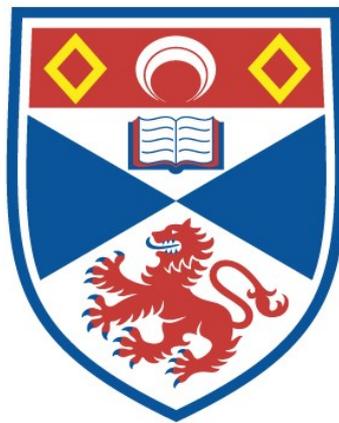


MECHANISTIC AND STRUCTURAL STUDIES ON MYO-
INOSITOL MONOPHOSPHATASE: THE EMERGING
TARGET FOR LITHIUM THERAPY

Roger David Pybus

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



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on *myo*-Inositol Monophosphatase**

The Emerging Target for Lithium Therapy

a thesis presented by

Roger David Pybus

to the

UNIVERSITY OF ST ANDREWS

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY



St Andrews

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ABSTRACT

Enzymic phosphate monoester hydrolysis by *myo*-inositol monophosphatase from bovine brain (EC.3.1.3.25) has been shown to occur *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 magnesium ions, one of which is buried deep in the active-site cleft (Mg1) and co-ordinated the phosphate moiety of the substrate. The second metal ion (Mg2) is located closer to the opening of the active site and co-ordinates to the alkyl-phosphate bridging oxygen bond of the substrate.

Detailed chemical and kinetic studies on the enzyme have defined many of the interactions of the natural substrate for the enzyme, inositol 1-phosphate, and has led to the proposal that Mg2, which is readily accessible from bulk solvent, should position and activate the nucleophilic water molecule through chelation. Independent X-ray crystallographic studies of protein-substrate complexes using inhibitory metal ions to prevent reaction has provided an almost identical picture of the active-site interactions. However, this study has placed the nucleophilic water molecule on Mg1. The two different sites for the nucleophile would give different stereochemical courses for phosphoryl transfer. A location on Mg1 would give inversion of configuration through an in-line displacement whereas attack by a water molecule chelated to Mg2 would give retention *via* an novel adjacent association and pseudorotation. In order to distinguish between these two mechanisms, the stereochemical course of the inositol monophosphatase reaction must be determined with respect to the phosphorus centre.

A synthetic route has been developed to produce the chirally labelled substrate analogues of inositol 1-phosphate which are required to perform such a study. These compounds, (*R_p*)- and (*S_p*)-inositol 1-[¹⁸O]phosphorothioate can now be produced with a high degree of isotopic enrichment. The absolute configuration at the phosphorus centre of each individual enantiomer has been determined indirectly using single crystal X-ray analysis.

Preliminary work has also been carried out on the hydrolysis of these substrate analogues by inositol monophosphatase using [¹⁷O]water and the subsequent derivatisation of the chiral inorganic [¹⁶O, ¹⁷O, ¹⁸O]phosphorothioate product. Derivatisation of the inorganic phosphorothioate product is necessary in order to determine its absolute configuration at phosphorus. A comparison of this result with the known configuration of the substrate analogue which was processed, will allow the stereochemical course of the inositol monophosphatase reaction to be determined.

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LIST OF ABBREVIATIONS

| Abbreviation | Meaning |
|--------------------|---------------------------------------|
| 2'AMP | Adenosine 2'-monophosphate |
| 2'AMP _S | Adenosine 2'-monophosphorothioate |
| A | Adenine |
| Ac | Acetyl |
| Ad | Adenosine |
| ADP | Adenosine 5'-diphosphate |
| ADPβS | Adenosine 5'-(2-thiodiphosphate) |
| Ala | Alanine |
| AMP | Adenosine 5'-monophosphate |
| Asp | Aspartate |
| ATPβS | Adenosine 5'-(2-thiotriphosphate) |
| ATPγS | Adenosine 5'-(3-thiotriphosphate) |
| Bn | Benzyl |
| br | broad |
| Bu | Butyl |
| calc. | calculated |
| cAMP | Cyclic adenosine monophosphate |
| camp. | camphanate |
| CI | Chemical ionisation |
| CMP-PA | Cytidine monophosphoryl-phosphatidate |
| d | doublet |
| Da | Daltons |
| DABCO | 1,4-Diazabicyclo[2,2,2]octane |
| DAG | Diacylglycerol |
| dd | doublet doublet |
| DEAE | Diethylaminoethyl |

| Abbreviation | Meaning |
|---------------------|--|
| decomp. | decomposed |
| DMAP | 4-Dimethylaminopyridine |
| DMF | <i>N,N</i> -Dimethylformamide |
| DMSO | Dimethyl sulfoxide |
| DTE | Dithioerythritol |
| EDTA | Ethylenediaminetetraacetic acid |
| EGTA | Ethylenebis(oxyethylenenitrilo)tetraacetic acid |
| EI | Electron impact |
| equiv. | equivalents |
| ES | Electrospray |
| Et | Ethyl |
| FAB | Fast atom bombardment |
| FPLC | Fast protein liquid chromatography |
| FT | Fourier transform |
| GDP | Guanosine diphosphate |
| GIP | Glycoinositol phosphate |
| Glu | Glutamate |
| Gly | Glycine |
| GTP | Guanosine triphosphate |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HPLC | High performance liquid chromatography |
| HRMS | High resolution mass spectrometry |
| Ile | Isoleucine |
| IMPase | Inositol monophosphatase |
| Ins(1,3)- P_2 | Inositol 1,3-bisphosphate |
| Ins(1,3,4)- P_3 | Inositol 1,3,4-trisphosphate |
| Ins(1,3,4,5)- P_4 | Inositol 1,3,4,5-tetrakisphosphate |
| Ins(1,4)- P_2 | Inositol 1,4-bisphosphate |

| Abbreviation | Meaning |
|---------------------|---|
| Ins(1,4,5)- P_3 | Inositol 1,4,5-trisphosphate |
| Ins(3,4)- P_2 | Inositol 3,4-bisphosphate |
| Ins1- P | Inositol 1-phosphate |
| Ins1- P_s | Inositol 1-phosphorothioate |
| Ins3- P | Inositol 3-phosphate |
| Ins4- P | Inositol 4-phosphate |
| IPTG | Isopropyl- β -D-thiogalactopyranoside |
| IR | Infra-red |
| IUPAC | International Union of Pure and Applied Chemistry |
| K_i | Enzyme inhibition constant |
| K_m | Michaelis constant |
| Leu | Leucine |
| lit. | literature value |
| m | multiplet |
| NAD ⁺ | Nicotinamide adenine dinucleotide |
| NADH | Reduced nicotinamide dinucleotide phosphate |
| NMR | Nuclear magnetic resonance |
| NOBA | Nitrobenzyl alcohol |
| NOE | Nuclear Overhauser Enhancement |
| Ⓟ | Phosphate group |
| PAGE | Polyacrylamide gel electrophoresis |
| Ph | Phenyl |
| P_i | Inorganic phosphate |
| PI-PLC | Phosphatidylinositol specific phospholipase C |
| Pr | Propyl |
| P_{si} | Inorganic phosphorothioate |
| PtdIns(4,5)- P_2 | Phosphatidylinositol 4,5-bisphosphate |
| Pv | Pivaloyl (trimethylacetyl) |

| Abbreviation | Meaning |
|---|---|
| q | quartet |
| R_f | Retention time |
| s | singlet |
| S_8 | Elemental sulfur |
| SDS | Dodecyl sulfate, sodium salt |
| t | triplet |
| TBAF | Tetra- <i>n</i> -butylammonium fluoride |
| TBDMSi | <i>tert</i> -Butyldimethylsilyl |
| TEAB | Triethylammoniumbicarbonate |
| THF | Tetrahydrofuran |
| Thr | Threonine |
| TLC | Thin-layer chromatography |
| Tris | Tris(hydroxymethyl)aminomethane |
| tRNA | Transfer ribonucleic acid |
| U | Uracil |
| UV | Ultra-violet |
| v/v | volume/volume |
| Val | Valine |
| $[\alpha^{18}\text{O}]\text{AMPS}$ | Adenosine 5'-(1- ^{18}O)monophosphorothioate) |
| $[\beta^{18}\text{O}]\text{ADP}\beta\text{S}$ | Adenosine 5'-(2- ^{18}O), 2-thiodiphosphate) |
| $[\gamma^{18}\text{O}]\text{ATP}\gamma\text{S}$ | Adenosine 5'-(3- ^{18}O), 3-thiotriphosphate) |
| ⊗ | Oxygen-17 atom |
| ⊙ | Oxygen-18 atom |

CHAPTER ONE

INTRODUCTION

1. Introduction

Bipolar disorder (or more commonly, manic-depression) is a debilitating disease which affects upto 1% of the world's population and is currently treated by administering lithium salts. The therapy is believed to be effective because of the ability of Li^+ to reduce signal transduction in the phosphatidylinositol intracellular signalling pathway. The cation is thought to exert its effect through its uncompetitive inhibition of the key enzyme in the pathway, inositol monophosphatase (IMPase). Inositol monophosphates produced by the signalling pathway are hydrolysed by this enzyme to liberate free *myo*-inositol which is used in the biosynthesis of a second messenger precursor, phosphatidylinositol 4,5-bisphosphate. Full elucidation of the chemical mechanism of IMPase and its mode of inhibition by lithium will allow the development of novel inhibitors of IMPase that might ultimately prove useful clinically by producing an agent with less side-effects than the currently available therapy.

1.1 The Inositols

Inositols are cyclohexane hexols and are members of a group of "carbocyclic carbohydrates" called cyclitols. There are nine possible stereoisomers; *allo*- **1**, (+)-*chiro*- **2**, (-)-*chiro*- **3**, *cis*- **4**, *epi*- **5**, *muco*- **6**, *myo*- **7**, *neo*- **8**, and *scyllo* - **9** (Figure 1.1).¹

1.1.1 *myo*-Inositol

Of the inositols, only *myo*-inositol **1** is common in nature and exhibits significant biological activity. It appears to be present in either free or combined form in all living species.² In animals and microorganisms the major portion of *myo*-inositol is present as inositol phospholipids which are components of cell membranes, and as its hexakisphosphate (phytic acid),³ where it acts as the major phosphorus source in plant seed.²

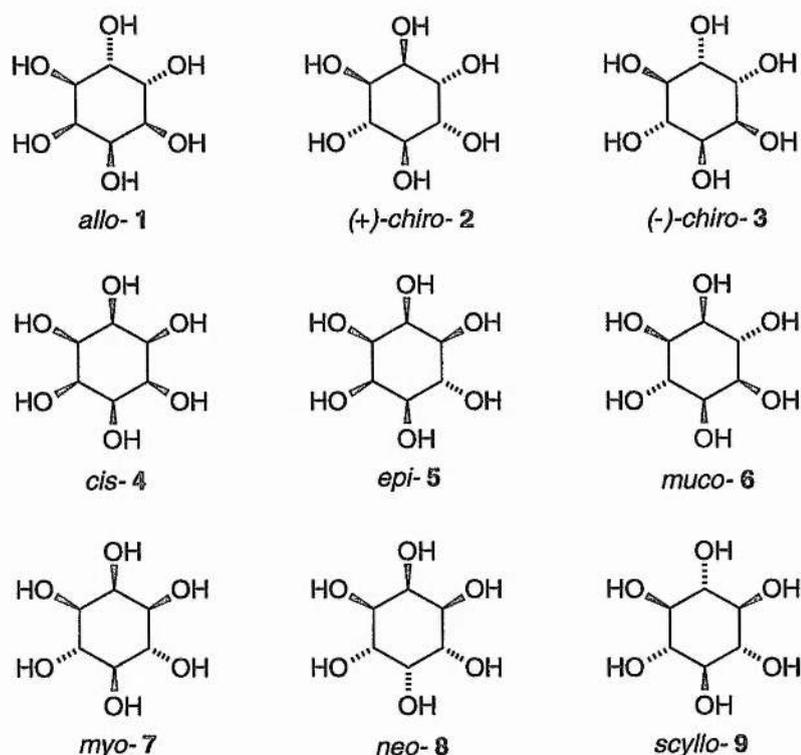


Figure 1.1: *The stereochemistry of the inositols*

In what is by far the more stable conformation in solution, *myo*-inositol has a single axial hydroxyl group (numbered by convention as position 2), and five equatorial hydroxyl groups. This leads to a plane of symmetry which runs through C-2 and C-5 (Figure 1.2).⁴ A useful mnemonic for remembering the structure of *myo*-inositol is Agranoff's turtle, a model where the axial 2-OH of *myo*-inositol represent the turtle's head and the remaining five equatorial hydroxyl groups represent the turtle's four legs and tail!⁵

Incorporation of a substituent at C-2 or C-5 of *myo*-inositol leads to an optically inactive *meso*- compound, whereas incorporation of a substituent at C-1 (enantiotopic to C-3) and / or C-4 (enantiotopic to C-6) leads to a pair of enantiomers. Thus inositol 1-phosphate* **10**, one of the natural substrates for inositol monophosphatase, exists as a pair of enantiomers (+)-**10** and (-)-**10** (Figure 1.2).

* "Inositol" or "Ins" is used throughout the text and refers to the *myo*-inositol isomer.

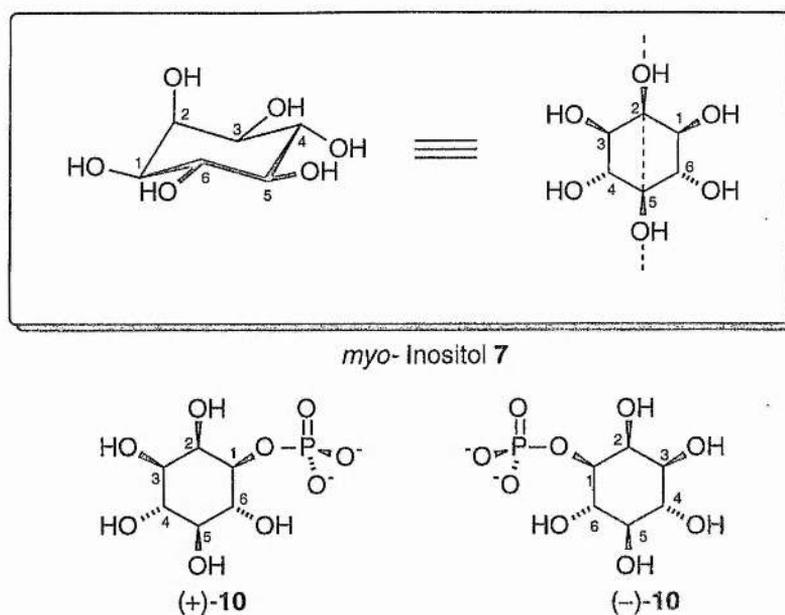
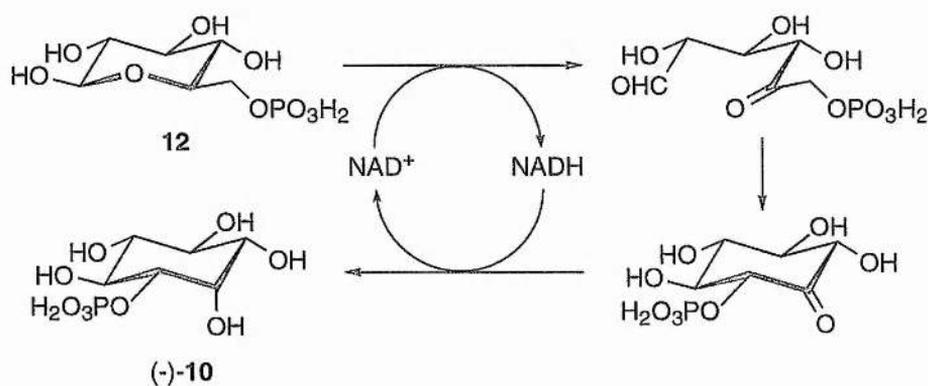


Figure 1.2: *myo*-Inositol and enantiomers of inositol 1-phosphate

1.1.2 Biosynthesis of *myo*-Inositol

Most inositol intake in humans is of dietary origin (mainly from plants), however, *de novo* biosynthesis does occur in animals and humans *via* the isomerization of D-glucose 6-phosphate **12** to L-inositol 1-phosphate (-)-**10**. The reaction is catalysed by the enzyme *L*-*myo*-inositol 1-phosphate synthase which is very abundant in mammalian testes and brain (Scheme 1.1).⁶



Scheme 1.1: Aldolase and coupled oxidoreductase activities of *L*-*myo*-inositol 1-phosphate synthase

The enzyme has attracted much mechanistic interest due to the coupled stereospecific ring closure and inosine reduction which it performs.⁷⁻⁹ The inositol 1-phosphate **10** produced is then hydrolysed to free inositol **7** by the action of the enzyme inositol monophosphatase (IMPase, EC 3.1.3.25).

1.2 Intracellular Signalling

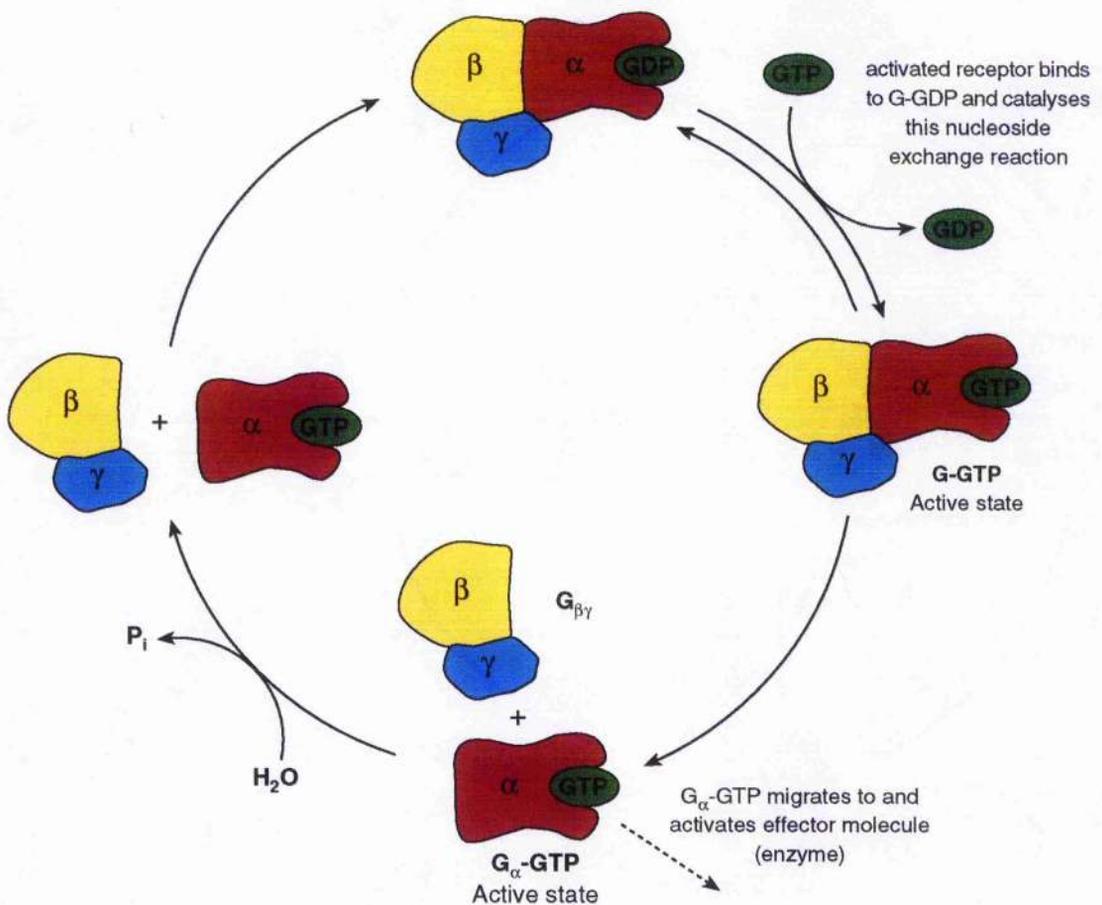
Complex organisms rely on communication between individual cells in order to maintain life. This communication involves the use of distinct chemicals to pass messages from one cell to another, and these chemical messengers can take many forms, being classified according to their function as neurotransmitters, hormones, growth factors, *etc.* These 'first' messengers interact with specific extracellular receptors spanning the plasma membrane of the receiving cells. Through a number of different mechanisms, this coupling produces a signal inside the cell which culminates in a change in cell behaviour, such as the regulation of enzyme activity, or in the case of muscle cells, physical contraction. This conversion of an extracellular chemical signal into an intracellular signal (a 'second' messenger) by passing information across the cell membrane is called *signal transduction*.¹⁰

In some of these transmembrane signalling systems the detection of an extracellular stimulus and the generation of an intracellular response are properties of the same protein or protein complex. For example, the receptor may contain or be closely linked to an ion channel which spans the plasma membrane. Binding the extracellular messenger to the receptor can open the channel to allow specific cations such as Na⁺ or K⁺ to pass across the cell membrane, resulting in a change in the intracellular concentration of that ion. Alternatively the receptor may contain some enzymatic activity; insulin when it binds to the extracellular domain of its receptor activates protein tyrosine kinase activity on the intracellular domain of the same protein. This phosphorylates specific tyrosine residues on target proteins within the cell.^{10, 11}

1.2.1 G-Protein Coupled Receptors

In many transmembrane signalling systems, however, a more complex class of signalling pathway is involved in which the sensor and intracellular effector are separate proteins that communicate using a guanine nucleotide-binding regulatory protein or G-protein.¹²⁻¹⁴

G-proteins exist as heterotrimeric structures containing one α -subunit, one β -subunit, and one γ -subunit, and are found associated with the internal surface of the cell membrane. The G-protein complex in its inactive form has guanosine diphosphate (GDP) bound to the α -subunit. Binding of an agonist to the receptor causes a conformational change in the receptor protein which causes it to bind to this form of the G-protein. This complex then instantly releases GDP and binds guanosine triphosphate (GTP) (Scheme 1.2).



Scheme 1.2: The G-protein cycle

Once in the GTP-bound form, the protein complex dissociates from the receptor, and then dissociates itself into a tightly bound non-covalent $\beta\gamma$ -dimer and an activated α -subunit with GTP bound. It is the G_{α} -GTP subunit which migrates to and activates the effector molecule, commonly one of two enzymes; adenylate cyclase or phosphoinositol lipase C.

Activation of the target enzyme brings about the release of internal 'second' messenger molecules and as many G_{α} -GTP molecules form for each bound agonist a significant amplification of the original signal is achieved. In time the GTP bound to the α -subunit is hydrolysed to GDP by the inherent GTPase activity of the α -subunit, and the resulting G_{α} -GDP dissociates from the effector thus re-setting the switch. The G_{α} -GDP complex then reassociates with the $\beta\gamma$ -dimer to return the system to the original state. (Scheme 1.2).¹³

1.2.2 Inositol Phosphates as Second Messengers

myo-Inositol 7, as a range of phosphate esters and phospholipids, has been shown to constitute a widely distributed second messenger system in the body, the phosphoinositide pathway.^{13, 15} This intracellular signalling system relies on an increase in the concentration of calcium in the target cell to evoke cellular responses and is entirely distinct from the more well known second messenger pathway which uses cyclic 3',5'-adenosine monophosphate (cAMP).¹⁶

The intracellular second messengers, formed by the activation of the phosphoinositide pathway, arise from phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)- P_2], a minor component of the cell membrane lipid. The phospholipid is hydrolysed by a G-protein controlled enzyme, phosphatidylinositol lipase C, to form two second messengers; diacylglycerol (DAG) which is hydrophobic and remains in the membrane, and D-inositol 1,4,5-trisphosphate [D-Ins(1,4,5)- P_3] which is released into the cytosol (Figure 1.3).^{17, 18}

DAG acts as a second messenger by binding to and activating a specific protein kinase, protein kinase C, promoting phosphorylation of target proteins in the cell. In addition,

DAG can be further metabolised to produce arachidonic acid, a starting point for the biosynthesis of prostaglandins.

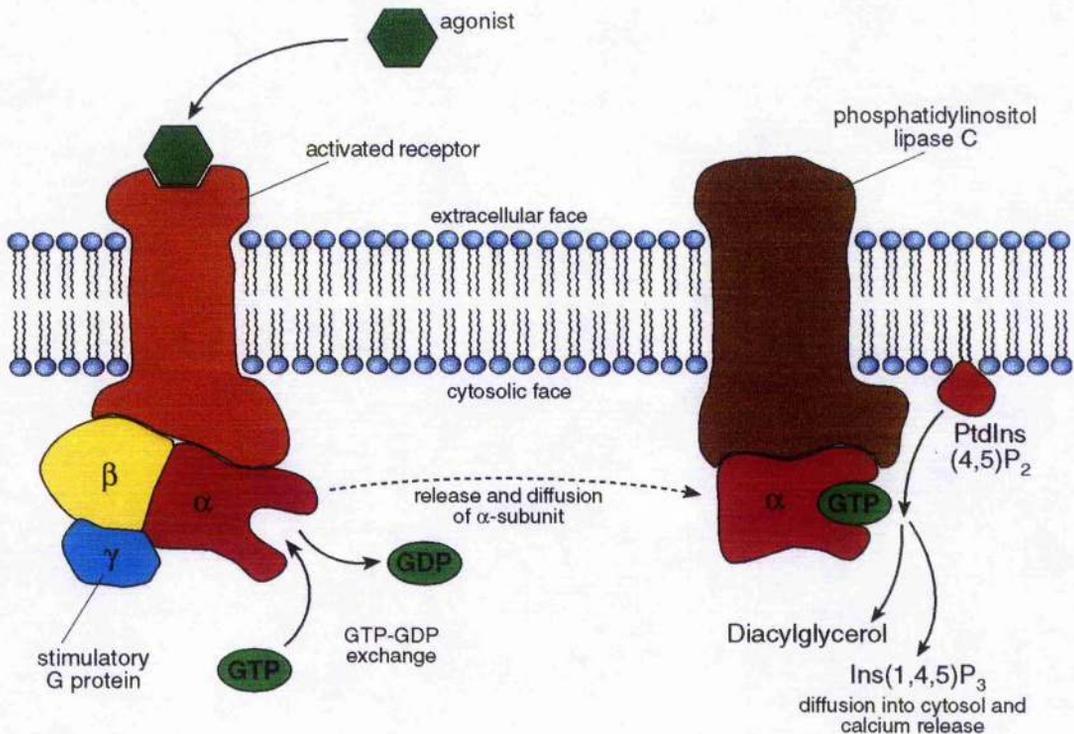


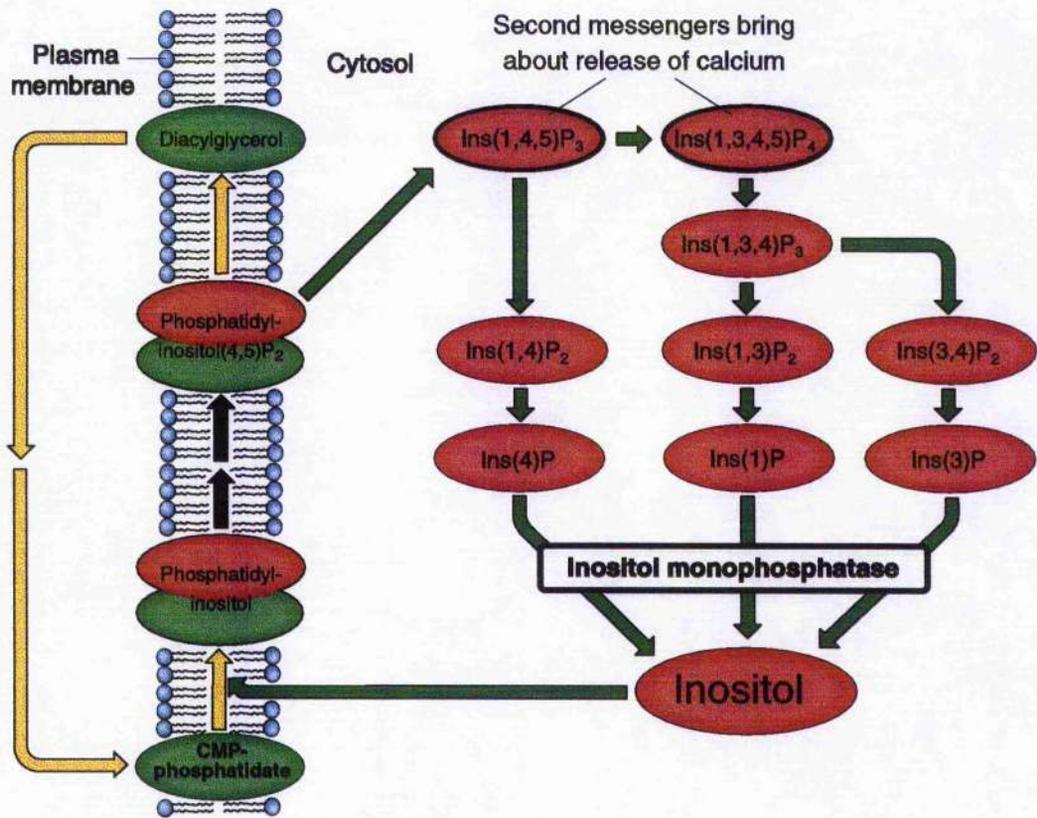
Figure 1.3: Formation of two second messengers diacylglycerol and D-inositol 1,4,5-trisphosphate from phosphatidylinositol 4,5-bisphosphate by the action of the G-protein controlled enzyme phosphatidylinositol lipase C

D-Ins(1,4,5)- P_3 diffuses through the cytosol and causes the rapid release of calcium ions from the endoplasmic reticulum. The elevated level of Ca^{2+} in the cell then triggers Ca^{2+} dependent processes such as smooth muscle contraction, glycogen breakdown and exocytosis.

Second messengers, once released inside the cell must be efficiently deactivated metabolically to terminate its action and return the cell to its prestimulated state.

1.2.3 Phosphoinositide Cycle

The second messenger breakdown processes comprise a lipid cycle in which DAG is converted into cytidine monophosphoryl-phosphatidate (CMP-PA), and a complex inositol phosphate cycle that regenerates free inositol from D-Ins(1,4,5)- P_3 (Scheme 1.3).



Scheme 1.3: The inositol cycle, comprising a lipid cycle (shown with yellow arrows) and inositol phosphate cycle (shown in green)

A closer look at the inositol phosphate cycle reveals two clear pathways for the metabolism of D-Ins(1,4,5)-P₃ (Scheme 1.3).

- 1) A specific 5-phosphatase removes the 5-phosphate group of D-Ins(1,4,5)-P₃, giving D-inositol 1,4-bisphosphate [D-Ins(1,4)-P₂] which is not active as a calcium mobilising agent as thus the signal is terminated. Further metabolism of D-Ins(1,4)-P₂ gives D-inositol 4-phosphate [D-Ins4-P].
- 2) The second pathway involves phosphorylation of D-Ins(1,4,5)-P₃ to D-inositol 1,3,4,5-tetrakisphosphate [D-Ins(1,3,4,5)-P₄] which is thought to be a second messenger in its own right,¹⁹ mediating the entry of extracellular Ca²⁺. The metabolic pathway continues *via* a second triphosphate, D-inositol 1,3,4-

trisphosphate [D-Ins(1,3,4)- P_3], and two further bisphosphates to give D-inositol 1-phosphate [D-Ins1- P] and D-inositol 3-phosphate [D-Ins3- P].

1.2.4 Inositol Monophosphatase and the Phosphatidylinositol Cycle

Metabolism of D-Ins(1,4,5)- P_3 by either pathway gives rise to three isomeric D-inositol monophosphates; D-Ins1- P , D-Ins3- P , and D-Ins4- P (Scheme 1.3). A single enzyme, inositol monophosphatase (IMPase) plays a pivotal role in the phosphatidylinositol cycle by hydrolysing all these compounds to free inositol which is then used to replenish cellular stores of phosphatidylinositol lipids and the messenger system based on these lipids.

1.3 Lithium and Bipolar Disorder

IMPase has been shown to be uncompetitively inhibited by Li^+ ions *in vitro*.²⁰ As recycling of inositol is of particular importance in the brain where inositol cannot readily cross the blood-brain barrier,²¹ and its *de novo* synthesis from glucose 6-phosphate is limited, it was proposed that IMPase may therefore be the target of lithium treatment* in bipolar-disorder.²²⁻²⁵ Inhibition of the enzyme *in vivo* would be expected to have a profound effect on the rate of cell signalling, particularly to those cells which are hyperactive in patients suffering from mania.²⁰ In addition, cells in the periphery would have relatively free access to plasma inositol, helping to explain the apparent selectivity of the drug for cells of the central nervous system. More recently, however, it has been suggested that differences in the distribution of *lithium*, rather than inositol may be the cause of the drugs specific action. Nerve cells, which possess voltage-dependent Na^+ channels, become much more permeable to Na^+ in the excited state. They may also become more permeable to the drug.²⁶

Although the depletion of free inositol and the accumulation of inositol monophosphates has been demonstrated in the brains of lithium treated rats,²⁷ it is only more recently that

* Lithium, in the form of its citrate or carbonate salts, has been used to treat a variety of disorders since the 19th century. John Cade was the first to describe the use of lithium in the treatment of mania (1949).

an actual link between Li^+ therapy and the *in vivo* inhibition of IMPase has been proven.²⁸⁻³¹

Lithium is now known to affect other aspects of phosphatidylinositol signal transduction than IMPase.³² However, inhibitors of the enzyme have been shown to mimic the effects of Li^+ on phosphatidylinositol cell signalling, and therefore IMPase has potential as a target for the treatment of bipolar-disorder.³³⁻³⁵ Such therapy is required as lithium is a toxic drug, it has a poor side-effect profile, and it has a narrow therapeutic window which requires the plasma concentration of Li^+ to be monitored.³⁶ Li^+ serum concentrations should be maintained at 0.5-1.0 mmol dm^{-3} , at which hand-tremor, weight gain and an increased urine production are experienced. Toxicity occurs at $>2 \text{ mmol dm}^{-3}$ and is characterised by nausea, fine tremors, ataxia, confusion and slurred speech. At $>4 \text{ mmol dm}^{-3} \text{ Li}^+$, coma or death may result.³⁷

1.4 Properties of Inositol Monophosphatase

myo-Inositol monophosphatase has been purified from a variety of tissue sources, including rat,³⁸ bovine,^{39, 40} and human brain. Recombinant strains of *Eschericia coli* which express the bovine and human brain enzymes have also been constructed.^{41, 42} The proteins have all been shown to be dimers of identical subunits and have similar native molecular masses of approximately 58,000 Da. The most intensive research has been conducted with bovine and human brain enzymes which show only minor differences in the amino acid sequence (see Section 1.16, page 31).

Inositol monophosphatase requires Mg^{2+} for activity, although Mn^{2+} , Co^{2+} , and Zn^{2+} have been demonstrated to support catalysis to different extents.^{38, 39, 43} Ca^{2+} , Mn^{2+} , Cu^{2+} , and Gd^{3+} are competitive inhibitors for Mg^{2+} .^{38, 39, 43, 44}

Li^+ , however, inhibits non-competitively with respect to Mg^{2+} and has been shown to be an uncompetitive inhibitor of inositol monophosphatase.^{43, 45, 46}

Inositol monophosphatase catalyses the hydrolysis of a broad range of monophosphate esters including both enantiomers of *myo*-inositol 1- and 4-phosphate (D-Ins1-P and L-

Ins1-*P*, **10**) and (D-Ins4-*P* and L-Ins4-*P*, **12**),⁴⁷ glycerol 2-phosphate **13**, and a range of nucleoside 2'-phosphates including adenosine 2'-monophosphate (2'AMP) **14**. The K_m for racemic **10** is $\approx 0.1 \text{ mmol dm}^{-3}$.⁴³ The enzyme has also been shown to process both L- and D-inositol 1-phosphorothioate (D-Ins1- P_s and L-Ins1- P_s , **15**) in the presence of Mg^{2+} ions (K_m - and K_i -values of $\approx 1.0 \text{ mmol dm}^{-3}$).⁴⁸

1.5 Early Kinetic Studies on Inositol Monophosphatase

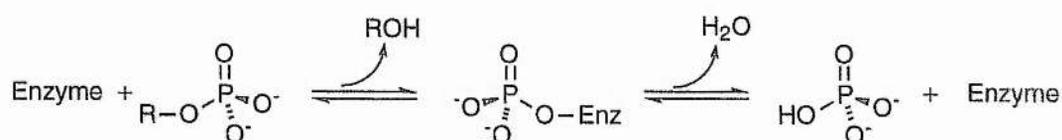
Preliminary work on the mechanism of inositol monophosphatase revealed that the uncompetitive nature of Li^+ inhibition only held at low lithium concentrations with the mode of Li^+ inhibition at higher concentrations more complex.⁴⁹ In addition, at saturating Li^+ , inositol was formed in a burst, faster than predicted from the steady-state rate for substrate turnover, and was followed by no further reaction.⁴⁹

In separate studies, it was established that Mg^{2+} binds to the enzyme after the substrate and dissociates from the enzyme before inorganic phosphate (P_i) is released. At low Mg^{2+} concentrations, the kinetic data suggested a co-operative mechanism where each monomer of the dimeric enzyme must bind one Mg^{2+} ion for full activity and where the binding of the first ion facilitated the binding of the second one. The fact that this observed co-operativity was dependent upon the structure of the substrate was in keeping with a mechanism in which the substrate bound to the enzyme first, in an ordered fashion, and modified or created the binding site for Mg^{2+} . At Mg^{2+} concentrations higher than 1 mmol dm^{-3} , Mg^{2+} behaved as an uncompetitive inhibitor for DL-Ins1-*P*, with the extent of inhibition by Mg^{2+} being substrate dependant, mirroring the sensitivities observed for inhibition by Li^+ . These results and the fact that Li^+ and Mg^{2+} inhibition are mutually exclusive, suggested that Li^+ binds to the site vacated by Mg^{2+} .

The order of product release was also determined by examination of the product inhibition properties of the components associated with hydrolysis. P_i was found to be a competitive inhibitor for DL-Ins1-*P* implying that P_i is released last. The K_i value for P_i inhibition decreased by a factor of approximately 25, from 8.0 mmol dm^{-3} at pH 6.5 to 0.3 mmol

dm^{-3} at pH 8.0 indicating that the enzyme binds to phosphate dianion more tightly at high pH. Inositol behaved as a non-competitive inhibitor for the hydrolysis of DL-Ins1-*P* ($K_{i \text{ apparent}} = 250 \text{ mmol dm}^{-3}$ at pH 6.5 and 400 mmol dm^{-3} at pH 8.0), in keeping with the notion that it is the first product to be released. These large K_i values suggested that inositol release was not slow.

From these results, it was proposed that inositol monophosphatase catalysed phosphate ester hydrolysis by a *substituted enzyme mechanism*, in keeping with phosphatases such as alkaline⁵⁰ and acid phosphatase⁵¹ which were known to involve such a pathway (Scheme 1.4). Ping-pong (or substituted enzyme) mechanisms operate through two half reactions. Typically, in the first half-reaction the phosphoryl group is transferred to a group on the enzyme to form a phosphorylated enzyme (E-P) intermediate and the first product. In the second half-reaction, the phosphate group is transferred to water to give the second product, P_i and regenerate the free enzyme. As the water molecule binds in the same site as the first product, the alcohol, it is possible to trap the phosphate group with alternative alcohols.

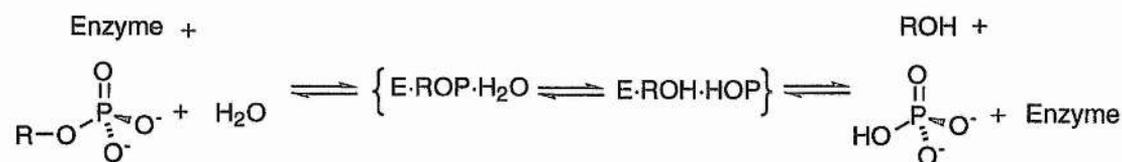


Scheme 1.4: *Substituted enzyme mechanism*

All attempts to detect an E-P intermediate for inositol monophosphatase failed.^{43, 48} In addition, phosphatase enzymes which proceed *via* a substituted enzyme intermediate can incorporate ^{18}O label from ^{18}O -water into a pool of unlabelled inorganic phosphate, as the second reaction can proceed to equilibrate the distribution of labelled oxygen atoms into phosphate in the absence of the first product. For inositol monophosphatase, however, the ^{18}O -phosphate ligand exchange reaction showed an absolute requirement for inositol (K_m for inositol was 190 mmol dm^{-3} at pH 8.0)⁵² The exchange reaction was also

Mg^{2+} -dependent, showed a similar pH optimum to that for the hydrolysis reaction, and was inhibited by Li^+ .

From these findings, it was evident that inositol monophosphatase did not follow a substituted enzyme mechanism as originally suggested,^{49, 53} and an alternative *ternary complex mechanism* was proposed in which the alcohol is directly displaced by a nucleophilic water molecule (Scheme 1.5).



Scheme 1.5: *Ternary complex mechanism*

Phosphate release from the enzyme after hydrolysis was shown to be partially rate limiting and cited as a possible explanation to account for the burst-phase formation of inositol, given that a substituted enzyme mechanism had been discounted.

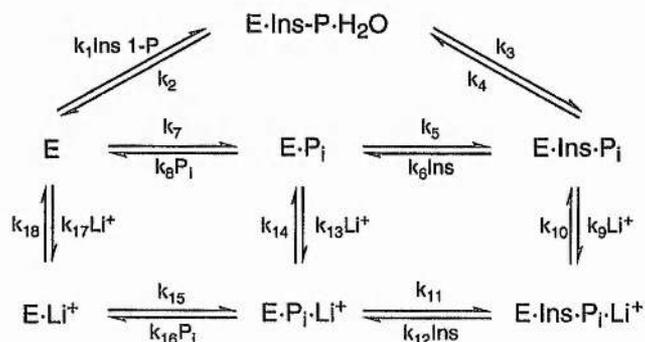
1.6 Mode of Inhibition by Lithium

Many groups have investigated the mode of inhibition of inositol monophosphatase by Li^+ with a wide range of different substrates.^{38-40, 43, 45, 46, 54} Universally, the metal ion was reported to act as a linear uncompetitive inhibitor at low Li^+ concentrations (K_i with Ins1-P as substrate was approx. 1 mmol dm^{-3}) indicating that Li^+ binds to enzyme-substrate and/ or enzyme-product complexes only and not to free enzyme. At these concentrations, it was also reported that Li^+ acted as a non-competitive inhibitor for Mg^{2+} with Ins1-P as the substrate, indicating that Li^+ does not prevent Mg^{2+} from binding to the enzyme and that Li^+ does not trap any $E\cdot Mg^{2+}$ complex.⁴³

At Li^+ concentrations above 5 mmol dm^{-3} , the mode of inhibition with respect to Ins1-P was non-competitive. This change in the mode of inhibition was initially interpreted to indicate that Li^+ can also bind to the free enzyme at high $[Li^+]$.

Inositol does not enhance or suppress the action of Li^+ , and the inhibitory effects of Li^+ and inositol were independent, suggesting that Li^+ could bind to a form of the enzyme to which inositol was bound, but in so doing, could not prevent inositol from debinding to give $\text{E}\cdot\text{P}_i\cdot\text{Li}^+$.

However, it was found that the inhibitory effect of P_i for the substrate was enhanced in the presence of Li^+ , and that Mg^{2+} caused a similar effect.⁴³ It was noted that this potentiating effect of P_i on Li^+ inhibition means that Li^+ may act as a more potent inhibitor of the enzyme *in vivo* than originally thought, since brain concentrations of P_i are 1–3 mmol dm^{-3} (Scheme 1.6).



Scheme 1.6: Mechanism accounting for the inhibition by lithium at low and high concentrations

1.7 Proposed Ternary Complex Mechanism

The conclusion from these observations was that inositol monophosphatase operated *via* a ternary complex mechanism in which phosphate release was partially rate-limiting and Li^+ binded selectively, at low concentrations, to the non-covalent species $\text{E}\cdot\text{P}_i$ in the site vacated by Mg^{2+} (Scheme 1.6).²⁶ The other partially rate limiting step in the mechanism was the catalytic step.⁴⁶

1.8 Structural Requirement Studies using Deoxy 1-Phosphate Analogues

To investigate the notable lack of substrate specificity of inositol monophosphatase, the structural requirements for catalysis and inhibition in substrates were investigated by the Merck, Sharp and Dohme group at Harlow, UK.⁵⁵⁻⁵⁷ Their elegant hydroxy group deletion studies defined many of the important binding interactions of the substrate D-Ins1-*P* with the enzyme and demonstrated that the 3- and 5-hydroxyl groups of the inositol ring were not required for binding or catalysis. Indeed, 3,5-bis-deoxyinositol **16** was a very good substrate for inositol monophosphatase, having an affinity for the enzyme greater than any of the natural substrates ($K_m=0.025 \text{ mmol dm}^{-3}$) and indicating that the 3- and 5-hydroxyl groups of Ins1-*P* actually have a detrimental effect on substrate binding.⁵⁷

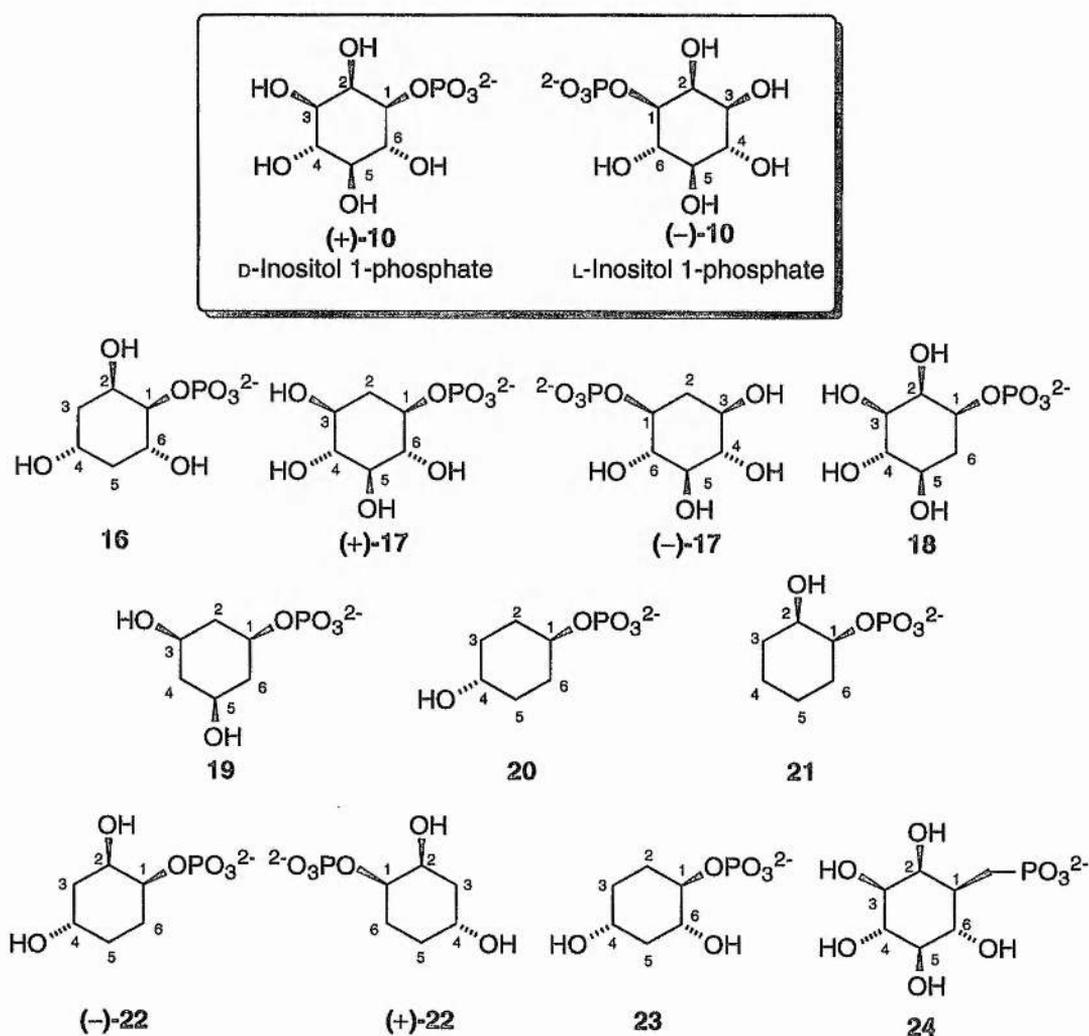


Figure 1.4: Structural formulae for substrates and inhibitors of IMPase

Replacement of the 2-hydroxyl group in D-Ins1-*P* (+)-**10** by a hydrogen gave (+)-2-deoxyinositol 1-phosphate (+)-**17**, a weak substrate for the enzyme ($V_{\max} = 0.78$ of V_{\max} for Ins1-*P*, $K_m = 1.3$ mmol dm⁻³, 10-times higher than K_m for Ins1-*P*), whereas the opposite enantiomer (-)-**17** was found to be a competitive inhibitor for the substrate ($K_i = 50$ μmol dm⁻³) (Figure 1.4).⁵⁵ (+/-)-6-Deoxyinositol 1-phosphate **18** was also found to be a competitive inhibitor ($K_i = 70$ μmol dm⁻³)⁵⁵ and these results taken together suggested that the α-hydroxyl groups had discrete and different roles in inositol monophosphatase activity towards substrates.⁵⁵ These studies led to the proposal that the 2-hydroxyl group in the D-Ins1-*P* was involved in a *binding* interaction while the 6-hydroxyl group was somehow involved in the *catalytic* mechanism (Figure 1.5).

The 2,4,6-tris-deoxyinositol 1-phosphate **19** was completely devoid of substrate or inhibitory activity whilst the *trans*-4-hydroxy- and (+/-)-*cis*-2-hydroxy-cyclohexanol phosphates, **20** and **21** respectively, did bind to the enzyme, revealing the importance of the 4-hydroxyl group for binding (Figure 1.4).

As a final investigation into the proposed binding role of the 2-hydroxyl group and the catalytic involvement of the 6-hydroxyl group of D-Ins1-*P*, compounds **22** and **23** were prepared in which both of the 3- and 5-hydroxyl groups and either the 2-hydroxyl group or the 6-hydroxyl group were deleted. As racemates, both **22** and **23** were found to be potent competitive inhibitors ($K_i = 7$ and 90 μmol dm⁻³ respectively), lacking any substrate activity. The more potent inhibitor **22** was resolved into its individual enantiomers (-)-**22** and (+)-**22**. As anticipated, (-)-**22**, which lacks the catalytic important group, was an extremely potent competitive inhibitor ($K_i = 3$ μmol dm⁻³) whereas its enantiomer (+)-**22** was a weak substrate (Figure 1.4).⁵⁶

Indeed, when comparisons were made of the three-dimensional structures of the enantiomers of the substrate **10**, using molecular modelling techniques, the phosphate group, both α-hydroxyl groups, and the 4-hydroxy group of each enantiomer could be superimposed. In this comparison, the 3- and 5-hydroxy groups could not be

superimposed, suggesting again that these groups may not be required for binding to the enzyme.⁵⁶

The role of the bridging 1-O was investigated by the synthesis of inositol 1-phosphonate **24**.⁵⁸ The compound showed no substrate activity or inhibitor activity, indicating that the alkyl phosphate bridging oxygen was also essential for substrate binding. Figure 1.5 shows the known structural interactions of the substrate, D-Ins 1-P, with inositol monophosphatase.

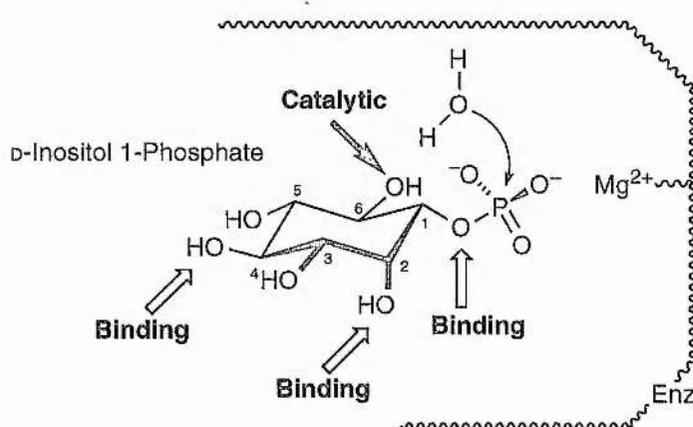


Figure 1.5: Structural features of D-Ins 1-P required for binding to and hydrolysis by inositol monophosphatase (active site nucleophilic water molecule is included)

1.9 Inhibitor Design

Inositol monophosphatase is able to catalyse the hydrolysis of the phosphate ester of a range of purine- and pyrimidine-containing 2'-nucleoside phosphates including adenosine 2'-monophosphate (2'-AMP) **14** which exhibits good substrate activity (V_{\max} is 90% of V_{\max} for Ins 1-P; $K_m=0.86$ mmol dm⁻³). The adenine group does not significantly reduce binding to the enzyme, implying a tolerance of steric bulk near the active site.⁵⁷

In an attempt to design a more potent inhibitor for inositol monophosphatase, molecular modelling was used to compare the 3-dimensional structures of the most successful deoxy-inositol 1-phosphate inhibitor (-)-**22** with 2'-AMP **14**.⁵⁷ The 2'-O atom, 3'-OH

and 5'-OH positions in **14** showed very close correspondence with the binding 1-O atom, 2-OH, and 4-OH of (-)-**22** implying similar binding functions and an involvement of the adenine group in catalysis (Figure 1.6) [for further elaboration see Section 1.11 on page 22].

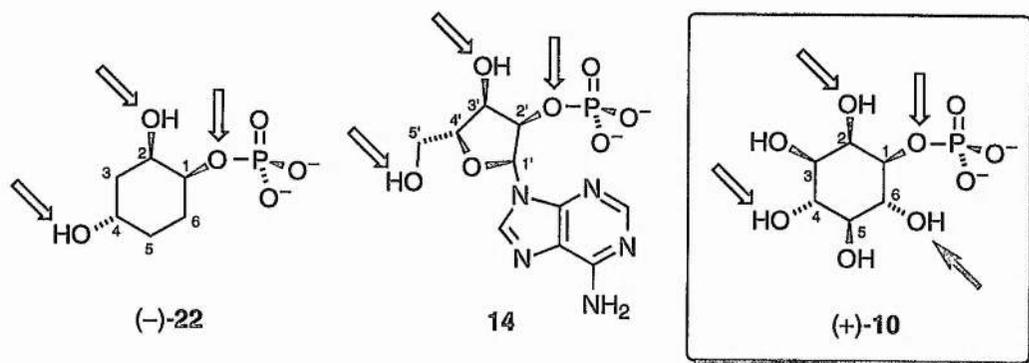


Figure 1.6: Proposed interactions of 2'-AMP and deoxyinositol inhibitor (-)-**22** with the enzyme active site (binding interactions shown with white arrows, catalytic interaction shown with a black arrow)

Hence, trisubstituted cyclohexyl phosphates (+/-)-**25** and (+/-)-**26** were synthesised and tested for inhibitory properties. The double objective of adding a lipophilic side chain to (+/-)-**22** being to increase its binding affinity by filling the active site space occupied by the adenine moiety of **14** and destroy the catalytic involvement of the 6-hydroxyl group in the hydrolysis of D-Ins1-P (Figure 1.7).⁵⁷

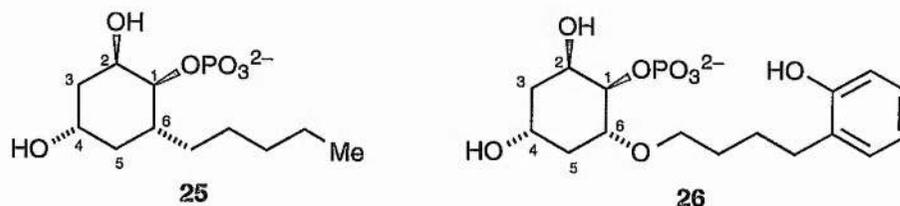


Figure 1.7: Deoxyinositol 1-phosphate inhibitors with modified side-chain

The *n*-pentyl substituent of **25** was well tolerated, affording a very good inhibitor ($K_i = 3 \mu\text{mol dm}^{-3}$). Results from **26** were much more dramatic, demonstrating a

significant positive binding contribution from the C-6 substituent in the form of an inhibitor 100 fold more potent than **22** ($K_i = 70 \text{ nmol dm}^{-3}$) (Figure 1.7).

The screening of a number of bisphosphonic acids revealed the hydroxymethylenebisphosphonic acid **27** to be a moderately potent and competitive inhibitor of inositol monophosphatase ($K_i = 0.18 \text{ mmol dm}^{-3}$), possibly by mimicking the action of phosphate in binding to the enzyme active-site.⁵⁹ This idea was tested by combining the bisphosphonate **27** with the structural features of deoxyinositol phosphate inhibitor (+/-)-**22** to produce (+/-)-3,5,6-trisdeoxyinositol-1,1-bisphosphonic acid **28**. Compound **28** proved to be a potent, competitive inhibitor of inositol monophosphatase ($K_i = 2.5 \text{ } \mu\text{mol dm}^{-3}$ for the racemate), but no improvement in affinity over **22**. Removal of the methyl group gave **29**, an inhibitor with slightly reduced affinity ($K_i = 7.4 \text{ } \mu\text{mol dm}^{-3}$ for the racemate, $K_i = 4.3 \text{ } \mu\text{mol dm}^{-3}$ for the enantiomer shown), and very considerable reduction in affinity was observed for the methylene analogue (+/-)-**30** ($K_i = 0.68 \text{ mmol dm}^{-3}$) relative to **22**, confirming that the oxygen at C-1 participates in an important interaction with the enzyme.⁵⁹

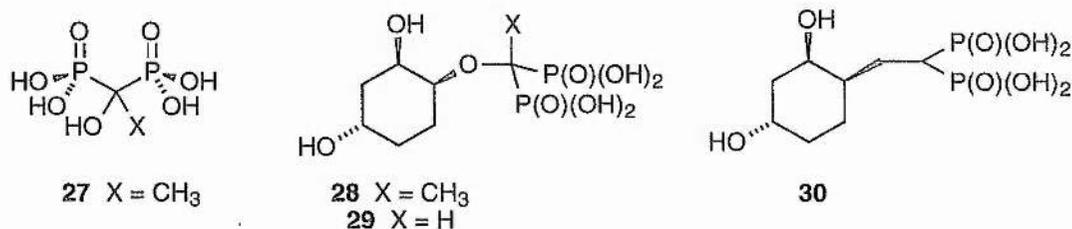


Figure 1.8: Bisphosphonic acid inhibitors

The deoxy analogues approach to inhibitor design is limited by the highly charged nature of such compounds, and their susceptibility to hydrolysis by non-specific phosphatase enzymes *in vivo* (hence the investigation of phosphonates such as **24** and **30**). Consequently, a range of inhibitors unrelated to the enzyme structure were investigated by substituting the deoxyinositol substituent on **28**.^{60, 61}

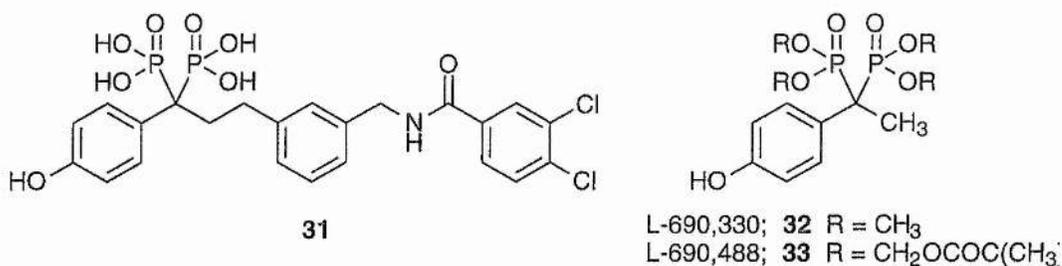


Figure 1.9: *Inhibitors of inositol monophosphatase unrelated to the enzyme substrate*

The most potent non-hydrolysable inhibitor to emerge was **31** with a $K_i = 0.08 \mu\text{mol dm}^{-3}$.⁶¹ A simpler but effective inhibitor (L-690,330) **32** ($K_i = 0.33 \mu\text{mol dm}^{-3}$) showed some *in vivo* inhibition of inositol monophosphatase and was selected for development into a prodrug.³⁴ Tetrapivaloyloxymethyl ester (L-690,448) **33** was chosen with the rationale that by reducing the polarity of L-690,330, the inhibitor would readily cross the cell membrane. Once inside the cell, the pivolate ester-linkages would be hydrolysed by esterases yielding the parent compound L-690,330. Prodrug L-690,488 proved to be much more potent than the parent compound L-690,330, indicating that the prodrug strategy used did indeed enhance cell penetrability. Moreover, the behaviour of L-690,488 mimicked the effect of lithium on the phosphoinositide cycle. Unfortunately, when L-690,488 was injected into animals, neither L-690,488 nor L-690,330 could be detected in either plasma or brain, suggesting that L-690,488 was too insoluble to leave the site of injection.³³

Other novel inhibitors for inositol monophosphatase unrelated to the natural substrate have been isolated from various fungal sources.⁶²⁻⁶⁴ These sesquiterpenic compounds (K-76) **34**⁶³ and (L-671,776) **35**⁶² are non-competitive inhibitors for D-Ins1-P and show higher potency than lithium ($K_i \approx 0.5 \text{ mmol dm}^{-3}$).

Recently, another class of potent inhibitors of inositol monophosphatase, α -hydroxytropolones, based on structure **36** have also been reported.⁶⁵

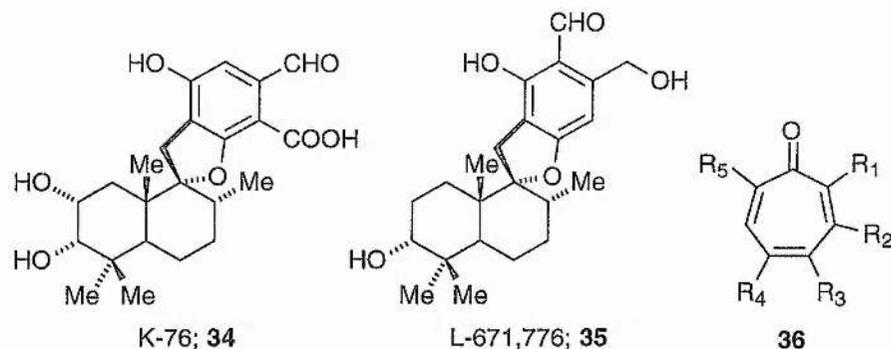


Figure 1.10: *Some novel inositol monophosphatase inhibitors*

1.10 Discrepancies with the Proposed Inositol Monophosphatase Mechanism

In spite of the success of some of the initial enzyme inhibitors, three important features of inositol monophosphatase remained difficult to rationalise with the proposed enzyme mechanism. These were:

- 1) the ability of the enzyme to process ribonucleoside 2'-phosphates such as 2'-AMP which were shown to lack an equivalent of the catalytically essential 6-hydroxyl group in D-Ins1-P (see Figure 1.6, page 18);
- 2) the optimum concentration of Mg^{2+} required for catalysis varied with the structure of the substrate and that Mg^{2+} binding was cooperative for some substrates but not for others;
- 3) the mode of inhibition of the enzyme by Li^+ changed from uncompetitive to non-competitive with increasing concentration and that K_i -values for uncompetitive inhibition by Li^+ depended acutely on the structure of the substrate.

Further studies based on the ability of inositol monophosphatase to recognise and process ribonucleotide 2'-phosphates, including 2'-AMP, and some other novel alternative substrates has led to the proposal that a key second Mg^{2+} cation is involved in the enzyme mechanism.^{66, 67}

1.11 Adenosine 2'-Monophosphate as a Substrate for Inositol Monophosphatase

2'-AMP **14** is a good substrate for inositol monophosphatase (V_{\max} is 90% of V_{\max} for Ins1-*P*; $K_m = 0.86 \text{ mmol dm}^{-3}$) and is hydrolysed to give adenosine and inorganic phosphate (P_i). The binding affinity of **14** ($K_i = 1.0 \text{ mmol dm}^{-3}$) when compared to DL-Ins1-*P* suggested that the interaction between the adenosine moiety and the active site did not quite compensate for the structural reorganisation required to accommodate the extra mass.⁴³

Functional group deletion studies on **14** by Leeson *et al.*⁶⁸ suggested that the 3'-hydroxyl group was required for binding to inositol monophosphatase, whereas the purine ring had no binding role. Additionally, the 4'-hydroxymethyl group had a binding role but its orientation was not optimal for interaction with the enzyme. Most importantly, the ribofuranosyl ring O-atom was found to be *essential* for efficient phosphate hydrolysis.

In stark contrast to inositol, adenosine was *not* found to mediate the incorporation of [¹⁸O]-label from [¹⁸O]-water into P_i and did not serve as a product inhibitor ($K_i > 4 \text{ mmol dm}^{-3}$).^{43, 52} From these findings it was inferred that adenosine was not recognised by inositol monophosphatase as the product of the enzymatic hydrolysis of **14**.

One possible explanation, the involvement of the adenosine moiety in an *alternative mechanism* for phosphate hydrolysis by the enzyme was discounted when no transphosphorylation products were detected.⁴³ A more convincing argument was that the product-binding complex for adenosine existed in a *high-energy state* so that free adenosine would be unable to bind in its product site.⁶⁶

2'-Adenosine monophosphorothioate (2'-AMP_S) **37** was also investigated for enzyme activity and in contrast to inositol 1-phosphorothioate **15**, did not serve as a substrate for inositol monophosphatase in the presence of Mg²⁺.^{66, 67} However, activity of around 20% that of **14** was observed when the compound was tested as a substrate in enzyme assays which contained thiophilic Mn²⁺ ions in place of Mg²⁺. It was concluded that a *strong*

primary binding interaction between the enzyme-bound metal ion and the phosphate or phosphorothioate group was necessary in order to off-set the energy required to reorganise the adenosine moiety into its active conformation and gave credence to the proposed high-energy arrangement of the adenosine system.^{66, 67}

As inositol is recognised as a product of the inositol monophosphatase reaction, it followed that the enzyme-bound form must exist in a *low-energy conformation* and it was reasoned that this would act as a *template* for deducing this arrangement of the adenosine system.⁶⁶

Examination of the structure of 2'-AMP revealed that the catalytic ribofuranosyl O-atom was relatively remote from the phosphate moiety of the substrate. Molecular modelling studies indicated that 2'-AMP must exist in a *very unfavourable conformation* to obtain congruence between the catalytic and binding groups of 2'-AMP and D-Ins1-P. In such an arrangement, the ribofuranosyl ring O-atom of 2'-AMP must move towards the 2'-O atom so that their respective lone-pairs point towards each other. Thus, the adenine moiety is forced into an unfavourable axial position causing an adverse 1,3-interaction between it and the 4'-hydroxymethyl group, and there is also a 1,2-interaction between the 2'-O and 3'-O atoms [Figure 1.11 (a)]. Calculations predicted that the strained structure would be as high as 100-105 kJ mol⁻¹ less stable than the unconstrained form, explaining why adenosine was not recognised by the enzyme.⁶⁷

It was proposed that this strained conformation would be substantially stabilised in the enzyme active site by the chelation to a *second* Mg²⁺ (Mg²⁺²) through the 2'-O and ribofuranosyl O-atoms, producing a *five membered metallocycle*. The second Mg²⁺ would also provide a possible site for a hydroxide ion suitably disposed for attacking the P-atom [Figure 1.11 (a)]. In such a system, the role of the existing *first* Mg²⁺ (Mg²⁺¹) would be to bind the substrate to the enzyme active-site cleft by co-ordinating to the phosphate moiety, and consequently, act as a Lewis acid to enhance the electrophilicity of the P-atom. Transposition of the interaction of Mg²⁺² with 2'-AMP onto the structure of D-Ins1-P gave a possible structure for the key binding interactions of both metal ions with

the substrate (note that the actual arrangement of the five-membered metallocycle in this model is inverted) [Fig. 1.11 (b)].

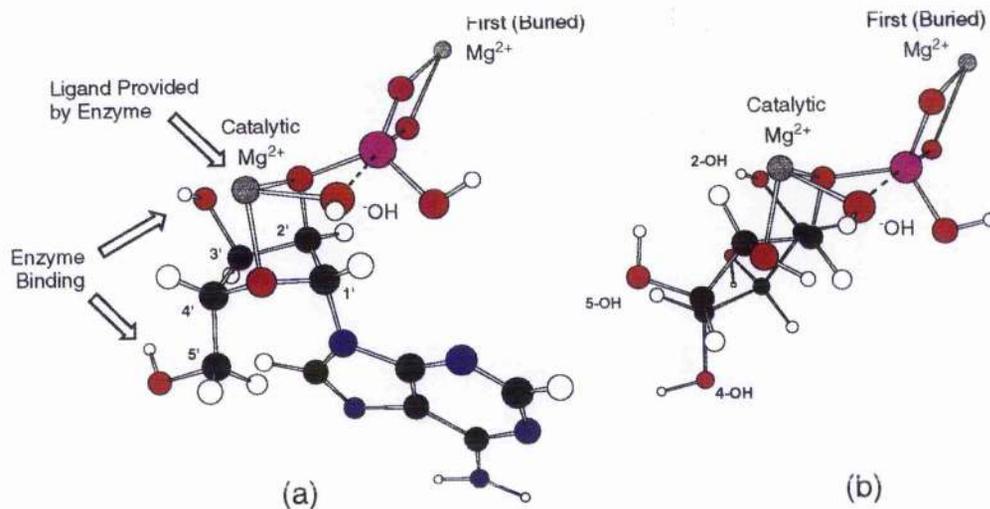


Figure 1.11: (a) Model showing the role of the second (catalytic) Mg^{2+} ion in stabilising the proposed active form of 2'-AMP through chelation by the 2'- and ribofuranosyl O-atoms. (b) The conformation of D-inositol 1-phosphate and the important catalytic and binding interactions with the two Mg^{2+} ions

The involvement of a second Mg^{2+} ion was completely consistent with all previously observed properties of substrates and inhibitors of inositol monophosphatase. Moreover, since Mg^{2+} was liganded by at least two O-atoms from the substrate, its involvement accounted for the variance of observed K_{Mg} -values for different substrates as well as the observed cooperativity effects that were displayed for Mg^{2+} with some substrates but not others.

1.12 Structure of Inositol Monophosphatase

In 1992, Bone *et al.*⁴⁴ determined the structure of human brain inositol monophosphatase to a resolution of 2.1 Å by using X-ray crystallography and confirmed that the enzyme exists as a homodimer of approximately 58,000 Da (Fig. 1.12).



Figure 1.12: *Ribbon representation of the secondary structure of the Gd³⁺-sulfate complex of inositol monophosphatase. The two identical subunits are shown in red and green*

Each subunit is folded into a five-layer sandwich of three α -helices and two β -sheets. The structure was solved with a lanthanide cation (Gd^{3+}) and sulfate bound at identical sites on each subunit, thereby establishing the positions of the active sites in the large hydrophobic cavern that is at the base of the two central helices where several segments of the secondary structure intersect. Gd^{3+} and sulfate are competitive inhibitors for Mg^{2+} and phosphate, respectively, and, therefore, it is reasonable to expect that the inhibitory ions bind in the same sites as Mg^{2+} and phosphate in the enzyme product complex.⁴⁴

The fact that the Gd^{3+} ion was found to be buried *deeper* in the active-site cleft than the sulfate group implied that magnesium should bind to inositol monophosphatase first before the substrate. This finding was inconsistent with the binding sequences of species which had been previously deduced from kinetic studies (Section 1.5, page 11), in which it was apparent that *Ins1-P* bound first to the enzyme active-site, followed by a magnesium ion. However, the proposed involvement of two magnesium ions in the enzyme reaction offered a valid reason for this observed inconsistency. Put simply, the two experimental approaches were each detecting one of two different metal ions.

More revealing X-ray structures of human inositol monophosphatase have been reported recently, one containing Gd^{3+} and either D- or L-*Ins1-P*,⁶⁹ and another containing inorganic phosphate and two Mn^{2+} ions.⁷⁰ The latter structure provides additional evidence for the involvement of two-metal ions in catalysis.

1.13 Binding of Alternative Substrates

As only two adjacent O-atoms interact with Mg^{2+} and the phosphate moiety interact with Mg^{2+} , ethane 1,2-diol 1-phosphate **38** was investigated as a possible minimum substrate for the enzyme. Modelling revealed that the molecule could bind in either or both of the two conformations proposed to be adopted by 2'-AMP and D-*Ins1-P* [Figure 1.11 (a) and (b)]. Indeed, **38** exhibited moderate activity ($V_{\text{max}} = 12\%$ of the V_{max} -value for *Ins1-P*, $K_{\text{m}} = 0.7 \text{ mmol dm}^{-3}$) with sensitivity to magnesium concentration, and behaved as a competitive inhibitor as expected on the basis of its properties as a substrate

($K_i = 1.0 \text{ mmol dm}^{-3}$).^{66, 71} The importance of the 2-OH group was verified by synthesising propyl phosphate **39** which proved to be completely inactive as a substrate and inhibitor.⁷¹

In order to probe the environment of Mg^{2+} , minimum substrate **38** was elaborated by incorporating extra oxygen-functionality into specific positions. (2*S*)-Pentane-1,2,5-triol 2-phosphate **40** served as a competitive inhibitor of inositol monophosphatase ($K_i = 0.12 \text{ mmol dm}^{-3}$). The observed 9-fold reduction in K_i over diol **38** implied that both the 1-OH and 5-OH groups interacted with the enzyme, presumably at the sites for the 2-OH and 4-OH groups of Ins1-*P* and the effective deoxyinositol inhibitor (-)-**22** (page 15). On the other hand, the (2*R*)-pentane-1,2,5-triol 2-phosphate **41** was expected to serve as a substrate by analogy to the weak deoxyinositol substrate (+)-**22** (page 15), but in the event, **41** served as a weak inhibitor ($K_i = 3.8 \text{ mmol dm}^{-3}$). The flexibility of the 5-OH group meant that it could likely bind to a site on Mg^{2+} and disrupt the geometry of the complex such that hydrolysis was prevented.⁷¹

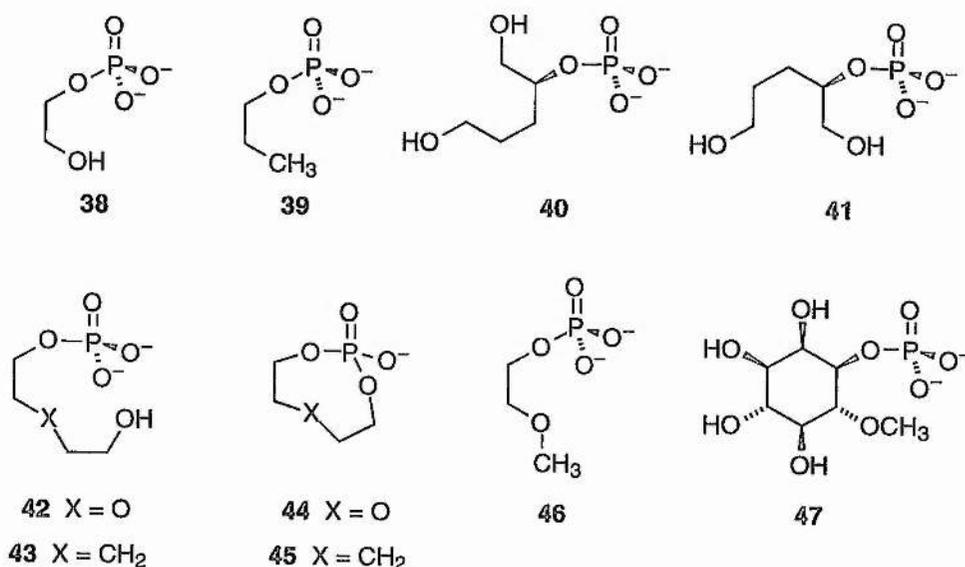


Figure 1.13: Compounds synthesised to probe the environment of the proposed second Mg^{2+} ion.

Another series of compounds, 42–46, were produced with the free hydroxyl group of diol 38 elaborated, so as to provide molecules that might displace the nucleophilic water molecule from its proposed site on Mg^{2+} . As expected, *none* of these compounds exhibited substrate activity.

Replacement of the 2-OH group of diol 38 with a methoxy group gave 2-methoxyethyl phosphate 46 which showed no activity as a substrate and exhibited a 25-fold reduction in binding affinity as an inhibitor ($K_i \geq 25 \text{ mmol dm}^{-3}$). This result correlated well with previously synthesised 6-*O*-methylinositol 1-phosphate 47 also shows no substrate activity and is an inhibitor.⁶⁸ These findings indicated that the ability of the O-atoms in the substrate (6-O in *D*-Ins1-*P* and 2-O in 38) to interact with Mg^{2+} was severely compromised by alkylation to give the methyl ether derivatives. It was proposed therefore, that steric crowding or adverse hydrophobic effects within the active site was preventing the lone-pairs of these O-atoms from assuming catalysis supporting positions. Only in the highly ordered conformation of 2'-AMP could an ether O-atom support catalysis.

Diethyleneglycol monophosphate 42 showed an 8-fold increase in binding affinity to inositol monophosphatase ($K_i = 3.5 \text{ mmol dm}^{-3}$) relative to 46. In a similar manner to the observed behaviour of triol monophosphate 41, this increase in affinity was ascribed to the direct interaction of the flexible 5-OH with Mg^{2+} , with the possible consequence of displacing the proposed nucleophilic hydroxide ligand. The affinity of 42 did not alter when the ether O-atom was replacement to give propane diol monophosphate 43, confirming the fact that the 5-OH was responsible for the major interaction with Mg^{2+} .

Cyclic ether diester 44 has a reduced negative charge on the phosphate moiety and was thus expected to bind less well to Mg^{2+} in the enzyme active-site than acyclic 42, and consequently decrease the affinity of the enzyme-bound species for Mg^{2+} . In the event 44 was an inhibitor, albeit a weak one with a K_i -value of 8 mmol dm^{-3} ; a remarkable finding for such a monoanionic compound. An altered binding arrangement of 44 with the enzyme as the surrogate for the nucleophile was now bonded directly to the P-atom,

was also cited as a reason for the low K_i -value.⁷¹ The fact that this compound is not a substrate for inositol monophosphatase is consistent with the proposed mechanism in which Mg^{2+2} rather than Mg^{2+1} provides the nucleophile. Again, removal of the ether O-atom of 44 to give cyclic propane diester 45 exerted little effect on the affinity of the compound.

From the general lack of interaction of the ether O-atoms of 42, 44, and 46 with Mg^{2+2} , and the observed substrate activity of ethane-1,2-diol phosphate 38, it was proposed that the catalytically essential O-atom in substrates did not interact directly with Mg^{2+2} . Instead, they provide a lone-pair to position the Mg^{2+2} -chelated nucleophile, water, properly and hold it in place on the metal ion.⁷¹ This arrangement would give a seven-membered trioxametallocycle containing an internal hydrogen-bond (see Figure 1.15).

1.14 Proposed Adjacent Association Mechanism for Inositol Monophosphatase and its Stereochemical Consequences

Many of the phosphate monoester probes studied which contained the two requisite oxygen functionalities for binding the second Mg^{2+} ion served as competitive inhibitors of inositol monophosphatase 41–43, 46, and 47. If Mg^{2+1} chelated and activated the nucleophilic water molecule, these compounds should have also served as substrates, with the Mg^{2+2} merely stabilising the leaving alkoxide group. Such a mechanism would predict an *in-line displacement at phosphorus with inversion of configuration*.

Conversely, if Mg^{2+2} provides the site for the nucleophile, *none* of these compounds which can either provide a ligand to displace the nucleophile, or cause the reorganisation of the coordination geometry about Mg^{2+2} , would be hydrolysed, as is observed. The many observations for inositol monophosphatase are in accord with a mechanism involving two Mg^{2+} ions in which the *second* Mg^{2+} ion chelates and activates the nucleophile. Such a mechanism predicts *adjacent displacement at phosphorus*. This route must then involve a *pseudorotation* to place the inositol leaving group into an apical position so that the $E-Mg^{2+1}-Ins-P_i-Mg^{2+2}$ product complex can form. The process gives *retention of configuration* at phosphorus (Section 1.23).⁷² While adjacent

displacement with pseudorotation at phosphorus has not yet been demonstrated for an enzymatic system, there is irrefutable chemical precedence for the mechanism.^{73, 74}

The proposed involvement of two Mg^{2+} ions in the enzyme mechanism was consistent with the reported X-ray crystal structure for a human inositol monophosphatase complex containing two divalent Mn^{2+} ions and phosphate.⁷⁰

1.15 Mechanism of Pseudorotation

The pseudorotation for compounds such as the pentacoordinate intermediate formed by the proposed adjacent displacement mechanism for inositol monophosphatase is defined as the intramolecular process where a trigonal-bipyramidal molecule is transformed by deforming bond angles in such a way that it appears to have been rotated by 90° about one of the interatomic bonds (Figure 1.14).⁷⁵

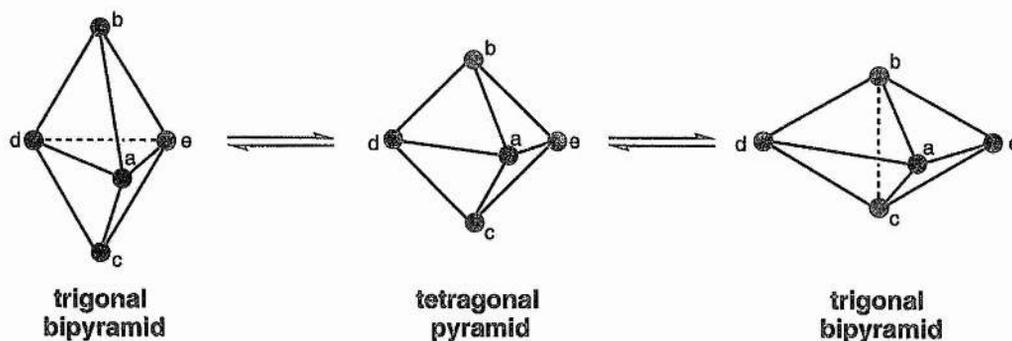


Figure 1.14: *Pseudorotation of a pentacoordinated compound*

In Figure 1.14 the substituent **a** that is towards the viewer remains fixed, while the vertical (apical) substituents **b** and **c** are pushed backwards and the horizontal (equatorial) substituents **d** and **e** pulled forward so as to produce a tetragonal pyramid where the fixed substituent **a** is at the apex. A continuation of the process leads to the second trigonal bipyramid, which appears to have been produced by rotating the first about the bond from substituent **a** (the 'pivot') to the central atom.

1.16 3-Dimensional Interactions between Inositol Monophosphatase and its Substrates and Metal Ion Cofactors

The proposed mechanism for D-Ins1-P was examined within the confines of the enzyme active-site by using the X-ray coordinates of the Gd^{3+} -sulfate form of the human enzyme and the available kinetic data (Appendix 4.1, page 246).⁷⁶ The Gd^{3+} ion in the crystal structure was replaced by Mg^{2+} and the phosphate group of the substrate was placed so that the P-atom occupied the position occupied by the S-atom of the sulfate dianion. The structure of the substrate was adjusted in accord with the findings of the kinetic studies, and the second Mg^{2+} ion was placed in a site created by the phosphate bridging O-atom of the substrate and three aspartate residues from the protein, but with no direct interaction with the 'catalytic' 6-OH (Fig. 1.15).

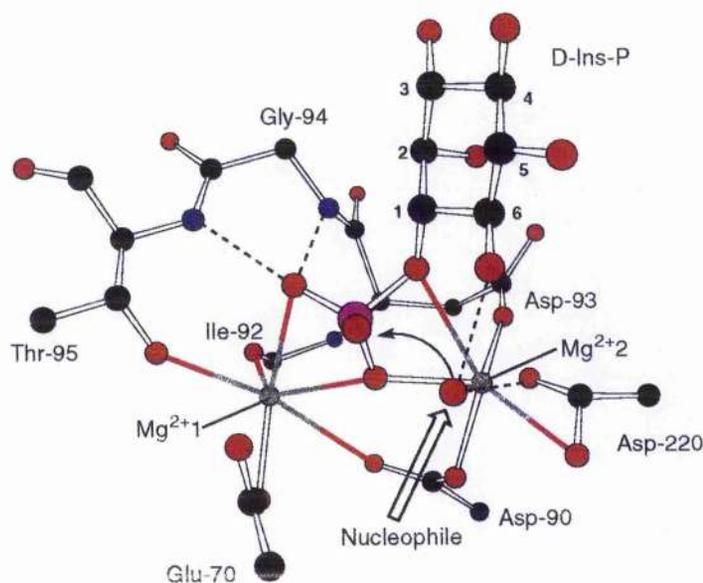


Figure 1.15: *Optimised active-site structure for the bound Mg^{2+} -D-Ins1-P complex showing the interactions with key amino acid residues. The nucleophilic water molecule on Mg^{2+} is perfectly setup to attack the phosphate moiety in an adjacent manner*

As most of the kinetic data was determined using the enzyme from bovine brain, there was initial concern that the mechanistic arguments founded on this enzyme would be less reliable in the human brain enzyme. Although it was found that the primary structure of each enzyme differed by 32 residues per subunit (11.5%), most changes were highly

conservative mutations and only four lay within 10 Å of either active site. By optimising the structures of both enzymes, it was demonstrated that none of these differences were likely to perturb the substrate-binding conformations or affect the proposed enzyme mechanism.⁷⁶

In the optimised structure, Mg^{2+1} coordinates to the side-chain carboxylate groups of Glu-70 and Asp-90, the hydroxy group of Thr-95 and to the carbonyl O-atom of Ile-92 as well as to two of the non-bridging phosphate O-atoms (Fig. 1.16). A water molecule which is visible in the Gd^{3+} -sulfate X-ray structure, completes the coordination sphere and is hydrogen bonded to Asp-47. This water molecule is distant from the phosphate moiety of the substrate and appears unable to interact. Moreover, the site for Mg^{2+1} lies at the bottom of the active-site cleft and after the first coordination sphere, is encapsulated by the protein. Access of bulk solvent to the site appears to be severely restricted by the protein backbone and the bound substrate. Thus it is *unlikely* that this water molecule is involved in substrate hydrolysis. This finding also implies that Mg^{2+1} would enter the active site through the top of the active-site cleft, before any of the other species, and would not be able to escape between individual catalytic events at saturating concentrations of substrates. This sequence of events is entirely consistent with the kinetic findings.

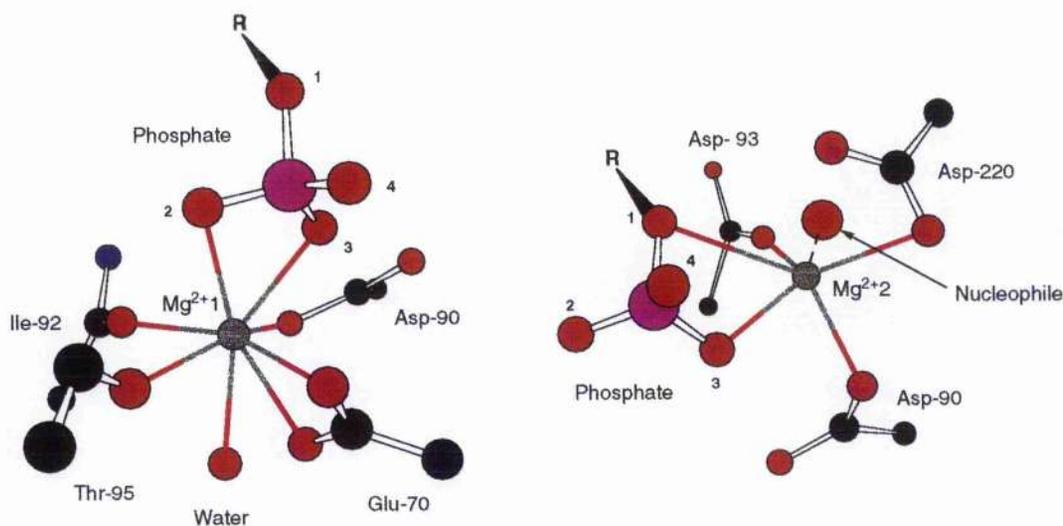


Figure 1.16: Primary coordination sphere of both Mg^{2+1} and Mg^{2+2} in the optimised Mg^{2+} -enzyme-D-Ins1-P complex

A metal ion site for Mg^{2+} is formed by coordination to the carboxylate groups of Asp-90, Asp-93, and Asp-220, and to the substrate through one of the three equivalent phosphate O-atoms and the bridging ester O-atom. Again, one face of the metal ion is free, but in this case it is *accessible* to bulk solvent (Figure 1.16).

The inositol ring binds in the active site cleft through its hydroxy groups by forming several hydrogen bonds, one from the 4-OH group to the carboxylate group of Glu-213, one from the 2-OH group to the side-chain of Asp-93, and one from the backbone NH of Ala-196 to the 2-O-atom. The residue Asp-220, in addition to providing a ligand for Mg^{2+} , also accepts a hydrogen bond from the 6-OH group. The phosphate ester binds to both metals and to backbone NH moieties of Thr-95 and Gly-94 (Figure 1.15).

The conformation revealed that the 'catalytic' 6-OH group was too distant from Mg^{2+} (4.02 Å) to make a direct contact, in keeping with the kinetic behaviour of several substrate analogues already discussed. Consequently, the catalytic hydroxy group is positioned to coordinate to a water molecule and that, in turn, completes the coordination shell of Mg^{2+} to give approximate octahedral geometry. The water molecule is well placed to function as the nucleophile. Moreover, the carboxylate group of Asp-220 may assist the reaction by acting as a base, forming a hydrogen-bond to the water molecule.⁷⁶ (Figure 1.15).

The N-terminals (positive poles)^{77, 78} of two α -helix are also directed at the phosphate moiety in the active-site. The first structure (residues 195-205) is directed at the phosphate ester group and could aid binding, whilst the second structure (residues 95-100) is directed at the bridging phosphate ester O-atom and at the nucleophilic water molecule. The interaction of this second helix would be expected to help stabilise the transition state for the hydrolytic reaction.⁷⁶

1.17 Adjacent Displacement Mechanism and the Enzyme Active Site

In the proposed adjacent displacement mechanism, the nucleophile is H-bonded to the 6-OH of the substrate and is activated and positioned by Mg^{2+} and Asp-220 (Figure

1.15, page 31 and Appendix 4.1, page 246). Attack on the phosphorus occurs from opposite the O-atom that is H-bonded to the backbone NH moieties of Gly-94 and Thr-95. The P-O bond lengths increase as the nucleophile attacks and the unbound phosphate O-atom moves towards Mg^{2+1} such that its position is stabilised by interactions between the side-chain hydroxy group of Thr-95 and Mg^{2+1} .

The O-1-P bond then begins to lengthen further and the other phosphate group P-O bonds shorten. The O-atom of the nucleophile moves out of the coordination sphere of Mg^{2+2} as O-1 of the substrate, stabilised as alkoxide, becomes protonated by (the now acidic) Asp-220, possibly *via* the intermediacy of the 6-OH group, to give the product complex.⁷⁶

In the complex, the side-chain of Thr-95 and Mg^{2+1} interact with the pseudorotated phosphate O-atom. Reassuringly, this arrangement of the phosphate group resembles the arrangement of phosphate observed in the X-ray crystal structure of the di- Mn^{2+} -phosphate product complex.⁷⁰

1.18 Proposed Alternative In-line Association Mechanism for Inositol Monophosphatase

Inositol monophosphatase has also been investigated independently by a group at Merck, Sharp and Dohme using a combination of X-ray crystallographic studies^{44, 69, 70} of protein-substrate complexes, and mutagenesis.⁷⁹⁻⁸⁴ An almost identical binding arrangement was proposed for D-Ins1-P within the enzyme active site involving two Mg^{2+} ions (Figure 1.17). However, the interpreted enzyme mechanism was different.⁸⁵

Figure 1.17 shows a more conventional in-line substitution mechanism in which the nucleophile is H-bonded to the 3-OH group of Thr-95 and is activated and positioned by Mg^{2+1} and Glu-70 (see also Appendix 4.2, page 247).⁸⁵ Attack on the phosphorus occurs from opposite the 1-O-atom of the substrate to give a stable, trigonal bipyramidal intermediate. The 1-O-atom of the substrate, stabilised as alkoxide, then moves away from the P-atom to give the product complex containing an inverted phosphate group.

The 1-O-atom of the substrate is subsequently protonated, possibly by bulk solvent in the active site, and the products are released.

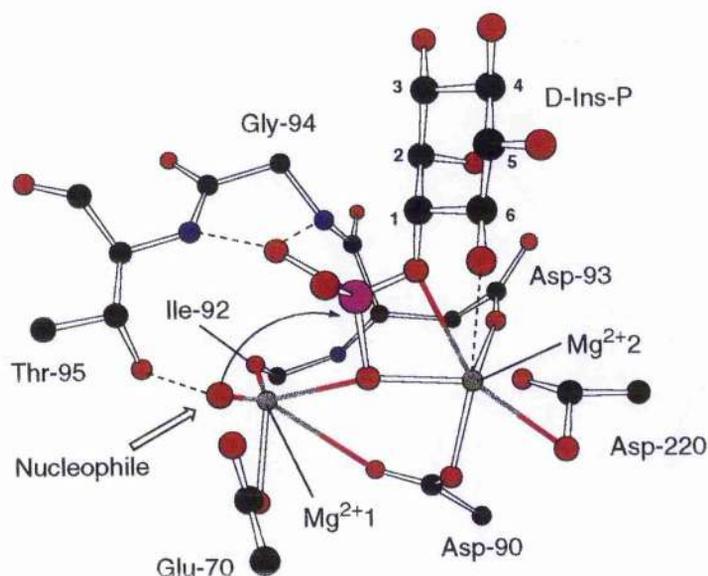


Figure 1.17: *Alternative optimised active-site structure for the bound Mg^{2+} -D-Ins1-P complex derived from X-ray crystallographical and mutagenesis studies. In-line attack of the nucleophilic water molecule on the phosphate moiety is proposed*

1.19 Comparison of Adjacent and In-line Association Mechanisms

Evidence for the proposed adjacent displacement mechanism comes from observations that the rate of inositol-mediated ^{18}O -exchange from water into inorganic phosphate was very fast ($70\% V_{max}$ for the hydrolysis reaction for DL-Ins1-P at saturating inositol and P_i).^{43, 52} It was argued that this fast exchange was much easier to accommodate if the nucleophilic water molecule was associated with Mg^{2+2} . The metal ion is easily accessible to the bulk solvent as it must debind from the enzyme with its associated hydration ligands, between every catalytic event, in order to allow phosphate to debind from the active site.⁷¹

Mg^{2+1} , on the other hand, is deeply buried in the active-site and, at high concentrations of substrates or P_i , is probably trapped there, restricting its access to bulk solvent.

Additionally, a water molecule associated with Mg^{2+} may be unsuitable as a nucleophile as it interacts strongly with the active site through hydrogen bonding to Glu-70 and Thr-95 residues.⁸⁵ The kinetics of the enzyme relative to the observed rate of ^{18}O -exchange also predict that for Mg^{2+} , the rapid exchange of water on the metal ion would only be observed if it occurred in the higher order substrate-bound complexes ($E-Mg^{2+}1-Ins1-P-Mg^{2+}2$ and $E-Mg^{2+}1-Ins1-P$). Although not impossible, such an exchange requires the co-ordination sphere of the most deeply buried Mg^{2+} ion to be easily accessible to bulk solvent in an enzyme conformation which is poised to catalyse its physiological reaction. The key species should be highly constrained in order to stabilise the transition state of the hydrolytic process and are thus unlikely to be unavailable to partake in side-reactions.

The fact that the phosphorothioate analogues of *Ins1-P* and 2'-AMP are processed by inositol monophosphatase, albeit slower than the phosphate versions, is another indication that inositol monophosphatase might operate *via* an adjacent displacement mechanism. Remembering that the first metal ion (Mg^{2+}) makes a strong binding interaction with the phosphate moiety of the substrate, one would expect a substantial reduction in rate with a phosphorothioate analogue due to the large size of the sulfur atom forcing the P-atom away from Mg^{2+} (Mn^{2+} in the case of 2'-AMP_s) and reducing the binding affinity. However, this effect would also increase steric hindrance for an in-line attack by a nucleophile located on Mg^{2+} and these two effects combined would be expected to cause a *much* more severe reduction in rate than is actually observed (typically 10–20% rate of hydrolysis for phosphomonoester substrate). Conversely, an adjacent attack mechanism would not encounter the second problem as the nucleophile could be positioned as to attack the phosphorothioate moiety from the opposite face to the sulfur atom.

Evidence supporting the alternative in-line association mechanism is based mainly on the results of site-specific mutagenesis experiments. Specifically, the alteration of Glu-70, which was proposed to activate the nucleophile on Mg^{2+} , to glutamine or aspartate resulted in a dramatic reduction in k_{cat} for the enzyme, suggesting the involvement of the residue in the hydrolytic mechanism.^{69, 85} However, this observed fall in the rate of

hydrolysis with the mutant enzyme could also be caused by a decrease in the binding affinity of the mutant enzyme for Mg^{2+} and the kinetic arguments associated with Glu-70 mutagenesis are not convincing.⁷⁶

Computational determination of the reaction pathways for models of these alternative mechanisms (see Appendix 4.1, page 246, and Appendix 4.2, page 247) has shown that it is not possible to differentiate between them on energetic grounds.⁸⁶ Moreover, both models give rise to a similar 'inverted' phosphate group in the product complex, which mimics the arrangement in the published di- Mn^{2+} -phosphate-enzyme X-ray structure.⁷⁰

The ability of the enzyme to be inhibited by compounds such as D-6-*O*-methylinositol 1-phosphate⁴⁷⁸⁷ and 6-deoxyinositol 1-phosphate¹⁸⁵⁵, which possess all the functional groups required for an in-line displacement, implies an adjacent-displacement mechanism for inositol monophosphatase. As each mechanism has a different stereochemical outcome (page 29), the validity of this hypothesis can only be tested by determining the stereochemical course of the inositol monophosphatase reaction with respect to the phosphorus atom.

