function.

The concentrations of inositol cycle components in the brain together with the uncompetitive nature of lithium on inositol monophosphatase and the fact that inositol is unable to cross the blood brain barrier suggest that the inositol cycle and the accompanied signalling processes are the therapeutic target for lithium. Note that lithium has little effect on cells in the periphery which has relatively free access to plasma inositol.

1.4 Inositol monophosphatase; Physiology and Biochemistry.

Inositol monophosphatase and the inositol cycle have been studied in a variety of tissue types from various sources but the most concentrated research has been conducted with bovine[50,51] and rat brain enzymes.[52] The enzyme has been purified from its natural sources and the gene cloned into *Escherichia coli*.[52] Inositol monophosphatase has been shown to be a homodimer of molecular weight 58,000 Daltons with the optimum pH for substrate hydrolysis being 7.8. It also requires magnesium cations for activity, but other metal ions are known to bind to the enzyme. These metal ions include calcium, manganese, cobalt, copper and zinc.[50,52] Zinc has been shown to support activity (around 20% that of magnesium) and cobalt also acts as a poor surrogate for magnesium (3.5 mM cobalt supports around 10% of the activity attainable with 3 mM magnesium with (+/-) Ins 1-P as the substrate).[50] Other metal ions gave at best 5% activity with (+/-) Ins 1-P as the substrate. A variety of natural substrates exist for the enzyme. In addition to both enantiomers of inositol 1- and inositol 4-phosphate, the enzyme is also capable of hydrolysing 2'-AMP, 2'-GMP, α-glycerophosphate and β-glycerophosphate.

Fluoride anions have been shown to inhibit the enzyme[50] in a manner which appears to be substrate dependent (competitive with respect to glycerol 2-phosphate, noncompetitive with respect to (+/-) Ins 1-P, and uncompetitive with respect to magnesium). It has been suggested that this substrate dependency arises because the enzyme bound fluoride can prevent glycerol 2-

phosphate from binding to the enzyme, but not inositol 1-phosphate due to it forming more specific interactions at the active site.[53]

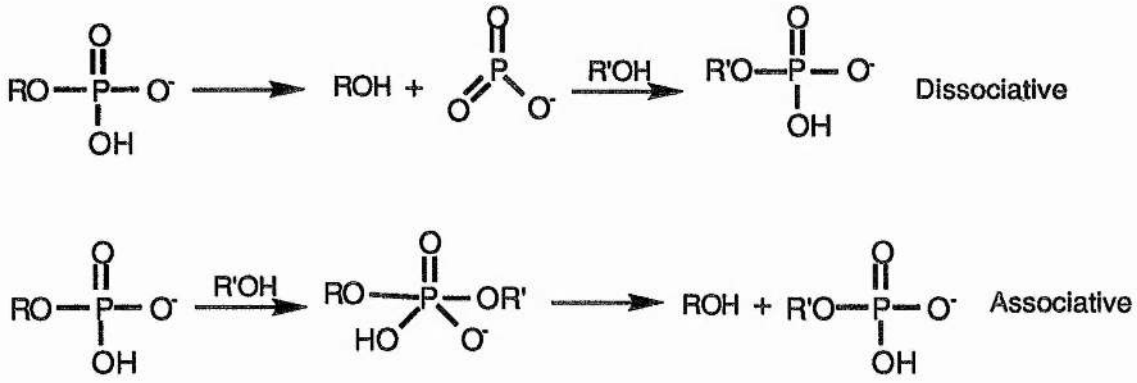
The order of product release has been determined by examination of the product inhibition properties of the components associated with hydrolysis. Inorganic phosphate is a competitive inhibitor for (+/-) Ins 1-P (K_i 0.5 mM at pH 7.8) and is the last product to debind. The K_i for phosphate inhibition decreases by a factor of approximately 25 from 8.0 mM at pH 6.5 to 0.3 mM (at pH 8.0), showing that the phosphate dianion binds more tightly to the enzyme at high pH. Inositol displays non-competitive inhibitor activity at high inositol concentrations ($K_{i\ apparent}$; 250 mM at pH 6.5, and 400 mM at pH 8.0 [49]) for the hydrolysis of (+/-) Ins 1-P, and is the first product to debind.

In the presence of saturating lithium, there is an initial rapid release of inositol followed by no further reaction occurring.[54] From these results, it was proposed that inositol monophosphatase catalysed phosphate ester hydrolysis by a ping pong mechanism (see section 1.6) and that inhibition by lithium occurred after hydrolysis.[54] Much research has since been focussed on the mode of lithium inhibition of inositol monophosphatase, including a study on the different isotopes of lithium[48] and their effects on the enzyme. As a result, it has been shown that phosphate increases the extent of lithium inhibition, a result of consequence *in vivo*.[55] Phosphate release from the enzyme after hydrolysis of a substrate is believed to be rate limiting for inositol monophosphates, and lithium has also been proposed to bind selectively to the site vacated by magnesium in binding to a non-covalent E.Pi complex.[49] This proposed mechanism is in accord with the initial rapid release of inositol observed in early studies of the hydrolysis mechanism.

A chemical modifier for arginine residues (phenyl glyoxal) has been shown to inactivate inositol monophosphatase.[56] The modified arginine residue has not been isolated. However, it has been shown that one arginine residue is modified in each subunit. It was thought unlikely that this residue is involved in the mechanism of hydrolysis as phosphoarginine residues have only previously been isolated from phosphoproteins, e.g. rat myelin basic protein,[57] where their role is not associated with any catalytic function. It is more likely that this residue has a substrate binding role.

1.5 Enzyme catalysed phosphoryl transfer.

Phosphoryl transfer reactions in biological systems are crucially important processes linked to energy transduction, metabolic regulation and a wide variety of signal transduction pathways.[58,59] Simple non-enzymic phosphoryl transfer reactions can occur by two main types of mechanism, associative ($S_N2(P)$) and dissociative ($S_N1(P)$).[60] (Scheme 1.5.1)



Scheme 1.5.1. Dissociative and associative mechanisms of phosphoryl group transfer.

Evidence suggests that both dissociative (*via* the metaphosphate intermediate)[61] and in-line associative mechanisms[62] can occur in aqueous non-enzymic hydrolyses of phosphate monoesters, but enzyme catalysed phosphoryl transfer reactions are believed to proceed *via* the in-line associative mechanism.[63]

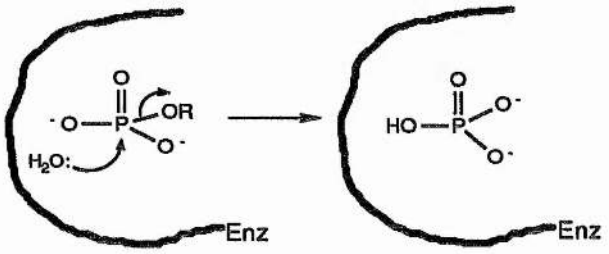
There are a number of different types of enzymic phosphoryl transfer reactions which depend on the level of substitution at the phosphorus centre and the nature of the enzyme in question. Many of these enzymes show an absolute requirement for a divalent metal ion (usually magnesium or zinc) to show catalytic activity. Metal ion coordination to a phosphate ester can neutralise negative charge on the phosphate group making it more susceptible to nucleophilic attack, stabilise the leaving group (RO^-), and with additional coordination to the acceptor molecule, orientate the reacting species in such a way as to make the reaction effectively intramolecular. The metal ion can also be involved in a more general role, in the stabilisation of the enzyme structure.

Enzymic phosphoryl transfer *via* in-line displacements is known to occur in one of three ways; a) a single step displacement by water, b) by a double displacement utilising an enzyme bound nucleophile forming a phosphorylated enzyme intermediate and c) by a double displacement utilising an adjacent hydroxyl group forming a cyclic diester intermediate (Scheme 1.5.2).

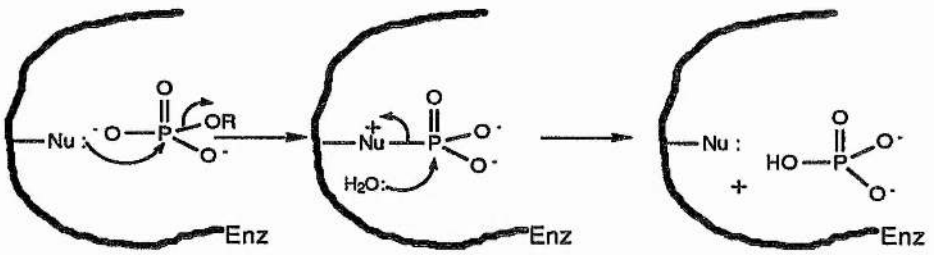
Three main categories of enzyme exist for the turnover of phosphate monoester substrates: phosphatase enzymes, where the phosphoryl donor is a phosphate monoester and the acceptor molecule is water; kinase enzymes, where the phosphoryl donor is a nucleoside triphosphate and the acceptor is some agent other than water; and the mutase enzymes where the phosphoryl acceptor is another functional group on the donor molecule itself. Other classes of phosphoryl transfer enzymes include those which process phosphate diesters (e.g. nucleases, phospholipases and phosphodiesterases) and those other than phosphokinases which utilise nucleoside triphosphates as substrates (e.g. ATP'ases and nucleotidyl cyclases) . Mechanistically, enzymes involved in processing phosphate monoesters fall into categories a) and b) (Scheme 1.5.2). The formation of cyclic phosphate diester intermediates (Scheme 1.5.2, c)) is a phenomenon associated with phosphate diester hydrolysis of nucleotides for example by ribonuclease, which catalyses the hydrolysis of RNA in the 3'->5' direction, giving the 3' monophosphate of the nucleotide.[64]

In the case of phosphatase enzymes, the double displacement mechanism (ping pong, scheme 1.5.2 b)) is by far the most common. The phosphatase enzyme that has received the most attention over the past forty years with regard to both characterisation and mechanistic determination is that of alkaline phosphatase from *E. coli*,[65,66] which involves the obligatory formation of a phosphoenzyme intermediate. This phosphatase is non-specific, suggesting its role may be to supply phosphate from phosphate esters under conditions of phosphate deprivation,[67] however other functions such as phosphate transport have also been suggested for this enzyme.[68] It is known that a maximum rate of synthesis of the enzyme occurs when phosphate concentrations within a cell become low enough to limit cell growth.[69] The evidence for the bi bi ping pong mechanism in alkaline phosphatase is unquestionable.[65] The enzyme has been shown to catalyse trans-

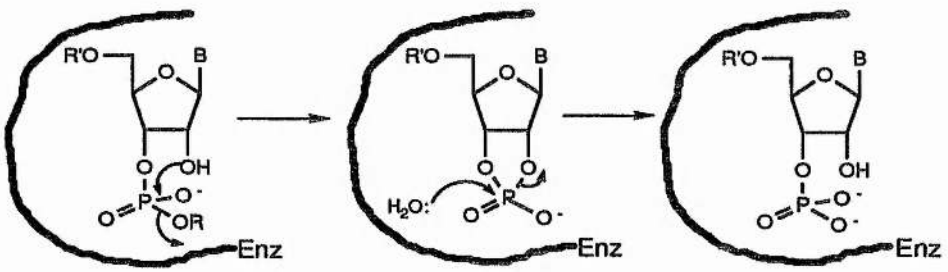
phosphorylation between alcohols (the alcohol being an alternative phosphoryl group acceptor to water, which is able to react with the phosphorylated enzyme intermediate), and catalyses ^{18}O-exchange between water and inorganic phosphate in the absence of any other acceptor molecule (where E-P forms as



a) Single step displacement mechanism.



b) Double displacement mechanism via enzyme bound nucleophile.



c) Double displacement mechanism via cyclic phosphate diester.

Scheme 1.5.2. The mechanisms of enzymic phosphoryl transfer reactions.

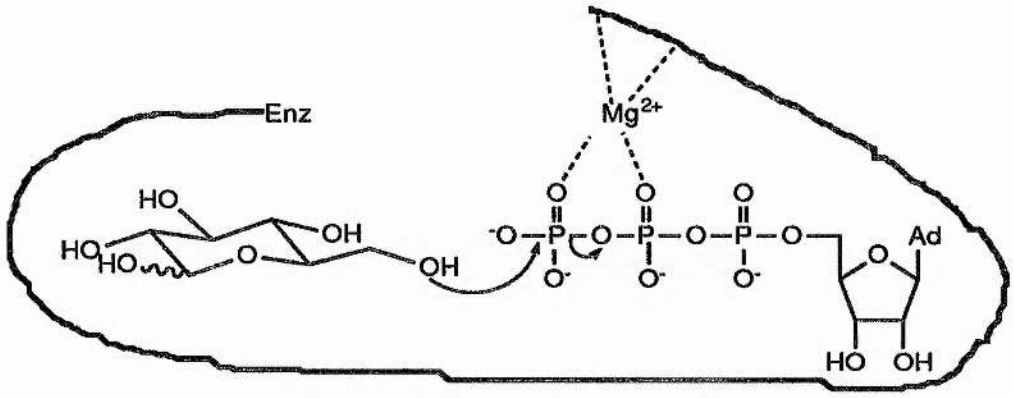
a result of the reverse hydrolysis reaction and is subsequently hydrolysed by [^{18}O]-water, see section 1.7). The phosphoenzyme intermediate has also been isolated from incubations with enzyme and inorganic phosphate and with

enzyme and substrate at low pH, and the initial transfer of the phosphoryl group has been shown to be to a serine residue at the active site.[70] Stereochemical analysis at the phosphorus centre (see section 1.7) shows overall retention of configuration, again indicating a two step mechanism. Acid phosphatase (another non-specific phosphatase) and glucose 6-phosphatase have also been shown to operate by this type of mechanism. Here, the phosphorylated enzyme intermediates are phosphorylated on histidine residues in each case.[71,72] Both alkaline and acid phosphatase show similar V_{max} values regardless of the substrate which indicates that the breakdown of the E-P complex is rate limiting. The alternative single step displacement mechanism is extremely rare for phosphatase enzymes, however examples have been reported. Subsequent to our own proposals, Knowles *et al.* have very recently shown that purple acid phosphatase (a diiron phosphatase) catalyses a direct phosphoryl group transfer to water. This conclusion was based on the analysis of the stereochemical course of the reaction with respect to the phosphorus centre.[73]

Evidence suggesting the involvement of a phosphorylated intermediate is not always as clear cut as in the case for alkaline phosphatase, and experimental data has been misinterpreted in some cases. The phosphokinase enzyme hexokinase, which is the initial enzyme involved in the sequence of reactions converting glucose into pyruvate with the concomitant production of ATP (glycolysis) operates by a random addition of substrates (glucose and ATP) to the enzyme with single step transfer of the phosphoryl group occurring after the formation of a ternary complex (Scheme 1.5.3).[74] Initial experimentation did however suggest the formation of a phosphorylated enzyme intermediate, with slow isotopic exchange being observed between ATP and ADP in the absence of glucose.[75] Such exchange has since been shown to occur due to the presence of cosubstrates and cosubstrate analogues such as water (the exchange reaction occurs at a rate about 10^4 times slower than the normal reaction).

In the third class of enzymes which involves catalysis of a phosphoryl transfer within the same molecule (the phosphomutases), the process generally involves phosphorylated enzyme intermediates. In the case of phosphoglucomutase, the enzyme responsible for the conversion of glucose 1-

phosphate to glucose 6-phosphate, an active site serine residue has been identified as the enzyme bound nucleophile.[76]



Scheme 1.5.3. Mechanism of hexokinase.

1.6 Stereochemical course of phosphoryl transfer reactions at the phosphorus centre.

Enzyme catalysed phosphoryl transfer reactions tend to be complex and kinetic data can often be misinterpreted. With regard to mechanistic determination of such reactions, much information can be obtained from studies involving the determination of the stereochemical course of the reaction at the phosphorus centre.[77] The stereochemical course of such a reaction can only be observed if the reaction involves a chiral starting material (with respect to phosphorus) leading to a chiral product (with respect to phosphorus), therefore, a substrate chiral at phosphorus must be produced. Sophisticated methods have been developed to analyse both substrates and products of such enzymic reactions.

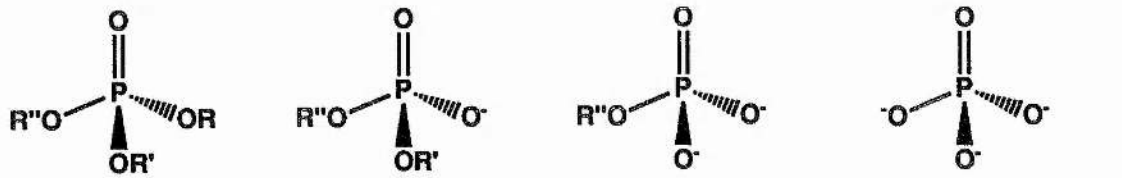


Figure 1.6.1. The stereochemical environment of phosphorus centres.

The different types of phosphoryl transfer reactions involving P-O bond cleavage and the stereochemical environment of both substrates and products are summarised in schemes 1.6.1 and 1.6.2. The production of chiral phosphate esters involves, in many cases, the incorporation of two or more of the stable isotopes of oxygen, ^{16}O-, ^{17}O- and ^{18}O-, and sulfur to produce phosphorothioate analogues, providing products of assessable stereochemical character. Many enzymes have been shown to accept phosphorothioate analogues as substrates,[78,79] often accompanied by significant reductions in rates of reaction, for example, inositol monophosphatase.[80] The reduction in rate

$ROPO_2OR'$ (prochiral) $\rightleftharpoons$ $ROPO_2OR''$ (prochiral) **(A)**

$ROPO_2OR'$ (prochiral) $\rightleftharpoons$ $ROPO_3$ (pro-prochiral) **(B)**

$ROPO_3$ (pro-prochiral) $\rightleftharpoons$ $R'OPO_3$ (pro-prochiral) **(C)**

$ROPO_3$ (pro-prochiral) $\rightleftharpoons$ PO_4 (pro-pro-prochiral) **(D)**

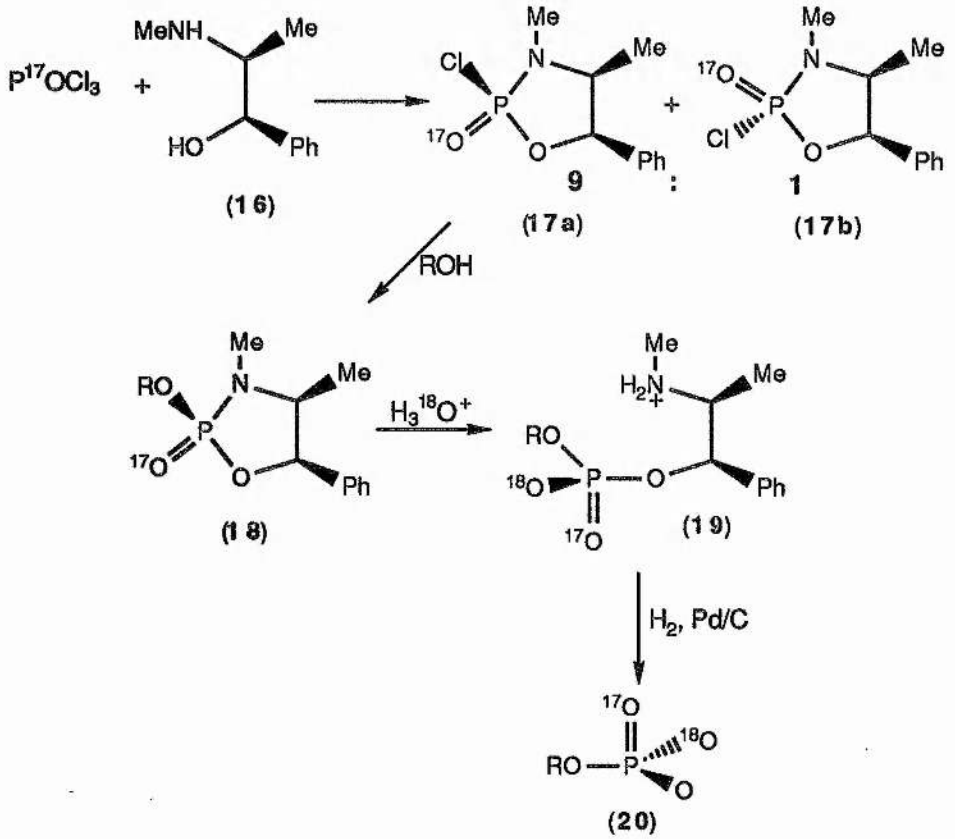
PO_4 (pro-pro-prochiral) $\rightleftharpoons$ PO_4 (pro-pro-prochiral) **(E)**

Scheme 1.6.2. Stereochemical environment of reactions at a phosphorus centre.

is probably due to the difference in size between sulfur and oxygen. The non-specific ping pong phosphatase enzyme alkaline phosphatase (which shows a wide range of substrate specificity for many phosphate monoesters with only minor changes in V_{max}) is not capable of hydrolysing phosphorothioates.[81]

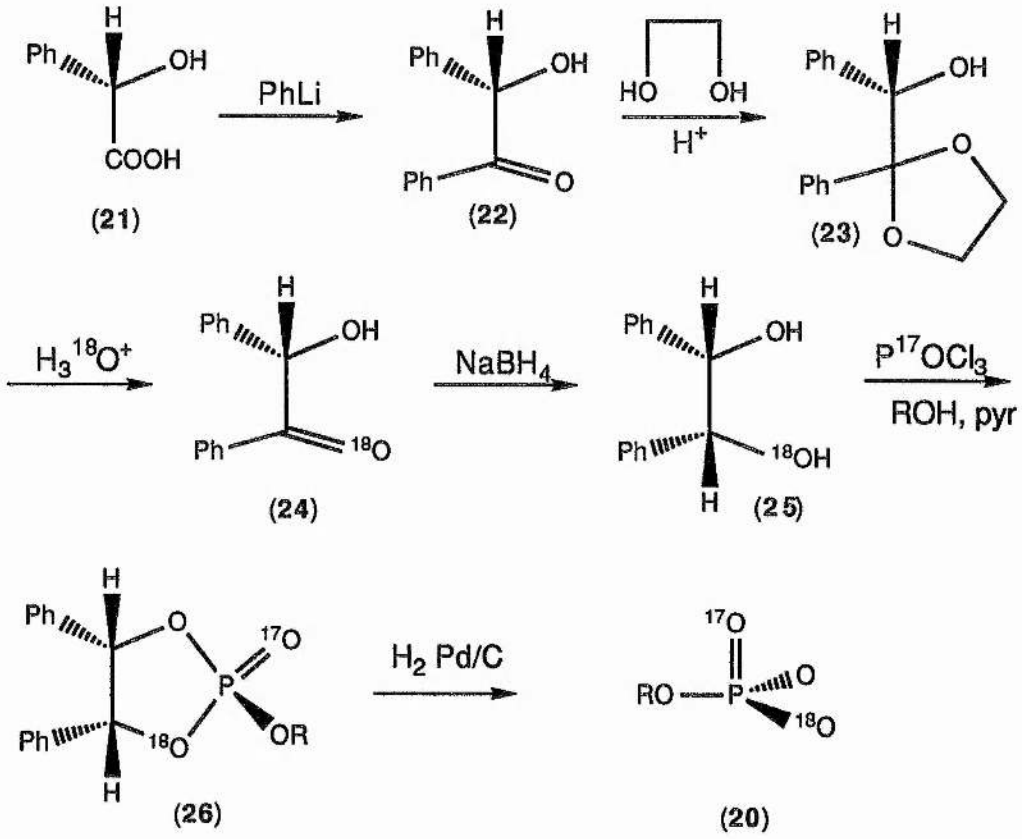
Since the size difference between isotopes of a particular atom is extremely small (even in the most extreme case of ^1H- and ^3H-),an isotopically chiral substrate will bind to the enzyme in an identical manner to the non-labelled substrate, and apart from possible rate changes arising from any isotope effects (which are insignificant between ^{16}O- and ^{18}O-), will react in exactly the same way. Enzymic reactions belonging to type C (Scheme 1.6.2) involving a pro-prochiral substrate leading to a pro-prochiral product as a result of phosphoryl group transfer to an acceptor molecule other than water (phosphokinase enzymes) have been studied in such stereochemical

investigations,[65] and a number of general methods for the production of chiral [$^{16}O^{17}O^{18}O$]-phosphate monoesters have been devised.[84-87] (Scheme 1.6.3 and 1.6.4.) The Lowe synthesis (Scheme 1.6.4) from (S)-mandelic acid (**31**) offers a route to both enantiomers of the chiral thiophosphate esters through control of the $POCl_3$ cyclisation.[87] The use of pyridine as a solvent gives solely the S_P enantiomer (**23**), as reversible ring opening of the phosphorochloridate intermediate leads to the production of the thermodynamic product. The use of two equivalents of pyridine leads solely to the R_P- enantiomer (the kinetic product). To date, all kinase enzymes have been shown to proceed with overall inversion of configuration as a result of direct transfer of the phosphoryl group to an acceptor molecule, and no involvement of a phosphorylated enzyme intermediate. Methods of synthesis and analysis of analogous chiral [$^{16}O^{17}O^{18}O$]-sulfate monoesters have also been developed for the study of both chemical and enzymic sulfuryl transfer reactions.[88-90]

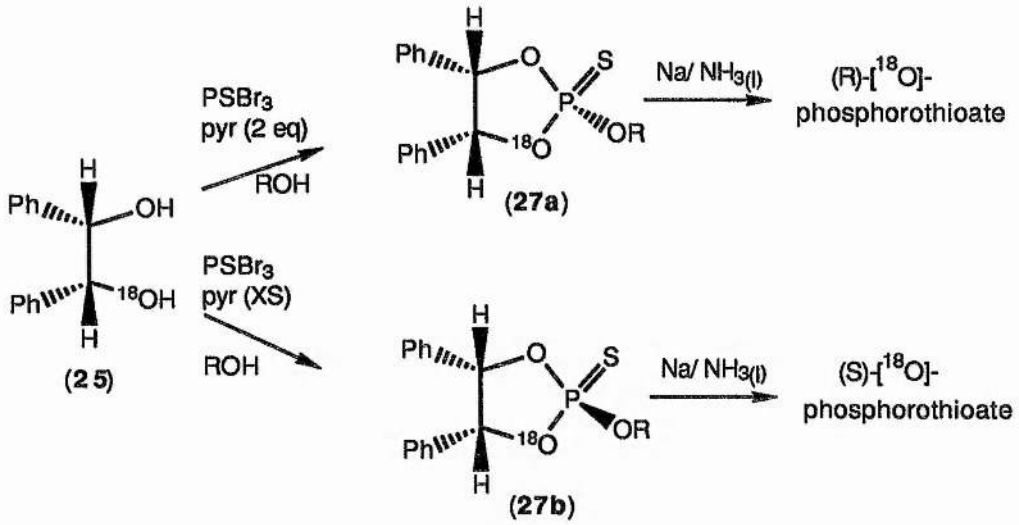


Scheme 1.6.3. Knowles synthesis of chiral [$^{16}O^{17}O^{18}O$]-phosphate monoesters.

Phosphatase enzymes belong to type D (Scheme 1.6.2), and involve a pro-prochiral phosphate monoester leading to a pro-pro-prochiral product (inorganic phosphate) , and in order to produce the product in a chirally labelled form, all three stable isotopes of oxygen and sulfur must be incorporated. The methods described for the production of [$^{16}O^{17}O^{18}O$]-phosphate monoesters can be manipulated to produce the analogous chiral [$^{16}O^{18}O$]-phosphorothioate monoesters[91] (Scheme 1.6.5). Incubation of the [$^{16}O^{18}O$]-phosphorothioate monoester with the enzyme under investigation in a ^{17}O-enriched aqueous medium, allows the production of chiral inorganic thiophosphate.



Scheme 1.6.4. Lowe synthesis of chiral [$^{16}O^{17}O^{18}O$]-phosphate monoesters.



Scheme 1.6.5 Synthesis of chiral [$^{16}O^{18}O$]-phosphorothioate monoesters.

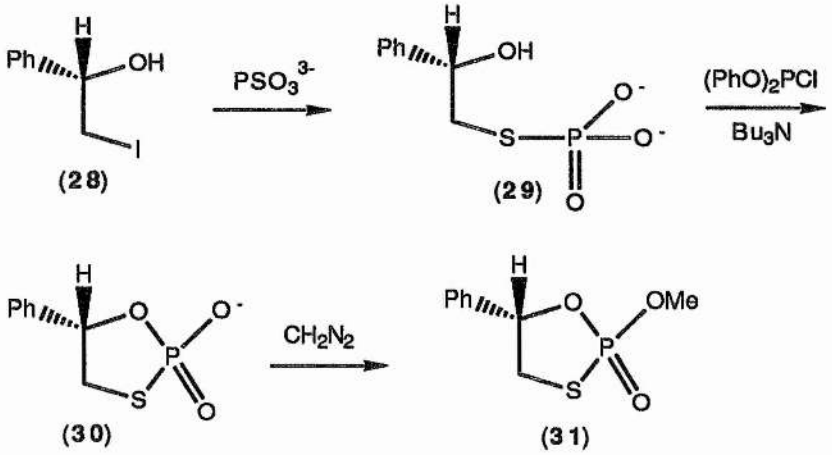
In 1980, Tsai reported the first stereochemical study developed specifically for a phosphatase enzyme[92] (alkaline phosphatase from *E.Coli* was the first phosphatase to receive such study, however since alkaline phosphatase also catalyses transesterification, its stereochemical course had been elucidated *via* a method developed for kinase enzymes[93]). In this study, it was shown that the hydrolysis of adenosine 5'-phosphorothioate to adenosine and inorganic thiophosphate catalysed by the snake venom 5'-nucleotidase (5'-ribonucleotide phosphohydrolase) occurred with overall inversion of configuration at phosphorus, indicating that the reaction did not involve the formation of a covalently bound phosphorylated intermediate. This result, although an unusual one for a phosphatase enzyme, agreed with previous experiments demonstrating the enzyme's inability to catalyse transphosphorylation[94] (type C, Scheme 1.6.2) or phosphate-oxygen isotope exchange[95] (type E, Scheme 1.6.2). The synthesis of the chiral adenosine 5'-phosphorothioate involved a combination of two chemical steps to achieve ^{18}O-label incorporation and two enzymic steps to give separately the two enantiomers.[92,96-98] Hydrolysis catalysed by venom 5'-nucleotidase in a ^{17}O-enriched aqueous medium gives the chiral product. Chiral analysis of the inorganic thiophosphate was achieved by a combination of enzymic and spectroscopic methods; firstly by coupling of the product to glyceraldehyde 3-

phosphate, followed by further kinase catalysed phosphoryl transfer reactions to ultimately give chiral adenosine 5'-[βS]-triphosphate which was analysed by ^{31}P-NMR spectroscopy. Two important properties of ^{17}O- and ^{18}O- are utilised in such analyses. Firstly, an ^{18}O- atom directly bonded to a phosphorus atom will result in a slight shift of the ^{31}P-NMR signal to higher field compared with that observed for a ^{16}O- atom directly bonded to phosphorus.[99] The magnitude of this shift is related to the fractional change in the mass of the isotope, the number of ^{18}O- atoms directly bonded to the phosphorus centre and also the bond order of the P-^{18}O bond. For example, adenosine 5'-[^{18}O]-monophosphate has a shift of 0.020 +/- 0.0003 ppm to higher field, and inorganic phosphate has a shift of 0.020 +/- 0.01 ppm to higher field for each ^{18}O- atom incorporated.[100] The shift associated with a doubly bonded P-^{18}O species is approximately twice that associated with a singly bonded P-^{18}O species. P-O-P Bridging species display smaller shifts as the effect is divided between two phosphorus atoms.[92] A ^{17}O- atom directly bonded to the phosphorus atom however, has a completely different effect. The effect of this isotope is to cause extensive line broadening of the ^{31}P-NMR signal due to quadrupolar relaxation.[101,102] The magnitude of this line broadening is so great that the signal is effectively reduced to a noise level, meaning that only species containing ^{16}O- and ^{18}O-label will be visible by NMR spectroscopy.

It has been shown that the chiral phosphate methyl ester produced by the Lowe synthesis (Scheme 1.6.4) described above exhibited circular dichroism, whereas the unlabelled methyl ester did not, giving a type of fingerprint for the chiral compound. Following this methodology, attempts have been made to obtain the circular dichroism spectrum of chiral inorganic thiophosphate to act as a possible method of enantiomeric analysis.[84] Although inorganic thiophosphate absorbs strongly at 220-230 nm, the circular dichroism spectrum was not observable in this region. It is very unlikely that normal methods for determining configuration could distinguish between chiral phosphate esters or chiral inorganic thiophosphate with any great accuracy, and alternative non-enzymic methods of analysis have been developed.

The most elegant method of chiral inorganic thiophosphate analysis was developed by Lowe *et al.* [103-105] and involves the coupling of the hydrolysis

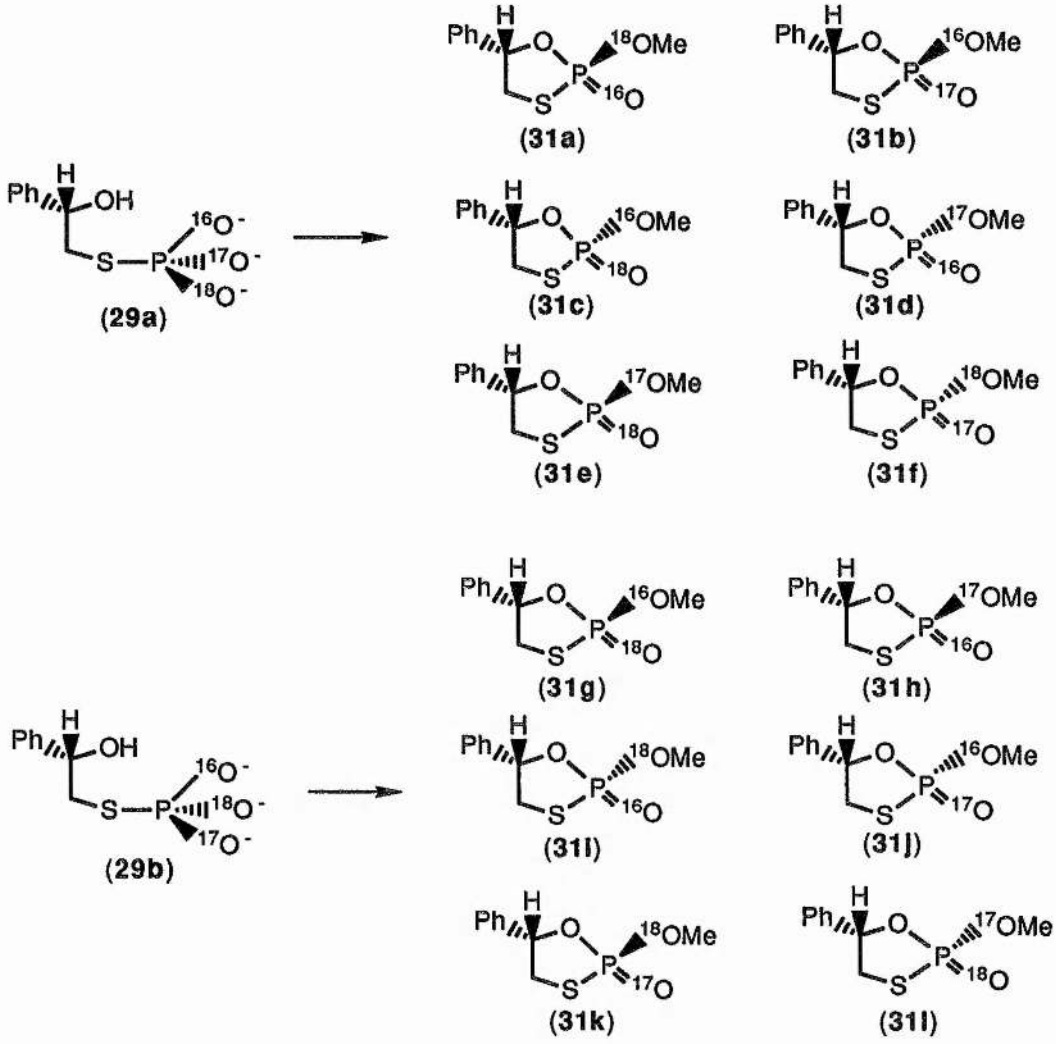
product (inorganic thiophosphate) to an enantiomerically pure derivative of (S)-mandelic acid (**21**) ((S)-2-iodo-1-phenylethanol (**28**))[103] (Scheme 1.6.6). Cyclisation of the coupled species followed by derivatisation as the methyl ester with diazomethane gives the species to be analysed by high resolution ^{31}P-NMR spectroscopy (Scheme 1.6.6). This method was successfully used to determine the stereochemical course of the activation of glutamate by glutamine synthase.[104]



Scheme 1.6.6 Chiral inorganic thiophosphate analysis species.

For the S_P- configuration of the alkylated thiophosphate (**29b**), there are six possible products formed on cyclisation (**31g-l**), three being *cis*- and three being *trans*-. For the R_P- configuration of the alkylated thiophosphate (**29a**), there are an alternative six products (**31a-e**), differing from the above in their isotopic distribution only. No two products from the R- and S- configurations are the same (Scheme 1.6.7.). Theoretically, only two of the aforementioned species will be detectable for each enantiomer of inorganic thiophosphate, and can be easily distinguished from each other by ^{31}P-NMR. The analytical technique becomes slightly more complicated by the fact that [^{17}O]-water is not commercially available in an isotopically pure form. Therefore some species initially thought to be ^{31}P NMR invisible will actually contain a ^{16}O-atom in the place of a ^{17}O-atom and will give rise to a signal. Calculations based on the isotopic abundance of the reagents used in the synthesis and the pattern expected for each enantiomer allows a theoretical value to be determined for the intensity of each peak. Comparison between theoretical and experimental

values have been shown to give excellent correlation, allowing the configuration of the thiophosphate to be determined.

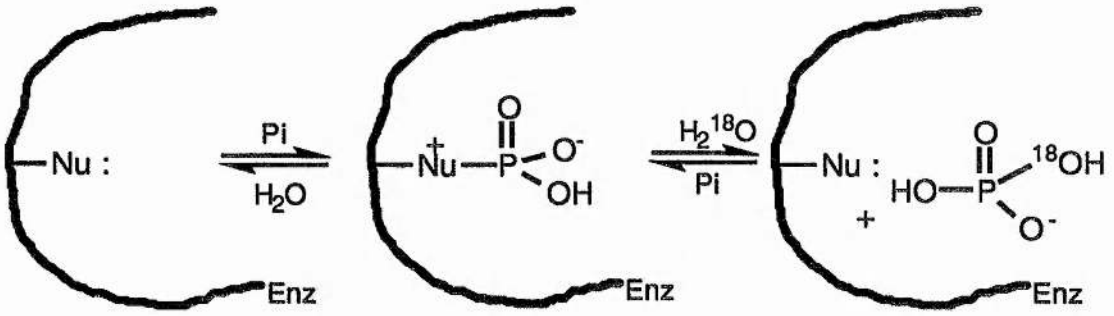


Scheme 1.6.7. Possible isotopic distribution products of the Lowe chiral inorganic thiophosphate analysis method.

1.7 Inositol monophosphatase catalysed phosphate-oxygen ligand exchange

Phosphatase enzymes like alkaline phosphatase and acid phosphatase, which operate *via* a substituted enzyme mechanism (ping pong mechanism) (see section 1.5) are able to catalyse the incorporation of ^{18}O-label from [^{18}O]-water into inorganic phosphate by means of the reverse hydrolysis

reaction[105,106] (Scheme 1.7.1). Isotope incorporation occurs in the absence of an acceptor molecule (other than water), and implicates the involvement of a phosphoenzyme intermediate, since ^{18}O-enrichment can occur as the result of phosphoenzyme hydrolysis.

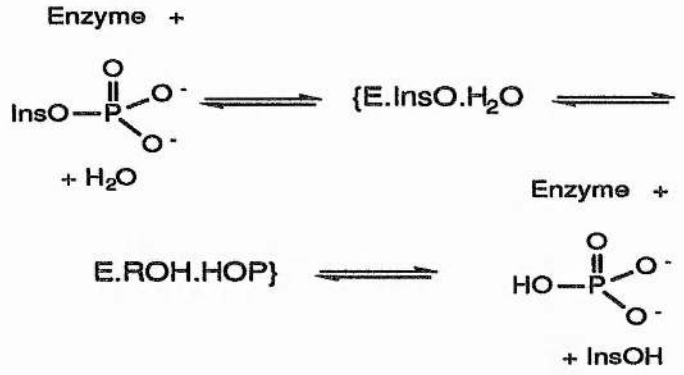


Scheme 1.7.1. Enzyme catalysed phosphate-oxygen ligand exchange.

Inositol monophosphatase is unable to catalyse such a reaction in the absence of inositol.[107] In the presence of inositol, however, an exchange reaction does occur, and shows similar pH and metal ion dependencies to the physiological phosphate ester hydrolysis. The exchange reaction is also inhibited by the lithium cation.[55,107] The observation of inositol dependent exchange (K_m 190 mM, V_{ex} (saturating phosphate) 60% that of V_{max} for (+/-) Ins 1-P hydrolysis) is inconsistent with a ping pong mechanism[54] as this would require inositol to bind to the enzyme before the water molecule derived from the enzyme phosphorylation (to form the proposed phosphorylated enzyme intermediate) is released. This would result in the formation of a ternary complex capable of binding both phosphoryl group acceptors simultaneously (Scheme 1.7.2).

Evidence suggests that the proposed ternary complex mechanism is of a non-substituted rather than a substituted nature, as attempts to detect a phosphorylated enzyme intermediate have failed.[55,107] Transesterification between 2'-AMP and [U^{14}C]-inositol and also between unlabelled inositol 1-phosphate and [U^{14}C]-inositol have proved unsuccessful. Also, attempts to trap the proposed E-P complex by incubation of 2'-AMP with enzyme and subsequent quenching with lithium chloride showed no modified protein.

Similar experiments using [^{32}P]-phosphate and (+/-) [^{35}S]-inositol 1-phosphorothioate resulted in no radiolabelled protein being detected.



Scheme 1.7.2. Ternary complex mechanism.

1.8 The structure of human inositol monophosphatase

Recently, the structure of human inositol monophosphatase has been solved to 2.1Å resolution, and has shown the enzyme to exist as a dimer of two identical subunits. Each subunit is folded into a five layered sandwich of three pairs of α-helices and two β-sheets. The structural determination has been determined with the lanthanide ion Gd^{3+} (a competitive inhibitor for magnesium) instead of the natural metal ion, and sulfate (a competitive inhibitor for the substrate) in place of the phosphate group.[108] If it is assumed that the position of the sulfate group in the crystal structure approximates that of the phosphate group of the substrate, and Gd^{3+} the position of magnesium, then it is evident that there are no amino acid residues at the active site with side chains that are correctly disposed to act as potential nucleophiles in a substituted enzyme mechanism.[109] Although the exact position of the phosphate group of the substrate is not known, the only amino acid side chain positioned to act as an internal nucleophile is the hydroxyl group of Thr-95. While it is possible that this residue is a catalytic nucleophile, it is still relatively distant with a separation of 4 Å between the hydroxyl oxygen of Thr-95 and the sulfur atom of the sulfate group in the crystal structure. The position of the threonine residue makes the proposed role of the hydroxyl group unlikely, and it is more likely that Thr-95 is involved in magnesium binding to facilitate polarisation of the phosphate group of the substrate (Thr-95 mutants

possessing alanine and serine residues have been shown to exhibit reductions in magnesium binding affinity). Examination of the amino acid residues with a single step displacement mechanism for the hydrolysis in mind reveals that the carboxylate group of Glu-70, at a distance of around 4.5 Å from the sulfur atom of the sulfate group, is positioned to possibly activate a water molecule. Although Glu-70 appears to be a metal ligand in the crystal structure, mutations replacing this residue with glutamine and aspartic acid result in 0.01% and 0.06% activity respectively compared with the wild type, with no change in K_m for the substrate or for magnesium.[109] Glu-70 could be less important in magnesium binding compared with that of Gd^{3+} (as in the crystal structure) due to the differences in coordination between the two metals. Figure 1.8.1 shows the positions of some of the active site amino acid residues relative to Gd^{3+} and sulfate in the crystal structure of inositol monophosphatase.

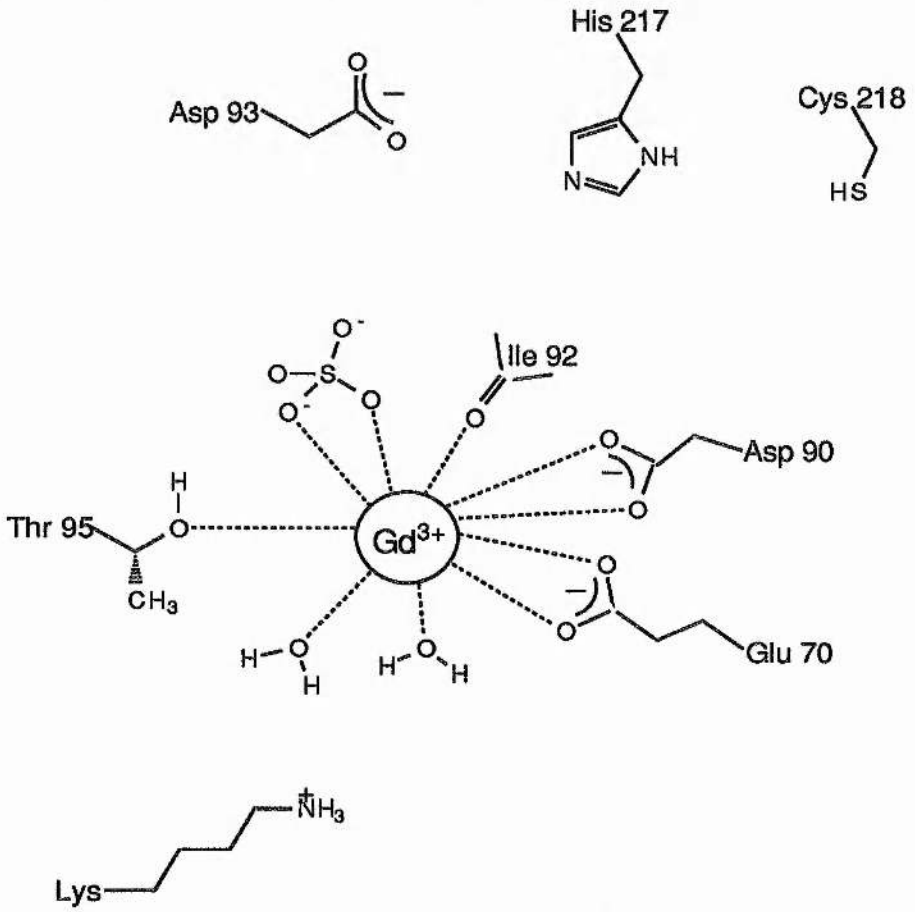


Figure 1.8.1 Active site amino acid residues of inositol monophosphatase (side chains within coordinating distance of the metal ion are indicated by dashed lines).

Chemical modifications of alternative amino acid residues at the active site have shown that the modification of Cys-218 (with N-ethyl maleimide)[110] and of His-217 (with diethyl pyrocarbonate)[111] also result in the loss of catalytic activity. The use of site directed mutagenesis to replace Cys-218 with an alanine residue proved that this residue is not essential for catalysis to occur, suggesting that the chemical modification inhibits the enzyme by steric effects.[110] Similarly, replacement of His-217 with a glutamine residue gives full activity in the mutant enzyme, again suggesting that the chemical modification is responsible for the loss of activity.[111]

1.9 Deoxyinositol 1-phosphate analogues.

Inositol monophosphatase shows a notable lack of substrate specificity in that it is able to catalyse the hydrolysis of both enantiomers of inositol 1- and inositol 4-phosphates as well as other polyol phosphates possessing a hydroxyl group α- to the phosphate group.[112] Inositol 1-phosphonate[114] (**32**) shows no substrate activity or inhibitor activity, indicating that the alkyl phosphate bridging oxygen is essential for substrate binding.

