Synthesis of Novel Phosphorothioate and Pyrophosphate Analogues of Inositol Phosphates.

A Thesis submitted for the Degree of

Doctor of Philosophy

by

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in the

Faculty of Science

of the

Department of Chemistry

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University of Leicester

JUNE 1992

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by Nicholas Jason Noble

Abstract.

A novel synthesis of cyclic pyrophosphates was developed. The vicinal bisphosphorothioate, 1-phenyl-ethane-1,2-bisphosphorothioate, was synthesised using phosphorus (III) chemistry. This model compound was used to investigate the formation of the cyclic 7-membered pyrophosphate; N-bromosuccinimide was found to be the reagent of choice, giving the cyclic pyrophosphate in moderate yield. Activation of the bisphosphorothioate moiety by S-methylation, increased the yield of the pyrophosphate, as did reducing the conformational mobility of the vicinal bisphosphorothioate. Isotopic labelling experiments were used to investigate the mechanism of the reaction. However, it was concluded that the reaction proceeded via a complex combination of reaction pathways. The synthesis of the bacterial metabolite cyclic-2,3-bisphosphoglycerate was attempted using the new method, unfortunately, cyclic-2,3-bisphosphoglycerate was not isolated.

In order to prepare pyrophosphate analogues of *myo*-inositol-1,4,5-trisphosphate, it was necessary to prepare compounds such as *myo*-Inositol-1-phosphate-4,5-bisphosphorothioate and 6-deoxy-D-*myo*-inositol-1,4,5-trisphosphorothioate. *myo*-Inositol-1,4-bisphosphate-5-phosphorothioate was synthesised from a precursor, the structure of which was determined by 2-dimensional NMR spectroscopy. *myo*-Inositol-1-phosphate-4,5-bisphosphorothioate and *myo*-inositol-1,4-bisphosphate-5-phosphorothioate were found to be potent inhibitors of the inositol-5-phosphatase enzyme, whereas 6-deoxy-D-*myo*-inositol-1,4,5-trisphosphorothioate was found to be a weak inhibitor of the inositol-5-phosphatase enzyme.

Initially, the synthesis of an inositol pyrophosphate was attempted where phosphate scrambling, a potential side reaction, could not compete with the formation of the pyrophosphate. To this end, the desulphurisation of 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol-1,4,5-trisphosphorothioate was attempted. The reaction gave the 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol-1-phosphate-4,5-pyrophosphate in good yield. *myo*-Inositol-1-phosphate-4,5-bisphosphorothioate was prepared from *myo*-inositol and was treated with N–bromosuccinimide. The result of the reaction was to give the target molecule *myo*-inositol-1-phosphate-4,5-pyrophosphate again in high yield. Unfortunately, the pyrophosphate was found not to be active at releasing Ca^{2+} nor active towards the phosphoinositide cycle enzymes.

Dedication.

To.....All of my Family,

To.....Mum, Dad, Melanie and especially

To.....William Henry Gravestock and Robert William Noble

Statement

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled "Synthesis of Novel Phosphorothioate and Pyrophosphate Analogues of Inositol Phosphates" is based on work conducted by the author primarily in the Department of Chemistry of the University of Leicester mainly during the period between October 1987 and November 1990. Subsequent work was conducted by the author in the School of Pharmacy and Pharmacology of the University of Bath during the period between December 1990 and June 1992.

All work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

Signed:..... Date:30 July 92.

Acknowledgements

I would like to extend my thanks to Barry Potter for his support, encouragement and supervision. I would also like to thank all of the staff in the Department of Chemistry, especially John Holloway, Paul Cullis, John Malpass, David Turner and Gerry Griffiths.

I would like to thank Stefan Nahorski and Steven Safrany for their guidance in the biological evaluation experiments.

I would also like to thank several other people at Leicester for their friendship. Mel Cooke, Tahir Yaqoob, Paul Nandra, Jaz Flora, Josh Howarth, Debby Sawyer, Nicola Ebert and lastly Carol Ekinsmyth for an email message.

I would like to thank the staff at the School of Pharmacy and Pharmacology at the University of Bath for their help, especially Noel Thomas and Nicola Howarth.

Lastly, I would like to acknowledge and thank all of my friends in Bath especially, John Lord, Elena "probably" De Angelis, Didier Dubreuil, Xavier [Coo, Coo] Doisy, Sam Light, Steve Mills, Det Lampe, Changsheng Liu and most importantly Lisa [Meep, Meep] Sidoli. Thanks for all that you have done to help me complete this Thesis.

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Abbreviations.

Ac	acetyl
ADP	adenosine 5'-diphosphate
cAMP	adenosine-3', 5-cyclic phosphate
ATP	adenosine 5'-triphosphate
ATPαS	adenosine 5'-*O*-(1-thiotriphosphate)
ATPαSMe	adenosine 5'-*O*-(*S*-methyl-1-thiotriphosphate)
BPG	2,3-bisphosphoglycerate
cBPG	cyclic 2,3-bisphosphoglycerate
Bn	benzyl
Bz	benzoyl
m-CPBA	*meta*-chloro-per benzoic acid
CDP	cytidine 5'-diphosphate
CMP	cytidine 5'-phosphate
CoA	coenzyme A
COSY	correlation spectroscopy
DCC	dicyclohexylcarbodiimide
dCDP	2'-deoxy cytidine 5'-diphosphate
1D	one dimensional
2D	two dimensional
DABCO	diazobicyclo-[2,2,2]-octane
DAST	diethyl amino sulphur trifluoride
DEAE	diethyl aminoethyl
DDQ	dichlorodicyanoquinone
DG	diacyl glycerol
DIBAL	diisobutylaluminium hydride
DMAP	4-dimethyl amino pyridine
DMBB	5-(1,3-dimethylbutyl)-5-ethyl barbituric acid
DMF	dimethyl formamide
DNA	deoxyribose nucleic acid
cDNA	copy deoxyribose nucleic acid
DNTB	5,5'-dithio-bis-(2-nitrobenzoic acid)
(-)-ephedrine	[(1R, 2S)-2-methylamino-1-phenylpropan-1-ol]
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene bis(oxyethylenenitrilo) tetra acetic acid
ER	endoplasmic reticulum

Et	ethyl
FAD	flavin adenine dinucleotide
FAR	3,7,11-trimethyl-2,6,10-dodectriene
GDP	guanosine 5'-diphosphate
GMP	guanosine 5'-phosphate
cGMP	guanosine cyclic-3',5'-diphosphate
GTP	guanosine 5'-triphosphate
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid)]
HMPA	hexamethyl phosphoric triamide
h.p.l.c	high pressure liquid chromatography
IANBD	*N*-[{2-(iodoacetoxy)ethyl}-*N*-methyl]amino-7-nitro-2,1,3-benzoxzdiazole
Ins(1,4,5)P_3	*myo*-inositol-1,4,5-trisphosphate
Ins(1)P	*myo*-inositol-1-phosphate
Ins(1,4)P_2	*myo*-inositol-1,4,-bisphosphate
Ins(2,4,5)P_3	*myo*-inositol-2,4,5-trisphosphate
Ins(c1:2,4,5)P_3	*myo*-inositol-1:2-cyclic, 4,5-trisphosphate
Ins(1,4:5 pyro)P_3	*myo*-inositol-1-phosphate-4,5-cyclic pyrophosphate
Ins(1,4,5)P_3-1S	*myo*-inositol-1-phosphorothioate-4,5-bisphosphate
Ins(1,4,5)P_3-5S	*myo*-inositol-1,4-bisphosphate-5-phosphorothioate
Ins(1,4,5)P_3-4,5S_2	*myo*-inositol-1-phosphate-4,5-bisphosphorothioate
Ins(1,4,5)PS_3	*myo*-inositol-1,4,5-trisphosphorothioate
6-deoxy-(23C)-Ins(1,4,5)PS_3	6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol-1,4,5-trisphosphorothioate
6-deoxy-Ins(1,4,5)PS_3	6-deoxy-D-*myo*-inositol-1,4,5-trisphosphorothioate
Ins(1,4,5)P_3-5Me	*myo*-inositol-1,4-bisphosphate-5-methylphosphonate
Ins(1,4,5)P_3-5CHF_2	*myo*-inositol-1,4-bisphosphate-5-difluoromethyl phosphonate
Ins(1,3,4,5)P_4	*myo*-inositol-1,3,4,5-tetrakisphosphate
Ins P_6	phytic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NBS	N-bromosuccinimide
NMR	nuclear magnetic resonance
NOBA	*m*-nitrobenzyl alcohol
OXDEP	*o*-xylylene diethylphosphoramidite

PA	phosphatidic acid
PI	[1-(3-*sn*-phosphatidyl)-D-*myo*-inositol]
PIP	[1-(3-*sn*-phosphatidyl)-D-*myo*-inositol-4-phosphate]
PIP_2	[1-(3-*sn*-phosphatidyl)-D-*myo*-inositol-4,5-bisphosphate]
PKC	protein kinase C
PLC	phopholipase C
ppm	parts per million
Pr^i	*iso*-propyl
Pr^n	*n*-propyl
RNA	ribonucleic acid
rt	room temperature
SEM	β-trimethylsilylethoxymethyl
TEAB	triethylammonium bicarbonate
TIPSCl	1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane
tlc	thin layer chromatography
ptsa	toluene-*p*-sulphonic acid monohydrate
UDP	uridine 5'-diphosphate

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Chapter One

General Introduction

1.1 Cellular Communication.

In order for a multicellular organism to function, cells must be able to respond to changes in their own environment and communicate this information. The information transfer is facilitated by the passage of chemical signals from one cell to another. These signals are classified by their function, such as neurotransmitters, hormones, growth factors *etc.* Certain molecules such as steroidal hormones are lipophilic and are able to pass directly through the plasma membrane, a hydrophobic membrane that acts as a physical barrier to most molecules. Once the external signal has passed through the cellular membrane of their target cell it is able to act on the intracellular environment. The internal response thus created causes flux in the biochemical state of the cell, effecting a change in cell behaviour, such as secretion, contraction (if the target cell is muscular) *etc.* Many of these primary or first messengers, however, are not able to pass through the plasma membrane, so a different system of information transfer has had to evolve. The primary messengers are received by specific receptors on the outer membrane of the target cell [Fig. 1]. These can then be divided into several types:

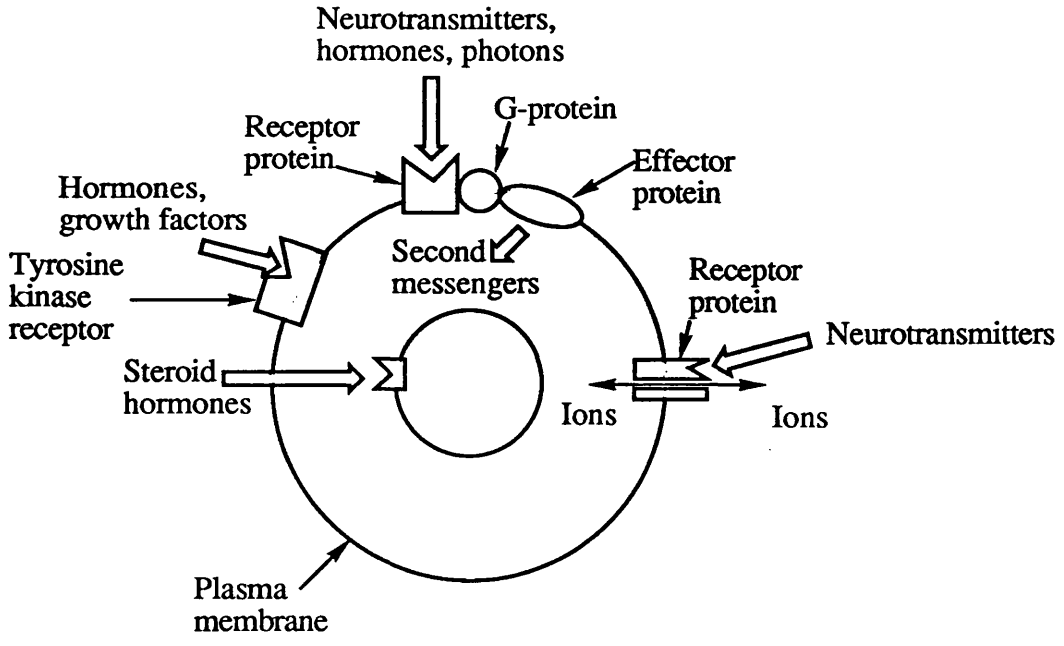


Figure 1

The first type of receptor contains or is closely linked to an ion channel that spans the membrane. Stimulation of the receptor alters the state of the ion channel so that the flow of specific ions is interrupted or encouraged. The second type of receptor are the membrane spanning tyrosine kinases. Binding of the primary messenger such as insulin[1], activates the enzyme which then phosphorylates a specific tyrosine residue in

target proteins inside the cell. The third class of receptor are transmembrane proteins that are coupled via a class of guanosine 5'-triphosphate (GTP) binding glycoproteins called 'G-proteins', which are in turn linked to proteins inside the cell[2], these effector proteins, upon activation, alter the state of a specific substrate: For example, a hydrolytic effector would cleave its substrate, an ion channel would alter the flow of ions. The receptors that have G-protein intermediaries have many agonists, such as a large array of biogenic amine, protein and polypeptide hormones; and neurotransmitters (*e.g.* adrenaline). Rhodopsin, a membrane-spanning light-as well as a G-protein linked receptor as apparently are those for various odourants[3]. The number of effector molecules known to be controlled by G-proteins is more modest: Interactions of adenylate cyclase and retinal cyclic GMP-specific phosphodiesterase with G-proteins is well understood, whereas the interactions between ion-channels and phosphatidylinositol phospholipase C with G-proteins are less understood[3]. The functions of G-proteins, heterotrimers that comprise of three subunits designated $G\alpha$, $G\beta$,$G\gamma$, are regulated cyclically by association of guanosine triphosphate (GTP) with one subunit, $G\alpha$, hydrolysis of GTP to guanosine diphosphate (GDP) and phosphate, followed by dissociation of the GDP. The two subunits $G\beta$ and $G\gamma$ are common to all G-proteins, the differences seen in G-proteins are reflected in the differences of the $G\alpha$ subunit. The binding of the GTP to $G\alpha$ brings about the activation of the other two subunits, $G\beta$ and $G\gamma$, and consequent regulation of the activity of the appropriate effector. Hydrolysis of GTP initiates deactivation, dissociation of the GDP being the rate limiting step. Where the effectors are enzymes, the enzymes act by releasing another discrete chemical signal into the cell cytosol, a 'second messenger' which elicits further change in the cell behaviour [Fig. 2].

AGONIST (FIRST MESSENGER)

↓

RECEPTOR

↓

TRANSDUCER (G-PROTEIN)

↓

AMPLIFIER (ENZYME)

↓

SECOND MESSENGER (cAMP, $InsP_3$)

↓

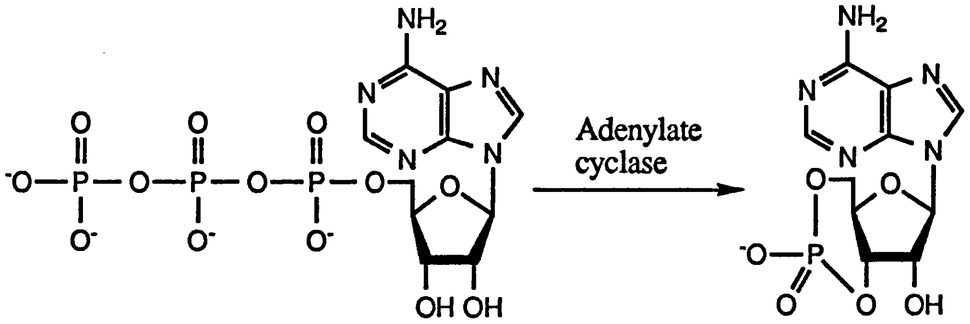
CELLULAR RESPONSE

(SECRETION, CONTRACTION, GROWTH etc.)

Figure 2

1.2 Cyclic Adenosine Monophosphate (cAMP)

In the late 1950's a a G-protein transduced second messenger system of this type was discovered. Cyclic 3', 5' adenosine monophosphate (cAMP) was found to be formed upon addition of adrenaline to particulate subcellular fractions obtained from rat liver[4]. The cAMP thus produced was found to induce the activation of the cytosolic enzyme glycogen phosphorylase. Subsequent demonstration that the active principal was cAMP, established this molecule as the first second messenger to be identified[5,6].



ATP cAMP

Figure 3

These discoveries were followed by the demonstration that most eukaryotic cells possess a plasma membrane-associated adenylate cyclase, which can produce cAMP from ATP

and that the activity of this enzyme can be regulated by various molecules[7], from low-molecular weight compounds such as noradrenaline and dopamine, to larger proteins such as adrenocorticotrophic hormone and thyroid-stimulating hormone[8]. Adenylate cyclase has been found to be modulated by inhibitory and stimulatory G-proteins linked to inhibitory and stimulatory receptors[9]. Since the enzyme can create many molecules of cAMP, stimulation of the appropriate receptor can result in many molecules of second messenger being produced, in effect the original signal has been amplified many fold. The cAMP thus produced is decomposed in order to retain the sensitivity of the second messenger system to changes in receptor stimulation. cAMP is hydrolysed by 3':5'-cyclic nucleotide phosphodiesterase[4]. In general, it appears that hormones and receptor agonists do not regulate the concentration of cAMP by directly controlling the activity of the 3':5'-cyclic nucleotide phosphodiesterase enzymes (there are sub types with differing specificities for cAMP verses cGMP)[10]. Hence, the major determinant of the cAMP concentration in a cell is the adenylate cyclase activity. In order to exert its effects in the cell, cAMP must interact with, and activate, an effector system which will convert the hormonal signal into a cellular response. A tetrameric protein kinase comprising of two catalytic and two regulatory subunits has been identified as being responsible for all of the known effects of cAMP in animal cells and is, therefore, the central regulatory component of all hormonal mechanisms using cAMP as a second messenger[5]. In its non-dissociated, tetrameric, form the cAMP-dependent protein kinase does not have catalytic activity. However, the catalytic activity of the protein kinase rapidly increases as the concentration of cAMP rises, the activated protein kinase then induces change in the properties of a substrate protein by phosphorylating it. The effected substrate is cell dependent, cAMP formed in liver cells promotes gluconeogenesis and glycogen breakdown, in adrenal cortex cells it leads to increased steroidal output and in many endocrine cells it facilitates an increase in hormone secretion rate.

1.3 Cyclic Guanosine Monophosphate (cGMP).

When cGMP was discovered in the early 1960's, the role of cAMP as an intracellular second messenger was becoming widely accepted. Whilst cGMP appears to possess many characteristics of a second messenger, the precise details of its action are not well understood. Like cAMP, cGMP is synthesised from GTP by a guanylate cyclase.

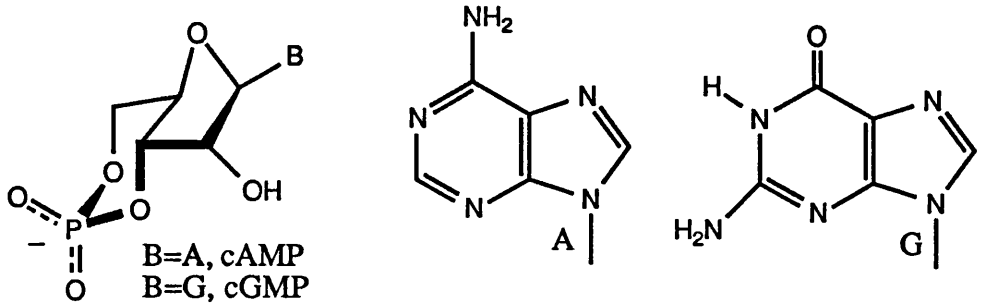


Figure 4

cGMP has emerged as a central regulatory molecule in the photosensitive rod cell (rhodopsin) of the vertebrate retina[11,12]. In this tissue, the soluble form of guanylate cyclase is very active and the concentration of cGMP is high under resting conditions. This is unusual; with cAMP, the basal level is low. However, the characteristics of the rod cell are unusual in that they respond to a decrease in cGMP levels. This has now been explained by that fact that the rod cell plasma membrane has an ion channel which is maintained in the open state under non-stimulating conditions (in the dark)[13]. This can be explained as follows: A photon of light is absorbed by rhodopsin which then promotes the activation of the G-protein transducin. The transducin catalytic sub unit dissociates and interacts with and activates a specific cGMP phosphodiesterase. cGMP is hydrolysed, as the concentration falls the plasma membrane ion channel closes halting the flow of both sodium and calcium ions. The rod-cell becomes hyperpolarized due to reduced ion influx which promotes a synaptic neurotransmitter release and nerve impulse propagation along the optic nerve to the brain. Return to the dark state is accomplished by deactivation of rhodopsin, regeneration of cGMP by guanylate cyclase, opening of the plasma-membrane ion channel and subsequent ionic equilibration.

1.4 The Cytosolic Role of Calcium Ions.

Ringer[14] found that he could induce contractions in heart muscle when he tested the action of a saline solution prepared with tap water, however, he could not induce any contractions when he tested the action of a saline solution prepared with distilled water. He concluded that the agents in the tap water responsible for the contractions of the heart ventricle were Ca^{2+} present in 38.3 parts per million and K^+ present in 7.1 parts per million, (the water contained 278.6 parts of solid per million). These initial observations led to the investigation of the cytosolic role of Ca^{2+}. Most of the Ca^{2+} present in mammals is found in bone and only about 5% is available to the remaining tissues. Nevertheless, this Ca^{2+} is necessary for maintenance of cell structure (by stabilization of membranes and regulation of the cytoskeleton), control of ion permeability (especially

sodium and potassium ions), and regulation of mobility and contraction, plus an enormous range of other functions which combine to control the state of activation of the cell. Proteins have evolved that can readily discriminate Ca^{2+} from all other common cations. In conjunction with these proteins, cells have developed sophisticated Ca^{2+} - buffering systems which involve Ca^{2+} transporters located both at the plasma membrane and on the membranes of intracellular organells, such as the endoplasmic reticulum. This enables them to regulate, very precisely, the concentration of Ca^{2+} in the cytosol, and it is this capability that allows Ca^{2+} to function effectively as a second messenger. Most of the cytosolic Ca^{2+} is not free and is bound to membranes and cytosolic proteins, so the concentration of free Ca^{2+} is low. The concentration of free Ca^{2+} in extracellular fluid is much greater. This means that there is a large Ca^{2+} gradient favouring Ca^{2+} influx into the cytosol. If a mechanism exists to regulate the rate of Ca^{2+} entry through the plasma membrane, the cell can readily employ this pathway as the means to transduce a primary messenger. Cells have developed various Ca^{2+} channels, *e.g.* channels that are hormonally controlled, channels that respond to agonist-induced changes in membrane potential. The distinction between the multiple types of calcium channel is relevant as to how cells employ calcium entry to regulate varied biological functions such as excitability, hormone or neurotransmitter release, metabolism or gene expression[15]. The response of the cell is dependent upon the type of cell targeted. For instance if a liver cell is stimulated by vasopressin, the resultant Ca^{2+} release will stimulate glycogenolysis, if the same agonist excites a smooth muscle cell the Ca^{2+} released will initiate vasoconstriction.

1.5 The Phosphatidylinositol Cycle

Even before cAMP was discovered by Sutherland, Hokin and Hokin in 1953 observed the receptor-mediated incorporation of radioactive phosphorus into inositol phospholipids[16]. Some twenty two years later on, Michell postulated that this event was linked to an increase in cytosolic calcium concentration[17]. These phenomena were then linked to the phospholipase C-catalysed cleavage of phosphatidyl inositol bisphosphate, into the two second messengers, *myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which mobilises[18] intracellular Ca^{2+}, and diacylglycerol [DG],which activates protein kinase C[19]. All of these factors led to the elucidation of a novel secondary messenger system, the receptor mediated synthesis of Ins(1,4,5)P_3 and DG by phospholipase C, and subsequent mobilization of cytosolic calcium[20].

1.6 Stereochemistry and Nomenclature of Inositol Derivatives.

Cyclohexanehexols, members of the cyclitol family are more familiarly known as inositols. Inositols consist of a ring of six tetrahedral carbon atoms joined by single bonds to form a chair. Each carbon is also bonded to a hydrogen atom and a hydroxyl group in either the axial or equatorial positions. These six secondary hydroxyl groups can be arranged among nine stereoisomeric forms with axial groups varying from none to three.

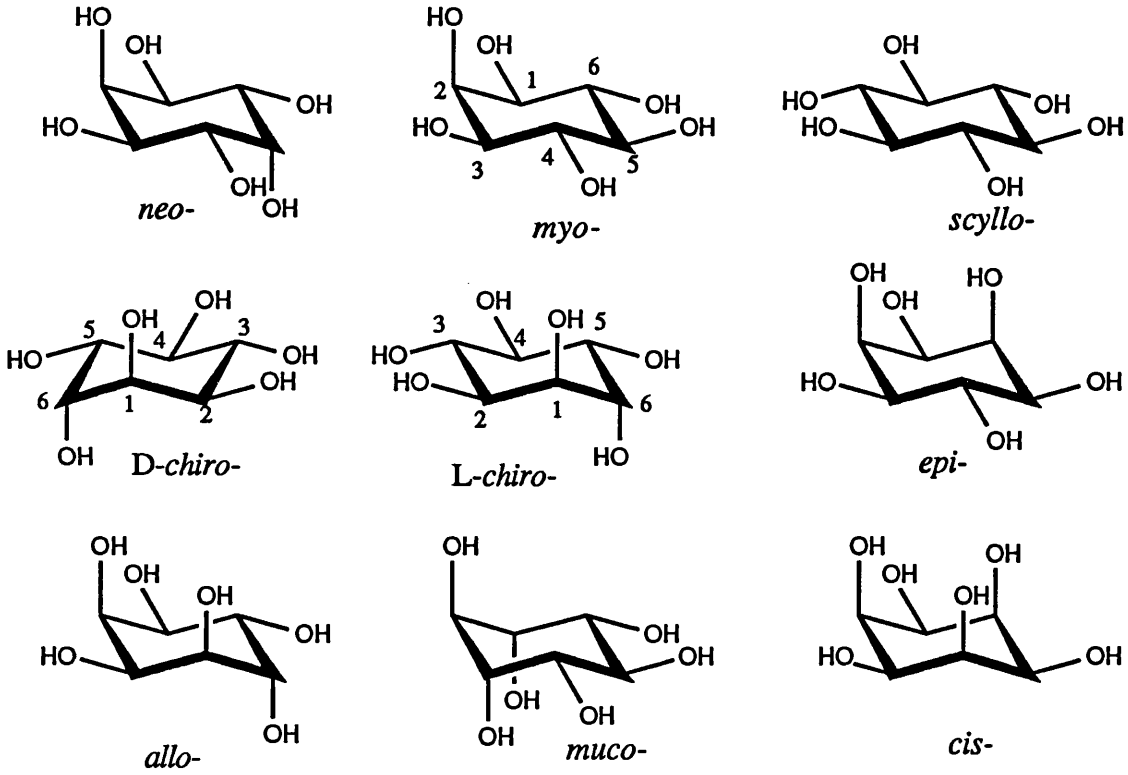


Figure 5

Referred to by their prefixes they are *scyllo-* (no axial); *myo-* (1); *neo-*, *epi-*,D-*chiro-*, and L-*chiro-* (2); *cis-*, *muco-* and *allo-* (3). Those with three axial hydroxyl groups can undergo chair to chair inter-conversions readily; those with less than three tend to favour one conformer with the greatest number of equatorial hydroxyl groups. Of the nine inositols, five have been found in nature[21]; *scyllo*-inositol has been isolated from marine animals and acorns. The 1-O- methyl ether of *muco*-inositol has been extracted from wood of *Phyllocladus trichomanides*. The methyl ether of D-*chiro*-inositol (+)-pinitol, was discovered in 1856 in the exudate of *Pinus lambertiana* and subsequently from many other plants. The methyl ether of L-*chiro*-inositol, quebrachitol, was first isolated in 1889 from the bark of *Aspidosperma quebracho-blanco*. Larger amounts have

been found in the latex of rubber trees.

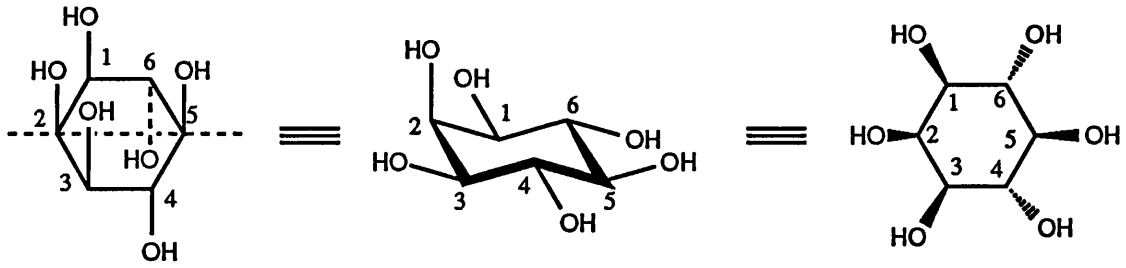


Figure 6

Inositols can be visualised using a projection more normally applied to carbohydrates, the Haworth projection [Fig. 6 (a)]: (i) The numbering is clockwise viewed from above the ring: (ii) The hydroxyl groups at C1 and C6 are *trans* to each other, with C1 and the majority of hydroxyls above the plane of the ring: (iii) If the previous rule cannot be applied then the ring is numbered so the the carbons bearing hydroxyls above the ring are given the lowest numbers. Consider *myo*-inositol [Fig. 6 (b)], with the C3-C4 edge closest to the viewer, with hydroxyl groups at C1, C2, C3, C5 *cis* to each other, as are those at C4 and C6, the two groups being *trans* to each other. However, the Haworth projection fails in one respect, because it cannot distinguish between axial and equatorial hydroxyl groups. This problem has been solved by applying information taken from the conformational diagram [Fig. 6 (c)]. Two further rules are required: (iv) in a vicinal *cis* pair of OH groups, if one is axial then the other must be equatorial, and conversely; (v) in a vicinal *trans* pair both must be axial or equatorial. *myo*-Inositol is unique in having an arrangement of three *cis* hydroxyl groups, the middle one of the three is axial and nominated as C2. Numbering clockwise or anti-clockwise in this case is not applicable as *myo*-inositol has a plane of symmetry between C2 and C5 [Fig. 6 (a)] and is therefore a *meso* compound. However, substitution at one of the stereogenic carbons (*i.e.*C3, C4, C6 or C1) disrupts the symmetry resulting in the formation of a chiral derivative. To illustrate the numbering of such a chiral *myo*-inositol consider the key metabolite *myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3].

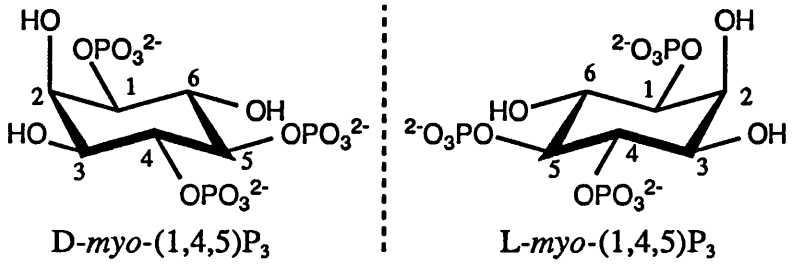


Figure 7

The axial hydroxyl group is nominated as position 2, numbering of the other groups from

1 is in accordance with the convention that the substituted positions be assigned the lowest possible number[22]. To meet that requirement the two possible enantiomers must be numbered in opposite directions from the axial two position, clockwise for L and anti-clockwise for D.

1.7 Inositol Phospholipid Synthesis.

Phospholipids are the major structural lipids of all biological membranes in eukaryotic and prokaryotic cells. A small percentage of the phospholipid found in animal cells, 2 to 8%, comprises of inositol-containing phospholipid. The structures of the three major phosphoinositide phospholipids are shown in [Fig. 8]. Over 90% of the phosphoinositide exists as [1-(3-*sn*-phosphatidyl)-D-*myo*-inositol], [Fig. 10], PI, (1) in the endoplasmic reticulum.

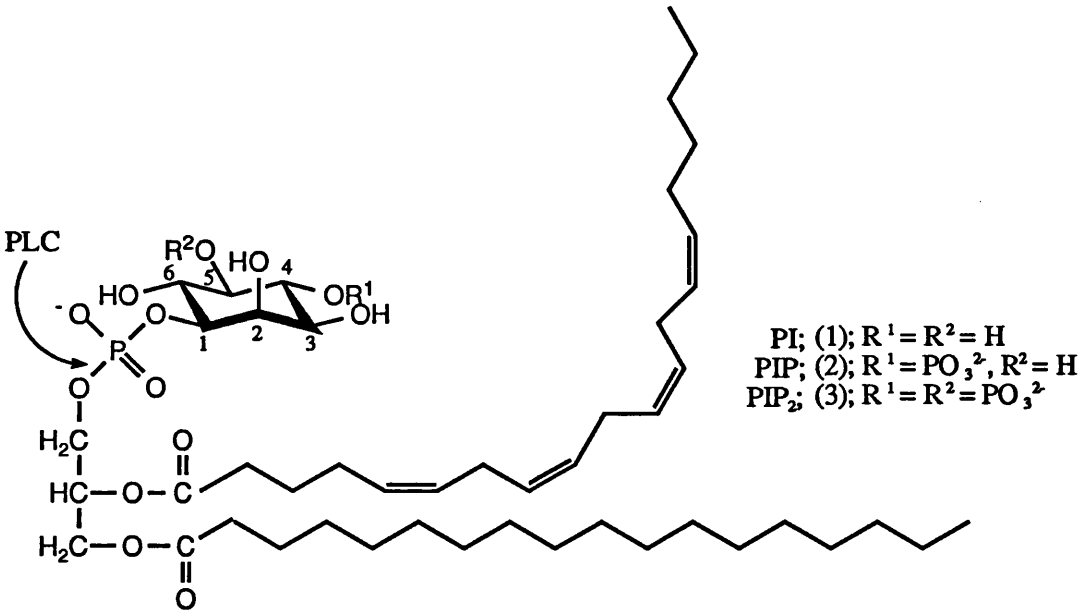


Figure 8

The structure of the phosphatidic acid moiety is not constant, it depends upon the source from which the PI is isolated from. PI from plants has the *sn*-2 glycerol carbon linked to the C_{18} of linoleic acid (18:2) and the *sn*-1 glycerol carbon linked to palmitic acid (16:0), stearic acid (18:0) or linoleic acid (18:2). The substitution of the glycerol in animal cells is simpler, arachidonic acid (20:4) at the C-2 position and stearic acid (18:0) at the C-1 position.

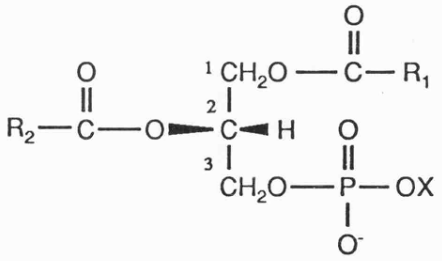


Figure 9: a general phospholipid, where R_1 and R_2 are fatty acids and X is a base

The interest in the phosphoinositides is focused on the inner leaflet of the plasma membrane, where phosphatidylinositol, PI, (1), phosphatidyl-4-phosphate, PIP, (2) and phosphatidyl-4,5-bisphosphate, PIP_2, (3) are located. Hokin and Hokin[16] in 1953 observed that cholinergic stimulation of pancreas slices caused the rapid incorporation of radioactive phosphorus, (^{32}P), into phosphatidyl inositol. It was suggested that receptor activation may be linked to the rapid hydrolysis of phosphoinositides and the mobilization of calcium[17, 23]. Rapid turnover of these lipids in a futile cycle of phosphatases and kinases has been observed in many tissues, in response to a number of varied external stimuli[20].

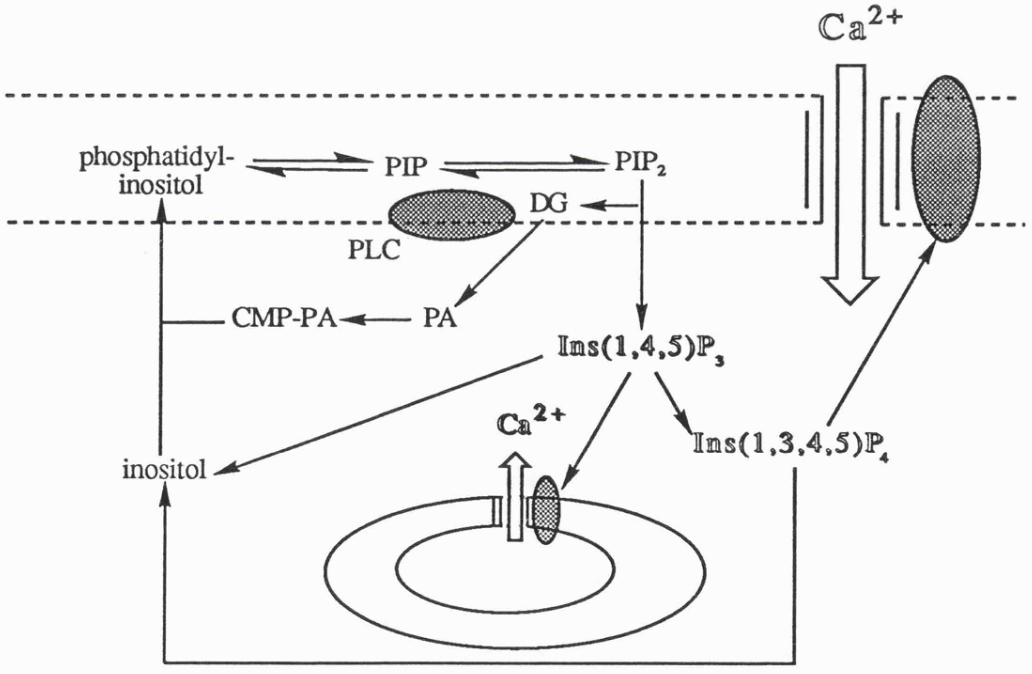


Figure 10.

PI is phosphorylated by specific kinases, phosphatidylinositol 4-kinase (PI 4-kinase), a membrane bound kinase in most tissues, that phosphorylates PI[24] to give PIP. This is then phosphorylated by phosphatidylinositol(4) 5-kinase (PIP 5-kinase), to give phosphatidylinositol 4,5-bisphosphate (PIP_2). The enzyme has been isolated in both

soluble and particulate fractions of cell homogenates[25] and is 100 fold more specific for PI(4)P than PI.

1.8 Second Messenger Synthesis.

Binding of a primary messenger to the receptor causes the associated G-protein to activate a specific membrane bound phospholipase, phospholipase C (PLC). PLC then cleaves PIP_2, (Fig. 10, 12), to yield two second messengers, diacylglycerol (DG) and *myo*-inositol 1,4,5-trisphosphate, Ins(1,4,5)P_3. Action of PLC on PI and PIP will also generate DG and two inositol phosphates; Ins(1,4)P_2 and Ins(1)P which are devoid of any known second messenger action. DG acts in the plane of the membrane as an activator for protein kinase C (PKC)[19]. The DG is then acted upon by either a kinase or by a lipase. The kinase phosphorylates the DG to phosphatidic acid (PA); the phosphatidic acid produced reacts with cytidine monophosphate, (CMP), to form CMP-phosphatidate (CMP-PA). The CMP-phosphatidate then combines with *myo*-inositol to resynthesise PI and eventually after a further two phosphorylations gives PIP_2. The action of the lipase is to metabolise the DG to monoacylglycerol and arachidonic acid. The arachidonic acid moiety is further metabolised to form various eicosanoids[26]. Ins(1,4,5)P_3 once produced, diffuses into the cytosol, where it then binds to specific receptors and stimulates release of calcium from ATP-dependent non-mitochondrial intracellular pools. These pools are principally associated with the endoplasmic reticulum, (ER)[18]. Ins(1,4,5)P_3 is able to mobilize only a third of the total endoplasmic reticular calcium. The phosphorylated derivative Ins(1,3,4,5)P_4 may act in concert with Ins(1,4,5)P_3 to mobilize Ins(1,4,5)P_3 insensitive stores of calcium from the ER or to mobilize extracellular calcium across the plasma membrane[19]. The subsequent rise in intracellular Ca^{2+} can be used to activate specialized Ca^{2+} /calmodulin (a Ca^{2+} binding protein) dependent protein kinases or multi-functional Ca^{2+} /calmodulin dependent protein kinases[27] which then activate, by phosphorylation, a given enzyme which then elicits the cellular response. For example; the binding of thrombin to a plasma membrane receptor of a blood platelet causes the cleavage of PIP_2 to Ins(1,4,5)P_3 and DG. The Ins(1,4,5)P_3 binds to the receptor on the ER and releases calcium, together the DG and the Ca^{2+} lead to the activation of protein kinase which then phosphorylates and activates a protein that secretes serotonin, resulting in the secretion of serotonin. Stimulation of the appropriate cell will lead via the same phosphoinositide pathway to growth, fertilization. A minor product is also formed by the PLC cleavage of PIP_2. This results from the attack of the 2-hydroxyl group of the inositol at phosphorus instead of hydrolysis by water, forming cyclic 1:2,4,5 Inositol trisphosphate Ins(c1:2,4,5)P_3. There is some controversy about this compound, it was reported to have some secondary messenger properties by

mobilising intracellular calcium to a similar or greater extent than Ins(1,4,5)P_3 in Swiss 3T3 cells[28]. Recent work[29,30] has cast doubt upon the role of Ins(c1:2,4,5)P_3 as a second messenger. The previous experimental conclusions may have been compromised by contamination from chemical hydrolysis of the compound to form a mixture of Ins(1,4,5)P_3 and Ins(2,4,5)P_3, Ins(1,4,5)P_3 thus produced elicited the observed release of calcium.

1.9 Metabolism of *myo*-Inositol 1,4,5-Trisphosphate.

Once a second messenger has effected its response, it must be quickly decomposed in order that the cell is not overly stimulated. Ins(1,4,5)P_3 is metabolised by two initial pathways, the simplest involves the successive dephosphorylation of the three phosphate groups via inactive intermediates to yield free *myo*-inositol. The free inositol is then recycled back into the membrane as a phosphatidylinositol. The second route involves the phosphorylation of Ins(1,4,5)P_3 by a kinase enzyme to Ins(1,3,4,5)P_4 inositol tetrakisphosphate, Ins(1,3,4,5)P_4, followed by dephosphorylation by phosphatases via less active intermediates to free inositol.

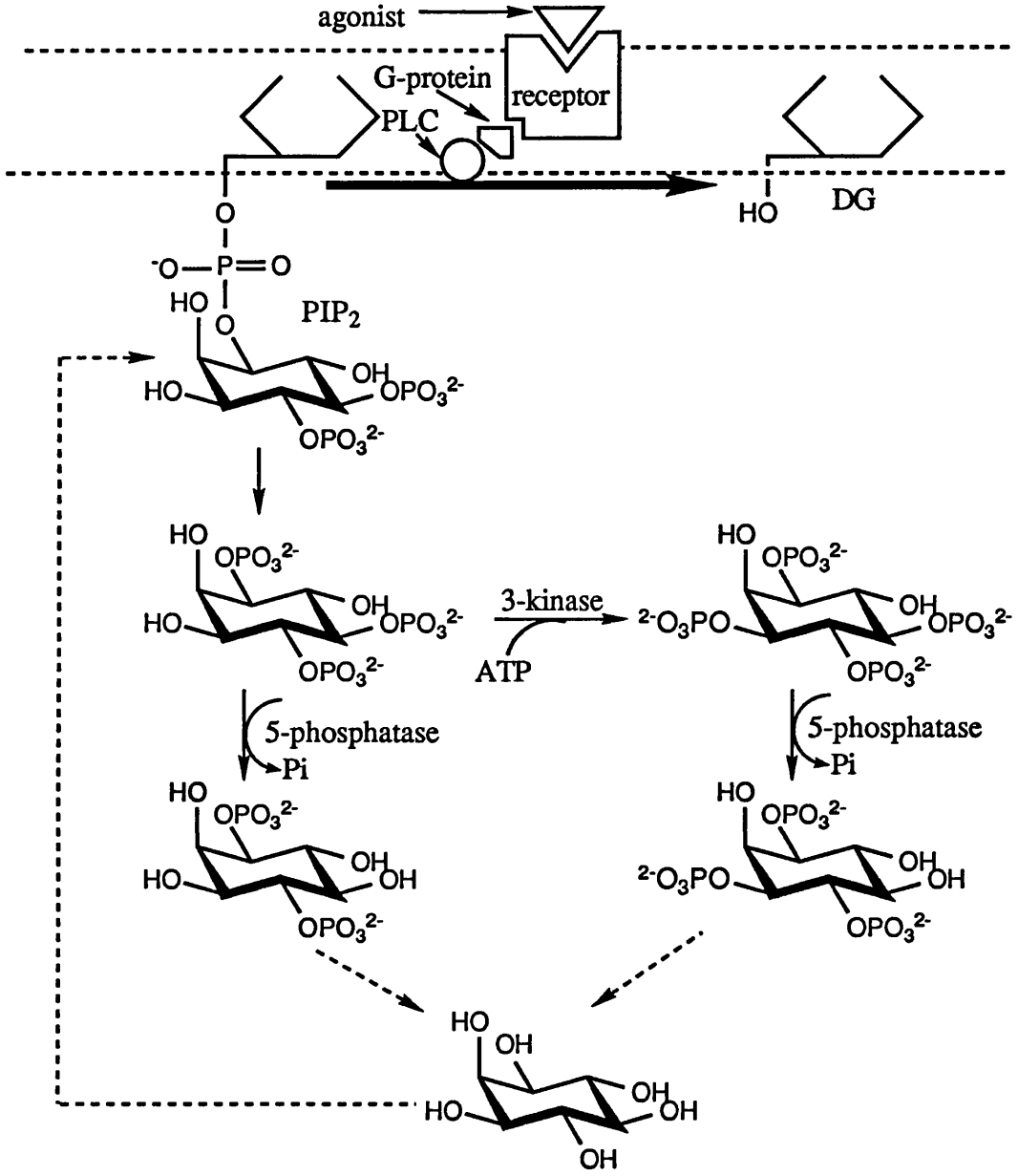
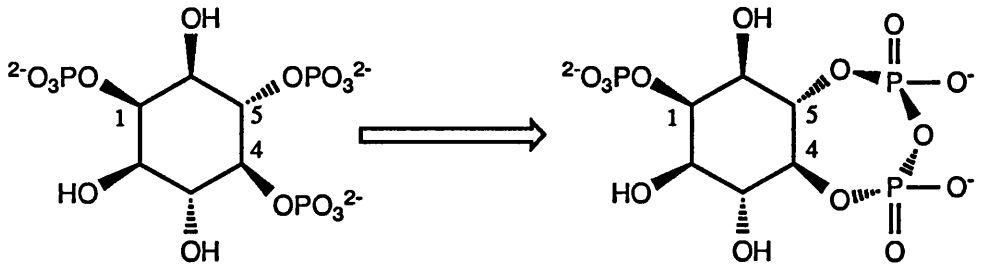


Figure 11. Metabolism of Ins(1,4,5)P_3

1.10 Aims Of This Thesis

Progress towards understanding the molecular basis of cellular signalling in the phosphatidylinositol cycle has been extremely rapid over the last nine years. It has become apparent that much biological work in this field has yielded useful information about the role of Ins(1,4,5)P_3 and its many metabolites in this process[31,32]. In recent years there has also been a great deal of interest in the development of synthetic routes to inositol phosphates[33]. With the considerable progress made, the emphasis towards the synthesis of novel analogues of Ins(1,4,5)P_3 including potential receptor antagonists and

inhibitors of the metabolic enzymes. Such compounds may be of therapeutic value. Modification of Ins(1,4,5)P_3, is already providing chemical tools that are giving us information about the other components of the cycle, substrate specificities, rates of reaction *etc.* Few analogues have, however, yet been prepared. It is for this reason, that we have chosen to synthesise analogues of Ins(1,4,5)P_3 that will help elucidate more details of the PI cycle.



Ins(1,4,5)P_3 (4) Ins(1,4:5 Pyro)P_3 (5)

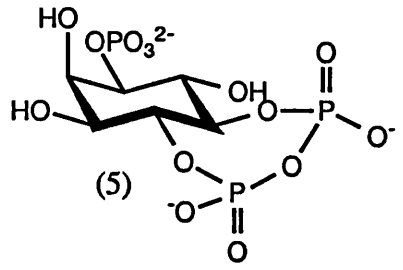
Figure 12

It has been found that the vicinal bisphosphate at the four and five positions of Ins(1,4,5)P_3 is of great importance to the binding of Ins(1,4,5)P_3 to the receptor on the ER[34,35], so it was of interest to synthesise analogues with different functionalities at the four and five positions. A particular aim of this thesis was to prepare the pyrophosphate. The pyrophosphate was chosen for this purpose as it is similar to the bisphosphate, differing only in charge and rotational flexibility. However, it would be very difficult, if not impossible, to synthesise this molecule using conventional chemistry as the presence of the neighbouring hydroxyl groups would lead to the formation of up to three cyclic five-membered phosphate diesters. These highly reactive five-membered cyclic phosphate diesters would rapidly ring open[36] to form a mixture of eight possible inositol trisphosphates. We have thus explored solutions to the synthesis of analogues of (4) and report here the successful outcome of these syntheses, the novel analogues thus prepared are listed below:

DL-*myo*-inositol-1-phosphate-4,5-cyclic pyrophosphate (5),
DL-*myo*-inositol-1-phosphate-4,5-bisphosphorothioate (6),
DL-*myo*-inositol-1,4-bisphosphate-5-phosphorothioate (7),
6-deoxy-D-*myo*-inositol-1,4,5-trisphosphorothioate (8),
6-deoxy-D-*myo*-inositol-1-phosphate-4,5-cyclic pyrophosphate (9),
[Fig. 13]

We also report here the successful development of a novel method for the synthesis of cyclic pyrophosphate moieties. This method involves the N-bromosuccinimide-mediated

oxidative desulphurisation of vicinal bisphosphorothioate compounds to give the corresponding cyclic pyrophosphate.



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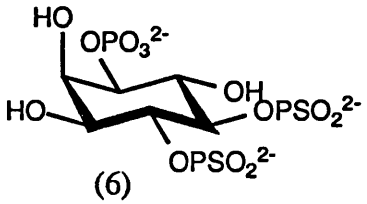

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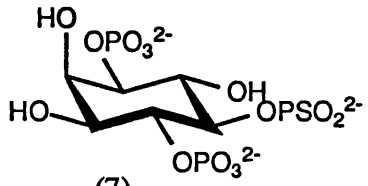

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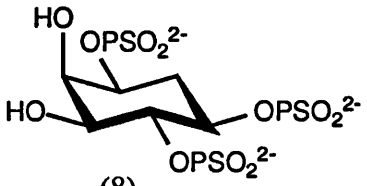

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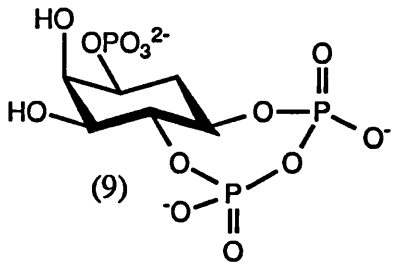


(9)

Figure 13

Chapter Two

Review of Biological Phosphates

2.1 Introduction.

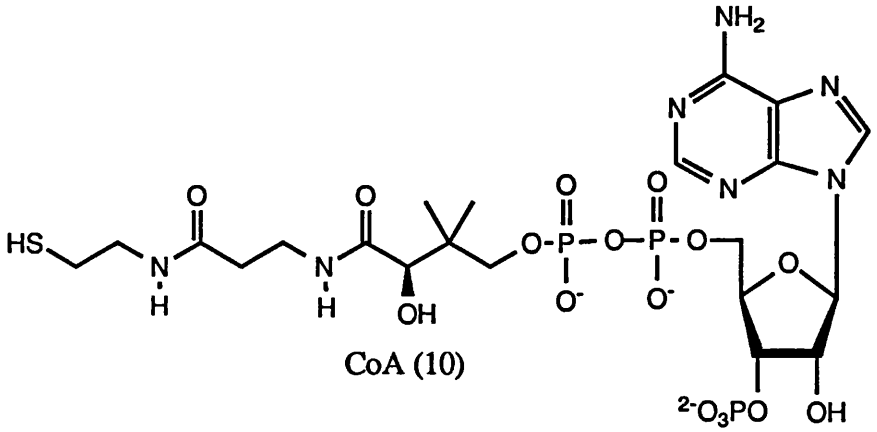
Phosphate esters have been found to occur in vast quantities in nature, and in order to study their biochemistry the development of effective syntheses is essential. Since, there are many examples of phosphate-containing molecules that have been successfully synthesised, only a few of the most ubiquitous will be discussed along with a selection of the more interesting ones.

2.2 Isolation of Biologically Important Phosphate Molecules

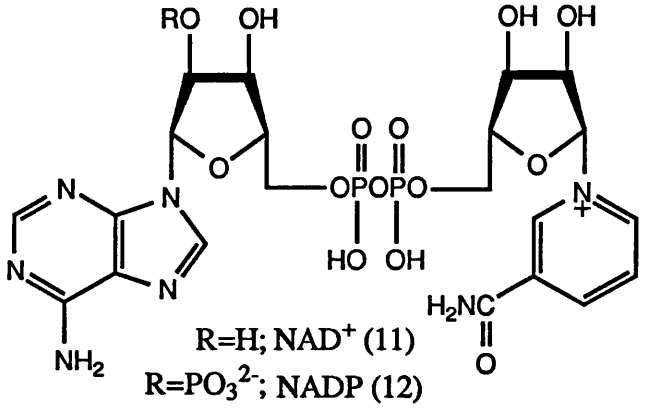
Many of the phosphate-containing molecules found in nature incorporate one or more nucleotide moieties. The nucleotides are invariably found as nucleotide anhydrides. These nucleotide anhydrides are best defined as nucleotide mixed anhydrides, that is anhydrides of nucleoside 5' phosphate and another acid which may be pyrophosphoric acid, phosphoric acid and mono esters thereof, carboxylic acids, amino acids or peptides. They are intimately involved in most biological processes and for this reason, much work has been directed towards their initial isolation, and subsequent syntheses.

2.2.1 Adenosine Phosphate Compounds.

Adenosine 5' triphosphate (ATP), the most essential of all biological compounds was first isolated from muscle extracts in 1929[37]. Other adenosine containing phosphate anhydrides have been also isolated from tissues; coenzyme A (CoA) (10) [Fig. 14] is a vitamin derivative found in liver[38], nicotinamide adenine dinucleotide (NAD) (11) [Fig. 14] was extracted from yeast[39], nicotinamide adenine dinucleotide phosphate (NADP) (12) [Fig. 14], from horse blood[40], and flavin adenine dinucleotide (FAD) (13) [Scheme 1] from yeast[41]. CoA acts as a carrier of activated acetyl or other acyl groups and is involved in the metabolism of many compounds such as, citrate, pyruvate, succinate, fatty acids and steroids. NAD and NADP act as coenzymes for a wide range of soluble dehydrogenase enzymes. NAD, NADP along with FAD form the basis of the respiratory chain of eukaryotic organisms[42].



CoA (10)



R=H; NAD^+ (11)

R=PO_3^{2-}; NADP (12)

Figure 14

2.2.2 Other Nucleoside Phosphate Compounds.

Nucleotide anhydrides of the other major nucleosides have also been isolated. For example, uridine 5' pyrophosphate derivatives like uridine 5' diphosphate glucose, (UDP Glucose) and uridine 5' diphosphate glucuronic acid, (UDP glucuronic acid) have been found. UDP glucose was the first nucleotide cofactor isolated that did not contain adenine. It is involved in part of the biosynthetic pathway leading to polysaccharide and cell wall synthesis in bacteria[43]. UDP glucuronic acid acts as a glucuronyl donor in the enzymic synthesis of ester, aliphatic and steroid ether glucuronides and it also transfers the glucuronic acid moiety to amino acids when forming N-glucosyluronic acid derivatives[44].

Another series of nucleotide anhydrides are cytidine 5' pyrophosphate derivatives. These are used in the biosynthesis of teichoic acids in certain bacteria. Teichoic acids are vital for the biosynthesis of bacteria membrane walls. Cytidine diphosphate choline, (CDP choline), cytidine 5' phosphate ethanolamine, (CDP ethanolamine)[45], and cytidine diphosphate glycerol, (CDP glycerol), are examples of teichoic acids. Cytidine

monophosphate on the other hand, has been found to play an important role in the phosphatidylinositol cycle. The diacylglycerol, produced by receptor-activated cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2), is phosphorylated to give phosphatidic acid (PA). The phosphatidic acid thus produced combines with cytidine monophosphate to form cytidine monophosphate phosphatidate (CMP-PA). Subsequently this combines with *myo*-inositol to reform phosphatidylinositol, which undergoes phosphorylation to produce PIP_2[20].

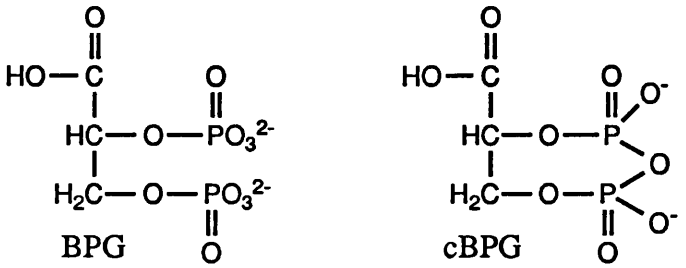
Finally, guanosine 5' diphosphate derivatives, such as guanosine 5' diphosphate mannose, (GDP mannose), have been found in yeasts[46]. Another derivative of this family, guanosine diphosphate fucose, (GDP fucose), has also been found in an *Aerobacter aerogenes* strain[47] which produces an L-fucose polysaccaharide from sheep's milk[48].

2.2.3 *Deoxynucleotide Anhydrides.*

Many 3' deoxynucleotide anhydride derivatives have been extracted from biological material, for example deoxycytidine diphosphate choline, (dCDP choline) and deoxycytidine diphosphate ethanolamine, (dCDP ethanolamine). dCDP choline has been found to catalyse the synthesis of lecithin as efficiently as CDP choline. dCDP ethanolamine is involved in the biosynthesis of the membrane phospholipid, phosphatidylethanolamine, but it is less effective than CDP ethanolamine[49].

2.2.4 *Sugar Phosphate Moieties.*

Many metabolites found in cells contain phosphorylated sugars. However, the scope of this thesis does not merit a full discussion of these compounds.



(14) (15)

Figure 15

The small molecule, 2,3–bisphosphoglycerate (BPG) (14) a key metabolite involved as a cofactor that promotes cooperative oxygen binding in haemoglobin. A compound which

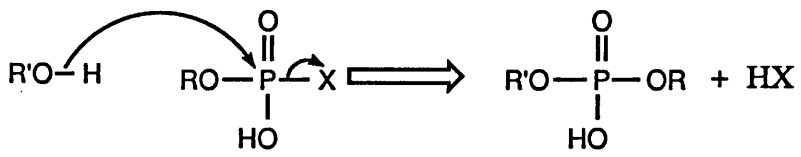
also acts as a weak inhibitor of inositol-5-phosphatase is of interest as it contains a vicinal bisphosphate moiety. The cyclic pyrophosphate analogue, cyclic 2,3-bisphosphoglycerate (cBPG) (15), a recently discovered bacterial metabolite which contains a cyclic seven-membered pyrophosphate linkage was thought to be an ideal target compound for synthesis using an oxidative desulphurisation of a vicinal bisphosphorothioate moiety a reaction which will be fully discussed in chapters 5 and 6.

2.3 Phosphorylation Methods.

In order to synthesise biologically important phosphate containing compounds it is vital to be able to phosphorylate precursors in a selective and high yielding manner to give the desired product. Much work has been conducted with this purpose in mind. Many varied techniques for the efficient synthesis of phosphates and pyrophosphates have been developed and will be reviewed here.

Phosphorylation is an aspect of acylation and it parallels the field of carboxylic acid chemistry in many respects, since, as in the synthesis of carboxylic esters and anhydrides, the formation of phosphate and pyrophosphate esters requires activation of one or both of the reactants. There are two general mechanisms of phosphorylation that encompass these factors and they are as follows.

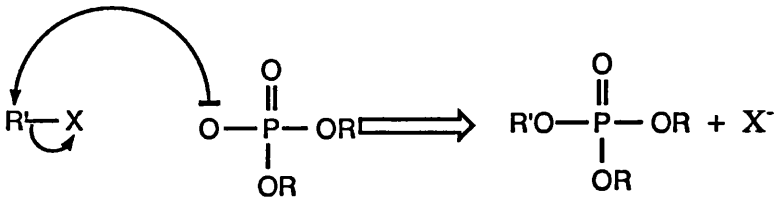
(i) nucleophilic attack of a hydroxyl group on an activated phosphoryl group, displacing a stable anion, [equation 1]. This is the most ubiquitous method. In general, phosphorylation is effected when the acid HX is stronger than the phosphate (RO)(R'O)POOH, the low pK_a of HX (where X^- is a good leaving group) ensures that the reaction forms the product: The group that follow this mechanism includes; phosphoryl trihalides and many phosphorohalidates; certain acyl phosphates and polyphosphates such as phosphoric oxide, phosphoric acid and neutral pyrophosphate esters. Heterolysis of the P-X bond may be achieved by activation of the X group at the time of the reaction. Thus phosphoramidates are stable as anions, but on protonation will act as mild phosphorylating agents.



Nucleophilic attack of a hydroxyl group upon an activated phosphoryl group.

Equation 1.

(ii) nucleophilic attack of a phosphate anion on the carbon of an alkyl halide, alkyl ester or similar compound [equation 2]. In the case of the alkyl ester the condensation must be performed in the presence of a suitable activating agent, such as a carbodiimide or arenesulphonyl chloride. There are obvious advantages in phosphorylation methods which involve the activation of an existing alkyl phosphate so that, for example, a naturally occurring phosphate could be used directly for the elaboration of more complex derivatives. One way this could be achieved is by conversion *in situ* to a mixed anhydride with a strong acid and subsequent addition of another alcohol or phosphate.



Nucleophilic attack of a phosphate anion on carbon.

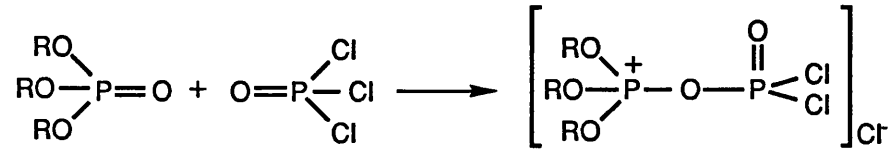
Equation 2.

Phosphorus exists in two main oxidation states, P(III) or P(V), and as a consequence of this there are many possible types of orthophosphate and polyphosphate esters. The main problem of phosphorylation lies in transforming the phosphoric acid molecule to a sufficiently powerful phosphorylating agent. Nevertheless this can be done by a variety of methods; for example by substitution of an electron withdrawing group such as chlorine for oxygen, to give the phosphorochloridate, or by alkylation at the oxygen atoms to form a less negatively charged moiety. These methods have the affect of making the phosphorus atom more electropositive or more accessible to nucleophiles, and as a result phosphorylation is made possible. Thus, pyrophosphoric acid is unable to phosphorylate under mild conditions due to the electrostatic repulsion, but by making the neutral tetraalkyl pyrophosphate it becomes accessible to nucleophiles and can phosphorylate many compounds.

2.3.1 Phosphorochloridates.

Phosphoryl chloride ($POCl_3$), has been used as a phosphorylating reagent since 1857[50]. Nucleoside 5'-phosphates have been synthesised in *circa* 90% yield using $POCl_3$ with trialkyl phosphate as the solvent[51]. Unprotected ribonucleoside taken with partially hydrolysed phosphoryl chloride at zero degrees in trimethyl or triethylphosphates has resulted in a selective synthesis of 5'-nucleotide. The active phosphorylating agent (16)

was probably the species below [Fig. 16]. This has been used to phosphorylate many compounds, and can be seen in the synthesis of 3'-amino-3'-deoxyadenosine 5'-phosphate[52].



(16)

Figure 16. Probable phosphorylating species

2.3.2 Diphenyl and Dibenzyl Phosphorochloridates.

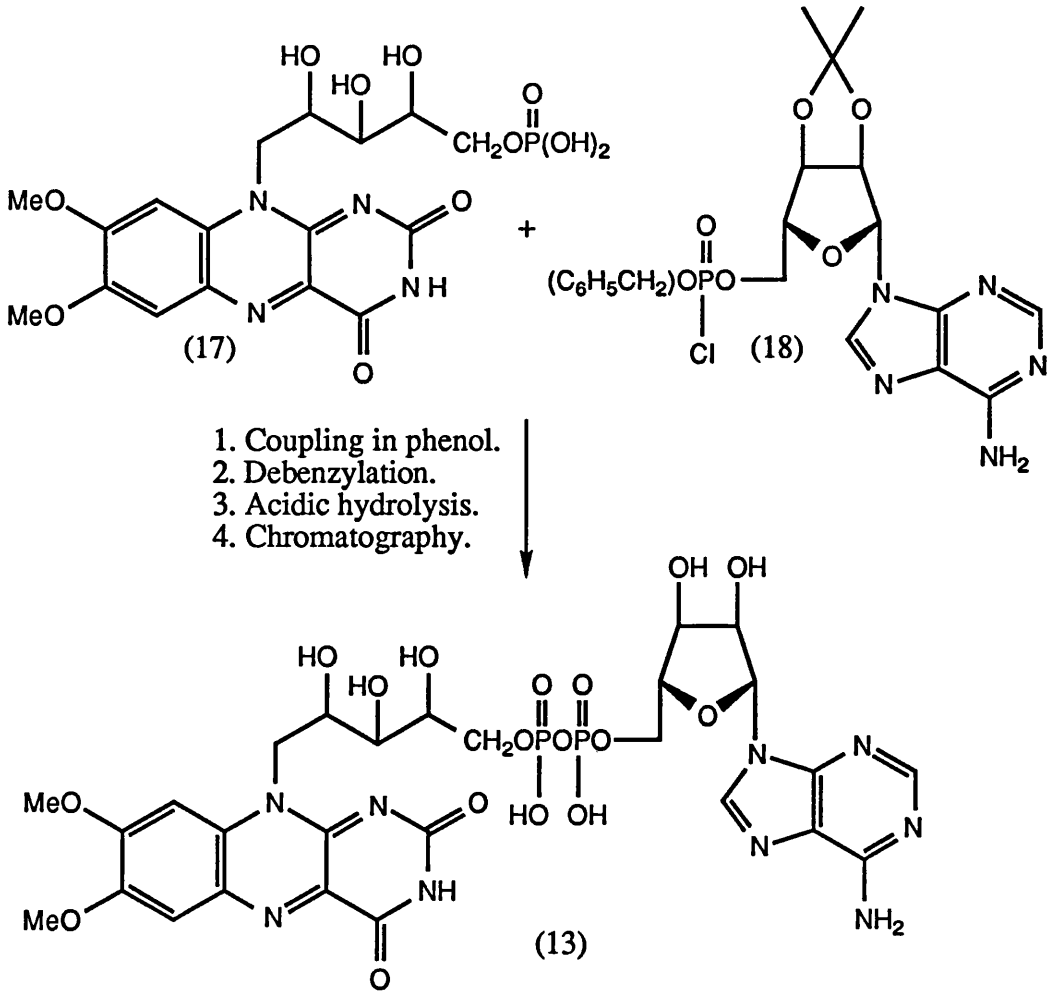
Diphenyl and dibenzyl phosphorochloridates have been used effectively for the preparation of many phosphate esters. They are easily synthesised, the former by the reaction of phosphoryl trichloride and phenol followed by fractional distillation and the latter by the oxidation of dibenzyl phosphite with N–chlorosuccinimide.

Diphenyl phosphorochloridate has been used to phosphorylate many alcohols in pyridine, at room temperature, in good yield. The deprotection can be subsequently effected by catalytic hydrogenolysis. This procedure was first used to synthesise glycerol phosphate and fructose phosphate[53]. Later on, Iselin used diphenyl phosphorochloridate to synthesise D-*myo*-inositol 2-phosphate[54]. Since then it has also been used to synthesise L-*myo*-inositol 1-phosphate[55,56]. Baer similarly took D-glyceric acid with diphenyl phosphorochloridate in order to form D–2,3-bisphosphoglycerate, (BPG) (14)[57]. Diphenyl phosphorochloridate, however, is often unsuitable for phosphorylating sterically hindered hydroxyl functions. Yields though, can be improved dramatically by the addition of 5-chloro-1-methylimidazole to the reactants in acetonitrile. The reactions can then be made to occur readily at room temperature[58].

The first synthesis of a nucleotide coenzyme, adenosine 5' pyrophosphate (ADP) was achieved in 1947[59], and this involved the use of dibenzyl phosphorochloridate. Following the removal of the ketal function in 2', 3'-O-isopropylidine adenosine-5' dibenzyl phosphate by mild acidic hydrolysis, phosphorylation of the silver salt with dibenzyl phosphorochloridate gave the adenosine 5' tribenzyl pyrophosphate. The benzyl groups were ultimately cleaved by catalytic hydrogenolysis, to yield the desired compound, adenosine 5'-pyrophosphate. This method has also been extended to give the adenosine 5' triphosphate (ATP)[60]. The adenosine 5' tribenzyl pyrophosphate was treated with N-methylmorpholine to remove one of the β–phosphorus benzyl groups.

The silver salt was again treated with dibenzyl phosphorochloridate. Subsequent catalytic hydrogenolysis gave adenosine 5' triphosphate. An alternative procedure[61], involved treatment of the silver salt of adenosine 5' phosphate with an excess of dibenzyl phosphorochloridate. This, after hydrogenolysis, yielded ATP, which was identical to natural ATP.

Flavin adenine dinucleotide (FAD) (13) was the first example of a diesterified pyrophosphate coenzyme to be made. It too was synthesised using the phosphorochloridate method whereby the monothallous salt of riboflavin-5'-phosphate (17) was treated with 2',3'-O-isopropylideneadenosine-5' benzyl phosphorochloridate (18) in phenol. After debenzylation, acidic hydrolysis and chromatography, pure FAD (13) was isolated in low yield, (6%), [Scheme 1][62].



Flavin adenine dinucleotide (FAD).

Scheme 1

As with all of these syntheses using partially protected or fully deprotected compounds, the presence of a free vicinal hydroxyl group in the molecule will probably lead to the

formation of a cyclic phosphate. Consider [Fig. 17] for example.

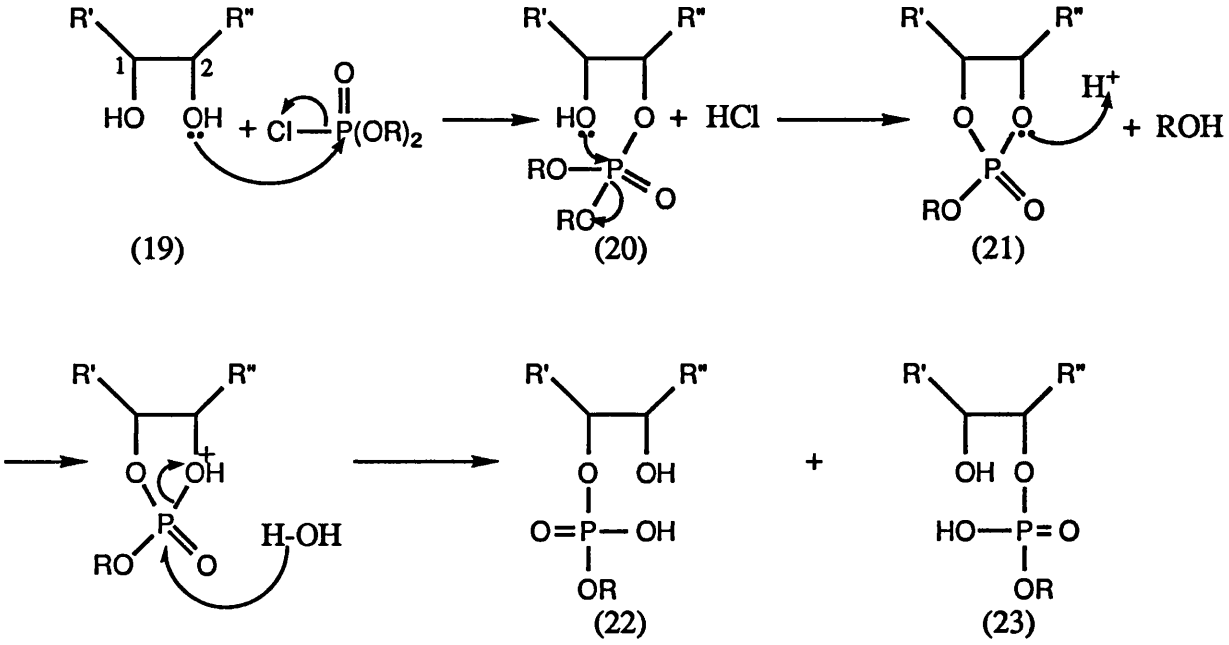


Figure 17

Monophosphorylation of (19) will lead to the formation of (20) which can cyclise to give (21). Hydrolysis of (21) will produce two different compounds (23) and (24) as a result of phosphate group scrambling, giving a mixture of compounds where the phosphate is on either the 1 (22) or the 2 position (23). Moffatt and Khorana encountered this problem during the synthesis of coenzyme A[63]. The cyclic phosphate intermediate was formed during the synthesis of pantetheine 4-phosphate when the disulphide dimer of pantetheine, pantethine, was dissolved in pyridine, and treated with dibenzyl phosphorochloridate in benzene. After work up and deprotection they obtained a mixture of D-pantetheine 4-phosphate (45%) and D-pantetheine 2,4-cyclic phosphate. Similarly when they took adenosine in boiling anhydrous pyridine, and added dibenzyl phosphorochloridate in benzene to the cooled solution, following deprotection, a mixture of three compounds was isolated; adenosine 5' phosphate (14%), adenosine 2',3',5'-triphosphate (6.1%) and adenosine bisphosphate (70%). The adenosine bisphosphate comprised of a mixture of adenosine 2',5'-bisphosphate and adenosine 3',5'-bisphosphate, which the resulted from the hydrolysis of the five membered cyclic intermediate.

2.3.3 Other Phosphorochloridates.

A good phosphorylation reagent must be powerful enough to phosphorylate the alcohol or phosphate but in addition the phosphate protecting groups must subsequently be removed easily and efficiently. There are many examples of such groups, the specific requirements of the reaction, determining which reagent is used. Apart from dibenzyl and diphenyl

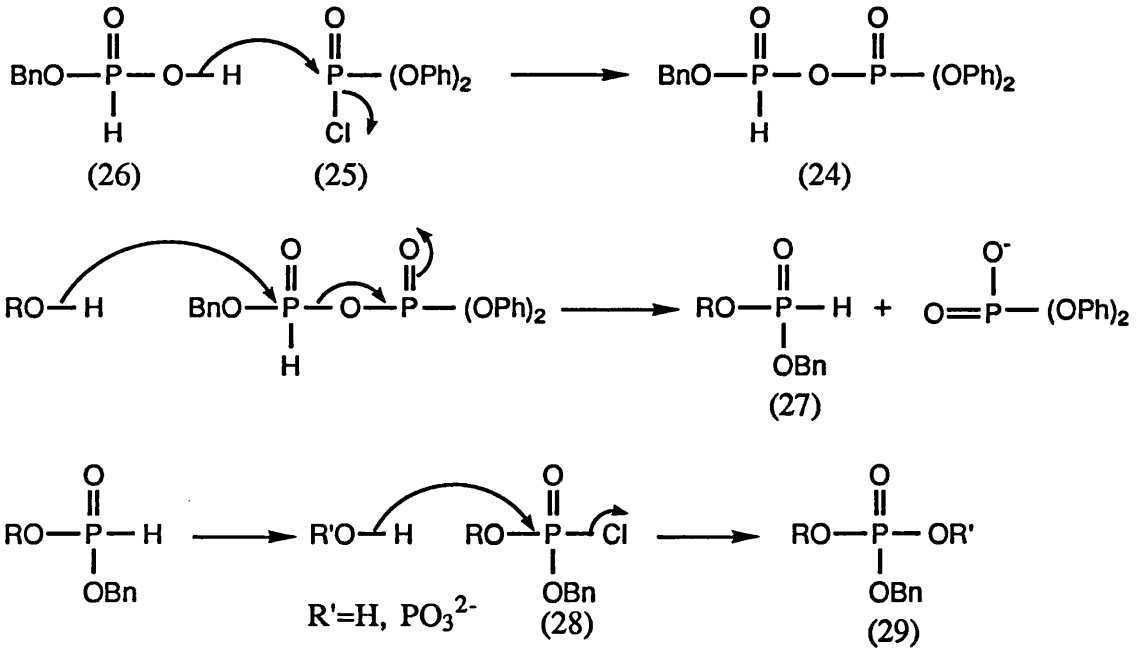
phosphorochloridate, other diaryl phosphorochloridates have been used as phosphorylating agents. Bis(*para*-nitrobenzyl)phosphorochloridate has been used for the phosphorylation of 5'-amino-5'-deoxynucleosides[64] and bis[*para*-nitrophenyl] phosphorochloridate, used in the synthesis of thymidine nucleotides[65], the deprotection being effected by alkaline hydrolysis. Bis (2,2,2-trichloroethyl)-phosphorochloridate has been used as a nucleotide phosphorylating agent[66]. It has been used to phosphorylate hydroxyl groups in greater than 80% yield at temperatures around 0°C in a pyridine solution. Ultimate deprotection of the 2,2,2-trichloroethyl group can be effected by various methods: Zinc dust in 80% acetic acid, rt for 1h[66]; Zinc/Copper couple in dimethyl formamide, 50 °C for 1 h[66]; Zinc with silver carbonate in pyridine containing 10 % (v/v) acetic acid[67,68]; electrolytic cleavage in methanol[69], or in dimethyl formamide[70]; tetrabutylammonium fluoride at rt for 30 min[71]; sodium borohydride under the catalysis of selenium in dimethyl formamide[72], sodium napthalene in hexamethylphosphoric triamide[73], Pd-catalysed hydrogenolysis in aqueous ethanol[74]. Preliminary experiments on the deprotection of 2,2,2-trichloroethoxymethyl ethers using lithium in liquid ammonia have been reported[75]. However, we report here the novel use of sodium in liquid ammonia as a convenient method for the deprotection of 2,2,2-trichloroethyl phosphate esters. Dianilino phosphorochloridates have also been used for phosphorylation[76], but removal of the protecting groups has proved to be difficult, so making the use of this reagent unfavorable. Finally, pyrophosphoryl chloride has been used as a selective phosphorylating agent for the primary hydroxyl of nucleosides[77]. Adenosine treated with pyrophosphoryl chloride in *m*-cresol gave adenosine 5'-phosphate in 72% yield after purification.

2.3.4 Phosphorodichloridates.

Linkage of two different moieties through a phosphate molecule is common in many biological systems, *e.g.* the inter-nucleotide bond of DNA or RNA. Thus, phosphorodichloridates have been developed in order to synthesise these phosphate diesters, phenyl phosphodichloridate has been used in the synthesis of dinucleoside phosphates, such as di(uridine-5') phosphate[78]. Methyl phosphorodichloridate has been used for DNA synthesis[79]. Phenyl and *para*-nitrophenyl phosphorodichloridate have been extensively used in the synthesis of phospholipids with two different lipids[80]. Dichlorophosphoric acid has also been used for inter-nucleotide bond formation and in syntheses of nucleoside 3', 5'-cyclic phosphates[81].

2.4 Anhydrides.

Polyphosphates and mixed anhydrides of phosphates have been found to be adequate phosphorylating reagents. Phosphorylation with non-specific phosphorylating agents such as orthophosphoric acid, phosphorus pentoxide and pyrophosphoric acids still continues to be used widely, especially on an industrial scale. Phosphoric acid[82], potassium hydrogen phosphate[83] and pyrophosphoric acid[84] have all been used as well in order to phosphorylate carbohydrates. Pyrophosphoric acid has also been used to phosphorylate *epi*- and *muco*-inositols[85]. However, use of these polymeric derivatives is restricted, due to their non-specific nature, which results in the formation of cyclic phosphate and other side products. Tetrabenzyl pyrophosphate has been successfully used for the phosphorylation of the 4 and 5 positions of inositol[86].

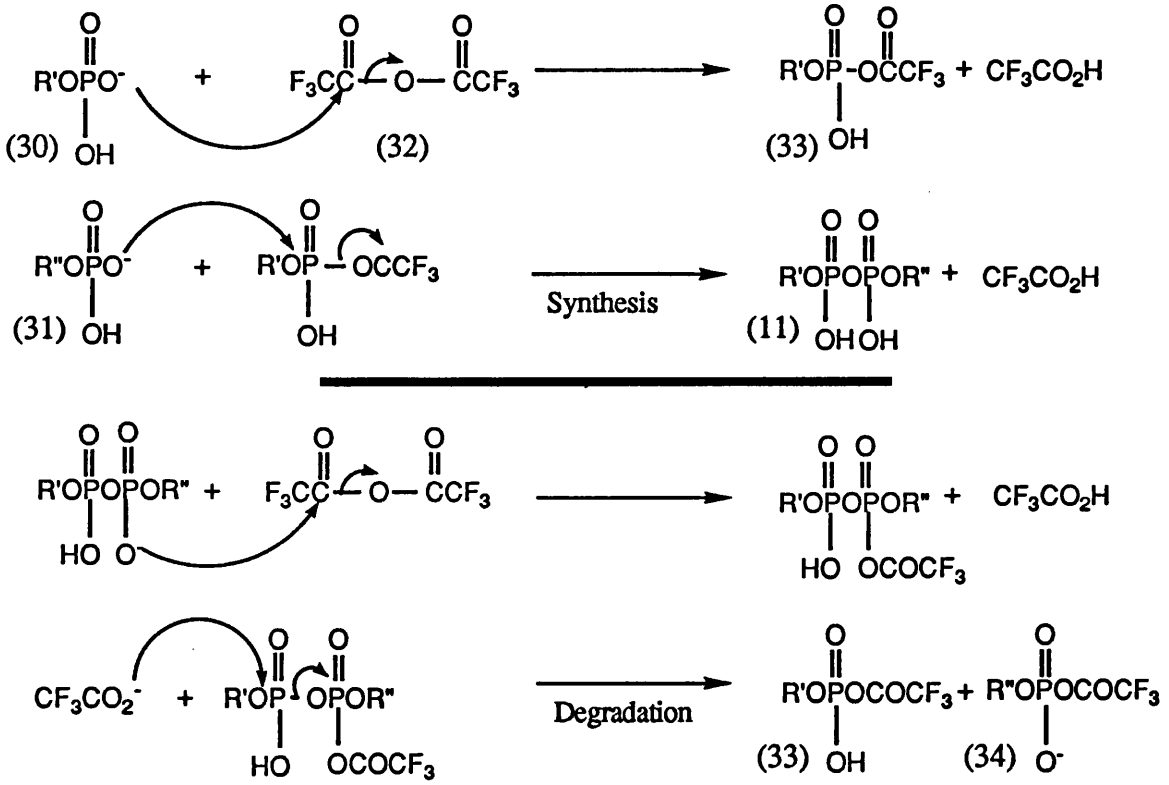


Reaction of *O*-Benzyl phosphorus-*O*-diphenyl phosphoric anhydride (24).

