Inositol phosphates and analogues: synthesis, stereochemistry and molecular recognition

Riley, Andrew M.

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INOSITOL PHOSPHATES AND ANALOGUES: SYNTHESIS, STEREOCHEMISTRY AND MOLECULAR RECOGNITION

submitted by Andrew M. Riley
for the degree of PhD of the University of Bath

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
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Abstract

The synthesis of inositol phosphates and novel analogues is described. Their effects at inositol 1,4,5-trisphosphate receptors and metabolising enzymes of the phosphoinositide pathway is reported. The enantiomers of *myo*-inositol 1,3,4-trisphosphate were prepared by a novel route from *myo*-inositol involving the optical resolution of DL-2,4,5-tri-*O*-benzyl-1-*O*-*p*-methoxybenzyl-*myo*-inositol. 1D-Ins(1,3,4)P_3 was shown to be essentially inactive at Ins(1,4,5)P_3 receptors, whereas 1L-Ins(1,3,4)P_3 was able to mobilise intracellular Ca^{2+}, in accordance with arguments based on structure-activity principles. The first five-membered ring mimic of Ins(1,4,5)P_3 [(1*R*, 2*R*, 3*S*, 4*R*, 5*S*)-3-hydroxy-1,2,4-trisphospho-5-vinylcyclopentane] was synthesised by a strategy involving zirconium-mediated diastereoselective ring contraction of a protected vinyl carbohydrate. A conformationally restrained cyclic phosphate analogue of Ins(1,4,5)P_3 [DL-6-deoxy-6-hydroxymethyl-*scyllo*-inositol 1:7-cyclic,2,4-trisphosphate] was synthesised from *myo*-inositol orthoformate *via* a versatile intermediate [2,4,6/3,5-pentahydroxy-3,5-di-*O*-*p*-methoxybenzyl-2,4,6-*O*-methylidene-cyclohexanone], and appeared to behave as a weak full agonist at Ins(1,4,5)P_3 receptors. Potentiometric and ^{31}P NMR studies showed the analogue to have markedly different acid-base properties to Ins(1,4,5)P_3. A *scyllo*-inositol-based analogue of Ins(1,4,5)P_3 [DL-6-deoxy-6-hydroxymethyl-*scyllo*-inositol-1,2,4-trisphosphate] bearing an hydroxymethyl group at a position analogous to that of this structure in adenophostin A. was synthesised and found to be equipotent to Ins(1,4,5)P_3 itself. The first disaccharide based Ca^{2+}-mobilising Ins(1,4,5)P_3 mimic, the C_2-symmetrical α,α-trehalose 3,4,3′,4′-tetrakisphosphate, was synthesised from α,α-trehalose by a route involving the simultaneous regioselective reduction of two benzylidene acetals. 1L-*myo*-inositol 1,3,4-trisphosphorothioate was synthesised and shown to be a very low intrinsic activity partial agonist at the Ins(1,4,5)P_3 receptors of platelets, and was able to inhibit Ins(1,4,5)P_3 - induced Ca^{2+} release in a dose-dependent manner. A novel symmetrical analogue of inositol 1,3,4,5-tetrakisphosphate [*scyllo*-inositol 1,2,3,5-tetrakisphosphate], intended for structure-activity investigations into the properties of Ins(1,3,4,5)P_4 binding proteins, was synthesised. A new rapid route to 1D-Ins(1,3,4,5)P_4 and its unnatural enantiomer 1L-Ins(1,3,4,5)P_4, *via* diastereoisomeric bis-(–)-ω-camphanate esters of *myo*-inositol orthoformate was developed, and the absolute configurations of the products proved by an X-ray crystallographic study of the intermediate 1D-2,6-di-*O*-[(–)-ω-camphanoyl]-1,3,5-*O*-methylidene-*myo*-inositol.

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Publications

Some of the work described in this thesis has already appeared in the following publications:

Unambiguous Total Synthesis of the Enantiomers of *myo*-Inositol 1,3,4-trisphosphate: 1L-*myo*-Inositol 1,3,4-trisphosphate Mobilizes Ca^{2+} in *Limulus* Photoreceptors. A M Riley, R Payne and B V L Potter, *J Med Chem* (1994) **37**, 3918-3927.

myo-Inositol 1,4,6-trisphosphorothioate and *myo*-Inositol 1,3,4-trisphosphorothioate: New Synthetic Ca^{2+}-mobilising Partial Agonists at the Inositol 1,4,5-trisphosphate Receptor. S J Mills, A M Riley, C T Murphy, A J Bullock, J Westwick and B V L Potter, *Bioorg Med Chem Lett* (1995) **5**, 203-208.

Pentagon IP3: Synthesis of a Ring-contracted Mimic of a Second Messenger. A M Riley, D J Jenkins and B V L Potter, *J Am Chem Soc* (1995) **117**, 3300-3301.

Synthesis of a Conformationally Restricted Cyclic Phosphate Analog of Inositol 1,4,5-trisphosphate. A M Riley and B V L Potter, *J Org Chem* (1995) **60**, 4970-4971.

Long-range ^{31}P-^{31}P Spin Coupling Constants in the ^{31}P NMR Spectra of Phosphite Triesters. A M Riley, S J Mills and B V L Potter, *Phosphorus, Sulfur and Silicon* (1996) **111**, 71.

Abbreviations

$[\alpha]_D$	specific rotation at 589 nm
Ac	acetyl
ADP	adenosine 5′-diphosphate
All	allyl
AMP	adenosine 5′-monophosphate
Anhyd	anhydrous
Ar	aryl
ATP	adenosine 5′-triphosphate
ATPase	adenosinetriphosphatase
9-BBN-H	9-borabicyclo[3.3.1]nonane
BAPTA	1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid
Bn	benzyl
bp	boiling point
br	broad (spectral)
Bu	butyl
Bu′	*tert*-butyl
Bz	benzoyl
°C	degrees Celsius
c	concentration in g/100mL
cADPR	cyclic adenosine diphosphate ribose
cald	calculated
cAMP	adenosine cyclic 3′,5′-phosphate
camph	(−)-ω-camphanate
CDPG	cytidine-5′-diphosphate-D-glucose
cGMP	guanosine cyclic 3′,5′-phosphate
CICR	calcium-induced calcium release
CIF	calcium influx factor
cm	centimetre(s)
CNDO	complete neglect of differential overlap
concd	concentrated
COSY	correlation spectroscopy
Cp	cyclopentadienyl
CRAC	calcium-release activated channel
δ	chemical shift
d	doublet (spectral)
DAG	1,2-di-*O*-acylglycerol
DCC	*N*,*N*-dicyclohexylcarbodiimide
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DEPT	distortionless enhancement by polarisation transfer
DHPR	dihydropyridine receptor
DIBALH	diisobutylaluminium hydride
DMAP	4-(dimethylamino)pyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenylhydrazine
EC_{50}	concentration producing 50% of maximal response
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis-(oxyethylenenitrilo)-tetraacetic acid
ER	endoplasmic reticulum
Et	ethyl
FAB	fast atom bombardment (mass spectrometry)
FT	Fourier transform
g	gram(s)
GAP	GTP-ase activator protein
Gluc(2′,3,4)P_3	(2-hydroxyethyl)-α-D-glucopyranoside-2′,3,4-trisphosphate
GTP	guanosine 5′-triphosphate
GTPase	guanosinetriphosphatase
h	hour
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrum
Hz	hertz
IC_{50}	concentration causing 50% inhibition of radioligand binding
I_{CRAC}	calcium-release activated calcium current
Ins	inositol
Ins(1,4,5)P_3	1D-*myo*-inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
IR	infrared
J	coupling constant (in NMR)
k	kilo
K_i	inhibitor constant
K_y	macroscopic protonation constant
k_i	microscopic protonation constant
L	litre(s)
μ	micro
m	multiplet (spectral), milli
M	moles per litre
m-CPBA	*m*-chloroperoxybenzoic acid
lit	literature (reference)
Me	methyl
MHz	megahertz
min	minute(s)
MIPP	multiple inositol polyphosphate phosphatase
mM	millimoles per litre
mol	mole(s)

mp	melting point
MS	mass spectrometry
m/z	mass to charge ratio (mass spectrometry)
NAD	nicotinamide adenine dinucleotide
NMDA	*N*-methyl-D-aspartate
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
OCRL	oculocerebrorenal syndrome of Lowe
Ph	phenyl
PH	pleckstrin homology
PLC	phospholipase C
PMB	*p*-methoxybenzyl
ppm	parts per million (in NMR)
Pr	propyl
Pr^i	isopropyl
PtdIns	phosphatidylinositol
PTSA	*para*-toluenesulphonic acid
q	quartet (spectral)
R_f	retention factor (in chromatography)
rt	room temperature
RYR	ryanodine receptor
SIP	sphingosine-1-phosphate
TEA^+	triethylammonium ion
TEAB	triethylammonium hydrogencarbonate
TBDMS	*tert*-butyldimethylsilyl
TFA	trifluoroacetic acid
THA^+	tetrahexylammonium ion
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMS	tetramethylsilane
UV	ultraviolet

Contents

1 Introduction

1.1 First and Second Messengers

This thesis is primarily concerned with 1D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3, Figure 1.1]. Ins(1,4,5)P_3 functions as a *second messenger* in cells.

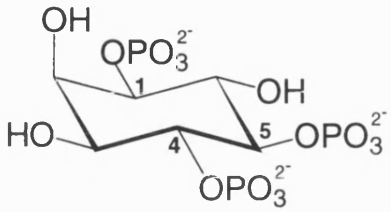


Figure 1.1 1D-*myo*-inositol 1,4,5-trisphosphate.

The cells in a multicellular organism must be able to communicate with one another in order to regulate their function, their growth and division, and their organisation into tissues. This intercellular communication is conducted by a range of extracellular signalling molecules or *first messengers,* which include hormones, neurotransmitters, and local mediators. Small hydrophobic signalling molecules, such as steroids and thyroid hormones, are able to pass through the cell membrane and activate receptor proteins within the cell, but the vast majority of signalling molecules are hydrophilic, and are denied entry to the cell by the lipid bilayer of the plasma membrane. Instead, they bind to receptors on the cell surface, and this interaction with the receptor somehow causes the production or release within the cell of a second messenger, which causes the cellular response. This concept is illustrated in Figure 1.2.

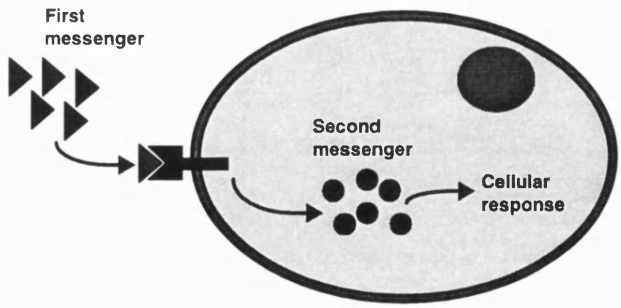


Figure 1.2 First and second messengers.

1.2 Calcium Signalling

The most common second messenger in cells, ranging from the simplest bacteria to the specialised neurones of the vertebrate nervous system,[1] is ionised calcium; Ca^{2+}. The reason for this choice of ion may be related to the changing compositions of the oceans

during the early stages of the evolution of life on Earth. When life first evolved, it is likely that the oceans were more alkaline than they are today and the concentration of dissolved Ca^{2+} was therefore low. As organisms proliferated, the accumulation of carbon dioxide and other acidic metabolites caused the pH to fall, and the solubility of Ca^{2+} increased.[2] Cells would have been unable to tolerate high levels of intracellular Ca^{2+} since it precipitates phosphate, which was already established as an energy currency.[3] They were therefore forced to develop mechanisms to control intracellular Ca^{2+} concentrations, either by pumping Ca^{2+} out of the cell or sequestering it in some way. Having created transmembrane Ca^{2+} gradients out of the need to avoid the toxic effects of Ca^{2+}, cells later evolved ways of exploiting these gradients. Mechanisms developed that could allow small amounts of Ca^{2+} to flow back into the cytosol, either from outside or from intracellular sequestering compartments, under the control of variables such as changes in membrane potential or extracellular stimuli. Proteins that originally functioned as Ca^{2+} buffers took on more specialised signal-transducing roles, involving conformational changes triggered by the binding of Ca^{2+}, and Ca^{2+}-binding motifs became incorporated into many different proteins.

It is now known that the complex mechanisms that have evolved for Ca^{2+} release from intracellular stores and for Ca^{2+} entry across the plasma membrane lead to an inherently oscillatory system.[4] The Ca^{2+} signals that are generated in response to external stimuli are not uniform, but pulsatile in nature, and take the form of repetitive spikes or waves which encode information by frequency modulation. Complex wave patterns with spherical, spiral and planar waves have been observed, and detailed mathematical models have been proposed to account for these.[5]

1.3 Calcium Channels

The flow of Ca^{2+}, either from the extracellular space or from internal stores is controlled by various types of Ca^{2+} channel (Figure 1.3). Entry from the outside is controlled by voltage-operated channels or receptor-operated channels (e.g. NMDA receptors). There are also calcium-release activated channels (CRAC) which are somehow triggered by emptying of the intracellular stores (Section 1.6) and there may be calcium influx channels gated by $Ins(1,4,5)P_3$ or other second messengers such as D-*myo*-inositol 1,3,4,5-tetrakisphosphate [$Ins(1,3,4,5)P_4$], or by Ca^{2+} itself. Two main families of intracellular Ca^{2+} channels are known; ryanodine receptors and $Ins(1,4,5)P_3$ receptors, and they share many similarities. A third type of intracellular Ca^{2+} channel, gated by

sphingolipids has recently been characterised.[6] The natural ligand may be sphingosine-1-phosphate (SIP).[7]

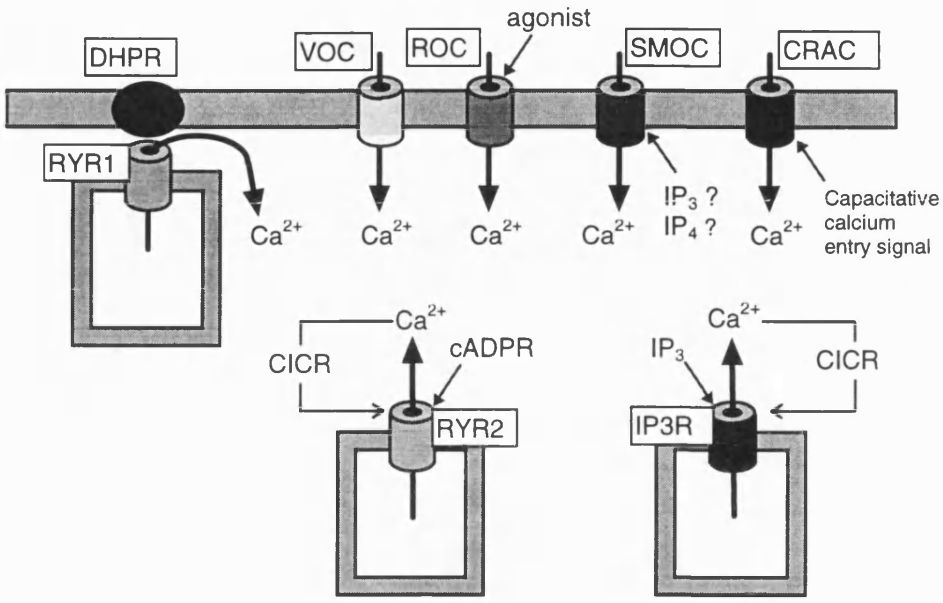


Figure 1.3 Calcium channels. **DHPR** = dihydropyridine receptor, **VOC** = voltage-operated channel, **ROC** = receptor-operated channel, **SMOC** = second messenger-operated channel, **CRAC** = calcium release-activated channel, **RYR1** = type 1 ryanodine receptor, **RYR2** = type 2 ryanodine receptor, **IP3R** = D-*myo*-inositol 1,4,5-trisphosphate receptor, **IP_3** = D-*myo*-inositol 1,4,5-trisphosphate, **IP_4** = D-*myo*-inositol 1,3,4,5-tetrakisphosphate, **CICR** = calcium-induced calcium release.

Ryanodine receptors were first identified through their high-affinity binding for the plant alkaloid ryanodine. Several subtypes have been found. The type 1 isoform (RYR1), predominantly found in skeletal muscle, is activated by depolarisation of transverse tubules (T-tubules), and this is an important step in excitation-contraction coupling of skeletal muscle. The bulbous head of RYR1 seems to interact through conformational coupling with the dihydropyridine receptor, which is the voltage sensor of the T-tubule membrane. RYR2, found in cardiac muscle, can also be opened by depolarisation of the cell membrane, but in this case the interaction is less direct, in that it is stimulated by a pulse of Ca^{2+}. Note that in this case, Ca^{2+} triggers its own release; an example of positive feedback. This process of calcium-induced calcium release (CICR) is of central importance in Ca^{2+} signalling, and it is a property of both ryanodine and Ins(1,4,5)P_3 receptors. A single mutation in RYR1 (Arg 615 → Cys) seems to be responsible for malignant hyperthermia, in which over-sensitivity of the receptor to Ca^{2+}

leads to uncontrolled CICR, with spasm of the muscles and potentially fatal overheating.[8]

Some cells express only ryanodine or only Ins(1,4,5)P_3 receptors, while others express both. Within an individual cell, individual Ca^{2+} stores may express only one of the receptors, both, or neither. As the phenomenon of CICR is increasingly seen as fundamental to the whole process of Ca^{2+} signalling, it may be more instructive to regard Ins(1,4,5)P_3 receptors and ryanodine receptors as two related families of CICR channels, the first of which is known to be modulated by Ins(1,4,5)P_3. Cyclic adenosine diphosphate ribose (cADPR, Figure 1.4),[9,10] a naturally occurring metabolite of nicotinamide adenine dinucleotide (NAD^+), triggers Ca^{2+} release through ryanodine receptors by sensitising them to CICR, and this molecule and/or its 2′-phosphate[11]may modulate ryanodine receptors in non-muscle cells.[12]

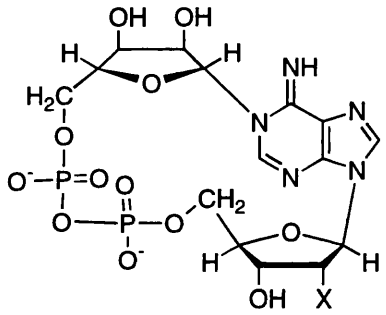


Figure 1.4 Cyclic ADP-ribose (X = OH) and its 2′-phosphate (X = OPO_3^{2-})

Finally, there is a recent report of intracellular Ca^{2+} release in *Xenopus* oocytes in response to microinjection of cytidine-5′-diphosphate-D-glucose (CDPG, Figure 1.5)[13] The site of action is currently unknown, but appears to be quite specific in its structural requirements. The closely-related uridine-glucose conjugate UDPG, for example, was less active and other nucleotide-glucose conjugates were inactive. The effect was not inhibited by heparin, suggesting a mechanism distinct from that of Ins(1,4,5)P_3.

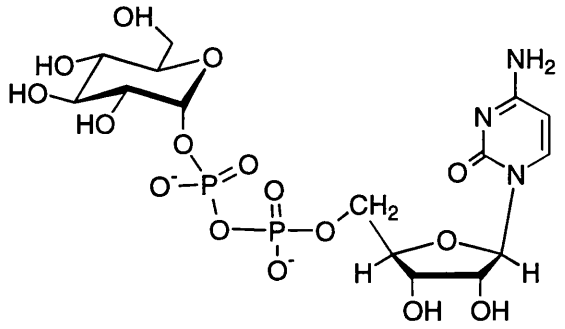


Figure 1.5 Cytidine 5′-diphosphate-D-glucose.

It is interesting to note a kind of family resemblance between cADPR (or its 2′-phosphate), CDGP and the adenophostins (Section 1.7). Whether this is a reflection of corresponding evolutionary relationships between their respective target proteins remains to be seen.

The rest of this thesis will be concerned with $Ins(1,4,5)P_3$ and other inositol phosphates. $Ins(1,4,5)P_3$ receptors will be discussed in Section 1.5. but first we will describe the origin of $Ins(1,4,5)P_3$, and how its production may be controlled by extracellular messengers.

1.4 Origin of $Ins(1,4,5)P_3$

$Ins(1,4,5)P_3$ is produced when the minor membrane phospholipid phosphatidylinositol 4,5-bisphosphate [$PtdIns(4,5)P_2$] is cleaved by a phosphodiesterase of the phospholipase C (PLC) type (Figure 1.6). Recent evidence suggests an additional source for $Ins(1,4,5)P_3$ (see Section 1.9.2) but the significance of this is unknown at present.

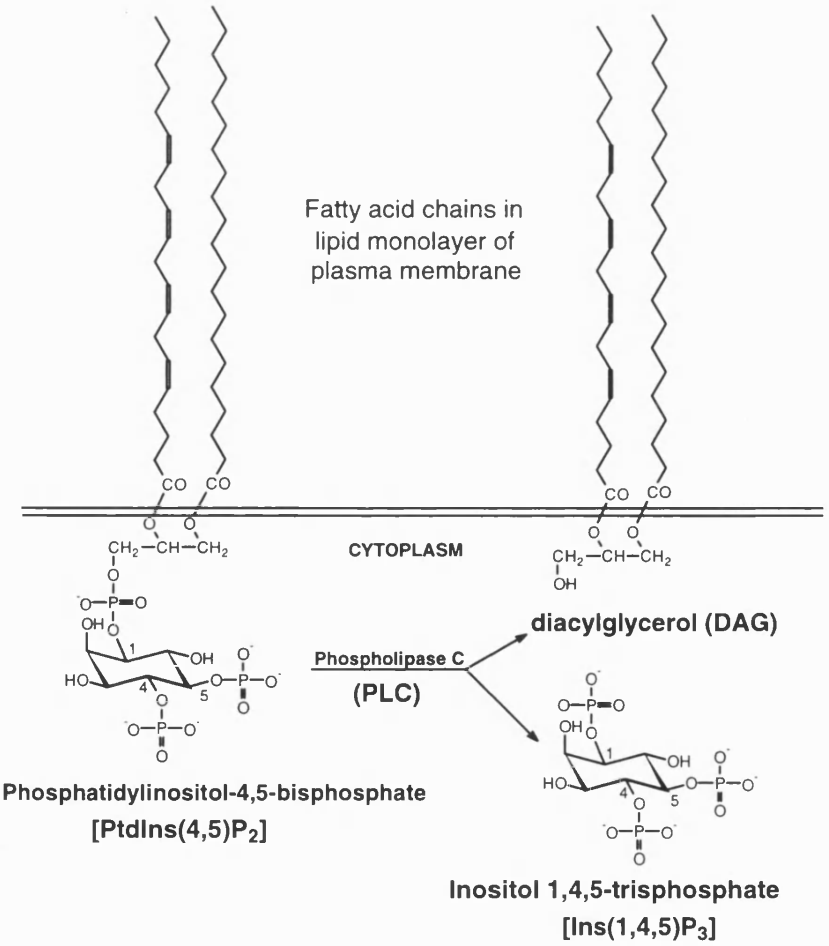


Figure 1.6 Hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C produces inositol 1,4,5-trisphosphate and diacylglycerol.

PLCs are classified into three types (PLC-β, PLC-γ and PLC-δ) and each type contains more than one subtype. Two main types of cell-surface receptor are able to initiate the production of Ins(1,4,5)P_3 by activating particular PLC isoforms. These are the G protein-coupled receptors and the tyrosine kinase-coupled receptors (Figure 1.7).

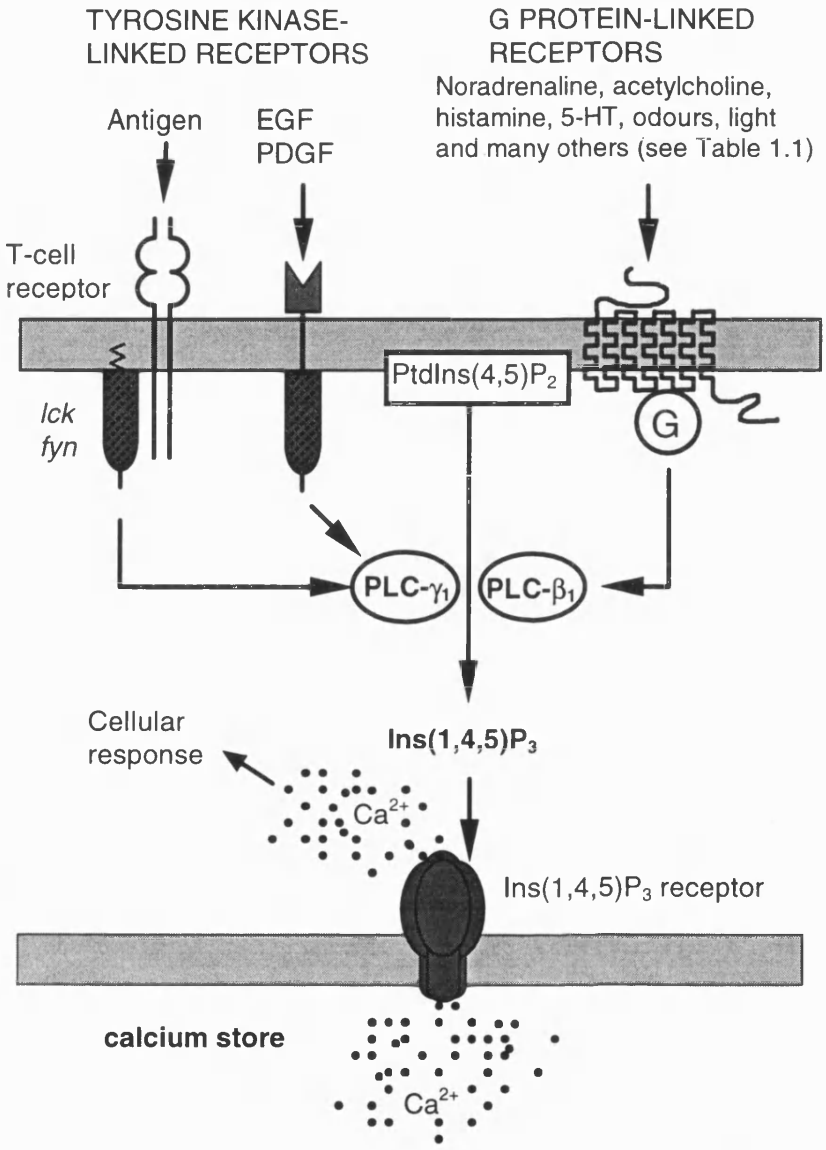


Figure 1.7 Tyrosine kinase-linked and G protein-linked receptors. EGF = epidermal growth factor, PDGF = platelet-derived growth factor, PLC = phospholipase C.

Many extracellular signals cause release of intracellular Ca^{2+} by interacting with cell-surface G-protein coupled receptors. Many of this large family of receptors, which all have seven transmembrane domains, are able to activate PLC-β_1 *via* $G_{q/11}$ subunits of their associated heterotrimeric G-proteins. The tyrosine kinase-linked receptors are simpler, having a single transmembrane domain, and relay information through a direct interaction between the receptor and PLC-γ_1. On binding their ligands, the receptors

dimerise and phosphorylate one another on tyrosine, creating docking sites for PLC-γ_1. PLC-γ_1 then binds to the receptor and is pulled onto the membrane, where it meets its substrate, PtdIns(4,5)P_2. The hydrophilic head-group of PtdIns(4,5)P_2 released by both these mechanisms is Ins(1,4,5)P_3, which then diffuses into the cytoplasm and causes the release of Ca^{2+} from intracellular stores by interacting with Ins(1,4,5)P_3 receptors. In general, the tyrosine-kinase-activated PLC-γs increase Ca^{2+} more slowly and for longer durations than do the G protein-mediated PLC-βs. It is important to stress that tyrosine kinase-linked receptors also activate other effectors such as phosphatidylinositol-3-kinase (Section 1.9.5), and GTPase-activator protein (GAP, Section 1.10.4). These other signalling pathways have been omitted from Figure 1.7 for the sake of clarity.

Via PLC-β	*Via* PLC-γ
Adrenoceptors α_{1A}, α_{1B},α_{1D}	Epidermal growth factor receptor
Angiotensin AT_1	Fibroblast growth factor receptor
Bombesin BB_1, BB_2	Platelet-derived growth factor receptor
Bradykinin B_1, B_2	T cell antigen receptor
Cholecystokinin CCK_A, CCK_B	
Endothelin ET_A, ET_B	
Glucagon	
Gonadotrophin releasing hormone	
Histamine H_1	
5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}	
Leukotriene BLT, $CysLT_1$	
Melatonin ML	
Metabotropic glutamate $mGlu_1$, $mGlu_5$	
Muscarinic M_1 ,M_3, M_5,	
Oxytocin	
Platelet-activating factor	
Prostanoid EP_1, EP_3, FP, TP	
Purinoceptors P_{2Y}, P_{2U}	
Tachykinin NK_1, NK_2, NK_3	
Thrombin	
Thromboxanes	
Thyrotropin releasing hormone	
Vasopressin V_{1A}, V_{1B}	

Table 1.1 Plasma membrane receptors activating phospholipase C.

Finally, IgM receptors in B-lymphocytes and the T-cell antigen receptor CD3 can also stimulate the production of Ins(1,4,5)P_3. They lack tyrosine kinase activity of their own but recruit members of the *src* proto-oncogene family such as the protein tyrosine kinases *fyn* and *lck* . Table **1.1** lists some of the plasma membrane receptors that are coupled to hydrolysis of PtdIns(4,5)P_2, either through activation of PLC-β or PLC-γ. The other product of hydrolysis of PtdIns(4,5)P_2 by PLCs is the lipophilic 1,2-di-*O*-acyl glycerol (DAG). DAG remains in the membrane, and can activate a family of protein kinase C isoenzymes, which catalyse protein phosphorylation. This part of the pathway is not shown in Figure 1.7. Many of the actions of DAG are mimicked by the phorbol esters, products of the *Euphorbaciae* family of higher plants.

1.5 Inositol 1,4,5-trisphosphate Receptors

The Ins(1,4,5)P_3 receptor is a tetramer, composed of four subunits surrounding a cationic pore which behaves as an ion channel. Each subunit binds one molecule of Ins(1,4,5)P_3 at a positively-charged domain close to the N-terminal. The transmembrane topology is thought to be as shown in Figure 1.8. Each subunit has six transmembrane helices towards the C-terminal which constitute the Ca^{2+} channel domain.

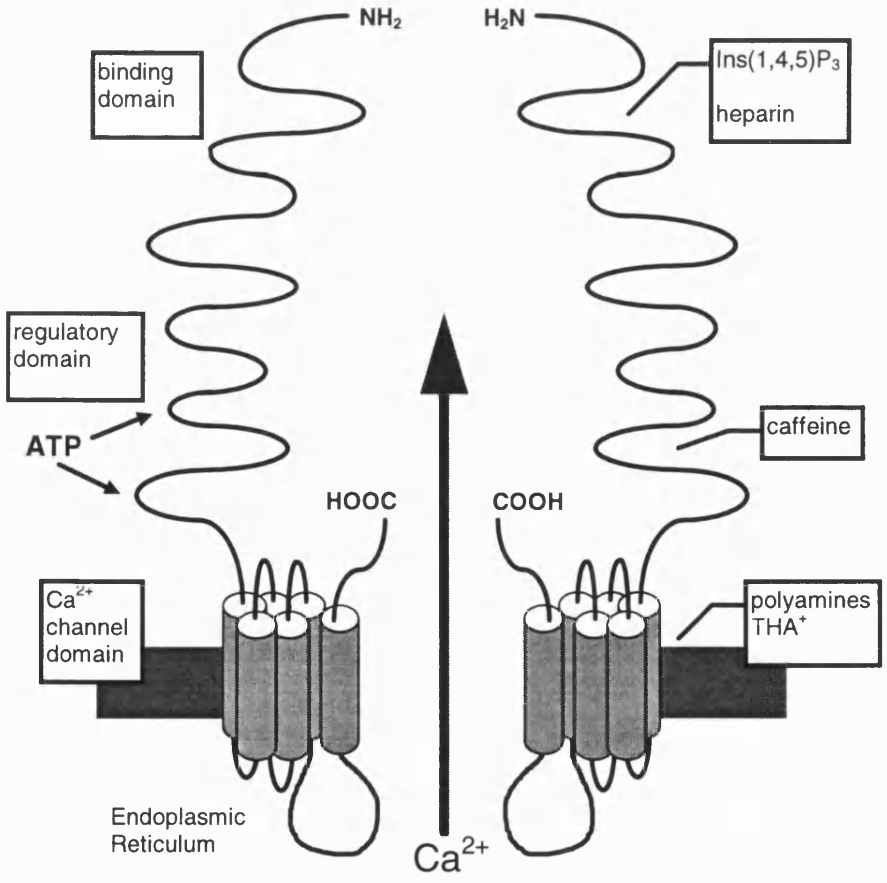


Figure 1.8 Cross-section of a generalised inositol 1,4,5-trisphosphate receptor, showing proposed site of action of antagonists.

The two domains are coupled by a large regulatory domain which contains sites for phosphorylation by many different protein kinases, and also two putative ATP binding sites. Note that Figure 1.8 represents a cross-section through a generalised Ins(1,4,5)P_3 receptor and therefore shows only two of the four subunits. There are at least three types of Ins(1,4,5)P_3 receptor subunits, derived from distinct genes and designated type 1 (IP_3R-1), type 2 (IP_3R-2) and type 3 (IP_3R-3), all of which share significant similarity to each other, partial homology with the ryanodine receptor and no significant homology with voltage-gated calcium channels. It is likely that this list will expand in the future. The distribution of isoforms differs considerably between tissues.[14] IP_3R-1, for example, is particularly enriched in cerebellum. Mutant mice lacking IP_3R-1 usually die *in utero*, and born animals have severe ataxia and epileptic seizures.[15] IP_3R-3 is expressed most abundantly in gastrointestinal tract and kidney[16] and is also the predominant isoform expressed in adult pancreatic islets. It may be responsible for mediating the effects of Ins(1,4,5)P_3 on insulin secretion.[17] For a recent review on the Ins(1,4,5)P_3 receptor family see Joseph (1996)[18]

It was originally thought that Ins(1,4,5)P_3 receptors were located only in the membranes of the endoplasmic reticulum, but more recently they have been found in the plasma membranes of various cells (e.g. lymphocytes[19,20]). This could indicate that, at least in some cell-types, Ins(1,4,5)P_3 receptors may have a role in Ca^{2+} entry from the extracellular space. Until very recently Ins(1,4,5)P_3 receptors were assumed to be homotetramers, composed of identical subunits, but evidence is now emerging that distinct types of subunits may assemble to form heterotetramers.[21,22] Heteroligomerisation of isoforms might provide a mechanism for generating greater diversity of Ins(1,4,5)P_3 receptors. The mixing of just two types of subunits, for example, would produce five kinds of Ins(1,4,5)P_3 receptor, i.e. two homotetramers and three kinds of heterotetramer. A mathematical model based on the random association of subunits with high and low affinities for Ins(1,4,5)P_3 in this way has been used to describe the kinetics of Ca^{2+} release from rat basophilic leukaemia cells and has been proposed as an explanation for the phenomenon of quantal Ca^{2+} release (see below).[23]

1.6 Capacitative Calcium Entry

The binding of Ins(1,4,5)P_3 to its receptor results in the release of Ca^{2+} stored in the endoplasmic reticulum (ER). Submaximal concentrations of Ins(1,4,5)P_3 release only a fraction of the Ins(1,4,5)P_3-sensitive Ca^{2+} store, and further increases in Ins(1,4,5)P_3

concentration can mobilise more Ca^{2+}. This phenomenon, which may also be a property of ryanodine receptors, is described as "quantal" Ca^{2+} release. The Ca^{2+} liberated by Ins(1,4,5)P_3 can activate a range of calcium-dependent processes by inducing conformational changes in various Ca^{2+}-binding proteins. Ca^{2+} is then pumped both out of the cell and back into the ER by Ca^{2+} ATPases. Thus there is a net loss of Ca^{2+} from the cell, and a small amount of extracellular Ca^{2+} must enter through the plasma membrane to make good this loss. This influx of Ca^{2+} from the environment is known as capacitative calcium entry, and it occurs regardless of whether the stores are emptied by Ins(1,4,5)P_3 or by blockade of the endoplasmic reticulum Ca^{2+} pump. Besides replenishing the intracellular Ca^{2+} stores this mechanism maximises Ca^{2+} concentrations near the plasma membrane, where specialised Ca^{2+}-dependent proteins are found. There is a growing awareness that capacitative calcium entry plays a central role in the functioning of non-excitable cells, which lack voltage-gated Ca^{2+} channels. A currently unsolved mystery is *why* Ca^{2+} entry through the plasma membrane inevitably follows Ca^{2+} release from the endoplasmic reticulum. This question is now a subject of great controversy in the field of cellular signalling.[24]

A specific depletion-activated Ca^{2+} current has now been identified and is termed I_{CRAC} (calcium-release-activated calcium current). I_{CRAC} has been shown to be highly selective for Ca^{2+}, not voltage-dependent, and is linked to depletion of intracellular Ca^{2+} stores, but neither Ins(1,4,5)P_3 nor D-Ins(1,3,4,5)P_4 are necessary for its activation.[25] The CRAC channel itself has not yet been identified, although there has been considerable excitement generated by reports that CRAC may be activated by a novel diffusible factor, released from the Ca^{2+} stores and acting as a kind of reverse messenger (Figure 1.9).[26,27] This putative messenger, now named calcium influx factor (CIF), was postulated to be a small anionic phosphorylated molecule and seemed to be present in extracts from cells in which the Ca^{2+} stores had been depleted. A more recent study, in which extracts from stimulated Jurkat T-lymphocytes caused Ca^{2+} influx in *Xenopus* oocytes suggests that the CIF activity may consist of at least two active components, one which can act from the outside and the other which works only when injected.[28] However, little progress has been made in identifying CIF, and alternative models of capacitative Ca^{2+} entry, based on protein-protein interactions, have been gaining ground recently.[24] It has been suggested, for example, that information is transmitted directly from Ins(1,4,5)P_3 receptors to CRAC channels by a conformational coupling mechanism (analogous to that which is thought to exist between RYR1 and the

dihydropyridine receptor, and perhaps involving specific Ins(1,4,5)P_3 receptor subtypes. It has been found that over-expression of type-3 Ins(1,4,5)P_3 receptors in *Xenopus* oocytes has no effect on Ins(1,4,5)P_3-induced Ca^{2+} release, but markedly enhances the magnitude and duration of calcium influx.[29] These two dominant theories of capacitative calcium entry, conformational coupling and CIF are summarised schematically in Figure 1.9. Either mechanism would be of interest to the medicinal chemist, as they both offer the possibility of pharmacological intervention in capacitative Ca^{2+} entry (either through CIF-analogues or selective IP_3R-3 ligands).

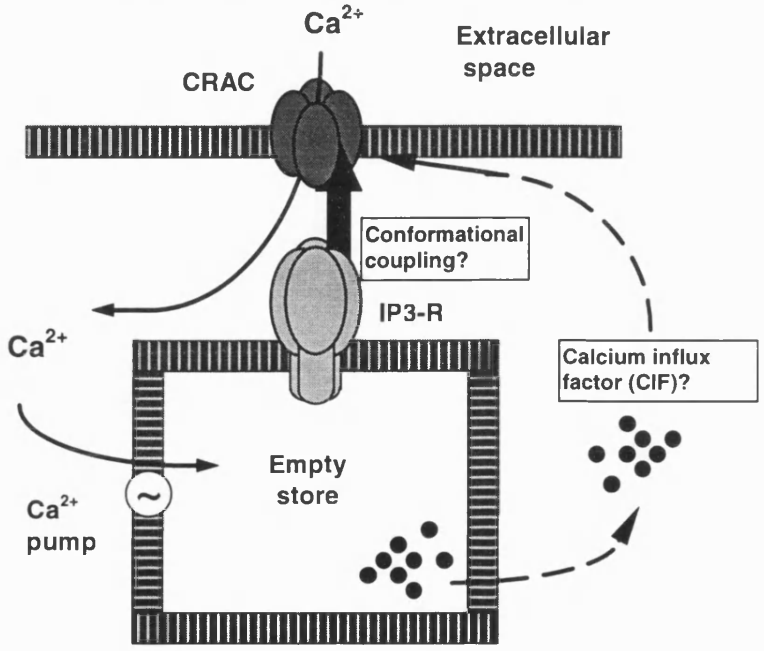


Figure 1.9 Two theories of capacitative Ca^{2+} entry: conformational coupling and a diffusible calcium influx factor (CIF).

A recent study claims that LU52396 (Figure 1.10) is the first pharmacological tool that selectively inhibits capacitative Ca^{2+} influx (K_i around 2μM in HeLa cells), although its selectivity is low, and it also inhibits Ca^{2+} fluxes through Ins(1,4,5)P_3 receptors, ryanodine receptors and Ca^{2+}-ATPases at higher concentrations.[30] LU52396 has three chiral centres, and the results were obtained with the mixture of eight stereoisomers, so there is the possibility that greater selectivity might be achieved when the individual isomers are synthesised and tested separately.

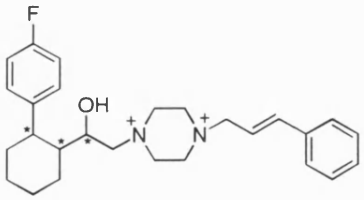


Figure 1.10 LU52396; recently claimed to be an inhibitor of capacitative Ca^{2+} entry.

1.7 Structure-Activity Relationships at Ins(1,4,5)P_3 Receptors

Axial 2-hydroxyl

The least important part of Ins(1,4,5)P_3. Of only minimal importance for binding.

> Can be deleted, changed to equatorial or replaced with fluorine with only slight effect on activity.
> C-2 can be replaced with pyranoside oxygen.
> Bulky substituents or phosphate group are tolerated.

> Equatorial 2-OH together with phosphorothioates at 1,4,5 and axial 3 or 6-OH gives low intrinsic activity partial agonists.

Equatorial 1-phosphate

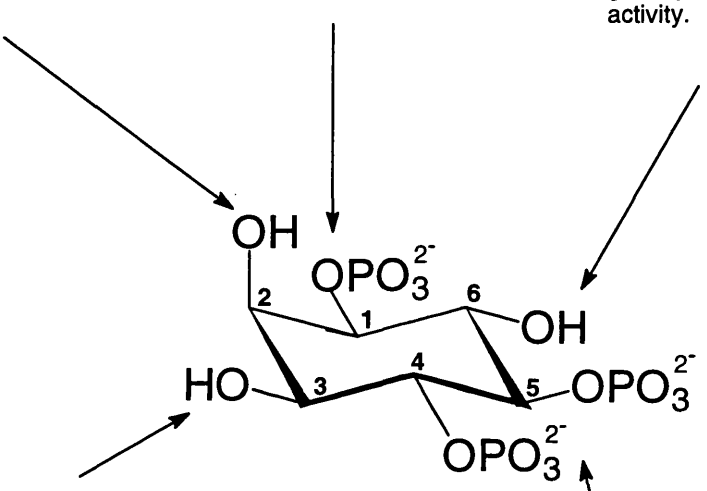
Less important than the 4- and 5-phosphates. Enhances binding, but not essential. May have a long-range interaction with the receptor binding site.

> Deletion greatly reduces activity, but replacement with axial phosphate or a phosphate more distant from the ring is tolerated.
> Large groups can be tolerated without major loss of activity.
> Replacement with phosphorothioate reduces activity only slightly.

Equatorial 6-hydroxyl

Very important for binding. May have H-bond interactions with binding site and/or affect conformation / ionisation state of 5-phosphate, probably *via* hydroxyl hydrogen rather than lone pair electrons.

> Deletion or changing to axial greatly reduces activity, but in combination with phosphorothioate at 1,4,5 gives partial agonists.
> Bulky substituents, equatorial phosphate or replacement with fluorine gives greatly reduced activity.



Equatorial 3-hydroxyl

Enhances binding, but less important than 6-hydroxyl. May have H-bond interactions with binding site probably *via* oxygen lone pair electrons.

> Deletion or changing to axial reduces activity, but less so than for the 6-hydroxyl.
> Replacement with fluorine reduces activity only slightly.
> Axial hydroxyl together with phosphorothioates at 1,4,5 gives a partial agonist.
> Increasing molecular volume of equatorial substituents is thought to correlate inversely with potency, but see Chapter 5.
> Equatorial or axial phosphate gives greatly reduced potency.

4,5-bisphosphate

The most important part of Ins(1,4,5)P_3 and essential for activity. Can be replaced with a bisphosphorothioate. Linking the 4- and 5-phosphates to give a cyclic pyrophosphate abolishes activity.

Equatorial 4-phosphate

Information is lacking on analogues selectively modified at this position.

> Deletion abolishes activity, changing to axial greatly reduces activity.
> Replacement with phosphorothioate has less effect on potency than replacing the 5-phosphate.

Equatorial 5-phosphate

Ionisation state correlates with the affinity of Ins(1,4,5)P_3 for its receptor.

> Deletion abolishes activity, effect of axial not known.
> Can be replaced with methylenephosphonate or phosphorothioate, to give metabolically resistant analogues causing prolonged Ca^{2+} release.

Figure 1.11 Structure-activity relationships for Ins(1,4,5)P_3 receptors.

Since 1986 there has been considerable interest in the synthesis of inositol polyphosphates and in establishing structure-activity relationships for the Ins(1,4,5)P_3 receptor. As a result, we now have a reasonable idea of which molecular features are associated with agonist activity, and which modifications will increase or decrease potency. Structure-activity relationships for the various enzymes involved in the interconversion of inositol phosphates have also been studied, but are currently less well-defined. The contemporary state of knowledge of these relationships has been comprehensively documented in a recent review[31] and there is no reason to reiterate it here. Instead, the important aspects of structure-activity relationships for the Ins(1,4,5)P_3 receptor are summarised in Figure 1.11, to which we will often return. The corresponding relationships for enzymes and other binding proteins will be discussed as they arise in the text.

The 4,5-bisphosphate appears to be the essential pharmacophore for activity at Ins(1,4,5)P_3 receptors. These two phosphate groups are vicinal and diequatorial, and the torsion angle O4–C4–C5–O5 is negative (Figure 1.12). It may be possible for vicinal phosphate groups positioned elsewhere in the *myo*-inositol ring (or in other rings, or even in some conformations of acyclic molecules) to mimic the 4,5-bisphosphate to greater or lesser extents, and this idea will be a recurring theme in what follows.

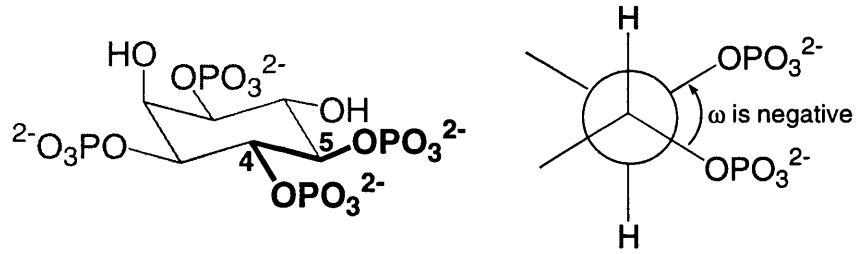
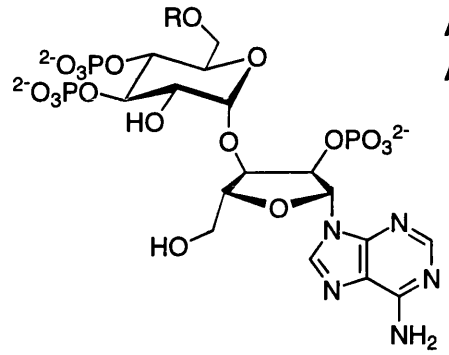


Figure 1.12 Definition of the term "4,5-bisphosphate".

In 1994 the discovery was announced of adenophostins A and B (Figure 1.13) from the culture broth of a fungus, *Penicillium brevicompactum*.[32] The adenophostins were reported to be astonishingly potent agonists at Ins(1,4,5)P_3 receptors, 100-fold more potent than Ins(1,4,5)P_3 itself. No compound had previously found to be *more* potent than Ins(1,4,5)P_3 and most modifications of the Ins(1,4,5)P_3 molecule had resulted in reduced potency. The publication of the structures of the adenophostins immediately confirmed most of the conclusions that had already been reached about the necessary features for Ins(1,4,5)P_3 receptor ligands, and which are detailed in Figure 1.11 (e.g. a 4,5-bisphosphate together with a third phosphate close to position 1, a 6-hydroxyl group, etc.) and yet the adenophostins also have some *additional* features.

At the time of writing, the significance of these additional components in the adenophostins, and the origin of their extreme potency is not understood. However, an explanation cannot be far away. The importance of the adenophostins is such that they are discussed separately, in Chapter 5.



Adenophostin A R = H

Adenophostin B R =$COCH_3$

Figure 1.13 Adenophostins A and B

The structure-activity principles outlined in Figure 1.11 are all derived from studies of analogues in which the 4,5-bisphosphate is on a six-membered ring. In Chapter 3 an excursion into new and uncharted territory is described: analogues based on a *five*-membered ring.

1.8 Antagonists at Ins(1,4,5)P_3 Receptors

The polysulphated polysaccharide heparin has been shown to be a potent competitive and reversible antagonist at Ins(1,4,5)P_3 receptors,[33] and it might seem likely that the anionic sulphate groups of heparin can somehow mimic the phosphate groups of Ins(1,4,5)P_3 at the receptor binding site. However, *myo*-inositol 1,4,5-trissulphate[34] has been shown to be inactive at Ins(1,4,5)P_3 receptors,[35] as has the hexadeoxy-1,4,5-tris(methylenesulphonic acid) analogue of Ins(1,4,5)P_3.[36] The design of antagonists based on heparin is made difficult by the large and flexible nature of the heparin molecule, and the use of heparin in studies of intact cells is complicated by the fact that heparin is also a potent inhibitor of Ins(1,4,5)P_3 3-kinase.[37] Heparin has also been reported to interact with the ryanodine receptor, causing Ca^{2+} release.[38] At millimolar concentrations, caffeine acts as an inhibitor of Ins(1,4,5)P_3-induced calcium release, probably by acting at an ATP binding site present on the cytoplasmic coupling domain of the receptor, rather than at the ligand binding site,[39] but like heparin, caffeine also interacts with ryanodine receptors.

It has recently been shown that physiological concentrations of polyamines can inhibit Ins(1,4,5)P_3-induced Ca^{2+} release and their potency is directly related to the number of positive charges on the molecule.[40] Furthermore, various tetraalkylammonium cations, particularly tetrahexylammonium ions (THA^+) have a similar effect, (higher concentrations of THA^+ (10-100μM) can *mobilise* Ca^{2+})[41] and may share a similar mechanism of action with polyamines, perhaps involving an interaction with negatively charged amino acids in the channel region of the receptor. It is not clear whether triethylammonium or cyclohexylammonium ions can have a significant effect on Ca^{2+} release, but as most of the compounds described in this thesis were isolated and used as the triethylammonium salts, the possibility must be borne in mind until experiments prove otherwise. It has also been reported that some reagents used as Ca^{2+} chelators may competitively antagonise binding of Ins(1,4,5)P_3 to the receptor.[42] This finding may be of importance, if only because these reagents (EDTA, EGTA, BAPTA, fura-2) are commonly used either to monitor or manipulate Ca^{2+} levels in biological studies of Ca^{2+} release, and may therefore give rise to experimental artefacts. Finally, the racemic 5-methylphosphonate analogue of Ins(1,4,5)P_3 was reported to antagonise Ins(1,4,5)P_3 -stimulated Ca^{2+} release in a pH-dependent manner in human platelets.[43] This finding would be of major significance if it could be confirmed, but further biological data are not available.

1.8.1 Partial Agonists at Ins(1,4,5)P_3 Receptors

It might be expected that Ins(1,4,5)P_3 analogues would show greater selectivity for the Ins(1,4,5)P_3 receptor than agents such as heparin, decavanadate or caffeine, and may therefore be better candidates as specific Ins(1,4,5)P_3 antagonists. Unfortunately it appears that, in general, the inositol phosphate analogues that bind to Ins(1,4,5)P_3 receptors also cause Ca^{2+} release, and must therefore be classified as agonists.* However, it sometimes happens that, while an analogue can fully displace [^{3}H]Ins(1,4,5)P_3 from its receptor, it induces only partial Ca^{2+} release compared to that releasable by Ins(1,4,5)P_3 alone (Figure 1.14). These compounds may be classified as partial agonists (or alternatively, partial antagonists).

* Evidence has recently been presented[54] that the naturally-occurring Ins(1,3,4,5,6)P_5 acts as a weak Ins(1,4,5)P_3 antagonist *in vitro* and that this may have physiological significance for Ins(1,4,5)P_3 receptor regulation *in vivo*.

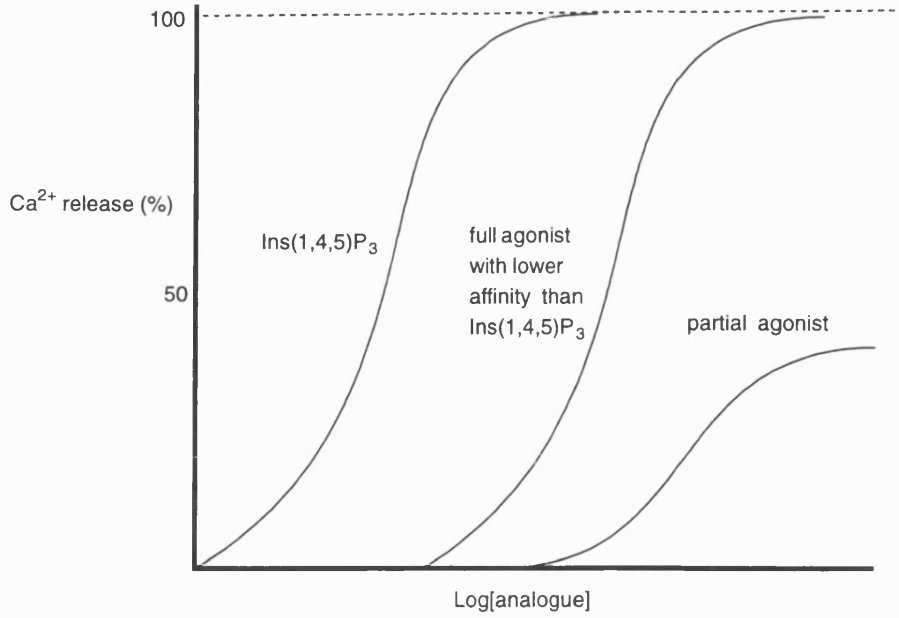
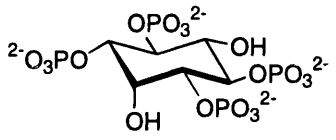


Figure 1.14 Theoretical dose-response relationships for full and partial agonists.

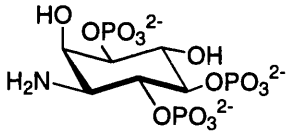
At least under some conditions, the symmetrical inositol tetrakisphosphate $Ins(1,3,4,6)P_4$ (Figure 1.15) has been found to behave in this way, releasing around 80% of the Ca^{2+} mobilised by $Ins(1,4,5)P_3$ when tested in SH-SY5Y neuroblastoma cells.[44] Again working with SH-SY5Y cells, it has been shown that when the maximally effective Ca^{2+}-releasing concentration of $Ins(1,3,4,6)P_4$ is administered together with $Ins(1,4,5)P_3$, the EC_{50} of the latter is increased.[44] This indicates that $Ins(1,3,4,6)P_4$ and $Ins(1,4,5)P_3$ are competing for the same site, and is strong evidence that, in this assay, $Ins(1,3,4,6)P_4$ is behaving as a true partial agonist. These observations were the stimulus for the synthesis of D-$Ins(1,3,4)P_3$ and D-$Ins(1,3,6)P_3$ [= L-$Ins(1,3,4)P_3$] described in Chapter 2, and also D-$Ins(1,4,6)P_3$ and D-$Ins(3,4,6)P_3$ (carried out by a colleague), in a collaborative attempt to identify which features of $Ins(1,3,4,6)P_4$ were responsible for its partial agonist properties. $Ins(1,3,4,6)P_4$ has since been tested in permeabilised platelets, which is the assay system used to evaluate most of the analogues described in this thesis, and appears to behave as a full agonist under these conditions,[45] further complicating the issue.* Unusual pH-dependent behaviour has been reported for D-3-amino-3-deoxy-*myo*-$Ins(1,4,5)P_3$ (Fig. 1.15) again in SH-SY5Y cells. Apparently this compound behaved as a full agonist at pH 7.2 and 7.6, but as a high intrinsic activity partial agonist (releasing about 80% of the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool) at pH 6.8.[46]

*However, the ability of $Ins(1,3,4,6)P_4$ to displace [H^3]$Ins(1,4,5)P_3$ from rat cerebellar membranes seems to indicate a binding affinity higher than would be expected from its moderate potency in the rabbit platelet assay for Ca^{2+}-release. The significance of this finding is not understood at present.

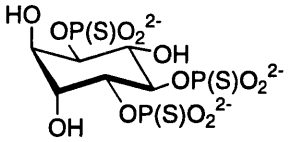
Two inositol *phosphorothioates*, L-*chiro*-inositol-2,3,5-trisphosphorothioate [L-*chiro*-Ins(2,3,5)PS_3] and D-6-deoxy-*myo*-inositol-1,4,5-trisphosphorothioate [D-6-deoxy-Ins(1,4,5)PS_3] have been shown to act as low intrinsic activity partial agonists, releasing only 34% and 42% of Ins(1,4,5)P_3-releasable Ca^{2+} respectively in SH-SY5Y cells.[47] Significantly, L-*chiro*-Ins(2,3,5)P_3 has now been tested in the rabbit platelet assay and shown to behave in a similar way, making it the first partial agonist at the platelet Ins(1,4,5)P_3 receptor.[45]



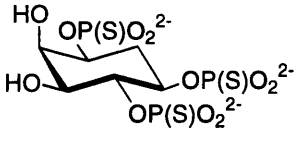
Ins(1,3,4,6)P_4



D-3-amino-3-deoxy-Ins(1,4,5)P_3



L-*chiro*-Ins(2,3,5)PS_3



D-6-deoxy-Ins(1,4,5)PS_3

Figure 1.15 Partial agonists at Ins(1,4,5)P_3 receptors.

All the partial agonists discussed above can be seen as Ins(1,4,5)P_3 analogues with modifications at C-3 or C-6, sometimes together with phosphorothioate substitution. It was these observations that led us to consider the synthesis of two phosphorothioate analogues, L-*myo*-inositol 1,3,4-trisphosphorothioate and DL-6-deoxy-6-hydroxymethyl-*scyllo*-inositol 1,2,5-trisphosphorothioate, described in Chapter 6, and also of DL-*myo*-inositol-1,4,6-trisphosphorothioate.[48,49]

Very recently, a study of three D-3-deoxy-3-fluoro-*myo*-Ins(1,4,5)P_3 phosphorothioate analogues provided the significant observations that their order of potency was as shown in Figure 1.16 and that of the three, only the 4,5-bisphosphorothioate appeared to be a partial agonist (in SH-SY5Y cells).[50] These results confirm the expectation that modification of the 5-phosphate is more perturbing for Ins(1,4,5)P_3 receptor binding than a similar modification of the 4-phosphate (see Chapter 4), and show that in this series, phosphorothioate substitution at both C-4 and C-5 was necessary for partial agonism.

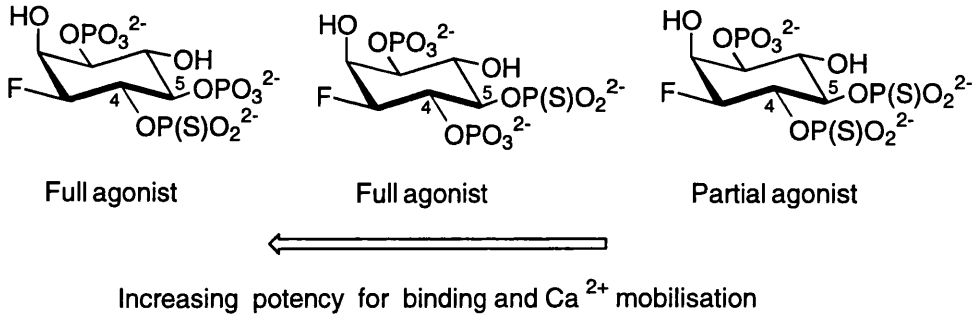


Figure 1.16 Consequences of selective phosphorothioate substitution at the 4,5-bisphosphate for Ins(1,4,5)P_3 receptor affinity and partial agonism. See Fauq *et al.* (1996)[50]

1.8.2 Antagonists and Partial Agonists: Conclusions

A major goal in the medicinal chemistry of inositol phosphates is the development of a specific competitive antagonist for Ins(1,4,5)P_3 receptors (and then, subtype-selective analogues). Unfortunately it appears that, for small-molecule Ins(1,4,5)P_3 receptor ligands, efficacy and affinity are difficult to disentangle, and it has not yet been possible to identify any structural feature that is related to one and not the other. Molecules that bind to the Ins(1,4,5)P_3 receptor with reasonably high affinity and yet appear to behave as partial agonists are therefore important lead compounds in the search for specific Ins(1,4,5)P_3 antagonists. However, it is becoming clear that Ins(1,4,5)P_3 -induced Ca^{2+} release is a complex phenomenon, and it may be necessary to exercise some caution in using the simple criteria illustrated in Figure 1.14 to identify these leads. It is known for example, that Ins(1,4,5)P_3 -sensitive Ca^{2+} stores can respond rapidly and transiently to a low concentration of agonist, and yet maintain the ability to release Ca^{2+} in response to higher concentrations (quantal Ca^{2+} release). This complicates investigation of the relationship between Ins(1,4,5)P_3 receptor occupation and Ca^{2+} release, and discrimination between agonists of different efficacy. Even a partial agonist may be able to deplete the Ca^{2+} stores given sufficient time,[47] particularly if it is resistant to metabolism, and only partial agonists with very low intrinsic efficacy will give a dose response curve similar to that in Figure 1.14. Finally, there may be problems involved in the detection of antagonistic effects when permeabilised, rather than intact, cells are used.[51]

1.9 Inositol Phosphate Metabolism

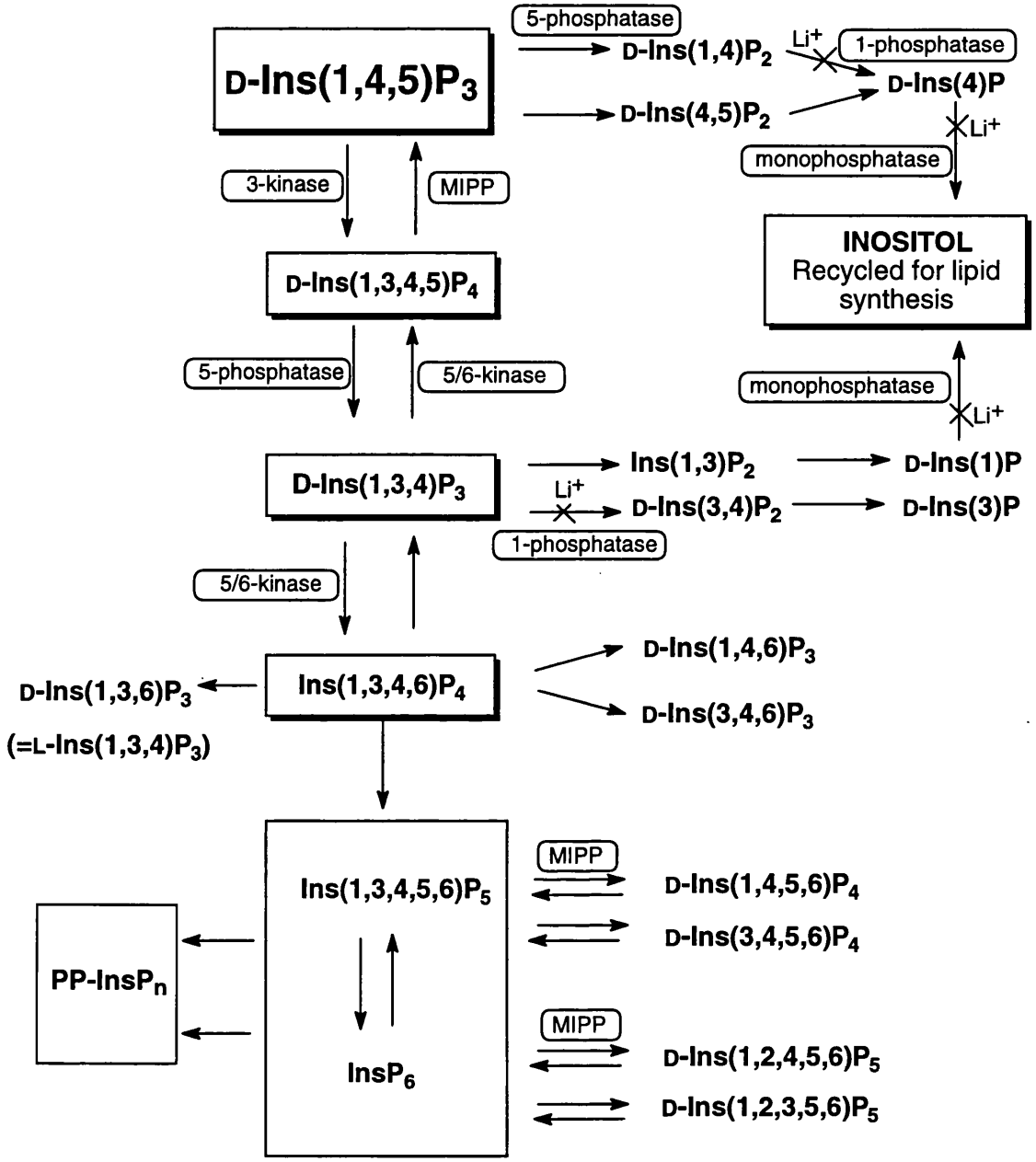


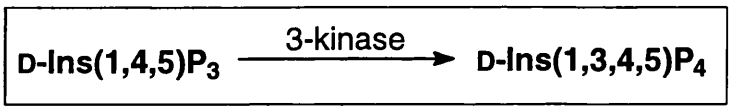
Figure 1.17 Metabolism of receptor-generated Ins(1,4,5)P_3 in mammalian cells.

Just as a cell must contain mechanisms for the generation of intracellular signals, it must also contain mechanisms for their termination. Cyclic nucleotides, (cAMP and cGMP), for example, are inactivated by the action of at least five classes of phosphodiesterase isoenzymes, and these enzymes are targets for therapeutic intervention in this signalling pathway. In a similar way, Ins(1,4,5)P_3 is a substrate for various enzymes, whose function may be seen as that of terminating the Ins(1,4,5)P_3 signal. However, it is now becoming clear that the metabolism of Ins(1,4,5)P_3 is complex, and the expanding number of metabolites, enzymes and isoenzymes reported

in the literature have brought this possibly simplistic view into question. Nearly half of the 63 possible monoesterified phosphate derivatives of inositol have been found in eukaryotic cells,[52] and it seems increasingly likely that some of these, particularly the higher inositol phosphates, will have important cellular functions of their own. Two of the most abundant inositol phosphates in cells are Ins(1,3,4,5,6)P_5 and $InsP_6$ for example, and many functions have been suggested for these highly charged molecules,[52-54] including the theory that $InsP_6$ may act as a binding site for iron, thus inhibiting iron-catalysed hydroxyl radical formation.[55] A new class of pyrophosphate-containing inositol polyphosphates (PP-$InsP_n$) has now been identified,[56] and a total synthesis of the enantiomers of 1-PP-$InsP_5$ has recently been reported.[57]

The remainder of this section provides a highly selective account of some of the enzymes involved in phosphoinositide metabolism. It deals only with those that are potential targets for the compounds synthesised in this work. The currently established pathways of Ins(1,4,5)P_3 metabolism in stimulated mammalian cells are diagrammed in Figure 1.17.

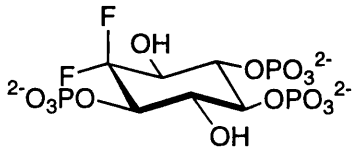
1.9.1 Inositol 1,4,5-trisphosphate 3-kinase



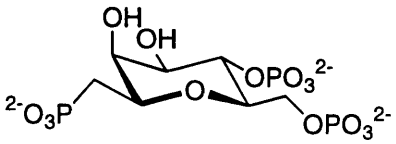
The enzyme D-*myo*-inositol 1,4,5-trisphosphate 3-kinase catalyses the phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4. Two isoenzymes, 3-kinase A and 3-kinase B have been identified and recent evidence suggests that the human forms are specifically expressed in different tissues and cells.[58] 3-Kinase seems to be highly selective for its substrates and there is increasing evidence that it is the key regulatory enzyme for Ins(1,4,5)P_3 metabolism. A number of mechanisms exist for the regulation of 3-kinase activity[59] and the enzyme is a substrate for the calcium-activated proteolytic enzyme calpain, which might provide a mechanism for regulation of 3-kinase levels.[60] Focal cerebral ischaemia in rats has been shown to result in a time-dependent irreversible decrease in 3-kinase activity, suggesting that 3-kinase is one of the target enzymes of cerebral ischaemia and that the resulting perturbation of Ins(1,4,5)P_3 metabolism may be an important factor underlying the changes in intracellular Ca^{2+} that lead to neuronal cell death.[61]

The specificity of 3-kinase poses problems for the design of inhibitors. Heparin inhibits the activity of bovine adrenal cortex cytosol 3-kinase in a non-competitive fashion[37] but, as noted above, has many disadvantages as a pharmacological tool. The

anthracycline antibiotic adriamycin, widely used in antineoplastic therapy has also been found to inhibit 3-kinase, but it too has numerous other biological effects.[62] Some 3-position-modified analogues of Ins(1,4,5)P_3 act as 3-kinase inhibitors, but also release calcium.[63] Unexpectedly, L-2,2-difluoro-2-deoxy-*myo*-inositol-1,4,5-trisphosphate is also a competitive inhibitor (K_i=11.9μM)[64] and a carbohydrate-based 3-kinase inhibitor (K_i=26.8μM) synthesised from mannose has also been reported.[65] These two molecules are therefore important leads in the search for non-calcium releasing, small-molecule inhibitors of 3-kinase.



L-2,2-F_2-Ins(1,4,5)P_3



mannose-based analogue

Figure 1.18 Small-molecule 3-kinase inhibitors

All the new compounds described in this thesis are currently being examined for their interaction with a purified 3-kinase preparation. In particular, an investigation is being carried out into the effect of 3-kinase on 6-deoxy-6-hydroxymethyl *scyllo*-inositol 1,2,4-trisphosphate (Figure 1.19). The synthesis of this analogue is described in Chapter 5.

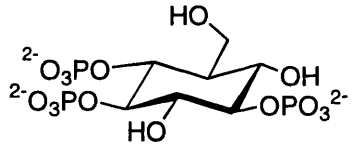
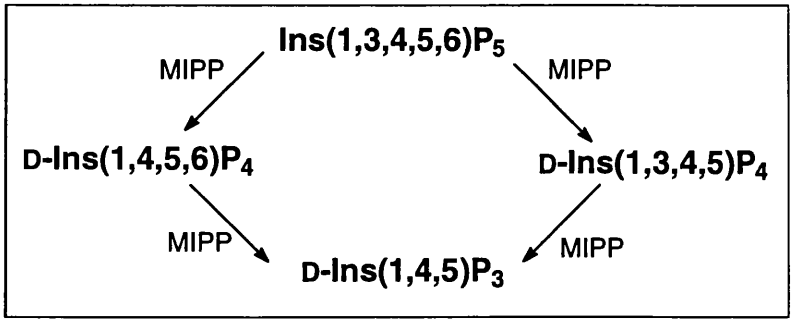


Figure 1.19 6-deoxy-6-hydroxymethyl *scyllo*-inositol 1,2,4-trisphosphate.

1.9.2 Multiple Inositol Polyphosphate Phosphatase (MIPP)



Ins(1,3,4,5)P_4 is a substrate for at least two metabolising enzymes, 5-phosphatase (see below) and a 3-phosphatase, both of which are potential targets for the design of inhibitors. The latter enzyme, (now re-named multiple inositol polyphosphate phosphatase, MIPP[66] has been shown to be capable of hydrolysing the 6-phosphate of

both Ins(1,3,4,5,6)P_5 and Ins(1,4,5,6)P_4, an observation which has been rationalised by considering the binding orientations in which these molecules can mimic Ins(1,3,4,5)P_4. This finding suggests that pathways might exist for the synthesis of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 independently of phospholipase C.[67]

Because Ins(1,3,4,5)P_4 is converted into Ins(1,4,5)P_3 *in vivo* by MIPP, it is possible that some effects apparently due to Ins(1,3,4,5)P_4 may result from its conversion to Ins(1,4,5)P_3. This problem has been addressed by the synthesis of the MIPP-resistant Ins(1,3,4,5)P_4 analogue *myo*-inositol-1,4,5-trisphosphate-3-phosphorothioate (Ins(1,3,4,5)P_4-3S).[68] In SH-SY5Y cells, this analogue was found to be essentially equipotent to Ins(1,3,4,5)P_4 in causing Ca^{2+} release,[69,70] providing evidence that, at least in this cell type, Ins(1,3,4,5)P_4 may be able to mobilise Ca^{2+} stores, independent of any conversion to Ins(1,4,5)P_3.

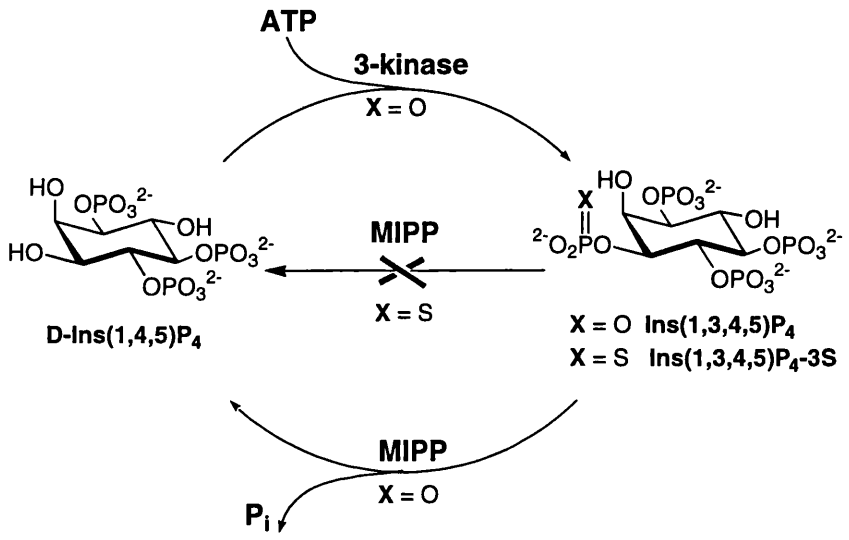
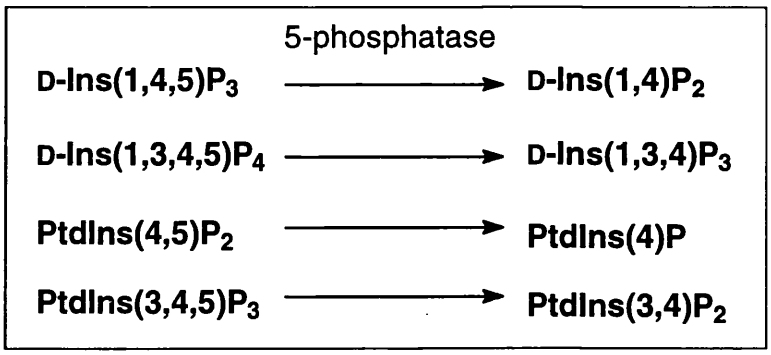


Figure 1.20 The resistance of the 3-phosphorothioate analogue of Ins(1,3,4,5)P_4 to the action of MIPP makes it a useful pharmacological tool.

In Chapter 7, a synthesis of the previously unknown *scyllo*-inositol analogue of Ins(1,3,4,5)P_4, is described. This symmetrical molecule, in which the equivalent to the 2-hydroxyl group is equatorial, rather than axial, should enable us to investigate the role of the 2-hydroxyl group in the mechanism of action of MIPP.

1.9.3 Inositol Polyphosphate 5-phosphatase



$Ins(1,4,5)P_3$ is dephosphorylated to the inactive D-$Ins(1,4)P_2$ by a family of specific 5-phosphatases. This metabolic step is therefore a highly effective method of inactivating the $Ins(1,4,5)P_3$ signal, as it removes the crucial 5-phosphate group, abolishing the pharmacophore in one step. There appear to be multiple types of 5-phosphatase, and both soluble, cytosolic forms and particulate, membrane-bound forms exist. For a review see Verjans *et al.* (1994).[71]

Three soluble, cytosolic 5-phosphatases have been identified and designated Type I 5-phosphatase. Type I 5-phosphatases have a mass of 40-45kDa and will hydrolyse both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$. A second, higher molecular mass group of cytosolic 5-phosphatases, which have reduced affinity for $Ins(1,3,4,5)P_4$, have been named Type II 5-phosphatase. Less is known about the particulate forms, which make up the majority of 5-phosphatase activity within the cell, although one such enzyme from human placental membranes has been purified.[72] Recently the cloning and expression of a cDNA encoding this enzyme has enabled its tissue distribution to be mapped, showing that this membrane-associated 5-phosphatase is predominantly expressed in heart, skeletal muscle and brain.[73]

A pattern seems to be emerging in which certain enzymes involved in the metabolism of inositol phosphates will also metabolise their inositol phospholipid counterparts. So for example, a Type II 75kDa 5-phosphatase from human platelets will also hydrolyse phosphatidylinositol 4,5-bisphosphate forming phosphatidylinositol 4-phosphate. The gene encoding this enzyme has been found to be highly homologous to the defective gene in Lowe's oculocerebrorenal syndrome (OCRL), a disease of unknown pathogenesis featuring defective development of the eyes, brain and kidney.[74,75] This discovery highlights the potential significance of the 5-phosphatases, and OCRL has become the first known example of an inborn defect in inositol polyphosphate metabolism.

It has been reported that inositol polyphosphate metabolism is deranged in lymphocytes infected with the HIV virus, and that the effect can be reversed by AZT therapy.[76] It seems that 5-phosphatase activity is reduced in patients infected with HIV, and that as the disease progresses, $Ins(1,3,4,5)P_4$-3-phosphatase (MIPP) is also decreased. In the later stages both activities are completely lost. A study of 5-phosphatase in normal and malignant haemopoietic cells also found that 5-phosphatase activity was significantly reduced or completely absent in subpopulations of cells taken from patients with various leukaemias.[77] The authors speculated that the half-life of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ may be increased in these cells, resulting in prolongation of Ca^{2+} signals. This might lead to aberrations in cellular regulatory processes, including those involved in cell proliferation and differentiation.

It has recently been reported that the 75kDa 5-phosphatase is also capable of hydrolysing phosphatidylinositol 3,4,5-trisphosphate, and growing evidence suggests that the potential signalling function of $PtdIns(3,4,5)P_3$ may be terminated by such an enzyme.[78] We can therefore imagine a pattern in which the initiation and termination of inositol phospholipid signals in the plasma membrane parallels that of inositol phosphate signals in the cytosol (Figure 1.21).

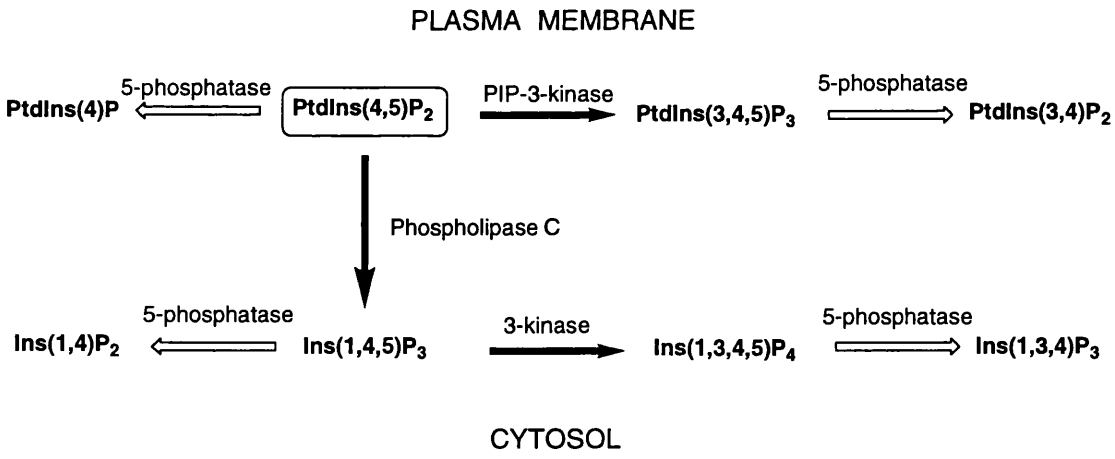


Figure 1.21 Parallels between phospholipid and inositol phosphate metabolism.

Very recently, the central region of synaptojanin, a nerve terminal protein, has been shown to be highly homologous to both type I and II 5-phosphatases.[79] The purified protein hydrolyses $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$ and $PtdIns(4,5)P_2$. The N-terminal domain of the same protein is similar to the cytosolic domain of the yeast Sac1 protein which is genetically implicated in inositol phospholipid metabolism. Synaptojanin therefore incorporates two separate domains which are linked to

phosphoinositide metabolism (hence the name, from the Roman god with two faces, Janus). The C-terminal domain binds the SH3 domain of amphysin, which is also bound by dynamin, a presynaptic protein implicated in endocytosis. The fact that synaptojanin is highly concentrated at the nerve terminal provides strong evidence for a link between phosphoinositide metabolism and synaptic vesicle recycling.

In contrast to Ins(1,4,5)P_3-3-kinase, it seems that 5-phosphatase is relatively non-specific. A range of phenothiazines, including chlorpromazine and trifluoperazine (which is also a potent calmodulin antagonist) inhibit both soluble and particulate 5-phosphatases, as does calmidazolinium chloride[80] (another calmodulin antagonist), disulfiram, and several of its analogues.[81]

The most potent 5-phosphatase inhibitor yet discovered is L-*chiro*-Ins(2,3,5)PS_3.[82] However, this compound is also a 3-kinase inhibitor and a partial agonist at Ins(1,4,5)P_3 receptors (see above). More selective inhibitors[83] include L-Ins(1,4,5)PS_3, the *meso*-compound Ins(1,3,5)PS_3, and (1*R*,2*R*,4*R*)-cyclohexane-1,2,4-tris(methylenesulphonate)[84] The most promising candidate so far in the search for a potent, selective 5-phosphatase inhibitor is L-*chiro*-inositol 1,4,6-trisphosphorothioate [L-*chiro*-Ins(1,4,6)PS_3] which, with its two axial phosphorothioate groups, seems to be structurally quite different from the other analogues. A possible rationalisation for the potency of L-*chiro*-Ins(1,4,6)PS_3 is that its three phosphorothioate groups may mimic the phosphate groups of Ins(1,4,5)P_3 if it were to bind with its ring orthogonal to the usual orientation. An alternative explanation may be that in solution under physiological conditions, a proportion of L-*chiro*-Ins(1,4,6)PS_3 may exist in the alternative chair form which has features in common with L-Ins(1,4,5)PS_3 (Figure 1.22).

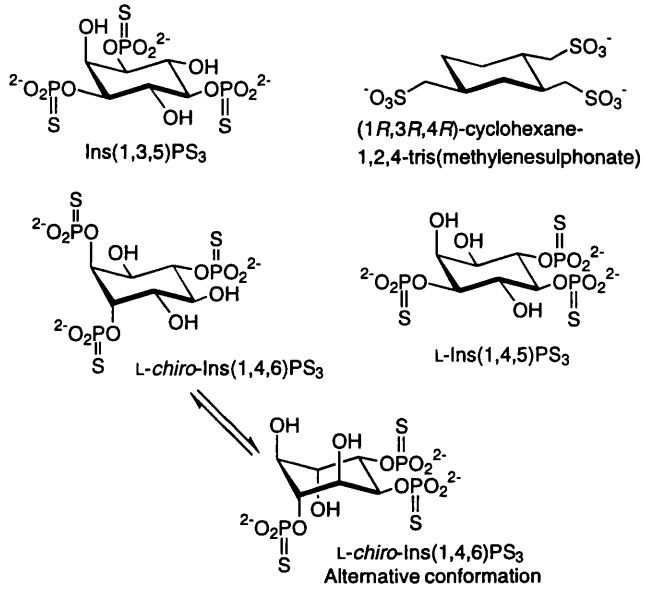
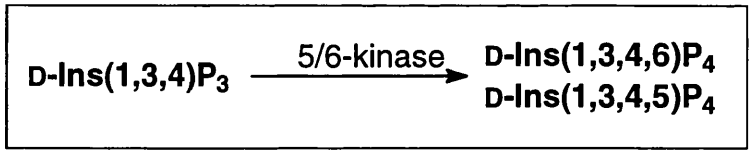


Figure 1.22 Small-molecule 5-phosphatase inhibitors.

1.9.4 Inositol 1,3,4-trisphosphate 5/6-kinase



In all vertebrate cells studied so far, D-Ins(1,3,4)P_3 is phosphorylated to produce Ins(1,3,4,6)P_4 and D-Ins(1,3,4,5)P_4. It is not established at present whether these two tetrakisphosphates have signalling functions of their own, or whether their cellular role is as precursors of Ins(1,3,4,5,6)P_5 and $InsP_6$. The activity responsible for phosphorylating D-Ins(1,3,4)P_3 has been purified and appears to be a single enzyme, which combines 6-kinase and 5-kinase activities in a ratio of roughly 5:1.[85] It is widely distributed and unaffected by Li^+, Ca^{2+}/calmodulin, protein kinase A or protein kinase C.[86] The affinity of this enzyme for D-Ins(1,3,4)P_3 is the highest yet determined for an inositol phosphate.

An initial structure-activity study of the interaction of the kinase with various inositol polyphosphates has been reported.[87] It was found that several inositol trisphosphate isomers inhibited D-Ins(1,3,4)P_3 kinase activity. They fell into two groups: $InsP_3$ isomers having vicinal phosphate groups at positions 5 and 6 [D-Ins(1,5,6)P_3 and D-Ins(3,5,6)P_3] were moderately potent inhibitors, whilst $InsP_3$ isomers containing a vicinal 4,5-bisphosphate were much weaker. Thus the essential pharmacophore for D-Ins(1,3,4)P_3 kinase may be a 3,4-bisphosphate, (the role of the third phosphate is not yet clear) but a 5,6-bisphosphate may be able to mimic this to some extent. Structure-activity principles therefore become, to some extent analogous to those already developed for Ins(1,4,5)P_3 analogues at the Ins(1,4,5)P_3 receptor. The suggested L-Ins(1,3,4)P_3 kinase pharmacophore is *enantiomorphic* with the minimal 4,5-bisphosphate pharmacophore identified for Ins(1,4,5)P_3 receptor (Figure 1.23).

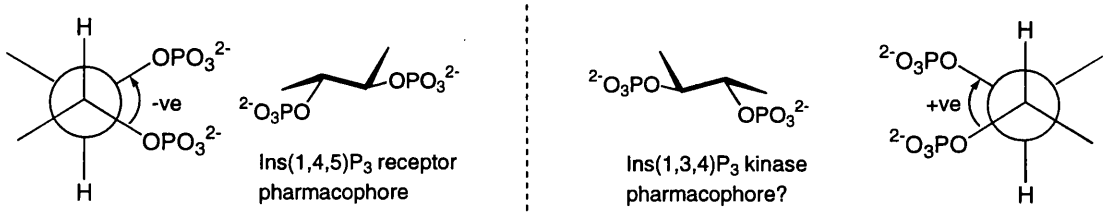


Figure 1.23 Pharmacophores for recognition by the Ins(1,4,5)P_3 receptor and by Ins(1,3,4)P_3 6-kinase may be enantiomorphic

This observation provides some justification for synthesising *both* enantiomers of novel polyphosphates, because, while one enantiomer may be recognised by the Ins(1,4,5)P_3 receptor, its non-calcium releasing antipode may be a D-Ins(1,3,4)P_3 kinase ligand. An excellent example of this principle is the case of Ins(1,3,4)P_3 itself (Chapter 2). The D-enantiomer has the highest affinity of any known molecule for the kinase and, as we will show, does not release calcium, while the L-enantiomer is an Ins(1,4,5)P_3 receptor agonist and (preliminary results indicate) interacts only weakly with the kinase.

The most potent competitive inhibitors of D-Ins(1,3,4)P_3 kinase were three inositol tetrakisphosphates; D-Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and D-Ins(3,4,5,6)P_4.[87] The first two are products of the kinase and may physiologically regulate its action through feedback inhibition. The third, and most potent of the three, D-Ins(3,4,5,6)P_4, is also present in the cytosol at physiologically relevant concentrations. Note that this molecule has both a 3,4- and a 5,6-bisphosphate, and its resistance to phosphorylation might be rationalised by visualising four possible binding modes which retain the 3,4-bisphosphate structure of D-Ins(1,3,4)P_3 (Figure 1.24). In each orientation, the area of the molecule corresponding to the site of action of the enzyme in Ins(1,3,4)P_3 is either blocked by the presence of phosphates or has vicinal *cis*-hydroxyl groups rather than *trans*.

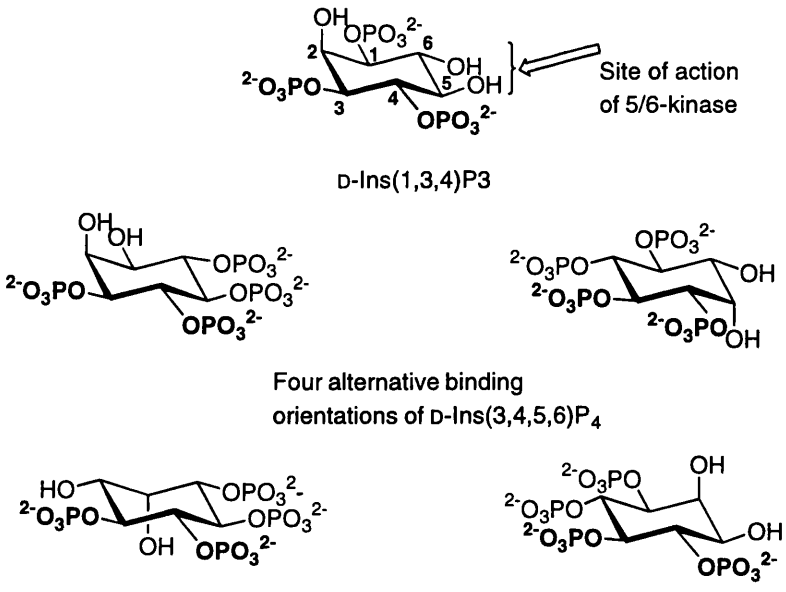
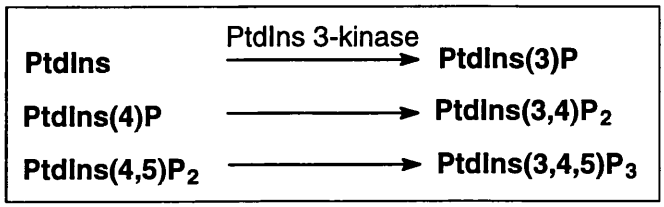


Figure 1.24 Possible binding modes of D-Ins(3,4,5,6)P_4 to Ins(1,3,4)P_3 6-kinase.

One can therefore imagine many potential inhibitors in which the recognition elements present in Ins(1,3,4)P_3 are retained, but this part of the molecule is modified (e.g. by deletion of hydroxyls or replacement with fluorine). It might also be possible to

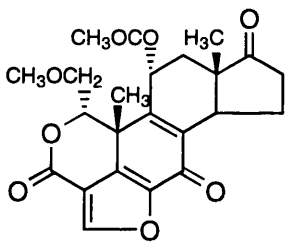
develop metabolically resistant, monosaccharide-based D-Ins(1,3,4)P_3 mimics. All the compounds described in this thesis are currently under evaluation as part of a major investigation into structure-activity relationships for Ins(1,3,4)P_3 5/6-kinase, and the synthesis of the natural substrate for this enzyme [D-Ins(1,3,4)P_3] is described in Chapter 2.

1.9.5 Phosphatidylinositol 3-kinase

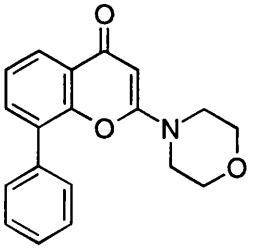


Phosphatidylinositol 3-kinase (PtdIns 3-kinase) phosphorylates the D-3-position of the inositol head-groups of phosphatidylinositol (PtdIns), phosphatidylinositol 4-monophosphate [PtdIns(4)P] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] giving D-3-phosphatidylinositol lipids, which are not substrates for phospholipase C. The enzyme is a heterodimer, comprising a 85kDa regulatory subunit and a 110kDa catalytic subunit. Evidence is accumulating that some or all of its 3-phosphorylated lipid products [and particularly PtdIns(3,4,5)P_3] may have second messenger functions of their own. Any attempt to summarise the rapidly expanding field of phospholipid signalling is outside the scope of this work, which is primarily concerned with the inositol phosphates, but the area has recently been reviewed.[88]

Inhibitors of PtdIns 3-kinase may help to clarify the role of this enzyme and its metabolic products in cells. The most widely used inhibitor at present is the fungal metabolite wortmannin, which is active at nanomolar concentrations, but also inhibits other enzymes (e.g. myosin light chain kinase and phospholipase D). The chromone derivative LY294002 (Figure 1.25) has recently been developed as the result of a structure-activity study of several chromones[89] and is claimed to be a specific inhibitor of PtdIns-3-kinase, although like wortmannin, it acts at the ATP site of the 110kDa catalytic subunit.



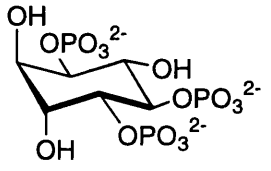
Wortmannin



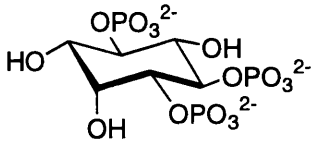
LY294002

Figure 1.25 Inhibitors of PtdIns 3-kinase currently in use as pharmacological tools.

In the light of the parallels that seem to exist between inositol phospholipid metabolism and the better-understood metabolism of the inositol phosphates, it might be instructive to examine the interaction of PtdIns 3-kinase with inositol phosphates and their analogues, many of which are now available. Such a study has been carried out recently.[90] Of the many analogues tested* most were inactive, but significantly, L-*chiro*-inositol 2,3,5-trisphosphate (and the corresponding trisphosphorothioate) were found to be inhibitors. Now L-*chiro*-Ins(2,3,5)P_3 can be considered as Ins(1,4,5)P_3 in which the equatorial 3-position hydroxyl group is replaced by an axial hydroxyl group. It may not be surprising that a structural alteration at the D-3 position, which is the site of phosphorylation by PtdIns 3-kinase, should have this effect, but D-Ins(1,4,6)P_3 was not recognised. A comparison of the structures of D-Ins(1,4,6)P_3 and L-*chiro*-Ins(2,3,5)P_3 (Figure 1.26) show that they differ only in the orientation of the hydroxyl group corresponding to the 2-hydroxyl of Ins(1,4,5)P_3. Thus there seems to be a requirement for axial hydroxyl groups at *both* 2- and 3- positions for inositol phosphates to inhibit PtdIns 3-kinase activity. One other analogue, benzene 1,2,4-trisphosphate (Bz(1,2,4)P_3), in which the inositol ring is replaced by the planar benzene ring† with three phosphate groups in a similar spatial arrangement to Ins(1,4,5)P_3, was also found to be an inhibitor.



L-*chiro*-Ins(2,3,5)P_3
Recognised



D-*myo*-Ins(1,4,6)P_3
Not Recognised

Figure 1.26 The orientation of the 2-hydroxyl group appears to be important to the recognition of inositol phosphates by PtdIns 3-kinase.‡

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* The syntheses of some of these are described in this thesis. Details are given in the appropriate chapters.

† A molecular modelling study has shown that benzene may not be as rigid as is sometimes supposed, and for small torsional deformations (<15°) it is as flexible as cyclohexane.[140]

‡ An X-ray crystal structure of Ins(1,4,5)P_3 bound to the PtdIns(4,5)P_2 binding site of mammalian PLC-δ has recently been published.[141] The axial 2-OH group of Ins(1,4,5)P_3 was seen to have a number of crucial

1.10 Syntheses of Two Naturally Occurring Inositol Phosphates

Chapter 2 describes the synthesis of the enantiomers of *myo*-inositol 1,3,4-trisphosphate, and in Chapter 7 a synthesis, strategically quite different, of the enantiomers of *myo*-inositol 1,3,4,5-tetrakisphosphate will be described. In each case there has been controversy in the biological literature over the roles of the naturally-occurring D-enantiomers of these compounds.

1.10.1 *myo*-Inositol 1,3,4-trisphosphate

We were interested in the enantiomers of Ins(1,3,4)P_3 (Figure 1.27) for two related reasons. First, there had been long-standing controversy in the literature as to the activity and role of the D-enantiomer, which is a major inositol phosphate in stimulated cells. Second, the previously unknown L-enantiomer has structural similarities to Ins(1,3,4,6)P_4, which had recently been found to behave as a partial agonist in SH-SY5Y neuroblastoma cells (see Section 1.8.1 and Chapter 6).

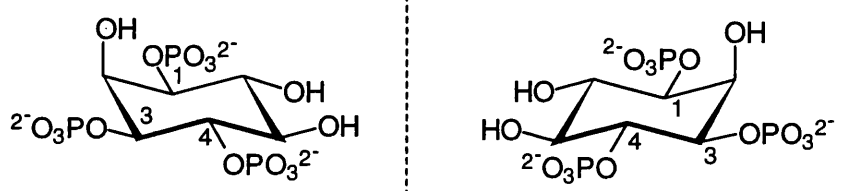

Figure 1.27 Enantiomers of *myo*-inositol 1,3,4-trisphosphate

1.10.2 D-*myo*-Inositol 1,3,4-trisphosphate

D-Ins(1,3,4)P_3 is well-established as one of the major inositol trisphosphates found in mammalian cells, and stands at a crucial branch-point in the metabolism of inositol phosphates. It has been isolated from biological sources and synthesised by various groups (see below). However, a survey of the literature presents us with a confusing, and sometimes contradictory, account of its biological activity (or lack of it) at Ins(1,4,5)P_3 receptors. The important features of the various literature reports are summarised below as a chronological list, in an attempt to minimise this confusion. The contradictions will quickly become apparent.

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interactions with amino acid residues at the active site (and also with one Ca^{2+} ion). Perhaps similar interactions explain the necessity for an axial 2-OH group in PtdIns(4,5)P_2-3-kinase ligands.

1986: D-Ins(1,3,4)P_3 is obtained by incubation of D-Ins(1,3,4,5)P_4 with human erythrocyte membranes. It is reported to release Ca^{2+} from Swiss 3T3 cells, with an EC_{50} of 9μmol. Speculation follows that D-Ins(1,3,4)P_3 may function to keep Ca^{2+} stores empty.[91]

1988: *Racemic* Ins(1,3,4)P_3 is synthesised. It releases Ca^{2+} from permeabilised aortic smooth muscle cells. The authors conclude that D-Ins(1,3,4)P_3 must be responsible.[92]

1988: Another group report that D-Ins(1,3,4)P_3 *does not* release Ca^{2+} from GH3 or Swiss 3T3 cells.[93]

1988: D-Ins(1,3,4)P_3 is found to be active in depolarising the ventral photoreceptors of the horseshoe crab (*Limulus polyphemus*) when injected intracellularly. It is reported to be "about half as potent as Ins(1,4,5)P_3." [94]

1988: D-Ins(1,3,4)P_3 is synthesised. It has a *negative* optical rotation.[95]

1988: [^{3}H]-D-Ins(1,3,4)P_3 and [^{3}H]-L-Ins(1,3,4)P_3 are synthesised. *Neither* shows binding displaceable by D-Ins(1,3,4)P_3 to rat brain receptor proteins.[96]

1992: D-Ins(1,3,4)P_3 is synthesised on a large scale by an enzymatic method.
It has a *positive* optical rotation. Authors comment on the discrepancy, but ". . . no further information on optically active Ins(1,3,4)P_3 is available".[97,98]

1992: When injected into *Limulus* photoreceptors, *some* samples of D-Ins(1,3,4)P_3 (including commercially available material) release Ca^{2+}. Others, produced by Gou and Chen, do not.[99]

While some of these contradictions might be explained by tissue-specific effects, or different experimental conditions, others, for example those involving *Limulus* photoreceptors, plainly cannot. It followed that some samples of D-Ins(1,3,4)P_3 were different from others. The obvious explanation is that some samples of D-Ins(1,3,4)P_3 were impure and/or that mistakes had been made regarding the absolute configuration of synthetic D-Ins(1,3,4)P_3. It was decided that the best way to provide a definitive solution to this problem would be to synthesise both enantiomers of Ins(1,3,4)P_3 in highly pure form, prove their absolute configurations beyond doubt, and then test them under

identical conditions in *Limulus* photoreceptors. In fact, these photoreceptors are sufficiently large that it would be possible to test both enantiomers *in the same cell* using a double-barrelled microelectrode.

1.10.3 L-*myo*-Inositol 1,3,4-trisphosphate: Relationship to Ins(1,3,4,6)P_4

The L-enantiomer of Ins(1,3,4)P_3 might also be useful in the search for partial agonists at Ins(1,4,5)P_3 receptors, because of its structural relationship to Ins(1,3,4,6)P_4. Recall that a 4,5-bisphosphate system has been accepted as a necessary feature for binding to the Ins(1,4,5)P_3 receptor (Section 1.7). Ins(1,3,4,6)P_4 plainly does not possess this structure, and therefore should not bind. However, it is possible to imagine Ins(1,3,4,6)P_4 in two different orientations relative to D-Ins(1,4,5)P_3, in which two of the phosphate groups in Ins(1,3,4,6)P_4 might mimic a 4,5-bisphosphate (a "pseudo-4,5-bisphosphate" arrangement[100,101]). The situation is best appreciated by the manipulation of computer-generated molecular graphics, but on paper, these orientations or binding modes can be represented as shown in Figure 1.28. In either one of the two binding modes shown, Ins(1,3,4,6)P_4 may be capable of presenting three phosphate groups to the receptor binding site in a way that mimics the spatial arrangement of the three phosphates in D-Ins(1,4,5)P_3. The extra phosphate now occupies a position analogous to position 2 in D-Ins(1,4,5)P_3, normally occupied by an axial hydroxyl group. The important differences between the two putative binding modes lies in the two hydroxyl groups which flank the pseudo-4,5-bisphosphate moiety. In binding mode (b) the equatorial 3-OH of D-Ins(1,4,5)P_3 is replaced by an axial OH group, while in (c) it is the 6-OH whose orientation is changed. It is not obvious, however, which of the two binding modes might be preferred by the receptor.

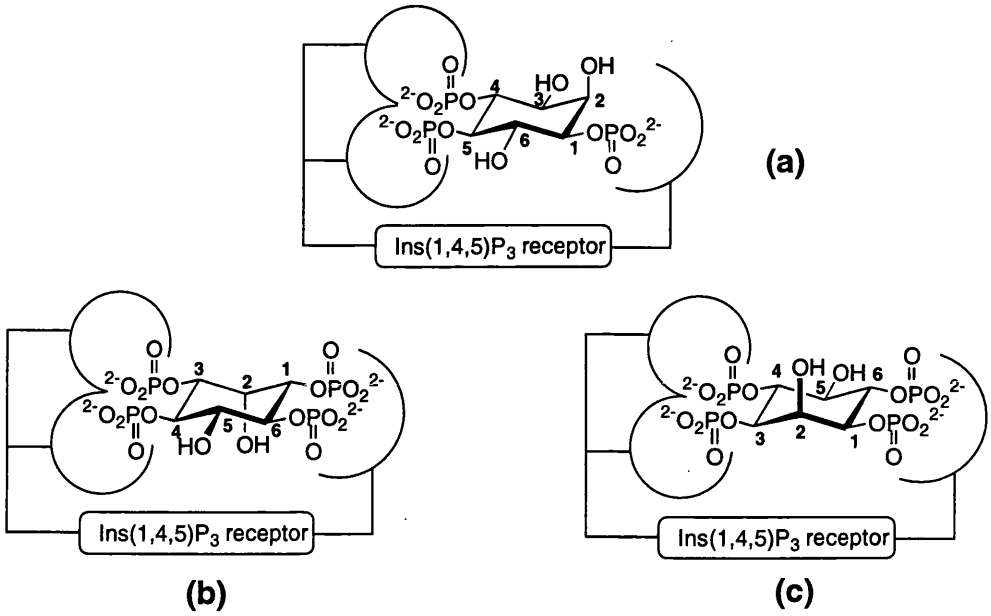

Figure 1.28 Possible binding orientations of Ins(1,3,4,6)P_4 at Ins(1,4,5)P_3 receptors.

Of course, it is possible that Ins(1,3,4,6)P_4 may bind to the receptor in *both* modes, it may bind to different conformational states[102] or isoforms of the receptor in different modes, and it is more than likely that this static view of binding is over-simplified. However, it might be possible to use Ins(1,3,4,6)P_4 as starting point for rational modification, with the aim of identifying the structural basis of its partial agonism and elucidating its mode of binding to the receptor. It might then be possible to design molecules with decreased Ca^{2+}-releasing activity, while maintaining or enhancing binding affinity.

Evidence exists that naturally occurring Ins(1,3,4,6)P_4 may be dephosphorylated[103] by enzymes that are still not well-characterised, to give D-Ins(1,3,4)P_3, D-Ins(1,4,6)P_3, D-Ins(1,3,6)P_3 [= L-Ins(1,3,4)P_3] and D-Ins(3,4,6)P_3 [= L-Ins(1,4,6)P_3] (Figure 1.29).

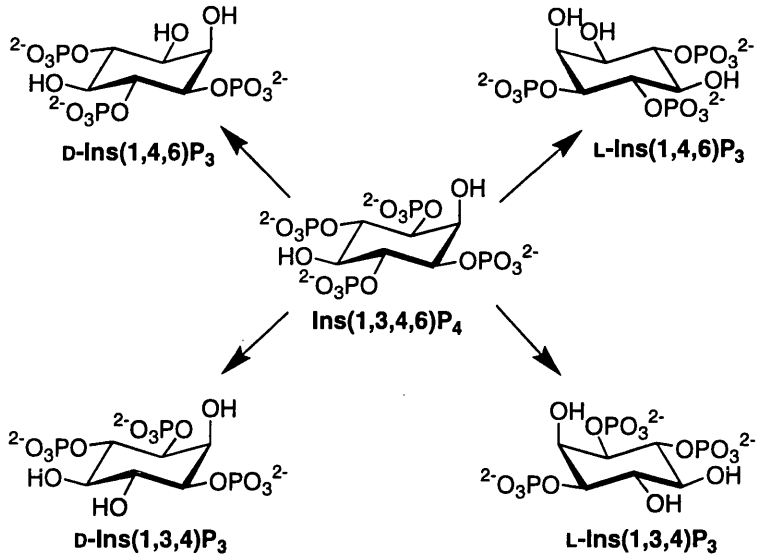

Figure 1.29 Relationship of the symmetrical Ins(1,3,4,6)P_4 to four chiral trisphosphates.