1.20 3-Dimensional Interactions between Inositol Monophosphatase and 2'-AMP and Metal Ion Cofactors

For comparison, Figure 1.18 represents the optimised active conformation of 2'-AMP within the confines of the enzyme active-site. The studies have shown that 2'-AMP exists in a less high-energy conformation than that originally proposed by Cole and Gani.^{66, 67} The catalytic ribofuranosyl 1'-O-atom no longer makes a direct contact with Mg^{2+} , but instead bonds to it through not one but two water molecules (OW1 and OW2). The structure, when removed from the active site, is only 20.1 kJ mol⁻¹ *less stable* than the ground-state conformation of 2'-AMP.⁷⁶

Close inspection of the structure reveals that the key H-bonding interactions between the peripheral hydroxy groups of the ribofuranosyl ring and the enzyme are similar to those observed for D-Ins1-*P*, and that the position of the second water molecule OW2 is

coincident with the 6-OH of D-Ins1-P. The nucleophilic water molecule (OW1) is again properly aligned for an attack on the phosphate group in an adjacent manner.

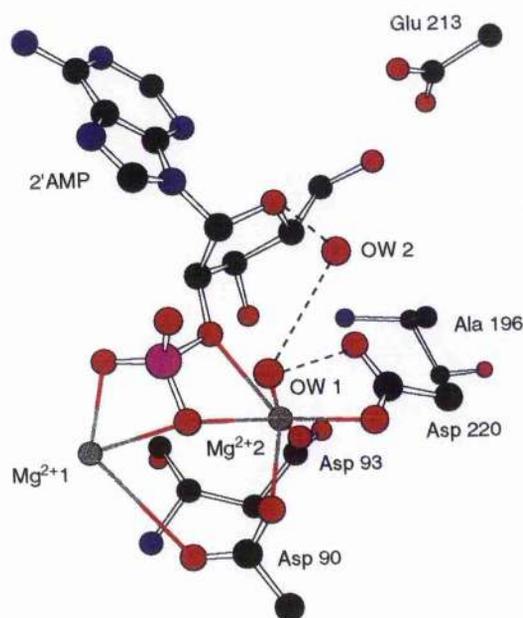


Figure 1.18: Optimised active-site structure for the Mg^{2+} -2'-AMP reactant complex

1.21 Lithium Inhibited Complex

Kinetic studies have shown that at the therapeutic levels of Li^+ , the metal ion acts as an uncompetitive inhibitor of inositol monophosphatase by blocking the release of the hydrolysis product, inorganic phosphate.^{26, 43} The full kinetic scheme for catalysis and inhibition in inositol monophosphatase can be found in Appendix 4.3, page 248, where this process is represented by step 13. The optimised structure of the lithium inhibited complex $E-Mg^{2+}1-HPO_4^{2-}-Li^+$ is shown in Fig. 1.19. Lithium occupies the site for $Mg^{2+}2$ and modelling studies have shown that it can interact with the four closest ligands for $Mg^{2+}2$ in the $E-Mg^{2+}1-HPO_4^{2-}-Mg^{2+}2$ product complex. These are the carboxylate O-atoms of Asp-90, Asp-93 and Asp-220, and one of the phosphate O-atoms of the product which form an approximate tetrahedral geometry in which the phosphate dianion is

slightly closer to the Mg^{2+1} ion. This may account for the kinetic observation that Li^+ binds better than Mg^{2+2} to the Mg^{2+1} -E- P_i complex.

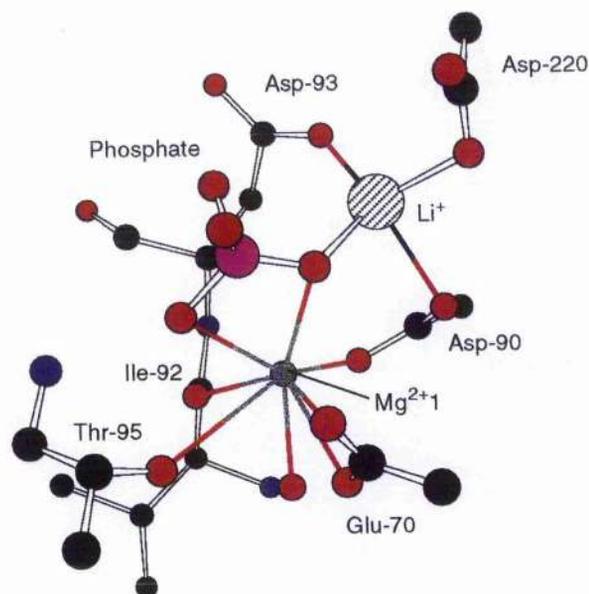


Figure 1.19: Optimised structure for the lithium-inhibited complex of inositol monophosphatase, $E-Mg^{2+1}$ - HPO_4^{2-} - Li^+

1.22 Second Generation Inositol Monophosphatase Inhibitors

The catalytic mechanism proposed by Cole and Gani^{66, 67, 71, 76} involved the adjacent nucleophilic attack on phosphorus by water co-ordinated to Mg^{2+2} . Consequently, a series of inositol derivatives based on deoxyinositol substrate **16** were designed to displace the nucleophile to give efficient, tight-binding inhibitors of inositol monophosphatase (Fig. 1.20).⁸⁸⁻⁹⁰ The compounds have been prepared *via* a chiral epoxide route,⁹⁰ or from (-)-quinic acid **51** which possesses much of the ring stereochemistry already.⁸⁹

Hydroxyethyl analogue **48** exhibited no substrate activity and proved to be a potent competitive inhibitor ($K_i = 1.8 \mu\text{mol dm}^{-3}$ for the racemate, $K_i = 0.5 \mu\text{mol dm}^{-3}$ for the enantiomer shown) implying that the pendant arm chelated Mg^{2+2} rather than hydrogen-bonding to a residue across the active-site (Ser-165) as partly predicted by

molecular modelling studies. Compounds **49** and **50** were designed to represent the intermediate and product respectively of a hypothetical intramolecular transesterification reaction if the pendant arm 2'-OH group of **48** were to act as the active-site nucleophile on Mg^{2+} . The inhibitory properties of both compounds were excellent,⁹⁰ and neither compound exhibited substrate activity – again strongly supporting the validity of the proposed enzyme mechanism. It was subsequently proven that transesterification is not in fact observed for **48**.

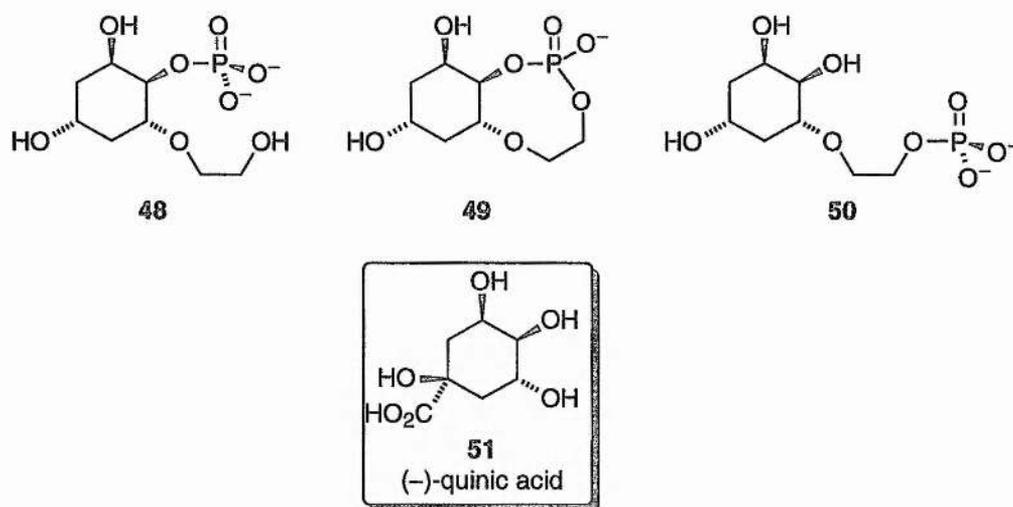


Figure 1.20: *Inhibitors of inositol monophosphatase based on the proposed adjacent displacement mechanism*

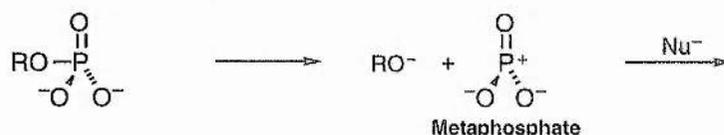
Kinetic and modelling studies have shown that two different environments in two different locations within the active site, one lipophilic (Val-40 and Leu-42), and one hydrophilic (the co-ordination sphere of Mg^{2+}), can be accessed by side-chains attached to the C-6 position of cyclitol analogues of D-Ins1-P. Work is continuing on potential inhibitors which could access both sites at the same time.

1.23 Stereochemical Significance of Phosphoryl Transfer

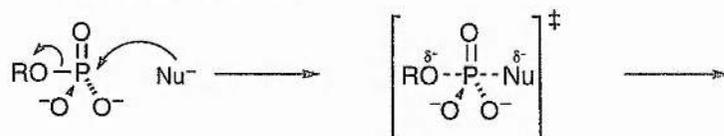
The transfer of phosphoryl groups from one entity to another is of crucial importance in nature, linked with energy transduction, metabolic regulation and as we have seen already, signal transduction.

Simple non-enzymatic phosphoryl transfer in phosphate monoesters can occur *via* one of four mechanistic extremes as depicted in Scheme 1.7, and each has a distinctive stereochemical course with respect to the phosphorus centre.^{86, 91, 92} The dissociative pathway (mechanism A), analogous to an S_N1 process in carbon chemistry, involves the formation of the highly reactive metaphosphate intermediate, which is then captured by the acceptor group. If this species is free and symmetrically solvated, *racemisation* at phosphorus can be predicted.^{93, 94}

A. Dissociative Ionisation
(*via* monomeric metaphosphate):



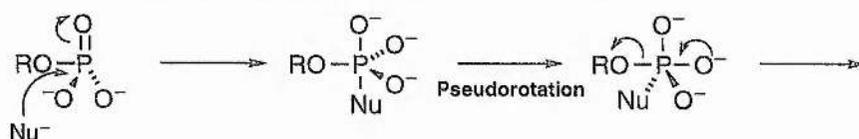
B. In-line Associative Substitution
(*via* a pentacoordinate transition state):



C. In-line Associative Addition
(*via* a pentacoordinate intermediate):



D. Adjacent associative Addition-Displacement
(*via* a pentacoordinate transition state and pseudorotation):



Scheme 1.7: Mechanisms of phosphoryl transfer

The in-line associative process which proceeds *via* a *pentacoordinate transition state* (mechanism B) is analogous to an S_N2 displacement in carbon chemistry and would give *inversion* of configuration at phosphorus. Mechanism C is also an in-line associative

process but gives rise to a *pentacoordinate stable intermediate* with fully formed bonds. Again, the displacement must occur with *inversion* of configuration at phosphorus. In the adjacent associative process (mechanism D), *retention* of configuration is observed as the stable intermediate that forms *must* undergo a pseudorotation so that the leaving group can leave from an apical position. Knowles and co-workers have demonstrated that such a mechanism can operate in free solution if the positions of the nucleophile and leaving group are constrained such that adjacent attack of the nucleophile is necessary.^{73, 95}

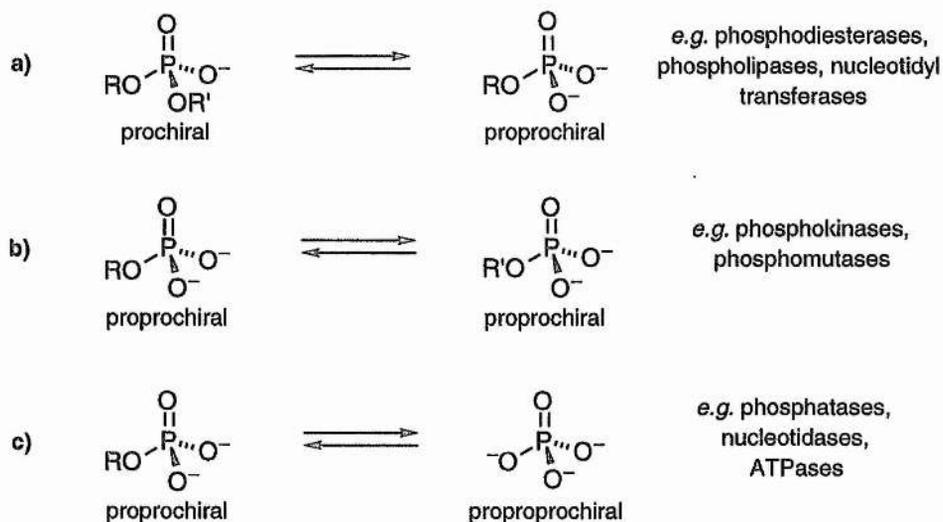
Enzyme catalysed phosphoryl transfer reactions are, however, thought to proceed exclusively *via* the in-line associative process (mechanism C),⁹⁶ a pattern which has emerged from the analysis of the stereochemical course of many phosphoryl transfer enzymes.⁹⁷ However, the picture is complicated by the fact that most, but not all, phosphoryl transfer enzymes operate by a double displacement utilising an enzyme bound nucleophile forming a phosphorylated enzyme intermediate and then attack of the intermediate by the nucleophile (a substituted enzyme mechanism, see Scheme 1.4, page 12), whilst some transfer the phosphoryl group directly to the incoming nucleophile in a single step (a direct displacement mechanism, see Scheme 1.5, page 13).⁹⁴ Therefore, although both types utilise the same putative in-line association mechanism, the overall stereochemical course for the enzymes are different; retention for the former, as two steps are involved; inversion for the latter enzyme type which uses only one step.

Nevertheless, the determination of the overall stereochemical course of the phosphoryl transfer reaction for an enzyme, when combined with results from kinetic studies, can be an extremely powerful tool in establishing enzyme mechanism.

1.24 Biological Reactions Involving Phosphate Monoesters

A phosphate monoester is an example of a compound containing a *propochiral* phosphorus centre, meaning that the centre requires two isotopic or heteroatomic substitutions at phosphorus to make the molecule chiral. Enzyme catalysed phosphoryl

transfer reactions involving a *proprochiral* phosphorus centre can be categorized into the following types, based on the stereochemistry involved (Scheme 1.8).



Scheme 1.8: *Enzyme catalyzed reactions involving a proprochiral phosphorus centre*

Phosphatase enzymes, including inositol monophosphatase, fit into category **c** where the phosphoryl donor molecule is a phosphate monoester (proprochiral) and the acceptor molecule is a water molecule (proproprochiral). Most phosphatase enzymes, but not inositol monophosphatase,^{43, 52} also catalyze phosphoryl transfer reaction **b** if an alcohol is used as an acceptor molecule.

1.25 General Approaches in the Elucidation of Reaction Stereochemistry

The general approach in elucidating the stereochemical course of an enzyme catalyzed reaction involves:

- 1) synthesis of the substrate chirally labelled at phosphorus;
- 2) use of the chirally labelled substrate to perform the reaction and isolation of the products;
- 3) determination of the absolute or relative configuration of the substrate and the product.

Oxygen is the lightest element to exist naturally as *three* stable isotopes, namely, ^{16}O , ^{17}O , and ^{18}O . A phosphate monoester can therefore be made chiral by labelling with ^{17}O and ^{18}O . Figure 1.21 shows a (R_p) - $[^{16}\text{O},^{17}\text{O},^{18}\text{O}]$ phosphate monoester **52a** and (S_p) - $[^{16}\text{O},^{17}\text{O},^{18}\text{O}]$ phosphate monoester **52b**. Such compounds are used to probe the stereochemistry of enzymes in which the phosphoryl group is transferred to another alcohol, where the chiral integrity of the phosphorus centre is not forfeited (Figure 1.21).

However, phosphatase enzymes that can only transfer the phosphoryl group to water cannot use this approach. The product, inorganic phosphate, can only be made chiral by labelling with ^{17}O , ^{18}O , and sulfur, giving (R_p) -inorganic $[^{16}\text{O},^{17}\text{O},^{18}\text{O}]$ phosphorothioate **53a** and (S_p) -inorganic $[^{16}\text{O},^{17}\text{O},^{18}\text{O}]$ -phosphorothioate **53b** (Figure 1.21).*

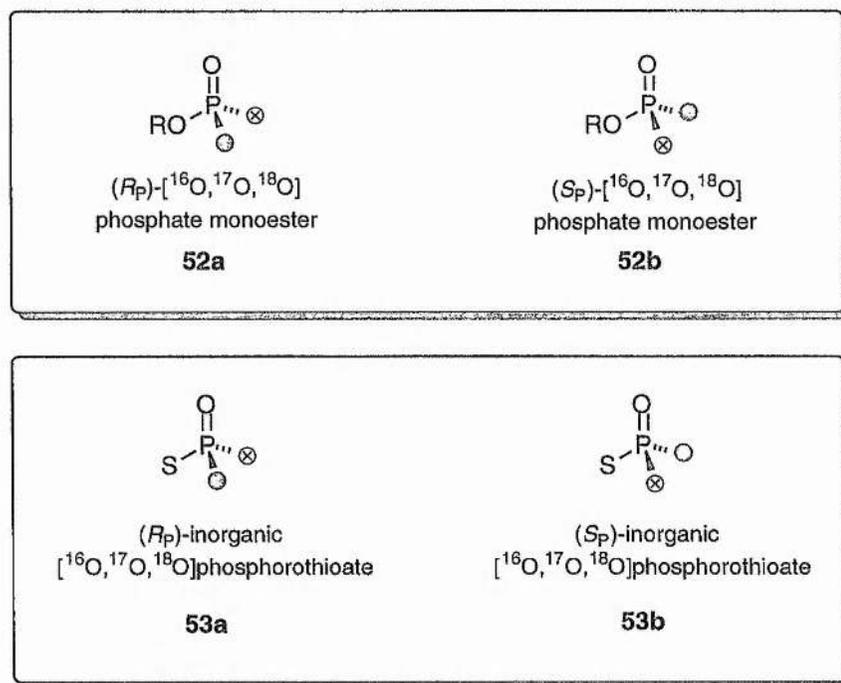


Figure 1.21: Chiral $[^{16}\text{O},^{17}\text{O},^{18}\text{O}]$ phosphate monoesters and chiral inorganic $[^{16}\text{O},^{17}\text{O},^{18}\text{O}]$ phosphorothioates

* ^{18}O is commonly represented by \odot and ^{17}O by \otimes . The stereochemical structural formulas for chiral phosphates and phosphorothioates are conventionally drawn without showing negative charges or double bonds.

1.25.1 Assignment of Chiral Phosphorothioates

The (*R*)- and (*S*)- configurational symbols specifying absolute configuration at phosphorus in biological molecules chiral at phosphorus are based on those used for a saturated carbon.⁹⁸ There are some modifications to take into account of a few special properties of phosphate and phosphorothioate:

- 1) electrostatic charges are ignored in assigning symbols;
- 2) bond orders are also ignored;

The priority order for substituents on phosphorus is $S > {}^{18}\text{O} > {}^{17}\text{O} > {}^{16}\text{O}$, with the heavy isotope priorities operating only as required.⁹⁹

1.25.2 Phosphorothioate Monoesters as Substrates for Enzymes

Inclusion of a sulfur atom as a 'surrogate fourth oxygen atom' represents a heteroatomic substitution. Sulfur is considerably different from oxygen in chemical and physical properties. Covalent bonding in phosphorothioate anions differs from that in an analogous phosphate anion. A higher P–O bond order is observed with the negative charge localized on sulfur where it is far more stable. The large size and polarizability of the sulfur atom permits low charge density relative to a phosphate ion and more than compensates for the reduced resonance delocalization of the charge on sulfur⁷²

For reactions such as the inositol monophosphatase reaction, where a *propiochiral* phosphorus centre (*i.e.* inorganic phosphate) is involved, the use of sulfur is unavoidable. The use of chiral phosphorothioates in enzyme reactions typically causes a decrease in reaction rate to less than 10% (in many cases below 1%).¹⁰⁰

Although it has been questioned whether the stereochemical course elucidated with a phosphorothioates reflects the real mechanism of a particular enzyme,¹⁰¹ for all enzymes that have been investigated by both chiral phosphate and chiral phosphorothioate substrates, the stereochemical outcome is the *same* without exception.¹⁰²⁻¹⁰⁵

1.26 Synthesis of Chiral [^{16}O , ^{17}O , ^{18}O]Phosphate Monoesters

Two syntheses of chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoesters have been reported, one by Knowles^{106, 107} and the other by Lowe.¹⁰⁸⁻¹¹³ The synthetic strategy for each route was constrained by the same considerations:

- 1) the method should be general enough to readily allow any chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoester to be synthesised;
- 2) the method should enable ^{17}O and ^{18}O to be incorporated from isotopically labelled water with minimal dilution of label;
- 3) the absolute configuration of the chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoester should follow from the method of synthesis.

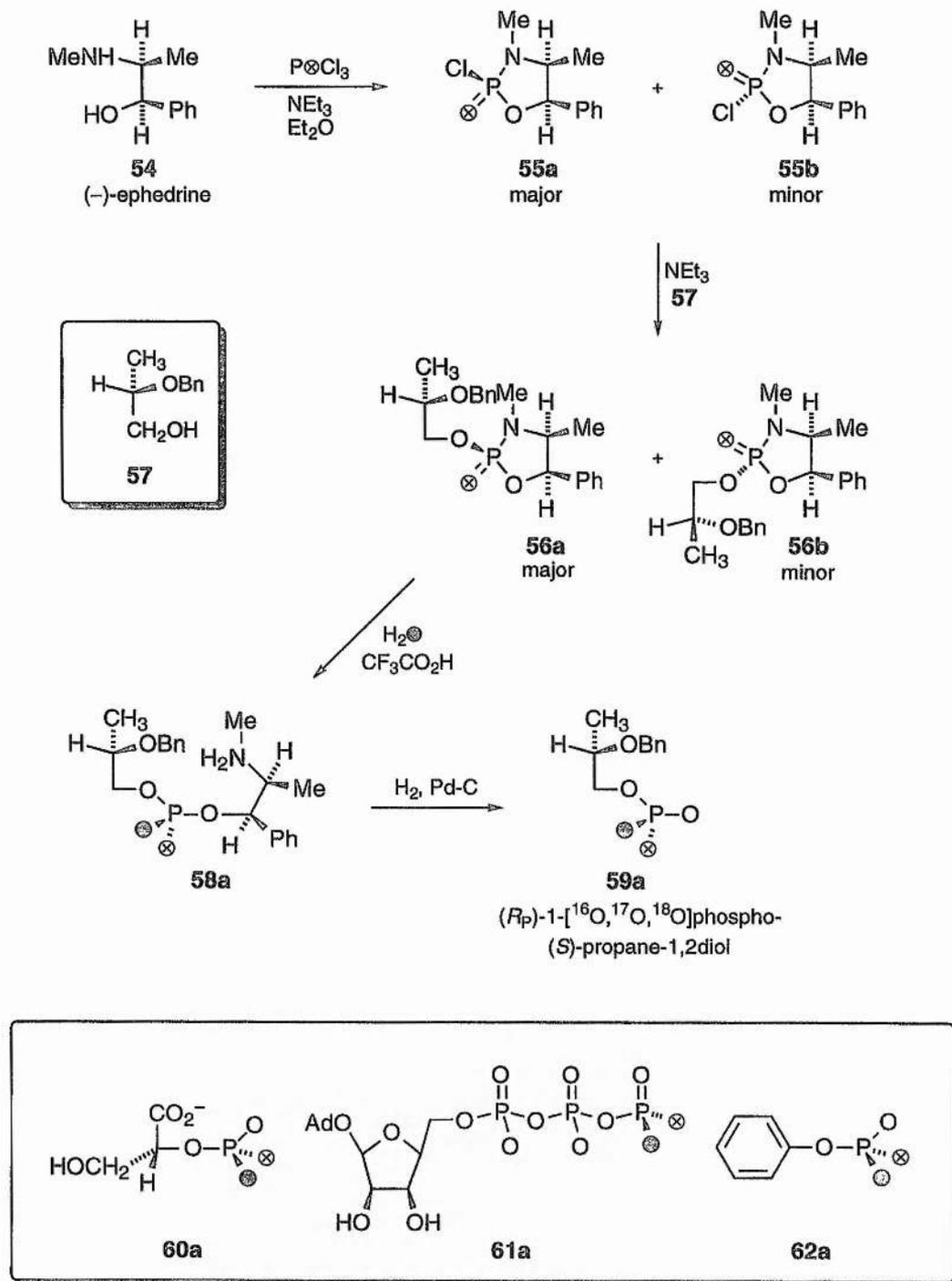
1.26.1 Knowles' Synthesis of Chiral [^{16}O , ^{17}O , ^{18}O]Phosphate Monoesters

The Knowles' synthetic method is outlined in Scheme 1.9.^{106, 107} (-)-Ephedrine **54** is treated with [^{17}O]- POCl_3 (from PCl_5 and H_2^{17}O) to yield two chloro adducts **55a** and **55b** (approximately 9:1).

These chloro adducts can be separated chromatographically or the major isomer **55a** can be fractionally crystallised,¹¹⁴ but more conveniently the mixture is converted to **56a** and **56b** by reaction with (*S*)-2-*O*-benzyl-propane-1,2-diol **57**.

Alcoholysis is known to proceed with *retention* of configuration at phosphorus.¹¹⁴ Chromatographic separation gives separate phosphoramidate diester diastereomers **56a** and **56b** in yields of 65% and 7% (based on POCl_3), respectively. Acid-catalyzed hydrolytic ring-opening of purified **56a** in [^{18}O]water yields **58a** (Scheme 1.9). The acid-catalyzed ring-opening has been shown to proceed by 'in-line' displacement resulting in an *inversion* of configuration at phosphorus.^{107, 114} Hydrogenolysis of **58a** gives (*R_p*)-1-[^{16}O , ^{17}O , ^{18}O]phospho-(*S*)-propane-1,2-diol **59a** in 72% yield from **56a**. By inverting the order in which ^{17}O and ^{18}O are introduced, the opposite isomer, (*S_p*)-1-[^{16}O , ^{17}O , ^{18}O]phospho-(*S*)-propane-1,2-diol **59b**, can be synthesized. Moreover,

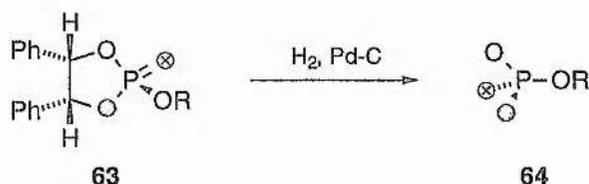
the synthetic method allows for a range of phosphate monoesters to be prepared, such as (*R_p*)-2-[¹⁶O, ¹⁷O, ¹⁸O]phospho-D-glycerate **60a**,¹¹⁵ (*R_p*)-adenosine γ-[¹⁶O, ¹⁷O, ¹⁸O]triphosphate **61a**,¹⁰³ and (*R_p*)-phenyl [¹⁶O, ¹⁷O, ¹⁸O]-phosphate **62a**¹¹⁶.



Scheme 1.9: Knowles' Stereoselective Synthesis of 1-(*R_p*)-[¹⁶O, ¹⁷O, ¹⁸O]phospho-(*S*)-propane-1,2-diol

1.26.2 Lowe's Synthesis of Chiral [^{16}O , ^{17}O , ^{18}O]Phosphate Monoesters

Lowe adopted the 2-substituted 2-oxo-4,5-diphenyl-1,3,2-dioxaphospholans **63** as the molecular framework into which the three oxygen isotopes could be incorporated, since catalytic hydrogenolysis of the benzylic oxygen bonds released the chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoester **64** without perturbing any of the phosphorus-oxygen bonds (Scheme 1.10).

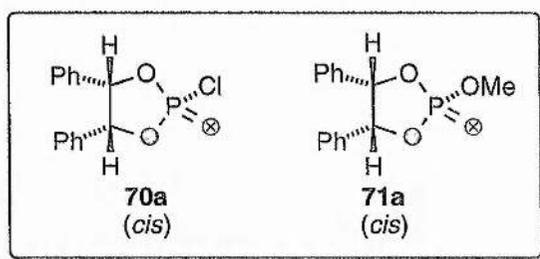
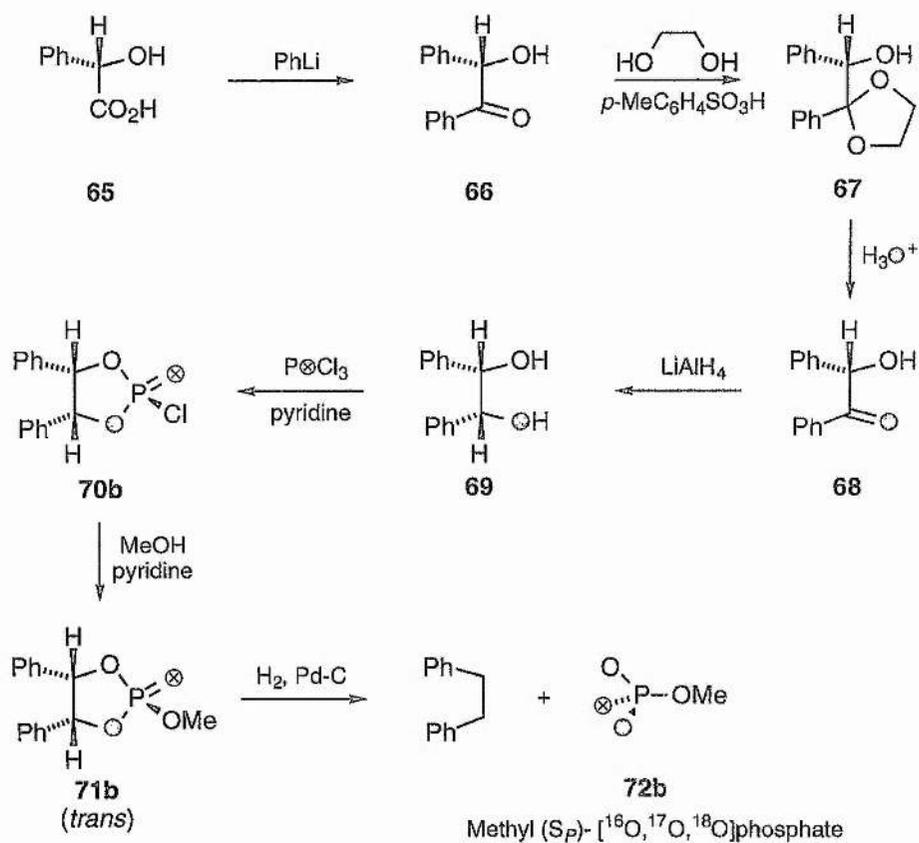


Scheme 1.10: 2-Substituted 2-oxo-4,5-diphenyl-1,3,2-dioxaphospholans undergo catalytic hydrogenolysis without perturbing any of the phosphorus-oxygen bonds

The Lowe route to chiral methyl (S_p)-[^{16}O , ^{17}O , ^{18}O]phosphate **72** is outlined in Scheme 1.11.¹⁰⁸⁻¹¹³ (S)-Benzoin **66** is synthesized by the reaction of (S)-mandelic acid **65** with phenyl lithium. Acid-catalyzed ketalization of **66** with ethylene glycol gives **67**, which is hydrolysed by [^{18}O]water under acidic conditions to give the (S_p)-[^{18}O]benzoin **68**. Introduction of the ^{18}O label by this method has been shown to proceed without loss of chirality and avoids the dilution of isotope that would accompany the acid-catalysed ^{18}O -exchange from [^{18}O]water into the carboxy-group of **65** and keto group of **66**.¹¹¹

Reduction of **68** with lithium aluminium hydride at 0 °C, or sodium borohydride is a classic example of asymmetric induction and gives exclusively *meso*-[^{18}O]hydrobenzoin **69**.¹¹⁷ Treatment of **69** with [^{17}O]- POCl_3 in pyridine gives the *trans*-dioxaphospholan **70b**. It is interesting to note when *meso*-[^{18}O]hydrobenzoin **69** reacts with POCl_3 in tetrahydrofuran with only 2 equiv. of base present, both diastereoisomers of the 2-chloro-dioxaphospholan **70a** and **70b** are formed. These observations cannot be explained by exocyclic nucleophilic substitution at phosphorus as this proceeds with retention of configuration.^{75, 118, 119} It appears that when 2 equiv. of base are present, kinetic control

leads to the predominant formation of the *cis*-diastereomer **70a**, but in the presence of an excess of pyridine, reversible ring-opening of the cyclic phosphorochloridate **70a** is possible leading to the thermodynamically more stable *trans*-diastereoisomer **70b**.^{111, 112}



Scheme 1.11: Lowe's stereoselective synthesis of methyl (S_P)-1- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]phosphate

Methanolysis of the *trans*-dioxaphospholan **70b** in pyridine gives 2- ^{17}O oxo-[1- ^{18}O]-1,3,2-dioxaphospholan **71b** as a crystalline product which on hydrogenolysis over palladium-charcoal or cleavage with sodium in liquid ammonia, gives methyl

(S_p)-[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphate **72b**. By using other alcohols to replace methanol, the route can be used as a general method of synthesis of chiral [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphate esters. (S_p)-Inorganic [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]pyrophosphate **73b**, (S_p)-glucose 6-[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphate **74b**, (S_p)-adenosine 5'-[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphate **75b**, (S_p)-glycerol-3-[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphate **76b**, (S_p)-2-[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phospho-(R)-glycerate **77b**, and (S_p)-adenosine 5'- γ -[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]triphosphate **78b** have all been synthesized by this route (Figure 1.22).¹¹¹ The alcoholysis reaction of **70a** or **70b** has been shown to proceed with retention of configuration, which is the expected result of such an exocyclic displacement at phosphorus in a five-membered cyclic phosphate derivative.⁷⁵

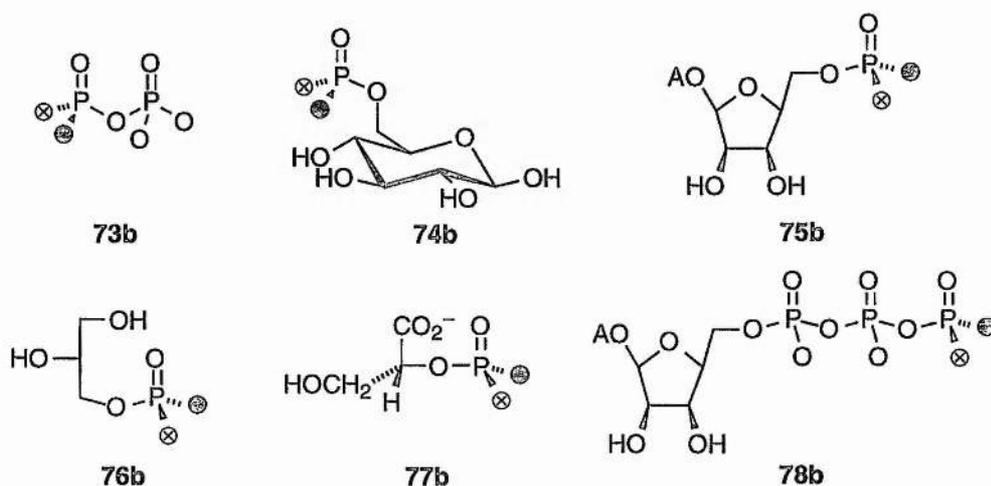


Figure 1.22: Chiral (S_p)-[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphate monoesters synthesized by the Lowe procedure

The absolute configuration of the chiral [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphate monoester can be assigned from the $^1\text{H-NMR}$ spectrum of unlabelled **70b** or **71b**.¹¹² In addition, the absolute configuration of the *trans*-2-[^{17}O]oxo-[1- ^{18}O]-1,3,2-dioxaphospholan **71b** was determined by X-ray crystallography.¹²⁰

As with the Knowles' synthetic route,^{106, 107} the isomers of opposite configuration at phosphorus can be obtained by inverting the order in which ^{17}O and ^{18}O are introduced or by using (R)-mandelic acid to synthesise the other isotopomer of *meso*-[^{18}O]benzoin **69**.

1.27 Configurational Analysis of Chiral [^{16}O , ^{17}O , ^{18}O]Phosphate Monoesters

Three methods have been developed to investigate the chirality of [^{16}O , ^{17}O , ^{18}O]phosphate monoesters, namely, chiroptical properties, mass spectrometry, and ^{31}P -NMR spectrometry.

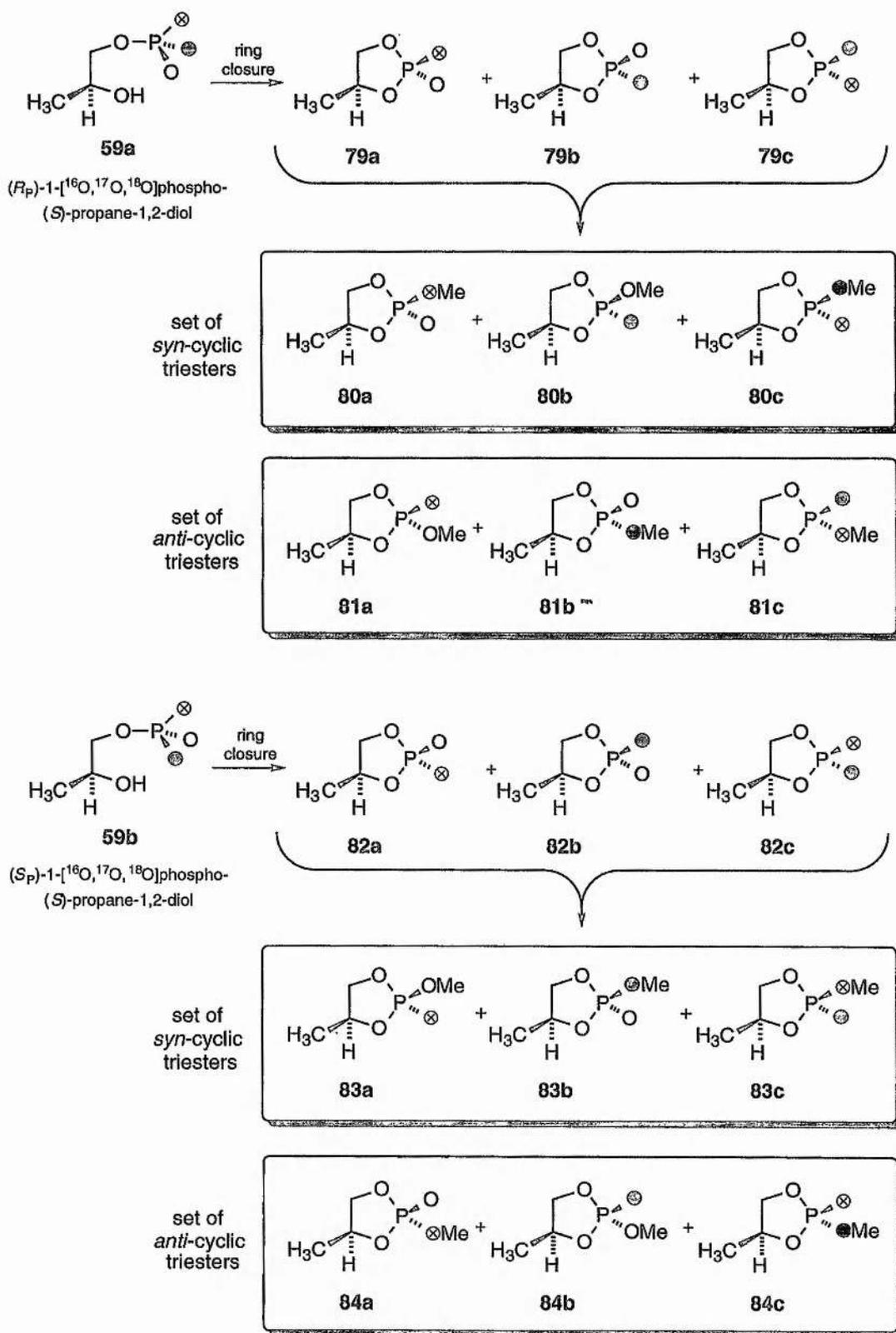
1.27.1 Circular Dichroism

Methyl (S_p)-[^{16}O , ^{17}O , ^{18}O]phosphate disodium salt **72b** has been shown to possess a measurable circular dichroic spectrum which results solely from the chiral disposition of the three isotopes.¹⁰⁸ Initially it was hoped that this technique would offer a convenient physical method for determining the configuration of chirally labelled phosphoryl groups and hence the stereochemical course of phosphoryl transfer reactions. However, the ellipticity was too small for it to be useful and the phosphate ester is required to be in a molecule which is otherwise achiral and free of chiral impurity.¹⁰⁹

1.27.2 Knowles' Configurational Analysis by Mass Spectrometry

Knowles developed a mass spectrometric analysis to determine the configuration of a chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoester.^{106, 107, 121}

When (R_p)-1-[^{16}O , ^{17}O , ^{18}O]phospho-(S)-propane-1,2-diol **59a** is treated with (diphenylphosphoryl)imidazole, the reaction proceeds with inversion of configuration to give an equimolar mixture of *three* isotopomeric cyclic diesters **79a-c**¹⁰⁷ (Scheme 1.12). In the cyclisation step, any one of the peripheral oxygen isotopes will be lost with equal probability as the kinetic isotope effects are negligible.¹⁰⁹ Cyclisation of (S_p)-1-[^{16}O , ^{17}O , ^{18}O]phospho-(S)-propane-1,2-diol **59b** gives the three epimers (at phosphorus) of the above species **82a-c** since the carbon skeleton is also chiral (at C-2) (Scheme 1.12).



Scheme 1.12: Sets of *syn*- and *anti*-cyclic triesters derived from 1-(*R*)- and 1-(*S*)-[¹⁶O,¹⁷O,¹⁸O]phospho-(*S*)-propane-1,2-diol

Methylation of the cyclic isotopomers **79a-c** occurs on either of the exocyclic oxygens to give two sets (*syn*- and *anti*-) of diastereomeric triesters **80a-c** and **81a-c** which are separated by chromatography and analysed by metastable ion mass spectrometry.^{106, 107} Related sets of diastereomeric triesters **83a-c** and **84a-c** are derived from **82a-c** (Scheme 1.12).

The mass spectra of the two *syn*-sets or two *anti*-sets resulting from 1-(*R*)- and 1-(*S*)- [¹⁶O, ¹⁷O, ¹⁸O]phospho-(*S*)-propane-1,2-diol **59a** and **59b** differ only in the disposition of the isotopic labels, and thus the mass spectra of each should be identical. However, they do differ in the relationship between individual daughter ions and their parents, and this has been measured using a linked-scan method.¹⁰⁷ Due to the technical difficulty of the mass spectrometric analysis and the susceptibility of the *syn*- and *anti*-isomers to hydrolysis, this method of configurational analysis has largely been superseded by a procedure based on ³¹P-NMR spectrometry.

1.27.3 ¹⁸O-Isotope Shift and ¹⁷O-Quadrupolar Effect in ³¹P-NMR Spectrometry

The configurational analysis of chiral [¹⁶O, ¹⁷O, ¹⁸O]phosphate monoesters by ³¹P-NMR spectrometry is based on the very different effects that ¹⁷O and ¹⁸O isotopes have on the phosphorus atom they are directly bonded to.⁹⁹

¹⁷O has a nuclear spin quantum number of $5/2$ and therefore possesses a nuclear electric quadrupole moment. When ¹⁷O is directly bonded to phosphorus, the ³¹P-nucleus will also be relaxed by virtue of its spin-spin coupling with ¹⁷O. Consequently, the ³¹P-resonance is 'broadened' and is not observed generally in the ³¹P-NMR spectrum.¹²² This is particularly true in biochemical phosphate molecules where the quadrupolar relaxation time of ¹⁷O is generally shorter due to the larger molecular size and a smaller degree of symmetry.^{99, 123, 124}

¹⁸O has a nuclear spin quantum number of zero and does not affect the relaxation time of the ³¹P-resonance. However, substitution by a heavier isotope causes the NMR signal of

the neighbouring nucleus to shift up-field. The magnitude of the shift is related to the fractional change in mass, the chemical shift range of the nucleus being observed, and the structure of the compound.^{123, 124} In the case of multiple substitution, the magnitude of shift is generally additive.¹²⁵ Typically, the magnitude of the ^{18}O shift on a ^{31}P -resonance of phosphate derivatives ranges from 0.01–0.04 ppm, and is dependent on the nature of the phosphorus to oxygen bond: the higher the bond order, the greater the isotopic shift.^{119, 125, 126} The magnitude of shift in the ^{31}P -NMR spectrum is defined as the 'S' value.⁹¹ The S-value for a P=O double bond is 0.038–0.044 ppm, whereas that for a P-O single bond is 0.015–0.025 ppm. The S-values of phosphorothioates are slightly greater than those of the corresponding phosphates.

1.27.4 Knowles' Configurational Analysis by ^{31}P -NMR Spectrometry

Knowles' method of configurational analysis of chiral [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]phosphate monoesters by ^{31}P -NMR requires the synthesis of the same sets of *syn*- and *anti*-cyclic isotopomers **80a-c** and **81a-c** as the Knowles' analysis by mass spectrometry (Section 1.27.2).^{106, 107} Each set of isomers derived from (R_p)-1- [$^{18}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]phospho-(*S*)-propane-1,2-diol consists of two species bearing a ^{17}O and a third which contains only ^{16}O and ^{18}O . Since ^{17}O 'quenches' the ^{31}P -NMR signal, only the species without ^{17}O **80b** and **81b** will show sharp ^{31}P -NMR signals.^{95, 127}

In the *syn*-isomer **80b**, the ^{18}O is non-bridging (P= ^{18}O), thus causes a larger isotope shift ($S = 0.043$ ppm). In the *anti*-isomer **81b**, the ^{18}O is located at the P-O-C bridging position, thus causing a smaller isotope shift ($S = 0.018$ ppm). An opposite pattern is observed for the *syn*- isomer **83b** and *anti*-isomer **84b** derived from (S_p)-1- [$^{18}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]phospho-(*S*)-propane-1,2-diol. In addition, the two diastereoisomers **80** and **81** show different chemical shifts which allows a direct analysis of the mixture without the need for chromatographic separation (Scheme 1.12).^{91, 127}

This analysis is based on the assumption that all the labelled sites are fully enriched. In practice, the sites labelled as ^{18}O will be about 99 atom % ^{18}O , but since ^{17}O is currently

available at only about 50 atom % enrichment, species will also be present in which sites labelled as ^{17}O in Scheme 1.12 will be ^{16}O and ^{18}O . This means that four *syn*- and four *anti*-triesters will be observed in each ^{31}P -NMR spectrum, namely $[\text{}^{16}\text{O}_2]$, $[\text{}^{16}\text{O}_{\text{syn}}, \text{}^{18}\text{O}_{\text{anti}}]$, $[\text{}^{18}\text{O}_{\text{syn}}, \text{}^{16}\text{O}_{\text{anti}}]$, and $[\text{}^{18}\text{O}_2]$. This, however, is an advantage since the $[\text{}^{16}\text{O}_2]$ - and $[\text{}^{18}\text{O}_2]$ -triesters provide the necessary internal reference signals for determining the isotopic shifts, and the ratio of the $[\text{}^{16}\text{O}_{\text{syn}}, \text{}^{18}\text{O}_{\text{anti}}]$ - and $[\text{}^{18}\text{O}_{\text{syn}}, \text{}^{16}\text{O}_{\text{anti}}]$ -triesters allow the stereochemistry of the chiral $[\text{}^{16}\text{O}, \text{}^{17}\text{O}, \text{}^{18}\text{O}]$ phosphate monoester to be determined.¹⁰⁹ Thus, **80b** and **81b** or **83b** and **84b** will predominate in the ^{31}P -NMR spectrum but will not be the exclusive resonances.

1.27.4.1 Determination of Stereochemical Course of Phosphoryl Group Transfer Using Knowles' Configurational Analysis Procedure

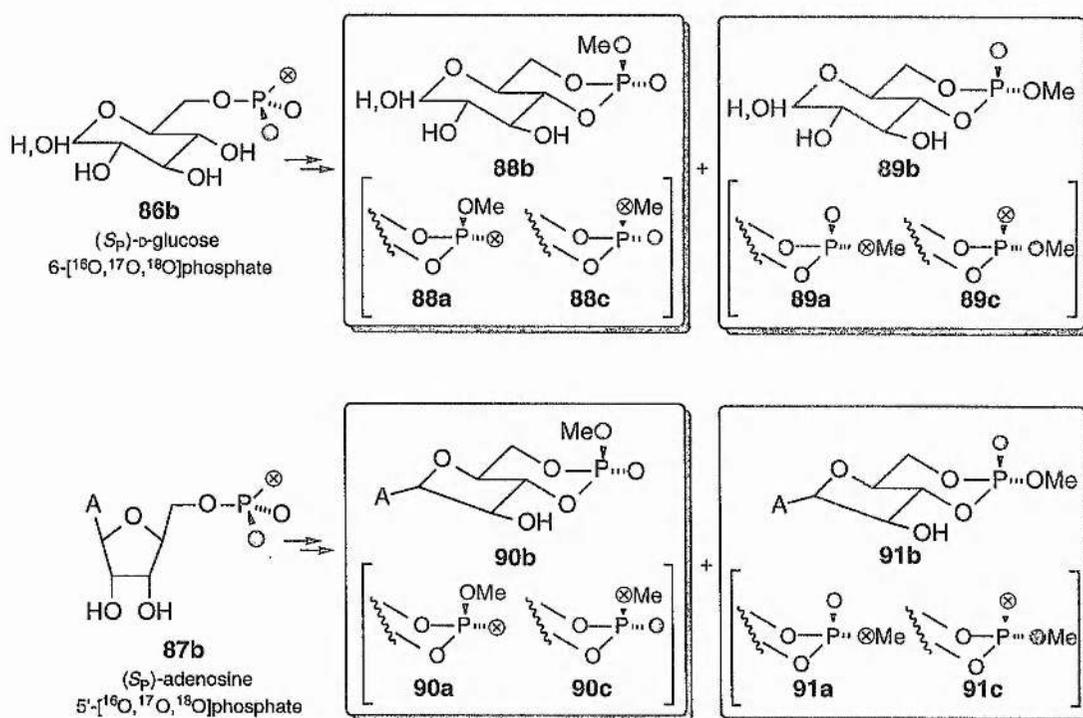
To determine the stereochemical course of any enzyme catalysing $[\text{}^{16}\text{O}, \text{}^{17}\text{O}, \text{}^{18}\text{O}]$ phosphoryl transfer using the Knowles' procedure, the chiral $[\text{}^{16}\text{O}, \text{}^{17}\text{O}, \text{}^{18}\text{O}]$ phosphoryl group in the product must be transferred to (*S*)-propane-1,2-diol.^{95, 106, 107, 121, 127} This is achieved using alkaline phosphatase, a non-specific phosphatase which transfers phosphoryl groups to alcohols as well as with water. The reaction has been shown to proceed with *retention* of configuration at phosphorus.^{116, 128}

This analysis must, however, be carried out on a fairly large scale as much of the chiral $[\text{}^{16}\text{O}, \text{}^{17}\text{O}, \text{}^{18}\text{O}]$ phosphoryl group is transferred to water (up to 80 %) by alkaline phosphatase and that which is transferred to (*S*)-propane-1,2-diol comprises a mixture of 1- and 2- $[\text{}^{16}\text{O}, \text{}^{17}\text{O}, \text{}^{18}\text{O}]$ phosphopropane-1,2-diol **59** and **85** that must be separated. Using this method, the stereochemical course of phosphoryl transfer enzymes such as creatine kinase,¹²⁹ glycerol kinase,^{102, 103, 130} hexokinase,^{102, 103, 130} pyruvate kinase^{102, 103, 130} and acetate kinase¹⁰² have been determined. All proceed with *inversion* of configuration at the phosphorus centre and in all cases, there was no evidence for the involvement of a phosphorylated enzyme intermediate. Results which have a strong implication that *inversion* is the preferred stereochemical course of enzyme-catalyzed

phosphoryl-group transfer, implying the involvement of an in-line association mechanism.

1.27.5 Lowe's Configurational Analysis Procedure by ^{31}P -NMR Spectrometry

Lowe's procedure for configurational analysis also depends on the stereospecific cyclisation of the chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoester of a chiral diol into a cyclic diester, which is then esterified (without perturbing the P-O bond) to give diastereomeric cyclic phosphate triesters which are analysed by ^{31}P -NMR spectrometry (Scheme 1.13).¹¹³



Scheme 1.13: Lowe's configurational analysis

In this case, D-glucose 6-[^{16}O , ^{17}O , ^{18}O]phosphate **86** or adenosine 5'-[^{16}O , ^{17}O , ^{18}O]phosphate **87** is cyclised with diphenyl phosphorochloridate and alkylated with methyl iodide.^{109, 110, 131-133} The diastereomeric six-membered cyclic phosphate triesters **88a-c** and **89a-c** or **90a-c** and **91a-c** are formed with *inversion* of configuration at

phosphorus.^{109, 112, 134} Their chemical shifts are well separated in the ³¹P-NMR spectrum, avoiding the need for physical separation. Scheme 1.13 shows this procedure for (*S_p*)-D-glucose 6-[¹⁶O,¹⁷O,¹⁸O]phosphate **86b** and (*S_p*)-adenosine 5'-[¹⁶O,¹⁷O,¹⁸O]phosphate **87b** (the non-¹⁷O containing, ³¹P-NMR active species are **88b** and **89b** or **90b** and **91b**).

1.27.5.1 Stereochemical Course of Phosphoryl Transfer Reactions using Lowe's Configurational Analysis Procedure

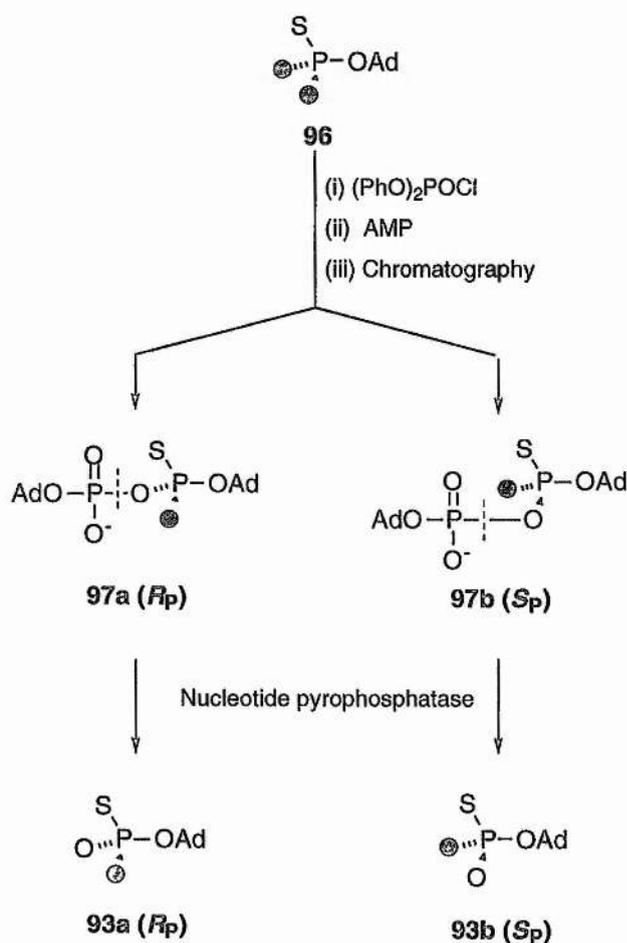
By synthesizing (*S_p*)-adenosine [γ -¹⁶O,¹⁷O,¹⁸O]triphosphate **78b**,¹¹¹ Lowe has been able to determine the stereochemical course of phosphoryl transfer catalyzed by hexokinase,¹³⁵ glucokinase,¹³⁶ and polynucleotide kinase,¹³⁷ and phenylalanyl-tRNA synthetase¹³⁸ by the ³¹P-NMR analysis of the glucose 6-[¹⁶O,¹⁷O,¹⁸O]phosphate **86** or adenosine 5'-[¹⁶O,¹⁷O,¹⁸O]phosphate **87** formed by the enzyme reaction. Extending this method, the stereochemical course of many phosphokinases has been investigated such as pyruvate kinase¹³⁹ and phosphofructose kinase,¹⁴⁰ as the common substrate for such enzymes is adenosine triphosphate. These too have been shown to proceed with *inversion* of configuration.

1.28 Chiral [¹⁸O]Phosphorothioate Monoesters

Enzymes which catalyse the hydrolysis of a phosphate monoester pose particular difficulties when it comes to determining the stereochemical course of the reaction. If the phosphomonoesterase also catalyses transphosphorylation to a substrate other than water, its stereochemical course can be elucidated by the methods previously described (Section 1.27.4.1 and 1.27.5.1). Many phosphomonoesterases including inositol monophosphatase, do not catalyse such transphosphorylation reactions. In this situation, the common approach to determining the stereochemical course of the enzyme is to synthesize the chiral (*R_p*)- and (*S_p*)-[¹⁸O]phosphorothioate analogues of the substrate **92a** and **92b**. Hydrolysis of these two substrates in ¹⁷O-water gives chiral (*R_p*)- or (*S_p*)-inorganic [¹⁶O,¹⁷O,¹⁸O]phosphorothioate (*P_{si}*) **53a** or **53b** (Scheme 1.14). The

1.29.1 (R_p)- and (S_p)-[$\alpha^{18}\text{O}$]AMPS

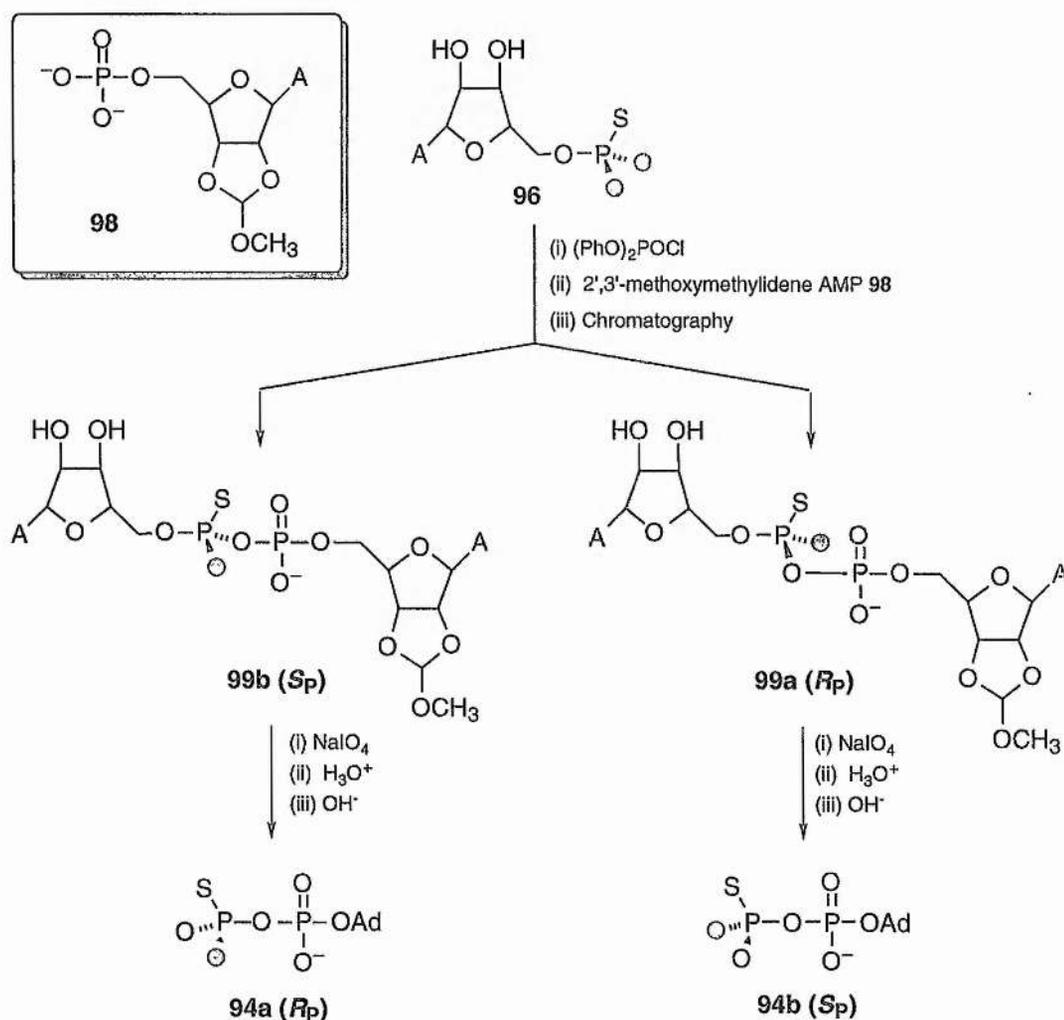
[$\alpha^{18}\text{O}_2$]AMPS **96**, prepared from adenosine, PSCl_3 , and [^{18}O]water,¹⁴³ is activated with diphenylphosphorochloridate and coupled to AMP (Scheme 1.15).¹⁴⁴ This is an example of the Michelson phosphoanhydride procedure.^{72, 145} The epimeric mixture of (R_p)- and (S_p)- P^1, P^2 -diadenosyl 1-thio[$1\text{-}^{18}\text{O}$]pyrophosphates **97a** and **97b** products are separated by chromatography. Hydrolysis of **97a** and **97b** by *C. adamanteus* nucleotide pyrophosphatase produces respectively (R_p)- and (S_p)-[^{18}O]AMP **93a** and **93b**.¹⁴⁴



Scheme 1.15: Synthesis of (R_p) and (S_p) [^{18}O]AMPS

1.29.2 (R_p)- and (S_p)-[$\beta^{18}\text{O}$]ADP βS

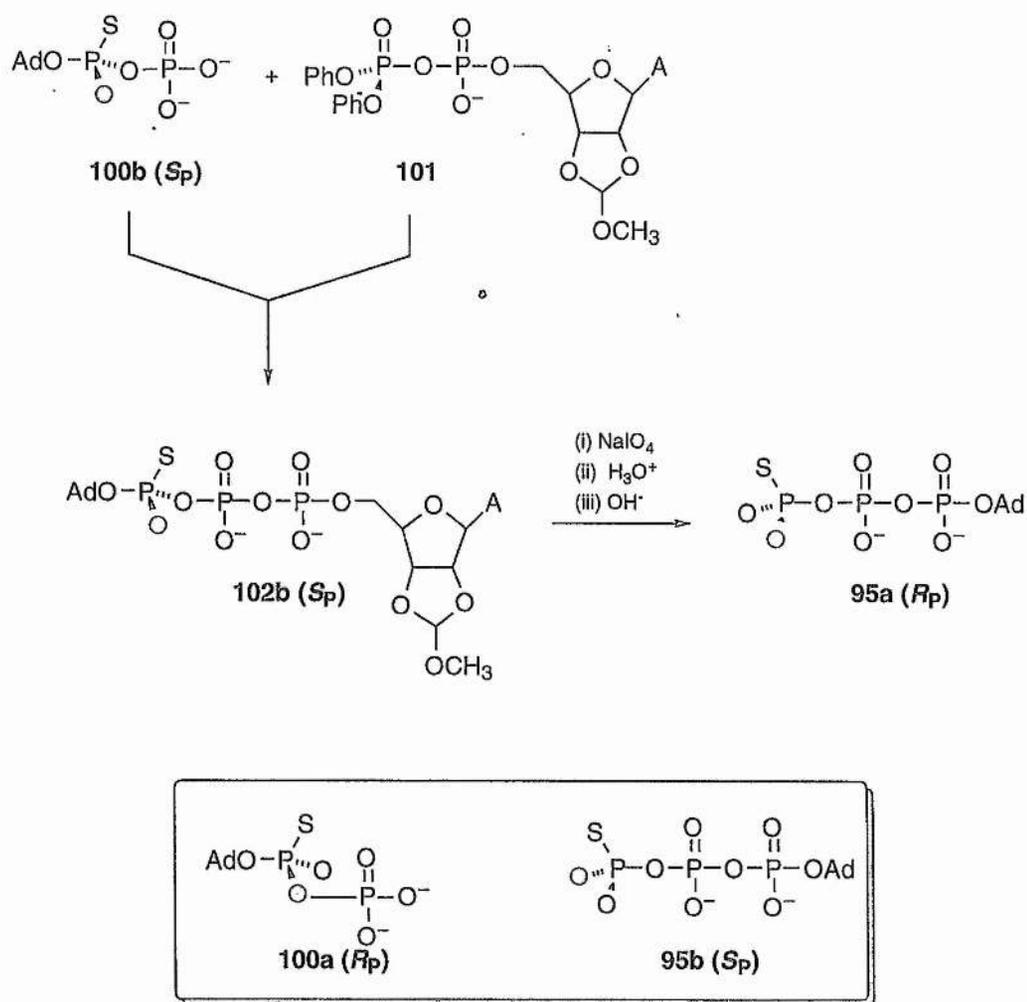
Scheme 1.16 shows the synthetic route to (R_p)- and (S_p)-[$\beta^{18}\text{O}$]ADP βS **94a** and **94b** devised by Richard *et al.*¹⁴⁶ Condensation of [$\alpha^{18}\text{O}_2$]AMPS **96** with 2',3'-methoxymethylidene-AMP **98** by the Michelson procedure¹⁴⁵ gives an epimeric mixture of (R_p)- and (S_p)- P^1 -adenosyl- P^2 -methoxymethylidene adenosyl-1-thio[1- ^{18}O]diphosphates **99a** and **99b**. The separated epimers are then converted into (R_p)- and (S_p)-[$\beta^{18}\text{O}$]ADP βS **94a** and **94b** by periodate cleavage of the unprotected ribosyl ring,¹⁴⁷ acidic hydrolysis of the methoxymethylidene protecting group and alkaline β -elimination of the cleaved adenosyl fragment.¹⁴⁶



Scheme 1.16: Synthesis of (R_p)- and (S_p)-[$\beta^{18}\text{O}$]ADP βS

1.29.3 (R_p)- and (S_p)-[$\gamma^{18}\text{O}$]ATP γS

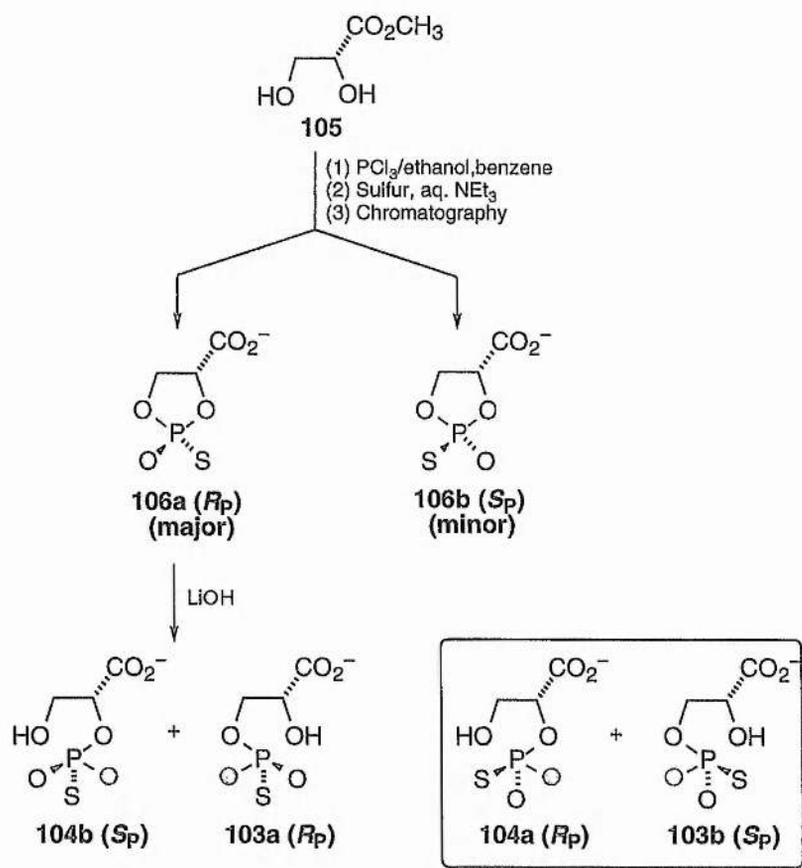
Scheme 1.17 shows the synthesis of (R_p)- and (S_p)-[$\gamma^{18}\text{O}$]ATP γS **95a** and **95b**.^{141, 148} (S_p)-[$\alpha^{18}\text{O}$]ADP αS **100b** is condensed with activated methoxymethylidene-AMP **101** producing (S_p)- P^1 -adenosyl- P^3 -methoxymethylidene adenosyl-1-thio[1- ^{18}O]tripolyphosphates **102b**.¹⁴¹ Periodate cleavage and work up gives (R_p)-[$\gamma^{18}\text{O}$]ATP γS **95a**. The other isotopomer, (S_p)-[$\gamma^{18}\text{O}$]ATP γS **95b**, is synthesised from (R_p)-[$\alpha^{18}\text{O}$]ADP αS **100a**.



Scheme 1.17: Synthesis of (R_p)- and (S_p)-[$\gamma^{18}\text{O}$]ATP γS

1.29.4 D-Glycerate [^{18}O]phosphorothioates

The 2- and 3- ^{18}O]phosphorothioate esters of D-glycerate **103** and **104** are prepared as shown in Scheme 1.18. Methyl-D-glycerate **105** is converted to a diastereomeric mixture of the (R_p)- and (S_p)-cyclic 2,3-phosphorothioates of D-glycerate **106a** and **106b** which are separated by chromatography. The major isomer **106a** is then reacted with lithium [^{18}O]hydroxide to produce a mixture of ($2R,R_p$)-glycerate-3- ^{18}O]phosphorothioate **103a** and ($2R,S_p$)-glycerate-2- ^{18}O]phosphorothioate **104b**.¹³⁰



Scheme 1.18: Synthesis of 2- and 3- ^{18}O]phosphorothioate esters of D-glycerate

The major diastereomer of the cyclic 2,3-phosphorothioates of D-glycerate produced in this synthesis has been shown by X-ray crystallography to be the syn (R_p) isomer.¹⁰⁴ Consequently, the configurations at phosphorus of the glycerate [^{18}O]phosphorothioates

has been determined by the fact that cleavage of 5-membered cyclic phosphates by hydroxide proceeds by an in-line mechanism with inversion of configuration at phosphorus.^{75, 130, 149} Using these compounds, the stereochemical course of thiophosphoryl-group transfer catalyzed by glycerokinase, pyruvate kinase, and hexokinase have been shown to proceed with inversion of configuration at phosphorus.^{104, 130}

The minor (S_p)-cyclic 2,3-phosphorothioate **106b** is used to synthesise ($2R,S_p$)-glycerate-3- ^{18}O]phosphorothioate **103b** and ($2R,R_p$)-glycerate-2- ^{18}O]phosphorothioate **104a**.

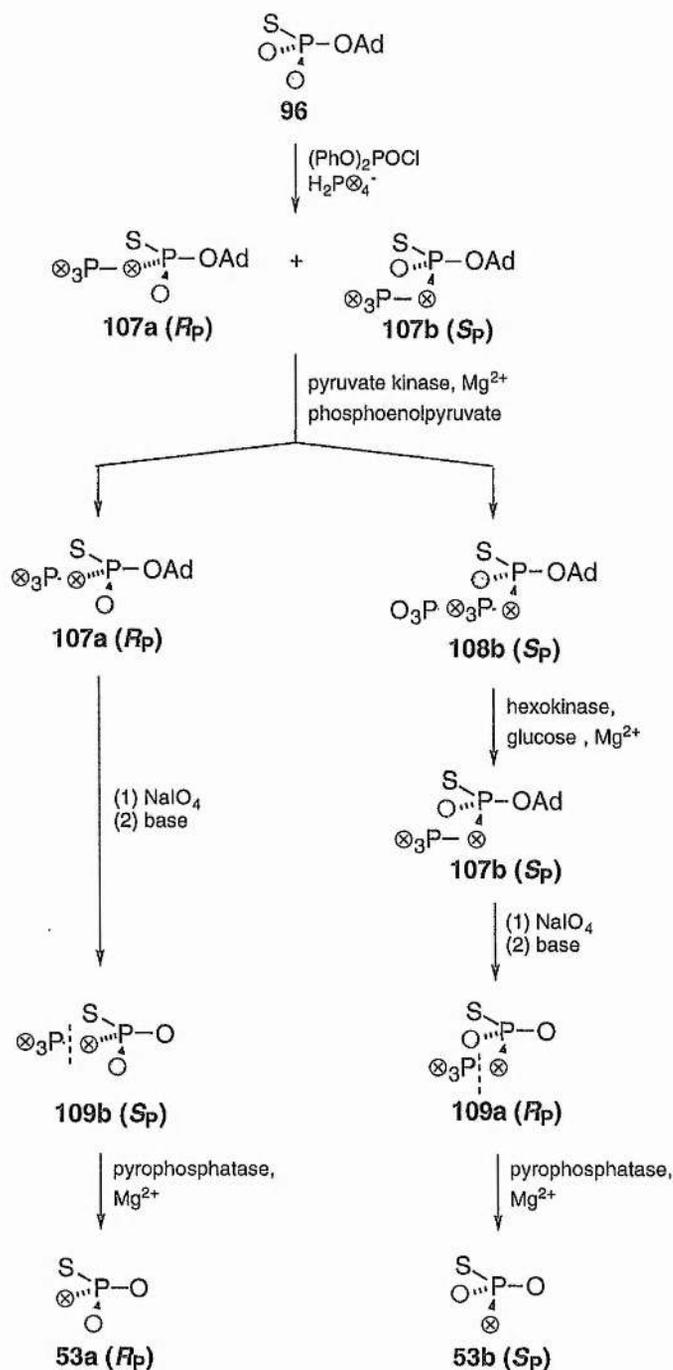
A convenient synthesis of (R_p)- and (S_p)- $[\gamma^{18}\text{O}]$ ATP γ S **95a** and **95b** involves the conversion of D-glycerate 2- ^{18}O]phosphorothioate **104** by the action of enolase to enolpyruvate 2- ^{18}O]phosphorothioate, which, in the presence of pyruvate kinase, transfers the ^{18}O]thiophosphoryl group to ADP with inversion of configuration to produce $[\gamma^{18}\text{O}]$ ATP γ S **95**.^{130, 141}

1.29.5 Chiral Inorganic $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]phosphorothioate

The synthesis of chiral inorganic (R_p)- and (S_p)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]phosphorothioate **53a** and **53b** of known configuration was first reported by Webb and Trentham (Scheme 1.19).¹⁵⁰ Activation of $^{18}\text{O}_2$]AMPS **96** with diphenylphosphorochloridate followed by coupling with inorganic $^{17}\text{O}_4$]phosphate gives a mixture of ^{17}O and ^{18}O enriched diastereomers of ADP α S **107a** and **107b**. Pyruvate kinase catalyzed phosphorylation of this mixture is specific for the (S_p)-diastereomer **107b** and the resulting labelled (S_p)ATP α S **108b** is readily separated by chromatography from the unreacted (R_p)-diastereomer **107a**. Hexokinase is used to dephosphorylate **108b** and regenerate (S_p)ADP α S **107b**.

The separated isomers **107a** and **107b** are degraded to the corresponding isomers of $^{17}\text{O}, ^{18}\text{O}$ -enriched pyrophosphorothioates **109a** and **109b** by periodate-cleavage and alkaline elimination. Retention of configuration was observed at the thiophosphoryl phosphorus.¹⁴⁶

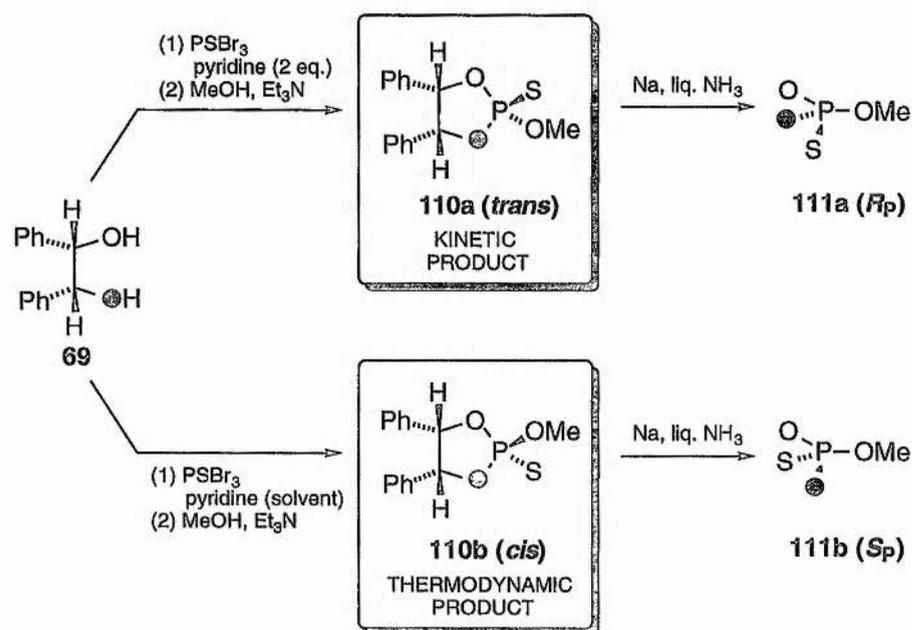
Yeast inorganic pyrophosphatase is then used to convert **109a** and **109b**, respectively, to chiral inorganic (S_P)- and (R_P)-[^{16}O , ^{17}O , ^{18}O]phosphorothioate (**53b** and **53a**). It has been shown that the oxygen atoms bonded to the thiophosphoryl phosphorus remain bonded to it.¹⁵⁰



Scheme 1.19: Combined chemical and enzymatic approach to the synthesis of chiral inorganic (R_P)- and (S_P)-[^{16}O , ^{17}O , ^{18}O]phosphorothioate

1.29.6 Lowe's General Synthesis of Chiral [^{18}O]Phosphorothioate Monoesters

The chiral [^{18}O]phosphorothioate monoesters described in this section were synthesized by a combination of chemical and enzymic reactions; the method however, was not applicable to the synthesis of [^{18}O]phosphorothioate monoesters in general. The only general route allowing the synthesis of chiral [^{18}O]phosphorothioate monoesters of known absolute configuration was developed by Lowe.¹¹⁹ The route is an adaption of the synthetic route to chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoesters previously described (Section 1.26.2).



Scheme 1.20: Lowe's general synthetic route to chiral [^{18}O]phosphorothioate monoesters

meso-[^{18}O]Hydrobenzoin **69** was converted into the diastereomeric phosphorothioate triesters **110a** and **110b** (Scheme 1.20). When two equivalents of pyridine were used, the predominant product after methanolysis was the *trans* diastereoisomer **110a**. However, when pyridine was used as a solvent, the predominant product was *cis* diastereoisomer **110b** after methanolysis. Again, it is likely that in the presence of an excess of pyridine, reversible ring opening of the cyclic phosphorobromidate is possible,

allowing the kinetically favoured product to be transformed to the thermodynamically more stable one.¹¹⁹

Reductive cleavage of the triesters with sodium in liquid ammonia gives the chiral (R_p)- and (S_p)-methyl[^{18}O]phosphorothioates **111a** and **111b**. The structure of *trans*-2-methoxy-4,5-diphenyl-1,3,2-dioxaphospholan **110a** has been established by X-ray crystallography, allowing the configuration of **111a** and **111b** to be determined. The stereochemistry at phosphorus of other [^{18}O]phosphorothioate monoesters synthesized by this method are determined by the relative ^{31}P -NMR chemical shifts of their triesters and the coupling constant between the phosphorus atom and the ring protons. Both [^{18}O]AMPS¹¹⁹ and inorganic [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphorothioate **53**¹⁵¹ have been synthesized by this method.

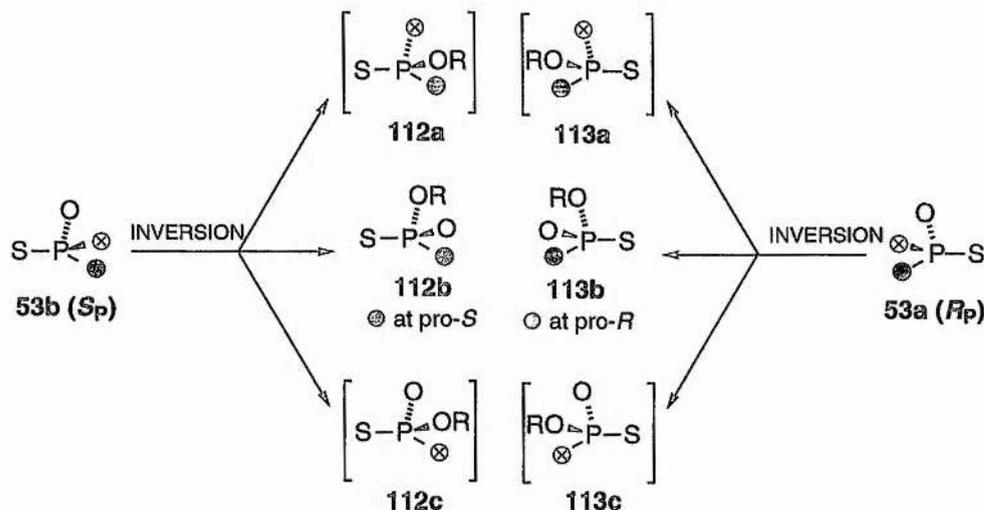
1.30 Configurational Analysis of Chiral Inorganic [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]Phosphorothioate

Two general methods are available for the configurational analysis of chiral inorganic [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]phosphorothioate, both involving the stereospecific derivatisation of the chiral P_{si} ; the first by an enzymatic approach¹⁵⁰, the second using a purely chemical protocol.^{151, 152}

1.30.1 Enzymatic Approach

Scheme 1.21 illustrates the rationale of configurational analysis for chiral P_{si} . Displacement of one of the three oxygen isotopes of (S_p)-[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$] P_{si} **53b** by a nucleophile (RO^-) gives a mixture of three inseparable, isotopically different species **112a-c**. Among them, two **112a** and **112c** (in square brackets in Scheme 1.21) contain an [^{17}O] isotope, which quenches the ^{31}P -NMR signal. The species which contains only ^{16}O and ^{18}O **112b** (^{18}O at the pro- S position) gives a sharp, unquenched ^{31}P -NMR signal. Analogously, the (R_p)-[$^{16}\text{O},^{17}\text{O},^{18}\text{O}$] P_{si} **53a** gives a corresponding non- ^{17}O containing species **113b** with ^{18}O at the pro- R position. The pro- R and pro- S positions can be distinguished by stereospecific derivatisation at one of the two positions. The $^{31}\text{P}(^{18}\text{O})$

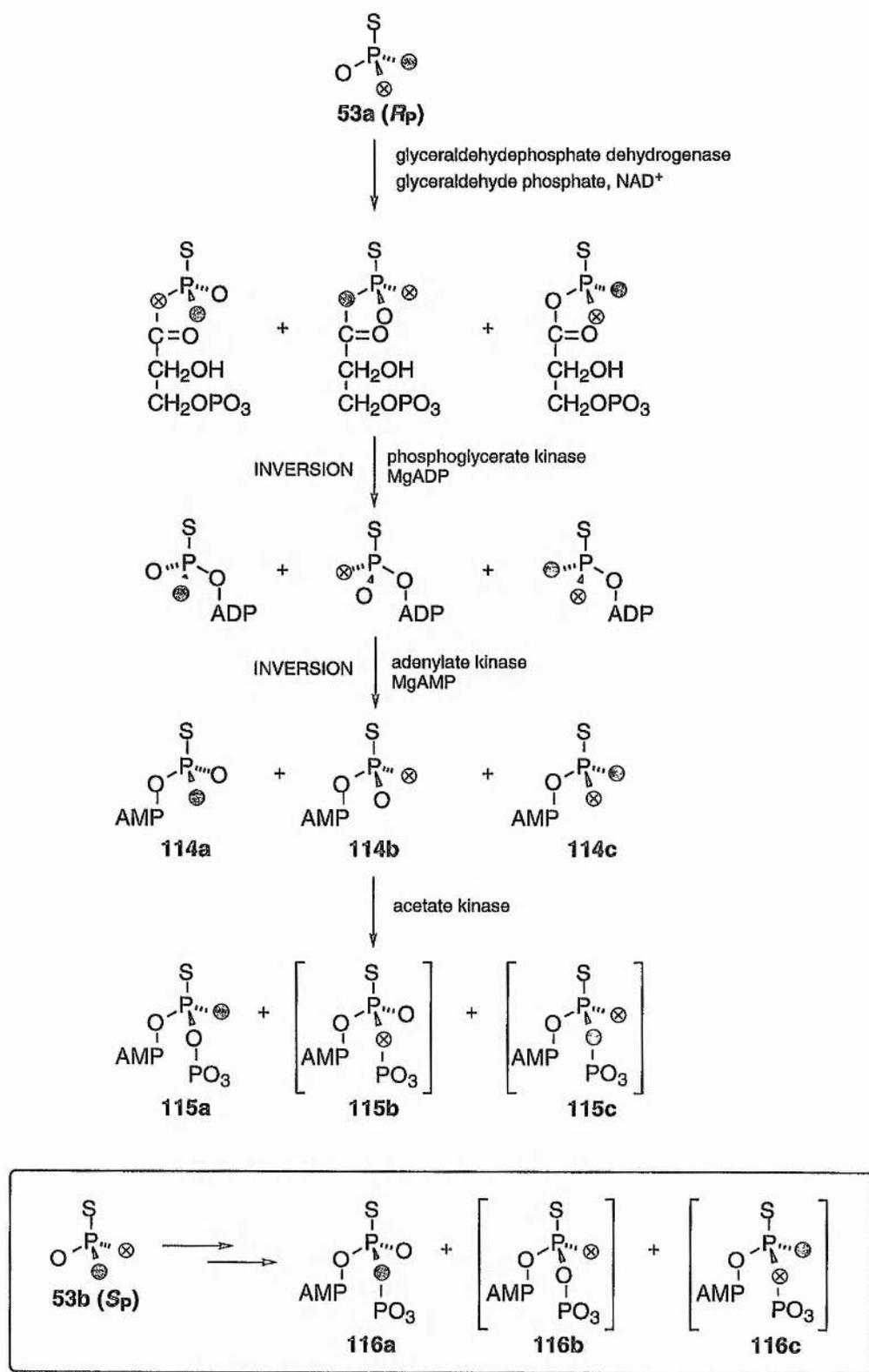
NMR method can then be employed to distinguish the bridging and non-bridging ^{18}O on the basis of the different magnitude of isotope shifts.



Scheme 1.21: Rationale of the configurational analysis for chiral inorganic $[^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]$ phosphorothioate

This process is achieved enzymatically by the actions of glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase (myokinase) which, with the required coenzymes and substrates, first incorporate $[^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]P_{si}$ into the β -position of ADP β S to give three isotopomers 114a-c (Scheme 1.22). The configuration at phosphorus is retained in this process as a result of the inversion in both the adenylate kinase and phosphoglycerate kinase reactions. The three isotopomers of ADP β S are then *stereospecifically* phosphorylated at the pro- R position by the action of acetate kinase to give three isotopomers of ATP β S 115a-c (Scheme 1.22).¹⁵³ Phosphoglycerate kinase can be used in place of acetate kinase, bringing about phosphorylation at the pro- R position of ADP β S and giving rise to a set of epimers.¹⁵⁰

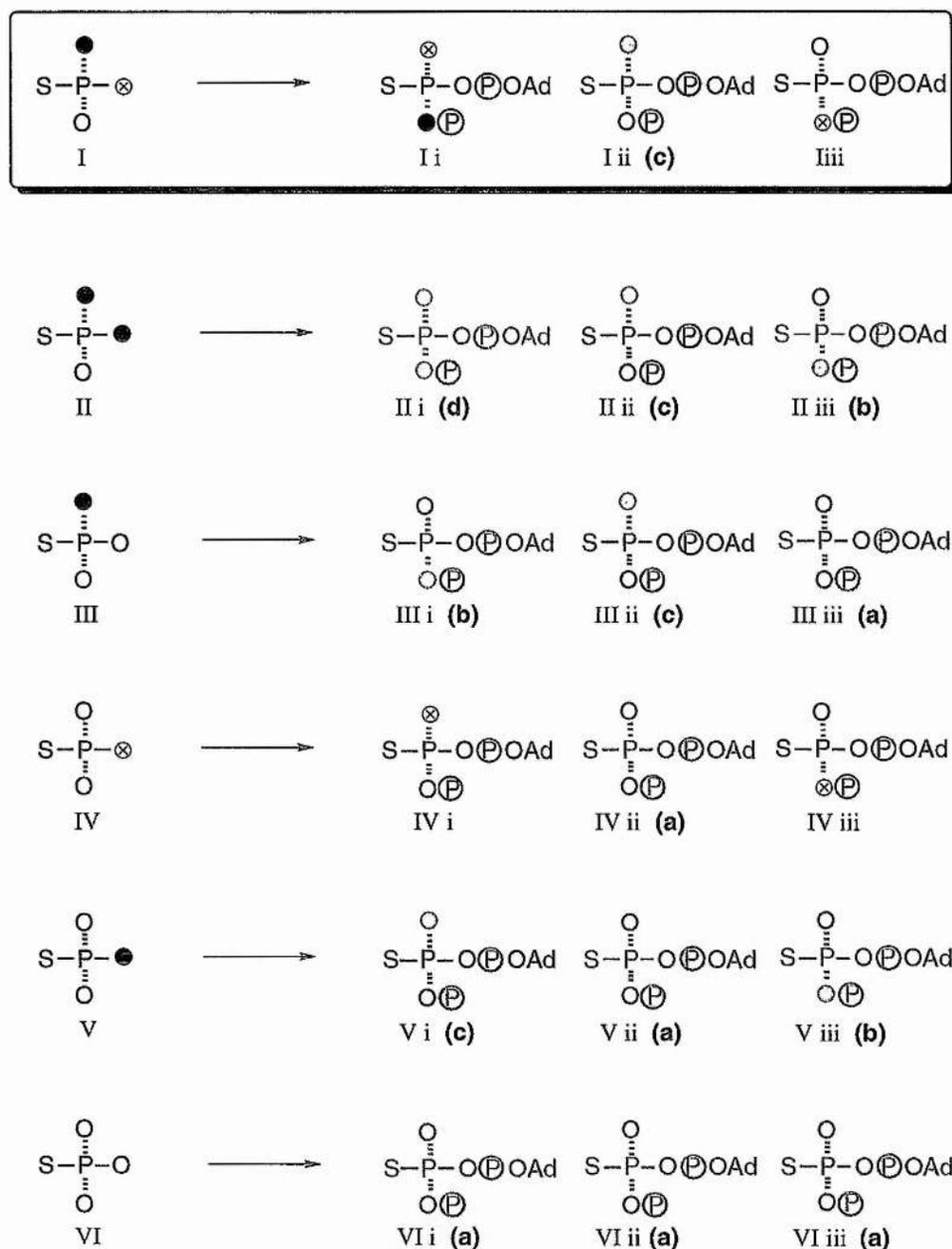
According to Scheme 1.22, (R_p)- $[^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]P_{si}$ 53a gives three species of ATP β S 115a-c, of these only $[^{18}\text{O}]$ ATP β S 115a with ^{18}O located specifically at the β -non-bridging position would give a visible signal in the ^{31}P -NMR spectrum. The (S_p)



Scheme 1.22: Stereospecific enzymatic incorporation of chiral inorganic (R_p)- and (S_p)- $[^{16}O, ^{17}O, ^{18}O]$ phosphorothioate **53a** and **53b** into ATP β S **115** and **116**

enantiomer would also give one specie of ATP β S visible in the ^{31}P -NMR spectrum, namely $[\beta^{18}\text{O}]\text{ATP}\beta\text{S}$ 116a which contains the ^{18}O label at the $\beta\gamma$ -bridging position. Configuration can thus be determined by examining the degree of isotopic shift of the β -phosphorus NMR signal caused by ^{18}O . Scheme 1.22 only shows the species that will form if the chiral P_{si} used has 100% isotopic purity but in practice this is impossible. Consequently, the position enriched with ^{18}O may also contain some ^{16}O , whereas the position enriched with ^{17}O may also contain some ^{18}O and some ^{16}O since ^{17}O -enriched water also contains ^{18}O . A chiral P_{si} sample therefore contains up to six isotopic species I–VI, as shown in the left column of Scheme 1.23; two of them are identical species, and each gives three ATP β S species i, ii, and iii. Fortunately, there are only four different non- ^{17}O containing species: non-labelled ATP β S (a) [red], $[\beta\gamma^{18}\text{O}]\text{ATP}\beta\text{S}$ (b) [green], $[\beta^{18}\text{O}]\text{ATP}\beta\text{S}$, (c) [blue] and $[\beta,\beta\gamma^{18}\text{O}]\text{ATP}\beta\text{S}$ (d) [magenta], and that all the non-chirally labelled P_{si} species (II–VI) contribute *equally* to species b and c. Only the chiral $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]P_{\text{si}}$ species gives specifically b or c, depending on whether the configuration is (S_{p}) or (R_{p}) respectively. The amounts of species a and d have to do with the isotopic enrichments, not configuration.^{99, 150}

The ratio of the peak heights b/c is defined as the 'F value'.¹⁵³ A (R_{p})- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]P_{\text{si}}$ sample should give $F < 1$. On the otherhand, a sample of the opposite enantiomer should contribute to b instead of c and give $F > 1$.



Scheme 1.23: Isotopically labelled ATP β S species formed in the enzymatic derivatisation of inorganic (R_p)-[^{16}O , ^{17}O , ^{18}O]phosphorothioate 53a

Figure 1.23 shows the β -phosphorus NMR signal of the ATP β S obtained from (R_p)- and (S_p)-[^{16}O , ^{17}O , ^{18}O]P $_{\text{si}}$ enantiomers on derivatisation *via* the acetate kinase route. The signal contains two overlapping doublets due to ^{31}P - ^{31}P coupling. Each half doublet

contains four lines due to the four species **a**, **b**, **c**, and **d**. The ratio of **b/c** deriving from **53a** and **53b** are clearly visible.