Scheme 2

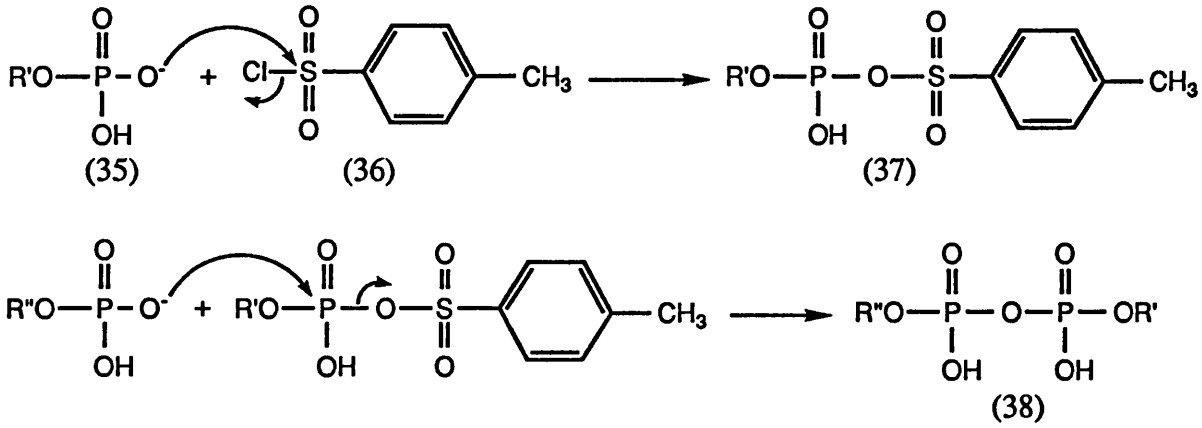
O-Benzyl phosphorus-*O*-diphenyl phosphoric anhydride (24), [Scheme 2], is made from the reaction of diphenyl phosphorochloridate (25) with benzyl phosphite (26) and has been used to synthesise nucleoside benzyl phosphonates (27)[87]. These, in common with dibenzyl phosphite, can be oxidised using N-chlorosuccinimide to form the phosphorochloridate (28). Subsequent treatment with triethylammonium dibenzyl phosphate forms the protected pyrophosphate (29). Similarly, triethylammonium tribenzyl pyrophosphate can be used instead of the triethylammonium dibenzyl phosphate to yield the triphosphate[88]. By choosing the appropriate anhydride one can obtain a

phosphate, pyrophosphate or polyphosphate. Trifluoroacetic acid and *para*-toluenesulphonyl chloride are able to form such phosphate and pyrophosphate esters. For example, Nicotinamide adenine dinucleotide (11) has been synthesised in low yield from adenosine 5' phosphate (30), nicotinamide nucleotide (31) and trifluoroacetic anhydride (32)[89] [Scheme 3]. The trifluoroacetic anhydride, forms the mixed adenosine 5' phosphate-trifluoroacetic anhydride (33). This is then attacked by the nicotinamide phosphate to give the nicotinamide adenine dinucleotide (11). However, the trifluoroacetate cleaves the pyrophosphate (11) to reform the mixed anhydrides (33) and (34). The trifluoroacetic anhydride, thus, cleaves the pyrophosphate as efficiently as it forms it.



Scheme 3. Where R' is adenine and R" is nicotinamide.

The acid chloride *p*-toluene sulphonyl chloride (35) will react with a benzyl phosphate (36) to form the intermediate mixed anhydride (37) which is then attacked by another benzyl phosphate to give dibenzyl pyrophosphate (38)[90] [as in the synthesis in Scheme 4].

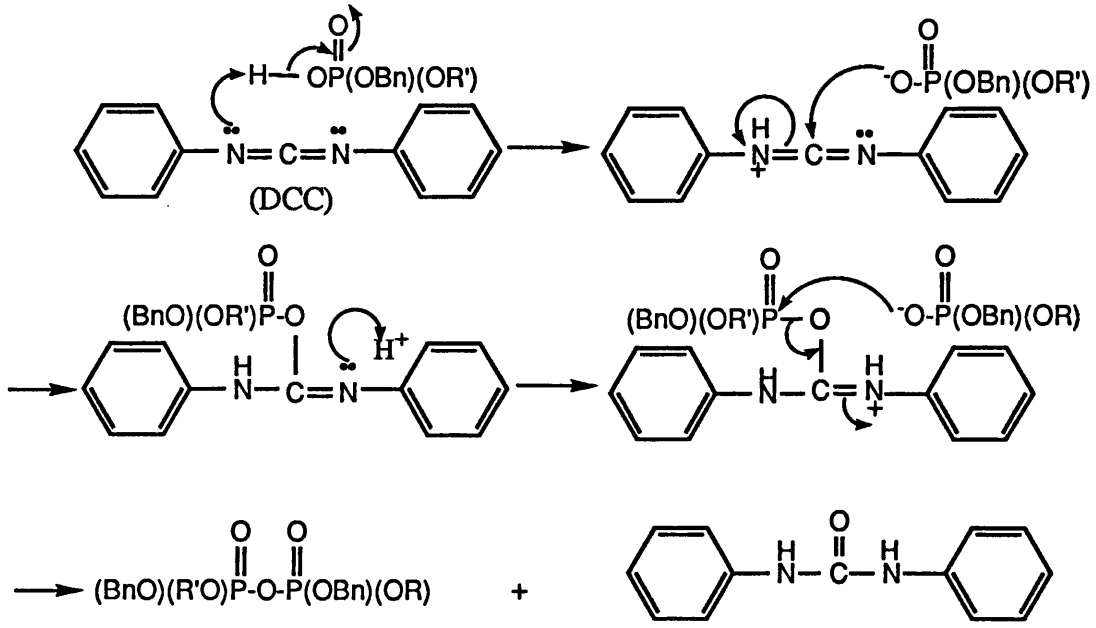


Scheme 4

Sulphonyl chlorides have formed the basis of many nucleotide 5' OH capping reagents in the phosphotriester method of DNA synthesis[91] [Scheme 4].

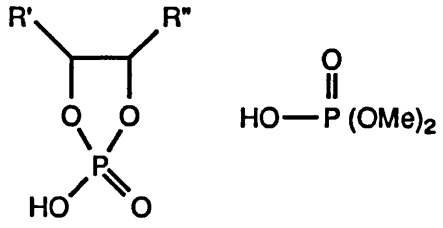
2.5 Carbodiimides.

Carbodiimides, as well as reactive anhydrides, can act as the activating agents capable of forming phosphate and pyrophosphate moieties. Since carbodiimides have been used to form carboxylic anhydrides from the corresponding aliphatic acid[92], they were an obvious choice for the synthesis of phosphate anhydrides. Dicyclohexylcarbodiimide (DCC), the most widely used carbodiimide, can form pyrophosphates from phosphoric acid and its esters in moderate yields.



Scheme 5

DCC has been extensively used to form nucleoside phosphate anhydrides[93]. However, given a pair of acids with anions of comparable nucleophilicity, all three possible anhydrides will be formed by DCC. In the presence of DCC pyrophosphate synthesis is generally favoured to the exclusion of esterification, unless there is hydroxyl group adjacent to the phosphate; in that case 5-membered cyclic phosphate formation superceeds the formation of the pyrophosphate. Nevertheless, intramolecular phosphorylation of suitably placed hydroxyl groups occurs readily, providing useful syntheses of cyclic phosphates. The ring size of the cyclic phosphate intermediate determines the stability of that compound in acidic or basic conditions. Consider the cyclic five membered phosphate (21), as opposed to the acyclic equivalent (39).



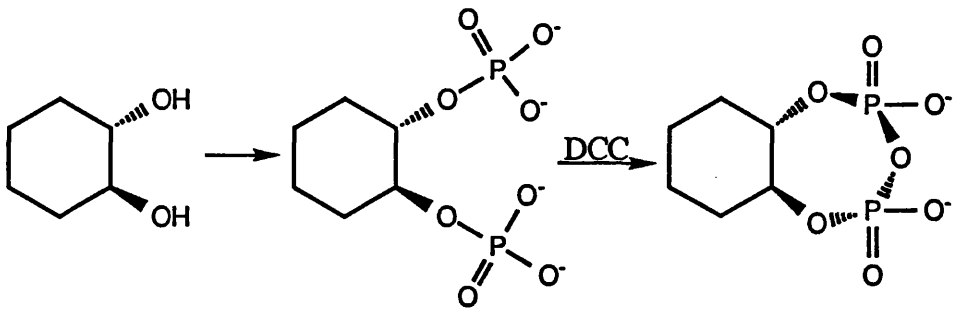
(21) (39)

Figure 18

The cyclic compound hydrolyses 10^6-10^7 times faster than the acyclic dimethyl phosphate in 0.5 M sodium hydroxide[36] Increasing the ring size to six reduces the reactivity of the cyclic intermediate, it hydrolyses less than ten times faster than dimethyl phosphate (39), in base[94]. Furthermore, increasing the ring size to seven results in a compound that is as stable as the acyclic equivalent to hydrolysis[94]. Similarly, this trend is reflected in the relative stabilities to acidic hydrolysis. Ballou found that of the two possible cyclic phosphates formed from *myo*-inositol 1 phosphate, the five membered cyclic phosphate containing two adjacent *cis* hydroxyl groups was more stable than the isomer with the cyclic phosphate containing two adjacent *trans* hydroxyl groups[95]. The difference in the stabilities is probably due to ring strain of the *trans*-fused 6:5 system and the steric effect of bond-bond interactions. It is a consequence of the reactivity of phosphate triesters that displacement at phosphorus readily occurs. This leads to the formation of the diester. Since the diester is less reactive, further displacement does not happen under mild conditions.

trans-Cyclohexane-1,2-cyclic pyrophosphate (40) has been synthesised initially by treatment of *trans*-cyclohexane 1,2-diol (41) with diphenylphosphorochloridate in pyridine to give the *trans*-cyclohexane 1,2-bisphosphate (42) in 73% yield after deprotection and purification. The triscyclohexylammonium salt of *trans*-cyclohexane 1,2-bisphosphate was then treated with DCC in DMF to give *trans*-cyclohexane-1,2-

cyclic pyrophosphate (40) as the biscyclohexylammonium salt in 98% yield[96].



(41) (42) (40)

Scheme 6

Nucleoside 5' polyphosphates are easily made by the condensation of phosphoric acid and the mononucleotide, using DCC. The complex mixtures produced can be simplified to some extent by varying the ratio of reactants and altering the experimental conditions. Thus by using carbodiimides the pyrophosphates and triphosphates of all the major natural nucleosides have been made[97]. Again, complex mixtures were produced, but the use of ion exchange chromatography has allowed for an effective separation of the various phosphates.

Nicotinamide adenine dinucleotide was successfully synthesised from a mixture of D-ribofuranosylnicotinamide-5' phosphate, adenosine 5' phosphate and DCC in aqueous pyridine[98]. Using anion exchange chromatography the mixture of mainly α, β, NAD, plus some P^1, P^2 - dinicotinamide nucleoside pyrophosphate and a small amount of diadenosine 5' pyrophosphate was separated to afford the β–anomer of NAD. This β-NAD was found to be identical to the natural coenzyme, [Fig. 14].

By taking the nucleoside phosphate, DCC and the appropriate alkyl phosphate many interesting metabolites have been made. This approach has been used in the synthesis of, adenosine, guanosine, cytosine and uridine diphosphate cholines[99]. Lipid metabolites, such as CDP ethanolamine, CDP dipalmitin and CDP dilaurin have also been synthesised. The latter two were made by the action of DCC on cytidine 5' phosphate and 1,2-O-isopropylidene-L-glycerol-3 phosphate. After removal of the isopropylidene group, the cytidine diphosphate glycerol was acylated to form CDP diglyceride[100].
Phosphate monoesters similarly can be used as phosphorylating agents if they are suitably activated. Protected alkyl phosphates have been used in conjunction with DCC to phosphorylate many alcohols. For example, phosphorylation of thymidine at the 3' position has been accomplished using a ß-cyanoethyl phosphate, DCC and 5'-trityl protected thymidine in quantitative yield. Subsequent removal of the trityl and ß-

cyanoethyl groups gave thymidine 3-phosphate in 88% yield[101]. Again, choice of the alkyl phosphate used, is dependent upon subsequent reactions that it will be exposed to.

A few phosphate esters do not have to be activated by DCC, the most commonly used leaving group of this type, employed to date, is the *para*-nitrophenyl group and thus *para*-nitrophenyl esters continue to find applications in nucleotide chemistry. For example, in the phosphorylation of the 5' position of a suitably protected nucleoside[102]. When 2',3'-*O*-isopropylidineguanosine was brought into reaction with *p*-nitrophenyl phosphate and DCC in DMF, 2',3'-*O*-isopropylidineguanosine-5'-*p*-nitrophenyl phosphate was obtained in 70% yield[103].

2.6 Phosphoramidates.

DCC has been used in the synthesis of an important class of phosphorylating reagents, the phosphoramidates.

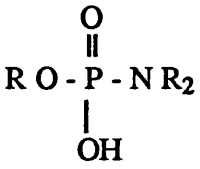
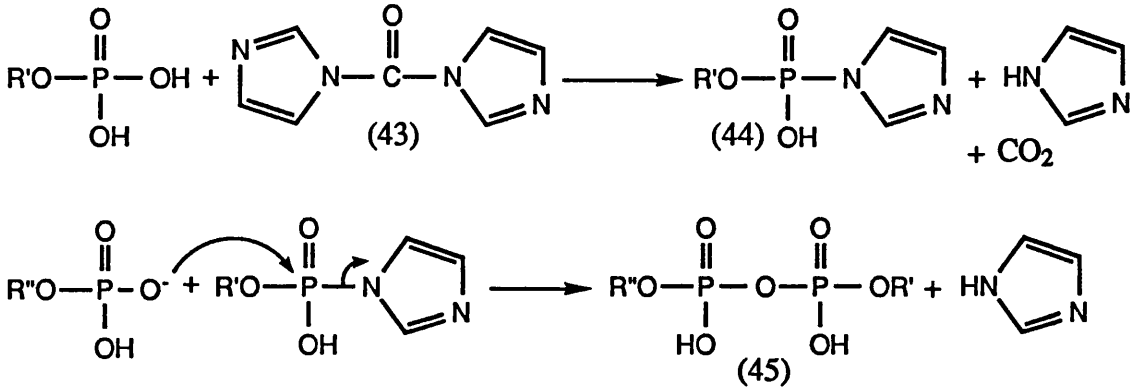


Figure 19 A phosphoramidate

Phosphoramidates have proven to be of great importance, as the reactive intermediates in the specific synthesis of pyrophosphates[104], as phosphoramidates generally form the pyrophosphate bond in good yield.

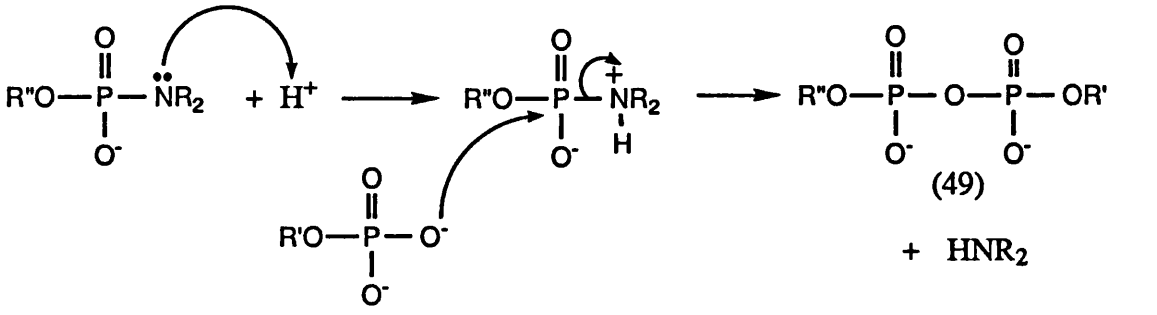
A means of activating a phosphate monoester group is by reaction with N,N'-carbonyl-diimidazole (43) and the reaction of the resulting phosphoramidate, phosphoryl-imidazole (44) with another nucleophile to give the product. If the nucleophile used is an alcohol this results in formation of the phosphate diester, however, if the nucleophile is another phosphate monoester then the pyrophosphate (44) is formed[105,106].

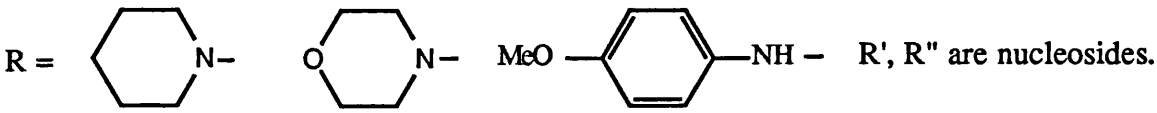


Scheme 7

2.6.1 *Phosphomorpholidates.*

Moffatt and Khorana tested three nucleoside 5' phosphoramidates; the phosphopiperidate (46), the phosphomorpholidate (47) and the phosphoanisidate (48). They found that the phosphomorpholidate (48) was the most suitable phosphoramidate of the three for the formation of nucleoside pyrophosphates (49)[107]. Whereas the phosphopiperidate was the most reactive (the half-reaction time for the formation of the nucleoside 5'-pyrophosphate was 3 minutes).

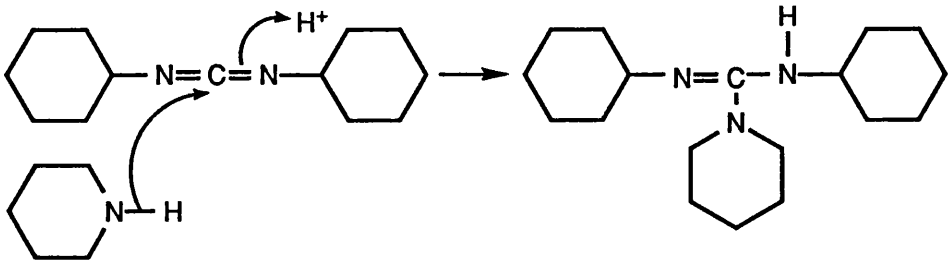




piperidine (46) | 4-morpholidine (47) | anisidine (48)

Scheme 8.

However, because of its strong basicity (pKa 11.22), the formation of a side product, N,N'-dicyclohexyl-4-piperidinecarboxamidine (50), effectively completed with the formation of the phosphoramidate.



(50)

Scheme 9

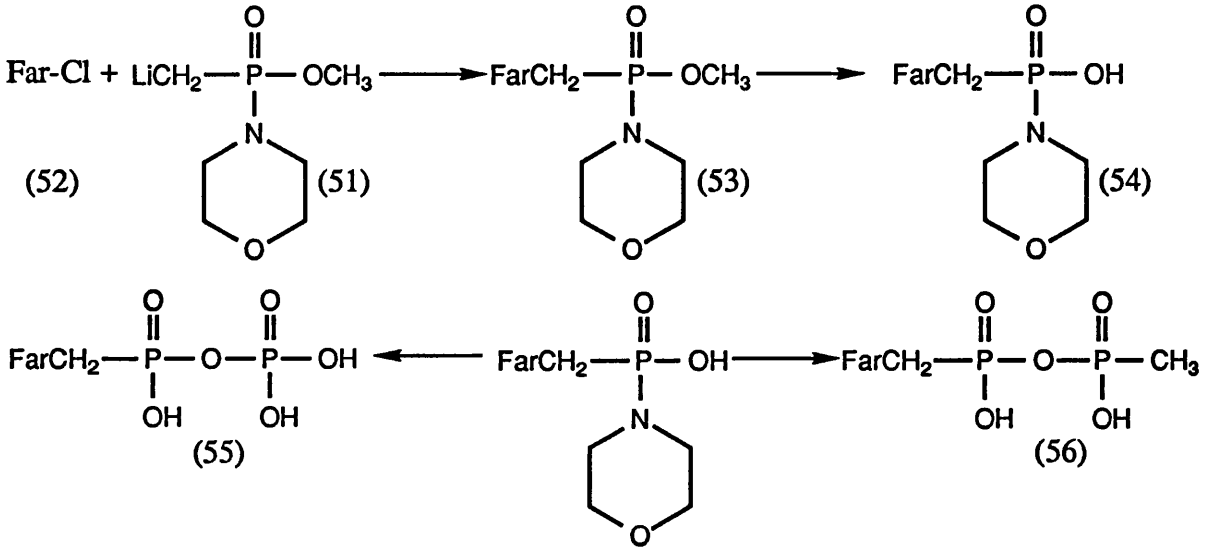
With the much weaker base, *p*-anisidine (pKa 5.29), the synthesis reaction went to completion, but the synthetic yield of the phosphoanisdate was low, only 20%. Therefore, the usefulness of this reagent was severely limited, as after seven hours the nucleoside 5'-pyrophosphate synthesis reaction had only gone to 10% of completion and the half-reaction time was 30 hours. However, because morpholine has a basicity

midway between the other two, (pKa 8.36), the synthesis of the phosphomorpholidate is not significantly affected by the formation of the trisubstituted gunanidine. The reaction also went to 95% completion and the half-reaction time for the synthesis of the nucleoside 5'-pyrophosphate was 6 minutes.

Therefore, the preparation of nucleoside di and triphosphates has been accomplished using the appropriate nucleoside phosphomorpholidate[108]. For example, synthesis of ATP was effected by taking adenosine 5'-phosphomorpholidate with the mono-tri-*n*-butylammonium salt of orthophosphoric acid in anhydrous pyridine. This created ADP in excellent yield after 24-48 hours. By taking the bis-tri-*n*-butylammonium salt of inorganic pyrophosphate, ATP was synthesised in 57% yield after 2 hours. However, longer reaction times caused the ATP to disproportionate to mainly ADP, (76% after 24 hours).

Phosphomorpholidates have also been used to prepare, in virtually quantitative yield, many ribonucleotide 5'-diphosphate derivatives. The general synthesis involved taking two equivalents of the appropriate phosphomonoester, as its soluble tri-*n*-octylammonium salt, and reacting this with the 4-morpholine NN'-dicyclohexylcarboxylidine salt of the nucleoside 5'-phosphomorpholidate in anhydrous pyridine. Thus when two and a half equivalents of mono (tri-*n*-octylammonium)-DL-glycerol-2-phosphate was reacted with 4-morpholine NN'-dicyclohexylcarboxaminium 5'-cytidine phosphomorpholidate in pyridine for 4 days, and after deprotection and purification, cytidine diphosphate glycerol was isolated in 70% yield as its calcium salt.

Recently, the reaction of methyl lithiomethylphosphonomorpholidate (51) with farnesyl chloride (1-chloro-*trans,trans*-3,7,11-trimethyl-2,6,10-dodecatriene) (52) has been reported[109]. The phosphonamidate (53) thus formed was demethylated, using trimethylsilyl bromide followed by tetra-*n*-butyl ammonium fluoride to give (54). Subsequent coupling with either tri-*n*-butyl ammonium phosphate or tri-*n*-butyl ammonium methyl-phosphonate in dry pyridine gave farnesylmethylphosphonic phosphoric anhydride (55) or farnesylmethylphosphonic methylphosphonic anhydride (56) respectively, both in about 40% yield.



Far= *trans,trans*-3,7,11-trimethyl-2,6,10-dodecatriene

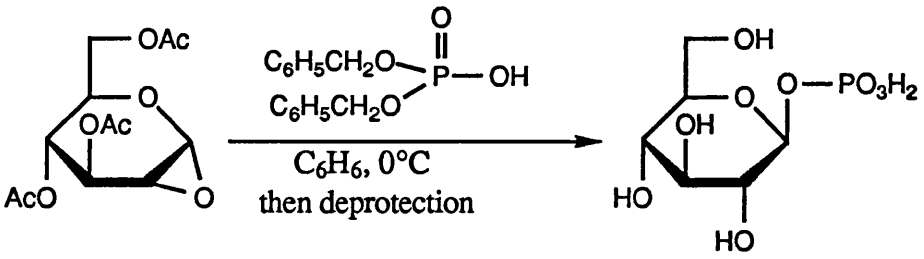
Scheme 10 Synthesis of farnesyl pyrophosphates

2.7 Tetraalkyl Pyrophosphate

Esterification of a preformed protected pyrophosphate has been successfully used to phosphorylate polyhydroxyl compounds[86,110]. The procedure involves taking tetrabenzyl pyrophosphate, which is a commercially available crystalline reagent, and subjecting this to the alkoxide ion of the alcohol. This method gives good yields and can be used to effectively phosphorylate vicinal diols. The benzyl esters are easily removed after phosphorylation by catalytic hydrogenolysis and without any phosphate migration being incurred. However, the main problem associated with this procedure is that of the formation of the alkoxide ion. This is usually effected by treatment of the alcohol with *n*-butyl lithium, potassium hydride or potassium hydroxide. However, the strong bases used can degrade the compound rather then form the alkoxide ion[111]. In summary, providing the compound is stable to the strong base, esterification of tetrabenzyl pyrophosphate can be a powerful method of phosphorylation and it has been used effectively in many syntheses, for example, inositol derivatives with vicinal diols were treated with *n*-butyl lithium then phosphorylated with tetrabenzyl pyrophosphate in 80% yield[86]. The alkoxide of 2,6-dibenzyl myo-inositol was phosphorylated with tetrabenzylpyrophosphate to give (±)-*myo*-inositol-1,3,4,5-tetrakisphosphate[111], this procedure being used in the preparation of (±)-*myo*-inositol-1,4,5-trisphosphate by Vacca *et.al.*[112].

2.8 Miscellaneous P(V) Reagents

Phosphorylations have been conducted using phosphate diesters. Some require the use of an activating agent, whereas others do not. The advantage of using a phosphate diester is that the reaction produces a fully protected phosphate triester. The stereospecific opening of epoxides or anhydrides with diaryl phosphate in the synthesis of sugar phosphates has been well know for some time[113,114], and this approach has been adopted for the synthesis of ß-D-glucopyranosyl phosphate (57) from 3,4,6-tris[*O*-acetyl]-1,2-anhydro-a-D-glucopyranose (58)[115].

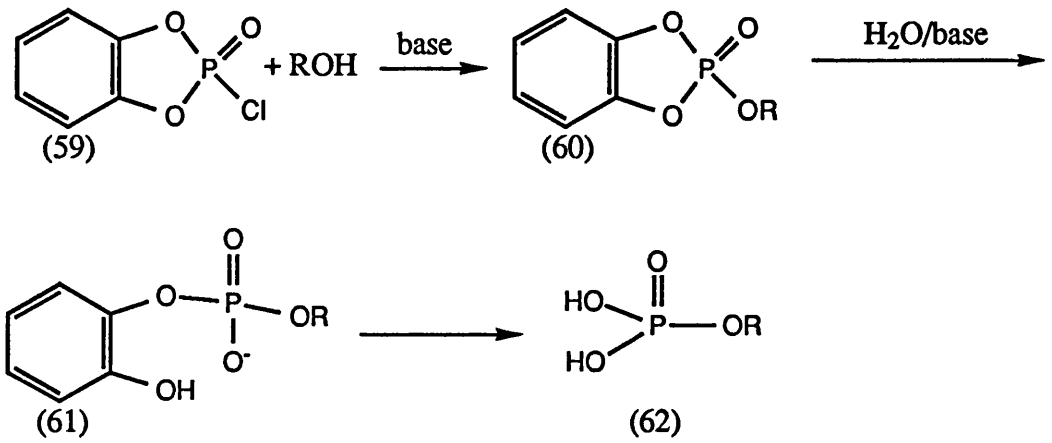


(57) (58)

Scheme 11

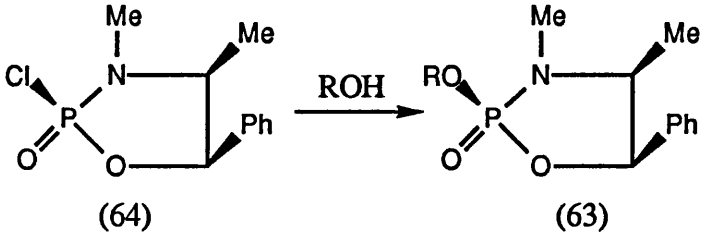
Mixed diesters of phosphoric acid have also been reported as phosphorylating agents. For example, phenyl 8-quinolyl phosphate reacts equally well with alcohols and amines to yield the corresponding alkyl phenyl phosphates and phosphoramidates[116].

Phosphorylation has been effected using 5 and 6-membered cyclic phosphorylating agents. 2-Chloro-2-oxo-P^V-1,3,2-benzodioxaphosphole (59) was investigated as a highly reactive phosphorylating species[117]. In the presence of triethylamine compound (59), was found to react rapidly with a stoichiometric amount of sterically hindered alcohols to give the intermediate 2-alkoxy derivative (60). The 2-alkoxy derivative is then quantitatively converted to the *o*-hydroxyphenyl phosphate ester (61), which are generally easily isolable crystalline salts, and oxidation with bromine/water then gives the phosphate monoester (62).



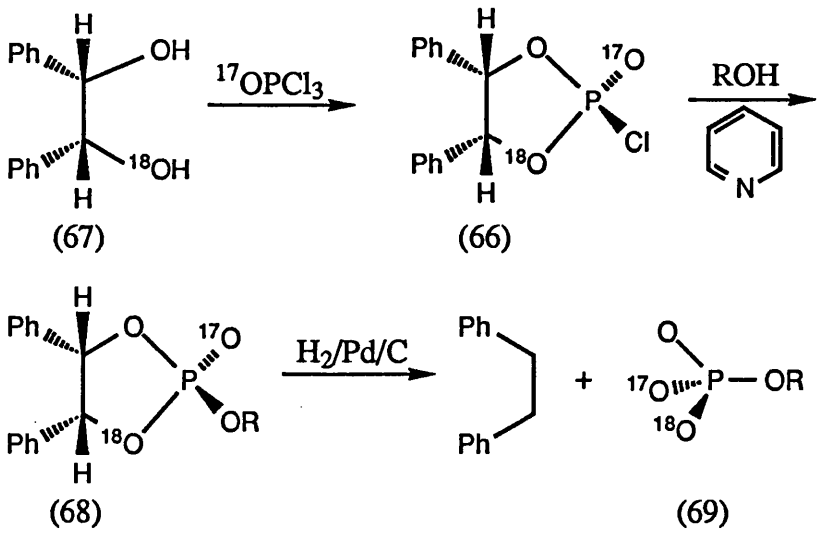
Scheme 12. Synthesis of 2-alkoxy-2-oxo-P^V-1,3,2-benzodioxaphosphole

Chiral cyclic phosphorus esters have been used to study the stereochemical course of various enzymic reactions. Examples of these chiral phosphorus esters are 2-substituted 1,3,2-oxazaphospholidine-2-ones (63). These compounds are easily derived from the reaction of 2-chloro-1,3,2-oxazaphospholidine-2-ones with an alkoxide, or with an alcohol and base[118]. In either reaction the configuration about phosphorus is retained. (2R, 4S, 5R) 2-Chloro-1,3,2-oxazaphospholidine-2-one (64), the major product from phosphoryl chloride and (-) ephedrine [(1R, 2S)-2-methylamino-1-phenylpropan-1-ol] is used to phosphorylate alcohols to give a chiral phosphorus ester. The enantiomer derived from (-)-ephedrine (64), tends to be used for the synthesis of substrates for enzymic investigation[118]. However, the enantiomer (2S, 4S, 5R) 2-Chloro-1,3,2-oxazaphospholidine-2-one (65) can be easily obtained in a similar manner from (+) ephedrine.



Scheme 13

Cullis *et al.*[119,120] have reported the use of various chiral phosphorchloridates for the synthesis of chiral phosphate monoesters. They treat an alcohol in dry pyridine with (2R, 4S, 5R)-2-chloro-2-[^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholan (66), derived from the [1-^{18}O] dihydroxyethane (67), to give the corresponding (2R, 4S, 5R)-2-alkoxy-2-[^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholan (68), which yields the chiral monophosphate (69) and 1,2-diphenyl ethane upon catalytic hydrogenolysis.



Synthesis of chiral phosphate monoesters.

Scheme 14.

2.9 Phosphorus (III) Reagents

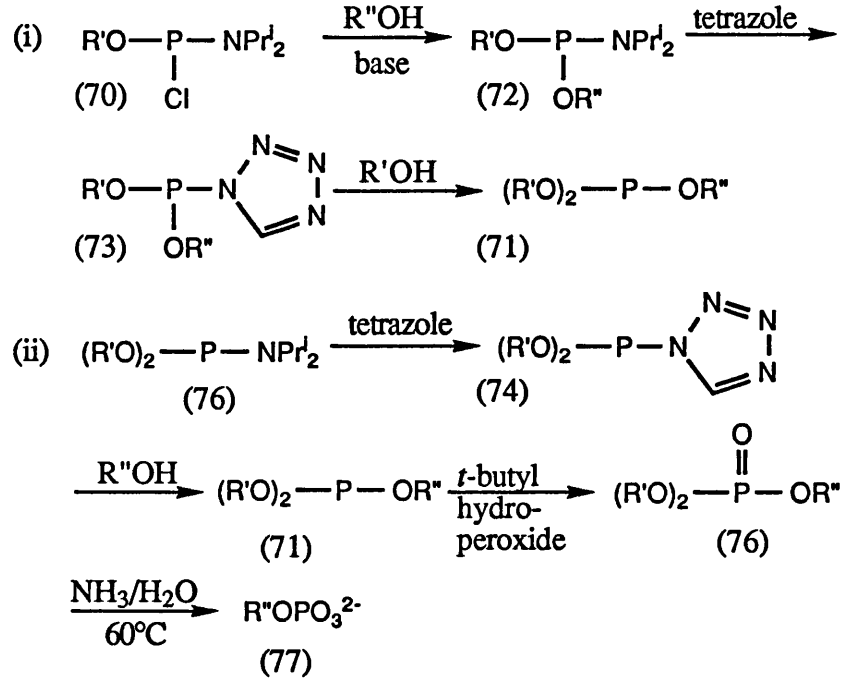
All of the methods so far discussed have revolved around phosphorus in the P(V) state. The advantage of using P(V) reagents for phosphorylation, is that the reagent and products are relatively stable. However, phosphorylation in certain situations is not possible, for example the phosphorylation of a vicinal diol, except when tetrabenzyl pyrophosphate is the reagent used. In order to phosphorylate these compounds it has become necessary to use more reactive phosphorylating agents, and so to this end, reagents with phosphorus in the P(III) state have been developed.

2.10 Phosphite Triesters.

The advantage of the P(III) over the P(V) approach lies primarily in the greater reactivity of phosphorus in its former oxidation state. Vicinal diols are unable to be phosphorylated using a P(V) reagent except for tetrabenzyl pyrophosphate, however, they are easily phosphorylated with a P(III) reagent.

The method known as the phosphite triester approach, has been extensively used in oligonucleotide synthesis[121,122] and in the phosphorylation of various polyols[123]. The phosphite triester method employs phosphorus (III) compounds to phosphorylate the polyol, the polyol displaces a halogen from the reagent of the type (70) to give the intermediate phosphite (71), via the phosphoramidite (72) and the phosphoryl-tetrazolide (73) [Scheme 15 (i)]. Or the polyol can displace tetrazole from the phororyl-tetrazolide (74) to give (71) when a reagent of the type (76) is used[124,125,126,127] [Scheme 15 (ii)]. The phosphite thus produced (71) can then be oxidised to phosphate triester (76),

which can be subsequently deprotected to give the phosphate monoester (77).



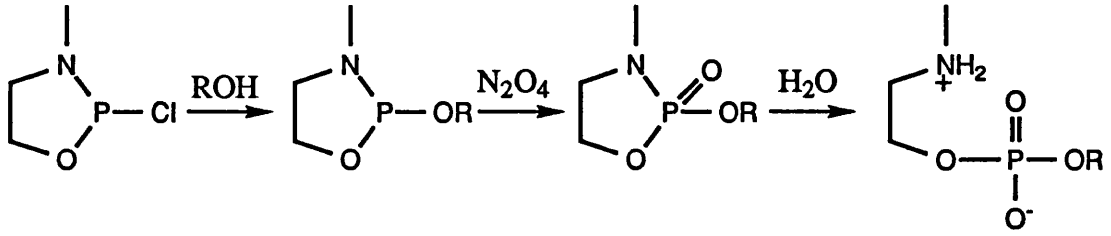
R'=2-cyanoethyl or benzyl R"=alkyl, Pr^i=*iso*-propyl

Scheme 15

2.10.1 Phosphorochloridites.

Phosphorochloridites (RR'PCl) are rather unstable, as they decompose on contact with moisture or oxidising reagents. However, these reagents are still commonly used. A reagent of this type is, chloro-(diisopropylamino)-2-cyanoethoxyphosphine (78) [Scheme 15], which has been used successfully to synthesise many inositol phosphate analogues. Namely DL-*myo*-inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] (4)[125].

2-Chloro-3-methyl-1,3,2-oxazaphosphacyclopentane (79) has been used in the field of lipid chemistry. Novel phospholipids have been prepared by a rapid three-step procedure. Oxazaphosphacyclopentane (79) reacts with alcohols to yield cyclic phosphoramidites in high yield. These are then oxidised to the corresponding phosphates with N_2O_4. The phosphates then undergo P-N cleavage, simply by treatment with water at room temperature, to give the phospholipid (81). The usefulness of this reagent is that the amino group is protected as the cyclic phosphonamidate, thus, obviating the use of triethylamine, normally used to introduce the terminal trimethylamino group. Triethylamine, tends to cause unwanted modifications of the labile diacylglycerol moiety and attack at phosphorus.



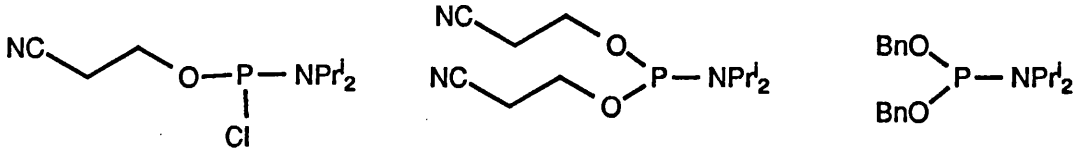
(79) (81)

Scheme 16

2.10.2 Phosphoramidites.

The phosphorochloridite reagents were difficult to use as hydrolysis readily occurred in moist air. So more stable phosphitylating reagents were investigated, phosphoramidites proved to be more stable than the phosphorochloridites and have been widely used in the phosphitylation of hydroxyl groups. The two amines commonly used are, diisopropylamine and morpholine. An added advantage of using a P(III) reagent is having the option to oxidise the phosphite with sulphur to the phosphorothioate. By taking chloro-(diisopropylamino)-2-cyanoethoxyphosphine (78) and substituting another 2-cyanoethyl group for the chlorine atom, the more stable phosphitylating reagent is isolated, (diisopropylamino)-bis(2–cyanoethyl) phosphoramidite (81). The added advantage of this reagent is that it is stable to purification by flash chromatography[128].

The reagent (diisopropylamino)-bis(benzyl) phosphoramidite (82)[129] is of similar reactivity to (diisopropylamino)-bis(2–cyanoethyl) phosphoramidite (81), however, it has the added advantage of producing phosphate triesters that are easily purified crystalline solids.



(78) (81) (82)

Figure 20

2.11 Summary

In conclusion, there are many procedures for phosphorylation, and agents capable of acting as phosphorylating reagents. The choice of the most appropriate phosphorylating reagent depends upon the system that is to be phosphorylated. In the case of a simple alcohol, phosphorylation with bis(2,2,2-trichloroethyl) phosphorochloridate to give an

easily purifiable phosphate triester. However, 2,2,2-trichloroethyl phosphate triesters are difficult and messy to deprotected to give the phosphate. Whereas, formation of a bisphosphate could not be accomplished using the former reagent so another reagent such as one of (78), (81) or (82) would have to be employed. Again, as with the synthesis of phosphates, the agent used to form a pyrophosphate is dependent upon the individual situation. Condensing two phosphates using DCC, is adequate in most simple situations, and this point will be fully discussed in chapter 5.

Chapter Three

Review of Inositol Chemistry

3.1 The Protection Chemistry of Inositols; a Brief Selective Overview.

Now that the role of D-Ins $(1,4,5)P_3$ as a second messenger and its consequent importance have been established. There has been a renaissance of interest in inositol phosphate synthesis. This has occurred after the initial interest in the isolation and synthesis of inositol phosphates, [see Cosgrove[130] for a review]. The isolation of these compounds from tissues is often complex and low yielding. In order to study the pharmacological and biochemical properties of these compounds and, in particular, chemically modified analogues thereof, it has therefore become necessary to develop efficient chemical syntheses of them.

Ever since the isolation of inositol phosphates[131] and long before this recent revival, many people have attempted to synthesise them[21,130,132]. The synthesis of inositol phosphates and their analogues from the readily available compound *myo*-inositol, poses many problems, however, and there are five major obstacles which must be overcome:

(i) Selective protection of several of the six hydroxyl groups of inositol to give a precursor suitable for phosphorylation.

(ii) Resolution of the protected inositol to give the respective D- and L- enantiomers.

(iii) Efficient phosphorylation of the appropriate hydroxyl groups without concomitant formation of a cyclic 5-membered phosphate between the vicinal 1,2-diols.

(iv) Removal of all of the various hydroxyl and phosphate protecting groups, avoiding any possibility of phosphate group migration.

(v) Effective purification of the resultant polyphosphate.

Some of these problems may be overcome by starting the synthesis from a compound other than *myo*-inositol. For example, the *Pseudomonas putida* oxidation of benzene affords *cis*-3,5-cyclohexadiene-1,2-diol. Protection of the diol followed by further oxidation leads to the synthesis of several natural products including (+)-pinitol, (+)-conduritol F, D-*myo*-inositol-1,4,5-trisphosphate and D-*myo*-inositol-1-phosphate[133]. Other strategies utilize the readily available compound (-)-quinic acid which after a suitable

asymmetric synthesis yields D-Ins (1,4,5)P_3[134], [see section 4.2 for details].

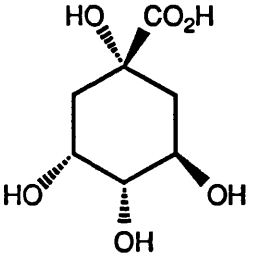
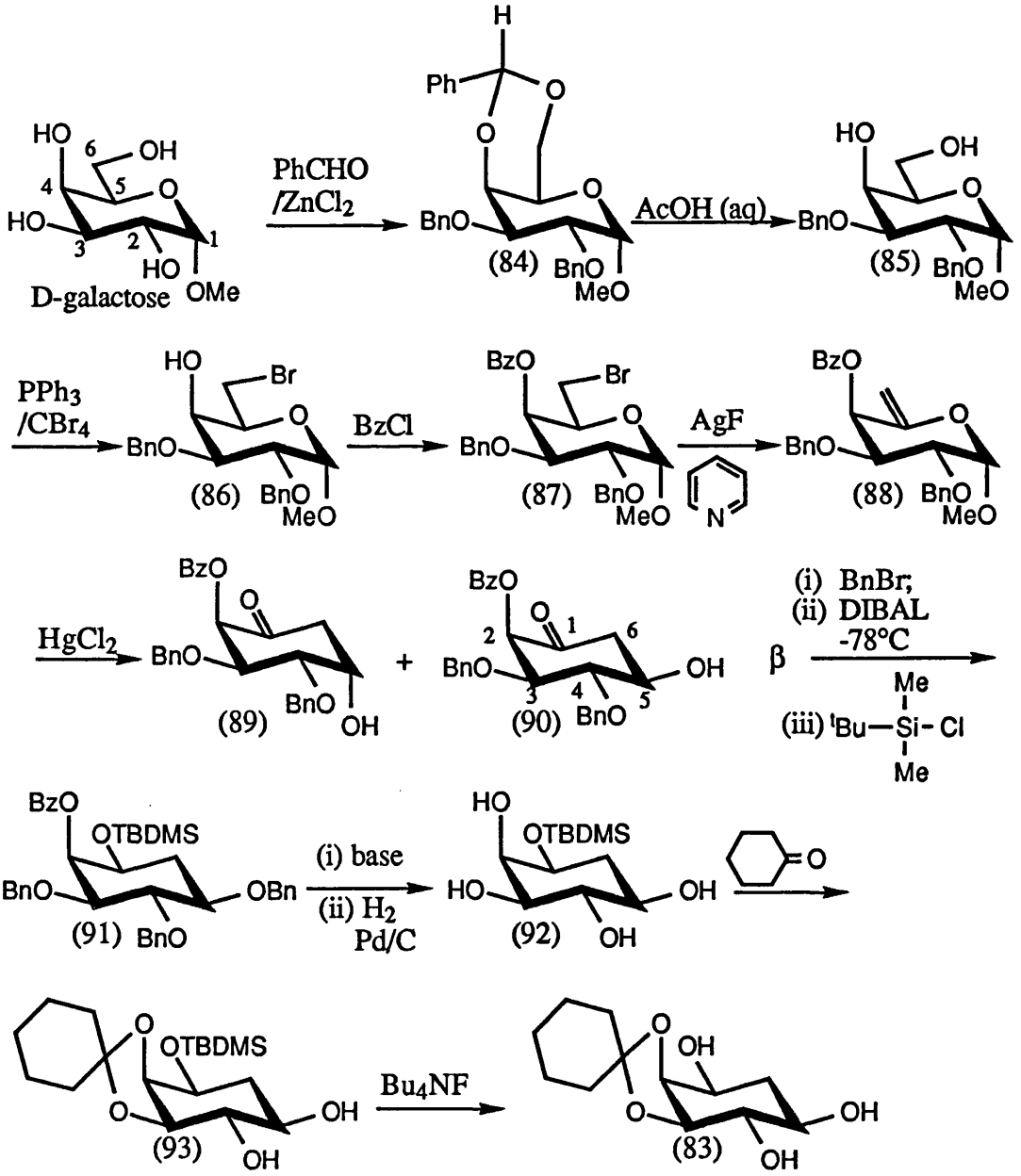


Figure 21. (-)-Quinic acid.

Other groups[111] have taken sugars and used those as the starting point of a synthesis of inositol phosphates.



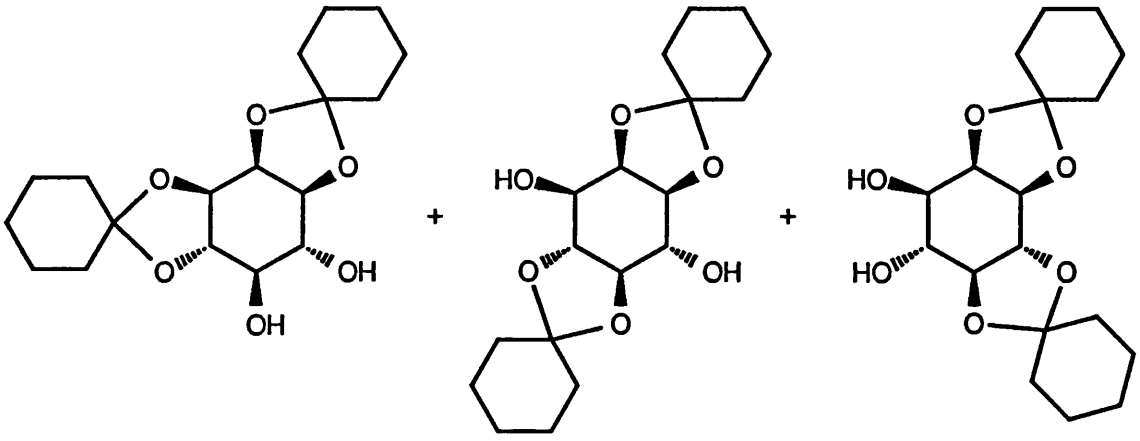
Scheme 17. Synthesis of 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol

For example, the precursor 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol (83) can be

prepared from D-galactose[111] and provides a suitable chiral precursor for the synthesis of 6-deoxy-D-*myo*-inositol-1,4,5-trisphosphorothioate (8) and 6-deoxy-D-*myo*-inositol-1-phosphate-4,5-cyclic pyrophosphate (9) *vide infra*. Treatment of D-galactose with benzaldehyde and zinc chloride gave the ketal with primary and secondary hydroxyl groups at positions 6 and 4 protected. This was followed by benzylation of the free hydroxyl groups at positions 2 and 3 which gave (84). Subsequent mild acidic hydrolysis of the ketal with aqueous acetic acid gave the free hydroxyl groups at positions 4 and 6 (85). Bromination with triphenylphosphine in carbon tetrabromide was effected almost exclusively at the primary hydroxyl group which gave (86). The hydroxyl group at position 4 was then benzoylated resulting in the formation of (87). The exocyclic alkene (88) was then formed by treatment with silver (I) fluoride. This then underwent an intramolecular aldol condensation, a Ferrier rearrangement, on treatment with mercury (II) chloride to give a mixture of the α (89) and β (90) deoxy inososes derivatives in the ratio of 3 α to 1 β. Since the β-conformer (90) has the desired *myo*-inositol stereochemistry, it was taken and the hydroxyl group at the 5-position was benzylated with benzyl bromide, the ketone function was then reduced to the alcohol with DIBAL. The alcohol thus produced at position 1 was protected as the *tert*-butyl dimethyl silyl (TBDMS) ether (91) by reaction with *tert*-butyl dimethyl silyl chloride (TBDMS-Cl). Debenzoylation with base followed by catalytic hydrogenolysis gave 1-*O*-TBDMS-6-deoxy-D-*myo*-inositol (92), which was then treated with cyclohexanone to give the compound with the 2 and 3 positions protected with the ketal (93). Removal of the TBDMS ether was effected by treatment with tetrabutyl ammonium fluoride to furnish the intermediate 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol (83) which can then be further manipulated [see chapter 7].

3.2 Selective Protection of Inositol Hydroxyl Groups.

The difficult problem of regiospecifically derivatising some of the six hydroxyl groups of inositol has been explored extensively and has been overcome by using many protecting groups, some of which were originally developed for use in carbohydrate chemistry. Several of the more commonly used groups will be discussed here. Due to the wide availability of pure *myo*-inositol, most protection strategies start from the parent cyclitol. Reaction of *myo*-inositol with one equivalent of cyclohexanone[135] or 1-ethoxycyclohexene[136], under acid catalysis affords the product 1,2-*O*-cyclohexylidene-*myo*-inositol where the ketal is preferentially formed on the *cis*-vicinal diol. Addition of a second equivalent of cyclohexanone gives a mixture of three bisketals which can be separated by column chromatography, [Fig. 22].



1:2, 3:4-bisketal 1:2, 4,5-bisketal 1:2, 5,6-bisketal.

Figure 22

Selective cleavage of the of the less stable *trans*-ketal can be effected by mild acidic hydrolysis[136]. Manipulation of the free hydroxyl groups of the bisketals followed by cleavage of the one or both of the bisketals leads to a series of inositols having four hydroxyl groups differentially protected[137].

The isopropylidene ketal had been used initially, however, it was found to be unsatisfactory because of poor and inconsistent yields[135]. Gigg revived the use of the isopropylidene group by using 2,2-dimethoxypropane instead of acetone, to generate the corresponding mixture of isopropylidene bisketals to that shown above. He subsequently published the synthesis of 1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (94)[138] which will be discussed later.

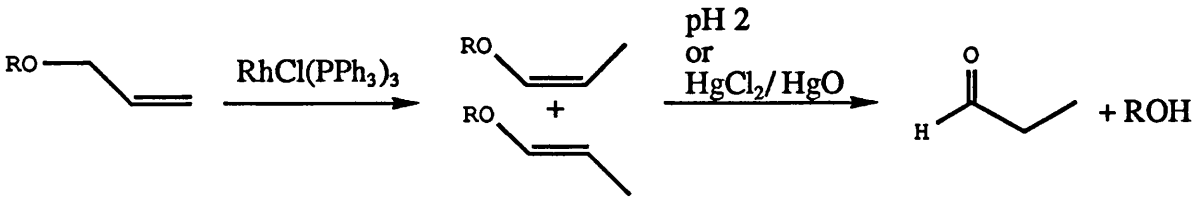
Gigg also pioneered the use of the allyl protecting group in carbohydrate chemistry[139]. An allyl ether can be easily formed from an alcohol, allyl bromide and base. Isomerisation of the allyl ether to the prop-1-enyl enol ether can be effected by one of several methods:

(i) Heating the allyl ether to reflux with diazobicyclooctane and bis(triphenylphosphine)rhodium chloride [Wilkinson's catalyst] in a solution of ethanol/water. The isomerisation in this case results in two compounds; one with the *cis*-prop-1-enyl group, the other with the *trans*-prop-1-enyl group in the ratio of 10 to 1 respectively[140].

(ii) Heating the allyl ether to 100 °C with potassium *tert*-butoxide in dimethyl sulphoxide; this again gives the *cis*-prop-1-enyl ether as the major product. However, the ratio of this compound to the *trans*-prop-1-enyl ether here is 95 to 5 respectively[141].

(iii) The *trans*-prop-1-enyl ether, though, can be prepared selectively, in greater than 90% yield by treatment with hydrogen activated 1,5-cyclooctadiene-bis[methyldiphenylphosphine]-iridium hexafluorophosphate in tetrahydofuran[142].

Removal of prop-1-enyl groups can be achieved by two main methods; cleavage by acidic hydrolysis[141] or by treatment with mercuric (II) chloride in the presence of mercuric (II) oxide[139]. In both cases the free hydroxyl group is produced.



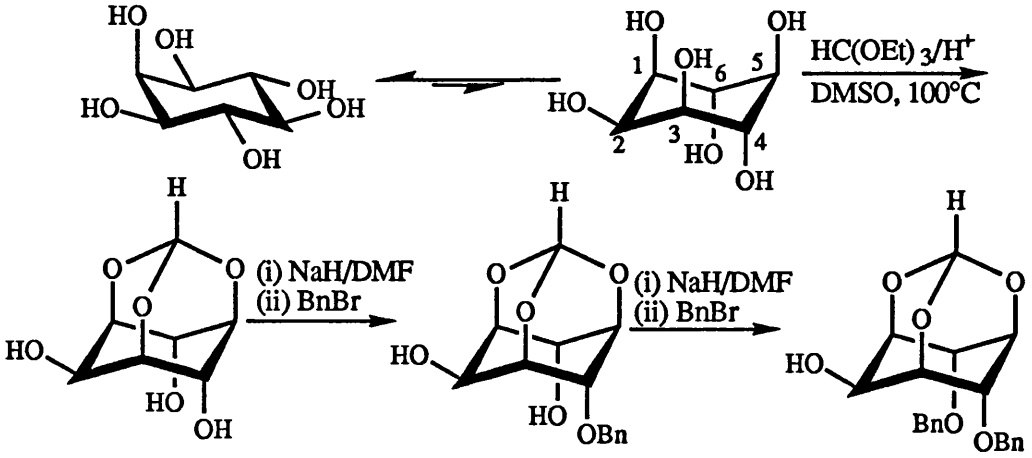
Scheme 18

Benzyl and *para*-methoxy benzyl ethers have featured in many inositol syntheses. They are very versatile as they are resistant to harsh acidic and basic conditions, but they can be simply removed by catalytic hydrogenolysis[143] or by sodium in liquid ammonia *(vide infra)*. The benzyl group can be introduced by one of two main methods. The first involves the formation of the alkoxide of the alcohol group to be benzylated with a strong base such as sodium hydride, followed by addition of benzyl chloride or benzyl bromide[138]. The second method involves benzylation under mild acidic conditions, *e.g.* the monobenzoate, 1,2:4,5-di-*O*-isopropylidene-3-benzoyl-*myo*-inositol, was benzylated in high yield, by treatment with benzyl trichloroacetamide in dichloromethane with a trace of trifluoromethane sulphonic acid[144]. The former method has found wide use in the field of inositol chemistry, the latter being useful where basic condition would be detrimental.

para-Methoxybenzylation can be achieved in the same manner as benzylation[145]. The *para*-methoxybenzyl groups can be removed subsequently under either: acidic hydrolysis (M HCl-ethanol 1:2 at reflux for three hours), or by treatment with dichlorodicyanoquinone (DDQ) in dichloromethane-water (15:1). The latter method is of greater use since it means that the *para*-methoxybenzyl group can be selectively removed in the presence of other protecting groups.

An orthoformate derivative of inositol is of considerable potential as a protected inositol; it has been used as an intermediate to form *myo*-inositol 2-phosphate, *myo*-inositol 1,3-bisphosphate, *myo*-inositol 1,3,5-trisphosphate and *myo*-inositol 1,3,4,5-

tetrakisphosphate[146]. The mono orthoformate of inositol provides a derivative of inositol where the hydroxyl groups at positions 1,3 and 5 are protected, and the axial/equatorial nature of the other three hydroxyls is reversed[86] [Scheme 19].

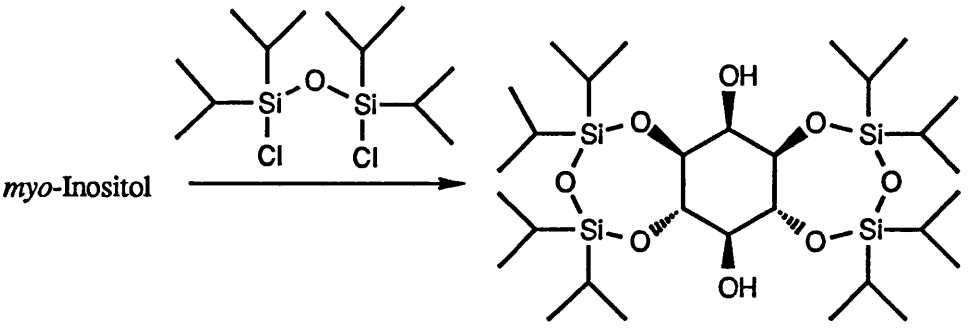


(95) (96) (97)

Scheme 19.

Subsequent reaction of this orthoformate (95) with one equivalent of sodium hydride in DMF gives the alkoxide at the 4(6) position which can then be selectively alkylated by a chelation-controlled alkylation with benzyl bromide or allyl bromide (96). Addition of another equivalent of sodium hydride/benzyl bromide leads to the 4,6 dibenzyl protected orthoformate (97). The orthoformate can be selectively removed using trifluoroacetic acid at room temperature[146].

1,3 Dichloro-1,1,3,3-tetraisopropyl disiloxane (TIPSCl), which has in the past been used as a nucleoside protecting group, has also been used with *myo*-inositol to give the crystalline *myo*-inositol 1,3:4,6-bis(1,1,3,3-tetraisopropyl-disiloxane) (98) [Scheme 20] in a simple one step reaction[147]. It was shown to be of great potential in the synthesis of *myo*-inositol 1,3,4,6-tetrakisphosphate[147].



Scheme 20

The TIPS group has also been used to protect the 3-azido-3-deoxy isostere of *myo*-

inositol to give 3-azido-3-deoxy-*myo*-inositol 1,6-(1,1,3,3-tetraisopropyl-disiloxane)[148], which following phosphorylation and deprotection yielded the 3-azido-3-deoxy-*myo*-inositol-2,4,5-trisphosphate[148].

Differentiation between the *cis*- and the *trans*-vicinal diols can be effected by making a stannylene derivative. David *et. al.*[149] described the tin-mediated alkylation of various polyols. They reported the first X-ray structure of a stannylene derivative in the solid state, a 2,3-O-dibutylstannylene derivative of methyl 4,6-O-benzylidene-α-D-glucopyranoside, and this was found to exist as depicted in [Fig. 23][150].

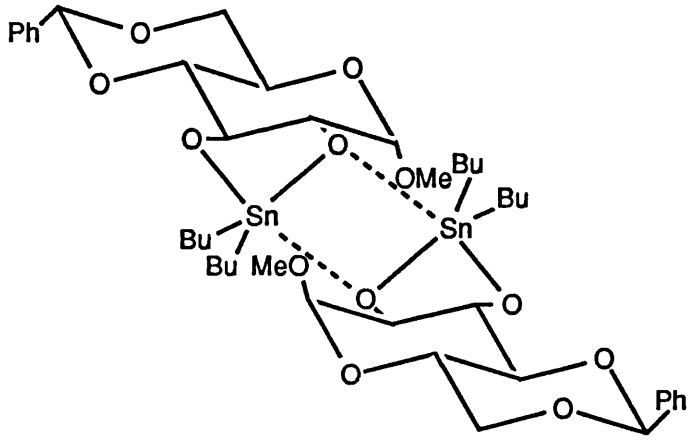


Figure 23

The tin adopts a trigonal bipyramidyl geometry where the two butyl groups and an oxygen atom are all equatorial (e), whilst the other oxygen is in the apical (a) position [Fig. 24].

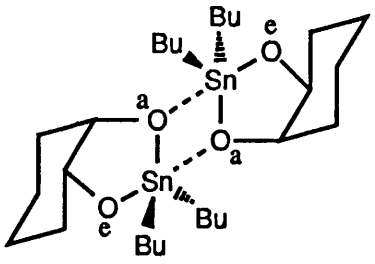


Figure 24

The coordination of apical oxygen to the other tin atom of the dimer has the effect of weakening the equatorially bound oxygen-tin bond. This results in selective alkylation of this position.

3.3 Resolution of Inositol Derivatives

myo-Inositol itself a *meso* compound. Upon derivatisation at one of the stereogenic carbons (carbons 1,3,4 and 6), it becomes chiral, as described in [Section 1.5]. Separation of enantiomers to give pure stereoisomers is of biological importance, since

one enantiomer may act physiologically completely different to the other. The presumption that a mixture of two different enantiomers will behave as if only one agent is involved is no longer tenable[151]. For example, the barbiturate 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid (DMBB) [Fig. 25] is chiral and the S-(-) enantiomer has been found to be a depressant, whereas the R-(+) enantiomer and the racemate have been found to be convulsants[151].

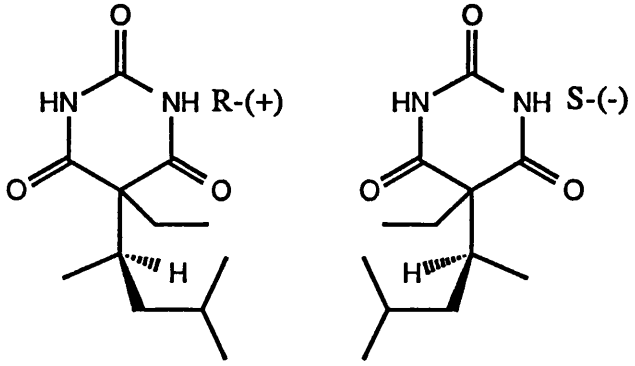


Figure 25. R-(+) and S-(-) DMBB

Resolution of protected inositols has for a long time been a problem. Initially, derivatisation with orthoesters of D-mannose was the only option[152]. Recently, the use of (+)-; and (-)-camphanic acids, monomenthoxyacetates, menthoxycarbonates, carbamates, acetylmandelates and tartrates has led to the synthesis of chiral inositol compounds. The most attractive route, for several reasons, involves the use of (-)-camphanic acid chloride. It is a stable crystalline reagent, which is readily available and it normally gives crystalline diastereoisomeric esters that can be separated by column chromatography, recrystallisation or h.p.l.c.[153]. Upon separation of isomers it can be removed effectively by saponification to give the pure enantiomers. Chiral h.p.l.c and g.l.c. columns[154] and enzymes have also been employed in order to separate enantiomers[129].

Other strategies towards overcoming this problem have involved the use of pure stereoisomers of inositol which can be isolated from natural sources. For example, derivatisation of D- and L-*chiro*-inositol from (+)-pinitol and quebrachitol respectively has resulted in the formation of D- and L-myo-inositol 1,4,5-trisphosphate[155,156]. *myo*-Inositol can give rise to a possible 15 dimethyl ethers. However, so far only two dimethyl ethers have actually been discovered in nature. These are dambonitol 1,3 dimethyl *myo*-inositol, found in the sap of rubber vines called "n'dambo" from Gabon[157] and L-liriodendritol 1,4-dimethyl L-*myo*-inositol isolated from the leaves and bark of the Virginian tulip tree *Liriodendron tulipifera*. Four monomethyl ethers of

myo-inositol have also been found, called sequoitol, D-bornesitol, L-bornesitol and D-ononitol. Sequoitol 5-methyl *myo*-inositol was discovered in 1929 in the wood of redwood trees *Sequoya sempervirens;* D and L-bornesitol 1-methyl *myo*-inositol were extracted from Borneo rubber sap the latter from the leaves of *Lathyrus vernus* respectively. D-Ononitol; 4-methyl *myo*-inositol was isolated from the leafy shoots of *Ononis natrix*. Exploitation of these natural ethers of inositol is dependent upon their availability, but protection strategies using these compounds can simplify the need for resolution and may reduce the number of steps required to reach target compounds.

3.4 Phosphorylation Strategies.

The requirements for phosphorylation of suitably protected inositol compounds are as follows

(i) The agent responsible for the phosphorylation of the inositol must be specific and capable of phosphorylating the hydroxyl groups, especially those that are in a vicinal diol arrangement.

(ii) The phosphate triester thus produced must be stable when subjected to purification.

(iii) The phosphate protecting groups must be easily removed, without concomitant phosphate migration via the formation of a cyclic phosphate intermediate.

There are several reagents that satisfy these criteria and that have been used in inositol syntheses; the three major phosphorylating reagents are (diisopropylamino) dibenzyl phosphoramidite (82)[124], (diisopropylamino) bis(2-cyanoethoxy)phosphoramidite (81)[125] and tetrabenzyl pyrophosphate[86] as discussed in chapter 2.

3.5 Isolation and Early Chemical Syntheses of Inositol Phosphates.

Phytic acid, *myo*-inositol-1,2,3,4,5,6-hexakisphosphate Ins P_6, was first isolated in the late 1870's from aleurone grains of wheat endosperm as the mixed calcium magnesium salt[131]. Ins P_6 was found upon hydrolysis to give inorganic orthophosphate and *myo*-inositol[158]. The first synthesis of Ins P_6 involved the treatment of *myo*-inositol with polyphosphoric acid[132]. However, when this work was repeated the product was found to differ from that isolated from seeds[159]. Manipulation of the conditions did, however,

allow a product to be obtained that was consistent with that of natural origin[159]. Lower phosphates of inositol have also been isolated from tissues. Ballou isolated Ins (1,4,5)P_3 by the chemical hydrolysis of bovine brain PIP_2[160].

Many of the inositol phosphates have been already synthesised and this early inositol chemistry is covered in two books[130,21].

3.6 Syntheses of Inositol Phosphates.

3.6.1 Inositol Monophosphates.

There are four possible *myo*-inositol monophosphates, the symmetrical 2 and 5 phosphates and the pair of enantiomers, the 1(3) and 4(6) phosphates. All of these monophosphates have been successfully synthesised. *myo*-Inositol-1-phosphate has been prepared by several routes. 1,2-Biscyclohexylidene-*myo*-inositol, easily obtained from *myo*-inositol (as discussed earlier) was benzylated and the ketal was removed to give 3,4,5,6-tetrabenzyl-*myo*-inositol. The greater reactivity of the equatorial 2-position over the axial 1-position was then exploited such that treatment with allyl bromide gave the 1-*O*-allyl-3,4,5,6-tetrabenzyl-*myo*-inositol. Subsequent benzylation of the 2-position, followed by removal of the allyl group left the hydroxyl group at the 1-position available for phosphorylation with diphenyl phosphorochloridate. Catalytic hydrogenolysis gives *myo*-inositol-1-phosphate, with 2.5% contamination of *myo*-inositol-2-phosphate[161], the latter probably arising from the opening of a cyclic phenyl phosphate ester.

myo-Inositol-2-phosphate was first synthesised by the phosphorylation of *myo*-inositol-1,3,4,5,6-pentaacetate with diphenyl phosphorochloridate[54]. The precursor is derived from the selective oxidation of the 2-position of *myo*-inositol with *Acetobacter suboxydans* to give *scyllo*-inosose[162]. Acetylation followed by reduction of the carbonyl gives *myo*-inositol-1,3,4,5,6-pentaacetate. Phosphorylation, then deprotection affords *myo*-inositol-2-phosphate. A more recent approach uses the orthoformate derivative (95). The orthoformate may be selectively benzylated at the 4 and 6-positions using the advantage of the chelation controlled reactions of the monoanions of (97)[163]. Phosphorylation of the free 2-position with sodium hydride and tetrabenzyl pyrophosphate, followed by subsequent deprotection gives *myo*-inositol-2-phosphate.

myo-Inositol-4-phosphate can be easily prepared from the orthoformate; again using a

chelation controlled reaction of the monoanion of the orthoformate to give selective phosphorylation at the 4-position with tetrabenzyl pyrophosphate in a single step. Subsequent catalytic hydrogenolysis of the benzyl groups, then acidic hydrolysis of the orthoformate ester gives *myo*-inositol-4-phosphate as the racemate[163]. Other routes have been employed in order to form *myo*-inositol-4-phosphate, both as the racemate and as single enantiomers, and the vast majority of these syntheses start with the 1:2, 4:5-bisketal. Benzylation of the more reactive 3-position followed by resolution with (-)-camphanic acid chloride gives a mixture of two diastereoisomers. Separation, hydrolysis of the camphanate and then phosphorylation and finally deprotection gives both enantiomers of *myo*-inositol 4-phosphate[163].

Both *myo*-inositol-5-phosphate and *myo*-inositol-6-phosphate have been synthesised in a similar manner[33].

3.6.2 Inositol Bisphosphates.

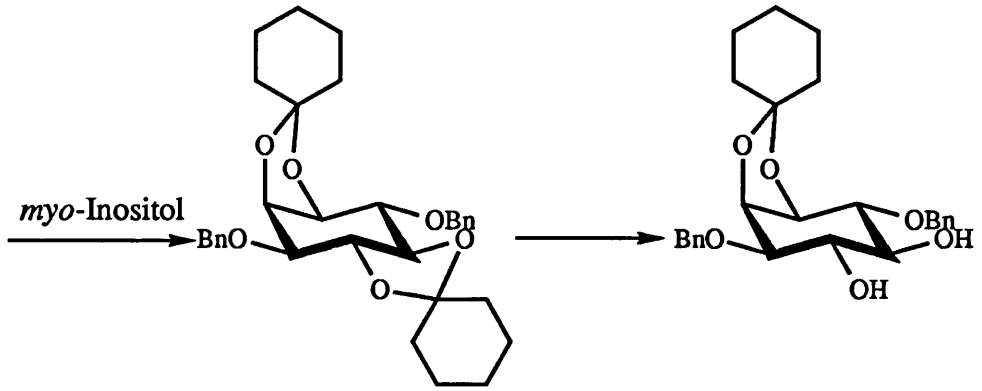
Interest in the physiological role of D-*myo*-inositol 1,4-bisphosphate, the product of receptor-mediated cleavage of Ins $(1,4,5)P_3$ has led to the search for effective syntheses of bisphosphates. DL-*myo*-Inositol 1,4-bisphosphate was synthesised from 1:2, 4:5-diisopropylidene-*myo*-inositol. This when treated with chloro-(diisopropylamino)-2-cyanoethoxyphosphine (78), followed by tetrazole/2-cyanoethanol gave the bisphosphite, which after subsequent oxidation and deprotection gave DL-*myo*-inositol 1,4-bisphosphate[164]. DL-*myo*-Inositol 4,5-bisphosphate has been prepared by the phosphorylation of 1,2,3,6-tetrabenzyl-*myo*-inositol using chloro-(diisopropylamino)-2-cyanoethoxyphosphine (78). Again after treatment of the phosphoramidite with tetrazole/2-cyanoethanol, the phosphite thus formed was oxidised with *meta*-chloroperbenzoic acid (mCPBA) to give DL-*myo*-inositol 4,5-bisphosphate[165,166]. Both DL-*myo*-inositol 1,3-bisphosphate and DL-*myo*-inositol 1,5-bisphosphate have also been synthesised from 2,4,6-tribenzyl *myo*-inositol (derived from the exhaustive benzylation of the orthoformate ester of *myo*-inositol). 2,4,6-Tribenzyl *myo*-inositol was phosphorylated with diphenyl phosphorochloridate to give a mixture of the 1,3- and the 1,5-bisphosphates in the ratio of 4:1 respectively. Separation of these two bisphosphates was effected by crystallisation; deprotection of these with lithium in liquid ammonia then gave DL-*myo*-inositol 1,3-bisphosphate and DL-*myo*-inositol 1,5-bisphosphate[33]. The pure enantiomers of *myo*-inositol 1,4-bisphosphate have been prepared by resolving the enantiomers of 2:3, 5:6-di-*O*-cyclohexylidene-*myo*-inositol with D-mannose, followed by phosphorylation with diphenyl phosphorochloridate after cleavage of the chiral auxiliary[167]. In a similar manner both enantiomers of *myo*-inositol

3,4-bisphosphate have been prepared from the appropriate enantiomers of 1:2, 5:6-di-*O*-cyclohexylidene-*myo*-inositol, proceeded by phosphorylation with diphenyl phosphorochloridate to give both D- and L-*myo*-inositol 3,4-bisphosphate after deprotection[167]. Phosphorylation of the resolved enantiomers of 2:3, 6:1-di-*O*-cyclohexylidene-*myo*-inositol has yielded optically pure D- and L-*myo*-inositol 4,5-bisphosphate. However, phosphorylation of the vicinal diol required was not possible with diphenyl phosphorochloridate. Dianilidophosphoryl chloride was used to phosphorylate both hydroxyl groups adequately, without any phosphate migration, but proved difficult to remove from the reaction mixture[168].

3.6.3 The First Two Complete Syntheses of myo*-Inositol 1,4,5-Trisphosphate.*

The obvious interest in the synthesis of Ins $(1,4,5)P_3$ has produced many varied routes that lead to both the racemic the pure D- and L- enantiomers of Ins $(1,4,5)P_3$.

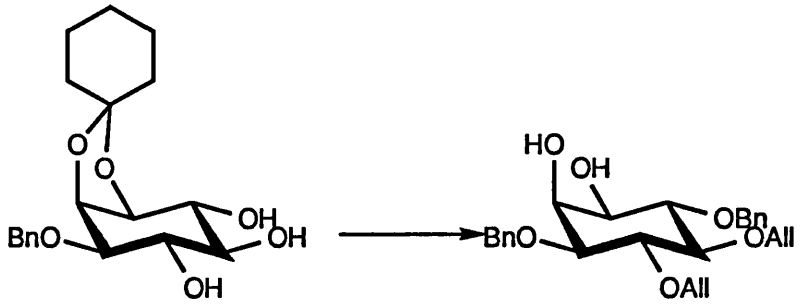
In 1986 Ozaki[169] reported the first complete synthesis of *D*-Ins $(1,4,5)P_3$ starting from *myo*-inositol. It involved firstly forming the 1,2:4,5-di-*O*-cyclohexylidene-3,6-di-*O*-benzyl-*myo*-inositol derivative (99), which is easily derived from *myo*-inositol. Selective removal of the less stable *trans*-4,5 ketal was effected by treatment with ethylene glycol in the presence of *para*-toluene sulphonic acid (ptsa) and gave 1,2-*O*-cyclohexylidene-3,6-di-*O*-benzyl-*myo*-inositol (100).



(99) (100)

Scheme 21

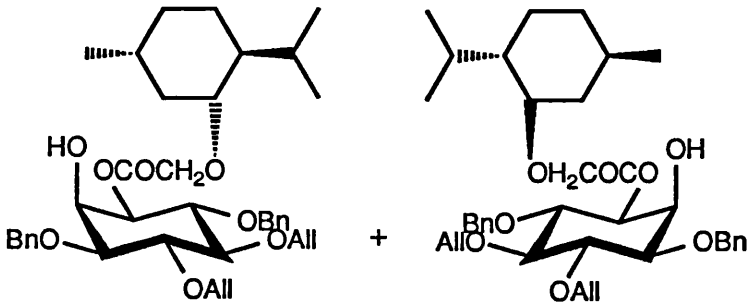
Allylation of the 4 and 5 positions using sodium hydride and allyl bromide followed by the cleavage of the 1,2 ketal with aqueous acetic acid yielded 3,6-di-*O*-benzyl-4,5-di-*O*-allyl-*myo*-inositol (101).



(100) (101)

Scheme 22

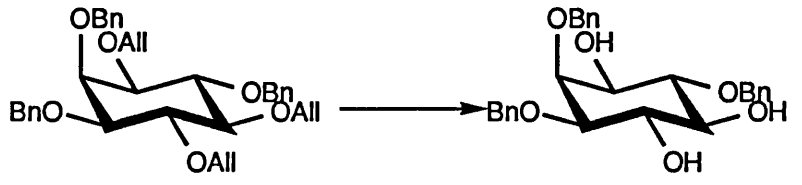
The racemic diol was then subjected to optical resolution. Treatment of the diol with 1-menthoxyacetylchloride in pyridine gave a mixture of the two diastereoisomers, esterified at the 1 position. Subsequent recrystallisation afforded the pure D- and L- 1-menthoxyacetal-3,6-di-*O*-benzyl-4,5-di-*O*-allyl-*myo*-inositol (102) and (103) respectively.



(102) (103)

Scheme 23

The optically pure diol obtained by basic hydrolysis of the ester, was selectively allylated at the 1 position, and then benzylated to furnish the fully protected 1,4,5-tri-*O*-allyl-2,3,6-tri-*O*-benzyl-*myo*-inositol (104). Isomerisation of the allyl groups to the prop-1-enyl groups was effected by treatment with tristriphenylphosphine rhodium(I) chloride (Wilkinson's Catalyst)[170] and subsequent acidic methanolysis yielded the key intermediate 2,3,6-tri-*O*-benzyl-*myo*-inositol (105).



(104) (105)

Scheme 24

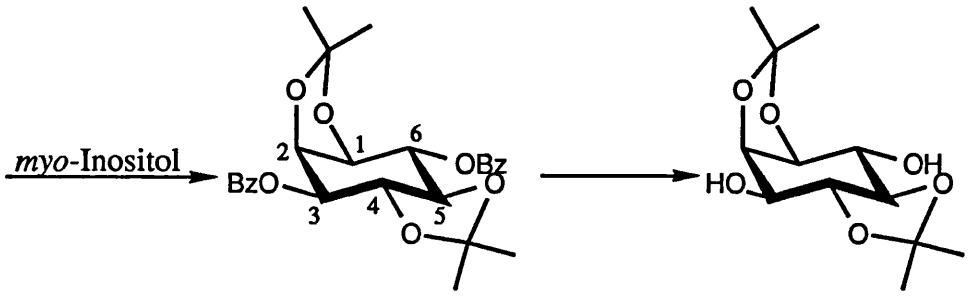
The triol was phosphorylated with dianilinophosphoric chloride in the presence of 1,4-dimethylamino pyridine (DMAP) to give the trisphosphate in moderate yield. The

phosphorylating reagent used, however, proved to be a poor choice as the phosphorylation and subsequent deblocking reactions were not satisfactory. Successive deprotection of the phosphate triesters using *iso*-amyl nitrite in pyridine-acetic acid-acetic anhydride (1:1:1) followed by debenzylation using hydrogen over palladium on charcoal gave the target compound of D-Ins(1,4,5)P_3 as the ammonium salt in *ca.* 4% overall yield.

3.6.4 *The Second Complete Synthesis of* myo-*Inositol 1,4,5-Trisphosphate.*

The key intermediate (115)[145], which features in the second and subsequent complete synthesis of *myo*-inositol 1,4,5-trisphosphate[125], was prepared as described below.

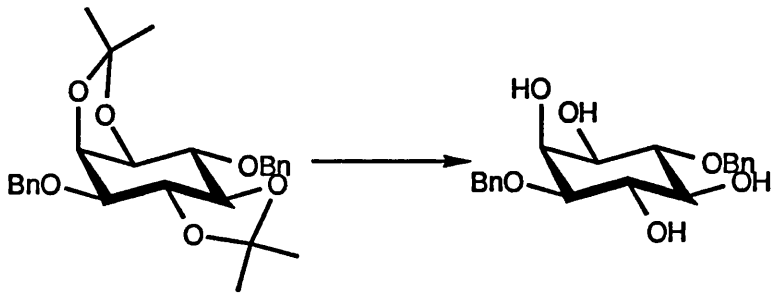
Reaction of *myo*-inositol with 2,2-dimethoxypropane and *para*-toluene sulphonic acid (ptsa) in DMF gave a mixture of three bis-diisopropylidene derivatives[138], the bisketals being in the same positions as those derived from cyclohexanone, (1,2:3,4; 1,2:4,5 and 1,2:5,6). Benzoylation of the mixture using benzoyl chloride in pyridine gave three dibenzoate regioisomers, (5,6; 3,6 and 3,4). Surprisingly, 1,2:4,5-di-*O*-isopropylidene, 3,6-di-benzoyl-*myo*-inositol (106), was found to be highly insoluble in most solvents, whereas the other two products were soluble in methanol. This fortuitous discovery enabled effective separation by subsequently washing with pyridine, water, acetone and ether to give (106) in 27% yield[138].



(106) (107)

Scheme 25

The benzoyl groups can be removed by basic hydrolysis to give 1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (107). The diol can be effectively benzylated as before by making the alkoxide, whereupon addition of benzyl chloride gives the 1,2:4,5-di-*O*-isopropylidene, 3,6-di-*O*-benzyl-*myo*-inositol (108). Mild acidic hydrolysis leads to the formation of the tetrol 1,4-di-*O*-benzyl-*myo*-inositol (109) in 77% yield from (106).



(108) (109)

Scheme 26

Reaction of (109) with tetrabutylammonium iodide and dibutyl tin oxide in toluene formed corresponding tin complex[Fig. 26].