As discussed above, D-Ins(1,3,4)P_3 is already well-known and there were no reports of it showing partial agonist activity. The activity of the other trisphosphates was unknown, although D-Ins(1,4,6)P_3 had been identified in WRK rat mammary tumour cells,[104] and L-Ins(1,3,4)P_3, (alternative name D-Ins(1,3,6)P_3) had been identified as a minor inositol trisphosphate in avian erythrocytes and a product of Ins(1,3,4,6)P_4 dephosphorylation by brain cytosol.[103]

Now Figure 1.29 also illustrates the structural relationships of these five molecules. The four chiral trisphosphates can be regarded as simplified analogues of Ins(1,3,4,6)P_4, each formed by the removal of a different phosphate group. It might therefore be possible to determine what feature of Ins(1,3,4,6)P_4 leads to partial agonist activity by comparing the biological activities of the four trisphosphates.

The study would also enable us to find out the effect of changing the orientations of the hydroxyl groups of Ins(1,4,5)P_3. It can be seen from Figure 1.30 that, by analogy with Ins(1,3,4,6)P_4, each of these molecules can bind to the receptor in one of two possible orientations. However, in each case, only one of these orientations mimics the positioning of the three phosphate groups in Ins(1,4,5)P_3. In the case of D-Ins(1,4,6)P_3 (Figure 1.30), this mode is **(b)** while for L-Ins(1,3,4)P_3 it is **(e)**.

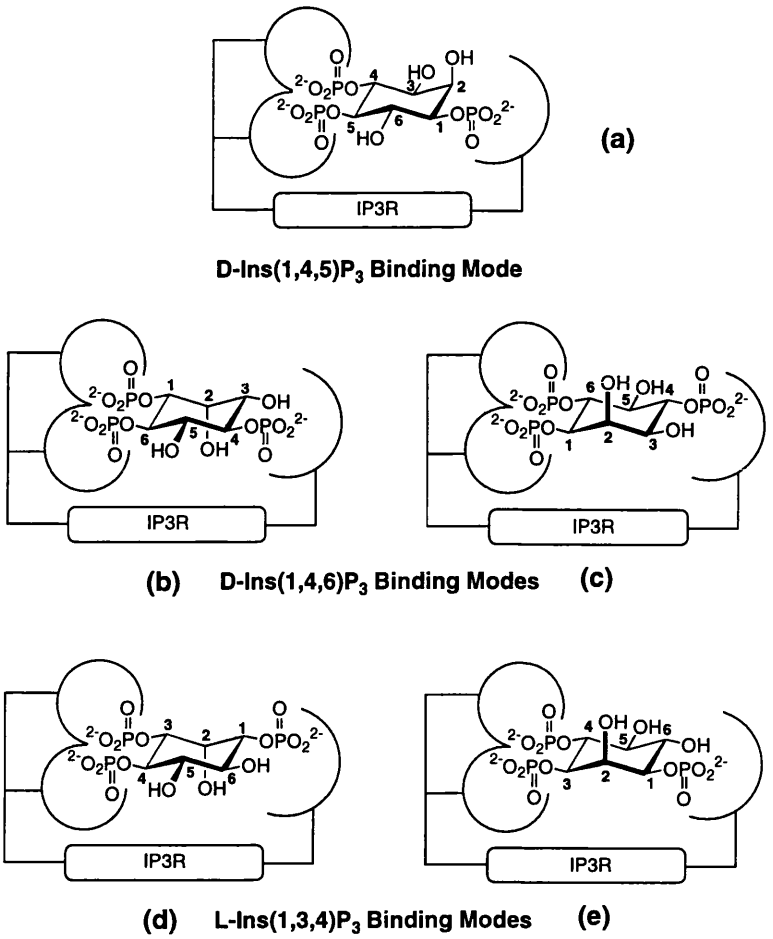

Figure 1.30 Possible binding modes (orientations) of D-Ins(1,4,6)P_3 and L-Ins(1,3,4)P_3 at the Ins(1,4,5)P_3 receptor.

Now in the D-Ins(1,4,6)P_3 binding mode (**b**), the axial hydroxyl group replaces the usual equatorial 3-hydroxyl group of Ins(1,4,5)P_3. In the L-Ins(1,3,4)P_3 binding mode (**e**), the positioning of the phosphates forces the axial hydroxyl group of L-Ins(1,3,4)P_3 into the equivalent position of the 6-hydroxyl group in Ins(1,4,5)P_3. The fact that these modes of binding have an equatorial rather than axial hydroxyl at the equivalent of Ins(1,4,5)P_3 position 2 is acceptable, because the 2-OH of Ins(1,4,5)P_3 has been shown to have a relatively insignificant role in receptor binding and Ca^{2+} release[105] with DL-*scyllo*-Ins(1,2,4)P_3 (which can be regarded as DL-*myo*-Ins(1,4,5)P_3 with an equatorial 2-OH) being only slightly less potent than Ins(1,4,5)P_3.[106]

Current opinion holds that the 3-OH group plays a minor role in receptor recognition while the 6-OH is critical (Figure 1.11). This has been deduced from the fact that D-3-deoxy-Ins(1,4,5)P_3[107,108] has a high affinity for the Ins(1,4,5)P_3 receptor while D-6-deoxy-Ins(1,4,5)P_3 is 70-fold less potent than D-Ins(1,4,5)P_3.[109] Thus we hypothesised that, if altering the orientation of an hydroxyl group has a similar effect to deleting it then, while both D-Ins(1,4,6)P_3 and L-Ins(1,3,4)P_3 should bind to the

Ins(1,4,5)P_3 receptor and release calcium, D-Ins(1,4,6)P_3 should bind with higher affinity.* Whether either of these molecules would also show partial agonist properties remained to be seen. This investigation is described in Chapter 2.

1.10.4 Inositol 1,3,4,5-tetrakisphosphate

The product of the action of 3-kinase on Ins(1,4,5)P_3 is D-*myo*-inositol 1,3,4,5-tetrakisphosphate. The level of controversy surrounding this molecule is currently very high, and often focuses on the detailed methodology of various biological assays, some of which seem to be giving conflicting results. However, the situation with respect to D-Ins(1,3,4,5)P_4 is far more complex than that already discussed for D-Ins(1,3,4)P_3, and only the briefest account will be given here.

Debate exists as to whether D-Ins(1,3,4,5)P_4 itself can mobilise intracellular Ca^{2+} stores by acting at Ins(1,4,5)P_3 receptors. Wilcox *et al.*[69,110,111] found that in SH-SY5Y cells, D-Ins(1,3,4,5)P_4 appears to cause Ca^{2+} release by direct interaction with the Ins(1,4,5)P_3 receptor, being a 40 fold weaker ligand and 20-fold weaker agonist than Ins(1,4,5)P_3. In contrast, using L1210 cells, Cullen *et al.*[112] found that D-Ins(1,3,4,5)P_4 caused no Ca^{2+} mobilisation in the absence of Ins(1,4,5)P_3. Gawler *et al.*[113] found that, using racemic Ins(1,3,4,5)P_4, there appeared to be a synergistic effect of Ins(1,3,4,5)P_4 in enhancing Ca^{2+} release by Ins(1,4,5)P_3, but also that Ins(1,3,4,5)P_4 was capable of releasing Ca^{2+} from intracellular stores by itself. Other workers have reported that D-Ins(1,3,4,5)P_4 acts synergistically with Ins(1,4,5)P_3 but is not itself able to induce Ca^{2+} release.[114]

Another issue is the possible existence of specific receptors for D-Ins(1,3,4,5)P_4.[115] The identification of an D-Ins(1,3,4,5)P_4-activated Ca^{2+} channel in the plasma membrane of endothelial cells[116] has lent weight to suggestions that D-Ins(1,3,4,5)P_4 may somehow modulate Ca^{2+} influx across the plasma membrane.[117] Specific binding sites for D-Ins(1,3,4,5)P_4 have been identified in several tissues, and a D-Ins(1,3,4,5)P_4 binding protein, purified from porcine platelets, has been demonstrated to be a member of the GAP1 family.[118] The affinity of this site, now designated $GAP1^{IP4BP}$, for various *myo*-inositol phosphates has been the subject of two recent

* But what of the remaining possible binding modes? The D-Ins(1,4,6)P_3 binding mode (**c**) seems very unlikely, because the axial hydroxyl group would be placed at position 6, but the situation is less clear in the case of the L-Ins(1,3,4)P_3 binding mode (**d**). In this orientation, the L-Ins(1,3,4)P_3 molecule can avoid placing its axial hydroxyl group in the equivalent of the 6-position, but only at the expense of placing its 1-phosphate group at the equivalent of position 2 in Ins(1,4,5)P_3. We know that an equatorial phosphate group is *tolerated* in this position, because *scyllo*-Ins(1,2,4,5)P_4 is highly active, but this does not allow us to deduce whether binding mode (**d**) or (**e**) would be lower energy for L-Ins(1,3,4)P_3.

investigations. [119,120] Another high-affinity D-Ins(1,3,4,5)P_4 binding site, recently purified from mouse cerebellum and designated IP4BP/synaptotagmin II, may be involved in synaptic function.[121]

1.10.5 Syntheses of Optically Active Ins(1,3,4,5)P_4 :Two Examples

Several syntheses of racemic Ins(1,3,4,5)P_4 [122-125] and D-Ins(1,3,4,5)P_4 [97,98,126-128] have been reported in the literature. Baudin *et al.*[129] developed a synthesis of *both* enantiomers of Ins(1,3,4,5)P_4 from *myo*-inositol orthoformate by the routes shown in Figure 1.31. This is the only reported synthesis of L-Ins(1,3,4,5)P_4. Regioselective protection of the sterically most accessible hydroxyl group of the three[130] gave the symmetrical silylated orthoformate **1.1** which was then benzylated to give the racemate **1.2ab**. Treatment of the racemate with (+)-(*R*)-1-phenylethyl isocyanate in the presence of BuLi at -78°C gave a mixture of the diastereoisomers **1.3a** and **1.3b** and some starting material. The starting material could not be separated from the carbamates, but desilylation followed by medium-pressure liquid chromatography gave the diastereoisomers **1.4a** and **1.4b** plus starting material which was recycled. Benzylation of each diastereoisomer with benzyl trichloroacetamidate in the presence of trifluoromethanesulphonic acid gave the diastereoisomers **1.5a** and **1.5b**. Removal of the orthoformate groups with aqueous CF_3COOH, followed by aqueous ammonia gave **1.7a** and **1.7b** which treated with NaOEt in ethanol giving the enantiomeric tetrols **1.8a** and **1.8b**. The absolute configurations of **1.8a** and **1.8b** were deduced by converting the monobenzyl orthoformate intermediate **1.3a** into the known 1L-4-*O*-benzyl-*myo*-inositol **1.6a**. Phosphitylation/oxidation of the tetrols followed by hydrogenolysis and treatment with cyclohexylamine gave D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4 as the cyclohexylammonium salts, each in around 10% yield from the orthoformate. The authors also reported an enzymatic route, based on selective monodeacylation of *meso*-derivatives of *myo*-inositol by pig liver diesterase, although this strategy involved more synthetic steps.

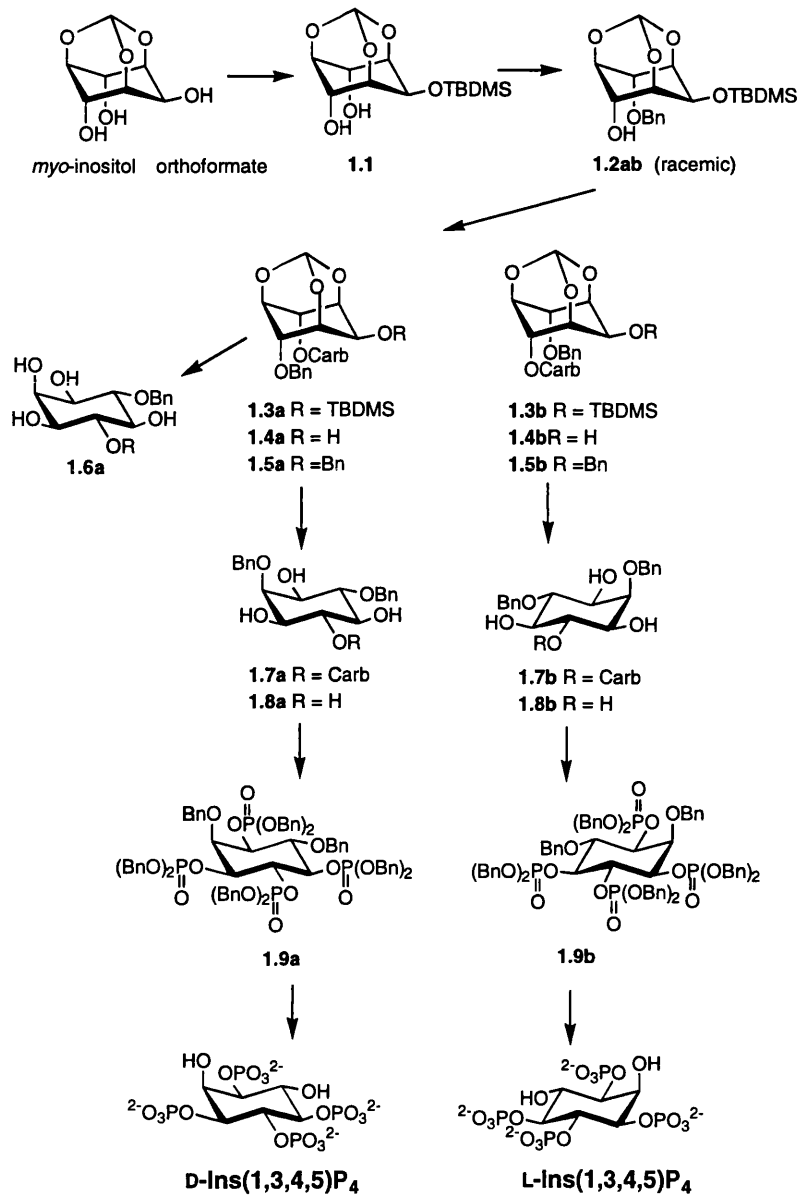

Figure 1.31 Synthesis of D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4 (Baudin *et al.*[129]).

More recently, a large-scale synthesis of D-Ins(1,3,4,5)P_4 has been reported by Gou and Chen[98](Figure 1.32). Regioselective stannylene-mediated acylation of racemic 1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (**DL-1.10**) gave the racemic butyrate ester **DL-1.11**. Enantiospecific hydrolysis of **DL-1.11** using porcine pancreatic lipase (PPL) gave 1D-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (**1.12**). Allylation followed by selective hydrolysis of the *trans* ketal gave **1.14,** which was also used for the synthesis of D-Ins(1,3,4)P_3 (see Chapter 2). Regioselective benzylation at the C-6 position *via* the *O*-stannylene acetal in the presence of CsF provided **1.15,** and allylation followed by removal of the 1,2-*O*-cyclohexylidene group gave **1.17**. 1-*O*-allylation followed by 2-*O*-benzylation furnished the fully-protected **1.19** and deallylation gave the known tetrol

1.8a. Phosphitylation/oxidation and finally debenzylation by catalytic hydrogenolysis gave D-Ins(1,3,4,5)P_4 in 38% overall yield from **1.12**.

This route, which was used to produce D-Ins(1,3,4,5)P_4 in multigram quantities, has been described in some detail because most commercially available material obtainable at present has been synthesised using this method by the University of Rhode Island Foundation Chemistry Group. Consequently, this has been the source of the samples used in many biological studies of Ins(1,3,4,5)P_4. The significance of this will be discussed in Chapter 7, where a very rapid and potentially large-scale synthesis of *both* enantiomers of Ins(1,3,4,5)P_4 is described, with particular reference to the purity of previously available D-Ins(1,3,4,5)P_4.

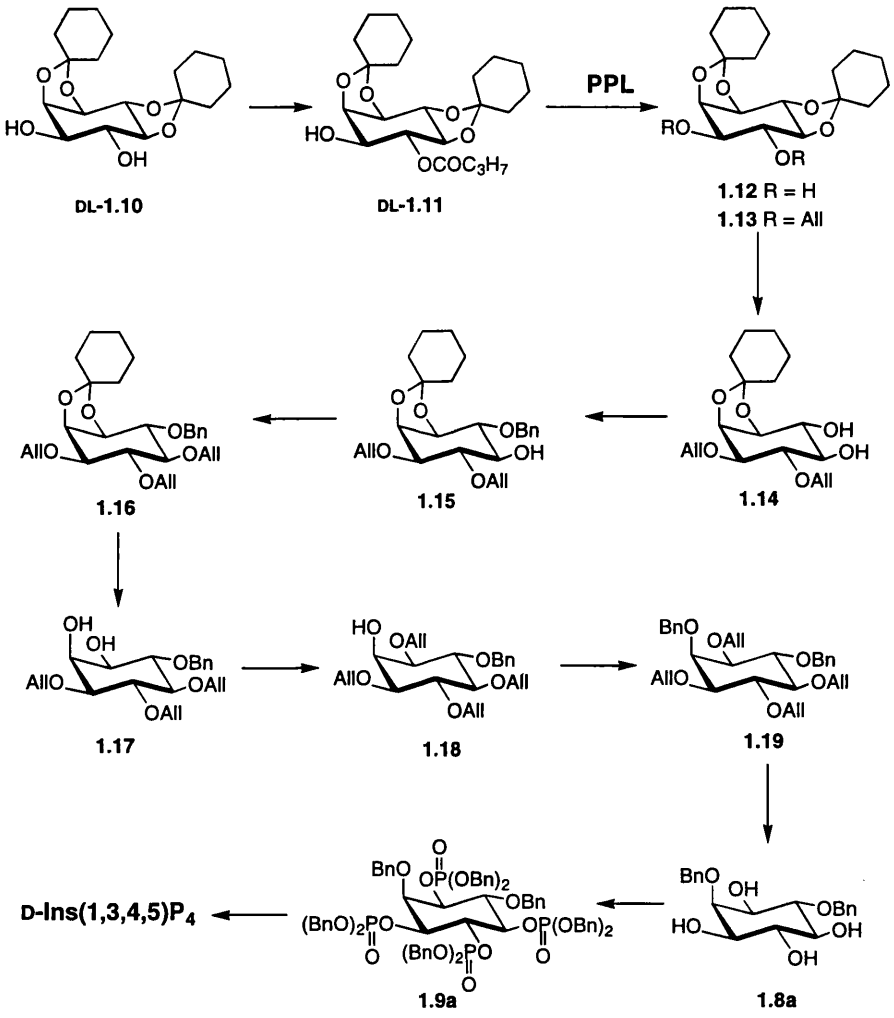

Figure 1.32 Chemoenzymatic route to D-Ins(1,3,4,5)P_4 (Gou and Chen [98])
PPL = porcine pancreatic lipase.

The routes discussed above also serve to illustrate some common features of most published syntheses of chiral inositol phosphates from *myo*-inositol. These can be summarised as:

1) **Selective protection** of hydroxyl groups.
2) **Resolution** of a protected intermediate (usually followed by more protection-deprotection steps).
3) **Phosphorylation** of exposed hydroxyl groups, either by P(V) or, more recently, P(III) methodology. The latter involves phosphitylation followed by oxidation.
4) **Deprotection** of hydroxyl groups and phosphate groups (sometimes simultaneously, sometimes in two stages).
5) **Purification** (e.g. by crystallisation or ion-exchange chromatography)

Much attention has been devoted to the first four of these steps over recent years (see References[131,31] for reviews) although little attention has been paid to step 5 (purification). It is now apparent that many inositol phosphates are biologically active at a range of sites, often in very low concentrations. It seems very likely that many of these activities are currently unknown, and even unsuspected. It will therefore become increasingly important that the samples used for biological investigations are subjected to a final purification step, (preferably ion-exchange chromatography or HPLC) and are demonstrated to be free of contaminants by a sensitive physical method. Failure to carry out these procedures may result in misleading biological results, and lead to confusion and unjustified speculation over the biological functions of naturally occurring inositol phosphates.

1.11 Ins(1,4,5)P_3 Conformation

No information is yet available on the three-dimensional structure of the active site of an Ins(1,4,5)P_3 receptor (although see below for a discussion of the recently-published X-ray structures of PH domains) and so we are presently limited to a study of the compounds that are active at these sites. In this situation, we attempt to make inferences about the active sites and the nature of the receptor-ligand interactions by systematically altering the structure of the natural ligand and observing the effects on activity. However, it is not always clear whether a change in activity, brought about by the modification of part of a molecule, results from a purely local effect, or whether there are more widespread consequences for the conformation or ionisation state of the molecule as a whole. Modification at C-3 of Ins(1,4,5)P_3, for example, can have various consequences for affinity and efficacy of analogues at Ins(1,4,5)P_3 receptors. Do these effects arise simply from the altered interaction of the 3-substituent with the receptor, or through effects on the orientation and ionisation state of the adjacent 4-phosphate

group? An additional phosphate group at this position, as in D-Ins(1,3,4,5)P_4 for example, must surely have dramatic effects on the 4-phosphate, and the evolution of a highly specific Ins(1,4,5)P_3 3-kinase may be associated with the functional consequences of these effects. We could even imagine cases in which modifications (e.g. an axial phosphate) could alter the overall conformation of the cyclohexane ring. This idea has been invoked to explain the unexpectedly high activity found for L-Ins(2,4,5)P_3 in one study.[132]

NMR studies can sometimes provide information on the relative proportions of equilibrating conformations in solution, but these may not necessarily resemble the receptor-bound conformation. The stabilising interactions in the receptor-ligand complex may be quite large, allowing considerable distortion of the ligand away from an energy minimum. This may be particularly relevant in the binding of a highly charged molecule such as Ins(1,4,5)P_3 with positively charged residues at the binding site, so that electrostatic interactions with the receptor may compensate the energetic penalty of large conformational changes. Molecular modelling studies can be used to predict energy minima in the conformational space available to a molecule, and information gained from NMR (e.g. NOE studies and coupling constants) can be used as constraints in theoretical calculations. By comparing a number of agonists with varying structures it may be possible to deduce a theoretical three-dimensional pharmacophore that is attainable at reasonable energetic cost by all active ligands. It is then possible to use this pharmacophore as a template for the design of receptor ligands.

In the case of Ins(1,4,5)P_3 however, we do not yet have available a range of active molecules that differ sufficiently in structure to make such a study feasible. The active molecules that we have are too similar to one another, and too conformationally mobile. In particular, the inositol phosphates that show significant affinity for the Ins(1,4,5)P_3 receptors all contain the 4,5-bisphosphate motif. This conformationally mobile system is regarded as essential for binding and Ca^{2+}-release, and yet little work has focused upon modification of this region of Ins(1,4,5)P_3.* Thus, little is known about the way in which these two phosphate groups interact with the receptor in the series of events that leads to the opening of the integral ion channel. Could it be, for

* After the synthesis of the conformationally restricted analogue described in Chapter 4, but before the publication of our results, a synthesis of 3-deoxy-D-*muco*-Ins(1,4,5)P_3, was published.[142] In this molecule, the configuration at C-4 is the opposite to that in Ins(1,4,5)P_3. The affinity of this analogue for the Ins(1,4,5)P_3 receptor is 3 orders of magnitude lower than that of Ins(1,4,5)P_3, further demonstrating the importance of the *trans* relationship of the 4- and 5-phosphates.

example, that the 4,5-bisphosphate binds to the receptor in one conformation, and then a mutual conformational change of receptor and ligand occurs during the opening of the ion channel? In that case, it might be possible to make an antagonist by fixing the bisphosphate in a conformation that binds to the receptor but cannot activate it (i.e. to separate affinity from efficacy).

The design and synthesis of a conformationally restricted analogue of Ins(1,4,5)P_3 are described in Chapter 4. Preliminary biological results are given, and an account of a detailed potentiometric and NMR investigation into its ionisation state and solution conformation.

1.12 PH Domains

PH domains are recently-discovered structural modules of around 100 amino acids that are present in many signal transduction proteins (e.g. protein kinases, phospholipases, PtdIns(4,5)P_2 3-kinase and GAP1^{IP4BP}). They also occur as part of some cytoskeletal proteins such as spectrin, and in dynamins,[133] which are involved in endocytotic vesicle formation. Many proteins implicated in human cancers and in developmental disorders contain PH domains, although the medical significance of this is currently not clear. Mutational changes in the PH domain of Bruton's tyrosine kinase are the cause of X-linked agammaglobulinaemia (XLA), a genetic disorder in which B lymphocytes fail to develop and consequently no circulating antibodies are produced, although the physiological ligand for this PH domain is presently unknown. A detailed review, dealing with the occurrence, structure and possible functions of PH domains has been published recently.[134]

In 1994 it was reported that the PH domain of pleckstrin bound PtdIns(4,5)P_2, and the ligand binding site was located to the N-terminus.[135] In November 1995, a study of an isolated PH domain from PLC-δ_1 showed that besides binding PtdIns(4,5)P_2, it was also able to bind inositol phosphates.[136] D-Ins(1,4,5)P_3 was bound stereospecifically and with highest affinity and various inositol tetrakisphosphates, including Ins(1,3,4,6)P_4 [but not D-Ins(1,3,4,5)P_4] also bound, as did D-Ins(2,4,5)P_3. Inositol mono- and bisphosphates and also D-Ins(1,3,4)P_3 were not recognised. Similar results have been reported for the β-spectrin PH domain.[137] All PH-domain-containing proteins are associated with membrane surfaces, and numerous lines of evidence now suggest that the PH domains are involved in reversible anchoring of these proteins to the membranes, often by binding to phosphoinositides. In the case of PLC-δ_1, for example,

a mechanism has been suggested [138] involving a negative feedback loop in which PLC-δ_1 uses its PH domain to bind PtdIns(4,5)P_2, in the cell membrane. The PLC enzyme than hydrolyses PtdIns(4,5)P_2 to give free Ins(1,4,5)P_3, which then inhibits the binding of further PtdIns(4,5)P_2 to the PH domain.

At the end of 1995, two groups independently published X-ray crystal structures of Ins(1,4,5)P_3 bound to pleckstrin homology (PH) domains.[137,139] These X-ray studies have allowed us to see, for the first time, the conformation of Ins(1,4,5)P_3 at one of its binding sites. This is shown schematically for the β-spectrin PH domain in Figure 1.33.

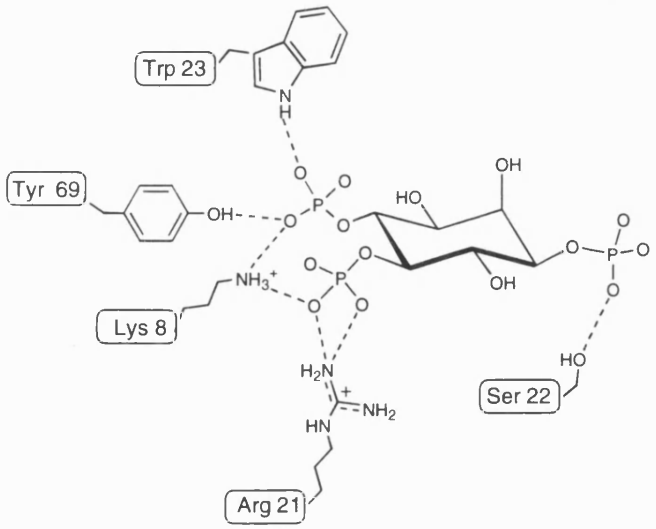

Figure 1.33 Schematic drawing of the Ins(1,4,5)P_3 binding site of the β-spectrin PH domain, showing hydrogen bonds and salt bridges between amino acid residues and ligand. Negative charges on phosphate groups are not shown. Adapted from Hyvönen *et al.* (1995)[137].

The first thing to notice is the importance of the 4 and 5-phosphate groups, which are anchored by salt bridges to positively charged amino acid residues, and by hydrogen bonds to Trp23 and Tyr69. The 1-phosphate group, in contrast, is linked by only one hydrogen bond to a Ser residue, and is mostly exposed to solvent. The upper face of the molecule, including the area around the axial 2-hydroxyl group is also open to solvent. Modelling of several inositol phosphates onto the bound Ins(1,4,5)P_3 ligand confirmed the importance if the 4,5-bisphosphate, and D-Ins(1,3,4)P_3 and L-Ins(1,4,5)P_3 did not fit the binding site. Although the 3-hydroxyl group was not close enough to Trp23 to interact with it directly, it did form an H-bond to one bound water molecule which was, in turn, H-bonded to the main-chain NH group of Trp23. It might be interesting to examine the binding affinity of the hydroxymethyl analogue **44** (Chapter 5) and the adenophostins at this site.

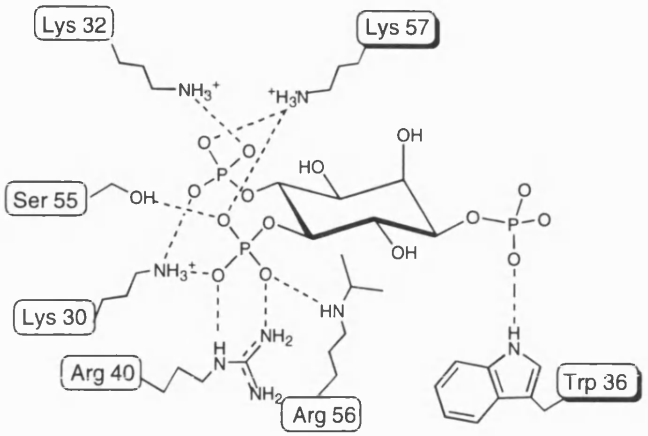

Figure 1.34 The Ins(1,4,5)P_3 binding site of the PLC-δ_1 PH domain. Adapted from Ferguson *et al.* (1995).[139]

The X-ray structure of Ins(1,4,5)P_3 at the PLC-δ_1 PH domain[139] shows that, by contrast with the β-spectrin PH domain, Ins(1,4,5)P_3 is significantly more buried within a binding pocket, and there are many more interactions with residues at the binding site, (Figure 1.34) particularly for the 5-phosphate (additional H-bonds *via* water molecules are not shown). This is reflected in the greater stability of the Ins(1,4,5)P_3-PLC-δ_1 complex. Again we see that the 4,5-bisphosphate is of primary importance for binding, while the 1-phosphate group forms a single hydrogen bond, this time to a Trp residue. The authors of this study make the remarkable observation that Trp 36 and Arg 40, which interact with the 1- and 5-phosphate groups of Ins(1,4,5)P_3 are analogous to two residues in the Bruton's kinase PH domain which, when altered by site-directed mutagenesis, result in XLA. Finally, both studies suggest that one function of the β-spectrin and PLC-δ_1 PH domains is to anchor the respective proteins to the cell membrane in a reversible way. This is illustrated schematically for the β-spectrin PH domain in Figure 1.35.

These two studies have been dealt with here in some detail because they represent an exciting new departure in the inositol phosphate field. For the first time, the interaction of Ins(1,4,5)P_3 with binding sites has been observed in detail, and the results are in excellent agreement with the conclusions reached about Ins(1,4,5)P_3 receptor binding as a result of structure-activity investigations conducted over a period of years. This is not to say that the Ins(1,4,5)P_3 binding site of these PH domains necessarily resembles that of Ins(1,4,5)P_3 receptors. Note, for example, that neither study showed any interactions of the 6-hydroxyl group of Ins(1,4,5)P_3 with the binding site, and yet this feature is known to be important for binding to Ins(1,4,5)P_3 receptors. However,

many of the structure-activity principles deduced for Ins(1,4,5)P_3 receptors can be accommodated by the binding site structures shown in Figures 1.33 and 1.34, and this may suggest that similar interactions and spatial relationships are involved.

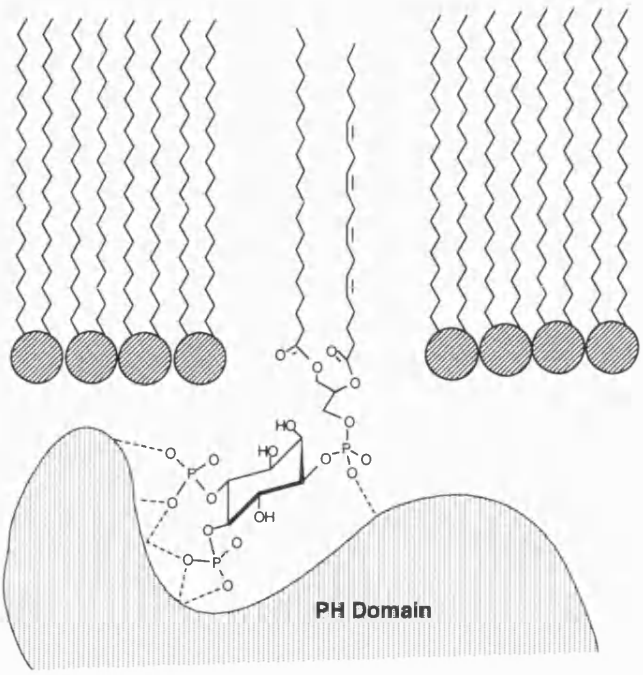

Figure 1.35 Schematic drawing of the proposed interaction of PtdIns(4,5)P_2 with the PH domain of β-spectrin. Adapted from Hyvönen *et al.* (1995)[137].

It will be interesting to investigate the interactions of a range of naturally-occurring and synthetic analogues with the binding sites of PH domains. Some of the compounds whose synthesis is described in this thesis are already being used in an NMR investigation of inositol phosphate binding by the PH domain of human dynamin.

2 *myo*-Inositol 1,3,4-Trisphosphate

2.1 Overview

D-*myo*-inositol 1,3,4-trisphosphate [D-Ins(1,3,4)P_3, Fig. 2.1] is produced in stimulated cells by the sequential action of the enzymes 3-kinase and 5-phosphatase. As discussed in Chapter 1, there had been controversy as to whether D-Ins(1,3,4)P_3 was active at Ins(1,4,5)P_3 receptors, and therefore confusion about its biological role. L-*myo*-inositol 1,3,4-trisphosphate [L-Ins(1,3,4)P_3] had not previously been investigated, and its biological activities were unknown. Structure-activity considerations predicted that, in contrast to the D-enantiomer, L-Ins(1,3,4)P_3 should be recognised by Ins(1,4,5)P_3 receptors and furthermore, arguments based on the possible ways in which the partial agonist Ins(1,3,4,6)P_4 might bind to Ins(1,4,5)P_3 receptors suggested that L-Ins(1,3,4)P_3 might show partial agonist properties.

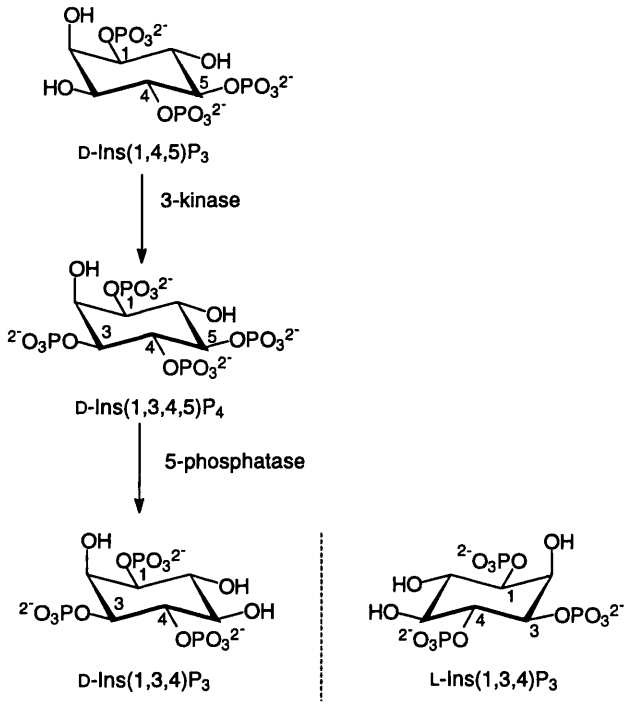

Figure 2.1 Origin of D-Ins(1,3,4)P_3 from D-Ins(1,4,5)P_3 *via* D-Ins(1,3,4,5)P_4. The enantiomer, L-Ins(1,3,4)P_3 [= D-Ins(1,3,6)P_3] is also shown.

We would begin by synthesising racemic Ins(1,3,4)P_3 in order to optimise the synthetic methods and to verify that this material could induce Ca^{2+}-release in our assay system (permeabilised rabbit platelets). Recall that racemic Ins(1,3,4)P_3 had previously been reported to cause Ca^{2+}-release from permeabilised aortic smooth muscle cells,[92] and the authors had concluded that D-Ins(1,3,4)P_3 was the active component (Chapter 1).

2.2 Synthesis of DL-*myo*-Inositol-1,3,4-trisphosphate

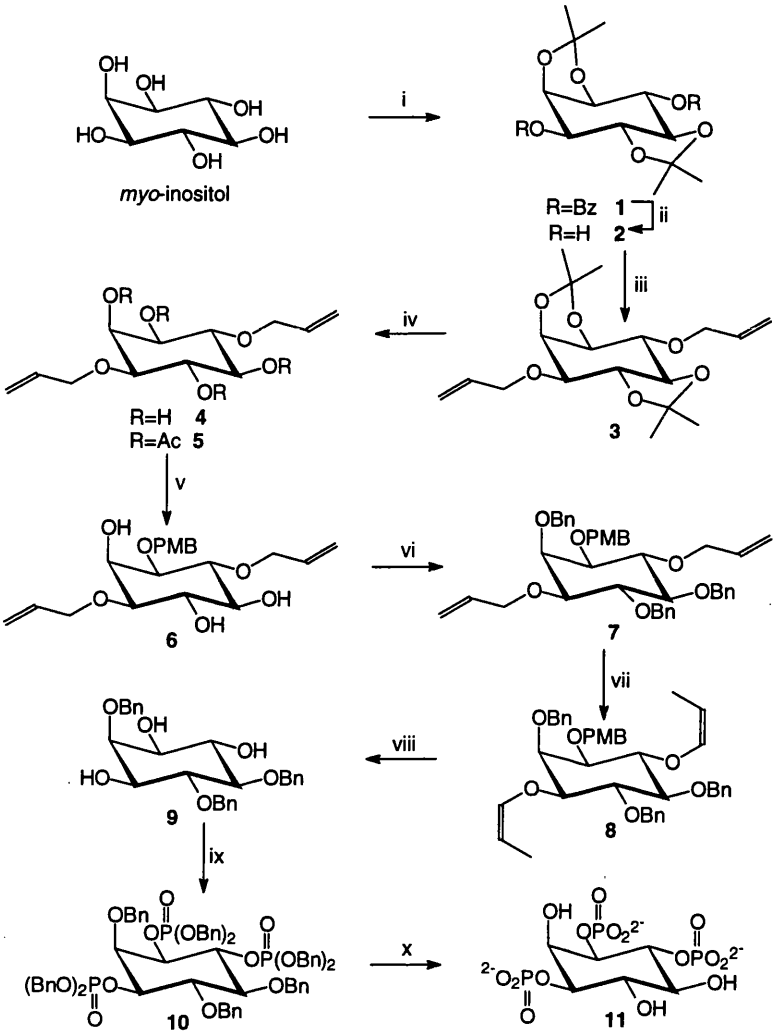

Figure 2.2 Synthesis of DL-*myo*-inositol-1,3,4-trisphosphate (**11**).
(i) (a) 2,2-Dimethoxypropane, PTSA DMF, reflux, (b) BzCl, pyridine; (ii) NaOH, MeOH, reflux; (iii) allyl bromide, NaH, DMF; (iv) AcOH/H_2O, 4:1, reflux; (v) (a) Bu_2SnO, toluene, reflux, (b) PMBCl, CsF, KI, DMF; (vi) BnBr, NaH, DMF; (vii) $KOBu^t$, DMSO, 50°C; (viii) MHCl/EtOH, 1:2, reflux; (ix) (a) $(BnO)_2PNPr^i_2$, 1*H*-tetrazole, CH_2Cl_2 (b) Bu^tOOH (x) Na/liquid NH_3. Bn, benzyl; Bz, benzoyl; PMB, *p*-methoxybenzyl. All compounds are racemic.

Racemic Ins(1,3,4)P_3 was synthesised (Figure 2.2) using a new route starting from *myo*-inositol The key fully-protected intermediate DL-1,4-di-*O*-allyl-2,5,6-tri-*O*-benzyl-3-*O*-*p*-methoxybenzyl-*myo*-inositol (**7**) was chosen for the following reasons:

First, partial deprotection of this compound leads to the known triol, 2,4,5-tri-*O*-benzyl-*myo*-inositol (**9**) which is then used as the precursor for phosphorylation. The absolute configurations of the enantiomers of this material had recently been assigned.[143] Second, the use of allyl protection at positions 1 and 4, together with *p*-methoxybenzyl at 3 allowed for two possible strategies of resolution using diastereoisomeric esters formed with a chiral resolving agent. The diastereoisomers must be capable of separation, either

by recrystallisation or by chromatography, and in many cases, neither is possible. The *p*-methoxybenzyl protecting group also gives versatility and the possibility of Ins(1,3,4)P_3 analogues modified at position 3.

2.2.1 Synthesis of a Key Intermediate

DL-1,4-di-*O*-allyl-2,5,6-tri-*O*-benzyl-3-*O*-*p*-methoxybenzyl-*myo*-inositol (**7**) was synthesised as shown in Figure 2.2 *via* the known DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (**2**). Following the procedure developed by Gigg *et al.*,[144] *myo*-inositol was treated with 2,2-dimethoxypropane in DMF with a catalyst of PTSA to give a mixture of three bisisopropylidene ketals (Figure 2.3).

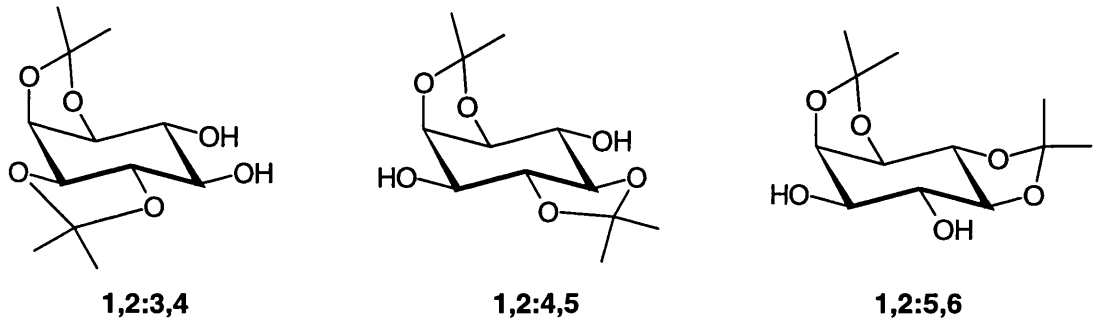

Figure 2.3 Reaction of *myo*-inositol with 2,2-dimethoxypropane gives a mixture of three bis-ketals.[144]

The three regioisomers were then converted into their dibenzoates by reaction with benzoyl chloride in pyridine. The dibenzoate **1** of the 1,2:4,5-bis-ketal is, in contrast to the other two dibenzoates, almost insoluble in pyridine, water, acetone and ether, and so it can be isolated by filtration and washing with these solvents. The benzoyl groups are then removed by refluxing in methanolic sodium hydroxide to give DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **2**.[144]

Allylation using sodium hydride in DMF followed by allyl bromide now gave the known 1,4-di-*O*-allyl-2,3:5,6-di-*O*-isopropylidene-*myo*-inositol (**3**).[145] Finally, the isopropylidene groups were removed by heating in acetic acid-water (4:1) to give the tetrol **4**.[149] The ^{1}H NMR spectrum of **4** initially posed some difficulties of interpretation due to overlapping signals from some of the inositol ring protons. **4** was therefore converted into the (highly crystalline) tetra-acetate **5**. In the ^{1}H NMR spectrum of this compound, the signals from protons at positions 2,3,5 and 6 were, as expected, shifted downfield, well away from those corresponding to H-1 and H-4. A 400MHz ^{1}H-^{1}H

COSY NMR spectrum of **5** allowed an unambiguous assignment of the ring protons, and interpretation of the original overlapping signals.

The next step was a selective *p*-methoxybenzylation of the tetrol at position 3. This involved the regioselective protection of one equatorial hydroxyl group in the presence of three other OH groups: one axial and two equatorial. The difference in the reactivity between the equatorial OH groups in inositol is slight, and the use of organotin derivatives has found a place in the selective activation of the equatorial OH of a vicinal equatorial-axial pair.[146] It has been shown that maximum yields in tin-mediated monoalkylations can be obtained by the use of caesium fluoride.[147] The authors of this study suggest that the polarisable caesium cation interacts with the halogen atom of the alkyl halide, causing its activation, and furthermore that Sn-O bonds are also activated by the formation of a pentacoordinate complex. The tetrol **4** was reacted with dibutyltin oxide by refluxing in toluene with azeotropic removal of water to give a dibutylstannylene derivative, which was not isolated, but reacted with *p*-methoxybenzyl chloride in DMF in the presence of caesium fluoride, to give the 3-*O*-*p*-methoxybenzyl ether **6** in 65% yield. Finally, **6** was benzylated using sodium hydride and benzyl bromide in DMF to give the fully-protected, crystalline intermediate DL-1,4-di-*O*-allyl-2,5,6-tri-*O*-benzyl-3-*O*-*p*-methoxybenzyl-*myo*-inositol (**7**).

2.2.2 Synthesis of DL-2,4,5-Tri-*O*-benzyl-*myo*-inositol

The next step was to generate the triol **9** by removing both the allyl and *p*-methoxybenzyl protecting groups from **7**. Allyl groups are similar to benzyl groups in their high stability to acidic and basic conditions, but in the presence of very strong base, isomerisation of allyl ethers to *cis*-prop-1-enyl ethers occurs. These enol ethers can then be cleaved by mild acid hydrolysis. The isomerisation was carried out using the standard conditions of potassium-*tert*-butoxide* in DMSO at 50°C.[145] The reaction proceeded smoothly and the highly crystalline *cis*-prop-1-enyl ether **8** was obtained in 83% yield.

In contrast to the unsubstituted benzyl ethers, *p*-methoxybenzyl ethers are acid labile, although less so than *cis*-prop-1-enyl ethers. Thus **8** can be converted into a diol by subjecting it to mild acid hydrolysis, or to the triol **9** by the use of harsher conditions. In the synthesis of racemic Ins(1,3,4)P_3, the triol was the desired product and so **8** was

* The potassium *t*-butoxide should be purified by sublimation for best results. When this step was omitted, the reaction was slower and poor yields were obtained.

deprotected using the relatively vigorous treatment of refluxing in 1M HCl-ethanol (2:1) for two hours, which resulted in complete conversion of **8** into the triol with no loss of benzyl protecting groups as judged by TLC. Flash chromatography removed the *p*-methoxybenzyl alcohol produced in the hydrolysis giving pure **9** in 90% yield.

There has been some disagreement in the literature as to the melting point of racemic **9**, with values of 126-128°C [148] and 135-137°C [145] being reported. It was found during the course of the current project that two different batches of **9**, both pure, had different melting points. On closer examination it was found that, when the temperature was very gradually increased as the crystals were observed on the hot stage microscope, they underwent a phase transition from plates to needles at about 128°C, with the needles then melting sharply at 135-136.5°C. If the temperature was increased slightly more rapidly, the plates melted at 128°C and then the liquid recrystallised to give needles which melted at the higher temperature. Thus it seems that racemic **9** can exist in at least two different crystalline forms (polymorphs) and the reason for the discrepancy in reported melting points is clear. Interestingly, another benzyl ether of *myo*-inositol (racemic-3,4-di-*O*-acetyl-1,2,5,6-tetra-*O*-benzyl-*myo*-inositol[149] has been shown to show unusual behaviour on heating ("jumping crystals"), and the effect is thought to be associated with solid-solid phase transitions.[150] X-ray crystal structures of the three crystalline phases have been published.[151]

2.2.3 Phosphorylation of DL-2,4,5-Tri-*O*-benzyl-*myo*-inositol

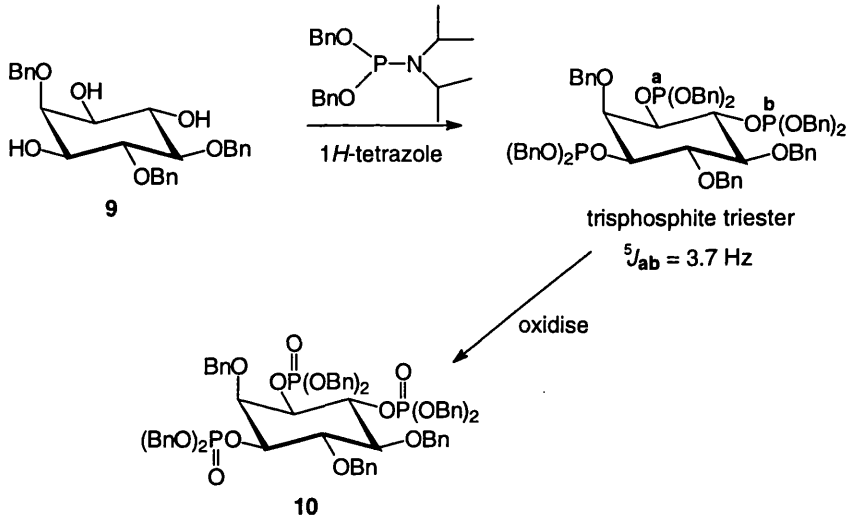

Figure 2.4 Phosphitylation of the triol **9**, gives a trisphosphite triester, which is oxidised to the trisphosphate triester **10**.

Because the triol possesses a vicinal diol, a P(III) approach to phosphorylation is appropriate. Direct phosphorylation of a vicinal diol using P(V) reagents, in which the

phosphorus atom is already in the +5 oxidation state, can cause difficulties, such as the formation of unwanted five-membered cyclic phosphates. Using P(III) methods, the hydroxyl groups are first phosphitylated and subsequently oxidised to give the protected phosphate ester. P(III) methodology can also be adapted to give phosphorothioates by the substitution of elemental sulphur for the oxidising agent in the second stage. The phosphitylation was carried out using the P(III) reagent bis-(benzyloxy)-*N*,*N*-diisopropylaminophosphine (Figure 2.4) which has to be catalytically activated by a weak acid, such as 1-*H* tetrazole, giving a reactive tetrazolide complex.

It is useful, and informative, to follow the course of the phosphitylation/oxidation sequence by ^{31}P NMR, if appropriate facilities are available. All such reactions reported in this thesis were monitored in this way by obtaining ^{31}P NMR spectra at various stages of the process, using a JEOL FX90 spectrometer. A low-field machine such as this is quite adequate for this purpose and may even have some advantages.* P(III) agents tend to be rather unstable, and by obtaining a ^{31}P NMR spectrum after adding the 1*H*-tetrazole, it is possible to check the purity of the phosphitylating agent-tetrazolide complex before committing (often precious) inositol derivative to the reaction (Figure 2.5 A). When the substrate is added, any vicinal phosphite triesters formed will show $^{5}J_{PP}$ spin couplings (Figure 2.5 B) and these couplings can give useful information, confirming the substitution pattern of the product. Finally, after oxidation (or sulphoxidation, which may be slower) the disappearance of all phosphite signals confirms that the reaction has gone to completion (Figure 2.5 C).

After 1*H*-tetrazole was added to a solution of the phosphitylating agent in dry dichloromethane, a proton-decoupled ^{31}P NMR spectrum showed a singlet at δ_P 127, confirming the presence of the phosphitylating agent-tetrazolide, and no impurities. The triol **9** was therefore added and now the ^{31}P NMR spectrum showed the appearance of signals at δ_P 140.39 (2P) and δ_P 142.21 (1P), corresponding to the trisphosphite triester. A signal at δ_P 127 (excess phosphitylating agent-tetrazolide) was also present. A high resolution, low sweep-width ^{31}P NMR spectrum of the signals close to δ_P 140 was able to resolve three signals, and two of these were doublets with a coupling constant of

*Spin couplings of low magnitude may be obscured in spectra acquired at high field as a result of signal broadening due to chemical shift anisotropy. This anistropic effect is reduced at low field strength (and high temperature).

3.7Hz. This arises from the $^{5}J_{PP}$ spin coupling between the P atoms at C-3 and C-4, and was clear evidence that the compound contained a vicinal bisphosphite.

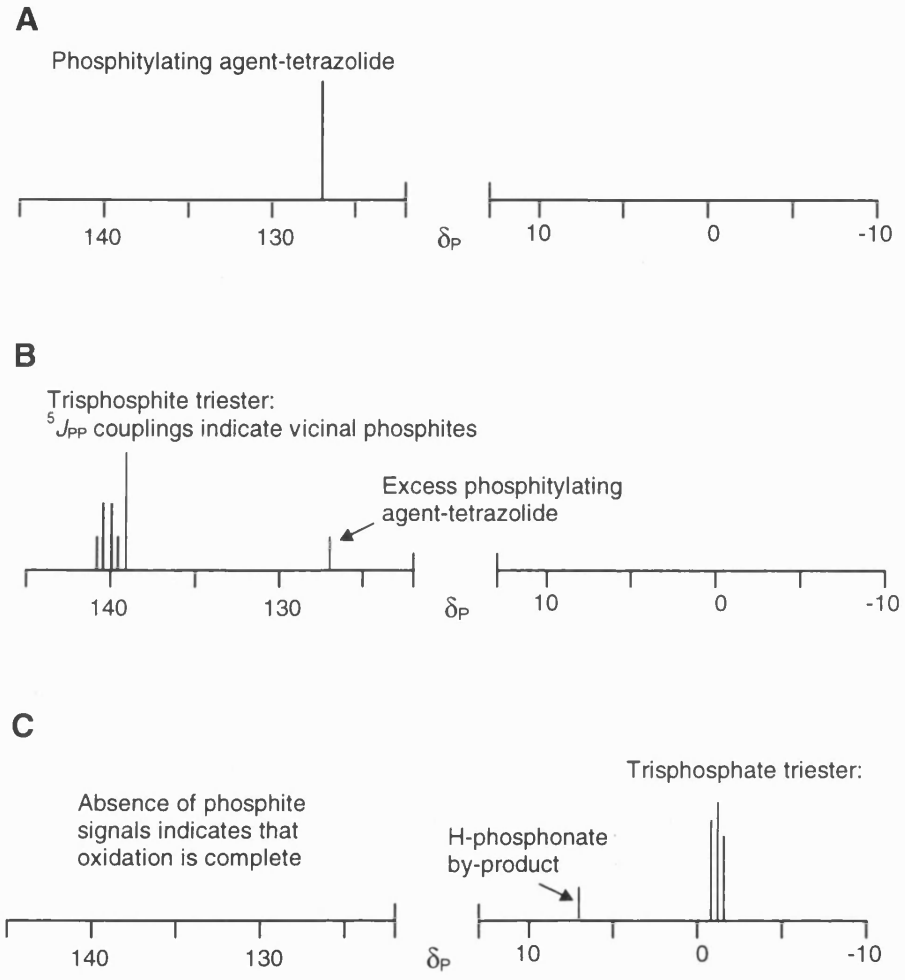

Figure 2.5 Proton-decoupled ^{31}P NMR is a useful technique for following the progress of phosphitylation oxidation. **A**: After addition of 1*H*-tetrazole to phosphitylating agent, **B**: After addition of triol, **C**: After oxidation with *tert*-butyl hydroperoxide.

A small amount of water was now added before oxidation. It was reasoned that this would react with excess phosphitylating agent-tetrazolide and therefore simplify the later purification stage. The oxidation was then carried out by adding excess *tert*-butyl hydroperoxide. ^{31}P NMR now showed that oxidation to the protected trisphosphate was complete, with the signals around δ_P 140 disappearing, to be replaced with three clear singlets at δ_P -1.42, -1.57, and -1.81. The only by-product detectable by ^{31}P NMR was an *H*-phosphonate at δ_P 7.5 and it was found that this could easily be removed by flash chromatography to give **10** in 73% yield. It was later found, in the course of phosphorylating a range of other compounds, that higher yields (80-90%) could usually be obtained by using *m*-CPBA as the oxidising agent.

2.2.4 Deprotection and Purification

The final deblocking of **10** involved the removal of the nine protecting benzyl groups using sodium in liquid ammonia. A number of attempts were necessary before this was carried out successfully and the procedure found to be effective is detailed in the experimental section. Subsequent experience with many deprotection reactions of this type have shown that the quality of the sodium used is a major factor influencing yield. The apparatus should be dried in an oven and assembled under nitrogen while hot. Only a small volume of liquid ammonia (20-30mL) should be used because the amount of sodium needed is then reduced accordingly. This, in turn, means that the ionic strength of the crude product is lower, and it can be loaded onto the ion-exchange column in a smaller volume of water, saving time during the purification stage.

An alternative deprotection strategy for this synthesis would have been to use hydrogenolysis, which is more reliable and usually gives near-quantitative yields. However the necessary apparatus was not available at this time. Note also that hydrogenolysis cannot be used to deprotect phosphorothioates (Chapter 6), compounds with ester protecting groups (Chapter 7) or unsaturated compounds (Chapter 3). Traces of paramagnetic ions from the catalyst (usually Pd) can cause extreme broadening of NMR signals, unless these are removed completely by ion exchange chromatography. The sodium in liquid ammonia method has wider applicability, but can give poor yields.

Trisphosphate **11** was purified using ion-exchange chromatography. After deprotection, the crude product contains large amounts of sodium salts and uncharged by-products, but these can easily be separated from the highly charged inositol phosphates by the ion-exchange column. Problems do occur, however, if other, similarly-charged inositol phosphates, which may arise from phosphate migrations during deprotection, are present (see Chapter 7). It is advisable to filter the solution of crude product before loading onto the column because any oily or particulate matter can cause blockage of filters and valves.

The ion-exchange column is eluted using a gradient of triethylammonium bicarbonate (TEAB) buffer from 0 to 1 M. It is not immediately apparent which fractions contain the target compound because, as there is usually no chromophore present, the usual post-column detection by UV absorption is not possible. The fractions containing phosphate were detected using a method that relies on the complexation of free phosphate with molybdate ions[152] and then subjected to ^{31}P NMR to determine which phosphate-containing fractions contained the desired product.

Because the inositol phosphates obtained by ion-exchange purification are isolated as their triethylammonium salts, whose stoichiometries may be different in each case, the formula mass is not known accurately and they cannot therefore be quantified on the basis of mass. Instead, a quantitative assay for phosphate must be carried out. The result of this assay is a quantity of phosphate (in μmoles) which is then divided by three for a trisphosphate, by two for a bisphosphate etc. The conventional method is to use the Briggs Phosphate Assay.[152]

The triethylammonium salt of racemic **11** was obtained as a colourless glass. The proton-coupled ^{31}P NMR spectrum of **11** confirmed the presence of the three phosphate groups with heteronuclear $^{3}J_{HCOP}$ coupling. The identity of **11** was confirmed by one- and two-dimensional ^{1}H NMR spectroscopy. It was possible to assign all the signals, including the separate assignments of H-1 and H-3 (see below) leaving no doubt remaining as to the identity of **11**.

2.2.5 Biological Testing of DL-Ins(1,3,4)P_3

Previous work has shown that intracellular injection of Ins(1,4,5)P_3 into the ventral photoreceptors of the horseshoe crab *Limulus polyphemus*, results in a transient depolarisation of the cellular membrane potential.[153,154] This depolarisation accompanies, and is caused by, a burst of Ca^{2+} release from intracellular stores.[155] Poorly-metabolisable active analogues of Ins(1,4,5)P_3, such as D-*myo*-inositol-1,4,5-trisphosphorothioate, induce a train of bursts of depolarisation due to bursts of Ca^{2+} release that persist for many minutes after injection.[156]

Racemic **11** was injected into *Limulus* ventral photoreceptors at a concentration in the injection pipette of 100μM. It was indeed, highly active, producing bursts of depolarisation that persisted for minutes after injection, suggesting that the active component of **11** was poorly metabolisable. (An alternative explanation of the prolonged effect would be that one enantiomer of **11** was capable of inhibiting the metabolism of the other).

11 Was also evaluated for its ability to mobilise $^{45}Ca^{2+}$ from saponin-permeabilised rabbit platelets, relative to Ins(1,4,5)P_3 and Ins(1,3,4,6)P_4. The results showed that racemic **11** was considerably less potent than Ins(1,4,5)P_3 but equal to, or slightly greater in potency than Ins(1,3,4,6)P_4 in its ability to mobilise $^{45}Ca^{2+}$. As discussed above, structure-activity considerations predict that this activity should reside in L-Ins(1,3,4)P_3, and not the D-enantiomer.

A preliminary assay showed that **11** was capable of inhibiting 5-phosphatase (K_i=8.8μM). Polokoff *et al.*[92] also found their racemic Ins(1,3,4)P_3 to behave in a similar way. It has previously been shown that D-Ins(1,3,4)P_3 is *not* a 5-phosphatase inhibitor,[157] so it follows that the L-enantiomer must be responsible. As discussed above, L-Ins(1,3,4)P_3 has been found to be present in avian erthyrocytes[103] but there is as yet no evidence that L-Ins(1,3,4)P_3 attains levels that would inhibit 5-phosphatase *in vivo*.[158]

It was at this stage in the project that Hirata *et al.* published a detailed biological study of D-Ins(1,4,6)P_3 and L-Ins(1,3,4)P_3 with no synthetic details.[159] Strangely, although their L-Ins(1,3,4)P_3 seemed active in binding studies in rat cerebellum (90-fold less potent than Ins(1,4,5)P_3), as would be expected, it was almost inactive in Ca^{2+}-release (3000 times weaker than Ins(1,4,5)P_3). The latter result is totally at odds with our finding that *racemic* **11** was moderately potent in Ca^{2+} release. These results strengthened our suspicions that this group had tested the wrong enantiomer of Ins(1,3,4)P_3, especially in the light of earlier discrepancies over the optical rotation of the D-enantiomer, and yet this could not explain the high potency in binding. The only other possibility seemed to be that the differences in Ca^{2+} release reflected differences in the Ins(1,4,5)P_3 receptors in different cell types. Our results, as explained above were, based on experiments using rabbit platelets (and *Limulus* photoreceptors) whereas Hirata *et al.* had used rat basophilic leukaemic (RBL) cells. Thus, it was important to continue with the original plan, synthesise both enantiomers of Ins(1,3,4)P_3, prove their absolute configurations, and evaluate their biological activities in our own assays.

2.3 Optical Resolution of 2,4,5-Tri-*O*-benzyl-1-*O*-*p*-methoxybenzyl-*myo*-inositol

The successful optical resolution of inositol derivatives *via* the formation of camphanate esters has been reported in various studies.[143,160-162] Both *S*-(−)- and *R*-(+)- camphanic acid chlorides are stable crystalline reagents available in high optical purities. The diastereoisomeric camphanate esters can sometimes be separated by crystallisation, or their polarities may be sufficiently different to allow separation by column chromatography. In other cases, neither method may be successful. The camphanate ester may also be used as a protecting group, allowing selective modification subsequent to resolution (see Chapter 7).

The decision was made to attempt the resolution *via* the formation of 3,6-bis-camphanates on the basis of a report that 1,2,4,5-tetra-*O*-benzyl-*myo*-inositol had been successfully resolved by this method.[143] Accordingly, racemic 2,4,5-tri-*O*-benzyl-3,6-di-

O-(*cis*-prop-1-enyl)-1-*O*-*p*-methoxybenzyl-*myo*-inositol **8** was subjected to mild acid hydrolysis using 1M HCl-acetone (1:10) at 50°C. TLC showed complete conversion to the 1,4-diol within 5 minutes, after which the acid was rapidly neutralised with aqueous sodium hydrogencarbonate. Experiments showed that if the reaction was continued beyond 30 min, then significant amounts of triol began to appear, arising from loss of the *p*-methoxybenzyl group at position 3. The racemic diol **12** was obtained in 84% yield after purification by flash chromatography. Racemic **12** was then converted into its bis-[(*1S*)-(–)-ω-camphanate] esters by reaction with (*1S*)-(–)-ω-camphanic acid chloride in pyridine with a catalyst of DMAP.

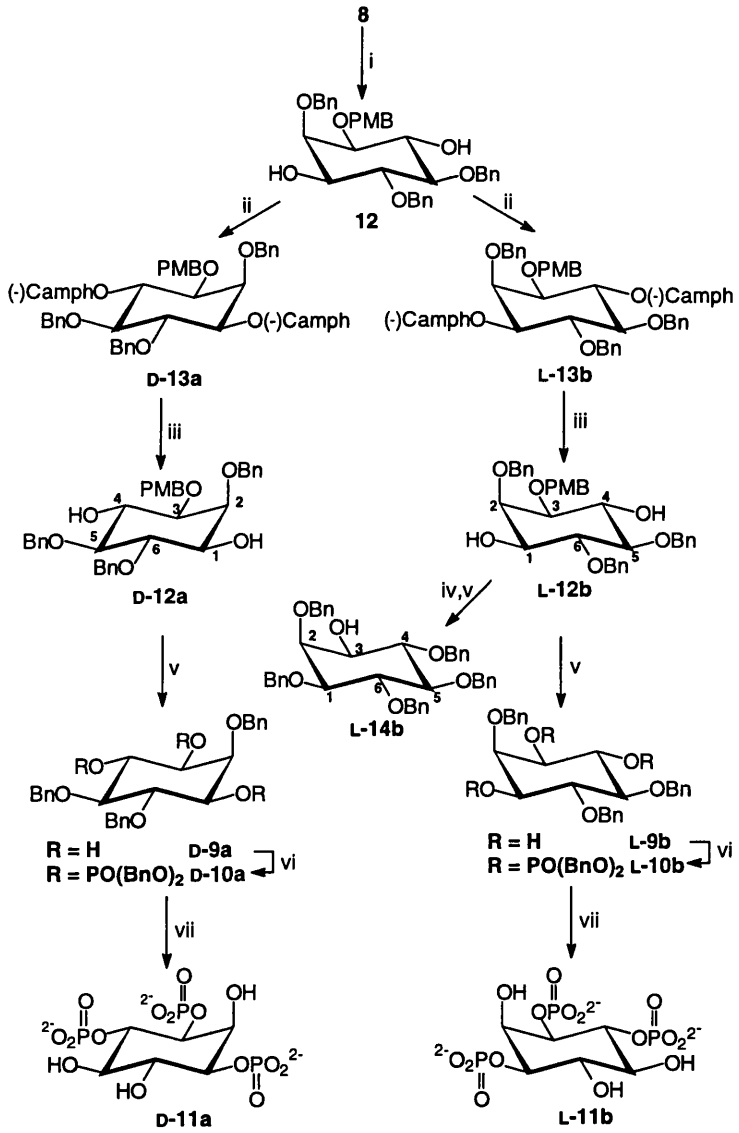

Figure 2.6 Optical resolution of **12** and conversion to D- and L-Ins(1,3,4)P_3.
(i) 1M HCl/acetone, 1:10, 50°C; (ii) (*S*)-ω-camphanic acid chloride, DMAP, pyridine; (iii) NaOH, MeOH, reflux; (iv) BnBr, NaH, DMF; (v) 1M HCl/EtOH 1:2, reflux; (vi) (a) $(BnO)_2PNPr^i_2$, 1*H*-tetrazole, (b) Bu^tOOH; (vii) Na/liquid NH_3. Bn, benzyl; PMB, *p*-methoxybenzyl; (–)Camph, (1*S*)-(–)-ω-camphanate.

Attempts to resolve the diastereoisomers by recrystallisation from various solvent systems (methanol, ethanol, ether, ethyl acetate/hexane) were not successful. The only remaining possibility was the use of chromatography, although the two diastereoisomers showed a ΔR_f of only 0.07. However, no better TLC system could be found and so the separation was attempted using chloroform/acetone (30:1). Perhaps surprisingly, this was successful, and excellent separation was achieved.

The individual diastereoisomers proved to be very different. One of these, the (+)-diastereoisomer, was highly crystalline, with a very low solubility in cold methanol allowing easy crystallisation from hot solvent. The (–)-diastereoisomer was much more soluble in methanol and ethanol, and could not be induced to form satisfactory crystals from any of a range of solvents, tending instead to precipitate as a gel, which then had to be dried under vacuum to remove solvent. Unfortunately, it was not possible to grow crystals of either diastereoisomer that were suitable for X-ray analysis.

The NMR spectra of the two diastereoisomers showed them to be pure, with no trace visible of the other diastereoisomer in each case (^{1}H NMR at 400MHz and ^{13}C NMR at 100MHz). The efficiency of the resolution could be judged by the ^{1}H NMR resonances of the camphanate methyl groups. Further evidence came from an examination of the H-2 signal, which occurred at very different chemical shifts (δ 4.11 and 4.23) in the two diastereoisomers. Saponification of the individual diastereoisomers by refluxing with sodium hydroxide in methanol then gave the enantiomeric diols **12a** and **12b**.

As it had not been possible establish the absolute configuration of either biscamphanate by X-ray crystallography, the remaining option was to convert one of the enantiomeric diols into a compound whose absolute configuration was already well-established. Fortunately, this had been anticipated in the design of the synthesis, and the (–)-diol **12b** was therefore converted into one of the enantiomers of the known 1,2,4,5,6-penta-*O*-benzyl-*myo*-inositol **14** by benzylation followed by removal of the *p*-methoxybenzyl group. The absolute configuration of the (+)-enantiomer as 1D-1,2,4,5,6-penta-*O*-benzyl-*myo*-inositol had been established by Shvets, in 1973, based on its conversion to (+)-bornesitol.[163] The product was found to have an ^{1}H NMR spectrum identical to that of racemic 1,2,4,5,6-penta-*O*-benzyl-*myo*-inositol[164], but an optical rotation of –11.5°, identifying it as (–)-1L-1,2,4,5,6-penta-*O*-benzyl *myo*-inositol **L-14b**. Thus the (–)-diol could be assigned as 1L-2,5,6-tri-*O*-benzyl-3-*O*-*p*-methoxybenzyl *myo*-

inositol **L-12b** allowing the absolute configurations of the diastereoisomeric biscamphanates and of the resolved enantiomers generated from them to be deduced.

However, at this stage in the project we encountered a problem. It emerged that there were, in fact, unacknowledged contradictions in the literature regarding the absolute configurations of the enantiomers of **14**. Two independent X-ray studies[165,166] carried out on the (1*S*)-(−)-ω-camphanic acid ester of the (+)-enantiomer had deduced the 1L-1,2,4,5,6-penta-*O*-benzyl-*myo*-inositol configuration for the (+)-enantiomer, the opposite of that found by Shvets. The authors did not remark on the discrepancy, and, partly because of their use of different nomenclature for **14**, we were unaware that Shvets' assignment was in question. The disagreement is reflected in the reported conversions of *both* enantiomers into the same 1D-*myo*-inositol-1-phosphate by phosphorylation and debenzylation.[163,165] Fortunately, a study published in 1994 by Aneja *et al.*[167] resolved this conflict unambiguously, showing beyond doubt that Shvets' original assignment was correct. Our assignments of the absolute configurations of (+) and (−)-**12**, and related compounds were therefore on solid ground.

The enantiomeric diols **D-12a** and **L-12b** were converted into the known triols **D-9a** and **L-9b** by removal of their *p*-methoxybenzyl groups as described for **9**. The optical rotations of these enantiomers were in agreement with, although larger than, those previously reported[143,98], thus confirming the absolute configurations proposed by Desai *et al.*[143]and of the D-Ins(1,3,4)P_3 synthesised by Gou and Chen.[98] Examination of a ^{1}H-^{1}H COSY NMR spectrum of **D-9a** allowed the signals corresponding to each of the three hydroxyl groups to be assigned, as well as the ring protons. The melting point behaviour of the optically pure triols paralleled that of the racemate. The phase change occurred at lower temperature (93°C) with the needles then melting at 104-106°C in each case. Thus the individual enantiomers exist in polymorphic forms.

The triols were phosphorylated as described for the racemic material, followed by deprotection and purification as before to give D- and L-*myo*-inositol-1,3,4-trisphosphates **D-11a** and **L-11b** respectively as the triethylammonium salts. The 162 MHz proton-coupled ^{31}P NMR spectra of **D-11a** and **L-11b** showed that they were free from contamination with other phosphates. Previously published NMR spectra of Ins(1,3,4)P_3 obtained from biological sources[168] showed the material to be contaminated, probably with Ins(1,4,5)P_3. In this respect it is also worth noting that, as L-Ins(1,3,4)P_3 is now known to occur naturally in at least some cell types (see above), it follows that any Ins(1,3,4)P_3 obtained from biological sources may contain L-

Ins(1,3,4)P_3, and of course, the two enantiomers would be indistinguishable by standard chromatographic analyses, or by NMR. However, as the *diastereoisomeric* biscamphanate intermediates in our route appeared pure by NMR, we can expect that neither **D-11a** nor **L-11b** would contain more than 1% of its enantiomer.

The NMR spectra of the enantiomers were identical with one another, and with that of racemic **11**. 400MHz ^{1}H spectra were obtained, and a 2D ^{1}H-^{1}H COSY spectrum of **L-11b**.The assignment of the ^{1}H NMR spectrum of **L-11b** (Figure 2.7) is explained below.

- H-2 appears as a narrow triplet (actually a dd) at δ_H 4.28. This is the only equatorial proton and it is deshielded relative to the axial protons. It can be deduced that this position is not phosphorylated because any $^3J_{HP}$ heteronuclear coupling to phosphorus would cause a doubling of the signal.

- H-4 is seen at δ_H 4.17. Three couplings are present; two axial-axial interactions with H-3 and H-5 and a $^3J_{HP}$ coupling to the phosphorus at C-4. The heteronuclear coupling constant has a similar magnitude to $^3J_{ax\text{-}ax}$, giving a quartet (or ddd). This proton cannot be H-5 because, given that C-1 and C-3 are also phosphorylated (see below) the molecule would then have a plane of symmetry.

- H-3 and H-1 each give a doublet of triplets (or ddd). If these positions were not phosphorylated, each signal would appear as a doublet of doublets. Again the pattern is a result of the heteronuclear coupling constant being similar in magnitude to the homonuclear $^3J_{ax\text{-}ax}$ coupling. It is not possible, from the 1D spectrum to say which of the two signals is H-1 and which is H-3, but in the ^{1}H-^{1}H COSY, the downfield signal has a cross-peak with H-4, establishing it as H-3.

- The quartet centred at δ_H 3.49 showed no cross-peaks with ring protons in the ^{1}H-^{1}H COSY spectrum. It arises from ethanol of crystallisation (or possibly $CH_3CH_2NR_2$ of an impurity in the triethylamine).

- Finally, H-6 and H-5 appear as triplets (dd) at high field, indicating that these positions are not phosphorylated. In *myo*-inositol derivatives, H-4 and H-6 tend to resonate downfield of H-5, given similar substituents, presumably as a result of a deshielding 1,3-diaxial interaction with the oxygen at position 2.

Thus, no doubt remained as to the identities of **D-11a** and **L-11b**.

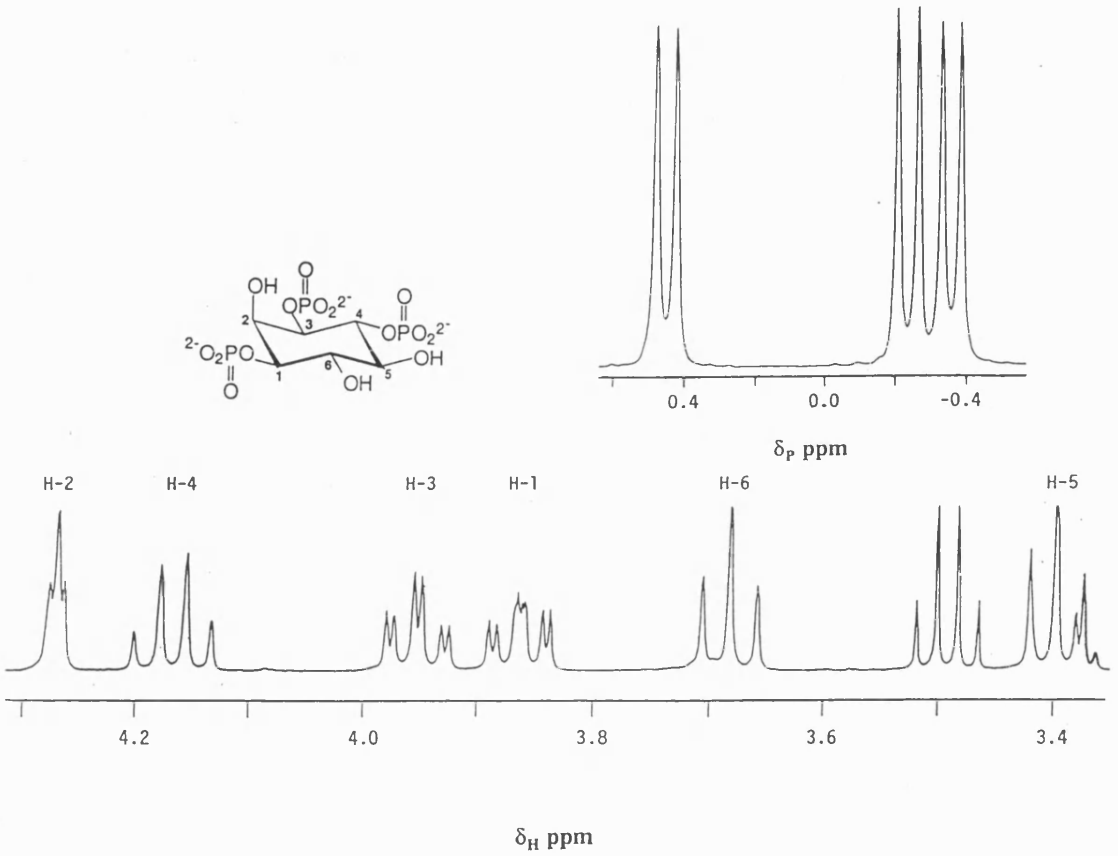

Figure 2.7 400 MHz ^{1}H NMR spectrum of **11b** in D_2O, pH~4.2. The inset shows the 162 MHz ^{1}H-coupled ^{31}P NMR spectrum of **11a** in D_2O, pH~4.2.

The complete ^{1}H NMR spectra showed the number of triethylammonium cations to be between 3 and 3.5. Solutions of the salts in water were acidic (pH 4.2), and their specific rotations, calculated for the free acid, were highly dependent on pH, being small (<10°) at acidic pH and large (>40°) at pH 10.6. A similar pH-dependency has been found for the optical rotations of D-Ins(1,4,5)P_3.[169] The rotations for D-Ins(1,3,4)P_3 were positive over this range and those for L-Ins(1,3,4)P_3 were negative. At pH 7.8 the values, calculated for the free acids, were +37° and -40° respectively (c = 0.42, TEAB buffer). The signs of the rotations therefore agreed with the positive rotation reported for the potassium salt of D-Ins(1,3,4)P_3 by Gou and Chen.[98] The larger magnitudes of our rotations may be attributed to pH differences. It is not clear why Ozaki *et al.*[95]obtained a *negative* rotation for their D-Ins(1,3,4)P_3 (ammonium salt). Had this group actually obtained the wrong enantiomer of Ins(1,3,4)P_3? In fact this now seems very unlikely, and the reasons will be discussed in detail below.

2.4 Biological Testing of D-Ins(1,3,4)P_3 and L-Ins(1,3,4)P_3

The structural considerations discussed in Chapter 1 predict that D-Ins(1,3,4)P_3 should show little ability to release Ca^{2+}. Our working hypothesis is that the essential requirement for Ca^{2+} release in inositol polyphosphates is the presence of a D-4,5-bisphosphate as found in D-Ins(1,4,5)P_3, or a pseudo-D-4,5-bisphosphate [e.g. in Ins(1,3,4,6)P_4]. L-Ins(1,3,4)P_3 possesses this motif and we therefore predicted that it should release Ca^{2+}. D-Ins(1,3,4)P_3 does not, and for this reason, despite the reports to the contrary cited in Chapter 1, we would expect it to show little, if any, activity as an agonist at the Ins(1,4,5)P_3 receptor.

2.4.1 Effects of D-Ins(1,3,4)P_3 and L-Ins(1,3,4)P_3 in *Limulus* Photoreceptors

The two enantiomers of Ins(1,3,4)P_3 **D-11a** and **L-11b** were injected into *Limulus* ventral photoreceptors in order to compare their effectiveness in a living cell.

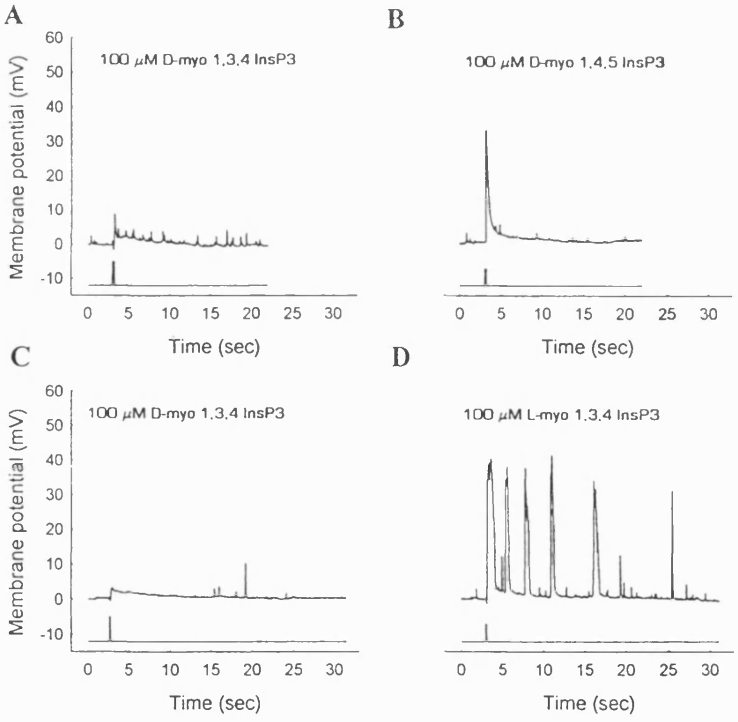

Figure 2.8 Injection of $InsP_3$ isomers into the rhabdomeral lobe of *Limulus* photoreceptors. Figures **A** and **B** show recordings of membrane potential made from a cell impaled with a double-barrelled micropipette containing 100 μM D-Ins(1,3,4)P_3 (**D-11a**) in one barrel and 100 μM Ins(1,4,5)P_3 in the other barrel. The pressure applied to either barrel was 60 psi for 100ms.Figures **C** and **D** show recordings of membrane potential made from another cell impaled with a double-barrelled electrode containing 100 μM D-Ins(1,3,4)P_3, (**D-11a**) in one barrel and 100 μM L-Ins(1,3,4)P_3 (**L-11b**) in the other. Pressure applied to the first barrel was 20 psi. for 200ms and to the second barrel was 38 psi applied for 200ms.

Ventral photoreceptors were impaled in their light-sensitive region with a double-barrelled micropipette, one barrel containing 100 μM D-Ins(1,3,4)P_3 (**D-11a**) and the other barrel either 100 μM D-Ins(1,4,5)P_3 or 100 μM L-Ins(1,3,4)P_3 (**L-11b**). These solutions were injected into the photoreceptor by brief pressure pulses delivering approximately equal volumes. Photoreceptors remained in darkness throughout the experiment. Injection of D-Ins(1,3,4)P_3 was found to be much less effective in inducing a rapid burst of depolarisation than injection of either D-Ins(1,4,5)P_3 or L-Ins(1,3,4)P_3 (Figure 2.8). In addition, unlike the single burst of depolarisation seen following injection of D-Ins(1,4,5)P_3 (Figure 2.8 B), repetitive bursts of depolarisation were induced by L-Ins(1,3,4)P_3 (Figure 2.8 D). These bursts continued for up to 5 minutes after injection of L-Ins(1,3,4)P_3, suggesting that L-Ins(1,3,4)P_3 is metabolised at a slower rate than D-Ins(1,4,5)P_3.

2.4.2 Effects of D- and L-Ins(1,3,4)P_3 in Permeabilised Rabbit Platelets

The enantiomers **D-11a** and **L-11b** were also examined for their ability to release $^{45}Ca^{2+}$ from permeabilised rabbit platelets (Figure 2.15). L-Ins(1,3,4)P_3 (**L-11b**) behaved as a full agonist and was found to be some 11-fold weaker at releasing $^{45}Ca^{2+}$ than D-Ins(1,4,5)P_3. However D-Ins(1,3,4)P_3 (**D-11a**) was almost inactive, releasing only 16% of the $^{45}Ca^{2+}$ at the highest concentration examined (100μM).

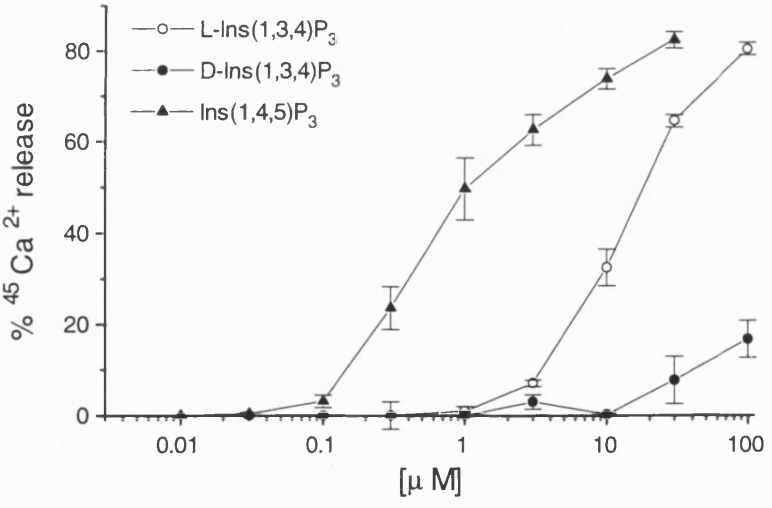

Figure 2.9 Calcium release from permeabilised rabbit platelets induced by Ins(1,4,5)P_3, L-Ins(1,3,4)P_3 (**L-11b**) and D-Ins(1,3,4)P_3 (**D-11a**). Values are mean ± S.E.M. for three separate experiments, each performed in triplicate.

The above findings were extended by examining the kinetics of Ca^{2+} release by the two enantiomers. Ca^{2+} release was monitored in the presence of the fluorescent dye fura-2 by spectrofluorimetry. Addition of 1μM Ins(1,4,5)P_3 caused release of Ca^{2+} from

the intracellular stores of platelets, detected as a rapid increase in the fluorescence of fura-2 free acid. The increase on fluorescence was transient, presumably due to metabolism of Ins(1,4,5)P_3 to inactive products, resulting in resequestration of Ca^{2+} back into the intracellular stores by Ca^{2+}-ATPase activity. Addition of **L-11b** [L-Ins(1,3,4)P_3] caused a dose-dependent release of Ca^{2+} from the intracellular stores. However, unlike the effect of Ins(1,4,5)P_3, Ca^{2+}-release by **L-11b** reached a maximum which was then maintained at a plateau phase over the time-course of the experiment (Figure 2.10). In agreement with the findings for $^{45}Ca^{2+}$-release, **D-11a** [D-Ins(1,3,4)P_3] was inactive, with no increase in fluorescence measured at 30μM of **D-11a**.

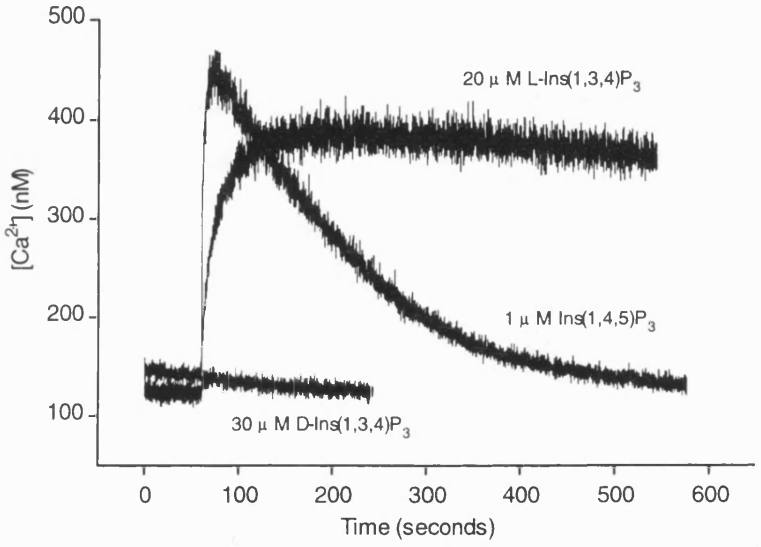

Figure 2.10 Ca^{2+} mobilisation induced by D-Ins(1,3,4)P_3 (**D-11a**) and L-Ins(1,3,4)P_3 (**L-11b**) monitored by spectrofluorimetry. Each trace is taken from a single experiment but is representative of 4 individual experiments.

As discussed in Chapter 1, heparin has previously been demonstrated to be a competitive antagonist at the Ins(1,4,5)P_3 binding site of the Ins(1,4,5)P_3 receptor.[33] Heparin was found to inhibit L-Ins(1,3,4)P_3-induced Ca^{2+} release, indicating that the effects of L-Ins(1,3,4)P_3 result from an interaction with the same site.

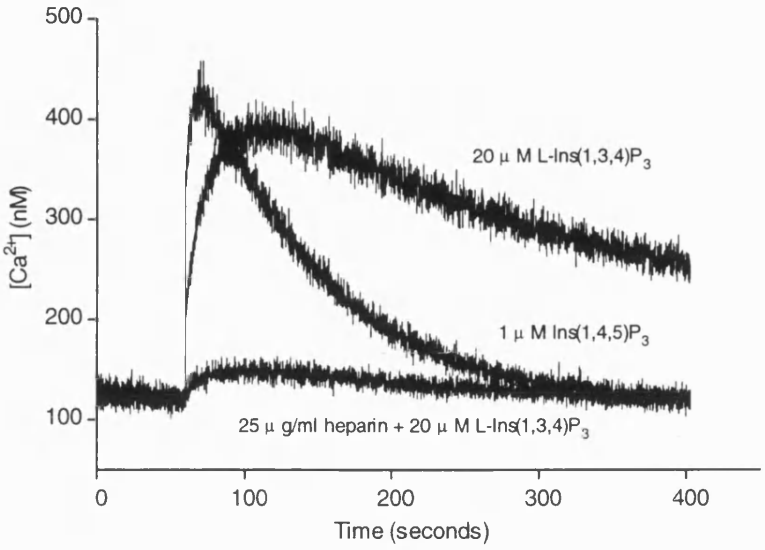

Figure 2.11 Effect of heparin on L-Ins(1,3,4)P_3-induced Ca^{2+}-release.

2.4.3 Binding Studies in Rat Cerebellar Membranes

[^{3}H]Ins(1,4,5)P_3 was readily displaced from specific binding sites on rat cerebellar membranes by cold Ins(1,4,5)P_3 with an IC_{50} of 0.045 ± 0.01μM. Ins(1,3,4,6)P_4 also displaced specifically bound [^{3}H]Ins(1,4,5)P_3 but was 36-fold weaker than Ins(1,4,5)P_3. L-Ins(1,3,4)P_3 was around 100-fold weaker than Ins(1,4,5)P_3 but D-Ins(1,3,4)P_3 was able to displace [^{3}H]Ins(1,4,5)P_3 only very weakly, even at the highest concentration examined (30μM) with an IC_{50} > 30μM (Figure 2.12).

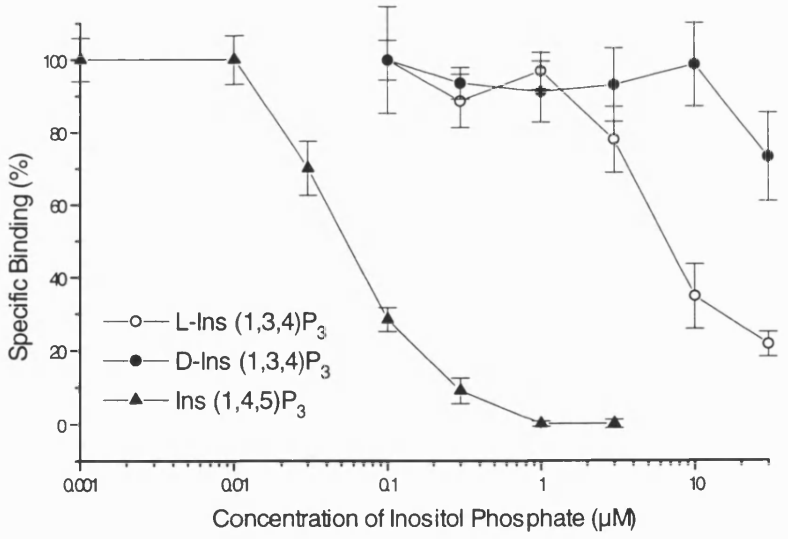

Figure 2.12 Displacement of specific [^{3}H]Ins(1,4,5)P_3 binding to rat cerebellar membranes by Ins(1,4,5)P_3, L-Ins(1,3,4)P_3 (**L-11b**) and D-Ins(1,3,4)P_3 (**D-11a**). Values are mean ± S.E.M. for three separate experiments, each performed in duplicate.

2.4.4 Comparison with Ins(1,3,4,6)P_4 and the Enantiomers of Ins(1,4,6)P_3

Table 2.1 summarises the results of the Ca^{2+}-release and binding assays for Ins(1,4,5)P_3, Ins(1,3,4,6)P_4 and the enantiomers of Ins(1,3,4)P_3 and Ins(1,4,6)P_3. In both assays, the activities of D-Ins(1,3,4)P_3 and L-Ins(1,4,6)P_3 were so low that EC_{40} and IC_{50} values could not be measured. Recall that both of these molecules would be expected to be essentially inactive because they lack any equivalent to a 4,5-bisphosphate.

The potency orders of the remaining compounds is as shown below:

Binding assay:

Ins(1,4,5)P_3 > D-Ins(1,4,6)P_3 = Ins(1,3,4,6)P_4 > L-Ins(1,3,4)P_3

Ca^{2+} release:

Ins(1,4,5)P_3 > D-Ins(1,4,6)P_3 > L-Ins(1,3,4)P_3 > Ins(1,3,4,6)P_4

Compound	Ins(1,4,5)P_3 receptor binding $IC_{50}/\mu M \pm$ S.E.M.	$^{45}Ca^{2+}$ release $EC_{40}/\mu M \pm$ S.E.M.
D-Ins(1,4,5)P_3	0.045 ± 0.01	0.69 ± 0.24
Ins(1,3,4,6)P_4	1.62 ± 0.39	28.50 ± 1.06
L-Ins(1,3,4)P_3 (L-11b)	**4.42 ± 1.29**	**8.05 ± 0.98**
D-Ins(1,3,4)P_3 (D-11a)	**> 30**	**> 100**
L-Ins(1,4,6)P_3	> 10	> 100
D-Ins(1,4,6)P_3	1.42 ± 0.34	1.56 ± 0.34

Table 2.1 Comparison of inositol phosphates for displacement of [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes and for release of $^{45}Ca^{2+}$ from permeabilised platelets. EC_{40} = concentration causing 40% $^{45}Ca^{2+}$-release. (n = 3-10).

In both assays, D-Ins(1,4,6)P_3 was more potent than L-Ins(1,3,4)P_3. These results agree with the predictions made on the basis of the binding-orientation arguments given in Chapter 1, and confirm that the 3-hydroxyl group of Ins(1,4,5)P_3 is more important for binding than the 6-hydroxyl group. The same conclusion was reached by Hirata *et al.* in their study of D-Ins(1,4,6)P_3 and L-Ins(1,3,4)P_3.[159]

All the analogues behaved as full agonists in the platelet Ca^{2+}-release assay, and yet, as discussed in Chapter 1, Ins(1,3,4,6)P_4 has been previously been demonstrated to behave as a partial agonist in SH-SY5Y cells. Both SH-SY5Y cells and platelets possess the type 1 Ins(1,4,5)P_3 receptor,[170,171] but while 99% of the Ins(1,4,5)P_3 receptors in SH-SY5Y cells are type 1, the abundance of this subtype in platelets is not known. It is

possible, then, that the difference may be related to different receptors in the two cell-types. It should also be borne in mind that there may be methodological problems in the detection of partial agonists (see Section 1.8.2 and Chapter 5) related to the details of the assay. It will therefore be necessary to investigate the effects of L-Ins(1,3,4)P_3 and D-Ins(1,4,6)P_3 in SH-SY5Y cells before we can come to any conclusions about the structural basis of the partial agonist behaviour of Ins(1,3,4,6)P_4. This study is now being carried out.

Finally, note that the orders of potency of the analogues are different for the binding and Ca^{2+}-release assays. Again, it must be remembered that the two assays were carried out in different cell-types, and it is also known that, even in the same cell-type, binding and Ca^{2+} release assays may not give the same results, due to differences in experimental conditions.[172] Ins(1,3,4,6)P_4 was more potent than L-Ins(1,3,4)P_3 in the binding assay, being equal to D-Ins(1,4,6)P_3 in its ability to displace [^{3}H]Ins(1,4,5)P_3 from cerebellar membranes. However, in Ca^{2+} release it was the least potent of the analogues tested. Thus Ins(1,3,4,6)P_4 seems to be weaker in Ca^{2+} release from platelets than would be expected from its binding affinity in cerebellum. The significance of this observation is not clear at the time of writing.

2.4.5 Interaction with Ins(1,3,4)P_3 5/6-kinase and Ins(1,4,5)P_3 3-kinase

Both enantiomers were tested for their ability to inhibit phosphorylation of [^{3}H]Ins(1,3,4)P_3 by a purified Ins(1,3,4)P_3 5/6-kinase preparation. As expected, D-Ins(1,3,4)P_3 was a potent inhibitor (IC_{50}= 5.25μM) and in this respect was identical to commercially available Ins(1,3,4)P_3. L-Ins(1,3,4)P_3 was almost inactive (IC_{50}>> 100μM), which is what would be predicted using the structural arguments described in Section 1.9.4

In contrast, when tested for interaction with Ins(1,4,5)P_3 3-kinase, D-Ins(1,3,4)P_3 was not a substrate, but L-Ins(1,3,4)P_3 was phosphorylated by the enzyme with approximately 0.4-fold the speed of the natural substrate, Ins(1,4,5)P_3. This finding is in agreement with the arguments given in Section 1.10.3, where it is argued that L-Ins(1,3,4)P_3 but not D-Ins(1,3,4)P_3 has structural similarities to Ins(1,4,5)P_3.

Finally, neither enantiomer was able to inhibit phosphatidylinositol 3-kinase. As for Ins(1,4,5)P_3 3-kinase, we would not expect D-Ins(1,3,4)P_3 to be recognised, because it does not resemble Ins(1,4,5)P_3. However, the fact that L-Ins(1,3,4)P_3 is unable to interact with PtdIns 3-kinase is significant, and is evidence for the hypothesis that an

axial 2-OH group is a structural requirement for inositol phosphates to be recognised by this enzyme (Section 1.9.5).[90] There are no possible binding orientations for L-Ins(1,3,4)P_3 in which it can mimic both the 4,5-bisphosphate and the axial 2-hydroxyl group of Ins(1,4,5)P_3.

2.5 Activities of D-Ins(1,3,4)P_3 and L-Ins(1,3,4)P_3: Conclusions

These results have clearly established that the Ca^{2+}-releasing ability of racemic Ins(1,3,4)P_3 resides in the L-enantiomer. In the biological assays described above, D-Ins(1,3,4)P_3 was essentially inactive. This is what we would expect from structure-activity considerations, and indeed, it is what seems to be most logical from a biological point of view. That is, the conversion of Ins(1,4,5)P_3 to D-Ins(1,3,4)P_3 by the sequential action of 5-phosphatase and 3-kinase abolishes its ability to release Ca^{2+} and therefore terminates this branch of the signalling pathway.

In *Limulus* photoreceptors, L-Ins(1,3,4)P_3 caused repetitive bursts of depolarisation, while in the fluorescence experiments with rabbit platelets it was seen to cause prolonged Ca^{2+} release. These observations both suggest that L-Ins(1,3,4)P_3 is only poorly metabolised, and therefore has a prolonged effect. In the study by Hirata *et al.*,[159] L-Ins(1,3,4)P_3 was found to be 90-fold weaker in displacement of [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes. In a similar assay, **L-11b** was 98-fold weaker than Ins(1,4,5)P_3. These results are in excellent agreement, and are very strong evidence that the absolute configuration of the L-Ins(1,3,4)P_3 synthesised by Hirata *et al.* was correctly assigned. As discussed above, we found that the optical rotations of **D-11a** and **L-11b** were highly dependent on pH, and this may account for the discrepancies noted earlier.

As mentioned above, some samples of D-Ins(1,3,4)P_3 tested by Dr Richard Payne, including those purchased from commercial sources, were consistently active in *Limulus* photoreceptors, while others, including **D-11a** were always inactive. We conclude that the active samples were *contaminated* with some unknown substance. In the case of the biologically-derived D-Ins(1,3,4)P_3 this contamination is likely to have been Ins(1,4,5)P_3. The activity was not due to L-Ins(1,3,4)P_3, because the active samples did not produce *repetitive* bursts of Ca^{2+} release. Similarly, we conclude that when D-Ins(1,3,4)P_3 was reported to release Ca^{2+} from Swiss 3T3 cells,[91] this too was contaminated, most likely with Ins(1,4,5)P_3. It was this report that led other workers[92] to infer that the activity of racemic Ins(1,3,4)P_3 in bovine aortic smooth muscle cells was due to D-Ins(1,3,4)P_3.

So the confusion over the activity of D-Ins(1,3,4)P_3, it seems, has been resolved. All absolute configurations were correctly assigned, but some samples of D-Ins(1,3,4)P_3 were not pure. Pure D-Ins(1,3,4)P_3 is essentially inactive at the Ins(1,4,5)P_3 receptors of rabbit platelets, rat cerebellar membranes, *Limulus* photoreceptors, GH3 cells and Swiss 3T3 cells. One puzzle, however, remains. Hirata *et al.* reported that their L-Ins(1,3,4)P_3 was almost inactive in Ca^{2+} release, being 3000-fold weaker than Ins(1,4,5)P_3.[159] (In fact this group found L-Ins(1,3,4)P_3 to be so weak that they were unable to measure an EC_{50} value). In the rabbit platelet assay, **L-11b** was only 12-fold weaker than Ins(1,4,5)P_3. What could be the reason for this anomaly? It is unlikely to result from the different preparations of L-Ins(1,3,4)P_4 used in the two studies, because the affinities for rat cerebellar membranes were the same. The Ca^{2+}-mobilising effect of L-Ins(1,3,4)P_3 cannot be unique to platelets, because it was also found in *Limulus* photoreceptors. Furthermore, we can infer from the results of Polokoff *et al.*[92] with racemic Ins(1,3,4)P_3 that L-Ins(1,3,4)P_3 was also quite potent in bovine aortic smooth muscle cells. It may be that the discrepancy somehow results from differences in assay conditions, but there is also the possibility that it is due to differences between the Ins(1,4,5)P_3 receptor subtypes in the different cell types.

Since this study was carried out, more information has emerged regarding the different distributions of Ins(1,4,5)P_3 receptor subtypes in various tissues.[170] Platelets possess the type 1 Ins(1,4,5)P_3 receptor, and this has been shown to be similar to the rat cerebellum type 1 receptor in its ligand-binding characteristics.[171] In contrast, RBL cells have now been shown to express receptors that are primarily of the type 2 family, with very low amounts of type 1.[172] The significance of Ins(1,4,5)P_3 receptor diversity has not been established, although some have been reported to have a different inositol phosphate specificity.* The apparent difference in potency of L-Ins(1,3,4)P_3 in RBL cells and platelets suggests the possibility that the Ins(1,4,5)P_3 receptor subtypes in these two cell types might respond differently to L-Ins(1,3,4)P_3.

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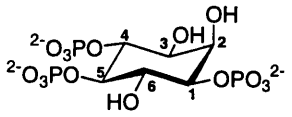
* An Ins(1,4,5)P_3 receptor associated with the plasma membrane of lymphocytes has been reported to show an unusually high affinity for Ins(1,3,4,5)P_4.[20] In another study, the Ins(1,4,5)P_3 receptor of olfactory cilia, also thought to be located in the plasma membrane, was found to bind Ins(2,4,5)P_3 with higher affinity than Ins(1,4,5)P_3 itself.[174]

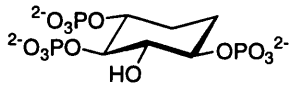
3 Acyclic and Ring-Contracted Analogues

3.1 An Acyclic Analogue of Ins(1,4,5)P_3

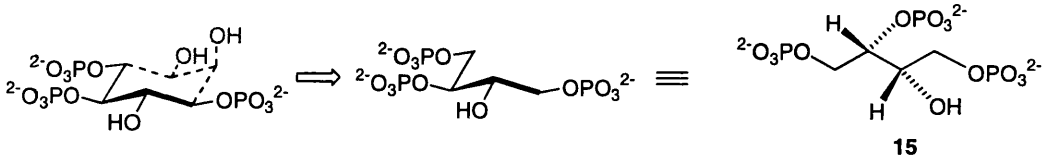
Various studies have demonstrated that the hydroxyl groups at positions 2 and 3 of Ins(1,4,5)P_3 play a relatively minor role in the recognition of this molecule by the Ins(1,4,5)P_3 receptor. The clearest illustration of this fact is the finding that 1D-2,3-dideoxy-Ins(1,4,5)P_3 (Figure 3.1) is only about six-fold weaker than Ins(1,4,5)P_3 in binding to bovine adrenal cortices and four-fold weaker in Ca^{2+} release from permeabilised SH-SY5Y cells.[107] It might therefore be interesting to delete this area of the cyclohexane ring in the Ins(1,4,5)P_3 molecule and examine the consequences for activity. The most drastic way to accomplish this would be to synthesise the acyclic trisphosphate **15** (Figure 3.1).



Ins(1,4,5)P3



1D-2,3-dideoxy-Ins(1,4,5)P3



15

Figure 3.1 Deletion of C-2 and C-3 in Ins(1,4,5)P_3 gives the acyclic analogue **15**.

This molecule retains a vicinal bisphosphate together with an adjacent hydroxyl group and a third phosphate group in an arrangement which is *topologically* equivalent to the important binding motifs of Ins(1,4,5)P_3. Of course, *conformationally*, **15** would be expected to be very different from Ins(1,4,5)P_3, and a molecular modelling simulation showed that many low energy conformers were possible. However, there was a report in the literature that L-glycerol 1,2-bisphosphate (but not glycerol 1,3-bisphosphate) could release Ca^{2+} in rat basophilic leukaemia cells at high concentrations (EC_{50} = 1.9mM).[132] Furthermore, 2,3-diphosphoglycerate has been reported to be a weak competitive antagonist of Ins(1,4,5)P_3 binding in rat cerebellar microsomes (IC_{50} = 400μM)[175] and, more recently, in calf cerebellar Ins(1,4,5)P_3 receptors.[176] This last observation implies that 2,3-diphosphoglycerate was recognised (albeit with very low affinity) by the

$Ins(1,4,5)P_3$ binding site and yet, perhaps as a result of its flexibility, was unable to cause the conformational changes in the $Ins(1,4,5)P_3$ receptor that result in opening of the ion channel. If it was possible to obtain a similar, or improved antagonist effect with **15**, then it might be possible to improve the binding affinity of the antagonist by further modifications.*

The acyclic analogue **15** can be related retrosynthetically to L-tartaric acid, but it was soon found that a more suitable starting material, (2*S*,3*S*)-(+)-2-benzyloxybutane-1,3,4-triol was commercially obtainable (Fluka), and could be used to synthesise **15** in only two steps. As this triol was readily available, the route would also provide an opportunity to evaluate a novel phosphitylating agent, bis-(4-chlorobenzyloxy)-*N*,*N*-diisopropylaminophosphine (Figure 3.2) which had been developed for the preparation of phosphorylated amino acids and peptides.[177] The advantages claimed for this reagent were that it is a solid, which is easy to handle, and that the 4-chlorobenzyl groups increased the crystallinity of the phosphorylated products, which are often oils and therefore difficult to purify.

