Synthesis of Various Carbohydrate Derivatives Towards the Elucidation and Inhibition of Mycobacterial Cell Wall Biosynthesis

A Thesis Submitted for the Degree of Doctor of Philosophy

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University of St Andrews

September 2000

Declaration

(i) I, Philip McGurk, hereby certify that this thesis, which is approximately 32,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any other previous application for a higher degree.

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DEDICATION

To my late brother Alec, a great friend who will always be missed.

ABSTRACT

Attempts were made to chemically synthesise UDP-Gal*f* in order to provide sufficient quantities of this product for enzymatic characterisation. Although the precursor Gal*f*-α-D-1-phosphate was successfully synthesised from galactonolactone, coupling of this product with UMP-morpholidate or UMP (using carbonyldiimidazole) was not succesful. This was thought to be due to either the degradation of what is a very labile product or the lack of product formation.

Biosynthesis of mycolylarabinogalactan takes place on a polyprenylpyrophospho- lipid carrier of this disaccharide and it was thought that synthesis of a simple analogue of this disaccharide may have provided a product with inhibitory properties towards galactan formation. The α- and β-octyl glycosides of this disaccharide were synthesised and tested for inhibitory activity. Neither of these compounds displayed inhibition towards the biosynthesis of galactan and this was thought to be due to either the lack of an anomeric phosphodiester or that the octyl chain was not sufficient to mimic the polyprenyl lipid carrier.

One of the possible biosynthetic routes from pRpp to DpA involves the epimerisation of the 2-hydroxyl of pRpp thus creating 5-phosphoribose-α-D-pyrophosphate (pApp). A chemical synthesis of this compound was attempted in order to confirm or deny whether this biosynthetic pathway was actually taking place in mycobacteria. Many attempts were made to synthesise this product and the most promising of these was nucleophilic attack of an arabinosyl chloride with tris(tetra-n-butylammonium) pyrophosphate to create the desired anomeric pyrophosphate. An anomeric pyrophosphate was observed in two different instances however deprotection conditions always resulted in degradation of this very labile product.

Since pApp was so difficult to synthesise, it was decided to make an analogue which would be more stable but which would involve the minimum change compared to the parent compound. Since glycosyl phosphonates have been shown to be biologically analogous to phosphates in certain instances, it was decided to substitute the troublesome anomeric pyrophosphate with a phosphono- analogue pAcpp. This compound was successfully synthesised in 12 steps with an overall yield of 3.4%, however, biological testing showed that the compound did not inhibit the conversion of radioactive pRpp into DpA. A number of factors could have been responsible for this

lack of activity. It is possible that pApp is not a biosynthetic intermediate or that the *in vitro* pH was not sufficient to effect the second ionisation of the phosphonate. It is also possible that precomplexation of the substrate with a divalent cation may be necessary for activity.

Testing of four commercially available phosphosugars (Glc-6-P, Rib-5-P, Man-6-P and Ara-5-P) showed, for the first time, that biosynthesis of DpA from pRpp could be inhibited without the use of detergent, albeit at concentrations of 5-10mM. It was not known exactly which enzyme or enzymes were being inhibited.

Finally, attempts to inhibit the phosphatase which converts 5-P-DpA into DpA using the methoxycarbonyl glycosides of arabinose- and ribose-5-phosphate were unsuccessful and this was thought to be due to the lack of an anomeric phosphodiester or insufficient analogy between an octyl chain and the native decaprenyl moiety.

CHAPTER 1:General Introduction

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1.1 OVERVIEW OF TUBERCULOSIS

1.1.1 Incidence and History

Tuberculosis kills more people every year than any other single infectious agent. A third of the worlds population is thought to be infected and 3 million people die of the disease annually, more than at any time in the history of tuberculosis.[1-3] In the developing world TB causes 25% of all avoidable adult deaths, 18.5% of all deaths in adults aged 15 to 59, and 6.7% of all deaths.[3]

The first observation of the decline in the incidence of TB was reported in 1939, before any effective therapies had been introduced.[4] Since then the disease had steadily declined until 1985 when this trend began to reverse.[5] In the US alone there have been over 40,000 extra cases between 1985 and 1994 than would have occurred had historical data continued.

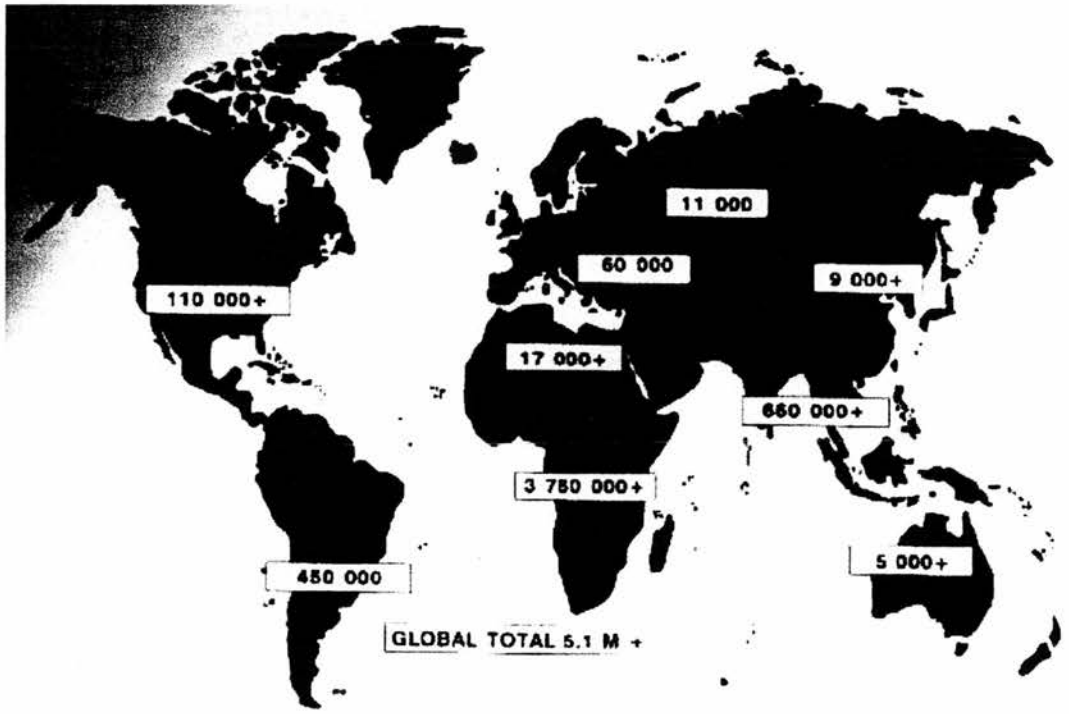

Figure 1: Estimated global distribution of adults who have been infected with HIV and TB as of mid 1993. Source of data is WHO Tuberculosis Programme

It is thought that the single greatest reason for this reversal of fortune is the deadly combination of AIDS with certain strains of tuberculosis.[5] Since one third of the population is infected with TB, a normal immune system can often prevent onset of the full pathogenic disease. However in immunocompromised individuals, defence to this disease is much reduced. In 1992, it was estimated that 14 million people had been

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infected by HIV world-wide and that over 5 million of these had dual HIV/TB infection.[6] Whereas the average person infected with TB has a 10% lifetime chance of developing the disease, someone co-infected by HIV and TB has about an 8% annual risk. As can be seen in Figure 1, the incidence of this dual infection is by far the greatest in sub-Saharan Africa followed by South-East Asia; the two places which already carry the highest risks of TB mortality.[6]

1.1.2 Different Species and Strains

Tuberculosis, the disease, results from infection by the pathogenic *Mycobacterium tuberculosis*. However, many of the different mycobacterial species have no consistent difference in cell envelope characteristics. Many of the mycobacterial species are opportunistic pathogens occurring naturally in the environment and only occasionally causing disease. These include *M. avium*, *M. intracellulare*, *M. chelonae*, *M. kansasii* and *M. fortuitum*. Of these it is the complex of *M. avium* and *M. intracellulare* that is the greatest threat to immunocompromised individuals.

Another important development in the rise of TB related deaths is the increase of multi drug-resistant strains (MDR-TB).[7] A strain is considered MDR if it is resistant to both rifampicin and isoniazid, the two most popular first-line anti-mycobacterial drugs. Of all cases tested in a New York City survey in 1991, one-third were resistant to one or more drugs.[3] In the same year, cases of TB were reported in all 50 US states and drug resistant cases were reported in 36 of these states.[3] One specific strain of MDR-TB (strain W) which infected more than 357 people in New York City between January 1990 and August 1993 cost at least $25M in hospital charges alone to bring under control.[8] Compared with an average cost of £25 per person for a six-month multi-drug regimen, the lack of research into new treatments and incomplete courses of current drugs could become very costly.

1.1.3 Infection and Development of Disease

The basic outline of the initial infection and progression of tuberculosis is presented in Figure 2. Firstly the transmission of active TB by the host, via coughing or sneezing, could result in inhalation by an unfortunate recipient, or not, as the case may be. Following inhalation the *M. tuberculosis* would travel into the lungs and be exposed to the alveolar macrophages, which may or may not result in phagocytosis (discussed

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later in more detail). The phagocytosed mycobacteria could be completely destroyed by the host's immune system or, as is common, pass into latent infection where the bacteria live in a dormant state inside granulomas.

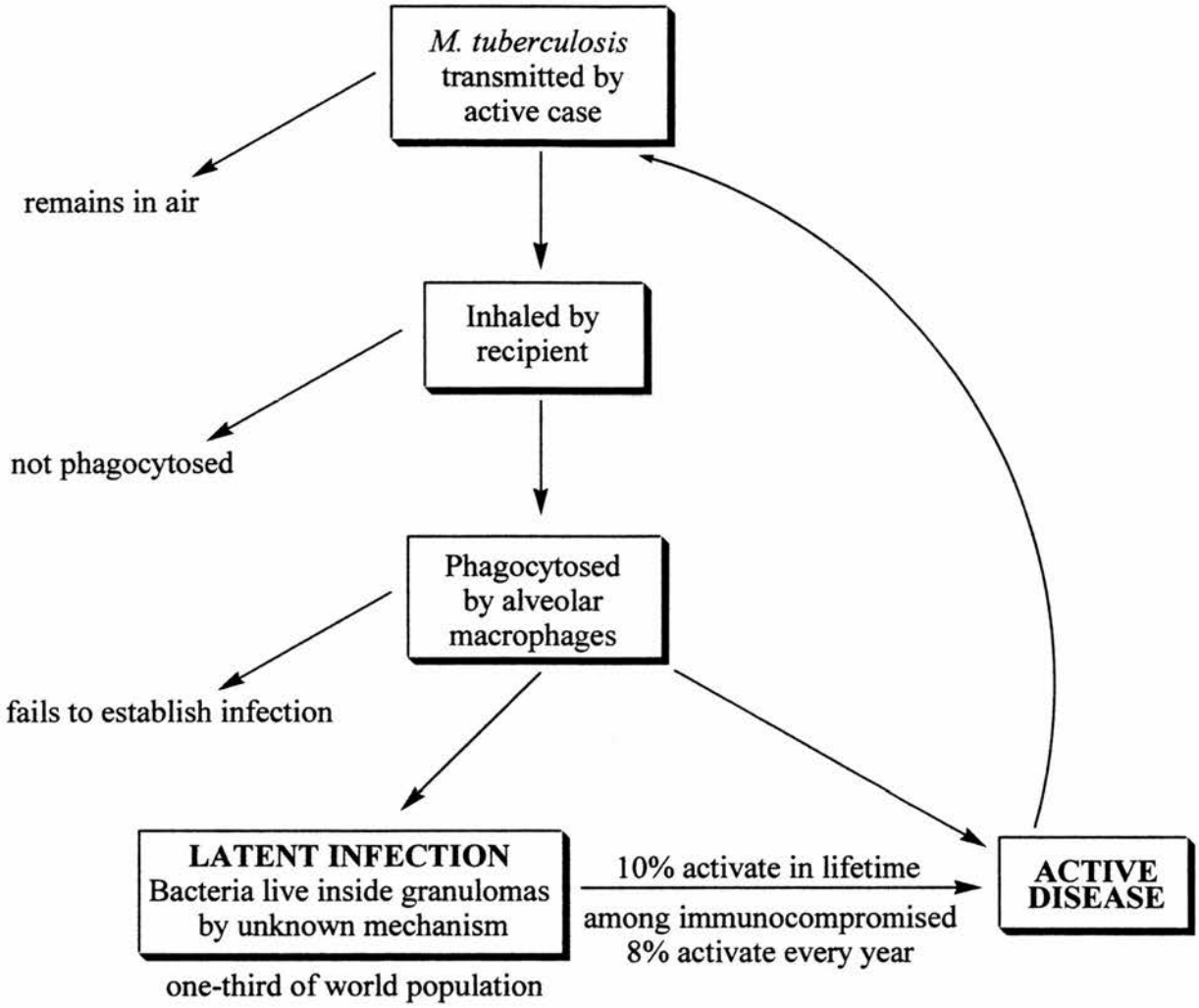

Figure 2: Process of Tuberculosis Infection and Development of Disease

Latent infection is the stage that a third of the world's population currently maintains and the mechanism by which the bacteria survive inside the granuloma is not known. It is possible that the bacteria enter a period of incredibly low growth accentuated by the low oxygen conditions of the granuloma. It is also possible that the host's immune system kills the bacteria faster than they can grow. Evidence[9] that shows the mycobacterial cell wall thickening under depleted oxygen conditions lends credence to the low growth theory as does the discovery[10] of a 'resuscitation promotion factor' involved in the reactivation of latent mycobacteria. However, the endogenous onset of TB in immunocompromised patients seems to support the latter theory.

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1.1.4 Macrophage Activation, Immune Responses and Mycobacterial Resistance Mechanisms[11]

Once inhaled into the lung, mycobacteria can bind to macrophages via a variety of cell surface molecules. These include lipoarabinomannan (LAM), which makes use of terminal mannose residues to bind macrophage mannose receptors. These receptors can also be indirectly bound by mycobacterial ligands of the integrin family (CR1 or CR2).

The macrophage then proceeds to internalise the mycobacterium inside a phagosomic vacuole within the cell. This process stimulates oxidative burst which results in the production of reactive oxygen intermediates (ROIs: H_2O_2, O_2^{2-} etc.), toxic chemicals designed to degrade foreign organisms. However, mycobacteria can avoid the production of ROI's by uptake via the CR1 ligand and can downregulate the production of ROI's by secreting LAM, sulfatides (ST's) and glycolipids (GL's). LAM has been shown to both scavenge ROI's and inhibit protein kinase C which downregulates oxidative burst.[12]

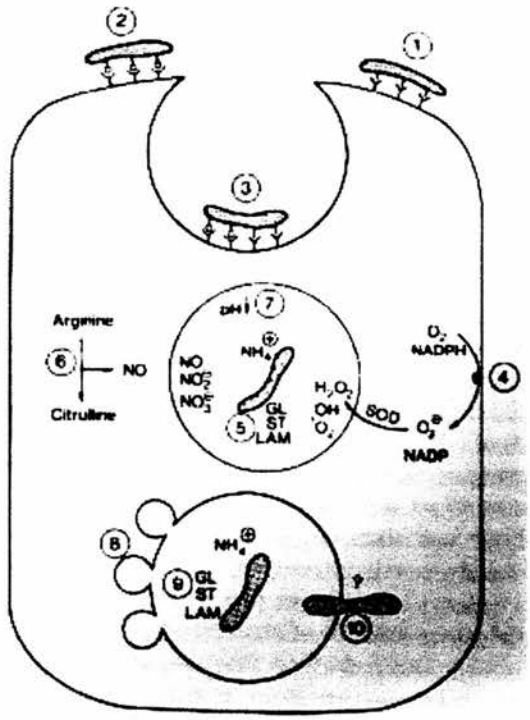

Figure 3: Phagocytosis of *M. tuberculosis* and its Mechanisms of Resistance. Binding of mycobacteria to the macrophage occurs via LAM **(1)** or integrins **(2)**. Phagocytosis **(3)** results in the internalisation of mycobacterium into a vacuole within the macrophage. Organism resists toxic effects of ROIs **(4)** and RNI's **(6)** by production of NH_4^+ (countering acidity of vacuole **(7)**), LAM, sulfatides and other molecules **(5)**. Mycobacteria can also resist lyosomal binding **(8)** by the production of these compounds **(9)** and can possibly escape from the vacuole into less toxic environment of the macrophage cytoplasm by producing haemolysin **(10)**.

Once stimulated by interferon γ (IFNγ) and tumour necrosis factor α (TNFα), the macrophage also produces reactive nitrogen intermediates (RNIs: $NO^{\cdot}$, $NO_2^{\cdot}$ etc.)

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which have optimal toxicity in the acidic environment of the vacuole. However, mycobacteria are equipped with powerful NH_4^+ production capabilities thus reducing the effectiveness of RNIs.[11]

Fusion of small organelles called lyosomes with phagosomes normally allows an influx of degradative enzymes directed towards the foreign organism, however, the production of NH_4^+, LAM, STs and GLs by the mycobacterium is thought to inhibit the fusion process.[11] It has also been proposed that mycobacteria can evade the toxicity of the phagosome by producing haemolysin to degrade the wall and escape into the more neutral environment of the macrophage cytoplasm.[11]

The cytokines involved in immunity and macrophage activation are many, however IFNγ and TNFα have been proposed as the most important of these.[11] CD4 and CD8 T-cells are also thought to be important and reduced levels of CD4 cells in HIV positive individuals is thought to contribute to the rise of tuberculosis among this population.[11]

Although tuberculosis possesses many mechanisms to overcome the immune response of the host, 90% of people infected with this agent never progress to the active disease stage. It would appear to be a straightforward solution to boost the immune system slightly and wipe out any susceptibility to this disease. However, the immunological studies outlined above mostly come from mouse models and it is not known how applicable the information is to humans. The immune response is also much more complex than has been outlined in this document and the fundamental issue of how the protective response differs in individuals who are susceptible compared with people who are resistant to TB remains unresolved.

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1.2 THE ENVELOPE OF MYCOBACTERIA

1.2.1 General Structure

It has been well established that the structural features of mycobacteria are generally very similar regardless of species. Studies using electron microscopy have shown the physical ultrastructure of the mycobacterial cell wall and one of these representations is shown in Figure 4.

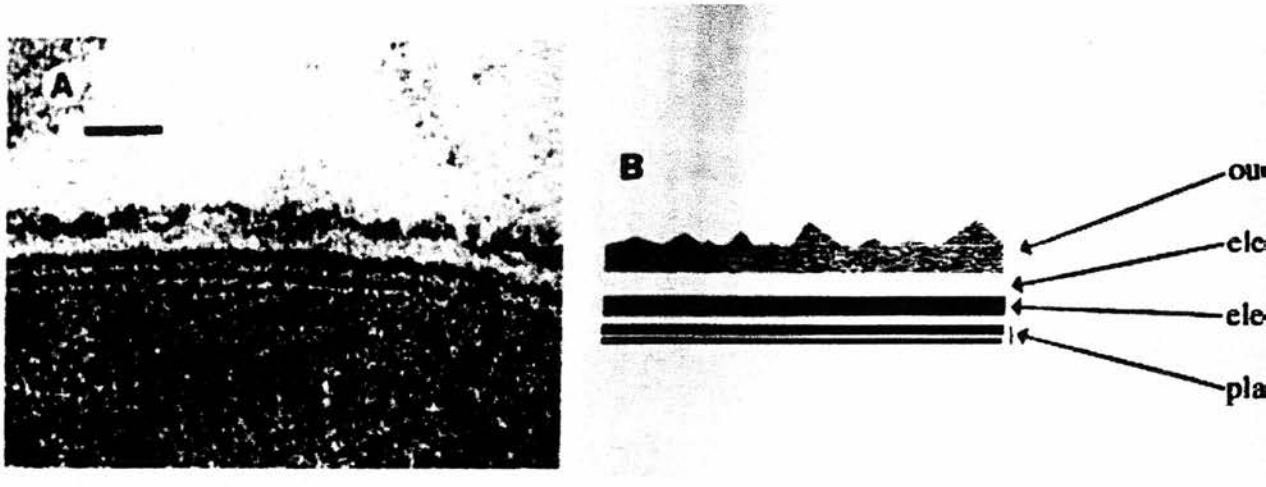

Figure 4: (A) Electron Micrograph of Envelope and Part of Cell Contents of *M. phlei* 425. (B) Interpretation of Panel (A)[13]

The innermost two layers represent the inner and outer lamina of the plasma membrane. The electron-dense layer is thought to reflect peptidoglycan which is known to have these staining properties from other gram positive bacteria. The electron transparent region is thought to be caused by lipoarabinogalactan, a large lipopolysaccharide known[14] to be connected via a disaccharide linker to peptidoglycan. The outer layer has not been conclusively elucidated but is thought to comprise of phenolic glycolipids (PGL's) or glycopeptidolipid.[1]

1.2.2 The Chemical Nature of the Cell Envelope

1.2.2.1 Plasma Membrane

Starting from the inner section of the mycobacterial cell envelope, the plasma membrane is the first part of the structure. As seen from the electron micrograph in Figure 4 the plasma membrane consists of a bilayer of which the inner layer appears thinner than the outer layer. It has been suggested that the outer layer of the plasma membrane is augmented by carbohydrates, specifically phosphatidylinositol mannosides (PIMs), resulting in the extra electron density seen by the microscope

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The phosphodiester linkage of the inositol to the lipid is located at the 1- position and the ring is mannosylated at the 2- and 6- positions (PIM_2). Higher homologues of PIM_2 involve elongation of the 6-mannose residue and PIM_3, PIM_4, PIM_5, and PIM_6 have been identified.[15]

Other components of the plasma membrane include polyterpene-based products thought to be involved in protection against photolytic damage and electron transport, and polyprenolphosphate sugars which are involved in cell wall biosynthesis and are discussed in greater detail later.