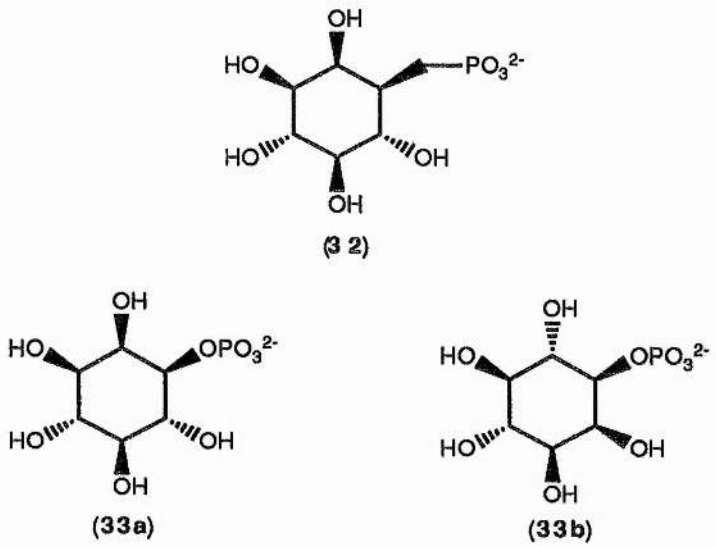


Figure 1.9.1. Inositol 1-phosphate and phosphonate.

By use of deoxy analogues of inositol 1-phosphate, the importance of the five free hydroxyl groups has been determined.[112] (+)-2-Deoxyinositol 1-

phosphate[115] (**34a**) has been shown to be a substrate for the enzyme (K_m 1.3 mM, V_{max} 78% that of (+/-) Ins 1-P), whereas (-)-2-deoxyinositol 1-phosphate (**34b**) is a good competitive inhibitor (K_i 50 μM). These results, accompanied by the fact that (+/-)-6-deoxyinositol 1-phosphate acts as a competitive inhibitor (K_i ~70 μM) and gives no detectable release of inorganic phosphate on enzyme incubation suggests that the α-hydroxyl groups have discrete and different roles in inositol monophosphatase activity towards substrates. Substrate recognition by inositol monophosphatase requires a binding interaction from one of the α-

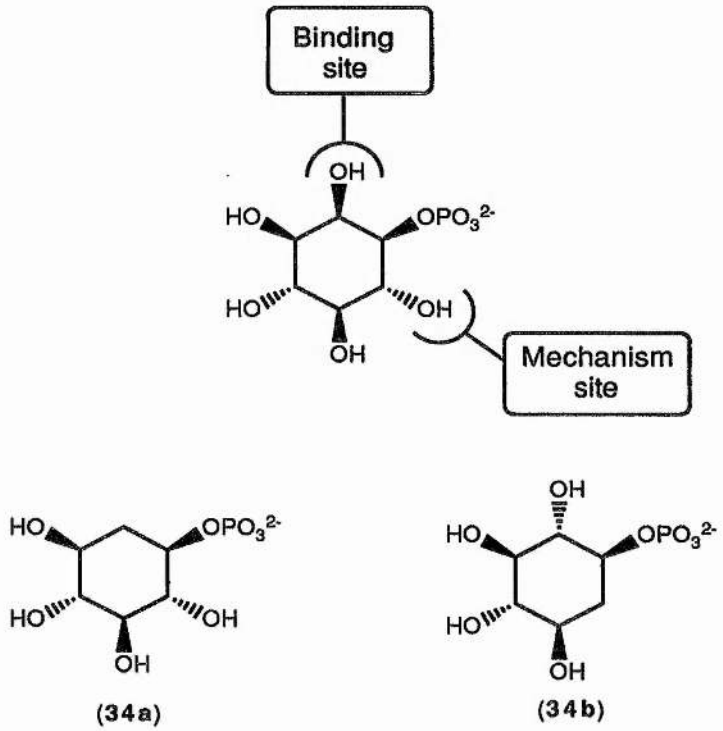
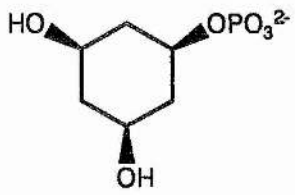


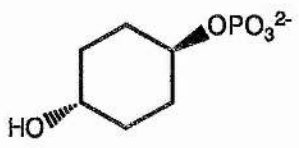
Figure 1.9.2. α-Hydroxyl interactions with inositol monophosphatase.

hydroxyl groups, with the second α-hydroxyl group being involved in the mechanism of phosphate ester hydrolysis. (+)-2-Deoxy Ins 1-P (**34a**) possesses the mechanistic hydroxyl group but not the binding hydroxyl group, resulting in its weak substrate activity and (-)-2-deoxy Ins 1-P (**34b**) possesses the binding hydroxyl group but not the mechanistic (or catalytic) hydroxyl group, resulting in its competitive inhibitor activity (Figure 1.9.2). Other substrates like α-glycerophosphate and β-glycerophosphate have at least one hydroxyl group

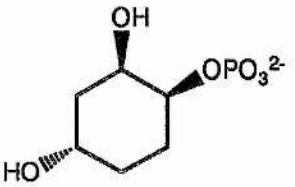
α– to the phosphate group, fulfilling the structural requirements for hydrolysis to occur. Further investigation into the roles of the hydroxyl groups of the inositol ring through comparison of the three dimensional structures of the two enantiomers of inositol 1-phosphate (**33a** & **33b**) using molecular modelling techniques has shown good overlap between the phosphate group, both α-hydroxyl groups and the 4-hydroxyl group. Superimposition of the 3- and 5-hydroxyl groups could not be achieved, suggesting that these groups are not of any great binding or catalytic significance. This prediction was confirmed through synthesis and incubation of *cis*-3,5-dihydroxycyclohexane phosphate (2,4,6-trisdeoxyinositol 1-phosphate) (**35**), which shows no substrate or inhibitor activity.[116] *trans*-4-Hydroxycyclohexane phosphate (**36**) does show some inhibitor activity, indicating that the 4-hydroxyl group is involved in binding. 2,3,5-Trisdeoxy- and 3,5,6-trisdeoxy- analogues of inositol 1-phosphate have also been synthesised, and exhibit the expected substrate and inhibitor properties.



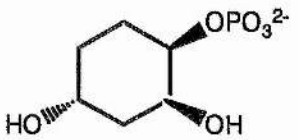
(**35**)
cis-3,5-dihydroxy
cyclohexane 1-phosphate



(**36**)
trans-4-hydroxy
cyclohexane 1-phosphate



(**37**)
1-(S)-phosphoryloxy-(2R, 4S)
dihydroxy cyclohexane ((-)PDC)



(**38**)
1-(S)-phosphoryloxy-(2S, 4R)
dihydroxy cyclohexane ((+)PDC)

Figure 1.9.3 Synthetic inhibitors and substrates of inositol monophosphatase.

1-(S)-Phosphoryloxy-(2R,4S)-dihydroxycyclohexane ((-)PDC) (**37**) which possesses the phosphate group and the two binding hydroxyl groups, but not the catalytic hydroxy group, has been shown to be an extremely potent competitive inhibitor of the enzyme (IC_{50} 3 μM) whereas its enantiomer ((+)PDC) (**38**) which possesses the catalytic hydroxyl group and a single binding hydroxyl group behaves as a weak substrate.[116] The binding and catalytic sites of D-inositol 1-phosphate are summarised in figure 1.9.4.

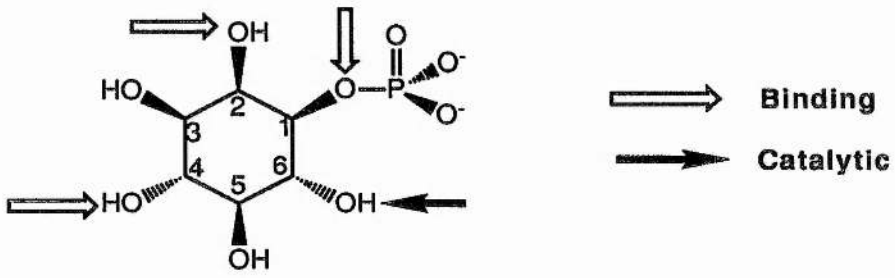
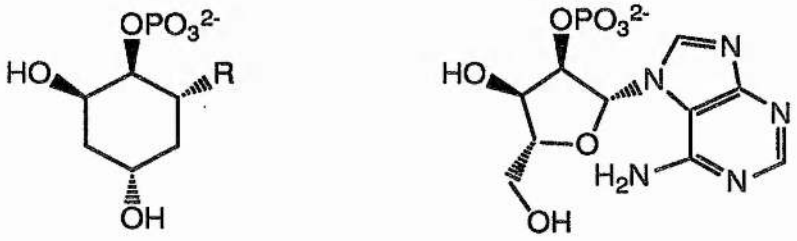


Figure 1.9.4. Binding and catalytic sites on D-myo-inositol 1-phosphate.

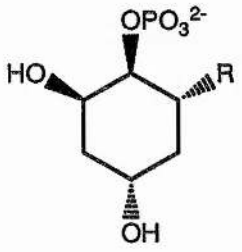
It is well known that inositol monophosphatase is able to hydrolyse 2'-nucleoside phosphates, including adenosine 2'-monophosphate (**40**)[113] (K_m 0.58 mM, V_{max} 1.58 that of (+/-) Ins 1-P). The adenine group is known not to significantly reduce binding to the enzyme, implying a tolerance of steric bulk near the active site. The use of molecular modelling techniques to compare the 3-dimensional structures of the deoxyinositol phosphates (**39**) and 2'-AMP (**40**) have shown good overlap between the phosphate group and the two hydroxy groups[117] of each compound (Figure 1.9.5).



(39) **(40)**

Figure 1.9.5. Comparison of inositol and adenosine functional groups.

The deoxyanalogue (**39**) R=H (Figure 1.9.5) is an extremely efficient competitive inhibitor[116] (no catalytic hydroxy group), which suggests that the adenine group may in some way be involved in the hydrolysis of 2'-AMP (See results and discussion section). The structural analysis indicates that the R group at the 6-position of the cyclohexane ring would occupy the same 3-dimensional space as the adenine moiety in 2'-AMP (**40**). The synthesis and testing of a number of deoxyinositol phosphate analogues possessing steric bulk at the 6-position have been reported,[117] the best of which inhibited with an IC_{50} value of 70 nM (**41d**) These reports confirm that there is a substantial tolerance of steric bulk at the active site, with a substituent at the 6-position providing significant extra binding affinity.



(**41a**) R = OH (K_m0.025 mM, V_{max} 0.61 that of (+/-) Ins 1-P).[117]

(**41b**) R = H (IC_{50} 3 μM).[116]

(**41c**) R = $(CH_2)_4CH_3$ (IC_{50} 3 μM).[117]

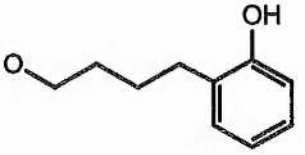
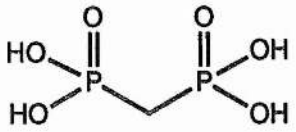
(**41d**) R =  (IC_{50} 70 nM).[117]

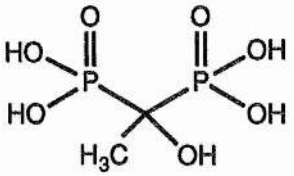
Figure 1.9.6 Side chain deoxyinositol phosphate inhibitors.

1.10 Non-phosphate inhibitors of inositol monophosphatase.

Although the deoxy analogues are potent specific inhibitors of inositol monophosphatase, their *in vivo* activity is compromised by their hydrolyses by non-specific phosphatase enzymes and also their lack of penetration into intact cells. Non-hydrolysable phosphonates have been shown to be devoid of substrate and inhibitory activity,[113] however, through random screening, some bis phosphonic acids have been identified as inhibitors. Methylene bis phosphonic acid (**42**) (the simplest example) was inactive, however hydroxyethylidine-1,1-bis phosphonic acid (**43**) was shown to be a reasonable competitive inhibitor (IC_{50} 110 μM).[118]



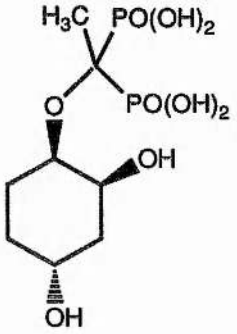
(42)



(43)

Figure 1.10.1. Bis-phosphonic acids.

Combination of the structural features of the deoxyinositol phosphate inhibitors and the bisphosphonic acid inhibitors resulted in the production of (+/-) 3,5,6-trisdeoxyinositol-1,1-bis phosphonic acid (**44**), a potent competitive inhibitor of inositol monophosphatase (IC_{50} 4 μM).[119]

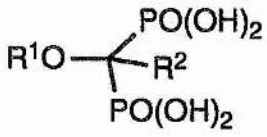


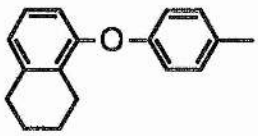
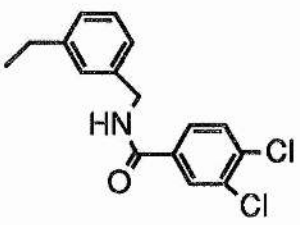
(44)

Figure 1.10.2. 3,5,6 trisdeoxy ethylidene 1,1-bisphosphonic acid.

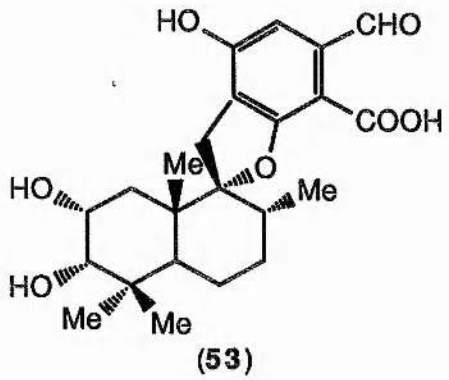
Substitution of the methyl group and the O-cyclohexane diol ring in compound (**44**) gave rise to a number of competitive inhibitors of varying degrees of efficiency (Table 1.10.1). The best of these is compound (**52**) (IC_{50} 0.08 μM), which is the most potent non-hydrolysable inhibitor of inositol monophosphatase reported to date,[119] and is structurally almost completely unrelated to the natural substrate. The *in vivo* activity of these compounds as yet remains unknown.

Table 1.10.1. Non-phosphate inhibitors of inositol monophosphatase.



Compound	R^1	R^2	IC_{50} (μM)
(45)	H	CH_3	110[118]
(46)	H	Ph	29[118]
(47)	H	Bn	38[118]
(48)	H		23[118]
(49)	H		0.61[118]
(50)		CH_3	0.33[119]
(51)		Bn	0.5[119]
(52)			0.08[119]

One further inhibitor unrelated to the natural substrate is the fungal metabolite K-76 monocarboxylic acid (**53**) (Figure 1.10.3), which is reported to be a competitive inhibitor for the substrate D-Ins 1-P (K_i 0.5 mM).[120]



(53)

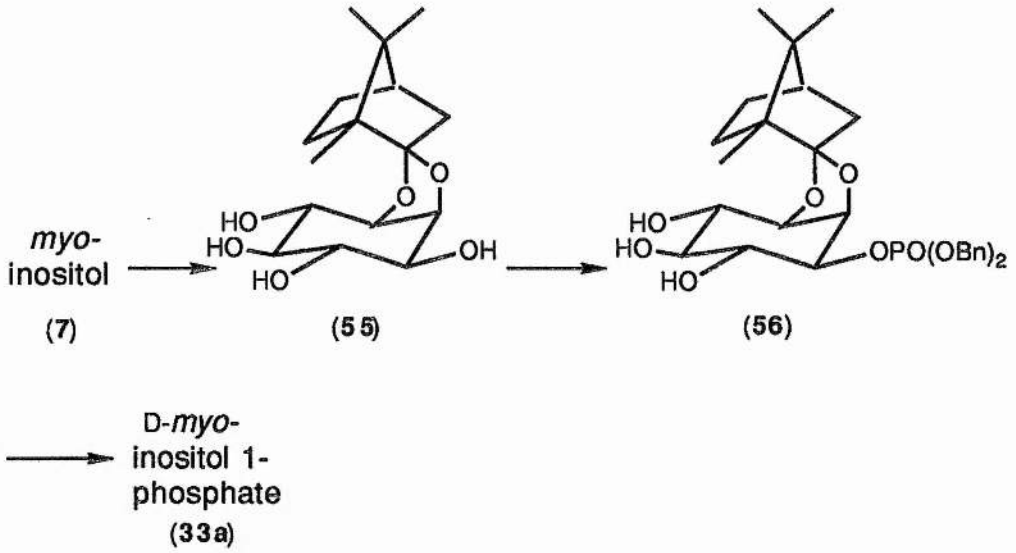
Figure 1.10.3. K-76 monocarboxylic acid.

1.11 The synthesis of inositol phosphates.

Interest in the inositol cycle and its specific enzymic transformations has presented the need for the natural substrates. Although they can be isolated from their natural sources, the ability to synthesise these compounds would result in the production of large amounts of the desired material, with the added advantage of being able to make minor chemical changes to investigate the enzymic processes more closely and at a mechanistic level.[121] Due to its commercial availability and low cost, the majority of inositol polyphosphate syntheses use the parent compound, inositol, as the initial starting material. There are, nevertheless, a number of problems associated with such syntheses. Firstly, *myo*-inositol (**7**) is an achiral compound, which becomes chiral upon unsymmetrical substitution. Syntheses therefore often require a resolution step in order to obtain the naturally occurring and biologically important stereoisomer. To combat this problem, a number of syntheses have been developed using chiral starting materials other than inositol, for example (-)-quinic acid[122] and conduritol B.[123] In addition, enzyme aided syntheses from achiral starting materials such as benzene have been developed.[124] A second problem arises due to the fact that specific regioisomers are required to be useful in research, meaning a number of specific protection steps are usually required to achieve phosphorylation at the desired position(s). To achieve

specific protection, one must exploit the differences in reactivity between the axial and equatorial hydroxyl groups and also the 1,2-*cis*- and 1,2-*trans*-relationship between adjacent hydroxyl groups.[125-129] With the hydroxyl groups all being secondary, further complications arise with efficient phosphorylation. The relative unreactivity of P(V) reagents for phosphorylation of secondary alcohols usually having large protection groups on adjacent carbon atoms has prompted the use of much more reactive phosphites and other P(III) related reagents for phosphitylation.[150] The use of phosphitylating agents has an added advantage in that they offer routes to both the natural phosphate esters and also the phosphorothioate analogues.[131,132] These have proved very useful in mechanistic investigations as they are often slower substrates and/ or inhibitors. The final problem is that of phosphoryl group migration and cyclic diester formation during deprotection (usually occurring under acidic conditions).

One synthesis of optically pure inositol 1-phosphate from inositol which appears to break the 'rules' for efficient inositol phosphate synthesis has however been reported[133] (Scheme 1.11.1). This three step synthesis does not

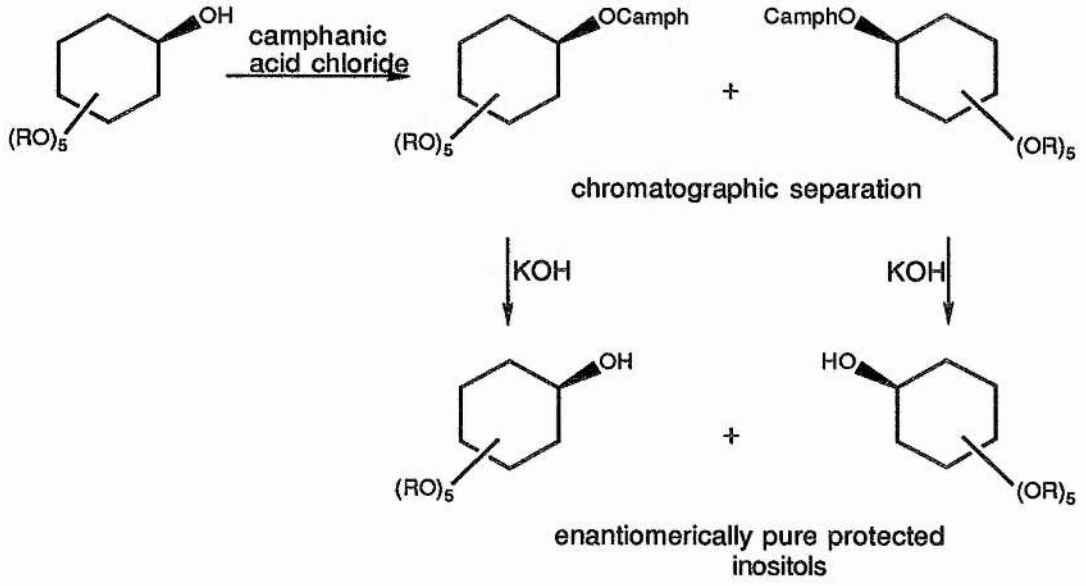


Scheme 1.11.1. Three step synthesis of D-myo-inositol 1-phosphate.

require complete protection of inositol (leaving a single hydroxyl group for phosphorylation), does not require any chromatographic resolution, uses a P(V) reagent for phosphorylation and involves an acidic final deprotection step. The

initial ketal formation (with camphor dimethylacetal[134]) is a precipitation driven equilibrium (performed in $CHCl_3$: CH_3OH : H_2O) which allows the isolation of the desired diastereoisomer (**55**) by simple filtration. (The use of this camphor monoacetal has been the subject of further investigation, with alternative enantiomerically pure inositol phosphates being produced after further protection steps[135]) Direct phosphorylation of the tetraol with dibenzylchlorophosphate results in the phosphate from reaction at C-1 OH (**56**) (reported to be the only component accumulating in any significant quantity). This selective phosphorylation of the polyol is possibly due to an α-effect. The strain involved in the five membered ring of the camphor ketal (**55**) results in a slight distortion of the inositol ring with C-1 OH not being completely equatorial, and the C-2 substituent not being entirely axial. This results in the interaction of the two oxygens, making C-1 OH slightly more nucleophilic than the other hydroxyl groups. Simple hydrogenation and acidic ketal deprotection gave no isomerisation or cyclic ester formation, and allowed the isolation of the target compound as the bis-cyclohexylammonium salt. This synthesis also offers equally straight forward access to L-*myo*-inositol 1-phosphate (**33b**) using the commercially available L-camphor.

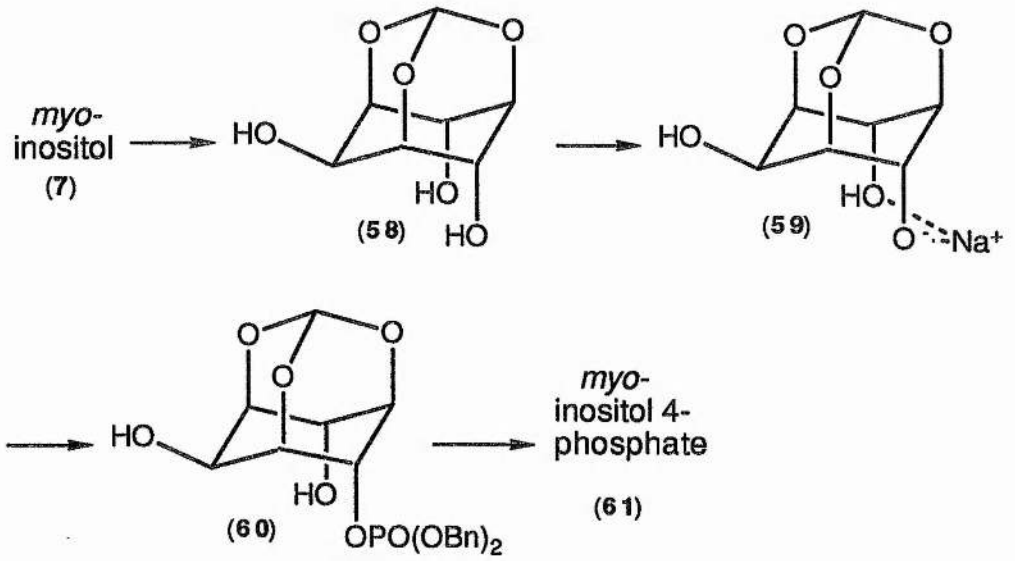
A more common method of obtaining enantiomerically pure inositol phosphates which does not rely on the fortune of selective crystallisation is that



Scheme 1.11.2. Camphanate diastereomeric resolution.

of diastereomeric resolution of a suitably protected *myo*-inositol derivative. Systems that have been used include the production of orthoesters of D-mannose, menthoxyacetates and camphanate esters (Scheme 1.11.2).[136] In the production of these diastereoisomers, the considerable bulk of the multichiral centred esters makes the diastereomeric components significantly different to allow relatively simple separation.

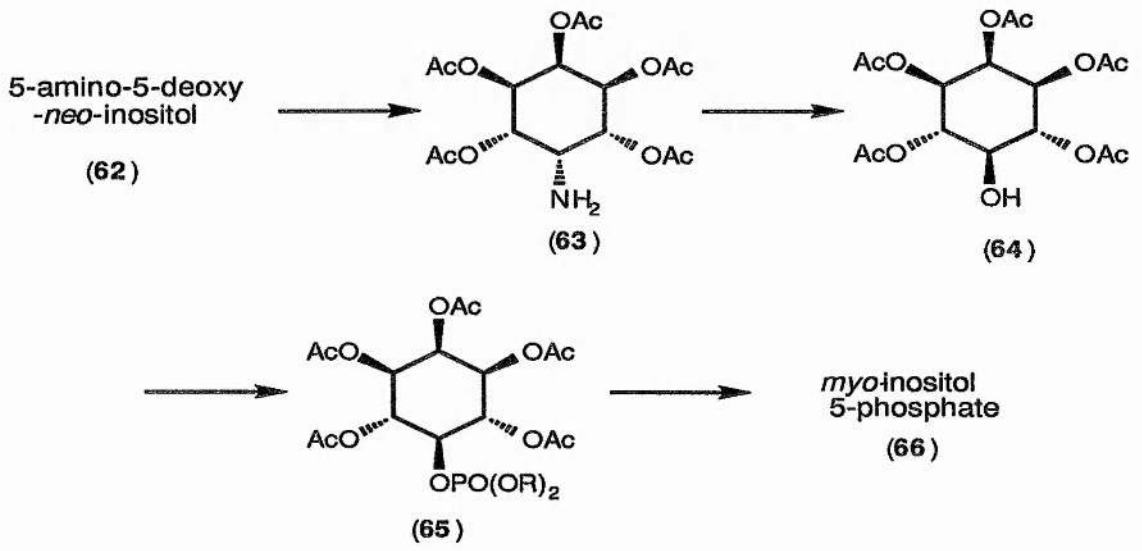
A similar synthesis to that outlined above of racemic inositol 4-phosphate (**61**) (similar in the fact that specific phosphorylation can be achieved from a non-fully protected inositol derivative) has been performed using mono-orthoformate inositol (**58**) which has hydroxyl groups at C-1, C-3 and C-5 simultaneously protected.[137] The axial/ equatorial relationship usually associated with *myo*-inositol (**7**) is now reversed, which allows specific 4-OH alkylations, or in this case phosphorylation, through deprotonation of one of the axial hydroxyl groups with stabilisation of the resulting alkoxide by coordination of the metal ion (Scheme 1.11.3.).



Scheme 1.11.3. Synthesis of racemic inositol 4-phosphate.

Phosphorylation is achieved with pentabenzylpyrophosphate, a reagent which has also been used with great efficiency in the polyphosphorylation of inositol derivatives. Phosphorylation of the orthoformate (**58**) can occur on either of the axial hydroxyl to give, on deprotection, racemic inositol 4-phosphate (**61**) (since (+) Ins 6-P = (-) Ins 4-P).

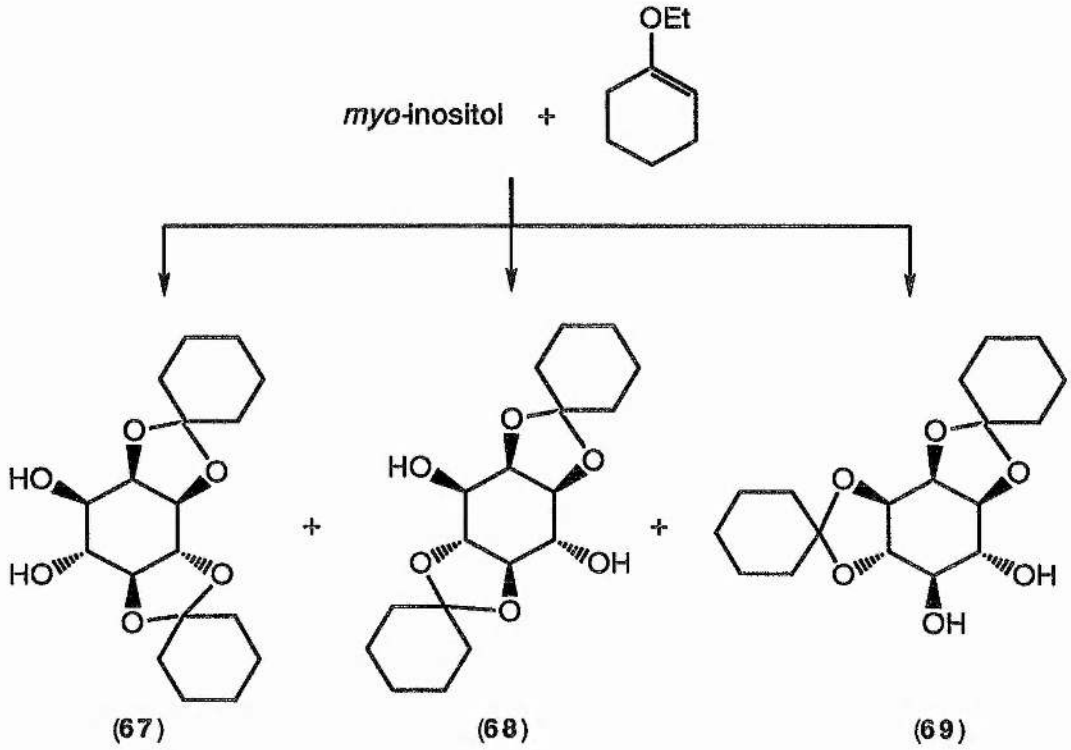
A relatively simple synthesis of an achiral inositol monophosphate not occurring in the inositol cycle (that of inositol 5-monophosphate (**66**)) has been reported[138] employing a derivative of *neo*-inositol (5-amino-5-deoxy-*neo*-inositol (**62**), obtained from the antibiotic hygromycin A[139,140]) as the initial starting material. The simplicity of this system arises because the hydroxyl group required for phosphorylation is only incorporated after full protection is complete. Protection as the penta-*O*-acetyl compound is followed by diazotisation and invertive hydrolysis giving the protected *myo*-inositol derivative for phosphorylation (**64**), (Scheme 1.11.4.). Although inositol 5-phosphate (**66**) is not involved in second messenger metabolism, it is of interest with regard to inositol monophosphatase, because it possesses an interesting 1,4 phosphate-OH *cis*-arrangement not present in inositol 1- and inositol 4-phosphate.



Scheme 1.11.4. Synthesis of inositol 5-phosphate.

A popular solution to the problem of acquiring suitably protected *myo*-inositol derivatives is the use of bis-ketals,[142] usually from cyclohexanone[143] or cyclohexanone precursors,[144,145] which yields a mixture of three different ketals that can be chromatographically separated or fractionally crystallised. (Scheme 1.11.5). Separation of the bis-ketal mixture immediately provides access to three inositol bis-phosphates (all be it in a racemic form), and further

manipulation by the use of specific protecting groups allows the synthesis of more complex and more interesting inositol phosphates.

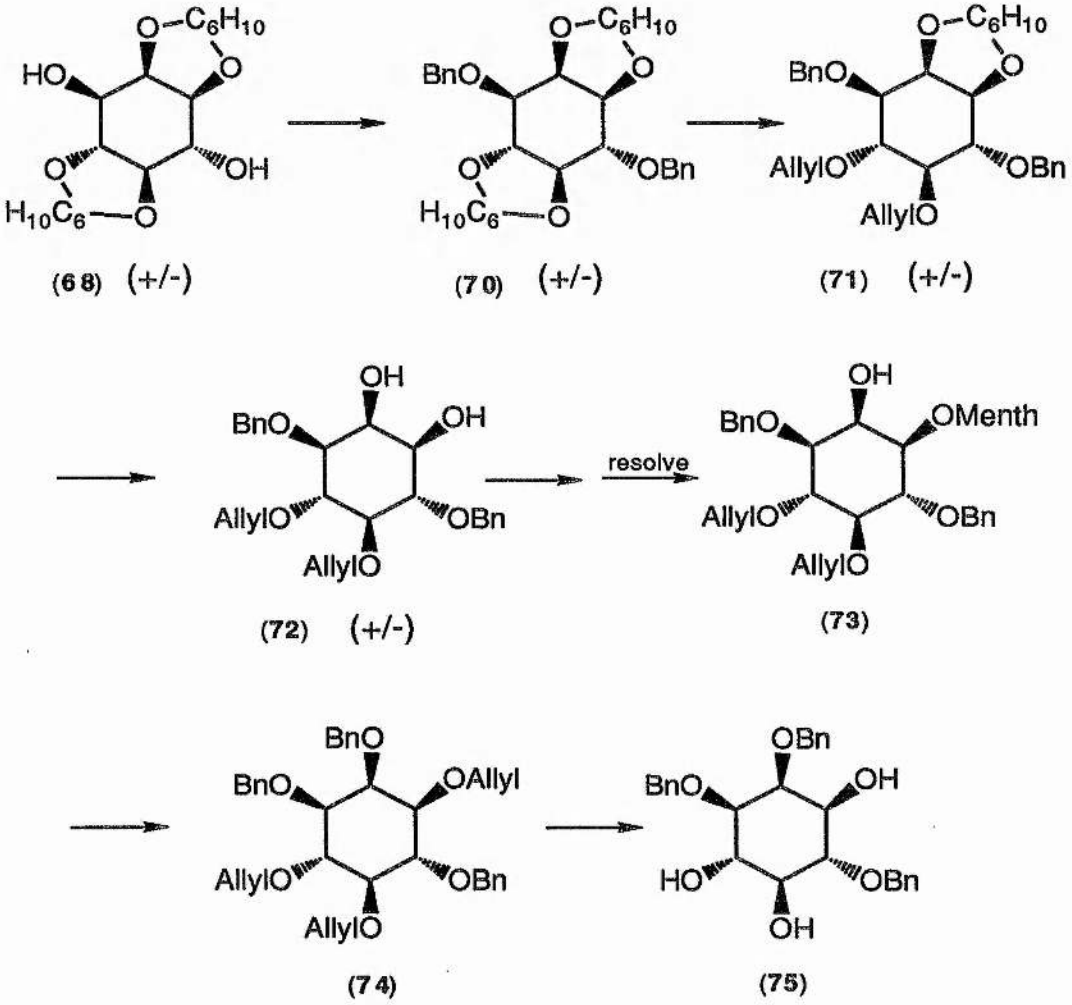


(67) (68) (69)

Scheme 1.11.5. Bis-ketal isomers of inositol.

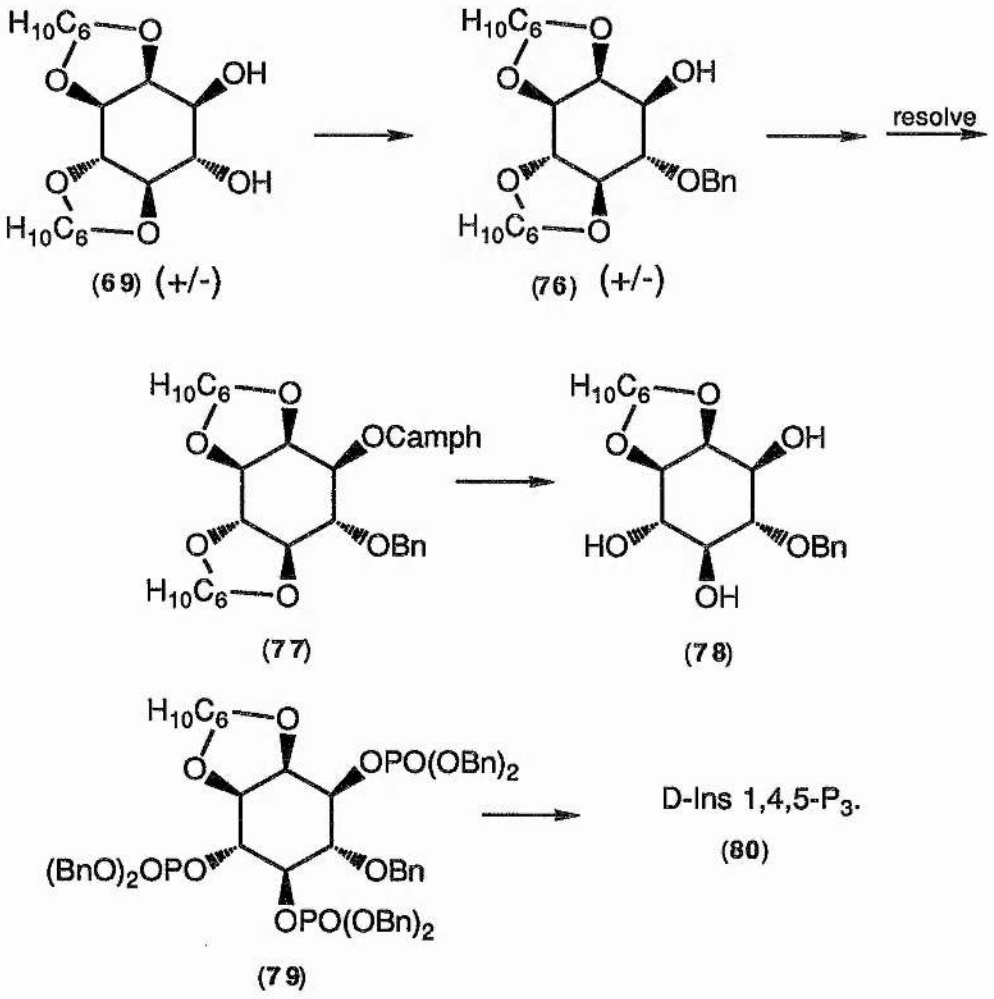
The mixture is also a readily available source of the most stable 1,2-monoketal, produced under mild hydrolysis conditions to remove the less stable and non specific ketal. The 1,2-monoketal (**81**) has recently been efficiently resolved into its enantiomeric components by enzymic esterification.[146] This involves the treatment of racemic 2,3-*cis*-cyclohexylidene *myo*-inositol in dioxane with the relevant enzyme (amano lipase C or amano lipase CES from *Pseudomonas sp.*) and acetic anhydride,[146] and results in selective acetylation at C-1 of the L-enantiomer.[148] The acetylated L-enantiomer and the non-acetylated D-enantiomer were easily separable by chromatography. D-*myo*-inositol 1,4,5-tris phosphate has since been synthesised in 30% yield from this enzymically resolved monoketal.[148] D-*myo* -inositol 1,4,5-trisphosphate (**80**) has received much attention as a synthetic target over recent years due to its importance in cell signalling. The first reported synthesis by Ozaki *et al* [146] although long and low yielding, incorporates protecting groups still used in

more recent syntheses, namely benzyl protection and allyl protection.[150,151] Both groups show acid and base stability and can be selectively removed in the presence of each other (see results and discussion section). Benzylation of the 1,2;4,5 bis-ketal (**68**) followed by partial ketal hydrolysis of (**70**) gave the racemic 4,5-diol. Bis-allylation followed by further ketal hydrolysis affords the 1,2-bishydroxy compound (**72**), from which chromatographic diastereomeric resolution was achieved *via* chiral ester formation on the more reactive and less hindered equatorial hydroxyl group. Ester hydrolysis and selective allylation, again on the equatorial hydroxyl group, followed by benzyl etherification of the C2-OH group gave a fully protected inositol derivative possessing allyl groups at C-1,C-4 and C-5 (**75**) (Scheme 1.11.6).



Scheme 1.11.6. Synthesis if D-1,4,5-tris-O-benzyl inositol.

Phosphorylation (using dianilidochlorophosphate), which occurred in low yield, and deprotection gave the target compound (**79**). Numerous problems have been associated with such phosphorylations due to the lability of the phosphorus protecting groups and subsequent cyclic ester formation. Ozaki *et al.* have since reported that cyclisations of monophosphorylated intermediates in the polyphosphorylation of a 1,2-diol similar to that described above occurs preferentially to give the five membered cyclic phosphate instead of further phosphorylation leading to the bisphosphate[152] (complex mixtures of mono and bis phosphate esters have been isolated in alternative syntheses of polyphosphates using dianilidochlorophosphate[153,154]).



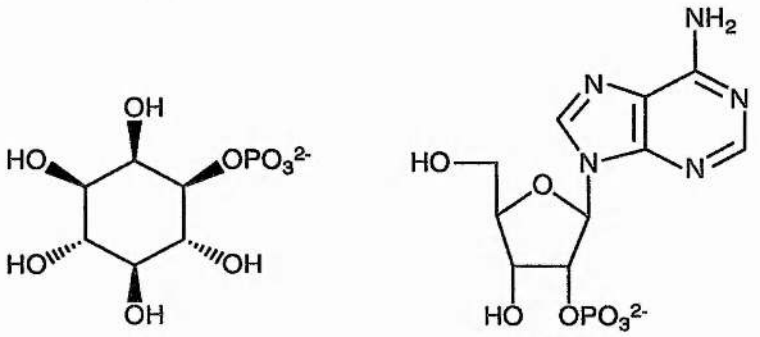
Scheme 1.11.7. Synthesis of D-myo-inositol 1,4,5-tris phosphate.

A more recent synthesis of D-Ins 1,4,5-P_3 combines the aforementioned bis-ketalisation with simple benzyl protection and effective phosphorylation to give the target compound in good yield.[148] Isolation of the 1,2;5,6 bis-ketal (**69**) followed by partially selective monobenzylation[155] affords the racemic 3-benzyl-4-hydroxy derivative (Scheme 1.11.7) which was resolved following camphanate ester formation. Partial ketal hydrolysis followed by ester hydrolysis gave the required protected inositol derivative (**78**) in just four steps from the bis-ketal. Tetrabenzyl pyrophosphate phosphorylation was achieved in excellent yield (> 80%).[148] Subsequent hydrogenation and ketal hydrolysis afforded the desired compound (**80**) (Scheme 1.11.7). The use of tetrabenzyl pyrophosphate appears to have eliminated the problems encountered in the earlier synthesis due to the relative inertness of the phosphate benzyl ester. A second advantage of this phosphate protecting group is that simultaneous phosphate benzyl ester and alkyl benzyl ether deprotection can be achieved in a 'one-pot' reaction, without the need for tedious ion exchange chromatography.

The development of synthetic routes to protected *myo*-inositols and *myo*-inositol phosphates has allowed the production of a number of related compounds such as phosphorothioate,[156] phosphonate,[157] sulfate[158] and sulfonate[159] analogues. In the case of inositol 1,4,5-trisphosphorothioate,[160,161] the analogue can fulfil the biological messenger function of the natural compound, however, is not accepted as a substrate for inositol 5-phosphatase. In many cases these analogues tend to be slow substrates and/or competitive inhibitors of varying degrees of potency. They are useful tools to be employed in the accumulation of mechanistic information, both for the enzymes involved in the inositol cycle and in elucidating the mechanisms of signal transduction.

2. RESULTS AND DISCUSSION

The aim of this project was to produce novel substrate analogues of inositol 1-phosphate (**33**), adenosine 2'-monophosphate (**40**) and glycerol phosphates, and by studying their behaviour as inhibitors and substrates for inositol monophosphatase, gain more information concerning the mode of action of the enzyme. By stereospecific incorporation of an ^{18}O-label into the phosphorothioate analogues of inositol 1-phosphate (**33**) and adenosine 2'-monophosphate (**40**) (Figure 2.1) the production of a substrate chiral at phosphorus (by means of isotopic substitution) was also investigated, with the aim of determining the stereochemical course of the phosphate ester hydrolysis at the phosphorus centre.



(**33**) (**40**)

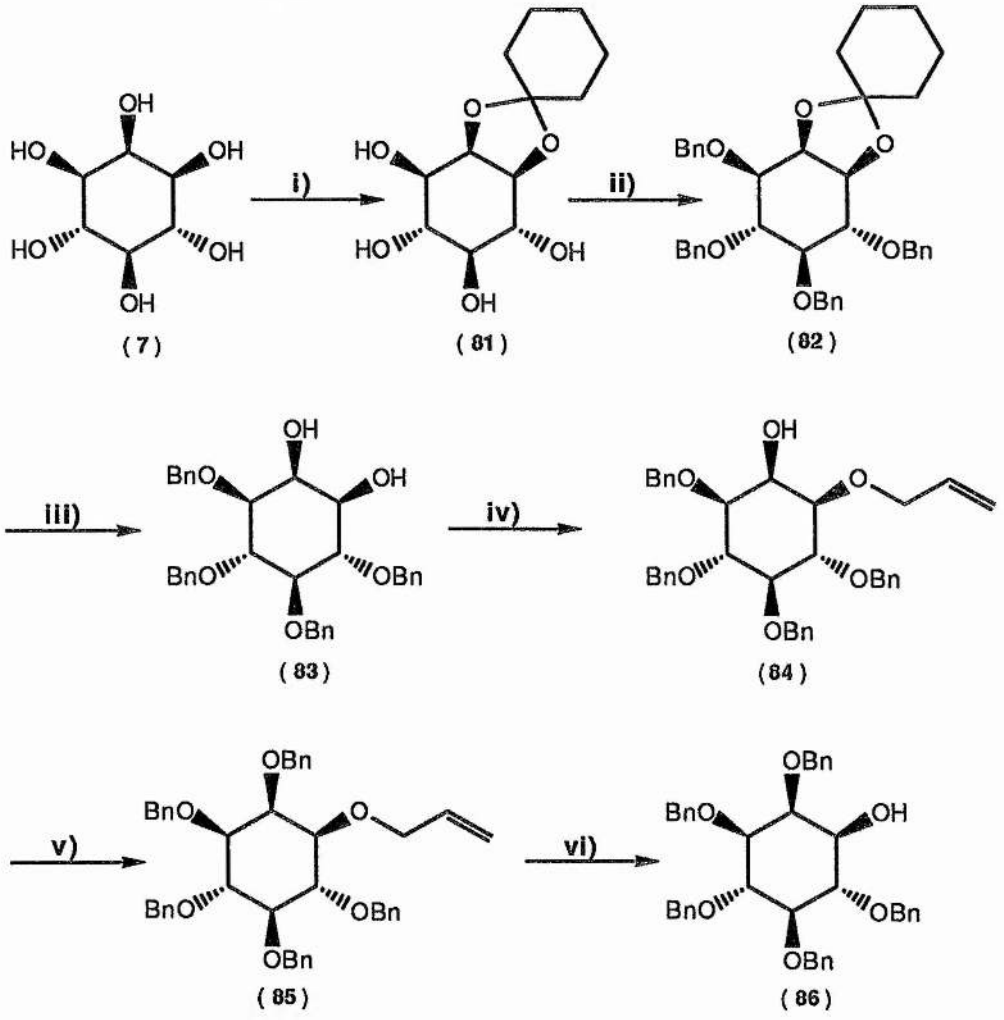
Figure 2.1. D-Inositol 1-phosphate and 2'-AMP.

Initial experimentation involved the production of the natural substrate, inositol 1-phosphate (**33**), which was required to determine the potency of synthetic inhibitors, and the production of the substrate analogue, inositol 1-phosphorothioate, with the aim of stereospecifically incorporating an oxygen label to produce R_P- and S_P-inositol 1-[^{18}O]-phosphorothioate.