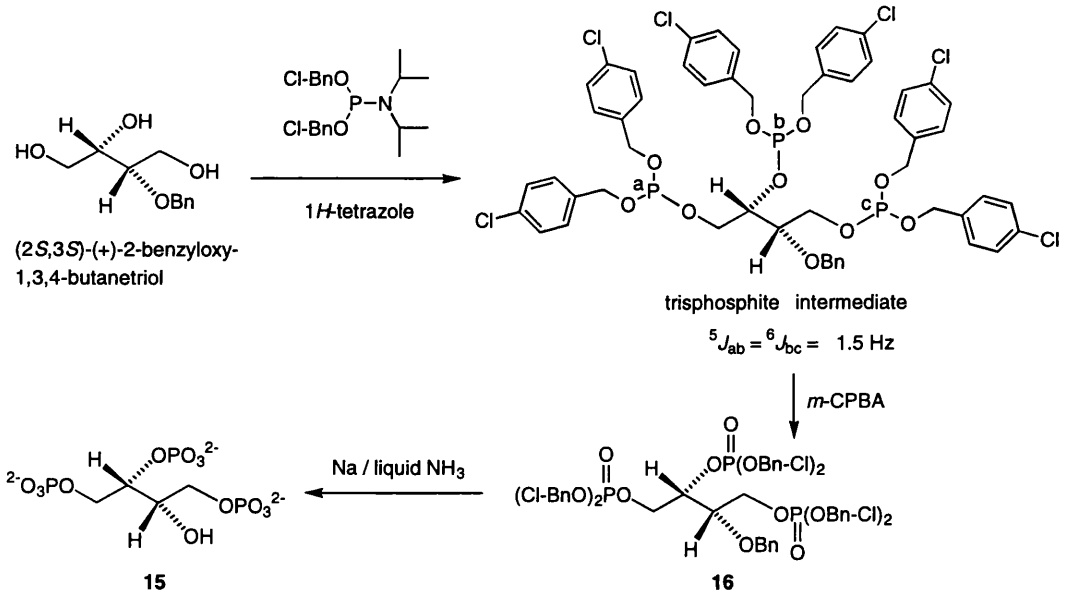


Figure 3.2 Synthesis of acyclic analogue **15**. Bn-Cl = 4-chlorobenzyl.

* For example, the 2′-AMP structure of the adenophostins appears to confer a hundred-fold increase in affinity for the receptor relative to analogues lacking this structure (See Chapter 5). Might it therefore be possible to design an acyclic analogue incorporating features of adenophostin, which would bind with high affinity, but not release Ca^{2+}?

The triol was phosphitylated using bis-(4-chlorobenzyloxy)-*N*,*N*-diisopropylaminophosphine / 1*H*-tetrazole in dichloromethane. The ^{31}P NMR spectrum of the intermediate trisphosphite triester showed very small $^{5}J_{PP}$ spin coupling constants (1.5 Hz), and unexpectedly, a $^{6}J_{PP}$ coupling of approximately the same magnitude was present. Thus, the ^{31}P NMR spectrum appeared as two doublets and a triplet. Oxidation gave the trisphosphate triester **16** which, disappointingly, was found to be an oil. Deprotection using sodium in liquid ammonia was successful, although the yield was low (42%) after purification by ion-exchange chromatography to give **15** as its triethylammonium salt.

3.1.1 Biological Evaluation

The acyclic trisphosphate **15** was tested for ability to release $^{45}Ca^{2+}$ from permeabilised rabbit platelets. Not surprisingly, it showed no effect over the usual concentration range (0 to 100μM) although slight $^{45}Ca^{2+}$ release was observed (19 ± 3.8%) at the highest concentration used (300μM). More significantly, **15** showed no ability to inhibit Ins(1,4,5)P_3 -induced $^{45}Ca^{2+}$-release (1μM Ins(1,4,5)P_3), and the slight $^{45}Ca^{2+}$-release effect at high concentrations was additive with the effect of Ins(1,4,5)P_3. Thus, **15** seemed to be acting as a very weak agonist, similar behaviour to that reported for L-glycerol 2,3-bisphosphate. It was decided not to pursue the investigation of acyclic analogues any further, and binding studies were not carried out.

These results suggest that a particular, relatively rigid, spatial arrangement of phosphate groups is required for recognition by the Ins(1,4,5)P_3 receptor. It might be reasoned that, although the acyclic analogue is *capable* of attaining the required conformation the suitable conformers are of relatively high energy, and are therefore present in very small amounts. An alternative interpretation is to invoke a more dynamic, induced-fit model, in which a flexible arrangement of phosphate and hydroxyl groups (even if it does mimic the appropriate binding conformation) will always be incapable of participating in a sequence of co-ordinated protein-ligand interactions which together constitute the process of ligand binding.

An examination of the ability of **15** to inhibit phosphorylation of Ins(1,3,4)P_3 by Ins(1,3,4)P_3 5/6-kinase showed that it was not recognised by the enzyme. The interaction of **15** with 5-phosphatase is under evaluation.

3.2 Five-membered Ring Analogues of Ins(1,4,5)P_3.

Returning now to the possibility of deleting part of the Ins(1,4,5)P_3 molecule, an approach based on ring-contraction might be more successful. In particular, a five-membered ring Ins(1,4,5)P_3 analogue that maintained the important recognition features and the conformational rigidity of Ins(1,4,5)P_3, but lacked any equivalent to positions 2 and 3 of Ins(1,4,5)P_3 might still be active. Of course, this ring-contracted version of Ins(1,4,5)P_3 would have to include an equivalent of the critical 4,5-bisphosphate feature, together with a surrogate for the important 6-hydroxyl group. A third phosphate group in a position corresponding to the 1-phosphate of Ins(1,4,5)P_3 should enhance binding. The simplest version of this is shown in Figure 3.3.

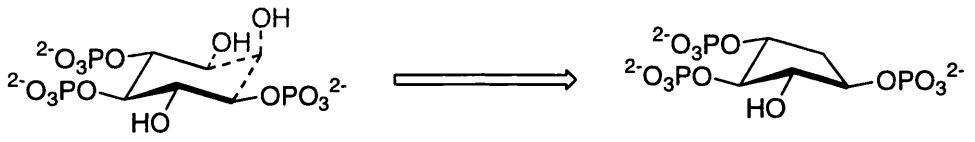


Figure 3.3 Deletion of the 2- and 3-hydroxyl groups of Ins(1,4,5)P_3 to give a five-membered ring analogue.*

The prospect of such a five-membered ring analogue was enticing because, despite the many modifications made to Ins(1,4,5)P_3 in the past, the requirement for a six-membered ring had never been questioned. Even in the adenophostins (see Chapter 5), which differ in many respects from Ins(1,4,5)P_3, the important 3,4-bisphosphate/2-hydroxyl triad, analogous to the 4,5-bisphosphate/6-hydroxyl arrangement of Ins(1,4,5)P_3, is contained within a six-membered pyranoside ring.

3.2.1 A Route to Five-Membered Rings from Glucose

This minimal structure did not seem to be easily accessible synthetically, and it was questionable whether the time and effort likely to be involved in developing a route was justified. At around this time a simple route to highly functionalised optically pure *vinyl*cyclopentanes from various carbohydrates was disclosed by Ito *et al.*[178] The method involved a zirconium-mediated diastereoselective ring contraction of vinyl carbohydrate derivatives. Two examples are shown in Figure 3.4.

* Note that the five-membered analogue shown in Figure 3.3 could be regarded as a conformationally-restrained analogue of the (inactive) acyclic analogue **15**.

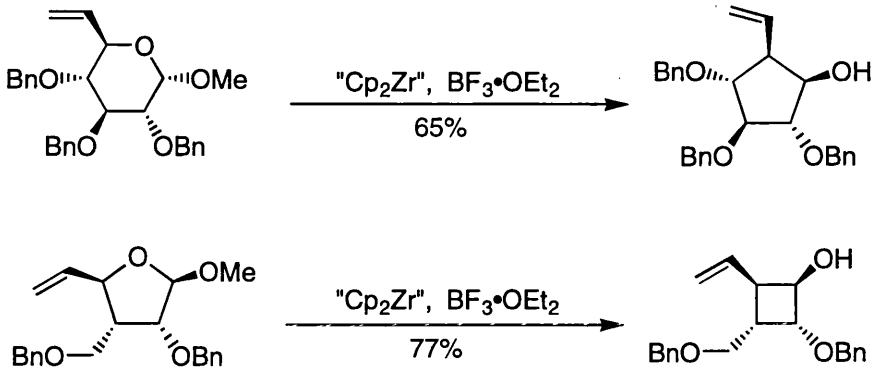


Figure 3.4 Diastereoselective ring contraction of vinyl carbohydrates using the zirconocene equivalent "Cp_2Zr" [178] (see text).

This prompted us to consider the feasibility of synthesising analogue **17**. Notice that **17** includes an hydroxymethyl group, which also occurs in adenophostin A (Chapter 5). Retrosynthetic analysis (Figure 3.5) showed that the required protected carbohydrate was the α-methyl-D-glucopyranoside derivative **18**, and it turned out that a colleague had already developed a route to this compound as part of a separate project. The appropriately protected pyranoside **18** was synthesised in 4 steps from α-methyl-D-glucopyranoside by this colleague.[179]

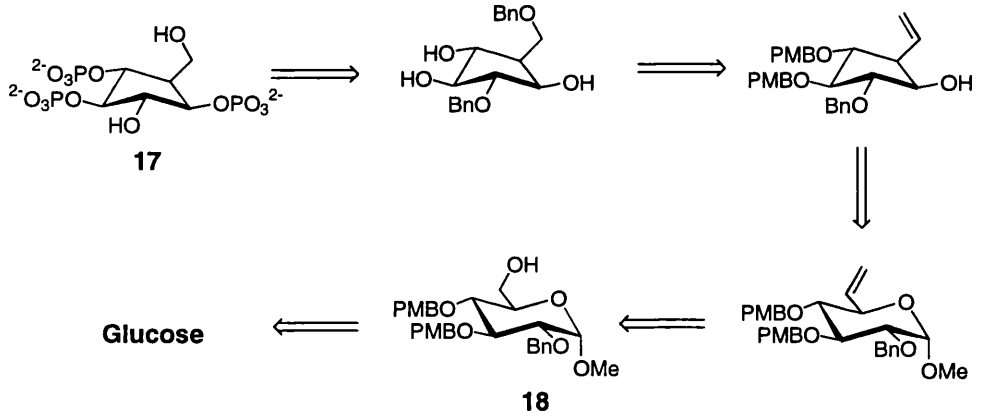


Figure 3.5. Retrosynthetic analysis suggests that the five-membered ring Ins(1,4,5)P_3 analogue **17** might be synthesised from glucose *via* the protected α-methyl-D-glucopyranoside derivative **18**.

3.2.2 Ring Contraction

The synthesis of the critical vinylcyclopentane derivative **21b** is shown in Figure 3.6. Swern oxidation of **18** with DMSO/oxalyl chloride gave the aldehyde **19.** This aldehyde was anticipated to be unstable and aqueous work-up was avoided. Instead the crude product was simply loaded onto a short column of silica and then eluted with ethyl acetate/hexane. Evaporation of solvents gave a yellow oil which was then used without

further purification. Wittig methylenation proceeded smoothly to give the required vinyl carbohydrate **20** in 75% overall yield from **18**. The ring contraction was carried out using the method described by Ito *et al.*,[178] in which the vinyl carbohydrate is treated with $Cp_2Zr(n\text{-}Bu)_2$ (prepared *in situ* from zirconocene dichloride and 2 equivalents of *n*-butyllithium), followed by $BF_3{\bullet}OEt_2$ in THF.

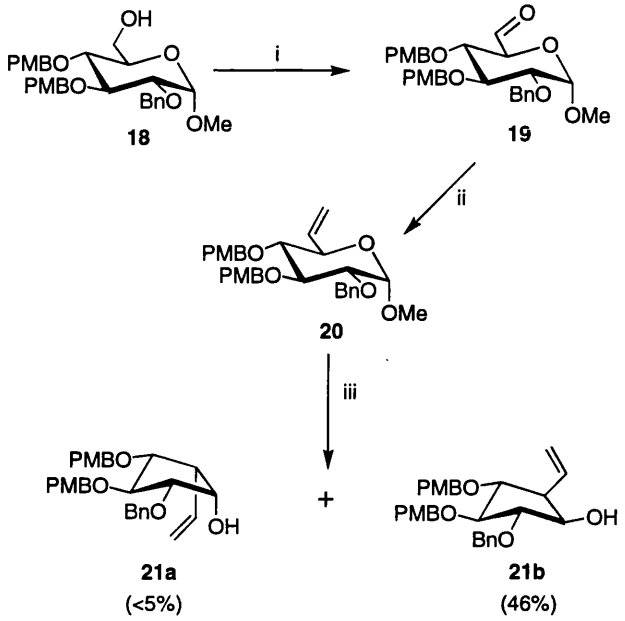


Figure 3.6 Synthesis of the vinylcyclopentane intermediate **21b**.
i) DMSO, $(COCl)_2$, CH_2Cl_2 then Et_3N, -60°C, ii) CH_3PPh_3, $KOBu^t$, THF, iii) "Cp_2Zr"/THF then $BF_3{\bullet}OEt_2$, -78°C to r.t. Bn = benzyl, PMB = *p*-methoxybenzyl.

The authors[178] propose a reaction mechanism (Figure 3.7) based on an NMR study of the reactive allylic zirconium intermediate, suggesting that the $BF_3{\bullet}OEt_2$ functions to accelerate the elimination of a methoxyl group by co-ordination to the methoxy oxygen of this intermediate. This gives two possible oxacarbenium ion transition states, one of which is of higher energy due an unfavourable steric interaction involving the protecting group at position 4 of the starting vinylpyranoside and a cyclopentadiene ring. The diastereoselectivity of the ring contraction is thought to originate in this energy difference. The stereochemistry of the new chiral centres in the product is thus strongly influenced by the stereochemistry at position 4 and the nature of the substituent. In this respect it was expected that a *p*-methoxybenzyl protecting group at this position would exert a similar influence to a benzyl group.

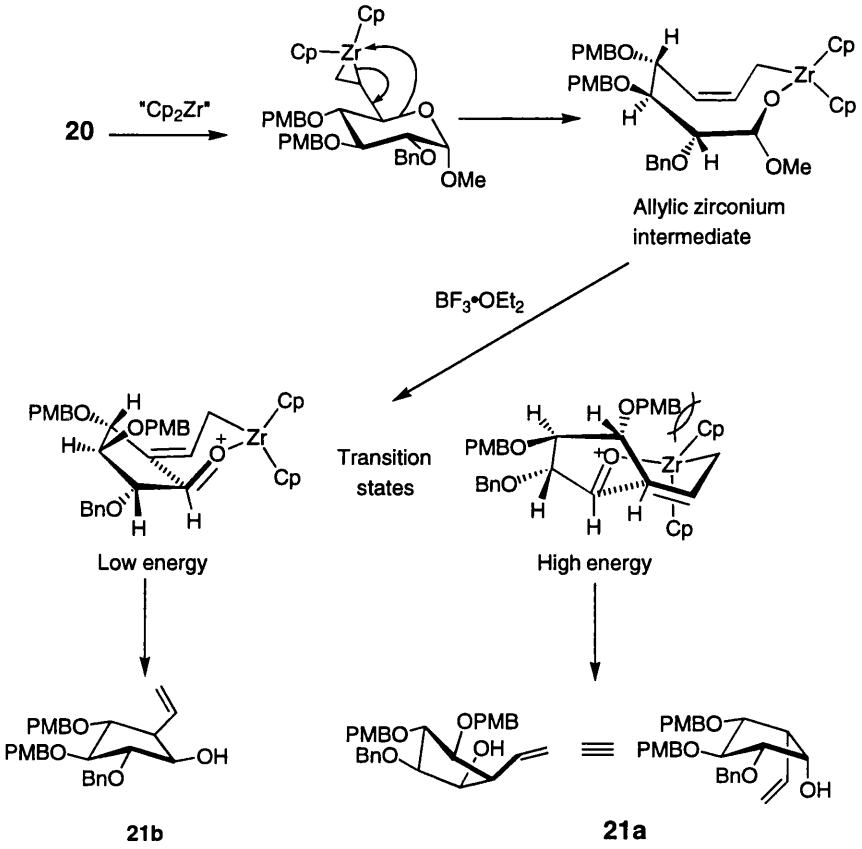


Figure 3.7 Proposed mechanism for the zirconium mediated ring-contraction [178] applied to the formation of the two vinylcyclopentanes **21a** and **21b**.

One complication encountered, however, was that gradual loss of *p*-methoxybenzyl protecting groups after the addition of BF_3•OEt_2 became a competing reaction. Nevertheless, the major product was obtained in fair (46%) yield as a waxy solid, together with a small amount of another product which had a very similar R_f, but could be isolated by careful chromatography, and then crystallised. The two products had almost identical infra-red spectra, but very different NMR spectra and optical rotations. It seemed likely that the two products were the diastereoisomers **21a** and **21b**, the minor isomer being the kinetically disfavoured product resulting from collapse of the higher energy pseudochair transition state.

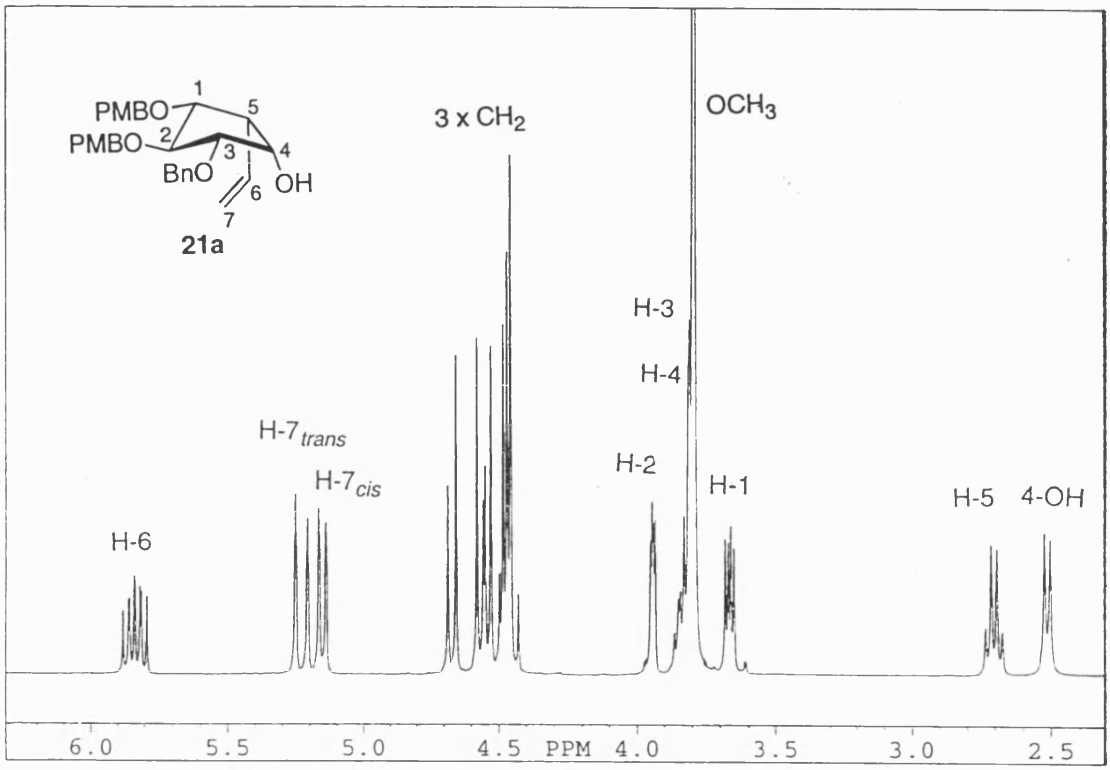
3.2.3 Assignment of Structures

At this stage it was not known which of the two products was the required structure **21b** although, on the basis of the mechanistic argument above, it seemed likely that **21b** would be the major product.

The ^{1}H and ^{13}C NMR spectra of this material closely resembled those obtained by Ito *et al.* for the related product with three benzyl protecting groups (Figure 3.4) while those for the minor product did not. However, Ito *et al.* did not isolate any of the minor product in the contraction of their glucopyranoside derivative, and so no data were available for comparison. X-ray crystal structures were out of the question, as the major product was a wax, and the other could be crystallised only with difficulty. However, the fact that we did have *both* diastereoisomers, meant that an NOE study was particularly appropriate.

^{1}H -^{1}H COSY NMR spectra were first obtained for both isomers and used to assign the ring protons in each case. The 1D ^{1}H NMR spectra, with the assignments are shown in Figure 3.8. A phase-sensitive 2D-NOESY was then obtained for the major product. Evidence from the 2D-NOESY was not conclusive. A definite strong NOE was seen between H-4 and H-5, for example, confirming that the substituents on the new C-C bond are *cis*, but this was compatible with either structure. All the expected strong NOEs, e.g. within the *p*-methoxybenzyl rings, within the vinyl group and between the ring protons at protected positions and the CH_2 protons of their respective protecting groups were visible, but other interactions within the five-membered ring were not clear.

The relative stereochemistries of **21a** and of **21b** were finally confirmed by NOE difference NMR spectroscopy. In each case, the H-5 proton was selectively irradiated and the NOE enhancements of the other ring protons were measured. The results are shown in Figure 3.9. This time, the expected NOEs could be seen, although there were some problems due to obscuring of signals by the *p*-methoxybenzyl methyl groups. However, the major product did indeed appear to be **21b**. The critical observations were the existence of an H-2/H-5 interaction in **21b** but not **21a**, and an H-1/H-5 interaction in **21a**, but not **21b**. This example illustrates the value of having both possible products when assigning structures on the basis of NOE experiments. The failure to observe an NOE, for example, is not in itself sufficient evidence for a particular structure. However, the observation of an NOE between two protons in one molecule, although not between the equivalent protons in another closely related structure, carries much more weight.



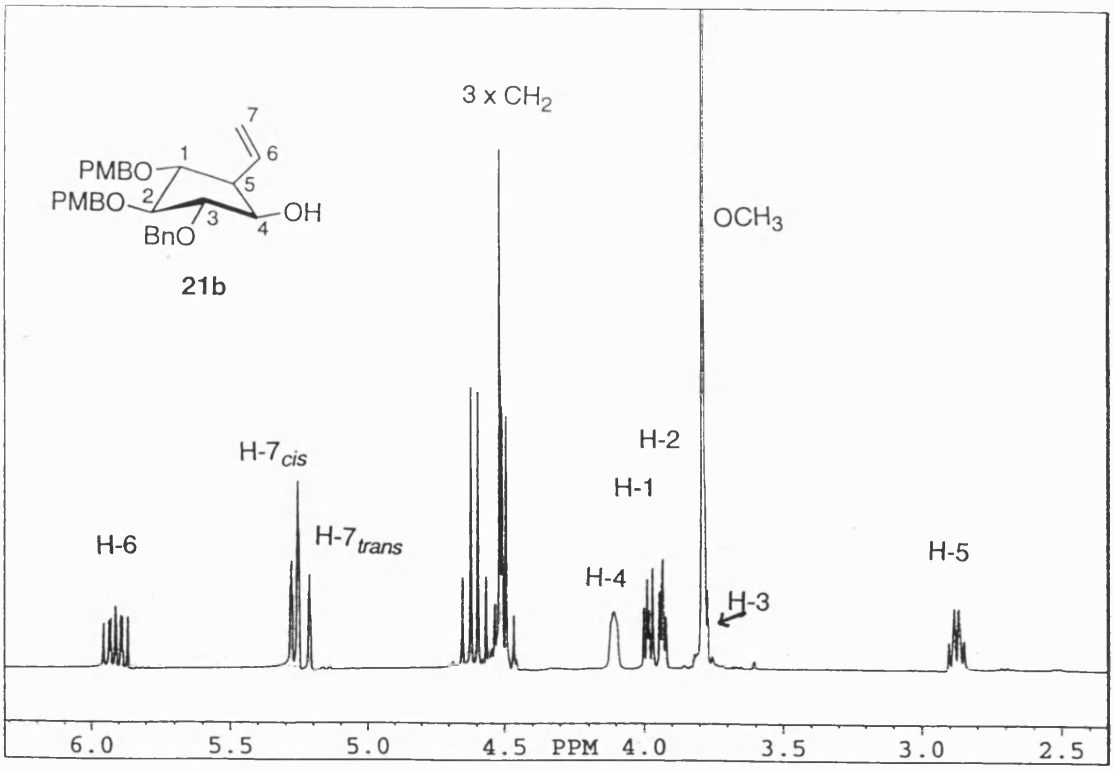
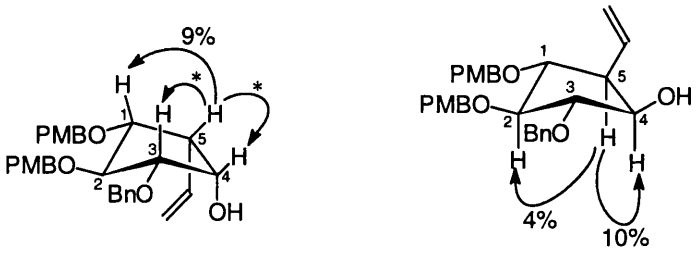


Figure 3.8 ^{1}H-^{1}H NMR spectra of vinylcyclopentanes **21a** and **21b** (400 MHz, $CDCl_3$)

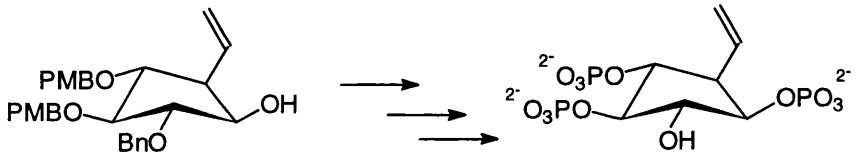


21a 21b

Figure 3.9 Results of NOE difference NMR spectroscopy of the diastereoisomeric vinylcyclopentanes **21a** and **21b**. Positive NOEs to H-3 and/or H-4 were observed for **21a**, but could not be quantified due to overlap of these signals with one another and with OCH_3 of PMBs.

3.2.4 A Prototype Vinylcyclopentane-based Analogue of Ins(1,4,5)P_3

At this stage, oxidative cleavage of the vinyl group and further protection/deprotection steps would be required to give the triol precursor for the target trisphosphate **17**. It was decided, with a relatively small amount of the ring-contracted intermediate **21b** in hand, to consider making the prototype compound **22**, which retains the vinyl group, and then to evaluate it biologically before continuing further with the synthesis of **17**.



21b 22

Figure 3.10 The intermediate **21b** could be converted to **22** in only three steps.

Molecular modelling showed that the vinyl group of **22** would be expected to occupy a position in space close to that of the axial 2-OH of Ins(1,4,5)P_3, and it is known that bulky substituents are tolerated in this region.[180] It therefore seemed logical to produce the immediately attainable trisphosphate **22**, and to find out whether this prototype molecule retained any biological activity, before attempting the more lengthy synthesis of **17**. If trisphosphate **22** did prove to be active, then the vinyl group of intermediate **21b** would provide a starting-point for modification to provide a variety of functional groups. including the original target trisphosphate **17**. Should this prototype prove to be totally inactive however, then the value of making other five-membered ring analogues might be called into question.

Removal of the *p*-methoxybenzyl protecting groups from the major product **21b** by acid hydrolysis gave the triol **23**. Phosphitylation using bis-(benzyloxy)-*N*,*N*-diisopropylaminophosphine and 1*H*-tetrazole, followed by oxidation of phosphites with *m*-chloroperoxybenzoic acid gave the fully protected trisphosphate **24**. ^{31}P NMR spectroscopy of the intermediate trisphosphite triester showed a large $^{5}J_{PP}$ coupling constant of 6.7 Hz [*cf.* 2.9 Hz and 3.4 Hz for precursors of Ins(4,5)P_2[181] and Ins(1,4,5)P_3[182] respectively], presumably reflecting the altered geometry of the P(III)-P(III) interaction in a five-membered ring.

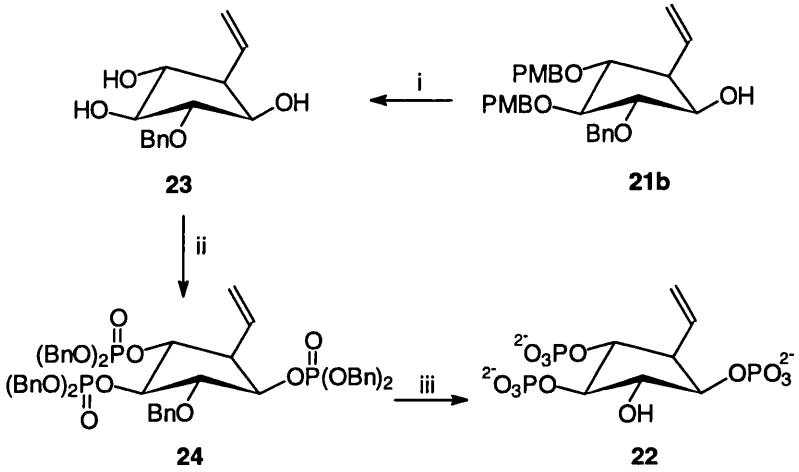


Figure 3.11 Conversion of **21b** to the prototype five-membered ring analogue **22.**
i) M HCl/EtOH 1:2, Δ, 3h, 87%; ii) a) $Pr^{i}_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2 b) *m*-CPBA, -78°C, 82%; iii) Na/ liquid NH_3, -78°C, 58%.

The ^{1}H-coupled ^{31}P NMR spectrum of the trisphosphate triester **24** (Figure 3.12) showed a surprising feature. In previous compounds of this type, which were all based on six-membered frameworks, the signals for the three phosphorus atoms appear as sextets. This pattern arises from heteronuclear $^{3}J_{HP}$ couplings to the four protons in the benzyl CH_2 groups and also to the single cyclitol ring proton at the position of phosphorylation. As these five protons are all chemically inequivalent, the splitting pattern is strictly a ddddd, but because the coupling constants are usually similar, each signal approximates to a sextet. In the spectrum of **24**, one of the three phosphorus atoms appears as a doubled sextet. The extra splitting (approximately 2.7Hz) must arise from a long-range ^{31}P -^{1}H coupling, probably related to the novel geometry of the five-membered ring, although it was not possible to locate this coupling in the complex ^{1}H

NMR spectrum. A 2D phosphorus-proton COSY experiment, which has not yet been carried out, should be able to identify the pair of atoms involved.

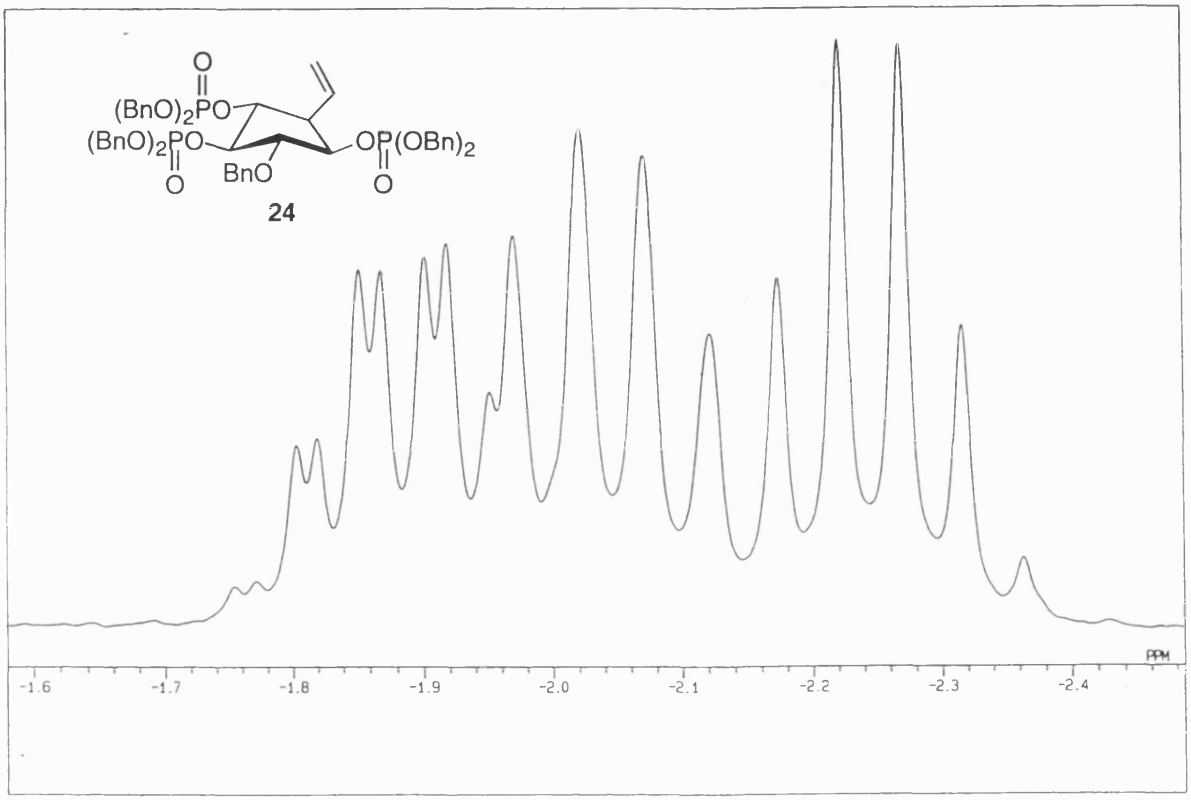


Figure 3.12 ^{31}P NMR spectrum of **24** (^{1}H-coupled, 400 MHz, $CDCl_3$).

Deprotection using sodium in liquid ammonia removed the seven benzyl protecting groups, leaving the vinyl group intact. Purification by ion-exchange chromatography of the crude product gave the trisphosphate **22**, which was isolated as the triethylammonium salt and quantified by phosphate assay. A ^{1}H-coupled ^{31}P NMR spectrum of **22** showed the expected three doublets. The long-range heteronuclear coupling seen in the spectrum of its protected precursor had now disappeared.

3.2.5 Biological Evaluation

Trisphosphate **22** was examined for Ca^{2+} mobilising activity at the platelet Ins(1,4,5)P_3 receptor using fluorescence techniques, and also using saponin-permeabilised platelets loaded with $^{45}Ca^{2+}$. It was found to be a full agonist, around 65-fold weaker than Ins(1,4,5)P_3 (Figure 3.13) and the effect was inhibited by addition of heparin.

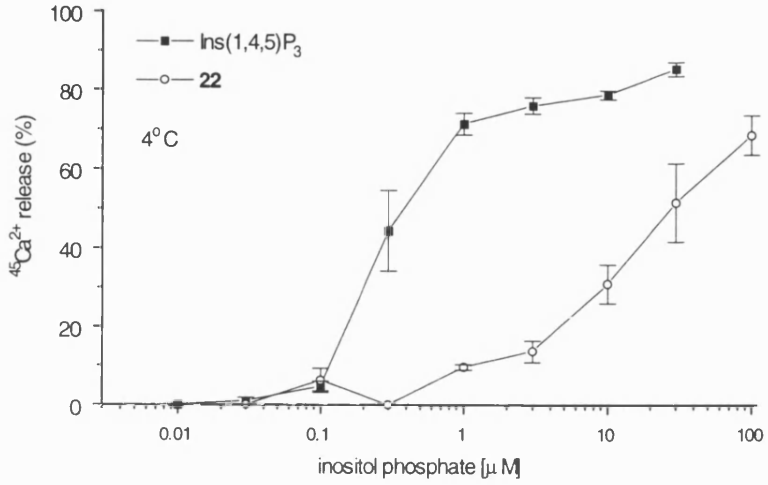


Figure 3.13 $^{45}Ca^{2+}$-release by **22** from permeabilised rabbit platelets (n=2).

These results demonstrated for the first time that potent Ins(1,4,5)P_3 receptor-mediated Ca^{2+} mobilisation does not necessarily require a six-membered ring. A smaller ring phosphate that retains crucial recognition elements of Ins(1,4,5)P_3, i.e. three appropriately orientated phosphates and a surrogate for the 6-hydroxyl group, is still recognised by the Ins(1,4,5)P_3 receptor.

The Ca^{2+}-releasing properties of **22** in SHSY-5Y cells and its interaction with a cloned 3-kinase are currently under investigation. As an optically pure analogue whose *trans*-bisphosphate has the D-4,5-configuration, we would not expect it to interact with D-Ins(1,3,4)P_3 5/6-kinase (Section 1.9.4), and biological testing has now confirmed that this enzyme does not recognise **22**.

3.3 Attempted Synthesis of Hydroxymethyl Analogue 17

It was not clear at this stage whether the considerably reduced potency of **22** relative to Ins(1,4,5)P_3* was related to the presence of the vinyl group or was a necessary consequence of the reduced ring size. In the five-membered ring, for example, molecular modelling shows that the torsion angle corresponding to O4–C4–C5–O5 of Ins(1,4,5)P_3 is expected to be larger,† and the relative position of the 1-phosphate group is altered. Increasing steric bulk at the 3-position of Ins(1,4,5)P_3 (see Chapter 5) reduces

* Strictly, **22** should be compared to D-3-deoxy-Ins(1,4,5)P_3, which is reported to be 4-fold weaker in Ca^{2+} release and 6-fold weaker in binding than Ins(1,4,5)P_3.[107]

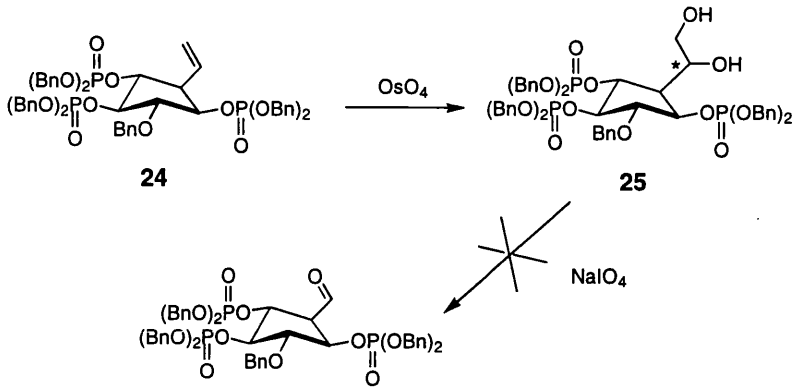
† The effect of increasing this torsion angle on biological activity could be explored by the synthesis of cyclohexene-based analogues (see section 4.5.2)

affinity for the receptor, and D-3-MeO-Ins(1,4,5)P_3 is 150-fold weaker in Ca^{2+}-release and 50-fold weaker in binding than Ins(1,4,5)P_3[183] while steric bulk at the axial position 2 is well-tolerated. As the molecular modelling of **22** showed its vinyl group to occupy an area of space somewhere *between* positions 2 and 3 it was difficult to predict the extent of its effect. However, having demonstrated reasonably potent activity for the prototype **22**, the next step was to synthesise and evaluate the hydroxymethylcyclopentane trisphosphate **17**.

The synthesis of **17** would require oxidative cleavage of the vinyl group in **21b** together with a number of protection/deprotection steps before phosphorylation and final deprotection. However, the intermediate **21b** was by now in short supply, and so it was decided to attempt a short-cut by cleaving the vinyl group of the phosphorylated intermediate **24** with osmium tetraoxide/ sodium metaperiodate followed by sodium borohydride.

A solution of **24** in ether was treated with an aqueous solution of osmium tetraoxide and sodium metaperiodate.[184] A TLC obtained 3 hours after addition of the OsO_4/$NaIO_4$ mixture showed only highly polar products together with one spot at longer R_f. What was worse, this product, which was assumed to be the aldehyde (although worryingly, it did not stain with DNP) appeared to be diminishing with time. When sodium borohydride was added the spot was unaffected. The reaction had obviously failed, and isolation of the only non-polar product by flash chromatography showed it to be a small amount of the *vic*-diol **25** (the stereochemistry at the position marked * is not established).

It seems, therefore that the initial dihydroxylation of the double bond had occurred, with the OsO_4 approaching exclusively from one face to give the glycol **25**. Presumably, **25** would then be cleaved over a period of hours, by the metaperiodate to give the aldehyde, hence the gradual diminution of the corresponding spot in the TLC. However, as no aldehyde was seen, it is tempting to conclude that it was unstable, and rapid conversion to polar products, with loss of phosphate esters had occurred.



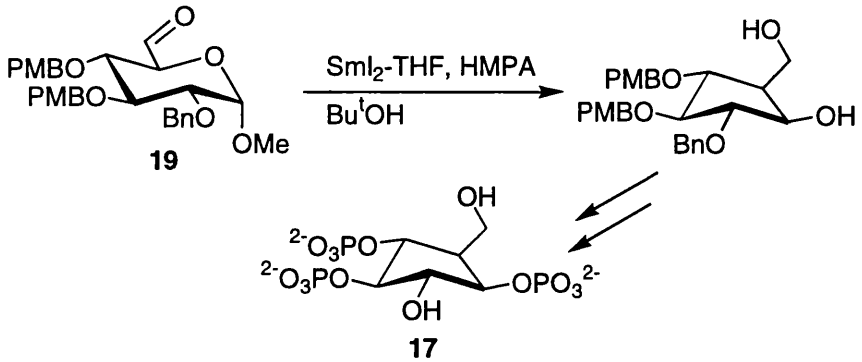
24 25

Figure 3.14 Unsuccessful attempt at oxidative cleavage of the vinyl group in **24**

3.3.1 Improved Route to the Hydroxymethyl Analogue

It would therefore be necessary to synthesise more ring-contracted intermediate **21b** and then carry out the cleavage of the vinyl group prior to phosphorylation. However, at about this time, a more direct route to **17** became available. A recently reported samarium (II) iodide-mediated ring contraction[185] could be used directly on aldehyde **19** to give the diol (Figure 3.15) required for the synthesis of **17**. The route would be far shorter than that *via* the vinylcyclopentane intermediate. The synthesis of **17** by this new method has now been successfully carried out by a colleague.

The vinylcyclopentane trisphosphate **22** and the hydroxymethyl analogue **17** were compared for release of $^{45}Ca^{2+}$ from permeabilised Jurkat T-lymphocytes (the rabbit platelet assay was no longer available). As expected, **17** showed greatly increased activity compared to vinylcyclopentane analogue **22**, being only two to four-fold weaker than Ins(1,4,5)P_3 in this assay.



19 17

Figure 3.15 SmI_2-mediated ring-contraction of an aldehyde[185] provides a rapid route to the diol required for the target trisphosphate **17**.

It is not yet clear whether this enhancement of activity in the hydroxymethyl analogue **17** is due simply to the removal of the hydrophobic vinyl group, or whether the primary hydroxyl group engages in favourable interactions with the Ins(1,4,5)P_3 receptor binding site. Another possibility is that the hydroxymethyl group influences the orientation of the nearby phosphate group by intramolecular hydrogen bonding. A molecular modelling simulation showed that, in many low energy conformations of both Ins(1,4,5)P_3 and **17**, the 4-phosphate group of Ins(1,4,5)P_3 and its equivalent in **17** did form H-bonds to the neighbouring hydroxyl (Figure 3.16), although it is difficult to say how significant such intramolecular interactions may be in solution and at the receptor binding site.

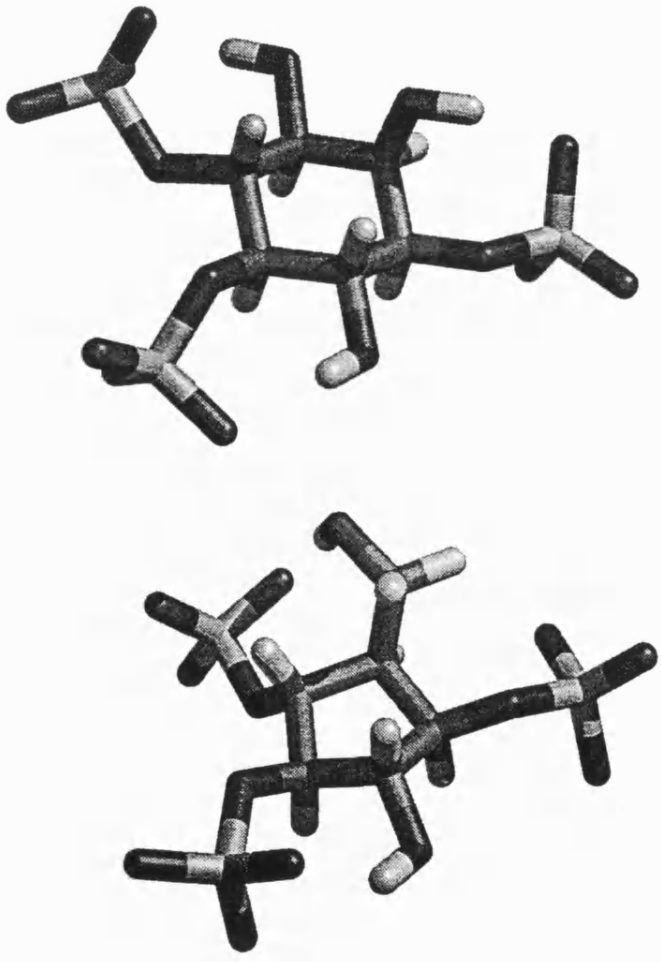


Figure 3.16 Low-energy conformations of Ins(1,4,5)P_3 and **17** show intramolecular H-bonding interactions. (See Experimental for details of modelling).

4 A Conformationally Restricted Analogue of Ins(1,4,5)P_3

4.1 Design

It was argued in Section 1.11 that it might be possible to gain more information about the Ins(1,4,5)P_3 receptor binding site by studies of conformationally restricted analogues. A problem inherent in the use of this strategy is that it is not possible to achieve the desired constraint without altering the molecule in some other way. It is likely that steric bulk will be added, for example, and that charge distribution and hydrogen bonding interactions will be disrupted. The ideal is to constrain some part of the molecule, which is thought to be significant in its binding to the receptor, in such a way as to minimise the disturbance to other regions that are known to be important for activity. This may be difficult to achieve, and, should the molecule prove to be inactive, it is not possible to infer with certainty that its inactivity is related to its conformational rigidity *per se.*

Considering now the Ins(1,4,5)P_3 molecule, it would be interesting to constrain the orientation of one of the phosphate groups. Apart from a study of the naturally occurring inositol 1:2-cyclic,4,5-trisphosphate,[186] which mobilises Ca^{2+}, and the synthetic inositol 1-phosphate-4,5-pyrophosphate,[187] which was inactive, this approach had not yet been explored for Ins(1,4,5)P_3. In the light of the discussion above, it might be particularly interesting to constrain the 4- or 5-phosphate group. To do this, we would need somehow to attach it to an adjacent position (Figure 4.1). Note that in the two analogues shown, the equivalent of the hydroxyl group at position 2 is equatorial rather than axial. This considerably simplifies the synthesis, as will be seen later. It was thought unlikely that the inversion of position 2 would significantly reduce activity, as *scyllo*-Ins(1,2,4)P_3 [106] is almost equipotent with Ins(1,4,5)P_3. Studies have shown that the Ins(1,4,5)P_3 receptor is intolerant of added steric bulk at position 6 [92] and that deletion of the hydroxyl group at 6 also reduces activity by a factor of 70.[109] On the other hand, the Ins(1,4,5)P_3 receptor can accommodate slightly increased bulk around the 3-position[188] and what is more, 3-deoxy-Ins(1,4,5)P_3 is highly active.[107,108]

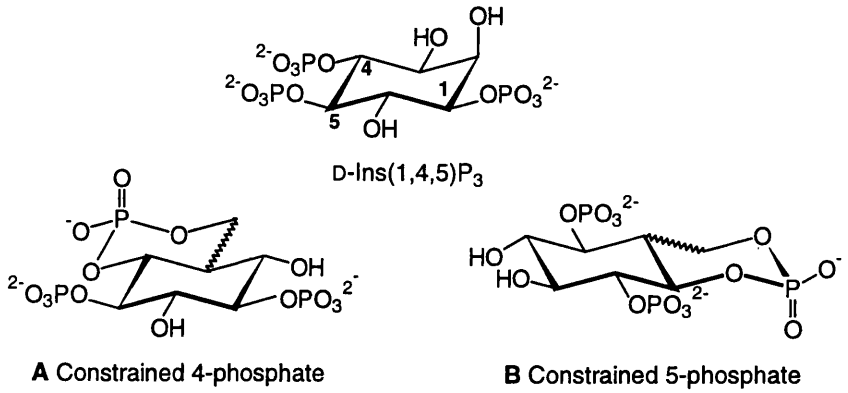


Figure 4.1 Two cyclic phosphate analogues of Ins(1,4,5)P_3

Finally, constraining a phosphate group in this way would necessarily result in a decrease of negative charge on the constrained phosphate. Now NMR studies have shown that the binding affinity of Ins(1,4,5)P_3 to its receptor correlates most closely with the ionisation state of the 5-phosphate group.[189] Taken together, then, these observations suggested that the initial target should be structure **A**, in which the 4-phosphate group is constrained.

There are two possible epimeric molecules corresponding to structure **A**, and these are shown in Figure 4.2. When molecular modelling studies of Ins(1,4,5)P_3 were carried out, the energy-minimised structures were consistently found to resemble the *trans*-fused structure **26**, in which the equivalent of the P4–O4–C4–H4 torsion angle* is positive and *gauche*, more than its *cis*-fused epimer.

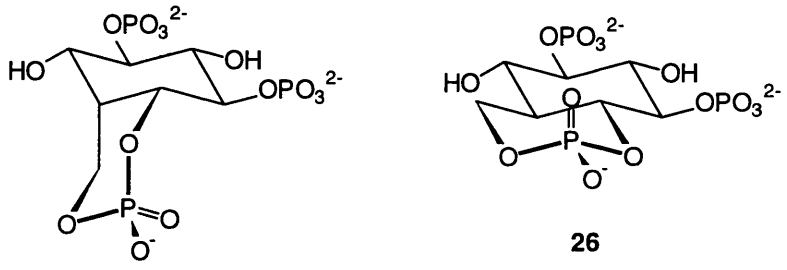


Figure 4.2 Two epimeric cyclic analogues of Ins(1,4,5)P_3. The phosphate group equivalent to the 4-phosphate of Ins(1,4,5)P_3 is constrained in one of two different orientations.

* Energy-minimised conformations of Ins(1,4,5)P_3 all had a P4–O4–C4–H4 torsion angle between 0° and +60°, but the size of the angle appeared to depend on the way in which the charges on the phosphate groups were simulated, on the treatment of the dielectric constant of the medium, and on whether the minimised structure showed intramolecular H-bonding. However, structures resembling the *cis*-fused epimer were *never* seen.

This arises because, at least in these simulations, the dominant factor in determining the conformation of the 4- and 5-phosphates is electrostatic repulsion. In some energy-minimised structures (e.g. Figure 3.16) a hydrogen-bonding interaction between the 4-phosphate and the 3-OH group was also seen, further stabilising a splayed arrangement of phosphate groups.

The decision was therefore made initially to develop a route leading to **26**, in which the two rings of the target bicyclic system are fused in a *trans* sense. Ideally the route should be capable of modification to give the *cis*-fused epimer, if required, so that the biological properties of the two epimers could be compared. Retrosynthetic analysis of analogue **26** led to a symmetrical precursor which could, in turn, be synthesised from *myo*-inositol orthoformate (**27**).

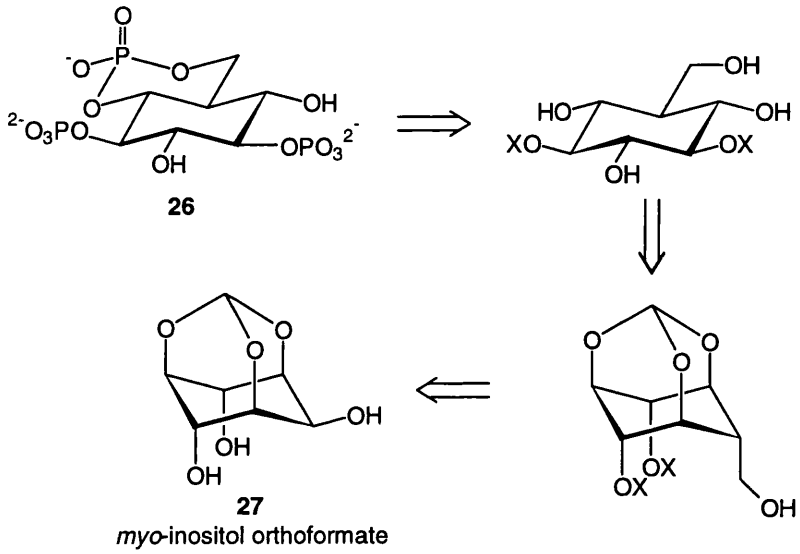


Figure 4.3 Retrosynthetic analysis of the cyclic analogue **26** suggests a synthesis from *myo*-inositol orthoformate.

4.2 Synthesis

The successful synthesis of **26** is shown in Figure 4.4. It has the advantage that the symmetrical nature of the intermediates is maintained well into the synthesis. This simplifies the interpretation of the NMR spectra, particularly for the orthoformate derivatives, whose ^{1}H NMR spectra can be complicated by high multiplicity of the signals due to long-range couplings, and the fact that all vicinal couplings are of similar magnitudes, making signals difficult to assign.

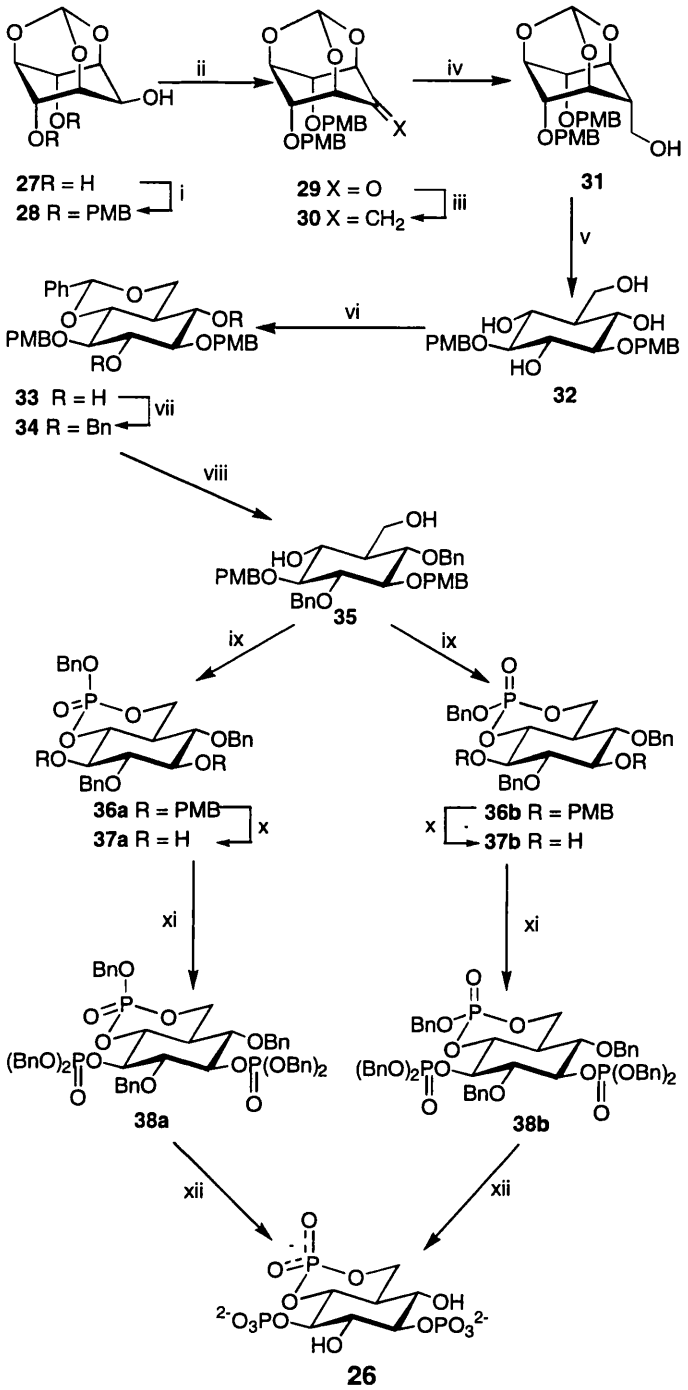


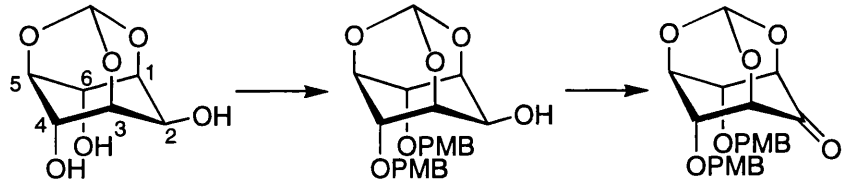
Figure 4.4 Synthesis of conformationally restrained analogue **26**
i) NaH (2.1equiv.), PMBCl (2.0 equiv.), DMF; ii) DMSO, $(COCl)_2$, CH_2Cl_2, -60°C then Et_3N; iii) CH_3PPh_3Br, *t*-BuOK, THF, reflux; iv) a) 9BBN-H, THF, 50°C b) H_2O_2, OH^-; v) 1M HCl/MeOH 1:10, 50°C; vi) $C_6H_5CH(OMe)_2$, DMF, *p*-TsOH, vii) NaH, BnBr, DMF; viii) 1M HCl/THF/MeOH 1:5:5, reflux; ix) a) $BnOP(NPr^i_2)_2$, 1*H*-tetrazole, b) *m*-CPBA, -78°C; x) DDQ, CH_2Cl_2, H_2O; xi) a) $(BnO)_2PNPr^i_2$, 1*H*-tetrazole, b) *m*-CPBA, -78°C; xii) Na/liq NH_3 PMB = *p*-methoxybenzyl; Bn = benzyl; All asymmetrical compounds are racemic.

It was intended that the methylenation step would be carried out using a Wittig reaction or by the use of Tebbe's reagent.[190] Difficulties had been reported in the use of Wittig reactions on inososes but apparently these could be overcome by introducing conformational restraint,[191] and the rigid structure of ketone **29** should be particularly

suited to this. Furthermore, it might be possible to exploit the structure of alkene **30** so as to determine the stereochemistry at the branch-point with high selectivity.

4.3 Synthesis of a Versatile Ketone Intermediate

p-Methoxybenzyl groups were chosen to protect positions 4 and 6 of *myo*-inositol orthoformate. The protecting groups at these positions were required to be of a semi-permanent nature and although, like the orthoformate ester, these groups are acid-labile, it was known that an orthoformate ester could selectively be cleaved in their presence.[83] Allyl groups, for example, were not suitable, because a hydroboration reaction might be required at a later stage. One route to the 4,6-di-*O*-alkylated compound **28** would be selectively to protect *myo*-inositol orthoformate at position 2 (e.g. with *tert*-butyldimethylsilyl chloride), carry out the *p*-methoxybenzylation, and then remove the protection at position 2. This strategy has been used for the synthesis of 4- and 6- substituted *myo*-inositol orthoformates.[129,130] However, the overall yield after these three steps is low and this arises mainly from inefficiency in the introduction of the silyl protecting group. There is a report in the literature that, benzylation of *myo*-inositol orthoformate using two equivalents of sodium hydride and two equivalents of benzyl bromide gave a mixture of mono-, di- and tri-*O*-benzylated orthoformates, with the 4,6-dibenzylated product as the major product (27% yield).[125] It was felt that, if this yield could be improved, then this strategy would be preferable to the three-step route, particularly for large-scale production of **28**.



27 **28** **29**

Figure 4.5 Regioselective alkylation of *myo*-inositol orthoformate followed by Swern oxidation gives the strained ketone **29**.

Reaction of **27** with 2.1 equivalents of *p*-methoxybenzyl chloride and 2.3 equivalents of sodium hydride gave the symmetrical 4,6-disubsituted alcohol **28** as the highly crystalline major product, easily recognisable from the NMR spectra by its plane of symmetry. With careful work-up and chromatography it was possible to increase the yield of **28** to around 40%. The monosubstituted by-product was found to be exclusively the axially substituted diol, and this could be recycled to give more **28**. No products substituted at position 2 were detected, apart from a small amount of the tri-*O*-*p*-

methoxybenzyl ether.[83] Alcohol **28** has obvious application as a precursor for position 2-modified analogues of Ins(1,3,4,5,6)P_5, and for the synthesis of position 2-modified Ins(1,4,6)PS_3 analogues, potential partial agonists at the Ins(1,4,5)P_3 receptor (see Chapter 6).

Swern oxidation of **28** using DMSO/oxalyl chloride was expected to give the inososе **29**, and the reaction appeared to be successful, as judged by TLC. However, after aqueous work-up, an infra-red spectrum of the product showed two bands at 3540 and 3440cm^{-1} suggesting two alcohol groups, together with a band at 1760cm^{-1}, corresponding to the expected ketone. The alcohol bands were not due to starting material, which showed a single band at 3500cm^{-1}. The ^1H NMR spectrum was confusing, showing a mixture of two compounds. Eventually it was realised that the desired ketone was present, but had been partially converted into the corresponding *gem*-diol (ketone hydrate **29a**, Figure 4.6) in the work-up. Part of the sample was refluxed in toluene, with azeotropic removal of water, and the IR spectrum then showed that the bands around 3500cm^{-1} had disappeared. ^1H NMR and ^{13}C NMR spectra were then obtained, and clearly showed the product to be the symmetrical ketone **29**.

The *gem*-diol **29a** could also be isolated by allowing a solution of the ketone in dioxane with a few drops of water to stand for a few days, followed by evaporation and re-crystallisation. The ^1H NMR spectrum of **29a** showed the two OH protons, exchangeable in D_2O within a molecule that maintained the same symmetry as the corresponding ketone. The chemical shifts of these protons were very different (δ 3.82 and δ 4.97, $CDCl_3$). It is likely that the signal at δ 4.97 corresponds to the proton of the axial hydroxyl group, as the higher-field signal is similar to that seen in the spectrum of **28,** and the corresponding hydroxyl proton of the *scyllo*-inositol orthoformate derivative **63** (Chapter 7) is also deshielded. The ^{13}C NMR spectrum showed the unusual feature of a quaternary carbon resonating at δ 88.67, corresponding to $C(OH)_2$.

The ease of hydration of ketone **29** may be attributable to strain effects in its rigid cage-like structure, resulting in destabilisation of sp^2-hybridized carbon relative to sp^3. The unusually high stretching frequency of the C=O bond in **29** (1760cm^{-1}) is further evidence of ring strain. Ketone **29** also reacted with methanol at room temperature to give a single hemiketal. The configuration of this product at C-2 has not yet been rigorously established, but it seems likely that it is **29b**, with the *O*-methyl group equatorial. Of the two alternatives, this epimer is sterically less congested, and

therefore likely to be the more thermodynamically stable. In the ^{1}H NMR of **29b**, the singlet corresponding to the hydroxyl proton is found at very low field (δ 4.92, $CDCl_3$), an observation which may be interpreted as evidence that hydroxyl group is axial, thus supporting the structure shown.

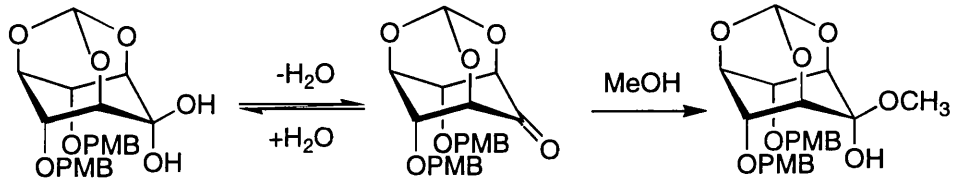


Figure 4.6 Conversion of the ketone **29** to *gem*-diol **29a** and hemiketal **29b**

Ketone **29** is highly crystalline, and stable if kept out of contact with moisture. Its carbonyl group shows high reactivity, and the fact that one face is sterically hindered by the *p*-methoxybenzyl groups, while the other is highly exposed to attack from a variety of reagents should make it a useful intermediate for stereoselective modification. An example of this is its use in the synthesis of *scyllo*-inositol 1,2,3,5-tetrakisphosphate (Chapter 7).

4.4 Wittig Methylenation: Observation of an Unusually Stable Intermediate.

The next step was the methylenation of ketone **29** to provide the symmetrical alkene **30**. It was planned to attempt this transformation initially by Wittig methylenation using methylenetriphenylphosphine, and then if this was unsatisfactory, to try Peterson olefination or the (much more expensive) Tebbe reagent. In a reported synthesis of conduritols from L-quebrachitol, Wittig methylenation of an inosose bearing cyclohexylidene and methyl ether protecting groups gave very poor yields, which the authors attributed to steric hindrance, although Peterson olefination was successful.[192] A comparative study of the Wittig and Tebbe reagents[190] claimed higher yields for the latter, and greater success with substrates that were sterically hindered. The strongly basic conditions of the Wittig reaction were not expected to be a problem, as neither orthoformate nor *p*-methoxybenzyl protecting groups are base-sensitive. Another important property of ketone **29** is that the protons α to the carbonyl group are not acidic, being located at bridgeheads, so there is no possibility of enolisation.

In the first attempts at Wittig methylenation using methyltriphenylphosphonium bromide, potassium *tert*-butoxide as base and heating to 50°C for two hours, the yields were consistently low (30 to 50%). During a final attempt to improve yields, it was

noticed that TLC of the crude product showed another polar by-product, besides triphenylphosphine oxide. In previous attempts, large quantities of highly polar, pale yellow solid material had been filtered off during work-up and discarded, assuming it to contain mainly triphenylphosphine oxide. However, one of the polar products stained purple with PMA, evidence that it contained *p*-methoxybenzyl groups. Some of the solid, still left in a Buchner funnel from the previous day's reaction was added to THF and heated to reflux. It rapidly decomposed to give large quantities of alkene **30**, and the cause of the low yields was immediately apparent. The intermediate oxaphosphetane formed by the reaction of ketone **29** with methylenetriphenylphosphorane was highly stable, and heating to 50°C was insufficient to decompose it fully within two hours. When the reaction was repeated, but with heating to reflux for two hours after adding ketone to Wittig reagent, **30** was obtained in 91% yield. In a later, large-scale run of the reaction, samples were taken and subjected to ^{31}P NMR. After addition of base to methyltriphenylphosphonium bromide, the canary yellow ylide appeared as a peak at δ_P 19.9 in the NMR spectrum. On addition of ketone, this was replaced by a single resonance at δ_P -68.9, corresponding to the oxaphosphetane. A sample of this material was kept for 2 days at 4°C, after which time ^{31}P NMR spectrum showed evidence of only slight decomposition.* The oxaphosphetane is assumed to have the structure shown in Figure 4.7, arising from attack at the less hindered face of the carbonyl group.

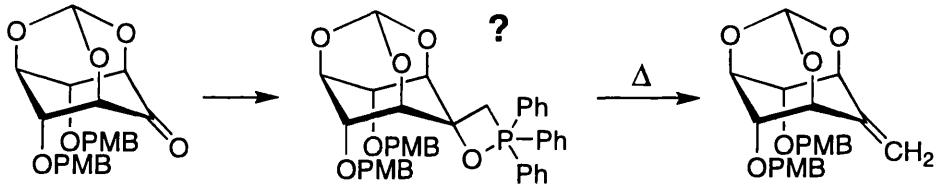
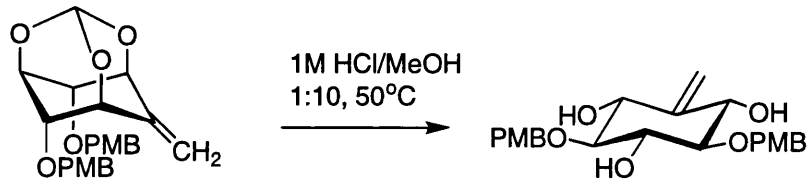


Figure 4.7 Wittig methylene of ketone **29** proceeds *via* a stable oxaphosphetane intermediate.

The orthoformate ester of **30** could be removed selectively using mild acid treatment to give the symmetrical crystalline triol **39**. This compound has not been further investigated to-date.

* A detailed low-temperature study of the intermediates in the Wittig reaction[193] has found that, although most oxaphosphetanes obtained from the reaction of aldehydes and ketones with $CH_2=PPh_3$ are unstable, being decomposed within a few minutes at -8°C, those derived from cyclobutanone and norbornanone require >0°C for conversion to the strained alkene.

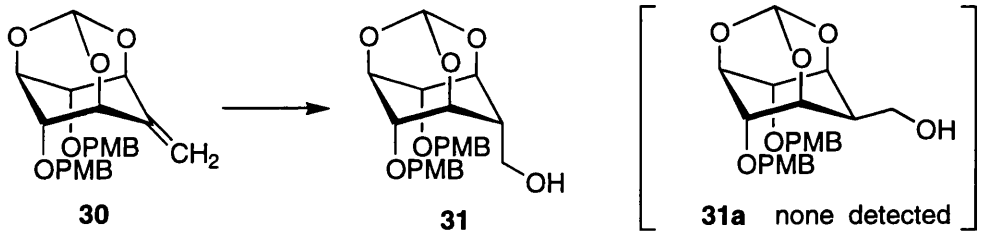


30 **39**

Figure 4.8 Selective removal of the orthoformate ester gives alkene **39.**

4.5 Diastereoselective Hydroboration/Oxidation

The alkene **30** was converted into alcohol **31** by hydroboration/oxidation using 9-BBN-H followed by alkaline hydrogen peroxide solution. It was hoped that the high steric demands of 9-BBN-H would give good regio- and stereoselectivity in this case. The results were even better than anticipated, and a single product was obtained in 97% yield after chromatography. Although the expected structure of this product was **31**, rather than the epimeric **31a**, this could not be deduced from the ^{1}H NMR spectrum simply by inspection of vicinal coupling constants. Both structures would show only axial-equatorial or equatorial-equatorial couplings which are of similar low magnitude, together with long-range-couplings, leading to rather complex or broadened signals from the ring protons. It would probably be possible to use NOESY to establish the correct structure of the product, but there was a more immediate way.



30 **31** **31a** none detected

Figure 4.9 Hydroboration-oxidation of alkene **30** with 9-BBN-H gives a single product.

It has been noted that the ^{1}H NMR spectrum of *myo*-inositol orthoformate and many of its derivatives shows a long-range 5-bond coupling (typically around 1 Hz) between the methylidine proton and the axial proton at C-2.[129] Thus, in the ^{1}H NMR spectrum of **28**, the methylidine proton appears as a narrow doublet with ^5J = 0.9Hz, whereas in the spectrum of the *scyllo*-inositol orthoformate derivative **63** (Figure 4.10 and Chapter 7), no coupling can be seen. Now the corresponding signal in the spectrum of the hydroboration/oxidation product was a sharp singlet. The fact that there was no coupling to H-2 was evidence that this proton was no longer axial, and that the product therefore had the desired structure **31**. It is important to note that this method of

predicting stereochemistry at C-2 of inositol orthoformate derivatives requires a well-resolved ^{1}H NMR spectrum. In the spectra of more polar derivatives, which were obtained using d_6-DMSO as solvent, all signals were broadened and small couplings were often not resolved.

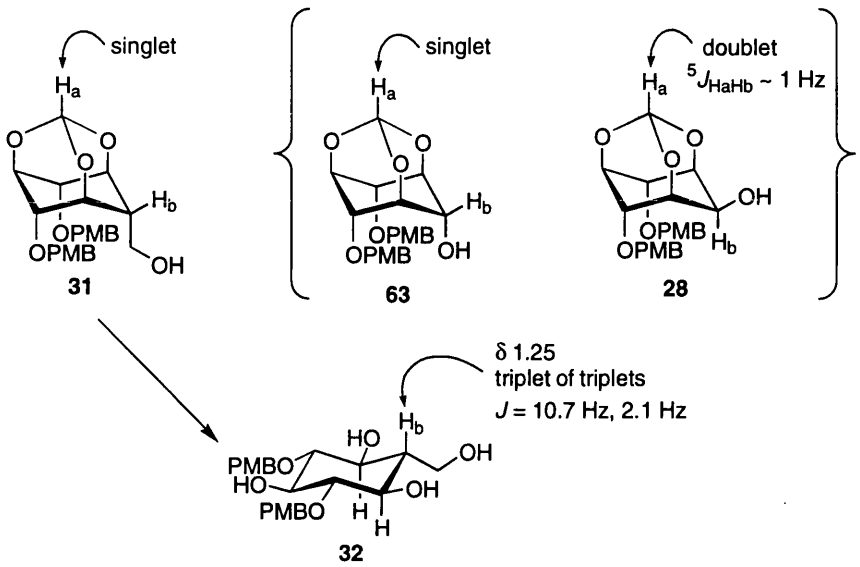


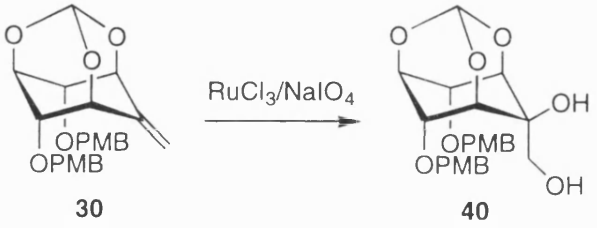
Figure 4.10 Determination of the structures of **28** and **32** by ^{1}H NMR.

Mild acid treatment cleaved the orthoformate ester without significant loss of *p*-methoxybenzyl groups to give the tetrol **32.** The structure of this molecule can be identified unequivocally by inspection of the ^{1}H NMR spectrum. The inositol ring, freed of the constraining orthoformate ester now flips into its alternative chair conformation with the two *p*-methoxybenzyl groups equatorial (Figure 4.10). Long-range couplings disappear and the signal from H_b moves to very high field (δ 1.25ppm), transforming itself into a clear triplet of triplets. The only couplings between the inositol ring protons are now large, and therefore axial-axial. No doubt remained as to the structure of **32** or its precursor **31.**

4.5.1 Ruthenium-Catalysed Dihydroxylation

The two most common reagents used for the *cis*-hydroxylation of alkenes are potassium permanganate and osmium tetraoxide, with the latter affording better yields, despite being both costly and highly toxic. Recently, a new "flash-dihydroxylation" method has been reported by Shing *et al.*, using ruthenium tetraoxide.[194] The procedure was reported to give rapid *cis*-hydroxylation of a variety of alkenes although its diastereoselectivity was, at the time, still under investigation. A sample of **30** was treated according to the published protocol, which is extremely straightforward, involving little more than

stirring a solution of the alkene in acetonitrile with $RuCl_3/NaIO_4$ in water. The reaction was successful at the first attempt, complete within 3 minutes, and was totally diastereoselective as judged by NMR of the crude product. Chromatography gave a crystalline diol in 77% yield. It seems highly likely that the structure is **40**, resulting from attack at the less-hindered face of the C=C bond (personal communication, Dr. T. K. M. Shing), but final confirmation of this will probably require NOESY. Time has not allowed further investigation of **40**, but it has obvious possibilities as a precursor for novel cyclitols and derivatives. The reaction illustrates the potential of alkene **30** for diastereofacioselective modification, as well as the value of the ruthenium method as a rapid and safe alternative to osmium tetraoxide.



30 **40**

Figure 4.11 Ruthenium-catalysed dihydroxylation of alkene **30**.

The reactions described in Sections 4.5 and 4.5.1 both exploit the fact that one diastereotopic face* of alkene **30** is exposed while the other is very hindered. A space-filling model of alkene **30** (Fig. 4.12) demonstrates the extreme steric hindrance of one face of the C=C bond by the two *p*-methoxybenzyl groups, while the other face is exposed.

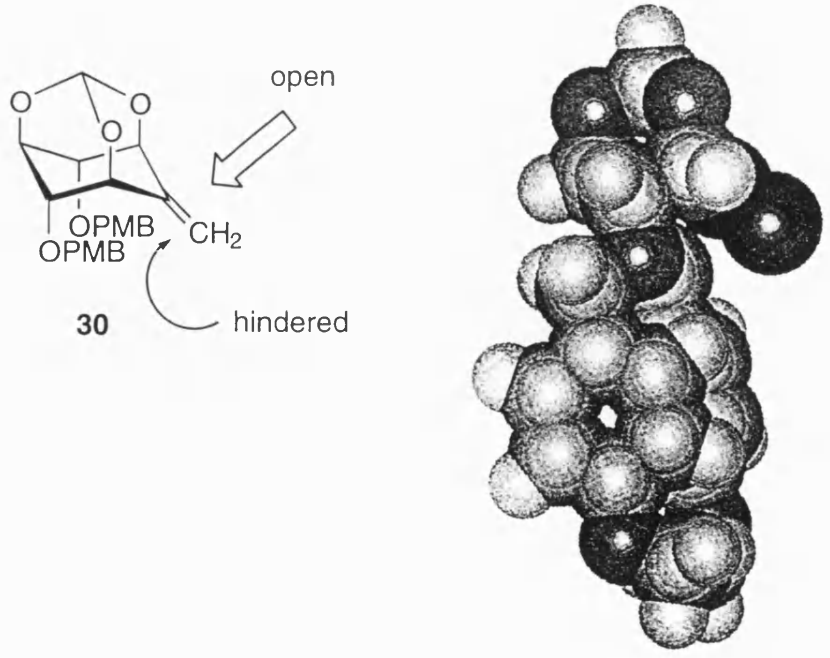


Figure 4.12 Space-filling model of alkene **30** showing hindrance of the C=C bond.

* Note that the faces of the C=C bond in alkene **30** are diastereotopic even though the products, **31** and **40** are not chiral. C-2 in alkene **30** (and also in ketone **29**) is therefore prostereogenic but not prochiral.

4.5.2 Investigation of an Alternative Route

Another route to the epimers of the alcohol **31** was also explored in parallel with the methylenation/hydroboration strategy described above. A Wittig reaction was used to convert ketone **29** into the asymmetrical enol ether **41** in 93% yield. It was thought that mild acid treatment could be used to hydrolyse **41** to an aldehyde (or mixture of aldehydes) which could then be epimerised using mildly basic conditions and reduced to give **31a**, the precursor for the *cis*-fused conformationally restrained analogue.

However, a series of experiments using a range of mildly acidic conditions all failed to give the required aldehyde, with TLC showing complete conversion to an unexpectedly polar compound. Under some conditions, an intermediate could be seen as a spot on the TLC plate, but rapid conversion to the polar product competed with its formation from **41**, and it could not be isolated. An IR spectrum of the product showed the expected strong C=O stretching band of an aldehyde, but the frequency was low (1685cm^{-1}), suggesting α,β-unsaturation. Alcohol groups were also present. The ^{1}H NMR spectrum clearly showed the aldehyde resonance as a singlet at δ 9.43 and the corresponding carbonyl signal in the ^{13}C NMR spectrum was shielded (δ_C 194.63) again indicating conjugation. ^{1}H and ^{13}C NMR both suggested the structure **42**, and this was confirmed by ^{1}H-^{1}H COSY. The proton at C-3 gave a highly distinctive doublet of triplets in the ^{1}H spectrum, caused by a long-range ^5J coupling to H-6, and H-2 was strongly deshielded (δ 6.54). The other vicinal couplings confirmed that the ring was now distorted well away from the chair conformation previously seen in saturated inositol derivatives.

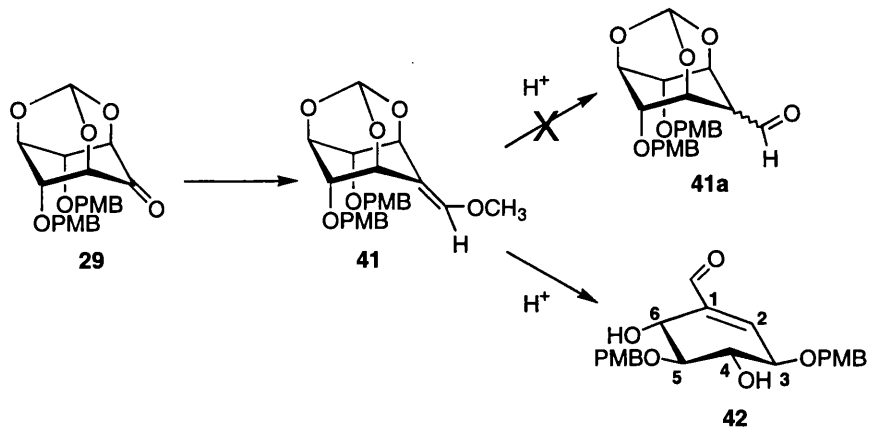


Figure 4.13 Acid hydrolysis of enol ether **41** always results in elimination to give the conjugated aldehyde **42**.

Conversion of the enol ether **41** to the saturated aldehyde proved to be impossible. Elimination to give the conjugated aldehyde **42** always occurred, even under very mildly acidic conditions. When the reaction was scaled up and carried out using 1 M HCl in THF (1:10), it was possible to isolate a small amount of the intermediate previously observed in the TLCs. The intermediate was shown to be the 4-formate ester **42a**, and showed a ^{1}H NMR spectrum very similar to that of **42**, except that the signal corresponding to H-4 was now shifted well downfield (by 1.29 ppm) establishing that this was the position of esterification. The distinctive singlet corresponding to the formate proton was at δ 8.11.

The failure to detect the saturated aldehyde **41a** as an intermediate suggests that the elimination proceeds by a one-step mechanism (Figure 4.14) rather than a two-step elimination *via* a saturated aldehyde. The orthoformate structure holds the C-O bond in an axial orientation, almost at right angles to the developing double bond, and therefore ideally placed for elimination to occur (Figure 4.14).*

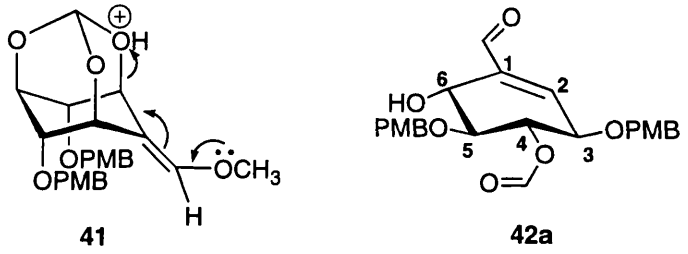


Figure 4.14 Proposed mechanism for formation of conjugated aldehyde **42**. Incomplete hydrolysis gave the formate ester **42a**.

α,β-Unsaturated aldehydes can themselves be valuable synthetic precursors, and **42** may therefore be a useful intermediate. It was found that crystalline **42** could be obtained in 82% yield by heating the enol ether **41** in 1M HCl / THF for 1 hour, and a large-scale synthesis would be straightforward. Treatment with sodium borohydride in methanol reduced the aldehyde group within 5 minutes, giving the racemic allylic alcohol **43** in 91% yield after crystallisation. A successful optical resolution of this material would provide two useful chiral intermediates, and the absolute configurations could easily be determined by hydrogenation to give the corresponding carbasugars, which are known.[195]

* Note that, if this one-step elimination mechanism is valid, then there is no need to assume that the required saturated aldehyde **41a** is itself prone to elimination. In fact elimination might be disfavoured, because it would have to proceed *via* a strained enol. It might still be possible, therefore, to make aldehyde **41a** by oxidation of the primary alcohol, followed by epimerisation and reduction to give the required alcohol. This route still awaits further investigation.

Deprotection of one enantiomer of **43**, for example, would give an epimer of the plant growth regulator streptol.[196] In the inositol phosphate field, **43** could prove to be a valuable precursor for a range of deoxygenated and unsaturated inositol phosphate analogues. Limitations of time did not allow further exploration of these possibilities, some of which are now being investigated by a colleague. Having developed a highly efficient route to the required symmetrical tetrol **31**, the priority was to continue with the planned route to the conformationally restricted analogue.

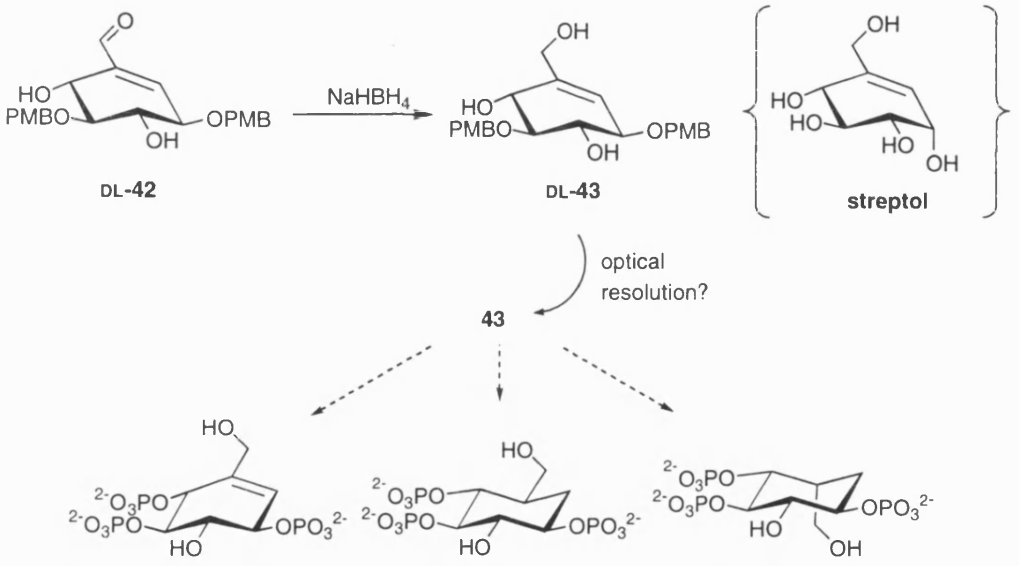
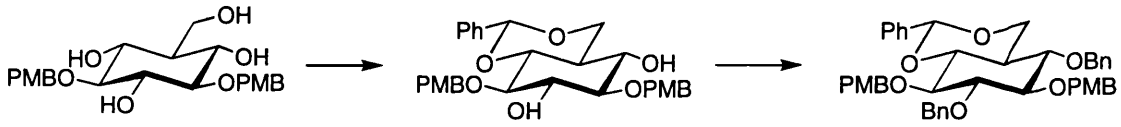


Figure 4.15 Reduction of the enal **42** with sodium borohydride gave allylic alcohol **43**, one enantiomer of which is related to the plant growth inhibitor streptol. An optical resolution of **43** would provide a precursor for novel analogues of Ins(1,4,5)P_3 .

4.6 Conversion to a Fully-Protected, Versatile Intermediate

Two protection steps were now used to produce the fully-protected **34**. The racemic benzylidene acetal **33** was initially prepared in 70% yield by the reaction of tetrol **32** with benzaldehyde dimethyl acetal (α,α-dimethoxytoluene) in dry DMF with a catalytic amount of PTSA at 70°C. However, in subsequent preparations in was found that the yield could be increased by applying a method described by Horton and Weckerle for the preparation of 2,3:4,6-di-*O*-benzylidene-α-D-mannopyranoside.[197] The key modification is simply to carry out the reaction with continuous removal of the liberated methanol. This can easily be achieved by fitting the reaction vessel with an air condenser attached to a water pump, so that the reactants and DMF are retained while methanol escapes, driving the equilibrium towards formation of the benzylidene acetal. This method was found to be faster, required a smaller excess of benzaldehyde dimethyl acetal, and consistently gave yields in excess of 90%.



32 **33** **34**

Figure 4.16 Protection of two hydroxyl groups in **32** as the benzylidene acetal gave racemic **33** which was converted to the fully-protected **34**.

Benzylation under standard conditions then gave fully-protected **34**. This is a versatile intermediate because either the benzylidene or *p*-methoxybenzyl groups can be removed chemoselectively, or the benzylidene ring can be reduced regioselectively in either direction (see Chapter 5). Intermediate **34** is currently being used by a colleague in the synthesis of a potential 3-kinase inhibitor.

4.7 Construction of the Cyclic Phosphate

The next step would be the introduction of the cyclic phosphate triester structure, strictly a 1,3,2-dioxaphosphorinane. There were reports in the literature of the construction of related structures based on carbohydrates[198] and *trans*-2-hydroxymethyl-1-cyclohexanol [199,200] in the course of stereochemical investigations, and P(V) methodologies had been employed. However, a more recent report[201] described the synthesis of some 5-membered cyclic phosphate triesters of *myo*-inositol using the bifunctional P(III) reagent benzyloxy-bis(*N*,*N*-diethylamino)phosphine and 1*H*-tetrazole, followed by oxidation with *m*-CPBA. The strained 5-membered rings are generally unstable in the presence of nucleophiles, and this property was exploited by treating them *in situ* with various alcohols, resulting in regioselective ring-opening. It the present case, it was reasoned that this P(III) approach should easily be adaptable to the construction of the more stable six-membered cyclic phosphate. The procedures were similar to that already used in previous work (Chapters 2 and 3), and employed the benzyl protecting group, which was ideal for the planned synthetic strategy. In the event, we decided to carry out the cyclic phosphitylation using a slightly different bifunctional phosphitylating agent, benzyloxy-bis(*N*,*N*-diisopropylamino)phosphine,[202] which was readily accessible by the reaction of bis(diisopropylamino)chlorophosphine with one equivalent of benzyl alcohol. This phosphitylating agent has been employed in the synthesis of an analogue of PtdIns(4,5)P_2,[202] and can be regarded as the bifunctional equivalent of the monofunctional bis(benzyloxy)-*N*,*N*-diisopropylamino-phosphine[203] described in Chapter 2.

The first step was to establish conditions for the selective removal of the benzylidene acetal from **34** with minimal loss of *p*-methoxybenzyl groups. It was found that 1M HCl/methanol at 50°C was effective, the reaction being complete within 30 minutes, giving diol **35**. Benzyloxy-bis(*N*,*N*-diisopropylamino)phosphine was synthesised according to Dreef *et al.*[202] and reaction of **35** with 1.2 equivalents of this reagent and 3 equivalents of 1*H*-tetrazole gave two cyclic phosphite triesters, epimeric at phosphorus as expected. These intermediates were visible as two spots close together on the TLC plate, and also as two distinct signals at δ_P 125.0 and δ_P 130.4 in the ^{31}P NMR spectrum. These signals disappeared and were replaced by peaks at δ_P –4.6 and δ_P –7.5 after addition of *m*-CPBA. It was possible to separate the two epimeric cyclic phosphate triesters by column chromatography, and isolate them as crystalline solids.

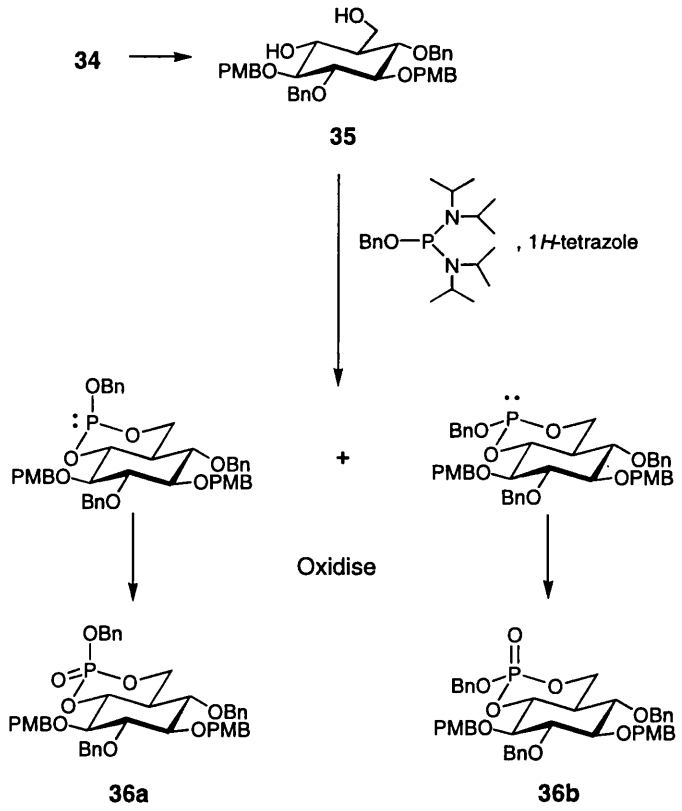
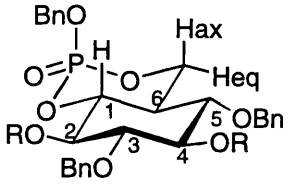


Figure 4.17 Formation of epimeric cyclic phosphite triesters by reaction of diol **35** with a bifunctional phosphitylating agent. Oxidation gives epimeric triesters **36a** and **36b**.

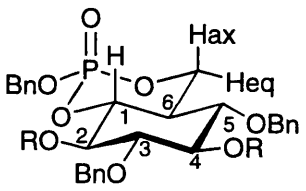
The configuration at phosphorus of the two epimers was determined by examination of their ^{31}P and ^{1}H NMR chemical shifts, $^{3}J_{HCOP}$ coupling constants and P=O stretching frequencies. Epimers **36a** and **36b** can be regarded as esters of 2-oxo-1,3,2-dioxaphosphorinanes, and studies have been published on simple related compounds.[198-199, 204] In all previous studies on isomeric pairs of phosphorinanes (see Reference[199] and references therein) the axially substituted epimer has an upfield ^{31}P

chemical shift. This identifies the less polar epimer (δ_P –7.5) as **36a** and the more polar isomer as the equatorially substituted **36b**. The P=O stretching frequency in the IR spectrum of **36b** is 21cm^{-1} lower than for **36a** ($\nu_{P=O}$ 1287cm^{-1}) confirming this assignment.

The *p*-methoxbenzyl ethers of **36a** and **36b** were successfully cleaved with DDQ giving the corresponding diols **37a** and **37b**. The ^{1}H NMR spectra of all four compounds were assigned completely with the aid of ^{1}H-^{1}H COSY. A detailed study of these spectra revealed further evidence for the assigned structures. In the equatorially protected epimers **36b** and **37b**, for example, H-1 and CH_2-ax are deshielded, as expected for protons in a 2,4-diaxial relationship with a P=O group. These protons therefore resonate downfield of the corresponding protons in **36a** and **37a**, while the CH_2-eq protons have similar chemical shifts in all four molecules (Table 4.1).



R = PMB **36a**
R = OH **37a**



R = PMB **36b**
R = OH **37b**

Table 4.1 Chemical shifts and $^3J_{HCOP}$ coupling constants for cyclic phosphate triesters **36a, 36b, 37a, 37b.**

Compound	Chemical Shifts/p.p.m.				Coupling Constants/Hz	
	δ_P	δ_{Hax}	δ_{Heq}	δ_{H1}	$^3J_{P\text{-}Hax}$	$^3J_{P\text{-}Heq}$
36a	–7.49	3.76	4.41	4.04	~0	24.2
37a	–7.27	3.81	4.43	3.86	~0	24.4
36b	–4.56	4.03	4.39	4.32	3.9	20.4
37b	–4.43	4.08	4.42	4.17	4.4	20.0

$^3J_{HCOP}$ coupling constants have been used to investigate the solution conformations of phosphorinanes,[199] and a Karplus-type relationship has been established for the HCOP dihedral angle and the magnitude of the coupling constant.[205] Thus the coupling constants for **36a** and **37a** are consistent with a normal chair conformation, because the large $^3J_{P\text{-}Heq}$ and small $^3J_{P\text{-}Hax}$ values indicate an *anti-* dihedral angle for $H_{eq}COP$ and a *gauche* dihedral angle for $H_{ax}COP$. In the equatorially protected esters **36b** and **37b**, the decrease in $^3J_{P\text{-}Heq}$ accompanied by increased $^3J_{P\text{-}Hax}$ is evidence for a slightly flattened

chair conformation, or a rapidly equilibrating mixture of undistorted chair and twist-boat conformations. Some relevant NMR data for the four compounds are summarised in Table 4.1. and the ^{1}H-coupled ^{31}P NMR spectra are shown in Figure 4.18.

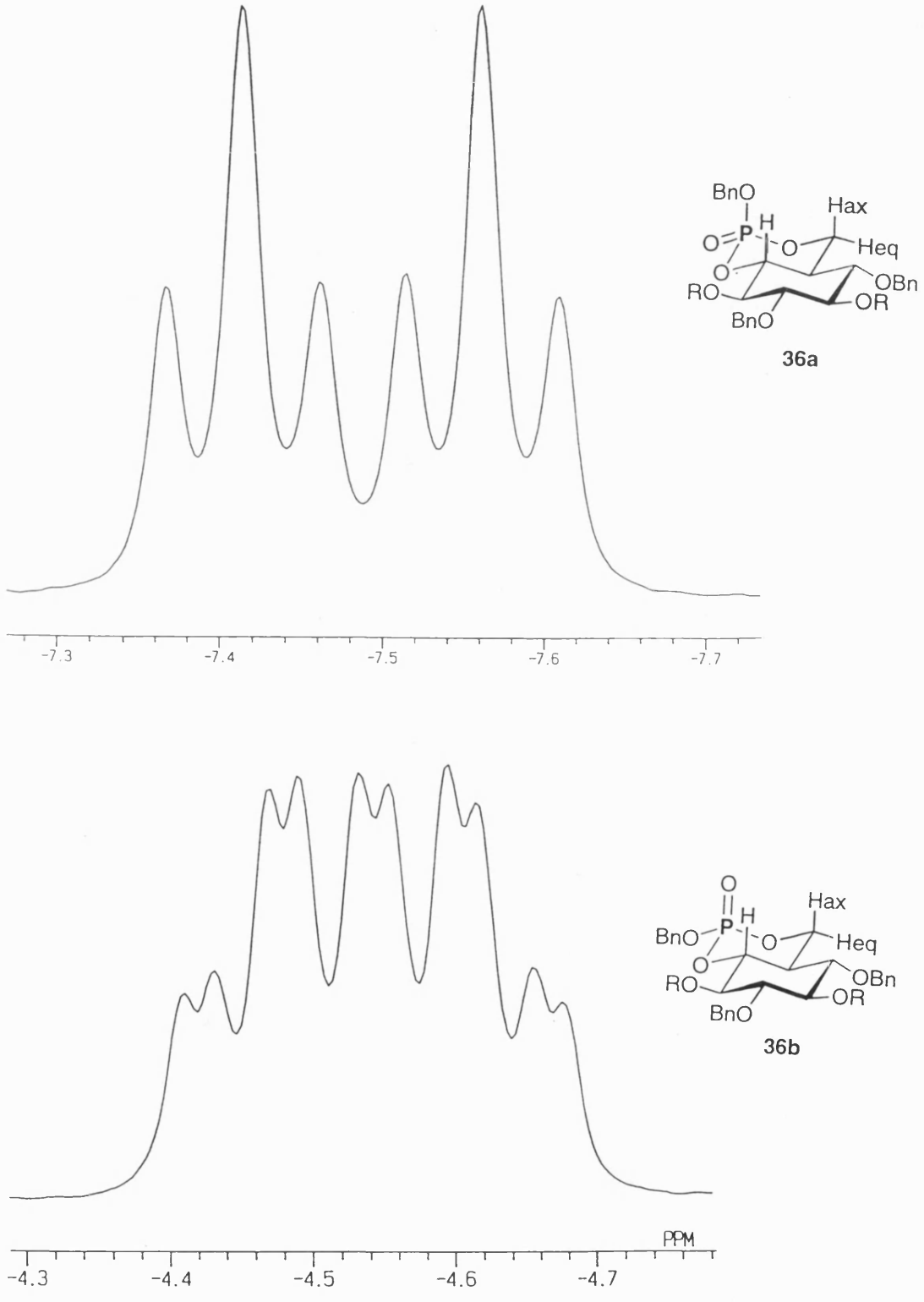


Figure 4.18 ^{31}P NMR spectra of **36a** and **36b** (162 MHz, ^{1}H-coupled, $CDCl_3$).

4.8 Phosphitylation/Oxidation: An Unexpected PIII-PV Spin-Spin Coupling

The diols **37a** and **37b** were then phosphitylated using the monofunctional phosphitylating agent bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine[203] with 1*H*-tetrazole. This step involves the formation of a phosphite triester at a position vicinal to an existing phosphate triester and in each case we were able to observe in the ^{31}P NMR spectrum a $^{5}J_{PP}$ spin-spin coupling of 1.2 Hz between the phosphorus atoms of neighbouring phosphate and phosphite groups. To the best of our knowledge, such a long-range P(III)-P(V) coupling has not been previously reported, although it is known in vicinal P(III)-P(III) systems. It is tempting to speculate that conformational restraint of the phosphate is responsible for this unusual effect, as no such coupling was observed in a mixed P(III)/P(V) species (Figure 4.19) leading the authors to conclude that two P(III) centres were necessary for the interaction.[206] Oxidation with *m*-CPBA and purification gave crystalline **38a** and **38b**, neither of which showed any phosphorus-phosphorus couplings.

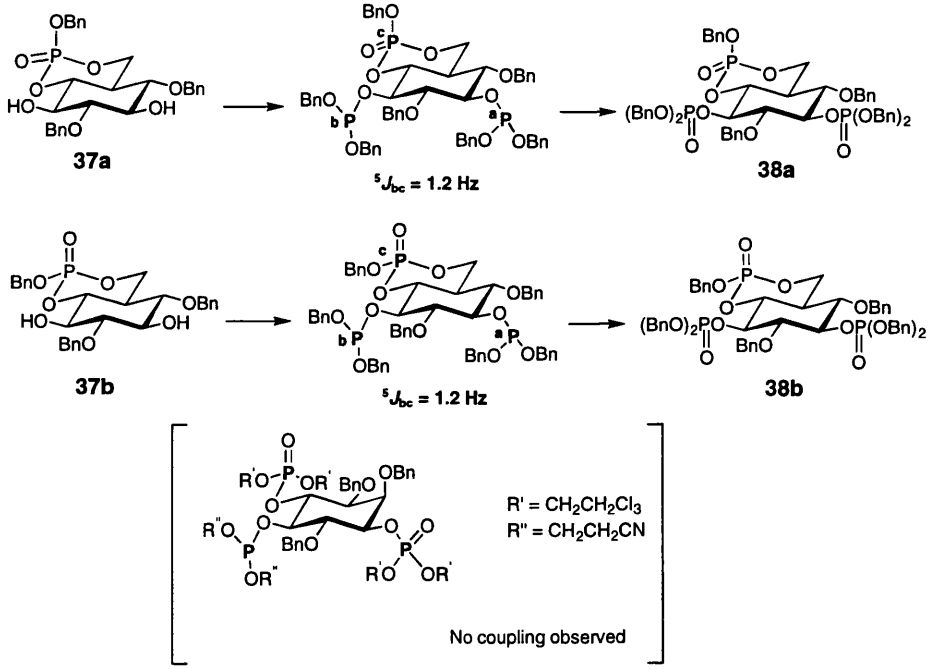


Figure 4.19 Phosphitylation of **37a** and **37b** gives mixed P(III)-P(V) intermediates, which both showed unusual P(III)-P(V) $^{5}J_{PP}$ spin couplings. A similar arrangement in a conformationally mobile system[206] showed no such coupling.

4.9 Deprotection

The planned deprotection using sodium in liquid ammonia was first tried out on the protected cyclic phosphate **36a**. An examination of a proton-coupled ^{31}P NMR spectrum of the crude product showed only one signal; a doublet with a large splitting (J > 20Hz). This splitting could only have resulted from the large heteronuclear coupling to the

equatorial proton in the 1,3,2-dioxaphosphorinane ring and therefore confirmed beyond doubt that the cyclic phosphate structure had remained intact. The fact that no other couplings were observed showed that the benzyl protecting group had been removed successfully. Thus is was established that the cyclic phosphate triester could be effectively deprotected using sodium in liquid ammonia.

The axially protected **38a** was deprotected using the same procedure and purification by ion-exchange chromatography at last gave the target compound **26** in 78% yield as the triethylammonium salt. The structure was confirmed by ^{1}H, ^{31}P and ^{13}C NMR. A ^{1}H-^{1}H COSY NMR allowed all the proton resonances to be assigned unequivocally. **26** Could also be crystallised as the cyclohexylammonium salt. A sample of the latter, left at room temperature for a year, showed no sign of decomposition. Some time later, when further supplies of **26** were required for a potentiometric study (see below), the equatorially protected **38b** was also deblocked with equal success.

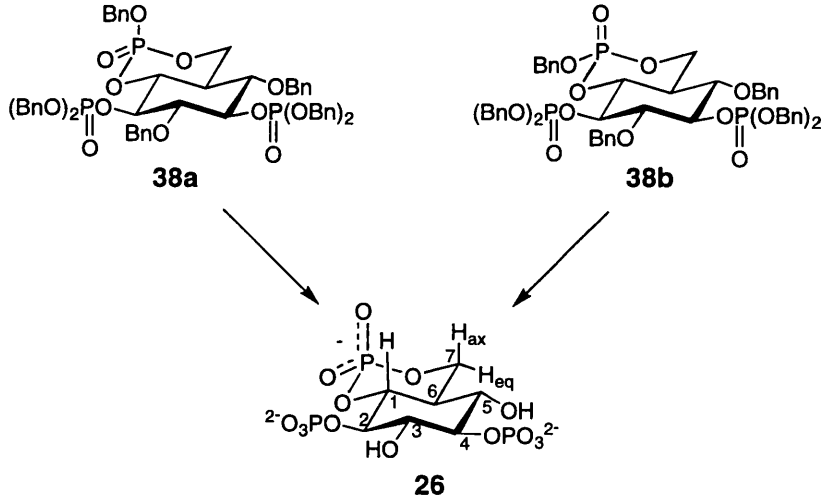


Figure 4.20 Deprotection of either epimer **38**a or **38b** gives the target compound **26**.

The proton-coupled ^{31}P NMR spectrum of **26** (Figure 4.21a) shows the expected heteronuclear spin couplings, with the phosphorus atoms in the unconstrained phosphate groups at positions 2 and 4 appearing as doublets with $^{3}J_{HP}$ approximately 7 Hz. However, notice that the signal from the cyclic phosphate phosphorus atom (δ_P −2.76) is also a doublet, with a large coupling constant ($^{3}J_{P\text{-}Heq}$ = 22.5 Hz). The predicted $^{3}J_{HP}$ coupling constant for a dihedral angle of 180° is approximately 23 Hz[205] and so we can conclude that the angle $H_{eq}COP$ is close to 180°. This suggests that the dioxaphosphorinane ring adopts a chair conformation as shown in Fig. 4.20 Further discussion of conformation, based on more detailed NMR analysis is given later in this section.