1.2.2.2 Peptidoglycan

The next layer outwards from the plasma membrane is peptidoglycan which is common to all gram positive bacteria. It consists of a number of linear alternating polymers of GlcNAc-(β1-4)-MurNAc (Figure 5) cross-linked via peptide bridges which are connected at the 3-position of MurNAc.

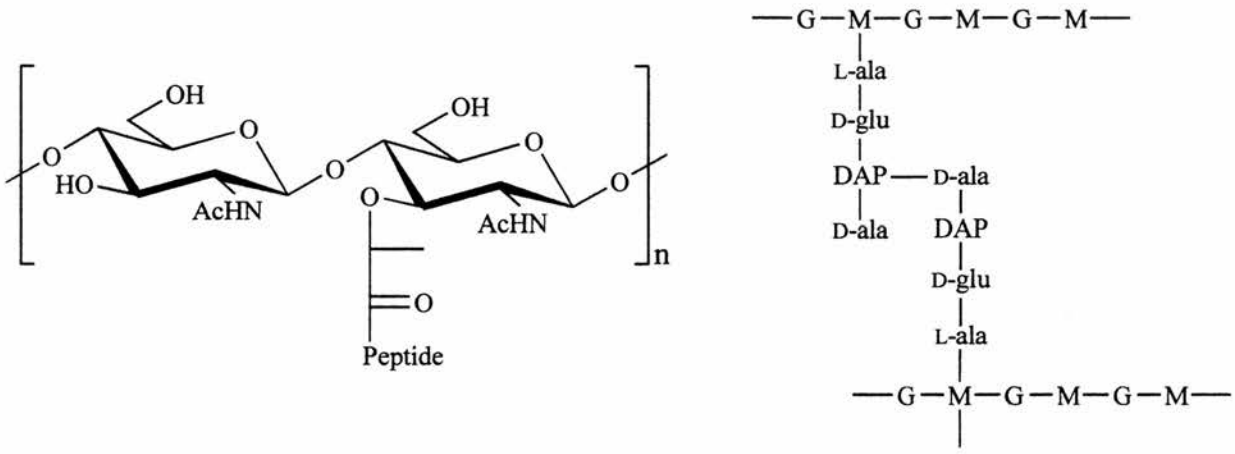

Figure 5: Peptidoglycan. The glycan moiety is a polymer of alternating GlcNAc-(β1-4)-MurNAc residues. Peptide bridges link the MurNAc residues via the 3-position and cross link between diamino acids (DAP's) to form a rigid network.

The peptidoglycan which is present in mycobacteria differs slightly from the standard model in that the muramic acid is *N*-glycolated and cross links involve bonds between diamino acids (DAPs) as well as between DAPs and D-alanine.

1.2.2.3 Arabinogalactan

Linked to the 6-position of about 10-12% of the muramic acid residues via a α-phosphodiester bridge, is the disaccharide Rha-(α1-3)-GlcNAc.[15] This is termed the

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linker region[14, 16] and forms the bridge between peptidoglycan and a large polymeric structure called arabinogalactan (AG).

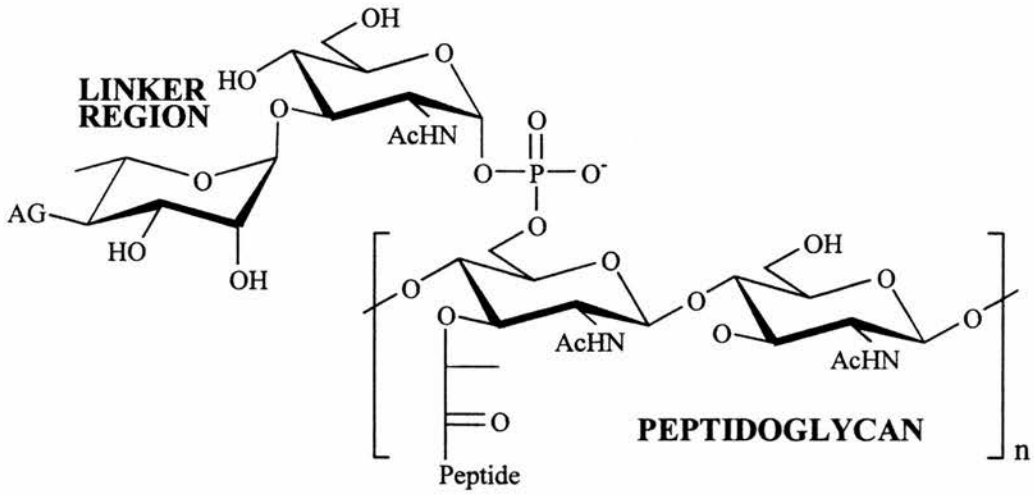

Figure 6: The Linker Region of Mycobacteria

Linked to the rhamnose residue at the 4-position is the linear galactan, a polymer of alternating (β1-5) and (β1-6) galactofuranose units. Branching outward from the linear galactan is the arabinan, a matrix of arabinofuranose units with (β1-2), (α1-3) and (α1-5) linkages. The arabinan connects in the α-orientation at the 5-position of some of the 6-linked galactofuranose units (see Figure 7) and then there is a linear chain of seven (α1-5) linked arabinofuranose residues.[15]

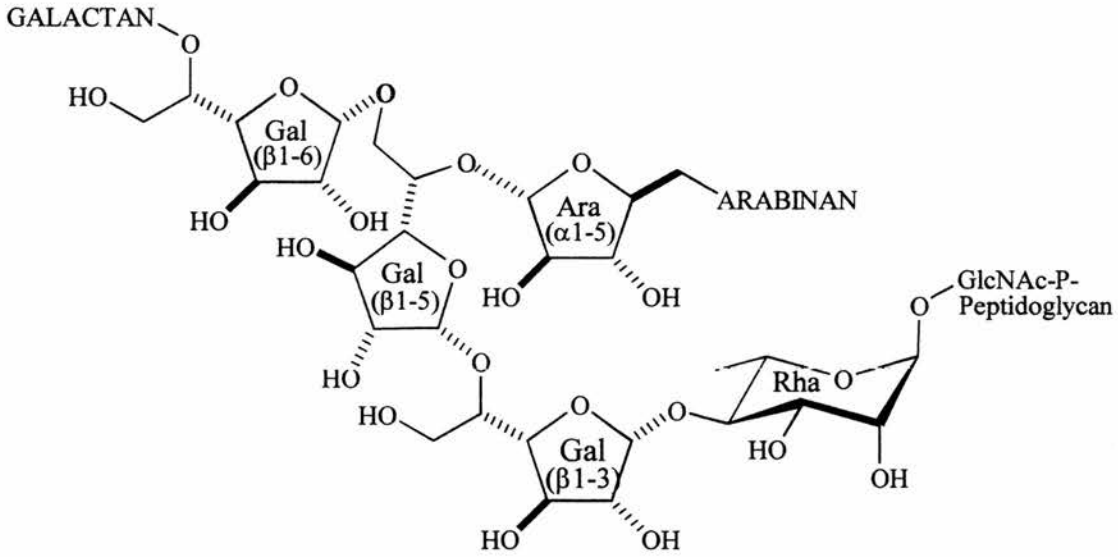

Figure 7: Linkage of Arabinan to Galactan and Galactan to Rhamnose. Ara*f* is α-linked to the 5-position of a 6-linked galactofuranose unit and the reducing Gal*f* is linked (β1-3) to rhamnose.

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The linear arabinan then branches from its 3- and 5-positions to form two four-residue (α1-5) chains which once more branch at the 3- and 5-positions to which are linked (β1-2) Ara*f* disaccharides. These arabinans have been found to attach only towards the reducing end of galactan.[15]

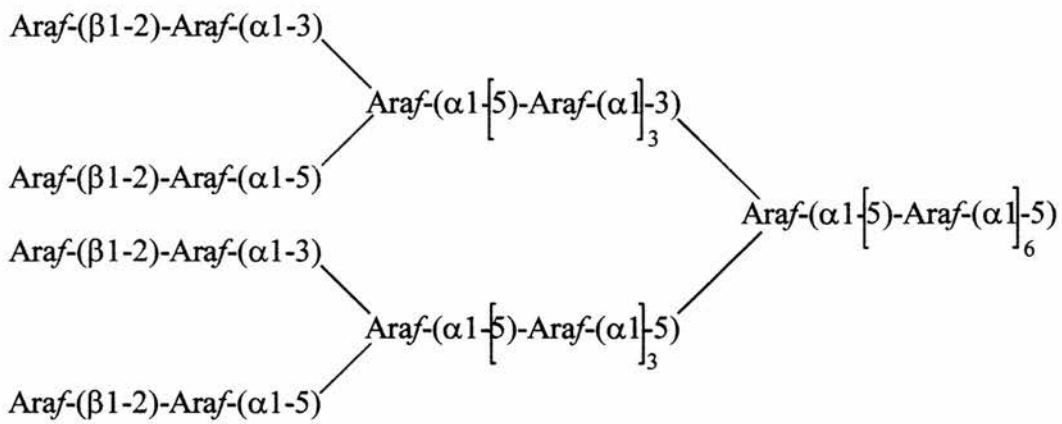

Figure 8: Branched Arabinan. The non-reducing termini are mycolated and the reducing ends are linked (α1-5) to some 6-linked galactofuranose units.

The overall picture of the arabinogalactan-linker is shown in Figure 9 and much of the information was derived from a large mild acid labile cell wall extract which contained the linker disaccharide, a 31-residue linear galactan and portions of the branched arabinan.

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●, 5-α-D-Araf; ◆, 3,5-α-D-Araf; ▮, T-α-D-Araf; ■, 2-α-D-Araf or 2-α-D-Araf mycolyl substituted; ▲, T-β-D-Araf mycolyl substituted; △, t-β-D-Galf; □, 6-β-D-Galf; ○, 5-β-D-Galf; ◇, 5,6-β-D-Galf.

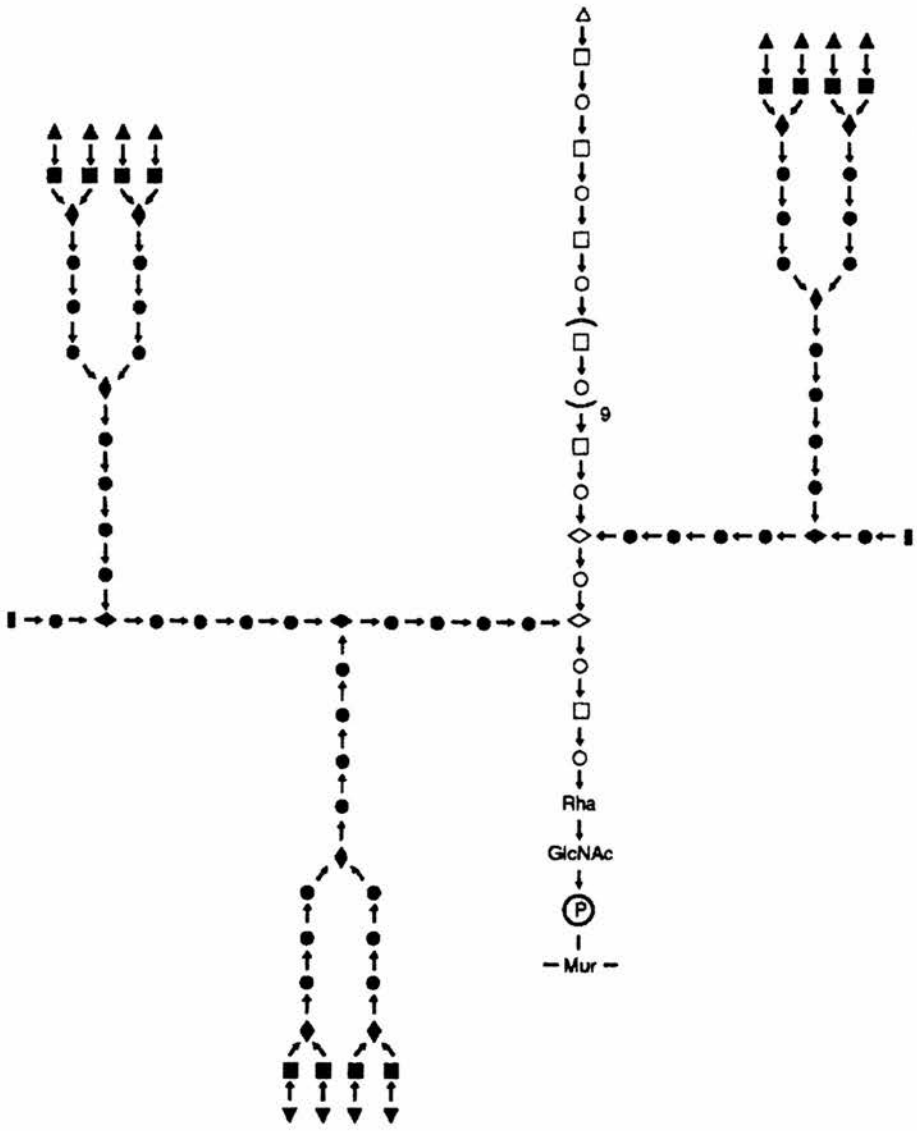

Figure 9: Proposed Overall Structure of the Arabinogalactan-Linker[17]

1.2.2.4 Mycolic Acids

At the non-reducing termini of the arabinan are located the mycolic acids which are responsible for the waxy appearance of mycobacteria. These are high molecular weight α-alkyl-β-hydroxy fatty acids esterified at the 5-position of arabinose. They have been extracted[17] from the cell walls as tetramycolylpentaarabinosyl clusters as shown in Figure 10. The mycolic acids range in length from C_{70} to C_{90} and the α-alkyl branch ranges from C_{20} to C_{25} which make them the largest of the bacterial mycolates. The other functionalities include cyclopropane rings, double bonds, methoxy groups,

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ketones and branched methyl groups which are capable of preventing linear, tight packing of the mycolates.

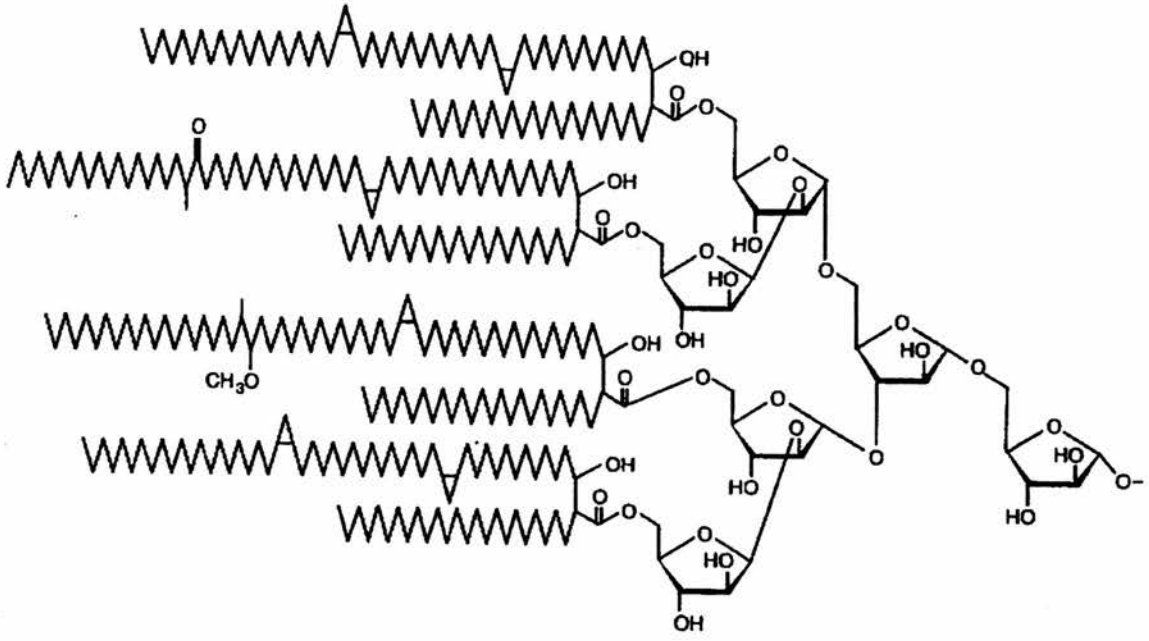

Figure 10: Structure of the Tetramycolylpentaarabinosyl Clusters

It is the very large structure of the mycolylarabinogalactan that dictates access and exit of molecules to and from the inner areas of mycobacteria. This is the barrier that any potential therapeutic drug must pass before any biological effectiveness would be seen.

1.2.2.5 Lipoarabinomannan (LAM)

Although not actually connected to the mycolylarabinogalactan-peptidoglycan structure detailed above, LAM is connected to the plasma membrane and is thought to protrude to the outside of the cell wall. LAM is thought to be a ligand involved in phagocytosis as well as being a key factor in the suppression of the hosts' immunity by the neutralisation of cytotoxic reactive oxygen intermediates and prevention of lyosomal-phagosomal binding.

LAM is based on monoacyl phosphatidylinositol, a member of the PIM family which contains inositol-2,6-dimannoside. A linear (α1-6) mannan with intermediate (α1-2) linked monomannoses extends from one of these mannosides and from the non-reducing end of the linear mannan, a branched arabinan containing (β1-2), (α1-5) and (α1-3) linkages is connected.

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1.2.2.6 Other Cell Wall Components

Many other extractable components of the mycobacterial cell wall have been discovered in the search for cell-surface antigens. These include various lipooligosaccharides (LOS's), glycopeptidolipids (GPL's) and phenolic glycolipids (PGL's).