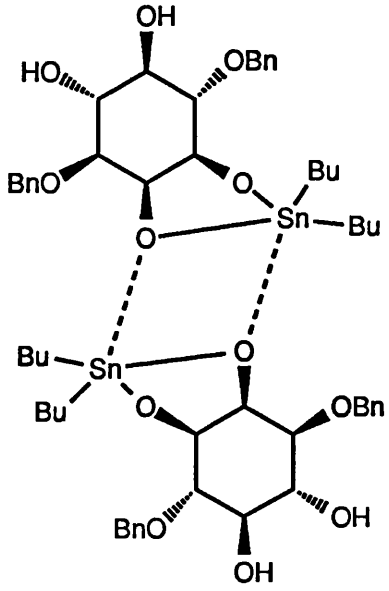
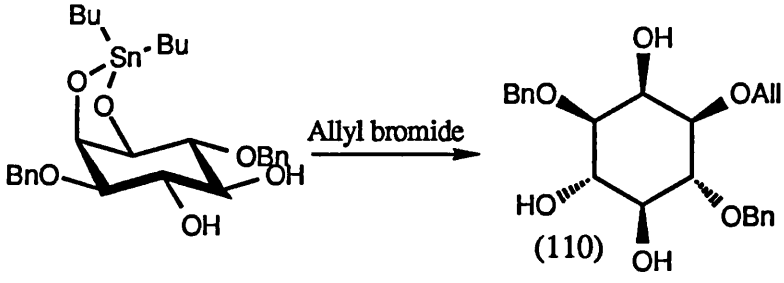


Figure 26

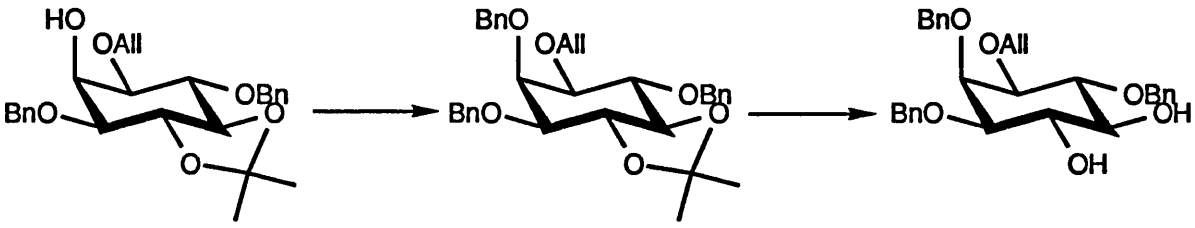
The tin complex formed from (109) reacts with allyl bromide at the equatorial tin-oxygen (position-1 of inositol), resulting in the regiospecific allylation of position-1 to give 1-*O*-allyl-3,6-di-*O*-benzyl-*myo*-inositol (110) in 45% yield. The precise mechanism of this reaction is not certain. However, steric factors may play an important role in the reaction as tin complexes form preferentially between *cis*-axial-equatorial vicinal diols rather than between *trans*-diequatorial vicinal diols [Scheme 27][150].



(110)

Scheme 27

The 4,5 position of the triol (110) was protected as before by the formation of the ketal using 2,2-dimethoxypropane and ptsa in acetone to give (111) in (20%) yield. After purification by flash chromatography the 2-position of the inositol ring was benzylated using a two fold excess of sodium hydride and benzyl bromide in DMF to yield (112).

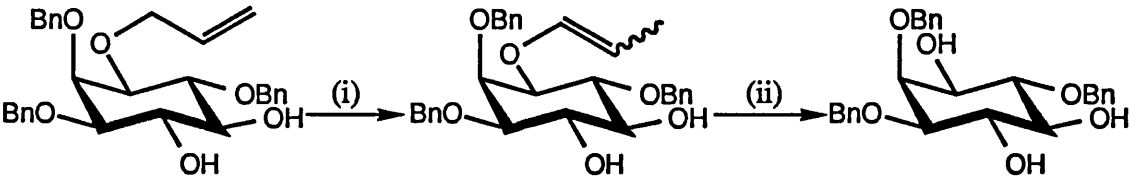


(111) (112) (113)

Scheme 28

Acidic hydrolysis gave (113), after which the allyl group was isomerised to the prop-1-enyl group upon heating to reflux in a solution of ethanol/water containing diazobicyclooctane and Wilkinsons catalyst [$RhCl(PPh_3)_3$]. The isomerisation resulted in the formation of two compounds; one with a *cis*-prop-1-enyl group, the other with a *trans*-prop-1-enyl group and they were produced in a ratio of 10 to 1 respectively. The base was present in order to inhibit the premature hydrolysis of the intermediate enol ether, since the free propionaldehyde reacts with $RhCl(PPh_3)_3$ to form the catalytically less active compound $RhCl(PPh_3)_2CO$[140].

The mercury (II) chloride/mercury (II) oxide[139] method was used to remove the prop-1-enyl moiety from 1-*O*-prop-1-enyl-2,3,6-tri-*O*-benzyl-*myo*-inositol (114) and this gave the desired DL-1,2,4-tri-*O*-benzyl-*myo*-inositol (115) in 51% yield from (111).



(i) $RhCl(PPh_3)_3$ (ii) $HgCl_2/HgO$

(113) (114) (115)

Scheme 29

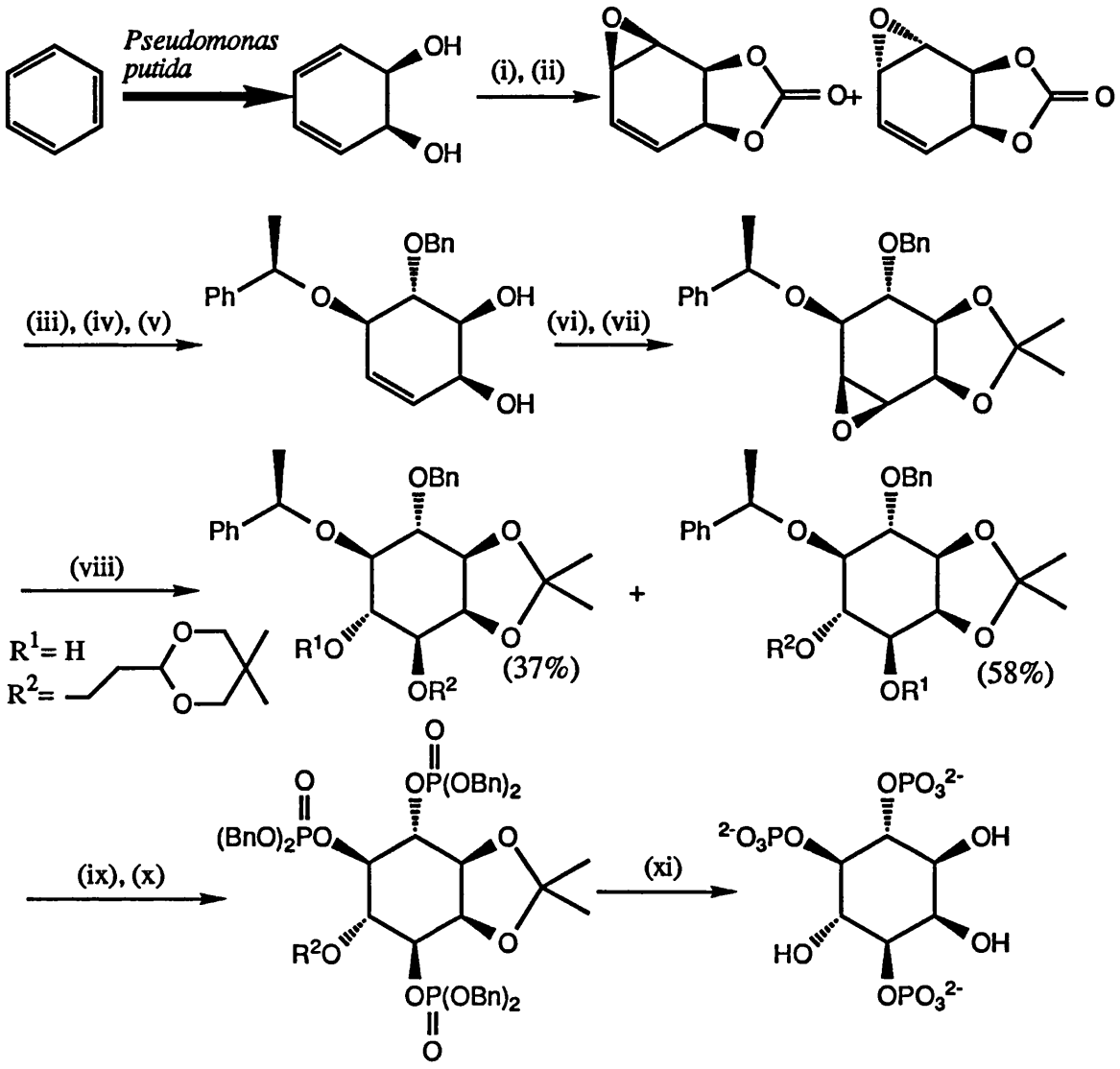
DL-1,2,4-Tri-*O*-benzyl-*myo*-inositol (115) was then phosphorylated using phosphite chemistry which involved treatment with chloro-(diisopropylamino)-2-cyanoethoxyphosphine (78), then tetrazole/2-cyanoethanol to give the trisphosphite triester, DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1,4,5-tris[di(2-cyanoethyl)phosphite] (116). Subsequent oxidation with *t*-butyl-hydroperoxide afforded quantitatively the trisphosphate triester DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1,4,5-tris[di(2-cyanoethyl)phosphate] (117). Finally, complete deblocking with sodium in liquid

ammonia, which effected reductive removal of benzyl groups and loss of the 2-cyanoethyl groups by ß-elimination and gave DL-Ins (1,4,5)P_3 in *ca.* 50% yield from (115).

3.6.5 Other Selected Syntheses of myo*-Inositol Trisphosphates.*

Several syntheses of both enantiomers of Ins (1,4,5)P_3 have been reported. One route utilizes the naturally occurring methyl ethers of D- and L-*chiro*-inositol, obtained from pinitol and quebrachitol respectively. They, after appropriate hydroxyl group protection, inversion of one of the two axial hydroxyl groups, phosphorylation and deprotection, yield D- and L-Ins (1,4,5)P_3[155]. Many syntheses use camphanic acid chloride to resolve the enantiomers of the suitably protected inositols, as in the synthesis of D- and L-Ins (1,4,5)P_3[146,153,171]. Another route has used the reagent ethylamino-bis(2–cyanoethyl) phosphine to synthesise racemic Ins(1,4,5) P_3 then optically pure *D*-Ins(1,4,5) P_3[172].

One interesting route to D-Ins (1,4,5)P_3 involved the use of *Pseudomonas putida* in a microbial oxidation of benzene to give *cis*-3, 5-cyclohexadiene-1,2-diol [Scheme 30][133]. The required cyclic epoxycarbonate (with the epoxide *trans* to the carbonate) was prepared by treatment with sodium methoxide and dimethyl carbonate, followed by stereoselective epoxidation using *m*-chloroperoxybenzoic acid.



Scheme 30 D-Ins (1,4,5)P_3

(i) $(MeO)_2CO$, NaOMe, MeOH; (ii) *m*CPBA, CH_2Cl_2; (iii) $HBF_4.OEt_2$, (R)-(+)-*sec*-phenethyl alcohol, CH_2Cl_2; (iv) BnBr, Ag_2O, DMF; (v) Et_3N/MeOH/H_2O; (vi) *m*CPBA, CH_2Cl_2; (vii) 2,2-dimethoxypropane, Cesium acetate, CH_2Cl_2; (viii) NaH, R^2OH; (ix) H_2, 10% Pd-C, EtOH; (x) *n*-BuLi, diisopropylamine, THF, tetrabenzylpyrophosphate; (xi) a 10% Pd-C, EtOH; b trifluroacetic acid; 88% overall yield.

Regiospecific ring opening of the required cyclic epoxycarbonate with (R)-(+)-*sec*-phenethyl alcohol and a catalytic amount of fluoboric acid in ether gave a mixture of diastereoisomers. Separation of the diastereoisomers, followed by benzylation and hydrolysis yielded the diol where the hydroxyl groups were ideally placed to aid in directing the subsequent epoxidation so that only the ß-epoxide was obtained (87% yield). Reaction of the epoxide with the hydroxyl equivalent, R^2OH gave a mixture of two

regioisomers; fortuitously, the desired one was formed in a greater yield than the other. Catalytic hydrogenolysis removed the benzyl ethers and the phenethyl group to give the triol. The trialkoxide was then phosphorylated with tetrabenzyl pyrophosphate to give D-Ins (1,4,5)P_3 after deprotection.

myo-Inositol 2,4,5-trisphosphate has also been synthesised as the racemate[146] and as the D-enantiomer, from 3,6-dibenzyl 1,2-cyclohexylidene-*myo*-inositol. After suitable protection, phosphorylation of the unreactive 2-position with PCl_3, then resolution of the 1-*l*-menthoxyacetyl derivatives, and finally phosphorylation of the diol with tetrabenzyl pyrophosphate the desired material maybe obtained[86]. Similarly, D-Ins (2,4,5)P_3 may be formed from D-*chiro*-inositol after protection, inversion and phosphorylation[155], and the L-enantiomer from L-*chiro*-inositol again after protection, inversion and phosphorylation[155].

D-*myo*-Inositol 1,3,4-trisphosphate has been synthesised from D-2,5,6-tribenzyl-*myo*-inositol,which after phosphorylation with tetrabenzyl pyrophosphate and subsequent deprotection gave D-*myo*-inositol 1,3,4-trisphosphate[143]. Similarly, L-*myo*-inositol 1,3,4-trisphosphate has been synthesised from L-2,5,6-tribenzyl-*myo*-inositol, again, phosphorylation was effected with tetrabenzyl pyrophosphate, yielding, after deprotection, D-*myo*-inositol 1,3,4-trisphosphate[171]. L-*myo*-Inositol 1,3,4-trisphosphate has also been prepared from the 2-methoxyethoxymethyl-4,5-dibenzyl-*myo*-inositol derivative, (which is obtained in the inversion reaction when converting protected D- and L-*chiro*-inositol to protected D- and L-inositols). After phosphorylation with (diisopropylamino) dibenzyl phosphoramidite (82) and deblocking, L-*myo*-inositol 1,3,4-trisphosphate is produced[155].

The orthoester of myo-inositol, after exhaustive benzylation, afforded the 2,4,6-tribenzyl-1,3,5-orthoformate of *myo*-inositol. Removal of the orthoformate gave the 2,4,6-tribenzyl-*myo*-inositol, which on phosphorylation of the trialkoxide with tetrabenzyl pyrophosphate gave *myo*-inositol 1,3,5-trisphosphate after deprotection[146].

Phosphorylation of D- 3,4,5-tribenzyl-*myo*-inositol with (diisopropylamino) dibenzyl phosphoramidite (82) or (diisopropylamino)-bis(2-cyanoethoxy) phosphoramidite (81) gave D-*myo*-Inositol 1,2,6 trisphosphate following deprotection[153].

3.6.6 Syntheses of Higher Phosphates of myo-*Inositol.*

Several higher phosphates of inositol have been synthesised due to the interest in the role

of inositol tetrakisphosphates in cellular signalling.

The orthoformate derivative of *myo*-inositol was used in the first synthesis of DL-*myo*-inositol-1,3,4,5-tetrakisphosphate. Monoallylation of the 6(4) position (95) was achieved under chelation-control. The other two positions were then benzylated, the orthoformate and allyl groups were then removed to give 1,3-dibenzyl-myo-inositol. The tetra-sodio alkoxide salt was then phosphorylated with tetrabenzyl pyrophosphate, in the presence of a catalytic amount of 18 crown-6 (in order to increase solubility), to give DL-*myo*-inositol-1,3,4,5-tetrakisphosphate after catalytic hydrogenation of the benzyl groups[110]. DL-*myo*-inositol-1,3,4,5-tetrakisphosphate has been prepared from 1,3,4,5-tetrabenzoyl *myo*-inositol, which is easily obtained from *myo*-inositol and benzoyl chloride as the major product. Benzylation of the 2- and 6-positions was accomplished using benzyl trichloroacetamide in the presence of trifluoromethane sulphonic acid. Cleavage of the benzoyl groups gave the tetrol which was phosphorylated using *o*-xylylene diethylphosphoramidite (OXDEP) [Fig. 27]. After oxidation and deprotection DL-*myo*-inositol-1,3,4,5-tetrakisphosphate was obtained[173].

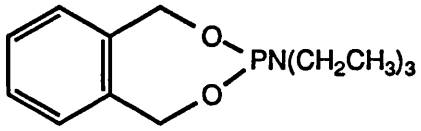


Figure 27 OXDEP

Phosphorylation of the 1,2-*O*-isopropylidene-myo-inositol dimethyl chlorophosphine, followed by demethylation with bromotrimethylsilane and concomitant removal of the ketal led to the synthesis of DL-*myo*-inositol-1,4,5,6-tetrakisphosphate[174].

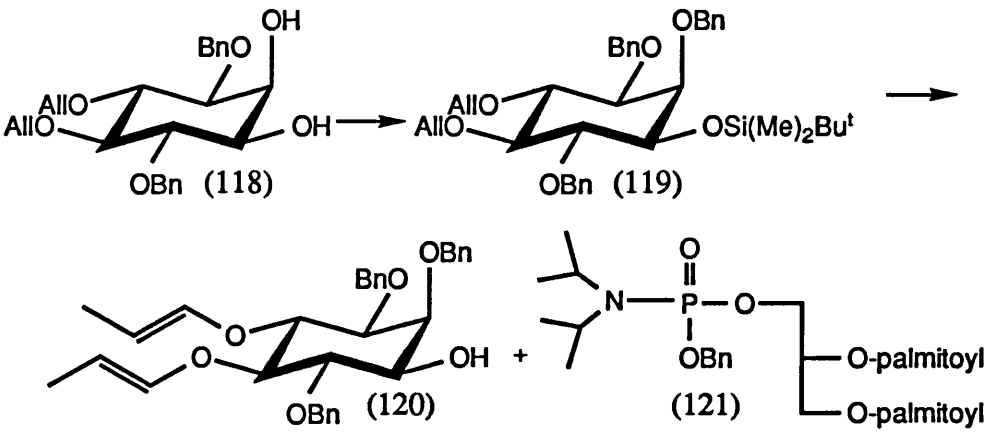
As mentioned earlier, treatment of *myo*-inositol with 1,3 dichloro-1,1,3,3-tetraisopropyl disiloxane (TIPSCl) gives the crystalline *myo*-inositol-1,3:4,6-bis(1,1,3,3-tetraisopropyl-disiloxane) (98). Benzoylation followed by removal of the TIPS groups leaves the tetrol which was phosphitylated with OXDEP. Oxidation proceeded by deprotection gave DL-*myo*-inositol-1,3,4,6-tetrakisphosphate[147], which is thought to be a partial agonist of Ins (1,4,5)P_3 receptor binding[175].

3.7 Synthesis of Inositol Phospholipids.

The synthesis of *myo*-inositol phospholipids has many problems in common with the synthesis of inositol phosphates: Production of a suitably protected enantiomerically pure *myo*-inositol derivative; phosphorylation/coupling step and finally deprotection without any phosphate group migration. Much of the work in this field has been

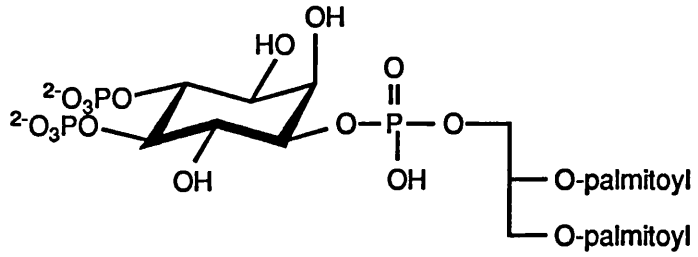
reviewed[176,177]. Protection strategies involve the use of mainly, benzyl, acetyl and cyclohexylidene groups, resolution as the (-)-camphanate or (-)-menthoxyacetate, whereas the phosphate tends to be protected as the benzyl, phenyl or 2,2,2-trichloroethyl ester.

Phosphatidyl inositol 4,5-bisphosphate (PIP_2) has been synthesised from optically pure D-3,6-dibenzyl-4,5-di-*O*-allyl-*myo*-inositol (118)[169]. Selective silylation gave the *t*-butyl dimethyl silyl ether at position-1. Subsequent benzylation at position-2 gave (119). Isomerisation of the allyl groups to prop-1-enyl groups followed by loss of the silyl group gave 2,4,5-tri-prop-1-enyl-3,6-dibenzyl-*myo*-inositol (120).



Scheme 31

This was then coupled with diacylglycerol phosphoramidite (121) which has been prepared from 1,2-di-*O*-palmitoyl-*sn*-glycerol and bis(diisopropylamino)benzyl phosphoramidite. Oxidation of the phosphite with *t*-butyl hydroperoxide to the phosphate triester followed by removal of the remaining prop-1-enyl groups, phosphitylation with diisopropylamino dibenzyl phosphoramidite and oxidation gave pure PIP_2 after catalytic hydrogenolysis[178].



(122)

Figure 28

Chapter Four

Review of Inositol Phosphate Analogues

4 *myo*-Inositol-1,4,5-Trisphosphate Analogues.

Analogues of biologically important molecules have helped in the understanding of many complex biological processes. There are many types of analogue, they are classified by their function, such as substrate analogues, reversible and irreversible inhibitors, transition-state inhibitors and suicide substrates.

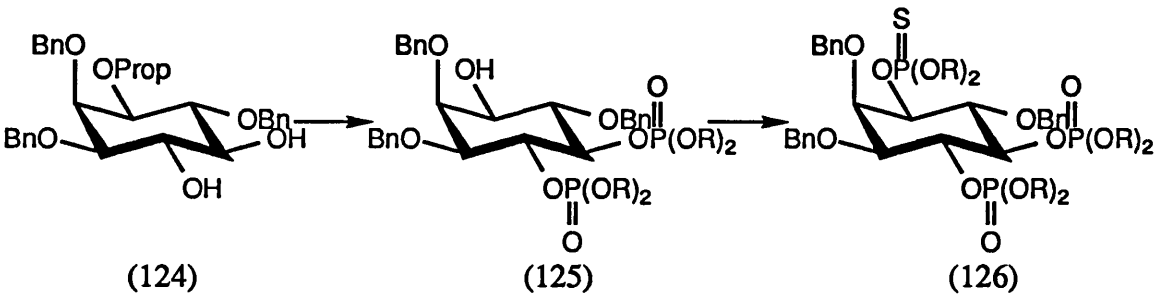
4.1 Phosphorothioate Analogues.

Phosphorothioate monoesters have provided useful reversible inhibitors of phosphate monoesters, they have been used extensively in the study of enzymic phosphoryl transfer reactions[179,180]. Nucleoside phosphorothioates were first described by Eckstein[181,182]. He took various nucleosides with trisimidazoyl-1-phosphinesulphide, to give the corresponding nucleoside phosphorothioates after acidic hydrolysis, thymidine 5'-phosphorothioate was synthesised in this manner. Replacement of an oxygen atom for a sulphur atom will result in changes in an inositol phosphate. The phosphorothioate will be a slightly more acidic than the phosphate since, the second dissociation constant of a phosphorothioate monoester is reduced by *ca.* 0.5 units. This can be seen reflected in the dissociation constants of thiophosphoric acid and phosphoric acid. Thiophosphoric acid is a somewhat stronger acid than phosphoric acid, with pK_a values of 1.67, 5.40 and 10.14, compared with values of 2.1, 7.2 and 12.3 for phosphoric acid. Around physiological pH the difference in the number of formal negative charges associated with the phosphate and the thiophosphate will be small[183]. The electronegativities of sulphur and oxygen are 2.5 and 3.5 respectively (Pauling index of electronegativities), the Van der Waals radii are 0.19 and 0.14 respectively. In a given phosphorothioate, the bond order of the sulphur to phosphorus bond in the phosphorothioate is around 1. Which is slightly less than that for the phosphorus to oxygen bond, both phosphorus to oxygen bonds being *ca* 1.5, with the charge localized on sulphur[183]. Substitution of oxygen for sulphur, increases the hydrophobicity of the molecule. The increased hydrophobicity may enhance the binding of the phosphorothioate molecule to the enzyme.

The first analogue of Ins(1,4,5)P_3 to be synthesised was *myo*-inositol-1,4,5-trisphosphorothioate Ins(1,4,5)PS_3. This compound was synthesised in a similar manner to Ins(1,4,5)P_3[125] [see chapter 3], again using a P(III) approach, the only difference being that the oxidation was carried out using sulphur in pyridine instead of *t*-butyl hydroperoxide to give Ins(1,4,5)PS_3[127]. Ins(1,4,5)PS_3 binds to Ins(1,4,5)P_3 receptor sites in brain[184] and in liver[185] and is only slightly less potent than Ins(1,4,5)P_3.

Moreover, it is a full and potent agonist for the release of Ca^{2+} from intracellular stores in a variety of systems, such as *Xenopus* oocytes[186] and permeablised Swiss 3T3 cells[186], with a potency only 3-4 fold down on Ins(1,4,5)P_3. Predictably Ins(1,4,5)PS_3 is resistant to Ins(1,4,5)P_3-5-phosphatase and is to date the most potent competitive inhibitor of Ins(1,4,5)P_3-5-phosphatase[187], for this reason Ins(1,4,5)PS_3 causes a sustained Ca^{2+}-release[188]. However, Ins(1,4,5)PS_3 is not bound by the Ins(1,4,5)P_3-3-kinase and does not compete with Ins(1,4,5)P_3 for the enzyme[188]. In summary, Ins(1,4,5)PS_3 is recognised with high affinity by Ins(1,4,5)P_3 receptors. It is a full agonist of Ca^{2+}-release, yet it is fully resistant to all known routes of metabolism. Several reviews cover this aspect in greater detail[31,32,123].

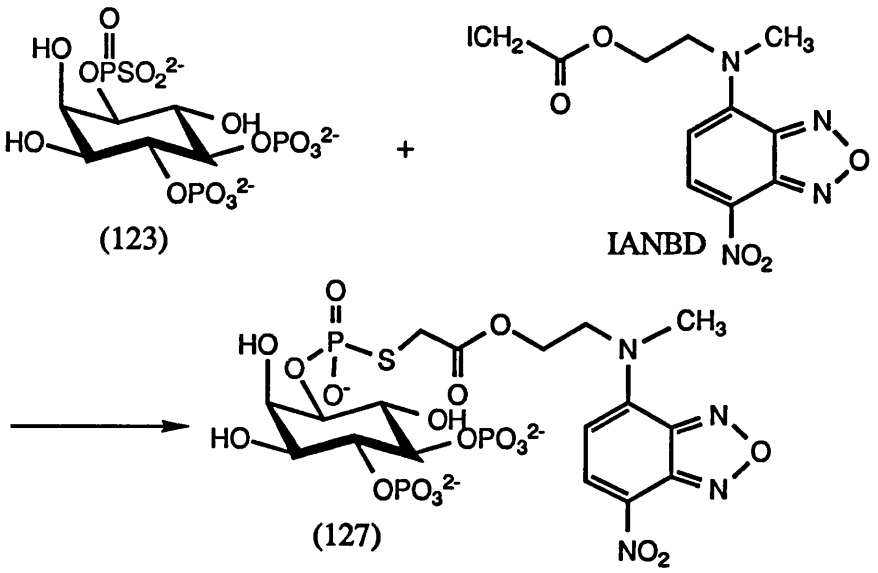
The phosphorothioate analogue of Ins(1,4,5)P_3 with the phosphate at position-1 replaced with a phosphorothioate, *myo*-inositol-1-phosphorothioate-4,5-bisphosphate Ins(1,4,5)P_3-1S (123) has been synthesised, again using a P(III) approach. The 1-prop-1-enyl-2,3,6-tribenzyl-*myo*-inositol (124) [prepared as described in chapter 7] was phosphitylated at the 4 and 5 positions with (diisopropylamino) bis(2-cyanoethoxy) phosphoramidite (81) and tetrazole. The bisphosphite (125) was then oxidised with *t*-butyl hydroperoxide, and the prop-1-enyl group was removed with mercuric chloride and mercuric oxide.



(124) (125) (126)

Scheme 32

The position-1 was phosphitylated as above, however, the oxidation was carried out using sulphur in pyridine which gave (126), this, after deprotection with sodium in liquid ammonia gave Ins(1,4,5)P_3-1S[189]. This analogue was then modified at sulphur to make a fluorescently tagged Ins(1,4,5)P_3 which should prove useful in probing the interaction between receptor and agonist. Ins(1,4,5)P_3-1S was treated with *N*-[{2'-(iodoacetoxy)ethyl}-*N*-methyl]amino-7-nitro-2,1,3-benzoxadiazole (IANBD) to give the adduct (127).

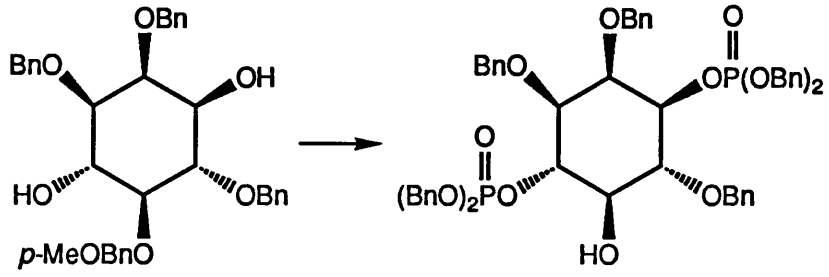


Synthesis of the labelled Ins(1,4,5)P_3-1S analogue (127)
Scheme 33.

4.2 Phosphonate Analogues.

The H-phosphonate analogue of Ins(1,4,5)P_3 was derived by the treatment of DL-1,2,4-tribenzyl-*myo*-inositol with ammonium phenyl phosphonate [activated with pivaloyl chloride], which after deprotection gave DL-*myo*-inositol-1,4,5-tris-H-phosphonate[190].

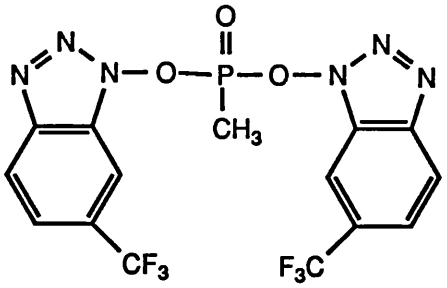
The racemic 5-phosphonate has been made from DL-1,2,4-tribenzyl-*para*-methoxybenzyl-*myo*-inositol (128). Phosphitylation of this compound with (diisopropylamino) dibenzyl phosphoramidite (82) in the presence of tetrazole was followed by oxidation to the phosphate (129) with *t*-butyl hydroperoxide. The *p*-methoxybenzyl group was removed by mild acid hydrolysis to afford the free hydroxyl group at position-5.



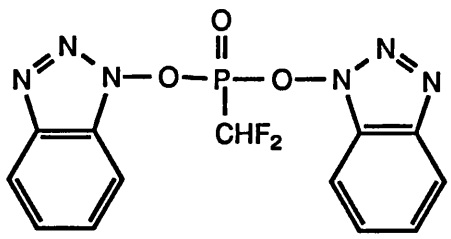
Scheme 34

This was then phosphonylated with one of three bifunctional agents, bis[6-(trifluoromethyl)benzotriazol-1-yl] methylphosphonate, bis(benzotriazol-1-yl)

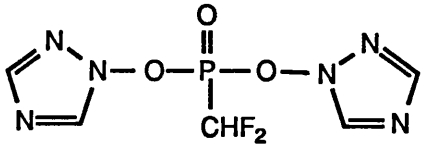
(difluoromethyl) phosphonate or (difluoromethyl) phosphonic di(1,2,4-triazolide) [Fig. 29].



bis[6-(trifluoromethyl)benzo triazol-1-yl] methylphosphonate



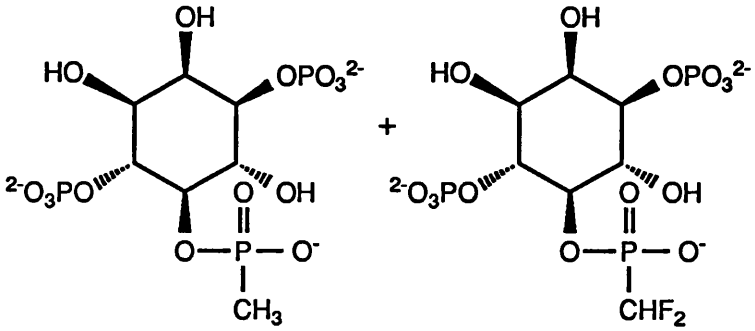
bis(benzotriazol-1-yl) (difluoromethyl) phosphonate



(difluoromethyl) phosphonic di(1,2,4-triazolide)

Figure 29

The former gave the methyl, the latter two gave difluoromethyl phosphonate intermediates, these were then treated with benzyl alcohol in the presence of *N*-methylimidazole to give the fully protected triesters. Subsequent catalytic hydrogenolysis gave DL-*myo*-inositol-1,4-bisphosphate-5-methylphosphonate Ins(1,4,5)P_3-5-Me and myo-inositol-1,4-bisphosphate-5-difluoromethylphosphonate Ins(1,4,5)P_3-5-CHF_2[191], [Fig. 30].

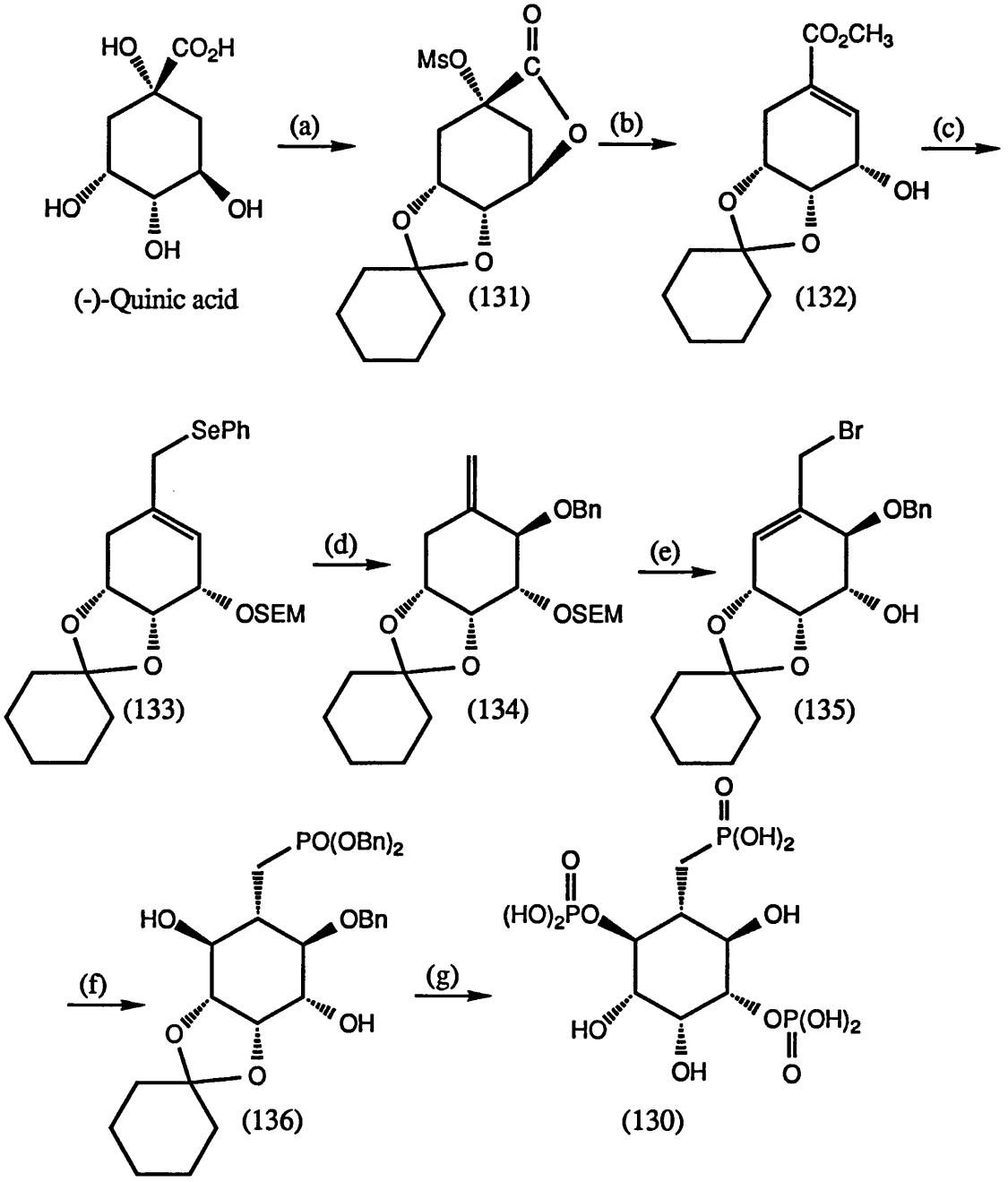


(Ins(1,4,5)P_3-5-Me) (Ins(1,4,5)P_3-5-CHF_2)

Figure 30

myo-Inositol-1,4-bisphosphate-5-methylenephosphonate (130) has been synthesised starting from the natural product (-)-quinic acid. (-)-Quinic acid had been previously used in the synthesis of optically pure Ins(1,4,5)P_3[134]. (-)-Quinic acid was taken and was converted to the lactone (131) by concomitant lactonisation/ketalisation using cyclohexanone and Amberlite IR 120 H^+resin followed by mesylation of the tertiary alcohol. Sequential methanolysis, pyridinium chlorochromate oxidation and triethylamine

induced mesylate elimination generated an enone from which ester (132) was generated by hydride delivery from the less hinderd β–face. The ester (132) was converted to the phenyl selenide (133) by the protection of the hydroxyl group at C-1 as its β–trimethylsilylethoxymethyl (SEM) ether, reduction of the ester using diisobutylaluminium hydride (DIBAL) at -78°C, and selenylation of the resultant primary alcohol with N-(phenylseleno)phthalimide in the presence of tributyl phosphine. Stereoselective *in situ* [2,3]-sigmatrophic rearrangement of the allylic selenoxide derivative from (133) and benzylation of the product gave (134) as the sole product. Fluoride ion desilylation of (134) in hexamethylphosphoric triamide (HMPA) and kinetically controlled addition of phenylselenyl bromide across the exocyclic alkene provided the anti-Markownikoff adduct. The latter underwent regiospecific oxidative elimination of the allylic bromide (135). This was subsequently phosphorylated with sodium dibenzyl phosphite in the presence of 18-crown-6 to improve the solubility of sodium dibenzyl phosphite in toluene. Hydroboration of the alkene followed by oxidative work-up using a peracid, as alkaline hydrogen peroxide hydrolyses the phosphonate, gave the diol (136). Phosphitylation of hydroxyl groups at positions-1 and -4 then oxidation with *m*-chloro-perbenzoic acid (*m*-CPBA) afforded the protected phosphate, deprotection was effected by catalytic hydrogenolysis to give the sodium salt of the 5-methylenephosphonate (130)[134] [Scheme 35].



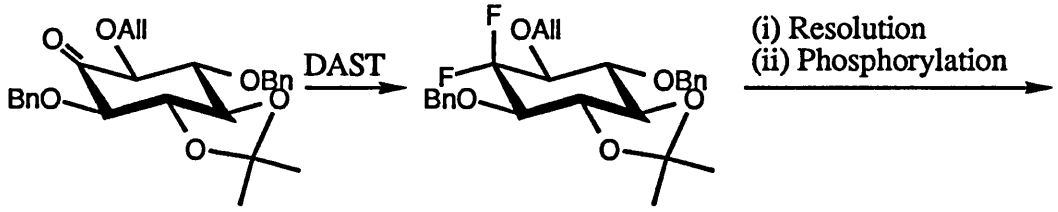
Scheme 35

(a) (i) cyclohexanone, IR-120: (ii). MsCl, Et_3N, 0°C: (b) (i) NaOMe, MeOH: (ii) PCC; Et_3N: (iii) $NaBH_4$, 0°C: (c) (i) SEM-Cl, Pr^i_2NEt: (ii) DIBAL, $PhCH_3$, -78°C: (iii) N-(PhSe)phth, Bu_3P, -15°C: (d) (i) $NaIO_4$, pH 7 buffer, 1,4-dioxan, 0°C: (ii) KH, BnBr: (e) (i) nBu_4NF, HMPA, 100°C, 4A sieves: (ii) PhSeBr, -78°C; mCPBA: pyridine, -78°C: (f) (i) $NaPO(OBn)_2$, 18-crown-6; (ii) BH_3,THF, mCPBA: (g) (i) $Pr^i_2NP(OBn)_2$, tetrazole: (ii) mCPBA: (iii) H_2, 50 psi, 10% Pd/C, 80% EtOH; $AcOH/H_2O$.

4.3 Fluorinated Analogues.

Isosteric replacement of the hydroxyl groups of inositol with fluorine has been effected at positions 1[192], 2[193], 3[194], 5[195] and 6[196] of *myo*-inositol. The C-F bond has bond lengths and polarisations almost identical to those of the C-OH bond. However, the C-F bond is not an H-bond acceptor.

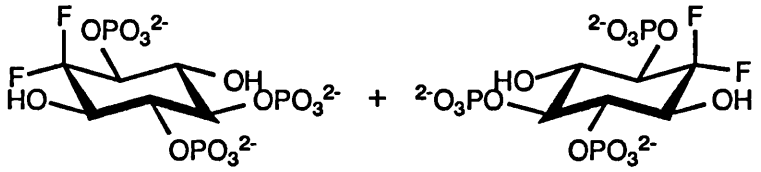
Both enantiomers of 2,2-difluoro-2-deoxy-*myo*-inositol-1,4,5-trisphosphate [2,2-F_2-Ins(1,4,5)P_3] have been synthesised by the (diethylamino) sulphur trifluoride (DAST) fluorination of 1-*O*-allyl-3,6-di-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-2-inosose (137).



(137) (138)

Scheme 36

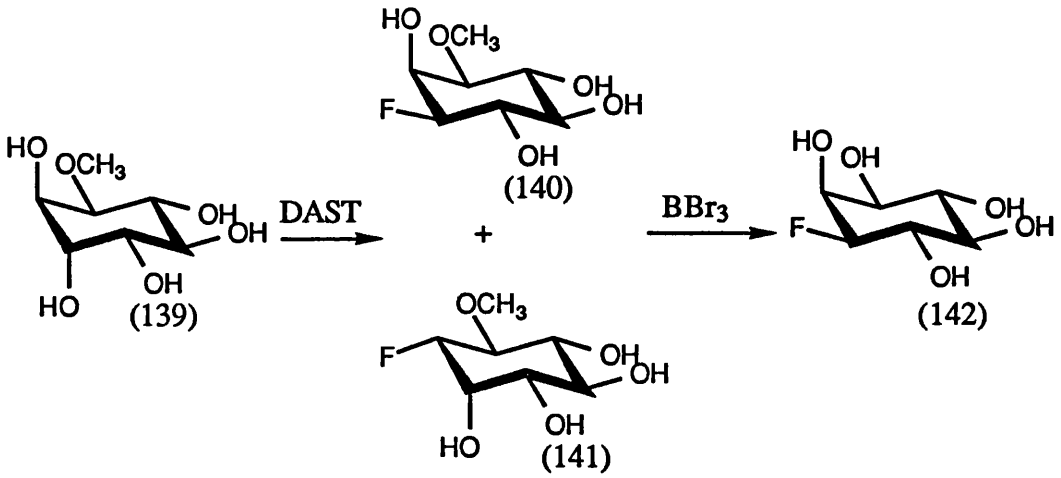
Subsequent removal of the allyl group (via the prop-1-enyl ether), followed by resolution of the (-)-camphanates, then removal of ester and ketal gave the two triols. The phosphorylation of which was effected by the tetrazole-mediated phosphitylation with bis(2-cyanoethyl) (diisopropylamino) phosphoramidite (81). Oxidation with *t*-butyl hydroperoxide and subsequent deprotection with sodium in liquid ammonia gave D- and L-2,2-F_2-Ins(1,4,5)P_3[185].



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

Figure 31

The 3-fluoro-analogue, 3-deoxy-3-fluoro-D-*myo*-inositol has been synthesised in two steps from quebrachitol (139)[194]. The quebrachitol (139) was fluorinated with (DAST) to give two products 3-deoxy-3-fluoro-1-*O*-methyl-*myo*-inositol (140) and 3-deoxy-3-fluoro-4-*O*-methyl-*myo*-inositol (141). Treatment of the mixture with BBr_3 in dichloromethane gave 3-deoxy-3-fluoro-D-*myo*-inositol (142) in 88% yield from quebrachitol [Scheme 37].



3-deoxy-3-fluoro-*myo*-Inositol

Scheme 37

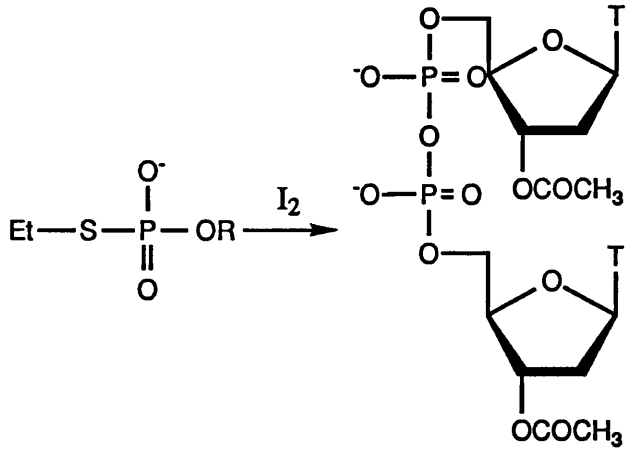
Chapter Five

Novel Synthesis of Cyclic Pyrophosphates

5 Introduction

The major aim of this thesis was to try to synthesise key cyclic 7-membered pyrophosphates. The classical method to do this would be by the use of a condensing agent such as dicyclohexylcarbodiimide DCC. However, this method was deemed to be inappropriate as DCC would not make the target molecule Ins(1,4:5pyro)P_3, DCC would form both the intermolecular pyrophosphate and the cyclic 5-membered phosphate in competition with the formation of the intramolecular pyrophosphate. DCC would condense the phosphates of Ins(1,4,5)P_3 with one of the vicinal hydroxyl groups present in the molecule at a greater rate than the rate of the cyclic pyrophosphate formation. One way to circumvent this cyclic 5-membered phosphate formation would be to protect the hydroxyl groups. This would then present the problem of deprotection of the hydroxyl groups without pyrophosphate cleavage, providing another problem that would then have to be solved.

The number of synthetic steps required to make a suitably protected compound with free phosphates is about the same as the number for the formation of a fully deprotected phosphorothioate, so to condense a suitably protected inositol phosphate with DCC would not have any advantages over the formation of the phosphorothioate analogue. It was therefore of interest to try to synthesise the cyclic pyrophosphate directly from the phosphorothioate compound. It had been shown that symmetrical pyrophosphates (143) could be formed by treatment of nucleoside S-ethyl phosphorothioates (144) with iodine in pyridine [Scheme 38][197], so desulphurisation of a bisphosphorothioate was an obvious place to start. We reasoned that desulphurisation of a vicinal bisphosphorothioate should be a facile way of preparing seven-membered cyclic pyrophosphates.



R=5'-nucleoside (144) (143)

Scheme 38

If this method proved to be useless, then the precursor could be phosphorylated to give the partially protected compound which could then be used in conjunction with DCC to form the pyrophosphate.

5.1 Pyrophosphate Synthesis.

Several methods have been employed to desulphurise phosphorothioates to form isotopically labelled phosphates. For example, methylation of sulphur with methyl iodide followed by displacement with $Na^{18}OH$[198], the direct reaction of the phosphorothioate with [^{18}O]chloral[199] or [^{18}O]styrene oxide[199]. The latter two have the disadvantage of having to prepare the labelled reagent. However, the most commonly used procedures are simple halogen mediated oxidative desulphurisations. Halogen mediated oxidative desulphurisations of phosphorothioates have been extensively used in the synthesis of nucleosides and nucleotides specifically labelled with isotopes of oxygen[200,201,202] the most commonly agents are iodine[203,204,197] bromine[205], cyanogen bromide[206] and N-bromosuccinimide[202]. Substitution at phosphorus with ^{18}O, ^{17}O, ^{16}O and S enables the reaction course to be followed by monitoring the configuration at phosphorus[207,208]. Phosphorothioate analogues of nucleotides are sometimes weak substrates for enzymes so it was of interest to develop methods that enabled the replacement of sulphur with oxygen. Nucleotides labelled stereospecifically with isotopes of oxygen have afforded useful probes, for the elucidation of the stereochemical course of certain enzyme catalysed reactions[209]. Of the reagents commonly used to desulphurise phosphorothioates, three were tested to see if a cyclic pyrophosphate could be synthesised in good yield by halogen mediated oxidative desulphurisations of a vicinal bisphosphorothioate.

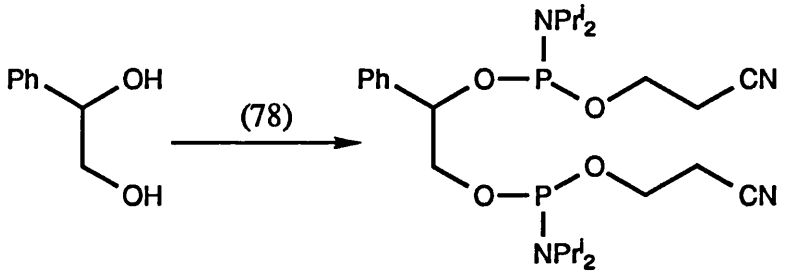
5.2 Synthesis of 1-Phenyl Ethane-1,2-Bisphosphorothioate

In order to develop a synthesis of seven-membered cyclic pyrophosphates via the oxidative desulphurisation of vicinal bisphosphorothioates. It was necessary to synthesis model compounds with a vicinal bisphosphorothioate moiety that could be used to screen various reagents for the ability to form cyclic pyrophosphates. 1-Phenyl ethane-1,2-bisphosphorothioate (145) was chosen as the first model compound, initial desulphurisation reactions were performed upon this compound and the results compared in order to find the most efficient cyclic pyrophosphate forming reagent. 1-Phenyl ethane-1,2-bisphosphorothioate (145) was synthesised by two similar routes. The two methods differing only in the choice of phosphitylation reagent used, either chloro (diisopropylamino)-2-cyanoethoxy phosphine (78) or (diisopropylamino) di(2-

cyanoethyl) phosphoramidite (81). Both routes led to the same intermediate, 1-phenyl ethane-1,2-bis[di(2-cyanoethyl) phosphite] (146).

The reagent chloro (diisopropylamino)-2-cyanoethoxy phosphine (78) was synthesised from phosphorus trichloride by treatment with 1 equivalent of 2-cyanoethanol, purified and then treated with an equivalent of NN-diisopropylamine and purified[210,211]. Subsequent treatment of (78) with another equivalent of 2-cyanoethanol afforded the second reagent di(2-cyanoethyl) (diisopropylamino) phosphoramidite (81). The latter can be prepared in two steps without preparing (78) as an intermediate[128].

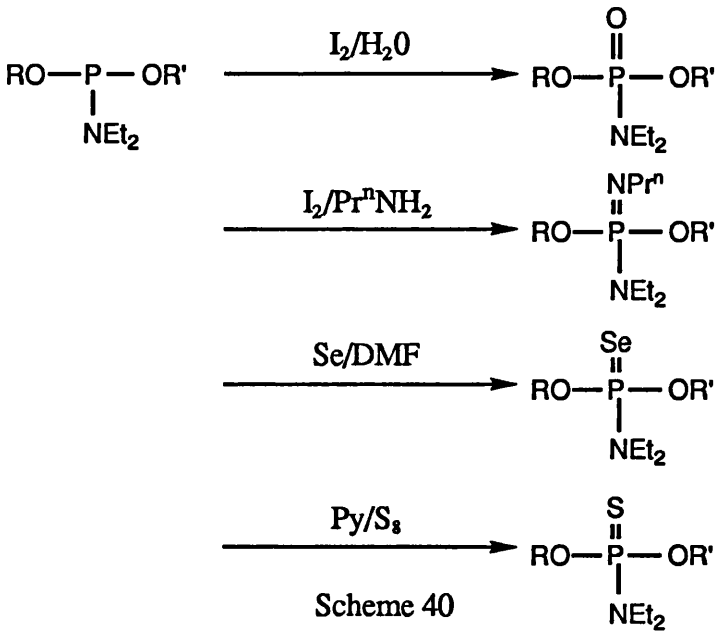
Treatment of 1-phenylethane-1,2-diol with chloro (diisopropylamino)-2-cyanoethoxy phosphine (78) gave the intermediate phosphoramidite, 1-phenyl ethane-1,2-bis[(2-cyanoethyl) (diisopropylamino) phosphoramidite] (147).



(147)

Scheme 39

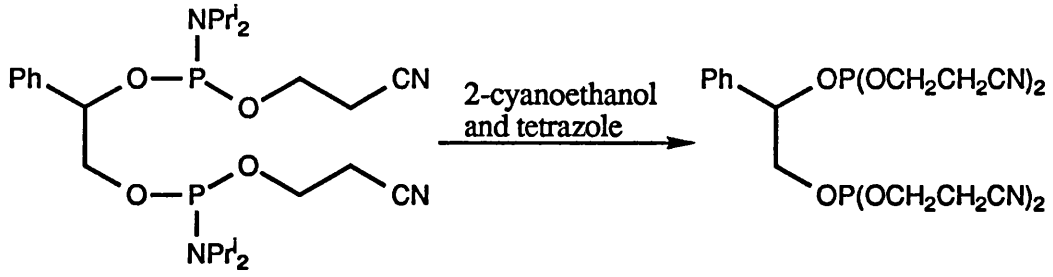
Phosphoramidites have been oxidised to yield a variety of products. For example, oxidation with iodine in water gives the phosphate, whereas iodine with propylamine gives the phosphoimine, selenium in dimethyl formamide gives the selenophosphoramidate and sulphur in pyridine gives thiophosphoramidite[212].



Scheme 40

Oxidation was effected upon the phosphite not the phosphoramidite, the phosphite triester was formed by the substitution of the amine by an alcohol, with tetrazole acting as a catalyst. The catalytic role of tetrazole has been investigated[213], and it was found that the coupling rate was dependent upon the order of the addition of alcohol and the tetrazole. It was also found that dialkylammonium salts inhibit the formation of the phosphite triesters.

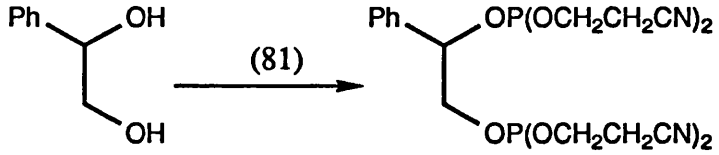
The phosphoramidite (147) was converted to the phosphite by the addition of 2-cyanoethanol under the catalysis of tetrazole to give 1-phenyl ethane-1,2-bis[di(2-cyanoethyl) phosphite] (146).



(147) (146)

Scheme 41

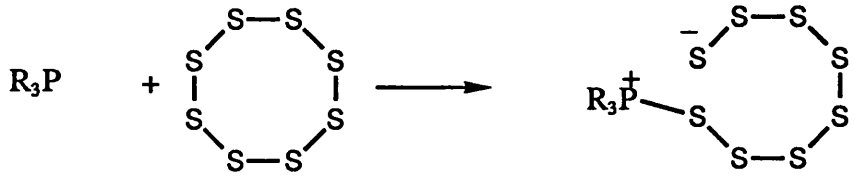
Reaction of 1-phenylethane-1,2-diol with di(2-cyanoethyl) (diisopropylamino) phosphoramidite (81) gave the bisphosphite triester (146) directly.



(146)

Scheme 42

The phosphite triester thus produced can then be oxidised to the phosphate using *t*-butyl hydroperoxide[214], or by sulphur in pyridine[215,204], to give the phosphate and the phosphorothioate respectively as glasses. The mechanism of the sulphur oxidation of triphenyl phosphine[216], and triphenyl phosphite[216,217], has been investigated and proposed as follows.



(i)

$R_3P^+SSSSSSSS^-$ ⟶ R_3PS + $R_3P^+SSSSSSS^-$, etc. (ii-vii)

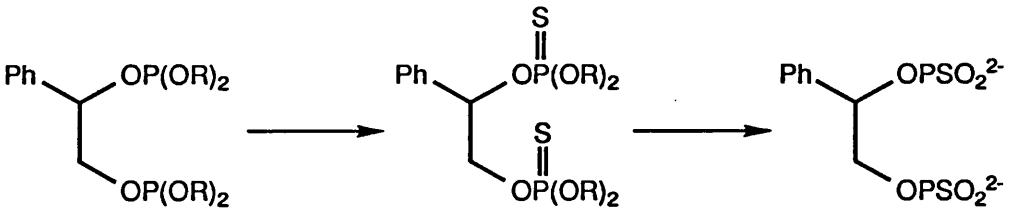
R_3P + $R_3P^+SS^-$ ⟶ $2R_3PS$ (viii)

Where R= Phenyl
or R=OPhenyl

Scheme 43

The rate determining step (i) represents the attack of triphenyl phosphite on S_8 followed by the opening of the eight-membered sulphur ring. This results in the dipolar intermediate $R_3P^+SSSSSSSS^-$, which, as a sulphenyl ylide, is more reactive toward R_3P than the cyclic sulphur molecule itself, thus favouring further nucleophilic displacement of sulphur on the intermediate to give ultimately the product R_3PS. Only the final step (viii) between R_3P and the disulphide $R_3P^+SS^-$ suggests that there was an electrostatic barrier to its accomplishment. However, this can be offset by the favorable entropy change upon the final release of the solvent molecules that were held in solvation by the negatively charged sulphur atom, and increase in entropy on formation of the two phosphorothioate molecules. The nucleophilicity of the P(III) species effects the rate of the rate limiting first step, triphenyl phosphite being a much weaker nucleophile due to the electron withdrawing effects of OPh, reacts significantly slower than triphenyl phosphine.

The bisphosphite thus produced using either reagent (78) or (81) was taken crude and oxidised with sulphur in pyridine to give 1-phenyl ethane-1,2-bis[(2-cyanoethyl) phosphorothioate] (148) in high yield from (146). Subsequent deprotection of (148) by heating to reflux in aqueous ammonia gave 1-phenyl ethane-1,2-bisphosphorothioate (145) again in high yield (90%) from (148). The overall yield of (145) from 1-phenyl ethane-1,2-diol using (78) was 22%.

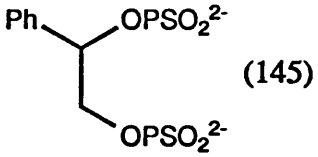


R=CH_2CH_2CN (146) (148) (145)

Scheme 44

5.3 Halogen Mediated Oxidative Desulphurisations of Vicinal Bisphosphorothioates.

All dephosphorylations were carried out upon an acyclic model system, phenylethane 1,2-bisphosphorothioate (145) was taken in each case and desulphurised.



(145)

Figure 32

Identification of the desired product, the cyclic pyrophosphate, by ^{31}P NMR was relatively simple. The signals of the pyrophosphates occur further upfield than phosphorothioates or phosphates around δ -6, cyclisation of the pyrophosphate causes the phosphorous resonance to shift further upfield to around δ -10. The two phosphorus atoms of the cyclic pyrophosphate couple together to form an AB system as the difference in chemical shift is the same order of magnitude as the coupling constant, with a P-O-P coupling constant of *ca.* 10-20 Hz, so the position and character of the phosphorus resonance gives a clear indication of the product in the NMR signal.

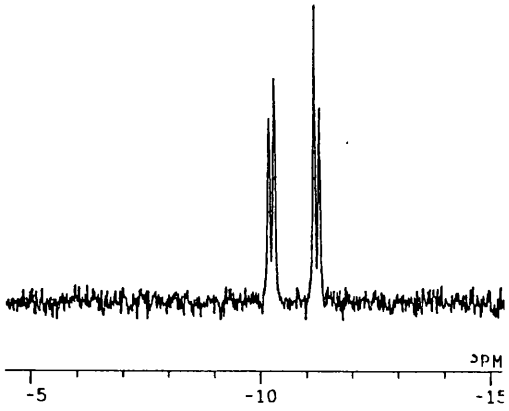
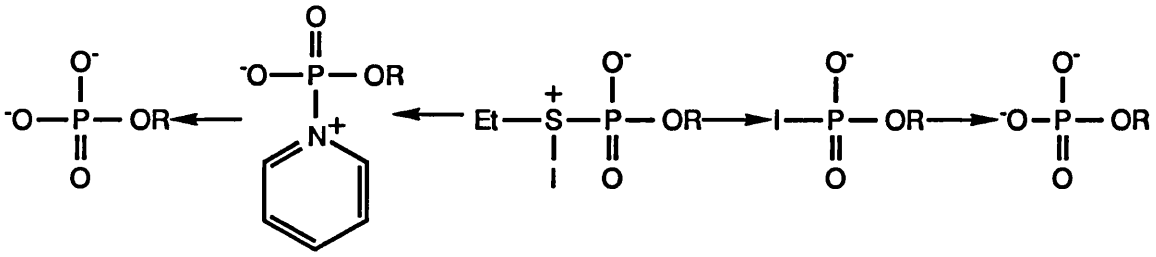


Figure 33. 161 MHz ^{31}P NMR Spectrum of (149) pH 9 in D_2O

Iodine was first tested as a saturated solution in aqueous pyridine, as it had been used for the oxidation of nucleoside S-ethyl phosphorothioates[197] to phosphate, however, more importantly, the symmetrical intermolecular pyrophosphate had been isolated as a by-product from the reaction. The mechanism of this reaction probably involves an intermediate iodosulphonium species (150) [Scheme 38] from the attack of iodine on (144). The iodosulphonium species thus produced can undergo P-S cleavage to give (151) followed by attack of water to give the monoester (152). Alternatively, (150) could undergo displacement by iodide ion to give a phosphoroiodate (153) which upon hydrolysis would give the phosphate monoester (152). The phosphate (152) formed could act as a nucleophile by attack on the active intermediate (151) or (153) to give the pyrophosphate (143) [Scheme 45].

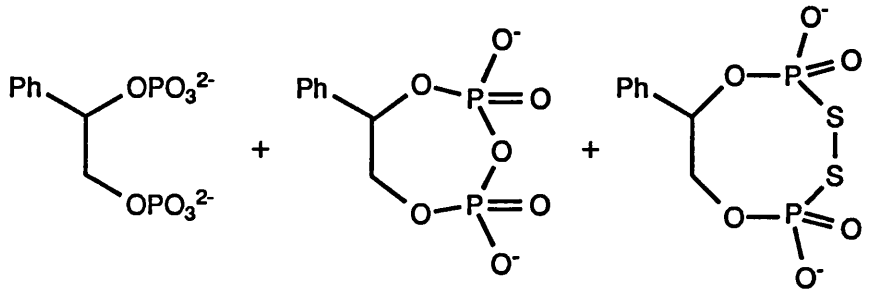


(152) (151) (150) (153) (152)

Scheme 45

Cummins [218] found that desulphurisation of (S_p)-5'-O-(2' deoxyadenosyl)-3'-O-thymidyl phosphorothioate with iodine in pyridine and water ($H_2{}^{18}O$) proceeded cleanly with epimerisation of the phosphorus, by way of activation of the sulphur by iodine. Followed by displacement of sulphur by pyridine, epimerisation, then base catalysed displacement of the pyridine by water.

We found that the iodine-mediated desulphurisation of phenylethane 1,2-bisphosphorothioate (145) resulted in the formation of three products [Fig. 34], double desulphurisation to the bisphosphate (154), formation of the cyclic disulphide (155) and the cyclic pyrophosphate (149). It is possible that the cyclic disulphide compound is an intermediate in pyrophosphate formation.

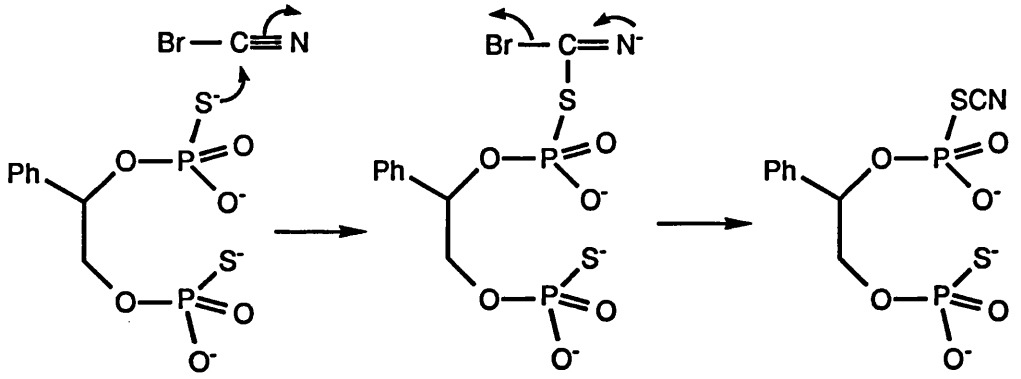


(154) (149) (155)

Figure 34

The yield of the pyrophosphate was low and after subsequent reactions, it was not significantly increased.

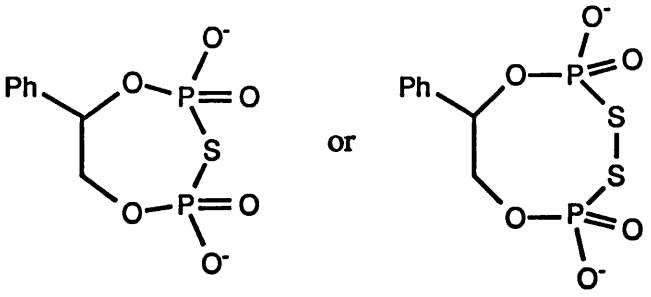
Synthesis of the cyclic pyrophosphate was attempted using cyanogen bromide to desulphurise phenylethane 1,2-bisphosphorothioate (145). The reaction proceeding by cyanylation of the sulphur by the cyanogen bromide at one or both phosphorothioate centres [Scheme 46], followed by its displacement as thiocyanate by water, phosphoryl oxygen or as cyanide by phosphoryl sulphur, the reaction probably going in a similar manner to that reported by Sammons[206], who found that cyanogen bromide easily desulphurised chiral phosphorothioates to the corresponding chiral phosphate.



Scheme 46

The electron withdrawing cyano group would markedly increase the reactivity of the phosphorus towards nucleophilic displacement by either one of the three nucleophiles mentioned above.

Our desulphurisation of 1-phenylethane-1,2-bisphosphorothioate (145) with cyanogen bromide again resulted in the formation of three products, the cyclic pyrophosphate (149), the bisphosphate (154) as well as the concomitant formation of the cyclic disulphide (155).



cyclic sulphide (156) cyclic disulphide (155)

Figure 35

There was some uncertainty as to whether the compound produced was in fact the disulphide or the sulphide compound. Since disulphide compounds can be reduced back to the parent bisphosphorothioate with 2-mercaptoethanol, the mixture was treated with 2-

mercaptoethanol, which resulted in the reduction of one of the components of the mixture back to starting material. Before reduction the compound resonated in the ^{31}P NMR as a simple AB system δ 17.8 and δ 19.9 ppm with a coupling constant of about 5 Hz. The implication is that the compound was the simple cyclic disulphide (155) and not the intermolecular disulphide, as the spectrum for the intermolecular disulphide would be complicated with several overlapping AB systems. These three products resulted from (i) the nucleophilic attack on phosphorus by phosphoryl oxygen to give the pyrophosphate ; (ii) the nucleophilic attack on phosphorus by water to give the bisphosphate; (iii) nucleophilic attack on the sulphur of the thiocyanate by phosphoryl sulphur to give the cyclic disulphide [Fig. 36]. Manipulation of reaction conditions did not lead to significant improvements in the yield of the pyrophosphate.

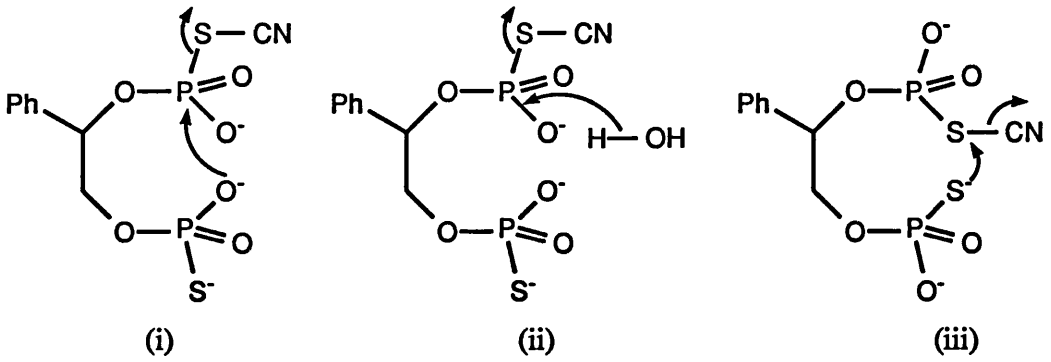


Figure 36

5.4 N-Bromosuccinimide-Mediated Desulphurisation.

N–bromosuccinimide (NBS) in dioxan water solution was first proposed by Connolly *et al.*[202] as a method for the clean conversion of a phosphorothioate to a phosphate. NBS was used to exchange sulphur for heavy isotopes of water in several nucleotide phosphorothioates[201,202].

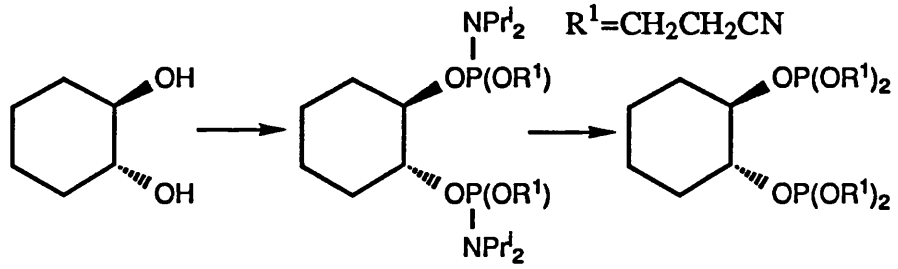
Our NBS-mediated desulphurisation of the 1-phenyl ethane 1,2-bisphosphorothioate (145) produced a mixture of 1-phenyl ethane 1,2-cyclic pyrophosphate (149) and 1-phenyl ethane 1,2-bisphosphate (154) as the only products[219]. Manipulation of the reaction conditions enabled the maximisation of the yield of the pyrophosphate to 40%, a greater yield than that from either iodine or cyanogen bromide. The quantification of the two phosphate fractions was achieved by Briggs' phosphate assay[220]. It was possible to determine the relative composition of the mixture by ^{31}P NMR as the ratio of the integrals was consistent with the absolute result from the phosphate assay. The moderate yield of the pyrophosphate was probably due to the free rotation of the phosphorothioate moieties

whilst the reaction progressed. It was therefore decided to try the desulphurisation of a conformationally more restrained system, a cyclic vicinal phosphorothioate to see if constraining the phosphorothioates would improve the yield of the NBS-mediated desulphurisation.

5.5 Synthesis of Cyclohexane Cyclic Pyrophosphate.

It was thought that by reducing in the degrees of freedom of the bisphosphorothioate, the yield of the pyrophosphate would be improved. To test this theory, NBS-mediated desulphurisation of the cyclic compound, cyclohexane 1,2-bisphosphorothioate (157) was attempted. The effect of the ring would be to reduce severely the amount of relative movement between the two phosphorothioate groups which could increase the yield of the desired pyrophosphate (40).

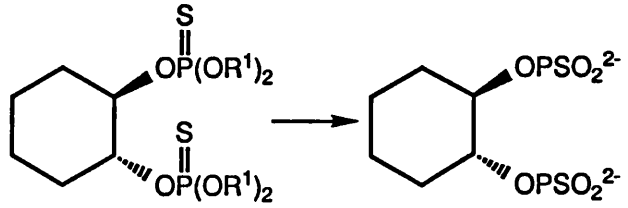
trans-Cyclohexane 1,2-diol (41) was phosphitylated with (78) to give the intermediate phosphoramidite (158) which on treatment with tetrazole and 2-cyanoethanol gave the intermediate cyclohexane 1,2-bisphosphite (159).



(41) (158) (159)

Scheme 47

Subsequent oxidation with sulphur in pyridine again afforded the bisphosphorothioate (160) in 95% yield from the diol.

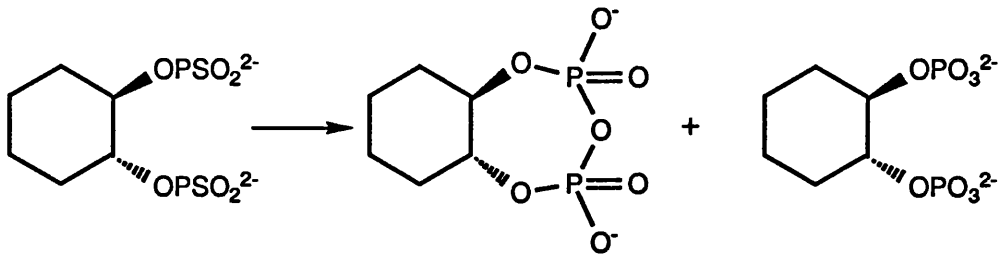


R^1=CH$_2$CH$_2$CN (160) (157)

Scheme 48

ß-Elimination of the 2-cyanoethyl groups was effected by heating to reflux for one hour a solution of (160) in aqueous ammonia to give *trans*-cyclohexane 1,2-bisphosphorothioate (157) as the cyclohexylammonium salt in 95% yield after purification.

Treatment of the tris-cyclohexylammonium salt of cyclohexane 1,2-bisphosphorothioate (157) with NBS gave a mixture of cyclohexane-1,2-bisphosphate (42) and cyclohexane-1,2-cyclic pyrophosphate (40). Manipulation of the reaction conditions namely, the excess of NBS and the solvent system used resulted in the maximisation of the pyrophosphate (40) content of the mixture, which after purification by anion exchange chromatography was separated into the phosphate (42) and the cyclic pyrophosphate (40) in 8% and 88% overall yield respectively.



(157) (40) (42)

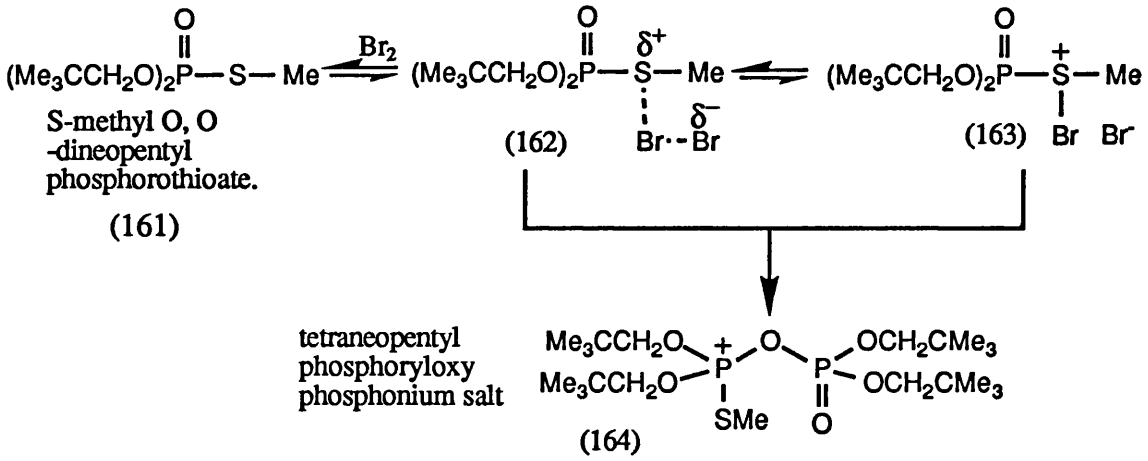
Scheme 49

The AB system associated with the cyclic pyrophosphate was not observed in the ^{31}P NMR because the molecule is symmetrical, the two phosphorus atoms are not in a magnetically different environment. This simple analogue of IP_3 lacking in the 1-position phosphate and hydroxyl groups at the 2, 3 and 6 positions was tested for any biological activity against the IP_3 cycle enzymes and receptor. Both (157) and (40) were very poor inhibitors of Ins(1,4,5)P_3-3-kinase, both with K_i values greater than 130 μM [*c.f.* K_m 3.2 μM for Ins(1,4,5)P_3]. The pyrophosphate was tested for Ca^{2+} release from Ins(1,4,5)P_3 sensitive intracellular stores, but was found not to have any Ca^{2+} release activity. To our surprise however, both (157) and (40) were found to inhibit Ins(1,4,5)P_3-5-phosphatase with values of K_i of the same order of magnitude as the K_m of the substrate Ins(1,4,5)P_3, 54 μM, 59 μM and 40 μM respectively. This result seems to imply that the enzyme is rather indiscriminate in its substrate specificity.

Cyclohexane 1,2-cyclic pyrophosphate has been synthesised from cyclohexane 1,2 bisphosphate by treatment with DCC in almost quantitative yield[96]. As stated earlier, the DCC method would be of little use for the synthesis of the target compound DL-*myo*-inositol-1-phosphate 4,5-cyclic pyrophosphate (5) due to the formation of cyclic phosphates.

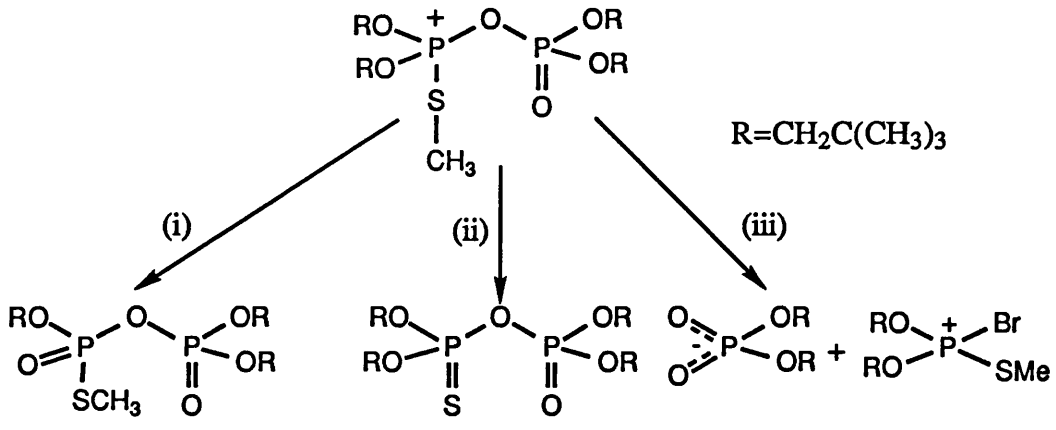
5.6 Mechanistic Analysis.

Much work has been conducted by Krawiecka and Michaliski[221,222,223] upon the halogen-mediated desulphurisation of phosphono- and phosphoro-thioates. They have studied the halogen-mediated oxidation of various phosphorothiolates. In their latest work[222] they took S-methyl O, O-dineopentyl phosphorothioate (161) with bromine in toluene and observed that the thiolester was in equilibrium with two compounds, a charge transfer molecular complex (162) and a bromosulphonium salt (163), and that the equilibrium favored the formation of the bromosulphonium salt (163). The two molecules (162) and (163) then reacted together to form the stable tetraneopentyl phosphoryloxy phosphonium salt (164).



Scheme 50

At room temperature this compound decomposed to form several different compounds via a number of routes [Schemes 51, 52, 53].



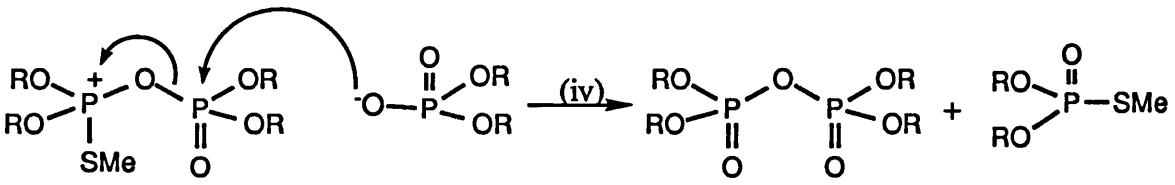
Scheme 51

In the reactions labeled (i to iii) the bromide ion acts as a nucleophile.

(i) This reaction involves the attack of the bromide ion upon the carbon attached to oxygen to give the S-methyl-O,O,O'-trineopentyl thiopyrophosphate (165)

(ii) In this reaction the bromide ion attacks the carbon attached to the sulphur atom to form tetraneopentyl thiopyrophosphate (166).

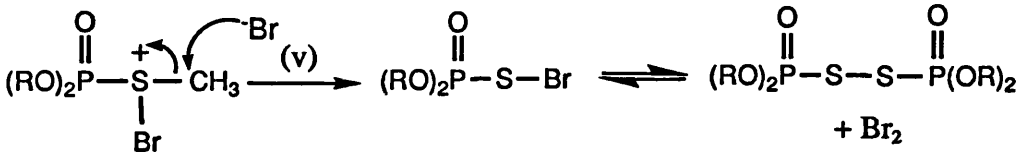
(iii) The bromide ion reacts with the phosphonium phosphorus to produce dineopentyl phosphate (167) and the bromophosphonium salt (168).



(164) (167) (169) (170)

Scheme 52

(iv) Dineopentyl phosphate acts as a nucleophile and attacks the phosphate phosphorus of (164) to produce tetraneopentyl pyrophosphate (169) and dineopentyl methyl phosphorothiolate (170).

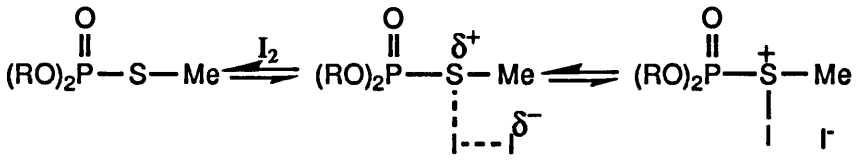


(163) (171) (172)

Scheme 53

(v) Again bromide ion acts as a nucleophile but attacks the bromosulphonium salt (163) to form the bromosulphide (171) which is in equilibrium with bis(dineopentoxy phosphoryl) disulphide (172).

When the reaction was repeated using iodine instead of bromine nothing seemed to have happened at room temperature. However, after 7 months ^{31}P NMR showed the presence of a charge transfer molecular complex (173), a iodosulphonium salt (174) [Scheme 54], and a small amount of bis(dineopentoxy phosphoryl) disulphide (172).



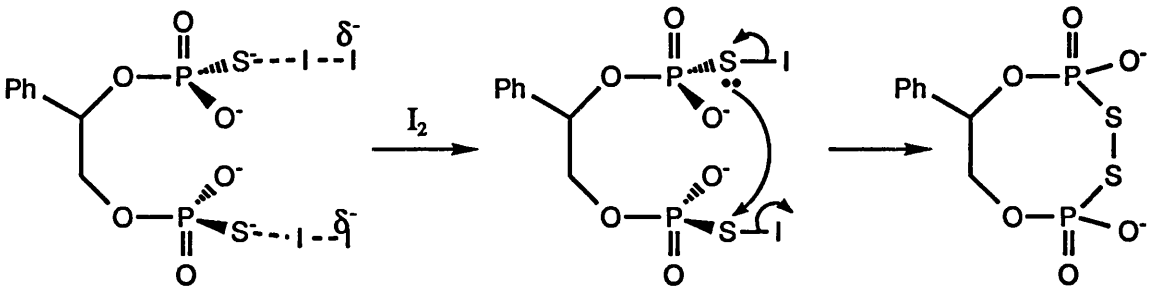
(161) (173) (174)

Scheme 54

The inference from this being that the equilibrium did not favor the formation of the products (173) and (174). The difference in reactivity between the two halide anions must therefore depend upon their nucleophilicity towards the electrophilic phosphorus centre.

