Polar group exchange receptor-ligand engineering of protein kinase B’s pleckstrin homology domain

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PhD thesis
ABSTRACT

A diverse range of cellular functions are influenced by the activity of protein kinase B (PKB, also known as Akt). PKB is involved in a number of physiological pathways, perturbation of which can lead to pathological conditions. PKB activation has been shown to involve binding of its pleckstrin homology (PH) domain to PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ generated on the inner face of the plasma membrane upon receipt of an external hormonal signal.

To study PKB independently of other inositol-phospholipid binding proteins which recognise the same lipid signals, their mutual dependence on the same ligand must be broken. It is proposed that this can be achieved by mutating PKB’s PtdInsPₙ binding PH domain to accept an unnatural ligand. By altering the inositol-phospholipid head-group to include an additional carboxylic acid substituent, its binding to the wild-type PH domains should be inhibited through steric compression. Disruption of a salt-bridge in the wall of the binding pocket by point mutation of glutamate 17 to alanine, E17A, introduces an additional positively charged pocket into the PH domain of PKB, which should bind with the tailored lipid, resulting in a polar group exchange receptor-ligand pair.

To this end, a novel 2-O-acetic acid functionalised analogue of Ins(1,3,4,5)P₄ was synthesised. The key steps were the protection/deprotection of myo-inositol’s hydroxyls, enabling selective functionalisation of the 2-hydroxyl, and phosphorylation of the 1,3,4,5-O positions. Using an allyl ether as a masked form of acetic acid allowed a divergent synthetic strategy to be employed to access additional 2-O analogues.

Both the wild-type and the E17A mutant of the PH domain were expressed in order to assess ligand binding, and determine whether an ion pair exchange receptor-ligand interaction occurred. Intrinsic tryptophan fluorescence spectrometry was initially used, but was unsuccessful due to denaturing problems arising from isolating the protein. Isothermal titration calorimetry suggested binding, but protein concentration could not be increased high enough for the binding energy to become significantly greater than the energy of mixing.

The analogues were also tested as potential inositol poly-phosphate phosphatase catalytic (IPPc) domain inhibitors.
ACKNOWLEDGEMENTS

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(N.B. This statement is not a reason for the relevant people to get a free pint...I’m skint...so tough).
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<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>2,4-DNP</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>AGC</td>
<td>cAMP dependent, cGMP dependent, and protein kinase C</td>
</tr>
<tr>
<td>AI</td>
<td>auto-induction</td>
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<tr>
<td>Ala/A</td>
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<td>Bam32</td>
<td>B cell adaptor molecule of 32 kDa</td>
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<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
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<tr>
<td>BOM</td>
<td>benzylxoxymethyl</td>
</tr>
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<td>benzyl</td>
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<tr>
<td>Bz</td>
<td>benzoyl</td>
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<tr>
<td>CAN</td>
<td>ceric ammonium nitrate</td>
</tr>
<tr>
<td>Cbz</td>
<td>carboxybenzyl</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>CDP-DAG</td>
<td>cytidinediphosphate-diacylglycerol</td>
</tr>
<tr>
<td>CSA</td>
<td>camphorsulfonic acid</td>
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<tr>
<td>Cys/C</td>
<td>cysteine</td>
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<tr>
<td>DAG</td>
<td>(sn)-1,2-diacylglycerol</td>
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<tr>
<td>DAPP1</td>
<td>dual adaptor of phosphotyrosine and 3-phosphoinositides</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<td>DEAD</td>
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<td>dimethylformamide</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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DNA-PK  DNA-dependent protein kinase
DP    differential power
DPPA  diphenylphosphoryl azide
DTT   dithiothreitol
ECL   enhanced chemiluminescence
EI    electron impact ionisation
ELISA enzyme linked immunosorbent assay
ENTH  epsin N-terminal homology
ER    endoplasmic reticulum
ES    electrospray
EST   expressed sequence tag
FAB   fast atom bombardment
FAB-MS fast atom bombardment mass spectrometry
FYVE  Fab1, YOTB, Vac1, and EEA1
Gln/Q glutamine
Glu/E glutamic acid
Gly/G glycine
GST   glutathione S-transferase
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hER   human estrogen receptor
His/H histidine
HPLC  high performance liquid chromatography
HRP   horseradish peroxidase
Ile/I isoleucine
Ins   inositol
InsPn/IPn inositol poly-phosphate
IPPC  inositol poly-phosphate phosphatase catalytic
IPTG  isopropyl β-D thiogalactoside
ITC   isothermal titration calorimetry
KLH   keyhole limpet hemocyanin
LB    lysogeny broth
Leu/L leucine
Lys/K lysine
MALDI matrix-assisted laser desorption/ionisation
<table>
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<tr>
<td>mCPBA</td>
<td>*meta-*chloroperbenzoic acid</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
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<tr>
<td>Met/M</td>
<td>methionine</td>
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<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>NMO</td>
<td>*N-*methylmorpholine-<em>N</em>-oxide</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>OMFP</td>
<td>*O-*methyl fluorescein phosphate</td>
</tr>
<tr>
<td>PBP</td>
<td>pyridinium bromide perbromide</td>
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<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<td>PEPP1</td>
<td>phosphoinositol 3-phosphate binding protein-1</td>
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<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>Phe/F</td>
<td>phenylalanine</td>
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<td>PHISH</td>
<td>3-phosphoinoside interacting SH2-containing protein</td>
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<tr>
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</tr>
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<td>PI5K</td>
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</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
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</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
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<td>phospholipase D</td>
</tr>
<tr>
<td>Pmb</td>
<td>*para-*methoxybenzyl</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
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<tr>
<td>Pro/P</td>
<td>proline</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
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<tr>
<td>Ptac</td>
<td><em>tac</em> promoter</td>
</tr>
<tr>
<td>Ptd</td>
<td>phosphatidyl</td>
</tr>
<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PtdOH</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PtdInsP&lt;sub&gt;n&lt;/sub&gt;/PIP&lt;sub&gt;n&lt;/sub&gt;</td>
<td>poly-phosphorylated inositol phospholipids</td>
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PTEN - phosphatase and tensin homologue deleted on chromosome ten
pTSA - para-toluenesulfonic acid
PVDF - polyvinylidene difluoride
PX - phox homology
$R_f$ - retention factor
RICTOR - rapamycin-insensitive companion of mTOR
RLE - receptor-ligand engineering
rpm - revolutions per minute
RTK - receptor tyrosine kinase
Sac1 - suppressor of actin-1
SDS-PAGE - sodium dodecyl sulfate – polyacrylamide gel electrophoresis
Ser/S - serine
SET - single electron transfer
SHIP - Src homology 2-containing inositol-5-phosphatase
SK1 - sphingosine kinase-1
SPR - surface plasmon resonance
Src - sarcoma
TBAF - tert-butyl ammonium fluoride
Tbdms - tert-butyl dimethyl silyl
Tbdps - tert-butyl diphenyl silyl
TFA - trifluoroacetic acid
THF - tetrahydrofuran
Thr/T - threonine
TipdsCl$_2$ - 1,3-dichloro-1,1,3,3-tetraiso propyl disiloxane
TLC - thin layer chromatography
Trp/W - tryptophan
Tyr/Y - tyrosine
Val/V - valine
VL1 - variable loop 1
VL2 - variable loop 2
VL3 - variable loop 3
wt - wild-type
1. BACKGROUND

1.1 OBJECTIVES

Protein kinase B (PKB, also known as Akt) participates in the regulation of a diverse range of cellular functions. It controls physiological pathways and is a key junction point that underlies pathological conditions that arise from perturbation to these pathways. PKB activation has been shown to involve binding of its pleckstrin homology (PH) domain to specific inositol phospholipids contained within the plasma membrane. 11213 PH domains in 9880 proteins have been reported across a range of species. Therefore, in addition to PKB, there are multiple other PH domain containing proteins that also interact with the same inositol phospholipids. Phosphoinositide-dependent protein kinase-1 (PDK1) is one of these proteins. In conjunction with PKB, PDK1 translocates to the cytoplasmic face of the plasma membrane where it then assists in the activation of PKB. This makes dissection of both PKB’s activation mechanism and the specific down-stream events it regulates very complex.

In order to study PKB independently of PDK1 it is necessary to break their mutual dependence on the same inositol phospholipids. It is proposed that this can be achieved by mutating PKB’s PH domain to accept an unnatural ligand utilising ion pair exchange methodology. By altering the inositol-phospholipid head-group to include an additional carboxylic acid substituent, its binding to the wild-type PH domains should be inhibited. Using site-directed mutation of E17 to A, an additional positively charged pocket can be introduced into the PH domain of PKB, which should bind with a tailored lipid.

To establish an ion pair exchange receptor-ligand interaction, the aim is to form an additional attractive interaction between the customised lipid head-group and the mutant PH domain. Studying the isolated interaction between the modified inositol head-group and the mutant PH domain will allow optimisation of the head-group-PH domain interaction. A synthetic lipid can then be developed that can then be used in combination with the full length PKB mutant expressed in vivo. It is predicted that inserting the unnatural inositol-phospholipid into this system will
allow PKB to be studied in isolation, and lead to identification of down-stream events that are purely dependent on PKB being activated.

To comprehend how PKB undergoes activation, it is important to understand how inositol phospholipids behave within biological systems.

1.2 INOSITOL PHOSPHOLIPIDS

Inositol phospholipids (PtdInsP₅/PIP₅) belong to the glycerophospholipid family, representing slightly under 10% of the total cellular levels of phospholipids. These compounds are made up of a sn-1,2-diacyl-glycerol-3-phosphate (phosphatidate) unit linked by a phosphodiester bond to a polar head-group. Inositol phospholipids are amphipathic; the head-group is hydrophilic and the long-chain fatty acid esters are hydrophobic. This amphipathic nature underlies the importance of phospholipids to biological membranes.

Abundant in all biological membranes, phospholipids make up a major component of membrane lipids. Membranes constitute a permeability barrier that regulates the transmission of information between a cell and its environment. Glycerophospholipid nomenclature is determined by the structure of its head-group. The main biological properties of the molecule are also a direct result of the head-group, and modifications of it.

![Figure 1. General molecular structure of PtdIns. The phosphatidyl subunit is highlighted in red and the myo-inositol head-group in yellow.](image)

Despite the fact inositol phospholipids have been studied since the 1930s, it was not until 1959 that their general structure was confirmed (Figure 1).
Phosphatidylinositol (PtdIns) consists of a phosphatidyl (Ptd) subunit - diacyl glycerol (DAG), bound to two long lipophilic saturated or unsaturated fatty acid esters - linked by a phosphate ester bond to the 1-O of the cyclohexane hexitol myo-inositol (Ins). The basic PtdIns structure provides the building block for a collection of biologically active compounds. These arise from reversible enzymatic phosphorylation and dephosphorylation of the hydroxyls.\(^7\) Compared to other membrane lipids, this makes PtdIns unique.

Potentially, all five of the free hydroxyl groups could undergo phosphorylation, generating a prospective thirty-one mono- and poly- phosphorylated compounds. However, within the biological context, only phosphorylation of the 3-, 4- and 5-hydroxyls has been documented.\(^8\) This produces seven phosphorylated PtdIns\(P_n\) species (Figure 2).

**Figure 2.** Molecular structures of the head-groups of the documented PtdIns\(P_n\) species found in eukaryotic cells.
While glycerophospholipids frequently function in a structural capacity, some are also actively involved in the intracellular signalling pathways of eukaryotic cells.\textsuperscript{9} Phosphatidylcholine (PC) undergoes catalytic hydrolysis by phospholipase D (PLD), releasing the soluble choline head-group into the cytosol and forming the signalling molecule phosphatidic acid (PtdOH) which acts to recruit sphingosine kinase 1 (SK1) to the membrane.\textsuperscript{10} Similarly the PtdInsP\textsubscript{n} family can also act in intracellular signalling events.

\textbf{1.3 INTRACELLULAR SIGNALLING}

Within complex organisms, it is vital for the cells to be able to communicate in order to sustain each other’s survival by mutual cooperation. Communication over varying distances, from a cell’s immediate vicinity to approaching the total length of the organism, needs to be spanned for successful survival. The predominant technique employs hormones, such as neurotransmitters, steroids and a range of peptides.

Due to the hydrophilicity of the majority of intercellular signalling molecules (with the significant exception of the steroid family of compounds) they are unable to directly cross a cell’s plasma membrane. So that the signal can be propagated into the cell’s interior, transmembrane proteins that traverse the plasma membrane act as receptors on the cell surface. Intracellular signalling events are initiated by conformational changes induced by association of the hormone with the extracellular binding pocket of the receptor. These changes transmit the signal to the inner leaflet of the plasma membrane. This change is detected at the cytoplasmic face of the transmembrane hormone receptor by a signal transducer, initiating a sequence of intracellular signalling events that eventually transforms a target protein, creating an alteration in the cell’s behaviour.

From the membrane’s inner leaflet, the signal is propagated through the cytosol by a combination of: (i) activating an intracellular signalling protein, which can either activate the next signalling protein in the signalling cascade, or generate second messengers; and (ii) second messengers – molecules of low molecular weight, generated from multiple ligands, that relay the signal to other areas within the cell.\textsuperscript{11}
In mammalian cells, the most abundant of the doubly phosphorylated PtdInsP₆ species is PtdIns(4,5)P₂. This lipid was first identified in the inositol phosphate signalling pathway as a precursor for the formation of the soluble second messenger Insof(1,4,5)P₃ and membrane bound sn-1,2-diacylglycerol (DAG). However, PtdInsP₆s also serve in an additional capacity, where they directly regulate cell functions. By acting as membrane docking sites for reversible recruitment of intracellular signalling proteins and house-keeping proteins from the cytosol (Figure 3), PtdInsP₆s operate as key elements in signalling cascades.

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**Figure 3.** Phosphoinositide mediators in the cytosol and cell membrane. Showing the synthesis of cytidinediphosphate-diacylglycerol from phosphatidic acid (PtdOH), and its reaction with inositol (Ins) to form phosphatidylinositol (PtdIns). The inter-conversions of the seven main signalling species are highlighted in white. The soluble inositol phosphates are included, demonstrating that the two signalling systems are only connected metabolically at two points – in the formation of PtdIns, and the cleavage of PtdIns(4,5)P₂ to form the secondary messengers Insof(1,4,5)P₃ and DAG.

### 1.4 PLECKSTRIN HOMOLOGY DOMAIN

For a protein to directly interact with PtdInsP₆, it must contain a phosphoinositide-binding domain. Various domains with this attribute have been recognized and studied, including ENTH, FYVE and PX domains; however the
pleckstrin homology (PH) domain is perhaps the most widely studied phosphoinositide binding region.

In 1993 the PH domain was originally identified in the PKC substrate pleckstrin.\textsuperscript{21} Since then PH domains have been identified in many other proteins. PH domain containing proteins can be divided into four main categories based on \textit{in vitro} experiments studying the strength of their affinity for each phosphoinositide species.\textsuperscript{18, 22, 23}

\textbf{Group 1:} Proteins containing PH domains that demonstrate highly specific binding with PtdIns(3,4,5)P\textsubscript{3}.

\textbf{Group 2:} Proteins whose PH domains have high affinity for both PtdIns(4,5)P\textsubscript{2} and PtdIns(3,4,5)P\textsubscript{3}.

\textbf{Group 3:} Proteins that possess PH domains whose affinity is greatest towards PtdIns(3,4)P\textsubscript{2} and PtdIns(3,4,5)P\textsubscript{3}.

\textbf{Group 4:} The largest of the four groups, it accounts for approximately 85\% of all PH domain containing proteins.\textsuperscript{18} These proteins exhibit relatively low binding affinities to the phosphoinositides, but are capable of binding non-phosphoinositide ligands.\textsuperscript{24}

The PH domain is a structurally conserved module of 100-120 amino acids.\textsuperscript{21} While PH domains exhibit low sequence homology, with many sharing only 7-23\% pairwise sequence identities, there are significant unifying secondary and tertiary structural motifs conserved across all PH domains (\textbf{Figure 4}).\textsuperscript{30-32} They are constructed from anti-parallel $\beta$-strands with $\beta$-meander motifs making up two near
orthogonal β-sheets; one sheet is made up of four strands, while the other has three. The two β-sheets form a β-sandwich. Because of the right handed twist commonly encountered in β-sheets, the two β-sheets in the sandwich only have close contact at two corners. The other two corners are called the ‘splayed’ corners, as they are where the two β-sheets are furthest from one another. One of the splayed corners is capped by a C-terminal amphipathic α-helix, the other corner is situated next to the three variable loops that link the β-strands together- variable loop 1 (VL1) between β-strands 1 and 2, variable loop 2 (VL2) between β-strands 3 and 4, and variable loop 3 (VL3) between β-strands 6 and 7. These loops are known as variable loops, as their structure and length is unique to the protein of interest. It is assumed that the variable loops bestow the PH domain’s specificity for its particular ligand, displaying a positively charged interface for the poly-anionic ligands to interact with.\textsuperscript{33}

\section*{1.5 \textbf{PROTEIN KINASE B}}

Protein kinase B (PKB, also referred to as Akt), is a serine/threonine kinase that was initially discovered in the transforming retrovirus AKT8.\textsuperscript{34} PKB\textsubscript{α} and PKB\textsubscript{β} were identified from a human clone library as the cellular homologues of AKT8’s ν-Akt oncogene.\textsuperscript{35} PKB has become the focal point of a great deal research as all three mammalian isoforms (PKB\textsubscript{α,β,γ}) have been identified as key mediators in signal transduction pathways. PKB assists in regulating a variety of cellular processes such as cell proliferation, glucose metabolism, and gene expression by phosphorylating a diverse range of substrates (\textbf{Figure 5}).\textsuperscript{36}

Regulation of apoptotic cell death is also one of PKB’s functions, deactivating pro-apoptotic factors by phosphorylation; because of this it plays a critical role in certain pathological conditions where the signalling pathways have become hyperactive, such as with the progression of ovarian and pancreatic cancers. When the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome ten) stops functioning correctly,\textsuperscript{37} PKB becomes over activated and this leads to unregulated proliferation of cancerous cells.\textsuperscript{38, 39}
Figure 5. PKB signalling. Binding of a hormone on the outer leaflet of the cell membrane by RTK induces a conformational change which leads to the activation of PI3K. PI3K phosphorylates PtdIns(4,5)P$_2$ to PtdIns(3,4,5)P$_3$ – a process which is reversed by PTEN. The increased concentration of PtdIns(3,4,5)P$_3$ causes co-recruitment of PKB and PDK1 to the inner leaflet of the cell membrane. At the membrane PKB undergoes phosphorylation initially by PDK1, and then PDK2 (now believed to be the mTOR/RICTOR complex). The di-phosphorylated, fully activated, PKB then helps regulates a diverse range of pathways including: Glycolysis, telomere extension, gene expression, nitric oxide synthesis, glucose uptake, apoptosis and cell survival.  

Although cancer caused by mutation of PKB had not been previously reported, a recent study has shown that an E17K mutation in the PKB PH domain has been found responsible for some forms of human breast, ovarian and colorectal cancers. This mutation modifies the binding interface between the PH domain and the phosphoinositide ligands, leading to PKB becoming localised on the plasma membrane without regulation, causing its over-activation and pathological downstream signalling.

Although the three PKB isoforms share common roles, each isoform also exhibits individual functionality. PKBα is expressed throughout the body. Its physiological roles, revealed by gene ablation in mice, have been identified as placental...
PKB is primarily expressed in insulin-responsive organs and tissues. It shares several functional roles with PKBα, such as lipogenesis and growth, but has also been shown to be involved in glucose metabolism. PKBγ is the least expressed of the three isoforms, with its highest concentration found in the brain. This correlates with it being associated with post-natal brain growth.

PKB is a member of the AGC (cAMP dependent, cGMP dependent, and protein kinase C) family of protein kinases. AGC protein kinases all contain sections of high sequence homology within their kinase domains and have related functionality. The entire crystal structure of PKB has not yet been solved; however the combined crystal structures of the individual domains have been used to produce a composite structure. In order to gain insight into how PKB functions it is necessary to consider its structure which can be separated into three key sections (Figure 6): (i) the N terminal PH domain, (ii) the kinase domain and (iii) the regulatory hydrophobic motif at the C terminal.
(i) The PH Domain

PKB has been identified as having a group 3 PH domain exhibiting high and similar affinity towards PtdIns(3,4)P\(_2\) and PtdIns(3,4,5)P\(_3\).\(^{47,48}\) This affinity arises from the interactions between the inositol head-group and residues in the variable loops and protein backbone of the PH domain (Figure 7).\(^{48,49}\) The 1-phosphate interacts with R23 and the backbone nitrogen of I19. The 3-phosphate interacts with K14, R23, R25 and N53; these contacts could account for PKB’s inability to bind PtdIns(4,5)P\(_2\). The 4-phosphate of the head-group interacts with K14, N53 and R86. The 5-phosphate does not form any interactions with the amino acids within the binding pocket, explaining why PtdIns(3,4)P\(_2\) and PtdIns(3,4,5)P\(_3\) bind with similar affinity.

![Figure 7. Sequence alignment of the three PKB isoforms. Highlighting (in red) the residues involved in binding PtdIns(3,4)P\(_2\) and PtdIns(3,4,5)P\(_3\). Dots represent residues the same as PKB, dashes represent an omission in the sequence.](image)

PtdIns(3,4)P\(_2\) and PtdIns(3,4,5)P\(_3\) concentrations within the plasma membrane are regulated by PI3K (phosphoinositide 3-kinase), PI5K (phosphoinositide 5-kinase) and the PtdIns(3,4,5)P\(_3\) phosphatases PTEN and SHIP (Src homology 2-containing inositol-5-phosphatase).\(^{50}\) PI3K is activated by cell-surface receptor stimulation from an external signal. Activated PI3K phosphorylates the 3-hyroxyl of PtdIns(4,5)P\(_2\) to form PtdIns(3,4,5)P\(_3\).\(^{51}\) PTEN deactivates this pathway by selectively dephosphorylating PtdIns(3,4,5)P\(_3\) at the 3-position,\(^{52}\) negating the effect of the PI3K (see Figure 5). Alternatively the 5 position can be dephosphorylated by SHIP,\(^{53}\) creating
PtdIns(3,4)P$_2$, removing the lipid from the PTEN inactivation pathway. PtdIns(3,4,5)P$_3$ is reformed from PtdIns(3,4)P$_2$ by PI5K mediated phosphorylation. When the concentrations of PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ on the inner leaflet of the plasma membrane reach a threshold level, PH domain mediated translocation of PKB from the cytosol to the membrane’s cytosolic face occurs. The binding interaction between PKB’s PH domain and the phosphoinositide induces a conformational change of its tertiary structure, noticeably in VL2 and at the N-terminus (Figure 8). Binding of the PH domain to phosphoinositides frees the kinase domain for subsequent phosphorylation. However, the structural reorganisation does not seem to activate the kinase domain towards phosphorylation, as it has been shown ΔPH-PKB is constitutionally active when co-expressed with PDK1.

(ii) The Kinase Domain

While PKB is in its inactive form its PH domain is tightly associated with the kinase domain. The PH domain-phosphoinositide interaction dissociates this complex and induces a conformational change that prepares the kinase domain for phosphorylation. Located within the kinase domain is the activation segment T-loop containing T308 (numbering for PKB$_{\alpha}$). Phosphorylation of this residue induces a second change in conformation, allowing substrate binding to occur and raising the rate of catalysis at least 30-fold.
Phosphorylation of T308 is undertaken by 3-phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 is also a member of the AGC family of protein kinases. The PH domains of PKB and PDK1 both belong to group 3. In the cytoplasm PKB and PDK1 interact to form an equilibrium between complexed and dissociated states (Figure 9). The complexed state allows co-recruitment, concentrating them together at the plasma membrane, permitting the associated, PDK1 to phosphorylate T308 rapidly. The phosphorylated kinase domain subsequently has reduced affinity for the PH domain, leaving it open for substrate binding.

Figure 9. PKB-PDK1 interaction and PKB activation. Within the cytoplasm PKB and PDK1 are in dynamic equilibrium between a complexed and dissociated state. Upon conversion of PtdIns(4,5)P2 to PtdIns(3,4,5)P3 by PI3K, the complex is recruited by its interaction with phosphoinositides (highlighted in red) to the plasma membrane. This shifts the PKB-PDK1 interaction further towards the associated form (PTEN inhibits this process). PKB’s PH domain interaction with the lipids causes a conformational change, allowing T308 to be accessed and phosphorylated by PDK1 and to be phosphorylated (denoted as yellow circles). S473 phosphorylation and loading of ATP leads to PKB dissociating from the plasma membrane in its active conformation. PKB is deactivated by dephosphorylation of T308 and S473.
(iii) The Hydrophobic Motif

A section of twenty-one residues makes up the hydrophobic motif at the C terminus of PKB. After the initial T308 activation event, this motif functions as an allosteric regulator of the catalytic action of PKB. This regulation occurs through phosphorylation of S473. Three theories have been proposed for this second phosphorylation event: (a) PKB undergoes autophosphorylation, (b) PDK1 performs a second phosphorylation at S473, or (c) it is carried out by a second, as yet unidentified distinct serine kinase “PDK2”. Recent publications have proposed a strong case for the third option being the mode of phosphorylation, with mTOR-RICTOR acting as the S473 kinase.\(^61\)

Phosphorylation of S473 amplifies PKB’s catalytic rate by a further 7-10 fold.\(^62\)

The difference in observed rate between the mono- and di-phosphorylated species is explained by the conformational change the second phosphorylation event triggers. Mono-phosphorylated PKB is not locked into its active conformation, but is instead in equilibrium with other non-catalytic conformations, with only about 10% of the enzyme in an active conformation at any one time. S473 phosphorylation promotes extensive reordering of the \(\alpha\)B and \(\alpha\)C helices, which induces a hydrophobic groove in the N-terminal lobe. Interactions between the hydrophobic motif and the groove act to hinder further conformational change and forces the majority of the PKB into the active conformation.\(^62\)

To fully comprehend how PKB operates within a biological system, it is necessary to have a range of techniques that allow it to be fully examined.

1.6 GENETIC TECHNIQUES

Dissecting complex biological systems by studying the functions and interactions of individual components, can achieve understanding of how organisms operate and provide insights into a range of diseases. It can be very difficult to completely understand even a small part of such systems because of the numerous participating components, how they interact with multiple partners, and how they are regulated. Classical genetic techniques have been extremely effective in elucidating pair-wise interactions for a wide range of biological processes.\(^63\) These techniques can be divided into two main categories: (i) forward genetics, and (ii) reverse genetics.
(i) Forward Genetics

Forward genetics (Figure 10) aims to identify the gene that underlies a phenotype of interest. It is possible to locate and identify the function of a gene by examining the effects and positions of mutations (usually deletions) on the phenotype. By mapping the mutant gene, the gene’s position in the genome and structure can then be determined. Once the sequence of the gene has been fully identified, the associated phenotype can be linked to the gene with a high level of assurance.63, 64

Figure 10. Forward genetic approach. Random mutagenesis is used to create cells with many different phenotypes. The mutant exhibiting the phenotype of interest is then selected. By mapping the mutant gene, its position in the genome and structure can then be determined.

(ii) Reverse Genetics

Reverse genetics can be considered the opposite of forward genetics. By generating a specific mutation in a gene of unknown function, it is possible to correlate this to any resultant phenotype and therefore deduce its function (Figure 11).63, 65

Figure 11. Reverse genetic approach. Point mutation a single gene of interest should lead to a change in the organism’s phenotype. By correlating the mutation to the observed phenotype it is possible to deduce the genes function.
Both approaches have several advantages and disadvantages. The forward approach generally provides both a gene and its function, but can be extremely time consuming. Since the Human Genome Project has provided the sequences of all ~30,000 human genes, reverse genetics has become increasingly popular. However, for this approach gene redundancy means that many genes may have been disabled but without having an observable effect on the phenotype. Also it is not possible to dissect the phenotype of mutations lethal to the organism.

To surmount these limitations alternative techniques have been sought. The use of small molecules to probe protein function has been adopted in many strategies; this is essentially classical pharmacological screening of small molecule libraries, but more recently has become known as chemical genetics. The basic premise of chemical genetics is that inactivation of a protein using a small molecule is functionally equivalent to the genetic inactivation of its parent gene by mutation.

Chemical genetics has several advantages over classical genetic techniques. In many cases, the same small molecule can be used across a range of organisms; this removes the need to repeatedly generate the same mutation in each new organism. Using small molecules also allows temporal control in dynamic processes on short timescales (seconds), compared to conditional gene activation (hours/days).

Reflecting classical genetics, chemical genetics can also be separated into two main fields: (i) forward chemical genetics, and (ii) reverse chemical genetics.

(i) Forward Chemical Genetics

Similar to forward genetics, in forward chemical genetics when a small molecule is added to a cell and elicits a phenotype of interest, it must be affecting a cellular pathway. Unlike classical genetics, the molecule can be added or removed, thus acting as a conditional switch, which although very useful in classical genetics is very difficult to achieve but in chemical genetics can be facile. It also allows for dose graduation and the study of embryonic lethal genetic loci.

When the protein that the molecule interacts with has been identified, the protein’s function within the system can then be probed using the new chemical switch. However, the target protein can be extremely hard to identify and this can be one of the most difficult aspects to forward chemical genetics, particularly if the protein has not previously been characterised.
(ii) Reverse Chemical Genetics

Reverse chemical genetics overcomes some of the problems of the forward approach, by starting with a protein of interest, and then identifying a small molecule that interacts selectively with it (Figure 13). The small molecule can then be used as a conditional switch to probe the phenotype of the target protein.

To diminish the possibility of the small molecule interacting with non-target proteins, and to limit the number of small molecules that need to be investigated, the fundamental idea of reverse chemical genetics has been extended to embrace the established field of receptor-ligand engineering.
Receptor-ligand engineering uses genetic techniques to create a mutant that expresses a protein of interest that can bind a modified ligand. The difficulty is then to find a small molecule that is complementary to the mutant protein, yet unable to bind to wild-type protein receptors (i.e. orthogonal). This is done through screening of a small library or through rational design. This general description covers several approaches. A commonly used technique is steric complementation, also known as the ‘bump-hole’ method (Figure 14). This steric technique modifies the shape of the complementary receptor-ligand interface. A ligand is created that is too sterically hindered fit into the wild-type receptor, but can interact with the engineered receptor into which a complementary cavity has been engineered. A characteristic of this technique is that the modified receptor often retains significant affinity for the natural ligand. Thus, although the modified ligand is orthogonal to the wild-type receptor, the mutant receptor is often not orthogonal to the natural ligand.

Should mutual orthogonality be desired, so that the modified receptor cannot bind with the natural ligand, several additional techniques have been developed. One way to overcome this has been to create dual ‘bump-hole’ plus ‘hole-bump’ combinations (Figure 15). Theoretically this provides better substrate selectivity and receptor discrimination, due to the further increase in steric interactions. However, there are two
potential difficulties arising from this technique: (i) too many mutations around the receptor can lead to over all distortion of the protein binding site and diminished functionality, and (ii) creating a ‘hole’ in a small molecule is very difficult to achieve without significantly distorting it, and therefore diminishing/destroying its specificity for the protein of interest.

Aside from steric complementation, the other main strategy for creating orthogonal receptor-ligand pairs is to introduce non-native polar receptor-ligand interactions. These polar methods can be utilised to engineer mutually orthogonal pairs. One important technique used for molecular recognition depends upon manipulating the hydrogen bonding at the receptor-ligand interface. By changing one amino acid, to reverse its hydrogen bonding characteristics, it is possible to disrupt recognition of the natural ligand by the receptor. A synthetic ligand is prepared that has functionality that is complementary to this mutation, then orthogonal ligand-receptor pairs, with reversed hydrogen bond patterns, can be created (Figure 16). This technique has one main advantage over the steric complementation techniques: It limits the extent to which the shape of the receptor-ligand interface is changed, minimising steric effects on the engineered complex’s activity, relative to that of the wild-type. However, this technique is difficult to implement as hydrogen bonds are rarely isolated, but form networks.
It is also possible to manipulate charged residues around the receptor-ligand interface to induce polar interactions. There are four basic techniques: (i) Introduction of new ion pairs (Figure 17A) - by engineering a new charged residue into the receptor-ligand interface it is possible to disrupt binding of the natural ligand; and, by including complementary functionality within the synthetic ligand, form an orthogonal pairing.\(^\text{75}\) (ii) Ion pair neutralisation (Figure 17B) - this is the opposite approach to introducing new ion pairs; by removing the existing ion pair interactions it is possible to induce discrimination against the natural ligand, and increase affinity towards a neutral synthetic ligand.\(^\text{76}\) (iii) Ion pair reversal (Figure 17C) - this technique is conceptually the same as reversed hydrogen bond patterns, only the magnitude of the electrostatic interactions is significantly larger.\(^\text{77}\) (iv) Ion pair exchange (Figure 17D) - this method requires the disruption of an intramolecular protein salt bridge, by mutagenesis of one of the charged residues.\(^\text{78, 79}\) This unpaired charged residue is stabilised by the formation of an intermolecular salt bridge between the receptor and a synthetic ligand to which the complementary half of the salt bridge is fused. In all these ion-pair methods the wild-type receptor will have reduced affinity for the modified ligand, arising from unnatural steric hinderance or electronic interference during binding.
There are also a few other techniques that have recently been developed. Creation of a de novo ligand binding site in the receptor-ligand interface requires an additional small molecule to be present for the protein to bind its ligand (Figure 18A).80 Another new approach is to increase the affinity between engineered receptor-ligand pairs by introducing functional groups, which could selectively react to form covalent complexes (Figure 18B).70 This overcomes the need to develop ligands with very high affinity and also helps avoid off target binding.

Figure 17. A Ion pair creation. A new ion pair interaction is created between the protein and its ligand. B Ion pair neutralisation. An existing ion pair interaction between the protein and its ligand is removed from the protein-ligand interface. C Ion pair reversal. The polarity of an existing ion pair interaction between the protein and its ligand is reversed. D Ion pair exchange. An intramolecular interaction within the protein’s binding pocket is replaced by an intermolecular interaction between the protein and its ligand. In all four cases the mutant proteins are not able to bind their natural ligand, but can bind their synthetic ligand. The wild-type proteins cannot bind the synthetic ligands.
Figure 18. A Creation of a de novo binding site. An additional binding site for a small molecule is engineered into both the protein and its ligand. The small molecule must be present for protein-ligand binding. B Engineered reactivity. The protein is functionalised near the binding interface. A ligand is synthesised with a group which will react with this functional group to form a covalent bond.

It is important that both the advantages and limitations of each technique are fully appreciated before deciding upon which approach to employ – it might be necessary to utilise a combination of techniques in order for the biological pathway to be fully dissected.
2. PROPOSED RESEARCH

It has been shown that PKB is a signalling kinase that regulates a diverse range of cellular functions. However, it has been difficult to elucidate all of its biological behaviour due to the dependence of both PKB and PDK1 on the same signalling phosphoinositides. Therefore, it is necessary to break the relationship between the two species in order to scrutinise the individual components of PKB’s activation pathway. Classical genetic techniques have been able to provide a lot of information about this pathway, but additional techniques are required for it to be fully understood. To achieve this, it has been proposed that the reverse chemical genetics technique of receptor-ligand engineering is employed.