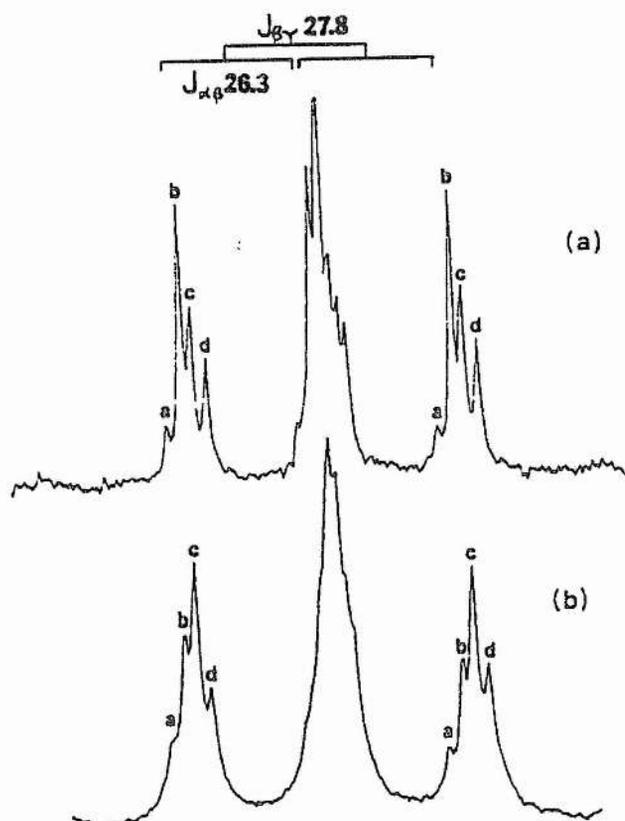
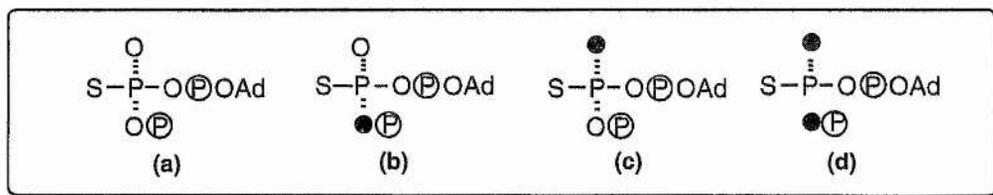


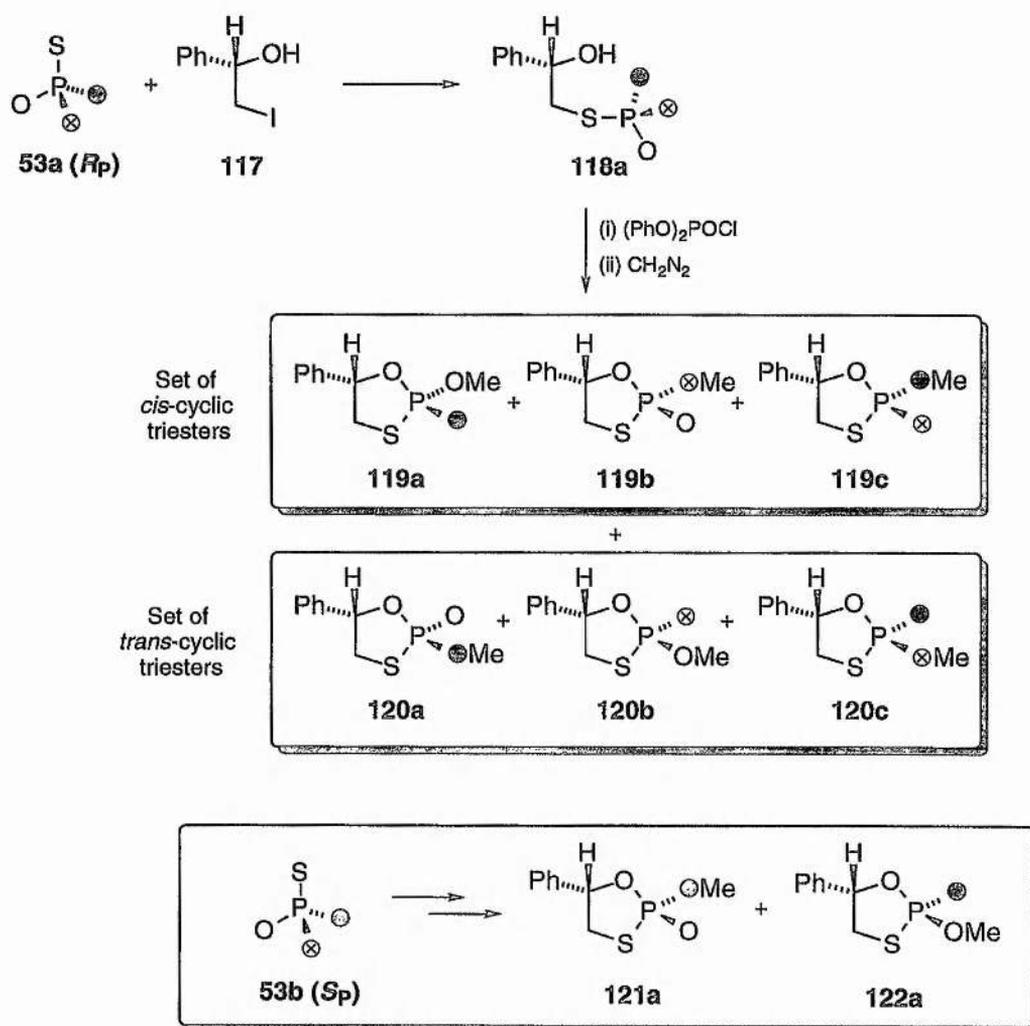
Figure 1.23: ^{31}P NMR spectra of the β -phosphorus atom of: (a) $\text{ATP}\beta\text{S}$ derived from $(S_p)\text{-}[^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{P}_{si}$ **53b**, and (b) $\text{ATP}\beta\text{S}$ derived from $(R_p)\text{-}[^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{P}_{si}$ **53a**

These methods have been applied to the stereochemical analysis of ATPases (myosin,¹⁵⁴ sarcoplasmic reticulum ATPase,¹⁵⁵ and beef heart mitochondrial ATPase¹⁵⁶), venom 5'-nucleotidase,¹⁵³ adenylosuccinate synthetase¹⁵⁷, and purple acid phosphatase.¹⁵⁸ Although this method of stereochemical analysis has general application, the enzymatic

incorporation of inorganic [^{16}O , ^{17}O , ^{18}O]phosphorothioate **53** into ATP βS **116/115** can suffer from the substantial loss of isotope and some racemization. This is thought to be due to the chemical instability of glycerate 1- ^{16}O , ^{17}O , ^{18}O]phosphorothioate 3-phosphate, an intermediate in the reaction. Consequently, an alternative method was developed by Lowe based on a purely chemical approach.^{151, 152}

1.30.2 Chemical Approach

The chemistry developed by Lowe for the stereochemical analysis of chiral inorganic (R_p)- ^{16}O , ^{17}O , ^{18}O]phosphorothioate **53a** is outlined in Scheme 1.24.



Scheme 1.24: Lowe's non-enzymatic configurational analysis of chiral inorganic (R_p)- and (S_p)- ^{16}O , ^{17}O , ^{18}O]phosphorothioate

Alkylation of **53a** by (*S*)-2-iodo-1-phenylethanol **117** yields (*R_p*)-(*S*)-1-phenylethanol-2-*S*-[¹⁶O, ¹⁷O, ¹⁸O]phosphonothioate **118a**, which is then cyclised with diphenylphosphorochloridate. This second step proceeds with loss of one peripheral oxygen to produce a mixture of cyclic phosphorothioate isotopomers which are methylated with diazomethane. The resulting mixture is of *cis*- and *trans*-triesters **119a-c** and **120a-c** which are epimeric at phosphorus and exhibit ³¹P-NMR chemical shifts differing by 2ppm.¹⁵¹

In a similar way to the procedure already described in Section 1.27.4, the ¹⁷O and ¹⁸O effects on the ³¹P-NMR signal of the components of the *cis*- and *trans*- isotopomers allow the original configuration of the incorporated [¹⁶O, ¹⁷O, ¹⁸O]P_{si} to be determined.

CHAPTER TWO

**RESULTS
AND
DISCUSSION**

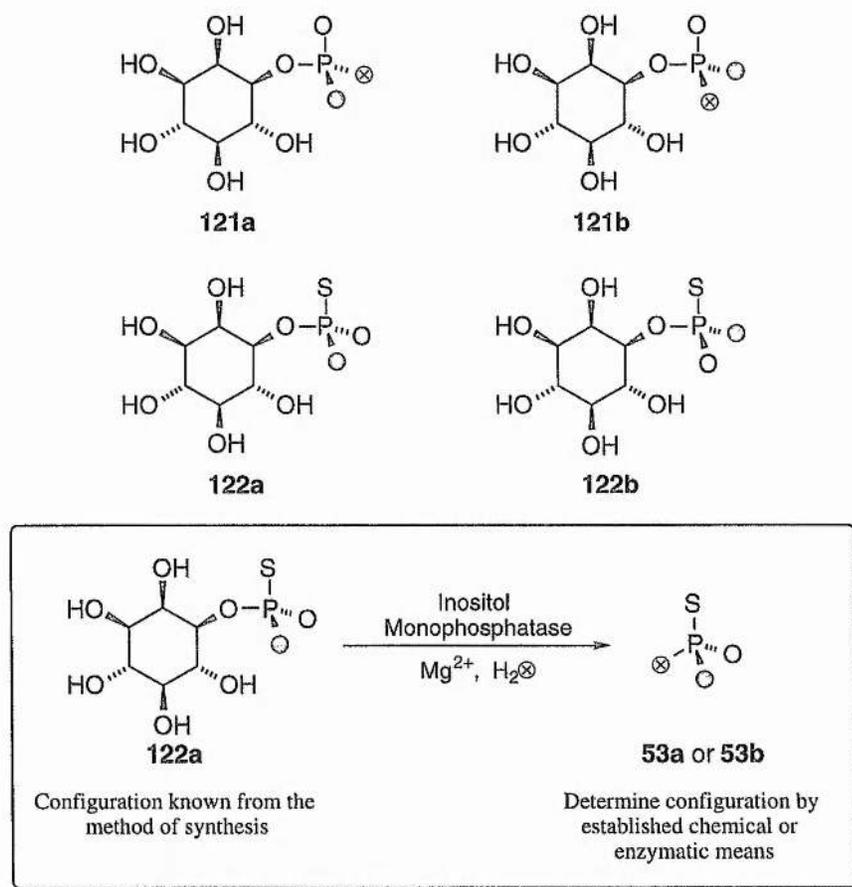
2. Results and Discussion

The aim of this project was to produce a suitable substrate analogue for inositol monophosphatase, chirally labelled at phosphorus (by means of isotopic substitution) and to use this compound to determine the stereochemical course of the phosphate ester hydrolysis reaction with respect to the phosphorus atom. Kinetic studies have shown that inositol monophosphatase operates by a ternary complex mechanism in which the phosphate group of the substrate, inositol 1-phosphate, is directly displaced by a nucleophilic water molecule.^{26, 43, 52} Moreover, there is strong evidence that this water molecule is positioned to attack the substrate in an adjacent manner, relative to the inositol leaving group, necessitating a pseudorotation and giving an overall *retention* of configuration at the phosphorus centre.^{66, 67, 71, 76} Some inhibitors based on this proposed mechanism have been produced within the group and the kinetic results are in accord with these predictions.^{88-90, 159} A more conventional in-line association mechanism for inositol monophosphatase has also been proposed, resulting in an overall *inversion* of configuration at the phosphorus centre.⁸⁵ The determination of the stereochemical course of the inositol monophosphatase reaction is therefore a very powerful tool in probing further the mechanism of the enzyme as it should resolve the present mechanistic ambiguities by distinguishing between these two proposals.

2.1 General Considerations in Determining the Stereochemical Course of the Inositol Monophosphatase Reaction

To determine the stereochemical course of the inositol monophosphatase reaction, a suitable substrate analogue, chiral at phosphorus, must be synthesised (Scheme 2.1). Extensive studies have shown that the enzyme *cannot* catalyse transphosphorylation to a second alcohol species,^{43, 52} and this finding precludes the use of chiral (R_p)- and (S_p)-[¹⁶O, ¹⁷O, ¹⁸O]inositol 1-phosphates **121a** and **121b** as suitable probes, as transfer of the phosphate moiety to water would bring about loss of chirality at the phosphorus centre. The only route available for inositol monophosphatase, therefore, is to synthesise suitable chiral (R_p)- and (S_p)-[¹⁸O]phosphorothioate substrate analogues, the ideal target molecules

being the chiral analogues of the natural substrate, namely (R_p)- and (S_p)-[^{18}O]inositol 1-phosphorothioate **122a** and **122b**. By performing the enzyme reaction on these chiral phosphorothioates (of known configuration at phosphorus) in [^{17}O]-enriched water and analysing the configuration of the chiral inorganic [^{16}O , ^{17}O , ^{18}O]phosphorothioate product, using one of the techniques previously described (Section 1.30.1, page 66 or Section 1.30.2, page 72), it was hoped that we would be able to determine the stereochemical course of the inositol monophosphatase reaction (Scheme 2.1).



Scheme 2.1: General approach in elucidating the stereochemical course of the inositol monophosphatase reaction

Both D- and L-inositol 1-phosphorothioate **D-123** and **L-123** have been previously synthesised in the group,⁴⁸ and are weak substrates for inositol monophosphatase ($K_i = 1.0 \text{ mmol dm}^{-3}$ and $K_m = 1.0 \text{ mmol dm}^{-3}$), being processed at a rate approximately 10% that of the analogous phosphates.^{67, 76} In fact, this represents a relatively 'high' rate

of hydrolysis for the phosphorothioate analogue of a natural substrate (Section 1.4, page 10). Moreover, phosphorothioate ester hydrolysis was found to be inhibited by lithium cation, and control experiments which contained the phosphorothioate but no enzyme, showed neither reaction nor decomposition at all.¹⁶⁰ These combined findings make the determination of the stereochemical course of inositol monophosphatase using (*R*_p)- and (*S*_p)-[¹⁸O]inositol 1-phosphorothioate **122a** and **122b** a viable option.

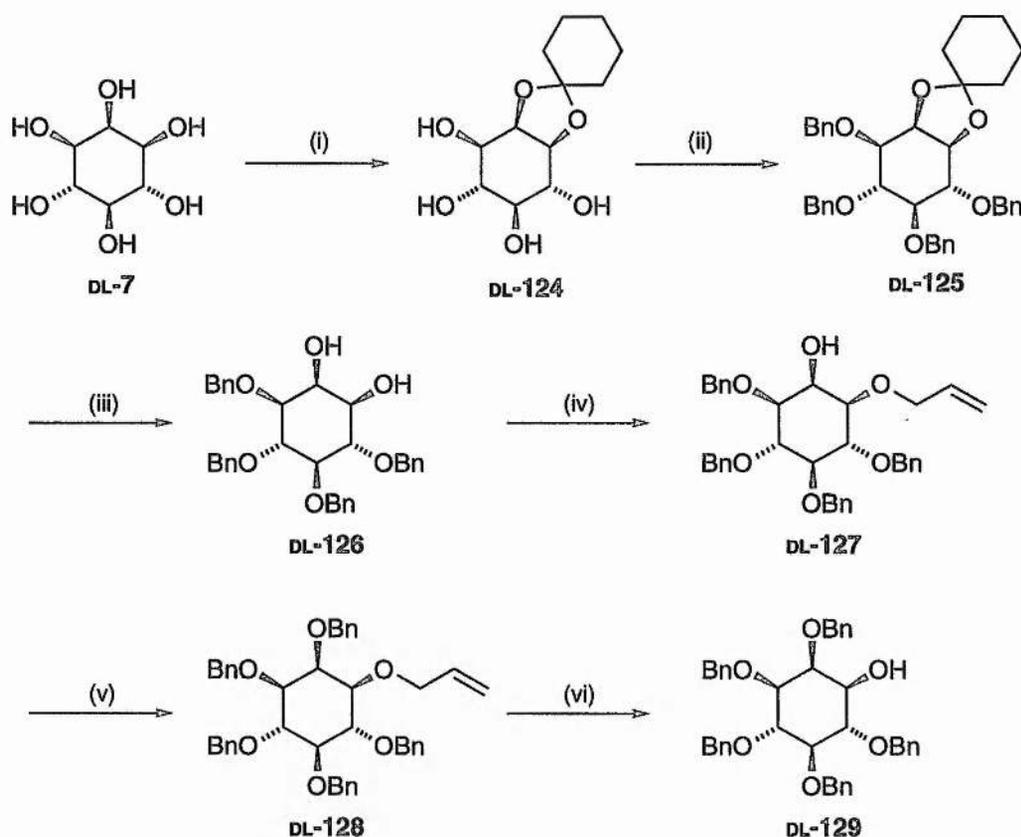
2.2 The Synthesis of DL-Inositol 1-Phosphate DL-10

The natural substrate for inositol monophosphatase, inositol 1-phosphate, was synthesised according to the method of Billington *et al.*⁴⁷ for use in the determination of enzyme activity. In addition, intermediates on the synthetic route, such as DL-3,4,5,6-tetrakis-*O*-benzyl *myo*-inositol **DL-126** and DL-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol **DL-129** have provided useful starting points to investigate the synthesis of the target chiral substrate analogues, (*R*_p)- and (*S*_p)-[¹⁸O]-inositol 1-phosphorothioate **122a** and **122b** (Scheme 2.2 and Scheme 2.5).

Due to the ready availability of pure *myo*-inositol **7** and its low cost, the synthesis of inositol 1-phosphate uses the parent cyclitol as a starting material (Scheme 2.2).

The acid catalysed reaction of inositol with cyclohexanone led to the formation of three isomeric bisacetals together with the trisacetal, all of which give the monoacetal **DL-124** on mild acid hydrolysis (86%).¹⁶¹ This is due to selective cleavage of the less stable *trans* acetal groups.^{1, 10} Benzylation of **DL-124** followed by acidic ketal deprotection yielded DL-3,4,5,6-tetra-*O*-benzyl *myo*-inositol **DL-126** (60% from the monoacetal) which was then specifically allylated at the C-1 position *via* the formation of a stannylidene intermediate using dibutyltin oxide under dehydrating conditions.^{48, 162} Previously, Gigg and Warren¹⁶¹ had exploited the naturally higher reactivity of the equatorial hydroxyl group in the diol to perform a direct allylation using allyl bromide. Under carefully controlled conditions,¹⁶¹ the reaction gave mainly the equatorial *O*-allyl compound **DL-127**. The use of dibutyltin oxide followed by allyl bromide, however, led to a much

greater regioselectivity for the reaction, with no 2-*O*-allyl- or bis-*O*-allyl- protected inositol derivatives being formed.¹⁶²

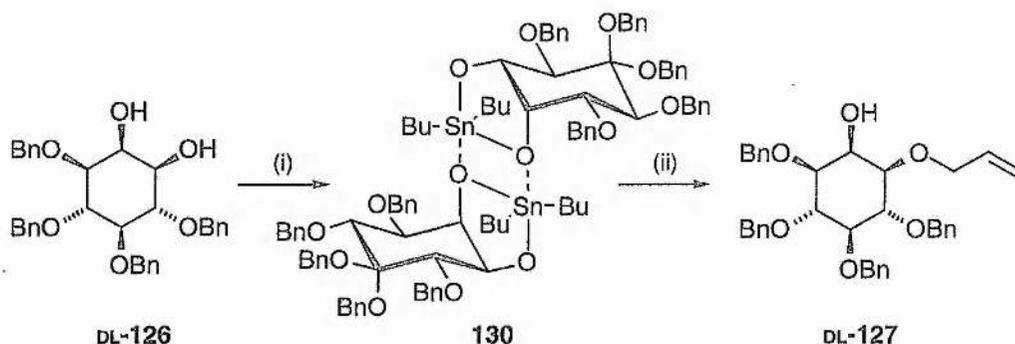


Reagents and conditions: (i) (a) $C_6H_{10}O$, $p-CH_3C_6H_4SO_3H$, $PhCH_3$, Dean-Stark reflux $150\text{ }^\circ\text{C}$, 2 h, then (b) $EtOH$, $p-CH_3C_6H_4SO_3H$, 86%; (ii) $BnCl$, KOH , reflux, 24 h, 72%; (iii) $AcOH$, H_2O , reflux, 2 h, 84%; (iv) (a) Bu_2SnO or $Bu_2Sn(OCH_3)_2$, C_6H_6 , Dean-Stark reflux, 24 h and 2 h respectively, then (b) Allyl bromide, Bu_4NBr , $60\text{ }^\circ\text{C}$, 48 h, 93%; (v) $BnBr$, NaH , DMF , $25\text{ }^\circ\text{C}$, 24 h, 88%; (vi) (a) $(Ph_3P)_3RhCl$, $DABCO$, $EtOH_{(aq)}$, reflux, 4 h, then (b) $AcOH_{(aq)}$, reflux, 4 h 83%.

Scheme 2.2: *Synthesis of DL-2,3,4,5,6-penta-O-benzyl myo-inositol DL-129*

The stannylidene intermediate which is thought to form in the solution phase of the reaction is the dimer **130** shown in Scheme 2.3. On examination of the dimer, it is evident that the two inositol oxygens are not equivalent. The C-2 hydroxyl is thought to be co-ordinated to both tin atoms in the dimer and is thus relatively electron deficient, as well as being sterically hindered. The C-1 hydroxyl, by contrast, is only coordinated to one tin atom in the dimer and is consequently more electron rich. 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 the C-1 hydroxyl.¹⁶³



Reagents and conditions: (i) $\text{Bu}_2\text{Sn}(\text{OCH}_3)_2$, C_6H_6 , Dean-Stark reflux, 2 h; (ii) Allyl bromide, Bu_4NBr , 60°C , 48 h, 93%.

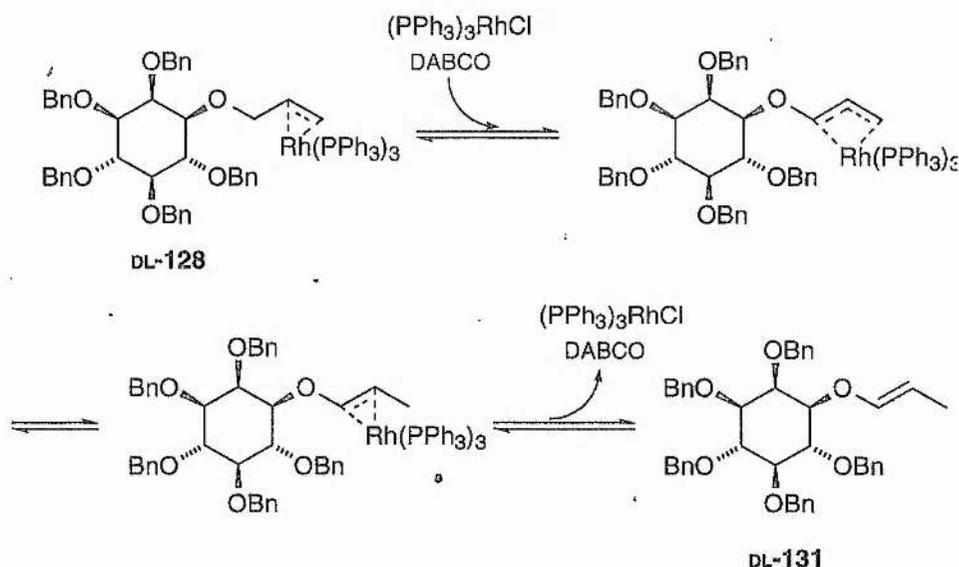
Scheme 2.3: Regioselective allylation of C-1 OH via a stannylidene intermediate.

Formation of the stannylidene dimer from dibutyltin oxide with azeotropic removal of water takes up to 24 h. It was found that by using the commercially available reagent dibutyltin dimethoxide in place of the tin oxide, stannylation time was greatly reduced. Formation of the dimer, as judged by infra-red spectroscopy, took typically less than 1 h under similar Dean-Stark conditions.¹⁶⁴ The overall yield of the allylation reaction using the modified procedure was unchanged, enabling the simplified and more rapid production of DL-1-O-allyl-3,4,5,6-penta-O-benzyl *myo*-inositol **DL-127**.

Following this selective equatorial allylation, the penta-protected inositol **DL-127** was further benzylated using benzyl bromide and sodium hydride in dimethylformamide (DMF) to give the fully protected inositol derivative **DL-128**. Significant increases in rates of reaction have been observed when DMF was used as a solvent in preference to tetrahydrofuran (THF).^{48, 165}

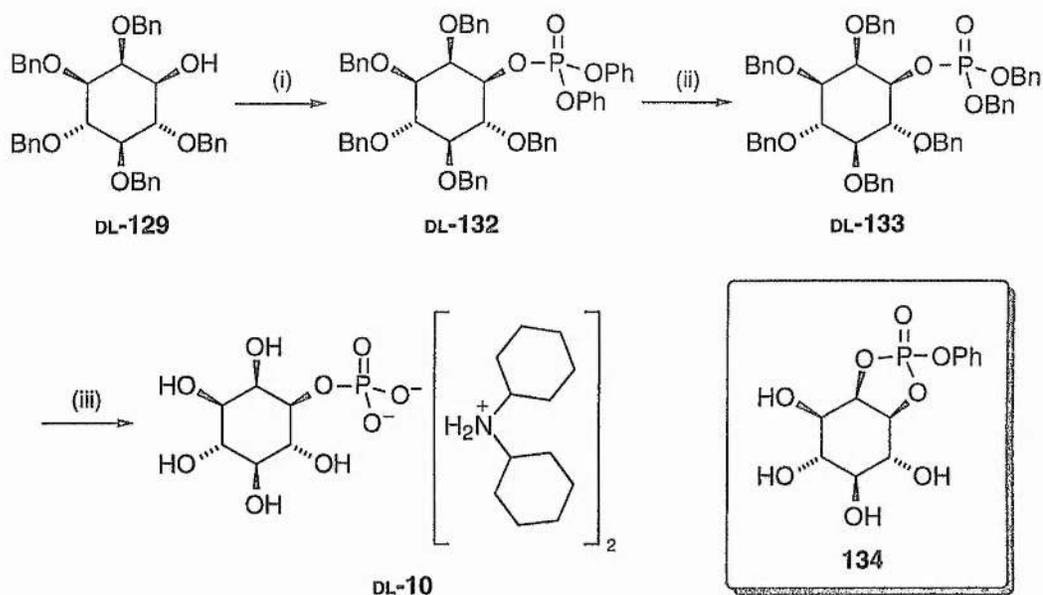
Removal of the allyl group from the fully protected inositol **DL-128** was achieved using the methodology of Corey and Suggs,¹⁶⁶ to give the desired protected precursor to inositol

1-phosphate **DL-129**. Catalytic amounts of both tris(triphenylphosphine) rhodium (I) chloride (Wilkinson's catalyst) and 1,4-diazabicyclo[2,2,2]octane (DABCO) were used in aqueous ethanol to accomplish the isomerisation of the allyl ether **DL-128** to the more thermodynamically stable enol ether **DL-131** (Scheme 2.4).



Scheme 2.4: Wilkinson's catalyst mediated deallylation

The enol ether **DL-131** was then easily hydrolysed in aqueous acid to give the key intermediate, DL-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol **DL-129**. To then produce inositol 1-phosphate **DL-10**, the key intermediate **DL-129** was phosphorylated using diphenylphosphorochloridate to give the penta-benzylated diphenyl phosphate **DL-132** (Scheme 2.5). Previous attempts to deprotect this compound by hydrogenolysis, first with a palladium catalyst to remove the benzyl ethers, followed by a platinum catalyst to remove the phenyl phosphate esters, resulted in the formation of a 98:2 mixture of DL-inositol 1-phosphate and DL-inositol 2-phosphate. This mixture arises *via* the formation and subsequent unselective ring opening of a cyclic phenyl phosphate ester **DL-134** formed from the intermediate diphenyl phosphate produced in the two-step deprotection procedure (see inset, Scheme 2.5).



Reagents and conditions: (i) $(\text{PhO})_2\text{POCl}$, DMAP, Et_3N , CH_2Cl_2 , 25 °C, 24 h, 83%; (ii) NaH, BnOH, THF, 25 °C, 18 h, 73%; (iii) (a) $\text{Na}/\text{NH}_3(l)$, -78 °C, 1 h, then (b) Amberlite IR118 (H^+), then (c) cyclohexylamine, H_2O , 67%.

Scheme 2.5: Synthesis of DL-inositol 1-phosphate DL-10

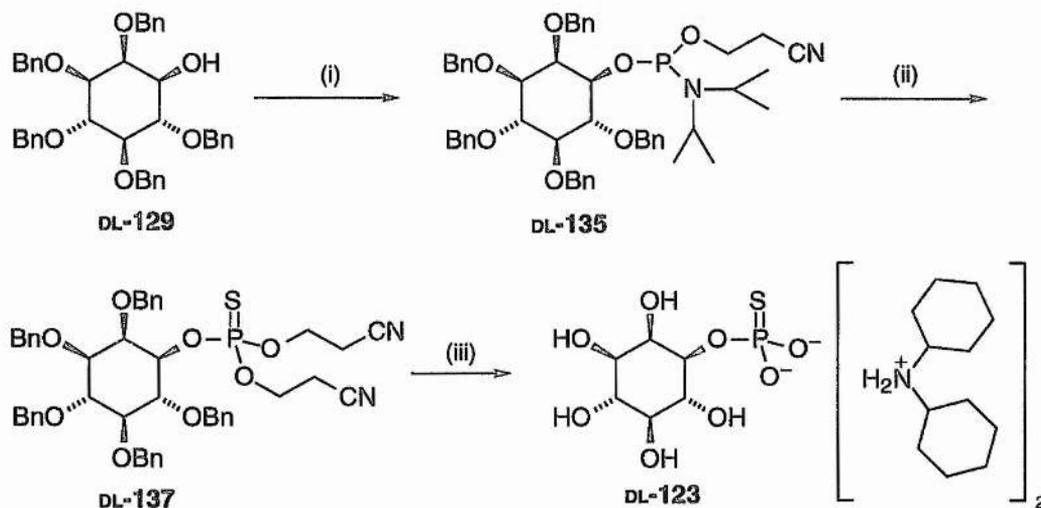
The general solution developed for this problem by Billington *et al.*^{47, 167} was to use benzyl esters as phosphate protecting groups, in place of phenyl esters. Transesterification of the fully protected compound DL-132 using the anion of benzyl alcohol⁴⁷ gives the dibenzyl phosphate DL-133 (Scheme 2.5) which can be deprotected by a single hydrogenolysis over a palladium catalysis.* We have utilised this procedure as far as the transesterification reaction, but then subsequently deprotected the fully protected dibenzyl phosphate product DL-133 using sodium/ liquid ammonia reduction to give DL-inositol 1-phosphate DL-10, which was converted to the bis-cyclohexylammonium salt, in 41% yield from DL-129.⁴⁸

2.3 The Synthesis of DL-Inositol 1-Phosphorothioate DL-123

The penta-benzylated precursor to inositol 1-phosphate DL-129 was also used in the synthesis of inositol 1-phosphorothioate by Baker *et al.*⁴⁸ Reaction of the alcohol DL-129

* The benzyl phosphate esters are cleaved much faster than the benzyl ethers, giving free phosphate which is not prone to migration during the slower hydrogenolysis of the benzyl ethers.

in dichloromethane with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in the presence of diisopropylethylamine under argon gave a solution of the pentabenzylated-inositol 1-phosphoramidite triester **DL-135** (Scheme 2.6).



Reagents and conditions: (i) *N,N*-diisopropyl(2-cyanoethyl)chlorophosphoramidite, diisopropylethylamine, THF, 0°C, 4 h; (ii) (a) 1*H*-tetrazole, 3-hydroxypropionitrile, CH₃CN, 25 °C, 16 h, then (b) S₈, pyridine, 25 °C, 15 min, 66% (over two steps); (iii) (a) NaOCH₃/CH₃OH, then (b) Na/NH_{3(l)}, -78 °C, then (c) Amberlite IR118 (H⁺), then (d) cyclohexylamine, H₂O, 89%.

Scheme 2.6: Synthesis of *DL*-inositol 1-phosphorothioate **DL-123**

Transesterification of triester **DL-135** *in situ* with an excess of 3-hydroxypropionitrile and 1*H*-tetrazole afforded the bis-cyanoethyl phosphite triester **DL-136** which was subsequently oxidised with elemental sulfur in pyridine using the method of Burgers and Eckstein¹⁶⁸ to give the pentabenzyl inositol 1-phosphorothioate triester **DL-137** in 66% overall yield from the alcohol **DL-129**.

Deprotection of triester **DL-137** was achieved in two stages. Treatment first with sodium methoxide in methanol, brought about the base-catalysed β -elimination of the phosphate moiety from both cyanoethyl groups to give the disodium salt of *DL*-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-phosphorothioate. This was then subjected to reductive cleavage with sodium in liquid ammonia and tetrahydrofuran to give the disodium salt of

myo-inositol 1-phosphorothioate* which was purified as for DL-inositol 1-phosphate, by ion exchange chromatography on Amberlite IR118 (H⁺) and then converted to the crystalline bis-cyclohexylammonium salt **DL-123**, in 89% overall yield from the protected inositol **DL-137**. The use of substrates in the form of their biscyclohexylammonium salts aids crystallisation, and hence purification, and also does not interfere in any way with the enzyme incubation.

2.4 Previous Studies on the Stereochemical Course of Inositol Monophosphatase Reaction

Previous attempts within the group to determine the stereochemical course of the inositol monophosphatase reaction have focussed on the production of (*R*_p)- and (*S*_p)-[¹⁸O]inositol 1-phosphorothioate **122a** and **122b**.

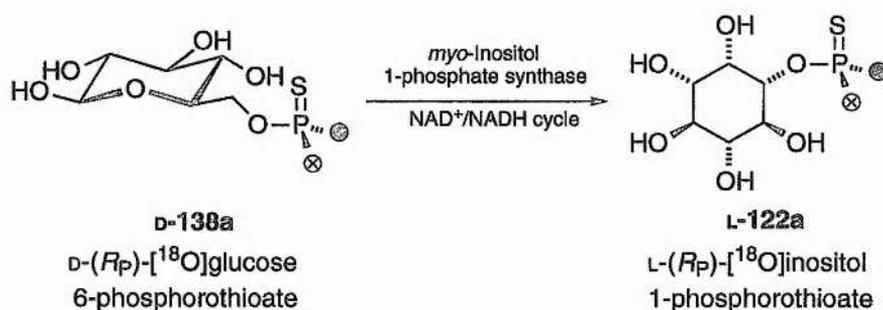
A general route to chiral [¹⁸O]phosphorothioates was developed by Lowe *et al.*¹¹⁹ (Section 1.29.6, page 65). To employ this route for the synthesis of **122a** and **122b** would, however, require the functionalisation of the secondary hydroxyl group of the *myo*-inositol intermediate **129**. Secondary alcohols react very slowly with Lowe's (chiral) thiophosphorylating agent,^{119, 160} and in this particular case, the secondary hydroxyl group of **129** is also sterically hindered by the adjacent bulky benzyl protecting-groups. Consequently, the synthesis of (*R*_p)- and (*S*_p)-[¹⁸O]inositol 1-phosphorothioate using this available method was not considered to be a viable option, and alternative routes to the chiral phosphorothioates were sought.

Earlier, within our own group, the potential of using a combined synthetic and enzymic route was explored.¹⁶⁰ *myo*-Inositol 1-phosphate synthase is the enzyme responsible for the *de novo* biosynthesis of inositol in animals *via* the isomerization of D-glucopyranose 6-phosphate **D-12** to L-inositol 1-phosphate **L-10** (Scheme 2.7, see also page 3). It was reasoned that if the enzyme could also convert D-glucopyranose 6-phosphorothioate to L-inositol 1-phosphorothioate, then the synthetic problems would be reduced to preparing

* The specific deprotection of the phosphorus protecting groups prior to reductive cleavage of the benzyl ethers again ensures that phosphoryl migration to adjacent hydroxyl groups does not occur.

suitably labelled glucopyranose 6-phosphorothioates **D-138a** and **D-138b**. Lowe's route could then be used to functionalise the primary 6-hydroxyl group of glucopyranose and produce these compounds.

Unfortunately, D-glucopyranose 6-phosphorothioate did not serve as a substrate for *myo*-inositol 1-phosphate synthase, precluding the possibility of using the enzyme in the synthesis of chiral target compounds L-(*R_p*)- and L-(*S_p*)-[¹⁸O]inositol 1-phosphorothioate **122a** and **122b**.¹⁶⁰

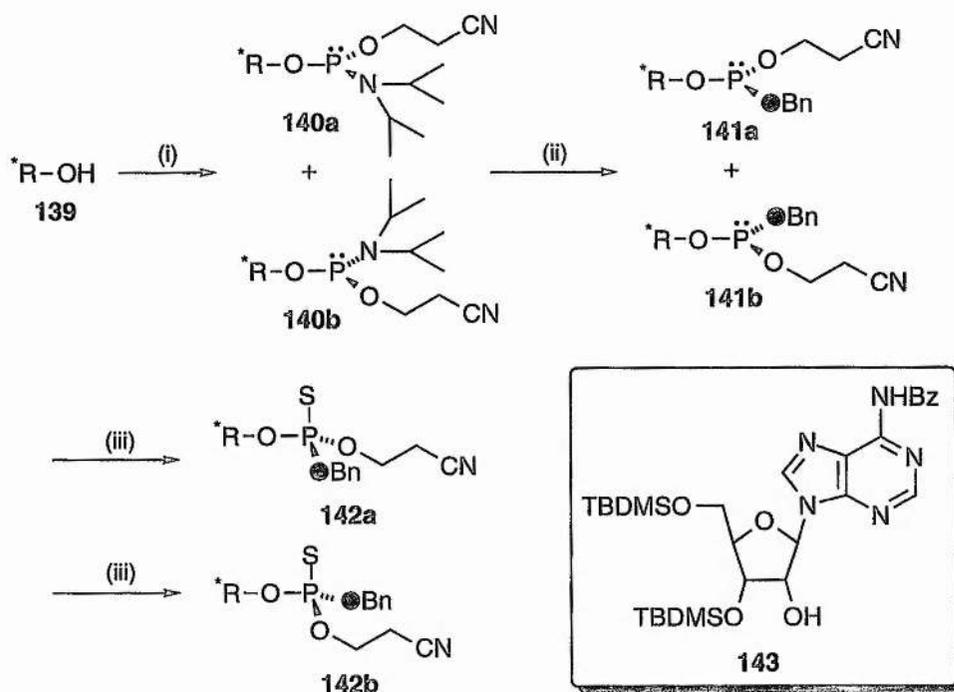


Scheme 2.7: Potential enzymic route to L-(*R_p*)- and L-(*S_p*)-[¹⁸O]inositol 1-phosphorothioate **L-122a** and **L-122b**

Though the enzymatic route, had it worked, would have potentially produced chiral phosphorothioates of high enantiomeric purity, the approach did suffer from the fact that only the L-inositol chiral phosphorothioates would have been produced **L-122a** and **L-122b**. As inositol monophosphatase processes both D- and L-inositol 1-phosphates, a complete study of the stereochemical course of the enzyme should involve using, individually, *both* chiral (*R_p*)- and (*S_p*)-[¹⁸O]inositol 1-phosphorothioates for *both* D- and L-forms of the compound.

More recently, Cole and Gani investigated the possibility of employing the previously established chemical synthesis of inositol 1-phosphorothioate¹⁶⁰ (Section 2.3, page 80) to incorporate an [¹⁸O]oxygen label.

Phosphitylation of a single enantiomer[†] of D- or L-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol D-129 or L-129, with racemic *N,N*-diisopropyl(cyanoethyl)chlorophosphoramidite and examination of the reaction intermediate 135 by thin layer chromatography revealed that the inositol phosphoramidite was present as an inseparable mixture of two diastereomers 140a and 140b ($R^* = \text{D-129 or L-129}$, Scheme 2.8).¹⁶⁹



Reagents and conditions: (i) *N,N*-diisopropyl(2-cyanoethyl)chlorophosphoramidite, diisopropylethylamine, DMAP, THF; (ii) BnOH , 1*H*-tetrazole, CH_3CN ; (iii) S_8 , pyridine.

Scheme 2.8: *Phosphoramidite transesterification and oxidation with sulfur*

Similarly, transesterification of intermediate 135 with benzyl alcohol, instead of 3-hydroxypropionitrile, gave an inseparable mixture of diastereomers. It was proposed that by using of [¹⁸O]benzyl alcohol in this reaction it would be possible to stereospecifically incorporate an [¹⁸O]-label, giving 141a and 141b ($R^* = \text{D-129 or L-129}$). Simple oxidation of the separate phosphite diastereomers, followed by deprotection would give access to (*R_p*)- and (*S_p*)-[¹⁸O]inositol 1-phosphorothioates 122a

[†] Resolved using (*S*)-(-)-camphanic acid chloride⁴⁷ (see Section 2.13, page 139).

and **122b**. The diastereomeric resolution of the phosphites was investigated accordingly, but in the case of inositol the compounds could not be separated.

As adenosine 2'-monophosphate (2'AMP) is also an excellent substrate for inositol monophosphatase, Cole and Gani also investigated the possibility of using chiral (R_p)- and (S_p)-[^{18}O]adenosine 2'-phosphorothioate as a suitable alternative probe for determining the stereochemical course of the enzyme reaction. A similar phosphorylation procedure on the protected adenosine derivative **143** gave a pair of diastereoisomers **140a** and **140b** ($R^* = \mathbf{143}$) which could be separated successfully (Scheme 2.8). Transesterification of separated diastereomers **140a** or **140b** with benzyl alcohol was found to be unselective, and gave a pair of (unlabelled) diastereomers **141a** and **141b** ($R^* = \mathbf{143}$). Synthesis of the nucleoside phosphorothioate in a chiral form therefore required the transesterification of the unpurified phosphoramidite diastereomers with [^{18}O]benzyl alcohol, followed by diastereomeric resolution, oxidation of separated phosphite triesters with sulfur and then final deprotection.

Cole and Gani found that adenosine 2'-monophosphorothioate exhibited no substrate activity under optimum hydrolysis conditions in the presence of Mg^{2+} . However, activity was observed when the more thiophilic metal ion Mn^{2+} was used in place of Mg^{2+} , and complete hydrolysis could be achieved.^{67, 71, 169} To deduce the stereochemical course of the phosphate ester hydrolysis, the chiral inorganic [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]phosphorothioate hydrolysis product must be isolated. Unfortunately, it was evident from these studies that levels of inorganic phosphorothioate had been depleted through complexation with the metal ion cofactor, evident as a pale cream precipitate, and as a result this approach was ultimately abandoned.

2.5 New Approach to Determining the Stereochemical Course of Inositol Monophosphatase Reaction

Due to the earlier failed attempts to synthesise (R_p)- and (S_p)-[^{18}O]inositol 1-phosphorothioates **122a** and **122b**, it was decided to focus on synthesising a simpler chiral [^{18}O]phosphorothioate substrate analogue which could then be used to probe the stereochemical course of the inositol monophosphatase reaction.

As discussed previously (Section 1.13, page 26), the minimum substrate for the enzyme is ethane 1,2-diol 1-phosphate **38**, exhibiting moderate activity (V_{max} 12% of the V_{max} -value for Ins1- P , $K_m = 0.7 \text{ mmol dm}^{-3}$, $K_i = 1.0 \text{ mmol dm}^{-3}$) which can be increased upon increasing the Mg^{2+} ion concentration to 5 mmol dm^{-3} .^{*71} Modelling studies have shown previously that the substrate, when docked into the active site of the enzyme, can bind in two different low-energy orientations (Figure 2.1) both of which are likely to be populated.⁷⁶

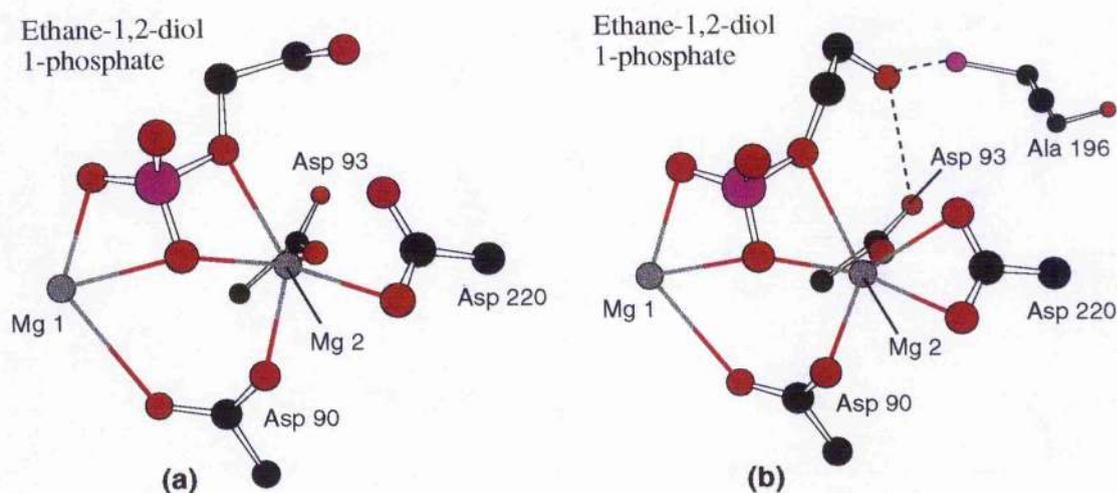


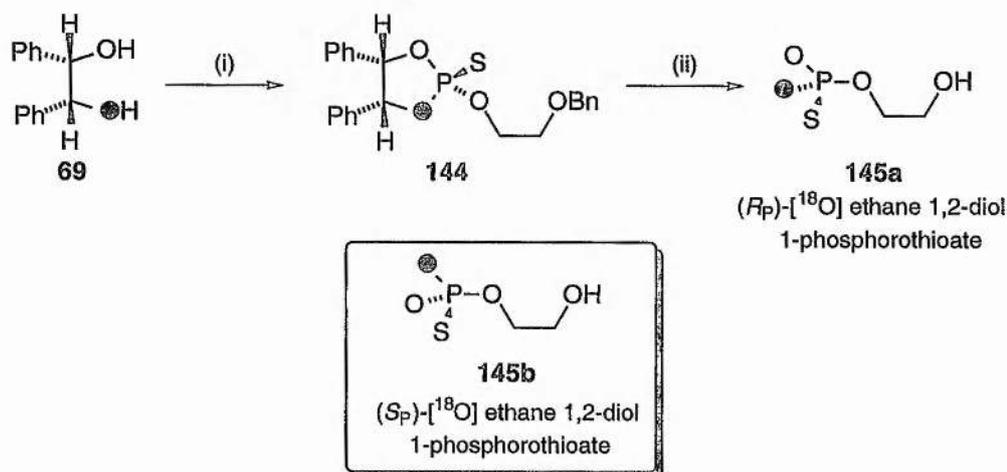
Figure 2.1: Minimised active-site structures for ethane 1,2-diol 1-phosphate; (a) bound as a substrate; (b) bound as an inhibitor

In one of these [Figure 2.1 (a)], the 2-OH group is positioned in the site for the 'catalytic' 6-OH group of D-Ins1- P such that compound **38** would act as a substrate. In the other

* At this high Mg^{2+} ion concentration, Mg^{2+} serves as an inhibitor when Ins1- P is the substrate.^{46, 170}

2-OH of D-Ins1-P (see Figure 1.15, page 31) such that the compound would act as an inhibitor.

From these studies, it appeared very likely that ethane 1,2-diol 1-phosphate was hydrolysed by inositol monophosphatase using the *same* mechanism as D-Ins1-P. If ethane 1,2-diol 1-phosphorothioate also proved to be a substrate for the enzyme, it was therefore envisaged that (*R_p*)- and (*S_p*)-[¹⁸O]ethane 1,2-diol 1-phosphorothioate **145a** and **145b** could be utilised as useful probes to determine the stereochemical course of the enzyme reaction. These compounds could be synthesised by employing Lowe's (chiral) thiophosphorylating agent (Section 1.29.6, page 65) on a suitably protected ethane 1,2-diol derivative, such as 1-*O*-benzyl ethane 1,2-diol **146** (Scheme 2.9).

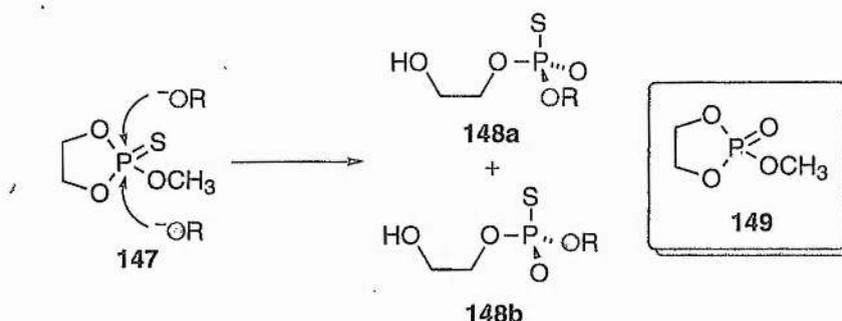


Reagents and conditions: (i) (a) PSBr_3 , pyridine (2 equiv.), then (b) $\text{BnCH}_2\text{CH}_2\text{OH}$ **146**, Et_3N ; (ii) $\text{Na}/\text{NH}_3(l)$, -78°C .

Scheme 2.9: Proposed synthesis of (*R_p*)- and (*S_p*)-[¹⁸O]-ethane 1,2-diol 1-phosphorothioate **145a** and **145b**

In order to avoid the lengthy synthesis of *meso*-[¹⁸O]hydrobenzoin **69**, we considered utilising the bis-functionality of ethane 1,2-diol to synthesise a cyclic phosphorothioate triester, such as **147**, which could then be ring-opened with an appropriate [¹⁸O]-labelled nucleophile to afford two chiral [¹⁸O]phosphorothioates **148a** and **148b**, the

configuration of which would be determined by the stereochemistry of the ring-opening reaction (Scheme 2.10).



Scheme 2.10: Proposed ring-opening reaction of ethane 1,2-diol 1,2-cyclic phosphorothioate triester

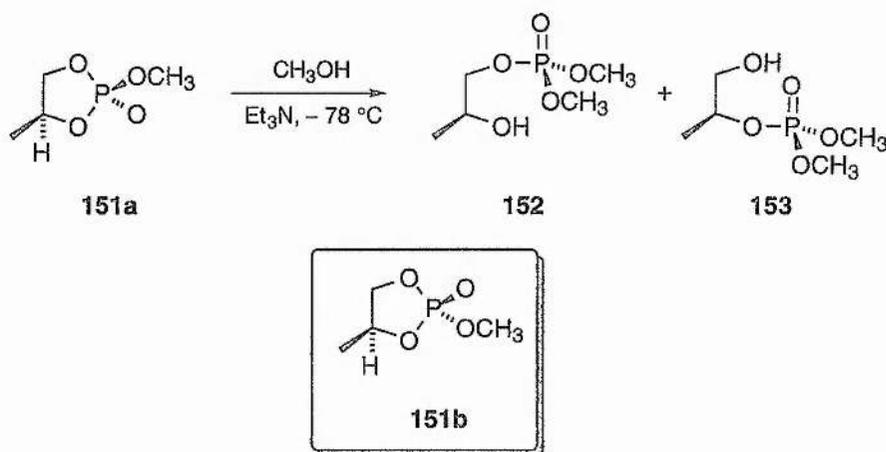
This seemed a reasonable assumption as analogous five-membered cyclic phosphate triesters are extremely sensitive to nucleophiles.⁷⁵ For example, methyl ethylene phosphate **149** is hydrolysed millions of times faster than acyclic phosphate triesters in acid or base. Ring-strain has been cited as the major cause of this effect, and X-ray crystallographic studies of **149** have revealed a small ring angle at phosphorus (99°), providing independent experimental evidence for this proposal.¹⁷¹

On closer examination of the reaction scheme (Scheme 2.10) it became evident that, although both target compounds **148a** and **148b** would be produced upon successful deprotection, the products are enantiomers and thus cannot be separated by conventional methods. As compounds of single known conformation at phosphorus are required for a successful stereochemical study, we sought a modified approach.

The original work by Knowles *et al.*^{106, 107} on the stereochemical analysis of chiral [¹⁶O, ¹⁷O, ¹⁸O]phosphate monoesters utilised the fact that the symmetry of ethane 1,2-diol can be disrupted by substitution at one of the carbon atoms (see Section 1.27.2, page 51). Consequently, (*S*)-(+)-propane 1,2-diol **150**^{*} was used, in place of ethane 1,2-diol, to synthesise *syn*- and *anti*-1,2-cyclic phosphate triesters **151a** and **151b** which were

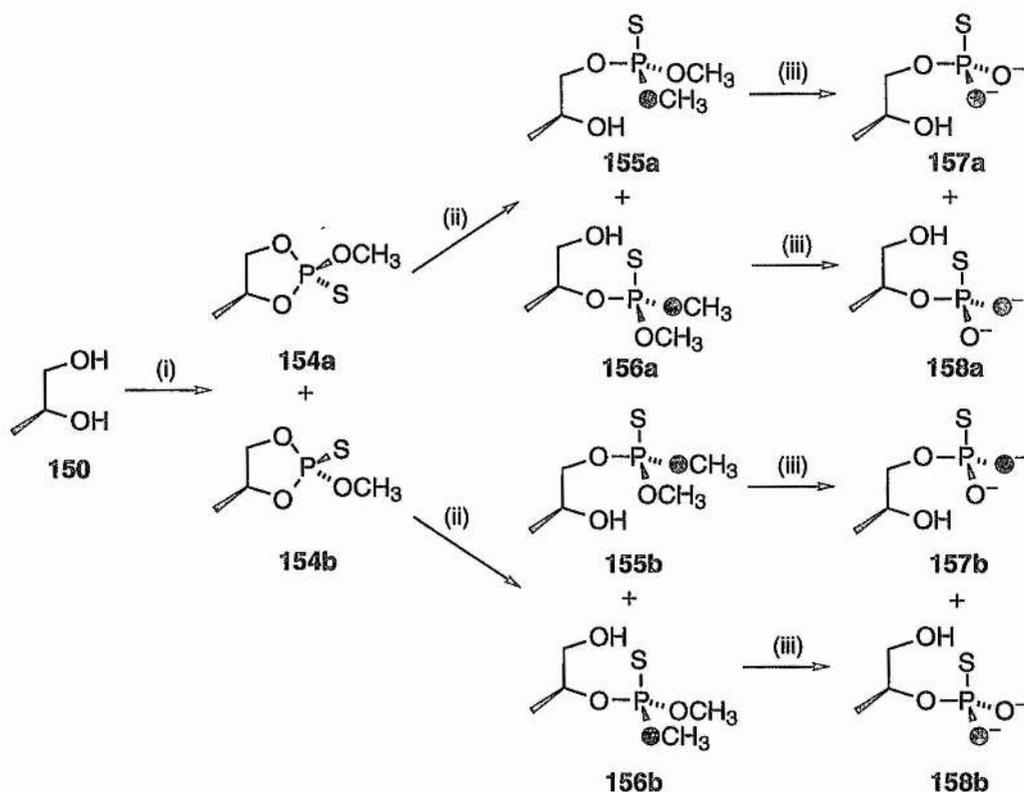
^{*} Both (*S*)-(+)- and (*R*)-(-)-propane 1,2-diol are commercially available; the former is by far the more affordable of the two enantiomers.

separated. Knowles then demonstrated that methanolysis of **151a** or **151b** gave rise to both 1- and 2-isomers of phosphopropane diol (**152** and **153**, Scheme 2.11).¹⁰⁷



Scheme 2.11: Methanolysis of syn-1,2-cyclic phosphate triester **151a**

Although Knowles made no attempt to separate **152** and **153**, these 'ring-opened' products are regioisomers and should be separable. We therefore reasoned that by synthesising the analogous *syn*- and *anti*-cyclic phosphorothioate triesters **154a** and **154b**, and by performing methanolysis on the separated compounds with [^{18}O]methanol, we should have a potentially short route to some small chiral [^{18}O]phosphorothioate probes, (**157a**, **157b**, **158a** and **158b**), which could be used to determine the stereochemical course of the inositol monophosphatase reaction (Scheme 2.12). As the proposed synthesis of triesters **154a** and **154b** utilised the same chemistry employed in the synthesis of Lowe's (chiral) thiophosphorylating agent (Section 1.29.6, page 65), there was also the possibility that each compound could be synthesised in a pure form by controlling the quantity of pyridine present in the reaction mixture [Step (i-a), Scheme 2.12].¹¹⁹ If successful, this would avoid one of the required separation procedures.



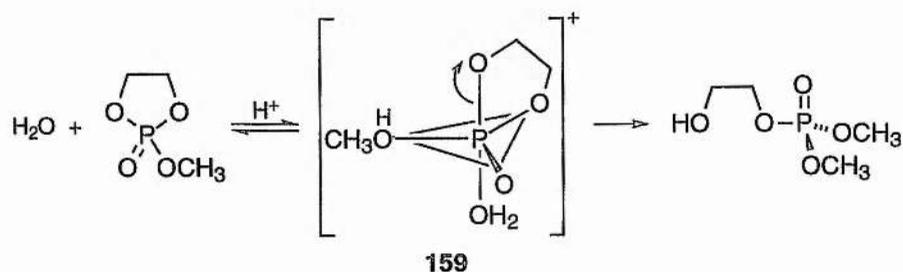
Reagents and conditions: (i) (a) PSBr_3 , pyridine, C_6H_6 , (b) CH_3OH , Et_3N , (c) chromatographic separation; (ii) (a) CH_3SH , Et_3N , -78°C , (b) chromatographic separation; (iii) $\text{N}(\text{CH}_3)_3$, C_6H_6 .

Scheme 2.12: Proposed route to chiral [^{18}O]phosphorothioate probes for inositol monophosphatase

In order to determine the absolute configuration of phosphorothioates **157a**, **157b**, **158a** and **158b**, both the structure of cyclic phosphorothioate triesters **154a** and **154b**, and the stereochemistry of the methanolysis reaction must be known.

The enantiomers of compounds **154a** and **154b** are known in the literature¹⁷² and the absolute structure of these cyclic phosphorothioate triesters and of their cyclic phosphite triester precursors¹⁷³ have been determined. In keeping with the later findings of Lowe,¹¹⁹ *trans*-cyclic phosphorothioates such as triester **154a** and their cyclic phosphite precursors resonate at a *lower* field in the ^{31}P -NMR spectrum than the corresponding *cis*-compounds.^{118, 172}

The hydrolytic ring-opening of cyclic phosphate triesters is thought to proceed with "in-line" stereochemistry, giving an *inversion* of configuration at phosphorus.⁷⁵ This stereochemistry is predicted on the basis of the 'preference rules'⁷⁵ which state that in the trigonal-bipyramidal intermediate that forms **159** (Scheme 2.13), (a) the five-membered ring of the cyclic phosphate triester are best placed so as to span one equatorial and one apical position,* and (b) that the more polar atoms preferentially occupy apical positions and the less polar atoms equatorial positions. In-line hydrolysis to a cyclic phosphate triester therefore proceeds through an intermediate that resembles the transition state of an S_N2 process in carbon chemistry, with one of the P–O bonds of the five-membered ring occupying the opposite apical position. The favourable breaking of this bond results in a ring-opened compound with inverted stereochemistry at phosphorus.⁷⁵



Scheme 2.13: Hydrolysis of a five-membered cyclic phosphate triester

Extensive studies by Cooper *et al.*¹¹⁴ have shown that the alcoholysis of cyclic phosphoramidites such as those employed in the Knowles synthesis of chiral [¹⁶O,¹⁷O,¹⁸O]phosphate monoesters (Section 1.26.1, page 46), also proceeds with inversion of configuration at phosphorus, under both acidic and basic conditions. In addition, Usher *et al.*¹⁴⁹ have proved that the ring-opening of a single diastereomer of uridine-2',3'-cyclic phosphorothioate using [¹⁸O]-enriched hydroxide ion involves in-line stereochemistry, and this observation has been used by Knowles to synthesise chiral D-glycerate [¹⁸O]phosphorothioates (Section 1.29.4, page 62).¹³⁰ Consequently, it seemed a reasonable assumption that the methanolysis of *syn*- and *anti*-cyclic phosphorothioate

* The formation of trigonal-bipyramidal intermediate **159** with an axial-equatorial arrangement of the cyclic phosphate triester and associated 90° angle at phosphorus relieves ring-strain, whereas a diequatorial arrangement and associated 120° angle at phosphorus causes a large increase in strain energy.

triesters **154a** and **154b** would also proceed in an in-line manner. Methanolysis of **154a** with [^{18}O]methanol would therefore give rise to (S_p)-[^{18}O]propane 1,2-diol 1-phosphorothioate **157a** and (R_p)-[^{18}O]propane 1,2-diol 2-phosphorothioate **158a**, with the [^{18}O]methanolysis of **154b** giving the isotopomers **157a** and **158b**.

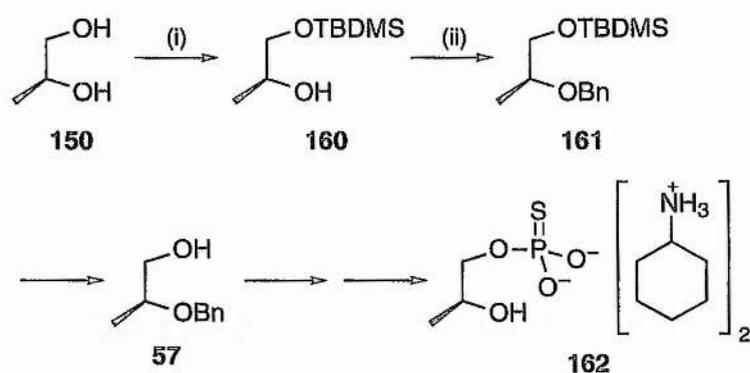
For this route to be feasible, it was necessary that methanolysis occurred with no loss of isotopic label or scrambling of chirality at phosphorus (*i.e.* no exchange of methoxyl groups). We therefore proposed to achieve this by using Knowles conditions of methanolysis ($\text{CH}_3^{18}\text{OH}$, Et_3N , $-78\text{ }^\circ\text{C}$).¹⁰⁷ Under these conditions, the methanolysis of *syn*- and *anti*-cyclic phosphate triesters **154a** and **154b** had already been shown to proceed as desired using CD_3OD for the methanolysis.¹⁰⁷

Importantly, deprotection of **155a**, **155b**, **156a** and **156b** also had to proceed with no loss of isotopic label. Methyl groups, however, can be removed from phosphorothioate triesters without disturbing the P–O bond using trimethylamine.¹¹⁸

Before pursuing this synthetic route, we had to investigate whether (*S*)-propane 1,2-diol 1-phosphorothioate **162** and/ or (*S*)-propane 1,2-diol 2-phosphorothioate **163** were acceptable substrates for inositol monophosphatase. As the phosphate analogue of compound **163** had been previously shown to have poor substrate activity ($K_m = 15\text{ mmol dm}^{-3}$, V_{\max} 8.4% of the V_{\max} -value for D-Ins1-*P*),⁴⁵ we initially focussed our efforts on the synthesis of (*S*)-propane 1,2-diol 1-phosphorothioate.

2.6 Synthesis of (*2S*)-Propane 1,2-Diol 1-Phosphorothioate **162**

The initial route we chose for the synthesis of (*2S*)-propane 1,2-diol 1-phosphorothioate **162** is shown in Scheme 2.14. (*2S*)-(+)-Propane 1,2-diol **150** is available with 99% enantiomeric excess. Selective protection of the primary alcohol of **150** with *tert*-butyldimethylsilyl chloride (TBDMSiCl) and imidazole afforded monoprotected alcohol **160** in 63% yield. To ensure that compound **160** was the sole product, the silyl ether formation was performed at $-10\text{ }^\circ\text{C}$ using just 1.1 equivalents of TBDMSiCl.

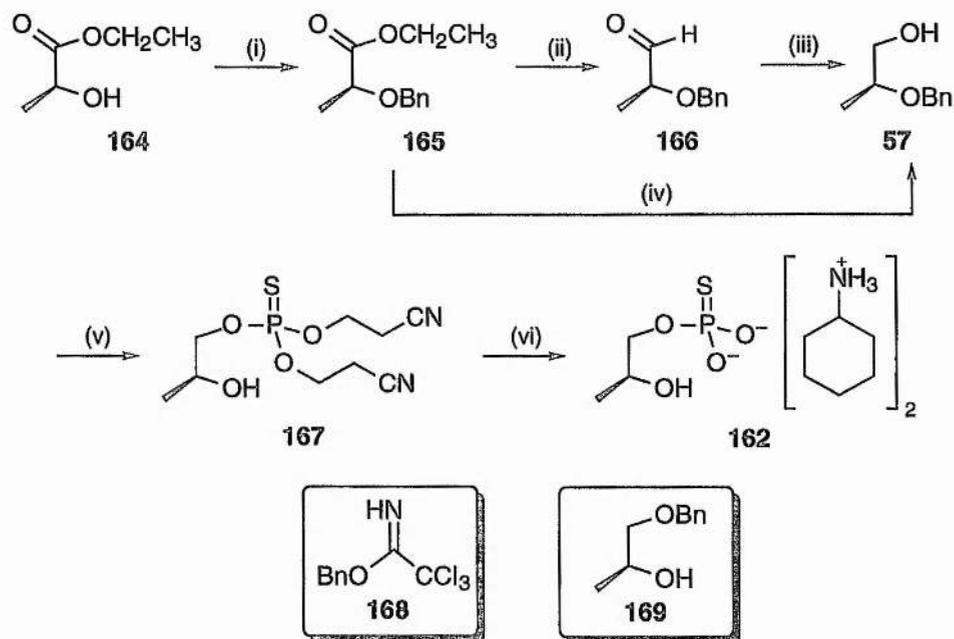


Reagents and conditions: (i) *t*-BDMSCl, imidazole, DMF, $-10 \rightarrow 25$ °C, 24 h, 63%;
(ii) BnBr, NaH, DMF, 25 °C, 24 h, (mixture of products).

Scheme 2.14: Attempted synthesis of (*S*)-propane 1,2-diol 1-phosphorothioate

Benzylation of **160** was then attempted using benzyl bromide and sodium hydride in DMF but the reaction did not proceed cleanly, giving a mixture of at least two inseparable products. The ^1H - and ^{13}C -NMR spectra of this mixture were consistent with the presence of (*S*)-1-*O*-benzyl 2-*O*-*t*-butyldimethylsilyl-propane 1,2-diol **163** in addition to **161**, indicating that under the basic conditions of the reaction, a facile intramolecular migration of the TBDMSi-group from the 1-*O* to the 2-*OH* appeared to be possible prior to benzylation. Therefore, rather than being able to simply cleave the silyl ether of **161** with tetra-*n*-butyl ammonium fluoride to give (*S*)-2-*O*-benzyl propane 1,2-diol **57**, it was necessary to seek another route to this intermediate. From compound **57**, we envisaged that by using the same phosphitylating and deprotection strategy used in the synthesis of inositol 1-phosphorothioate we could also synthesise (*S*)-propane 1,2-diol 1-phosphorothioate **162**.

Kobayashi *et al.*¹⁷⁴ had previously synthesised optically pure (*S*)-(2-*O*-benzyl)-propanal **166** from (*S*)-ethyl lactate **164** via (*S*)-ethyl-(2-*O*-benzyl)-propionate **165** (Scheme 2.15). It was therefore proposed to synthesise (*S*)-2-*O*-benzyl propane 1,2-diol **57** by the complete reduction of ester **165**.



Reagents and conditions: (i) *O*-benzyl trichloroacetimidate **168**, CF₃SO₃H (cat.), cyclohexane-dichloromethane (7:1 v/v), 25 °C, 20 h, 82%; (ii) *i*-Bu₂AlH, (CH₃CH₂)₂O, -78 → 25 °C, 1 h, 84%; (iii) NaBH₄, CH₃CH₂OH, 0 °C, 2 h, 79%; (iv) NaBH₄, EtOH, 0 °C, 65%; (v) (a) *N,N*-diisopropyl(2-cyanoethyl)chlorophosphoramidite, diisopropylethylamine, THF, 0 °C, 4 h, then (b) 1*H*-tetrazole, 3-hydroxypropionitrile, CH₃CN, 25 °C, 16 h, then (c) S₈, pyridine, 71%; (vi) (a) NaCO₃/MeOH, then (b) Na/NH₃₍₀₎, -78 °C, then (c) Amberlite IR118 (H⁺), (d) cyclohexylamine, H₂O, 53%.

Scheme 2.15: Synthesis of (*S*)-2-*O*-benzyl propane 1,2-diol

Takai *et al.*¹⁷⁵ had found that the direct benzylation of lactate **164** under basic conditions such as those employing benzyl bromide and sodium hydride in DMF, gave a low yield of **165** and resulted in considerable racemisation. Kobayashi demonstrated, however, that **165** could be prepared in excellent yield and without racemisation by treating the lactate with *O*-benzyl trichloroacetimidate **168** in the presence of a catalytic amount of trifluoromethanesulfonic acid.¹⁷⁴

O-Benzyl trichloroacetimidate **168** is particularly well suited to the formation of benzyl ethers in compounds where the usual strongly alkaline benzylation conditions can cause the loss of an alkali-labile protecting groups or the abstraction of an acidic proton (as was

the case with compound **164**).^{176, 177} Though this alternative milder reagent could have potentially been used for the benzylation step of mono-protected diol **160**, it was chosen instead to modify the method of Kobayashi¹⁷⁴ and attempt the synthesis of **57** in two steps from **164** without racemisation (Scheme 2.15).

The benzylation of lactate **164** proceeded cleanly giving compound **165** in a yield of 82%. The optical rotation measurement of purified **165** confirmed that under the reaction conditions, racemisation had not occurred, $\{[\alpha]_D^{20} -74.9 (c 2.94 \text{ in } \text{CHCl}_3) \text{ lit.},^{178} [\alpha]_D^{20} -74.5 (c 2.94 \text{ in } \text{CHCl}_3)\}$.

The complete reduction of protected lactate **165** was achieved using sodium borohydride to give the alcohol **57** as the major product of the reaction. However, the unexpected transbenzylation product **169** also appeared to be formed in moderate yield.* Though, with difficulty, these two products could be separated by column chromatography [$R_f = 0.17$ (20% ethyl acetate/petroleum ether) for alcohol **57**], the two stage reduction of compound **165** was also investigated to see if this would decrease the quantity of side-product that formed.

Compound **165** was reduced to the aldehyde **166** with diisobutylaluminium hydride at -78°C in 84% yield.¹⁷⁴ Following purification of the aldehyde, it was then reduced further to the alcohol **57** using sodium borohydride. Less transbenzylation product was observed to form, and thus allowing the alcohol **57** to be more easily isolated. Measurements of the optical rotation of the aldehyde **166** and samples of the alcohol **57** formed from both reduction strategies were in agreement with literature values. The optical purity of the alcohol **57** was also confirmed by examining the ¹H- and ¹³C-NMR spectra of its (-)-(1*R*,4*S*)-camphanate ester **170**.⁴⁷ The ¹H-NMR spectrum of the ester **170** showed no signals corresponding to the other diastereomer.

* Selected data for benzyl-group migration product **169**: δ_{H} (300 MHz) 1.13 (3 H, d, $J_{2,3}$ 6.3, 3-CH₃), 1.96 (1 H, br s, OH), 3.40–3.66 (3 H, m, 1-CH₂ and 2-CH), 4.43 and 4.60 (2 H, 2 x d, J_{AB} 11.7, CH₂Ph), 7.10–7.40 (5 H, m, Ar-H); δ_{C} (75.4 MHz; C²HCl₃) 15.59 (3-CH₃), 66.34 (1-CH₂), 68.86 (CH₂Ph), 75.71 (2CH), 128.51 and 128.74 (Ar-CH) and 140.75 (Ar-C quaternary); $R_f = 0.23$ (20% ethyl acetate/petroleum ether).

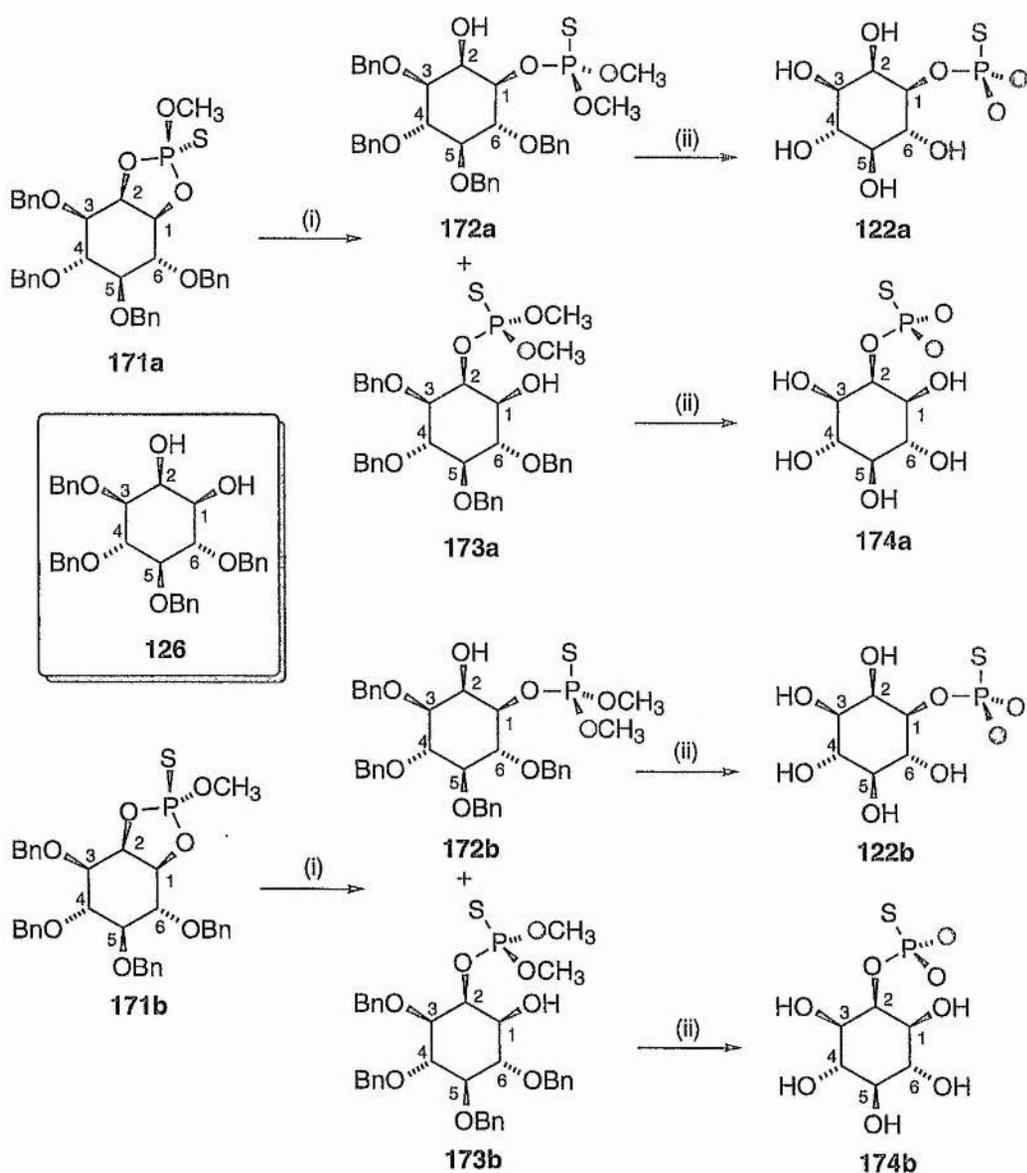
(*S*)-2-*O*-Benzyl propane 1,2-diol **57** was then phosphitylated using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite and transesterified with 3-hydroxypropionitrile to give (*S*)-1-*O*-bis(2'-cyanoethyl)phosphoro-thioate-2-*O*-benzyl propane-1,2-diol **167** in 71% yield $\{[\alpha]_D^{25} +1.7 (c\ 0.5\ \text{in}\ \text{CHCl}_3)\}$.⁴⁸ The phosphorothioate triester was deprotected first with sodium methoxide and secondly by reductive cleavage with sodium in liquid ammonia and tetrahydrofuran to give the disodium salt of (*S*)-propane 1,2-diol 1-phosphorothioate which was purified as for inositol 1-phosphorothioate, by ion exchange chromatography on Amberlite IR118 (H^+) and then converted to the crystalline bis-cyclohexylammonium salt **162**, in 39% overall yield from (*S*)-ethyl lactate **164** $[\delta_p(121.5\ \text{MHz};\ \text{C}^2\text{HCl}_3) 43.93]$. The compound could not be recrystallised from acetone/water and was instead recovered by lyophilisation. It was hoped that the specific deprotection of the phosphorus protecting groups prior to reductive cleavage of the benzyl ether would ensure that phosphoryl migration to the adjacent hydroxyl groups did not occur. Spectral data was indeed consistent with the formation of the 1-*O*-thiophosphorylated compound. The ¹³C-NMR spectrum of the salt **162** exhibited a coupling of both 1-C and 2-C to the phosphorus atom [²*J*_{1C-P} 5.4, ³*J*_{2C-P} 7.6] but no coupling of the 3-C methyl signal.

During the course of this work, it was realised that the chiral analogues of the natural substrate, namely (*R*_p)- and (*S*_p)-[¹⁸O]inositol 1-phosphorothioate **122a** and **122b**, could potentially be synthesised by adapting the cyclic phosphorothioate triester and methanolysis chemistry proposed for (2*S*)-propane 1,2-diol (Scheme 2.12, page 90). As these compounds are the most suitable substrate with which to probe the stereochemistry of the inositol monophosphatase reaction, it was decided to concentrate our efforts on this area.

2.7 Synthesis of Chiral (*R*_p)- and (*S*_p)-[¹⁸O]Inositol 1-Phosphorothioates via Cyclic Phosphorothioate Triester Intermediates

The synthesis of both DL-inositol 1-phosphate **DL-10** (Section 2.2, page 76) and DL-inositol 1-phosphorothioate **DL-123** (Section 2.3, page 80) involve a common

intermediate, DL-3,4,5,6-tetra-*O*-benzyl *myo*-inositol DL-129, which contains a *cis*-diol functionality. It was reasoned that if the cyclic phosphorothioate triesters 171a and 171b, similar to those proposed for (2*S*)-propane 1,2-diol, could be synthesised and separated, then methanolysis of each compound with [¹⁸O]methanol in an in-line manner under the conditions of Knowles¹⁰⁷ should lead to two ring-opened compounds, each chiral at phosphorus, 172a and 173a or 172b and 173b (Scheme 2.16).



Reagents and conditions: (i) (a) MeOH, Et₃N, CHCl₃, -78 °C, (b) chromatographic separation; (ii) Li/NH_{3(l)}, -78 °C.

Scheme 2.16: Proposed synthesis of *D*-(R_p)- and *D*-(S_p)-[¹⁸O]inositol 1-phosphorothioate 122a and 122b

The subsequent separation of **172a** from **173a** and **172b** from **173b** followed by deprotection would give access to both (R_p)- and (S_p)-[^{18}O]inositol 1-phosphorothioate **122a** and **122b**. The separation of regioisomers **172** and **173** by simple silica column chromatography was considered possible by virtue of the fact that each contained a fully protected phosphorothioate moiety in a different arrangement relative to the inositol ring; equatorial for compound **172**, axial for compound **173**.

myo-Inositol 1,2-cyclic-phosphates are well known in nature. For example, the inositol 1,2-cyclic phosphate moiety is thought to be found as part of a putative second messenger, a glycosylinositol phosphate (GIP), which has been implicated in mediating the action of insulin in insulin-sensitive tissues such as muscle, liver and fat,¹⁷⁹ and inositol 1,2-cyclic 4,5-trisphosphate is also a second messenger. The latter compound is formed by the action of the enzyme phosphatidylinositides-specific phospholipase C (PI-PLC),¹⁸⁰ which is additionally responsible for the hydrolysis of phosphatidylinositol and its phosphorylated derivatives (Scheme 1.3, page 8) to inositol 1,2-cyclic phosphate, inositol 1-phosphate and diacylglycerol.¹⁸¹

To investigate the mechanism of PI-PLC, *exo*- and *endo*-inositol 1,2-cyclic phosphorothioates **175a*** and **175b** had been previously synthesised and characterised by Lin *et al.* (Figure 2.2).¹⁸²

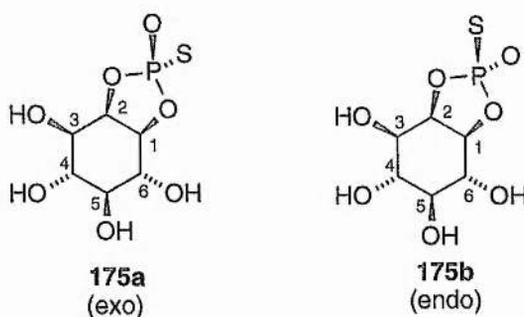


Figure 2.2: *Exo- and endo-inositol 1,2-cyclic phosphorothioates 175a and 175b synthesised by Lin. et al.*¹⁸²

* The *exo*- form of **175** is defined as the form in which sulfur and the inositol ring are on opposite sides of the five-membered ring.

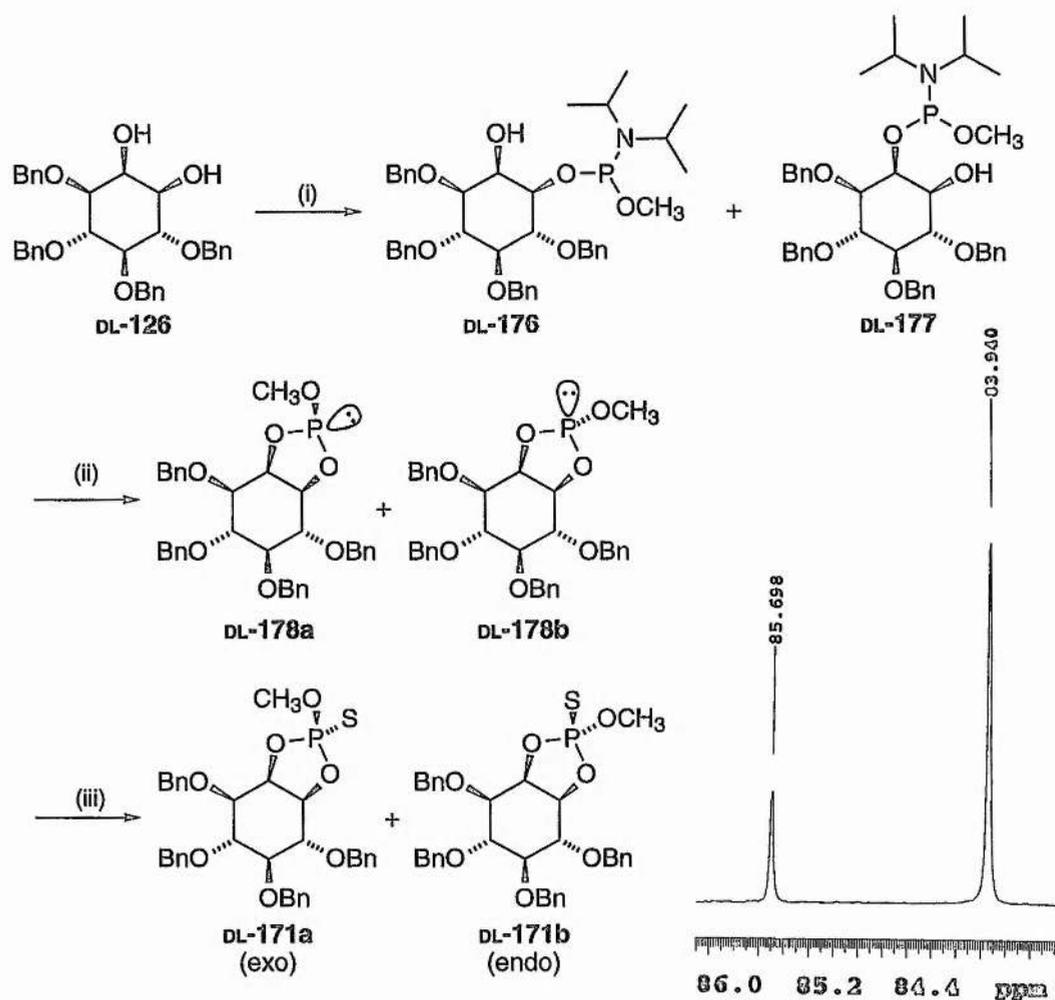
In this synthesis, the protected precursors to these compounds are in fact the same cyclic phosphorothioate triesters **171a** and **171b** which were required to attempt the synthesis of (*R_p*)- and (*S_p*)-[¹⁸O]inositol 1-phosphorothioate **122a** and **122b** (Scheme 2.16, page 97).

2.8 Synthesis of *exo*- and *endo*-Inositol 1,2-Cyclic Phosphorothioate Triesters **DL-171a** and **DL-171b**

Cyclic phosphorothioate triesters **DL-171a** and **DL-171b** were therefore synthesised from DL-3,4,5,6-tetra-*O*-benzyl *myo*-inositol **DL-126** (prepared by established procedures, Scheme 2.2, page 77) according to the method of Lin *et al.*¹⁸²⁻¹⁸⁴ (Scheme 2.17). Compound **DL-126** was phosphorylated with *N,N*-diisopropylmethylphosphonamidic chloride [CIP(OCH₃)N(*i*-Pr)₂] in the presence of diisopropylethylamine to give phosphoramidites **DL-176** and **DL-177**. After removal of excess diisopropylethylamine, 4 equiv. 1*H*-tetrazole in tetrahydrofuran-acetonitrile was added to the reaction mixture to form two cyclic phosphite triesters **DL-178a** and **DL-178b** *via* a novel intramolecular cyclisation. Without isolation, **DL-178** was oxidized by an excess of elemental sulfur (S₈) in toluene to give **DL-171a** [δ_p (121.5 MHz) 85.69 ppm (*exo*), *i.e.* D-(*R_p*) and L-(*S_p*)] and **DL-171b** [δ_p (121.5 MHz) 83.94 ppm (*endo*), *i.e.* D-(*S_p*) and L-(*R_p*)] which were separated by column chromatography. It can be seen from the ³¹P-NMR spectrum in Scheme 2.17 that by using the literature conditions,¹⁸³ the major product of the reaction was the *endo*-cyclic phosphorothioate triester **DL-171b** formed, typically, in a ratio of 10:1 relative to the *exo*-compound **DL-171a** [respective yields of 40% and 4%, based on **DL-126**].

Chromatographic separation of these products proved troublesome, requiring repeated column chromatography which in turn caused some loss of product due to decomposition by prolonged contact with the silica [Fluka Kieselgel (220–440 mesh) silica gel]. Improved separation and thereby higher yields were achieved by the use of TLC grade silica [Kieselgel N] and eluting the column with 20% ethyl acetate in hexane. As the isolation of **DL-171a** and **DL-171b** free from contamination by the other compound was

paramount for the synthesis of (*R_p*)- and (*S_p*)-[¹⁸O]inositol 1-phosphorothioates **122a** and **122b** (Scheme 2.16, page 97) with high configurational integrity at phosphorus, other purification methods were considered in order to try and achieve this.



Reagents and conditions: (i) $\text{CIP(OCH}_3\text{)N}(i\text{-Pr)}_2$, $i\text{-Pr}_2\text{NEt}$, CH_2Cl_2 , 25 °C, 0.5 h; (ii) 1*H*-tetrazole, $\text{THF-CH}_3\text{CN}$, 25 °C, 4 h; (iii) excess S_8 , toluene, 25 °C, 48 h, 44% overall (ratio *exo:endo* 1:10).

Scheme 2.17: Synthesis of *exo*- and *endo*-inositol 1,2-cyclic phosphorothioate triesters **DL-171a** and **DL-171b**. ^{31}P -NMR of the crude reaction mixture indicated the ratio of products formed

The *endo*-triester **DL-171b** was found to recrystallise well from ethanol, whereas the *exo*-triester **DL-171a** oiled out. Consequently, it was possible to isolate sizable portions of pure *endo*-triester from the crude reaction mixture, prior to column chromatography,

through fractional crystallisation from ethanol. To synthesise both 122a and 122b, however, both *exo*- and *endo*-cyclic phosphorothioate triesters were required and it proved to be very difficult to isolate substantial quantities of purified *exo*-cyclic phosphorothioate triester.

Lowe¹¹⁹ had previously shown that the configuration at phosphorus of cyclic phosphate and phosphorothioate triesters was subject to thermodynamic control during formation (Section 1.26.2, page 47 and Section 1.27.1.6, page 62). By altering the quantity of base (pyridine) present in the cyclisation reaction, one cyclic phosphate or phosphorothioate triester could be favoured. The 1*H*-tetrazole catalysed intramolecular cyclisation reaction of DL-176 and DL-177 was therefore studied further to see if it was also subject to thermodynamic control. If this was the case, reaction conditions could be developed which would favour the formation of the *exo*-cyclic phosphorothioate triester.

Accordingly, the synthesis of DL-171a and DL-171b was performed using a literature procedure¹⁸²⁻¹⁸⁴ but the quantities of 1*H*-tetrazole present in the intramolecular cyclisation step varied. The ratios of crude DL-171a and DL-171b were judged by ³¹P-NMR (Table 2.1).

Table 2.1: Ratios of *exo*- and *endo*-inositol 1,2-cyclic phosphorothioate triesters DL-171a and DL-171b formed by varying the quantity of 1*H*-tetrazole present during the intramolecular cyclisation step

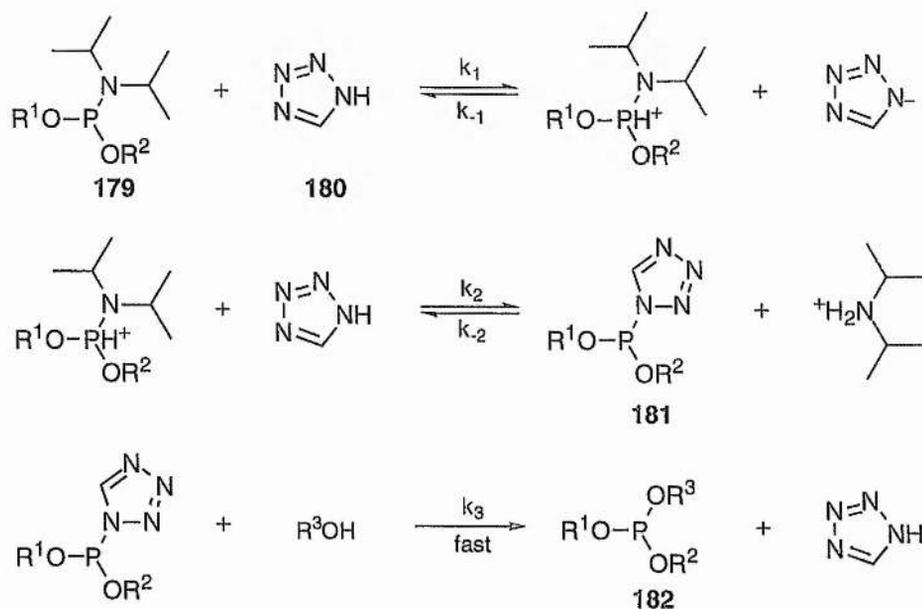
| Equiv. 1 <i>H</i> -tetrazole | Relative proportions of <i>endo</i> : <i>exo</i> | |
|---------------------------------|---|-----|
| 4 | 12 | 1 |
| 10* | 20 | 1 |
| 1.5 | 1 | 1.5 |

* 10 Equivalents of 1*H*-tetrazole represented the solubility limit of the catalyst under the conditions of the reaction.

It was found that the overall reaction *could* be controlled to a certain extent by varying the quantity of 1*H*-tetrazole present. Excess 1*H*-tetrazole* favoured the formation of the *endo*-triester almost exclusively, whilst 1.5 equiv. of the catalyst resulted in the formation of similar quantities of both cyclic triesters, permitting more *exo*-triester to be isolated than previously.

The results imply that cyclic phosphoramidite **DL-178a** represents the kinetic product of the intramolecular cyclisation step, whilst **DL-178b** is the more thermodynamically stable of the two intermediates. This has been confirmed with molecular modelling calculations by Lin *et al.*¹⁸³ which have categorically demonstrated that **DL-178b** is more thermodynamically stable than **DL-178a** and fits with the general observation that the least sterically hindered form of the phosphite should be **DL-178b**.¹⁸²

Although phosphoramidites containing a *N,N*-diisopropyl group exhibit an advantageous lack of reactivity towards nucleophiles (mainly water),¹⁸⁵ they can be activated to undergo coupling reactions with alcohols by using 1*H*-tetrazole **180**, a weak acid ($pK_a = 4.76$).¹⁸⁶ The mechanism of activation has been thoroughly investigated and is thought to involve both acid catalysis and nucleophilic catalysis by the heterocycle (Scheme 2.18).¹⁸⁵⁻¹⁸⁷



Scheme 2.18: The role of 1*H*-tetrazole in the activation of phosphoramidites

Quick protonation of the phosphoramidite **179** is followed by a slow formation of the tetrazolophosphane intermediate **181** which is the rate determining step of the catalysis cycle. Intermediate **181** then reacts with alcohols in a facile manner and irreversibly, to give the triester product **182**.

The nucleophilic nature of the tetrazolium anion is also the likely cause of the thermodynamic control observed for the favourable formation of cyclic phosphite triester **DL-178b** over **DL-178a** in the presence of excess *1H*-tetrazole. Simply put, the anion may facilitate the reversible ring-opening of the formed cyclic phosphite triesters, leading to the thermodynamically more stable product being favoured.

2.8.1 Configuration of *exo*- and *endo*-Inositol 1,2-Cyclic Phosphorothioate Triesters **DL-171a** and **DL-171b**

Knowledge of the configuration at phosphorus of the cyclic phosphorothioate triesters **DL-171a** and **DL-171b** is essential to allow the configuration of the subsequent ring opened [¹⁸O]phosphorothioate triesters and [¹⁸O]phosphorothioates to be determined (Scheme 2.16, page 97).

The absolute configuration of **DL-171a** and **DL-171b** was determined by Lin *et al.*¹⁸³ based on four criteria:

1. The predominant form **171b** should be *endo*- since the predominant form of the phosphite **178** should be the least sterically hindered form **178b**, and oxidation by sulfur is known to proceed with retention of configuration at phosphorus.^{118, 172, 173} The observed effect of varying the concentration of *1H*-tetrazole on the formation of **178a** and **178b** was in accord with this finding.
2. The relative ³¹P-NMR signals for **171a** and **171b** as assigned are consistent with that of the known cyclic phosphorothioate triesters **183a** and **183b** (83.0 and 80.5 ppm, respectively) in that the trans (*exo*) form is more downfield (Figure 2.3).^{118, 172}

3. The three bond coupling constants between P and 1-H are 18.4 and 9.7 ppm for **DL-171a** and **DL-171b** respectively (our measurements were 18.0 and 10.2 ppm respectively). These are consistent with the data for **183a** and **183b** and related compounds where $^3J_{4H,P}$ for the trans (*exo*) configuration (**183a**) is consistently larger than that measured for the cis (*endo*) form (**183b**).¹¹⁸
4. Irradiation of the 2-H resulted in detectable nuclear Overhauser enhancement (NOE) on the methyl proton resonance in **DL-171b** but not **DL-171a**, whereas irradiation of the 4-H resulted in a detectable NOE on the methyl proton resonance in **DL-171a** but not **DL-171b**.¹⁸³ These observed couplings were also confirmed by our own two dimensional NMR work on compounds **DL-171a** and **DL-171b**.

Although these detailed arguments for the assignment of the phosphorus configuration of **DL-171a** and **DL-171b** are plausible, the only definitive way to determine the absolute structure of the triesters is by X-ray crystal structural analysis. In spite of repeated efforts, however, suitable crystals of **DL-171b** could not be grown. The cyclic phosphorothioate triester recrystallised consistently as very fine fibrous or needle-like crystals inappropriate for such analysis.

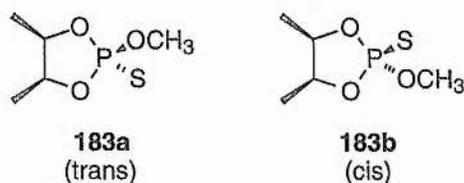


Figure 2.3: Model phosphorothioates used by Lin et al.¹⁸³ for the configurational assignment of cyclic phosphorothioate triesters **171a** and **171b**

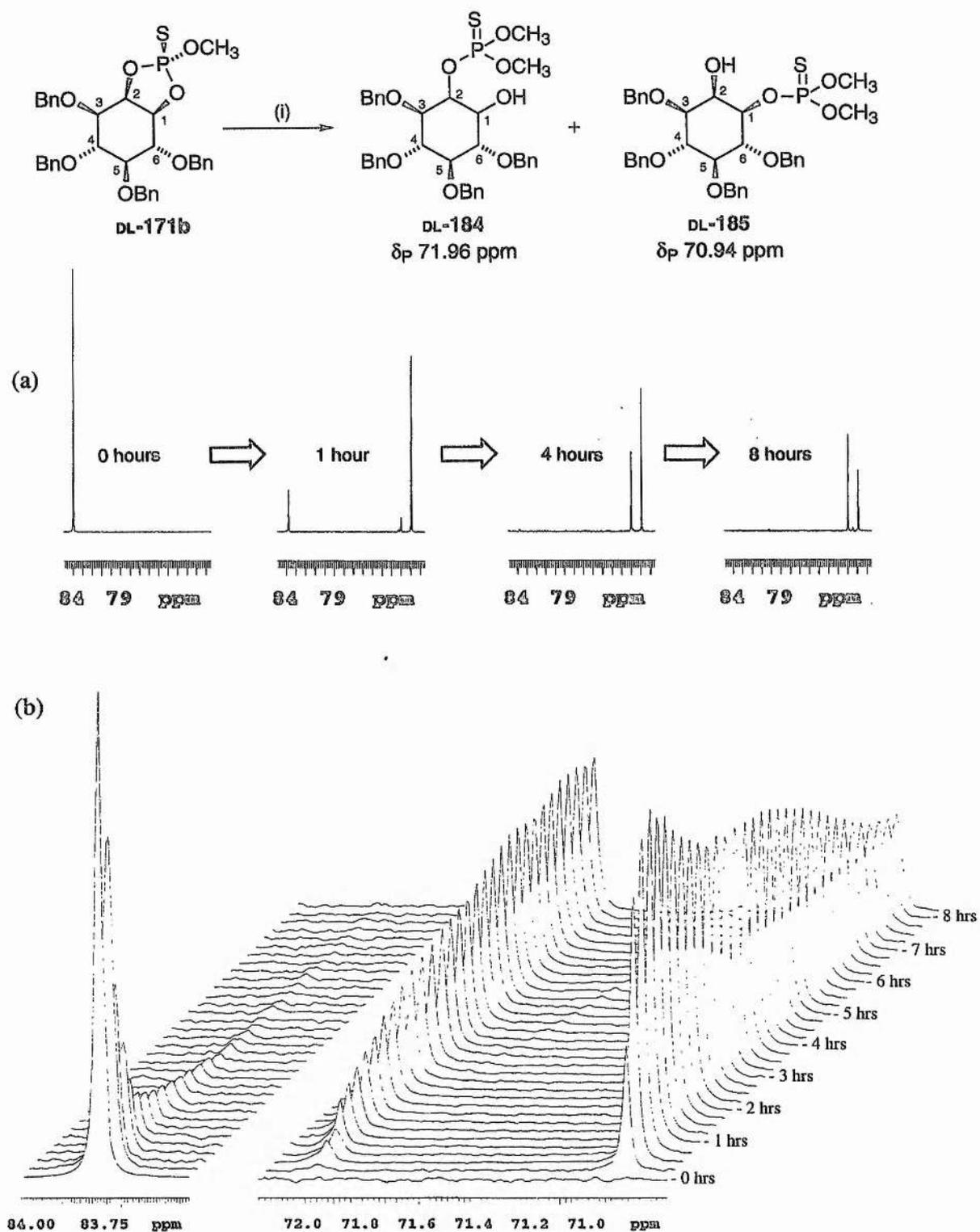
2.8.2 Methanolysis Studies

The *endo*-cyclic phosphorothioate **DL-171b** [δ_p (121.5 MHz) 83.94 ppm] was subjected to methanolysis [10 equiv. methanol, 10 equiv. triethylamine in dichloromethane at 25 °C] and the reaction monitored by ^{31}P -NMR spectrometry. Two products were seen to form

rapidly, consistent with the cyclic phosphorothioate having undergone a ring-opening by the alcohol to give a minor product **DL-184** [δ_p (121.5 MHz) 71.96 ppm] and a major product **DL-185** [δ_p (121.5 MHz) 70.94 ppm] (Scheme 2.19). Unfortunately, full ^{31}P -NMR analysis of the reaction course clearly showed that the two ring-opened products that form were not stable under the reaction conditions and appeared to be able to isomerise. For several hours after the cyclic phosphorothioate had reacted, the signal for **DL-184** [δ_p (121.5 MHz) 71.96 ppm] was seen to *increase* further at the expense of the signal for **DL-185** [δ_p (121.5 MHz) 70.94 ppm] until a final ratio of 1.4:1 was reached. Over extended reaction periods, other products were also observed to form albeit at a slower rate [δ_p (121.5 MHz) 62.55, 60.40, 57.55 and 57.05 ppm], possibly a result of the stepwise demethylation of **DL-184** and **DL-185** by triethylamine. These products were not characterised further.