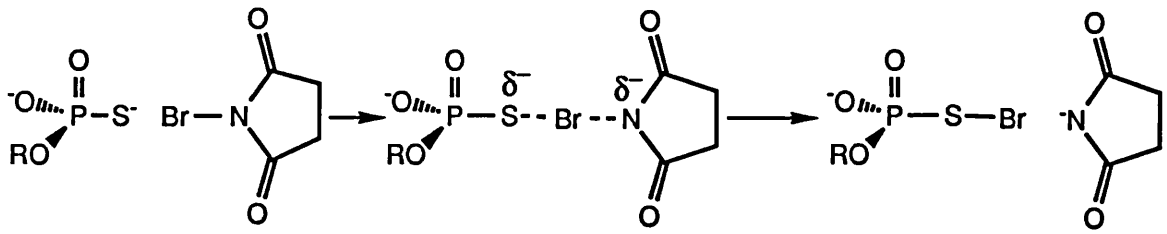
Under the conditions used, the formation of the halosulphonium salt must have been the rate determining step. Iodine is more able to form molecular donor-acceptor complexes than bromine[224] and as a consequence iodine does not tend to form the iodosulphonium salt as readily as bromine tends to form the bromosulphonium salt.

Desulphurisation of phenylethane 1,2-bisphosphorothioate (145) by iodine in pyridine probably proceeded via a similar activation mechanism to that reported by Krawiecka and Michaliski, namely, the formation of a charge transfer molecular complex followed by the formation of the iodosulphonium salt. Once activated in this manner, the reaction followed a combination of mechanisms (1) to (3), as described later. However, the presence of the disulphide implies that another mechanism also featured in the reaction. It is likely that the formation of the disulphide followed the mechanism detailed in [Scheme 55].



Scheme 55

Obviously the mechanism could go by an ionic or a radical mediated pathway to produce either the cyclic disulphide monomer or dimer resulting from an intra- or inter-molecular reaction. Similarly, the reaction with CNBr starts with the formation of a charge transfer molecular complex, the cyanide then cyanylates the sulphur. Nucleophilic attack on the thiocyanate activated phosphorus by a hydroxyl group or water ensues to give the pyrophosphate and phosphate respectively. Again the formation of the disulphide may be due to a similar reaction as in [Scheme 55]. It is probable that our NBS-mediated desulphurisation of 1-phenyl ethane 1,2-bisphosphorothioate proceeds initially via a charge transfer complex (175), the sulphur then attacks the NBS resulting in the formation of phosphorothiobromide (176) and succinimide.



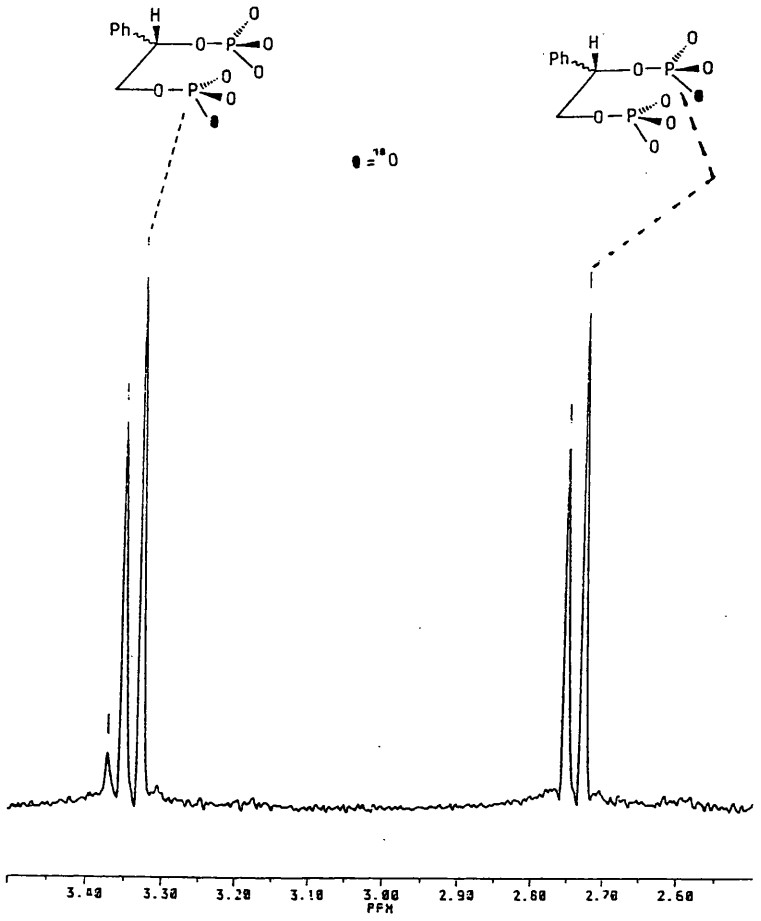
(175) (176)

Scheme 56

The activated phosphorothioate then reacted by a combination of pathways as detailed below [mechanisms 1,2 and 3].

5.7 Isotopic Labelling Experiment.

Isotope labelling experiments were undertaken in order to determine the mechanism of the NBS mediated desulphurisation reaction. The desulphurisations were conducted in dioxan, with NBS, phenylethane 1,2-bisphosphorothioate, and a 1:1 mixture of oxygen 18 water ($H_2{}^{18}O$), and oxygen 16 water. It is known that for a given molecule the substitution of a certain isotope for another influences the molecular properties, resulting in a change in the Larmor frequency υ of that molecule which is reflected in its NMR spectrum, since, $\upsilon=(\gamma B_0)/2\pi$ where γ is the magnetogyric ratio and B_0 is the applied magnetic field. This change of Larmor frequency has been observed in the central nuclei of oxyanions upon oxygen isotope substitution[225]. A relationship has been proposed[226] that describes the observed oxygen induced isotope effects in oxyanions. The theory predicts a lower Larmor frequency , if in the molecule with the heavier isotope the vibrational energies are smaller than in the molecule with the lighter one. Substitution of a heavier oxygen isotope has the effect of shifting the Larmor frequency of the central nuclei of the oxyanion to a lower frequency. The bond characteristics of that heavier isotope will also determine the value of υ, as the vibrational energies of P-O-P are less than that of P=O, so the value of υ will be less for P-O-P than for P=O. The value for the chemical shift δ of a nucleus is proportional to the Larmor frequency, $\delta=(\upsilon-\upsilon_o)$ ppm, the result of substituting ^{18}O for ^{16}O would be a shift to a higher field. So in summary, the bonding environment of phosphorus is characterised by its NMR signal, $P=^{18}O$ and $P-^{18}O-P$ would be shifted upfield from the ^{16}O equivalents, with $P=^{18}O$ being shifted further upfield as it has a lower vibrational energy.



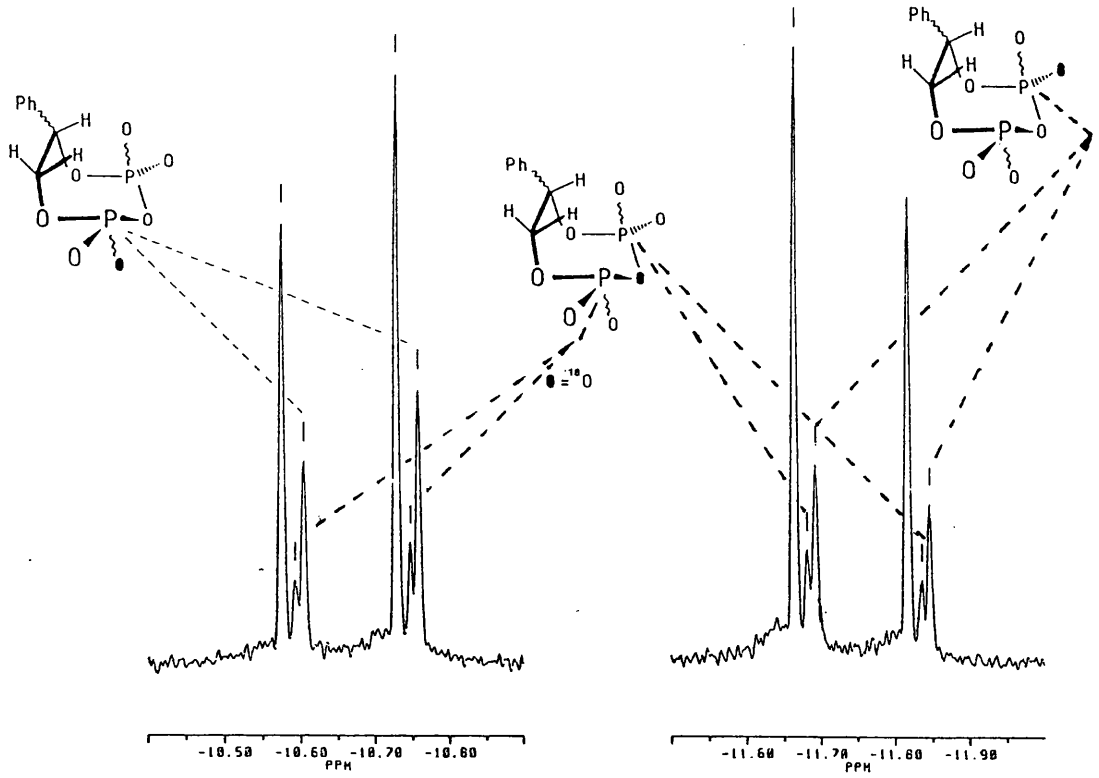
D_2O pH 9, 121 MHz, *ca.* 25 μM, $H_2{}^{18}O$:H_2O, 1:1

Desulphurisation of (145) in the presence of labelled water to form the bisphosphate.

Figure 37

Assignment of the resonances was achieved by conducting a proton coupled experiment. This experiment revealed that each of the signals at δ 3.36 and δ 3.34 ppm coupled to give a triplet, confirming that the phosphate was at position-2. The two signals at δ 2.76 and δ 2.74 ppm proton coupled to give doublets, confirming their position as 1.

Dealing with the formation of the bisphosphate first, both of the phosphate groups contained ^{18}O, the labelling occurred in the ratio of 1:2 labelled to unlabelled, the ^{18}O isotope effected a change in the chemical shift of the phosphorus, the signal was shifted upfield by 0.0235 ppm.



D_2O pH 9, 121 MHz, *ca.* 25 μM, $H_2{}^{18}O$:H_2O, 1:1

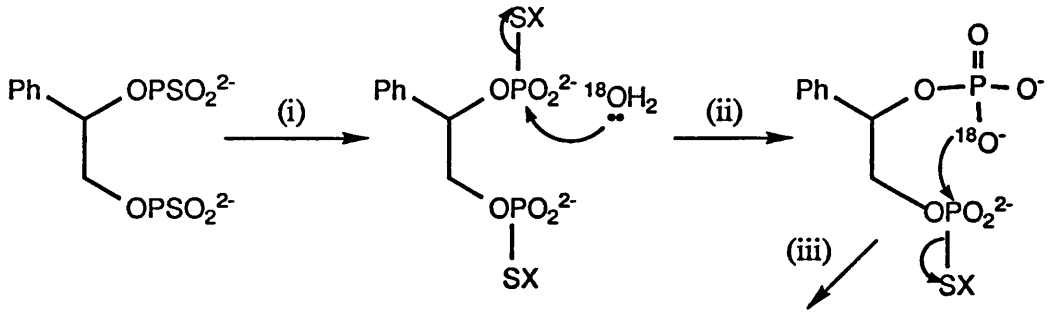
Desulphurisation of (145) in the presence of labelled water to form the pyrophosphate.

Figure 38

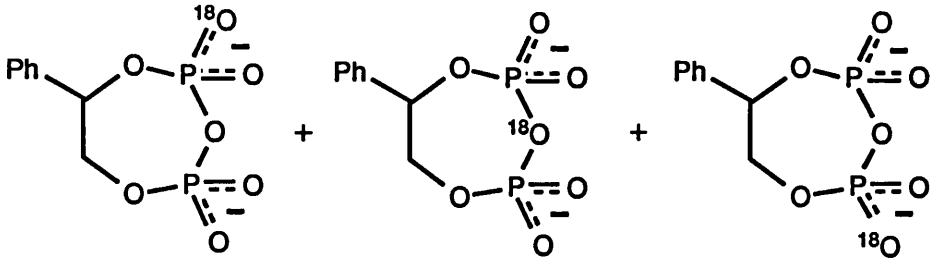
Three mechanisms have been proposed to try to explain the isotopic distribution incurred during the formation of the cyclic pyrophosphate.

Mechanism 1.

These schemes, [57 and 58] involves the attack of the labelled water upon either of the two phosphorothioates.



Scheme 57



Ratio 1:1:1

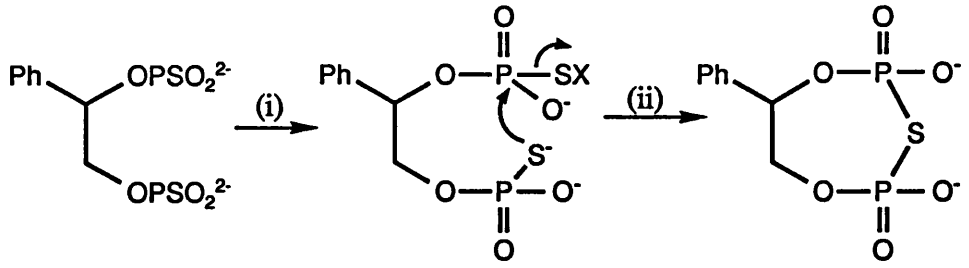
Scheme 58

(i) initial activation of the sulphur of the two phosphorothioate groups on addition of NBS in $H_2{}^{18}O$, subsequent thermal equilibration involving torsional rotation around the P-OCH bond.
(ii) nucleophilic attack (S_N2) of $H_2{}^{18}O$ upon one of the activated, and hence more electrophilic phosphorus, with release of XS^-. [Attack shown at position-1, the mechanism is the same reaction if initial attack occurs at position-2].
(iii) subsequent nucleophilic attack (S_N2) on the activated phosphorus by either the ^{18}O or one of the other two oxygens from the other phosphate.

The reaction resulting in the formation of the pyrophosphate and bisphosphate, with the ^{18}O equally distributed between the bridgehead position and either of the two phosphates of the pyrophosphate. In the interests of simplicity, the subsequent schemes will illustrate only the initial activation of the sulphur of the phosphorothioate at position-1, obviously the reverse sequence of activation may play a considerable part in the mechanism. However, there maybe slight differences between the two phosphorothioates resulting in some preferential activation

Mechanism 2.

The second mechanism involves the formation of the cyclic sulphide compound (156) possessing a thiopyrophosphate moiety.
(i) treatment with NBS results in the activation of either one of the two phosphorothioate sulphurs.
(ii) subsequent attack by sulphur on the activated phosphorus in an S_N2 manner, forms the cyclic sulphide (156).

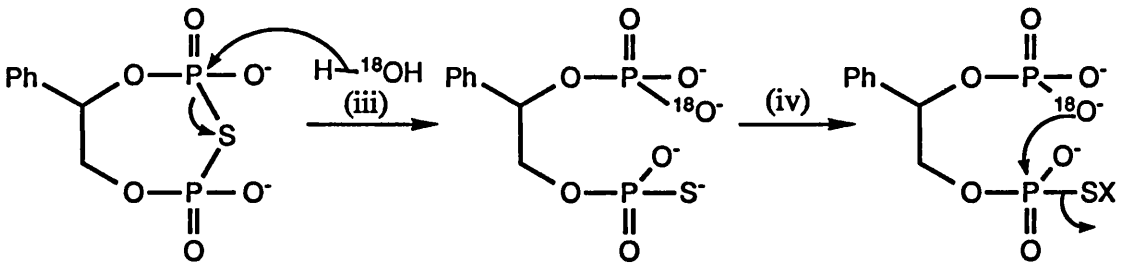


(145) (156)

Scheme 59

(iii) S_N2 attack by labelled water on either of the two phosphates results in a compound with a phosphorothioate at one position and a labelled phosphate at the other. Torsional rotation around the COP bonds then leads to thermal equilibrium.

(iv) subsequent nucleophilic attack on phosphorus by either the labelled oxygen or one of the two unlabelled oxygens results in the formation of pyrophosphate. Again with the label equally distributed between the two phosphates and the pyrophosphate bridge. Attack with additional water will lead to the generation of the bisphosphate.

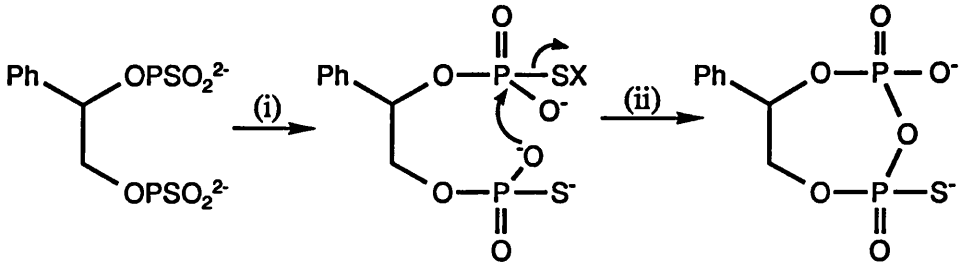


Scheme 60

Mechanism 3.

(i) the initial step of this mechanism as with all three, involves activation of either of the phosphorothioate sulphurs with NBS.

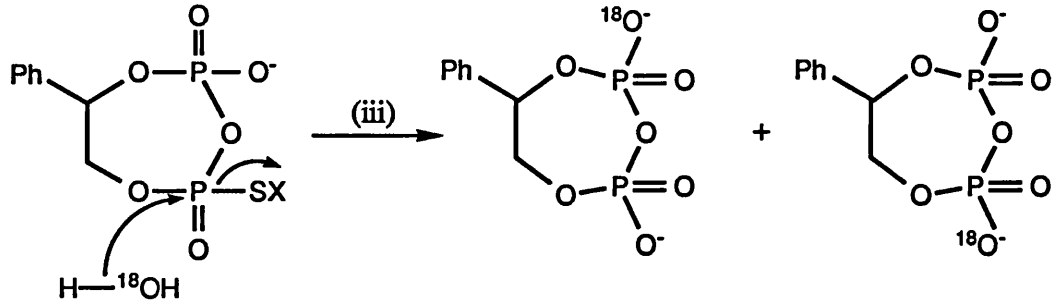
(ii) S_N2 attack upon the phosphate by oxygen in preference to S_N2 attack upon the phosphate by sulphur to form the thiopyrophosphate (177).



Scheme 61 (177)

(iii) the thiopyrophosphate (177) thus produced is then activated by more NBS and then

hydrolysed to the pyrophosphate by nucleophilic attack of the labelled water.
Two products are formed in this manner, one with the label at P_1 (178) the other with label at P_2 (179), no label is found in the pyrophosphate bridge position.



(178) (179)

Scheme 62

Several labelling experiments were conducted, all having the same result [see Fig. 38]. The interpretation of which proved to be complex, since ^{18}O was found in the molecule in three possible positions. Considering the phosphorus at position-1, three signals are evident. The unlabelled pyrophosphate, a signal δ 0.02 ppm upfield and one δ 0.03 ppm upfield, the latter two in a ratio of 1:2. As discussed earlier it can be assumed that the first resonance is due to a P-^{18}O-P bond, with the bond order *ca.* 1 implying that the label is in the pyrophosphate bridge position[225,226]. The further upfield signal which is due to a P=^{18}O bond, with the bond order *ca.* 1.5 implying that one of the two free oxygens is labelled. Similarly, the phosphorus at position-2 has a signal δ 0.02 ppm upfield due to the presence of a pyrophosphate with a P-^{18}O-P bond in the pyrophosphate bridge and a signal δ 0.03 ppm upfield with one of the two free oxygens labelled. Again, the ratio between the two signals was 1:2 respectively. The other point to note was that there was a slight selectivity in the disposition of the label between the two phosphorus atoms in the ratio of 1:1.1 for position-1 relative to position-2 [Fig. 38].

It is possible therefore to conclude that the reaction does not proceed exclusively via one mechanism but rather via a combination of the mechanisms. However, it is possible to determine that mechanism 3 is not the exclusive mechanism as the pyrophosphate bridge position is labelled, a result that is not possible if mechanism 3 is followed exclusively.

5.8 Ionic Chelation Experiments.

To try to reduce the rotational freedom of the two phosphorothioate groups it was decided to try the NBS-mediated desulphurisation in the presence of various bidentate metal ions. Initially a soft Lewis acid was chosen in order to chelate the sulphur atoms of the

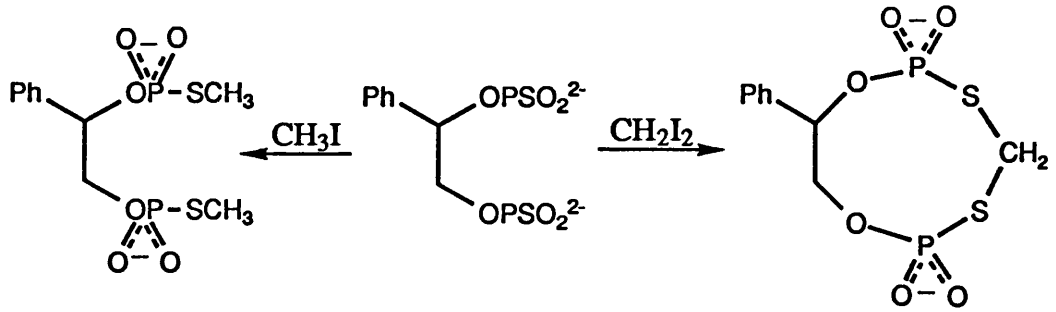
phosphorothioates, holding them closer together to reduce the rotation and thus increase the yield of the pyrophosphate (149). Desulphurisation of 1-phenyl ethane-1,2-bisphosphorothioate (145) was conducted in a solution of manganese (II) chloride. A small amount of the pyrophosphate (149) was formed, however, this was significantly less than in the absence of the metal ions. The manganese may have acted by intermolecularly chelating phosphorothioates from different molecules leading to mainly the bisphosphate. Or alternatively, the intramolecular chelation of the two phosphorothioates by the metal ion may have reduced the availability of the phosphorus to the other phosphoryl oxygen leading to an increased formation of the bisphosphate. Desulphurisation was conducted in the presence of a hard Lewis acid[227], the rationale being that the metal ion would chelate an oxygen from both of the phosphorothioate groups and thus enable the expedient desulphurisation by the NBS to the pyrophosphate. However, the effect was to reduce the amount of the pyrophosphate produced to almost zero. The implications of the chelation experiments are that the two phosphorothioate groups are held by the metal ions in an unfavorable alignment, the phosphoryl oxygen atom is less able to attack the neighbouring phosphorus. It seems to be irrelevant whether the ion chelates the sulphur or the oxygen as both seem to reduce the pyrophosphate formation.

5.9 Synthesis of Miscellaneous S-Alkylated Phosphorothioates

S-Methyl phosphorothiolates differ in two major respects from the parent phosphorothioate, namely:
(i) the alkylated sulphur increases the electrophilicity of the phosphorus attached to it, thus making the phosphorus more susceptible to nucleophilic attack.
(ii) phosphorothiolates have one less charge than the corresponding phosphorothioate.
(iii) the phosphorothiolate moiety is larger than the phosphorothioate.
We decided to consider the effect of S-alkylation upon the NBS-mediated desulphurisation. To this end, two bisphosphorothiolate compounds were synthesised, 1-phenyl ethane 1,2-bis [S-methyl-phosphorothiolate] (180) and 1-phenyl ethane-1,2-[cyclic-S,S-methylene] bisphosphorothiolate (181). Compound (180) was chosen to see if the increased electrophilicity of the phosphorus and the reduced charge of the molecule would favour the pyrophosphate synthesis over straight desulphurisation. The second compound (181) was thought may act as an analogue of a metal ion chelated between the two phosphorothioates of (145). It was also thought that the methylene ring may hold the two phosphorus atoms in a favourable orientation for pyrophosphate formation.

Alkylation of sulphur has been employed in the development of probes for enzymic nucleotide binding sites[228]. Specifically, the S-methyl phosphorothiolate analogue, both diastereoisomers of adenosine 5'-*O*-(*S*-methyl 1-thiotriphosphate) (ATPαSMe) have been prepared from the parent phosphorothioate (ATPαS) and used to study the metal-ATPαS and metal-ATPαSMe binding in various kinase enzymes[228].

Methylation of (145) was easily accomplished by the treatment of a methanolic solution of (145) with methyl iodide to give 1-phenyl ethane 1,2-bis [S-methyl-phosphorothiolate] (180) in quantitative yield. Alkylation of a solution of (145) in acetonitrile and triethylamine with diiodomethane gave the 1-phenyl ethane 1,2-[cyclic-S,S-methylene] bisphosphorothiolate (181) in 83 % yield.



(180) (145) (181)

Scheme 63

It was possible to confirm the structure of (180) by conducting several irradiation experiments.

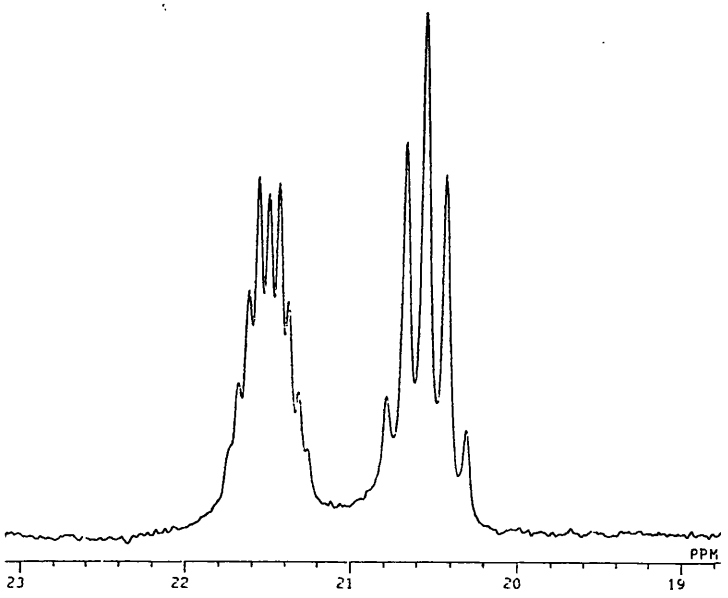
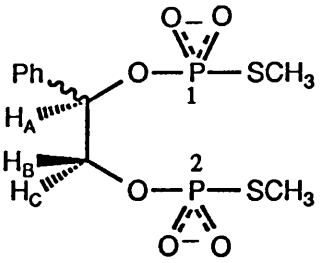


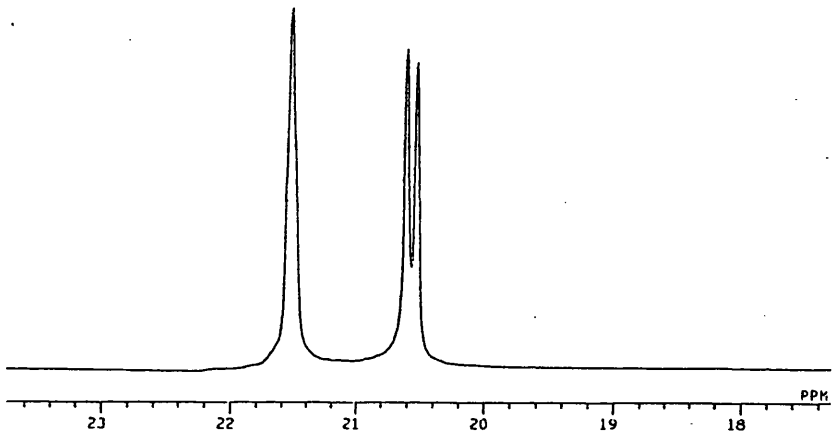
Figure 39. 109 MHz ^{31}P-^{1}H NMR of (180) (D_2O, pH 9, *ca.* 100 μM)



(180)

Figure 40

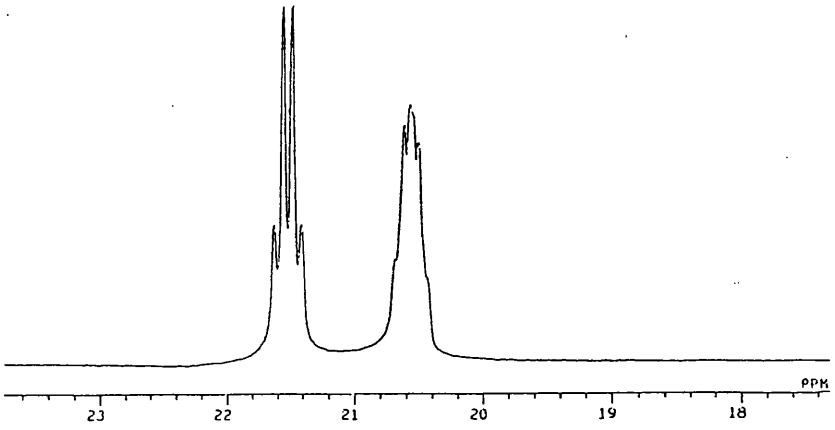
The phosphorus at position-1 couples to 4 protons to give a doublet of quartets. Coupling to the phosphorus at position-2 results in a double doublet of quartets. Assignment of [Fig. 39] is possible by conducting an irradiation experiment, observing the proton coupled phosphorus spectrum whilst selectively irradiating the two S-methyl groups. The result of this is displayed [Fig. 41].



109 MHz ^{31}P-^{1}H NMR of (180) (D_2O, pH 9, *ca.* 100 μM) irradiating both S-methyl groups.

Figure 41

The signal at δ 20.26 ppm has collapsed to a doublet, the phosphorus is coupling to the proton H_A [Fig. 40] with a coupling constant of 9 Hz. The double doublet of quartets centred at δ 21.59 ppm has collapsed to an apparent singlet. The signal is broad, 14 Hz wide at its mid-point. For this reason, the triplet of phosphorus at position-2 is not resolved.

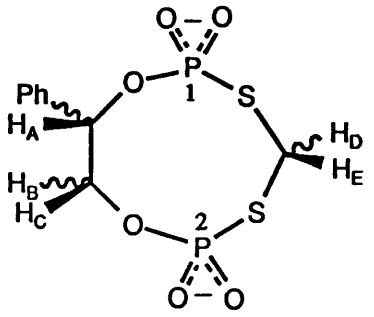


109 MHz ^{31}P-^{1}H NMR of (180) (D_2O, pH 9, *ca.* 100 μM) irradiating both H_B and H_C.

Figure 42

Irradiation of both H_B and H_C caused the signal at δ 21.59 ppm to collapse to give a quartet with a the phosphorus coupling to the methyl group, with a ^{31}P-^{1}H coupling constant of 9 Hz. The phosphorus at position-1 is unaffected by this irradiation and remains as a double quartet. It is possible therefore to confirm that the compound is 1-phenyl-1,2-bis [S-methyl-phosphorothiolate] (180).

In a similar manner the structure of 1-phenyl ethane 1,2-[cyclic-S,S-methylene] bisphosphorothiolate (181) was elucidated by selective proton irradiation in the proton coupled phosphorus NMR spectrum [Fig. 44].



(181)

Figure 43

Irradiation of H_A caused the signal at δ 18.65 ppm to collapse to a double doublet, whilst the signal at δ 19.40 ppm remained unaffected. We tried to irradiate H_D and H_E selectively, however, H_C was also irradiated. The resonance at δ 18.65 ppm was collapsed to a doublet, the other signal appeared as a broad singlet. The singlet was *c.a.* 4 Hz wide at its mid point, the implication was that the coupling to H_C was not obscured. Combining all of the spectroscopic data it was possible to determine that the compound

was as depicted [Fig. 43], and not an intermolecular polymer.

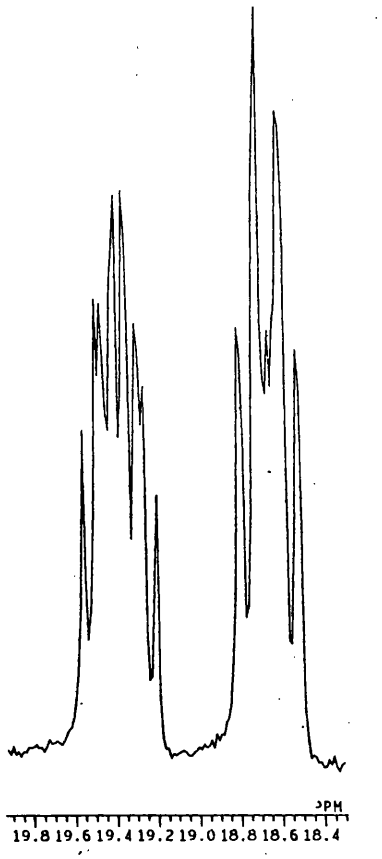
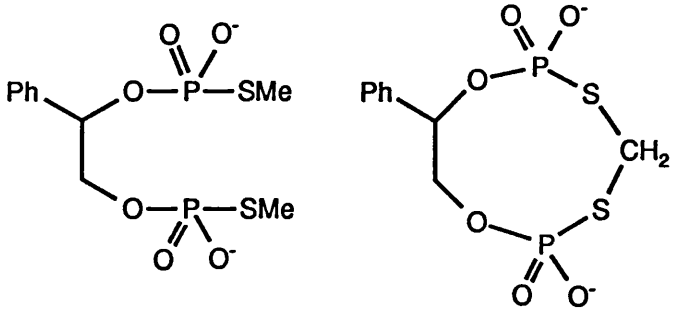


Figure 44. ^{31}P-^{1}H NMR of (181) (D_2O, pH 9, 109 MHz *c.a.* 40 μM)

5.10 Desulphurisation of Phosphorothiolates

Alkylation of the sulphur of a phosphorothioate increases the electrophilicity of the phosphorus atom. Desulphurisation reactions were conducted on two phosphorothiolate compounds, 1-phenyl ethane 1,2-bis(S-methyl phosphorothiolate) (180) and 1-phenyl ethane 1,2-(cyclic-S,S-methylene)bis-phosphorothiolate (181).



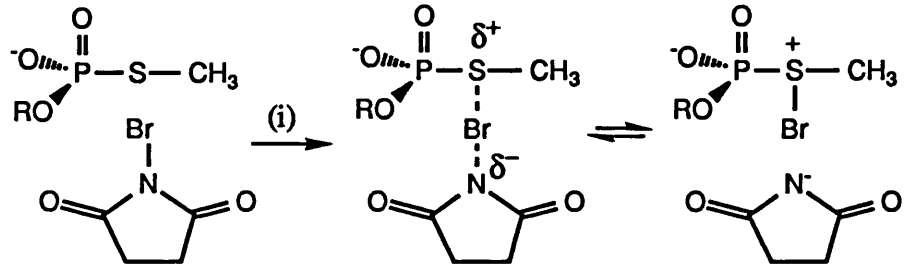
(180) (181)

Figure 45

Treatment of (180) with NBS resulted in the formation of two products as expected, the

bisphosphate (154) and the pyrophosphate (149). The yield of the pyrophosphate, however, was improved upon that of the desulphurisation of the corresponding bisphosphorothioate, from 38% to *ca.* 55%, a significant yield improvement. Again the reaction mechanism was studied by conducting the desulphurisation in equimolar oxygen-18, oxygen-16 water. However, it was not possible to achieve baseline resolution at the pyrophosphate AB system. So it was not possible to say if there was any evidence of label in the pyrophosphate bridge position.

NBS desulphurisation of (181) was less successful, the yield of the pyrophosphate was slightly less than that obtained from the phosphorothioate. It seems probable that the mechanism for the desulphurisation of (180) proceeds via (i) the formation of a charge transfer complex (182), which is in equilibrium with the bromosulphonium salt (183).



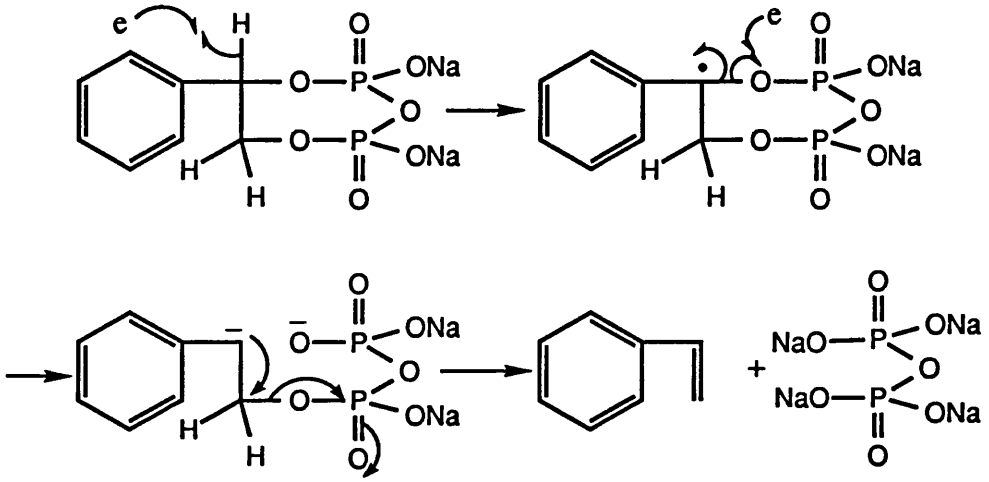
(182) (183)

Scheme 64

The increased electrophilicity of phosphorus due to the charge upon sulphur of the intermediate (183) reduces the lifetime of the activated intermediate. The transient intermediate has therefore a short existence, only long enough for nucleophiles that are close by to attack. The closest nucleophile would probably be the phosphoryl oxygen of the neighbouring phosphorus moiety, reaction of this would lead to pyrophosphate synthesis upon the loss of MeSBr. However, water does play a part in the attack upon the transient intermediate as there is evidence of straight desulphurisation to bisphosphate, albeit less bisphosphate than in the desulphurisation of the non alkylated bisphosphorothioate.

Considering the desulphurisation of (181) it seems probable that the methylene ring holds the two thiophosphate moieties in an unfavorable orientation. An orientation that hinders the attack by the neighbouring phosphoryl oxygen. One that favours attack by water to give the bisphosphate and not the pyrophosphate. In a similar manner to the chelation effect of the divalent metal ions in which they reduce the yield of pyrophosphate.

The stability of the cyclic seven-membered pyrophosphate ring to acid and base was investigated. 1-Phenyl ethane-1,2-cyclic pyrophosphate (149) was treated with 1M HCl for 2 days at room temperature and was found to be unaffected. However, elevation of the temperature to 100°C led to hydrolysis of the pyrophosphate to the bisphosphate within 5 min. The stability of 1-phenyl ethane-1,2-cyclic pyrophosphate (149) to base mirrored the stability to acid. 1M Sodium hydroxide at room temperature did not significantly hydrolyse the pyrophosphate after 5 days. Whereas the solution to 100°C hydrolysed the pyrophosphate within 2 hours. Treatment of 1-phenyl ethane-1,2-cyclic pyrophosphate (149) with sodium in liquid ammonia resulted in dephosphorylation to give pyrophosphate and styrene.



Scheme 65

The mechanism for this reaction starts with the abstraction of the benzylic proton to give a benzylic and a proton radical. Subsequent P-O bond fission gives the carbanion and the phosphoryl radical. The phosphoryl radical picks up another electron and then forms the sodium salt, the carbanion redistributes the charge and eliminates styrene to give the pyrophosphate. This represents a novel method of pyrophosphate synthesis, however, as it stands it is not of obvious synthetic use.

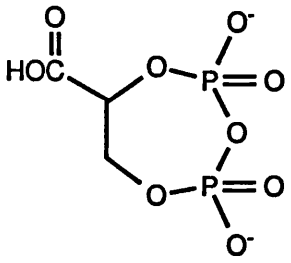
Cyclohexane-1,2-cyclic pyrophosphate (40) was also tested for stability of the pyrophosphate ring to acid, base and sodium in liquid ammonia, in the same way as (149) above. Hydrolysis in molar acid or base at room temperature did not occur within 3 days. Elevation of the temperature to 100°C effected cleavage of the pyrophosphate to give the bisphosphate (42) within 15 min and 5 hours for acidic and basic hydrolysis respectively. Cleavage of the pyrophosphate was also effected by treatment with sodium in liquid ammonia to give the bisphosphate (42).

Chapter Six

Bisphosphoglycerate Analogues

6 Cyclic 2,3-Bisphosphoglycerate

The novel metabolite cyclic 2,3-bisphosphoglycerate (cBPG) (15) has been isolated from several genera of archaebacteria[229]. It is of special interest as it contains a cyclic 7-membered pyrophosphate linkage. It was decided that this would be an interesting molecule to attempt to synthesise using our novel NBS-mediated desulphurisation of a vicinal bisphosphorothioate.



(15)

Figure 46

6.1 Biochemistry of cBPG

Methanogens such as *Methanobacterium thermoautotrophicum* are fastidious anaerobes whose metabolism centres around the reduction of carbon dioxide to methane. It was originally suggested that cBPG could act in a number of ways:

(i) as a store of phosphorus[229] (much like other polyphosphates in other bacteria);

(ii) as a store of energy[230], in a similar role to that of creatine phosphate in muscle, where the latter compound provides a reserve of high-energy phosphoryl groups and keeps the adenylate system of muscle buffered at high "energy charge"

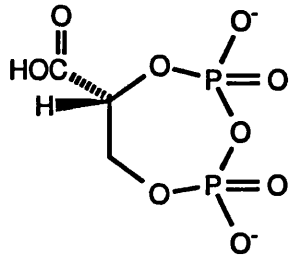
(iii) as a source of carbon[231];

(iv) or act as an allosteric effector of some critical enzyme. cBPG (15) may act in a similar manner to 2,3-bisphosphoglycerate BPG (14), BPG is present in human erythrocytes in the same molar concentration as haemoglobin. It binds to the haemoglobin and reduces the oxygen affinity twenty six fold, enabling the efficient exchange of carbon dioxide and oxygen in the capillary beds[232]. BPG has also been found to inhibit weakly the activity of D-*myo*-inositol 1,4,5-trisphosphate 5-phosphatase[187].

Subsequent work[233] has dismissed some of these hypotheses, cBPG does not seem to represent a storage compound for ATP or phosphate, the 2 moles of ATP consumed during the synthesis of cBPG cannot be recovered because the reverse reactions are not detected. The formation of the phosphate anhydride ATP from the phosphate ester BPG is thermodynamically unfavorable. Further studies of the function of cBPG and its degradation are required as the role of cBPG remains uncertain[233].

6.2 Analysis of cBPG

In vivo phosphorus NMR experiments have shown high concentrations cBPG[230] which has been purified and its configuration elucidated. The absolute configuration was determined by stereospecific enzymic digestion of cBPG to *sn*-glycerol 3-phosphate, the quantitative conversion of cBPG to *sn*-glycerol 3-phosphate established the structure of the methanogen pyrophosphate as the D (or *R*) enantiomer[234].

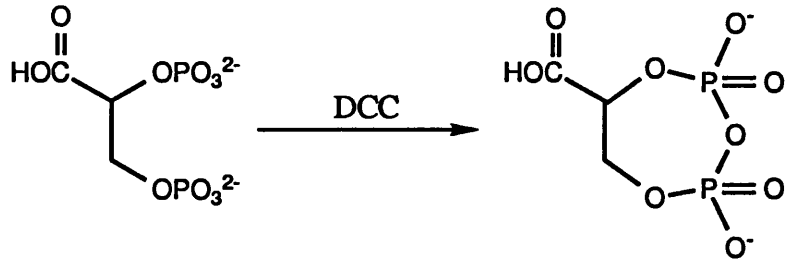


D (*R*)-cBPG

Figure 47

The isolation and subsequent chemical synthesis[235] of cBPG represents the first reported biological occurrence and synthesis of a pyrophosphate group in a seven-membered ring[230]. Molecular modeling of cBPG has shown that there are only two possible conformations[234], neither conformation minimizes the electrostatic repulsions between the four non-ring anionic oxygen atoms of the pyrophosphate group. Both conformations bring two non-bridging atoms close together, thereby generating significant electrostatic repulsion, implying that the pyrophosphate group is unable to assume the characteristic staggered pyrophosphate conformation.

The synthesis involved the condensation of 2,3-bisphosphoglycerate (14) with dicyclohexylcarbodiimide, however, the yield was poor and the product was neither purified nor isolated[230]. The tentative proof for its synthesis was provided by the enhancement of four peaks in the ^{31}P NMR on addition of a small amount of cBPG obtained from *Methanobacterium thermoautotrophicum.*



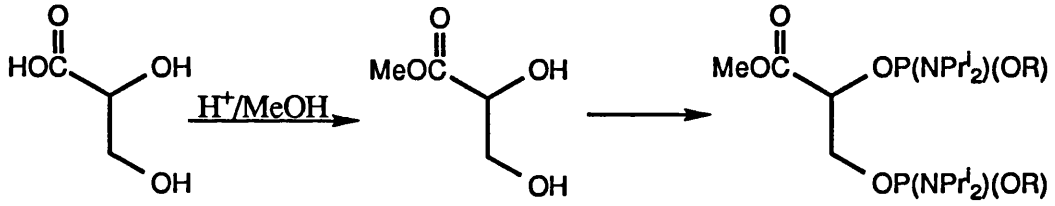
BPG cBPG

Scheme 66

Purification of small quantities of intramolecular pyrophosphate from the corresponding intermolecular pyrophosphates and bisphosphates is extremely difficult, so another route that would lead to significant amounts of cBPG has been sought.

6.3 Synthesis of 2,3-Bisthiophosphoglycerate

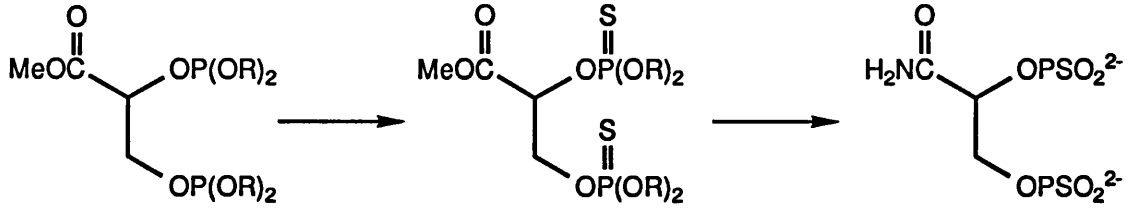
In order to protect the carboxylic acid we decided to esterify (±)-glyceric acid (184) with methanol to give the methyl ester (185). The methyl ester thus produced was then phosphytilated with chloro (diisopropylamino)-2-cyanoethoxy phosphine to give (±)-1-methyl 2,3-bis[di(2-cyanoethyl) (diisopropylamino) phosphoramidoglycerate] (186).



(184) (185) (186)

Scheme 67

The crude product (186) was then converted to the phosphite triester (187) by treatment with 2-cyanoethanol and tetrazole and subsequently oxidized with sulphur in pyridine to afford (±)-1-methyl 2,3-bis[di(2-cyanoethyl) thiophosphoglycerate] (188) in 45% yield from (±)-glyceric acid.



(187) (188) (189)

Scheme 68

Removal of the protecting groups presented a problem, initially the 2-cyanoethyl groups

and the ester were removed by basic hydrolysis, the former by a ß-elimination. Heating (188) in a solution of aqueous ammonia (35%) to 65°C for an hour cleaved the phosphorothioate protecting groups to give the bisphosphorothioate monoester, however, the ester was probably cleaved to give the amide (189) not the carboxylic acid. Deprotection of the 2-cyanoethyl groups was attempted using sodium methoxide (1M), conversion to the diester was simple enough, however, formation of the monoester was only accomplished after prolonged reaction time *ca.* one month.

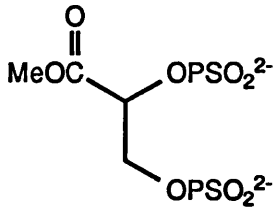
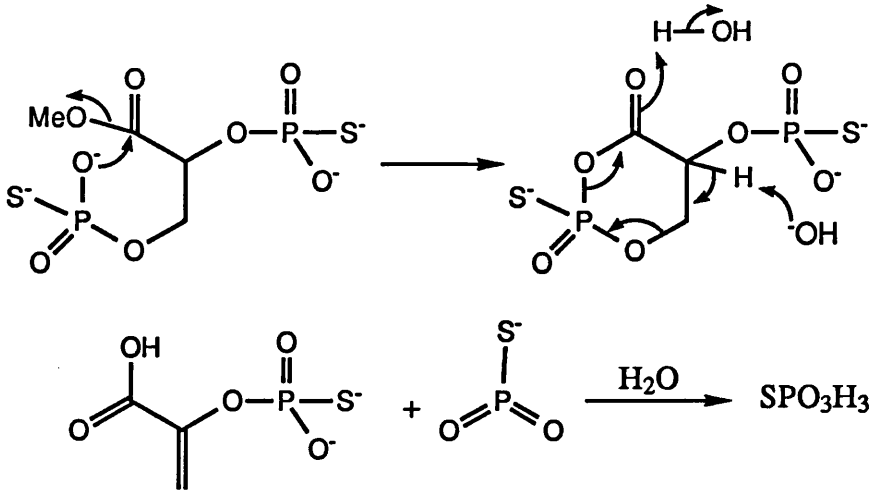


Figure 48

(190)

Selective deprotection of the bisphosphorothioate was eventually effected by treatment of a methanolic solution of (188) with sodium methoxide to give (190). After dilution with water the product decomposed upon standing at high pH (*ca.*11). The ^{31}P NMR signal of (190) δ 42.8 ppm reduced in intensity with the concomitant appearance of a signal at δ 33 ppm, the former gave an apparent triplet on proton coupling, whereas the latter did not apparently proton couple. The evidence that the signal at δ 33 ppm did not proton couple implies that thiophosphoric acid was produced in the reaction. This could have been the result of the phosphoryl oxygen nucleophilically attacking the ester to form a six-membered ring with concomitant releasing of methoxide.



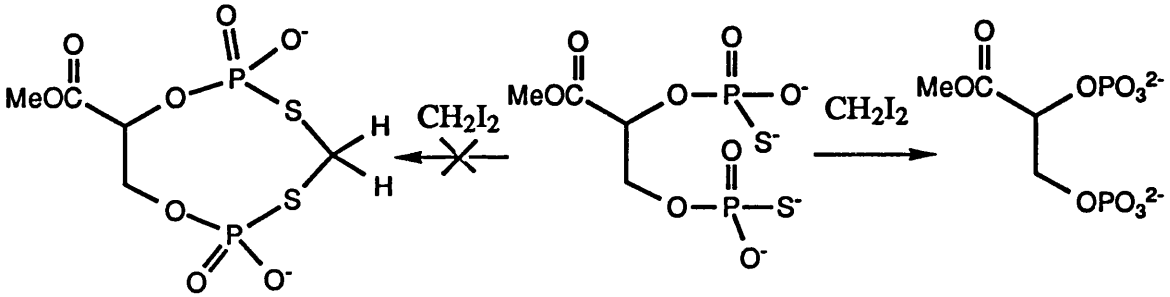
(191) (192)

Scheme 69

The mixed anhydride compound thus produced then decomposes to give thiophosphoenol pyruvate (191) and meta-thiophosphate (192), a transient species which is quickly trapped

by its solvent cage to give thiophosphoric acid. The decomposition was easily avoided by taking the reaction to neutral pH by treatment with H^+-Dowex resin as soon as the deprotection was complete to give (190) in 58% yield. However, attempts to purify further the impure (190) by anion-exchange chromatography resulted in the atmospheric oxidation to a mixture of the phosphate and phosphorothioate. Attempts were made to synthesise the title compound from (190) by trimethyl silyl iodide demethylation, unfortunately this reaction resulted in some oxidation of the phosphorothioates to the disulphide compound and the corresponding bisphosphate. The small amount of the desired (±)-2,3-bisthiophosphoglycerate (193) proved too difficult to purify.

An attempt was made to form the cyclic S,S-methylene derivative (194) by treatment of (192) with diiodomethane, however, the product was not formed. Iodine formed by the decomposition of excess trimethyl silyl iodide to iodine, oxidised (192) to the bisphosphate (195).

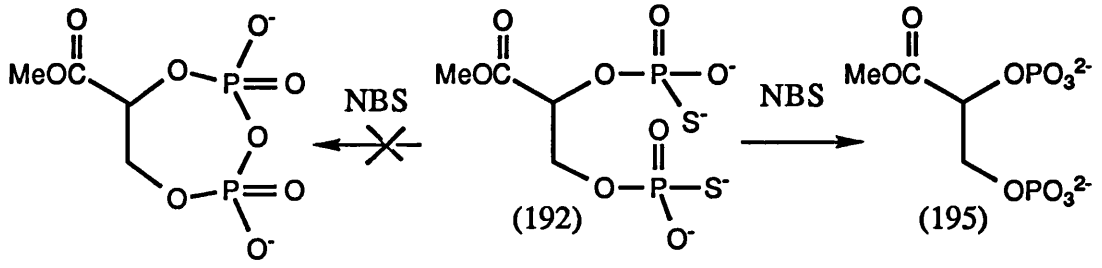


(194) (192) (195)

Scheme 70

6.4 Synthesis of Cyclic 2,3-Bisphosphoglycerate

Initial experiments to synthesise cBPG by the NBS-mediated desulphurisation of (189) were attempted. However, they did not result in the formation of the pyrophosphate, they resulted in the formation of a phosphate compound that resonated in the ^{31}P NMR at δ 3.1 ppm and coupled to protons as an apparent triplet ^3J 6.5 Hz, it was not identified. It was decided to try the desulphurisation upon the methyl ester of (±)-1-methyl 2,3-bisthiophosphoglycerate (192). Unfortunately all reaction conditions resulted in the exclusive formation of the 1-methyl-2,3-bisphosphoglycerate (195).



(192) (195)

Scheme 71

It seems probable that the molecule is too conformationally mobile for the intramolecular attack of a nucleophile upon the activated phosphorus to compete effectively with the attack of water thus yielding the bisphosphate.

As the target molecule cBPG could not be satisfactorily synthesised from the vicinal bisphosphorothioate via our NBS-mediated desulphurisation, we decided to abandon this project. However, 2,3-bisthiophosphoglycerate may have potential uses as a non-hydrolysable BPG analogue. For example, BPG is a haemoglobin co-factor which upon binding enables the co-operative binding of oxygen molecules. BPG is metabolised in this capacity and has to be added to stores of blood kept for transfusion. It is possible that 2,3-bisthiophosphoglycerate may find a use in extending the life span of stored blood.

Chapter Seven

Synthesis Of Inositol Pyrophosphates And Other Analogues

7 Introduction.

All of the analogues of Ins(1,4,5)P_3 synthesised to date have had a phosphate-like moiety at both the 4- and 5-positions. It was hoped that the compound with a pyrophosphate group linking these two positions would prove to be similar enough for the compound to be recognized by the Ins(1,4,5)P_3 receptor and bound. It was also hoped that it would be sufficiently different to Ins(1,4,5)P_3 such that receptor does not release Ca^{2+} ions. Another point about the pyrophosphate is that it may be metabolised in whole cells by cytosolic pyrophosphatases to give Ins(1,4,5)P_3. The Ins(1,4,5)P_3 thus produced would release Ca^{2+} after an initial lag phase that would be dependent upon the promiscuity of the cellular pyrophosphatases.

The linking together of the two phosphate moieties of the phosphatidylinositol cycle metabolite, Ins(1,4,5)P_3 (4) to form the cyclic pyrophosphate analogue, Ins(1,4:5 Pyro)P_3 (5) results in several physical differences, namely;

(i) the reduction in the number of formal negative charges on the molecule, from 6 to 4.

(ii) the difference in the pK_a values of Ins(1,4,5)P_3 and Ins(1,4:5 Pyro)P_3 resulting in different charges upon the two molecule at a given pH.

(iii) the reduction of number of degrees of freedom of the phosphate moieties at the 4 and 5 positions by constraining them in a 7-membered ring.

(iv) perturbations to the conformation of the *myo*-inositol ring caused by the formation of the *trans*-fused pyrophosphate ring.

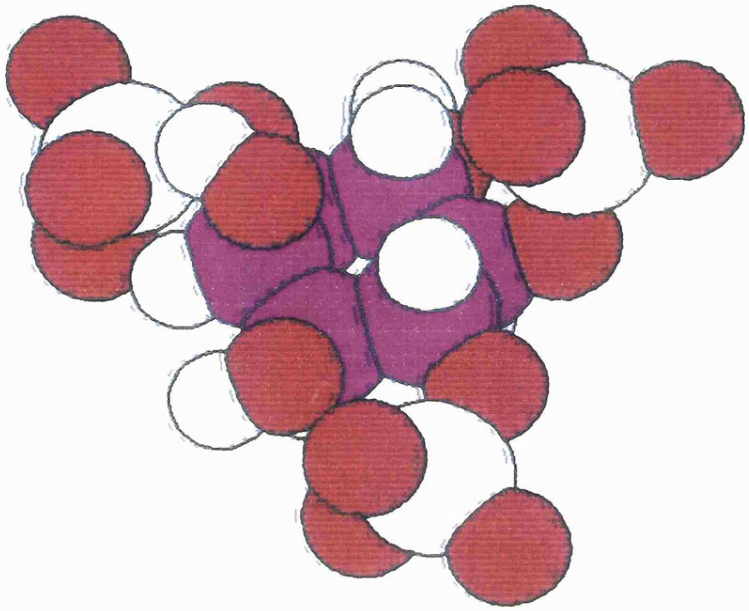


Figure 49. Minimised conformer of D-Ins(1,4,5)P_3

It is possible to see some of the differences in conformation between Ins(1,4,5)P_3 (4) and Ins(1,4:5 Pyro)P_3 (5) by careful consideration of [Figures 49 and 50].

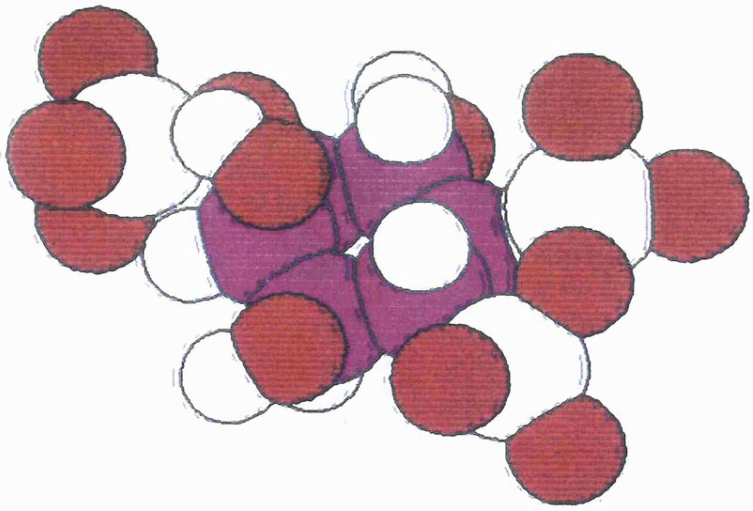


Figure 50. Minimised conformer of D-Ins(1,4:5 pyro)P_3.

The inositol ring of the pyrophosphate has adopted a slightly different shape to that of the trisphosphate. The pyrophosphate group is more compact than the bisphosphate of the Ins(1,4,5)P_3. This latter fact may play an important role in the compound's ability to bind to the Ins(1,4,5)P_3 receptor and Ins(1,4,5)P_3 metabolising enzymes.

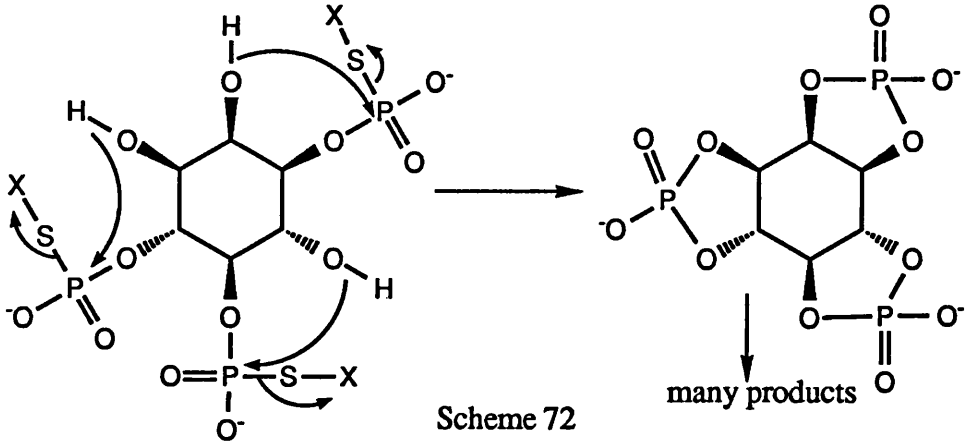
It was hoped that the pyrophosphate analogue (5) would bind to the Ins(1,4,5)P_3

receptor. Ideally, we thought that the act of binding of the pyrophosphate (5) to the receptor would not elicit Ca^{2+} release. The pyrophosphate (5) would be acting as an antagonist of the receptor. It was thought that the pyrophosphate (5) would be a close enough analogue of Ins(1,4,5)P_3 for it to bind to the Ins(1,4,5)P_3 receptor. However, the reduction in the charge on the molecule and the rigidity of the pyrophosphate ring, would be sufficiently different such that the pyrophosphate (5) would not release Ca^{2+}. The other interesting aspect of this molecule, is in the potential of cellular pyrophosphatases to cleave the pyrophosphate to give Ins(1,4,5)P_3. So Ins(1,4:5 pyro)P_3 could be introduced into cells without activating Ca^{2+} release. Subsequent action of cellular pyrophosphatases could then produce Ins(1,4,5)P_3 which would then release Ca^{2+}. So the introduction of the pyrophosphate would cause Ca^{2+} to be released after a delay, a lag-phase.

Since it has been shown that it is possible to synthesise cyclic 7-membered pyrophosphate ring systems from the corresponding vicinal bisphosphorothioate [chapter 5] by NBS-mediated oxidative desulphurisation[219], the successful synthesis of inositol pyrophosphates was attempted using this novel reaction.

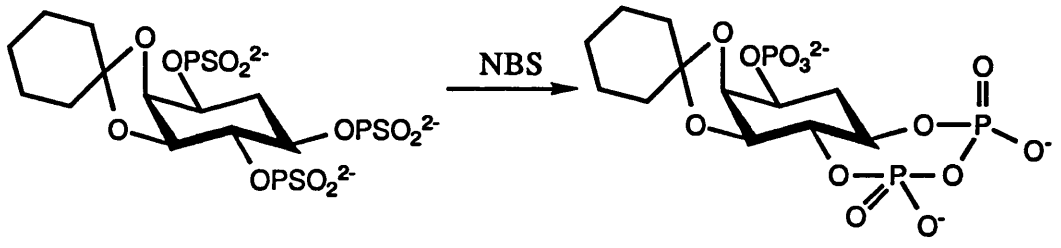
7.1 NBS-Mediated Desulphurisation of 6-Deoxy-2,3-Cyclohexylidene-D-*myo*-Inositol-1,4,5-Trisphosphorothioate (196)

Oxidative desulphurisation of inositol 1,4,5-trisphosphorothioate would result in the formation of up to three cyclic 5-membered phosphates. The unstable 5-membered cyclic phosphate diesters would rapidly hydrolyse to give all manner of Ins P_3 molecules, the phosphate groups migrating to give many combinations of phosphate monoesters. The chance of forming the desired 4,5-cyclic pyrophosphate would probably be remote and possible separation by ion-exchange chromatography would be arduous.



Scheme 72

We decided initially to attempt the desulphurisation of model inositol phosphorothioate compound. A compound that has been modified at the 3- and 6-positions. These modifications should prevent any chance of cyclic 5-membered phosphate formation and consequent side reactions. To this end we attempted the desulphurisation 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol 1,4,5-trisphosphorothioate (196). The phosphorothioate at position-1 would be unable to form a cyclic phosphate due to the presence of the ketal at position-2, and because of the absence of a hydroxyl group at position-6, desulphurisation would hopefully result in the formation of the phosphate at position-1. Similarly, the phosphorothioate at position-4 would not be able to react at position-3 due to the presence of the ketal, the phosphorothioate at position-5 would not be able to react with position-6 since the hydroxyl group is absent. The 4- and 5-positions would have no option but to react together to form the cyclic pyrophosphate (197) or desulphurise to the give the trisphosphate (198).

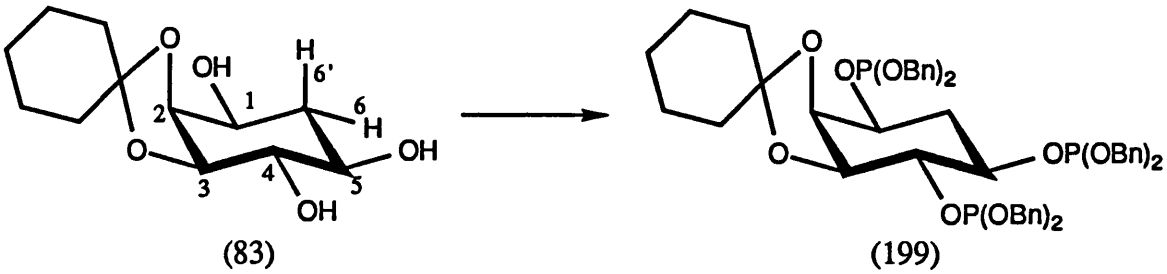


(196) (197)

Scheme 73

7.1.1 Synthesis of 6-Deoxy-2,3-Cyclohexylidene-D-myo-Inositol-1,4,5-Trisphosphorothioate (196)

2,3-*O*-Cyclohexylidene-6-deoxy-D-*myo*-inositol (83), a kind gift from Didier Dubreuil [synthesis as described in chapter 3], was phosphitylated with (diisopropylamino) dibenzyl phosphoramidite (82) to give 2,3-*O*-cyclohexylidene-6-deoxy-D-*myo*-inositol - 1,4,5-tris[(di-*O*-benzyl) phosphite] (199).

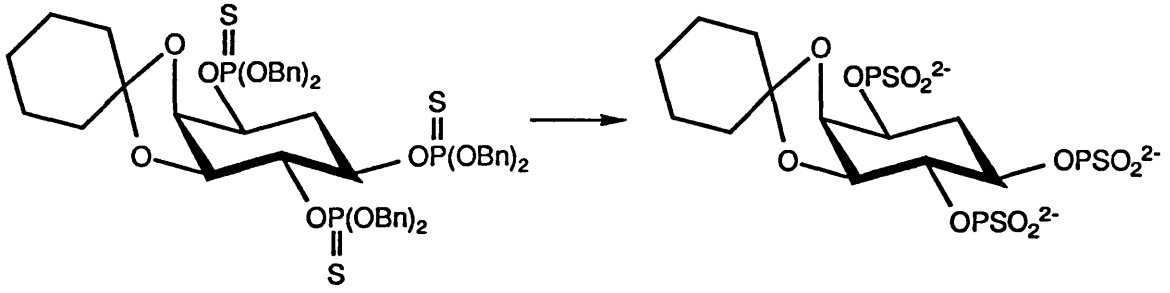


(83) (199)

Scheme 74

The trisphosphite (199) was oxidised with sulphur in pyridine to give the trisphosphorothioate (200) in 70% yield after recrystallisation. The 2,3-*O*-cyclohexylidene-6-deoxy-D-*myo*-inositol -1,4,5-tris[(di-*O*-benzyl) phosphorothioate]

(200) thus produced was then deprotected by treatment with sodium in liquid ammonia to give the 2,3-*O*-cyclohexylidene-6-deoxy-D-*myo*-inositol -1,4,5-trisphosphorothioate (196) in 60% yield.

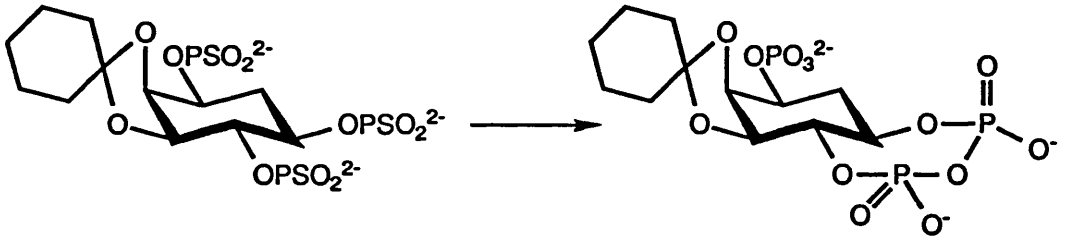


(200) (196)

Scheme 75

7.1.2 Desulphurisation of (196)

The phosphorothioate at position-1 did desulphurise to the phosphate as was expected and intramolecular coupling of the bisphosphorothioate formed the cyclic pyrophosphate; NBS treatment of (196) gave 2,3-*O*-cyclohexylidene-6-deoxy-D-*myo*-inositol-1-phosphate-4,5-cyclic pyrophosphate (197) almost exclusively by ^{31}P NMR.



(196) (197)

Scheme 76

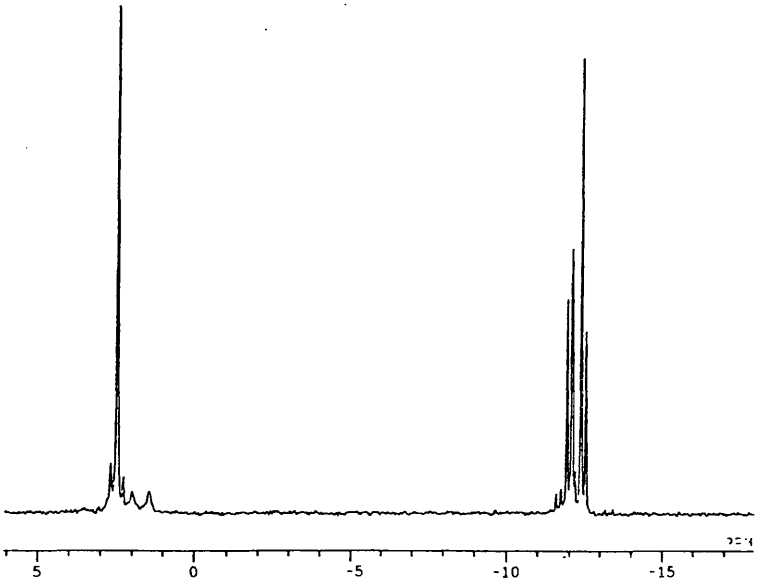


Figure 51. ^{31}P NMR of crude (197) (D_2O, pH 8, 109 MHz)

Subsequent purification by anion-exchange chromatography gave the pyrophosphate (197) as a pure product [Fig. 52]

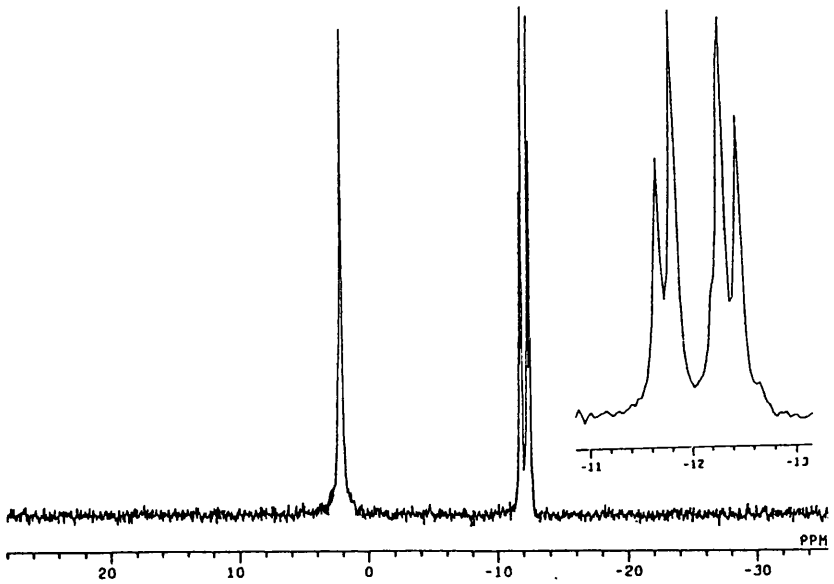
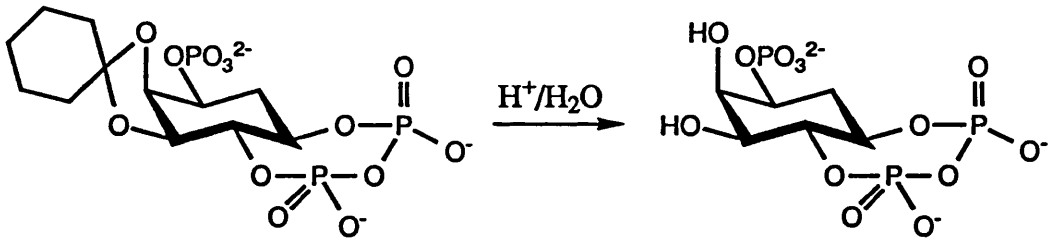


Figure 52. ^{31}P NMR of pure (197) (D_2O, pH 8, 109 MHz)



(197) (9)

Scheme 77. Removal of the ketal.

Removal of the ketal proved to be difficult. Removal using the same method as used in the synthesis of 6-deoxy-D-*myo*-inositol 1,4,5-trisphosphorothioate (8) ***vide infra i.e.*** stirring with H^+-Amberlite anion-exchange resin for several hours resulted in cleavage of the pyrophosphate bond. Stirring a solution of (197) pH 3 at room temperature for 24 hours, however, was sufficient to remove the ketal without effecting the pyrophosphate linkage, thus yielding (9). During the reaction a signal upfield of the phosphate and another AB system downfield of the pyrophosphate signal appeared as the signals of the ketal protected pyrophosphate disappeared. After 24h all of the ketal had been removed as is evident [Fig. 53].

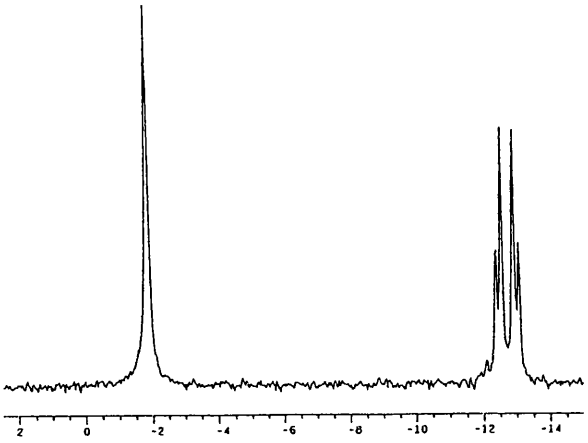
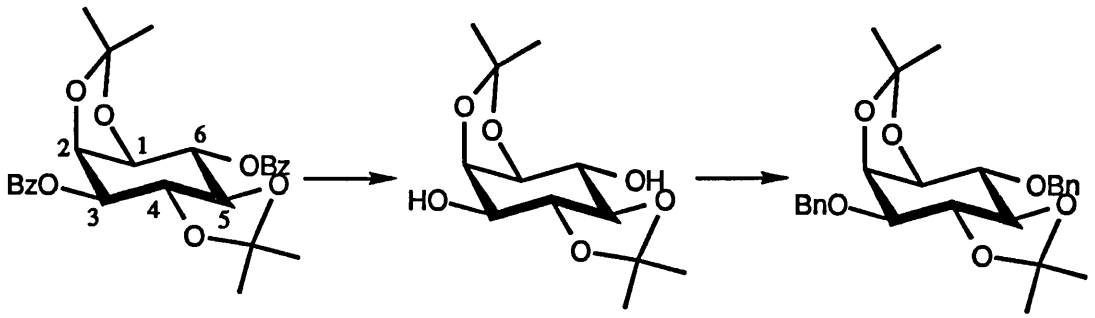


Figure 53. ^{31}P NMR of pure (9) (D_2O, pH 8, 109 MHz)

7.2 Synthesis of DL-*myo*-Inositol-1-Phosphate-4,5-Bisphosphorothioate (6)

DL-*myo*-Inositol-1-phosphate-4,5-bisphosphorothioate [Ins(1,4,5)P_3-4,5S_2] (6) is an important synthetic target compound. This novel inositol phosphorothioate is of biological importance as an Ins(1,4,5)P_3 analogue, since it may act as an Ins(1,4,5)P_3-5-phosphatase inhibitor. We also envisage that it is the key compound in the synthesis of DL-*myo*-inositol-1-phosphate-4,5-pyrophosphate (5), and several other biologically interesting molecules. We report here the successful synthesis of Ins(1,4,5)P_3-4,5S_2 using a mixed P(V) and P(III) approach, the initial synthetic steps based upon literature precedents [as described in section 3.10]. However, the authors only report melting points and microanalysis of these compounds. We thought that the lack of spectroscopic data was an important omission. We therefore decided that it would be useful to report here the proton and carbon NMR data of these compounds.

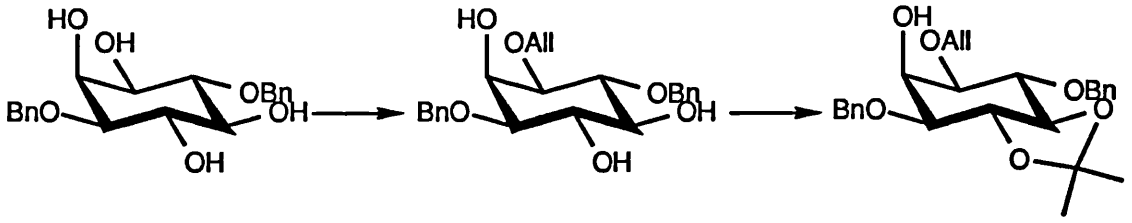
myo-Inositol was treated with 2,2-dimethoxypropane under acidic catalysis to give a mixture of bisketals[138]. Benzoylation of the mixture with benzoyl chloride enabled the separation of the desired highly insoluble 1,2:3,4-di-*O*-isopropylidene-3,6-dibenzoyl-*myo*-inositol (106) in 27% yield, from the other two methanol soluble regioisomers[138].



(106) (107) (108)

Scheme 78

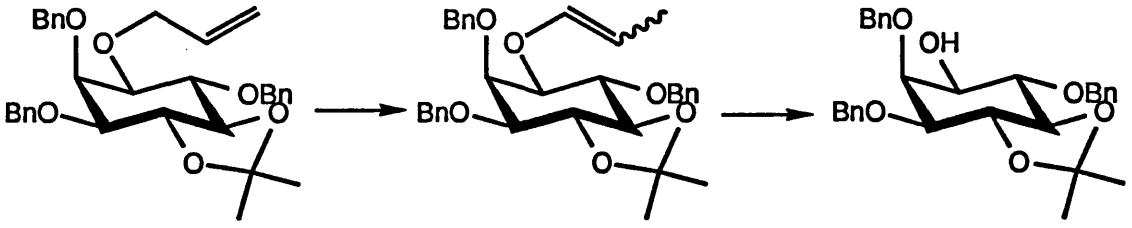
Basic hydrolysis gave the diol (107), the dialkoxide of which was then benzylated with benzyl chloride to give 1,2:3,4-di-*O*-isopropylidene-3,6-di-*O*-benzyl-*myo*-inositol (108) in 67% yield from (106)[138]. The dibenzyl ether upon mild acidic hydrolysis gave, in 81% yield, the tetrol 1,4-di-*O*-benzyl-*myo*-inositol (109)[138], which formed a tin complex upon reflux with dibutyl tin oxide in the presence of tetrabutylammonium iodide. Regiospecific allylation of the tin-complex at position-1 with allyl bromide gave 1-*O*-allyl-3,6-di-*O*-benzyl-*myo*-inositol (110) in 43% yield[236]. The vicinal diol was then protected by forming the ketal by treatment with 2,2-dimethoxypropane again under acidic catalysis to give 1-*O*-allyl-3,6-di-*O*-benzyl-4,5-isopropylidene-*myo*-inositol (111) in 65% yield[236].



(109) (110) (111)

Scheme 79

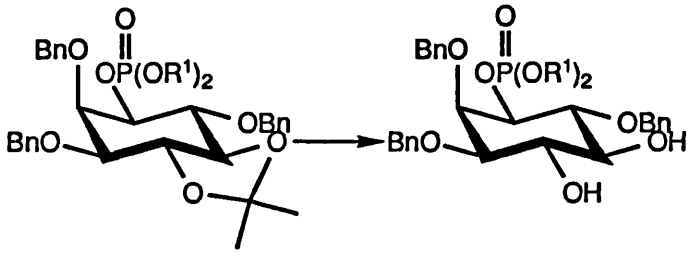
Benzylation of the 2-position was effected by treatment of the alkoxide of (111) with benzyl bromide to give 1-*O*-allyl-2,3,6-tri-*O*-benzyl-4,5-isopropylidene-*myo*-inositol (112) in 65% yield[237]. Isomerisation of the allyl group to the prop-1-enyl group was effected by treatment with tris(triphenylphosphine)rhodium(I)chloride [Wilkinson's catalyst][140]. This gave a mixture of the *cis*-prop-1-enyl and *trans*-prop-1-enyl ethers in ratio of 10:1 respectively, the overall yield of 1-*O*-prop-1-enyl-2,3,6-tri-*O*-benzyl-4,5-isopropylidene-*myo*-inositol (201) was 78%. Removal of the prop-1-enyl ether was accomplished by treatment with mercuric chloride and mercuric oxide[139] to form in 78% 2,3,6-tri-*O*-benzyl-4,5-isopropylidene-*myo*-inositol (202) as an oil that would not crystallise after a prolonged time, which is contrary to that reported in the literature[237,138].



(112) (201) (202)

Scheme 80

Phosphorylation of (202) was achieved using the P(V) reagent bis(2,2,2-trichloroethyl) phosphorochloridate to give 2,3,6-tri-*O*-benzyl-4,5-isopropylidene-*myo*-inositol-1-[di(2,2,2-trichloroethyl)phosphate] (203). This was taken and the ketal was removed by acidic hydrolysis to give 2,3,6-tri-*O*-benzyl-*myo*-inositol-1-[di(2,2,2-trichloroethyl)phosphate] (204) in overall 90% yield from (202).