Bump-hole engineering has had wide success in the case of Src family of kinases. Src kinases are involved in a range of signal transduction pathways within the cell - influencing processes such as growth, differentiation, adhesion and transcription. Being able to selectively activate an individual member of the family provides valuable information about its function.

Liu et al. developed an ATP analogue – \(N^6\)-benzyl ATP (Figure 19B) – that was preferentially bound by the I338G mutant of the \(v\)-Src kinase. Due to the high degree of sequence homology within the kinase domain of the Src family, applying the equivalent mutation to different Src proteins makes it possible to utilise the same compound to study a range of different proteins.

In order to apply this methodology to PKB it is necessary to identify a suitable site for mutation within the PH domain. The main prerequisites for choosing which site to mutate are: (i) The residue must be on the surface of the binding interface so that a cavity is formed to accommodate the synthetic ligand; (ii) The residue must not be structurally important, so that the tertiary structure of the protein is maintained; and (iii) The residue should not be functionally important as mutation would then be likely to lead to reduced affinity for the ligand.

Taking this all into consideration, locating a suitable site for mutation within the PH domain is difficult due to the high degree of structural residues within the receptor pocket. An additional problem in the case of PKB is that bump-hole engineered receptors tend to discriminate poorly between the natural and synthetic ligand, so would not help to separate the effects of PKB from PDK1, as they share...
the same natural ligands. This could be overcome by adopting the dual bump-hole/hole-bump approach, but would require identifying a second site for mutation.

![Image of ATP and N6-(benzyl) ATP](image)

**Figure 19.** A The structure of natural ATP. B N6-(benzyl) ATP. The benzyl group (highlighted in yellow) provides the steric ‘bump’ in the synthetic ligand complementary to the ‘hole’ created by the I338G mutation in v-Src kinase.

Because the steric complementation techniques are unsuitable in this situation, an alternative technique needs to be applied. Polar techniques require careful consideration of the receptor-ligand interface in order to identify potential sites for mutation. Study of the crystal structures of the apo and holo forms of the PH domain of PKB\(\alpha\) reveal an intramolecular salt bridge (**Figure 20**). Located on the surface of the phosphoinositide ligand binding interface, the salt bridge is formed between E17 and R86 upon ligand binding. Because of this it could be ideal for polar group exchange receptor-ligand engineering.
Shi and Koh\textsuperscript{78} and Tedesco et al.\textsuperscript{79} have both reported previous success with polar group exchange receptor-ligand engineering of estrogen to selectively regulate gene expression.

From the co-crystal structure of the estradiol-bound form of human estrogen receptor (hER), it was seen that hydrogen bonding holds the 3-hydroxyl of estrogen next to an intramolecular E353-R394 salt bridge adjacent to the ligand-binding pocket (Figure 21A). The intramolecular salt bridge was disrupted by the E353A mutation; a carboxylate functionalized ligand acted in lieu of the glutamic acid to form an intermolecular salt bridge (Figure 21B). The E353A mutation is favoured over the E353G mutation, as while glycine is a smaller residue, its use can decrease the structural rigidity of the protein, and lead to mis-folding of the protein.

The mutant hER had a greatly reduced affinity for the natural ligand. However, the synthetic ligand can induce a transcriptional response in hER(E353A) expressing cells, creating a tool that can be used for remote regulation of gene expression.
Figure 21. A The natural interactions between the binding residues in hER and the E2 molecule. B The ion pair exchange receptor-ligand engineered interaction between the hER(E353A) mutant and the E2 analogue ES7.

The identification of a similar, suitably located, intramolecular salt bridge, between E17 and R86 (highlighted in red in Figure 22) in the PKBα PH domain, suggests polar group exchange receptor-ligand engineering is a suitable technique for studying PKB. These residues are conserved across all three isoforms, allowing the potential for using polar group exchange receptor-ligand engineering to help differentiate the roles of PKBα, β and γ.
Polar group exchange receptor-ligand engineering of protein kinase B’s pleckstrin homology domain

Figure 22. Protein sequence of PKBα PH domain, highlighting (in red) the E17 and R86 residues involved in formation of the salt bridge.

Theoretically either side of the salt bridge could be removed through point mutation. There are several reasons why the E17A mutation is favoured over the R86A mutation: (i) The R86 residue is involved in recognition of the 4-phosphate, so mutagenesis could lead to reduced affinity between the mutant domain and the synthetic ligand; (ii) creating a carboxyl ligand is potentially less synthetically challenging than the corresponding guanidine or amine functionality; and (iii) The protonated amine or guanidine functionality might reduce attraction to the interior of the binding pocket due to the cationic surface presented by the variable loops. In contrast to the E17K mutation, it is anticipated that the E17A mutant will have substantially reduced activity because, complementary to the known R86A mutant, this mutant also lacks one half of the key salt-bridge that is formed in the active conformation.

Figure 23. A E17–R86 salt bridge. B Effect of proposed E17A mutation and its interaction with a functionalized ligand.

Point mutation to convert the E17 residue (Figure 23A) into an alanine residue disrupts the intramolecular salt bridge. Including a glutamic acid-like carboxylate group within a synthetic ligand can provide complementary binding to the R86 residue (Figure 23B).
Polar group exchange receptor-ligand engineering of protein kinase B’s pleckstrin homology domain

It is rationalised that this would be a favourable pairing due to several key points. The wild-type PH domain will not be able to bind the synthetic ligand because of unfavourable steric and electrostatic interactions with the additional functionalisation on the ligand. The mutant PH domain should discriminate against binding to the natural lipid; although natural PtdIns(3,4,5)P$_3$ should be not be disfavoured for steric reasons, it is hypothesized that binding of PtdIns(3,4,5)P$_3$ will not induce the loop remodelling that normally occurs between the natural ligand and the wild-type PH domain due to the mutant PH domains’ incapability to form an internal salt bridge. The additional polar interaction between the synthetic ligand and the R86 residue should form a functional orthogonal receptor-ligand pair due to the alterations conferring increased selectivity. However, whether the unnatural protein-ligand pairing will have as high (or greater) an affinity for each other as the natural pair is uncertain. Although there will clearly be a new strong polar association in the artificial complex, this will be counter-balanced by the need to desolvate the exposed polar groups during binding. The total free energy for this will consist of enthalpic and entropic components individually far greater than for the natural pairing. Hence it is impossible to predict the structure of the optimum ligand, and it is expected that a second generation of ligands will be required.

PKB’s dependence on natural phosphoinositide lipids will be removed by the introduction of the mutant PH domain, breaking the link between PKB and PDK1 activation.

Rational design suggests that the synthetic ligand should retain as many functional similarities to the natural ligand as possible in order to preserve the maximum number of original molecular contacts and maintain high affinity and specificity in the engineered system.

The affinity between PKB’s PH domain and PtdIns(3,4,5)P$_3$ underlies recruitment to the plasma membrane; the association between the natural ligand and the wild-type receptor is specific for the phosphorylation pattern on the inositol head-group. In the engineered ligand it is essential that the distribution of phosphate groups is retained. Ins(1,3,4,5)P$_4$ is known *in vitro* to compete with PtdIns(3,4,5)P$_3$ for binding to PKB’s PH domain. Therefore syntheses will initially focus on the head-group. The 1-phosphate mimics the phosphodiester linkage in the natural lipid. The 3- and 4-phosphates are directly involved in recognition by the PH domain. The 5-
phosphate is thought to be unimportant for binding as both Ins(3,4)P$_2$ and Ins(3,4,5)P$_3$ are bound with similar affinity.

This leaves either the hydroxyls at the 2- and 6-positions, or the inositol ring, for functionalisation. The choice of functional group is dictated by the E17A mutation that requires the engineered ligand to contain a carboxylate similar to the glutamic acid side chain, so that it can form a replacement intermolecular salt bridge with the remaining R86 residue.

The X-ray crystal structure of Ins(1,3,4,5)P$_4$ bound to the wild-type receptor shows that the orientation adopted by Ins(1,3,4,5)P$_4$ means it presents its 2-hydroxyl and protons in the 4- and 6-positions towards the binding interface.
Assuming the chosen centre is functionalized with an acetic acid group, then the distances of the 2-\textit{O}, 4-\textit{C} and 6-\textit{C} to the central carbon of the R86 guanidine correspond to the distance between the \(\beta\) carbon of the E17 carboxylate and the central carbon of the R86 guanidine. The distance between the \(\beta\) carbon of the E17 carboxylate and the central carbon of the R86 guanidine is 5.31 Å. The distances for the 2-\textit{O}, 4-\textit{C} and 6-\textit{C} of \(\text{Ins}(1,3,4,5)\text{P}_4\) are 5.52 Å, 4.86 Å and 5.59 Å respectively (Figure 24). Therefore for the functionalized ligand - assuming a similar distance should be maintained so that normal binding of the phosphates occurs - this discounts the 4 position. When considering the remaining two sites, the difference in bond length between carbon-carbon bonds and oxygen-carbon bonds needs to be taken into account. As oxygen-carbon bonds tend to be longer, functionalisation of the 2-\textit{O} should provide the best interaction. This is re-enforced by consideration of how the 6-\textit{C} acetic acid would bind the mutant. Assuming the synthetic ligand adopts the same orientation with the mutant domain as the natural ligand with the wild-type domain, then the 6-\textit{C} acetic acid would need to bind across the face of the inositol ring, resulting in unfavourable steric interactions with the 4-\textit{CH}. Therefore, the first generation of engineered ligands will be of the structure 1 (Figure 25).

Figure 25. Proposed structure for target molecule. \(\text{Ins}(1,3,4,5)\text{P}_4\) with additional acetic acid moiety attached to the 2-\textit{O} (highlighted in yellow).
3. BIO-PHYSICAL TECHNIQUES

A range of bio-physical techniques have been developed to study protein-protein and protein-ligand interactions. Each technique has its advantages and limitations concerning the type and quality of information derived from each experiment. These techniques can be used at several stages in developing an engineered receptor-ligand pair.

Initially, receptor-ligand engineering is assisted by having a good structural understanding of the target protein to identify potential sites for mutation. This information can be established from either X-ray crystallography or from structural calculations based upon nuclear magnetic resonance (NMR) spectroscopy. X-ray crystallography offers high resolution structures, but proteins can be difficult to crystallise, and it can be questionable whether the solid state data reflect the protein’s conformation in solution. NMR spectroscopy generates a composite structure from various solution conformations. Because of this, specific folds will only be observable if the data from the various structures converge, if this does not occur the conformation will be unresolved.

Later in the process - once a potential receptor-ligand pair has been produced - there is a range of techniques that can be used to probe whether they interact, and to what extent. Commonly used techniques are circular dichroism (CD), dual polarisation interferometry, fluorescence spectroscopy and isothermal titration calorimetry (ITC). The choice of technique is dependent upon the type of data required.

PKBα’s PH domain has previously been studied using intrinsic tryptophan fluorescence spectroscopy.

3.1 INTRINSIC TRYPTOPHAN FLUORESCENCE SPECTROSCOPY

The basic intrinsic tryptophan fluorescence spectroscopy experiment requires single wavelength excitation of tryptophan. The resulting fluorescence – measured at 90° to the path of excitation to minimise incidental light from the excitation source – is then measured using a photo detector (Figure 26).
Figure 26. Simple schematic of a fluorescence spectrometer. Emissions from the excitation source pass through a monochromator. The photons excite the sample in the cell, and the resulting fluorescence is passed through a second monochromator and then measured by a photo detector.

There are 3 amino acid residues that are naturally fluorescent: tryptophan (W), tyrosine (Y) and phenylalanine (F). Each residue can be excited at a different wavelength, allowing selective excitation and hence detection. The W residues are excited in the 290-305 nm wavelength range. W is unusual as, unlike the F and Y residues, its fluorescence parameters are especially sensitive to the environment it is in. This means that, as long as there is a suitably placed W residue, it is possible to study conformational changes arising from protein-ligand interactions.

W’s ability to act as an environmentally-sensitive fluorophore mainly occurs due to photon absorption causing a large redistribution of electron density across its indole ring system. Studies have shown that excitation leads to a large increase in the dipole moment of the excited indole, creating a localised non-equilibrium state in the environment surrounding the residue. This arises from loss of electron density from the N1 and Cγ atoms and a corresponding increase at the Cε3, Cζ2, and Cδ2 atoms of the indole ring (Figure 27).89 The same type of redistribution is not observed in F or Y residues as their side chains possess higher order symmetry than the indole ring.
The shape and maxima ($\lambda_{\text{max}}$) of the steady-state tryptophan fluorescence spectrum are energetic parameters that represent the energy gap between the excited and ground state of W (Figure 28). Photon absorption causes excitation from the electronic ground state to one of the vibrational states of in the electronic excited state. Relaxation of the vibrational states occurs until the lowest energy level of the electronic excited state is reached, at which point fluorescence occurs when the electron returns to its ground state with a corresponding photon emission.\textsuperscript{90}
The polarity, flexibility and chemical nature of the environment surrounding the W residue influences how the environment adjusts to the redistribution of electron density upon excitation. Because of this a combination of different universal and specific interactions can occur with the excited state, resulting in additional modifications to the spacing between the energy levels of the excited and ground states. Hence the steady-state fluorescence spectrum highlights changes in the protein’s tryptophan environments.

The main universal interaction occurs due to the redistribution of electron density in the indole ring inducing dipole-dipole relaxation of the surrounding protein groups and solvent molecules.\(^9\) This relaxation process causes lowering of the energy of the excited state and therefore causes lengthening of the observed \(\lambda_{\text{max}}\) in the fluorescence spectra. The scale of the difference between the wavelength of absorption and observed fluorescence is influenced by several factors: (i) the magnitude of the dipole difference between the ground and excited states; (ii) the refractive index and dielectric constant of the environment; and (iii) the lifetime of fluorescence when compared to the time of dipole relaxation of the W and its surrounding dielectric.

This also explains why the Y and F residues are insensitive to their surroundings, as the differences between the dipoles of their ground and excited states are essentially zero.

This effect does not solely explain the shift in fluorescence; the W residue also can undergo specific interactions with a few select groups in its surrounding environment. These can be due to charge transfer reactions or from the formation of weak hydrogen bonds. Specific interactions can also give rise to significant shifts in the emission spectra.\(^9\)

Besides \(\lambda_{\text{max}}\), the other important parameter that can be extracted from the steady-state fluorescence spectrum is the quantum yield (\(\Phi\)) – the efficiency of conversion of photons absorbed into fluorescence. This varies from around 0.4 to the limits of detectability.

Used together \(\lambda_{\text{max}}\) and \(\Phi\) can be used to characterise the environment a W residue is in. However, while this gives some information about the environment the tryptophans occupy, taken on its own this does not explain how quantitative data can be determined for protein-ligand interactions.
In order to acquire this type of data there are some pre-requisites for the protein-ligand interaction: (i) that binding of the ligand induces a conformational change in the protein; or (ii) that the presence of the ligand causes a change to the universal or specific interactions within the environment of the binding pocket; and (iii) that there is at least one suitably placed W residue that experiences this shift in environment, leading to either quenching or enhancement of the fluorescence.

This is best illustrated by example: M. Frech et al. used intrinsic tryptophan fluorescence spectroscopy to determine the stoichiometry (n) and dissociation constant (K_d) for PKBα’s PH domain interacting with various inositol phosphates and phosphoinositides. As already discussed, upon binding of PtdIns(3,4,5)P_3, PKB’s PH domain undergoes a conformational change. This is reflected by a corresponding change in the steady-state tryptophan fluorescence spectrum (Figure 29).

**Figure 29.** Fluorescence spectra of PKBα’s PH domain with, and without, addition of dioctanoyl PtdIns(3,4,5)P_3. Excitation was carried out at 290 nm. The higher curve is the emission spectrum of the PH domain alone; the lower curve is the emission spectrum of the PH domain in the presence of an excess of dioctanoyl PtdIns(3,4,5)P_3. The emissions are expressed in arbitrary units.

The protein was excited at 290 nm, and a corresponding emission λ_max is observed at 345 nm. Addition of excess ligand led to quenching of the signal by ~30%, but no change in the value of λ_max. PKBα’s PH domain contains four W residues – W11, W22, W80 and W98. Unpublished mutational studies carried out by Dr. P. Free in the Gaffney group suggest that only the W80 significantly contributes to the observed quenching in the fluorescence spectrum upon ligand addition –
corresponding to W80 undergoing the greatest degree of reorientation of upon ligand binding, as seen by comparing X-ray crystal structures.\(^{25, 57}\)

Figure 30. A Titration of the PKB\(_\alpha\)’s PH domain with sequentially increased concentration of dioctanoyl PtdIns(3,4,5)P\(_3\). Excitation carried out at 290 nm, and emission measured at 345 nm. B Scatchard plot of the data from A.\(^{92}\)

Once the \(\lambda_{\text{max}}\) values of the bound and unbound states have been determined (in this case they are the same), the next step is to carry out a titration, plotting the change in fluorescence intensity (\(\Delta F\)) against the relative amount of ligand added to protein. This gives a graph where the \(\Delta F\) values tend towards \(\Delta F_{\text{MAX}}\) at high ligand concentrations (Figure 30A). A non-linear fit to a hyperbolic function is used to determine the \(\Delta F_{\text{MAX}}\) value.

\[
\frac{\Delta F}{\Delta F_{\text{MAX}}} = n - K_d\left\{\frac{(\Delta F/\Delta F_{\text{MAX}})}{[\text{PtdIns(3,4,5)P}_3]}\right\}
\]  
\text{(eq. 1)}

A Scatchard plot of eq. 1 is then employed to analyse the data,\(^{93}\) plotting the relative change in the difference between \(\Delta F\) and \(\Delta F_{\text{MAX}}\) per increase in ligand concentration against the relative change in the difference between \(\Delta F\) and \(\Delta F_{\text{MAX}}\) (Figure 30B). The linear fit of this data set gives both the binding stoichiometry for the interaction (\(n\)) – the intercept on the y axis – and \(K_d\) is given by the slope of the line.

In the case of PKB\(_\alpha\)’s PH domain binding dioctanoyl PtdIns(3,4,5)P\(_3\) this gives the binding stoichiometry as 1:0.92 for the PH domain-PtdIns(3,4,5)P\(_3\) complex, which shows good agreement with the expected value of 1:1, and a \(K_d\) of 0.40 (±0.03) \(\mu\)M.
While intrinsic tryptophan fluorescence spectroscopy is known to work for the wild-type PH domain of PKBα, to probe a mutant PH domain requires that a similar response is observed upon binding to a synthetic ligand. While a positive result is proof of concept, a negative result will not necessarily mean binding is not occurring. Because the formation of the intramolecular salt bridge is being disrupted, it is possible that even with ligand binding, the conformational change observed in the wild-type protein will not occur in the mutant.
Hence a second technique is required to either verify a positive result, or establish a negative result as genuine. ITC is a potential technique to give this verification.

3.2 ISOThermal Titration CALorimetry

Isothermal titration calorimetry (ITC), like intrinsic tryptophan fluorescence spectroscopy, is a technique that can be used to determine the \( K_d \) and binding stoichiometry (n) of a protein-ligand interaction. Additionally, it determines the enthalpy changes (\( \Delta H \)) of the interaction. Since the interaction is studied at a controlled temperature, it is also then possible to use \( K_d, n \) and \( \Delta H \) to calculate the Gibbs free energy change (\( \Delta G \)) and the entropy change (\( \Delta S \)). Because of this ITC is the only bio-physical technique that can determine all of these parameters with a single experiment, and nearly completely characterise the thermodynamic properties of the interaction.

The standard experimental equipment (MicroCal VP-ITC MicroCalorimeter) (Figure 31) is formed of two identical coin shaped cells fashioned from the highly efficient heat conducting metal Hastelloy® alloy C-276. The cells are then encased in an adiabatic jacket. This allows thermostatted heating/cooling to maintain equal constant temperature between the two cells. The differential power (DP) signal between the reference cell and the sample cell is the power required to maintain temperature equilibrium, and is measured using sensitive thermopile/thermocouple circuits. This signal is calibrated electrically by applying a known amount of power through a resistive heater element positioned on the cell. When the ligand is titrated into a solution of its macromolecular receptor it is typically accompanied by the evolution of heat within the sample cell. This results in a negative change in the DP as less power is needed due to the heat contribution
from the exothermic chemical interactions. For endothermic reactions the opposite is true.

Raw data from the experiment is displayed as a series of spikes of heat flow (power) - each spike a consequence of an injection of ligand (Figure 32A). Because DP has units of power (μcal/s), the time integral of the spike gives a measurement of ∆H. The amount of binding is directly proportional to the heat which is absorbed or released, with the heat signal diminishing to only the background heat of dilution as the macromolecule becomes saturated with ligand.
Figure 32. Representative ITC data. A Raw ITC data. Twenty injections of ligand solution added to a solution of macromolecule in the sample ITC cell B Binding isotherm. The area of each injection spike equals the total heat released from that injection. A plot of the integrated heat against the molar ratio of ligand added to macromolecule - the binding isotherm for the interaction - is acquired. A non-linear least squares fit gives a best fit curve (in red) from which the thermodynamic parameters can be determined.\(^\text{34}\)

Once the raw data has been collected, Origin® software is used to analyse the data. An iterative mathematical curve fitting process – a non-linear least squares fit – is used to create a curve through the plotted integrated peak areas. The type of equation created is dependent upon the type of interaction being observed. The mathematical parameters of the binding isotherm are then used to determine \(n\), \(K_d\), and \(\Delta H\). In the case of the “one set of sites” model (APPENDIX A), as for the example below (Figure 32B), \(n\) is the point of inflection of the curve. \(K_d\) is the inverse of the slope at the point of inflection. \(\Delta H\) is the difference between \(H_{\text{MIN}}\) and \(H_{\text{MAX}}\).
Plot of ITC isotherms for various values of $c$: At low values the isotherm is a near horizontal line. As $c$ increases the isotherm takes on a sigmoid form. At high values of $c$ the isotherm takes on a rectangular form.

The shape of the binding isotherm is determined by the parameter $c$. $c$ is a unitless constant, defined by eq. 2:

$$c = \frac{[M_{\text{tot}}]n}{K_d}$$  \hspace{1cm} (eq. 2)

Where $[M_{\text{tot}}]$ is the total macromolecule concentration at the start of the experiment, $n$ the stoichiometry parameter, and $K_d$ the dissociation constant. Since $n$ and $K_d$ are constant for any given interaction, $[M_{\text{tot}}]$ is the only parameter that can be varied. If the initial $[M_{\text{tot}}]$ is very low (i.e. $c \leq 0.1$), then the isotherm is essentially featureless, appearing as a near horizontal line (Figure 33), making characterisation of the interaction’s thermodynamic properties impracticable. As $[M_{\text{tot}}]$ is increased the equivalence point begins to be defined, and the calculated $\Delta H$ converges towards the true value of $\Delta H$. At very high concentrations of $M_{\text{tot}}$ (i.e. $c > 5000$), there is very tight binding, and the isotherm takes on a rectangular form with the sharp drop occurring precisely at $n$ and its height exactly corresponding to $\Delta H$. However it is not then possible to accurately determine the $K_d$ value for the interaction. This gives rise to an “experimental window” for determining $K_d$, where $c$ is high enough for the isotherm to have definition, yet low enough so that $K_d$ can
be extracted from the curve fit. It has been experimentally determined that the middle of the window $5 \leq c \leq 500$ is ideal for measuring $K_d$.

However, $K_d$ is not the only parameter required when designing an ITC experiment. It is also necessary to have some understanding of the expected $\Delta H$ value. This arises from the limitations in the sensitivity of the ITC equipment. For accurate measurements at least 3-5 μcal of heat should be evolved/absorbed within the sample cell with each injection. Hence, while ITC can be a very powerful technique, with the potential to characterise many thermodynamic parameters with a single experiment, it is limited by the need to have a strong enough understanding of the interaction so that a reasonable estimate of $K_d$ and $\Delta H$ can be made before starting the experiment. These estimates are needed to ensure $[M_{tot}]$ is high enough so that the heat evolved/absorbed is accurately observable. This is why ITC is often used in parallel with intrinsic tryptophan fluorescence spectroscopy as it independently provides a $K_d$ value, which can then be fed into the calculations to determine the required $[M_{tot}]$ for the ITC experiments.

One advantage of ITC, when compared to intrinsic tryptophan fluorescence spectroscopy, is that it does not require suitably placed W residues within the protein in order to study an interaction. This means that ITC can be used to study a wider range of interactions inaccessible to intrinsic tryptophan fluorescence spectroscopy. However, if there is a suitable W residue, intrinsic tryptophan fluorescence spectroscopy is a significantly more sensitive technique, allowing much lower concentrations of protein and ligand to be used per experiment. Because of this, interactions with very high $K_d$ values can be studied, which would not be possible using classical ITC techniques as the requisite $[M_{tot}]$ would be unobtainable.

While ITC has not previously been used to study the PKBα PH domain’s interactions with inositol phosphates/phosphoinositides, it has been used to study other PH domains. Kavran et al. studied several different PH domains using – amongst other techniques – ITC.\(^\text{95}\) One of the PH domains studied was that of EST (expressed sequence tag) 684797. ITC of its PH domain titrated into solutions of $\text{Ins}(1,3,4)P_3$, $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ produced three binding isotherms (Figure 34). All three inositol phosphates show binding to the EST 684797 PH domain. However both $\text{Ins}(1,3,4)P_3$ and $\text{Ins}(1,3,4,5)P_4$ exhibit higher $\Delta H$ and lower
$K_d$ than Ins(1,4,5)P$_3$, placing it in group 3 (section 1.4) similar to PKB’s PH domain. EST 684797 has subsequently been identified as the B cell antigen receptor (BCR) downstream signalling regulator DAPPI (dual adaptor of phosphotyrosine and 3-phosphoinositides), also known as PHISH (3-phosphoinositide interacting SH2-containing protein) or Bam32 (B cell adaptor molecule of 32 kDa). While its PH domain is in the same group as PKBs, they only share ~18% pair-wise sequence identity. This would account for the differences in the X-ray crystal structures of each domains holo conformation when binding Ins(1,3,4,5)P$_4$. The inositol ring is rotated approximately 45° in the plane of the ring between the two structures; additionally the ring is closer to the core of the PH domain in the case of PKB.

![Figure 34. ITC results of inositol phosphates binding the EST 684797 PH domain. 50 mM PH domain titrated into 5 mM solutions of Ins(1,3,4,5)P$_4$ (■), Ins(1,3,4)P$_3$ (●), or Ins(1,4,5)P$_3$ (▲), at 25°C. Best fits curves assume a single class of binding site. $K_d$ for Ins(1,4,5)P$_3 > 1$ μM, Ins(1,3,4,5)P$_4 = 43$ nm and Ins(1,3,4)P$_3 = 49$ nM, in a 1:1 interaction. $\Delta H$ values measured at 25°C: Ins(1,3,4,5)P$_4 = 28.3$ kcal/mol and Ins(1,3,4)P$_3 = 24.9$ kcal/mol.](image)

This shows how ITC can be used to probe a PH domain’s interaction with inositol phosphates, and should be transferable for studying the interactions of both wild-type and mutant PKBα PH domains with natural and functionalised inositol phosphates.
4. SYNTHESSES OF PHOSPHORYLATED MYO-INOSITOL DERIVATIVES

A range of biological processes have been shown to be influenced by phosphorylated myo-inositol and inositol phospholipid compounds. This has created demand for a wide range of synthetic derivatives and analogues.

There are five main issues that arise when considering the synthesis of the Ins(1,3,4,5)P$_4$ analogue 1: (i) Choice of starting material; (ii) enantiomeric purity; (iii) how to achieve the desired functionalisation at the 2-O position; (iv) phosphorylation technique; and (v) choice of protecting groups.

Scheme 1. (i) Hydroxyl groups protection/deprotection;$^{98-101}$ (ii) Ferrier reaction;$^{102-105}$ (iii) SmI$_2$-mediated cyclisation;$^{106-108}$ (iv) Pseudomonas putida mediated hydroxylation followed by dihydroxylation reactions;$^{109}$ (v) hydroxylation.$^{110-112}$
The first two points can be taken - to some extent - in parallel. Inositol derivatives have been synthesised from a variety of sources (Scheme 1).\textsuperscript{98-113} Most commonly used is myo-inositol itself (2) and other naturally occurring cyclitols (3 and 4). However, carbohydrates (5-9), chiral acids (10), and benzene derivatives (11) have also been employed. Chiral, non-racemic, compounds (5-10) provide routes to optically active inositol derivatives, but, compared to myo-inositol, require several additional reactions to establish the myo-stereochemistry of the incipient hydroxyls around the 6-membered ring. The choice of starting material therefore depends on the importance of chirality versus yield and minimal number of synthetic steps.

Neither \textit{in vitro} nor \textit{in vivo} studies of the L-enantiomers of \textit{Ins}(1,3,4)P\textsubscript{3} and \textit{Ins}(1,3,4,5)P\textsubscript{4} interacting with the PH domain of PKB have been reported. However studies of the PH domain of PDK1 binding a range of PtdInsP\textsubscript{n} suggest that the PH domain of PKB should exhibit a high level of discrimination between the $D$- and $L$-enantiomers of both \textit{Ins}(1,3,4,5)P\textsubscript{4} and \textit{Ins}(1,3,4)P\textsubscript{3}.\textsuperscript{114} If this holds true for the E17A mutant PH domain of PKB then there is no immediate need for a chiral synthesis of 1 – the D-enantiomer will bind, but the L-enantiomer will not do so to any significant extent. This premise can be verified by the biophysical studies for this project – both intrinsic tryptophan fluorescence spectroscopy and isothermal titration calorimetry give a constant of stoichiometry. Using a racemic mixture, assuming that only the D-enantiomer interacts, should give an \textit{n} value of 2, as only half the mixture partakes in the interactions. An \textit{n} value significantly lower than 2 would show that both enantiomers are interacting, and that both isomers should be investigated in their own rights.

If enantiomeric purity is not of prime importance then myo-inositol, is an ideal starting point for preparing inositol polyphosphate analogues, as the correct stereochemistry is already in place around the 6-membered ring. This is re-enforced by retrosynthetic analysis of the target molecule.

### 4.1 RETROSYNTHETIC ANALYSIS

The first consideration in the retrosynthetic analysis of 1 (Scheme 2) is the creation of the phosphate moieties. Free phosphates are difficult to handle, and their protected forms are sensitive to nucleophiles, so they should only be formed in the final step of the synthesis to
Polar group exchange receptor-ligand engineering of protein kinase B's pleckstrin homology domain

avoid unwanted side reactions. Because of the need for myo-inositol and inositol phospholipid derivatives and analogues, a variety of polyol phosphorylation techniques have been investigated.

\[
\begin{align*}
\text{HO} & \quad \text{OR} \\
\text{O} & \quad \text{OP} \quad \text{OP} \quad \text{OP} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}
\]

1

\[
\begin{align*}
\text{OR} & \quad \text{OP} \quad \text{OP} \quad \text{OP} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}
\]

13

\[
\begin{align*}
\text{OR} & \quad \text{OP} \quad \text{OP} \quad \text{OP} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}
\]

14

\[R = \text{Acetic acid or a protected/masked form}\]
\[\text{PG}^3 = \text{Phosphate protecting group}\]
\[\text{PG}^6 = \text{Protecting group for 6-O}\]

Scheme 2. Initial retrosynthetic analysis of 1.

4.2 PHOSPHORYLATION

P(III) Reagents

15

17

19

21

P(V) Reagents

16

18

20

22

Figure 35. A selection of commonly employed P(III) and P(V) phosphorylation reagents.
Phosphorylating reagents can be split into two main classes: (i) P(III), and (ii) P(V) reagents (Figure 35). P(V) reagents react to form phosphate triesters directly, so the products are already in the desired oxidation state. P(III) reagents can react via several different routes to form either an intermediate phosphite triester – which then requires oxidation to the P(V) state – or a phosphate triester, if the oxidant is also the activator.

The one-step formation of phosphate triesters from phosphites requires the presence of an oxidising agent such as I₂, or pyridinium bromide perbromide (PBP). I₂ oxidises the P(III) reagent in situ to form a P(V) phosphoryl iodide intermediate (25) which then react with the alcohol.¹¹⁵ PBP reacts with the P(III) reagent to form a bromophosphonium salt intermediate (27), which then reacts with the alcohol to form the phosphate triester via a bromophosphorane¹¹⁶ (Scheme 3).

![Scheme 3](image)

**Scheme 3.** Phosphorylation reaction mechanisms for the reaction of trialkyl phosphites with an alcohol, with either I₂ or PBP as an oxidising reagent.

The main issue that arises with phosphorylation of inositol polyols, and helps dictate the choice of reagent, is the phosphate/phosphite triester’s ability to migrate (Scheme 4). Attack on the P(III/V) centre by an adjacent alcohol leads to the formation of cyclic intermediate 30. This cyclic intermediate (after oxidation if via the
Phosphate triesters are less sensitive to nucleophilic attack than their phosphite counterparts, so when phosphorylating a diol the absolute rate of formation of 30 would be expected to be slower for the phosphate than for the corresponding phosphate triester. This would appear to make it advantageous to use P(V) reagents compared to P(III) reagents. However P(V) reagents are much less reactive than the corresponding P(III) species, so the rate of phosphorylation of the second, now more hindered, alcohol is much slower for P(V) than for the P(III) reagents. Consequently, the ratio of the rate of formation of 30 to its rate of phosphorylation, is much greater for P(V) than P(III) reagents. Hence P(III) reagents can often be used for phosphorylation where P(V) reagents are unsuccessful. This is the reason why P(III) reagents have become favoured for inositol chemistry.

Additionally, while the P(V) compound tetrabenzyl pyrophosphate (TBPP, 20) - with sodium hydride and catalytic imidazole or 18-crown-6 in THF - effected tetraphosphorylation in the first reported synthesis of Ins(1,3,4,5)P₄,¹¹⁷ it requires full conversion of the hydroxyls into alkoxides. However, many phosphoinositide related targets contain functional groups sensitive to these strongly basic conditions (e.g. fatty acid esters). Hence they have been almost completely superseded by P(III) reagents in more recently reported syntheses.¹¹⁸ P(III) reagents can be split into two sub-groups: Phosphorochloridites and phosphoramidites.

Phosphorochloridites have been widely employed in the synthesis of phosphorylated peptides and oligonucleotides. They have also been used for the preparation of poly-phosphorylated inositols - di(2-cyanoethyl)phosphorochloridite (15) was used to establish the phosphoryl centres in the total synthesis of PtdIns(3,4,5)P₃.¹¹⁹ However, in the case of dialkyl/diaril phosphorochloridite reagents, it has been shown that product purity is a function of the purity of the reagent, and the phosphorochloridites can disproportionate over time, leading to variable yields.¹²⁰ Because of this the less reactive phosphoramidites have become favoured.
By themselves phosphoramidites do not phosphitylate hydroxyls. In order for the reaction to proceed, the phosphoramidite reagent needs to be activated by protonation (Scheme 5). There are several reagents that can achieve this, but the most commonly employed is the weakly acidic 1-\(H\)-tetrazole (34, pK\(_a\) ca. 5). Tetrazole protonates the amide nitrogen and the tetrazolide anion then displaces the ammonium leaving group. The activated P(III) centre is next attacked by the weakly nucleophilic alcohol (35) to form a phosphite triester (36); the tetrazole released at this stage forms a salt with the more basic dialkylamine (pK\(_a\) ca. 10) liberated in the initial activation.