Methanolysis of the *exo*-cyclic phosphorothioate **DL-171a** [δ_p (121.5 MHz) 85.69 ppm] under the same conditions gave rise to a similar reaction course, with the ^{31}P -NMR spectrum exhibiting major signals at 71.97 ppm and 71.06 ppm in a final ratio of 1.9:1 together with other minor signals [δ_p (121.5 MHz) 61.91, 59.65, 55.66 and 55.59 ppm].

In order to identify the two initial products of methanolysis **DL-184** and **DL-185** as assigned, the reaction was repeated with **DL-171b** as above but stopped immediately after all the starting material had been used up by evaporation of the methanol, triethylamine and dichloromethane *in vacuo*. The stabilised mixture of products in a ratio of 1:2.1 was then subjected to full deprotection by dissolving metal reduction using lithium in tetrahydrofuran-liquid ammonia at $-78\text{ }^\circ\text{C}$ in an identical procedure to that used for the sodium metal tetrahydrofuran-liquid ammonia deprotection step in the synthesis of DL-Ins1-*P* **DL-10** and DL-Ins1-*P_S* **DL-123**.¹⁸² In this type of deprotection, it is believed that the benzyl groups are removed by a radical reductive cleavage and the methyl groups are removed by an $\text{S}_{\text{N}}2$ nucleophilic attack of ammonia; neither would affect the configuration at phosphorus if [^{18}O]methanol were to be used in the methanolysis step.¹⁸³ As in the synthesis of **DL-123**, the reaction time was kept to a minimum (10 min) to avoid any risk of desulfurization.



Reagents and conditions: (i) MeOH, Et₃N, CH₂Cl₂, 25 °C, 8 h.

Scheme 2.19: Methanolysis of *endo*-cyclic phosphorothioate triester *DL-171b* monitored by ³¹P-NMR spectroscopy; (a) shows four spectra of the reaction taken at various time intervals, and (b) shows the same reaction viewed as an arrayed series of spectra

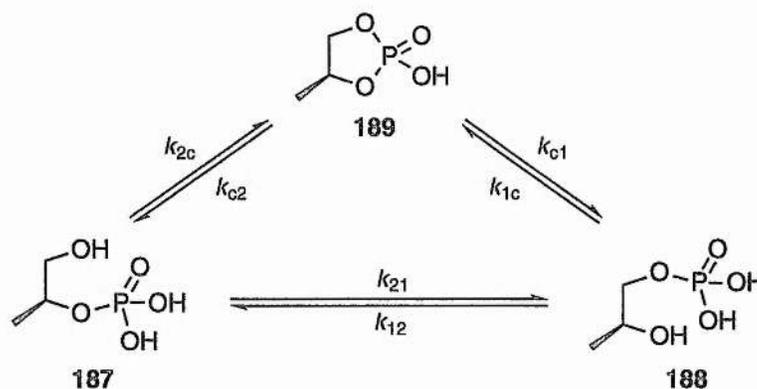
The deprotected mixture was then subjected to anion exchange chromatography on Amberlite IR-118 (H^+) and worked up as a mixture of dicyclohexylammonium salts. 1H - and ^{13}C -NMR analysis of the salt mixture confirmed that deprotection of the compounds was complete and indicated that inositol 1-phosphorothioate **DL-123** was present as the main component of the mixture (by comparison to spectra of an authentic sample), with some smaller signals being consistent with the presence of a second inositol-containing compound, putatively inositol 2-phosphorothioate **DL-186**. The ^{31}P -NMR spectrum of the mixture was in agreement with this analysis, exhibiting two main signals at 46.15 and 44.47 ppm [δ_p (121.5 MHz, 2H_2O)], the latter being the major component and having a similar chemical shift to the biscyclohexylammonium salt of **DL-123** [δ_p (121.5 MHz, 2H_2O) 44.48 ppm] and the former also possessing a chemical shift characteristic of a phosphorothioate monoester.

Though the two main products of methanolysis looked separable by standard silica column chromatography ($R_f = 0.24$ and 0.16 for **DL-184** and **DL-185** respectively using 30% ethyl acetate/petroleum ether), this was not attempted. Due to the detected isomerization of ring-opened products **DL-184** and **DL-185** under the reaction conditions, it was concluded that there was a risk that the planned methanolysis of **DL-171a** and **DL-171b** with [^{18}O]methanol could result in products [**172** and **173**] with the chirality at phosphorus scrambled.

This conclusion came from an observation by Knowles *et al.*^{73, 95} that the phospho group of 2-phosphopropane 1,2-diol **187** can migrate to the 1-position **188** by two routes under certain conditions (Scheme 2.20). The direct route is believed to proceed *via* a pentacoordinate intermediate that must, by the preference rules,^{*} pseudorotate at least once to yield the product.⁷⁵ This is an example of nucleophilic displacement at phosphorus, where the entering nucleophile is constrained to attack phosphorus 'adjacent' to the ligand that will become the leaving group. The second 'phosphodiester route' involves the formation of a cyclic 1,2-phosphodiester intermediate **189**. The stereochemistry of the direct route has been shown to proceed with *retention* of configuration at phosphorus

* See page 91.

whereas both the cyclisations of **187** and **188** (k_{2c} and k_{c1}) and the ring-opening reactions (k_{c2} and k_{c1}) should proceed with 'in-line' geometry.



Scheme 2.20: Pathways for the isomerization of 2-phosphopropane 1,2-diol **187** and 1-phosphopropane 1,2-diol **188**

From this work it can be seen that the chiral integrity of ring-opened [^{18}O]phosphorothioate triesters **172** and **173** would *only* be maintained if the observed isomerization between the triesters during methanolysis was due solely to pseudorotation via a pentacoordinate intermediate. If, however, the isomerization of **172** and **173** was to proceed *via* the formation of a cyclic phosphorothioate triester, one would envisage loss of chiral integrity at the phosphorus centre by virtue of the fact that either [^{16}O]- or [^{18}O]methanol could be lost on cyclisation.

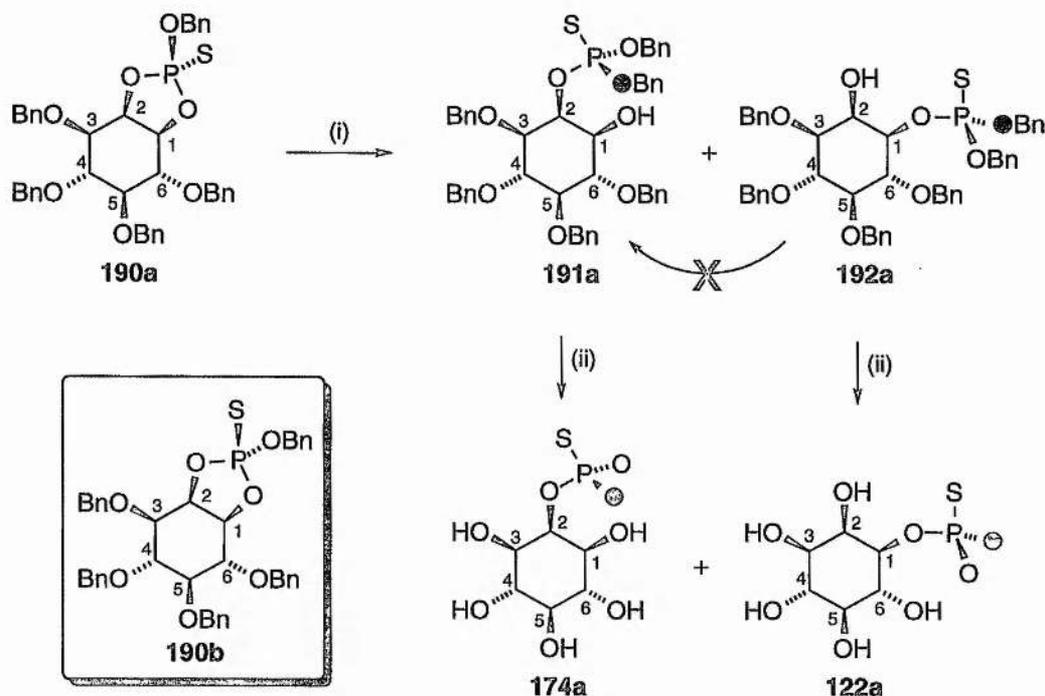
For practical reasons, it was decided not to repeat the methanolysis of **DL-171a** or **DL-171b** at $-78\text{ }^\circ\text{C}$ (the conditions previously used by Knowles¹⁰⁷, page 92) to investigate the temperature dependence of the isomerization reaction. Instead, other approaches were examined in an attempt to 'stabilise' the products of ring-opening at room temperature.

2.8.3 Alternative Approaches to the Controlled Ring-opening of Cyclic Phosphorothioate Triesters

The synthesis of the alternative *exo*- and *endo*-cyclic phosphorothioates triesters **DL-190a** and **DL-190b** was considered (Scheme 2.21). It was argued that the ring-opening of

these compounds with [^{18}O]benzyl alcohol [10 equiv. [^{18}O]benzyl alcohol, 10 equiv. triethylamine in dichloromethane at 25 °C] would produce two ring-opened compounds **191** and **192** which might be less prone to isomerization due to their more sterically hindered nature preventing the approach of the adjacent nucleophilic hydroxyl group (Scheme 2.21, shown for the *exo*-isomer **190a**).

For the *exo*-triester, it was hoped that the chromatographic separation of phosphorothioates **191a** and **192a** followed by mild deprotection of **192a** by hydrogenolysis [H_2 , (50 atm.), Pd on C] or dissolving metal-reduction would yield D-(R_p)-[^{18}O]inositol 1-phosphorothioate **122a** with the configuration at phosphorus known and intact (Scheme 2.21). A similar procedure on the ring-opened products of the *endo*-triester would yield D-(S_p)-[^{18}O]inositol 1-phosphorothioate **122b**.

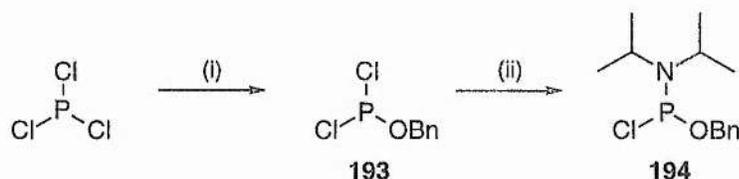


Reagents and conditions: (i) (a) BnOH , Et_3N , CH_2Cl_2 , 25 °C, then (b) chromatographic separation; (ii) $\text{Na}/\text{NH}_3(l)$, -78 °C.

Scheme 2.21: Alternative approach to the synthesis of D-(R_p)-[^{18}O]inositol 1-phosphorothioate **122a** via cyclic phosphorothioate triesters **190a**. A similar operation on triester **190b** would give rise to D-(S_p)-[^{18}O]inositol 1-phosphorothioate **122b**

The synthesis of cyclic phosphorothioate triesters **190a** and **190b** was proposed to be carried out from 3,4,5,6-tetrakis-*O*-benzyl *myo*-inositol **126** in a manner similar to that for the cyclic phosphorothioate triesters **171a** and **171b** (Scheme 2.17, page 100) by substituting *N,N*-diisopropylbenzylphosphonamidic chloride **194**^{188, 189} for *N,N*-diisopropylmethylphosphonamidic chloride [CIP(OCH₃)N(*i*-Pr)₂].

Phosphoramidite **194** was prepared according to the method of Prestwich *et al.*¹⁸⁸ (Scheme 2.22). As all attempts to purify **194** by distillation under reduced pressure resulted in significant decomposition, the crude product [δ_p (121.5 MHz, C²HCl₃) 179.89] was used without purification.



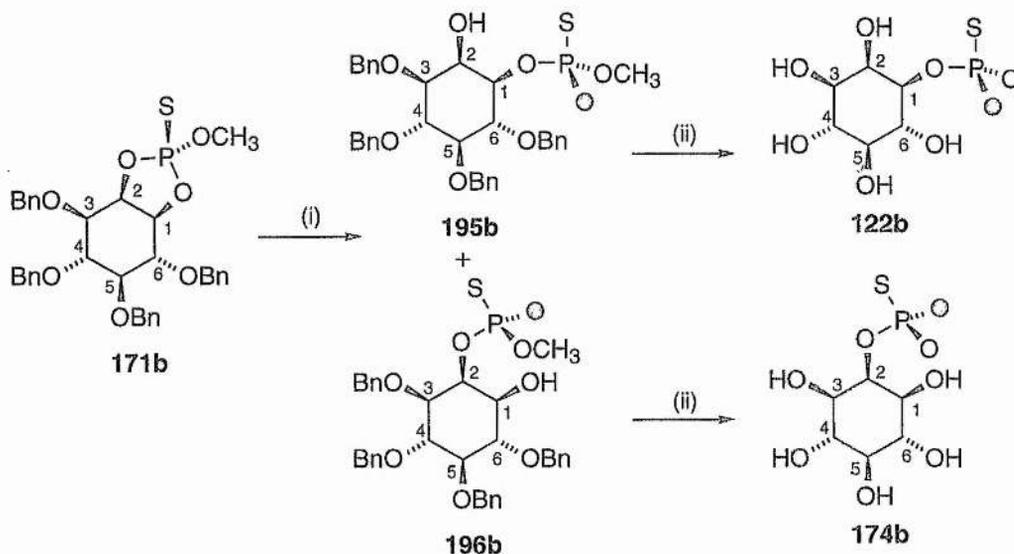
Reagents and conditions: (i) 1 equiv. BnOH, 1 equiv. pyridine, Et₂O, -20 °C, 20 min; (ii) 2 equiv. HN(*i*-Pr)₂, CH₂Cl₂, -20 °C to 25 °C, 1 h.

Scheme 2.22: Synthesis of *N,N*-diisopropylbenzylphosphonamidic chloride **194**

Unfortunately, under the conditions of Lin *et al.*^{182, 183} phosphoramidite **194** exhibited extremely poor reactivity towards diol **126** as judged by ¹H-NMR spectroscopy, and the cyclic phosphorothioates **190a** and **190b** did not form. After several failed attempts, this approach was discontinued. A possible cause for the observed difference in reactivity of phosphoramidite **194** and *N,N*-diisopropylmethylphosphonamidic chloride towards the hindered 2° hydroxyl groups of *DL*-**126** was the added steric bulk of the benzyl group in reagent **194**.

Another approach to circumvent the isomerization problem was to attempt the ring-opening of cyclic phosphorothioate triesters *DL*-**171a** and *DL*-**171b** with [¹⁸O]hydroxide instead of [¹⁸O]methanol (Scheme 2.23, shown for the *endo*-isomer *DL*-**171b**). It was

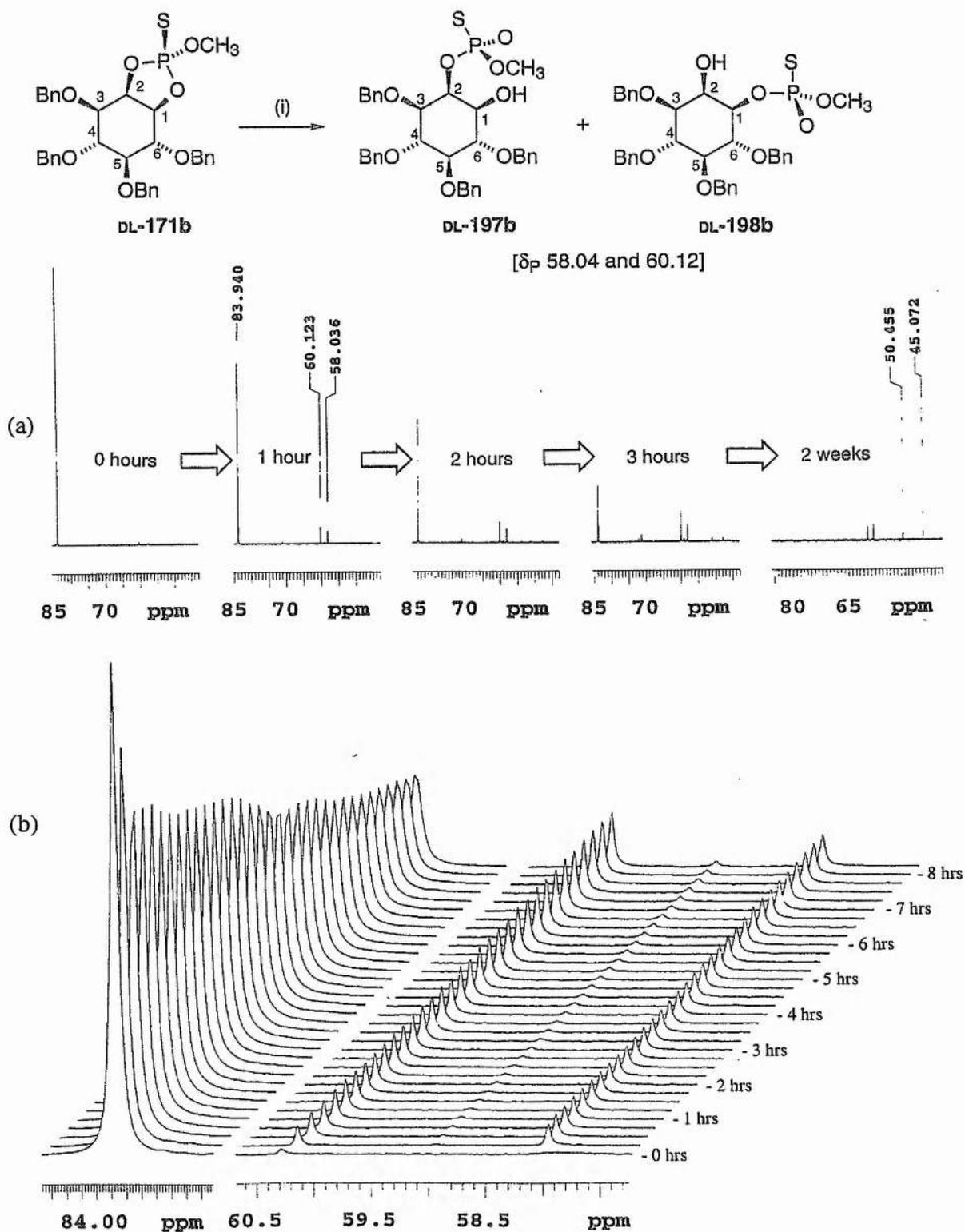
thought that the negative charge on the resultant ring-opened phosphorothioate diesters **195** and **196** might prevent the nucleophilic attack of the adjacent hydroxyl group and hence isomerization.¹³⁰ The approach, if successful, had the added benefit in that [¹⁸O]water could be used directly (by reaction with sodium), rather than having to synthesise and isolate a [¹⁸O]-labelled alcohol and introduce the risk of isotopic dilution.



Reagents and conditions: (i) (a) $\text{NaOH}_{(\text{aq})}$, $(\text{CH}_3)_2\text{SO}$, 25 °C, then (b) chromatographic separation; (ii) $\text{Na}/\text{NH}_3(\text{l})$, -78 °C.

Scheme 2.23: Proposed synthesis of *D*-(*S_p*)-[¹⁸O]inositol 1-phosphorothioate **122b**

Ring-opening of the *endo*-cyclic phosphorothioate **DL-171b** [δ_{p} (121.5 MHz) 83.94 ppm] was attempted with unlabelled hydroxide [10 equiv. NaOH (2.0 mol dm^{-3}) in dimethylsulfoxide at 25 °C] and the reaction monitored by ³¹P-NMR spectroscopy. Two products were seen to form quickly, consistent with the cyclic phosphorothioate having undergone a ring-opening reaction by the hydroxide ion to give products **DL-197b** and **DL-198b** [δ_{p} (121.5 MHz) 58.04 and 60.12, ratio 2.1:1, though the individual compounds were not identified] (Scheme 2.24). This time, full ³¹P-NMR analysis of the reaction course revealed that the ratio of signals of the two ring-opened products, under the reaction conditions, remained *constant* during the reaction and for several hours



Reagents and conditions: (i) NaOH (aq.), $(\text{CH}_3)_2\text{SO}$, 25 °C.

Scheme 2.24: Ring-opening of *endo*-cyclic phosphorothioate triester *DL-171b* with hydroxide monitored by ^{31}P -NMR spectrometry; (a) shows five spectra of the reaction taken at various time intervals, and (b) shows the same reaction viewed as an arrayed series of spectra

thereafter (Scheme 2.24). This suggested that compounds **DL-197b** and **DL-198b** were much less prone to isomerization. However, over extended periods, these products were slowly lost from the system and two new species were observed to form [δ_p (121.5 MHz) 45.07 and 50.45, ratio 1:2.4] possibly due to demethylation of **DL-197b** and **DL-198b**.

It is also interesting to note that the initial observation of only two signals in the ^{31}P -NMR spectrum implies that the initial ring-opening reaction is stereospecific as anticipated. Compounds **DL-197b** and **DL-198b** are *chiral* at phosphorus (unlike the products of methanolysis) and one would expect to see *four* ^{31}P -NMR signals if the chirality at phosphorus was being scrambled. Indeed, when the ring-opening reaction by hydroxide was attempted on the *exo*-cyclic phosphorothioate **DL-171a** [δ_p (121.5 MHz) 85.69 ppm] two different products were observed to form [δ_p (121.5 MHz) 55.35 and 60.70], presumably the opposite diastereomers **DL-197a** and **DL-198a**.

Although these initial results were promising, the ring-opened products could not be separated by standard silica column chromatography (chromatography, 0-10% methanol/dichloromethane, in 2% steps) and the route was abandoned at this stage.

In order to avoid the many practical difficulties experienced with the various attempted ring-opening reactions, an altogether new approach to the synthesis of (R_p)- and (S_p)- ^{18}O inositol 1-phosphorothioates **122a** and **122b** was sought (Section 2.10, page 118).

2.9 Synthesis of Enantiomerically Pure D- and L-3,4,5,6-Tetrakis-O-Benzyl *myo*-Inositol

It should be noted that throughout the 'ring-opening' studies discussed in Section 2.8, racemic (DL) forms of the various compounds were used since this had no effect on the observed chemical shifts. However, in order to ultimately synthesise the four compounds required for a full stereochemical study of inositol monophosphatase, namely D-(R_p)- and D-(S_p)- ^{18}O inositol 1-phosphorothioates **D-121a** and **D-121b** and L-(R_p)- and L-(S_p)- ^{18}O inositol 1-phosphorothioates **L-121a** and **L-121b** from cyclic phosphorothioate intermediates *via* ring-opening, it was necessary to develop a synthetic route to the

individual enantiomers of diol **126**, D- and L-3,4,5,6-tetrakis-O-benzyl *myo*-inositol **D-126** and **L-126**.

Approaches to the resolution of alcohols **D-126** and **L-126** were based on work by Billington *et al.*⁴⁷ who synthesised the individual enantiomers of inositol 1-phosphate, **D-10** and **L-10**. They demonstrated that the intermediate alcohol DL-2,3,4,5,6-penta-O-benzyl *myo*-inositol **DL-129** could be resolved by preparing the diastereomeric camphanate esters **D-199a** and **L-199b**, separating them by flash chromatography, and then removing the camphanate moiety by simple hydrolysis. The absolute configuration of the less polar camphanate ester was established by single crystal X-ray analysis,⁴⁷ permitting the configuration of the resolved alcohols **D-129** and **L-129** to be determined. This procedure is covered in greater detail in Section 2.13 on page 139.

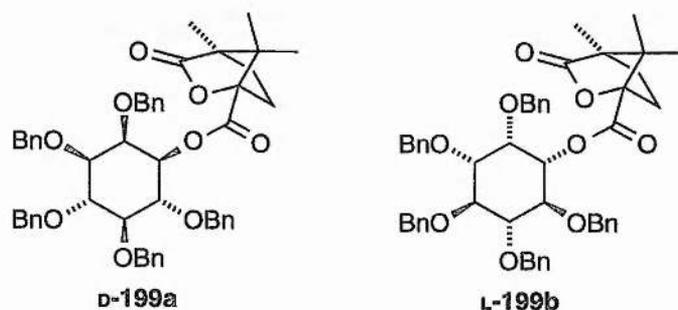
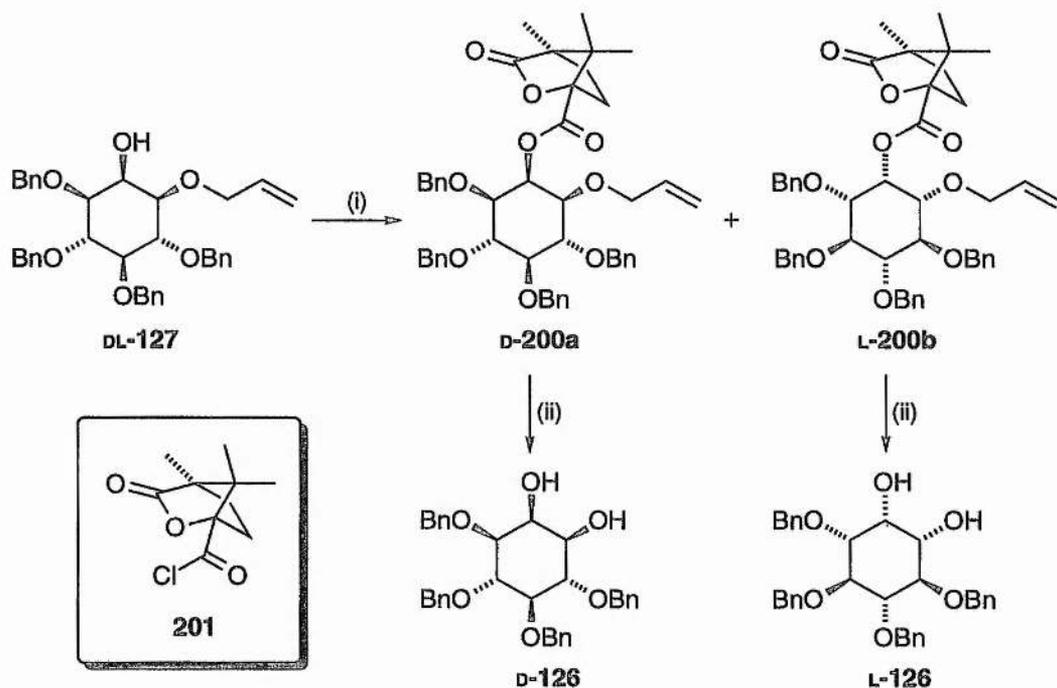


Figure 2.4: Diastereomeric camphanate esters synthesised by Billington *et al.*

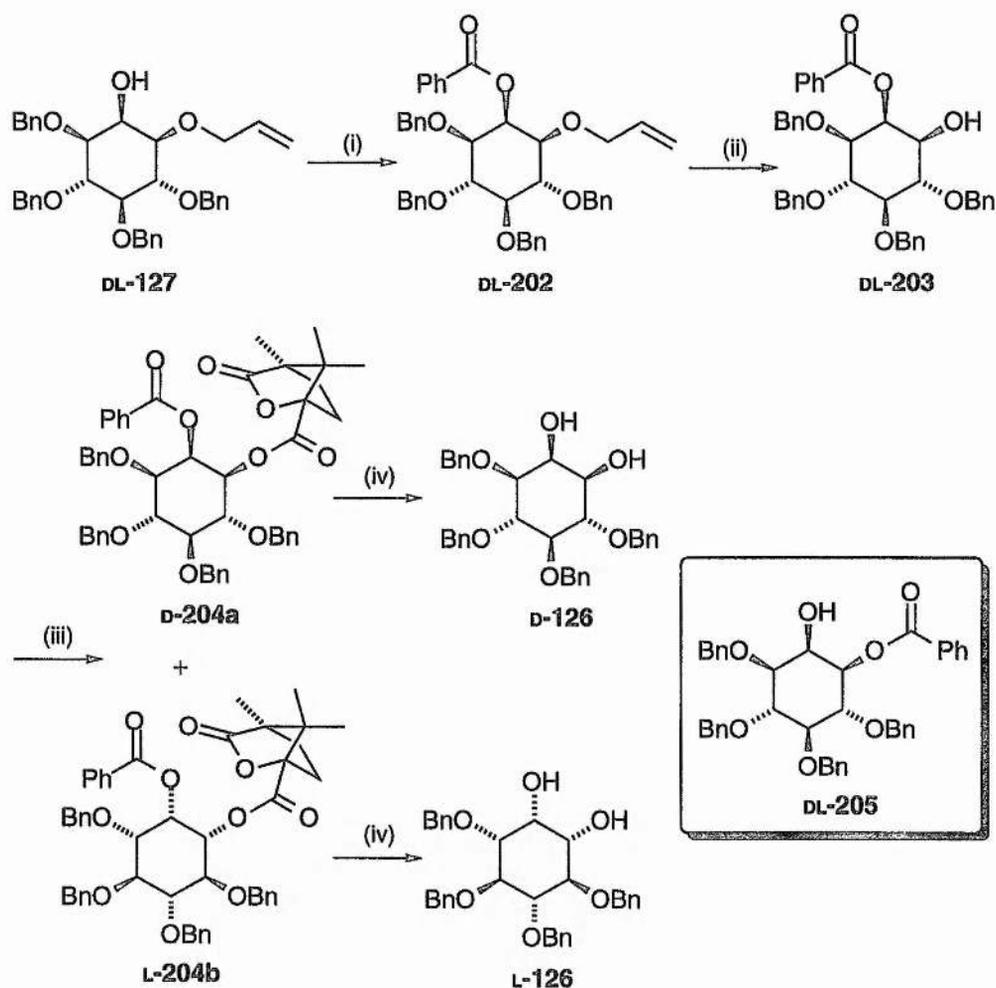
The proposed route to resolve diol **DL-126** is shown in Scheme 2.25. DL-1-O-Allyl-3,4,5,6-tetrakis-O-benzyl *myo*-inositol **DL-127**, an intermediate in the synthesis of DL-Ins1-P and DL-Ins1-P_s (Scheme 2.2, page 77), was treated with (1*S*,4*R*)-(-)-camphanic acid chloride **201** using the conditions of Billington *et al.*⁴⁷ to yield the diastereomeric camphanate esters **D-200a** and **L-200b** as a mixture [65%, *m/z* (CI⁺) 759 (4%, [M - H]⁺), *R_f* = 0.63 and *R_f* = 0.66 (2% diethyl ether/dichloromethane)].



Reagents and conditions: (i) (a) (1S,4R)-(-)-camphanic acid chloride **201**, CH_2Cl_2 , Et_3N , DMAP, $0\text{ }^\circ\text{C} \rightarrow 25\text{ }^\circ\text{C}$, 24 h, then (b) column chromatography (65%, mixture); (ii) (a) $(\text{Ph}_3\text{P})_3\text{RhCl}$, DABCO, $\text{EtOH}_{(\text{aq})}$, reflux, 4 h, then (b) AcOH, H_2O , reflux, 4 h, then (c) KOH, EtOH, $25\text{ }^\circ\text{C}$, 24 h.

Scheme 2.25: First attempt at the resolution of diol DL-126

Though ^1H - and ^{13}C -NMR spectra were consistent with the presence of a pair of diastereomeric products, the compounds could not be separated by silica column chromatography (0-3% diethyl ether/dichloromethane, in 1% steps). It was possible that the axial position of the camphanate group relative to the inositol ring was contributing to the poor separation of the diastereomers, and an alternative approach *via* diastereomeric camphanate esters **D-204a** and **L-204b** was attempted (Scheme 2.26). As these compounds resemble more closely the camphanate esters **D-199a** and **L-199b** synthesised by Billington *et al.*⁴⁷ it was hoped that they would be separable by column chromatography. Simple alkaline hydrolysis of both ester functionalities after separation would then yield both enantiomers of the diol.



Reagents and conditions: (i) benzoyl chloride, CH_2Cl_2 , pyridine, $-10\text{ }^\circ\text{C} \rightarrow 0\text{ }^\circ\text{C}$, 24 h, 85%; (ii) (a) $(\text{Ph}_3\text{P})_3\text{RhCl}$, DABCO, $\text{EtOH}_{(\text{aq})}$, reflux, 4 h, then (b) $\text{AcOH}_{(\text{aq})}$, reflux, 4 h, 67%; (iii) (a) (1*S*,4*R*)-(-)-camphanic acid chloride **201**, CH_2Cl_2 , Et_3N , DMAP, $0\text{ }^\circ\text{C} \rightarrow 25\text{ }^\circ\text{C}$, 24 h, then (b) column chromatography, 45% and 44%; (iv) KOH, EtOH, $25\text{ }^\circ\text{C}$, 24 h, 83% and 76%.

Scheme 2.26: Synthetic scheme for the successful resolution of diol DL-126

Alcohol DL-127 was therefore alternatively esterified with benzoyl chloride to give the fully protected *myo*-inositol DL-202 in good yield (85%).¹⁹⁰ The allyl group was then removed using the methodology of Corey and Suggs¹⁶⁶ (Scheme 2.4, page 79) to give DL-2-*O*-benzoyl-3,4,5,6-tetrakis-*O*-benzyl *myo*-inositol DL-203 as the major product [67%, $R_f = 0.24$ (20% ethyl acetate/petroleum ether)]. Unfortunately, the deprotection step was accompanied by the partial migration of the benzoyl group to the more stable 1-*O*

position,¹⁹⁰ giving the by-product DL-1-*O*-benzoyl-3,4,5,6-tetrakis-*O*-benzyl *myo*-inositol DL-205 [20%, $R_f = 0.33$ (20% ethyl acetate/petroleum ether)]* (Scheme 2.26).

Note that in the ¹H-NMR spectroscopic data for DL-205, the 1-CH signal is shifted *downfield* and the 2-CH signal is shifted *upfield* when compared to the ¹H-NMR spectroscopic data for DL-203 (page 205). This is consistent with the single benzoyl moiety being present on the 1-*O* position. The desired 2-*O*-benzoyl product DL-203 was easily isolated from this minor trans-benzoylation by-product DL-203 by silica column chromatography (ethyl acetate/petroleum ether). It was subsequently treated with (1*S*,4*R*)-(-)-camphanic acid chloride 201 under the conditions of Billington *et al.*⁴⁷ to yield the diastereomeric camphanate esters D-204a and L-204b [$R_f = 0.31$ and 0.35 (3% diethyl ether/dichloromethane), configurations unknown] which were successfully resolved by silica column chromatography (0-2% diethyl ether/dichloromethane, in 1% steps).

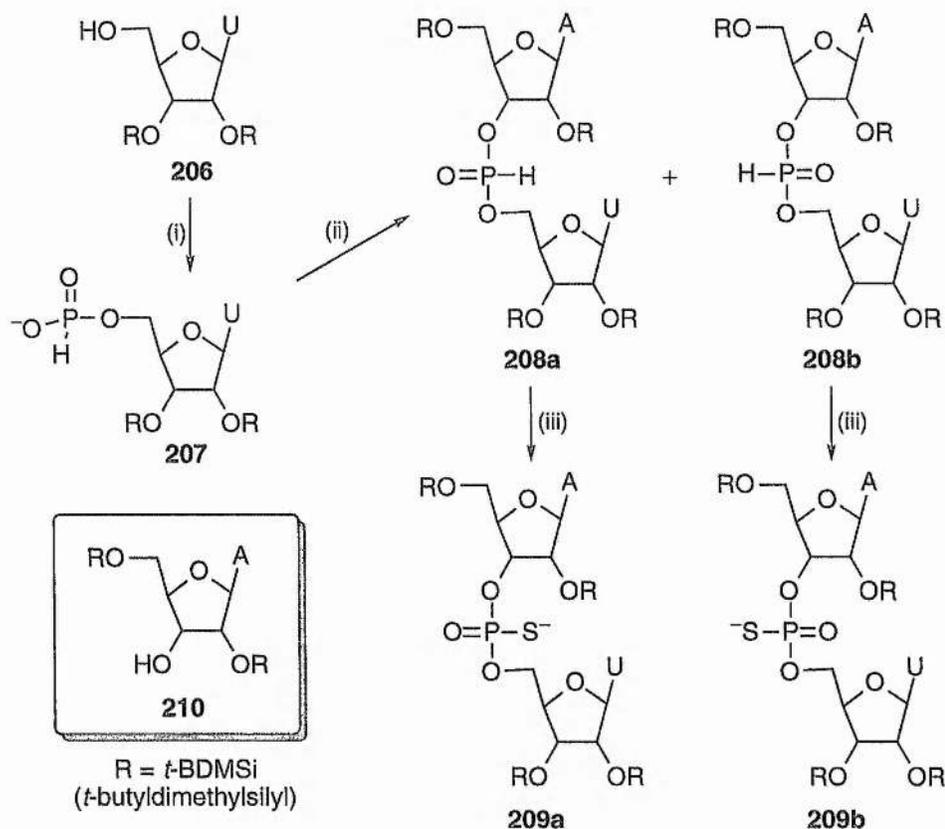
It was proposed to determine the configuration of the resolved diols by single crystal X-ray analysis, but initial attempts to recrystallise both camphanate ester from ethanol or methanol were unsuccessful. The individual diesters D-204a and L-204b were hydrolysed efficiently by ethanolic potassium hydroxide to yield the resolved diols which were purified by silica column chromatography (diol from higher R_f diastereomer 83%, diol from lower R_f diastereomer 76%; ¹H- and ¹³C-NMR spectra for both products being identical to that obtained for the racemic compound DL-126). No further attempts were made to determine the configurations of the resolved diols as the 'ring-opening' approach to the synthesis of (*R*_p)- and (*S*_p)-[¹⁸O]inositol 1-phosphorothioates 122a and 122b was ultimately abandoned.

* Selected data for benzoyl-group migration product DL-205: δ_H (300 MHz, C²HCl₃) 3.61 (1 H, t, Ins-CH), 3.63 (1 H, dd, $J_{2,3}$ 2.7, $J_{3,4}$ 9.3, 3-CH), 4.01 (1 H, t, Ins-CH), 4.24 (1 H, t, Ins-CH), 4.43 (1 H, t, $J_{1,2}$ 2.7, $J_{2,3}$ 2.7, 2-CH), 4.71–4.90 (8 H, m, 4 x CH₂Ph), 5.11 (1 H, dd, $J_{6,1}$ 10.2, $J_{1,2}$ 2.7, 1-CH), 7.11–7.32 (20 H, m, Benzyl Ar-CH) and 7.45, 7.57 and 8.08 (5 H, 3 x m, Benzoyl Ar-CH); δ_C (75.4 MHz, C²HCl₃) 67.93 (Ins-CH), 72.87 (CH₂Ph), 73.84 (Ins-CH), 75.74 and 75.97 (CH₂Ph), 79.04, 80.03, 81.21 and 83.20 (Ins-CH), 127.61, 127.67, 127.97, 128.10, 128.29, 128.43, 128.52 and 128.62 (Benzyl Ar-CH), 129.88 and 133.31 (Benzoyl Ar-CH), 137.60, 138.21, 138.60 and 138.67 (Ar-C quaternary) and 166.10 (C=O).

2.10 The Synthesis of Chiral Phosphorothioates using 'H-Phosphonate' Methodology

It was decided to consider again DL-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol DL-129 as a possible starting point in the synthesis of (*R*_p)- and (*S*_p)-[¹⁸O]inositol 1-phosphorothioates 122a and 122b. The major attraction of this approach was that the alcohol could be resolved by a simple two-step procedure (Section 2.13 on page 139) and the configuration of the individual enantiomers was already solved.⁴⁷

Almer *et al.*¹⁹¹ had demonstrated that a stereochemically homogenous diribonucleoside phosphorothioate could be prepared in an efficient manner *via* the stereospecific sulfurization of a *H*-phosphonate diester (Scheme 2.27).



Reagents and conditions: (i) H_3PO_3 , PvCl , pyridine, 25 °C; (ii) (a) 210, PvCl , pyridine, then (b) chromatography; (iii) S_8 , pyridine.

Scheme 2.27: Synthesis of stereochemically homogenous diribonucleoside phosphorothioates using *H*-phosphonate methodology

The procedure involved the initial formation of a *H*-phosphonate salt **207** which was then coupled to the 2° alcohol of a second ribonucleoside unit **210** to yield a pair of diastereomeric *H*-phosphonate diesters **208a** and **208b** which were found to be readily separable by ordinary silica gel chromatography. As each diribonucleoside *H*-phosphonate could then be sulfurized in a *completely stereospecific manner* to give **209a** and **209b** respectively,¹⁹¹ an efficient route became available for the synthesis of chiral phosphorothioate diesters with intact stereochemistry at phosphorus (Scheme 2.27).

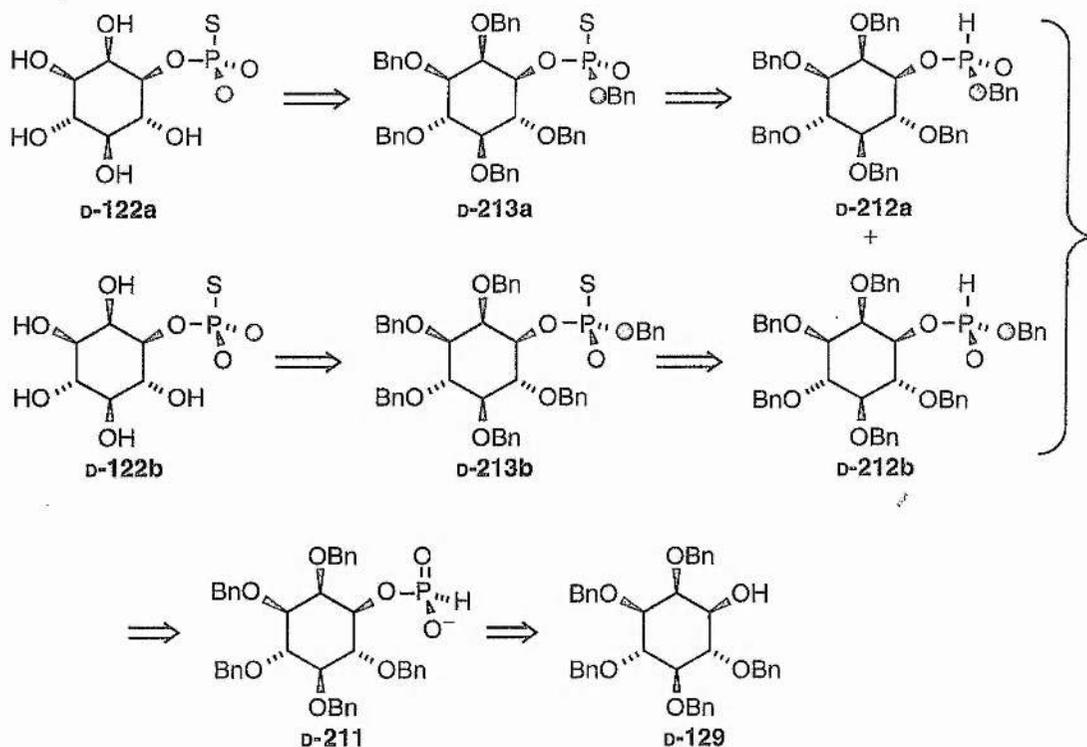
Most methods prior to this development necessitated either separation of a pair of chiral phosphorothioate diastereomers after a final deprotection step, or separation of the chiral phosphorothioate precursors followed by deprotection.^{130, 192-194} In either case, the resolution step was laborious due to the presence of the charge on sulfur. As the '*H*-phosphonate' approach conveniently avoids this problem by introducing the sulfur atom *after* the separation step, it has rapidly become the method of choice for the preparation of phosphorothioate diesters where the optical purity of the chiral phosphorus centre is a stringent requirement.^{195, 196}

It became apparent that if one was to replace the first ribonucleoside unit in Almers' synthesis **206** with D- or L-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol **D-129** or **L-129** and the second ribonucleoside unit **210** with [¹⁸O]benzyl alcohol, a pair of [¹⁸O]benzyl *H*-phosphonate diastereomers **D-212a** and **D-212b** could be produced *via H*-phosphonate salt **D-211** (Scheme 2.28). If these diastereomers were readily separable by column chromatography and the sulfurization of each diastereomer was shown to proceed in a stereospecific manner, the protected [¹⁸O]phosphorothioates **D-213a** and **D-213b** would be formed. Deprotection of each phosphorothioate by mild metal-reduction would then give D-(*R*_p)- and D-(*S*_p)-[¹⁸O]inositol 1-phosphorothioates **D-122a** and **D-122b** or L-(*R*_p)- and L-(*S*_p)-[¹⁸O]inositol 1-phosphorothioates **L-122a** and **L-122b** in four steps from the relevant alcohol (Scheme 2.28).

The procedure involved the initial formation of a *H*-phosphonate salt **207** which was then coupled to the 2° alcohol of a second ribonucleoside unit **210** to yield a pair of diastereomeric *H*-phosphonate diester **208a** and **208b** which were found to be readily separable by ordinary silica gel chromatography. As each diribonucleoside *H*-phosphonates could then be sulfurized in a *completely stereospecific manner* to give **209a** and **209b** respectively,¹⁹¹ an efficient route became available for the synthesis of chiral phosphorothioate diesters with intact stereochemistry at phosphorus (Scheme 2.27).

Most methods prior to this development necessitated either separation of a pair of chiral phosphorothioate diastereomers after a final deprotection step, or separation of the chiral phosphorothioate precursors followed by deprotection.^{130, 192-194} In either case, the resolution step was laborious due to the presence of the charge on sulfur. As the '*H*-phosphonate' approach conveniently avoids this problem by introducing the sulfur atom *after* the separation step, it has rapidly become the method of choice for the preparation of phosphorothioate diesters where the optical purity of the chiral phosphorus centre is a stringent requirement.^{195, 196}

It became apparent that if one was to replace the first ribonucleoside unit in Almers' synthesis **206** with D- or L-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol **D-129** or **L-129** and the second ribonucleoside unit **210** with [¹⁸O]benzyl alcohol, a pair of [¹⁸O]benzyl *H*-phosphonate diastereomers **D-212a** and **D-212b** could be produced *via H*-phosphonate salt **D-211** (Scheme 2.28). If these diastereomers were readily separable by column chromatography and the sulfurization of each diastereomer was shown to proceed in a stereospecific manner, the protected [¹⁸O]phosphorothioates **D-213a** and **D-213b** would be formed. Deprotection of each phosphorothioate by mild metal-reduction would then give D-(*R_p*)- and D-(*S_p*)-[¹⁸O]inositol 1-phosphorothioates **D-122a** and **D-122b** or L-(*R_p*)- and L-(*S_p*)-[¹⁸O]inositol 1-phosphorothioates **L-122a** and **L-122b** in four steps from the relevant alcohol (Scheme 2.28).



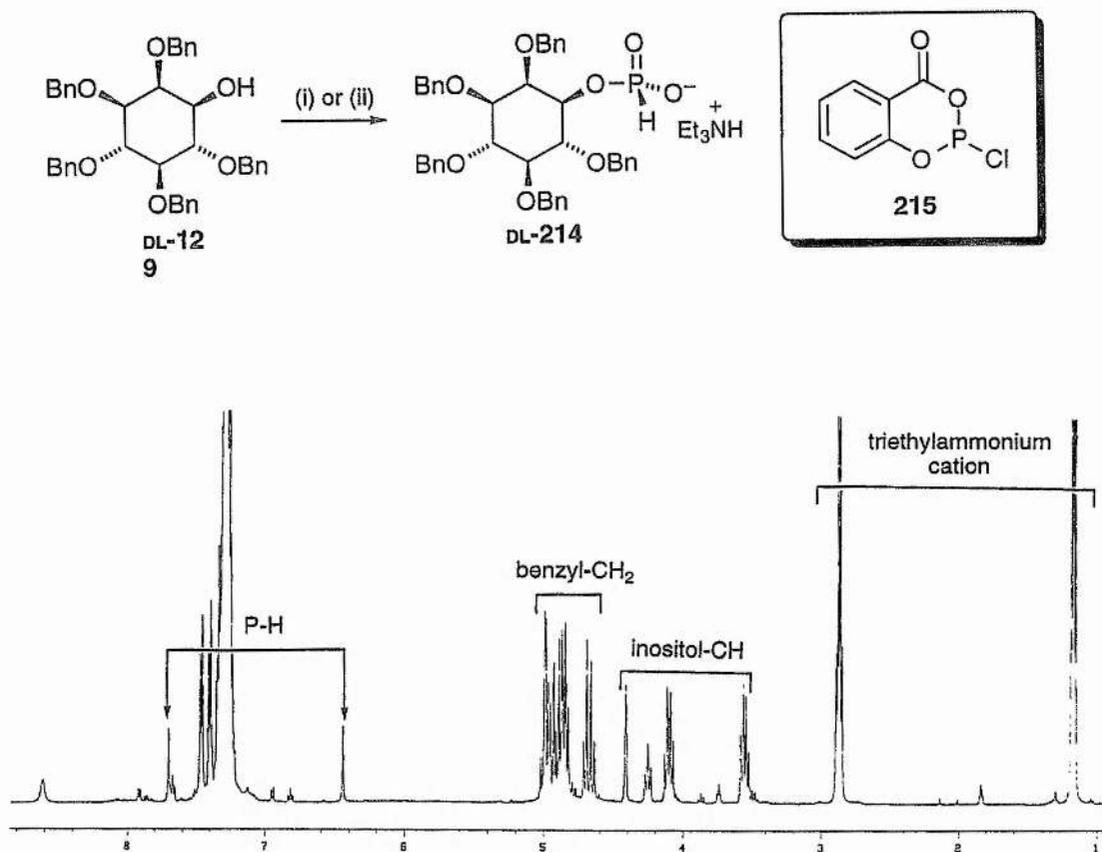
Scheme 2.28: Rationale behind the synthesis of *D*-(R_p)- and *D*-(S_p)-[¹⁸O]inositol 1-phosphorothioates *D*-122a and *D*-122b from alcohol *D*-129 using *H*-phosphonate methodology

2.11 Synthesis of *D*- and *L*-Inositol 1-Phosphorothioate using *H*-Phosphonate Methodology

The racemic alcohol *DL*-129 was used in the initial exploratory work for the proposed *H*-phosphonate route since this had no effect on the observed chemical shifts.

DL-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol *DL*-129 was successfully condensed with phosphorous acid (H₃PO₃) in the presence of pivaloyl chloride (PvCl, trimethylacetyl chloride) according to the method of Almer,¹⁹¹ and the reaction quenched with triethylammonium bicarbonate buffer (TEAB buffer, 2.0 mol dm⁻³)¹⁹⁷ to obtain the *H*-phosphonate building block *DL*-214 as its stable triethylammonium salt [δ_p (121.5 MHz; C²HCl₃) 4.47]. Purification of the salt was accomplished by silica column chromatography using a stepwise gradient of methanol in dichloromethane (2–10%, containing 0.1% triethylamine) to give the product *DL*-214 in a 48% yield. The ¹H-NMR

spectrum revealed an extremely large $^1J_{\text{PH}}$ coupling constant, [δ_{H} (500 MHz; C^2HCl_3) 7.07 (1 H, d, $J_{\text{P-H}}$ 629.0, P-H)], which is a characteristic of all *H*-phosphonates (Scheme 2.29).



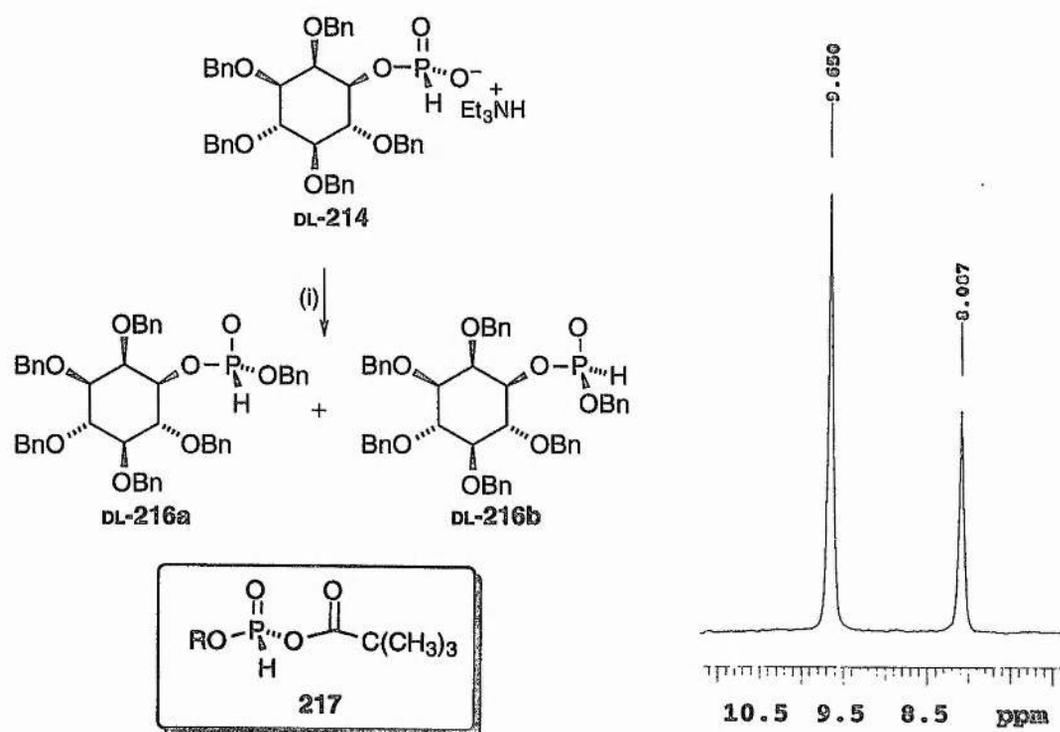
Reagents and conditions: (i) (a) H_3PO_3 , PvCl , pyridine, 25 °C, 3 h, then (b) TEAB, 20 min, 48%; (ii) (a) 2-chloro-4*H*-1,3,2-benzo-dioxaphosphorin-4-one **215**, pyridine:THF (1:4, v/v), 0–25 °C, 15 min, then (b) water, 10 min, then (c) TEAB, 20 min, 75%.

Scheme 2.29: *The synthesis of inositol H-phosphonate building block DL-214 (the $^1\text{H-NMR}$ spectrum (500 MHz) of the purified compound is shown)*

In an attempt to improve the yield of DL-214, alcohol DL-129 was reacted with 2-chloro-4*H*-1,3,2-benzo-dioxaphosphorin-4-one (salicylchlorophosphate) **215** in the presence of pyridine (THF:pyridine, 4:1 v/v, 0 °C).^{198, 199} The reagent was developed as an alternative

to phosphorous acid (H_3PO_3) in the synthesis of *H*-phosphonate monoesters such as DL-214, due to its stable crystalline nature and ease of handling.²⁰⁰⁻²⁰² The reaction proceeded rapidly to give the product, after quenching with TEAB (2.0 mol dm^{-3}), in only 15 min. After column chromatography (as above) the purified product was recovered in a 75% yield. The spectroscopic data was identical to that of *H*-phosphonate DL-214.

Formation of the *H*-phosphonate diesters DL-216a and DL-216b was accomplished via the condensation of DL-214 with benzyl alcohol (Scheme 2.30).



Reagents and conditions: (i) (a) BnOH, PvCl, pyridine:THF (1:1, v/v), 25 °C, 8 h, then (b) TEAB, 20 min, 85% for the mixture.

Scheme 2.30: Coupling reaction of inositol *H*-phosphonate salt DL-214 with benzyl alcohol (only the *D*-enantiomers are shown for clarity)

The reaction was carried out in THF:pyridine (1:1, v/v, 25 °C) using pivaloyl chloride as the coupling reagent,^{203, 204} to give two enantiomeric pairs of *H*-phosphonates in good yield [higher R_f pair DL-216a, $R_f = 0.21$ (30% ethyl acetate/petroleum ether), $\delta_p(121.4$

MHz; C^2HCl_3) 8.08, 31%; lower R_f pair DL-216b, $R_f = 0.16$ (30% ethyl acetate/petroleum ether), $\delta_p(121.4 \text{ MHz; } C^2HCl_3) 9.65, 55\%]^*$ (Figure 2.5).

The mechanism of activation of such coupling reactions has been previously investigated and the course of the reaction is thought to involve the intermediate formation of a mixed phosphonate-carboxylic anhydride 217.²⁰⁵ The creation of the lower R_f H -phosphonate enantiomeric pair DL-216b (Figure 2.5) was always favoured by the coupling reaction (ratio DL-216a to DL-216b 1:1.8) as can be seen from the ^{31}P -NMR spectrum of the crude mixture (Scheme 2.30). The stereoselectivity during condensation is probably due to steric influence from the adjacent O -benzyl groups in the inositol ring.

The two pairs of H -phosphonates diastereomers were successfully separated by silica column chromatography (Figure 2.5).

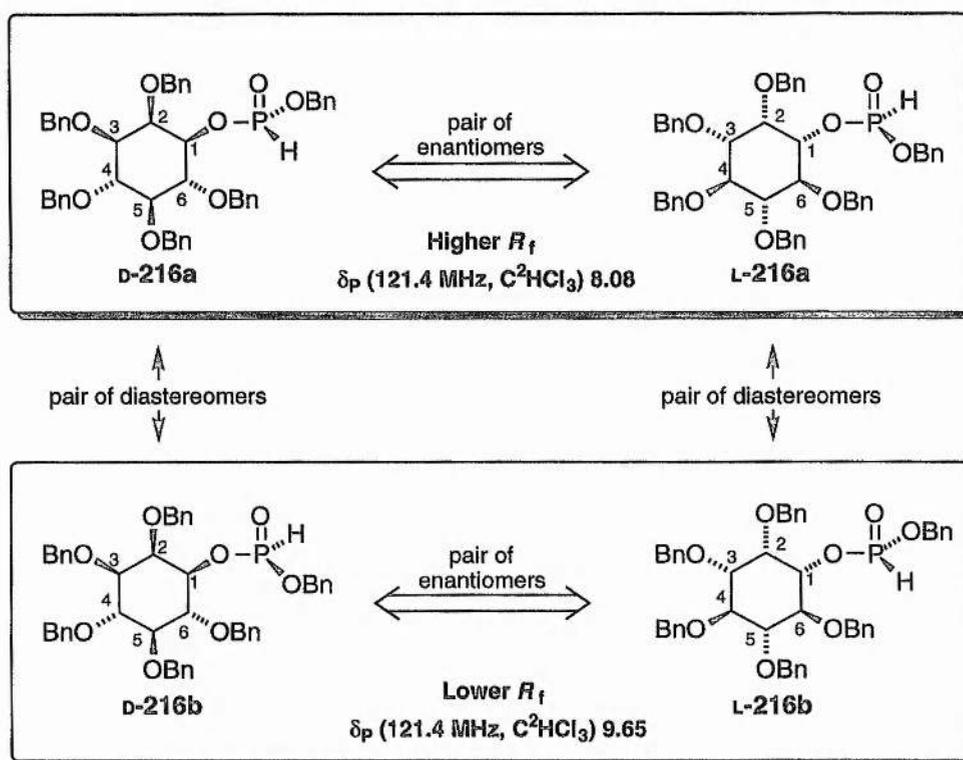


Figure 2.5: Complex relationship between the H -phosphonate diesters formed from the racemic alcohol DL-129

* The absolute configuration at phosphorus of the benzyl H -phosphonates was determined by single crystal X-ray analysis (see Section 2.12, page 129 and Appendix 4.4, page 249).

The best separation was achieved using a stepwise gradient of ethyl acetate in dichloromethane (0-10%, in 2% steps) as the eluent. Unfortunately, as some of the excess pivaloyl chloride and benzyl alcohol was found to partially co-elute with the *H*-phosphonates, it was first necessary to purify the crude diastereomeric mixture. This was carried out using a short silica column and eluting with a gradient of ethyl acetate in petroleum ether (0-35%, in 5% seps). The pivaloyl chloride and benzyl alcohol were found to elute well before the *H*-phosphonates, allowing the latter to be recovered as an unseparated, but impurity free, mixture of diastereomers.

Occasionally, during the course of the coupling reaction, a third product was detected by TLC ($R_f = 0.34$, 30% ethyl acetate/petroleum ether) which was readily isolated by the column chromatography described above. The compound has spectral characteristics consistent with the bicyclic structure shown in Figure 2.6. The putative formation of the dimer **218** may be caused by an excess of pivaloyl chloride activating the further coupling of the benzyl *H*-phosphonate product molecules to each other, but no clear evidence for its source was obtained.

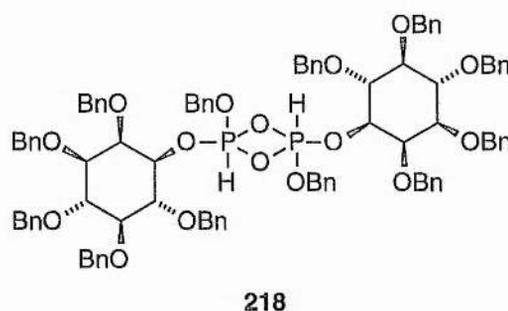
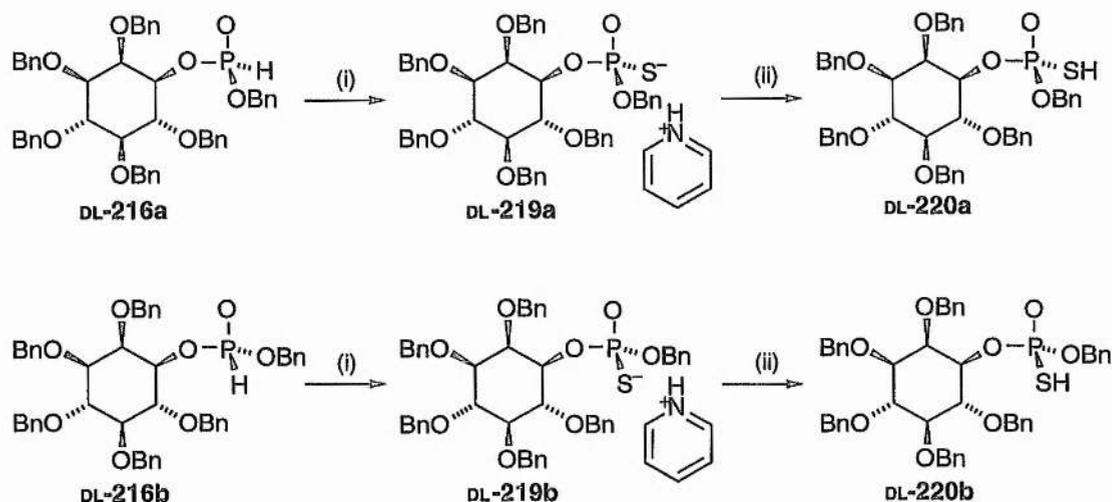


Figure 2.6: Putative by-product in the *H*-phosphonate coupling reaction

* Selected data for putative dimer by-product **218**: δ_H (300 MHz; C^2HCl_3) 3.39 (1 H, dd, 3-CH), 3.48 (1 H, t, Ins-CH), 3.49 (1 H, dt, 1-CH), 3.88 (1 H, t, Ins-CH), 3.99 (1 H, t, Ins-CH), 4.22 (1 H, t, 2-CH), 4.61–5.07 (12 H, m, 5 x CH_2Ph and $POCH_2Ph$), 7.08 (1 H, d, J_{P-H} 736, P-H) and 7.26–7.42 (30 H, m, Ar-H); δ_C (75.4 MHz; C^2HCl_3) 65.81 (d, J_{C-P} 6.5, $POCH_2Ph$), 72.26, 72.81, 75.89, 75.95 and 76.02 (CH_2Ph), 72.40 (d, $J_{C,P}$ 7.6, Ins-C), 78.19 (d, $J_{C,P}$ 3.2, Ins-C), 78.73, 81.17 and 82.88 (Ins-C), 127.70, 127.88, 127.90, 128.02, 128.08, 128.23, 128.32, 128.36, 128.41 and 128.54 (Ar-CH) and 137.49, 137.60 and 138.55 (Ar-C quaternary); δ_P (121.4 MHz; C^2HCl_3) 8.21; m/z (CI^+) 1569 (1%, $[M + H]^+$), 785 (25%, $[C_{48}H_{49}O_8P + H]^+$), 205 (100%, $[Inositol + 2H + Na]^+$).

The separate pairs of enantiomers of DL-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-(*O*-benzyl)-hydrogen phosphonate DL-216 were then sulfurized with elemental sulfur using pyridine as the solvent (0.5 mol dm⁻³ solution, typically 20 equivalents relative to DL-216) (Scheme 2.31).



Reagents and conditions: (i) S₈, pyridine, 25 °C, 16 h; (ii) Florisil[®], column chromatography (2-10% CH₃OH/DCM), overall 91%.

Scheme 2.31: Stereospecific sulfurization of *H*-phosphonate diesters DL-216a and DL-216b, to yield phosphorothioate diesters DL-219a and DL-219b respectively (only the *D*-enantiomers are shown for clarity)

By monitoring the process by ³¹P-NMR spectrometry, it was established that the sulfurization reactions proceeded in a clean manner and with *complete stereospecificity*, affording the corresponding pure isomers of DL-219 as anticipated (Figure 2.7)*.

Sulfurization of the higher *R_f* *H*-phosphonate diester DL-216a [δ_p (121.4 MHz; C²HCl₃) 8.08] gave the corresponding phosphorothioate pyridinium salt DL-219a [δ_p (121.4 MHz; C²HCl₃) 61.08], whereas the reaction performed on the lower *R_f* *H*-phosphonate diester DL-216b [δ_p (121.4 MHz; C²HCl₃) 9.65] gave phosphorothioate pyridinium salt DL-219b [δ_p (121.4 MHz; C²HCl₃) 59.81]. By mixing the two phosphorothioates

* The absolute configuration at phosphorus of the phosphorothioates was also determined by single crystal X-ray analysis (see Section 2.12, page 129 and Appendix 4.5, page 254).

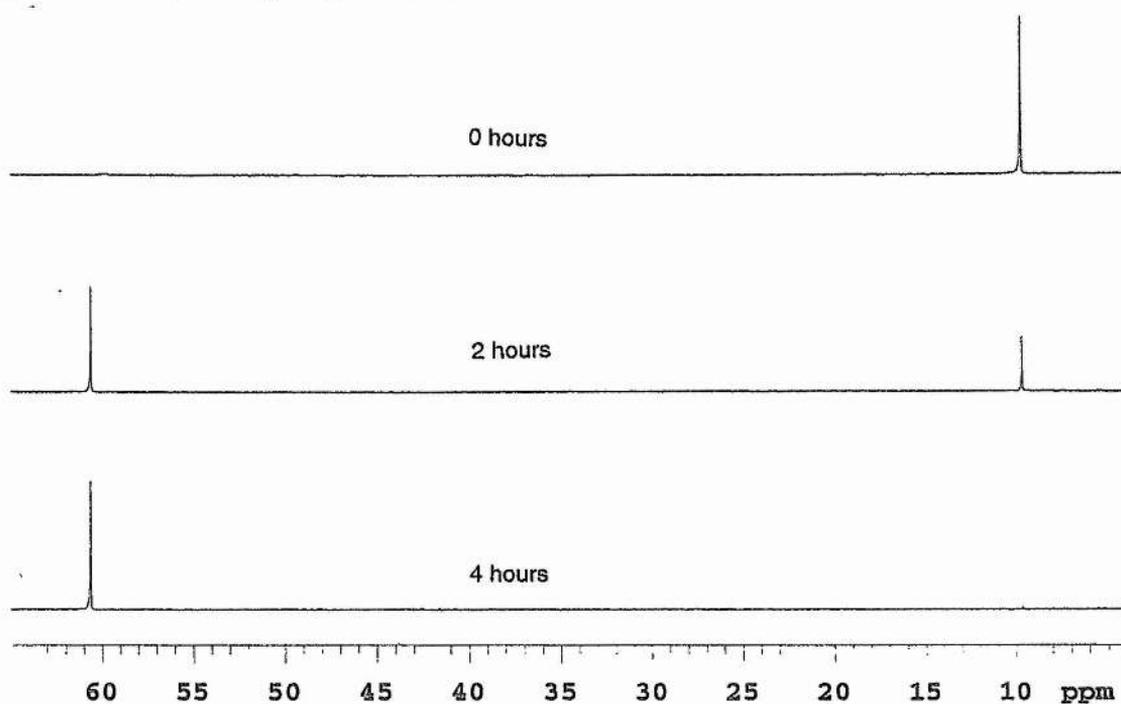
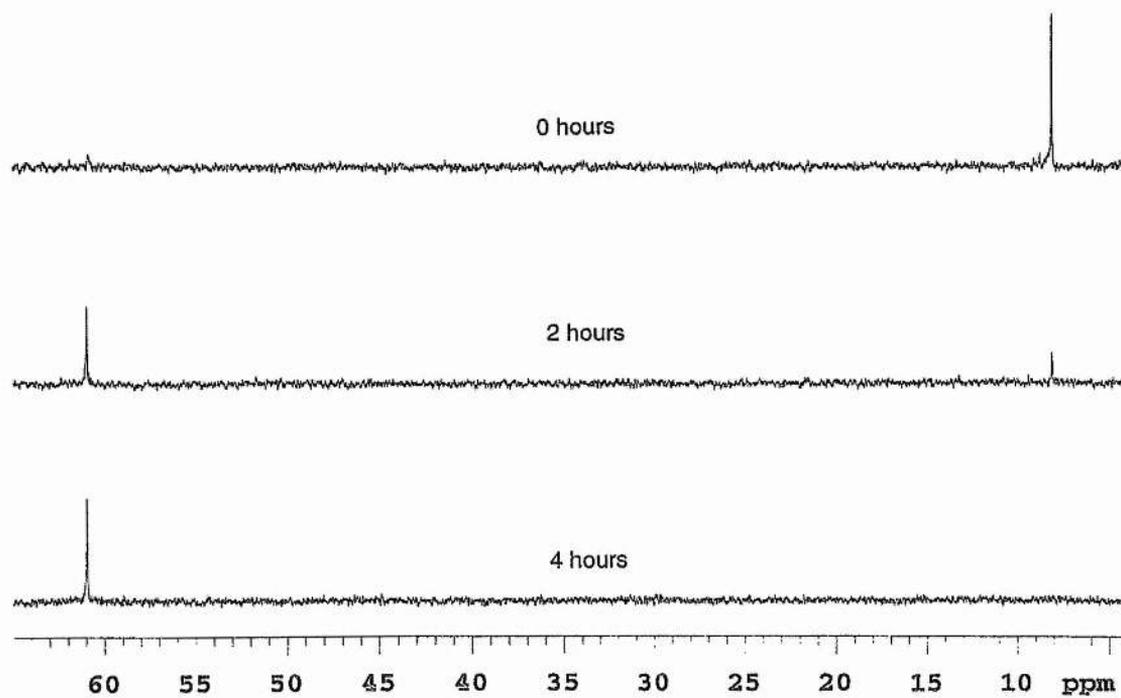
(a) Sulfurization of *H*-phosphonate diester DL-216a:(b) Sulfurization of *H*-phosphonate diester DL-216b:

Figure 2.7: Stereospecific sulfurization of *H*-phosphonate diesters DL-216a and DL-216b in pyridine, as monitored by ^{31}P -NMR spectroscopy

samples together, it was confirmed that each sulfurization reaction did give rise to a different phosphorothioate enantiomeric pair.

After completion of each sulfurization reaction [16 h, as judged by TLC (10% methanol/dichloromethane)], the solvents were removed and the crude residue redissolved in dichloromethane, filtered to remove the excess sulfur, and solvents removed again under reduced pressure to give the respective phosphorothioates as their crude pyridinium salts. Purification of each compound was attempted by column chromatography using the conditions of Almer *et al.*¹⁹¹ [flash silica, gradient of 2–20% methanol/dichloromethane in 2% steps] but significant decomposition of the phosphorothioates occurred, probably due to the acidity of the silica. Florisil[®] is an activated magnesium silicate commonly used as an adsorbent in gas chromatography, but is suitable for use in column chromatography as an alternative to silica.²⁰⁶ When this support was used and the column eluted with a gradient of methanol in dichloromethane (2–10%, in 2% steps) there was no evidence of decomposition, and the phosphorothioates were recovered as their respective thiophosphoric acids DL-220a and DL-220b in excellent yield (typically greater than 90%).²⁰⁷

Evidence for the formation of the thiophosphoric acids (probably due to the acidity of the Florisil[®]) was seen in an upfield shift and broadening of the ³¹P-NMR signal caused by the loss of the deshielding affect of the pyridinium cation (for example DL-219a to DL-219a; δ_p (121.4 MHz; C²HCl₃) 61.08 to 54.34) and in the lack of pyridinium signals in the ¹H and ¹³C-NMR spectra.

It is interesting to note that as the putative by-product from the *H*-phosphonate coupling reaction 218 (Scheme 2.30, page 122 and Figure 2.6, page 124) also contains a P-H bond, as observed in the ¹H-NMR spectrum, one would expect the compound to undergo a similar sulfurization reaction. When the compound was dissolved in pyridine and elemental sulfur added, a single phosphorothioate species was indeed observed to form [δ_p (121.4 MHz; C²HCl₃) 61.15 for the crude pyridinium salt].

Both thiophosphoric acids **DL-220a** and **DL-220b** were then successfully deprotected by reductive cleavage with sodium in liquid ammonia and tetrahydrofuran to give the *same* disodium salt of *DL-myo*-inositol 1-phosphorothioate which was purified as previously by ion exchange chromatography on Amberlite IR118 (H⁺) and converted to the crystalline bis-dicyclohexylammonium salt **DL-221** [δ_p (121.4 MHz; C²HCl₃) 44.46], in 44% overall yield. ¹H and ¹³C-NMR data was in agreement with the spectral data for inositol 1-phosphorothioate **DL-123** previously synthesised according to the method of Baker *et al.*⁴⁸ (Section 2.3, page 80).

As the removal of the benzyl groups of the thiophosphoric acids **DL-220a** and **DL-220b** by reductive cleavage would not be expected to perturb the stereochemistry at the phosphorus centre,¹⁰⁶ the successful synthesis of inositol 1-phosphorothioate using *H*-phosphonate methodology demonstrated that this route *could* be used to synthesise the chiral target molecules (*R*_p)- and (*S*_p)-[¹⁸O]inositol 1-phosphorothioates **122a** and **122b** by simply substituting the benzyl alcohol with the [¹⁸O]-labelled alcohol.

2.12 Configurational Determination D-(R_p)-, D-(S_p)-, L-(R_p)- and L-(S_p)- [^{18}O]Inositol 1-Phosphorothioates

In order for the D-(R_p)-, D-(S_p)-, L-(R_p)- and L-(S_p)-[^{18}O]inositol 1-phosphorothioates **D-122a**, **D-122b**, **L-122a** and **L-122b** produced by this route to be of any use in the stereochemical analysis of inositol monophosphatase, the configuration at phosphorus for each chiral molecule *must* be known. Due to the often crystalline nature of inositol derivatives, it was proposed to achieve this by single crystal X-ray analysis.

Though an ^{18}O -atom itself is indistinguishable from an ^{16}O -atom by X-ray analysis, by using the *H*-phosphonate approach the ^{18}O -atom is introduced into the molecule as [^{18}O]benzyl alcohol and as a consequence the ^{18}O -atom becomes visible by virtue of the attached benzyl group. In other words, the benzyl group on the phosphorus of *H*-phosphonate **216** or phosphorothioate **219** acts an easily removable 'tag' with which to locate the ^{18}O -atom in the molecule. Therefore, if the absolute structure of a *single enantiomer* of phosphorothioate **219a** or **219b** could be determined by X-ray crystallographic analysis, then the location of the [^{18}O]atom in the molecule when [^{18}O]benzyl alcohol is used in the synthesis would be known. As the final reductive step to remove the benzyl group does *not* involve phosphorus-oxygen bond fission,¹⁰⁶ the stereochemistry at phosphorus of the deprotected final product should be *identical* to that of the protected phosphorothioate precursor. Hence, by indirect methods, the absolute configuration of (R_p)- and (S_p)-[^{18}O]inositol 1-phosphorothioates **122a** and **122b** could be determined. Moreover, since the sulfurization reactions were shown to be completely stereospecific and such processes are thought to proceed with *retention of configuration* at phosphorus (see Figure 2.12, page 137), determination of the absolute structure of a *single enantiomer* of the *H*-phosphonate precursors **216a** or **216b** would also allow the absolute configuration of (R_p)- and (S_p)-[^{18}O]inositol 1-phosphorothioates **122a** and **122b** to be predicted. This second approach was of interest as phosphorothioates often give crystals of poor diffracting quality and/ or crystals that decay significantly during measurement.²⁰⁸

Therefore, as an initial approach to the problem, each of the four *H*-phosphonate diesters **D-216a**, **D-216b**, **L-216a** and **L-216b** (Figure 2.5, page 123) were synthesised from the resolved alcohols **D-129** and **L-129** in an identical manner to that for the racemic compounds. The recrystallisation of each diester was then attempted using a range of solvent systems (methanol, ethanol, dichloromethane/methanol, toluene, diethyl ether, ethyl acetate/petroleum ether, ethyl acetate/hexane) at 4 °C, but in all cases the individual *H*-phosphonate diesters only formed oils. Consequently, each diester was individually sulfurised in an identical manner to that for the racemic compound, and purified to generate the corresponding thiophosphoric acids **D-220a**, **D-220b**, **L-220a** and **L-220b**. Each thiophosphoric acid diester was then dissolved in methanol/dichloromethane (5:1, v/v) and a small quantity of pyridine added.²⁰⁸⁻²¹⁰ Though the pyridinium salt of each diester was observed to form on standing at 4 °C (seen by a downfield shift of the phosphorus signal in the ³¹P-NMR spectrum), the compounds were unfortunately all recovered as oils.

Due to the failure to recrystallise **216** and **219**, it was proposed to substitute the benzyl alcohol used in the synthetic route for an alternative alcohol with a crystalline nature. It was hoped that by having such a group present at the phosphorus centre, the resulting *H*-phosphonates and phosphorothioates might be more likely to form solids. The choice of alcohol was restricted to those containing a benzylic hydrogen to ensure that the new group could be removed by the reductive cleavage step.

Consequently *p*-nitrobenzyl alcohol (mp 92–94 °C) was initially chosen as a suitable compound and was coupled to the *L*-*H*-phosphonate salt **L-214** in an identical manner to that for the *O*-benzyl *H*-phosphonates **216a** and **216b**.²¹¹ The resultant pair of diastereomers **L-222a** and **L-222b** [$\delta_p(121.5 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 7.72 and 9.78 respectively] were separated by repeated silica column chromatography (0-6% ethyl acetate/dichloromethane, in 2% steps), but all attempts at the recrystallisation of each compound were unsuccessful (Figure 2.8). Each diastereomer was subsequently sulfurized in an identical manner to that for the *O*-benzyl *H*-phosphonates **216a** and **216b** to give the corresponding phosphorothioate pyridinium salt diastereomers **L-223a**

and **L-223b** [$\delta_p(121.5 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 60.01 and 59.91 respectively] which were not subjected to column chromatography (Figure 2.8). Instead, the compounds were each purified from excess sulfur by tritiation with dichloromethane. It was not possible to recrystallise either phosphorothioate.

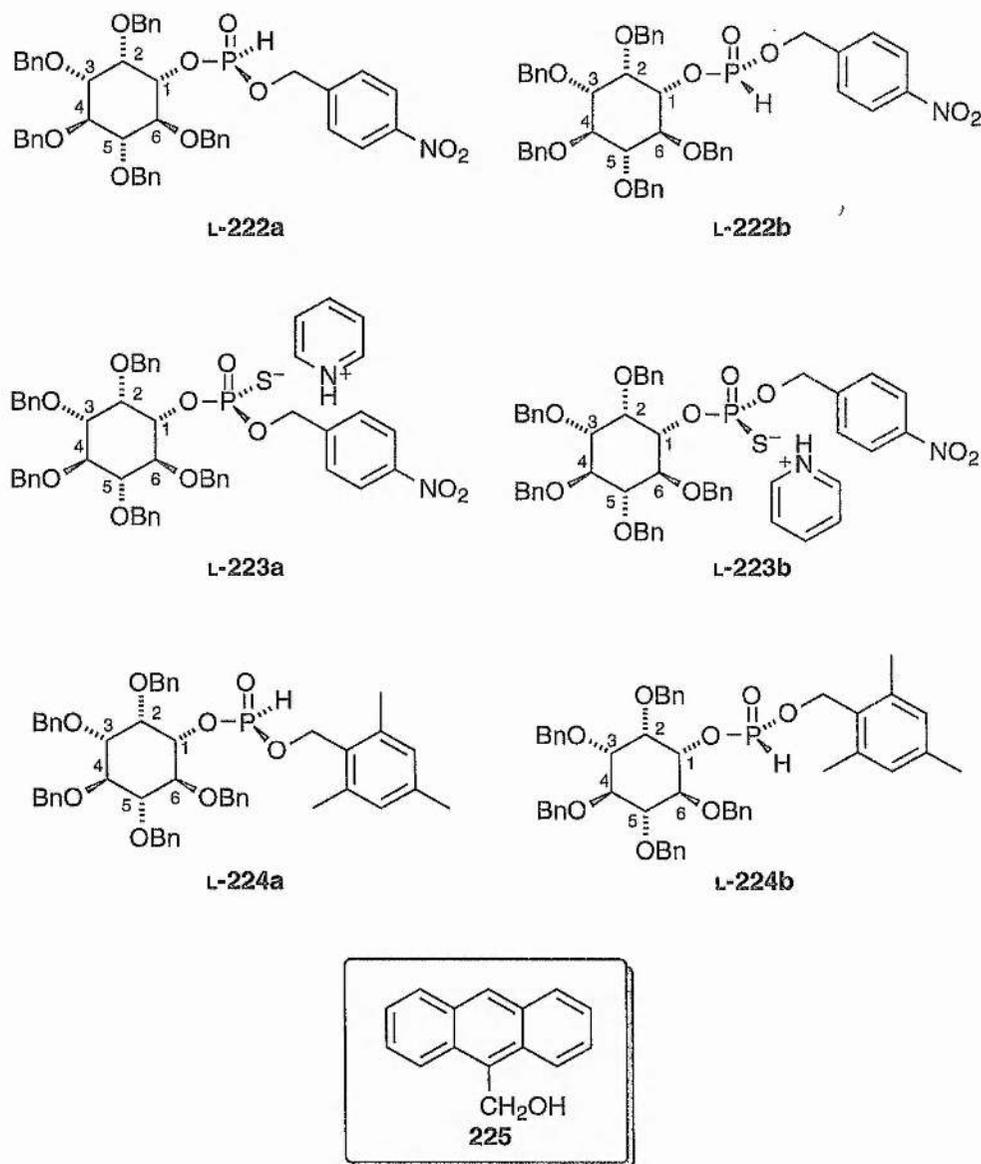


Figure 2.8: Alternative H-phosphonate and phosphorothioate diesters

Consequently, the coupling procedure was attempted between the highly crystalline 2,4,6-trimethylbenzyl alcohol (87–89 °C) and L-H-phosphonate salt **L-214** to give H-phosphonate diastereomers **L-224a** and **L-224b** [$\delta_p(121.5 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 8.03 and

9.91 respectively] (Figure 2.8). The compounds proved extremely difficult to separate by silica column chromatography (0–15% ethyl acetate/dichloromethane, in 5% steps) and this approach was discontinued.

The coupling reaction was also attempted between the more bulky 9-anthracenemethanol **225** (mp 162–164 °C) and *L-H*-phosphonate salt **L-214** but no reaction occurred, probably due to steric effects (Figure 2.8).⁷⁵

As single enantiomer inositol derivatives, such as alcohols **D-129** and **L-129**, tend to be more difficult to recrystallise than their racemic counterpart, in this example **DL-129**, the recrystallisation of the *racemic O*-benzyl *H*-phosphonate diesters **DL-216a** or **DL-216b** was therefore attempted. This time, it proved possible to grow crystals of the lower R_f *H*-phosphonate diester **DL-216b** from ethanol (4 °C) which were suitable for single crystal X-ray analysis (colourless, block, 0.5 x 0.4 x 0.2 mm, monoclinic, see Appendix 4.4, page 249 for the full experimental data). The higher R_f *H*-phosphonate **DL-216a** formed as an oil from ethanol.

The crystal structure which was determined for **DL-216b** is represented in Figure 2.9, showing the *D*-enantiomer. As the crystal contains both enantiomers, the structure *only* demonstrates the *relative stereochemistry* of the phosphorus centre to the inositol ring (S_p for the *D*-enantiomer, R_p for the *L*-enantiomer). However, it was realised during the course of this work that the absolute configuration at phosphorus of the four *H*-phosphonate diesters **D-216a**, **D-216b**, **L-216a** and **L-216b** (Figure 2.5, page 123), could be determined indirectly by combining several pieces of information.

By knowing which alcohol is used in the synthesis {the absolute configurations of **D-129** and **L-129** have already been determined by the work of Billington *et al.*,⁴⁷ (see Section 2.13, page 139)} the absolute configuration of the inositol ring of the *H*-phosphonate enantiomer in question is therefore known. If the ³¹P-NMR signal for this compound is then matched to one of the racemic compound [either δ_p (121.4 MHz; C²HCl₃) 8.08 for the higher R_f diester, 9.65 for the lower R_f diester] then the configuration of its phosphorus centre relative to the inositol ring can be determined from the X-ray crystal

structure. It follows, therefore, that as the stereochemistry of the inositol ring is already known, the absolute configuration of the phosphorus centre can be determined.¹⁰⁴

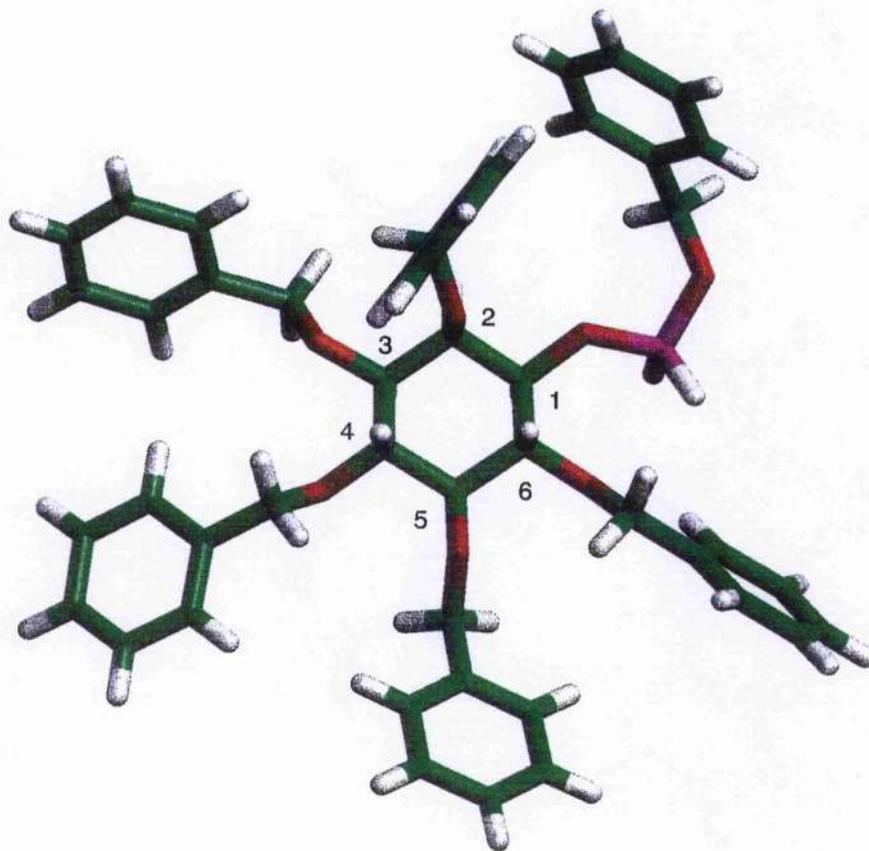


Figure 2.9: Representation of the racemic lower R_f H-phosphonate *DL-216b* generated from crystallographic data

By examining the crystal packing scheme for *DL-216b* in Figure 2.10, a possible explanation can be found for why only crystals of the racemic compound could be readily grown. The arrangement clearly shows the opposite enantiomers 'paired-up' in the structure, with two of these sets being found within the dimensions of the unit cell [symmetry operators: (1) X, Y, Z ; (2) $\frac{1}{2}-X, \frac{1}{2}+Y, -Z$; (3) $-X, -Y, -Z$; and (4) $\frac{1}{2}+X, \frac{1}{2}-Y, Z$]. If a favourable interaction were to exist between each pair of enantiomers, for example, by intermolecular π -stacking of some of the benzyl aromatic rings, one could

envisage the packing energy required to form the centrosymmetric space group being possibly lowered to the point where crystallisation becomes possible. Conversely, for the single enantiomeric compounds to form crystals, the molecules must arrange themselves into a chiral space group. In such an arrangement, the molecules might not experience as many favourable interactions, making the packing energy too high for crystals to form.

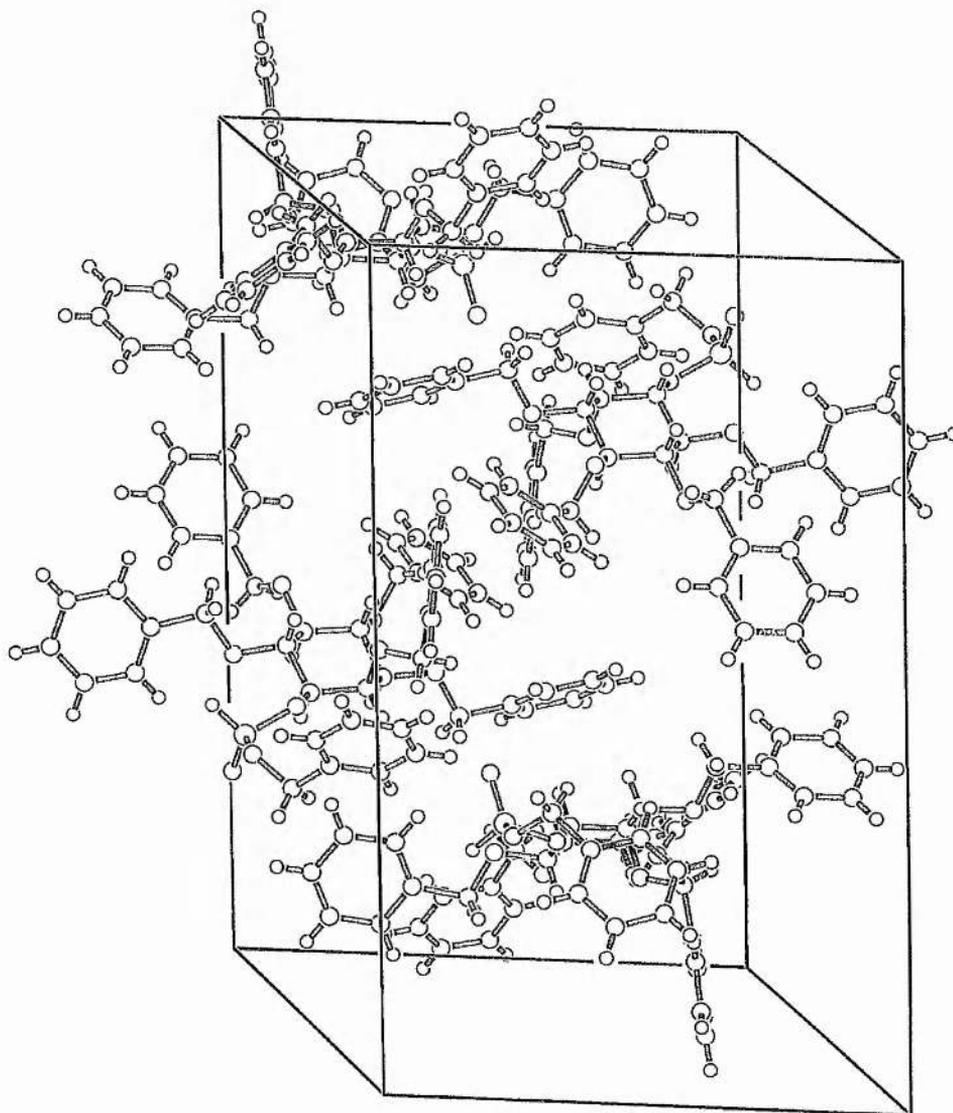


Figure 2.10: Crystal packing scheme for the racemic lower R_f H -phosphonate **DL-216b** showing the pairing up of opposite enantiomers

Though it was highly likely that the stereospecific reaction of H -phosphonates **216a** and **216b** with sulfur did proceed with *retention of configuration* at phosphorus,^{191, 212}

allowing the structure of the phosphorothioate products **219a** and **219b** (and hence the structure of the chiral [^{18}O]inositol 1-phosphorothioate probe for inositol monophosphatase) to be *predicted* from the X-ray crystal structure of **216b**, the stereochemistry of the reaction could *not* be proved categorically without also obtaining a X-ray crystal structure of one of the phosphorothioate products.

As crystallisation of the individual phosphorothioate pyridinium salts **D-219a**, **D-219b**, **L-219a** and **L-219b** had already been attempted without success, the recrystallisation of the racemic phosphorothioates **DL-219a** and **DL-219b** was considered.

Crystallisation of the thiophosphoric acid **DL-220b** resulting from the crystalline *H*-phosphonate **DL-216b** was attempted first. The compound was dissolved in methanol/dichloromethane and a small quantity of pyridine added,^{209, 213} but unfortunately the compound could not be recrystallised after prolonged standing at 4 °C. However, when the procedure was repeated using cyclohexylamine as the base, very fine white needles of the cyclohexylammonium salt **DL-226b** were recovered [δ_{p} (121.4 MHz; C^2HCl_3) 55.83]. Unfortunately, the crystals were unsuitable for single crystal X-ray analysis, and the procedure was repeated again, this time using the more bulky dicyclohexylamine.²⁰⁷ Fortunately colourless needle-type crystals of the dicyclohexylamine salt **DL-227b** [δ_{p} (121.4 MHz; C^2HCl_3) 54.89] were recovered which were suitable for single crystal X-ray analysis (0.5 x 0.25 x 0.2 mm, triclinic, see Appendix 4.5, page 254 for the full experimental data). Due to decay of the crystal during measurement, it was necessary to collect the X-ray crystal data at a temperature of -78 °C.

The crystal structure of phosphorothioate **DL-227b** is represented in Figure 2.11, showing the *D*-enantiomer. Again, the structure *only* demonstrates the *relative stereochemistry* of the phosphorus centre to the inositol ring (R_{p} for the *D*-enantiomer, S_{p} for the *L*-enantiomer)* but as for *H*-phosphonate **DL-216b**, the absolute configuration of single enantiomers of the phosphorothioates can be determined by also knowing their ^{31}P -

* Though the configurations at phosphorus of *H*-phosphonate **D-216b** and dicyclohexylammonium salt **D-227b** have been shown to be the *same*, the *R,S* designations of the chiral centres are reversed due to the difference in priority of the H- and S-atoms.

NMR signal [$\delta_p(121.5 \text{ MHz; C}^2\text{HCl}_3)$ 60.01 for the phosphorothioate pyridinium salt **DL-219a** derived from the higher R_f *H*-phosphonate diester and 59.91 for the phosphorothioate pyridinium salt **DL-219b** derived from the lower R_f *H*-phosphonate diester].

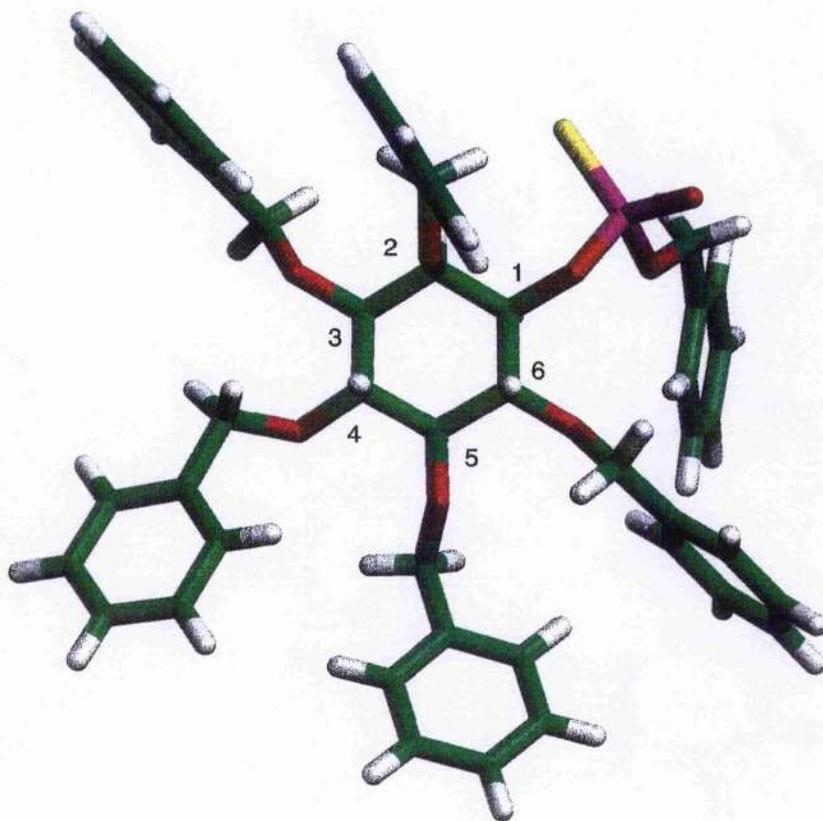


Figure 2.11: Representation of the dicyclohexylammonium salt **DL-227b** generated from crystallographic data (the dicyclohexylamine cation has been omitted for clarity)

It can be clearly seen from this image that the configuration at phosphorus of phosphorothioate **DL-227b** is the same as the *H*-phosphonate precursor **DL-216b** (Figure 2.9, page 133), confirming that the sulfurization reaction does proceed with retention of configuration at phosphorus (Figure 2.12).