2.1 The synthesis of (+/-) inositol 1-phosphorothioate.

As described in section 1.11, the synthesis of specific isomers of inositol monophosphates requires a number of specific protection steps to ensure phosphorylation occurs in the correct position. Two syntheses of inositol 1-phosphorothioate have been reported,[132,162] one of which has potential in terms of stereospecifically incorporating an oxygen label. The production of a penta-protected inositol derivative possessing a free hydroxyl group at the 1-

position was performed according to the method of Baker *et al.*.[132] Initial formation of the mono-*cis*-ketal (**81**)[1] (section 1.11), followed by tetrabenzylation and acidic ketal deprotection yields (+/-)-3,4,5,6-tetra-*O*-benzyl *myo*-inositol (**83**) in 52% yield from *myo*-inositol (**7**) (Scheme 2.1.1). Specific allylation at the C-1 position was achieved *via* the formation of a stannylidene intermediate using dibutyl tin oxide under dehydrating conditions.[132]

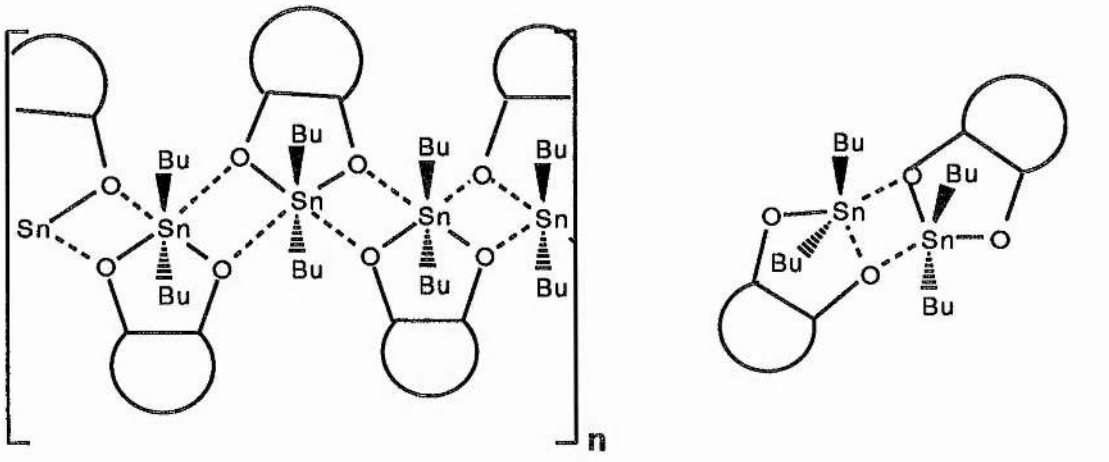


Reagents: **I)** $C_6H_{10}O$, $PhCH_3$, 65%; **II)** BnCl, KOH, 84%; **III)** AcOH, H_2O, 82%; **Iv)** a) Bu_2SnO, PhH, b) Allyl bromide, 94%; **v)** BnBr, NaH, DMF, 93%; **vI)** a) $(Ph_3P)_3RhCl$, DABCO, EtOH, H_2O; b) AcOH, H_2O, 91%;

Scheme 2.1.1. Synthesis of 3,4,5,6-tetra-O-benzyl myo-inositol.

Previously, a partial allylation reaction of a similarly protected compound had

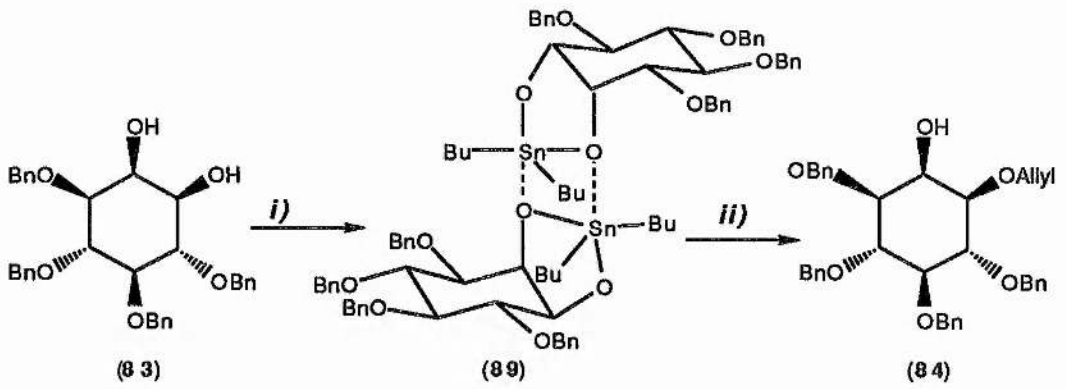
been used to protect the equatorial C-1 hydroxyl group.[144] This reaction, apart from being low yielding, was reported not to be completely regioselective. The use of dibutyl tin oxide followed by allyl bromide gave the desired product (**84**) in 94% yield after chromatography, with no 2-*O*-allyl- or bis-*O*-allyl- protected inositol derivatives being detected. The reaction has previously been studied by ^{119}Sn NMR spectroscopy, and the intermediates characterised by X-ray crystallography and mass spectrometry. In the solid phase, the stannylidene intermediate (**87**) (Figure 2.1.2), exists apparently as an infinite chain of dimeric units joined by weak Sn-O bonds.



Solid phase structure (**87**) Solution phase structure (**88**)

Figure 2.1.2. Structure of stannylidene intermediate.

In the solution phase, the species is thought to exist as a single dimer (**88**) in which the two inositol oxygens are not equivalent. C-2 OH Is thought to be coordinated to both tin atoms in the dimer (Scheme 2.1.3) and is relatively electron deficient, as well as being sterically hindered. C-1 OH on the other hand is only two coordinate, and more electron rich (only being bonded to one tin atom in the dimer). It has been proposed that a combination of these factors together with the fact that the axial hydroxyl group is less nucleophilic than the equatorial hydroxyl group, results in the preferential allylation of C-1 OH.[163] Having achieved selective equatorial allylation (Figure 2.1.3), the penta-*O*-protected inositol (**84**) was further benzylated using benzyl bromide and sodium hydride in THF or in DMF. Significant increases in rates of reaction have been observed when DMF was used as a solvent in preference to THF.[57]

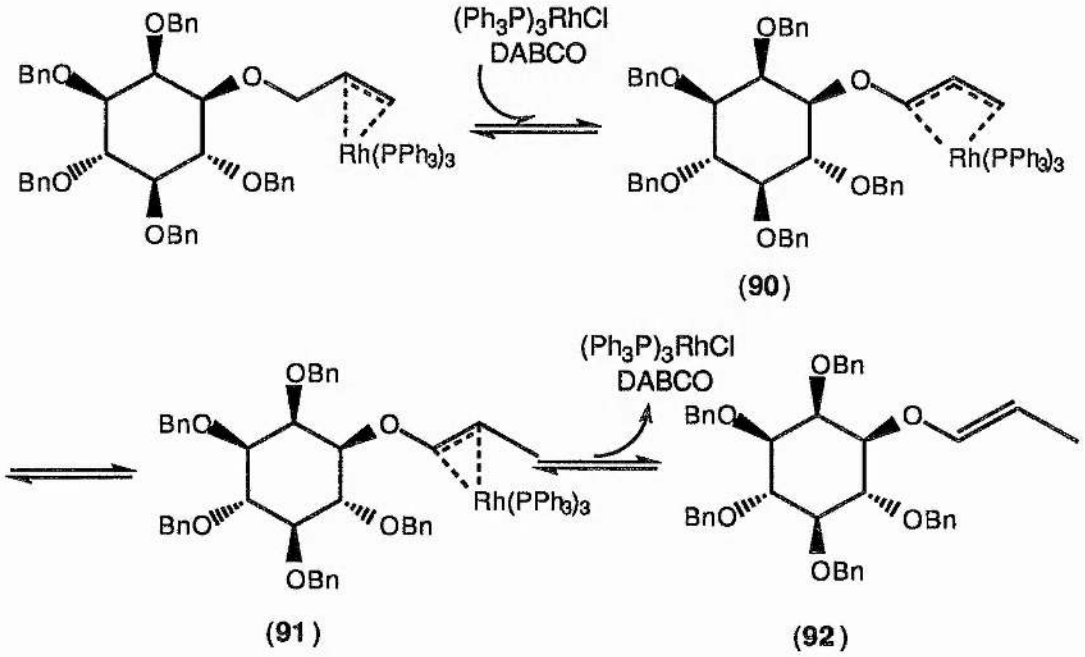


(83) (89) (84)

Reagents: I) Bu_2SnO, Benzene, (- H_2O); II) Allyl bromide, 94%.

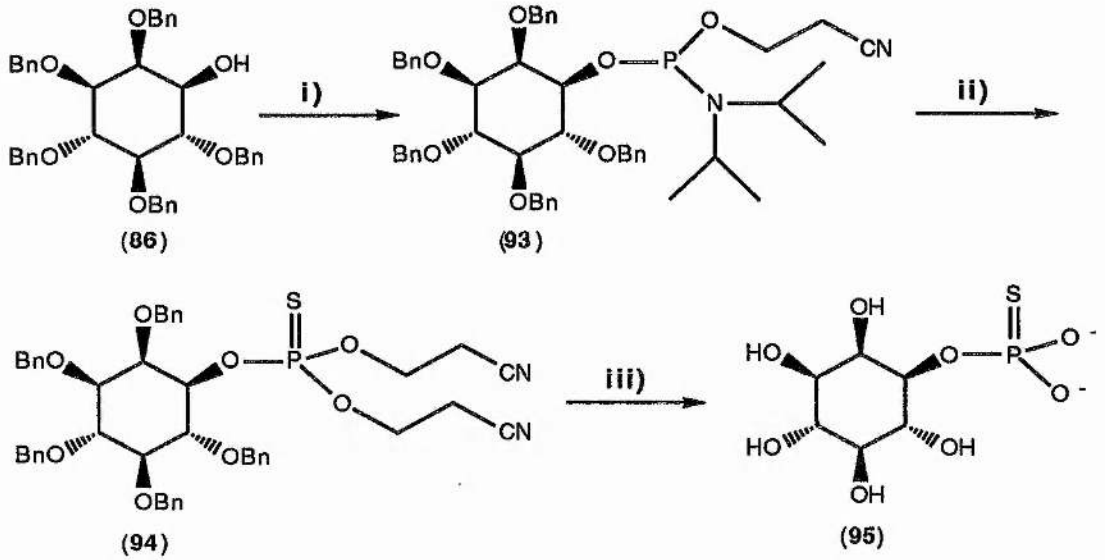
Scheme 2.1.3. C-1 OH allylation via a stannylidene intermediate.

De-allylation to produce the desired protected precursor to inositol 1-phosphorothioate (**86**) was achieved following the methodology of Corey and Suggs.[164] The use of catalytic amounts of both Wilkinson's catalyst (tris triphenylphosphine rhodium (I) chloride) and DABCO in aqueous ethanol resulted in the isomerisation of the allyl ether (**85**) to the more thermodynamically stable enol ether (**92**) (Scheme 2.1.4).



Scheme 2.1.4. Wilkinson's catalyst mediated deallylation.

The enol ether (**92**) is easily hydrolysed in aqueous acid to give the precursor to both inositol 1-phosphate and inositol 1-phosphorothioate. Incorporation of the phosphorothioate functionality is achieved using the phosphitylating agent N,N'-diisopropyl(2-cyanoethyl)chlorophosphoramidite, in its racemic form. Transesterification of (**93**) with an excess of 3-hydroxypropionitrile forms the bis-cyanoethyl phosphite which was oxidised with elemental sulfur in pyridine to afford the fully protected phosphorothioate (**94**) (Scheme 2.1.5).



Reagents: **I)** N'N-diisopropyl(2-cyanoethyl)chlorophosphoramidite, diisopropylethyl amine,THF; **II)** a) 1-H tetrazole, 3-hydroxypropionitrile, CH_3CN,b)S_8, pyridine, 65%; **III)** a) NaOMe/ MeOH, b) Na/ $NH_{3(l)}$, THF, c) Amberlite IRA 118$(H)^+$, d) cyclohexylamine, H_2O, 58%.

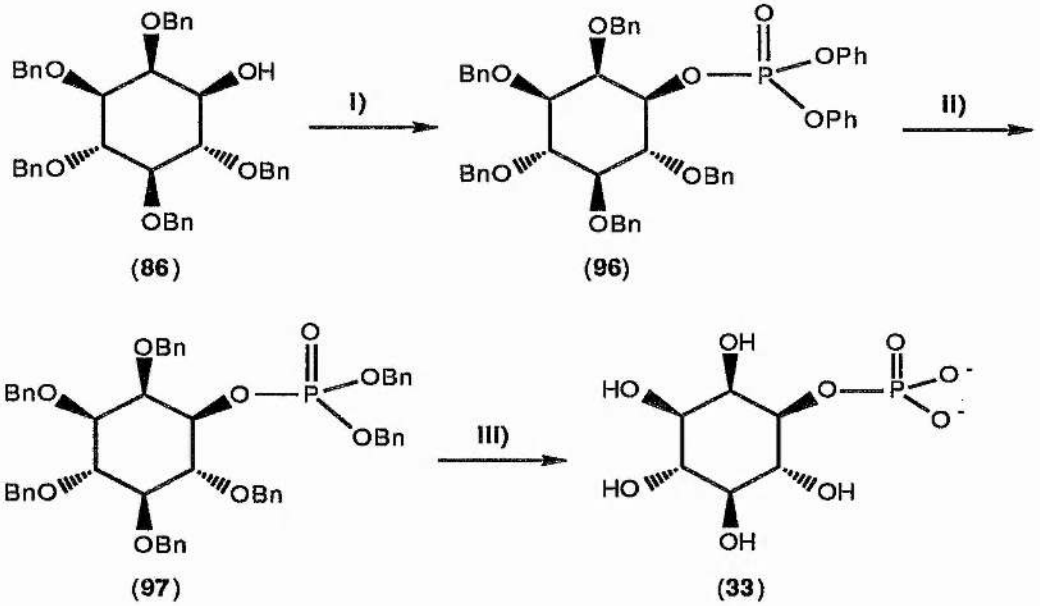
Scheme 2.1.5. Synthesis of inositol 1-phosphorothioate.

Deprotection is achieved firstly by treatment with sodium methoxide in methanol (removing the cyanoethyl groups by production of the volatile side-product acrylonitrile), and secondly by a dissolving metal reduction (sodium metal in liquid ammonia) removing the benzyl ethers to produce pure inositol 1-phosphorothioate (**95**). Ion exchange chromatography (Amberlite IRA 118 $(H)^+$) and conversion to the bis-cyclohexylammonium salt gives the desired product in 58% yield from (**94**). The use of substrates in the form of their bis-cyclohexylammonium salts aids crystallisation, and hence purification, and also does not interfere in any way with the enzyme incubation.

Phosphitylation of a single enantiomer of 2,3,4,5,6-penta-*O*-benzyl *myo*-inositol ([α_D] +10.4°, (c 0.3 in $CHCl_3$), {lit.,[165] [α_D] +9.1°, (c 0.3 in $CHCl_3$)}) with racemic N,N'-diisopropyl(2-cyanoethyl)chlorophosphoramidite and examination of the reaction intermediate (**93**) by thin layer chromatography revealed that the inositol phosphoramidite was apparently present as an inseparable mixture of two diastereomers. Similarly, transesterification with benzyl alcohol instead of cyanoethyl alcohol (3-hydroxypropionitrile) gave an inseparable mixture of diastereomers as judged by TLC. Diastereomeric resolution of such species was investigated as a possible route to stereospecifically incorporate an ^{18}O-label (by use of [^{18}O]-cyanoethyl alcohol or [^{18}O]-benzyl alcohol) however in the case of inositol, this does not seem to be a valid possibility.

2.2 Synthesis of (+/-) inositol 1-phosphate.

The production of inositol 1-phosphate (**33**) for use in the determination of enzyme activity and inhibitor potency of synthetic inhibitors was performed according to the method of Billington *et al.*[165] and involved the phosphorylation of (**86**) with diphenylphosphorochloridate (Scheme 2.2.1).



Reagents: **I)** $(PhO)_2POCl$, DMAP, Et_3N: CH_2Cl_2,68% **II)** NaH, BnOH, THF, 63%. **II)** a) Na/ $NH_{3(l)}$, -78°C,b) Amberlite IRA 118 $(H)^+$, c) cyclohexylamine, H_2O, 69%.

Scheme 2.2.1. Synthesis of (+/-) inositol 1-phosphate.

Transesterification with benzoxide, followed by sodium/ liquid ammonia reduction gave inositol 1-phosphate (**33**), which was converted to the bis-cyclohexylammonium salt as in the case of inositol 1-phosphorothioate (Scheme 2.2.1) in 69% yield from (**97**).

For the purpose of following the enzymic reaction course of synthetic substrates with inositol monophosphatase by ^{1}H-NMR spectroscopy, the amount of activity lost on lyophilisation of an enzyme solution was investigated. Inositol 1-phosphate was dissolved in deuteriated assay buffer (20 mM NH_4HCO_3 and 2 mM $MgCl_2 \cdot 6H_2O$ in $^{2}H_2O$, pH 8.0) and a lyophilised enzyme sample redissolved in deuteriated assay buffer added. Incubation at 37 °C and analysis by ^{1}H-NMR spectroscopy revealed that the lyophilised protein was capable of completely processing the substrate. Parallel experiments comparing the initial rate of enzymic hydrolysis of inositol 1-phosphate using a normal and a freeze dried enzyme sample by the colormetric assay (see experimental section) showed that lyophilisation does not result in the loss of enzyme activity.

2.3 Adenosine as a substrate for inositol monophosphatase.

As inositol 1-phosphorothioate is known to be a substrate for inositol monophosphatase,[57] it was hoped that this compound could be produced in a form chiral at the phosphorus centre in order to examine the stereochemical course of the phosphate ester hydrolysis. Diastereomeric resolution of a suitably protected inositol 1-phosphite or inositol 1-phosphoramidite could not be achieved, as determined by TLC analysis (see page 48), so the synthesis of a thiophosphate analogue of an alternative substrate for the enzyme, namely 2'-AMP (**40**), was investigated. In addition to the synthesis of the substrate analogue, the ability of adenosine to act as a substrate for inositol monophosphatase with regard to oxygen-phosphate ligand exchange between the solvent and inorganic phosphate in an ^{18}O-enriched aqueous medium was assessed. As inositol was known to act as a substrate for the exchange reaction, all incubations for probable but unknown substrates were run in parallel with identical incubations containing inositol in addition to controls containing no enzyme. In initial studies, [^{18}O]-water was added to an assay mixture (30 mM Tris·HCl, 20 mM inorganic phosphate, 2 mM $MgCl_2$, 30 mM

substrate (inositol or adenosine)), and aliquots removed periodically, quenching in liquid nitrogen. On lyophilisation, the inorganic phosphate was isolated by extraction into acidic methanol (2%) and derivatised to the trimethyl ester by treatment with freshly prepared ethereal diazomethane. On evaporation of the solvent, the trimethyl phosphate samples were analysed by g.c. mass spectrometry using a 50% phenyl methyl silicone capillary column at an initial temperature of 60 °C. Surprisingly, in the incubations containing adenosine, no oxygen isotope incorporation into phosphate was observed. The experiments were repeated in duplicate with a higher atom % of $[^{18}O]$-H_2O (up to a maximum of 45%) and extended incubation periods (up to 24 hours). Those containing inositol showed the expected parent ion at 140, and an additional signal at 142 assigned as $(MeO)_2P^{18}OMe$. The inositol incubation showed the expected isotope incorporation whereas no incorporation could be detected in incubations containing adenosine. Only the parent ion of trimethyl phosphate an 140 was observed, with no additional signals present as a result of oxygen isotope incorporation. Examination of the lower molecular weight fragments (*e.g.* 110 (M^+ - MeO) shows multiple incorporations in the case of inositol with the adenosine case being identical to the control (Figures 2.3.1-2.3.4, pages 51 & 52). This result suggests that there is either a mechanistic difference in the enzymic hydrolysis of inositol monophosphates and adenosine 2'-monophosphate (*i.e.* adenosine is not the true product of enzymic hydrolysis), or that adenosine is such a weak substrate that exchange between inorganic phosphate and the solvent is too slow to be observed. Slow exchange could result from the alcohol (adenosine) not being recognised by the enzyme in the absence of the phosphate group.

In separate studies, adenosine was tested as an inhibitor for inositol monophosphatase. The nucleoside showed no inhibitory properties at almost saturating concentrations (up to 30 mM) with regard to the hydrolysis of (+/-) inositol 1-phosphate or adenosine 2'-monophosphate. (Inhibition studies were performed at a substrate concentration of 0.1 mM for inositol 1-phosphate and a substrate concentration of 5 mM for adenosine 2'-monophosphate.)

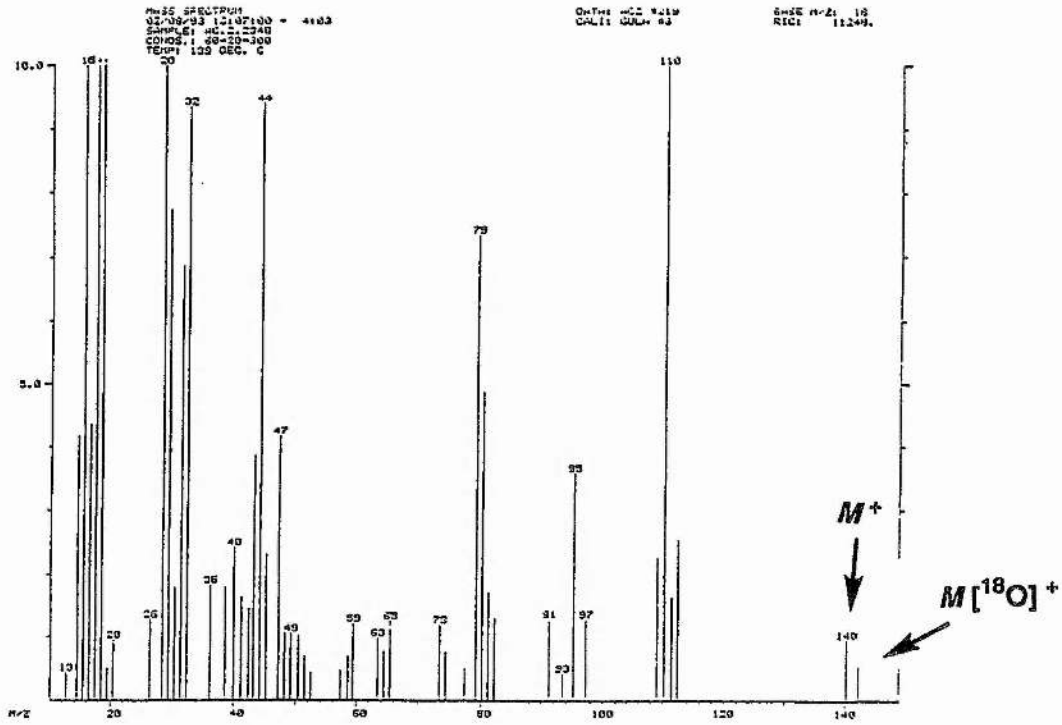


Figure 2.3.1. Expanded mass spectrum of derivatised inorganic phosphate isolated from enzymic incubations containing inositol and [^{18}O]-water; Also see Figure 2.3.3.

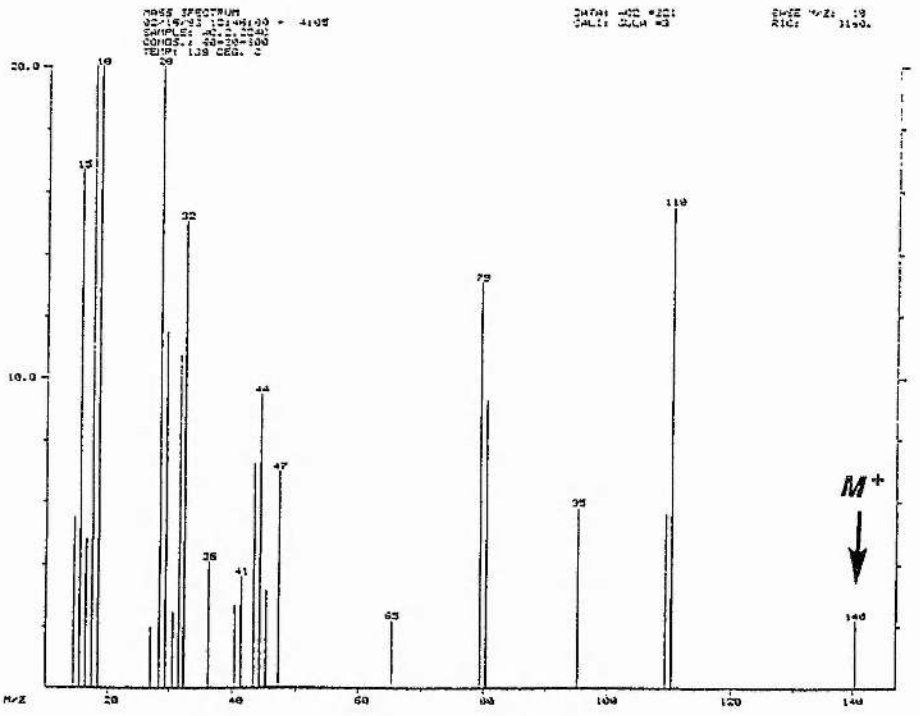


Figure 2.3.2. Expanded mass spectrum of derivatised inorganic phosphate isolated from enzymic incubations containing adenosine and [^{18}O]-water; Also see Figure 2.3.4.

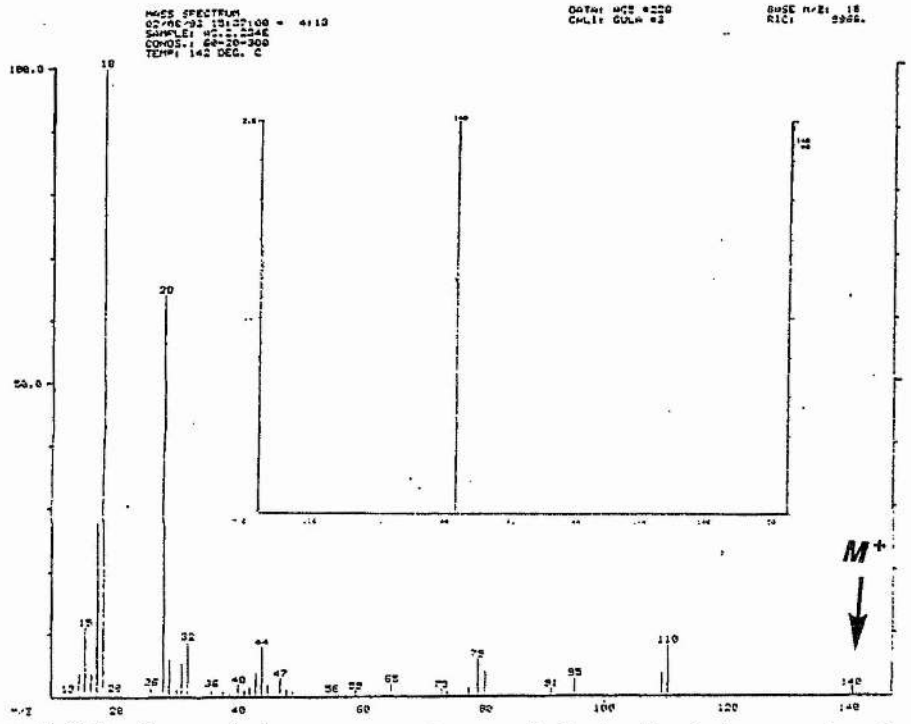


Figure 2.3.3. Expanded mass spectrum of derivatised inorganic phosphate isolated from control incubations containing inositol and [^{18}O]-water.

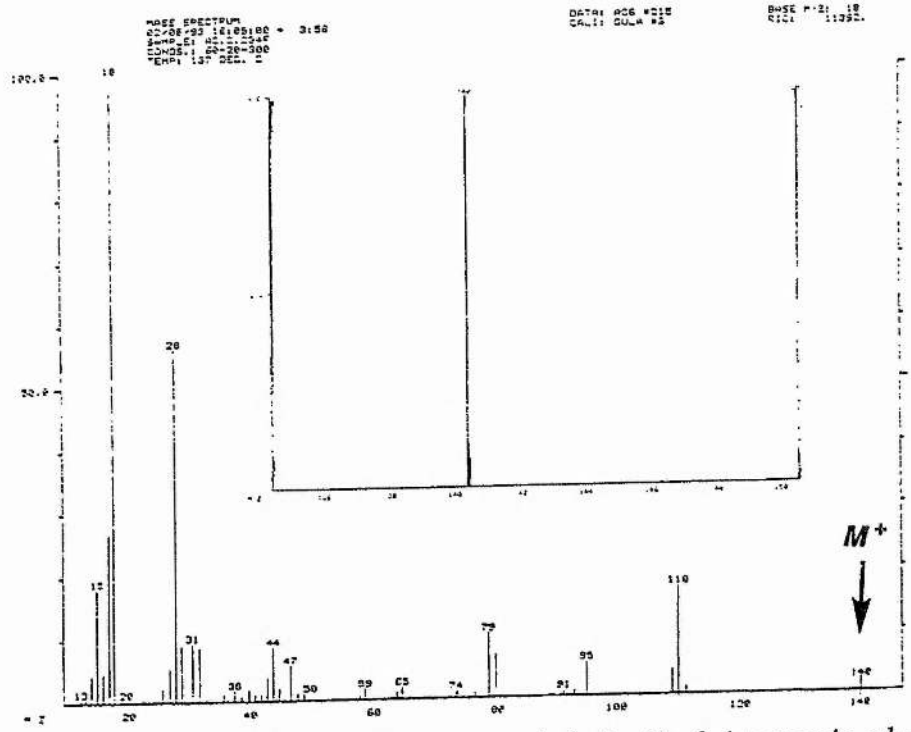
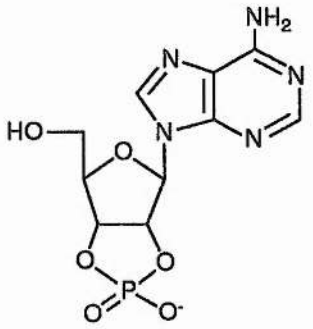


Figure 2.3.4. Expanded mass spectrum of derivatised inorganic phosphate isolated from control incubations containing adenosine and [^{18}O]-water.

In addition to the isotope exchange experiments, adenosine 2',3'-cyclic monophosphate (**c40**, Figure 2.3.5) was tested as a possible substrate. Although the formation of nucleoside cyclic phosphates is a phenomenon normally associated with nucleotidases (Scheme 1.5.2, type c), the possible involvement of the 3'-OH was considered. If the hydrolysis did occur through such a cyclic intermediate, the enzyme should be capable of processing 2',3'-cAMP (**c40**). Incubation (in a 50 mM Tris·HCl, 150 mM KCl, 2 mM $MgCl_2$ buffer system) at 37 °C with concentrations of cAMP up to 100 mM showed no formation of inorganic phosphate, even with extended incubation periods.



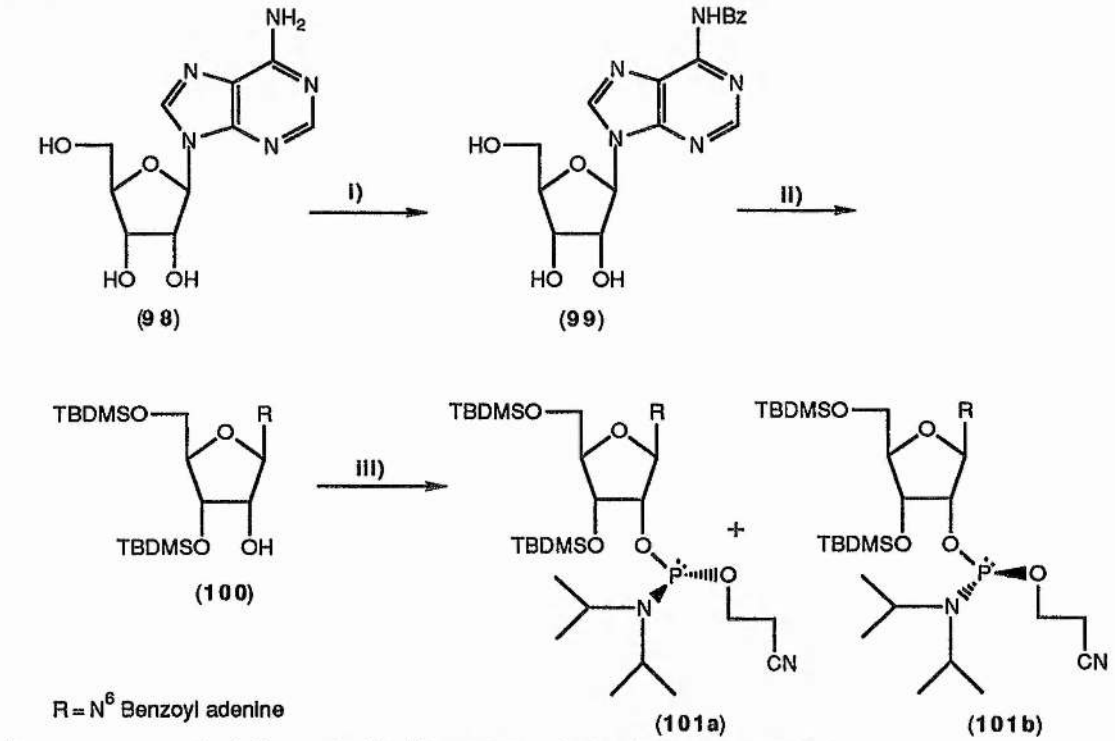
(c40)

Figure 2.3.5. Adenosine 2',3'-cyclic monophosphate.

2.4 Synthesis of adenosine 2'-phosphorothioate.

As in the case of the inositol phosphate synthesis, specific protection of the parent alcohol is required to ensure the production of the desired isomer of the thiophosphate. Treatment of adenosine (**98**) with excess benzoyl chloride in pyridine followed by base hydrolysis (aqueous sodium hydroxide in pyridine) of the undesired benzoyl esters afforded N^6-benzoyl adenosine (**99**). The desired product (**99**) was separated from the unwanted benzoic acid by acidic precipitation and was recrystallised from aqueous methanol. 3',5'-*t*-Butyldimethylsilyl bis-etherification[166,167] was carried out in low yield to give the fully protected alcohol for phosphitylation (**100**). Due to the lack of selectivity of secondary alcohol silylation, an isomeric resolution step is required to remove the unrequired 2'5'-bis-*O*-protected adenosine derivative. Initial attempts to obtain a protected alcohol suitable for phosphitylation with trimethylsilyl chloride proved unsuccessful. Treatment of N^6-benzoyl adenosine (**99**) with 2.2 equivalents of trimethylsilyl chloride gave an isomeric mixture of

the 3',5'- and 2',5'-bis-*O*-protected adenosine derivatives (as observed by TLC). After separation of the components of the mixture by silica chromatography, the desired product was found to be unstable, readily isomerising in solution (most rapidly in methanol and DMSO). The *t*-butyldimethylsilyl ethers proved to be stable, and showed only slow isomerisation in polar solvents such as methanol. (TLC 2',3',5'-tris-*O*-TBDMS N^6-benzoyl adenosine, R_f = 0.93, 2',5' bis-*O*-TBDMS N^6-benzoyl adenosine, R_f = 0.67, 3',5' bis-*O*-TBDMS N^6-benzoyl adenosine, R_f = 0.58 (50% ethyl acetate/ petroleum ether)). The alcohol (3',5' bis-*O*-TBDMS-N^6-benzoyl adenosine (**100**)) was phosphitylated with N,N'-diisopropyl-(2-cyanoethyl)chlorophosphoramidite in its racemic form (Scheme 2.4.1), which resulted in the formation of a pair of diastereomers which were separated by silica gel chromatography (0-30% ethyl acetate/ petroleum ether) on triethylamine basified silica. (High R_f diastereomer, δ_H 4.86 (1H, dt, 2'H), Low R_f diastereomer δ_H 4.70 (1H, dt, 2'H)).



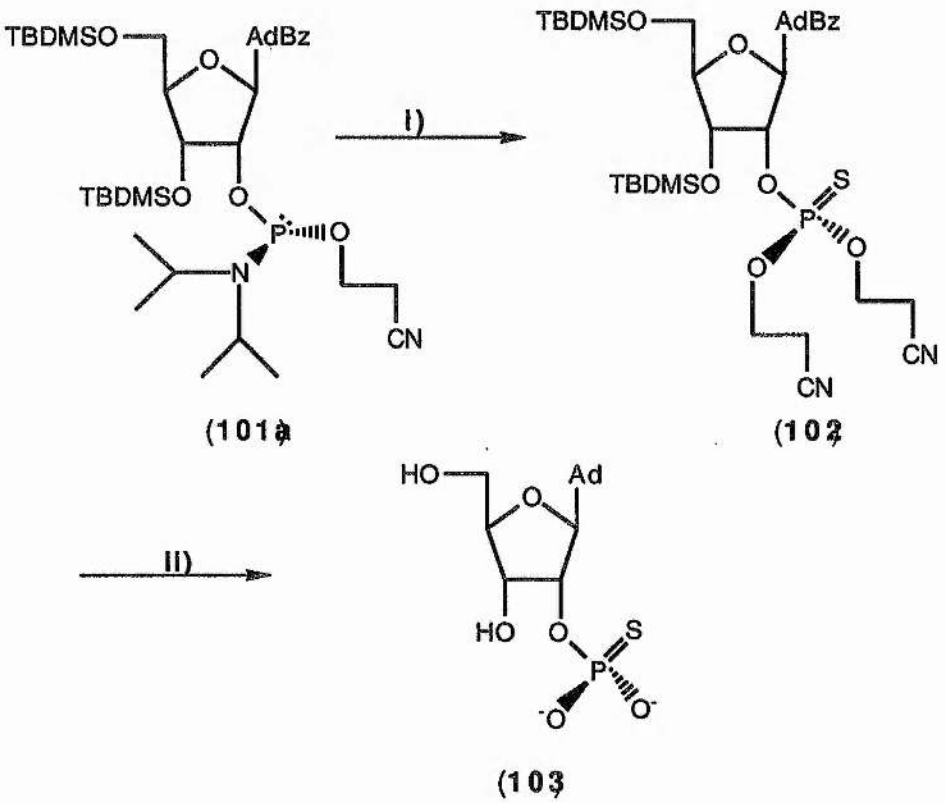
Reagents: I) a) Benzoyl chloride, pyridine, b) NaOH, pyridine, 71%;
II) a) TBDMSCl, imidazole, DMF, b) separate isomers, 39%,
III) a) N'N-diisopropyl(2-cyanoethyl)chlorophosphoramidite, DMAP, *i*-Pr_2NEt, THF, b) separate diastereomers, 76%.

Scheme 2.4.1. Synthesis of N'N-diisopropyl cyanoethyl adenosine diastereomers.

Initially transesterification was performed on the racemic phosphoramidite using cyanoethyl alcohol and 1-H tetrazole in dry acetonitrile. Analysis of the phosphite intermediate by ^{1}H-NMR spectroscopy showed that the reaction had proceeded to at least 70% with respect to the desired product (based on the integration of the 1'-H doublets in the crude reaction mixture). Re-dissolution of the residue in pyridine and treatment with an excess of elemental sulfur (re-sublimed) gave the fully protected phosphoramidite, proceeding quantitatively as judged by ^{1}H-NMR spectroscopy. Isolation of the desired product by silica column chromatography on triethylamine basified silica gave the desired compound in disappointing yield (48%). Decomposition of the bis-cyanoethyl phosphoramidite was considered as a possible cause of the low yield (given that the desired product accounted for approximately 70% of the total nucleoside content in the reaction mixture as determined by ^{1}H-NMR spectroscopy). The transesterification and oxidation were repeated using each of the separated diastereomers and shown to give the same compound (as shown by TLC ^{1}H- and ^{13}C-NMR spectroscopy), again in disappointing yield. Purification of the reaction mixture at the phosphite stage did not result in an improved overall yield for the reaction. Production of the protected phosphorothioate without isolation of the phosphoramidite diastereomers gave (**102**) in 39% yield from (**100**). Deprotection of compound (**102**) was undertaken, initially with alcoholic ammonia/ concentrated ammonia using a modification of the method of Usman *et al.*[168] to remove the cyanoethyl groups and cleave the N^{6}-amide. The reaction was conducted at 60 °C in a pressurised vessel to prevent evaporation of the dissolved ammonia gas. Decyanoethylation appeared to be rapid, whereas debenzoylation was slow. Isolation of the silyl protected nucleoside phosphorothioate was achieved by simple evaporation of the solvent followed by treatment of the residue with diethyl ether. The desired product formed a white precipitate which was desilylated without further purification. Tetra-*n*-butyl ammonium fluoride was employed as the desilylating agent, and on completion, the solvent was removed *in vacuo* and the product dissolved in water and applied to an ion exchange column of Amberlite IRA 118 $(H)^{+}$ (pretreated with cyclohexylamine and washed to neutral pH with water). Initial attempts to isolate the product as the free acid (by ion exchange on Amberlite IRA 118 $(H)^{+}$) proved unsuccessful,

possibly due to the insolubility of adenosine phosphorothioate in the form of the free acid in a neutral aqueous medium. (Adenosine 2'-monophosphate free acid has an extremely low solubility product in water.) Isolation of the compound as the bis-cyclohexylammonium salt gave adenosine 2' monophosphorothioate (**103**) in 53% yield from (**102**) (Scheme 2.4.2).

In order to make the substrate analogue chiral at the phosphorus centre, the possible manipulation of the separated phosphoramidite diastereomers (**101a & 101b**) was investigated. If the transesterification step was stereospecific, transesterification of a single diastereomer using ^{18}O-labelled cyanoethyl alcohol would allow the introduction of a single chirality at the phosphorus centre. The production of cyanoethyl alcohol was therefore required in a manner that would allow isotopic incorporation in good yield.

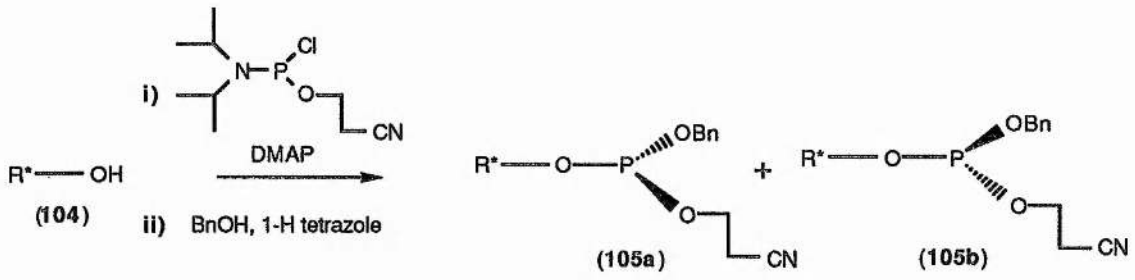


Reagents: I) a) 3-hydroxypropionitrile, 1-H tetrazole, CH_3CN, b) S_8, pyridine,39%, II) a) NH_4OH/ EtOH (3:1), b) TBAF, THF, c) Amberlite 118 (cyclohexylammonium)$^+$, 53%.

Scheme 2.4.2. Synthesis of adenosine 2'-monophosphorothioate.

Hence, Michael condensation,[169] of acrylonitrile with [^{18}O]-water was investigated as a method of producing [^{18}O]-3-hydroxy propionitrile. Reaction of acrylonitrile with water in the presence of triethylamine gave none of the desired material. In addition, extensive acrylonitrile polymerisation was observed when the reaction was conducted in the light. The eventual use of a catalytic quantity (< 5%) of sodium metal with a 1:1 ratio of acrylonitrile to water in acetonitrile in the dark gave the desired product in 81% yield after distillation. The use of an excess acrylonitrile resulted in the formation of unwanted bis-cyanoethyl ether (as determined by the change in boiling point and the reduction in intensity of the acidic proton in the ^{1}H-NMR spectrum).

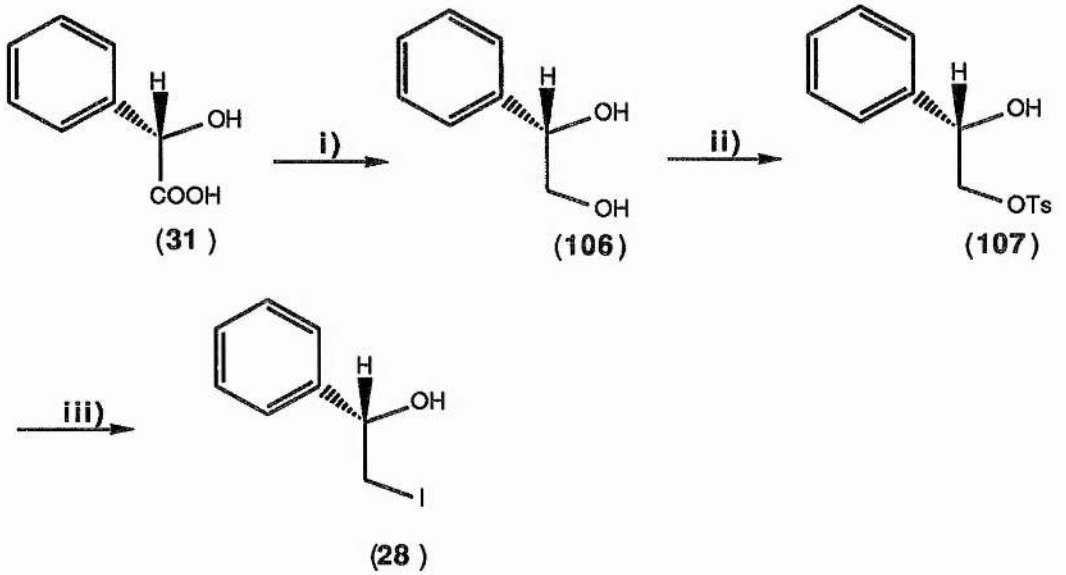
To determine the stereoselectivity of transesterification, a single phosphoramidite diastereomer (**101a or 101b**) was reacted with benzyl alcohol in the presence of 1-H tetrazole. This resulted in the formation of a pair of phosphite diastereomers (**105a &105b**) identical to those obtained from reaction of a mixture of the phosphoramidite diastereomers (**101a & b**) with benzyl alcohol in the presence of 1-H tetrazole. Synthesis of the nucleoside thiophosphate in a chiral form would therefore require transesterification of the unpurified phosphoramidite diastereomers with, for example [^{18}O]-benzyl alcohol,[86] followed by diastereomeric resolution (Scheme 2.4.3). Simple oxidation of the separate phosphite diastereomers, followed by deprotection would give access to R_P- and S_P-adenosine 2'-phosphorothioate.



Scheme 2.4.3 Alcohol phosphitylation and phosphoramidite transesterification.

For the purpose of stereochemical analysis of chiral thiophosphate, (S)-2-iodophenylethanol (**28**) was synthesised from (S)-mandelic acid (**31**) by the method of Bethell and Lowe (Scheme 2.4.4).[103] Lithium aluminium hydride

reduction of mandelic acid (**31**) followed by regioselective pyridine catalysed tosylation of the primary alcohol yields 1-(S)-phenylethanol 2-tosylate (**107**) in 71% from (**31**). S_N2 tosyl displacement by iodide (in the form of tetra-*n*-butyl ammonium iodide) gives (S)-2-iodophenylethanol (**28**), to which the inorganic thiophosphate hydrolysis product can be coupled (See section 1.6).[103] In addition, inorganic thiophosphate was synthesised by the method of Akerfeldt[170] (δ_P 32.3 ppm) to couple to compound (**28**). S_N2 Displacement of iodide by the sulfur atom of inorganic thiophosphate (See page 22) allows the production of TLC and NMR standards for the alkylated thiophosphate.



Reagents: I) $LiAlH_4$, THF, 83%, II) TsCl, pyridine, 86%;
III) tetra-*n*-butyl ammonium iodide, PhH, 76%.

Scheme 2.4.4 Synthesis of (S)-iodophenylethanol.

2.5 Adenosine 2'-phosphorothioate as a substrate for inositol monophosphatase.

Incubation of the adenosine 2'-phosphorothioate with inositol monophosphatase under optimum hydrolysis conditions in the presence of Mg^{2+} did not lead to a reaction as judged by ^{1}H-NMR spectroscopy in comparison to controls containing no enzyme. Extended incubation times led to minimal hydrolysis (*ca.* 10%) as determined by the appearance of a signal at δ_H 5.95 ppm assigned as 1'-H of adenosine.

Knowles *et al.* have reported the use of alternative metal ions in the enzymic processing of phosphorothioate substrates by magnesium dependent phosphatase enzymes.[171] Thus utility of the thiophilic metal ion Co^{2+} in the hydrolysis of adenosine 2'-phosphorothioate by inositol monophosphatase resulted in an increase in the rate of reaction in comparison to similar incubations containing magnesium. The initial rate of hydrolysis of adenosine 2'-phosphorothioate in the presence of Co^{2+} was approximately 50 fold slower than the hydrolysis of 2'-AMP in the presence of Mg^{2+}. In addition to increased rates of hydrolysis, the formation of a black precipitate was also observed. Separate experiments involving the solvation of differing amounts of inorganic thiophosphate (in the form of its tri-lithium salt) in the cobalt containing buffer gave an identical black precipitate. This suggests that a cobalt-thiophosphate complex forms between the hydrolysis product (inorganic thiophosphate) and the cobalt cofactor. In accord with this, it was noted that as the precipitate became more dense, the rate of hydrolysis slowed dramatically, even in the presence of excess substrate.