Once formed, the phosphite triester needs to be oxidized to form the desired phosphate. The most commonly used oxidising agents are \(m\)CPBA,\(^{118}\) \('\)BuOOH\(^{119}\) or I\(_2\)/water.\(^{121}\) \(m\)CPBA has been shown to give cleaner oxidations of dibenzylphosphite triesters,\(^{122}\) while \('\)BuOOH has been favoured for di(2-cyanoethyl)phosphites, so the choice of oxidising agents is dependent upon the protecting groups used in the phosphorylating reagent, and the other functional groups present (e.g. double bonds are not epoxidised by \('\)BuOOH).

The free phosphate is subsequently formed by removal of the protecting group.

4.3 PROTECTING GROUPS

Protecting group strategies play a central role in the synthesis of phosphorylated myo-inositol and inositol phospholipid compounds. The choices of protecting groups influence every stage of the synthesis.

To minimize the number of synthetic steps to target 1, it would be simplest if the protecting groups for the phosphates (PG\(^6\)) could be removed by the same conditions as for the protecting group on the 6-\(O\) (PG\(^6\)). To make a suitable choice
for these protecting groups, how the 2,6-<i>O</i> substitution pattern of the tetrol might be established should be considered.

No reactions have been reported for selective 2-<i>O</i>-alkylation of myo-inositol, and this axial ring substituent is the least reactive of the six hydroxyls. Therefore, for the synthesis of the 2-<i>O</i> acetic acid Ins(1,3,4,5)<i>P</i><sub>4</sub> derivate 1, a protecting group strategy is required that isolates the 2-hydroxyl for functionalisation.

![Scheme 6](image)

Acetal chemistry has been widely employed in the synthesis of inositol derivatives to protect 1,2-diols. However the regioselectivity of these reactions – forming a 1(3),2-acetal (Scheme 6) – makes them unsuitable for the synthesis of 1 without the inclusion of additional deprotection and functionalisation steps. Several alternative protecting groups have been utilised that leave the 2-hydroxyl accessible.
The reaction of myo-inositol with an excess of either 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane (TipdsCl₂), or with 2,2,3,3-tetramethoxybutane and trimethyl orthoformate in the presence of catalytic camphorsulfonic acid (CSA), produce myo-inositol based 2,5-diols (39 and 40). Alternatively the reaction with bis-dihydropyran (42) produces a 1(3),2-diol (41).

Orthoesters also provide a route to isolate the 2-hydroxyl for functionalisation. Orthoester formation results in the simultaneous protection of the 1-, 3- and 5-hydroxyls, creating a 2,4,6-triol (43).¹²⁹

Both types of diol and the orthoesters fulfill the initial prerequisite for synthesising 1. The choice between them is dictated by the 1,3,4,5 phosphorylation pattern of the target compound. Deprotection of functionalized Tipds or bis(butane-2,3-diacetal) compounds would subsequently require selective reprotection of the 6-hydroxyl before phosphorylation. The same would also be true for the 1(3),2-diol based compounds. However this reprotection step would not be necessary in the case of the orthoesters.

The first reported synthesis of a myo-inositol orthoester (Scheme 9) was in 1966, when Luk’yanov and Tolkachev claimed to have synthesised an orthoformate of myo-inositol. However subsequent research by Lee and Kishi showed that while they probably achieved the protection, they mis-assigned its structure (44).¹²⁹
Myo-inositol orthoesters are formed by exchanging myo-inositol with either the trimethyl- or triethyl-orthoester of the corresponding carboxylic acid under catalytic acidic conditions. Because the six hydroxyls have similar reactivity, the protecting group can initially react with any of the hydroxyls. Orthoester exchange around the inositol ring leads to a series of intermediate structures that equilibrate to the thermodynamically most favoured structure – the 1,3,5-O-triester (43).

Using this protecting group influences the choice of PG₆ and PG⁴, as PG₆ must be orthogonal to the deprotection conditions of the orthoester. Using either 4:1 trifluoroacetic acid (TFA)-water,¹³⁰ or refluxing hydrochloric acid in methanol,¹³¹ causes acid hydrolysis of orthoesters (Scheme 10). Potter et al. formed a mixture of 1- and 3-O benzoate carboxyl esters by acid hydrolysis to deprotect inositol-2,6-O-dibenzyl orthobenzoate.¹³² Since none of the 5-O benzoate ester was observed, this implies that acid hydrolysis of orthoesters proceeds via initial scission of the bond to the 5-O. This is followed by largely unselective cleavage of the bond to either the 1- or 3-O, to give a mixture of 1-and 3-O acylated intermediates (48). In the case of the orthoformates, these acyl intermediates then undergo spontaneous hydrolysis.
so that all three hydroxyls are revealed. If an orthoester with a more stable corresponding acyl ester is used then it is possible to isolate this intermediate for the synthesis of compounds such as Ins(1,4,5)P\(_3\) and PtdIns(3,4,5)P\(_3\). Alternatively the acyl group can then be displaced by nucleophilic attack to recover all three hydroxyls. Studies of the rates of hydrolysis of 2,4,10-trioxaadamantanes suggest that, if the same holds true for inositol orthoesters, substitution at the orthoester bridgehead influences the rate of hydrolysis.\(^{133}\) The rate determining step is the ring-flip of the cyclohexane ring (46) into the boat conformation (47). This change in conformation allows water to access the carboxonium ion for hydrolysis to occur. Since the stability of the carboxonium ion increases with the size of bridgehead substituent, there is a corresponding decrease in the rate of hydrolysis.

Since the immediate precursor to the target 1 will need to be protected on the 6-\(O\), PG\(^6\) must be stable to strong acid conditions. Furthermore if \(R^1\neq H\), it will also need to withstand the subsequent nucleophilic displacement of the acyl ester after initial hydrolysis of the orthoester.

### 4.4 INOSITOL ORTHOESTER CHEMISTRY

#### 4.4.1 Functionalisation

As well as protecting the 1-, 3- and 5-hydroxyls, the orthoester imposes an adamantane-like structure upon the compound. This forces the 2-hydroxyl into an equatorial orientation relative to the carbocycle, and the 4- and 6-hydroxyls to adopt axial dispositions. This creates a reactivity differential between the 2- and 4-/6- hydroxyls. Taking advantage of this difference has allowed the development of techniques to selectively react either: (i) just the equatorial 2-hydroxyl, (ii) just an axial 4(6)-hydroxyl, (iii) both the 2- and 4(6)-hydroxyls simultaneously, or (iv) both axial hydroxyls simultaneously.

As a general rule, the regioselectivity of functionalisation of myo-inositol orthoesters is controlled by the strength of base used in the reaction. Using a mild base (Scheme 11), such as pyridine or imidazole, leads to selective reaction of the 2-hydroxyl. However these bases can only generally be used to enable functionalisation with acyl, sulfonyl or silyl protecting groups, but not alkylation.
Acylation with one equivalent of reagent in pyridine is selective for the 2-hydroxyl. The reaction proceeds via a bulky but reactive acyl pyridinium ion intermediate. Because the hydroxyl group is mostly un-ionised in the reaction transition state, product formation is governed by steric factors, leading to preferential acylation of the least hindered 2-hydroxyl. \(^{134}\)

\[
\begin{align*}
 &\begin{array}{c}
 \text{HO} \\
 \text{O} \\
 \text{O} \\
 \text{R} \\
 \text{HO} \\
 \end{array} \\
 &\begin{array}{c}
 \text{HO} \\
 \text{O} \\
 \text{O} \\
 \text{BzO} \\
 \text{HO} \\
 \end{array}
\end{align*}
\]

\(R = \text{H, Me}\)


With one equivalent of a reactive alkylating agents, bases such as \(\text{Et}_3\text{N}\) are sufficient. Otherwise metal hydrides need to be used. However these bases lead to the 4(6)-hydroxyl being favoured due to the stabilisation of the axial alkoxide by neighbouring group participation from the other axial hydroxyl (Scheme 12). \(^{135}\) The 4(6)-alkoxide is stabilised by a hydrogen bonding interaction with the 6(4)-hydroxyl, combined with chelation in the case of metal ion containing bases, of the metal cation in the di-axial 4,6-\(O\) pocket. Since the 2-alkoxide would not be similarly stabilised, these conditions lead to selective functionalisation of the 4(6)-hydroxyl.

\[
\begin{align*}
 &\begin{array}{c}
 \text{HO} \\
 \text{O} \\
 \text{O} \\
 \text{H} \\
 \text{HO} \\
 \end{array} \\
 &\begin{array}{c}
 \text{HO} \\
 \text{O} \\
 \text{O} \\
 \text{BzO} \\
 \text{HO} \\
 \end{array}
\end{align*}
\]

\(43a\)

Scheme 12. Synthesis of 4(6)-O-benzoyl-myoinositol-1,3,5-orthoformate. (i) BzBr, NaH (1 eq.).
A di-substituted compound is formed by increasing the number of equivalents of reagents to 2. The substitution pattern of the product is again defined by choice of base. Consideration of **Scheme 13** explains these patterns if $R_2=\text{n}$, strong base; (ii) $R_2\text{X}$, mild base; (iii) $R_3\text{X}$, strong base; (iv) $R_3\text{X}$, mild base. When using a weak base the reaction proceeds by route BE, initially forming intermediate 54. The second equivalent of reagent then reacts with one of the axial hydroxyls for selective formation of the unsymmetrical alcohol 55a. Selectivity is diminished by using a stronger base. Functionalisation can follow either route AC – to form the symmetrical alcohol 53a – or route AD – to create the unsymmetrical alcohol 55a.

The ratio of 53a:55a is determined by several factors. The presence of a group on the 4(6)-OH means the formation of the second alkoxide ion cannot be stabilised by intramolecular hydrogen bonding, decreasing the selectivity for the second axial hydroxyl and increasing the yield of 55a. 1,3-Diaxial steric interactions, especially with bulky reagents, increase the level of 55a formed. If metal ion containing bases are used, the choice of ion can increase selectivity for the 6(4)-hydroxyl; the small Li$^+$ cation binds more tightly in the 4,6-O pocket than Na$^+$, increasing the chelation effect and favouring 53a.

If the substitution reactions are separated into individual steps, the chemistry creates compounds with different functional groups on each oxygen atom.

Because of the symmetrical nature of 54, the product of reacting the 4,6-diol under either weak or strongly basic conditions (route E) can only break this symmetry.
The unsymmetrical diol (52) can be functionalised in one of two ways; (i) using a weak base (route D) favours the 2-hydroxyl in the same manner as for the triol (43); (ii) the use of strong bases has the same problems with selectivity as for the one-pot di-functionalisation, creating a mixture of 53b and 55b.

Both diols 52 and 54 can be exhaustively di-functionalised, depending on the desired functionality, using both weak or strong base and an excess of reagent. Similarly the already di-substituted compounds (53 and 55) can be functionalised at their remaining hydroxyl in the same way, to create a fully protected myo-inositol.

### 4.4.2 Reductive orthoester removal

![Scheme 14. Reaction mechanism for DIBAL-H reduction of orthoesters.](image-url)
As already discussed, the orthoesters can be cleaved by acid hydrolysis. However there are two other main routes that have been developed for manipulating orthoesters: (i) reduction with diisobutyl aluminium hydride (DIBAL-H), or (ii) using Grignard reagents or trimethyl aluminium to insert an alkyl group into the C-O bond.

DIBAL-H offers greater control of the intermediate products that can be generated when compared to acid hydrolysis (Scheme 14 vs. Scheme 10). Using 2 equivalents of DIBAL-H selectively generates a bridging 1,3-O acetal (56). Similar to acid hydrolysis of orthoesters the 5-O reacts preferentially, but in this case it is clearly due to being the least sterically hindered of the three orthoester oxygens. This acetal can then be hydrolysed to form 49. Using an excess of DIBAL-H inserts a second hydride, but this time unselectively, leading to the formation of a mixture of the 1- and 3-O isomers of the ether (57).  

Scheme 19. Reaction mechanism for Grignard reagent reduction of orthoesters.
Use of limiting Grignard reagents or trimethyl aluminium to cleave myo-inositol orthoesters also generates a bridging acetal. Unlike DIBAL-H, where the 1,3-\(O\) acetal is preferentially formed, these reagents create a 1(3),5-\(O\) acetal (58). In the case of trimethyl aluminium, this difference in region-selectivity has been attributed to its smaller steric bulk compared to DIBAL-H allowing it to access the more sterically hindered 3(1)-\(O\). With Grignard reagents, their activation and orientation of attack is dictated by the chelation of a magnesium ion in the myo-inositol-1,2,3-\(O\)-trioxa pocket (Scheme 15). 138

Using an excess of Grignard reagent leads to the generation of a 5-\(O\)-alkyl derivative (59). Both these techniques can be used to create the desired tetrol 49. However acid hydrolysis, particularly of the orthoformate, provides the most efficient route as it requires the fewest synthetic steps.

### 4.5 PROPOSED SYNTHETIC ROUTE

Combining the retrosynthetic analysis to the 2,6-\(O\) substituted tetrol 14 (Scheme 2) with the chemistry of inositol orthoesters, suggests a synthetic route to the Ins(1,3,4,5)\(P_4\) analogue 1 (Scheme 16).

Since direct alkylation of the 2-\(O\) is not possible, the longer route via temporary protection – i.e. using a protecting group that controls regiochemistry, but which is not retained up to the final global deprotection – of the 4-hydroxyl needs to be adopted. The orthoester dictates the selection of subsequent protecting groups. Strongly basic conditions can be used to set up the desired substitution pattern, in which \(\text{PG}^6\) could be an alkyl, acyl or sulfonyl group. As a permanent protecting group – i.e. a group that is retained up to the final global deprotection step – it must be stable to the strong acid conditions used to remove the orthoester and, if \(R^1\neq H\), the subsequent nucleophilic displacement of the \(R^1\) acyl ester. It must also be stable to the deprotection conditions for \(\text{PG}^4\).

This discounts acyl protecting groups for the role of \(\text{PG}^6\) as they would be displaced during removal of the orthoester (\(R^1\neq H\)). Because of this alkyl reagents, particularly allyl and benzyl (Bn) groups, have been widely employed for this purpose due to their stability towards both acidic and basic conditions. The criteria for \(\text{PG}^4\) are less stringent. As a temporary protecting group the main requirement is that its removal does not affect \(\text{PG}^6\). Using an acid labile protecting group would allow for a one-pot deprotection to form the tetrol (14) directly from the fully protected inositol (62).
The choice of protecting group for the phosphates is influenced by PG\(^6\). By using a group with a deprotection compatible with that of PG\(^6\), a final one-pot global deprotection can be used. Although base labile groups such as cyanoethyl could be used in a two-step final unblocking, the obvious choice is for PG\(^6\)=PG\(^p\)=Bn to provide a one-pot global deprotection. Alternatively, other groups susceptible to reductive conditions can be used [e.g. benzyloxymethyl (BOM) or para-methoxybenzyl (Pmb)]. To minimise the steric interactions when trying to phosphitylate multiple hydroxyls, the phosphoramidite N,N-diethylamino-5,6-benzo-1,3,2-dioxaphosphepane (section 4.2, 19), with a bridging protecting group, has been developed.\(^{142}\)

The choice of R\(^2\) initially seems quite simple, as direct formation of the 2-\(O\) acetic acid is possible using chloroacetic acid. However this would need to be protected during the...
phosphorylation step. Additionally it does not allow alternative analogues from a divergent synthetic strategy to be explored. Using an allyl group as a masked form of the acid would allow the potential synthesis of a wide range of compounds.

4.6 MANIPULATIONS OF ALLYL GROUPS

Although allyl ethers are most commonly used as protecting groups, they can also be used as a form of masked functionality. The desired acetic acid functionality can be generated by a variety of techniques (Scheme 17), either in a “one-pot” reaction or via isolation of the aldehyde intermediate 66.

However, the double bond of the allyl group need not only be oxidised but can be converted into a diverse range of other functionalities as well. The range of chemistry that can be applied means that, at least in theory, nearly any commonly utilised functionality should be accessible. In addition to the aldehyde and acid functionalities it is also possible to directly access: alcohols, ethers, 1,2-diols, 1,2-halohydrins, 1,2-dihalides, halides, and the products of pericyclic reactions such as cyclopropanes. Further compounds can then be synthesised from these intermediates.

The simplest inter-conversion is the reduction of the double bond to give a propyl group 67. Alternatively using the acetaldehyde 66, with subsequent reduction instead of oxidation, allows access to the 2-hydroxyethyl ether 68. Ins(1,3,4,5)P₄ analogues with these functionalities at the 2-O substituent can act as control molecules for the receptor-ligand engineering studies, testing the effectiveness of a polar group exchange interaction instead of steric complementation. The alcohol also allows for the formation of further analogues. Using the Mitsunobu reaction, the alcohol can be replaced by a wide range of nucleophiles.
The alcohol can also undergo further substitution reactions, such as PEGylation (Scheme 18).

![Scheme 18. Manipulations of the allyl group. (i) Reduction of allyl; (ii) oxidation of the allyl to an aldehyde; (iii) reduction of aldehyde to hydroxyethyl; (iv) Mitsunobu reaction; (v) PEGylation.](image)

 Longer carbon chain lengths can also be accessed from the allyl ether. Hydroboration-oxidation of the allyl ether forms a 3-hydroxypropyl ether (71). The alcohol can be manipulated as for the 2-hydroxyethyl ether above, or can be oxidised to the aldehyde (72) or carboxylic acid (73, Scheme 19). The organoborane also increases the options for functionalisation, allowing the introduction of halides and amines, or can be used in carbon-carbon bond forming reactions.

![Scheme 19. (i) Hydroboration/oxidation; (ii) oxidation of hydroxypropyl to the aldehyde; (iii) oxidation to propionic acid.](image)

From the allyl ether the chain length might be extended even further by the use of olefin metathesis with the Grubbs or Schrock catalysts (Scheme 20). A suitable choice of R group allows a wide range of further functionalities to be introduced.

![Scheme 20. Cross-metathesis reaction.](image)
This demonstrates that using an allyl group to form the acetic acid functionality on the 2-O creates a versatile synthetic route that can be readily adapted to create a library of 2-O substituted Ins(1,3,4,5)P$_4$ analogues.
5. CHEMISTRY RESULTS & DISCUSSION

5.1 MRes RESEARCH

This section summarises the research undertaken during the MRes year of the 4 year MRes/PhD, for which an MRes qualification was awarded. It is included to provide background information relevant to the subsequent PhD research.

The first consideration for the synthesis of 1 is the choice of the primary protecting group. The orthoformate affords the shorter synthetic route - requiring only a single step deprotection. It also provides a higher degree of freedom with the choices for subsequent protecting groups - since there is no nucleophilic deprotection step, it is possible for PG\(^6\)=acyl. However this needs to be weighed against the flexibility of the synthetic route.

A divergent strategy allows for a range of compounds to be synthesised from a shared intermediate. Acidic hydrolysis of a substituted orthoester allows selective conversion to the 1-\(O\) acyl derivative. This can then be used as an intermediate in the synthesis of PtdInsP\(_n\) analogues. DIBAL-H reduction of orthobenzoates forms a mixture of the 1- and 3-\(O\) Bn compounds. Consideration of the proposed synthetic route (see section 4.5) suggests that if PG\(^6\)=Bn, then the 3-\(O\) Bn provides a succinct route to the Ins(1,4,5)P\(_3\) analogues. Because of this the orthobenzoate was chosen over the orthoformate, and the Bn group was picked as PG\(^6\). Giving the 2-\(O\)-functionalised 6-\(O\)-benzyl-\textit{myo}-inositol-1,3,5-orthobezoate (75) as a key intermediate target molecule (Figure 36).

Acid catalysed reaction of \textit{myo}-inositol with trimethyl orthobenzoate (Scheme 21)\(^{147}\) can be carried out in either dimethylformamide\(^{117}\) or dimethyl sulfoxide.\(^{129}\) Published studies of the formation of other orthoesters have reported that the
reaction in DMF gives a cleaner product.\textsuperscript{135} However in the case of the orthobenzoates, previous work in the Gaffney group gave better results when DMSO was used.

Recrystallisation of triol (43c) from ethyl acetate allowed it to be obtained in high purity and with a reasonable yield. Rather than isolating the triol it was decided to take the crude the myo-inositol orthobenzoate on to the next step. This acted to increase the overall yield for the entire synthesis, since it avoided product loss due to the recrystallisation procedure. Reacting the crude triol (43c) with one equivalent of NaH in DMF selectively formed the 6-alkoxide ion. Subsequent addition of BnBr (Scheme 22) gave a racemic mixture of the asymmetric diol (76) in good yield and as the only isolatable compound.\* 

The next step was to use a temporary protecting group to protect the 4-hydroxyl. The protecting group needs to be either acid labile – so that it can removed during acid hydrolysis of the orthoester – or removable by another means that is orthogonal to the other functionalities present. para-Methoxybenzyl (Pmb) has previously been used in inositol syntheses and fulfils both these requirements.\textsuperscript{148} The Pmb protecting group can either be

\* All compounds in this synthesis are a racemic mixture. For ease of nomenclature all subsequent numbering will be as for the single biologically active D-enantiomer of myo-inositol.
removed by acidic hydrolysis or by single electron transfer (SET) reagents such as ceric ammonium nitrate (CAN) or 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ). Reacting 76 with one equivalent of NaH formed a mixture of the desired 4-O-, the 2-O- and the di-substituted 2,4-O-Pmb ether derivatives. Using a range of protecting groups Devaraj et al. showed that selectivity for the alkylation of the 4-hydroxyl can be increased by chelating the 4-alkoxide with the smaller lithium ion. This study did not include the Pmb group. Trying to reproduce their observed selectivity alkylation with this protecting group did not prove successful, and a similar yield of each product was observed as for using NaH. In an attempt to improve this, the order of substitution was reversed (Scheme 23).

![Scheme 23. Dual synthetic routes for the preparation of 4-O-para-methoxybenzyl-6-O-benzyl-myoinositol-1,3,5-O-orthobenzoate. (i) 1 eq. NaH, 1 eq. BnBr, DMF; (ii) 1 eq. NaH, 1 eq. PmbCl, DMF.](image)

<table>
<thead>
<tr>
<th>Product</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-inositol-1,3,5-O-orthobenzoate</td>
<td>21</td>
</tr>
<tr>
<td>2-O-para-methoxybenzyl-myoinositol-1,3,5-O-orthobenzoate</td>
<td>13</td>
</tr>
<tr>
<td>4-O-para-methoxybenzyl-myoinositol-1,3,5-O-orthobenzoate</td>
<td>42</td>
</tr>
<tr>
<td>di-para-methoxybenzylated compounds</td>
<td>17</td>
</tr>
<tr>
<td>2,4,6-O-tri-para-methoxybenzyl-myoinositol-1,3,5-O-orthobenzoate</td>
<td>2</td>
</tr>
<tr>
<td>Non-reclaimed material</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 1.** Yields of mono-, di- and tri- substituted products from para-methoxybenzylaion of myo-inositol-1,3,5-O-orthobenzoate.
It was hoped that first reacting 43c with one equivalent of NaH and PmbCl would selectively form the 4-O-Pmb ether. Then using one equivalent of BuLi and BnBr, this should exhibit similar selectivity for the formation of the 6-O Bn ether as reported by Devaraj et al. When the mono-alkylation of triol 43c with PmbCl was attempted, it failed to reproduce the selectivity and yield achieved in the corresponding benzylation reaction. A mixture of the mono-, di- and tri- para-methoxybenzylated myo-inositol orthoesters formed (Table 1), as well as unreacted starting material. Since PmbCl is more reactive than the reagents that Devaraj et al. used, the combination of results from the para-methoxybenzylation reactions imply that it is not as dependent on the stabilised alkoxide ion as other alkylations in order to react.

Comparisons of the cumulative yields established that the initial route (benzylation followed by para-methoxybenzylation) gave the most efficient route to 78 (cumulative yield of 54% versus 24% for the alternative route).

The final step to fully functionalise the inositol ring is to introduce the masked acid group. The allyl functionality was chosen as it has the potential for divergent synthesis to create several other alternative final inositol phosphate analogues (see section 4.6). Since there is only one alcohol left to functionalise, reacting 78 with an excess of NaH and allyl bromide, cleanly produced 79 in high yield (Scheme 24).

The next synthetic step was to oxidise the allyl ether to the carboxylic acid functionality (Scheme 25). A range of different reagents were employed to try to achieve this. Direct formation was attempted first. Treating alkenes with ozone produces a highly reactive mol-ozonide, which undergoes a rapid rearrangement to form an ozonide. Treatment with hydrogen peroxide causes oxidation of the ozonide into the corresponding ketone or carboxylic acid species. A reductive work-up with dimethyl sulfide creates two aldehydes. However, both reactions gave a mixture of products, with
only trace amounts of the desired acid present. An alternative method that has been employed to convert allyl functionality into an acetic acid is to use sodium periodate with a ruthenium catalyst.\textsuperscript{151, 152} However, no reaction was observed, so a stepwise approach was adopted instead.

Osmium tetroxide \emph{cis}-dihydroxylated the alkene (81). Using excess oxidant (\textit{N}-methylmorpholine-\textit{N}-oxide, NMO) regenerated the OsO\textsubscript{4}, so only catalytic levels were required.\textsuperscript{153} Sodium periodate was then used to cleave the diol\textsuperscript{154} to create aldehyde 82. It was then necessary to oxidise the aldehyde to the acid. Several different oxidising reagents were tested: Potassium permanganate oxidised the aldehyde, but secondary oxidation at the benzyl ether was also observed; silver oxide\textsuperscript{155} showed no sign of reaction, as did oxone.\textsuperscript{156}

Due to the problems with oxidising the aldehyde, it was decided that direct alkylation with chloroacetic acid (\textbf{Scheme 26}) would be used so that synthesis of the primary synthetic target (1) could be investigated in parallel to resolving these issues. This method proceeded as a relatively clean reaction to give desired acid 80; however, due to difficulties handling the acid, it was decided to protect the acid and form the methyl ester 83.
Scheme 26. Synthesis of 2-O-acetic acid-4-O-para-methoxybenzyl-6-O-benzyl-myoinositol-1,3,5-O-orthobenzoate and its conversion to its methyl ester. (i) 2.2 eq. NaH, 1 eq. chloroacetic acid, DMF; (ii) 0.1 eq. TSA, MeOH.

Scheme 27. Mechanism for SET deprotection of Pmb ethers by DDQ.
Once the fully functionalised myo-inositol had been produced, the next step was to deprotect the 1-, 3-, 4-, and 5-O positions to regenerate the free hydroxyls ready for phosphorylation. The standard orthoester deprotection conditions were employed, as the strongly acidic conditions should allow a ‘one-pot’ reaction that would also cleave the Pmb ether and return the methyl ester to a carboxylic acid. However, preliminary attempts using 4:1 TFA-water gave a complex mixture of products. In order to minimise the potential for side reactions it was decided to remove the Pmb group prior to orthoester deprotection. DDQ (85) can be used to selectively remove Pmb ethers in the presence of Bn ethers due to their differences in reactivity. This difference arises from the para-methoxy substituent on the benzyl group being able to stabilise the intermediate cation after H-atom transfer, leading to reaction times of minutes (for Pmb) rather than days (for Bn).

Treating alcohol 90 from Pmb deprotection with acid initially seemed promising, with negative ion FAB-MS showing a peak at \( m/z = 431 \) corresponding to the formation of the isomeric benzoate ester. Subsequent treatment with sodium methoxide removed the benzoyl ester, but the target compound (91) could not be isolated, and instead a mixture of compounds was formed (Scheme 28), believed to derive from intramolecular esterification (92). Attempts at reforming the acid functionality were unsuccessful.

Scheme 28. (i) DDQ SET Pmb cleavage: 4 eq. DDQ, 10:1 DCM-water; (ii) 4:1 TFA-water; (iii) MeONa, MeOH.
A dual approach was decided upon to overcome these difficulties: (i) the orthoester removal conditions needed to be properly established in isolation from the acid functionality, and (ii) further study of the allyl ether as a potential route to the acid was pursued. To cover the first point it was decided that the natural Ins(1,3,4,5)P₄ would be synthesised using the same strategies as employed for the synthetic ligand. Allyl ethers are stable towards the orthoester deprotection conditions, so transforming the allyl ether into the acetic acid after phosphorylation of the tetrol would allow the acid to be formed without the risk of intramolecular esterification.

5.2 PhD RESEARCH

5.2.1 Synthesis of myo-inositol tetrol precursor to Ins(1,3,4,5)P₄

The synthesis of Ins(1,3,4,5)P₄ serves two purposes: (i) It allows the deprotection of the orthobenzoate to be studied without the complication of the acid functionality, and (ii) it can be used as a control molecule for the receptor-ligand binding studies.

Billington et al. developed a synthetic route to Ins(1,3,4,5)P₄ from myo-inositol orthoformate.¹³⁵ It was decided to use a modified form of the synthesis to establish reaction conditions for the modified ligand.

Direct di-benzylolation of the orthoformate triol has been reported to produce an unfavourable 1:5 mixture of the 2,6- and 4,6-O-diethers.¹⁵⁸ The first step is therefore to protect the 4-hydroxyl so that only the 2,6-O-dibenzyl ether is formed. Pmb has already been shown to display poor selectivity for only the 4-O position; therefore an alternative temporary protecting group was required. Since the 2-O is not functionalised in the natural product, the
allyl group can be used in this role. Allylation followed by benzylation of the inositol orthobenzoate gave the fully protected inositol (94) in good yield. There are several methods by which the allyl ether can be deprotected, but the chosen method must be orthogonal to the other protecting groups. Gigg et al. showed that allyl ethers of a wide range of myo-inositol compounds may be removed using a relay strategy. The allyl ether is activated for deprotection by isomerising it to the Z-1-propenyl ether with potassium tert-butoxide ('BuOK) in DMSO at elevated temperatures. Isomerisation places the double bond into conjugation with the oxygen atom, creating an enol ether that can then be removed under acid hydrolytic conditions to expose the free hydroxyl (Scheme 30).

Although there was the potential for a ‘one-pot’ reaction to hydrolyse both the 1-propenyl ether (95) and the orthobenzoate using acidic hydrolysis conditions, this was not employed as it would be counter-productive for determining optimum deprotection conditions for the orthobenzoate. Hence the dibenzyl orthobenzoate 96 was isolated first, and then subjected to the literature 4:1 TFA-water acid hydrolysis conditions.
Scheme 31. Synthesis of 2,6-O-dibenzyl-myoinositol. (i) 4:1 TFA-water; (ii) MeONa, MeOH.

Acidic hydrolysis gave a mixture of the 1- and 3-O benzoyl esters. The crude reaction mixture was evaporated to dryness and then taken up in dry methanol. Sodium methoxide was added until pH 11 was obtained. This induced transesterification of the benzoyl ester, leading to the formation of the putative sodium salt of 2,6-O-dibenzyl-myoinositol and methyl benzoate 100 (Scheme 31). To achieve good yields, the reaction mixture was neutralised with hydrochloric acid to convert any inositol alkoxide ions into the tetrol (99), with sodium chloride byproduct. It was discovered that 99 could then be obtained in a quantitative yield, and high purity, using a two step process: (i) after evaporating to dryness, the crude mixture was partitioned between chloroform and water. Because of the hydrophilicity of the tetrol (99), it joins the NaCl in the aqueous phase, while methyl benzoate (100) moves into the organic phase. (ii) The aqueous phase was then evaporated to dryness, and taken up in dry MeOH. The NaCl formed a precipitate, while the tetrol dissolved in the supernatant, allowing filtration to isolate the product with no requirement for further purification.

While the 2,6-O-dibenzyl-myoinositol was being prepared, alternative uses for dibenzyl orthobenzoate compound 96 were explored.
5.2.2 Synthesis of myo-inositol triol precursor to Ins(1,4,5)P₃

The 4-O tert-butyl diphenyl silyl (Tbdps) and tert-butyl dimethyl silyl (Tbdms) derivatives were synthesised by silylation of the remaining axial hydroxyl of 96 (Scheme 32). Both reactions proceeded with complete conversion of the hydroxyl to the corresponding silyl ether; however the Tbdps derivative (101b) was contaminated with TbdpsOH, which could not be readily removed by flash chromatography. This was overcome by the discovery that the TbdpsOH could be sublimed from the product mixture at low pressure and high temperature, to leave a pure sample of the fully protected orthobenzoate 101b.

Scheme 32. Synthesis of 2,6-O-dibenzyl-4-O-(1,3,5-orthobenzoate). (i) 1.5 eq. RCl, 2.2 eq. imidazole, 1 eq. Et₃N, DMF, 100 °C.

Scheme 33. Synthesis of 1,2,6-O-tribenzyl-myoinositol and 2,3,6-O-tribenzyl-myoinositol. (i) 3.5 eq. DIBAL-H, DCM; (ii) 4.75 eq. TBAF, THF. This reaction sequence was carried out by J. Swarbrick.
These compounds were synthesised for collaborative work with Joanna Swarbrick, to investigate the ability of a range of sterically bulky substituents to influence the regioselectivity of DIBAL-H reduction of myo-inositol orthobenzoate compounds.\textsuperscript{141} Treating silyl ethers 101\textsubscript{a} or 101\textsubscript{b} with excess DIBAL-H led to the reduction of the orthobenzoate to a benzyl ether. In both cases a 5:4 mixture of the 1-\textit{O} Bn (102) and 3-\textit{O} Bn (103) compounds was created. The fact that there was no difference between the ratios of products obtained from these two compounds was attributed to repulsion between the bulky 4-\textit{O}-silyl ether and the 5-\textit{O}-DIBAL adduct. This forces the inositol ring into an inverted chair-like conformation similar to the orthoester, which acts to reduce the contrast between the steric environments at the 1- and 3-\textit{O} as the distance between the silyl 4-\textit{O} and 3-\textit{O} acetal linkage is increased (\textbf{Scheme 34}).\textsuperscript{161}

These compounds were separated by flash chromatography, and then reacted with tert-butyl ammonium fluoride (TBAF) to remove the silyl group (\textbf{Scheme 33}, reaction sequence carried out by J. Swarbrick.). Triol 105 has the correct regiochemistry to be a precursor for the synthesis of Ins(1,4,5)P\textsubscript{3}. Like Ins(1,3,4,5)P\textsubscript{4}, this will also be used as a control molecule for the receptor-ligand binding studies.

\begin{center}
\textbf{Scheme 34.} Rationale for the observed poor selectivity of DIBAL-H reduction of 101. For the reaction mechanism see \textbf{Scheme 14}. The chair-like conformation of 107 means there is poor discrimination between the 1-\textit{O} and 3-\textit{O} and DIBAL-H can co-ordinate to either oxygen equally.
\end{center}
### 5.2.3 Synthesis of 2-**O**-allyl-6-**O**-benzyl-**myo**-inositol

**Scheme 35.** Synthesis of 2-allyl-6-**O**-benzyl-**myo**-inositol. (i) 4 eq. CAN, 9:1 MeCN-water; (ii) 4:1 TFA-water; (iii) MeONa, 1:1 CHCl₃-MeOH.