1.2.3 Current Drugs and Future Outlook

Among the many broad spectrum antibacterials that target the biosynthesis of peptidoglycan (cycloserine, vancomycin, teicoplanin, β-lactams, and cephalosporins) only cycloserine is effective in antimycobacterial therapy.[18] This is thought to be due to the impermeable nature of the mycobacterial cell wall.[18] The two most commonly used treatments for tuberculosis are rifampicin and isoniazid. Rifampicin binds to RNA polymerase thus preventing transcription[19] and isoniazid is known[17] to inhibit mycolic acid biosynthesis. Ethionamide has also been shown to inhibit mycolic acid biosynthesis.[17]

Another commonly used antimycobacterial, ethambutol, has been shown to interrupt the biosynthesis of arabinogalactan by primarily inhibiting the transfer of (α1-3) arabinofuranosyl residues.[20] Due to the extensive Ara(α1-3) linkages at the branching points of arabinan, this drug prevents synthesis of arabinan and thus the mycolic acids cannot bind to the terminal residues and cell viability is reduced.[20]

Strains of mycobacteria which confer resistance to all of these drugs have been discovered and therefore it is imperative that new therapeutic agents be developed to combat the increase in world-wide tuberculosis. The great increase in the knowledge of the structure and biosynthesis of mycobacterial cell walls over the past 10-15 years has identified many new drug targets against which potential therapeutic agents may be screened. As was recently stated about the mycobacterial cell envelope:

"This highly complex and well organised structure, unique to the mycobacteria, represents the best overall target for novel antimycobacterial agents."[19]

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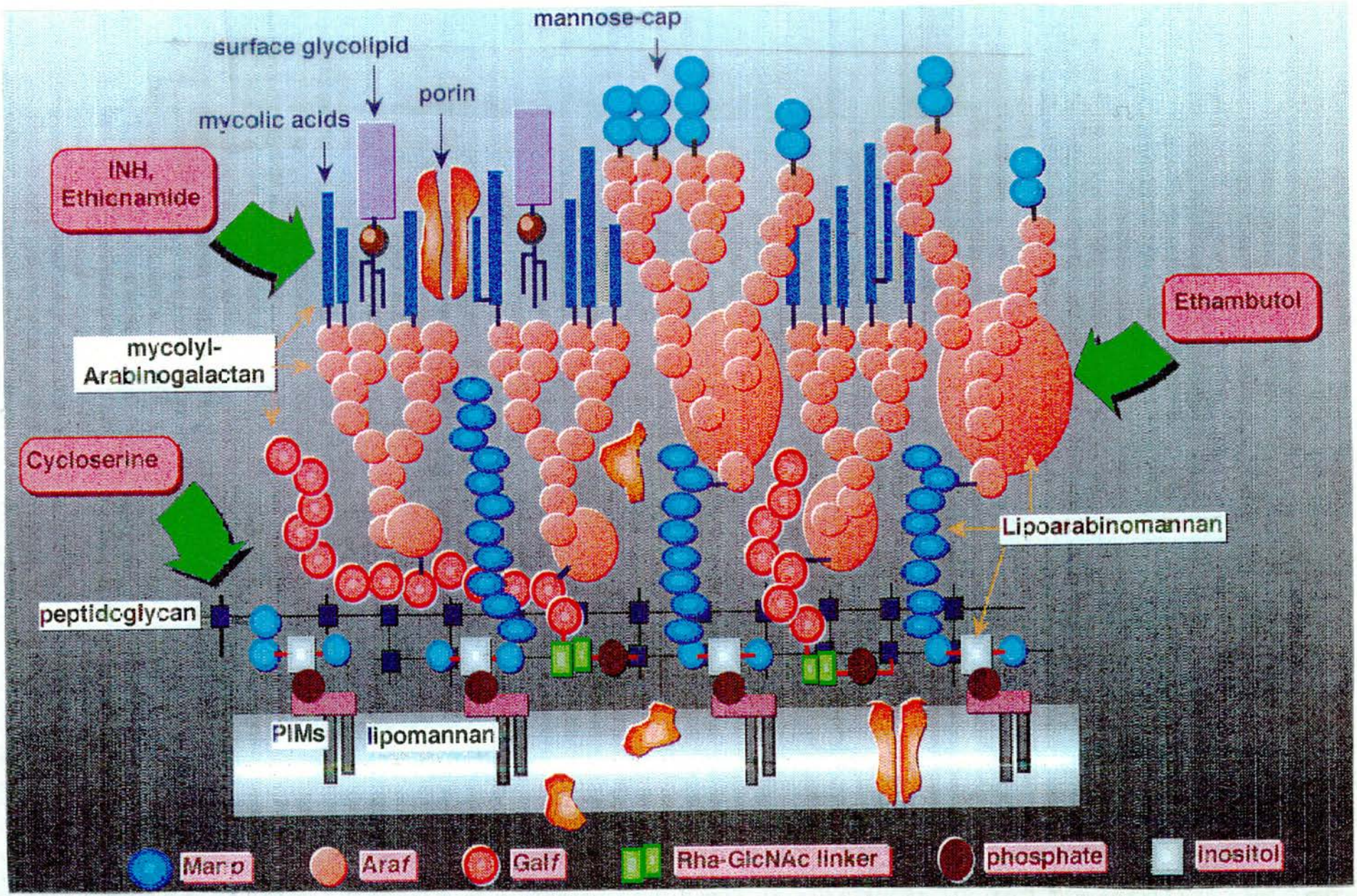
Figure 11: Mycobacterial Cell Wall



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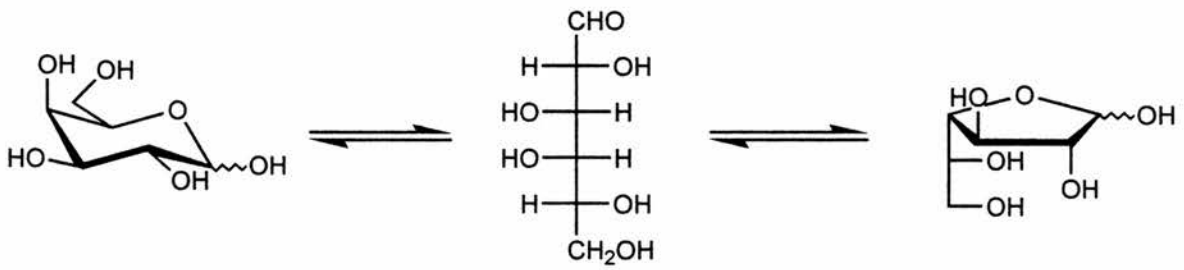
CHAPTER 2: Attempted Synthesis of UDP-Gal*f*

2.1 INTRODUCTION

2.1.1 Occurrence of Galactofuranose

2.1.1.1 General background

In nature D-galactose exists in a variety of oligosaccharides, polysaccharides and glycoconjugates. However, in mammalian systems only the six-membered ring pyranose form **(1)** is found. Galactofuranose, the five-membered ring form **(3)**, is restricted to bacteria[1-4] (e.g. arabinogalactans in *Mycobacterium tuberculosis*), fungi[5,6] (e.g. galactomannans in *Penicillium* varieties) and protozoa[7] (e.g. in trypanosomatids). The occurrence of this unusual monosaccharide in the glycoconjugates of the aforementioned lifeforms has made it the subject of study in many disease areas.



(1) **(2)** **(3)**

Scheme 1: The different forms of D-galactose. (1). Galactopyranose (Gal*p*); (2). open chain form; (3). galactofuranose (Gal*f*)

2.1.1.2 Relevant diseases

Tuberculosis is a chronic infectious disease in humans and animals caused by tubercle bacilli, *M. tuberculosis* or *M. bovis*. On gaining access to the lung of a new host, the bacilli are quickly engulfed by white blood cells but rather than die, they are more likely to grow and multiply, very slowly, invade neighbouring cells, and form a tubercle.[8] These tubercles can then grow, spread and consume the lungs, often resulting in death.

Chagas' disease,[7] or American trypanosomiasis, is endemic to Central and South America where 25% of the population are at risk. It is an acute and chronic disease caused by the pathogenic hemoflagellate, *Trypanosoma cruzi,* and is transmitted to man via the infected faeces of blood-sucking triatoma bugs. Symptoms such as liver and spleen enlargement, facial oedema and irreversible tissue damage can occur. Annually, one million people are infected, 45,000 die of the disease and it currently afflicts 16-18

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million people, one third of whom will contract the incurable lesions and chronic complications 10-20 years after the initial acute phase.[7]

Leishmaniasis,[7] an infectious disease of which there are four types is transmitted by blood-sucking sand flies. The parasites penetrate the skin, causing deep ulcers (cutaneous leishmaniasis) and they can also affect the organs (visceral leishmaniasis), causing fever, spleen and liver enlargement. It is a world-wide disease but is concentrated in Asia, the Middle East and North Africa, and affects 12 million people, with up to two million new cases each year.[8]

2.1.1.3 Galactofuranose in bacteria

It is thought[5] that in tuberculosis, leprosy and other mycobacterioses, the host recognises certain components of the insoluble cell-wall matrix. This cell-wall matrix is thought to consist of two major entities, the mycolyl-D-arabino-D-galactan (mAG) and peptidoglycan (PG) (Figure 12).

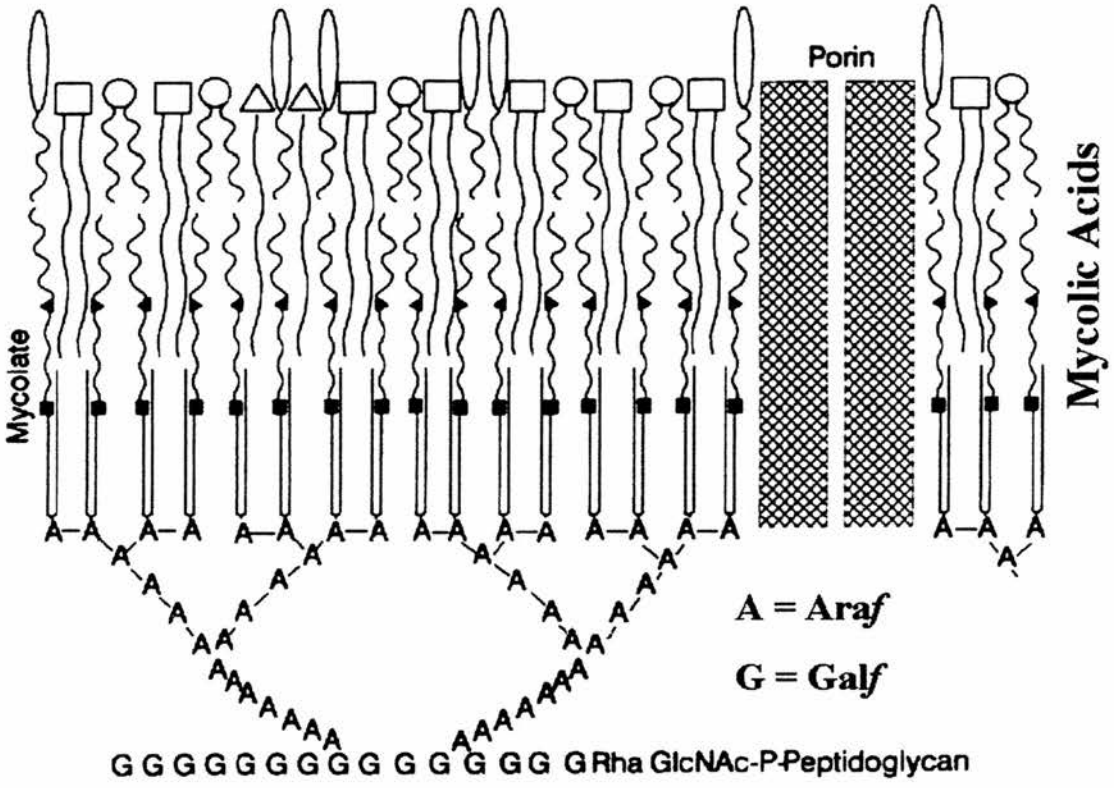

Figure 12: The mycobacterial cell wall matrix.[9]

The arabinan part of mAG is a branched oligomer of arabinofuranosides which, at the non-reducing ends are linked to the mycolic acids. At the reducing end is

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the galactan which is a chain of alternating β1→5 and β1→6 linked galactofuranose residues.

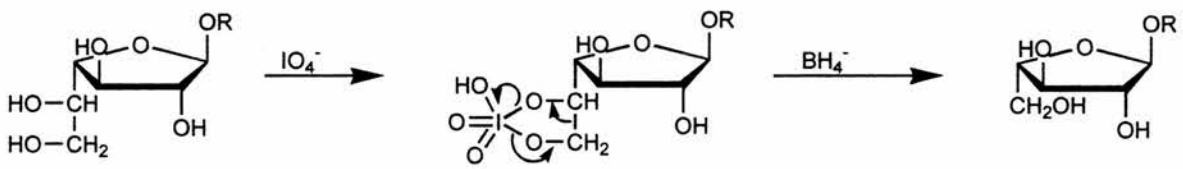
Between the mAG and the PG there is a linking disaccharide: [→4-L-Rha-α-1→3-D-GlcNAc-α]. The GlcNAc is linked anomerically to a phosphate which bridges the oligosaccharide to the PG. The overall structure of the linkage region is represented in Figure 13.

D-Gal*f*-β1→4-L-Rha-α1→3-D-GlcNAc-α-1→P→Peptidoglycan

Figure 13: Linkage region of the Mycobacterial Cell Wall

2.1.1.4 Galactofuranose in protozoa

Two protozoa[7] in which Gal*f* is found are *Trypanosoma cruzi*, which is responsible for Chagas disease, and *Leishmania* species, which are responsible for leishmaniasis. In *T. cruzi*, Gal*f* has been found in the lipopeptidophosphoglycan (LPPG) which is a glycoconjugate with a glycoinositolphospholipid (GIPL) structure isolated from a trypanosomatid. Galactofuranose was detected in this structure by periodate oxidation followed by borohydride reduction[7] (Scheme 2). This oxidises *cis* 1,2-diols and cleaves the bond between them. In the case of galactofuranose, the process effectively removes the C-6 group, leaving the C-5 L-arabinofuranoside after acid hydrolysis.



(4) **(5)** **(6)**

Scheme 2: Periodate oxidation converting a galactofuranoside (4) into an arabinofuranoside (6)

In LPPG, galactofuranose is terminal, non-reducing and is always found β1→3 linked to mannose. This is mostly as a part of the linear oligosaccharide core although also as a branch to the core, which can be seen in Figure 14.

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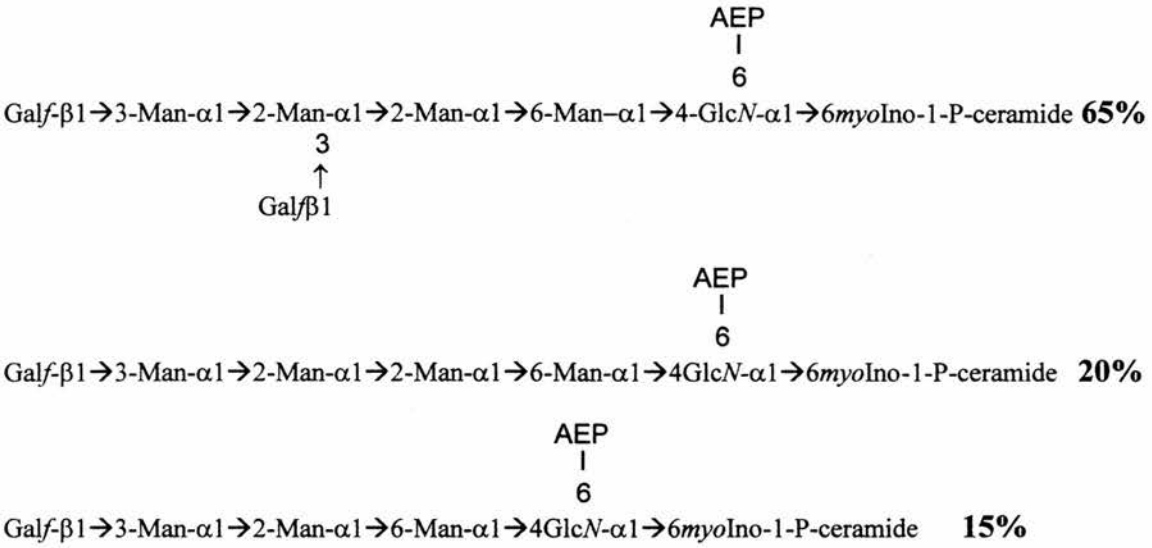

Figure 14: **Structures of the LPPG in *T. cruzi*.** The percentages reflect the proportions of each LPPG found.

There are two glycoconjugates which, together, play an important role in the pathogenesis of leishmaniasis, LPPG and GIPL.[7] LPPG (lipopeptidophosphoglycan) consists of four distinct regions: 1. A lysoalkylphosphoinositol, 2. A hexasaccharide core, 3. A repeating phosphoglycan and 4. A small oligosaccharide cap. Galactofuranose was found in the middle of the hexasaccharide core which makes this occurrence differ from *T. cruzi* which contain only terminal Gal*f* (Figure 15).[7]

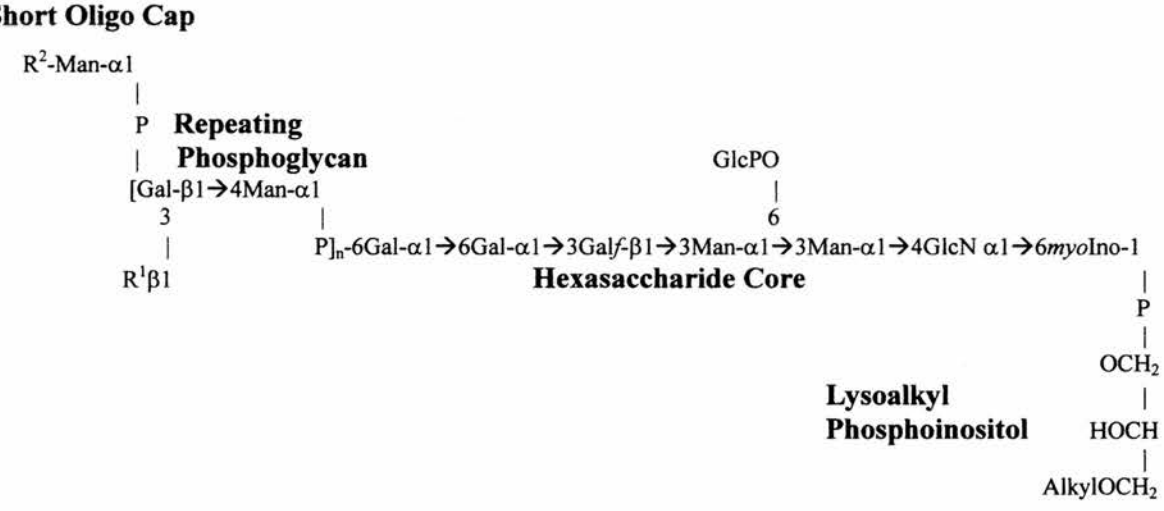

Figure 15: **Structure of LPG from *Leishmania*:** R^1 may be H, Glc, Gal or a short oligosaccharide. R^2 is Manα1→2 or a short oligosaccharide.

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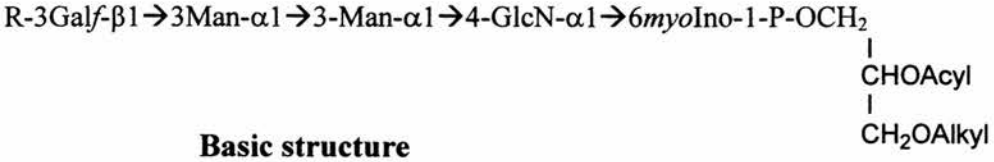
A second type of glycoconjugate found in *Leishmania* species is the GIPL structures (Figure 16). These molecules are important constituents of the plasma membranes and in each case the structures contain one Gal*f* unit, but only GIPL-1 of *L. major* contains a terminal Gal*f* unit. Type-2 GIPLs of *L. major* and *L. mexicana* are thought to be biosynthetic precursors to LPG.



Basic structure

R	GIPL	PARASITE
H	GIPL-1	*L. major*
Gal-α1→	GIPL-2	*L. major and L. mexicana*
Gal-α1→6Gal-α1→	GIPL-3	*L. major and L.mexicana*
Gal-β1→3Gal-α1→	GIPL-A	*L. major*

Figure 16: Structures of GIPLs from *Leishmania*.

2.1.1.5 Galactofuranose in fungi

The thermally dimorphic fungus *Paracoccidioides brasiliensis*, which is prevalent among rural farm workers in South and Central America and causes paracoccidioidomycosis (PCM), produces another galactofuranose containing glycoconjugate.[5] The antigen isolated from *Paracoccidioides brasiliensis* was shown to contain a phosphoglyceroglycolipid with a core glycan structure containing of Gal*f* β1→6 linked to mannose as a terminal non-reducing residue as shown in Figure 17.

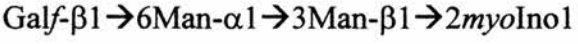


Figure 17: The basic structure of the antigenic tetrasaccharide in *Paracoccidioides brasiliensis*.

The glycolipid which contains Gal*f* in *P. brasiliensis* is antigenic and it was found that removal of the Gal*f* residue resulted in 60-80% loss of antigenicity in the sera of all patients with PCM. This shows the Gal*f* residue to be highly antigenic and that at least 60% of the antibodies produced by the host against the glycolipid antigen are

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directed to the terminal Gal*f*. Since Gal*f* is not naturally occurring in mammalian systems this is perhaps no great surprise, however the antigenicity of Gal*f* has been commented on before[7] and one hypothesis[5] is that Gal*f* may be involved in the invasion of macrophages by different parasites and fungi through specific receptors. This process is an important pathogenic step in PCM.

Oligomers of galactofuranose, called galactocaralose, are present in various *Penicillium* varieties.[6] These exist as 9 or 10 β1→5 linked galactofuranose units that are biosynthesised from glucose. The biological function of this polymer is unknown.

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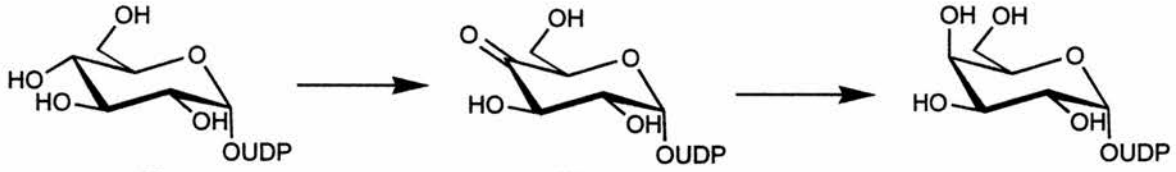
2.1.2 Biosynthesis of Galactofuranose and Galactofuranose-Containing Glycoconjugates

2.1.2.1 Biosynthesis of galactofuranose

Experiments on the formation of the T1 antigen of *Salmonella typhimurium*[10] (another galactofuranose containing glycoconjugate), demonstrated that the source of galactofuranose was neither free galactose nor galactose-1-phosphate, but was UDP-galactopyranose **(9)**. This was synthesised from UDP-glucose **(7)** *in situ* using the enzyme UDP-glucose-4-epimerase (Scheme 3).