Additional metal ion systems used with adenosine 2'-phosphorothioate and inositol monophosphatase included Zn^{2+}, a Zn^{2+} and Mg^{2+} cocktail (1:1) and Mn^{2+}. The Zn^{2+} and the Zn^{2+}/ Mg^{2+}assays showed minimal hydrolysis of the substrate analogue. Monitoring the incubation containing Mn^{2+} by NMR spectroscopy was difficult due to manganese possessing a nuclear quadrupolar moment. This resulted in extensive broadening of all the signals in the ^{1}H-NMR spectrum, and meant that identification of the 1'-H doublets of the substrate analogue and the hydrolysis product (adenosine) could not be achieved. The formation of a pale cream precipitate was however evident in the adenosine 2'-phosphorothioate/ Mn^{2+} enzymic reaction mixture. The dissolution of inorganic thiophosphate in the manganese containing buffer gave an identical pale cream precipitate, again indicating that precipitation results from the formation of a metal ion-thiophosphate complex. Subsequent examination of the ^{1}H-NMR spectrum revealed that all of the starting material had been processed to adenosine, with no line broadening present. The ^{1}H-NMR spectrum of the control reaction, which contained no enzyme could not be accurately interpreted due to the Mn^{2+}-dependent line broadening. However, analysis of

the control reaction by TLC showed the absence of hydrolysis product. Although complete hydrolysis had been achieved, it was evident that levels of inorganic thiophosphate had been depleted through complexation with the metal ion co-factor. The deduction of the stereochemical course of the phosphate ester hydrolysis would require the isolation of the inorganic thiophosphate hydrolysis product, and as a result of these studies, the synthesis of the chiral phosphorothioate substrate analogue was not investigated further.

Other adenosine phosphates that were tested with the enzyme were adenosine 5'-monophosphorothioate[172] (produced in a crude form contaminated by adenosine), 5'-AMP and 2',3'-cyclic AMP, none of which showed any change in comparison with controls containing no enzyme.

2.6 Hydrogen bonded water molecule in the hydrolysis of inositol 1-phosphate.

Examination of the results from the Merck Sharpe and Dohme laboratories[114-117] which highlight the roles of the α-hydroxyl groups of inositol 1-phosphate (**33**) (see section 1.9) shows an analogy between adenosine 2'-monophosphate and inositol 1-phosphate. There is good overlap between C-2 OH and C-4 OH of D-inositol 1-phosphate with C-3' OH and C-5' OH of 2'-AMP respectively as determined by molecular modelling. This suggests that these groups on nucleoside 2'-phosphates are involved in binding to the enzyme (Figure 2.6.1). The absence of a catalytic hydroxyl group on 2'AMP (**40**) and also the apparent inability of the enzyme to catalyse phosphate-oxygen ligand exchange in the presence of adenosine (see section 2.3) appears to suggest a difference in the mechanism of hydrolysis of the two substrates by inositol monophosphatase. The presence of C-6 OH in D-Ins 1-P is known to be an absolute requirement for catalysis.[116] Comparison of (**33**) and (**40**) (Figure 2.6.1) with regard to the three dimensional arrangement of the functional groups presents two possibilities. Either the ribose ether oxygen is acting as a surrogate for the catalytic hydroxyl group of inositol 1-phosphate, and has a similar 'mechanistic' role, or the nucleoside base (adenine) is in some way involved in the hydrolysis reaction.

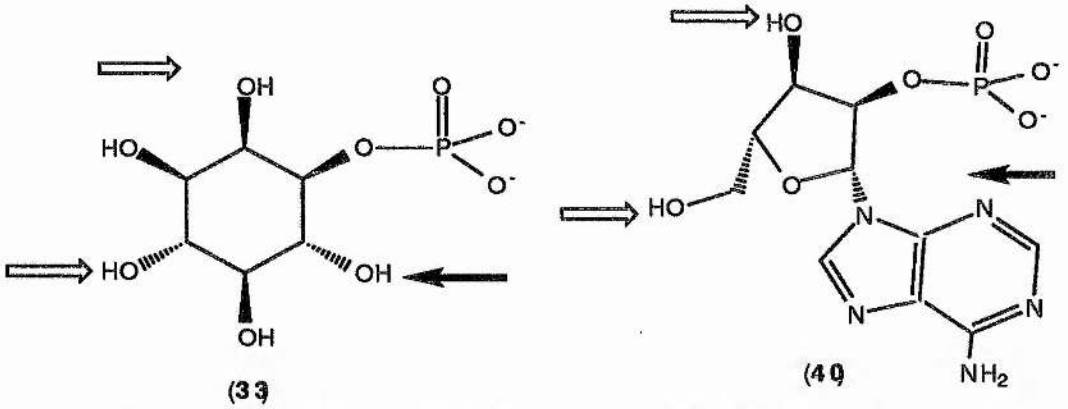


Figure 2.6.1. Proposed binding groups of D-inositol 1-phosphate and adenosine 2'-monophosphate.

Due to the fact that C-6 OH is required for catalytic activity in the hydrolysis of D-Ins 1-P, it seems reasonable that its function is to hydrogen bond a water molecule, which is actually the attacking species in phosphate ester hydrolysis. This would result in the formation of a seven membered ring, with nucleophilic attack by water being essentially intramolecular. Also, molecular modelling has shown that the adenine N^3-nitrogen of 2'-AMP (**40**) occupies the same three dimensional space as the proposed attacking water molecule, resulting in a similar seven membered ring being formed (Figure 2.6.2).

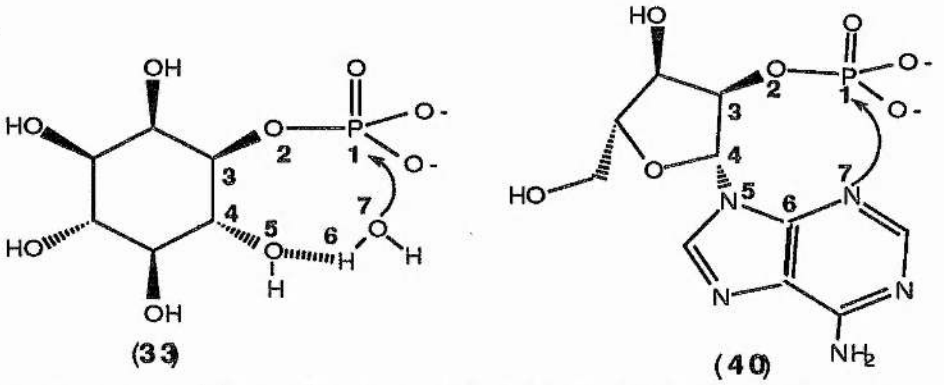
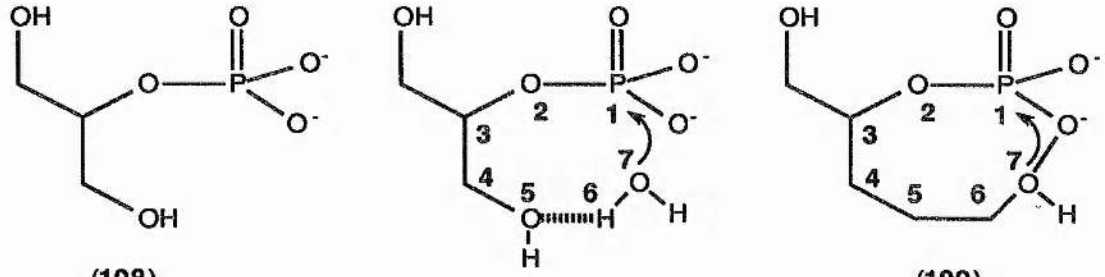


Figure 2.6.2. Possible mechanism of hydrolysis of inositol 1-phosphate and adenosine 2'-monophosphate.

To probe the mechanism of the enzyme further, a number of analogues of inositol and glycerol phosphates have been synthesised. In addition, to determine whether a C-6 OH hydrogen bonded water molecule is present and involved in the hydrolysis, analogues of glycerol 2-phosphate (**108**) replacing the catalytic hydroxyl group by a hydroxyethylene group (Figure 2.6.3) were

synthesised (**109**) (See section 2.8). If there is a hydrogen bonded water molecule involved as hypothesised, enzyme catalysed phosphoryl transfer between 2-OH and 5-OH should be detectable in compound (**109**) by ^{1}H-NMR spectroscopy.

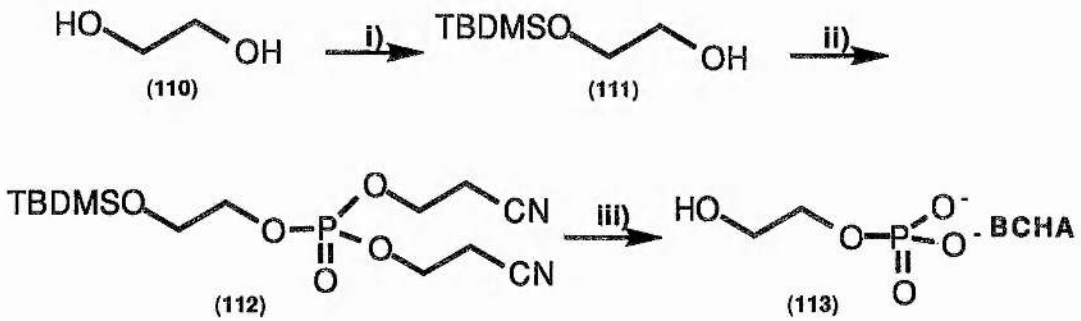


(**108**) (**109**)

Figure 2.6.3. Phosphoryl transfer through replacement of one of the possible catalytic hydroxyl group of glycerol 2-phosphate.

2.7 Synthesis and incubation of ethane 1,2-diol monophosphate.

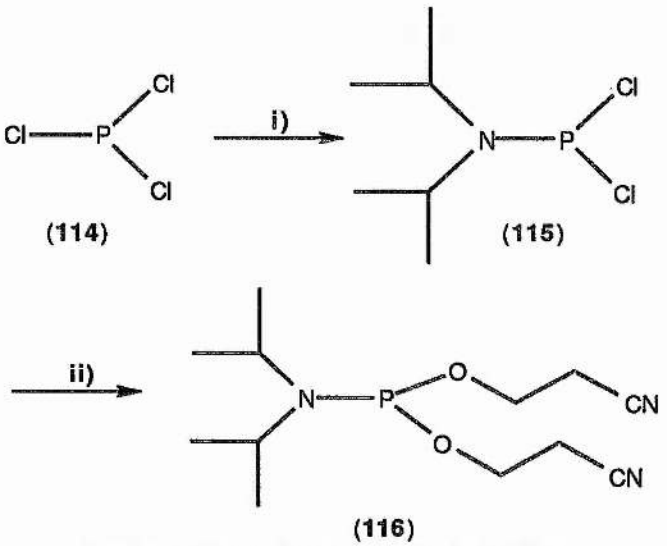
The simplest example of a monophosphate that should be hydrolysed by the enzyme is that of ethane 1,2-diol monophosphate (**113**). The presence of a single α-hydroxyl group should allow hydrolysis to occur, however, the absence of further hydroxyl groups to fulfil the structural binding requirements means the substrate should be weak. The important feature is that ethane 1,2-diol monophosphate (**113**) will be processed by the enzyme, which will show that the presence of essentially no binding hydroxyl groups (comparable to 2-OH and 4-OH of D-inositol 1-phosphate) will not cause complete inactivation.



Reagents: **I)** TBDMSCl, imidazole, DMF; **ii)** a) N'N-diisopropyl biscyanoethyl phosphoramidite, 1-H tetrazole, CH_3CN; b) *m*-CPBA, CH_2Cl_2, 54%; **iii)** a) NaOMe, MeOH; b)TBAF, THF, c) cyclohexylamine, H_2O60%.

Scheme 2.7.1. Synthesis of ethane 1,2-diol monophosphate.

Initial protection as the mono *t*-butyldimethyl silyl ether (**111**) conducted with a five fold excess of ethylene glycol (**110**) to ensure that the monoprotected glycol was the sole product was followed by immediate phosphitylation using N'N-diisopropyl bis-cyanoethyl phosphoramidite (**116**) (Scheme 2.7.2) The resulting phosphite was oxidised to the phosphate with *m*-CPBA (Scheme 2.7.1). The bis-cyanoethyl phosphate (**112**) was purified by silica column chromatography, and deprotected firstly with sodium methoxide and secondly with tetra-*n*-butyl ammonium fluoride. Ion exchange chromatography (Amberlite 118 $(H)^+$) allowed isolation of the compound as the free acid, and the removal of the tetra-*n*-butyl ammonium counter-ion. Conversion to the biscyclohexylammonium salt and recrystallisation from water and acetone yielded the desired compound in 33% yield from ethylene glycol as the di-hydrate. Incubation of the glycerol 2-phosphate analogue (**113**), showed, as expected slow substrate activity as determined by both NMR spectroscopy and by the colormetric assay.[173]



Reagents: I) *i*-Pr_2NH, Et_2O, 68%; II) $HO(CH_2)_2CN$, Et_3N, CH_2Cl_2, 87%.

Scheme 2.7.2. Synthesis of N,N'-diisopropyl biscyanoethyl phosphoramidite phosphitylating agent.

The substrate analogue (**113**) was processed by the enzyme at a rate (V_{max}) of between 10-12% that of (+/-) inositol 1-phosphate (**33**), and a K_m value of approximately 0.7 mM (Figure 2.7.4) (compared with around 0.1 mM for (+/-) Ins 1-P, Figure 2.7.3). When following the reaction course by ^{1}H-NMR

spectroscopy, the initial rate can be thought of as comparable to V_{max} since the substrate is present at a concentration of 50 mM (several tens of times K_m). The rate of hydrolysis becomes increasingly slower with time due to product inhibition caused by inorganic phosphate (K_i = 0.5 mM), which is present in an equimolar amount with respect to substrate after hydrolysis has proceeded to 50%. As expected ethane 1,2-diol monophosphate (**113**) was also a weak competitive inhibitor for the enzyme (K_i = 1.0 mM) (Figure 2.7.5).

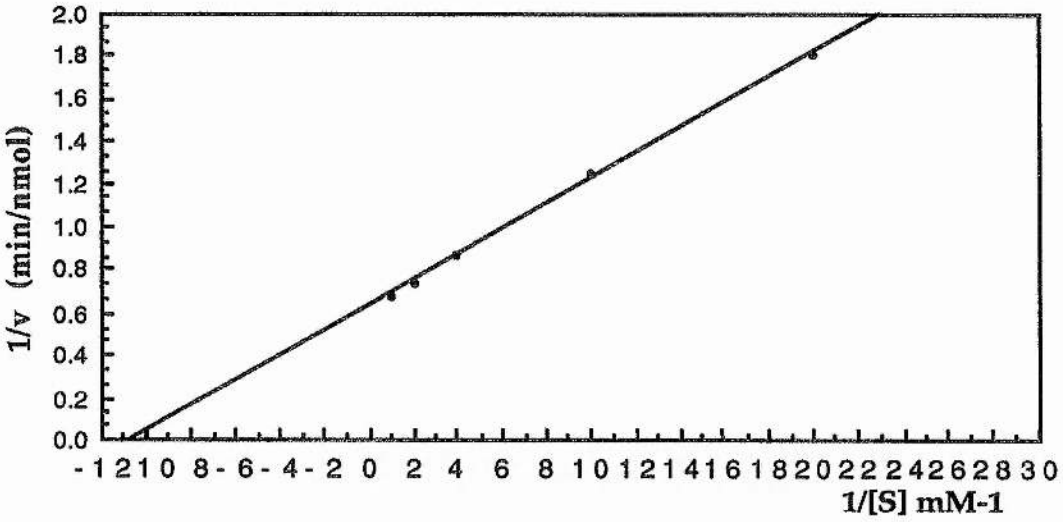


Figure 2.7.3 Lineweaver-Burk double reciprocal plot for (+/-) inositol 1-phosphate.

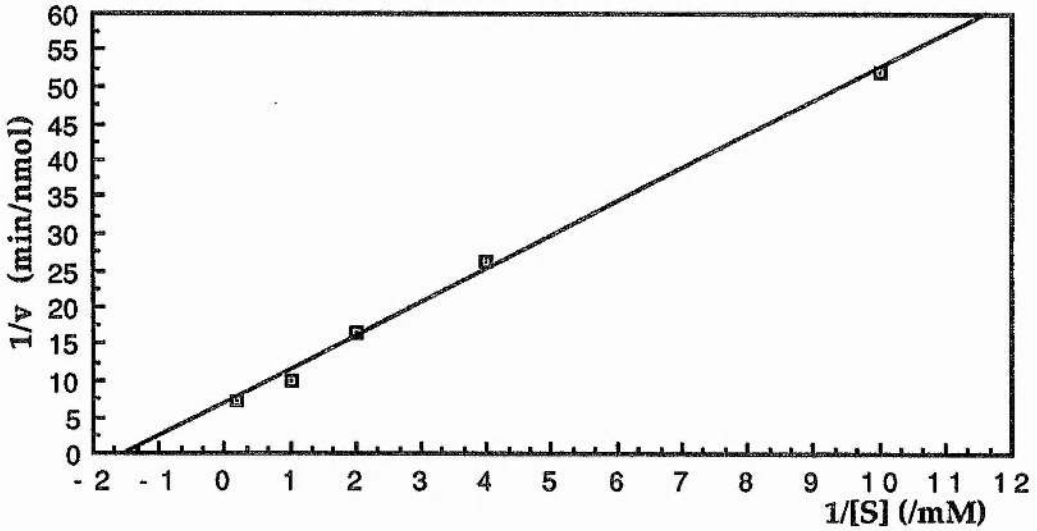


Figure 2.7.4 Lineweaver-Burk double reciprocal plot for ethane 1,2-diol monophosphate.

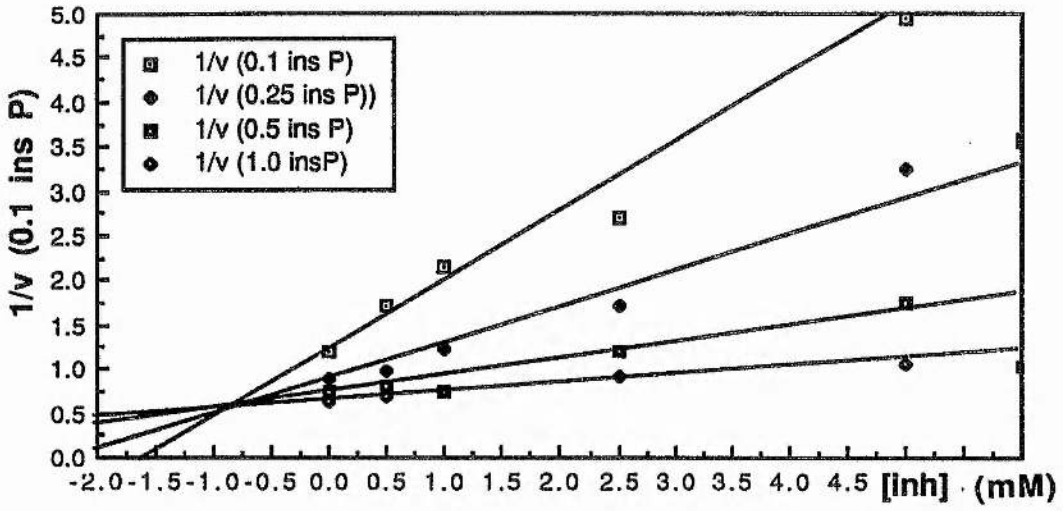


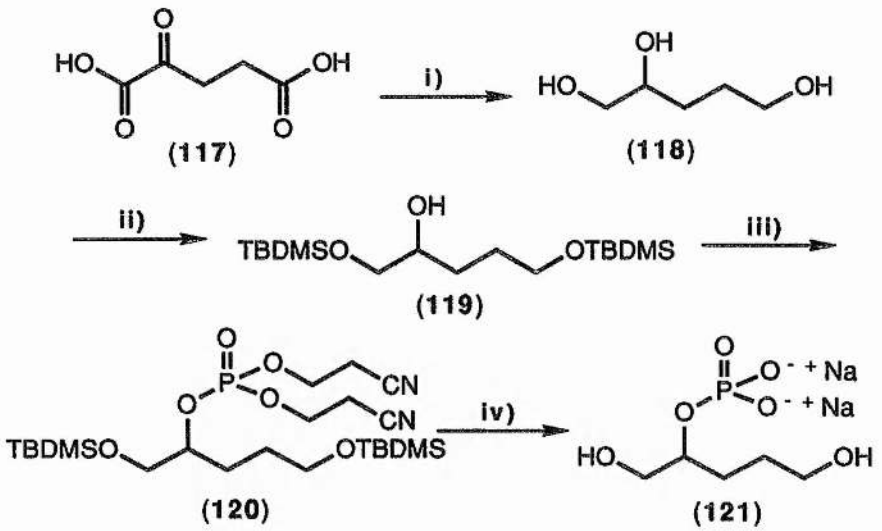
Figure 2.7.5 Dixon plot for (+/-) inositol 1-phosphate inhibited by ethane 1,2-diol monophosphate.

2.8 Synthesis and incubation of pentane 1,2,5-triol 2-phosphate

To determine whether a C-6 OH hydrogen bonded water molecule was present and involved in the hydrolysis of D-inositol 1-phosphate (Section 2.6), analogues of glycerol 2-phosphate ((SR)-, (S)- and (R)-pentane 1,2,5-triol 2-phosphate) were synthesised. The replacement of C-1 OH of glycerol 2-phosphate by a hydroxyethylene group should result in the substrate analogue occupying the same three-dimensional space as glycerol 2-phosphate possessing a water molecule hydrogen bonded to the C-1 OH. (See figure 2.6.3, page 62). If there is a hydrogen bonded water molecule involved as hypothesised, enzyme catalysed phosphoryl transfer between 2-OH and 5-OH should be detectable in compound (**109**) by ^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopy. Phosphate transfer would be evident from the upfield shift and loss of P,H coupling of the C-2H and C-2 signals in the ^{1}H- and ^{13}C-NMR spectra respectively.

Initial attempts to synthesise the racemic material started from allyl acetic (**122**) acid. Reduction with lithium aluminium hydride yielded pent-4-ene-1-ol in 84% yield after distillation (b.pt. 130 °C). Protection of the alcohol as the benzyl ether followed by treatment with metachloroperbenzoic acid gave 1-*O*-benzylpentane-4,5-epoxide (*m/z* (Found: $[M + H]^+$ 193.1229. $C_{12}H_{17}O_2$ requires

193.1228)). Opening of the epoxide at C-5 with benzyl alcohol and sodium hydride (benzoxide) would have given the required protected pentane triol for phosphorylation. Extended reaction times and the application of heat to the reaction mixture gave none of the desired product, so the use of alternative starting materials was investigated. α-Keto glutaric acid (**117**) was identified as a suitable starting material with respect to the arrangement of the oxygen functionality. Complete reduction with lithium aluminium hydride gave the parent alcohol, pentane 1,2,5-triol (**118**) in moderate yield (45%). Soxhlet extraction of the inorganic precipitate with acetone did not give a substantial increase in the overall yield of the reaction. Protection of the two secondary alcohols with TBDMSCl and imidazole afforded the fully protected alcohol (**119**) (Scheme 2.8.1). Phosphitylation as in the synthesis of ethane 1,2-diol monophosphate (**113**) with N,N'-diisopropyl bis-cyanoethyl phosphoramidite (**116**) followed by oxidation gave the crude fully protected phosphate (**120**), (δ_H 1.65, (4H, t, 3-H_2 and 4-H_2) 2.75 (4H, t, cyanoethyl CH_2CN), 3.65 (4H, m, 1-H_2 and 5-H_2), 4.30 (5H, m, $POCH_2$ and 2-H)). Attempted purification of the bis-cyanoethyl phosphate by silica column chromatography on triethylamine basified silica gave a very poor recovery of material (< 5%). Deprotection of the crude bis-cyanoethyl phosphate (**120**) with sodium methoxide in methanol followed by tetra-*n*-butyl ammonium fluoride desilylation resulted in the production of the crude phosphate (**121**). Ion exchange chromatography on Amberlite IRA 118 $(Na)^+$ gave the product as the bis- sodium salt (**121**) (Scheme 2.8.1), which proved difficult to crystallise from aqueous acetone. Although a crystalline material was obtained, it was evident that inorganic phosphate arising from the phosphitylation step was present as an impurity (determined by the addition of acidic malachite green solution which detects the total phosphate present in a system).



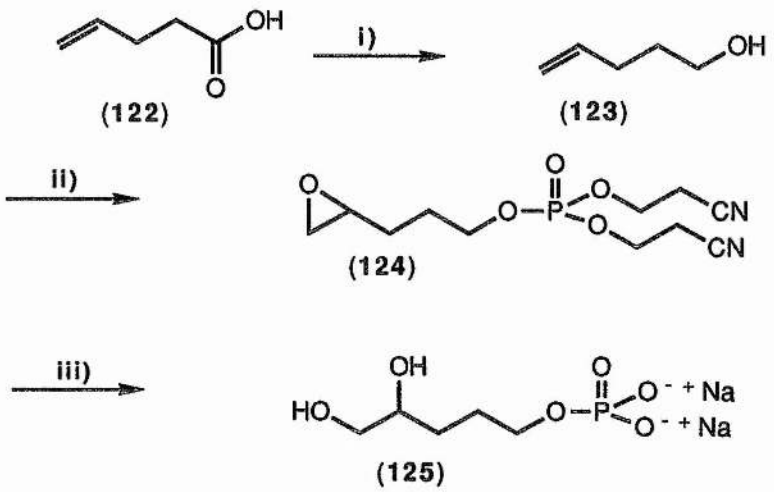
Reagents: **i)** $LiAlH_4$, THF, **ii)** TBDMSCl, imidazole, DMF, **iii)** a) $^{i}Pr_2NP(OCH_2CH_2CN)_2$ tetrazole, CH_3CN, b) *m*-CPBA, CH_2Cl_2 **iv)** a) MeONa/ MeOH, b) TBAF, THF, c) Amberlite 118 $(Na)^+$

Scheme 2.8.1. Synthesis of (+/-) pentane 1,2,5-triol 2-phosphate.

Enzymic assay of the crude material with inositol monophosphatase, following the reaction course by NMR spectroscopy showed no detectable transfer of the phosphate functionality to the terminal hydroxyl group or any appreciable hydrolysis. Due to the contamination by phosphate, and the apparent instability the bis-cyanoethyl phosphate (**120**) on silica, an alternative phosphitylating agent was used, namely N,N'-diisopropyl bis-benzyl phosphoramidite (**116a**) (which was prepared from diisopropyl dichloro-phosphoramidite (**115**) and benzyl alcohol). The resulting 1,5-bis-*O*-TBDMS-2-*O*-dibenzyl phosphate was stable to silica gel chromatography, and the product was isolated in good yield (78%). Deprotection firstly under an atmosphere of hydrogen gas in the presence of 10% palladium on activated charcoal catalyst, in methanol afforded complete debenzylation. Desilylation was again achieved with TBAF in THF, and the product isolated as the free acid (by ion exchange chromatography). Conversion to the bis-cyclohexylammonium salt followed by recrystallisation from water and acetone yielded the desired compound as the

dihydrate in the form of white needles (m.pt. 165-167 °C)

Assaying of this phosphate against the enzyme showed no detectable substrate properties as judged by colormetric assay[173] and ^{1}H-NMR spectroscopy. In addition, transesterification was not detected. For TLC and NMR comparison, racemic pentane 1,2,5-triol 5-phosphate (**125**) was synthesised in three steps from allyl acetic acid (**122**). Reduction to pent-4-ene-1-ol (**123**) as described previously was followed by phosphitylation using N,N'-diisopropylbiscyanoethyl phosphoramidite (**116**). Oxidation of the phosphite to the phosphate was accompanied by epoxidation of the olefin (Scheme 2.8.2). The phosphate epoxide was purified by column chromatography on neutral alumina, and deprotected under basic conditions. Due to the fact that epoxides are relatively stable to nucleophilic attack under basic conditions, the removal of the cyanoethyl groups was achieved with sodium hydroxide in aqueous acetone, leaving the epoxide intact. On complete decyanoethylation, the solvent was removed *in vacuo* to prevent the formation of cyanoethyl alcohol through Michael attack by hydroxide on acrylonitrile. Epoxide opening was achieved using excess sodium hydroxide to give pentane 1,2,5-triol 5-phosphate (**125**).



(122) (123) (124) (125)

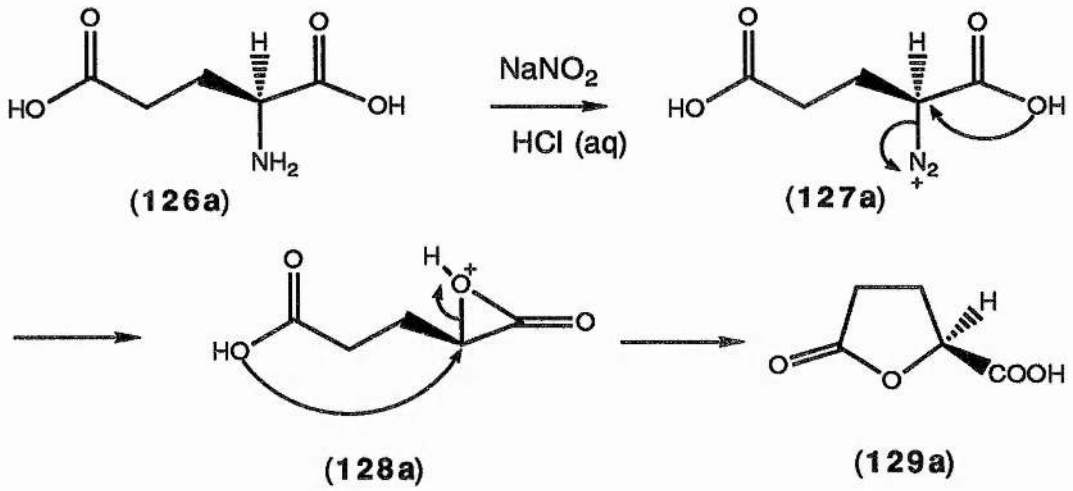
Reagents: I) $LiAlH_4$, THF, 86%, II) a) $^iPr_2NP(OCH_2CH_2CN)_2$ tetrazole, CH_3CN, b) *m*-CPBA, CH_2Cl_2, 63%; III) a) H_2O/ NaOH/ acetone, b) Amberlite 118 $(Na)^+$, 68%.

Scheme 2.8.2 Synthesis of pentane 1,2,5-triol 5-phosphate.

Initial purification firstly by ion exchange chromatography followed by conversion to the bis-cyclohexylammonium salt gave the product as a gum which resisted crystallisation. NMR spectroscopic and TLC analysis showed no terminal 5-phosphate was formed in the pentane 1,2,5-triol 2-phosphate enzyme incubation mixture. To determine whether the proposed transphosphorylation between the 2-OH and 5-OH was an equilibrium balanced in favour of the initial starting material (**121**), pentane 1,2,5-triol 5-phosphate was incubated with the enzyme and the course of the reaction followed. No changes were detected.

The inhibitory properties of racemic pentane 1,2,5-triol 2-phosphate (**121**) were assessed by a radiochemical assay using a known concentration of unlabelled inositol 1-phosphate and a small amount of [U^{14}C]-L-inositol 1-phosphate (with respect to concentration) (see experimental section) of known activity. The racemic material was shown to be a weak inhibitor of the enzyme in comparison to controls containing no inhibitor. To study the inhibitory properties in more detail, the syntheses of the (R)- and (S)-enantiomers were undertaken. Two methods of synthesis of the enantiomerically pure pentane 1,2,5 triol 2-phosphates were considered. Firstly, the formation of the camphanate ester of 1,5-bis-*O*-TBDMS pentane 1,2,5-triol (**119**) with camphanoyl chloride, followed by chromatographic diastereomeric resolution and chiral ester hydrolysis would allow the production of the chiral phosphate precursors (**119a & b**). Simple phosphorylation of the enantiomeric components, followed by deprotection, as in the case of the racemic material, would give the required compounds. Alternatively, (S)- and (R)-glutamic acid (**126**) could be used as chiral starting materials to provide the stereocontrol. Diazotisation of (S)- or (R)-glutamic acid using sodium nitrite in aqueous acid, would results in a two step double invertive process giving the lactone (**129**) resulting from attack by the γ-acid.[175,176] (Scheme 2.8.3). Complete reduction of the lactone (**129**) affords the parent alcohol, pentane 1,2,5-triol in a chiral form.

Hence, lactonisation by a modification of the method of Ravid *et al.*, followed by complete reduction with lithium aluminium hydride yields (R)- and (S)- pentane 1,2,5-triol, from which the desired phosphates were synthesised in an identical manner to that described for the racemic material



Scheme 2.8.3 Mechanism of lactonisation of glutamic acid.

Incubation of the individual enantiomers of pentane 1,2,5-triol 2-phosphate with inositol monophosphatase revealed that as with the racemic material, neither compound underwent transphosphorylation from C-2 to C-5. In addition, neither (S)- or (R)-pentane 1,2,5 triol 2-phosphate exhibited any significant substrate activity. Extremely slow enzymic hydrolysis of the pentane triol phosphates was observed in comparison to control reactions containing no enzyme, however, the rates of reaction were slow enough for the compounds to be classed as non-substrates. Examination of the inhibitory properties of the individual enantiomers revealed that the (2S)-enantiomer (**121a**) was a good competitive inhibitor (K_i = 0.12 mM, Figure 2.8.4), whereas the (2R)-enantiomer (**121b**) was a weak competitive inhibitor (K_i = 3.8 mM, Figure 2.8.5). These results are in total agreement with the inhibition data for the deoxyinositol phosphate analogues[114-117] (section 1.9) and show that although the potency of the inhibitors is reduced, non cyclic phosphate monoesters with the correct stereochemical arrangement of hydroxyl groups can bind effectively to the enzyme.

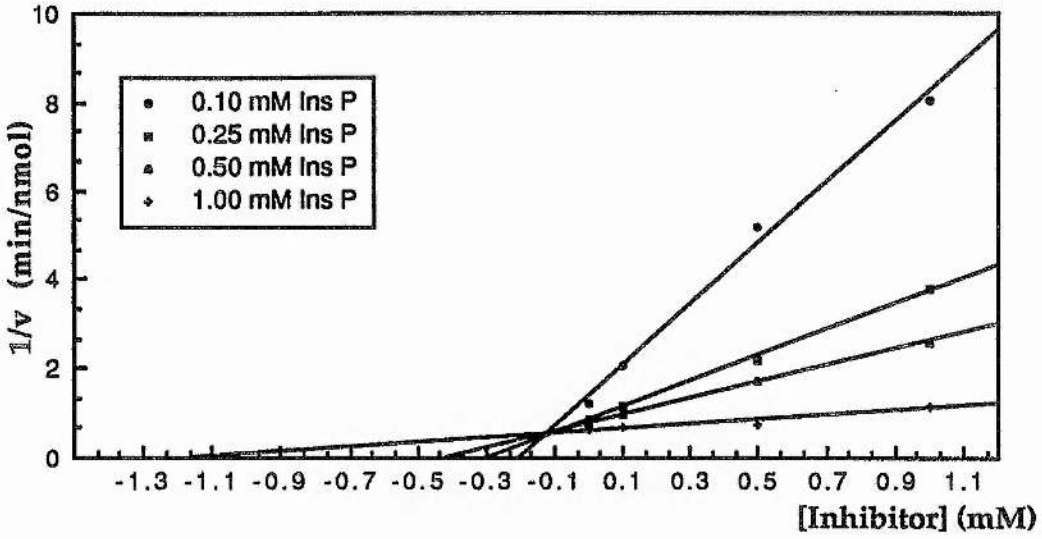


Figure 2.8.4 Dixon plot for (+/-) inositol 1-phosphate inhibited by (S)-pentane 1,2,5-triol 2-phosphate.

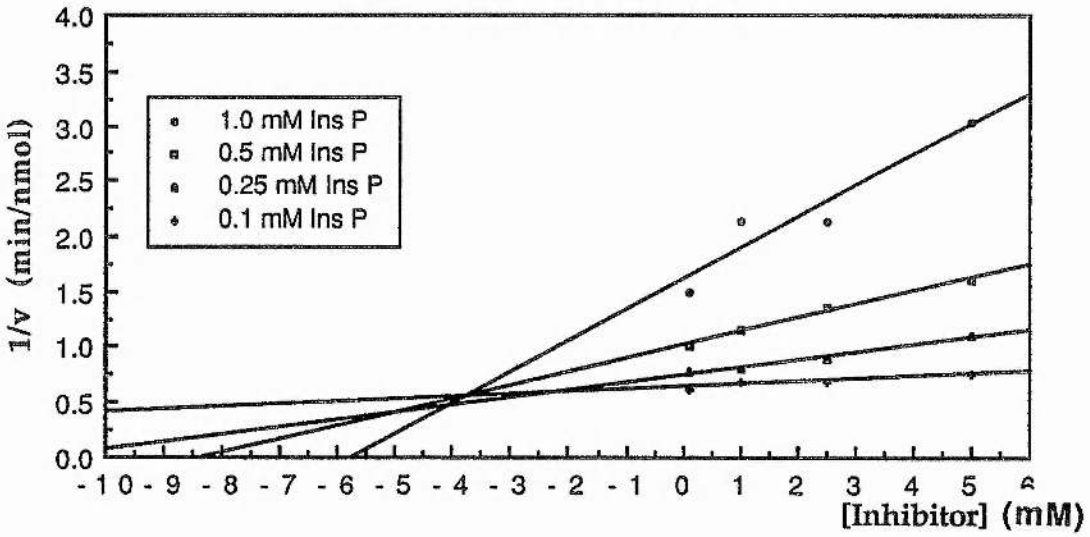
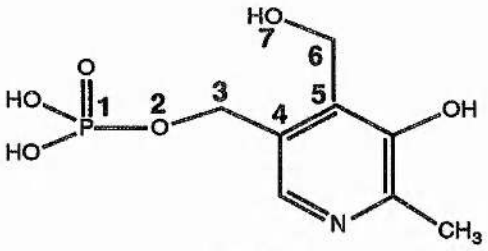


Figure 2.8.5. Dixon plot for (+/-) inositol 1-phosphate inhibited by (R)-pentane 1,2,5-triol 2-phosphate.

2.9 Alternative transphosphorylation substrates

Pyridoxol 5'-phosphate (**130**, figure 2.9.1) was assessed as a possible 1,7-transphosphorylation substrate for inositol monophosphatase. Reduction of pyridoxal 5'-phosphate with sodium borohydride in water yielded pyridoxol 5'-phosphate (**130**) (δ_H (^2H_2O) 4.80 (2H, s, 4'H_2), δ_P (^2H_2O) 3.98), with

contamination arising from boric acid (approximately 20%, as judged by ^{1}H-NMR spectroscopy using a known quantity of N'N-dimethyl formamide as an integration standard).



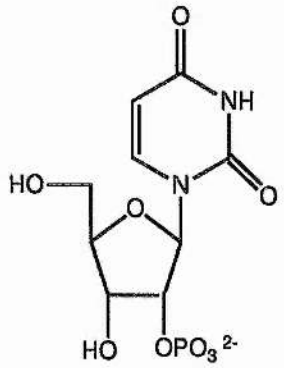
(130)

Figure 2.9.1 Pyridoxol phosphate.

On incubation of pyridoxol 5'-phosphate (**130**) with inositol monophosphatase, transphosphorylation was not detected. Analysis of the mixture as an inhibitor of the enzyme (by the radiochemical assay) in parallel with incubations containing known concentrations of boric acid revealed that pyridoxol phosphate showed no appreciable inhibitory properties and that boric acid acted as a weak inhibitor (probably inhibiting in the same manner as inorganic phosphate).

2.10 New nucleoside substrates of inositol monophosphatase.

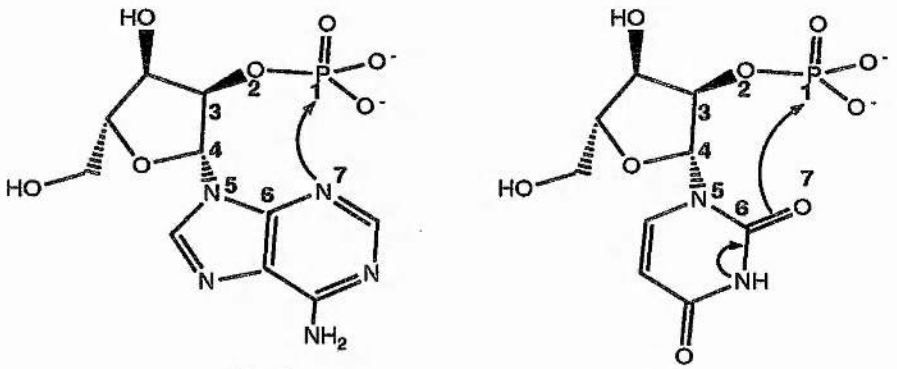
To investigate the possible involvement of the N^3-ntrogen of the adenine ring in the hydrolysis of adenosine 2'-monophosphate (**40**), uridine 2'-monophosphate (**131**, figure 2.10.1) was tested with inositol monophosphatase.



(131)

Figure 2.10.1 Uridine 2'-monophosphate.

Nucleophilic attack at the phosphorus centre by N^3- of adenine in the case of 2'-AMP (**40**), is analogous to attack by the urea carbonyl oxygen of uracil in the case of uridine 2'-monophosphate (**131**). Both systems involve the possible formation of similar seven membered ring intermediates (Figure 2.10.2).



(40) **(131)**

Figure 2.10.2 Comparison of structures of 2'-AMP and 2'-UMP.

The proposed phosphorylated uracil ring system (**132**) was predicted to be longer lived than the analogous pyridinium type phosphate species in the case of 2'-AMP. As uridine 2'-monophosphate is only commercially available as the di-lithium salt, cation exchange chromatography was performed prior to incubation as lithium is known to be an inhibitor of the enzyme (see section 1.3) when 2'-AMP and inositol 1-phosphate are substrates. Incubations ran in parallel contained the following:

1) uridine 2'-monophosphate di-sodium salt with enzyme;
2) uridine 2'-monophosphate di-lithium salt with enzyme;
3) uridine 2'-monophosphate di-sodium salt without enzyme;
4) uridine 2'-monophosphate di-lithium salt without enzyme;

Assignment of the ^{1}H-NMR spectra recorded at regular time intervals, revealed that hydrolysis of 2'-UMP by inositol monophosphatase was rapid, as shown by the reduction in intensity of the 2'-H multiplet of 2'-UMP at 4.65 ppm, and the appearance of an additional signal at 4.20 ppm, assigned as 2'H of uridine (Figure 2.10.3). In addition, the phosphate monoester and the parent alcohol (uridine) were the only species detected (Figure 2.10.3). Assay 2 was slow, showing that 2'-UMP hydrolysis is inhibited by lithium, whereas assays 3 and 4 showed no change by ^{1}H- or ^{31}P-NMR spectroscopy. Intramolecular nucleophilic displacement of phosphate was not observed.

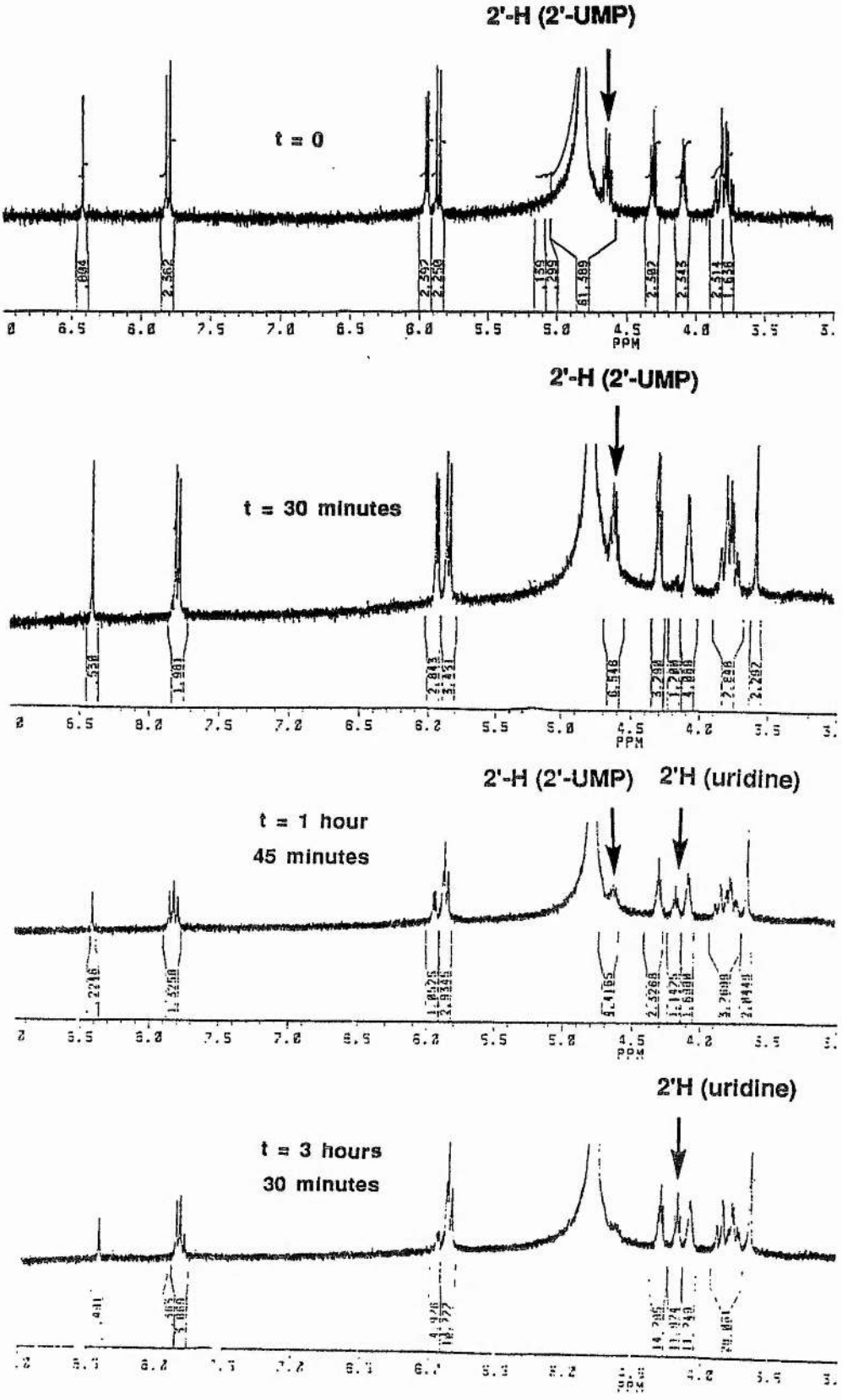
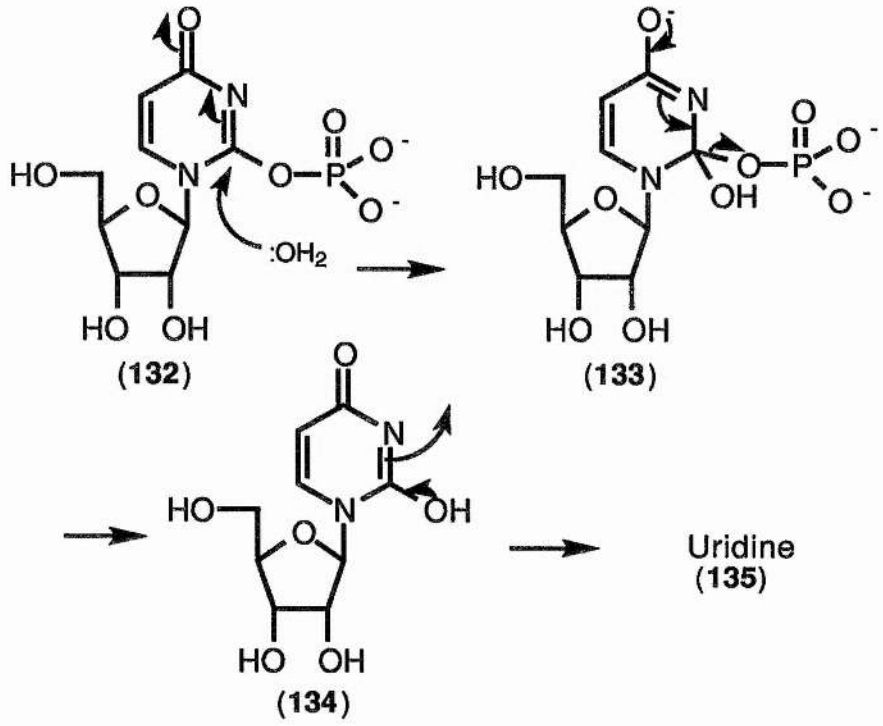


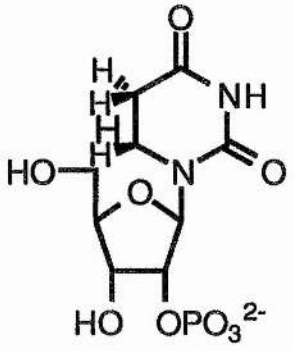
Figure 2.10.3. Uridine 2'-monophosphate as a substrate for inositol monophosphatase.