Having established suitable conditions for the synthesis of the **Ins**(1,3,4,5)**P**₄ precursor 2,6-**O**-dibenzyl-**myo**-inositol (99), the same approach was used for the synthesis of 2-**O**-allyl-6-**O**-benzyl-**myo**-inositol (109). The first step was to remove the Pmb group from the fully protected allyl compound (79). CAN removes the Pmb group by a SET mechanism similar to DDQ (see **Scheme 27**), but was found to give the higher yield of the two reagents. This was ascribed to the differences in the work-up procedures for the two reactions. Using the conditions developed for dibenzyl ether 99, the orthobenzoate of inositol alcohol 108 was deprotected to cleanly form 2-**O**-allyl-6-**O**-benzyl-**myo**-inositol 109 (**Scheme 35**).

Once these conditions had been confirmed as a practical route to 109, it was decided to revisit the ‘one-pot’ option to shorten the number of synthetic steps, and potentially increase the overall yield. The initial attempt adopted the same conditions as were previously used for orthobenzoate deprotection: the acidic step seemed to proceed as expected, but when preparing the crude mixture for the methoxide treatment, it formed a gum which was not soluble in methanol. Since this was not observed in the other deprotections it was attributed to the Pmb group. The gum could either be formed of debris from the removal of the Pmb group, or by...
other unwanted by-products. To establish which option was true, a suitable solvent was needed to dissolve the gum. Working through the standard solvents in the laboratory showed it to be most soluble in chloroform. The second step of the deprotection was thus modified so that the crude mixture was taken up in 1:1 chloroform-methanol. Although treating chloroform with sodium methoxide risked the formation of dichlorocarbene, a test reaction gave only the expected products. Using the work-up established for the previous deprotections removed the Pmb debris into the organic phase, along with the methyl benzoate, and gave the tetrol (109) in quantitative yield, and in high purity.

5.2.4 Phosphorylation

The 2-O-allyl-6-O-benzyl-myoinositol, 2,6-O-dibenzyl-myoinositol and 2,3,6-O-tribenzyl-myoinositol were all then ready for phosphorylation. Baudin et al. used N,N-diisopropyl dibenzyl phosphoramidite (section 4.2, 17) in the synthesis of Ins(1,3,4,5)P₄. Using this reagent, activated with 1-H-tetrazole (34), allows the phosphitylation of polyols under mild conditions. Subsequent oxidation of the P(III) centre creates a dibenzyl phospho-triester. Since both the alcohol of the final product and the phosphates are benzyl protected, this allows for a single ‘one-pot’ global deprotection strategy to be employed to create the target compound. It was decided that the synthesis of Ins(1,3,4,5)P₄ would again be used to establish conditions for synthesising the 2-O-acetic acid derivative (1).

The first step was to synthesise the activating agent. Using a Sandmeyer reaction, 5-aminotetrazole monohydrate (110) was treated with hypophosphorus acid and sodium nitrite in an aqueous solution. This initially converts the amine into a nitrosamine (111). The acid then converts the nitrosamine into a diazonium ion (112). Via a radical mechanism the hypophosphorus acid then operates as a reducing agent to remove the diazonium ion and form 34 (Scheme 36).

- 85 -
1-$H$-Tetrazole (34) was initially separated from the crude reaction mixture by trituration in ethyl acetate. The crude heterocycle was then purified by sublimation, using a Kugelröhr heater and a drying piston. Due to the explosive nature of 34 (decomposes rapidly and explosively if heated above its melting point of 155-157 °C), careful control of pressure ($\sim$0.01 mmHg) and temperature ($<$ 130 °C) was required to safely control the deposition of the purified 1-$H$-tetrazole. Additionally only small samples of the crude 1-$H$-tetrazole ($< 2$ g) were purified each time in a purpose-built high-pressure laboratory.

Reaction of 2,6-O-dibenzyl-myoinositol tetrol 99 with $N,N$-diisopropyl dibenzyl phosphoramidite 17 activated by 1-$H$-tetrazole 34 in acetonitrile-dichloromethane created the intermediate tetrakis-phosphite 113. The $N,N$-diisopropyl dibenzyl phosphoramidite was first dissolved in DCM and then added dropwise to the solution in order to avoid increased viscosity during activation of the phosphitylating reagent. It was then necessary to oxidise the P(III) centres up to phosphates. The choice of oxidising reagent was influenced by the functionality in the target molecule. For 114 either $m$CPBA$^{118}$ or $t$BuOOH$^{119}$ can be used (Scheme 37), with $m$CPBA being the better choice since it has been shown to give a cleaner reaction. However in the case of the 2-$O$-allyl derivative there is the potential for additional epoxidation of the double bond in the allyl group.
The tetrakis-phosphitylated myo-inositol compound 113 was oxidised using both mCPBA and tBuOOH. The crude reaction mixtures were then compared by proton decoupled $^{31}$P-NMR which showed the mCPBA oxidation (Figure 37B) to be significantly cleaner, with the four expected peaks from the product clearly visible (-0.05, -0.29, -0.47, -0.80 ppm), and the additional peaks derived from the excess
phosphitylating reagent. The reaction with 1BuOOH (Figure 37A) exhibited a considerable number of extra peaks, arising from side reactions. TLC also implied that, in the case of the 1BuOOH reaction, separation by flash chromatography would prove difficult. It was therefore decided to use mCPBA if possible for all the oxidation steps. Oxidation with mCPBA is assumed to occur through a concerted mechanism similar to the “butterfly mechanism” familiar from its reaction with olefins (Scheme 38).

The final step in the synthesis of Ins(1,3,4,5)P₄ was the global deprotection of the fully benzyl protected precursor 114 (Scheme 39). A wide range of procedures have been developed for the cleavage of benzyl ethers and phosphate esters. The most commonly employed in the synthesis of inositol poly-phosphates are hydrogenolysis conditions using a Pd based catalyst.¹⁶⁶-¹⁶⁸ Attempted deprotection using catalytic 10% Pd on carbon under a 60 psi atmosphere of H₂ only partially removed the benzyl groups, giving a mixture of products. Instead Pd-black (Pd nanoparticles) in tert-butanol-water under ambient pressure of hydrogen,¹⁶⁹ plus 8 equivalents of NaHCO₃ was used to successfully unblock precursor 114. The hydrogen carbonate was added to the reaction in order to neutralise the free acids that are generated during the deprotection, preventing a decrease in pH that can lead to cyclisation and migration of the phosphates.¹⁷⁰ After filtration to remove solids, any chelated Pd²⁺ was removed by passing a concentrated aqueous solution of the Ins(1,3,4,5)P₄ through a DOWEX 50WX8-200 H⁺ column. Acidic fractions from the column were combined and then neutralised with aqueous ammonia before evaporating to dryness to give the Ins(1,3,4,5)P₄ ammonium salt (115) in a quantitative yield.
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Using the conditions established in the synthesis of Ins(1,3,4,5)P₄, Ins(1,4,5)P₃ 117 was synthesised from the 2,3,6-O-tribenzyl-myo-inositol 105 (Scheme 40).

Scheme 40. Synthesis of Ins(1,4,5)P₄ from 2,3,6-O-tribenzyl-myo-inositol. (i) 10 eq. 1-H-tetrazole, 7 eq. 17, 5:2 MeCN-DCM; (ii) 6.5 eq. mCPBA; (iii) 15 eq. Pd-black, 1 atm. H₂, 6 eq. NaHCO₃, 6:1 'BuOH-water.

Because of the potential for the allyl ether being epoxidised by mCPBA during the oxidation of the P(III) centres, oxidation with 'BuOOH was attempted first. The same problems were experienced using this reagent as for the Ins(1,3,4,5)P₄ precursor; however a small sample of the desired product 118 was isolated. Using the established mCPBA conditions, phosphorylation of the 2-O-allyl-6-O-benzyl-
myo-inositol was then attempted (Scheme 41). Since the $^1$H-NMR spectra of the product from this reaction are complex, it was not possible to be certain that this material was purely allyl tetraphosphate 118, and was not contaminated with epoxide 119. For this reason, the allyl ether was explicitly further oxidised with mCPBA to convert all the vinyl functionality to the diastereomeric epoxides. Notably, epoxide 119 was separable from the starting allyl ether by TLC, and it was found that this second oxidation took considerably longer than the phosphite oxidation reaction. Thus, using the epoxide, it was possible to carefully monitor the oxidation step by TLC. The reaction was quenched once full conversion from the tetrakis-phosphites to -phosphates was achieved, but before epoxide formation became detectable. Following the reaction in this manner meant that the desired 1,3,4,5-O-tetrakis(dibenzylxyrophosphoryl)-2-O-allyl-6-O-benzyl-myoinositol 118 was formed, with NMR confirming that there was no formation of the epoxidised side product 119.

**Scheme 41.** Phosphorylation of 2-O-allyl-6-O-benzyl-myoinositol. (i) 14 eq. 1-H-tetrazole, 7 eq. 17, 5:2 MeCN-DCM; (ii) 14 eq. mCPBA, 1 h; (iii) 14 eq. 'BuOOH; (iv) 3 eq. mCPBA, CHCl$_3$, overnight.

Hydrogenolysis of 118 using the previously established conditions (Scheme 42) removed the benzyl protecting groups as expected, and also reduced the allyl ether.
to a propyl moiety. This compound is useful for the receptor-ligand binding studies as the propyl group should enter the binding pocket in the same way as the acid. However, since it is not charged there can be no polar group exchange interaction. Hence, 120 acts as a control molecule to explore whether binding of 1 is due to polar interactions rather than steric complementation.

![Scheme 42](image)

Scheme 42. Global deprotection and reduction of the allyl ether to form 2-O-propyl-Ins(1,3,4,5)P₄. (i) 20 eq. Pd-black, 1 atm. H₂, 8 eq. NaHCO₃, 6:1 tBuOH-water.

It was now possible to explore potential routes to the 2-⁰-acetic acid from the allyl ether 118.

5.2.5 2-⁰-Acetic acid formation

![Scheme 43](image)

Scheme 43. Oxidation of the allyl ether: (i) to the 2-⁰-acetic acid by ozonolysis with an oxidative work-up, and (ii) to the 2-⁰-acetaldehyde by ozonolysis with a reductive work-up.

It was decided to initially revisit ozonolysis as a way of accessing the acetic acid moiety (Scheme 43). Using an oxidative work-up should provide a direct route to
the acid (121).\textsuperscript{150} Mass spectrometry of the crude product mixture from this reaction revealed the presence of the desired product. However, \textsuperscript{31}P-NMR showed the presence of several additional phosphorylated species, and there was poor mass recovery, so a stepwise approach was attempted instead. Ozonolysis with a reductive work-up to form the aldehyde 122 appeared to proceed more cleanly, and provided a pure enough sample to explore its oxidation up to the acid. However, once more the mass recovery was poor. It was then noted that ozonolysis has been reported to remove benzyl ethers (Scheme 44).\textsuperscript{171} Although the conditions reported (0°C) are far more forcing than those used here (-78°C), the benzyl phosphate esters may be more reactive than benzyl ethers as the benzyl C-H is more polarised by the electron withdrawing phospho-triester. Partial deprotection of the phosphates would then explain the poor mass recovery, as any product could have been water-soluble, and would certainly have a low R\textsubscript{f}. Therefore an alternative approach was needed.

Based on the earlier attempts to oxidize allyl ethers during the MRes phase of this research, osmium tetroxide was used to catalyse the cis-dihydroxylation of the allyl ether of tetraphosphate 118. Sodium periodate was then used to oxidatively cleave the glycer-1-yl diol, to create aldehyde 122 successfully and with good mass recovery (Scheme 45). These two reactions were telescoped in order to minimise compound lost during any intermediate work-up.
A mild oxidising agent is required to convert the aldehyde into the acid without concomitant side reactions with the other functionalities around the inositol ring. Poly-phospho-triesters are particularly sensitive to nucleophiles, so care needs to be taken when choosing a suitable reagent. The Pinnick reaction is a very mild oxidation of aldehydes to carboxylic acids, which uses sodium chlorite in a sodium dihydrogen phosphate buffered solution in the presence of 2-methyl-2-butene. The sodium chlorite acts as the oxidising agent, while the 2-methyl-2-butene acts as a chlorine scavenger. Treatment of aldehyde 122 with the Pinnick reagents readily converted it into the desired nona-benzyl protected 2-O-acetic acid Ins(1,3,4,5)P₄ derivative (121, Scheme 46).

Scheme 46. Synthesis of 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2-O-acetic acid-6-O-benzyl-myoinositol. Pinnick conditions: NaClO₂, aq. NaH₂PO₄, 2-methyl-2-butene, 'BuOH.

The final step was the global deprotection to remove the benzyl groups. This was carried out under the same conditions as for Ins(1,3,4,5)P₄, and gave 2-O-acetic acid-myoinositol-1,3,4,5-O-tetraphosphate 1 in quantitative yield (Scheme 47). ³¹P-NMR originally exhibited 8 peaks, suggesting two species. This was attributed to the protonated and deprotonated forms of the acid. Addition of deuterated acetic acid to the deuterium oxide NMR solvent caused the secondary peaks to disappear as the 2-O-acetic acid was converted into only its protonated form.
5.2.6 Synthesis 2-\(O\)-(2-hydroxyethyl) derived ligands

Using acetaldehyde tetraphosphate 122, the synthesis of alternative Ins(1,3,4,5)P\(_4\) analogues was explored. It was hoped that the aldehyde 122 could be reduced to the corresponding alcohol 128 (Scheme 48) which could then be activated under Mitsunobu conditions to be displaced by a range of nucleophiles. The choice of reducing agent was important so that the phosphates were not reduced. Sodium borohydride (NaBH\(_4\)) was considered too nucleophilic a reducing agent, so milder reagents were studied. Sodium cyanoborohydride is familiar for converting imines to amines under mildly acidic conditions.\(^{174}\) However it can also be used to reduce aldehydes and ketones.\(^{174}\) The reaction required careful monitoring because leaving the reaction too long led to overreaction, with trace levels H-phosphonates observable in the \(^{31}\)P-NMR spectrum of the crude product. However, too short a time and the reaction does not go too completion, but reaction progress is difficult to monitor by TLC. Although a range of solvent systems were investigated, obtaining a good separation of the spots corresponding to starting aldehyde 122 and alcohol 128 was not achieved. Thus a chemoselective stain was used to estimate the extent of reaction. 2,4-Dinitrophenylhydrazine (2,4-DNP) selectively stains compounds containing aldehyde or ketone moieties, to give an orange spot. Following the decrease in intensity, and eventual disappearance, of this spot allowed the reaction to be quenched when all of the starting aldehyde had been consumed.
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Scheme 48. Reduction of the 2-O-acetaldehyde to a 2-O-(2-hydroxyethyl) ether.

(i) 1 eq. NaBH₃CN, acetic acid, MeOH.

Hydroxyethyl tetraphosphate 128 was then globally deprotected to give the 2-O-(2-hydroxyethyl)-myo-inositol-1,3,4,5-O-tetraphosphate 129 (Scheme 49). Similar to the propyl analogue (120), this compound was also designed to act as a control molecule to establish if any observed interaction between 1 and the E17A mutant of the PH domain of PKB could be due to polar interactions rather than steric complementation.

Scheme 49. Global deprotection to form 2-O-(2-hydroxyethyl)-Ins(1,3,4,5)P₄.

(i) 20 eq. Pd-black, 1 atm. H₂, 8 eq. NaHCO₃, 6:1 tBuOH-water.

Conversion of the hydroxyl of the nona-benzyl derivative 128 to nitrogen based functionalities was now addressed. The first target considered was 2-O-(2-aminoethyl)-myo-inositol-1,3,4,5-O-tetraphosphate 139. The E17K mutant of PKBα’s PH domain suggests that the presence of a positively charged functionality on the ligand could elicit an interaction with the E17A mutant. Additionally, although the R86A mutant was discounted as being the less favourable of the two possible polar group exchange mutations (see section 2), the 2-aminoethyl derivative is also a potential ligand if the initially preferred E17A interaction is unsuccessful.

Amines can be synthesised by hydrogenolysis of an azide precursor. The most common approach for the conversion of hydroxyls into azides involves a two step protocol: (i)
Transforming the alcohol into a suitable leaving group, such as a sulfonate or halide; then (ii) displacement of the leaving group by nucleophilic substitution with the azide anion.\textsuperscript{175} When this was attempted with the 2-hydroxyethyl tetrakisphosphate \textit{128}, the mesylate (\textit{130}) was successfully formed, although in low yield (31\%). Subsequent displacement of the mesylate by sodium azide was not observed (Scheme \textit{50}). Forcing conditions significantly above room temperature were not employed to drive the azidation due to concerns over the stability of the protected phosphates.

Alternative routes to the azide were explored in parallel to the mesylation experiments. Alkyl azides can be formed in a single reaction using a modification of the Mitsunobu reaction. The Mitsunobu reaction is commonly used to convert primary and secondary alcohols into a wide variety of other functionalities.\textsuperscript{176} The alcohol is first activated \textit{in situ} as a leaving group by reacting it with a phosphonium intermediate generated from triphenylphosphine (PPh\textsubscript{3}) and diethyl azodicarboxylate (DEAD) or the less toxic \textit{diisopropyl} azodicarboxylate (DIAD, \textit{133}), and is then displaced by a nucleophile with a \textit{pK}_a of 10 or less (Scheme \textit{51}). This approach has been employed to produce azides using hydrazoic acid (HN\textsubscript{3}) as the acidic nucleophile, but HN\textsubscript{3} is a volatile and extremely explosive liquid at room temperature and pressure, as well as being highly toxic. Therefore a related route was required that did not use HN\textsubscript{3}. 
Diphenylphosphoryl azide (DPPA, 132) has been used in Mitsunobu azidations as an alternative source for the azide ion. The success of Mitsunobu reactions has been shown to be highly dependent upon the order of addition. First the DIAD was added to a solution of PPh₃ in tetrahydrofuran (THF) to form the betaine intermediate 134. Then the hydroxyethyl compound (128) was added to the solution, converting the alcohol into a leaving group. Finally the DPPA was added to the solution, where the azide ion displaced the leaving group and formed the 2-O-(2-azidoethyl) compound (131, 48%).

**Scheme 51.** Reaction mechanism for the modified Mitsunobu azidation reaction. R is the nona-benzyl functionalised inositol ring.

Hydrogenolysis of fully protected 2-azidoethyl tetrakisphosphate 131 served two roles: As with the other ligands it globally deprotected the benzyl ethers, but in addition to this it also reduced the azide to an amine. Mass spectrometry of the isolated product showed the major peak to be the desired molecular ion. $^{31}$P-NMR of the sodium chelated compound showed four peaks, but the Ins-$H$ signals all occur in the region 3.5-4.5 ppm, and the overlap made designation of individual resonances difficult. Previously, conversion of the sodium salt to an ammonium salt overcame this problem. However in this case the $^1$H-NMR was broad and the $^{31}$P-NMR suggested...
the presence of multiple phosphorylated species. These were attributed to the compound being in several different protonation and metal chelation states.

![Diagram](image)

**Scheme 52.** Global deprotection to form 2-O-(2-aminoethyl)-Ins(1,3,4,5)P₄.
(i) 20 eq. Pd-black, 1 atm. H₂, 8 eq. NaHCO₃, 6:1 'BuOH-water.

Based on the success of the Mitsunobu reaction it was decided to also synthesise the guanidinium based analogue. To insert the guanidinium functionality into the compound, a suitably protected form of this group must first be synthesised so that only one of the nitrogen centres undergoes reaction, and the product is easily handleable. Dodd and Kozikowski reported the synthesis of a range of protected guanidines from the corresponding protected S-methylisothiourea for use in Mitsunobu reactions.¹⁷⁹ Consideration of the available starting materials, and the conditions used in the final deprotection, suggested the carboxybenzyl (Cbz) protected form (140) as being the most suitable, as the hydrogenolysis step would also be expected to deprotect it.

![Diagram](image)

**Scheme 53.** Synthesis of N,N'-bis(benzyloxycarbonyl)guanidine. (i) NH₃, MeOH.

Reacting *isothiourea* 139 with ammonia in methanol rapidly formed the desired bis-Cbz guanidine 140 (Scheme 53). In an improvement on the published procedure, this was easily isolated by recrystalisation of the crude product in dichloromethane-methanol. Using the conditions already established for the Mitsunobu azidation, the protected guanidinium (140) was reacted with hydroxyethyl ether 128 (Scheme 54).
While the $^1$H and $^{31}$P-NMR of the crude reaction mixture showed full conversion of the hydroxyethyl ether (128) to the protected guanidine (141), all attempts at purifying the product were unsuccessful. TLC showed only one spot after flash chromatography, but $^{31}$P-NMR showed trace amounts of other phosphorus species attributable to side reactions. Additional purification using high performance liquid chromatography (HPLC) failed to fully remove these impurities. Because of this the final global deprotection (Scheme 55) was not attempted.
5.3 CHARACTERISATION OF THE SYNTHETIC LIGANDS

5.3.1 myo-Inositol orthobenzoates

![Structure of 76](image)

Figure 37. Structure of 76 with the "w" $^4J_{1,5}$ coupling highlighted in red. There are three other "w" couplings between the five equatorial protons - $^4J_{1,3}$, $^4J_{3,5}$ and $^4J_{4,6}$.

<table>
<thead>
<tr>
<th>Inositol proton</th>
<th>$^3J$ coupling</th>
<th>Number of additional $^4J$ &quot;w&quot; couplings</th>
<th>Observed Splitting Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dd</td>
<td>2 ($^4J_{1,3}$, $^4J_{1,5}$)</td>
<td>dq (3, 1.5 Hz)</td>
</tr>
<tr>
<td>2</td>
<td>t</td>
<td>0</td>
<td>t  (1.5 Hz)</td>
</tr>
<tr>
<td>3</td>
<td>dd</td>
<td>2 ($^4J_{1,3}$, $^4J_{3,5}$)</td>
<td>dq (3, 1.5 Hz)</td>
</tr>
<tr>
<td>4</td>
<td>t</td>
<td>1 ($^4J_{4,6}$)</td>
<td>td (3, 1.5 Hz)</td>
</tr>
<tr>
<td>5</td>
<td>t</td>
<td>2($^4J_{1,5}$, $^4J_{3,5}$)</td>
<td>tt (3, 1.5 Hz)</td>
</tr>
<tr>
<td>6</td>
<td>t</td>
<td>1 ($^4J_{4,6}$)</td>
<td>td (3, 1.5 Hz)</td>
</tr>
</tbody>
</table>

Table 2. Expected splitting patterns for each Ins-H based on $^3J$ coupling, and observed splitting pattern because of long range "w" couplings.

Orthoesters fix the inositol ring into a rigid conformation. Like myo-inositol itself, the inositol orthobenzoate (43c) possesses $\sigma_h$ symmetry. Alkylation of the 4-O
breaks this symmetry. The combination of a fixed conformation and the lack of symmetry often allow the substitution patterns around the inositol ring of orthoesters to be unambiguously defined by nuclear magnetic resonance (NMR). Not only does NMR strongly reflect the altered stereochemistry of 76 compared to inositol orthobenzoate 43c, the asymmetry places all six hydroxyls in different chemical environments. The restricted conformation means that up to four long range 4-bond “w” couplings can be observed (Figure 37). The actual and observed couplings are dependent upon similarities in chemical environments, and the resolution of the NMR machine used.

This is demonstrated by considering each inositol proton in turn (Table 2). Ins 1-H is split by the equatorial Ins 6-H (~3 Hz) and the axial Ins 2-H (~1.5 Hz). This would be expected to give a double doublet (dd). However, the additional long range $^4J_{1,3}$ and $^4J_{1,5}$ “w” coupling further split the signal for Ins 1-H (~1.5 Hz). This would be expected to give a double double double doublet (dddd), but because $^4J_{1,3} \approx^4J_{1,5} \approx^3J_{1,2}$, and the NMR machine cannot resolve the slight differences that exist, so a double quartet (dq) is usually observed. The same argument can be applied to Ins 3-H. Ins 2-H is the only inositol proton in an axial position, so there is no potential for “w” coupling. This means it only experiences coupling (~1.5 Hz) to Ins 1-H and Ins 3-H, and since $^3J_{1,2} \approx^3J_{2,3}$ an apparent triplet (t) is observed.

In the case of Ins 4-H, it is split by the equatorial Ins 3-H and Ins 5-H. Since its geometrical relationships to both these protons are very similar $^3J_{3,4} \approx^3J_{4,5}$ and this gives an apparent t (~3 Hz). The additional $^4J_{4,6}$ “w” coupling (~1.5 Hz) in asymmetrical orthoesters then splits this into a triple doublet (td). Very similar geometry also explains the observed splitting pattern for Ins 6-H. The Ins 5-H is observed as a triple triplet (tt). This arises from $^3J_{4,5} \approx^3J_{5,6}$ (~3 Hz) creating a large initial t, and then $^4J_{1,5} \approx^4J_{3,5}$ (~1.5 Hz) which splits each peak further leading to the formation of an observed tt.

If the NMR is carried out in DMSO, it is possible to also observe any free hydroxyl as a d around 5 ppm. The d arises from coupling (~6 Hz) between the proton of the alcohol and the adjacent proton on the inositol ring. In the case of 76 this means Ins 2-H is further split into a double triplet (dt) and the Ins 4-H becomes a double triple doublet (ddtd), enabling differentiation between the Ins 4- and 6-H.
5.3.2 Allyl ethers

The $^1$H-NMR spectra of allyl compounds (Figure 38) exhibit a characteristic ABMXY splitting pattern (Table 3). $H_A$ and $H_B$ give a complex signal. Each proton is split into a $d$ by geminal coupling to the other ($\sim 13$ Hz). This $d$ is then split again by $H_M$ ($\sim 5$ Hz) to create a ddd. Finally there is long range couplings to $H_X$ and $H_Y$ ($\sim 1.5$ Hz), creating two sets of double double triplets (ddt). Because $H_A$ and $H_B$ are in similar chemical environments their signals occur at a similar chemical shift, with the roofing effect further complicating the pattern of observed peaks. $H_M$ is split into a $d$ from trans coupling to $H_X$ ($\sim 17$ Hz), into a $d$ from cis coupling to $H_Y$ ($\sim 10$ Hz), and into a further $t$ by coupling to $H_A$ and $H_B$ ($\sim 5$ Hz), creating an observable ddt. Similarly $H_X$ and $H_Y$ both produce ddt or a dq from $^3J$ coupling to $H_M$, vinylic geminal coupling to each other ($\sim 1.5$ Hz) and then long range coupling to $H_A$ an $H_B$ ($\sim 1.5$ Hz).

![Figure 38](image)

**Figure 38.** Structure of an allyl ether with each proton designated in the ABMXY nomenclature.

<table>
<thead>
<tr>
<th>Proton</th>
<th>Coupling</th>
<th>Observed Splitting Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$^2J_{A,B}$, $^3J_{A,M}$, $^4J_{A,X}$ ≈ $^4J_{A,Y}$</td>
<td>ddt (13, 5, 1.5 Hz)</td>
</tr>
<tr>
<td>B</td>
<td>$^2J_{A,B}$, $^3J_{B,M}$, $^4J_{B,X}$ ≈ $^4J_{B,Y}$</td>
<td>ddt (13, 5, 1.5 Hz)</td>
</tr>
<tr>
<td>M</td>
<td>$^3J_{A,M}$ ≈ $^3J_{B,M}$, $^3J_{M,X}$, $^3J_{M,Y}$</td>
<td>ddt (17, 10, 5 Hz)</td>
</tr>
<tr>
<td>X</td>
<td>$^2J_{X,Y}$, $^3J_{M,X}$, $^4J_{A,X}$ ≈ $^4J_{B,X}$</td>
<td>ddt/dq (17, 1.5, 1.5 Hz)</td>
</tr>
<tr>
<td>Y</td>
<td>$^2J_{X,Y}$, $^3J_{M,Y}$, $^4J_{A,Y}$ ≈ $^4J_{B,Y}$</td>
<td>ddt/dq (10, 1.5, 1.5 Hz)</td>
</tr>
</tbody>
</table>

**Table 3.** Observed splitting pattern arising from allyl ether ABMXY splitting pattern, with real $^1$H-NMR examples.
5.3.3 Inositol tetrols

Removal of the orthobenzoate protecting group creates an inositol tetrol (Figure 39). Since the conformation is no longer fixed by the protecting group, the compound adopts the lowest energy conformation – a chair conformation with the 1-, 3-, 4- and 5-hydroxyls and 6-O substituent positioned equatorially, and the 2-O substituent in an axial position. Compared to the inositol orthoester conformation, the inositol protons are now in different environments.

![Figure 39. Structure of myo-inositol tetrol.](image)

<table>
<thead>
<tr>
<th>Inositol proton</th>
<th>$^3J$ coupling</th>
<th>Observed Splitting Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$^3J_{1,2}, ^3J_{1,6}$</td>
<td>$dd$ (10, 2.5 Hz)</td>
</tr>
<tr>
<td>2</td>
<td>$^3J_{1,2} \approx ^3J_{2,3}$</td>
<td>$t$ (2.5 Hz)</td>
</tr>
<tr>
<td>3</td>
<td>$^3J_{2,3}, ^3J_{3,4}$</td>
<td>$dd$ (10, 2.5 Hz)</td>
</tr>
<tr>
<td>4</td>
<td>$^3J_{3,4} \approx ^3J_{4,5}$</td>
<td>$t$ (10 Hz)</td>
</tr>
<tr>
<td>5</td>
<td>$^3J_{4,5} \approx ^3J_{5,6}$</td>
<td>$t$ (10 Hz)</td>
</tr>
<tr>
<td>6</td>
<td>$^3J_{1,6} \approx ^3J_{5,6}$</td>
<td>$t$ (10 Hz)</td>
</tr>
</tbody>
</table>

Table 4. Observed proton splitting patterns for inositol tetrols due to $^3J$ couplings. The representative peaks are taken from real spectra, because of this in some cases peaks overlap. To assist visualising the splitting patterns the red lines illustrate where an obscured peak would be expected to occur.
The Ins 1-\(H\) is initially split into a \(d\) by the anti-periplanar \(3J_{1,6}\) coupling (~10 Hz). This \(d\) is then split by the smaller gauche \(3J_{1,2}\) coupling (~2.5 Hz) to form a \(dd\). The Ins 2-\(H\) is split onto an observed \(t\) due to two approximately equal gauche \(3J\) couplings. The Ins 3-\(H\) forms a \(dd\) for the same geometric reasons as the Ins 1-\(H\). The Ins 4-\(H\), Ins 5-\(H\) and Ins 6-\(H\) inositol protons all give an observed \(t\). The protons are split by two approximately equal anti-periplanar \(3J\) couplings (~10 Hz).

### 5.3.4 Inositol poly-phosphates

The introduction of the P atoms into the myo-inositol compounds provides a useful analytical tool for confirming the substitution pattern around the myo-inositol ring. \(^{31}\)P has a spin \(\frac{1}{2}\) nucleus and is the only stable phosphorus isotope, so is 100% abundant. \(^{31}\)P-NMR confirms the number of phosphates on the ring. Additionally, 3-bond \(^1H-^{31}P\) coupling is observed in the \(^1H\)-NMR spectra, and \(^{13}C-^{31}P\) coupling is observed in the \(^{13}C\)-NMR spectra. This additional coupling assists in confirming the regiochemistry of phosphorylation in the final compounds (Table 5). The \(^1H-^{31}P\) coupling is similar enough in magnitude to the myo-inositol anti-periplanar \(3J_{HH}\) constant that the observed splitting pattern is increased by an order of one. Thus, observed in the range of Ins(1,3,4,5)P\(_4\) derivatives prepared above, the Ins 1-\(H\) is initially split into a \(d\) by the anti-periplanar \(3J_{1,6}\) coupling (~10 Hz), now forms a \(t\) due to the additional \(3J_{PH}\) coupling. This \(t\) is then split by the smaller gauche \(3J_{1,2}\) coupling (~2.5 Hz) to form a \(td\). Since the 2-\(O\) is not phosphorylated, the Ins 2-\(H\) experiences no \(3J_{PH}\) coupling, resulting in only a \(t\) (~2.5 Hz) as observed for the tetrols, from the two approximately equal gauche \(3J_{HH}\) couplings. The Ins 3-\(H\) forms a \(td\) for the same reasons as the Ins 1-\(H\). Both the Ins 4-\(H\) and Ins 5-\(H\) inositol protons give quartets (\(q\)); in each case the proton is split into a \(t\) by two approximately equal anti-periplanar \(3J_{HH}\) couplings (~10 Hz), and then into the \(q\) by the \(3J_{PH}\) coupling. Like the Ins 2-\(H\), the Ins 6-\(H\) does not experience \(3J_{PH}\) coupling, so only a \(t\) from the two anti-periplanar \(3J_{HH}\) couplings (~10 Hz) is seen.
<table>
<thead>
<tr>
<th>Inositol proton</th>
<th>$J_{HH}$ coupling</th>
<th>$^3J_{PH}$ couplings</th>
<th>Observed Splitting Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$^3J_{1,2}$, $^3J_{1,6}$</td>
<td>$^3J_{PH} \approx ^3J_{1,6}$</td>
<td>$td$ (10, 2.5 Hz)</td>
</tr>
<tr>
<td>2</td>
<td>$^3J_{1,2} \approx ^3J_{2,3}$</td>
<td>-</td>
<td>t (2.5 Hz)</td>
</tr>
<tr>
<td>3</td>
<td>$^3J_{2,3}$, $^3J_{3,4}$</td>
<td>$^3J_{PH} \approx ^3J_{3,4}$</td>
<td>$td$ (10, 2.5 Hz)</td>
</tr>
<tr>
<td>4</td>
<td>$^3J_{3,4} \approx ^3J_{4,5}$</td>
<td>$^3J_{PH} \approx ^3J_{3,4} \approx ^3J_{4,5}$</td>
<td>q (10 Hz)</td>
</tr>
<tr>
<td>5</td>
<td>$^3J_{4,5} \approx ^3J_{5,6}$</td>
<td>$^3J_{PH} \approx ^3J_{4,5} \approx ^3J_{5,6}$</td>
<td>q (10 Hz)</td>
</tr>
<tr>
<td>6</td>
<td>$^3J_{1,6} \approx ^3J_{5,6}$</td>
<td>-</td>
<td>t (10 Hz)</td>
</tr>
</tbody>
</table>

Table 5. Observed proton splitting patterns in Ins(1,3,4,5)P$_4$, its 2-O functionalised analogues and their fully protected precursors, due to $J_{HH}$ and $^3J_{PH}$ couplings. The representative peaks are taken from real spectra, because of this in some cases peaks overlap. To assist visualising the splitting patterns the red lines illustrate where an obscured peak would be expected to occur.