Scheme 3: Metabolism of UDP-glucose to UDP-galactose by UDP-glucose-4-epimerase

Since UDP-galactose was thought to be the precursor to galactofuranose, it was thought that an enzyme was involved in isomerising galactopyranose to galactofuranose whilst still bound to the UDP nucleotide. This was recently confirmed[11] by the discovery of two genes from the *Escherichia coli* K-12 *rfb* region. *E. coli* K-12 was isolated in 1922 and is perhaps the most thoroughly studied of all organisms.[11] However, adaptation to the laboratory environment led to mutations which resulted in a loss of O-antigen biosynthesis. A strain was constructed which had all the O-antigen genes intact and this strain was found to produce an antigen which contained galactofuranose (Figure 18). Gal*f* is β1→6 linked to glucose which is then linked α1→3 to a disaccharide which is identical to the disaccharide which links mAG and PG in mycobacterial cell walls.

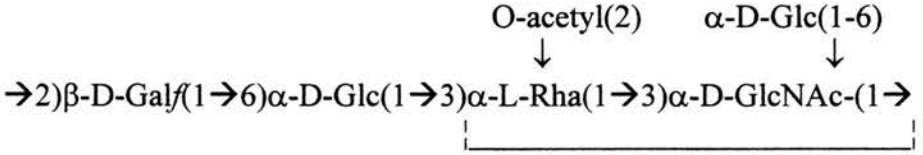
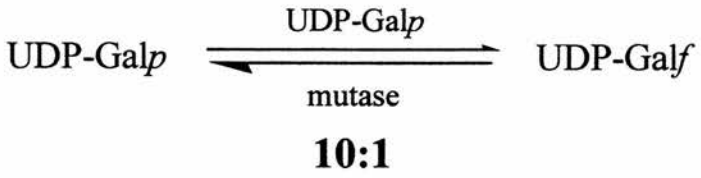


Figure 18: O-antigens of *E. coli* K-12. The marked region indicates homology to the *Mycobacterium* linkage region

References are on page 68

There were only two unassigned genes left in the *E. coli* rfb cluster and these were assumed to be the UDP-galactose mutase, and the UDP-galactofuranosyltransferase (see 2.1.2.2). Both of these genes, *orf* 6 and *orf*8, were cloned recently[12] from *E. coli* K-12 and *orf* 6 was characterised as being the UDP-galactopyranose mutase (*glf*). Incubations of *glf* with UDP-galactopyranose led to 11% conversion to UDP-galactofuranose in an equilibrium reaction (Scheme 4).



Scheme 4: Conversion of UDP-Gal*p* to UDP-Gal*f* from incubation with UDP-galactose mutase.

The purified enzyme extract contains the co-factor flavin adenine dinucleotide (FAD), the function of which is unknown. It had been suggested[11] that the mechanism for conversion proceeds via a 2-keto intermediate in a manner analogous to the isomerisation of UDP-glucose to UDP-galactose (Scheme 4), however it is not clear how this could occur. Recently,[12] UDP-Gal*f* has been prepared enzymatically using the *glf*. This has been performed on a 1mg scale with isolation of 30μg of pure UDP-Gal*f* giving a 3% overall yield. Although this is an exciting development, there is still a need for a chemical method of synthesis to produce milligram quantities of material.

2.1.2.2 Biosynthesis of galactofuranose containing glycoconjugates

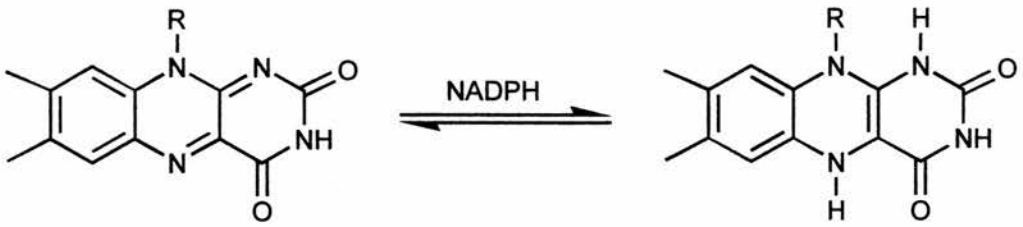
It is thought that the remaining gene (*orf* 8) is UDP-galactofuranosyltransferase which would be responsible for the transfer of Gal*f* from UDP-Gal*f* to the K-12 O-antigen polysaccharide. However as yet there has been little study of this enzyme.

References are on page 68

2.1.3 Putative mechanisms for UDP-Gal isomerisation

2.1.3.1 Reactivity of FAD

Flavins are known to mediate a variety of redox processes, including electron transfer, and the activation of molecular oxygen for oxygenation reactions. They can undergo one and two electron transfer processes, but many of the mechanisms are not fully understood.[13] A review[14] on the known and hypothesised mechanisms contains detailed information regarding these processes. The most basic two states in which FAD exists are in the oxidised form **(10)**, which is just termed FAD, and in the reduced form **(11)** which is termed $FADH_2$.



(10) **(11)**

Scheme 5: Redox equilibrium of FAD

2.1.3.2 Glutathione reductase

One interesting mechanism which could be relevant to UDP-galactose mutase is the reaction catalysed by glutathione reductase.[14] The basic reaction involved and the proposed mechanism which includes the role of the flavin co-factor are represented in Scheme 6. The part of the reaction which is of relevance to Gal*f* is the reduction of the redox-labile disulfide link in **(13)**.

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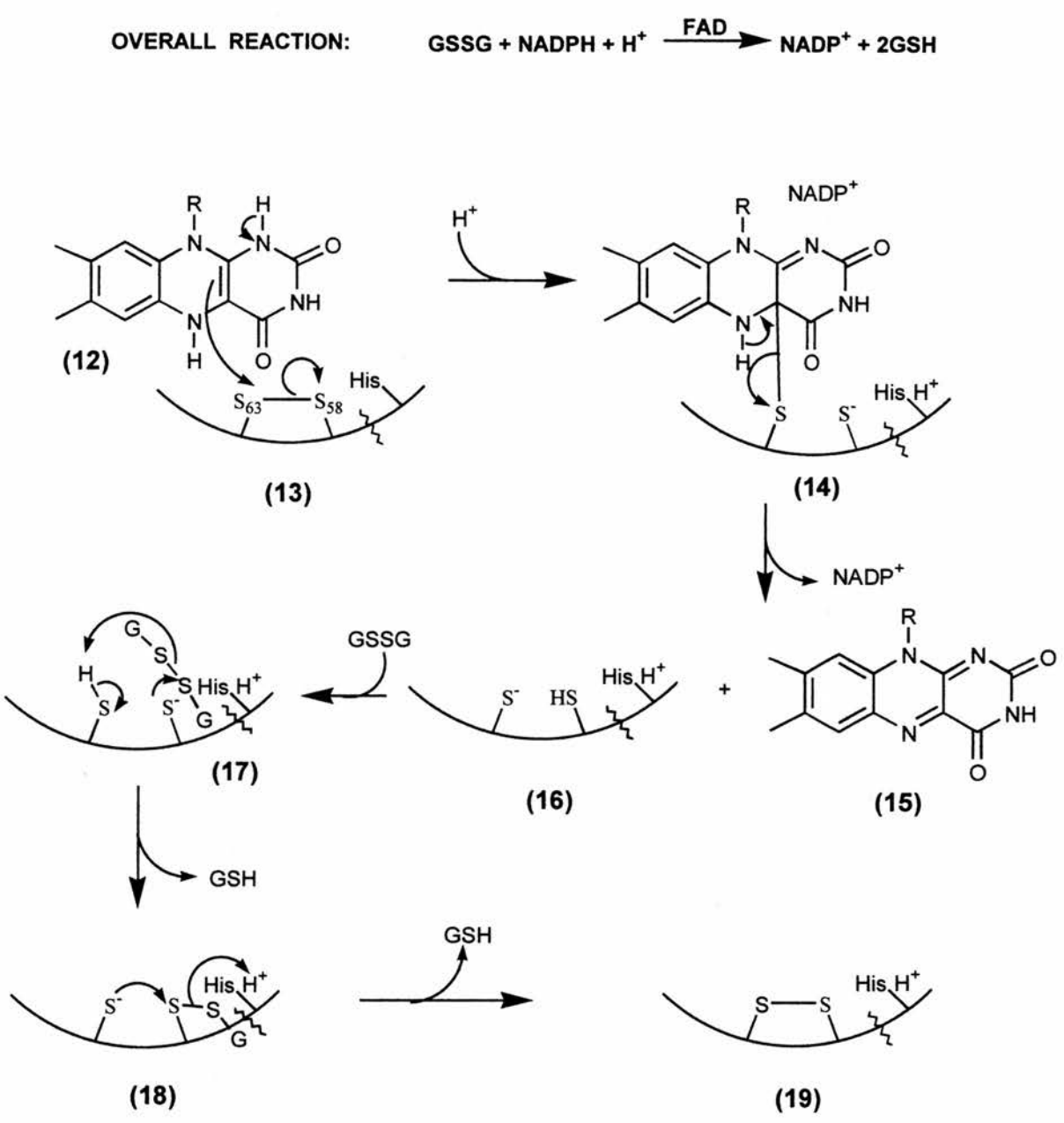


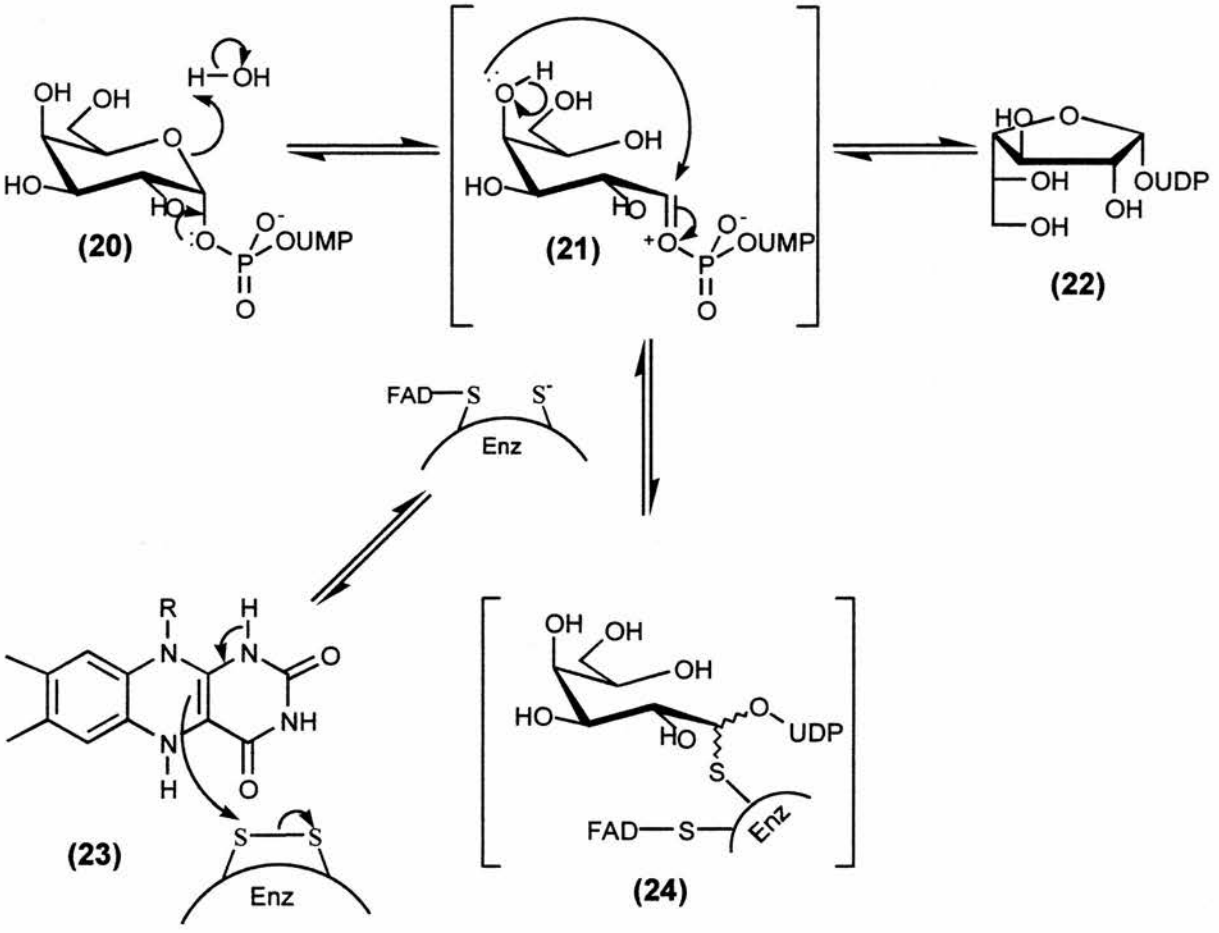
Scheme 6: FAD involvement in the mechanism of glutathione reductase[14]

2.1.3.3 Possible mutase mechanism 1: sulfur stabilised oxocarbenium ion intermediate

One of the possible mechanisms for UDP-Gal*p* isomerisation in the presence of FAD is linked to both of the mechanisms in Scheme 6 and Scheme 8, and is shown in Scheme 7. The proposal is that an oxocarbenium ion intermediate similar to **(26)** is formed **(21)**, the difference being that in **(21)** a carbon to oxygen bond is broken whereas in **(26)** a carbon to carbon double bond is broken. The presence of FAD could possibly be due to similar reasons as for glutathione reductase, that is the hydrolysis and activation of a disulfide link. The residual enzyme-bound sulfur nucleophile could then possibly act

References are on page 68

in a similar way to cysteine-115 in enol-pyruvyl transfer **(27)**, in stabilising the oxocarbenium ion through a covalently bound intermediate, whilst some of the UDP-galactose reorientates itself for furanose ring closure **(21)** to form ~11% UDP-Gal*f*.

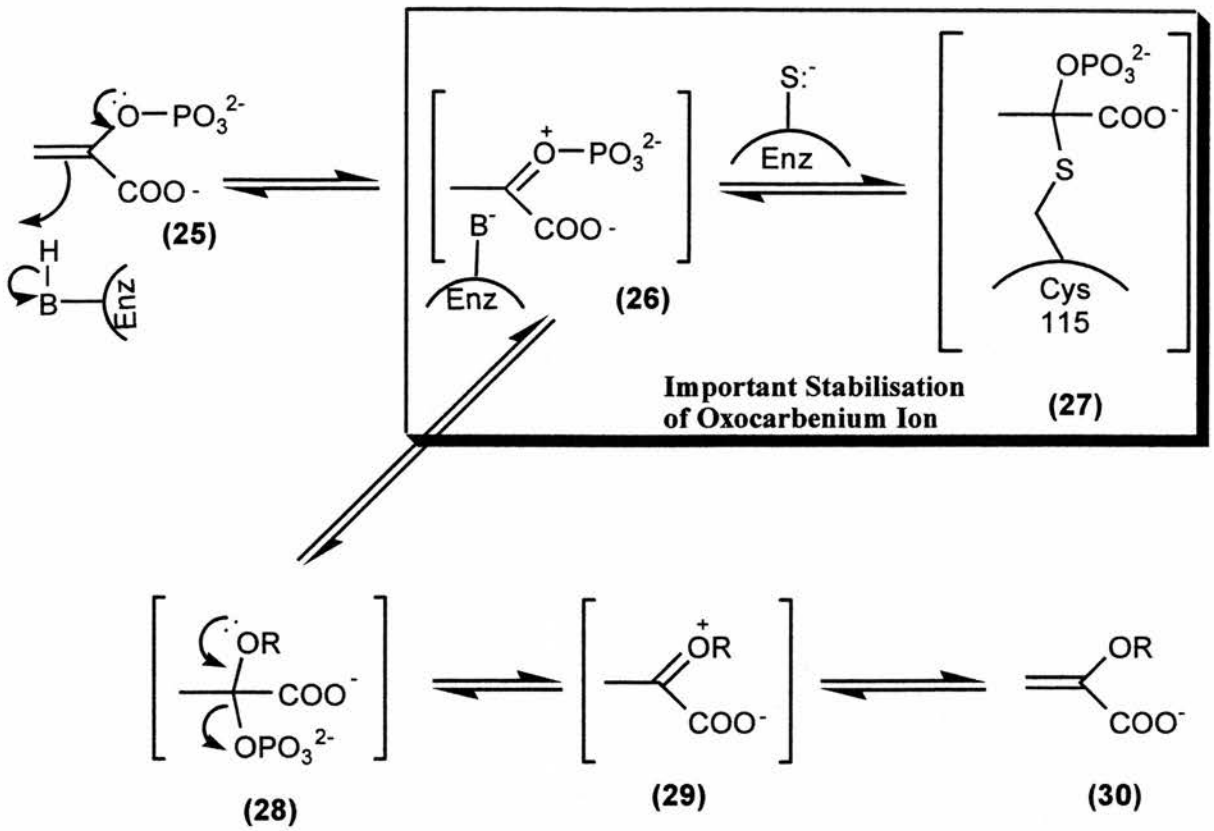


Scheme 7: Prospective mutase mechanism 1: sulfur stabilised oxocarbenium anion intermediate

2.1.3.4 Enol pyruvyl transfer in bacterial cell wall biosynthesis

In order to shows the relevance of this mechanism to the isomerisation of UDP-galactose, another mechanism must be discussed, this time pertaining to enol pyruvyl transfer in bacterial cell wall biosynthesis.[15,16]

References are on page 68



Scheme 8: Cysteine-115 stabilisation in the enolpyruvyl transfer step of bacterial cell wall biosynthesis[13]

In this mechanism, an oxocarbenium ion intermediate **(26)** is proposed, but due to the unstable nature of the adduct, it is proposed that it is stabilised by cysteine-115 which forms the covalently bound intermediate **(27)**. Cys-115 has been shown to be essential for catalysis and the antibiotic fosfomycin **(30)** acts by alkylating this residue.[15,16]

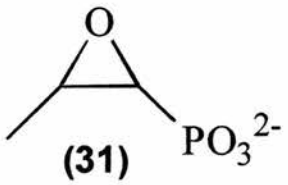
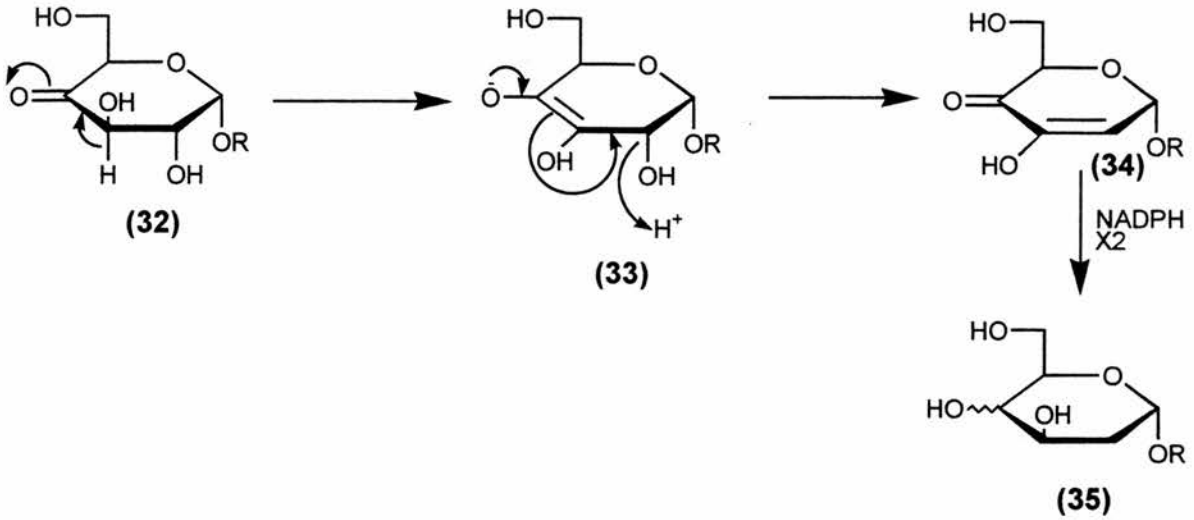


Figure 19: Structure of Fosfomycin

2.1.3.5 Biosynthesis of novel deoxy sugars by bacteria

Another possible mechanism was inspired by the pathways involved in the biosynthesis of novel deoxy sugars by bacteria.[17] A typical mechanism (Scheme 9) involves oxidation of one position to a ketone, followed by enolisation **(33)**, then a *retro*-Michael type reaction, to give enone **(34)**, followed by hydride transfer to form the deoxy sugar **(35)**.