If formed, the uracil phosphate intermediate (**132**) may have been sufficiently unstable in aqueous solution to undergo immediate non-enzymic hydrolysis to uridine and inorganic phosphate. The possible mechanism of breakdown of the proposed intermediate (**132**) *via* attack at C-2 (analogously to phosphoenol pyruvate hydrolysis) was investigated, by conducting the hydrolysis reaction in an [^{18}O]-enriched aqueous medium. The assay was incubated at 37 °C for 24 hours to ensure complete hydrolysis, and the products analysed by high field ^{13}C- and ^{31}P-NMR spectroscopy and by mass spectrometry. If hydrolysis *via* attack at C-2 had occurred, an isotope shift[177] would be evident in the ^{13}C- NMR spectrum. A single peak was present for C-2 of uridine at 150 MHz. ^{31}P-NMR spectroscopic analysis at 242 MHz did however show an upfield isotope shift of 0.018 ppm (of the expected intensity with respect to the atom % of [^{18}O]-H_2O used in the incubation). In addition, mass spectroscopic analysis of the nucleoside hydrolysis product showed no incorporation of [^{18}O]-label, with the enzyme derived sample showing identical mass ions and fragments to authentic uridine (**135**).



Scheme 2.10.4. Possible mechanism of uridine phosphate breakdown.

5,6-Dihydrouridine 2'-monophosphate (**136**, figure 2.10.5) was also tested as a substrate for inositol monophosphatase, in the hope that the breakdown of the proposed phosphouracil type intermediate would be more informative. Hydrogenation of the uridyl double bond of 2'-UMP with rhodium on alumina as the catalyst, under mildly acidic conditions[178] gave (**136**) in good yield. Similarly, a sample of uridine was reduced to dihydro uridine (**137**) to serve as an NMR and TLC standard for analysis of the incubation mixture.



(136)

Figure 2.10.5 5,6-dihydrouridine 2'-monophosphate

5,6-Dihydrouridine 2'-monophosphate (**136**) was found to be a substrate for the enzyme (as determined by NMR spectroscopy) however at a greatly reduced rate in comparison to that of uridine 2'-monophosphate (**131**). No material other than the starting material, the parent alcohol (dihydrouridine) and inorganic phosphate was detected.

The kinetic parameters of the nucleoside phosphates are shown in table 2.10.6 and figures 2.10.7 - 2.10.9.

Table 2.5.6 Kinetic parameters of 2'-UMP and 5,6-(2H)-2'-UMP.

	V_{max}	K_m (mM)
Uridine 2'-monophosphate	230% that of2'-AMP	4.0
5,6-(2H)-Uridine 2'-monophosphate	70% that of2'-AMP	1.4
Adenosine 2' -mpnophsophate	2'-AMP	0.5

In the case of adenosine 2'- and uridine 2'-monophosphate, the nucleoside base is essentially planar. All carbon and nitrogen atoms are sp^2 hybridised. Upon hydrogenation of the C-C double bond of uridine, the introduction of two sp^3 centres makes the base more bulky in its out of plane dimension. This may account for the difference in rates of reaction for the two substrates.

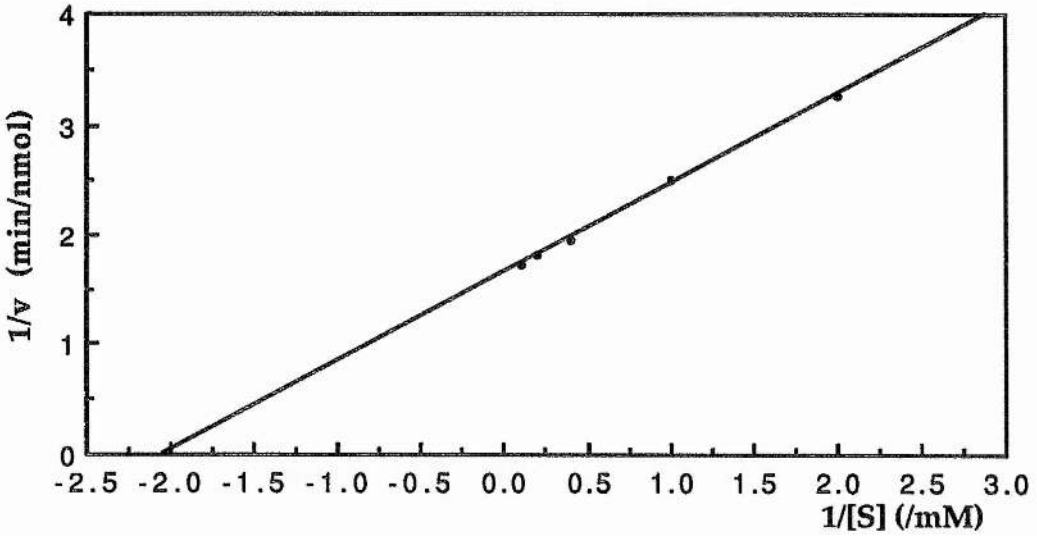


Figure 2.10.7 Lineweaver-Burk double reciprocal plot for Adenosine 2'-monophosphate.

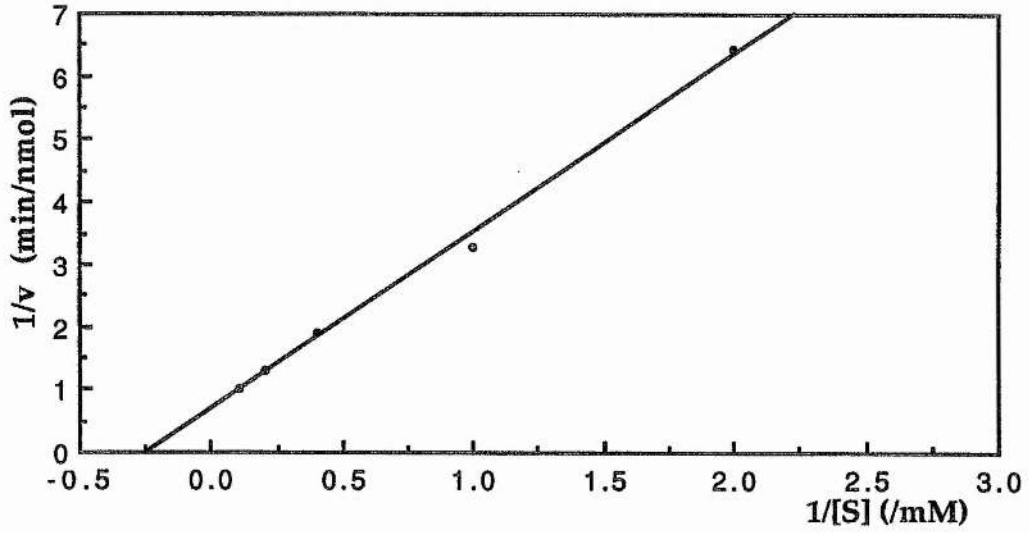


Figure 2.10.8 Lineweaver-Burk double reciprocal plot for uridine 2'-monophosphate.

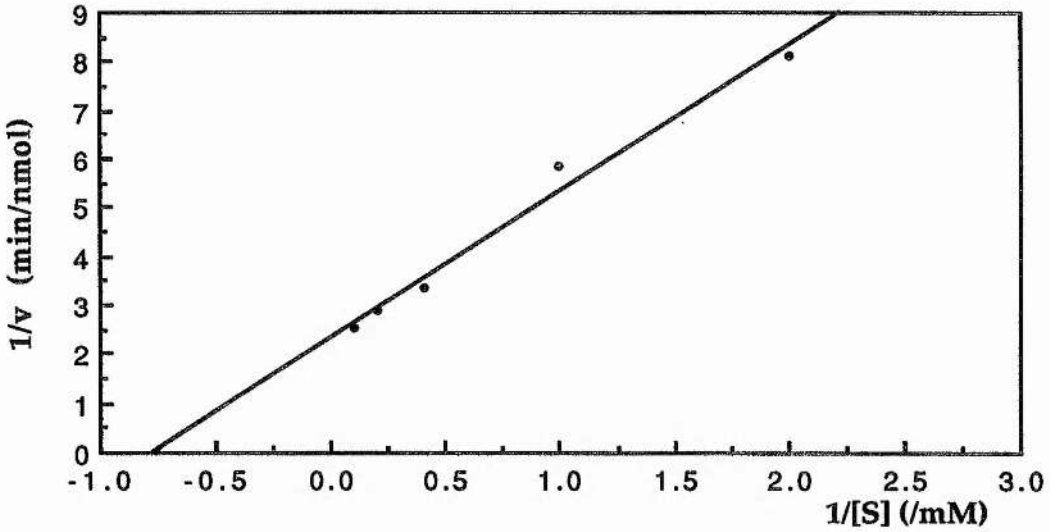
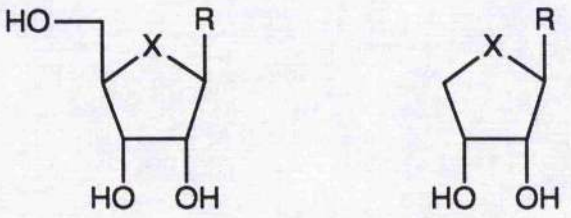


Figure 2.10.9 Lineweaver- Burk double reciprocal plot for dihydro uridine 2'-monophosphate.

2.11 Role of the nucleoside base in the hydrolysis of nucleoside 2'-monophosphates by inositol monophosphatase.

Very recent results from the Merck Sharp and Dohme laboratories have shown that deletion of the nucleoside base of 2'-AMP in compound (**138**), (figure 2.11.1) does not reduce the rate of hydrolysis by inositol monophosphatase.[179] Furthermore, additional deletion of the ribofuranosyl oxygen gave a non-hydrolysable phosphate monoester (**139**), indicating that the ribofuranosyl oxygen may be involved in the mechanism of nucleoside 2'-monophosphate hydrolysis by inositol monophosphatase. Also, deletion of the 5'-hydroxymethylene group (**140**) gave a weaker substrate, providing further evidence that the 5'-OH group of 2'-AMP has a binding function (binding in the same position at the active site as the 4-OH group of inositol 1-phosphate). Thus, these results indicate that ribofuran 2'-phosphate hydrolysis is facile in the absence of the nucleoside base, and that there is a specific requirement for the ribofuranosyl oxygen atom. Comparison of the structures of 2'-AMP (**40**) and Ins 1-P (**33**) reveals that the 1,4-phosphorus-catalytic oxygen relationship in Ins 1-P is repeated in 2'-AMP, with the ribofuranosyl oxygen acting as a surrogate catalytic hydroxyl group.



(**40**) R = Adenine, X = O
(**138**) R = H, X = O
(**139**) R = H, X = CH_2

(**140**) R = H, X = O
(**141**) R = H, X = CH_2

Figure 2.11.1 Adenosine 2'-monophosphate substrate analogues.

Examination of the structure of adenosine 2'-monophosphate shows that the catalytic ribofuranosyl oxygen atom[179] is relatively distant from the phosphate moiety of the substrate. Molecular modelling studies in this laboratory have revealed that in order to obtain congruence between the catalytic and the binding groups of D-Ins 1-P (**33a**) and 2'-AMP (**40**), 2'-AMP must exist in a very unfavourable conformation with a 1,3-interaction between the adenine and the 4-hydroxymethylene groups and a 1,2-interaction between the 2'-O and 3'-O atoms (Figure 2.11.2).

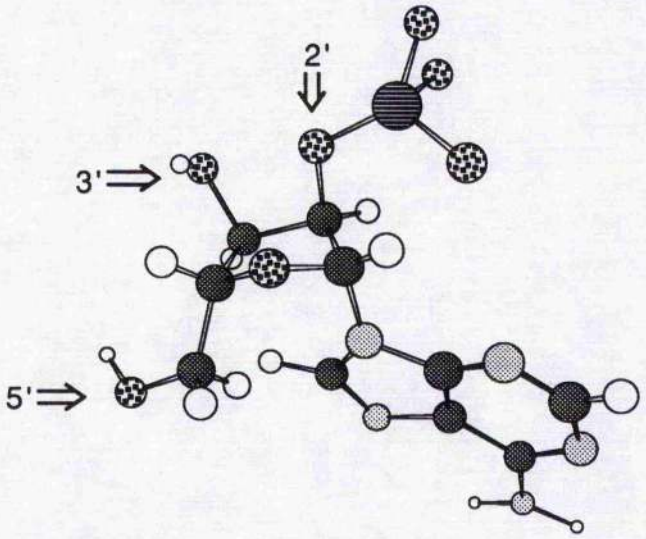


Figure 2.11.2 Proposed active conformation of adenosine 2'-monophosphate.

By analogy with the Ins 1-P system, which this strained conformation now accurately mimics, the adenosine 2'-O atom and the ribofuranosyl-O atom are key binding and catalytic functionalities, and must, therefore, interact with other

species on the enzyme. Neither of the O-atoms possess a hydrogen atom so, H-bonding cannot stabilise the system. However, the chelation of a second Mg^{2+} ion to produce a five membered metallocycle would substantially stabilise the system (Figure 2.11.3), and also provide a possible site on the Mg^{2+} ion for water or hydroxide ion suitably disposed for attacking the phosphorus atom.

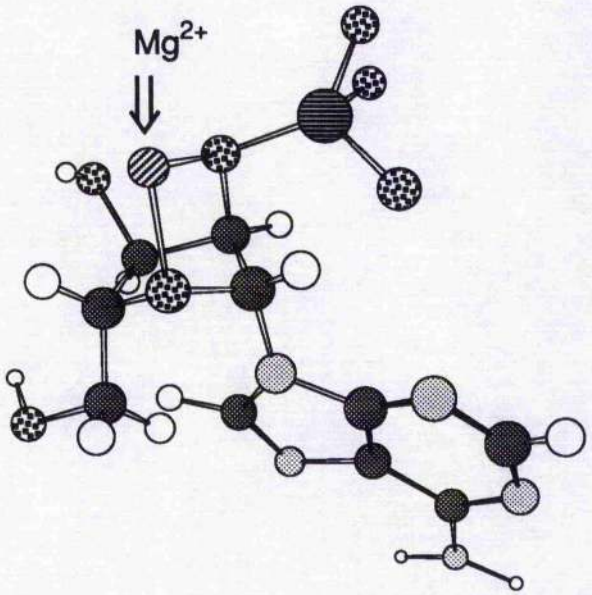


Figure 2.11.3 Proposed active conformation of adenosine 2′-monophosphate incorporating stabilisation by Mg^{2+} coordination to the 2′-O and ribofuranosyl-O atoms.

2.12 Requirement for two magnesium ions in enzymic hydrolyses by inositol monophosphatase.

In 1990, Ganzhorn and Chanal proposed a mechanism for the inhibition of inositol monophosphatase by lithium involving acid-base catalysis and alignment of two metal ions in the active site of the enzyme (Figure 2.12.1).[53] The requirement for two metal ions per subunit with regard to substrate hydrolysis is now considered. The role of the second metal ion can be many fold. It can orientate the phosphate and catalytic hydroxyl groups, and also the hydrolysis nucleophile itself (water or hydroxide) making the hydrolysis effectively intramolecular. Additional binding of an active site amino acid residue to the second Mg^{2+} ion is also possible (see page 82).

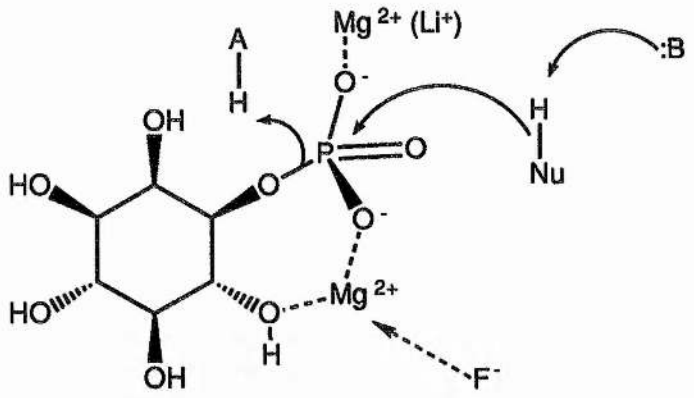
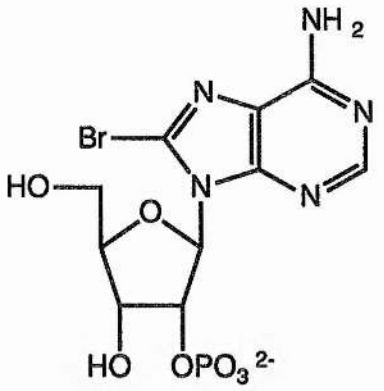


Figure 2.12.1. Ganzhorn and Chanal double metal ion mechanism for the inhibition of inositol monophosphatase by magnesium and lithium.

The assessment of adenosine as an inhibitor for inositol monophosphatase at almost saturating concentration (30 mM) showed that the nucleoside was not active. Calculations predict that adenosine in the proposed enzyme bound conformation is 100-105 kJ/ mol less stable than the unconstrained form which would certainly explain why adenosine is not recognised by the enzyme. To determine whether this conformation could be achieved in free solution with the assistance of Mg^{2+}, 2'-AMP was dissolved in ^2H_2O and its ^{1}H-NMR spectrum recorded. Magnesium chloride in ^2H_2O was introduced and the Mg^{2+} concentration gradually increased to a maximum of 250 mM. After each addition the ^{1}H-NMR spectrum was recorded. The binding of magnesium in the 'active' conformation would have resulted in a significant change in coupling constants for the ribose sugar protons. No change in frequency or coupling constant was observed in the presence of a vast excess of Mg^{2+}, suggesting that 2'-AMP requires additional interaction with the active site to achieve the 'active' conformation.

The introduction of substantial bulk in the nucleoside base was undertaken as a possible way of increasing the energy of the proposed conformation through additional steric interaction between the 4'-hydroxymethylene group and the nucleoside base. 8-Bromoadenosine 2'-monophosphate (**142**, figure 2.12.2) was considered a suitable target, being synthesised from 2'-AMP (**40**) and bromine.[180] (δ_H 5.15 (1H, m, 2'-H), 6.15 (1H, d, $J_{1',2'}$ 6.8 Hz, 1'-H), 8.10 (1H, s, adenine-H); δ_P 3.26).



(142)

Figure 2.12.2 8-Bromoadenosine 2'-monophosphate.

Incubation of 8-Bromoadenosine 2'-monophosphate with inositol monophosphatase, in parallel with similar incubations containing 2'AMP (**40**), showed no noticable changes in rates of hydrolysis. Closer examination of the nucleoside substrates by molecular modelling compared the relative energies of minimised 2'-AMP (**40**) and minimised (8-Br)-2'-AMP (**142**) in the gas phase with and without stabilisation from a metal ion *via* coordination to the ribifuranosyl and 2'-O atoms. Molecular modelling has shown that the introduction of a bromine atom on the adenine ring does not increase the energy of the already high energy conformation for 2'-AMP. With metal ion coordination, (8-Br)-2'-AMP (**142**) is in fact slightly lower in energy than 2'-AMP (**40**) in the minimised gas phase structures. Also, experimentation has shown that the introduction of a bromine atom on the adenine ring does not decrease the rate of hydrolysis of 2'-AMP.

Figure 2.12.3 shows the proposed model for 2'-AMP binding incorporating two Mg^{2+} ions. The role of the first (buried) Mg^{2+} ion is that of coordination to the phosphate moiety of the substrate at the active site. The role of the second (catalytic) Mg^{2+} ion in stabilising the conformation of the active form of 2'-AMP (and other nucleoside 2'-monophosphates), through chelation to the 2'-O and ribofuranosyl O-atoms is also represented. Other ligands for the second Mg^{2+} are possibly the nucleophilic water (hydroxide) and Asp-220.

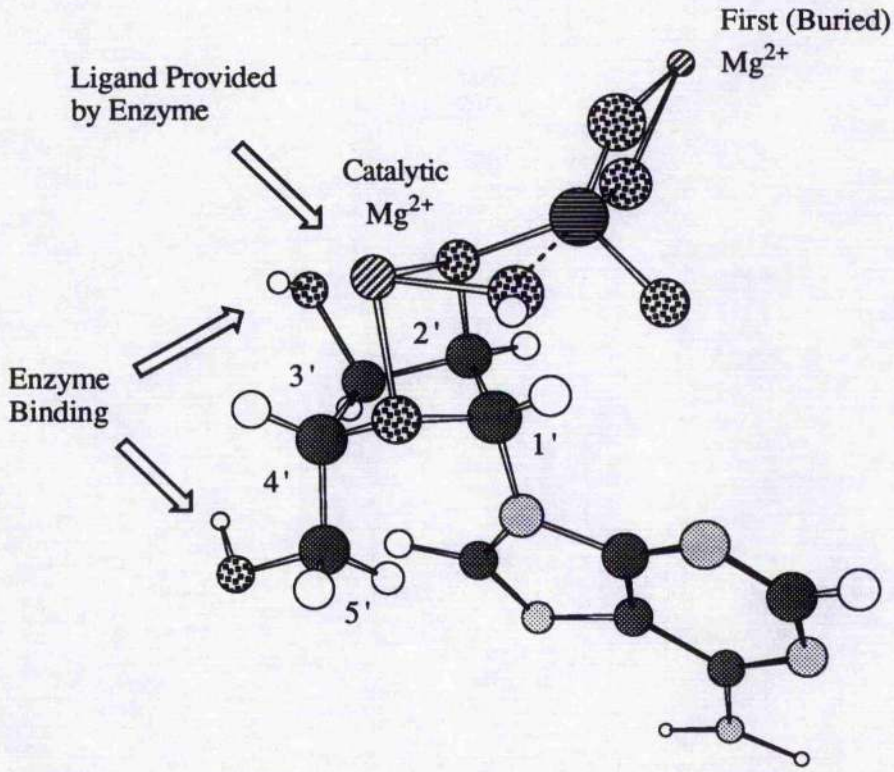


Figure 2.12.3. Mg^{2+} stabilised active conformation of 2'-AMP.

Molecular modelling studies incorporating two Mg^{2+} metal ions at the active site have shown the following in the non solvated minimised structure of the enzyme-inositol 1-phosphate complex. The binding group C-4 OH of inositol 1-phosphate is within good coordination distance of Glu 513 (3.11 and 2.93 Å from each oxygen of Glu 513 and C-4 OH). In addition, the second binding group on the inositol ring (C-2 OH of D-Ins 1-P) is positioned 2.82 Å from Asp 393. With regard to coordination of the Mg^{2+} ions to the active site residues, the first (buried) metal ion is positioned 2.02 and 3.14 Å from two of the phosphate oxygens and also appears to coordinate to Asp 390 (2.08 Å separation) in addition to further residues deeper within the protein. The same aspartate residue (Asp 390) is also positioned 2.03 Å from the second (catalytic) metal ion. Asp 520 appears to interact with the catalytic hydroxyl group (C-6 OH of D-Ins 1-P) with a separation of 2.48 Å and also to the second (catalytic) metal ion, with a separation of 2.0 Å. Water 683 (2.09 Å from the second metal ion) appears to be the catalytic species causing hydrolysis (Figures 2.12.4 & 2.12.5)

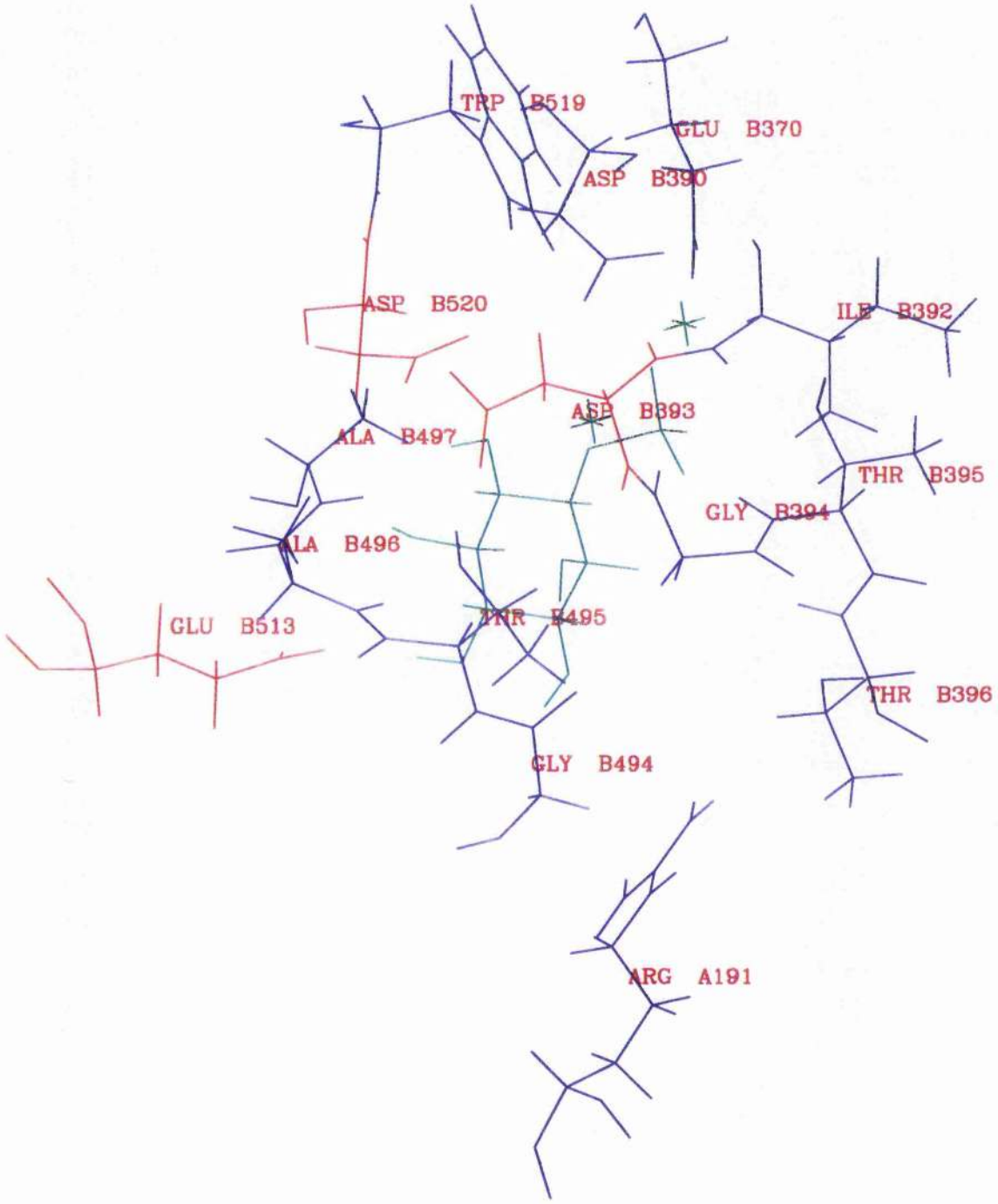


Figure 2.12.4. Minimised active site residue interactions with inositol 1-phosphate.

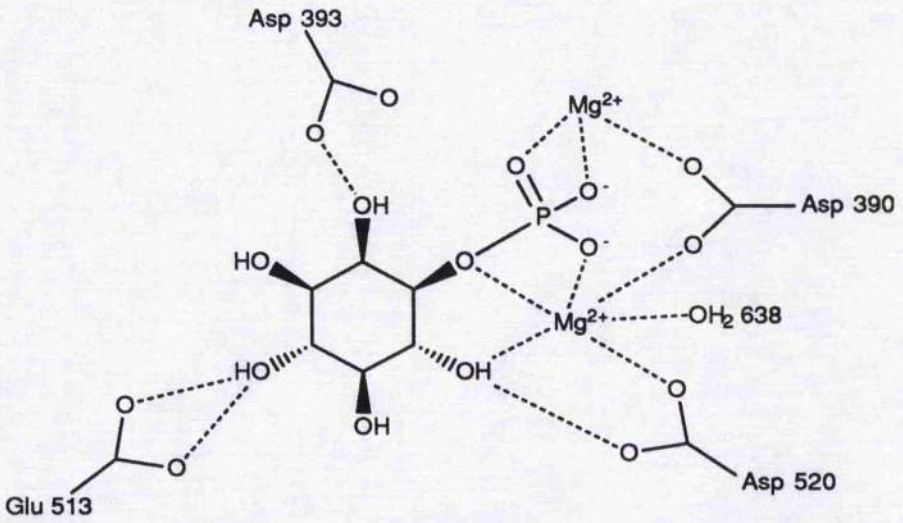


Figure 2.12.5 Active site interactions of D-inositol 1-phosphate.

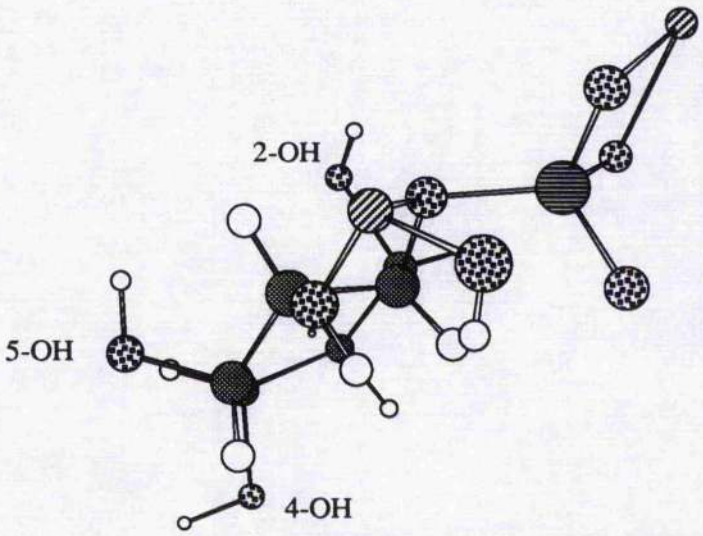


Figure 2.12.6 Active conformation of D-inositol 1-phosphate showing stabilisation through coordination of a second Mg^{2+} metal ion.

Comparison of figure 2.12.3 with figure 2.12.6 which depicts the conformation of D-inositol 1-phosphate and the important catalytic and binding interactions with the two Mg^{2+} ions, shows good overlap between the catalytic and binding functionality of the nucleoside and inositol substrates.

Closer examination of the three dimensional structure of inositol monophosphatase shows that further stabilisation of the developing negative charge may arise from the positive dipole of the Thr-195-Thr-205 α-helix (Figure 2.12.7) and also from the Thr-95-His-100 α-helix (Figure 2.12.8) in modelled enzyme-substrate complexes.

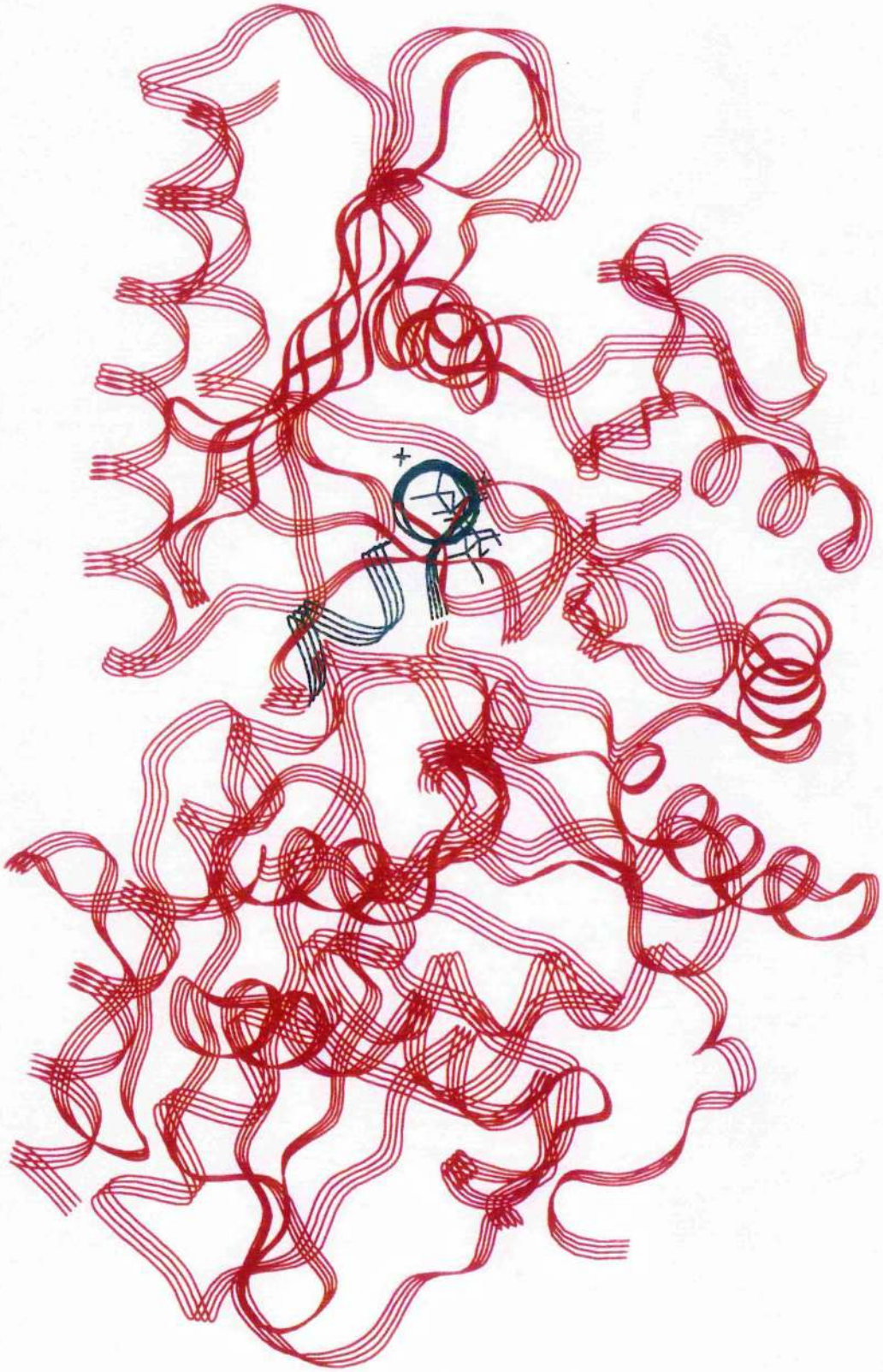


Figure 2.12.7. Inositol monophosphatase showing possible stabilisation of the phosphate group from the Thr 195-Thr 205 α-helix (highlighted in green).

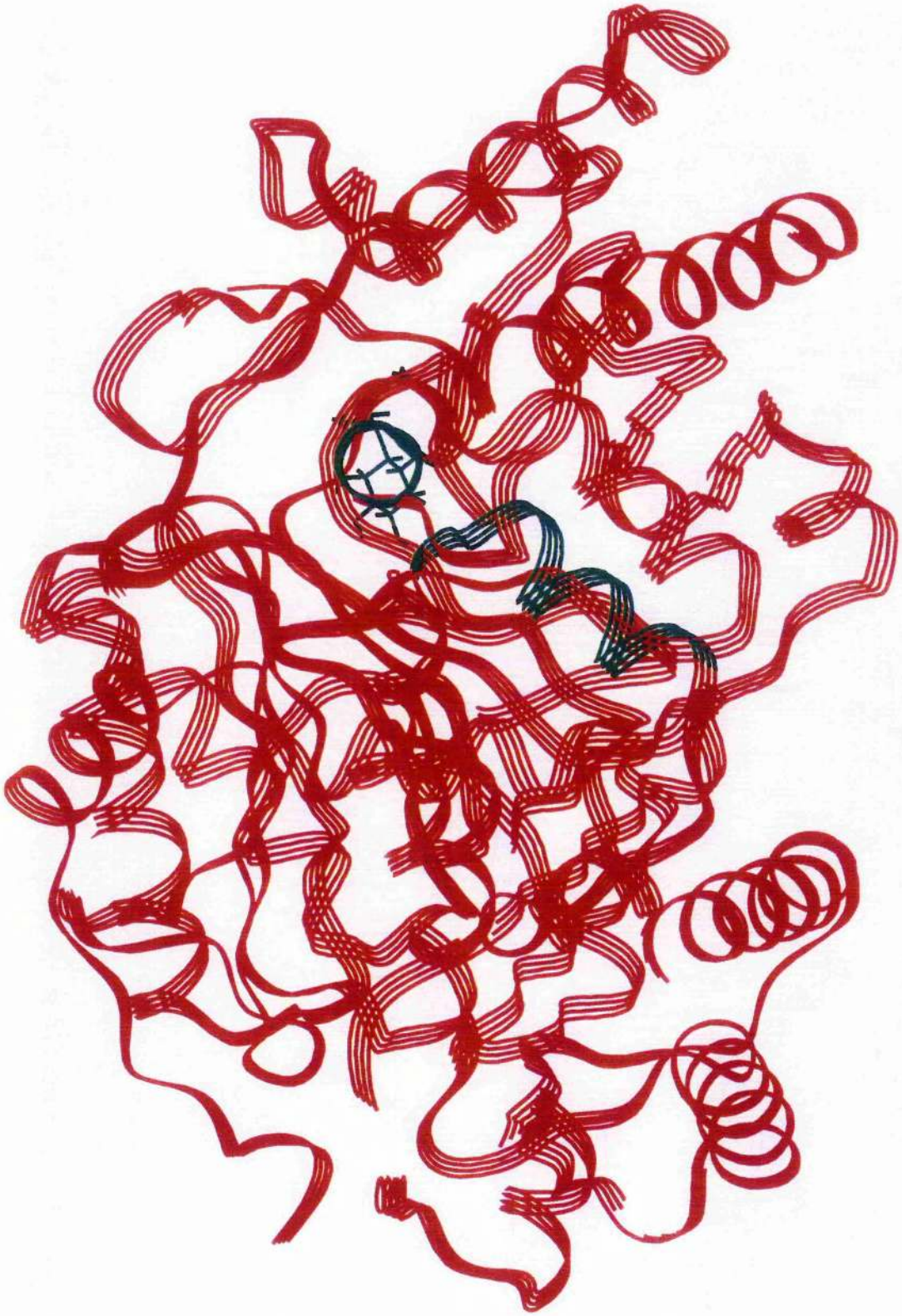


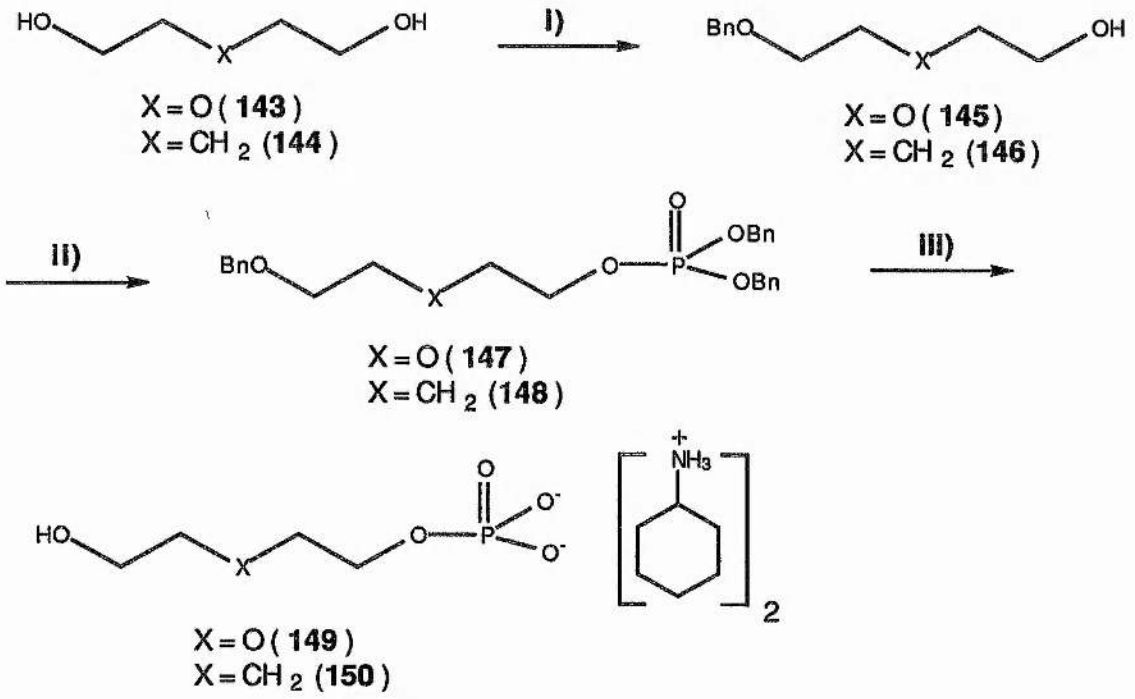
Figure 2.12.8. Inositol monophosphatase showing possible stabilisation of the alkoxide leaving group from the Thr 95-His 100 α-helix (highlighted in green).

The Thr-195-Thr-205 α-helix (highlighted in green in figure 2.12.7, viewing down the helix to reveal the substrate) points directly at the sulfate anion in the published crystal structure.[108] In modelled enzyme-substrate complexes, the helix points directly towards the phosphate group of Ins 1-P, leading to additional stabilisation of the phosphate monoester dianion. Also the Thr-95-His-100 α-helix (highlighted in green in figure 2.12.8, viewing down the helix to reveal the substrate), points directly towards the leaving 1-O atom of the inositol moiety in the same modelled enzyme-substrate complex. This allows stabilisation of the developing negative of the inositol alkoxide leaving group.

To determine the validity of the proposed active structure of inositol monophosphatase, a number of mechanism designed inhibitors were produced. The free hydroxyl group of ethane 1,2-diol monophosphate (**113**) was further elaborated to provide molecules that might displace the nucleophilic water molecule (or hydroxide ion) from its site on the second Mg^{2+} ion. Accordingly, diethylene glycol phosphate (**149**) and diethylene glycol cyclic phosphate diester (**157**) were produced. The free hydroxyl group of diethylene glycol phosphate (**149**) should be capable of displacing the hydrolysis nucleophile *via* coordination to the catalytic Mg^{2+} ion. If the hydrolysis nucleophile was displaced, 1,5-phosphoryl group transfer, but not phosphate monoester hydrolysis, would be possible. The cyclic phosphate diester (**157**) should also displace the hydrolysis nucleophile and thus cause inhibition. For comparison, pentane 1,5-diol 1-phosphate (**150**) and pentane 1,5-diol cyclic phosphate diester (**158**) were also prepared, and their inhibition properties studied.

Diethylene glycol phosphate (**149**) and pentane 1,5-diol 1-phosphate (**150**) were synthesised from diethylene glycol (**143**) and pentane 1,5-diol (**144**) respectively. A five fold excess of the parent alcohols, (**143**) and (**144**) was treated with benzyl bromide and sodium hydride in DMF, and on completion (as judged by TLC) the reaction mixture quenched with water. The monobenzylated diols were extracted into diethyl ether and washed with water to remove the excess alcohol. Phosphitylation with N,N'-diisopropyl bis-benzyl phosphoramidite was performed without further purification, followed by phosphite oxidation with *m*-CPBA and purification by silica column chromatography to yield the fully benzylated alkyl phosphates. (Benzyl

diethylene glycol phosphate dibenzyl ester (**147**) *m/z* (Found: $[M + H]^+$ 457.1780. $C_{25}H_{30}O_6P$ requires 457.1780); benzyl pentane 1,5-diol 1-phosphate dibenzyl ester (**148**) (Found: $[M + H]^+$ 455.1990. $C_{26}H_{32}O_5P$ requires 455.1987)). Palladium on activated charcoal catalysed deprotection under an atmosphere of hydrogen gas followed by conversion to the bis-cyclohexylammonium salts and recrystallisation from water/ acetone afforded diethylene glycol monophosphate (**149**) and pentane 1,5-diol monophosphate (**150**) as white solids (Scheme 2.12.9). (Diethylene glycol phosphate (derivatised as the dimethyl ester) *m/z* (Found: $[M + H]^+$ 215.0685. $C_6H_{16}O_6P$ requires 215.0685); pentane 1,5-diol 1-phosphate (derivatised as the dimethyl ester) (Found: $[M + H]^+$ 213.0890. $C_7H_{18}O_5P$ requires 213.0892)).

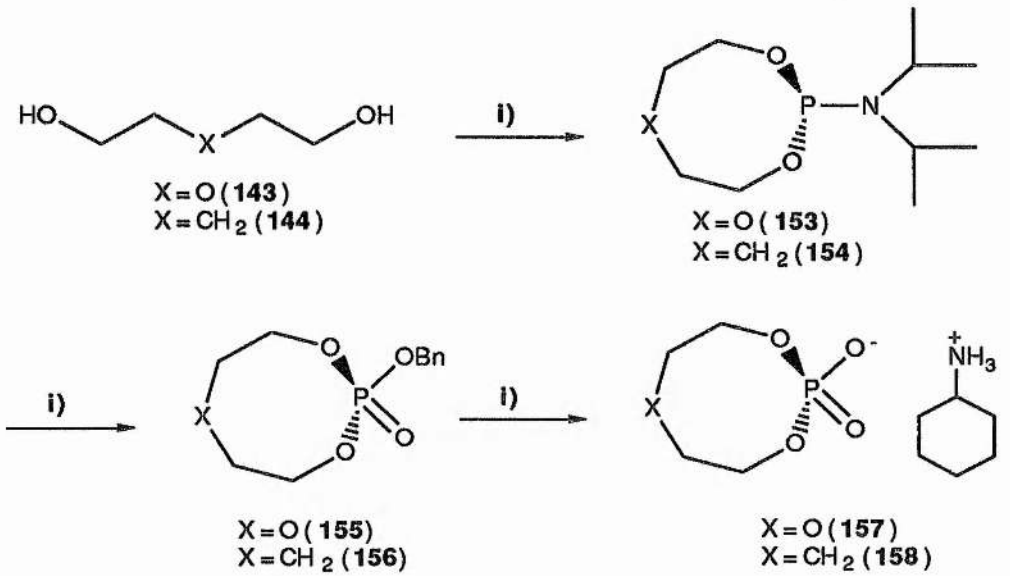


Reagents: I)BnBr, NaH, DMF; II) a) N,N'-diisopropyl bisbenzyl phosphoramidite, 1-H tetrazole, CH_3CN, b) *m*-CPBA, CH_2Cl_2; III) a) H_2, Pd/ C, b) cyclohexylamine, H_2O.

Scheme 2.12.9. Synthesis of phosphate monoester inhibitors of inositol monophosphatase.

The cyclic diester substrate analogues (**157**) and (**158**) were

synthesised in three steps, again from the parent alcohols. Treatment of the diols with N,N'-diisopropyl dichloro phosphoramidite and triethylamine in dichloromethane gave the cyclic phosphoramidites (**153**) and (**154**) from which the corresponding benzyl phosphites were produced *via* the phosphitylation of benzyl alcohol. Oxidation with *m*-CPBA and rapid debenzylation (by hydrogenation) and conversion to the mono-cyclohexylammonium salts yielded (**157**) (*m/z* (Found: [*M* (free acid) + H]$^+$ 169.0266. $C_4H_{10}O_5P$ requires 169.0266)) and (**158**) (*m/z* (Found: [*M* (free acid) + H]$^+$ 167.0470. $C_5H_{12}O_4P$ requires 167.0473)) as white crystalline solids (Scheme 2.12.10).



Reagents: **I)** a) N,N'-diisopropyl dichlorophosphoramidite, Et_3N, CH_2Cl_2;
II) a) 1-H tetrazole, BnOH, CH_3CN, b) *m*-CPBA, CH_2Cl_2;
III) a) H_2, Pd/ C, b) cyclohexylamine, H_2O.

Scheme 2.12.10 Synthesis of cyclic phosphate diester inhibitors of inositol monophosphatase.

For comparison, 2-methoxyethanol 1-phosphate (**160**) and propyl phosphate (**162**) were also prepared (Figure 2.12.11).

(**160**) (**162**)

Figure 2.12.11 Methoxyethanol phosphate and propyl phosphate.

Each of the compounds were tested as potential substrates and inhibitors for the enzyme, and none acted as a substrate, as expected (with the exception of diethylene glycol monophosphate (**149**) which showed extremely slow hydrolysis in comparison to controls containing no enzyme. The rate of enzymic hydrolysis was approximately 10-fold slower than that of ethane 1,2-diol monophosphate (**113**)).