When combined with 2-D correlation spectroscopy (COSY) $^1$H-NMR, the splitting patterns arising from the presence of $^{31}$P allows unambiguous designation of each peak. While the $^3J_{PH}$ coupling is useful in the final compounds, it is rather less so for the benzyl protected forms where the CH$_2$s of eight of the benzyl ethers also experience this coupling. Since these protons resonate in a similar range to the inositol protons (3.5-4.5 ppm) the additional coupling acts to further complicate already complex spectra.
Inositol carbon & $^2J_{PC}$ coupling & $^3J_{PC}$ coupling & Observed Splitting Pattern \\
1 & $^2J_{1-P,1-C}$ & - & d \\
2 & - & - & \\
3 & $^2J_{3-P,3-C}$ & $^3J_{4-P,3-C}$ & dd \\
4 & $^2J_{4-P,4-C}$ & $^3J_{4-P,4-C}$ & dt or ddd \\
5 & $^2J_{5-P,5-C}$ & $^3J_{4-P,5-C}$ & dd \\
6 & - & $^3J_{1-P,6-C}$, $^3J_{5-P,6-C}$ & dd \\

Table 6. Observed $^{13}$C splitting patterns in Ins(1,3,4,5)P$_4$, and its 2$^-$$^3$O functionalised analogues due to $^2J_{PC}$ and $^3J_{PC}$ couplings.

In addition to the $^1$H-$^3$P couplings in $^1$H-NMR, $^{13}$C-NMR exhibits $^1$H-$^3$C-$^3$P coupling for both the protected and deprotected families of phosphorylated compounds. Both $^2J_{PC}$ and $^3J_{PC}$ couplings can be observed in these spectra. Similar to the $^1$H-$^3$P couplings, when the complications arising from the presence of the benzyl protecting groups are removed, these couplings can also provide a convenient analytical technique for assigning each of the inositol carbons (Table 6). Ins 1-C is split by short range $^2J_{PC}$ coupling (~5.5 Hz) to the phosphorus attached to the 1-O, giving rises to a d. In the case of the Ins 2-C a t or dd would be expected from the two longer range $^2J_{1-P,2-C}$ and $^3J_{3-P,2-C}$ couplings. However, only a singlet (s) peak is observed, a fact that is not easily explained by a Karplus relationship, which for $J_{PC}$ coupling is not fully understood. A dd is observed for the Ins 3-C signal, first from splitting due to $^2J_{3-P,3-C}$ coupling (~6 Hz) then additional splitting arising from a smaller $^3J_{4-P,3-C}$ coupling (~2 Hz). Ins 4-C is observed as either a dt or a ddd, since it is split by the $^2J_{4-P,4-C}$ coupling (~6 Hz), and then by two $^2J_{PC}$ couplings (~3.5 Hz). The Ins 5-C again exhibits a dd for the same reasons as the Ins 3-C. The Ins 6-C is split by two unequal $^3J_{PC}$ couplings (~6 Hz and ~2.5 Hz) so forms a dd.

These general relationships were verified by 2-D NMR techniques such as heteronuclear single quantum correlation (HSQC) $^1$H-$^1$C-NMR.
Having confirmed that the target compounds had been correctly synthesised, the final consideration was the effect of functionalizing the 2-0 on the conformation of the inositol ring. Although there have not been any published studies that specifically identify the relationship between the magnitude of the $^{2}J_{PC}$ and $^{3}J_{PC}$ coupling constants and the conformation of the myo-inositol ring, a relationship between these two parameters has been established for studying the conformation of nucleic acids (NA). Assuming that a similar relationship does exist for the myo-inositol compounds, it is reasonable to assume that compounds sharing similar $^{2}J_{PC}$ and $^{3}J_{PC}$ coupling constants also share a similar conformation, and this assumption is reinforced by the $^{1}$H-NMR. Comparing these values for the three synthetic ligands to those of Ins(1,3,4,5)P$_{4}$ (Table 7) shows that all three compounds share similar values to the natural ligand, with only the 2-O-acetic acid exhibiting slight variances. Because of this, combined with the $^{1}$H-NMR data, it appears that substitution at the 2-O does not cause the myo-inositol ring to adopt a significantly different conformation to Ins(1,3,4,5)P$_{4}$, which could reduce binding to the PH domain of PKB.

<table>
<thead>
<tr>
<th>Inositol carbon</th>
<th>Ins(1,3,4,5)P$_{4}$</th>
<th>1-0-acetic acid</th>
<th>2-0-propyl</th>
<th>2-0-(2-hydroxyethyl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.5 Hz</td>
<td>5.3 Hz</td>
<td>5.4 Hz</td>
<td>5.4 Hz</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>5.6, 2.1 Hz</td>
<td>5.8, 2.0 Hz</td>
<td>5.9, 2.1 Hz</td>
<td>6.0, 1.9 Hz</td>
</tr>
<tr>
<td>4</td>
<td>6.7, 4.1, 2.5 Hz</td>
<td>5.9, 4.6 Hz</td>
<td>6.0, 3.3 Hz</td>
<td>5.8, 2.9 Hz</td>
</tr>
<tr>
<td>5</td>
<td>6.0, 3.1 Hz</td>
<td>6.2, 3.1 Hz</td>
<td>6.8, 3.0 Hz</td>
<td>6.3, 2.8 Hz</td>
</tr>
<tr>
<td>6</td>
<td>5.8, 2.8 Hz</td>
<td>5.2, 2.2 Hz</td>
<td>6.3, 2.8 Hz</td>
<td>6.0, 2.8 Hz</td>
</tr>
</tbody>
</table>

Table 7. $J_{PC}$ coupling constants for Ins(1,3,4,5)P$_{4}$ and its 2-O functionalised analogues.
6. PROTEIN EXPRESSION AND BIO-PHYSICAL TESTING

6.1 PROTEIN EXPRESSION

Once the synthesis of the ligands had been achieved, samples of the wild-type and E17A mutant of the PH domain of PKB\(\alpha\) were required for bio-physical testing to study any receptor-ligand interactions which maybe occurring.

Bacterial plasmids naturally occur in *Escherichia coli* (*E. coli*). They are self-replicating, circular, extrachromosomal DNA molecules that can confer new characteristics to the bacteria, such as antibiotic resistance or production of restriction enzymes. Because of this, engineered plasmids are often used for the production of unnatural proteins.

It is possible to express target proteins by introducing a plasmid containing the sequence for the desired protein into *E. coli*. A versatile technique for the expression, purification and detection of fusion proteins is the glutathione-S-transferase (GST) gene fusion system. By inserting a gene or gene fragment into the multiple cloning site (MCS) of a pGEX vector (Figure 40) it is possible to construct GST fusion proteins for synthesis in *E.coli*. The vector can be split into five main regions:

(i) The *tac* promoter (Ptac): This is a hybrid promoter, constructed from sequences of the trp and the lac UV5 promoters, to induce expression of the GST fusion protein. Ptac directs transcription approximately 11 times more efficiently than the de-repressed parental lac UV5 promoter and approximately 3 times more efficiently than the trp promoter in the absence of the trp repressor.

(ii) The gene encoding for the GST tag: The tag is roughly 26 KDa in size, and allows separation and purification of GST fusion proteins by binding to agarose beads coated with glutathione (known as GST beads).

(iii) The MCS: This is a sequence of DNA encoded with the recognition sequence for a site-specific cleavage (in the case of pGEX-4T-1 a thrombin cleavage site) followed by a short segment containing many restriction sites for the introduction of the sequence for the desired protein.
(iv) The gene for ampicillin resistance: Transformed bacteria, unlike their untransformed counterparts, continue to grow on a medium containing the antibiotic ampicillin, allowing isolation and growth of only cells that have successfully been transformed.

(v) The lac I\(^{\text{E.coli}}\) repressor gene: The lac I repressor protein binds to the lac UV5 promoter region of Ptac, thus repressing its expression. Expression of Ptac is induced by the lactose analogue \textit{iso}propyl \textbeta-D thiogalactoside (IPTG). IPTG works by displacing the lac I repressor protein, allowing expression of Ptac and inducing the expression of the GST fusion protein.

![Figure 40. pGEX-4T-1 vector. Showing the regions for: (i) the \textit{lac} promoter; (ii) the GST tag; (iii) the MCS containing the thrombin cleave site, and highlighting the BamH1 and EcoR1 restriction sites; (iv) the ampicillin resistance gene; and (v) the lac I\(^{\text{E.coli}}\) repressor gene.](image)

The pGEX-4T-1 vector containing the cloned human PKB\(\alpha\) PH domain (pGEX-PKB\(\alpha\)-PH) was kindly donated by the Alessi group, and Dr. P. Free produced the mutant pGEX-PKB\(\alpha\) PH domain containing plasmid (pGEX-E17A) by using a Quickchange II Site-Directed Mutagenesis Kit (\textit{Stratagene}) to perform in \textit{vitro} site-specific mutations using the pGEX-PKB\(\alpha\)-PH vector as a template for the polymerase chain reaction (PCR). Confirmation of the accurate mutation and transformation was then obtained through high-resolution electrophoretic gel
dsDNA sequencing (carried out by Dr. P. Free, Figure 41). For pGEX-PKBα-PH position 56 shows the appropriate adenosine (A) base, while position 55 of pGEX-E17A shows the cytosine (C) mutant base. The shift from 56 to 55 does not show an alteration in the actual base positions but instead reflects the differences in numbering by the sequencing display program.

A: pGEX-PKBα-PH

B: pGEX-E17A

Figure 41. High-resolution electrophoretic gel dsDNA sequencing, provided by Dr. P Free. A pGEX-PKBα-PH: position 56 shows the appropriate adenosine (A) base (circled in red). B pGEX-E17A: position 55 of shows the cytosine (C) mutant base (circled in red).

Initially protein expression was attempted using the BL21 strain of E.coli. It is a protease-deficient E.coli host strain ideal for optimal expression of recombinant proteins. The BL21 E.coli were transformed with either the pGEX-PKBα-PH or pGEX-E17A plasmid by electroporation, and grown on ampicillin LB/Agar plates. The transformed cells were then transferred in to lysogeny broth (LB) media containing ampicillin. The transformed bacteria were able to grow due to the plasmid-conferred ampicillin resistance, while the non-transformed bacteria could not survive in those conditions. The potential for protein expression is at a maximum during the mid-log phase, and is before overcrowding and nutrient competition cause the bacteria to enter their stationary phase. Once the E.coli had grown to mid-log phase, treatment with IPTG induced expression of the GST fusion protein. After a suitable expression period, the fusion protein was then purified from bacterial lysates by immobilized glutathione affinity chromatography. This purification process preserves protein antigenicity and function.
Protein behavior during purification chromatography with a gel depends upon its size, charge and shape, as well as the pore size of the gel. It is possible to simplify these parameters and allow separation of proteins based upon molecular size by denaturing the protein. This is achieved by using sodium dodecyl sulfate (SDS) in conjunction with disulfide bond reducing agents (such as 2-ME or DTT) and heating. Using SDS – polyacrylamide gel electrophoresis (PAGE) with a Coomassie stain it was possible to confirm that a protein of the correct size was being isolated (Figure 42).

The information from the SDS-PAGE combined with the DNA sequencing of the pGEX-PKBα-PH and pGEX-E17A plasmids, implies that there is successful expression of the two PKBα PH domains. To confirm this conclusion the protein was tested using a Western blot (also known as a protein immunoblot). The protein was isolated using SDS-PAGE, and then transferred on to a polyvinylidene difluoride (PVDF) membrane by electroblotting. The PVDF was then washed in a dilute solution of non-fat dry milk to block non-specific binding of the antibodies used for detection on to the PVDF. The membrane was then incubated with a suitable primary antibody, washed, and then incubated with a secondary antibody. A range of PKBα PH domain antibodies are commercially available. The choice of
antibody is dictated by the E17A mutation, as mutant protein must also be detectable by the antibody. Because of this the rabbit anti-Akt proto-oncogene product (PKB) polyclonal antibody (Millipore) was employed as the primary antibody. This was raised against a 13 residue synthetic peptide [sequence: FHVETPEEREEWT(C)] based on residues 88-100 of the human PKBα isoform, with the cysteine (C) residue added to couple the peptide to keyhole limpet hemocyanin (KLH). Because the peptide is designed to interact with only a short stretch of the PH domain located at the opposite end of the domain to the E17A mutation, both the mutant and wild-type forms of the PH domain could be studied using the same antibody. A rabbit anti-KLH antibody conjugated to horseradish peroxidase (HRP) was then used as the secondary antibody. The HRP allowed the enhanced chemiluminescence (ECL)-based immunodetection technique to be used to show that both the wild-type and E17A forms of the PH domain of PKBα were successfully expressed (Figure 43).

![Figure 43. Western blot of wild-type PKBα PH domain and E17A mutant PH domain, using a rabbit anti-Akt proto-oncogene product (PKB) polyclonal antibody as a primary antibody.]

The next step was to quantify the level of expression. This was done using the colorimetric Bradford protein assay. This uses the Coomassie reagent which is converted from its red form into its blue form as it complexes with proteins. The amount of blue dye present in the solution is a measure of the protein concentration. The bound form of the dye has an absorption spectrum maximum at 595 nm. Hence, by measuring the increase of absorbance at this wavelength, the amount of bound dye and therefore protein concentration of the sample can be calculated. Multiple dilutions of the protein solution are often required for two reasons: (i) taking readings at various concentrations allows the concentration of the parent solution to be confirmed by multiple measurements; and (ii) the assay is only linear over a short range (2-120 µg/ml).

Using the Bradford assay to measure the protein concentrations of the wild-type and E17A PH domains of PKB showed the concentration to be below the minimum level
accessible by the assay (< 2 µg/ml). It was therefore necessary to consider how the level of expression could be increased.

The first consideration was the strain of *E. coli* used. *Stratagene* supply three different strains of BL21 competent cells that provide different levels of expression: (i) BL21(DE3) competent cells allow high-level protein expression with induction, but can suffer from uninduced expression of proteins; (ii) The BL21(DE3)pLysS cells provide tighter control of protein expression, but exhibit slight inhibition of expression with comparison to the BL21(DE3) cells; and (iii) BL21 cells provide the tightest control of protein expression, but the induction process is more cumbersome and less efficient than for the DE3 derivatives.

Since the proteins being expressed are not toxic, the control of expression that the BL21 cells afford is not required. It was therefore decided that BL21(DE3) cells should be adopted to increase the level of expression.

The second consideration was the choice of growth media. Expression in LB media requires induction by IPTG. The point of induction is determined by monitoring the medium until the *E. coli* reach mid-log phase. The requirement for monitoring can be removed by using auto-induction (AI) medium. AI medium has been designed to induce expression when the cells reach their optimum stage for expression; it also negates the use of IPTG.¹⁸¹ AI media works by using glucose lactose and glycerol as food sources for the cells. The cells initially consume the glucose; the glucose acts to support cell growth and inhibit the entry of the lactose into the cells. Once the glucose has been fully consumed the bacteria utilise the glycerol alternative food source; this allows the lactose to enter the cells. The presence of lactose displaces the lac I repressor protein within the plasmid and causes expression of Ptac, thus inducing the expression of the GST fusion protein at optimal cell density.

These two considerations were combined with using the Ultra Yield™ flask, which was kindly donated by Karin Ellis of *Thomson Instrument Company*. The flask has a baffled base and its walls are more vertical than with traditional shake flask designs (*Figure 44*). These flasks have been shown to generate an equivalent amount of protein per unit cell culture density as the traditional flasks, but at a substantially greater amount per unit volume.¹⁸²
BL21(DE3) competent cells were transformed using the heat shock protocol to insert the pGEX-PKBα-PH or pGEX-E17A plasmids. Using the Ultra Yield™ flasks, the transformed cells were grown in AI media containing ampicillin. The resulting expressed protein was isolated using the same techniques as before, to give considerably higher yields of both the wild-type and E17A GST fusion proteins bound to GST beads.

6.2 THROMBIN DIGEST

The next stage was to remove the wild-type and E17A mutant PH domains of PKB from the GST beads. There are two routes through which this can be achieved: (i) thrombin digest, and (ii) glutathione elution. A thrombin digest only gives the desired protein, while glutathione elution releases the entire fusion protein from the GST beads (Figure 45). The choice of technique is dependent upon how the protein is going to be employed. In this case the presence of multiple W residues in the GST tag make the fusion protein unsuitable for use in the intrinsic tryptophan fluorescence experiments as the additional residues would greatly reduce the observed change in emission on binding, hence decreasing the accuracy of any data obtained.
Because of this thrombin cleavage was employed. The thrombin cleavage site is a sequence of six amino acids (LVPRGS) that is used to link the purification tag to the protein of interest. It is recognised by the serine protease thrombin, which selectively cleaves between the R and G residues in the cleavage site, allowing the protein to enter the supernatant, while the GST tag remains bound to the GST beads.

SDS-PAGE showed that cleavage of the two PH domains appeared to proceed with good conversion of the fusion proteins to the corresponding bound GST tag and cleaved PH domain (Figure 46). However Bradford protein assay of the supernatant once more showed only trace levels of the protein. A combination of the Bradford assay and SDS-PAGE revealed that the majority of the PH domain was still located on the GST beads. Repeated washing of the beads failed to elute any additional protein. Since the protein of interest no longer contains a GST tag this was attributed to non-specific binding of the protein to the beads. An internet search of biochemistry forums for this problem suggested either using a phosphate buffer (pH 8) or carbonate buffer (pH 11) as a way of breaking the protein bead interaction. Both have their positive and negative aspects. The high pH of the carbonate buffer risks denaturing the proteins. There is less risk of this occurring with the lower pH.

**Figure 45.** Cleavage of expressed protein from GST beads. Thrombin digest makes use of the thrombin cleavage site within the GST fusion protein to give the isolated protein of interest. Glutathione elution disassociates the whole fusion protein from the GST bead.
of the phosphate buffer, but the PH domain is an inositol phosphate binding protein, so the potential exists for the buffer blocking the binding site for the ligand. It was therefore decided to use the carbonate buffer and then use a concentrator to transfer the protein into a more suitable buffer.

![Figure 46](image)

**Figure 46.** 12% SDS-PAGE gel, run at 200 volts for 40 mins, visualised by Coomassie Blue, showing thrombin cleavage of GST-PH domain fusion proteins. Lane 1 – markers; Lane 2 – wt PH domain on-beads; Lane 3 – wt PH domain beads after thrombin cleavage; Lane 4 – supernatant from thrombin cleavage of wt PH domain beads; Lane 5 – E17A PH domain on-beads; Lane 6 – E17A PH domain beads after thrombin cleavage; Lane 7 – supernatant from thrombin cleavage of E17A PH domain beads.

6.3 INTRINSIC TRYPTOPHAN FLUORESCENCE SPECTROSCOPY

As already discussed Fench *et al.* have used intrinsic tryptophan fluorescence spectroscopy to study the wild-type PH domain of PKB\(\alpha\) binding various inositol phosphates and phosphoinositides, including \text{Ins}(1,3,4,5)\text{P}_4.^{82} \text{Reproducing these results using the synthetic \text{Ins}(1,3,4,5)\text{P}_4 and the expressed wild-type PH domain allows direct verification of the use of carbonate buffer for disrupting the non-specific binding between the PH domain and the GST beads. It also establishes a protocol for the subsequent study of the interactions of E17A mutants.
Therefore after the wild-type PH domain was removed from the GST beads a 5000 MW spin concentrator was used to transfer the protein from the carbonate buffer into 20 mM HEPES/NaOH, pH 7.0, 10 mM MgCl₂. This process led to the formation of a protein precipitate, lowering the yield of the PH domain. However enough protein remained dissolved in the buffer solution to allow testing to proceed. The wild-type PH domain was diluted to a concentration 2.5 µg/ml and placed in a sample cell in the spectrometer. The fluorescence spectrum of the PH domain was obtained at 25 °C by exciting the sample at 290 nm and recording its emission spectrum. Comparison of the spectrum to that published (Figure 47) showed the emission maximum at a lower wavelength than expected, suggesting that the tryptophans of the expressed PH domain are not in the same environment as in Fench’s research, and that the protein has been denatured by the carbonate buffer.

To confirm this, binding of the PH domain to Ins(1,3,4,5)P₄ was attempted. If binding occurred, a decrease in emission should have been observed. However, no decrease was seen and an additional peak with a maximum at 340 nm appeared (Figure 48). Addition of Ins(1,3,4,5)P₄ to the buffer, in the absence of protein, showed this peak to be due to the ligand. There could be two potential reasons for this peak: Either trace impurities introduced in the buffer; or it is due to the ammonium salt. Re-purification should remove any contaminants, if this does not correct the problem, conversion back to the sodium salt should. However, it was decided that an alternative approach was needed to isolate the protein so that it is not denatured in the process.
6.4 GLUTATHIONE ELUTION

As previously discussed, there are two ways to remove the protein from the GST beads (Figure 45). Since thrombin cleavage was unsuccessful, glutathione elution was utilised. Adding reduced glutathione to the beads in Tris pH 8.0 releases the whole GST fusion protein into the solution. Repeated treatment allowed both the wild-type and E17A mutant PH domains to be isolated in good yield (Figure 49).

Figure 48. **A** Emission spectrum of wt PKBα PH domain with addition of Ins(1,3,4,5)P₄. **B** Emission spectra of isolated of wt PKBα PH domain (blue) and isolated Ins(1,3,4,5)P₄ (green) super-imposed to show individual components of **A**.

Figure 49. 12% SDS-PAGE gel, run at 200 volts for 40 mins, visualised by Coomassie Blue, showing glutathione elution of the GST-PH fusion proteins. Lane 1 – markers; Lane 2 – wt GST-PH domain off the beads; Lane 3 – E17A GST-PH domain off the beads.
6.5 ISOTHERMAL TITRATION CALORIMETRY

The presence of the GST tag means that an alternative approach is required as the presence of the additional W residues render intrinsic tryptophan fluorescence spectroscopy impracticable. As already discussed, isothermal titration calorimetry offers a useful alternative technique for the study of protein ligand binding. While it has not been previously employed to study the interactions between the PH domain of PKBα with various inositol phosphates and phosphoinositides, Lemmon et al. have used it to investigate the binding patterns of a wide range of other PH domains.

The first consideration is the amount of protein needed per experiment. As discussed in the bio-physical techniques (section 2.3.2) the shape of the binding isotherm is determined by the unitless constant $c$.

$$c = ([M_{tot}]n)/K_d \tag{eq. 2}$$

The recommended minimum value of $c$ is 5. Since experimental values are only available for the wild-type protein these were used for initial calculations. For the interaction of the PH domain of PKBα with Ins(1,3,4,5)P₄, the $n$ value would be expected to be 1, and a value of 0.95 has been determined experimentally. The same research used intrinsic tryptophan fluorescence spectroscopy to determine the $K_d$ value to be 1.5 (±0.2) μM.$^82$ Adding all these values into the above equation give:

$$5 = ([M_{tot}] \times 0.95)/1.5 \ (±0.2) \ \text{μM} \tag{eq. 3}$$

$$7.5 \ (±1.0) \ \text{μM} = [M_{tot}] \times 0.95 \tag{eq. 4}$$

$$7.9 \ (±1.1) \ \text{μM} = [M_{tot}] \tag{eq. 5}$$

This gives the desired molar concentration for the wild-type PH domain. Both the wild-type and E17A mutant PH domains have a molecular weight of ~14 kDa, and the GST tag an additional ~25 kDa. This gives a minimum required concentration of ~230 μg/ml per experiment.
The wild-type fusion protein was transferred from the Tris pH 8.0 buffer into 25 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.8, 100 mM NaCl and 1 mM dithiothreitol (DTT) prior to the experiment, once again using a 5000 MW spin concentrator, and then adjusted to the required concentration. The protein sample was then carefully injected into the sample cell and sequential aliquots (10μl) of Ins(1,3,4,5)P₄ in water were injected, under computer control, to cover a range of Ins(1,3,4,5)P₄-PH domain molar ratios of 0:1 to 6:1 [taking into account that the sample of Ins(1,3,4,5)P₄ is racemic]. A control experiment for the heat of dilution of the Ins(1,3,4,5)P₄ into the buffer was performed in a separate titration, and data corrected accordingly (Figure 50).

Comparison of these results (Figure 50A vs. Figure 50B) show significant differences, implying the potential occurrence of binding, but the relative intensity of the observed peak compared to just the background heat of mixing implied either
the protein had once more become denatured, or a secondary factor was influencing the shape of the observed binding isotherm. MicroCal report in their user's manual\textsuperscript{94} that as well as consideration of the \( c \) value, the sensitivity of the equipment must also be taken into account. The equipment has a limiting sensitivity of \( \sim 0.1 \) \( \mu \text{cal} \), so each injection into the cell should have an average heat absorbed or evolved of 3-5 \( \mu \text{Cal} \). Hence if the \( \Delta H \) value for the interaction is low then the minimum concentration of protein can be significantly higher than that implied by calculations from \( c \). To investigate whether this could be the cause an increased concentration of protein was needed. Attempting to increase the concentration lead to the formation of protein precipitate, and no significantly higher protein concentrations could be achieved. This suggests an alternative approach is needed to deal with the insolubility issues arising with the protein so that the RLE interactions can be investigated. However, due to time restraints this could not be fully explored.

6.6 ADDITIONAL BIOLOGICAL TESTING

The following research was carried out in collaboration with Jessica Knott, using the \( \text{Ins}(1,3,4,5)P_4 \) analogues synthesised for the RLE studies. While the above testing was being attempted, the compounds were also undergoing testing as phosphatase inhibitors. Synaptojanin is a type II phosphatase that has been shown to be involved in the uncoating of vesicles in neurons.\textsuperscript{183} It is an important lipid phosphatase that dephosphorylates the 5-\( O \) of \( \text{PtdIns}(3,4,5)P_3 \) and \( \text{PtdIns}(4,5)P_2 \), as well as \( \text{Ins}(1,4,5)P_3 \) and \( \text{Ins}(1,3,4,5)P_4 \). It possesses two phosphatase domains: A central inositol 5-phosphatase domain (IPPc), that dephosphorylates both \( \text{PtdIns}(3,4,5)P_3 \) and \( \text{PtdIns}(4,5)P_2 \), as well as \( \text{Ins}(1,4,5)P_3 \) and \( \text{Ins}(1,3,4,5)P_4 \); and a phosphatidylinositide phosphatase Sac1 homology domain at its N-terminal, of which the main substrate is \( \text{PtdIns}(4)P \).\textsuperscript{184} To investigated the possible models and mechanisms to explain the lipid based Sac/IPPc domain co-dependence a suitable IPPc domain inhibitor needed to be identified. The 2-\( O \) substituted analogues of \( \text{Ins}(1,3,4,5)P_4 \) synthesised for polar group exchange RLE of protein kinase B’s pleckstrin homology domain (Figure 51), were tested as potential synaptojanin inhibitors. Because \( \text{Ins}(1,4,5)P_3 \) and \( \text{Ins}(1,3,4,5)P_4 \) are both substrates for only the IPPc domain, it was believed that the
analogues would also only selectively interact with the IPPc domain, and not interact or inhibit the Sac active site. However their additional functional moieties could disrupt the hydrolysis mechanism, leading to inhibition of the active site.

![Figure 51. Structures of Ins(1,3,4,5)P₄ analogues tested as potential IPPc domain inhibitors.](image)

Initially it needed to be confirmed that none of these compounds were substrates for either the IPPc or Sac domains. Using a malachite green assay, it is possible to measure the activity of Sac/IPPc towards each compound compared to the IPPc domain hydrolysing Ins(1,4,5)P₃ (Figure 52). The malachite green assay measures the level of free phosphate released into the solution by inositol phosphate ester hydrolysis.

This showed that within the detection limits of the assay, none of the compounds tested exhibited significant formation of free phosphate. Based on this it can be assumed that none of them are substrates for IPPc/Sac.
Figure 52. Free phosphate levels produced relative to Ins(1,4,5)P₃ from the incubation of IPPc/Sac with 200 µM of the inositol phosphate analogue compounds.

Figure 53. The activity of Sac/IPPc on 100µM Ins(1,4,5)P₃ in the presence of 100 µM inositol phosphate analogues (as a percentage of the control). The control is the hydrolysis of Ins(1,4,5)P₃ in the absence of any inositol phosphate analogue.

Once this had been established, each compound’s ability to inhibit the IPPc domain of IPPc/Sac was tested. The compounds were each incubated with the IPPc/Sac before the protein’s activity towards Ins(1,4,5)P₃ was measured (Figure 53). The activity of the IPPc/Sac phosphatase in the presence of each analogue was expressed as a percentage of the domain’s activity towards just Ins(1,4,5)P₃. Based
on these results it can be seen that the 2-\(O\)-propyl InsP\(_4\) and 2-\(O\)-(2-hydroxyethyl) InsP\(_4\) inhibit the IPPc domain by more than 50% at 100 \(\mu\)M.

The inhibition mechanism was then investigated using a Lineweaver-Burke plot (Figure 54) of the Sac/IPPc hydrolysis of Ins(1,4,5)P\(_3\) compared to those of Sac/IPPc in the presence of the strongest inhibitor - the 2-\(O\)-(2-hydroxyethyl) InsP\(_4\). If the ligand inhibits an enzyme in a competitive manner the plot shows a shift in \(K_M\) with the \(V_{MAX}\) value staying constant. For non-competitive inhibition \(K_M\) stays constant but a drop in \(V_{MAX}\) is observed. Figure 54 shows no shift in the \((1/V)\) axis intercept, corresponding to \(V_{MAX}\) remaining constant in each case; and a shift in the projected intersect on the \((1/[S])\) axis, showing a change in \(K_M\). It can therefore be stated that the mechanism of IPPc inhibition by 2-\(O\)-(2-hydroxyethyl) InsP\(_4\) is competitive in nature.

![Figure 54](image_url)

**Figure 54.** A Lineweaver-Burke plot of the Sac/IPPc hydrolysis of Ins(1,4,5)P\(_3\) in the presence of (i) 2-\(O\)-(2-hydroxyethyl) InsP\(_4\) (blue); and (ii) in the absence of an inositol phosphate analogue (black).

To determine if these analogues acted as inhibitors in a dose dependent manner, increasing concentrations of 2-\(O\)-propyl InsP\(_4\) and 2-\(O\)-(2-hydroxyethyl) InsP\(_4\), were incubated with the IPPc/Sac protein, and the activity of the IPPc domain towards Ins(1,4,5)P\(_3\) measured in each case (Figure 55).
The 2-O-propyl InsP₄ inhibited up to 50% of IPPc activity, with the level of inhibition staying constant after ~100 μM. Only the 2-O-(2-hydroxyethyl) InsP₄ showed a dose-dependent behaviour that allowed inhibition of 100% of IPPc’s activity.

To validate these compounds’ potential as selective inhibitors of the IPPc domain in the presence of the Sac phosphatase, they were assayed with both the IPPc/Sac active synaptojanin and the IPPc-dead and Sac-dead mutants in the presence of either PtdIns(4)P, Ins(1,4,5)P₃ or O-methyl fluorescein phosphate (OMFP) (Figure 56).

In each case the compounds inhibit the IPPc/Sac hydrolysis of PtdIns(4)P by ~35%, and similar levels of inhibition are observed in the IPPc dead mutant, showing that these
compounds exhibit poor selectivity for just the IPPc domain. It is worth noting however that significantly less inhibition is observed using the artificial substrate OMFP. The inhibition of the Sac dead mutant and Sac/IPPc hydrolysing Ins(1,4,5)P$_3$ was comparable in each case studied, suggesting that the compounds do not bind to the Sac active site, but cause additional inhibition via conformational changes upon binding. Hence while the 2-O-(2-hydroxyethyl) InsP$_4$ analogue initially seemed promising as an inhibitor of the IPPc domain, it is not selective enough to be employed for probing the IPPc/Sac protein.
7. CONCLUSIONS AND OUTLOOK

The preceding research can be split into three sections: (i) chemical synthesis of a range of 2-O functionalised Ins(1,3,4,5)P₄ derivatives; (ii) the expression and purification of both the wild-type and E17A mutant forms of the PH domain of PKBα; and (iii) bio-physical testing of any interactions between the PH domains and the synthetic ligands in order to establish the potential for polar group exchange RLE of protein kinase B’s pleckstrin homology domain.

The first part can be considered successful with the synthesis of the 2-O-acetic acid Ins(1,3,4,5)P₄. Adopting a divergent synthetic strategy allowed the synthetic route to be adapted for the synthesis of 2-O-propyl and 2-O-(2-hydroxyethyl) compounds. These compounds are designed to act as control molecules to ensure that polar group exchange RLE is occurring rather than a steric complementation interaction. Using the 2-O-(2-hydroxyethyl) compound also showed potential for further functionalisation of the 2-O side-chain. The Mitsunobu reaction was used to convert the alcohol selectively into both an azide and a protected guanidinium. However further development of the reaction conditions is required to allow purification of the final compound. One potential route to overcome this problem could be to employ polymer-supported triphenylphosphine to allow easier separation of the reagent debris from the product.

Although it was not explored as part of this research, there are two key intermediates that would allow for this small library of compounds to be expanded. The first is the 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2-O-allyl-6-O-benzyl-myoinositol (118): The allyl moiety can be expanded beyond the ethyl chain length explored here, to propyl, and longer chain length, derivatives. The second main derivative is the 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2-O-(2-hydroxyethyl)-6-O-benzyl-myoinositol (128): As already seen, in combination with the Mitsunobu reaction, this allows the introduction of a variety of moieties into this derivative, including phenols, anilines and carbon nucleophiles.

In addition to these compounds there is the route to Ins(1,4,5)P₃ which was discussed in section 3.2.2. Applying this methodology to myoinositol-2-O-allyl-6-O-benzyl-1,3,5-O-orthobenzoate (108), would allow access to the Ins(1,4,5)P₃ analogues similarly to the Ins(1,3,4,5)P₄ compounds.
Expression of both the wild-type and E17A mutant of the PH domain of PKBα was successfully achieved, and both proteins isolated in yields comparable to those reported in current literature, but subsequent purification was less successful. Trying to use the thrombin cleavage site for purification led to non-specific binding of the protein to the GST beads, and the conditions that allowed disruption of the binding also caused denaturation of the protein. These restrictions meant that the use of intrinsic tryptophan fluorescence spectroscopy had to be discarded due to the presence of the GST tag after cleavage with reduced glutathione. Theoretically the GST tag could be removed post cleavage, but the issues with solubility already encountered suggest that removing the tag – which is known to increase protein solubility in many cases – would be unwise. Since this solubility issue arose again when trying to probe the interaction using ITC, an alternative approach is needed. Ingley and Hemmings have developed a large-scale method for the expression and purification of a form of PKBα’s PH domain with increased solubility. This solubility is conferred on the protein by the presence of three additional lysines on the C-terminus of the protein. It is proposed that this method is employed to create samples of both the wild-type and E17A mutant in significant yields, and at high enough concentrations to facilitate study by ITC. Identification and verification of the cause of the observed compound peak in intrinsic tryptophan fluorescence spectroscopy should also allow this method to be revisited. This approach is going to be continued by Sarah Rosen as part of her PhD research.