References are on page 68



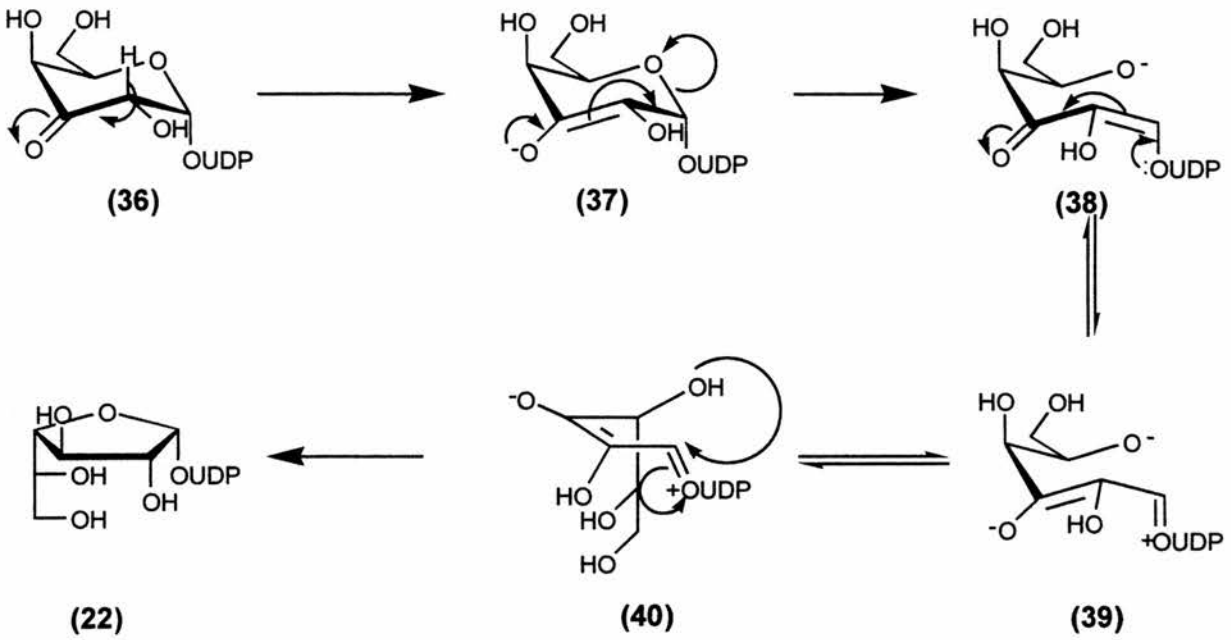
Scheme 9: Typical mechanism for deoxy sugar formation, R = nucleotide.

2.1.3.6 Possible mutase mechanism 2: enolisation of a 3-keto intermediate followed by a *retro* Michael reaction

An analogous mechanism for the formation of UDP-Gal*f* is outlined in Scheme 10. FAD is known as a redox co-factor, and there has been a reference[11] to the possibility of a 2-keto intermediate in the biosynthesis of UDP-Gal*f*, therefore the mechanism shown, incorporates these factors (albeit with a 3-keto intermediate). FAD would have to act upon the UDP-Gal*p* to form the 3-keto adduct **(36)**. Enolisation to form a double bond between the 2 and 3 position **(37)** followed by a *retro*-Michael type reaction could break the carbon to oxygen bond, allowing ring closure at the 4-OH position **(40)**, to yield UDP-Gal*f*.

At this point, these are merely hypotheses, as there have been no reported experiments into the actual mechanisms of isomerisation.

References are on page 68



Scheme 10: Prospective mutase mechanism 2: enolisation of a 3-keto intermediate, followed by a *retro* Michael reaction breaks the C-O bond allowing reformation in the furanose form.

References are on page 68

2.1.4 Aims and Objectives

Since the equilibrium position of the galactopyranose mutase reaction lies so far in favour of the pyranose form, it would be desirable to have milligram quantities of UDP-Gal*f* to study the conversion of furanose to pyranose.

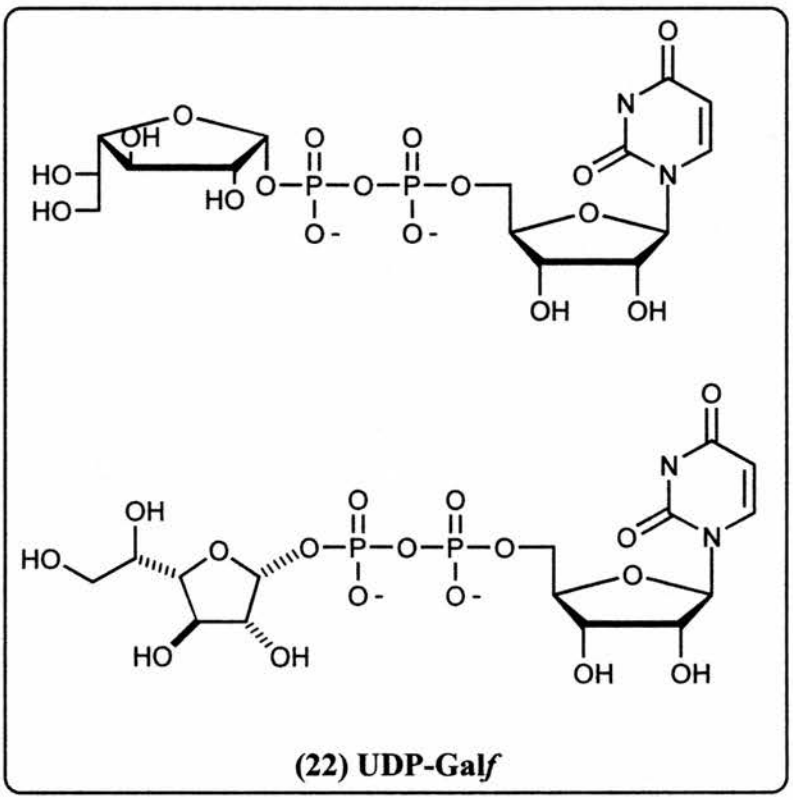


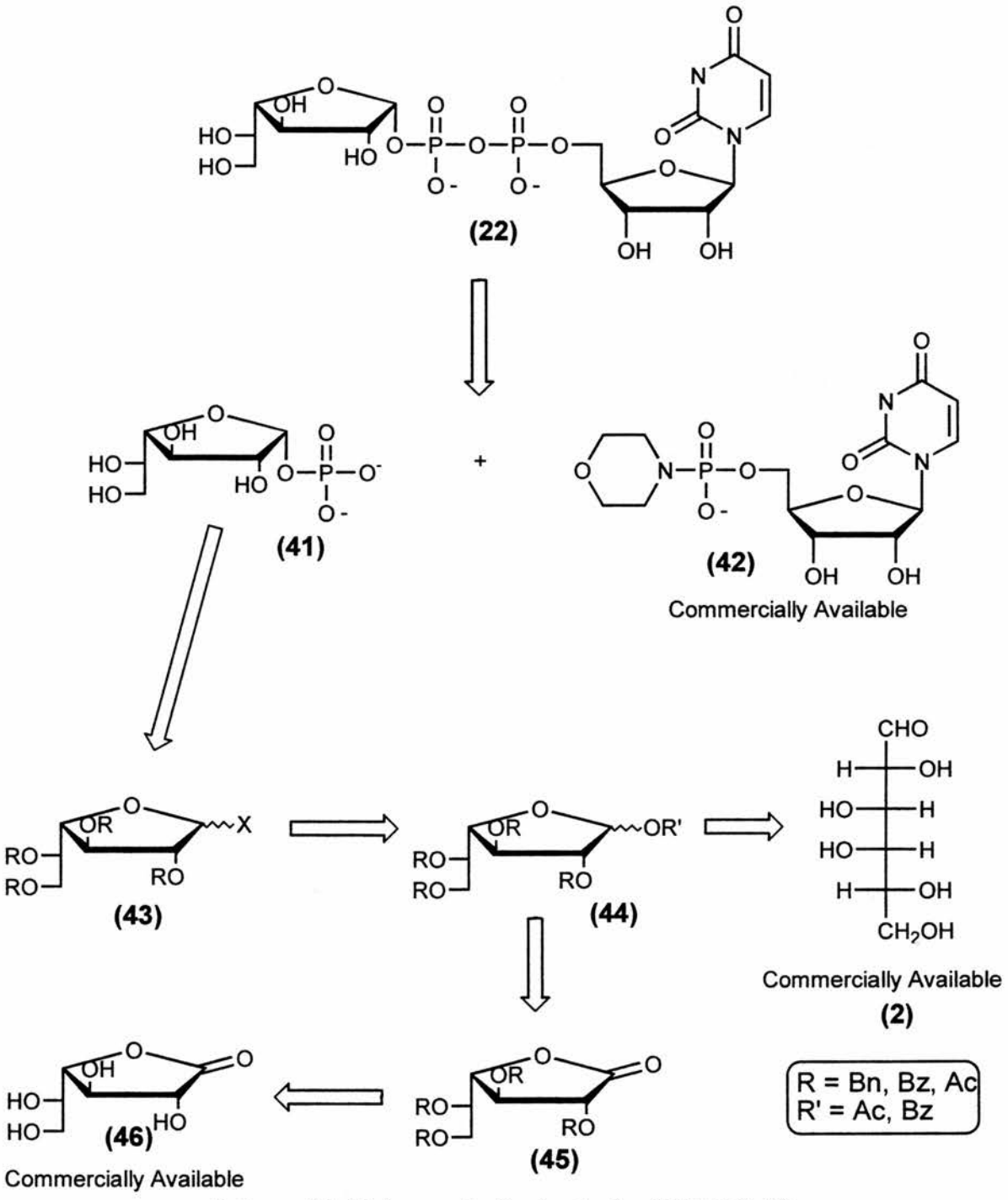
Figure 20: Target Compound (2 different drawing styles)

References are on page 68

2.2 RESULTS AND DISCUSSION

2.2.1 Attempted synthesis of UDP-Gal*f*

The obvious starting point for a retrosynthetic disconnection of **(22)** would be the pyrophosphate group which could be synthesised by a coupling of galactofuranosyl phosphate **(41)** and the commercially available uridine monophosphomorpholidate **(42)**.

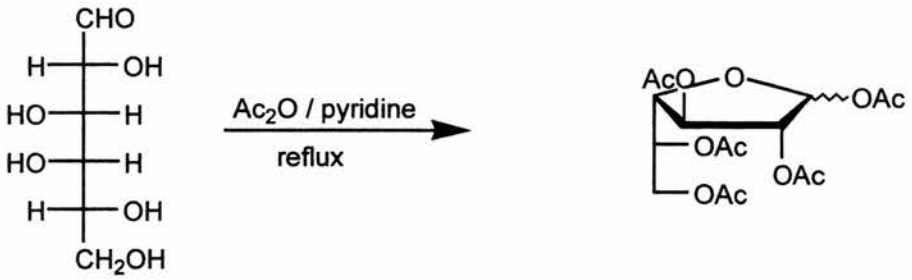


Scheme 11: Retrosynthetic Analysis of UDP-Gal*f*

References are on page 68

Glycosyl phosphates are commonly synthesised[18-20] from anomeric esters via glycosyl halides. The anomeric ester could possibly be made directly from D-galactose via a high temperature acylation or from reduction and acylation of the lactone **(45)**.

2.2.1.1 Attempted synthesis of penta-*O*-acetyl-α/β-D-galactofuranose



(2) **(47)**

Scheme 12: High temperature acetylation of galactose

Initially, it was decided to attempt the synthesis of fully protected galactofuranose using free galactose as the starting material. The problem with this strategy was overcoming the fact that the furanose product is less thermodynamically favourable than the pyranose. The literature on the synthesis of these compounds was found to be quite limited, although some general methods did exist. n-Pentenyl galactofuranosides[21] have been synthesised by treating galactose with a mixture of DMSO, n-pentenyl alcohol and camphorsulfonic acid at high temperature (90-100°C) to yield 43% of the furanoside product (β-60%, α-21%). n-Octyl galactofuranosides[22] (α-16%, β-84%) were made in THF using $FeCl_3$ as promoter and $CaCl_2$ as an additive, this time at room temperature. In a similar vein, galactofuranosides (α-100%) were obtained[23] using anomeric alkylation with either dodecyl sulfate, benzyl bromide or allyl bromide in DMPU with yields around 60%. Pentaacetylated galactofuranose[24] (α,β mixture, no data) was made by a high temperature acetylation in refluxing pyridine with a low yield of 14% although another source[25] reported a higher yield of 35%, (α-50%, β-50%). Pentabenzoylated galactofuranose (α-56%, β-44%) was also made using high temperatures, this time in pyridine at 60°C.[26]

Since the most desirable product would have been the peracetylated galactofuranose **(47)**, (for ease of subsequent bromination, introduction of an anomeric phosphate group, and ease of deprotection), the high temperature acetylation was attempted. Two components were observed by TLC, and the faster running spot was

 References are on page 68

thought to be the mixed furanoses as the slower running component co-eluted with an authentic sample of pentaacetyl-β-D-galactopyranose obtained via the iodine catalysed acetylation of galactose.[27] The fast running component was isolated using a combination of crystallisation and flash-column chromatography, then analysed by NMR spectroscopy. Initially, it was thought that a mixture of a furanose and a pyranose was present due to the presence of a far downfield unsplit anomeric proton (δ_H 6.28) which was characteristic of furanose ^{1}H spectra), and the sharp doublet and double doublet (δ_H 5.62, 5.02) more characteristic of pyranose spectra. Due to the puckering of the ring in a furanose, the functionalities are known not to be strictly axial and equatorial as in the pyranose configuration but rather have been termed *quasi* axial and *quasi* equatorial therefore, ^{1}H NMR signals of the ring protons tend to be less well resolved than in a pyranose molecule. However, the ^{13}C spectrum showed no peaks in the range δ 73-82 where C-4, 3 and 2 would have been found in pentaacetyl-α,β-D-galactofuranose.[25]

The experiment was repeated, however, samples were taken every five minutes and after work-up were analysed by NMR without further purification. There were two new signals in the anomeric region of the ^{1}H spectrum which corresponded very well with the literature values[25] for the α & β Gal*f* pentaacetates. Therefore, the faster running component corresponded to a mixture of the α & β pyranoses whereas the slower running component (more difficult to isolate) corresponded to the mixture of furanoses which were quantified by ^{1}H NMR to be 39% of the whole mixture (α/β, 40/60).

At the time of the experiment, the data presented was not so clear. It was clear, however, that purification of this mixture would prove time-consuming, therefore a different synthetic strategy was adopted. It was not until data emerged later (i.e. NMR data from a separate synthesis of pentaacetylgal*f*) that the conclusions of this experiment became clear.

2.2.1.2 Synthesis of α-D-Gal*f*-1-phosphate

The title compound was made using literature[28-32] procedures as outlined in Scheme 13. Care had to be taken in the benzoylation of **(46)** as elimination across the two-three positions was known to occur[28] with prolonged reaction times. Reduction of **(48)** was carried out using disiamylborane. Attempts to use borane-trimethylamine complex (a less unpleasant reagent) in place of borane-dimethylsulfide complex to prepare disiamylborane, failed. Anomeric benzoylation of **(49)** was a straightforward

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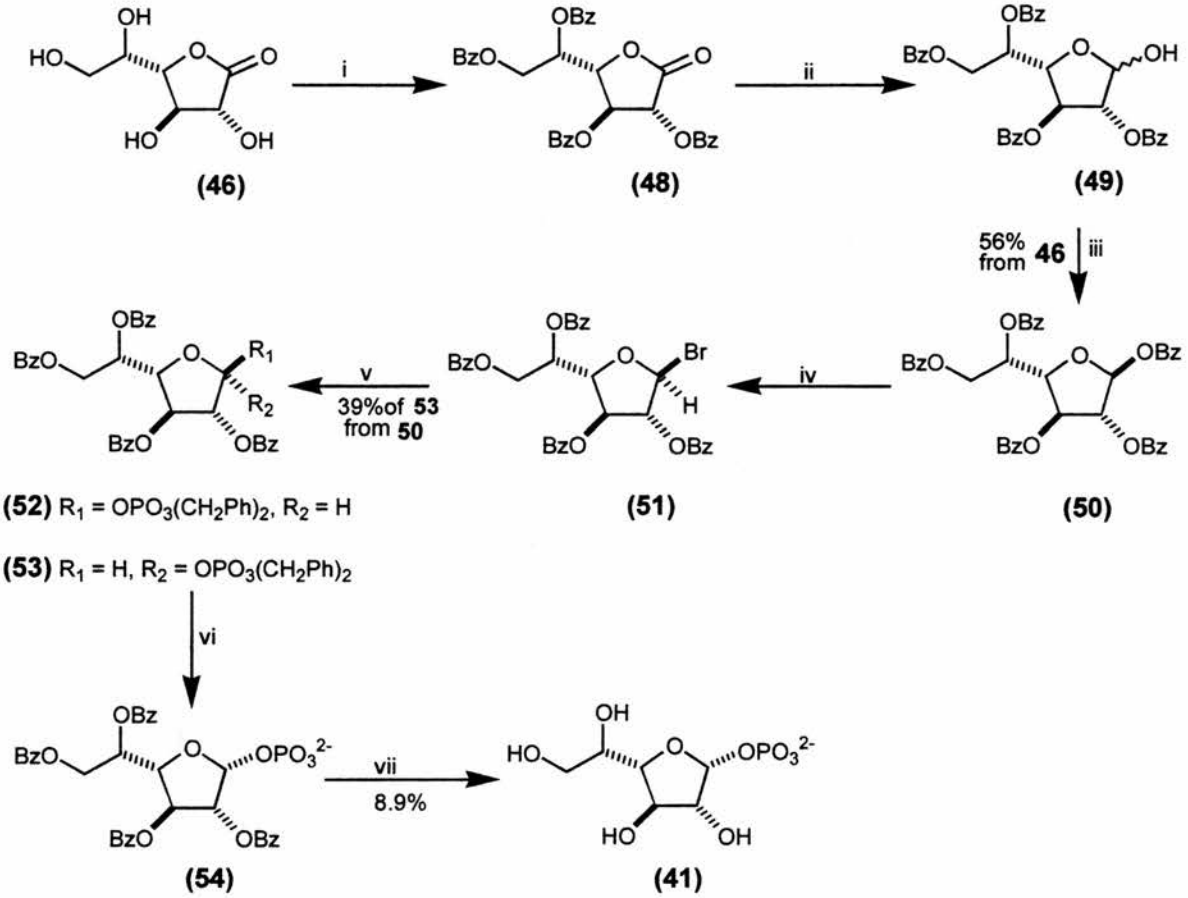
reaction in benzoyl chloride and pyridine and gave exclusively the β-benzoate. Bromination was found to be more efficient using HBr in HOAc than the literature[19] procedure which used TMS bromide. The phosphate **(53)** was prepared using triethylammonium dibenzyl phosphate and the anomeric mixture was separated by flash column chromatography. In the deprotection of **(54)** it was concluded by ^{1}H-NMR that only 65% of the starting material proceeded to completion and the remaining 35% yielded 6-*O*-benzoyl-α-D-galactofuranose-1-phosphate. However, only a small amount of the desired, fully deprotected product **(41)** could be crystallised. The desired crystalline anomeric phosphate **(41)** was obtained in 7 steps with a 1.9% overall yield .



Reagents: (i) BzCl, pyridine; (ii) 2-methyl-2-butene, $BH_3.(CH_3)_2S$, CH_2Cl_2; (iii) BzCl, pyridine; (iv) HBr in HOAc; (v) $(CH_2Ph)_2PO_3H$, Et_3N, toluene; (vi) H_2, 10% Pd on C, EtOAc, Et_3N; (vii) $MeOH/H_2O/Et_3N$, 5:2:1.