Their competitive modes of inhibition and the values of the inhibition constants are informative and are consistent with the proposed mechanism of hydrolysis. For example, the replacement of the 2-OH group in ethane 1,2-diol monophosphate (**113**) by OMe, to give (**160**), led to a 25 fold reduction in binding affinity ($K_i \geq 25$ mM) (Propyl phosphate (**162**) was devoid of inhibitory activity). Replacement of the methyl group by a 2-hydroxyethyl group, compound (**149**), led to an 8-fold increase in binding affinity ($K_i = 3.5$ mM). This increase in affinity is ascribed to the ability of the inhibitor to provide extra ligands for the second Mg^{2+}, by displacing the nucleophilic hydroxide ligand. The replacement of the ether O-atom to give compound (**150**) did not affect the K_i value ($K_i = 3.8$ mM) as would be expected if the ω-hydroxyl group binds to the Mg^{2+} ion. The fact that neither compound served as a substrate (to any significant extent) excludes the possibility that the ω-hydroxyl group binds in the site for the 5'-OH of 2'-AMP.

The cyclic diester (**157**) was a weak inhibitor ($K_i = 8$ mM). Nevertheless, this latter result is remarkable given that the compound is a monoanion and that K_i for sulfate is 20 mM.[50] As predicted on the basis of the properties of diethylene glycol phosphate (**149**) and pentane 1,5-diol phosphate (**150**), removal of the ether O-atom in the monoanion (**157**) to give (**158**) had little effect on K_i ($K_i = 7.5$ mM). As mentioned above, none of the compounds showed any appreciable activity as substrates, as determined by ^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopy, and notably the cyclic diesters were not cleaved to give monoesters. These results give clues to the location of the active site nucleophile. Since each of the compounds contains a phosphate ester group and at least the two requisite oxygen functionalities for binding to the second Mg^{2+} ion on the enzyme (of which one interactions stabilises the leaving group), the compounds which serve as competitive inhibitors should also serve as substrates, if the first Mg^{2+} ion chelates and activates the nucleophilic water

molecule. Conversely, if the second Mg^{2+} ion provides the site for the nucleophile (water or hydroxide) neither of the compounds which can provide a ligand to displace the nucleophile would be hydrolysed. Thus, the results of the study are in accord with a mechanism involving two Mg^{2+} ions, and also indicate that the second Mg^{2+} ion chelates and activates the nucleophile, hydroxide ion. Given that two Mg^{2+} ions are required and that Li^{+} and Mg^{2+} (at high Mg^{2+}concentration) serve as mutually exclusive uncompetitive inhibitors[50] and bind to the $E.P_i$ product complex, 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,[55] 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 $E.P_i$ product complex. 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) 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.

Conclusion:

As a result of this research, a mechanism for phosphate monoester hydrolysis by inositol monophosphatase has been proposed that is in accord with all published kinetic data. Hydrolysis occurs *via* the direct displacement of phosphate by water, rather than the more common substituted enzyme mechanism, and two Mg^{2+} ions per subunit are required for hydrolysis. The inability of inositol monophosphatase to catalyse phosphate-oxygen ligand exchange in the presence of adenosine, and the lack of inhibitory activity of adenosine suggests that the active form of adenosine in 2'-AMP hydrolysis cannot be achieved in the absence of the phosphate moiety. 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 conformation of adenosine. The discovery of new nucleoside 2'-monophosphate substrates and the use of molecular modelling has identified the conformation of the active form of adenosine, which exhibits an adverse 1,3-interaction between the adenine and the hydroxymethyl groups and also a 1,2-

interaction between the 2-O and 3-O atoms. Calculations predict that the active structure of adenosine is 100-105 kJ/ mol less stable than the unconstrained form ($K \sim 10^{-17}$). This high energy conformation explains the lack of phosphate-oxygen ligand exchange in the presence of adenosine and also the lack of inhibitory activity of adenosine. The proposed mechanism demonstrates distinct roles for the catalytic hydroxyl group[116] and the 1-O atom of inositol 1-phosphate in coordinating to the second (catalytic) Mg^{2+} ion, and orientating the hydrolysis nucleophile (water or hydroxide), with analogous Mg^{2+} coordination to the ribofuranosyl- and the 2'-O atoms in the hydrolysis of 2'-AMP. Synthetic mechanism based inhibitors (diethylene glycol monophosphate and diethylene glycol cyclic monophosphate), which have no binding interactions comparable to C-2 OH and C-4 OH of D-inositol 1-phosphate, have been shown to bind to the enzyme. Notably, cyclic phosphate diester substrate analogues have been shown to bind to the enzyme despite suffering from reduced coordination to the first (buried) Mg^{2+} ion. The proposed mechanism is in accord with earlier results from this laboratory demonstrating cooperativity effects observed for Mg^{2+} with inositol 1-phosphate and adenosine 2'-monophosphate as substrates.[55] In the $E.P_i$ product complex, Li^+ inhibition occurs by Li^+ occupying the catalytic Mg^{2+} site. At higher concentration Li^+ is able to bind into the first Mg^{2+} (buried) site 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.

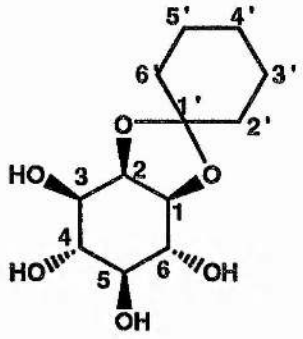
The understanding of the mode of action of inositol monophosphatase now allows the design and synthesis of more potent inhibitors incorporating functional groups capable of chelating to the Mg^{2+} ions in addition to binding groups comparable to C-2 OH and C-4 OH of D-inositol 1-phosphate.

CHAPTER THREE
EXPERIMENTAL

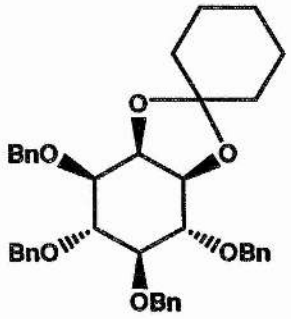
3. EXPERIMENTAL.

NMR Spectra were recorded on a Bruker AM-300 f.t. spectrometer (^{1}H, 300 MHz; ^{13}C, 75 MHz; ^{31}P, 121.5 MHz) and a Varian Gemini f.t. spectrometer (^{1}H, 200 MHz; ^{13}C, 50 MHz). High field NMR spectra were obtained on an S.E.R.C. service basis at the University of Warwick (^{1}H, 400 MHz) and the University of Edinburgh (^{13}C, 150 MHz; ^{31}P, 242 MHz) ^{1}H NMR spectra were referenced on chloroform, TMS, methanol or DMSO, ^{13}C NMR spectra were referenced on chloroform, TMS, methanol or DMSO and ^{31}P spectra on external H_3PO_4. NMR spectra are described in parts per million downfield shift from TMS and are reported consecutively as position (δ_H or δ_C), relative integral, multiplicity (s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, dd-doublet of doublets and b-broad), coupling constant ($J_{X,Y}$ Hz if applicable) and assignment. Infrared spectra were recorded using a Perkin Elmer 1420 ratio recording spectrometer and a Perkin Elmer 1710 f.t. i.r. 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. Melting points were measured using 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. Mass spectra were recorded on a Kratos MS50, and obtained on an S.E.R.C. service basis at the University of Swansea using a VG ZAB E. Major fragments are given as percentages of the base peak intensity. GC/ MS spectra were recorded on a Hewlett Packard 5890A 6C. U.V./ 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.[181] Flash chromatography was performed according to the procedure of Still[182] using Sorbisil C60 (40-60 μm) silica gel. Analytical thin layer chromatography 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, aqueous potassium permanganate, acidic palladium chloride or ninhydrin. Radiochemical data of ^{14}C-compounds was

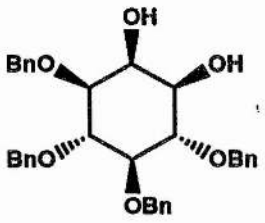
obtained using a Packard Tri-Carb 4000 scintillation counter.



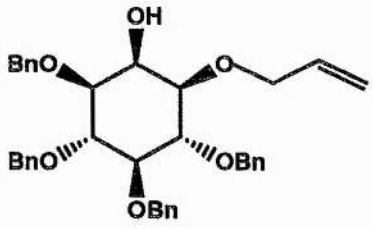
(+/-)-*cis*-1,2-Cyclohexylidene-*myo*-inositol (81):[132] *myo*-Inositol (**7**) (10 g, 55.5 mmol) was added to a mixture of cyclohexanone (100 ml), toluene (50 ml) and DMF (10 ml) with stirring, and the mixture refluxed (Dean-Stark apparatus, 155 °C) under a nitrogen atmosphere for 2 hours to dry the reagents. *p*-Toluene sulphonic acid (90 mg) was added and the mixture refluxed for a further 24 hours, until no further water was produced. On cooling to 35 °C, toluene (50 ml), petroleum ether (50 ml) and ethanol (25 ml) were added to the resulting clear solution. *p*-Toluene sulphonic acid (0.6 g) was added and the solution stirred at 4 °C for 2 hours. Triethylamine (0.6 ml) was added, and the solution allowed to stand at -20 °C for a further 24 hours. On filtering, the resultant paste was heated in a mixture of ethanol (180 ml) and triethylamine (1 ml) for 1 hour (100 °C), hot filtered, and on cooling the white crystalline product collected by filtration (9.39 g, 65%); m.pt. 181-183 °C (dec) (lit.,[132] 184-186 °C (dec)); *m/z* (Found: $[M + H]^+$ 261.1338. $C_{12}H_{21}O_6$ requires 261.1338); υ_{max} (nujol)/ cm^{-1} 3000-3600 sb (OH), 1240 m, 1120 s, 1040 s (C-O) and 1005 s; δ_H (300 MHz; ^2H_2O) 1.15-1.85 (10H, m, C_6H_{10}), 3.25 (1H, t, $J_{4,5}$ 9.0 Hz, $J_{5,6}$ 10.6 Hz, 5-H), 3.60 (1H, dd, $J_{5,6}$ 10.6 Hz, $J_{1,6}$ 8.0 Hz, 6-H), 3.65 (1H, t, $J_{4,5}$ 9.0 Hz, $J_{3,4}$ 9.6 Hz, 4-H), 3.85 (1H, dd, $J_{3,4}$ 9.6 Hz, $J_{2,3}$ 4.1 Hz, 3-H), 4.05 (1H, dd, $J_{1,2}$ 5.0 Hz, $J_{1,6}$ 8.0Hz, 1-H) and 4.50 (1H, t, $J_{1,2}$ 5.0 Hz, $J_{2,3}$ 4.1 Hz, 2-H); δ_C (75.5 MHz; ^2H_2O) 26.1, 26.4, 27.1, (3'-C, 4'-C and 5'-C), 37.4, 40.2 (2'-C and 6'-C), 72.4, 75.1, 75.5, 77.9, 78.5, 80.1 (Ins-CH) and 114.3 (1'-C); *m/z* (CI) 261 ($[M + H]^+$,100%) and 217 (5).



(+/-)-*cis*-1,2-Cyclohexylidene-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (82):[132] Potassium hydroxide (55.5 g) was added to a mixture of (+/-) *cis*-1,2-cyclohexylidene-*myo*-inositol (**81**) (9.11 g, 35 mmol) and benzyl chloride (90 ml), and the resulting mixture heated (140°C) under a nitrogen atmosphere for 24 hours. Toluene (50 ml) and water (150 ml) were added with stirring, and the phases separated. The aqueous phase was extracted with toluene (50 ml) and the combined organic extracts washed with water (3 x 50 ml, until neutral), saturated brine (50 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo*, and the product purified by silica column chromatography (0-10% ethyl acetate/ petroleum ether, in 5% steps) and recrystallised from ethanol to give white crystals (18.25 g, 84%); m.pt. 82-85 °C, (lit.,[132] 84-86 °C); *m/z* (Found: $[M + NH_4]^+$ 638.3480. $C_{40}H_{48}O_6N$ requires 638.3481); υ_{max} (nujol)/ cm^{-1} 1490 w, 1360 m, 1100 m, 1070 s, 930 m and 700 s; δ_H (300 MHz; C^2HCl_3) 3.40 (1H, t, $J_{4,5}$ 8.5 Hz, $J_{5,6}$ 9.8 Hz, 5-H), 3.70 (1H, dd, $J_{3,4}$ 8.4 Hz, $J_{2,3}$ 3.8 Hz, 3-H), 3.83 (1H, dd, $J_{5,6}$ 9.8 Hz, $J_{1,6}$ 6.9 Hz, 6-H), 3.94 (1H, t, $J_{3,4}$ 8.4 Hz, $J_{4,5}$ 8.5 Hz, 4-H), 4.10 (1H, t, $J_{1,2}$ 5.6 Hz, $J_{1,6}$ 6.9 Hz, 1-H), 4.30 (1H, dd, $J_{1,2}$ 5.6 Hz, $J_{2,3}$ 3.8 Hz, 2-H), 4.70-5.00 (8H, m, Benzyl CH_2) and 7.20 (20H, m, Ar-H); δ_C (75.5 MHz; C^2HCl_3) 26.4, 26.7, 27.8 (3'-C, 4'-C and 5'-C), 37.8, 40.2 (2'-C and 6'-C), 75.9, 76.7, 77.8, 78.0 (Benzyl CH_2), 76.8, 80.2, 81.5, 83.7, 84.9, 85.7 (Ins-CH), 113.2 (1'-C) and 130.2-131.1 (Ar-C); *m/z* (CI) 638 ($[M + NH_4]^+$,100%), 621 ($[M + H]^+$,5%), 351 (20), 108 (25, [OBn + H^+]) and 91 (10, $PhCH_2^+$); TLC R_f = 0.43 (20% ethyl acetate/ petroleum ether).

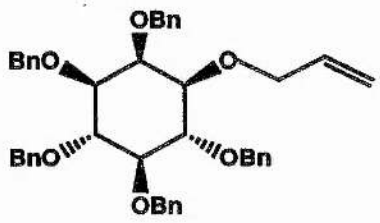


(+/-)-3,4,5,6-Tetra-*O*-benzyl-*myo*-inositol (83):[132] (+/-)-*cis*-1,2-Cyclohexylidene-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (**82**) (12.4 g, 20 mmol) was refluxed in acetic acid (160 ml) and water (40 ml) for 2 hours. The solvents were removed *in vacuo* and the resulting oil dried azeotropically using toluene. The crude product was recrystallised from methanol to give a white solid. (9.62 g, 88%); m.pt. 113-115 °C, (lit.,[132] 113-115 °C); *m/z* (Found: $[M + NH_4]^+$ 558.2855. $C_{34}H_{40}O_6N$ requires 558.2855); υ_{max} (nujol)/ cm^{-1} 3390 sb (OH), 3300 sb (OH), 1495 w, 1460 s, 1320 m, 1300 w, 1130 m, 1060 s and 930 s; δ_H (400 MHz; C^2HCl_3) 2.44 (1H, s, OH), 2.53 (1H, s, OH), 3.48 (3H, m, Ins-CH), 3.85 (1H, t, Ins-CH), 3.97 (1H, t, Ins-CH), 4.20 (1H, t, Ins-CH), 4.68-4.98 (8H, m, Benzyl CH_2) and 7.28 (20H, m, Ar-H); δ_C (75.5 MHz; C^2HCl_3) 70.1, 72.6 (Ins-CH), 73.6, 76.5, 76.7 (Benzyl CH_2), 80.8, 82.2, 82.5, 84.1 (Ins-CH), 128.5, 128.6, 128.8, 129.3, 129.4, 138.7, 139.4 and 139.5 (Ar-C); *m/z* (CI) 558 ($[M + NH_4]^+$, 100%), 541 (20, $[M + H]^+$), 449 (30, $[M + H - BnH]^+$), 359 (45, $[M + H - 2Bn]^+$) and 108 (60, $[OBn + H]^+$); TLC R_f = 0.13 (20% ethyl acetate/ petroleum ether).

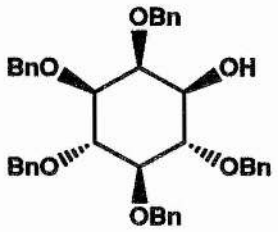


(+/-)-1-*O* -Allyl-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (84):[132] 3,4,5,6-Tetra-*O*-benzyl-*myo*-inositol (**83**) (9.19 g, 17 mmol), in dry benzene (250 ml) was treated with dibutyl tin oxide (4.22 g, 1.0 mmol) and the mixture refluxed

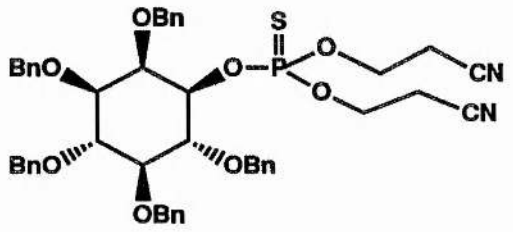
under a nitrogen atmosphere (Dean Stark apparatus) for 24 hours until formation of the stannylidene complex was complete as judged by infra-red spectroscopy. The volume of the solvent was reduced to one half of its original volume by distillation, and the reaction mixture cooled to 60 °C. Tetrabutyl ammonium bromide (5.48 g, 17 mmol) and allyl bromide (4.43 ml, 51 mmol) were added and the reaction was stirred for a further 48 hours until no starting material remained. Water (3.5 ml) was added and the reaction mixture stirred for a further 1 h. On removal of the solvents *in vacuo*, the product was purified by silica column chromatography (0-30% ethyl acetate/ petroleum ether, in 5% steps) and recrystallised from ethyl acetate and heptane to give a white solid. (9.32 g, 94%); m.pt. 61-63 °C, (lit.,[114] 63-66 °C); *m/z* (Found: $[M + H]^+$ 581.2900. $C_{37}H_{41}O_6$ requires 581.2903); υ_{max} (nujol)/ cm^{-1}, 3600-3300 mb, (O-H), 2760 w, 1760 w, 1640 w, 1340 w, 1220 w, 1080 m, 1040 m, 740 s and 710 s; δ_H (300 MHz; C^2HCl_3) 2.47 (1H, s, OH), 3.30 (1H, dd, *J* 2.7 Hz, *J* 9.6 Hz, Ins-H), 3.41 (1H, dd, *J* 2.7 Hz, *J* 9.7 Hz, Ins-H), 3.42-3.77 (1H, dd, *J* 9.5 Hz, *J* 2.7Hz, Ins-H), 3.95 (1H, t, *J* 9.6Hz, Ins-H), 3.99 (1H, t, *J* 9.6Hz, Ins-H), 4.18 (2H, dd, $J_{1',2'}$ 5.7 Hz, $J_{1',3'cis}$ 1.3 Hz, C1'H_2), 4.23 (1H, t, *J* 2.7 Hz, Ins-2H), 4.70-4.91 (8H, m, Benzyl CH_2), 5.17-5.20 (1H, dd, $J_{3'cis,2'}$ 10.4 Hz, $J_{3'cis,3'trans}$ 1.5 Hz, 3'Hcis), 5.28 (1H, dd, $J_{2',3'trans}$ 17.2 Hz, $J_{3'cis,3'trans}$ 1.5 Hz, 3'H trans), 5.94 (1H, m, $J_{2',3'trans}$ 17.2 Hz, $J_{2',3'cis}$ 10.4 Hz, $J_{1',2'}$ 5.7 Hz, 2'-H) and 7.32 (20H, m, Ar-H); δ_C (75.5 MHz; C^2HCl_3) 67.7 (2-C), 71.9, 72.8, 75.9 (Benzyl-CH_2 and 1'-C), 79.7, 79.9, 81.2, 83.2 (Ins-C), 117.4 (3'-C), 127.6, 127.8, 127.9, 128.0, 128.1, 128.3 (Ar-C), 134.7 (2'-C); *m/z* (CI) 598 ($[M + NH_4]^+$,100%), 581 (60, $[M + H]^+$), 498 (25, $[M + H - BnH]^+$), 399 (25, $[M + H - 2Bn]^+$), 108 (25, $[OBn + H]^+$) and 91 (10, Bn^+); TLC R_f = 0.16 (20% ethyl acetate/ petroleum ether).



(+/-)-1-*O*-Allyl-2,3,4,5,6-penta-*O*-benzyl-*myo*-inositol (85):[132] (+/-)-1-*O*-Allyl-3,4,5,6-tetra-*O*-Benzyl-*myo*-inositol (**84**) (8.71 g, 15 mmol) in dry DMF (360 ml) was added dropwise to a suspension of sodium hydride (1.44 g, 30 mmol (50% dispersion in oil)) and benzyl bromide (3.3 ml, 30.6 mmol) under a nitrogen atmosphere. The reaction was stirred at room temperature for 24 hours, water (90 ml) added with extreme caution and the solvents removed *in vacuo*. The residual oil was partitioned between water (90 ml) and ethyl acetate (250 ml) and the organic phases collected. The aqueous phase was extracted with ethyl acetate (3 x 50 ml) and the combined organic extracts washed with saturated brine (250 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo*, the product purified by silica column chromatography (0-20% ethyl acetate/ petroleum ether, in 5% steps) and recrystallised from ethanol to give white crystals. (9.31 g, 93%); m.pt. 60-62 °C, (lit.,[57] 60-62 °C); (Found: C, 78.65; H, 6.9. Calc. for $C_{44}H_{46}O_6$: C, 78.8; H, 6.9%); *m/z* (Found: $[M + H]^+$ 671.3370. $C_{44}H_{47}O_6$ requires 671.3372); υ_{max} (nujol)/ cm^{-1} 3010 m, 2880 m, 1500 m, 1460 m, 1370 s, 1080 s and 700 s; δ_H (300 MHz; C^2HCl_3) 3.26 (1H, dd, *J* 9.8 Hz, *J* 2.3 Hz, InsH), 3.36 (1H, dd, *J* 9.8 Hz, *J* 2.3 Hz, InsH), 3.47 (1H, t, *J* 9.3 Hz, InsH), 4.10 (5H, m, 3 x InsH and 1'-H_2), 4.60-4.95 (10H, m, Benzyl CH_2), 5.15-5.20 (1H, dd, $J_{3'cis,2'}$ 10.4 Hz, $J_{3'cis,3'trans}$ 1.5 Hz, 3'-H cis), 5.30 (1H, dd, $J_{2',3'trans}$ 17.3 Hz, $J_{3'cis,3'trans}$ 1.5 Hz, 3'-H trans), 5.92 (1H, m, $J_{2',3'\ trans}$ 17.3 Hz, $J_{2',3'\ cis}$ 10.4 Hz, $J_{2',1'}$ 5.7 Hz, 2'-H) and 7.32 (25H, m, Ar-H); δ_C (50.3 MHz; C^2HCl_3) 72.2 (2-C) 73.3, 74.5, 74.7, 76.2, 76.4 (Benzyl-CH_2 & 1'-C), 81.2, 81.4, 82.2, 84.1 (Ins-C), 117.2 (3'-C), 127.9 ,128.0, 128.1, 128.3, 128.5, 128.6, 128.9 (Ar-C), 135.4 (2'-C); *m/z* (CI) 688 ($[M + NH_4]^+$, 35%), 671 (65, $[M + H]^+$), 108 (100, $[OBn + H]^+$) and 91 (50, Bn^+); TLC R_f = 0.57 (20% ethyl acetate/ petroleum ether).

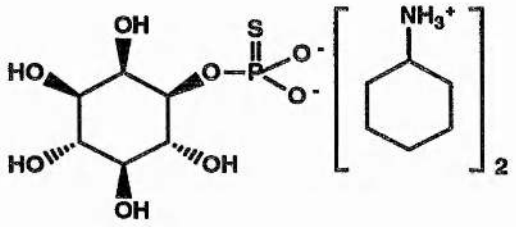


(+/-)-2,3,4,5,6-Penta-*O*-benzyl-*myo*-inositol (86):[132] To (+/-)-1-*O*-Allyl-2,3,4,5,6-penta-*O*-benzyl-*myo*-inositol (**85**) (5.0 g, 7.5 mmol) in ethanol/ water (9:1 v/v, 90 ml) was added Wilkinson's complex (0.7 g, 0.8 mmol) and DABCO (0.27 g, 2.4 mmol) and the mixture refluxed under a nitrogen atmosphere for 4 hours. The suspension was hot filtered and the precipitate washed with ethanol. The solvents were removed *in vacuo* to give an oil which was refluxed in a mixture of acetic acid (120 ml), water (40 ml) and THF (80 ml) for 4 hours. The solvents were removed *in vacuo* and the product purified by silica column chromatography (0-40% ethyl acetate/ petroleum ether, in 5% steps) and recrystallised from ethanol to give white crystals. (4.29 g, 91%); m.pt. 90-92 °C, (lit.,[132] 91-93 °C); *m/z* (Found: $[M + H]^+$ 631.3060. $C_{41}H_{43}O_6$ requires 631.3059); υ_{max} (nujol)/ cm^{-1} 3600 sb (O-H), 3010 m, 2880 m, 1500 m, 1460 s, 1370 s, 1040 s, 1070 s and 700 s; δ_H (300 MHz; C^2HCl_3) 2.20 (1H, bs, OH), 3.30-3.56 (3H, m, Ins-H), 3.64-3.98 (1H, t, *J* 9.6 Hz, Ins-H), 3.90-4.15 (2H, m, Ins -H), 4.55-5.05 (10H, m, Benzyl CH_2) and 7.30 (25H, m, Ar-H); δ_C (75.5 MHz; C^2HCl_3) 72.5 (2-C), 73.0, 74.8, 75.5, 75.7, 75.9 (Benzyl-CH_2), 77.2, 81.2, 81.9, 82.2, 83.6 (Ins-C), 127.5, 127.6, 127.7, 127.8, 128.0, 128.1, 128.3, 128.4 and 128.5 (Ar-C); *m/z* (CI) 648 ($[M + NH_4]^+$, 30%), 631 (15, $[M + H]^+$), 108 (100, [OBn + H]$^+$) and 91 (40, Bn^+); TLC R_f = 0.16 (20% ethyl acetate/ petroleum ether).



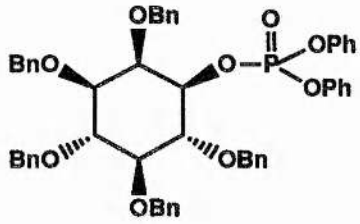
(+/-)-2,3,4,5,6-Pentakis-*O*-benzyl-*myo*-inositol-biscyanoethyl phosphorothioate (95):[132] To 2,3,4,5,6-penta-*O*-benzyl-*myo*-inositol (**86**) (2.5 g, 4 mmol) and N'N-diisopropylethylamine (1.0 ml, 6 mmol) in anhydrous acetonitrile (15 ml) was added N'N-diisopropyl(2-cyanoethyl)chloro-phosphoramidite (1.57 g, 1.4 ml, 6 mmol) at 0 °C under an argon atmosphere and the reaction mixture stirred for 4 hours. 3-Hydroxypropionitrile (1.3 ml, 18 mmol) and 1-H tetrazole (0.4 g, 6 mmol) in dry acetonitrile (15 ml) were added and the reaction mixture stirred for 16 hours at room temperature. The solvent was removed *in vacuo*, and the residue partitioned between ethyl acetate (100 ml) and 100 mM phosphate buffer (50 ml, pH 7, 100 mM). The aqueous fraction was extracted with ethyl acetate (2 x 50 ml) and the combined organic extracts washed with saturated brine (100 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo*, the residual oil redissolved in pyridine (100 ml), and sulphur (1.9 g, 60 mmol) added. The reaction mixture was stirred at room temperature for 15 minutes. The solvent was removed *in vacuo* and the residue extracted in toluene. Excess sulphur was removed by filtration. On removal of the solvents the residual oil was redissolved in toluene (5 ml), purified by silica column chromatography on triethylamine basified silica (0-50% ethyl acetate/ petroleum ether, in 5% steps) and recrystallised from methanol to give white crystals. (2.16 g, 65%); m.pt. 96-97 °C, (lit.,[132] 97-98 °C); (Found, C, 67.8; H, 5.95; N, 3.35. Calc. for $C_{47}H_{49}N_2O_8PS$, C, 68.1; H, 6.1; N, 3.35 %); υ_{max} (nujol)/ cm^{-1} 3050 w, 2900 w, 2270 w (CN), 1500 m, 1460 m, 1370 m, 1070 vs, 1030 vs, 950 m, 800 m (P=S) and 700 s; δ_H (C^2HCl_3; 300 MHz) 2.15-2.58 (4H, m, 2"H_2), 3.48 (1H, dd, $J_{2,3}$ 2.3 Hz, $J_{3,4}$ 9.6 Hz, 3-H), 3.55 (1H, t, $J_{4,5}$ 9.2 Hz, $J_{5,6}$ 9.2 Hz, 5-H), 4.00 (6H, m, 1"H_2 and 4-H and 6-H), 4.36 (1H, td, $J_{1,2}$ 2.5 Hz, $J_{2,3}$ 2.3 Hz, 2-H), 4.70-5.05 (10H, m, Benzyl CH_2) and 7.33 (25H, m, Ar-H); δ_C (75.5 MHz; C^2HCl_3), 19.8, 19.9, 20.1, 20.2 (2"C), 63.1, 63.2, 63.3 (1"C), 73.9 (2-C), 75.8, 76.3, 76.7, 76.9 (Benzyl CH_2), 80.3, 80.4, 80.6, 80.7, 81.3, 82.2, 84.1 (Ins-C), 117.3 (CN), 128.3, 128.4, 128.6, 128.7, 128.1, 128.7, 128.8 and 129.2 (Ar-C); δ_P (C^2HCl_3,

121.5 MHz) 66.5; *m/z* (CI), 850 ([*M* + NH_4]$^+$, 10%), 833 (10, [*M* + H]$^+$), 201 (10), 181 (20), 105 (30) and 91 (100, Bn^+); TLC R_f = 0.09 (20% ethyl acetate/ petroleum ether).



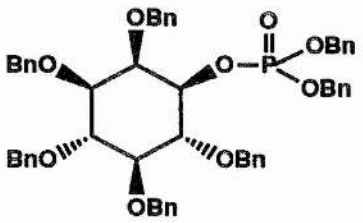
(+/-)-*myo*-Inositol 1-phosphorothioate bis-cyclohexylammonium salt (95a);[132] A freshly prepared solution of sodium methoxide (4 mmol) in dry methanol (10 ml) was added to (+/-)-2,3,4,5,6-pentakis-*O*-benzyl-*myo*-inositol-bis-cyanoethyl phosphorothioate (**95**) (0.83 g, 1 mmol) in dry methanol (10 ml) at 4 °C under an argon atmosphere, and stirred for 4 hours. The solvent was removed *in vacuo* to give disodium salt, which showed the expected NMR parameters. The crude 2,3,4,5,6-penta-*O*-benzyl-*myo*-inositol 1-phosphorothioate disodium salt was dissolved in THF (50 ml), and this solution added dropwise with stirring to a solution of sodium (50 mg) in liquid ammonia (100 ml), at -78 °C, under an argon atmosphere. When the blue colouration of the ammonia solution became faint, further small pieces of sodium were added to the ammonia solution followed by further amounts of the disodium salt solution. After the final addition of this solution the reaction mixture was stirred for a further 30 minutes and dry methanol (10 ml) added. The ammonia was allowed to evaporate and the solvents removed *in vacuo*. The residue was then partitioned between water (100 ml) and diethyl ether (50 ml). The aqueous layer was removed, reduced to a volume of approximately 10 ml and subjected to chromatography on Amberlite IR-118 (H^+) ion exchange resin, eluting with water. The acid fractions containing the product were collected and freshly distilled cyclohexylamine (3 ml) added. The mixture stirred at room temperature for 4 hours, and the aqueous solution extracted with diethyl ether (3 x 50 ml) to remove the excess cyclohexylamine. The solution was lyophilised overnight and the residue recrystallised from water/ acetone to give a white solid (2.16 g,

65%); m.p. 165-170 °C (dec.), (lit.,[132] 165-170 °C (dec.)); υ_{max} (nujol)/ cm^{-1} 3500-2500 bs (O-H and N-H), 1608 m, 1523 m, 1114 s, 1040 s, 979 s, 811 s and 722 s; δ_H (200 MHz; ^2H_2O) 1.00-2.00 (20H, m, cyclohexylamine-CH_2), 3.08 (2H, m, cyclohexylamine-CH), 3.28 (1H, t, $J_{5,6}$ 9.2 Hz, $J_{4,5}$ 9.0 Hz, 5-H), 3.52 (1H, dd, $J_{2,3}$ 2.8 Hz, $J_{3,4}$ 9.7 Hz, 3-H), 3.62 (1H, t, $J_{3,4}$ 9.7 Hz, $J_{4,5}$ 9.0 Hz, 4-H), 3.73 (1H, t, $J_{1,6}$ 9.7 Hz, $J_{5,6}$ 9.2 Hz, 6-H), 4.08 (1H, td, $J_{1,P}$ 11.0 Hz, $J_{1,6}$ 9.7 Hz, $J_{1,2}$ 2.7 Hz, 1-H) and 4.20 (1H, t, $J_{1,2}$ 2.7 Hz, $J_{2,3}$ 2.8 Hz, 2-H); δ_C (75.5 MHz; ^2H_2O) 26.74, 27.24, 33.61, (cyclohexyl-CH_2), 53.12, (cyclohexyl-CH) 73.68, 74.01, 74.92, 75.16, 77.29 and 77.63 (Ins-CH); δ_P (121.5 MHz; ^2H_2O) 44.4; *m/z* (FAB) 475 ([*M* + H]$^+$, 2%), 376 (6, [*M* - $C_6H_{13}N$ + H]$^+$) and 100 (100, cyclohexyl-ammonium$^+$).



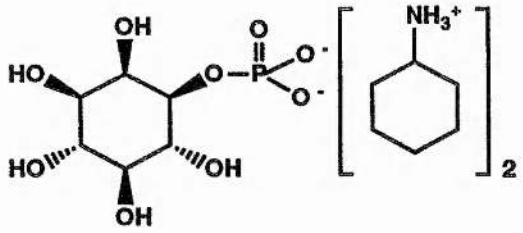
(+/-) 2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol bis-phenyl phosphate (96):[165] (+/-) 2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol (**86**) (4.5 g, 7.2 mmol), dimethyl amino pyridine (90 mg, 0.72 mmol) and dry triethylamine (3.0 ml, 21.4 mmol) were dissolved in dry dichloromethane (25 ml) under an argon atmosphere. Diphenylphosphorochloridate (2.28 ml, 11.1 mmol) was added and the reaction mixture stirred at room temperature for 24 hours. The solvent was removed *in vacuo*, redissolved in diethyl ether (100 ml) and washed with water (50 ml). The aqueous phase was washed with diethyl ether (3 x 50 ml), the combined organic fractions washed with saturated brine (40 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo* to give a pale brown oil that was purified by silica column chromatography (25% ethyl acetate/ petroleum ether) and recrystallised from hexane to give white crystals (4.23 g, 68%); m.pt. 96-99 °C, (lit.,[165] 97-99 °C); *m/z* (Found: [*M* + H]$^+$ 863.3350. $C_{53}H_{52}O_9NP$ requires 863.3349); υ_{max} (nujol)/ cm^{-1} 3000 s, 2850 s (CH), 1600 s, 1200 m, 1050 s (C-

O) and 970 s; δ_H (300 MHz; C^2HCl_3) 3.45 (2H, m, Ins-H), 4.00 (1H, t, *J* 9.3 Hz, Ins-H), 4.05 (1H, t, *J* 9.3 Hz, Ins-H), 4.25 (1H, t, *J* 2.1 Hz Ins-H), 4.45 (1H, m, 1-H), 4.75 (10H, m, Benzyl CH_2) and 7.20 (35H, m, Ar-H); δ_C (75.5 MHz; C^2HCl_3) 72.8, 74.9, 75.4, 75.8, 75.9, 76.6 (Benzyl CH_2), 79.7, 79.8, 80.4, 81.1, 83.0 (Ins-C), 119.8, 120.0, 125.1, 127.3, 127.4, 127.9, 128.2, 128.3, 129.6 (Ar-CH), 137.9, 138.1, 138.3, 138.4, 150.3 and 150.4 (Ar-C quaternary); δ_P (121.5 MHz; C^2HCl_3) -12.0; *m/z* (CI) 863 ([*M* + H]$^+$, 10%), 108 (35, $BnOH^+$) and 91 (100, Bn^+).



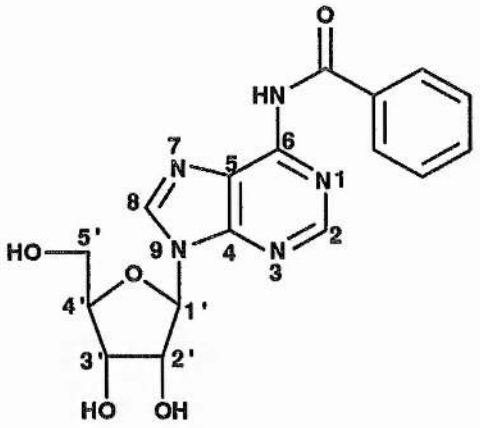
(+/-) 2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol bis benzyl phosphate (97):[165] Benzyl alcohol (0.51 ml, 4.8 mmol) in dry THF (20 ml) was added dropwise to a solution of (+/-) 2,3,4,5,6-penta-*O*-benzyl *myo*-inositol bis phenyl phosphate (**96**) (2.15 g, 2.45 mmol) and sodium hydride (0.27 g, 45% dispersion in oil, 5.04 mmol) in dry THF (20 ml). The reaction mixture was stirred at room temperature for 18 hours and on completion saturated ammonium chloride solution (10 ml) added with extreme caution. The solvent was removed *in vacuo*, the residue redissolved in dichloromethane (100 ml) and washed with water (3 x 50 ml). The aqueous phase was washed with dichloromethane (3 x 30 ml) and the combined organic fractions dried ($MgSO_4$). The solvent was removed *in vacuo* and the resulting pale brown oil purified by silica column chromatography (15-45% ethyl acetate/ petroleum ether, in 10% steps) to give a pale yellow oil (1.35 g, 63%); υ_{max} (nujol)/ cm^{-1} 3030 s, 3000 s, 2800 s (C-H), 1500 m, 1450 s, 1370 s, 1180 s, 1030 s (C-O) and 700 s; δ_H (200 MHz; C^2HCl_3) 3.50 (2H, m, Ins-H), 4.10 (2H, m, Ins-H), 4.30 (2H, m, Ins-H), 4.90 (14H, m, Benzyl CH_2) and 7.30 (35H, m, Ar-H); δ_C (50.5 MHz; C^2HCl_3) 69.7, 69.8, 69.9, 73.2, 75.4, 76.0, 76.8 (Benzyl CH_2), 78.2, 79.1, 79.2, 7806, 80.7, 81.0, 81.8,

83.6 (Ins-C), 127.9, 128.0, 128.2, 128.3, 128.5, 128.6, 128.8, 129.0, 129.1 (Ar-C), 136.2, 136.3, 139.0 and 139.2 (Ar-C quaternary); δ_P (121.5 MHz; C^2HCl_3) -1.1; *m/z* (CI), 892 ([*M* + H]$^+$, 20%), 784 (5, [*M* + H -BnO]$^+$), 678 (5, [*M* + H - 2BnO]$^+$), 255 (7, [*M* + H - 7Bn]$^+$), 108 (20, $BnOH^+$) and 91 (40, Bn^+).



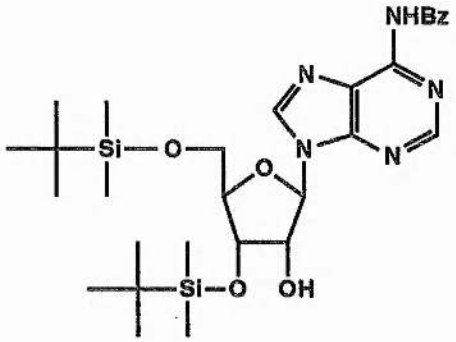
(+/-) *myo*-Inositol 1-phosphate bis cyclohexylammonium salt (33):[128] 2,3,4,5,6-Penta-*O*-benzyl-*myo*-inositol bis benzyl phosphate (**97**) (1.20 g, 1.35 mmol) was dissolved in THF (30 ml), and this solution added dropwise to a solution of sodium (50 mg) in liquid ammonia (100 ml) at -78 °C under an argon atmosphere, with stirring. When the blue colouration of the ammonia solution became faint, further small pieces of sodium were added to the ammonia solution followed by further amounts of protected phosphate solution. After the final addition of this solution the reaction mixture was stirred for a further 30 minutes and dry methanol (10 ml) added. The ammonia was allowed to evaporate and the solvents removed *in vacuo*. The residue was dissolved in water (10 ml) and subjected to chromatography on Amberlite IR-118 (H^+) ion-exchange resin, eluting with water. The acid fractions containing the product were collected, and freshly distilled cyclohexylamine (5 ml) added and the reaction mixture stirred at room temperature for 4 hours. The aqueous solution was extracted with diethyl ether (3 x 50 ml) to remove the excess cyclohexylamine and the sample lyophilised. The residue was recrystallised from water/ acetone to give a white solid. (0.43 g, 69%); m.p. 185-195 °C (dec.), (lit.,[165] 185-190 °C (dec.)); (Found: C, 45.55; H, 8.7; N, 5.7. Calc. for $C_{18}H_{39}N_2O_9P{\cdot}H_2O$: C, 45.4; H, 8.7; N, 5.9%); υ_{max} (nujol)/ cm^{-1} 3500-2000 bvs (O-H and N-H), 1640 s, 1560 s, 1260-950 bs, 840 s and 740 s; δ_H (200 MHz; ^2H_2O) 1.0-2.1 (20H, m, cyclohexyl-CH_2), 3.13 (2H, m, cyclohexyl-CH), 3.33 (1H,

t, $J_{4,5}$ 9.2 Hz, $J_{5,6}$ 9.2 Hz, 5-H), 3.56 (1H, dd, $J_{3,4}$ 9.9 Hz, $J_{2,3}$ 2.8 Hz, 3-H), 3.63 (1H, t, $J_{3,4}$ 9.5 Hz, $J_{4,5}$ 9.5 Hz, 4-H), 3.74 (1H, t, $J_{1,6}$ 9.6 Hz, $J_{5,6}$ 9.6 Hz, 6-H), 3.89 (1H, td, $J_{1,P \text{ and } 1,6}$ 9.1 Hz, $J_{1,2}$ 2.8 Hz, 1-H) and 4.21 (1H, t, $J_{1,2 \text{ and } 2,3}$ 2.8 Hz, 2-H); δ_C (50.5 MHz; ^2H_2O) 21.8, 22.3, 28.4 (cyclohexyl-CH_2), 48.4 (cyclohexyl-CH), 69.0, 69.2, 69.7, 69.8, 70.2, 70.4, 72.3 and 72.5 (Ins-C); δ_P (121.5 MHz; ^2H_2O) 4.3; *m/z* (FAB) 459 ([*M* + H]$^+$, 24%), 360 (2, [*M* - $C_6H_{13}N$ + H]$^+$) and 100 (100, [cyclohexylamine + H]$^+$).



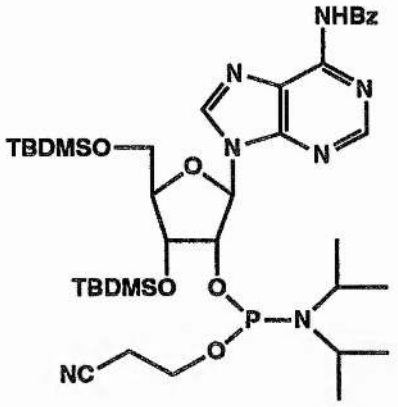
N^6-Benzoyl adenosine (99);[166] Benzoyl chloride (18.5 g, 15 ml, 131 mmol) was added to a suspension of adenosine (**98**) (6.30 g, 25.0 mmol) in dry pyridine (40 ml) at 0 °C, and the reaction mixture stirred under a nitrogen atmosphere for 4 hours. The resulting pink solution was poured into iced water (*ca.* 60 ml) and the adenosine derivative extracted into dichloromethane (3 x 100 ml). The combined organic extracts were washed with water (100 ml) and saturated sodium bicarbonate solution (100 ml) and the solvent removed *in vacuo*. The resulting yellow oil was redissolved in ethanol (150 ml) and pyridine (100 ml) and this solution treated with a mixture of sodium hydroxide (200 ml, 2 M) and ethanol (100 ml), and stirred at room temperature for 5 minutes. The solvent was removed *in vacuo*, and the resulting solid redissolved in water (100 ml). Hydrochloric acid (150 ml, 2 M) was added to form a white precipitate, which was filtered and washed with diethyl ether to remove any benzoic acid present. Recrystallisation from methanol afforded white crystals (6.60 g, 71%); m.pt. 152 °C (dec); (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 372.1308); $[\alpha]_D$ -49.5° (c 0.052 in CH_3OH); υ_{max} (nujol)/ cm^{-1} 3500 w, 3420 w, 3340 w, 3100 w (OH and NH), 1700 s, (amide C=O), 1300 m, 1220 m, 1050 m and 700 m; δ_H (200 MHz; DMSO) 3.70 (2H, ABX, $J_{5'A,5'B}$ 12 Hz, $J_{4',5'A}$ 4 Hz, $J_{4',5'B}$ 3 Hz, 5'-H_2), 4.05 (1H, q, $J_{4',3'}$ 4 Hz, $J_{4',5'}$ 4 Hz, 4'-H), 4.24 (1H, t, $J_{3',4'}$ 4 Hz, $J_{2',3'}$ 4 Hz, 3'-H), 4.65 (1H, t, $J_{2',3'}$ 4 Hz, $J_{1',2'}$ 5 Hz, 2'-H), 6.13 (1H, d, $J_{1',2'}$ 5 Hz, 1-'H), 7.60 (3H, m, Ar-H meta and para), 8.08-8.12 (2H, m, Ar-H ortho), 8.87 (1H, s, adenine CH)) and 9.11 (1H, s, adenine CH); δ_C (200 MHz; DMSO) 61.6 (C-5'), 70.7 (C-3'), 74.0 (C-2'), 86.0 (C-4'), 87.9 (C-1'), 126.1 (5-C), 128.8 (Ar-C meta and para), 132.7 (Ar-C ortho), 133.7 (Ar-C quaternary), 143.4 (2-C), 150.8, 151.9, 152.6 (8-C, 4-C and 6-C) and 166.1 (amide C=O); *m/z* (EI) 371 (M^+, 5%), 238 (8, benzoyl adenine), 135 (100, $[adenine-H]^+$), 105 (95, PhCO) and 77 (85, Ph^+); TLC R_f = 0.62 (50% ethyl acetate/ methanol).



N^6-Benzoyl 3',5'-*O*-*t*butyl dimethylsilyl adenosine (100);[167] N^6-Benzoyl adenosine (**99**) (4.0 g, 10.8 mmol) was dried azeotropically by the addition and evaporation of aliquots of dry DMF (3 x 20 ml). The resulting residue and imidazole (4.40 g, 64 mmol) were dissolved in dry DMF (15 ml) under an argon atmosphere, and *t*-butyldimethylsilyl chloride (4.54 g, 32.3 mmol) added. The reaction mixture was stirred at room temperature for 2 hours and quenched by the addition to ammonia solution (40 ml, 5%). The silylated adenosine derivatives were extracted into ethyl acetate (3 x 50 ml) and the extracts dried ($MgSO_4$). The solvents were removed *in vacuo* to give a mixture of four compounds as a yellow oil. The products were separated by short silica

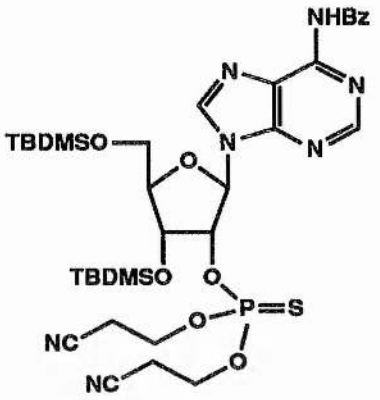
column chromatography, (0-35% ethyl acetate/ petroleum ether, in 5% steps) to give the desired product as a white foam (3.89 g, 36%); (Found: C, 57.6; H, 7.95; N, 11.4: Calc. for $C_{29}H_{45}N_5O_5Si_2$: C, 58.05; H, 7.55; N, 11.65%); *m/z* (Found: $[M+H]^+$ 600.3040. $C_{29}H_{46}N_5O_5Si_2$ requires 600.3037); $[\alpha]_D$ -21° (c 0.33 in Et_2O); υ_{max} (nujol)/ cm^{-1} 3300 m (O-H), 2900 s (C-H), 1710 s (amide C=O), 1450 s, 1240 m, 1050 m and 850 m; δ_H (200 MHz; C^2HCl_3) 0.03 (3H, s), 0.05 (3H, s), 0.13 (3H, s) and 0.14 (3H, s) ($SiCH_3$), 0.87 (9H, s) and 0.93 (9H, s) (Si^tBu H_3's), 3.85 (2H, ABX, $J_{5'A,5'B}$ 12 Hz, $J_{4',5'A}$ 3 Hz, $J_{4',5'B}$ 3 Hz, 5'H_2), 4.11 (1H, q, $J_{4',3'}$ 4 Hz, $J_{4',5'}$ 3 Hz 4'H), 4.57 (2H, m, 2'H and 3'H), 6.07-6.09 (1H, d, $J_{1',2'}$ 4 Hz, 1'H), 7.53 (3H, m, Ar-H meta and para), 8.02 (2H, m, Ar-H ortho), 8.21 (1H, s, adenine H), 8.76 (1H, s, adenine H) and 9.24 (1H, bs, amide NH); δ_C (75 MHz; C^2HCl_3) -10.2 - -10.3 ($SiCH_3$'s), 18.6, 18.9 (Si*t*-Bu, quaternary C), 26.4, 26.9 (Si^tBu CH_3's), 64.7 (5'-C), 72.1, 75.6, 86.0, 89.4 (ribose ring C), 123.8 (5-C), 128.4, 129.3 (Ar-C mata and para), 133.2 (Ar-C ortho), 134.2 (Ar-C quaternary), 142.1 (8-C), 150.1, 152.1, 153.1 (2-C, 4-C and 6-C), and 165.3 (amide C=O); *m/z* (CI) 600 ($[M+H]^+$, 70 %), 343 (5, [TBDMSO_2 ribose + H]$^+$), 240 (100, [AdBz + H]$^+$) and 121 (10, $BzNH_2^+$); TLC: 2',3',5'-tris-*O*-TBDMS-N^6BzAd, R_f = 0.93; 2',5'-bis-*O*-TBDMS-N^6Bz Ad, R_f = 0.67; 3',5'-bis-*O*-TBDMS-N^6Bz Ad, R_f = 0.58 and 5'-*O*-TBDMS-N^6Bz Ad, R_f = 0.48 (50% ethyl acetate/ diethyl ether).