While a successful polar group exchange RLE pair has not yet been identified the key components are in place. The ligand designed for this purpose has been synthesised. The problems with isolation of the expressed proteins have been identified, and a strategy for surmounting these problems has been proposed. Both intrinsic tryptophan fluorescence spectroscopy and ITC have been shown to be applicable to studying any interaction that occurs, and the problems encountered experimentally should be removed by the new expression strategy, combined with either re-purification, or changing the salt of the Ins(1,3,4,5)P₄ analogues.
8. EXPERIMENTAL

8.1 CHEMISTRY

8.1.1 General experimental

Unless otherwise stated, the following protocols were used for each reaction:

- All reactions were conducted under a nitrogen atmosphere, at room temperature and in dry glassware.
- Chemicals were purchased from *Sigma-Aldrich Ltd.* or *Acros Organics*, and used directly from the supplier’s container.
- A water-cooled Büchi rotary evaporator was used for the evaporation of organic solvents.
- Solvents were dried over a suitable reagent, and then distilled, before use. Distilled solvents were stored under nitrogen, and over 4 Å molecular sieves if suitable, in sealed containers.

Depending upon availability, $^1$H-, $^{13}$C- and $^{31}$P-NMR spectra were recorded on Bruker AV-500, AV-400 or AC-270 spectrometers. Chemical shifts (δ) are recorded in parts per million (ppm), referenced to an internal solvent standard, $\delta_{CH}$ (CHCl$_3$) 7.25 ppm, $\delta_{CH}$ (d$_6$-DMSO) 2.50 ppm, $\delta_{HOD}$ (HOD) 4.60 ppm, $\delta_C$ (CDCl$_3$) 77.50 ppm, $\delta_C$ (d$_6$-DMSO) 39.43 ppm, or an external reference, $\delta_P$ (H$_3$PO$_4$) 0.00 ppm. Splitting patterns are recorded using standard abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), b (broad) and combinations of the above. Coupling constants (J) are quoted in Hertz (Hz). $^{13}$C peaks are assigned as C (quaternary), CH (tertiary), CH$_2$ (secondary) and CH$_3$ (primary). $^1$H- and $^{13}$C-NMR assignments were made with the aid of DEPT-90 and -135, HSQC, COSY and NOESY experiments. $J_{PC}$ coupling constants were assigned using resolution enhancement in *MestRe-C*. $^{13}$C- and $^{31}$P-NMR spectra are proton decoupled. Deuterated solvents were purchased from *Merck*, with the exception of d$_6$-DMSO (*Apollo Scientific Ltd.*).
Mass spectra were recorded on a VG AutoSpec-Q (CI and FAB), a Micromass AutoSpec Premier (CI and EI), a Micromass LCT Premier (ES) or a Micromass MALDI micro MX (MALDI). 

*Merck* silica gel 60 F₂₅₄ glass-backed plates were used for analytical TLC. The plates were visualised using UV light or after treatment with a chemo-selective stain. Flash chromatography was carried out using flash silica from *Merck*.

A general method was designed for the global deprotection of Ins(1,3,4,5)P₄ and its 2-Ο analogues

**General method 1: Global deprotection to form Ins(1,3,4,5)P₄ and its 2-Ο analogues**

1,3,4,5-Ο-tetrakis(dibenzyl phosphoryl)-2,6-Ο-dibenzyl-myo-inositol (114, 110 mg, 78 μmol) was taken up in 1BuOH-water (6:1 v/v, 14 ml), then sodium bicarbonate (53 mg, 0.62 mmol) and Pd-black (167 mg, 1.57 mmol) were added. The reaction vessel was flushed with N₂ (× 3) followed by H₂ (× 3). The reaction mixture was stirred under 1 atm. H₂ for 24 h. The solution was filtered, and the remaining solids washed with water (15 ml), and the combined filtrates evaporated under reduced pressure. The residue was taken up in water (15 ml) and washed with CHCl₃ (15 ml). The aqueous layer was evaporated under reduced pressure to give the sodium salt.

**General method 2: Ion exchange of Ins(1,3,4,5)P₄ and its 2-Ο analogues**

The sodium salt was passed through a DOWEX 50WX8-200 (H⁺ form) ion exchange column, and the acidic fractions of eluent were combined, neutralised with aq. NH₃ and then freeze dried, giving the ammonium salt.

**8.1.2 MRes research**

This section contains the characterisation of a selection of compounds that were synthesised during the MRes year of research. They are included here as they are involved in the final synthetic route to the target compounds.
myo-Inositol-1,3,5-O-orthobenzoate (43c)

\[
\begin{align*}
\text{HO} & \quad \text{O} & \quad \text{O} & \quad \text{Ph} \\
\text{HO} & \quad \text{O} & \quad \text{O} & \quad \text{BnO}
\end{align*}
\]

myo-Inositol (2, 5.00 g, 28 mmol) was suspended in DMSO (23 ml), then trimethyl orthobenzoate (7.2 ml, 42 mmol) and toluene sulfonic acid (55 mg, cat.) were added. The suspension was stirred at 100 °C for 5 h. The solution was quenched with Et$_3$N (40 μl, 1 eq./TSA), and the solvents were evaporated under reduced pressure to give the crude product. This was purified by recrystallisation from EtOAc to afford the title compound (43c, 4.28 g, 58%) as colourless crystals. $R_f$ (EtOAc) 0.55; $\delta$$_T$ (500 MHz; d$_6$-DMSO) 7.58-7.53 (2H, m), 7.37-7.30 (3H, m) (5 × Ph-H), 5.50 (2H, s, Ins 4-OH + Ins 6-OH), 5.32 (1H, d, J 6.2, Ins 2-OH), 4.40 (2H, t, J 3.6, Ins 4-H + Ins 6-H), 4.20 (1H, tt, J 3.2, 1.6, Ins 5-H), 4.15 (2H, dq, J 3.6, 1.5, Ins 1-H + Ins 3-H), 4.08 (1H, dt, J 6.2, 1.5, Ins 2-H); $\delta$C (125 MHz; d$_6$-DMSO) 137.9 (Ph C), 129.0, 127.5 (2C), 125.4 (2C) (5 × Ph CH), 106.4 (PhCO$_3$), 75.8 (2C), 70.1, 67.2 (2C), 57.8 (6 × Ins CH); MS (FAB+) $m/z$ (%): [M+H]$^+$ 267 (100), [PhCO]$^+$ 105 (56); HRMS (FAB+) $m/z$ (%): found [M+H]$^+$ 267.0878 (100), C$_{13}$H$_{15}$O$_6$ requires 267.0869.

6-O-Benzyl-myoinositol-1,3,5-O-orthobenzoate (76)

Crude myo-inositol-1,3,5-O-orthobenzoate (43c, 1.00 g, 3.8 mmol) was evaporated from MeCN (3 × 5 ml) then taken up in DMF (20 ml). The solution was cooled to -15 °C and NaH (60% dispersion in mineral oil, 150 mg, 3.8 mmol) was added. The reaction was stirred for 15 min at -15 °C, and a further 30 min at room temperature. Benzyl bromide (434 μl, 3.8 mmol) was added and the reaction stirred overnight. The reaction was quenched with water, stirred for a further 20 min, then the solvent was evaporated under reduced pressure. The remaining solids were taken up in EtOAc (20 ml) and washed with water (3 × 15 ml), then brine (10 ml). The organic layer was dried over MgSO$_4$, filtered and the filtrate evaporated under reduced pressure. The residue was fractionated by flash column chromatography, eluting with with hexane-EtOAc (1:1 v/v) to give the title compound (76, 1.20 g, 90%) as a
colourless oil. R\textsubscript{f} (hexane-EtOAc, 1:4 v/v) 0.65; \delta\textsubscript{H} (500 MHz; d\textsubscript{6}-DMSO) 7.58-7.53 (2H, m), 7.37-7.30 (8H, m) (10 × Ph-H), 5.33 (1H, d, J 6.2, Ins 2-OH), 5.28 (1H, d, J 5.1, Ins 4-OH), 4.72 (1H, d, J 11.7), 4.58 (1H, d, J 11.7) (OCH\textsubscript{2}Ph), 4.46 (1H, td, J 3.4, 1.6, Ins 6-H), 4.43 (1H, dtd, J 6.1, 3.5, 1.4, Ins 4-H), 4.32 (1H, tt, J 3.8, 1.8, Ins 5-H), 4.25 (1H, dq, J 3.3), 4.13 (1H, dq, J 3.9, 2.0, Ins 3-H), 4.08 (1H, dt, J 6.0, 1.7, Ins 2-H); \delta\textsubscript{C} (125 MHz; d\textsubscript{6}-DMSO) 138.2, 137.9 (2 × Ph-C), 129.1, 128.3 (2C), 127.6 (2C), 127.6 (3C), 125.4 (2C) (10 × Ph CH), 106.9 (PhCO\textsubscript{3}), 76.0, 74.1, 73.6 (3 × Ins CH), 70.2 (OCH\textsubscript{2}Ph), 69.6, 67.0, 58.2 (3 × Ins CH); MS (FAB+) m/z (%): [M+H]\textsuperscript{+} 357 (100), [PhCO]\textsuperscript{+} 105 (63), [PhCH\textsubscript{2}]\textsuperscript{+} 91 (95); HRMS (FAB+) m/z (%): found [M+H]\textsuperscript{+} 357.1333 (100), C\textsubscript{20}H\textsubscript{21}O\textsubscript{6} requires 357.1338.

4-O-para-Methoxybenzyl-6-O-benzyl-myoinositol-1,3,5-O-orthobenzoate (78)

6-O-Benzyl-myoinositol-1,3,5-O-orthobenzoate (76, 11.21 g, 31.5 mmol) was evaporated from MeCN (3 × 20 ml) then taken up in DMF (75 ml). The solution was cooled to -15 °C and NaH (60% dispersion in mineral oil, 1.26 g, 31.5 mmol) was added. The reaction was stirred for 15 min at -15 °C, and a further 30 min at room temperature. para-Methoxybenzyl chloride (4.26 ml, 31.5 mmol) was added and the reaction stirred overnight. The reaction was quenched with water, stirred for a further 20 min, then the solvent was evaporated under reduced pressure (oil pump). The remaining solids were taken up in DCM (30 ml) and washed with water (3 × 30 ml), then brine (30 ml). The organic layer was dried over MgSO\textsubscript{4}, filtered and the filtrate evaporated under reduced pressure. The residue was fractionated by flash column chromatography, eluting with with hexane-DCM-EtOAc (3:2:1 v/v/v/v) to give the title compound (78, 9.03 g, 60%) as a colourless oil. R\textsubscript{f} (EtOAc-hexane, 1:1 v/v) 0.55; \delta\textsubscript{H} (400 MHz; d\textsubscript{6}-DMSO) 7.56-7.52 (2H, m), 7.38-7.26 (8H, m), 7.21 (2H, d, J 8.5), 6.82 (2H, d, J 8.5) (14 × Ar-H), 5.40 (1H, d, J 6.2, ex. → s, Ins 2-OH), 4.75-4.68 (1H, m, Ins 5-H), 4.69 (1H, d, J 11.6), 4.61 (1H, d, J 11.6), 4.60 (1H, d, J 11.2), 4.53 (1H, d, J 11.2) (2 × OCH\textsubscript{2}Ar), 4.37-4.26 (4H, m, 4 × Ins-H), 4.06 (1H, bd, J 6.2, ex. → s, Ins 2-H\textsubscript{2}), 3.72 (3H, s, OCH\textsubscript{3}); \delta\textsubscript{C} (100 MHz; d\textsubscript{6}-DMSO) 158.7, 138.1, 137.7, 130.0 (4 × Ar C), 129.3 (2C), 129.1, 128.2 (2C), 127.6 (2C), 127.6 (2C), 127.5, 125.4 (2C), 113.6 (2C) (14 × Ar CH), 107.1 (PhCO\textsubscript{3}), 73.9, 73.1, 73.6, 73.3 (4 × Ins CH), 70.5, 70.3 (2 × OCH\textsubscript{2}Ar), 67.8,
58.5 (2 × Ins CH), 55.0 (OCH₃); MS (FAB+) m/z (%): [M+H]⁺ 477 (54), [MeOC₆H₄CH₂]⁺ 121 (100), [PhCH₂]⁺ 91 (46); HRMS (FAB+) m/z (%): found [M+H]⁺ 477.1934 (100), C₂₈H₂₉O₇ requires 477.1913.

2-O- Allyl-4- O-para- methoxybenzyl- 6- O- benzyl- myo- inositol- 1,3,5- O- orthobenzoate (79)

4-O-para- Methoxybenzyl- 6- O- benzyl- myo- inositol- 1,3,5- O- orthobenzoate (78, 9.03 g, 18.9 mmol) was evaporated from MeCN (3 × 20 ml) then taken up in DMF (50 ml). The solution was cooled to -15 °C and NaH (60% dispersion in mineral oil, 1.13 g, 28.4 mmol) was added. The reaction was stirred for 15 min at -15 °C, and a further 30 min at room temperature. Allyl bromide (2.45 ml, 28.4 mmol) was added, and the reaction stirred overnight. The reaction was quenched with water, stirred for a further 20 min, then the solvent was evaporated under reduced pressure (oil pump). The remaining solids were taken up in DCM (20 ml) and washed with water (3 × 15 ml), then brine (15 ml). The organic layer was dried over MgSO₄, filtered and the filtrate evaporated under reduced pressure. The residue was fractionated by flash column chromatography, eluting with hexane-DCM-EtOAc (3:2:1 v/v/v) to give the title compound (79, 9.30 g, 95%) as a colourless oil. Rᵋ (hexane-EtOAc, 1:1 v/v) 0.65; δᵥ (400 MHz; d₆-DMSO) 7.53-7.49 (2H, m), 7.39-7.30 (8H, m), 7.24 (2H, d, J 8.6), 6.86 (2H, d, J 8.6) (14 × Ar-H), 5.92 (1H, ddt, J 17.2, 10.5, 5.3, OCH₂CH₂H₂), 5.30 (1H, dq, J 17.2, 1.6), 5.17 (1 H, dq, J 10.4, 1.2) (OCH₃CHCH₂), 4.73-4.68 (1H, m, Ins 5-H), 4.70 (1H, d, J 11.6), 4.64 (1H, d, J 11.6), 4.63 (1H, d, J 11.3), 4.56 (1H, d, J 11.3) (2 × OCH₂Ar), 4.52 (1H, dq, J 3.5, 1.7), 4.49 (1H, dq, J 3.5, 1.7), 4.41-4.34 (2H, m) (4 × Ins-H), 4.03-4.15 (2H, m, OCH₂CH₂H₂), 3.89 (1H, bs, Ins 2-H), 3.75 (3H, s, OCH₃); δᵥ (100 MHz; d₆-DMSO) 159.3, 138.6, 138.0 (3 × Ar C), 135.7 (OCH₂CH₂H₂), 130.5 (Ar C), 129.8 (2C), 129.7, 128.7 (2C), 128.2 (2C), 128.1 (2C), 128.0, 125.7 (2C) (12 × Ar CH), 117.3 (OCH₂CH₂H₂), 114.1 (2C, Ar CH), 107.5 (PhCO₃), 74.0, 73.7, 71.6, 71.5 (4 × Ins CH), 71.1, 70.9, 69.5 (3 × CH₂), 68.6, 66.4 (2 × Ins CH), 55.5 (OCH₃); MS (FAB+) m/z (%): [M+H]⁺ 517 (28), [MeOC₆H₄CH₂]⁺ 121 (100), [PhCH₂]⁺ 91 (31); HRMS (FAB+) m/z (%): found [M+H]⁺ 517.2223 (100), C₃₁H₃₃O₇ requires 517.2226.
8.1.3 PhD research

4-O- Allyl-myo-inositol-1,3,5-O-orthobenzoate (93)

Crude myo-inositol-1,3,5-O-orthobenzoate (43c, 3.00 g, 11.2 mmol) was evaporated from MeCN (3 × 5 ml) then taken up in DMF (30 ml). The solution was cooled to -15 °C and NaH (60% dispersion in mineral oil, 450 mg, 11.2 mmol) was added. The reaction was stirred for 15 min at -15 °C, and then stirred for a further 30 min at room temperature. Allyl bromide (972 μl, 11.2 mmol) was added, and the reaction stirred overnight. The reaction was quenched with water, stirred for a further 20 min, then the solvent was evaporated under reduced pressure. The remaining solids were then taken up in DCM (20 ml) and washed with water (3 × 20 ml) and then with brine (20 ml). The organic layer was dried over MgSO₄, filtered and the filtrate evaporated under reduced pressure. The residue was fractionated by flash column chromatography, eluting with hexane-EtOAc (1:1 v/v) to give the title compound (93, 3.06 g, 89%).

2,6-O-Dibenzyl-4-O-allyl-myo-inositol-1,3,5-O-orthobenzoate (94)
(60% dispersion in mineral oil, 977 mg, 24.4 mmol) was added. The reaction was stirred for 15 min at -15 °C, and then stirred for a further 30 min at room temperature. Benzyl bromide (2.90 ml, 24.4 mmol) was added to the reaction, which was then stirred overnight. The reaction was carefully quenched with water (5 ml) and stirred for a further 20 min and then the solvent was evaporated. The remaining solids were taken up in DCM (20 ml) and washed with water (3 × 15 ml), then brine (15 ml). The organic layer was dried over MgSO₄, filtered and the filtrate evaporated under reduced pressure. The residue was fractionated by flash column chromatography, eluting with hexane-EtOAc (9:1 v/v) to give the title compound (94, 3.81 g, 96%).

\[ R_f (\text{hexane-EtOAc, } 9:1 \text{ v/v}) = 0.35; \]

\[ \delta_H (500 \text{ MHz; } CDCl_3) = 7.71-7.66 \text{ (2H, m), } 7.45 \text{ (2H, bd, } J = 7.2), 7.40-7.29 \text{ (11H, m) (15 × Ph-H)}, 5.87 \text{ (1H, ddt, } J = 17.0, 10.7, 5.5), 5.26 \text{ (1H, dq, } J = 17.4, 1.5), 5.20 \text{ (1H, dq, } J = 10.4, 1.4) \]

(15 × PhC₂H₂), 4.77-4.71 (2H, m), 4.68 (1H, d, J = 11.8) (3 × OCH₂CH), 4.58-4.53 (2H, m, 2 × Ins-H), 4.55 (1H, d, J = 12.1, OCH₂Ph), 4.50 (1H, dq, J = 3.7, 1.8, Ins-H), 4.47 (1H, td, J = 3.7, 1.6, Ins-H), 4.42 (1H, td, J = 3.6, 1.6, Ins-H), 4.13 (1H, ddt, J = 12.7, 5.4, 1.3, OCH₂CHCH₂), 4.10 (1H, t, J = 1.6, Ins 2-H), 4.04 (1H, ddt, J = 12.7, 5.6, 1.2, OCH₂CHCH₂); \[ \delta_C (125 \text{ MHz; } CDCl_3) = 138.2, 137.8, 137.2 \text{ (3 × Ph C), } 134.2 \text{ (OCH₂CHCH₂), } 129.4, 128.5 \text{ (5C), } 128.0 \text{ (2C), } 127.9 \text{ (3C), } 127.8, 127.6, 125.4 \text{ (2C) (15 × CH), } 117.6 \text{ (OCH₂CHCH₂), } 107.9 \text{ (PhCO₃), } 73.9, 73.8, 72.1, 71.9 \text{ (4 × Ins CH), } 71.5, 71.3, 70.7 \text{ (3 × CH₂), } 69.1, 66.4 \text{ (2 × Ins CH); HRMS (Cl+), m/z (%): found [M+H]⁺ 487.2130 (100), C₃₀H₃₁O₆ requires 487.2121.}

2,6-O-Dibenzyl-4-O-(prop-Z-1-eny)-myo-inositol-1,3,5-O-orthobenzoate (95)

\[
\begin{align*}
\text{Ph} & \quad \text{BnO} \\
\text{BnO} & \quad \text{BnO}
\end{align*}
\]

2,6-O-Dibenzyl-4-O-allyl-myoinositol-1,3,5-O-orthobenzoate (94, 3.00 g, 6.17 mmol) was evaporated from MeCN (3 × 3 ml) then taken up in DMSO (6.15 ml). Potassium tert-butoxide (1.38 g, 12.3 mmol) was then added and the solution stirred at 100 °C for 3 h. The reaction was allowed to cool, was quenched with water, and then the solvent was evaporated. The remaining solids were taken up in EtOAc (20 ml). The organic layer was washed with water (4 × 15 ml) and brine (15 ml). The combined aqueous layers were then back extracted with EtOAc (20 ml), and the previous washing procedure repeated. The combined organic layers were dried over MgSO₄, filtered and the filtrate evaporated under
reduced pressure. The crude title compound (95, 3.00 g, 100%) was isolated as a yellow oil. 

\(R_t\) (hexane-EtOAc, 1:1 v/v) 0.76; \(\delta_H\) (400 MHz; CDCl\(_3\)) 7.61-7.68 (2H, m), 7.22-7.46 (13H, m) (15 × Ph-H), 6.03 (1H, dq, \(J = 6.1, 1.7\), OCHCH\(_3\)), 4.72 (2H, s, OCH\(_2\)Ph), 4.67-4.62 (1H, m, Ins-H), 4.66 (1H, d, \(J = 11.8\)) (OCH\(_2\)Ph), 4.57-4.42 [5H, m, (4 × Ins-H) + OCHCH\(_3\)], 4.06 (1H, t, \(J = 1.6\), Ins 2-H), 1.39 (3H, dd, \(J = 6.8, 1.5\), OCHCH\(_3\)); \(\delta_C\) (100 MHz; CDCl\(_3\)) 143.3 (OCHCH\(_3\)), 137.8, 137.6, 136.9 (3 × Ph-C), 129.4, 128.4 (2C), 128.3 (2C), 128.0 (2C), 127.9 (2C), 127.8 (2C), 127.5 (2C), 125.4 (2C) (15 × Ph CH), 107.8 (PhCO\(_3\)), 104.0 (OCHCH\(_3\)), 75.2, 73.5, 71.9, 71.7 (4 × Ins CH), 71.5, 71.1 (2 × OCH\(_2\)Ph), 69.1, 65.6 (2 × Ins CH), 14.2 (OCHCH\(_3\)); HRMS (CI+) \(m/z\) (%): found [M+H]\(^+\) 487.2130 (100), \(\text{C}_{30}\text{H}_{31}\text{O}_6\) requires 487.2121.

2,6-O-Dibenzyl-myoinositol-1,3,5-O-orthobenzoate (96)

\[ \text{Ph} \]
\[ \text{BnO} \]
\[ \text{BnO} \]
\[ \text{OH} \]

2,6-O-Dibenzyl-4-O-(prop-Z-1-enyl)-myo-inositol-1,3,5-O-orthobenzoate (95, 3.00 g, 6.17 mmol) was taken up in MeCN-water (9:1 v/v, 18 ml). \(p\)TSA (117 mg, 0.617 mmol) was added to the vigorously stirred solution. After 48 h \(p\)TSA (117 mg, 0.617 mmol) was added again and the reaction stirred for a further 24 h. The reaction was quenched with \(\text{Et}_3\)N, then taken up in EtOAc (20 ml), washed with sat. aq. NaHCO\(_3\) (15 ml), water (2 × 15 ml) and brine (15 ml). The organic layer was dried over MgSO\(_4\), filtered and the filtrate evaporated under reduced pressure. The residue was fractionated by flash column chromatography, eluting with hexane-DCM-EtOAc (6:2:1 v/v/v) to give the title compound (96, 2.20 g, 80%) as a waxy colourless solid. \(R_t\) (hexane-EtOAc, 4:1 v/v) 0.50; \(\delta_H\) (400 MHz; CDCl\(_3\)) 7.69-7.66 (2H, m), 7.47-7.34 (11H, m), 7.24-7.21 (2H, m) (15 × Ph-H), 4.82 (1H, d, \(J = 12.4\)), 4.71 (1H, d, \(J = 12.4\)) (OCH\(_2\)Ph), 4.61-4.55 [4H, m, (2 × Ins-H) + OCH\(_2\)Ph], 4.54-4.45 (2H, m, 2 × Ins-H), 4.41 (1H, tt, \(J = 3.5, 1.7\), Ins 5-H), 3.97 (1H, bs, Ins 2-H), 3.65 (1H, d, \(J = 9.8\), Ins-OH); \(\delta_C\) (100 MHz; CDCl\(_3\)) 137.9, 136.9, 136.0 (3 × Ph-C), 129.5, 128.9 (2C), 128.8, 128.6 (2C), 128.2 (2C), 128.0 (5C), 125.4 (2C) (15 × Ph CH), 107.4 (PhCO\(_3\)), 74.5, 73.5 (2 × Ins CH), 73.0 (OCH\(_2\)Ph), 71.3 (Ins CH), 71.0 (OCH\(_2\)Ph), 68.7, 68.0, 65.1 (3 × Ins CH); MS (FAB+) \(m/z\) (%): [M+H]\(^+\) 447 (16), [PhCH\(_2\)]\(^+\) 91 (100); HRMS (FAB+) \(m/z\) (%): found [M+H]\(^+\) 447.1822 (100), \(\text{C}_{27}\text{H}_{27}\text{O}_6\) requires 447.1808.
2,6-<i>O</i>-Dibenzy1-<i>myo</i>-inositol (99)

2,6-<i>O</i>-Dibenzy1-<i>myo</i>-inositol-1,3,5-<i>O</i>-orthobenzoate (96, 500 mg, 1.12 mmol) was dissolved in TFA-water (4:1 v/v, 10 ml), and stirred overnight. The reaction mixture was then evaporated to dryness and re-evaporated from MeOH (3 × 5 ml). The residue was taken up in MeOH (10 ml), NaOMe (25 wt. % solution in MeOH) was added drop-wise until the reaction solution attained pH 11, then the reaction was stirred overnight. The solution was neutralised by the dropwise addition of 0.1 M HCl and the solvent was evaporated. The crude product was partitioned between CHCl<sub>3</sub> (10 ml) and water (10 ml), and the aqueous layer collected. The organic layer was then washed with water (3 × 10 ml), and the combined aqueous layers evaporated to dryness. The crude product was then taken up in MeOH (10 ml). The solution was filtered, and the filtrate evaporated to afford the title compound (99, 403 mg, 100%) as a colourless oil, which was used without further purification. 

R<sub>f</sub> (EtOAc) 0.25; δ<sub>H</sub> (400 MHz; CD<sub>3</sub>OD) 7.46-7.40 (4H, m), 7.33-7.27 (4H, m), 7.26-7.21 (2H, m) (10 × Ph-<i>H</i>), 4.90-4.87 (2H, m), 4.86 (1H, d, J 10.9), 4.83 (1H, d, J 10.9) (2 × OCH<sub>2</sub>Ph), 4.89 (1H, t, J 2.5, Ins 2-<i>H</i>), 3.70 (1H, t, J 9.6), 3.66 (1H, t, J 9.3), 3.58 (1H, dd, J 9.8, 2.5), 3.42 (1H, dd, J 9.9, 2.5) (4 × Ins-<i>H</i>), 3.31 (1H, t, J 9.4, Ins 5-<i>H</i>); δ<sub>C</sub> (100 MHz; CD<sub>3</sub>OD) 140.6, 140.5 (2 × Ph C), 129.3 (2C), 129.2 (2C), 129.1 (2C), 128.9 (2C), 128.5, 128.3 (10 × Ph CH), 83.4, 82.8, 76.8 (3 × Ins CH), 76.4, 76.0 (2 × OCH<sub>2</sub>Ph), 74.9, 73.8, 73.7 (3 × Ins CH); HRMS (ES+): m/z (%) found [M+Na]<sup>+</sup> 383.1486 (100), C<sub>20</sub>H<sub>24</sub>O<sub>6</sub>Na requires 383.1471.

2,6-<i>O</i>-Dibenzy1-4-<i>O</i>-(tert-butyldimethylsilyl)-<i>myo</i>-inositol-1,3,5-<i>O</i>-orthobenzoate (101a)

2,6-<i>O</i>-Dibenzy1-<i>myo</i>-inositol-1,3,5-<i>O</i>-orthobenzoate (96, 300 mg, 0.672 mmol) and imidazole (99 mg, 1.48 mmol) were evaporated from MeCN (3 × 1 ml), then taken up in DMF (5 ml) and Et<sub>3</sub>N (93 μl, 0.672 mmol) was added. The solution was cooled to 0 °C,
before adding TbdmsCl (0.152 g, 1.01 mmol). The temperature was then raised to 100 °C and the reaction stirred for 48 h. The reaction was quenched with water (1 ml), stirred for a further 20 min and the solvent was evaporated. The remaining solids were taken up in DCM (10 ml) and washed with sat. aq. NaHCO₃ (10 ml), water (10 ml), then brine (10 ml). The organic layer was dried over Na₂SO₄, filtered and the filtrate evaporated under reduced pressure. The residue was fractionated by flash column chromatography, eluting with hexane-DCM (9:1 → 5:5 v/v), when the title compound (101a, 286 mg, 76%) eluted hexane-DCM (6:4 v/v). Rᵥ(hexane-DCM, 1:2 v/v) 0.55; δH (400 MHz; CDCl₃) 7.72-7.67 (2H, m), 7.47-7.43 (2H, m), 7.40-7.29 (9H, m), 7.28-7.22 (2H, m) (15 × Ph-H), 4.77 (1H, d, J 12.6), 4.73 (1H, d, J 12.6) (OCH₂Ph), 4.67-4.63 (1H, m, Ins-H), 4.64 (1H, d, J 11.8, OCH₂Ph), 4.51-4.49 (1H, m, Ins-H), 4.49 (1H, d, J 11.8, OCH₂Ph), 4.44 (1H, td, J 3.7, 1.6, Ins-H), 4.38-4.33 (2H, m, 2 × Ins-H), 4.12 (1H, t, J 1.5, Ins 2-H), 0.80 [9H, s, SiC(CH₃)₃], 0.06 [6H, s, Si(CH₃)₂]; δC (100 MHz; CDCl₃) 138.1, 137.8, 137.3 (3 × Ph C), 129.4, 128.5 (2C), 128.4 (2C), 128.1 (2C), 128.0 (2C), 127.9, 127.8, 127.6 (2C), 125.4 (2C) (15 × Ph CH), 107.7 (PhCO₂), 74.3, 74.1, 72.0 (3 × Ins CH), 71.5 (OCH₂Ph), 71.2 (Ins CH), 71.0 (OCH₂Ph), 68.3, 65.3 (2 × Ins CH), 25.6 (3C, SiC(CH₃)₃), 17.8 (SiC(CH₃)₃), -4.8, -5.1 (Si(CH₃)₂); HRMS (ES+) m/z (%): found [M+H]+ 561.2677 (83), C₃₃H₄₁O₆Si requires 561.2672, found [M+Na]+ 583.2501 (100), C₃₃H₄₀O₆SiNa requires 583.2492.

2,6-O-Dibenzyl-4-O-(tert-butyldiphenylsilyl)-myo-inositol-1,3,5-O-orthobenzoate (101b)

2,6-O-Dibenzyl-myositol-1,3,5-O-orthobenzoate (96, 500 mg, 1.12 mmol) and imidazole (164 mg, 2.46 mmol) were evaporated from MeCN (3 × 1 ml), then taken up in DMF (3 ml) and Et₃N (155 µl, 1.12 mmol) was added. The reaction was cooled to 0 °C, before adding TbdpsCl (1.16 ml, 4.48 mmol). The reaction was heated to 100 °C and stirred for 72 h. The reaction was quenched with water (0.5 ml), stirred for a further 20 min and the solvent was evaporated. The remaining solids were taken up in DCM (10 ml) and washed with sat. aq. NaHCO₃ (10 ml), water (10 ml), then brine (10 ml). The organic layer was dried over Na₂SO₄, filtered and the filtrate evaporated. The residue
was fractionated by flash column chromatography, eluting with hexane-EtOAc (9:1 v/v), followed by sublimation of the residual TbdpsOH contaminant (oil pump, heat gun) to give the title compound (101b, 645 mg, 84%). $R_f$ (hexane-EtOAc, 4:1 v/v) 0.52; $\delta$H (500 MHz; CDCl3) 7.71-7.67 (2H, m), 7.66-7.63 (4H, m), 7.52-7.47 (4H, m), 7.46-7.32 (13H, m), 7.31-7.27 (2H, m) (25 × Ph-H), 4.70 (1H, td, J 3.9, 1.7, Ins-H), 4.70 (1H, d, J 12.2), 4.67 (1H, d, J 12.6), 4.60 (1H, d, J 12.0) (3 × OCH2HHPh), 4.48 (1H, dq, J 3.9, 1.9, Ins-H), 4.48 (1H, d, J 12.0, OCH2HHPh), 4.39 (1H, td, J 3.6, 1.6, Ins-H), 4.24 (1H, tt, J 3.5, 1.7, Ins 5-H), 4.22 (1H, t, J 1.7, Ins 2-H), 4.19 (1H, dq, J 3.9, 1.9, Ins-H), 0.97 (9H, s, SiC(CH3)3); $\delta$C (125 MHz; CDCl3) 138.1, 137.7, 137.2 (3 × Ph-C), 135.8 (2C), 135.7 (2C) (4 × Ph CH), 133.2, 133.1 (2 × Ph-C), 130.2, 130.1, 129.4, 128.6 (2C), 128.5 (2C), 128.2 (2C), 127.9 (8C), 127.8 (2C), 125.4 (2C) (21 × Ph CH), 107.6 (PhCO3), 74.4, 73.6 (2 × Ins CH), 72.1 (OCH2Ph), 72.0 (Ins CH), 71.0 (OCH2Ph), 70.9, 68.7, 65.3 (3 × Ins CH), 26.8 (3C, SiC(CH3)3), 19.14 (SiC(CH3)3); HRMS (ES+) m/z (%): found [M+Na]+ 707.2792 (94), $C_{43}H_{44}O_6SiNa$ requires 707.2805, found [M+H]+ 685.2970 (100), $C_{43}H_{45}O_6Si$ requires 685.2985.