Scheme 13: Synthesis of Gal*f*-1-phosphate

A number of other methods were employed in an attempt to deprotect compound **(54)**. Sodium methoxide, a hard nucleophile for the deprotection of ester groups, was tried in a concentration of 0.05M at 10-100mol%, from room temperature to reflux, without any change. It was also used at reflux in 5M strength but decomposition occurred in this

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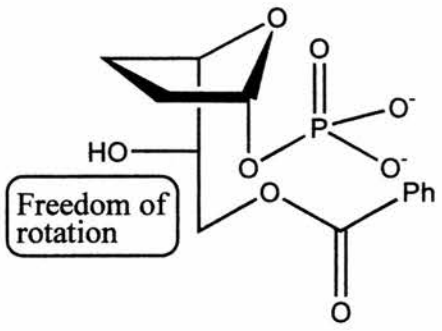
experiment. $Ba(OMe)_2$ was known[33] to deacetylate sugars and it was thought that the divalent nature of the cation could possibly associate across with the two anions of the phosphate with the effect of weakening a possible interaction of the phosphate to the benzoate. However this did not work. The presence of a multiplet at δ=4.49 in the ^{1}H-NMR which was shown to correspond with H-6,6' by C-H correlation and ^{1}H-COSY 2-dimensional techniques, indicated that the monoprotected impurity was benzoylated at the 6-position. It was unclear exactly why the 6-*O*-benzoyl group was so persistent since it should be the most reactive of all the groups because of the secondary rather than tertiary substitution. A crude molecular model of the compound showed the benzoyl group to be potentially in very close proximity to the anomeric phosphate group, therefore it was possible that some interaction was occurring between them.



(55)

Figure 21: Schematic Representation of 6-*O*-Benzoyl-α-D-Gal*f*-1-phosphate. Bond angles are not accurate as the figure is intended only to show approximate orientations.

The nature of this possible interaction is unclear, however the only plausible way of explaining the deactivation of the carbonyl centre in the 6-O-benzoyl protecting group was through an interaction with the phosphate group.

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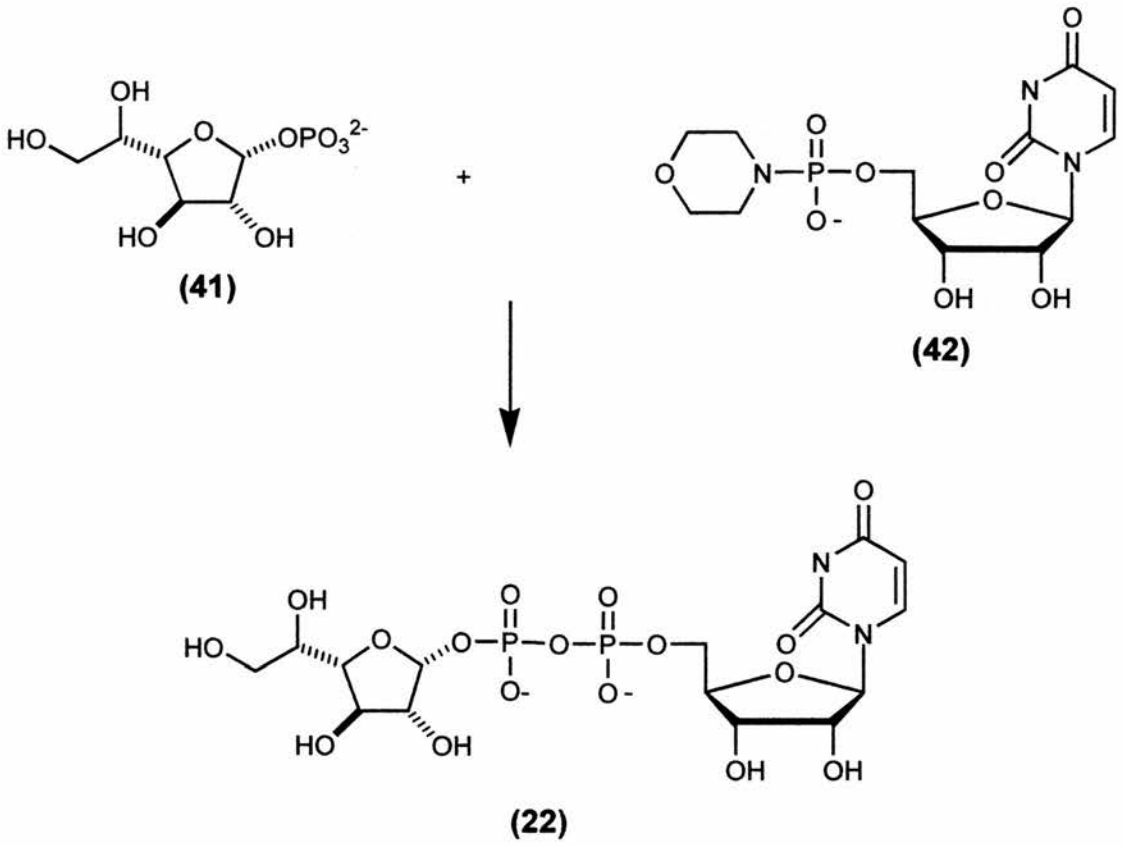
2.2.1.3 Attempted synthesis of UDP-Gal*f*

It is interesting to note that UDP-Gal*f* has never been made before. De Lederkremer and co-workers[19] reported the synthesis of α-D-Gal*f*-1-phosphate, and since this is a compound of little importance alone, presumably they had problems in coupling this with UMP to make UDP-Gal*f*.



Scheme 14: Attempted synthesis of UDP-Gal*f*

The coupling procedure[20] used for the synthesis of GDP-fucose from guanosine monophosphate (a nucleotide) and fucose-1-phosphate (a sugar-1-phosphate) was followed due to the similar nature of the phosphate coupling. This required stringent anhydrous conditions which involved repeated coevaporations of the starting materials in freshly distilled pyridine.

In our hands, the first experiment of this type gave little, if any product as judged by TLC. However, in the second reaction two new components were visible by TLC and it was attempted to purify these for NMR analysis. One of the two components at $R_f = 0.3$ & 0.58 was thought to possibly be the product. The components at $R_f = 0.7$ & 0.74 (solvent system: $EtOH/H_2O/Et_3N$ 5:3:1) were thought to be UMP adducts and the spots at $R_f = 0.82$ and 0.44 co-spotted with UMP and Gal*f*-1-phosphate respectively. The

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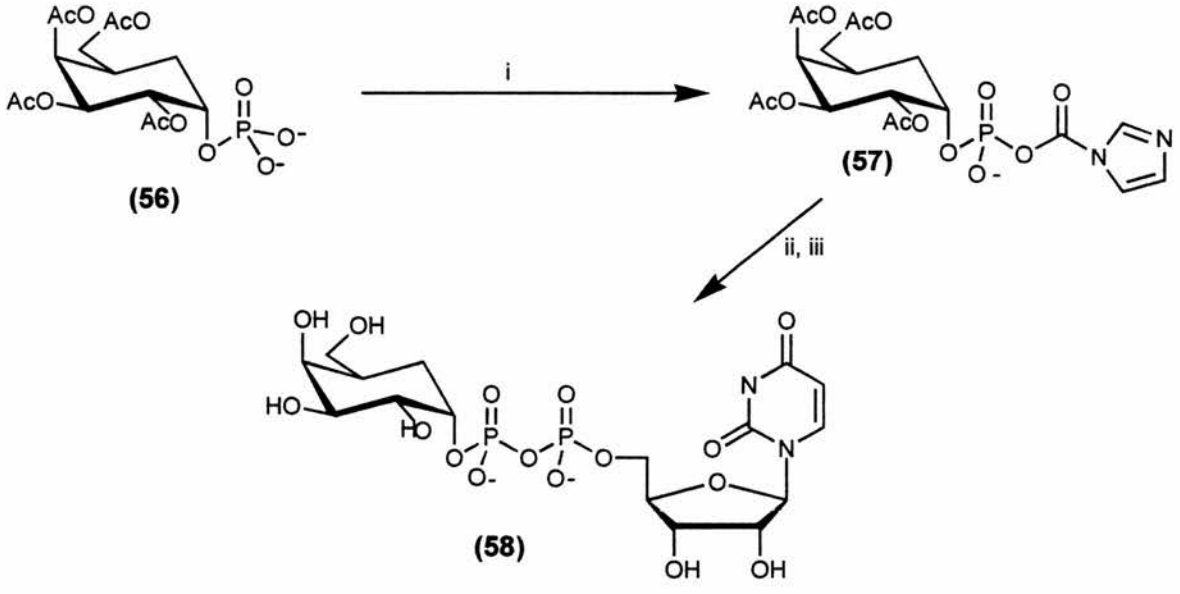
spot at $R_f = 0.13$ was thought to be unimportant as it was only UV active and was very, very feint. Purification through Dowex 50X8 (H^+) resin, using ammonium bicarbonate as the eluent was attempted for two reasons. Firstly because it had been used for the purification of a carbocyclic UDP-galactose analogue[34] (Scheme 15), and secondly because the ammonium bicarbonate could be neutralised easily with acidic resin, releasing NH_3 and CO_2, thereby avoiding lengthy desalting procedures. However, running the column resulted in the candidate spots being eluted in the same fractions as Gal*f*-1-phosphate. Incidentally, the compounds eluted at such a dilute concentration that observation by TLC was difficult, even with orcinol staining. Since the possible product spots were quite feint compared with the Gal*f*-1-phosphate starting materials and that the scale was so small, it was considered impractical to try further purification, and it was decided to attempt other coupling procedures.

Carbonyl diimidazole had been used in the aforementioned[34] carbocyclic UDP-Gal analogue **(58)** synthesis to activate the anomeric phosphate for attack by free UMP. This strategy was the opposite of the first coupling attempt where Gal*f*-1-phosphate was the nucleophile and UMP-morpholidate the electrophile.



Reagents: (i) 1,1' carbonyldiimidazole, acetone; (ii) UMP, DMF; (iii) MeOH/H_2O/Et_3N (3:1:7)

Scheme 15: Synthesis of the carbocyclic analogue of UDP-Gal

It was decided to apply this strategy to the synthesis of UDP-Gal*f* as it was thought that the reactivity of the phosphorus as an electrophilic centre could be similar. However, this turned out not to be the case. The conversion of tetra-*O*-benzoyl-α-D-

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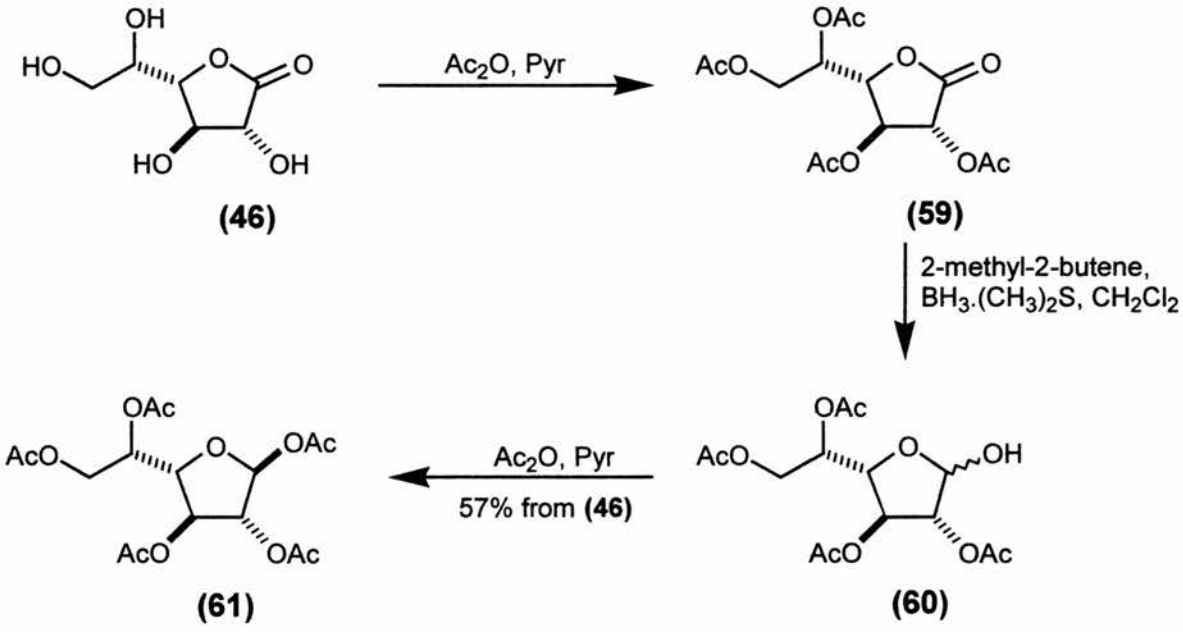
gal*f*-1-phosphate to the active imidazolate appeared, by TLC, to go very smoothly. In the reaction of the imidazolate with free UMP, only tetra-*O*-benzoylgal*f*-1-phosphate **(54)** and UMP were observed by TLC, therefore it was thought that the imidazolate had decomposed before any coupling reaction could take place. This reaction was attempted twice, however the same negative results were obtained both times.

To date, attempts to make UDP-Gal*f* have been extremely time consuming and fraught with a lack of success and the latter procedures have been difficult to deal with due to scale. The lack of a reliable method for the production of α-D-galactofuranosyl-1-phosphate also inhibits full investigation of different coupling procedures. Therefore, it was decided to go back and repeat the synthesis using acetyl protecting groups instead of benzoyl, due to their greater lability.

2.2.1.4 Synthesis of penta-O-acetyl-β-D-galactofuranose

The methods for the synthesis of pentabenzoate **(50)** were followed and a 57% yield of **(61)** from the unprotected galactono-γ-lactone was observed. The NMR data (both ^{1}H and ^{13}C) corresponded almost exactly with the literature data[24,25] for penta-O-acetyl-β-D-galactofuranose and both the melting point and the optical rotation confirmed the anomeric configuration.



Scheme 16: Synthesis of Penta-*O*-acetyl-β-D-galactofuranose

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This β-selectivity was in accordance with the results for the benzoylated compound. Such selectivity is highly unusual in the pyranose systems when electron withdrawing groups are put on the anomeric position since the α-isomer is the thermodynamically more stable due to the anomeric effect.

The bromination of acetate **(61)** was attempted using HBr in acetic acid, however, only the hemi-acetal was formed. Normally such an occurrence would be due to moisture in the system, therefore the reaction was repeated but strictly anhydrous conditions were observed, and a newer batch of HBr in acetic acid was used, the same product was obtained. Acetic anhydride was added to regenerate the anomeric acetate *in situ* to produce the bromide but although more of the acetate was observed by TLC, only very little bromide was obtained. Clearly this was not a satisfactory method for the bromination.

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2.2.2 Conclusions

Attempts to synthesise galactofuranosyl derivatives using high temperature acylation of galactose, although successful in creating the desired products, were discontinued due to the complexity of purification.

A more straightforward method of synthesising penta-*O*-benzoyl-β-D-galactofuranose was via the commercially available galactono-γ-lactone. Bromination and phosphorylation of this compound also proceeded smoothly, however deprotection was not so facile. Many attempts were made to remove a single persistent benzoyl group, however these were unsuccessful.

Crystallisation provided a small amount of the Gal*f*-α-D-1-phosphate and this was used in experiments attempt the synthesis of UDP-Gal*f*. Two methods were used in these attempts: firstly the standard sugar nucleotide coupling procedure utilising UMP-morpholidate and secondly a less common procedure utilising UMP and carbonyldiimidazole. Both of these attempts were unsuccessful. It appeared by TLC that new products had formed, however, purification by ion-exchange did not yield the desired products.

It was thought possible that the extremely labile nature of furanosyl pyrophosphates may cause unavoidable degradation during the purification process. It was also possible that the reaction conditions were not amenable to product formation.

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2.3 Experimental

TLC was performed on silica gel 60 GF_{254} (Merck) and compounds were detected with UV light or charring with dilute sulfuric acid as appropriate. Flash-column chromatography was performed on silica gel 60 (230-400 mesh, Merck) and anion exchange chromatography was performed on Dowex 50X8 (H^+) resin unless otherwise specified. NMR spectra were recorded on a Bruker 300 (300 MHz; FTNMR) spectrometer usually using deuteriochloroform as the solvent unless otherwise specified and Me_4Si as internal reference. Where necessary, assignments were made using ^1H-COSY and C-H correlation techniques and coupling constants are given in Hz. Melting points were determined on an electrothermal melting point apparatus and are uncorrected. The solvents used were either distilled or of analytical grade. Solvents were dried according to literature procedures.[35] Pyridine and dichloromethane were distilled over CaH_2; THF was distilled over sodium/benzophenone under nitrogen. All solid reagents were dried over P_2O_5 overnight *in vacuo* prior to use. Sodium hydride was used as a 60% dispersion in oil, which was washed with hexane immediately before use. Standard extraction procedures were carried out by diluting with dichloromethane and washing sequentially with equal volumes of saturated aqueous sodium hydrogen carbonate solution and water. For procedures using pyridine the solution in dichloromethane was first washed with 2M hydrochloric acid. Organic extracts were dried using either anhydrous sodium sulfate or anhydrous magnesium sulfate.

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Attempted synthesis of D-galactofuranose penta-O-acetate **(47)**

D-Galactose (20g, 0.11mol) was suspended in pyridine (300cm^3), stirred, and the temperature was raised to reflux. Acetic anhydride (82.5cm^3, 0.82mmol) was then carefully added and after 3 minutes, the galactose was observed to have dissolved, and the reaction was terminated by the addition of MeOH after 12 minutes. TLC (toluene/EtOAc 3:1) indicated complete consumption of starting material, and two components were present, R_f: 0.31 and 0.36. The reaction mixture was concentrated *in vacuo* and subjected to a standard aqueous work-up. The resultant sticky solid was dissolved in a minimum of warm ethanol, cooled to room temperature, seeded with penta-*O*-acetyl-β-D-galactopyranose, and cooled to 5°C for 24h. The solution was filtered and 10.9g of crystals were obtained, m.p. 110-115°C. A solution of these crystals ran parallel with penta-*O*-acetyl-β-D-galactopyranose (mp[2] 142-144°C) by TLC (toluene/EtOAc 3:1), however there was still significant amounts of the faster running material which could not be removed by recrystallisation. The impure mother liquor was subjected to flash column chromatography. The column was eluted with a gradient of EtOAc (3-15%) in toluene. Pure fractions of the fast-running component were combined and evaporated to dryness, (356mg). ^{13}C NMR data corresponded to literature values[36] for α and β penta-*O*-acetylated galactopyranoses. δ_H ($CDCl_3$), 2.07-1.9 (30H, overlapping s, *Ac*), 4.07-3.99 (4H, m, H-6,6'$_\alpha$, H-6,6'$_\beta$), 4.27-4.1 (2H, m, H-5$_\alpha$, H-5$_\beta$), 5.02 (1H, dd, $J_{2,3}$ 10.4, $J_{3,4}$ 3.4, H-3$_\beta$), 5.27-5.20 (3H, m, H-2$_{\alpha,\beta}$, H-3$_\alpha$), 5.33 (1H, d, *J* 3.2, H-4$_\beta$), 5.41 (1H, t, *J* 1.5, H-4$_\alpha$), 5.62 (1H, d, $J_{1,2}$ 8.2, H-1$_\beta$), 6.28 (1H, d, $J_{1,2}$ 4.4, H-1$_\alpha$); δ_C ($CDCl_3$) 20.8-20.4 (*Ac*), 61.0 (C-6$_\beta$), 61.1 (C-6$_\alpha$), 66.4 (C-4$_\alpha$) , 66.6 (C-4$_\beta$), 67.3 (C-3$_\alpha$), 67.3 (C-2$_\alpha$), 67.8 (C-2$_\beta$), 68.7 (C-5$_\alpha$), 70.2 (C-3$_\beta$), 71.6 (C-5$_\beta$), 89.6 (C-1$_\alpha$), 92.1 (C-1$_\beta$).

Attempted synthesis of D-galactofuranose penta-O-acetate **(47)**

Method as above but the reaction was left to continue for 45mins and samples were taken at 5min intervals and analysed by TLC (chloroform/methanol/water 3:3:1). Since no change occurred after 5min, this sample which contained two TLC components, was worked-up and analysed by ^{1}H-NMR. The peaks in the anomeric region corresponded to α and β penta-*O*-acetylated galactofuranoses[25] (gfα, gfβ) as well as α and β penta-*O*-acetylated galactopyranoses[2] (gpα, gpβ). δ_H ($CDCl_3$) 6.36 (1H, s, H-1$_{gf\alpha}$), 6.31 (1H, d, $J_{1,2}$ 4.7, H-1$_{gp\alpha}$), 6.17 (1H, s, H-1$_{gf\beta}$), 5.69 (1H, d, $J_{1,2}$ 7.7, H-1$_{gp\beta}$), 5.55-5.04 (~12H, m,

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H-2, 3, 2xH-4_{gp}, 2xH-5_{gf}), 4.36-4.02 (~12H, m, H-6,6', 2xH-4_{gf}, 2xH-5_{gp}), 2.34-1.80 (~60H, m, *Ac*)

2,3,5,6-Tetra-O-benzoylgalactono-1,4-lactone **(48)**

The title compound was prepared using the procedure as described by Varela and co-workers.[37] Galactono-1,4-lactone (5g, 28mmol) was dissolved in pyridine ($50cm^3$), benzoyl chloride ($14.4cm^3$ in $25cm^3$ CH_2Cl_2) was added slowly and the reaction was stirred for 3h. TLC (toluene/EtOAc 9:1) indicated one main component ($R_f = 0.55$). A standard aqueous work-up was undertaken and the organic extract was evaporated to dryness giving a yellow foam (>18g). The impure benzoate was used without further purification.