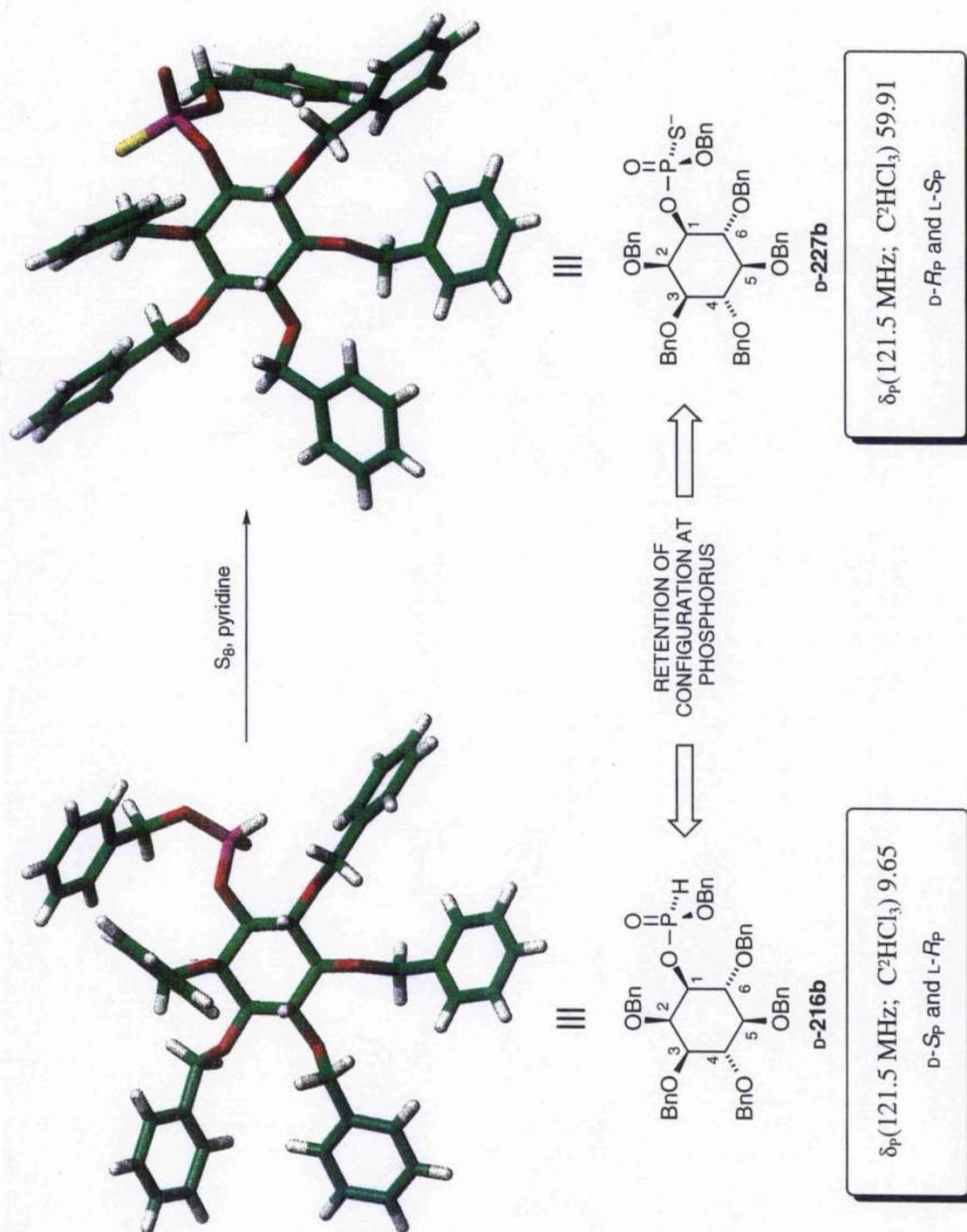


Figure 2.12: Stereospecific sulfurization reaction of H-phosphonate *DL*-216b to phosphorothioate *DL*-227b shown by X-ray crystallography and ^{31}P -NMR spectroscopy

The crystal packing scheme for the phosphorothioate dicyclohexylammonium salt **DL-227b** in Figure 2.13 shows a single pair of enantiomers in the unit cell sharing two dicyclohexylammonium cations [symmetry operators: (1) X, Y, Z ; and (2) $-X, -Y, -Z$].

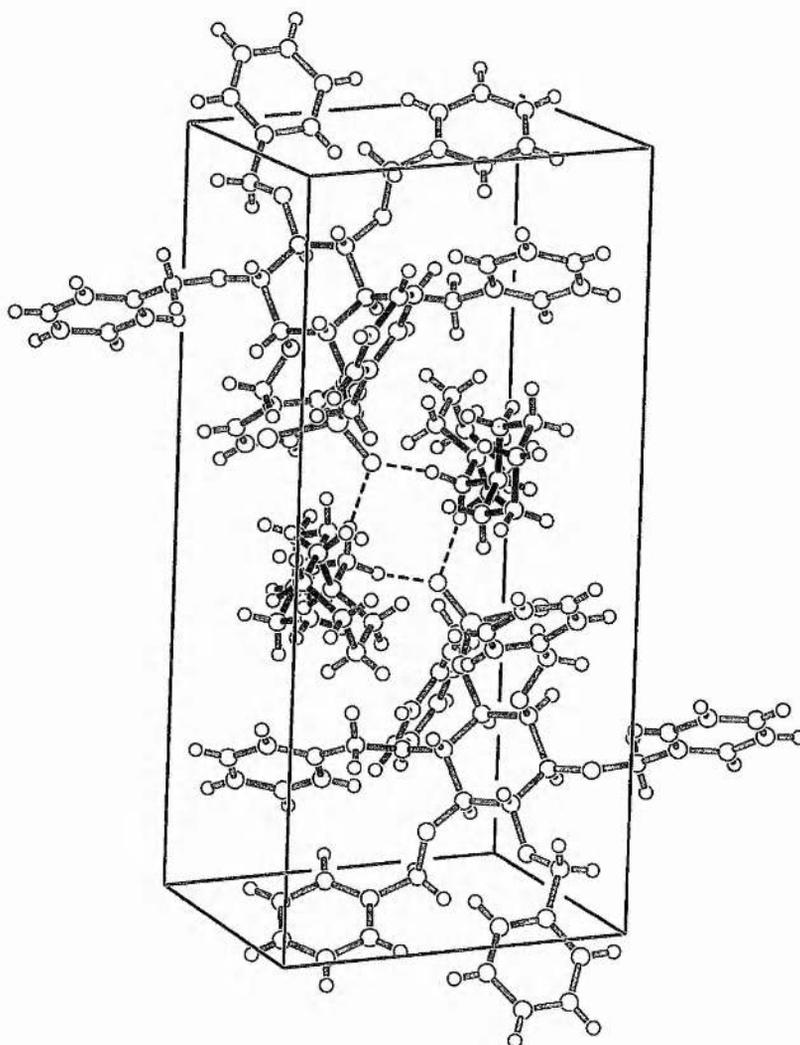


Figure 2.13: Crystal packing scheme for the racemic dicyclohexylammonium salt **DL-227b**

Interestingly, the S-atom is not involved in hydrogen bonding whereas P=O⁻ accepts two hydrogen bonds. This finding is in agreement with the structural determination of the diester *R_p*-uridine 3',5'-cyclic phosphorothioate by Hinrichs *et al.*²¹⁴ and other structures of phosphorothioate diesters.^{208, 210}

2.13 Resolution of DL-2,3,4,5,6-Penta-O-Benzyl myo-Inositol

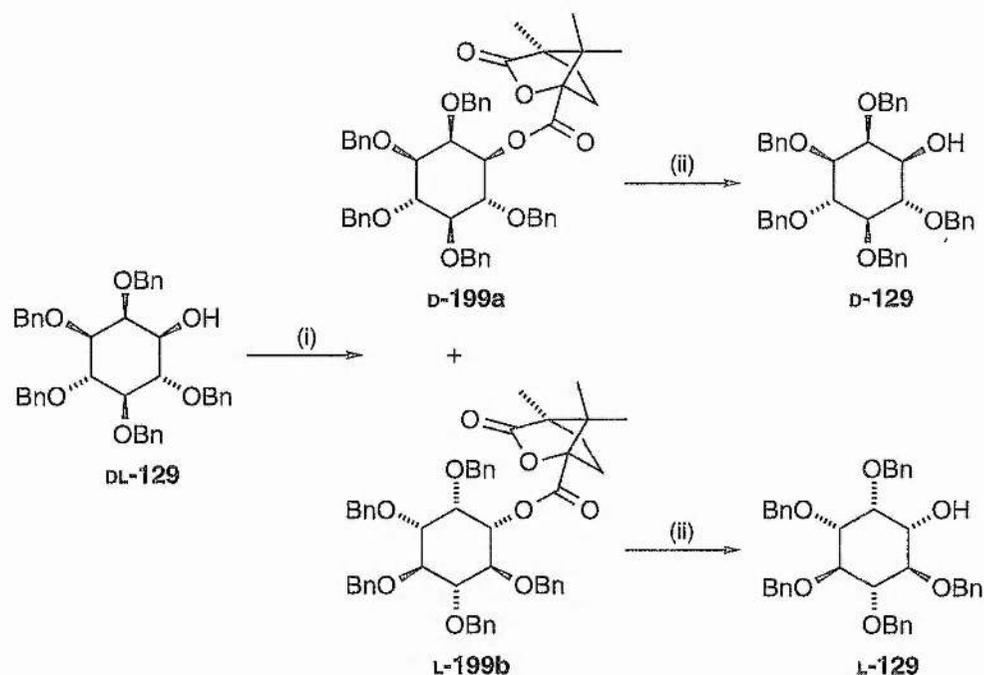
In order for this *H*-phosphonate chemistry to be used successfully to synthesise each chiral phosphorothioate probe for inositol monophosphatase with its absolute configuration correctly assigned [D-(*R*_p)- and D-(*S*_p)-¹⁸O]inositol 1-phosphorothioates **D-122a** and **D-122b** and L-(*R*_p)- and L-(*S*_p)-¹⁸O]inositol 1-phosphorothioates **L-122a** and **L-122b**], the absolute stereochemistry of the resolved intermediate alcohol **D-129** or **L-129** used in the synthesis had to be known.

As mentioned on page 114, alcohol **DL-129** was first resolved by Billington *et al.*,⁴⁷ via its treatment with '*R*-(*-*)-camphanic acid chloride' to produce diastereomeric camphanate esters **D-199a** and **L-199b**. Though this assignment for the acid chloride used in the esterification reaction is misleading, the literature scheme clearly indicates that the reagent (1*S*)-(*-*)-camphanic acid chloride **201** [in other words (1*S*, 4*R*)-(*-*)-camphanic acid chloride] was used (Scheme 2.32).^{1,47} It is also unlikely that the opposite isomer, (1*R*)-(*+*)-camphanic acid chloride, would have been chosen as the resolving agent, as it is grossly more expensive (by a factor of 12) than reagent **201**.

According to Billington, camphanate esters **D-199a** and **L-199b** were then separated by silica column chromatography (1% diethyl ether/dichloromethane) and each diastereomer was recrystallised from ethanol. Single X-ray crystal analysis of the 'less-polar' diastereomer established the absolute configuration of the compound as **D-199a**, though the X-ray structure was not shown in the literature. Hydrolysis of each diastereomer **D-199a** and **L-199b** {[α]_D²⁵ +12.5 and -17.8 (*c* 0.5 in CHCl₃), respectively} by ethanolic potassium hydroxide gave the corresponding single enantiomers **D-129** and **L-129** of the original alcohol {[α]_D²⁵ +9.1 and -8.7 (*c* 0.3 in CHCl₃), respectively} (Scheme 2.32).

The resolution of alcohol **DL-129** was therefore attempted according to the method of Billington *et al.* using the (1*S*)-(*-*)-camphanic acid chloride as the resolving agent. Following hydrolysis of the separated camphanate esters, each single enantiomer of the alcohol was recovered in excellent overall yield {[α]_D²⁵ +9.2 for the alcohol derived from

the higher R_f camphanate ester and -9.2 for the alcohol derived from the lower R_f camphanate ester (c 0.3 in CHCl_3), respectively; 41% for each enantiomer from a theoretical maximum of 50%} (Scheme 2.32).



Reagents and conditions: (i) (a) (*S*)-(-)-camphanic acid chloride **201**, CH_2Cl_2 , Et_3N , DMAP, $0\text{ }^\circ\text{C} \rightarrow 25\text{ }^\circ\text{C}$, 24 h, then (b) column chromatography, 42% and 45% respectively; (ii) KOH, EtOH, $25\text{ }^\circ\text{C}$, 24 h, 97% and 91% respectively.

Scheme 2.32: Resolution of DL-2,3,4,5,6-penta-O-benzyl myo-inositol DL-129

Because so much depended on the assignment of the resolved alcohols being correct, it was decided to verify the X-ray crystallographic analysis performed by Billington *et al.* Consequently, crystals of both separated camphanate esters were grown from ethanol ($4\text{ }^\circ\text{C}$). The lower R_f camphanate ester gave extremely fine white crystals which were unsuitable for single crystal X-ray analysis. Fortunately, the higher R_f camphanate ester formed colourless plate-like crystals ($0.4 \times 0.25 \times 0.1\text{ mm}$) which diffracted well.

The crystal structure that was determined for the higher R_f camphanate ester is shown in Figure 2.14 (see Appendix 4.6, page 261 for the full experimental data).

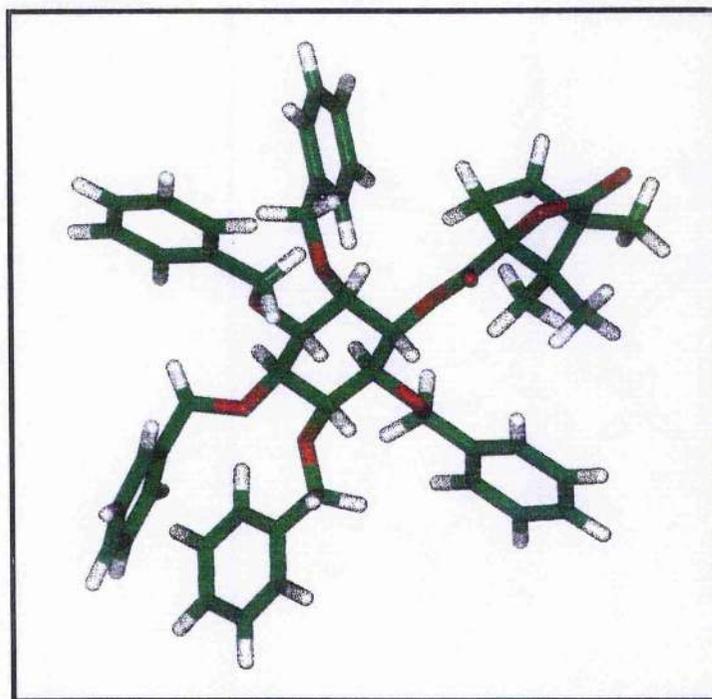
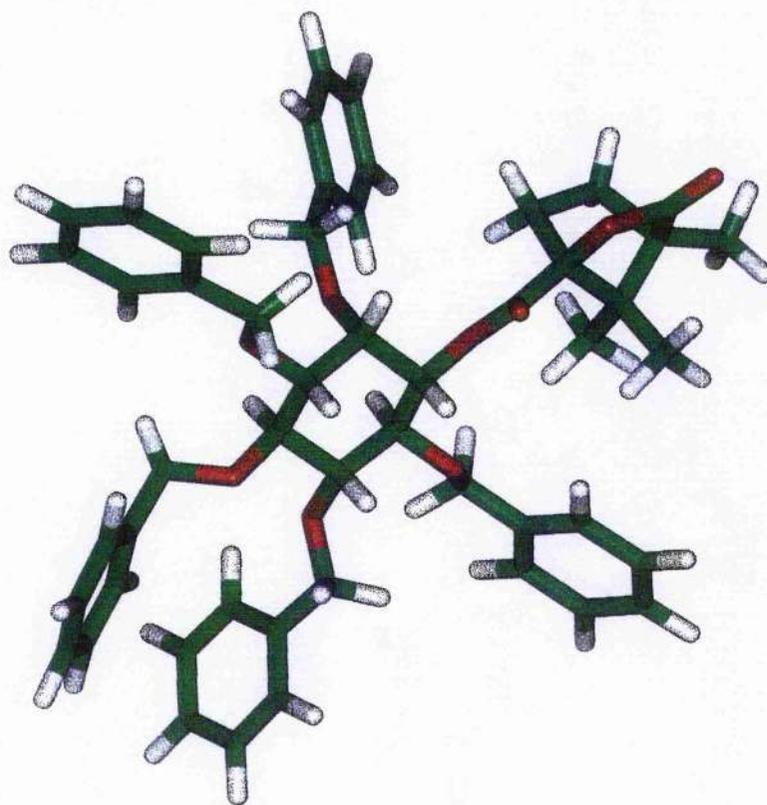


Figure 2.14: Representation of the higher R_f camphanate ester L-199b generated from crystallographic data. The identical structure represented the box was generated from the atomic coordinates deposited by Billington et al. at the Cambridge Crystallographic Data Centre

Surprisingly, the data *disagreed* with the assignment of Billington *et al.* and established the higher R_f compound as **L-199b** derived from the L-alcohol **L-129**, in spite of the fact that the optical rotations of the respective recrystallised camphanate esters $\{[\alpha]_D^{25} +11.95 \pm 0.4$ for the higher R_f camphanate ester and -17.65 ± 0.35 for the R_f camphanate ester (c 0.3 in CHCl_3) $\}$ were found to *agree* with literature values.¹

In an attempt to solve this anomaly, the original set of coordinates for the X-ray structure collected by Billington *et al.* was obtained from the Cambridge Crystallographic Database (Figure 2.14). When the structures were compared to each other, they were found to be *identical* [St Andrews structure for $\text{C}_{51}\text{H}_{54}\text{O}_9$; $M = 810.98$, monoclinic, $a = 6.143(5)$ Å, $b = 23.989(5)$ Å, $c = 15.044(3)$ Å, $\beta = 98.68(3)^\circ$, $U = 2191(1)$ Å³, space group $P2_1$, $Z = 2$ and $D_{\text{calc}} 1.229$ g cm⁻³. Structure by Billington *et al.* for $\text{C}_{51}\text{H}_{54}\text{O}_9$; $M = 810.98$, monoclinic, $a = 6.147(1)$ Å, $b = 23.976(6)$ Å, $c = 15.030(3)$ Å, $\beta = 98.68(2)^\circ$, $U = 2189.65$ Å³, space group $P2_1$, $Z = 2$, $D_{\text{calc}} 1.230$ g cm⁻³]. Therefore, it was apparent that the configurational assignment for the higher R_f camphanate ester as diastereomer **L-199b** was correct, and that the X-ray crystal structure produced by Billington *et al.* had simply been misinterpreted in the original work.

To save confusion, the D- and L- notations which have been used to designate the camphanate ester diastereomers **D-199b** and **L-199b** and the alcohol single enantiomers **D-129** and **L-129** (and all other inositol derivatives in this text) were assigned according to the actual configuration of the inositol ring found in the molecule rather than the measured optical rotation of the compound. If the ring configuration resembled that found in the D-enantiomer of the natural substrate for inositol monophosphatase, inositol 1-phosphate **10**, then the compound was also given the D-notation. Likewise, if the ring configuration resembled that found in L-inositol 1-phosphate, then the compound was also assigned the L-notation. The absolute configuration of D- and L-inositol 1-phosphate **10** was determined in 1960 by the synthesis of the (-) form from galactinol **228**, a natural product which was known to have the absolute configuration shown in Figure 2.15.²¹⁵

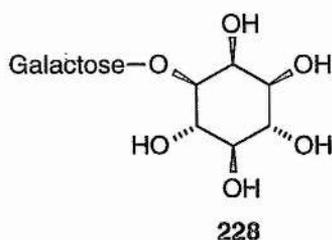


Figure 2.15: *The natural product galactinol 228, used to determine the absolute configuration of D- and L-inositol 1-phosphate, natural substrate of inositol monophosphatase*

It can be seen, however, that the optical rotation measurements of **D-199a** and **L-199b**, and **D-13** and **L-13** are all *reversed* relative to their inositol ring-assignments, implying that at some stage during the synthesis of D- or L-inositol 1-phosphate, the optical rotation of the molecules must switch. Due to their incorrect assignment of **L-199b**, this phenomenon appears not to have been detected by Billington *et al.* It is interesting to note that during the synthesis of the triethylammonium salt of L-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-hydrogen phosphonate **L-214** from alcohol **L-129** (Section 2.12, page 129 and Section 2.15, page 147) such a change in the optical rotation measurements of the respective compounds was observed $\{[\alpha]_{\text{D}}^{25} +9.2$ (*c* 0.3 in CHCl_3) for the L-alcohol, and $[\alpha]_{\text{D}}^{25} -26.5$ (*c* 1 in CHCl_3) for **L-214**}. $\}$

2.14 Synthesis of [¹⁸O]Benzyl Alcohol

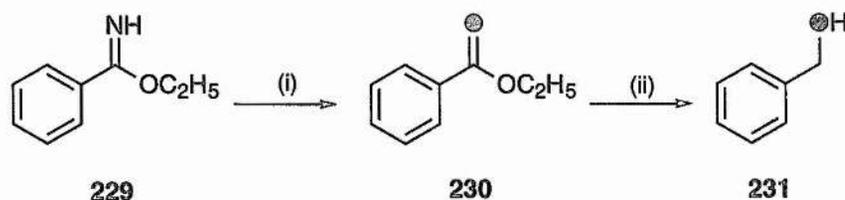
It was proposed initially to attempt the synthesis of [¹⁸O]benzyl alcohol by exchanging benzaldehyde with ¹⁸O-enriched water and then reducing the labelled aldehyde with sodium borohydride to give the alcohol. A solution of benzaldehyde in anhydrous THF was stirred for two days in the presence of 5.5 fold excess of ¹⁸O-enriched water (97 atom % ¹⁸O) under slightly acidic conditions, according to the method of Ludeman *et al.*,²¹⁶ and the labelled benzaldehyde isolated.

The ¹⁸O-enriched aldehyde was subsequently reduced by adding sodium borohydride to a methanolic solution of the compound. Disappointingly, the recovered [¹⁸O]benzyl alcohol was found to have a ¹⁸O incorporation of only 84.7% {estimated by mass spectrometry (EI⁺) from the ratio of the peak heights of the C₆H₅CH₂●H⁺ [110] and C₆H₅CH₂OH⁺ [108] ions²¹⁷}.

On closer examination, however, it was realised that this measured enrichment was approaching the theoretical maximum attainable for the reaction from the quantity of labelled water which was used in the synthesis. The acid-catalyzed ¹⁸O-exchange from [¹⁸O]water into the carbonyl group of an aldehyde or ketone, is always accompanied by a dilution of isotope due to the presence of the unlabelled O-atom in the initial benzaldehyde.

To be able to determine the stereochemical course of the inositol monophosphatase reaction successfully, the chiral [¹⁸O]inositol 1-phosphorothioate substrate probes must be produced with a high degree of isotopic incorporation. Consequently, an alternative route was sought for the synthesis of [¹⁸O]benzyl alcohol which would allow an isotopically richer alcohol to be produced.

Although the acid-catalyzed hydrolysis of an acetal of benzaldehyde with ¹⁸O-enriched water would have lead to a better isotopic incorporation [this approach was adopted by Lowe in the synthesis of (*S*)-[¹⁸O]benzoin **68** (Section 1.26.2, page 48)],¹¹¹ an alternative route, developed by Hermanns *et al.*,²¹⁸ was chosen instead to produce [¹⁸O]benzyl alcohol with excellent isotopic enrichment (Scheme 2.33).



Reagents and conditions: (i) H_2^{18}O , 80 °C, 45 min, 78%; (ii) LiAlH_4 , diethyl ether, reflux, 1 h, 58%.

Scheme 2.33: Synthesis of [^{18}O]benzyl alcohol

The route involved the synthesis of the labelled alcohol **231** via the a reaction between ethyl benzimidate hydrochloride **229** and a 5 fold excess of ^{18}O -enriched water (97 atom % ^{18}O), resulting in labelled ethyl benzoate **230** with ^{18}O exclusively in the carbonyl group. Labelled ethyl benzoate **230** was then reduced by lithium aluminium hydride to give the [^{18}O]benzyl alcohol **231** in a 45% overall yield.

The extent of ^{18}O incorporation in the recovered alcohol was determined by mass spectrometry (EI^+) and shown to be typically 92.4 atom % (Figure 2.16). The [^{18}O]benzyl alcohol was subsequently used to successfully synthesise the chiral substrate probes L-(R_p)- and L-(S_p)-[^{18}O]inositol 1-phosphorothioate L-122a and L-122b.

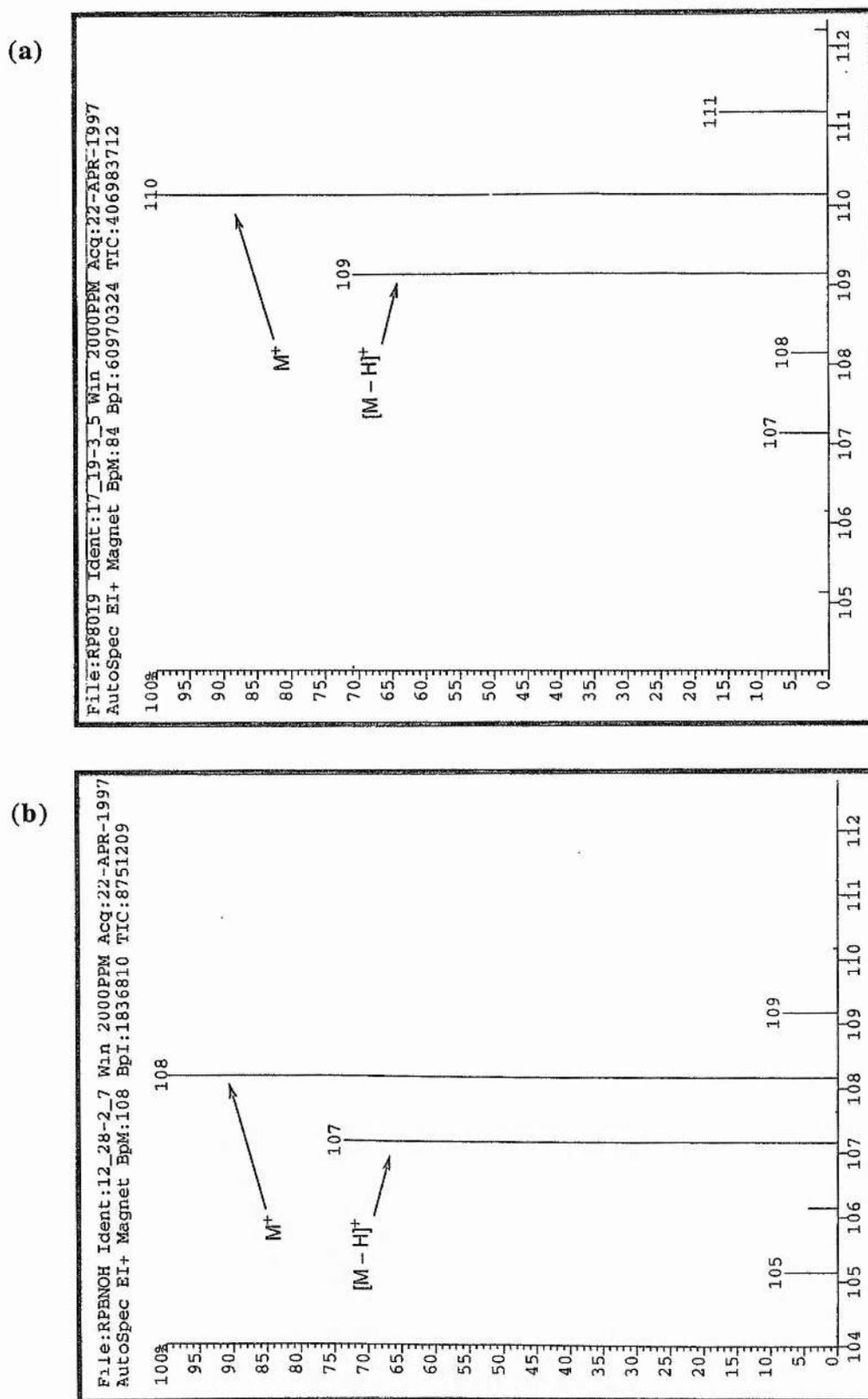


Figure 2.16: Expanded region of the mass-spectra of (a) [^{18}O]benzyl alcohol 231 synthesized according to the method of Hermanns et al.,²¹⁸ and (b) unlabelled benzyl alcohol for comparison

2.15 Synthesis of L-(*R_p*)- and L-(*S_p*)-[¹⁸O]inositol 1-Phosphorothioate

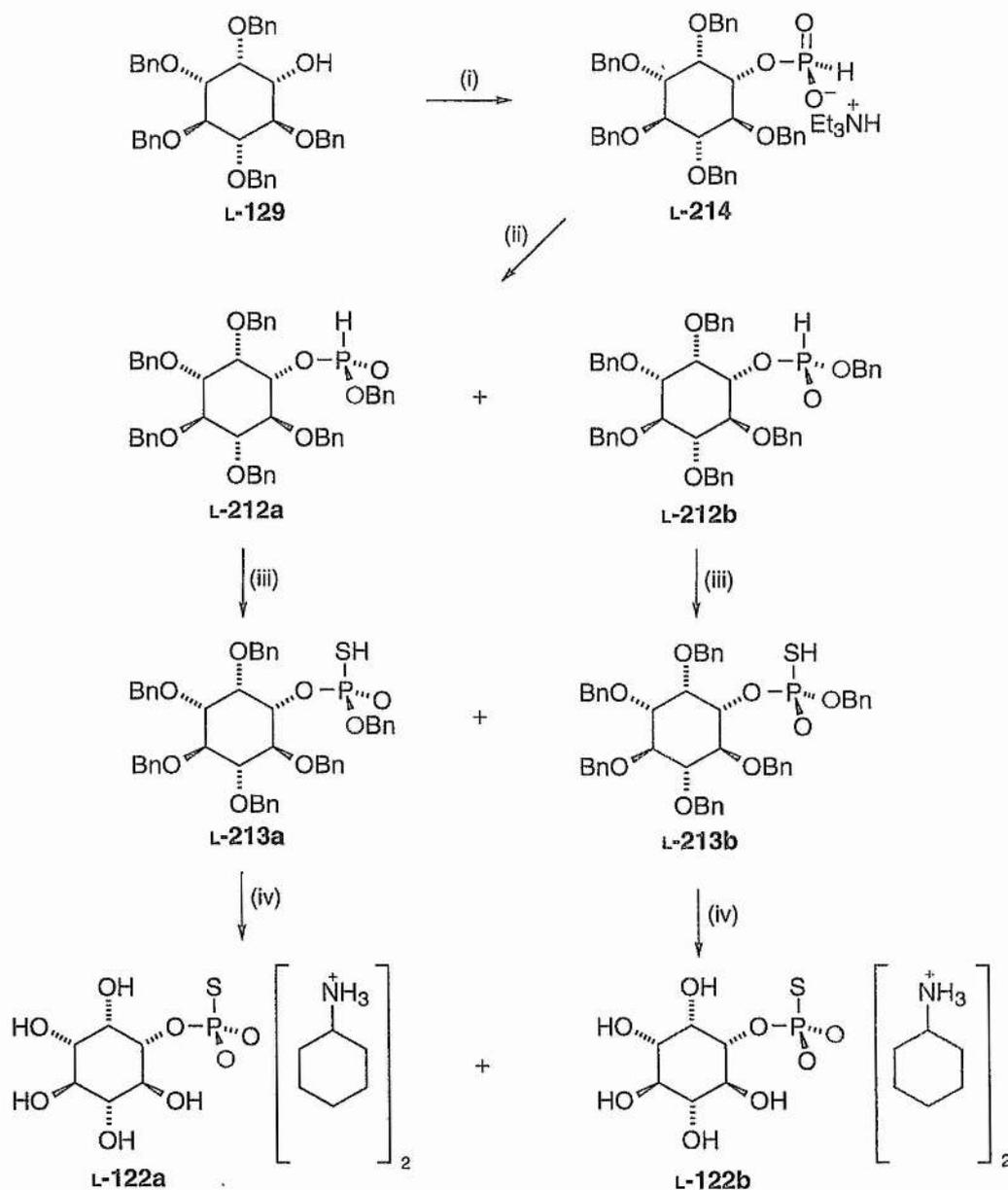
The isolated alcohol enantiomer L-129, derived from the higher *R_f* camphanate ester L-199b, was coupled to 2-chloro-4*H*-1,3,2-benzo-dioxaphosphorin-4-one 215 in a manner identical to that for the racemic alcohol DL-129 (page 121) to produce the L-*H*-phosphonate triethylammonium salt L-214 in good yield {[α]_D²⁵ -26.5 (*c* 1, CHCl₃); δ_p (121.4 MHz; C²HCl₃) 4.31; 81%)} (Scheme 2.34).

Next, the *H*-phosphonate salt was condensed with previously synthesised [¹⁸O]benzyl alcohol (92.4 atom % (Section 2.14, page 144) using pivaloyl chloride as the coupling reagent, to give the pair of ¹⁸O-labelled *H*-phosphonate diastereomers L-(*S_p*)-212a and L-(*R_p*)-212b which were separated by silica column chromatography {for the higher *R_f* diastereomer L-(*S_p*)-212a, 30% from the salt; [α]_D²⁵ -6.0 (*c* 0.5, CHCl₃); δ_p (121.4 MHz; C²HCl₃) 8.07; *m/z* (FAB⁺) 810 (3%, [M + H + Na]⁺) and 107 (100, OCH₂Ph⁺); *R_f* = 0.21 (30% ethyl acetate/petroleum ether); for the lower *R_f* diastereomer L-(*R_p*)-212b, 53% from the salt; (HRMS: Found, [M + H]⁺, 787.3311. Calc for C₄₈H₅₀O₇¹⁸OP: 787.3286); δ_p (121.4 MHz; C²HCl₃) 9.59; *m/z* (FAB⁺) 787 (6%, [M + H]⁺) and 181 (100, [Inositol + H]⁺); *R_f* = 0.16 (30% ethyl acetate/petroleum ether)}.

Each separated ¹⁸O-labelled *H*-phosphonate diastereomer L-(*S_p*)-212a or L-(*R_p*)-212b was then stereospecifically oxidised by elemental sulfur in pyridine to give the corresponding ¹⁸O-labelled phosphorothioate which was subsequently purified to produce each compound as its thiophosphoric acid L-(*R_p*)-213a {78% from *H*-phosphonate L-(*S_p*)-212a, δ_p (121.4 MHz; C²HCl₃) 54.60; *m/z* (FAB⁺) 841 (1%, [M + H + Na]⁺)* and 322 (100)} or L-(*S_p*)-213b {74% from *H*-phosphonate L-(*R_p*)-212b, δ_p (121.4 MHz; C²HCl₃) 53.91; *m/z* (ES⁺) 841 (2%, [M + H + Na]⁺)* and 101 (100, [C₃H₅N - H + Na]⁺)}.
* M is the molecular weight of the phosphorothioate anion.

The single purified ¹⁸O-labelled thiophosphoric acids L-(*R_p*)-213a and L-(*S_p*)-213a were each deprotected using sodium/liquid ammonia reduction to give the desired chiral

* M is the molecular weight of the phosphorothioate anion.



Reagents and conditions: (i) (a) 2-chloro-4*H*-1,3,2-benzo-dioxaphosphorin-4-one **215**, pyridine:THF (1:4, v/v), 0–25 °C, 15 min, then (b) water, 10 min, then (c) TEAB, 20 min, 81%; (ii) (a) BnOH, PvCl, pyridine:THF (1:1, v/v), 25 °C, 8 h, then (b) TEAB, 20 min, 30% and 53%, respectively, then (c) silica column chromatography; (iii) (a) S₈, pyridine, 25 °C, 16 h, then (b) Florisil[®], column chromatography (2–10% CH₃OH/DCM), 78% and 74%, respectively; (iv) (a) Na/NH_{3(l)}, –78 °C, then (b) Amberlite IR118 (H⁺), then (c) cyclohexylamine, H₂O, 48% and 62% respectively.

Scheme 2.34: Synthesis of *L*-(R_p)- and *L*-(S_p)-[¹⁸O]inositol 1-phosphorothioate *L*-122a and *L*-122b

substrate probes L-(*R*_p)- and L-(*S*_p)-[¹⁸O]inositol 1-phosphorothioate respectively, which were converted into their stable biscyclohexylammonium salts **L-122a** and **L-122b**,⁴⁸ {for **L-122a**, 48% from the thiophosphoric acid **L-213a**; $\delta_p(121.4 \text{ MHz}; ^2\text{H}_2\text{O})$ 44.53; m/z (ES⁺) 479 (1%, [M + 3H + 2C₆H₅NH₃]⁺) and 100 (100, C₆H₅NH₃⁺); for **L-122b**, 62% from the thiophosphoric acid **L-213b**; $\delta_p(121.4 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 44.46; m/z (ES⁺) 479 (1%, [M + 3H + 2C₆H₅NH₃]⁺) and 100 (100, C₆H₅NH₃⁺)}. The mass spectra of the recovered salts confirmed that the deprotection step had proceeded *without* major loss of the ¹⁸O-label, although due to the low intensity of the signals it was not possible to quantify the exact level of isotopic incorporation for each compound.

2.16 Preliminary Investigations into the Configurational Analysis of Chiral Inorganic [^{16}O , ^{17}O , ^{18}O]Phosphorothioate

In order to determine the stereochemical course of the inositol monophosphatase reaction using these synthesised chiral [^{18}O]phosphorothioate probes, the subsequent enzymic hydrolysis of each compound has to be performed in ^{17}O -enriched water and the chiral (R_p)- or (S_p)-inorganic [^{16}O , ^{17}O , ^{18}O]phosphorothioate product (**53a** or **53b**) stereospecifically derivatised such that its absolute configuration can be determined (Section 1.30, page 66).

As mentioned previously, there are two general methods available for the stereospecific derivatisation of a single isotopomer of **53**;

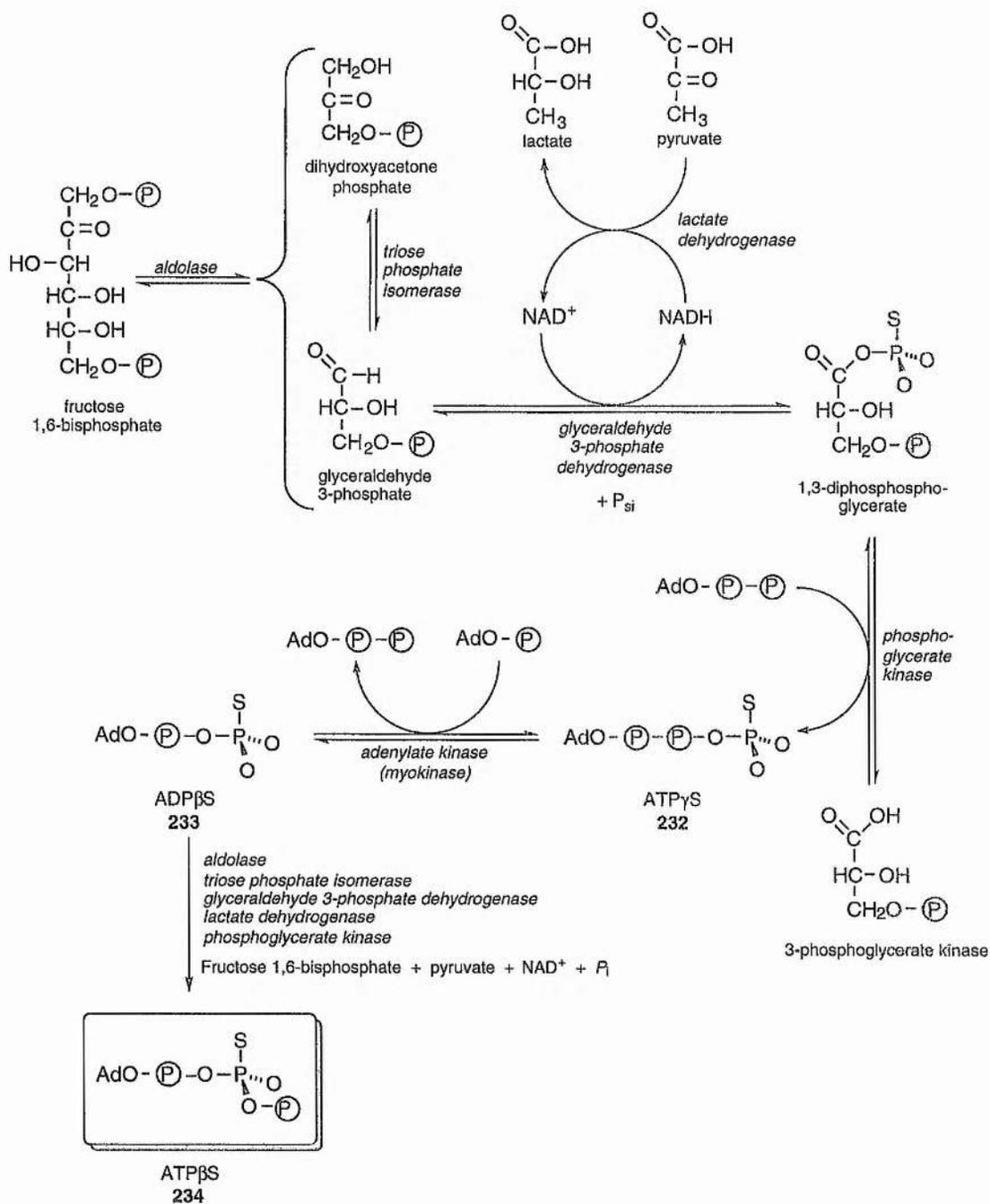
1. the approach developed by Webb and Trentham¹⁵⁰ in which a series of enzyme reactions incorporates **53** into the β -position of an ATP molecule (Section 1.30.1, page 66),
2. the chemical approach developed by Lowe *et al.*^{151, 152} in which **53** is initially alkylated with (*S*)-2-iodo-1-phenylethanol **117** to produce a phosphonothioate which then undergoes further reaction (Section 1.30.2, page 72).

In both cases, the absolute configuration of the incorporated isotopomer of **53** is then determined by analysing the ^{31}P -NMR spectrum of the final product of derivatisation.

The chemical approach (2) for the derivatisation of **53** has been investigated previously in the group for its application to the inositol monophosphatase reaction, but without success.¹⁶⁹ Although (*S*)-2-iodo-1-phenylethanol was synthesized and then coupled to unlabelled inorganic phosphorothioate according to the literature procedure,^{151, 152} it was found that the subsequent cyclisation reaction of the resulting phosphonothioate with diphenylphosphorochloridate could *not* be performed and the work was abandoned at this stage (Scheme 1.24, page 72).

Accordingly, it was decided to utilise the alternative enzymic approach of Webb and Trentham (1) to derivatise **53** resulting from the inositol monophosphatase reaction, and preliminary investigations were carried out using unlabelled inorganic phosphorothioate

(P_{si}). The relationship between the individual enzyme reactions and the production of ATP β S 234 is shown in Scheme 2.35.



Scheme 2.35: Full enzyme reaction scheme for the derivatisation of inorganic phosphorothioate into ATP β S 234 by glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase

According to the method of Webb and Trentham,¹⁵⁰ unlabelled P_{si} (commercially available as sodium thiophosphate dodecahydrate) is first converted into ATP γ S 232 by the joint action of glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase.¹⁵³ To perform this procedure, inorganic phosphorothioate was incubated with glyceraldehyde phosphate dehydrogenase, 3-phosphoglycerate kinase, aldolase, triose phosphate isomerase and lactate dehydrogenase in the presence of fructose 1,6-bisphosphate, ADP, NAD^+ , pyruvate, and magnesium (50 mmol dm⁻³ Tris, pH 8.0, 30 °C). The formation of ATP γ S 232 was monitored by ³¹P-NMR spectrometry, and after 5 hours the conversion was judged to be almost complete. The reaction mixture was then quickly cooled (0-2 °C), and applied to an anion exchange column [DEAE-Sephadex A-25, eluting with a gradient system of triethylammonium bicarbonate (TEAB) buffer (0.1–0.6 mol dm⁻³, pH 7.5, 4 °C)] to separate the various nucleotides arising from the incubation.⁷² Each product was located by UV absorption at 260 nm and identified by ³¹P-NMR spectrometry (Figure 2.17).

The ATP γ S 232 product was recovered in an overall 17% yield from inorganic phosphorothioate [δ_p (121.4 MHz; ²H₂O) -22.78 (dd, ² $J_{\alpha\beta}$ 19.4, ² $J_{\beta\gamma}$ 29.1, P_β), -10.73 (d, ² $J_{\alpha\beta}$ 19.4, P_α) and 33.81 (d, ² $J_{\beta\gamma}$ 29.1, P_γ)].¹⁴⁴ It is likely that the desulfurization of P_{si} and/ or ATP γ S during the incubation contributed to the poor recovery of the product.^{219, 220} Evidence for this process having occurred is the sizeable quantity of ATP which was also recovered regularly from the enzyme incubation, despite the extensive efforts which were taken to choose supplies of enzymes, substrates and buffers which contained the lowest possible level of inorganic phosphate. Fortunately, the nucleoside phosphate, because of its slightly higher pK_a value,¹⁹³ was eluted well ahead of ATP γ S 232, permitting the latter compound to be isolated in its pure form (Figure 2.17).

The next step of the process was to transfer the thiophosphoryl group of ATP γ S 232 to AMP, by the action of adenylate kinase (myokinase), to produce ADP β S 233.^{150, 153} Consequently, the isolated ATP γ S 232 was incubated with adenylate kinase in the presence of AMP and magnesium (100 mmol dm⁻³ Hepes, pH 7.5, 30 °C), and the

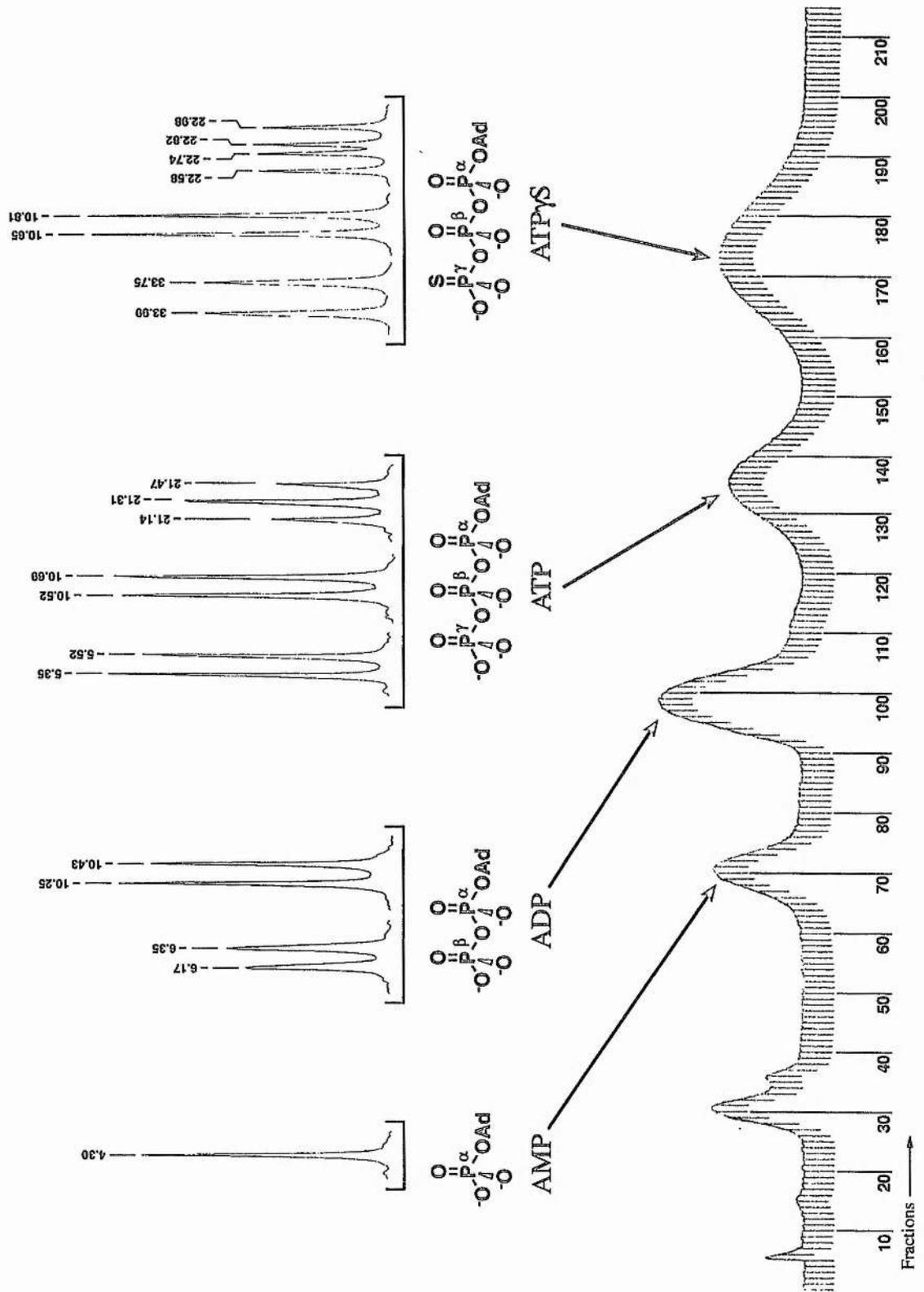


Figure 2.17: Anion exchange chromatography of the products arising from the incubation of inorganic phosphorothioate with glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase

formation of the ADP β S **233** was monitored by ^{31}P -NMR spectrometry. After 3 hours, the reaction mixture was rapidly cooled (0-2 °C) to stop the enzyme reaction, and the products separated by anion exchange chromatography using the same conditions as for ATP γ S **232** [DEAE-Sephadex A-25, TEAB gradient, 4 °C] (Figure 2.18). ADP β S **233** was isolated in 74% yield [δ_{p} (121.4 MHz; $^2\text{H}_2\text{O}$) -11.18 (d, $^2J_{\alpha\beta}$ 32.1, P_{α}), and 33.56 (d, $^2J_{\alpha\beta}$ 32.1, P_{β})].

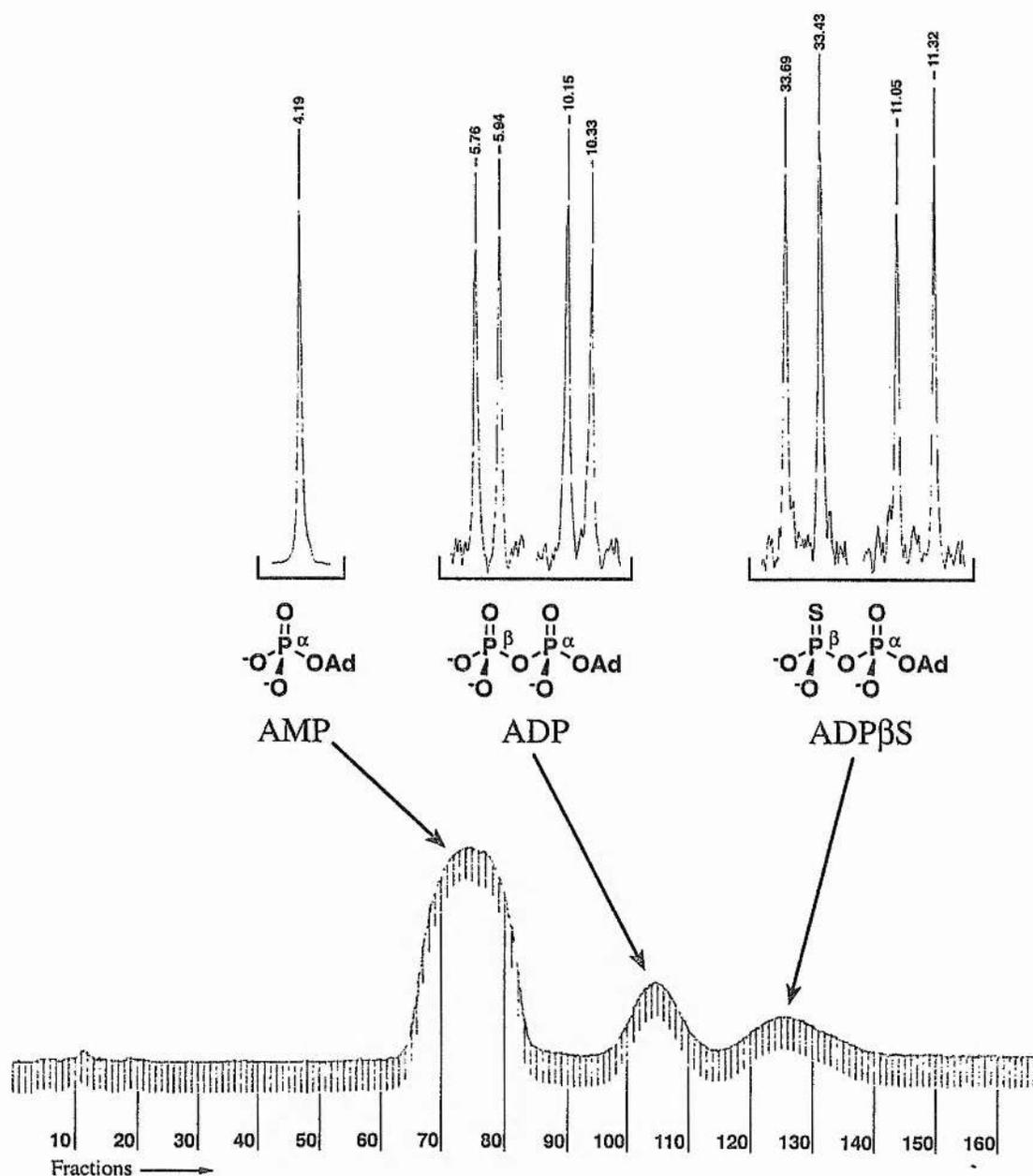


Figure 2.18: Anion exchange chromatography of the products arising from the incubation of ATP γ S with adenylate kinase and ADP

The final step in the derivatisation of inorganic phosphorothioate requires the stereospecific phosphorylation of the product ADP β S 233 at the *pro-S* oxygen to give ATP β S 234 (when the entire derivatisation procedure is performed with chiral [^{16}O , ^{17}O , ^{18}O]inorganic phosphorothioate, it is this compound which is then analysed by ^{31}P -NMR spectrometry in order to determine the absolute configuration of the phosphorothioate isotopomer which was incorporated). This phosphorylation reaction was achieved, according to the method of Webb and Trentham,¹⁵⁰ by employing the same mixture of enzymes which was used in the first step to derivatise P_{si} to ATP γ S 232, but replacing the supply of ADP with the product ADP β S 233, and P_{si} with P_{i} . Consequently, ADP β S 233 was incubated with glyceraldehyde phosphate dehydrogenase, 3-phosphoglycerate kinase, aldolase, triose phosphate isomerase and lactate dehydrogenase in the presence of inorganic phosphate, fructose 1,6-bisphosphate, NAD^+ , pyruvate, and MgCl_2 (50 mmol dm $^{-3}$ Tris, pH 8.0, 33 °C). The incubation was performed for 8 hours to compensate for poorer substrate activity of the nucleotide phosphorothioate *cf.* ADP, and then the products were separated, as for the previous two stages, by anion exchange chromatography (DEAE-Sephadex A-25, TEAB gradient, 4 °C). The product ATP β S 234 was isolated in 44% yield [δ_{p} (121.4 MHz; $^2\text{H}_2\text{O}$) -12.23 (d, $^2J_{\alpha\beta}$ 27.9, P_{α}), -6.66 (d, $^2J_{\beta\gamma}$ 29.1, P_{γ}) and 27.85 (t, $^2J_{\alpha\beta}$ 27.9, $^2J_{\beta\gamma}$ 29.1, P_{β})]. Much less ATP was observed to form during this incubation step, suggesting that ADP β S 233 might be more resistant to desulfurization than ATP γ S 234 under the same incubation conditions (Figure 2.19).

The observed ^{31}P -NMR spectrum of ATP β S 234 was consistent with the literature observation that the phosphorus-phosphorus coupling constants $^2J_{\alpha\beta}$ and $^2J_{\beta\gamma}$ are similar, but not identical, causing the lines of the P_{β} signal to appear as a triplet with a broadened central line.¹⁵¹ The ^{31}P -NMR spectra of this compound and all other nucleoside phosphate and phosphorothioates which were isolated from the three enzyme incubation reactions are shown in Figure 2.19 and Figure 2.20, page 157.

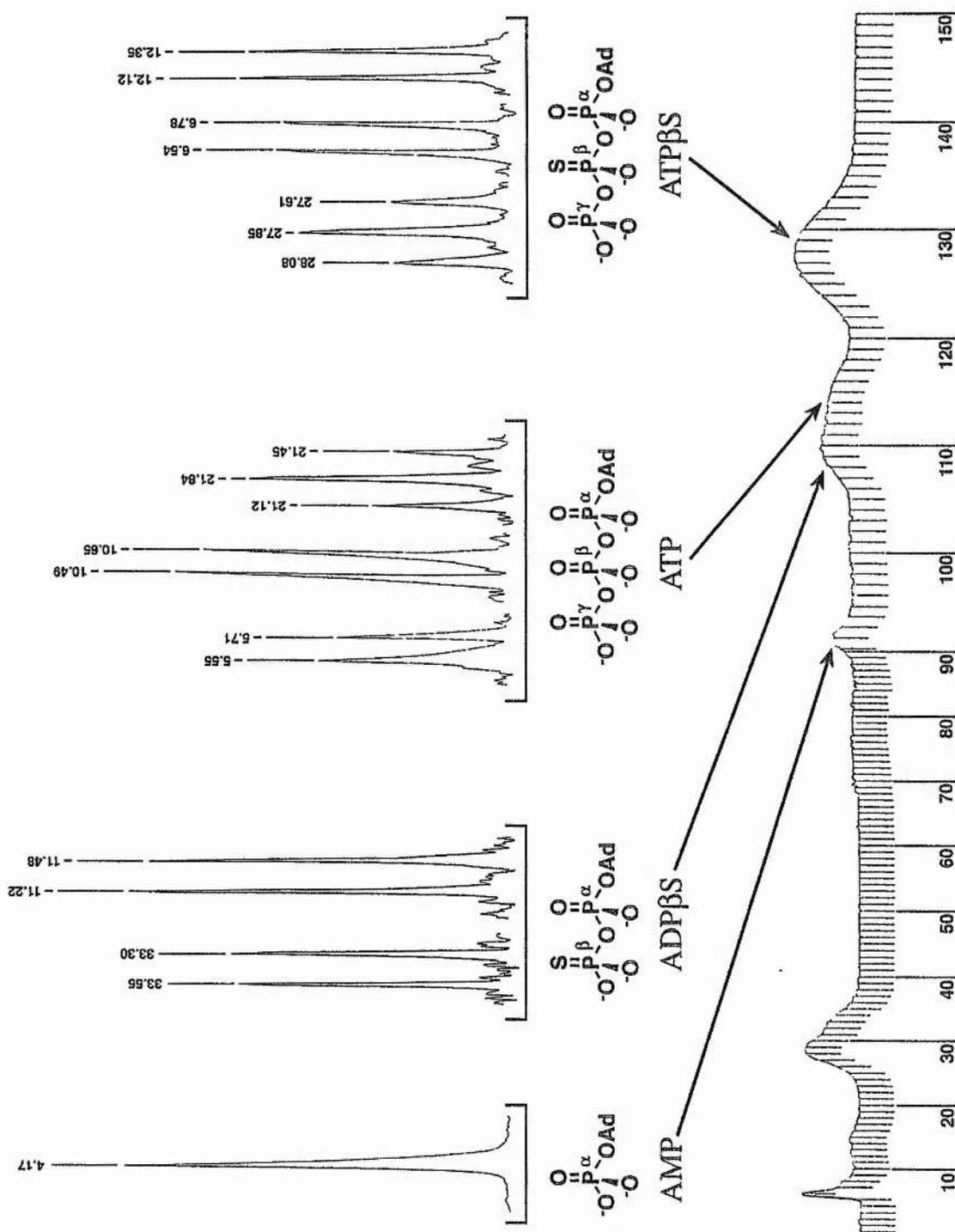


Figure 2.19: Anion exchange chromatography of the products arising from the incubation of ADP β S 234 with glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase

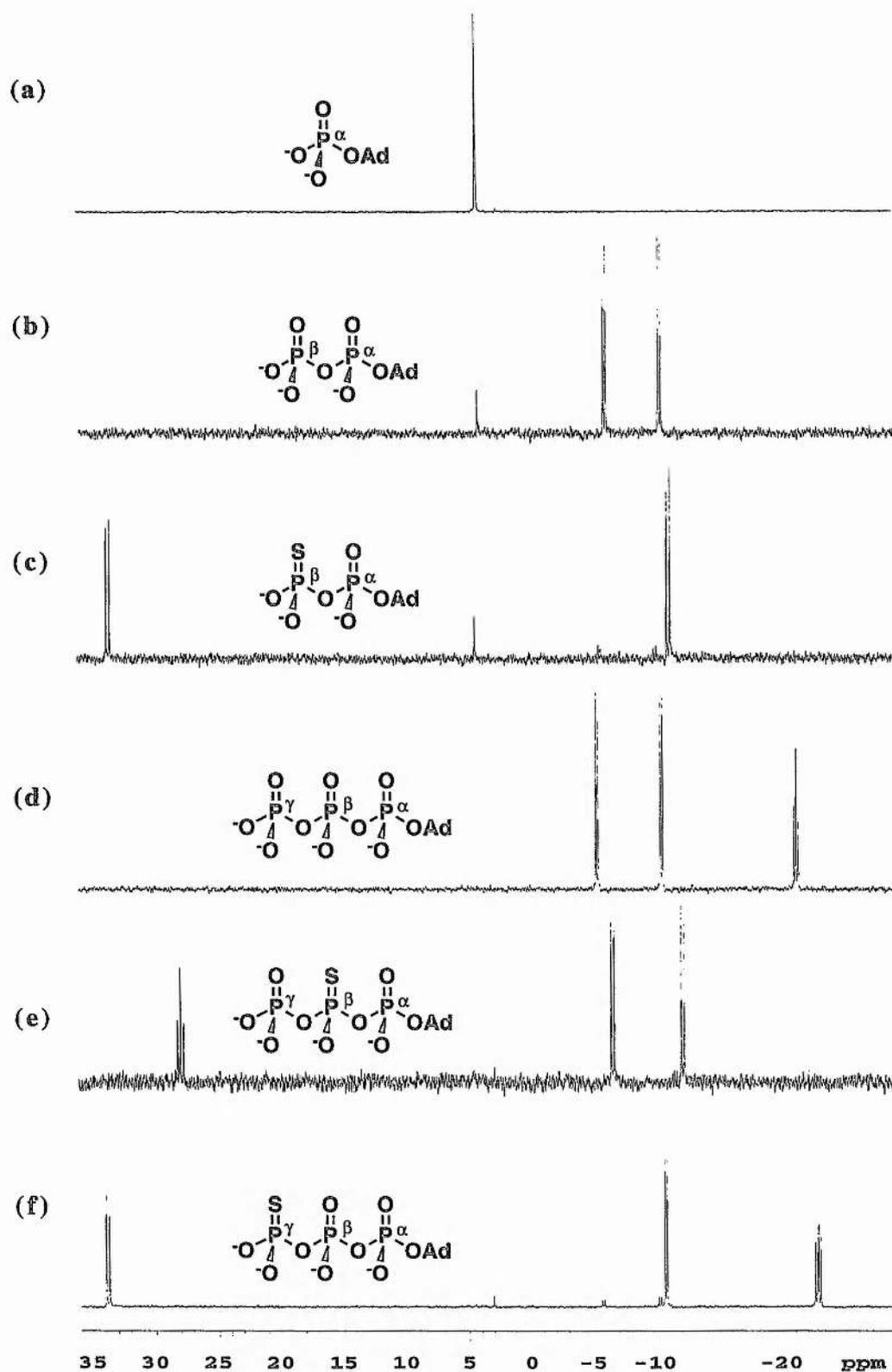


Figure 2.20: ^{31}P -NMR spectra of (a) AMP, (b) ADP, (c) ADP β S, (d) ATP, (e) ATP β S, and (f) ATP γ S. All nucleotides were isolated by anion exchange chromatography from enzymic incubation reactions

This successful isolation of ATP β S 234 from the final incubation step demonstrated that providing the synthesised L-(R_p)- and L-(S_p)-[¹⁸O]inositol 1-phosphorothioate probes L-122a and L-122b could be hydrolysed by inositol monophosphatase in the presence of [¹⁷O]water to give a reasonable quantity of chiral [¹⁶O,¹⁷O,¹⁸O]inorganic phosphorothioate (40 μ mol), then it should be possible to determine the stereochemical course of the enzyme reaction.

2.17 Purification of Inositol Monophosphatase

Bovine brain *myo*-inositol monophosphatase was purified from a recombinant strain of *Escherichia coli* according to a procedure based on the method developed by Leech *et al.*⁴³ Purification to the end of Step 4 (Sephadex G-100 Gel Exclusion Chromatography, see Section 3, page 241) was performed in an identical manner to that in the literature, and the resulting concentrated semi-purified protein solution was stored at -78 °C until required.

The concentrated protein solution was then subjected to further purification by anion exchange HPLC (Poros 20 HQ quaternized polyethyleneimine) using a BioCad Sprint™ Perfusion Chromatography® System. The column was equilibrated with buffer E (50 mmol dm⁻³ Tris/HCl, pH 8.5) before use.

To give the best purification factor, the protein solution (at approximately 150 mmol dm⁻³ KCl and 50 mmol dm⁻³ Tris/HCl) was first desalted by several cycles of dilution with 20 cm³ buffer D (80 mmol dm⁻³ KCl, 50 mmol dm⁻³ Tris/HCl, pH 8.5) and concentration by ultrafiltration at 4 °C. The solution was then applied to the column (2 cm³ portions) and washed with buffer E (5 cm³), before running a linear gradient of 0–200 mmol dm⁻³ KCl in buffer E (13 cm³), followed by a linear gradient of 200–400 mmol dm⁻³ KCl in buffer E (40 cm³). Active enzyme* eluted between 235 and 255 mmol dm⁻³ KCl. The active fractions were pooled and concentrated to a volume of *ca.* 5 cm³ by ultrafiltration, and the purified enzyme solution stored at -78 °C until required (Figure 2.21).

* A colorimetric assay was used to determine the activity of the enzyme in column eluates and confirm that the observed phosphatase activity was due solely to inositol monophosphatase (see Section 3, page 243).

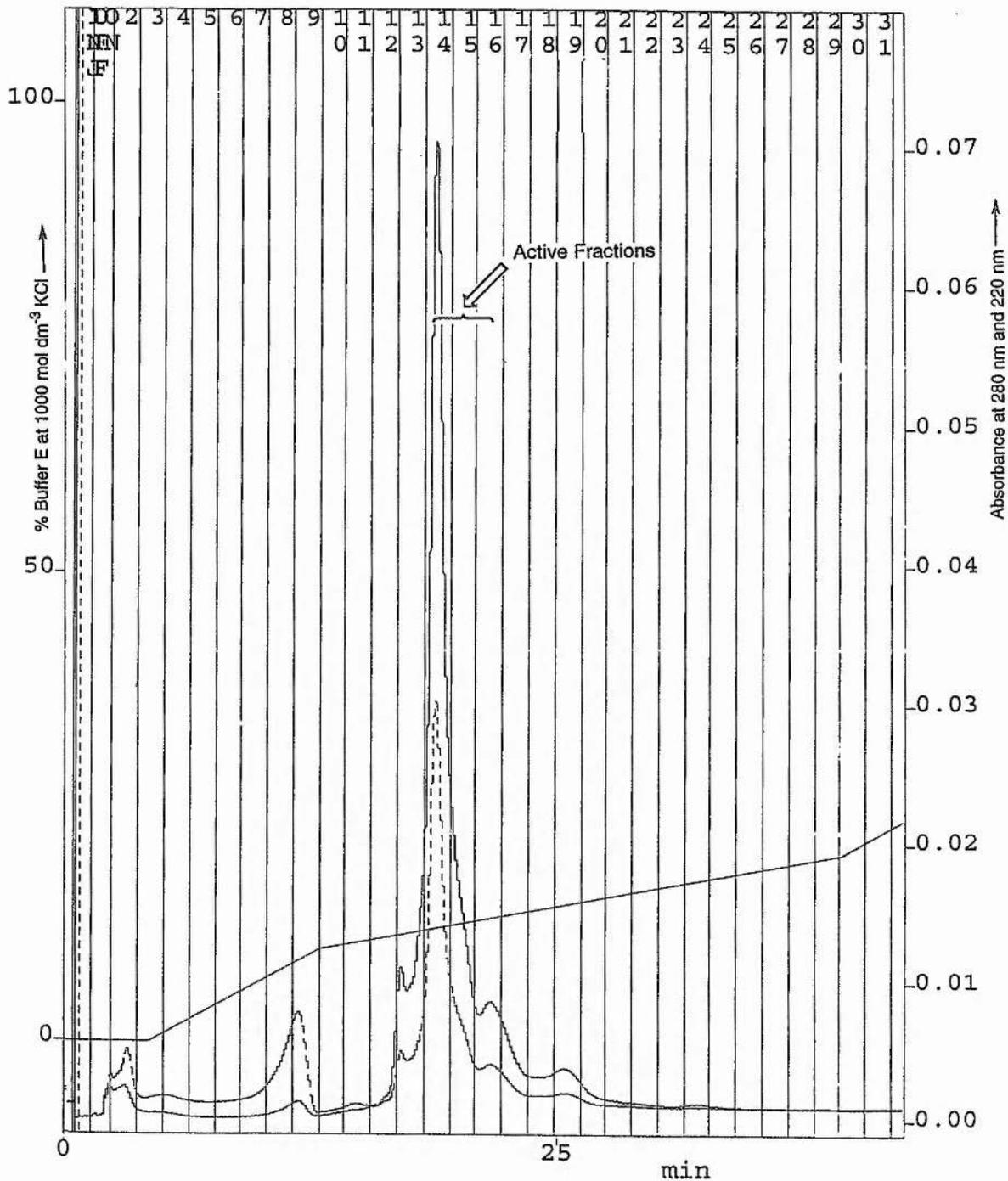


Figure 2.21: HPLC Poros 20 HQ anion exchange chromatography of recombinant inositol monophosphatase. Fractions 14 and 15 were strongly active [absorbance at 280 nm is represented by the solid line, absorbance at 220 nm is represented by the dashed line]

Recombinant inositol monophosphatase was shown to be homogenous by discontinuous SDS polyacrylamide gel electrophoresis (PAGE) using both silver stain and coomassie blue stain with a single band being visible for the HPLC purified enzyme (Figure 2.22, gel stained with coomassie blue). A sample of inositol monophosphatase previously purified to homogeneity by FPLC anion exchange chromatography (DEAE TSK-5PW) was included for comparison and corresponded exactly with the single band observed for the HPLC purified enzyme. From the molecular weight standards used in the gel, it was possible to estimate the molecular weight of the band as $\approx 29,000$ kDa. This value corresponds well with the molecular weight of the inositol monophosphatase monomer (29,000 kDa).^{43, 221}

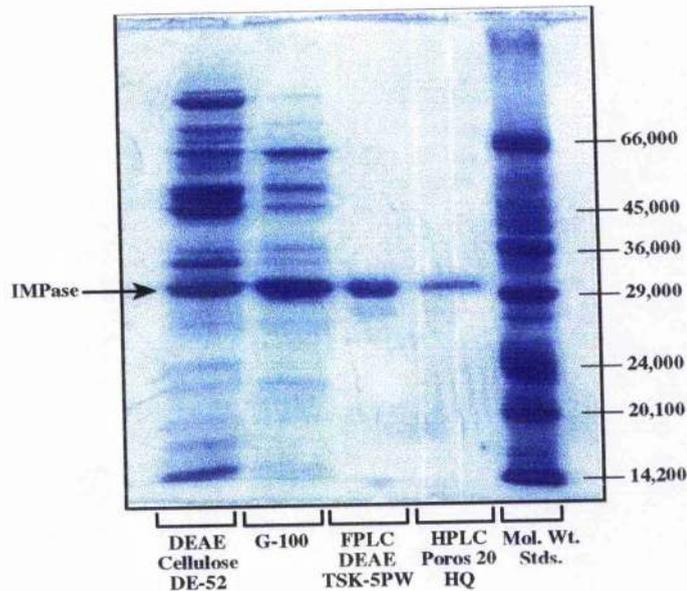


Figure 2.22: SDS PAGE of samples from recombinant inositol monophosphatase purification

The specific activity of the recombinant enzyme recovered from the HPLC purification step was found to be at $46,500 \text{ nmol min}^{-1} \text{ mg}^{-1}$,* a value which compared well with the specific activity for DEAE TSK-5PW purified enzyme (Table 2.2).^{43, 165}

* The activity of purified inositol monophosphatase was determined using a colorimetric assay for inorganic phosphate (see Section 3, page 245).

Table 2.2: Purification of bovine brain myo-inositol monophosphatase from recombinant bacteria. †Previous literature values included for comparison

| Purification Step | Total Protein (mg) | Total Activity (nmol min ⁻¹) | Specific Activity (nmol min ⁻¹ mg ⁻¹) |
|--------------------|--------------------|--|--|
| HPLC HQ Poros 20 | 3.75 | 174,500 | 46,530 |
| FPLC DEAE TSK-5PW* | 2.24 [†] | 116,800 [†] | 52,100 [†] |

2.18 Inositol 1-Phosphorothioate as a Substrate of Inositol Monophosphatase

In order to determine the stereochemical course of the inositol monophosphatase reaction, the synthesised probes L-(*R*_p)- and L-(*S*_p)-[¹⁸O]inositol 1-phosphorothioate **L-122a** and **L-122b** must be hydrolysed by the enzyme in the presence of [¹⁷O]water to produce a reasonable quantity of chiral [¹⁶O,¹⁷O,¹⁸O]inorganic phosphorothioate (this would then be incorporated into a molecule of ATPβS and the ³¹P-NMR spectrum of the compound analysed) (Section 1.30.1, page 66, and Section 2.16, page 150).

To perform an enzyme hydrolysis reaction in [¹⁷O]water, it is necessary to first prepare the enzyme in the buffer required for the reaction [in the case of inositol monophosphatase this is the standard assay buffer (200 mmol dm⁻³ KCl, 2 mmol dm⁻³ MgCl₂·6H₂O, 50 mmol dm⁻³ Tris.HCl, pH 7.8)] and then reduced the volume of the solution by ultrafiltration to that required for the enzyme reaction (typically less than 1 cm³*). The remaining water is then removed by lyophilization and replaced by an identical volume of [¹⁷O]water (46 atom %) to give a suitable system in which a chiral [¹⁸O]-labelled substrate can be hydrolysed to produce the chiral [¹⁶O,¹⁷O,¹⁸O]inorganic phosphorothioate required for configurational analysis.

* As this volume is replaced directly by H₂¹⁷O, the relative scale of the enzyme reaction is mainly dictated by the high cost of the labelled water.

Due to time constraints, it was only possible to carry out some preliminary work into developing the conditions required for this process to be carried out successfully with inositol monophosphatase. Unlabelled inositol 1-phosphorothioate **DL-123** and water were used throughout.

A solution of inositol monophosphatase (15 units) was therefore made up to a nominal volume (20 cm³) containing 200 mmol dm⁻³ KCl, 2 mmol dm⁻³ MgCl₂·6H₂O, 50 mmol dm⁻³ Tris.HCl [pH 7.8] and then reduced down to 1 cm³ by ultrafiltration, before being lyophilised. In order to allow the subsequent enzyme incubation to be monitored by NMR spectroscopy, the removed water was replaced with the same volume of ²H₂O. The enzyme was used *immediately* after lyophilisation to minimise any loss of activity. Inositol 1-phosphorothioate **DL-123** (95 mg, 200 μmol, as its biscyclohexylammonium salt) was added to the solution and the rate of reaction was monitored by observing the formation of inositol in the ¹H-NMR spectrum. It was hoped that by using 200 μmol of substrate, a sufficient quantity of inorganic phosphorothioate would be produced (≈40 μmol) such that it could then be successfully incorporated into ATPβS. Unfortunately, the substrate was turned over extremely slowly (30% converted to inositol after 7 days of incubation) and was accompanied by protein precipitation (Figure 2.23).

A smaller scale incubation was attempted (20 mg, 42 μmol inositol 1-phosphorothioate) with more enzyme (33 units), but an equally slow turnover was observed (5% after 11 hours) and the reaction was again accompanied by protein precipitation.

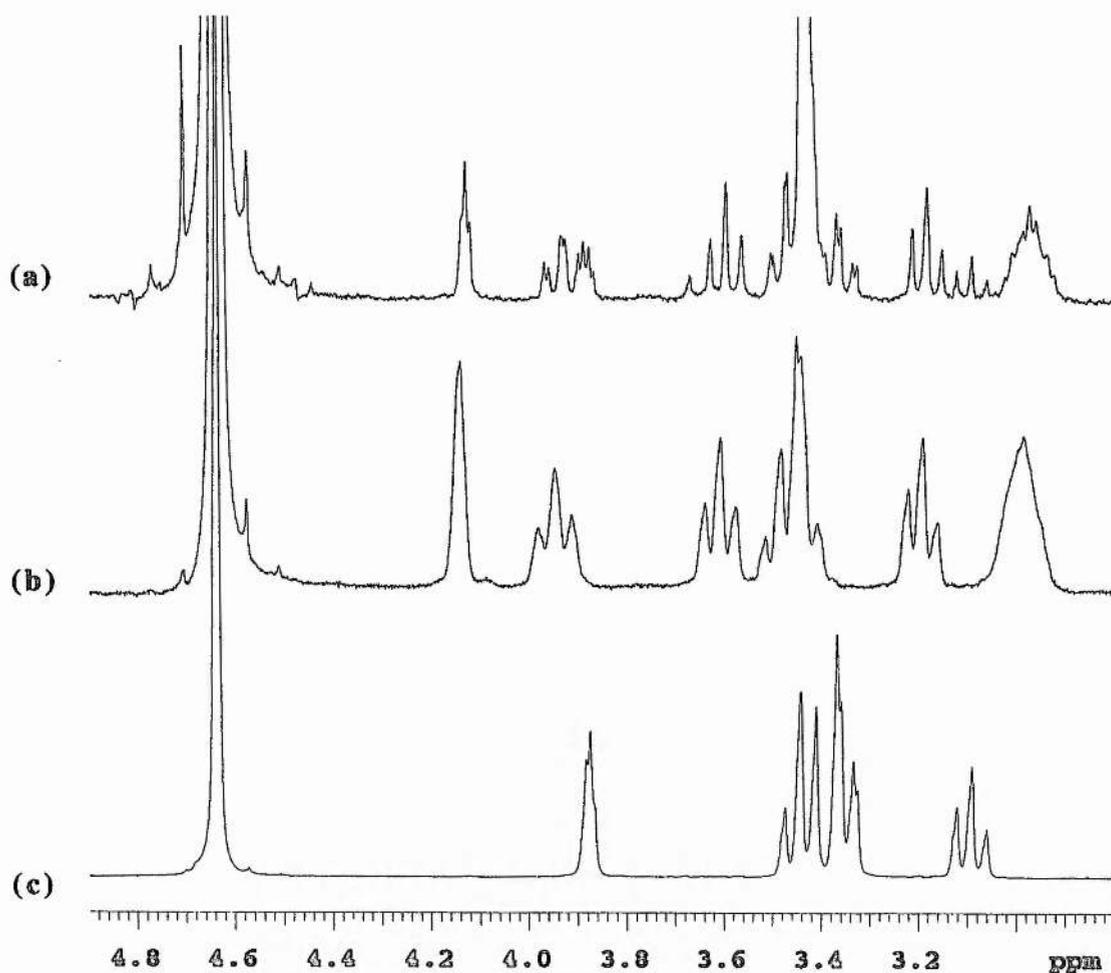


Figure 2.23: Expanded $^1\text{H-NMR}$ spectra of the incubation residue from the hydrolysis of inositol 1-phosphorothioate by inositol monophosphatase after 7 days (using lyophilised enzyme); (a) *DL-Ins1-P*, $2 \text{ mmol dm}^{-3} \text{ MgCl}_2$, $200 \text{ mmol dm}^{-3} \text{ KCl}$, $50 \text{ mmol dm}^{-3} \text{ Tris.Base}$, pH 7.8, 15 units IMPase; (b) *DL-Ins1-P*, $2 \text{ mmol dm}^{-3} \text{ MgCl}_2$, $200 \text{ mmol dm}^{-3} \text{ KCl}$, $50 \text{ mmol dm}^{-3} \text{ Tris.Base}$, pH 7.8, no IMPase; (c) *myo-inositol*, $2 \text{ mmol dm}^{-3} \text{ MgCl}_2$, $200 \text{ mmol dm}^{-3} \text{ KCl}$, $50 \text{ mmol dm}^{-3} \text{ Tris.Base}$, pH 7.8

In an initial attempt to increase the rate of turnover of inositol 1-phosphorothioate by inositol monophosphatase, Mg^{2+} was replaced by the more thiophilic Mn^{2+} cation ($2 \text{ mmol dm}^{-3} \text{ MnCl}_2 \cdot 4\text{H}_2\text{O}$),^{66, 67, 160} and the incubation repeated in a manner identical in every other way to that above (11 hours). Prior to NMR analysis of the incubation

residues, the Mn^{2+} had to be removed by cation exchange chromatography [Amberlite IR-118 (H^+)], as it was previously demonstrated that the presence of manganese results in extensive broadening of all signals in the $^1\text{H-NMR}$ spectrum due to its nuclear quadrupolar moment.¹⁶⁹ Subsequent analysis demonstrated that the Mn^{2+} ion gave a moderately *faster* turnover rate (15% after 11 hours *cf.* 5% for the similar incubation with Mg^{2+}) (Figure 2.24). It was decided, however, that the risk of perturbing the mechanism of the enzyme reaction by changing the cations present in the active site was too great to proceed further with incubations involving the Mn^{2+} cation.¹⁰¹

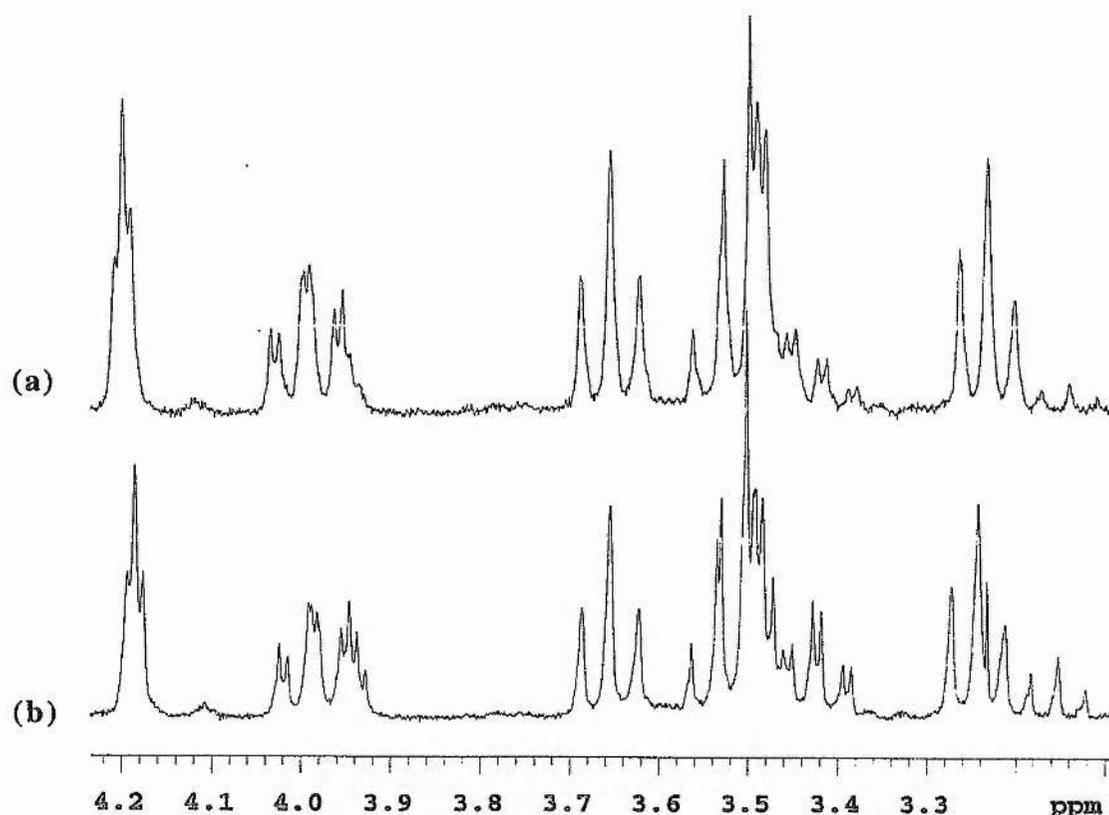


Figure 2.24: Expanded $^1\text{H-NMR}$ spectra of the incubation residues from the hydrolysis of inositol 1-phosphorothioate by inositol monophosphatase showing the effect of changing the divalent metal ion, incubation time 11 h; (a) DL-Ins1-P_s , $2 \text{ mmol dm}^{-3} \text{MgCl}_2$, $200 \text{ mmol dm}^{-3} \text{KCl}$, $50 \text{ mmol dm}^{-3} \text{Tris.Base}$, pH 7.8, 33 units IMPase; (b) DL-Ins1-P_s , $2 \text{ mmol dm}^{-3} \text{MnCl}_2$, $200 \text{ mmol dm}^{-3} \text{KCl}$, $50 \text{ mmol dm}^{-3} \text{Tris.Base}$, pH 7.8, 33 units IMPase

Although the cause of the slow rate of hydrolysis was unclear, due to time constraints this area was not investigated further, and instead a more expedient solution to the problem was sought.

Previous studies had shown that V_{\max} for the minimum substrate for inositol monophosphatase, ethane-1,2-diol 1-phosphate **38**, could be increased 1.5-fold upon increasing the Mg^{2+} ion concentration to 5 mmol dm^{-3} (Section 2.5, page 86).⁷¹ Consequently, inositol 1-phosphorothioate (10 mg, $21 \mu\text{mol}$) was incubated with lyophilised enzyme at *three* different magnesium concentrations (2, 5, and 15 mmol dm^{-3} $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 200 mmol dm^{-3} KCl, 50 mmol dm^{-3} Tris.Base, pH 7.8, 11 units IMPase) and the formation of inositol monitored by $^1\text{H-NMR}$ spectrometry. By increasing the Mg^{2+} ion concentration to 15 mmol dm^{-3} , a moderate 20% increase in V_{\max} for inositol 1-phosphorothioate was observed (Figure 2.25).

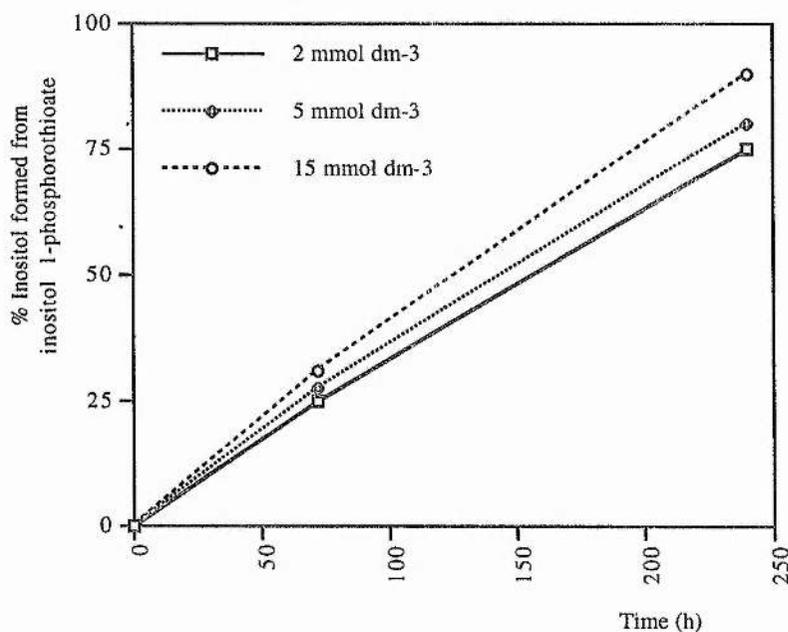


Figure 2.25: *The effect of varying the Mg^{2+} ion concentration on the rate of hydrolysis of inositol 1-phosphorothioate by inositol monophosphatase*

After 240 hours, an attempt was made to convert the product P_{si} from the 15 mmol dm^{-3} Mg^{2+} incubation reaction (which had gone to 90% completion) directly into $\text{ATP}\gamma\text{S}$ without isolation. The incubation reaction mixture was therefore mixed with an identical enzyme solution (7 cm^3) to that used previously to derivatise inorganic phosphorothioate (Section 2.16, page 150) [glyceraldehyde phosphate dehydrogenase (2000 units), 3-phosphoglycerate kinase (2000 units), aldolase (8 units), triose phosphate isomerase (200 units) and lactate dehydrogenase (200 units) containing Tris (50 mmol dm^{-3}), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (10 mmol dm^{-3}), fructose 1,6-bisphosphate (15 mmol dm^{-3}), ADP (15 mmol dm^{-3}), pyruvate (40 mmol dm^{-3}), NAD^+ (0.5 mmol dm^{-3}) and DTE (1 mmol dm^{-3}), pH 8.0] and incubated for 6 hours ($30 \text{ }^\circ\text{C}$). Following incubation, the reaction mixture was quickly cooled ($0\text{--}2 \text{ }^\circ\text{C}$) and the various nucleotide products were separated previously by anion exchange chromatography [DEAE-Sephadex A-25, eluting with a gradient system of triethylammonium bicarbonate (TEAB) buffer ($0.1\text{--}0.6 \text{ mol dm}^{-3}$, pH 7.5, $4 \text{ }^\circ\text{C}$)]. The desired product $\text{ATP}\gamma\text{S}$ was successfully isolated from the incubation mixture [$\delta_p(121.4 \text{ MHz}; ^2\text{H}_2\text{O}) -22.85$ (dd, $^2J_{\alpha\beta} 19.8$, $^2J_{\beta\gamma} 29.6$, P_β), -10.76 (d, $^2J_{\alpha\beta} 19.8$, P_α) and 33.86 (d, $^2J_{\beta\gamma} 29.6$, P_γ)]. Unfortunately, by comparison to previous experiments (Section 2.16, page 150) it was clear that an insufficient quantity of $\text{ATP}\gamma\text{S}$ had been isolated to enable its further derivatisation to $\text{ATP}\beta\text{S}$ and this was not attempted (approximately $7 \text{ }\mu\text{mol}$ of $\text{ATP}\gamma\text{S}$ is required; the estimated quantity of $\text{ATP}\gamma\text{S}$ which was recovered was $< 0.5 \text{ }\mu\text{mol}$).

Though the isolation of $\text{ATP}\gamma\text{S}$ was encouraging, it was imperative that the inorganic phosphorothioate should be produced by inositol monophosphatase as rapidly as possible and incorporated into $\text{ATP}\gamma\text{S}$ immediately in order to *minimise* the risk of *isotopic dilution* when chiral [^{16}O , ^{17}O , ^{18}O]inorganic phosphorothioate is ultimately involved. Consequently, inositol 1-phosphorothioate (29 mg , $61 \text{ }\mu\text{mol}$) was incubated this time with a much greater quantity of lyophilised enzyme (350 units) and at high Mg^{2+} ion concentration (15 mmol dm^{-3}), and after 8 h, the hydrolysis reaction was shown by ^{31}P -NMR spectrometry to have proceeded almost to completion (Figure 2.26).

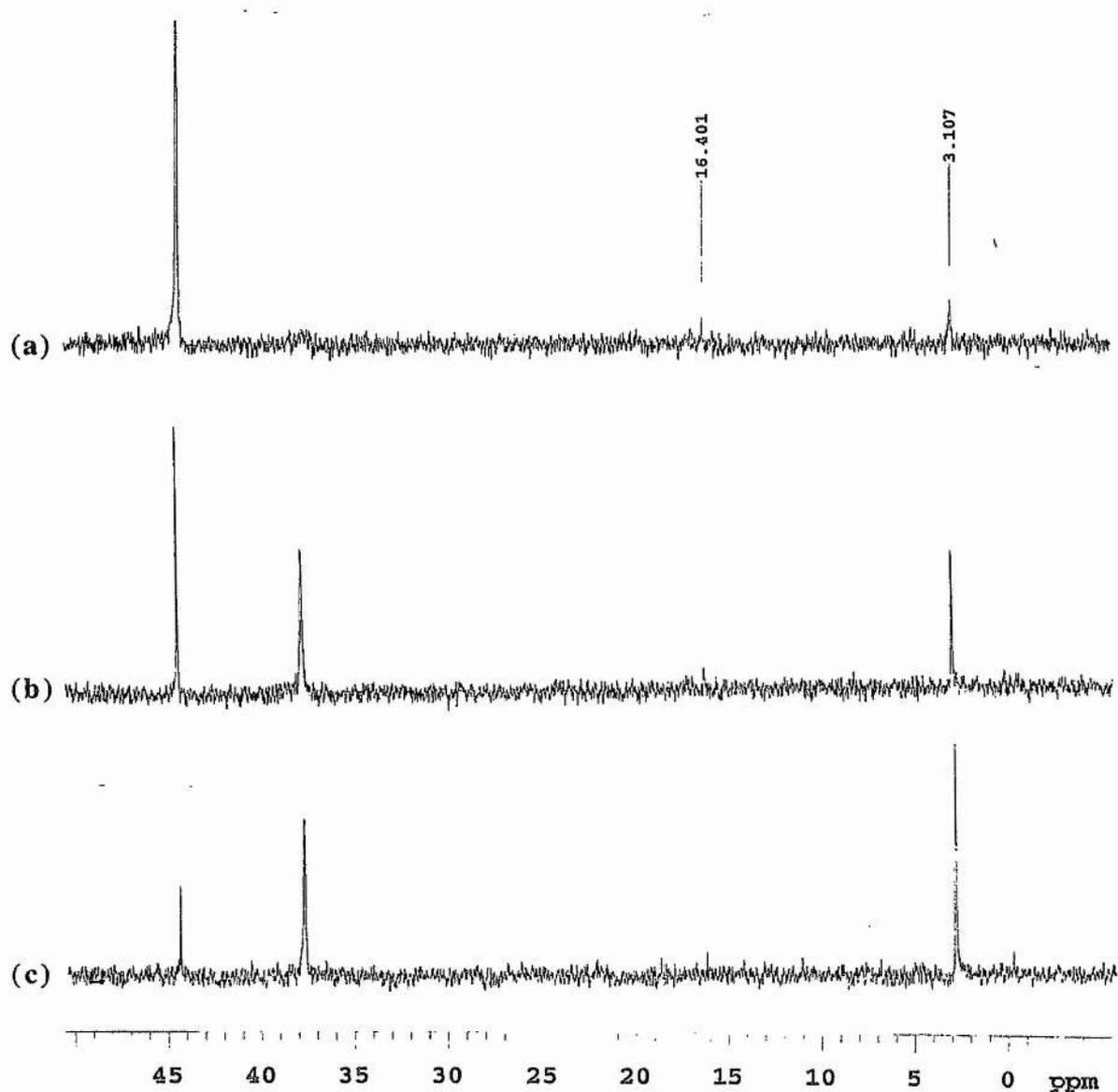


Figure 2.26: Expanded ^{31}P -NMR spectra of the incubation residues from the hydrolysis of inositol 1-phosphorothioate by inositol monophosphatase using a much larger quantity of enzyme, after; (a) 20 min; (b) 4 h; and (c) 8 h

Unfortunately, it can be seen from the ^{31}P -NMR spectra taken during the course of the enzyme reaction that inorganic phosphorothioate [δ_{p} (121.4 MHz; $^2\text{H}_2\text{O}$) 37.98] was not the only product (Figure 2.26). A slightly larger quantity of inorganic phosphate [δ_{p} (121.4 MHz; $^2\text{H}_2\text{O}$) 3.11] also formed during the reaction, and on closer examination

of this and similar incubations, it was apparent that the product P_{si} was undergoing a gradual desulfurization reaction under the conditions of the enzyme reaction to form at least some of the observed inorganic phosphate. The formation of both P_{si} and P_i was reflected in the subsequent enzymic derivatisation of the incubation mixture, as both ATP and a small quantity of ATP γ S were recovered. Further studies revealed that this desulfurization process could occur under the conditions of incubation with or without the presence of inositol monophosphatase, but appeared to be accelerated by the presence of enzyme. The inclusion of inositol (320 mmol dm^{-3}) in the incubation mixture had no effect on the observed acceleration.

To date it has not been possible to perform an inositol monophosphatase hydrolysis of inositol 1-phosphorothioate using lyophilised enzyme and derivatise the products such that a sufficient quantity of ATP β S can be isolated from the subsequent enzymic derivatisation reactions for ^{31}P -NMR analysis. Preliminary studies have indicated that the enzymes involved in the derivatisation of P_{si} are *insensitive* to the presence of the Li^+ ion at 20 mmol dm^{-3} , a concentration at which the action of inositol monophosphatase is inhibited. Therefore, it may be possible to utilise the selective inhibitory effect of Li^+ for inositol monophosphatase to effectively 'quench' the hydrolysis reaction at the moment of maximal inorganic phosphorothioate production to allow more to be incorporated enzymatically. This area of work awaits further investigation.