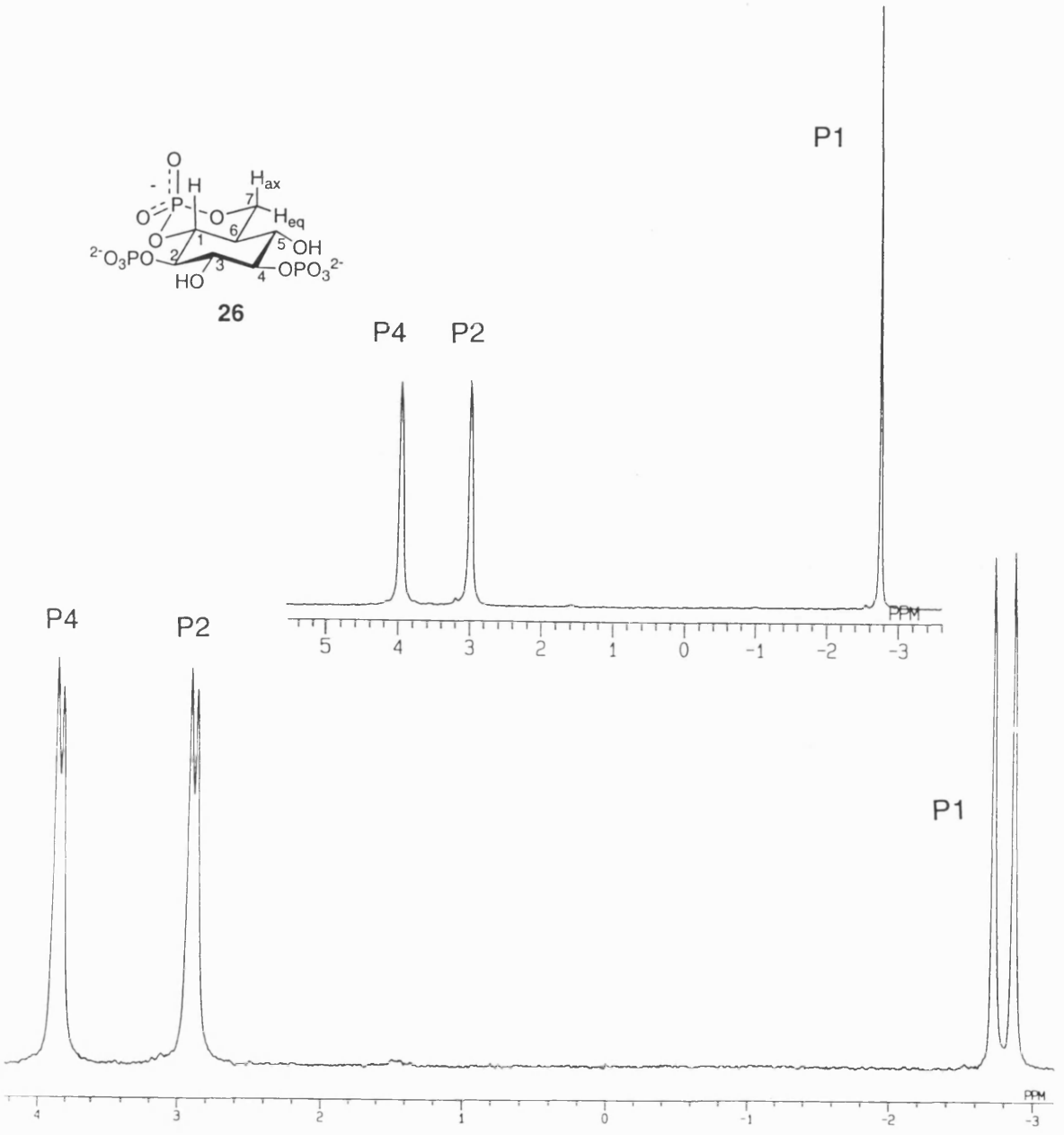


Figure 4.21 ^{1}H-coupled ^{31}P NMR spectra of the racemic conformationally restricted analogue **26** in D_2O (162, MHz, pH~4). The inset shows the ^{1}H-decoupled spectrum.

Figure 4.21 also demonstrates an unusual effect: In the proton-decoupled spectrum (inset) the cyclic phosphate resonance at δ_P –2.76 is much more intense (approximately three times more by integration) than either signal from the phosphorus atoms at positions 2 and 4. A categorical explanation for this observation awaits further NMR studies, but the finding that the effect disappears when the decoupler is switched off may suggest the involvement of heteronuclear NOEs.[207]

4.10 Biological Properties

Racemic **26** was examined for Ca^{2+}-mobilizing activity at the platelet Ins(1,4,5)P_3 receptor using saponin-permeabilised platelets loaded with $^{45}Ca^{2+}$. The results are shown in Figure 4.22. It appears that **26** behaves as a full agonist, although with an EC_{50} around 40-70 fold higher than Ins(1,4,5)P_3. Assuming that only one enantiomer is active, this would imply a thrty-fold reduction in potency relative to Ins(1,4,5)P_3.

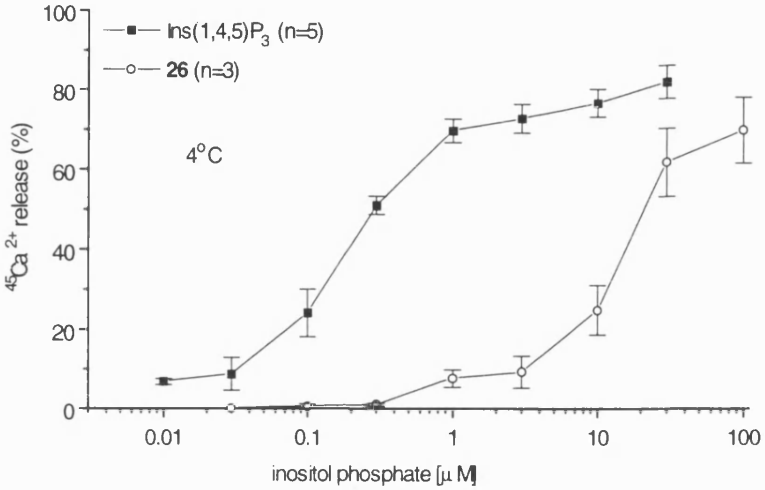


Figure 4.22 $^{45}Ca^{2+}$-release by the conformationally restricted analogue **26** in permeabilised rabbit platelets.

Binding studies in rat cerebellar membranes (Fig. 4.23) show that the affinity of the analogue for the Ins(1,4,5)P_3 receptor is much reduced (200 to 300-fold). The interpretation and possible significance of these results will be discussed later in this Chapter.

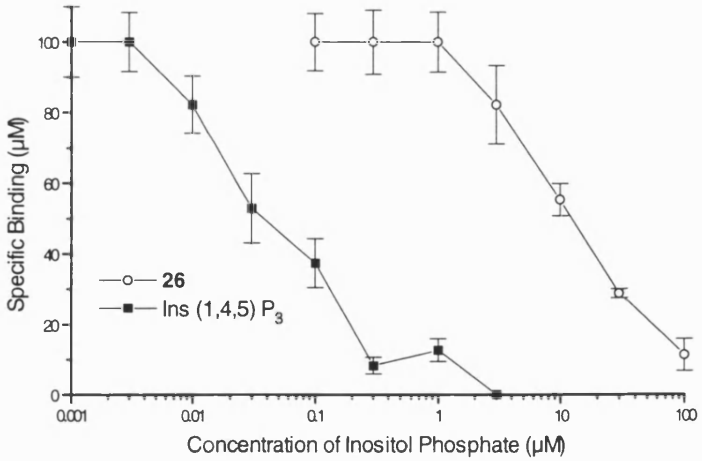


Figure 4.23 Inhibition of [^{3}H]Ins(1,4,5)P_3 binding to rat cerebellar membranes by **26**.

4.11 Protonation Sequence

A larger sample of **26** was synthesised (this time by deprotection of the equatorially protected epimer **38b**) and used for detailed NMR and potentiometric titrations, which were carried out by Professor B. Speiss and his group at the Université Louis Pasteur, Strasbourg.

4.11.1 Potentiometric Titration

A sample of **26** (triethylammonium salt) was first converted into the free acid and then titrated with potassium hydroxide solution. The titrations were carried out at 37°C in the presence of 0.2 M KCl, in order to mimic the intracellular medium. The potentiometric titration allows the calculation of macroscopic protonation constants, and for **26** the values obtained were $\log K_1 = 6.70$ and $\log K_2 = 5.60$. These values are much lower than those found for Ins(1,4,5)P_3 under similar conditions ($\log K_1 = 7.85$, $\log K_2 = 6.40$).[208] The K_y values allow the calculation of the percentage of each ionic species as a function of pH, although it is not possible at this stage to say which phosphate groups are protonated. In Figure 4.24 then, L represents the fully ionised species, LH the total monoprotonated species etc., but the actual locations of the protons in LH, LH_2 and LH_3 are unknown.

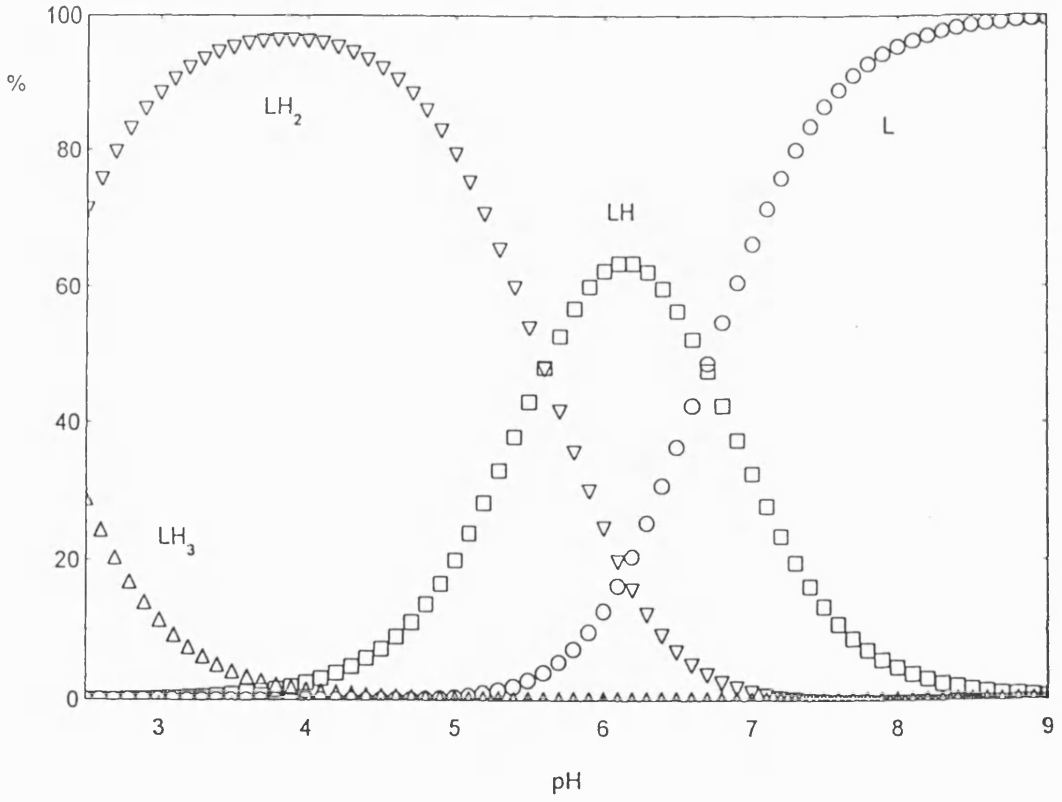


Figure 4.24 Distribution curves of various species of **26** plotted against pH. For clarity the charges are omitted.

When Figure 4.24 is compared to a similar diagram for Ins(1,4,5)P_3, it is evident that under these conditions, at physiological pH, **26** is less protonated than Ins(1,4,5)P_3. Thus at pH 7.4, over 80% of **26** is in the fully ionised form L, while for Ins(1,4,5)P_3 the predominant species is the monoprotonated form LH.[208]

4.11.2 ^{31}P NMR Titration

A ^{31}P NMR titration was now carried out under similar conditions, and accurate measurements of ^{31}P chemical shifts and coupling constants were obtained over a pH range from 1.65 to 11.67. The phosphorus resonances of P2 and P4 were later assigned by performing a phosphorus-proton 2D COSY experiment. Figure 4.25 shows the ^{31}P chemical shifts of P1, P2 and P4 plotted against pH. We see that the chemical shift of the cyclic phosphate phosphorus atom P1 does not change at all over the pH range, implying that the basicity of the cyclic phosphate is very weak, so that it is protonated only at very low pH.. The chemical shifts of the phosphate atoms P2 and P4 in the two phosphate monoesters become progressively shielded with decreasing pH. The curves are monophasic, showing that P2 and P4 do not significantly interact (a similar lack of interaction is seen between the 1 and 4 phosphates of Ins(1,4)P_2 [189]). In each case, the change in chemical shift is related to the gain of one proton by each phosphate group as pH decreases (the second protonation occurs at pHs below 3).

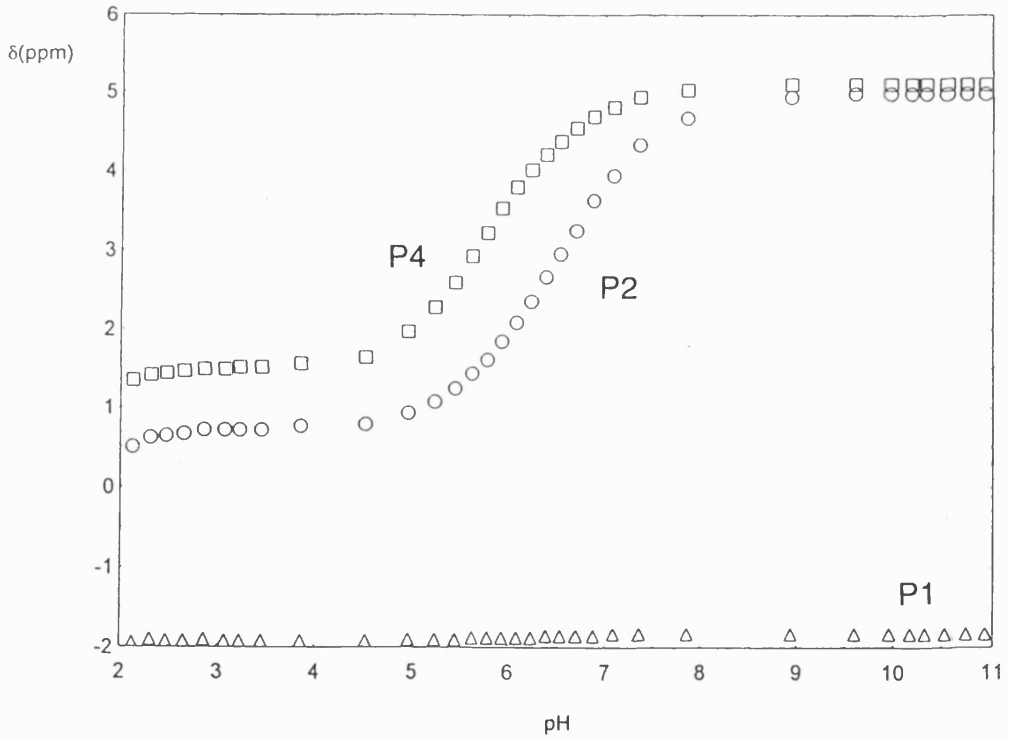


Figure 4.25 Chemical shifts δ from ^{31}P NMR titration of **26**, plotted as a function of pH.

Now the chemical shift observed for any signal δ_i^{obs} is just the weighted average of the shifts for the protonated and deprotonated forms:

$$\delta_i^{obs} = (f_{i,p} \times \delta_{i,p}) + (f_{i,d} \times \delta_{i,d}) \qquad (1)$$

where $f_{i,p}$ and $f_{i,d}$ respectively correspond to the protonated and deprotonated fractions at position i, and $\delta_{i,p}$ and $\delta_{i,d}$ are the chemical shifts of the monoprotonated and fully deprotonated forms of the phosphate. But $f_{i,p} + f_{i,d} = 1$, and so equation (1) becomes:

$$f_{i,p} = (\delta_i^{obs} - \delta_{i,d}) / (\delta_{i,p} - \delta_{i,d}) \qquad (2)$$

Equation (2) now allows the calculation of the protonated fractions $f_{i,p}$ from the chemical shift information. The result is shown in Figure 4.26. There is no curve for P1 as this phosphate group is fully deprotonated ($f_{i,p} = 0$) over the whole pH range.

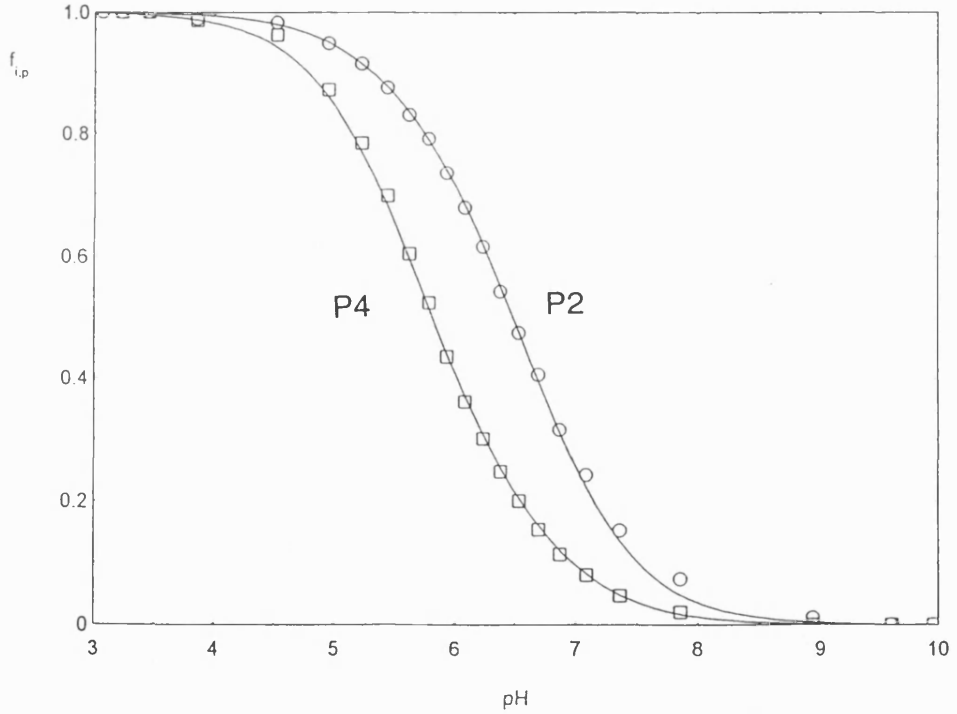


Figure 4.26 Protonation fraction $f_{i,p}$ plotted against pH for **26**.

Now equations (1) and (2) are based on an assumption that the measured chemical shifts are largely dependent on the protonation state of the phosphate groups. It is now necessary to demonstrate that this assumption is valid for **26**. The mean number of protons, $\bar{p}$, bound per mole of **26**, according to the NMR calculations, is just the sum of the protonation fractions.

$$\bar{p} = \sum f_{i,p} = f_{2,p} + f_{4,p} \qquad (3)$$

However, $\bar{p}$ can also be obtained directly from the potentiometric measurements by applying equation (4):

$$\bar{p} = \frac{C_H - [H^+] + [OH^-]}{C_L} \qquad (4)$$

where C_H and C_L are the analytical concentrations of acid and ligand respectively.

Figure 4.27 shows values of $\bar{p}$ calculated according to both equations, plotted against pH. It is clear that, for **26**, the two values are in excellent agreement.

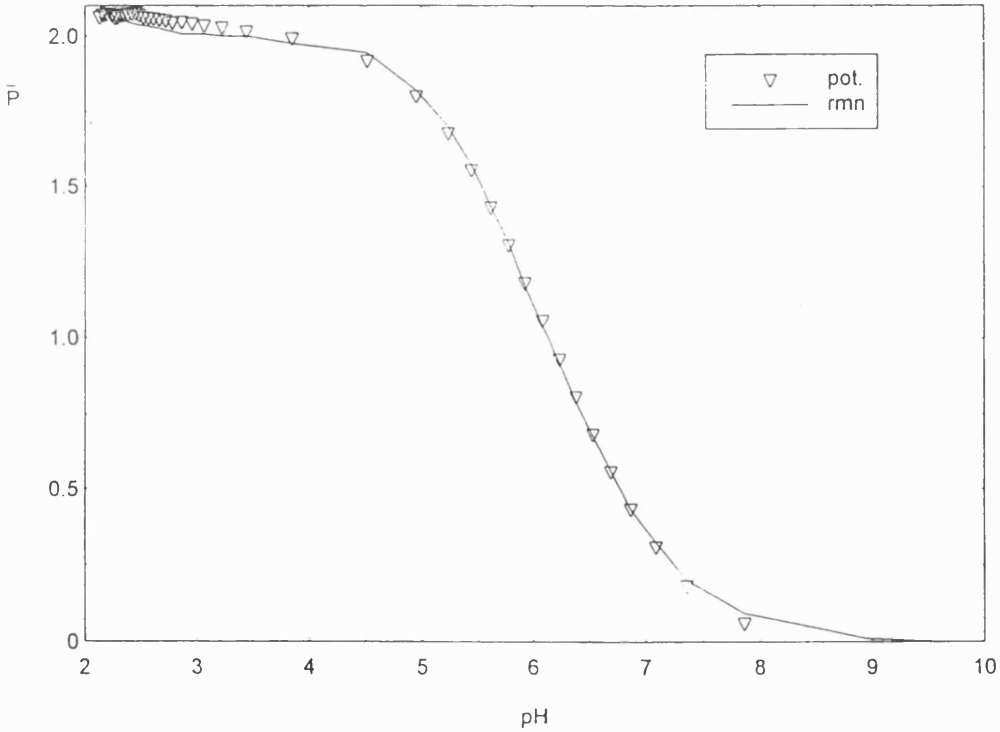


Figure 4.27 Plots of $\bar{p}$ (the mean number of protons bound per mole of **26**) versus pH. Values of $\bar{p}$ calculated from the potentiometric titration are in excellent agreement with those derived from the ^{31}P NMR titration.

Now the macroscopic protonation constants, calculated from the potentiometric titration can be considered to be composites of microscopic constants reflecting the behaviour of the individual phosphate groups (in this case the phosphates at positions 2 and 4). The macro- and micro-constants for **26**, are related by equations (5)-(7) and the protonation scheme is shown in Figure 4.26.

$$K_1 = k_2 + k_4 \qquad (5)$$

$$1/K_2 = 1/k_{24} + 1/k_{42} \qquad (6)$$

$$K_1K_2 = k_2 \text{ x } k_{24} = k_4 \text{ x } k_{42} \qquad (7)$$

Given the macroscopic constants (from the potentiometric titration) and the values $f_{2,p}$ and $f_{4,p}$ (from the NMR experiments) it is now possible to calculate the microscopic constants k_2, k_4, k_{24} and k_{42} (equations not shown). This is relatively straightforward for **26** because, as it behaves as a diprotic acid over this pH range, there are only three different protonated forms to be considered. The situation is more complicated in the case of an inositol trisphosphate with three phosphate triesters (such as Ins(1,4,5)P_3), which will have seven protonated forms under similar conditions. The macroscopic and microscopic protonation constants for **26** are given in Table 4.2.

y	$\log K_y \pm \sigma$	i	$\log k_i \pm \sigma$	ii'	$\log k_{ii'} \pm \sigma$
1	6.70 ± 0.01	2	6.57 ± 0.01	24	5.73 ± 0.02
2	5.60 ± 0.01	4	6.10 ± 0.01	42	6.20 ± 0.02

Table 4.2 Macroscopic and microscopic protonation constants for **26**

From these results, k_2/k_4 is calculated to have a value of $10^{(6.57-6.10)} = 2.95$. This is an indication of the lower basicity (higher acidity) of the P4 phosphate, which may be due to a greater probability of hydrogen bonding with the two neighbouring equatorial hydroxyl groups. The P4 phosphate is also less basic than the P1 phosphate of either Ins(1)P_1 or Ins(1,4)P_2, which are flanked by one equatorial and one axial hydroxyl group, and is more similar to the P4 phosphate of Ins(1,4)P_2. Thus, the fact that **26** has the *scyllo*- rather than the *myo*- configuration may have resulted in P4 being less basic than the equivalent P1 of Ins(1,4,5)P_3. The fact that this difference does not greatly influence Ins(1,4,5)P_3 receptor binding is reflected in the high potency of *scyllo*-Ins(1,2,4)P_3 and 6-deoxy-6-hydroxymethyl *scyllo*-Ins(1,2,4)P_3 (Chapter 5), and also in the observation that Ins(1,4,5)P_3 receptor binding, when measured over a range of pH values,[175] does not correlate with the ^{31}P NMR chemical shift of P1 in Ins(1,4,5)P_3.[209]

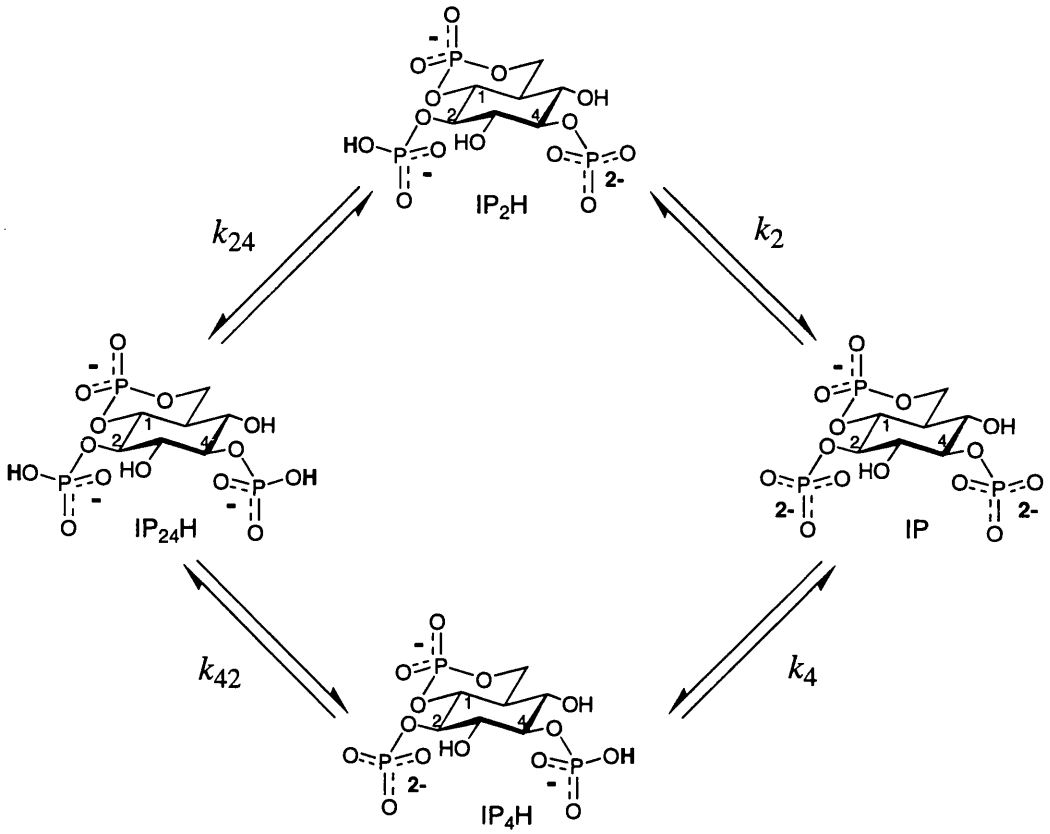


Figure 4.28 Protonation scheme for **26**. For interpretation of symbols, see text.

The cyclic phosphate of **26**, as we have seen, has very low basicity and is therefore ionised over the whole pH range, while the 2-phosphate of **26**, which is intended to mimic the 5-phosphate of Ins(1,4,5)P_3, has logk_2 = 6.57. While, as explained above, microscopic protonation constants are not available for Ins(1,4,5)P_3, which behaves as a more complex system, the equivalent logk_5 for Ins(4,5)P_2 is 8.15.[189] The 2-phosphate group of **26** is therefore $10^{(8.15-6.57)}$ = 38 times less basic (more acidic) than its equivalent in a vicinal phosphate pair. Thus, although conformational restraint of one phosphate in the vicinal pair necessarily reduces its charge, the ionisation of the other phosphate is enhanced. The first effect would be expected to reduce binding affinity, and the second to enhance it. It would seem that the decision to constrain the equivalent of the 4-phosphate (rather than the 5-phosphate) was well made, as constraining this less important phosphate group increases the ionisation of its vicinal partner at physiological pH.

The microscopic protonation constants can now be used to calculate the proportion α of each species present at a given pH (Figure 4.29). For example, at pH 10, **26** is fully deprotonated, and when the pH is reduced, the proportion of the fully-ionised species gradually decreases as protons are added to the P2 and P4 phosphates. At

physiological pH, around 80% of **26** is still in the fully-ionised form, while the rest is monoprotonated. The predominant monoprotonated form has the proton on P2, and the proportion of this species rises to a maximum at around pH 6, when it forms 50% of the total. Below this pH, the proportion of the 2,4-diprotonated form increases, approaching 100% at pH 3. The basicity of the cyclic phosphate P1, and the second basicities of the P2 and P4 are so weak that they are only protonated at lower pH.

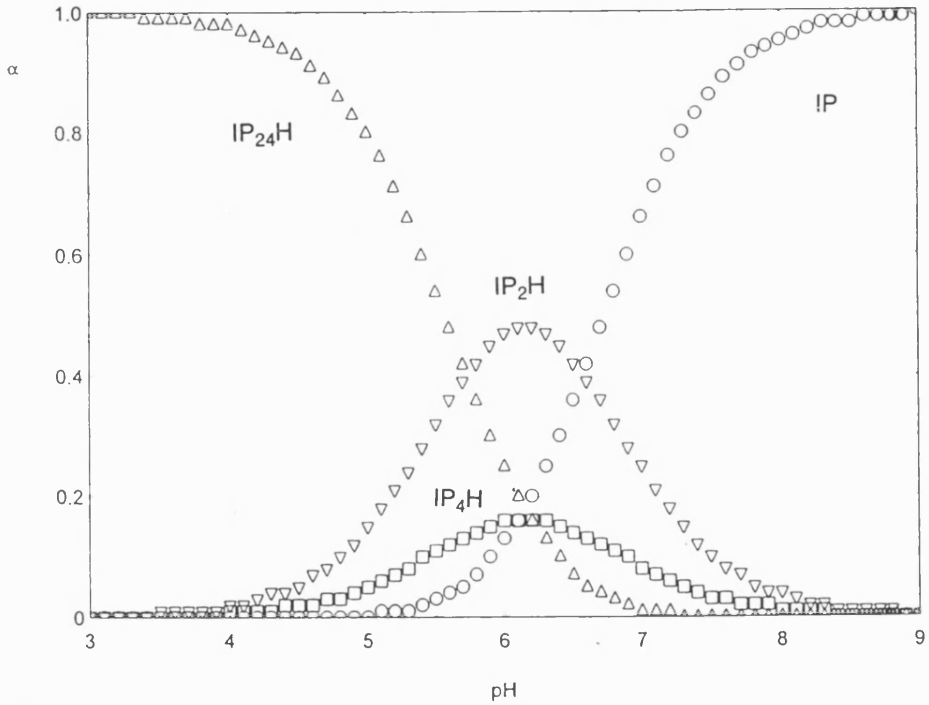


Figure 4.29 Distribution curves of the various species of **26** plotted against pH.

4.11.3 ^{1}H NMR Titration

Finally, a proton NMR titration was carried out under similar conditions. Figure 4.30 shows the chemical shifts of protons at positions 3, 5 and 6 in **26**, plotted against pH. These protons are at unphosphorylated positions, and perhaps not surprisingly, they are little affected by pH. Notice however that H3, which is vicinal to two phosphate monoesters, is most strongly influenced, and is deshielded by 0.1ppm at high pH.

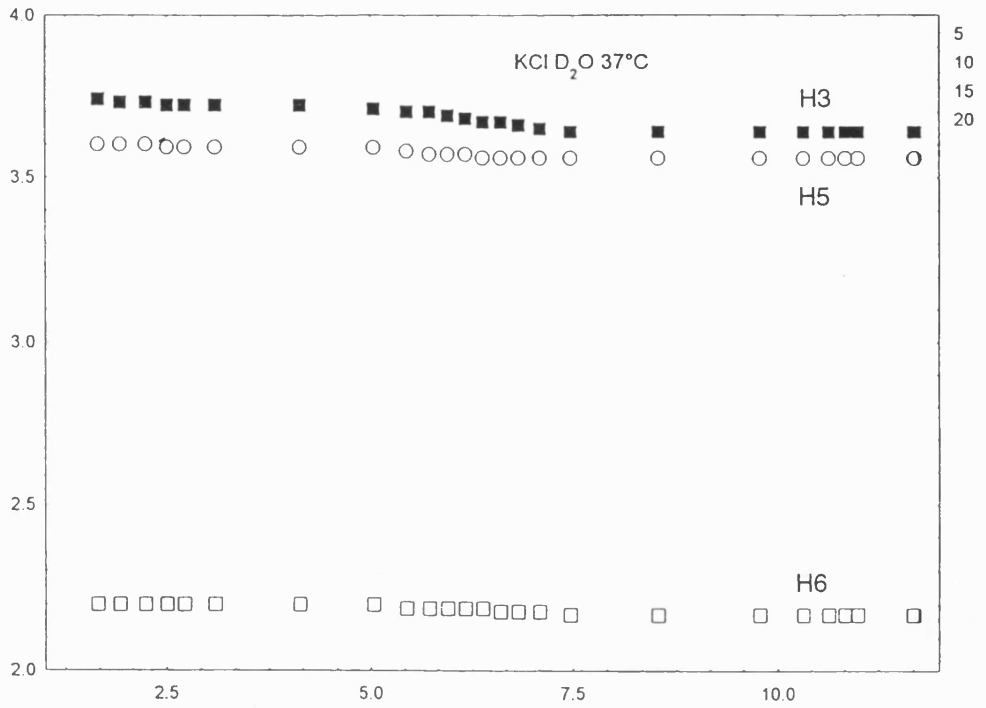


Figure 4.30 ^{1}H NMR chemical shifts δ of H3, H5 and H6 in **26** plotted against pH.

Figure 4.31 shows a similar plot for the remaining five protons (note the larger scale). The chemical shifts of H2 and H4, which are at positions bearing phosphate monoesters, are pH-dependent, and follow a biphasic relationship. If we follow the plot for H2, from right to left, for example, we see that decreasing pH has no effect until around pH 7.6, after which it is progressively deshielded. This effect seems to be related to the first protonation of P2, and is also reflected in the ^{31}P NMR plot (Figure 4.25). After a plateau, the deshielding begins again at pH 2.6, presumably as the second proton is added to P2. H_{ax} and H_{eq} are only slightly influenced by pH, behaving in a similar way to H5 and H6, and this is in accordance with the finding from the ^{31}P NMR titration that the cyclic phosphate P1 is ionised over the whole pH range. H1 shows a moderate pH dependency, and it seems likely that its chemical shift is influenced by the ionisation state of the adjacent P2. Thus it appears that the effect of pH on the chemical shifts of the protons in **26** is mediated by the ionisation state of the phosphate monoesters. The very weakly basic cyclic phosphate diester seems to have negligible effect.

Measurements of all ^{3}J vicinal coupling constants were also obtained. The effect of pH on these values seemed slight, which is what we would expect for a conformationally restrained bicyclic molecule such as **26**. Some values did appear to steadily increase or decrease with pH, but the significance of these small changes, and whether they can be related to conformational changes is not obvious at present.

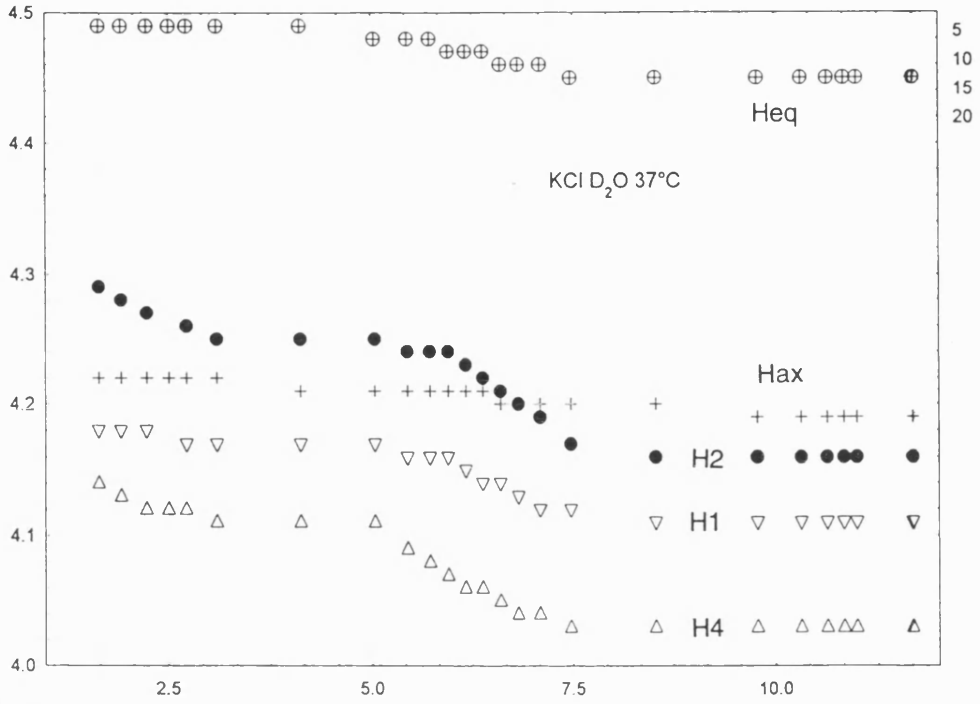


Figure 4.31 ^{1}H NMR chemical shifts δ of H1, H2, H4, H_{ax} and H_{eq} in **26** plotted against pH

More definite conclusions can be drawn from the average values of the heteronuclear $^{3}J_{HCOP}$ coupling constants, which were measured in both the ^{1}H NMR and proton-coupled ^{31}P NMR spectra. The mean values, together with standard deviations are given in Table 4.3.

	H_{ax}-P1	H_{eq}-P1	H1-P1	H2-P2	H4-P4
$^{3}J_{HCOP}$ / Hz	2.00	22.66	1.33	7.85	8.48
σ / Hz	0.00	0.13	0.08	1.06	1.12

Table 4.3 Mean and standard deviation (σ) values for phosphorus-proton coupling constants in **26**.

4.11.4 Conformation

According to Lankhorst *et. al.*,[205] $^{3}J_{HCOP}$ is dependent on the dihedral angle φ = H–C–O–P, and the relationship can be described by the Karplus-type equations given below:

$$^{3}J_{HCOP} = 18.1\cos^{2}\varphi - 4.8\cos\varphi \qquad (\text{for } 0 < \varphi < 90°) \qquad (8)$$

$$^{3}J_{HCOP} = 15.3\cos^{2}\varphi - 6.1\cos\varphi + 1.6 \quad (\text{for } 90 < \varphi < 180°) \qquad (9)$$

First consider the geometry of the cyclic phosphate in **26**. Solving equations (8) and (9) for the mean $^3J_{HCOP}$ values given in table yields $\varphi = 61°$ for H_{ax}–C–O–P1, $\varphi = 64°$ for H1–C–O–P1 and $\varphi = 172°$ for H_{eq}–C–O–P1. These values are good evidence that the cyclic phosphate adopts a chair-like conformation. The very small variations in the magnitudes of the $^3J_{HCOP}$ values for P1 suggest that the conformation remains much the same over the entire pH range.

The conformation of the cyclic phosphate can be deduced from the coupling constants with a fair degree of confidence, even though solution of equations (8) and (9) actually yields four possible dihedral angles for each coupling constant. For example, the possible solutions for $^3J_{HCOP} = 2$ Hz give values of +61°, +93°, -61° and -93° for the angle H_{ax}–C–O–P1. However, only one of these values (+61°) is both geometrically possible in the six-membered ring, and consistent with the other coupling constants. The situation with respect to the phosphate monoesters is far more complex. First notice that there seems to be greater variation in their $^3J_{HCOP}$ values over the pH range, suggesting that their conformation is influenced by pH. This is what we would expect, because, as the protonation state of these phosphate changes with pH, their hydrogen-bonding interactions with neighbouring hydroxyl groups will be affected, as will the electrostatic repulsion between phosphates. Now application of equations (8) and (9) yields the φ values of around +35°, -35°, +120°, and -120° for both H2–C2–O2–P2 and H4–C4–O4–P4. Similar results have been obtained for P4 and P5 of Ins(1,4,5)P_3.[210] If we take the coupling constants to arise from a single predominant rotamer then we can probably discard the last two solutions, which represent eclipsed conformations. We would expect the 2-phosphate group to be orientated away from the 1 phosphate (due to electrostatic repulsion) and towards the 3-hydroxyl (due to hydrogen bonding) and so we might tentatively suggest a value of close to +35° for the angle H2–C2–O2–P2 (and also for the 4-and 5-phosphate groups of Ins(1,4,5)P_3). There may be some additional support for this value in the $^3J_{CCOP}$ couplings measured from the ^{13}C NMR of **26**. However, many assumptions have now been made in arriving at this value, not least of which is the assumption that the coupling constant reflects the geometry of a particular predominant conformation, rather than a time-averaged value arising from a population of rapidly interconverting rotamers.

4.12 Conclusions

The conformationally restricted analogue can be viewed physicochemically as a simplified version of Ins(1,4,5)P_3. Because it is conformationally restricted, we can be reasonably sure of the orientation of one of its phosphate groups, and NMR studies confirm that this phosphate is held at a dihedral angle close to +60°, that is, a positive *gauche* orientation. An unexpected advantage of introducing the cyclic phosphate has been that the acid-base properties of **26** have been simplified. Thus, **26** behaves essentially as a diprotic acid and it has therefore been possible to carry out a detailed analysis of the protonation state of the individual phosphate groups, something that has not so far been possible with Ins(1,4,5)P_3.

Turning now to the biological results, the finding that **26** appears to behave as a full agonist, but with considerably reduced potency, presents us with some problems, which would not have arisen had the analogue been highly active, completely inactive, or a partial agonist. Experience with D-Ins(1,3,4)P_3 (Chapter 2) and D-Ins(1,3,4,5)P_4 (Chapter 7) shows that in the inositol phosphate field, when a biological assay demonstrates moderate full agonist activity for a compound, we must consider an important (and painful) question. The question is "Could the apparent activity of analogue X be due to the presence of a small amount of a second, highly potent, full agonist Y?" The problem is particularly acute if there is some obvious way in which the agonist Y can arise in the synthesis or metabolism of X. For example, phosphate migration during the synthesis of D-Ins(1,3,4,5)P_4 can lead to D-Ins(1,2,4,5)P_4, and metabolism *in vivo* can lead to Ins(1,4,5)P_3 (Chapter 7). Opening of the cyclic phosphate ring of the (presumably) active enantiomer of the conformationally restrained analogue can, in principle, lead to two trisphosphates (Figure 4.32).

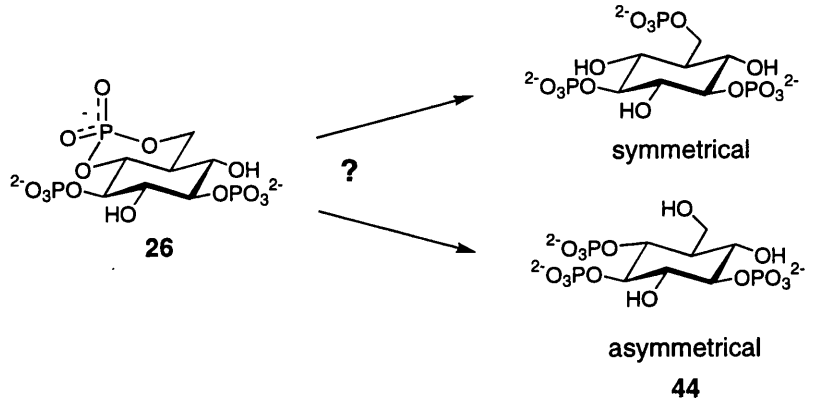


Figure 4.32 Ring-opening of the cyclic phosphate in **26** could give two possible products, one of which (**44**) is now known to be highly active.

The symmetrical compound lacks a vicinal bisphosphate and is therefore expected to be inactive (this is currently under investigation), but we have now shown the alternative product, **44** to be highly potent in our assay systems (see Chapter 5).

We must therefore consider whether the apparent activity of **26** could conceivably be due to the presence of **44**. It is difficult to see how the synthetic route could lead to the production of **44**, unless some opening of the cyclic phosphate ring occurred on deprotection. Furthermore, the product was subsequently purified by ion exchange, and appeared to be pure by NMR. The FAB mass spectrum of **26** showed no evidence of contamination with **44**. The six-membered cyclic phosphate structure fused to a six-membered ring would be expected to be highly stable to hydrolysis, and as already mentioned, a sample of the cyclohexylammonium salt showed no deterioration after a year at room temperature (samples for biological testing were kept at -20°C). It must be borne in mind, however, that neither NMR nor mass spectroscopy are ideal methods for the assay of inositol phosphates. The method of choice is currently HPLC with complexometric metal-dye detection,[257] and it is hoped to apply this methodology to the analysis of **26** (and other analogues) in the near future.

There is also the possibility that enzymatic hydrolysis of the cyclic phosphate by non-specific phosphodiesterases during the biological assays could generate **44**. A thorough evaluation of this possibility would involve consideration of the time-course of the assays, enzyme kinetics, and of the particular preparations used (whole cells, membranes, purified receptors etc.). An initial investigation, however, might involve comparison of an untreated sample of **26** to one pre-incubated with cell lysate or a purified phosphodiesterase.

With these reservations in mind, the finding that **26** retains full agonist activity might have significant implications for the understanding of Ins(1,4,5)P_3 receptor function and the design of ligands. These involve considerations of *affinity* for the Ins(1,4,5)P_3 receptor, *efficacy* in activation of Ca^{2+}-release and the relation of these to the *conformation* of the ligand.

1) Conformational restraint together with charge reduction of the equivalent of the 4-phosphate of Ins(1,4,5)P_3 decreases *affinity* for the Ins(1,4,5)P_3 receptor. It is hardly surprising that charge-reduction causes this, but it does not necessarily follow from conformational restraint. Some rigid analogues of other natural ligands (e.g. of acetylcholine, to take a classical example) have higher affinity and greater selectivity

than the natural ligand itself. However, in the present case, it is difficult to disentangle the biological impact of conformational restraint from the effect of reducing the charge on the constrained phosphate. The potentiometric and NMR investigations suggest that the reduction of charge compared to the equivalent phosphate of Ins(1,4,5)P_3 may not be so great at physiological pH, and the ionisation of the other phosphate groups is increased.

2) The conformationally restrained analogue appears not to be an antagonist, nor a partial agonist. Therefore it does not seem that the path to an Ins(1,4,5)P_3 antagonist lies in conformational restraint of the inositol ring nor of a phosphate group (at least not of the 4-phosphate in the positive *gauche* orientation). Assuming that the observed activity *is* due to **26**, then conformational restraint and charge reduction of the 4-phosphate do not reduce *efficacy*. Therefore channel-opening cannot require the inositol ring to flip, and may not require a dramatic change in the orientation of the 4-phosphate.

3) Again assuming that **26** itself is recognised by the Ins(1,4,5)P_3 receptor, the 4-phosphate group may be constrained in a way that is close to the conformation of Ins(1,4,5)P_3 at the receptor binding site. This might be taken to suggest that in the active conformation of Ins(1,4,5)P_3, the dihedral angle P4–O4–C4–H4 is positive and synclinal ($\varphi = +30°$ to $+90°$). Note that the 4,5-pyrophosphate analogue (Figure 4.29) which mimics a high-energy conformation of Ins(1,4,5)P_3, was totally inactive.[187] It might still be interesting to synthesise the epimeric analogue, in which the phosphate is constrained in an *anti* orientation, but until the question of ring-opening is settled, the results could still be open to different interpretations.

Very recently, and since the work described in this Chapter was carried out, X-ray crystal structures, showing Ins(1,4,5)P_3 bound to β-spectrin[137] and PLC-δ_1[139] PH domains, have been published (See Chapter 1). In both these structures, the bound conformations of Ins(1,4,5)P_3 appear to be similar, and are close to that predicted by our early molecular modelling, which was the basis for the design of **26**. The dihedral angle P4–O4–C4–H4 does indeed appear to be positive and synclinal, as in **26**, and apparently* with $\varphi < +60°$.

* Co-ordinates had not yet been deposited with the appropriate databases at the time of writing and so measurements could not be made.

Of course, the conformation of Ins(1,4,5)P_3 at the Ins(1,4,5)P_3 receptor itself need not necessarily resemble these structures. The Ins(1,4,5)P_3 receptor performs a very different function from these PH domains, which are now thought to act as membrane-anchoring modules. However, the similarity between these X-ray structures of bound Ins(1,4,5)P_3, and the picture of Ins(1,4,5)P_3 derived from the theoretical and experimental investigations described above is quite striking. No doubt, X-ray structures of Ins(1,4,5)P_3 at other binding sites, and NMR studies, which will give more dynamic information about the process of binding, will be published in the near future.

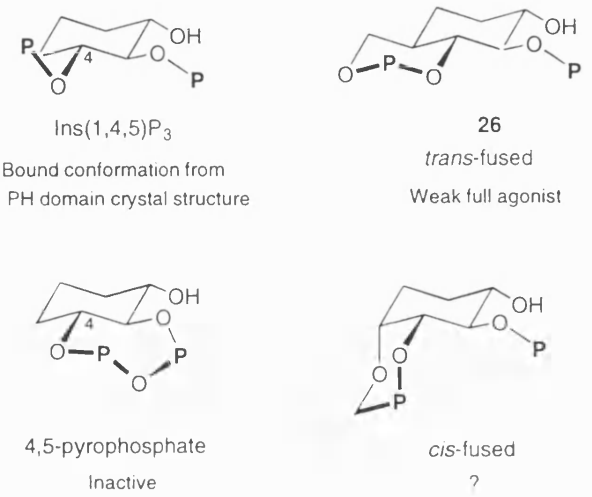


Figure 4.32 Orientation of the 4-phosphate group in the X-ray crystal structure of Ins(1,4,5)P_3 bound to PH-domains, and its equivalent in various conformationally restrained analogues.

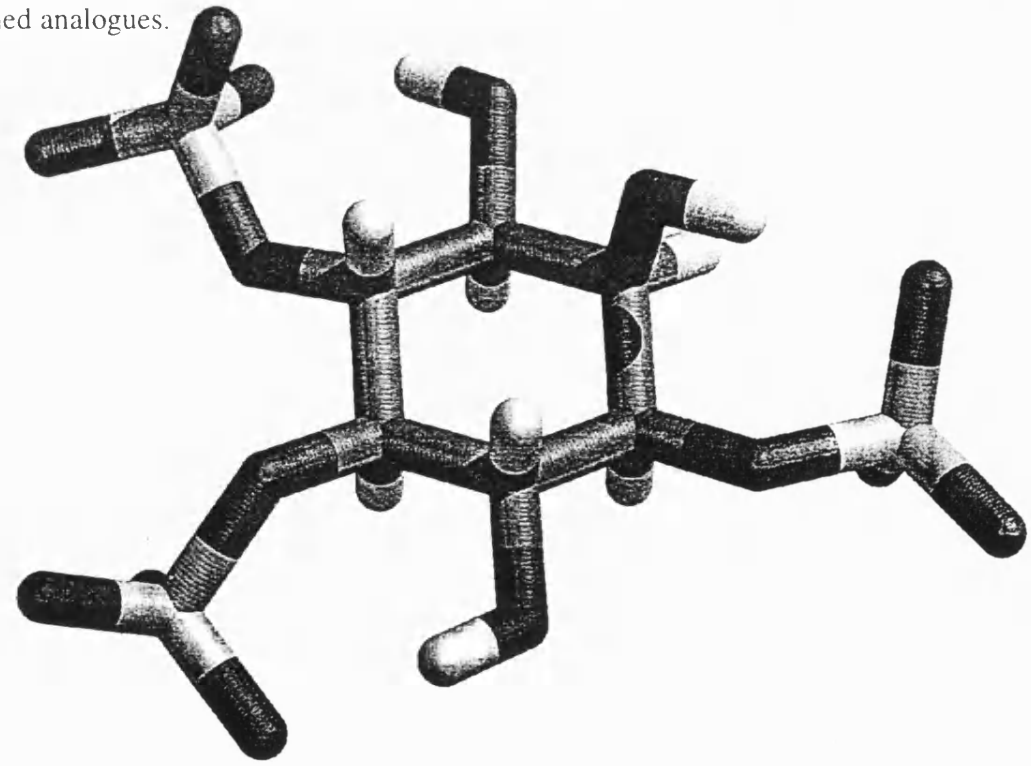


Figure 4.33 Energy-minimised structure of Ins(1,4,5)P_3. For details of molecular modelling, see Experimental section.

5 Adenophostins: Initial Structure-Activity Investigations

5.1 Adenophostins A and B

In 1994, two highly potent Ins(1,4,5)P_3 receptor agonists were identified through screening of compounds for inhibition of [^{3}H]Ins(1,4,5)P_3 binding.[211] Adenophostins A and B, found in the culture broth of *Penicillium brevicompactum* were reported to show very high potency in biological assays, being 100-fold more potent than Ins(1,4,5)P_3 in Ca^{2+} release and producing detectable effects in cerebellar microsomes at concentrations as low as 1 nM. Their effect could be totally blocked by heparin. They appeared to be completely resistant to phosphorylation or dephosphorylation by the Ins(1,4,5)P_3-metabolising enzymes 3-kinase and 5-phosphatase, and highly selective for Ins(1,4,5)P_3 receptors. The adenophostins, in contrast to Ins(1,4,5)P_3 itself, showed no ability to inhibit [^{3}H]Ins(1,3,4,5)P_4-binding to a purified Ins(1,3,4,5)P_4 -binding protein.[32]

5.1.1 Biological Effects of Adenophostin A

At this time there was no independent evidence for these surprising claims. Before embarking on a programme of research into synthetic adenophostin analogues it was important to establish whether the adenophostins had similar effects in our own biological assays. We were able to obtain a small amount (6mg) of adenophostin A from the discoverers and compare its activity to Ins(1,4,5)P_3. A sample was first quantified accurately by UV assay and then accurately divided into 20nmole aliquots, while another (2mg) portion was used for ^{1}H NMR studies.*

An initial test for biological activity was carried out in permeabilised rabbit platelets, and calcium release was monitored in the presence of the fluorescent dye fura-2 by spectrofluorimetry. As can be seen from Figure 5.1a, adenophostin A was indeed astonishingly potent, causing measurable Ca^{2+} release at concentrations as low as 2nM. Figure 5.1b confirms that the effect was blocked by heparin. Note that the effect of adenophostin A, unlike that of Ins(1,4,5)P_3 is *prolonged*, confirming its resistance to metabolism. Thus, what had seemed initially to be a small sample of adenophostin A was in fact sufficient for many biological studies, and the results of some of these, including dose-response and radioligand binding curves are given below.

* The high-resolution 400 MHz ^{1}H NMR spectrum of this sample in D_2O confirmed the assigned structure, although in this spectrum, two signals showed greater than the expected multiplicity. Whether this showed an impurity in the sample, an experimental artefact or something more significant, is currently not clear. Further studies with synthetic adenophostin are planned.

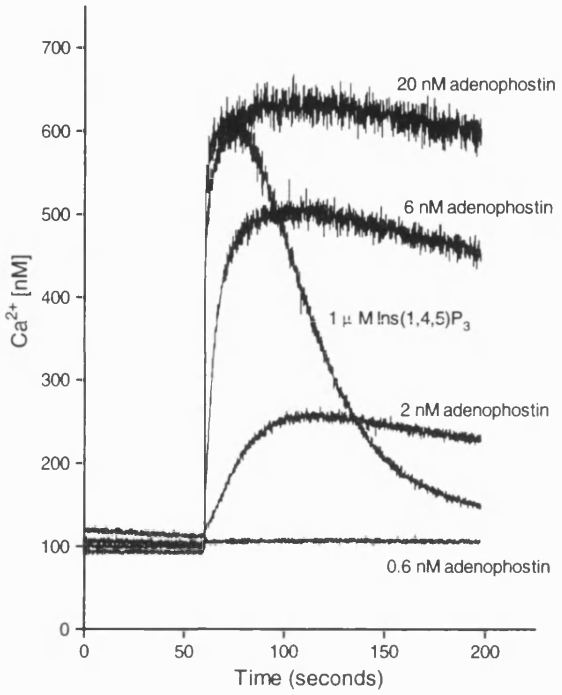


Figure 5.1a Adenophostin A-induced Ca^{2+}-release from permeabilised rabbit platelets, monitored by spectrofluorimetry.

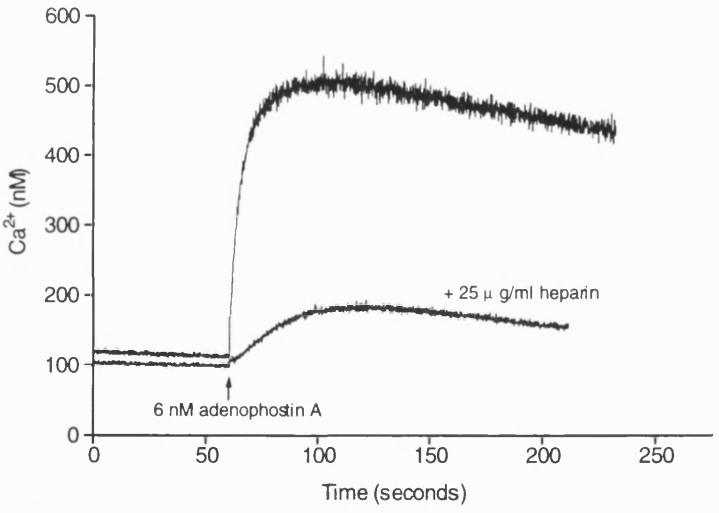
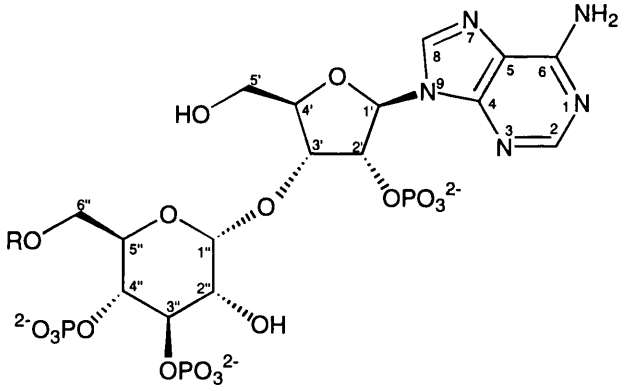


Figure 5.1b Inhibition of adenophostin-induced Ca^{2+}-release by heparin.

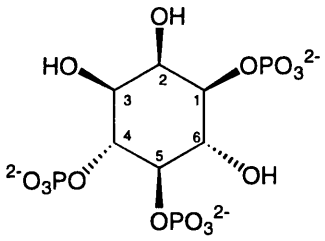
5.2 Structures of the Adenophostins

The publication of the structures[212] of the adenophostins (Figure 5.2) came as a shock. At first glance they appear to be strikingly different from Ins(1,4,5)P_3 and, like cyclic ADP-ribose, they are adenosine derivatives. However, the Ca^{2+}-releasing potency of cyclic-ADP ribose, which is thought to act *via* ryanodine receptors (see Chapter 1), is much lower than that of the adenophostins, and is not inhibited by heparin. On closer examination, the resemblances to Ins(1,4,5)P_3 become more apparent. Most importantly,

the 3″,4″-bisphosphate of the adenophostins is analogous to the 4,5-bisphosphate of Ins(1,4,5)P_3. As we have seen, this structural motif seems to be an essential feature of all agonists at Ins(1,4,5)P_3 receptors. One cannot help thinking that the finding of this feature in a molecule discovered by mass screening, and so far removed from Ins(1,4,5)P_3 or from any other Ins(1,4,5)P_3 receptor ligands previously known, is a testament to its importance as a recognition feature for Ins(1,4,5)P_3 receptors. The bisphosphate is in a six-membered ring, albeit a glucopyranose rather than an inositol ring, and is accompanied by an equatorial hydroxyl group at C-2″, which can be seen as equivalent to the 6-hydroxyl of Ins(1,4,5)P_3. Here the direct structural correspondence ends, but some other features of adenophostin A, and its 6″-acetylated homologue adenophostin B, can be shown to be compatible with existing structure-activity principles derived from studies of synthetic Ins(1,4,5)P_3 analogues.



Adenophostin A R = H
Adenophostin B R = $COCH_3$



D-*myo*-Ins(1,4,5)P_3

Figure 5.2 Structures of adenophostins A and B compared to Ins(1,4,5)P_3 .

First, consider the axial 2-hydroxyl group of Ins(1,4,5)P_3. Studies of 2-modified analogues have established that this group is of little or no importance in binding to the Ins(1,4,5)P_3 receptor.[213] Thus, *scyllo*-Ins(1,4,5)P_3 [106] [strictly *scyllo*-Ins(1,2,4)P_3] and 2-deoxy-Ins(1,4,5)P_3 [214] is only slightly less potent than Ins(1,4,5)P_3. In the adenophostins, the equivalent position is occupied by the pyranoside oxygen. Second, it is known that, although a third phosphate group is not essential for activity in inositol phosphates, it greatly enhances it. Thus Ins(4,5)P_2 is a 460-fold weaker agonist than Ins(1,4,5)P_3.[215] Now in Ins(1,4,5)P_3, this extra phosphate group is at the equatorial position 1, but

moving it to the axial position 2 [as in Ins(2,4,5)P_3] causes little reduction in activity* and an axial phosphate at position 1 as in D-*chiro*-Ins(1,3,4)P_3 will greatly enhance activity relative to Ins(4,5)P_2.[132] We can conclude that the location of the third phosphate group is less critical than that of the first two (perhaps the interaction with the binding site is *long-range*, as suggested by some workers[215]) and a phosphate group located somewhere within a fairly large region of space will enhance binding to the receptor. Very significantly, in the adenophostins, removal of the phosphate group at position 2′ of the ribose ring causes a 1000-fold reduction in activity. Now the 2′-phosphate group of the adenophostins does seem to be in a strange position, but it is known that many disaccharides and higher saccharides have rather rigid shapes, and it may be that the 2′-phosphate is held in a precise position with respect to the glucopyranose ring. Our own preliminary molecular modelling simulations seemed to show that, in most low-energy conformations, the 2′-phosphate occupies a position similar to that of the 1-phosphate of Ins(1,4,5)P_3, but in a different orientation and slightly further away from the ring. An example of one of these conformations is shown in Figure 5.3a. In other words, it is possible that 2′-phosphate may be positioned to interact with a similar area of the Ins(1,4,5)P_3 binding site to the 1-phosphate of Ins(1,4,5)P_3, but more effectively than in Ins(1,4,5)P_3 itself. Of course, it is also possible that the 2′-phosphate of the adenophostins interacts with a different area of the Ins(1,4,5)P_3 binding site from the 1-phosphate of Ins(1,4,5)P_3.

The adenosine component of the adenophostins is probably the most surprising, and currently the most mysterious feature of these molecules. Its steric bulk alone requires that we invoke the idea of some kind of binding pocket or vacant area of the receptor to accommodate it. It is known that very large substituents at position 2 of Ins(1,4,5)P_3 can be tolerated with minimal effect on potency, and it may be that this area of the binding site is open to solvent. This might lead us to consider an alternative binding orientation for the glucopyranose ring of the adenophostins as illustrated in Figure 5.3b. In this case, the above arguments still apply, but the equivalent of the 6-hydroxyl in Ins(1,4,5)P_3 is now occupied by an hydroxymethyl group, (at present there is no information on the effect of a 6-hydroxymethyl group in Ins(1,4,5)P_3 derivatives) and

* It may be more useful to consider the axial 2-phosphate in Ins(2,4,5)P_4 as equivalent to an axial 1-phosphate at the receptor binding site, by visualising Ins(2,4,5)P_3 in an inverted binding orientation, so that the 1-phosphate is axial. It is interesting to note here that Ins(1,4,5)P_3 receptors from rat olfactory membranes are reported to respond to a 100-fold lower concentration of Ins(2,4,5)P_3 relative to Ins(1,4,5)P_3.[247]

the third phosphate group is held in an area of space close to that occupied by the 2-phosphate of Ins(2,4,5)P_3. Binding orientation 5.3a requires an adenosine binding pocket in the area "below" position 1 of Ins(1,4,5)P_3, but there is currently no evidence against this, and indeed, the idea of a specific binding pocket, that may interact strongly with the adenosine moiety may be more appealing. Figures 5.3a, 5.3b and 5.3c are also intended to illustrate the possibility of extended and hairpin conformations, in which the adenine is either orientated well away from the glucopyranose ring (5.3a and 5.3b) or held close to it as in 5.3c.

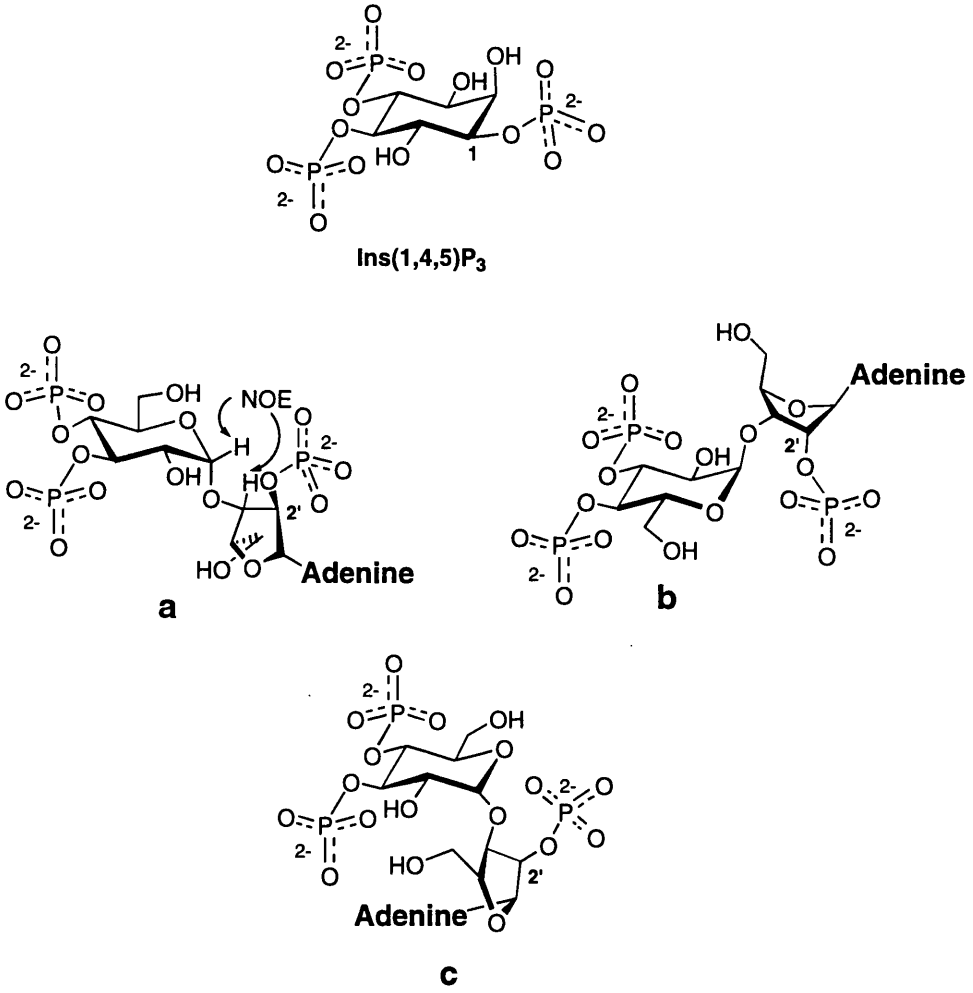


Figure 5.3 Examples of binding conformations and orientations in which adenophostin A might mimic Ins(1,4,5)P_3. See text for discussion.

Of the three conformations shown, only 5.3a conforms with the *exo*-anomeric effect, which is usually the dominant factor in determining conformation around a glycosidic bond. Furthermore, an NOE between H-3′ of ribose and H-1″ of glucose has been reported,[212] and these two protons can be seen to be close together in 5.3a, while at a maximal separation in 5.3c. * We still do not know whether the adenosine moiety itself

* This was actually reported for adenophostin B, which the authors used for the detailed NMR studies.

is directly involved in the extraordinary potency of the adenophostins or whether it serves to orientate another part of the molecule (e.g. the 2′-phosphate?) in a particularly favourable way.

5.2.1 Is 2′-AMP Active at Ins(1,4,5)P_3 Receptors?

If the Ins(1,4,5)P_3 receptor has a binding pocket for 2′-AMP close to the Ins(1,4,5)P_3 binding site, can 2′-AMP itself bind to the Ins(1,4,5)P_3 receptor? 2′-AMP is readily available and quite surprisingly, it has been found to be a substrate for inositol monophosphatase[216]. Molecular modelling studies have been carried out on 2′-AMP in connection with research into the design of inositol monophosphatase inhibitors, and recently a study of the active conformation of 2′-AMP at the enzyme's active site has been published.[217]

A sample of 2′-AMP free acid was converted to the triethylammonium salt in order to increase its aqueous solubility, and after the purity had been checked by ^{31}P NMR and HPLC, it was tested for ability to release Ca^{2+} in permeabilised rabbit platelets. As might be expected, it showed no ability to release Ca^{2+}, even at concentrations up to 1mM. More importantly, however, 2′-AMP did not inhibit Ins(1,4,5)P_3 -induced Ca^{2+} release at these concentrations. This finding, although not unexpected, demonstrates that 2′-AMP alone, separated from the phosphate-bearing glucopyranose ring does not have significant affinity for the Ins(1,4,5)P_3 binding site of the Ins(1,4,5)P_3 receptor. The activity of the isolated "other half" of adenophostin A, i.e. glucose 3,4-bisphosphate, is presently not known, although this molecule could easily be synthesised from a protected glucose intermediate.

5.2.2 The 5″-CH_2OH Group

There is one structural feature of the adenophostins that is not easily accommodated by structure-activity relationships based on Ins(1,4,5)P_3 analogues, namely the presence of an hydroxymethyl group at a position corresponding to the 3-hydroxyl (or conceivably the 6-hydroxyl) group of Ins(1,4,5)P_3. In a study designed to explore the steric tolerance of the Ins(1,4,5)P_3 receptor at the equatorial 3-position a series of racemic 3-*O*-alkylated analogues have been synthesised.[188] When these compounds were evaluated as Ca^{2+}-mobilising agonists in permeabilised SH-SY5Y cells, activity was found to decrease dramatically with increasing chain length (R = Me, Et, Pr^n). When R=Me, for example, potency was 25 times reduced, while for R = Et potency was reduced by greater than

500-fold. However, when R = CH_2COO^- the reduction in potency was less, despite the steric bulk of this substituent.[218]

Another study of 3-position-substituted Ins(1,4,5)P_3 mimics, including halogenated analogues,[219] used regression analysis to establish a negative, linear correlation between steric volume of an equatorial 3-substituent and activity at Ins(1,4,5)P_3 receptors.[183] However, further analysis of the data showed that Ins(1,3,4,5)P_4, 3PS-Ins(1,4,5)P_3 and Ins(1,4,5)P_3 itself were more active than would be predicted by this correlation based on purely steric considerations, and therefore fell into a second group (see Figure 5.4).

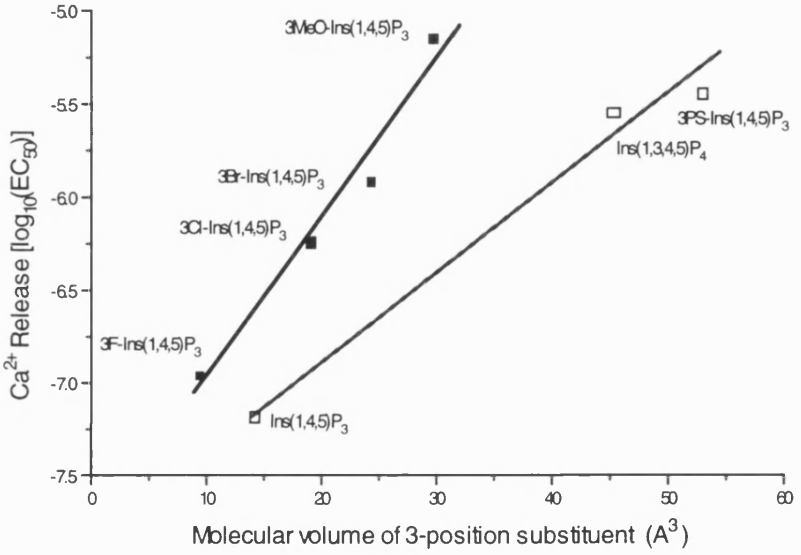


Figure 5.4 Potency of 3-position-substituted Ins(1,4,5)P_3 analogues plotted against molecular volume ($Å^3$) of the substituent. Adapted from Reference.[183]

No analogue with a CH_2OH group at the equivalent of position 3 had been synthesised, but based on the considerations above, its activity would be expected to fall somewhere between that of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (i.e. considerably weaker than Ins(1,4,5)P_3). And yet the equivalent of a 3-hydroxymethyl group is present in adenophostin A in the form of the 5″-hydroxymethyl, and adenophostin B has the even more unwieldy acetate at this position. Does this mean that the adenophostins must bind to the Ins(1,4,5)P_3 receptor in a different way to Ins(1,4,5)P_3?

After the publication of the structures of the adenophostins, it was quickly realised that intermediate **34**, discussed in Chapter 4, could be used to synthesise the hydroxymethyl analogue **44** (Figure 5.5) in a few steps. Analogue **44**, which can be considered as the 3-deoxy-3-hydroxymethyl *scyllo*-inositol analogue of Ins(1,4,5)P_3, would represent the first step away from Ins(1,4,5)P_3 towards an adenophostin-related

structure. True, **44** would (at this stage) be racemic, but it would enable us to investigate the effect of introducing an hydroxymethyl group at a position adjacent to the 4,5-bisphosphate in a molecule closely related to Ins(1,4,5)P_3.

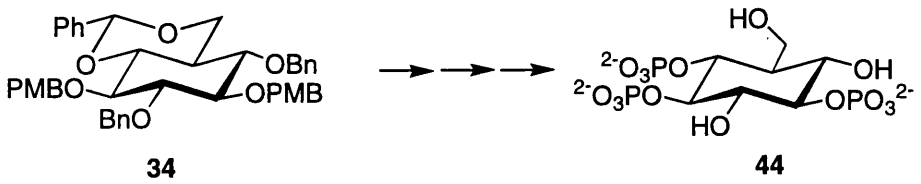


Figure 5.5 The intermediate **34**, from the synthesis of the conformationally restrained analogue (Chapter 4) can be used to synthesise an Ins(1,4,5)P_3 analogue **44**, which bears an equatorial hydroxymethyl group, as in adenophostin A.

5.3 Synthesis of a 3-Hydroxymethyl Analogue of Ins(1,4,5)P_3

The 3-hydroxymethyl analogue **44** (strictly 6-deoxy-6-hydroxymethyl-*scyllo*-inositol 1,2,4-trisphosphate) was synthesised in four steps from intermediate **34**, as shown in Figure 5.6.

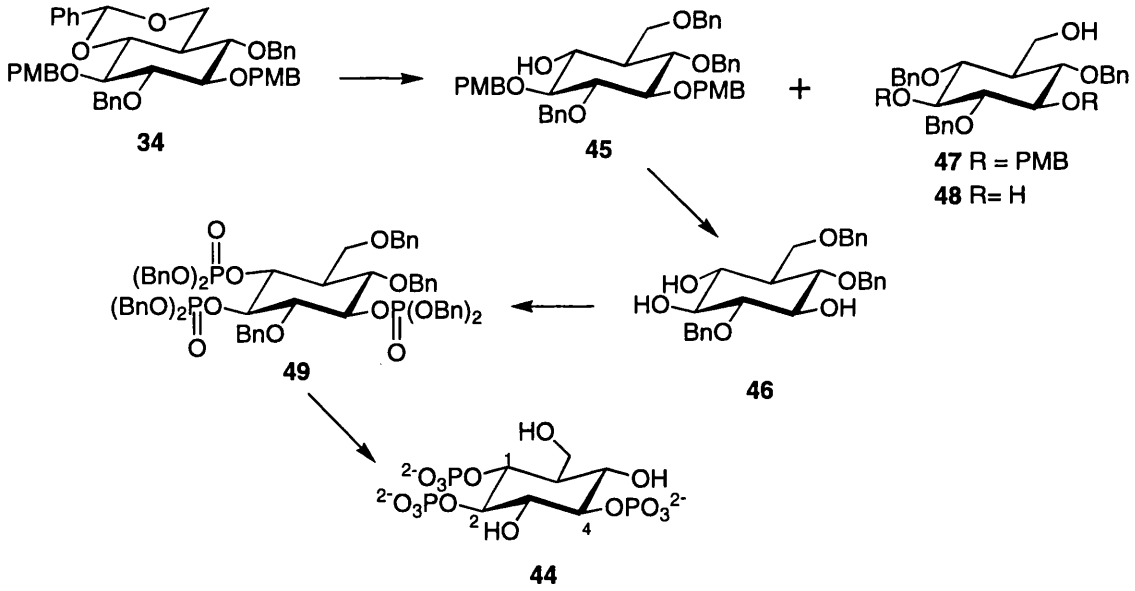


Figure 5.6 Synthesis of 6-deoxy-6-hydroxymethyl *scyllo*-inositol 1,2,4-trisphosphate (**44**).

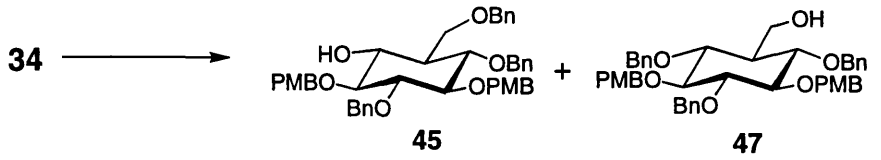
5.3.1 Regioselective Cleavage of a Benzylidene Acetal

Benzylidene acetals are versatile protecting groups in that they can be removed completely by acid hydrolysis to give a diol (see Chapter 4), or reductively cleaved in either direction to give primary or secondary benzyl ethers. In the synthesis of **44**, the required product was **45**, in which the acetal has been reduced regioselectively to a primary benzyl ether, exposing a secondary alcohol group. Reductions of this type have

been reported for benzylidene acetals of carbohydrates, and have employed aluminium chloride/trimethylamine-borane complex,[220] or sodium cyanoborohydride/hydrogen chloride,[221, 222](see below) and more recently, trifluoroacetic acid / triethylsilane.[223]

It was decided to try the first method, using aluminium chloride/trimethylamine-borane complex to reduce intermediate **34**. The results were disappointing at first, and the reaction seemed sluggish, eventually yielding a mixture of products, some of which resulted from loss of *p*-methoxybenzylidene groups. Rather than separating and individually characterising the products, the crude mixture was treated with refluxing 1M HCl/ethanol 2:1, giving only two products, which were shown to be the triol **46** (41% from **34**) and the (unwanted) symmetrical triol **48** (27% from **34**). It seems likely that the unwanted hydrolysis of the *p*-methoxybenzyl ethers, had interfered with the regioselectivity of the ring-opening.

A literature search located another more recent application of the same method in carbohydrate chemistry, with the modification that the reaction was carried out in the presence of 4Å molecular sieves.[224] When the reaction on **34** was attempted again, but this time with the addition of 4Å sieves, the results were much improved and the required alcohol **45** was obtained in 65% yield. A small amount of the symmetrical alcohol **47** (5%) was also isolated, and the TLC still showed quantities of more polar products resulting from *p*-methoxybenzyl ether cleavage. However, with sufficient quantities of **45** now in hand, no further attempts were made to optimise the procedure. It was later shown that the regioselectivity of the benzylidene cleavage could be reversed by reduction using diisobutylaluminium hydride (DIBAL-H) in toluene,[225] this time giving the symmetrical alcohol **47** as the major product (72%) together with a small amount of **45** (5%).



Conditions	**45** (% yield)	**47** (% yield)
$BH_3NMe_3/AlCl_3$, THF, 0°C	65	5
DIBAL-H, toluene, 0°C	5	72

Table 5.1 Regioselective reduction of **34**.

5.3.2 Phosphitylation/Oxidation and Deprotection

The *p*-methoxybenzyl protecting groups of the asymmetrical alcohol **45** were removed by acid hydrolysis giving triol **46**, which was phosphitylated using bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine/1*H*-tetrazole. The ^{31}P NMR spectrum of the intermediate trisphosphite triester showed a large $^{5}J_{PP}$ coupling of 6.1Hz between the phosphorus atoms of the vicinal phosphite groups (values of 3 or 4 Hz are typical for vicinal phosphites in an inositol ring). Oxidation as usual with *m*-CPBA gave the fully protected trisphosphate triester **49** which was a low-melting point crystalline solid. Deprotection using sodium in liquid ammonia, and purification by ion exchange chromatography went smoothly and the trisphosphate **44** was obtained as the pure triethylammonium salt.

5.4 Biological Activity of 6-deoxy-6-hydroxymethyl-*scyllo*-inositol 1,2,4-trisphosphate

The ability of racemic **44** to release Ca^{2+} from permeabilised rabbit platelets was examined. It appeared that **44** was equal in potency to Ins(1,4,5)P_3, despite being racemic. In an assay of this type, it is possible for a non-metabolisable compound to show artefactually high potency relative to Ins(1,4,5)P_3 due to prolonged $^{45}Ca^{2+}$ release. However, when **44** was tested for its ability to displace [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes, the results, shown in Figure 5.7 confirmed the $^{45}Ca^{2+}$-release data, with racemic **44** being equipotent to Ins(1,4,5)P_3. Assuming that only the L-enantiomer* of **44** is active, these binding results suggest that it should be one of the most potent synthetic Ins(1,4,5)P_3 analogues yet identified.

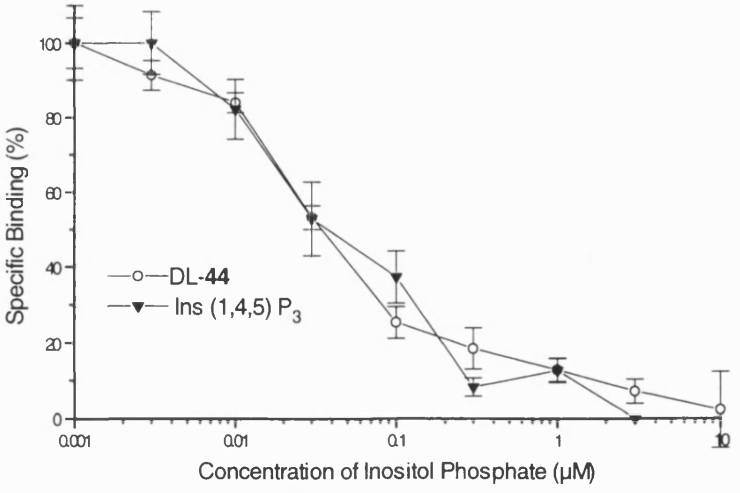


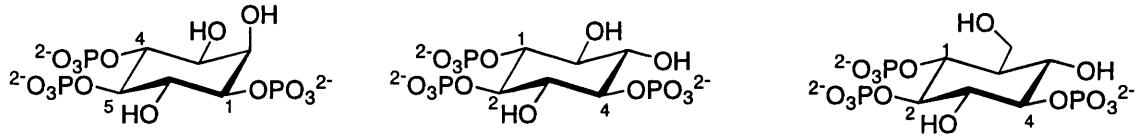
Figure 5.7 Displacement of [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes by **DL-44**.

* The enantiomer of **44** whose structure is analogous to Ins(1,4,5)P_3 is the L-enantiomer. See Appendix 1 for an explanation of this nomenclature.

The observation that racemic **44** is equipotent with Ins(1,4,5)P_3 implies that the CH_2OH component, which is not present in Ins(1,4,5)P_3 itself, is tolerated by the Ins(1,4,5)P_3 receptor despite the additional steric bulk. This finding seems to be at odds with the results of studies on 3-position modified Ins(1,4,5)P_3 analogues (Figure 5.4), which found that any increase in steric volume in this area reduced affinity for the Ins(1,4,5)P_3 receptor, and yet it is keeping with the high potency of the adenophostins. It would be interesting to establish whether analogues of **44** esterified at the primary hydroxyl group retained activity (as does adenophostin B) and if so, what limitations exist on the size and nature of the ester. Clearly, the potency of the adenophostins does not result from the CH_2OH group. It must somehow be related to the 2′-AMP component, as discussed earlier, but equally well, there is no need to imagine an unusual binding conformation for the glucopyranose ring of adenophostin A to allow the receptor to accommodate the CH_2OH group.

5.4.1 Comparison with *scyllo*-Inositol 1,2,4-trisphosphate

Is a CH_2OH at this position simply *tolerated* by the Ins(1,4,5)P_3 receptor, or can it actually enhance binding? Unfortunately, **44** differs from Ins(1,4,5)P_3 in two ways: it is modified at the equivalent of position 3, but also, it is strictly an analogue of *scyllo*- rather than *myo*-Ins(1,4,5)P_3 (Figure 5.8).



***myo*-Ins(1,4,5)P_3** ***scyllo*-Ins(1,2,4)P_3** **44**

Figure 5.8 Comparison of the structure of **44** with Ins(1,4,5)P_3 and *scyllo*-Ins(1,2,4)P_3.

We therefore need to exercise some care in drawing conclusions from direct comparisons between **44** and Ins(1,4,5)P_3. However, it would be valid to draw conclusions from a comparison of **44** with racemic *scyllo*-Ins(1,2,4)P_3. The two analogues were therefore compared for ability to release $^{45}Ca^{2+}$ from rabbit platelets, and the results are shown in Figure 5.9. It appears that **44** is significantly more potent than *scyllo*-Ins(1,2,4)P_3. Finally, racemic **44** was compared to racemic *scyllo*-Ins(1,2,4)P_3 and Ins(1,4,5)P_3 for ability to displace [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes. Again, it was significantly more potent than racemic *scyllo*-Ins(1,2,4)P_3, and equipotent to Ins(1,4,5)P_3. The results of these experiments are summarised in Table 5.2. We can conclude that, in the *scyllo*-series at least, replacement of the

secondary hydroxyl group at position 3 (strictly now position 6) with a primary hydroxyl group enhances affinity for the Ins(1,4,5)P_3 receptor.

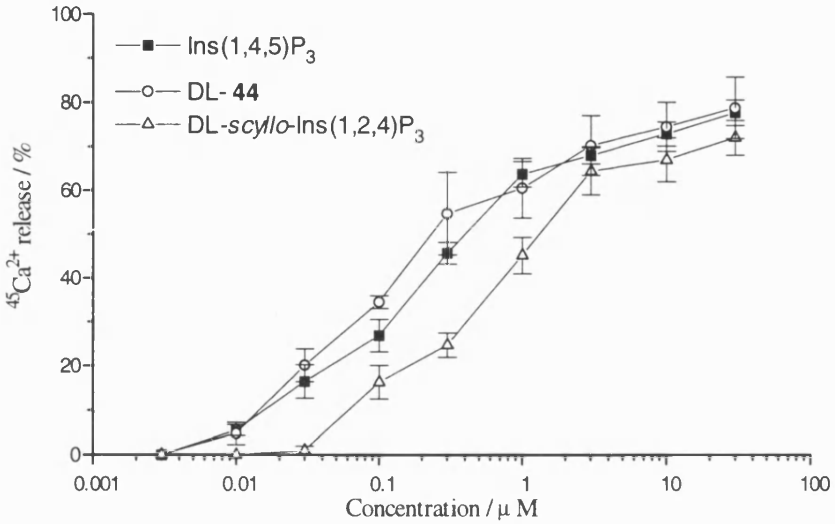


Figure 5.9 $^{45}Ca^{2+}$-release by racemic **44**, racemic *scyllo*-Ins(1,2,4)P_3 and Ins(1,4,5)P_3.

Compound	Ins(1,4,5)P_3 receptor binding at 4°C $IC_{50}/\mu M \pm$ S.E.M. (n=3)	$^{45}Ca^{2+}$ release at 4°C $EC_{50}/\mu M \pm$ S.E.M. (n=6)
D-Ins(1,4,5)P_3	0.04 ± 0.01	0.404 ± 0.11
DL-*scyllo*-Ins(1,2,4)P_3	0.15 ± 0.018	1.67 ± 0.35
DL-44	0.027 ± 0.01	0.44 ± 0.26

Table 5.2 Comparison of inositol phosphates for displacement of [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes and for release of $^{45}Ca^{2+}$ from permeabilised platelets.

5.4.2 Interaction with Ins(1,4,5)P_3 3-Kinase

DL-*scyllo*-Ins(1,2,4)P_3 is known to be a substrate for 3-kinase,[213] although the position of phosphorylation, and indeed, which enantiomer is metabolised, is not yet established. It might therefore be interesting to investigate the interaction of 3-kinase with **44.** A sample of racemic **44** was incubated with a purified 3-kinase preparation and the product analysed by HPLC with metal dye detection.[257] The results showed, perhaps surprisingly, that half of the sample had been phosphorylated, to give *two* unidentified tetrakisphosphates. This suggests that one enantiomer of **44** is being phosphorylated by the enzyme, but at two different positions. Although the specificity of 3-kinase for its natural substrate Ins(1,4,5)P_3 is known to be very high, we cannot assume that it is necessarily the Ca^{2+} -releasing enantiomer that is phosphorylated. Benzene 1,2,4-trisphosphate[226] and a mannose-based L-Ins(1,4,5)P_3 analogue (Chapter 1) are

recognised by 3-kinase, for example, but are inhibitors. Other possibilities exist; one enantiomer could be a 3-kinase inhibitor while the other is phosphorylated, for example, as was the case with racemic 2,2-difluoro-*scyllo*-Ins(1,4,5)P_3.[64] Thus, the tetrakisphosphate products could have any one of five possible structures. How can they be identified?

The ideal solution is to resolve an intermediate in the synthesis of **44** (see below), synthesise optically pure **D-** and **L-44**, incubate the two enantiomers separately with the 3-kinase, and identify the product by NMR. However there is another strategy which might be able to establish which enantiomer is phosphorylated and at what positions (Figure 5.10).

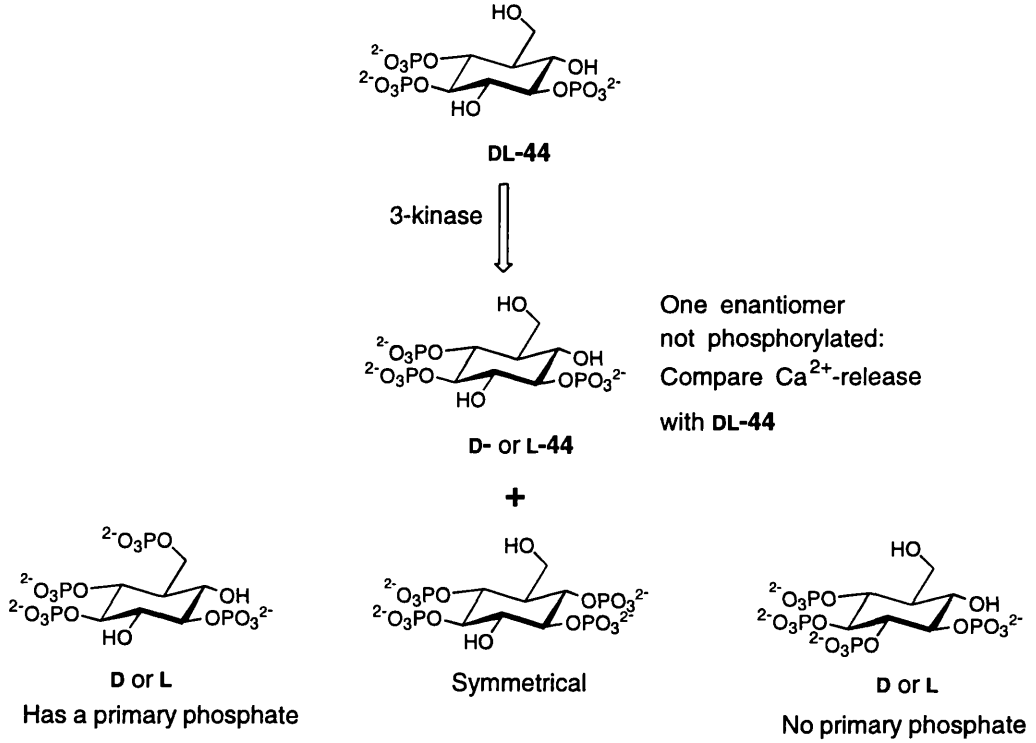


Figure 5.10 Proposed strategy to identify the products of phosphorylation of **44** by Ins(1,4,5)P_3 3-kinase.

If a larger quantity of racemic **44** is incubated with the enzyme, then it should be possible to separate the tetrakisphosphate products from the unphosphorylated trisphosphate enantiomer using ion exchange chromatography. There is little doubt that the potent Ca^{2+} -releasing enantiomer of **44** must have the D-4,5-bisphosphate configuration. If the Ca^{2+}-releasing activity of the unphosphorylated trisphosphate is then compared to racemic **44**, the assay must show that either its apparent activity has increased or decreased. The configuration of the phosphorylated enantiomer can then be deduced. A ^{1}H-coupled ^{31}P NMR of the tetrakisphosphate product should then be able

to identify the relative configuration of the tetrakisphosphates, even if they cannot be completely separated. A triplet in the spectrum implies phosphorylation of the primary alcohol, four doublets means phosphorylation at position 3, while two doublets identifies the product as the symmetrical tetrakisphosphate resulting from phosphorylation at position 5. Putting this information together gives the absolute configuration of the products. Accordingly, a larger sample of racemic **44** was synthesised, and this study is now in progress.

5.4.3 Lack of Interaction with Ins(1,3,4)P_3 5/6-kinase and PtdIns 3-kinase

When the interaction of racemic **44** with a purified D-Ins(1,3,4)P_3 5/6-kinase[87] was examined, racemic **44** was found to be completely inactive in inhibiting phosphorylation of [^{3}H]Ins(1,3,4)P_3. This result is quite remarkable, considering that all other racemic inositol phosphate analogues tested and all optically active analogues having the L-4,5 bisphosphate [or equivalent, e.g. as in D-Ins(1,3,4)P_3, see Section 1.9.4] were recognised by the enzyme, and yet in the case of **44**, neither enantiomer was recognised. This finding might be rationalised by a consideration of the binding orientation of **D-44** that might mimic the structure of D-Ins(1,3,4)P_3, the natural substrate of 5/6-kinase (see Figure 5.11).

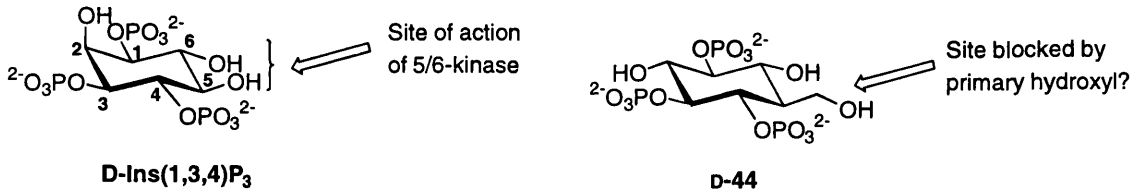


Figure 5.11 **D-44** appears not to be recognised by Ins(1,3,4)P_3 5/6-kinase, despite its similarities to D-Ins(1,3,4)P_3.

Neither racemic **44** nor racemic *scyllo*-Ins(1,2,4)P_3 were able to inhibit PtdIns 3-kinase. This observation can be explained by the fact that the D-enantiomers of these molecules bear no resemblance to Ins(1,4,5)P_3, while the L-enantiomers, although they may mimic the arrangement of phosphate groups in Ins(1,4,5)P_3 , have no equivalent to the axial 2-hydroxyl group (see Section 1.9.5).

5.4.4 Optical Resolution

An attempt to resolve an intermediate in the synthesis of **26** and **44** by formation of bis-(−)-ω-camphanate esters of diol **33** (Figure 4.4) was unsuccessful. The two diastereoisomeric biscamphanates, were inseparable by either chromatography or recrystallisation. Another attempt at resolution *via* monocamphanate esters of alcohol **45**

(Figure 5.6) also failed. However, when **45** was reacted with (*S*)-(+)-acetylmandelic acid (activated by DCC), the two products were well separated on the TLC plate. No more of the alcohol **45** remained at this stage, and so the reaction could not be scaled-up, but it is clear that the required optical resolution can be carried out at this stage, enabling the synthesis of the enantiomers of **44**. This is now being carried out by another worker.

Should it not prove possible to obtain crystals suitable for an X-ray study, then reduction of the free alcohol group of (+)- or (−)-**45** followed by hydrogenation will give the corresponding pseudosugar (either carba-β-D-glucopyranose, or its enantiomer). The absolute configuration can then be deduced from the direction of the optical rotation ($[\alpha]_D^{20} = +13.0°$ for the D-enantiomer in water[227]). Note that the enantiomers of **45** are precursors for optically active position-4 modified analogues of **44**.

5.5 Phosphorylation of Carbohydrates

The L-enantiomer of **44** resembles both a *scyllo*-inositol derivative and a derivative of β-D-glucopyranose. The fact that this structure showed highly potent activity at Ins(1,4,5)P_3 receptors suggested that a phosphorylated glucose derivative would also be active, even without the additional 2′-AMP feature found in the adenophostins. This may seem obvious in retrospect, but it must be remembered that the prevailing opinion at this time was that bulky substituents placed adjacent to the vicinal bisphosphate of Ins(1,4,5)P_3 analogues would inevitably cause a reduction in potency.

The idea of producing inositol phosphate mimics based on carbohydrates is not new. A synthesis of a phosphonate-containing D-Ins(1,4,5)P_3 analogue (Figure 5.12) from D-galactose has been described, for example.[228] No biological results have been reported for this analogue, but in the light of our present knowledge it seems unlikely that it would be very active at Ins(1,4,5)P_3 receptors. The galactose-based analogue retains an axial hydroxyl group, intended to mimic the 2-hydroxyl of *myo*-inositol, but the important 6-hydroxyl is replaced with pyranoside oxygen. It is now apparent, from studies of position 2-modified Ins(1,4,5)P_3 analogues, from the discovery of the adenophostins, and from the potent activity of the hydroxymethyl analogue **44** described above, that the 2-hydroxyl group is of low importance with respect to Ins(1,4,5)P_3 receptor binding of trisphosphate Ins(1,4,5)P_3 analogues. It can be changed to an equatorial hydroxyl, replaced with a pyranoside oxygen or removed completely. What is more, there is no need to retain a secondary hydroxyl group at position 3, and an hydroxymethyl group at this position is tolerated with no loss in activity. Thus, we

would expect that analogues derived from D-glucose, D-xylose (or even D-mannose) would be better mimics of Ins(1,4,5)P_3 at Ins(1,4,5)P_3 receptors.

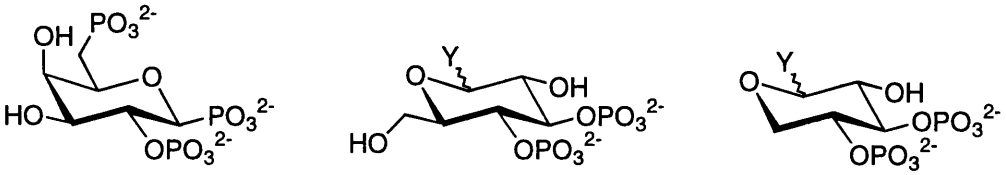


Figure 5.12 Ins(1,4,5)P_3 analogue based on D-galactose.[228] Structure-activity considerations (see text) now suggest that analogues based on D-glucose or D-xylose may be better Ins(1,4,5)P_3 receptor ligands. Y could have various structures (see below).

A synthesis of D-mannose 1,4,6-trisphosphate, intended as an L-Ins(1,4,5)P_3 mimic, has also been reported (quoted in Reference[65]) but it was found that the anomeric phosphate was chemically and stereochemically unstable. The fact that phosphates formed at the anomeric position (Y = OPO_3^{2-} in Figure 5.12) are intrinsically unstable poses severe limitations on the use of phosphorylated simple monosaccharides as inositol polyphosphate mimics. (A *phosphonate* formed at the anomeric position is stable, but in general, phosphonates have not been found to be good phosphate mimics at the Ins(1,4,5)P_3 receptor). If we were to phosphorylate some of the hydroxyl groups in a glycoside, or even a disaccharide, then the anomeric position would not be a problem. Disaccharides could give plenty of scope for novel polyphosphates, but the difficulties of protecting the hydroxyl groups selectively would be much greater. One solution is to protect selected hydroxyl groups in two monosaccharides and then couple the two together (ideally in a stereospecific way). Another solution might be to choose a disaccharide that is easy to manipulate because of its symmetry. This reasoning led us to consider the use of α,α-trehalose.

5.5.1 An Ins(1,4,5)P_3 Analogue Based on Trehalose

Trehalose is the general name for D-glucosyl D-glucosides. Since the glucose residues are joined at their anomeric carbon atoms, there are three possibilities; α,α-, β,β- and α,β-trehaloses. The first of these, α,α-trehalose (α-D-glucopyranosyl 1,1′-α′-D-glucopyranoside) is the only isomer known to occur in Nature, although α,β- and β,β-forms have been synthesised. The glucose residues in all three known trehaloses are in the pyranose form. α,α-Trehalose is found in "Trehalamanna", the cocoons of a parasitic beetle, and it also occurs in some fungi, and a variety of plants and bacteria. In insects, α,α-trehalose is the main sugar found in the blood, and in adult flies it is the

sole source of energy for flight. It may also help to protect enzymes and membranes in low-temperature and desiccated environments.[229]

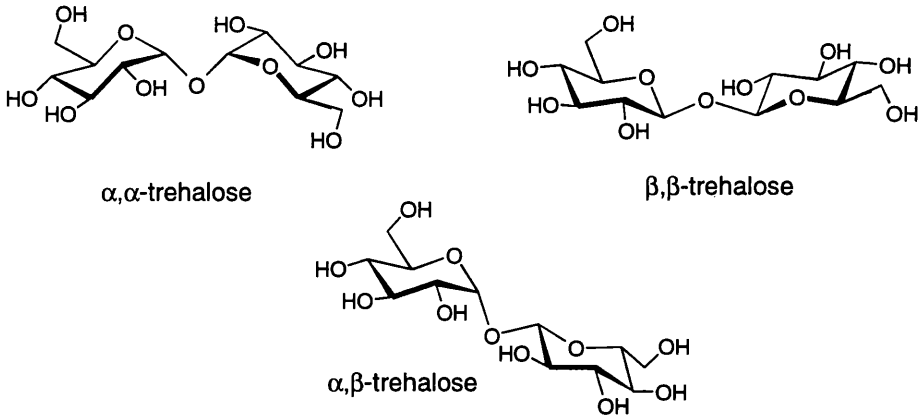


Figure 5.13 The trehaloses. Only the α,α-form is known to occur naturally.

A series of trehalose-based mycobacterial cell surface antigens have been isolated from *Mycobacterium tuberculosis*, the causative agent of tuberculosis. These glycolipids have been identified as a series of 2,3-di-*O*-acyl-α,α-trehaloses[230] and the related *M. fortuitum*, which is an opportunistic pathogen*, has also been found to contain 2,3,4- and 2,3,6-tri-*O*-acylated forms. These findings have stimulated renewed chemical interest in trehalose, and strategies have been developed for the regioselective protection of its hydroxyl groups.[231] A major component of the *M. fortuitum* lipid antigens has recently been synthesised from α,α-trehalose using these methods.[232]

It is interesting to note that sulphated β-maltosyl trehalose has recently been shown to have heparin-like antiproliferative effects and yet unlike heparin itself, has no (antithrombin III-mediated) anticoagulant properties.[233] The authors hypothesise, on the basis of molecular modelling experiments, that the trehalose moiety is responsible for the biological activity of the molecule, although its site of action is not yet known. Although little information is available on trehalose phosphates, it is now thought that α,α-trehalose-6-phosphate plays an important part in the regulation of yeast glycolysis, by feedback inhibition of hexokinase II, a role similar to that of glucose 6-phosphate in higher organisms.[234] Syntheses of α,α-trehalose-6,6′-phosphate and the four monophosphates of α,α-trehalose have been published recently.[235, 236]

An important property of α,α-trehalose is that it possesses a C_2 axis of symmetry, and no other symmetry elements. It therefore belongs to the $\mathbf{C_2}$ point group. Corresponding atoms e.g. C-1 and C-1′, C-2 and C-2′ etc., are homotopic and are

* *M. fortuitum* is becoming increasingly important because of its effects on AIDS patients.

therefore identical chemically and indistinguishable by NMR. This gives some important advantages:

- The synthesis of symmetrical trehalose derivatives is greatly simplified.
- Interpretation of NMRs is straightforward.
- Molecular modelling studies require fewer computations and therefore less time.

The C_2 symmetry of α,α-trehalose does lead to one disadvantage; that of determining its conformation and that of its derivatives by NMR. Because corresponding atoms in the two glucose residues are homotopic, the NMR equivalence of the two residues precludes determination of the solution conformation of symmetrical trehaloses by the measurement of chemical shifts, relaxation times, coupling constants or NOEs.[237] However, X-ray structures are available for various trehalose derivatives (summarised in Reference[238]) and a detailed molecular modelling study has been carried out.[238] The preferred conformations of the *C*-disaccharide equivalents of the trehaloses, in which the two methylene bridge protons are magnetically inequivalent, have recently been determined directly by NMR.[239] These studies suggest that α,α-trehalose is less flexible than the other trehaloses, and probably more conformationally rigid than most other disaccharides, a factor which may be important in its various biological roles.[238]

The observation that the adenophostins interact potently with the Ins(1,4,5)P_3 receptor binding site implies that the receptor can accommodate considerable steric bulk in an area of the binding site close to that normally occupied by Ins(1,4,5)P_3. Exactly which area of space, relative to the inositol ring, is involved is currently unknown but, if the adenophostins bind in an extended conformation, then this suggests that the receptor should also be able to accommodate a disaccharide having an α,α-linkage (Figure 5.14).

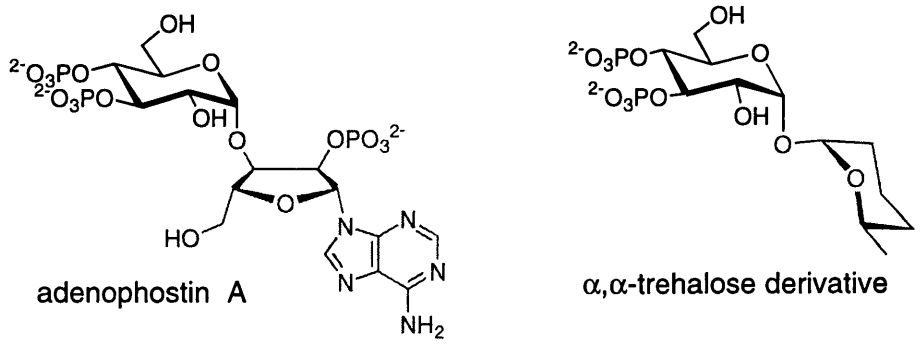
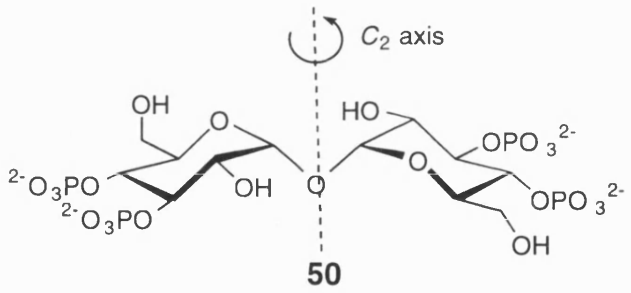


Figure 5.14 The fact that the Ins(1,4,5)P_3 receptor can accommodate the steric bulk of adenophostin A suggests that it might also be able to bind trehalose derivatives.