2,3,5,6-Tetra-O-benzoyl-α,β-D-galactofuranose **(49)**

Borane-dimethylsulfide complex (2.0M solution in diethyl ether, $49.6cm^3$, 99.2mmol) was dissolved in freshly distilled dichloromethane ($40cm^3$) under N_2. 2-Methyl-2-butene ($20cm^3$, 189mmol) in dichloromethane ($55cm^3$) was carefully added to the solution at 0°C over 15 minutes. The reaction mixture was allowed to warm to room temperature and was stirred for a total of 1.5 hours. 2,3,5,6-Tetra-*O*-benzoylgalactono-1,4-lactone (14.68g 24.7mmol) was dissolved in dichloromethane ($30cm^3$) and added dropwise to the reaction mixture at 0°C. The reaction mixture was then heated to 32°C for 18 hours. TLC (toluene/EtOAc 9:1) showed that the reaction had stopped before going to completion therefore water ($40cm^3$) was carefully added and the solution was stirred for 1.5 hours. The organic layer was separated and the aqueous layer was extracted with $3x50cm^3$ of dichloromethane whereupon the organics were combined and evaporated to a yellow gum. The gum was coevaporated with small volumes of water and ethanol and put under high vacuum overnight. Purification by flash chromatography (eluent - toluene/ethyl acetate 10:1) gave the title compound as a white solid (8.4g, 57%) which was used directly in the next step.

Penta-O-benzoyl-β-D-galactofuranose **(50)**

The title compound was prepared using the procedure described by Varela[37] and co-workers. 2,3,5,6-Tetra-*O*-benzoyl-D-galactofuranose (8.4g, 14.1mmol) was dissolved in pyridine ($50cm^3$) and cooled to 0°C on an ice-bath under nitrogen. Benzoyl chloride ($21.3cm^3$, 183mmol, 13eq) was slowly added to the solution via a syringe. The solution

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was allowed to warm to room temperature and stirred until completion whereupon it was diluted with dichloromethane and subjected to a standard aqueous work-up. The resultant yellow oil was purified by flash-chromatography using toluene/ethyl acetate 10:1 as the eluent and a clear oil was obtained which was treated with small volumes of ethanol and filtered whereupon a free flowing white powder was obtained (9.64g, 98%); m.p. 159-160°C (Lit.[37] 159-160°C); $[\alpha]_D$ -27 (*c* 0.1, $CHCl_3$) (Lit.[37] *c* 1.0, $CHCl_3$ -25), δ_H ($CDCl_3$), 4.80 (3H, m, H-6, H-6', H-4), 5.8 (2H, m, H-2, H-3), 6.15 (1H, dt, H-5), 6.8 (1H, dd, $J_{1,2}$ 0.9, H-1), 7.1-8.2 (25H, m, *Ar*); δ_C ($CDCl_3$), 63.6 (C-6), 70.3 (C-5), 77.4 (C-3), 81.0 (C-2), 84.3 (C-4), 99.8 (C-1), 128.3-133.7 (*Ar*), 164.5 (C=O), 164.7 (C=O), 165.0(C=O), 165.4 (C=O), 166.0 (C=O).

2,3,5,6-Tetra-O-benzoyl-β-D-galactofuranosyl bromide **(51)**

Acetic acid ($4cm^3$) was added to **(50)** (2g, 2.85mmol) under N_2 and the suspension was cooled to 0°C. HBr ($4cm^3$, 14.83mmol, 30% w/v in acetic acid) was added and the solution was slowly warmed to ambient temperature whereupon it was stirred for 2 hours. TLC (toluene/EtOAc 9:1) showed completion of reaction. The reaction mixture was poured into iced water ($200cm^3$), extracted into dichloromethane ($200cm^3$), washed with iced saturated sodium bicarbonate solution ($2x200cm^3$), and dried over Na_2SO_4. This work-up was completed in less than ten minutes. The solution was then filtered and evaporated to a light brown solid (1.23g, 65%) which was use directly in the preparation of compound **(53)**.

Dibenzyl 2,3,5,6-tetra-O-benzoyl-α-D-galactofuranosyl-1-phosphate **(53)**

Compound **(51)** (1.232g, 1.86mmol) was dissolved in dry toluene ($10cm^3$) under N_2. Dibenzyl phosphate (776mg, 1.5eq) was dissolved in Et_3N (392 µl, 1.5eq) and immediately transferred to this solution, which was stirred for 3 hours at room temperature. TLC (toluene/EtOAc 9:1) showed two components running at R_f = 0.23 and R_f = 0.13. The slower running component was the α-phosphate[19] and this was isolated by flash chromatography (gradient elution: EtOAc 0-10% in toluene) which gave **53** as a white foam (1.029g, 65%). NMR data corresponded with literature values.[19] m.p. 109-111°C (Lit.,[2] 112-113°C); $[\alpha]_D$ +48.7 (*c* 0.1, $CHCl_3$) (Lit.,[19] *c* 1.0, $CHCl_3$, +54.6); δ_H ($CDCl_3$) 5.02-4.56 (7H, m, H-4,6,6' & $C\underline{H}_2Ph$), 5.71 (1H, dd, $J_{2,3}$ 10.9, $J_{1,2}$ 5.6, H-2), 5.82 (1H, dd, $J_{5,6}$ 10.3, $J_{5,6'}$ 5.3, H-5), 6.16 (1H, t, *J* 7.5, H-3), 6.32 (1H, t, $J_{H\text{-}1,P}$ 5.4, H-1),

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8.13-7.03 (~30H, m, Ar); δ_C ($CDCl_3$) 62.7 (C-6), 69.2 ($\underline{C}H_2Ph$), 70.8 (C-5), 73.4 (C-3), 76.6 (C-2), 79.9 (C-4), 97.8 (C-1), 133.6-127.6 (Ar), 165.1 (C=O), 165.2 (2xC=O), 165.4 (C=O).

2,3,5,6-O-Benzoyl-α-D-galactofuranosyl-1-phosphate triethylammonium salt hydrate **(54)**

Compound **(53)** (50mg, 58.3μmol) was dissolved in EtOAc (1.5cm^3), to which was added Et_3N (60μl) and 10% palladium on charcoal (20mg). The solution was hydrogenated at atmospheric pressure and room temperature overnight whereupon it was filtered through Celite and the filtrate was evaporated to give a white foam (41mg, 51.6μmol, 88%). After crystallisation from ethanol/ether it gave m.p. 88-89°C (Lit.[19] 88-89°C); [α_D] +66 (*c* 1.0, $CHCl_3$) (Lit.,[19] *c* 1.0, $CHCl_3$, +68); δ_H ($CDCl_3$) 4.54 (1H, t, *J* 1.6, H-4), 4.74 (2H, m, H-6,6'), 5.62 (1H, dd, $J_{2,3}$ 10.8, $J_{1,2}$ 5.4, H-2), 5.83 (1H, dd, $J_{5,6}$ 10.1, $J_{5,6'}$ 5.4, H-5), 6.11 (1H, t, *J* 7.2, H-3), 6.13 (1H, dd, $J_{H\text{-}1,P}$ 7.1, $J_{1,2}$, H-1), 8.13-7.03 (20H, m, Ar); δ_C ($CDCl_3$) 8.4 ($CH_3\underline{C}H_2N$), 45.3 ($\underline{C}H_3CH_2N$), 63.1 (C-6), 71.6 (C-5), 74.4 (C-3), 76.8 (C-2), 79.0 (C-4), 96.0 (C-1), 128.1-133.5 (Ar), 165.1 (C=O), 165.3 (C=O), 165.6 (C=O), 165.9 (C=O).

Attempted synthesis of α-D-galactofuranosyl-1-phosphate triethylammonium salt hydrate **(41)**

The procedure described by De Lederkremer and co-workers[19] was followed for the synthesis of **(41).** Compound **(54)** (840mg, 2.24mmol) was dissolved in 5:2:1 MeOH-water-triethylamine (50cm^3) and stirred at 30°C for 25h. Four components were detected by TLC (ethanol/aqueous ammonia (28%)/water 5:3:1) at R_f= 0.43, 0.62, 0.70, 0.81. The component at R_f = 0.43 was the product (not UV active) but the reaction was incomplete. The temperature was increased to 55°C for 3 hours but no further reaction was observed, therefore the mixture was refluxed for three hours and TLC indicated two components R_f=0.43 and 0.62. The solvent was evaporated *in vacuo* and coevaporated several times with methanol. 5:2:1 MeOH-water-triethylamine (10cm^3) was added and the mixture was stirred for 25h at 30°C but TLC indicated no further reaction. The solution was again concentrated and the sticky brown solid was dissolved in methanol (4cm^3), cyclohexylamine (4cm^3) was added and the solution was taken to reflux for 4h. No change was observed by TLC. The solution was evaporated to dryness, coevaporated

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several times with methanol and partitioned between $CHCl_3$ and water ($20cm^3$ each). The aqueous layer was washed 3 times with $CHCl_3$ and evaporated to dryness. The product was crystallised from $MeOH/Et_2O$ in several fractions to give pure α-D-galactofuranosyl-1-phosphate bis(cyclohexylammonium salt) (88mg, 0.2mmol, 8.9%). The NMR data corresponded with the literature.[19] m.p. 139-140°C (Lit.,[19] 137-138°C); $[\alpha]_D$ +35 (*c* 0.1, H_2O) (Lit.,[19] *c* 1.0, H_2O, +41); δ_H (CD_3OD) 1.14-1.96 (22H, m, $2xC_6H_{11}$), 3.13 (2H, m, N<u>H</u> partially exchanged), 3.52-3.77 (4H, m, H-4, H-5, H-6, H-6'), 4.05 (1H, m, H-2), 4.22 (1H, t, H-3), 5.46 (1H, t, H-1); δ_C (CD_3OD) 50.4, 30.4, 24.4, 23.9 (cyclohex), 62.6 (C-6), 71.9 (C-5), 74.5 (C-3), 77.1 (C-2), 81.3 (C-4), 96.1 (C-1).

The mixture was also analysed by NMR and was found to contain **(41)** and **(55)** (6-*O*-benzoyl-α-D-galactofuranosyl-1-phosphate) in a 65:35 ratio. The subscripts after each proton designation refer to which of these two compounds is being referred to. δ_H (CD_3OD) 3.74 (3H, m, $H\text{-}5_{41}$, $H\text{-}6_{41}$, $H\text{-}6'_{41}$), 3.87 (1H, dd, $J_{4,5}$ 7.3, $J_{3,4}$ 3.0, $H\text{-}4_{41}$), 3.94 (1H, dd, $J_{4,5}$ 7.3, $J_{3,4}$ 3.5, $H\text{-}4_{55}$), 4.07 (3H, m, $H\text{-}5_{55}$, $H\text{-}2_{55}$, $H\text{-}2_{41}$), 4.34 (1H, t, $J_{2,3}$ 8.1, $H\text{-}3_{41}$), 4.42 (1H, t, $J_{2,3}$ 7.8, $H\text{-}3_{55}$), 4.49 (2H, m, $H\text{-}6_{55}$, $H\text{-}6'_{55}$), 5.62 (2H, m, $H\text{-}1_{55}$, $H\text{-}1_{41}$), 7.72-7.44 (6.4H, m, Ar), 8.14-8.02 (3.6H, m, Ar); δ_C 8.94, 58.1 (<u>Et_3</u>NH), 64.5 ($C\text{-}6_{41}$), 66.8 ($C\text{-}6_{55}$), 70.1 ($C\text{-}5_{55}$), 72.7 ($C\text{-}5_{41}$), 75.1 ($C\text{-}3_{55}$), 75.2 ($C\text{-}3_{41}$), 78.8 ($C\text{-}2_{55}$), 79.0 ($C\text{-}2_{41}$), 83.2 ($C\text{-}4_{55}$), 83.4 ($C\text{-}4_{41}$), 98.4 ($C\text{-}1_{55}$), 98.6 ($C\text{-}1_{41}$), 134.4-128.7 (Ar), 167.6 (C=O).

2,3,5,6-Tetra-O-acetyl-D-galactono-1,4-lactone **(59)**

Following the procedure described by Kartha *et al*,[27] galactono-1,4-lactone (10g, 56mmol) was stirred in acetic anhydride ($50cm^3$, 9.5eq), I_2 (500mg) was added and the reaction was stirred vigorously until dissolution had occurred (20min). The reaction mixture was poured into CH_2Cl_2 ($300cm^3$), washed with sodium thiosulfate, saturated sodium bicarbonate, dried over anhydrous sodium sulfate then filtered and the filtrate was concentrated *in vacuo* followed by coevaporation with toluene several times. A yellow oil was obtained and this was pumped overnight at high vacuum (19.27g, 55.6mmol, 99%): δ_H ($CDCl_3$) 1.95-1.84 (12H, *Ac*), 4.02 (1H, dd, $J_{6,6'}$ 9.5, $J_{5,6'}$ 5.2, H-6'), 4.41 (1H, dd, $J_{6,6'}$ 8.2, $J_{5,6}$ 5.2, H-6), 4.45 (1H, dd, $J_{4,5}$ 6.9, H-4), 5.10 (1H, m, H-5), 5.22 (1H, t, $J_{2,3}$ 7.0, H-3), 5.42 (1H, d, $J_{2,3}$ 7.1, H-2); δ_C ($CDCl_3$) 20.5-20.3 (*Ac*), 61.6 (C-6), 68.3 (C-5), 72.0 (C-2), 72.2 (C-3), 77.4 (C-4), 169.8 (C=O), 169.9, (C=O), 170.0 (2xC=O), 170.2 (C=O).). ^1H NMR and ^{13}C NMR data corresponded with the literature.[30]

2,3,5,6-Tetra-O-acetyl-α,β-D-galactofuranose **(60)**

Borane-dimethylsulfide complex (2.0M solution in diethyl ether, 100cm^3, 200mmol) was added to freshly distilled CH_2Cl_2 (50 cm^3) under N_2. 2-Methyl-2-butene (44.2cm^3, 417mmol) in CH_2Cl_2 (40cm^3) was carefully added to the solution at 0°C over 15 min. The reaction mixture was allowed to warm to room temperature and was stirred for a total of 1.5 h. Compound **(59)** (19g, 54.9mmol), was dissolved in CH_2Cl_2 (40cm^3) and added dropwise to the reaction mixture at 0°C. The reaction mixture was then heated to 32°C for 18 h and was then quenched with H_2O (50cm^3) and stirred for 2 h. The organic layer was separated and the aqueous layer was extracted with 3x50cm^3 of CH_2Cl_2 whereupon the organics were combined and evaporated to a mobile yellow liquid. This was coevaporated with toluene (3x100cm^3) and small volumes (~3x10cm^3) of diethyl ether were added and removed under high vacuum. The yellow foam obtained (18.38g) was used directly in the next step.

Penta-O-acetyl-β-D-galactofuranose **(61)**

Compound **(60)** was dissolved in pyridine (20cm^3) and cooled to 0°C whereupon acetic anhydride was added (12.5cm^3, 2.5eq). The reaction mixture was allowed to warm to room temperature and stirred overnight. TLC indicated completion of reaction. A standard aqueous work-up was carried out and a yellow oil was obtained. This was treated with small volumes of cold methanol whereupon white crystals formed. These were filtered whereupon the filtrate was concentrated and the method repeated. This was carried out several times and the crystals were washed in small volumes of cold methanol and dried over P_2O_5 (12.55g, 57% from **59**). ^{1}H NMR and ^{13}C NMR data corresponded with the literature.[27,36]: m.p. 93-95°C (Lit.,[37] 96-97°C), $[\alpha]_D$ +57.4 (*c* 0.1, $CHCl_3$) (Lit.,[37] *c* 1.0, $CHCl_3$, +61.2). δ_H ($CDCl_3$) 2.09-2.01 (15H, *Ac*), 4.17 (1H, dd, *J* 12.0, $J_{5,6'}$ 6.9, H-6'), 4.33-4.27 (2H, 2xdd, $J_{4,5}$ 3.9, H-4, $J_{6,6'}$ 12.4, $J_{5,6}$ 4.1, H-6), 5.04 (1H, dd, $J_{3,4}$ 5.4, $J_{2,3}$ 1.9, H-3), 5.14 (1H, dd, $J_{2,3}$ 1.9, $J_{1,2}$ 0.6, H-2), 5.32 (1H, dt, $J_{4,5}$ 6.8,$J_{5,6}$ 4.1, H-5), 6.14 (1H, s, H-1); δ_C 21.0-20.7 ($\underline{C}H_3CO$), 62.5 (C-6), 69.2 (C-5), 76.3 (C-3), 80.5 (C-2), 82.1 (C-4), 99.1 (C-1), 169.0 (C=O), 169.2 (C=O), 169.5 (C=O), 170.2 (C=O), 170.5 (C=O).

Attempted synthesis of 2,3,5,6-tetra-O-acetyl-β-Dgalactofuranosyl bromide

Compound **(61)** (100mg, 256mmol) was stirred in glacial acetic acid (200µl) and cooled to 0°C. HBr (30% w/w) in acetic acid (200µl) was then added under N_2 and the reaction

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was allowed to warm to room temperature. Stirring was continued for 3h. TLC (toluene/EtOAc 2:1) indicated two products: R_f = 0.19 & 0.35. Acetic anhydride (200µl) was added and stirring was continued for 2h. TLC indicated the presence of slightly more pentaacetate, and some bromide but still mostly the hemi-acetal, which was confirmed by ^{1}H-NMR.

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CHAPTER 3:Synthesis of Disaccharide Acceptor Analogue for DpA

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3.1 INTRODUCTION

3.1.1 The Linker Region of the Mycobacterial Cell Wall

Between the mycolylarabinogalactan and peptidoglycan in the cell wall structure of mycobacteria lies a disaccharide **(62)** which is the single linker between these two important regions.

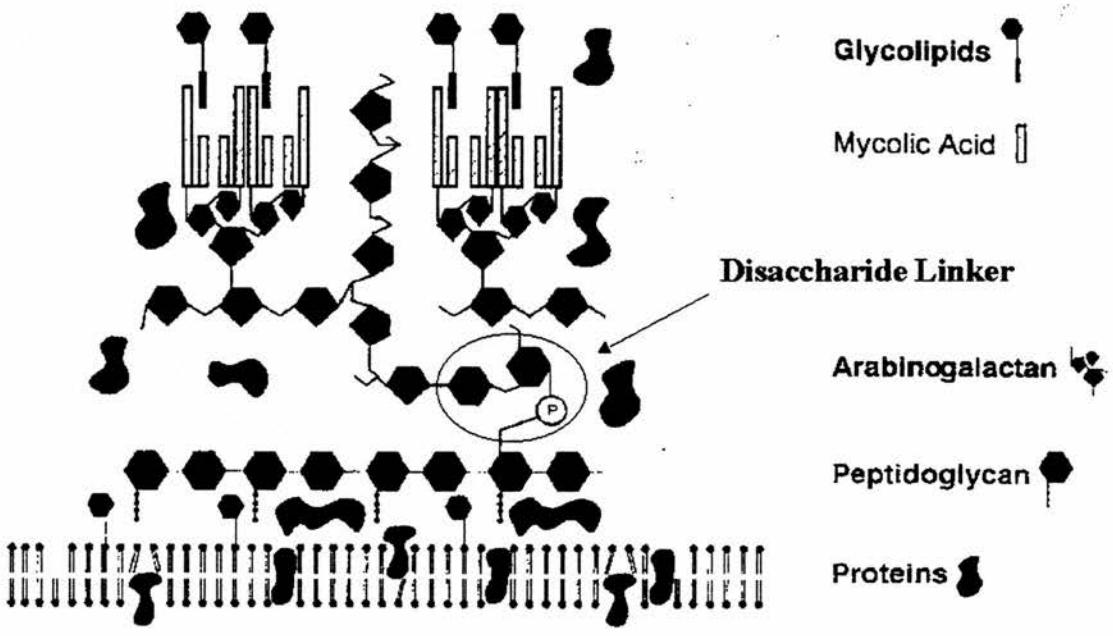

Figure 22: Linker Region Within the Mycobacterial Cell Wall Matrix[1]

As both the peptidoglycan and the arabinogalactan are large crosslinked three dimensional structures, an unbranched disaccharide linker moiety appears to be an attractive 'weak link' for biosynthetic inhibition. Without the linker it is very likely that cell viability would be much reduced.