(203) (204)

Scheme 81

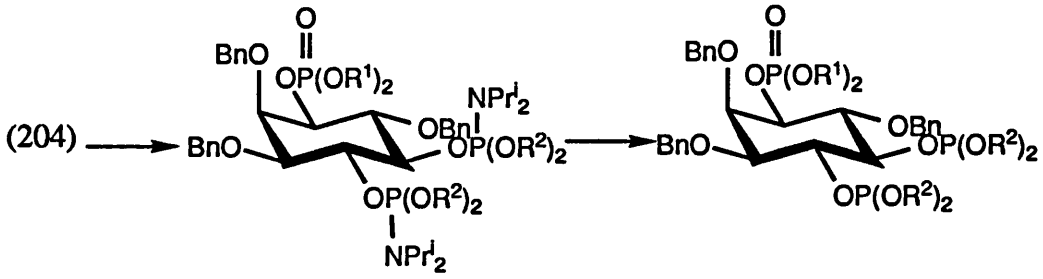
Phosphitylation of (204) was effected by three reagents, chloro (diisopropylamino)-2-cyanoethoxyphosphine (78), di(2-cyanoethyl) (diisopropylamino) phosphoramidite (81) and dibenzyl (diisopropylamino) phosphoramidite (82).

Initially, (78) was used to phosphitylate (204) to form DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1-[di(2,2,2-trichloroethyl)phosphate]-4,5-bis[diisopropylamino (2-cyanoethyl) phosphoramidite] (205).

The phosphoramidite (205) was converted to the phosphite by the addition of 2-cyanoethanol under the catalysis of tetrazole to give DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1-[di(2,2,2-trichloroethyl)phosphate]-4,5-bis[di(2-cyanoethyl) phosphite] (206). Reaction of (204) with (81) gave the bisphosphite triester (206) directly. The compound (204) was also phosphitylated with dibenzyl (diisopropylamino) phosphoramidite (82), which afforded DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1-[di(2,2,2-trichloroethyl)phosphate]-4,5-bis[dibenzyl phosphite] (207).

Both of the bisphosphites, (206) and (207) were oxidised *in situ* with sulphur in pyridine to give the fully protected phosphorothioate triesters, DL-2,3,6-tri-*O*-benzyl-*myo*-

inositol-1-[di(2,2,2-trichloroethyl)phosphate]-4,5-bis[di(2-cyanoethyl) phosphorothioate] (208) from (206) and DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1-[di(2,2,2-trichloroethyl)phosphate]-4,5-bis[dibenzyl phosphorothioate] (207) from (209).

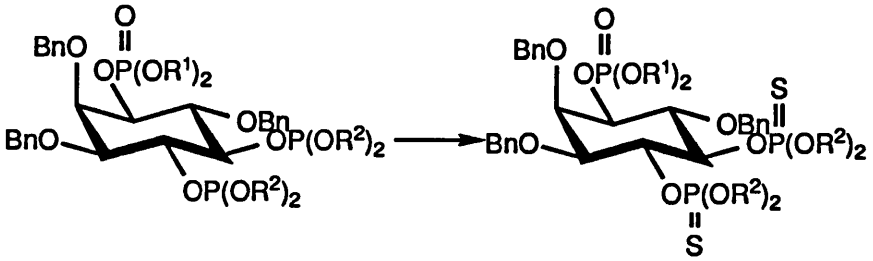


R^1=2,2,2-trichloroethyl
R^2=2-cyanoethyl, (205)

R^1=2,2,2-trichloroethyl
R^2=2-cyanoethyl, (206)
R^2=benzyl, (207)

Scheme 82

The three phosphitylation reagents used have certain merits and disadvantages, the chlorophosphine (78), for example is more reactive than the phosphoramidite (81). However, the chlorophosphine (78) is a moisture sensitive viscous oil that hydrolyses on prolonged storage and as such, requires careful handling. The phosphoramidite (81) on the other hand is as active a phosphitylating agent as the chlorophosphine (78) and is a relatively stable solid that can be purified by flash chromatography. Both reagents form the bisphosphorothioates in good yield, in 73% and 81% respectively. The advantage of using the 2-cyanoethoxy protecting group is its ease of deprotection, removal of both 2-cyanoethoxy groups being effected by mild basic hydrolysis. The third reagent, dibenzyl (diisopropylamino) phosphoramidite (82), a viscous oil, was used to form 2,3,6-tri-*O*-benzyl-*myo*-inositol-1-[di(2,2,2-trichloroethyl) phosphate]-4,5-bis(dibenzyl phosphorothioate) (209) in 42% overall yield. The bisphosphorothioate (208) appeared after purification as a pale yellow glass. Whereas the the bisphosphorothioate (209) was a colourless crystalline solid that was easily recrystallised to give pure a compound. Therefore purification of (209) was more effective than the purification of the glass (208).



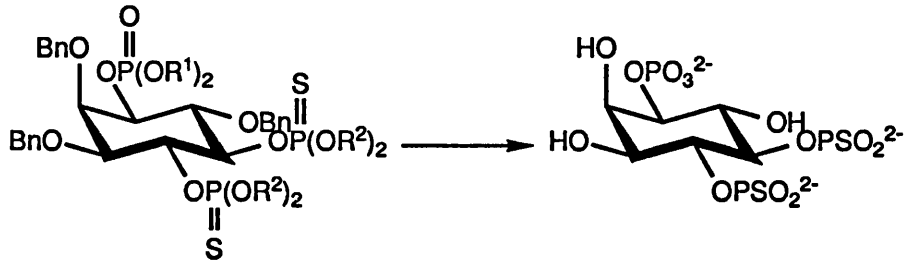
R^1=CH_2CCl_3

R^2=CH_2CH_2CN (206)
R^2=OBn (207)

R^2=CH_2CH_2CN (208)
R^2=OBn (209)

Scheme 83

The position of the phosphate at ring position -1 and the position of the two phosphorothioates at ring positions-4 and -5 were confirmed by two dimensional correlation experiments.



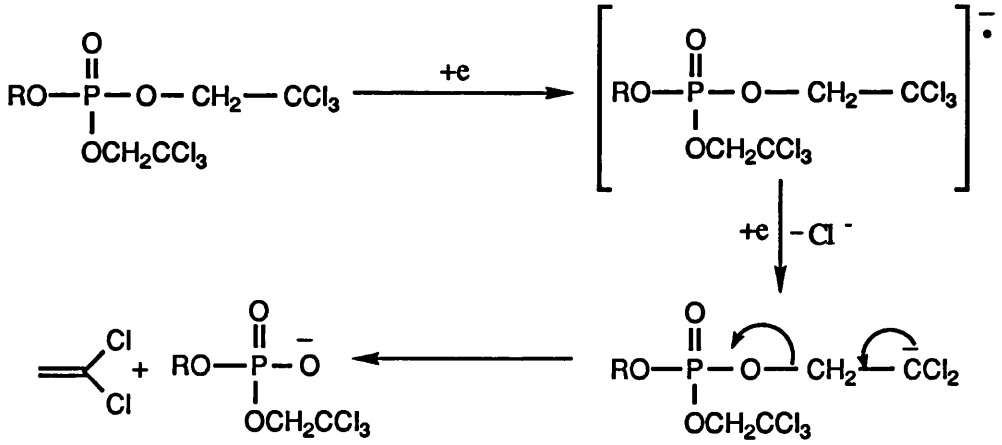
R^1=2,2,2-trichloroethyl (6)

R^2=2-cyanoethyl, (208)

R^2=benzyl, (209)

Scheme 84

All the protecting groups were removed from both (208) or (209) in a one pot reaction to give in both cases DL-*myo*-inositol-1-phosphate-4,5-bisphosphorothioate [Ins(1,4,5)P_3-4,5S_2] (6). Cleavage of benzyl ethers, benzyl esters, 2-cyanoethyl esters and 2,2,2-trichloroethyl esters was effected by treatment with sodium in liquid ammonia. The proposed mechanism for the cleavage of the 2,2,2-trichloroethyl group involves electron transfer from the medium to one of the C-Cl bonds, formation of a carbanion and subsequent ß-elimination of 1,2-dichloroethene [Scheme 85]. Sodium in liquid ammonia also removes 2-cyanoethyl groups by ß-elimination, the base presumably being NH_2^-.

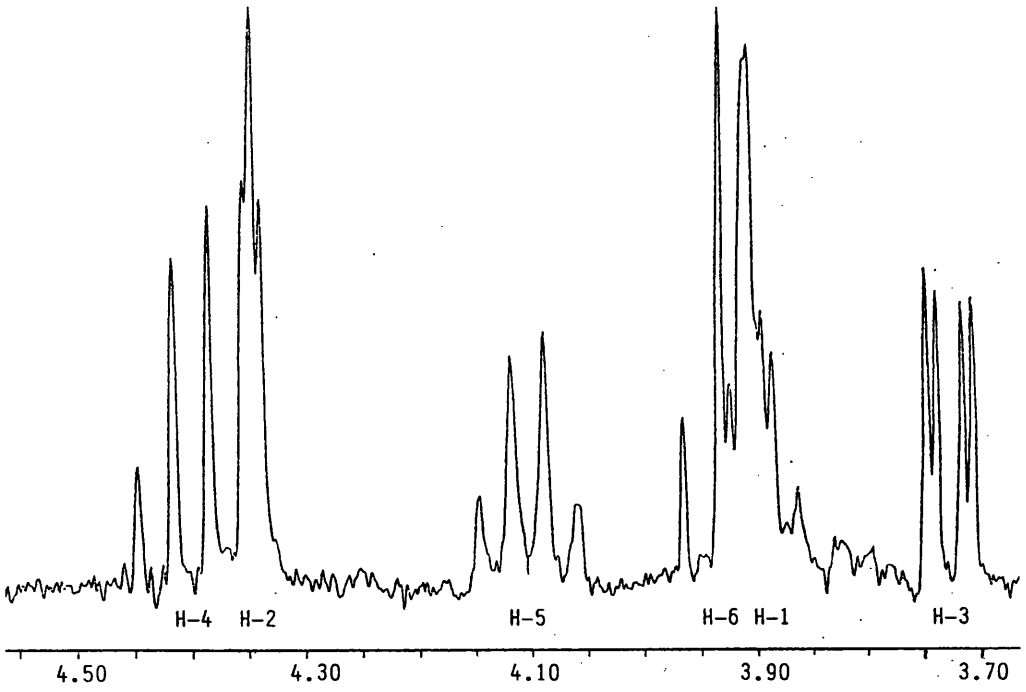


Scheme 85

This method is doubly advantageous where there are benzyl protected phosphorothioate triesters as it concomitantly removes any benzyl protecting groups reductively and overcomes the difficult procedure of catalytic hydrogenolysis normally used to remove benzyl groups, which would be complicated by the presence of phosphorothioates, since sulphur acts as a poison to conventional hydrogenolysis catalysts. Treatment of either (208) or (209) with sodium in liquid ammonia gave *myo*-inositol 1-phosphate-4,5-

bisphosphorothioate (6) as the crude ammonium salt. Purification of the crude product was effected by anion-exchange chromatography, using a linear gradient of triethylammonium bicarbonate eluent passing through a column packed with diethylaminoethyl-Sephadex or Q-Sepharose fast flow gel. The product thus obtained was quantified using a Briggs' phosphate assay[220]. Deprotection and subsequent purification gave the tris-salt in 83-85% yield. The difference in the purity of the two triesters (208) and (209) was reflected the differences in the yield of (6) obtained.

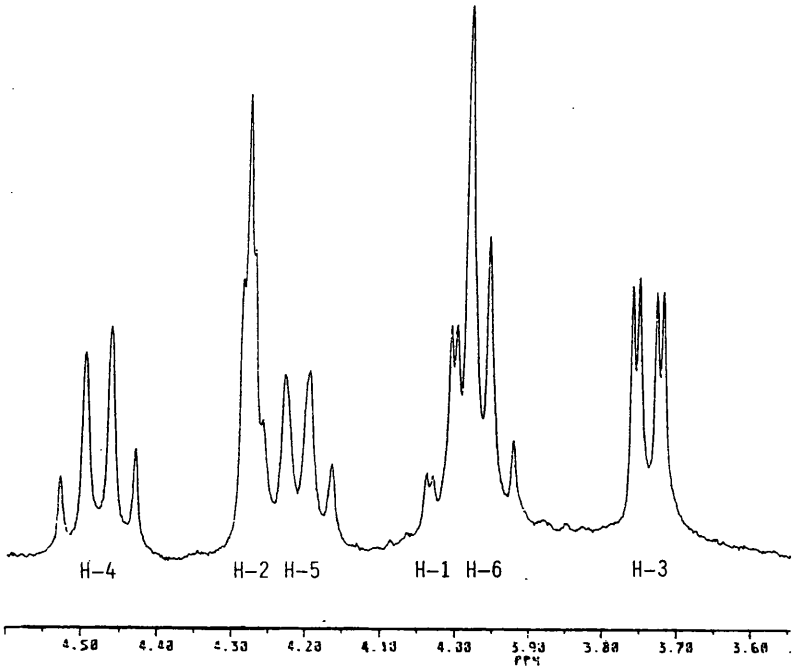
The locations of the phosphate at position-1 and the two phosphorothioate moieties at positions-4 and -5 of (6) can be confirmed by considering the one and two dimensional correlation NMR experiments discussed below.



300 MHz ^{1}H NMR of the triethylammonium salt of (6) in D_2O pH~8

Figure 54

It should be noted that the chemical shift of an inositol phosphate ring proton varies as a function of the pH the NMR experiment. A proton spectrum of the sample sample of (6) ran at a different time at a slightly different pH (the error of pH assessment using pH paper was ±1 unit) shows differences in each of the ring proton resonances. Consider the two spectra [Figs. 54 and 55], both spectra are of the same compound taken at different times with pH *ca.* 9.

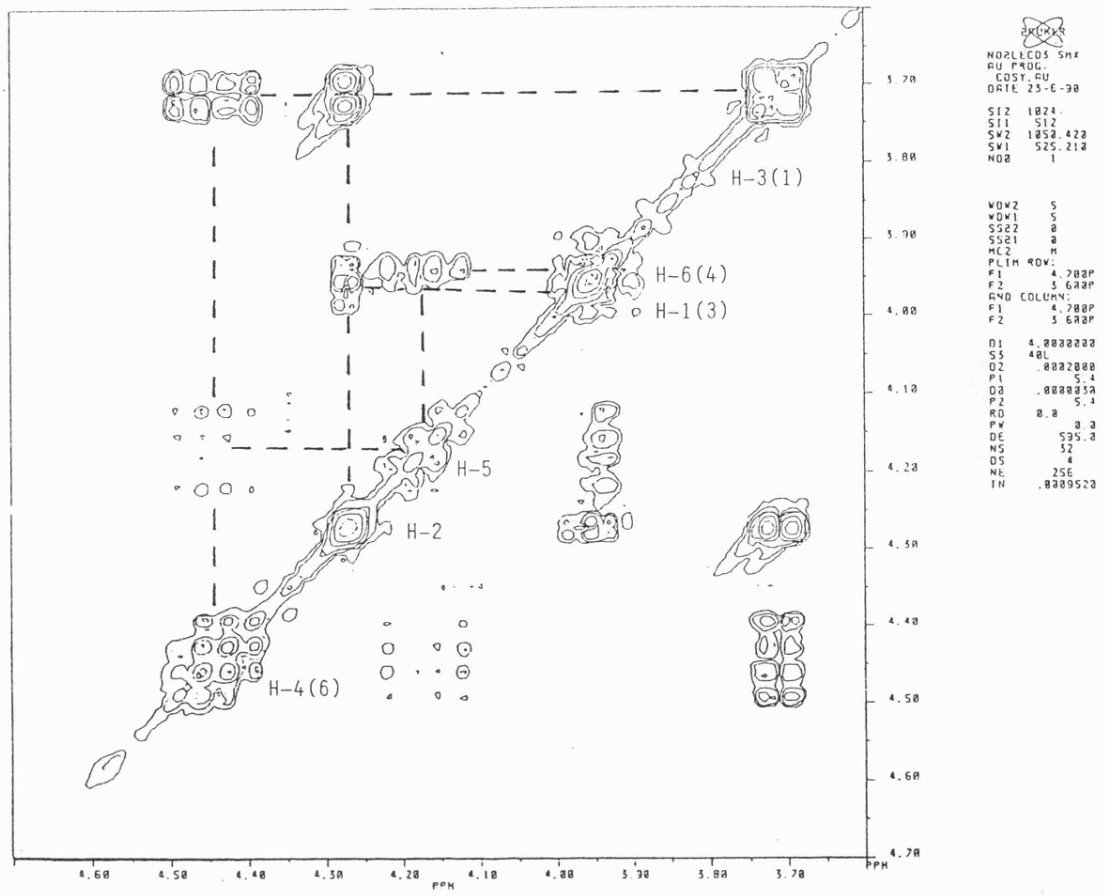


300 MHz ^{1}H NMR of the triethylammonium salt of (6) in D_2O pH~8

Figure 55

The most obvious change is in that of the resonance of H-2. It was shifted downfield by 0.067 ppm from H-5 and overlapped with the signal from H-4. The resonances of two protons that are bonded through carbon and oxygen to a phosphorothioate phosphorus moved by the greatest amount, 0.11 ppm and 0.08 ppm. There are several factors that could account for this phenomenon, the change in ionisation of the phosphate may effect the electron distribution of the proton-carbon bond and resulting in change in the shielding of the proton, *ie* shifts move to a lower field on increasing the pH. Other factors that should also be considered are temperature and ionic strength. Irregularities in these factors result in the movement of proton resonances.

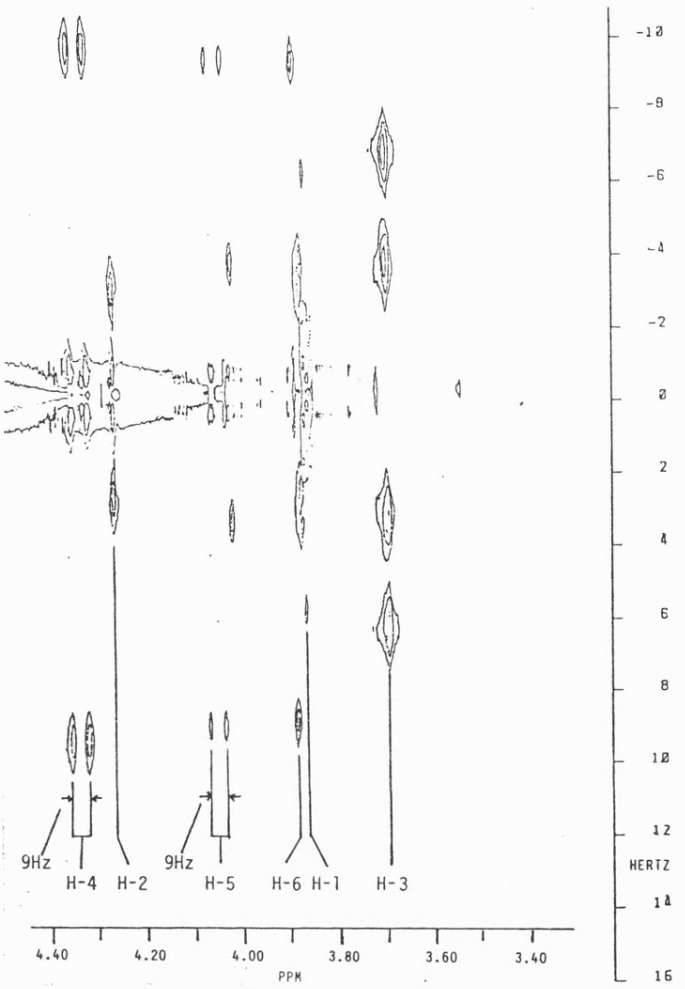
Consideration of the 1D proton NMR of the (6) [Fig. 55] does not enable the unambiguous assignment of the proton resonances. However, it is possible to assign the resonance at δ 4.28 as H-2 as it has two small coupling constants, both 2.7 Hz. By using the relationship derived by Karplus, the small value can be assumed due to be a gauche coupling, either a diequatorial (J_{ee}) or an axial-equatorial coupling (J_{ae}). The value of a diaxal coupling (J_{aa}) would be much larger, in the order of 8-10 Hz. It is possible to assign the resonances of the remaining protons on the strength of the ^{1}H-^{1}H COSY, J-resolved and the ^{31}P-^{1}H correlation experiments. The position of H-2 at δ 4.28 ppm can be determined [Fig. 55], applying this information to the ^{1}H-^{1}H COSY [Fig. 56] two possible sequences for the assignment of protons is possible.



300 MHz ^{1}H-H COSY NMR of the triethylammonium salt of (6) in D_2O pH 8, run on the same sample as the 300 MHz ^{1}H NMR [Fig. 55]

Figure 56

The first sequence assumes that the first cross peak encountered at H-2 relates to proton 3, the sequence will then be δ 4.28 H-2, δ 3.96 H-3, δ 3.93 H-4, δ 4.17 H-5, δ 4.45 H-6, δ 3.71 H-1, δ 4.28 H-2. The second sequence assumes that the first cross peak encountered at H-2 is due to the spin-spin interaction of H-1, resulting the following assignment; δ 4.28 H-2, δ 3.96 H-1, δ 3.93 H-6, δ 4.17 H-5, δ 4.45 H-4, δ 3.71 H-3, δ 4.28 H-2.

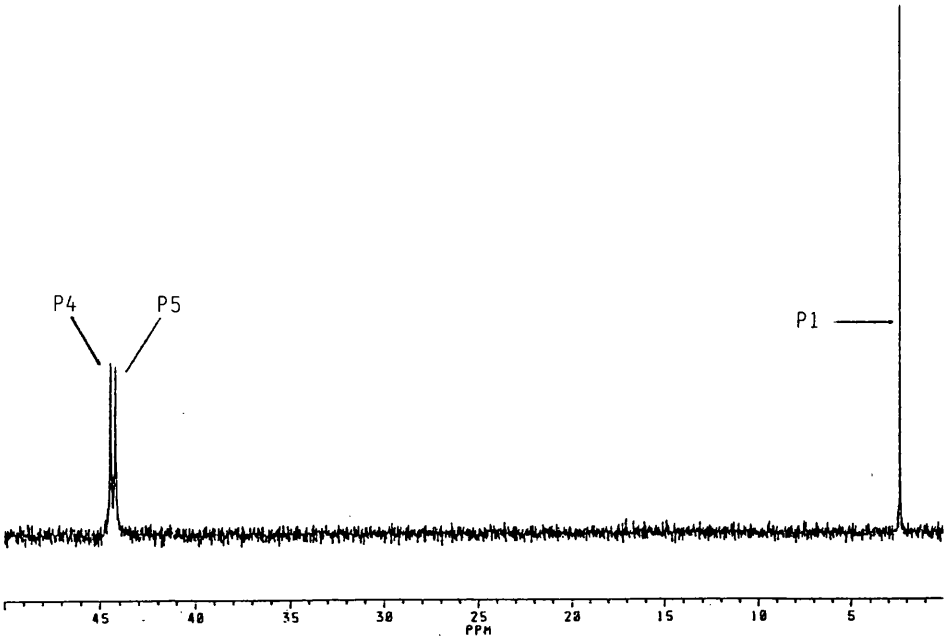


121 MHz ^{1}H-^{1}H 2D J-resolved NMR of the triethylammonium salt of (6) in D_2O, pH 8 run on the same sample as the 300 MHz ^{1}H NMR [Fig. 55]

Figure 57

In 2D J-resolved ^{1}H spectroscopy, the choice of quantum mechanical terms for the evolution and detection periods allow unique characterisation of each spectral line. The conventional chemical shift axis, f_2, can be arranged to contain only chemical shifts; splitting due to J-coupling appears along a perpendicular axis, f_1. The homonuclear J-resolved spin-echo pulse sequence gives two dimensional spectra that depend only upon the effects of homonuclear J-couplings during τ. The routine proton 2D J-resolved experiment involves only proton pulses, couplings to heteronuclei *i.e.* ^{31}P, are not phase modulated by the π-pulse, so they appear as chemical shifts in f_2. By considering the Homonuclear J-resolved experiment, [Fig. 57] it is possible to dismiss the first sequence as incorrect as the proton at δ 3.71 ppm has no heteronuclear coupling in the f_2 domain, so it must be position 3. The J-res spectrum [Fig. 57] clearly shows two heteronuclear

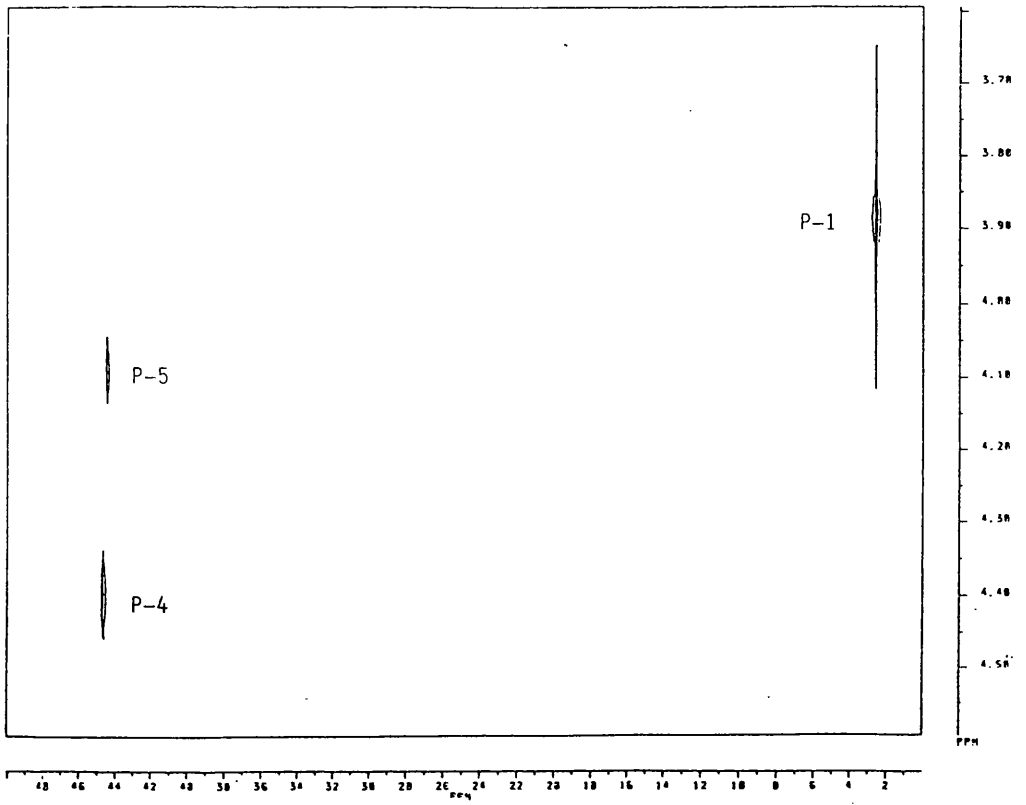
couplings of *ca.* 9-10 Hz at δ 4.34 and δ 4.05 ppm due to coupling to the two phosphorothioates, the heteronuclear coupling to the phosphate is smaller *ca.* 4 Hz and is therefore harder to see, but it is at δ 3.86 ppm. Combining this information it is possible to determine that the inositol was phosphorylated at positions -1, -4 and -5.



109 MHz ^{31}P NMR of the triethylammonium salt of (6) in D_2O, pH 8 run on the same sample as the 300 MHz ^{1}H NMR [Fig. 55]

Figure 58

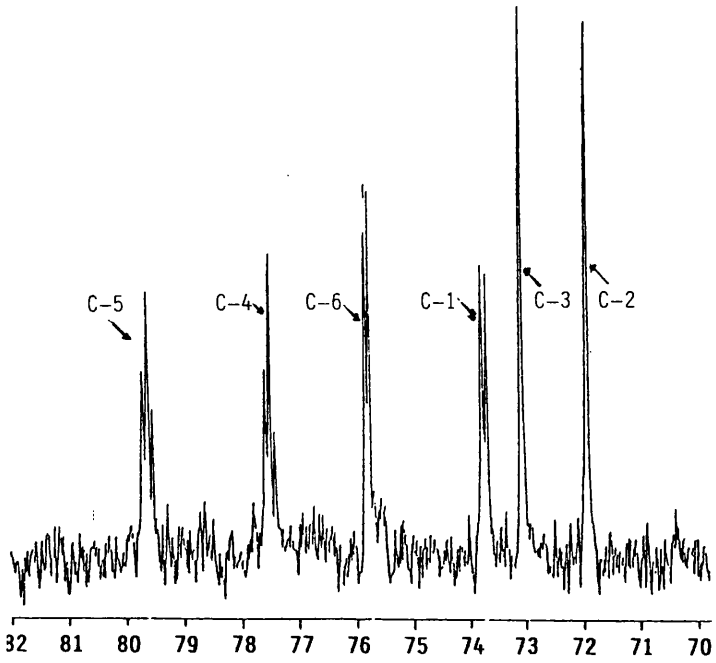
It can be seen from the 1-D ^{31}P NMR [Fig. 58] that the molecule contains two phosphorothioates δ 44.64 and δ 44.56 ppm and one phosphate δ 2.30 ppm. By running a ^{31}P-^{1}H correlation experiment it was possible to correlate each phosphorus with a ring proton.



109 MHz ^{1}H-^{31}P Shift Correlation NMR of the triethylammonium salt of (6) in D_2O, pH 8 run on the same sample [Fig. 54]

Figure 59

The phosphate δ 2.30 ppm couples with the proton at δ 3.89 ppm, which is H-1, the phosphorothioate at δ 44.56 ppm correlates to the proton at t δ 4.10 ppm, which is H-5 and finally, the phosphorothioate at δ 44.64 ppm correlates to the proton at δ 4.41 ppm, which is H-4. A ^{13}C-^{1}H correlation experiment enabled the assignment of the ^{13}C spectrum [Fig. 60].

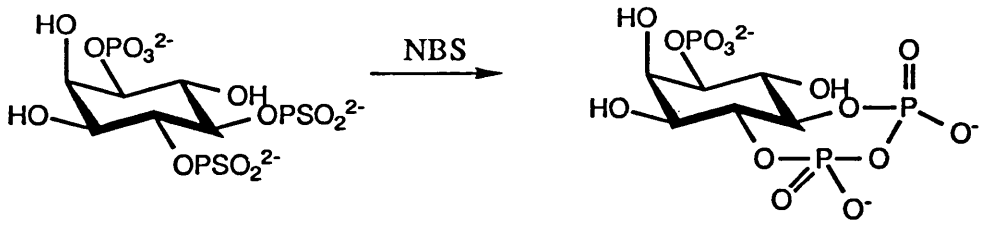


75 MHz broad band proton decoupled ^{13}C NMR of the triethylammonium salt of (6) in D_2O pH 8. Figure 60

The broad band proton decoupled spectra show coupling to phosphorus. The coupling is dependent upon the dihedral angle and give some information about the conformation of the inositol ring. Combining all of the spectroscopic data it is possible to assign unambiguously the compound (6) as DL-*myo*-inositol-1-phosphate-4,5-bisphosphorothioate [Ins(1,4,5)P_3-4,5S_2].

7.3 Synthesis of DL-*myo*-Inositol-1-Phosphate 4,5-Cyclic Pyrophosphate (5)

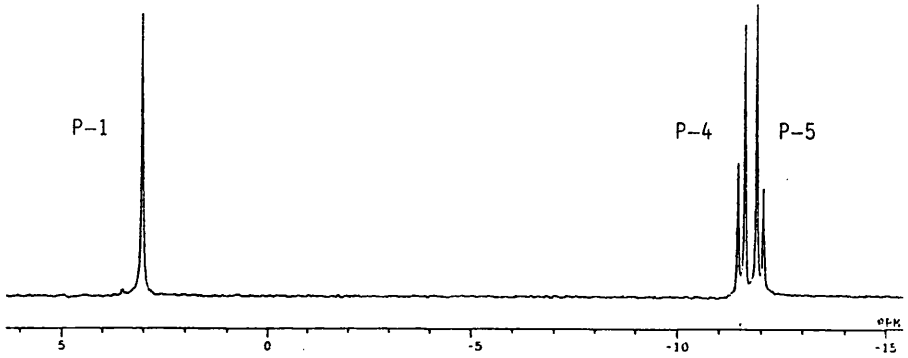
In order to avoid some of the problems resulting from the desulphurisation of a trisphosphorothioate, it was decided to try the desulphurisation of DL-*myo*-inositol-1-phosphate-4,5-bisphosphorothioate (6) since the phosphate at position-1 would not participate in the NBS reaction. Compound (6) was prepared as described in the preceding section and was desulphurised with NBS to give the crude pyrophosphate (5) in high yield[238]. This was surprising as we expected that the presence of the vicinal hydroxyl groups would promote the formation of 5-membered cyclic phosphate leading to phosphate migration. There was some evidence of phosphate migration (24%) but this was much less than we expected. A small amount of apparent straight desulphurisation to Ins (1,4,5)P_3 (7%) also occurred as was predicted.



(6) (5)

Scheme 86

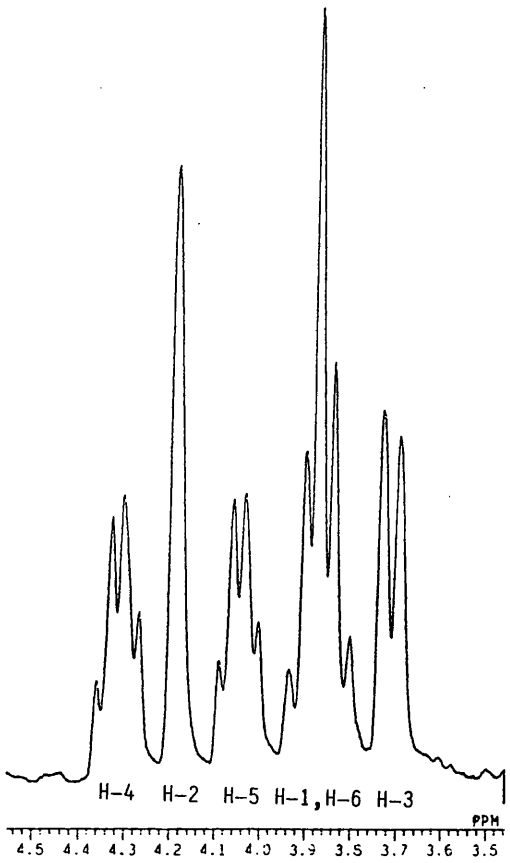
Subsequent purification by anion exchange chromatography gave the triethylammonium salt of (5) (67% yield).



109 MHz ^{31}P NMR of (5) pH 8 in D_2O

Figure 61

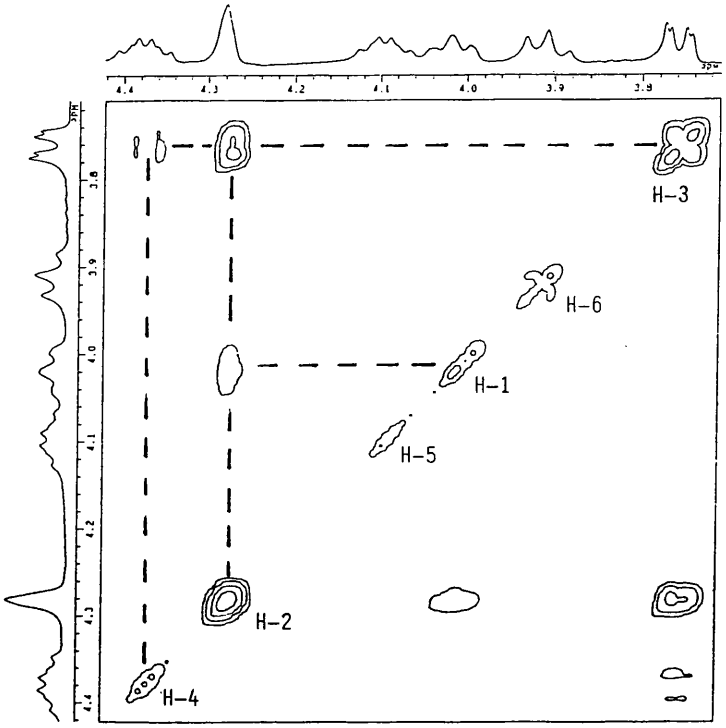
Compound (5) exhibited a ^{31}P NMR spectrum [Fig. 61] showing clearly the presence of the phosphate at position-1 (δ 3.04 ppm) and the 4,5-cyclic pyrophosphate [δ -11.57 (pos. 4) and -11.98 ppm (pos. 5), $^{2}J_{pp}$ 16.8 Hz], the latter resonating as the expected AB system.



270 MHz ^1H NMR of (5) pH 8 in D_2O

Figure 62

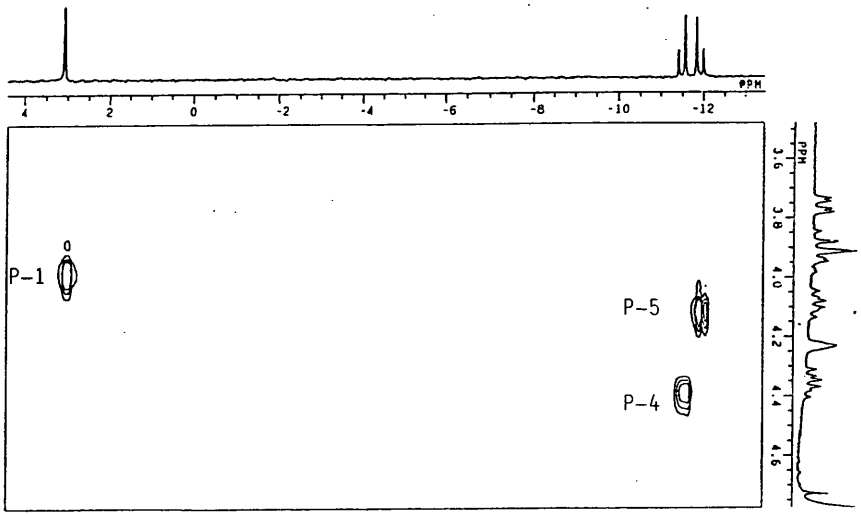
The proton NMR of (5) [Fig. 62] does not show all of the couplings present. The double doublet of H-2 which normally appears as a triplet, appears as a singlet. The double doublet of H-3 is present as a simple doublet. Knowing the position of H-2 at δ 4.28 ppm it is possible to assign the ^1H-^1H COSY experiment depicted [Fig. 63].



270 MHz ^{1}H-^{1}H COSY NMR of (5) pH 8 in D_2O

Figure 63

Due to the poor resolution of the experiment, two of the protons H-5 and H-6 do not show any correlation to other protons. However, both protons appear [Fig. 63] as double doublets, *i.e.* coupling to two other protons, this point should be kept in mind when considering [Fig. 63]. It is possible to obtain the sequence δ 4.28 H-2, δ 3.75 H-3, δ 4.37 H-4 and δ 4.20 H-1; δ 3.90, 4.09 H-6,5. This sequence is confirmed when considering the ^{31}P-^{1}H correlation experiment shown [Fig. 64].



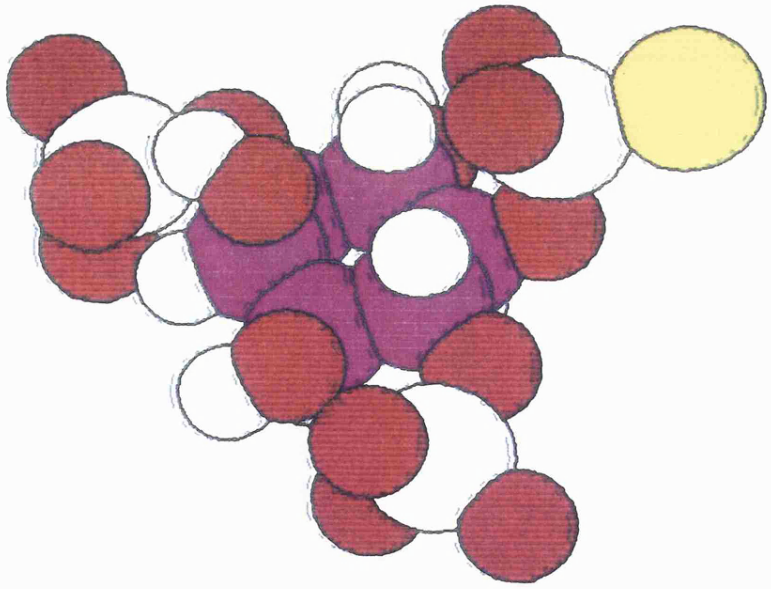
270 MHz ^{1}H-^{31}P Shift Correlation NMR of (5) pH 8 in D_2O

Figure 64

The phosphorus at δ 3.04 ppm in the phosphorus domain, does not correlate with the signal at δ 3.74 ppm in the proton domain. This phosphorus however, couples with the proton at δ 4.20 ppm. The inference of these observations is that the cross peak that corresponds with δ 3.74 is H-3, and that the signal at δ 4.20 is H-1. Furthermore, one of the phosphate atoms of the pyrophosphate linkage at δ −11.57 correlates with H-4 at δ 4.38 and the other phosphorus of the pyrophosphate at δ -11.98 correlates with a proton at δ 4.10, H-5.

7.4 Synthesis of DL-*myo*-Inositol-1,4-Bisphosphate-5-Phosphorothioate (7)

After the biological properties of DL-Ins(1,4,5)PS_3 had been investigated [see chapter 8], it became clear that analogues where one or two of the phosphates are replaced by phosphorothioates would be of considerable importance. The analogue with a phosphorothioate at the 5 position would be an Ins(1,4,5)P_3 analogue of great interest, since this would be nearer in structure to Ins(1,4,5)P_3 but would be resistant to phosphatase action [Fig. 65].

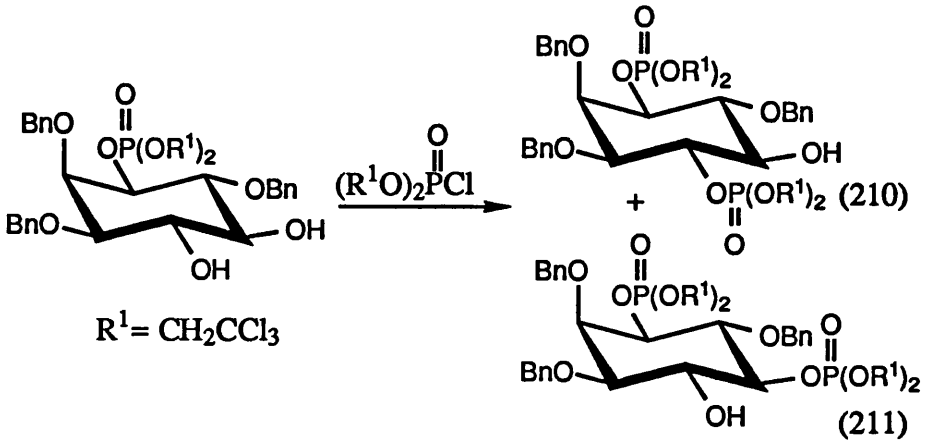


Minimised conformer of D-Ins(1,4,5)P_3-5S (7)

Figure 65

Thus we have developed a synthetic route to DL-*myo*-Inositol-1,4-bisphosphate-5-phosphorothioate [Ins(1,4,5)P_3-5S] (7)[240,241,242]. Ins(1,4,5)P_3-5S (7) was synthesised from the protected intermediate DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1-[di(2,2,2-trichloroethyl)phosphate] (204) which was prepared as described [section 7.2].

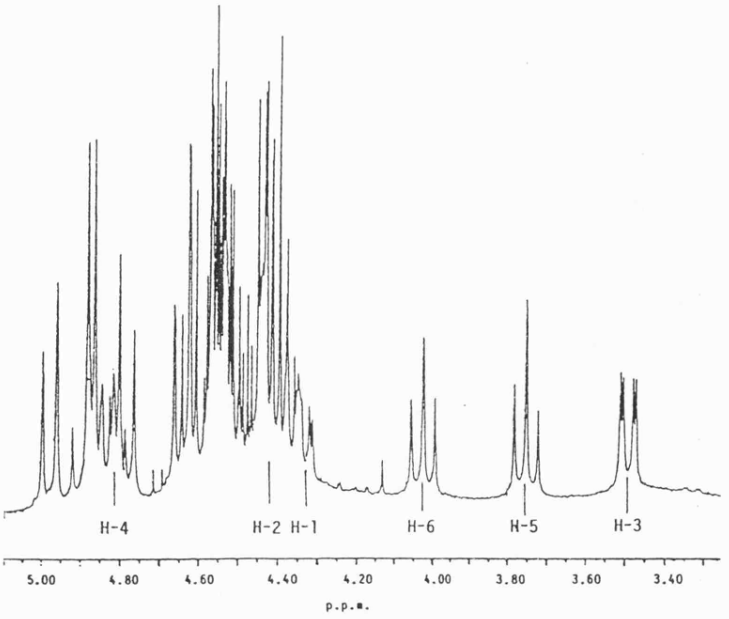
Phosphorylation of (204) with bis(2,2,2-trichloroethyl) phosphorochloridate (2.1 eq.) in dichloromethane and pyridine gave a mixture of two regioisomers, the 1,4-bisphosphate (210) and the 1,5-bisphosphate (211). Separation of the two compounds was not achieved by simple short column chromatography. However, trituration of the solid mixture with pentane gave a solid product that was recrystallised from ethyl acetate-pentane and which proved to be one pure regioisomer *vide infra.*



Scheme 87

Analysis of the supernatant after trituration, by ^{1}H and ^{31}P NMR spectroscopy revealed the mixture of the 1,4- and 1,5-bisphosphates remaining, it was possible to measure the ratio of these two compounds by the difference in integration of proton resonances from the inositol ring (position-3) in each case, the ratio was found to be 2:1 for the 1,5- to 1,4- respectively. Further attempts to remove more of (210) from the mixture by further trituration were not successful.

Subsequent identification of the regioisomer isolated was difficult as the proton NMR in the region of interest, around three of the inositol ring protons [Fig. 66] was significantly obscured by overlapping AB and ABX spin systems from the benzyl and phosphotriester moieties respectively.



300 MHz ^{1}H NMR of (210) in $CDCl_3$

Figure 66

From the ^{1}H spectrum it can be seen that the signal at δ 3.48 ppm has two coupling constants which can be measured; one of 2.6 Hz, the other of 9.8 Hz, the former a gauche coupling, J_{ee} or J_{ae}; the larger latter value due to a J_{aa} coupling. As the proton at position 2 is the only ring proton that is equatorial, protons 1 and 3 are the only ring protons that will have a J_{ae} and a J_{aa} coupling constant.

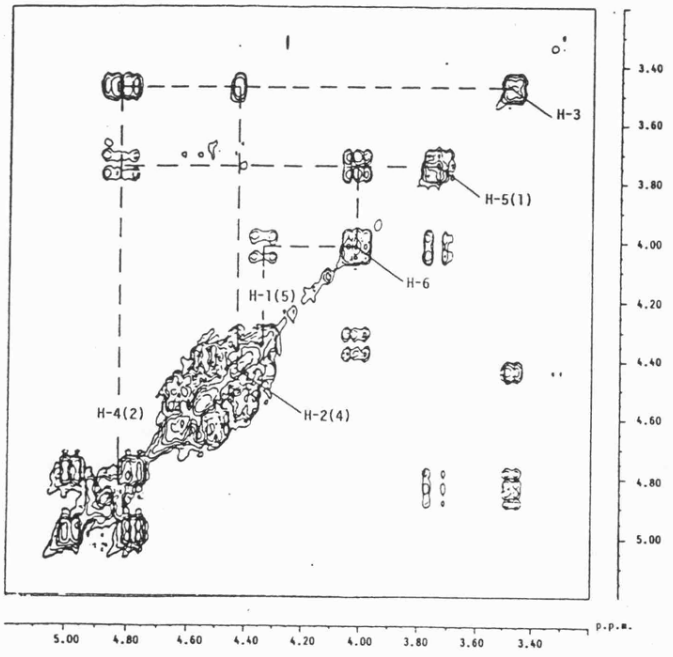
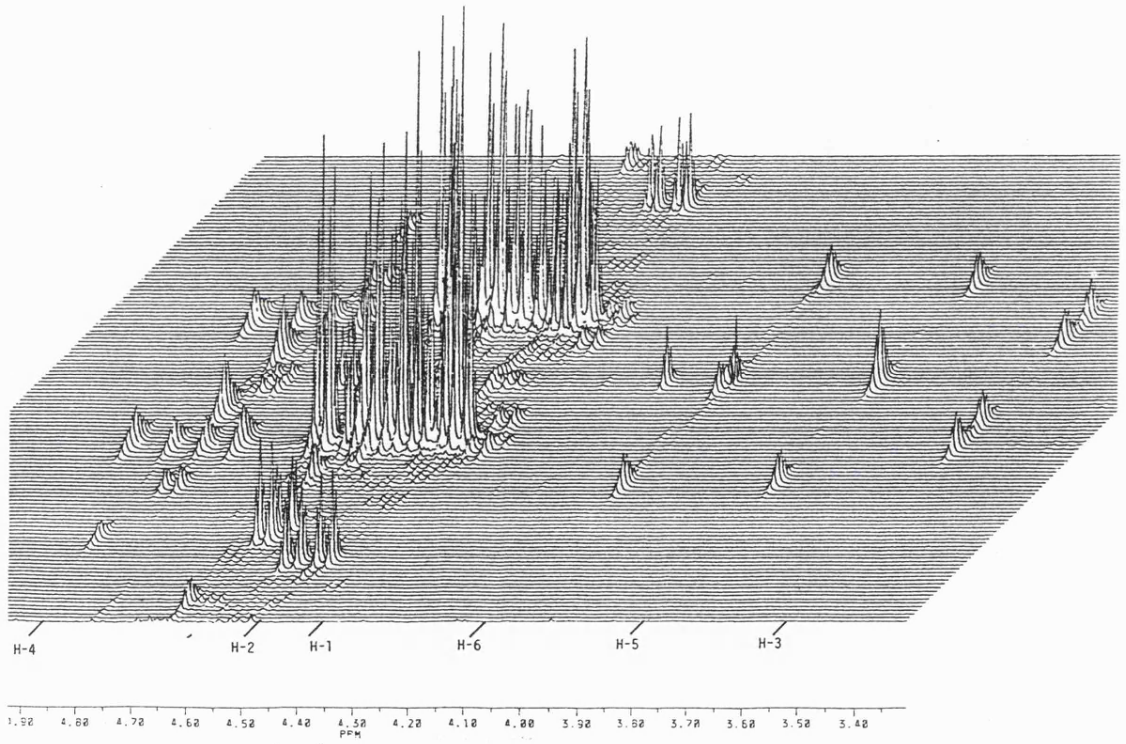


Figure 67. 300 MHz ^{1}H-^{1}H COSY NMR of (210) in $CDCl_3$

Applying this information to the $^1H^1H$ COSY [Fig. 67], it is plausible to assume that if the signal at δ 3.48 is H-1 there are are two possible assignments, arising from the fact that the first cross peak encountered, δ 4.41, could be the result of correlation with H-2 or H-6. The resultant two sequences are as follows: 3.48 H-1, 4.41 H-2, 4.34 H-3, 4.01 H-4, 3.73 H-5, 4.83 H-6, 3.48 H-1; and 3.48 H-1, 4.41 H-6, 4.34 H-5, 4.01 H-4, 3.73 H-3, 4.83 H-2, 3.48 H-1. Similarly, by assuming that the signal at δ 3.48 is H-3, there are another two plausible sequences possible; 3.48 H-3, 4.41 H-4, 4.34 H-5, 4.01 H-6, 3.73 H-1, 4.83 H-2, 3.48 H-3; or 3.48 H-3, 4.41 H-2, 4.34 H-1, 4.01 H-6, 3.73 H-5, 4.83 H-4, 3.48 H-3.

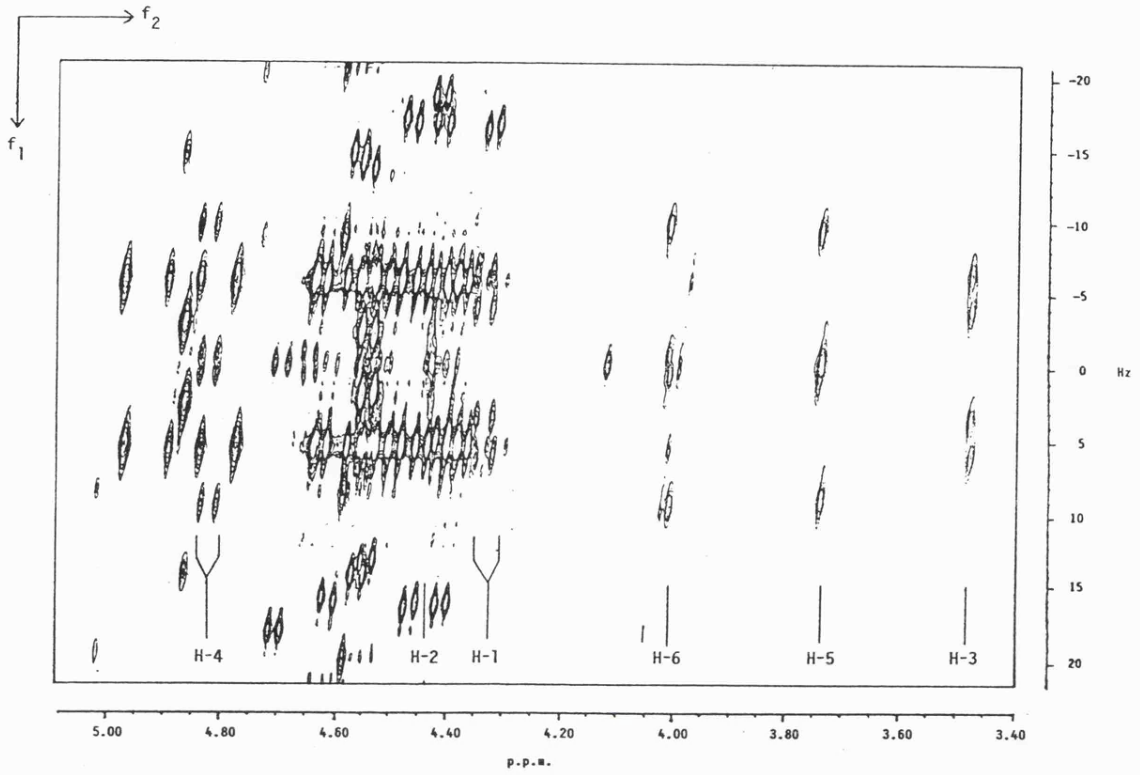
To determine which sequence was the correct one, it was necessary to conduct another NMR experiment to obtain a 2D J-resolved ^1H spectrum.



300 MHz ^1H-^1H J-res NMR of (210) in $CDCl_3$ [stacked display]

Figure 68

Examining the 2D J-resolved ^1H experiment [Fig. 68]; The signal at δ 3.48 is clearly a double doublet with two homonuclear couplings of 2.6 Hz and 9.8 Hz; the smaller representing a diequatorial coupling (J_{ee}) or an axial-equatorial coupling (J_{ae}), the larger value being due to a diaxal coupling (J_{aa}). As there is no evidence of any heteronuclear coupling along the f_2 axis, it is possible to assign the signal as H-3. This can be confirmed by consideration of the contour representation of the J-res NMR of (210) [Fig. 69].



300 MHz ^{1}H-^{1}H J-res NMR of (210) in $CDCl_3$ [contour display]

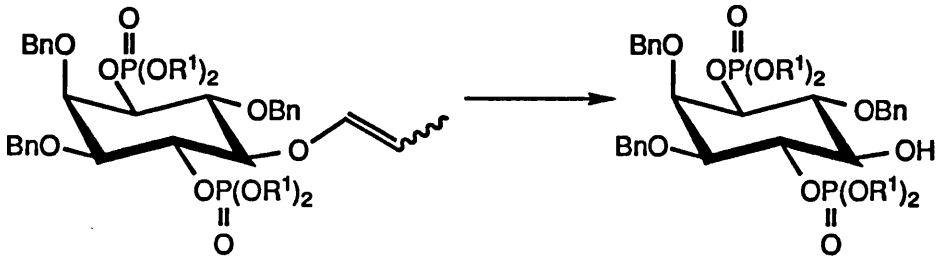
Figure 69

The signal at δ 3.74 is seen as a triplet with one homonuclear coupling of $^3J_{aa}$ 9.2 Hz. The signal at δ 4.01 is a doublet of doublets with two homonuclear couplings of $^3J_{aa}$ 9.2 and 9.7 Hz respectively. The signal at δ 4.34 is split by three couplings to give a double doublet of doublets. Two of the couplings are in the f_1 plane and are due to homonuclear couplings of 2.6 Hz, (J_{ee} or J_{ae}) and 9.7 Hz, J_{aa} respectively. The coupling in the f_2 direction being due to a heteronuclear coupling of $^3J_{PH}$ 8.8 Hz. Considering these data it is possible to assign the signal at δ 4.34 as H-1. The apparent triplet at δ 4.41 ppm with two equivalent homonuclear couplings ^{3}J 2.6 Hz can be assigned to H-2.

The protons on each of the four (2,2,2-trichloroethyl) phosphate protecting groups are coupled to give an ABX system. The first ABX system can be seen δ 4.42. The two protons couple together as an AB system with $^2J_{AB}$ 11.2 Hz and both are also split by a heteronuclear coupling to the phosphorus $^3J_{PH}$ 6.1 Hz. The other three (2,2,2-trichloroethyl) phosphate groups are seen at δ 4.48, $^2J_{AB}$ 11.2 Hz, $^3J_{PH}$ 6.1 Hz; δ 4.56, $^2J_{AB}$ 11.2 Hz, $^3J_{PH}$ 6.5 Hz; δ 4.57, $^2J_{AB}$ 11.2 Hz, $^3J_{PH}$ 6.1 Hz. The CH_2 signals of the three benzyl protecting groups at positions 2,3 and 6 appear as three AB systems, the first at δ 4.60, $^2J_{AB}$ 11.2 Hz, the second at δ 4.87, $^2J_{AB}$ 11.2 Hz, the third at δ 4.88, $^2J_{AB}$

11.2 Hz.

Finally, an assignment of the key proton at δ 4.83 is possible. As with H-1 this proton resonates as a double doublet of doublets with two homonuclear couplings, J_{aa} 9.2 and 9.8 Hz and a heteronuclear coupling $^3J_{PH}$ 8.5 Hz. Combining the information from both 2D experiments there is a choice of two possible regioisomers, 2,5-bisphosphate or 1,4-bisphosphate. Clearly the first regioisomer is not possible as the signal at δ 4.34 has been assigned as position of H-1. The sequence of the $^1H^1H$ COSY [Fig. 67] is as follows: δ 3.48 H-3, 4.41 H-2, **4.34 H-1**, 4.01 H-6, 3.73 H-5, **4.83 H-4.** The phosphorus coupling to H-1 and H-4 enables the unambiguous assignment of the compound as 2,3,6-tri-*O*-benzyl-*myo*-inositol 1,4-bis-[di(2,2,2-trichloroethyl) phosphate] (210).



(212) (210)

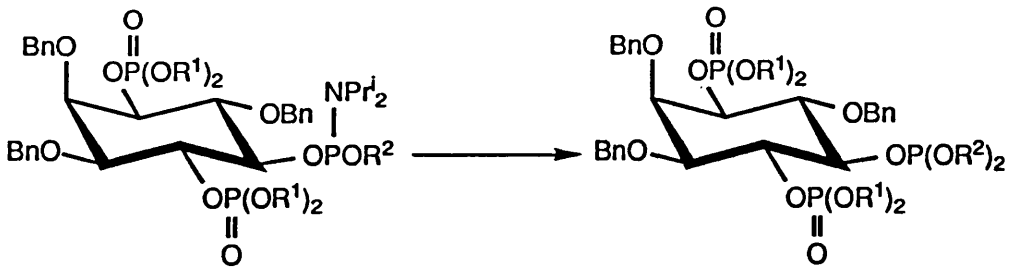
$R^1=CH_2CCl_3$

Scheme 88

Compound (210) was prepared by a different route, via the deprotection of DL-2,3,6-tri-*O*-benzyl-5-*O*-prop-1-enyl-*myo*-inositol 1,4-bis-[di(2,2,2-trichloroethyl) phosphate] (212). This proved to be a difficult task as the normal deprotection conditions for the removal of a prop-1-enyl group resulted in the formation of the cyclic 5-membered phosphate diester. The cyclic phosphate upon hydrolysis led to phosphate group migration to yield products very difficult to purify and use. This cyclisation reaction was promoted by the presence of acid or base, which meant that the mercuric chloride/mercuric oxide method of deprotection was inapplicable. Heating (212) in the presence of mercuric chloride gave (210). The (210) thus produced was affected by the presence of the base and elevated temperature, the net result of which led to the phosphate group scrambling. So deprotection of the prop-1-enyl was attempted by taking a stirred solution of (212) in acetone/water with mercuric chloride at rt for an extended period. The prop-1-enyl group was removed after 4 days. However, problems were encountered when trying to remove the mercury residue without concomitant phosphate migration.

Phosphitylation of the hydroxyl group at the 5-position was effected by treatment of (210) with chloro (diisopropylamino)-2-cyanoethoxy phosphine (78) to give 2,3,6-tri-*O*-benzyl-*myo*-inositol-1,4-bis[di(2,2,2-trichloroethyl)phosphate]-5-[diisopropylamino (2-

cyanoethyl) phosphoramidite] (213)

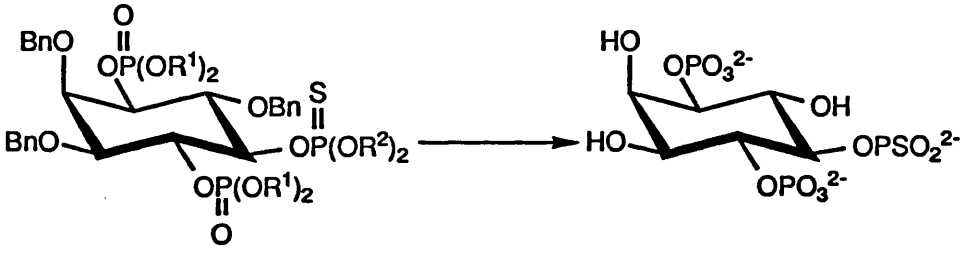


$R^1=CH_2CCl_3$

$R^2=CH_2CH_2CN$ (213) (214)

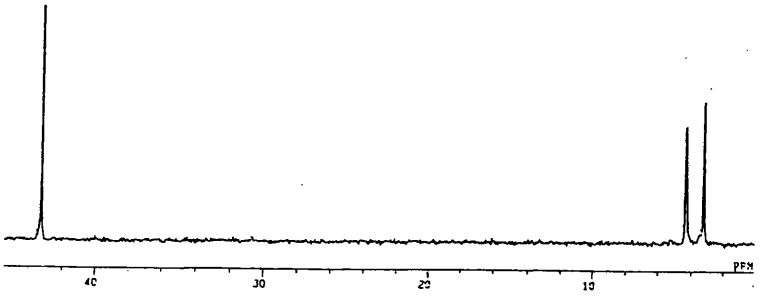
Scheme 89

Reaction of (213) *in situ* with 2-cyanoethanol-tetrazole gave DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1,4-bis[di(2,2,2-trichloroethyl)phosphate]-5-[di(2-cyanoethyl) phosphite] (214), which was not isolated but oxidised directly to the mixed phosphate / phosphorothioate with sulphur in pyridine to give DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1,4-bis[di(2,2,2-trichloroethyl)phosphate]-5-[di(2-cyanoethyl) phosphorothioate] (215). Deprotection of this compound was again effected by treatment with sodium in liquid ammonia to give Ins(1,4,5)P_3-5S (7) as a glass in 88% yield.



R^1=2,2,2-trichloroethyl

R^2=2-cyanoethyl (215) Scheme 90 (7)



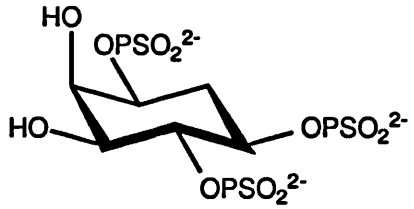
109 MHz ^{31}P NMR of (7) in D_2O pH 9

Figure 70

The presence of one phosphorothioate at δ 43.24 pos.-5 and two phosphates at δ 3.25, 4.35 pos. -1 and -4 can be clearly seen [Fig. 70], thus confirming the structure of (7) as DL-*myo*-inositol-1,4-bisphosphate-5-phosphorothioate.

7.5 Synthesis of 6-Deoxy-D-myo-Inositol-1,4,5-Trisphosphorothioate (8)

The 6-deoxy analogue of Ins(1,4,5)P_3, 6-deoxy-Ins(1,4,5)P_3, has been found to be a full agonist of Ins(1,4,5)P_3 receptor-mediated intracellular calcium release. However, the 6-deoxy-Ins(1,4,5)P_3 was 70 fold less potent than Ins(1,4,5)P_3, implying that the 6-hydroxyl group of Ins(1,4,5)P_3 is important for receptor binding and Ca^{2+} release, but it is not an essential feature. 6-Deoxy-Ins(1,4,5)P_3 was found to not be a substrate for the Ins(1,4,5)P_3-5-phosphatase but a moderately potent inhibitor and a probable substrate for Ins(1,4,5)P_3-3-kinase[218]. It was decided to make the phosphorothioate analogue of 6-deoxy-Ins(1,4,5)P_3 for several reasons. It would be interesting to see the cumulative effect of removing the 6-hydroxyl and altering the three phosphate moieties upon its biological properties. Moreover, since phosphorothioate substitution appears to enhance binding to 5-phosphatase[187], 6-deoxy-Ins(1,4,5)PS_3 (8) might be expected to be a better phosphatase inhibitor than 6-deoxy-Ins(1,4,5)P_3 and yet be a weaker releaser of Ca^{2+}. Such properties would be highly desirable, since the present most effective inhibitor Ins(1,4,5)PS_3 is a potent Ca^{2+} mobiliser. Synthesis of 6-deoxy-Ins(1,4,5)PS_3 was easily accomplished by the removal of the ketal from 6-deoxy-2,3-cyclohexylidine-D-*myo*-inositol -1,4,5-trisphosphorothioate by simple acidic hydrolysis using a strong ion-exchange resin. This was followed by purification by anion-exchange chromatography to give 6-deoxy-D-*myo*-inositol -1,4,5-trisphosphorothioate (8) as the tris-triethylammonium salt in 97% yield from (196).



(8)

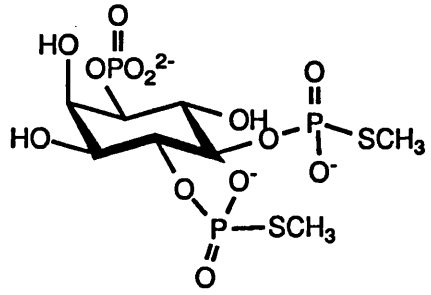
Figure 71

7.6 Synthesis of S-Alkylated *myo*-Inositol-Phosphorothioates

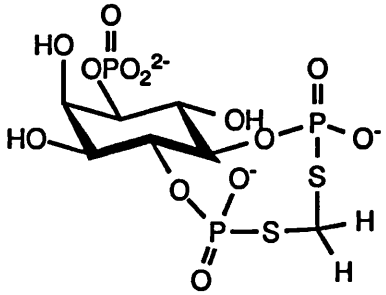
Alkylation of sulphur has been employed in the development of probes for enzymic nucleotide binding sites[228]. Specifically, the S-methyl phosphorothiolate analogue, both diastereoisomers of adenosine 5'-*O*-(*S*-methyl 1-thiotriphosphate) (ATPαSMe) have been prepared from the parent phosphorothioate (ATPαS) and used to study the metal-ATPαS and metal-ATPαSMe binding in various kinase enzymes[228].

In order to provide more tools for the study of inositol phosphate biology, we decided to try to synthesise *myo*-inositol-1-phosphate-4,5-bis-[methyl-phosphorothiolate] (216) and *myo*-inositol-1-phosphate-4,5-[cyclic-S,S-methylene]-bisphosphorothiolate (217).
The S-methyl phosphorothiolate differs in two major respects from the parent phosphorothioate, namely:
(i) the alkylated sulphur increases the electrophilicity of the phosphorus.
(ii) the phophorothiolate thus produced is a diester and consequently has one less charge than the phosphorothioate monoester.
(iii) the phosphorothiolate moiety is somewhat larger than the phosphorothioate monoester.
We decided to see if inositol phosphorothiolates would be of use as Ins(1,4,5)P_3 analogues. We also thought that it would be possible to increase the yield of Ins(1,4:5 pyro)P_3 buy taking the phosphorothiolate and using it for the NBS-mediated desulphurisation.

Both of these compounds were obtained from *myo*-inositol-1-phosphate-4,5-bisphosphorothioate by treatment with iodomethane or diiodomethane respectively. The reaction of (204) with diiodomethane produced a small amount of the cyclic pyrophosphate (5) as a side product. In both reactions isolation and purification of the products by anion-exchange chromatography proved to be arduous, the cyclic compound proved impossible to isolate in a pure state as the elution profile of the (217) was very close to that of the pyrophosphate due to the similar shape and charge.



(216)



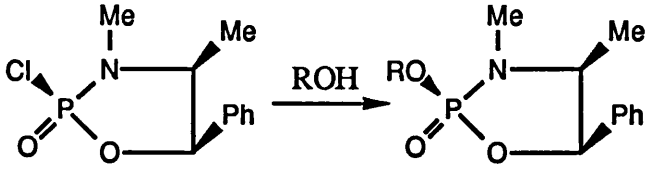
(217)

Figure 72

7.7 Miscellaneous Syntheses

In order to address the two key problems of resolution and phosphorylation, we decided that it would be interesting to try to phosphorylate (202) with a chiral phosphorylating agent. Thus both problems of phosphorylation and resolution could be addressed

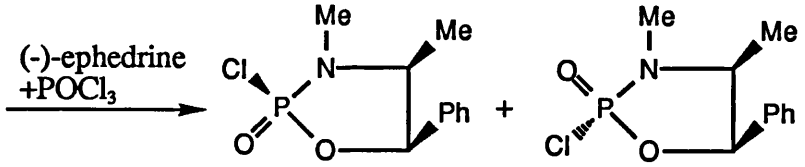
simultaneously. Efficient resolution with the chiral phosphorus reagent would significantly reduce the number of synthetic steps required to produce pure enantiomers of the desired inositol phosphate. To this end the diasteromeric reagents, the 2-chloro-1,3,2-oxazaphospholidine-2-ones were synthesised. As previously mentioned [see chapter 2], these compounds have been used to phosphorylate alcohols with retention[242] of configuration about phosphorus to give chiral phosphates.



(64) (63)

Scheme 91

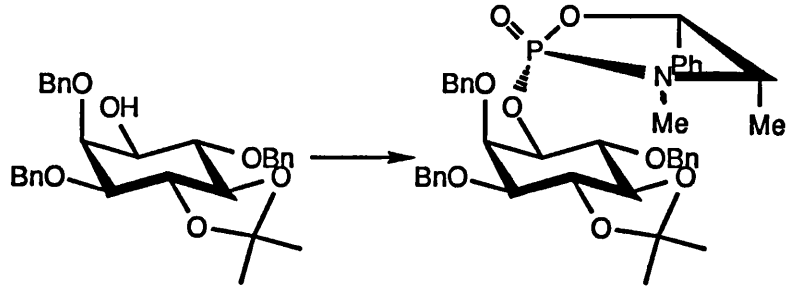
These chiral phosphates have been used to study the stereochemical course of several enzymic reactions[243]. The reagent (2R,4S,5R)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-one (64) was thus chosen as the chiral phosphorylating agent. The compound (64) was easily obtained as the major product from the reaction of phosphoryl chloride and (-)-ephedrine [(1R, 2S)-2-methylamino-1-phenylpropan-1-ol] to give (64) in 67% yield, plus the other diastereoisomer (2S, 4S, 5R)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-one (65) in 6% yield[118].



(64) (65)

Scheme 92

The latter product (65) could be obtained in a similar manner starting from (+) ephedrine instead of (-)-ephedrine[118].



(202) (218)

Scheme 93

A sample of (202) in diisopropylamine was stirred with the reagent (64) and after 30 min. the reaction was seen to be finished by NMR. In the proton NMR, part of which is

shown [Fig. 72], both diastereoisomers can be identified in the crude mixture in the ratio of 1:1.2 along with unreacted phosphorochloridate. The three N-methyl groups can clearly be seen, one at δ 2.85 ppm $^3J_{PH}$ 12 Hz from excess reagent, the other two from the pair of diastereoisomers, at δ 2.69 and 2.70 $^3J_{PH}$ 9 Hz ppm [Fig. 73].

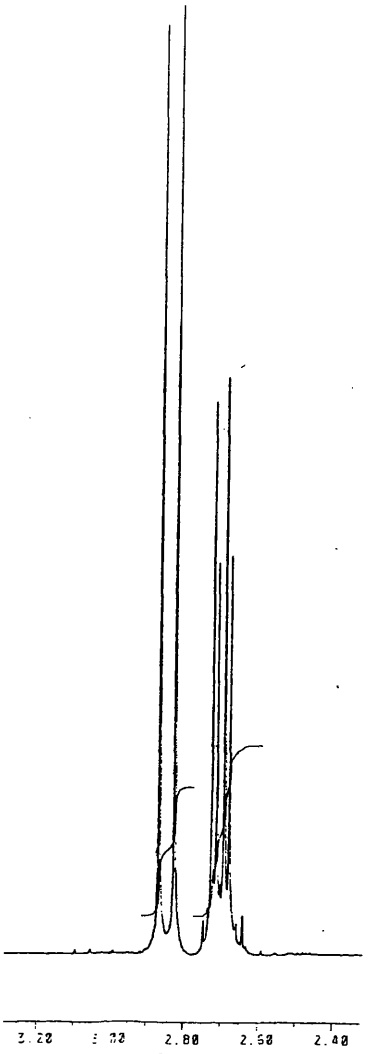


Figure 73. 300 MHz ^1H NMR of (218) in $CDCl_3$

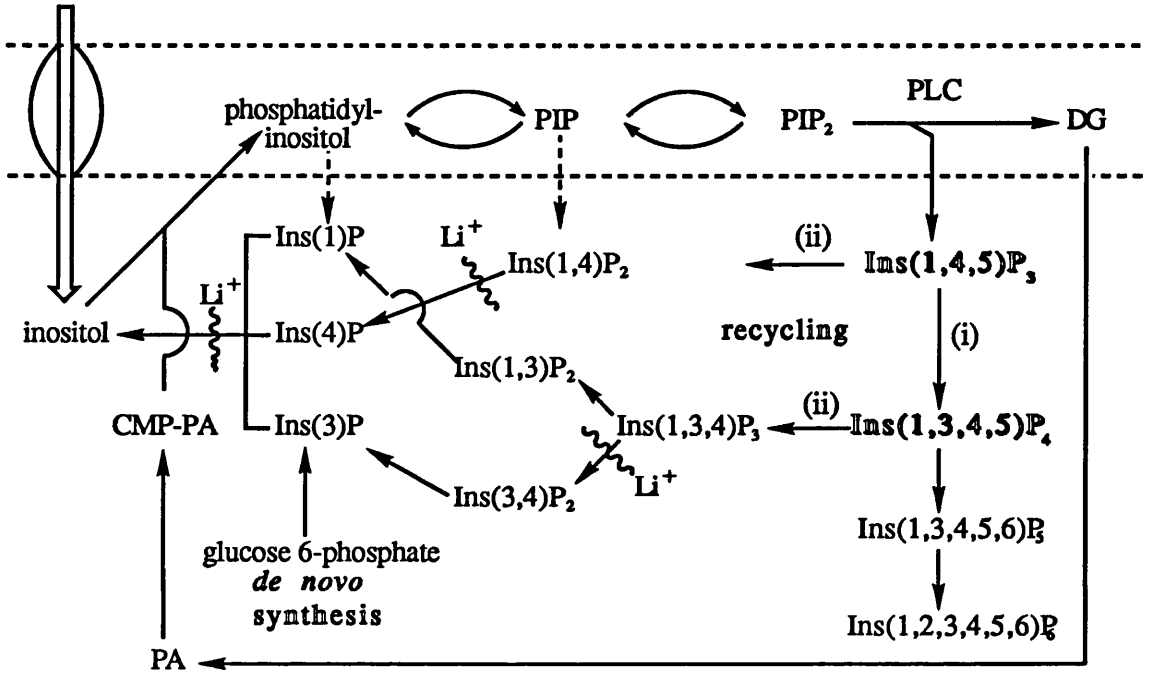
The phosphorus resonance of (218) is shifted upfield by *ca.* 3 ppm as compared to the signal of (64). However, it is not possible to see two distinct signals of the two diastereoisomers. Separation of these two promising diastereoisomers will lead to the first reported chiral synthesis of Ins(1,4,5)P_3 -45S_2, Ins(1,4,5)P_3 -5S and Ins(1,4:5 pyro)P_3 plus other interesting inositol phosphates.

Chapter Eight

Pharmacology

8.1 Introduction.

Once the second messenger has elicited its response it must be metabolised to an inactive intermediate that can be recycled back to a precursor of the second messenger. $Ins(1,4,5)P_3$ is metabolised by two distinct pathways, one initially anabolic, the other catabolic.



The phosphatidyl inositol cycle.

Figure 74

8.2 Inositol Polyphosphate 5-Phosphatase.

The catabolic pathway starts by the dephosphorylation of $Ins(1,4,5)P_3$ at the 5-position by a 5-phosphatase, no metabolite produced via this pathway posses Ca^{2+} mobilising activity. There are two soluble 5-phosphatases, type I, type II and one membrane bound 5-phosphatase, all of which have been isolated from human platelets[244], and have the same substrates, namely $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$ and cyclic 1:2,4,5 inositol trisphosphate Ins(c 1:2,4,5)P_3. All of the 5-phosphatases act by cleaving off the phosphate at the 5-position to yield *myo*-inositol -1,4-bisphosphate $Ins(1,4)P_2$, *myo*-inositol-1,3,4-trisphosphate $Ins(1,3,4)P_3$ and cyclic 1:2,4 inositol trisphosphate Ins(c 1:2,4)P_2 respectively. $Ins(1,4)P_2$ and Ins(c1:2,4)P_2 are inactive at releasing calcium from intracellular stores, $Ins(1,3,4)P_3$ is 10 to 30 fold less active at releasing calcium than $Ins(1,4,5)P_3$. The major difference between the two soluble phosphatases is in their substrate specificities, kinetic behaviour and apparent molecular masses. Type I 5-

phosphatase is very similar to the membrane-bound 5-phosphatase; both have a much greater affinity for Ins(1,3,4,5)P_4, (K_m 1 μM), than for Ins(1,4,5)P_3,(K_m 10 μM)[245,246]; the presence of 1μM Ins(1,3,4,5)P_4 inhibits the dephosphorylation of Ins(1,4,5)P_3 or Ins(c1:2,4,5)P_3 by the type I or the membrane-bound 5-phosphatase. However, the maximal rate of dephosphorylation of Ins(1,3,4,5)P_4 is very low, approximately 2% of that of Ins(1,4,5)P_3[247]. This probably leads to an extended presence of Ins(1,3,4,5)P_4. The apparent molecular mass of both type I and the membrane-bound form is 38 kDa by size-exclusion chromatography. However, the mass was altered by the presence of detergent, the molecular mass increased to 51 kDa, due to abnormal binding of the detergent molecule cholate. Type II 5-phosphatase has less of an affinity for Ins(1,3,4,5)P_4, (K_m > 150 μM) than for Ins(1,4,5)P_3 (K_m 72 μM). The apparent molecular mass for the type II phosphatase was much larger than the other two proteins, at 115 kDa.

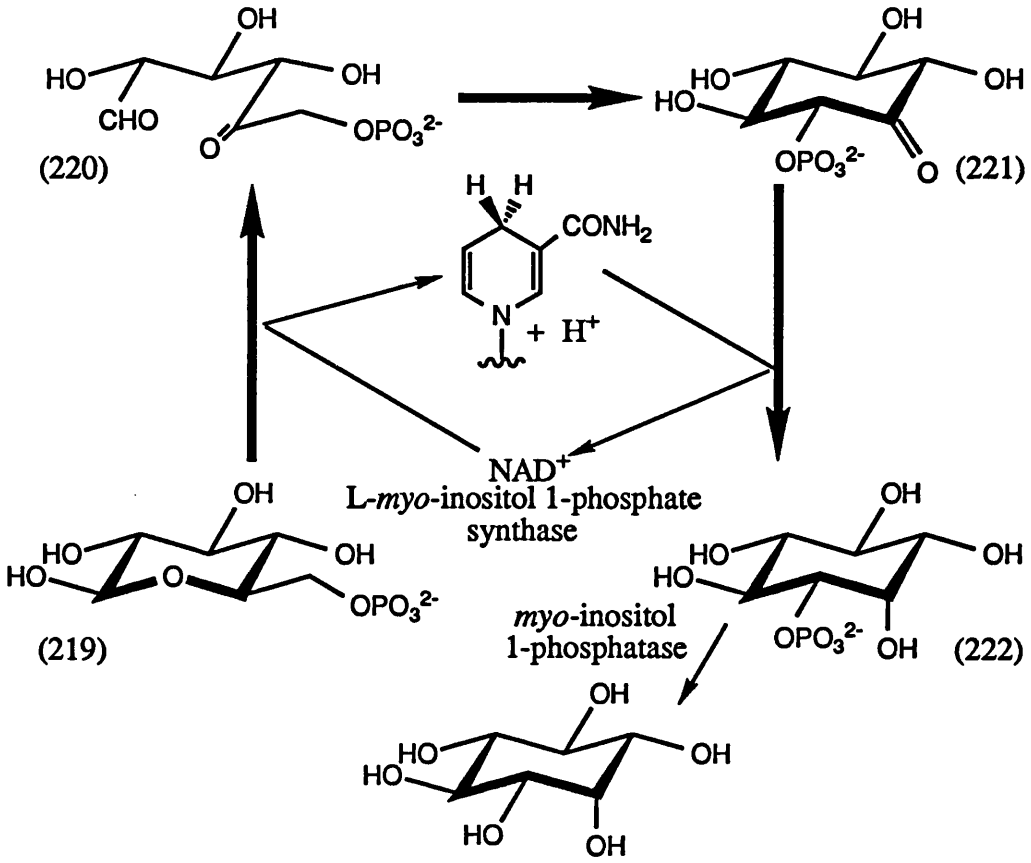
8.3 Ins (1,4,5) P_3 3-Kinase.

The other inactivation pathway of Ins(1,4,5)P_3 involves the phosphorylation of Ins(1,4,5)P_3 at the 3-position by a specific 3-kinase to Ins(1,3,4,5)P_4. The Ins(1,3,4,5)P_4 thus formed may act by gating extracellular Ca^{2+} across the cell membrane[248]. Ins(1,4,5)P_3 3-kinase phosphorylates Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 in the presence of ATP and magnesium ions[249]. In most tissues the enzyme is soluble except for turkey erythrocytes where it is membrane-bound[250]. Since, Ins(1,4,5)P_3 3-kinase has a high affinity for Ins(1,4,5)P_3, (K_m 0.2 to 1.5 μM)[251], which is greater than that of the Ins(1,4,5)P_3 5-phosphatase, it effectively completes for Ins(1,4,5)P_3 and forms Ins(1,3,4,5)P_4. The 3-kinase protein has been purified to homogeneity from rat brain and is composed of two 53 kDa catalytic subunits plus calmodulin[252].