The C_2 symmetry of α,α-trehalose makes it an ideal starting point for a molecule that can be used to test this hypothesis because to some extent, the same protecting

group strategies can be applied that have been developed for α-methyl-D-glucopyranoside. Furthermore, the glycosidic linkage in α,α-trehalose is known to be resistant to α-glycosidase, a fact that should confer some metabolic resistance upon trehalose-based analogues. The initial target disaccharide chosen was the C_2-symmetric 3,4,3′,4′-α-D-glucopyranosyl-α'-D-glucopyranoside tetrakisphosphate **50**. Note that, if we define the Ins(1,4,5)P_3 binding pharmacophore as described in Chapter 1, this molecule possesses *two* copies of it. Contrast this with Ins(1,3,4,6)P_4 which has a symmetry plane rather than axis, and belongs to the $\mathbf{C_S}$ ($\mathbf{C_{1h}}$) point group. In Ins(1,3,4,6)P_4, positions 1 and 3 are enantiotopic, as are 4 and 6. They are therefore not equivalent in a chiral environment such as the binding site of a receptor or enzyme, although they are chemically equivalent as far as NMR is concerned (in the absence of any chiral environment). Ins(1,3,4,6)P_4 therefore has only one vicinal bisphosphate that is equivalent to the 4,5-bisphosphate of Ins(1,4,5)P_3.



50

Figure 5.15 Proposed C_2-symmetrical tetrakisphosphate analogue based on α,α-trehalose.

X-ray crystal structures of α,α-trehalose itself, and many derivatives generally show the torsion angles ϕ and ψ to be negative and synclinal, with values between –40 and –60°. This observation is a direct manifestation of the *exo*-anomeric effect, which in trehalose will be an important factor in determining both ϕ and ψ. Applying this to the tetrakisphosphate **50** gives a conformation (Figure 5.16) in which the 3′ and 4′ phosphates are positioned such that neither corresponds to the crucial 2′ phosphate of the adenophostins in the conformations shown in Figure 5.3.

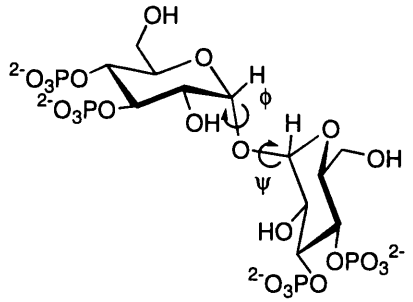


Figure 5.16 Theoretical conformation for **50** based on a consideration of the *exo*-anomeric effect, so that the torsion angles ϕ and ψ are both negative and synclinal.

There is no available information on the conformations of trehalose phosphates. (Note that it would not be possible to detect the expected NOE between H-1 and H-1′, because these protons are homotopic). Although it is certainly true that the *exo*-anomeric effect does govern the conformations of most (but not all[240]) α,α-trehalose derivatives studied to-date, it seems possible that the influence of the charged phosphate groups may exert a major influence, and this is likely to vary with pH, ionic strength and counter-ion in solution. The symmetrical **50** seemed to be an immediately accessible, and aesthetically appealing starting point for an investigation into phosphorylated disaccharides.

Synthesis of the symmetrical tetrakisphosphate **50** required a protected precursor such as **56**. A literature search showed that the diol **53** was known, and could be made in good yield from trehalose in two steps. The key synthetic strategy would be the regioselective reduction of both benzylidene acetals together to give **56**. It was thought that the symmetry of **53** and its expected rigidity would mean that the two acetals could be regarded as being equivalent, and essentially independent from one another. The outcome of the reduction would be easy to determine by NMR because, of the three possible products, the required **56** is symmetrical and has no primary hydroxyl group.

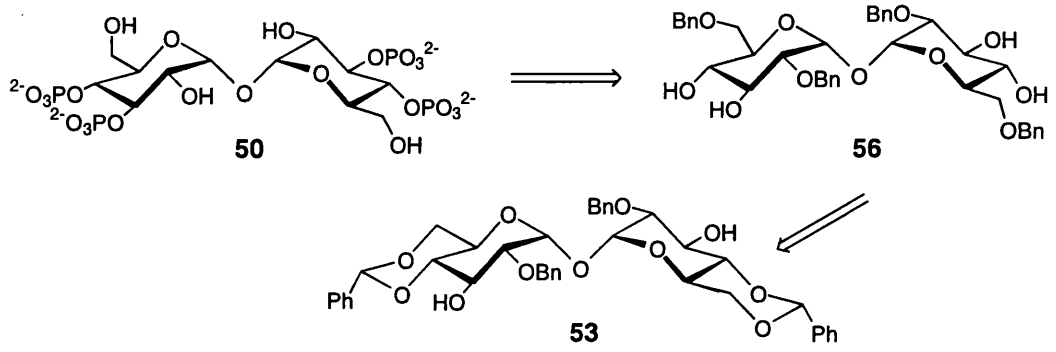


Figure 5.17 Retrosynthetic analysis of the symmetrical tetrakisphosphate **50** suggests a synthesis from **53**.

The synthesis of **50** would also be expected to produce some of the asymmetrical 2,3′-di-*O*-benzylated **52** as a by-product, and this could be used to make another analogue, α,α-trehalose 2,4,3′,4′-tetrakisphosphate (Figure 5.18) This molecule preserves the pharmacophoric bisphosphate on one glucose residue, while the other bears a phosphate at the 2′-position, which was expected to be a better mimic of the adenophostin 2′-phosphate. Should **50** show biological activity, then, it would be interesting to compare it to the asymmetrical regioisomer.

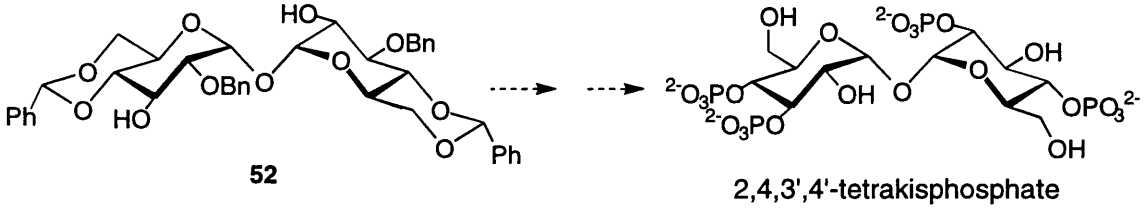


Figure 5.18 The asymmetrical product of the benzylation reaction (**52**) could be used to make another, potentially active, asymmetrical tetrakisphosphate.

5.5.2 Other carbohydrate-based Ins(1,4,5)P_3 /adenophostin mimics.

It was suggested that the planned synthesis was over-long, and that a direct route to tetrol **56**, or a similarly protected precursor would be preferable. Accordingly, a series of attempts were made to prepare a precursor for phosphorylation by direct dibutyltin-mediated alkylation and acylation of trehalose. These experiments, which were carried out by a colleague, were unsuccessful, and attempts to synthesise **50** were abandoned.

Later, a report was published of the synthesis of a phosphorylated glucose derivative intended as an Ins(1,4,5)P_3 /adenophostin A mimic[241]. This analogue showed potent activity at Ins(1,4,5)P_3 receptors, as would be predicted from the results for the hydroxymethyl analogue **44**, and by the reasoning given in Section 5.6. 2-Hydroxyethyl α-D-glucopyranoside 2′,3,4-trisphosphate [Gluc(2′,3,4)P_3, Figure 5.19] was found to be a full agonist at the Ins(1,4,5)P_3 receptors of rabbit platelets, but ten-fold weaker than Ins(1,4,5)P_3.

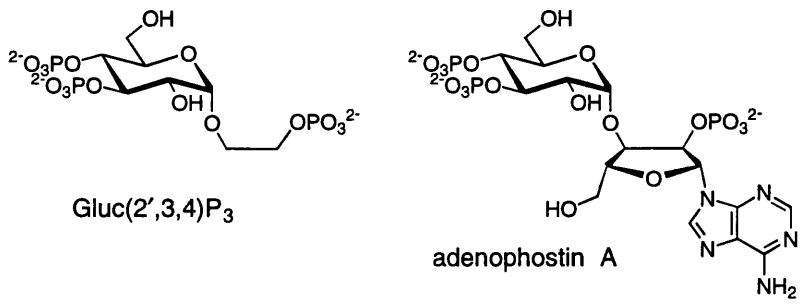


Figure 5.19 Structure of 2-hydroxyethyl-α-D-glucopyranoside-2′,3,4-trisphosphate, [Gluc(2′,3,4)P_3], showing relationship to adenophostin A.

It seemed likely that the reduced potency of Gluc(2′,3,4)P_3 relative to adenophostin A, Ins(1,4,5)P_3 and to the hydroxymethyl analogue **44** could be due to the flexibility of the ethylphosphate structure. A molecular dynamics simulation of Gluc(2′,3,4)P_3 supported this idea and as expected, low-energy conformations tended to be of the extended type, so that the primary phosphate group was positioned further away from the glucose ring than the equivalent 2′-phosphate of adenophostin A.

A comprehensive biological study of Gluc(2′,3,4)P_3 was published by another group,[242] confirming its activity in SH-SY5Y neuroblastoma cells and MDCK cells. Binding studies were also carried out in pig cerebellum, and enzyme assays showed that Gluc(2′,3,4)P_3, like adenophostin A, was resistant to metabolism by 3-kinase and 5-phosphatase. These authors also carried out a molecular dynamics simulation, confirming our unpublished results and again showing that the ethylphosphate was too conformationally mobile and too distant from the glucose ring to be a good mimic of the 2′-phosphate in adenophostin A. The authors also hypothesised that adenophostin A must interact with the Ins(1,4,5)P_3 receptor in a different orientation than does Ins(1,4,5)P_3 in order to minimise the negative effect of the hydroxymethyl group at position 5″. However, our results for the hydroxymethyl analogue **44** (see above) imply that no such assumption is necessary, unless of course, **44** *also* interacts with the Ins(1,4,5)P_3 receptor in a different orientation to Ins(1,4,5)P_3.

Shortly afterwards, a third group published the syntheses of more analogues similar to Gluc(2′,3,4)P_3 but based on xylopyranosides.[243] Their reported potencies were similar to that of Gluc(2′,3,4)P_3 (i.e. roughly ten times weaker than Ins(1,4,5)P_3) although the β-D-xylopyranoside with n = 3 appeared to be significantly weaker than the others. It would be interesting to compare the α-D-xylopyranosides with the glucopyranoside Gluc(2′,3,4)P_3 in the same biological assay so as to determine the contribution of the hydroxymethyl group to activity.

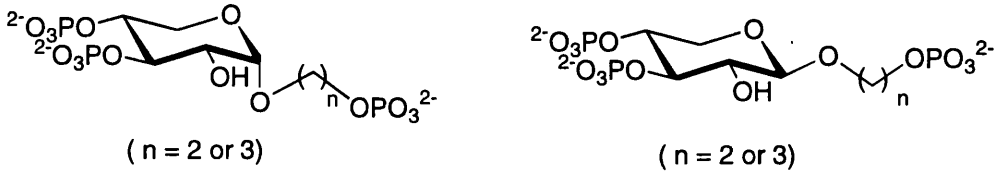


Figure 5.20 α- and β-xylopyranoside-based Ins(1,4,5)P_3 mimics.[243]

5.6 Synthesis of α,α-trehalose-3,4,3′,4′-tetrakisphosphate

The biological results reported for these phosphorylated carbohydrates did not bode well for the planned trehalose-based analogue, whose synthesis was now resumed. It seemed that the positioning of the third phosphate group was indeed a critical determinant of activity. (This did not imply, of course, that precise positioning of the phosphate group *alone* would necessarily give highly potent activity). As explained above, if we make that assumption that the *exo*-anomeric effect would be dominant in determining the conformation of **50**, and that the molecule would be quite rigid, then *neither* phosphate of the additional glucose ring would be in the right place. On the other hand, the published minimised energy conformation of adenophostin A, which was similar to that shown in Figure 5.3b, did not show any *exo*-anomeric effect either, and the idea of a disaccharide analogue with two copies of the essential pharmacophore was still attractive. At least the trehalose phosphate would be more rigid than the phosphorylated carbohydrate analogues discussed above, and if it showed any activity at all, it should be possible to produce various analogues in which the diequatorial bisphosphate motif was maintained on one ring while one or more phosphate groups were placed at various positions in the second. Finally, **50** and other trehalose polyphosphates might have interesting biological properties outside the phosphoinositide field. The synthesis of **50** was finally carried out, and is shown in Figure 5.21.

5.6.1 Regioselective Benzylation of 4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose

The di-*O*-benzylidene acetal **51** was prepared according to the improved procedure of Baer and Radatus.[244] The positive ion FAB mass spectrum of this compound, and of all subsequent derivatives in this synthesis showed a characteristic peak at [(M/2)–8]/*z* corresponding to the positive fragment ion produced by cleavage of the glycosidic bond. Regioselective benzylation according to Vicent *et al.*,[245] using two equivalents of dibutyltin oxide, excess benzyl bromide and a catalyst of *N*-methylimidazole gave the symmetrical 2,2′-di-*O*-benzyl-4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose (**53**) in 54% yield and the asymmetrical 2,3′-di-O-benzylated derivative **52** in 32% yield after column chromatography. The authors of the original study reported that unchanged starting material (11%) was recovered, although in our hands, the conversion was complete as judged by TLC, and more of the asymmetrical **52** was obtained. Both **52** and **53** could be crystallised easily from ethyl acetate/hexane (**52** had previously been reported to be a syrup). The isolation of substantial amounts of the unsymmetrically substituted **52** was

useful, because it was a precursor for an asymmetrical trehalose tetrakisphosphate (Figure 5.18). The respective identities of **52** and **53** were immediately apparent from the ^{1}H and ^{13}C NMR spectra, in that **53** possesses a symmetry element, which is lacking in **52**. The very simple ^{1}H NMR spectrum of **53** could be assigned completely, confirming the positions of the benzyl ethers.

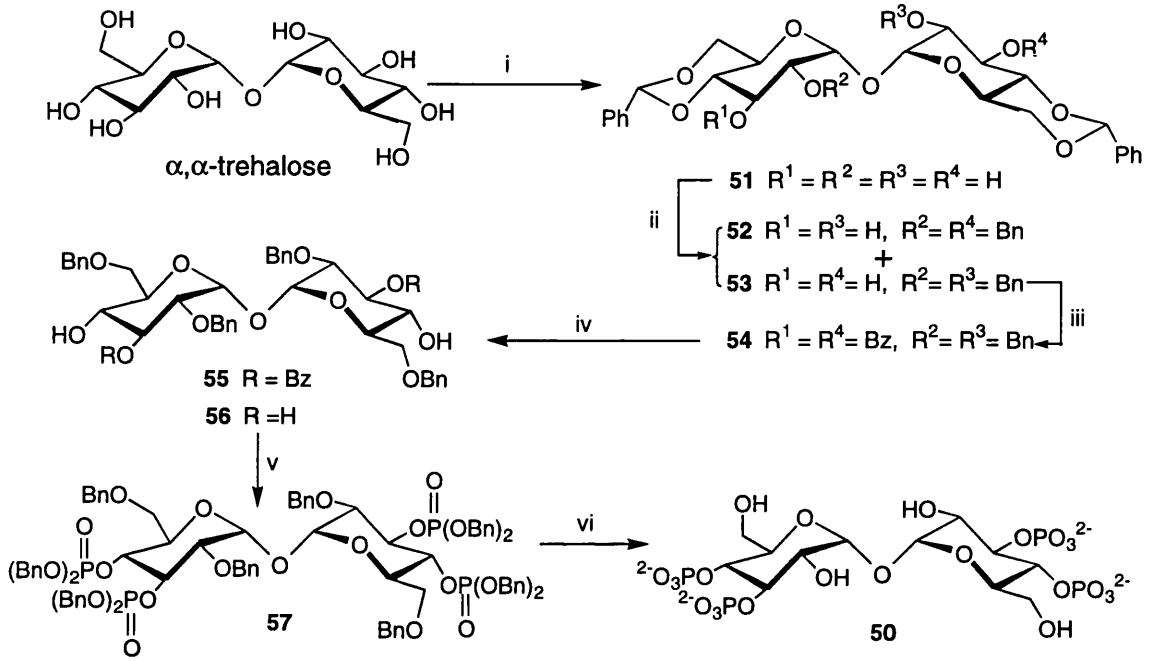


Figure 5.21 Synthesis of α,α-trehalose-3,4,3′,4′-tetrakisphosphate **50**.
i) $C_6H_5CH(OMe)_2$, DMF, *p*-TsOH, 75°C; ii) a) Bu_2SnO, 3Å sieves, CH_3CN, 120°C b) BnBr, *N*-methylimidazole; iii) BzCl, DMAP, pyridine; iv) $NaCNBH_3$, THF, 3Å sieves, dry HCl; v) a) $(BnO)_2PNPr^i_2$, 1*H*-tetrazole, b) *m*-CPBA, -78°C; vi) Na/liq NH_3. Bn=benzyl, Bz=benzoyl.

5.6.2 Regioselective Reduction of Benzylidene Acetals

The next step would be a regioselective reduction of both benzylidene acetals in the symmetrical derivative **53**. As discussed above, the borane-trimethylamine/aluminium chloride method had been successful when used in the case of the protected inositol **34**, but the yield had been modest, and regioselectivity was sometimes poor. It was therefore decided to attempt the reaction using the method of Garegg *et al.*[221], which employs sodium cyanoborohydride-hydrogen chloride, and if this was not successful, to try the newly reported method of DeNinno *et al.* using triethylsilane-trifluoroacetic acid.[223] In both of these methods, the carbohydrate model compounds used by the authors to investigate the regioselectivity of their method had protecting groups at position 3 (or its equivalent), although it was not clear what might be the effect of a free hydroxyl group at this position. It was particularly important in this case that the regioselectivity of the reaction was maximised so as to avoid obtaining a mixture of three products, which may be difficult to separate. (In fact a small-scale trial of the reaction directly on **53** did give

at least three products, which still await positive identification). It was therefore decided to protect this position temporarily as a benzoate ester. It was expected that this would also aid identification of the product, as the benzoylated 3-position should be easily recognisable in ^{1}H NMR spectra.

Accordingly **53** was benzoylated under standard conditions to give the highly crystalline symmetrical derivative **54**. Reduction using sodium cyanoborohydride and hydrogen chloride in dry ether according to the published procedure[221] was successful at the first attempt, with total conversion to a single product within 5 minutes. When the reaction was scaled up, the pure diol **55** was obtained in 80% yield after flash chromatography. The simple ^{1}H NMR spectrum confirmed the symmetry of the product, and as expected, the distinctive H-3 resonance, a pseudo-triplet, was shifted well downfield away from other signals, resulting in a first-order spectrum that could be completely assigned. Saponification using methanolic sodium hydroxide gave the required tetrol **56** in 85% yield.

5.6.3 Phosphorylation and Deprotection

The tetrol was phosphitylated with bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine / 1*H*-tetrazole, following the usual procedure. The ^{31}P NMR of the intermediate trisphosphite was a single AB system with $^5J_{PP}$ = 4.9 Hz. This confirmed beyond any doubt the structure of the tetrol precursor, in that such a spectrum could only arise if the precursor contained vicinal alcohol groups and was symmetrical. It is worth mentioning here that phosphitylation of polyols could be a useful technique for assigning their structure in situations when the ^{1}H NMR spectrum is complex, or where the hydroxyl groups cannot be seen. It enables immediate identification of vicinal diols, and the phosphites derived from isolated alcohol groups appear as singlets. In six-membered rings, the coupling constant can often be used to judge whether the relative stereochemistry of two hydroxyl groups is *trans* or *cis*.

Oxidation with *m*-CPBA gave the tetrakisphosphate triester **57.** The proton-coupled ^{31}P NMR spectrum of this compound simply consists of two pseudo-sextets (Figure 5.22a). Deprotection using sodium in liquid ammonia removed all benzyl protecting groups, leaving the glycosidic linkage intact and the target tetrakisphosphate was obtained as the pure triethylammonium salt after ion exchange chromatography. The proton-coupled ^{31}P NMR spectrum of **50** is shown in Figure 5.22b.

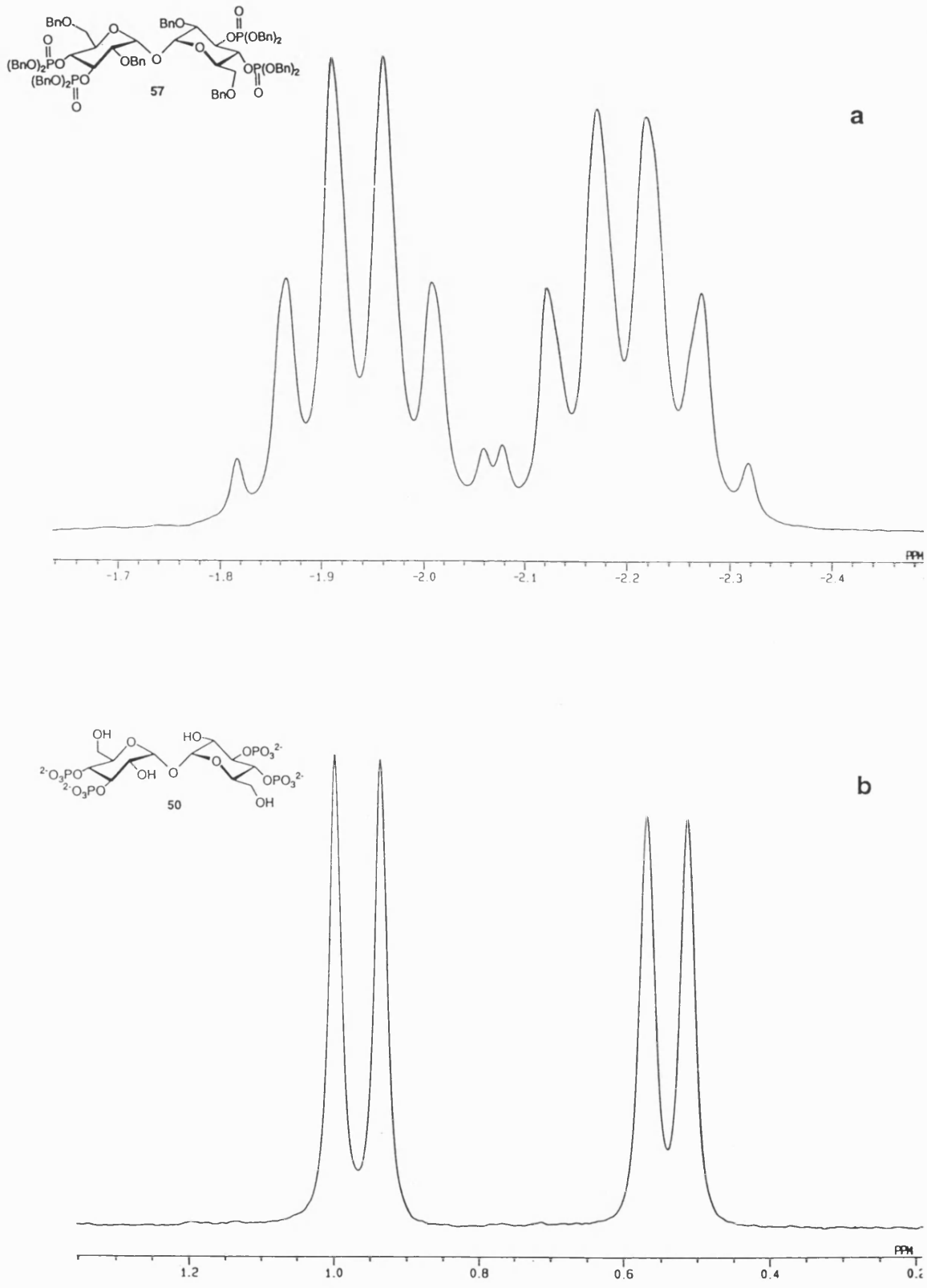


Figure 5.22 ^{31}P NMR spectra (^{1}H-coupled, 162 MHz) of **a**) protected tetrakisphosphate triester **57** in $CDCl_3$ **b**) α,α-trehalose-3,4,3′,4′-tetrakisphosphate **50** in D_2O, pH~7.

5.6.4 Ca^{2+}-mobilising Ability of Trehal(3,4,3′,4′)P_4

α,α-Trehalose-3,4,3′,4′-tetrakisphosphate (**50**) was tested for ability to release $^{45}Ca^{2+}$ in permeabilised rabbit platelets. The results (Figure 5.23) showed that **50** was able to release Ca^{2+} although with a potency around 100-fold lower than Ins(1,4,5)P_3. Thus **50**, although relatively weak, is the first known example of a disaccharide-based analogue active at Ins(1,4,5)P_3 receptors. The results do not allow us to judge whether **50** is behaving as a full agonist or as a high-efficacy partial agonist in this assay.

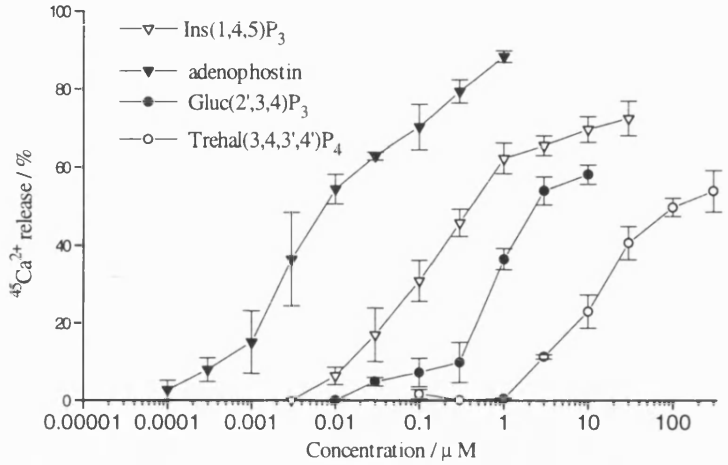


Figure 5.23 $^{45}Ca^{2+}$-release from permeabilised rabbit platelets induced by adenophostin A, Ins(1,4,5)P_3 , Gluc(2′,3,4)P_3 and Trehal(3,4,3′,4′)P_4 (**50**).

The time-course of Ca^{2+}-release induced by **50** can be seen in Figure 5.24. The effect is prolonged, indicating that **50** is poorly metabolised and is probably resistant to 3-kinase and 5-phosphatase. The effect of **50** is inhibited by heparin (Figure 5.25), which is good evidence that it is acting at the Ins(1,4,5)P_3 receptor binding site rather than by some other non-specific mechanism.

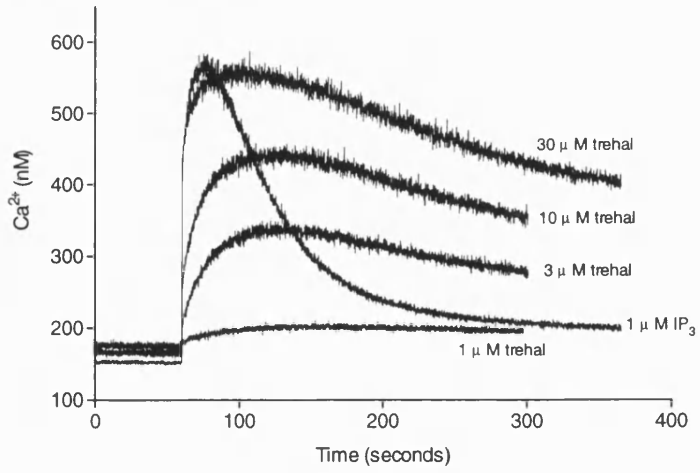


Figure 5.24 Time-course of Ca^{2+}-mobilisation by Trehal(3,4,3′,4′)P_3 (**50**) monitored by spectrofluorimetry.

Judging by these results, the affinity of **50** for the Ins(1,4,5)P_3 receptor appeared to be quite low, and this might be expected on the basis of the molecular modelling results discussed above. The results do show however, that the receptor is capable of accommodating the steric bulk of a second glucose residue, as predicted.

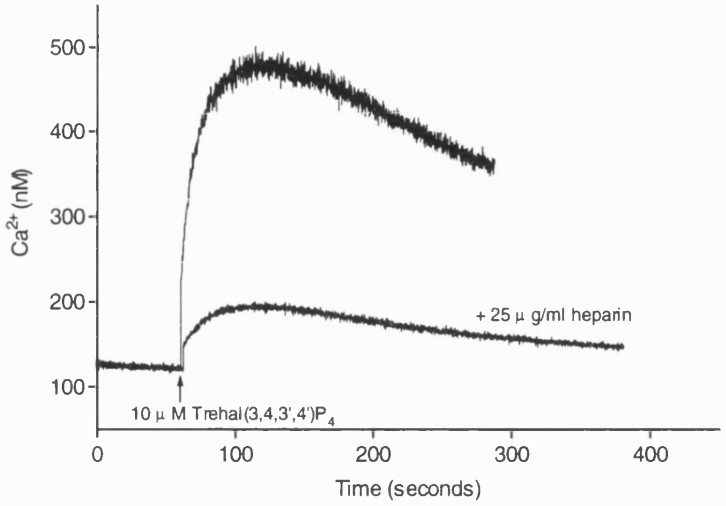


Figure 5.25 Spectrofluorimetry shows that the action of Trehal(3,4,3′,4′)P_4 (**50**) is inhibited by heparin.

5.6.5 Preliminary Binding Assay

Preliminary binding assays have recently been carried out for **50** (Figure 5.26). These results are puzzling, in that **50** appears to displace [^{3}H]Ins(1,4,5)P_3 with a potency similar to that of Gluc(2′,3,4)P_3. Thus the affinity of **50** for the rat cerebellar Ins(1,4,5)P_3 receptor appears to be surprisingly high, only 10-fold weaker than Ins(1,4,5)P_3 itself. On the basis of these results, **50** would be classified as a potent Ins(1,4,5)P_3 receptor ligand, and yet its ability to release Ca^{2+} release from rabbit platelets is low. Speculation as to the significance of this anomaly will be avoided until the findings can be confirmed.

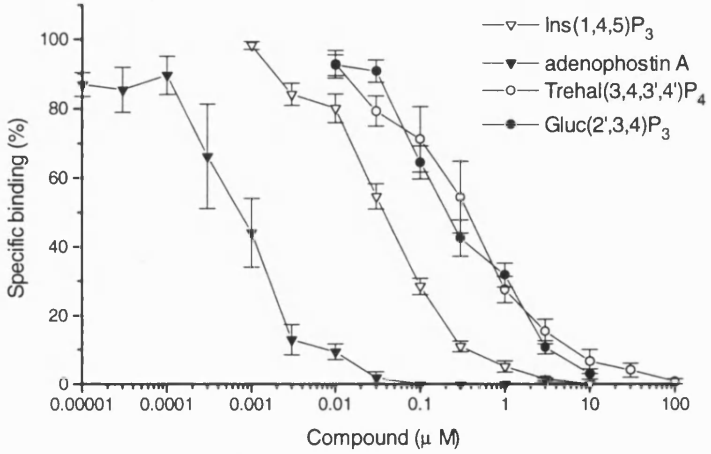


Figure 5.26 Displacement of [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes by adenophostin A, Ins(1,4,5)P_3, Gluc(2′,3,4)P_3 and Trehal(3,4,3′,4′)P_4 (**50**).

Table 5.3 Binding and $^{45}Ca^{2+}$ release data* for adenophostin A, Ins(1,4,5)P_3, Gluc(2′,3,4)P_3 , Trehal(3,4,3′,4′)P_4 (**50**) and 2′-AMP.

Compound	Binding ($IC_{50}/\mu M$)	$^{45}Ca^{2+}$ release ($EC_{50}/\mu M$)
adenophostin A	0.00074 ± 0.00042	0.0073± 0.0036
2′-AMP	—	no release (1mM)
Ins(1,4,5)P_3	0.038 ± .005	0.4 ± 0.08
Gluc(2′,3,4)P_3	0.21 ± 0.07	2.05 ± 0.3
Trehal(3,4,3′,4′)P_4	0.37 ± 0.17	100 ± 65

*Displacement of specific [^{3}H]Ins(1,4,5)P_3 binding from rat cerebellar membranes, and $^{45}Ca^{2+}$ release from permeabilised rabbit platelets were used to determine the EC_{5o} and IC_{50} values respectively. Values are mean ± S.E.M. (n = 3 - 10).

5.7 Summary

The discovery of the adenophostins has provided an important new stimulus to research in an area of medicinal chemistry where lead compounds were scarce and ideas were beginning to run out. The adenophostins possess the essential structural motifs that have been deduced for Ins(1,4,5)P_3 mimics by application of the classical active analogue approach over a period of years, and they also confirm our existing ideas as to which features of Ins(1,4,5)P_3 are not essential for activity. It was not previously appreciated that an hydroxymethyl group, and perhaps even larger structures, can be tolerated by the Ins(1,4,5)P_3 receptor in the region that normally binds the 3-hydroxyl group of Ins(1,4,5)P_3, but the high potency of 6-hydroxymethyl *scyllo*-Ins(1,2,4)P_3 has shown that this is indeed the case, and the presence of a similar structure in adenophostin A is not mysterious.

Attempts to mimic the 2′-phosphate of the adenophostins with conformationally mobile primary phosphate groups have so far met with limited success, producing analogues weaker than Ins(1,4,5)P_3 , but have established that simple carbohydrate-based analogues can show high potency. By comparing Gluc(2′,3,4)P_3 to 6-deoxy-6-hydroxymethyl-*scyllo*-Ins(1,2,4)P_3 we can deduce that the ethylphosphate of Gluc(2′,3,4)P_3 is not acting as a good surrogate for the 2′-phosphate, nor of the 1-phosphate of Ins(1,4,5)P_3. However, it is almost certainly better than no phosphate at all. The fact that Gluc(2′,3,4)P_3 is far more potent than Ins(4,5)P_2 , and the observation that the activity of the xylopyranoside-based Ins(1,4,5)P_3 mimics (Figure 5.20) is dependent on the orientation and chain-length of the alkylphosphates, suggest that the

conformationally mobile phosphate groups of these analogues enhance affinity for the Ins(1,4,5)P_3 receptor binding site but that their interactions with it are sub-optimal. An attempted short-cut to a conformationally more rigid analogue **50** based on trehalose seems to have been less successful in terms of agonistic effect, but the preliminary binding assay apparently shows high-affinity binding for **50**; an exciting result if it can be confirmed. Trehal(3,4,3′,4′)P_4 is the first synthetic disaccharide active at Ins(1,4,5)P_3 receptors, and its other biological properties await investigation.

We still do not know whether high potency can be achieved with a minimal structure lacking adenine, but the next logical step is obviously to include a degree of conformational restraint in the structure of Gluc(2′,3,4)P_3 , and Desai *et al.*[246] have very recently reported a synthesis of methyl 2-*O*-allyl-5-*O*-benzyl-*β*-D-ribofuranoside intended for the synthesis of such an analogue (Figure 5.27A). Our molecular modelling suggests that, if the 4′-hydroxymethyl group is not important, then a similar effect might be achieved by an even simpler structure (Figure 5.27B), based on a conjugate of glucose with 1,4-anhydroerythritol. Comparison of the activities of these analogues would establish the importance of the 4′-hydroxymethyl group, which up to now, has received little or no attention.

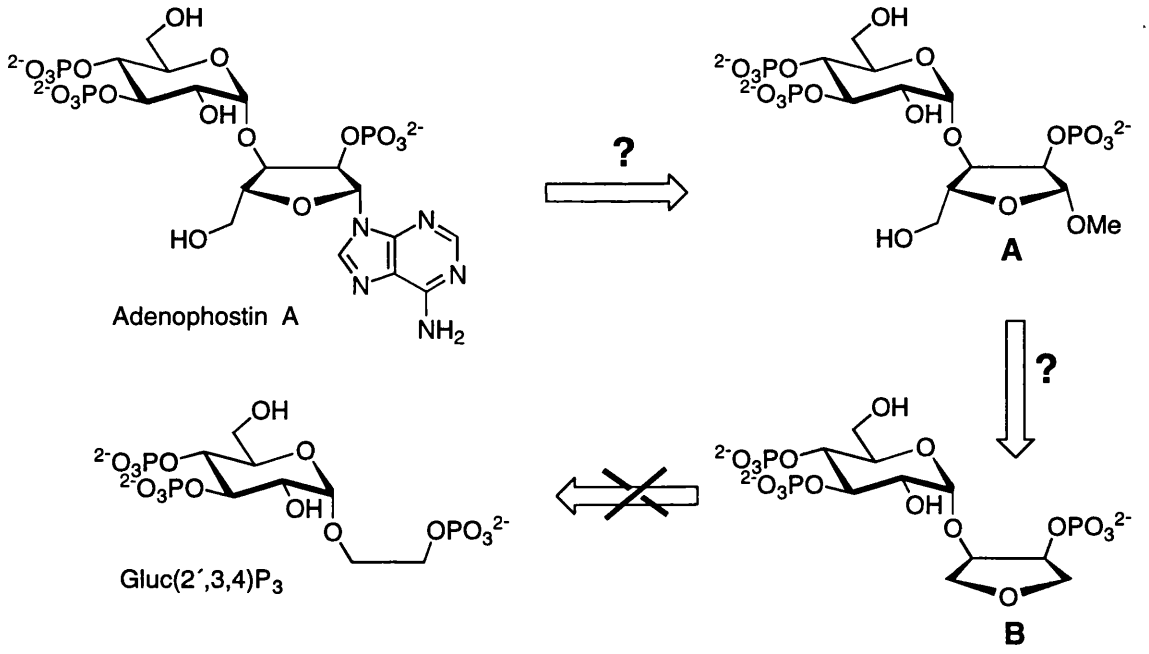


Figure 5.27 Attempt to establish the structural basis for the extreme potency of adenophostin A. Testing of **A** should establish whether the adenine is necessary. If not, the next step would be to synthesise and evaluate the even more simple **B** (or its cyclopentane-based equivalent). It is already established that Gluc(2′,3,4)P_3, with its conformationally mobile ethylphosphate does not retain high potency.

6 Phosphorothioates as Partial Agonists

6.1 Overview

As discussed in Chapter 1, Ins(1,3,4,6)P_4 has been shown to behave as a partial agonist at the Ins(1,4,5)P_3 receptor of SH-SY5Y neuroblastoma cells.[44] An attempt to determine the structural basis of this property by the synthesis and testing of L-Ins(1,3,4)P_3 and D-Ins(1,4,6)P_3 has not yet given conclusive results, because it now seems that Ins(1,3,4,6)P_4 itself does not behave as a partial agonist in our biological assay system (permeabilised rabbit platelets). Although these analogues are now being evaluated in SH-SY5Y cells, the results are not yet available. So far, therefore, no inositol phosphate has demonstrated partial agonist properties in the rabbit platelet assay.

However, one inositol *phosphorothioate*, namely L-*chiro*-inositol-2,3,5-trisphosphorothioate,[45] had been found to be a partial agonist in rabbit platelets. During the course of our work on L-Ins(1,3,4)P_3, described in Chapter 2, a colleague synthesised racemic Ins(1,4,6)PS_3, and this analogue too was found to be a partial agonist with very low intrinsic efficacy in platelets. Ins(1,4,6)PS_3 was also demonstrated to inhibit Ins(1,4,5)P_3-induced Ca^{2+}-release in a concentration-dependent fashion.[48] It was assumed that the D-enantiomer was responsible for this effect, and this can be rationalised by considering possible binding orientations at the Ins(1,4,5)P_3 receptor as described for D-Ins(1,4,6)P_3 in Chapter 1. Another low-intrinsic activity phosphorothioate partial agonist in SH-SY5Y cells, D-6-deoxy-*myo*-inositol-1,4,5-trisphosphorothioate,[47] has not been tested in platelets.

It was possible to discern a pattern to these results (Figure 6.1), in which replacement of phosphate groups with phosphorothioates, coupled with the reorientation or deletion of the hydroxyl group at position 3 or 6 gives a partial agonist. Notice that two analogues are missing from this pattern and to the best of our knowledge, they have not so far been synthesised. Now in the suggested binding mode for L-Ins(1,3,4)P_3 (Chapter 1), the hydroxyl group corresponding to position 6 of Ins(1,4,5)P_3 is also reorientated, so that it is now axial rather than equatorial, and in addition, the hydroxyl group corresponding to position 2 of Ins(1,4,5)P_3 has now been changed from axial to equatorial, as in the suggested orientation for D-Ins(1,4,6)PS_3. It was therefore thought that L-Ins(1,3,4)PS_3 might also be a partial agonist.

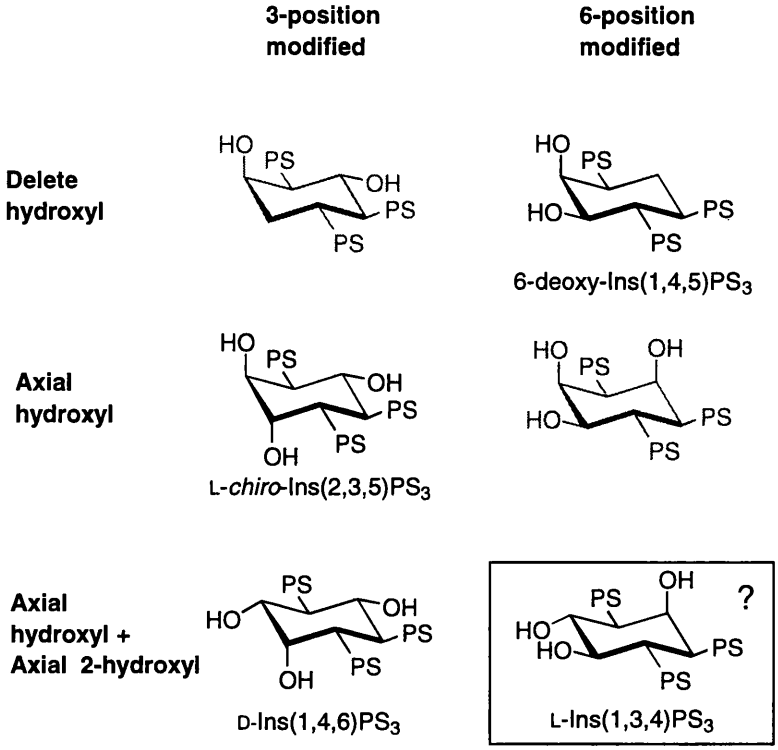


Figure 6.1 The finding that L-*chiro*-Ins(2,3,5)PS_3, 6-deoxy-Ins(1,4,5)PS_3 and D-Ins(1,4,6)PS_3 are partial agonists suggested that L-Ins(1,3,4)PS_3 might also share this property. Two other compounds are also predicted to be partial agonists from this pattern, but have not been synthesised. [PS = phosphorothioate, $OP(S)O_2^{2-}$].

6.2 Synthesis of DL-*myo*-Inositol 1,3,4-trisphosphorothioate

It was therefore decided to convert a small sample of the racemic triol **9** into DL-*myo*-inositol-1,3,4-trisphosphorothioate (**59**). If the racemic phosphorothioate showed interesting activity, then pure L-Ins(1,3,4)PS_3 [and if necessary, D-Ins(1,3,4)PS_3] could easily be made from the corresponding optically pure triols.

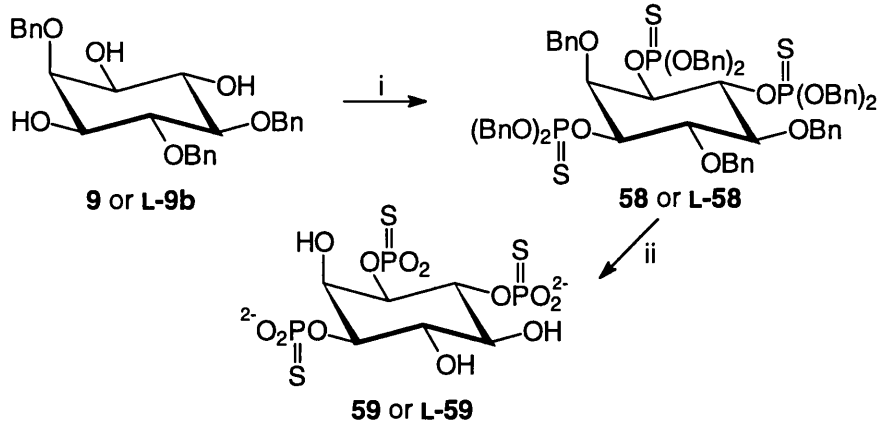


Figure 6.2 Syntheses of racemic Ins(1,3,4)PS_3 (**59**) and L-Ins(1,3,4)PS_3 (**L-59**)
i) a) $(BnO)_2PNPr^i_2$, 1*H*-tetrazole, b) S_8; ii) Na / liquid NH_3. See Experimental section for details.

9 Was phosphitylated as before and then stirred overnight with elemental sulphur in dry pyridine. After the solvents were removed, purification by column chromatography gave the protected trisphosphorothioate **58** as an oil in modest (50%) yield. Deprotection using sodium in liquid ammonia, and purification by ion exchange chromatography gave racemic Ins(1,3,4)PS_3 (**59**) in 61% yield.

6.2.1 Biological Evaluation

The racemic trisphosphorothioate was found to have very low efficacy at the Ins(1,4,5)P_3 receptor of rabbit platelets, releasing only 20% of the Ins(1,4,5)P_3 -sensitive Ca^{2+} pool, even at concentrations above 1mM (Fig. 6.3).

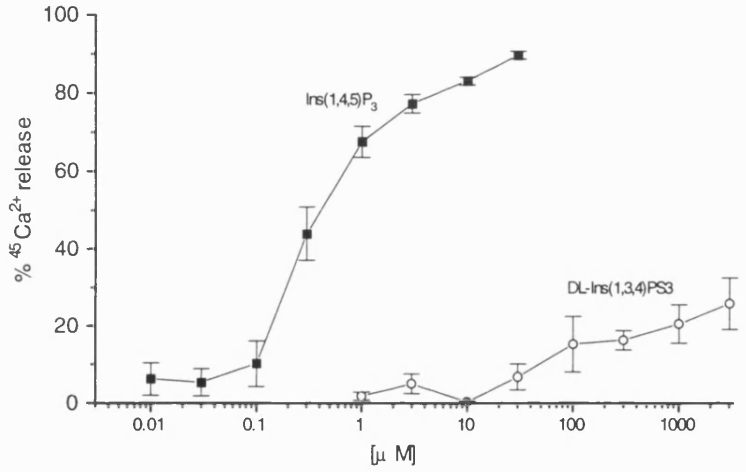


Figure 6.3 $^{45}Ca^{2+}$ release by DL-Ins(1,3,4)PS_3 (**59**) from permeabilised rabbit platelets.

Now this result, taken in isolation, does not show that DL-Ins(1,3,4)PS_3 is a partial agonist; an extremely weak full agonist could give similar results. However, when platelets were treated with Ins(1,4,5)P_3 at a concentration of 1μM, together with increasing concentrations of DL-Ins(1,3,4)PS_3 a definite inhibition of Ins(1,4,5)P_3-stimulated Ca^{2+} release was observed (Figure 6.4). These results demonstrate that DL-Ins(1,3,4)PS_3 was acting as a true partial agonist. The effect of Ins(1,4,5)P_3 is reduced as the concentration of DL-Ins(1,3,4)PS_3 is increased, until it eventually falls to a level approaching the intrinsic efficacy of DL-Ins(1,3,4)PS_3 itself (the DL-Ins(1,3,4)PS_3 - induced Ca^{2+} release curve is shown again for comparison).

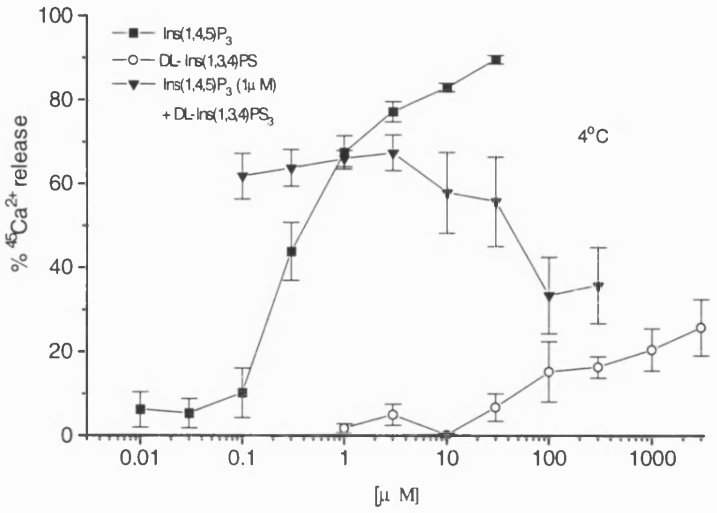


Figure 6.4 Inhibition of Ins(1,4,5)P_3 -induced $^{45}Ca^{2+}$-release by DL-Ins(1,3,4)PS_3

6.3 Synthesis and Evaluation of L-*myo*-Inositol 1,3,4-trisphosphorothioate

It was assumed that the partial agonist activity of racemic DL-Ins(1,3,4)PS_3 resided in the L-enantiomer, but it was important to demonstrate that this was true. Accordingly a sample of optically active triol **L-9b** was phosphitylated as previously described. This time the sulphoxidation was carried out using an improved method, developed by a colleague.[49] After the presence of the trisphosphite triester had been confirmed by ^{31}P NMR, the dichloromethane was removed by evaporation under reduced pressure and DMF-pyridine (2:1) was added, followed by 3 equivalents of sulphur per phosphite group. Sulphoxidation was complete within 10 min as judged by ^{31}P NMR. Note that this method is much faster than the use of sulphur in pyridine, which requires a few hours for complete conversion and can give poor yields. The small excess of sulphur was filtered off and the solvents evaporated *in vacuo* to give a syrup, which was purified by flash chromatography giving the fully protected trisphosphorothioate triester **L-58** in 71% yield. **L-58** was deprotected as for the racemic compound and purification gave L-Ins(1,3,4)PS_3 (**L-59**).

The ability of L-Ins(1,3,4)PS_3 to displace [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes was examined, and the results are shown in Figure 6.5. The observation that L-Ins(1,3,4)PS_3 shows roughly twice the affinity of the racemic mixture in this assay confirms that the activity does indeed reside in the L-enantiomer.

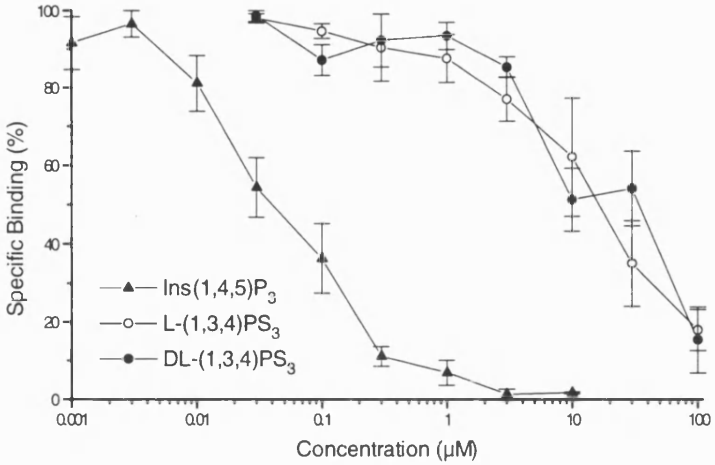


Figure 6.5 Displacement of [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes by DL-Ins(1,3,4)PS_3 (**59**) and L-Ins(1,3,4)PS_3 (**L-59**).

D-Ins(1,4,6)PS_3, which was synthesised in a parallel project by a colleague, showed even higher affinity for the Ins(1,4,5)P_3 receptor in binding studies, an observation which can be rationalised by similar structure-activity arguments to those given in Chapter 1 for D-Ins(1,4,6)P_3 and L-Ins(1,3,4)P_3. Like L-Ins(1,3,4)PS_3 this analogue also shows very low efficacy, releasing less than 20% of the Ins(1,4,5)P_3 - sensitive Ca^{2+} pool (Figure 6.6). D-Ins(1,4,6)PS_3 therefore constitutes an important lead compound in the search for Ins(1,4,5)P_3 receptor antagonists, and it should be possible to produce a series of analogues of this compound, selectively modified at the axial 2-position from the *myo*-inositol orthoformate derivative **28** (Chapter 4).

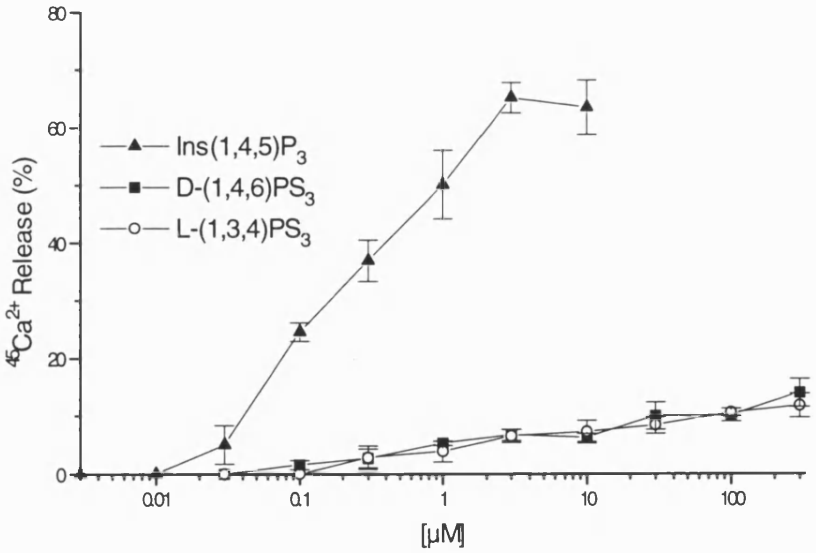


Figure 6.6 $^{45}Ca^{2+}$-release from permeabilised rabbit platelets induced by D-Ins(1,4,6)PS_3 and L-Ins(1,3,4)PS_3 (**L-59**).

A further study was carried out in which Ca^{2+}-release curves for Ins(1,4,5)P_3 in rabbit platelets were obtained in the presence of increasing concentrations of **L-59**. The results, shown in Figure 6.7 demonstrate that **L-59** shows the classical antagonist behaviour, causing a progressive shifting of the Ca^{2+}-release curves to the right as the dose is increased.

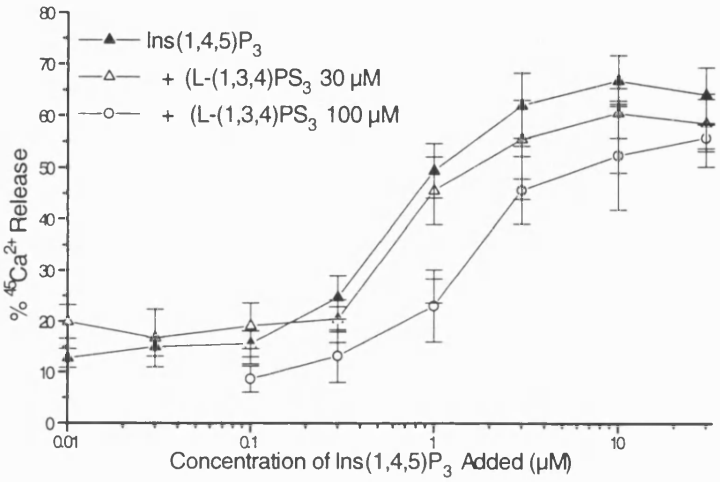


Figure 6.7 Progressive shifting of the dose-response curve for Ins(1,4,5)P_3 -induced $^{45}Ca^{2+}$ release by increasing concentrations of L-Ins(1,3,4)PS_3 (**L-59**).

6.4 6-Deoxy-6-hydroxymethyl-*scyllo*-inositol-1,2,4-trisphosphorothioate

As discussed in Section 6.3, D-Ins(1,4,6)PS_3 is currently the most promising lead compound in the search for partial agonists at Ins(1,4,5)P_3 receptors. Its intrinsic efficacy is very low, and yet it also binds with high affinity to Ins(1,4,5)P_3 receptors, a property inherited from its parent compound, D-Ins(1,4,6)P_3. This suggested that a simple strategy for the design of a high affinity, low efficacy partial agonist might be to;

1) Identify a 3-position modified Ins(1,4,5)P_3 analogue that shows high affinity for the Ins(1,4,5)P_3 receptor, and then

2) Reduce its efficacy by replacing phosphates with phosphorothioates.*

Now the only difference between D-Ins(1,4,6)PS_3 and L-chiro-Ins(2,3,5)PS_3 is the orientation of one hydroxyl group, and yet this appears to lower the efficacy of D-Ins(1,4,6)PS_3 without reducing its affinity for the receptor. Ideally then, a candidate for a

* This strategy has since been shown to be valid. The 4,5-bisphosphorothioate of the highly potent D-3-deoxy-3-fluoro-*myo*-Ins(1,4,5)P_3 has recently been found to be a partial agonist in SH-SY5Y cells.[50]. If 3-deoxy-Ins(1,4,5)PS_3 itself is a partial agonist, then a xylose-based bisphosphorothioate conjugated with 2′-AMP (by analogy with the adenophostins) might be worthy of investigation.

partial agonist should also have this feature. Taken together these arguments, summarised in Figure 6.8, suggest that the phosphorothioate analogue **60** of the highly potent hydroxymethyl analogue **44** (Chapter 5) should be a promising candidate.

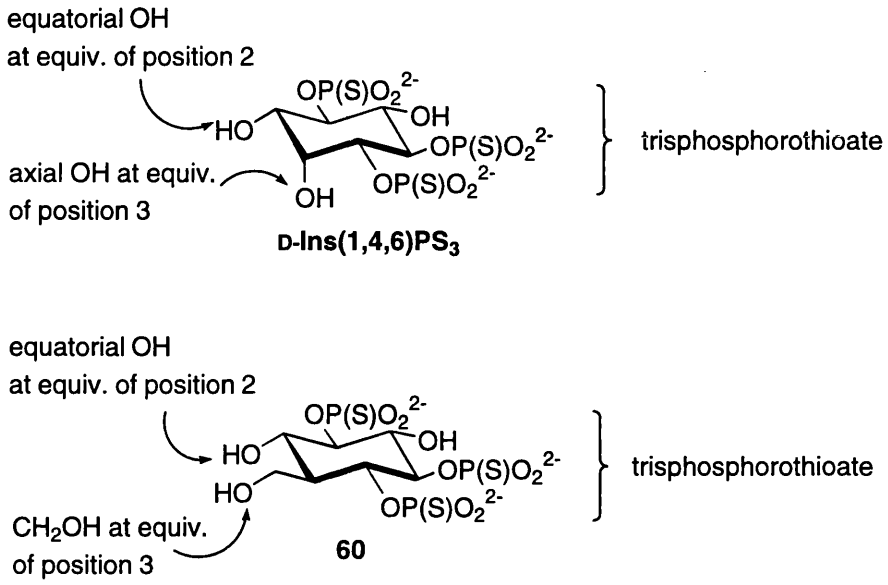


Figure 6.8 Structural considerations suggest that, by analogy with D-Ins(1,4,6)PS_3, the trisphosphorothioate **60** might also be a partial agonist.

Accordingly, racemic **60** was synthesised from the triol **46** (Figure 6.9). The structure of **60** was confirmed beyond doubt by ^{1}H-^{1}H COSY NMR, ^{31}P NMR and high resolution FAB mass spectrometry. The ^{1}H-coupled ^{31}P NMR is shown in Figure 6.10. Note that the heteronuclear $^{3}J_{HCOP}$ coupling constants are quite large (11.9 Hz, 12.6 Hz and 9.9 Hz). This seems to be a general feature in the ^{31}P NMR spectra of phosphorothioates. The signals occur at lower field than those of the corresponding trisphosphate as a result of the greater electronegativity of sulphur compared to oxygen.

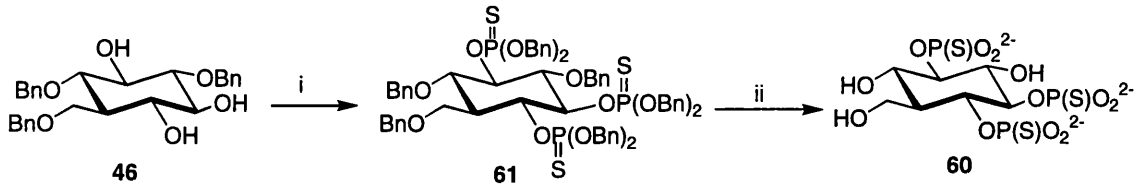


Figure 6.9 Synthesis of racemic 6-deoxy-6-hydroxymethyl-*scyllo*-inositol 1,2,4-trisphosphorothioate (**60**)
i) a) $(BnO)_2PNPr^{i}_2$, 1*H*-tetrazole, CH_2Cl_2 b) S_8; DMF/pyridine; ii) Na / liquid NH_3.

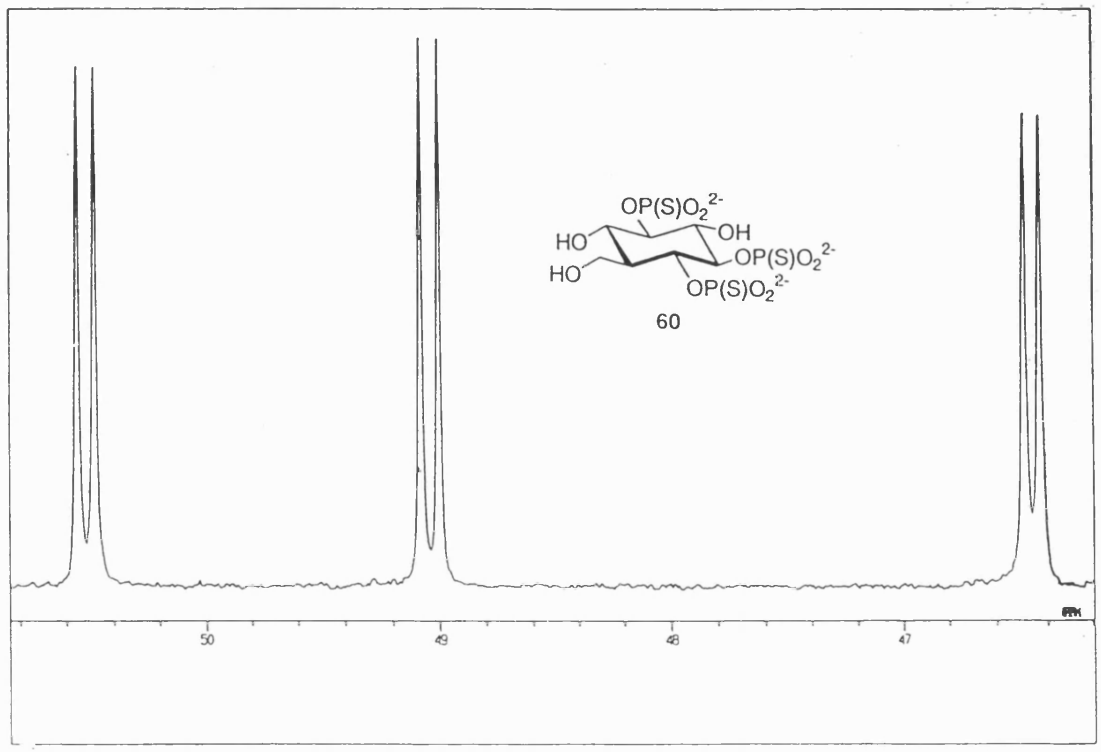


Figure 6.10 ^{31}P NMR spectrum of **60** (^{1}H-coupled, 162 MHz, D_2O).

6.4.1 Biological Evaluation

The ability of racemic trisphosphorothioate **60** to release $^{45}Ca^{2+}$ from permeabilised rabbit platelets was evaluated (Figure 6.11).

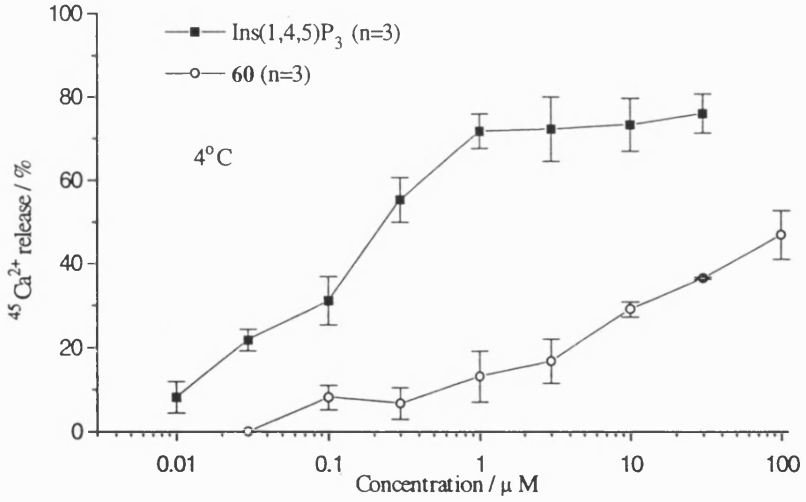


Figure 6.11 $^{45}Ca^{2+}$ release by racemic **60** from permeabilised rabbit platelets.

It appears that phosphorothioate substitution in **60** results in a dramatic reduction in ability to release Ca^{2+} (more than 500-fold), relative to the trisphosphate **44**. The

situation is very different from that found for Ins(1,4,5)P_3, whose trisphosphorothioate appears to be a full agonist and is only three to four times weaker than the parent compund.[93] It is not possible to say whether **60** behaves as a full agonist or not from these results, but it does appear to have higher efficacy than L-Ins(1,3,4)PS_3, in that it can be seen to be releasing more than half of the Ins(1,4,5)P_3 -sensitive Ca^{2+} pool at a concentration of 100μM. Whether the drastic reduction in the potency of **60** is reflected in a similar reduction in its affinity for the Ins(1,4,5)P_3 receptor awaits the results of [^{3}H]Ins(1,4,5)P_3 binding assays, but disappointingly, a preliminary assay appears to show that it does not inhibit Ins(1,4,5)P_3 -induced Ca^{2+}-release from rabbit platelets. Studies of the interaction of **60** with 3-kinase and 5/6-kinases are in progress.

6.5 Conclusions

The only low efficacy partial agonists at Ins(1,4,5)P_3 receptors discovered to date have been phosphorothioates. L-Ins(1,3,4)PS_3 can now be added to this small group of analogues, but D-Ins(1,4,6)PS_3, which has higher affinity for the Ins(1,4,5)P_3 receptor and yet maintains very low efficacy, is the most important lead compound. The behaviour of 6-deoxy-6-hydroxymethyl-*scyllo*-inositol-1,2,4-trisphosphorothioate (**60**) is perplexing. The parent compound **44** (Chapter 5) showed one of the highest binding affinities to the Ins(1,4,5)P_3 receptor of any Ins(1,4,5)P_3 analogue yet synthesised and yet replacement of the phosphate groups with phosphorothioates seems, at present, to have given only a very weak, high-efficacy agonist. It is not known why the presence of the hydroxymethyl group should affect the activity of a trisphosphorothioate in this way, but this finding may have implications for the design of adenophostin-based partial agonists.

Finally, it should be noted that L-Ins(1,3,4)PS_3 and D-Ins(1,4,6)PS_3 only appeared to behave as partial agonists at 4°C. The effect disappeared when the experiments were repeated at 20°C, and this finding underscores the reservations that were expressed in Chapter 1 about our criteria for partial agonism. One of the hallmarks of quantal Ca^{2+} release is that it disappears at low temperature,* and it has been argued that the phenomenon of quantal Ca^{2+} release may obscure partial agonist behaviour.[47]

* This effect is in accordance with the kinetics predicted from a model based on stochastic recombination of high- and low-affinity Ins(1,4,5)P_3 receptor subunits to give heterotetramers.[23]

7 *myo*-Inositol 1,3,4,5-tetrakisphosphate

7.1 Overview

Chapter 2 dealt with the synthesis and properties of the enantiomers of a naturally occurring inositol trisphosphate, Ins(1,3,4)P_3. Recall that there had been disagreements in the literature as to whether D-Ins(1,3,4)P_3 was active at Ins(1,4,5)P_3 receptors, and therefore controversy as to its biological role. It was shown that pure D-Ins(1,3,4)P_3 is essentially inactive, and it was suggested that studies finding otherwise had used contaminated Ins(1,3,4)P_3. Today there are few workers in this field who would claim that D-Ins(1,3,4)P_3 has any direct role in releasing intracellular Ca^{2+}.

In contrast, the debate surrounding another, structurally-related and naturally-occurring inositol phosphate, D-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] is currently growing more complex by the week, and this molecule will be the subject of this final chapter. There are now many suggested biological roles for Ins(1,3,4,5)P_4 and many putative Ins(1,3,4,5)P_4 binding proteins. There is also the question of whether Ins(1,3,4,5)P_4 is recognised by Ins(1,4,5)P_3 receptors, by only certain subtypes of Ins(1,4,5)P_3 receptors, or not recognised by Ins(1,4,5)P_3 receptors at all. As this thesis was nearing completion (April 1996) a new study was published claiming that, not only does Ins(1,3,4,5)P_4 *not* release Ca^{2+} at physiologically relevant levels, but that at least in mouse lachrymal acinar cells, Ins(1,3,4,5)P_4 or perhaps one of its metabolites, actually *inhibits* Ca^{2+} release, and therefore functions to turn *off* the Ins(1,4,5)P_3 signal.[51]

In this chapter we describe a new and rapid synthesis of both enantiomers of Ins(1,3,4,5)P_4, and some problems encountered relating to the purity and biological activity of the D-enantiomer. In contrast to the study of D-Ins(1,3,4)P_3, no firm conclusions can be given, because the compounds are still under biological evaluation, and the significance of the results is still being discussed. Clearer answers may be available in a few months time. We begin however with a synthesis of the previously unknown *scyllo*-inositol analogue of Ins(1,3,4,5)P_4.

7.2 Stereochemical Relationships of D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4

Various Ins(1,3,4,5)P_4 binding proteins have been identified in recent years, and some of these have been discussed briefly in Chapter 1. The beginnings of a structure-activity

relationship are being established for an Ins(1,3,4,5)P_4 binding protein purified from porcine platelets, and now designated GAP1^{IP4BP}. It has been reported[120] that at this site, phosphate groups at positions 3 and 5 of *myo*-inositol are essential for binding, but position 4 is not needed. A third phosphate in the 1-position enhances binding, but phosphorylation of the axial 2-position abolishes activity. Notice how different these proposed requirements (Figure 7.1) are from those of the Ins(1,4,5)P_3 receptor (Figure 1.11).

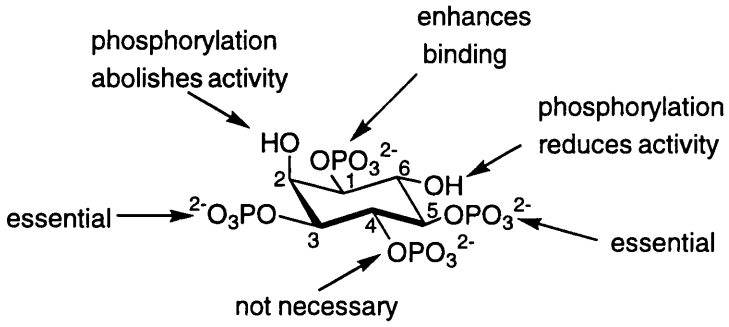
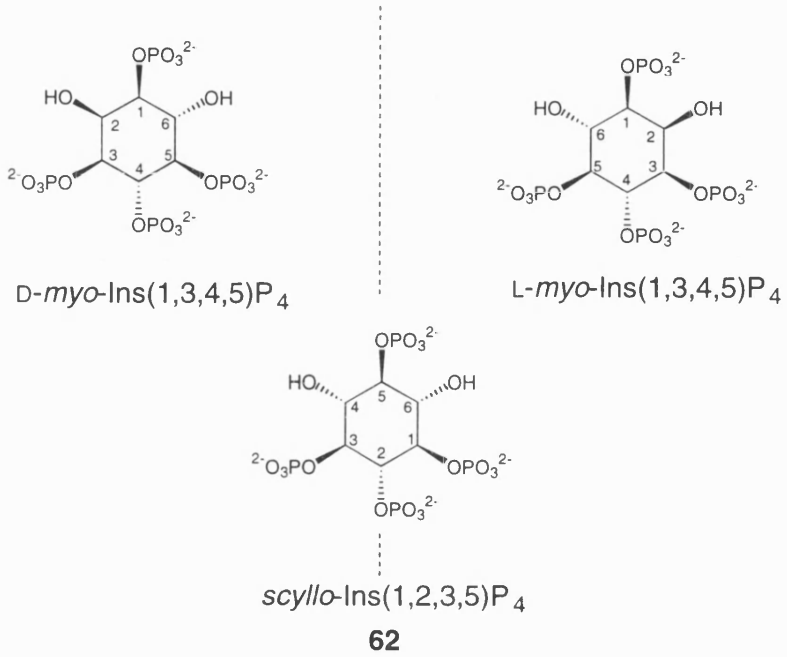


Figure 7.1 Proposed structure-activity relationships for GAP1^{IP4BP}

It is interesting to note that L-Ins(1,3,4,5)P_4 [129] appears to have only a 13-fold lower affinity for GAP1^{IP4BP} than D-Ins(1,3,4,5)P_4.[119] This is very different from the interaction of Ins(1,4,5)P_3 with Ins(1,4,5)P_3 receptors, where L-Ins(1,4,5)P_3 has at least 1000 times lower affinity than its enantiomer.[111] The explanation for this finding may simply be that L-Ins(1,3,4,5)P_4 is more similar to D-Ins(1,3,4,5)P_4 than L-Ins(1,4,5)P_3 is similar to D-Ins(1,4,5)P_3. D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4, although enantiomers, have identical arrangements of phosphate groups. L-Ins(1,3,4,5)P_4 could therefore be regarded as an analogue of D-Ins(1,3,4,5)P_4, in which the orientations of the hydroxyl groups at positions 2 and 6 have been interchanged (Figure 7.2). This is analogous to the relationship between D-Ins(1,4,5)P_3 and L-Ins(1,3,4)P_3, and from the point of view of a binding protein, must surely be more significant than the fact that D- and L-Ins(1,3,4,5)P_4 are related to one another as an object is to its mirror image. Thus the concept of some "stereospecificity" as a property of the binding protein, and the notion that the Ins(1,3,4,5)P_4 receptor may be "less stereospecific" than the Ins(1,4,5)P_3

receptor, without a consideration of the symmetry properties of the phosphate configurations in their respective ligands, may be misleading.*



62

Figure 7.2 Stereochemical interrelationships of D-Ins(1,3,4,5)P_4, L-Ins(1,3,4,5)P_4 and *scyllo*-Ins(1,2,3,5)P_4 (**62**).

To-date, all the inositol phosphates evaluated at Ins(1,3,4,5)P_4 binding sites have been of the *myo*-configuration, possessing an axial group at the 2-position. The *scyllo*-inositol analogue of Ins(1,3,4,5)P_4 (strictly *scyllo*-inositol 1,2,3,5-tetrakisphosphate, **62**, Figure 7.2), in which this group is equatorial, will allow the importance of this feature to be explored at the various putative Ins(1,3,4,5)P_4 binding sites. The *meso*-compound *scyllo*-Ins(1,2,3,5)P_4 can be seen as an analogue of both D- and L-Ins(1,3,4,5)P_4. It is epimeric with both, but because it possesses a plane of symmetry it is identical with its enantiomer. Thus D-Ins(1,3,4,5)P_4 , L-Ins(1,3,4,5)P_4 and *scyllo*-Ins(1,2,3,5)P_4 all possess the structural elements identified as important for $GAP1^{IP4BP}$ binding as shown in Figure 7.1, but the different affinities of the D- and L-enantiomers show that hydroxyl group orientations must also be important, as they are at the Ins(1,4,5)P_3 receptor (Chapter 2). In *scyllo*-Ins(1,2,3,5)P_4, only the orientation of the 2-hydroxyl group is changed, and it

* In opposition to this argument, it might be pointed out that L-Ins(1,3,4,5)P_4 has been reported to be almost inactive in releasing $^{45}Ca^{2+}$ from permeabilised SH-SY5Y cells, while D-Ins(1,3,4,5)P_4 appeared to be only 20-fold weaker than Ins(1,4,5)P_3.[111] Thus D-Ins(1,3,4,5)P_4 seems to be a more potent agonist at the Ins(1,4,5)P_3 receptors of SH-SY5Y cells than would be predicted from the arguments given above.

will be interesting to examine the effect of this single modification on GAP1^{IP4BP} binding.

D-Ins(1,3,4,5)P_4 is also recognised with high affinity by the enzyme multiple inositol polyphosphate phosphatase (MIPP) which removes its 3-phosphate group, generating Ins(1,4,5)P_3, and this has sometimes caused problems in establishing the true effects of Ins(1,3,4,5)P_4 (Chapter 1). Whether the adjacent 2-hydroxyl group of Ins(1,3,4,5)P_4 is involved in the mechanism of hydrolysis is not known, but it may be enlightening to establish whether *scyllo*-Ins(1,2,3,5)P_4 is recognised by MIPP, and if so, whether it can be dephosphorylated by this enzyme. Similarly, the effect of 5-phosphatase on *scyllo*-Ins(1,2,3,5)P_4 should be studied, as inactivation of Ins(1,3,4,5)P_4 by 5-phosphatase, which dephosphorylates it to Ins(1,3,4)P_3 is also a problem in studies of Ins(1,3,4,5)P_4.

7.3 Synthesis of *scyllo*-Inositol 1,2,3,5-tetrakisphosphate

The symmetry of *scyllo*-Ins(1,2,3,5)P_4 makes it a particularly accessible analogue of Ins(1,3,4,5)P_4 synthetically, in that neither a stereospecific synthesis nor an optical resolution is required. The synthesis (Figure 7.3) began with the versatile ketone **29** (Chapter 4), which was reduced rapidly and stereoselectively with sodium borohydride to the *scyllo*-inositol orthoformate derivative **63** in 89% yield.

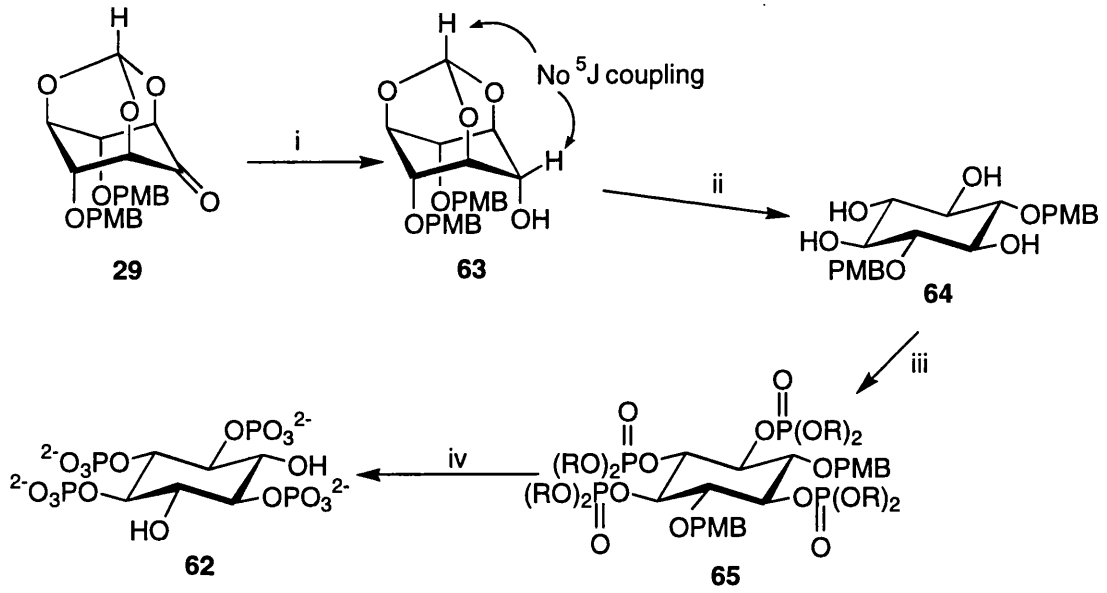


Figure 7.3: i) $NaBH_4$ / MeOH / THF; ii) a) 1M HCl / MeOH 1:10, Δ; b) aqueous NH_3; iii) a) $Pr^i_2NP(OR)_2$, 1*H*-tetrazole, CH_2Cl_2 b) *m*-CPBA, -78°C iii) Na/liquid NH_3, -78°C. PMB = *p*-methoxybenzyl, R=CH_2CH_2CN.

The fact that the product had the *scyllo*-configuration was immediately apparent from the ^{1}H NMR spectrum of **63**, because the signal corresponding to the methylidene proton was a singlet, while in the epimeric *myo*-analogue **28** (Chapter 4) it is doubled, due to a long-range spin coupling. The axial OH proton resonated at low field (δ 4.12, $CDCl_3$) and showed a very large $^3J_{HCOH}$ value of 12.45 Hz. Selective cleavage of the orthoformate ester by mild acid hydrolysis gave the tetrol **64** in 68% yield.* Phosphitylation with bis(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphine / 1*H*-tetrazole, followed by oxidation of the intermediate tetrakisphosphite triester with *m*-CPBA gave the fully-protected **65** (85%). Deprotection using sodium in liquid ammonia caused some problems in that the *p*-methoxybenzyl protecting groups proved more resistant to cleavage by this method than the more usual benzyl groups. This was apparent during the purification process by ion exchange chromatography as the target compound, which contains no chromophore, appeared to show some UV absorbance as it eluted from the ion-exchange column. A ^{31}P NMR spectrum showed small impurity peaks, and the ^{1}H NMR spectrum showed small but characteristic signals due to the *p*-disubstituted aromatic ring protons. The deprotection reaction was therefore repeated, but left for 5 minutes rather than the usual 2 minutes before quenching. This time, deprotection was complete and pure **62** was obtained as the triethylammonium salt (71%) giving a 37% overall yield from **29**.

Scyllo-Ins(1,2,3,5)P_4 is intended as a probe for Ins(1,3,4,5)P_4 binding sites, rather than Ins(1,4,5)P_3 receptors, but its ability to mobilise intracellular Ca^{2+} in SH-SY5Y neuroblastoma cells has now been examined. **62** Appears to be some four-fold weaker than commercially available D-Ins(1,3,4,5)P_4. The significance of these results is not yet clear, because the true activity of D-Ins(1,3,4,5)P_4 is still in question, as will be seen later in this Chapter. A study of the interaction of **62** with GAP1^{IP4BP} is now underway. **62** Showed potent ability to inhibit phosphorylation of Ins(1,3,4)P_3 by the 5/6-kinase enzyme as might be expected, because three of its phosphate groups can be superimposed upon those of D-Ins(1,3,4)P_3 (Figure 7.4).

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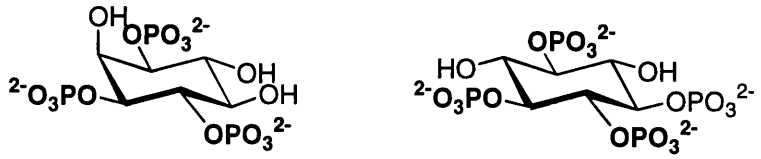
* These two steps were carried out by a final-year undergraduate project student, who also converted compound **63** into its tri-*O*-*p*-methoxybenzyl ether. The fact that the NMR spectra of this compound showed *three*-fold symmetry was irrefutable evidence that **63** had the *scyllo* configuration.

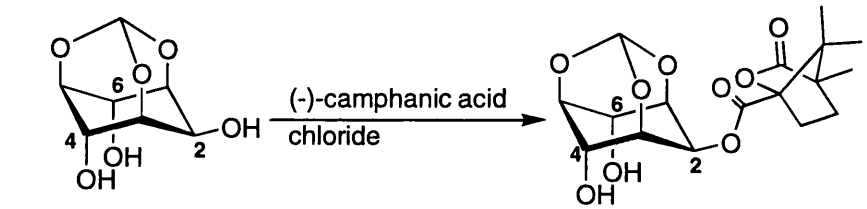


D-Ins(1,3,4)P_3 *scyllo*-Ins(1,2,3,5)P_4

Figure 7.4 *scyllo*-Ins(1,2,3,5)P_4 may be recognised by Ins(1,3,4)P_3 5/6-kinase because it is capable of mimicking certain aspects of the natural substrate Ins(1,3,4)P_3

7.4. A Short Route to D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4

As part of an investigation into long-range ^{31}P-^{31}P spin-spin coupling in the NMR spectra of phosphite triesters,[248] an orthoformate ester of *myo*-inositol was required in which the two axial hydroxyl groups were chemically inequivalent. Thus it was necessary to break the symmetry of the molecule. It was thought that this might be achieved by reacting *myo*-inositol orthoformate with 1.1 equivalents of (*1S*)-(−)-ω-camphanic acid chloride in pyridine with a catalyst of DMAP. The expectation was that the 2-camphanate ester **66** would be the major product, and that this would be sufficiently dissymmetric to make possible the direct observation of any spin-spin coupling between phosphite esters formed at positions 4 and 6.



myo-inositol orthoformate
Symmetrical

66
Asymmetrical

Figure 7.4a Reaction of the symmetrical *myo*-inositol orthoformate with 1 equivalent of (1*S*)-(−)-ω-camphanic acid chloride gives an unsymmetrical ester.

The reaction was carried out and when the major product was isolated, it was indeed found to be the 2-camphanate ester. The position of substitution was established beyond doubt by removing the orthoformate ester and obtaining a ^1H NMR of the product **66a** (Figure 7.5). In this molecule, the proton at position 2 is easily identified by its distinctive splitting pattern (a narrow triplet or strictly, a narrow doublet of doublets), and in **66a** this signal was shifted well downfield. Compound **66** was therefore used for the planned NMR study.

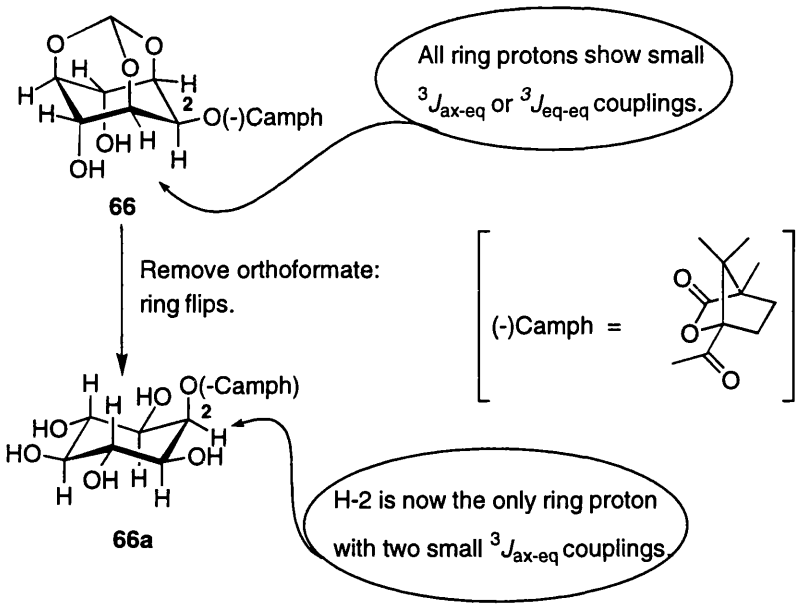


Figure 7.5 Confirmation of the structure of the 2-camphanate **66** by ^{1}H NMR.

The TLC also showed two minor products, close together at longer R_f. These were isolated and shown to be the 2,6-biscamphanate and the 2,4-biscamphanate. It was impossible to tell which was which by NMR. None of the 4,6-disubstituted product was detected.

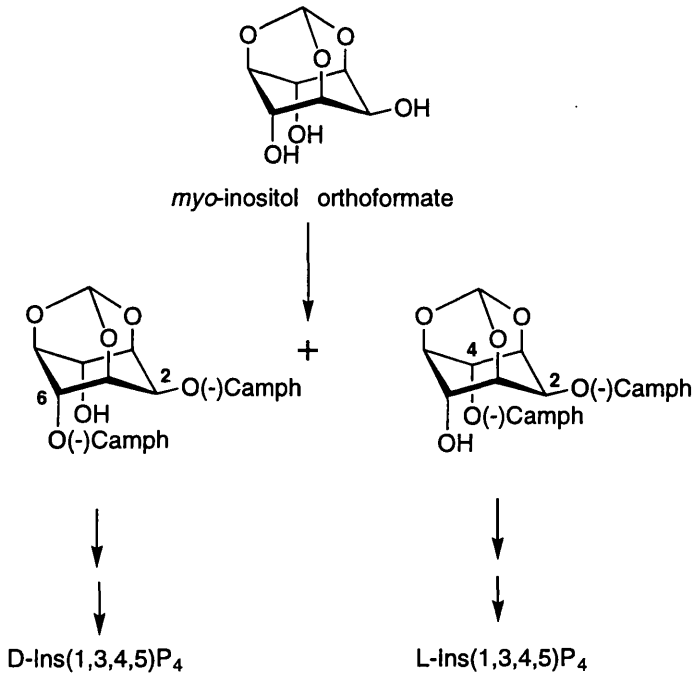


Figure 7.6 Reaction of *myo*-inositol orthoformate with 2 equivalents of (1*S*)-(−)-ω-camphanic acid chloride gives mainly the 2,6- and 2,4-biscamphanate esters, which are precursors for D- and L-Ins(1,3,4,5)P_4 respectively.

Note that this situation is quite different to the *alkylation* of *myo*-inositol orthoformate (Chapter 4) in which the disubstituted product was exclusively the 4,6-di-*O*-*p*-methoxybenzyl ether, with no 2,4- or 2,6- disubstituted ethers detectable. It was quickly realised that the biscamphanates could provide a short and large-scale route to the enantiomers of Ins(1,3,4,5)P_4 (Figure 7.6).

7.5 Synthesis of D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4

Several syntheses of D-Ins(1,3,4,5)P_4 have been published, although many are long or employ special techniques, such as the use of enzymes. Two examples were described in Chapter 1. The inconsistencies in the reports of the activity of D-Ins(1,3,4,5)P_4 in releasing Ca^{2+} might lead us to suspect that some samples were not pure and indeed, a study published by Gawler *et al.* in 1990 [113] showed that when commercially available samples of D-Ins(1,3,4,5)P_4, from more than one source, were treated with 3-kinase, their activity in SH-SY5Y neuroblastoma cells was markedly decreased. This is strong evidence that these samples contained Ins(1,4,5)P_3.

The unnatural enantiomer, L-Ins(1,3,4,5)P_4 is increasingly being used as a biological tool in studies of D-Ins(1,3,4,5)P_4. [111, 114, 119] There is only one reported synthesis of L-Ins(1,3,4,5)P_4 ,[129] and to the best of our knowledge, all the material used has come from this source. It is agreed that L-Ins(1,3,4,5)P_4 has only low activity at Ins(1,4,5)P_3 receptors (which would be expected from the structure-activity relationships outlined in Figure 1.11) and yet has moderate affinity for Ins(1,3,4,5)P_4 receptors. Therefore, it is argued, effects induced by L-Ins(1,3,4,5)P_4 must be mediated by the Ins(1,3,4,5)P_4 receptor. This strategy has been used to distinguish putative Ins(1,3,4,5)P_4 binding sites from Ins(1,4,5)P_3 receptors but has led workers to rather different conclusions. Wilcox *et al.*[111] have argued that, because D-Ins(1,3,4,5)P_4 caused Ca^{2+} release, but L-Ins(1,3,4,5)P_4 did not then D-Ins(1,3,4,5)P_4 must cause Ca^{2+} release *via* Ins(1,4,5)P_3 receptors. In contrast, Loomis-Husselbee *et al.*[114] claim that both D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4 can induce Ca^{2+} release by acting synergistically with Ins(1,4,5)P_3, * but that, because both enantiomers have this synergistic effect with Ins(1,4,5)P_3, this effect must be mediated by Ins(1,3,4,5)P_4 receptors.

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* D-Ins(2,4,5)P_3, which is active at Ins(1,4,5)P_3 receptors but 10-fold less so than Ins(1,4,5)P_3 , was used instead of Ins(1,4,5)P_3 in this study so as to obviate the possibility of conversion to Ins(1,3,4,5)P_4.

Whatever the validity of these biological arguments, it is clear that such studies require a reliable source of both D- and L-Ins(1,3,4,5)P_4, which must be optically pure and free from contamination with other, potentially active, inositol phosphates. A synthesis of both enantiomers using the strategy outlined in Figure 7.6 might achieve this in the minimum number of synthetic steps.

7.5.1 Absolute Configurations of Biscamphanates: X-Ray Study

The esterification reaction was repeated, using 2.2 equivalents of *S*-(−)-ω-camphanic acid chloride. Now, as expected, the major products were the two biscamphanates. These two diastereoisomers were quite distinct in their physical properties. The less polar diastereoisomer (minor product) was easily recrystallised from ethyl acetate/hexane, whereas the more polar major product showed low solubility in all but the most polar solvents (DMF, acetonitrile, pyridine, but not water). It was eventually found that this isomer could be recrystallised easily from DMF/water. In order to find out which biscamphanate was which an X-ray crystal structure would be required or failing this, it would be necessary to convert one of them to a known reference compound.

The crystals of the less polar diastereoisomer appeared to be the better candidates for X-ray analysis, and so larger crystals were grown slowly from a solution in propan-2-ol. The crystals proved to be of a suitable type and an X-ray study was carried out. The X-ray crystal structure (Figure 7.7a) showed this compound to be the 1D-2,6-biscamphanate ester of *myo*-inositol orthoformate **67a**. Within the rigid adamantane-like cage structure all C-C bond lengths were in the range 1.502-1.538 Å and all C-O bond lengths between 1.394 and 1.448 Å, with bond angles ranging from 105.8° to 114.3°. An examination of the supramolecular structure of **67a** (Figure 7.7b) revealed that the lattice was dominated by one-dimensional linear polymers as a result of intermolecular hydrogen-bonding between the free hydroxyl groups and the lactone carbonyl oxygens. This interaction probably accounts for the high crystallinity of this material compared to diastereoisomer **67b**. For full details, see Appendix 2.

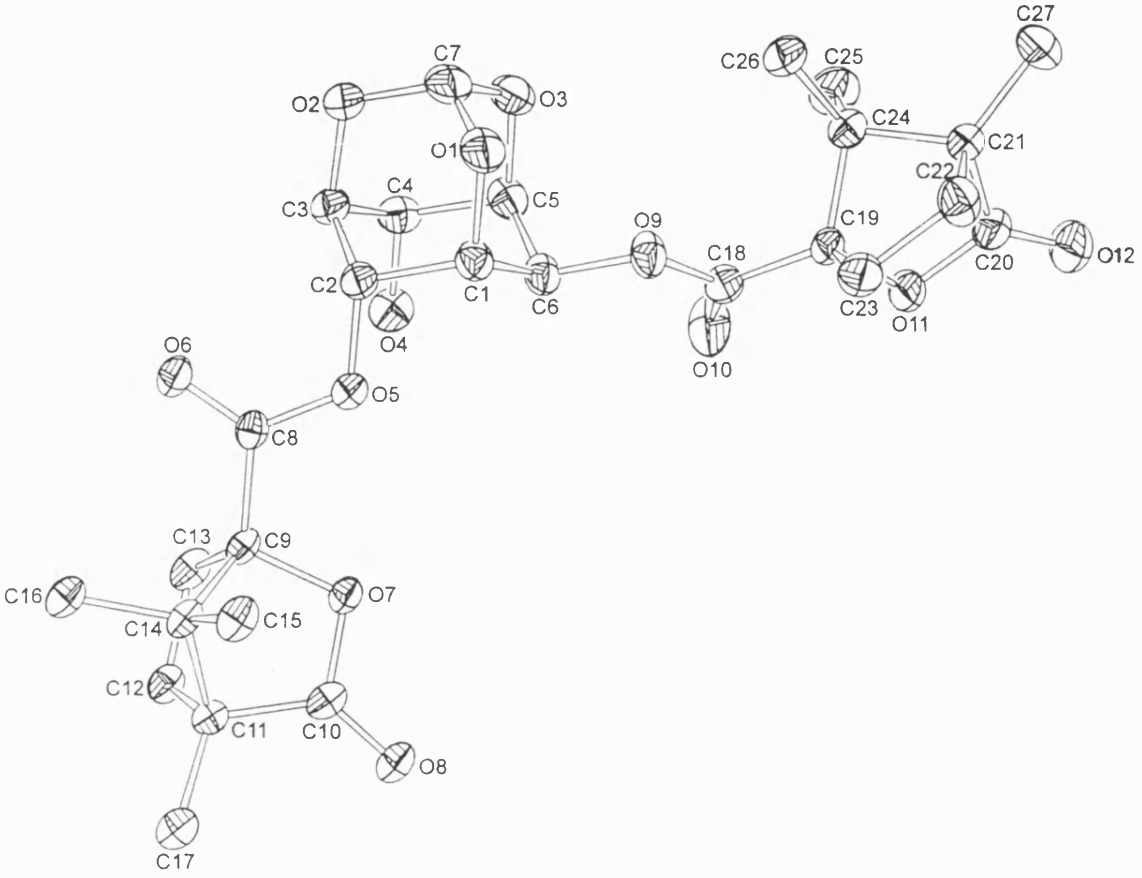
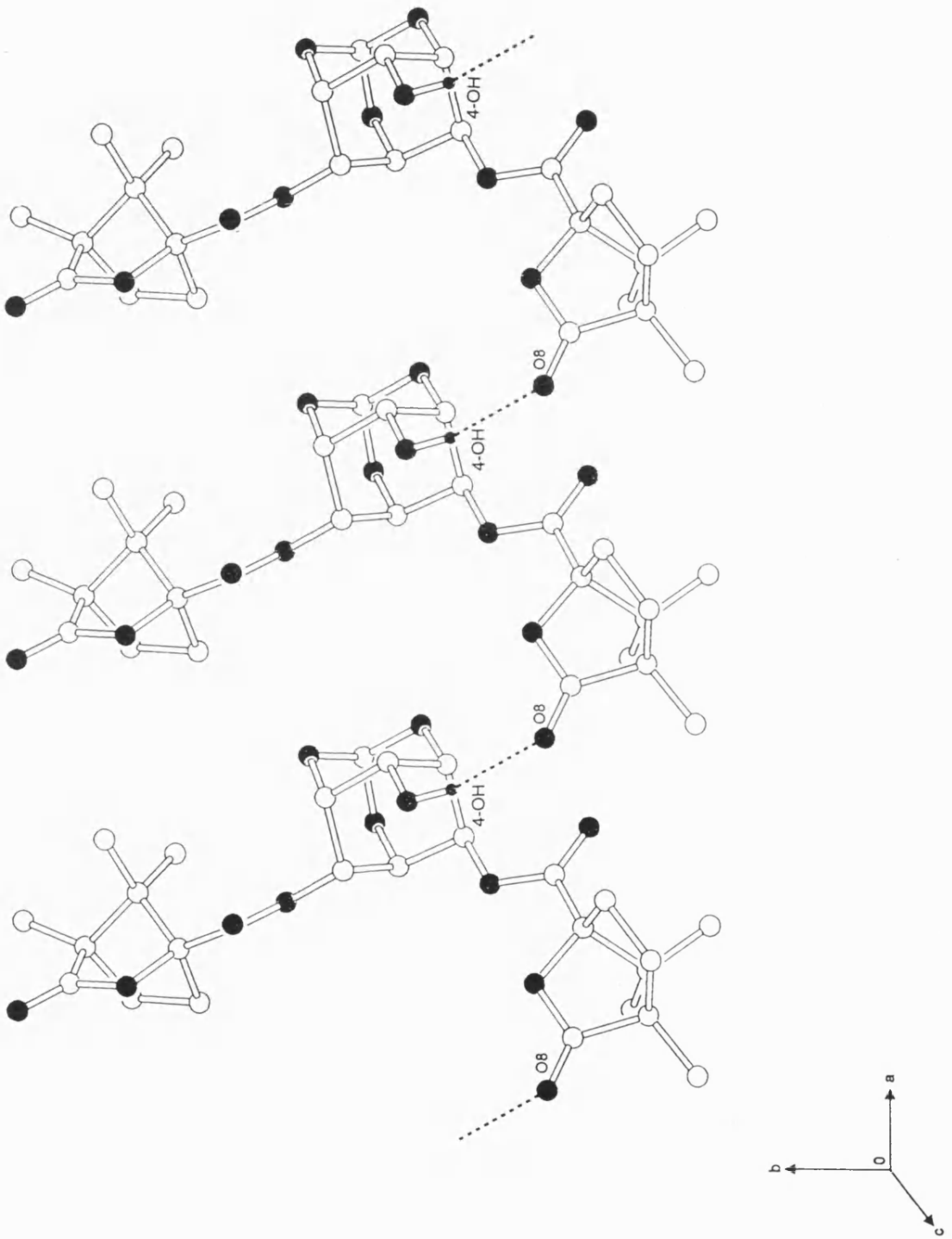


Figure 7.7a X-ray crystal structure of the 1D-2,6-biscamphanate ester **67a**. Note: the numbering of atoms used in this representation is not the same as that used in the rest of this thesis.

7.5.2 Optimising the Yield of the 2,6-Biscamphanate

Diastereoisomer **67a** was therefore a synthetic precursor to D-Ins(1,3,4,5)P_4, and the more polar, major product **67b** would provide L-Ins(1,3,4,5)P_4. It seemed likely that demand for the natural D-enantiomer of Ins(1,3,4,5)P_4 would exceed that for the L-enantiomer, and so attempts were made to alter the selectivity of the esterification reaction by using different conditions.



7.7 b) The supramolecular structure of **67a** shows intermolecular hydrogen bonding between the 4-hydroxyl groups and lactone carbonyl oxygens, giving a one-dimensional, linear polymeric structure.

The reaction was repeated, replacing the pyridine with dichloromethane, together with a small amount of triethylamine to act as base. Again, DMAP was used as the catalyst. This worked well, and although *myo*-inositol orthoformate is only very slightly soluble in dichloromethane the reaction went smoothly, with the suspended orthoformate rapidly disappearing as esterification progressed. The reaction was complete within half an hour, and this time, TLC showed that the major product was the desired 2,6-biscamphanate, with a reduced amount of 2,4-ester and almost no monocamphanate. However significant amounts of the unwanted 2,4,6-triscamphanate were now produced.

The reaction was repeated a number of times, using various amounts of camphanic acid chloride and adjusting the volume of solvent and the temperature. Optimised conditions provided a respectable 60% yield of the 2,6-biscamphanate after flash chromatography. Note that the yield of this regioselective esterification exceeds the maximum possible theoretical yield (50%) that could be obtained by a conventional optical resolution of a racemate (e.g. in the synthesis of D- and L- Ins(1,3,4)P_3 , Chapter 2). The 2,4-disubstituted product was obtained in 23% yield. It was found that no work-up was needed, and these yields could be achieved with minimal effort by simply removing the solvents in a rotary evaporator and purifying the crude residue using flash chromatography. Careful recrystallisation of the two products at this stage was carried out to increase their optical purity further.

A 400 MHz COSY spectrum of the 2,6-biscamphanate **67a** is shown in Figure 7.8. Having established the absolute configuration, it was now possible to assign the spectrum completely. Note the narrow signals corresponding to the ring protons, which have only small vicinal couplings, and the long-range ^5J coupling between H-1 and H-3. This interaction is not seen directly in the spectra of any of the symmetrical inositol orthoformate derivatives (Chapter 4) because in these compounds H-1 and H-3 are enantiotopic, and therefore isochronous. The small (0.98 Hz) long-range coupling between the methylidine proton (δ 5.54) and H-2 (δ 5.30) is not seen as a cross-peak in this spectrum, but is clearly resolved in the one-dimensional spectrum (Figure 7.8b).

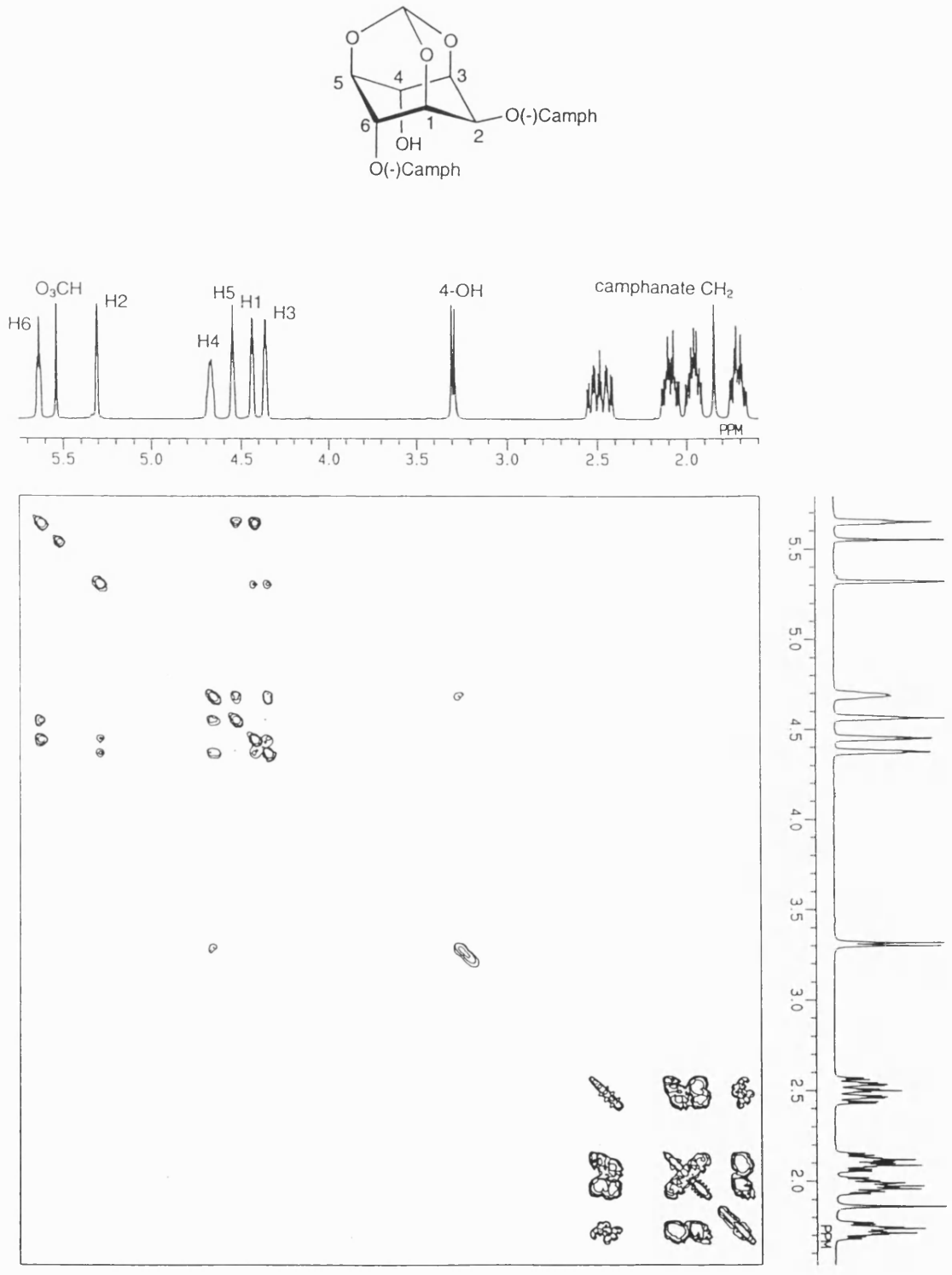


Figure 7.8 a) Part of the ^{1}H-^{1}H COSY NMR spectrum of the 1D-2,6-biscamphanate **67a** ($CDCl_3$, 400 MHz).