2-O- Allyl-6-O-benzyl-my0-inositol-1,3,5-O-orthobenzoate (108)

2-O- Allyl-4-O-para-methoxybenzyl-6-O-benzyl-my0-inositol-1,3,5-O-orthobenzoate (79, 1.90 g, 3.6 mmol) was taken up in MeCN-water (9:1, 100 ml). The solution was cooled to 0 °C and CAN (8.0 g, 14.7 mmol) was added. The reaction was stirred for 5 min at 0 °C, and then a further 45 min at room temperature. The solvent was evaporated, and the reaction mixture then taken up in CHCl3 (50 ml). This solution was washed with water (3 × 50 ml), then sat. aq. NaHCO3 (2 × 50 ml) and finally with brine (50 ml). The organic layer was dried over MgSO4, filtered and the filtrate evaporated. The residue was fractionated by flash column chromatography, eluting with hexane-DCM-EtOAc (6:2:1 v/v/v) to give the title compound (108, 1.04g, 72%) as a waxy colourless solid. $R_f$ (hexane-DCM-EtOAc, 3:2:1 v/v/v) 0.33; $\delta$H (400 MHz; CDCl3) 7.67-7.63 (2H, m), 7.45-7.28 (8H, m) (10 × Ph-H), 6.02 (1H, ddt, J 15.7, 10.2, 5.2, OCH2CHCH2), 5.39 (1H, dq, J 15.7, 2.1), 5.28 (1H, dq, J 10.2, 2.1) (OCH2CHCH2), 4.73 (1H, d, J 12.1), 4.68 (1H, d, J 12.1) (OCH2Ph), 4.62 (1H, ddt, J 10.1, 3.7, 1.8, Ins 4-H), 4.57-4.52 (3H, m, 3 × Ins-H), 4.43 (1H, tt, J 3.5, 1.8, Ins 5-H), 4.23
(1H, ddt, \( J = 13.1, 5.5, 1.5\)), 4.18 (1H, ddt, \( J = 13.1, 5.5, 1.5\)) (OCH\(_2\)CHCH\(_2\)), 3.95 (1H, t, \( J = 1.7\), Ins 2-H), 3.72 (1H, d, \( J = 13.1\), 5.5, 1.5), 3.72 (1H, ddt, \( J = 13.1, 5.5, 1.5\)), 4.18 (1H, ddt, \( J = 13.1, 5.5, 1.5\)), 3.95 (1H, t, \( J = 1.7\), Ins 2-H), 3.72 (1H, d, \( J = 10.0\), Ins 6-OH); \( \delta^C \) (100 MHz; CDCl\(_3\)) 136.9, 136.2 (2 × PhC), 134.7 (OCH\(_2\)CHCH\(_2\)), 129.5, 129.0 (2C), 128.9, 128.2 (2C), 128.0 (2C), 125.4 (9 × PhCH), 117.8 (OCH\(_2\)CHCH\(_2\)), 107.4 (PhCO\(_3\)), 74.7, 73.5 (2 × Ins CH), 73.3 (OCH\(_2\)Ph), 71.4 (OCH\(_2\)CHCH\(_2\)), 70.4, 68.7, 68.0, 65.4 (4 × Ins CH); MS (FAB+) \( m/z \) (%): [M+H]\(^+\) 397 (47), [PhCO]\(^+\) 105 (55), [PhCH\(_2\)]\(^+\) 91 (100); HRMS (FAB+) \( m/z \) (%): found [M+H]\(^+\) 397.1657 (100), C\(_{23}\)H\(_{25}\)O\(_6\) requires 397.1651.

2-O-Allyl-6-O-benzyl-myoinositol (109)

\[
\begin{align*}
\text{Method 1:} & \quad 2\text{-O-Allyl-6-O-benzyl-myoinositol-1,3,5-O-orthobenzoate (79, 500 mg, 1.26 mmol) was dissolved in TFA-water (4:1 v/v, 10 ml) and stirred overnight. The reaction mixture was then evaporated to dryness and re-evaporated from MeOH (3 × 5 ml). The residue was taken up in MeOH (10 ml), NaOMe (25 wt. % solution in MeOH) was added dropwise until the reaction solution attained pH 11, then the reaction was stirred overnight. The solution was neutralised by the dropwise addition of 0.1 M HCl and the solvent was evaporated. The crude product was partitioned between CHCl\(_3\) (10 ml) and water (10 ml), and the aqueous layer separated collected. The organic layer was then washed with water (3 × 10 ml), and the combined aqueous layers evaporated to dryness. The crude product was taken up in MeOH (10 ml). The solution was filtered, and the filtrate evaporated to afford the \textit{title compound} (109, 391mg, 100%) as a colourless oil, which was used without further purification. \\
\text{Method 2:} & \quad 2\text{-O-Allyl-4-O-para-methoxybenzyl-6-O-benzyl-myoinositol-1,3,5-O-orthobenzoate (108, 9.30 g, 18.0 mmol) was dissolved in TFA-water (4:1 v/v, 50 ml) and stirred overnight. The reaction mixture was evaporated to dryness and then re-evaporated from MeOH-CHCl\(_3\) (1:1 v/v, 3 × 20 ml). The residue was taken up in MeOH-CHCl\(_3\) (1:1 v/v, 50 ml), NaOMe (25 wt. % solution in MeOH) was added dropwise until the reaction solution attained pH 11, and the reaction was stirred overnight. The solution was neutralised by the dropwise addition of 0.1 M HCl and the solvent was evaporated. The crude product was partitioned between CHCl\(_3\) (25 ml) and water (25 ml), and the aqueous layer collected. The organic layer was then washed with water (3 × 20 ml), and the combined aqueous layers
\end{align*}
\]
evaporated to dryness. The crude product was taken up in MeOH (10 ml). The solution was filtered, and the filtrate evaporated to afford the title compound (109, 5.59 g, 100%) as a colourless oil, which was used without further purification. \( R_f \) (EtOAc) 0.25; \( \delta_H \) (400 MHz; CD$_3$OD) 7.43 (2H, bd, J 7.2), 7.30 (2H, bt, J 7.2), 7.24 (1H, tt, J 7.2, 1.4) (5 × Ph-H), 6.00 (1H, ddt, J 17.3, 10.4, 5.7, OCH$_2$CH$_2$), 5.25 (1H, dq, J 17.3, 1.8), 5.11 (1H, ddt, J 10.4, 2.1, 1.3) (OCH$_2$CH$_2$), 4.86 (1H, d, J 10.7), 4.84 (1H, d, J 10.7) (OCH$_2$Ph), 4.37-4.29 (2H, m, OCH$_2$CH$_2$), 3.77 (1H, t, J 2.6, Ins 2-H), 3.64 (1H, t, J 9.5), 3.60 (1H, t, J 9.4), 3.52 (1H, dd, J 9.8, 2.6), 3.38 (1H, dd, J 9.9, 2.6) (4 × Ins-H), 3.29 (1H, t, J 9.1, Ins 5-H); \( \delta_C \) (100 MHz; CD$_3$OD) 140.5 (Ph C), 137.2 (OCH$_2$CH$_2$), 129.2 (2C), 129.1 (2C), 128.5 (5 × Ph CH), 116.5 (OCH$_2$CH$_2$), 83.5 (Ins CH), 82.3 (Ins 2 CH), 76.7 (Ins 5 CH), 76.0 (OCH$_2$Ph), 75.6 (OCH$_2$CH$_2$), 74.8, 73.6, 73.6 (3 × Ins CH); MS (FAB+) \( m/z \) (%): [M+H]$^+$ 333 (100), [PhCH$_2$]$^+$ 91 (29); HRMS (ES+) \( m/z \) (%): found [M+Na]$^+$ 333.1317 (70), \( C_{16}H_{22}O_6Na \) requires 333.1314.

1-H-Tetrazole (34)

![Figure 57. Sublimation apparatus.](image)

Sodium nitrite (69 g, 1.0 mol) in water (200 ml) was slowly added to a mechanically stirred suspension of 5-aminotetrazole monohydrate (103 g, 1.0 mol) and 50% aqueous hypophosphous acid (120.5 ml, 1.1 mol) in water (500 ml). The reaction mixture was
maintained at 35 °C (initially by cooling, then by heating) for 30 min after addition of the sodium nitrite. After cooling the pH was adjusted to ~3.5 with 5 M sodium hydroxide solution, and the reaction mixture evaporated to dryness. The residue was refluxed for 15 min in EtOAc (250 ml), and then the supernatant was decanted. Extraction by refluxing and decanting was repeated (4 × 100 ml), and the extracts were combined, then evaporated to dryness to afford crude 1-H-tetrazole. A drying pistol with its end cap filled with batches of ~2 g crude 1-H-tetrazole – with glass wool to hold it in place – was attached to a high vacuum oil pump, and heated using a Kugelröhr heater (Figure 57). Careful control the temperature (< 130 °C), and pressure (~0.01 mmHg) allowed the crude 1-H-tetrazole to be sublimed to afford the pure product (34).

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2,6-O-dibenzyl-myoinositol (114)

2,6-O-Dibenzyl-myoinositol (99, 50 mg, 0.14 mmol) was evaporated from MeCN (2 × 1 ml) and then tetrazole (246 mg, 1.94 mmol) was added and the mixture evaporated again from MeCN (1 ml). The mixture was taken up in MeCN (2.5 ml) and N,N-diisopropyl dibenzyl phosphoramidite (320 µl, 0.97 mmol) in DCM (2.5 ml) was added drop-wise. The reaction was stirred for 2 h. The reaction was cooled to -20 °C, before adding mCPBA (77% max., 435 mg, 1.94 mmol) in DCM (2.5 ml). The solution was then stirred for a further 1 h at 0 °C. The reaction mixture was then taken up in DCM (15 ml) and washed with 10% aq. sodium thiosulfate (2 × 10 ml), sat. aq. NaHCO₃ (2 × 7.5 ml), water (7.5 ml), then brine (7.5 ml). The organic layer was dried over Na₂SO₄, filtered and the filtrate evaporated. The residue was fractionated by flash column chromatography, eluting with hexane-EtOAc (1:1 v/v), to give the title compound (114, 101 mg, 52%). Rₜ (hexane-EtOAc, 2:3 v/v) 0.55; δH (500 MHz; CDCl₃) 7.39-7.36 (2H, m), 7.29-7.09 (46H, m), 6.98-6.96 (2H, m) (50 × Ph-H), 5.09-4.73 [20H, m, (19 × OCH₂Ph) + Ins 4-H], 4.69 (1H, t, J 1.9, Ins 2-H), 4.64 (1H, dd, J 11.8, 8.8, POCH₂Ph), 4.46 (1H, q, J 9.2, Ins 5-H), 4.32 (1H, td, J 9.8, 1.9, Ins 1-H), 4.25 (1H, td, J 9.4, 1.6, Ins 3-H), 4.09 (1H, t, J 9.5, Ins 6-H); δP (162 MHz; CDCl₃) -0.05, -0.29, -0.47, -0.80; δC (125 MHz; CDCl₃) 138.1, 138.0, 136.1 (d, J 7.5), 136.0 (d, J 6.9), 135.9 (d, J
Polar group exchange receptor-ligand engineering of protein kinase B's pleckstrin homology domain

7.4), 135.7 (d, J 7.1), 135.6 (d, J 6.9), 135.5 (2C, d, J 7.2), 135.4 (d, J 6.7) (10 × Ph C), 128.6-127.1 (m, 50 × Ph CH), 78.1-77.9 (m, Ins 5 CH), 77.7 (d, J 7.7, Ins 6 CH), 77.2 (Ins 2 CH), 77.1 (d, J 5.8, Ins 1 CH), 76.0 (dt, J 8.6, 5.3, Ins 4 CH), 75.8 (OCH2Ph), 75.4-75.3 (m, Ins 3 CH), 74.6 (OCH2Ph), 69.75 (d, J 5.7), 69.58 (d, J 5.4), 69.52 (d, J 5.6), 69.50 (d, J 5.7), 69.48 (2C, d, J 5.6), 69.28 (d, J 5.4), 69.22 (d, J 5.2) (8 × POCH2Ph); MS (FAB+) m/z (%): [M+H]+ 1401 (56), [M-PhCH2]+ 1310 (30), [PhCH2]+ 91 (100).

myo-Inositol-1,3,4,5-O-tetrakisphosphate (115)

Hydrogenolysis of 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2,6-O-dibenzyl-myoinositol (114, 110 mg, 79 μmol) was effected using general method 1 followed by general method 2 to give the ammonium salt of the title compound (115, 53 mg, 100%). δH (500 MHz; D2O) 4.26 (1H, q, J 9.5, Ins 4-H), 4.25 (1H, t, J 2.7, Ins 2-H), 3.98 (1H, td, J 9.8, 2.8, Ins 3-H), 3.92 (1H, td, J 9.3, 2.5, Ins 1-H), 3.91 (1H, q, J 9.2, Ins 5-H), 3.77 (1H, t, J 9.6, Ins 6-H); δC (202 MHz; D2O) 1.49, 1.13, 0.59, 0.42; δC (125 MHz; D2O) 78.2 (dd, J 6.1, 3.1, Ins 5 CH), 76.0 (ddd, J 6.5, 4.8, 3.1, Ins 4 CH), 74.5 (d, J 5.5, Ins 1 CH), 74.3 (dd, J 5.7, 2.1, Ins 3 CH), 70.9 (dd, J 5.8, 2.8, Ins 6 CH), 70.5 (Ins 2 CH); HRMS (ES-) m/z (%): found [M-H]- 498.9274 (100), C6H15O18P4 requires 498.9209.

1,4,5-O-Tris(dibenzyloxyphosphoryl)-2,3,6-O-trisbenzyl-myoinositol (116)

2,3,6-O-Dibenzyl-myoinositol (105, 76 mg, 0.17 mmol) was evaporated from MeCN (2 × 1 ml) and then tetrazole (118 mg, 1.68 mmol) was added and the mixture evaporated again from MeCN (1 ml). The mixture was taken up in MeCN (2 ml) and N,N-diisopropyl
dibenzyl phosphoramidite (277 µl, 0.84 mmol) in DCM (2 ml) was added drop-wise. The reaction was stirred for 2 h. The reaction was cooled to -20 °C, before adding mCPBA (77% max., 245 mg, 1.09 mmol) in DCM (2 ml). The solution was then stirred for a further 1 h at 0 °C. The reaction mixture was taken up in DCM (10 ml) and washed with 10% aq. sodium thiosulfate (2 × 10 ml), sat. aq. NaHCO₃ (2 × 7.5 ml), water (7.5 ml), then brine (7.5 ml). The organic layer was dried over Na₂SO₄, filtered and the filtrate evaporated. The residue was fractionated by flash column chromatography, eluting with hexane-EtOAc (1:1 v/v), to give the title compound (116, 114 mg, 55%).

**myo-Inositol-1,4,5-O-trisphosphate (117)**

\[
\text{HO} - \text{O} - \text{P} - \text{O} - \text{O} - \text{P} - \text{O} - \text{HO}
\]

1,4,5-O-Tris(dibenzylxylophosphoryl)-2,3,6-O-tribenzyl-mylo-inositol (116, 88 mg, 72 µmol) was taken up in 'BuOH-water (6:1 v/v, 7 ml), then sodium bicarbonate (36 mg, 0.43 mmol) and Pd-black (114 mg, 1.07 mmol) were added. The reaction vessel was flushed with N₂ (× 3) followed by H₂ (× 3). The reaction mixture was stirred under 1 atm. H₂ for 24 h. The solution was filtered, the remaining solids washed with water (15 ml), and the combined filtrates evaporated under reduced pressure. The residue was taken up in water (15 ml) and washed with CHCl₃ (15 ml). The aqueous layer was evaporated under reduced pressure to
give the sodium salt. Using general method 2 then gave the ammonium salt of the title compound (117, 53 mg, 100%). δ_H (400 MHz; D_2O) 4.15 (1H, t, J 2.8, Ins-2-H), 4.15 (1H, q, J 9.2, Ins-H), 3.90 (1H, td, J 9.7, 2.7, Ins 1-H), 3.89 (1H, q, J 8.9, Ins-H), 3.79 (1H, t, J 9.5, Ins 6-H), 3.61 (1H, dd, J 9.8, 2.7, Ins 3-H); δ_P (162 MHz; D_2O) 2.06, 1.32, 0.20; δ_C (125 MHz; CDCl_3) 78.1 (t, J 5.3), 76.5 (dd, J 6.1, 3.4), 75.0 (d, J 5.6), 71.1 (t, J 4.5), 70.6-70.5 (2C, m) (6 × Ins CH); HRMS (ES- m/z (%): found [M-H] 418.9566 (100), C_8H_14O_15P_3 requires 418.9546.

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-allyl-6-O-benzyl-myo-inositol (118)

2-O-Allyl-6-O-benzyl-myo-inositol (109, 380 mg, 1.22 mmol) was evaporated from MeCN (2 × 2 ml) and then tetrazole (1.20 g, 17.15 mmol) was added and the mixture evaporated again from MeCN (2 ml). The mixture was taken up in MeCN (10 ml) and N,N-diisopropyl dibenzyl phosphoramidite (2.82 ml, 8.58 mmol) in DCM (10 ml) was added. The reaction was stirred for 2 h, then cooled to -20 °C, before adding mCPBA (77% max., 2.46 g, 11.03 mmol) in DCM (10 ml). The solution was then stirred for a further 1 h at 0 °C. The reaction mixture was taken up in DCM (20 ml) and washed with 10%aq. sodium thiosulfate (2 × 15 ml), sat. aq. NaHCO_3 (2 × 10 ml), water (10 ml), then brine (10 ml). The organic layer was dried over Na_2SO_4, filtered and the filtrate evaporated. The residue was fractionated by flash column chromatography, eluting with hexane-EtOAc (1:1 v/v), to give the title compound (118, 721 mg, 44%). R_t (hexane-EtOAc, 1:2 v/v) 0.45; δ_H (400 MHz; CDCl_3) 7.39-7.35 (2H, m), 7.32-7.09 (41H, m), 6.99-6.95 (2H, m) (45 × Ph-H), 5.84 (1H, ddt, J 17.2, 10.6, 5.3, OCH_2CHCH_3), 5.17 (1H, dq, J 17.2, 1.7, OCH_2CHCHH), 5.09-4.73 [19H, m, (17 × OCH/HPH) + OCH_2CHCHH + Ins 4-H], 4.66 (1H, dd, J 11.8, 8.8, POCH/PH), 4.53 (1H, t, J 2.3, Ins 2-H), 4.43 (1H, q, J 9.2, Ins 5-H), 4.25 (1H, ddd, J 9.9, 7.4, 2.5, Ins 1-H), 4.24-4.21 (2H, m, OCH_2CHCH_3), 4.17 (1H, ddd, J 9.9, 7.5, 2.4, Ins 3-H), 4.05 (1H, t, J 9.5, Ins 6-H); δ_P (162 MHz; CDCl_3) -0.68, -0.95, -1.11, -1.45; δ_C (100 MHz; CDCl_3) 138.1, 136.08 (d, J 7.6), 136.07 (d, J 6.8), 135.96 (d, J 7.4), 135.79 (d, J 7.1),
135.62 (d, J 6.9), 135.56 (d, J 7.4), 135.54 (d, J 7.4), 135.49 (d, J 7.1) (9 × Ph C), 134.51 (OCH₂CHCH₂), 128.6-127.2 (m, 45 × Ph CH), 116.6 (OCH₂CHCH₂), 78.2-78.0 (m, Ins 5 CH), 77.72 (d, J 7.2, Ins 6 CH), 77.16 (d, J 6.0, Ins 1 CH), 76.8 (Ins 2 CH), 76.2-75.8 (m, Ins 4 CH), 75.6-75.3 (m, Ins 3 CH), 74.7, 74.6 (2 × CH₂), 69.75 (d, J 5.9), 69.61 (d, J 5.4), 69.54-69.47 (3C, m), 69.53 (d, J 5.5), 69.31 (d, J 5.9), 69.25 (d, J 6.1) (8 × PO₂CH₂Ph); MS (FAB+) m/z (%): [M+H]+ 1351 (15), [PhCH₂]+ 91 (56).

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-(2,3-epoxypropyl)-6-O-benzyl-myoinositol (119)

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-allyl-6-O-benzyl-myoinositol (118, 66 mg, 49 μmol) was dissolved in CHCl₃ (1 ml), and cooled to 0 °C. mCPBA (77% max., 33 mg, 147 μmol) was added, and the reaction mixture allowed to warm to room temperature. The solution was then stirred for 48 h. The reaction mixture was taken up in DCM (5 ml) and washed with 10% aq. sodium thiosulfate (25 ml), sat. aq. NaHCO₃ (25 ml), water (5 ml), then brine (5 ml). The organic layer was dried over Na₂SO₄, filtered and the filtrate evaporated. The residue was fractionated by flash column chromatography, eluting with hexane-EtOAc (1:2 v/v), to give an inseparable ~2:1 mixture of diastereoisomers of the title compound (118, 24 mg, 35%). Rᵣ(hexane-EtOAc, 1:2 v/v) 0.30; δᵣ (400 MHz; CDCl₃) 7.41-7.36 (2H, m), 7.34-7.10 (41H, m), 7.01-6.96 (2H, m) (45 × Ph -H), 5.12-4.60 [19H, m, (18 × OCH₂HPh) + Ins 4-H], 4.55 (1H, d, J 9.2, Ins 2-H), 4.45 (1H, q, J 9.2, Ins 5-H), 4.26 (1H, td, J 10.0, 2.2, Ins-H), 4.18 (1H, td, J 8.6, 2.0, Ins-H), 4.07 (0.3H, t, J 9.4, Ins 6-H), 4.06 (0.7H, t, J 9.6, Ins 6-H), 3.90 (0.3H, dd, J 11.4, 3.3), 3.88 (0.7H, dd, J 11.4, 3.3), 3.65 (0.7H, dd, J 11.4, 5.6), 3.55 (0.3H, dd, J 11.2, 5.3) (OCH₂CHOCH₂), 3.10-3.05 (1H, m, OCH₂CHOCH₂), 2.66 (1H, t, J 4.4), 2.45 (0.7H, dd, J 5.0, 2.7), 2.40 (0.3H, dd, J 5.0, 2.6) (2 × OCH₂CHOCH₂); δᵣ (162 MHz; CDCl₃) -1.32 (1P), -1.58 (1P), -1.85 (0.3P), -1.79 (0.7P), -2.16 (1P); MS (FAB+) m/z (%): [M+H]+ 1368 (100).
myo-Inositol-1,3,4,5-O-tetrakisphosphate-2-O-propyl (120)

Hydrogenolysis of 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2-O-allyl-6-O-benzyl-myoinositol (118, 50 mg, 37 μmol) was effected using general method 1 followed by general method 2, to give the ammonium salt of the title compound (120, 25 mg, 100%). \( \delta_H \) (500 MHz; D_2O) 4.36 (1H, q, \( J = 9.4 \), Ins 4-H), 4.16 (1H, t, \( J = 2.6 \), Ins 2-H), 4.05 (1H, td, \( J = 9.9, 2.6 \), Ins 3-H), 3.98 (1H, q, \( J = 9.0 \), Ins 5-H), 3.97 (1H, td, \( J = 9.5, 2.8 \), Ins 1-H), 3.82 (1H, t, \( J = 9.6 \), Ins 6-H), 3.78-3.70 (2H, m, OCH_2CH_2CH_3), 1.55 (2H, sextet, \( J = 7.1 \), OCH_2CH_2CH_3), 0.85 (3H, t, \( J = 7.4 \), OCH_2CH_2CH_3); \( \delta_C \) (125 MHz; D_2O) 78.4 (dd, \( J = 6.8, 3.0 \), Ins 5 CH), 78.1 (Ins 2 CH), 76.5 (dt, \( J = 6.0, 3.3 \), Ins 4 CH), 75.9 (OCH_2CH_2CH_3), 74.7 (d, \( J = 5.4 \), Ins 1 CH), 74.4 (dd, \( J = 5.9, 2.1 \), Ins 3 CH), 71.1 (dd, \( J = 6.3, 2.8 \), Ins 6 CH), 22.6 (OCH_2CH_2CH_3), 9.9 (OCH_2CH_2CH_3); HRMS (ES-) m/z (%): found [M-H]^– 540.9670 (100), C_{9}H_{21}O_{18}P_{4} requires 540.9678.

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-acetaldehyde-6-O-benzyl-myoinositol (122)

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-allyl-6-O-benzyl-myoinositol (118, 754 mg, 0.558 mmol) was taken up in acetone-water (9:1 v/v, 8 ml) and N-methylmorpholine-N-oxide (226 mg, 1.67 mmol), followed by OsO_4 (4% in water, 226 μl) was added. The reaction was stirred for 16 h. The solvent was evaporated and the residue suspended in ether (10 ml), then washed with water (2 × 10 ml), then brine (10 ml). The organic layer was dried
over Na$_2$SO$_4$, filtered and the filtrate evaporated. This crude mixture was dissolved in dioxane (9 ml) and sat. aq. NaIO$_4$ (3 ml) was added. The solution was stirred for 1 h. The solvent was evaporated, and the residue was taken up in DCM (20 ml) and washed with sat. aq. NaHCO$_3$ (3 × 20 ml), then brine (20 ml). The organic layer was dried over Na$_2$SO$_4$, filtered and the filtrate evaporated. The residue was fractionated by flash column chromatography, eluting with DCM-EtOAc (9:1 v/v), to give the title compound (122, 619 mg, 82%). $R_f$ (DCM-EtOAc, 9:1 v/v) 0.65; $\delta_H$ (400 MHz; CDCl$_3$) 9.61 (1H, bs, OCH$_2$CHO), 7.44-7.41 (2H, m), 7.40-7.12 (41H, m), 7.06-7.03 (2H, m) (45 × Ph-H), 5.15-4.68 [19H, m, (9 × OCH$_2$Ph) + Ins 4-H], 4.62 (1H, t, J 2.0, Ins 2-H), 4.60 (1H, q, J 9.2, Ins 5-H), 4.32 (1H, td, J 8.7, 2.0, Ins 1-H), 4.24 (1H, td, J 8.6, 1.9, Ins 3-H), 4.22 (2H, s, OCH$_2$CHO), 4.15 (1H, t, J 9.6, Ins 6-H); $\delta_D$ (162 MHz; CDCl$_3$) -1.26, -1.51, -1.71, -2.08; $\delta_C$ (100 MHz; CDCl$_3$) 199.9 (CHO), 138.0, 136.2 (d, J 7.6), 136.02 (d, J 7.0), 135.98 (d, J 7.4), 135.8 (d, J 7.0), 135.6 (d, J 6.8), 135.44 (d, J 7.0), 135.41 (2C, m) (9 × Ph C), 128.7-127.3 (45 × Ph CH), 78.9 (OCH$_2$CHO), 78.8 (Ins 2 CH), 78.1-77.9 (m, Ins 5 CH), 77.6 (d, J 8.7, Ins 6 CH), 76.9 (d, J 6.8, Ins 1 CH), 75.9 (dt, J 9.2, 4.8, Ins 4 CH), 75.1 (d, J 3.9, Ins 3 CH), 74.8 (OCH$_2$Ph), 70.0 (d, J 5.7), 69.9 (d, J 5.4), 69.8 (d, J 5.6), 69.66-69.63 (3C, m), 69.62 (d, J 5.4), 69.4 (d, J 5.2) (8 × POCH$_2$Ph); MS (MALDI+) m/z (%): [M+K]$^+$ 1416 (82), [M+Na]$^+$ 1376 (100).

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-acetic acid-6-O-benzyl-myoinositol (121)
used with no further purification. \( R_f \) (DCM-EtOAc, 9:1 v/v) 0.15; \( \delta_H \) (400 MHz; CDCl3-dacetic acid) 7.38-7.09 (43H, m), 7.03-6.98 (2H, m) (45 × Ph-H), 5.10-4.64 [19H, m, (9 × OCH2Ph) + Ins 4-H], 4.59 (1H, bs, Ins 2-H), 4.60-4.52 (1H, bs, Ins 5-H), 4.40-4.25 (2H, m, 2 × Ins-H), 4.35 (2H, s, OCH2COOH), 4.17 (1H, t, J 9.5, Ins 6-H); \( \delta_C \) (100 MHz; CDCl3) -2.06, -2.18, -2.47, -2.71; \( \delta_C \) (100 MHz; CDCl3) 177.5 (OCH2COOH), 138.0, 135.8-135.2 (8C, m) (9 × Ph-C), 128.7-127.3 (45 × Ph-CH), 78.5 (Ins 2 CH), 78.1-77.9 (m, Ins 5 CH), 77.5 (dd, J 7.6, 1.9, Ins 6 CH), 76.9 (d, J 5.9, Ins 1 CH), 76.0 (dt, J 8.2, 4.5, Ins 4 CH), 75.0-74.9 (m, Ins 3 CH), 74.8 (OCH2Ph), 70.7 (OCH2COOH), 70.3 (d, J 5.9), 70.2 (d, J 5.6), 70.12-70.01 (2C, m), 70.00 (d, J 5.8), 69.93-69.87 (2C, m), 69.75 (d, J 5.3) (8 × POCH2Ph); MS (ES+) \( m/z \) (%): [M+Na]+ 1391 (40), [M+H]+ 1369 (93).

**myo-Inositol-1,3,4,5-O-tetrakisphosphate-2-O-acetic acid (1)**

![myo-Inositol-1,3,4,5-O-tetrakisphosphate-2-O-acetic acid (1)](image)

Hydrogenolysis of 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2-O-acetic acid-6-O-benzylmyo-inositol (121, 14 mg, 10 \( \mu \)mol) was effected using *general method 1* followed by *general method 2*, to give the ammonium salt of the *title compound* (1, 8 mg, 100%). \( \delta_H \) (500 MHz; D2O) 4.25 (1H, q, J 9.3, Ins 4-H), 4.24 (1H, d, J 15.4), 4.17 (1H, d, J 15.4) (OCH2COOH), 4.10 (1H, t, J 2.1, Ins 2-H), 3.94 (1H, td, J 9.9, 2.6, Ins 3-H), 3.91-3.81 (3H, m, 3 × Ins-H); \( \delta_C \) (202 MHz; D2O) 3.49, 3.33, 3.05, 2.85; \( \delta_C \) (100 MHz; D2O) 178.5 (OCH2COOH), 79.0 (Ins 2 CH), 78.3 (dd, J 6.2, 3.1, Ins 5 CH), 76.1 (dt, J 5.9, 4.6, Ins 4 CH), 74.2 (d, J 5.3, Ins 1 CH), 74.0 (dd, J 5.8, 2.0, Ins 3 CH), 72.6 (OCH2COOH), 71.5 (dd, J 5.2, 2.2, Ins 6 CH); HRMS (ES-) \( m/z \) (%): found [M-H]- 556.9276 (65), \( C_{81}H_{17}O_{20}P_4 \) requires 556.9264.
1,3,4,5-\(O\)-Tetrakis(dibenzyloxyphosphoryl)-2-\(O\)-(2-hydroxyethyl)-6-\(O\)-benzyl-myoinositol (128)

1,3,4,5-\(O\)-Tetrakis(dibenzyloxyphosphoryl)-2-\(O\)-acetaldehyde-6-\(O\)-benzyl-myoinositol (122, 482 mg, 0.36 mmol) was taken up in MeOH (30 ml). Acetic acid (300 \(\mu\)l) was added, followed by NaBH\(_3\)CN (1 M in THF, 356 \(\mu\)l, 0.36 mmol). The solution was stirred for ~2 h, monitoring extent of reaction by TLC and staining with 2,4-DNP. When the reaction had gone to completion it was taken up in DCM (20 ml), washed with sat. aq. NaHCO\(_3\) (2×15 ml), and brine (15 ml). The organic layer was dried over MgSO\(_4\), filtered and the filtrate evaporated. The title compound (128, 309 mg, 64%) was used with no further purification. 

\(R_f\) (DCM-EtOAc, 9:1 v/v) 0.65; \(\delta_H\) (500 MHz; CDCl\(_3\)) 7.41-7.37 (2H, m), 7.34-7.13 (41H, m), 7.04-7.01 (2H, m) (45 × Ph-\(H\)), 5.10-4.67 [19H, m, (9 × OCH\(_2\)Ph) + Ins 4-\(H\)], 4.57 (1H, t, J 2.4, Ins 2-\(H\)), 4.46 (1H, q, J 9.3, Ins 5-\(H\)), 4.29 (1H, ddd, J 10.1, 7.5, 2.5, Ins 1-\(H\)), 4.21 (1H, ddd, J 9.8, 7.5, 2.3, Ins 3-\(H\)), 4.08 (1H, t, J 9.3, Ins 6-\(H\)), 3.88-3.82 (2H, m, OCH\(_2\)CH\(_2\)O), 3.65 (2H, t, J 4.4, OCH\(_2\)CH\(_2\)O); \(\delta_P\) (202 MHz; CDCl\(_3\)) -1.22, -1.64, -1.73, -2.03; \(\delta_C\) (125 MHz; CDCl\(_3\)) 138.0, 136.02 (d, J 7.2, 135.98 (d, J 7.2), 135.94 (d, J 7.4), 135.78 (d, J 7.0), 135.58 (d, J 6.9), 135.54 (d, J 7.2), 135.50 (d, J 7.4), 135.45 (d, J 7.0) (9 × Ph C), 128.6-127.3 (m, 45 × Ph CH), 78.0-77.9 (m, Ins 5 CH), 77.8 (d, J 6.4, Ins 6 CH), 77.2 (d, J 6.1, Ins 1 CH), 76.9 (Ins 2 CH), 76.0 (dt, J 8.3, 5.0, Ins 4 CH), 75.41 (OCH\(_2\)CH\(_2\)O), 75.35 (d, J 4.7, Ins 3 CH), 74.7 (OCH\(_2\)Ph), 69.93 (d, J 5.6), 69.85 (d, J 5.3), 69.71 (d, J 5.5), 69.61 (2C, d, J 5.8), 69.59 (d, J 5.9), 69.56 (d, J 5.8), 69.38 (d, J 5.1) (8 × POCH\(_2\)Ph), 61.9 (OCH\(_2\)CH\(_2\)O); MS (ES+) \textit{m/z} (%): [M+Na]\(^+\) 1377 (95), [M+H]\(^+\) 1355 (100).
myo-Inositol-1,3,4,5-O-tetrakisphosphate-2-O-(2-hydroxyethyl) (129)

Hydrogenolysis of 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2-O-(2-hydroxyethyl)-6-O-benzyl-myoinositol (128, 80 mg, 59 µmol) was effected using general method 1 followed by general method 2, to give the ammonium salt of the title compound (129, 40 mg, 100%).

\[ \delta_{H} (400 \text{ MHz}; D_{2}O) 4.31 (1H, q, J 9.4, \text{Ins} 4-H), 4.12 (1H, t, J 2.6, \text{Ins} 2-H), 4.02 (1H, td, J 9.9, 2.6, \text{Ins} 3-H), 3.96 (1H, td, J 9.9, 2.5, \text{Ins} 1-H), 3.91 (1H, q, J 9.0, \text{Ins} 5-H), 3.84 (2H, t, J 4.6, OCH_{2}CH_{2}O), 3.82 (1H, t, J 9.4, \text{Ins} 6-H), 3.70-3.60 (2H, m, OCH_{2}C_{6}H_{5}O); \delta_{P} (162 \text{ MHz}; D_{2}O) 2.75, 2.49, 1.71, 1.41; \delta_{C} (100 \text{ MHz}; D_{2}O) 78.7 (\text{Ins} 2 \text{ CH}), 78.3 (\text{dd}, J 6.3, 2.8, \text{Ins} 5 \text{ CH}), 76.2 (\text{dt}, J 5.8, 2.9, \text{Ins} 4 \text{ CH}), 74.7 (d, J 5.4, \text{Ins} 1 \text{ CH}), 74.5 (OCH_{2}CH_{2}O), 74.3 (\text{dd}, J 6.0, 1.9, \text{Ins} 3 \text{ CH}), 71.1 (\text{dd}, J 6.0, 2.8, \text{Ins} 6 \text{ CH}), 61.0 (OCH_{2}CH_{2}O); \text{HRMS (ES-)} m/z (%): found \text{[M-H]}^{-} 542.9456 (92), C_{8}H_{19}O_{19}P_{4} \text{requires} 542.9471.