8.4 Other Inositol Polyphosphate Kinases and Phosphatases.

The products formed by the action of the 3-kinase and the 5-phosphatase are further metabolised by a number of inositol polyphosphate kinases and phosphatases eventually to form *myo*-inositol. An inositol polyphosphate 4-phosphatase converts Ins(1,3,4)P_3 and Ins(3,4)P_2 to Ins(1,3)P_2 and Ins(3)P respectively. This enzyme is metal ion independent and has a mass of 110 kDa by gel filtration and 105 kDa by SDS-PAGE filtration. As inositol polyphosphate 4-phosphatase and inositol polyphosphate 1-phosphatase act on Ins(1,3,4)P_3, the relative activities of these two enzymes determine the levels of Ins(1,3)P_2 and Ins(3,4)P_2. In all tissues except brain the polyphosphate 4-

phosphatase accounts for 5 to 20% of Ins(1,3,4)P_3 hydrolysis. However, in brain this enzyme accounts for 70% of Ins(1,3,4)P_3 hydrolysis which implies that polyphosphate 4-phosphatase may play a role in neuronal function. Inositol polyphosphate 3-phosphatase has been found to hydrolyse Ins(1,3)P_2 to Ins(1)P but it does not act upon Ins(1,3,4)P_3, Ins(3,4)P_2 or Ins(3)P, again this enzyme is metal ion independent. Inositol polyphosphate 1-phosphatase hydrolyses Ins(1,3,4)P_3 and Ins(1,4)P_2 to Ins(3,4)P_2 and Ins(4)P respectively. This enzyme has a mass of 40 to 46 kDa as determined by gel filtration and requires Mg^{2+} ions and is inhibited by Ca^{2+} and Mn^{2+} ions. Li^+ uncompetitively inhibits inositol polyphosphate 1-phosphatase hydrolysis of both Ins(1,3,4)P_3 and Ins(1,4)P_2 with differing K_i, 0.3 and 6 mM, implying that the Li^+ binds to the phospho-enzyme complex. There is one final phosphatase, inositol monophosphate phosphatase. This enzyme is less specific for its substrates, it hydrolyses the phosphate from the 1-, 3- ,4- and 5-positions on inositol. It also has a similar affinity for some non-inositol substrates. This enzyme is a dimer and a cDNA clone isolated implies that this enzyme is unrelated to any other known protein[253]. The enzyme requires Mg^{2+} and is competitively inhibited by other divalent cations. It is uncompetitively inhibited by lithium ions, as with the Li^+ inhibition of inositol polyphosphate 1-phosphatase it is thought that the Li^+ binds to the phospho-enzyme complex thus preventing the hydrolysis of the product[254]. The understanding of the Li^+ inhibition of these two enzymes is of great importance, as Li^+ has been extensively used in the treatment of manic-depressive disorders. The presence of Li^+ in brain prevents the resynthesis of inositol from inositol monophosphates by uncompetitively inhibiting the inositol polyphosphate 1-phosphatase. Li^+ also prevents the *de novo* synthesis of inositol from glucose 6-phosphate. The brain relies upon this pathway for its inositol as inositol cannot cross the blood-brain barrier. So Li^+ effectively reduces the cycling of the Ins(1,4,5)P_3 to such en extent that the radical mood swings of mania and depression are alleviated in such afflicted patients.



Scheme 94

D-glucose 6-phosphate (219) is oxidised to 5-keto-glucose 6-phosphate (220) by the NAD^+-dependent oxidoreductase L-*myo*-inositol 1-phosphate synthase. The aldolase activity of this enzyme then closes the ring stereospecifically to form L-*myo*-inosose-2,1-phosphate (221). The keto group is then stereospecifically reduced by the synthase using NADH to give L-*myo*-inositol 1 phosphate [D-Ins(3)P] (222). The action of the Li^+ is to stop the hydrolysis by inositol polyphosphate 1-phosphatase or inositol monophosphate phosphatase, of the monophosphate to inositol and thus prevent the resynthesis of the PIP_2 and thus stop the second messenger function by which neurotransmitters alter electrical activity in brain.

There are a number of kinases present in cells that act on the higher phosphates of inositol. Inositol (1,3,4) P_3 6-kinase, Inositol (1,3,4,6) P_4 5-kinase and Inositol (3,4,5,6) P_4 1-kinase, the first enzyme acts on Ins(1,3,4)P_3 to give Ins(1,3,4,6)P_4. The other two enzymes act on Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 respectively to give Ins(1,3,4,5,6)P_5. It can be seen that the inositol lipid cycle produces a plethora of inositol phosphates, only a few of these phosphates are used for cellular signalling, namely; Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4,the other compounds produced have varying cellular functions, Ins(1,3,4,5,6)P_5 has been found to modulates the oxygen affinity of

haemoglobin in birds[255]. Phytic acid Ins(1,2,3,4,5,6)P_6, plays an important role in plants, it acts as an antioxidant and as a storage molecule for phosphorus and divalent cations.

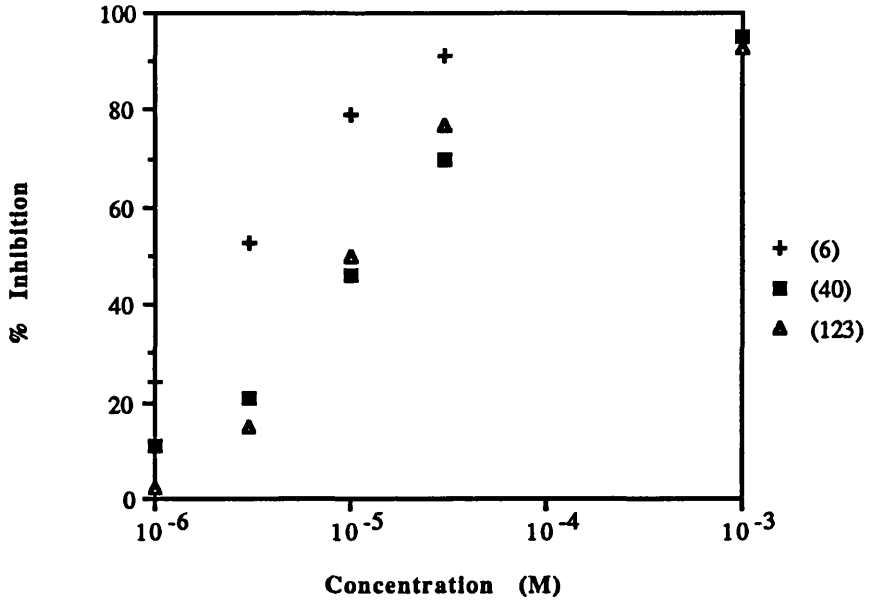
8.5 Biological Activity of Inositol Analogues

The analogues synthesised thus far have been tested for inhibitory activity upon three main systems: for effects upon the 5-phosphatase, 3-kinase and intracellular Ca^{2+} release. We report here the assessment of the inhibition of the 5-phosphatase enzyme for various analogues. This work was conducted in the Department of Pharmacology, Leicester University in conjunction with Steven Safrany and S. R. Nahorski. The assessment of the 3-kinase and Ca^{2+} release activity was performed by Steven Safrany and will only be briefly reported here. Ins(1,4,5)P_3-4,5-pyro (5) was tested for Ca^{2+} release by microinjection into invertebrate photoreceptors of the horseshoe crab *Limulus* by Prof. R. Payne, University of Maryland, U.S.A.

The analogue DL-*myo*-inositol-1,4,5-trisphosphorothioate Ins(1,4,5)PS_3 was found to be active in binding to specific brain receptors[184] with only a small reduction in potency as compared to Ins(1,4,5)P_3, and capable of releasing intracellular Ca^{2+} with only a four-fold reduction in potency as compared to Ins(1,4,5)P_3[186]. The presence of the phosphorothioate at the 5-position of Ins(1,4,5)PS_3 does confer resistance to the membrane-bound 5-phosphatase[184] and is a potent inhibitor of the enzyme[187]. Ins(1,4,5)PS_3 is not bound by the 3-kinase and does not compete with the binding of Ins(1,4,5)P_3[256].

8.5.1 Ins(1,4,5)P_3-5-Phosphatase Activity

We decided to try to probe the binding requirements of the membrane-bound 5-phosphatase by testing several of the analogues for their ability to inhibit 5-phosphatase. The inhibition of membrane-bound 5-phosphatase activity relies on the ability of the test ligand to compete effectively for binding to the active site of the enzyme in competition to the natural substrate. The competition between the two ligands is monitored by the generation of "hot" inorganic phosphate formed by the 5-phosphatase-mediated cleavage of D-5-[^{32}P]-Ins(1,4,5)P_3 to Ins(1,4)P_2 and [^{32}P]-phosphate.



Plot of the inhibition of Ins(1,4,5)P_3-5 phosphatase

Figure 75

By determining the value of K_m for the 5-phosphatase as 40 μM[187,256] it was possible to calculate the inhibition constants for the compounds tested and some others.

	K_i
DL-*myo*-inositol 1-phosphate-4,5-pyrophosphate (5)	300 μM < 50% inhibition
DL-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate (6)	1.3 ± 0.3 μM (n=4).
cyclohexane 1,2-cyclic pyrophosphate (40)	59 ±12 μM (n=3)
cyclohexane 1,2-bisphosphorothioate (123)	54 ±7 μM (n=3)
DL-*myo*-inositol 1,4-bisphosphate-5-phosphorothioate (7)	6.8 μM
DL-*myo*-inositol 1,4,5-trisphosphorothioate	1.7 μM
D-2,3-bisphosphoglycerate	978 μM

The analogue Ins(1,4,5)P_3-4,5S_2 (6) having a phosphorothioate groups at the 4 and 5 positions resembles the natural substrate more than Ins(1,4,5)PS_3 and was found to have a K_i =1.3 μM, whereas the analogue Ins(1,4,5)P_3-5S (7), a closer structural analogue was found have K_i =6.8 μM[256]. It appears that the phosphorothioate groups of an inositol phosphorothioate, having lower pK_a values than phosphate groups, can bind with a higher affinity to Ins(1,4,5)P_3-5-phosphatase. This would also explain why Ins(1,4,5)PS_3, which contains 3 phosphorothioate groups, and why Ins(1,4,5)P_3-4,5S_2, which contains 2 phosphorothioate groups are more potent inhibitors of Ins(1,4,5)P_3-5-phosphatase than is Ins(1,4,5)P_3-5S, which contains only one phosphorothioate group.

In addition, because phosphorothioate groups are more hydrophobic than phosphate groups, the lower K_i values for Ins(1,4,5)PS_3 and Ins(1,4,5)P_3-4,5S_2 relative to that of Ins(1,4,5)P_3-5S, may reflect enhanced hydrophobic interactions between the Ins(1,4,5)P_3-5-phosphatase and the analogues possessing more than one phosphorothioate group.

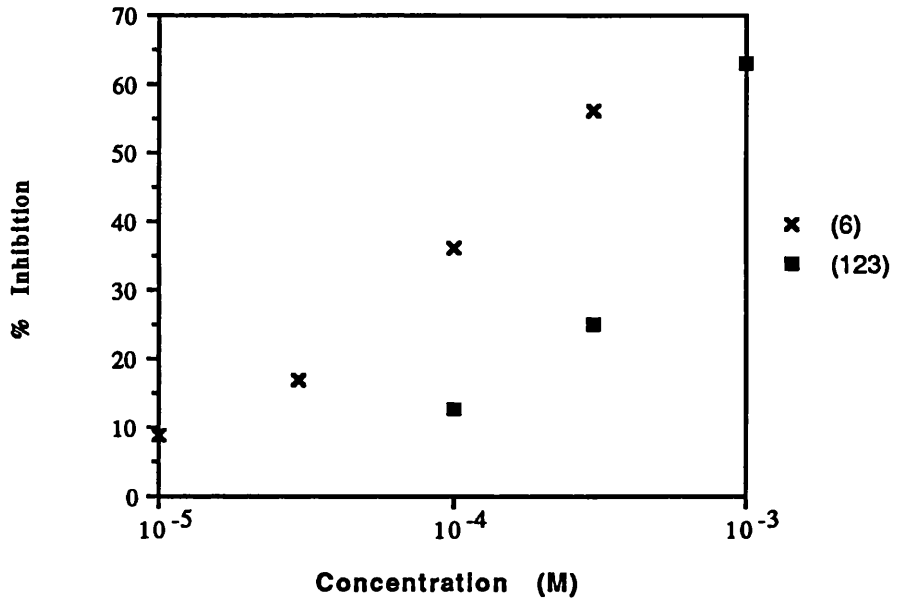
Two further results are rather surprising, two compounds which resemble the substrate only remotely, have K_i values of the order of the K_m of Ins(1,4,5)P_3. The other analogues tested were; cyclohexane 1,2 bisphosphorothioate (123), which had 3 hydroxyl groups and one phosphate group of Ins(1,4,5)P_3 absent and had a K_i =59 μM. The other analogue cyclohexane-1,2-pyrophosphate (40) had 3 hydroxyl groups and one phosphate group of Ins(1,4,5)P_3 absent and a pyrophosphate linkage instead of two phosphate groups, and yet this compound had a K_i =59 μM. This is a strange result since the pyrophosphate analogue of Ins(1,4,5)P_3, Ins(1, 4:5 pyro)P_3 (5) does not seem to inhibit the 5-phosphatase, less than 50% inhibition at 300 μM.

2,3-Bisphosphoglycerate, (BPG) was found to inhibit competitively the 5-phosphatase enzyme weakly. The conformationally mobile BPG is able to rotate to achieve approximately the desired conformation of the two phosphates for binding. Whereas the inositol phosphorothioates with the correct *trans*-vicinal arrangement bind to the 5-phosphatase with a greater affinity than the natural substrate D-Ins(1,4,5)P_3 (K_m 40 μM). It is possible that at the pH of the experiments (7.2) the phosphorothioate moieties were more charged than the phosphates, thus leading to a stronger binding of the phosphorothioates. When considering the binding of the 5-phosphorothioate as compared to the trisphosphorothioate, this idea is supported, as the mono-phosphorothioate has an intermediate binding between the two. However, the bisphosphorothioate has an inhibition constant very similar to that of the trisphosphorothioate. This may mean that the strength of the binding is determined by the interaction between the phosphate and the enzyme at both the 4- and the 5- positions, whereas the type of phosphate moiety at position-1 is not of as much importance as the position-4 moiety. Both the cyclohexane-1,2-bisphosphorothioate (123) and cyclohexane1,2-pyrophosphate (40) bind and inhibit but not to the extent of the other analogues. Nevertheless, it is of interest that these analogues are far removed from Ins(1,4,5)P_3 in structure and yet is still are moderately potent 5-phosphatase inhibitors. This is a testimony to the promiscuity of the 5-phosphatase in binding many compounds, whereas the 3-kinase appears to be much more selective[256,257].

8.5.2 *Ins(1,4,5)P_3-3-Kinase Activity*

The potential inhibitor compounds were tested for their ability to compete with tritium labelled substrate [^{3}H]-Ins(1,4,5)P_3 for the binding to the 3-kinase enzyme. After incubation of 3-kinase with inhibitor and [^{3}H]-Ins(1,4,5)P_3, the reaction was stopped and the resultant mixture of [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-Ins(1,3,4,5)P_4 was separated into the two components. Since the amount of the [^{3}H]-Ins(1,4,5)P_3 left unaltered was a measure of the inhibition of the 3-kinase by the inhibitor, it was possible to determine the K_i for the various inhibitors.

Ins(1,4,5)P_3-4,5S_2 was tested for 3-kinase activity and was found to have a K_i =46 μM, approximately 10 fold less than the affinity for the natural substrate, K_m =3.2 μM.



Plot of the inhibition of Ins(1,4,5)P_3-5 kinase

Figure 76

3-Kinase activity

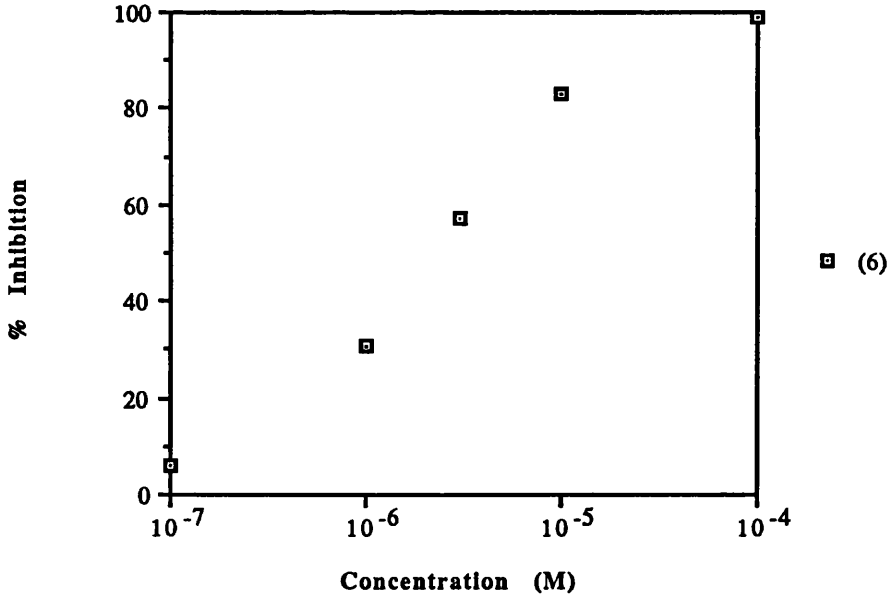
	K_i
DL-*myo*-inositol 1-phosphate-4,5-pyrophosphate (5)	>> 600 μM
DL-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate (6)	46 ± 12 μM (n=3).
cyclohexane 1,2-cyclic pyrophosphate (40)	>250 μM (n=2)
cyclohexane 1,2-bisphosphorothioate (123)	134 ±3 μM (n=2)

Cyclohexane 1,2-bisphosphorothioate (123), cyclohexane 1,2-pyrophosphate (40) and

Ins(1,4:5 pyro)P_3 (5) were found to be very poor inhibitors of the 3-kinase. A possible explanation for this result is that the enzyme cannot recognise the potential analogues as the differences between these molecules and the natural substrate are too great for the enzyme to accommodate them in its active site.

8.5.3 Ins(1,4,5)P_3-Mediated Ca^{2+}-Release Activity

It was hoped that the introduction of Ins(1,4:5 pyro)P_3 into a whole cell system would lead to the production of Ins(1,4,5)P_3 after an initial lag phase. To this end a sample of Ins(1,4:5 pyro)P_3 (5) was microinjected into the photoreceptor cells of the crab *Limulus*. However, intracellular phosphatases and pyrophosphatases did not cleave the pyrophosphate bond. Consequently, there was no resultant Ins(1,4,5)P_3-mediated release of Ca^{2+}. Ins(1,4:5 pyro)P_3 and other analogues were tested for their ability to release Ca^{2+} in an electroporated cell system.



Plot of Ca^{2+} release from Ins(1,4,5)P_3 sensitive stores mediated by (6)

Figure 77

The potential inhibitors for Ca^{2+} release were tested according to Safrany *et. al.*[256].

	EC_{50}
D-*myo*-inositol 1,4,5-trisphosphate	0.1 μM
DL-*myo*-inositol 1,4-bisphosphate-5-phosphorothioate (7)	0.8 ± 0.2 μM
DL-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate (6)	1.4 ± 0.4 μM
DL-*myo*-inositol 1-phosphate-4,5-pyrophosphate (5)	>> 30 μM
cyclohexane 1,2-cyclic pyrophosphate (40)	no Ca^{2+} release

It can be seen that the non-hydrolysable phosphorothioate analogues release Ca^{2+} with differing efficacies. The greater the structural analogy to Ins(1,4,5)P_3, the greater the Ca^{2+} release. DL-*myo*-Inositol 1,4-bisphosphate-5-phosphorothioate (7), only having one phosphate group altered *c.f.* Ins(1,4,5)P_3, releases Ca^{2+} with the lowest EC_{50}. Secondly, DL-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate (6) has two phosphates groups replaced with phosphorothioates moieties, and has the next lowest EC_{50}. Finally, the trisphosphorothioate analogue has the largest EC_{50} of the group. Both of the pyrophosphate analogues tested, DL-*myo*-inositol 1-phosphate-4,5-pyrophosphate (5) and cyclohexane 1,2-cyclic pyrophosphate (40) did not seem to have any Ca^{2+} release activity. This is probably due to a requirement of the receptor for phosphate groups at position-4 and -5 to have rotational mobility.

8.6 Experimental

8.6.1 Preparation of D-Ins(1,4,5)P_3-5-Phosphatase

Human blood (80 ml) in citrate anticoagulant (3.2%, trisodium salt, 10 ml) was spun at 2500g for 5 min. The supernatant liquid and "buffy coat" were aspirated and the red cells were resuspended in isotonic saline/HEPES buffer (NaCl 0.154 M, HEPES 1.5 mM, pH 7.2 with NaOH). The cells were again centrifuged and the supernatant liquid was aspirated. This process was repeated 4 times until no "buffy coat" was present.

The cells were stirred for 30 min. in large quantities of ice-cold, low ionic strength Tris-buffer (Tris 10 mM, EDTA 1 mM, pH 7.0 with HCl) so that lysis occurred. The membranes were centrifuged (50 000g, 30 min., 4 °C). The red supernatant liquid was poured off leaving the erthrocyte ghosts above a dark red pellet of unlysed cells. The membranes were resuspended in the buffer and centrifuged as previously described, the process was repeated four more times and the membranes were transferred to a clean centrifuge tube. The membranes were washed a further four times in HEPES buffer (HEPES 2.5 mM, EGTA 1 mM, pH 7.2 with NaOH) until the membranes were white and the supernatant liquid was colourless. After removal of the supernatant, the pellets were resuspended in isotonic saline (15 ml), centrifuged again resuspended in isotonic saline (15 ml) and stored at -40 °C. Lowery protein assay[258] showed the protein concentration to be 2.5 mg ml^{-1}.

8.6.2 *Determination of K_m and V_{max} for D-Ins(1,4,5)P_3-5-Phosphatase*

Ins(1,4,5)P_3 (from 10-200 μM, 10 μl) and D-5-[^{32}P]-Ins(1,4,5)P_3 (*ca.* 3 pmol, 10 μl) were dissolved in magnesium buffer (30 mM HEPES, 2 mM $MgSO_4$, 20 μl). The reaction was started by the addition of the membrane preparation (10 μl, 25 μg protein) prepared as described earlier, and incubated at 37 °C for 30 min. The reaction was quenched by the addition of ice cold buffer (200 μl) and $HClO_4$ (250 μl, 2M). The protein was removed by centrifugation at 16 000g for 3 min. The supernatant liquid was removed and a solution of ammonium molybdate (10 μl of a 0.4 g ml^{-1} soln. in water) was added. The complex of phosphorus and molybdenum thus produced was separated from the inositol phosphates by adding isobutyl alcohol (400 ml) and toluene (400 μl), perturbation of the layers by vortex and subsequent separation of the layers by centrifugation gave the phospho-molybdate complex in the upper layer. The complex was then taken with scintillation fluid (optiphaseX, 4.5 ml) and the number of disintegrations was counted in a scintillation counter over 5 min. Since less than 20% of the substrate was turned over in this experiment linear kinetics were observed, so it was possible to determine the observed binding constant (K_m) and velocity constant (V_{max}) as 40 μM and 1.1 nmol min^{-1} mg^{-1} of protein respectively.

8.6.3 *D-Ins(1,4,5)P_3-5-Phosphatase Inhibition Assay*

A solution of D-Ins(1,4,5)P_3 (200 μmol, 200 μM, 10 μl) was spiked with D-5-[^{32}P]-Ins(1,4,5)P_3 (10 000 d.p.m., 0.6 μl). A sample of this (200 μmol, 200 μM, 10 μl) was taken with the analogue to be tested, various concentrations of the the analogue were made up in magnesium buffer (30 mM HEPES, 2 mM $MgSO_4$, 20 μl) to give final assay concentrations of 0-1 mM. The reaction was started by the addition of the erythrocyte membranes (20 μl, 25 μg protein). The incubation, quenching and subsequent counting as above.

A plot of percentage inhibition against concentration of inhibitor, gives the value of the concentration required to displace 50 % of the radioactive substrate from the enzyme, (IC_{50}). From the value of the IC_{50} it is possible to calculate the inhibition constant (K_i) by using the relationship below, where K_m is the Michaelis-Menten constant for the substrate and [S] is the concentration of inhibitor.

$K_i = IC_{50}/(1+[S]/K_m)$.

Chapter Nine

Experimental Section

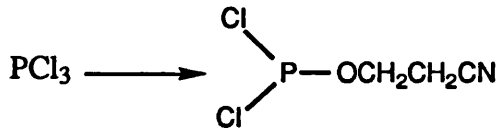
9 Analytical and Preparative Methods

Thin layer chromatography (tlc) was performed on precoated plates (Merck silica 60F): products were visualised by UV or by spraying phosphomolybdic acid in methanol or 5% palladium chloride in M HCl followed by heating. Flash chromatography was performed on silica gel (SORBSIL C60). ^{1}H-NMR spectra were recorded on a Bruker AM-300 spectrometer or on Jeol FX90Q, JMN-GX270 or JMN-GX400 spectrometers. Chemical shifts are referenced to internal tetramethylsilane. ^{13}C-NMR spectra were recorded on a Bruker AM-300 spectrometer or on Jeol JMN-GX270 or JMN-GX400 spectrometers. Chemical shifts are referenced to external 1,4-dioxan. ^{31}P-NMR spectra were recorded on a Bruker AM-300 spectrometer or on Jeol FX60, FX90Q, JMN-GX270 or JMN-GX400 spectrometers. Chemical shifts are referenced to external 85% phosphoric acid and are positive when down field to this reference. Two-dimensional NMR experiments, ^{1}H-^{1}H Correlation (COSY), heteronuclear (^{1}H-^{13}C, ^{1}H-^{31}P) shift correlation and homonuclear ^{1}H-^{1}H J-resolved (J-res) experiments were acquired using the standard pulse sequences[259,260] at either, 270 MHz, 300 MHz or 400 MHz, using the Jeol JMN-GX270, Bruker AM-300 and the Jeol JMN-GX400 spectrometers respectively. ^{1}H-^{1}H (COSY) spectra were acquired with quadrature detection in both dimensions, and complete phase cycling. Routinely, 32 scans were accumulated in 512 blocks of 1024 data points. The second dimension being zero-filled once, both windows subject to sine bell multiplication and subsequently made symmetrical along the diagonal. Mass spectra were recorded at the SERC Mass Spectrometry Service Centre, Swansea or by the Mass Spectrometry Service , University of Bath. FAB mass spectra were carried out using *m*-nitrobenzyl alcohol (NOBA), glycerol or thioglycerol matrices. Melting points (uncorrected) were determined using a Reichert-Jung Thermo Galen Kofler Block micro melting point apparatus. Ion-exchange chromatography was performed on an LKB-Pharmacia automated medium pressure liquid chromatography system equipped with a 'GP250 plus' gradient programmer, using columns packed with Q-Sepharose Fast Flow or DEAE Sephadex A-25 resin and eluting with a gradient of triethylammonium bicarbonate (TEAB) buffer pH 8.0. Quantitative analysis of phosphate was achieved using the Briggs' phosphate assay[220], quantitative analysis of phosphorothioate was performed using a 5,5'-dithio-bis-(2-nitrobenzoic acid) (DNTB) assay [261]. UV measurements for the colorimetric assays were carried out on Shimadzu UV-240 and Perkin-Elmer Lambda 3B instruments. Microanalysis was performed by the Microanalysis Service, University of Bath. All chemicals were obtained from Aldrich Chemical Company, except authentic samples of DL-2,3,6-tri-*O*-benzyl-*myo*-inositol 1-di(2,2,2-trichloroethyl) phosphate (204), DL-2,3,6-tri-*O*-benzyl-*myo*-inositol 1,4-

bis[di(2,2,2-trichloroethyl) phosphate] (210) and DL-2,3,6-tri-*O*-benzyl-5-propenyl-*myo*-inositol 1,4-bis[di(2,2,2-trichloroethyl) phosphate] (212) which were provided by Dr. R. Gigg, NIMR, Mill Hill. The sample of 2,3-cyclohexylidene-6-deoxy-D-*myo*-inositol was a gift from Dr D. Dubreuil, University of Bath.

9.1 Synthesis of Phosphitylation Reagents

9.1.1 Synthesis of Dichloro-2-Cyanoethoxyphosphine (223)



(223)

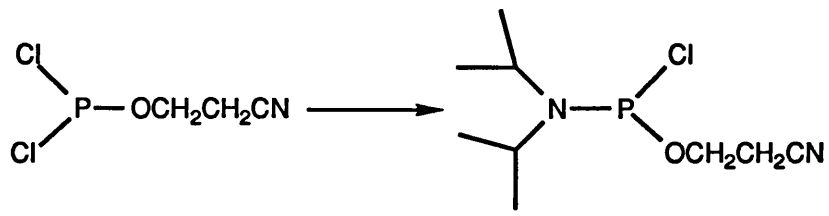
Scheme 95

Dichloro-2-cyanoethoxy-phosphine (223) was prepared according to the procedure of Ogilvie[210]. 2-Cyanoethanol (34 ml, 0.5 mol) in ether (50 ml) was added slowly under nitrogen to a cooled solution (-78°C) of pyridine (40 ml, 0.5 mol) in ether (150 ml) containing phosphorus trichloride (44.0 g, 0.5 mol). After a total of 1h at -78°C, ether (100 ml) was added to the solution. Removal of the pyridine hyrochloride by filtration and removal of the ether by evaporation under reduced pressure gave a clear liquid that was distilled under reduced pressure to give dichloro-2-cyanoethoxy phosphine (57.94 g, 52%).

b.p 69-73°C, 0.25 mm Hg {lit.[210] b.p. 70°C 0.4 mm Hg}.

δ_P (ether, 36 MHz) 178.29 (s). {lit.[210] δ_P 175.94 ppm}.

9.1.2 Synthesis of Chloro-(Diisopropylamino)-2-Cyanoethoxyphosphine (78)



(223) (78)

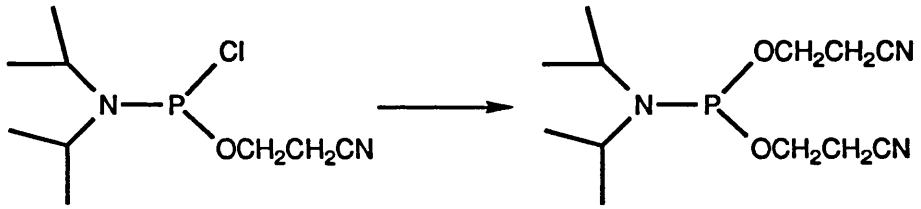
Scheme 96

Chloro diisopropylamino (2-cyanoethoxy) phosphine (78) was prepared according to the procedure of Sinha[211]. To a stirred solution of (223) (40.0 g, 263 mmol) in ether (120 ml) at -78°C, a solution of diisopropylamine (50.3 g, 526 mmol) in ether (60 ml) was added over 1.5h under nitrogen. Addition of ether (150 ml) precipitated the colourless solid diisopropylamino-hydrochloride which was removed by filtration. Solvent was removed by evaporation *in vacuo*, the crude yellow oil was distilled under reduced pressure to give chloro diisopropylamino (2-cyanoethoxy) phosphine (78) (40.22 g, 63%).

b.p. 95-96°C 0.09 mm Hg {lit.[211] b.p. 103-104°C 0.8 mm Hg}.

δ_P (ether, 36 MHz) 179.34 (s). {lit.[211] δ_P 179.82 ppm}.

9.1.3 Synthesis of Di(2-Cyanoethoxy) (Diisopropylamino) Phosphoramidite (81)



(78) (81)

Scheme 97

To a stirred solution of (78) (4.21 g, 17.8 mmol) in acetonitrile (4 ml) under an atmosphere of dry nitrogen at 0°C, diisopropylethylamine (2.56 g, 20 mmol) and 2-cyanoethanol (1.42 g, 20 mmol) were added over 30 min, then left for 2h. Flash chromatography of the crude material down a short column of silica gel with diethyl ether-pentane (3:1) (300 ml) gave pure (81) (3.402 g, 70%).

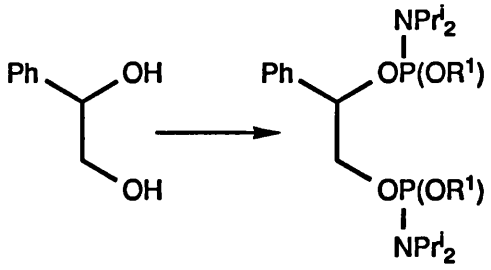
R_f 0.44 (diethyl ether-pentane; 3:1).

δ_P (ether, 36 MHz) 148.77 (s).

9.2 Synthesis of 1-Phenyl Ethane-1,2-Bisphosphorothioate

Syntheses of intermediates that were not isolated nor fully characterised due to their reactive nature are described in a subsection.

9.2.1 *Synthesis of 1-Phenyl Ethane-1,2-Bis[(2-Cyanoethoxy) (Diisopropylamino) Phosphoramidite] (147)*



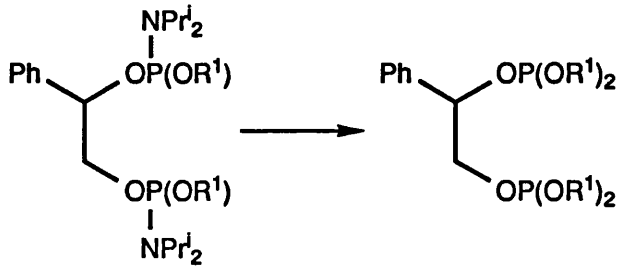
(147)

Scheme 98. $R^1=CH_2CH_2CN$

A sample of 1-phenyl ethane 1,2-diol (281 mg, 2.03 mmol) was dried by co-evaporation of dry acetonitrile, then redissolved in dry acetonitrile (2 ml) and N,N-diisopropylethylamine (529 mg, 4.4 mmol). To the solution under an atmosphere of dry nitrogen, (78) (1090 mg, 4.6 mmol) was added over 1 min. with stirring. After 1h this gave 1-phenyl ethane 1,2-bis[(2-cyanoethoxy) (diisopropylamino) phosphoramidite] (147).

δ_P (CH_3CN, 36 MHz) 180.11 (s, excess reagent), 147.64 (s).

9.2.2 *Synthesis of 1-Phenyl Ethane 1,2-Bis[Di(2-Cyanoethyl) Phosphite] (146)*



(147) (146)

Scheme 99 $R^1=CH_2CH_2CN$

A sample of (147) in acetonitrile, the crude product from the previous reaction was taken and stirred with tetrazole (341 mg, 4.9 mmol) and 2-cyanoethanol (365 mg, 5.1 mmol) and kept under nitrogen. After 1h at rt the product 1-phenyl ethane 1,2-bis[di(2-cyanoethyl) phosphite] (146) was formed.

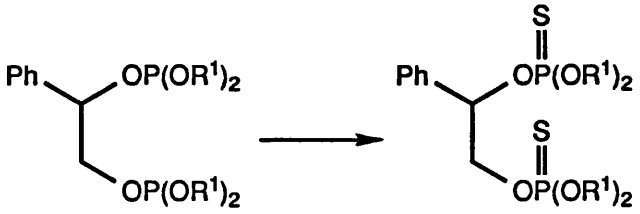
δ_P (CH_3CN, 36 MHz) 138.51.

The product was formed directly using di(2-cyanoethoxy) (diisopropylamino) phosphoramidite (81) instead of chloro (diisopropylamino)-2-cyanoethoxy phosphine (78), thus avoiding the synthesis of the intermediate phosphoramidite (146).

A mixture of 1-phenyl ethane 1,2-diol (6.9 mg, 50 μmol) and tetrazole (4.2 mg, 60 μmol) were dried by co-evaporation of dry acetonitrile, the mixture was then redissolved in dry acetonitrile (2 ml) and di(2-cyanoethyl) (diisopropylamino) phosphoramidite (81) (16.4 mg, 60 μmol) was added with stirring over 1 min. After 30 min. the reaction was seen to have been completed by NMR.

δ_P (CH_3CN, 36 MHz) 148.2 (s, excess reagent), 138.85 (s), 7.8 (s, phosphonate).

9.2.3 Synthesis of 1-Phenyl Ethane 1,2-Bis[Di(2-Cyanoethyl) Phosphorothioate] (148)



(146) (148)

Scheme 100 $R^1=CH_2CH_2CN$

A crude sample of (146) (1.036 g, 2.03 mmol) was dissolved in dry pyridine (5 ml), to this solution sulphur (0.5 g, 15.62 mmol) was added over 1 min. with stirring. After 8h excess sulphur was removed by filtration and the solvents were evaporated *in vacuo* to give crude 1-phenyl ethane 1,2-bis[di(2-cyanoethyl) phosphorothioate] (148). The crude product was then purified by flash column chromatography on silica gel, ($CHCl_3$-ethyl acetate, 1:4) to give pure (148) (1.028 g, 1.89 mmol, 93%) as a colourless glass.

R_f 0.35 ($CHCl_3$-ethyl acetate, 1:4)

δ_H ($CDCl_3$, 300 MHz) 2.77 (8H, m, $CH_2C\underline{H}_2CN$), 4.21-4.43 (10H, m, 4 $C\underline{H}_2CH_2CN$, $C\underline{H}_2OPS$), 5.72 (1H, ddd, ^3J 3.6, 7.5 Hz, $^3J_{PH}$ 12 Hz, PhC$\underline{H}$OPS)

δ_C ($CDCl_3$, 76 MHz), 18.50, 19.80 (2t, $\underline{C}H_2CN$), 62.29-63.20 (t, O$\underline{C}H_2CH_2CN$),

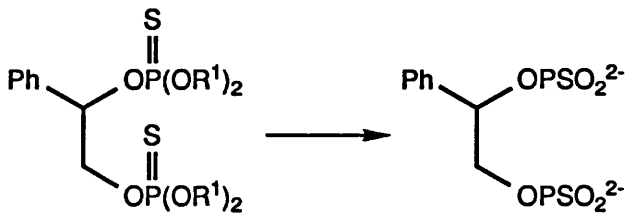
70.03-70.08 (t, $\underline{C}H_2OPS$), 80.01-80.06 (d, $\underline{C}HOPS$), 116.80, 117.00, 117.20, 117.31 (4s, $\underline{C}N$), 127.00, 129.20, 130.00 (3d, Ph), 135.00 (s, Ph).

δ_P ($CDCl_3$, 121 MHz) 64.04 (s, $CHO\underline{P}$), ^{31}P-^{1}H (m, ^{3}J 2.7, 12 Hz); 64.39 (s, $CH_2O\underline{P}$), ^{31}P-^{1}H (m ^{3}J 9.1 Hz).

m/z (FAB, +ve ion, NOBA), 323 (mono-dephosphorothiolation, 10%), 149 [(SH)(OH)PO(OR),100].

E.I. 323 (18%), 322 (81), both due to dephosphorothiolation.

9.2.4 Deprotection of 1-Phenyl Ethane 1,2-Bis[Di(2-Cyanoethyl) Phosphorothioate] (148) to give 1-Phenyl Ethane 1,2-Bisphosphorothioate (145)



(148) (145)

Scheme 101 $R^1=CH_2CH_2CN$

A sample of (148) (1.00 g, 1.8 mmol) was put into two pressure vials (2 x 5 ml), with aqueous ammonia (10 ml, 35%) and heated at 60 °C for 3h. The aqueous ammonia was then removed *in vacuo* and the resultant oil was taken up in water (10 ml) and stirred with a cation-exchange resin (H^+-Dowex 50-W-X8 20-50 mesh) for 30 minutes. The residue was washed with ethyl acetate (2 x 20 ml), then triethylamine (2 ml), removal of solvent under reduced pressure yielded a pale yellow glass of the triethylammonium salt of (145) (876 mg, 90%). This was further purified by passage through a DEAE-Sephadex A-25 anion-exchange chromatographic column with a linear gradient of triethylammonium bicarbonate buffer, pH 7.2; 40 mM-600 mM. The title compound (145) eluted at 250 mM TEAB removal of buffer gave a glass. Treatment of the glass with cyclohexylamine (1 ml) followed by acetone (50 ml) gave a colourless solid, the triscyclohexylammonium salt of 1-phenyl ethane 1,2 bisphosphorothioate (214 mg, 406 μmol, 22%) from 1-phenyl ethane diol via the phosphoramidite intermediate (147).

δ_H ($CDCl_3$, 270 MHz), 3.88, 4.00 (2H, m, ^{3}J 6, $^{3}J_{PH}$ 12 Hz, $C\underline{H}_2OP$), 5.28 (H, dt, ^{3}J 6, 12 Hz, $C\underline{H}OP$), 7.34 (3H, m, Ar-$\underline{H}$), 7.49 (2H, m, Ar-$\underline{H}$).

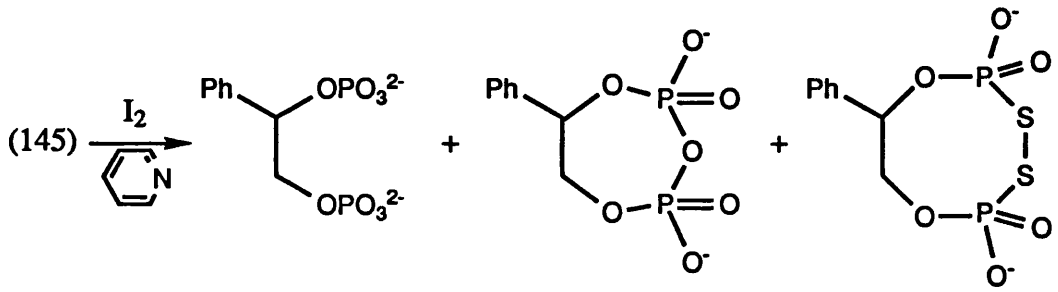
δ_C ($CDCl_3$, 67.80 MHz), 67.69 (t, $\underline{C}H_2OPS$), 74.86 (d, $\underline{C}HOPS$), 126.78, 127.14, 127.76 (3d, Ar-$\underline{C}$), 140.28 (s, Ar-$\underline{C}$).

δ_P ($CDCl_3$, 161.8 MHz), 42.58 (s), ^{31}P-^{1}H (d, ^{3}J 12 Hz, pos. 1); 42.70 (s), ^{31}P-^{1}H (t, ^{3}J 6 Hz, pos. 2).

m/z (FAB -ve ion NOBA), 659 [$(2M-H)^-$,15%], 329 [$(M-H)^-$,100].
(FAB +ve ion NOBA), 530 (17%), 431 (85), 100 [$(C_6H_{11}NH_3)^+$,100].

9.3 Halogen-Mediated Oxidative Desulphurisation of (145)

9.3.1 Desulphurisation of (145) using a Saturated Solution of Iodine in Pyridine



(154) (149) (155)

Scheme 102

To a stirred solution of 1-phenyl ethane 1,2-bisphosphorothioate (145) (31 mg, 50 μmol) in water (0.5 ml) at rt, a saturated solution of iodine in dry pyridine (0.5 ml) was added over 1 min. After stirring for 2h the solvents were removed *in vacuo* to produce a glass. The glass was washed with water, extracted with ether to remove the iodine, and again washed with water, the solvents were removed under reduced pressure and the glass produced was dried *in vacuo* over P_2O_5, to give three products; the bisphosphate (154) (53%), the pyrophosphate (149) (13%) and a possible cyclic disulphide (155) (34%).

δ_P (D_2O, pH 9, 36 MHz) -10.08 (m), 3.83 (d), 15.93 (m).

The doublet at δ 3.83 ppm due to the presence of bisphosphate (154), the signal at δ-10.08 ppm due to the trace of pyrophosphate (149), however, there was not enough sample to see the AB system. In order to determine if the signal at δ 15.93 ppm was due to the presence of the cyclic disulphide (155), the sample was taken with 2-

mercaptoethanol (25 µl) stirred for a further hour. Solvents were removed *in vacuo*.and the NMR was taken.

δ_P (D_2O, pH 9, 36 MHz) –10.68 (m), 3.83 (d), 42.15 (m), 43.97 (m), 44.78 (m).

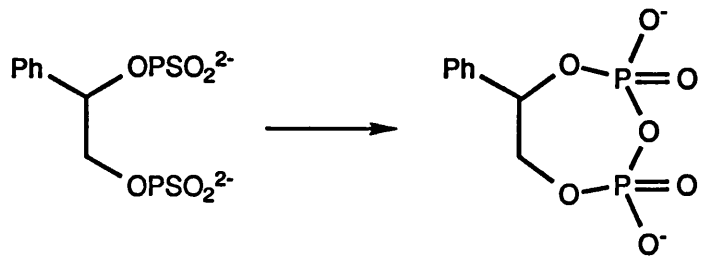
The compound responsible for the signal at δ_P 15.93 ppm was reduced by the 2–mercaptoethanol implying that the compound was in fact the cyclic disulphide (155).

9.3.2 Desulphurisation of (145) using Cyanogen Bromide

1-Phenyl ethane 1,2-bisphosphorothioate (145) (26 mg, 40 µmol) was taken in dioxan (1.6 ml) and water (0.4 ml) then stirred at rt. To this solution cyanogen bromide (85 mg, 802 µmol, 20 fold excess) was added over 1 min. After 15 minutes at rt the reaction was quenched by the addition of 2–mercaptoethanol (54 µl) which reduced the excess cyanogen bromide. Dilution with triethylammonium bicarbonate buffer (TEAB) (10 mM, 7 ml, pH 8.5) followed 1 min. later. Solvents were removed *in vacuo*, the resultant glass was washed twice with water (20 ml) and resulted in mixture of three products, the pyrophosphate (149) (22%), the bisphosphate (154) (10%), the cyclic disulphide (155) (58%) and starting material (10%).

δ_P (H_2O, pH 9, 36 MHz) δ_1 –11.64, δ_2 –10.59 [dd, $^2J_{1,2}$ 18.5 Hz, (149)], 3.3 [s, (154)], 3.4 [s, (154)], δ_1 17.76, δ_2 19.89 [dd, $^2J_{1,2}$ 4.9 Hz, (155)], 42.64 [s, (145)], 42.75 [s, (145)].

9.3.3 Desulphurisation of (145) using N–Bromosuccinimide.



(145) (149)

Scheme 103

1-Phenyl ethane 1,2-bisphosphorothioate (145) (31.3 mg, 50 µmol), was dissolved in dioxan and water (4:1) and an eight fold excess of NBS was added. The mixture was shaken and after 1 min excess NBS was reduced by addition of 2–mercaptoethanol (25

μl). After a further minute the reaction was diluted with TEAB buffer and solvent was removed *in vacuo* to yield a crude mixture of 1-phenyl ethane 1,2-cyclic pyrophosphate (149) and 1-phenyl ethane 1,2-bisphosphate (154). The products were dissolved in TEAB buffer, (100 ml, 50 mM, pH 7.3) and loaded onto a DEAE-sephadex A 25 chromatographic ion exchange column, (20 x 2.5 cm). A linear gradient 50 mM to 600 mM of TEAB buffer was eluted through the column separating the two products.

The triethylammonium salt of 1-phenyl ethane 1,2-cyclic pyrophosphate (149) eluted over a range of 390 to 410 mM TEAB as a pale yellow glass, yield 38%.

δ_H (D_2O, pH 9, 270 MHz) 4.11 (1H, m, ^2J 11.3 Hz, $^3J_{PH}$ 7.4 Hz, HCHOP), 4.21 (1H, m, ^2J 11.3 Hz, $^3J_{PH}$ 7.4 Hz, HCHOP), 5.52 (1H, m, ^3J 7.4 Hz, $^3J_{PH}$ 6.4 Hz, PhCHOP) 7.48 (5H, m, Ar-H).

δ_C (D_2O, pH 9, 68 MHz) 58.57 (d, CHOP), 76.54 (t, CH_2OP), 126.07, 128.47 (2d, Ph), 132.17 (s, Ph).

δ_P (D_2O, pH 9, 161 MHz) -11.20 (dd, $^2J_{1,2}$ 18.8 Hz); ^{31}P-^1H (d, $^3J_{PH}$ 6.4 Hz, pos. 1), –10.23 (dd, $^2J_{1,2}$ 18.8 Hz); ^{31}P-^1H (t, $^3J_{PH}$ 7.4 Hz, pos. 2).

m/z, (FAB, -ve ion thioglycerol), 559 [$(2M-H)^-$, 10%], 279 [$(M-H)^-$, 100%], 177 (7), 159 (29).

The by-product 1-phenyl ethane 1,2-bisphosphate (154) eluted over a range of 550 to 560 mM TEAB as colourless glass, yield 62%.

δ_H (D_2O, pH 9, 270 MHz) 3.90 (2H, dd, ^3J 6, 6.5 Hz, CH_2OP), 5.20 (1H, dd, ^3J 6, 8 Hz, PhCHOP), 7.40 (5H, m, J 6.75, 7.50, 9.00 Hz, Ph).

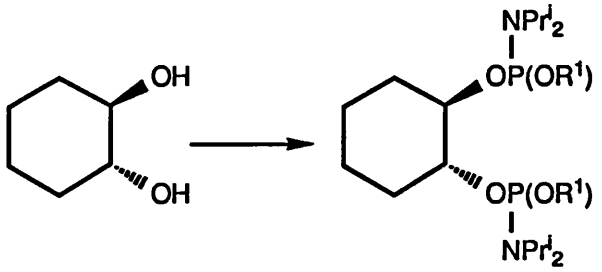
δ_C (D_2O, pH 9, 68 MHz) 67.89 (t, CH_2OP), 75.31 (d, PhCHOP), 126.36, 127.47, 127.95 (3d, Ph), 139.47 (s, Ph).

δ_P (D_2O, pH 9, 121 MHz) 1.58 (s); ^{31}P-^1H (d, $^3J_{PH}$ 8.0 Hz, pos. 1), 2.63 (s); ^{31}P-^1H (t, $^3J_{PH}$ 6.5 Hz, pos. 2),.

m/z, (FAB, -ve ion NOBA), 595 [(M+three cyclohexylamine)$^-$, 35%]. 497 (35), 319 (35), 297 [$(M-H)^-$, 100], 199 (33), 181 (48).

9.4 Synthesis of Cyclohexane Cyclic Pyrophosphate (40)

9.4.1 Synthesis of Cyclohexane 1,2-Bis[Di(2-Cyanoethyl) (Diisopropylamino) Phosphoramidite] (158)



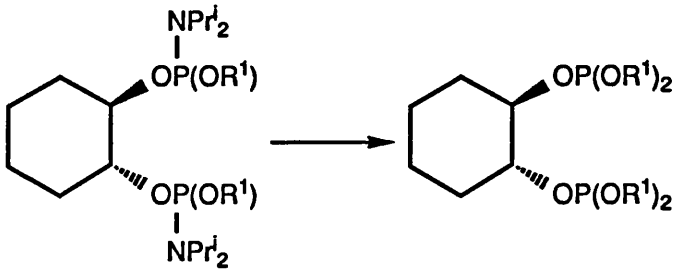
(158)

Scheme 104 $R^1=CH_2CH_2CN$

trans-Cyclohexane 1,2-diol (237 mg, 2.04 μmol) was dried by co-evaporation of dry solvent, then redissolved in diisopropylethylamine (569 mg, 4.4 μmol) and dry acetonitrile (2 ml) and stirred at 20°C, (78) (1.19 g, 5.03 mmol) was added dropwise over 5 min. After 1h. the formation of cyclohexane 1,2-bis[(2-cyanoethyl) (diisopropylamino) phosphoramidite] (158) was seen to have been complete by NMR.

δ_P (CH_3CN, 36 MHz) 180.11 (s, excess reagent), 146.42 (s).

9.4.2 Synthesis of Cyclohexane 1,2-Bis[Di(2-Cyanoethyl) Phosphite] (159)



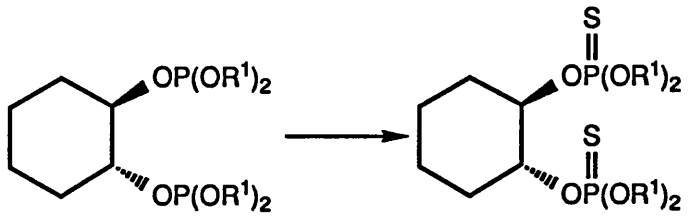
(158) (159)

Scheme 105 $R^1=CH_2CH_2CN$

A sample of (158) as the crude solution from the previous reaction, was taken and to this tetrazole (420 mg, 6 μmol) and 2-cyanoethanol (426 mg, 6 μmol) were added over 1 min. Stirring for 1h resulted in the production of cyclohexane 1,2-bis[di-(2-cyanoethyl) phosphite] (159).

δ_P (CH_3CN, 36 MHz) 138.76 (s).

9.4.3 *Oxidation of* trans-*Cyclohexane 1,2-Bis[Di(2-Cyanoethyl) Phosphite] (159)*



(159) (160)

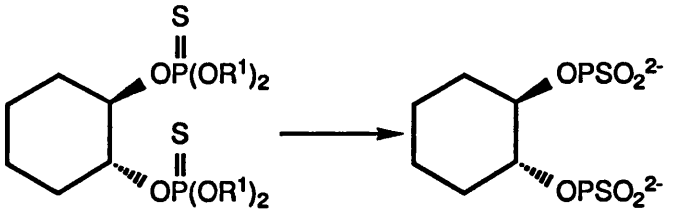
Scheme 106 $R^1=CH_2CH_2CN$

The crude product (159) in solution from above was oxidised *in situ.* for 3h by the addition of elemental sulphur (0.5 g, 16 mmol) in dry pyridine (5 ml). Purification by flash column chromatography on silica gel with ethyl acetate-chloroform (1:5) eluent, to give gave cyclohexane 1,2-bis[di-(2-cyanoethyl) phosphorothioate] (160) as a glass (1.008 g, 1.938 mmol, 95%, from *trans*-cyclohexane-1,2-diol.

R_f 0.35 (ethyl acetate-chloroform, 1:5)

δ_P (CH_3CN, 36 MHz) 65.95 (s)

9.4.4 *Deprotection of* trans-*Cyclohexane 1,2-Bis[Di(2-Cyanoethyl) Phosphite] (160)*



(160) (157)

Scheme 107 $R^1=CH_2CH_2CN$

A sample of (160) (1.000 g, 1.923 mmol) was divided in two and then put into pressure vials (5 ml). Aqueous ammonia (2 x 5 ml, 35%) was added and the mixture was heated to 65°C under pressure for 11h. The aqueous ammonia was then removed *in vacuo,* the oil was taken up in water (150 ml), stirred with a cation-exchange resin (H^+-Dowex 50-W-X 8 20-50 mesh) for 30 min., after removal of resin by filtration, the resultant oil was washed with ethyl acetate (2 x 20 ml). The solvents were removed under reduced pressure and the glass thus produced was dried *in vacuo* over P_2O_5 and NaOH.

Addition of water (1 ml) and cyclohexylamine (3 ml) produced a viscous oil. The tris cyclohexylammonium salt of cyclohexane 1,2 bisphosphorothioate (157) was precipitated as a colourless solid upon addition of acetone (70 ml), (0.708 g, 61%).

Anal. Calc. for $C_6H_{14}O_6P_2S_2$.4 cyclohexylamine. 1 H_2O: C, 49.83; H, 9.49; N 7.75. Found: C, 49.8; H, 9.62; N, 7.80%.

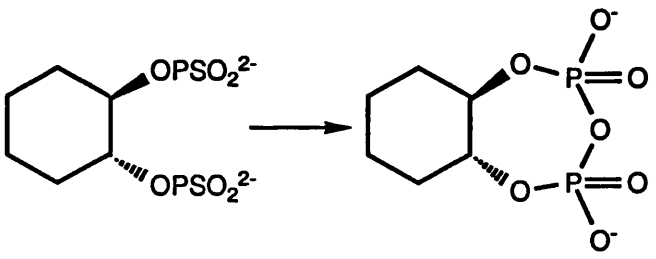
δ_H (D_2O, pH 7, 270 MHz), 1.05-1.40 (8H, m, ring-H), 3.02-3.61 (2H, m, CHOPS).

δ_C (D_2O, pH 7, 68 MHz), 23.28 (t, ring-CH_2), 29.84 (t, ring-CH_2), 49.72 (d, CHOPS).

δ_P (D_2O, pH 9, 161.8 MHz) 41.52 (s, CHOPS); ^{31}P-^{1}H (d, $^{3}J_{PH}$ 8 Hz, CHOPS).

m/z (FAB -ve ion NOBA) 615 [$(2M-H)^-$, 10%], 501 (15), 307 [$(M-H)^-$, 100], 273 (14). (FAB +ve ion NOBA),508 (12%), 409 (48), 100 [$(C_6H_{11}NH_3)^+$, 100].

9.4.5 NBS-Mediated Desulphurisation of Cyclohexane-1,2-Bisphosphorothioate (157)



(157) (40)

Scheme 108

Cyclohexane 1,2-bisphosphorothioate (157) (30.2 mg, 50μmol) was dissolved in water (0.4 ml) and dry dioxan (1.6 ml), to this stirred solution NBS (80 mg, 449 μmol, 9 fold excess) was added at rt. The reaction was quenched after 2 min. by the addition of 2-mercaptoethanol (25 μl), then TEAB (10 ml, 10 mM) 1 minute later. Evaporation of solvents under reduced pressure was followed by ion exchange chromatography to give the cyclic pyrophosphate (40) as a colourless solid of the biscyclohexylammonium salt (20 mg, 88%),

δ_H (D_2O, pH 9, 270 MHz) 2.38 (1H, d, ^{3}J 3.0 Hz), 2.43 (4H, t, ^{3}J 4.2 Hz), 2.75 (1H, s), 2.85 (2H, t, ^{3}J 6.0 Hz), 3.83 (2H, t, ^{3}J 6.0 Hz).

δ_C (D_2O, pH 9, 68 MHz) 58.77 (t, ring-C), 49.00, 48.10 (2d, CHOP), 39.53, 33.76, 32.49 (3t, ring-C).

δ_P (D_2O, pH 9, 109.25 MHz) -11.69 (s); ^{31}P-^{1}H (m).

m/z, (FAB, -ve ion glycerol), 487 (12%), 384 (20), 359 [$(M+Et_3N)^-$, 60], 256 [$(M-2H)^-$, 100], 127 (54)

The bisphosphate (42) (1.9 mg, 8%) was isolated as a colourless triethylammonium salt.

δ_P (D_2O, pH 9, 121 MHz) 2.40, 2.42 (2s).

9.5 Desulphurisation of (145) in the presence of $H_2{}^{18}O$

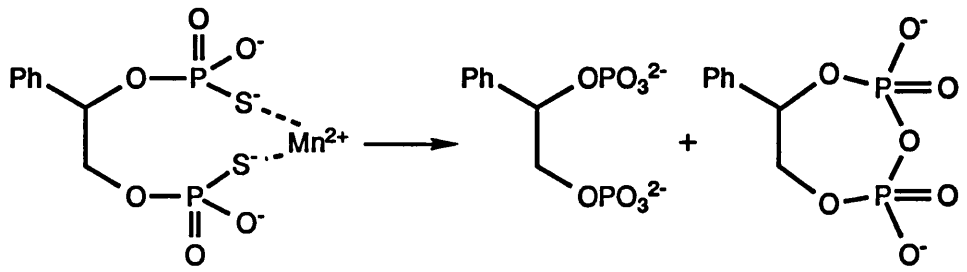
A sample of (145) (31.3 mg, 50 μmol) was taken and dried by repeated co-evaporation of dry dioxan. The dry sample was then dissolved in dioxan (1.6 ml), water (0.2 ml) and oxygen-18 water ($H_2{}^{18}O$) (0.2 ml). The solution was stirred at rt for 2 min until the solid had fully dissolved. NBS (77 mg, 432 μmol) was added, stirred for 2 min. and then the reaction was halted by the addition of 2-mercaptoethanol (25 μl), followed 1 min. later by TEAB (10 mM, 10 ml). The solvents were removed *in vacuo* and the products were separated by ion-exchange chromatography to give labelled pyrophosphate (149) (32%).

δ_P (D_2O, pH 9, 121 MHz) -11.81 (dd, $^2J_{1,2}$ 18.6 Hz), -10.73 (dd, $^2J_{1,2}$ 18.6 Hz), -11.80 (dd, $^2J_{1,2}$ 18.7 Hz), -10.72 (dd, $^2J_{1,2}$ 18.7 Hz), -11.78 (dd, $^2J_{1,2}$ 18.8 Hz), -10.70 (dd, $^2J_{1,2}$ 18.8 Hz).

and labelled bisphosphate (154) (68%)

δ_P (D_2O, pH 9, 121 MHz) 2.74, 2.76 (2s), 3.34, 3.36 (2s)

9.6 Ionic Chelation Experiments



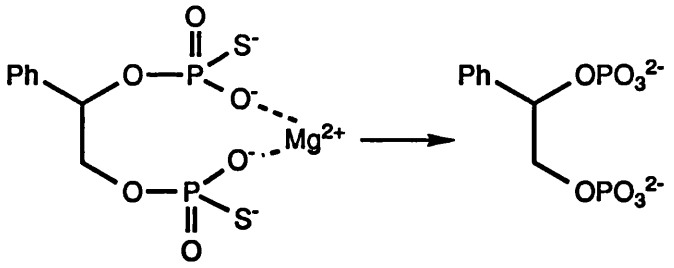
(145) (154) (149)

Scheme 109

Desulphurisation of (145) (12.5 mg, 20 μmol) was effected in a stirred solution of dioxan (1.6 ml) and $MnCl_2$ (0.4 ml, 10 mM) by the addition of NBS (29 mg, 160 μmol). The reaction was quenched by the addition of 2-mercaptoethanol (25 μl) after 2 min. This was followed by the addition of TEAB (10 ml, 10 mM). Removal of the solvents under reduced pressure, gave a mixture of the bisphosphate (154) (85%) and the pyrophosphate (149) (15%), the products were not separated, the yield was estimated by NMR

δ_P (D_2O, pH 9, 36 MHz) -9.8 (m), 2.40 (s), 2.4 (s)

The above reaction was repeated but with a different metal ion, magnesium (II) as the cation present as opposed to Mn(II).



(145) (154)

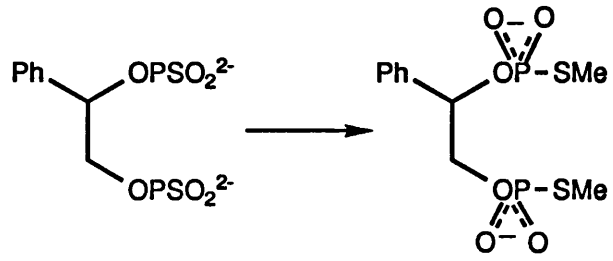
Scheme 110

A sample of (145) (19 mg, 31 μmol) was taken in a solution of dioxan (1.6 ml) and $MgCl_2$ (1 ml, 10 mM), then treated with NBS (58 mg, 326 μmol) and worked up in the normal manner. The yield of the pyrophosphate (149) was virtually zero, whereas the yield of the bisphosphate (154) was in excess of 95%.

δ_P (D_2O, pH 9, 36 MHz) 2.40 (s), 2.4 (s)

9.7 NBS-Mediated Desulphurisation of Phosphorothiolates

9.71 Synthesis of 1-Phenyl Ethane 1,2-Bis[S-Methyl Phosphorothiolate] (180)



(145) (180)

Scheme 111

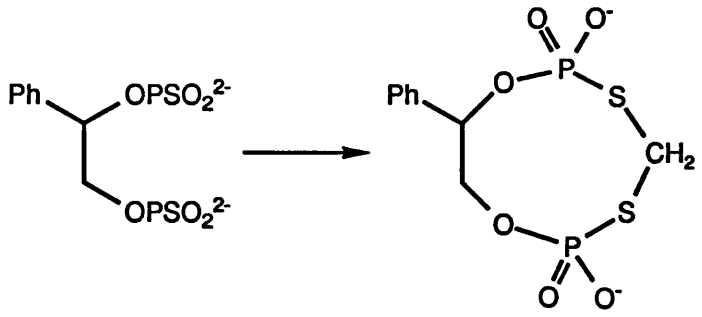
A sample of (145) (33 mg, 52 μmol) was dried azeotropically, then dissolved in methanol (500 μl) and stirred at rt. To this solution methyl iodide (52 μl, 832 μmol) was added over 1 min. After 2h the solvents were removed *in vacuo* to give an oil. This was taken up in water (20 ml) and washed with ethyl acetate (20 ml), aqueous sodium chloride solution (10 ml). The solution dried *in vacuo,* then redissolved in water (30 ml), the solution was taken and stirred with H^+-Dowex 50-W-X8 20-50 mesh cation exchange resin to pH 7, triethylamine (0.1 ml) was added to the solution, the resin was filtered and the solvents were removed under reduced pressure to give as glass, the triethylammonium salt of (180) (28.0 mg, 51 μmol, 98%).

δ_H (D_2O, pH 7, 270 MHz) 1.96, 2.19 (6H, 2 d, $^3J_{PH}$ 9 Hz, PSC$\underline{H}_3$), 4.11 (2H, m, C$\underline{H}_2$OPSMe), 5.35 (H, m, C$\underline{H}$OPSMe), 7.40-7.55 (5H, m, Ph).

δ_C (D_2O, pH 7, 68 MHz) 11.14, 11.44 (2 q, PS$\underline{C}H_3$), 68.48 (t, $^3J_{CP}$ 6.6 Hz, $\underline{C}H_2$OPS), 76.23 (d, $\underline{C}$HOPS), 126.62, 128.10, 128.21 (3 d, Ar-$\underline{C}$), 138.06 (s, Ar-$\underline{C}$).

δ_P (D_2O, pH 9, 36 MHz) 20.26 (s); ^{31}P-^1H (dq, ^3J 9, 9 Hz), 21.59 (s); ^{31}P-^1H (brd ddq).

9.7.2 Synthesis of 1-Phenyl Ethane-1,2-[Cyclic-S,S-Methylene] Bisphosphorothiolate (181)



(145) (181)

Scheme 112

A sample of (145) (15 mg, 24 μmol) was dried by co-evaporation of dry acetonitrile, then redissolved in acetonitrile (50 μl), triethylamine (17 μl, 120 μmol) and methanol (250 μl). Diiodomethane (10 μl, 120 mmol) was added. The stirred solution was then left at 20 °C for 4h. After which time the solvents were removed under reduced pressure and the residue taken up in water (20 ml). The solution was washed with ethyl acetate (10 ml), aqueous sodium chloride solution (20 ml) and then stirred with H^+-Dowex 50-W-X8 20-50 mesh cation exchange resin until the pH reached neutrality. The resin was removed by filtration and the resultant solution was diluted with water (40 ml). The solution was then loaded onto a Q-sepharose fast-flow gel (1.5 x 15 cm) anion exchange column and eluted with a linear gradient of TEAB (0-500 mM) to give the triethylammonium salt of (181) (11 mg, 20 μmol) as a colourless glass.

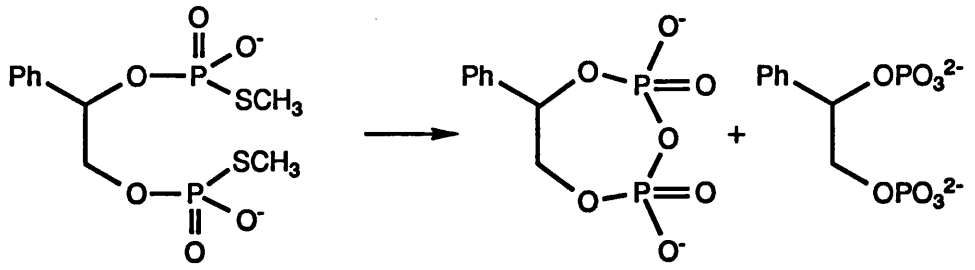
δ_H (D_2O, pH 7, 270 MHz) 3.98-4.16 (3H, m, $C\underline{H}_2OPS$, $SC\underline{H}_2$), 4.29 (1H, ddd, ^3J 9, 12.5, 14 Hz, $C\underline{H}_2OPS$), 5.46-5.53 (1H, m, $C\underline{H}OPS$), 7.28-7.40 (5H, m, Ar-$\underline{H}$).

δ_H (D_2O, pH 7, 400 MHz) ^1H irradiated at 5.4 ppm. 3.98-4.16 (3H, m, $C\underline{H}_2OPS$, $SC\underline{H}_2$), 4.29 (1H, dd, ^3J 12.5, 14 Hz, $C\underline{H}_2OPS$), 7.28-7.40 (5H, m, Ar-$\underline{H}$).

δ_C (D_2O, pH 7, 68 MHz) 17.80 (t, $S\underline{C}H_2$), 29.50 (d), 65.00 (t, $\underline{C}H_2OPS$), 77.27 (d, $\underline{C}HOPS$), 126.04, 128.21, 128.44 (3 d, Ar-$\underline{H}$).

δ_P (D_2O, pH 9, 109 MHz) 18.65 (s); ^{31}P-^1H (ddd, ^3J 4.3, 6.4, 6.7 Hz), 19.40(s); ^{31}P-^1H (brd dddd)

9.7.3 *Desulphurisation of (180)*



(180) (149) (154)

Scheme 113

A sample of (180) (14 mg, 25 μmol) was taken up in dioxan (1.6 ml) and water (0.4 ml) and desulphurised with NBS (35 mg, 197 μmol) in the normal manner. Consequent work up as above led to a mixture of 2 products the pyrophosphate (149) 55% and the bisphosphate (154) 45%, the relative yield was determined by NMR. Previous work comparing the ratio of (149) to (154) by ^{31}P NMR to the result obtained by Briggs phosphate assay, supports the idea that these two compounds have a sufficiently similar relaxation time for the integral of the ^{31}P NMR signal to be a valid reflection of the composition of the mixture.

δ_P (D_2O, pH 9, 121 MHz) -11.93 (dd, $^2J_{1,2}$ 18.8 Hz); ^{31}P-^1H (d, $^3J_{PH}$ 6.4 Hz, pos.-1), -10.96 (dd, $^2J_{1,2}$ 18.8 Hz); ^{31}P-^1H (t, $^3J_{PH}$ 7.4 Hz, pos.-2), 1.58 (s); ^{31}P-^1H (d, $^3J_{PH}$ 8.0 Hz, pos.-1), 2.63 (s); ^{31}P-^1H (t, $^3J_{PH}$ 6.5 Hz, pos.-2),.

9.7.4 *Desulphurisation of (180) in the presence of $H_2{}^{18}O$*

A sample of (180) (32 mg, 57 μmol) was dried by co-evaporation of dry dioxan. The sample was then redissolved in a solution of dioxan (1.6 ml), $H_2{}^{16}O$ (0.2 ml) and $H_2{}^{18}O$ (0.2 ml), then left to stir for 5 minutes to equilibrate. NBS (86 mg, 486 μmol) was quickly added. The reaction was then quenched after 1 min. by the addition of 2-mercaptoethanol. Addition of TEAB (10 ml, 10 mM) followed after a further 2 min. The solvents were then removed *in vacuo* and the residual glass was dissolved in a solution of D_2O (0.5 ml) with EDTA (3 mg) and triethylamine (2 drops, pH 9). Quantitative analysis of the mixture by NMR gave the ratio of pyrophosphate (149) to phosphate (154) to be 55% to 45% respectively.

δ_P (D_2O, pH 9, 109 MHz) -12.23 (dd, $^2J_{1,2}$ 18.4 Hz, pos.-1'), -11.19 (dd, $^2J_{1,2}$ 18.4 Hz, pos.-2'), -12.20 (dd, $^2J_{1,2}$ 18.4 Hz, pos.-1), -11.16 (dd, $^2J_{1,2}$ 18.4 Hz, pos.-2),

2.26 (s, pos. 1), 2.28 (s, pos. 1'), 2.82 (s, pos. 2), 2.84 (s, pos. 2'),.
pos n' denotes isotopically labelled compound.

9.8 Stability of 1-Phenyl Ethane-1,2-Cyclic Pyrophosphate

1-Phenyl ethane cyclic-1,2-pyrophosphate (10 mg, 21 μmol) (149) was taken in HCl (20 ml; 1M) and stirred at rt for 2 days, after which little hydrolysis of the pyrophosphate had been observed by ^{31}P NMR. When the temperature was elevated to 100 °C, hydrolysis of the pyrophosphate bond to the 1-phenyl ethane 1,2-bisphosphate (154) occurred within 5 min.

Similarly, when 1-Phenyl ethane-1,2-cyclic pyrophosphate (10 mg, 21 μmol) (149) was taken in sodium hydroxide (10 ml; 1M), no appreciable hydrolysis of the pyrophosphate was seen in 5 days at rt. Elevation of the temperature to 100 °C resulted in the complete conversion to the bisphosphate (154) after 2h.

Sodium (0.5 g, 22 mmol) was added to distilled ammonia (30 ml) at -78 °C. Subsequently, 1-Phenyl ethane-1,2-cyclic pyrophosphate (20 mg, 42 μmol) (149) was added with stirring over 1 min. The reaction was quenched by the addition of methanol after 60 s, the solvents were then removed under reduced pressure. The residue was taken up in water (10 ml) and stirred with H^+-Dowex 50-W-X8 20-50 mesh cation exchange resin until the pH reached neutrality. Triethylamine (1 drop) was added to increase the pH to 9.

δ_P (D_2O, pH 9, 121 MHz) -5.72 (s, O_3POPO_3); ^{31}P-^1H (s).

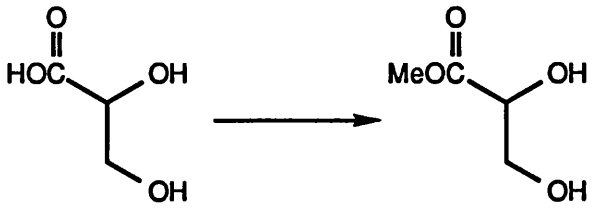
9.9 Stability of Cyclohexane-1,2-Cyclic Pyrophosphate.

Cyclohexane-1,2-cyclic pyrophosphate (14 mg, 30 μmol) (40) was treated to 1 M acid and 1 M base in the same manner as 1-phenyl ethane-1,2- cyclic pyrophosphate (149). Hydrolysis to the bisphosphate (42) did not occur at rt after 3 days, however, hydrolysis occurred at 100 °C within 15 min in the case of the acidic hydrolysis and after 5h in the case of the basic hydrolysis.

A sample of (40) (14 mg, 30 μmol) was subjected to treatment with sodium (0.5 g, 22 mmol) in liquid ammonia (30 ml) at -78°C for 1 min. The reaction was quenched after 1 min by the addition of methanol (10 ml), then diluted with TEAB (10 ml,10 mM) 60 s later. The solvents were removed *in vacuo*. to give a glass.

δ_P (D_2O, pH 9, 121 MHz) 0.47 (s); ^{31}P-^{1}H (q, ^{3}J 6.5 Hz): 3.33 (s, 242); ^{31}P-^{1}H (d, ^{3}J 7.23 Hz): 3.57 (s, 242); ^{31}P-^{1}H (d, ^{3}J 6.2 Hz).

9.10 Synthesis of (±)-1-Methyl Glycerate (185)



(184) (185)

Scheme 114

To a stirred solution of (±)-glyceric acid hemi calcium salt (12.5 g, 100 mmol) (184) in acetone (40 ml) and methanol (15 ml) at 20°C, concentrated sulphuric acid (1.5 ml) in methanol (20 ml) was added over 30 min. and left for a further 30 min. until the conversion of starting material from the base-line to product was seen by tlc. Dilution with water (50 ml), filtration and subsequent evaporation of solvents under reduced pressure gave a crude product, which after distillation under reduced pressure (14 mmHg) gave (±)-1-methyl glycerate (185) (7.05 g, 59 mmol, 59%) as a liquid.

R_f 0.5 (MeOH)

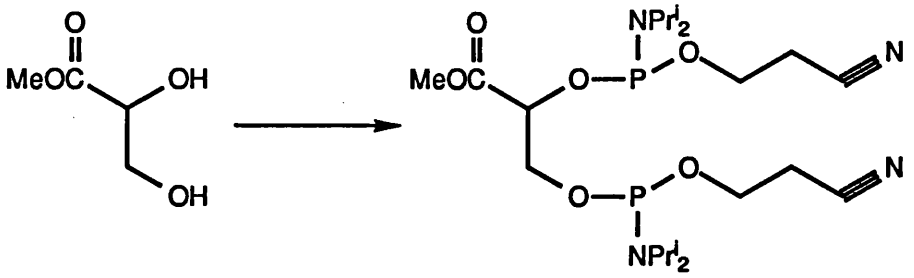
b.p. 119-120 °C 14 mmHg [Lit.[262] b.p. 239-244 °C]

δ_H (CD_3OD, 300 MHz) 3.86 (3H, s, $OC\underline{H}_3$), 3.9 (2H, d, ^{3}J 4 Hz, $C\underline{H}_2OH$), 4.46 (1H, t, ^{3}J 4 Hz, $C\underline{H}OH$), 4.85 (2H, brd, OH).

δ_C (CD_3OD, 75 MHz) 52.85 (q, $O\underline{C}H_3$), 65.00 (t, $\underline{C}H_2OH$), 73.19 (d, $\underline{C}HOH$), 174.65 (s, $\underline{C}O$).

9.11 Synthesis of (±)-1-Methyl-2,3-Bis[Di-(2-Cyanoethyl) Thiophosphoglycerate] (188)

9.11.1 Synthesis of (±)-1-Methyl-2,3-Bis[Di(2-Cyanoethyl) (Diisopropylamino) Phosphoramidoglycerate] (186)



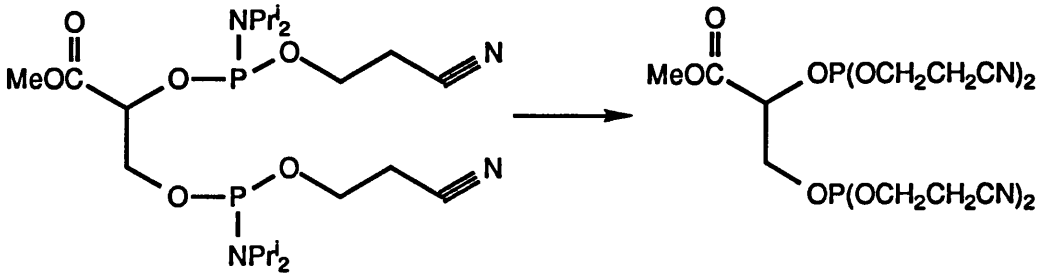
(185) (186)

Scheme 115

Diisopropylethylamine (2.042 g, 15.8 mmol) and (±)-methyl glycerate (185) (0.804 g, 6.69 mmol) were azeotropically dried. The resultant liquid was then dissolved in acetonitrile (5 ml) and kept at 20°C. To the stirred solution (209) (3.59 g, 15.15 mmol) was added dropwise over 2 min under nitrogen to give (±)-1-methyl-2,3-bis[di(2-cyanoethyl) (diisopropylamino) phosphoramidoglycerate] (186).

δ_P (CH_3CN, 36 MHz) 179.90 (s, excess reagent), 149.00 (s) 148.40 (s) 138.80 (m, $(NCCH_2CH_2O)_3P$).

9.11.2 Synthesis of (±)-1-Methyl-2,3-Bis[Di(2-Cyanoethyl) Glycerophosphite] (187)



(186) (187)

Scheme 116

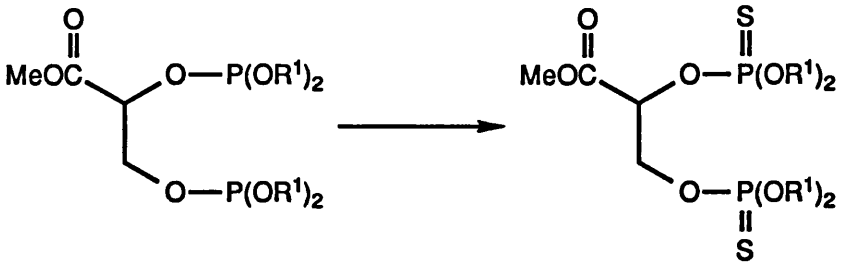
A solution of (186) in dry acetonitrile (5 ml), the product of the previous reaction, was taken without purification and treated with 2-cyanoethanol (1.34 g, 18.8 mmol) under the catalysis of tetrazole (1.32 g, 18.8 mmol). After 1h the synthesis of (±)-1-methyl-2,3-

bis[di(2-cyanoethyl) glycerophosphite] (187) was effected.

R_f 0.05 (pentane-ethyl acetate, 2:1)

δ_P (CH_3CN, 36 MHz) 143.20 (s, 218), 142.80 (s, 218).

9.11.3 Oxidation of (187) to give (±)-1-Methyl-2,3-Bis[Di-(2-Cyanoethyl) Thiophosphoroglycerate] (188)



$R^1=CH_2CH_2CN$ (187) (188)

Scheme 117

A sample of (187) was taken crude from the previous reaction. The solution was then diluted with pyridine (5 ml) and stirred. To this sulphur (0.5 g, 16 mmol) was added and stirred at 20°C. After 8 h (187) was seen by tlc to have been completely converted to (±)-1-methyl-2,3-bis[di(2-cyanoethyl) thiophosphoroglycerate] (188). Partial purification was effected by flash column chromatography on silica gel (pentane-ethyl acetate, 2:1) to give (188) as a pale yellow glass (1.86 g, 3.03 mmol, 45%) from (185).

R_f 0.3 (pentane-ethyl acetate, 2:1)

δ_H ($CDCl_3$, 300 MHz) 2.83 (8H, t, ^3J 6 Hz, $CH_2\underline{CH_2}CN$), 3.85 (3H, s, OCH_3), 4.27-4.66 (11H, m, $CHOP$, CH_2OP, 4 OCH_2CH_2).

δ_C ($CDCl_3$, 74 MHz) 19.33, 19.44, 19.54 (3t, 3 $CH_2\underline{C}H_2CN$), 53.30 (q, $O\underline{C}H_3$), 63.00 (t, $O\underline{C}H_2CH_2CN$), 67.71 (t, $\underline{C}H_2OPS$), 75.30 (d, $^2J_{CP}$ 4 Hz, $\underline{C}HOPS$), 116.73, 116.84, 116.89 (3s, 3 $\underline{C}N$), 166.83 (d, $^2J_{CP}$ 4 Hz, $\underline{C}O$).

δ_P ($CDCl_3$, 121.496 MHz) 67.59 (s); ^{31}P-^1H (m, $^3J_{PH}$ 9.5 Hz), 67.53 (s); ^{31}P-^1H (m, $^3J_{PH}$ 9.5 Hz).