2.19 Conclusions and Future Work

As a result of this research, an efficient route has been developed for the synthesis of D-(*R_p*)-, D-(*S_p*)-, L-(*R_p*)- and L-(*S_p*)-[¹⁸O]inositol 1-phosphorothioate **D-122a**, **D-122b**, **L-122a** and **L-122b**, chiral analogues of the natural substrate for inositol monophosphatase which are suitable for use in determining the stereochemical course of the enzyme reaction. The final result of such a study should identify *which* of the two proposed magnesium ions in the enzyme active site binds and activates the catalytically important water molecule.

The *H*-phosphonate methodology, which this route encompassed, (Section 2.11, page 120), has permitted *both* target diastereomers to be efficiently synthesised at the *same* time from the corresponding resolved alcohol **L-129**. Use of the opposite enantiomer **D-129** would lead to the production of the sister pair of diastereomers **D-122a** and **D-122b**, although this was not attempted. Moreover, the simple chromatographic separation of the [¹⁸O]benzyl *H*-phosphonate diester intermediates **212a** and **212b** during the synthesis (Scheme 2.28, page 120) has ensured that diastereomers **L-122a** and **L-122b** were produced with a high degree of diastereomeric purity. Another major benefit of this synthetic route was the late introduction of the expensive [¹⁸O]-label as [¹⁸O]benzyl alcohol. In this form, the label was found to be stable to isotopic dilution, the isotopic enrichment of the [¹⁸O]-label could be accurately monitored (by mass spectrometry, Figure 2.16, page 146), and the excess [¹⁸O]-label could be recovered from the crude reaction mixture by simple column chromatography. As the analysis of this excess [¹⁸O]benzyl alcohol indicated that very little isotopic dilution had occurred, it was possible to reuse the compound in further coupling reactions.

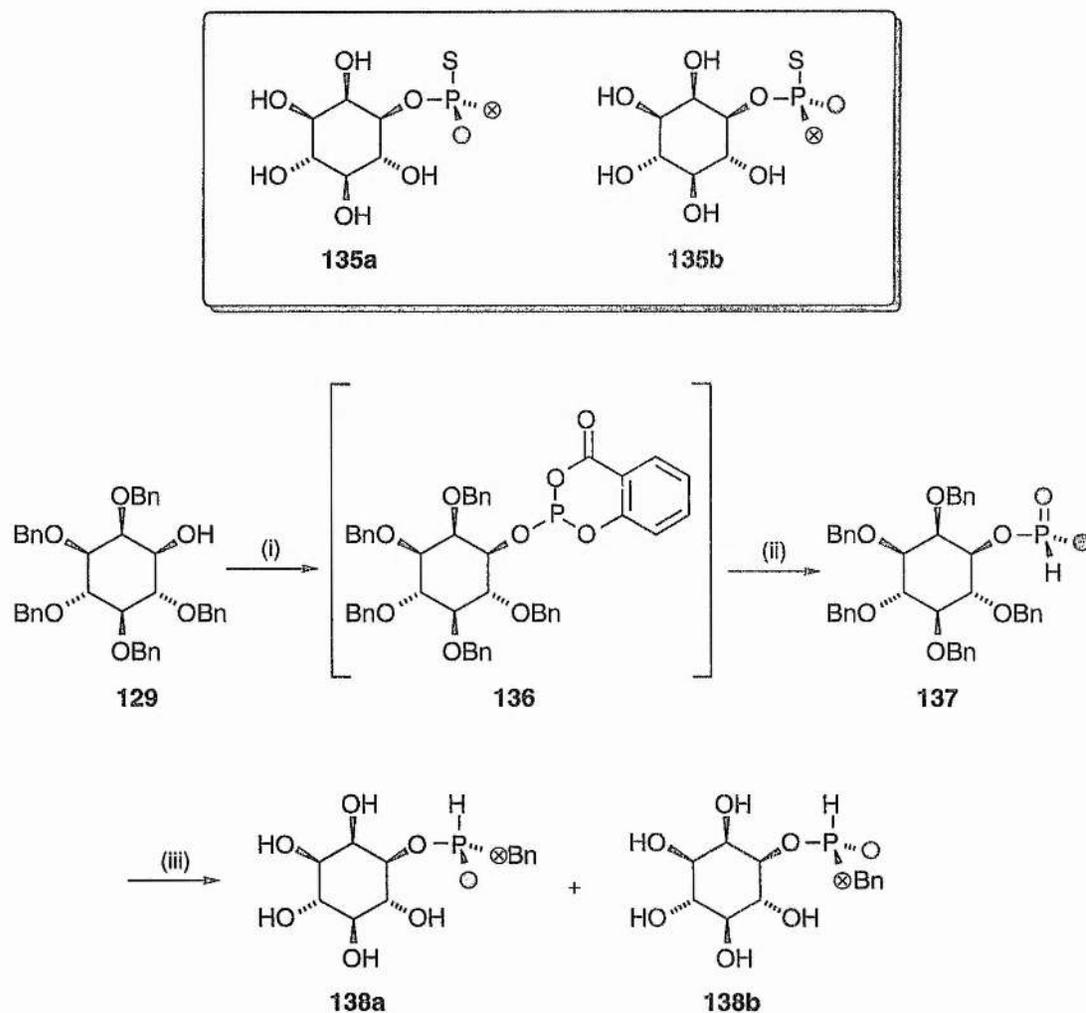
The absolute configuration of the chiral target compounds **122a** and **122b** was determined indirectly by single crystal X-ray analysis of the dicyclohexylammonium salt (**227b**) of fully protected thiophosphoric acid precursor **220b** (Section 2.12, page 129), using the benzyl group attached to the [¹⁸O]-label to indicate its position in the compound. In addition, by combining the X-ray crystallographic analysis with ³¹P-NMR data, it has

been possible to prove that the sulfurization of *H*-phosphonate diesters **216a** and **216b** by elemental sulfur in pyridine to produce thiophosphoric acids **220a** and **220b** did proceed as predicted with *complete stereospecificity* and *retention of configuration* at the phosphorus centre. During the course of this work, it also became apparent that the original assignment of camphanate esters **D-199a** and **L-199b** by Billington *et al.*⁴⁷ was in error (Section 2.13, page 139). This was a crucial discovery as their correct assignment was paramount in the production of L-(*R*_p)- and L-(*S*_p)-[¹⁸O]inositol 1-phosphorothioate **L-122a** and **L-122b** with their absolute configuration known.

Now that target compounds **L-122a** and **L-122b** have been synthesised and their absolute configuration determined, they can be used to determine the stereochemical course of the inositol monophosphatase reaction.

Preliminary work has demonstrated that inositol monophosphatase will process inositol 1-phosphorothioate **DL-123** and that the inorganic phosphorothioate product can then be incorporated into a molecule of ATP_γS using the procedure developed by Webb and Trentham.¹⁵⁰ This is the first of three enzymatic derivatisation steps which is required to allow the chiral inorganic [¹⁶O,¹⁷O,¹⁸O]phosphorothioate (which will be ultimately produced by inositol monophosphatase in the stereochemical study) to be derivatised such that its configuration can be determined by ³¹P-NMR spectroscopy. However, the quantity of ATP_γS which has been produced so far is insufficient for the further two incorporation steps to be performed successfully. It has become apparent that DL-inositol 1-phosphorothioate **DL-123** may be a weaker substrate for inositol monophosphatase than previously thought (Section 2.18, page 161), although it is likely that the small volume of the enzyme incubation reaction (typically < 1 cm³) could also be contributing to the observed poor production of inorganic phosphorothioate (a small volume incubation is a prerequisite when performing an enzyme reaction in [¹⁷O]water, as is required for the stereochemical study, due to the extremely high cost of the labelled water). P_i is a competitive inhibitor of inositol monophosphatase (Section 1.5, page 11) and it has been observed to form during the enzymic hydrolysis of inositol 1-phosphorothioate (Figure 2.26, page 167). Its formation becomes more significant at the small volume used for the

chemistry of the procedure remains unchanged, the configuration at phosphorus of these compounds would be known from the X-ray structural work already performed (Section 2.12, page 129).



Reagents and conditions: (i) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one **215**, pyridine:THF (1:4, v/v), 0–25 °C, 15 min; (ii) [¹⁸O]water, 10 min, then (c) TEAB, 20 min; (iii) [¹⁷O]BnOH, PvCl, pyridine:THF (1:1, v/v), 25 °C, 8 h, then (b) TEAB, 20 min.

Scheme 2.36: Proposed synthetic route to alternative labelled phosphorothioates **135a** and **135b** utilising the previously developed H-phosphonate methodology

It is also worth noting that the isolation of nucleoside phosphates and phosphorothioates from the enzymic derivatisation of inorganic phosphorothioate which was performed by

conventional anion exchange column chromatography [DEAE-Sephadex A-25, eluting with a gradient system of triethylammonium bicarbonate (TEAB) buffer (0.1–0.6 mol dm⁻³, pH 7.5, 4 °C), Section 2.16, page 150] could be potentially improved by employing reversed phase HPLC on the recently developed SUPELCOSIL™ LC-18-T silica column.²²² A special treatment of the silica surface of the column reduces the potential for silanol or metal ion interactions with small, charged, very hydrophilic compounds such as nucleotide phosphates. Using this system, an excellent resolution has already been observed for mixtures of AMP, ADP and ATP.²²²

CHAPTER THREE
EXPERIMENTAL

3. Experimental Section

Elemental analyses were performed in the departmental microanalytical laboratory. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 500 (^1H , 500.3 MHz), a Varian Gemini 300 (^1H , 300 MHz; ^{13}C , 75.4 MHz), a Varian Gemini 200 (^1H , 200 MHz; ^{13}C , 50.31 MHz) or a Bruker AM-300 (^1H , 300 MHz; ^{13}C , 74.8 MHz) spectrometers. Chemical shifts are described in parts per million downfield shift from SiMe_4 and are reported consecutively as position (δ_{H} or δ_{C}), relative integral, multiplicity ((s = singlet, d = doublet, t = triplet, q = quartet, dd = double of doublets, m = multiplet, and br = broad), coupling constant (J/Hz , if of practical importance) and assignment (numbering according to the IUPAC nomenclature for the compound). ^1H NMR spectra were referenced internally on ^2HOH (δ 4.68) or C^2HCl_3 (δ 7.27). ^{13}C NMR spectra were referenced on the central resonance of C^2HCl_3 (δ 77.0). ^{31}P NMR spectra were recorded on either a Varian Gemini 300 (^{31}P , 121.4 MHz) and chemical shifts are described in parts per million upfield or downfield shift from external reference 85% $^2\text{H}_3\text{PO}_4$.

Infra-red spectra were recorded on a Perkin-Elmer 1710 FT-IR spectrometer. The samples were prepared as Nujol mulls, solutions in chloroform or thin films between sodium chloride discs. The frequencies (ν) as absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. Mass spectra and accurate mass (HRMS) measurements were recorded on a VG 70-250 SE, a Fisons Instruments VG Platform E/S Mass Spectrometer or by the SERC service at Swansea using a VG AZB-E. Fast atom bombardment spectra were recorded using NOBA or glycerol as a matrix. Major fragments were given as percentages of the base peak intensity (100%) and multiple signals arising through the presence of ^{18}O isotope are all given.

Melting points were taken on an Electrothermal Gallenkamp or Reichert melting point apparatus and are uncorrected. Optical rotations were measured at 20 °C or at room temperature (25 °C) depending on the literature requirement on a Optical Activity AA-1000 polarimeter using a 10 cm path length cell and are given in $10^{-1} \text{ deg cm}^2 \text{ g}^{-1}$.

Reagents were used without purification unless otherwise stated. Quantities of reagents were calculated from the manufacturers' stated purities. Experiments were conducted at room temperature (20–25 °C) unless otherwise stated. All reactions that employed organometallic reagents or other moisture sensitive reagents were performed in dry solvent under an atmosphere of dry nitrogen or argon in oven-dried and/ or flame-dried glassware. Solutions in organic solvents were dried over anhydrous magnesium sulfate, filtered and concentrated or evaporated under reduced pressure on a Büchi rotary evaporator unless otherwise stated. All oils, after rotary evaporation, were further dried on a high-vacuum line.

Flash chromatography was performed according to the method of Still *et al.*²²³ using Fluka Kieselgel (220–440 mesh) silica gel or Florisil® (100–200 mesh). Analytical thin layer chromatography (TLC) was carried out on 0.25 mm precoated silica gel plates (Whatman PE SIL G/UV₂₅₄) and compounds were visualised using UV fluorescence, ethanolic phosphomolybdic acid or aqueous permanganate solution. Preparative cation exchange column chromatography was performed using Amberlite IR118 (H⁺) ion-exchange resin (120 x 25 mm), eluting with distilled water at a rate of 1 drop every 3 seconds. The fresh resin was prepared by washing with distilled water until no more coloration was observed, loaded into a glass column and then washed stepwise, at a rate of 1 drop every 1 second, with two bed volumes of 0.5 mol dm⁻³ NaOH, two bed volumes of distilled water, two bed volumes of 0.5 mol dm⁻³ and finally distilled water until the eluant was neutral. Used resin was regenerated in a similar manner.

The solvents used were either distilled or of analar quality and petroleum ether refers to that portion boiling between 40 and 60 °C. Solvents were purified and dried according to the method of Perrin and Armarego.²²⁴ Ethanol and methanol were dried over magnesium turnings. DMF, dichloromethane, acetonitrile, triethylamine and pyridine were distilled over CaH₂. Diisopropylethylamine was distilled from ninhydrin, and then from potassium hydroxide pellets. THF and diethyl ether were dried over sodium-benzophenone and distilled under nitrogen. Toluene was dried over sodium and distilled under nitrogen. Phosphorous acid and all *H*-phosphonate intermediates were rendered

anhydrous before use by repeated evaporation of pyridine under high vacuum.¹⁹¹ All other chemicals were of analytical grade or were recrystallised or distilled before use.

Water-¹⁸O, normalized, 97% atom % ¹⁸O and water-¹⁷O, normalized, 46% atom % ¹⁷O were obtained from Euriso-top, Gif-Sur-Yvette, France.

Glyceraldehyde 3-phosphate dehydrogenase (EC.1.2.1.12, from porcine muscle, 100 units mg⁻¹), aldolase (EC.4.1.2.13, type IV from rabbit muscle, 11 units mg⁻¹), L-lactic dehydrogenase (EC.1.1.1.27, type II from rabbit muscle, 900 units mg⁻¹), adenylate kinase (myokinase) (EC.2.7.4.3, from rabbit muscle, 2000 units mg⁻¹) and triose phosphate isomerase (EC.5.3.1.1, type XII from porcine muscle, 5900 units mg⁻¹) were purchased from Sigma. 3-Phosphoglycerate kinase (EC.2.7.2.3, from yeast, 450 units mg⁻¹) was purchased from Boehringer Mannheim. All the enzymes were obtained as crystalline suspensions in 3.2 mol dm⁻³ ammonium sulfate which were desalted prior to use by dialysis against Tris buffer (3 x 1000 cm³, 20 mmol dm⁻³ Tris, 1.0 mmol dm⁻³ DTE, 1.0 mmol dm⁻³ EDTA, pH 8.0) except for myokinase which was desalted by dialysis against Hepes buffer (3 x 1000 cm³, 20 mmol dm⁻³ Hepes, 1.0 mmol dm⁻³ DTE, 1.0 mmol dm⁻³ EDTA, pH 7.5). The resultant enzyme solutions were used directly. AMP (sodium salt, from yeast), ADP (sodium salt, from bacterial source), D-fructose 1,6-diphosphate (tetrasodium salt), β-NAD (from yeast), pyruvate (sodium salt), magnesium chloride hexahydrate and DTE were all from Sigma and of the highest purity available. Sodium thiophosphate was obtained as the dodecahydrate from Aldrich.

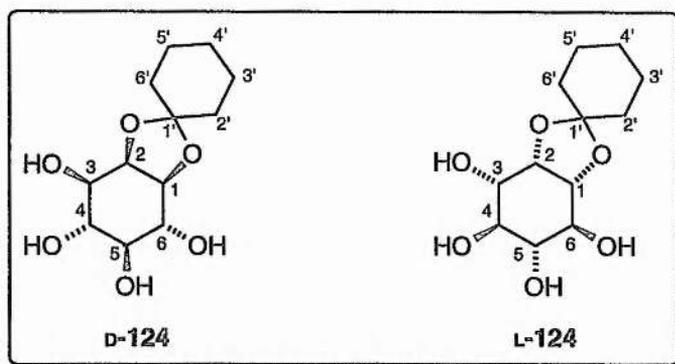
Triethylammonium bicarbonate buffer (TEAB buffer) was prepared by passing a stream of CO₂ gas through a cooled (ice-water bath) solution of triethylamine in deionized water (at the desired molarity) until the solution became neutral.¹⁹⁷

A DEAE-Sephadex A-25 anion exchange column (500 mm x 26 mm, 4 cm³ min⁻¹, 4 °C) was used for routine column chromatography to separate nucleotides, P_i, and P_{si}. The column was equilibrated before use with TEAB buffer (0.1 mol dm⁻³, pH 7.5). The column was developed using a linear gradient of 0.1–0.6 mol dm⁻³ TEAB buffer (960 cm³, pH 7.5), followed by steady elution with TEAB buffer (0.6 mol dm⁻³,

pH 7.5), collecting 14 cm³ fractions until all products had eluted.¹⁵³ The TEAB buffer gradient was controlled using a LKB 2113 Ultrograd Gradient Mixer and the various components in the eluant observed using a LKB 2238 Uvicord SII, measuring UV absorbance at 260 nm.

The DEAE-Sephadex A-25 was regenerated after use by washing the anion exchange resin successively with 3 x 500 cm³ H₂O, then 2 x 500 cm³ sodium hydroxide solution (0.01 mol dm⁻³), then 2 x 500 cm³ hydrochloric acid solution (0.2 mol dm⁻³), then 2 x 500 cm³ H₂O, and then finally 3 x 500 cm³ TEAB buffer (0.1 mol dm⁻³). The column was repacked every time before use to achieve maximum flow rates.

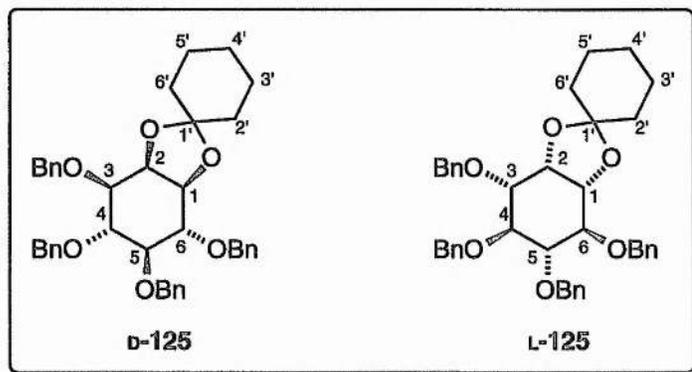
For consistency and clarity throughout this text, the notation D- and L- when describing *myo*-inositol derivatives refers to the ring configurations found in the enantiomers of the natural substrate for inositol monophosphatase, D- and L-inositol 1-phosphate. This configuration has been determined absolutely by the independent synthesis of D- and L-inositol 1-phosphate from several natural products of known configuration.¹⁰ It must be noted, however, that the optical rotation of enantiomers of intermediate *myo*-inositol derivatives described in this text sometimes differ from that inferred by the D- and L-notation of the ring.

DL-*cis*-1,2-*O*-Cyclohexylidene *myo*-inositol DL-124⁴⁸

myo-Inositol **7** (20 g, 111.0 mmol) was added to cyclohexanone (200 cm³), toluene (100 cm³) and DMF (20 cm³) with stirring, and the mixture refluxed (Dean-Stark apparatus, 150 °C) under a nitrogen atmosphere for 2 h to dry the reagents. *p*-Toluene sulfonic acid (0.6 g, 3.15 mmol) was added and the mixture refluxed until no further water was collected (24 h). On cooling to 35 °C, toluene (100 cm³), petroleum ether (100 cm³) and ethanol (50 cm³) were added to the resulting clear solution. On the addition of *p*-toluene sulfonic acid (1.2 g, 6.3 mmol) a precipitate was formed immediately, and the solution was stirred at 4 °C for 2 h. Triethylamine (1.2 cm³) was added, and the solution allowed to stand at -20 °C for a further 24 h. On filtering, the resultant paste was heated in ethanol (200 cm³) and triethylamine (1.0 cm³) for 1 h (100 °C), hot filtered, and on cooling the white crystalline product was collected by filtration (25.0 g, 86%); mp 179–183 °C (decomp.) (lit.,⁴⁸ 184–186 °C (decomp.)) ν_{\max} (Nujol)/cm⁻¹ 3020–3550 br s (OH), 1713 w, 1377 s, 1246 m, 1148 m, 1114 m, 1044 m (C-O), 1002 m, 929 w, 899 w, 730 w; δ_{H} (300 MHz; ²H₂O) 1.38–1.77 (10 H, m, cyclohexyl-CH₂), 3.26 (1 H, t, $J_{4,5}$ 9.8, $J_{5,6}$ 10.3, 5-CH), 3.57 (1 H, dd, $J_{5,6}$ 10.3, $J_{6,1}$ 7.8, 6-CH), 3.64 (1 H, t, $J_{3,4}$ 9.7, $J_{4,5}$ 9.8, 4-CH), 3.84 (1 H, dd, $J_{2,3}$ 4.2, $J_{3,4}$ 9.7, 3-CH), 4.05 (1 H, dd, $J_{6,1}$ 7.8, $J_{1,2}$ 4.9, 1-CH) and 4.47 (1 H, t, $J_{1,2}$ 4.9, $J_{2,3}$ 4.2, 2-CH); δ_{C} (75.4 MHz; ²H₂O) 26.08, 26.38 and 27.09 (3'-C, 4'-C and 5'-C), 37.40 and 40.27 (2'-C and 6'-C), 72.40, 75.08, 75.45, 77.88, 78.41 and 80.98 (Ins-CH) and 114.32 (1'-C); m/z (EI⁺) 260 (63%, M⁺) 231 (47), 217 (76), 181 (3, [Inositol + H]⁺), 127 (41), 109 (64), 99 (78, [Cyclohexanone + H]⁺),

81 (82) and 55 (100); m/z (FAB⁺) 307 (31%, [M - H + 2Na]⁺), 283 (54, [M + Na]⁺), 261 (100, [M + H]⁺), 217 (42) and 109 (92).

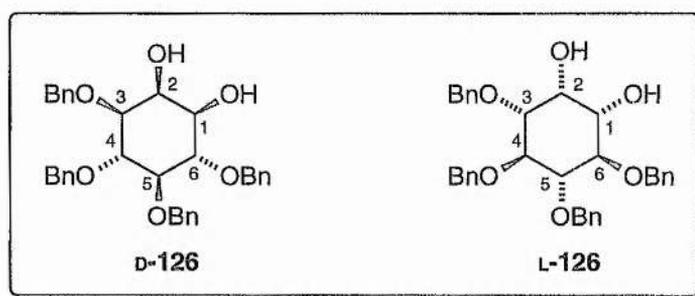
**DL-*cis*-1,2-*O*-Cyclohexylidene-3,4,5,6-tetra-*O*-benzyl
myo-inositol DL-125⁴⁸**



To DL-*cis*-1,2-*O*-cyclohexylidene *myo*-inositol DL-124 (2.0 g, 7.68 mmol) was added benzyl chloride (20 cm³, 0.17 mol) and potassium hydroxide (12.0 g, 0.21 mol). The resulting mixture was refluxed under a nitrogen atmosphere for 24 h (mechanical stirrer necessary) and then allowed to cool to room temperature. Toluene (15 cm³) and water (40 cm³) were then added with stirring, and the phases separated. The aqueous phase was extracted with toluene (15 cm³) and the combined organic phases washed with water (3 x 50 cm³) until the pH of the wash-water was neutral, then saturated brine (50 cm³), and dried (MgSO₄). The solvent was removed under reduced pressure, and the residue oil purified by silica column chromatography (0–10% ethyl acetate/petroleum ether, in 5% steps) and recrystallised from ethanol to give the fully protected *myo*-inositol DL-125 as white crystals (3.45 g, 72%); mp 83–86 °C, (lit.,⁴⁸ 84–86 °C); ν_{\max} (Nujol)/cm⁻¹ 1495 w, 1377 m, 1071 w, 1038 w, 738 w, 695 m; δ_{H} (300 MHz; C²HCl₃) 1.38–1.89 (10 H, m, cyclohexyl-CH₂), 3.44 (1 H, dd, $J_{4,5}$ 8.5, $J_{5,6}$ 9.8, 5-CH), 3.73 (1 H, dd, $J_{2,3}$ 3.9, $J_{3,4}$ 8.4, 3-CH), 3.85 (1 H, dd, $J_{5,6}$ 9.8, $J_{6,1}$ 7.0, 6-CH), 3.97 (1 H, t, $J_{3,4}$ 8.4, $J_{4,5}$ 8.5, 4-CH), 4.13 (1 H, dd, $J_{6,1}$ 7.0, $J_{1,2}$ 5.8, 1-CH), 4.31 (1 H, dd, $J_{1,2}$ 5.8, $J_{2,3}$ 3.9, 2-CH), 4.61–4.97 (8 H, m, 4 x CH₂Ph) and 7.22–7.47 (20 H, m, Ar-H);

δ_c (75.4 MHz; C^2HCl_3) 23.60, 23.85 and 25.00 (3'-C, 4'-C and 5'-C), 34.98 and 37.34 (2'-C and 6'-C), 73.11 and 73.96 (CH_2Ph), 74.02 (Ins-CH), 75.08 and 75.22 (CH_2Ph), 77.29, 78.79, 80.94, 82.15 and 82.92 (Ins-CH's), 110.50 ($1'CH_2$), 127.53, 127.59, 127.66, 127.82, 128.04, 128.30, 128.35 and 128.42 (Ar-CH) and 138.39 and 138.71 (Ar-C quaternary); m/z (Cl^+) 643 (12%, $[M + Na]^+$), 621 (23, $[M + H]^+$), 422 (19), 351 (11), 271 (8), 181 (100, $[Inositol + H]^+$), 165 (28), 107 (24, $PhCH_2O^+$); $R_f = 0.43$ (20% ethyl acetate/petroleum ether).

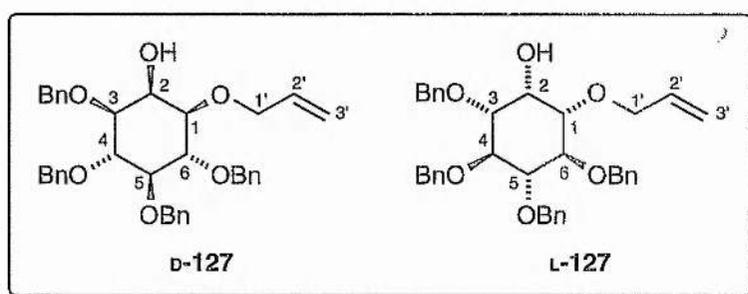
DL-3,4,5,6-Tetrakis-*O*-benzyl *myo*-inositol DL-126¹⁶¹



DL-*cis*-1,2-*O*-Cyclohexylidene-3,4,5,6-tetra-*O*-benzyl *myo*-inositol DL-125 (1.0 g, 1.6 mmol) was refluxed in acetic acid (15 cm³) and water (5 cm³) for 2 h. The solvents were removed under reduced pressure and the resulting oil dried azeotropically using toluene. The crude product was purified by silica column chromatography (0–30% ethyl acetate/petroleum ether, in 5% steps) to give the tetra-benzyl *myo*-inositol as a white solid DL-126 after recrystallisation from methanol (0.74 g, 84%), mp 114–115 °C (lit.,¹⁶¹ 113–115 °C) (Found: C, 75.8; H, 6.5. Calc. for $C_{34}H_{36}O_6$: C, 75.5; H, 6.7%) (HRMS: found: $[M + H]^+$, 541.2597. Calc. for $C_{34}H_{37}O_6$: 541.2590); ν_{max} (Nujol)/cm⁻¹ 3382 br s (OH), 3235 br s (OH), 1497 w, 1377 s, 1303 w, 1136 m, 1063 s; δ_H (300 MHz; C^2HCl_3) 2.44 (1 H, br s, OH), 2.51 (1 H, br s, OH), 3.45–3.52 (3 H, m, 3 x Ins-CH), 3.85 (1 H, t, Ins-CH), 3.98 (1 H, t, Ins-CH), 4.21 (1 H, t, 2-CH), 4.68–4.98 (8 H, m, 4 x CH_2Ph), 7.26–7.34 (20 H, m, Ar-H); δ_c (75.4 MHz; C^2HCl_3) 69.17 and 71.75 (Ins-CH), 72.74, 75.59, 75.69 and 75.94 (CH_2Ph), 80.01, 81.32, 81.66 and 83.22 (Ins-

CH), 127.68, 127.88, 127.95, 128.02, 128.44, 128.60 and 128.64 (Ar-CH) and 137.86, 138.58 and 138.71 (Ar-C quaternary); m/z (Cl^+) 541 (10%, $[M+H]^+$), 539 (14, $[M-H]^+$), 449 (35, $[M+H-CH_3Ph]^+$), 359 (31, $[M+H-2CH_2Ph]^+$), 269 (31, $[M+2H-3CH_2Ph]^+$), 181 (100, $[Inositol+H]^+$), 179 (44, $[Inositol-H]^+$), 107 (12, OCH_2Ph^+) and 91 (37, CH_2Ph^+); $R_f = 0.15$ (20% ethyl acetate/petroleum ether).

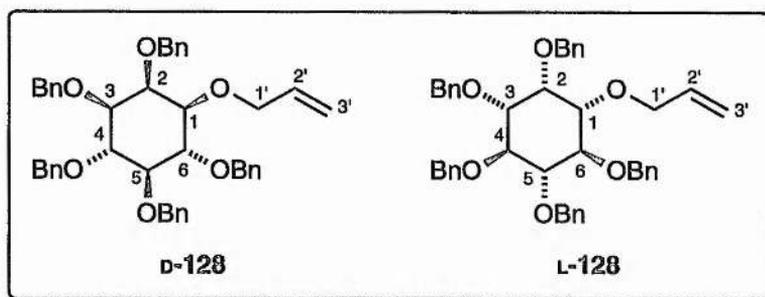
DL-1-O-Allyl-3,4,5,6-tetrakis-O-benzyl myo-inositol DL-127¹⁶¹



Procedure 1: Following the method of David *et al.*,¹⁶² a solution of DL-3,4,5,6-tetrakis-O-benzyl myo-inositol DL-126 (10.0 g, 18.5 mmol) in dry benzene (200 cm³) was treated with dibutyltin oxide (4.7g, 18.5 mmol) and the mixture refluxed under a nitrogen atmosphere (Dean-Stark apparatus) for 24 h until formation of the stannylidene complex was complete as judged by the disappearance of the O-H stretches of uncomplexed DL-126 in the infra-red spectrum. The solution was reduced to one half of its original volume by distillation, and the reaction mixture was cooled to 60 °C. Tetrabutyl ammonium bromide (6.0 g, 18.5 mmol) and allyl bromide (5 cm³, 57.6 mmol) were added and the reaction was stirred for a further 48 h until no starting material remained (as judged by TLC). Water (4 cm³) was added and the reaction mixture stirred for a further 1 h. On removal of the solvents, the product was purified twice by column chromatography on silica gel (0–40% ethyl acetate/petroleum ether, in 5% steps) to give allyl ether DL-127 as a clear oil (10.3 g, 96%).

Procedure 2: A solution of DL-3,4,5,6-Tetrakis-O-benzyl myo-inositol DL-126 (1.0 g, 1.85 mmol), in dry benzene (25 cm³) was treated with dibutyltin dimethoxide

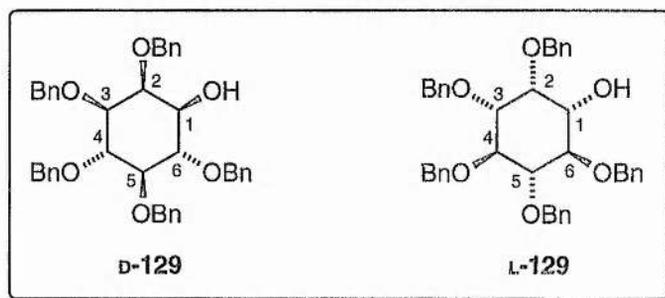
(0.55 g, 1.85 mmol) and the mixture refluxed under a nitrogen atmosphere (Dean-Stark apparatus).¹⁶⁴ Formation of the stannylidene complex was greatly accelerated, being complete after 1 h (infra-red spectroscopy). The solution was reduced to one half of its original volume by distillation, and the reaction mixture was cooled to 60 °C. From this point the allyl ether **DL-127** was prepared from the stannylidene complex in an identical manner to procedure 1 (0.79 g, 93%), (HRMS: found: $[M + H]^+$, 581.2903. Calc. for $C_{37}H_{41}O_6$: 581.2912); ν_{\max} (thin film, $CHCl_3$)/ cm^{-1} 3600-3200 br m (O-H), 3064 m, 3031 m, 2872 m (C-H), 1727 m, 1497 m, 1454 s, 1360 s, 1129 s, 1087 vs, 1028 s, 911 m, 734 vs and 697 vs; δ_H (300 MHz, C^2HCl_3) 2.55 (1H, s, OH), 3.33 and 3.45 (2 H, 2 x dd, J 2.7, J 9.6, 1-CH and 3-CH), 3.49 (1 H, t, J 9.5, Ins-CH), 4.00 (1 H, t, J 9.6, Ins-CH), 4.03 (1 H, t, J 9.6, Ins-CH), 4.22 (2 H, dd, $J_{1,2}$ 5.7, $J_{1,1'}$ 0.9, 1'-CH₂), 4.27 (1 H, t, $J_{1,2}$ 2.7, $J_{2,3}$ 2.7, 2-CH), 4.76–4.95 (8 H, m, 4 x CH₂Ph), 5.21 (1 H, dd, $J_{2',3'cis}$ 10.5, $J_{3'cis,3'trans}$ 1.5, 3'-CH_{cis}H_{trans}), 5.32 (1 H, dd, $J_{2',3'trans}$ 17.4, $J_{3'cis,3'trans}$ 1.5, 3'-CH_{cis}H_{trans}), 5.96 (1H, m, $J_{1,2}$ 5.7, $J_{2',3'cis}$ 10.5, $J_{2',3'trans}$ 17.4, 2'-CH) and 7.27–7.41 (20 H, m, Ar-H); δ_C (75.4 MHz; C^2HCl_3), 67.54 (2-CH), 71.79, 72.69 and 75.89 (CH₂Ph and 1'-CH₂), 79.51, 79.81, 81.11 and 83.02 (Ins-CH), 117.48 (3'-CH₂), 127.56, 127.60, 127.85, 127.87, 128.05, 128.10, 128.36 and 128.48 (Ar-CH), 134.67 (2'-CH) and 137.96 and 138.72 (Ar-C quaternary); m/z (Cl^+) 581 (26%, $[M + H]^+$), 489 (6, $[M - CH_2Ph]^+$), 399 (4, $[M - 2CH_2Ph]^+$), 107 (100, $PhCH_2O^+$), 91 (13, $PhCH_2^+$); R_f = 0.15 (20% ethyl acetate/petroleum ether).

DL-1-*O*-Allyl-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol DL-128¹⁶¹

A solution of DL-1-*O*-allyl-3,4,5,6-tetrakis-*O*-benzyl *myo*-inositol DL-127 (8.9 g, 15.3 mmol) in dry DMF (360 cm³) under a nitrogen atmosphere was treated with sodium hydride (1.2 g, 60% dispersion in oil, 30.0 mmol) and benzyl bromide (3.3 cm³, 30.6 mmol). The reaction was stirred at room temperature for 24 h. Water (90 cm³) was added with care and the solvents then removed under reduced pressure. The residual oil was partitioned between water (90 cm³) and ethyl acetate (250 cm³) and the organic phase collected. The aqueous phase was extracted with ethyl acetate (3 x 50 cm³) and the combined organic phases washed with saturated brine (50 cm³) and dried (MgSO₄). On removal of the solvents under reduced pressure, the product was purified by silica column chromatography (0–20% ethyl acetate/petroleum ether, in 5% steps) and recrystallised from ethanol to give the pentabenzyl allylated *myo*-inositol DL-128 as white crystals (9.0 g, 88%), m.pt 60–62 °C (lit.,⁴⁸ 60–62 °C) (HRMS: found: [M + H]⁺, 671.3348. Calc. for C₄₄H₄₇O₆: 671.3373); ν_{\max} (thin film)/cm⁻¹ 3030 m, 2868 m, 1729 w, 1497 m, 1454 s, 1359 s, 1087 vs, 734 vs and 697 vs; δ_{H} (300 MHz; C²HCl₃) 3.35 and 3.46 (2 H, 2 x dd, *J* 9.9, *J* 2.1, 1-CH and 3-CH), 3.56 (1 H, t, *J* 9.6, Ins-CH), 4.10–4.22 (5 H, m, 3 x Ins-CH and 1'-CH₂), 4.67–5.02 (10 H, m, 5 x CH₂Ph), 5.24 (1 H, dd, *J*_{2',3'cis} 10.5, *J*_{3'cis,3'trans} 1.5, 3'-CH_{cis}H_{trans}), 5.37 (1 H, dd, *J*_{2,3'trans} 17.2, *J*_{3'cis,3'trans} 1.5, 3'-CH_{cis}H_{trans}), 5.98 (1 H, ddt, *J*_{1',2'} 5.7, *J*_{2',3'cis} 10.5, *J*_{2',3'trans} 17.2, 2'-CH) and 7.32–7.68 (25 H, m, Ar-CH); δ_{C} (75.4 MHz; C²HCl₃) 71.54, 72.70, 73.96, 75.74 and 75.77 (CH₂Ph and 1'-CH₂), 74.17, 80.63, 80.83, 81.59 and 83.58 (Ins-CH), 116.61 (3'-CH₂), 127.33, 127.44, 127.48, 127.51, 127.55, 127.76, 127.82, 128.05, 128.11, 128.28 and 128.34 (Ar-CH), 134.92 (2'-CH) and 138.44, 138.90 and 138.98 (Ar-C quaternary); *m/z* (CI⁺)

671 (11%, $[M + H]^+$), 579 (4, $[M - CH_2Ph]^+$), 489 (2, $[M + H - 2CH_2Ph]^+$), 399 (4, $[M + 2H - 3CH_2Ph]^+$), 271 (11), 221 (12), 181 (44, $[Inositol + H]^+$), 107 (100, $PhCH_2O^+$), 91 (68, $PhCH_2^+$); $R_f = 0.55$ (20% ethyl acetate/petroleum ether).

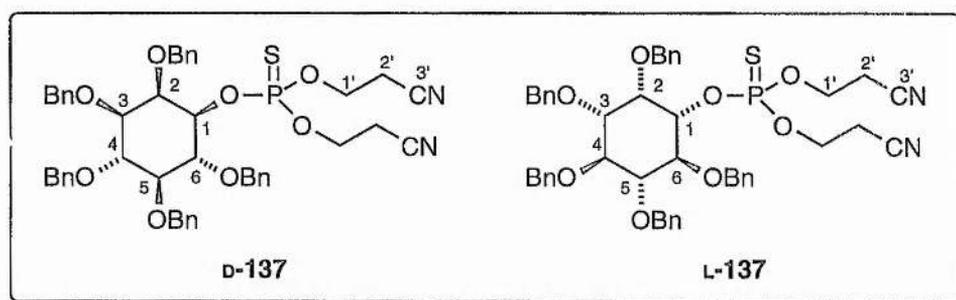
DL-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol DL-129¹⁶¹



To a stirred solution of DL-1-*O*-allyl-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol **DL-128** (5.0 g, 7.5 mmol) in ethanol/water (90 cm³, 9:1 v/v) was added Wilkinson's complex (0.7 g, 0.8 mmol) and DABCO (0.27 g, 2.4 mmol) and the mixture refluxed under nitrogen atmosphere for 4 h. The suspension was hot filtered and the pad washed with ethanol. The solvents were removed under reduced pressure to give an oil which was refluxed in a mixture of acetic acid/water/THF (240 cm³, 3:1:2 v/v) for 4 h. The solvents were removed under reduced pressure and the product purified by silica column chromatography (0–40% ethyl acetate/petroleum ether, in 5% steps) and recrystallised from ethanol to give the alcohol **DL-129** as a white crystalline solid (3.4 g, 83%); mp 90–92 °C, (lit.¹⁶¹ 91–93 °C (methanol)) (Found: C, 78.1; H, 6.45. Calc. for C₄₁H₄₂O₆: C, 78.1; H, 6.7%) (HRMS: found: $[M + H]^+$, 631.3060. Calc. for C₄₁H₄₃O₆: 631.3057); ν_{max} (thin film, CHCl₃)/cm⁻¹ 3560 br w (OH), 3016 s, 2873 m, 2402 w, 1497 m, 1455 m, 1361 m, 1217 s, 1131 s, 1071 s, 930 w, 756 br vs, 699 s and 669 s; δ_H (300 MHz, C²HCl₃) 2.26 (1 H, br s, OH), 3.51–3.59 (3 H, m, Ins-CH), 3.89 (1 H, t, *J* 9.6, Ins-CH), 4.08 (1 H, t, *J* 3.0, 2-CH), 4.11 (1 H, t, *J* 9.6, Ins-CH), 4.74–5.06 (10 H, m, 5 x CH₂Ph), 7.31–7.40 (25 H, m, Ar-CH); δ_C (75.4 MHz, C²HCl₃) 72.30 (2-CH), 72.88, 74.67, 75.43, 75.67 and 75.8, (CH₂Ph), 77.04, 81.04, 81.83, 82.07 and 83.52

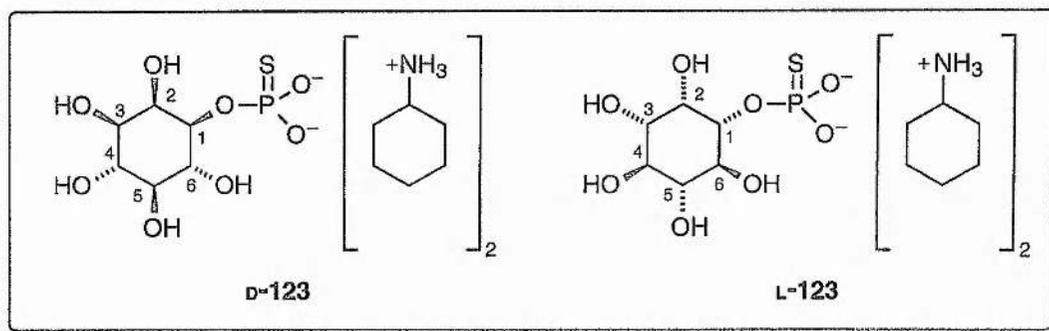
(Ins-CH), 127.59, 127.63, 127.70, 127.82, 127.86, 128.05, 128.07, 128.36, 128.39, 128.44 and 128.50 (Ar-CH) and 138.27, 138.63, 138.67 and 138.76 (Ar-C quaternary); m/z (Cl^+) 631 (100, $[\text{M} + \text{H}]^+$), 539 (57, $[\text{M} - \text{CH}_2\text{Ph}]^+$), 449 (36, $[\text{M} + \text{H} - 2\text{CH}_2\text{Ph}]^+$), 359 (20, $[\text{M} + 2\text{H} - 3\text{CH}_2\text{Ph}]^+$), 181 (51, $[\text{Inositol} + \text{H}]^+$), 107 (35, OCH_2Ph^+) and 91 (30, CH_2Ph^+); $R_f = 0.17$ (20% ethyl acetate/petroleum ether).

DL-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-[bis-(2'-cyanoethyl)]-phosphorothioate DL-137⁴⁸



To a solution of DL-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol DL-129 (0.5 g, 0.79 mmol) and *N,N*-diisopropylethylamine (0.11 cm³, 1.19 mmol) in anhydrous acetonitrile (15 cm³) was added 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (280 mm³, 1.19 mmol) at 0 °C under an argon atmosphere, and the reaction mixture stirred for 4 h. A solution of 3-hydroxypropionitrile (0.25 cm³, 3.6 mmol) and 1-*H*-tetrazole (0.08 g, 1.19 mmol) in dry acetonitrile (5 cm³) were added and the reaction mixture stirred for 16 h at room temperature.¹⁸⁷ The solvent was removed under reduced pressure, and the residue partitioned between ethyl acetate (50 cm³) and potassium dihydrogen phosphate buffer (25 cm³, pH 7, 0.1 mol dm⁻³). The aqueous fraction was extracted with ethyl acetate (2 x 25 cm³) and the combined organic extracts washed with saturated brine (25 cm³) and dried (MgSO_4). The solvent was removed under reduced pressure, the residual oil redissolved in pyridine (30 cm³), and sulfur (0.38 g, 11.9 mmol) added. The reaction mixture was stirred at room temperature for 15 min. The solvent was removed under reduced pressure and the residue extracted in toluene. Excess sulfur was removed by filtration. On removal of the solvents the residual oil was redissolved in toluene (2 cm³)

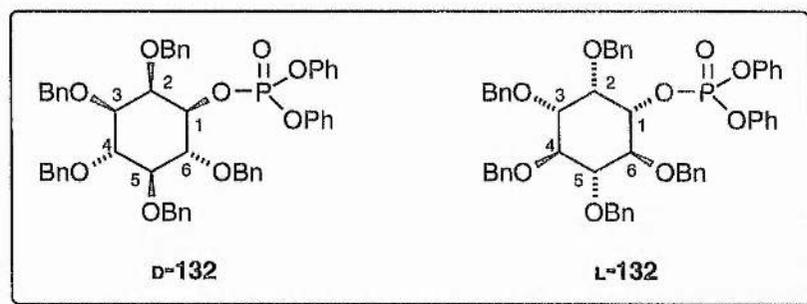
and purified by silica column chromatography (0-40% ethyl acetate/petroleum ether, short column in 5% steps). The recovered product was recrystallised from methanol to yield the phosphorothioate DL-137 as a white crystalline solid (0.43 g, 66%), mp 96-98 °C, (lit.,⁴⁸ 97-98 °C) (Found: C, 67.95; H, 5.95; N, 3.3. Calc. for C₄₇H₄₉O₈N₂PS: C, 67.75; H, 5.95; N, 3.35%); ν_{\max} (thin film, CHCl₃)/cm⁻¹ 3030 m, 2872 m, 2256 w (CN), 1497 m, 1455 m, 1360 m, 1217 s, 1072 vs, 1029 vs, 944 m, 801 m (PS), 755 vs, 698 s and 668 m; δ_{H} (300 MHz; C²HCl₃) 2.21-2.54 (4 H, m, 2'-CH₂), 3.50 (1 H, dd, $J_{2,3}$ 2.1, $J_{3,4}$ 9.9, 3-CH), 3.54 (1 H, t, $J_{4,5}$ 9.3, $J_{5,6}$ 9.3, 5-CH), 3.96-4.14 (6 H, m, 1'-CH₂ and 4-CH and 6-CH), 4.30 (1 H, t, $J_{1,2}$ 2.1, $J_{2,3}$ 2.1, 2-CH), 4.34 (1 H, dt, $J_{6,1}$ 10.2, $J_{1,2}$ 2.1, $J_{1,p}$ 10.2, 1-CH), 4.68-5.01 (10 H, m, 5 x CH₂Ph) and 7.24-7.44 (25 H, m, Ar-CH); δ_{C} (75.4 MHz; C²HCl₃) 18.75 and 18.96 (2 x d, $J_{2\text{C-P}}$ 8.7, 2'-CH₂), 62.16 and 62.22 (2 x d, $J_{1\text{C-P}}$ each 9.7, 1'-CH₂), 72.80, 74.35, 75.29, 75.67 and 75.84 (CH₂Ph), 76.04 (Ins-CH), 79.27 (d, $J_{\text{C-P}}$ 7.54, 1-CH or 6-CH), 79.61 (d, $J_{\text{C-P}}$ 6.49, 1-CH or 6-CH), 80.24 and 81.19 (Ins-CH), 83.07 (2-CH), 116.46 (3'-CN), 127.44, 127.54, 127.61, 127.71, 127.78, 127.87, 128.22, 128.27 and 128.34 (Ar-CH) and 138.05, 138.28, 138.41 and 138.61 (Ar-C quaternary); δ_{P} (121.5 MHz; C²HCl₃) 66.61; m/z (CI⁺) 833 (13%, [M + H]⁺), 741 (5), 431 (6), 311 (19), 271 (18, [Inositol - H + CH₂Ph]⁺), 219 (31), 181 (57, [Inositol + H]⁺), 144 (8), 123 (24), 107 (100, OCH₂Ph⁺); m/z (FAB⁺) 855 (10%, [M + Na]⁺), 271 (7, [Inositol - H + CH₂Ph]⁺), 203 (6, [P(S)(OCH₂CH₂CN)₂]⁺), 181 (100, [Inositol + H]⁺) and 105 (45); R_f = 0.29 (25% ethyl acetate/petroleum ether).

DL-*myo*-Inositol 1-phosphorothioate, bis-cyclohexylammonium salt**DL-123⁴⁸**

A freshly prepared solution of sodium methoxide (0.27 g, 4.8 mmol) in dry methanol (10 cm³) was added dropwise to a solution of [bis(2'-cyanoethyl)] phosphorothioate DL-137 (1.0 g, 1.2 mmol) in dry methanol (20 cm³) at 4 °C under an argon atmosphere, and stirred for 4 h. The solvent was removed under reduced pressure to give the disodium salt, which showed the expected NMR parameters [δ_p (121.5 MHz; C²HCl₃) 44.65]. The crude (+/-)-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-phosphorothioate disodium salt was dissolved in THF (30 cm³), and this solution added dropwise with stirring to a solution of sodium (0.5 g) in liquid ammonia (100 cm³), 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 min and dry methanol (10 cm³) added. The ammonia was allowed to evaporate and the solvents were removed under reduced pressure. The residue was then partitioned between water (100 cm³) and diethyl ether (50 cm³). The aqueous layer was removed, reduced to a volume of approximately 10 cm³ by rotary evaporation and subjected to column 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 cm³) added. The mixture was stirred at room temperature for 4 h, and the aqueous solution was then extracted with diethyl ether (3 x 50 cm³) to remove the cyclohexylamine. The solution was lyophilised overnight and the residue recrystallised from water/acetone to give the phosphorothioate

DL-123 as an amorphous white solid (0.51 g, 89%), mp 162–169 °C (decomp.) (lit.,⁴⁸ 165–170 °C(decomp.)) (Found: C, 43.05; H, 8.25; N, 5.4. Calc. for $C_{18}H_{39}O_8N_2PS \cdot \frac{3}{2}H_2O$: C, 43.1; H, 8.45; N, 5.6%); ν_{max} (Nujol)/ cm^{-1} 3500–2500 br s (O-H and N-H), 1624 w, 1530 w, 1123 m, 1070 m, 1050 m, 977 m, 817 w and 723 s (P=S); δ_H (300 MHz; 2H_2O) 1.04–1.89 (20 H, m, cyclohexyl- CH_2), 3.04–3.06 (2 H, m, cyclohexyl-CH), 3.25 (1 H, t, $J_{4,5}$ 9.3, $J_{5,6}$ 9.3, 5-CH), 3.48 (1 H, dd, $J_{2,3}$ 2.4, $J_{3,4}$ 10.2, 3-CH), 3.54 (1 H, t, $J_{3,4}$ 10.2, $J_{4,5}$ 9.3, 4-CH), 3.67 (1 H, t, $J_{5,6}$ 9.3, $J_{6,1}$ 9.9, 6-CH), 4.00 (1 H, dt, $J_{6,1}$ 9.9, $J_{1,2}$ 2.7, $J_{1,P}$ 11.7, 1-CH) and 4.20 (1 H, t, $J_{1,2}$ 2.7, $J_{2,3}$ 2.4, 2-CH); δ_C (75.4 MHz; 2H_2O) 23.77, 24.29 and 30.63 (cyclohexyl- CH_2), 50.24 (cyclohexyl-CH), 70.84 (Ins-CH), 71.54 (d, J 2.2, Ins-CH), 72.1 (d, J 3.2, Ins-CH), 72.30 and 74.45 (Ins-CH), 74.75 (d, J 5.4, Ins-CH); δ_p (121.5 MHz; 2H_2O) 44.48; m/z (FAB⁺) 497 (2%, [M + Na]⁺), 494 (2), 475 (5, [M + H]⁺), 376 (4, [M - $\Rightarrow C_6H_{14}N + 2H$]⁺), 297 (15), 234 (4), 192 (2), 147 (3) and 100 (100, $C_6H_{11}NH_3^+$).

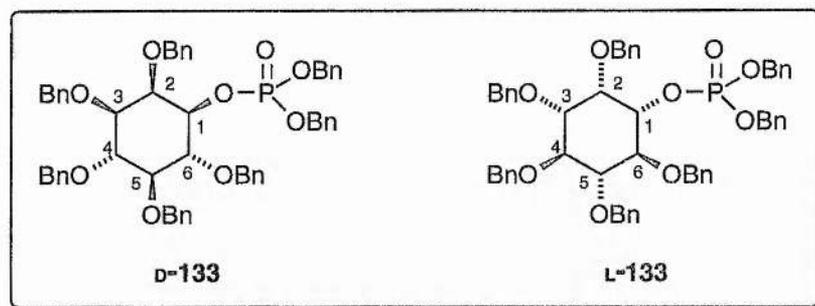
DL-2,3,4,5,6-Penta-O-benzyl myo-inositol 1-[bis-(O-phenyl)]-phosphate
DL-132⁴⁷



DL-2,3,4,5,6-Penta-O-benzyl myo-inositol **DL-129** (0.5 g, 0.79 mmol), DMAP (10 mg, 0.08 mmol) and dry triethylamine (0.4 cm^3 , 2.8 mmol) were dissolved in dry dichloromethane (10 cm^3) under an argon atmosphere. Diphenylphosphorochloridate (0.3 cm^3 , 1.45 mmol) was added dropwise and the reaction mixture stirred for 24 h. The solvent was removed under reduced pressure, the residue redissolved in diethyl ether (15 cm^3) and washed with water (10 cm^3). The aqueous phase was washed with diethyl

ether ($3 \times 10 \text{ cm}^3$) and the combined organic fractions washed with saturated brine (10 cm^3) and dried (MgSO_4). The solvents was removed under reduced pressure to give a pale brown oil that was purified by silica column chromatography (0–25% ethyl acetate/petroleum ether, in 5% steps) and recrystallised from hexane to give the diphenyl phosphate **DL-132** as clear crystals (0.57 g, 83%), mp 97–99 °C (lit.,⁴⁷ 97–99 °C) (Found: C, 73.55; H, 5.95. Calc. for $\text{C}_{53}\text{H}_{51}\text{O}_9\text{P}$: C, 73.75; H, 5.95%); ν_{max} (thin film, CHCl_3)/ cm^{-1} 3019 s, 2929 m, 2872 m (C-H), 2400 m, 1735 w, 1592 m, 1491 s, 1285 (P=O), 1214 vs (P–O-aryl), 1071 s, 1027 vs, 963 vs, 739 vs, 669 s and 625 m; δ_{H} (300 MHz; C^2HCl_3) 3.53 (1 H, dd, $J_{2,3}$ 2.4, $J_{3,4}$ 9.9, 3-CH), 3.56 (1 H, t, J 9.3, Ins-CH), 4.11 (1 H, t, J 9.3, Ins-CH), 4.14 (1 H, t, Ins-CH), 4.16 (1 H, t, Ins-CH), 4.34 (1 H, t, $J_{1,2}$ 2.4, $J_{2,3}$ 2.4, 2-CH), 4.57 (1 H, dt, $J_{6,1}$ 9.9, $J_{1,2}$ 2.4, $J_{1,P}$ 12.6, 1-CH) 4.58–4.95 (10 H, m, 5 x CH_2Ph), 7.14–7.41 (35 H, m, Ar-H); δ_{C} (75.4 MHz; C^2HCl_3) 72.87, 74.91, 75.42, 75.79 and 75.92 (CH_2Ph), 76.66, 79.78, 79.87, 80.41 and 81.20 (Ins-CH), 83.04 (d, $J_{\text{C,P}}$ 2.2, Ins-CH), 119.9, 119.98, 120.10, 120.16, 125.24, 125.27, 125.46, 127.36, 127.45, 127.53, 127.56, 127.68, 127.76, 127.82, 128.04, 128.17, 128.32, 128.41, 129.69 and 129.77 (Ar-CH), 138.08, 138.35, 138.49, 138.62 and 138.68 (Ar-C quaternary) and 150.57 (m, OPh-C quaternary); δ_{P} (121.5 MHz; C^2HCl_3) –12.02; m/z (Cl^+) 863 (59%, $[\text{M} + \text{H}]^+$), 773 (11, $[\text{M} + 2\text{H} - \text{CH}_2\text{Ph}]^+$), 679 (9), 631 (10, $[\text{M} + 2\text{H} - \text{P}(\text{O})(\text{OPh})_2]^+$), 327 (14), 271 (6, $[\text{Inositol} + \text{CH}_2\text{Ph}]^+$), 251 (28), 181 (20, $[\text{Inositol} + \text{H}]^+$) and 107 (100, OCH_2Ph^+); $R_f = 0.43$ (20% ethyl acetate/petroleum ether).

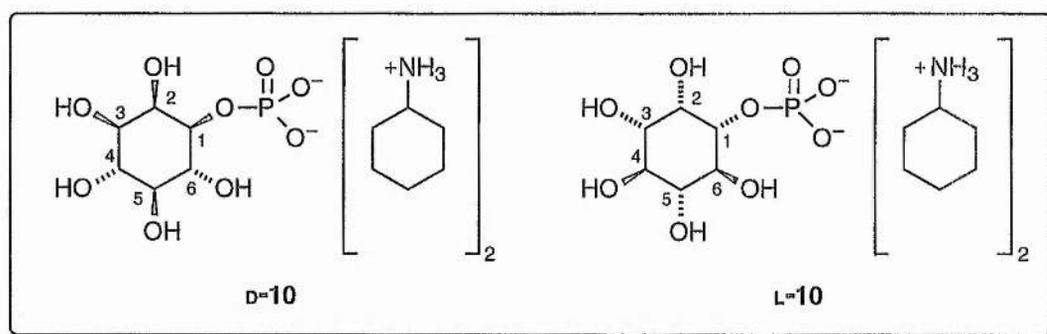
DL-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-[bis-(*O*-benzyl)]-phosphate
DL-133⁴⁷



Benzyl alcohol (0.08 cm³, 0.8 mmol) in dry THF (5cm³) was added dropwise to a solution of DL-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-(bis-phenyl) phosphate **DL-132** (0.35 g, 0.4mmol) and sodium hydride (0.03 g, 60% dispersion in oil, 0.72 mmol) in dry THF (15 cm³). The reaction mixture was stirred for 18 h and on completion saturated ammonium chloride solution (10 cm³) added with caution. The solvent was removed under reduced pressure, the residue redissolved in dichloromethane (15 cm³) and washed with water (3 x 10 cm³). The aqueous phase was washed with dichloromethane (3 x 10 cm³) and the combined organic fractions dried (MgSO₄). The solvent was removed under reduced pressure and the resulting pale brown oil purified by silica column chromatography (0–45% ethyl acetate/petroleum ether, in 5% steps) to give the transesterified phosphate **DL-133** as a clear oil (0.26 g, 73%), $\nu_{\max}(\text{neat})/\text{cm}^{-1}$ 3067 s, 3019 vs, 2935 m, 2872 m (C-H), 2401 m, 1490 s, 1455 s, 1360 s, 1222 vs (P=O), 1127 s, 1021 vs (C-O), 928 m, 759 vs, 697 vs, 671 vs and 598 s; $\delta_{\text{H}}(300 \text{ MHz}, \text{C}^2\text{HCl}_3)$ 3.51 (1 H, dd, $J_{2,3}$ 2.1, $J_{3,4}$ 9.6, 3-CH), 3.58 (1 H, t, J 9.3, Ins-CH), 4.16 (1 H, t, J 9.3, Ins-CH), 4.17 (1 H, t, J 9.6, Ins-CH), 4.36 (1 H, dt, $J_{6,1}$ 9.9, $J_{1,2}$ 2.4, $J_{1,P}$ 12.3, 1-CH), 4.43 (1 H, t, $J_{1,2}$ 2.4, $J_{2,3}$ 2.1, 2-CH), 4.68–5.11 (14 H, m, 7 x CH₂Ph) and 7.25–7.46 (35 H, m, Ar-CH); $\delta_{\text{C}}(75.4 \text{ MHz}, \text{C}^2\text{HCl}_3)$ 69.12 and 69.26 (2 x d, $J_{\text{C-P}}$ 5.4, POCH₂Ph) 72.61, 74.93, 75.45, 75.75 and 75.88 (CH₂Ph), 76.25 (Ins-CH), 78.53 (d, $J_{\text{C-P}}$ 5.4, Ins-CH), 80.05 (d, $J_{\text{C-P}}$ 7.5, Ins-CH), 80.44, 81.20 and 83.05 (Ins-CH), 127.38, 127.42, 127.49, 127.53, 127.61, 127.69, 127.76, 127.82, 128.02, 128.17, 128.22, 128.29, 128.32, 128.35, 128.40, 128.48 and 128.52 (Ar-CH) and 135.73, 135.81,

138.14, 138.51, 138.68 and 138.77 (Ar-C quaternary); $\delta_p(121.5 \text{ MHz, C}^2\text{HCl}_3) -1.13$; $m/z (\text{CI}^+) 891 (7\%, [\text{M} + \text{H}]^+)$, $631 (8, [\text{M} + 2\text{H} - \text{P}(\text{O})(\text{OCH}_2\text{Ph})_2]^+)$, $539 (9, [\text{M} + \text{H} - \text{P}(\text{O})(\text{OCH}_2\text{Ph})_2 - \text{CH}_2\text{Ph}]^+)$, $449 (9, [\text{M} - \text{P}(\text{O})(\text{OCH}_2\text{Ph})_2 - 2\text{CH}_3\text{Ph}]^+)$, $359 (10)$, $271 (19, [\text{Inositol} + \text{OCH}_2\text{Ph}]^+)$, $181 (61, [\text{Inositol} + \text{H}]^+)$ and $(100, \text{OCH}_2\text{Ph}^+)$; $R_f = 0.16$ (20% ethyl acetate/petroleum ether).

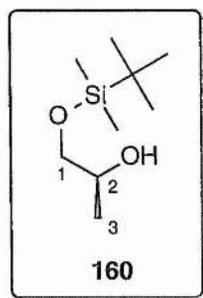
DL-*myo*-Inositol 1-phosphate, bis-cyclohexylammonium salt DL-10¹⁹⁰



DL-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-(bis-benzyl) phosphate **DL-133** (0.30 g, 0.34 mmol) was dissolved in THF (30 cm³), and this solution added dropwise to a solution of sodium (2 mg, 0.87 mmol), in liquid ammonia (50 cm³) at $-78 \text{ }^\circ\text{C}$ 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 the protected phosphate solution. After the final addition of this solution the reaction mixture was stirred for a further 30 min and dry methanol (5 cm³) added. The ammonia was allowed to evaporate and the solvents were removed under reduced pressure. The residue was dissolved in water (5 cm³) and subjected to chromatography on Amberlite IR-118 (H⁺) ion-exchange resin, eluting with water. The acidic fractions containing the product were collected, and freshly distilled cyclohexylamine (3 cm³) added and the reaction mixture stirred at room temperature for 4 h. The aqueous solution was extracted with diethyl ether (3 x 20 cm³) to remove the excess cyclohexylamine and the sample lyophilised. The residue was recrystallised from water/acetone to give phosphate **DL-10** as a white solid (0.11 g, 67%); mp 186–193 $^\circ\text{C}$

(decomp.) (lit.,⁴⁷ 185–190 °C (decomp.)) (Found: C, 44.75; H, 8.6; N, 5.6. Calc. for $C_{18}H_{39}O_9PN_2 \cdot \frac{3}{2}H_2O$: C, 44.55; H, 8.7; N, 5.75%); $\nu_{\max}(\text{Nujol})/\text{cm}^{-1}$ 3550–2000 br vs (O-H and N-H), 1715 s, 1629 w, 1546 w, 1289 br s (P=O), 1111 s, 1047 s, 968 m, 836 w and 722 m; $\delta_{\text{H}}(300 \text{ MHz}; ^2\text{H}_2\text{O})$ 1.07–1.87 (20 H, m, cyclohexyl- CH_2), 3.04 (2 H, m, cyclohexyl-CH), 3.22 (1 H, t, $J_{4,5}$ 9.6, $J_{5,6}$ 9.3, 5-CH), 3.46 (1 H, dd, $J_{2,3}$ 2.7, $J_{3,4}$ 9.9, 3-CH), 3.50 (1 H, t, $J_{3,4}$ 9.9, $J_{4,5}$ 9.6, 4-CH), 3.63 (1 H, t, $J_{5,6}$ 9.3, $J_{6,1}$ 9.6, 6-CH), 3.79 (1 H, dt, $J_{6,1}$ 9.6, $J_{1,2}$ 2.7, $J_{1,P}$ 9.6, 1-CH) and 4.11 (1 H, t, $J_{1,2}$ 2.7, $J_{2,3}$ 2.7, 2-CH); $\delta_{\text{C}}(75.4 \text{ MHz}; ^2\text{H}_2\text{O})$ 23.71, 24.20 and 30.27 (cyclohexyl- CH_2), 50.31 (cyclohexyl-CH), 70.84 (Ins-CH), 71.68 (d, $J_{\text{C-P}}$ 2.2, Ins-CH), 72.22 (d, $J_{\text{C-P}}$ 4.1, Ins-CH), 72.33 (Ins-CH), 74.36 (d, $J_{\text{C-P}}$ 5.3, Ins-CH) and 74.41 (Ins-CH); $\delta_{\text{P}}(121.5 \text{ MHz}, ^2\text{H}_2\text{O})$ 4.21; m/z (FAB⁺) 481 (15%, $[\text{M} + \text{Na}]^+$), 459 (24, $[\text{M} + \text{H}]^+$), 360 (14, $[\text{M} - \text{C}_6\text{H}_{14}\text{N} + 2\text{H}]^+$), 305 (20), 283 (37), 261 (13, $[\text{M} - 2\text{C}_6\text{H}_{14}\text{N} + 3\text{H}]^+$), 253 (14), 181 (33, $[\text{Inositol} + \text{H}]^+$), 154 (100) and 136 (82).

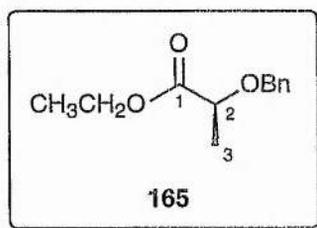
1-*O*-*tert*-butyldimethylsilyl (2*S*)-propane-1,2-diol **160**



tert-Butyl dimethyl silyl chloride (4.4 g, 28.9 mmol) was added to a solution of (*S*)-(+)-propane 1,2-diol **150** (2.0 g, 26.3 mmol, 99% enantiomeric excess) and imidazole (1.9 g, 28.9 mmol) in anhydrous DMF (150 cm³) at –10 °C. The reaction mixture was stirred at room temperature under an argon atmosphere for 24 h. The solution was added to 5% sodium bicarbonate solution (50 cm³) and this aqueous solution was extracted with diethyl ether (3 x 50 cm³). The pooled organic extracts were washed with saturated brine (50 cm³) and dried (MgSO₄). The solvent was removed under

reduced pressure to give a pale yellow oil which was purified by silica column chromatography (0-10% ethyl acetate/petroleum ether, in 5% steps) to give the TBDMSi-protected alcohol **160** as a clear oil (3.15 g, 63%); (Found: C, 57.05; H, 11.85. $C_9H_{22}O_2Si$ requires: C, 56.8; H, 11.65%) $[\alpha]_D^{25} +12.7$ (*c* 3 in $CHCl_3$); $\nu_{max}(neat)/cm^{-1}$ 3250–3600 (OH), 3680 w, 3581 m, 3019 s, 2932 s, 2859 s (C-H), 1464 s, 1384 s ($C(CH_3)_3$), 1363 s ($C(CH_3)_3$), 1331 s, 1256 vs, 1219 vs, 1093 vs, 931 s, 839 vs, 780 vs and 671 s; $\delta_H(300\text{ MHz}; C^2HCl_3)$ 0.06 (6 H, s, $Si(CH_3)_2$), 0.89 (9 H, s, $SiC(CH_3)_3$), 1.10 (3 H, d, $J_{2,3}$ 6.3, 3- CH_3), 2.53 (1 H, br d, J_{2-OH} 3.3, OH), 3.34 (1 H, dd, $J_{1A,1B}$ 9.9, $J_{1A,2}$ 7.8, 1- CH_AH_B), 3.57 (1 H, dd, $J_{1A,1B}$ 9.9, $J_{1B,2}$ 3.6, 1- CH_AH_B) and 3.79 (1 H, m, $J_{1A,2}$ 7.8, $J_{1B,2}$ 3.6, $J_{2,3}$ 6.3, J_{2-OH} 3.3, 2-CH); $\delta_C(75.4\text{ MHz}; C^2HCl_3)$ -5.55 ($SiCH_3$), 18.14 ($SiC(CH_3)_3$), 25.59 (3- CH_3), 25.76 ($SiC(CH_3)_3$), 67.90 (1- CH_2) and 68.51 (2-CH); $R_f = 0.33$ (20% ethyl acetate/petroleum ether).

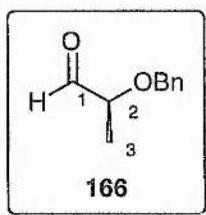
Ethyl 2-benzyloxy-(2*S*)-propionate **165**¹⁷⁸



Trifluoromethanesulfonic acid (0.10 cm^3 , 1.13 mmol) was added to a solution of (2*S*)-ethyl lactate **164** (3.50 g, 29.6 mmol) and *O*-benzyl trichloroacetimidate (11.0 cm^3 , 59.2 mmol) in cyclohexane-dichloromethane (80 cm^3 , 7:1 v/v) at room temperature. After stirring for 20 h, the mixture was diluted with water (20 cm^3) and hexane (80 cm^3), then stirred for an additional 3 h. The resulting white precipitate was removed by filtration. The aqueous layer of the filtrate was separated and the organic layer was washed with saturated sodium bicarbonate solution (50 cm^3) and saturated brine (50 cm^3) and then dried ($MgSO_4$). The solvent was removed under reduced pressure to give an oily residue which was purified by column chromatography on silica gel (0–4% ethyl acetate/petroleum ether, in 1% steps) to yield the ester **165** a colourless oil (5.1 g, 82%),

(Found: C, 69.2; H, 7.8. Calc. for $C_{12}H_{16}O_3$: C, 69.2; H, 7.75%) $[\alpha]_D^{20} -74.9$ (c 2.94 in $CHCl_3$) (lit.,¹⁷⁸ $[\alpha]_D^{20} -74.5$ (c 2.94 in $CHCl_3$)); $\nu_{max}(\text{neat})/\text{cm}^{-1}$ 2985 s, 1745 s (ester CO), 1497 s, 1455 s, 1372 s, 1271 s, 1200 s, 1142 s, 1065 s, 1027 s, 908 m, 860 m, 739 s and 698 s; $\delta_H(300 \text{ MHz}; C^2HCl_3)$ 1.32 (3 H, t, $J_{1,2}$ 7.2, 2'- CH_3), 1.47 (3 H, d, $J_{2,3}$ 6.6, 3- CH_3), 4.08 (1 H, q, $J_{2,3}$ 6.6, 2-CH), 4.24 (2 H, q, $J_{1,2}$ 7.2, 2'- CH_2), 4.48 and 4.73 (2 H, 2 x d, J_{AB} 11.7, CH_2Ph) and 7.31–7.39 (5 H, m, Ar-H); $\delta_C(75.4 \text{ MHz}; C^2HCl_3)$ 14.04 (3'- CH_3), 18.50 (3- CH_3), 60.69 (2'- CH_2), 71.88 (CH_2Ph), 73.99 (2-CH), 127.81, 128.95 and 128.40 (Ar-CH), 137.65 (Ar-C quaternary) and 173.31 (ester CO); m/z (CI^+) 209 (19%, $[M + H]^+$), 131 (9, $[M - Ph]^+$), 102 (39, $[CH_3CH_2OC(O)CHCH_3 + H]^+$), 91 (100, CH_2Ph^+) and 74 (5, $CH_3CH_2OC(O)^+$); $R_f = 0.20$ (5% ethyl acetate/petroleum ether).

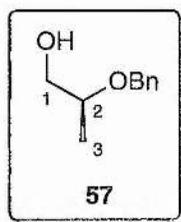
2-benzyloxy-(2S)-propanal **166**¹⁷⁸ °



A solution of diisobutylaluminium hydride in hexane (7.20 cm^3 , 7.20 mmol, 1.0 mol dm^{-3}) was added slowly to a solution of the ester **165** (1.00 g, 4.80 mmol) in diethyl ether (50 cm^3) at -78 °C. After stirring at the same temperature for 10 min., the mixture was diluted successively with methanol (10 cm^3) and water (10 cm^3), then allowed to warm up to room temperature. After stirring for an additional 1 h, the resulting suspension was filtered through a pad of celite and the residue was washed with diethyl ether (30 cm^3). The combined filtrates were dried ($MgSO_4$) and concentrated under reduced pressure. The resulting residue was purified by silica column chromatography (0–10% ethyl acetate/petroleum ether, in 2% steps) to give aldehyde **166** as a colourless oil (0.66 g, 84%), $[\alpha]_D^{20} -66.2$ (neat) (lit.,¹⁷⁸ $[\alpha]_D^{20} -65.85$ (neat)); $\nu_{max}(\text{neat})/\text{cm}^{-1}$ 3448 w, 3032 m, 2982 m, 2869 m, 1735 s (aldehyde C=O), 1497 m, 1454 m, 1375 m, 1093

s, 739 s and 689 s; δ_{H} (300 MHz; C^2HCl_3) 1.23 (3 H, d, $J_{2,3}$ 6.9, 3- CH_3), 3.79 (1 H, dq, $J_{1,2}$ 1.8, $J_{2,3}$ 6.9, 2-CH), 4.50 and 4.56 (2 H, 2 x d, J_{AB} 11.7, CH_2Ph), 7.28 (5 H, s, Ar-H) and 9.57 (1 H, d, $J_{1,2}$ 1.8, 1-CH); δ_{C} (75.4 MHz; C^2HCl_3) 14.99 (3- CH_3), 71.78 (CH_2Ph), 79.25 (2-CH), 127.82, 127.95 and 128.43 (Ar-CH), 137.36 (Ar-C quaternary) and 203.32 (C=O); m/z (Cl^+) 181 (23%, $[\text{M} - \text{H} + \text{NH}_4]^+$), 165 (4, $[\text{M} + \text{H}]^+$), 163 (3, $[\text{M} - \text{H}]^+$), 135 (25), 107 (6, OCH_2Ph^+), 91 (100, CH_2Ph^+); $R_f = 0.40$ (20% ethyl acetate/petroleum ether).

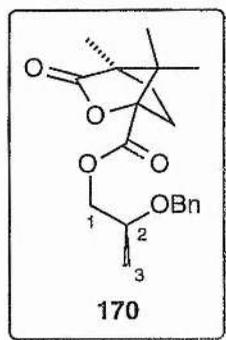
2-O-benzyl-(2S)-propane-1,2-diol 57¹⁷⁴



Sodium borohydride (0.22 g, 5.75 mmol) was added to a solution of **166** (0.37 g, 2.25 mmol) in ethanol (5 cm^3) at 0 °C. After stirring for 2 h at this temperature, the mixture was diluted with hydrochloric acid (10 cm^3 , 1.0 mol dm^{-1}) and extracted with ethyl acetate (3 x 20 cm^3). The combined extracts were washed successively with saturated brine (20 cm^3), saturated sodium bicarbonate solution (20 cm^3) and saturated brine (20 cm^3), and then dried (MgSO_4). The solvents were removed under reduced pressure to give an oily residue which was purified by silica column chromatography (0–25% ethyl acetate/petroleum ether, in 5% steps), giving alcohol **57** a colourless oil (0.30 g, 79%); (Found: C, 70.85; H, 8.6. Calc. for $\text{C}_{10}\text{H}_{14}\text{O}_2 \cdot \frac{1}{5}\text{H}_2\text{O}$: C, 70.75; H, 8.55%) $[\alpha]_{\text{D}}^{25} +43.0$ (c 3 in CHCl_3) (lit.,²²⁵ $[\alpha]_{\text{D}}^{25} +43.1$ (c 3 in CHCl_3)); ν_{max} (neat)/ cm^{-1} 3437 br s (O-H), 3031 s, 2870 s, 1953 w, 1878 w, 1812 w, 1605 w, 1587 w, 1454 m, 1206 m, 1063 (s), 914 m, 859 m, 799 m, 737 s and 698 s; δ_{H} (300 MHz; C^2HCl_3) 1.17 (3 H, d, $J_{2,3}$ 6.3, 3- CH_3), 2.24 (1 H, br s, OH), 3.46–3.72 (3 H, m, 1- CH_2 and 2-CH), 4.49 and 4.64 (2 H, 2 x d, J_{AB} 11.7, CH_2Ph) and 7.26–7.38 (5 H, m, Ar-H); δ_{C} (75.4 MHz; C^2HCl_3) 15.71 (3- CH_3), 66.24 (1- CH_2), 70.72 (CH_2Ph), 75.49 (2-CH), 127.72 and

128.45 (Ar-CH) and 138.52 (Ar-C quaternary); m/z (Cl^+) 166 (7%, M^+), 135 (20, $[\text{M} - \text{CH}_2\text{OH}]^+$), 107 (100, PhCH_2O^+), 91 (100 PhCH_2^+), $R_f = 0.17$ (20% ethyl acetate/petroleum ether).

1-O-[(1*S*,4*R*)-Camphanoyl]-2-O-benzyl-(2*S*)-propane-1,2-diol 170⁴⁷

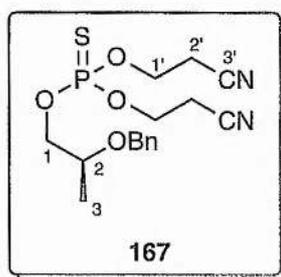


To a stirred solution of the *O*-benzyl diol **57** (0.19 g, 1.1 mmol), triethylamine (0.30 cm³, 2.5 mmol) and DMAP (0.02 g, 0.16 mmol) in anhydrous dichloromethane (20 cm³) at 0 °C under an atmosphere of nitrogen, was added, dropwise, a solution of (-)-(1*S*,4*R*)-camphanoyl chloride (0.5 g, 2.3 mmol) in dichloromethane (5 cm³). The mixture was stirred at 0 °C for 1 h and then at 25 °C for 24 h. The solution was washed with water (3 x 20 cm³), saturated sodium bicarbonate solution (2 x 20 cm³) and then dried (MgSO_4). The solvent was removed under reduced pressure to give a clear oil which was purified by silica column chromatography (0–30% ethyl acetate/petroleum ether, in 5% steps), giving camphanate ester **170** a colourless oil. (0.36 g, 95%), (Found: C, 69.7; H, 7.9. $\text{C}_{20}\text{H}_{26}\text{O}_5$ requires: C, 69.35; H, 7.55%) $[\alpha]_D^{25} -6.1$ (c 3 in CHCl_3); ν_{max} (neat)/cm⁻¹ 3560 w, 2971 s, 2876 s, 1786 s (ester C=O), 1751 s (ester C=O), 1454 s, 1398 s, 1378 s, 1340 s, 1316 s, 1273 s, 1105 s, 740 s and 699 s; δ_{H} (300 MHz; C^2HCl_3)^{*} 0.93, 1.02 and 1.09 (9 H, 3 x s, Camp- CH_3), 1.23 (3 H, d, $J_{2,3}$ 6.3, 3- CH_3), 1.66 (1 H, m, Camp- CH_2), 1.95 (2 H, m, Camp- CH_2), 2.41 (1 H, m, Camp- CH_2), 3.81 (1 H, m, $J_{1A,2}$ 6.0, $J_{1B,2}$ 3.9, $J_{2,3}$ 6.3, 2-CH), 4.12 (1 H, dd, $J_{1A,1B}$ 11.4 $J_{1A,2}$ 6.0, 1- CH_AH_B), 4.30 (1 H, dd,

^{*} The ¹H- and ¹³C-NMR crude product spectra showed no signals corresponding to the (-)-(1*S*,4*R*)-camphanate ester diastereomer of (*R*)-2-benzyloxy-2-propanol.

$J_{1A,1B}$ 11.4 $J_{1B,2}$ 3.9, 1- CH_AH_B), 4.55 and 4.60 (2 H, 2 x d, J_{AB} 11.7, CH_2Ph) and 7.32 (5 H, s, Ar-H); δ_c (75.4 MHz; C^2HCl_3)⁺ 9.51 (Camp- CH_3), 14.01, 16.54 and 16.75 (Camp- CH_3 and 3- CH_3), 28.79 and 30.51 (Camp- CH_2), 54.05 and 54.68 (Camp-C quaternary), 67.83 (1- CH_2), 70.86 (CH_2Ph), 72.28 (2-CH), 91.09 (Camp-C quaternary), 127.59 and 128.38 (Ar-CH), 138.28 (Ar-C quaternary) and 167.41 and 178.18 (Camp-C=O); m/z (CI^+) 347 (16%, $[M + H]^+$), 239 (16, $[M - OCH_2PH]^+$), 227 ($M - OCH_2PH]^+$), 209 (53), 193 (25, $PhCH_2OCH(CH_3)CH_2OC(O)^+$), 181 (100, $C(O)C_9H_{13}O_2^+$), 167 (44, $[PhCH_2OCH(CH_3)CH_2OH + H]^+$), 107 (76, $PhCH_2O^+$) and 91 (70, $PhCH_2^+$); $R_f = 0.52$ (30% ethyl acetate/petroleum ether).

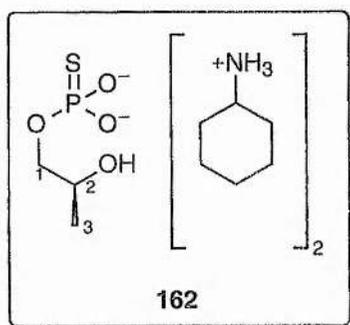
2-O-benzyl-(2S)-propane-1,2-diol 1-O-[bis-(2'-cyanoethyl)]-phosphorothioate 167⁴⁸



This compound was prepared in manner identical to that for **DL-137** (see page 185) using 2-O-benzyl-(2S)-propane-1,2-diol **57** (0.45 g, 2.7 mmol) to give a crude yellow oil which was purified by column chromatography on silica gel (0–30% ethyl acetate/petroleum ether, in 5% steps) to yield the phosphorothioate **167** as a clear oil (0.70 g, 71%), (Found: C, 52.3; H, 5.85; N, 7.6. $C_{16}H_{21}O_4N_2PS$ requires: C, 52.15; H, 5.75; N, 7.6%) $[\alpha]_D^{25} +1.7$ (c 0.5 in $CHCl_3$); ν_{max} (thin film, $CHCl_3$)/ cm^{-1} 3684 w, 3020 s (C-H), 2400 m ($C\equiv N$), 2259 w, 1522 m, 1454 m, 1416 m, 1262 m, 1222 vs, 1035 vs (P–O-alkyl), 930 s, 751 vs, 669 s (P=S) and 626 m; δ_H (300 MHz; C^2HCl_3) 1.25 (3 H, d, $J_{2,3}$ 6.3, 3- CH_3), 2.67 (4 H, t, $J_{1,2}$ 6.0, 2 x 2'- CH_2), 3.81 (1 H, m, 2-CH), 4.12 (2 H, m, 1- CH_2), 4.15–4.28 (4 H, m, $J_{1,2}$ 6.0, 2 x 1'- CH_2), 4.56 and 4.63 (2 H, d, J_{AB} 11.7, CH_2Ph) and 7.28–7.38 (5 H, m, Ar-H); δ_c (75.4 MHz; C^2HCl_3)

16.08 (3-CH₃), 19.06 (d, J_{2C-P} 7.5, 2'-CH₂), 62.28 (br s, 1'-CH₂), 70.82 (CH₂Ph), 71.22 (d, J_{1C-P} 6.5, 1-CH₂), 72.95 (d, J_{2C-P} 7.5, 2-CH), 116.48 (3'-CN), 127.63, 127.68 and 128.32 (Ar-CH) and 138.15 (Ar-C quaternary); δ_p (121.5 MHz; C²HCl₃) 67.87; m/z (CI⁺) 368 (30%, M⁺), 311 (70, [M - CN - S + H]⁺), 221 (18, [M - PhCH₂OCH(CH₃)C]⁺), 107 (8, PhCH₂O⁺), 91 (92, PhCH₂⁺) and 83 (100, [P(OH)₃ + H]⁺); R_f = 0.49 (30% ethyl acetate/petroleum ether).

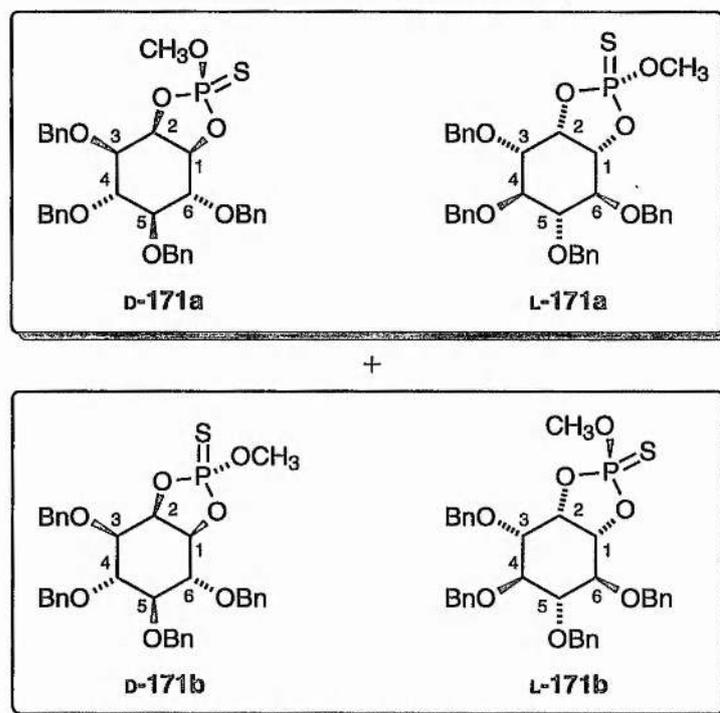
(2S)-propane-1,2-diol 1-phosphorothioate, bis-cyclohexylammonium salt
162⁴⁸



This compound was prepared in an identical manner to that for **DL-123** (see page 187) using (*S*)-1-*O*-bis(2'-cyanoethyl)phosphorothioate-2-*O*-benzyl-propane-1,2-diol **167** (0.50 g, 1.35 mmol). The resulting aqueous solution was lyophilised and the residue precipitated from water/acetone to give the phosphorothioate **162** as an amorphous white solid (0.27 g, 53%), ν_{max} (Nujol)/cm⁻¹ 3550–2450 br w (O-H and N-H), 1775 w, 1732 w, 1169 w, 1074 w, 881 m (P=S), 722 w and 702 w; δ_H (300 MHz; C²HCl₃) 1.10–2.18 (20 H, m, cyclohexyl-CH₂), 1.93 (3 H, d, $J_{2,3}$ 2.7, 3-CH₃), 3.18 (2 H, m, cyclohexyl-CH), 3.61–3.72 (1 H, m, 1-CH₂), 3.77–3.83 (1 H, m, 1-CH₂) and 3.98–4.05 (1 H, m, 2-CH); δ_C (75.4 MHz; C²HCl₃) 18.91 (3-CH₃), 24.35, 28.12 and 29.66 (cyclohexyl-CH₂), 49.94 (cyclohexyl-CH), 67.22 (d, J_{2C-P} 7.6, 2-CH), 69.78 (d, J_{1C-P} 5.4, 1-CH); δ_p (121.5 MHz; C²HCl₃) 43.93; m/z (FAB⁺) 393 (11%, [M + Na]⁺), 371 (21, [M + H]⁺), 329 (20), 307 (13), 295 (16, [M - C₆H₁₄N + 2H + Na]⁺), 272 (19,

$[M - C_6H_{14}N + 2H]^+$, 176 (71), 173 (19, $[M - 2C_6H_{14}N + 3H]^+$), 154 (100) and 136 (75).

DL-*exo*-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol 1,2-cyclic (*O*-methyl)-phosphorothioate DL-171a and DL-*endo*-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol 1,2-cyclic (*O*-methyl)-phosphorothioate DL-171b^{182, 184}



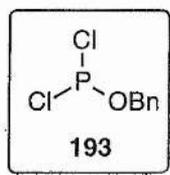
Racemic cyclic phosphorothioates DL-171a and DL-171b were prepared according to the procedure of Tsai *et al.*^{182, 183} DL-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol DL-126 (2.2 g, 4.0 mmol) was dried by the evaporation of anhydrous pyridine *in vacuo* (3 x 5 cm³) and then dissolved in anhydrous dichloromethane (10 cm³). *N,N*-Diisopropylethylamine (2.3 cm³, 6.0 mmol) was added *via* a cannula and then *N,N*-diisopropylmethylphosphonamidic chloride (1.0 g, 1.0 cm³, 4.6 mmol; 95% purity) added dropwise to the stirred solution. After 30 min, the reaction was complete as judged by TLC, and the solvent and excess *N,N*-diisopropylethylamine were removed *in vacuo*. The reaction was further dried *in vacuo* for a further 30 min. Thoroughly dried 1*H*-tetrazole (0.56 g, 16.0 mmol)

tetrazole (0.56 g, 16.0 mmol) was dissolved in tetrahydrofuran-acetonitrile (1:1 v/v, 10 cm³, warming to 40 °C was necessary) and the solution added to the above reaction mixture *via* a cannula. The reaction mixture was stirred at room temperature for 4 h and then evaporated to dryness *in vacuo*. To the resulting residue, anhydrous toluene (20 cm³) and an excess of elemental sulfur (0.3 g, powder, sublimed) were added at room temperature. The suspension was then stirred at room temperature for 2 days. The reaction mixture was diluted with toluene (10 cm³), filtered to remove the excess sulfur, and then washed with saturated NaHCO₃ (25 cm³). After the solvents were removed under reduced pressure, the crude products were purified and separated by column chromatography using TLC-grade silica (20% ethyl acetate/hexane) to give the higher *R_f* racemic *exo* cyclic phosphorothioate **DL-171a** as an oil (0.11 g, 4% based on **DL-126**) and the lower *R_f* *endo* cyclic phosphorothioate **DL-171b** as an oil (1.11 g, 40% based on **DL-126**). The *exo*-phosphorothioate **DL-171a** resisted all attempts at recrystallisation, however the *endo*-phosphorothioate **DL-171b** was recovered from ethyl acetate/heptane as fine white needle crystals.

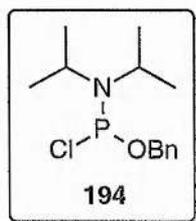
DL-*exo*-cyclic phosphorothioate DL-171a: (HRMS: found, [M + H]⁺, 633.2089. Calc. for C₃₅H₃₈O₇PS: 633.2076) (Found: C, 66.3; H, 6.1. Calc. for C₃₅H₃₇O₇PS: C, 66.45; H, 5.9%); ν_{\max} (thin film, CHCl₃)/cm⁻¹ 2955, 2927 and 2873 3 x m (C-H), 1952 w, 1880 w, 1812 w, 1727 br w, 1497 s, 1455 s, 1360 s, 1067 vs (P-O-alkyl), 1028 vs, 989 vs, 906 vs 825 vs and 699 s (P=S); δ_{H} (300 MHz; C²HCl₃) 3.46 (1 H, dd, *J*_{4,5} 7.2, *J*_{5,6} 9.6, 5-CH), 3.74 (1 H, ddd, *J*_{2,3} 3.9, *J*_{3,4} 7.5, *J*_{3-P} 1.2, 3-CH), 3.78 (3 H, d, *J*_{H-P} 15.0, POCH₃), 3.87 (1 H, t, *J*_{3,4} 7.5, *J*_{4,5} 7.2, 4-CH), 4.21 (1 H, dd, *J*_{5,6} 9.6, *J*_{6,1} 8.4, 6-CH), 4.60 (1 H, ddd, *J*_{6,1} 8.4, *J*_{1,2} 3.9, *J*_{1-P} 18.0, 1-CH), 4.69–4.90 (9 H, m, 2-CH and 4 x CH₂Ph) and 7.25–7.38 (20 H, m, Ar-CH); δ_{C} (75.4 MHz; C²HCl₃) 56.29 (d, *J*_{C-P} 5.4, POCH₃), 73.26, 74.56, 74.58 and 75.11 (CH₂Ph), 75.88 (d, *J*_{3C-P} 6.5, 3-CH), 76.95 (2-CH), 79.48 (4-CH), 81.15, 81.35 and 81.50 (Ins-CH), 127.84, 127.93, 128.00, 128.04, 128.09, 128.39, 128.46, 128.50 and 128.56 (Ar-CH), 137.35, 137.88, 138.01 and 138.14 (Ar-C quaternary); δ_{P} (121.5 MHz; C²HCl₃) 85.69; *m/z* (CI⁺) 633 (100%, [M + H]⁺), 541 (12, [M - CH₂Ph]⁺), 435 (8, [M - CH₂Ph - OCH₂Ph + H]⁺), 419

(5, $[M - 2OCH_2Ph + H]^+$), 279 (23), 205 (23), 181 (17, $[Inositol + H]^+$), 107 (58, $PhCH_2^+$) and 91 (54, $PhCH_2^+$); m/z (FAB⁺) 633 (2%, $[M + H]^+$), 602 (1, $[M - CH_3O + H]^+$), 525 (2, $[M - OP(O)OCH_3 + 3H]^+$), 181 (100, $[Inositol + H]^+$), 107 (42 $PhCH_2O^+$), 102 (81); m/z (ES⁺) 672 (1%, $[M + H + K]^+$), 671 (1, $[M + K]^+$), 656 (3, $[M + H + Na]^+$), 655 (3, $[M + Na]^+$), 634 (6, $[M + H]^+$), 633 (6, M^+), 102 (100); $R_f = 0.40$ (20% ethyl acetate/hexane).

DL-endo-cyclic-phosphorothioate DL-171b: mp 79–81 °C (HRMS: found, $[M + H]^+$, 633.2060. Calc. for $C_{35}H_{38}O_7PS$: 633.2076) (Found: C, 66.5; H, 5.95. Calc. for $C_{35}H_{37}O_7PS$: C, 66.45; H, 5.9%); ν_{max} (thin film, $CHCl_3$)/ cm^{-1} 2953, 2923 and 2872 3 x m (C-H), 1955 w, 1878 w, 1814 w, 1752 br w, 1497 m, 1455 s, 1361 m, 1267 m, 1067 br s (P–O-alkyl), 1019 br s, 899 s and 698 s (P=S); δ_H (300 MHz; C^2HCl_3) 3.44 (1 H, dd, $J_{4,5}$ 6.6, $J_{5,6}$ 10.2, 5-CH), 3.74 (1 H, dd, $J_{2,3}$ 3.3, $J_{3,4}$ 5.7, 3-CH), 3.84 (3 H, d, J_{H-P} 14.4, $POCH_3$), 3.86 (1 H, t, $J_{3,4}$ 5.7, $J_{4,5}$ 6.3, 4-CH), 4.33 (1 H, dd, $J_{5,6}$ 10.2, $J_{6,1}$ 7.2, 6-CH), 4.58 (1 H, ddd, $J_{6,1}$ 7.2, $J_{1,2}$ 6.9, J_{1-P} 10.2, 1-CH), 4.55–4.93 (9 H, m, 2-CH and 4 x CH_2Ph) and 7.20–7.44 (20 H, m, Ar-CH); δ_C (75.4 MHz; C^2HCl_3) 55.55 (d, J_{C-P} 5.4, $POCH_3$), 73.63, 73.84, 74.78 and 74.92 (CH_2Ph), 75.85 (d, $J_{3,P}$ 6.5, 3-CH), 76.88 (d, $J_{2,P}$ 2.1, 2-CH), 80.51 (4-CH), 81.14 (5-CH), 81.18 (d, $J_{6,P}$ 5.4, 6-CH), 81.75 (d, $J_{1,P}$ 3.2, 1-CH), 127.71, 127.86, 127.89, 127.92, 127.95, 128.04, 128.27, 128.31, 128.34, 128.38, 128.51 and 128.54 (Ar-CH) and 137.62, 137.79 and 138.28 (Ar-C quaternary); δ_P (121.5 MHz; C^2HCl_3) 83.94; m/z (CI⁺) 675 (8%), 633 (100, $[M + H]^+$), 541 (14, $[M - CH_2Ph]^+$), 435 (9, $[M - CH_2Ph - OCH_2Ph + H]^+$), 181 (6, $[Inositol + H]^+$) and 107 (28, $PhCH_2^+$); m/z (ES⁺) 671 (4%, $[M + K]^+$), 656 (9, $[M + H + Na]^+$), 655 (9, $[M + Na]^+$), 634 (36, $[M + H]^+$), 633 (37, M^+), 148 (100), 102 (63); $R_f = 0.32$ (20% ethyl acetate/hexane).