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-(2-methanesulfonyloxyethyl)-6-O-benzyl-myoinositol (130)

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-(2-hydroxyethyl)-6-O-benzyl-myoinositol (128, 105 mg, 78 µmol) was taken up in pyridine (1 ml), and cooled to 0 °C. MsCl (7.80 µl, 97 µmol) was added, and the solution stirred for 2 h at room temperature. The solvent was evaporated, and the residue resuspended in DCM (10 ml), then washed with sat. aq. NaHCO_{3} (10 ml), water (10 ml), then brine (10 ml). The organic layer was dried over MgSO_{4}, filtered and the filtrate evaporated. The residue was fractionated by flash column
chromatography, eluting with hexane-EtOAc (1:3 v/v), to give the title compound (130, 34 mg, 31%). \( R_t \) (hexane-EtOAc, 1:3 v/v) 0.80; \( \delta_H \) (400 MHz; CDCl3) 7.46-7.11 (43H, m), 7.02-6.99 (2H, m) (45 × Ph-H), 5.10-4.65 [19H, m, (9 × OCH2Ph) + Ins 4-H], 4.57 (1H, t, \( J \) 2.2, Ins 2-H), 4.44 (1H, q, \( J \) 9.3, Ins 5-H), 4.26 (1H, ddd, \( J \) 10.1, 7.4, 2.4, Ins 1-H), 4.21-4.14 (3H, m, OCH2CH2O + Ins 3-H), 4.03 (1H, t, \( J \) 9.3, Ins 6-H), 3.94-3.83 (2H, m, OCH2CH2O), 2.99 (3H, s, SO2CH3); \( \delta_F \) (162 MHz; CDCl3) -1.29, -1.58, -1.77, -2.03; \( \delta_C \) (100 MHz; CDCl3) 138.0, 135.97 (d, \( J \) 6.8), 135.93 (d, \( J \) 6.7), 135.92 (d, \( J \) 6.9), 135.72 (d, \( J \) 7.0), 135.54-135.46 (2C, m), 135.43 (d, \( J \) 6.9), 135.42-135.37 (m) (9 × Ph C), 129.5-127.3 (45 × Ph CH), 78.0-77.9 (m, Ins 5 CH), 77.7 (Ins 6 CH), 77.5 (d, \( J \) 8.3, Ins 1 CH), 77.3 (Ins 2 CH), 77.0 (d, \( J \) 8.3, Ins 4 CH), 75.0 (d, \( J \) 4.7, Ins 3 CH), 74.8 (OCH2Ph), 71.4 (OCH2CH2O), 69.98 (d, \( J \) 5.7), 69.90 (d, \( J \) 5.6), 69.75 (d, \( J \) 5.7), 69.62 (2C, d, \( J \) 5.7), 69.60 (2C, d, \( J \) 5.7), 69.39 (d, \( J \) 5.3) (8 × POCH2Ph), 68.8 (OCH2CH2O), 37.5 (SO2CH3); MS (ES+) m/z (%): [M+Na]+ 1455 (100), [M+H]+ 1433 (87).

1,3,4,5-O-Tetrakis(dibenzyl oxyphosphoryl)-2-O-(2-azidoethyl)-6-O-benzyl-myoinositol (131)

[Diagram of the compound]

DIAD (52.9 µl, 0.27 mmol) was added dropwise to a cooled (-5 °C) solution of Ph3P (65 mg, 0.25 mmol) in THF (0.4 ml). After 30min, a solution of 1,3,4,5-O-tetrakis(dibenzyl oxyphosphoryl)-2-O-(2-hydroxyethyl)-6-O-benzyl-myoinositol (128, 140 mg, 0.10 mmol) in THF (0.3 ml) was added. After a further 10 min, diphenyl phosphorazidate (DPPA, 52.5 µl, 0.24 mmol) was added and the reaction mixture was allowed to attain room temperature. After stirring overnight, the reaction was taken up in DCM (10 ml) and washed with sat. aq. NaHCO3 (2 × 10 ml), then brine (10 ml). The organic layer was dried over Na2SO4, filtered and the filtrate evaporated. The residue was fractionated by flash column chromatography, eluting with hexane-EtOAc (1:3 v/v), to give the title compound (131, 68 mg, 48%). \( R_t \) (hexane-EtOAc, 1:1 v/v) 0.50; \( \delta_H \) (400 MHz; CDCl3) 7.42-7.38 (2H, m), 7.35-7.12 (41H, m), 7.01-6.98 (2H, m) (45 × Ph-H), 5.13-4.79
[18H, m, (17 ∗ OCH(Ph)) + Ins 4-H], 4.66 (1H, dd, J 11.8, 8.9, POCH(Ph)), 4.57 (1H, t, J 2.5, Ins 2-H), 4.46 (1H, q, J 9.3, Ins 5-H), 4.28 (1H, ddd, J 10.0, 7.7, 2.3, Ins 1-H), 4.20 (1H, ddd, J 9.9, 7.5, 2.4, Ins 3-H), 4.08 (1H, t, J 9.6, Ins 6-H), 3.90 (1H, ddd, J 10.3, 5.8, 3.5), 3.81 (1H, ddd, J 10.4, 7.0, 3.4) (CH₂CH₂), 3.27 (1H, ddd, J 13.4, 7.1, 3.5), 3.16 (1H, ddd, J 13.4, 5.8, 3.4) (CH₂CH₂); δp (162 MHz; CDCl₃) -1.37, -1.52, -1.81, -2.07; δc (100 MHz; CDCl₃) 138.1, 136.02 (2C, d, J 7.5), 135.94 (d, J 7.9), 135.53 (d, J 6.4), 135.48 (d, J 7.2), 135.46 (d, J 7.2), 135.41 (d, J 6.9) (9 ∗ Ph C), 128.6-127.1 (45 ∗ Ph CH), 78.0-77.9 (m, Ins 5 CH), 77.7 (Ins 2 CH), 77.5 (dd, J 8.0, 1.8, Ins 6 CH), 76.8 (d, J 5.7, Ins 1 CH), 75.9 (dt, J 8.5, 5.5, Ins 4 CH), 75.3 (dt, J 5.5, 1.9, Ins 3 CH), 74.7 (OCH₃Ph), 73.1 (CH₂CH₂), 69.84 (d, J 5.7), 69.73 (d, J 5.5), 69.59 (d, J 5.8), 69.53 (d, J 5.7), 69.52 (2C, d, J 5.8), 69.41 (d, J 5.5), 69.25 (d, J 5.2) (8 ∗ POCH₂Ph), 50.7 (CH₂CH₂); MS (ES+) m/z (%): [M+Na]⁺ 1402 (54), [M+H]⁺ 1380 (44).

myo-Inositol-1,3,4,5-O-tetrakisphosphate-2-O-(2-aminoethyl) (138)

Hydrogenolysis of 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2-O-(2-azidoethyl)-6-O-benzyl-myoinositol (131, 80 mg, 59 µmol) was effected using general method 1, to give the sodium salt of the title compound (139, 40 mg, 100%). δH (400 MHz; D₂O) 4.26 (1H, q, J 9.2, Ins 4-H), 4.18 (1H, bs, Ins 2-H), 4.07-3.98 (2H, m, CH₂CH₂), 3.98-3.76 (4H, m, 4 ∗ Ins-H), 3.17-3.07 (2H, m, CH₂CH₂); δp (162 MHz; D₂O) 3.33, 3.20, 2.76, 1.46; HRMS (ES-) m/z (%): found [M-H]⁻ 541.9625 (100), C₈H₂₀NO₁₈P₄ requires 541.9631, found [M+Na-2H]⁻ 563.9452 (32), C₈H₁₉NO₁₈P₄Na requires 563.9450.
N,N'-Bis(benzyloxycarbonyl)guanidine (140)

N,N'-Bis(benzyloxycarbonyl)-S-methylisothiourea (1.0 g, 2.79 mmol) was dissolved in MeOH (27 ml) and 0.88 ammonia (3 ml), and stirred overnight. The precipitate was separated from the supernatant, then recrystallised from MeOH-DCM to give the title compound (141, 731 mg, 80%). δH (400 MHz; CDCl3) 9.50-8.00 (2H, bs, NH2), 7.58-7.12 (10H, m, 10 × Ph-H), 5.10 (4H, s, 2 × OC2H5Ph); δC (100 MHz; CDCl3) 158.90 (2C, 2 × CO), 135.58 (2C, 2 × Ph C), 128.52 (4C), 128.28 (2C), 128.01 (4C) (10 × Ph CH), 67.45 (2C, 2 × OC2H5Ph); mp 148 – 150 °C; HRMS (ES+) m/z (%): found [M+H]+ 328.1309 (100), C17H18N3O4 requires 328.1297.

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-[2-[N,N'-bis(benzyloxycarbonyl)guanidino]ethyl]-6-O-benzyl-myoinositol (141)

DIAD (39.1 µl, 0.20 mmol) was added dropwise to a cooled (-5 °C) solution of Ph3P (48 mg, 0.18 mmol) in THF (0.3 ml). After 30 min, a solution of 1,3,4,5-O-tetrakis(dibenzyloxyphosphoryl)-2-O-(2-hydroxyethyl)-6-O-benzyl-myoinositol (128, 100 mg, 74 μmol) in THF (250 µl) was added. After a further 10 min, N,N'-bis(benzyloxycarbonyl)guanidine (141, 58 mg, 0.18 mmol) was added and the reaction mixture was allowed to attain room temperature. After stirring overnight, the reaction was taken up in DCM (10 ml) and washed with sat.aq. NaHCO3 (2 × 10 ml), then brine (10 ml). The organic layer was dried over Na2SO4, filtered and the filtrate evaporated. The residue was fractionated by flash column chromatography, eluting with hexane-EtOAc (1:3 v/v), to give the impure title compound (141, 49 mg, 40%). Rf (hexane-EtOAc, 1:1 v/v) 0.45; δH (400 MHz; CDCl3) 7.42-7.02 (53H, m), 6.93-6.90 (2H, m) (55 × Ph-H), 5.33 (1H, d, J 12.3),
5.24 (1H, d, J 12.3) (OCH$_3$Ph), 5.12-4.52 [21H, m, (10 × OCH$_3$Ph) + Ins 4-H], 4.46-4.38 (2H, m, Ins 2-H + Ins 5-H), 4.27-4.19 (3H, m, CH$_2$CH$_2$ + Ins 1-H), 4.20 (1H, ddd, J 10.0, 7.9, 2.4, Ins 3-H), 3.93-3.85 (3H, m, CH$_2$CH$_2$ + Ins 6-H); $\delta_p$ (162 MHz; CDCl$_3$) -0.03, -0.33, -0.44, -0.65; $\delta_C$ (100 MHz; CDCl$_3$) 163.5, 160.4, 155.8 [(2 × CO) + C=N], 138.2, 136.7, 136.00 (d, J 7.5), 135.95 (d, J 7.9), 135.86 (d, J 7.0), 135.85 (d, J 6.4), 135.51-135.46 (m), 135.45-135.41 (m), 135.41-135.37 (m), 135.37-135.33 (m), 135.0 (11 × Ph C), 128.7-127.0, (55 × Ph CH), 78.1-78.0 (m), 77.8-77.7 (m), 77.23-77.15 (m), 77.13-77.04 (m), 76.2-76.1 (m), 75.2-75.1 (m) (6 × Ins CH), 74.6, 72.1 (2 × OCH$_3$Ph), 69.84 (d, J 5.7), 69.74 (d, J 5.5), 69.65-69.59 (m), 69.58-69.54 (m), 69.53-69.49 (m), 69.49-69.45 (m), 69.44-69.38 (m), 69.27 (d, J 5.1) (8 ×POCH$_3$Ph), 69.1 (OCH$_3$Ph), 667.0 (CH$_2$CH$_2$), 44.6 (CH$_2$CH$_2$); MS (ES+) m/z (%): [M+H]$^+$ 1664.6 (100).

8.2 BIOCHEMISTRY

The following protocols where either: Adapted from the standard protocols used within the Mann group; adapted from those provided by G. Rosenberg (née Busch); or protocols provided with the the reagents.

8.2.1 Transformation by electroporation

Transformation of BL21 *E.coli*, with either the pGEX-PKBα-PH or pGEX-E17A plasmids were carried out using the electroporation protocol:

- Take out *E.coli* BL21 from -80 °C freezer and thaw on ice.
- Put cuvettes on ice.
- Switch on the electroporation machine. Set to 25, 200 (black knob) and set the voltage to 2.2-2.3.
- Calibrate machine by applying a pulse across the empty cuvette holder.
- Take 1 μl of DNA and add it into the 50 μl aliquot of *E.coli*.
- Set the Gilson to about 60 μl, mix carefully and transfer into chilled cuvettes. Make sure not to get bubbles. If you get this wrong or there is too much salt it will spark. If you have a spark then the cuvette must be thrown away.
Set up the cuvettes on the electroporation machine (make sure bottom is covered with mixture) and apply the pulse.

- Add 1ml of LB into the cuvettes, mix and transfer back into an Eppendorf tube.
- Spin and remove about a half of supernatant, then resuspend the rest.
- Plate it onto LB + ampicillin and leave in 37 °C overnight. Clean cuvettes thoroughly and dry in the 37 °C room.

8.2.2 Transformation by heat shock

Transformation of BL21(DE3) *E.coli*, with either the pGEX-PKBα-PH or pGEX-E17A plasmids were carried out using the heat shock protocol:

- Take out *E.coli* BL21(DE3) from -80 °C freezer and thaw on ice.
- Take 1 µl of DNA, add it into the 50 µl aliquot of *E.coli* and swirl gently.
- Incubate the reactions on ice for 30 min.
- Preheat 1 ml LB in a 42 °C water bath.
- Heat-pulse each transformation reaction in a 42 °C water bath for 45 s. The duration of the heat pulse is critical for optimal transformation efficiencies.
- Incubate the reactions on ice for 2 min.
- Add 0.9 ml of preheated (42 °C) LB to the transformation reaction and incubate the reactions at 37 °C for 1 h with shaking at 225–250 rpm.
- Using a sterile spreader, plate ≤ 200 µl onto LB + ampicillin and leave in 37 °C overnight.

8.2.3 LB preparation of GST fusion protein

GST fusion proteins where expressed and isolated from BL21 *E.coli* in LB:

Bacterial expression

- Incubate a colony of transformed BL21 *E.coli* in three litres of LB, containing 100 µg/ml ampicillin, at 30 °C and 225 rpm for 16 h.
- Induce protein expression by adding 1 ml/l of 1M IPTG (in freezer).
- Incubate for a further 16h at 18 °C and 225 rpm.

GST fusion protein isolation

- Collect the cells from the LB into a single sample:
  - Spin samples for 5 min at 5000 rpm, 4 °C.
  - Remove supernatant and collect pellet.
- Resuspend pellet in NETN and vortex. N.B. As NETN contains detergent (i.e. NP-40), it could develop foam. Try not to get the flask full of bubbles.
- Keep on ice and sonicate at power 18 for 2 × 30 s.
- Spin for 5 min at 5000 rpm at 4 °C (If the sonication has worked well the pellet volume should be reduced compared to the initial big pellet and the supernatant solution should be brown/yellow).
- In the meantime, equilibrate glutathione sepharose beads (from Amersham).
  - Take 1.5 ml of beads from the stock into 50 ml Falcon tube.
  - Add 50ml of NETN .
  - Spin 2000 rpm, 4 °C, 2 min. Remove supernatant.
- To these beads add the supernatant from the lysed culture.
- Mix on the wheel at 4 °C for at least 1 h.
- Perform 3 × washes.
- Spin to 2000 rpm for 1 min at 4 °C.
- Remove supernatant.
- Add 40ml NETN
- Mix by inversion and repeat.
- Resuspend the beads into 15 ml of NETN and transfer into a fresh Falcon tube.
- Pulse down microfuge (up to 13000 rpm and stop immediately)
- Reduce the volume to about 2-3 ml of NETN.
- Add an equal volume of 80% glycerol.
- Let it sit on ice for about 30 min so that glycerol will be distributed evenly throughout the liquid volume, preventing the protein from freezing.
- Store at -20 °C unless running on a gel.
<table>
<thead>
<tr>
<th>Component of NETN solution</th>
<th>Volume of stock per 1 l of NETN (ml)</th>
<th>Stock concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% NP40</td>
<td>50</td>
<td>10%</td>
</tr>
<tr>
<td>20 mM Tris-HCl, pH 8</td>
<td>10</td>
<td>2 M</td>
</tr>
<tr>
<td>0.1 M NaCl</td>
<td>20</td>
<td>5 M</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>2</td>
<td>0.5 M</td>
</tr>
</tbody>
</table>

### 8.2.4 AI media preparation of GST fusion protein

GST fusion proteins where expressed and isolated from BL21(DE3) *E.coli* in AI media:

**Bacterial expression**

- Incubate a colony of transformed BL21(DE3) *E.coli* in three litres of AI media (40 mM Na₂HPO₄, 20 mM KH₂PO₄, 90 mM NaCl, 2% w/v tryptone, 0.5% w/v yeast extract), containing 100 μg/ml ampicillin and 0.6% v/v glycerol, 0.05% w/v glucose, and 0.2% w/v lactose, at 30 °C and 225 rpm for 16 h.
- Incubate for a further 16 h at 18 °C and 225 rpm.

**GST fusion protein isolation**

As for previous (section 8.2.3)

### 8.2.5 Thrombin digest

- Wash ~100 μl of GST prep (containing ~50 μl beads) 3-4 times in assay buffer.
- Adjust final volume to 200 μl.
- Add 0.5U (2 μl) of thrombin to sample.
- Incubate at room temperature for 2-3 h.
- Pulse down and remove supernatant from beads.
- Pulse supernatant down once more and remove to ensure no beads are left in sample.
- Adjust so all protein samples are stored in 50% glycerol.

_Thrombin Assay Buffer_ (made up fresh on day of use)

- 20mM Tris pH 8.0
- 150mM NaCl
- 2.5mM CaCl₂
- 10% Glycerol

### 8.2.6 SDS-PAGE and Western blot

<table>
<thead>
<tr>
<th></th>
<th>Resolving gel 12.5% (ml)</th>
<th>Stacking gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total vol.</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Ac</td>
<td>4.25</td>
<td>Ac 0.85</td>
</tr>
<tr>
<td>Tris 8.8</td>
<td>1.9</td>
<td>Tris 6.8 0.3</td>
</tr>
<tr>
<td>H₂O</td>
<td>3.65</td>
<td>H₂O 3.8</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2</td>
<td>10% SDS 0.05</td>
</tr>
</tbody>
</table>

- Load samples (2-40 µl), and markers (3 µl).
- Run at 200 volts in running buffer (can be run at lower voltage to extend the time).

**Staining gels**

- After running the gel, disassemble the apparatus and remove the stacking gel.
- Place gel in staining solution (Coomassie Brilliant Blue) for 30 min.
- Rinse the gel with warm water.
Semi-dry Western blot transfer procedure

- Cut 6-10 sheets of Whatman 3MM filter paper the same size as the PAGE gel.
- Cut membrane (PVDF) to the size of the gel.
- Membrane activation:
  - Wet PVDF membrane in methanol for 15 s.
  - Transfer to a container of ddH₂O for 5 min.
  - Soak membrane in 1 × transfer buffer for 10 min while preparing transfer sandwich.
- Soak Whatman filter paper in transfer buffer for several minutes to avoid trapping of air bubbles.
- Assemble sandwich (bottom up on the surface of wet semi-transfer plate):
  - 3-5 sheets of wet Whatman filter paper
  - Membrane
  - Gel
  - 3-5 sheets of wet Whatman filter paper
- Use a roller to roll over sandwich gently to remove trapped air butbbles.
- Apply a few ml of transfer buffer on top of sandwich to avoid drying out membrane during transfer.
- Transfer at 14 volts for 40-60 min.

Westerns

- After transferring the protein to membrane, the membrane can be cut (best whilst wet and then allowed to dry).
- Make up milk solution 5% milk powder in PBST.
- Place membrane in the milk solution and then add primary antibody (1:500-1:2000 dilution of anti-Akt1/PKB, PH Domain) and incubate overnight at 4 °C.
- Rinse membrane in water.
- Make milk solution 5% milk powder in PBST.
- Incubate the membrane in the secondary reagent (a goat anti-rabbit HRP conjugated IgG) in PBST-milk for 1.5 h at room temperature.
- Rinse twice with water.
- Wash the membrane in PBS-0.1% Tween 20 for 3-5 minutes.
- Rinse the membrane in 4-5 changes of water.
- Prepare ECL reagent - Black first then White (do not cross contaminate).
- Also need: tissue, tweezers, Saran wrap and timer.
- Check Fuji SLA-3000 is on and cooled.
- Remove PBS and add ECL to the membrane for 1 min.
- Remove excess ECL by blotting membrane on tissue.
- Wrap in Saran wrap.
- Set exposure times 1 or 2 min and then assess if you need longer exposure.
- Take a white light image so that the markers can be visualized (F2.8 exposure 1/100 s).

**SDS PAGE loading buffer**

<table>
<thead>
<tr>
<th>50 ml 2×</th>
<th>10 ml 5×</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6 ml</td>
<td>2M Tris pH6.8</td>
</tr>
<tr>
<td>6 ml</td>
<td>80% glycerol</td>
</tr>
<tr>
<td>10 ml</td>
<td>10% SDS</td>
</tr>
<tr>
<td>2.5 ml</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>30 ml</td>
<td>water</td>
</tr>
<tr>
<td>1-2 drops</td>
<td>bromophenol blue</td>
</tr>
</tbody>
</table>

**Running buffer**

For 2.5 l of 10×
- 75 g Tris base
- 360 g glycine
- 25 g SDS (measure in fume cupboard)
Transfer buffers

ETB
For 2.5 l of 10×
75 g Tris base
360 g glycine
25 ml 10% SDS
for 1× need to add 10% methanol

Carbonate

For 2 l of 10×
6.36 g disodium carbonate
16.8 g sodium hydrogen carbonate
20 ml 10% SDS
for 1× need to add 10% methanol

Coomassie Blue Stain

0.25% Coomassie Blue Dye (be careful it gets everywhere and it stains)
10% Methanol
10% Acetic Acid
Use dH₂O – must be added before adding acid

Destain

10% Methanol
10% Acetic Acid
Use dH₂O – must be added before adding acid

PBST

Add 2 ml of Tween 20 to make up 2 l of PBST (i.e. 0.1%). Use a glass pipette and when adding Tween 20, pipette up and down to wash the inside of pipette.
Make PBS as normal (200 ml of 10× PBS and make up to 2 l with dH₂O).

8.2.7 Bradford assay

To prepare in advance:

1× stock of Bradford reagent

- Dilute stock in fridge 1:5 with distilled water.
- Keep in tin foil to protect from light.
- Make on day of use.

BSA standards

- Range of known BSA concentrations from 0.125-1 mg/ml.
- Make up in distilled water.

Sample protein stock dilutions

- Dilute your sample over a range so that optimum concentrations can be obtained.
- For high concentrations dilute up to 1:100/1:1000.

Method

- Add 5 μl of each BSA standard to 3 wells of a clear 96 well plate.
- Add 5 μl of sample protein, including the neat stock and dilutions, into 3 wells (i.e. each sample in triplicate).
- Add 250 μl of 1× Bradford stock to each well.
- Incubate at room temperature for 5 min.
- Take to plate reader and measure absorbance at 595 nm.
8.2.8 Glutathione elution

- Wash beads in 100 mM Tris pH 8 2×.
- Elute in 300 μl 20 mM reduced glutathione (in 100 mM Tris pH 8) for 5 min at room temperature, vortexing once or twice. Make sure the pH of the Tris is 8 and that the glutathione is made up freshly just before use.
- Pulse down the beads and transfer the supernatant into a fresh tube.
- Repeat elution several times (3-4) and check which fractions are best on a gel - also check the beads to see what is left on them (can be lots).
- Dialyze against your favorite buffer to remove the glutathione/Tris if necessary.
9. APPENDIX A – ITC EQUATIONS

Taken from the ITC Data Analysis in Origin® Tutorial Guide:

Equations used for fitting ITC data

General considerations

It will be assumed throughout that the macromolecule M is in the cell at an initial bulk concentration \( M_0 \) (mol/l) before the first injection, and the ligand X to be injected is initially at zero concentration in the cell. The working volume (cross-hatched area below) of the lollipop-shaped cell is \( V_0 \), the size of the \( i^{th} \) injection is \( \Delta V_i \) and the total liquid which has been injected at any point during the experiment, \( \Delta V \), is simply the sum of the individual \( \Delta V_i \) for all injections.

![Diagram](image)

**Figure 58.** Initial volume \( (V_0) \), and increase in volume \( (\Delta V) \) on addition of ligand to sample cell.

At the beginning of an experiment, both the cell and the long communication tube are filled with macromolecule solution, but it is only that contained within \( V_0 \) that is sensed calorimetrically. Because of the total-fill nature of the cell each injection acts to drive liquid out of the working volume and up into the inactive tube as shown by the darkened portion representing \( \Delta V \). Thus, the concentration of macromolecule in \( V \) changes a small amount with each injection since the total number of moles of macromolecule initially in \( V \) (i.e. \( M_0 \times V_0 \)) at the beginning of the experiment is later distributed in a larger volume, \( V_0 + \Delta V \). Since the average bulk concentration of macromolecule in \( \Delta V \) is the mean of the beginning
concentration \( M^0_t \) and the present concentration \( M_t \) in the active volume, then conservation of mass requires that:

\[
M^0_t V_0 = M_t V_0 + \frac{1}{2}(M_t + M^0_t) AV
\]  
(eq. 6)

so that:

\[
M_t = M^0_t \left[ 1 - \left(\frac{AV}{2V_0}\right) \right] \left[ 1 + \left(\frac{AV}{2V_0}\right) \right]^{-1}
\]  
(eq. 7)

Using similar reasoning, it is easily shown that the actual bulk concentration of ligand in \( V_0 \), \( X_t \), is related to the hypothetical bulk concentration \( X^0_t \) (assuming that all of the injected ligand remained in \( V_0 \)) as follows:

\[
X^0_t V_0 = X_t V_0 + \frac{1}{2} X_t AV
\]  
(eq. 8)

\[
X = X^0_t \left[ 1 - \left(\frac{AV}{2V_0}\right) \right]
\]  
(eq. 9)

The above expressions for \( M_t \) and \( X_t \) are used by Origin to correct for displaced volume effects which occur with each injection.

Single Set of Identical Sites

In the following equations,

\[ K_b = \text{Binding constant;} \]

\[ n = \text{number of sites;} \]

\[ V_0 = \text{active cell volume;} \]

\( M_t \) and \([M]\) are bulk and free concentration of macromolecule in \( V_0 \);

\( X_t \) and \([X]\) are bulk and free concentration of ligand, and
\( \Theta = \text{fraction of sites occupied by ligand } X. \)

\[
K_b = \frac{\Theta}{(1 - \Theta) [X]} \tag{eq. 10}
\]

\[X_t = [X] + n\Theta M_t \tag{eq. 11}\]

Combining equations (eq. 10) and (eq. 11) above gives:

\[
\Theta^2 - \Theta [1 + (X_t/nM_t) + (1/nK_b M_0)] + (X_t/nM_t) = 0 \tag{eq. 12}
\]

The total heat content \( Q \) of the solution contained in \( V_0 \) (determined relative to zero for the unliganded species) as fractional saturation \( \Theta \) is:

\[
Q = n\Theta M_0 \Delta H V_0 \tag{eq. 13}
\]

where \( \Delta H \) is the molar heat of ligand binding. Solving the quadratic equation (eq. 12) for \( \Theta \) and then substituting into eq. 13 gives:

\[
Q = \frac{(nM_0 \Delta H V_0/2) [1 + (X_t/nM_t) + (1/nK_b M_0)] - [(1 + (X_t/nM_t) + (1/nK_b M_0))^2 - (4X_t/nM_t)]^{1/2}} \tag{eq. 14}
\]

The value of \( Q \) above can be calculated (for any designated values of \( n, K_b, \) and \( \Delta H \)) at the end of the i\(^{th}\) injection and designated \( Q(i) \). The parameter of interest for comparison with experiment, however, is the change in heat content from the completion of the i-1\(^{th}\) injection to completion of the i\(^{th}\) injection. The expression for \( Q \) in equation (eq. 14) only applies to the liquid contained in volume \( V_0 \). Therefore, after completing an injection, it is obvious that a correction must be made for displaced volume (i.e., \( \Delta V_i = \text{injection volume} \)) since some of the liquid in \( V_0 \) after the i-1\(^{th}\) injection will no longer be in \( V_0 \) after the i\(^{th}\) injection, even though it will contribute to the heat effect (assuming the kinetics of reaction and mixing are fast) before it passes out of the working volume \( V_0 \). The liquid in the displaced volume contributes about 50% as much heat effect as an equivalent volume.
remaining in $V_0$. The correct expression then for heat released, $\Delta Q(i)$, from the $i^{th}$ injection is:

$$\Delta Q(i) = Q(i) + \frac{(dV_i/V_0)((Q(i)+\Delta Q(i-1))/2)}{2} - Q(i-1) \quad \text{(eq. 15)}$$

The process of fitting experimental data then involves: (i) initial guesses (which most often can be made accurately enough by Origin) of $n$, $K_b$, and $\Delta H$; (ii) calculation of $\Delta Q(i)$ for each injection and comparison of these values with the measured heat for the corresponding experimental injection; (iii) improvement in the initial values of $n$, $K_b$, and $\Delta H$ by standard Marquardt methods; and (iv) iteration of the above procedure until no further significant improvement in fit occurs with continued iteration.
10. APPENDIX B – NMR DATA

The spectra included here are presented as illustrations of the characteristic signals observed in the $^1$H-, and $^{31}$P-NMR spectra of compounds at various stages of the synthesis. They were taken on either 270 MHz, 400 MHz or 500 MHz machines, depending on availability. Because of the differences in the machines there are noticeable difference in the quality of the spectra and therefore the quality of the possible interpretation. The spectra of the following compounds have been supplied:

- **myo-Inositol-1,3,4,5-tetrakisphosphate-2-O-acetic acid** (1)
- **2,6-O-Dibenzyld-4-O- (tert-butyldiphenylsilyl)-myo-inositol-1,3,5-O-orthobenzoate** (101b)
- **2-O-Allyl-6-O-benzyl-my-o-inositol-1,3,5-O-orthobenzoate** (108)
- **2-O-Allyl-6-O-benzyl-my-o-inositol** (109)
- **myo-Inositol-1,3,4,5-tetrakisphosphate** (115)
- **myo-Inositol-1,4,5-trisphosphate** (117)
- **1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-allyl-6-O-benzyl-my-o-inositol** (118)
- **myo-Inositol-1,3,4,5-tetrakisphosphate-2-O-propyl** (120)
- **1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-acetaldehyde-6-O-benzyl-my-o-inositol** (122)
- **myo-Inositol-1,3,4,5-tetrakisphosphate-2-O-(2-hydroxyethyl)** (129)
- **1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-(2-azidoethyl)-6-O-benzyl-my-o-inositol** (131)
- **myo-Inositol-1,3,4,5-tetrakisphosphate-2-O-(2-aminoethyl)** (138)
- **1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O- {2-[N,N’-bis(benzylxycarbonyl)guanidino]ethyl} -6-O-benzyl-my-o-inositol** (141)
**Polar group exchange receptor-ligand engineering of protein kinase B’s pleckstrin homology domain**

**H-NMR (D₂O):**

**31P-NMR (D₂O):**

Ins 4-\(H\) (q)

myo-inositol 1,3,4,5-tetra-phosphate 2.0 acetic acid (1)
Polar group exchange receptor-ligand engineering of protein kinase B’s pleckstrin homology domain

'H-NMR (CDCl3):

Sublimation removes this compound, confirmed by peak disappearing in the spectrum.
2-O-(2-Acrylamido)-3,4,5-O-tri-O-benzyl-β-D-glucopyranose (108)

'H-NMR (CDCl3):
2-O-Allyl-6-O-benzyl-myoinositol (109)
Polar group exchange receptor-ligand engineering of protein kinase B's pleckstrin homology domain

$^1$H-NMR (D$_2$O):

$^{31}$P-NMR (D$_2$O):

myo-Inositol-1,3,4,5,6-pentakisphosphate (I15)
1,3,4,5-Tetrakis(dibenzyl oxyphosphoryl)-2-O-allyl-6-O-benzyl-myoinositol (118)
Polar group exchange receptor-ligand engineering of protein kinase B's pleckstrin homology domain

$^1$H-NMR (CDCl$_3$):

$^{31}$P-NMR (CDCl$_3$):
Polar group exchange receptor-ligand engineering of protein kinase B’s pleckstrin homology domain

'H-NMR (D₂O):

'P-NMR (D₂O):
Polar group exchange receptor-ligand engineering of protein kinase B's pleckstrin homology domain

1,3,4,5-O-Tetrakis(dibenzyloxophosphoryl)-2-O-(2-azidoethyl)-6-O-benzyl-myoinositol (131)
Polar group exchange receptor-ligand engineering of protein kinase B's pleckstrin homology domain

myo-Inositol-1,3,4,5-tetakisphosphate-2-O-(2-aminoethyl) (138)
Polar group exchange receptor-ligand engineering of protein kinase B's pleckstrin homology domain

1,3,4,5-O-Tetrakis(dibenzyloxyphosphoryl)-2-O-[2-[N,N'-bis(benzyloxycarbonyl)guanidino]ethyl]-6-O-benzyl-myoinositol (141)

$^{1}H$-NMR (CDCl$_3$):

$^{31}P$-NMR (CDCl$_3$):
11. APPENDIX C – SYNAPTOJANIN ASSAYS

These are the assays that were employed for the study of the Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ analogues in section 4.6.

**Malachite Green Assay**

The activity of the phosphatases was determined by evaluating the levels of phosphate released during the course of the reaction with a malachite green dye.

- Compounds and enzyme (SAL) were incubated at room temperature for 15 min with 100 mM Tris, 4 mM MgCl$_2$, pH 7.4.
- Boiled enzyme was used as a negative control.
- The final volume of the assay was 80 µl.
- Ins(1,4,5)P$_3$ was added to start the reaction and samples were incubated for 30 min at 37 °C.
- To stop the enzyme reaction, an equal volume of the acidic malachite green dye was added.
- The mixture was allowed to develop for 5 min.
- An ELISA reader was used to read the absorption of the dye at a wavelength of 625 nm.

**O-Methyl Fluorescein Phosphate (OMFP) Fluorescence Assay**

The activity of the phosphatases towards OMFP was determined by measuring the fluorescence of O-methyl fluorescein (OMF), exciting at 485 nm and detecting emission at 525 nm.

- Compounds and enzyme were incubated at room temperature for 15 min with 100 mM Tris, 4 mM MgCl$_2$, pH 7.4.
- Boiled enzyme was used as a negative control.
- OMFP was added to start the reaction.
- Continuously monitoring the fluorescence, with the assay thermostatted at 37 °C on a Cary Eclipse fluorimeter was used to study the reaction.
12. REFERENCES