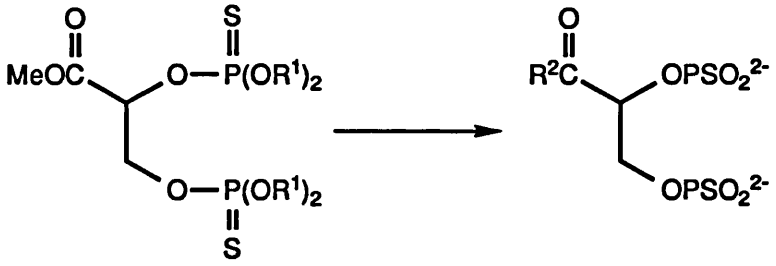
Plus tri(2-cyanoethyl) phosphorothioate (1.64 g) (224).

δ_H ($CDCl_3$, 300 MHz) 2.83 (8H, t, ^3J 6 Hz, $CH_2\underline{C}H_2CN$), 4.27-4.66 (8H, m, $OC\underline{H}_2CH_2$).

δ_C ($CDCl_3$, 74.495 MHz) 19.33, 19.44, 19.54 (3t, 3 $CH_2\underline{C}H_2CN$), 63.00 (t, $O\underline{C}H_2CH_2CN$), 116.73, 116.84, 116.89 (3 s, 3 $\underline{C}N$).

δ_P ($CDCl_3$, 121.496 MHz) 67.01 (s); ^{31}P-^1H (m, $^3J_{PH}$ 9.5 Hz).

9.12 Deprotection of (±)-1-Methyl-2,3-Bis[Di(2-Cyanoethyl) Thiophosphoroglycerate] (188)



$R^1=CH_2CH_2CN$ (188) $R^2=OH$ (194)

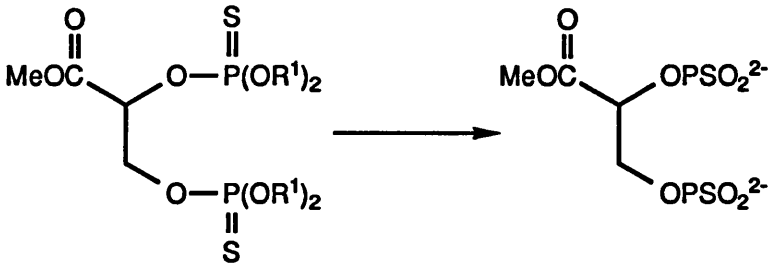
$R^2=NH_2$ (189)

Scheme 118

A sample of (188) (60 mg, 98 µmol) was taken with aqueous ammonia (5 ml, 35%) and heated with stirring to 65°C in a sealed vessel for 1h. Subsequent removal of solvent under reduced pressure gave a crude oil containing phosphorothioate monoester, disulphide and phosphate. It is likely that the compound present was the amide (189) and not the free acid (194).

δ_P (D_2O, pH 9, 36 MHz) 2.80 (s), 15.35 (m), 43.27 (s).

9.13 Synthesis of (±)-1-Methyl-2,3-Bisthiophosphoglycerate (190)



$R^1=CH_2CH_2CN$ (188) (190)

Scheme 119

Initial investigations using 1M NaOMe gave the diester after several hours and afforded the monoester only after a prolonged reaction time *i.e.* 1 month at rt. We decided therefore to use a concentrated solution of NaOMe. This was produced by taking methanol (5 ml) and adding sodium metal until the sodium metal remained. The NaOMe thus produced was then decanted off and used immediately.

Sodium methoxide (3 ml) as described above was taken and added to a stirred solution of (188) (172 mg, 329 μmol) at 20 °C, precipitation of a pale yellow solid ensued. After precipitation was complete, the suspension was dissolved in water (50 ml) and the resultant solution was stirred with a cation exchange resin (H^+-Dowex 50-W-X8 20-50 mesh) until the pH reached neutrality. The resin was removed by filtration and triethylamine (0.1 ml) was added. The solution stirred for 1 min, the solvents were then removed *in vacuo* to give the title compound (190) (118 mg, 193 μmol, 58%) as a pale yellow glass.

δ_H (D_2O, pH 9, 90 MHz) 3.50 (3H,s, $OC\underline{H}_3$), 4.60-4.81 (2H, m, $C\underline{H}_2OPS$), 4.96 (1H, ^{3}t, J 9.9 Hz, $C\underline{H}OPS$).

δ_P (D_2O, pH 9, 36 MHz) 42.80 (s).

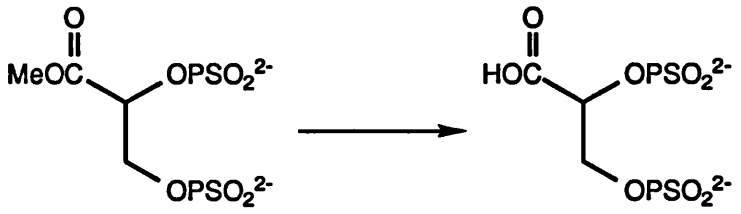
The compound was further purified by passage through an anion-exchange column (DEAE-sephadex A 25, 20 x 2.5 cm) and eluted with a linear gradient of triethylammonium bicarbonate (0-600 mM). The phosphorothioate (190) eluted between 216 and 288 mM, however, when this was analysed by NMR there was evidence that some of the compound had decomposed by atmospheric oxidation to the bisphosphate (196) whilst on the column.

δ_P (D_2O, pH 9, 36 MHz) 2.40 (s), 0.02 (s), 42.80 (s).

n.b. If the sample of (190) is left at high pH for any length of time it decomposes to a phosphorothioate monoester probably thiophosphoenol pyruvate (191) and thiometaphosphate (192) which then decomposes to thiophosphoric acid. Also produced were the bisphosphate (195) and also the disulphide.

δ_P (D_2O, pH 9, 121 MHz) 2.96, 3.21 (2s); ^{31}P-^1H (s), 15.57-17.32 (m); ^{31}P-^1H (m), 33.00 (s); ^{31}P-^1H (s)., 43.36 (s); ^{31}P-^1H (t, $^3J_{PH}$ 8.0 Hz).

9.14 Synthesis of (±)-2,3-Bisthiophosphoglycerate (193).



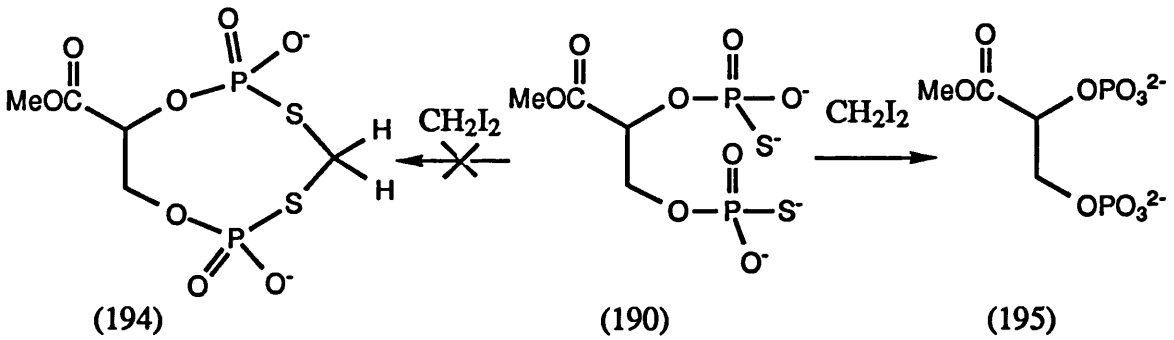
(190) (193)

Scheme 120

A sample of (190) (13 mg, 21 μmol) was taken in acetonitrile (2 ml), trimethylsilyliodide (4.6 μl, 32 μmol) was slowly added with stirring over 5 min. Oxidation of the phosphorothioate to (±)-2,3-bisphosphoglycerate (14) accompanied hydrolysis of the ester. The yield of the bisthiophosphoglycerate (193) was low and proved impossible to purify.

δ_P (D_2O, pH 9, 36 MHz) 2.89 (s), 16.56 (s), 43.14 (s).

9.15 (±)-1-Methyl-2,3-[Cyclic-S,S-Methylene] Bisthiolophosphoglycerate (194)



(194) (190) (195)

Scheme 121

A sample of (190) (23 mg, 37 μmol) was dried by co-evaporation of dry solvent. It was then dissolved in a solution of acetonitrile (50 μl) and diisopropylamine (27 ml, 185 μmol) and stirred. Subsequently, diiodomethane (15 μl, 185 μmol) was added and the solution was stirred for 10 min. at 20 °C. Removal of solvents *in vacuo* gave a crude solid. The residue taken up in D_2O (1 ml) and basified with triethylamine. Alkylation (190) with diiodomethane did not result in the formation of (±)-1-methyl-2,3-[cyclic-S,S-methylene] bisthiolophosphoglycerate (194), it resulted in oxidation to a phosphate monoester, probably the bisphosphate (195).

δ_P (D_2O, pH 9, 36 MHz) -0.06 (s), 0.33 (s).

9.16 Desulphurisation of (189)

The crude sample of (189) (60 mg, 97 μmol) from an earlier reaction was dissolved in water (0.4 ml) and dioxan (1.6 ml) and stirred at rt. NBS (136 mg, 768 μmol) was added, the reaction was quenched after 1 min. by the addition of 2-mercaptoethanol (25 μl). After a further 2 min. TEAB (10 ml, 10 mM) was used to dilute the mixture. Removal of solvents under reduced pressure gave a glass that did not contain any of the pyrophosphate, it contained a phosphate monester, probably the bisphosphate.

δ_P (D_2O, pH 9, 36 MHz) 3.10 (s); ^{31}P-^{1}H (m, $^{3}J_{PH}$ 6.5 Hz).

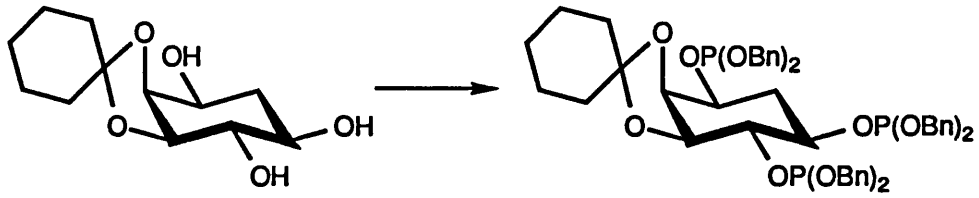
9.17 Desulphurisation of (±)-1-Methyl-2,3-Bisthiophosphoglycerate (190)

A solution of (190) (73 mg, 118 μmol) in dioxan (1.6 ml) and water (0.4 ml) was stirred with NBS (168 mg, 944 μmol) for 1 min. The reaction was quenched by the addition of 2-mercaptoethanol (25 μl) and diluted 1 min. latter by the addition of TEAB (10 ml, 10 mM). Removal of solvent under reduced pressure gave a crude solid that did not contain any pyrophosphate, it contained only phosphate, probably 1-methyl-2,3-bisphosphoglycerate (195).

δ_P (D_2O, pH 9, 36 MHz) 2.89 (s), 3.23 (s).

9.18 Synthesis of 2,3-Cyclohexylidene-6-Deoxy-D-*myo*-Inositol-1,4,5-Tris[(Di-*O*-Benzyl) Phosphorothioate] (200)

*9.18.1 Synthesis of 2,3-Cyclohexylidene-6-Deoxy-D-*myo*-Inositol-1,4,5-Tris[(Di-O-Benzyl) Phosphite] (199)*



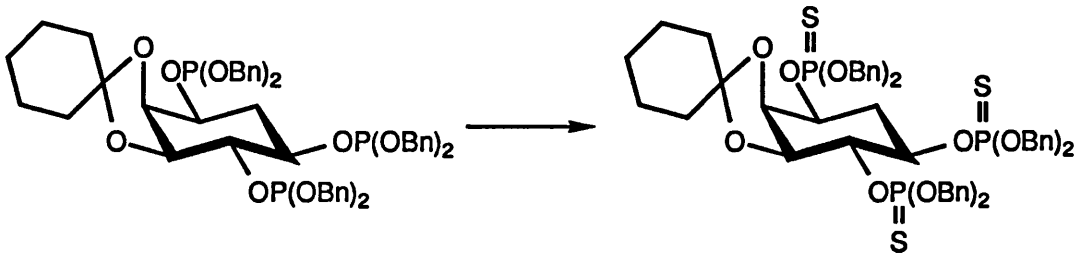
(83) (199)

Scheme 122

6-Deoxy-2,3-cyclohexylidene-D-*myo*-inositol (83) (110 mg, 451 μmol) was taken with tetrazole (504 mg, 7.2 mmol) and dried by co-evaporation of dry acetonitrile. The mixture was then dissolved in dry acetonitrile (2 ml), (diisopropylamino) dibenzyl phosphoramidite (1.2 g, 3.6 mmol) was added with stirring and left at 20°C for 1h to give the intermediate trisphosphite (199).

δ_P ($CDCl_3$, 32.61 MHz) 139.40(m)

*9.18.2 Oxidation of 2,3-Cyclohexylidene-6-Deoxy-D-*myo*-Inositol-1,4,5-Tris[(Di-O-Benzyl) Phosphite] (199)*



(199) (200)

Scheme 123

The crude mixture of (199) in acetonitrile from the previous reaction was taken crude. This was subsequently diluted with pyridine (2 ml) followed by the addition of sulphur (1 g, 32 mmol) with stirring. After 8h at rt the bisphosphite was oxidised to the

trisphosphorothioate (200). The solution was filtered and extracted with ethyl acetate (20 ml), then dried over $MgSO_4$. The solvents were removed and purification was effected by flash column chromatography on silica gel with a stepwise elution [ethyl acetate-pentane, 1:19 (500 ml), then 1:9 (500 ml)]. Crystallisation from the ethyl acetate-pentane solution was initiated by the addition of methanol to give pure (200) (338 mg, 315 μmol, 70%).

m.p. 85-86°C.

$[\alpha]_D \approx 0$ ($CHCl_3$, c=1).

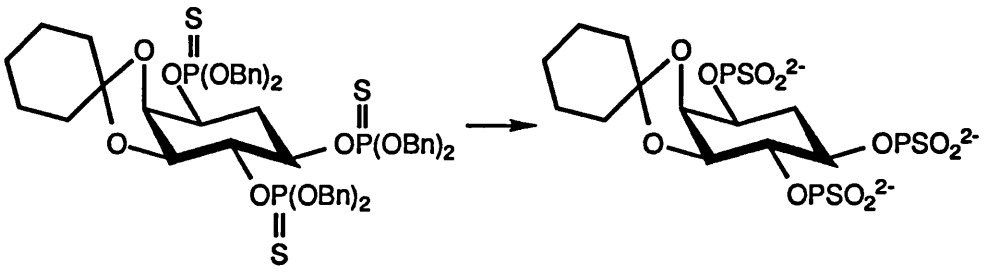
Anal. Calc. for $C_{54}H_{59}O_{11}P_3S_3.0.5\ H_2O$ C, 59.93; H, 5.59. Found: C, 60.10; H, 5.45%.

δ_H ($CDCl_3$, 270 MHz) 1.38-1.55 (6H, m, ketal), 1.60-1.70 (2H, m, ketal), 1.78-1.80 (2H, m, ketal), 2.22 (H, dd, ^3J 7.6, 7.4 Hz, 6D-Ins-H, pos. 6'), 2.40-2.48 (H, m, 6D-Ins-H, pos. 6), 4.09 (H, dd, ^3J 2.5, 5 Hz, 6D-Ins-H, pos. 3), 4.35 (H, t, ^3J 2.5 Hz, 6D-Ins-H, pos. 2), 4.45 (H, ddd, ^3J 4.4, 7.6, 13.5 Hz, 6D-Ins-H, pos. 5), 4.70 (H, dt, ^3J 2.5, 7.6 Hz, 6D-Ins-H, pos. 1), 4.75-4.84 (H, m, 6D-Ins-H, pos. 4), 4.40-5.80 (12H, m, 6 CH_2Ph), 7.20-7.36 (30H, m, Ar-H).

δ_C ($CDCl_3$, 68 MHz) 23.55, 23.94, 24.88, 31.2, 35.35, 37.14 (6t, ketal), 69.57, 69.83, 70.03 (3t, CH_2Ph), 71.45, 73.79, 74.54, 77.16, 81.54 (5d, 6D-Ins-C), 111.87 (s, C), 127.83, 127.96, 128.12, 128.41, 128.54 (5d, Ph), 135.58 (s, Ph).

δ_P (CH_3CN, 36 MHz) 68.32 (s), 67.20 (s), 66.69 (s).

9.19 Synthesis of 6-Deoxy-D-*myo*-Inositol-2,3-Cyclohexylidene-1,4,5-Trisphosphorothioate (196)



(200) (196)

Scheme 124

Ammonia (50.0 ml) was condensed onto sodium and stirred for 5 min. The freshly dried and distilled ammonia (30.0 ml) was then transferred into another vessel containing sodium (200 mg, 8.7 mmol) giving a deep blue colour and kept at -78°C. To this, a solution of (200) (100 mg, 104 μmol) in dry dioxan (0.5 ml) was added over 5 min. The reaction was stirred for 2 min, and then quenched by the addition of isopropanol until the blue colour was lost. The solution was allowed to warm to rt. and the solvents were then removed *in vacuo*. The solid thus produced was taken up in water (50 ml) and mixed with H^+-Dowex 50-W-X8 20-50 mesh cation exchange resin until the pH reached neutrality. The resin was removed and the solution basified with triethylamine (pH 9) to give the triethylammonium salt of (196).

δ_P (D_2O, pH 9, 36 MHz) 41.53 (s); ^{31}P-^1H (d, ^3J 9.8 Hz), 42.33 (s); ^{31}P-^1H (d, ^3J 12.2 Hz), 43.01 (s); ^{31}P-^1H (d, ^3J 14.7 Hz).

Further purification was effected by anion exchange chromatography Q-sepharose fast-flow gel (1.5 x 15 cm), TEAB buffer (300-1000 mM), the title compound (196) eluted around 800 mM as the tris-triethylammonium salt (32 mg, 63 μmol, 60 %).

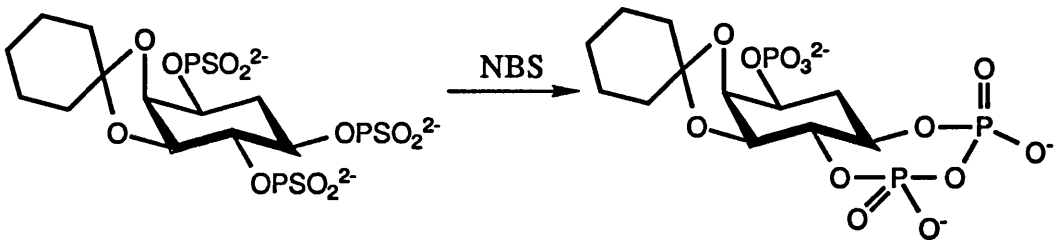
δ_H (D_2O, pH 7, 270 MHz) 1.08-1.75 (10H, m, ketal), 1.74 (H, b, 6D-Ins-H, pos. 6') 2.08-2.44 (H, m, 6D-Ins-H, pos. 6), 4.20 (H, b, 6D-Ins-H, pos. 3), 4.22 (H, b, 6D-Ins-H, pos. 2), 4.38-4.41 (H, b, 6D-Ins-H, pos. 5), 4.51 (H, d, ^3J 9 Hz, 6D-Ins-H, pos. 1), 4.58 (H, m, 6D-Ins-H, pos. 4).

δ_C (D_2O, pH 7, 68 MHz) 22.73, 22.12, 23.87 (3t, ketal), 32.07, 34.08, 34.34, (3 t, 2 ketal, 6D-Ins-C, pos. 6), 67.14, 72.16, 75.41, 77.68, 79.72 (5 d, 6D-Ins-C), 111.02 (s, C, ketal).

δ_P (D_2O, pH 7, 36 MHz) 43.68 (s); ^{31}P-^1H (d, ^3J 9.8 Hz), 46.10 (s); ^{31}P-^1H (d, ^3J 12.3 Hz), 47.52 (s); ^{31}P-^1H (m).

m/z (FAB -ve ion, NOBA), 531 [$(M-H)^-$, 81%], 526 (65), 515 [$(M-OH)^-$, 80], 497 (48), 343 (30), 232 (45), 95 [$(SPO_2)^-$, 100].

9.20 Synthesis of 6-Deoxy-D-*myo*-Inositol-1-Phosphate-2,3-Cyclohexylidene-4,5-Cyclic Pyrophosphate (197)



(196) (197)

Scheme 125

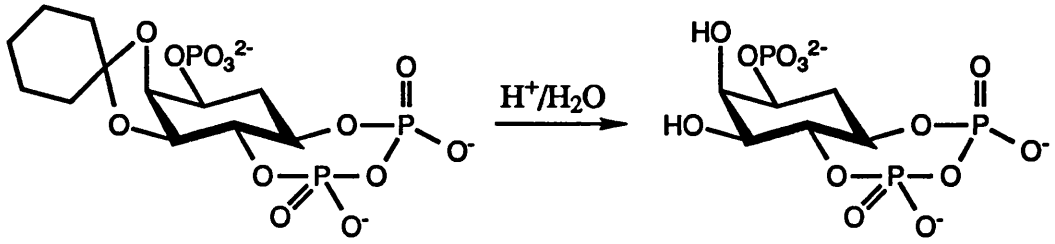
A sample of (196) (18.6 mg, 26 μmol) dissolved in a solution of water (0.4 ml) and dioxan (1.6 ml). NBS (73 mg, 204 μmol) was then added to the stirred solution and left at rt for 1 min. After this time 2-mercaptoethanol (25 μl) was added, followed after a further min. by TEAB (10 ml, 10 mM). Subsequent removal of solvents under reduced pressure gave (197) as a glass. Purification by extraction with ether (2 x 50 ml) and water (50 ml) gave (197) as a pale yellow glass (17 mg, 25 μmol) as the dicyclohexylammonium salt.

δ_H (D_2O, pH 7, 270 MHz) 1.07-2.00 (10H, m, ketal), 1.98 (1H, m, 6D-Ins-H, pos. 6'), 2.42 (1H, m, 6D-Ins-H, pos. 6), 3.6 (1H, m, 6D-Ins-H), 4.18-4.41 (4H, m, 6D-Ins-H)

δ_P (D_2O, pH 8, 109 MHz) 2.15 (s, pos. 1), δ_1 -11.99, δ_2 -12.42 (dd, $J_{1,2}$ 16.8 Hz, pos. 4 and 5).

m/z (FAB -ve ion NOBA), 465 [$(M-H)^-$, 13%], 458 (14), 403 (18), 264 (21), 250 (75), 195 (20), 96.9 [$(H_2PO_3)^-$, 100].
(FAB +ve ion NOBA), 566 [$(M+C_6H_{11}NH_3)^+$, 3%], 490 (8), 450 (8), 100 [$(C_6H_{11}NH_3)^+$, 100].

9.21 Synthesis of 6-Deoxy-D-*myo*-Inositol-1-Phosphate-4,5-Cyclic Pyrophosphate (9)



(197) (9)

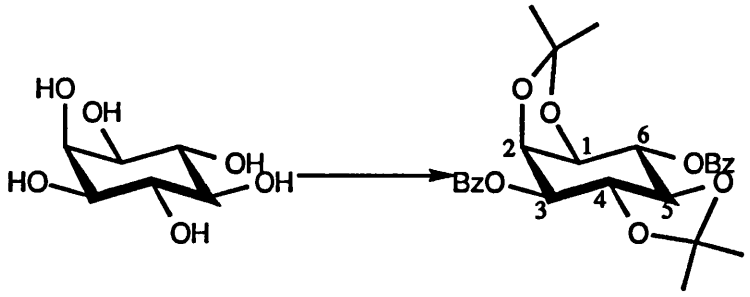
Scheme 126

A solution of (197) (17 mg, 25 μmol) was acidified with HCl and stirred at pH 3 for 24h at rt. The solution was neutralised with triethylamine and the solvents were removed under reduced pressure to give (9) as a glass (8 mg, 20 μmol), a quantity too small to purify..

δ_H (D_2O, pH 7, 270 MHz) 2.01 (1H, m, 6D-Ins-H, pos. 6'), 2.50 (1H, m, 6D-Ins-H, pos. 6), 3.66 (1H, m, 6D-Ins-H), 4.00-4.35 (4H, m, 6D-Ins-H)

δ_P (D_2O, pH 8, 109 MHz) -1.84 (s, pos. 1), δ_1 -12.52, δ_2 -13.02 (dd, $J_{1,2}$ 16.8 Hz, pos. 4 and 5).

9.22 Synthesis of DL-1,2:4,5-Di-*O*-Isopropylidene-3,6-Di-*O*-Benzoyl-*myo*-Inositol (106)



myo-inositol (106)

Scheme 127

The title compound was prepared according to Gigg *et al.*[138].

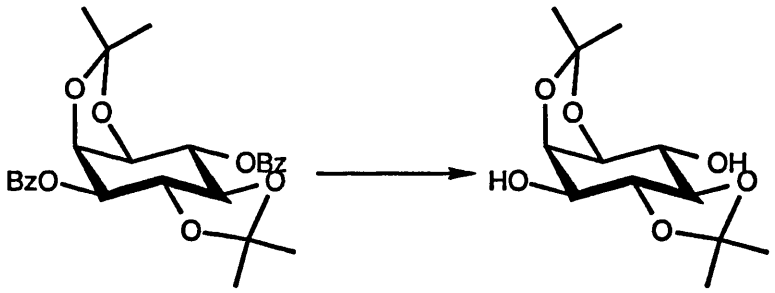
A mixture of *myo*-inositol (50.0 g, 278 mmol), 2,2-dimethoxypropane (150 ml, 1220 mmol) and toluene-*p*-sulphonic acid monohydrate (1.0 g, 5 mmol) in dry DMF (200 ml) were stirred and heated at 200°C for 2h until little solid remained. Triethylamine (10 ml)

was added to the cooled solution, the solid was removed by filtration, addition of toluene (25 ml) was followed after 5 min by the evaporation of the triethylamine and toluene *in vacuo*. Pyridine (150 ml) was added to the DMF solution followed by benzoyl chloride (200 ml) dropwise with stirring and cooling over 20 min. After 9h the solid was collected and washed successively with pyridine, water, acetone, and ether, then dried *in vacuo* over P_2O_5 to give DL-1,2:4,5-di-*O*-isopropylidene-3,6-di-*O*-benzoyl-*myo*-inositol (106) (34.6 g, 27%).

m.p. 326-329°C {Lit.[138] m.p.328-330°C}.

δ_H ($CDCl_3$, 270 MHz) 1.25, 1.40, 1.48, 1.60 (12H, 4s, 2 $C(C\underline{H}_3)_2$), 3.70 (H, dd, ^3J 9.8, 10.5 Hz, Ins-H, pos. 5), 4.31 (2H, m, Ins-H, pos. 1,4), 4.75 (H, t, ^3J 4.5, Ins-H, pos. 2), 5.50 (2H, m, Ins-H, pos. 3,6), 7.40 (6H, m, Ar-H), 8.00 (4H, m, Ar-H).

9.23 Synthesis of DL-1,2:4,5-Di-*O*-Isopropylidene-*myo*-Inositol (107)



(106) (107)

Scheme 128

The title compound was prepared according to Gigg *et al.*[138].

A mixture of (106) (25.0 g, 53 mmol), sodium hydroxide (10.0 g, 250 mmol) and methanol (625 ml) was heated under reflux for 45 min. The clear solution was cooled and neutralised with solid carbon dioxide, then diluted with water (200 ml) and then evaporated to dryness. The colourless solid, sodium bicarbonate, was then washed with dichloromethane (3x 150 ml) and removed by filtration. The dichloromethane was removed under reduced pressure, the solid produced was then recrystallised from ethyl acetate and dried *in vacuo* to give (201) (13.1 g, 95%).

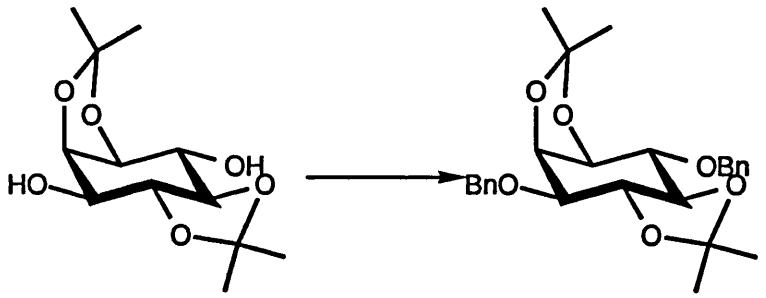
m.p. 171-173°C [Lit.[138] m.p. 171-173°C].

δ_H ($CDCl_3$, 270 MHz) 1.38, 1.46, 1.48, 1.54 (12H, 4s, 2 $C(C\underline{H}_3)_2$), 3.05 (1H, brd,

OH), 3.33 (1H, dd, ^{3}J 9, 10.5 Hz, Ins-H, pos. 5), 3.45 (1H, brd, OH), 3.87 (2H, m, Ins-H, pos. 3,6), 4.28 (2H, m, Ins-H, pos. 1,4), 4.47 (H, t, ^{3}J 4.7 Hz, Ins-H).

δ_C ($CDCl_3$, 68 MHz) 26.79, 27.93, 27.99 (3q, $C(\underline{C}H_3)_2$), 69.54, 74.54, 77.58, 77.78, 78.10, 81.77 (6d, Ins-C), 110.12, 112.52 (2s, $Me_2\underline{C}$).

9.24 Synthesis of DL-1,2:4,5-Di-*O*-Isopropylidene-3,6-Di-*O*-Benzyl-*myo*-Inositol (108)



(107) (108)

Scheme 129

The title compound was prepared according to Gigg *et al.*[138].

Compound (107) in DMF (200 ml), under an atmosphere of dry nitrogen, was treated with sodium hydride (4.0 g, 160 mmol), an excess of benzyl bromide (17.1 g, 100 mmol) over 20 min. The mixture was stirred at 20°C for 2h, after which the reaction was quenched with methanol (20 ml), then diluted with water yielding a colourless solid that was taken up in ether and recrystallised from petroleum ether (60-80), the ether was removed to give the colourless solid (108) (12.0 g, 70%).

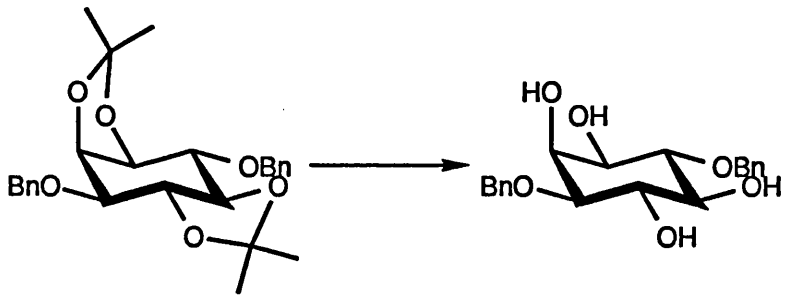
m.p. 150-153°C {Lit.[138] m.p. 153-155}.

δ_H ($CDCl_3$, 270 MHz) 1.32, 1.38, 1.46, 1.49 (12H, 4s, 2 $C(\underline{C}H_3)_2$), 3.34 (1H, dd, ^{3}J 9.3, 10.6 Hz, Ins-H, pos. 5), 3.67 (1H, dd, ^{3}J 6.6, 10.6 Hz, Ins-H, pos. 4), 3.73 (1H, dd, ^{3}J 4.5, 10.5 Hz, Ins-H, pos. 1), 4.03 (2H, m, Ins-H, pos. 6 and 3), 4.29 (1H, t, ^{3}J 4.5 Hz, Ins-H, pos. 2), δ_1 4.80, δ_2 4.83 (4H, $J_{1,2}$ 12.6 Hz, 2 $C\underline{H}_2Ph$), 7.33 (10H, m, Ar-H).

δ_C ($CDCl_3$, 68 MHz) 25.82, 26.99, 27.86 (3q, $\underline{C}H_3$), 71.75, 71.86 (2t, 2 $Ph\underline{C}H_2$), 74.24, 76.61, 77.06, 78.78, 79.76, 80.99 (6d, Ins-C), 109.82, 112.06 (2s, $Me_2\underline{C}$),

127.37, 127.76, 127.86, 128.09, 128.25, 128.31 (6d, Ph), 137.82, 138.14 (2s, Ph).

9.25 Synthesis of DL-1,4-Di-*O*-Benzyl-*myo*-Inositol (109).



(108) (109)

Scheme 130

The title compound was prepared according to Gigg *et al.*[138].

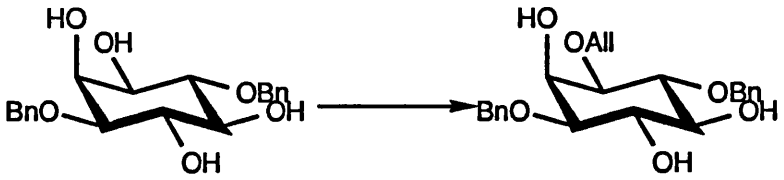
A solution of (108) (11.0 g, 25 mmol) in 80% acetic acid (150 ml) was taken to reflux for 20 min. Addition of ice water precipitated out (±) 1,4-di-*O*-benzyl-*myo*-inositol (109), which was filtered and subsequently washed with water then recrystallised from ethanol to give (109) as a colourless solid. (7.3 g, 81%).

m.p. 201-206°C {Lit.[138] m.p. 205-207°C}

δ_H (DMSO-d_6, 270 MHz) 3.15 (2H, m, Ins-H), 3.32 (1H, m, Ins-H), 3.44 (1H, m, Ins-H), 3.64 (1H, m, Ins-H), 3.99 (1H, brd, OH), 4.63 (3H, m, PhCH_2), 4.78-4.86 (5H, m, Ins-H, 1 PhCH_2, 3 OH), 7.34 (10H, m, 3 Ar-H).

δ_C ($CDCl_3$, 68 MHz) 69.89 (d, Ins-C), 70.89 (t, PhCH_2), 71.58 (d, Ins-C), 72.42 (d, Ins-C), 73.75 (t, PhCH_2), 75.21 (d, Ins-C), 79.95 (d, Ins-C), 81.96 (d, Ins-C), 127.08 (d, Ph), 127.24 (d, Ph), 127.66 (d, Ph), 128.02 (d, Ph), 128.15 (d, Ph), 139.44 (s, Ph), 140.08 (s, Ph).

9.26 Synthesis of DL-1-*O*-Allyl-3,6-Di-*O*-Benzyl-*myo*-Inositol (110).



(109) (110)

Scheme 131

The title compound was prepared according to Gigg *et al.*[236].

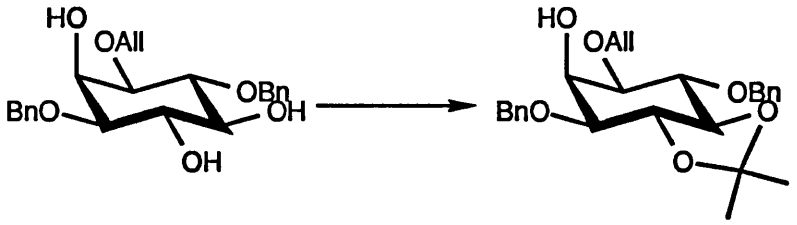
Compound (109) (6.0 g, 16.6 mmol), was taken with tetrabutylammonium iodide (6.2 g, 16.8 mmol) and dibutyl tin oxide (4.5 g, 18 mmol) in toluene (150 ml) at reflux for 3h with concomitant azeotropic removal of water. Allyl bromide (3 ml, 35 mmol) was added over 15 min. with cooling, after which the mixture was refluxed for a further hour. Evaporation of toluene under reduced pressure, preceded washing with HCl (100 ml, 2M) followed by saturated KCl solution (100 ml). It was then stirred with saturated $NaHCO_3$ solution (50 ml), filtered through celite and dried over $MgSO_4$, yielding (110) as a colourless solid (2.9 g, 43%).

m.p. 107-108 {Lit.[236] m.p. 108-110}

δ_H ($CDCl_3$, 270 MHz) 2.93 (3H, b, O<u>H</u>), 3.21 (1H, dd, ^3J 3, 9.75 Hz, Ins-<u>H</u>, pos. 1 or 3), 3.22 (1H, dd, ^3J 3, 9.34 Hz, Ins-<u>H</u>, pos. 1 or 3), 3.38 (1H, t, ^3J 9.34 Hz, Ins-<u>H</u>, pos. 5), 3.76 (1H, t, ^3J 9.34 Hz, Ins-<u>H</u>, pos. 6 or 4), 3.94 (1H, t, ^3J 9.3 Hz, Ins-<u>H</u>, pos. 6 or 4), 4.13 (2H, m, C<u>H</u>$_2$CHCH$_2$), 4.20 (1H, t, ^3J 3 Hz, Ins-<u>H</u>, pos. 2), δ_1 4.68, δ_2 4.71 (2H, $J_{1,2}$ 12.8 Hz, PhC<u>H</u>$_2$), δ_1 4.75, δ_2 4.91 (2H, $J_{1,2}$ 11.2 Hz, PhC<u>H</u>$_2$), 5.18 (1H, ^2J 1.5, ^3J 10.5 Hz, CHC<u>H</u>H'), 5.27 (1H, ^2J 1.5, ^3J 18 Hz, CHCH<u>H</u>'), 5.86 (1H, m, CH$_2$C<u>H</u>CHH'), 7.31 (10H, m, Ar-<u>H</u>).

δ_C ($CDCl_3$, 68 MHz) 67.14 (d, Ins-<u>C</u>) 71.33 (t, <u>C</u>H$_2$Ph), 71.88 (d, Ins-<u>C</u>), 72.23 (t, <u>C</u>H$_2$Ph), 74.15 (d, Ins-<u>C</u>), 75.38 (t, <u>C</u>H$_2$CHCH$_2$), 79.04, 79.56, 80.34 (3d, Ins-<u>C</u>), 117.48 (t, CH$_2$CH<u>C</u>H$_2$), 127.63, 127.86, 127.92, 128.38, 128.48 (5d, Ph), 134.48 (d, CH$_2$<u>C</u>HCH$_2$), 137.69, 138.66 (2s, Ph).

9.27 Synthesis of DL-1-*O*-Allyl-3,6-Di-*O*-Benzyl-4,5-*O*-Isopropylidene-*myo*-Inositol (111)



(110) (111)

Scheme 132

The title compound was prepared according to the method of Gigg *et. al.*[236].

Compound (110) (2.5 g, 6.25 mmol) was taken with 2,2-dimethoxypropane (2 ml, 16.3 mmol), ptsa (0.25 g, 1.25 mmol) in acetone (50 ml) and stirred for 2h after which time tlc (ether-pentane, 1:1) showed almost complete conversion of (110) from the base-line to the isopropylidene derivative (111). The products were extracted into dichloromethane and the crude product was flash chromatographed on silica gel ether-pentane (1:1) to give product (111) (1.79 g, 65%).

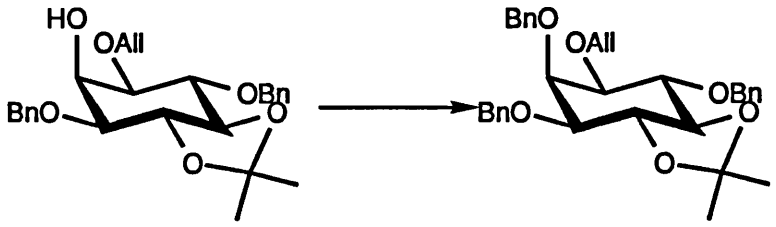
m.p. 102-103°C {Lit.[236] m.p. 103-105°C}.

R_f 0.30 (ether-pentane, 1:1)

δ_H ($CDCl_3$, 270 MHz) 2.17, 2.18 (6H, 2s, $C(C\underline{H}_3)_2$), 3.35 (2H, m, Ins-H), 3.57 (1H, dd, ^3J 3, 9.9 Hz, Ins-H, pos. 1 or 3), 3.94 (1H, t, ^3J 9.7 Hz, Ins-H), 4.09 (1H, t, ^3J 9.9 Hz, Ins-H), 4.14-4.25 (3H, m, Ins-H, pos. 2, $C\underline{H}_2CHCH_2$), 4.84 (4H, m, 2 $PhC\underline{H}_2$), 5.18 (1H, dd, ^2J 1.5, ^3J 10.5 Hz, $CH_2CHC\underline{H}H'$, *cis*), 5.26 (1H, dd, ^2J 1.5, ^3J 17.25 Hz, $CH_2CHCH\underline{H}'$, *trans*), 5.90 (1H, m, $CH_2C\underline{H}CHH'$), 7.34 (10H, m, Ar-H).

δ_C ($CDCl_3$, 68 MHz), 31.49, 31.56 (2q, $C(\underline{C}H_3)_2$), 74.44 (d, Ins-C), 80.47 (t, $\underline{C}H_2Ph$), 81.05 (d, Ins-C), 81.54 (t, $\underline{C}H_2Ph$), 81.64, 81.77 (2d, Ins-C), 82.00 (t, $\underline{C}H_2Ph$), 83.75, 85.37 (2d, Ins-C), 116.25 (s, $Me_2\underline{C}$), 122.12 (s, $Me_2\underline{C}$), 131.91, 132.14, 132.30, 132.50, 132.76, 132.95 (6d, Ph), 139.11 (s, Ph).

9.28 Synthesis of DL-1-*O*-Allyl-2,3,6-Tri-*O*-Benzyl-4,5-*O*-Isopropylidene-*myo*-Inositol (112)



(111) (112)

Scheme 133

The title compound was prepared according to the method of Gigg *et. al.*[237].

To a solution of (111) (1124 mg, 2.56 mmol) in DMF under an atmosphere of dry nitrogen, sodium hydride (134 mg, 5.6 mmol) and benzyl bromide (666 µl, 5.6 mmol) were added with stirring and left at 20°C for 2h at which time tlc indicated the disappearance of (111) and appearance of (112). The crude product was taken up in methanol (50 ml), diluted with water (50 ml) and extracted with ether (3x 50 ml). The crude product was flash chromatographed on silica gel ether-pentane (1:3) and recrystallised from pet. ether (40-60) to give product (112) (887 mg, 65%).

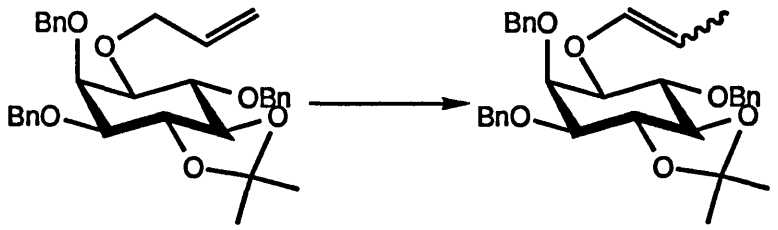
m.p. 82-83°C {Lit.[237] m.p 82-84°C}.

R_f 0.31 (ether-pentane, 1:3)

δ_H ($CDCl_3$, 300 MHz) 1.45, 1.46 (6H, 2s, $C(C\underline{H}_3)_2$), 3.30 (1H, dd, ^3J 2.8, 8.9 Hz, Ins-H pos. 1 or 3), 3.37 (1H, t, ^3J 9.6, Ins-H), 3.54 (1H, dd, ^3J 2.7, 10.3 Hz, Ins-H, pos. 1 or 3), 3.99-4.20 (5H, m, $C\underline{H}_2CHCH_2$, 3 Ins-H), 4.75 (6H, m, $PhC\underline{H}_2$), 5.14 (1H, ddd, ^2J 1.5, ^3J 10.4, ^4J 3 Hz, $CH_2CHC\underline{H}H'$, *cis*), 5.27 (1H, ddd, ^2J 1.5, ^3J 17.2, ^4J 3.8 Hz, $CH_2CHCH\underline{H}'$, *trans*), 5.87 (1H, ddt, ^3J 5.4, 10.4, 17.2 Hz, $CH_2C\underline{H}CHH'$), 7.31 (15H, m, Ar-H).

δ_C ($CDCl_3$, 76 MHz) 26.98, 27.06 (2s, $C(\underline{C}H_3)_2$), 71.67, 72.36, 73.30 (3d, Ph), 75.18 (t, $\underline{C}H_2CHCH_2$), 76.59, 77.013, 77.53, 78.37, 79.72, 81.84 (6d, Ins-C), 111.48 (s, $Me_2\underline{C}$), 116.59 (t, $CH_2CH\underline{C}H_2$), 127.21, 127.31, 127.40, 127.44, 127.53, 127.93, 128.04, 128.09, 128.23 (9d, Ph), 134.91 (d, $CH_2\underline{C}HCH_2$), 138.46, 138.88, 138.96 (3s, Ph).

9.29 Synthesis of DL-1-*O*-Prop-1-enyl-2,3,6-Tri-*O*-Benzyl-4,5-*O*-Isopropylidene-*myo*-Inositol (201)



(112) (201)

Scheme 134

The title compound was prepared according to the methods of Gigg *et. al.*[236,237].

Compound (112) (770 mg, 1.45 mmol) was taken up in ethanol-water (55 ml, 9:1) and taken to reflux. At reflux, diazobicyclo-[2,2,2]-octane (0.33 g, 3 mmol)[DABCO] and tristriphenylphosphine rhodium(I) chloride (Wilkinson's Catalyst)[170] (0.1 g, 108 μmol) were added and again heated to reflux for a further 2h. After cooling the reaction mixture was diluted with water (50 ml) and extracted into ether (3 x 50 ml). The ether was then washed with aqueous sodium chloride solution (50 ml, pH 2), water (50 ml) and dried over anhydrous $MgSO_4$. The products were purified by flash chromatography on silica gel ether-pentane (1:3) to yield (201) as a mixture of *cis* to *trans* isomers in the ratio of 10:1, (598 mg, 78%).

m.p. 112-115°C {Lit.[237] m.p. 115-116°C}.

R_f 0.77 (ether-pentane, 1:1).

IRν_{MAX} (propenyl) 1670 cm^{-1}

cis-isomer

δ_H ($CDCl_3$, 300 MHz) 1.45, 1.46 (6H, 2s, $C(C\underline{H}_3)_2$), 1.65 (3H, dd, ^3J 6.8, ^4J 1.6 Hz, $CHCHC\underline{H}_3$ *cis*), 3.36 (H, t, ^3J 9.5, Ins-H, pos. 5), 3.58 (2H, m, Ins-H), 4.08 (2H, m, Ins-H), 4.14 (1H, dd, ^3J 9.5, 10.2 Hz, Ins-H), 4.42 (1H, dq, ^3J 6.2, 6.8 Hz, CHCHMe), 4.74 (6H, m, $PhC\underline{H}_2$), 6.08 (1H, dd, ^3J 6.2, ^4J 1.6 Hz, $C\underline{H}CHCH_3$ *cis*), 7.32 (15H, m, Ar-H).

δ_C ($CDCl_3$, 76 MHz) 9.38 (q, $CHCH\underline{C}H_3$), 26.95, 27.05 (2q, $C(\underline{C}H_3)_2$), 71.56, 73.27, 75.57 (3t, $Ph\underline{C}H_2$), 76.99, 77.44, 78.58, 79.44 (4d, Ins-C), 84.31(d, Ins-C, pos.-1),

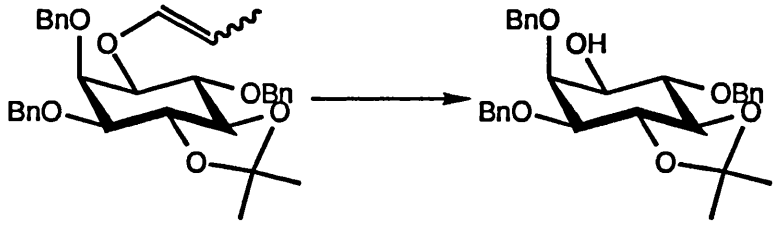
100.56 (d, CHCHMe, *cis*), 111.71 (s, Me_2C), 127.30, 127.39, 127.44 , 127.72, 127.87, 128.08, 128.26 (7d, Ph), 138.34, 138.53, 138.64 (3s, Ph), 145.95 (d, OCHCHMe, *cis*).

trans-isomer

δ_H ($CDCl_3$, 300 MHz) 1.45, 1.46 (6H, 2s, $C(CH_3)_2$), 1.54 (3H, dd, ^3J 6.8, ^4J 1.6 Hz, $CHCHCH_3$ *trans*), 3.36 (H, t, ^3J 9.5, Ins-H, pos. 5), 3.58 (2H, m, Ins-H), 4.06 (2H, m, Ins-H), 4.14 (1H, dd, ^3J 9.5, 10.2 Hz, Ins-H), 4.42 (1H, dq, ^3J 6.2, 6.8 Hz, CHCHMe), 4.73 (6H, m, $PhCH_2$), 6.13 (^{1}H, dd, ^3J 6.2, ^4J 1.6 Hz, $CHCHCH_3$ *trans*), 7.32 (15H, m, Ar-H).

δ_C ($CDCl_3$, 76 MHz) 9.38 (q, $CHCHCH_3$), 26. 95, 27.05 (2q, $C(CH_3)_2$), 71.56, 73.27, 75.57 (3t, $PhCH_2$), 76.99, 77.44, 78.58, 79.44 (4d, Ins-C), 84.31(d, Ins-C, pos.-1), 101.16 (d, CHCHMe, *trans*), 111.71 (s, Me_2C), 127.30, 127.39, 127.44 , 127.72, 127.87, 128.08, 128.26 (7d, Ph), 138.34, 138.53, 138.64 (3s, Ph), 146.55 (d, OCHCHMe, *trans*).

9.30 Synthesis of DL-2,3,6-Tri-*O*-Benzyl-4,5-*O*-Isopropylidene-*myo*-Inositol (202)



(201) (202)

Scheme 135

Cleavage of the prop-1-enyl ether of (201) was facilitated by taking a sample of (201) (530 mg, 1.00 mmol) in acetone-water (30 ml, 10:1) with mercury (II) oxide (900 mg, 4.15 mmol). Mercury (II) chloride (900 mg, 3.32 mmol) in acetone-water (10 ml, 10:1) was added dropwise over 20 min. When the addition was finished tlc showed complete conversion of starting material to (202) and 2-chloromercuripropionaldehyde. The HgO was removed by filtration through celite. After evaporation of the acetone, the residue was taken up in ether and washed with saturated potassium iodide (2 x 50 ml), dried over K_2CO_3. The solvent was then evaporated under reduced pressure. Purification by silica gel chromatography (pentane-ethyl acetate, 3:1) gave (202) as a light yellow coloured glass (381 mg, 78%).

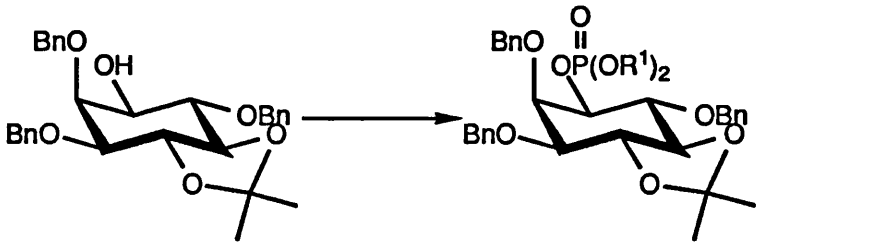
R_f 0.6 (ether-pentane, 1:1)

δ_H ($CDCl_3$, 300 MHz) 1.45, 1.46 (6H, 2s, $C(C\underline{H}_3)_2$), 2.64 (1H, brd, O<u>H</u>), 3.34 (H, t, ^3J 9.6 Hz, Ins-<u>H</u>, pos.-5), 3.49 (1H, dd, ^3J 2.8, 9 Hz, Ins-<u>H</u>, pos.-1), 3.56 (1H, dd, ^3J 2.8, 10.3 Hz, Ins-<u>H</u>, pos.-3), 3.77 (1H, dd, ^3J 9, 9.6 Hz, Ins-<u>H</u>, pos.-6), 3.99 (1H, t, ^3J 2.8, Ins-<u>H</u>, pos.-2), 4.11 (1H, dd, ^3J 9.6, 10.3 Hz, Ins-<u>H</u>, pos.-4), 4.78 (6H, m, $PhC\underline{H}_2$), 7.30 (15H, m, Ar-<u>H</u>).

δ_C ($CDCl_3$, 76 MHz) 26.95, 27.03 (2q, $C(\underline{C}H_3)_2$), 71.11, 72.82 (2t, $Ph\underline{C}H_2$), 73.80 (d, Ins-<u>C</u>), 75.63 (t, $Ph\underline{C}H_2$), 77.70, 77.80, 78.70, 79.60 (4d, Ins-<u>C</u>), 111.61 (s, $Me_2\underline{C}$), 127.39, 127.44, 127.55, 127.75, 127.85, 128.19, 128.24 (7d, Ph), 138.30, 138.49, 138.53 (3s, Ph).

2-chloromercuripropionaldehyde R_f 0.17 (ether-pentane, 1:1).

9.31 Synthesis of DL-2,3,6-Tri-*O*-Benzyl-4,5-*O*-Isopropylidene-*myo*-Inositol-1-[Di(2,2,2-Trichloroethyl) Phosphate] (203)



(202) (203) $R^1=CH_2CCl_3$

Scheme 136

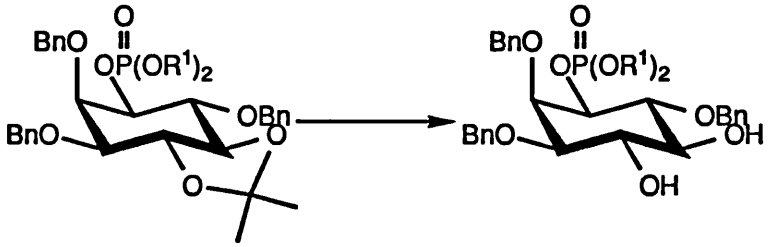
Bis(2,2,2-trichloroethyl) phosphorochloridate (24.0 mg, 62.5 µmol) was added to a solution of the 2,3,6-tri-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inositol (202) (24.5 mg, 50 µmol) in dry pyridine (5 ml). After 12h at rt tlc showed complete conversion of the starting material into product (203). Water (0.5 ml) was added and the solution was kept at rt for 1h. It was then further diluted with water (30 ml) and extracted into ether. The ether extract was washed successively with ice cold hydrochloric acid (100 ml, 1M), saturated potassium chloride solution (100 ml) and saturated sodium hydrogen carbonate solution (100 ml). The residue was then dried over magnesium sulphate, the solvent was then removed *in vacuo* to give (203) (38 mg, 45 µmol, 90%) as a brown solid.

R_f 0.55 (ether-pentane, 1:1)

δ_H ($CDCl_3$, 270 MHz), 1.48 (6H, 2s, 2 $CC\underline{H}_3$), 3.37 (1H, t, ^3J 9.5 Hz, Ins-$\underline{H}$, pos.-3), 3.63 (1H, dd, ^3J 1.9, 10.4 Hz, Ins-$\underline{H}$), 4.00-5.04 (14H, m, 3 $C\underline{H}_2Ph$, 2 $C\underline{H}_2CCl_3$, 4 Ins-$\underline{H}$), 7.30-7.33 (15, m, Ar-$\underline{H}$)

δ_p ($CDCl_3$, 121 MHz) -5.18 (s)

9.32 Synthesis of DL-2,3,6-Tri-*O*-Benzyl-*myo*-Inositol 1-[Di(2,2,2-Trichloroethyl) Phosphate] (204)



(203) (204)

Scheme 137. $R^1=CH_2CCl_3$

The sample of (203) (37 mg, 44 µmol) was taken up in a mixture of acetone-methanol-1M HCl (5:6:1, 50 ml) and kept at 20°C for 1h after which time tlc (ether-pentane., 2:1) showed complete conversion of (203) to product (204). Anhydrous sodium acetate (100 mg) was added and the solvents were evaporated. Water (50 ml) and ether (50 ml) were added to the residue and the ether layer was separated, washed with saturated potassium chloride solution (2 x 50 ml) and dried over $MgSO_4$ Evaporation of the solvents gave crystalline product, which was recrystallised from ethyl acetate-pentane (3:10) to give pure (204) (35 mg, 44 µmol, 100%)

m.p. 141-143°C.

R_f 0.2 (ether-pentane., 2:1)

Anal. Calc. for $C_{31}H_{33}Cl_6O_9P$: C, 46.94; H, 4.19. Found: C, 47.00; H, 4.15%.

δ_H ($CDCl_3$, 270 MHz), 2.90, 2.97 (2H, 2 brd, O$\underline{H}$), 3.25 (1H, dd, ^3J 2 , 9.5 Hz, Ins-$\underline{H}$, pos. 3), 3.49 (1H, t, ^3J 9.1 Hz, Ins-$\underline{H}$), 4.00 (2H, dt, ^3J 9.5, 11.7 Hz, Ins-$\underline{H}$, pos. 4 and 5), 4.30-4.96 (12H, m, 3 $PhC\underline{H}_2$, 2 $OC\underline{H}_2CCl_3$, 2 Ins-$\underline{H}$), 7.33 (15H, m, Ar-$\underline{H}$).

δ_C ($CDCl_3$, 68 MHz) 72.14 (d, Ins-$\underline{C}$), 72.26 (t, $Ph\underline{C}H_2$ or $O\underline{C}H_2CCl_3$), 74.79 (d, Ins-$\underline{C}$), 75.09, 76.90 (2t, $Ph\underline{C}H_2$, $O\underline{C}H_2CCl_3$), 79.24, 79.79 (2d, Ins-$\underline{C}$), 94.60 (s, $OCH_2\underline{C}Cl_3$), 127.60, 127.70, 127.90, 128.05, 128.28, 128.51, 128.57 (7d, 3 Ph),

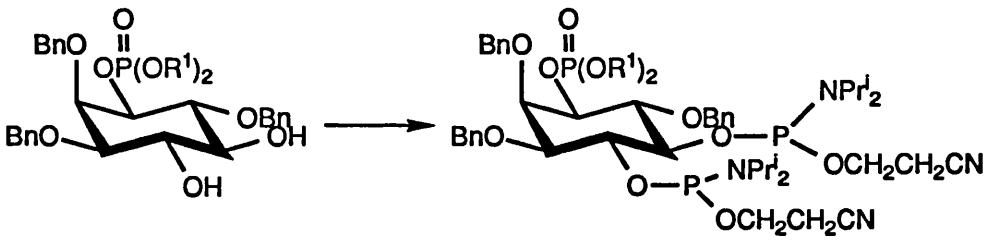
137.27, 138.01, 138.11 (3s, 3 Ph).

δ_P ($CDCl_3$, 121 MHz) -6.75 (s); ^{31}P-^{1}H (apparent quintet ^{3}J 6.7 Hz)

m/z, multiple peaks found when Cl present in fragment.
(FAB +ve ion NOBA), 793 [(M, 3 $^{37}Cl)^+$, 3.5%; plus M^+ with 1,2,4,5,6 ^{37}Cl], 361 [$(HOPO(OCH_2CCl_3)_2)^+$, 0.5], 181 [$(PhCH_2CH_2Ph)^+$,18], 91 [$(PhCH_2)^+$,100].

9.33 Synthesis of DL-2,3,6-Tri-*O*-Benzyl-*myo*-Inositol-1-[Di(2,2,2-Trichloroethyl) Phosphate]-4,5 Bis[(2-Cyanoethyl) Phosphorothioate] (208)

*9.33.1 Synthesis of DL-2,3,6-Tri-O-Benzyl-*myo*-Inositol-1-[Di (2,2,2-Trichloroethyl) Phosphate]-4,5-Bis [Diisopropylamino (2-Cyanoethoxy) Phosphoramidite] (205)*



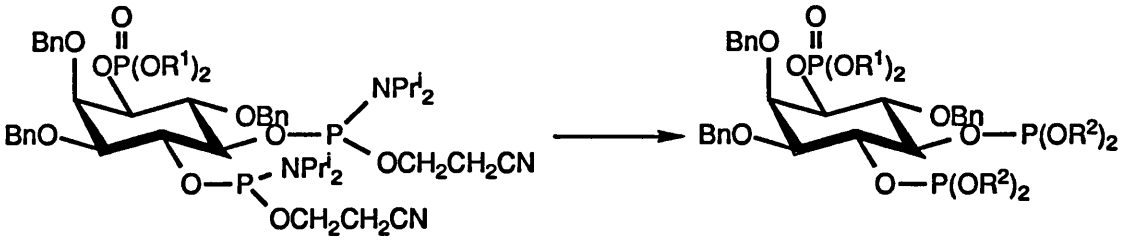
(204) (205)

Scheme 138 $R^1=CH_2CCl_3$

A sample of (204) (197 mg, 250 μmol) was dried by coevaporation of dry acetonitrile. It was then dissolved in a solution of diisopropylethylamine (230 mg, 1.78 mmol) and dry acetonitrile (2 ml). The solution was stirred at 20°C and (78) (341 mg, 1.4 mmol) was added under nitrogen. After stirring for *ca.* 5 min. the formation of the phosphoramidite (205) had been effected.

δ_P (MeCN, 36 MHz) -6.10 (s), 138.30 (s, $[NCCH_2CH_2O]_3P$), 149.20 (s), 150.65 (s), 179.9 (s, excess reagent).

9.33.2 Synthesis of DL-2,3,6-Tri-O-Benzyl-myo-Inositol 1-[Di(2,2,2-Trichloroethyl) Phosphate]-4,5 Bis[(2-Cyanoethyl) Phosphite] (206)



(205) (206)

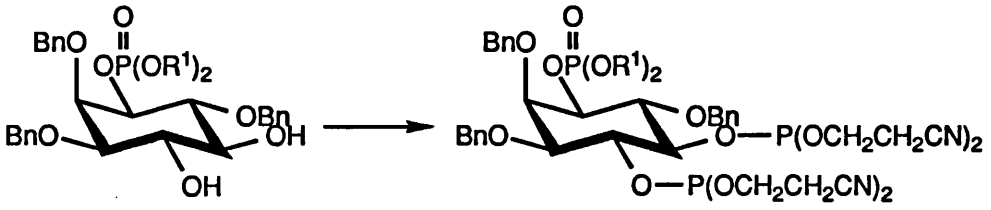
$R^1=CH_2CCl_3$, $R^2=CH_2CH_2CN$

Scheme 139

The solution of (205) in acetonitrile, from the previous reaction was taken crude. The solution of (205) was stirred under nitrogen and treated with tetrazole (200 mg, 2.3 mmol) then 2-cyanoethanol (200 mg, 2.3 mmol). The intermediate phosphite (206) was formed seen after approximately 5 min.

δ_P (MeCN, 36 MHz) -6.10 (s), 140.10 (s), 141.01 (s).

The product was obtained in a slightly simpler reaction by using the reagent (diisopropylamino) bis(2-cyanoethyl) phosphoramidite (81) to give the intermediate bisphosphite (206).



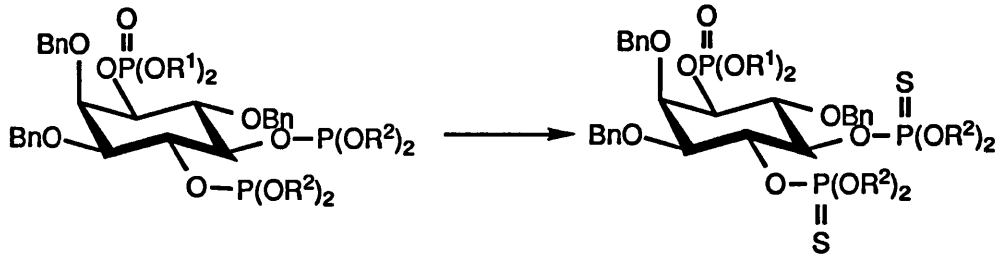
(204) (206)

Scheme 140. $R^1=CH_2CCl_3$

To a stirred solution of (204) (79.3 mg, 100 μmol) and tetrazole (17 mg, 240 μmol) in acetonitrile (2 ml). A sample of (81) (65.5 mg, 240 μmol) was added over 2 min. to give intermediate phosphite (206) after *ca.* 5 min.

δ_P (MeCN, 36 MHz) -5.52 (s), 7.81 (s, $[RO]_2POH$), 140.13 (d), 148.14 (s, excess 210)

9.33.3 *Oxidation of DL-2,3,6-Tri-O-Benzyl-myo-Inositol-1-[Di(2,2,2-Trichloroethyl) Phosphate]-4,5 Bis[(2-Cyanoethyl) Phosphite] (206)*



(206) (208)

$R^1=CH_2CCl_3$, $R^2=CH_2CH_2CN$

Scheme 141

Again the crude mixture of (206) in acetonitrile from an earlier experiment was taken under nitrogen and diluted with dry pyridine (2 ml). Subsequent addition of sulphur (0.5 g, 16 mmol) effected the complete oxidation of the bisphosphite (206) to the bisphosphorothioate (208) within 24h. The product was initially purified by short column silica chromatography ($CHCl_3$-EtOAc, 10:1), to remove base-line contamination and slight contamination with traces of tris(2-cyanoethyl)phosphorothioate (224) and tris(2-cyanoethyl)phosphate still present. The partially purified sample was then loaded onto a chromatotron plate (silica, 2.0 mm), eluted with chloroform and visualised with U.V. light to give (208) as a pale yellow glass (219 mg, 183 μmol, 73%) from (204) using the chlorophosphine reagent (78) via the bisphosphoramidite intermediate (205). The yield of (208) from (204) using the phosphoramidite reagent (81) was (81%).

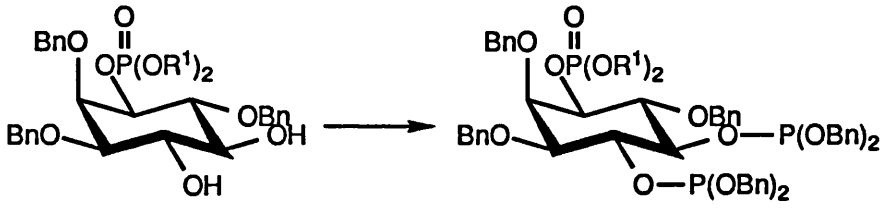
R_f 0.58 ($CHCl_3$-EtOAc, 10:1)

δ_H ($CDCl_3$, 300 MHz) 2.55 (8H, t, ^3J 6.1 Hz, $OCH_2\underline{CH_2}CN$), 3.10 (2H, brd, Ins-H), 3.64 (H, dd, ^3J 1.7, 9.6 Hz, Ins-H, pos.-3), 3.71-3.76 (2H, m, Ins-H), 3.80 (8H, t, ^3J 6.1 Hz, $O\underline{CH_2}CH_2CN$), 3.98-4.94 (10H, m, 4 $O\underline{CH_2}CCl_3$, 6 $O\underline{CH_2}Ph$), 5.18 (H, dt, ^3J 9.6, 11.6 Hz, Ins-H, pos.-4), 7.22-7.42 (15H, m, Ph).

δ_P ($CDCl_3$, 121 MHz) -7.86 (s, pos.-1); ^{31}P-^1H (m ^3J 6.5 Hz), 63.94 (s, pos.-4 or -5); ^{31}P-^1H (m), 64.14 (s, pos. -4 or -5); ^{31}P-^1H (m) .

9.34 Synthesis of DL-2,3,6-Tri-*O*-Benzyl-*myo*-Inositol 1-[Di(2,2,2-Trichloroethyl) Phosphate]-4,5 Bis[Di-*O*-Benzyl) Phosphorothioate] (209)

*9.34.1 Synthesis of DL-2,3,6-Tri-O-Benzyl-*myo*-Inositol 1-[Di(2,2,2-Trichloroethyl) Phosphate]-4,5 Bis[Di-O-Benzyl) Phosphite] (207)*



(204) (207)

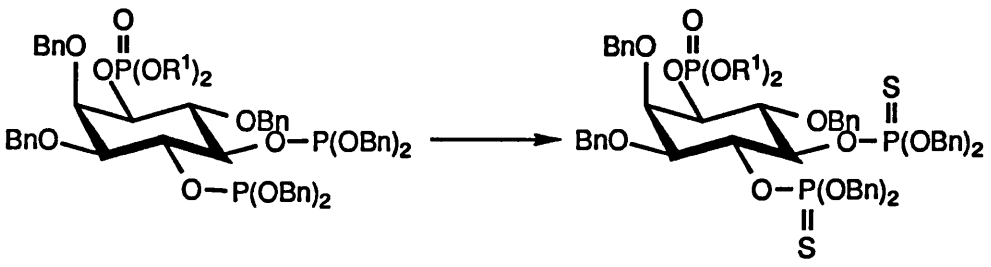
Scheme 142. $R^1=CH_2CCl_3$

A sample of di-*O*-benzyl (diisopropylamino) phosphoramidite (790 mg, 2.35 mmol) (82) was taken with tetrazole (172 mg, 2.46 mmol) and dried by co-evaporation of solvent. The mixture was then dissolved in acetonitrile (2 ml) and a solution of (204) (397 mg, 500 μmol) in acetonitrile (2 ml) was carefully added to the stirred solution. After *c.a.* 10 min. at rt the the intermediate phosphite (207) was formed.

R_f 0.01 (pentane-EtOAc; 1:4)

δ_P ($CDCl_3$, 32.61 MHz) -5.68 (s), 138.72(m)

*9.34.2 Oxidation of DL-2,3,6-Tri-O-Benzyl-*myo*-Inositol 1-[Di(2,2,2-Trichloroethyl) Phosphate]-4,5 Bis[Di-O-Benzyl) Phosphite] (207)*



(207) (209)

Scheme 143. $R^1=CH_2CCl_3$

The crude sample of (207) in acetonitrile solution from the above reaction was diluted with pyridine (5 ml). The bisphosphite (207) was then oxidised by the addition of sulphur (0.5 g, 16 mmol). After 4h the reaction was shown to be complete by tlc. Purification was effected by elution through a silica gel column with a stepwise elution gradient. The elution stared with pentane (100 ml), to remove excess tetrazole, then pentane-EtOAc; 10:1 (500 ml) to remove excess di-*O*-benzyl (diisopropylamino) phosphorothioate and finally pentane-EtOAc; 4:1, after which the crude product was dried *in vacuo* and recrystallised from pentane to give (209) (673 mg, 42%),

m.p. 103-104° C

R_f 0.30 (pentane-EtOAc; 1:4)

Anal. Calc. for $C_{59}H_{59}Cl_6O_{13}P_3S_2$: C, 52.65; H, 4.36. Found: C, 52.7; H, 4.36%.

δ_H ($CDCl_3$, 400 MHz) 3.59 (H, ddd, ^3J 2, 9.2 Hz, Ins-H, pos.-3), 4.16-4.35 (4H, m), 4.36 (H, t, ^3J 2 Hz, Ins-H, pos.-2), 4.43 (H, dd, ^3J 7, 11 Hz), 4.45 (H, dd, ^3J 2, 11 Hz, Ins-H, pos.-1), 4.52-4.77 (6H, m), 4.81-5.02 (7H,m), 5.17 (2H, dt, ^3J 7.3, 11.3 Hz), 5.38 (H, dt, ^3J 9.2, 11.9 Hz, Ins-H, pos.-4), 6.88-7.42 (35H, m, Ph).

Assignments of ring protons were confirmed using a ^1H-^1H COSY spectrum at 400 MHz, and a P-H chemical shift correlation spectrum at 109.25 MHz.

δ_C ($CDCl_3$, 68 MHz) 69.80 (t), 69.87 (d, Ins-C), 72.14 (t), 74.31 (d, Ins-C), 74.57 (t), 77.00 (d, Ins-C), 77.49 (t), 78.01 (d, Ins-C), 78.27 (t), 79.08, 79.17 (2d, 2 Ins-C), 126.60, 127.11, 127.34, 127.63, 127.76, 127.93, 127.99, 128.09, 128.25, 128.35, 128.41 (11d, Ph), 135.94, 137.40, 137.98 (3s, Ph).

δ_P ($CDCl_3$, 161.8 MHz) -5.45 (s, pos.-1); ^{31}P-^1H (m), 68.39 (s, pos.-4 or -5); ^{31}P-^1H (m), 68.44 (s, pos.-4 or -5); ^{31}P-^1H (m).

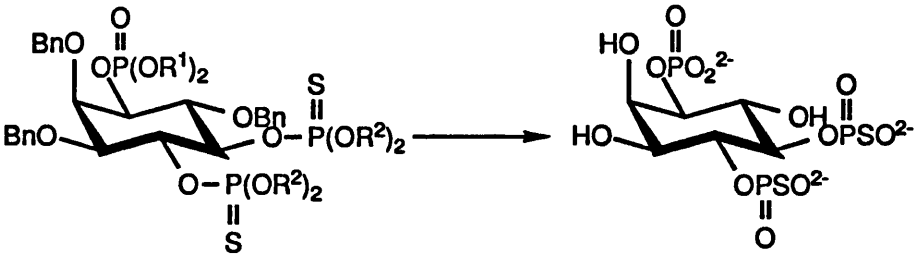
m/z, multiple peaks found when Cl present in fragment.

(FAB +ve ion NOBA), 1347 [(M, 3 $^{37}Cl)^+$, 3%; also M^+ with 1,2,4,5 ^{37}Cl], 1256 [(M-CH_2Ph), 0.5], 181 [$(PhCHCH_2Ph)^+$, 100], 91 [$(CH_2Ph)^+$, 95].

(FAB -ve ion NOBA), 1255 [$(M\text{-}CH_2Ph)^-$, 9%], 1212 [$(M\text{-}OCH_2CCl_3)^-$, 15], 293

[$(OPS\{OBn\}_2)^-$, 100], 95 [$(SPO_2)^-$, 28]

9.35 Synthesis of DL-*myo*-Inositol 1-Phosphate-4,5-Bisphosphorothioate (6)



$R^1=CH_2CCl_3$ $R^2=CH_2CH_2CN$ (208)

$R^2=Bn$ (209) (6)

Scheme 144

The title compound (6) was prepared by the deprotection of samples of (208) and (209), the deprotection reaction being a reductive cleavage by sodium in liquid ammonia.

Ammonia (50 ml) was condensed onto sodium, the resultant deep blue colour was stirred for 5 min, the ammonia (30 ml) was then distilled into another vessel containing sodium (0.2 g, 9 mmol) again to give a deep blue colour, the ammonia solution was then kept at -78°C to avoid evaporation. To this solution, the sample to be deprotected either (208) or (209) in dry dioxan (5 ml) solution was carefully added over 2 min under nitrogen. The reaction was left for 2 min at -78°C, the excess sodium was then quenched by the addition of isopropanol. The white solid produced was taken up in water (50 ml) and stirred with H^+-Dowex 50-W-X8 20-50 mesh cation exchange resin until pH reached 7. Removal of the resin by filtration and subsequent addition of triethylamine (0.1 ml) increased the pH of the solution to 9.

Deprotection of (208) (358 mg, 300 μmol) gave a crude mixture.

δ_P (D_2O, pH 9, 121.497 MHz) 2.95 (s), 3.28 (s), 3.71 (m), 3.79 (m), 4.24 (s), 44.75 (s), 44.80 (s), 45.23 (s), 45.72 (s).

Separation was effected using a DEAE-sephadex A 25, 20 x 2.5 cm chromatographic column, linear gradient of TEAB buffer 0-800 mM to give (6) (189 mg, 250 μmol, 83%) as a glass.

Deprotection of a sample of (209) (140 mg, 104 μmol) as above resulted in the formation of (6) (88 μmol, 85%) with little or no contamination.

δ_P (D_2O, pH 9, 36 MHz) 2.89 (s), 44.76 (s), 45.03 (s).

However, further purification was effected by anion exchange chromatography using Q-sepharose fast-flow gel (1.5 x 15 cm) column, again with a linear gradient of TEAB buffer (0.2-1 M) to give (6) (88.5 μmol, 85%) as a glass.

δ_H (D_2O, pH 7, 300 MHz) 3.74 (H, dd, ^3J 2.7, 9.7 Hz, Ins-H, pos. -3), 3.96 (H, dd, ^3J 9.5, 9.7 Hz, Ins-H, pos. -6), 4.02 (H, dd, ^3J 2.7, 9.7 Hz, Ins-H, pos. -1), 4.21 (H, dd, ^3J 9.5, 9.7 Hz, Ins-H, pos. -5), 4.28 (H, t, ^3J 2.7 Hz, Ins-H, pos. -2), 4.48 (H, dd, ^3J 9.5, 9.7 Hz, Ins-H, pos. -4).

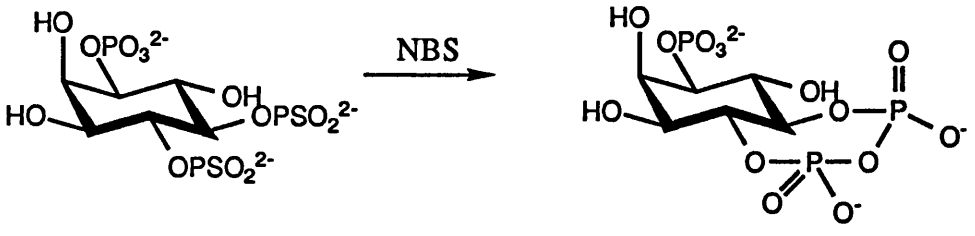
δ_C (D_2O, pH 7, 76 MHz) 72.92 (d, Ins-C, pos. -2), 74.06 (d, Ins-C, pos. -3), 74.72 (d, $^2J_{CP}$ 6.7 Hz, Ins-C, pos. -1), 76.78 (d, $^2J_{CP}$ 5.1 Hz, Ins-C, pos. -6), 78.49 (d, $^2J_{CP}$ 6.4 Hz, Ins-C, pos. -4), 80.62 (d, $^2J_{CP}$ 6.5 Hz, Ins-C, pos. -5).

δ_P (D_2O, pH 9, 121 MHz), 2.30 (s, pos. -1); ^{31}P-^1H (d, ^3J 6.2 Hz), 44.56 (s, pos. -5); ^{31}P-^1H (d, ^3J 9.5 Hz), 44.64 (s, pos. -4); ^{31}P-^1H (d, ^3J 9.6 Hz).

Assignments were made with reference to $^1H^1H$ COSY, $^1H^{13}C$, $^1H^{31}P$ and J resolved ^1H, correlated spectra, [Chapter 7 Figs. 56, 57, 59 and 60].

m/z (FAB +ve ion NOBA) 656 [(M+2 $HNEt_3)^+$, 73%], 554 [(M+$HNEt_3)^+$, 35], 102 [$(HNEt_3)^+$, 100].

9.36 Synthesis of DL-*myo*-Inositol-1-Phosphate-4,5-Cyclic Pyrophosphate (5)



(6) (5)

Scheme 145

Addition of NBS (77 mg, 432 μmol, 8 fold excess) to a stirred solution of Ins(1,4,5)P_3-4,5S_2 (41 mg, 54 μmol) (6) in dry dioxan (1.6 ml) water (0.4 ml) at rt was followed after 1 min. by the reduction of excess NBS with 2-mercaptoethanol (30 μl). The products were diluted with TEAB (10 ml, 10 mM). Excess solvents were removed *in vacuo* and the products separated by anion exchange chromatography on a 10 cm Q-sepharose fast flow column. A linear gradient of TEAB (0.2 to 1 M) eluted three phosphate bands: Ins(1, 4:5 Pyro)P_3 (5), 550 to 645 mM (21.8 mg, 36 μmol, 67%).

δ_H (D_2O, pH 9, 400 MHz), 3.75 (1H, dd, ^3J 2.6, 9.5 Hz, Ins-H, pos. -3), 3.90 (1H, t, ^3J 9.0 Hz, Ins-H, pos. -6), 4.20 (1H, m, Ins-H, pos. -1), 4.09 (1H, dd, ^3J 9.0, 14.5 Hz, Ins-H, pos. -5), 4.28 (1H, brd, Ins-H, pos. -2), 4.37 (1H, dd, ^3J 9.5, 14.5 Hz, Ins-H, pos. -4).

δ_C (D_2O, pH 9, 68 MHz) 68.62 (d, $^3J_{CP}$ 7 Hz, Ins-C), 69.60 (d, $^2J_{CP}$ 4 Hz, Ins-C), 70.18 (d, Ins-C), 74.00, 78.54, 78.64 (3d, 3 Ins-C).

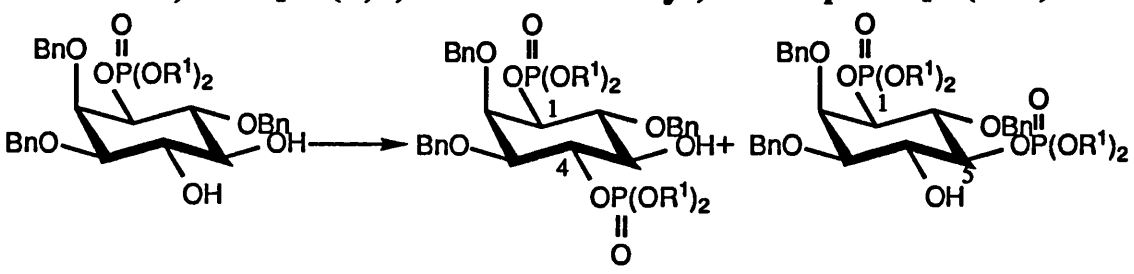
δ_P (D_2O, pH 8, 109 MHz) δ_1 -11.98 (pos. -5); ^{31}P-^1H (d, $^3J_{PH}$ 6.7 Hz), δ_2 -11.57 (dd, $J_{1,2}$ 16.8 Hz, pos. -4); ^{31}P-^1H (d, $^3J_{PH}$ 6.7 Hz), 3.04 (s, pos. -1); ^{31}P-^1H (m, $^3J_{PH}$ 6.7 Hz).

m/z, (FAB, -ve ion NOBA), 401 [(M-H)$^-$, 100%], 177 (5), 159 (14).

The other two bands that separated and comprised of several unidentified Ins P_3; 674 to 735 mM (9.7 mg, 24%) and (ii) 755 to 775 mM, (2.7 mg, 7%)

δ_P (D_2O, pH 8, 121 MHz) 0.87 (s), -0.25 (s, 2P).