(Benzyloxy)dichlorophosphine 193²²⁶

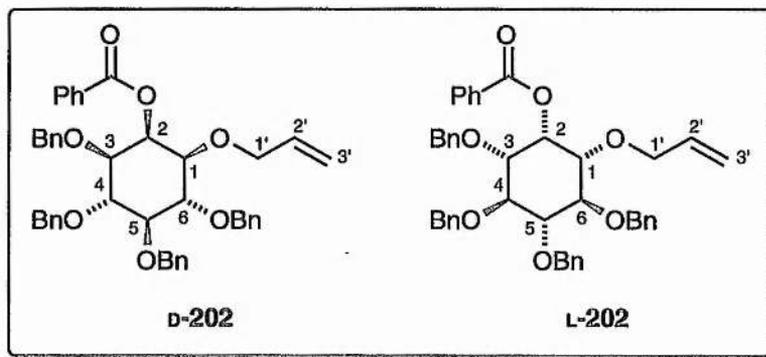
A solution of benzyl alcohol (10.3 cm³, 0.1 mol) in dry diethyl ether (50 cm³) was added dropwise over 90 min to a stirred solution of PCl₃ (13.73 g, 0.1 mol) and pyridine (8.1 cm³, 0.1 mol) in dry diethyl ether (100 cm³) at -78 °C under a nitrogen atmosphere. The mixture was stirred for a further 30 min and then the resultant precipitate was removed by filtration under nitrogen and washed twice with diethyl ether (2 x 100 cm³). The filtrates were combined and the solvent was removed under reduced pressure to give an oily residue which was distilled under reduced pressure to give (benzyloxy)dichlorophosphine **193** as a colourless oil which was unstable and used immediately (13.8 g, 66%), bp 80 °C (0.8 mmHg) (lit.,¹⁸⁸ 79 °C (0.75 mmHg)); δ_H (300 MHz, C²HCl₃) 5.27 (2 H, d, CH₂Ph) and 7.39–7.43 (5 H, m, Ar-CH); δ_C (75.4 MHz, C²HCl₃) 69.61 (d, J_{C,P} 9.7, CH₂Ph), 128.43, 128.62, and 128.88 (Ar-CH) and 137.10 (Ar-C quaternary); δ_p (121.5 MHz, C²HCl₃) 177.99.

***N,N*-diisopropylbenzylphosphonamidic chloride 194^{188, 189}**

A solution of diisopropylamine (4.8 cm³, 3.5 g, 34.6 mmol) in dichloromethane (5 cm³) was added dropwise over 30 min to a stirred solution of (benzyloxy)dichlorophosphine **193** (3.75 g, 17.3 mmol) in dichloromethane (10 cm³) at -20 °C. The mixture was allowed to warm up to room temperature over 1 h, and then stirred at room temperature for a further 30 min. Diisopropylammonium chloride was removed by filtration and

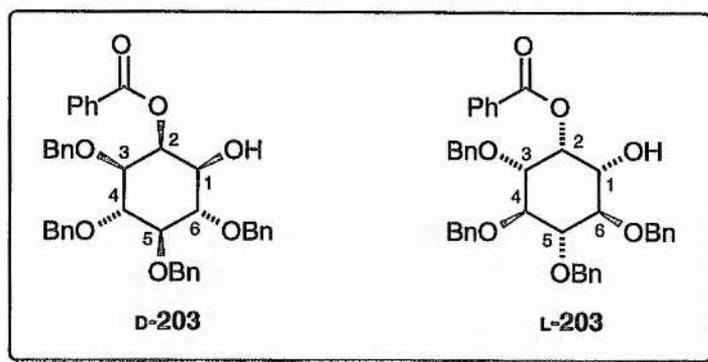
washed with methylene chloride ($3 \times 5 \text{ cm}^3$). The filtrates were combined and the solvent was removed under reduced pressure. The resultant yellow oil was dissolved in dry diethyl ether (25 cm^3) and the additional ammonium salt that precipitated was removed by filtration. Removal of the solvent under reduced pressure yielded the crude phosphine product which was used directly without further purification, δ_{H} (300 MHz, C^2HCl_3) 1.32 (12 H, d, diisopropyl- CH_3), 3.27 (2 H, m, diisopropyl-CH), 4.98 (2 H, m, CH_2Ph) and 7.27–7.31 (5 H, m, Ar-CH); 5.27 (2 H, d, CH_2Ph) and 7.39–7.43 (5 H, m, Ar-CH); δ_{C} (75.4 MHz, C^2HCl_3) 19.08 (diisopropyl- CH_3), 22.83 (diisopropyl-CH), 67.21 (d, $J_{\text{C,P}}$ 5.4, CH_2Ph), 128.23, 128.48 and 128.72 (Ar-CH) and 135.69 (Ar-C quaternary); δ_{P} (121.5 MHz, C^2HCl_3) 179.89.

DL-1-O-Allyl-2-O-benzoyl-3,4,5,6-tetrakis-O-benzyl *myo*-inositol DL-202



A mixture of anhydrous pyridine and dichloromethane (1:1, 20 cm^3) was cooled to $-10 \text{ }^\circ\text{C}$ and a previously cooled solution of benzoyl chloride (0.2 cm^3 , 1.72 mmol) in dry dichloromethane (20 cm^3) was added. DL-1-O-Allyl-3,4,5,6-tetrakis-O-benzyl *myo*-inositol DL-127 (0.5 g, 0.86 mmol) was dissolved in anhydrous dichloromethane (10 cm^3) and the solution added dropwise to the vigorously stirred benzoylating reagent from above at a rate which maintained the temperature below $0 \text{ }^\circ\text{C}$. The solution was allowed to stand at $0 \text{ }^\circ\text{C}$ for 24 h and then diluted with dichloromethane (50 cm^3) and washed with several portions of sulfuric acid (50 cm^3 , 2.0 mol dm^{-3}), saturated sodium bicarbonate solution (50 cm^3) and then water (50 cm^3). The organic phase was dried

(MgSO₄), filtered, and the solvents were removed under reduced pressure. The product was purified by silica column chromatography (0-20% ethyl acetate/petroleum ether, in 5% steps) to yield benzoylated product **DL-202** as a clear oil (0.51 g, 85%), (HRMS: found $[M + H]^+$, 685.3153. C₄₄H₄₅O₇ requires: 685.3165); ν_{\max} (thin film, CHCl₃)/cm⁻¹ 3063 w, 3032 w, 2914 w, 2868 w, 1723 s (C=O), 1497 w, 1453 m, 1360 w, 1271 s, 1094 s, 1070 s, 736 m and 697 m; δ_{H} (500 MHz, C²HCl₃) 3.52 and 3.61 (2 H, 2 x dd, J 2.7, J 9.6, 1-CH and 3-CH), 3.57 (1 H, t, $J_{4,5}$ 9.6, $J_{5,6}$ 9.6, 5-CH), 3.98 and 4.00 (2 H, 2 x t, J 9.6, 4-CH and 6-CH), 4.11 and 4.26 (2 H, 2 x dd, $J_{1,2'}$ 5.7, $J_{1',1}$ 1.5, 1'-CH₂) 4.58–4.95 (8 H, m, 4 x CH₂Ph), 5.16 (1 H, dd, $J_{2,3'cis}$ 10.2, $J_{3'cis,3'trans}$ 1.8, 3'-CH_{cis}H_{trans}), 5.29 (1 H, dd, $J_{2,3'trans}$ 17.4, $J_{3'cis,3'trans}$ 1.8, 3'-CH_{cis}H_{trans}), 5.91 (1 H, m, $J_{1,2'}$ 5.7, $J_{2,3'cis}$ 10.2, $J_{2,3'trans}$ 17.4, 2'-CH), 6.04 (1 H, t, $J_{1,2}$ 2.7, $J_{2,3}$ 2.7, 2-CH) 7.27–7.39 (20 H, m, Benzyl Ar-CH), 7.49, 7.61 and 8.10 (5 H, 3 x m, Benzoyl Ar-CH); δ_{C} (75.4 MHz, C²HCl₃) 67.49 (2-CH), 71.22 (1'-CH₂), 72.09, 76.31 and 75.88 (CH₂Ph), 78.18 and 78.43 (1-CH and 3-CH), 81.46 and 81.49 (2-CH and 4-CH), 82.90 (5-CH), 117.39 (3'-CH₂), 127.59, 127.64, 127.74, 127.78, 128.04, 128.11, 128.24, 128.35 and 128.47 (Benzyl Ar-CH), 129.96, 130.12 and 133.13 (Benzoyl Ar-CH), 134.58 (2'-CH), 137.78, 138.60 and 138.74 (Ar-C quaternary) and 165.98 (C=O); m/z (CI⁺) 685 (100%, $[M + H]^+$), 595 (36, $[M + 2H - CH_2Ph]^+$), 577 (53, $[M - OCH_2Ph]^+$), 503 (33, $[M + H - 2CH_2Ph]^+$), 487 (78, $[M + H - CH_2Ph - OCH_2Ph]^+$), 397 (84, $[M + 2H - 2CH_2Ph - OCH_2Ph]^+$), 221 (16, $[Inositol + CH_2CH=CH_2]^+$), 181 (12, $[Inositol + H]^+$) 107 (55, OCH₂Ph⁺) and 91 (19, CH₂Ph⁺); R_f = 0.63 (25% ethyl acetate/petroleum ether).

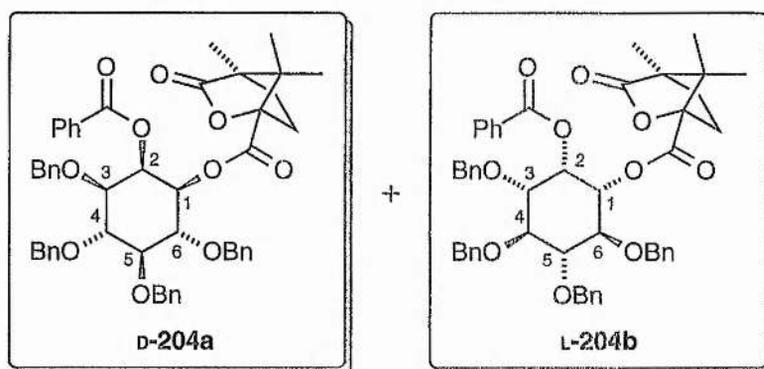
DL-2-*O*-benzoyl-3,4,5,6-tetrakis-*O*-benzyl *myo*-inositol DL-203

This compound was prepared in a manner identical to that for DL-129 (page 184), using DL-1-*O*-allyl-2-*O*-benzoyl-3,4,5,6-tetrakis-*O*-benzyl *myo*-inositol DL-202 (0.86 g, 1.2 mmol). The crude product was purified by silica column chromatography (0-20% ethyl acetate/petroleum ether, in 5% steps) to give alcohol DL-203 as a clear oil (0.54 g, 67%*), (Found: C, 74.4; H, 6.85. $C_{12}H_{16}O_3 \cdot H_2O$ requires: C, 74.3; H, 6.4%); ν_{\max} (thin film, $CHCl_3$)/ cm^{-1} 3600–3350 br w (O-H), 3064 m and 3092 m (C-H), 2815 m and 2873 m (C-H), 1726 vs (C=O), 1453 s, 1267 vs, 1094 vs, 1071 vs, 1027 s, 737 vs and 704 vs; δ_H (500 MHz, C^2HCl_3) 3.57 (1 H, t, $J_{4,5}$ 9.3, $J_{5,6}$ 9.6, 5-CH), 3.60 (1 H, dd, $J_{2,3}$ 3.0, $J_{3,4}$ 9.6, 3-CH), 3.67 (1 H, dd, $J_{6,1}$ 9.9, $J_{1,2}$ 2.7, 1-CH), 3.87 (1 H, t, $J_{5,6}$ 9.6, $J_{6,1}$ 9.9, 6-CH), 3.95 (1 H, t, $J_{3,4}$ 9.6, $J_{4,5}$ 9.3, 4-CH), 4.50–4.98 (8 H, m, 4 x CH_2Ph), 5.19 (1 H, t, $J_{1,2}$ 2.7, $J_{2,3}$ 3.0, 2-CH), 7.19–7.32 (20 H, m, Benzyl Ar-CH), 7.44, 7.55 and 8.03 (5 H, 3 x m, Benzoyl Ar-CH); δ_C (75.4 MHz, C^2HCl_3) 70.01 (2-C), 70.40 (1-C), 72.00, 75.55, 75.88 and 76.11 (CH_2Ph), 78.50 (3-C), 81.41 (6-C), 81.96 (4-C), 83.16 (5-C), 127.63, 127.74, 127.89, 127.99, 128.03, 128.16, 128.23, 128.36, 128.46, 128.55 and 128.62 (Benzyl Ar-CH), 129.95 and 133.20 (Benzoyl Ar-CH), 137.70, 138.33 and 138.62 (Ar-C quaternary) and 166.23 (C=O); m/z (Cl^+) 645 (90%, $[M + H]^+$), 555 (16, $[M + 2H - CH_2Ph]^+$), 537 (5, $[M - OCH_2Ph]^+$), 433 (8, $[M + H - CH_2Ph - OC(O)Ph]^+$), 465 (13, $[M + 3H - 2CH_2Ph]^+$), 375 (19, $[M + 4H - 3CH_2Ph]^+$),

* Low yield due to partial transfer of the benzoyl group to the 1-hydroxyl group during deprotection to give DL-1-*O*-benzoyl-3,4,5,6-tetrakis-*O*-benzyl *myo*-inositol DL-205 as a significant side-product. These isomers were readily separated by column chromatography and their identities determined by 1H and ^{13}C NMR spectrometry.

285 (13, $[M + 5H - 4CH_2Ph]^+$), 181 (10, $[Inositol + H]^+$), 123 (44, $[HOC(O)Ph + H]^+$) and 107 (100, OCH_2Ph^+); $R_f = 0.24$ (20% ethyl acetate/petroleum ether).

D- and L-1-O-[(1S,4R)-camphanoyl]-2-O-benzoyl-3,4,5,6-tetrakis-O-benzyl myo-inositol D-204a and L-204b



These compound was prepared in an identical manner to that for **D-199a** and **L-199b** (page 219), using DL-2-O-benzoyl-3,4,5,6-tetrakis-O-benzyl myo-inositol **DL-203**.

Chromatographic resolution of the diastereomers: The crude reaction mixture was applied to a silica column (220 x 30 mm) in dichloromethane. The product diastereomers were eluted with 250 cm³ of dichloromethane, 500 cm³ of 1% and 500 cm³ of 2% diethyl ether in dichloromethane collecting 15 cm³ fractions. The higher R_f ester was eluted in fractions 46–64 and the lower R_f ester in fractions 57–73. The fractions containing one compound only were pooled and the solvents were removed under reduced pressure to yield clear oils, higher R_f ester 0.22 g, 45%; lower R_f ester 0.21 g, 44%; mixture 30 mg. The mixed fraction residues were further purified by column chromatography on silica gel. The pure ester residues resisted all attempts at recrystallisation.

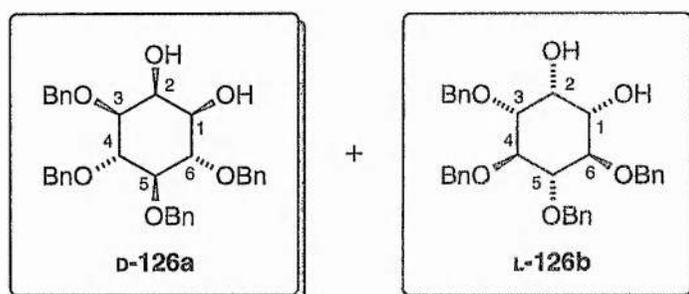
Higher R_f camphanate ester: (Found: C, 72.85; H, 6.55. $C_{51}H_{52}O_{10} \cdot H_2O$ requires: C, 72.65; H, 6.4%) $[\alpha]_D^{25} -5.4$ (c 0.5 in $CHCl_3$); ν_{max} (thin film)/cm⁻¹ 3065 m, 2969 m, 2932 m, 2251 w, 1792 vs (ester C=O), 1744 vs (ester C=O), 1732 vs (ester C=O), 1602 m, 1452 s, 1268 vs, 1169 s, 1097 vs, 1069 vs, 911 s and 734 vs; δ_H (300 MHz, C^2HCl_3)

0.80, 0.98 and 1.03 (9 H, 3 x s, 3 x Camp-CH₃), 1.61 (1 H, m, Camp-CH₂), 1.83 (2 H, m, Camp-CH₂), 2.33 (1 H, m, Camp-CH₂), 3.67 (1 H, t, *J* 9.3, Ins-CH), 3.75 (1 H, dd, *J*_{2,3} 2.7, *J*_{3,4} 9.6, 3-CH), 4.00 (1 H, t, *J* 9.6, Ins-CH), 4.17 (1 H, t, *J* 9.6, Ins-CH), 4.53–4.96 (8 H, m, 4 x CH₂Ph), 5.13 (1 H, dd, *J*_{6,1} 9.9, *J*_{1,2} 2.7, 1-CH), 6.12 (1 H, t, *J*_{1,2} 2.7, *J*_{2,3} 2.7, 2-CH), 7.25–7.36 (20 H, m, Benzyl Ar-CH), 7.49, 7.62 and 8.02 (5 H, 3 x m, Benzoyl Ar-CH); δ_c(75.4 MHz, C²HCl₃) 9.50, 16.12 and 16.32 (Camp-CH₃), 28.64 and 30.67 (Camp-CH₂), 54.26 and 54.77 (Camp-C quaternary), 67.86 (Ins-CH), 72.24 (CH₂Ph), 73.27 (Ins-CH), 75.54, 75.98 and 76.41 (CH₂Ph), 78.30, 79.08, 81.26 and 82.74 (Ins-CH), 90.86 (Camp-C quaternary), 127.63, 127.66, 127.82, 127.94, 128.02, 128.18, 128.25, 128.38, 128.55, 128.68, 129.56 and 129.79 (Ar-CH), 137.45, 138.15, 138.18 and 138.49 (Ar-C quaternary) and 165.86, 167.35 and 178.17 (C=O); *m/z* (FAB⁺) 847 (1%, [M + Na]⁺), 627 (1, [M - CO₂(C₉H₁₃O₂)]⁺), 271 (2, [Inositol + CH₂Ph]⁺), 181 (17, [Inositol + H]⁺ and [C(O)(C₉H₁₃O₂)]⁺), 107 (25, PhCH₂O⁺) and 105 (100); *R*_f = 0.35 (3% diethyl ether/dichloromethane).

Lower *R*_f camphanate ester: (Found: C, 72.50; H, 5.9. C₅₁H₅₂O₁₀·H₂O requires: C, 72.65; H, 6.4%) [α]_D²⁵ -8.3 (c 0.5 in CHCl₃); ν_{max}(thin film)/cm⁻¹ 3064 m, 2967 m, 2930 m, 2251 w, 1792 vs (ester C=O), 1744 vs (ester C=O), 1732 vs (ester C=O), 1602 m, 1459 s, 1268 vs, 1169 s, 1095 vs, 1070 vs, 911 s and 734 vs; δ_H(300 MHz, C²HCl₃) 0.87, 0.95 and 1.05 (9 H, 3 x s, 3 x Camp-CH₃), 1.61 (1 H, m, Camp-CH₂), 1.87 (2 H, m, Camp-CH₂), 2.30 (1 H, m, Camp-CH₂), 3.68 (1 H, t, *J* 9.3, Ins-CH), 3.77 (1 H, dd, *J*_{2,3} 2.4, *J*_{3,4} 9.6, 3-CH), 4.04 (1 H, t, *J* 9.3, Ins-CH), 4.14 (1 H, t, *J* 9.9, Ins-CH), 4.55–4.97 (8 H, m, 4 x CH₂Ph), 5.11 (1 H, dd, *J*_{6,1} 10.2, *J*_{1,2} 2.7, 1-CH), 6.14 (1 H, t, *J*_{1,2} 2.7, *J*_{2,3} 2.4, 2-CH), 7.25–7.31 (20 H, m, Benzyl Ar-CH), 7.51, 7.64 and 8.05 (5 H, 3 x m, Benzoyl Ar-CH); δ_c(75.4 MHz, C²HCl₃) 9.41, 16.17 and 16.39 (Camp-CH₃), 28.82 and 30.83 (Camp-CH₂), 53.92 and 54.62 (Camp-C quaternary), 67.66 (Ins-CH), 72.18 (CH₂Ph), 73.34 (Ins-CH), 75.68, 75.95 and 76.45 (CH₂Ph), 78.09, 78.96, 81.25 and 82.79 (Ins-CH), 90.73 (Camp-C quaternary), 127.53, 127.72, 127.85, 127.95, 128.04, 128.11, 128.27, 128.41, 128.57, 128.70, 129.53 and 129.86

(Ar-CH), 137.40, 138.02, 138.16 and 138.48 (Ar-C quaternary) and 165.95, 167.23 and 178.06 (C=O); $R_f = 0.31$ (3% diethyl ether/dichloromethane).

D- and L-3,4,5,6-Tetrakis-O-benzyl myo-inositol D-126 and L-126

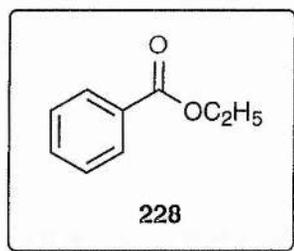


To a stirred suspension of the appropriate camphanoyl ester **D-204a** or **L-204b** (0.20 g, 0.24 mmol) in absolute alcohol (15 cm³), was added potassium hydroxide (0.14 g, 2.4 mmol) and the mixture stirred for 48 h at 25 °C to produce a clear solution. The solvent was removed under reduced pressure and the products were partitioned between water (10 cm³) and diethyl ether (30 cm³). The ethereal layer was washed with water (3 x 10 cm³), saturated brine (10 cm³) and then dried (MgSO₄). The solvent was removed to give the alcohol **D-126** or **L-126** which was purified by silica column chromatography (0-40% ethyl acetate/petroleum ether, in 5% steps).

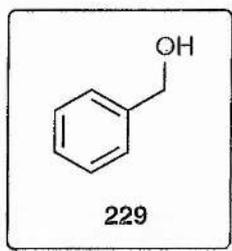
Alcohol* from higher R_f camphanoyl ester: (0.11 g, 83%), spectroscopic data was identical to that obtained for the racemic compound **DL-126**.

Alcohol* from lower R_f camphanoyl ester: (0.10 g, 76%), spectroscopic data was identical to that obtained for the racemic compound **DL-126**.

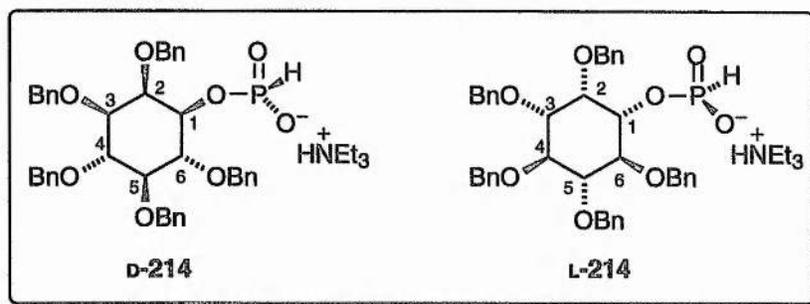
* The configurations of the resolved alcohols was not determined.

Ethyl-[^{18}O]carboxy benzoate 228 218 

To a small Schlenk flask with a stirring magnet, ethyl benzimidate hydrochloride 227 (0.40 g, 2.13 mmol) and H_2^{18}O (0.20 g, 10.02 mmol, 97% enrichment) were added.²²⁷ The flask was sealed and heated for 30 min at a temperature of 80 °C. The resulting organic layer was transferred *via* a canular to a dry test tube. An additional quantity of ethyl benzimidate (0.09 g, 0.47 mmol) was added to the reaction mixture, which was treated in the same way for 15 min. Again, the organic layer was removed, and then the two organic fractions were pooled. Hexane (30 cm³) was added and the resultant precipitate was removed by filtration. Finally, the hexane solution was dried (MgSO_4), filtered, and the solvents were removed under reduced pressure to give ester 228 as an oily residue which was used in the next step without further purification (0.31 g, 78%, isotopic purity of 94.3 atom % was calculated), ν_{max} (neat)/cm⁻¹ 3422 w, 2982 m, 1719 (ester C=O), 1603 m, 1584 m, 1452 m, 1276 s, 1109 m, 1071 m, 1029 m and 710 s; δ_{H} (300 MHz; C^2HCl_3) 1.39 (3 H, t, J 7.2, $\text{CH}_3\text{CH}_2\text{O}$), 4.38 (2 H, q, J 7.2, $\text{CH}_3\text{CH}_2\text{O}$), 7.40–7.46 (2 H, m, Ar-H), 7.51–7.57 (1 H, m, Ar-H) and 8.04–8.08 (2 H, m, Ar-H); δ_{C} (75.4 MHz; C^2HCl_3) 14.11 ($\text{CH}_3\text{CH}_2\text{O}$), 60.78 ($\text{CH}_3\text{CH}_2\text{O}$), 128.28, 129.51 and 132.77 (Ar-CH), 130.52 (Ar-C quaternary) and 166.61 (ester CO); m/z (CI^+) 153 (100%, $[\text{M} + \text{H}]^+$ ^{18}O -labelled), 152 (5, M^+ ^{18}O -labelled), 151 (3, $[\text{M} + \text{H}]^+$ unlabelled), 150 (1, M^+ unlabelled), 134 (10, $[\text{M} - \text{O}]^+$), 103 (8) and 43 (38).

[¹⁸O]Benzyl alcohol 229²¹⁸

A solution of ethyl-[¹⁸O]carboxy benzoate **228** (0.52 g, 3.46 mmol) in dry diethyl ether (15 cm³) was slowly added to a mixture of LiAlH₄ (0.21 g, 5.20 mmol) in dry diethyl ether (10 cm³)²²⁸ and the mixture was then refluxed until the reduction was judged complete by TLC (toluene) (1 h). The excess of LiAlH₄ was decomposed by cautious addition of ethyl acetate. The reaction mixture was poured out into diluted sulfuric acid (20 cm³, 0 °C, 1.0 mol dm⁻³). The ethereal layer was separated, extracted with sodium hydroxide solution (3 x 20 cm³, 0.5 mol dm⁻³) and washed with water until the pH of the wash-water was neutral. The product was purified by silica column chromatography (0–30% ethyl acetate/petroleum ether, in 5% steps), giving labelled alcohol **229** as a colourless oil (0.22 g, 58%, isotopic purity of 92.4 atom % was calculated), $\nu_{\text{max}}(\text{neat})/\text{cm}^{-1}$ 3335 s (⊖H), 3030 s, 2875 s, 1952 m, 1876 m, 1810 m, 1702 s, 1607 s, 1454 s, 1208 s, 1080 s, 1019 s (C-⊖), 913 s, 817 s, 734 s and 697 s; $\delta_{\text{H}}(300 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 2.73 (1 H, br s, ⊕H), 4.59 (2 H, s, CH₂Ph) and 7.25–7.37 (5 H, m, Ar-H); $\delta_{\text{C}}(75.4 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 64.92 (CH₂Ph), 126.96, 127.53 and 128.48 (Ar-CH) and 140.90 (Ar-C quaternary); m/z (CI⁺) 110 (85%, M⁺ ¹⁸O-labelled), 108 (7, M⁺ unlabelled), 91 (100, PhCH₂⁺), 79 (21, [PhH + H]⁺) and 77 (6, Ph⁺).

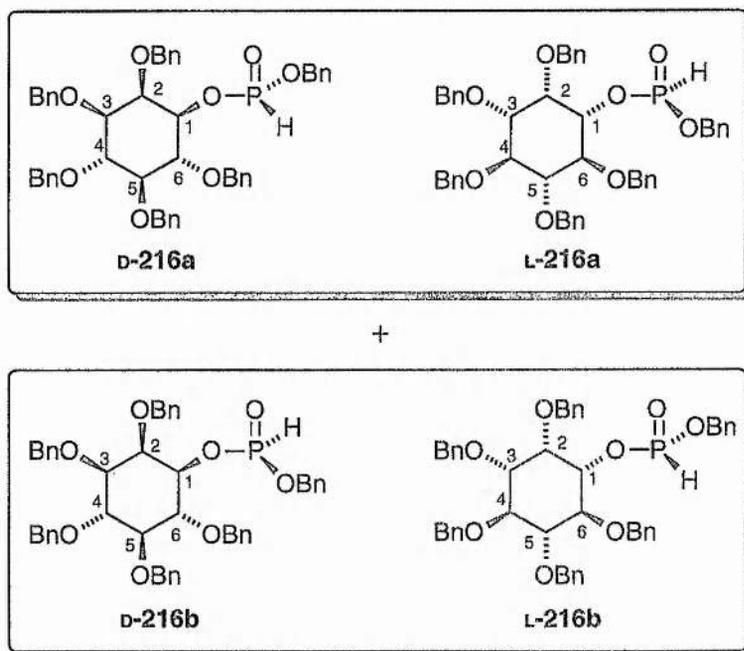
DL-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-hydrogen phosphonate, triethylammonium salt DL-214¹⁹¹

Procedure 1: To a solution of phosphorous acid (0.33 g, 4.0 mmol) and DL-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol DL-129 (0.50 g, 0.79 mmol) in anhydrous pyridine (5 cm³) was added pivaloyl chloride (0.29 cm³, 2.4 mmol) with stirring. The reaction was quenched after 3 h by careful addition of triethylammonium bicarbonate buffer (TEAB, 2 cm³, 2.0 mol dm⁻³). After evolution of carbon dioxide ceased the reaction mixture was evaporated to dryness and the residue partitioned between dichloromethane (20 cm³) and TEAB (10 cm³, 0.5 mol dm⁻³). The aqueous layer was extracted with dichloromethane (3 x 10 cm³) and the combined organic extracts were dried (MgSO₄), filtered, and evaporated under reduced pressure to leave an orange-brown oil. The residue was purified on a short silica gel column using a stepwise gradient of methanol in dichloromethane (2–10%, containing 0.1% triethylamine) to yield the H-phosphonate salt DL-214 as a foamy white solid (0.30 g, 48%).

Procedure 2: DL-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol DL-129 (0.83 g, 1.3 mmol) was dissolved in a mixture of anhydrous THF and pyridine (4:1, 20 cm³). The solution was cooled on ice under an atmosphere of nitrogen and a solution of 2-chloro-5,6-benzo-4*H*-1,3,2-dioxaphosphorin-4-one 215 in dry THF (6.3 cm³, 3.1 mmol, 0.5 mol dm⁻¹) was added dropwise with stirring. The reaction mixture was left for 15 min at room temperature. Water (1 cm³) was added and the reaction mixture kept at room temperature for a further 10 min. TEAB (20 cm³, 2.0 mol dm⁻³) was added and after evolution of carbon dioxide ceased the reaction mixture was evaporated to dryness and the residue partitioned between dichloromethane (50 cm³) and TEAB (25 cm³, 0.5 mol dm⁻³). The

aqueous layer was extracted with dichloromethane ($3 \times 25 \text{ cm}^3$) and the combined organic extracts were dried (MgSO_4) and concentrated under reduced pressure to leave an orange-brown oil. The residue was purified on a short silica gel column using a stepwise gradient of methanol in dichloromethane (2–10%, containing 0.1% triethylamine) to yield the *H*-phosphonate salt **DL-214** as a foamy white solid (0.78 g, 75%), (Found: C, 68.15; H, 7.25; N, 1.45. $\text{C}_{47}\text{H}_{58}\text{O}_8\text{NP}\cdot 2\text{H}_2\text{O}$ requires: C, 67.85; H, 7.5; N, 1.7%); ν_{max} (thin film, CHCl_3)/ cm^{-1} 2398 m (C-H), 2928 m (C-H), 2300–2750 m (N-H), 2398 m (P-H), 1593 s, 1462 s, 1216 s (P=O), 1071 vs, 1029 s (P–O-alkyl) and 758 vs; δ_{H} (500 MHz; C^2HCl_3) 1.17 (9 H, t, $J_{1,2}$ 7.3, $\text{CH}_3\text{CH}_2\text{N}$), 2.87 (6 H, q, $J_{1,2}$ 7.3, $\text{CH}_3\text{CH}_2\text{N}$), 3.54 (1 H, t, $J_{4,5}$ 9.9, $J_{5,6}$ 9.3, 5-CH), 3.57 (1 H, dd, $J_{2,3}$ 2.4, $J_{3,4}$ 9.6, 3-CH), 4.09 (1 H, t, $J_{5,6}$ 9.3, $J_{6,1}$ 9.9, 6-CH), 4.11 (1 H, t, $J_{3,4}$ 9.6, $J_{4,5}$ 9.9, 4-CH), 4.24 (1 H, dt, $J_{1,P}$ 10.7, $J_{1,6}$ 9.9, $J_{1,2}$ 2.1, 1-CH), 4.41 (1 H, t, $J_{1,2}$ 2.1, $J_{2,3}$ 2.4, 2-CH), 4.64–5.02 (10 H, m, $5 \times \text{CH}_2\text{Ph}$), 7.07 (1 H, d, $J_{\text{P-H}}$ 629.0, P-H), 7.22–7.47 (25 H, m, Ar-H) and 8.61 (1 H, br s, $\text{CH}_3\text{CH}_2\text{NH}$); δ_{C} (75.4 MHz; C^2HCl_3) 8.13 ($\text{CH}_3\text{CH}_2\text{N}$), 45.05 ($\text{CH}_3\text{CH}_2\text{N}$), 72.29 and 74.57 (CH_2Ph), 74.82 (d, $J_{\text{Cl-P}}$ 5.4, 1-C), 75.16, 75.57 and 75.67 (CH_2Ph), 77.34 (2-C), 80.74 (3-C), 80.94 (d, $J_{\text{C6-P}}$ 4.3, 6-C), 81.43 (4-C), 83.19 (5-C), 127.01, 127.23, 127.27, 127.33, 127.74, 127.80, 127.97, 128.10 and 128.14 (Ar-CH) and 138.52, 138.72, 138.82, 138.86 and 139.33 (Ar-C quaternary); δ_{P} (121.5 MHz; C^2HCl_3) 4.47; m/z (CI^+) 795 (5%, M^+), 767 (11, $[\text{M} + \text{H} - \text{CH}_3\text{CH}_2]^+$), 733 (16, $[\text{M} - (\text{CH}_3\text{CH}_2)_3\text{N} + \text{K}]^+$), 717 (5, $[\text{M} - (\text{CH}_3\text{CH}_2)_3\text{N} + \text{Na}]^+$), 694 (15, $[\text{M} - (\text{CH}_3\text{CH}_2)_3\text{N}]^+$), 631 (49, $[\text{M} - (\text{CH}_3\text{CH}_2)_3\text{N} - \text{P}(\text{O})(\text{O})\text{H}]^+$), 569 (71, $[\text{C}_{34}\text{H}_{34}\text{O}_6\text{P}]^+$), 539 (42, $[\text{C}_{34}\text{H}_{34}\text{O}_6\text{H}]^+$), 479 (100, $[\text{C}_{27}\text{H}_{28}\text{O}_6\text{P}]^+$), 449 (24, $[\text{C}_{27}\text{H}_{28}\text{O}_6\text{H}]^+$), 181 (68, $[\text{Inositol} + \text{H}]^+$) and 101 (100, $[(\text{CH}_3\text{CH}_2)_3\text{N} + \text{H}]^+$); m/z (FAB^+) 733 (15%, $[\text{M} - (\text{CH}_3\text{CH}_2)_3\text{N} + \text{K}]^+$), 717 (5, $[\text{M} - (\text{CH}_3\text{CH}_2)_3\text{N} + \text{Na}]^+$) and 118 (100, $[(\text{CH}_3\text{CH}_2)_3\text{N} + \text{NH}_3]^+$); m/z (ES^+) 796 (29%, $[\text{M} + \text{H}]^+$) and 101 (100, $[(\text{CH}_3\text{CH}_2)_3\text{N}]^+$); $R_f = 0.46$ (10% methanol/dichloromethane).

[D-(R_p)- and L-(S_p)-] and [D-(S_p)- and L-(R_p)-] 2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-(*O*-benzyl)-hydrogen phosphonate DL-216a and DL-216b¹⁹¹



To a stirred solution of *H*-phosphonate salt DL-214 (0.69 g, 0.86 mmol) and benzyl alcohol (0.19 cm³, 1.73 mmol) in anhydrous THF and pyridine (1:1, 20 cm³) was added, dropwise with stirring, pivaloyl chloride (0.21 cm³, 1.73 mmol) dissolved in THF (5 cm³). After 8 h (when TLC analysis indicated that the reaction was complete), triethylammonium bicarbonate buffer (TEAB, 10 cm³, 2.0 mol dm⁻³) was added. The reaction mixture was concentrated and the residue partitioned between dichloromethane (20 cm³) and TEAB (10 cm³, 0.5 mol dm⁻³). The organic layer was collected, dried (MgSO₄), filtered, and concentrated under reduced pressure. The crude diastereomeric mixture was first purified by silica column chromatography (0–35% ethyl acetate/petroleum ether, short column in 5% steps) to remove excess benzyl alcohol and pivaloyl chloride. The resulting mixture of diastereomers was then separated by silica column chromatography (0–10% ethyl acetate/dichloromethane, in 2% steps) to give the desired product as two pairs of enantiomers (two separated pairs of diastereomers). The higher R_f pair of enantiomers DL-216a were recovered as a clear oil which resisted recrystallisation. The lower R_f pair of enantiomers DL-216b were recovered as a white

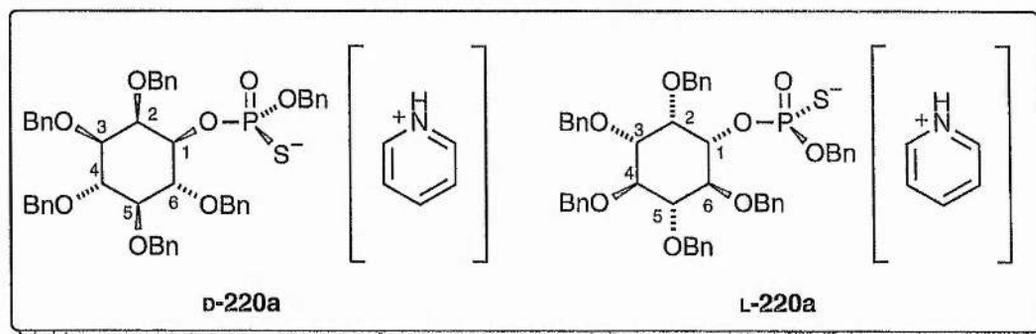
solid which was recrystallised from ethanol to give clear crystals which were submitted for X-ray crystallographic analysis. The ratio DL-216a:DL-216b was estimated as 1:1.8 by observing the ^{31}P -NMR spectra of the unseparated crude mixture (0.58 g, 86% for the purified mixture).

Higher R_f pair of enantiomers DL-216a: (Found: C, 73.70; H, 6.0. $\text{C}_{48}\text{H}_{49}\text{O}_8\text{P}$ requires: C, 73.45; H, 6.3%) (HRMS: found $[\text{M} + \text{H}]^+$, 785.3268. $\text{C}_{48}\text{H}_{50}\text{O}_8\text{P}$ requires: 785.3243); ν_{max} (thin film, CHCl_3)/ cm^{-1} 3020 m (C-H), 2980 m, 2932 m, 2390 m (P-H), 1487 m, 1455 s, 1418 m, 1368 s, 1273 s (P=O), 1217 s, 1113 s, 1029 vs (P-O-alkyl), 904 m, 757 vs, 698 vs and 657 m; δ_{H} (500 MHz; C^2HCl_3) 3.41 (1 H, dd, $J_{2,3}$ 2.1, $J_{3,4}$ 9.9, 3-CH), 3.50 (1 H, t, $J_{4,5}$ 9.6, $J_{5,6}$ 9.3, 5-CH), 4.10 (1 H, t, $J_{5,6}$ 9.3, $J_{6,1}$ 10.2, 6-CH), 4.11 (1 H, t, $J_{3,4}$ 9.9, $J_{4,5}$ 9.6, 4-CH), 4.12 (1 H, t, $J_{1,2}$ 2.4, $J_{2,3}$ 2.1, 2-CH), 4.23 (1 H, dt, $J_{6,1}$ 10.2, $J_{1,2}$ 2.4, $J_{1,P}$ 10.2, 1-CH), 4.65–5.06 (12 H, m, 5 x CH_2Ph and POCH_2Ph), 6.85 (1 H, d, $J_{\text{P-H}}$ 716.9, P-H) and 7.26–7.46 (30 H, m, Ar-H); δ_{C} (75.4 MHz; C^2HCl_3) 67.04 (d, $J_{\text{C-P}}$ 6.5, POCH_2Ph), 72.83, 74.78, 75.54, 75.83 and 75.85 (CH_2Ph), 76.00 (d, $J_{\text{C}_2\text{-P}}$ 2.5, 2-C), 76.73 (d, $J_{\text{C}_1\text{-P}}$ 7.6, 1-C), 79.91 (d, $J_{\text{C}_6\text{-P}}$ 4.4, 6-C), 80.46 (3-C), 81.27 (4-C), 83.17 (5-C), 127.52, 127.62, 127.77, 127.96, 128.02, 128.30, 128.38, 128.45 and 128.70 (Ar-CH) and 138.08, 138.25, 138.45, and 138.61 (Ar-C quaternary); δ_{P} (121.4 MHz; C^2HCl_3) 8.08; m/z (FAB $^+$) 807 (7%, $[\text{M} + \text{Na}]^+$), 785 (14, $[\text{M} + \text{H}]^+$), 271 (7, $[\text{Inositol} + \text{Bn}]^+$), 181 (100, $[\text{Inositol} + \text{H}]^+$) and 107 (41, PhCH_2O^+); R_f = 0.21 (30% ethyl acetate/petroleum ether).

Lower R_f pair of enantiomers DL-216b: mp 118–119 °C (Found: C, 73.35; H, 6.20. $\text{C}_{48}\text{H}_{49}\text{O}_8\text{P}$ requires: C, 73.45; H, 6.3%) (HRMS: found, $[\text{M}]^+$, 784.3146. $\text{C}_{48}\text{H}_{49}\text{O}_8\text{P}$ requires: 784.3165. Found, $[\text{M} + \text{H}]^+$, 785.3310. $\text{C}_{48}\text{H}_{50}\text{O}_8\text{P}$ requires: 785.3243); ν_{max} (thin film, CHCl_3)/ cm^{-1} 3030 m (C-H), 2893 m, 2887 m, 2464 m (P-H), 1497 m, 1455 s, 1400 w, 1362 s, 1262 s (P=O), 1246 s, 1216 m, 1127 s, 1073 vs, 1029 vs (P-O-alkyl), 974 s, 958 s, 738 s and 698 vs; δ_{H} (500 MHz; C^2HCl_3) 3.48 (1 H, dd, $J_{2,3}$ 2.7, $J_{3,4}$ 9.9, 3-CH), 3.54 (1 H, t, $J_{4,5}$ 9.3, $J_{5,6}$ 9.9, 5-CH), 4.07 (1 H, t, $J_{5,6}$ 9.9, $J_{6,1}$ 10.0, 6-CH), 4.08 (1 H, t, $J_{3,4}$ 9.9, $J_{4,5}$ 9.3, 4-CH), 4.12 (1 H, t, $J_{1,2}$ 2.4, $J_{2,3}$ 2.7, 2-CH), 4.37 (1 H, dt, $J_{6,1}$ 10.0, $J_{1,2}$ 2.4, $J_{1,P}$ 10.2, 1-CH), 4.64–5.06 (12 H, m,

5 x CH_2Ph and POCH_2Ph), 6.87 (1 H, d, $J_{\text{P-H}}$ 721.3, P-H) and 7.26–7.40 (30 H, m, Ar-H); δ_{C} (75.4 MHz; C^2HCl_3) 66.65 (d, $J_{\text{C-P}}$ 5.4, POCH_2Ph), 72.90, 74.95, 75.32 and 75.88 (CH_2Ph), 76.74 (d, $J_{\text{C1-P}}$ 6.5, 1-C), 76.88 (d, $J_{\text{C2-P}}$ 3.2, 2-C), 79.99 (d, $J_{\text{C6-P}}$ 3.2, 6-C), 80.51 (3-C), 81.46 (4-C), 83.31 (5-C), 127.58, 127.64, 127.75, 127.88, 128.07, 128.25, 128.40, 128.44, 128.65 and 128.77 (Ar-CH) and 138.09, 138.20, 138.25, 138.39, 138.47 and 138.61 (Ar-C quaternary); δ_{P} (121.5 MHz; C^2HCl_3) 9.65; m/z (FAB⁺) 785 (1%, $[\text{M} + \text{H}]^+$), 723 (13), 675 (7), 633 (100), 631 (20, $[\text{M} - \text{P}(\text{O})(\text{H})\text{OCH}_2\text{Ph} + 2\text{H}]^+$), 539 (15, $[\text{M} - \text{P}(\text{O})(\text{H})\text{OCH}_2\text{Ph} - \text{Ph} + \text{H}]^+$), 449 (12, $[\text{M} - \text{P}(\text{O})(\text{H})\text{OCH}_2\text{Ph} - 2\text{Ph} + 2\text{H}]^+$), 359 (10, $[\text{M} - \text{P}(\text{O})(\text{H})\text{OCH}_2\text{Ph} - 3\text{Ph} + 3\text{H}]^+$), 269 (14, $[\text{M} - \text{P}(\text{O})(\text{H})\text{OCH}_2\text{Ph} - 4\text{Ph} + 4\text{H}]^+$), 263 (36), 181 (46, $[\text{Inositol} + \text{H}]^+$) and 107 (48, PhCH_2O^+); m/z (ES⁺) 824 (5%, $[\text{M} + \text{H} + \text{K}]^+$), 808 (4, $[\text{M} + \text{H} + \text{Na}]^+$), 786 (35, $[\text{M} + 2\text{H}]^+$), 212 (5), 196 (13), 181 (19, $[\text{Inositol} + \text{H}]^+$) and 91 (100, PhCH_2^+); $R_f = 0.16$ (30% ethyl acetate/petroleum ether).

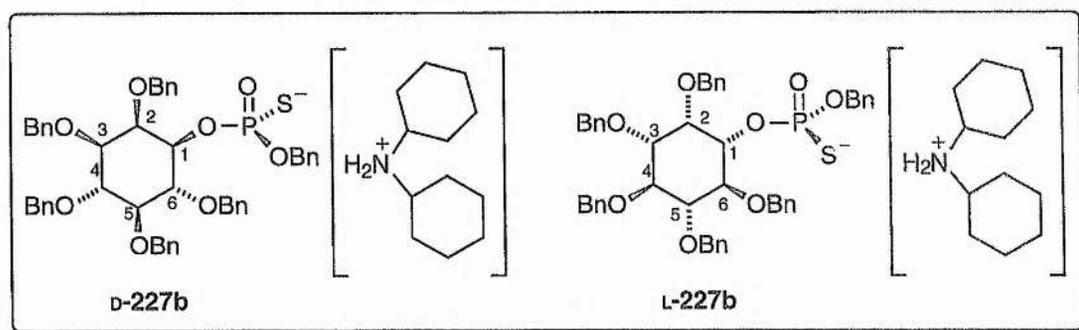
D-(S_p)- and L-(R_p)-2,3,4,5,6-Penta-O-benzyl *myo*-inositol 1-(O-benzyl)-thiophosphoric acid DL-220a¹⁹¹



The higher R_f pair of enantiomers of DL-2,3,4,5,6-penta-O-benzyl *myo*-inositol 1-(benzyl)-hydrogen phosphonate **DL-216a** (0.2 g, 0.25 mmol) was dissolved in pyridine (20 cm^3) under an argon atmosphere, and elemental sulfur (0.16 g, 5.0 mmol) was added. After 16 h the pyridine was removed under reduced pressure. The crude residue was then dissolved in dichloromethane, filtered to remove the excess sulfur, and solvents were removed under reduced pressure to give the product phosphorothioate as its

pyridinium salt **DL-219a** [$\delta_p(121.4 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 61.08]. The residue was further purified by column chromatography on Florisil[®] (2-10% methanol/dichloromethane, short column in 2% steps) to yield the thiophosphoric acid **DL-220a** as a clear oil which resisted all attempts at recrystallisation (0.19 g, mmol, 91%), $\delta_H(300 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 2.92 (1 H, br s, P-SH), 3.78–3.88 (2 H, m, 2 x Ins-CH), 4.16–4.30 (2 H, m, Ins-CH), 4.62–5.18 (14 H, m, 2 x Ins-CH and 6 x CH_2Ph) and 7.15–7.44 (30 H, m, Ar-CH); $\delta_C(75.5 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 68.52 (br s, POCH_2Ph), 72.05, 75.05, 75.73, 75.76 and 76.13 (CH_2Ph), 75.51 (br s, 2 x Ins-CH), 79.97 (Ins-CH), 80.71 (d, J 7.3, Ins-CH), 81.68 and 82.85 (Ins-CH), 127.23, 127.56, 128.73, 127.78, 128.05, 128.09, 128.29, 128.34 and 128.91 (Ar-CH) and 137.35, 138.29, 138.49, 138.82 and 139.31 (Ar-C quaternary); $\delta_p(121.4 \text{ MHz}; \text{C}^2\text{HCl}_3)$ 54.34 (br s); m/z (ES^+)^{*} 856 (39, $[\text{M} + 2\text{H} + \text{K}]^+$), 840 (14, $[\text{M} + 2\text{H} + \text{Na}]^+$), 817 (3, $[\text{M} + \text{H}]^+$), 212 (100, $[\text{C}_6\text{H}_{12}\text{O}_6\text{P} + \text{H}]^+$) and 102 (39, $[\text{C}_6\text{H}_{11}\text{NH}_3 + \text{H}]^+$); $R_f = 0.47$ (10% methanol/dichloromethane).

D-(R_p)- and L-(S_p)-2,3,4,5,6-Penta-O-benzyl myo-inositol 1-(O-benzyl)-phosphorothioate, dicyclohexylammonium salt **DL-227b¹⁹¹**



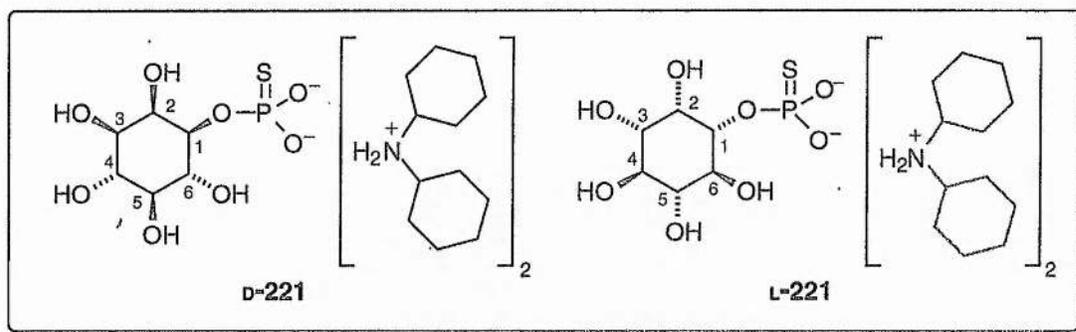
The lower R_f pair of enantiomers of DL-2,3,4,5,6-penta-O-benzyl myo-inositol 1-(benzyl)-hydrogen phosphonate **DL-216b** (0.1 g, 0.13 mmol) was dissolved in pyridine (10 cm^3) under an argon atmosphere, and elemental sulfur (82 mg, 2.5 mmol) was added. After 16 h the pyridine was removed under reduced pressure. The crude residue was then dissolved in dichloromethane, filtered to remove the excess sulfur, and

* M is the molecular weight of the phosphorothioate anion.

the solvents were removed under reduced pressure to give the product phosphorothioate as its pyridinium salt DL-227b [δ_p (121.4 MHz; C^2HCl_3) 59.81]. The crude residue was further purified by column chromatography on Florisil[®] (2-10% methanol/dichloromethane, short column in 2% steps) to yield the thiophosphoric acid DL-220b as a clear oil (85 mg, 82%). The thiophosphoric acid was then redissolved in a mixture of methanol and dichloromethane (3 cm³, 5:1), dicyclohexylamine (65 mm³, 0.32 mmol) was added, and the solution left to stand at 0 °C. The product was recovered as the crystalline dicyclohexylamine salt DL-227b (98 mg, 77%). The clear crystals of DL-227b were submitted for X-ray crystallographic analysis, mp 297–310 °C (decomp.) (Found: C, 71.9; H, 7.3; N, 1.35. $C_{60}H_{72}O_8NPS$ requires: C, 72.2; H, 7.3; N, 1.4%) (HRMS: found, $[M + H + K]^+$, 855.3275. $C_{48}H_{49}O_8PSK$ requires: 855.2523)*; ν_{max} (thin film, $CHCl_3$)/cm⁻¹ 3120-2400 br m (N-H), 2934 vs (C-H), 2861 vs (C-H), 1497 s, 1455 s, 1216 s (P=O), 1054 s (P-OCH₂) and 752 vs (P=S); δ_H (500 MHz; C^2HCl_3) 0.98-1.98 (20 H, m, Dicyclohexylamine-CH₂), 2.86 (2 H, m, Dicyclohexylamine-CH), 3.49 (1 H, t, $J_{4,5}$ 9.3, $J_{5,6}$ 9.6, 5-CH), 3.50 (1 H, dd, $J_{2,3}$ 2.1, $J_{3,4}$ 9.6, 3-CH), 4.06 (1 H, t, $J_{3,4}$ 9.6, $J_{4,5}$ 9.3, 4-CH), 4.07 (1 H, t, $J_{5,6}$ 9.6, $J_{6,1}$ 9.9, 6-CH), 4.32 (1 H, dt, $J_{6,1}$ 9.9, $J_{1,2}$ 2.1, $J_{1,P}$ 10.4, 1-CH), 4.88 (1 H, t, 2-H), 4.59–5.20 (12 H, m, 6 x CH₂Ph), 7.18–7.41 (30 H, m, Ar-CH) and 8.63 (2 H, br s, Dicyclohexylamine-NH₂⁺); δ_C (75.5 MHz; C^2HCl_3) 24.49, 24.69 and 29.54 (Dicyclohexylamine-CH₂), 53.56 (Dicyclohexylamine-CH), 68.1 (d, J_{C-P} 1.4, POCH₂Ph), 72.11, 74.88, 75.34, 75.67 and 75.97 (CH₂Ph), 76.37 (2-C), 77.31 (d, J_{1C-P} 7.5, 1-C), 80.50 (3-C), 81.04 (d, J_{6C-P} 6.5, 6-C), 81.54 (4-C), 83.43 (6-C), 127.22, 127.34, 127.52, 127.77, 128.03, 128.10, 128.21 and 128.34 (Ar-CH) and 138.60, 138.83, 139.04, 139.23 and 139.66 (Ar-C quaternary); δ_p (121.4 MHz; C^2HCl_3) 54.89; m/z (FAB⁺) 855 (6%, $[M + H + K]^+$), 181 (8, [Inositol + H]⁺), 149 (100) and 107 (16, OCH₂Ph⁺); R_f = 0.55 (10% methanol/dichloromethane).

* Accurate mass error of 88 ppm in spite of all other data, including X-ray crystallography coordinates, being in accordance with the structure shown for DL-227b. M is the molecular weight of the phosphorothioate anion.

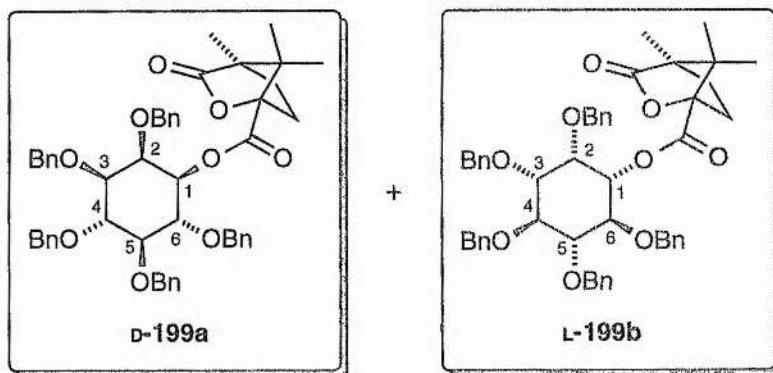
DL-*myo*-Inositol 1-phosphorothioate, bis-dicyclohexylammonium salt
DL-221



The diester **DL-220a** or **DL-220b** (15 mg, 15 nmol) was dissolved in THF (5 cm³), and this solution added dropwise to a solution of sodium (10 mg, 0.44 mmol), in liquid ammonia (25 cm³) 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 the protected phosphate solution. After the final addition of this solution the reaction mixture was stirred for a further 30 min and then dry methanol (5 cm³) was added. The ammonia was allowed to evaporate and the solvents were removed under reduced pressure. The residue was redissolved in water (10 cm³) and concentrated under reduced pressure again. The residue was dissolved in water (5 cm³) 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 dicyclohexylamine (1 cm³) was added and the reaction mixture stirred at room temperature for 4 h. The aqueous solution was extracted with diethyl ether (3 x 5 cm³) to remove the excess dicyclohexylamine and the sample lyophilised to give phosphorothioate **DL-221** as an off-white solid (4.2 mg, 44%), δ_{H} (300 MHz; ²H₂O) 0.93–1.95 (40 H, m, Dicyclohexylamine-CH₂), 2.97–3.12 (4 H, m, Dicyclohexylamine-CH), 3.18 (1 H, t, *J* 9.0, Ins-CH), 3.40–3.48 (2 H, m, Ins-CH), 3.60 (1 H, t, *J* 9.6, Ins-CH), 3.93 (1 H, dt, *J* 9.9, *J* 3.6, 1-CH) and 4.12 (1 H, t, *J* 2.4, 2-CH); δ_{C} (75.4 MHz; ²H₂O) 22.05, 22.65 and 27.17 (Dicyclohexylamine-CH₂), 51.33 (Dicyclohexylamine-CH), 68.88 (Ins-CH), 69.31 (d, *J* 5.2, Ins-CH), 69.46 (d, *J* 9.0, Ins-CH), 70.38 (Ins-CH), 71.94 (d, *J* 3.0, Ins-CH) and 72.30 (Ins-CH); δ_{P} (121.4 MHz;

C^2HCl_3) 44.46; All data was consistent with the successful and complete deprotection of DL-220a or DL-220b.

D- and L-1-O-[(1S,4R)-Camphanoyl]-2,3,4,5,6-penta-O-benzyl myo-inositol D-199a and L-199b⁴⁷



The alcohol DL-129 (1.0 g, 1.6 mmol), triethylamine (0.5 cm³, 3.5 mmol) and DMAP (33 mg, 0.3 mmol) were dissolved in anhydrous dichloromethane (20 cm³) at 0 °C under an atmosphere of nitrogen. To this solution was added dropwise (-)-(1S,4R)-camphanoyl chloride (0.69 g, 3.2 mmol) in dichloromethane (4 cm³). The reaction was stirred at 0 °C for 1 h and then at 25 °C for 24 h. The solution was washed with water (3 x 10 cm³) and saturated sodium bicarbonate solution (2 x 10 cm³), and then dried (MgSO₄). The solvent was removed under reduced pressure to give a white solid in quantitative recovery. The ¹H-NMR spectrum of the crude residue showed that only the two product diastereomeric esters were present.

Chromatographic resolution of the diastereomers: The crude reaction mixture was applied to a silica gel column (320 x 40 mm) in dichloromethane. The product enantiomers were eluted with 1 dm³ of dichloromethane, 3 dm³ of 1% and then 3 dm³ of 2% diethyl ether in dichloromethane collecting 50 cm³ fractions. Ester L-199b was eluted in fractions 62–80 and ester D-199a in fractions 72–106. The pure fractions were pooled and the solvent was removed under reduced pressure to yield white solids, ester L-199b and ester D-199a. The mixed fractions were purified by further silica gel column

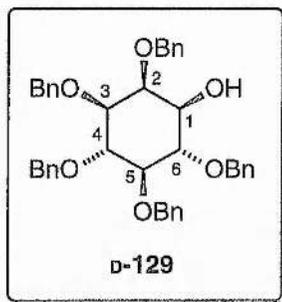
chromatography and the pure ester residues finally recovered by recrystallisation from ethanol giving **L-199b** (0.59 g, 45%)* and **D-199a** (0.55 g, 42%)* with remaining mixture (0.19 g, 13%). The less polar ester **L-199b** formed suitable crystals for X-ray crystallographic analysis. The more polar ester **D-199a** formed as an amorphous white solid.

Higher R_f diastereomer L-199b: mp 146–147 °C (ethanol) {lit.⁴⁸ 146–148 °C (ethyl acetate/petroleum ether)} (Found: C, 75.6; H, 6.85. Calc. for $C_{51}H_{54}O_9$: C, 75.5; H, 6.7%) $[\alpha]_D^{25} +11.95 \pm 0.4$ (*c* 0.5 in $CHCl_3$) {lit.⁴⁸, $[\alpha]_D^{25} +12.5$ (*c* 0.5 in $CHCl_3$)}; ν_{max} (thin film, $CHCl_3$)/ cm^{-1} 3065, 2960, 2995 and 2875 4 x m (C-H), 1787 s (C=O), 1735 s (C=O), 1604 w, 1498 m, 1454 s, 1360 m, 1316 m, 1265 s, 1100 vs, 1068 vs and 700 s; δ_H (300 MHz, C^2HCl_3) 0.91, 1.00 and 1.09 (3 x 3 H, 3 x s, 3 x Camp- CH_3), 1.63 (1 H, m, Camp- CH_2), 1.83 (2 H, m, Camp- CH_2), 2.29 (1 H, m, Camp- CH_2), 3.58 (1 H, dd, *J* 9.6, *J* 2.1, Ins-CH), 3.58 (1 H, t, *J* 9.6, Ins-CH), 4.11 (1 H, t, *J* 9.6, Ins-CH), 4.14 (1 H, t, *J* 2.1, Ins-CH), 4.18 (1 H, t, *J* 9.6, Ins-CH), 4.65–5.01 (11 H, m, CH_2Ph and Ins-CH) and 7.26–7.40 (25 H, m, Ar-CH); δ_C (75.4 MHz; C^2HCl_3) 9.55, 16.52 and 16.67 (Camp- CH_3), 28.82 and 30.65 (Camp- CH_2), 54.16 and 54.74 (Camp-C quaternary), 73.06 and 74.99 (CH_2Ph), 75.01 (Ins-CH), 75.23 and 75.92 (CH_2Ph), 75.98, 79.11, 80.92, 81.47 and 83.46 (Ins-CH), 90.84 (Camp-C quaternary), 127.32, 127.46, 127.56, 127.59, 127.65, 127.78, 127.87, 128.09, 128.29, 128.41 and 128.50 (Ar-CH), 135.55, 138.15, 138.38, 138.49 and 138.68 (Ar-C quaternary) and 167.45 and 178.11 (Camp-CO); *m/z* (FAB⁺) 833 (10%, $[M + Na]^+$), 809 (2, $[M - H]^+$), 271 (15, $[Inositol + PhCH_2]^+$), 181 (100, $[Inositol + H]^+$ and $C(O)C_9H_{13}O_2^+$), 154 (27), 136 (24), 107 (20, $PhCH_2O^+$) and 105 (19); $R_f = 0.17$ (3% diethyl ether/dichloromethane).

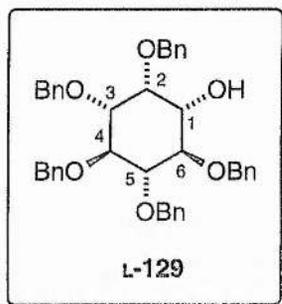
Lower R_f diastereomer D-199a: mp 162–164 °C (ethanol) {lit.⁴⁸ 161–164 °C (ethyl acetate/petroleum ether)} (Found: C, 75.35; H, 6.75. Calc. for $C_{51}H_{54}O_9$: C, 75.5; H, 6.7%) $[\alpha]_D^{25} -17.65 \pm 0.35$ (*c* 0.5 in $CHCl_3$) (lit.⁴⁸, $[\alpha]_D^{25} -17.8$ (*c* 0.5 in $CHCl_3$)); ν_{max} (thin film, $CHCl_3$)/ cm^{-1} 3067, 2963, 2994 and 2874 4 x m (C-H), 1787 s (C=O),

* The yields quoted for **D-199a** and **L-199b** represent the total quantity of products isolated with two sequential chromatographic separations.

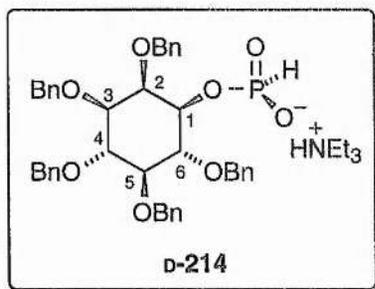
1732 s (C=O), 1603 w, 1497 m, 1454 s, 1360 m, 1316 m, 1266 s, 1099 vs, 1070 vs and 700 s; δ_{H} (300 MHz, C^2HCl_3) 0.85, 0.97 and 1.08 (3 x 3 H, 3 x s, 3 x Camp- CH_3), 1.67 (1 H, m, Camp- CH_2), 1.89 (2 H, m, Camp- CH_2), 2.87 (1 H, m, Camp- CH_2), 3.58 (1 H, dd, J 9.6, J 2.1, Ins-CH), 3.58 (1 H, t, J 9.3, Ins-CH), 4.12 (1 H, t, J 9.6, Ins-CH), 4.18 (1 H, t, J 10.2, Ins-CH), 4.22 (1 H, t, J 2.1, Ins-CH), 4.65–5.00 (11 H, m, CH_2Ph and Ins-CH), 7.26–7.50 (25 H, m, Ar-CH); δ_{C} (75.4 MHz; C^2HCl_3) 9.53, 16.48 and 16.53 (Camp- CH_3), 28.87 and 30.85 (Camp- CH_2), 54.04 and 54.73 (Camp-C quaternary), 73.07 and 74.66 (CH_2Ph), 75.21 and 75.25 (Ins-CH), 75.28, 75.90 and 75.97 (CH_2Ph), 78.88, 80.96, 81.41 and 83.53 (Ins-CH), 90.87 (Camp-C quaternary), 127.27, 127.47, 127.57, 127.63, 127.77, 127.89, 128.09, 128.35, 128.41 and 128.51 (Ar-CH) and 138.36, 138.42, 138.46 and 138.66 (Ar-C quaternary) and 167.58 and 178.05 (Camp-C=O); m/z (FAB⁺) 833 (64%, $[\text{M} + \text{Na}]^+$), 809 (1, $[\text{M} - \text{H}]^+$), 271 (14, $[\text{Inositol} + \text{PhCH}_2]^+$), 181 (100, $[\text{Inositol} + \text{H}]^+$ and $\text{C}(\text{O})\text{C}_9\text{H}_{13}\text{O}_2^+$), 154 (34), 136 (31), 107 (20, PhCH_2O^+) and 105 (25); $R_f = 0.09$ (3% diethyl ether/dichloromethane).

D-2,3,4,5,6-penta-O-benzyl myo-inositol D-129⁴⁷

To a suspension of camphanoyl ester **D-199a** (0.40 g, 0.49 mmol) in absolute ethanol (10 cm³) was added potassium hydroxide (0.3 g, 5.3 mmol) and the resulting mixture was stirred for 20 h at 25 °C to produce a clear solution. The solvent was removed under reduced pressure and the products were partitioned between water (5 cm³) and diethyl ether (20 cm³). The ethereal layer was washed with water (3 x 5 cm³), saturated brine (5 cm³) and then dried (MgSO₄). The solvent was removed under reduced pressure to give a solid residue which was recrystallised from hexane (0.3 g, 97%), mp 62–63 °C {lit.¹⁶⁵ 63–64 °C (hexane)} (Found: C, 77.8; H, 6.5. Calc. for C₄₁H₄₂O₆: C, 78.05; H, 6.7%) [α]_D²⁵ +9.2 ± 0.23 (c 0.3 in CHCl₃) {lit.⁴⁷, [α]_D²⁵ +9.1 (c 0.3 in CHCl₃)}. All other data was the same as for the racemic compound **DL-129**.

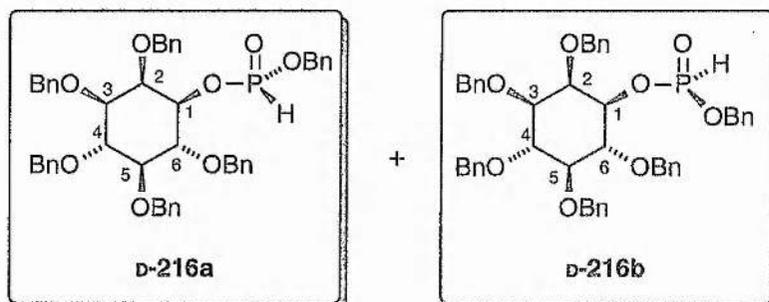
L-2,3,4,5,6-penta-O-benzyl myo-inositol L-129⁴⁷

Alcohol **L-129** was prepared in an identical manner to that for **D-129** using camphanoyl ester **L-199b** (0.31 g, 0.38 mmol) and recrystallised from hexane (0.22 g, 91%), mp 62–64 °C {lit.¹⁶⁵ 63–65 °C (hexane)} (Found: C, 77.75; H, 6.7. Calc. for C₄₁H₄₂O₆: C, 78.05; H, 6.7%) [α]_D²⁵ -9.2 ± 0.23 (c 0.3 in CHCl₃) {lit.⁴⁷, [α]_D²⁵ -8.7 (c 0.3 in CHCl₃)}. All other data was the same as for the racemic compound **DL-129**.

D-2,3,4,5,6-Penta-O-benzyl myo-inositol 1-hydrogen phosphonate, triethylammonium salt D-214¹⁹¹

This compound was prepared in a manner identical to that for **DL-214** (see page 211) using separated enantiomer **D-2,3,4,5,6-penta-O-benzyl myo-inositol D-129** (0.30 g, 0.48 mmol). The H-phosphonate triethylammonium salt **D-214** was recovered as a clear oil (0.34 g, 90%). δ_p (121.4 MHz; C²HCl₃) 4.48; All other spectroscopic data was identical to that of the racemic compound **DL-214**.

D-(*S_p*)-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-(*O*-benzyl)-hydrogen phosphonate **D-216a and D-(*R_p*)-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-(*O*-benzyl)-hydrogen phosphonate **D-216b**¹⁹¹**

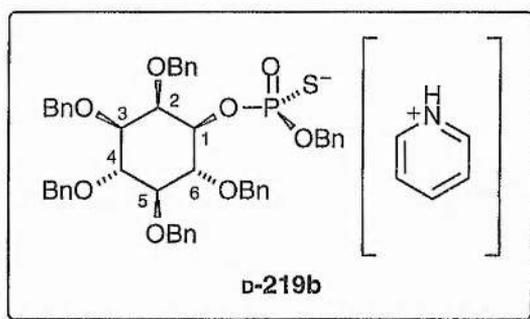


The separated enantiomer of the triethylammonium salt of D-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-hydrogen phosphonate **D-214** (0.18 g, 0.23 mmol) was coupled to benzyl alcohol (23 mm³, 0.23 mmol) using the pivaloyl coupling methodology described for the racemic compound **DL-216** (see page 213). The crude diastereomeric mixture (**D-216a** and **D-216b** (ratio of diastereomers 1:1.8, as estimated ³¹P-NMR spectrometry) was purified as previously described to give the separated *H*-phosphonates **D-216a** and **D-216b**. Both diastereomers were recovered as clear oils which resisted all attempts at recrystallisation. (**D-216a** 0.05 g, 27%; **D-216b** 0.08 g, 48%).

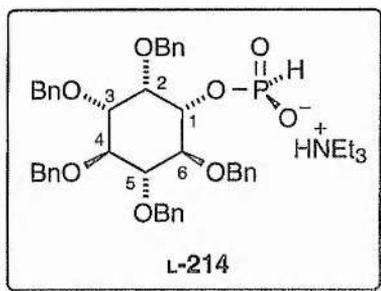
Higher *R_f* diastereomer D-216a: $\delta_p(121.4 \text{ MHz; C}^2\text{HCl}_3)$ 8.06; All other spectroscopic data was identical to that of the racemic compound **DL-216a**.

Lower *R_f* diastereomer D-216b: $\delta_p(121.4 \text{ MHz; C}^2\text{HCl}_3)$ 9.65; All other spectroscopic data was identical to that of the racemic compound **DL-216b**.

D-(R_p)-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-(*O*-benzyl)-phosphorothioate, pyridinium salt **D-219b¹⁹¹**



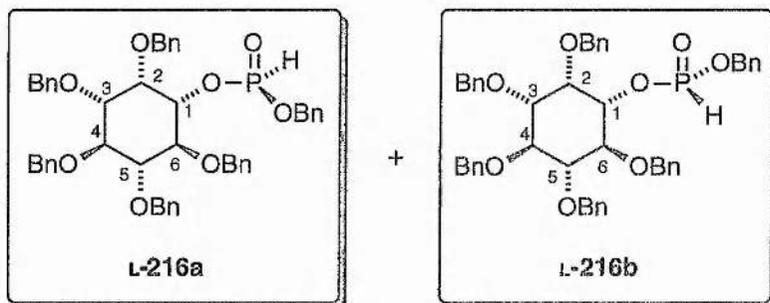
The separated lower R_f diastereomer **D-216b** (80 mg, 0.10 mmol) was stereospecifically sulfurised using the methodology described for the racemate **DL-219b**. The crude residue was dissolved in dichloromethane, filtered to remove the excess sulfur, and the solvents were removed under reduced pressure to yield the pyridinium salt **D-219b**. The residue was further purified by column chromatography on Florisil® (2-10% methanol/dichloromethane, short column in 2% steps) to yield the thiophosphoric acid **D-220b** as a clear oil (75 mg, 89%) [δ_p (121.4 MHz; C^2HCl_3) 55.81 (br s)]. The thiophosphoric acid was then redissolved in a mixture of methanol and dichloromethane (3 cm³, 5:1) and after the addition of pyridine (50 mm³, 0.62 mmol) was left to stand at 0 °C. The reformed pyridinium salt **D-219b** failed to recrystallise and the solvents and excess pyridine were removed under reduced pressure; δ_H (300 MHz; C^2HCl_3) 3.51–3.57 (2 H, m, 3-CH and 5-CH), 4.07–4.17 (2 H, m, 4-CH and 6-CH), 4.47 (1 H, dt, $J_{6,1}$ 10.4, $J_{1,2}$ 2.1, $J_{1,p}$ 10.4, 1-CH), 4.85 (1 H, t, 2-H), 4.57–5.28 (12 H, m, 6 x CH₂Ph), 7.16 (2 H, t, Pyr-CH), 7.18–7.41 (30 H, m, Ar-CH) 7.89 (1 H, t, Pyr-CH), 8.61 (2 H, d, Pyr-CH) and 11.83 (1 H, br s, Pyridine-NH⁺); δ_C (75.5 MHz; C^2HCl_3) 68.10 (d, J_{C-P} 4.3, POCH₂Ph), 72.14, 74.66, 75.20, 75.63 and 75.86 (CH₂Ph), 76.20 (2-C), 77.83 (d, J_{1C-P} 7.5, 1-C), 80.47 (3-C), 880.77 (d, J_{6C-P} 6.5, 6-C), 81.39 (4-C), 83.23 (6-C), 124.94 (Pyridine-CH), 126.90, 127.03, 127.40, 127.50, 127.80, 127.89, 128.01, 128.11 and 128.25 (Ar-CH) and 138.37, 138.71, 138.88, 139.07 and 139.49 (Ar-C quaternary) and 139.93 and 145.83 (Pyridine-CH); δ_p (121.4 MHz; C^2HCl_3) 59.81; R_f = 0.55 (10% methanol/ dichloromethane).

L-2,3,4,5,6-Penta-O-benzyl *myo*-inositol 1-hydrogen phosphonate, triethylammonium salt L-214¹⁹¹

This compound was prepared in a manner identical to that for DL-214 (see page 211) using separated enantiomer L-2,3,4,5,6-penta-O-benzyl *myo*-inositol L-129 (0.81 g, 1.3 mmol). The *H*-phosphonate triethylammonium salt L-214 was recovered as a foamy white solid (0.82 g, 81%). Selected data: $[\alpha]_D^{25} -26.5 \pm 0.01$ (*c* 1 in CHCl_3)*; δ_p (121.4 MHz; C^2HCl_3) 4.31; *m/z* (ES⁺) 796 (29%, [M + H]⁺), 717 (2, [M - N(C₂H₅)₃ + Na]⁺), 419 (5, [M - N(C₂H₅)₃ - 3Ph]⁺), 403 (25, [M - N(C₂H₅)₃ - 2Ph - OPh]⁺), 234 (26), 214 (100), 183 (55), 157 (64) and 150 (96). All other spectroscopic data was identical to that of the racemic compound DL-214.

* After repeated attempts at purification, C-H-N microanalysis indicated that L-214 remained slightly impure. Optical rotation is therefore only included to show the approximate degree of rotation of the compound.

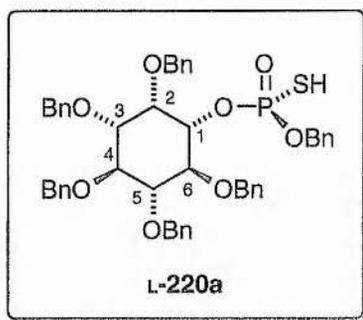
L-(*S_p*)-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-(*O*-benzyl)-hydrogen phosphonate L-216a and L-(*R_p*)-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-(*O*-benzyl)-hydrogen phosphonate L-216b¹⁹¹



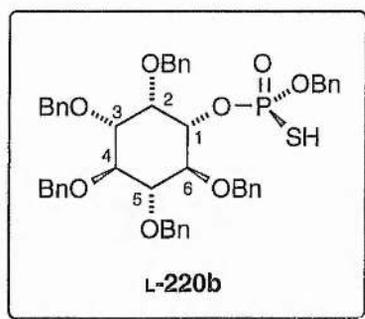
The separated enantiomer of the triethylammonium salt of L-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-hydrogen phosphonate L-214 (0.34 g, 0.43 mmol) was coupled to benzyl alcohol (60 mm³, 0.60 mmol) using the pivaloyl coupling methodology described for the racemic compound DL-216 (see page 213). The crude diastereomeric mixture L-216a and L-216b (ratio of diastereomers 1:1.7, as estimated by ³¹P-NMR spectrometry), was purified as previously to give the separated *H*-phosphonates L-216a and L-216b. Both diastereomers were recovered as clear oils which resisted all attempts at recrystallisation. (L-216a 0.08 g, 25%; L-216b 0.15 g, 44%).

Higher *R_f* diastereomer L-216a: $\delta_p(121.4 \text{ MHz; C}^2\text{HCl}_3)$ 8.11; All other spectroscopic data was identical to that of the racemic compound DL-216a.

Lower *R_f* diastereomer L-216b: $\delta_p(121.4 \text{ MHz; C}^2\text{HCl}_3)$ 9.64; All other spectroscopic data was identical to that of the racemic compound DL-216b.

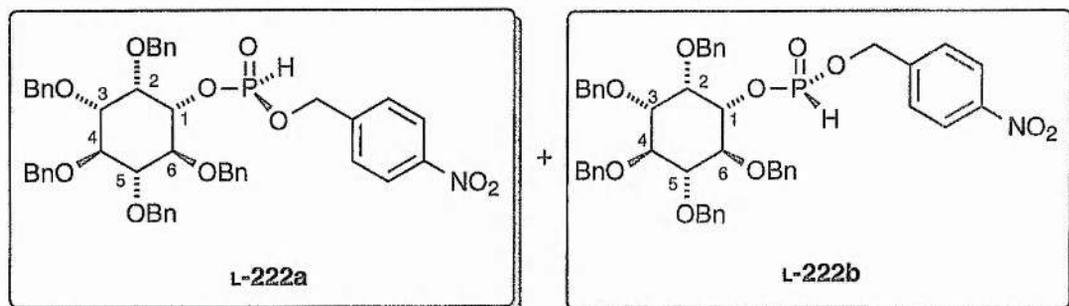
L-(R_p)-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-(*O*-benzyl)-thiophosphoric acid L-220a¹⁹¹

The separated higher R_f diastereomer **L-216a** (38 mg, 0.048 mmol) was stereospecifically sulfurised using the methodology described for the racemate **DL-220a**. The crude residue was dissolved in dichloromethane, filtered to remove the excess sulfur, and solvents were removed under reduced pressure to yield the pyridinium salt **L-219a**. The residue was further purified by column chromatography on Florisil[®] (2-10% methanol/dichloromethane, short column in 2% steps) to yield the thiophosphoric acid **L-220a** as a clear oil (35 mg, 89%). The thiophosphoric acid was then redissolved in a mixture of methanol and dichloromethane (3 cm³, 5:1), and after the addition of pyridine (30 mm³, 0.37 mmol) the solution was left to stand at 0 °C. The reformed pyridinium salt **L-219a** failed to recrystallise and the solvents and excess pyridine were removed under reduced pressure. For the purified thiophosphoric acid **L-220a**; δ_p (121.4 MHz; C²HCl₃) 55.34; m/z (ES⁺) 855 (1%, [M + H + K]⁺) and 212 (100).

L-(S_p)-2,3,4,5,6-Penta-O-benzyl myo-inositol 1-(benzyl)-thiophosphoric acid L-220b¹⁹¹

The separated lower R_f diastereomer **L-216b** (66 mg, 0.085 mmol) was stereospecifically sulfurised using the methodology described for the racemate **DL-220b**). The crude residue was dissolved in dichloromethane, filtered to remove the excess sulfur, and solvents were removed under reduced pressure to yield the pyridinium salt **L-219b**. The residue was further purified by column chromatography on Florisil® (2-10% methanol/dichloromethane, short column in 2% steps) to yield the thiophosphoric acid **L-220b** as a clear oil (59 mg, 86%). The thiophosphoric acid was then redissolved in a mixture of methanol and dichloromethane (3 cm³, 5:1) and after the addition of pyridine (50 mm³, 0.62 mmol) the solution was left to stand at 0 °C. The reformed pyridinium salt **L-219b** failed to recrystallise and the solvents and excess pyridine were removed under reduced pressure. For the purified thiophosphoric acid **L-220b**; δ_p (121.4 MHz; C²HCl₃) 56.12.

L-(S_p)-2,3,4,5,6-Penta-O-benzyl myo-inositol 1-(O-p-nitrobenzyl) hydrogen phosphonate L-222a and L-(R_p)-2,3,4,5,6-penta-O-benzyl myo-inositol 1-(O-p-nitrobenzyl)-hydrogen phosphonate L-222b¹⁹¹



The separated enantiomer of the triethylammonium salt of L-2,3,4,5,6-penta-O-benzyl myo-inositol 1-hydrogen phosphonate L-214 (0.38 g, 0.48 mmol) was coupled to *p*-nitrobenzyl alcohol (80 mg, 0.52 mmol) at -25 °C using the pivaloyl coupling methodology described for the racemic compound DL-216 and benzyl alcohol (page 213). The crude diastereomeric mixture (L-222a and L-222b, ratio of diastereomers 1:1.4, as estimated ³¹P-NMR spectrometry) was purified and separated by repeated silica column chromatography (0–6% ethyl acetate/dichloromethane, in 2% steps). Both diastereomers were recovered as clear yellow oils which resisted all attempts at recrystallisation. (L-222a 99 mg, 25%*; L-222b 139 mg, 35%*).

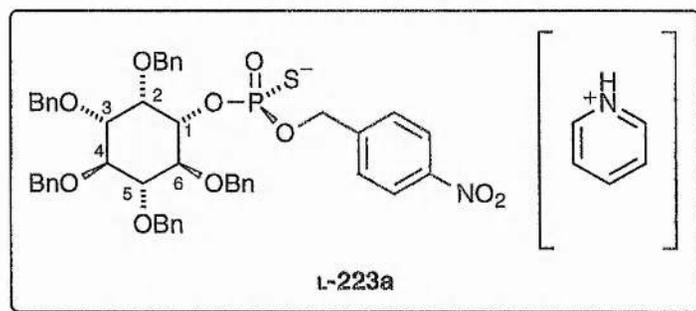
Higher R_f diastereomer L-222a: δ_H(300 MHz; C²HCl₃) 3.47 (1 H, dd, *J* 9.9, *J* 2.4, 3-CH), 3.51 (1 H, t, *J* 9.3, Ins-CH), 4.07 (1 H, t, *J* 9.6, Ins-CH), 4.10 (1 H, t, *J* 9.9, Ins-CH), 4.20 (1 H, t, *J* 2.4, 2-CH), 4.34 (1 H, dt, *J* 9.9, *J* 2.4, 1-CH), 4.69–5.11 (12 H, m, 5 × CH₂Ph and 1 × *p*-Nitrobenzyl CH₂), 6.74 (1 H, d, *J*_{P-H} 724, P-H), 7.19–7.34 (25 H, m, Benzyl Ar-CH), 7.37 (2 H, d, *J*_{o-c-m-c} 8.4, 2 × *p*-Nitrobenzyl *m*-Ar-CH) and 8.09 (2 H, d, *J*_{o-c-m-c} 8.4, 2 × *p*-Nitrobenzyl *o*-Ar-CH); δ_C(75.5 MHz; C²HCl₃) 65.11 (d, *J*_{C-P} 5.4, *p*-Nitrobenzyl-CH₂), 73.06, 74.81 and 75.36 (CH₂Ph), 75.92 (Ins-CH), 75.36 (2 × CH₂Ph), 76.70 (d, *J*_{C-P} 7.5, Ins-CH), 79.81 (d, *J*_{C-P} 4.4, Ins-CH), 80.51, 81.34 and 83.20 (Ins-CH), 123.73 (*p*-Nitrobenzyl *o*-Ar-CH), 127.36, 127.57, 127.63, 127.66,

* DL-2,3,4,5,6-penta-O-benzyl myo-inositol DL-129 was recovered as a side-product from the coupling reaction, indicating that a degree of dephosphorylation had occurred.

127.71, 127.74, 127.80, 127.83, 127.86, 128.07, 128.13, 128.35, 128.37, 128.43, 128.46 and 128.52 (Ar-CH) and 138.00, 138.27, 138.31 and 138.53 (Ar-C quaternary); δ_p (121.5 MHz; C^2HCl_3) 7.72.

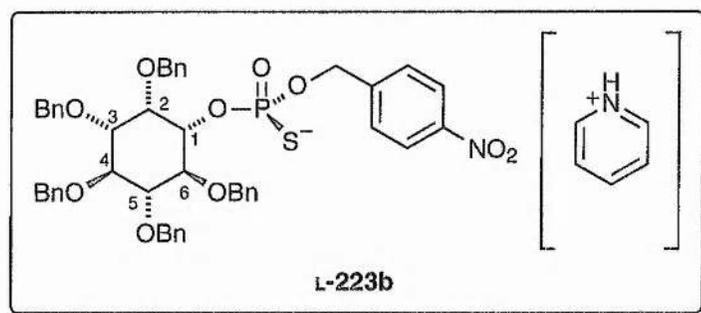
Lower R_f diastereomer L-222b: δ_H (300 MHz; C^2HCl_3) 3.50 (1 H, dd, J 9.6, J 2.4, 3-CH), 3.55 (1 H, t, J 9.3, Ins-CH), 4.09 (2 H, 2 x t, J 9.6, 2 x Ins-CH), 4.13 (1 H, t, J 2.4, 2-CH), 4.36 (1 H, dt, J 10.2, J 2.4, 1-CH), 4.63–5.29 (12 H, m, 5 x CH_2Ph and 1 x p -Nitrobenzyl CH_2), 6.87 (1 H, d, J_{P-H} 728, P-H), 7.23–7.34 (25 H, m, Benzyl Ar-CH), 7.39 (2 H, d, $J_{o-C-m-C}$ 8.4, 2 x p -Nitrobenzyl m -Ar-CH) and 8.09 (2 H, d, $J_{o-C-m-C}$ 9.0, 2 x p -Nitrobenzyl o -Ar-CH); δ_p (121.5 MHz; C^2HCl_3) 9.78.

L-(R_p)-2,3,4,5,6-Penta- O -benzyl *myo*-inositol 1-(O - p -nitrobenzyl) phosphorothioate, pyridinium salt L-223a¹⁹¹



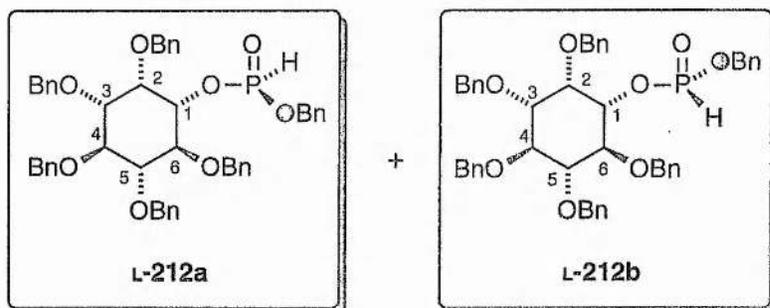
The separated higher R_f diastereomer L-222a (021 mg, 0.025 mmol) was stereospecifically sulfurised using the methodology described for the benzyl-racemate DL-220a. The crude residue was dissolved in dichloromethane, filtered to remove the excess sulfur, and the solvents were removed under reduced pressure to yield the crude pyridinium salt L-223a as a clear yellow oil which failed to recrystallise (19 mg, 82%); δ_p (121.4 MHz; C^2HCl_3) 60.01.

L-(S_p)-2,3,4,5,6-Penta-O-benzyl myo-inositol 1-(O-p-nitrobenzyl) phosphorothioate, pyridinium salt L-223b¹⁹¹



The separated lower R_f diastereomer **L-222b** (29 mg, 0.035 mmol) was stereospecifically sulfurised using the methodology described for the benzyl-racemate **DL-220b**. The crude residue was dissolved in dichloromethane, filtered to remove the excess sulfur, and solvents were removed under reduced pressure to yield the crude pyridinium salt **L-223b** as a clear yellow oil (28 mg, 85%). The residue was further purified by column chromatography on Florisil® (2-10% methanol/dichloromethane, short column in 2% steps) to yield the thiophosphoric acid of **L-223b** as a clear oil [δ_p (121.4 MHz; C²HCl₃) 55.90]. The thiophosphoric acid was then redissolved in a mixture of methanol and dichloromethane (3 cm³, 5:1) and after the addition of pyridine (30 mm³, 0.37 mmol) the solution was left to stand at 0 °C. The reformed pyridinium salt **L-223b** failed to recrystallise and the solvents and excess pyridine were removed under reduced pressure; δ_p (121.4 MHz; C²HCl₃) 59.91; m/z (ES⁺) 901 (11%, [M + H + K]⁺), 885 (3, [M + H + Na]⁺), 863 (1%, [M + H]⁺), 862 (1, M⁺), 212 (100) and 186 (47).

L-(*S_p*)-2,3,4,5,6-Penta-*O*-benzyl *myo*-inositol 1-(*O*-[¹⁸O]-benzyl) hydrogen phosphonate L-212a and L-(*R_p*)-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-(*O*-[¹⁸O]-benzyl) hydrogen phosphonate L-212b¹⁹¹



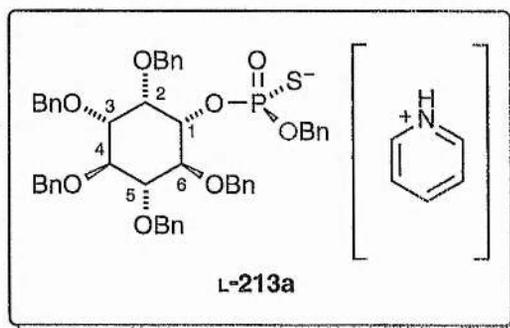
The separated enantiomer of the triethylammonium salt of L-2,3,4,5,6-penta-*O*-benzyl *myo*-inositol 1-hydrogen phosphonate L-214 (0.57 g, 0.7 mmol) was coupled to [¹⁸O]-benzyl alcohol 231 (0.15 cm³, 1.4 mmol) using the pivaloyl coupling methodology described for the racemic unlabelled compound DL-216 (see page 213). The crude diastereomeric mixture L-212a and L-212b (ratio of diastereomers 1:1.8, as estimated ³¹P-NMR spectrometry), was purified as previously by silica column chromatography (0–35% ethyl acetate/petroleum ether, short column in 5% steps) to remove excess [¹⁸O]-benzyl alcohol and pivaloyl chloride. Mass-spectral analysis of the recovered [¹⁸O]-benzyl alcohol revealed no discernable loss of isotopic purity, permitting the use of the [¹⁸O]-benzyl alcohol in further labelling reactions. The resulting pair of diastereomers was then separated by silica column chromatography (0–10% ethyl acetate/dichloromethane, in 2% steps) to give the desired [¹⁸O]-labelled *H*-phosphonates L-212a and L-212b. Both diastereomers were recovered as clear oils which resisted all attempts at recrystallisation. (L-212a 0.17 g, 30%; L-212b 0.30 g, 53%).

Higher *R_f* diastereomer L-212a: Selected data: (Found: C, 73.05; H, 6.6. C₄₈H₄₉O₇¹⁸OP requires: C, 73.25; H, 6.3%) [α]_D²⁵ –6.0 (*c* 0.5 in CHCl₃); δ_p(121.5 MHz; C²HCl₃) 8.07; *m/z* (FAB⁺) 810 (3%, [M + H + Na]⁺), 181 (34, [Inositol + H]⁺) and 107 (100, OCH₂Ph⁺); *m/z* (ES⁺) 826 (4%, [M + H + K]⁺), 810 (3, [M + H + Na]⁺), 788 (17, [M + 2H]⁺), 752 (6), 212 (9), 196 (18), 182 (23, [Inositol + 2H]⁺) and

91 (100, PhCH_2^+); $R_f = 0.21$ (30% ethyl acetate/petroleum ether). All other spectroscopic data was identical to that of the racemic compound **DL-216a**.

Lower R_f diastereomer L-212b: Selected data: (Found: C, 72.1; H, 6.5. $\text{C}_{48}\text{H}_{49}\text{O}_7^{18}\text{OP} \cdot \frac{1}{2}\text{H}_2\text{O}$ requires: C, 72.45; H, 6.2%) (HRMS: found, $[\text{M} + \text{H}]^+$, 787.3311. Calc. for $\text{C}_{48}\text{H}_{50}\text{O}_7^{18}\text{OP}$: 787.3286); δ_p (121.5 MHz; C^2HCl_3) 9.59; m/z (FAB $^+$) 809 (5%, $[\text{M} + \text{Na}]^+$), 787 (6, $[\text{M} + \text{H}]^+$), 355 (2, $[\text{M} - \text{P}(\text{O})(\text{H})^{18}\text{OCH}_2\text{Ph} - 2\text{CH}_2\text{Ph} - \text{CH}_3\text{Ph}]^+$), 271 (9, $[\text{Inositol} + \text{CH}_2\text{Ph}]^+$), 181 (100, $[\text{Inositol} + \text{H}]^+$), 107 (21, PhCH_2^+) and 105 (23); m/z (ES $^+$) 826 (6%, $[\text{M} + \text{H} + \text{K}]^+$), 810 (7, $[\text{M} + \text{H} + \text{Na}]^+$), 788 (25, $[\text{M} + 2\text{H}]^+$), 752 (1), 212 (21), 196 (16), 182 (33, $[\text{Inositol} + 2\text{H}]^+$) and 91 (100, PhCH_2^+); $R_f = 0.16$ (30% ethyl acetate/petroleum ether). All other spectroscopic data was identical to that of the unlabelled racemic compound **DL-216b**.

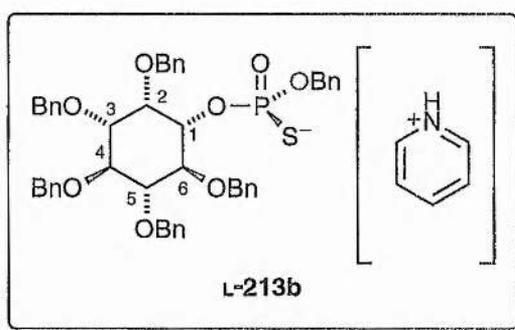
L-(R_p)-2,3,4,5,6-Penta-O-benzyl *myo*-inositol 1(O-[^{18}O]-benzyl)-phosphorothioate, pyridinium salt L-213a¹⁹¹



The separated higher R_f diastereomer of the [^{18}O]-benzyl hydrogen phosphonate **L-212a** (0.14 g, 0.18 mmol) was stereospecifically sulfurised using the methodology described for the unlabelled racemate **DL-220a**. The crude residue was dissolved in dichloromethane, filtered to remove the excess sulfur, and the solvents were removed under reduced pressure to yield the pyridinium salt **L-213a** as a clear oil. The residue was further purified by column chromatography on Florisil[®] (2-10% methanol/dichloromethane, short column in 2% steps) to yield the thiophosphoric acid of **L-213a** as a clear oil (0.11 g, 78%) [δ_p (121.5 MHz; C^2HCl_3) 54.60]. For pyridinium

L-213a as a clear oil (0.11 g, 78%) [δ_p (121.5 MHz; C^2HCl_3) 54.60]. For pyridinium salt **L-213a**; δ_p (121.5 MHz; C^2HCl_3) 61.07; m/z (FAB $^+$) * 841 (1%, [M + H + Na] $^+$), 322 (100), 181 (16, [Inositol + H] $^+$), 147 (37) and 109 (39, PhCH $_2^{18}O^+$) and 107 (32, PhCH $_2O^+$); m/z (ES $^+$) * 857 (4%, [M + H + K] $^+$), 841 (45, [M + H + Na] $^+$), 836 (5, [M + H + NH $_4$] $^+$), 819 (22, [M + 2H] $^+$), 204 (33, [Inositol + H + Na] $^+$), 181 (10, [Inositol + H] $^+$) and 101 (100, [C $_5$ H $_5$ N - H + Na] $^+$). All other spectroscopic data was identical to that of the unlabelled racemic compound **DL-220a**.