N^6-Benzoyl 3',5'-*O*-^{t}Butyl dimethylsilyl-adenosine-2'-*O*-(N,N'-diisopropyl-cyanoethyl) phosphoramidite (101):[168,183] Diisopropyl ethyl amine (2.97 ml, 16.9 mmol), DMAP (106 mg, 1.69 mmol) and N'N-diisopropyl(cyanoethyl)chlorophosphoroamidite (1.31 g, 0.97 ml, 5.5 mmol) were dissolved in THF (40 ml) under an argon atmosphere, and the mixture stirred at room temperature for 10 minutes. A solution of N^6-Benzoyl 3',5'-*O*-^{t}butyl dimethylsilyl adenosine (**100**) (2.5 g, 4.24 mmol) in THF (30 ml) was added dropwise, and the reaction mixture was stirred at room temperature for 3 hours. The solvents were removed *in vacuo*, and the resulting oil partitioned between ethyl acetate (50 ml) and saturated sodium bicarbonate solution (50 ml). The organic layer was washed with saturated sodium bicarbonate solution (3 x 30 ml) and dried ($MgSO_4$) The solvents were removed *in vacuo*, and the residue applied to a triethylamine basified silica column (35% ethyl acetate/ petroleum ether) giving the desired product as two separate diastereomers (2.11 g, 62%); Lower R_f diastereomer; *m/z* (Found, $[M+Na]^+$ 822.3935. $C_{38}H_{62}N_7O_6Si_2Na$ requires 822.3935); $[\alpha]_D$ -62.2° (c 0.8 in CH_3OH); υ_{max} (nujol)/ cm^{-1} 3200 bs, 1700 s (amide C=O), 1611 s, 1460 s, 1250 s and 1073 s; δ_H (200 MHz; C^2HCl_3), 0.09 (3H, s, $SiCH_3$), 0.11 (3H, s, $SiCH_3$), 0.13 (3H, s, $SiCH_3$) and 0.14 (3H, s, $SiCH_3$), 0.92 (9H, s, Si^{t}BuCH$_3$'s) and 0.94 (9H, s, Si^{t}BuCH$_3$'s), 1.13 (12H, dd, $J_{iPrCH3,P}$ 14.6 Hz, $J_{iPrCH3,iPrH}$ 6.6 Hz, ^{i}PrCH$_3$'s), 3.58 (4H, m, CH_2CN), 3.90 (2H, ABX, $J_{5'A,5'B}$ 11.4 Hz, $J_{4',5'A}$ 3.6 Hz, $J_{4',5'B}$ 3.4 Hz, 5'-H_2), 4.15 (1H, q, $J_{4',3'}$ 3.6 Hz, $J_{4',5'}$ 4 Hz C4'-H), 4.55 (1H, t, $J_{4',3'}$ 4 Hz, $J_{2',3'}$ 4.4 Hz C3'-H), 4.70 (1H, dt, $J_{2',3'}$ 4.5 Hz, $J_{1',2'}$ 5.2Hz, $J_{2',P}$ 10 Hz, 2'-H), 6.35 (1H, d, $J_{1',2'}$ 4.9 Hz, 1'-H), 7.58 (3H, m, Ar-H meta and para), 8.05 (2H, m,

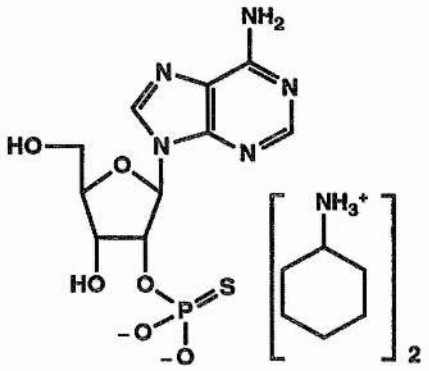
Ar-H ortho), 8.39 (1H, s, adenine-H) and 8.80 (1H, s, adenine-H) and 9.14 (1H, s, amide NH); δ_C (75 MHz; C^2HCl_3) -4.9 - -3.9 ($SiCH_3$'s), 18.6 (Si^tBu, quaternary C), 18.9 (Si^tBu, quaternary C), 23.1 ($^iPrCH_3$), 23.2 ($^iPrCH_3$) 24.9 (Si^tBu CH_3's), 43.5 (^iPrC-H), 43.9 (^iPrC-H), 58.4 ($J_{P,C}$ 18 Hz, $POCH_2$), 62.8 (5'-C), 70.8 (4'-C), 77.0, 77.2 (2'-C $J_{P,C}$ 15 Hz), 86.0, 87.9 (1'-C and 4'-C), 116.9 (CN), 123.7 (5-C), 128.4, 129.3 (Ar-C meta and para), 133.2 (Ar-C ortho), 134.3 (Ar-C quaternary), 142.6 (8-C), 149.9, 152.3, 152.9 (2-C, 4-C and 6-C) and 165.3 (amide C=O); δ_P (121.5 MHz; C^2HCl_3) 150.4; *m/z* (FAB) 823 ([*M* + Na]$^+$, 45%), 801 (10, [*M* + H]$^+$), 699 (25, [*M* - $^iPr_2NH$]$^+$), 343 (45, [(TBDMSO)$_2$ ribose + H]$^+$), 262 (15, [AdBz + Na]$^+$) and 240 (45, [AdBz + Na]$^+$); R_f = 0.32 (50 % ethyl acetate/ petroleum ether).

Higher R_f diastereomer; *m/z* (Found: [*M* + Na]$^+$ 822.3935. $C_{38}H_{62}N_7O_6Si_2Na$ requires 822.3935); $[\alpha]_D$ -54.4° (c 0.8 in CH_3OH); υ_{max} (nujol)/ cm^{-1} 3200 bs, 1700 s (amide C=O), 1611 s, 1456 s, 1255 s, 1073 s and 839 s; δ_H (200 MHz ; C^2HCl_3), 0.10 (3H, s, $SiCH_3$), 0.11 (3H, s, $SiCH_3$), 0.15 (3H, s, $SiCH_3$) and 0.20 (3H, s, $SiCH_3$), 0.92 (9H, s, Si^tBu CH_3), 0.95 (9H, s, Si^tBu CH_3), 1.15 (12H, dd, $J_{iPrCH3,P}$ 14.6 Hz, $J_{iPrCH3,iPrH}$ 6.6 Hz, $^iPrCH_3$'s), 3.60 (4H, m, CH_2CN), 4.00 (3H, m, 5'-H_2 and 4'-H), 4.55 (1H, t, $J_{4',3'}$ 4 Hz, $J_{2',3'}$ 4.4Hz 3'-H),4.86 (1H, dt, $J_{2',3'}$ 4.5 Hz, $J_{1',2'}$ 5.2 Hz, $J_{2',P}$ 10 Hz, 2'-H), 6.25 (1H, d, $J_{1',2'}$ 4.9 Hz, 1'-H), 7.58 (3H, m, Ar-H meta and para), 8.05 (2H, m, Ar-H ortho), 8.39 (1H, s, adenine CH), 8.80 (1H, s adenine CH) and 9.12 (1H, bs, amide NH); δ_C (75 MHz; C^2HCl_3) -4.9 - -3.9 ($SiCH_3$'s), 17.0, 17.3 (Si^tBu, quaternary C), 23.1, 23.2 ($^iPrCH_3$) 24.7, 25.0 (Si^tBuCH_3's), 41.9, 42.1 (^iPrC-H), 57.1, 57.3 ($J_{P,C}$ 18 Hz, $POCH_2$), 61.0 (5'-H_2), 69.9 (4'-C), 74.9, 75.1 (2'-C $J_{P,C}$ 15 Hz), 84.3, 86.4 (1'-C and 4'-C), 115.6 (CN), 122.1 (5-C), 126.6, 127.7 (Ar-C meta and para), 131.6 (Ar-C ortho), 132.7 (Ar-C quaternary), 140.8 (8-C), 148.3, 150.4, 151.5 (2-C, 4-C and 6-C) and 165.4 (amide C=O); δ_P (121.5 MHz; C^2HCl_3) 150.6; *m/z* (FAB) 823 ([*M* + Na$^+$, 90%), 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/ petroleum ether).



N^6-Benzoyl 3',5'-bis-*O*-*t*-Butyldimethylsilyl adenosine-(2'-bis-cyanoethyl) phosphorothioate (102):[132] To 1-H tetrazole (0.3 g, 4.2 mmol) in dry acetonitrile (10 ml) under an argon atmosphere was added 3-hydroxy propionitrile (0.41 ml, 6.0 mmol). The protected phosphoramidite nucleoside (**101**) (1.58 g, 2.0 mmol) in dry acetonitrile (10 ml) was added, and the reaction mixture stirred at room temperature for 3 hours. The solvent was removed *in vacuo* and the resulting oil redissolved in dry pyridine (20 ml). Sulfur (0.64 g, 20 mmol) was added and the reaction mixture stirred at room temperature for 15 minutes. Excess sulfur was removed by filtration, using ethyl acetate as a solvent for the phosphorothioate. The product was further purified by silica column chromatography on triethylamine basified silica gel (65% ethyl acetate/ petroleum ether) to give the product as a white foam. (0.75 g, 48%); *m/z* (Found: $[M + NH_4]^+$ 802.3003. $C_{35}H_{53}N_7O_7PSSi_2$ requires 802.3003); $[\alpha]_D$ -50.6° (c 0.6 in CH_3OH); υ_{max} (nujol)/ cm^{-1} 2520 w, 1700 s, 1580 s, 1550 s, 1300 s, 1150 s, 1000 s, 950 w, 930 w, 890 w, 830 m, 790 m and 725 s; δ_H (200 MHz; C^2HCl_3) 0.12 (3H, s, Si-CH_3), 0.13 (3H, s, Si-CH_3), 0.17 (3H, s, Si-CH_3), 0.19 (3H, s, Si-CH_3), 0.93 (9H, s, Si-^{t}BuCH_3), 0.96 (9H, s, Si-^{t}BuCH_3), 2.62 (2H, t, CH_2CN), 2.69 (2H, t, CH_2CN), 3.90 (2H, ABX, $J_{A,B}$ 11.6 Hz, $J_{A,X}$ 2.9 Hz, $J_{B,X}$ 2.3 Hz, 5'-H), 4.20 (5H, m, 4'-H and $POCH_2$), 4.60 (1H, t. $J_{4',3'}$ 3.1 Hz, $J_{2',3'}$ 4.3 Hz, 3'-H), 5.40 (1H, dq, $J_{1',2'}$ 6.2 Hz, $J_{2',3'}$ 4.7 Hz, $J_{2',P}$ 10.6 Hz, 2'-H), 6.40 (1H, d, $J_{1',2'}$ 6.2 Hz 1'-H), 7.55 (3H, m, Ar-H meta and para), 8.05 (2H, d, *J* 6.8 Hz, Ar-H ortho), 8.81 (1H, s, adenine-H) and 8.82 (1H, s, adenine-H); δ_C (75.5 MHz; C^2HCl_3) -4.8, -4.4, -4.0 (Si-CH_3's), 18.9, 19.3 (Si^{t}Bu quaternary C), 20.0, 20.1, 20.2 (CH_2CN), 26.5, 26.7, 26.8 (Si^{t}BuCH_3), 60.3, 62.2, 62.4, 62.8 (5'-C

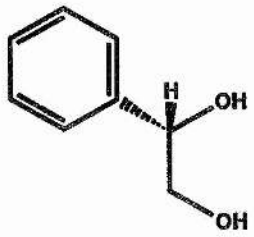
and $POCH_2$), 72.0, 80.4, 86.3, 87.2 (ribose C-H), 117.0, 117.1 (CN), 123.9 (5-C), 128.7, 129.6 (Ar-C meta and para), 133.6 (Ar-C ortho), 134.3 (Ar-C quaternary), 142.5 (8-C), 150.4, 152.7, 153.3 (2-C, 4-C and 6-C) and 165.6 (amideC=O); δ_P (121.5 MHz; C^2HCl_3) 66.5; *m/z* (FAB) 824 ([*M* + Na]$^+$, 100%), 802 (55, [*M* + H]$^+$) and 600 (5, [*M* + H - $PS(OR)_2$]$^+$).



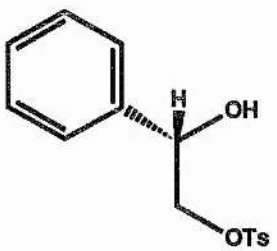
Adenosine 2'-phosphorothioate bis-cyclohexylamine salt (103):[168] N^6-Benzoyl 3',5'-bis-*O*-*t*-Butyldimethylsilyl adenosine-(2'-bis-cyanoethyl) phosphorothioate (**102**) (300 mg, 0.375 mmol) was dissolved in an ethanolic ammonia/ concentrated ammonia solution (1:3 v/v; 5 ml) in a sealed glass vial, and stirred at 60 °C for 20 h. The solvent was removed *in vacuo* and the residue partitioned between 5% ammonia solution (5 ml) and diethyl ether (10 ml). The organic phase was washed with water (3 x 5 ml) and the combined aqueous phases concentrated under reduced pressure. The resulting oil was dissolved in dry THF (2.0 ml) and tetra-*n*-butyl ammonium fluoride (1.0 M solution in THF, 0.8 ml) added. The reaction mixture was stirred for 10 hours and the solvents removed *in vacuo*. The residue was redissolved in water (2 ml) and the product subjected to ion exchange chromatography on Amberlite IR 118 (cyclohexylammonium)$^+$. The uv active fractions were combined and lyophilised to give a white solid (35 mg, 53%); m.pt. >250°C; (Found: C, 47.65; H, 7.25; N, 17.1. Calc. for $C_{22}H_{40}N_7O_6PS$: C, 47.1; H, 7.2; N, 17.45%); $[\alpha]_D$ -41.2° (c 1.4 in CH_3OH); υ_{max} (nujol)/ cm^{-1} 3400 bs, 2200 w, 1700 s, 1300 s, 1150 s and 700 s; δ_H (200 MHz; ^2H_2O), 0.9-2.1 (cyclohexyl CH_2), 3.10 (cyclohexyl CH), 3.85 (2H,

m, 5'-H), 4.28 (1H, m, 4'-H), 4.38 (1H, m, 3'-H), 5.20 (1H, m, 2'-H), 6.00 (1H, d, $J_{1',2'}$ 6.0 1'-H), 8.2 (1H, s, adenine-H) and 8.4 (1H, s, adenine-H); δ_C (75.5 MHz; ^2H_2O) 26.7, 27.3, 33.3 (cyclohexyl CH_2), 53.2 (cyclohexyl CH), 64.7 (5'-C), 73.6 (4'-C), 79.1 (3'-C), 88.5 (1'-C), 90.5 (2'-C) 121.8 (5-C), 144.0 (8-C), 151.5, 155.2 and 158.2 (2-C, 4-C and 6-C); δ_P (121.5 MHz; ^2H_2O) 45.2; *m/z* (FAB) 463 ([*M* - $C_6H_{14}N$ + H]$^+$, 2%), 430 (4, [*M* (bis sodium salt) + Na]$^+$), 408 (10, [*M* (mono sodium salt) + Na]$^+$), 386 (15, [*M* (mono sodium salt) + H]$^+$), 364 (15, [*M* (free acid) + H]$^+$) and 272 (25, [*M* - thiophosphate + Na]$^+$).

3-Hydroxy propionitrile:[186] To water (1.0 g, 1.0 ml, 55.6 mmol) in dry acetonitrile (5 ml) was added dry acrylonitrile (3.7 ml, 56 mmol) and the reaction mixture stirred at room temperature under an argon atmosphere. Freshly cut sodium metal (50 mg) was added with extreme caution, and the reaction mixture stirred at room temperature for 6 days. Hydrochloric acid (0.36 ml, 6 M) was then added and the solvents removed *in vacuo*. The residue was resuspended in acetone (30 ml) and the salts removed by filtration. The solvent was once again removed *in vacuo* to give a pale yellow liquid (crude yield 94%), which was purified by distillation at reduced pressure (3.20 g, 81%); b.pt. 105 °C, 10 mm/ Hg, (lit.,[186] 228 °C); υ_{max} (nujol)/ cm^{-1} 3470 bs (OH), 2250 s, 1680 s, 1410 s, 1350 s, 1200 s and 1070 s; δ_H (200 MHz; C^2HCl_3), 1.70 (1H, bs, OH), 2.60 (2H, t, CH_2CN) and 3.70 (2H, t, OCH_2); δ_C (50.5 MHz; C^2HCl_3), 19.3 ($\underline{C}H_2CN$), 66.3 (OCH_2) and 112.8 (CN); *m/z* (EI), 71 (*M*$^+$, 55%), 54 (70, [*M* - OH]$^+$) and 45 (58, [*M* - CN]$^+$).

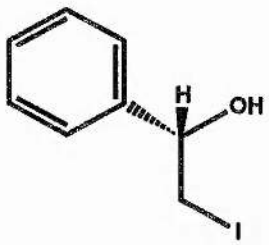


(+)-(S)-Phenyl ethane-1,2-diol (106):[184] (+)-(S)-Mandelic acid (**31**) (7.5 g, 50 mmol) in dry THF (100 ml) was added over a period of 2 hours to a stirred suspension of lithium aluminium hydride (2.0 g, 55 mmol) in dry THF (100 ml) under a nitrogen atmosphere. The reaction mixture was stirred under reflux for 4 hours, and then stirred overnight at room temperature. Water (5 ml) was added with extreme caution, and the resulting suspension filtered through celite. The precipitate was washed with diethyl ether (50 ml) and the solvent removed *in vacuo* to yield a solid residue that was recrystallised from toluene and hexane to give white crystals (5.68 g, 83%); m.pt. 65-66°C, lit.,[184] 67 °C; *m/z* (Found: M^+ 138.0681. $C_8H_{10}O_2$ requires 138.0681); $[\alpha]_D$ +58.2° (c 0.97 in Et_2O) (lit.,[184] $[\alpha]_D^{20}$ +60.7° (c 0.97 in Et_2O)); υ_{max} (nujol)/ cm^{-1} 3350 bs, 3210 bs (O-H), 1530 m, 1200 w, 1305 w, 820 m, 680 w and 650 w; δ_H (200 MHz; C^2HCl_3), 3.65 (2H, m, 1-OH and 2-OH), 3.65 (2H, ABX, $J_{A,B}$ 11.6 Hz, $J_{A,X}$ 3.6 Hz, $J_{B,X}$ 8.1 Hz, 2-H_2), 4.75 (1H, q, $J_{A,X}$ 3.6 Hz, $J_{B,X}$ 8.1 Hz, 1-H) and 7.30 (5H, m, Ar-H); δ_C (50.5 MHz; C^2HCl_3), 68.3 (2-C), 75.1 (1-C), 126.6, 128.4, 129.0 (Ar-C) and 141.0 (Ar-C quaternary); *m/z* (EI) 138 (M^+, 20%), 107 (100, $[M-CH_2OH]^+$) and 77 (90, Ph^+).



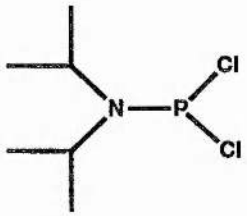
1-(S)-Phenylethanol-2-tosylate (107):[103] (+)-S-Phenyl ethane-1,2-diol (**106**) (4.80 g, 35 mmol) was dissolved in pyridine (10 ml) under an argon

atmosphere, and cooled to 0 °C. *p*-Toluene sulphonyl chloride (7.15 g, 37.5 mmol) was added, and the reaction mixture stirred at 4°C for 3 days. The solution was poured into ice water (70 ml), and extracted with diethyl ether (3 x 70 ml). The combined organic fractions were washed with hydrochloric acid (2 x 70 ml, 1 M) and water (70 ml) and dried (Na_2SO_4). The solvent was removed *in vacuo* to give an off white solid that was recrystallised from toluene and hexane to give white needles (8.76g, 86%); m.pt. 71-72°C, (lit.,[103] 72 °C); (Found, C, 61.9; H, 5.5: Calc. for $C_{15}H_{16}O_4S$: C, 61.65; H, 5.65%); *m/z* (Found: $[M + NH_4]^+$ 310.1110. $C_{15}H_{20}O_4SN$ requires 310.1113); $[\alpha]_D$ +31.2° (c 4.22 in CH_3OH), (lit.,[103] $[\alpha]_D$ +30.3° (c 3.35 in CH_3OH)); υ_{max} (nujol)/ cm^{-1} 3540 s (O-H), 1730 w, 1370 s, 1180 s (OSO_2), 1000 s, 925 s, 850 s, 816 s, 760 s, 685 s and 651 s; δ_H (200 MHz; C^2HCl_3), 2.45 (3H, s, Ar-CH_3), 2.60 (1H, bs, OH), 4.10 (2H, ABX, $J_{A,B}$ 10.3 Hz, $J_{A,X}$ 3.5 Hz, $J_{B,X}$ 8.4 Hz, 2-H_2), 4.95 (1H, q, $J_{A,X}$ 3.5 Hz, $J_{B,X}$ 8.4 Hz, 1-H), 7.35 (7H, m, Ar-H) and 7.75 (2H, d, *J* 8.4 Hz, tosyl Ar-H ortho to sulfur); δ_C (50.5 MHz; C^2HCl_3), 21.5 (Ph-CH_3), 71.8 (2-C), 74.2 (1-C), 126.0, 127.8, 128.4, 128.5, 129.8 (Ar-C), 132.4 (Ph-Me quaternary C)138.1 (Ar-C quaternary) and 144.9 (Ph-SO_2 quaternary C); *m/z* (CI) 310 ($[M + NH_4]^+$, 100%), 292 (5, M^+), 275 (50, $[M + H - H_2O]^+$), 156 (60, $MePhSO_2^+$) 120 (60, $[M + H - CH_2OTs]^+$), 91 (70, $PhCH_3^+$) and 78 (15, $[Ph + H]^+$).



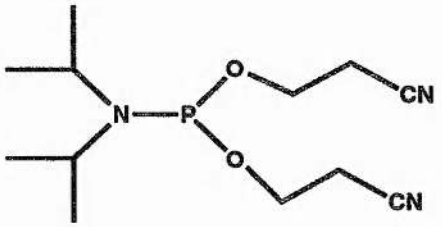
(S)-2-Iodophenylethanol (28):[103] 1-(S)-Phenylethanol-2-tosylate (**107**) (4.0 g, 14 mmol) and tetra-*n*-butyl ammonium iodide (10.0 g, 28 mmol) were dissolved in dry benzene (60 ml) and heated under reflux, under an argon atmosphere for 5 hours. On cooling, the solution was washed with water (100 ml), 5% sodium bisulphite solution (2 x 100 ml), saturated sodium bicarbonate solution (100 ml) and water (100 ml). The organic phase was dried ($MgSO_4$)

and the solvent removed *in vacuo* to give a brown oil, which was recrystallised from petroleum ether to give pale yellow needles (2.56g, 76%); m.pt. 28-29 °C, (lit.,[103] 30 °C); (Found: C, 38.7; H, 3.65. Calc. for C_8H_9OI; C, 38.75 H, 3.65%); *m/z* (Found: $[M + NH_4 - H_2O]^+$ 247.9940. $C_8H_{11}ON$ requires 247.9938); $[\alpha]_D$ + 47.8° (c 0.62 in $CHCl_3$), (lit.,[103] $[\alpha]_D$ + 43.6° (c 1.58 in $CHCl_3$)); υ_{max} (nujol)/ cm^{-1}, 3800 bs (O-H), 1560 m, 1505 s, 1250 m and 570 s (C-I); δ_H (200 MHz; C^2HCl_3), 2.50 (1H, bs, OH), 3.45 (2H, ABX, $J_{A,B}$ 10.3 Hz, $J_{A,X}$ 3.7 Hz, $J_{B,X}$ 8.6 Hz, 2-H_2), 4.82 (1H, q, $J_{A,X}$ 3.7 Hz, $J_{B,X}$ 8.6 Hz, 1-H) and 7.40 (5H, m, Ar-H); δ_C (50.5 MHz, C^2HCl_3), 15.9 (2-C), 74.5 (1-C), 126.2, 128.9, 129.2, (Ar-C) and 141.6 (Ar-C quaternary); *m/z* (CI) 248 ($[M + NH_4 - H_2O]^+$, 15%), 231 (10, $[M + H - H_2O]^+$), 138 (30, $[M + NH_4 - I]^+$), 122 (40, $[M + H - I]^+$) and 108 (25, $[M + H - CH_2I]^+$).

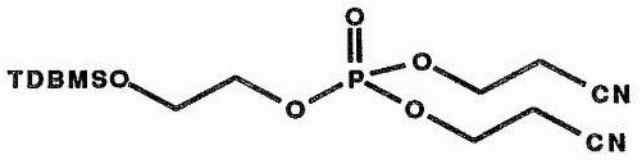


N,N'-diisopropyldichlorophosphoramidite (115):[130] Dry diisopropylamine (31.8g, 44.3 ml, 0.155 mol) in dry diethyl ether (75 ml) was added dropwise to a vigorously stirred solution of phosphorus trichloride (**114**) (21.3 g, 0.155 mol) in dry diethyl ether (75 ml), at -20 °C under a nitrogen atmosphere. After 2 hours the reaction was allowed to warm to room temperature and stirred for a further 1 hour. The salts were removed by filtration and solvent removed *in vacuo*. The residue was fractionally distilled at reduced pressure to yield the product as a colourless liquid which solidified on cooling. (18.8 g, 60 %), b.pt. 80-81°C, 8 mm Hg, (lit.,[57] 72-74°C, 7 mm Hg); *m/z* (Found: $[M]^+$ 201.0241. $C_6H_{14}NPCl_2$ requires 201.0241); υ_{max} (nujol)/ cm^{-1} 2800 s (C-H), 1500 m, 1450 m, 1200 s and 1050 s; δ_H (200 MHz; C^2HCl_3) 1.25 (12H, d, $J_{Me,H}$ 6.3 Hz, ^{i}Pr-CH_3) and 3.90 (2H, dsept, $J_{P,H}$ 12.5 Hz, $J_{Me,H}$ 6.3 Hz, ^{i}Pr-H);

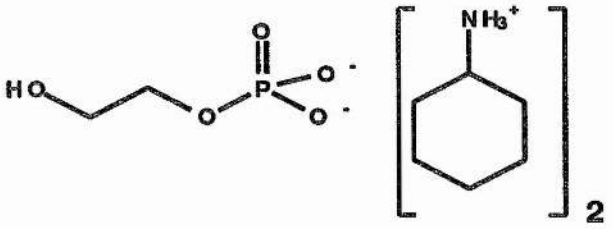
δ_C (50 MHz; C^2HCl_3) 23.7 (d, $J_{P,C}$ 8 Hz, Me-C) and 48.4 (d, $J_{P,C}$ 14.8 Hz, ^{i}Pr-C); δ_P (121.5 MHz; C^2HCl_3) 169; *m/z* (EI) 203 (M^+ $^{35}Cl^{37}Cl$, 6%), 201 (15, M^+ $^{35}Cl^{35}Cl$), 186 (65, $[Cl_2PN^iPr_2\text{-Me}]^+$), 166 (46, $ClPN^iPr_2^+$), 144 (48, $Cl_2P^iPr^+$), 124 (18, $ClPNH^iPr^+$), 101 (8, Cl_2P^+), 88 (32, PN^iPr^+) and 43 (95, $^iPr^+$).



N,N-Diisopropyl-bis-cyanoethyl phosphoramidite (116):[57,130] To N,N-diisopropyl dichlorophosphoramidite (**115**) (5.05 g, 25 mmol) in dry dichloromethane (20 ml), at -10 °C under a nitrogen atmosphere was added a solution of 3-hydroxypropionitrile (4.6 g, 65 mmol) and anhydrous triethylamine (6.6 g, 65 mmol) in dry dichloromethane (20 ml). The reaction mixture was stirred for 30 minutes, warmed to room temperature and stirred for a further 5 hours. The reaction was diluted with dichloromethane (50 ml), washed with sodium bicarbonate solution (50 ml, 5%), saturated brine (50 ml), dried ($MgSO_4$) and the solvent removed *in vacuo* to yield a colourless oil (3.9 g, 61%); *m/z* (Found: $[M]^+$ 271.1452. $C_{12}H_{22}N_3O_2P$ requires 271.1450); υ_{max} (nujol)/ cm^{-1} 3000 s, 2280 w (CN), 1030 vs and 730 s; δ_H (200 MHz; C^2HCl_3) 1.20 (12H, d, $J_{Me\text{-}H}$ 7 Hz, ^{i}Pr-CH_3), 2.62 (4H, t, $J_{1,2}$ 6.4 Hz, CH_2 CN) and 3.38 (7H, m, ^{i}Pr-H and CH_2OP); δ_C (50 MHz; C^2HCl_3) 20.5 (d $J_{P,2}$ 6.7 Hz, $\underline{C}H_2$ CN), 24.6 (d, $J_{P,iPr\text{-}Me}$ 8.1 Hz, ^{i}Pr-CH_3), 43.5 (d, $J_{P,iPr\text{-}CH}$ 13.4 Hz, ^{i}Pr-C), 58.6 (d, $J_{P,2}$ 18.8 Hz, CH_2OP) and 118.0 (CN); δ_P (121.5 MHz; $CDCl_3$) 159; *m/z* (EI) 271 (M^+, 7%), 256 (35, $[M - CH_3]^+$), 201(25, $[M - O(CH_2)_2CN]^+$), 171 (95, $P(O(CH_2)_2CN)_2^+$), 70 (15, $O(CH_2)_2CN^+$) and 54 (100, $(CH_2)_2CN^+$).

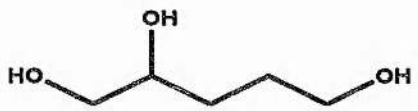


1-*O*-TBDMS-2-biscyanoethane 1,2-diol phosphate (112): *t*-Butyl dimethylsilyl chloride (1.5 g, 10 mmol) was added to a solution of ethylene glycol (**110**) (2.5 g, 50 mmol) and imidazole (1.85 g, 20 mmol) in dry DMF (30 ml) under an nitrogen atmosphere, and the solution stirred at room temperature for 3 hours. The reaction was quenched by the addition of sodium bicarbonate solution (30 ml, 5%), and extracted into diethyl ether (3 x 30 ml). The pooled organic fractions were washed with water (20 ml) and saturated brine (30 ml), dried (Na_2SO_4) and the solvent removed *in vacuo* to yield a colourless liquid which showed the expected NMR parameters. The silyl protected glycol was redissolved in dry acetonitrile (30 ml), and 1-H tetrazole (0.7 g, 10 mmol) added. A solution of N,N'-diisopropyl bis-cyanoethylphosphoramidite (**116**) (3.50 g,13 mmol) was added and the reaction mixture stirred at room temperature under a nitrogen atmosphere for 1 hour. The reaction mixture was cooled to -10 °C and a solution of *m*-CPBA (50-60%, 5.3 g, 15 mmol) in dry dichloromethane (50 ml) added, and stirred for a further 1 hour. The reaction mixture was diluted with dichloromethane (30 ml), washed with sodium sulfite solution (3 x 50 ml, 10%) and saturated brine (30 ml) and dried (Na_2SO_4). The solvent was removed *in vacuo* to yield a pale yellow oil which was purified by chromatography on triethylamine treated silica (ethyl acetate) to yield a colourless liquid (1.94 g, 54%); *m/z* (Found, $[M + H]^+$ 363.1505, $C_{14}H_{28}N_2O_5PSi$ requires 363.1505); υ_{max} (nujol)/ cm^{-1} 3000 s (C-H), 1285 s (P=O), 1050 s and 820 s; δ_H (200 MHz; C^2HCl_3) 0.08 (6H, s, $SiCH_3$), 0.90 (9H, s, $^tBuCH_3$), 2.85 (4H, t, *J* 6.2Hz, CH_2CN), 3.82 (2H, t, *J* 4.7Hz, 1-H_2), 4.20 (2H, m, 2-H_2) and 4.28, (4H, m, cyanoethyl $POCH_2$); δ_C (50.5 MHz; $CDCl_3$) -4.9 ($SiCH_3$), 10.6 (^{t}Bu quaternary), 20.1 ($\underline{C}H_2CN$), 26.3 ($^tBuCH_3$), 62.4 (1-C), 62.6 (*J* 5.5Hz, cyanoethyl $POCH_2$), 70.2 (*J* 6.2 Hz, 2-C) and 116.8 (CN); δ_P (121.5 MHz; C^2HCl_3) -1.95; *m/z* (CI) 380 ($[M + NH_4]^+$, 45%), 363 (100, $[M + H]^+$), 310 (10, $[M + NH_4$ - cyanoethyl$]^+$), 266 (10, $[M + NH_4$ - TBDMS$]^+$) and 249 (15, $[M + H$-TBDMS$]^+$).



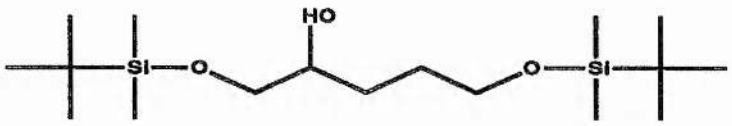
Ethane 1,2-diol monophosphate bis-cyclohexylammonium salt (113): 1-*O*-TBDMS 2-biscyanoethylphosphate (**112**) (0.5 g, 1.38 mmol) was dissolved in dry methanol (5 ml), and sodium methoxide in methanol (5.0 ml, 1.0 M) added. The reaction mixture was stirred at room temperature for 4 hours and the solvent removed *in vacuo*. The residue was redissolved in dry THF (1 ml) and a solution of tetra-*n*-butyl ammonium fluoride (1.4 ml, 1.0 M in THF) added. The solution was stirred overnight at room temperature and the solvent removed *in vacuo*. The residue was redissolved in water (5 ml) and subjected to chromatography on Amberlite IRA 118 $(H)^+$ ion exchange resin, eluting with water. The acidic fractions were combined, concentrated to a volume of *ca.* 10 ml, and excess cyclohexylamine (4 ml) added. The mixture was stirred at room temperature for 4 hours, and extracted with diethyl ether (3 x 30 ml) to remove excess cyclohexylamine. The aqueous phase was lyophilised and recrystallised from aqueous acetone to yield white needles (0.31 mg, 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 %); m.pt. 134-136 °C; υ_{max} (nujol)/ cm^{-1} 3400-2400 bs (O-H and N-H), 1270 (P=O), 1100 s, 970 s and 750 s; δ_H (200 MHz; ^2H_2O) 1.00-2.00 (20H, m, cyclohexyl CH_2), 3.05 (2H, m, cyclohexyl CH), 3.65 (2H, t, *J* 4.5Hz, 2-H_2), 3.80 (2H, m, 1-H_2); δ_C (50.5 MHz; ^2H_2O) 26.6, 27.1, 33.1 (cyclohexyl CH_2), 53.0 (cyclohexyl CH), 64.7 (2-C) and 67.8 (*J* 5.8 Hz, 1-C); δ_P (121.5 MHz; ^2H_2O) 3.98; *m/z* (FAB) 341 ($[M + H]^+$, 3%), 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, $[CHA + H]^+$).

4-Penten-1-ol (123): Allyl acetic acid (**122**) (5.0 g, 50 mmol) in dry THF (50 ml) was added over a period of 30 minutes to a stirred suspension of lithium aluminium hydride (2.1 g, 55 mmol) in dry THF (50 ml) under a nitrogen atmosphere. The reaction mixture was stirred for a further 30 minutes, water (5 ml) in THF (20 ml) added with extreme caution, and the resulting suspension filtered. The precipitate was washed with diethyl ether (100 ml) and the solvent removed *in vacuo* to give a pale yellow liquid that was kugelrohr distilled to give a colourless liquid (3.7 g, 43.1 mmol, 86 %); b.pt. 130-132 °C (lit.,[187] 134-137 °C); υ_{max} (nujol)/ cm^{-1} 3150 bs (O-H), 3490 s (C-H), 3080 m (vinyl C-H), 1641 s (C=C) and 1050 s (C-O); δ_H (200 MHz; C^2HCl_3) 1.65 (2H, m, 2-H_2), 1.95 (1H, bs, OH), 2.10, (2H, m, 3-H_2) 3.60 (2H, t, $J_{1,2}$ 6.6 Hz, 1-H_2), 4.95 (2H, m, 5-H_2) and 5.80, (1H, m, 4-H); δ_C (50.5 MHz; C^2HCl_3) 30.5, 32.0 (2-C and 3-C), 62.8 (1-C), 115.4 (4-C) and 138.6 (1-C); *m/z* (EI) 86 (M^+, 1%), 68 ([M - H_2O]$^+$, 70%) and 67 (100, [M - H_2O - H]$^+$); R_f = 0.25 (20% ethyl acetate/ petroleum ether). .

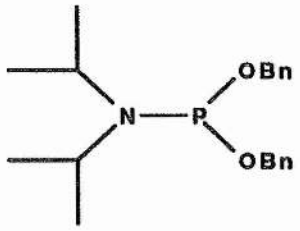


Pentane-1,2,5-triol (118):[184] 2-Ketoglutaric acid (**117**) (2.5 g, 17.1 mmol) in dry THF (50 ml) was added over a period of 30 minutes to a stirred suspension of lithium aluminium hydride (1.63 g, 42.9 mmol) in dry THF (75 ml) under a nitrogen atmosphere. The reaction mixture was stirred for a further 1 hour. Water (2.5 ml) in THF (10 ml) was added with extreme caution, and the resulting suspension filtered. The precipitate was washed with acetone (100 ml) and the solvent removed *in vacuo*. The residue was redissolved in acetone, and dried ($MgSO_4$). The solvent was removed *in vacuo* to give a colourless liquid (0.93 g, 45%); *m/z* (Found: [M + NH_4]$^+$ 138.1130. $C_5H_{16}O_3N$ requires 138.1130); υ_{max} (nujol)/ cm^{-1} 3350 bs (O-H), 2900 s (C-H) and 1075 s (C-O); δ_H (200 MHz; ^2H_2O),1.45 (4H, m, 3-H_2 and 4-H_2), 3.50 (4H, m, 1-H_2 and 5-H_2) and 3.60 (1H, m, 2-H); δ_C (50.5 MHz; ^2H_2O) 30.3, 31.6 (3-C and 4-C), 64.4 (5-C),

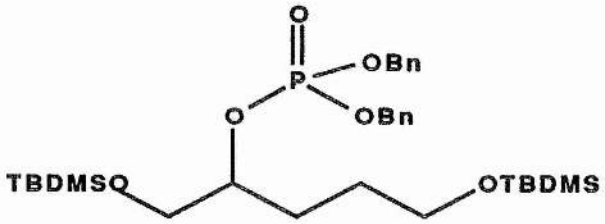
68.2 (1-C) and 74.4 (2-C); *m/z* (CI) 138 ($[M + NH_4]^+$, 100%), 121 (100, $[M + H]^+$), 103 (5, $[M + H - H_2O]^+$) and 85 (10, $[M + H - 2H_2O]^+$).



1,5-Bis-*O*-*t*-butyldimethylsilyl pentane-1,4,5-triol (119): To pentane-1,4,5-triol (**118**) (0.72 g, 6.0 mmol) and imidazole (0.9 g, 13.4 mmol) in dry DMF (20 ml) was added *t*-butyl dimethyl silyl chloride (2.0 g, 13.4 mmol). The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 24 hours. The solution was added to sodium bicarbonate solution (30 ml, 5%), and extracted into diethyl ether (3 x 50 ml). The organic phase was washed with saturated brine (30 ml) and dried (Na_2SO_4). The solvent was removed *in vacuo* to yield a pale yellow liquid that was purified by silica column chromatography (10% ethyl acetate/ petroleum ether) to give a colourless viscous liquid (1.43 g, 68%); (Found: C, 58.7; H, 11.7. Calc. for $C_{17}H_{40}O_3Si_2$: C, 58.6; H, 11.6%); *m/z* (Found: $[M + H]^+$ 349.2594. $C_{17}H_{41}O_3Si_2$ requires 349.2594); υ_{max} (nujol)/ cm^{-1} 3400 bs (O-H), 2950 s (C-H), 1100 bs (Si-O), 840 s and 790 s; δ_H (200 MHz; C^2HCl_3) 0.05 and 0.06 (12H, 2 x s, $SiCH_3$), 0.89 and 0.90 (18H, 2 x s, $^tBuCH_3$), 1.30-1.75 (4H, m, 3-H_2 and 4-H_2), 2.70 (1H, bs, OH), 3.45 (1H, dt, 2-H) and 3.60 (4H, m, 1-H_2 and 5-H_2); δ_C (50.5 MHz; C^2HCl_3) -4.9 ($SiCH_3$), 18.8 (^{t}Bu quaternary), 26.4 (^{t}Bu CH_3) 28.4 (4-C), 30.2, (3-C), 63.7 (5-C), 67.7 (1-C) and 77.2 (2-C); *m/z* (CI) 349 ($[M + H]^+$, 100 %), 331 (10, $[M + H - H_2O]^+$), 235 (5, $[M + NH_4$ - TBDMSO$]^+$), 217 (15, $[M + H$ - TBDMSOH$]^+$), 132 (15, TBDMSOH$^+$) and 58 (3, ^{t}Bu + H^+).

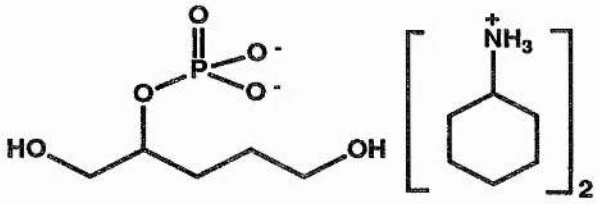


N,N'-diisopropyl bisbenzylphosphoramidite (116a):[130,185] This was prepared in an identical manner to compound (**116**) from N,N'-diisopropyl dichloro phosphoramidite (**115**) and benzyl alcohol to produce a pale yellow oil (5.2 g, 72%); *m/z* (Found: $[M + H]^+$ 346.1936. $C_{20}H_{29}NO_2P$ requires 346.1936); υ_{max} (nujol)/ cm^{-1} 3000 s (C-H), 1010 s (P-O), 750 and 700 s (C-H Ar out of plane); δ_H (200 MHz; C^2HCl_3), 1.30 (12H, d, J_{Me-H} 6.8 Hz, ^{i}Pr-CH_3), 3.75 (2H, dsept, J_{Me-H} 6.8 Hz, J_{P-H} 6.4 Hz ^{i}Pr-H), 4.80 (2H, m, CH_2OP) and 7.35 (10H, m, Ar-H); δ_C (50 MHz; C^2HCl_3) 25.0 (^{i}Pr-CH_3), 43.6 (d, $J_{P,C}$ 12.2 Hz, ^{i}Pr-CH), 65.9 (d, $J_{P,C}$ 18.1 Hz, $BnCH_2$), 127.5, 127.7, 128.8 (Ar-C) and 140.1 (Ar-C quaternary); δ_P (121.5 MHz; C^2HCl_3) 148.1; *m/z* (CI) 346 ($[M + H]^+$, 100%), 238 (5, $[M - BnO]^+$) and 91 (10, Bn^+).



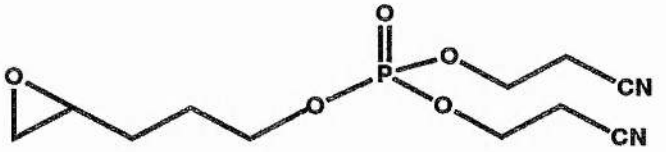
(+/-)-1,5-Bis-*O*-*t*-butyl dimethylsilyl pentane-1,2,5-triol-dibenzylphosphate (120a):[130] (+/-)-1,5-bis-*O*-TBDMS pentane-1,2,5-triol (**119**) (0.80 g, 2.29 mmol) and 1-H-tetrazole (0.32 g, 4.59 mmol) were dissolved in dry acetonitrile (10 ml) under an argon atmosphere and a solution of N,N'-diisopropyl bisbenzylphosphoramidite (**116a**) (1.03 g, 2.98 mmol) in dry acetonitrile (5 ml) added. The reaction mixture was stirred at room temperature for 2 hours and cooled to -10 °C. A solution of *m*-CPBA (55-60% purity, 1.08 g, 3.44 mmol) in dichloromethane (10 ml) was added dropwise, and the resulting

solution stirred at 0 °C for 1 hour. The reaction mixture was diluted with dichloromethane (60 ml), washed with 10% sodium sulfite solution (3 x 30 ml), sodium bicarbonate solution (2 x 20 ml, 5%), saturated brine (30 ml) and dried over (Na_2SO_4). The solvent was removed *in vacuo* to give a pale yellow oil which was purified by silica column chromatography on triethylamine basified silica (30% ethyl acetate/ petroleum ether) to give a colourless liquid (1.09 g, 78%); (Found: C, 61.0; H, 9.2: Calc. for $C_{37}H_{56}O_6PSi_2$: C, 61.15; H, 8.8%); *m/z* (Found: $[M + H]^+$ 609.3200. $C_{31}H_{57}O_6PSi_2$ requires 609.3196); υ_{max} (nujol)/ cm^{-1} 2800 s (C-H), 1500 s, 1275 s (P=O), 1100 s and 1000 s; δ_H (200 MHz; C^2HCl_3) 0.08 (12H, s, $SiCH_3$), 0.88 (18H, s, $^tBuCH_3$), 3.60 (2H, t, *J* 5.9 Hz, 5-H_2), 3.70 (2H, ABX, J_{AB} 10.6 Hz, J_{AX} 4.8 Hz, 1-H_2), 4.40 (1H, m, 2-H), 5.05 (4H, d, $J_{P,H}$ 7.7 Hz, Benzyl CH_2) and 7.35 (10H, m, Ar-H); δ_C (50 MHz; C^2HCl_3) -4.9, -4.8 ($SiCH_3$), 18.8 (^{t}Bu quaternary), 26.3, 26.4 ($^tBuCH_3$), 28.6, 29.0 (3-C and 4-C), 63.2 (5-C), 65.2 (1-C), 69.6 ($J_{P,C}$ 5.7 Hz, Benzyl CH_2), 80.1 ($J_{P,C}$ 6.4 Hz, 2-C), 128.3, 128.8, 128.9 (Ar-C) and 136.5 (Ar-C quaternary); δ_P (121.5 MHz; C^2HCl_3) -1.1; *m/z* (CI) 609 ($[M + H]^+$, 10%), 132 (5, $[TBDMSO + H]^+$) and 58 (60, $[^tBu + H]^+$).