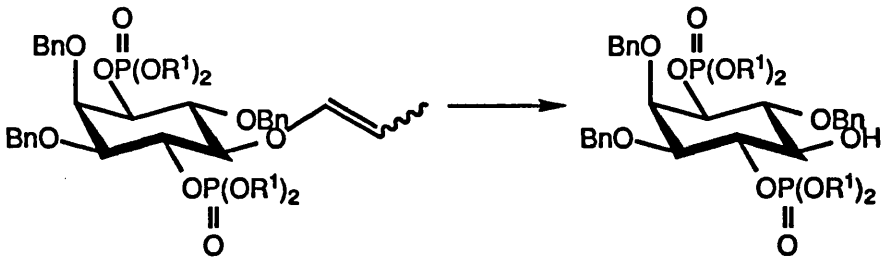
9.37 Synthesis of DL-2,3,6-Tri-*O*-Benzyl-*myo*-Inositol 1,4-Bis[Di(2,2,2-Trichloroethyl) Phosphate] (210)



(204) (210) (211)

Scheme 146. R^1=CH_2CCl_3

Dry pyridine (0.2 ml, 2.5 mmol) was added to a stirred solution of of the diol (204) (70 mg, 87 μmol) and bis(2,2,2-trichloroethyl) phosphorochloridate (70 mg, 185 μmol) in dry dichloromethane (15.0 ml). After 6h the conversion of (204) to a major product was seen by tlc. The solution was stirred with water (0.2 ml) for 30 min. and then washed successively with HCl (10.0 ml, 1M) saturated NaCl (10 ml) and dried with $MgSO_4$. The solvents were evaporated under reduced pressure and the crude product was chromatographed on a short silica gel column, as rapidly as possible to reduce formation of the cyclic 5-membered phosphate. The elution was performed in a stepwise manner initially with ether-pentane (1:2), to remove the by product of the phosphorylating reagent (not occurring on tlc) and finally ether-pentane (1:1) to give a mixture of the two possible bisphosphates (210) and (211), (73.5 mg, 65 μmol, 65%). The mixture was triturated with pentane and the solid material was filtered off and recrystallised from EtOAc-pentane to give (210) (25 mg, 22 μmol, 29%).



(212) (210)

Scheme 147. $R^1=CH_2CCl_3$

The title compound (210) was prepared in a slightly different manner. A sample of DL-2,3,6-tri-*O*-benzyl-5-propenyl-*myo*-inositol 1,4-bis-[di(2,2,2-trichloroethyl) phosphate] (212) (113 mg, 100 μmol) was taken in acetone-water (20 ml; 10:1) at rt. To this solution, mercury (II) chloride (200 mg, 740 μmol) in acetone-water (10 ml; 10:1) was added over 5 min. with stirring. After 4 days tlc. (ether-pentane; 1:4) showed the conversion of (212) to product. Solvents were removed *in vacuo* and the residue taken up in ether (30 ml), and washed with saturated potassium iodide (4 x 50 ml), water (2 x 50 ml) and dried over $MgSO_4$. Purification was effected by expeditious short column flash chromatography (ether-pentane; 1:4) to give (210) (90 mg, 80%), again care was needed to avoid the formation of the cyclic 5 membered phosphate.

m.p. 149-151°C.

R_f 0.26 (ether-pentane, 2:1)

Anal. Calc. for $C_{35}H_{35}Cl_{12}O_{12}P_2$: C, 37.04; H, 3.11. Found: C, 36.91; H, 3.08%.

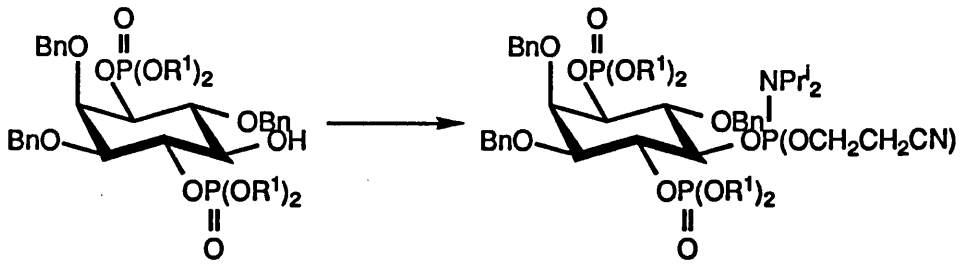
δ_H ($CDCl_3$, 300 MHz) 3.48 (1H, dd, ^3J 2.6, 9.8 Hz, Ins-H, pos.- 3), 3.73 (1H, t, ^3J 9.2 Hz, Ins-H, pos. -5), 4.01 (1H, dd, ^3J 9.2, 9.7 Hz, Ins-H, pos. -6), 4.31-4.716 (12H, m, CH_2Ph, 4 CH_2CCl_3, 2 Ins-H), δ_1 4.69, δ_2 5.08 (2H, $J_{1,2}$ 11.1 Hz, CH_2Ph), 4.83 (1H, dd, ^3J 9.2, 9.8 Hz, Ins-H, pos. -4), δ_1 4.83, δ_2 4.91 (2H, $J_{1,2}$ 11.5 Hz, CH_2Ph), 7.26-7.43 (15H, m, Ar-H).

δ_P ($CDCl_3$, 121 MHz) -6.58 (s); ^{31}P-^1H (m, ^3J 8.6 Hz), -7.9 (s); ^{31}P-^1H (dt ^3J 6.5, 6.8 Hz)

m/z (FAB +ve ion, NOBA), 1135 [(M, 3 $^{37}Cl)^+$, 5%; also M^+ with 1,2,4,5,6,7 ^{37}Cl], 1044 [$(M\text{-}CH_2Ph)^+$, 2], 954 [$(M\text{-}2CH_2Ph)^+$, 2], 361 {$[HOPO(OCH_2CCl_3)_2]^+$, 0.5}, 181 [$(PhCHCH_2Ph)^+$,10], 91 [$(PhCH_2)^+$,100].

9.38 Synthesis of DL-2,3,6-Tri-*O*-Benzyl-*myo*-Inositol-1,4-Bis[Di(2,2,2-Trichloroethyl) Phosphate]-5-[Di-(2-Cyanoethyl) Phosphorothioate] (215)

*9.38.1 Synthesis of DL-2,3,6-Tri-O-Benzyl-*myo*-Inositol-1,4-Bis[Di(2,2,2-Trichloroethyl) Phosphate]-5-[Diisopropylamino (2-Cyanoethyl) Phosphoramidite] (213)*



(210) (213)

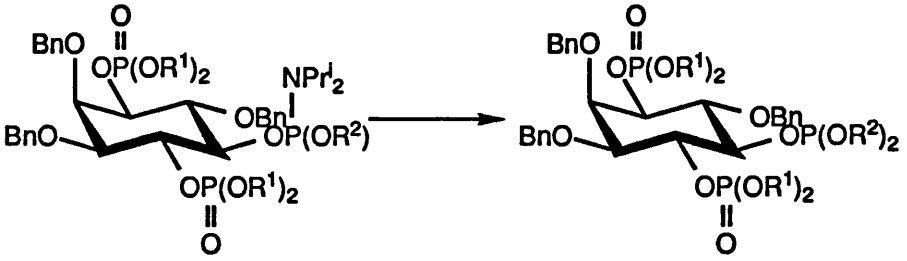
Scheme 148. R^1=CH_2CCl_3

A sample (210) (182 mg, 160 μmol) was dried by repeated co-evaporation with dry solvent. The sample was then dissolved in a solution of acetonitrile (2 ml) with diisopropylethylamine (45 μl, 250 μmol) and stirred under nitrogen at rt. The phosphitylating agent (78) (53 mg, 224 μmol) was carefully added dropwise to give the

mixed phosphate / phosphoramidite (213).

δ_P (MeCN, 36 MHz) -5.79 (s), -5.59 (s), 13.39 (s), 138.63 (s, $(R^2O)_3P$), 150.08 (s), 151.63 (s), 180.03 (s, excess reagent).

9.38.2 Synthesis of DL-2,3,6-Tri-O-Benzyl-myo-Inositol-1,4-Bis[Di(2,2,2-Trichloroethyl) Phosphate]-5-[Di(2-Cyanoethyl) Phosphite] (214)



(213) (214)

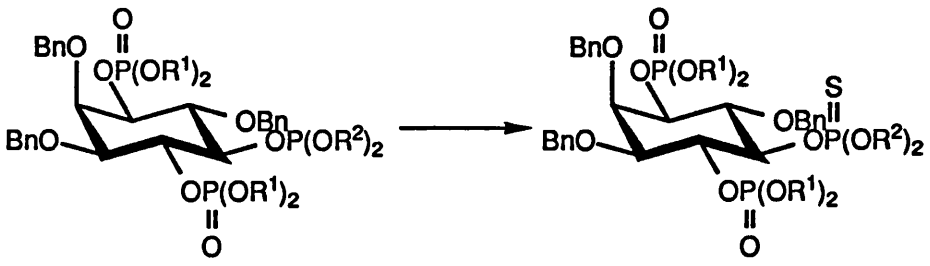
$R^1=CH_2CCl_3$, $R^2=CH_2CH_2CN$

Scheme 149

The crude acetonitrile solution of (213) from the above reaction was taken with tetrazole (21 mg, 300 μmol) and 2-cyanoethanol (22 μl, 300 μmol). The mixture was stirred for 1h after which time the mixed phosphate / phosphite (214) was formed.

δ_P (MeCN, 36 MHz) -5.65 (s), -5.45 (s), 13.80(s), 138.37 (s, $(R^2O)_3P$), 141.40 (s),

9.38.3 Oxidation of DL-2,3,6-Tri-O-Benzyl-myo-Inositol-1,4-Bis[Di(2,2,2-Trichloroethyl) Phosphate]-5-[Di-(2-Cyanoethyl) Phosphite] (214)



(214) (215)

$R^1=CH_2CCl_3$, $R^2=CH_2CH_2CN$

Scheme 150

The crude acetonitrile solution of (214) was diluted by the addition of pyridine (5 ml) and oxidised by the subsequent addition of sulphur (0.5 g, 16 mmol). Purification by flash column chromatography over silica, eluent (pentane-EtOAc, 1:2), gave the mixed phosphate / phosphorothioate triester (215) as a pale yellow glass (94 mg, 70 μmol, 44%).

R_f 0.53 (pentane-EtOAc, 1:2)

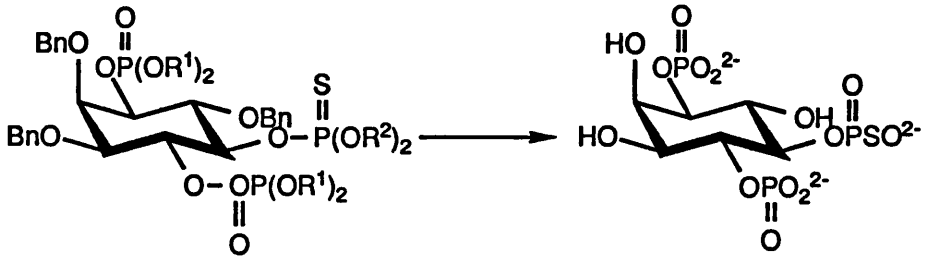
δ_H ($CDCl_3$, 270 MHz), 2.70-2.78 (4H, m, $OCH_2\underline{C}H_2CN$), 3.57 (H, dd, ^3J 2.0, 11.25 Hz, Ins-H, pos. 3), 3.66-3.80 (2H, m, 2 Ins-H), 4.00-4.75 (18H, m, $C\underline{H}_2Ph$, $OC\underline{H}_2CCl_3$, Ins-H), 4.80-5.00 (3H, m), 7.26-7.44 (15H, m, Ph).

δ_C ($CDCl_3$, 67.80 MHz), 18.46 (t, J_{HP} 8.9 Hz, $OCH_2\underline{C}H_2CN$), 19.33 (t, J_{HP} 8.9 Hz, $OCH_2\underline{C}H_2CN$), 62.57, 62.03, 62.89 (3 t), 72.36, 74.60 (2 d, Ins-C), 75.77 (t), 76.77 (d, Ins-C), 76.87, 77.20 (2 t), 77.62, 79.08, 79.17 (3 d, 3 Ins-C), 94.55 (s, $\underline{C}Cl_3$), 126.14, 127.70, 128.09, 128.22, 128.51, 128.74 (6 d, Ph), 136.42, 137.69 (2 s, Ph).

δ_P ($CDCl_3$, 109.37 MHz), -5.98 (s, pos. -1 or -4); ^{31}P-^1H (m, ^3J 6.7 Hz), -5.92 (s, pos. -1 or -4); ^{31}P-^1H (m, ^3J 6.7 Hz), 67.00 (s, pos.-5); ^{31}P-^1H (m, ^3J 6.7 Hz).

m/z, multiple peaks found when Cl present in fragment.
(FAB + ve ion NOBA), 1341 [(M, 5 $^{37}Cl)^+$, 6%; also M^+, with 2,3,4,6,7 ^{37}Cl], 1306 $[(M\text{-}Cl)^+$, 1], 1249 $[(M\text{-}H\text{-}CH_2Ph)^+$, 1.2], 91 $[(CH_2Ph)^+$, 100].

9.39 Synthesis of DL-*myo*-Inositol 1,4-Bisphosphate 5-Phosphorothioate (7)



(215) (7)

R^1=CH_2CCl_3, R^2=CH_2CH_2CN

Scheme 151.

Deprotection of (215) to give DL-*myo*-inositol 1,4-bisphosphate 5-phosphorothioate, Ins(1,4,5)P_3-5S (7) was effected by a sodium in liquid ammonia reaction.

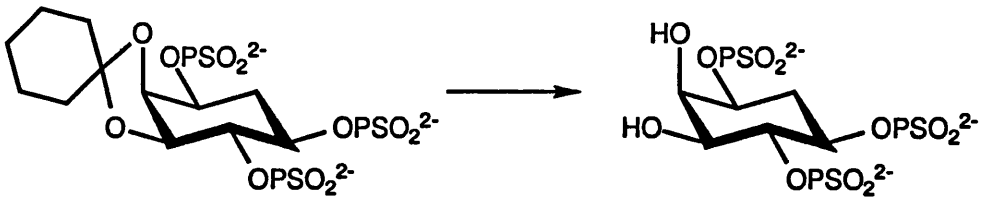
Ammonia (50.0 ml) was condensed onto sodium and stirred for 5 min. The ammonia (40.0 ml) was distilled into another vessel containing sodium (200.0 mg, 8.7 mmol) giving a deep blue colour and was kept at -78°C. To this a solution of (215) (80.0 mg, 60 μmol) in dry dioxan (0.2 ml) was added over 5 min. The reaction was stirred for 2 min, and then quenched by the addition of isopropanol until the blue colour was lost. The solution was allowed to warm to rt and the solvents were then removed *in vacuo*. The solid thus produced was taken up in water (50.0 ml) and mixed with H^+-Dowex cation-exchange resin until the pH reached neutrality. The resin was removed by filtration and the solution basified with triethylamine (pH 9). The salt was further purified by anion-exchange chromatography on DEAE Sephadex-A25 resin using triethylammonium bicarbonate buffer (0.1-1M), the product eluting *ca.* 750 mM, (19.6 mg, 26 μmol, 88%) as a pale yellow glass.

δ_H (D_2O, pH 9, 300 MHz) 3.70 (1H, dd, ^3J 2.6, 9.5 Hz, Ins-H, pos. -3), 3.90 (2H, m, 2 Ins-H), 4.10-4.20 (2H, m, 2 Ins-H), 4.30 (1H, t, ^3J 2.6, Ins-H, pos. -2).

δ_P (D_2O, pH 9, 109.4 MHz) 3.25 (s, pos. -1 or -4); ^{31}P-^1H (d, ^3J 6.7 Hz), 4.35 (s, pos. -1 or -4); ^{31}P-^1H (d, ^3J 6.7 Hz), 43.24 (s, pos. -5); ^{31}P-^1H (d, ^3J 6.7 Hz).

m/z (FAB -ve ion thioglycerol) 435 [$(M-H)^-$, 100%], 401 (8), 321 (9), 177 (19), 159 (20).

9.40 Synthesis of 6-Deoxy-D-*myo*-Inositol-1,4,5-Tris-Phosphorothioate (8).



(196) (8)

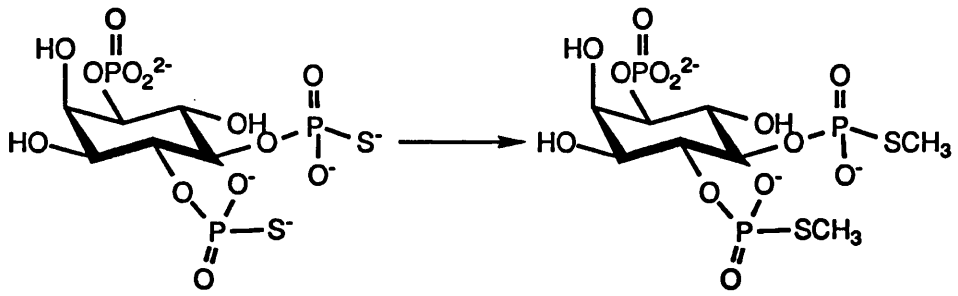
Scheme 152

A stirred solution of (196) (30 mg, 57 μmol) in water (50 ml) at rt was treated with Amberlite IR-120(plus) ion-exchange resin for 1h. Subsequent removal of the resin by filtration, followed by the evaporation of solvent *in vacuo* gave (8) as a glass. Purification by anion-exchange chromatography using Q-sepharose fast-flow gel (1.5 x 15 cm) TEAB (0.3 M to 1 M) gave (8) as the triethylammonium salt (25 mg, 55 μmol, 97%) as a glass.

δ_H (D_2O, pH 7, 270 MHz) 1.96-1.98 (H, m, 6D-Ins-H, pos. 6'), 2.37-2.41 (H, m, 6D-Ins-H, pos. 6), 3.66 (H, dd, ^3J 2.3, 9 Hz, 6D-Ins-H) 4.17-4.30 (3H, m, 6D-Ins-H), 4.33 (H, t, ^3J 9 Hz, 6D-Ins-H).

δ_P (D_2O, pH 7, 270 MHz) 42.19 (s), 54.37-45.84 (s)

9.41 Synthesis of *myo*-Inositol 1- Phosphate 4,5-Bis [Methyl-Phosphorothiolate] (216)



(6) (216)

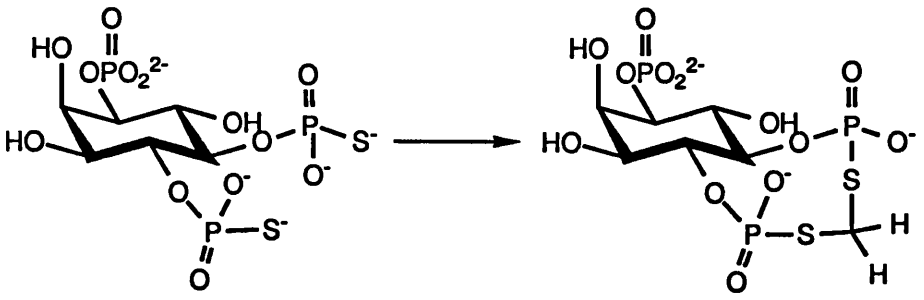
Scheme 153

A sample of (6) (18.6 mg, 27 μmol) as the tris-triethylammonium salt was taken and dissolved in dry methanol (0.5 ml). Iodomethane (30 μl, 540 μmol) was added to the stirred solution and the solution was left at rt. for 8h. The solvents were removed under reduced pressure and the resultant colourless glass was taken up in water (20 ml) and washed with EtOAc (20 ml), then brine (20 ml).

δ_P (D_2O, pH 9, 109 MHz) -10.15 (s), 1.83 (s), 2.05 (s) 2.96 (s), 16.25 (d), 21.43 (s).

The sample was then loaded onto a Q-Sepharose fast flow gel anion-exchange column and eluted with TEAB buffer (0.2 to 1 M) to give (216) as a glass (6.8 mg, 9.5 μmol, 51%). However, characterisation of the product proved to be difficult due to the small amount of product.

9.42 Synthesis of *myo*-Inositol 1- Phosphate 4,5-[Cyclic-S,S-Methylene] Bis-Phosphorothiolate (217)



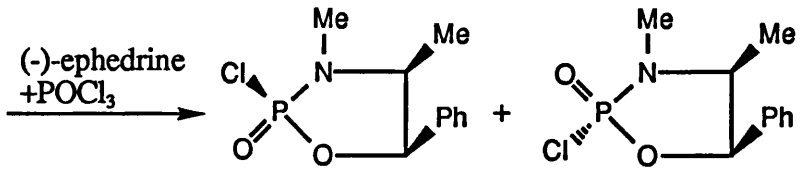
(6) (217)

Scheme 154

A sample of (6) (27 mg, 36 μmol) was dissolved in acetonitrile (500 μl), methanol (250 μl) and triethylamine (25 μl, 180 μmol). After the subsequent addition of diiodomethane (15 μl, 180 μmol) the reaction was left at rt for 4h. Removal of solvents under reduced pressure gave a glass which was taken up in water (50 ml) and loaded onto a Q-sepharose fast flow gel, anion exchange column (1.5 x 15 cm) and eluted with TEAB (0.2 to 1 M). However, the product co-eluted with the pyrophosphate present in the mixture. It was impossible to separate the two because of the small amounts of compounds.

δ_P (D_2O, pH 9, 109 MHz) δ_1 -8.82, δ_2 -8.43 (dd, $J_{1,2}$ 18 Hz), 4.85-7.13 (m), 17.70-19.03 (m)

9.43 Synthesis of (2R, 4S, 5R) and (2S, 4S, 5R) -2-Chloro-3,4 Dimethyl-5-Phenyl-1,3,2-Oxazaphospholidin-2-ones (64) and (65)



(64) (65)

Scheme 155

This compound was prepared according to the procedure of Cooper *et al.*[118]. Phosphorus oxychloride (2 ml, 21 mmol) was slowly added to a stirred solution of (-) ephedrine HCl [(1R, 2S)-2-methylamino-1-phenylpropan-1-ol] (4.1 g, 20 mmol) in a

mixture of dry benzene (100 ml) and triethylamine (14 ml, 99 mmol) and left at rt for 2h until the reaction was seen to be complete by tlc. After filtration of triethylamine hydrochloride and removal of solvent under reduced pressure, a brown oil was isolated which was then purified by silica gel flash column chromatography to give (64) (3.3 mg, 67%)

R_f 0.38 ($CHCl_3$-EtOAc; 9:1)

m.p.87-88 °C {Lit.[118] m.p. 88-89 °C}

δ_P (D_2O, pH 9, 36 MHz) 21.34 (s)

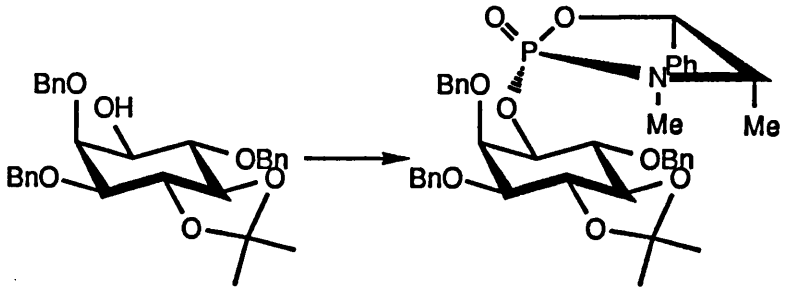
and (65) (0.3 g, 6%)

R_f 0.52 ($CHCl_3$-EtOAc; 9:1)

m.p. 108-112 °C {Lit.[118] m.p. 111-113 °C}

δ_P (D_2O, pH 9, 36 MHz) 23.31 (s)

9.44 Synthesis of (2R, 4S, 5R) -2'-(-1-[2,3,6-Tri-*O*-Benzyl]-4,5-*O*-Isopropylidene-*myo*-inositol)-3',4'-Dimethyl-5'-Phenyl-1',3',2'-Oxazaphospholidin-2'-one (218)



(202) (218)

Scheme 156

A sample of (202) (115 mg, 235 µmol) was dried by co-evaporation of dry solvent. The sample was then dissolved in diisopropylamine (5 ml) and to this stirred solution of (202), the reagent (64) (87 mg, 352 µmol) was added over 1 min. After 30 min at rt the reaction was seen to be complete by NMR.

δ_P ($CHCl_3$, diisopropylamine, 36 MHz) 17.50 (s).

The solvent was removed under reduced pressure, the residue extracted into ether (20 ml) and washed with brine (20 ml) and water (20 ml) to give both diastereoisomers of (218) (95 mg, 62%) as a glass.

δ_H ($CDCl_3$, 300 MHz) 0.75, 0.84, 0.85 (9H, 3d, ^3J 6 Hz, NCC$\underline{H}_3$), 1.35, 1.37, 1.39, 1.41 (12H, 4s, CC$\underline{H}_3$), 2.03 (1H, d, J 4 Hz), 2.69, 2.70 (6H, 2d, $^3J_{PH}$ 9 Hz, PNC$\underline{H}_3$), 2.85 (3H, d, $^3J_{PH}$ 12 Hz, PNC$\underline{H}_3$), 3.55-3.95 (13H, m, 10 Ins-$\underline{H}$, 3 MeC$\underline{H}$CHPh), 4.12 (2H, dd, ^3J 7.4, 7.5 Hz, Ins-$\underline{H}$), 4.53-4.67 (12H, m, 6 PhC$\underline{H}_2$), 5.63, 5.64, 5.85 (3H, 3d, ^3J 6 Hz, PhC$\underline{H}$CMe), 7.25-7.45 (45 H, m, Ar-$\underline{H}$)

δ_P ($CDCl_3$, 121 MHz) 18.68 (s); ^{31}P-^1H (m).

Summary

The emphasis of this thesis has been to provide a synthetic route to 7-membered cyclic pyrophosphates, namely *myo*-inositol-1-phosphate-4,5-pyrophosphate. To this end we have developed a novel synthesis using a N-bromosuccinimide (NBS) mediated oxidative desulphurisation of vicinal bisphosphorothioates.

Several halogen containing oxidising agents were tested for their ability to form cyclic pyrophosphates. However, NBS was found to give the highest yield of pyrophosphate. The synthesis involves the treatment of a solution of a vicinal bisphosphorothioate in dioxan-water, (4:1) respectively, with an 8-fold excess of NBS. In order to try to elucidate the mechanism of the NBS-mediated desulphurisation reaction, the reaction was conducted in the presence of an equimolar mixture of oxygen-18 water ($H_2{}^{18}O$) and oxygen-16 water. The mechanistic studies revealed that the reaction proceeded by a complex combination of reaction pathways. It was possible to determine that activation of one sulphur atom followed by nucleophilic replacement by labeled water preceded pyrophosphate ring closure. The evidence for this was provided by the presence of ^{18}O label in the pyrophosphate bridge position. We have discovered that the yield of the pyrophosphate was increased by reducing the rotational freedom of the two phosphorothioate moieties. This was achieved by making a cyclic vicinal bisphosphorothioate, thus constraining the two phosphorothioates to a less conformationally mobile system. The result of this rotational constraint was to increase the yield of the cyclic pyrophosphate produced by *c.a.* 50%. The yield of the pyrophosphate was also increased by altering the electrophilicity of the phosphorus atoms. This was effected by alkylating the phosphorothioates to give S-alkyl phosphorothiolates. NBS-mediated desulphurisation of vicinal bisphosphorothiolates increased the yield of the pyrophosphate by *ca.* 20 %.

The discovery of a cyclic 7-membered pyrophosphate, cyclic bisphosphoglycerate, in methanogenic bacteria has provided us with a biologically important molecule to synthesise using our novel synthetic reaction. However, the synthesis of the vicinal bisphosphorothioate intermediate, 2,3-bisphosphorothioglycerate proved to be difficult. The desulphurisation of this molecule to give the pyrophosphate was unfortunately unsuccessful.

Synthesis of a cyclic pyrophosphate from a deoxy cyclitol polyphosphorothioate seemed to be a more achievable target.. We decided to try the desulphurisation of a

phosphorothioate-containing molecule that was a closer structural analogue to *myo*-inositol-1-phosphate-4,5-pyrophosphate. To this end 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol-1,4,5-trisphosphorothioate was synthesised. Desulphurisation of this molecule yielded the desired product, 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol-1-phosphate-4,5-pyrophosphate. Removal of the ketal gave another Ins(1,4,5)P_3 analogue, 6-deoxy-D-*myo*-inositol-1-phosphate-4,5-pyrophosphate. Since the synthesis of 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol-1-phosphate-4,5-pyrophosphate went as anticipated, we decided to attempt the desulphurisation of *myo*-inositol-1-phosphate-4,5-bisphosphorothioate. This molecule was synthesised from the parent cyclitol, *myo*-inositol, in a long and complicated linear synthesis. Some of the early intermediates had been reported before[138,236,237]. However, characterisation of those compounds was rudimentary, with only melting points and microanalysis being reported, and there was neither proton nor carbon NMR data. In order to rectify this omission we report here both proton and carbon NMR of these key intermediates. Desulphurisation of *myo*-inositol-1-phosphate-4,5-bisphosphorothioate, as was hoped, produced *myo*-inositol-1-phosphate-4,5-pyrophosphate in surprisingly high yield. However, there was some evidence of straight desulphurisation to Ins(1,4,5)P_3 and some phosphate migration due to cyclic phosphate hydrolysis. The analogue *myo*-inositol-1-phosphate-4,5-pyrophosphate was tested for biological activity, unfortunately, it was found be inactive at releasing Ca^{2+} and to be inactive towards the 5-phosphatase and 3-kinase enzymes. However, several of the intermediates prepared in this pathway proved to be interesting Ins(1,4,5)P_3 analogues.

Phosphorylation of the intermediate DL-2,3,6-tri-*O*-benzyl-*myo*-inositol-1-[di(2,2,2-trichloroethyl)phosphate] with bis(2,2,2-trichloroethyl) phosphate gave a mixture of two regioisomers, the 1,4-bisphosphate and the 1,5-bisphosphate. Trituration of the mixture gave a pure sample of one of the bisphosphates. Identification of the regioisomer isolated by spectroscopic methods was not trivial; it involved conducting several 2-dimensional NMR studies. Fortuitously, the isolated regioisomer was the desired 1,4-bisphosphate, which was then phosphitylated and sulphoxidised to give *myo*-inositol-1,4-bisphosphate-5-phosphorothioate after deprotection and purification. This molecule has been found to be an important inhibitor of Ins(1,4,5)P_3-5-phosphatase.

The key compound, *myo*-inositol-1-phosphate-4,5-bisphosphorothioate Ins(1,4,5)P_3-4,5S_2, synthesised as the penultimate intermediate in the synthesis of Ins(1,4:5-pyro)P_3 was found to be of great importance as an inhibitor of the Ins(1,4,5)P_3-5 phosphatase enzyme. Ins(1,4,5)P_3-4,5S_2 inhibits the 5-phosphatase with a similar K_i to that of the trisphosphorothioate, 1.3 μM as compared to 1.7 μM, but less than Ins(1,4,5)P_3-5S which has a K_i of 6.8 μM. Both Ins(1,4,5)P_3-4,5S_2 and Ins(1,4,5)P_3-5S inhibit the 3-

kinase with a greater K_i than the trisphosphorothioate, with values of 46 μM, 230 μM for Ins(1,4,5)P_3-4,5S_2 and Ins(1,4,5)P_3-5S respectively, as compared to the K_i of 5 μM for Ins(1,4,5)PS_3. Like Ins(1,4,5)PS_3, Ins(1,4,5)P_3-4,5S_2 is a poor substrate for the 3-kinase. Both Ins(1,4,5)P_3-5S and Ins(1,4,5)P_3-4,5S_2 were found to be full agonists of Ca^{2+} release, Ins(1,4,5)P_3-4,5S_2 was found to be more potent than Ins(1,4,5)PS_3 at releasing Ca^{2+}.

Two 6-deoxy phosphorothioate molecules were synthesised as part of the synthesis of the 6-deoxy pyrophosphate analogue. Namely, 6-deoxy-2,3-cyclohexylidene-D-*myo*-inositol-1,4,5-trisphosphorothioate and 6-deoxy-D-*myo*-inositol-1,4,5-trisphosphorothioate. Both 6-deoxy-D-*myo*-inositol-1,4,5-trisphosphorothioate and the trisphosphate analogue proved to be very weak agonists for Ca^{2+} release.

The phosphorothioate analogues of Ins(1,4,5)P_3 synthesised thus far have played a crucial role in the unravelling of the biology of the inositol lipid cycle. It is hoped that the analogues described in this thesis will further the understanding of this complex and highly interesting biological signalling system.

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Appendix

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Received 20 June 1988

Measurement of intracellular inositol 1,4,5-trisphosphate concentrations in unstimulated and vasopressin-stimulated rat hepatocytes using a novel inositol 1,4,5-trisphosphate-specific binding assay

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Ca^{2+}-mobilizing stimuli activate the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol, each of which have a second messenger role (see Downes & Michell, 1985, for a review). Ins(1,4,5)P_3 stimulates the release of Ca^{2+} into the cytosol from an intracellular store, probably the endoplasmic reticulum (Berridge & Irvine, 1984), by a mechanism which is proposed to involve a specific intracellular membrane receptor (Burgess *et al.*, 1984*a*). Evidence for a specific intracellular inositol trisphosphate (InsP_3) binding site has been obtained for a variety of systems including bovine adrenal microsomes (Guillemette *et al.*, 1987). We have examined the characteristics of the InsP_3 binding site in this system and found it to show stereospecificity. This preparation is therefore suitable for use as a binding protein for the determination of Ins(1,4,5)P_3 concentrations in cell extracts.

A radioligand binding assay was developed in which aliquots of the binding protein (containing 500–1000 mg of protein) were incubated at pH 9 with [^{3}H]Ins(1,4,5)P_3 (3000 c.p.m. = 52 fmol = 0.5 nM). Specific binding varied between 25 and 40% of total radioactivity in the incubation with non-specific binding not exceeding 10%.

The specific binding of Ins(1,4,5)P_3 to the microsomal preparation was rapid, reversible and saturable. Scatchard analysis of the [^{3}H]Ins(1,4,5)P_3 binding data demonstrated a single population of binding sites with an apparent dissociation constant (K_d) of 6.82 ± 2.3 nM and a binding capacity ($B_{max.}$) of 370 ± 38 fmol/mg of protein (combined data from seven determinations using three preparations of binding protein). The EC_{50} of Ins(1,4,5)P_3 binding was estimated to be 5.9 ± 0.9 nM which is similar to that observed by other workers using similar preparations (Baukal *et al.*, 1985; Guillemette *et al.*, 1987).

Competition studies were performed using a range of inositol polyphosphates (see Fig. 1). These experiments demonstrated a clear specificity of the binding site for Ins(1,4,5)P_3. The EC_{50} values of inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] (a naturally occurring inositol tetrakisphosphate) and Ins(2,4,5)P_3 (a synthetic Ca^{2+}-mobilizing inositol trisphosphate) were similar to each other, being approximately 19-fold greater than that of Ins(1,4,5)P_3. Ins(1,3,4)P_3 at concentrations up to 5 μM was without effect on the binding of [^{3}H]Ins(1,4,5)P_3. These results indicate that the vicinal 4- and 5-phosphate groups are required for binding. Other inositol phosphates and structurally similar molecules found intracellularly showed only limited competition with [^{3}H]Ins(1,4,5)P_3 binding.

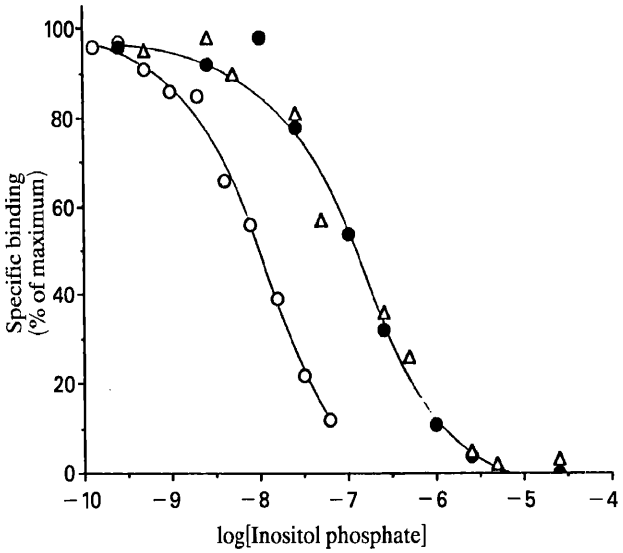

Fig. 1. *Competition of inositol polyphosphates for [^{3}H]Ins(1,4,5)P_3 binding sites*

[^{3}H]Ins(1,4,5)P_3-binding to bovine adrenocortical microsomes was determined in the presence of increasing concentrations of unlabelled Ins(1,4,5)P_3 (○), Ins(1,3,4,5)P_4 (●) or Ins(2,4,5)P_3 (△). Results are means (n = 3–6) for combined data of four experiments. S.D. values (which did not exceed 10%) have been omitted for clarity.

Using this radioligand binding assay, the concentration of Ins(1,4,5)P_3 in neutralized acid extracts of control and vasopressin-stimulated hepatocytes was determined. The addition of neutralized acid extracts prepared in the absence of hepatocytes was without effect on the radioligand-binding assay. By measuring the hepatocyte intracellular volume (Shears & Kirk, 1984), we have calculated the intracellular concentration of Ins(1,4,5)P_3.

While some variation was observed between hepatocyte preparations, basal concentrations of Ins(1,4,5)P_3 were generally in the order of 0.2 μM (0.22 ± 0.15 μM, combined data from six hepatocyte preparations). Stimulation of hepatocytes with vasopressin (230 nM) resulted in a 6–10-fold increase in Ins(1,4,5)P_3 concentration (2.5 ± 1.8 μM, n = 12, combined data from four hepatocyte preparations) which peaked at 5–15 s before returning towards basal levels.

These concentrations are an order of magnitude lower than those calculated using isotopic techniques (e.g. 5.6 μM basal, 38 μM stimulated — calculated from the data of Burgess *et al.*, 1985), but are much closer to what might be expected in view of the fact that the EC_{50} for Ins(1,4,5)P_3-induced Ca^{2+} release in permeabilized guinea-pig hepato-

Abbreviations used: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(2,4,5)P_3, inositol 2,4,5-trisphosphate; InsP_3, inositol trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate.

cytes is 0.1 μM (Burgess *et al.*, 1984*b*). Furthermore, similar values have been obtained for Ins(1,4,5)P_3 concentration in neutrophils by competing [^{32}P]Ins(1,4,5)P_3 with cellular Ins(1,4,5)P_3 in permeabilized cells (Bradford & Rubin, 1986) and in platelets by separation of InsP_3 isomers and determination of the specific activity (Daniel *et al.*, 1987).

Parts of this work were supported by grants from the Cancer Research Campaign and the Medical Research Council. Thanks to Robin Irvine for the generous supply of inositol polyphosphates.

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Received 20 June 1988

myo-Inositol 1,4-bisphosphate-5-phosphorothioate: chemical synthesis and biochemical properties

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It is now generally agreed that D-*myo*-inositol 1,4,5-trisphosphate (InsP_3) (Fig. 1*a*), released by receptor-mediated phospholipase C-catalysed cleavage of the minor membrane phospholipid phosphatidylinositol 4,5-bisphosphate, is a second messenger linking the spatially separated events of receptor stimulation and mobilization of calcium from internal stores (Berridge, 1987).

Metabolism of InsP_3 occurs via two major pathways: specific 5-phosphatase-catalysed breakdown and deactivation to D-inositol 1,4-bisphosphate [Ins(1,4)P_2], which is then further degraded (Majerus *et al.* 1988) and 3-kinase-mediated phosphorylation to D-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] (Irvine *et al.*, 1986), which has been proposed to regulate calcium entry across the plasma membrane (Irvine & Moor, 1986; Morris *et al.*, 1987). We have sought to develop synthetic routes to inositol phosphates (Hamblin *et al.*, 1987*a*; Cooke *et al.*, 1987*a*) and especially to develop novel non-hydrolysable analogues such as phosphorothioates (Hamblin *et al.*, 1987*b*), which can be used to modify and investigate phosphoinositide metabolism. Our recent synthesis of *myo*-inositol 1,4,5-trisphosphorothioate (Ins$P$[S]$_3$) (Fig. 1*b*) (Cooke *et al.*, 1987*b*) has provided a molecule which is recognized by a specific cerebellar InsP_3 binding site, which may be the InsP_3 receptor (Worley *et al.*, 1987; Willcocks *et al.*, 1987), and is a potent mobilizer of intracellular calcium (Taylor *et al.*, 1988; Strupish *et al.*, 1988) and yet is resistant to 5-phosphatase-catalysed deactivation (Willcocks *et al.*, 1988) and is therefore able to generate a prolonged calcium signal (C. W. Taylor, M. J. Berridge, A. M. Cooke & B. V. L. Potter, unpublished work). It is clear

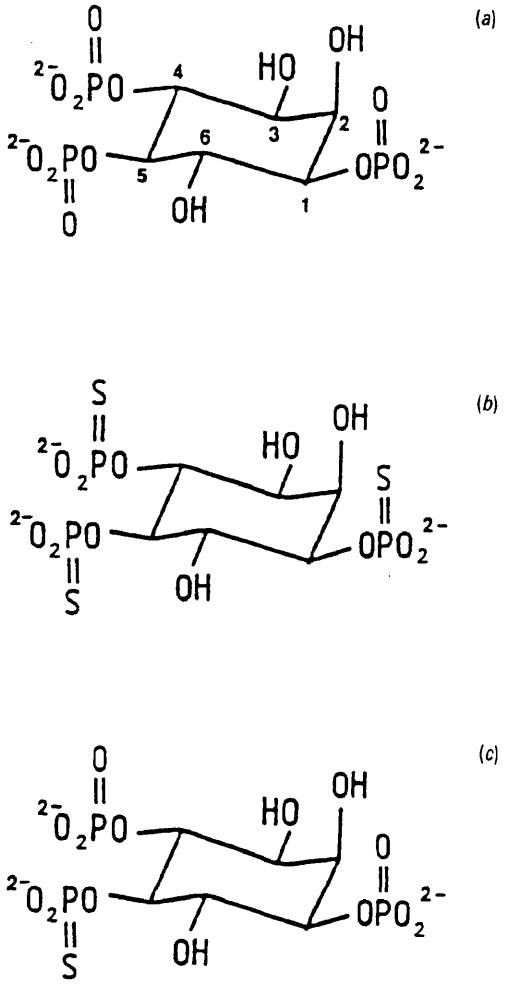

Fig. 1. *Structures of myo-inositol 1,4,5-trisphosphate (a) and phosphorothioate analogues thereof (b, c)*

Single D-enantiomers are shown in each case.

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Abbreviations used: InsP_2, InsP_3, inositol bis- and tris-phosphate respectively, with locants designated where appropriate; Ins$P$[S]$_3$, inositol 1,4,5-trisphosphorothioate; Ins(1,4)P_2(5′)P[S], inositol 1,4-bisphosphate-5-phosphorothioate.

that such analogues possess considerable potential for investigations of the phosphoinositide signalling pathway.

Ins$P$[S]$_3$ possesses all three phosphate groups replaced by phosphorothioates. It is desirable, however, to synthesize an

analogue nearer in structure to InsP_3 and yet incorporating the advantages of 5-phosphatase resistance. Such requirements would be satisfied by the novel molecule *myo*-inositol 1,4-bisphosphate-5-phosphorothioate {Ins(1,4)P_2(5)P[S]} (Fig. 1*c*), wherein only the 5-phosphate group has been replaced by phosphorothioate. We thus sought to develop a synthetic route to Ins(1,4)P_2(5)P[S], and make a preliminary investigation of its biological properties.

DL-1,2,4-Tri-*O*-benzyl-isopropylidene-*myo*-inositol (Gigg *et al.*, 1987) was phosphorylated with bis-(2,2,2-trichloroethyl)phosphorochloridate and the isopropylidene group removed by acid treatment to give DL-1-[bis-(2,2,2-trichloroethyl)phospho]-2,3,6-tri-*O*-benzyl-*myo*-inositol. This was phosphorylated again to give a mixture of isomers possessing, in addition to the phosphorylated 1-position, a phosphotriester at either the 4- or 5-position. One of these isomers crystallized and its structure was investigated by 2D-J resolved ^{1}H n.m.r. spectroscopy in combination with correlated spectroscopy. Fortuitously, the crystalline isomer was found to be that with the desired 1,4 arrangement of phosphotriesters. Phosphitylation of the free hydroxyl group at the 5-position using *N*,*N*-di-isopropylamino-(2-cyanoethyl)chlorophosphine as previously reported (Cooke *et al.*, 1987*a*) followed by oxidation with t-butyl hydroperoxide yielded DL-1,4-[bis-(2,2,2-trichloroethyl)phospho]-2,3,6-tri-*O*-benzyl-5-[bis-(2-cyanoethylphospho)]-*myo*-inositol. Removal of protecting groups was accomplished in one step using sodium in liquid ammonia. Thus, the benzyl groups and 2,2,2-trichloroethyl groups were removed reductively and the 2-cyanoethyl groups by β-elimination. The resulting DL-*myo*-inositol-1,4,5-trisphosphate was purified by column chromatography and was obtained in 44% yield. This material was equipotent as previously synthesized DL-InsP_3 (Cooke *et al.*, 1987*a*) in displacing [^{3}H]InsP_3 from specific InsP_3 cerebellar recognition sites and at releasing calcium from intracellular stores in saponin-permeabilized Swiss 3T3 cells.

If the synthesis was modified at the final oxidation stage by using sulphur instead of t-butyl hydroperoxide and deblocking carried out as before, the novel DL-Ins(1,4)P_2(5)P[S] (Fig. 1*c*) could be obtained. This was examined for its affinity to bind to cerebellar 'receptors' and for its ability to release calcium. In both these respects its potency was very similar to that of Ins$P$[S]$_3$. The resistance of Ins(1,4)P_2(5)P[S] to the action of human erythrocyte 5-phosphatase-catalysed degradation was also examined. It was not deactivated by this enzyme under conditions where >90% of InsP_3 activity was destroyed.

We thank S.E.R.C., The Wellcome Foundation and the Research Corporation Trust for financial support. B.V.L.P. is a Lister Institute Research Fellow.

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Received 3 June 1988

A rise in intracellular calcium is not by itself sufficient signal to trigger the acetylcholine-induced intracellular acidosis in rabbit mandibular salivary gland acinar cells

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Stimulus–response coupling in a wide range of cells involves inositol phospholipid hydrolysis and an increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$) mediated by inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (Berridge & Irvine, 1984). It has been suggested that, in some exocrine gland acinar cells, the Ins(1,4,5)P_3-mediated rise in $[Ca^{2+}]_i$ which follows cholinergic stimulation may activate apical Cl^- channels (Marty, 1987). We have recently shown that acetylcholine (ACh) causes a transient, receptor-mediated intracellular acidosis in acinar cells from the rabbit mandibular salivary gland (Brown *et al.*, 1988*b*). The acidosis appears to be caused by HCO_3^- efflux from the cell, possibly through anion channels permeable to HCO_3^- as well as Cl^- (Brown *et al.*, 1988*b*; Booth *et al.*, 1988). We have now investigated the activation of the mechanisms responsible for the acidosis.

$[Ca^{2+}]_i$ and the intracellular pH (pH_i) were measured in suspensions of isolated acini using fura-2 and 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein, respectively. Resting $[Ca^{2+}]_i$ was 120–160 nM. Fig. 1 shows that ACh (1 μM) caused $[Ca^{2+}]_i$ to increase to a peak value 2–3-fold higher than control, followed by a partial recovery to a plateau. A similar change in $[Ca^{2+}]_i$ could be evoked by the calcium ionophore ionomycin (0.25 μM). Stimulation with 1 μM-ACh also evoked a transient intracellular acidosis of around 0.1 pH unit (Fig. 1). In contrast, no change in pH_i was observed on stimulation with 0.25 μM-ionomycin. We were also unable to elicit an acidosis with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) at a concentration of 1 μM.

The failure of ionomycin to cause an acidosis similar to that evoked by ACh, despite the similarity of the increase in $[Ca^{2+}]_i$ produced by the two interventions, indicates that the

Abbreviations used: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; ACh, acetylcholine; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate.

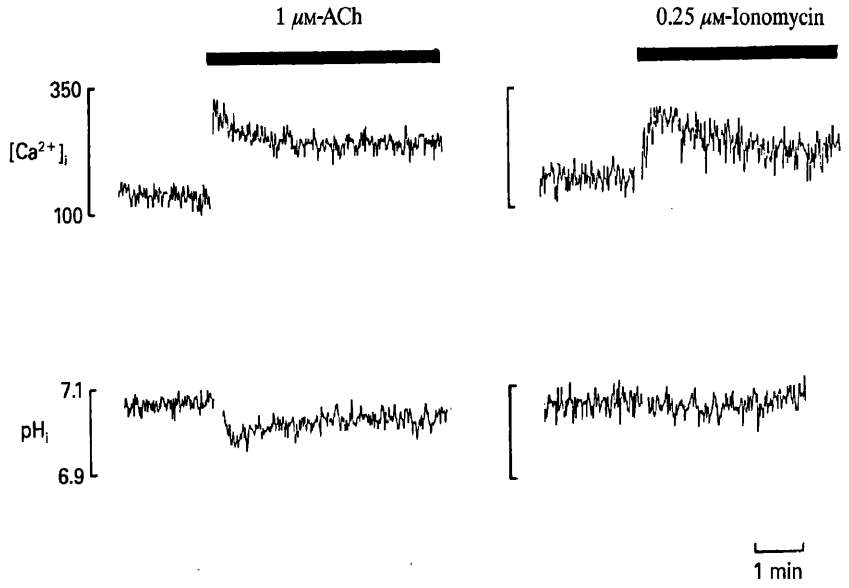

Fig. 1. *Effects of 1 μM-ACh and 0.25 μM-ionomycin on $[Ca^{2+}]_i$ and pH_i in suspensions of isolated acini from rabbit mandibular salivary gland at 37°C*

The methods used for isolating acini (by collagenase digestion), dye loading and fluorescence measurements are described in detail elsewhere (Brown *et al.*, 1988*a*, *b*; Lau *et al.*, 1988).

ACh-induced rise in $[Ca^{2+}]_i$ is not by itself sufficient to activate the HCO_3^- efflux pathway(s). We therefore suggest that other events triggered by activation of the ACh receptor may be involved. The experiments with TPA suggest that activating protein kinase C alone is also insufficient to activate HCO_3^- efflux. It may be that interaction of the Ca^{2+} pathway with the protein kinase C pathway, or indeed with some other messenger system, is necessary for HCO_3^- efflux to occur. Whether this result has implications for the transport of anions other than HCO_3^- remains to be determined.

Supported by the M.R.C. and the Wellcome Trust

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Received 14 June 1988

Effects of phorbol ester on inositol trisphosphate production and secretion in permeabilized HL 60 cells

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Agonist-induced stimulation of neutrophils and HL60 cells results in the generation of a number of intracellular messengers two of which, diacylglycerol (DAG) and inositol trisphosphate ($InsP_3$), arise from the hydrolysis of phosphatidylinositol bisphosphate by polyphosphoinositide phosphodiesterase (PPI-pde). The membrane receptors for such agonists have been suggested to be coupled to the PPI-pde by a putative G-protein, G_p [1].

Experiments were conducted in which secretion (of β-glucuronidase) and PPI-pde activity [formation of inositol polyphosphates ($InsP_2$) and ($InsP_3$)] were simultaneously measured in streptolysin-O permeabilized HL60 cells. The cells were permeabilized with streptolysin-O (0.4 i.u./ml) and incubated for 10 min in buffer (pH 6.8) containing 137 mM-NaCl, 2.7 mM-KCl, 20 mM-Pipes, 5.6 mM-glucose, 1 mM-MgATP, 10 mM-LiCl, 1 mg of albumin/ml and Ca^{2+} (pCa 8–pCa 5), buffered with 3 mM-EGTA. The cells had been prelabelled for 48 h with 1 μCi of [^{3}H]inositol/ml. After the incubation period, the cells were quenched and samples were taken from the supernatant for measurement of β-glucuronidase and inositol phosphates.

The effect of guanosine 5'-[γ-thio]triphosphate (GTP[S]) is to greatly potentiate both secretion and PPI-pde activity; both responses show a similar sensitivity to Ca^{2+}. Secretion is a function of two effectors, GTP[S] and Ca^{2+} (pCa 5).The effect of GTP[S] on secretion cannot be completely accounted for, however, by its ability to generate either $InsP_3$ or DAG. In these experiments, the effect of $InsP_3$ on intracellular Ca^{2+} mobilization can be disregarded, since Ca^{2+} is already provided in the form of Ca^{2+} buffers. DAG would, however, still function as an activator of protein kinase C (PKC). We have recently demonstrated that the potent and stable activator of PKC, phorbol 12-myristate 13-acetate

Abbreviations used: DAG, diacylglycerol; $InsP_2$, $InsP_3$, inositol bis- and tris-phosphate, respectively; PPL-pde, polyphosphoinositide phosphodiesterase; GTP[S], guanosine 5'-[γ-thio]triphosphate; PKC, protein kinase C.

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Issue 5 1989

Synthesis of *myo*-Inositol 1,4-Bisphosphate-5-phosphorothioate

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Synthesis of *myo*-Inositol 1,4-Bisphosphate-5-phosphorothioate

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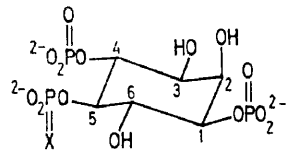
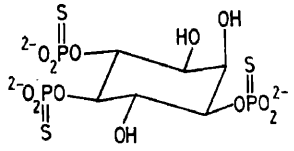
Synthesis of *myo*-insoitol 1,4,5-triphosphate and *myo*-inositol 1,4,-bisphosphate-5-phosphorothioate, a phosphatase-resistant analogue of a biological second messenger, have been accomplished using a novel combination of P^{III} and P^{V} chemistry and a new deprotection method for the 2,2,2-trichloroethyl group.

It is now generally accepted that D-*myo*-inositol 1,4,5-trisphosphate (IP_3) (**1**), released by receptor-mediated phospholipase C-catalysed cleavage of phosphatidylinositol 4,5-bisphosphate, is the second messenger linking the spatially separated events of receptor stimulation and release of calcium from cellular internal stores.[1] IP_3 is metabolised *via* two pathways: deactivation by a 5-phosphatase[2] to 1,4-IP_2 and phosphorylation by a 3-kinase to 1,3,4,5-IP_4,[3] whose function remains controversial.

We have sought to develop synthetic routes to inositol phosphates[4,5] and especially to prepare novel non-hydrolysable analogues such as phosphorothioates.[6] Our recent synthesis of *myo*-inositol 1,4,5-trisphosphorothioate (IPS_3) (**2**)[7] has provided an analogue that is a potent releaser of calcium[8,9] and yet is resistant to phosphatase-catalysed deactivation.[10] It is clear that such analogues offer considerable potential for investigation and modification of the complex metabolism of IP_3.



(1) X = O
(3) X = S



(2)

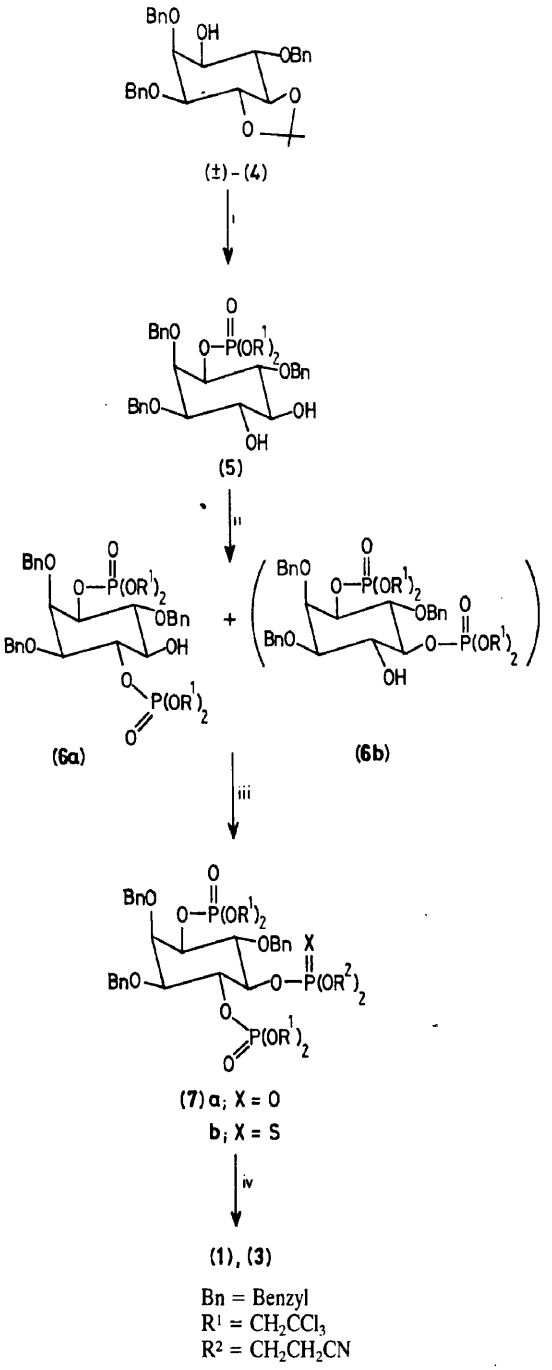
IPS_3 has all three phosphates replaced by phosphorothioates. We wished to devise a synthetic route to the novel analogue *myo*-inositol 1,4-bisphosphate-5-phosphorothioate (**3**), which has only the 5-phosphate position substituted by phosphorothioate and will be nearer in structure to the natural messenger whilst still enjoying the advantages of metabolic stability.

(±)-2,3,6-Tri-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inositol (**4**)[11] was phosphorylated in 90% yield at the free hydroxyl group using bis(2,2,2-trichloroethyl)phosphorochloridate to 2,3,6-tri-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inositol-1-(bis-2,2,2-trichloroethyl)phosphate (m.p. 114–115 °C) (Scheme 1). The isopropylidene group was removed quantitatively to give the corresponding diol (m.p. 141–143 °C) (**5**), which was phosphorylated to the mixed bisphosphate triesters (**6a**) and (**6b**) possessing 1,4- and 1,5-substitution patterns, respectively. It was clearly not possible to bisphosphorylate (**5**) under these conditions, presumably on account of the steric crowding which would accompany the formation of *trans*-vicinal bis(bis-2,2,2-trichloroethylphosphate) triesters. Only a small amount of pyridine was used in this phosphorylation, which was carried out in dichloromethane, since the normal use of pyridine as solvent was found to result in the formation of the five-membered cyclic phosphate, which decomposed on work-up to give a mixture of phosphate diesters at the 4- and 5-positions. Trituration of the mixed bisphosphate triesters with petrol after rapid chromatography on silica (to avoid cyclisation) afforded one bisphosphate in crystalline form† [m.p. 151–153 °C; δ_P ($CDCl_3$) (proton decoupled) −6.59, −7.94 p.p.m., 40% yield].

The structure of the crystalline bisphosphate could not readily be deduced from its complex ^1H n.m.r. spectrum on account of overlap of benzyl $-CH_2-$ protons and the complex ABX systems of the four trichloroethyl groups with three of the inositol ring protons. The latter, however, were readily located from the 2D J-resolved ^1H n.m.r. spectrum, which also distinguished those protons experiencing heteronuclear coupling to phosphorus. A 2D ^1H n.m.r. COSY spectrum was then sufficient to deduce the ring connectively and assign the structure to the 1,4-bisphosphate triester (**6a**).

Compound (**6a**) was then phosphitylated at the free 5-hydroxyl using our P^{III} approach[4,5] and oxidised either to 2,3,6-tri-*O*-benzyl-*myo*-inositol-1,4-bis(bis-2,2,2-trichloroethylphosphate)-5-(bis-2-cyanoethylphosphate) (**7a**) or the corresponding 5-(bis-2-cyanoethylphosphorothioate) (**7b**) using t-butyl hydroperoxide or sulphur in pyridine, respectively.

† Intermediates up to and including this stage gave satisfactory elemental analyses.



Scheme 1. *Reagents and conditions:* i, (a) $(CCl_3CH_2O)_2POCl$ (1.25 equiv.), pyridine, 12 h, (b) H_3O^+; ii, $(CCl_3CH_2O)_2POCl$ (2 equiv.), pyridine (4 equiv.), CH_2Cl_2, 6 h; iii, (a) $(NCCH_2CH_2O)P(NPr^i_2)Cl$ (1.2 equiv.), $EtNPr^i_2$ (1 equiv.), MeCN, 1 h, (b) $NCCH_2CH_2OH$, tetrazole (2.5 equiv. of each), MeCN, 1 h, (c) for X = O, Bu^tOOH (3 M solution in toluene), 0 °C, 15 min; X = S, sulphur in pyridine, 16 h; iv, Na in liq. NH_3. All compounds are racemic.

Deblocking of protecting groups was accomplished using sodium in liquid ammonia, which removed the cyanoethyl groups by β-elimination and reductively removed the benzyl groups and the 2,2,2-trichloroethyl groups, the latter cleavage presumably involving initial C–Cl bond cleavage. Although several methods have been devised for deblocking of 2,2,2-trichloroethyl groups[12] none is entirely satisfactory. To the best of our knowledge, the use of sodium in liquid ammonia has not previously been reported and therefore represents a new procedure for this purpose. *myo*-Inositol 1,4,5-trisphos-

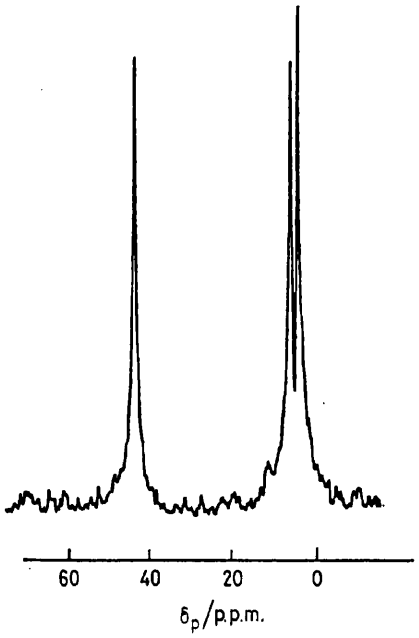


Figure 1. 24.15 MHz ^{31}P N.m.r. spectrum of *myo*-inositol 1,4-bisphosphate-5-phosphorothioate (*ca.* 70 mM solution in D_2O, pH 8). N.m.r. parameters were: sweep width, 10 kHz; pulse width, 8 μs; collected over 4 K; no. of transients, 1000; broad band proton decoupling; referenced to external H_3PO_4.

phate (**1**) and 1,4-bisphosphate-5-phosphorothioate (**3**) were obtained as triethylammonium salts by anion exchange chromatography in *ca.* 44% yield based upon (**6a**). The ^{31}P n.m.r. spectrum of (**3**) (Figure 1) clearly shows the presence of two phosphate groups [δ_P (D_2O) 2.62, 4.64 p.p.m.] and one phosphorothioate group (δ_P 44.98 p.p.m.) in the molecule; *m/z* [fast atom bombardment (FAB)] 435 $(M-H)^-$. DL-IP_3 prepared by this route was equipotent as previously synthesized material[5] at releasing calcium from permeabilized Swiss 3T3 cells[9] and displacing [^{3}H]-IP_3 from a specific IP_3 binding site in cerebellum.[13] *myo*-Inositol 1,4-bisphosphate-5-phosphorothioate was found to be very similar to IPS_3 in calcium release and 5-phosphatase resistance experiments. These novel biological properties will be reported in detail elsewhere.

We thank Dr. D. L. Turner for advice concerning the 2D n.m.r. experiments and Professor S. R. Nahorski for biological testing. This work was supported by the S.E.R.C., the Research Corporation Trust, and in part by Merck Sharp and Dohme. B. V. L. P. is a Lister Institute Research Fellow.

Received, 11th July 1988; Com. 8/02760l

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Issue 171989

Desulphurisation of Vicinal Bisphosphorothioates: a Novel Synthetic Route to Substituted Cyclic Pyrophosphates

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Desulphurisation of Vicinal Bisphosphorothioates: a Novel Synthetic Route to Substituted Cyclic Pyrophosphates

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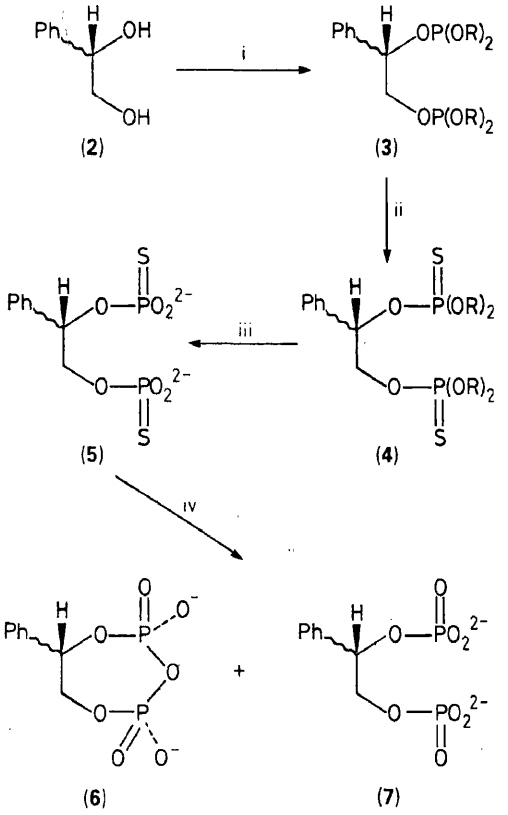
N-Bromosuccinimide-mediated desulphurisation of vicinal bisphosphorothioates offers a novel route for the synthesis of P^1, P^2-disubstituted 7-membered cyclic pyrophosphates.

Considerable work has been carried out on the formation of pyrophosphate linkages during the development of nucleotide synthesis,[1] and much of the classical chemistry has involved the use of activating agents such as phosphorochloridates[1] and carbodiimides.[2] Recently , the novel metabolite, D-2,3-bisphosphoglycerate cyclic pyrophosphate (**1**), a seven-membered cyclic pyrophosphate, has been found to be the major phosphate component in *Methanobacterium thermoautotrophicum* and certain *methanobrevibacteria*,[3,4] and is the first biological example of this system.[3] Its function is, however, still uncertain. It may act as a phosphate reserve or, more likely, be a chelator of potassium ions. We report here a novel route directed towards the synthesis of such 7-membered cyclic pyrophosphates.

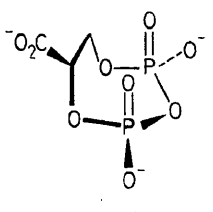
Phosphorothioate analogues are of considerable use in nucleotide chemistry and enzymology.[5] Recent work in this laboratory has addressed the synthesis and exploitation of novel molecules of biological interest possessing vicinal bisphosphorothioates.[6,7] *N*-Bromosuccinimide (NBS) *inter alia* has been used to desulphurise phosphorothioate esters and anhydrides to form oxygen isotope-labelled phosphates.[5,8] We reasoned that if a phosphorothioate moiety in a vicinal bisphosphorothioate were to be activated, *e.g.* by NBS, then a neighbouring phosphorothioate might effectively compete with the solvent and couple with the activated system to form a cyclic P^1,P^2-disubstituted pyrophosphate.

An NBS-mediated desulphurisation was carried out on two model systems, 1-phenylethane-1,2-bisphosphorothioate (**5**) and *trans*-cyclohexane-1,2-bisphosphorothioate (**8**), which were synthesised according to Scheme 1 from their respective diols using a P^{III} approach.[6,9] 1-Phenylethane-1,2-diol (**2**) was phosphitylated with *N*,*N*-di-isopropylamino(2-cyanoethoxy)chlorophosphine to the bisphosphoramidite (^{31}P n.m.r., δ_P 147.4 p.p.m.) and reaction with tetrazole and 3-hydroxypropionitrile produced 1-phenylethane-1,2-bis(di-2-cyanoethoxy)phosphite (**3**) (δ_P 138.5 p.p.m.). Addition of sulphur gave 1-phenylethane-1,2-bis(di-2-cyanoethoxy)-phosphorothioate (**4**) in 65% yield after purification by flash chromatography (δ_P 66.5 p.p.m.). Treatment with aqueous ammonia removed the cyanoethyl groups to give the ammonium salt of 1-phenylethane-1,2-bisphosphorothioate (**5**) (δ_P 43.4 p.p.m.). Subsequent treatment of the free acid with cyclohexylamine generated the triscyclohexylammonium salt of (**5**). *trans*-Cyclohexane-1,2-bisphosphorothioate (**8**) was prepared in a similar fashion.

The desulphurisation reaction was rapid and simple to execute. The 1,2-bisphosphorothioate (**5**) was dissolved in water and dioxane (1 : 4) and an eight-fold excess of NBS was added (other desulphurising agents were tried and were found to be less efficient). The mixture was shaken and after 1 min excess of NBS was reduced by 2-mercaptoethanol. After a further minute the reaction mixture was diluted with triethylammonium hydrogen carbonate (TEAB) buffer and solvent was removed *in vacuo* to yield a crude mixture of pyrophos-

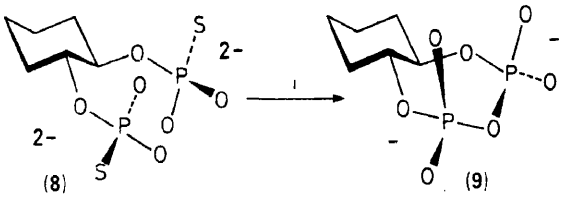


Scheme 1. Synthesis and desulphurisation of 1-phenylethane-1,2-bisphosphorothioate. R = $-CH_2CH_2CN$.
Reagents: i, $ClP(NPr^i_2)OR$. $HNPr^i_2$ (2 equiv. of each in dry MeCN, 1 h at 0 °C) then ROH–tetrazole (2.4 equiv.); ii, S_8–dry pyridine, 2 h at room temp.; iii. NH_4OH_{aq}, 3 h at 65 °C; iv, *N*-bromosuccinimide (8 equiv.)–dioxane–water. 1 min at room temp., then $HSCH_2CH_2OH$, 10 mM TEAB, pH 7.1.



(**1**)

Figure 1



Scheme 2. Synthesis of cyclohexane-1,2-cyclic-pyrophosphate.
Reagents: i, *N*-bromosuccinimide (8 equiv.)–dioxane–water, 1 min at room temp., then $HSCH_2CH_2OH$, 10 mM TEAB, pH 7.1.

phate (**6**) (δ_P −10.7, −11.5 p.p.m., $^2J_{PP}$ 18 Hz), [f.a.b., 279 $(M - H)^-$] and the bisphosphate (**7**) (δ_P 2.8, 3.0 p.p.m.) as a glass. The products were purified by anion exchange chromatography using DEAE-Sephadex (DEAE = diethylaminoethyl) eluted with TEAB buffer.

The relative yield of pyrophosphate depended on which bisphosphorothioate was used. When (**5**) was desulphurised the pyrophosphate (**6**) was formed in *ca.* 40% yield, the remaining 60% being the bisphosphate (**7**) resulting from double desulphurisation. Yields were estimated by ^{31}P n.m.r. spectroscopy and quantitative phosphate analysis. Use of cyclohexane-1,2-bisphosphorothioate (**8**), however, increased the yield of the cyclic pyrophosphate (**9**) (δ_P, −11.4 p.p.m.), [f.a.b., 257$(M-H)^-$] to 85—90% (Scheme 2). The ratio of pyrophosphate to phosphate formed is presumably a function of the relative conformation of the two phosphorothioate groups. Owing to stereochemical constraints imposed by the cyclohexane ring, the juxtaposition of the two phosphorothioate groups is presumably more favourable for pyrophosphate formation.

Using isotopic labelling, a preliminary investigation of the mechanism of this novel coupling reaction was undertaken. When desulphurisation was carried out in $H_2{}^{18}O$ and the ^{18}O-labelled pyrophosphate was examined by ^{31}P n.m.r. spectroscopy, a significant proportion (45%) of the ^{18}O was found in the bridging P–O–P linkage, as determined by $^{31}P^{18}O$ isotope shift analysis (data not shown). This indicates that although the situation is undoubtedly complex and several mechanisms are possible, significant desulphurisation at one centre must have occurred, followed by torsional rotation of the resulting ^{18}O-labelled phosphate, before its capture by the other activated phosphorothioate.

Seven-membered cyclic pyrophosphates are stable to both acid and alkaline conditions in the cold. Treatment of (**9**) with 1 M HCl at room temperature for three days yielded little hydrolysis, but boiling at 100 °C hydrolysed the pyrophosphate, within 15 min. Similarly, with 1 M NaOH at room temperature no hydrolysis was detected. Boiling at 100 °C for 5 h resulted in total hydrolysis to the bisphosphate. It is clear, however, that intracellular enzymes are capable of efficiently hydrolysing such pyrophosphates, as (**1**) is rapidly hydrolysed in neutral cell extracts to 2,3-bisphosphoglycerate.[10] These systems could thus be interesting 'pro drug' precursors of biologically active molecules possessing vicinal bisphosphates and exploitation of this reaction could lead to the synthesis of less hydrophilic biologically interesting cyclic pyrophosphates such as those which might be derived from second messenger polyphosphates of *myo*-inositol.[11]

We thank the S.E.R.C., Merck Sharp and Dohme Research, and the European Social Fund for financial support (N. J. N.). B. V. L. P. is a Lister Institute Research Fellow.

Received, 23rd March 1989; Com. 9/01266D

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Bioorganic & Medicinal Chemistry Letters, Vol.2, No.5, pp. 471-476, 1992
Printed in Great Britain

0960-894X/92 $5.00 + .00

TOTAL SYNTHESIS OF *MYO*-INOSITOL-1-PHOSPHATE-4,5-PYROPHOSPHATE, A NOVEL SECOND MESSENGER ANALOGUE, VIA *MYO*-INOSITOL-1-PHOSPHATE-4,5-BISPHOSPHOROTHIOATE

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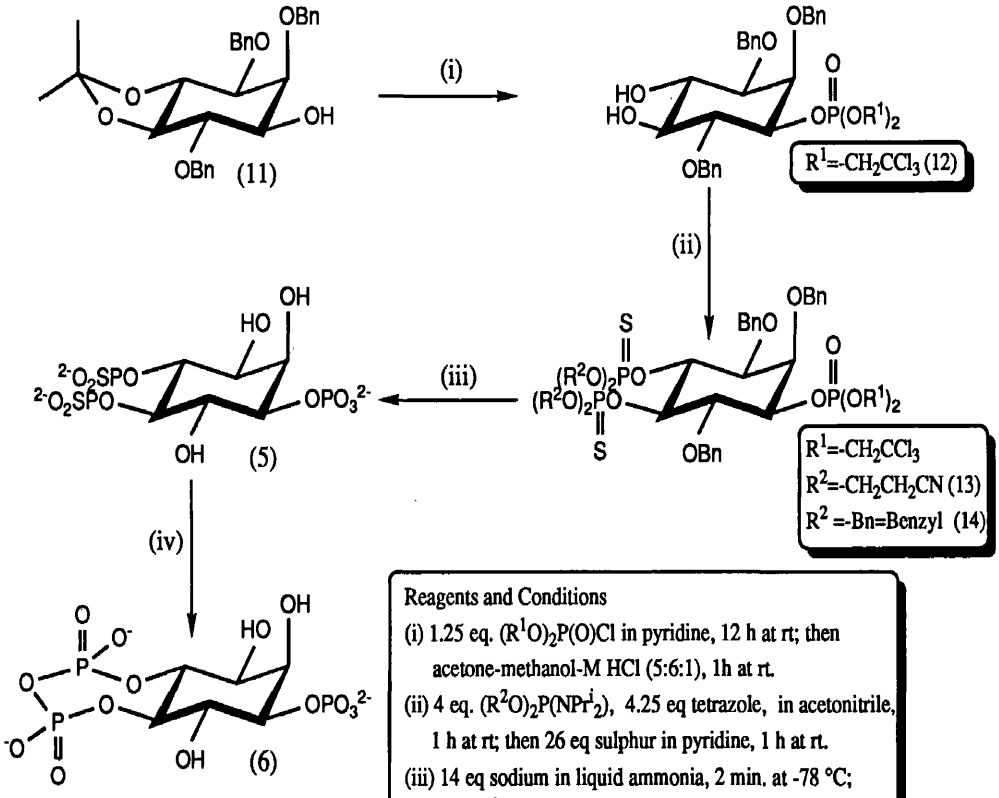
(*Received* 28 *February* 1992)

Abstract: The synthesis of the novel analogues of *myo*-inositol 1,4,5-trisphosphate, *myo*-inositol 1-phosphate 4,5-bisphosphorothioate and *myo*-inositol 1-phosphate 4,5-pyrophosphate is reported; the latter was prepared via desulphurisation and intramolecular coupling.

INTRODUCTION

It is now generally accepted that D-*myo*-inositol 1,4,5-trisphosphate (IP_3) (**1**) (Fig. 1), released by receptor-mediated phospholipase C-catalysed cleavage of phosphatidylinositol 4,5-bisphosphate, is the second messenger linking the spatially separated events of receptor stimulation and release of intracellular calcium from internal stores[1,2]. IP_3 is metabolised *via* two pathways[3]: deactivation by a 5-phosphatase to 1,4-IP_2 or phosphorylation by a 3-kinase to 1,3,4,5-IP_4. The function of the latter still remains controversial and IP_4 may gate a plasma membrane Ca^{2+} channel[4]. IP_3 acts through an intracellular receptor which has been isolated[5], cloned and sequenced[6,7] and reconstituted[8].

We have sought to develop synthetic routes to inositol phosphates[9] and especially to prepare non-hydrolysable analogues such as phosphorothioates[9,10,11]. Our synthesis of *myo*-inositol 1,4,5-trisphosphorothioate (IPS_3) (**2**)[12] (Fig. 1) has provided an analogue that is a potent releaser of calcium[13-15] and yet is resistant to phosphatase-catalysed deactivation[16]. Other biologically potent Ca^{2+}-mobilising synthetic phosphorothioate analogues include *myo*-inositol 1-phosphorothioate 4,5-bisphosphate (**3**)[17] and *myo*-inositol 1,4-bisphosphate 5-phosphorothioate (**4**)[18,19]. It is clear that such analogues offer considerable potential for investigation and modification of the complex metabolism of IP_3 and this has been recognized by other groups[20,21].



Reagents and Conditions

(i) 1.25 eq. $(R^1O)_2P(O)Cl$ in pyridine, 12 h at rt; then acetone-methanol-M HCl (5:6:1), 1h at rt.

(ii) 4 eq. $(R^2O)_2P(NPr^i_2)$, 4.25 eq tetrazole, in acetonitrile, 1 h at rt; then 26 eq sulphur in pyridine, 1 h at rt.

(iii) 14 eq sodium in liquid ammonia, 2 min. at -78 °C; then Pr^iOH.

(iv) 8 eq. N-bromosuccinimide in dioxan-water (4:1); 1 min at rt, then 2-mercaptoethanol (30 µl).

Scheme 2

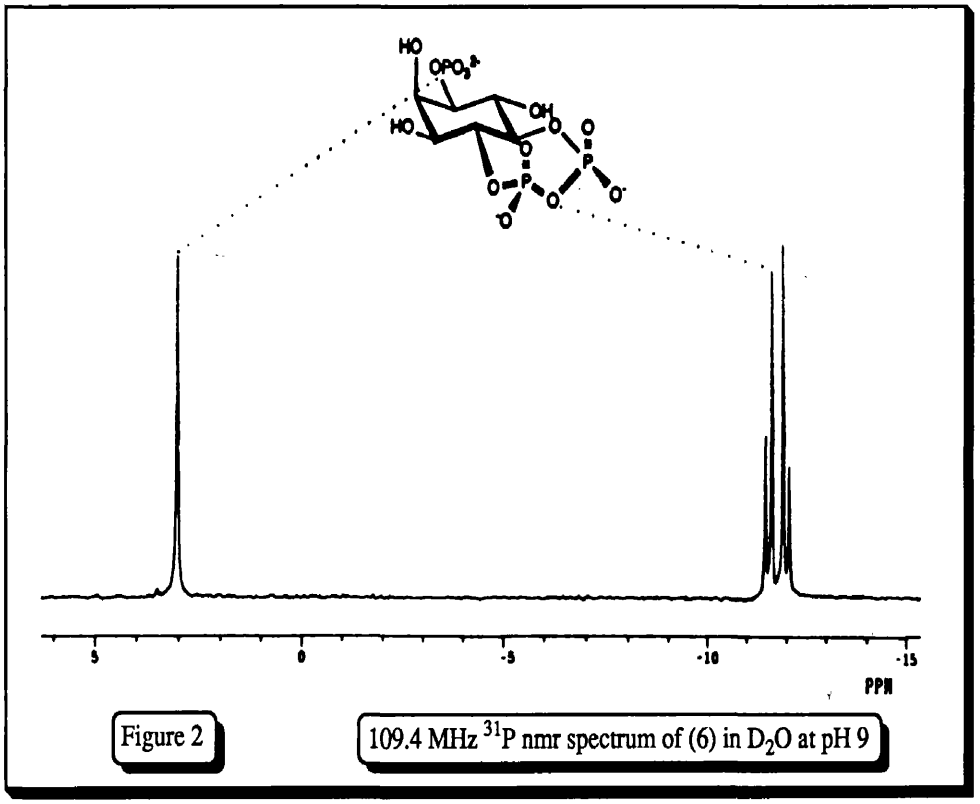


Figure 2 — 109.4 MHz ^{31}P nmr spectrum of (6) in D_2O at pH 9

sodium in liquid ammonia as above gave crude DL-*myo*-inositol-1-phosphate-4,5-bisphosphorothioate (**5**) which was purified by ion-exchange chromatography on either DEAE Sephadex A-25 or Q-Sepharose to give the pure triethylammonium salt of (**5**), quantified by Briggs phosphate assay[30], in 83% yield.

DL-(**5**) Was a potent agonist for intracellular Ca^{2+} mobilisation in permeabilised SH-SY5Y neuroblastoma cells[31]. It mobilised Ca^{2+} with a potency slightly less than IP_3 and similar to IPS_3 (**2**). Having a 5-phosphorothioate group, (**5**) is resistant to degradation by IP_3-5-phosphatase and stimulated a persistant release of Ca^{2+} like IPS_3 [3,31]. It inhibited IP_3-5 phosphatase potently with a K_i of 1.3 $\pm$ 0.3 μM similar to IPS_3 [32] suggesting that D-(**5**) would have a submicromolar K_i for this enzyme. Full biological details will be published elsewhere.

(**5**) Was desulphurised with NBS and gave the crude pyrophosphate (**6**) in high yield with some evidence of phosphate migration (24%) and straight desulphurisation to IP_3 (7%). (**6**) Was purified by anion exchange chromatography on Q-Sepharose as above to give the triethylammonium salt (67% yield). It exhibited a ^{31}P n.m.r spectrum (Figure 2) showing clearly the presence of the 1-phosphate (δ 3.04 ppm) and the 4,5-pyrophosphate [δ -11.61 (pos. 4) and -12.02 ppm (pos. 5), $^2J_{pp}$ 16.8 Hz], the latter resonating as the expected AB system. Biological evaluation of (**6**) is in progress.

ACKNOWLEDGEMENTS

This work was supported by SERC (Molecular Recognition Initiative) and in part by Merck Sharp and Dohme (UK). We thank The Royal Society for a European Exchange Fellowship (DD) and an equipment grant, S Safrany and Professor S R Nahorski for preliminary biological evaluation of analogues, Dr R Gigg for an authentic sample of (**12**), Drs Cleophax and Gero for an authentic sample of (**7**) and S Alston for manuscript preparation. BVLP is a Lister Institute Fellow.

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