L-(S $_p$)-2,3,4,5,6-Penta-O-benzyl myo-inositol 1-(O-[^{18}O]-benzyl)-phosphorothioate, pyridinium salt L-213b 191

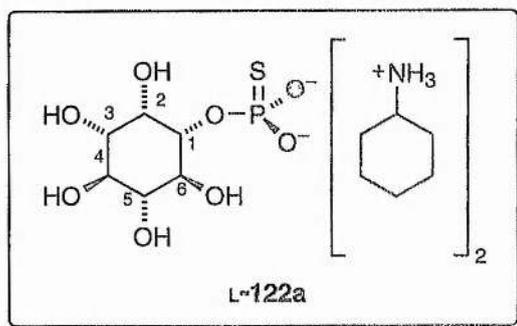


The separated lower R_f diastereomer of the [^{18}O]-benzyl hydrogen phosphonate **L-212b** (0.15 g, 0.19 mmol) was stereospecifically sulfurised using the methodology described for the unlabelled racemate **DL-220b**. The crude residue was dissolved in dichloromethane, filtered to remove the excess sulfur, and the solvents were removed under reduced pressure to yield the pyridinium salt **L-213b**. The residue was further purified by column chromatography on Florisil $^{\circledR}$ (2-10% methanol/dichloromethane, short column in 2% steps) to yield the thiophosphoric acid of **L-213b** as a clear oil (0.11 g, 74%) [δ_p (121.5 MHz; C^2HCl_3) 53.91]. For pyridinium salt **L-213b**; δ_p (121.5 MHz; C^2HCl_3) 59.60; m/z (ES $^+$) * 857 (1%, [M + H + K] $^+$), 841 (2, [M + H + Na] $^+$), 836 (3, [M + H + NH $_4$] $^+$), 819 (14, [M + 2H] $^+$), 181 (7, [Inositol + H] $^+$) and 101 (100, [C $_5$ H $_5$ N -

* M is the molecular weight of the phosphorothioate anion.

H + Na]⁺). All other spectroscopic data was identical to that of the unlabelled racemic compound DL-220b.

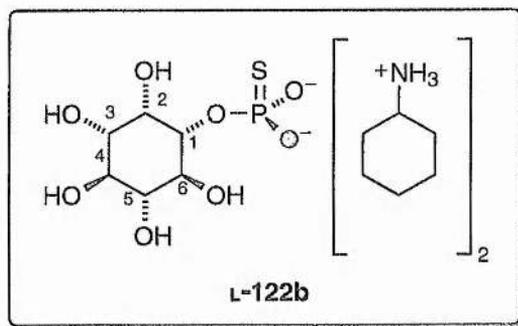
L-(R_P)-myo-Inositol 1-[¹⁸O]phosphorothioate, bis-cyclohexylammonium salt L-122a



The diester L-213a (100 mg, 120 nmol) was fully deprotected using the identical procedure to the unlabelled racemate DL-220a (page 218) and the sample was lyophilised to give phosphorothioate L-122a as an off-white solid (28 mg, 48%), δ_{H} (300 MHz; ²H₂O) 1.01–1.83 (20 H, m, Cyclohexyl-CH₂), 2.91–3.01 (2 H, m, Cyclohexyl-CH), 3.23 (1 H, t, $J_{4,5}$ 8.7, $J_{5,6}$ 9.3, 5-CH), 3.46 (1 H, dd, $J_{2,3}$ 2.4, $J_{3,4}$ 9.6, 3-CH), 3.49 (1 H, t, $J_{3,4}$ 9.6, $J_{4,5}$ 8.7, 4-CH), 3.64 (1 H, t, $J_{5,6}$ 9.3, $J_{6,1}$ 9.6, 6-CH), 3.97 (1 H, dt, $J_{6,1}$ 9.6, $J_{1,2}$ 2.4, $J_{1,P}$ 10.2, 1-CH) and 4.17 (1 H, t, $J_{1,2}$ 2.4, $J_{2,3}$ 2.4, 2-CH); δ_{C} (75.4 MHz; ²H₂O) 23.80, 24.31 and 30.70 (Cyclohexyl-CH₂), 50.23 (Cyclohexyl-CH), 70.84 (Ins-CH), 71.53 (d, $J_{\text{C-P}}$ 3.2, Ins-CH), 72.09 (d, $J_{\text{C-P}}$ 4.3, Ins-CH), 72.30 (Ins-CH), 74.44 (Ins-CH) and 74.76 (d, $J_{\text{C-P}}$ 5.4, Ins-CH); δ_{P} (121.5 MHz, ²H₂O) 44.53; m/z (ES⁺)^{*} 479 (1%, [M + 3H + 2C₆H₅NH₃]⁺), 401 (1, [M + 2H + C₆H₅NH₃ + Na]⁺), 378 (2, [M + 2H + C₆H₅NH₃]⁺), 323 (1, [M + H + 2Na]⁺), 302 (4, [M + 3H + Na]⁺), 278 (1, [M + 2H]⁺) and 100 (100, C₆H₅NH₃⁺).

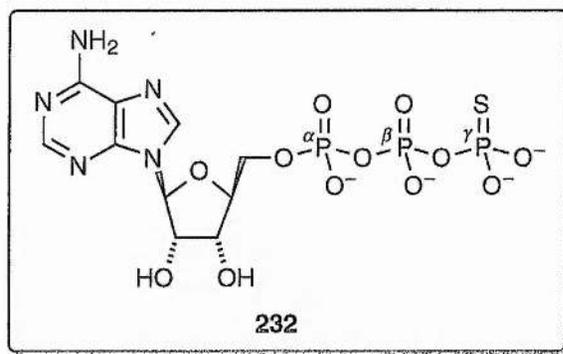
^{*} M is the molecular weight of the phosphorothioate dianion.

L-(S_p)-myo-inositol 1-[¹⁸O]phosphorothioate, bis-cyclohexylammonium salt L-122b

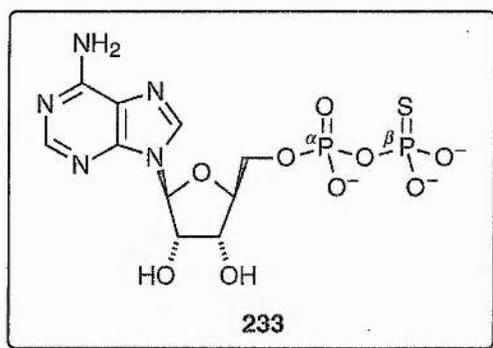


The diester **L-213b** (100 mg, 120 nmol) was fully deprotected using the identical procedure to the unlabelled racemate **DL-220b** (page 218) and cyclohexylamine to form the bis-cyclohexylammonium salt **L-213b**. The sample was lyophilised to give phosphorothioate **L-213b** as an off-white solid (36 mg, 62%); δ_{H} (300 MHz; ²H₂O) 0.98–1.83 (20 H, m, Cyclohexyl-CH₂), 2.90–3.02 (2 H, m, Cyclohexyl-CH), 3.17 (1 H, t, *J* 8.7, 5-CH), 3.38–3.48 (2 H, m, 3-CH and 4-CH), 3.58 (1 H, t, *J* 9.3, 6-CH), 3.92 (1 H, dt, 1-CH) and 4.11 (1 H, t, 2-CH); δ_{P} (121.5 MHz, ²H₂O) 44.46; *m/z* (ES⁺)^{*} 479 (1%, [M + 3H + 2C₆H₅NH₃]⁺), 478 (1, [M + 2H + 2C₆H₅NH₃]⁺), 401 (2, [M + 2H + C₆H₅NH₃ + Na]⁺), 378 (3, [M + 2H + C₆H₅NH₃]⁺), 323 (2, [M + H + 2Na]⁺), 301 (4, [M + 2H + Na]⁺) and 100 (100, C₆H₅NH₃⁺).

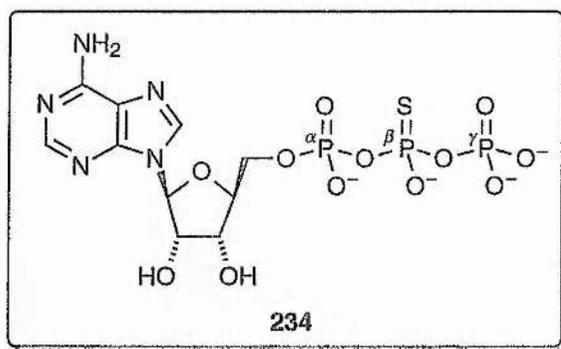
^{*} M is the molecular weight of the phosphorothioate dianion.

Adenosine 5'- γ -thiotriphosphate **232**^{150, 153}

The title compound was synthesised by a modification of the method of Webb and Trentham.¹⁵⁰ ADP (58.5 mg, 120 μmol), NAD^+ (1.3 mg, 2 μmol), magnesium chloride hexahydrate (13.0 mg, 64 μmol), DTE (12.3 mg, 80 μmol), D-fructose 1,6-diphosphate (81.5 mg, 160 μmol), sodium pyruvate (44.0 mg, 400 μmol), and sodium thiophosphate dodecahydrate (15.8 mg, 40 μmol) were dissolved in water (4 cm^3) and the solution adjusted to pH 8.0 by the addition of Tris-base and warmed to 30 $^\circ\text{C}$. To this solution was added the following enzyme mixture; glyceraldehyde phosphate dehydrogenase (2000 units), 3-phosphoglycerate kinase (2000 units), aldolase (8 units), triosephosphate isomerase (200 units) and lactate dehydrogenase (200 units), which had been previously dialysed against 20 mmol dm^{-3} Tris, 1.0 mmol dm^{-3} DTE, 1.0 mmol dm^{-3} EDTA, pH 8.0. The mixture was incubated at 30 $^\circ\text{C}$ for 5 h, after which ^{31}P -NMR showed that most of the inorganic phosphorothioate was present as $\text{ATP}_\gamma\text{S}$. The reaction mixture was quickly cooled to 0-2 $^\circ\text{C}$ and applied directly to a column of DEAE-Sephadex A-25 and eluted with the triethylammonium bicarbonate gradient (see page 176). Product containing fractions were combined and solvent was removed under reduced pressure (<35 $^\circ\text{C}$ waterbath) to give the phosphorothioate **232** as a clear glass (10.5 mg, 17%), δ_{p} (121.4 MHz, $^2\text{H}_2\text{O}$) -22.78 (dd, $J_{\alpha\beta}$ 19.4, $J_{\beta\gamma}$ 29.1, P_β), -10.73 (d, $J_{\alpha\beta}$ 19.4, P_α) and 33.81 (d, $J_{\beta\gamma}$ 29.1, P_γ).

Adenosine 5'- β -thiodiphosphate **233**¹⁵³

The purified ATP γ S **232** from the previous incubation (10.5 mg, 20 μ mol) (page 238) was dissolved in buffer (5 cm³, 0.1 mol dm⁻³ HEPES, 50 mmol dm⁻³ KCl, 25 mmol dm⁻³ MgCl₂·6H₂O, 1.0 mmol dm⁻³ DTE, 1.0 mmol dm⁻³ EDTA, pH 7.5) and AMP (116 mg, 300 μ mol) and adenylate kinase (myokinase) (2000 units) were added. The reaction mixture was incubated at 30 °C and formation of ADP β S monitored by ³¹P-NMR. After 3 h formation of ADP β S was judged complete and the reaction mixture was quickly cooled to 0-2 °C and applied directly to a column of DEAE-Sephadex A-25 and eluted with the triethylammonium bicarbonate gradient (see page 176). Product containing fractions were combined and the solvent was removed under reduced pressure (<35 °C waterbath) to give the phosphorothioate **233** as a clear glass (6.6 mg, 74%), δ_p (121.4 MHz, ²H₂O) -11.18 (d, $J_{\alpha\beta}$ 32.2, P_α) and 33.56 (d, $J_{\alpha\beta}$ 32.2, P_β).

Adenosine 5'- β -thiotriphosphate 234¹⁵⁰

The purified ADP β S 233 from the previous incubation (6.6 mg, 15 μ mol) (page 239) was phosphorylated to ATP β S in a reaction catalysed by phosphoglycerate kinase. The ADP β S was dissolved in water (4 cm³) and NAD⁺ (1.3 mg, 2 μ mol), magnesium chloride hexahydrate (13.0 mg, 64 μ mol), DTE (12.3 mg, 80 μ mol), D-fructose 1,6-diphosphate (81.5 mg, 160 μ mol), sodium pyruvate (44.0 mg, 400 μ mol), and potassium dihydrogen phosphate (54.0 mg, 400 μ mol) were added. The solution was adjusted to pH 8.0 by the addition of Tris-base and warmed to 30 °C. The following enzyme mixture was added; glyceraldehyde phosphate dehydrogenase (2000 units), 3-phosphoglycerate kinase (2000 units), aldolase (8 units), triosephosphate isomerase (200 units) and lactate dehydrogenase (200 units), which had been previously dialysed against 20 mmol dm⁻³ Tris, 1.0 mmol dm⁻³ DTE, 1.0 mmol dm⁻³ EDTA, pH 8.0. The reaction mixture was then incubated at 30 °C for 8 h to compensate for the poorer substrate activity of ADP β S *cf.* ADP. The reaction mixture was quickly cooled to 0-2 °C and applied directly to a column of DEAE-Sephadex A-25 and eluted with the triethylammonium bicarbonate gradient (see page 176). Product containing fractions were combined and the solvent was removed under reduced pressure (<35 °C waterbath) to give the phosphorothioate 234 as a clear glass (3.4 mg, 44%), δ_p (121.4 MHz, ²H₂O) -12.23 (d, $J_{\alpha\beta}$ 27.9, P $_{\alpha}$), -6.66 (d, $J_{\beta\gamma}$ 28.2, P $_{\gamma}$) and 27.85 (t, $J_{\alpha\beta}$ 27.9, $J_{\beta\gamma}$ 28.2, P $_{\beta}$).

Purification of Bovine Brain *myo*-Inositol Monophosphatase (ex. *E. Coli*)

Bovine brain *myo*-inositol monophosphatase was purified from a recombinant strain of *Escherichia coli* BL21-DE3⁴¹ using a procedure based on the method of Leech *et al.*⁴³

All the protein purification steps were carried out at 0–4 °C (except the h.p.l.c. step which was conducted at room temperature). All materials used were of AR quality.

Centrifugations were carried out on a CENTRIKON C-124 centrifuge. Pooled fractions from various steps (see below) were concentrated by ultrafiltration using an AMICON apparatus (43 or 76 mm diameter), through a YM 10 or a YM 30 membrane, using N₂.

Step 1: Culture Growth and Harvesting. Ampicillin (20 mg l⁻¹) was added to four sterilised 500 cm³ portions of Luria-Bertani culture broth²²⁹ in 2000 cm³ shake flasks. The media were inoculated with the recombinant *E. Coli* and were incubated at 37 °C in an orbital shaker at 70 revolutions min⁻¹. After approximately 12 h (when A₆₀₀ was ≈0.9) isopropyl-thio-β-D-galactoside (IPTG) inducer was added (110 mg l⁻¹, 86% pure) and the cultures were incubated for a further 4 h at the faster shaking rate of 110 revolutions min⁻¹. The cells were cooled on ice to terminate growth, and the cells were then harvested by centrifugation (5500 × g, 4 °C, 20 min).

Step 2: Cell Lysis and Ammonium Sulfate Fractionation. The cell paste pellets were washed and then resuspended in 100 cm³ buffer A (25 mmol dm⁻³ Tris.HCl, pH 7.5, 20 °C, 0.5 mmol dm⁻³ EGTA) containing the serine protease inhibitor phenylmethylsulfonyl fluoride (175 mg l⁻¹). Lysozyme (20 mg) was added, the cells were digested at 4 °C for 30 min, and then sonicated on ice. The cell debris was removed by centrifugation (12000 × g, 4 °C, 30 min) and the supernatant was fractionated at 4 °C with ammonium sulfate at 40% and 60% of saturation, removing the precipitated protein by centrifugation at 21000 × g for 30 min. The 40–60% fraction pellet was dissolved in buffer B (50 mmol dm⁻³ Tris/HCl, pH 8.0, 300 cm³) at 4 °C and then dialysed against the same buffer (2 × 8000 cm³) for 20 h to give a final volume of 370 cm³.

Step 3: *DEAE-Cellulose Anion Exchange Chromatography.* The dialysed protein was applied onto a Whatman-DEAE-cellulose (DE-52) anion-exchange resin (250 x 26 mm) which had been previously equilibrated with buffer B at 4 °C. The column was washed with six column volumes of buffer B, and then developed with a three column volume linear gradient of KCl (0–200 mmol dm⁻³) in buffer B, at a flow rate of 50 cm³ h⁻¹. Fractions containing Li⁺ inhibitable activity (eluting at ≈100 mmol dm⁻³ KCl) were pooled (300 cm³) and were concentrated to ca. 20 cm³ by ultrafiltration using an Amicon YM 30 (30-kDa cutoff) membrane.

Step 4: *Sephadex G 100 Gel Exclusion Chromatography.* The concentrated protein solution was subjected to gel filtration on a column of Sephadex G 100 (Pharmacia, 950 x 26 mm) which had been pre-equilibrated in buffer C (150 mmol dm⁻³ KCl, 50 mmol dm⁻³ Tris/HCl, pH 8.0, 0.1 mmol dm⁻³ EGTA) at a flow rate of 20 cm³ h⁻¹ at 4 °C. Upon elution, the pooled active fractions were concentrated by ultrafiltration to ca. 5 cm³ using an Amicon YM 30 membrane. The protein solution was stored at -78 °C in this form.

The column was repacked every time before use to achieve the maximum flow rate (20 cm³ h⁻¹).

Step 5: *Poros 20 HQ Anion Exchange HPLC* The concentrated protein solution was desalted by several cycles of dilution with 20 cm³ buffer D (80 mmol dm⁻³ KCl, 50 mmol dm⁻³ Tris/HCl, pH 8.5) and concentration by ultrafiltration at 4 °C. It was then applied to a Poros 20 HQ anion exchange column (quaternized polyethyleneimine, 100 mm x 46 mm, 2.0 cm³ min⁻¹, 20 °C) on a Biocad Sprint™ Perfusion Chromatography® System which had been previously equilibrated with buffer E (50 mmol dm⁻³ Tris/HCl, pH 8.5). The column was washed with buffer E (5 cm³), then developed using a linear gradient of 0–200 mmol dm⁻³ KCl in buffer E (13 cm³), followed by a linear gradient of 200–400 mmol dm⁻³ KCl in buffer E (40 cm³). Fractions

containing the active enzyme eluted at 235–255 mmol dm⁻³ KCl. The active fractions were pooled and concentrated to a volume of *ca.* 5 cm³ by ultrafiltration using as Amicon YM 10 membrane, and stored at -78 °C until required.

To determine the activity of the enzyme in column eluates and confirm that the observed phosphatase activity was due solely to inositol monophosphatase, several 70 mm³ samples of the protein solution were each added to 2'AMP (100 mmol dm⁻³, 30 mm³) in assay buffer (500 mmol dm⁻³ KCl, 6 mmol dm⁻³ MgCl₂, 100 mmol dm⁻³ Tris/HCl, pH 7.8, 200 mm³) with or without 150 mmol dm⁻³ LiCl. Each sample was incubated at 37 °C and the reactions were quenched after 15 min by the addition of the acidic Malachite Green colorimetric assay reagent (2.5 cm³) (see page 246). The colour was allowed to develop for 30 min when A_{660} was determined. Comparison of the absorbance for the quenched samples allowed the purified inositol monophosphatase eluates to be identified.

The accurate analysis of inositol monophosphatase activity in the solutions of purified enzyme was carried out using the colorimetric assay of inorganic phosphate developed by Itaya *et al.*²³⁰ The procedure for inositol monophosphatase is described in detail on page 246.

Protein Concentration Assay: Protein concentrations were determined by the method of Lowry *et al.*²³¹ Appropriately diluted samples of the protein solution were mixed with Coomassie blue dye reagent (100 mg of Coomassie brilliant blue dissolved in 50 cm³ of 95% ethanol to which 100 cm³ of 85 % of phosphoric acid has been added and the whole diluted to 1 l with water). After 5 min the absorbance was measured at 595 nm. Standard curves were prepared by using bovine serum albumin.

Discontinuous SDS Polyacrylamide Gel Electrophoresis: Purification of inositol monophosphatase was judged for homogeneity by SDS-PAGE using the methodology of Laemmli²³² on a discontinuous medium (10% acrylamide resolving gel, 80 x 100 mm, 0.75 mm thick) using Sigma molecular weight marker kit 'Dalton Mark VII' (bovine albumin, 66,000; egg albumin, 45,000; glyceraldehyde 3-phosphate

dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen inhibitor, 24,000; trypsinogen inhibitor, 20,100; and α -lactalbumin, 14,200) as standards. A Hoefer SE 200 Series "Mighty-Small Multi-Vertical" unit was used.

Determination of the Activity of Purified Inositol Monophosphatase²³⁰

Colorimetric Assay Reagent. Malachite green (1.5 g) was dissolved in hydrochloric acid (25 cm³, 5.0 mol dm⁻³) and diluted with water (750 cm³). To this solution was added ammonium molybdate (10.5 g) in hydrochloric acid (225 cm³, 5.0 mol dm⁻³), and the solution stirred at room temperature for 10 min. The solution was filtered by gravity, and stored in the dark for periods of upto one month.

Assay buffer. 200 mmol dm⁻³ KCl, 2 mmol dm⁻³ MgCl₂·6H₂O, 50 mmol dm⁻³ Tris·Base, pH 7.8.

Assay. Incubation samples comprised of the following:

- assay buffer A 240 mm³
- substrate* in assay buffer A 30 mm³
- enzyme solution 30 mm³

The assay samples were incubated at 37 °C in triplicate and the reaction quenched by the addition of colorimetric assay reagent (2.0 cm³) at the required time (relative to the addition of the enzyme solution). The colour was allowed to develop over a period of approximately 30 min, and measured for optical absorption at 660 nm. In addition, known concentrations of inorganic phosphate (potassium phosphate) 0-200 μmol dm⁻³ were prepared in triplicate, and treated with the colorimetric assay reagent (2.0 cm³) to give a standard phosphate concentration curve from which accurate concentrations of product phosphate could be calculated.

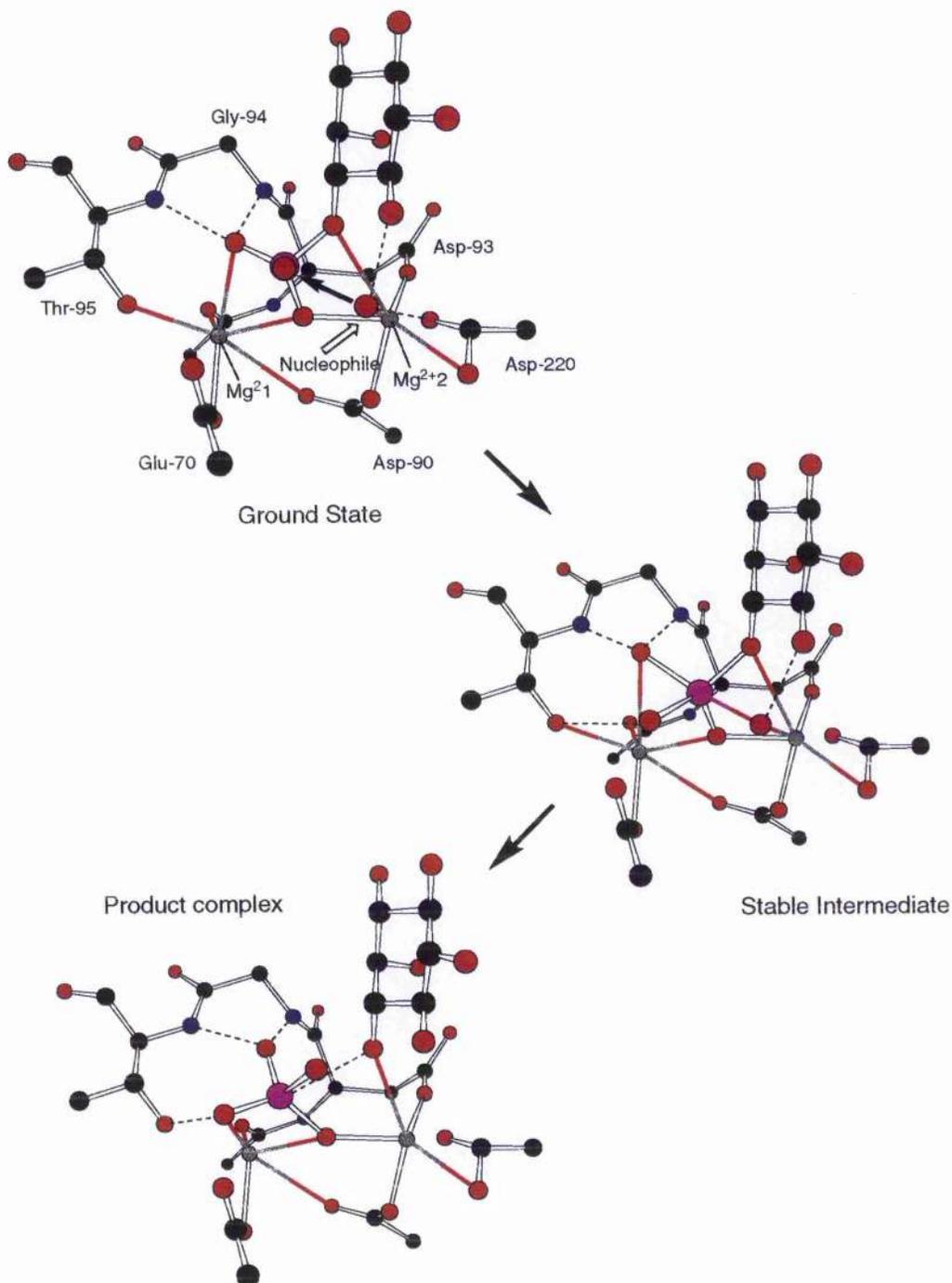
* Either synthesised DL-inositol 1-phosphate or adenosine 2'-monophosphate was used, and the enzyme activity calculated accordingly. The standard substrate concentration used was 100 mmol dm⁻³.

CHAPTER FOUR

APPENDICES

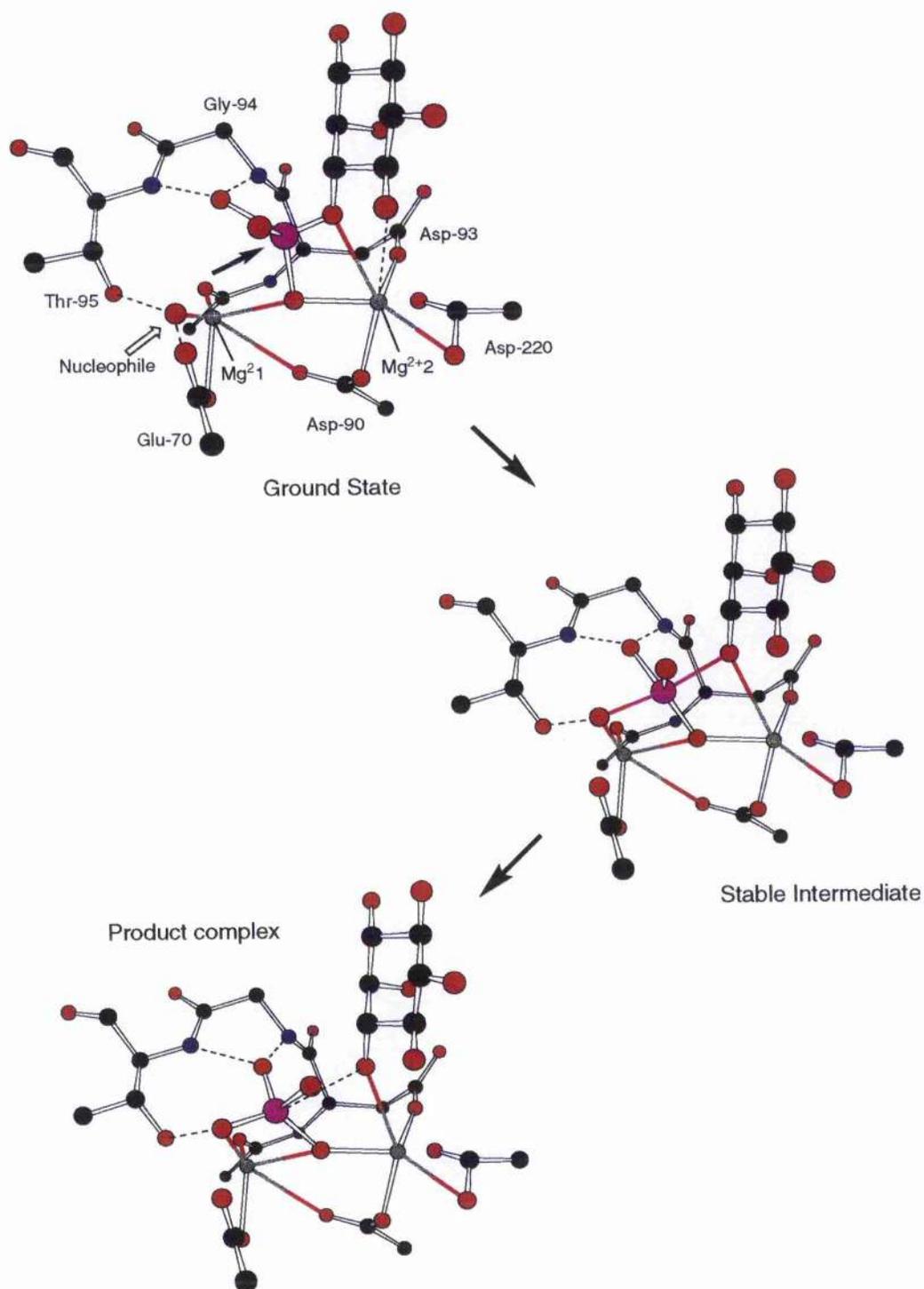
4. Appendices

4.1 Appendix 1: Proposed Adjacent Displacement Mechanism for IMPase



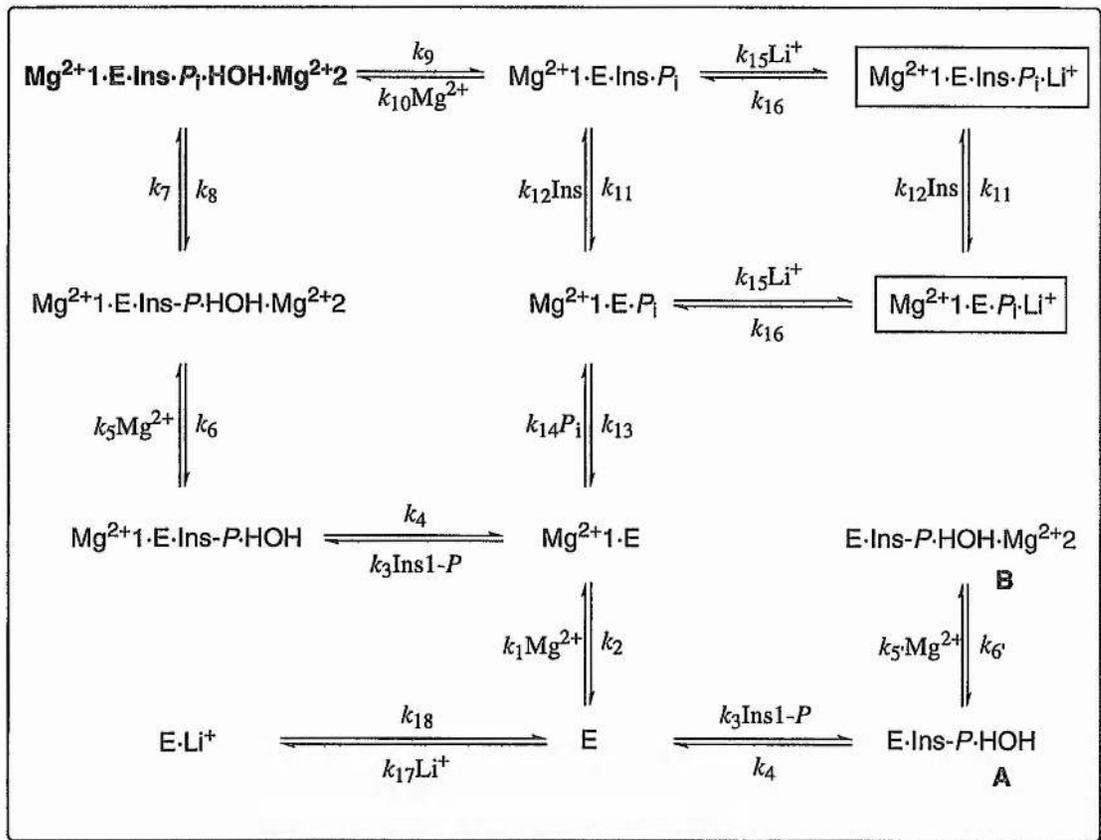
Scheme 4.1: Proposed adjacent displacement (with pseudorotation) mechanism for inositol monophosphatase [full description Section 1.17, page 33]. One water ligand for Mg^{2+1} is omitted for clarity.

4.2 Appendix 2: Proposed In-line Association Mechanism for IMPase

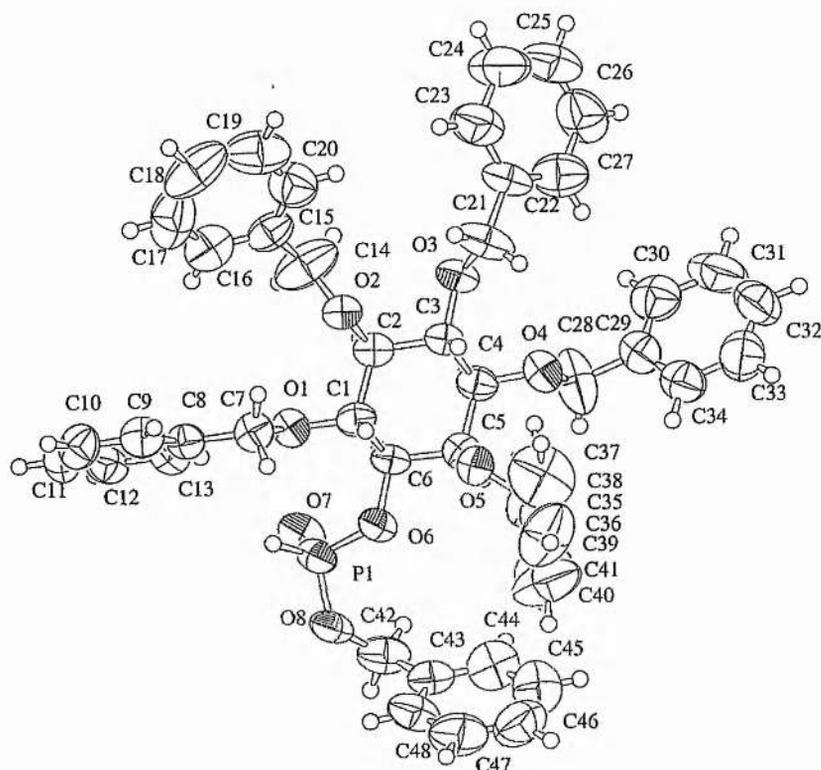


Scheme 4.2: *Alternative in-line displacement mechanism for inositol monophosphatase [full description Section 1.18 on page 34]. One water ligand for Mg²⁺₁ is omitted for clarity.*

4.3 Appendix 3: Full Kinetic Scheme of Inositol Monophosphatase



Scheme 4.3: Full kinetic scheme for catalysis and for Li^+ inhibition. The concentration of the bold complex increases at high Mg^{2+} concentration and causes inhibition. Inositol can probably dissociate from this complex to give $\text{Mg}^{2+}\text{I-E-P}_i\text{-Mg}^{2+2}$. The boxed complexes form only in the presence of Li^+ where Li^+ binds in the site of Mg^{2+2} . The complex E-Li^+ forms only at very high Li^+ concentrations ($\geq 5 \text{ mmol dm}^{-3}$) where Li^+ binds in the site for Mg^{2+1} . The concentration of complexes **A** and **B** become significant for mutant enzymes which possess a low binding affinity for Mg^{2+} at site 1. These dead-end complexes are inactive and reduce the apparent value of k_{cat} although the actual magnitude of the rate constant for the hydrolytic step (k_7) could be identical in the wild-type and mutant enzymes. Steps 7 and 13 are each partially rate-limiting.

4.4 Appendix 4: Crystallographic Data for Compound DL-216b²³³

$C_{48}H_{49}O_8P$, $M = 784.88$, monoclinic, space group $P2_1/a$ (#14), $a = 14.046(10)$, $b = 23.644(7)$, $c = 14.341(9)$ Å, $\beta = 115.78(4)^\circ$, $V = 4288(3)$ Å³, $Z = 4$, $D_c = 1.216$ g cm⁻³, $T = 293$ K. 2808 unique reflections were collected on a Rigaku AFC7S diffractometer employing Mo-K α radiation ($\lambda = 0.71069$ Å) of which 2990 observations [$I > 3.00\sigma(I)$] were used for refinement. Convergence at $R(F) = 8.2\%$, $R_w(F) = 5.6\%$ for 515 variables.

Table 4.1: Bond lengths (Å) for compound DL-216b

| atom | atom | distance | atom | atom | distance |
|------|-------|-----------|------|------|-----------|
| P(1) | O(6) | 1.607(5) | P(1) | O(7) | 1.465(6) |
| P(1) | O(8) | 1.564(6) | O(1) | C(1) | 1.448(9) |
| O(1) | C(7) | 1.433(8) | O(2) | C(2) | 1.428(8) |
| O(2) | C(14) | 1.41(1) | O(3) | C(3) | 1.428(8) |
| O(3) | C(21) | 1.420(10) | O(4) | C(4) | 1.439(9) |
| O(4) | C(28) | 1.400(10) | O(5) | C(5) | 1.405(9) |
| O(5) | C(35) | 1.421(10) | O(6) | C(6) | 1.453(8) |
| O(8) | C(42) | 1.43(1) | C(1) | C(2) | 1.544(10) |
| C(1) | C(6) | 1.496(10) | C(2) | C(3) | 1.54(1) |

| atom | atom | distance | atom | atom | distance |
|-------|-------|-----------|-------|-------|-----------|
| C(3) | C(4) | 1.522(10) | C(4) | C(5) | 1.530(10) |
| C(5) | C(6) | 1.538(10) | C(7) | C(8) | 1.53(1) |
| C(8) | C(9) | 1.37(1) | C(8) | C(13) | 1.36(1) |
| C(9) | C(10) | 1.40(1) | C(10) | C(11) | 1.36(1) |
| C(11) | C(12) | 1.37(1) | C(12) | C(13) | 1.40(1) |
| C(14) | C(15) | 1.49(1) | C(15) | C(16) | 1.37(1) |
| C(15) | C(20) | 1.34(1) | C(16) | C(17) | 1.35(2) |
| C(17) | C(18) | 1.35(2) | C(18) | C(19) | 1.37(2) |
| C(19) | C(20) | 1.38(2) | C(21) | C(22) | 1.50(1) |
| C(22) | C(23) | 1.37(1) | C(22) | C(27) | 1.36(1) |
| C(23) | C(24) | 1.38(2) | C(24) | C(25) | 1.32(2) |
| C(25) | C(26) | 1.36(2) | C(26) | C(27) | 1.39(1) |
| C(28) | C(29) | 1.52(1) | C(29) | C(30) | 1.38(1) |
| C(29) | C(34) | 1.35(1) | C(30) | C(31) | 1.43(2) |
| C(31) | C(32) | 1.34(2) | C(32) | C(33) | 1.34(2) |
| C(33) | C(34) | 1.38(1) | C(35) | C(36) | 1.50(1) |
| C(36) | C(37) | 1.35(1) | C(36) | C(41) | 1.33(1) |
| C(37) | C(38) | 1.36(2) | C(38) | C(39) | 1.31(2) |
| C(39) | C(40) | 1.31(2) | C(40) | C(41) | 1.39(2) |
| C(42) | C(43) | 1.51(1) | C(43) | C(44) | 1.38(1) |
| C(43) | C(48) | 1.37(1) | C(44) | C(45) | 1.37(1) |
| C(45) | C(46) | 1.36(1) | C(46) | C(47) | 1.37(2) |
| C(47) | C(48) | 1.40(1) | | | |
| P(1) | H(49) | 1.21 | C(1) | H(1) | 0.95 |
| C(2) | H(2) | 0.95 | C(3) | H(3) | 0.95 |
| C(4) | H(4) | 0.95 | C(5) | H(5) | 0.95 |
| C(6) | H(6) | 0.95 | C(7) | H(7) | 0.95 |
| C(7) | H(8) | 0.95 | C(9) | H(9) | 0.95 |
| C(10) | H(10) | 0.95 | C(11) | H(11) | 0.95 |
| C(12) | H(12) | 0.95 | C(13) | H(13) | 0.95 |
| C(14) | H(14) | 0.95 | C(14) | H(15) | 0.95 |
| C(16) | H(16) | 0.95 | C(17) | H(17) | 0.95 |
| C(18) | H(18) | 0.95 | C(19) | H(19) | 0.95 |
| C(20) | H(20) | 0.95 | C(21) | H(21) | 0.95 |
| C(21) | H(22) | 0.95 | C(23) | H(23) | 0.95 |
| C(24) | H(24) | 0.95 | C(25) | H(25) | 0.95 |
| C(26) | H(26) | 0.95 | C(27) | H(27) | 0.95 |
| C(28) | H(28) | 0.95 | C(28) | H(29) | 0.95 |

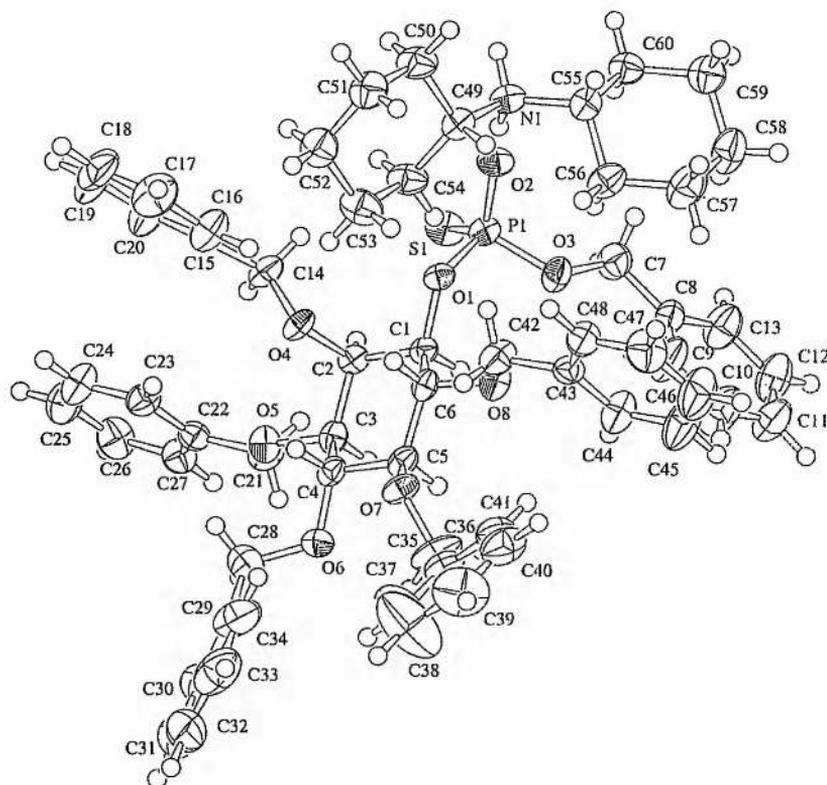
| atom | atom | distance | atom | atom | distance |
|-------|-------|----------|-------|-------|----------|
| C(30) | H(30) | 0.95 | C(31) | H(31) | 0.95 |
| C(32) | H(32) | 0.95 | C(33) | H(33) | 0.95 |
| C(34) | H(34) | 0.95 | C(35) | H(35) | 0.95 |
| C(35) | H(36) | 0.95 | C(37) | H(37) | 0.95 |
| C(38) | H(38) | 0.95 | C(39) | H(39) | 0.95 |
| C(40) | H(40) | 0.95 | C(41) | H(41) | 0.95 |
| C(42) | H(42) | 0.95 | C(42) | H(43) | 0.95 |
| C(44) | H(44) | 0.95 | C(45) | H(45) | 0.95 |
| C(46) | H(46) | 0.95 | C(47) | H(47) | 0.95 |
| C(48) | H(48) | 0.95 | | | |

Table 4.2: Bond Angles ($^{\circ}$) for compound DL-216b

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|----------|-------|-------|-------|----------|
| O(6) | P(1) | O(7) | 114.6(3) | O(6) | P(1) | O(8) | 101.3(3) |
| O(7) | P(1) | O(8) | 118.2(4) | C(1) | O(1) | C(7) | 112.1(6) |
| C(2) | O(2) | C(14) | 112.7(6) | C(3) | O(3) | C(21) | 112.5(6) |
| C(4) | O(4) | C(28) | 112.8(6) | C(5) | O(5) | C(35) | 114.7(6) |
| P(1) | O(6) | C(6) | 119.8(5) | P(1) | O(8) | C(42) | 120.7(6) |
| O(1) | C(1) | C(2) | 108.4(6) | O(1) | C(1) | C(6) | 106.1(6) |
| C(2) | C(1) | C(6) | 108.4(7) | O(2) | C(2) | C(1) | 107.7(6) |
| O(2) | C(2) | C(3) | 109.8(6) | C(1) | C(2) | C(3) | 111.2(7) |
| O(3) | C(3) | C(2) | 108.1(6) | O(3) | C(3) | C(4) | 109.2(6) |
| C(2) | C(3) | C(4) | 109.0(7) | O(4) | C(4) | C(3) | 106.8(6) |
| O(4) | C(4) | C(5) | 111.0(7) | C(3) | C(4) | C(5) | 110.1(7) |
| O(5) | C(5) | C(4) | 111.7(7) | O(5) | C(5) | C(6) | 107.1(6) |
| C(4) | C(5) | C(6) | 107.0(7) | O(6) | C(6) | C(1) | 110.8(6) |
| O(6) | C(6) | C(5) | 106.3(6) | C(1) | C(6) | C(5) | 110.6(7) |
| O(1) | C(7) | C(8) | 107.9(6) | C(7) | C(8) | C(9) | 116.6(8) |
| C(7) | C(8) | C(13) | 121.7(8) | C(9) | C(8) | C(13) | 121.7(8) |
| C(8) | C(9) | C(10) | 119.0(8) | C(9) | C(10) | C(11) | 120.3(9) |
| C(10) | C(11) | C(12) | 120.1(8) | C(11) | C(12) | C(13) | 120.3(8) |
| C(8) | C(13) | C(12) | 118.7(8) | O(2) | C(14) | C(15) | 109.5(8) |
| C(14) | C(15) | C(16) | 117(1) | C(14) | C(15) | C(20) | 124(1) |
| C(16) | C(15) | C(20) | 118(1) | C(15) | C(16) | C(17) | 120(1) |
| C(18) | C(19) | C(20) | 117(1) | C(15) | C(20) | C(19) | 122(1) |
| O(3) | C(21) | C(22) | 109.3(8) | C(21) | C(22) | C(23) | 120(1) |
| C(21) | C(22) | C(27) | 120(1) | C(23) | C(22) | C(27) | 118(1) |

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|-----------|-------|-------|-------|----------|
| C(16) | C(17) | C(18) | 120(1) | C(17) | C(18) | C(19) | 120(1) |
| C(22) | C(23) | C(24) | 121(1) | C(23) | C(24) | C(25) | 119(1) |
| C(24) | C(25) | C(26) | 120(1) | C(25) | C(26) | C(27) | 120(1) |
| C(22) | C(27) | C(26) | 118(1) | O(4) | C(28) | C(29) | 107.5(8) |
| C(28) | C(29) | C(30) | 119(1) | C(28) | C(29) | C(34) | 119(1) |
| C(30) | C(29) | C(34) | 121.0(10) | C(29) | C(30) | C(31) | 118(1) |
| C(30) | C(31) | C(32) | 118(1) | C(31) | C(32) | C(33) | 123(1) |
| C(32) | C(33) | C(34) | 118(1) | C(29) | C(34) | C(33) | 120(1) |
| O(5) | C(35) | C(36) | 107.9(8) | C(35) | C(36) | C(37) | 122(1) |
| C(35) | C(36) | C(41) | 120(1) | C(37) | C(36) | C(41) | 116(1) |
| C(36) | C(37) | C(38) | 122(1) | C(37) | C(38) | C(39) | 116(1) |
| C(38) | C(39) | C(40) | 125(1) | C(39) | C(40) | C(41) | 116(1) |
| C(36) | C(41) | C(40) | 122(1) | O(8) | C(42) | C(43) | 110.7(8) |
| C(42) | C(43) | C(44) | 119(1) | C(42) | C(43) | C(48) | 121(1) |
| C(44) | C(43) | C(48) | 118(1) | C(43) | C(44) | C(45) | 120(1) |
| C(44) | C(45) | C(46) | 120(1) | C(45) | C(46) | C(47) | 120(1) |
| C(46) | C(47) | C(48) | 119(1) | C(43) | C(48) | C(47) | 120(1) |
| O(6) | P(1) | H(49) | 107.4 | O(7) | P(1) | H(49) | 107.4 |
| O(8) | P(1) | H(49) | 107.4 | O(1) | P(1) | H(1) | 111.3 |
| C(2) | C(1) | H(1) | 111.2 | C(6) | C(1) | H(1) | 111.3 |
| O(2) | C(2) | H(2) | 109.4 | C(1) | C(2) | H(2) | 109.4 |
| C(3) | C(2) | H(2) | 109.4 | O(3) | C(3) | H(3) | 110.2 |
| C(2) | C(3) | H(3) | 110.2 | C(4) | C(3) | H(3) | 110.2 |
| O(4) | C(4) | H(4) | 109.7 | C(3) | C(4) | H(4) | 109.7 |
| C(5) | C(4) | H(4) | 109.6 | O(5) | C(5) | H(5) | 110.3 |
| C(4) | C(5) | H(5) | 110.3 | C(6) | C(5) | H(5) | 110.3 |
| O(6) | C(6) | H(6) | 109.7 | C(1) | C(6) | H(6) | 109.7 |
| C(5) | C(6) | H(6) | 109.7 | O(1) | C(7) | H(7) | 109.9 |
| O(1) | C(7) | H(8) | 109.9 | C(8) | C(7) | H(7) | 109.8 |
| C(8) | C(7) | H(8) | 109.8 | H(7) | C(7) | H(8) | 109.5 |
| C(8) | C(9) | H(9) | 120.5 | C(10) | C(9) | H(9) | 120.5 |
| C(9) | C(10) | H(10) | 119.8 | C(11) | C(10) | H(10) | 119.9 |
| C(10) | C(11) | H(11) | 120.0 | C(12) | C(11) | H(11) | 120.0 |
| C(11) | C(12) | H(12) | 119.9 | C(13) | C(12) | H(12) | 119.8 |
| C(8) | C(13) | H(13) | 120.6 | C(12) | C(13) | H(13) | 120.7 |
| C(15) | C(14) | H(15) | 109.6 | C(15) | C(14) | H(15) | 109.4 |
| C(15) | C(14) | H(14) | 109.4 | C(15) | C(14) | H(15) | 109.5 |

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|-------|-------|-------|-------|-------|
| O(2) | C(14) | H(14) | 109.4 | O(2) | C(14) | H(15) | 109.4 |
| H(14) | C(14) | H(15) | 109.6 | C(16) | C(17) | H(17) | 119.6 |
| C(17) | C(16) | H(16) | 119.8 | C(16) | C(17) | H(17) | 119.8 |
| C(18) | C(17) | H(17) | 119.6 | C(16) | C(17) | H(17) | 119.6 |
| C(19) | C(18) | H(18) | 119.8 | C(18) | C(19) | H(19) | 121.4 |
| C(20) | C(19) | H(19) | 121.4 | C(15) | C(20) | H(20) | 118.5 |
| C(19) | C(20) | H(20) | 118.6 | O(3) | C(21) | H(21) | 109.5 |
| O(3) | C(21) | H(22) | 109.5 | C(22) | C(21) | H(21) | 109.5 |
| C(22) | C(21) | H(22) | 109.5 | H(21) | C(21) | H(22) | 109.5 |
| C(22) | C(23) | H(23) | 119.4 | C(24) | C(23) | H(23) | 119.3 |
| C(23) | C(24) | H(24) | 120.1 | C(25) | C(24) | H(24) | 120.2 |
| C(24) | C(25) | H(25) | 119.6 | C(26) | C(25) | H(25) | 119.6 |
| C(25) | C(26) | H(26) | 119.8 | C(27) | C(26) | H(26) | 119.6 |
| C(22) | C(27) | H(27) | 120.6 | C(26) | C(27) | H(27) | 120.6 |
| O(4) | C(28) | H(28) | 110.0 | O(4) | C(28) | H(29) | 110.0 |
| C(29) | C(28) | H(28) | 109.9 | C(29) | C(28) | H(29) | 109.9 |
| H(28) | C(28) | H(29) | 109.6 | C(29) | C(30) | H(30) | 121.0 |
| C(31) | C(30) | H(30) | 121.0 | C(30) | C(31) | H(31) | 120.8 |
| C(32) | C(31) | H(31) | 120.8 | C(31) | C(32) | H(32) | 118.3 |
| C(33) | C(32) | H(32) | 118.4 | C(32) | C(33) | H(33) | 120.6 |
| C(34) | C(33) | H(33) | 120.6 | C(29) | C(34) | H(34) | 119.8 |
| C(33) | C(34) | H(34) | 119.8 | O(5) | C(35) | H(35) | 109.9 |
| O(5) | C(35) | H(36) | 109.8 | C(36) | C(35) | H(35) | 109.9 |
| C(36) | C(35) | H(36) | 109.8 | H(35) | C(35) | H(36) | 109.5 |
| C(36) | C(37) | H(37) | 118.8 | C(38) | C(37) | H(37) | 118.8 |
| C(37) | C(38) | H(38) | 121.5 | C(39) | C(38) | H(38) | 121.6 |
| C(38) | C(39) | H(39) | 117.4 | C(40) | C(39) | H(39) | 117.3 |
| C(39) | C(40) | H(40) | 122.0 | C(41) | C(40) | H(40) | 121.9 |
| C(36) | C(41) | H(41) | 118.9 | C(40) | C(41) | H(41) | 118.8 |
| O(8) | C(42) | H(42) | 109.1 | O(8) | C(42) | H(43) | 109.1 |
| C(43) | C(42) | H(42) | 109.2 | C(43) | C(42) | H(43) | 109.2 |
| H(42) | C(42) | H(43) | 109.5 | C(43) | C(44) | H(44) | 119.7 |
| C(45) | C(44) | H(44) | 119.7 | C(44) | C(45) | H(45) | 119.7 |
| C(46) | C(45) | H(45) | 119.8 | C(45) | C(46) | H(46) | 119.9 |
| C(47) | C(46) | H(46) | 120.0 | C(46) | C(47) | H(47) | 120.3 |
| C(48) | C(47) | H(47) | 120.3 | C(43) | C(48) | H(48) | 119.8 |
| C(47) | C(48) | H(48) | 119.7 | | | | |

4.5 Appendix 5: Crystallographic Data for Compound DL-227b²³³

$C_{60}H_{72}NO_8SP$, $M = 998.26$, triclinic, space group $P 1$ (#2), $a = 13.464(9)$, $b = 21.24(1)$, $c = 9.557(4)$ Å, $\alpha = 95.42(4)^\circ$, $\beta = 91.87(5)^\circ$, $\gamma = 87.67(5)^\circ$, $V = 2716(2)$ Å³, $Z = 2$, $D_c = 1.220$ g cm⁻³, $T = 200$ K. 5886 unique reflections were collected on a Rigaku AFC7S diffractometer employing Mo-K α radiation ($\lambda = 0.71069$ Å) of which 4076 observations [$I > 3.00\sigma(I)$] were used for refinement. Convergence at $R(F) = 4.8\%$, $R_w(F) = 4.8\%$ for 640 variables.

Table 4.3: Bond lengths (Å) for compound DL-227b

| atom | atom | distance | atom | atom | distance |
|------|------|----------|------|-------|----------|
| S(1) | P(1) | 1.953(2) | P(1) | O(1) | 1.587(3) |
| P(1) | O(2) | 1.498(3) | P(1) | O(3) | 1.599(3) |
| O(1) | C(1) | 1.450(5) | O(3) | C(7) | 1.417(5) |
| O(4) | C(2) | 1.430(5) | O(4) | C(14) | 1.415(5) |
| O(5) | C(3) | 1.430(5) | O(5) | C(21) | 1.426(5) |
| O(6) | C(4) | 1.415(5) | O(6) | C(28) | 1.423(6) |
| O(7) | C(5) | 1.426(5) | O(7) | C(35) | 1.423(5) |

| atom | atom | distance | atom | atom | distance |
|-------|-------|-----------|-------|-------|----------|
| O(8) | C(6) | 1.428(5) | O(8) | C(42) | 1.412(5) |
| N(1) | C(49) | 1.508(5) | N(1) | C(55) | 1.509(6) |
| C(1) | C(2) | 1.507(6) | C(1) | C(4) | 1.507(6) |
| C(2) | C(3) | 1.542(6) | C(3) | C(4) | 1.507(6) |
| C(4) | C(5) | 1.525(6) | C(5) | C(6) | 1.521(6) |
| C(7) | C(8) | 1.503(6) | C(8) | C(9) | 1.361(7) |
| C(8) | C(13) | 1.387(7) | C(9) | C(10) | 1.392(7) |
| C(10) | C(11) | 1.371(8) | C(11) | C(12) | 1.355(8) |
| C(12) | C(13) | 1.389(7) | C(14) | C(15) | 1.499(6) |
| C(15) | C(16) | 1.382(7) | C(15) | C(20) | 1.380(7) |
| C(16) | C(17) | 1.399(7) | C(17) | C(18) | 1.354(8) |
| C(18) | C(19) | 1.353(8) | C(19) | C(20) | 1.394(8) |
| C(21) | C(22) | 1.498(6) | C(22) | C(23) | 1.380(6) |
| C(22) | C(27) | 1.400(6) | C(23) | C(24) | 1.376(7) |
| C(24) | C(25) | 1.373(7) | C(25) | C(26) | 1.383(8) |
| C(26) | C(27) | 1.377(7) | C(28) | C(29) | 1.482(7) |
| C(29) | C(30) | 1.395(8) | C(29) | C(34) | 1.382(8) |
| C(30) | C(31) | 1.370(9) | C(31) | C(32) | 1.36(1) |
| C(32) | C(33) | 1.390(10) | C(33) | C(34) | 1.388(8) |
| C(35) | C(36) | 1.504(7) | C(36) | C(37) | 1.349(8) |
| C(36) | C(41) | 1.376(7) | C(37) | C(38) | 1.395(8) |
| C(38) | C(39) | 1.362(9) | C(39) | C(40) | 1.342(9) |
| C(40) | C(41) | 1.391(8) | C(42) | C(43) | 1.491(6) |
| C(43) | C(44) | 1.365(6) | C(43) | C(48) | 1.395(6) |
| C(44) | C(45) | 1.370(7) | C(45) | C(46) | 1.387(8) |
| C(46) | C(47) | 1.376(8) | C(47) | C(48) | 1.366(7) |
| C(49) | C(50) | 1.530(6) | C(49) | C(54) | 1.510(7) |
| C(50) | C(51) | 1.522(7) | C(51) | C(52) | 1.510(7) |
| C(52) | C(53) | 1.523(7) | C(53) | C(54) | 1.519(7) |
| C(55) | C(56) | 1.519(6) | C(55) | C(60) | 1.517(6) |
| C(56) | C(57) | 1.522(7) | C(57) | C(58) | 1.528(7) |
| C(58) | C(59) | 1.523(7) | C(59) | C(60) | 1.520(7) |
| N(1) | H(49) | 0.95 | N(1) | H(50) | 0.95 |
| C(1) | H(1) | 0.95 | C(2) | H(2) | 0.95 |
| C(3) | H(3) | 0.95 | C(4) | H(4) | 0.95 |
| C(5) | H(5) | 0.95 | C(6) | H(6) | 0.95 |
| C(7) | H(7) | 0.95 | C(7) | H(8) | 0.95 |

| atom | atom | distance | atom | atom | distance |
|-------|-------|----------|-------|-------|----------|
| C(9) | H(9) | 0.95 | C(10) | H(10) | 0.95 |
| C(11) | H(11) | 0.95 | C(12) | H(12) | 0.95 |
| C(13) | H(13) | 0.95 | C(14) | H(14) | 0.95 |
| C(14) | H(15) | 0.95 | C(16) | H(16) | 0.95 |
| C(17) | H(17) | 0.95 | C(18) | H(18) | 0.95 |
| C(19) | H(19) | 0.95 | C(20) | H(20) | 0.95 |
| C(21) | H(21) | 0.95 | C(21) | H(22) | 0.95 |
| C(23) | H(23) | 0.95 | C(24) | H(24) | 0.95 |
| C(25) | H(25) | 0.95 | C(26) | H(26) | 0.95 |
| C(27) | H(27) | 0.95 | C(28) | H(28) | 0.95 |
| C(28) | H(29) | 0.95 | C(30) | H(30) | 0.95 |
| C(31) | H(31) | 0.95 | C(32) | H(32) | 0.95 |
| C(33) | H(33) | 0.95 | C(34) | H(34) | 0.95 |
| C(35) | H(35) | 0.95 | C(35) | H(36) | 0.95 |
| C(37) | H(37) | 0.95 | C(38) | H(38) | 0.95 |
| C(39) | H(39) | 0.95 | C(40) | H(40) | 0.95 |
| C(41) | H(41) | 0.95 | C(42) | H(42) | 0.95 |
| C(42) | H(43) | 0.95 | C(44) | H(44) | 0.95 |
| C(45) | H(45) | 0.95 | C(46) | H(46) | 0.95 |
| C(47) | H(47) | 0.95 | C(48) | H(48) | 0.95 |
| C(49) | H(62) | 0.95 | C(50) | H(63) | 0.95 |
| C(50) | H(64) | 0.95 | C(51) | H(65) | 0.95 |
| C(51) | H(66) | 0.95 | C(52) | H(67) | 0.95 |
| C(52) | H(68) | 0.95 | C(53) | H(69) | 0.95 |
| C(53) | H(70) | 0.95 | C(54) | H(71) | 0.95 |
| C(54) | H(72) | 0.95 | C(55) | H(51) | 0.95 |
| C(56) | H(52) | 0.95 | C(56) | H(53) | 0.95 |
| C(57) | H(54) | 0.95 | C(57) | H(55) | 0.95 |
| C(58) | C(56) | 0.95 | C(58) | H(57) | 0.95 |
| C(59) | H(58) | 0.95 | C(59) | H(59) | 0.95 |
| C(60) | H(60) | 0.95 | C(60) | H(61) | 0.95 |

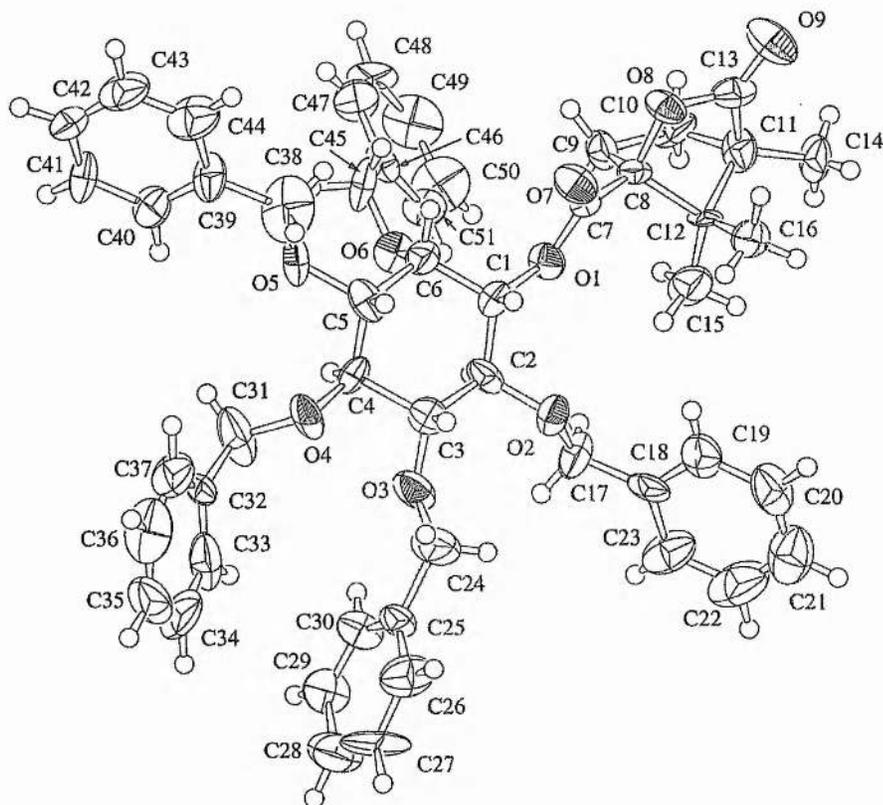
Table 4.4: Bond Angles ($^{\circ}$) for compound DL-227b

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|----------|-------|-------|-------|----------|
| S(1) | P(1) | O(1) | 113.8(1) | S(1) | P(1) | O(2) | 116.2(1) |
| S(1) | P(1) | O(3) | 111.9(1) | O(1) | P(1) | O(2) | 105.6(2) |
| O(1) | P(1) | O(3) | 98.7(2) | O(2) | P(1) | O(3) | 109.1(2) |
| P(1) | O(1) | C(1) | 123.6(3) | P(1) | O(3) | C(7) | 123.1(3) |
| C(2) | O(4) | C(14) | 112.3(3) | C(3) | O(5) | C(21) | 114.5(3) |
| C(4) | O(6) | C(28) | 117.1(4) | C(5) | O(7) | C(35) | 115.4(3) |
| C(6) | O(8) | C(42) | 114.4(3) | C(49) | N(1) | C(55) | 116.0(3) |
| O(1) | C(1) | C(2) | 110.2(3) | O(1) | C(1) | C(6) | 107.7(4) |
| C(2) | C(1) | C(6) | 112.4(4) | O(4) | C(2) | C(1) | 110.2(4) |
| O(4) | C(2) | C(3) | 107.9(4) | C(1) | C(2) | C(3) | 110.4(4) |
| O(5) | C(3) | C(2) | 109.5(4) | O(5) | C(3) | C(4) | 108.5(4) |
| C(2) | C(3) | C(4) | 111.5(4) | O(6) | C(4) | C(3) | 113.2(4) |
| O(6) | C(4) | C(5) | 107.3(4) | C(3) | C(4) | C(5) | 110.0(4) |
| O(7) | C(5) | C(4) | 110.9(4) | O(7) | C(5) | C(6) | 108.1(4) |
| C(4) | C(5) | C(6) | 112.4(4) | O(8) | C(6) | C(1) | 109.7(4) |
| O(8) | C(6) | C(5) | 109.0(4) | C(1) | C(6) | C(5) | 111.8(4) |
| O(3) | C(7) | C(8) | 108.9(4) | C(7) | C(8) | C(9) | 121.9(5) |
| C(7) | C(8) | C(13) | 119.5(5) | C(9) | C(8) | C(13) | 118.6(5) |
| C(8) | C(9) | C(10) | 120.6(5) | C(9) | C(10) | C(11) | 120.0(5) |
| C(10) | C(11) | C(12) | 120.1(5) | C(11) | C(12) | C(13) | 119.9(5) |
| C(8) | C(13) | C(12) | 120.7(5) | O(4) | C(14) | C(15) | 110.8(4) |
| C(14) | C(15) | C(16) | 121.7(4) | C(14) | C(15) | C(20) | 120.4(5) |
| C(16) | C(15) | C(20) | 117.8(5) | C(15) | C(16) | C(17) | 120.6(5) |
| C(16) | C(17) | C(18) | 120.5(5) | C(17) | C(18) | C(19) | 119.7(5) |
| C(18) | C(19) | C(20) | 120.8(5) | C(15) | C(20) | C(19) | 120.6(5) |
| O(5) | C(21) | C(22) | 109.9(4) | C(21) | C(22) | C(23) | 122.6(5) |
| C(21) | C(22) | C(27) | 118.5(4) | C(23) | C(22) | C(27) | 118.8(4) |
| C(22) | C(23) | C(24) | 120.5(5) | C(23) | C(24) | C(25) | 120.9(5) |
| C(24) | C(25) | C(26) | 119.2(5) | C(25) | C(26) | C(27) | 120.6(5) |
| C(22) | C(27) | C(26) | 120.0(5) | O(6) | C(28) | C(29) | 110.7(4) |
| C(28) | C(29) | C(30) | 121.4(6) | C(28) | C(29) | C(34) | 120.7(6) |
| C(30) | C(29) | C(34) | 117.8(6) | C(29) | C(30) | C(31) | 121.8(7) |
| C(30) | C(31) | C(32) | 119.1(8) | C(31) | C(32) | C(33) | 121.6(8) |
| C(32) | C(33) | C(34) | 118.3(7) | C(29) | C(34) | C(33) | 121.3(7) |
| O(7) | C(35) | C(36) | 109.1(4) | C(35) | C(36) | C(37) | 121.8(6) |
| C(35) | C(36) | C(41) | 120.7(5) | C(37) | C(36) | C(41) | 117.5(5) |

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|----------|-------|-------|-------|----------|
| C(36) | C(37) | C(38) | 121.9(6) | C(37) | C(38) | C(39) | 119.4(7) |
| C(38) | C(39) | C(40) | 119.9(6) | C(39) | C(40) | C(41) | 120.2(6) |
| C(36) | C(41) | C(40) | 121.0(6) | O(8) | C(42) | C(43) | 111.2(4) |
| C(42) | C(43) | C(44) | 123.7(5) | C(42) | C(43) | C(48) | 117.6(5) |
| C(44) | C(43) | C(48) | 118.5(4) | C(43) | C(44) | C(45) | 120.7(5) |
| C(44) | C(45) | C(46) | 121.2(5) | C(45) | C(46) | C(47) | 117.9(5) |
| C(46) | C(47) | C(48) | 121.1(5) | C(43) | C(48) | C(47) | 120.6(5) |
| N(1) | C(49) | C(50) | 111.6(4) | N(1) | C(49) | C(54) | 110.7(4) |
| C(50) | C(49) | C(54) | 110.5(4) | C(49) | C(50) | C(51) | 109.6(4) |
| C(50) | C(51) | C(52) | 112.0(4) | C(51) | C(52) | C(53) | 110.5(4) |
| C(52) | C(53) | C(54) | 110.3(4) | C(49) | C(54) | C(53) | 109.8(4) |
| N(1) | C(55) | C(56) | 110.1(4) | N(1) | C(55) | C(60) | 108.2(4) |
| C(56) | C(55) | C(60) | 111.9(4) | C(55) | C(56) | C(57) | 110.6(5) |
| C(56) | C(57) | C(58) | 111.1(4) | C(57) | C(58) | C(59) | 111.6(5) |
| C(58) | C(59) | C(60) | 111.1(4) | C(55) | C(60) | C(59) | 111.6(4) |
| C(49) | N(1) | H(49) | 107.8 | C(49) | N(1) | H(50) | 107.8 |
| C(55) | N(1) | H(49) | 107.8 | C(55) | N(1) | H(50) | 107.8 |
| H(49) | N(1) | H(50) | 109.5 | O(1) | C(1) | H(1) | 108.8 |
| C(2) | C(1) | H(1) | 108.8 | C(6) | C(1) | H(1) | 108.8 |
| O(4) | C(2) | H(2) | 109.4 | C(1) | C(2) | H(2) | 109.4 |
| C(3) | C(2) | H(2) | 109.4 | O(5) | C(3) | H(3) | 109.1 |
| C(2) | C(3) | H(3) | 109.1 | C(4) | C(3) | H(3) | 109.1 |
| O(6) | C(4) | H(4) | 108.8 | C(3) | C(4) | H(4) | 108.8 |
| C(5) | C(4) | H(4) | 108.8 | O(7) | C(5) | H(5) | 108.4 |
| C(4) | C(5) | H(5) | 108.4 | C(6) | C(5) | H(5) | 108.4 |
| O(8) | C(6) | H(6) | 108.8 | C(1) | C(6) | H(6) | 108.8 |
| C(5) | C(6) | H(6) | 108.8 | O(3) | C(7) | H(7) | 109.6 |
| O(3) | C(7) | H(8) | 109.6 | C(8) | C(7) | H(7) | 109.6 |
| C(8) | C(7) | H(8) | 109.6 | H(7) | C(7) | H(8) | 109.5 |
| C(8) | C(9) | H(9) | 119.9 | C(10) | C(9) | H(9) | 119.7 |
| C(9) | C(10) | H(10) | 120.0 | C(11) | C(10) | H(10) | 120.0 |
| C(10) | C(11) | H(11) | 119.9 | C(12) | C(11) | H(11) | 119.9 |
| C(11) | C(12) | H(12) | 120.1 | C(13) | C(12) | H(12) | 120.0 |
| C(8) | C(13) | H(13) | 119.7 | C(12) | C(13) | H(13) | 119.7 |
| O(4) | C(14) | H(14) | 109.1 | O(4) | C(14) | H(15) | 109.1 |
| C(15) | C(14) | H(14) | 109.1 | C(15) | C(14) | H(15) | 109.1 |
| H(14) | C(14) | H(15) | 109.5 | C(15) | C(16) | H(16) | 119.7 |
| C(17) | C(16) | H(16) | 119.7 | C(16) | C(17) | H(17) | 119.7 |

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|-------|-------|-------|-------|-------|
| C(18) | C(17) | H(17) | 119.8 | C(17) | C(18) | H(18) | 120.2 |
| C(19) | C(18) | H(18) | 120.2 | C(18) | C(19) | H(19) | 119.6 |
| C(20) | C(19) | H(19) | 119.5 | C(15) | C(20) | H(20) | 119.7 |
| C(19) | C(20) | H(20) | 119.7 | O(5) | C(21) | H(21) | 109.4 |
| O(5) | C(21) | H(22) | 109.4 | C(22) | C(21) | H(21) | 109.3 |
| C(22) | C(21) | H(22) | 109.3 | H(21) | C(21) | H(22) | 109.5 |
| C(22) | C(23) | H(23) | 119.7 | C(24) | C923) | H(23) | 119.8 |
| C(23) | C(24) | H(24) | 119.5 | C(25) | C(24) | H(24) | 119.6 |
| C(24) | C(25) | H(25) | 120.5 | C(26) | C(25) | H(25) | 120.4 |
| C(25) | C(26) | H(26) | 119.7 | C(27) | C(26) | H(26) | 119.7 |
| C(22) | C(27) | H(27) | 120.0 | C(26) | C(27) | H(27) | 120.0 |
| O(6) | C(28) | H(28) | 109.2 | O(6) | C(28) | H(29) | 109.2 |
| C(29) | C(28) | H(28) | 109.1 | C(29) | C(28) | H(29) | 109.2 |
| H(28) | C(28) | H(29) | 109.4 | C(29) | C(30) | H(30) | 119.1 |
| C(31) | C(30) | H(30) | 119.1 | C(30) | C(31) | H(31) | 120.4 |
| C(32) | C(31) | H(31) | 120.5 | C(31) | C(32) | H(32) | 119.2 |
| C(33) | C(32) | H(32) | 119.2 | C(32) | C(33) | H(33) | 120.8 |
| C(34) | C(33) | H(33) | 120.9 | C(29) | C(34) | H(34) | 119.3 |
| C(33) | C(34) | H(34) | 119.3 | O(7) | C(35) | H(35) | 109.6 |
| O(7) | C(35) | H(36) | 109.5 | C(36) | C(35) | H(35) | 109.6 |
| C(36) | C935) | H(36) | 109.6 | H(35) | C(35) | H(36) | 109.4 |
| C(36) | C(37) | H(37) | 119.0 | C(38) | C(37) | H(37) | 119.0 |
| C(37) | C(38) | H(38) | 120.3 | C(39) | C(38) | H(38) | 120.3 |
| C(38) | C(39) | H(39) | 120.1 | C(40) | C(39) | H(39) | 120.0 |
| C(39) | C(40) | H(40) | 119.9 | C(41) | C(40) | H(40) | 119.9 |
| C(36) | C(41) | H(41) | 119.5 | C(40) | C(41) | H(41) | 119.5 |
| O(8) | C(42) | H(42) | 109.0 | O(8) | C(42) | H(43) | 109.1 |
| C(43) | C(42) | H(42) | 109.0 | C(43) | C(42) | H(43) | 109.0 |
| H(42) | C(42) | H(43) | 109.5 | C(43) | C(44) | H(44) | 119.6 |
| C(45) | C(44) | H(44) | 119.6 | C(44) | C(45) | H(45) | 119.4 |
| C(46) | C(45) | H(45) | 119.4 | C(45) | C(46) | H(46) | 121.1 |
| C(47) | C(46) | H(46) | 121.0 | C(46) | C(47) | H(47) | 119.5 |
| C(48) | C(47) | H(47) | 119.4 | C(43) | C(48) | H(48) | 119.7 |
| C(47) | C(48) | H(48) | 119.7 | N(1) | C(49) | H(62) | 108.0 |
| C(50) | C(49) | H(62) | 108.0 | C(54) | C(49) | H(62) | 108.0 |
| C(49) | C(50) | H(63) | 109.4 | C(49) | C(50) | H(64) | 109.4 |
| C(51) | C(50) | H(63) | 109.5 | C(51) | C(50) | H(64) | 109.4 |
| H(63) | C(50) | H(64) | 109.5 | C(50) | C(51) | H(65) | 108.8 |

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|-------|-------|-------|-------|-------|
| C(50) | C(51) | H(66) | 108.8 | C(52) | C(51) | H(65) | 108.9 |
| C(52) | C(51) | H(66) | 108.9 | H(65) | C(51) | H(66) | 109.5 |
| C(51) | C(52) | H(67) | 109.2 | C(51) | C(52) | H(68) | 109.2 |
| C(53) | C(52) | H(67) | 109.2 | C(53) | C(52) | H(68) | 109.2 |
| H(67) | C(52) | H(68) | 109.5 | C(52) | C(53) | H(69) | 109.3 |
| C(52) | C(53) | H(70) | 109.3 | C(54) | C(53) | H(69) | 109.2 |
| C(54) | C(53) | H(70) | 109.3 | H(69) | C(53) | H(70) | 109.4 |
| C(49) | C(54) | H(71) | 109.4 | C(49) | C(54) | H(72) | 109.4 |
| C(53) | C(54) | H(71) | 109.3 | C(53) | C(54) | H(72) | 109.3 |
| H(71) | C(54) | H(72) | 109.5 | N(1) | C(55) | H(51) | 108.9 |
| C(56) | C(55) | H(51) | 108.8 | C(60) | C(55) | H(51) | 108.9 |
| C(55) | C(56) | H(52) | 109.2 | C(55) | C(56) | H(53) | 109.2 |
| C(57) | C(56) | H(52) | 109.2 | C(57) | C(56) | H(53) | 109.2 |
| H(52) | C(56) | H(53) | 109.5 | C(56) | C(57) | H(54) | 109.1 |
| C(56) | C(57) | H(55) | 109.0 | C(58) | C(57) | H(54) | 109.1 |
| C(58) | C(57) | H(55) | 109.0 | H(54) | C(57) | H(55) | 109.4 |
| C(57) | C(58) | H(56) | 109.0 | C(57) | C(58) | H(57) | 109.0 |
| C(59) | C(58) | H(56) | 108.9 | C(59) | C(58) | H(57) | 108.9 |
| H(56) | C(58) | H(57) | 109.5 | C(58) | C(59) | H(58) | 109.0 |
| C(58) | C(59) | H(59) | 109.1 | C(60) | C(59) | H(58) | 109.1 |
| C(60) | C(59) | H(59) | 109.1 | H(58) | C(59) | H(59) | 109.5 |
| C(55) | C(60) | H(60) | 109.0 | C(55) | C(60) | H(61) | 108.9 |
| C(59) | C(60) | H(60) | 108.9 | C(59) | C(60) | H(61) | 108.9 |
| H(60) | C(60) | H(61) | 109.4 | | | | |

4.6 Appendix 6: Crystallographic Data for Compound L-199b²³³

$C_{51}H_{54}O_9$, $M = 810.98$, monoclinic, space group $P 2_1$ (#4), $a = 6.143(5)$, $b = 23.989(5)$, $c = 15.044(3)$ Å, $\beta = 98.68(3)^\circ$, $V = 2191(1)$ Å³, $Z = 2$, $D_c = 1.229$ g cm⁻³, $T = 293$ K. 3142 unique reflections were collected on a Rigaku AFC7S diffractometer employing Mo- $K\alpha$ radiation ($\lambda = 0.71069$ Å) of which 1669 observations [$I > 3.00\sigma(I)$] were used for refinement. Convergence at $R(F) = 7.3\%$, $R_w(F) = 4.7\%$ for 540 variables.

Table 4.5: Bond lengths (Å) for compound L-199b

| atom | atom | distance | atom | atom | distance |
|------|-------|----------|------|-------|----------|
| O(1) | C(1) | 1.42(2) | O(1) | C(7) | 1.37(2) |
| O(2) | C(2) | 1.42(2) | O(2) | C(17) | 1.41(2) |
| O(3) | C(3) | 1.47(2) | O(3) | C(24) | 1.32(2) |
| O(4) | C(4) | 1.46(2) | O(4) | C(31) | 1.42(2) |
| O(5) | C(5) | 1.47(2) | O(5) | C(38) | 1.41(2) |
| O(6) | C(6) | 1.44(2) | O(6) | C(45) | 1.41(2) |
| O(7) | C(7) | 1.16(2) | O(8) | C(8) | 1.44(2) |
| O(8) | C(13) | 1.43(2) | O(9) | C(13) | 1.19(2) |

| atom | atom | distance | atom | atom | distance |
|-------|-------|----------|-------|-------|----------|
| C(1) | C(2) | 1.49(2) | C(1) | C(6) | 1.51(2) |
| C(2) | C(3) | 1.49(2) | C(3) | C(4) | 1.55(2) |
| C(4) | C(5) | 1.46(2) | C(5) | C(6) | 1.55(2) |
| C(7) | C(8) | 1.48(2) | C(8) | C(9) | 1.56(2) |
| C(8) | C(12) | 1.57(2) | C(9) | C(10) | 1.60(2) |
| C(10) | C(11) | 1.61(2) | C(11) | C(12) | 1.58(2) |
| C(11) | C(13) | 1.48(2) | C(11) | C(14) | 1.50(2) |
| C(12) | C(15) | 1.52(2) | C(12) | C(16) | 1.48(2) |
| C(17) | C(18) | 1.48(2) | C(18) | C(19) | 1.37(2) |
| C(18) | C(23) | 1.41(2) | C(19) | C(20) | 1.42(3) |
| C(20) | C(21) | 1.40(3) | C(21) | C(22) | 1.34(3) |
| C(22) | C(23) | 1.39(3) | C(24) | C(25) | 1.56(2) |
| C(25) | C(26) | 1.37(2) | C(25) | C(30) | 1.30(2) |
| C(26) | C(27) | 1.39(2) | C(27) | C(28) | 1.31(2) |
| C(28) | C(29) | 1.39(2) | C(29) | C(30) | 1.30(2) |
| C(31) | C(32) | 1.46(2) | C(32) | C(33) | 1.35(3) |
| C(32) | C(37) | 1.44(3) | C(33) | C(34) | 1.27(3) |
| C(34) | C(35) | 1.40(3) | C(35) | C(36) | 1.34(3) |
| C(36) | C(37) | 1.35(3) | C(38) | C(39) | 1.53(3) |
| C(39) | C(40) | 1.35(2) | C(39) | C(44) | 1.39(2) |
| C(40) | C(41) | 1.36(2) | C(41) | C(42) | 1.32(2) |
| C(42) | C(43) | 1.36(2) | C(43) | C(44) | 1.34(2) |
| C(45) | C(46) | 1.55(2) | C(46) | C(47) | 1.33(2) |
| C(46) | C(51) | 1.35(2) | C(47) | C(48) | 1.37(3) |
| C(48) | C(49) | 1.37(3) | C(49) | C(50) | 1.37(3) |
| C(50) | C(51) | 1.34(2) | | | |
| C(1) | H(1) | 0.95 | C(2) | H(2) | 0.95 |
| C(3) | H(3) | 0.95 | C(4) | H(4) | 0.95 |
| C(5) | H(5) | 0.95 | C(6) | H(6) | 0.95 |
| C(9) | H(7) | 0.95 | C(9) | H(8) | 0.95 |
| C(10) | H(9) | 0.95 | C(10) | H(10) | 0.95 |
| C(14) | H(11) | 0.96 | C(14) | H(12) | 0.95 |
| C(14) | H(13) | 0.95 | C(15) | H(14) | 0.95 |
| C(15) | H(15) | 0.95 | C(15) | H(16) | 0.95 |
| C(16) | H(17) | 0.95 | C(16) | H(18) | 0.95 |
| C(16) | H(19) | 0.95 | C(17) | H(20) | 0.95 |
| C(17) | H(21) | 0.95 | C(19) | H(22) | 0.95 |
| C(20) | H(23) | 0.95 | C(21) | H(24) | 0.95 |

| atom | atom | distance | atom | atom | distance |
|-------|-------|----------|-------|-------|----------|
| C(22) | H(25) | 0.95 | C(23) | H(26) | 0.95 |
| C(24) | H(27) | 0.95 | C(24) | H(28) | 0.95 |
| C(26) | H(29) | 0.95 | C(27) | H(30) | 0.95 |
| C(28) | H(31) | 0.95 | C(29) | H(32) | 0.94 |
| C(30) | H(33) | 0.95 | C(31) | H(34) | 0.94 |
| C(31) | H(35) | 0.95 | C(33) | H(36) | 0.95 |
| C(34) | H(37) | 0.96 | C(35) | H(38) | 0.95 |
| C(36) | H(39) | 0.95 | C(37) | H(40) | 0.95 |
| C(38) | H(41) | 0.95 | C(38) | H(42) | 0.94 |
| C(40) | H(43) | 0.95 | C(41) | H(44) | 0.95 |
| C(42) | H(45) | 0.95 | C(43) | H(46) | 0.95 |
| C(44) | H(47) | 0.95 | C(45) | H(48) | 0.95 |
| C(45) | H(49) | 0.95 | C(47) | H(50) | 0.95 |
| C(48) | H(51) | 0.95 | C(49) | H(52) | 0.95 |
| C(50) | H(53) | 0.95 | C(51) | H(54) | 0.95 |

Table 4.6: Bond Angles ($^{\circ}$) for compound *L-199b*

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|--------|-------|-------|-------|--------|
| C(1) | O(1) | C(7) | 118(1) | C(2) | O(2) | C(17) | 117(1) |
| C(3) | O(3) | C(24) | 116(1) | C(4) | O(4) | C(31) | 115(1) |
| C(5) | O(5) | C(38) | 114(1) | C(6) | O(6) | C(45) | 115(1) |
| C(8) | O(8) | C(13) | 106(1) | O(1) | C(1) | C(2) | 108(1) |
| O(1) | C(1) | C(6) | 110(1) | C(2) | C(1) | C(6) | 111(1) |
| O(2) | C(2) | C(1) | 110(1) | O(2) | C(2) | C(3) | 114(1) |
| O(4) | C(4) | C(3) | 107(1) | O(4) | C(4) | C(5) | 110(1) |
| C(3) | C(4) | C(5) | 109(1) | O(5) | C(5) | C(4) | 107(1) |
| O(5) | C(5) | C(6) | 108(1) | C(4) | C(5) | C(6) | 111(1) |
| O(6) | C(6) | C(1) | 109(1) | O(6) | C(6) | C(5) | 109(1) |
| C(1) | C(6) | C(5) | 108(1) | O(1) | C(7) | O(7) | 121(1) |
| O(1) | C(7) | C(8) | 109(1) | O(7) | C(7) | C(8) | 128(1) |
| O(8) | C(8) | C(7) | 105(1) | O(8) | C(8) | C(9) | 107(1) |
| O(8) | C(8) | C(12) | 103(1) | C(7) | C(8) | C(9) | 119(1) |
| C(7) | C(8) | C(12) | 116(1) | C(9) | C(8) | C(12) | 103(1) |
| C(8) | C(9) | C(10) | 101(1) | C(9) | C(10) | C(11) | 101(1) |
| C(10) | C(11) | C(12) | 102(1) | C(10) | C(11) | C(13) | 104(1) |
| C(10) | C(11) | C(14) | 114(1) | C(12) | C(11) | C(13) | 101(1) |
| C(12) | C(11) | C(14) | 117(1) | C(13) | C(11) | C(14) | 114(1) |

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|--------|-------|-------|-------|--------|
| C(8) | C(12) | C(11) | 90(1) | C(8) | C(12) | C(15) | 115(1) |
| C(11) | C(12) | C(16) | 111(1) | C(15) | C(12) | C(16) | 109(1) |
| O(8) | C(13) | O(9) | 121(1) | O(8) | C(13) | C(11) | 105(1) |
| O(9) | C(13) | C(11) | 132(1) | O(2) | C(17) | C(18) | 108(1) |
| C(17) | C(18) | C(19) | 124(1) | C(17) | C(18) | C(23) | 117(1) |
| C(19) | C(18) | C(23) | 117(1) | C(18) | C(19) | C(20) | 120(2) |
| C(19) | C(20) | C(21) | 121(2) | C(20) | C(21) | C(22) | 116(1) |
| C(21) | C(22) | C(23) | 124(2) | C(18) | C(23) | C(22) | 119(2) |
| O(3) | C(24) | C(25) | 114(1) | C(24) | C(25) | C(26) | 116(1) |
| C(24) | C(25) | C(30) | 121(1) | C(26) | C(25) | C(30) | 121(1) |
| C(25) | C(26) | C(27) | 117(1) | C(26) | C(27) | C(28) | 121(1) |
| C(27) | C(28) | C(29) | 116(1) | C(28) | C(29) | C(30) | 123(1) |
| C(25) | C(30) | C(29) | 119(1) | O(4) | C(31) | C(32) | 110(1) |
| C(31) | C(32) | C(33) | 117(2) | C(31) | C(32) | C(37) | 125(2) |
| C(33) | C(32) | C(37) | 115(1) | C(32) | C(33) | C(34) | 125(2) |
| C(33) | C(34) | C(35) | 119(2) | C(34) | C(35) | C(36) | 119(2) |
| C(35) | C(36) | C(37) | 119(2) | C(32) | C(37) | C(36) | 120(2) |
| O(5) | C(38) | C(39) | 107(1) | C(38) | C(39) | C(40) | 126(1) |
| C(38) | C(39) | C(44) | 117(1) | C(40) | C(39) | C(44) | 115(1) |
| C(39) | C(40) | C(41) | 124(1) | C(40) | C(41) | C(42) | 117(1) |
| C(41) | C(42) | C(43) | 120(1) | C(42) | C(43) | C(44) | 121(2) |
| C(39) | C(44) | C(43) | 120(1) | O(6) | C(45) | C(46) | 111(1) |
| C(45) | C(46) | C(47) | 116(1) | C(45) | C(46) | C(51) | 120(1) |
| C(47) | C(46) | C(51) | 122(1) | C(46) | C(47) | C(48) | 120(2) |
| C(47) | C(48) | C(49) | 117(1) | C(48) | C(49) | C(50) | 119(2) |
| C(49) | C(50) | C(51) | 121(2) | C(46) | C(51) | C(50) | 117(1) |
| O(1) | C(1) | H(1) | 108.8 | C(2) | C(1) | H(1) | 108.7 |
| C(6) | C(1) | H(1) | 108.9 | O(2) | C(2) | H(2) | 108.7 |
| C(1) | C(2) | H(2) | 108.9 | C(3) | C(2) | H(2) | 108.6 |
| O(3) | C(3) | H(3) | 107.1 | C(2) | C(3) | H(3) | 107.0 |
| C(4) | C(3) | H(3) | 107.0 | O(4) | C(4) | H(4) | 110.2 |
| C(3) | C(4) | H(4) | 110.0 | C(5) | C(4) | H(4) | 109.9 |
| O(5) | C(5) | H(5) | 109.8 | C(4) | C(5) | H(5) | 109.3 |
| C(6) | C(5) | H(5) | 109.6 | O(6) | C(6) | H(6) | 109.9 |
| C(1) | C(6) | H(6) | 109.6 | C(5) | C(6) | H(6) | 109.6 |
| C(8) | C(9) | H(7) | 111.3 | C(8) | C(9) | H(8) | 111.1 |
| C(10) | C(9) | H(7) | 111.6 | C(10) | C(9) | H(8) | 111.6 |
| H(7) | C(9) | H(8) | 109.7 | C(9) | C(10) | H(9) | 111.2 |

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|-------|-------|-------|-------|-------|
| C(9) | C(10) | H(10) | 111.3 | C(11) | C(10) | H(9) | 111.4 |
| C(11) | C(10) | H(10) | 111.2 | H(9) | C(10) | H(10) | 109.6 |
| C(11) | C(14) | H(11) | 109.3 | C(11) | C(14) | H(12) | 109.6 |
| C(11) | C(14) | H(13) | 109.9 | H(11) | C(14) | H(12) | 109.1 |
| H(11) | C(14) | H(13) | 109.1 | H(12) | C(14) | H(13) | 109.8 |
| C(12) | C(15) | H(14) | 109.5 | C(12) | C(15) | H(15) | 109.5 |
| C(12) | C(15) | H(16) | 109.5 | H(14) | C(15) | H(15) | 109.5 |
| H(14) | C(15) | H(16) | 109.3 | H(15) | C(15) | H(16) | 109.4 |
| C(12) | C(16) | H(17) | 109.7 | C(12) | C(16) | H(18) | 109.5 |
| C(12) | C(16) | H(19) | 109.8 | H(17) | C(16) | H(18) | 109.2 |
| H(17) | C(16) | H(19) | 109.4 | H(18) | C(16) | H(19) | 109.3 |
| O(2) | C(17) | H(20) | 110.0 | O(2) | C(17) | H(21) | 109.8 |
| C(18) | C(17) | H(20) | 109.7 | C(18) | C(17) | H(21) | 109.8 |
| H(20) | C(17) | H(21) | 109.4 | C(18) | C(19) | H(22) | 120.0 |
| C(20) | C(19) | H(22) | 119.3 | C(19) | C(20) | H(23) | 119.1 |
| C(21) | C(20) | H(23) | 119.2 | C(20) | C(21) | H(24) | 121.8 |
| C(22) | C(21) | H(24) | 121.9 | C(21) | C(22) | H(25) | 118.2 |
| C(23) | C(22) | H(25) | 117.8 | C(18) | C(23) | H(26) | 120.4 |
| C(22) | C(23) | H(26) | 119.6 | O(3) | C(24) | H(27) | 107.9 |
| O(3) | C(24) | H(28) | 108.1 | C(25) | C(24) | H(27) | 107.8 |
| C(25) | C(24) | H(28) | 108.3 | H(27) | C(24) | H(28) | 109.8 |
| C(25) | C(26) | H(29) | 121.2 | C(27) | C(26) | H(29) | 121.2 |
| C(26) | C(27) | H(30) | 119.2 | C(28) | C(27) | H(30) | 119.5 |
| C(27) | C(28) | H(31) | 121.5 | C(29) | C(28) | H(31) | 121.7 |
| C(28) | C(29) | H(32) | 118.5 | C(30) | C(29) | H(32) | 118.4 |
| C(25) | C(30) | H(33) | 120.1 | C(29) | C(30) | H(33) | 120.2 |
| O(4) | C(31) | H(34) | 109.1 | O(4) | C(31) | H(35) | 108.7 |
| C(32) | C(31) | H(34) | 108.9 | C(32) | C(31) | H(35) | 109.9 |
| H(34) | C(31) | H(35) | 109.7 | C(32) | C(33) | H(36) | 119.4 |
| C(34) | C(33) | H(36) | 115.6 | C(33) | C(34) | H(37) | 121.9 |
| C(35) | C(34) | H(37) | 118.4 | C(34) | C(35) | H(38) | 122.5 |
| C(36) | C(35) | H(38) | 118.0 | C(35) | C(36) | H(39) | 122.1 |
| C(37) | C(36) | H(39) | 118.0 | C(32) | C(37) | H(40) | 117.7 |
| C(36) | C(37) | H(40) | 122.2 | O(5) | C(38) | H(41) | 109.9 |
| O(5) | C(38) | H(42) | 109.8 | C(39) | C(38) | H(41) | 109.5 |
| C(39) | C(38) | H(42) | 109.6 | H(41) | C(38) | H(42) | 110.1 |
| C(39) | C(40) | H(43) | 117.9 | C(41) | C(40) | H(43) | 117.9 |
| C(40) | C(41) | H(44) | 121.5 | C(42) | C(41) | H(44) | 121.0 |

| atom | atom | atom | angle | atom | atom | atom | angle |
|-------|-------|-------|-------|-------|-------|-------|-------|
| C(41) | C(42) | H(45) | 120.1 | C(43) | C(42) | H(45) | 119.8 |
| C(42) | C(43) | H(46) | 119.9 | C(44) | C(43) | H(46) | 118.8 |
| C(39) | C(44) | H(47) | 119.1 | C(43) | C(44) | H(47) | 120.8 |
| O(6) | C(45) | H(48) | 109.0 | O(6) | C(45) | H(49) | 109.0 |
| C(46) | C(45) | H(48) | 109.2 | C(46) | C(45) | H(49) | 109.0 |
| H(48) | C(45) | H(49) | 109.5 | C(46) | C(47) | H(50) | 119.6 |
| C(48) | C(47) | H(50) | 119.7 | C(47) | C(48) | H(51) | 120.6 |
| C(49) | C(48) | H(51) | 121.4 | C(48) | C(49) | H(52) | 120.6 |
| C(50) | C(49) | H(52) | 120.1 | C(49) | C(50) | H(53) | 119.3 |
| C(51) | C(50) | H(53) | 119.0 | C(46) | C(51) | H(54) | 120.9 |
| C(50) | C(51) | H(54) | 121.3 | | | | |

CHAPTER FIVE

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