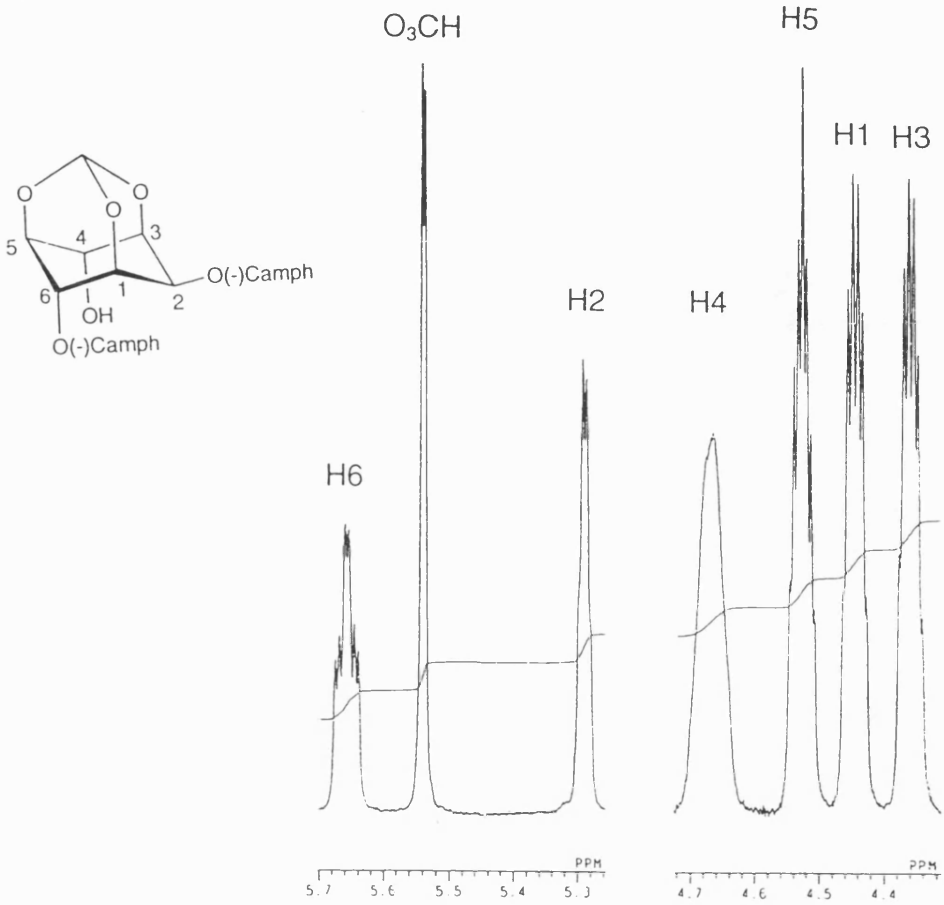


Figure 7.8b An expansion of the part of the 270 MHz ^{1}H NMR spectrum of **67a**, shows complex, narrow signals with splittings caused by long-range couplings. Note that the methylidine proton signal (δ 5.54) is a narrow doublet, and couples to H-2.

7.5.3 Removal of Orthoformate Esters and Phosphitylation/Oxidation

It was now necessary to remove the orthoformate esters, while leaving the camphanate esters, with their lactone rings, intact. This was found to be straightforward for the 2,6-biscamphanate, and was carried out using a 1 to 10 refluxing mixture of 1M HCl and methanol, without significant loss or migration of camphanate groups. The use of higher concentrations of acid was not successful, causing extensive loss of camphanates.

The 2,4-biscamphanate (which would lead to L-Ins(1,3,4,5)P_4) posed much more of a problem. Although it was only slightly soluble in methanol or ethanol, it was thought that this would not preclude the use of the usual HCl/methanol or HCl/ethanol conditions. This was not the case, and almost no reaction was detectable after 5 to 6 hours of reflux. Increasing the concentration of acid only caused loss of camphanate

esters. The reaction was next attempted in refluxing 80% acetic acid, and this strategy was more successful. After 7 hours, most of the starting material was consumed and a clear solution was formed. However, the conversion was still not complete, with significant quantities of at least one intermediate formate ester being visible on the TLC. The reaction seemed to progress no further after this stage and the yield of tetrol was low.

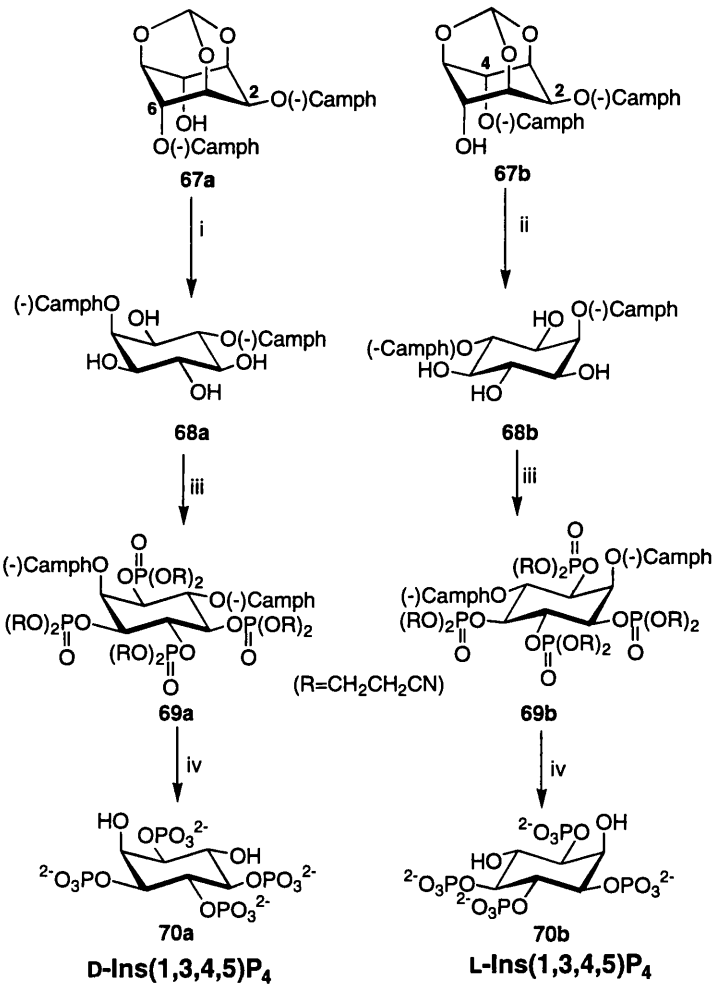


Figure 7.9 Synthesis of D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4 from *myo*-inositol orthoformate biscamphanates **67a** and **67b**. i) 1M HCl/MeOH 1:10, reflux (88%); ii) TFA/H_2O 4:1, 40h (85%); iii) a) $Pr^i_2NP(OCH_2CH_2CN)_2$, 1*H*-tetrazole, b) *m*-CPBA,78°C (80-84%) iv) Conc. NH_3 solution, 60°C, 6 h (90-93%).

Another method of removing the orthoformate ester is to use 80% TFA at room temperature. This method had been avoided because it had previously been found to give significant amounts of formate esters, although these can be removed in a second stage, usually by stirring with methanolic ammonia.[129] However, as other methods had

failed, the reaction was attempted. The 2,4-biscamphanate was, encouragingly, very soluble in 80% TFA, and the reaction was left at room temperature for 22 hours. TLC after this time showed that the reaction was not complete, and although the starting material had mostly been consumed, two intermediates, presumably formate esters were still present. This partial cleavage of an orthoester in compounds such as **67a** and **67b** could provide a rapid route to Ins(1,4,5)P_3 or other chiral trisphosphates, and this prospect is now under investigation by a colleague. As expected, treatment with ammonia solution, even for a short time resulted in loss of the camphanate groups.

Finally it was found that **67b** could be deprotected most successfully by simply stirring in 80% TFA at room temperature for a longer time. After 40 hours, the reaction was almost complete, with little formate remaining. This could easily be removed by flash chromatography and the pure tetrol **68b** was at last obtained, in 85% yield.

Both of the diastereoisomeric tetrols **68a** and **68b** could easily be recrystallised (from propan-2-ol and DMF/water respectively). Note that, with a conventional optical resolution, the chiral resolving agent is normally removed immediately after the resolution step. The intermediates subsequent to this stage are therefore *enantiomeric*, and contamination with the unwanted enantiomer will not be apparent by NMR or TLC. Nor will chromatography give any increase in optical purity. At each step in this synthesis, the precursors to D-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4 were *diastereoisomeric,* having very different NMR spectra and different R_fs. Any contamination would therefore be obvious, especially in the camphanate methyl resonances of the ^{1}H NMR spectra. No such contamination was visible in any of the NMR spectra.

The next stage was phosphitylation/oxidation, which was carried out without problems using bis(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphine / 1*H*-tetrazole followed by *m*-CPBA. This phosphitylating agent was chosen because the cyanoethyl protecting groups can be removed by alkaline hydrolysis and so could be removed together with the camphanate esters in a single deprotection step. It would eventually become clear that this choice was a serious mistake (see below).

The two intermediate tetrakisphosphite triesters, gave quite different ^{31}P NMR spectra but both showed the expected five-bond couplings between the P(III) atoms of

the vicinal phosphites and the values of $^{5}J_{PP}$ differed in magnitude for the two intermediates. This characteristic pattern of a triplet (P4), two doublets (P3 and P5) and a singlet (P1) in each confirmed the 1,3,4,5 arrangement of phosphites.

Oxidation gave the fully-protected tetrakisphosphate triesters **69a** and **69b**. Again it is important to remember that these molecules are diastereoisomeric, as was apparent from their very different ^{31}P , ^{13}C, and ^{1}H NMR spectra. The ^{1}H NMR spectrum reconfirmed the substitution pattern, with the characteristic signals for H-2 and H-6 in compound **69a**, and for H-2 and H-4 in compound **69b**, being shifted well downfield away from all other resonances. The ^{13}C NMR spectra of both compounds were complicated by extensive ^{31}P - ^{13}C couplings. Both compounds took the form of brittle white foams, so no further recrystallisations were possible, but their NMR spectra testified to their chemical and optical purity.

7.5.4 Deprotection: Problems with Phosphate Migration

A small-scale trial established that total deprotection of **69a** could easily be achieved by heating at 60°C in concentrated aqueous ammonia solution in a sealed container for 3 hours. A ^{1}H-coupled ^{31}P NMR of the product showed four clear doublets, confirming that the deprotection was complete. When the reaction was scaled up, the main problem was one of how to carry out this reaction safely, and the solution adopted was to carry out the reaction in a thick-walled, 100mL glass autoclave bottle with a screw-cap. The foam (2g of **69a**) was compressed into a fine powder, which was transferred to the bottle, followed by the ammonia solution and a magnetic stirring bead. The bottle was sealed and lowered into a water bath with a thermostatically-controlled heater-stirrer behind a safety screen. The thermostat was set to 60°C, and with the screen safely in place and the fume-cupboard closed, the heater stirrer was switched on. After the temperature had reached 60°C, heating was continued for 6 hours, and at the end of the reaction, the bottle was allowed to cool fully, before opening.

The product would now be in the form of the ammonium salt together with the hydrolysis products from the camphanate groups. A previous trial of the deprotection method using only ethyl camphanate and ammonia solution had established that the hydrolysis product, which was water-soluble, could be removed by acidification followed by extraction into dichloromethane or ether. This strategy was successfully

employed, with Dowex-50 H^+ resin being used to reduce the pH to ~2, prior to extraction. The aqueous layer was then titrated to pH 10 with 1M KOH. A final purification stage, designed to remove any excess KOH, was to re-dissolve the product in a small volume of de-ionised water and then add methanol, thus precipitating the potassium salt of D-Ins(1,3,4,5)P_4 , and leaving residual KOH in solution. This stage also served to remove a slight brownish colour which had originated from the Dowex resin. The precipitate was dissolved once more in de-ionised water and lyophilised to give the potassium salt of D-Ins(1,3,4,5)P_4 in 94% yield.

A smaller quantity of **69b** was deprotected in exactly the same way to give L-Ins(1,3,4,5)P_4 (potassium salt) in 90% yield. The ^1H and ^{13}C NMRs of the two enantiomers **D-70a** and **L-70b** were identical and fully agreed with the previously published NMR data[168]. The 400 MHz ^1H NMR spectrum of **L-70b** and the corresponding 100 MHz ^{13}C NMR spectrum are shown in Figure 7.10.

However, the 162 MHz ^{31}P NMR spectra did show some slight impurity in each case although this was not seen at lower field. It was not clear at this stage how the impurities arose, as the precursors **69a** and **69b** appeared pure by high field ^{31}P NMR. The potassium salts are not highly crystalline, tending instead to precipitate as fine or fibrous amorphous material from a number of solvent systems, and no improvement could be obtained by recrystallisation. A portion of the D- enantiomer was converted to both the ammonium and the cyclohexylammonium salts, and although the latter could be crystallised (with some difficulty), the impurity seen in the ^{31}P NMR persisted. These attempts at purification, involving conversion to free acid, titrations and various attempts at crystallisations, occupied many weeks. High field NMR spectra had to be obtained after each attempt, each time adjusting pH to separate the signals, and often the sample had to be treated to remove paramagnetic ions (with Chelex-100 resin or EDTA), meaning that it could not be re-used. Time was running out, the originally large supply of D-Ins(1,3,4,5)P_4 was dwindling, and the impurity would not go.

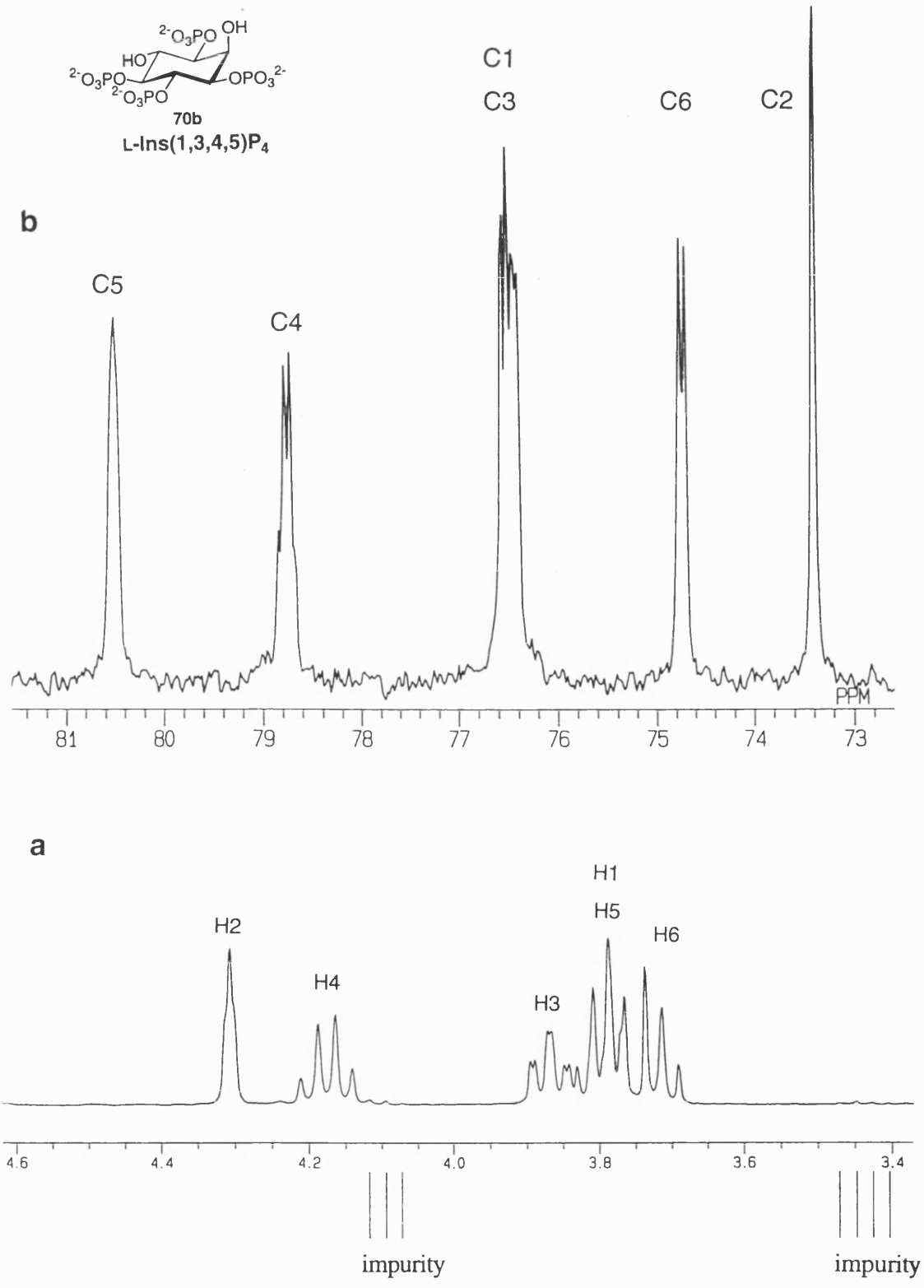


Figure 7.10 **a)** 400MHz ^{1}H NMR spectrum and **b)** 100 MHz ^{13}C NMR spectrum of L-Ins(1,3,4,5)P_4 (potassium salt, D_2O, pH 8-9).

7.5.5 Preliminary Biological Testing

The slightly impure potassium salts of D- and L-Ins(1,3,4,5)P_4 were evaluated for Ca^{2+} release from permeabilised SH-SY5Y cells. As expected, the L-Ins(1,3,4,5)P_4 proved to be weak (results not shown), and was able to release only about 37% of the Ca^{2+}-pool at a concentration of 100μM. The D-Ins(1,3,4,5)P_4 proved to be almost identical in its effect to the commercially available material, that is, around forty times weaker than Ins(1,4,5)P_3. Interestingly, *scyllo*-Ins(1,2,3,5)P_4 was considerably less active. Now as discussed above, there has been much controversy as to whether D-Ins(1,3,4,5)P_4 can mobilise Ca^{2+} *via* Ins(1,4,5)P_3 receptors, and this has caused concern as to the purity of the D-Ins(1,3,4,5)P_4 used. In this case, it was *known* that the D-Ins(1,3,4,5)P_4 contained an unidentified impurity, and yet it behaved in an almost identical way to commercially available Ins(1,3,4,5)P_4.

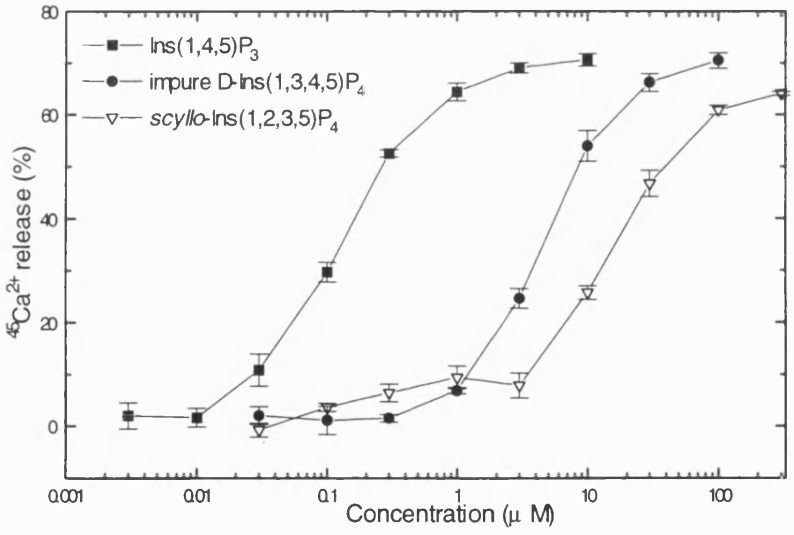


Figure 7.11 $^{45}Ca^{2+}$-release from SH-SY5Y neuroblastoma cells induced by Ins(1,4,5)P_3 (potassium salt), *scyllo*-Ins(1,2,3,5)P_4 (**62**, triethylammonium salt) and impure D-Ins(1,3,4,5)P_4 (**D-70a**, potassium salt). $^{45}Ca^{2+}$-release is given relative to the maximal release induced by Ins(1,4,5)P_3.

It was intended to repeat the large-scale route and optimise it as a method of producing pure D- and L-Ins(1,3,4,5)P_4 at a later stage, but time was pressing. and it seemed that the best course of action would be to purify the D-Ins(1,3,4,5)P_4 by ion-exchange chromatography, and then test it again. A small sample (100mg) of the impure D-Ins(1,3,4,5)P_4 was subjected to careful ion-exchange chromatography, taking only the fractions eluting above 700mM buffer strength, so as to exclude the possibility of

overlap with any trisphosphate. However, the ion-exchange purified D-Ins(1,3,4,5)P_4 *still* contained around 2-3% of an impurity as judged by high-field phosphorus NMR. At low pH the D-Ins(1,3,4,5)P_4 peaks were broad and no impurities could be seen (Figure 7.12a), but after adding EDTA to chelate paramagnetic ions, impurities were visible in the ^{31}P NMR spectrum (Figure 7.12b). In order to spread the peaks as far as possible and therefore minimise overlap, the pH was raised to ~10 by addition of KOH solution (Figure 7.12c). Four small impurity peaks could now be seen, suggesting a tetrakisphosphate, and of course, this finding was what might be expected for a compound that co-eluted with Ins(1,3,4,5)P_4. Significantly, the fact that there were four peaks and not two, ruled out the possibility that the contaminant was a symmetrical tetrakisphosphate such as Ins(1,3,4,6)P_4. It seemed very likely, therefore, that the contaminant was an asymmetrical tetrakisphosphate arising from phosphate migration at some stage during or after the deprotection. Figures 7.12a to 7.12c illustrate the importance of careful adjustment of conditions if ^{31}P NMR is to be used as a criterion for the purity of inositol phosphates. The 400MHz ^{1}H NMR spectrum also showed irregularities in the baseline (as in Figure 7.10a) that suggested the presence of a second inositol phosphate, although overlap with other peaks made it impossible to identify the contaminant.

Now a consideration of the possible asymmetrical migration products obtainable from D-Ins(1,3,4,5)P_4 shows that the production of D-Ins(1,2,4,5)P_4 is a distinct possibility. A literature search revealed that another group had previously encountered problems with phosphate migration (under *acidic* conditions) and they reported that migration to an unprotected 2-position was particularly favoured.[249] Thus, the expected migration products from D-Ins(1,3,4,5)P_4 are D-Ins(2,3,4,5)P_4 and D-Ins(1,2,4,5)P_4. Now D-Ins(1,2,4,5)P_4 is highly active at Ins(1,4,5)P_3 receptors, being almost equipotent with Ins(1,4,5)P_3.[250-252] It was therefore possible that the apparent activity of our D-Ins(1,3,4,5)P_4 (Figure 7.11) could result from around 2-3% contamination with D-Ins(1,2,4,5)P_4. However, this would mean that commercially available D-Ins(1,3,4,5)P_4 was also contaminated with a Ca^{2+}-mobilising material.

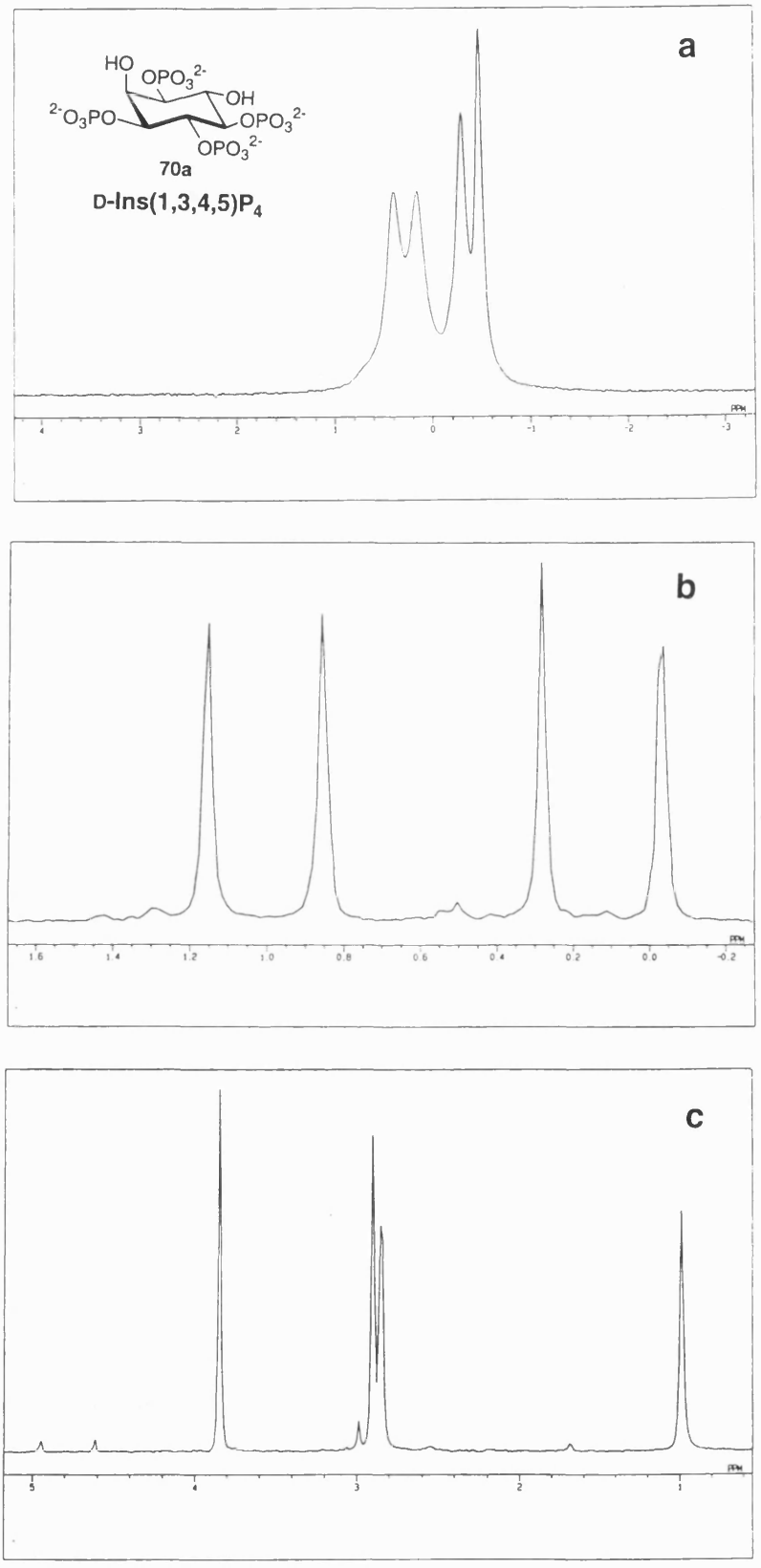


Figure 7.12 162 MHz ^{31}P NMR spectra of ion-exchange purified **D-70a** (triethylammonium salt in D_2O). **a**) pH 4-5; **b**) pH 4-5, EDTA added; **c**) pH ~10, EDTA added.

It seemed possible that the migration had occurred as a result of acidification with Dowex-H^+ resin, and so the deprotection was repeated, and the crude product purified immediately by ion exchange with no work-up procedure. However, the impurity was still present, suggesting that migration was taking place at the deprotection stage, which employed *alkaline* conditions. It is known that, in the cleavage of cyanoethyl groups under alkaline conditions by β-elimination, the first cyanoethyl group is removed very rapidly, while the second is then cleaved slowly. Perhaps migration was occurring at this prolonged intermediate stage, and the presence of cyanoethyl groups or the use of a slow deprotection method was the source of the problem.

More tetrol **68a** was synthesised and this time phosphorylated using bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine, giving **71a** which was deprotected using sodium in liquid ammonia. It was not known how rapidly camphanate groups would be cleaved by this method, and so the reaction was quenched after five minutes, rather than the usual one or two minutes. This method gave better results, and all protecting groups were cleaved within this time, but a small amount of migration product was still visible in the NMR spectra after ion-exchange chromatography. Now migration had not been detected in previous deprotections of this type, notably in the synthesis of Ins(1,3,4)P_3, which also has a vacant 2-position, so it seemed likely that it was the presence of camphanate esters at positions 2 and 6 that were causing the problem. Perhaps the esters were being cleaved more rapidly than the phosphate protecting groups, so that unprotected 2- and 6-hydroxyl groups were available as targets for phosphate migration during the deprotection. Presumably, the mechanism of migration would then be as shown in Figure 7.13. If this were so then it would seem that the camphanate groups were the cause of the difficulties. However, the selective protection of positions 2 and 6- with camphanate esters was the basis of the synthetic strategy, and if the problems they entailed were insurmountable, then that strategy would be intrinsically flawed.

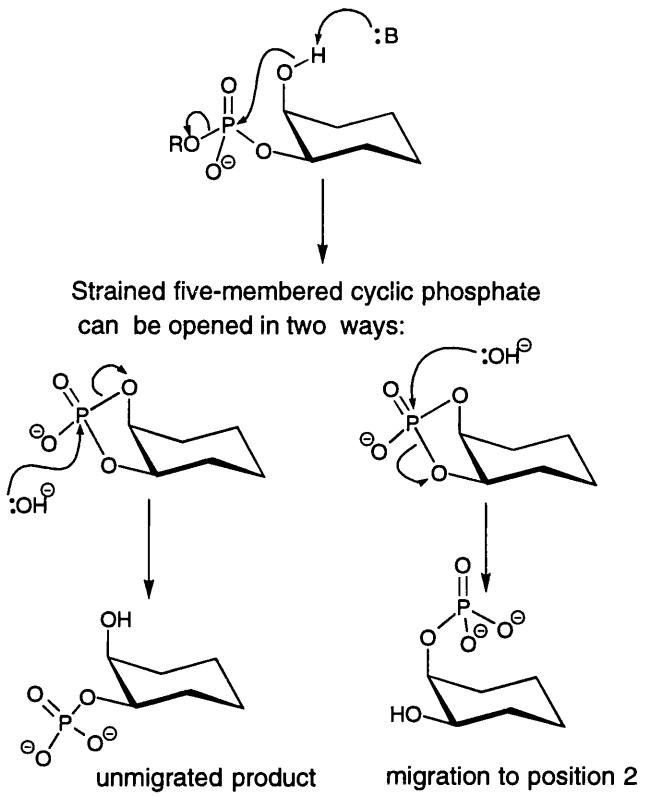


Figure 7.13 Possible mechanism for base-catalysed migration of a phosphate group to the axial 2-position *via* a five-membered cyclic phosphate intermediate. R = benzyl or 2-cyanoethyl.

7.5.6 Another Strategy: Two-Step Deprotection

There was one method remaining that should make migration impossible. It was to deprotect the phosphate groups first, and then remove the camphanate esters (Figure 7.14). The fully protected compound **71a** was therefore hydrogenolysed using a catalyst of palladium on carbon, and hydrogen at 50 p.s.i. overnight.

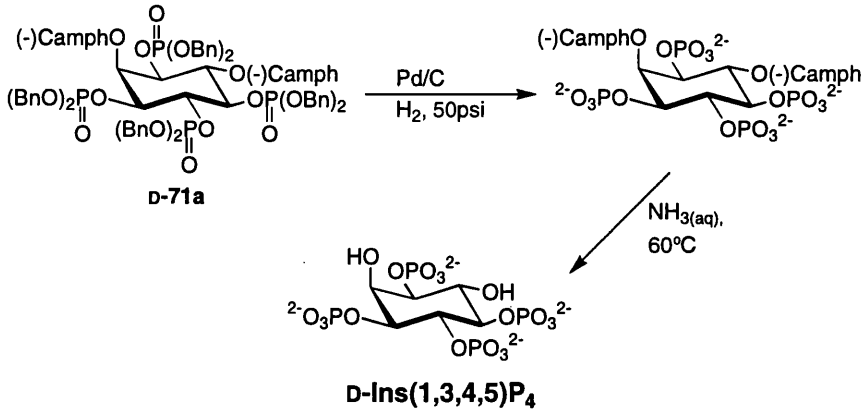


Figure 7.14 Two-step deprotection of **D-71a** avoids migration of phosphate groups.

It was impossible to tell whether the intermediate tetrakisphosphate was pure because residual palladium ions caused severe broadening of the NMR spectra, and could not be removed with EDTA. The camphanate esters were removed from this intermediate by heating at 60°C in concentrated ammonia solution as before and the crude product purified by ion exchange giving the triethylammonium salt of D-Ins(1,3,4,5)P_4. The impurities that had been seen in all the previous samples of **D-70a** were no longer visible in the ^{31}P NMR (Figure 7.15) and the baseline in the 400 MHz NMR was now smooth.

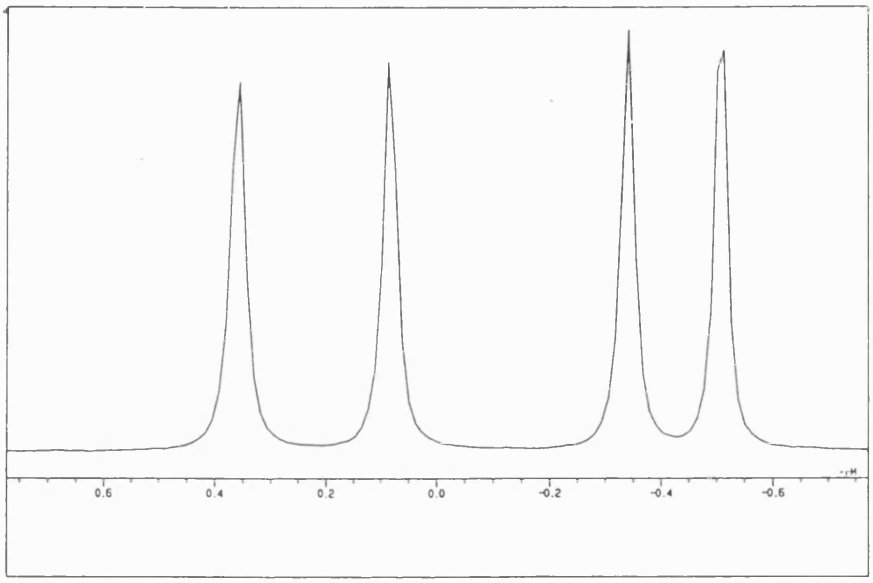


Figure 7.15 162 MHz ^{31}P NMR spectrum of D-Ins(1,3,4,5)P_4 produced by the two-step deprotection method (triethylammonium salt in D_2O, pH 4-5; EDTA added). Compare with Figure 7.12b.

Now that the problems with this synthesis appear to have been solved, it should provide a rapid and simple access to D- and L-Ins(1,3,4,5)P_4. The strategy should enable either enantiomer of Ins(1,3,4,5)P_5 to be synthesised from *myo*-inositol orthoformate, using procedures that require only a few days work. It now only remains to repeat the route using the optimised methods and produce larger amounts of both enantiomers. The methodology could also be adapted to give a rapid route to [^{3}H]Ins(1,3,4,5)P_4. Oxidation of the unprotected 4-position in **67a** followed by stereoselective reduction of the resulting ketone with [^{3}H]$NaBH_4$ should yield **67a** tritiated at position 4, which can then be converted to optically pure D-[^{3}H]Ins(1,3,4,5)P_4 in only three steps, using the methods already developed for **67a**.

The remaining impure D-Ins(1,3,4,5)P_4 is currently being put to good use in a potentiometric/NMR investigation of Ins(1,3,4,5)P_4 in the hope of gaining information on the ionisation state and solution conformation of Ins(1,3,4,5)P_4.* It should be particularly interesting to establish how the addition of a phosphate group to position 3 of Ins(1,4,5)P_3 may affect the conformation and ionisation state of the adjacent 4,5-bisphosphate. Samples of the purified D-Ins(1,3,4,5)P_4 are being used in an NMR study of their interaction with the isolated PH domain of human dynamin.

The purified D-Ins(1,3,4,5)P_4 is now being evaluated in SH-SY5Y cells under the same conditions as before, and compared to the impure sample. Commercially available D-Ins(1,3,4,5)P_4, HPLC-purified commercial D-Ins(1,3,4,5)P_4, and HPLC-purified biologically-derived D-Ins(1,3,4,5)P_4 are also being compared in the same assay. Further tests are in progress, and full binding assays are still to be carried out before firm conclusions can be drawn. It is hoped that the complete results, which should be available soon, will allow a clearer understanding of the effects of D-Ins(1,3,4,5)P_4 and its role in cell signalling.

* A preliminary ^{31}P NMR titration curve for **D-70a** is now available. See Appendix 3.

8 Experimental

8.1 General Methods

Chemicals were purchased from Aldrich and Fluka. Dichloromethane was dried over phosphorus pentoxide, distilled and kept over 4 Å molecular sieves. Pyridine was dried by refluxing with sodium hydroxide pellets, followed by distillation, and stored over 5 Å sieves. Dimethylformamide was stored over 4 Å sieves. Tetrahydrofuran (THF) was dried by passing through activated alumina to expel peroxide radicals, followed by distillation from sodium in the presence of benzophenone ketyl.

TLC was performed on pre-coated plates (Merck TLC aluminium sheets silica F_{254}, Art. no. 5554) with detection by UV light or with methanolic phosphomolybdic acid followed by heating. Flash-column chromatography was performed on silica gel (Sorbsil C60).

^{1}H and ^{13}C NMR spectra (internal Me_4Si as reference) were recorded with a Jeol GX270 or EX400 NMR spectrometer, and ^{31}P NMR spectra (external aq. 85% phosphoric acid as reference) were recorded with a Jeol EX400 or Jeol FX90Q spectrometer. Mass spectra were recorded at the SERC Mass Spectrometry Service Centre, Swansea, and at the University of Bath. Microanalysis was carried out by the Microanalysis Service, University of Bath. FAB-mass spectra were carried out using *m*-nitrobenzyl alcohol as the matrix. Melting points (uncorrected) were determined using a Reichert-Jung Thermo Galen Kofler Block. Optical rotations were measured using an Optical Activity Ltd. AA-10 polarimeter. Ion-exchange chromatography was performed on an LKB-Pharmacia Medium Pressure Ion Exchange Chromatograph using DEAE Sepharose or Sepharose Q Fast Flow by elution with a gradient of triethylammonium hydrogencarbonate (TEAB) buffer as eluent. Quantitative analysis of phosphate was performed using a modification of the Briggs phosphate assay.[152, 83]

Dichloro-*N*,*N*-diisopropylaminophosphine was prepared by the method of Tanaka *et al.*[253] by adding 2 equiv. of *N*,*N*-diisopropylamine to a solution of PCl_3 in dry ether at −78°C. The crude product (δ_P 166.4) was purified by distillation under reduced pressure, and reaction with 2 equiv. of benzyl alcohol in the presence of 2 equiv. of triethylamine afforded bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine[254] (δ_P 145.24) which was purified by flash chromatography.

Benzyloxy-bis(*N*,*N*-diisopropylamino)phosphine was prepared by the method of Dreef *et al.*[202] Chloro-bis(*N*,*N*-diisopropylamino)phosphine (2.67g, 10.0mmol) was dissolved

in dry ether (20mL) and cooled to 0°C under N_2. Dry triethylamine (1.4mL, 10.0mmol) was added followed by anhydrous benzyl alcohol (1.39mL, 10.0mmol). The mixture was allowed to reach room temperature and stirred for a further 30 min. The mixture was then poured into pentane at 0°C (30mL) and rapidly filtered to remove the precipitated triethylammonium chloride. The filtrate was evaporated *in vacuo* giving benzyloxy-bis(*N*,*N*-diisopropylamino)phosphine as a colourless oil (3.17g, 9.37mmol, 94%).

^{1}H NMR ($CDCl_3$, 270MHz): δ 1.17 (12 H, d, J = 6.8 Hz, 4 x CH_3), 1.19 (12 H, d, J = 6.8 Hz, 4 x CH_3), 3.55 (2 H, septet, J = 6.8 Hz, 2 x CH), 3.59 (2 H, septet, J = 6.8 Hz, 2 x CH), 4.65 (2 H, d, $^3J_{HP}$ = 7.1 Hz, $CH_2C_6H_5$), 7.30 - 7.40 (5 H, m, C_6H_5).

^{31}P NMR ($CDCl_3$, 36 MHz, ^{1}H-decoupled): δ 123.6.

Bis(4-chlorobenzyloxy)-*N*,*N*-diisopropylaminophosphine was prepared according to de Bont *et al.*[177]

8.1.2 Computer assisted Molecular Modelling

Energy minimisations of structures and molecular dynamics simulations were carried out using the AMBER or MM+ forcefields within the software packages Discover 2.95 (Biosym Technologies, San Diego, USA) running on a Silicon Graphics workstation or Hyperchem release 4 for Windows (Hypercube Inc.) on a 486DX266 personal computer. Partial charges for the fully ionised molecules were calculated using a semi-empirical method (CNDO or AM1). Molecular dynamics simulations were conducted at 900K for 50psec after an initial equilibration period of 0.1psec.

8.1.3 Biological Assays

The investigations involving $^{45}Ca^{2+}$ release from rabbit platelets, the spectrofluorimetric studies of Ca^{2+} release from platelets and the binding assays in rat cerebellar membranes were carried out by or under the supervision of Dr C. Murphy, in the Group of Prof. J. Westwick, School of Pharmacy and Pharmacology, University of Bath. Experimental details are given in Reference.[255]

Assays for inhibition of phosphatidylinositol 3-kinase were carried out by Dr S. Ward in the School of Pharmacy and Pharmacology, University of Bath. Full details of procedures are given in Reference.[90]

Testing of D- and L-Ins(1,3,4)P_3 in *Limulus* ventral photoreceptors was carried out by Dr R. Payne of the Dept of Zoology, University of Maryland, USA. Experimental details are given in Reference.[256]

Investigations into the effects of Ins(1,3,4,5)P_4 analogues in SH-SY5Y neuroblastoma cells were undertaken by Dr R. A. Wilcox, Dept. of Cell Physiology and Pharmacology, University of Leicester. Full details of procedures are given in Reference.[110]

Preliminary assays of the interaction of analogues with Ins(1,3,4)P_3 5/6-kinase were performed by Dr P. J. Hughes at the Centre for Clinical Research in Immunology and Signalling, The Medical School, University of Birmingham. For details of procedures see Reference.[87]

The compounds described in this thesis are being examined for their interaction with a purified Ins(1,4,5)P_3 3-kinase preparation, with analysis of the phosphorylation products by HPLC with metal-dye detection.[257] These studies are being conducted by Dr G. W. Mayr and Dr U. Bertsch at the Institute for Physiological Chemistry, University of Hamburg, Germany.

8.1.4 Potentiometric and ^{31}P NMR Investigations

These studies were carried out by Professor Bernard Speiss and his group at the Université Louis Pasteur, Strasbourg. Details of procedures are given in Reference.[189]

8.1.5 X-ray Crystallography

The X-ray crystallographic study of compound **67a** was carried out by Dr M. Mahon in the Dept of Chemistry, University of Bath. Full details are given in Appendix 2.

8.2 D- and L-*myo*-Inositol 1,3,4-trisphosphate

DL-1,4-Di-*O*-benzoyl-2,3:5,6-di-*O*-isopropylidene-*myo*-inositol (1)

A mixture of *myo*-inositol (100g, 0.55mol), *N,N*-dimethylformamide (500mL), 2,2-dimethoxypropane (400mL), and toluene-*p*-sulphonic acid monohydrate (2g) was stirred at 100-120°C for 2 h. Triethylamine (20mL) was added to the cooled solution and the low-boiling solvents were evaporated at 50°C under reduced pressure. Pyridine (400mL) was added, followed by benzoyl chloride (400mL) dropwise with stirring and cooling over 25 min. The mixture was left to stand for a further 2 h, and then the solid was removed by filtration and washed successively with water, triethylamine, acetone and ether to give **1** (41.3g, 94mmol, 17%).

Mp: 328 - 330°C (from DMF) (lit.[144] 328 - 330° C)

^{1}H NMR ($CDCl_3$, 270MHz): δ 1.30, 1.44, 1.51, 1.64 (12 H, 4 s, 4CH_3); 3.72 (1 H, dd, J = 11.0 Hz, 9.3 Hz, C-5-H), 4.40 (1 H, dd, J = 10.6 Hz, 9.3 Hz, C-6-H); 4.40 (1 H, dd, J

= 6.6 Hz, 4.6 Hz, C-3-H); 4.79 (1 H, dd, J = 4.6 Hz, 4.6 Hz, C-2-H), 5.43 (1 H, dd, J = 10.6 Hz, 4.4 Hz, C-1-H); 5.61 (1 H, dd, J = 11.0 Hz, 6.6 Hz, C-4-H), 7.41-7.51 (4 H, m, C_6H_5COO), 7.53-7.63 (2 H, m, C_6H_5COO, 8.08-8.18 (4 H, m, C_6H_5COO).

DL-1,2:4,5-Di-*O*-isopropylidene-*myo*-inositol (2)

A mixture of **1** (20g, 45mmol), sodium hydroxide (6g), and methanol (250ml) was heated under reflux for 30 min producing a clear solution which was allowed to cool. The solution was neutralised with solid carbon dioxide and the resulting white paste was then diluted with water (100mL) and evaporated to dryness under reduced pressure. The residue was extracted with dichloromethane (6 x 150mL), dried ($MgSO_4$) and evaporated under reduced pressure to give a solid, which was recrystallised from ethyl acetate giving **2** as colourless crystals (9.7g, 37mmol, 82%).

Mp: 166 - 170°C (from ethyl acetate) (lit.[144] 171-173°C).

^{1}H NMR ($CDCl_3$, 270MHz): δ 1.38, 1.46, 1.49, 1.54 (12 H, 4 s, 4 CH_3), 2.48 (1 H, d, J = 8.6 Hz, D_2O ex, C-3-OH), 2.67 (1 H, d, J = 3.1 Hz, D_2O ex, C-6-OH), 3.33 (1 H, dd, J = 10.6 Hz, 9.4 Hz, C-5-H), 3.84 (1 H, dd, J = 10.1 Hz, 10.1 Hz, C-4-H), 3.90 (1 H, ddd, J = 10.6 Hz, 6.0 Hz, 3.0 Hz, C-6-H), 4.03 (1 H, ddd, J = 10.0 Hz, 8.6 Hz, 4.4 Hz, C-3-H), 4.08 (1 H, dd, J = 6.5 Hz, 5.0 Hz, C-1-H), 4.49 (1 H, dd, J = 5.0 Hz, 5.0 Hz, C-2-H).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 25.85, 26.89, 28.06 (3 q, 4 CH_3), 69.74, 74.76, 77.56, 77.97, 81.28, 81.83 (6d, inositol ring C), 110.28, 112.71 (2 s, 2 $C(CH_3)_2$).

DL-1,4-Di-*O*-allyl-2,3:5,6-di-*O*-isopropylidene-*myo*-inositol (3)

To a solution of diol **2** (18.2g, 70mmol) and sodium hydride (4.8g, 200mmol) in *N,N*-dimethylformamide (250mL) at 0°C was added dropwise allyl bromide (13mL, 150mmol), with stirring. The reaction mixture was stirred for 2 h at 20°C. Excess sodium hydride was destroyed with ethanol and the mixture evaporated to dryness under reduced pressure. Water (200mL) was added and the product extracted with ether (3 x 200mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure to give **3** (22g, 65mmol, 93% yield).

Mp: 83 - 85°C (from hexane); (lit.[145] 85 - 87°C).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 1.38, 1.43, 1.46, 1.54 (12 H, 4 s, 4 CH_3), 3.34 (1H, dd J = 10.5 Hz, 9.8 Hz, C-5-H), 3.66 (1 H, dd, J = 10.6 Hz, 6.4 Hz, C-4-H), 3.80 (1 H, dd, J = 10.3 Hz, 4.2 Hz, C-1-H), 3.98 (1 H, dd, J = 10.1 Hz, 10.1 Hz, C-6-H), 4.10 (1 H, dd J

= 6.1 Hz, 4.6 Hz C-3-H), 4.21-4.37 (4 H. m, OCH_2), 4.46 (1 H, dd, J = 4.4 Hz, 4.4 Hz, C-2-H), 5.17-5.35 (4 H, m, $=CH_2$), 5.90-5.60 (2 H, m, -CH=)

^{13}C NMR ($CDCl_3$, 68 MHz): δ 25.91, 26.95, 28.06 (3q, $4CH_3$), 71.16, 71.26 (2t, OCH_2), 74.76, 76.42, 76.84, 78.56, 80.11, 81.38 (6d, inositol ring), 109.99, 112.10 (2 s, $C(CH_3)_2$), 117.38, 118.16 (2 t, $=CH_2$), 134.67, 134.74 (2d, -CH=).

DL-1,4-Di-*O*-allyl-*myo*-inositol (4)

Compound **3** (21.0g, 61.7mmol) was dissolved in acetic acid-water (4:1 , 200mL) and the solution was refluxed for 30 min. The solvents were then evaporated under reduced pressure. The white residue was recrystallised from ethanol to give **4** (15.0g, 57.6mmol, 93%).

Mp: 133-136°C (from ethanol); Lit[149] 137-139°C.

^1H NMR (d_6-DMSO, 270 MHz): δ 2.96 (1 H, dd, J = 9.7 Hz, 2.6 Hz, C-1-H), 3.03 (1 H, ddd, J = 9.0 Hz, 9.0 Hz, 4.8 Hz, D_2O ex gives dd, J = 9.0, 9.0, C-5-H), 3.16 - 3.28 (2 H, m, C-3-H and C-4-H), 3.46 (1 H, ddd, J = 9.6 Hz, 9.3 Hz, 4.6 Hz, D_2O ex gives dd, J = 9.6 Hz, 9.3 Hz, C-6-H), 3.86 (1 H, ddd, J = 3.2 Hz, 2.6 Hz, 2.6 Hz, D_2O ex gives dd J = 2.6 Hz, 2.6 Hz, C-2-H), 3.97 - 4.12, (2 H, m, $CH_2CH=CH_2$), 4.16 - 4.26 (2 H, m, $CH_2CH=CH_2$), 4.54 (1 H, d, J = 6.0 Hz, D_2O ex, OH), 4.61 (1 H, d, J = 3.7 Hz, D_2O ex, C-2-OH), 4.64 (1 H, d, J = 4.8 Hz, D_2O ex, OH), 4.69 (1 H, d, J = 4.8 Hz, D_2O ex, OH), 5.01 - 5.29 (4 H, m, $CH_2CH=CH_2$), 5.80 - 5.98 (2 H, m , $CH_2CH=CH_2$)

^{13}C NMR (d_6-DMSO, 68 MHz): δ 69.83, 71.42, 72.20, 75.02, 79.53, 81.41 (6d, inositol ring *C*), 70.09, 72.94 (2 t, $CH_2CH=CH_2$), 115.30, 115.99 (2 t, $CH_2CH=CH_2$), 136.23, 137.06 (2 d, $CH_2CH=CH_2$)

DL-2,3,5,6-Tetra-*O*-acetyl-1,4-di-*O*-allyl-*myo*-inositol (5)

To a solution of **4** (1.3g, 5 mmol) in pyridine (10mL) were added acetic anhydride (2.8mL, 30mmol) and DMAP (100mg). The mixture was stirred at room temperature for 1 h, after which TLC (ether) showed conversion to a major product at R_f 0.60. The liquid was evaporated under reduced pressure and the product dissolved in dichloromethane (100mL), washed with sat. sodium hydrogencarbonate solution (100mL) then water (100mL), dried ($MgSO_4$) and evaporated to give an off-white solid which was recrystallised from hexane / ethyl acetate yielding **5** (1.70g, 3.97mmol, 79%).

Mp: 118.5 - 119.5°C (from hexane / ethyl acetate).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.04, 2.05, 2.06, 2.16 (12H, 4s, 4 CH_3), 3.55 (1 H, dd, J = 10.3 Hz, 2.9 Hz, C-1-H), 3.84 (1 H, dd, J = 10.3 Hz, 9.8 Hz, C-4-H), 3.89 - 4.17 (4 H, m, $CH_2CH=CH_2$). 4.87 (1 H, dd, J = 10.3 Hz, 2.9 Hz, C-3-H), 5.07 (1 H, dd, J = 9.8 Hz, 9.8 Hz, C-5-H), 5.11 - 5.26 (4 H, m, $CH_2CH=CH_2$), 5.31 (1 H, dd, J = 10.3 Hz, 10.3 Hz, C-6-H), 5.64 (1 H, dd, J = 2.9 Hz, 2.9 Hz, C-2-H), 5.71 - 5.84 (2 H, m, $CH_2CH=CH_2$).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 20.69 (q, 4 CH_3), 67.37, 71.06, 71.26, 72.52, 74.41, 76.61 (6 d, inositol ring *C*), 70.97, 73.92 (2 t, $CH_2CH=CH_2$), 116.70, 117.67 (2 t, $CH_2CH=CH_2$), 133.63, 134.28 (2 d, $CH_2CH=CH_2$), 169.73, 169.90, 170.06 (3 s, 4 *C*=O)

MS: *m/z* (+ve ion FAB, rel intensity) 429 [$(M + H)^+$, 80%] 371 [$(M-CH_2CHCH_2O^-)^+$, 80%], 369 [$(M-CH_3COO^-)^+$, 100%]

Anal. Calcd for $C_{20}H_{28}O_{10}$ (428.44): C, 56.07; H, 6.59. Found: C, 56.0; H, 6.64.

DL-1,4-Di-*O*-allyl-3-*O*-*p*-methoxybenzyl-*myo*-inositol (6)

A mixture of 1,4-di-O-allyl-*myo*-inositol **4** (13.0g, 50mmol), dibutyltin oxide (14.9g, 60mmol) and toluene (200mL) were heated under reflux for 3h in a Dean and Stark apparatus. The mixture was allowed to cool and the toluene removed by evaporation *in vacuo* giving an off-white solid. Caesium fluoride (19.0g, 125mmol), potassium iodide (12.5g, 75mmol) and DMF (200mL) were added, followed by *p*-methoxybenzyl chloride (10.2mL, 75mmol). After stirring for 3h at rt TLC (ethyl acetate) showed a major product at R_f 0.30. The mixture was left overnight at room temperature and then evaporated *in vacuo*. The residue was taken up in dichloromethane, washed with water, stirred with sodium hydrogencarbonate solution (10% w/v) for 30 min, and washed with water again. The insoluble tin derivatives were removed by filtration through Celite and the solution was dried ($MgSO_4$), and evaporated to give an oil which was chromatographed on silica gel (ethyl acetate) giving **6** (12.4g, 32.6mmol, 65%).

Mp: 94-96°C (from ethyl acetate / hexane).

^{1}H NMR ($CDCl_3$, 270MHz): δ 2.62 (1 H, br s, D_2O ex., OH), 3.11-3.16 (3 H, m, D_2O ex. gives 1H, dd, J = 9.5 Hz, 2.6 Hz, 2 OH and C-1-H or C-3-H), 3.32 (1 H, dd, J = 9.5 Hz, 2.8 Hz, C-1-H or C-3-H), 3.36 (1 H, ddd, J = 9.5 Hz, 9.5 Hz, 1.5 Hz, D_2O ex gives dd, J = 9.5 Hz, 9.5 Hz, C-5-H), 3.66 (1 H, dd, J = 9.5 Hz, 9.5 Hz, C-4-H), 3.88 (1 H, br dd, J = 9.5 Hz, 9.5 Hz, D_2O ex gives dd, C-6-H), 4.07 - 4.45 (5 H, m, 2 $CH_2CH=CH_2$ and C-2-H), 4.63, (2 H, br s, CH_2Ph), 5.15 - 5.32 (4 H, m, 2 $CH_2CH=CH_2$), 5.86 - 6.05 (2 H, m, 2 $CH_2CH=CH_2$), 6.86 - 6.90 (2 H, m, *Ph*OMe), 7.27 - 7.31 (2 H, m, *Ph*OMe)

^{13}C NMR ($CDCl_3$, 68 MHz): δ 55.27 (q, OCH_3), 67.01, 71.55, 74.05, 78.85, 79.34, 80.08 (6d, inositol ring *C*), 71.29, 72.27 (2 t, $CH_2CH=CH_2$), 74.24 (t, CH_2Ph), 113.89, 129.51 (2 d, *Ph*OMe) 116.83, 117.87 (2 t, $CH_2CH=CH_2$), 129.94 (s, *Ph*OMe) 134.51, 135.26 (2 d, $CH_2CH=CH_2$), 159.35 (s, *Ph*OMe)

MS: *m/z* (+ve ion FAB, rel intensity) 379 [$(M-H)^+$, 5%], 259 [$(M-CH_2C_6H_4OCH_3)^+$, 2%], 137(12) 121 [$(CH_2C_6H_4OCH_3)^+$, 100%] ;

MS: *m/z* (–ve ion FAB, rel intensity) 759 [$(2M-H)^-$, 20%)], 533 [$(M+NBA)^-$, 92%], 379 [$(M-H)^-$, 100%], 339(25), 322(20), 249(21).

Anal. Calcd for $C_{20}O_7H_{28}$ (380.44): C, 63.14; H, 7.42. Found: C, 62.9; H, 7.42.

DL-1,4-Di-*O*-allyl-2,5,6-tri-*O*-benzyl-3-*O*-*p*-methoxybenzyl-*myo*-inositol (7)

To a solution of triol **6** (6.85g, 18mmol) in dry *N*-*N*-dimethylformamide (100mL) was added sodium hydride (2.0g, 83mmol). The mixture was cooled to 0°C and benzyl bromide (7.0mL, 59mmol) was added dropwise with stirring. The mixture was stirred at room temperature for 2 h, after which TLC (hexane/ether 1:1) showed the reaction to be complete, with a major product at R_f 0.43. The excess sodium hydride was carefully destroyed with water and the mixture concentrated *in vacuo*. The residue was dissolved in dichloromethane (100mL), the solution was washed successively with water, 0.1MHCl, sat. $NaHCO_3$ solution and water (100mL of each), dried ($MgSO_4$) and evaporated *in vacuo* to give 11.8g of a solid which was recrystallised from hexane giving **7** (10.2g, 15.7mmol, 87%).

Mp: 72 - 74°C (from hexane).

^1H NMR ($CDCl_3$, 270MHz): δ 3.23 (1H, dd J = 9.9 Hz, 2.2 Hz, C-1-H or C-3-H), 3.28 (1H, dd J = 9.9 Hz, 2.2 Hz, C-1-H or C-3-H), 3.39 (1H, dd, J = 9.3 Hz, 9.3 Hz, C-5-H), 3.81 (3H, s, OCH_3), 3.91 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-4-H or C-6-H), 3.97 (1 H, dd, J = 2.2 Hz, 2.2 Hz, C-2-H), 3.98 (1 H, dd, J = 9.7 Hz, 9.7 Hz, C-4-H or C-6-H), 4.07 - 4.14 (2 H, m $OCH_2CH=CH_2$) , 4.27 - 4.44 (2 H, m $OCH_2CH=CH_2$), 4.54, 4.59 (2 H, AB, J_{AB} 11.4 Hz, $CH_2C_6H_4OMe$), 4.76 - 4.90 (6 H, m 3 $OCH_2C_6H_5$), 5.13 - 5.35 (4 H, m $=CH_2$), 5.83 - 6.06 (2 H, m, $-CH=$), 6.86 - 6.90 (2 H, m, $CH_2C_6H_4OMe$), 7.24 - 7.42 (17 H, m, 3 $CH_2C_6H_5$ and $CH_2C_6H_4OMe$)

^{13}C NMR ($CDCl_3$, 68 MHz): 55.27 (q, OCH_3), δ 71.62, 72.54, 73.95, 74.54, 75.80 (6t, OCH_2), 74.44, 80.50, 80.63, 81.41, 81.57, 83.65 (6 d, inositol ring *C*), 113.72 (d, $CH_2C_6H_4OMe$), 116.54 and 116.60 (2 t, $=CH_2$), 127.28, 127.50, 127.79, 127.92, 128.09,

128.31 (6 d, $CH_2C_6H_5$ and $CH_2C_6H_4OMe$), 130.62 (s, $CH_2C_6H_4OMe$), 134.93 and 135.48 (2 d, 2-*C*H=), 138.92 and 138.98 (2 s, $CH_2C_6H_5$), 159.13 (s, $CH_2C_6H_4OMe$)

MS: *m/z* (+ve ion FAB, rel intensity) 649 [$(M-H)^+$, 1.5%], 121 [$(CH_2C_6H_4OCH_3)^+$, 100%] 91 [$(M-C_7H_7)^+$, 70%].

MS: *m/z* (–ve ion FAB, rel intensity) 803 [$(M+NBA)^-$, 100%], 529 [$(M-CH_2C_6H_4OCH_3)^-$, 10%].

Anal. Calcd for $C_{41}H_{46}O_7$ (650.81): C, 75.67; H, 7.12. Found: C, 75.6; H, 7.09.

DL-2,4,5-tri-*O*-Benzyl-1-*O*-*p*-methoxybenzyl-3,6-di-*O*-(*cis*-prop-1-enyl)-*myo*-inositol (8)

A solution of **7** (1.0g, 1.54mmol) and freshly sublimed potassium *tert*-butoxide (2.0g, 17.8mmol) in dry DMSO (50mL) was stirred for 3 h at 50°C, after which TLC (hexane/ether 1:1) showed complete conversion from starting material (R_f 0.44) to a product at R_f 0.56. Water (50mL) was added to the cooled brown solution, which was then extracted with ether (3 x 100mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure to give an off-white solid which was recrystallised from ethanol giving **8** (0.83g, 1.28mmol, 83%).

Mp: 110-112°C (from ethanol).

^{1}H NMR ($CDCl_3$, 400MHz): δ 1.64 (3 H, dd, J = 6.8 Hz, 1.5 Hz, CH=CH-CH_3), 1.66 (3 H, dd, J = 6.8 Hz, 1.5 Hz, CH=CH-CH_3), 3.33 (1 H, dd, J = 9.8 Hz, 2.4, C-1-H or C-3-H), 3.44 (1 H, dd, 9.3 Hz, 9.3 Hz, C-5-H), 3.53 (1 H, dd, 9.8 Hz, 2.4 Hz, C-1-H or C-3-H), 3.81 (3 H, s, OCH_3), 3.99 (1 H, dd, J = 2.4 Hz, 2.4 Hz, C-2-H), 4.03 (1H, dd, J = 9.8 Hz, 9.8 Hz, C-4-H or C-6-H), 4.15 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-4-H or C-6-H), 4.35 (1 H, dq, J = 6.8 Hz, 6.8 Hz, CH=C*H*-CH_3), 4.44 (1 H, dq, J = 6.8 Hz, 6.8 Hz, CH=C*H*-CH_3), 4.51, 4.56 (2 H, AB, J_{AB} 11.7 Hz, CH_2Ph), 4.72, 4.80 (2 H, AB, J_{AB} 10.3 Hz, CH_2Ph), 4.73, 4.80, (2 H, AB, J_{AB} 10.7 Hz, CH_2Ph), 4.82 (2 H, br s, CH_2Ph), 6.08 (1 H, dq, J = 6.8 Hz, 1.5Hz, C*H*=CH-CH_3), 6.27 (1 H, dq, J = 6.8 Hz, 1.5 Hz, C*H*=CH-CH_3), 6.85 - 6.88 (2 H, m, $CH_2C_6H_4$OMe), 7.24 - 7.35 (15 H, m, $CH_2C_6H_5$), 7.37 - 7.42 (2 H, m, $CH_2C_6H_4$OMe)

^{13}C NMR ($CDCl_3$, 100 MHz): δ 9.36, 9.42 (2 q, CH=CH-*C*H_3), 55.29 (q, $CH_2C_6H_4OCH_3$), 72.29, 74.47, 75.69, 75.78, (4 t, *C*H_2Ph), 75.88, 78.35, 80.71, 82.55, 84.40, 82.94 (6d, inositol ring), 98.19. 100.62, (2 d, CH=*C*H-CH_3), 113.72 (d, $CH_2C_6H_4$OMe), 127.38, 127.61, 127.83, 128,13, 129.26 (6 d, $CH_2C_6H_5$ and

$CH_2C_6H_4OMe$), 130.34 (s, $CH_2C_6H_4OMe$), 138.50, 138.61, 138.71 (s, $CH_2C_6H_5$), 145.66, 147.77 (2 d, OCH=CH), 159.18 (s, $CH_2C_6H_4OMe$)

MS: *m/z* (+ve ion FAB, rel intensity) 649 [$(M-H)^+$, 0.4%)], 559 [$(M-C_7H_7)^+$, 0.5%)], 121 [$(CH_2C_6H_4OCH_3)^+$, 100%], 91 [$(C_7H_7)^+$, 80%];

MS: *m/z* (–ve ion FAB, rel intensity) 803 [$(M+NBA)^-$, 100%], 696 (32%) 559 [$(M-C_7H_7)^-$ 10%] 529 [$(M-CH_2C_6H_4OCH_3)^-$, 10%]

Anal. Calcd for $C_{41}H_{46}O_7$ (650.81): C, 75.67; H, 7.12; Found; C, 75.7; H, 7.17.

DL-2,4,5-Tri-*O*-benzyl-*myo*-inositol (9)

The *cis*-propenyl ether **8** (500mg, 0.77mmol) was refluxed in 1M HCl - ethanol (1:2) for 2h, after which TLC (ethyl acetate/hexane 4:1) showed conversion to a major product at R_f 0.34. After cooling, the mixture was evaporated to dryness under reduced pressure. The residue was taken up in dichloromethane (50mL), washed with sat. $NaHCO_3$ and water (50mL of each), dried ($MgSO_4$) and evaporated to give a white solid, which was purified by column chromatography (ethyl acetate/ hexane 4:1) giving **9** (310mg, 0.69mmol, 90%).

Mp: 135 - 136.5°C with a phase change at 127 - 128°C (from ethyl acetate/ hexane); Lit. [145] 135-137°C; Lit.[124] 126-128°C.

^1H NMR ($CDCl_3$, 270MHz): δ 2.38 (1 H, d, J = 5.4 Hz, D_2O ex, C-3-OH), 2.48 (1H, dd, J = 5.9 Hz, D_2O ex, C-1-OH), 2.63 (1 H, br s, D_2O ex, C-6-OH), 3.32 (1 H, dd, J = 9.3 Hz, 9.3 Hz C-5-H), 3.44 (1 H, ddd, J = 9.7 Hz, 5.9 Hz, 2.8 Hz, D_2O ex gives dd, J = 9.7 Hz, 2.8 Hz, C-1-H), 3.57 (1 H, ddd, J = 9.7 Hz, 5.4 Hz, 2.6, D_2O ex gives dd, J = 9.7 Hz, 2.6 Hz, C-3-H), 3.78 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-4-H), 3.83 (1 H, br dd, D_2O ex gives dd, J = 9.5 Hz, 9.5 Hz, C-6-H), 3.98 (1 H, dd, J = 2.7 Hz, 2.7 Hz, C-2-H), 4.75-4.94 (6H, m, $OCH_2C_6H_5$), 7.26 - 7.38 (15H, m, $OCH_2C_6H_5$)

^{13}C NMR ($CDCl_3$, 68 MHz): δ 72.33, 72.70, 74.02, 78.89, 81.78, 82.95 (6 d, inositol ring C), 75.17, 75.23, 75.41 (3 t, $OCH_2C_6H_5$), 127.76, 127.79, 127.89, 128.04, 128.46, 128.54 (6 d, 3 $CH_2C_6H_5$) 138.35, 138.48 (2 s, 3 $CH_2C_6H_5$)

MS: *m/z* (+ve ion FAB, rel intensity) 451 [$(M+H)^+$, 3%], 181(20%), 91 [$(C_7H_7)^+$, 100%]

MS: *m/z* (–ve ion FAB, rel intensity) 616 (70%), 603 [$(M + NBA)^-$, 92%], 496 (30%), 449 [$(M - H)^-$, 100%], 359 [$(M - C_7H_7)^-$, 23%].

DL-2,5,6-Tri-*O*-benzyl-*myo*-inositol 1,3,4-tris(dibenzylphosphate) (10)

To a solution of bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (920mg, 2.66mmol) in dry dichloromethane (3mL) was added 1*H*-tetrazole (280mg, 4.00mmol). The mixture was stirred at room temperature for 10mins. **9** (200mg, 0.44mol) was added, and stirring continued for a further 1h. TLC (ethyl acetate) showed complete conversion of the triol (R_f 0.44) to a product (R_f 0.70) and ^{31}P NMR spectroscopy showed phosphite triester peaks at 140.39 (2P) and 142.2 (1P) . Water (5mL) was added and after stirring for a further 5min, *tert*-butyl hydroperoxide (1mL of a 70% solution in water). Stirring was continued overnight after which TLC showed conversion of the trisphosphite to a new product (R_f 0.64). The solvents were removed by evaporation *in vacuo* and then ethanol (20mL), water (20mL) and sodium metabisulphite (1.5g) were added. After stirring at rt. for 15min the mixture was evaporated to give a paste which was taken up in ether (100mL), washed with water (2 x 50mL), dried ($MgSO_4$), and evaporated to give an oil (680mg). This was purified by column chromatography (ethyl acetate) giving **10** (398mg, 0.323mmol, 73%) as a colourless oil.[124]

^{1}H NMR ($CDCl_3$, 270 MHz): δ 3.45 (1 H, dd, J = 9.4 Hz, 9.4 Hz C-5-H), 4.06 (1 H, dd. J = 9.4 Hz, 9.4 Hz, C-6-H), 4.22-4.36 (2 H, m, C-1-H and C-3-H), 4.62 (1 H, dd, J = 2.3 Hz, 2.3 Hz, C-2-H), 4.74 - 5.05 (19 H, m, 9 $CH_2C_6H_5$ and C-4-H), 7.12 - 7.38 (45 H, m, 9 $CH_2C_6H_5$).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 69.20, 69.28, 69.36, 69.49, 69.74, 69.82 (6 t, 6 PO-$CH_2C_6H_5$), 75.27, 75.51, 75.74 (3 t, 3 O$CH_2C_6H_5$), 77.63, 77.92, 78.04, 79.37, 79.47, 80.53, (6 d, inositol ring C), 127.21, 127.29, 127.36, 127.42, 127.53, 127.65, 127.73, 127.79, 127.96, 128.07, 128.12, 128.25, 128.46, 128.72, 130.80 (15 d, 9 $CH_2C_6H_5$), 135.47, 137.93, 138.19 (3 s, 9 $CH_2C_6H_5$)

^{31}P NMR ($CDCl_3$, 162MHz) δ 1.41, -1.56 and -2.01.

MS: *m/z* (+ve ion FAB, rel intensity) 1231 [$(M + H)^+$, 5%], 1230(6%), 181 (10%), 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1383 [$(M + NBA)^-$, 5%], 1139 [$(M - C_7H_7)^-$, 30%], 1138(35%), 1138 (35%), 277 [$(C_6H_5CH_2O)_2PO_2)^-$, 100%].

DL-*myo*-Inositol-1,3,4-trisphosphate (11)

Ammonia was condensed into a three neck flask at -78°C. An excess of sodium was added to dry the liquid ammonia, which was then distilled into a second three neck flask and kept at -78°C. Sodium was added until the solution remained blue. Compound **10**

(100mg, 81.2mmol) was dissolved in anhydrous dioxane (2mL) and added to the sodium - liquid ammonia mixture. After stirring for 3 min the reaction was quenched with ethanol. Ammonia and ethanol were evaporated. The residue was dissolved in de-ionised water (500mL) and purified by ion-exchange chromatography on Q Sepharose Fast Flow, eluting with a gradient of triethylammonium bicarbonate buffer (0 to 1M), pH 8.0. The triethylammonium salt of **11** eluted between 400 mM and 470 mM. Yield 26mmol, 32%).[124, 258]

^{1}H NMR (D_2O, 400 MHz, pH ~4.2): δ 3.40 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-5-H), 3.68 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-6-H), 3.85 (1 H, ddd, J = 9.3 Hz, 8.8 Hz, 2.4 Hz, C-1-H), 3.95 (1 H, ddd, J = 9.8 Hz, 9.8 Hz, 2.5 Hz, C-3-H), 4.16 (1 H, ddd, J = 9.3 Hz, 9.3 Hz, 9.3 Hz, C-4-H), 4.40 (1 H, dd, J = 2.4 Hz, 2.4 Hz, C-2-H)

^{31}P NMR (D_2O, 162 MHz, pH ~7) δ 0.13 (1 P, d, J = 8.6 Hz), 0.24 (1 P, d, J = 9.5 Hz), 0.97 (1 P, d, J = 8.5 Hz).

MS: *m/z* (+ve ion FAB, rel intensity) 102 [$(C_2H_5)_3NH^+$, 100%]

MS: *m/z* (–ve ion FAB, rel intensity) 838 [$2M^-$, 12%], 419 [M^-, 100%], 97 [$H_2PO_4^-$, 10%]

MS: *m/z* 418.954 $(M–H)^-$ (calcd for $C_6H_{14}O_{15}P_3$, 418.955).

DL-2,4,5-Tri-*O*-benzyl-1-*O*-*p*-methoxybenzyl-*myo*-inositol (12)

A solution of **8** (0.83g, 1.28mmol) in acetone (45mL) was warmed to 50°C with stirring. 1M HCl (5mL) was added, and stirring continued for 10mins, after which TLC (ether) showed complete conversion of starting material (R_f 0.76) to a major product at R_f 0.56. Sodium hydrogen carbonate (1g) was added, and stirring continued for a further 10min, as the solution was allowed to cool to rt. The solvents were removed under reduced pressure and the residue was taken up in dichloromethane (100mL). The suspension was washed with water (2 x 100mL), dried ($MgSO_4$), and evaporated to give an oil which was purified by column chromatography (ether) to give **12** (0.69g, 1.21mmol, 94%).

Mp: 100 - 102°C (from hexane / ethyl acetate).

^{1}H NMR ($CDCl_3$, 270MHz): δ 2.27 (1 H, d, J = 6.4 Hz, D_2O ex. C-3-OH), 2.50 (1 H, d, J = 1.5 Hz, D_2O ex, C-6-OH), 3.26 (1 H, dd, J = 9.5 Hz, 2.2 Hz, C-1-H), 3.38 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-5-H), 3.51 (1 H, ddd, J = 9.3 Hz, 6.4 Hz, 2.2 Hz, D_2O ex gives dd, J = 9.3 Hz, 2.2 Hz, C-3-H), 3.78 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-4-H), 3.81 (3 H, s, $C_6H_4OCH_3$), 4.04 (1 H, dd, J = 2.2 Hz, 2.2 Hz, C-2-H), 4.13 (1 H, ddd, J = 9.3 Hz, 9.3Hz, 1.5, D_2O ex gives dd, J = 9.3 Hz, 9.3 Hz, C-6-H), 4.53, 4.61 (2H, AB, J_{AB}, 11.4,

OCH_2Ph), 4.67 - 4.93 (6 H, m, OCH_2Ph), 6.88 (2 H, br d, J = 8.2Hz, $CH_2C_6H_4OMe$), 7.24 - 7.74 (17 H, m, $CH_2C_6H_4OMe$ and 3 $CH_2C_6H_5$).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 55.27 (q, OCH_3), 72.15, 74.62, 75.20, 75.46, (4 t, OCH_2Ph), 72.51, 73.04, 76.14, 80.03, 81.87, 83.23 (6 d, inositol ring C), 113.98 (d, $OCH_2C_6H_4OCH_3$), 127.65, 127.78, 127.91, 128.04, 128.35, 128.41, 128.48, 129.45 (8 d, 3 $CH_2C_6H_5$ and $CH_2C_6H_4OCH_3$), 129.32 (s, $CH_2C_6H_4OMe$), 138.04, 138.17 (2 s, 3 $CH_2C_6H_5$), 158.90 (s, $CH_2C_6H_4OMe$).

MS: *m/z* (+ve ion FAB, rel intensity) 569 [$(M-H)^+$, 7%], 479 [$(M-C_7H_7)^+$, 2%], 449 [$(M-CH_2C_6H_4OCH_3)^+$, 10%], 181(60%), 121 [$(CH_2C_6H_4OCH_3)^+$, 90%], 91 [$(C_7H_7)^+$, 100%];

MS: *m/z* (–ve ion FAB, rel intensity) 723 [$(M+NBA)^-$, 100%], 569 [$(M-H)^-$, 50%], 322 (42%), 140 (52%).

Anal. Calcd for $C_{35}H_{38}O_7$ (570.68) C, 73.66; H 6.71, Found C, 73.8; H, 6.65.

Resolution of DL-2,4,5-tri-*O*-benzyl-1-*O*-*p*-methoxybenzyl-*myo*-inositol

A mixture of **12** (1.35g, 2.37mmol) and (1*S*,4*R*)-(–)-ω-camphanic acid chloride (1.54g, 7.11mmol) and DMAP (50mg, 0.41mmol) in dry pyridine (10mL) was stirred for 1h at rt, after which TLC (chloroform/acetone 30:1) showed complete conversion from starting material (R_f 0.13) to two products (R_f 0.17 and R_f 0.24). The mixture was cooled in an ice bath, and water (1mL) was added. Stirring was continued for a further 1h. Ether (100mL) and dichloromethane (50mL) were added, and the organic phase was washed successively with sat. KCl, ice-cold 1M HCl, sat. KCl, and sat. $NaHCO_3$ (100mL of each), dried ($MgSO_4$), and evaporated to give the mixture of diastereoisomers (2.16g, 2.32mmol, 98%).

The mixture of diastereoisomers was separated by column chromatography (chloroform/acetone 30:1) into two fractions, which were recrystallised from methanol to give the two diastereoisomers; **13a** , R_f 0.17 (683mg, 0.734mmol, 63.3% of this diastereoisomer) and **13b**, R_f 0.24 (796mg, 0.855mmol, 73.7% of this diastereoisomer).

1D-2,5,6-Tri-*O*-benzyl-1,4-di-*O*-[(–)-ω-camphanoyl]-3-*O*-*p*-methoxybenzyl-*myo*-inositol (13a)

Mp: 186-188°C (from methanol)

^1H NMR ($CDCl_3$, 270MHz): δ 0.74, 0.84 (6 H, 2s, camph-CH_3), 0.96 (6 H, br s, camph-CH_3), 1.04, 1.07 (6 H, 2s, camph-CH_3), 1.60-1.74 (2 H, m, camph-CH_2), 1.78 - 1.96 (4

H, m, camph-CH_2), 2.21 - 2.37 (2 H, m, camph-CH_2), 3.63 (1 H, dd, J = 10.8 Hz, 1.5 Hz, C-3-H), 3.70 (1 H, dd, J = 9.5 Hz, 9.5 Hz, C-5-H), 3.79 (3 H, s, $CH_2C_6H_4OCH_3$), 4.23 (1 H, br s, C-2-H), 4.28 (1 H, dd, J = 9.7 Hz, 9.7 Hz, C-6-H), 4.47 - 5.05 (9 H, m, 4 CH_2Ph and C-1-H), 5.85 (1 H, dd, J = 9.9 Hz, 9.9 Hz C-4-H), 6.86 (2 H, br d, J = 8.4 Hz, C_6H_4OMe), 7.20 - 7.44 (17 H, m, C_6H_4OMe and 3 C_6H_5).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 15.72, 15.97, 16.06, (3 q, camph-CH_3), 28.35, 30.33, 30.47 (3 t, camph-CH_2), 53.42, 53.57, 54.18 (3 s, camph), 54.70 (q, OCH_3), 71.62, 74.03, 74.18, 74.63 (4 t, CH_2Ph), 73.24, 73.47, 74.41, 77.85, 78.22, 80.10 (6 d, inositol ring C), 90.17, 90.43 (2 s, camph), 113.31 (d, C_6H_4OMe), 126.37, 126.61, 126.82, 126.92, 127.10, 127.16, 127.70, 127.76, 128.23 (9 d, 3 $CH_2C_6H_5$ and C_6H_4OMe), 137.30, 137.40 (2 s, 3 $CH_2C_6H_5$), 158.78 (s, C_6H_4OMe), 166.17, 166.83, 177.21, 177.70, (4 s, camph C=O).

MS: *m/z* (+ve ion FAB, rel intensity) 929 [$(M-H)^+$, 0.7%], 839 [$(M-C_7H_7)^+$, 0.7%], 809 [$(M-CH_2C_6H_4OCH_3)^+$, 1.2%], 121 [$(CH_2C_6H_4OCH_3)^+$, 100%], 91 [$(C_7H_7)^+$,68%]

MS: *m/z* (–ve ion FAB, rel intensity) 1083 [$(M + NBA)^-$, 7%], 197 [$(camphO)^-$,100%]

$[\alpha]_D^{27} = -11$ (c = 1 in $CHCl_3$)

Anal. Calcd for $C_{55}H_{62}O_{13}$ (931.09); C, 70.95; H 6.71, Found C, 70.8; H, 6.76.

1L-2,5,6-Tri-*O*-benzyl-1,4-di-*O*-[(–)-ω-camphanoyl]-3-*O*-*p*-methoxybenzyl-*myo*-inositol (13b)

Mp: 194-195°C (from methanol).

^{1}H NMR ($CDCl_3$, 400MHz): δ 0.82, 0.88, 0.96, 1.00, 1.05, 1.08 (18 H, 6 s, camph-CH_3), 1.54 - 1.71 (2 H, m, camph-CH_2), 1.72 - 1.88 (4 H, m, camph-CH_2), 2.34 - 2.31 (2 H, m, camph-CH_2), 3.62 (1H, dd, J = 10.4 Hz, 2.1 Hz C-3-H), 3.68 (1 H, dd, J = 9.5 Hz, 9.5 Hz, C-5-H), 3.79 (3 H, s, $C_6H_4OCH_3$), 4.12 (1 H, dd, J = 2.1Hz, 2.1 Hz, C-2-H), 4.24 (1 H, dd, J = 9.5 Hz, 9.5 Hz, C-6-H), 4.47, 4.58 (2 H, AB, J_{AB} 11.3 Hz, $CH_2C_6H_4OCH_3$), 4.59 - 4.88 (6 H, m, 3 $CH_2C_6H_5$), 5.00 (1 H, dd, J = 10.4 Hz, 2.4 Hz, C-1-H), 5.82 (1 H, dd, J = 9.8 Hz, 9.8 Hz, C-4-H), 6.86 (2 H, br d, J = 8.9 Hz, C_6H_4OMe), 7.19 - 7.38 (17 H, m, C_6H_4OMe and 3 C_6H_5)

^{13}C NMR ($CDCl_3$, 68 MHz): δ 16.45, 16.59, 16.72, (3 q, camph-CH_3), 28.85, 30.73, 30.85 (3 t, camph-CH_2), 54.18, 54.77, 54.83 (3 s, camph), 55.27 (q, OCH_3), 71.88, 75.01, 75.20 75.27 (4 t, CH_2Ph), 74.07, 74.77, 74.81, 77.88, 78.77, 81.23 (6 d, inositol ring C), 90.75, 91.13 (2 s, camph), 113.88 (d, C_6H_4OMe), 127.21, 127.26, 127.49, 127.55, 127.67, 128.25, 128.36, 128.82 129.38 (9d, 3 $CH_2C_6H_5$ and C_6H_4OMe), 137.96

138.14 (2 s, 3 $CH_2C_6H_5$), 159.32 (s, C_6H_4OMe), 166.73, 167.29, 177.84, 178.46, (4 s, camph C=O).

MS: *m/z* (+ve ion FAB, rel intensity) 929 [$(M-H)^+$, 0.8%], 839 [$(M-C_7H_7)^+$, 1.1%], 809 [$(M-CH_2C_6H_4OCH_3)^+$, 1.2%], 121 [$(C_7H_6OCH_3)^+$, 100%], 91 [$(C_7H_7)^+$, 58%]

MS: *m/z* (–ve ion FAB, rel intensity) 1083 [$(M + NBA)^-$, 11%], 197 [$(camph\text{-}O)^-$, 100%]

$[\alpha]_D^{27} = +5$ (c =1 in $CHCl_3$)

Anal. Calcd for $C_{55}H_{62}O_{13}$ (931.09); C, 70.95; H 6.71, Found C, 70.6; H, 6.70

(+)-1D-2,5,6-Tri-*O*-benzyl-3-*O*-*p*-methoxybenzyl-*myo*-inositol (12a)

The (–)-biscamphanate ester **13a** (570mg, 0.612mmol) was dissolved in methanol (100mL) containing sodium hydroxide pellets (4g) and refluxed for 30min. The mixture was cooled and then neutralised with solid carbon dioxide. After the solvents were removed by evaporation under reduced pressure, the residue was taken up in water (100mL), extracted with chloroform (2 x 100mL), dried ($MgSO_4$) and evaporated to give a white solid. This was recrystallised from ethyl acetate/ hexane giving **12a** (334mg, 0.585mmol, 96%)

Mp: 95- 96.5°C (from ethyl acetate / hexane).

$[\alpha]_D^{24} = +4$ (c = 1, $CHCl_3$)

Anal. Calcd for $C_{35}H_{38}O_7$ (570.68); C, 73.66; H 6.71, Found C, 73.6; H, 6.64

NMR and mass spectrometry data were identical to those for the racemic diol **12.**

(–)-1L-2,5,6-Tri-*O*-benzyl-3-*O*-*p*-methoxybenzyl-*myo*-inositol (12b)

The (+)-biscamphanate ester **13b** (770mg,0.827mmol) was converted to the diol **12b** as described for compound **13a**. Yield 461mg, 0.808mmol, 98%.

Mp: 95- 97°C (from ethyl acetate / hexane).

$[\alpha]_D^{22} = -4$ (c = 1, $CHCl_3$)

Anal. Calcd for $C_{35}H_{38}O_7$ (570.68); C, 73.66; H 6.71, Found C, 73.6; H, 6.66

NMR and mass spectrometry data were identical to those for the racemic diol **12.**

(–)-1L-1,2,4,5,6-Penta-*O*-benzyl-*myo*-inositol (14b)

The absolute configuration of **12b** was determined by converting it to the known pentabenzyl ether **14b.** A sample of the (–)-diol **12b** (60mg, 0.105mmol) was dissolved in dry DMF (5mL), and sodium hydride (20mg of a 60% dispersion in oil, 0.50mmol)

was added. After stirring at room temperature for 10min, benzyl bromide (0.05mL, 0.42mmol) was added and stirring continued for 2 h. The excess sodium hydride was destroyed by addition of water (5mL) and the mixture concentrated under reduced pressure. The residue was taken up in dichloromethane (20mL), washed with 0.1M HCl (20mL) and evaporated. The residue was then refluxed in methanol / 1M HCl 2:1 for 5 h after which time TLC showed complete conversion to the penta-*O*-benzyl ether. The solvents were removed by evaporation *in vacuo* and the residue was taken up in dichloromethane (30mL), washed with sat. $NaHCO_3$ and water (20mL of each), dried ($MgSO_4$) and evaporated. Column chromatography (pentane / ether 1:1) gave the pure penta-*O*-benzyl ether, which had an ^1H NMR spectrum identical to an racemic 1,2,4,5,6-penta-*O*-benzyl-*myo*-inositol,[164] but a specific rotation of −11.5°, allowing its absolute configuration to be assigned as **L-14b**. (52mg, 0.0824mmol, 78% from **12b**).

$[\alpha]_D^{22}$ = −11.5 (*c* = 2 in $CHCl_3$) [lit. [163] $[\alpha]_D$ = −13.5 (*c* = 0.5, $CHCl_3$); lit. [259] $[\alpha]_D$ = +10.0 (*c* =1, $CHCl_3$) for the enantiomer; Lit.[167] $[\alpha]_D$ = +9.7 (*c* = 1.5, $CHCl_3$) for the enantiomer].

^1H NMR ($CDCl_3$, 270MHz): δ 2.22 (1 H, d, *J* = 6.0Hz, D_2O ex., C-3-OH); 3.43 - 3.53 (3H, m, C-1-H, C-3-H, C-5-H); 3.81 (1 H, dd, *J* = 9.5 Hz, 9.5 Hz, C-6-H or C-4-H); 4.03 (1 H, dd, *J* = 2.2 Hz, 2.2 Hz, C-2-H); 4.06 (1 H, dd, *J* = 9.5 Hz, 9.5 Hz, C-6-H or C-4-H); 4.70 - 5.02 (10 H, m, $CH_2C_6H_5$); 7.25 - 7.38 (25 H, m, C_6H_5).

(−)-1D-2,5,6-Tri-*O*-benzyl-*myo*-inositol (9a)

The (+)-diol **12a** (300mg, 0.526mmol) was dissolved in ethanol (80mL) and 1M HCl (40mL) was added. The mixture was refluxed for 4h and the solvents evaporated under reduced pressure. The residue was taken up in dichloromethane (50mL), washed with water, saturated $NaHCO_3$ and brine (30mL of each) and dried ($MgSO_4$). The solvents were removed and the residue purified by flash chromatography (ethyl acetate/ hexane 4:1) to give a white solid which was recrystallised from ethyl acetate/ hexane giving the triol **9a** (213mg, 0.473mmol, 90%).

Mp: 104 - 106°C with a phase change at 92 - 93°C (from ethyl acetate / hexane); Lit.[143] 103 - 105°C

$[\alpha]_D^{23}$ = −32 (*c* = 1, $CHCl_3$) [Lit. [23] $[\alpha]_D^{25}$ = −27 (*c* = 1, $CHCl_3$). Lit. [24] $[\alpha]_D$ = −25 (*c* = 0.5, $CHCl_3$)].

NMR and mass spectrometry data were identical to those for the racemic triol **9**.

(+)-1L-2,5,6-Tri-*O*-benzyl-*myo*-inositol (9b)

The (–)-diol **12b** (350mg, 0.613mmol) was treated as described for the enantiomer above to give the (+)-triol **9b** (250mg, 0.555mmol, 91%).

Mp: 104- 106°C with a phase change at 93 - 94°C (from ethyl acetate / hexane); Lit.[143] 104 - 106°C

$[\alpha]_D^{21}$ = +32 (c = 1, $CHCl_3$). [Lit.[143] $[\alpha]_D^{25}$ = + 25 (c = 1, $CHCl_3$)].

NMR and mass spectrometry data were identical to those for the racemic triol **9**.

(+)-1D-2,5,6-Tri-*O*-benzyl-*myo*-inositol 1,3,4-tris(dibenzylphosphate) (10a)

The (–)-triol **9a** (60mg, 0.133mmol) was phosphitylated as described for the racemic material. Oxidation and purification as before gave **10a** as a colourless oil (126mg, 0.102mmol, 77%).

$[\alpha]_D^{20}$ = +6 (c = 1, $CHCl_3$)

Anal. Calcd for $C_{69}H_{69}O_{15}P_3$; C, 67.31; H 5.65, Found C, 67.3; H, 5.70

NMR and mass spectrometry data were identical to those for racemic **10**.

(–)-1L-2,5,6-Tri-*O*-benzyl-*myo*-inositol 1,3,4-tris(dibenzylphosphate) (10b)

The (+)-triol **9b** (60mg, 0.133mmol) was phosphitylated as described for the racemate giving **9b** as a colourless oil. Yield 123mg, 0.100mmol, 75%.

$[\alpha]_D^{20}$ = –6 (c = 1, $CHCl_3$) Lit.[143] $[\alpha]_D^{25}$ = –5.8 (c = 1, $CHCl_3$)

NMR and mass spectrometry data were identical to those for racemic **10**.

(+)-1D-*myo*-inositol-1,3,4-trisphosphate (11a)

The fully-protected trisphosphate triester **10a** (80mg, 65μmol) was deprotected and purified as described for the racemic material to give **11a** as the glassy triethylammonium salt. Yield 34μmol, 52%

$[\alpha]_D^{26}$ = +37 (c = 0.42, TEAB buffer, pH 7.8) Calculated for the free acid. Lit.[95] $[\alpha]_D$ = –6 (c = 0.5, H_2O), Lit.[98] $[\alpha]_D$ = +13.6 (c = 2, H_2O, pH 8.2, potassium salt).

MS: m/z 418.956 $(M-H)^-$ (calcd for $C_6H_{14}O_{15}P_3$, 418.955).

NMR data were identical to those for the racemate **11**.

(–)-1L-*myo*-inositol-1,3,4-trisphosphate (11b)

Compound **104b** (100mg, 81.2μmol) was deprotected and purified as described for the racemic material giving the triethylammonium salt **11b**. Yield 31μmol, 38%).

$[\alpha]_D^{26}$ = –40 (c = 0.42, TEAB buffer, pH 7.8) Calculated for the free acid.

MS: *m/z* 418.957 $(M-H)^-$ (calcd for $C_6H_{14}O_{15}P_3$, 418.955).

NMR data were identical to those for **11**.

8.3 Acyclic Analogue

(2*S*,3*S*)-butane-1,2,3,4-tetrol-1,2,4-tris[bis(4-chlorobenzyl)phosphate)] (16)

To a solution of bis(4-chlorobenzyloxy)-*N*,*N*-diisopropylaminophosphine[177] (1.24g, 2.99mmol) in dry dichloromethane (5mL) was added 1*H*-tetrazole (420mg, 5.99mmol). The mixture was stirred at room temperature for 10 min and then (2*S*,3*S*)-(+)-2-benzyloxybutane-1,3,4-triol (106mg, 0.499mmol) was added. The mixture was stirred for a further 0.5 h, after which a 90 MHz ^{31}P NMR spectrum showed signals around 139ppm (two doublets and a triplet, J_{PP} = 1.5 Hz) corresponding to the trisphosphite triester. The mixture was cooled to -78°C and *m*-CPBA (570mg, 3.30 mmol) was added. The mixture was warmed to room temperature, with stirring, and then diluted with ether (50mL). The solution was washed with 10% sodium sulphite solution, sat. $NaHCO_3$ and brine (50mL of each), dried ($MgSO_4$) and evaporated *in vacuo* to give an oil. Purification by column chromatography (ether, then ethyl acetate) gave the trisphosphate triester **16** as a colourless oil (437mg, 0.364mmol, 73%).

R_f 0.54 (Ethyl acetate); $[\alpha]_D^{20}$ ~0 (*c* = 1, $CHCl_3$).

^1H NMR ($CDCl_3$, 400 MHz): δ 3.76 (1 H, dt, *J*= 4.6 Hz, 4.6 Hz, C-3-H), 4.03-4.09 (1 H, m, CH_2OP), 4.13-4.19 (1 H, m, CH_2OP), 4.21-4.26 (2 H, m, CH_2OP), 4.47, 4.59 (2 H, AB_q, J_{AB} = 11.6 Hz, OC$H_2C_6H_5$), 4.68 (1 H, m, C-2-H), 4.85-4.98 (12 H, m, 6 x OC$H_2C_6H_4$Cl), 7.11-7.29 (29 H, m, 6 x C_6H_4Cl and C_6H_5).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 65.14 (t, broad, CH_2OP), 69.93 (t, broad, CH_2OP), 68.64, 68.73, 68.76 (overlapping triplets with J_{COP} coupling, 6 x POCH_2Ar), 73.12 (t, OC$H_2C_6H_5$), 75.86 (d, J_{COP} = 5.5Hz, 7.3 Hz, C-2 or C-3), 76.12 (d, J_{COP} = 5.5Hz, 5.6 Hz, C-2 or C-3), 127.83, 128.11, 128.22, 128.49, 128.77, 128.82, 129.04, 129.21, 129.24, 129.30, 129.55 (d, C_6H_5 and C_6H_4Cl), 133.80, 133.87, 133.94, 134.55, 134.60, 134.66 (6 s, 6 x C-1 and C-4 of C_6H_5Cl), 137.01 (s, *C*-1 of C_6H_5).

^{31}P NMR ($CDCl_3$, 162MHz, ^1H-decoupled) δ -0.96, -1.13 and -1.52.

MS: *m/z* (+ve ion FAB, rel intensity) 1202(30%), 1201(76%), 1200(42%) 1199(82%), 1198(16%), 1197(34%) [isotopomers of $(M+H)^+$], 127 [$(C_7H_6{}^{37}Cl)^+$, 100%], 125 [$(C_7H_6{}^{35}Cl)^+$,33%].

MS: *m/z* (–ve ion FAB, rel intensity) 1073 [$(M–C_7H_6{}^{35}Cl)^-$, 85% and various isotopomer peaks], 345 [$(^{35}ClC_7H_6O)_2PO_2)^-$, 100%].

Anal. Calcd for $C_{53}H_{49}O_{13}P_3Cl_6$ (1199.60); C, 53.07; H 4.12, Found C, 52.8; H, 4.14.

(2*S*,3*S*)-butane-1,2,3,4-tetrol-1,2,4-trisphosphate (15)

Ammonia (~100mL) was condensed into a three-neck flask at –78°C. An excess of sodium was added to dry the liquid ammonia and the deep blue solution was stirred at –78°C for 30min. A small volume of the dry ammonia (~30mL) was then distilled into a second three-neck flask and kept at –78°C. Sodium was added until the solution remained blue-black for 10 min. A solution of **16** (95mg, 79μmol) in dry dioxane (2mL) was added to the vigorously-stirring sodium - liquid ammonia mixture. After 60 - 90 sec the reaction was carefully quenched with methanol, followed by de-ionised water. Ammonia and solvents were then removed by evaporation *in vacuo*. The residue was dissolved in de-ionised water (500mL) and purified by ion-exchange chromatography on Q Sepharose Fast Flow Resin, eluting with a gradient of triethylammonium bicarbonate buffer (0 to 1M), pH 8.0. The glassy triethylammonium salt of **15** eluted between 300 mM and 360 mM. Yield 33μmol, 42%).

[The NMR spectra of **15** were difficult to interpret, due to broad signals in the ^{31}P NMR spectrum at acidic pH, and obscuring of signals in the ^1H NMR spectrum by peaks from the triethylammonium ions. **15** was therefore converted to its Na^+ salt by treatment with H^+ Dowex resin, followed by Chelex-100 (Na^+ form)].

$[\alpha]_D^{20}$ ~0 (c = 0.2, H_2O).

^1H NMR (D_2O, 400 MHz, Na^+ salt, pH 6-7): δ 3.68-3.76 (1 H, m, CH_2OP), 3.77-3.86 (3 H, m, CH_2OP), 3.86-3.91 (1 H, m, C-3-H), 4.11-4.18 (1 H, m, C-2-H).

^{31}P NMR (D_2O, 161.7 MHz, ^1H-coupled, Na^+ salt, pH 6-7) δ 0.96 (1 P, d, J_{HP} = 9.4 Hz, P-2), 1.92 (1 P, t, J_{HP} = 5.8 Hz), 2.07 (1 P, t, J_{HP} = 6.5 Hz)

8.4 Five-membered ring Analogue

Methyl 2-*O*-benzyl-6,7-dideoxy-3,4-di-*O*-*p*-methoxybenzyl-α-D-*gluco*-hept-6-enopyranoside (1,5) (20)

Into a dry three-neck flask under N_2 was placed oxalyl chloride (3.2mL of a 2M solution in dichloromethane, 6.4mmol) followed by dry dichloromethane (10mL). The apparatus was cooled to −60°C and a solution of anhydrous dimethylsulphoxide (0.90mL, 12.7mmol) in dry dichloromethane (15mL) was carefully injected over a period of 5 min. The mixture was stirred for 5 min and then a solution of methyl 2-*O*-benzyl-3,4-di-*O*-*p*-methoxybenzyl-α-D-glucopyranoside (**18**) (3.00g, 5.72mmol) in dry dichloromethane (10mL) was added dropwise over 5 min. Stirring was continued at −60 °C for a further 20 min and then dry triethylamine (5mL) was added. After stirring for 5 min, the reaction was allowed to reach room temperature. The solvents were removed by evaporation *in vacuo* and the residue was taken up in dichloromethane (15mL) to give a slurry, which was loaded onto a short column of silica and eluted with ethyl acetate/hexane 3:2, giving the crude aldehyde as a pale yellow oil (2.6g). This was used for the next step without further purification.

A dry 100mL flask was charged with methyltriphenylphosphonium bromide (3.70g, 10.36mmol), previously dried *in vacuo* at 60°C. Dry THF (10mL) was added and the suspension was cooled to −10°C, with stirring, under N_2. Potassium *tert*-butoxide (9.8mL of a 1.0M solution in dry THF, 9.8mmol) was added, and the yellow suspension was allowed to reach room temperature. After stirring for a further 10 min the suspension was cooled to −10°C once more, and a solution of the crude aldehyde (2.6g) in dry THF (5mL) was added. The colour darkened to deep orange almost immediately, and the mixture was allowed to warm to room temperature. Stirring was continued for a further 1 h and then the solvent was removed by evaporation *in vacuo* giving a brown oil. Purification by column chromatography (ethyl acetate/pentane 1:3) gave pure **20** as a waxy solid (2.23g, 4.28mmol, 75% from **18**.

^{1}H NMR ($CDCl_3$, 270MHz): δ 3.21 (1 H, dd, J = 9.3 Hz, 9.2 Hz, C-4-H), 3.37 (3 H, s, OCH_3), 3.50 (1 H, dd J = 9.7 Hz, 3.2 Hz, C-2-H), 3.79, 3.80 (6 H, 2 s, 2 x $ArOCH_3$), 3.96 (1 H, dd, J = 9.5 Hz, 9.2 Hz, C-3-H), 4.02 (1 H, dd, J = 9.3 Hz, 6.8 Hz, C-5-H), 4.51-4.90 (6 H, m, 3 x $ArCH_2O$ AB systems), 4.58 (1 H, d, J = 3.2 Hz, C-1-H), 5.26 (1 H, br d, J = 10.1 Hz, C-7-H, *cis*), 5.40 (1 H, br d, J = 17.2 Hz, C-7-H, *trans*), 5.88 (1H, ddd, J = 17.2 Hz, 10.1 Hz, 6.8 Hz, C-6-H), 6.83–7.36 (13 H, m, aromatic CH).

^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 55.11, 55.25 (2 q, OCH_3), 71.41 (d, *C*H), 73.38, 74.75, 75.51 (3 t, Ar*C*H_2O), 79.87, 81.44, 81.95 (3 d, *C*H), 98.08 (d, C-1), 113.75, 113.80 (2 d, 2 x C-3 and C-5 of PMB rings), 118.01 (t, C-7), 127.84, 128.05, 128.41, 129.56, 129.59 (5 d, aromatic *C*H), 130.42, 131.07 (2 s, 2 x C-1 of PMB rings), 135.32 (d, C-6), 138.24 (s, C-1 of benzyl ring), 159.18, 159.26 (2 s, 2 x C-4 of PMB rings).

MS: *m/z* (+ve ion FAB, rel intensity) 519 [$(M-H)^+$, 2.0%)], 121 [$(CH_2C_6H_4OCH_3)^+$, 100%], 91 [$(C_7H_7)^+$ 20%];

MS: *m/z* (–ve ion FAB, rel intensity) 673 [(M + NBA)$^-$, 25%], 399 [(M–$CH_2C_6H_4OCH_3)^-$, 100%].

$[\alpha]_D^{23} = -23$ (c = 1, $CHCl_3$)

Anal. calcd. for $C_{31}H_{36}O_7$ (520.62) C, 71.52; H, 6.97 Found: C, 71.5; H, 7.07.

Zirconium-Mediated Ring Contraction[178]

Zirconocene dichloride (672mg, 2.30mmol) was placed into a dry 100mL flask under N_2, followed by dry THF (10mL). The suspension was cooled to –78°C and *n*-butyllithium (1.84mL of a 2.5M solution in hexane, 4.60mmol) was added. The mixture was stirred at –78°C for 1 h and then a solution of the vinyl pyranoside **20** (1.00g, 1.92mmol) in dry THF (8mL) was added. The clear yellow solution was allowed to reach room temperature and stirring was continued for 3 h, during which time it gradually darkened to reddish orange. The solution was cooled to 0°C and a solution of boron trifluoride etherate (0.45mL, 3.7mmol) in dry THF (5mL) was added. Stirring was continued at room temperature, and TLC (dichloromethane/ethyl acetate 10:1) showed conversion of starting material (R_f 0.72) to a major product (R_f 0.56) within 30min. After 45min, TLC showed that PMB groups were being lost. 1M HCl (50mL) was added and the mixture extracted with dichloromethane (2 x 50mL). The organic extracts were combined, washed with brine (100mL), dried ($MgSO_4$) and evaporated in *vacuo* giving a yellow oil, which was purified by column chromatography (dichloromethane/acetone 30:1) to give the major diastereoisomer **21b** (433mg, 0.883mmol, 46%) as a waxy solid. A small amount of the minor diastereoisomer **21a** was also isolated (~30mg, 0.06mmol, 3%).

(1*R*, 2*S*, 3*S*, 4*R*, 5*S*)-3-Benzyloxy-4-hydroxy-1,2-di-*p*-methoxybenzyloxy-5-vinylcyclopentane (Major Diastereoisomer) (21b)

R_f = 0.32 (dichloromethane/acetone 30:1)

^{1}H NMR($CDCl_3$, 400MHz): δ 1.99 (1 H, d, J = 4.22 Hz, D_2O ex, C-4-OH), 2.86 (1 H, ddd, J = 7.8 Hz, 7.8 Hz, 6.2 Hz, C-5-H), 3.77, 3.78 (6H, 2s, 2 x OCH_3), 3.77 (1 H, obscured by OCH_3, C-3-H), 3.93 - 4.01 (2 H, m, C-1-H and C-2-H), 4.11 (1 H, m, D_2O ex gives dd, J = 6.0 Hz, 3.1 Hz, C-4-H), 4.45 - 4.65 (6 H, m, 3 x ArCH_2O AB systems), 5.23 (1 H, br d, J = 17.2 Hz, $=CH_2$, *trans*), 5.25 ((1 H, br d, J = 10.6 Hz, $=CH_2$, *cis*), 5.91 (1 H, ddd, J = 17.2 Hz, 10.8 Hz, 8.0 Hz, CH=), 6.83 - 6.87 (4 H, m, C_6H_4OMe), 7.20 - 7.25 (4 H, m, C_6H_4OMe), 7.28 - 7.35 (5 H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 50.96 (d, C-5), 55.20 (q, 2 x OCH_3), 71.57 (t, OCH_2Ar), 71.60 (t, 2 x OCH_2Ar), 75.36, 84.49, 87.17, 87.91 (4 d, C-1, C-2, C-3, C-4), 113.67, 113.72 (2 d, C-3 and C-5 of PMB rings), 118.76 (t, $=CH_2$), 127.65, 127.73, 128.33, 129.40, 129.51 (5 d, aromatic *C*H), 130.13, 130.36 (2 s, 2 x C-1 of PMB rings), 134.69 (d, *C*H=), 138.06 (s, C-1 of benzyl ring), 159 21, 159.13 (2 s, C-4 of PMB rings).

MS: *m/z* (+ve ion FAB, rel intensity) 489 [$(M-H)^+$, 1.2%)], 369 [$(M-CH_2C_6H_4OCH_3)^+$, 6.0%), 121 [$(CH_2C_6H_4OCH_3)^+$, 100%];

MS: *m/z* (–ve ion FAB, rel intensity) 643 [$(M + NBA)^-$, 100%]

$[\alpha]_D$ = +9 (c = 1, $CHCl_3$)

Anal. Calcd. for $C_{30}H_{34}O_6$: C, 73.45; H, 6.99 Found: C, 73.2; H, 7.05.

(1*R*, 2*S*, 3*S*, 4*S*, 5*R*)-3-Benzyloxy-4-hydroxy-1,2-di-*p*-methoxybenzyloxy-5-vinylcyclopentane (Minor Diastereoisomer) (21a)

R_f = 0.36 (dichloromethane/acetone 30:1)

Mp: 83-85°C (from ethanol)

^{1}H NMR ($CDCl_3$, 400MHz): δ 2.53 (1 H, d, J = 8.3 Hz, D_2O ex, C-4-OH), 2.71 (1 H, ddd, J = 8.3 Hz, 7.8 Hz, 7.8 Hz, C-5-H), 3.66 (1 H, dd J = 7.8 Hz, 4.4 Hz, C-1-H), 3.79, 3.80 (6 H, 2s, 2 x OCH_3), 3.79 - 3.87 (2 H, m, partly obscured by OCH_3, C-3-H and C-4-H), 3.94 (1 H, dd, J = 4.3 Hz, 2.44 Hz, C-2-H), 4.43 - 4.69 (6 H, m, 3 x ArCH_2O AB systems), 5.16 (1 H, br d, J = 10.3 Hz, $=CH_2$, *cis*), 5.23 (1 H, br d, J = 17.1, $=CH_2$, *trans*), 5.84 (1 H, ddd, J = 17.1 Hz, 10.3 Hz, 7.8 Hz, CH=), 6.85 - 6.89 (4 H, m, C_6H_4OMe), 7.31 - 7.37 (4 H, m, C_6H_4OMe), 7.31 - 7.37 (5 H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 53.74 (d, C-5), 55.19 (q, 2 x OCH_3), 71.45, 71.62, 71.75 (3t, OCH_2Ar), 73.79, 81.75, 85.21, 86.13 (4 d, C-1, C-2, C-3, C-4), 113.67, 113.78 (2 d, C-3 and C-5 of PMB rings), 117.12 (t, $=CH_2$), 127.79, 127.89, 128.44, 129.35, 129.38 (5 d, aromatic *C*H), 129.93, 130.24 (2 s, 2 x C-1 of PMB rings), 137.59 (s, C-1 of benzyl ring), 137.82 (d, *C*H=), 159 14, 159.29(2 s, C-4 of PMB rings).