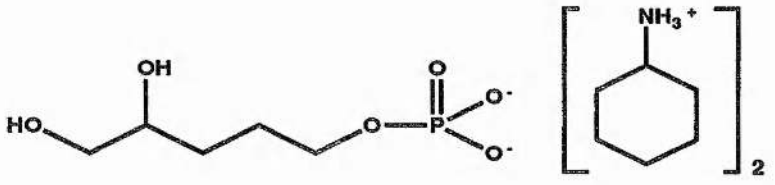
(+/-)-Pentane-1,2,5-triol-2-phosphate bis cyclohexylammonium salt (121): (+/-)-1,5-bis-*O*-TBDMS pentane-1,2,5-triol-dibenzylphosphate (**120a**) (0.80 g, 1.31 mmol) was dissolved in methanol (10 ml) and palladium on activated charcoal (80 mg, 10 %) added with stirring. The reaction vessel was flushed with hydrogen gas (approx. 500 ml) and the reaction mixture stirred under a hydrogen atmosphere at room temperature for 16 hours. The mixture was filtered through celite and the solvent removed *in vacuo*. The residue was redissolved in THF (5 ml) and TBAF (1.0 M solution in THF, 1.31 ml) added. The mixture was stirred for a further 4 hours, and the solvent removed *in vacuo*. The

residue was redissolved in water (5 ml) and subjected to chromatography on Amberlite 118 $(H)^+$ ion exchange resin eluting with water. The acidic fractions were combined, treated with freshly distilled cyclohexylamine (3 ml, 26.5 mmol) and the reaction mixture stirred at room temperature for 4 hours. The aqueous solution was extracted with diethyl ether (3 x 50 ml) to remove the excess cyxlohexylamine, and the sample lyophilised. The residue was recrystallised from water/ acetone to give white crystals. (0.36 g, 69 %); m.pt. 165-167 °C; (Found: C, 46.7; H, 9.8; N, 6.1. Calc. for $C_{17}H_{39}N_2O_6 \cdot 2H_2O$: C, 47.0; H, 10.0; N, 6.45%); υ_{max} (nujol)/ cm^{-1} 3200-2400 bs (OH and H-bonding), 1280 m (P=O), 1055 s, and 880 s; δ_H (300 MHz; ^2H_2O) 1.00-2.00 (24H, m, cyclohexyl CH_2 and 3-H_2 & 4-H_2), 3.15 (2H, m, cyclohexyl CH), 3.50-3.70 (4H, m, 1-H_2 and 5-H_2) and 4.10 (1H, m, 2-H); δ_C (75 MHz; ^2H_2O) 26.5, 27.0 (cyclohexyl CH_2), 30.0 (4-C), 30.8 (3-C), 35.0 (cyclohexyl CH), 64.4 (5-C), 67.5 (1-C) and 78.0 ($J_{P,C}$ 5.4 Hz, 2-C); δ_P (121.5 MHz; C^2HCl_3) 2.6; *m/z* (FAB) 300 ([*M* - $C_6H_{14}N$ + H]$^+$, 95%), 223 (50, [*M* - 2 ($C_6H_{14}N$) + Na]$^+$), 143 (5, [*M* - phosphate + Na]$^+$) and 121 (100, [*M* - phosphate + H]$^+$).



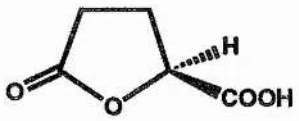
Pentane-1-biscyanoethyl phosphate-4,5-epoxide (124): Penten-1-ol (**123**) (0.30 g, 3.48 mmol) was dissolved in dry acetonitrile (10 ml) under an argon atmosphere, and 1-H tetrazole (0.24 g, 3.48 mmol) added. A solution of N,N'-diisopropyl bis-cyanoethyl phosphoramidite (**116**) (1.18 g, 4.35 mmol) was added and the solution stirred at room temperature for 2 hours. The solvent was removed *in vacuo* and the residue redissolved in dichloromethane. The resulting solution was cooled to -10 °C and a solution of *m*-CPBA (50-60% purity, 4.2 g, 12.0 mmol) in dichloromethane (50 ml) added dropwise, and the solution stirred for 1 hour. The reaction mixture was diluted with dichloromethane (60 ml), washed with sodium sulfite solution (3 x 30 ml, 10%), sodium bicarbonate solution (2 x 20 ml, 5%), saturated brine (30 ml) and dried

(Na_2SO_4). The solvent was removed *in vacuo* to give a pale yellow oil which was purified by silica column chromatography on triethylamine basified silica (ethyl acetate) to give a colourless liquid (0.63 g, 63 %); *m/z* (Found: $[M + H]^+$ 289.0953. $C_{11}H_{18}N_2O_5P$ requires 289.0953); υ_{max} (neat)/ cm^{-1} 3000 s (C-H), 1260 s (P=O), 1050 s and 950 s; δ_H (200 MHz; C^2HCl_3) 1.3-1.9 (4H, m, 2-H_2 and 3-H_2), 2.45 (1H, q, 5-H), 2.75 (5H, m, 5-H and cyanoethyl CH_2CN), 2.9 (1-H, m, C4H), and 4.2 (6H, m, 1-H_2 and cyanoethyl $POCH_2$); δ_C (50 MHz; C^2HCl_3) 20.2 ($\underline{C}H_2CN$), 27.2 (2-C) 28.9 (3-C), 47.4 (5-C), 51.0 (4-C), 62.8 ($J_{P,C}$ 5.3 Hz, cyanoethyl $POCH_2$), 68.8 ($J_{P,C}$ 6.2 Hz, 1-C), and 117.2 (CN); δ_P (121.5 MHz; C^2HCl_3) -1.98; *m/z* (CI) 306 ($[M + NH_4]^+$, 70%), 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 + $H]^+$).

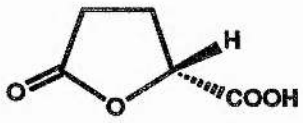


(+/-)-Pentane 1,2,5-triol 5-phosphate biscyclohexylammonium salt (125); Pentane 1-biscyanoethyl phosphate 4,5-epoxide (**124**) (85 mg, 0.3 mmol) was dissolved in acetone (0.5 ml) and sodium hydroxide solution (0.44 ml, 0.88 mmol, 2 M) added. The solution was stirred at room temperature for 2 hours, the solvent removed *in vacuo*. Excess sodium hydroxide solution (2.5 ml, 2M) was added and the reaction mixture stirred for a further 3 hours.The solvent was once again removed *in vacuo* and the residue subjected to chromatograph on Amberlite 118 $(H)^+$ ion exchange resin eluting with water. The acidic fractions were combined, treated with freshly distilled cyclohexylamine (1 ml, 8.8 mmol) and stirred at room temperature for 4 hours. The aqueous solution was extracted with diethyl ether (3 x 50 ml) to remove the excess cyxlohexylamine, and the solution was lyophilised to yield 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 229.0841); υ_{max} (nujol)/

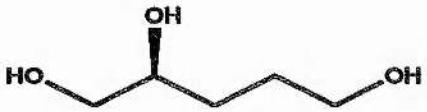
cm^{-1} 3200-2400 bs (OH and N-H), 1270 m (P=O), 955 s, and 900 s; δ_H (200 MHz; ^2H_2O) 1.00-2.00 (24H, m, cyclohexyl CH_2 and 3-H_2 & 4-H_2), 3.00 (2H, m, cyclohexyl CH), 3.40 (2H, m, 5-H_2) and 3.50-3.90 (3H, m, 1-H_2 and 2-H); δ_C (75 MHz; ^2H_2O) 26.7, 27.2 (cyclohexyl CH_2), 29.0 (4-C), 31.6 (3-C), 33.2 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 67.5 ($J_{P,C}$ 5.0 Hz, 5-C), 68.2 (1-C) and 74.4 (2-C); δ_P (121.5 MHz; C^2HCl_3) 2.66 ; *m/z* (CI) (as the dimethyl ester) 243 ([*M* + NH_4]$^+$, 40%), 229 (100, [*M* + H]$^+$), 214 (10, [*M* + H - Me]$^+$), 114 ([dimethyl phosphate + NH_4]$^+$), 127 ([dimethyl phosphate + H]$^+$).



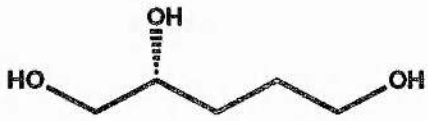
(S)-2-Oxo 5-tetrahydrofurancarboxylic acid (129a):[174] (S)-Glutamic acid (**126a**) (10.0 g, 70.0 mmol) was dissolved in hydrochloric acid (100 ml, 1 M) and cooled to -10 °C. Sodium nitrite (6.21 g, 90 mmol) was added portionwise over a period of 1 hour, and the reaction mixture stirred at room temperature for a further 16 hours. The solvent was removed *in vacuo* and the residue stirred in hot acetone (200 ml). The inorganic salts were removed by filtration and the solvent removed *in vacuo*. The residue was stirred in hot ethyl acetate (200 ml) for 1 hr, the solution decanted, and once again the solvent removed *in vacuo* to give the desired product which crystallised on cooling to give white crystals (7.78 g, 76 %); m.pt. 70-71°C (lit.,[174] 72-73°C); *m/z* (Found: [*M* + NH_4]$^+$ 148.0610. $C_5H_{10}NO_4$ requires 148.0610); υ_{max} (nujol)/ cm^{-1} 3000 bs (O-H), 1725 s, 1750 s (C=O lactone and acid), 1470 s and 1490 s; $[\alpha]_D$ +16° (c 6 in CH_3OH), (lit.,[174] $[\alpha]_D$ +14° (c 6 in CH_3OH)); δ_H (200 MHz; ^2H_2O) 2.05-2.35 (1H, m, 4-H), 2.40-2.65 (3H, m, 4-H & 3-H_2) and 4.90-5.05 (1H, q, $J_{A,X}$ 1.6 Hz $J_{B,X}$ 7.0 Hz 5-H); δ_C (50 MHz; ^2H_2O) 26.5, 28.4 (3-C & 4-C), 78.0 (5-C), 175.1 (2-C) and 181.8 (COOH); *m/z* (CI) 148 ([*M*+ NH_4]$^+$, 50%), 102 (20, [*M*+ H - CO_2H_2]$^+$) and 85 (30, [*M* - CO_2H]$^+$).



(R)-2-Oxo-5-tetrahydrofurancarboxylic acid (129b):[174] This was prepared in an identical manner to compound (**129a**) from (R)-glutamic acid (**126b**). $[\alpha]_D$ -14° (c 6 in CH_3OH), (lit.,[174] $[\alpha]_D$ -14° (c 6 in CH_3OH)); All other spectral data was identical to that obtained for the (S)-enantiomer (**129a**).



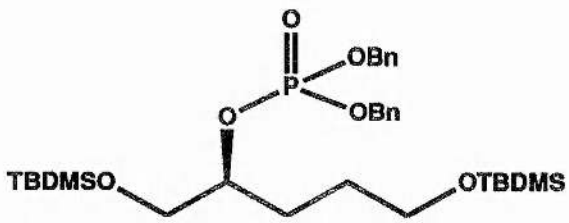
(S)-1,2,5-Pentane triol (118a):[184] (S)-2-Oxo-5-tetrahydrofurancarboxylic acid (**118a**) (2.0 g, 15.4 mmol) in dry THF (30 ml) was added dropwise with stirring under a nitrogen atmosphere to a suspension of lithium aluminium hydride (1.17 g, 30.8 mmol) over a period of 30 minutes. The reaction mixture was stirred for 1 hour, and water (3 ml) in THF (15 ml) added with extreme caution. The resulting suspension was filtered and the solvent removed *in vacuo*. The residue was redissolved in acetone (50 ml) and dried (Na_2SO_4). The solvent was removed *in vacuo* to yield a colourless viscous liquid (0.84 g, 45%); $[\alpha]_D$ -8.0° (c 1.7 in CH_3OH); All other spectral data was identical to that obtained for the racemic compound (**118**).



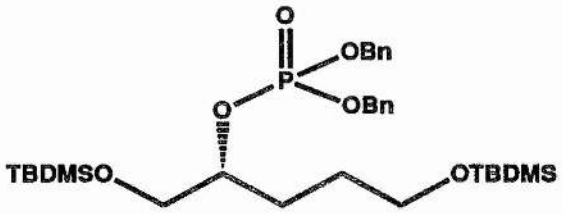
(R)-Pentane-1,4,5-triol (118b): This was prepared in an identical manner to compound (**118a**) from (R)-2-oxo-5-tetrahydrofurancarboxylic acid (**129b**). $[\alpha]_D$ +7.5° (c 2.05 in CH_3OH); All other spectral data was identical to that obtained for the racemic compound (**118**).

(S)-1,5-Bis-*O*-*t*-butyldimethylsilyl pentane1,4,5-triol (119a): This was prepared in an identical manner to compound (**119**) from (S)-Pentane-1,4,5-triol (**118a**). $[\alpha]_D$ -7.9 ° (c 5 in Et_2O); All other spectral data was identical to that obtained for the racemic compound (**119**).

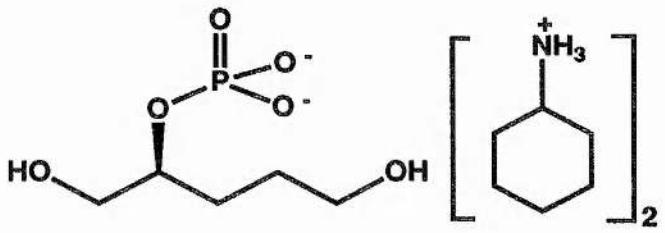
(R)-1,5-Bis-*O*-*t*-butyldimethylsilyl pentane1,4,5-triol (119b): This was prepared in an identical manner to compound (**119**) from (R)-Pentane-1,4,5-triol (**118b**). $[\alpha]_D$ +8.5° (c 4.2 in Et_2O); All other spectral data was identical to that obtained for the racemic compound (**119**).



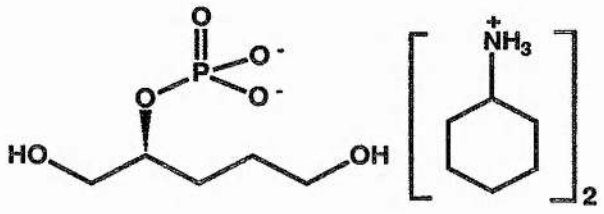
(S)-1,5-Bis-*O*-TBDMS pentane 1,2,5-triol-dibenzylphosphate (120a): This was prepared in an identical manner to compound (**120a**) from (S)-1,5-bis-*O*-^{t}butyldimethylsilyl pentane 1,4,5-triol (**119a**). $[\alpha]_D$ -10.2° (c 2.5 in Et_2O); All other spectral data was identical to that obtained for the racemic compound (**120a**).



(R)-1,5-bis-*O* -TBDMS pentane-1,2,5-triol-dibenzylphosphate (120b): This was prepared in an identical manner to compound (**120a**) from (R)-1,5-bis-*O*-*t*-butyldimethylsilyl pentane1,4,5-triol (**119b**). $[\alpha]_D$ +10.3° (c 2.5 in Et_2O); All other spectral data was identical to that obtained for the racemic compound (**120a**).

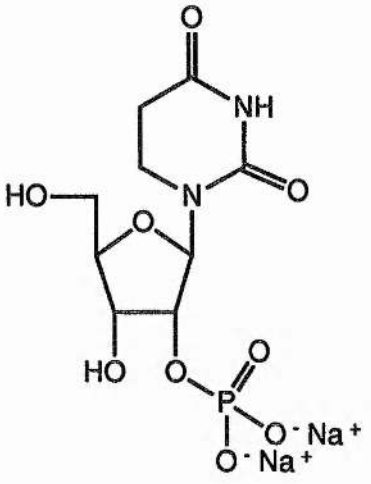


(S)-Pentane 1,2,5-triol-2-phosphate bis cyclohexylammonium salt (121a): This was prepared in an identical manner to compound (**121**) from (S)-1,5-bis-*O* -TBDMS pentane-1,2,5-triol 2-dibenzylphosphate (**120a**). $[\alpha]_D$ -2.1° (c 1.4 in H_2O); All other spectral data was identical to that obtained for the racemic compound (**120a**).

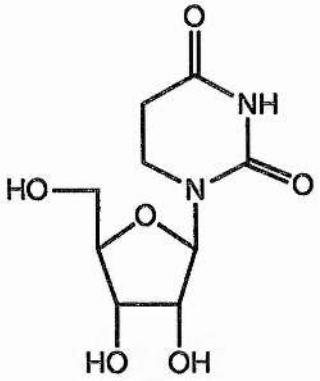


(R)-Pentane 1,2,5-triol 2-phosphate bis-cyclohexylammonium salt (121b): This was prepared in an identical manner to compound (**121**) from (R)-1,5-bis-*O*-TBDMS pentane 1,2,5-triol 2-dibenzylphosphate (**120b**). $[\alpha]_D$ +1.7° (c 1.4 in H_2O); All other spectral data was identical to that obtained for the

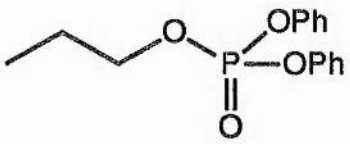
racemic compound (**121**).



Dihydrouridine 2'-phosphate bis-sodium salt (136);[178] Uridine 2'-phosphate bis-lithium salt (200 mg, 0.59 mmol) was dissolved in aqueous hydrochloric acid (20 ml, 1 mM) and 5% rhodium on alumina added. The reaction vessel was flushed with hydrogen gas (*ca.* 500 ml), and the mixture stirred under an atmosphere of hydrogen for 12 hours. On completion, the reaction mixture was filtered through celite and the solvent removed *in vacuo*. The residue was redissolved in water (5 ml) and subjected to ion exchange chromatography on Amberlite 118 $(Na)^+$, eluting with water. The eluant was lyophilised to give yield a white powder (170 mg, 88 %); m.pt. 145-155 °C (dec); $[\alpha]_D$ -32.3° (c 1.0 in H_2O) υ_{max} (nujol)/ cm^{-1} 3400-2400 bs (O-H and N-H), 1650 and 1670 (C=O), 1270 (P=O), 1100 s, 970 s and 750 s; δ_H (200 MHz; ^2H_2O) 2.70 (2H, t, *J* 6.5 Hz, CH_2CO), 3.50 (2H, t, *J* 6.4 Hz, CH_2NR), 3.65 (2H, m, 5'-H_2), 3.95 (1H, m, 4'-H), 4.15 (1H, m, 3'-H), 4.45 (1H, m, 2'-H) and 5.80 (1H, d, *J* 6.2 Hz, 1'-H); δ_C (50.5 MHz; ^2H_2O) 32.9 ($\underline{C}H_2CO$), 39.5 (CH_2NR_2), 64.1 (5'-C), 72.9 (4'-C), 75.5 (3'-C), 86.0 (1'-C), 89.8 ($J_{P,C}$ 9.7 Hz, 2'-C), 157.5 (NC(O)N) and 176.8 ($CH_2\underline{C}(O)N$); δ_P (121.5 MHz; ^2H_2O) 3.90; *m/z* (FAB) 393 ($[M + Na]^+$, 5%), 371 (5, $[M + H]^+$), 137 (15, [dihydrouracil + $Na]^+$) and 115 (10, [dihydrouracil + $H]^+$).

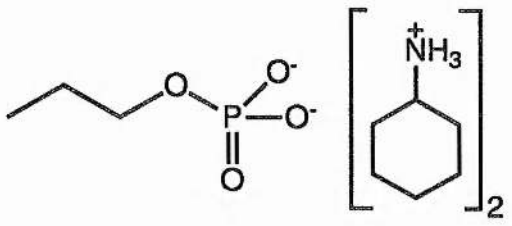


Dihydrouridine (137);[178] Uridine (**135**) (250 mg, 1.02 mmol) was hydrogenated in the presence of 5% rhodium on alumina catalyst as described for compound (**131**). On filtering through celite, the solvent was removed in vacuo to yield a white gum which resisted crystallisation (210 mg, 83%); *m/z* (Found: $[M + H]^+$ 247.0930. $C_9H_{15}N_2O_6$ requires 247.0930); $[\alpha]_D$ -25.3° (c 1.0 in H_2O) δ_H (200 MHz; ^2H_2O) 2.70 (2H, t, *J* 6.8 Hz, CH_2CO), 3.50 (2H, m, CH_2NR), 3.70 (2H, m, 5'-H_2), 3.95 (1H, m, 4'-H), 4.10 (1H, m, 2'-H), 4.25 (1H, m, 3'-H) and 5.80 (1H, d, *J* 6.4 Hz, 1'-H); δ_C (50.5 MHz; ^2H_2O) 33.0 (CH_2NR), 39.3 ($\underline{C}H_2CO$), 64.2 (5'-C), 72.9 and 73.2 (2'-C and 3'-C), 86.3 (4'-C), 90 3 (1'-C), 157.6 (NC(O)N) and 176.8 ($CH_2\underline{C}(O)N$); *m/z* (CI) 264 ($[M + NH_4]^+$, 5%), 247 (100, $[M + H]^+$), 132 (5, [Uracil + NH_4]$^+$) and 115 (10, [Uracil + H]$^+$).

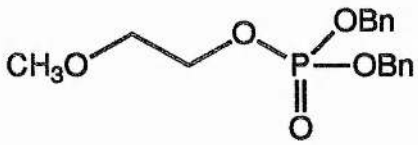


Diphenyl propyl phosphate (161);[165] *n*-Propanol (0.5 g, 8.33 mmol), DMAP (50 mg) and triethylamine (2.1 ml, 1.55 g, 15.4 mmol) were dissolved in dry THF under an argon atmosphere and the reaction mixture cooled to -10 °C. Diphenyl chlorophosphate (1.88 g, 145 ml, 7.0 mmol) was added dropwise and the reaction mixture stirred at room temperature for 2 hours. The salts were removed by filtration and the solvent removed *in vacuo*. The product was purified by silica column chromatography (30% ethyl acetate/ petroleum ether)

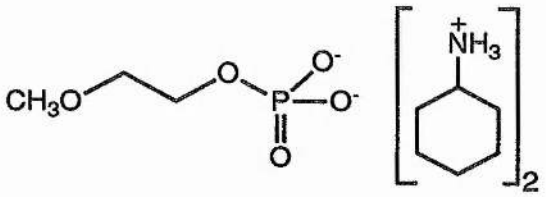
to yield a colourless liquid (1.58 g, 84 %), *m/z* (Found: M^+ 292.0864 $C_{15}H_{17}O_4P$ requires 292.0864); υ_{max} (nujol)/ cm^{-1} 2900 s (C-H), 1600 s (Ar), 1490 s, 1300 s, 1200 s (P=O), 1100 s, 1050 s, 790 s and 595 s; δ_H (200 MHz; C^2HCl_3) 0.95 (3H, t, $J_{2,3}$ 7.4 Hz, CH_3), 3.55 (2H, m, $C\underline{H}_2CH_3$), 4.25 (2H, m, CH_2OP) and 7.20 (10H, m, Ar-H); δ_C (50.5 MHz; C^2HCl_3) 9.9 (CH_3), 23.5 ($\underline{C}H_2CH_3$), 70.8 ($J_{P,C}$ 6.5 Hz, CH_2OP), 120.0, 125.3, 129.8 (Ar-C) and 136.4 ($J_{P,C}$ 5.9 Hz, Ar-C quaternary); δ_P (121.5 MHz; C^2HCl_3) -11.4; *m/z* (CI) 310 ([*M* + NH_4]$^+$, 75%), 293 (100, [*M* + H]$^+$) and 93 (20, PhO^+); TLC R_f = 0.47 (30% ethyl acetate/ petroleum ether)



Propyl phosphate bis cyclohexylammonium salt (162); This was prepared in an identical manner to compound (**97**) from diphenyl propyl phosphate (**161**) and benzyl alcohol, followed by hydrogenation over Pc/ C catalyst and recrystallisation from water and acetone (0.42 g, 59 %); m.pt. 96-99 °C; *m/z* (Found: [*M* (derivatised as the dimethyl ester) + H]$^+$ 169.0630. $C_5H_{14}O_4P$ requires 169.0630); υ_{max} (nujol)/ cm^{-1} 2850 s (N-H and C-H), 1210 m (P=O), 1050 s (C-O), 790 w and 700 w; δ_H (200 MHz; ^2H_2O) 0.95 (2H, t, $J_{2,3}$ 7.5 Hz, CH_3), 1.10-2.00 (22H, m, cyclohexyl CH_2 and $\underline{C}H_2CH_3$), 3.10 (2H, m, cyclohexyl CH) and 3.70 (2H, m, CH_2OP); δ_C (50.5 MHz; ^2H_2O) 12.5 (CH_3) 26.6, 27.1, 33.1 (cyclohexyl CH_2 and $\underline{C}H_2CH_3$), 53.1 (cyclohexyl CH), 68.9 ($J_{P,C}$ 4.4 Hz, CH_2OP); δ_P (121.5 MHz; ^2H_2O) 4.10; *m/z* (derivatised as the dimethyl ester) (CI) 186 (*M* + NH_4]+, 40%) and 169 (100, [*M* + H]+).

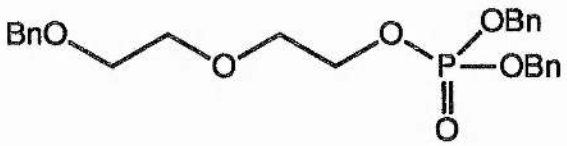


Methoxyethanol dibenzyl phosphate (159); This was prepared in an identical manner to compound (**120a**) from methoxyethanol, and purified by silica column chromatography (50% ethyl acetate/ petroleum ether) to give the desired compound as a colourless oil (1.31 g, 78 %); *m/z* (Found: $[M + H]^+$ 337.1205. $C_{17}H_{22}O_5P$ requires 337.1205); υ_{max} (neat)/ cm^{-1} 2900 s (C-H), 1485 s, 1260 s (P=O), 1050 s (C-O), 760 s and 700 s; δ_H (200 MHz; C^2HCl_3) 3.38 (3H, s, OCH_3), 3.55 (2H, m, CH_2OMe), 4.15 (2H, m, CH_2OP), 5.05 (4H, d, *J* 7.9 Hz, Benzyl CH_2) and 7.35 (10H, m, Ar-H); δ_C (50.5 MHz; C^2HCl_3) 59.5 (OCH_3), 67.1 (CH_2OMe), 69.8 ($J_{P,C}$ 5.7 Hz, Benzyl CH_2),71.7 ($J_{P,C}$ 7.2 Hz,CH_2OP), 128.3, 128.5, 129.0, 129.1 (Ar-C) and 136.4 (Ar-C quaternary); δ_P (121.5 MHz; C^2HCl_3)-0.50; *m/z* (CI) 354 ($[M + NH_4]^+$, 3%), 337 (100, $[M + H]^+$).



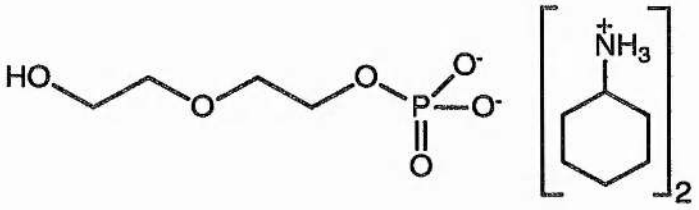
Methoxy ethanol 2-phosphate bis-cyclohexylammonium salt (160); This was prepared in an identical manner to compound (**121**) from methoxy ethanol dibenzyl phosphate (**159**) (0.72 g, 68%); m.pt. 191-195 °C; *m/z* (Found: $[M$ (free acid) + $H]^+$ 157.0266. $C_3H_{10}O_5P$ requires 157.0266); υ_{max} (nujol)/ cm^{-1} 2850 s (N-H and H-bonding), 1200 m (P=O), 1050 s (C-O), 770 w and 720 w; δ_H (200 MHz; C^2HCl_3) 1.10-2.00 (20H, m, cyclohexyl CH_2), 3.0 (2H, m, cyclohexyl CH), 3.27 (3H, s, OCH_3), 3.50 (2H, t, $J_{1H,2H}$ 4.7 Hz, CH_2OMe) and 3.50 (2H, dt, $J_{1H,2H}$ 4.6 Hz, $J_{P,C}$ 4.9 Hz CH_2OP); δ_C (50.5 MHz; C^2HCl_3) 26.6, 27.1, 33.1 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 60.8 (OCH_3), 65.6 (CH_2OMe) and 75.0

($J_{P,C}$ 8.1 Hz, Benzyl CH_2); δ_P (121.5 MHz; C^2HCl_3) 3.52; *m/z* (EI) 157 ([*M* + H]$^+$, 5%), 99 (5, [phosphate + H]$^+$) and 45 (100, $MeOCH_2^+$).



5-*O*-Benzyl diethylene glycol-1-bisbenzyl phosphate (147); Diethylene glycol (**143**) (2.0 g, 18.9 mmol) and benzyl bromide (0.64 g, 3.78 mmol) were dissolved in dry DMF (30 ml) under a nitrogen atmosphere, and sodium hydride (0.36 g, 7.5 mmol (50% dispersion in oil)) added portionwise over a period of 30 minutes. The reaction mixture was stirred at room temperature for 2 hours and quenched by the addition of water (30 ml). The product was extracted into diethyl ether (3 x 50 ml), washed with sodium bicarbonate solution (3 x 30 ml, 5%) and saturated brine (30 ml), and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue added to 1-H tetrazole (0.53 g, 7.5 mmol), and dissolved in dry acetonitrile (20 ml). A solution of N,N'-diisopropylbisbenzyl phosphoramidite (**116a**) (1.68 g, 4.9 mmol) in dry acetonitrile (10 ml) was added and the reaction mixture stirred at room temperature under a nitrogen atmosphere for 30 minutes. The reaction mixture was cooled to -10°C and a solution of *m*-CPBA (1.72 g, 4.9 mmol, 50%) in dichloromethane (30 ml) added dropwise over a period of 10 minutes at -10 °C. The reaction mixture was diluted with dichloromethane and washed with 10% sodium sulfite solution (3 x 30 ml), sodium bicarbonate solution (30 ml, 5%), saturated brine (30 ml), and dried (Na_2SO_4). The solvent was removed *in vacuo* and the residue purified by silica column chromatography on triethylamine basified silica (60% ethyl acetate/ petroleum ether) to yield a colourless oil. (0.76 g, 44% (from diethylene glycol)); *m/z* (Found: [*M* + H]$^+$ 457.1780. $C_{25}H_{30}O_6P$ requires 475.1780); υ_{max} (neat)/ cm^{-1} 2900-3100 s (C-H), 1280 s (P=O), 1050 s (C-O), 750 s and 700 s; δ_H (200 MHz; C^2HCl_3) 3.60 (6H, m, diethylene glycol CH_2), 4.15 (2H, m, CH_2OP), 4.50 (2H, s, Benzyl ether CH_2), 5.05 (4H, d, *J* 8 Hz, Benzyl ester CH_2) and 7.30 (15H, m, Ar-H); δ_C (50.5 MHz, ^2H_2O) 67.2, 67.3, 69.7, 69.9, 70.3, 70.5, 71.2 (CH_2), 128.1, 128.2, 128.5,

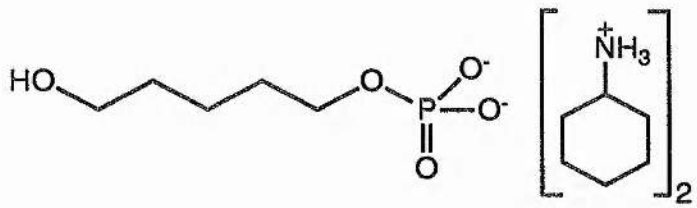
128.9, 129.0 (Ar-C) and 136.3, 138.8 (Ar-C quaternary); δ_P (121.5 MHz; C^2HCl_3) -0.55; *m/z* (CI) 457 ([*M* + H]$^+$, 100%), 277 (10, [*M* (free acid) + H]$^+$), 259 (5, [*M* + H - 2 BnO]$^+$), 108 (85, BnOH) and 91 (55, Bn$^+$); TLC R_f = 0.32 (60% ethyl acetate/ petroleum ether).



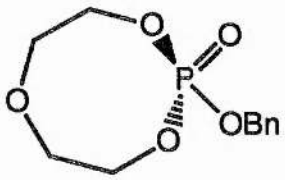
Diethylene glycol monophosphate bis cyclohexylammonium salt (149); This was prepared in an identical manner to compound (**121**) from 1-*O*-benzyl diethylene glycol-1-bisbenzyl phosphate (**147**) (0.28 g, 78 %); m.pt. 135-145 °C (phase transition); *m/z* ((derivatised as the dimethyl ester); Found: [*M* + H]$^+$ 215.0685. $C_6H_{16}O_6P$ requires 215.0685); υ_{max} (nujol)/ cm^{-1} 3300 bs (O-H), 2850 bs (N-H and C-H), 1210 s (P=O), 1070 s (C-O), 770 w and 700 w; δ_H (200 MHz; ^2H_2O) 1.00-2.00 (20H, m, cyclohexyl CH_2), 3.00 (2H, m, cyclohexyl CH_2), 3.65 (6H, m, diethylene glycol CH_2), 4.15 (2H, m, CH_2OP); δ_C (50.5 MHz, ^2H_2O) 26.6, 27.1, 33.2 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 63.1 (3-C), 65.9 (2-C), 73.4 ($J_{P,C}$ 7.7 Hz 1-C) and 74.5 (4-C); δ_P (121.5 MHz; C^2HCl_3) 2.96; *m/z* (CI) (derivatised as the dimethyl ester) 232 ([*M* + NH_4]$^+$, 65%), 215 (100, [*M* + H]$^+$), 183 (5, [*M* - OMe]$^+$) and 153 (7, [*M* - 2 OMe]$^+$).

5-*O*-benzyl pentane 1,5-diol 1-bis benzyl phosphate (148); This was prepared in an identical manner to compound (**147**) from pentane1,5-diol (**144**) (0.62 g, 28%); (Found: [*M* + H]$^+$ 455.1990. $C_{26}H_{32}O_5P$ requires

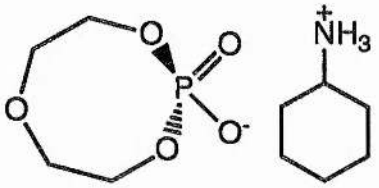
455.1987); υ_{max} (neat)/ cm^{-1} 2900 s (C-H), 1270 s (P=O), 1050 s (C-O), 750 s and 700 s; δ_H (200 MHz; C^2HCl_3) 1.30-1.65 (6H, m, 2-H_2, 3-H_2 and 4-H_2), 3.45 (2H, t, $J_{4,5}$ 6.2 Hz, 5-H_2), 4.00 (2H, dt, $J_{1,2}$ 6.3 Hz, $J_{1,P}$ 6.6 Hz, 5-H_2), 4.50 (2H, s, Benzyl ether CH_2), 5.05 (4H, d, $J_{Benzyl\ H2,P}$ 8.2 Hz, Benzyl ester CH_2) and 7.30 (15H, m, Ar-H); δ_C (50.5 MHz, C^2HCl_3) 22.7 (3-C), 29.7 (4-C), 30.5 (2-C), 68.3, 69.6, 70.5, 73.4 (1-C, 5-C and benzyl CH_2), 128.0, 128.1, 128.4, 128.9, 129.1 (Ar-C) and 136.4, 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^+); tlc R_f = 0.51 (60% ethyl acetate/ petroleum ether).



Pentane 1,5-diol monophosphate bis cyclohexylammonium salt (150); This was prepared in an identical manner to compound (**121**) from 5-*O*-benzyl pentane 1,5-diol 1-bis benzyl phosphate (**148**) (0.31 g, 83%); m.pt. 149-151 °C *m/z* ((derivatised as the dimethyl ester); Found: $[M + H]^+$ 213.0890. $C_7H_{18}O_5P$ requires 213.0892); υ_{max} (nujol)/ cm^{-1} 3300 bs (O-H), 2850 bs (N-H), 1200 m (P=O), 1050 s (C-O), 770 m and 700 m; δ_H (200 MHz; ^2H_2O) 1.00-2.00 (26H, m, cyclohexyl CH_2, 2-C, 3-C & 4-C), 3.00 (2H, m, cyclohexyl CH_2), 3.45 (2H, m, 5-CH_2) and 3.65 (2H, m, 1-H_2); δ_C (50.5 MHz, ^2H_2O) 24.3 (5-C), 26.6, 33.1 (cyclohexyl CH_2), 27.0, 32.6 (2-C & 4-C), 27.1 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 64.5 (5-C) and 67.3 ($J_{P,C}$ 5.0 Hz, 1-C); δ_P (121.5 MHz; ^2H_2O) 3.57; *m/z* (CI) (derivatised as the dimethyl ester) 230 ($[M + NH_4]^+$, 35%), 213 (100, $[M + H]^+$) and 181 (3, $[M - OMe]^+$).

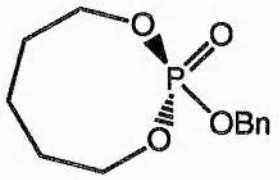


Diethylene glycol cyclic phosphate benzyl ester (155); This was prepared in an identical manner to compound (**116**) from diethylene glycol and N,N'-diisopropyl dichloro phosphoramidite (**115**) followed by transesterification with benzyl alcohol, and oxidation with *m*-CPBA in an identical manner to compound (**120**) to give the desired compound as a colourless oil (0.75 g, 46%); *m/z* (Found, $[M + H]^+$ 259.0735. $C_{11}H_{16}O_5P$ requires 259.0735); υ_{max} (nujol)/ cm^{-1}2900 bs (C-H) 1485 s, 1250 (P=O), 1100 s, 1050 s, 900 s and 720 s; δ_H (200 MHz, C^2HCl_3) 3.60 (2H, m, 2-H and 4-H), 4.00 (4H, m, 1H, 2-H, 3-H and 4-H), 4.40 (2H, m, 1-H and 4-H), 5.10 (2H, d, $J_{P,H}$ 7.2 Hz, Benzyl CH_2) and 7.35 (5H, m, Ar-H); δ_C (50.5 MHz, C^2HCl_3) 69.5, 69.7 (1-C, 5-C and Benzyl CH_2), 73.1 (2-C and 4-C), 128.2, 128.5 (Ar-C) and 139.5 (Ar-C quaternary) δ_P (121.5 MHz, C^2HCl_3) -0.79; *m/z* (CI) 517 ($[2M + H]^+$, 5%), 276 (3, $[M + NH_4]^+$), 259 (100, $[M + H]^+$), 108 (4, BnOH) and 91(5, $PhCH_2^+$).

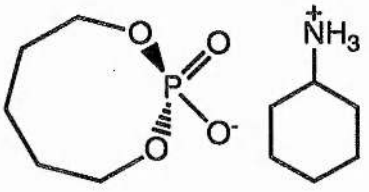


Diethylene glycol cyclic phosphate diester cyclohexylammonium salt (157); This was prepared in an identical manner to compound (**121**) from diethylene glycol cyclic phosphate benzyl ester (**155**) to give the desired compound as a white solid (0.52 g, 86%); m.pt. 190-193 °C; *m/z* (Found: [*M* (free acid) + H]$^+$ 169.0266. $C_4H_{10}O_5P$ requires 169.0266); υ_{max} (nujol)/ cm^{-1} 2900 s (C-H), 1270 (P=O), 1100 s, 1050 s and 750 s; δ_H (200 MHz; ^2H_2O) 1.00-2.00 (10H, m, cyclohexyl CH_2), 3.00 (1H, m, cyclohexyl CH_2), 3.75 (4H, m, 2-H_2

and 4-H_2), 3.95 (4H, m, 1-H_2 and 5-H_2); δ_C (50.5 MHz, ^2H_2O) 26.6, 27.1, 33.1 (cyclohexyl CH_2), 53.1 (cyclohexyl CH), 69.5 ($J_{P,C}$ CH_2OP), 73.7 (CH_2OCH_2); δ_P (121.5 MHz,C^2HCl_3) -0.25; *m/z* (CI) 337 ([2*M* + H]$^+$, 5%), 186 (100, [*M* + NH_4]$^+$) and 169 (100, [*M* + H]$^+$).



Pentane 1,5 diol cyclic phosphate monobenzyl ester (156); This was prepared in an identical manner to compound (**155**) from pentane 1,5-diol (**144**) to give the desired compound as a colourless oil (0.35 g, 34%); *m/z* (Found: *M*$^+$ 256.0864. $C_{12}H_{17}O_4P$ requires 256.0864); υ_{max} (neat)/ cm^{-1} 2860 bs (N-H and H-bonding), 1220 (P=O), 1050 s, 900 s, 760 m and 750 m; δ_H (200 MHz, C^2HCl_3) 1.60-2.10 (6H, m, 2-H_2, 3-H_2 and 4-H_2), 4.20 (4H, m, CH_2OP), 5.15 (2H, d, $J_{P,C}$ 8.5 Hz, Benzyl CH_2); δ_C (50.5 MHz, C^2HCl_3) 24.2, 29.6 (2-C, 3-C and 4-C), 69.4, 69.6 (1-C, 5-C and Benzyl CH_2), 128.4, 129.0 (Ar-C) and 137.5 (Ar-C quaternary); δ_P (121.5 MHz,C^2HCl_3) -0.80; *m/z* (CI) 274 ([*M* + NH_4]$^+$, 15%), 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 (158); This was prepared in an identical manner to compound (**121**) from pentane 1,5 diol cyclic phosphate benzyl ester (**156**) to give the desired compound as a

white solid (0.41 g, 85 %); m.pt. 160-162 °C; *m/z* (Found: [*M* (free acid) + NH_4]$^+$ 184.0739. $C_5H_{15}NO_4P$ requires 184.0738); υ_{max} (nujol)/ cm^{-1} 2850 bs (N-H and H-bonding), 1210 (P=O), 1050 s, 950 s and 790 s; δ_H (200 MHz, ^2H_2O) 1.0-2.0 (16H, m, cyclohexyl CH_2, 2-H_2, 3-H_2 and 4-H_2), 3.00 (1H, m, cyclohexyl CH), 3.85 (4H, m, CH_2OP); δ_C (50.5 MHz; ^2H_2O) 26.1, 26.6, 27.0, 31.4, 33.1 (2-C, 3-C, 4-C and cyclohexyl CH_2), 53.1 (cyclohexyl CH_2) and 70.3 ($J_{P,C}$ 6.1 Hz, $POCH_2$); δ_P (121.5 MHz, C^2HCl_3) 0.70; *m/z* (CI) 333 ([2 *M* + H]$^+$, 15%), (100, [*M* + NH_4]$^+$) and 167 (30, [*M* + H]$^+$).

Inositol as a substrate for inositol monophosphatase.[107]

50 µl Assay buffer (150 mM Tris HCl, pH 8, RT, 100 mM KH_2PO_4).

25 µl 30 mM aqueous inositol solution (for control, use 25µl distilled water).

25 µl 20mM $MgCl_2$.

100 µl enzyme solution, 0.44 mg/ ml bovine inositol monophosphatase, 1 mg/ ml BSA, S.A. = 34,400 nmol/ min/ mg.

The above samples were incubated at 37 °C for 30 minutes to achieve steady state conditions, and [^{18}O]-water (60% atom excess, 50 µl) added to each. Samples were quenched in liquid nitrogen at t = 0, 40 and 80 minutes, and the control (containing no inositol) quenched at t = 80 minutes. The water removed by lyophilisation, and the residues extracted with HCl in methanol (100 µl, 0.2 M) by vortexing for 30 seconds. Excess ethereal diazomethane was added to each sample until the yellow colouration persisted. Excess diazomethane was removed by bubbling nitrogen gas through the solution, and the supernatant separated from the residue by decanting after centrifugation. The derivatised trimethyl phosphate samples were analysed by GC/MS.

Adenosine as a substrate for inositol monophosphatase.

a) The above experiment was repeated using adenosine (30 mM, 25 µl / sample) in place of inositol.

b) The above experiment was repeated using adenosine (30 mM, 25 µl / sample) inplace of inositol, in addition to a greater overall concentration of [^{18}O]-water (45 atom %) and greater incubation periods (t_{max} = 24 hrs).

(All incubations containing adenosine were run in parallel with incubations containing inositol under identical conditions)

Colorimetric Assay.[173]

Colorimetric Assay reagent: Malachite green (1.5 g) was dissolved in hydrochloric acid (25 ml, 5 M) and diluted with water (750 ml). To this solution was added ammonium molybdate (10.5 g) in hydrochloric acid (225 ml, 5 M), and the solution stirred at room temperature for 10 minutes. The solution was

filtered by gravity, and stored in the dark for periods of up to one month.

Assay:

Assay buffer A: 200 mM KCl, 2 mM $MgCl_2$, 50 mM Tris.HCl, pH 7.8

Assay buffer B: 200 mM KCl, 2 mM $MgCl_2$, 150 mM LiCl, l50 mM Tris.HCl, pH 7.8

Incubation samples comprised of the following:

1). -assay buffer A - 210 µl
-substrate in assay buffer - 30 µl
-inhibitor in assay buffer - 30 µl (in the absence of an inhibitor, this addition was substituted with plain assay buffer (30 µl))
-enzyme solution - 30 µl

Parallel incubations were performed in the presence of Li^+ to account for any background phosphate present as follows:

2). -assay buffer B - 210 µl
-substrate in assay buffer - 30 µl
-inhibitor in assay buffer - 30 µl (in the absence of an inhibitor, this addition was substituted with plain assay buffer (30 µl))
-enzyme solution - 30 µl

The assay samples were incubated at 37 °C and the reaction quenched by the addition of colorimetric assay reagent (2.0 ml) at the required time (relative to the addition of the enzyme solution). The colour was allowed to develop over a period of approximately 30 minutes, and the optical density measured at 660 nm. In addition, known concentrations of inorganic phosphate 0-200 µM were prepared in triplicate, and treated with the colorimetric assay reagent (2.0 ml) to give a standard phosphate concentration curve from which accurate concentrations of product phosphate could be calculated .

Radiochemical Assay.[55]

[U^{14}C]-D-*myo*-inositol 1-phosphate stock solution (50 µl, 1.25 µCi) was diluted with assay buffer A (200 mM KCl, 2 mM $MgCl_2 \cdot 6H_2O$, 50 mM Tris HCl, pH 7.8) (450 µl).

Incubation samples comprised of the following:

-assay buffer A - 270 µl
-unlabelled inositol 1-phosphate in assay buffer - 30 µl
-labelled inositol 1-phosphate (diluted form) - 60 µl
-inhibitor in assay buffer - 60 µl

A 70 µl aliquot was removed and added to sodium hydroxide solution (50 µl, 1.0 M) (to act as a t = 0 quench). The relevant enzyme solution (150 µl) was added, and the sample incubated at 37 °C. At regular time intervals, aliquots (100 µl) were removed, and quenched by addition to sodium hydroxide solution (50 µl, 1.0 M). The quenched assay solutions were diluted with water (1.0 ml) and applied to a 0.5 ml column of Dowex-1 (1 x 8, 400 mesh) formate form. The [^{14}C] inositol was washed off the columns with water (1 ml), and the elutant emulsified with 10 ml scintillation fluid. Disintegrations were measured in a Packard Tri-Carb 4330 scintillation counter.

Reaction course of synthetic substrates:

assay buffer C	20 mM NH_4HCO_3, 2 mM $MgCl_2$, pH 8.0 in D_2O
enzyme solution	50 µl (0.44 mg/ ml bovine inositol monophosphatase, 1 mg/ ml BSA, S.A. = 34,400 nmol/ min/ mg) lyophilised and redissolved in assay buffer C (100 µl).

The substrate was dissolved in assay buffer C (500 µl) to a concentration of 60 mM. The ^{1}H NMR spectrum was recorded, and the enzyme solution (100 µl) added. 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, and the ^{1}H NMR and ^{31}P spectra recorded at regular time intervals.

Reaction course of uridine 2'-monophosphate hydrolysis:

Uridine 2'-monophosphate was dissolved in assay buffer C (300 µl) and [^{18}O]-H_2O (200 µl) added, and the reaction was incubated at 37 °C for 24 hours. Half of the reaction mixture was lyophilised, and the resulting uridine analysed my CI mass spectroscopy. The remainder of the solution was analysed by high resolution ^{31}P NMR spectroscopy.

Inhibition studies.

Synthetic substrates/ inhibitors were tested using the colorimetric and/ or radiochemical assays, over a range of substrate and inhibitor concentrations.

4. Appendix 1.

Publications.

1. Andrew G. Cole and David Gani.

   'Active' Conformation of the Inositol Monophosphatase Substrate, Adenosine 2'-Phosphate: Role of the Ribofuranosyl O-atoms in Chelating Magnesium Ion.
   J. Chem. Soc., Chem.Comm., Submitted for publication.

2. Andrew G. Cole and David Gani.

   Structure of the Catalytic Complex of Substrate, Magnesium Ion and Enzyme in Inositol Monophosphatase and Mechanism of the Hydrolytic Process.
   J. Chem. Soc., Chem.Comm., Submitted for publication.

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