MS: *m/z* (+ve ion FAB, rel intensity) 489 [$(M-H)^+$, 1.0%)], 369 [$(M-CH_2C_6H_4OCH_3)^+$, 5.0%), 121 [$(CH_2C_6H_4OCH_3)^+$, 100%]; 91 [$(C_7H_7)^+$, 10%]

$[\alpha]_D = -26$ (c = 1, $CHCl_3$)

Anal. Calcd. for $C_{30}H_{34}O_6$: C, 73.45; H, 6.99 Found: C, 73.6; H, 7.02.

(1*R*, 2*S*, 3*S*, 4*R*, 5*R*)-3-Benzyloxy-1,2,4-trihydroxy-5-vinylcyclopentane (23)

The alcohol **21b** (175mg, 0.357mmol) was dissolved in ethanol (40mL) and 1M HCl (20mL) was added. The solution was refluxed for 3 h and then the solvents were removed by evaporation *in vacuo*. The residue was taken up in ethyl acetate (50mL), and washed with sat. $NaHCO_3$ and brine (25mL of each). The combined aqueous layers were re-extracted with ethyl acetate (50mL) and the combined organic layers were then dried ($MgSO_4$) and evaporated *in vacuo* to give a yellow oil. Purification by column chromatography (chloroform/methanol 5:1) gave the triol **23** as a waxy solid (78mg, 0.312mmol, 87%).

Mp: 64-66°C (from ether)

^1H NMR ($CDCl_3$, 400MHz): δ 2.55 (1 H, ddd, J = 9.3 Hz, 7.8 Hz, 7.3 Hz, C-5-H), 2.79 (1 H, br s, D_2O ex, OH), 2.92 (1 H, br s, D_2O ex, OH), 3.65 (1 H, dd J = 5.4 Hz, 2.0 Hz, C-3-H), 3.88 (1 H, br dd, D_2O ex gives dd, J = 7.3 Hz, 5.4 Hz, C-2-H), 3.95 - 4.00 (2 H, br m, sharpens on D_2O ex, C-1-H and C-4-H), 4.10 (1 H, br s, D_2O ex, OH), 4.54, 4.58 (2 H, AB, J_{AB} = 11.7 Hz, $OCH_2C_6H_5$), 5.20 (1H, dd, J = 17.1 Hz, 1.6 Hz, $=CH_2$, *trans*), 5.23 (1 H, dd, J = 10.3 Hz, 1.6 Hz, $=CH_2$, *cis*), 5.82 (1 H, ddd, J = 17.1 Hz, 10.3 Hz, 7.8 Hz, CH=), 7.22 - 7.32 (5 H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 100.4 MHz): δ 51.47 (d, C-5), 71.84 (t, $OCH_2C_6H_5$), 74.73, 76.67, 81.46, 89.09 (4 d, C-1, C-2, C-3, C-4), 119.56 (t, $=CH_2$), 127.86, 127.91, 128.48 (3 d, aromatic *C*H), 133.77 (d, *C*H=), 137.79 (s, C-1 of benzyl ring)

MS: *m/z* (+ve ion FAB, rel intensity) 501 [$(2M + H)^+$, 2.0%], 249 [$(M-H)^+$, 5.0%)], 149 (30%), 91 [$(C_7H_7)^+$, 100%]

MS: *m/z* (–ve ion FAB, rel intensity) 403 [$(M + NBA)^-$, 100%], 249 [$(M–H)^-$, 50%)], 149 (50%).

$[\alpha]_D = +53$ ($c = 1$, $CHCl_3$)

Anal. Calcd. for $C_{14}H_{18}O_4$: C, 67.18; H, 7.25 Found: C, 66.9; H, 7.44.

(1*R*, 2*R*, 3*S*, 4*R*, 5*S*)-3-Benzyloxy-1,2,4-tris(dibenzyloxyphosphoryloxy)-5-vinylcyclopentane (24)

To a solution of bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (670mg, 1.94mmol) in dry dichloromethane (2mL) was added 1*H*-tetrazole (200mg, 2.85mmol). The mixture was stirred at room temperature for 10 min and then the triol **23** (80mg, 0.320mmol) was added. The mixture was stirred for a further 1 h, after which a 90 MHz ^{31}P NMR spectrum showed signals around 139ppm (a singlet and an AB quartet with $J = 6.7$ Hz) corresponding to the trisphosphite triester. The mixture was cooled to –78°C and *m*-CPBA (517mg, 3.00mmol) was added. The mixture was warmed to room temperature, with stirring, and then diluted with ethyl acetate (50mL). The solution was washed with 10% sodium sulphite solution, 1M HCl, sat. $NaHCO_3$ and brine (50mL of each), dried ($MgSO_4$) and evaporated *in vacuo* to give an oil. Purification by column chromatography (chloroform/acetone 10:1) gave the trisphosphate triester **24** as a colourless oil (271mg, 0.263mmol, 82%).

^1H NMR ($CDCl_3$, 400MHz): δ 3.02 (1 H, m, C-5-H), 4.20 (1 H, br s, C-3-H), 4.50 (2 H, AB, J_{AB} = 11.2 Hz, $OCH_2C_6H_5$), 4.68 (1 H, br dd, J = 7.8 Hz, 4.9 Hz, C-4-H), 4.87 - 5.08 (14 H, m, 6 x $OCH_2C_6H_5$, C-1-H and C-2-H), 5.18 (1 H, d, J = 10.3 Hz, 1.5 Hz, $=CH_2$, *cis*), 5.26 (1 H, d, J = 17.1 Hz, 1.5 Hz, $=CH_2$, *trans*), 5.86 (1 H, ddd, J = 17.1 Hz, 10.3 Hz, 8.3 Hz, CH=), 7.17 - 7.33 (35 H, m, 7 x C_6H_5)

^{13}C NMR ($CDCl_3$, 100.4 MHz): δ 50.85 (d, C-5), 69.24, 69.37, 69.48, 69.57, 71.96 (5 t, 7 x $OCH_2C_6H_5$), 81.18, 84.05, 85.57, 86.23 (4 d, C-1, C-2, C-3, C-4), 120.72 (t, $=CH_2$), 127.43, 127.52, 127.74, 127.89, 127.98, 128.13, 128.18, 128.29, 128.46 128.86 (10 d, aromatic *C*H), 131.38 (d, *C*H=), 135.40, 135.48, 135.53, 135.59, 135.66, 135.75, 137.29 (7 s, 7 x C-1 of benzyl ring)

^{31}P NMR ($CDCl_3$, 161.7 MHz): δ –1.97, –2.12, –2.34

MS: *m/z* (+ve ion FAB, rel intensity) 1031 [$(M + H)^+$, 2.5%)], 149 (20%), 91 [$(C_7H_7)^+$, 100%]

MS: *m/z* (–ve ion FAB, rel intensity) 938(14), 277 [$(C_6H_5O)_2PO_2^-$, 100%]

$[\alpha]_D^{26} = +3.5$ ($c = 1$, $CHCl_3$)

Anal. Calcd. for $C_{56}H_{57}O_{13}P_3$ C, 65.24; H, 5.57 Found: C, 65.0; H, 5.60.

(1*R*, 2*R*, 3*S*, 4*R*, 5*S*)-3-Hydroxy-1,2,4-trisphospho-5-vinylcyclopentane (22)

The trisphosphate triester **24** (80mg, 77.6μmol) was deprotected as described for compound **15**. Purification by ion-exchange chromatography on Q Sepharose Fast Flow Resin, as before gave the glassy triethylammonium salt of **22**, which eluted between 300 mM and 400 mM TEAB. Yield 45μmol, 58%)

^{1}H NMR (D_2O, 400 MHz, pH 3.2): δ 2.76 (1 H, ddd, J = 8.9 Hz. 7.9 Hz, 7.9 Hz, C-5-H), 3.98 (1 H, dd, J = 3.7 Hz, 3.7 Hz, C-3-H), 4.14 - 4.21 (2 H, m, C-2-H, C-4-H), 4.37 (1 H, ddd, J = 8.9 Hz, 8.9 Hz, 5.2 Hz, C-1-H), 5.07 (1 H, br d, J = 10.4 Hz, =CH_2, *cis*), 5.12 (1 H, br d, J = 17.4 Hz, =CH_2, *trans*), 5.75 (1 H, ddd, J = 17.4 Hz, 10.4 Hz, 7.9 Hz, C*H*=)

^{31}P NMR (D_2O, 161.7 MHz, pH 3.2, ^{1}H-coupled) δ 1.62 (1 P, d, J_{HP} = 9.0 Hz), 1.86 (1 P, d, J_{HP} = 8.8 Hz), 2.20 (1 P, d, J_{HP} = 9.0 Hz)

MS: *m/z* (+ve ion FAB, rel intensity) 102 [$(C_2H_5)_3NH^+$, 100%]

MS: *m/z* (–ve ion FAB, rel intensity) 798 [$2M^-$, 10%], 399 [M^-, 100%]

$[\alpha]_D = -8$, $[\alpha]_{436} = -28$ (c = 0.36, TEAB buffer pH 7.8) calcd for the free acid

MS: *m/z* 398.963 (M^-) calcd for $C_7H_{14}O_{13}P_3^-$, 398.965.

Attempted oxidative cleavage of vinyl group

The attempted oxidative cleavage of vinylcyclopentane **24** according to a reported procedure[184] gave only a small amount of **25** isolated as an oil and identified as (1*R*, 2*R*, 3*S*, 4*R*, 5*S*)-3-Benzyloxy-1,2,4-tris(dibenzylphosphoryloxy)-5-(1,2-dihydroxyethyl)-cyclopentane. NMR data for **25** are given below.

^{1}H NMR ($CDCl_3$, 270MHz): δ 2.57 (1 H, br t, C-5-H), 3.58 (1 H, dd, J = 11.5 Hz, 6.6 Hz, C-7-H), 3.74-3.86 (2 H, m, C-6-H and C-7-H), 4.13 (1 H, br s, C-3-H), 4.37, 4.43 (2 H, AB, J_{AB} = 11.7 Hz, OC$H_2C_6H_5$), 4.82 - 5.05 (15 H, m, 6 x OC$H_2C_6H_5$, C-1-H, C-2-H and C-4-H), 7.16 - 7.32 (35 H, m, 7 x C_6H_5)

^{13}C NMR ($CDCl_3$, 100.4 MHz): δ 50.10 (d, C-5), 64.32 (t, C-7), 68.85 (d, C-6), 69.37, 69.47, 69.77, 69.95, 70.24 (5 t, 6 x PO$CH_2C_6H_5$), 71.65 (t, $OCH_2C_6H_5$), 79.97, 83.16, 86.83 (3 d, C-1, C-2, C-4), 84.30 (d, C-3), 127.67, 127.80, 127.85, 127.91, 128.00, 128.03, 128.07, 128.13, 128.31, 128.51, 128.69, 128.78, 128.98, 129.22, 129.31 (15 d, C_6H_5), 135.20, 135.28, 135.40, 135.46, 135.53, 135.61 137.14 (7 s, 7 x C-1 of benzyl rings).

^{31}P NMR ($CDCl_3$, 109.4 MHz): δ −1.75, −0.77, 0.52

MS: *m/z* (+ve ion FAB, rel intensity) 1065 [$(M + H)^+$, 50%)], 281(80%), 91 [$(C_7H_7)^+$, 100%]

MS: *m/z* (−ve ion FAB, rel intensity) 973[$M-C_7H_7]^-$, 85%], 277 [$(C_6H_5O)_2PO_2^-$,100%]

8.5 Conformationally-Restricted Analogue

1,3,5-*O*-Methylidene-*myo*-inositol [= *myo*-inositol orthoformate] (27)[130]

This compound was synthesised according to the improved procedure of Baudin *et al.*[129] *myo*-Inositol (50.0g, 0.278mol) and toluene-*p*-sulphonic acid (13.8g, 0.073mol) were suspended in dry DMF (500mL). Triethyl orthoformate (83mL, 0.50mol) was added dropwise under N_2 at 100°C. The mixture was stirred at 100°C for 4 h , after which the reaction was judged to be complete by TLC (acetonitrile, product at R_f 0.44). DMF was distilled off under reduced pressure at 50°C. The residue was treated with 10% sodium hydrogencarbonate solution (100mL), stirred for 15min at rt, diluted with water (1L), and extracted with chloroform (3 x 250mL). The aqueous phase was evaporated to dryness under reduced pressure at 40°C, and the residue was then stirred with methanol (2L) at 50°C. The suspension was filtered and evaporated to give a sticky off-white solid.(~70g). This was dissolved in the minimum amount of water, and purified by flash chromatography (acetonitrile) to give **27** (40.5g, 0.213mol, 77%)

Mp: 297-300°C (from methanol/ethyl acetate) (lit.[130] 300-302°C).

^{1}H NMR (d_6-DMSO, 270MHz): δ 3.95 (2 H, dd, J = 3.0 Hz, 1.7 Hz, C-1-H, C-3-H), 3.99 (1 H, br s, C-2-H), 4.06 (1 H, m, C-5-H), 4.27 (2 H, br s, D_2O ex gives dd, J = 3.8 Hz, 3.8 Hz, C-4-H, C-6-H), 5.32 (1H, br s, D_2O ex, C-2-OH), 5.44 (1H, d, J =1.1 Hz, CO_3H), 5.47 (2H, br s, D_2O ex, C-4-OH, C-6-OH).

4,6-Di-*O*-*p*-methoxybenzyl-1,3,5-*O*-methylidene-*myo*-inositol (28)

To a solution of *myo*-inositol orthoformate **27** (15.0g, 78mmol) in dry DMF (300mL) at 0°C was added sodium hydride (7.2g of a 60% dispersion in oil, 180mmol). The mixture was stirred for 20 min at 0°C and then *p*-methoxybenzyl chloride (22.5mL, 166mmol) was added. The mixture was stirred for a further 2h at rt, after which TLC (dichloromethane/ethyl acetate 2:1) showed a major product at R_f 0.49 and minor products (mono- and tri-*O*-*p*-methoxybenzylated material) at R_f 0.22 and 0.64. Water (10mL) was added carefully to quench the reaction. Solvents were evaporated *in vacuo* and the residue partitioned between water (200mL) and dichloromethane (400mL). The

organic layer was washed with brine (200mL), dried ($MgSO_4$), and evaporated to give an oil which was purified by flash chromatography (dichloromethane/ethyl acetate 5:1) giving, in order of elution; the tri-*O*-*p*-methoxybenzylated ether, di-*O*-*p*-methoxybenzylated material, and finally, mono-substituted products. Recrystallisation of the second fraction from ethyl acetate/hexane gave **28** (13.5g, 31.4mmol, 40% yield).

Mp: 120 - 121°C (from ethyl acetate/hexane).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.26 (1 H d, J = 11.3 Hz , D_2O ex, C-2-OH), 3.79 (6 H, s, 2 x $CH_2C_6H_4OC\mathit{H}_3$), 4.16 (1 H, br d, J = 11.2 Hz, D_2O ex gives br s, C-2-H), 4.18-4.20 (2 H, m, C-1-H and C-3-H) 4.32 (2 H, dd, J = 3.4 Hz, 3.4 Hz, C-4-H and C-6-H), 4.40 (1 H, m, C-5-H), 4.48, 4.56 (4 H, AB, J_{AB} = 11.0 Hz, 2 x $C\mathit{H}_2C_6H_4OCH_3$) 5.46 (1 H, d, J = 0.9 Hz, O_3CH), 6.81 (4 H, d, J = 8.9 Hz, 2 x $CH_2C_6\mathit{H}_4OCH_3$), 7.17 (4 H, d, J = 8.6 Hz, 2 x $CH_2C_6\mathit{H}_4OCH_3$)

^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 55.14 (q, 2 x $C_6H_4OC\mathit{H}_3$), 61.12 (d, inositol ring C), 67.74 (d, inositol ring C), 71.21 (t, 2 x $C\mathit{H}_2C_6H_4OCH_3$), 72.90 (d, 2 inositol ring C), 73.38 (d, 2 inositol ring C), 103.24 (d, $O_3C\mathit{H}$), 113.75 (d, 2 x $CH_2\mathit{C}_6H_4OCH_3$), 129.27 (d, 2 x $CH_2\mathit{C}_6H_4OCH_3$), 129.53 (s, 2 x $CH_2\mathit{C}_6H_4OCH_3$), 159.29 (s, 2 x C-OCH_3)

MS: *m/z* (+ve ion FAB, rel intensity) 431 [$(M+H)^+$, 12%], 309 [$(M-C_7H_6OCH_3)^+$, 15%], 121 [$(C_7H_6OCH_3)^+$, 100%].

Anal. Calcd for $C_{23}H_{26}O_8$ (430.45); C, 64.18; H 6.09, Found C, 63.9; H, 6.05

2,4,6/3,5-Pentahydroxy-3,5-di-*O*-*p*-methoxybenzyl-2,4,6-*O*-methylidene-cyclohexanone (29)

Dry dichloromethane (40mL) was placed into a 250mL flask under an atmosphere of N_2. A solution of oxalyl chloride in dry dichloromethane (12.8mL of a 2M solution, 25.6mmol) was injected, and the flask was cooled to −60°C using a chloroform/solid CO_2 bath. Anhydrous DMSO (3.6mL, 51mmol) dissolved in dry dichloromethane (5mL) was added dropwise over 5 min (*care! rapid evolution of gas*) and stirring continued for 5min. A solution of **28** (10.0g, 23.2mmol) in dry dichloromethane (30mL) was added dropwise over 5min and stirring continued for an additional 20min, maintaining a temperature of −55 to −60°C. Dry triethylamine (15mL) was added dropwise over 2min and the reaction was stirred for a further 5min before allowing it to warm to rt. The mixture was stirred with water (100mL) for 10min, and then dichloromethane (200mL) was added. The organic layer was separated, and the aqueous layer re-extracted with a further 200mL of dichloromethane. The combined organic

layers were then washed successively with, sat. NaCl, 1% HCl, water, 10%$NaHCO_3$, and water (200mL of each), dried ($MgSO_4$), and evaporated to give a white solid consisting of a mixture of the ketone **29** and its hydrated *gem*-diol **29a**. The mixture was dissolved in toluene (300mL) and refluxed with azeotropic removal of water in a Dean and Stark apparatus for 3 h. The toluene was removed by evaporation *in vacuo* and the residue recrystallised from ethyl acetate/hexane to give the ketone **29** (9.14g, 21.3mmol, 92%).

Mp: 125 - 126°C (from ethyl acetate/hexane).

IR: $\nu_{C=O}$ 1760cm^{-1}

^1H NMR ($CDCl_3$, 270 MHz) δ 3.78 (6 H, s, 2 x $C_6H_4OCH_3$), 4.39 (2 H, dd, J = 2.7 Hz, 1.0 Hz, 2 inositol ring C-H), 4.47 - 4.56 (7 H, m, 3 inositol ring C-H and 2 x $CH_2C_6H_4OCH_3$), 5.63 (1 H, s, O_3CH), 6.81 (4 H, d, J = 8.2 Hz, 2 x $C_6H_4OCH_3$), 7.16 (4 H, d, J = 8.4 Hz, 2 x $C_6H_4OCH_3$)

^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 55.22 (q, 2 x $C_6H_4OCH_3$), 68.88 (d, C-4), 71.18 (t, 2 x $CH_2C_6H_4OCH_3$), 76.37 (d, 2 inositol ring C), 77.97 (d, 2 inositol ring C), 102.64 (d, O_3CH), 113.85 (d, 2 x $CH_2C_6H_4OCH_3$), 128.90 (s, 2 x $CH_2C_6H_4OCH_3$), 129.50 (d, 2 x $CH_2C_6H_4OCH_3$), 159.47 (s, 2 x C-OCH_3), 199.23 (s, C=O).

MS: *m/z* (+ve ion FAB, rel intensity) 447 [$(M+H_2O+H)^+$, 0.3%], 429 [$(M+H)^+$, 1.2%], 121 [$(C_7H_6OCH_3)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 580 [$(M+NBA-H)^-$, 75%], 427 [$(M-H)^-$, 30%], 322 (80), 303(100), 287 (60%).

Anal. Calcd for $C_{23}H_{24}O_8$ (428.44); C, 64.48; H 5.65, Found C, 64.6; H, 5.66

2-*C*-Hydroxy-4,6-di-*O*-*p*-methoxybenzyl-1,3,5-*O*-methylidene-*myo*-inositol (*gem*-diol, 29a)

The ketone **29** (400mg, 0.924mmol) was dissolved in dioxan (4mL) and water (0.4mL) was added. The solution was left at room temperature for 3 days and then water was added dropwise until crystals began to appear. After another day at room temperature the crystals were filtered off, and were found to consist of pure *gem*-diol **29a** (315mg, 0.706mmol, 76%).

Mp: 129-131°C. (from ethyl acetate/hexane).

^1H NMR ($CDCl_3$, 270 MHz) δ 3.79 (6H, s, $C_6H_4OCH_3$), 3.82 (1H, s, D_2O ex, C-2-OH), 4.13 (2H, br d, J = 1.5 Hz, C-1-H and C-3-H), 4.41 - 4.60 (7 H, m, 2 x CH_2Ar , C-4-H, C-6-H and C-5-H), 4.97 (1 H, s, D_2O ex, C-2-OH), 5.50 (1 H, s, O_3CH) 6.80 (4 H, d, J = 8.6 Hz, $C_6H_4OCH_3$), 7.12 (4 H, d, J = 8.6 Hz, $C_6H_4OCH_3$).

^{13}C NMR ($CDCl_3$, 68 MHz) δ 55.25 (q, 2 x $C_6H_5OCH_3$), 67.51 (d, C-5), 71.42 (t, 2 x $CH_2C_6H_4OCH_3$), 73.32 (d, 2 x inositol ring C), 73.56 (d, 2 x inositol ring C), 88.67 (s, $C(OH)_2$), 102.30 (d, O_3CH), 113.95 (d, C-3 and C-5 of *p*-methoxyphenyl rings), 128.74 (s, C-1 of *p*-methoxyphenyl rings), 129.76 (d, C-2 and C-6 of *p*-methoxyphenyl rings), 159.58 (s, C-4 of *p*-methoxyphenyl rings).

MS: *m/z* (+ve ion FAB, rel intensity) 447 [$(M+H)^+$, 2%], 121 [$(C_7H_6OCH_3)^+$, 100%]

Anal. Calcd for $C_{23}H_{26}O_9$ (446.45); C, 61.88; H 5.87, Found C, 61.8; H, 5.89

2-*C*-Hydroxy-4,6-di-*O*-*p*-methoxybenzyl-2-*O*-methyl-1,3,5-*O*-methylidene-*myo*-inositol (hemiketal 29b)

A solution of the ketone **29** in methanol/THF under N_2 was kept at room temperature for one week. The formed crystals were removed by filtration and found to be the hemiketal **29b**. (Method not optimised).

Mp: 134-146°C. (from ethyl acetate/hexane).

^1H NMR ($CDCL_3$, 270 MHz) δ 3.48 (3 H, s, OCH_3), 3.79 (6 H, s, 2 x $C_6H_4OCH_3$), 4.26-4.29 (2 H, m, C-1-H and C-3-H), 4.47 (2 H, dd, J = 3.7 Hz, 3.7 Hz, C-4-H and C-6-H), 4.48-4.58 (1 H, m, C-5-H), 4.54, 4.56 (4 H, AB_q, J_{AB} = 10.4 Hz, 2 x OCH_2Ar), 4.92 (1 H, s, D_2O ex, C-2-OH), 5.52 (1 H, s, O_3CH) 6.76-6.84 (4 H, m, $C_6H_4OCH_3$), 7.10-7.18 (4 H, m, $C_6H_4OCH_3$).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 48.28 (s, OCH_3), 55.23 (q, 2 x $C_6H_5OCH_3$), 67.72, 70.08, 73.57 (3 d, 5 inositol ring *C*H), 71.38 (t, 2 x $CH_2C_6H_4OCH_3$), 90.91 (s, *C*-2), 102.30 (d, O_3CH), 113.88 (d, C-3 and C-5 of *p*-methoxyphenyl rings), 128.71 (s, C-1 of *p*-methoxyphenyl rings), 129.83 (d, C-2 and C-6 of *p*-methoxyphenyl rings), 159.54 (s, C-4 of *p*-methoxyphenyl rings).

MS: *m/z* (+ve ion FAB, rel intensity) 461 [$(M+H)^+$, 2%], 121 [$(C_7H_6OCH_3)^+$, 100%]

Anal. Calcd for $C_{24}H_{28}O_9$ (446.45); C, 62.6; H 6.13, Found C, 62.5; H, 6.03

2,4-Di-*O*-*p*-methoxybenzyl-6-methylidene-1,3,5-*O*-methylidene-cyclohexane-1,3,5/2,4-pentol (30)

Methyltriphenylphosphonium bromide (6.13g, 17.2mmol), previously dried *in vacuo* at 70°C, was suspended in dry THF (20mL) under N_2 at 0°C. Potassium *tert*-butoxide (16.3mL of a 1M solution in THF, 16.3mmol) was added. The resulting yellow suspension was allowed to reach rt and was then stirred at rt for 10min. A solution of ketone **29** (3.50g. 8.17mmol) in dry THF (30mL) was added. [At this stage, a ^{31}P NMR spectrum of a sample taken from the yellow suspension showed the presence of an

oxaphosphetane intermediate (δ_P –68.9ppm). This signal could still be observed in the NMR sample after several days at 4°C]. The mixture was refluxed for 2 h, after which its colour had darkened to orange and TLC (ethyl acetate/hexane 1:1) showed the reaction to be complete, with the product at R_f 0.52. The solvent was removed by evaporation *in vacuo* and the residue was taken up in ether (100mL), the solution washed with brine (100mL), dried ($MgSO_4$) and evaporated to give a clear brown oil. Purification by flash chromatography (ethyl acetate/hexane 1:2) gave the alkene **30** as a white crystalline solid (3.17g, 7.42mmol, 91%).

Mp: 95-97°C (from ethanol).

^{1}H NMR ($CDCl_3$, 270 MHz) δ 3.80 (6 H, s, 2 x OCH_3), 4.23 (2 H, dd, J = 3.6 Hz, 3.6 Hz, C-2-H, C-4-H), 4.31 (1 H, tt, J = 3.6 Hz, 1.7 Hz, C-3-H), 4.40 (2 H, dd, J = 3.6 Hz, 1.7 Hz, C-1-H, C-5-H), 4.54, 4.58 (4 H, AB_q, J = 11.8 Hz, 2 x $OCH_2C_6H_4OMe$), 5.25 (2 H, s, $=CH_2$), 5.57 (1 H, s, O_3CH), 6.82-6.87 (4 H, m 2 x C_6H_4OMe), 7.22-7.26 (4 H, m, 2 x C_6H_4OMe).

^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 55.19 (q, 2 x OCH_3), 68.94, 73.55, 74.26 (3 d, 5 x inositol ring C), 71.05 (t, $OCH_2C_6H_4OMe$), 103.73 (d, O_3CH), 113.75, 129.43 (2 d, 2 x C_6H_4OMe), 114.27 (t, $C=CH_2$), 129.82 (s, $C=CH_2$), 137.15 (s, 2 x C_6H_4OMe), 159.32 (s, 2 x C_6H_4OMe).

MS: *m/z* (+ve ion FAB, rel intensity) 427 [$(M+H)^+$, 1%], 305[$(M-C_7H_6OCH_3)^+$, 6%], 121 [$(C_7H_6OCH_3)^+$, 100%].

Anal. Calcd for $C_{24}H_{26}O_7$ (426.47); C, 67.59; H 6.15, Found C, 67.3; H, 6.15.

(1,3,5/2,4,6)-6-Hydroxymethyl-1,3,5-*O*-methylidene-2,4-di-*O*-*p*-methoxybenzyl-cyclohexane-1,2,3,4,5-pentol (31)

A dry 250mL three-necked flask was charged with the alkene **30** (6.0g, 14.0mmol), previously dried at 60°C *in vacuo*. 9-BBN-H (60mL of a 0.5M solution in THF, 30mmol) was added under an atmosphere of N_2 at room temperature. The temperature was increased to 50°C and the mixture was stirred under N_2 for 2h. The mixture was allowed to cool to room temperature and then further cooled to 0°C in an ice bath. Ethanol (20mL), 6M NaOH (5mL) and 30% H_2O_2 (10mL) were added dropwise (*care! exothermic reaction with rapid evolution of gas*), and then the temperature was increased to 50°C. After stirring at 50°C for 30min, the mixture was cooled to room temperature and the aqueous layer was saturated with K_2CO_3. The organic layer was

removed, dried ($MgSO_4$) and evaporated *in vacuo* to give a colourless oil which was purified by column chromatography giving **31** as a white solid (6.04g,13.6mmol, 97%).

Mp: 81-82°C (from ethanol or ethyl acetate/hexane)

^{1}H NMR ($CDCl_3$, 270 MHz) δ 1.58 (1 H, t, J = 5.9 Hz, D_2O ex., OH), 2.97 (1 H, br t, J = 8.2 Hz, C-6-H), 3.80 (6 H, s, 2 x OCH_3), 4.08 (2 H, dd, J = 8.2 Hz, 5.9 Hz, D_2O ex gives d, J = 8.2 Hz, CH_2OH), 4.27 (2 H, br s, C-1-H, C-5-H), 4.36 (2 H, br s, C-2-H, C-4-H), 4.49, 4.59 (4 H, AB_q, J = 10.8 Hz, 2 x $CH_2C_6H_4OMe$), 4.53 (1 H, m, C-3-H), 5.58 (1 H, s, O_3CH), 6.79-6.82 (4 H, m, 2 x C_6H_4OMe), 7.15-7.26 (4 H, m, 2 x C_6H_4OMe).

^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 42.88 (d, C-6), 55.17 (q, 2 x OCH_3), 59.89 (t, CH_2OH), 68.50 (d, C-3), 69.02, 73.06 (2 d, C-1,2,4,5), 71.41 (t, 2 x $CH_2C_6H_4OMe$), 103.84 (d, O_3CH), 113.78 (d, 2 x C_6H_4OMe), 129.29 (d, 2 x C_6H_4OMe), 129.63 (s, 2 x C_6H_4OMe), 159.26 (s, 2 x C_6H_4OMe).

MS: *m/z* (+ve ion FAB, rel intensity) 445 [$(M+H)^+$, 1.2%], 323 [$(M-C_7H_6OCH_3)^+$, 3.1%], 121 [$(C_7H_6OCH_3)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 597 [$(M+NBA)^-$, 100%], 323 [$(M-C_7H_6OCH_3)^-$, 90%].

Anal. Calcd for $C_{24}H_{28}O_8$ (444.48); C, 64.85; H 6.35, Found C, 65.1; H, 6.47

(1,3,5/2,4,6)-6-Hydroxymethyl -2,4-di-*O*-*p*-methoxybenzyl-cyclohexane-1,2,3,4,5-pentol (32)

Alcohol **31** (2.32g, 5. 22mmol) was dissolved in methanol (100mL) and heated to 50°C. 1M HCl (10mL) was added and the mixture stirred at 50°C for 30min. Excess concentrated ammonia solution was added and the mixture was allowed to cool. Stirring was continued at room temperature for a further 30min, and the solvents were removed by evaporation *in vacuo*. The residue was extracted with hot ethyl acetate (2 x 100mL) and the combined extracts evaporated *in vacuo* to give a white solid which was purified by flash chromatography ($CHCl_3$/MeOH 100:0 - 50:50) giving tetrol **32** (1.98g, 4.56mmol, 87%).

Mp: 136-137°C (from ethyl acetate/hexane)

^{1}H NMR (d_6-DMSO, 400 MHz) δ 1.25 (1 H, tt, J = 10.7 Hz, 2.1 Hz, C-6-H), 3.09 (2 H, dd, J = 9.3 Hz, 9.3 Hz, C-2-H, C-4-H), 3.20 (1 H, dt, J = 9.2 Hz, 5.4 Hz, D_2O ex gives t, J = 9.2 Hz, C-3-H), 3.29 (2 H, ddd, J = 10.7 Hz, 9.3 Hz, 5.9 Hz, D_2O ex gives dd, J = 10.7 Hz, 9.3 Hz, C-1-H, C-5-H), 3.69 (2 H, dd, J = 5.4 Hz, 2.1 Hz, D_2O ex gives d, J = 2.1 Hz, CH_2OH), 3.73 (6 H, s, 2 x OCH_3), 4.27 (1 H, t, J = 5.4 Hz, D_2O ex, CH_2OH),

4.71 (4 H, s, 2 x $CH_2C_6H_4OCH_3$), 4.74 (2 H, d, J = 5.9 Hz, D_2O ex, C-1-O*H*, C-5-O*H*), 4.92 (1 H, d, J = 5.4 Hz, D_2O ex, C-3-O*H*), 6.83-6.87 (4 H, m, 2 x $C_6H_4OCH_3$), 7.29-7.36 (4 H, m, 2 x $C_6H_4OCH_3$)

^{13}C NMR (d_6-DMSO, 67.8 MHz) δ 48.13 (d, C-6), 55.49 (q, 2 x OCH_3), 57.54 (t, CH_2OH), 69.00 (d, 2 inositol ring C), 73.87 (d, C-3), 73.88 (t, 2 x $CH_2C_6H_4OCH_3$), 85.90 (d, 2 inositol ring C), 113.78 (d, 2 x $C_6H_4OCH_3$), 129.80 (d, 2 x $C_6H_4OCH_3$), 132.07 (s, 2 x $C_6H_4OCH_3$), 158.91 (s, 2 x $C_6H_4OCH_3$)

MS: *m/z* (+ve ion FAB, rel intensity) 433 [$(M-H)^+$, 2.0%], 313 [$(M-C_7H_6OCH_3)^+$, 7.0%], 121 [$(C_7H_6OCH_3)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 587 [$(M+NBA)^-$, 100%], 433 [$(M-H)^-$, 100%], 313 [$(M-C_7H_6OCH_3)^-$, 20%]

Anal. Calcd for $C_{23}H_{30}O_8$ (434.49); C, 63.58; H 6.96, Found C, 63.4; H, 6.94

DL-(1,3,5/2,4,6)-1,7-*O*-Benzylidene-6-hydroxymethyl-2,4-di-*O*-*p*-methoxybenzyl-cyclohexane-1,2,3,4,5-pentol (33)

The tetrol **32** (2.00g, 4.60mmol) was dissolved in dry DMF (10mL) in a 100mL round bottomed flask. A catalytic amount of toluene-*p*-sulphonic acid (50mg) was added, followed by benzaldehyde dimethyl acetal (0.80mL, 5.33mmol). The flask was fitted with a 250mm air condenser connected to a filter pump and the solution was stirred at 65 - 75°C under reduced pressure for 1h, after which TLC (ethyl acetate) showed the reaction to be complete. The solution was cooled to room temperature and triethylamine (1mL) added. After stirring for 30min at room temperature the solvents were removed by evaporation *in vacuo*. The residue was taken up in dichloromethane (100mL), washed with brine (50mL), dried ($MgSO_4$) and evaporated *in vacuo* to give a solid which was purified by column chromatography (ethyl acetate/chloroform 1:1) giving the diol **33** as a white solid (2.24g, 4.29mmol, 93%).

Mp: 158 - 160°C (from ethyl acetate/hexane).

^1H NMR ($CDCl_3$, 270 MHz) δ 1.92 (1 H, dddd, J = 11.3 Hz, 11.3 Hz, 10.1 Hz, 4.4 Hz, C-6-H), 2.36 (1 H, br s, D_2O ex, OH), 2.69 (1 H, br s, D_2O ex, OH), 3.22 (1 H, br dd, D_2O ex gives dd, J = 11.0 Hz, 8.8 Hz, C-3-H), 3.33 (1 H, dd, J = 8.8 Hz, 8.6 Hz, C-4-H), 3.50-3.66 (3 H, m, C-1-H, C-2-H, C-3-H), 3.68 (1 H, dd, J = 11.1 Hz, 11.1 Hz, C-7-H_{ax}), 3.78 (6 H, br s, 2 x OCH_3), 4.49 (1 H, dd, J = 11.2 Hz, 4.4 Hz, C-7-H_{eq}), 4.61, 4.97 (2 H, AB_q, J = 11.2 Hz, $CH_2C_6H_4OCH_3$), 4.63, 4.96 (2 H, AB_q, J = 11.0 Hz,

$CH_2C_6H_4OCH_3$), 5.53 (1 H, s, C*H*Ph), 6.84-6.92 (4 H, m, $C_6H_4OCH_3$), 7.24-7.32 (4 H, m, $C_6H_4OCH_3$), 7.34-7.54 (5 H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 39.51 (d, C-6), 55.27 (q, 2 x OCH_3), 69.39 (t, *C*-7), 69.41, 74.68, 80.03, 82.03, 84.33 (5 d, inositol ring *C*), 74.62 (t, $CH_2C_6H_4OCH_3$), 74.75 (t, $CH_2C_6H_4OCH_3$), 101.10 (d, *C*HPh), 113.97, 114.09 (2 d, $C_6H_4OCH_3$), 125.98, 128.27, 128.87, 129.74, 129.87 (5 d, $C_6H_4OCH_3$ and C_6H_5), 130.47, 130.62 (2 s, $C_6H_4OCH_3$), 138.06 (s, C_6H_5), 159.42, 159.45 (2 s, $C_6H_4OCH_3$).

MS: *m/z* (+ve ion FAB, rel intensity) 523 [$(M+H)^+$, 1.0%], 522 [M^+, 2.0%], 401 [$(M-C_7H_6OCH_3)^+$, 2.0%], 121 [$(C_7H_6OCH_3)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1043 [$(2M-H)^-$, 30%], 828(10%), 688(10%), 675 [$(M+NBA)^-$, 100%], 521 [$(M-H)^-$, 100%], 401 [$(M-C_7H_6OCH_3)^-$, 40%]

Anal. Calcd for $C_{30}H_{34}O_8$ (522.60); C, 68.95; H 6.56, Found C, 68.8; H, 6.53.

DL-(1,3,5/2,4,6)-1,3-Di-*O*-benzyl-5,7-*O*-benzylidene-6-hydroxymethyl-2,4-di-*O*-*p*-methoxybenzyl-cyclohexane-1,2,3,4,5-pentol (34)

The diol **33** (1.00g, 1.91mmol) was dissolved in dry DMF and sodium hydride (250mg of a 60% dispersion in oil, 6.25mmol) was added. The suspension was stirred for 20min at room temperature and then benzyl bromide (0.50mL, 4.60mmol) was added, and stirring was continued for 2h, after which TLC (ethyl acetate/hexane 1:1) showed the reaction to be complete. Excess NaH was carefully destroyed by dropwise addition of water and the solvents were removed by evaporation *in vacuo*. The residue (which had a very low solubility in ether) was taken up in dichloromethane (50mL), washed with brine (2 x 50mL), dried ($MgSO_4$) and evaporated *in vacuo* to give a white solid which was washed with pentane and then recrystallised from hot ethanol yielding **34** (1.26g, 1.79mmol, 94%) as colourless crystals.

Mp 135 - 137°C (from ethanol).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.98 (1 H, dddd, J = 11.2 Hz, 11.2 Hz, 10.8 Hz, 4.4 Hz, C-6-H), 3.21 (1 H, dd, J = 10.8 Hz, 9.3 Hz, C-1-H), 3.45 - 3.73 (5 H, m, C-2-H, C-3-H, C-4-H, C-5-H, C-7-H_{ax}), 3.76 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 4.42 (1 H, dd, J = 11.2 Hz, 4.4 Hz, C-7-H_{eq}), 4.51 - 4.97 (8 H, m, 4 x CH_2Ar), 5.48 (1 H, s, C*H*Ph), 6.77 - 6.81 (4 H, m, $C_6H_4OCH_3$), 7.20 - 7.50 (19 H, m, $C_6H_4OCH_3$ and C_6H_5).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 39.69 (d, C-6), 55.21 (q, 2 x OCH_3), 69.31 (t, $CHCH_2OCHPh$), 74.93, 75.20, 75.62, 76.06 (4 t, OCH_2Ar), 77.94, 80.03, 82.77, 83.19, 85.70 (5 d, inositol ring C), 100.95 (d, *C*HPh), 113.73, 113.84 (2 d, $C_6H_4OCH_3$),

125.91, 127.60, 127.78, 127.96, 128.05, 128.18, 128.38, 128.51, 128.77, 129.59, 129.83 (11 d, $C_6H_4OCH_3$ and C_6H_5), 130.58, 130.74 (2 s, $C_6H_4OCH_3$), 138.00, 138.04, 138.64 (3 s, C_6H_5), 159.20, 159.23 (2 s, $C_6H_4OCH_3$).

MS: *m/z* (+ve ion FAB, rel intensity) 703 [$(M+H)^+$, 0.5%], 702 [M^+, 0.7%], 611 [$(M-C_7H_7)^+$, 0.3%], 581 [$(M-C_7H_6OCH_3)^+$, 3.5%], 121 [$(C_7H_6OCH_3)^+$, 100%], 91 [$(C_7H_7)^+$, 22%].

MS: *m/z* (−ve ion FAB, rel intensity) 855 [$(M+NBA)^-$, 100%], 611 [$(M-C_7H_7)^-$, 40%], 581 [$(M-C_7H_6OCH_3)^-$, 20%].

Anal. Calcd for $C_{44}H_{46}O_8$ (702.84); C, 75.19; H 6.60, Found C, 75.2; H, 6.62

DL-(1,3,5/2,4,6)-1,3-Di-*O*-benzyl-6-hydroxymethyl-2,4-di-*O*-*p*-methoxybenzyl-cyclohexane-1,2,3,4,5-pentol (35)

Compound **34** (1.00g, 1.42mmol) was dissolved in a mixture of THF (25mL) and methanol (25mL). 1M HCl (5mL) was added and the solution was refluxed for 30min, after which TLC (ethyl acetate) showed that no starting material remained. Excess $NaHCO_3$ (1g) was added and the mixture was allowed to cool to rt with stirring before the solvents were removed by evaporation *in vacuo*. The residue was taken up in dichloromethane (100mL), washed with water (50mL) and brine (50mL), dried ($MgSO_4$) and evaporated *in vacuo* to give a solid which was purified by flash chromatography yielding the diol **35** (720mg, 1.17mmol, 82%).

Mp 116 - 117.5°C (from ethanol).

^1H NMR ($CDCl_3$, 270 MHz) δ 1.65 (1 H, dddd, J = 9.3 Hz, 9.3 Hz, 4.5 Hz, 3.0 Hz, C-6-H), 2.27 (1 H, br dd, J = 5.1 Hz, 5.1 Hz, D_2O ex, C-7-OH), 2.77 (1 H, d, J = 1.5 Hz, D_2O ex, C-5-O*H*), 3.34 - 3.51 (4 H, m, 4 x inositol ring H), 3.63 (1 H, dd, J = 9.3 Hz, 9.3 Hz, inositol ring H), 3.73 (1 H, br m, D_2O ex gives dd, J = 11.2 Hz, 4.5 Hz, C-7-H), 3.89 (1 H, br m, D_2O ex gives dd, J = 11.2 Hz, 3.0 Hz, C-7-H), 3.77 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 4.59 - 4.97 (8 H, m, 4 x CH_2Ar), 6.79 - 6.87 (4 H, m, $C_6H_4OCH_3$), 7.18 - 7.23 (4 H, m, $C_6H_4OCH_3$), 7.29 - 7.38 (10 H, m, 2 x C_6H_5).

^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 46.07 (d, C-6), 55.20 (q, 2 x OCH_3), 60.22 (t, *C*-7), 70.37, 77.52, 83.20, 84.80, 85.95 (5 d, inositol ring C), 75.10, 75.20, 75.43, 75.57 (4 t, CH_2Ar), 113.80, 114.03 (2 d, $C_6H_4OCH_3$), 127.63, 127.84, 127.99, 128.41, 129.37, 129.53 (6 d, C_6H_5 and $C_6H_4OCH_3$), 130.49, 130.60 (2 s, $C_6H_4OCH_3$), 138.14, 138.45 (2 s, C_6H_5), 159.14, 159.37 (2 s, $C_6H_4OCH_3$).

MS: *m/z* (+ve ion FAB, rel intensity) 615 [$(M+H)^+$, 0.5%], 614 [M^+, 0.4%], 523 [$(M-C_7H_7)^+$, 0.2%], 493 [$(M-C_7H_6OCH_3)^+$, 2.8%], 121 [$(C_7H_6OCH_3)^+$, 100%], 91 [$(C_7H_7)^+$, 20%].

MS: *m/z* (–ve ion FAB, rel intensity) 767 [$(M+NBA)^-$, 60%], 613 [$(M-H)^-$, 100%), 493 [$(M-C_7H_6OCH_3)^-$, 35%].

Anal. Calcd for $C_{37}H_{42}O_8$ (614.74); C, 72.29; H 6.89, Found C, 72.3; H, 6.88.

Cyclic Phosphorylation

Benzyloxy-bis(*N*,*N*-diisopropylamino)phosphine[202] (285mg, 0.84mmol) was placed in a dry round-bottomed flask and dichloromethane (5mL) was added, followed by *1H*-tetrazole (150mg, 2.14mmol). The suspension was stirred for 10min and then cooled to 0°C. The diol **35** (430mg, 0.70mmol, previously dried *in vacuo* at 60°C) was added and stirring was continued at 0°C for 2h. ^{31}P NMR spectroscopy now showed signals at δ_P 125.0 and 130.4ppm, corresponding to the two cyclic phosphite triester invertomers. The mixture was cooled to –78°C and *m*-CPBA (240mg, 1.4mmol) was added. The clear solution was now allowed to warm to room temperature and then diluted with ethyl acetate (50mL), washed with 10% Na_2SO_3, 1MHCl, saturated $NaHCO_3$ and brine (50mL of each), dried ($MgSO_4$) and evaporated *in vacuo* to give a colourless oil. Purification by flash chromatography (ethyl acetate/hexane 1:1) gave the two cyclic phosphate triesters **36a**, R_f 0.30 (246mg, 0.32mmol) and **36b**, R_f 0.18 (214mg, 0.28mmol) corresponding to a total yield of 0.60mmol (86% from **35**).

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-hydroxymethyl-2,4-di-*O*-*p*-methoxybenzyl-cyclohexane-1,2,3,4,5-pentol 1,7-(benzylphosphate) (Epimer 36a)

Mp: 130 - 132°C (from ethyl acetate/hexane).

IR: (KBr disk) $\nu_{P=O}$ 1287cm^{-1}

^1H NMR ($CDCl_3$, 270 MHz) δ 2.19 (1 H, dddd, J = 11.3 Hz, 11.3 Hz, 11.3 Hz, 4.5 Hz, C-6-H), 3.08 (1 H, dd, J = 11.2 Hz, 9.2 Hz, C-5-H), 3.40 (1 H, dd, 9.3 Hz, 9.3 Hz, C-3-H), 3.63 (1 H, dd, J = 9.4 Hz, 9.4 Hz, C-2-H or C-4-H), 3.66 (1 H, dd, J = 9.4 Hz, 9.4 Hz, C-2-H or C-4-H), 3.76 (1 H, dd, J = 11.3 Hz, 11.3 Hz, C-7-H_{ax}), 3.768 (3 H, s, OCH_3), 3.773 (3 H, s, OCH_3), 4.04 (1 H, dd, J = 11.2 Hz, 9.2 Hz, C-1-H), 4.41 (1 H, ddd, J = 24.2 Hz, 11.4 Hz, 4.5 Hz, C-7-H_{eq}), 4.41 (1 H, d, J = 11.2 Hz, part of a broad AB system, CH_2Ar), 4.68 - 4.93 (7 H, m, OCH_2Ar), 5.06, 5.11 (2 H, ABX, J_{AB} =11.7

Hz, J_{HP} = 7.7 Hz, 7.7 Hz, P(O)OCH_2C$_6$H$_5$), 6.79 - 6.83 (4 H, m, C$_6H_4$OCH$_3$), 7.15 - 7.36 (19 H, m, C$_6H_4$OCH$_3$ and C$_6$H$_5$).

^{13}C NMR (CDCl$_3$, 68 MHz) δ 40.28 (d, C-6), 55.20 (q, 2 x OCH$_3$), 68.93 (t, P(O)O*C*H$_2$Ar), 70.26 (t, *C*-7), 75.02, 75.59, 76.01 (3 t, 4 x *C*H$_2$Ar), 76.29, 81.22, 82.30, 82.60, 84.93 (5 d, inositol ring C), 113.77, 113.83 (2 d, *C*$_6$H$_4$OCH$_3$), 127.58, 127.65, 127.97, 128.13, 128.38, 128.56, 128.67, 128.72, 129.50, 129.76 (10 d, *C*$_6$H$_4$OCH$_3$ and *C*$_6$H$_5$), 130.15, 130.24 (2 s, *C*$_6$H$_4$OCH$_3$), 135.44 (s, P(O)OCH$_2$*C*$_6$H$_5$), 137.30, 138.25 (2 s, CHOCH$_2$*C*$_6$H$_5$), 159.30 (s, *C*$_6$H$_4$OCH$_3$).

^{31}P NMR (CDCl$_3$, 162 MHz) –7.49 (ddd, J_{HP} = 24.2 Hz, 7.7 Hz, 7.7 Hz).

MS: *m/z* (+ve ion FAB, rel intensity) 767 [(M+H)$^+$, 1.2%], 675 [(M–C$_7$H$_7$)$^+$, 1.2%], 645 [(M–C$_7$H$_6$OCH$_3$)$^+$, 1.4%], 121 [(C$_7$H$_6$OCH$_3$)$^+$, 100%], 91 [(C$_7$H$_7$)$^+$, 28%].

MS: *m/z* (–ve ion FAB, rel intensity) 919 [(M+NBA)$^-$, 80%], 765 [(M–H)$^-$, 30%), 675 [(M–C$_7$H$_7$)$^-$,100%], 645 [(M–C$_7$H$_6$OCH$_3$)$^-$, 30%], 187 [C$_7$H$_7$OPO$_3$H)$^-$, 80%], 97 [(H$_2$PO$_4$)$^-$, 45%].

Anal. Calcd for C$_{44}$H$_{47}$O$_{10}$P (766.82); C, 68.92; H 6.18, Found C, 69.1; H, 6.11.

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-hydroxymethyl-2,4-di-*O*-*p*-methoxybenzyl-1,2,3,4,5-cyclohexanepentol 1,7-(benzylphosphate) (Epimer 36b)

Mp 101 - 102.5°C (from ethanol).

IR (KBr disk) $\nu_{P=O}$ 1266cm^{-1}

^{1}H NMR (CDCl$_3$, 270 MHz) δ 2.17 (1 H, dddd, J = 11.2 Hz, 11.2 Hz, 11.2 Hz, 4.8 Hz, C-6-H), 3.18 (1 H, dd, J = 11.0 Hz, 9.0 Hz, C-5-H), 3.44 (1 H, dd, 9.0 Hz, 9.0 Hz, C-3-H), 3.51 (1 H, dd, J = 9.1 Hz, 9.1 Hz, C-2-H or C-4-H), 3.60 (1 H, dd, J = 9.0 Hz, 9.0 Hz, C-2-H or C-4-H), 3.76 (3 H, s, OCH$_3$), 3.78 (3 H, s, OCH$_3$), 4.03 (1 H, ddd, J = 11.2 Hz, 11.2 Hz, 3.9 Hz, C-7-H$_{ax}$), 4.32 (1 H, br dd, J = 11.2 Hz, 9.2 Hz, C-1-H), 4.39 (1 H, ddd, J = 20.4 Hz, 11.2 Hz, 4.7 Hz, C-7-H$_{eq}$), 4.45 - 4.90 (8 H, m, OCH_2Ar), 5.08, 5.13 (2 H, ABX, J_{AB} = 11.9 Hz, J_{HP} = 9.9 Hz, 9.9 Hz, P(O)OCH_2C$_6$H$_5$), 6.78 - 6.84 (4 H, m, C$_6H_4$OCH$_3$), 7.17 - 7.38 (19 H, m, C$_6H_4$OCH$_3$ and C$_6$H$_5$).

^{13}C NMR (CDCl$_3$, 68 MHz) δ 40.41 (d, C-6), 55.19, 55.22 (2 q, 2 x OCH$_3$), 69.47 (t, P(O)O*C*H$_2$Ar), 70.40 (t, *C*-7), 75.12, 75.59, 76.06 (3 t, 4 x *C*H$_2$Ar), 76.47, 81.14, 82.29, 82.65, 85.04 (5 d, inositol ring C), 113.65, 113.85 (2 d, *C*$_6$H$_4$OCH$_3$), 127.63, 127.71, 127.86, 128.17, 128.26, 128.35, 128.62, 128.65, 129.50, 129.72 (10 d, *C*$_6$H$_4$OCH$_3$ and *C*$_6$H$_5$), 130.18, 130.26 (2 s, *C*$_6$H$_4$OCH$_3$), 135.45 (s, P(O)OCH$_2$*C*$_6$H$_5$), 137.28, 138.30 (2 s, CHOCH$_2$*C*$_6$H$_5$), 159.22, 159.30 (2 s, *C*$_6$H$_4$OCH$_3$).

^{31}P NMR ($CDCl_3$, 162 MHz) δ –4.56 (dddd, J_{HP} = 20.3 Hz, 9.9 Hz, 9.9 Hz, 3.9 Hz).

MS: *m/z* (+ve ion FAB, rel intensity) 767 [$(M+H)^+$, 2.0%], 675 [$(M-C_7H_7)^+$, 1.0%], 645 [$(M-C_7H_6OCH_3)^+$, 4.0%], 121 [$(C_7H_6OCH_3)^+$, 100%], 91 [$(C_7H_7)^+$, 22%].

MS: *m/z* (–ve ion FAB, rel intensity) 919 [$(M+NBA)^-$, 20%], 765 [$(M-H)^-$, 10%), 675 [$(M-C_7H_7)^-$,100%], 645 [$(M-C_7H_6OCH_3)^-$, 12%], 187 [$C_7H_7OPO_3H)^-$, 70%], 97 [$(H_2PO_4)^-$, 45%].

Anal. Calcd for $C_{44}H_{47}O_{10}P$ (766.82); C, 68.92; H 6.18, Found C, 69.1; H, 6.13.

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-hydroxymethyl-cyclohexane-1,2,3,4,5-pentol 1,7-(benzylphosphate) (Epimer, 37a)

To a solution of **36a** (300mg, 0.39mmol) in dichloromethane (10mL) was added water (1mL) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (355mg, 1.56mmol). The mixture was stirred at rt for 2.5 h, after which TLC (ethyl acetate/ hexane 2:1) showed the reaction to be complete. Dichloromethane (60mL) was added and the organic layer was washed with 10% Na_2SO_3 solution (3 x 50mL), sat $NaHCO_3$ solution, and brine (50mL of each), dried ($MgSO_4$) and evaporated *in vacuo* to give a yellow oil which was purified by column chromatography (ethyl acetate/pentane 3:2) giving the diol **37a** (137mg, 0.260mmol, 66%).

Mp 145-149°C (from ethyl acetate/ hexane).

R_f 0.45 (ethyl acetate/ dichloromethane 1:1), R_f 0.31 (ethyl acetate/ hexane 2:1).

IR (KBr disk) $\nu_{P=O}$ 1280cm^{-1}

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.07 (1 H, dddd, J = 11.2 Hz, 10.7 Hz, 10.7 Hz, 4.4 Hz, C-6-H), 2.83 (1 H, d, J = 1.96 Hz, D_2O ex., C-4-OH), 3.07 (1 H, dd, J = 10.7 Hz, 9.3 Hz, C-5-H), 3.20 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-3-H), 3.55 (1 H, d, J = 2.9 Hz, D_2O ex. C-2-OH), 3.63 - 3.70 (2 H, m, C-2-H, C-4-H), 3.81 (1 H, dd, J = 11.2 Hz, 11.2 Hz, C-7-H_{ax}), 3.86 (1 H, dd, J = 10.7 Hz, 9.3 Hz, C-1-H), 4.43 (1 H, ddd, J = 24.4 Hz, 11.2 Hz, 4.4 Hz, C-7-H_{eq}), 4.48 - 5.13 (6 H, 3 x AB systems, 3 x $CH_2C_6H_5$), 7.25 - 7.38 (15 H, m, 3 x C_6H_5).

^{13}C NMR ($CDCl_3$, 100.4 MHz): δ 39.60 (d, C-6), 69.15, 70.45 (2 t, 2 x $POCH_2$), 74.35, 75.17 (2 t, 2 x $OCH_2C_6H_5$), 74.70, 75.85, 76.69, 80.90, 81.32 (5 d, inositol ring *C*), 127.99, 128.06, 128.13, 128.28, 128.39, 128.52, 128.59, 128.72, 128.85 (9 d, C_6H_5), 135.28 (s, $POCH_2C_6H_5$), 137.59, 138.17 (2 s, 2 x $OCH_2C_6H_5$).

^{31}P NMR ($CDCl_3$, 162 MHz) δ –7.27 (1 P, ddd, J_{HP} = 24.2 Hz, 8.1 Hz, 8.1 Hz)

MS: *m/z* (+ve ion FAB, rel intensity) 527 [$(M+H)^+$, 22%], 435 [$(M-C_7H_7)^+$, 3%], 391 (10), 181(6), 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 679 [$(M+NBA)^-$, 90%], 525 [$(M-H)^-$, 100%), 435 [$(M-C_7H_7)^-$,90%], 187 [$C_7H_7OPO_3H)^-$, 40%], 97 [$(H_2PO_4)^-$, 20%].

Anal. Calcd for $C_{28}H_{31}O_8P$ (526.52); C, 63.87; H 5.93, Found C, 63.7; H, 6.00

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-hydroxymethyl-cyclohexane-1,2,3,4,5-pentol 1,7-(benzylphosphate) (Epimer 37b)

The *p*-methoxybenzyl groups were removed from **36b** (260mg, 0.34mmol) using the same procedure as for **36a**. Purification by column chromatography (ethyl acetate/ dichloromethane 1:1) gave **37b** (125mg, 0.237mmol, 70%).

Mp 160 - 162°C (from ethyl acetate/ hexane).

R_f 0.25 (ethyl acetate/dichloromethane 1:1), R_f 0.15 (ethyl acetate/hexane 2:1).

IR: (KBr disk) $\nu_{P=O}$ 1263cm^{-1}

^1H NMR ($CDCl_3$, 400 MHz): δ 2.13 (1 H, dddd, J = 11.2 Hz, 10.8 Hz, 10.7 Hz, 4.9 Hz, C-6-H), 2.93 (1 H, d, J = 2.4 Hz, D_2O ex., OH), 3.13 (1 H, dd, J = 10.7 Hz, 9.3 Hz, C-5-H), 3.25 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-3-H), 3.50 (1 H, br s, D_2O ex., OH), 3.63 - 3.69 (2 H, br m, C-2-H and C-4-H), 4.08 (1 H, ddd, J = 11.2 Hz, 11.2 Hz, J_{HP} = 4.4 Hz, C-7-H_{ax}), 4.17 (1 H, dd, J = 10.8 Hz, 10.7 Hz, C-1-H), 4.42 (1 H, ddd, J = 20.0 Hz, 10.7 Hz, 4.9 Hz, C-7-H_{eq}), 4.51 - 5.12 (6 H, AB systems, 3 x $CH_2C_6H_5$), 7.25 - 7.40 (15 H, m, 3 x C_6H_5).

^{13}C NMR ($CDCl_3$, 100.4 MHz): δ 40.13 (d, C-6), 70.16, 70.86 (2 t, 2 x $POCH_2$), 74.81, 75.54 (2 t, 2 x $OCH_2C_6H_5$), 75.39, 76.56, 77.13, 80.08, 81.94 (5 d, inositol ring *C*), 128.32, 128.48, 128.55, 128.72, 128.87, 128.90, 129.05 (7 d, C_6H_5), 135.41 (s, $POCH_2C_6H_5$), 137.90, 138.56 (2 s, 2 x $OCH_2C_6H_5$).

^{31}P NMR ($CDCl_3$, 162 MHz) δ –4.43 (1P, br dddd, J_{HP} = 20 Hz, 8 Hz, 8 Hz, 4 Hz)

MS: *m/z* (+ve ion FAB, rel intensity) 527 [$(M+H)^+$, 20%], 435 [$(M-C_7H_7)^+$, 4%], 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1051 [$(2M-H)^-$, 4%], 960(5), 679 [$(M+NBA)^-$, 40%], 525 [$(M-H)^-$, 80%), 435 [$(M-C_7H_7)^-$,100%], 187 [$C_7H_7OPO_3H)^-$, 50%], 97 [$(H_2PO_4)^-$, 22%].

Anal. Calcd for $C_{28}H_{31}O_8P$ (526.52); C, 63.87; H 5.93, Found C, 63.6; H, 6.01.

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-hydroxymethyl-cyclohexane-1,2,3,4,5-pentol 1,7-(benzylphosphate)-2,4-bis(dibenzylphosphate) (Epimer 38a)

To a solution of bis(benzyloxy)-*N,N*-diisopropylaminophosphine (345mg, 1.00mmol) in dry dichloromethane (3mL) was added 1*H*-tetrazole (140mg, 2.00mmol). The mixture was stirred at rt for 20min and then the diol **37a** (130mg, 0.247mmol) was added. Stirring was continued for 30min, after which ^{31}P NMR showed signals at δ 143 (1 P, s, phosphite at C-4), 142 (1 P, d, $^{5}J_{PP}$ = 1.2 Hz, phosphite triester at C-2), –7.9 (1 P, d, $^{5}J_{PP}$ = 1.2 Hz, cyclic phosphate triester). The mixture was cooled to –78°C, *m*-CPBA (345mg, 2.00mmol) was added, and the cooling bath was removed. The mixture was allowed to reach rt and then diluted with ethyl acetate (50mL). The clear solution was washed with 10% Na_2SO_3, 1MHCl, sat. $NaHCO_3$ and brine (50mL of each) dried ($MgSO_4$) and evaporated *in vacuo* giving a solid residue. Purification by column chromatography (ethyl acetate/dichloromethane 1:2) afforded **38a** (192mg, 0.183mmol, 74%) as a white solid.

Mp 171 - 172.5°C (from ethanol).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 2.20 (1 H, dddd, J = 11.2 Hz, 11.0 Hz, 11.0 Hz, 4.4 Hz, C-6-H), 3.21 (1 H, dd, J = 11.0 Hz, 9.0 Hz, C-5-H), 3.58 (1 H, dd, J = 9.2 Hz, 9.2 Hz, C-3-H), 3.68 (1 H, dd, J = 11.2 Hz, 11.0 Hz, C-7-H_{ax}), 4.10 (1 H, br dd, J = 11.2 Hz, 9.4 Hz, C-1-H), 4.32 (1 H, ddd, J = 24.2 Hz, 11.0 Hz, 4.4 Hz, C-7-H_{eq}), 4.33 (1 H, d, J = 11.4 Hz, part of a broad AB system, C$H_2C_6H_5$), 4.50 - 4.68 (2 H, m, C-2-H and C-4-H), 4.72 - 5.10 (13 H, m, C$H_2C_6H_5$ AB systems), 6.95 - 7.42 (35 H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 39.0 (d, *C*-6), 69.04 (t, *C*-7), 69.40, 69.49, 69.60, 69.68 (4 t, 5 x PO*C*$H_2C_6H_5$) 74.16 (t, 2 x O*C*$H_2C_6H_5$) 75.04, 78.20, 78.75, 79.38, 80.81 (5 d, inositol ring *C*), 127.36, 127.44, 127.84, 127.89, 128.09, 128.17, 128.23, 128.26, 128.36, 128.39, 128.51, 128.67, 128.77 (13 d, C_6H_5), 135.51, 135.63, 136.75, 137.54 (4 s, 7 x C_6H_5)

^{31}P NMR ($CDCl_3$, 162 MHz) δ –8.33 (1 P, ddd, J = 24.1 Hz, 7.6 Hz, 7.6 Hz), –1.81, –1.65 (2 P, overlap to give m in ^{1}H- coupled spectrum).

MS: *m/z* (+ve ion FAB, rel intensity) 1047 [$(M+H)^+$, 5%], 181(10), 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1199 [$(M+NBA)^-$, 8%], 1045 [$(M-H)^-$, 3%] 955 [$(M-C_7H_7)^-$,55%], 277 [$(C_6H_5O)_2PO_2^-$, 100%], 187(38%), 97 [$H_2PO_4^-$, 10%].

Anal. Calcd for $C_{56}H_{57}O_{14}P_3$ (1046.98); C, 64.24; H 5.49, Found C, 63.9; H, 5.46.

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-hydroxymethyl-cyclohexane-1,2,3,4,5-pentol 1,7-(benzylphosphate)-2,4-bis(dibenzylphosphate) (Epimer 38b)

Compound **37b** (110mg, 0.209mmol) was phosphorylated using the procedure described for **37a**. Purification by flash chromatography (ethyl acetate/dichloromethane 1:3) afforded **38b** (190mg, 0.182mmol, 87%) as a white solid.

Mp 136 - 138°C (from ethyl acetate/hexane).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.24 (1 H, dddd, J = 11.0 Hz, 11.0 Hz, 11.0 Hz, 4.6 Hz, C-6-H), 3.33 (1 H, dd, J = 9.8 Hz, 9.8 Hz, C-5-H), 3.62 (1 H, dd, J = 9.2 Hz, 8.9 Hz, C-3-H), 4.00 (1 H, br dd, J = 11.0 Hz, 11.0 Hz, C-7-H_{ax}), 4.31 (1 H, ddd, J = 21.4 Hz, 11.0 Hz, 4.6 Hz, C-7-H_{eq}), 4.39 (1 H, d, J = 11.3 Hz, part of a broad AB system, $CH_2C_6H_5$), 4.44 (1 H, br dd, J = 10.7 Hz, 10.1 Hz, C-1-H), 4.61 (1 H, ddd, J = 9.2 Hz, 9.2 Hz, 8.9 Hz, C-2-H or C-4-H), 4.67 - 5.27 (14 H, m, $CH_2C_6H_5$ AB systems and C-2-H or C-4-H), 6.99 - 7.40 (35 H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 100.4 MHz): δ 39.3 (d, *C*-6), 68.88 (t, *C*-7), 69.17, 69.54, 69.59, 70.82 (4 t, $POCH_2C_6H_5$), 74.18, 74.34 (2 t, 2 x $CH_2C_6H_5$), 75.31, 77.14, 78.82, 79.44, 81.03 (5 d, inositol ring *C*), 127.27, 127.32, 127.72, 127.89, 128.00, 128.13, 128.22, 128.33, 128.42, 128.60, 128.71 (11 d, C_6H_5), 134.88, 135.54, 135.66, 135.73, 135.83, 136.76, 137.65 (7 s, 7 x C_6H_5)

^{31}P NMR ($CDCl_3$, 162 MHz) δ −5.28 (1 P, dddd, J = 21.4 Hz, 7.6 Hz, 7.6 Hz, 2 Hz), −1.58, −1.51 (2 P, overlap to give m in ^{1}H- coupled spectrum).

MS: *m/z* (+ve ion FAB, rel intensity) 1047 [$(M+H)^+$,70%], 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (−ve ion FAB, rel intensity) 1212(65), 1199 [$(M+NBA)^-$, 30%], 1046(50), 955 [$(M-C_7H_7)^-$,100%], 277 [$(C_6H_5O)_2PO_2^-$, 60%]

Anal. Calcd for $C_{56}H_{57}O_{14}P_3$ (1046.98); C, 64.24; H 5.49, Found C, 64.4; H, 5.55

DL-(1,3,5/2,4,6)-6-Hydroxymethyl-cyclohexane-1,2,3,4,5-pentol 1:7-cyclic,2,4-trisphosphate (= DL-6-deoxy-6-hydroxymethyl-*scyllo*-inositol 1:7-cyclic,2,4-trisphosphate) (26)

Either compound **38a** or **38b** (95mg, 91μmol) was deprotected as described for compound **15**. Purification by ion-exchange chromatography on Q Sepharose Fast Flow Resin, as before gave the glassy triethylammonium salt of **26**, which eluted between 500 mM and 650 mM TEAB. Yield 78 - 82%)

^{1}H NMR (D_2O, 400 MHz, Na^+ salt, pH 8): δ 1.94 (1 H, dddd J = 10.7 Hz, 10.7 Hz, 10.7 Hz, 4.4 Hz, C-6-H), 3.32 (1 H, dd, J = 10.7 Hz, 9.3 Hz, C-5-H), 3.41 (1 H, dd, J = 9.3

Hz, 8.8 Hz, C-3-H), 3.80 (1 H, ddd, J = 8.8 Hz, 8.8 Hz, 7.8 Hz, C-4-H), 3.85 - 3.98 (3 H, m, C-1-H, C-2-H, C-7-H_{ax}), 4.21 (1 H, ddd, J = 22.9 Hz, 11.3 Hz, 4.4 Hz, C-7-H_{eq})

^{13}C NMR (D_2O, 100.4 MHz, Na^+ salt, pH 8) 41.67 (d, *C*-6), 69.15 (t, *C*-7), 70.58, 74.37, 77.57, 78.14, 80.21 (5 d, inositol ring *C*).

^{31}P NMR (D_2O, 162 MHz, ^{1}H-coupled, Na^+ salt, pH 8) δ −2.76 (1 P, d, J_{HP} = 22.5 Hz, P1), 2.99 (1 P, d, J_{HP} = 7.1 Hz, P2), 3.96 (1 P, d, J_{HP} = 6.8 Hz, P4).

MS: *m/z* (−ve ion FAB, rel intensity) 831 [$(2M-H)^-$, 20%], 415 [$(M-H)^-$, 90%], 159(100), 97 [$H_2PO_4^-$, 83%].

MS: *m/z* 414.962 $(M-H)^-$ (calcd for $C_7H_{14}O_{14}P_3^-$, 414.960).

8.6 Various Compounds from the Route to 26

2,4-Di-*O*-*p*-methoxybenzyl-6-methylidene-cyclohexane-1,3,5/2,4-pentol (39)

The alkene **30** (4.0g, 9.38 mmol) was suspended in methanol (100mL) and heated to reflux. 1M HCl (10mL) was added, and heating continued for 30 min, after which TLC (ethyl acetate/hexane 2:1) showed that almost all the starting material (R_f 0.67) had been consumed. The solution was allowed to cool, concentrated ammonia solution (5mL) was added, and the mixture was stirred at room temperature for a further 1 h. The solvents were removed by evaporation *in vacuo* giving a white solid, which was dry-extracted with hot ethyl acetate (2 x 100mL). The combined extracts were evaporated in vacuo to give a white solid which was purified by flash chromatography, (ethyl acetate/ dichloromethane 1:1) yielding the allylic alcohol **39** (2.66g, 6.39mmol, 68%).

Mp: 161.5-163°C (from ethyl acetate/hexane); IR: $\nu_{C=C}$ 1660cm^{-1}

^{1}H NMR (d_6-DMSO, 400 MHz) δ 2.92 (2 H, dd, J = 9.5 Hz, 9.2 Hz, C-2-H and C-4-H), 3.47 (1 H, dt, J = 9.2 Hz, 5.8 Hz, D_2O ex gives t, J = 9.2 Hz, C-3-H), 3.74 (6 H, s, 2 x OCH_3), 3.87 (2 H, dd, J = 9.5 Hz, 5.5 Hz, D_2O ex gives d, J = 9.5 Hz, C-1-H and C-5-H), 4.72, 4.75 (4 H, AB_q, J_{AB} = 11.0 Hz, 2 x OCH_2Ar), 5.04 (1 H, d, J = 5.8 Hz, D_2O ex, C-3-OH), 5.18 (2 H, d, J = 5.50 Hz, D_2O ex, C-1-OH, C-5-OH), 5.22 (2 H, s, $=CH_2$), 6.88 (4 H, d, J = 8.6 Hz, C_6H_4OMe), 7.37 (4 H, d, J = 8.6 Hz, C_6H_4OMe).

^{13}C NMR (d_6-DMSO, 100 MHz) δ 55.03 (q, 2 x OCH_3), 71.23 (d, C-2 and C-4), 73.52 (t, OCH_2Ar), 73.83 (d, C-3), 85.68 (d, C-1 and C-5), 105.59 (t, $=CH_2$), 113.27 (d, C-3 and C-5 of *p*-methoxyphenyl rings), 129.26 (d, C-2 and C-6 of *p*-methoxyphenyl rings),), 131.64 (s, C-1 of *p*-methoxyphenyl rings), 149.14 (s, C-6), 158.45 (s, C-4 of *p*-methoxyphenyl rings).

MS: *m/z* (+ve ion FAB, rel intensity) 415(35), 295[$(M-C_7H_6OCH_3)^+$, 100%], 173(100)

MS: *m/z* (–ve ion FAB, rel intensity) 582(100), 569[$(M+NBA)^-$, 50%], 415[$(M-H)^-$, 60%],

Anal. Calcd for $C_{23}H_{28}O_7$ (416.47); C, 66.3; H 6.78, Found C, 66.2; H, 6.66

2-*C*-Hydroxymethyl-1,3,5-*O*-methylidene-4,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol (40)

To a vigorously stirred solution of the alkene **30** (426mg, 1.0mmol) in ethyl acetate/acetonitrile (6mL of each) at 0°C was added a solution of $RuCl_3.3H_2O$ (15mg, 0.072mmol) and $NaIO_4$ (320mg, 1.5mmol) in distilled water (2mL). The two-phase mixture was stirred vigorously for 3 min and then quenched with sat. Na_2SO_3 solution (10mL). The aqueous layer was separated and extracted with ethyl acetate (2 x 20mL). The combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to give a brown oil. Purification by flash chromatography (ethyl acetate/hexane 1:1) gave pure **40** as a white crystalline solid (356mg, 0.733mmol, 77%).

Mp: 120-121.5°C (from ethyl acetate/hexane).

^{1}H NMR ($CDCl_3$, 270 MHz) δ 2.13 (1 H, t, J = 6.9 Hz, D_2O ex., CH_2OH), 3.77 (1 H, s, D_2O ex, OH), 3.80 (6 H, s, 2 x OCH_3), 4.09 (2 H, d, J = 6.8 Hz, D_2O ex gives s, CH_2OH), 4.17 (2 H, m, 2 x inositol ring C-H), 4.42 (3 H, m, 2 x inositol ring C-H and C-5-H), 4.54 (4 H, br s, 2 x $CH_2C_6H_4OMe$), 5.52 (1 H, s, O_3CH), 6.81 (4 H, d, J = 8.6 Hz, 2 x C_6H_4OMe), 7.16 (4 H, d, J = 8.6 Hz, 2 x C_6H_4OMe).

^{13}C NMR ($CDCl_3$, 68 MHz) δ 55.21 (q, 2 x OCH_3), 63.49 (t, CH_2OH), 67.89 (d, C-5), 70.16 (s, C-2), 71.52 (t, 2 x CH_2Ar), 72.69, 74.13 (2d, C-1, C-3, C-4, C-6), 103.19 (d, O_3CH), 113.82 (d, C-3 and C-5 of *p*-methoxyphenyl rings), 129.53 (d, C-2 and C-6 of *p*-methoxyphenyl rings), 132.68 (s, C-1 of *p*-methoxyphenyl rings), 158.35 (s, C-4 of *p*-methoxyphenyl rings).

MS: *m/z* (+ve ion FAB, rel intensity) 461 [$(M+H)^+$, 54%], 339 [$(M-C_7H_6OCH_3)^+$, 14%], 121 [$(C_7H_6OCH_3)^+$, 100%]

Anal. Calcd for $C_{24}H_{28}O_9$ (460.48); C, 62.60; H 6.13, Found C, 62.4; H, 6.11

DL-2,4-Di-*O*-*p*-methoxybenzyl-6-(methoxymethylidene)-1,3,5-*O*-methylidene-cyclohexane-1,3,5/2,4-pentol (41)

Methoxymethyltriphenylphosphonium chloride (3.50g, 10.2mmol) was suspended in dry THF (10mL) under N_2 at 0°C. Potassium *tert*-butoxide (10.1mL of a 1M solution in

THF, 10.1mmol) was added. The resulting orange suspension was allowed to reach room temperature and stirred for 1 h before cooling again to 0°C. A solution of ketone **29** (2.14g 5.00mmol) in dry THF (10mL) was added over 5 min. The mixture was refluxed for 2 h, after which its colour had darkened and TLC (ethyl acetate/hexane 1:1) showed the reaction to be complete, with the product at R_f 0.44. The mixture was allowed to cool and the solvent was removed by evaporation *in vacuo.* The orange residue was taken up in ether (100mL), the solution washed water then brine (50mL of each), dried ($MgSO_4$) and evaporated to give a clear brown oil. Purification by flash chromatography (ethyl acetate/pentane 2:3) gave the enol ether **41** as a white crystalline solid (2.12, 4.63mmol, 93%).

Mp: 110-111°C (from ethanol)

^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.67 (3H, s, $=OCH_3$), 3.79 (3 H, s, OCH_3), 3.80 (3 H, s, OCH_3), 4.16 (1 H,ddd, J = 3.4 Hz, 3.4 Hz, 1.2 Hz, inositol ring C-H), 4.21 - 4.23 (2 H, m, 2 x inositol ring C-H), 4.32 (1 H, appears as tt, J = 3.4 Hz, 1.5 Hz, C-3-H), 4.50, 4.58 (2 H, AB_q, J = 11.9 Hz, $OCH_2C_6H_4OMe$), 4.51, 4.64 (2 H, AB_q, J = 11.6 Hz, $OCH_2C_6H_4OMe$), 5.05 (1 H, ddd, J = 3.4 Hz, 1.5 Hz, 1.5 Hz, C-1-H or C-5-H), 5.58 (1 H, s, O_3CH), 6.24 (1 H, s, $=CHOCH_3$), 6.79-6.87 (4 H, m C_6H_4OMe), 7.18-7.29 (4 H, m, C_6H_4OMe).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.49 (q, 2 x OCH_3), 60.47 (q, $=CHOCH_3$), 66.98, 69.94, 72.10, 72.92, 73.29 (5 d, inositol ring *C*), 71.13 (t, 2 x $OCH_2C_6H_4OMe$), 104.94 (d, O_3CH), 106.72 (s, *C*-6), 113.96, (d, 2 x C_6H_4OMe), 129.43 (d, 2 x C_6H_4OMe), 130.35 (s, 2 x C_6H_4OMe), 145.98 (d, $=CHOCH_3$), 159.52 (s, 2 x C_6H_4OMe).

MS: *m/z* (+ve ion FAB, rel intensity) 335[$(M-C_7H_6OCH_3)^+$, 1%], 121 [$(C_7H_6OCH_3)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 610 [$(M+NBA)^-$, 50%], 471(60), 291(100).

Anal. Calcd for $C_{24}H_{28}O_8$ (456.49); C, 65.78; H 6.18, Found C, 65.6; H, 6.16

DL-3,5/4,6-Tetrahydroxy-3,5-di-*O*-*p*-methoxybenzyl-cyclohex-1-ene-1-carbaldehyde (42)

The enol ether **41** (1.00g, 2.19mmol) was dissolved in THF (20mL) and 1M HCl (2mL) was added. The solution was heated at reflux for 1 h and allowed to cool. The solution was stirred with 10% $NaHCO_3$ solution (5mL) for 5 min and the solvents were removed by evaporation *in vacuo.* The residue was taken up in dichloromethane (50mL), washed with water and brine (50mL of each), dried ($MgSO_4$) and evaporated in vacuo to give a

white solid which was purified by flash chromatography (ethyl acetate/dichloromethane 1:5) giving the enal **42** (748mg, 1.80mmol, 82%). (A small amount of the formate **42a** (~ 70mg) was also isolated as an oil).

Mp: 131-133°C (from ethyl acetate/hexane)

IR: $\nu_{C=O}$ 1685cm^{-1}

^{1}H NMR ($CDCl_3$, 270 MHz) δ 2.92 (1H, br s, D_2O ex, OH), 3.55 (1 H, dd, J = 10.3 Hz, 7.3 Hz, C-5-H), 3.67 (1 H, br s, D_2O ex, OH), 3.73 (1 H, br dd, D_2O ex gives dd, J = 10.3 Hz, 8.2 Hz, C-4-H), 3.80 (6 H, br s, 2 x OCH_3), 4.26 (1 H, ddd (appears as dt), J = 8.2 Hz, 2.4 Hz, 2.2 Hz, C-3-H), 4.68 (1 H, buried, C-6-H), 4.69, 4.82 (2 H, AB_q, J_{AB} = 11.4 Hz, $CH_2C_6H_4OMe$), 4.69, 4.99 (2 H, AB_q, J_{AB} = 11.0 Hz, $CH_2C_6H_4OMe$), 6.54 (1H, dd (appears as t), J ~ 2 Hz, 1.5 Hz, C-2-H), 6.86-6.92 (4 H, m, C_6H_4OMe),), 7.29-7.33 (4 H, m, C_6H_4OMe), 9.43 (1 H, s, CHO).

^{13}C NMR ($CDCl_3$, 68 MHz) δ 55.22 (q, 2 x OCH_3), 70.90, 73.30, 77.45, 81.87 (4 d, inositol ring *C*), 72.99, 74.54 (2 t, OCH_2Ar), 113.91 (d, C-3 and C-5 of *p*-methoxyphenyl rings), 129.64, 129.77 (2 d, C-2 and C-6 of *p*-methoxyphenyl rings), 130.24 (s, C-1 of *p*-methoxyphenyl rings), 139.45 (s, C-1), 148.05 (d, C-2), 159.37, 159.43 (2 s, C-4 of *p*-methoxyphenyl rings), 194.63 (d, conjugated C=O).

MS: *m/z* (+ve ion FAB, rel intensity) 413(1%), 121 [$(C_7H_6OCH_3)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 567 [(M+NBA)$^-$, 100%], 292(60), 233(50), 112(45).

Anal. Calcd for $C_{23}H_{26}O_7$ (414.46); C, 66.65; H 6.32, Found C, 65.4; H, 6.34

DL-4-O-Formyl-3,5/4,6-tetrahydroxy-3,5-di-*O*-*p*-methoxybenzylcyclohex-1-ene-1-carbaldehyde (42a)

^{1}H NMR ($CDCl_3$, 270 MHz) δ 3.64 (1H, br s, OH), 3.68 (1 H, dd, J = 10.5 Hz, 7.4 Hz, C-5-H), 3.79 (3 H, s, OCH_3), 3.80 (3 H, s, OCH_3), 4.38 (1 H, ddd (appears as dt), J = 8.4 Hz, 2.3 Hz, 2.0 Hz, C-3-H), 4.58, 4.63 (2 H, AB_q, J_{AB} = 11.2 Hz, $CH_2C_6H_4OMe$), 4.74 (1 H, br m, C-6-H), 4.66, 4.83 (2 H, AB_q, J_{AB} = 11.1 Hz, $CH_2C_6H_4OMe$), 5.02 (1 H, dd, J = 10.5 Hz, 8.4 Hz, C-4-H), 6.56 (1 H, dd (appears as t), J ~ 2 Hz, 1.5 Hz, C-2-H), 6.83-6.92 (4 H, m, C_6H_4OMe), 7.22-7.28 (4 H, m, C_6H_4OMe), 8.11 (1 H, s, OCHO), 9.43 (1 H, s, CHO).

DL-5-Hydroxymethyl-1,3-di-*O*-*p*-methoxybenzyl-cyclohex-5-ene-1,3/2,4-tetrol (43)

The enal **42** (414mg, 1.0mmol) was dissolved in methanol (20mL) and THF (10mL), and sodium borohydride (38mg, 1.0mmol) was added. TLC (ethyl acetate) after 5 min

showed that the reaction was complete with total conversion of enal (R_f 0.29) to a product with R_f 0.60. Water (5mL) was added and then the solvents were removed by evaporation *in vacuo*. The residue was taken up in ethyl acetate (50mL), washed with water (50mL) and brine (50mL) and dried ($MgSO_4$). Evaporation of solvents in vacuo gave a white solid which was recrystallised from ethyl acetate/hexane giving the allylic alcohol (380mg, 0.912mmol, 91%).

Mp: 142-145 (from ethyl acetate/hexane).

^{1}H NMR ($CDCl_3$, 270 MHz) δ 2.34 (1 H, br t, J ~ 6 HZ, D_2O ex, CH_2OH), 2.70-2.73 (2 H, m, D_2O ex, 2 x OH), 3.47 (1 H, dd, J = 10.4 Hz, 7.9 Hz, C-3-H), 3.76 (1 H, ddd, J = 10.3 Hz, 7.7 Hz, 2.0 Hz, D_2O ex gives dd, J = 10.3 Hz, 7.7 Hz, C-2-H), 3.80 (3 H, s, OCH_3),), 3.81 (3 H, s, OCH_3), 4.08 (1 H, m, C-1-H), 4.17 (2 H, br m, CH_2OH), 4.39 (1 H, br m, D_2O ex gives ddd, J = 7.9 Hz, ~2 Hz, ~2 Hz, C-4-H), 4.65 (2 H, br s, $CH_2C_6H_4OMe$), 4.76, 4.84 (2 H, AB_q, J_{AB} = 11.2 Hz, $CH_2C_6H_4OMe$), 5.64 (1 H, br s, C-6-H), 6.87-6.91 (4 H, m, C_6H_4OMe), 7.26-7.33 (4 H, m, C_6H_4OMe).

^{13}C NMR (d_6-DMSO, 100 MHz) δ 55.13 (q, 2 x OCH_3), 60.69 (t, C-7), 71.55, 74.59, 79.41, 84.72 (4 d, C-1, C-2, C-3, C-4), 70.69, 73.52 (2 t, OCH_2Ar), 113.41, 113.63 (2d, C-3 and C-5 of *p*-methoxyphenyl rings), 120.25 (d, C-6), 129.23, 129.38 (2 d, C-2 and C-6 of *p*-methoxyphenyl rings), 131.13, 131.64 (2 s, C-1 of *p*-methoxyphenyl rings), 141.30 (s, C-5), 158.58, 158.66 (2 s, C-4 of *p*-methoxyphenyl rings).

MS: *m/z* (+ve ion FAB, rel intensity) 415(30), 295(10), 121 [$[C_7H_6OCH_3)^+$, 100%]

MS: *m/z* (–ve ion FAB, rel intensity) 582(100), 569(100), 415 [$(M–H)^-$, 60%]

Anal. Calcd for $C_{23}H_{28}O_7$ (416.47); C, 66.3; H 6.78, Found C, 66.4; H, 7.01

8.7 DL-6-Deoxy-6-hydroxymethyl-*scyllo*-inositol-1,2,4-trisphosphate

DL-(1,3,5/2,4,6)-1,3-Di-*O*-benzyl-6-benzyloxymethyl-2,4-di-*O*-*p*-methoxybenzyl-cyclohexane-1,2,3,4,5-pentol (45)

To an ice-cold mixture of compound **34** (300mg, 0.427mmol) and 4Å molecular sieves (2g) and THF (10mL) under N_2 were added borane-trimethylamine complex (190mg, 2.60 mmol) and freshly pulverised aluminium chloride (345mg, 2.59 mmol). The mixture was stirred at 0°C for 23 h, after which time TLC (ethyl acetate/hexane 1:1) showed the reaction to be complete with conversion of starting material (R_f 0.57) to a product (R_f 0.49). Ether (50mL) was added followed by ice-water (50mL) and 1M HCl (10mL). The organic layer was removed and the aqueous layer re-extracted with a further 50mL of ether. The combined organic extracts were washed with brine (100mL),

dried ($MgSO_4$) and evaporated under reduced pressure to give an oily residue. Purification by flash chromatography (dichloromethane/ethyl acetate 20:1) gave the alcohol **45** as a colourless oil which slowly solidified (195mg, 0.271mmol, 65%).

Mp 95-97°C (from hexane).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 1.67 (1 H, m, C-6-H), 2.66 (1 H, d, J = 2.0 Hz, D_2O ex., C-5-OH), 3.37 (1 H, dd, J = 9.2 Hz, 9.2 Hz, C-H), 3.49-3.85 (6 H, m, 4 x inositol C-H and C-7-H_2), 4.41-4.96 (10 H, m, AB systems of $CH_2C_6H_5$ and $CH_2C_6H_4OMe$), 6.78-6.88 (4 H, m, C_6H_4OMe) 7.18-7.36 (19 H, m, C_6H_5 and C_6H_4OMe).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 45.46 (d, C-6), 55.16 (q, 2 x OCH_3), 65.65 (t, C-7), 69.48 (d, inositol ring *C*H), 73.06, 75.09, 75.35, 75.59 (4t, 5 x CH_2Ar), 77.44, 83.07, 85.22, 85.92 (4d, inositol ring *C*H), 113.73, 113.93 (2d, C-3 and C-5 of *p*-methoxyphenyl rings), 127.40, 127.45, 127.53, 127.66, 127.78, 128.85, 128.33 (7d, C_6H_5 and C_6H_4OMe), 130.74 (s, 2 x C-1 of *p*-methoxyphenyl rings), 138.27, 138.50, 138.56 (3s, C_6H_5 *ipso*), 159.08, 159.26 (2s, C-4 of *p*-methoxyphenyl rings).

MS: *m/z* (+ve ion FAB, rel intensity) 705[$(M+H)^+$, 24%], 584[$(M-PMB)^+$,28%], 211(80), 121[$(CH_2C_6H_4OMe)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 857 [$(M+NBA)^-$, 100%], 703 [$(M-H)^-$, 40%], 470(78), 303(60), 140(80), 121(80).

Anal. Calcd for $C_{44}H_{48}O_8$ (704.86); C, 74.98; H 6.80, Found C, 74.7; H, 6.86

DL-(1,3,5/2,4,6)-1,3-Di-*O*-benzyl-6-benzyloxymethyl-cyclohexane-1,2,3,4,5-pentol (46)

The alcohol **45** (200mg, 0.284mmol) was dissolved in ethanol (60mL) and 1M HCl (30mL) added. The mixture was heated at reflux for 5 h and then the solvents removed by evaporation under reduced pressure. The residue was dissolved in dichloromethane (50mL), washed with sat $NaHCO_3$ and brine (50mL of each) and evaporated to give an oily residue. Purification by flash chromatography (ethyl acetate/hexane 1:1) gave the triol as a white solid (115mg, 0.248mmol, 87%).

R_f 0.24 (ethyl acetate/hexane 1:1)

Mp: 93-95°C (from hexane)

^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.70 (1 H, dddd, J = 10.7 Hz, 10.7 Hz, 4.4 Hz, 2.4 Hz, C-6-H), 2.59 (1 H, d, J = 1.95 Hz, D_2O ex., OH), 2.83 (1 H, br s, D_2O ex., OH), 3.19 (1 H, d, J = 2.9 Hz, D_2O ex, OH), 3.22 (1 H, dd, J = 9.76 Hz, 9.28 Hz, C-3-H), 3.38-3.48 (2 H, m, C-1-H and C-4-H), 3.57-3.68 (3 H, m, C-2-H, C-5-H and C-7-H_a), 3.85 (1 H,

dd, J = 9.28 Hz, 2.4 Hz, C-7-H_b), 4.46, 4.50 (2 H, AB q, J_{AB} = 11.7 Hz, OCH_2C$_6$H$_5$), 4.58, 4.78 (2 H, AB q, J_{AB} = 10.7 Hz, OCH_2C$_6$H$_5$), 4.86 (2 H, s, OCH_2C$_6$H$_5$), 7.24-7.38 (15H, m, 3 x C$_6$H$_5$).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 44.92 (d, C-6), 66.60 (t, C-7), 70.58 (d, inositol ring CH), 73.25, 74.79, 74.83 (3t, CH_2C$_6$H$_5$), 76.69, 77.05, 77.65, 81.88 (4d, inositol ring CH), 127.68, 127.75, 127.86, 127.92, 127.99, 128.39, 128.43, 128.54 (9d, C_6H_5), 137.95, 138.40, 138.53 (3s, C_6H_5 *ipso*).

MS: *m/z* (+ve ion FAB, rel intensity) 465[$(M+H)^+$, 6%], 181(15), 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 617 [$(M+NBA)^-$, 80%], 463 [$(M-H)^-$, 100%].

Anal. Calcd for $C_{28}H_{32}O_6$ (464.56); C, 72.39; H 6.94, Found C, 72.3; H, 6.90

(1,3,5/2,4,6)-1,3,5-Tri-*O*-benzyl-6-hydroxymethyl-2,4-di-*O*-*p*-methoxybenzyl-cyclohexane-1,2,3,4,5-pentol (47)

The fully-protected compound **34** (351mg, 0.5mmol) was suspended in dry toluene (2mL) and DIBALH (1.7mL of a 1.5 M solution in toluene, 2.55mmol) was added dropwise at 0°C. Stirring was continued at 0°C for 20 h after which TLC (chloroform/acetone 20:1) showed that starting material (R_f 0.38) had been almost completely converted to a major product (R_f 0.13). A minor product with R_f 0.26 was also present. Methanol (10mL) was added (fizzing!) followed by 10% NaOH (10mL). The mixture was stirred for 10 min and then the solvents removed by evaporation under reduced pressure. The residue was taken up in ethyl acetate (50mL), washed with water and brine (50mL of each) and dried ($MgSO_4$). Evaporation under reduced pressure gave a colourless oil, which was purified by flash chromatography (dichloromethane/ethyl acetate 10:1) giving the symmetrical alcohol **47** (252mg, 0.358mmol, 72%) as a white solid.

Mp: 118.5-120.5°C (from ethanol).*

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.91 (1 H, t, J = 5.6 Hz, D_2O ex, CH_2OH), 1.58 (1 H, br t, J ~ 11 Hz, C-6-H), 3.42 (1 H, dd, J = 10.8 Hz, 9.2 Hz, C-1-H and C-5-H), 3.46 (1 H, t, J = 9.0 Hz, C-3-H), 3.60 (1 H, dd, J = 9.2 Hz, 9.0 Hz, C-2-H and C-4-H), 3.70 (2 H, dd, J = 5.6 Hz, 2.4 Hz, D_2O ex. gives d J = 2.4 Hz, C-7-H_2), 3.78 (6 H, s, 2 x OCH_3), 4.65, 4.91 (4H, AB q, J_{AB} = 11.0 Hz, CH_2Ar), 4.80, 4.85 (4 H, AB q, J_{AB} = 10.4 Hz, CH_2Ar),

* The crystals contained 0.5mole of EtOH per mole of **47**, but a second ^{1}H NMR spectrum taken some months later showed that the ethanol had now disappeared.

4.92 (2H, s, $CH_2C_6H_5$), 6.82 (4H, d, J = 8.6 Hz, C_6H_4OMe), 7.21 (4H, d, J = 8.6 Hz, C_6H_4OMe), 7.26-7.40 (15H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 45.56 (d, C-6), 55.25 (q, 2 x OCH_3), 58.30 (t, C-7), 69.48 (d, inositol ring *C*H), 75.04, 75.44 (2t, 4 x CH_2Ar), 75.82 (t, OCH_2Ar at C-5), 76.66 (d, 2 x inositol ring *C*H), 83.30 (d, C-3), 85.88 (d, 2 x inositol ring *C*H), 113.81, (d, C-3 and C-5 of *p*-methoxyphenyl rings), 127.56, 127.63, 127.98, 128.27, 128.42, 128.58, 129.49 (7d, C_6H_5 and C_6H_4OMe), 130.72 (s, C-1 of *p*-methoxyphenyl rings), 138.26, (s, 2 x C_6H_5 *ipso*), 138.68 (s, C_6H_5 *ipso*), 159.16(s, C-4 of *p*-methoxyphenyl rings).

MS*: m/z* (+ve ion FAB) 703[$(M-H)^+$, 30%], 583[$(M-PMB)^+$,50%], 211(80), 121[$(CH_2C_6H_4OMe)^+$, 100%].

MS*: m/z* (–ve ion FAB) 857 [$(M+NBA)^-$, 100%], 470(40), 289(45), 135(60), 140(80), 121(50).

Anal. Calcd for $C_{44}H_{48}O_8$ (704.86); C, 74.98; H 6.86, Found C, 74.7; H, 6.86

(1,3,5/2,4,6)-1,3,5-Tri-*O*-benzyl-6-hydroxymethyl-cyclohexane-1,2,3,4,5-pentol (48)

The *p*-methoxybenzyl protecting groups of the symmetrical alcohol **47** (200mg, 0.284mmol) were cleaved using the same procedure as that for the symmetrical alcohol **45**. Purification by flash chromatography (ethyl acetate/hexane 1:1) gave the symmetrical triol **48** as a white solid (108mg, 0.232mmol, 81%).

R_f 0.46 (ethyl acetate/hexane 1:1)

Mp: 88-90°C (from hexane/ethyl acetate 10:1)

^1H NMR ($CDCl_3$, 270 MHz): δ 1.07 (1 H, t, J = 5.5 Hz, D_2O ex., CH_2OH), 1.62 (1 H, tt, J = 11 Hz, 3.8 Hz, C-6-H), 2.54 (2 H, d, J = 2.2 Hz, D_2O ex., C-2-OH, C-4-OH), 3.23 (1 H, t, J = 9.3 Hz, C-3-H), 3.38 (2 H, dd, J = 11 Hz, 9.3 Hz, C-1-H and C-5-H), 3.66 (2 H, ddd, J = 9.3 Hz, 9.3 Hz, 2.2 Hz, D_2O ex. gives dd, J = 9.3 Hz, 9.3 Hz, C-2-H and C-4-H), 3.78 (2 H, dd, J = 5.5 Hz, 2.8 Hz, D_2O ex. gives d, J = 2.7 Hz, C-7-H_2), 4.74, 4.83 (4 H, AB q, J_{AB} = 11.4 Hz, 2 x $OCH_2C_6H_5$), 4.87 (2 H, s, $OCH_2C_6H_5$), 4.86 (2 H, s, $OCH_2C_6H_5$), 7.32-7.39 (15 H, m, 3 x C_6H_5).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 45.78 (d, C-6), 58.19 (t, C-7), 74.42, (t, 2 x $CH_2C_6H_5$), 74.92 (t, $CH_2C_6H_5$), 76.74 (d, 2 x inositol ring CH), 76.94, (d, 2 x inositol ring CH), 82.37 (d, C-3), 127.86, 127.91, 127.97, 128.20, 128.57 (5d, C_6H_5), 138.24, (s, 2 x C_6H_5 *ipso*), 138.47 (s, C_6H_5 *ipso*).

MS: *m/z* (+ve ion FAB, rel intensity) 465[$(M+H)^+$, 2.3%], 181(10), 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 617 [(M+NBA)$^-$, 50%], 463 [(M–H)$^-$, 100%].

Anal. Calcd for $C_{28}H_{32}O_6$ (464.56); C, 72.39; H 6.94, Found C, 72.1; H, 6.98.

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-benzyloxymethyl-cyclohexane-1,2,3,4,5-pentol 1,2,4-tris(dibenzylphosphate) (49)

To a solution of bis(benzyloxy)diisopropylaminophosphine (356mg, 1.03mmol) in dry dichloromethane (3mL) was added 1*H*-tetrazole (144mg, 2.06mmol). The mixture was stirred at room temperature for 20min and then the triol **46** (80mg, 0.172mmol) was added. and stirring was continued for 30min. The mixture was cooled to -78°C, *m*-CPBA (200mg, 1.16mmol) was added, and the cooling bath was removed. The mixture was allowed to reach rt and then diluted with ethyl acetate (50mL). The clear solution was washed with 10% Na_2SO_3, 1M HCl, sat. $NaHCO_3$ and brine (50mL of each) dried ($MgSO_4$) and evaporated in vacuo giving an oily residue. Purification by column chromatography (chloroform acetone 10:1) afforded **49** (183mg, 0.147mmol, 85%) as a colourless oil which slowly crystallised.

Mp: 87.5 - 88.5°C (from hexane).

R_f 0.20 (chloroform/acetone 10:1)

^{1}H NMR ($CDCl_3$, 400MHz): δ 1.93 (1 H, br t, J = 10.7 Hz, C-6-H), 3.63 (1 H, dd, J = 8.9 Hz, 8.6 Hz, C-3-H or C-5-H), 3.71-3.77 (2 H, m, C-7-H_a and C-3-H or C-5-H), 3.84 (1 H, dd, J = 9.5 Hz, 2.1 Hz, C-7-H_b), 4.18, 4.44 (2 H, AB q, J_{AB} = 11.6 Hz, $OCH_2C_6H_5$), 4.42 (1 H, half of AB system of $OCH_2C_6H_5$), 4.53-5.07 (18 H, AB systems of $OCH_2C_6H_5$, C-1-H, C-2-H, C-4-H), 6.93-7.01 (6 H, m, C_6H_5), 7.08-7.26 (37H, m, C_6H_5), 7.39-7.41 (2 H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 44.75 (d, C-6), 63.66 (t, C-7), 69.24, 69.29, 69.35, 69.40, 69.71, 69.77 (6t, P(O)OCH_2), 72.56, 73.90, 74.34 (3t, CHO$CH_2C_6H_5$), 74.98, 75.20, 78.86, 80.23, 81.84 (5d, inositol ring *C*H), 127.05, 127.41, 127.52, 127.61, 127.80, 127.91, 127.98, 128.02, 128.11, 128.18, 128.27, 128.33, 128.38, 128.47 (14d, C_6H_5), 135.77, 135.83, 135.90, 135.97, 136.03, 136.10 (6s, C_6H_5 *ipso*), 138.06, 138.20, 138.24 (3s, C_6H_5 *ipso*).

^{31}P NMR ($CDCl_3$, 162 MHz, ^{1}H-decoupled): δ –2.12 (1P), –1.67(1P), –1.53(1P).

MS: *m/z* (+ve ion FAB, rel intensity) 1245 [(M+H)$^+$, 65%], 271(10), 181(10), 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1397 [(M+NBA)$^-$, 80%], 1153 [(M–C_7H_7)$^-$, 100%], 277 [$(C_6H_5CH_2O)_2P(O)O^-$, 100%].

Anal. Calcd for $C_{70}H_{71}O_{15}P_3$ (1245.25); C, 67.52; H 5.75, Found C, 67.4; H, 5.64

DL-(1,3,5/2,4,6)-6-Hydroxymethyl-cyclohexane-1,2,3,4,5-pentol 1,2,4-trisphosphate (= DL-6-Deoxy-6-hydroxymethyl-*scyllo*-inositol-1,2,4-trisphosphate 44)

The trisphosphate triester **49** (60mg, 48mmol) was deprotected as described for compound **15**. Purification by ion-exchange chromatography on Q Sepharose Fast Flow Resin, as before gave the glassy triethylammonium salt of **44**, which eluted between 450 mM and 550 mM TEAB. Yield 34μmol, 71%

^{1}H NMR (D_2O, 400MHz): δ 1.47 (1 H, dddd, J = 11 Hz, 11 Hz, 2 Hz, C-6-H), 3.38 (1 H, dd, J = 9.5 Hz, 9.2 Hz, C-3-H), 3.48 (1 H, dd, J = 11 Hz, 9.5 Hz, C-5-H), 3.68 (2 H, ABX, CH_2), 3.80 (1 H, ddd, J = 9.5 Hz, 9.2 Hz, 8.5 Hz, C-4-H), 3.87 (1 H, ddd, J = 10.4 Hz, 10.4 Hz, 10.4 Hz, C-1-H), 4.00 (1 H, ddd, J = 9.2 Hz, 9.2 Hz, 9.2 Hz, C-2-H).

^{31}P NMR (D_2O, 162 MHz): 0.15 (1P, J_{HP} = 10.0 Hz), 0.45 (1P, J_{HP} = 8.1 Hz), 0.96 (*1*P, J_{HP} = 9.0 Hz)

MS: *m/z* (+ve ion FAB, rel intensity) 102 [$(C_2H_5)_3NH^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 867 [$(2M-H)^-$, 10%], 433 [$(M-H)^-$, 100%], 159(10), 97 [$H_2PO_4^-$, 35%].

MS (accurate mass FAB$^-$): *m/z* 432.9708 $(M-H)^-$ (calcd for $C_7H_{16}O_{15}P_3^-$, 432.9702)

8.8 α,α-Trehalose 3,4,3′,4′-tetrakisphosphate

4,6:4′,6′-Di-*O*-benzylidene-α,α-trehalose (51)

This was prepared according to the procedure of Baer and Radatus.[244]

α,α-Trehalose dihydrate (10.0g, 26.4mmol) was dehydrated by heating a suspension in absolute ethanol (60mL) at reflux for 30min followed by evaporation of solvents *in vacuo*. The dry residue was dissolved in dry DMF (60mL) in a round-bottomed flask, and benzaldehyde dimethyl acetal (4.0mL, 27mmol) was added, together with a catalytic amount of *p*-toluenesulphonic acid (250mg). The flask was fitted with an air condenser and the mixture was heated in an oil bath, with stirring, at 100°C for 10 min. The flask was then attached to a rotary evaporator for 5 min (bath temperature 60°C). The heating procedures in oil bath and on the evaporator were repeated twice, each time with the addition of fresh benzaldehyde dimethyl acetal (4.0mL and 1mL respectively). By this time, no solid material remained and TLC (ethyl acetate/dichloromethane 3:1) showed total conversion to a product at R_f 0.4. Most of the DMF was removed by evaporation *in*

vacuo to give a syrup, and toluene (60mL) was then added. Crystals began to form within a few minutes and were filtered off and washed with toluene. Further crops of crystals were obtained from the mother liquor by partial evaporation, addition of more toluene, and cooling in a refrigerator to 4°C. The combined crops were stirred with 10% sodium hydrogen carbonate solution for 20 min and then filtered off and washed successively with water and hexane. The product could be recrystallised by dissolving in boiling ethanol, followed by addition of hot water and slow cooling.* Considerable care was necessary to avoid the formation of an oil, but **51** was eventually obtained as colourless crystals of the hemihydrate (10.7g, 20.3mmol, 77%)

Mp:198-200°C (from ethanol/water) Lit [260] 195°C, Lit [261] 199-200°C.

$[\alpha]_D^{18}$ = +81 (*c* = 1, MeOH) (lit.[260] +80.3, lit.[244] +81.3).

^{1}H NMR (d_6-DMSO, 270MHz): δ 3.36-3.50 (4 H, br m obscured by H_2O, D_2O ex gives dd, *J* = 9.3 Hz, 3.7 Hz, C-2-H and C-2′-H, and dd *J* = 9.3 Hz, 9.2 Hz, C-4-H and C-4′-H), 3.65 (2 H, br m, D_2O ex gives dd, *J* = 9.9 Hz, 9.9 Hz, C-3-H and C-3′-H), 4.00-4.15 (4 H, m, C-6-H_2 and C-6′-H_2), 4.94 (2 H, d, *J* = 3.8 Hz, C-1-H and C-1′-H), 5.26 (2 H, d, *J* = 5.0 Hz, D_2O ex, 2 x C-O*H*), 5.32 (2 H, d, *J* = 5.9 Hz, D_2O ex, 2 x C-O*H*), 5.54 (2 H, s, C*H*Ph), 7.35-7.44 (10 H, m, aromatic H)

^{13}C NMR (d_6-DMSO, 68 MHz) δ 62.74, 69.86, 72.36, 81.73 (4 d, C-2/2′, C-3/3′, C-4/4′, C-5/5′), 68.69 (t, C-6/6′), 95.29 (d, C-1/1′), 101.42 (d, *C*HPh), 126.90, 128.62, 129.46 (3 d, C_6H_5), 138.20 (s, C_6H_5 *ipso*).

MS: *m/z* (+ve ion FAB, rel intensity) 1037 [$(2M+H)^+$, 45%], 613(80), 519 [$(M+H)^+$, 100%], 251 [+ve fragment ion from cleavage of glycosidic bond, 45%].

MS: *m/z* (–ve ion FAB, rel intensity) 1202(30), 1123(40), 957(80), 684(100), 517 [$(M-H)^-$, 80%].

Regioselective Dibenzylation of 4,6:4′,6′-Di-*O*-benzylidene-α,α′-trehalose

This was carried out according to Vicent *et al.*[245]

The tetrol **51** (2.00g, 3.46mmol) was placed in a dry three-neck flask, together with powdered molecular sieves (3Å, 10g), dibutyltin oxide (2.40g, 4.82mmol) and dry acetonitrile (100mL). The mixture was stirred under N_2 at 120°C for 24 h. Benzyl bromide (4.6mL, 38.6mmol) and *N*-methylimidazole (0.60mL, 7.6mmol) were added, and stirring was continued at 110°C. TLC (chloroform/acetone 10:1) showed the

* **51** could be recrystallised far more easily from propan-2-ol. The crystals so formed contained 1 equivalent of propan-2-ol, visible in the NMR spectra.

reaction to be complete after 70 h, with products at R_f 0.22 and R_f 0.30. The mixture was allowed to cool, and filtered to remove the molecular sieves, which were washed with chloroform. The combined filtrate and washings were concentrated by evaporation *in vacuo*. Column chromatography of the residue (chloroform/acetone 10:1) gave first the 2,3′-*O*-benzylated derivative **52** R_f 0.30 (785mg, 1.12mmol, 32%) and then the symmetrical 2,2′-*O*-benzylated product **53** (1.32g, 1.88mmol, 54%).

2,3′-Di-*O*-benzyl-4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose (52)

Mp: 185-187°C (from ethyl acetate/hexane) (lit.[245] syrup).

$[\alpha]_D^{19}$ = +88 (*c* = 1, $CHCl_3$) [lit[32] $[\alpha]_D$ = +76 (*c* = 1.1, $CHCl_3$)]

^{1}H NMR ($CDCl_3$, 270MHz): δ 2.38 (1 H, d, *J* = 5.1 Hz, C-2′-OH), 2.69 (1 H, d, *J* = 2.2 Hz, C-3-OH), 3.50 (1 H, dd, *J* = 9.5 Hz, 9.3 Hz, C-4-H), 3.55 (1 H, dd, *J* = 9.3 Hz, 3.7 Hz, C-2-H), 3.63-3.79 (4 H, m, C-2′-H, C-4′-H, C-6-H_{ax}, C-6′-H_{ax}), 3.95 (1 H, dd, *J* = 9.2 Hz, 9.2 Hz, C-3′-H), 4.09-4.29 (5 H, m, C-3-H, C-5-H, C-5′-H, C-6-H_{eq}, C-6′-H_{eq}), 4.69-5.00 (4 H, 2 AB systems, PhCH_2), 5.17 (1 H, d, *J* = 3.8 Hz, C-1-H or C-1′-H), 5.19 (1 H, d, *J*= 3.8 Hz, C-1-H or C-1′-H), 5.51 (1 H, s, PhC*H*), 5.55 (1 H, s, PhC*H*), 7.23-7.52 (20 H, m, C_6H_5)

^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 62.71, 63.31 (2 d, *C*H), 68.84 (t, *C*-6 and *C*-6′), 70.35, 71.60 (2 d, *C*H), 73.30, 74.97 (2 t, O*C*$H_2C_6H_5$), 78.61, 78.83, 81.23, 82.14 (4 d, *C*H), 93.87, 95.38 (2 d, *C*-1-H and *C*-1′-H), 101.28, 101.88 (2 d, Ph*C*H), 126.12, 126.29, 127.84, 127.92, 128.00, 128.05, 128.17, 128.26, 128.44, 128.65, 128.94, 129.16 (12 d, C_6H_5), 137.05, 137.36, 137.46, 138.32 (4 s, C_6H_5 *ipso*).

MS: *m/z* (+ve ion FAB, rel intensity) 699 [$(M+H)^+$, 15%], 341[+ve fragment ion from cleavage of glycosidic bond, 4%], 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 697 [$(M-H)^-$, 52%], 625(40), 612(45), 308(44), 274(42).

2,2′-Di-*O*-benzyl-4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose (53)

Mp: 196-199°C (from ethyl acetate/hexane) (lit.[245] 197-199°C).

$[\alpha]_D^{19}$ = +99 (*c* = 1, $CHCl_3$) [lit[32] $[\alpha]_D$ = +90 (*c* = 2, $CHCl_3$)]

^{1}H NMR ($CDCl_3$, 400MHz): δ 2.57 (2 H, d, *J* = 1.95 Hz, D_2O ex., 3/3′-OH), 3.49 (2 H, dd, *J* = 9.8 Hz, 9.8 Hz, C-4/4′-H), 3.54 (2 H, dd, *J* = 9.3 Hz, 3.4 Hz, C-2/2′-H), 3.66 (2 H, dd, *J* = 10.7 Hz, 10.3 Hz, C-6/6′-ax), 4.11 (2 H, dd, *J* = 10.3 Hz, 4.9 Hz, C-6/6′-eq),

4.20-4.27 (4 H, m, simplifies on D_2O shake, C-3/3′-H and C-5/5′-H), 4.75, 4.78 (4 H, ABq, J_{AB} = 11.7 Hz, $OCH_2C_6H_5$), 5.18 (2 H, d, J = 3.4 Hz, C-1/1′-H), 5.49 (2 H, s, PhC*H*), 7.25-7.48 (20 H, m, C_6H_5).

^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 62.76 (d, 2 x *C*H), 68.84 (t, C-6/6′), 70.27 (d, 2 x *C*H), 73.29 (t, 2 x $OCH_2C_6H_5$), 78.95, 81.30 (2d, 4 x *C*H), 94.40 (d, C-1/1′-H), 101.98 (d, 2 x PhCH), 126.46, 127.76, 127.97, 128.23, 128.62, 129.21 (6d, C_6H_5), 137.10, 137.57 (2s, 4 x C_6H_5 *ipso*).

MS: *m/z* (+ve ion FAB, rel intensity) 1397[$(2M+H)^+$, 30%], 1090(60), 830(65), 781(56), 699 [$(M+H)^+$, 20%], 341 [+ve fragment ion from cleavage of glycosidic bond, 40%], 94(75).

MS: *m/z* (–ve ion FAB, rel intensity) 697 [$(M-H)^-$, 20%], 625(60), 612(65), 308(70), 274(60).

3,3′-Di-*O*-benzoyl-2,2′-di-*O*-benzyl-4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose (54)

The symmetrical diol **53** (500mg, 0.716mmol) was dissolved in dry pyridine (4mL). A catalytic amount of DMAP (50mg) was added, followed by benzoyl chloride (0.19mL, 1.64mmol). The mixture was stirred overnight at room temperature and then water (1mL) was added and stirring continued for a further 10min. The solvents were removed by evaporation under reduced pressure. The residue was taken up in dichloromethane and washed with 0.1M HCl, sat. $NaHCO_3$ and brine (50mL of each) then dried over $MgSO_4$. Evaporation under reduced pressure gave a solid which was purified by flash chromatography (ethyl acetate/hexane 1:1) giving **54** (586mg, 0.646mmol, 90%) as a white solid.

Mp: 230-231°C (from ethyl acetate/hexane).

$[\alpha]_D^{20}$ = +78 (*c* = 1, $CHCl_3$).

^1H NMR ($CDCl_3$, 400MHz): δ 3.68 (2 H, dd, J = 10.3 Hz, 9.8 Hz, C-6/6′-H_{ax}) 3.73 (2 H, dd, J = 9.8 Hz, 9.8 Hz, C-4/4′-H), 3.86 (2 H, dd, J = 9.8 Hz, 3.9 Hz, C-2/2′-H), 4.11 (2 H, dd, J = 10.3 Hz, 4.9 Hz, C-6/6′-H_{eq}), 4.37 (2 H, ddd, J = 9.8 Hz, 9.8 Hz, 4.9 Hz, C-5/5′-H), 4.62, 4.69 (4 H, AB_q, J_{AB} = 12.2 Hz, CH_2Ph), 5.28 (2 H, d, J = 3.9 Hz, C-1/1′-H), 5.43 (2 H, s, C*H*Ph), 5.92 (2 H, dd, J = 9.8 Hz, 9.8 Hz, C-3/3′-H), 7.17-7.46 (24 H, m, C_6H_5), 7.56 (2 H, t, J = 7.3 Hz, $OC(O)C_6H_5$ *para*), 8.05 (4 H, d, J = 7.8 Hz, $OC(O)C_6H_5$ *ortho*)

^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 62.95 (d, 2 x *C*H), 68.84 (t, C-6/6′), 71.65 (d, 2 x *C*H), 72.64 (t, 2 x O*C*H$_2$C$_6$H$_5$), 76.17, 79.55 (2d, 2 x *C*H), 94.79 (d, C-1/1′), 101.61 (d, 2 x PhCH), 126.35, 127.91, 128.02, 128.25, 128.49, 128.84, 129.86 (7 d, C_6H_5) 130.17 (s, 2 x OC(O)C_6H_5 *ipso*), 132.84 (d, 2 x OC(O)C_6H_5 *para*), 136.96, 137.20 (2s, C_6H_5 *ipso*), 165.09 (s, 2 x *C*=O).

MS: *m/z* (+ve ion FAB, rel intensity) 907 [(M+H)$^+$, 60%], 445 [+ve fragment ion from cleavage of glycosidic bond, 60%], 323(80), 91 [(C_7H_7)$^+$, 100%].

Anal. Calcd for $C_{54}H_{50}O_{13}$ (906.98); C, 71.51 H 5.56, Found: C, 71.6; H, 5.56.

Found: C, 71.6; H, 5.56. Calcd. for $C_{54}H_{50}O_{13}$: C, 71.51; H, 5.56%

3,3′-Di-*O*-benzoyl-2,6,2′,6′-tetra-*O*-benzyl-α,α-trehalose (55)

To a solution of sodium cyanoborohydride in THF (14mL of a 1M solution) containing 3Å molecular sieves was added compound **54** (500mg, 0.551mmol). The mixture was stirred under N_2 at room temperature, and a solution of hydrogen chloride in dry ether was added dropwise until evolution of gas ceased. TLC (chloroform/acetone 10:1) showed the reaction to be complete with total conversion of starting material (R_f 0.55) to a product at R_f 0.24. The mixture was diluted with dichloromethane (50mL), washed with water, sat. $NaHCO_3$ and brine (50mL of each) and dried over $MgSO_4$. Evaporation under reduced pressure gave an oil which was purified by flash chromatography to provide the symmetrical **55** as a white foam (404mg, 0.439mmol, 80%).

$[\alpha]_D^{25}$ = +158 (c = 1, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 270MHz): δ 2.99 (2 H, d, J = 6.4 Hz, D_2O ex. C-4/4′-OH), 3.59 (4 H, d AB q, J_{AB} ~ 11 Hz, C-6/6′-H). 3.69 (4 H, dd, J = 9.8 Hz, 3.5 Hz, C-2/2′-H), 3.74, (2 H, m, D_2O ex. gives dd, J = 9.7 Hz, 9.2 Hz, C-4/4′-H), 4.21 (2 H, ddd, J = 10 Hz, 3.4 Hz, 3.4 Hz, C-5/5′-H), 4.50 (4 H, AB s, O*C*H$_2$C$_6$H$_5$), 4.57, 4.63 (4 H, AB q, J_{AB} = 12.3 Hz, O*C*H$_2$C$_6$H$_5$), 5.35 (2 H, d, J = 3.5 Hz, C-1/1′-H), 5.63 (2 H, dd, J = 9.5 Hz, 9.5 Hz, C-3/3′-H), 7.14-7.19 (10 H, m, CH$_2$C$_6$*H*$_5$), 7.24-7.29 (10 H, m, CH$_2$C$_6$*H*$_5$) 7.45 (4 H, dd, J = 7.9 Hz, 7.1 Hz, OC(O)C$_6$*H*$_5$ *meta*), 7.59 (2 H, tt, J = 7.1 Hz, 1.5 Hz, OC(O)C$_6$*H*$_5$ *para*), 8.04 (4 H, dd, J = 7.9 Hz, 1.5 Hz, OC(O)C$_6$*H*$_5$ *ortho*)

^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 68.89 (t, C-6/6′), 69.38, 71.73, 72.52 (3 d, 6 x *C*H), 73.64, 75.22 (2 t, 4 x O*C*H$_2$C$_6$H$_5$), 76.17 (d, 2 x *C*H), 93.45 (d, C-1/1′), 127.60, 127.70, 127.83, 127.89, 128.33 (5d, C_6H_5), 129.76 (s, C(O)C_6H_5 *ipso*), 129.93 (d, OC(O)C_6H_5

ortho), 133.23 (d, OC(O)C_6H_5 *para*), 137.88, 136.99 (2 s, $CH_2C_6H_5$ *ipso*), 167.14 (s, 2 x *C*=O).

MS: *m/z* (+ve ion FAB, rel intensity) 911 [M^+, 40%], 537(80), 447 [+ve fragment ion from cleavage of glycosidic bond, 10%], 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1063 [(M+NBA-H)$^-$, 35%], 121 [$(C_7H_5O_2)^-$, 100%].

Anal. Calcd for $C_{54}H_{54}O_{13}$ (911.01) C, 71.19; H, 5.97; Found C, 71.0; H, 5.97.

2,6,2′,6′-Tetra-*O*-benzyl-α,α-trehalose (56)

Sodium hydroxide pellets (100mg, 2.5mmol) were added to a solution of **55** (400mg, 0.439mmol) in methanol (25mL) and the mixture was heated at reflux for 30 min. TLC (chloroform methanol 5:1) showed complete conversion of starting material (R_f 0.82) to a product (R_f 0.50). The solution was allowed to cool and then neutralised by bubbling CO_2 gas through it overnight. The solvents were removed by evaporation under reduced pressure and the solid residue was extracted with ethyl acetate (3 x 50mL). The combined extracts were washed with water (100mL), dried over $MgSO_4$ and evaporated to give an oil. Purification by flash chromatography (ethyl acetate) gave **56** (263mg, 0.374mmol, 85%) as a white crystalline solid.

R_f 0.2 (ethyl acetate)

Mp: 135-137°C (from ethyl acetate/hexane).

$[\alpha]_D^{18}$ = +127 (*c* = 1, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 270MHz): δ 3.37 (2 H, dd, *J* = 9.7 Hz, 3.1 Hz, C-2/2′-H), 3.53 (2 H, ddd, *J* = 9.5 Hz, 9.5 Hz, ~5Hz, D_2O ex gives dd, *J* = 9.5 Hz, 9.5 Hz, C-4/4′-H), 3.52 - 3.65 (4 H, m, C-6/6′-H_2), 3.64, (2 H, br d, *J* ~ 5Hz, D_2O ex., C-4/4′-OH), 4.04 (2 H, ddd, *J* = 9.3 Hz, 9.3 Hz, 3.4 Hz, D_2O ex gives dd, *J* = 9.3 Hz, 9.3 Hz, C-3/3′-H), 4.10 (2 H, ddd, *J* = 9.5 Hz, 4 Hz, 3.4 Hz, C-5/5′-H), 4.20 (2 H, br d, *J* ~ 3.5 Hz, D_2O ex, C-3/3′-OH), 4.44 - 4.66 (8 H, 2 x AB systems, 4 x O*C*$H_2C_6H_5$), 5.12 (2 H, d, *J* = 3.1 Hz, C-1/1′-H), 7.22-7.27 (20 H, m, $CH_2C_6H_5$).

^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 69.31 (t, C-6/6′), 70.72, 71.28, 72.41 (3 d, 6 x CH), 72.54, 73.55 (2 t, 4 x O*C*$H_2C_6H_5$), 78.09 (d, 2 x CH), 93.92 (d, C-1/1′), 127.57, 127.66, 128.04, 128.09, 128.30 (6 d, C_6H_5), 137.05, 137.96 (2 s, 4 x $CH_2C_6H_5$ *ipso*).

MS: *m/z* (+ve ion FAB, rel intensity) 703 [(M+H)$^+$, 10%], 685(56), 433(24), 343 [+ve fragment ion from cleavage of glycosidic bond, 92%], 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1404 [$2M^-$, 20%], 869(100), 855 [$(M+NBA)^-$, 65%], 701 [$(M-H)^-$, 30%].

Anal. Calcd. for $C_{40}H_{64}O_{11}$: (702.80) C, 68.36; H, 6.60; Found: C, 68.2; H, 6.6.

2,6,2′,6′-Tetra-*O*-benzyl-α,α-trehalose-3,4,3′,4′-tetrakis(dibenzylphosphate) (57)

To a solution of bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (550mg, 1.59mmol) in dry dichloromethane (2mL) was added 1*H*-tetrazole (223mg, 3.18mmol). The mixture was stirred at room temperature for 10 min and then the tetrol **56** (140mg, 0.199mmol) was added. The mixture was stirred for a further 1 h, after which a 90 MHz ^{31}P NMR spectrum showed signals at δ 140.7 and 141.1 ppm (AB system $^5J_{PP}$ = 4.9Hz). The mixture was cooled to –78°C and *m*-CPBA (288mg, 1.67mmol) was added. The mixture was allowed to reach room temperature and then diluted with ethyl acetate (50mL). The solution was washed with 10% sodium sulphite solution, 1M HCl, sat. $NaHCO_3$ and brine (50mL of each), dried ($MgSO_4$) and evaporated *in vacuo* to give an oil. Purification by column chromatography (chloroform/acetone 10:1) gave **57** as a colourless oil (315mg, 0.181mmol, 91%).

R_f 0.26 (chloroform/acetone 10:1)

$[\alpha]_D^{26}$ = +46 (*c* = 1, $CHCl_3$)

^1H NMR ($CDCl_3$, 400MHz): δ 3.28 (2 H, br d, *J* ~ 11 Hz, C-6/6′-H), 3.47 (2 H, dd, *J* = 11 Hz, 3.5 Hz, C-6/6′-H), 3.63 (2 H, dd, *J* = 9.8 Hz, 3.7 Hz, C-2/2′-H), 4.21 (2 H, obscured by AB system, C-5/5′-H), 4.20, 4.37 (4 H, ABq, J_{AB} = 11.9 Hz, 2 x $OCH_2C_6H_5$), 4.52, 4.60 (4 H, ABq, J_{AB} = 11.9 Hz, 2 x $OCH_2C_6H_5$), 4.70 (2 H, ddd, *J* = 9.5 Hz, 9.5 Hz, 9.5 Hz, C-3/3′-H or C-4/4′-H), 4.84-5.13 (18 H, m, CH_2OP AB systems and C-3/3′-H or C-4/4′-H), 5.16 (2 H, d, *J* = 3.7 Hz, C-1/1′-H), 7.07-7.27 (60 H, m, 12 x C_6H_5).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 67.34 (t, *C*-6/6′-H), 69.18 (t, J_{COP} = 5.5 Hz, 4 x CH_2OP), 69.42 (t, J_{COP} = 5.5 Hz, 2 x CH_2OP), 69.66 (t, J_{COP} = 5.5 Hz, 4 x CH_2OP), 69.69 (d, 2 x *C*H), 72.59 (t, 2 x $OCH_2C_6H_5$), 73.05 (t, 2 x $OCH_2C_6H_5$), 74.07 (d, J_{COP} = 5.5 Hz, 3.7 Hz, C-3/3′ or C-4/4′), 76.89 (d, 2 x CH), 78.39 (d, (d, J_{COP} = 5.5 Hz, 3.7 Hz, C-3/3′ or C-4/4′), 93.44 (d, C-1/1′), 127.22, 127.33, 127.46, 127.59, 127.79, 127.88, 127.93, 127.99, 128.12, 128.23, 128.28, 128.37 (12 d, C_6H_5), 135.69 (s, J_{COP} = 7.4 Hz, 2 x $POCH_2C_6H_5$ *ipso*), 135.89, 136.00, 136.12 (3 s, J_{COP} = 7.3 Hz, 6 x $POCH_2C_6H_5$ *ipso*), 137.33, 137.90 (2 s, 4 x $OCH_2C_6H_5$ *ipso*).

^{31}P NMR ($CDCl_3$, 162 MHz): δ −1.94 (2 P), −2.20(2 P).

MS: *m/z* (+ve ion FAB, rel intensity) 1744 [$(M+H)^+$, 85%], 1654 (20), 459(40), 307(85), 221(50), 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (−ve ion FAB, rel intensity) 1742 [$(M-H)^-$, 30%], 1652 [$(M-C_7H_7)^-$, 80%], 277 [$((C_6H_5CH_2O)_2PO_2)^-$, 100%].

α,α-Trehalose 3,4,3′,4′-tetrakisphosphate (50)

The trisphosphate triester **57** (135mg, 77.4μmol) was deprotected as described for compound **15**. Purification by ion-exchange chromatography on Q Sepharose Fast Flow Resin, as before gave the glassy triethylammonium salt of **50**, which eluted between 500 mM and 600 mM TEAB. Yield 50.5μmol, 65%.

$[\alpha]_D^{20}$ = +124 (*c* = 0.3, H_2O, pH 5-6 , calculated for the free acid)

^{1}H NMR (D_2O, 400 MHz, pH 7) δ 3.54-3.61 (4 H, m, C-2/2′-H and C-6/6′-H), 3.67-3.73 (4 H, m, C-5/5′-H and C-6/6′-H), 3.86 (2 H, ddd, *J* = 9.8Hz, 9.3Hz, 9.3Hz, C-4/4′-H), 4.36 (2 H, ddd, *J* = 9.3Hz, 9.3Hz, 8.8Hz, C-3/3′-H), 5.03 (2 H, br s, C-1/1′-H).

^{31}P NMR (D_2O, 162 MHz, pH 7, ^{1}H-coupled) δ 0.58 (2 P, d, J_{HP} = 8.9Hz, 3/3′-P), 0.99 (2 P, d, J_{HP} = 10.1 Hz, 4/4′-P).

MS*:* *m/z* (+ve ion FAB) 255[$((C_2H_5)_3N^+H$ +NBA), 85%], 102 [$(C_2H_5)_3N^+H$, 100%].

MS*:* *m/z* (−ve ion FAB) 1323 [$2M^-$, 80%], 661 [M^-, 100%].

Accurate mass FAB$^-$: *m/z* 660.982 (M^-) calcd for $C_{12}H_{25}O_{23}P_4^-$, 660.974.

8.9 L-*myo*-Inositol 1,3,4-trisphosphorothioate

DL-2,5,6-Tri-*O*-benzyl-*myo*-inositol 1,3,4-tris(dibenzylthiophosphate) (58)

To a solution of bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (460mg, 1.33mmol) in dry dichloromethane (3mL) was added tetrazole (140mg, 2.00mmol). The mixture was stirred at rt for 10mins. The racemic triol **9** (100mg, 0.222mmol) was added and stirring continued for a further 30mins. Dry pyridine (3mL) and sulphur (100mg, 3.13mmol) were added, and stirring continued for a further 18 h. The solvents were removed by evaporation under reduced pressure and the residue was purified by column chromatography (ethyl acetate/hexane 1:4) to give **58** (141mg, 0.110mmol, 50%) as an oil.

^{1}H NMR ($CDCl_3$, 400MHz): δ 3.50 (1 H, dd, J 9.4 Hz, 9.4 Hz, C-5-H), 4.11 (1H, dd, J 9.4 Hz, 9.4 Hz, C-6-H), 4.41-4.48 (2H, m, C-1-H and C-3-H), 4.63-5.13 (19H, m, $CH_2C_6H_5$ and C-2-H), 5.32 (1H, m, C-4-H), 6.98-7.30 (45H, m, $CH_2C_6H_5$)

^{13}C NMR ($CDCl_3$, 100 MHz): δ 69.60-70.34 (triplets showing $^3J_{COP}$ coupling, overlapping, 6 x $POCH_2$), 75.05, 75.60, 75.61 (3 t, $CHOCH_2C_6H_5$), 76.46, 77.34, 78.07, 78.14, 79.817, 80.67 (6 d, inositol ring *C*) 127.08, 127.16, 127.27, 127.34, 127.45, 127.80, 127.80, 127.87, 127.94, 128.09, 128.14, 128.22, 128.29, 128.36, 128.40, 128.47, 128.53 (16d, $CH_2C_6H_5$) 135.39-136.19 (singlets showing $^3J_{COP}$ coupling, overlapping, 6 x $POCH_2C_6H_5$ *ipso*), 138.06, 138.29, 138.50 (3s, $COCH_2C_6H_5$ *ipso*).

^{31}P NMR ($CDCl_3$, 162MHz) δ 66.91 (1 P, app. as sextet, J_{HP} = 9.5 Hz), 67.75(1 P, app. as sextet, J_{HP} = 10.0 Hz), 69.80(1 P, app. as sextet, J_{HP} = 9.5 Hz).

MS: *m/z* (+ve ion FAB, rel intensity) 1279[$(M+H)^+$, 1.6%], 181(80), 91[$(CH_2C_6H_5)^+$, 100%]

MS:*m/z* (–ve ion FAB, rel intensity) 1277[$(M-H)^-$, 64%], 1185(25), 293 [$OP(S)(OCH_2C_6H_5)_2^-$, 100%], 95(30).

Anal. Calcd for $C_{69}H_{69}O_{11}S_3P_3$ (1279.40); C, 64.78; H 5.44, Found C, 65.0; H, 5.45

DL-*myo*-Inositol-1,3,4-trisphosphorothioate (59)

Compound **58** (90mg, 70μmol) was deprotected as described for compound **15** and purified by ion-exchange chromatography on Q Sepharose Fast Flow Resin, eluting with a gradient of triethylammonium bicarbonate buffer (0 to 1M), pH 8.0. The triethylammonium salt of **59** eluted between 800 mM and 930 mM. Yield 43μmol, 61%).

MS: *m/z* 466.887 $(M-H)^-$ (calcd for $C_6H_{14}O_{12}P_3S_3^-$, 466.886).

^{1}H NMR (D_2O, 400MHz): δ 3.39 (1H, dd, J = 9.3 Hz, 9.3 Hz, C-5-H), 3.67 (1H, dd, J = 9.8 Hz, 9.8 Hz, C-6-H), 4.00 (1H, ddd, J = 11.7 Hz, 9.8 Hz, 2.5 Hz, C-1-H), 4.10 (1H, ddd, J = 12.2 Hz, 9.8 Hz, 2.5 Hz, C-3-H), 4.34 (1H, br s, C-2-H), 4.35 (1H, ddd, J = 10.7 Hz, 9.8 Hz, 9.3 Hz, C-4-H).

^{31}P NMR (D_2O, 162 MHz): δ 46.02 (1 P, J_{HP} = 11.2 Hz), 48.16 (1P, J_{HP} = 11.7 Hz), 48.83 (1P, J_{HP} = 11.2 Hz).

MS: *m/z* (+ve ion FAB, rel intensity) 102 [$(C_2H_5)_3NH^+$, 100%].

MS: *m/z* (+ve ion FAB, rel intensity) 467 [$(M-H)^-$, 100%], 113 [$H_2PO_3S^-$, 10%].

1L-2,5,6-Tri-*O*-benzyl-*myo*-inositol 1,3,4-tris(dibenzylthiophosphate) (L-58)

To a solution of bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (334mg, 0.966mmol) in dry dichloromethane (2mL) was added 1*H*-tetrazole (135mg, 1.93mmol). The mixture was stirred at room temperature for 20 min and then the (+)-triol **L-9b** (72mg, 0.160mmol) was added. and stirring was continued for 30min. A ^{31}P NMR spectrum now showed an AB system centred around δ_P 141.2ppm (J_{AB} = 3.7Hz) and a singlet at 140.25ppm. Dry DMF (2mL) and dry pyridine(1mL) were added, followed by sulphur (46mg, 1.4mmol), and stirring was continued at rt for 10min. ^{31}P NMR now showed 3 singlets around δ_P 67ppm. No phosphite signals remained. The solvents were removed by evaporation under reduced pressure at room temperature. The residue was taken up in dichloromethane (50mL), washed with brine (50mL) and dried($MgSO_4$). The solution was concentrated by evaporation under reduced pressure to a volume of about 20mL. This was left at 4°C overnight to crystallise out some of the excess sulphur, which was then filtered off. Purification by column chromatography (pentane/ethyl acetate 4:1) afforded **L-58** (144mg, 0.113mmol, 71%) as a colourless oil.

$[\alpha]_D^{18}$ = +1.6 (*c* = 1.2, $CHCl_3$).

Anal. Calcd for $C_{69}H_{69}O_{11}S_3P_3$ (1279.40); C, 64.78; H 5.44, Found C, 65.0; H, 5.55

NMR and mass spectroscopic data were identical to those for the racemic compound.

1L-1,3,4-*myo*-Inositol trisphosphorothioate (L-59)

L-58 (100mg, 78.2μmol) was deprotected as described for the racemic compound **59** and purified by ion exchange chromatography as before. Yield 41μmol, 52%).

$[\alpha]_D^{22}$ = −14 (*c* = 0.35, TEAB buffer, pH 8.0) Calculated for the free acid.

NMR and mass spectroscopic data were identical to those for the racemic compound.

Accurate mass FAB$^-$: m/z 466.887 $(M-H)^-$ (calcd for $C_6H_{14}O_{12}P_3S_3^-$, 466.886).

8.10 DL-6-Deoxy-6-hydroxymethyl-*scyllo*-inositol 1,2,4-trisphosphorothioate

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-benzyloxymethyl-cyclohexane-1,2,3,4,5-pentol 1,2,4-tris(dibenzylthiophosphate) (61)

The triol **46** (75mg, 0.161mmol) was thiophosphorylated as described for compound **L-59**. Purification by column chromatography (pentane/ethyl acetate 5:1) afforded **61** (163mg, 0.126mmol, 78%) as a colourless oil.

Anal. Calcd for $C_{70}H_{71}O_{12}P_3S_3$ (1293.43); C, 65.00; H 5.53, Found C, 65.0; H, 5.38

^{1}H NMR ($CDCl_3$, 270MHz): δ 2.19 (1 H, m, C-6-H), 3.65 (2 H, ABX system (broad), C-7-H_2), 3.89 (1 H, dd, J = 10.9 Hz, 7.4 Hz, C-5-H), 4.00 (1 H, dd, J = 4.7 Hz, 4.7 Hz, C-3-H), 4.18, 4.40 (2 H, AB q, J_{AB} = 11.5 Hz, OCH_2C$_6$H$_5$), 4.42 (1 H, half of AB system of OCH_2C$_6$H$_5$), 4.62-5.25 (18 H, AB systems of OCH_2C$_6$H$_5$, C-1-H, C-2-H, C-4-H), 7.04-7.32 (45 H, m, C$_6H_5$.

^{13}C NMR ($CDCl_3$, 68 MHz): δ 43.90 (d, C-6), 64.69 (t, C-7), 69.59-70.04 (6t, P(O)OCH_2), 72.30, 72.74, 73.79 (3t, CHOCH_2C$_6$H$_5$), 75.62, 76.45, 79.30, 81.74, 81.85 (5d, inositol ring *C*H), 127.06, 127.18, 127.32, 127.37, 127.55, 127.76, 127.89, 127.97, 128.00, 128.07, 128.12, 128.15, 128.28, 128.31, 128.35, 128.39 (16d, C_6H_5), 135.59, 135.63, 135.71, 135.84 (4s, 6 x C$_6$H$_5$ *ipso*), 137.93, 138.29 (2s, 3 x C$_6$H$_5$ *ipso*).

^{31}P NMR ($CDCl_3$, 162 MHz): δ 67.76 (2P), 68.23 (1P).

MS: *m/z* (+ve ion FAB) 1293 [(M+H)$^+$, 70%], 305(50), 271(10), 181(15), 91 [(C_7H_7)$^+$, 100%].

MS: *m/z* (–ve ion FAB) 1201 [(M–C_7H_7)$^-$, 80%], 293 [($C_6H_5CH_2O)_2P(O)S^-$, 100%].

DL-(1,3,5/2,4,6)-6-Hydroxymethyl-cyclohexane-1,2,3,4,5-pentol 1,2,4-trisphosphorothioate (= DL-6-Deoxy-6-hydroxymethyl-*scyllo*-inositol-1,2,4-trisphosphorothioate 60)

Compound **61** (90mg, 70μmol) was deprotected as described for **15** and purified by ion-exchange chromatography on Q Sepharose Fast Flow Resin, eluting with a gradient of triethylammonium bicarbonate buffer (0 to 1M), pH 8.0. The triethylammonium salt of **60** eluted between 880 mM and 1000 mM. Yield 45mmol, 64%)

Accurate mass FAB$^-$: m/z 480.9025 (M–H)$^-$ (calcd for $C_7H_{16}O_{12}P_3S_3^-$, 432.9017).

^{1}H NMR (D_2O, 400MHz): δ 1.43 (1 H, br t, J ~ 11 Hz, 9.2 Hz, C-6-H), 3.36 (1 H, dd, J = 9.3 Hz, 9.3 Hz, C-3-H), 3.47 (1 H, dd, J = 10.3 Hz, 9.8 Hz, C-5-H), 3.62, 3.62 (1 H, br d, J = 12.2 Hz, C-7-H_a), 3.77 (1 H, br d, J = 12.2 Hz, C-7-H_b), 3.94-4.08 (2 H, m, C-1-H, C-4-H), 4.27 (1 H, ddd, J =11.7 Hz, 9.8 Hz, 9.3 Hz, C-2-H).

^{31}P NMR (D_2O, 162 MHz): 46.47 (1 P, J_{HP} = 9.9 Hz), 49.05 (1 P, J_{HP} = 12.6 Hz), 50.50 (1 P, J_{HP} = 11.9 Hz)

MS: *m/z* (+ve ion FAB) 102 [($C_2H_5)_3NH^+$, 100%].

MS: *m/z* (–ve ion FAB) 481 [(M–H)$^-$, 100%], 447(5), 385(5), 113 [$H_2PO_3S^-$, 10%].

8.11 *scyllo*-Inositol 1,2,3,5-tetrakisphosphate

2,4-Di-*O*-*p*-methoxybenzyl-1,3,5-*O*-methylidene-*scyllo*-inositol (63)

The ketone **29** (1.98g, 4.63mmol) was dissolved in a mixture of THF (20mL) and methanol (80mL). Sodium borohydride (430mg, 11.6 mmol) was added gradually and the mixture stirred at room temperature. for a further 30 min. TLC showed complete conversion to a product with R_f 0.44 (ethyl acetate/hexane 1:1). Water (100mL) was added and the product extracted with dichloromethane (3 x 100mL). The combined organic phases were washed with brine and dried over $MgSO_4$. Evaporation of solvent under reduced pressure gave a white solid which was recrystallised from ethyl acetate/hexane to provide **63** (1.77g, 4.12 mmol, 89%).

Mp: 125-126°C (from ethyl acetate/hexane or ethanol).

^{1}H NMR ($CDCl_3$, 270MHz): δ 3.79 (6 H, s, 2 x OCH_3), 4.10 (1 H, d, J = 12.45 Hz, D_2O ex 6-OH), 4.34-4.42 (3 H, m, narrows on D_2O ex, C-6-H and 2 x C-H), 4.43-4.48 (2 H, m, 2 x C-H), 4.56 (4 H, AB s, $CH_2C_6H_5$), 4.56 (1 H, m, buried, C-3-H), 5.49 (1 H, s, O_3CH), 6.77-6.84 (4 H, m, C_6H_4OMe), 7.08-7.16 (4 H, m, C_6H_4OMe).

^{13}C NMR ($CDCl_3$, 68 MHz): δ 55.15 (q, 2 x OCH_3), 66.71, 68.73, 69.18, 72.85 (4 d, 6 x inositol ring *C*), 71.36 (t, 2 x $CH_2C_6H_4OMe$), 102.40 (d, O_3CH), 113.83 (d, C-3 and C-5 of *p*-methoxyphenyl rings), 129.56 (d, C-2 and C-6 of *p*-methoxyphenyl rings), 129.72 (s, C-1 of *p*-methoxyphenyl rings), 159.42 (s, C-4 of *p*-methoxyphenyl rings).

MS: *m/z* (+ve ion FAB, rel intensity) 431 [$(M+H)^+$, 1%], 309 (8%), 121 [$(CH_2C_6H_4OCH_3)^+$, 100%].

Anal. Calcd for $C_{23}H_{26}O_8$, C, 64.18; H, 6.09%), Found: C, 63.9; H, 6.08.

1,3-Di-*O*-*p*-methoxybenzyl-*scyllo*-inositol (64)

To a solution of **63** (1.0g, 2.32mmol) in methanol (50mL) was added 1M HCl (5mL). The mixture was heated at reflux for 30 min after which TLC showed that most of the starting material (R_f 0.48, ethyl acetate/ hexane 1:1) had been consumed. The heating source was removed and concentrated ammonia solution (1mL) was added. Stirring was continued for a further 30 min at room temperature and then the solvents were removed by evaporation *in vacuo* to give a solid residue which was extracted with hot ethyl acetate (5 x 50mL). Evaporation of the combined extracts, followed by crystallisation from methanol/ethyl acetate gave **64** (666mg, 1.58mmol, 68%).

Mp: 161-163°C (from ethyl acetate/methanol).

^{1}H NMR (d_6-DMSO, 270MHz): δ 2.98–3.19 (5 H, m, C-1-H, C-3-H, C-4-H, C-6-H and C-5-H or C-2-H), 3.29 (1 H, m, D_2O ex gives t, J = 9.2 Hz, C-2-H or C-5-H), 3.73 (6 H, s, 2 x OCH_3), 4.70 (4 H, s, 2 x OCH_2Ar), 4.84 (3 H, d, J = 3.5 Hz, D_2O ex, 3 x OH), 4.98 (1 H, d, J = 5.3 Hz, D_2O ex, OH), 6.86 (4 H, d, J = 8.4 Hz, C_6H_4OMe), 7.34 (4 H, d, J = 8.4 Hz, C_6H_4OMe).

^{13}C NMR (d_6-DMSO, 68 MHz): δ 55.09 (q, 2 x OCH_3), 73.43 (t, 2 x OCH_2Ar), 73.67 (d, inositol ring C), 73.79 (d, 2 x inositol ring C), 74.50 (d, inositol ring C), 82.95 (d, 2 x inositol ring C), 113.33 (d, C-3 and C-5 of *p*-methoxyphenyl rings), 129.27 (d, C-2 and C-6 of *p*-methoxyphenyl rings), 131.75 (s, C-1 of *p*-methoxyphenyl rings), 158.49 (s, C-4 of *p*-methoxyphenyl rings).

MS: *m/z* (+ve ion FAB, rel intensity) 419 [$(M-H)^+$, 3%], 299 (12), 149 (15), 121 [$(CH_2C_6H_4OCH_3)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 573 [$(M+NBA)^-$, 95%], 419 [$(M-H)^-$, 100], 291 (43), 118 (32).

Anal. Calcd for $C_{22}H_{28}O_8$ (420.46):C, 62.85; H, 6.71%, Found: C, 62.5; H, 6.69.

4,6-Di-*O*-*p*-methoxybenzyl-*scyllo*-inositol 1,2,3,5-tetrakis[bis(2-cyanoethyl)-phosphate] (65)

To a solution of bis(cyanoethoxy)-*N*,*N*-diisopropylaminophosphine (516mg, 1.90mmol) in dry dichloromethane (2mL) was added 1*H*-tetrazole (267mg, 3.80mmol). The mixture was stirred at room temperature for 10 min and then the tetrol **64** (100mg, 0.238mmol) was added. The mixture was stirred for a further 1 h, after which a ^{31}P NMR spectrum showed a complex pattern of signals around 141ppm. The mixture was cooled to –78°C and *m*-CPBA (360mg, 2.09mmol) was added. The mixture was allowed to reach room temperature, and then diluted with ethyl acetate (50mL). The solution was washed with 10% sodium sulphite solution, sat. $NaHCO_3$ and brine (50mL of each), dried ($MgSO_4$) and evaporated *in vacuo* to give an oil. Purification by column chromatography (ethyl acetate/ethanol 5:1) gave the tetrakisphosphate triester **65** as a colourless oil (237mg, 0.202mmol, 85%).

^{1}H NMR ($CDCl_3$, 400MHz): δ 2.36-2.58 (8 H, m, 4 x CH_2CN), 2.72 (4 H, br t, J ~ 6 Hz, CH_2CN), 2.84 (4H, br t, J ~ 6 Hz, CH_2CN), 3.80 (6 H, s, 2 x)CH_3), 3.86 (2 H, dd, J = 7.6 Hz, 7.6Hz, C-4/6-H), 3.97-4.47 (16 H, m, 8 x OCH_2CH_2CN), 4.61-4.71 (4 H, m, C-1/3-H, C-2-H and C-5-H), 4.79 (4 H, AB system, J_{AB} = 11.3 Hz, OCH_2Ar), 6.90 (4 H, d, J = 8.5 Hz, C_6H_4OMe), 7.39 (4 H, d, J = 8.5 Hz, C_6H_4OMe).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.32 (t, J_{COP}= 7.3 Hz, 4 x CH_2CN), 19.55 (t, J_{COP} = 9.2 Hz, 2 x CH_2CN), 19.70 (t, J_{COP}= 7.3 Hz, 2 x CH_2CN), 55.34 (q, 2 x OCH_3), 62.67 (t, J_{COP} = 5.6 Hz, 2 x OCH_2CH_2CN), 62.85 (t, J_{COP} = 3.6 Hz, 2 x OCH_2CH_2CN), 63.08 (t, J_{COP} = 5.5 Hz, 2 x OCH_2CH_2CN), 63.28 (t, J_{COP} = 5.5 Hz, 2 x OCH_2CH_2CN), 73.92 (t, 2 x $OCH_2C_6H_4OMe$), 76.50, 77.27, (2 d, J_{COP} unreadable, 3 x inositol ring C), 78.20 (d, inositol ring C-4/6), 78.60 (d, J_{COP} = 7.4 Hz, C-5), 113.81 (d, C-3 and C-5 of *p*-methoxyphenyl rings), 116.66, 116.90, 117.12, 117.43 (4 s, 8 x CN), 128.93 (d, C-2 and C-6 of *p*-methoxyphenyl rings), 129.35 (s, C-1 of *p*-methoxyphenyl rings), 159.34 (s, C-4 of *p*-methoxyphenyl rings).

^{31}P NMR ($CDCl_3$, 162 MHz): –2.79 (1 P), –3.11 (3 P).

MS: *m/z* (+ve ion FAB, rel intensity) 1165 [$(M+H)^+$, 40%], 1043(60), 281(80), 121 [$(CH_2C_6H_4OCH_3)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1367(30), 1330(80), 1110(100), 203[$((NCC_2H_5O)_2PO_2)^-$, 45%].

scyllo-Inositol-1,2,3,5-tetrakisphosphate (62)

Compound **65** (100mg, 85.8μmol) was deprotected as described for **15**, with the modification that the reaction was allowed to proceed for 5 min before quenching with methanol. Purification by ion-exchange chromatography on Q Sepharose Fast Flow Resin, eluting with a gradient of triethylammonium bicarbonate buffer (0 to 1M), pH 8.0 gave the glassy triethylammonium salt of **62**, which eluted between 670 mM and 780 mM. Yield 61μmol, 71%)

^1H NMR (D_2O, 400 MHz, pH 4): δ 3.48 (2 H, dd, J = 9.76 Hz, 8.55 Hz, C-4/6-H), 3.83 (1 H, dt, J = 8.85 Hz , 9.16 Hz, C-5-H), 3.90-4.02 (3 H, m, C-1/3-H and C-2-H).

^{31}P NMR (D_2O, 162 MHz, pH 4): δ 0.11 (2 P, d, J_{HP} = 8.85 Hz, P-1 and P-3), 0.38 (1 P, d, J_{HP} = 8.85 Hz), 0.53 (1 P, d, J_{HP} = 8.85 Hz).

MS: *m/z* (+ve ion FAB, rel intensity) 102 [$(C_2H_5)_3NH^+$, 100%]

MS: *m/z* (–ve ion FAB, rel intensity) 999 [$(2M-H)^-$, 80%], 499 [$(M-H)^-$, 100%]

Accurate mass FAB$^-$: *m/z* 498.919 $(M-H)^-$ calcd for $C_6H_{15}O_{18}P_4^-$, 498.921.

8.12 D- and L-*myo*-Inositol 1,3,4,5-tetrakisphosphate

2-*O*-[(–)-ω-Camphanoyl]-1,3,5-*O*-methylidene-*myo*-inositol (66)

To a solution of *myo*-inositol orthoformate (**27**) (1.00g, 5.26mmol) in dry pyridine (10mL) was added (1*S*,4*R*)-(–)-ω-camphanic acid chloride (1.25g, 5.78mmol and a catalytic amount of DMAP (50mg). The solution was stirred for 30 min at room temperature, after which TLC (dichloromethane/ethyl acetate 2:1) showed conversion to a major product (R_f 0.26) and two minor products (R_f 0.40 and 0.48). The solvents were removed by evaporation *in vacuo* and the residue was taken up in dichloromethane. (100mL), washed with 1M HCl (50mL) and brine (50mL), dried ($MgSO_4$). Evaporation *in vacuo* followed by purification using flash chromatography (dichloromethane/ethyl acetate 3:1) gave the 2-camphanate **66** (1.19g, 3.21mmol, 61%) and smaller amounts of the 2,4- and 2,6-biscamphanates (see below).

R_f 0.26 (dichloromethane/ethyl acetate 2:1)

Mp: 241-250°C (from ethyl acetate)

^{1}H NMR (d_6-DMSO, 400 MHz) δ 0.87, 1.02, 1.07 (9 H, 3 s, camph-CH_3), 1.54-1.61 (1 H, m, camph-CH_2), 1.96-2.06 (2 H, m, camph-CH_2), 2.40-2.47 (1 H, m, camph-CH_2), 4.16-4.20 (3 H, br m, C-4-H, C-5-H, C-6-H), 4.36 (2 H, br m, C-1-H and C-3-H), 5.41 (1 H, br s, C-2-H), 5.59 (1 H, br s, O_3CH), 5.70 (2 H, br s, D_2O ex, C-4-OH, C-6-OH)

^{13}C NMR (d_6-DMSO, 100 MHz) δ 9.87, 16.64, 16.82 (3q, camph-*C*H_3), 28.84, 30.72 (2 t, camph-*C*H_2), 54.88, 54.64 (2 s, camph-*C*), 65.20, 67.32, 69.99, 71.96 (4 d, inositol ring *C*), 91.40 (s, camph-*C*), 102.25 (d, O_3*C*H), 167.22, 178.75 (2 s, 2 x camph *C*=O).

MS: *m/z* (+ve ion FAB, rel intensity) 741 [$(2M+H)^+$, 90%)], 371 [$(M+H)^+$, 100%)], 173 [(M–camphanate)$^+$, 40%]

MS: *m/z* (–ve ion FAB, rel intensity) 536(100), 523 [$(M+NBA)^-$, 80%], 416(30), 369 [$(M-H)^-$, 40%], 197 [(camphanate)$^-$,30%].

Anal. Calcd for $C_{17}H_{22}O_9$ (370.36); C, 55.13; H 5.99, Found C, 55.1; H, 6.08

Bis[(–)-ω-camphanate] Esters of *myo*-Inositol Orthoformate: Optimised Method

To a stirred suspension of *myo*-inositol orthoformate **27** (2.00g, 10.5mmol) in dry dichloromethane (40mL) at 0°C were added triethylamine (3.3mL, 23.7mmol) and a catalytic amount of DMAP (80mg). A solution of (1*S*,4*R*)-(–)-ω-camphanic acid chloride (4.55g, 21.0mmol) in dry dichloromethane (10mL) was added dropwise under N_2 at 0°C. The cooling bath was removed after 15min and stirring continued for further

45min, after which time almost no solid was present and TLC (dichloromethane/ethyl acetate 3:1)showed two major products at R_f 0.32 and 0.23. The solvent was removed by evaporation *in vacuo* and the residue purified by flash chromatography (dichloromethane/ethyl acetate 4:1) giving first the 2,6-biscamphanate **67a** (3.46g, 6.28mmol, 60% yield) and secondly the 2,4-biscamphanate **67b** (1.35g, 2.45mmol, 23% yield).

1D-2,6-Di-*O*-[(–)-ω-camphanoyl]-1,3,5-*O*-methylidene-*myo*-inositol (67a)

R_f 0.32 (dichloromethane/ethyl acetate 3:1); $[\alpha]_D = -15$ (c = 1, $CHCl_3$), $[\alpha]_D^{26} = -21$ (c = 1, DMF).

Crystals from ethyl acetate/hexane or propan-2-ol. Gradual melting with sublimation above 235°C with phase change to needles, melting at 274-275°C.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.00, 1.03, 1.09, 1.10, 1.12, 1.14 (18 H, 6 s, camph-CH_3), 1.65-1.75 (2 H, m, camph-CH_2), 1.92-2.03 (2 H, m, camph-CH_2), 2.06-2.18 (2 H, m, camph-CH_2), 2.41-2.56 (2 H, m, camph-CH_2), 3.29 (1 H, d, J = 6.8 Hz, D_2O ex., 4-OH), 4.36 (1 H, dddd, appears as dq, J = 4 Hz, 2 Hz, 2 Hz, 2 Hz, C-3-H), 4.43 (1 H, dddd, appears as dq, J = 4 Hz, 2 Hz, 2 Hz, 2 Hz, C-1-H), 4.55 (1 H, dddd, appears as tt, J = 4 Hz, 4 Hz, 2 Hz, 2 Hz, C-5-H), 4.66-4.68 (1 H, br m, C-4-H), 5.30 (1 H, ddd, appears as partially resolved dt, J ~ 2 Hz, 2 Hz, 1 Hz, C-2-H), 5.54 (1 H, d, J = 0.98 Hz, O_3CH), 5.63 (1 H, ddd, appears as dt, J = 4 Hz, 4 Hz, 1.5 Hz, C-6-H).

^{13}C NMR (d_6-DMSO, 100 MHz) δ 9.40,9.45, 15.93, 16.11, 16.18, 16.35 (6 q, camph-CH_3), 28.22, 28.29, 30.21, 30.65 (4 t, camph-CH_2), 53.91, 54.02, 54.26, 54.37 (4s, camph *C*), 64.28, 65.76, 68.14, 68.43, 69.38, 71.08 (6 d, inositol ring *C*), 90.67 (s, 2 x camph *C*), 102.05 (d, O_3CH), 166.44, 166.16, 177.63 (3 s, 4 x camph *C*=O).

MS: *m/z* (+ve ion FAB, rel intensity) 1101 [$(2M+H)^+$, 100%)], 687(60), 551 [$(M+H)^+$, 80%)], 353 [$(M-camphanate)^+$, 20%]

MS: *m/z* (–ve ion FAB, rel intensity) 1100[$2M^-$, 40%], 989(80), 716(100), 703 [$(M+NBA)^-$, 75%], 197 [$(camphanate)^-$,30%].

Anal. Calcd for $C_{27}H_{34}O_{12}$ (550.56); C, 58.90; H 6.22, Found C, 58.7; H, 6.23

1D-2,4-Di-*O*-[(–)-ω-camphanoyl]-1,3,5-*O*-methylidene-*myo*-inositol (67b)

R_f 0.23 (dichloromethane/ethyl acetate 3:1); $[\alpha]_D^{25} = +7$ (c = 1, DMF).

Mp: 270-272°C (from DMF/water).

^{1}H NMR (d_6-DMSO, 400 MHz) δ 0.86, 0.88, 1.01, 1.02, 1.05, 1.08 (18 H, 6s, camph-CH_3), 1.52-1.61 (2 H, m, camph-CH_2), 1.84-2.07 (4 H, m, 2 x camph-CH_2), 2.38-2.47 (2 H, m, camph-CH_2), 4.23 (1 H, br s, C-H), 4.42 (3 H, br s, 3 x C-H), 5.32 (1 H, br s, C-2-H), 5.44 (1 H, br s, C-4-H), 5.73 (1 H, s, O_3CH), 5.96 (1 H, d, J = 3.36 Hz, D_2O ex., C-6-OH).

^{13}C NMR (d_6-DMSO, 68 MHz) δ 9.47,9.52, 16.13, 16.25, 16.44, 16.54 (6 q, camph-*C*H_3), 28.28, 28.41, 30.31, (3 t, 4 x camph-*C*H_2), 53.90, 54.07, 54.31, 54.41 (4 s, camph *C*), 64.46, 65.81, 67.95, 68.39, 68.87, 71.05 (6 d, inositol ring *C*), 90.73, 90.86 (2 s, camph *C*), 102.12 (d, O_3*C*H), 165.81, 166.57, 177.61 (3 s, 4 x camph *C*=O).

MS: *m/z* (+ve ion FAB, rel intensity) 1101 [$(2M+H)^+$, 55%)], 687(65), 551 [$(M+H)^+$, 100%)], 353 [(M–camphanate)$^+$, 30%]

MS: *m/z* (–ve ion FAB, rel intensity) 716(70), 703 [$(M+NBA)^-$, 40%], 197 [(camphanate)$^-$,100%].

Anal. Calcd for $C_{27}H_{34}O_{12}$ (550.56); C, 58.90; H 6.22, Found C, 58.6; H, 6.31

1D-2,6-Di-*O*-[(–)-ω-camphanoyl]-*myo*-inositol (68a)

To a stirred suspension of 1D-2,6-di-*O*-[(–)-ω-camphanoyl]-1,3,5-*O*-methylidene-*myo*-inositol **67a** (3.0g, 5.45mmol) in methanol (100mL) was added 1.0M HCl (10mL). The mixture was heated at reflux for 6 h, after which time TLC ($CHCl_3$/MeOH 5:1) showed that almost all the starting material (R_f 0.76) had been converted to a major product (R_f 0.40). The clear solution was allowed to cool, and then evaporated to dryness under reduced pressure, leaving a white residue which was purified by flash chromatography ($CHCl_3$/MeOH 5:1) to give the tetrol **68a** (2.58g, 4.77mmol, 88% yield).

R_f 0.23 ($CHCl_3$/MeOH 5:1)

$[\alpha]_D^{25} = -8$ (c = 1, DMF).

Mp: 211-213°C (from propan-2-ol)

^{1}H NMR (d_6-DMSO, 400 MHz) δ 0.82, 0.87, 0.96 (9 H, 3 s, 3 x camph-CH_3), 0.99 (6H, s, 2 x camph-CH_3), 1.03 (3 H, s, c camph-CH_3), 1.48-1.58 (2 H, m, camph-CH_2), 1.83-2.05 (4 H, m, 2 x camph -CH_2), 2.33-2.44 (2 H, m, camphanate-CH_2), 3.22 (1 H, m, D_2O ex. gives dd, J = 9.28 Hz, 9.27 Hz, C-5-H), 3.38 (1 H, m, D_2O ex. gives dd, J = 9.28 Hz, 9.27 Hz, C-4-H), 3.49 (1 H, m, D_2O ex. gives dd, J = 9.76 Hz, 2.93 Hz, C-3-H), 3.76 (1 H, m, after D_2O ex. signal obscured by H_2O, C-1-H), 4.98 (1 H, dd, J = 10.26 Hz, 9.76 Hz, C-6-H), 5.12 (1 H, d, J = 4.88 Hz, D_2O ex., C-4-OH), 5.19 (2 H, br

s, D_2O ex., C-3-OH and C-5-OH), 5.34 (1 H, dd, J = 2.45 Hz, 2.44 Hz, C-2-H), 5.41 (1 H, d, J = 5.37 Hz, D_2O ex., C-1-OH).

^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 9.60, 9.65, 16.15, 16.41, 16.59 (5 q, 6 x camph-CH_3), 28.48, 30.29 (2 t, 4 x camph-CH_2), 53.99, 54.03, 54.37, 54.44 (4 s, 4 x camph-*C*), 67.02, 68.94, 72.08, 73.48, 75.80, 76.22 (6 d, inositol ring *C*H), 91.19, 91.38 (2 s, camph-*C*), 166.26, 178.10 (2 s, 4 x camph-*C*=O).

MS: *m/z* (+ve ion FAB, rel intensity) 1081 [$(2M+H)^+$, 40%], 694(40), 677(80), 541 [$(M+H)^+$, 100%], 343 [(M–camphanate)$^+$, 15%.

MS: *m/z* (–ve ion FAB, rel intensity) 1246(65), 1233 [$(2M+NBA)^-$, 10%], 1145(15), 1079 [(2M-H), 45%], 979(80), 706(100), 693 [$(M+NBA)^-$, 70%], 539 [$(M-H)^-$, 28%], 197 [(camphanate)$^-$, 60%].

Anal. Calcd for $C_{26}H_{36}O_{12}.2H_2O$ (576.60), C 54.2; H 6.99, Found C, 54.5; H, 6.89.

1D-2,4-Di-*O*-[(–)-ω-camphanoyl]-*myo*-inositol (68b)

A solution of the alcohol **67b** (520mg, 0.944mmol) in TFA (8mL) and water (2mL) was stirred at room temperature. After 40 h, TLC showed almost complete conversion to the tetrol. The solvents were removed by evaporation under reduced pressure. Water was added (10mL) and removed by evaporation under reduced pressure. This procedure was repeated twice to remove residual TFA, and the dry solid white residue was purified by flash chromatography (chloroform/methanol 5:1) to give **68b** (436mg, 0.807mmol, 85%).

R_f 0.23 ($CHCl_3$/MeOH 5:1)

$[\alpha]_D^{25}$ = +12 (c = 1, DMF).

Mp: 238-241°C (from DMF/water).

^1H NMR (d_6-DMSO, 400 MHz) δ 0.87, (6 H, s, 2 x camph-CH_3), 0.997, 1.00, 1.02, 1.11 (12 H, 4 s, 4 x camph-CH_3), 1.51-1.57 (2 H, m, camph-CH_2), 1.87-2.03 (4 H, m, 2 x camph-CH_2), 2.36-2.46 (2 H, m, camph-CH_2), 3.25 (1 H, m, D_2O ex. gives dd, J = 9.46 Hz, 9.16 Hz, C-5-H), 3.35 (1 H, m, D_2O ex. gives dd, J = 9.16 Hz, 9.15 Hz, C-6-H), 3.49 (1 H, m, D_2O ex. gives br d, J = 9.16 Hz, C-1-H), 3.74 (1 H, m, D_2O ex gives br d, J = 10.07 Hz, C-3-H), 5.03-5.13 (4 H, m, D_2O ex. gives 1 H, dd, J = 9.77 Hz, 9.46 Hz, C-4-H and 3 x OH), 5.37 (1 H, br s, C-2-H), 5.38 (1 H, br s, D_2O ex, OH).

^{13}C NMR ($CDCl_3$, 100.4 MHz) δ 9.73, 16.37, 16.42, 16.59, 16.66 (5 q, 6 x camph-CH_3), 28.56, 28.62, 30.47 (3 t, 4 x camph-CH_2), 54.11, 54.19, 54.53, 54.44 (3 s, 4 x camph-C), 66.99, 69.18, 72.09, 73.24, 76.22, 76.39 (6 d, inositol ring CH), 91.27, 91.53 (2 s, camph-C), 166.38, 166.42, 178.21, 178.25 (4 s, 4 x camph-C=O).

MS: *m/z* (+ve ion FAB, rel intensity) 1081 [$(2M+H)^+$, 45%], 694(50), 677(80), 541 [$(M+H)^+$, 100%], 343 [(M–camphanate)$^+$, 10%.

MS: *m/z* (–ve ion FAB, rel intensity) 1246(60), 1145(15), 1079 [(2M-H), 50%], 979(70), 706(100), 693 [$(M+NBA)^-$, 65%], 539 [$(M-H)^-$, 32%], 197 [$(camphanate)^-$, 80%].

1D-2,6-Di-*O*-[(–)-ω-camphanoyl]-*myo*-inositol 1,3,4,5-tetrakis[bis(2-cyanoethyl)phosphate] (69a)

The tetrol **68a** (1.0g, 1.85mmol) was phosphitylated using bis(cyanoethoxy)-diisopropylaminophosphine as described for the synthesis of **64**. The ^{31}P NMR spectrum of the intermediate tetrakisphosphite showed signals at δ 138.4 (1P, s,), 139.7 (1P, d, $^5J_{PP}$ = 7.3 Hz), 141.3 (1P, d, $^5J_{PP}$ = 7.3 Hz), 142.1 (1P, dd, $^5J_{PP}$ = 7.3 Hz, 7.3 Hz). Oxidation and work-up as before, followed by purification using flash chromatography (chloroform/methanol 5:1) afforded **69a** (1.99g, 1.55mmol, 84%) as a brittle white foam.

$[\alpha]_D^{22} = -11$ (c = 1, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.97, 1.05 (6 H, 2s, camph-CH_3), 1.10 (6 H, s, 2 x camph-CH_3), 1.13, 1.15 (6 H, 2s, camph-CH_3), 1.71-1.76 (2 H, m, camph-CH_2), 1.91-2.05 (2 H, m, camph-CH_2), 2.13-2.18 (1 H, m, camph-CH_2), 2.24-2.30 (1 H, m, camph-CH_2), 2.40-2.47 (1 H, m, camph-CH_2), 2.51-2.58 (1 H, m, camph-CH_2), 2.75-2.98 (16 H, m, 8 x CH_2CN), 4.32-4.46 (16 H, m, 8 x CH_2CH_2CN), 4.66 (1 H, ddd, J = 9.46 Hz, 9.46 Hz, 9.15 Hz, C-4-H or C-5-H), 4.73-4.86 (3 H, m, C-1-H, C-3-H and C-4-H or C-5-H), 5.54 (1 H, dd, J = 9.77 Hz, 9.76 Hz, C-6-H), 6.19 (1 H, br s, C-2-H).

^{13}C NMR ($CDCl_3$, 100.4 MHz) δ 9.62, 9.73, 16.70, 16.73 (4 q, 6 x camph-CH_3), 19.73 (t, 8 x CH_2CN), 28.80, 28.86, 30.65, 31.65 (4 t, camph-CH_2), 54.81, 54.94, 55.0 (3 s, 4 x camph-C), 63.09-63.55 (overlapping triplets showing $^2J_{COP}$ coupling, 8 x $POCH_2$), 69.9, 70.26, 71.94, 72.68, 74.83, 75.64 (6 d, inositol ring C), 90.95, 91.04 (2 s, camph-C), 117.12, 117.24, 117.34, 117.45 (4 s, 8 x CN), 166.24, 167.15, 177.81, 178.44 (4 s, camphanate-C=O)

^{31}P NMR ($CDCl_3$, 162 MHz) δ –3.83, –3.74, –3.10, –2.85

MS: *m/z* (+ve ion FAB, rel intensity) 1285 [$(M+H)^+$, 100%], 1081 [$(M-(NCC_2H_5O)_2PO_2^-)^+$, 12%] 288(40), 169(45), 94(60).

MS: *m/z* (–ve ion FAB, rel intensity) 1450(50), 1437 [$(M+NBA)^-$, 25], 1230 [$(M-CH_2CH_2CN^+)^-$, 100], 203 [$(NCC_2H_5O)_2PO_2^-$, 48%].

Anal. Calcd for $C_{50}H_{64}O_{24}P_4N_8$ (1284.99); C, 46.7; H 5.02, N 8.72, Found C, 46.4; H, 5.00, N 8.40.

1D-2,4-Di-*O*-[(–)-ω-camphanoyl]-*myo*-inositol 1,3,5,6-tetrakis[bis(2-cyanoethyl)phosphate] (69b)

The tetrol **68b** (380mg, 0.703mmol) was phosphitylated as described for **68a**. A ^{31}P NMR of the intermediate tetrakisphosphite showed signals at δ 139.3 (1P, d, $^5J_{PP}$ = 6.7 Hz), 139.5 (1P, s), 141.3 (1P, d, $^5J_{PP}$ = 7.9 Hz), 142.0 (1P, dd, $^5J_{PP}$ = 7.9 Hz, 6.7 Hz). After oxidation and work-up as before, purification by flash chromatography (chloroform/methanol 10:1) afforded **69b** (723mg, 0.563mmol, 80%) as a brittle white foam.

$[\alpha]_D^{20} = -6$ (c = 1, $CHCl_3$).

^1H NMR ($CDCl_3$, 400 MHz) δ 1.01, 1.03, 1.11, 1.12, 1.14, 1.17 (18 H, 6s, camph-CH_3), 1.69-1.78 (2 H, m, camph-CH_2), 1.92-2.06 (2 H, m, camph-CH_2), 2.14-2.25 (2 H, m, camph-CH_2), 2.43-2.58 (2 H, m, camph-CH_2), 2.77-3.05 (16 H, m, 8 x CH_2CN), 4.27-4.51 (16 H, m, 8 x CH_2CH_2CN), 4.70-4.87 (4 H, m, C-1-H, C-3-H, C-5-H, C-6-H), 5.61 (1 H, dd, J = 9.77 Hz, 9.27 Hz, C-4-H), 6.15 (1 H, br s, C-2-H).

^{13}C NMR ($CDCl_3$, 100.4 MHz) δ 9.66, 16.70, 16.77, 16.86 (4 q, 6 x camph-CH_3), 19.66, 19.73 (2 t, 8 x CH_2CN), 28.80, 28.86, 30.93, 31.29 (4 t, camph-CH_2), 54.55, 54.68, 54.77, 54.93 (4 s, camph-*C*), 64.31-63.57 (overlapping triplets showing $^2J_{COP}$ coupling, 8 x $POCH_2$), 69.70, 70.64, 72.29, 72.84, 74.40, 75.46 (6 d, inositol ring *C*), 90.87, 90.97 (2 s, camph-*C*), 116.95, 117.08, 117.30, 117.35, 117.41, 117.46, 117.65 (7 s, 8 x *C*N), 166.31, 166.82, 177.78, 178.35 (4 s, camph-*C*=O)

^{31}P NMR ($CDCl_3$, 109.4 MHz) δ –1.84, –2.05, –2.85, –3.01.

MS: *m/z* (+ve ion FAB, rel intensity) 1285 [$(M+H)^+$, 100%], 288(30), 169(40), 94(50).

MS: *m/z* (–ve ion FAB, rel intensity) 1450(50), 1437 [$(M+NBA)^-$, 30%], 1230 [$(M-CH_2CH_2CN^+)^-$, 100%], 203 [$(NCC_2H_5O)_2PO_2^-$, 55%].

Anal. Calcd for $C_{50}H_{64}O_{24}P_4N_8$ (1284.99); C, 46.7; H 5.02, N 8.72, Found C, 46.5; H, 5.00, N 8.40.

1D-*myo*-Inositol-1,3,4,5-tetrakisphosphate (70a)

The fully-protected tetrakisphosphate **69a** (2.00g, 1.56mmol) was placed into a thick-walled Pyrex 100mL autoclavable bottle with a screw-cap and magnetic stirrer bead, followed by concentrated ammonia solution (50mL). The bottle was sealed and heated (*Care!! Use a safety screen*) in a water bath at 60°C for 6 hours. During this time the suspension first changed to a clear solution and then to a white suspension. The bottle was cooled in an ice bath and then the contents were transferred to a round-bottomed flask, together with aqueous washings. The mixture was evaporated to dryness under reduced pressure and the residue dissolved in de-ionised water (50mL). Dowex 50 resin (H^+ form, previously well-washed with de-ionised water) was added to the stirred solution until the pH had fallen to approximately pH 2. The solution was then washed with dichloromethane (3 x 50mL) and ether (3 x 50mL) before titrating with 1M potassium hydroxide solution to pH 10. The solution was then lyophilised and the solid residue redissolved in the minimum volume of de-ionised water. Methanol (60mL) was added to precipitate the potassium salt which was separated by centrifugation, and the supernatant liquid discarded. The precipitate was redissolved in de-ionised water (20mL) and lyophilised to give the potassium salt of **70a** (1.17g, 1.45mmol, 93% yield).

$[\alpha]_D^{22} = -3.5$ to -4.0 ($c = 2$, H_2O) (potassium salt) ; Lit.[98] -3.5 for the potassium salt

$[\alpha]_D^{20} = -3$ ($c = 2$, H_2O) (cyclohexylammonium salt); Lit.[129] -2.5 for the cyclohexylammonium salt.

^1H NMR (D_2O, 400 MHz) δ 3.71 (1 H, dd app. as t, J = 9.8 Hz, 8.8 Hz, C-6-H), 3.77-3.83 (2 H, m, C-1-H and C-5-H), 3.87 (1 H, ddd app. as dt, J = 9.3 Hz, 8.8 Hz, ~2.5 Hz, C-3-H), 4.18 (1 H, ddd app. as q, J = 9.8 Hz, 9.3 Hz, 9.3 Hz, C-4-H), 4.31 (1 H, dd app. as poorly resolved t, C-2-H).

^{13}C NMR (D_2O, 100 MHz) δ 73.40 (C-2), 74.74 (C-6), 76.47, 76.54 (C-1 and C-3), 78.77 (C-4), 80.51 (C-5).

^{31}P NMR (D_2O, 162 MHz) δ 3.07, 3.66, 3.94, 4.58.

MS: *m/z* (+ve ion FAB, rel intensity, cyclohexylammonium salt) 100 [$C_6H_{14}N^+$, 100%]

MS: *m/z* (–ve ion FAB, rel intensity, cyclohexylammonium salt) 999 [$(2M-H)^-$, 20%], 499 [$(M-H)^-$, 100%]

Accurate mass FAB^-: *m/z* 498.921 M^- calcd for $C_6H_{15}O_{18}P_4^-$, 498.921.

1L-*myo*-Inositol-1,3,4,5-tetrakisphosphate (70b)

The fully-protected L-trisphosphate **69b** (600mg, 0.467mmol) was deprotected in the same way as described for **69a**, and treated similarly to give the potassium salt of **70b** (340mg, 0.422mmol, 90%).

$[\alpha]_D^{22}$ = +3.5 to +4.0 (c = 2, H_2O) (potassium salt)

(Lit[129] +2.6 for the cyclohexylammonium salt.

NMR and mass spectroscopic data were identical to those for the enantiomer **70a**.

Accurate mass FAB$^-$: m/z 498.925 M^- calcd for $C_6H_{15}O_{18}P_4^-$, 498.921.

8.13 Alternative Route to D-*myo*-Inositol 1,3,4,5-tetrakisphosphate

1D-2,6-Di-*O*-[(–)-ω-camphanoyl]-*myo*-inositol-1,3,4,5-tetrakis(dibenzylphosphate) (71a)

To a solution of bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (765mg, 2.22mmol) in dry dichloromethane (4mL) was added 1*H*-tetrazole (311mg, 4.43mmol). The mixture was stirred at room temperature for 20min and then the tetrol **68a** (150mg, 0.277mmol) was added. Stirring was continued for 30min, after which ^{31}P NMR showed a complex pattern of signals around δ_P 140ppm. The mixture was cooled to –78°C, *m*-CPBA (420mg, 2.44mmol) was added, and the cooling bath was removed. The mixture was allowed to reach room temperature and then diluted with dichloromethane (50mL). The clear solution was washed with 10% Na_2SO_3, and sat. $NaHCO_3$ (50mL of each) dried ($MgSO_4$) and evaporated *in vacuo*. Purification of the residue by flash chromatography (chloroform/methanol 10:1) afforded **71a** (377mg, 0.238mmol, 86%) as a colourless oil.

$[\alpha]_D^{21}$ = –6 (c = 1, $CHCl_3$).

^1H NMR ($CDCl_3$, 270 MHz) δ 0.90 (3 H, s, camphanate-CH_3), 0.95 (6 H, s, 2 x camphanate-CH_3), 1.03 (3 H, s, camphanate-CH_3), 1.05 (3 H, s, camphanate-CH_3), 1.08 (3 H, s, camphanate-CH_3), 1.45-2.50 (8 H, m, 4 x camphanate-CH_2), 4.17 (1 H, ddd, J = 10 Hz, 8 Hz, 2.5 Hz, C-1-H or C-3-H), 4.26-4.38 (2 H, m, 2 x phosphorylated inositol ring C-H), 4.89-5.09 (17 H, m, 8 x $CH_2C_6H_5$ and C-4-H) 5.59 (1 H, dd, J = 9.8. Hz, 9.8 Hz, C-6-H), 6.29 (1 H, dd, J = 2.5 Hz 2.5 Hz, C-2-H), 7.12-7.36 (40 H, m, 8 x C_6H_5).

^{13}C NMR ($CDCl_3$, 68 MHz) δ 9.58, 16.40, 16.50, 16.77 (4 q, 6 x camph-CH_3), 28.87 (t, 2 x camph-CH_3), 30.55, 31.14 (2 t, camph-CH_3), 69.87, 70.34 (2 t, 8 x OCH_2Ar), 70.61, 71.63, 72.69, 75.15, 75.60 (5 d, 6 x inositol ring C), 90.87, 90.90 (2 s, 4 x camph-C), 127.84, 128.20, 128.28, 128.38, 128.57, 128.82 (6 d, C_6H_5), 135.29, 135.37, 135.48,

135.58, 135.68, 135.77 (6 s, 8 x C_6H_5), 165.48, 166.90 (2 s, 2 x camph-*C*=O), 177.66, 178.07 (2 s, 2 x camph-*C*=O)

^{31}P NMR ($CDCl_3$, 109 MHz) δ –1.04, –0.98, –0.92, –0.61

MS: *m/z* (+ve ion FAB, rel intensity) 1581[$(M+H)^+$, 80%], 271(50), 181(16), 91 [$(C_7H_7)^+$, 100%].

MS: *m/z* (–ve ion FAB, rel intensity) 1746(80), 1732(40), 1489 [$(M-C_7H_7)^-$,50%], 277 [$(C_6H_5O)_2PO_2^-$, 100%]

Two-Stage Deprotection of 71a

A solution of **71a** (180mg, 114μmole) in ethanol/water (4:1, 50mL) was hydrogenolysed at room temperature over Pd/C (10%, 200mg) at a pressure of 50 p.s.i. for 18 h. The suspension was filtered through Celite to remove the catalyst and then split into two portions. The first portion was adjusted to pH 10 with cyclohexylamine and then lyophilised and kept at -20°C for later use. The second portion was dissolved in concentrated ammonia solution and stirred at 60°C in a sealed container for 6 h. The solution was allowed to cool and washed with ether (25mL). A ^{31}P NMR at this stage was extremely broad, due to the presence of paramagnetic ions, and showed no detail. The sample was made up to 50mL with de-ionised water and purified by ion-exchange chromatography on Q Sepharose Fast Flow Resin, eluting with a gradient of triethylammonium bicarbonate buffer (0 to 1M), pH 8.0. The triethylammonium salt of **70a** eluted between 730 mM and 850 mM. (Method not optimised).

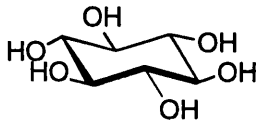
$[\alpha]_D^{20}$ = –3 (*c* = 2, TEAB buffer, pH 8) (triethylammonium salt) Lit[129] –2.5° for the cyclohexylammonium salt.

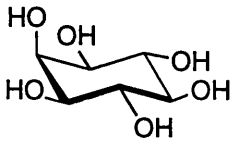
^{1}H NMR (D_2O, 400 MHz) δ 3.73 (1 H, dd, *J* = 9.8 Hz, 9.5 Hz, C-6-H), 3.86-3.93 (2 H, m, C-1-H and C-5-H), 3.98 (1 H, ddd, *J* = 9.8 Hz, 9.8 Hz, 2.4 Hz, C-3-H), 4.24 (1 H, dd *J* ~ 2 Hz, app. as poorly resolved t, C-2-H), 4.28 (1H, ddd, *J* = 9.8 Hz, 9.5 Hz, 9.5 Hz, C-4-H)

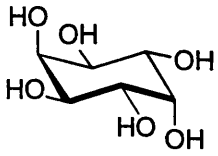
^{31}P NMR (D_2O, 162 MHz, ^{1}H-decoupled) δ –0.51, –0.34, 0.08, 0.35 (no impurities visible).

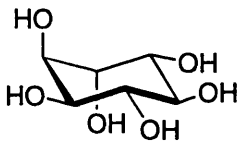
Appendix 1 Notes on Nomenclature

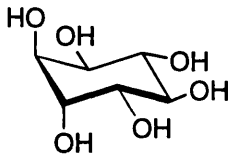
The inositols are cyclohexane-1,2,3,4,5,6-hexols. Individual inositols are differentiated by the use of an italicised prefix and hyphen. There are eight possible diastereoisomeric inositols, of which seven are achiral *meso* compounds possessing an internal symmetry plane (*scyllo*, *myo*, *neo*, *epi*, *muco*, *cis*, *allo*) and the eighth (*chiro*-inositol) is chiral. The symmetry plane may not be obvious in *allo*-inositol, in that the individual chair forms are chiral, but chair-inversion leads to the enantiomer.

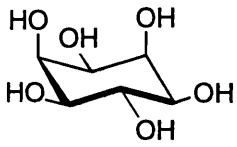

scyllo

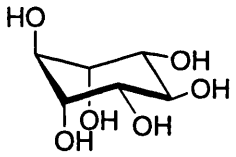

myo

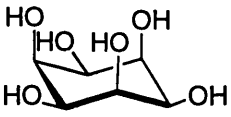

neo

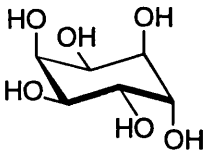

D-*chiro*


L-*chiro*


epi


muco


cis


allo

Assignment of Locants (Positional Numbers)[262]

Locants are assigned to the carbon atoms of the ring. The substituents above the plane of the ring constitute a set, and those below the plane another set. **Lowest locants** are related to one set of substituents according to a sequence of criteria which are applied successively until a decision is reached:

(i) to the substituents considered as a numerical series, without regard to configuration;

(ii) if one set of substituents is more numerous than the other, to the more numerous;

(iii) if the sets are equally numerous and one of them can be denoted by lower numbers, to that set;

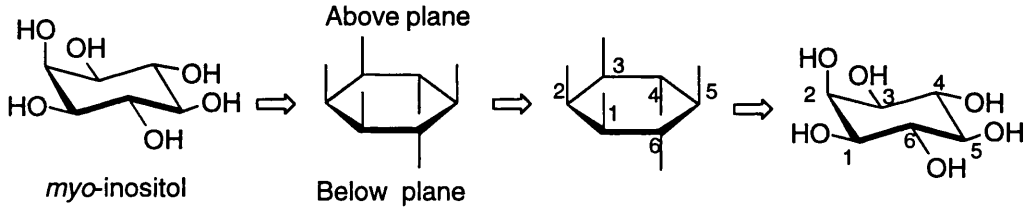
(iv) to substituents other than unmodified hydroxyl groups;

(v) to the substituent first in alphabetical order;

(vi) —for *meso*-compounds only—to those positions that lead to an L rather than a D designation when the **absolute configuration rule** is applied to the lowest-numbered asymmetric carbon atom (see below).

Note: Lowest numbers are those that, when considered as a single ascending series, contain the lower number at the first point of difference, e.g. 1,2,3,6 is lower than 1,2,4,5.

These criteria are generally applicable to cyclitol nomenclature. For unsubstituted inositols, only criteria (ii) and (vi) are required. Application of these criteria to *myo*-inositol, for example, gives the numbering shown below, in which the axial hydroxyl group is designated as the 2-position.



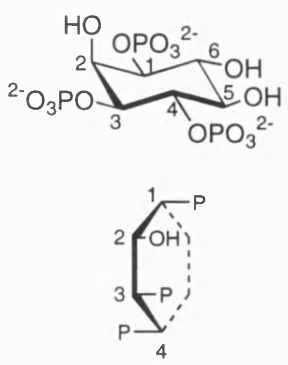
myo-inositol

Derivatives of Inositols

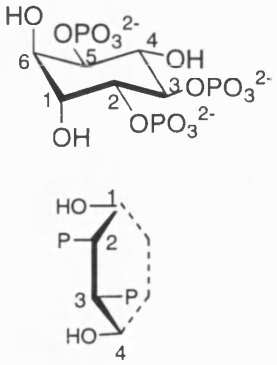
The numberings of the parent inositols are retained for derivatives. Within this framework, criteria (iv) and (v) are used to decide between alternatives. These arise because (a) in several of the parent inositols (*scyllo*, *neo*, *muco*, *cis*, *chiro*) there are two or more fully equivalent starting points (related by a C_n symmetry axis) for the numbering, that may not be equivalent in the derivatives, and (b) criterion (vi) does not apply to chiral derivatives of inositols having a symmetry plane (*scyllo*, *myo*, *neo*, *epi*, *muco*, *cis*, *allo*). The application of criteria (iv) and (v) to a pair of enantiomers gives each a pair of mirror-related positions of the same numbering. Typically, one enantiomer will be numbered clockwise, the other anti-clockwise.

Absolute Configuration Rule[262]

The absolute configuration of each enantiomer is specified by making a vertical Fischer projection of the structure with C-1 at the top and with C-2 and C-3 on the front edge of the ring. The configuration is then designated D if the hydroxyl group of the lowest-numbered stereogenic centre (or other substituent if no hydroxyl group is present) projects to the right, and as L- if it points to the left:

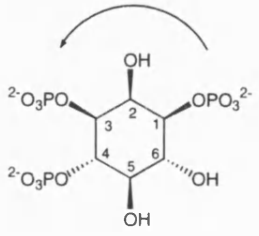


1D-*myo*-inositol 1,3,4-trisphosphate

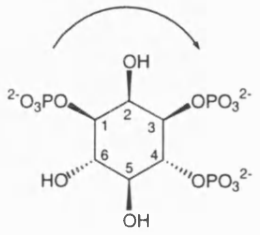


1L-*chiro*-inositol 2,3,5-trisphosphate

For *myo*-inositol derivatives, this rule has the consequence that derivatives numbered anticlockwise have the prefix D-, and clockwise numbering leads to the L- prefix:



D-*myo*-Ins(1,3,4)P_3



L-*myo*-Ins(1,3,4)P_3

The mere absence of a prefix D, L or DL indicates that the compound has a *meso*-configuration (e.g. Ins(1,3,4,6)P_4) and should therefore not be omitted. However, see below.

Relaxation of Lowest Locant Rule

A more recent recommendation[263] allows that, when necessary, the lowest-locant rule may be relaxed, so that a compound that by this rule belongs to the 1L series may be given the 1D numbering if this shows the relationships that the author wishes to stress. For example, in the metabolic conversion:

1D-*myo*-inositol 1,3,4-trisphosphate → 1L-*myo*-inositol 1,6-bisphosphate,

it may not be obvious that the step is simply a hydrolysis at C-1, but this is immediately apparent if the conversion is written as:

1D-*myo*-inositol 1,3,4-trisphosphate → 1D-*myo*-inositol 3,4-bisphosphate.

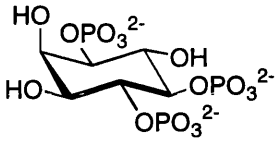
The Symbol Ins

The recommendation[263] also allows that Ins should be taken to mean *myo*-inositol with the numbering of the 1D configuration unless other prefixes are explicitly added. Thus, 1D-*myo*-inositol 1,4,5-trisphosphate may be symbolised as Ins(1,4,5)P_3. In this thesis, D-

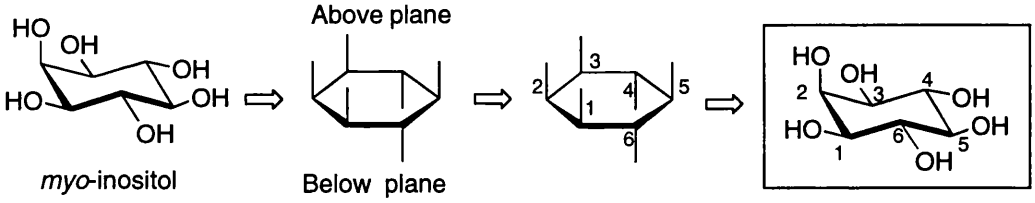
and L-prefixes are often used, even when not strictly required, and the lowest locant rule applied, so as to emphasise particular stereochemical relationships, e.g.

"D-Ins(1,3,4)P_3 and L-Ins(1,3,4)P_3" rather than "Ins(1,3,4)P_3 and Ins(1,3,6)P_3".

Example

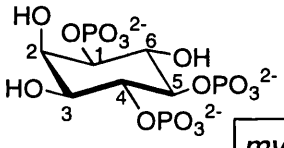
To name: 

1 Numbering of parent inositol:



[Criteria (ii) and (vi)]

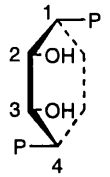
2 Allocate lowest numbers to phosphate groups within this framework:



myo-inositol 1,4,5-trisphosphate

[Criterion (iv)]
Criterion (vi) no longer applies, and therefore direction of numbering changes to give lowest locants.

3 Determine absolute configuration:



1D-*myo*-inositol 1,4,5-trisphosphate

[Absolute Configuration Rule]

Appendix 2 X-Ray Crystallography Data for 67a

Note: The labelling scheme for atoms in **67a** is that given in Figure 7.7a.

A crystal of approximate dimensions 0.3 x 0.3 x 0.3 mm was used for data collection.

Crystal data: $C_{27}H_{34}O_{12}$, M = 550.54, Monoclinic , a = 6.755(1), b = 31.139(2), c = 7.035(1) Å , β = 117.800(10)°, U = 1309.0(3) Å^3, space group $P2_1$, Z = 2, D_c = 1.397 gcm^{-3}, (μMo-K_α) = 0.110 mm^{-1}, F(000) = 584. Crystallographic measurements were made at 293(2)° K on a CAD4 automatic four-circle diffractometer in the range 2.61<θ<21.93 . Data (1795 reflections) were corrected for Lorentz and polarization but not for absorption.

In the final least squares cycles all atoms were allowed to vibrate anisotropically. Hydrogen atoms were included at calculated positions where relevant, except in the case of H1A which was located in the penultimate difference Fourier and refined at a distance of 0.96 Å from O4. Examination of the supramolecular structure revealed that the lattice is dominated by one dimensional linear polymers parallel to the *a* axis as a consequence of hydrogen bonding. Typically, H1A (attached to O4) interacts with O8 of the molecule generated via the operator *-1+x, y, z.* [H1A-O8, 2.14(3) Å; O4-H1A-O8, 157(6) °].

The solution of the structure (SHELX86)[1] and refinement (SHELX93)[2] converged to a conventional [i.e. based on 1326 with $Fo>4\sigma(Fo)$] $R1$ = 0.0318 and $wR2$ = 0.0755. Goodness of fit = 1.029. The max. and min. residual densities were 0.185 and -0.175 eÅ^{-3} respectively.

1. Sheldrick G.M., Acta Cryst., A46, 467-73, 1990.
2. Sheldrick G.M., J.Appl.Cryst., 1995 (In preparation.)

Table 1. Crystal data and structure refinement

Identification code	95FARM2
Empirical formula	$C_{27} H_{34} O_{12}$
Formula weight	550.54
Temperature	293(2)°K
Wavelength	0.70930 A
Crystal system	Monoclinic
Space group	$P2_1$
Unit cell dimensions	a = 6.755(1)Å
	b = 31.139(2)Å beta = 117.80(1)°
	c = 7.035(1)Å
Volume	1309.0(3) $Å^3$
Z	2
Density (calculated)	1.397 Mg/m^3
Absorption coefficient	0.110 mm^{-1}
F(000)	584
Crystal size	0.3 x 0.3 x 0.3 mm
Theta range for data collection	2.61 to 21.93 °.
Index ranges	-7<=h<=0; 0<=k<=32; -6<=l<=7
Reflections collected	1795
Independent reflections	1638 [R(int) = 0.1331]
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	1630 / 2 / 362
Goodness-of-fit on F^2	1.029
Final R indices [$I>2\sigma(I)$]	R1 = 0.0318 wR2 = 0.0755
R indices (all data)	R1 = 0.0593 wR2 = 0.1193
Absolute structure parameter	-1(2)
Largest diff. peak and hole	0.185 and -0.175 $eÅ^{-3}$
Weighting scheme	calc $w=1/[\s^2(Fo^2)+(0.0502P)^2+0.6641P]$
	where P=(Fo^2^+2Fc^2^)/3
Extinction coefficient	0.0187(27)
Extinction expression	$Fc^*=kFc[1+0.001xFc^2\lambda^3/\sin(\theta)]^{-1/4}$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

Atom	x	y	z	U(eq)
O(1)	-255(8)	5365(2)	10997(6)	64(1)
O(2)	-3838(8)	5078(2)	9334(7)	66(1)
O(3)	-3254(8)	5782(2)	8695(7)	67(1)
O(4)	-4273(7)	5171(1)	3953(6)	63(1)
O(5)	-518(6)	4652(1)	6815(6)	48(1)
O(6)	-2121(7)	4025(1)	6876(6)	53(1)
O(7)	1069(6)	4373(1)	4277(5)	43(1)
O(8)	3352(6)	4304(2)	2804(6)	51(1)
O(9)	903(7)	5933(2)	8669(6)	59(1)
O(10)	74(11)	6264(2)	5571(7)	98(2)
O(11)	2818(8)	6933(2)	7690(7)	64(1)
O(12)	3831(9)	7625(2)	8288(9)	85(2)
C(1)	200(9)	5224(2)	9282(8)	48(2)
C(2)	-1127(10)	4817(2)	8392(9)	47(2)
C(3)	-3592(10)	4924(2)	7521(9)	50(2)
C(4)	-4433(10)	5285(2)	5835(9)	57(2)
C(5)	-2947(11)	5670(2)	6852(10)	57(2)
C(6)	-517(10)	5572(2)	7602(9)	50(2)
C(7)	-2541(14)	5444(2)	10204(11)	69(2)
C(8)	-1083(8)	4243(2)	6232(8)	39(1)
C(9)	-298(8)	4062(2)	4734(8)	38(1)
C(10)	2196(9)	4135(2)	3469(8)	41(2)
C(11)	1597(8)	3669(2)	3509(8)	41(1)
C(12)	-848(9)	3647(2)	1708(9)	46(1)
C(13)	-2145(9)	3925(2)	2538(9)	50(2)
C(14)	1270(8)	3672(2)	5553(8)	38(1)
C(15)	3405(9)	3763(2)	7640(9)	49(2)
C(16)	187(10)	3265(2)	5859(9)	51(2)
C(17)	3192(10)	3353(2)	3352(9)	53(2)
C(18)	1041(12)	6256(2)	7500(10)	58(2)
C(19)	2430(10)	6610(2)	8982(9)	46(2)
C(20)	3356(11)	7301(2)	8881(11)	60(2)

C(21)	3306(9)	7199(2)	10961(10)	50(2)
C(22)	5365(10)	6905(2)	12144(11)	63(2)
C(23)	4740(10)	6494(2)	10779(11)	62(2)
C(24)	1349(9) ·	6868(2)	10101(9)	48(2)
C(25)	-901(11)	7066(3)	8529(13)	77(2)
C(26)	1091(12)	6630(2)	11874(10)	64(2)
C(27)	3188(13)	7588(2)	12185(12)	76(2)

Table 3. Bond lengths [Å] and angles [°]

O(1)-C(7)	1.397(9)
O(1)-C(1)	1.445(6)
O(2)-C(7)	1.394(8)
O(2)-C(3)	1.443(7)
O(3)-C(7)	1.410(8)
O(3)-C(5)	1.448(7)
O(4)-C(4)	1.422(7)
O(5)-C(8)	1.338(7)
O(5)-C(2)	1.445(6)
O(6)-C(8)	1.205(6)
O(7)-C(10)	1.362(6)
O(7)-C(9)	1.472(6)
O(8)-C(10)	1.203(6)
O(9)-C(18)	1.329(7)
O(9)-C(6)	1.441(7)
O(10)-C(18)	1.201(7)
O(11)-C(20)	1.365(8)
O(11)-C(19)	1.459(7)
O(12)-C(20)	1.192(8)
C(1)-C(2)	1.510(8)
C(1)-C(6)	1.508(8)
C(2)-C(3)	1.520(9)
C(3)-C(4)	1.538(9)
C(4)-C(5)	1.512(9)
C(5)-C(6)	1.502(9)
C(8)-C(9)	1.494(7)
C(9)-C(13)	1.526(8)
C(9)-C(14)	1.538(8)
C(10)-C(11)	1.511(8)
C(11)-C(17)	1.501(8)
C(11)-C(12)	1.545(7)
C(11)-C(14)	1.553(7)
C(12)-C(13)	1.529(8)
C(14)-C(16)	1.528(8)
C(14)-C(15)	1.530(7)
C(18)-C(19)	1.506(9)
C(19)-C(23)	1.524(9)
C(19)-C(24)	1.529(8)

C(20)-C(21)	1.513(9)
C(21)-C(27)	1.509(9)
C(21)-C(22)	1.545(9)
C(21)-C(24)	1.559(8)
C(22)-C(23)	1.534(9)
C(24)-C(25)	1.532(9)
C(24)-C(26)	1.529(8)

C(7)-O(1)-C(1)	110.2(4)
C(7)-O(2)-C(3)	110.6(5)
C(7)-O(3)-C(5)	110.7(5)
C(8)-O(5)-C(2)	115.5(4)
C(10)-O(7)-C(9)	105.3(4)
C(18)-O(9)-C(6)	119.3(5)
C(20)-O(11)-C(19)	105.6(4)
O(1)-C(1)-C(2)	106.6(4)
O(1)-C(1)-C(6)	108.7(5)
C(2)-C(1)-C(6)	111.5(5)
O(5)-C(2)-C(1)	106.9(4)
O(5)-C(2)-C(3)	114.8(5)
C(1)-C(2)-C(3)	108.1(5)
O(2)-C(3)-C(2)	105.8(5)
O(2)-C(3)-C(4)	106.6(5)
C(2)-C(3)-C(4)	114.3(5)
O(4)-C(4)-C(5)	108.4(5)
O(4)-C(4)-C(3)	112.0(5)
C(5)-C(4)-C(3)	106.8(5)
O(3)-C(5)-C(6)	108.8(5)
O(3)-C(5)-C(4)	105.9(5)
C(6)-C(5)-C(4)	112.4(5)
O(9)-C(6)-C(5)	112.2(5)
O(9)-C(6)-C(1)	105.1(4)
C(5)-C(6)-C(1)	108.7(5)
O(1)-C(7)-O(2)	112.5(6)
O(1)-C(7)-O(3)	111.0(5)
O(2)-C(7)-O(3)	110.8(6)
O(6)-C(8)-O(5)	123.6(5)
O(6)-C(8)-C(9)	120.9(5)
O(5)-C(8)-C(9)	115.5(5)
O(7)-C(9)-C(8)	111.8(4)
O(7)-C(9)-C(13)	105.2(4)

C(8)-C(9)-C(13)	115.4(4)
O(7)-C(9)-C(14)	102.0(4)
C(8)-C(9)-C(14)	116.3(4)
C(13)-C(9)-C(14)	104.7(5)
O(8)-C(10)-O(7)	121.0(5)
O(8)-C(10)-C(11)	131.0(5)
O(7)-C(10)-C(11)	108.0(4)
C(17)-C(11)-C(10)	114.9(4)
C(17)-C(11)-C(12)	116.1(5)
C(10)-C(11)-C(12)	102.7(4)
C(17)-C(11)-C(14)	119.3(5)
C(10)-C(11)-C(14)	99.6(4)
C(12)-C(11)-C(14)	101.6(4)
C(13)-C(12)-C(11)	104.2(4)
C(9)-C(13)-C(12)	101.7(4)
C(16)-C(14)-C(15)	108.5(4)
C(16)-C(14)-C(9)	114.7(4)
C(15)-C(14)-C(9)	112.9(5)
C(16)-C(14)-C(11)	114.2(5)
C(15)-C(14)-C(11)	114.3(4)
C(9)-C(14)-C(11)	91.6(4)
O(10)-C(18)-O(9)	123.9(6)
O(10)-C(18)-C(19)	127.0(6)
O(9)-C(18)-C(19)	109.0(5)
O(11)-C(19)-C(18)	107.9(4)
O(11)-C(19)-C(23)	105.5(5)
C(18)-C(19)-C(23)	117.9(5)
O(11)-C(19)-C(24)	103.1(5)
C(18)-C(19)-C(24)	116.7(5)
C(23)-C(19)-C(24)	104.3(5)
O(12)-C(20)-O(11)	122.2(6)
O(12)-C(20)-C(21)	130.3(7)
O(11)-C(20)-C(21)	107.4(5)
C(20)-C(21)-C(27)	114.5(6)
C(20)-C(21)-C(22)	103.0(5)
C(27)-C(21)-C(22)	116.3(5)
C(20)-C(21)-C(24)	99.2(5)
C(27)-C(21)-C(24)	119.4(5)
C(22)-C(21)-C(24)	101.8(5)
C(23)-C(22)-C(21)	104.0(5)
C(19)-C(23)-C(22)	101.5(5)

C(19)-C(24)-C(25)	113.0(5)
C(19)-C(24)-C(26)	115.4(5)
C(25)-C(24)-C(26)	109.4(5)
C(19)-C(24)-C(21)	91.5(4)
C(25)-C(24)-C(21)	112.9(5)
C(26)-C(24)-C(21)	113.7(5)

Table 4. Anisotropic displacement parameters ($Å^2 \times 10^3$)
The anisotropic displacement factor exponent takes the form:
$-2\ pi^2\ [\ h^2\ a^*\ 2\ U11 + ... + 2\ h\ k\ a^*\ b^*\ U12\ ]$

Atom	U11	U22	U33	U23	U13	U12
O(1)	89(4)	60(3)	44(2)	-5(2)	34(2)	-10(3)
O(2)	87(3)	63(3)	72(3)	-13(2)	58(3)	-14(3)
O(3)	90(3)	54(3)	69(3)	-5(3)	48(3)	6(2)
O(4)	63(3)	73(3)	43(2)	-3(2)	17(2)	-9(2)
O(5)	54(2)	47(3)	52(2)	-1(2)	33(2)	-6(2)
O(6)	58(3)	50(3)	59(3)	0(2)	35(2)	-12(2)
O(7)	44(2)	43(3)	46(2)	5(2)	25(2)	-4(2)
O(8)	47(2)	56(3)	55(2)	8(2)	30(2)	-5(2)
O(9)	82(3)	49(3)	46(2)	-4(2)	30(2)	-19(2)
O(10)	155(5)	93(4)	45(3)	-4(3)	48(3)	-51(4)
O(11)	95(3)	51(3)	66(3)	-5(2)	55(3)	-19(2)
O(12)	116(4)	58(3)	100(4)	5(3)	68(3)	-20(3)
C(1)	52(3)	47(4)	45(3)	-4(3)	21(3)	-5(3)
C(2)	60(4)	47(4)	42(3)	0(3)	30(3)	-5(3)
C(3)	56(4)	54(4)	49(3)	-3(3)	31(3)	-6(3)
C(4)	51(4)	70(5)	51(4)	-5(4)	24(3)	3(3)
C(5)	76(5)	49(4)	45(3)	0(3)	27(3)	0(3)
C(6)	66(4)	46(4)	40(3)	-4(3)	25(3)	-12(3)
C(7)	102(6)	67(5)	54(4)	-12(4)	51(4)	-8(5)
C(8)	31(3)	41(4)	38(3)	2(3)	11(3)	-3(3)
C(9)	36(3)	47(4)	34(3)	6(2)	18(3)	-5(3)
C(10)	29(3)	59(4)	31(3)	5(3)	10(3)	-1(3)
C(11)	36(3)	47(4)	40(3)	0(3)	20(3)	-2(3)
C(12)	41(3)	56(4)	38(3)	-3(3)	17(3)	-10(3)
C(13)	30(3)	73(4)	37(3)	7(3)	7(3)	-6(3)
C(14)	32(3)	49(4)	33(3)	7(3)	14(3)	-5(3)
C(15)	36(3)	59(4)	45(3)	9(3)	13(3)	-2(3)
C(16)	54(4)	57(4)	50(3)	10(3)	30(3)	-4(3)
C(17)	49(4)	59(4)	56(3)	2(3)	29(3)	-3(3)
C(18)	89(5)	54(5)	51(4)	1(3)	49(4)	-11(4)
C(19)	57(4)	43(4)	46(3)	-2(3)	31(3)	-13(3)
C(20)	67(4)	50(4)	76(5)	2(4)	43(4)	-5(4)
C(21)	53(4)	47(4)	60(4)	-3(3)	34(3)	-4(3)
C(22)	42(4)	60(5)	74(4)	-9(4)	15(3)	0(3)

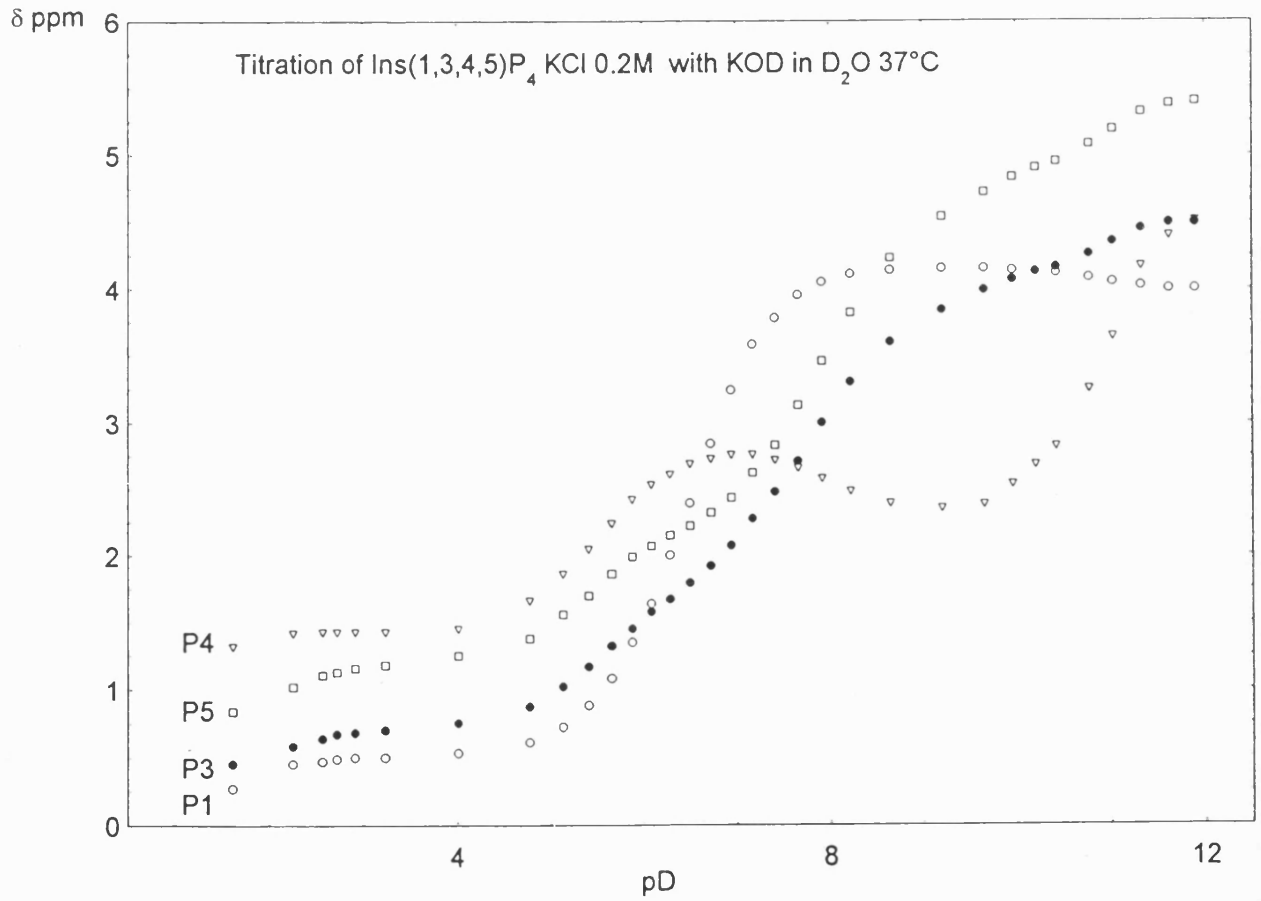
C(23)	55(4)	52(4)	86(5)	3(4)	38(4)	8(3)
C(24)	43(3)	53(4)	48(3)	2(3)	21(3)	-1(3)
C(25)	51(4)	88(6)	87(5)	6(4)	28(4)	12(4)
C(26)	76(5)	70(5)	61(4)	-3(4)	45(4)	-14(4)
C(27)	101(6)	58(5)	82(5)	-21(4)	51(5)	-10(4)

Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$ x 10^3)

Atom	x	y	z	U(eq)
H(1A)	-5099(95)	4921(13)	3199(94)	75
H(1)	1802(9)	5165(2)	9848(8)	58
H(2)	-696(10)	4607(2)	9556(9)	57
H(3)	-4499(10)	4665(2)	6931(9)	60
H(4)	-5987(10)	5356(2)	5455(9)	69
H(5)	-3418(11)	5909(2)	5831(10)	68
H(6)	-285(10)	5479(2)	6390(9)	60
H(7)	-2772(14)	5536(2)	11418(11)	82
H(12A)	-976(9)	3760(2)	370(9)	55
H(12B)	-1394(9)	3353(2)	1477(9)	55
H(13A)	-2822(9)	4171(2)	1613(9)	60
H(13B)	-3297(9)	3761(2)	2668(9)	60
H(15A)	4108(35)	4018(7)	7471(23)	74
H(15B)	4413(28)	3524(6)	7971(33)	74
H(15C)	3034(12)	3804(12)	8789(16)	74
H(16A)	-31(58)	3295(5)	7107(36)	77
H(16B)	1144(29)	3023(3)	6047(59)	77
H(16C)	-1232(29)	3219(7)	4616(27)	77
H(17A)	2730(38)	3066(2)	3459(64)	79
H(17B)	4671(15)	3402(8)	4500(39)	79
H(17C)	3195(51)	3387(9)	1998(28)	79
H(22A)	6697(10)	7036(2)	12208(11)	76
H(22B)	5623(10)	6844(2)	13594(11)	76
H(23A)	5787(10)	6437(2)	10224(11)	75
H(23B)	4678(10)	6248(2)	11590(11)	75
H(25A)	-1385(46)	7261(14)	9288(22)	116
H(25B)	-1992(26)	6843(3)	7885(66)	116
H(25C)	-734(24)	7220(15)	7428(53)	116
H(26A)	-49(56)	6415(10)	11243(12)	96
H(26B)	676(76)	6830(3)	12665(48)	96
H(26C)	2485(26)	6496(13)	12828(44)	96
H(27A)	3189(87)	7497(3)	13490(41)	115
H(27B)	1840(44)	7745(9)	11319(34)	115
H(27C)	4460(47)	7769(9)	12524(73)	115

Appendix 3 ^{31}P NMR Titration of Ins(1,3,4,5)P_4

A preliminary ^{31}P NMR titration for **D-70a** indicates a complex micro-protonation process due to interactions of the phosphate groups. A slight upfield shift is observed for P1 on deprotonation, an effect also observed for Ins(1,4,5)P_3.[189] However it is clear that the behaviour of P4 is very different from that of P4 in Ins(1,4,5)P_3. Further experiments are in progress.



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