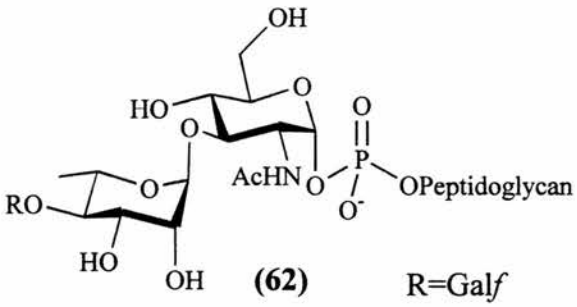

Figure 23: Disaccharide Linker in the Mycobacterial Cell Wall

The terminal galactofuranose of the linear galactan is connected (β1-4) to rhamnose which is then linked (α1-3) to GlcNAc which, via an α-phosphodiester, is connected to a

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muramic acid residue which is part of the peptidoglycan. In three dimensions the disaccharide linker is present at intervals between peptidoglycan and mycolylarabinogalactan and about 10-12% of the muramic acid residues in peptidoglycan are linked to it.[2]

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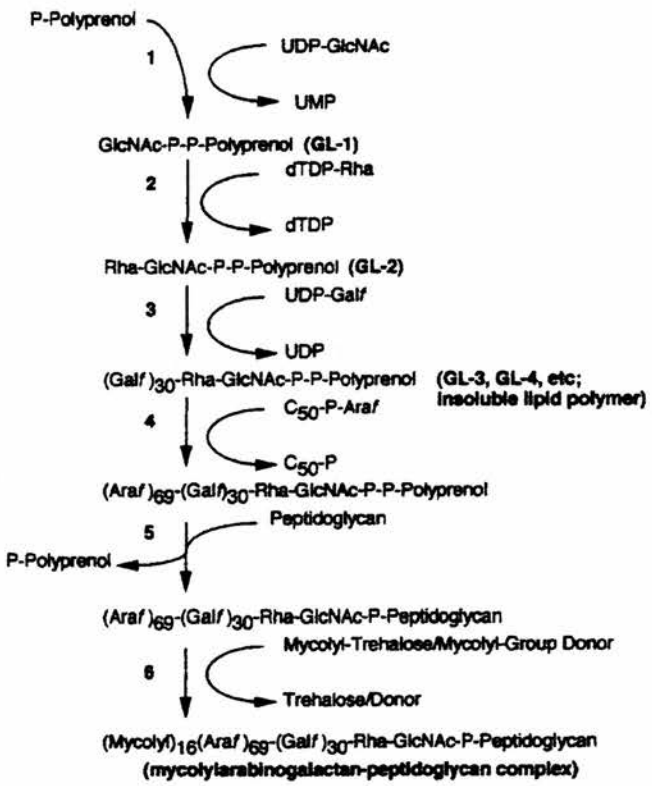
3.1.2 Biosynthesis of th e Linker Disaccharide

Experiments using the radioactively labelled donor UDP-[^{14}C]GlcNAc have shown that incorporation of this sugar unit into a lipid soluble form is the first step in mycobacterial cell wall biosynthesis.[3] It was also concluded from these same experiments that the lipid soluble compound was polyprenol-P-P-GlcNAc which then acts as the acceptor for rhamnose.



Scheme 17: Stages of Mycobacterial Cell Wall Biosynthesis

It is thought that as many as 30 galactofuranosyl residues are then transferred onto this polyprenyl-P-P-disaccharide **(63)** from UDP-Gal*f* using galactofuranosyltransferases.[4]

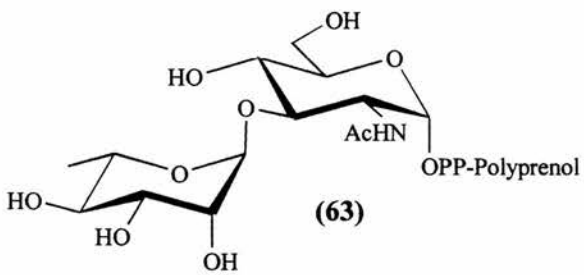


Figure 24: Biological Galactofuranose Acceptor

The arabinan is then thought to be biosynthesised on the lipid carrier resulting in a polyprenyl-P-P-oligosaccharide containing over 100 sugar units.[4] This arabinogalactan-

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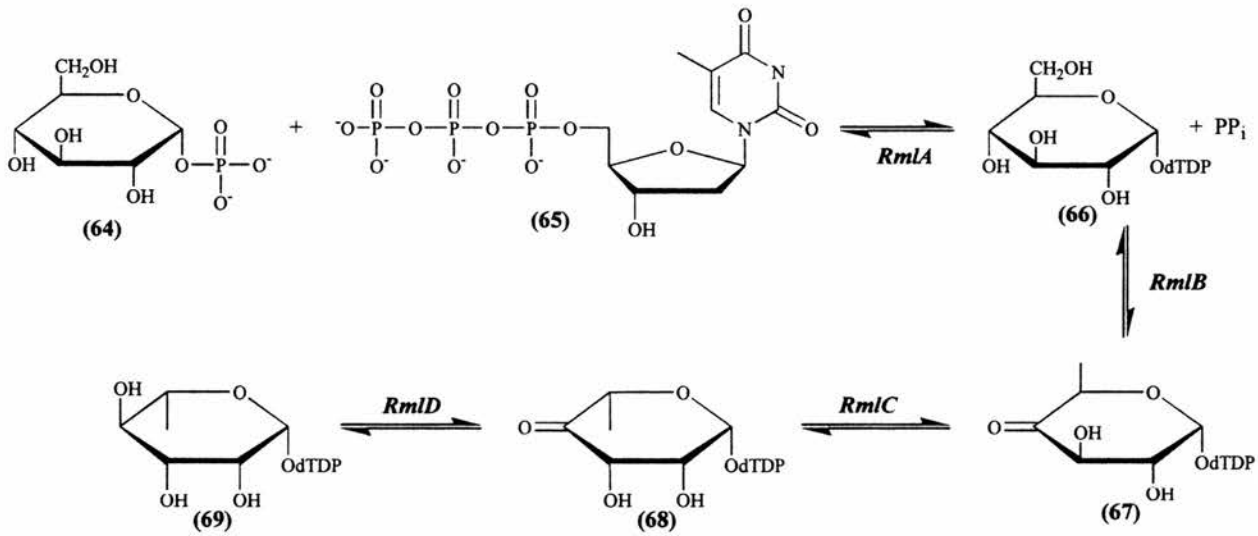
linker is then thought to be transferred from the lipid carrier onto a muramic acid residue of the peptidoglycan thus creating the arabinogalactan-peptidoglycan complex which is then mycolated.

The location of a reducing end on the GlcNAc (in an acid degraded fraction) rather than on the more acid labile Rha suggested a phosphodiester linkage between GlcNAc and peptidoglycan. This was confirmed by ^{31}P NMR of an AGP fragment which showed about 75% of phosphodiester phosphorus (δ~-1.7) and upon exposure to mild acid, this converted to phosphomonoester phosphorus (δ~4).[5]

3.1.2.1 Biosynthesis of the rhamnose donor

As rhamnose is not found in mammalian systems, inhibition of the biosynthesis of this sugar could compromise mycobacterial cell viability whilst causing minimal side effect in the host.

The rhamnose donor dTDP-Rha is biosynthesised in many gram negative bacteria[6] by the sequential action of four enzymes on Glc-1-P. Enzymes which have been shown[6] to convert Glc-1-P into the rhamnose donor dTDP-Rha, in other bacteria, were found to be present in *M. smegmatis*.[7]



Scheme 18: Biosynthesis of dTDP-rhamnose

Glucose-1-phosphate **(64)** is combined with 2'-deoxy-thymidine-5'-triphosphate **(65)** by the enzyme RmlA to produce dTDP-Glc **(66)** with the loss of inorganic pyrophosphate. RmlB then oxidises the 4-position of dTDP-Glc as well as reducing the primary alcohol to a methyl group resulting in the formation of dTDP-D-*xylo*hex-4-ulose **(67)**. Inversion

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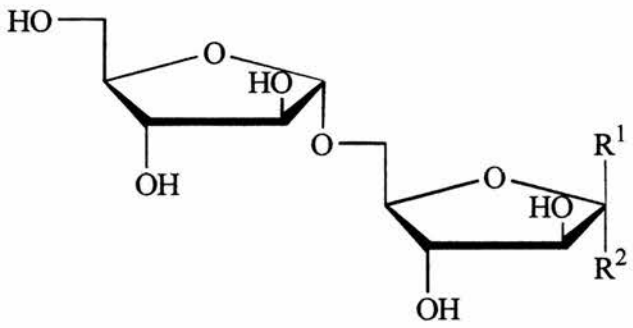
of the 5-methyl group is performed by RmlC changing the compound from a D- to an L-form and thus from α to β. This bifunctional enzyme also inverts the 3-OH, forming dTDP-L-*lyxo*hex-4-ulose **(67)**. The final enzyme, RmlD reduces the 3-carbonyl of **(68)** to produce β-L-dTDPrhamnose **(69)**.

In other bacteria, the genes responsible for the production of RmlA-D are clustered in the same area of the genome, however, sequencing of the DNA surrounding RmlA in *M. tuberculosis* found no genes with homology to other Rml biosynthetic enzymes.[7]

3.1.3 Aims and Objectives

Since the galactofuranosyltransferase has neither been purified or characterised and since the synthesis of the donor UDP-Gal*f* was unsuccessful, a change in focus towards the acceptor as a means of manipulating the transferase was decided upon.

Transferases involved in the synthesis of arabinan in mycobacteria have been subject to a similar strategy using acceptor analogues.[8-12] In Lee's work[8], a number of synthetic arabinoside disaccharide acceptors with different anomeric configurations at the reducing end were tested for activity (Figure 25). The biological results reflected a high degree of tolerance for the nature and orientation of the aglycone. The difference between β **(71)** and α **(72)** was only a factor of two as was the difference between O-alkyl **(71)** and S-alkyl **(73)**. Shortening the alkyl chain to a methyl with mixed anomeric configuration **(75)** merely resulted in a fourfold loss in activity



Compounds in decreasing order of acceptor activity (italics show relative activity):

(70) R^1= H, R^2= OHex *(1)*
(71) R^1= OOct, R^2= H *(0.5)*
(72) R^1= H, R^2= OOct *(1)*
(73) R^1= H, R^2= SOct *(0.5*
(74) R^1= H, R^2= O-dec-1-enyl *(0.75)*
(75) R^1 or R^2 = OMe *(0.25)*

Figure 25: Disaccharide Acceptor Analogues for Arabinan Biosynthesis[8]

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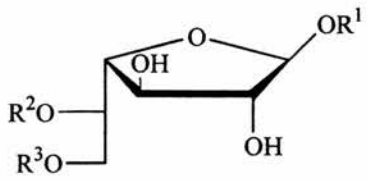
Therefore, it seemed reasonable to propose a similar strategy for the design of analogues based on the linker disaccharide.

Galactofuranose disaccharides (Figure 26) have been synthesised[13,14] by the Besra group and have proven to be functional as acceptor analogues for galactofuranosyl transferases. Therefore, it was decided to synthesise a compound which would both model the linker disaccharide and be more straightforward to synthesise than galactofuranosyl disaccharides.



(76) R^1= dec-1-enyl, R^2= β-Gal*f*1-, R^3= H
(77) R^1= dec-1-enyl, R^2= H, R^3= β-Gal*f*1-
(78) R^1= allyl, R^2= β-Gal*f*1-, R^3= H

Figure 26: Galactofuranose Disaccharide Acceptor Analogues

The two main problems with the biological acceptor **(63)** were a labile anomeric pyrophosphate bridge which would render any analogue difficult to work with and a C_{35} octahydroheptaprenol which would make NMR characterisation difficult as well as creating detergent problems in working with any analogue.

Since the reducing end of the arabinose acceptor analogues seemed to be very tolerant to substitution, it was decided to eliminate both the pyrophosphate bridge and the polyprenol constituent and use the simple octyl glycosides **(79)** and **(80)** as analogues. These compounds would possess the exact disaccharide configuration of the biological acceptor whilst being much simpler to synthesise and easier to work with.

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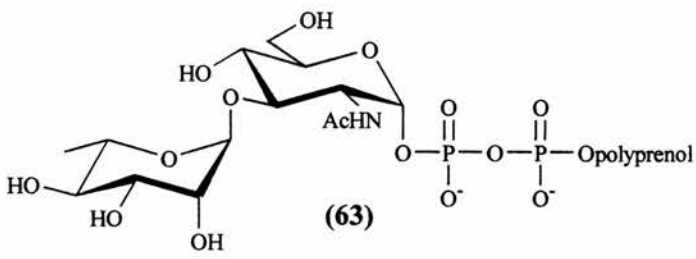
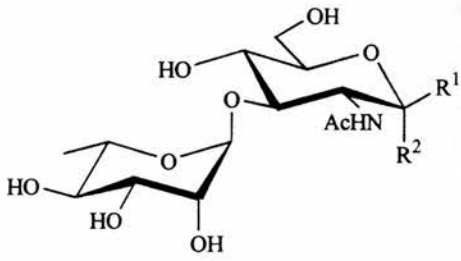


(63)



(79) R^1 = O-Oct, R^2 = H

(80) R^1 = H, R^2 = O-Oct

Figure 27: Biological Acceptor and Target Compounds

As this work was part of a collaboration with the G.S. Besra group at Colorado State University, it was decided that the α-configured compound **(80)** would be synthesised in his laboratory and that the β-configured compound **(79)** would be synthesised by the author.

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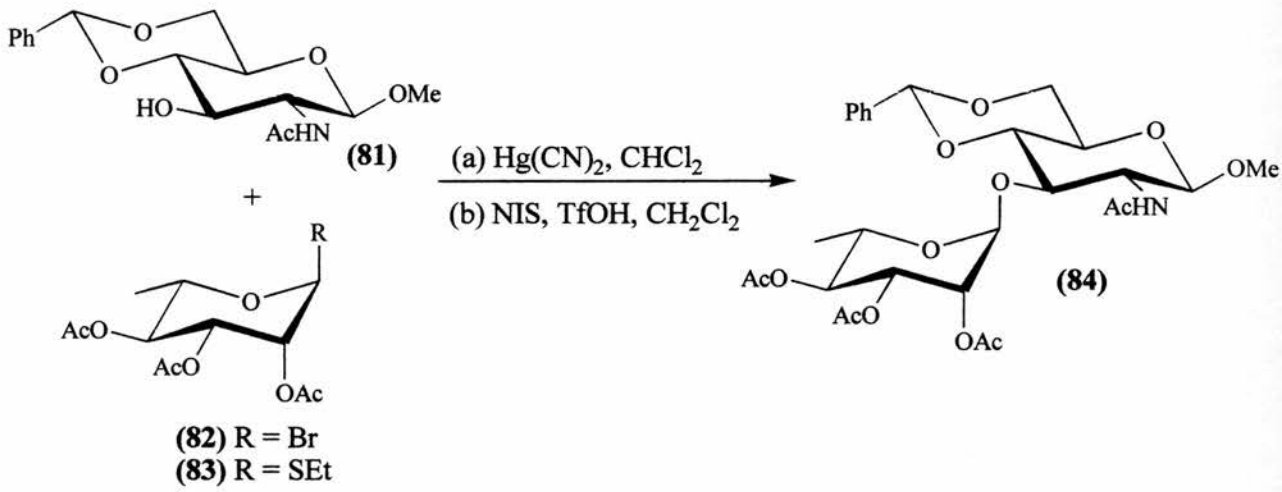
3.2 RESULTS AND DISCUSSION

3.2.1 Synthesis of the β-Octyl Disaccharide Acceptor Analogue

The disaccharide Rha(α1-3)GlcNAc has been synthesised previously[15,16] as shown in Scheme 19.



Scheme 19: Previous Synthesis of a Protected Rha(α1-3)GlcNAc Derivative

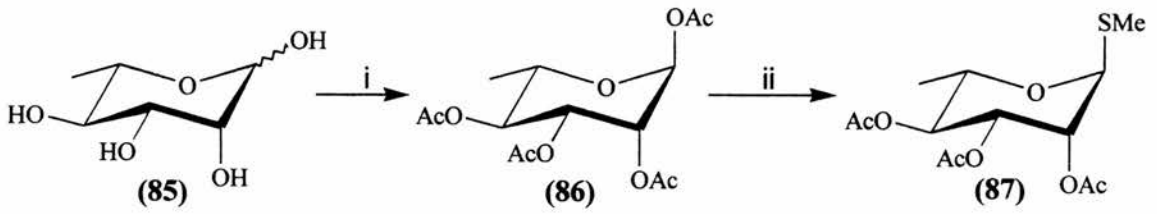
It was decided that a synthesis of the disaccharide fragment with a suitable substitute for peptidoglycan at the reducing end would give a useful compound. Octyl chains have been commonly used as aglycons in acceptor analogue syntheses and since the synthesis of octyl GlcNAc had already been carried out in the Field group,[17] it was decided to use this compound.

In a previous synthesis of a similar disaccharide from the *Shigella flexneri* Y polysaccharide antigen, the acceptor **(81)** was commercially available and the donor could be made in two steps. Both a bromo **(82)** and thioethyl **(83)** donor were used in the published synthesis; the latter was used as the method of choice due to an easier work-up.

3.2.1.1 Synthesis of the Rhamnose Donor

In our hands, a straightforward acetylation of rhamnose produced exclusively the α-anomer **(86)** in a near quantitative yield.The thiomethyl rhamnoside **(87)** was synthesised using thiomethyltrimethylsilane as the source of the methylthio group and ZnI_2 as the promoter.

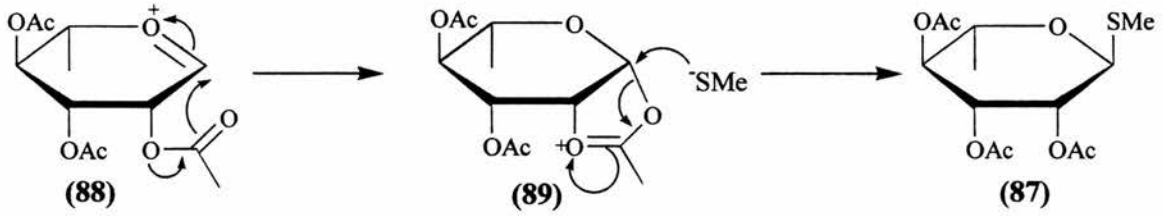
References are on page 90



Reagents: (i) Ac_2O, pyr; (ii) TMS-SMe, ZnI_2, DCE

Scheme 20: Synthesis of the Rhamnose Donor

Again, the α-anomer was produced exclusively and this was due to an anchimeric effect illustrated in Scheme 21. The acetate on position 2 of **(88)** reacts with the oxocarbonium centre at position 1 creating a 5-membered ring as shown in **(89)**.



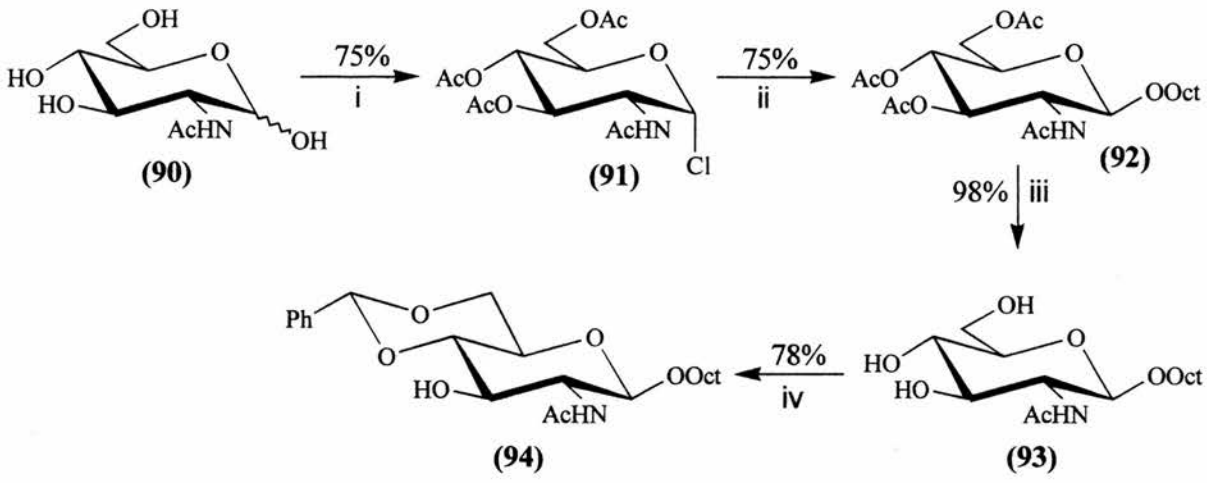
Scheme 21: Mechanism of the Thiomethyl Rhamnoside Synthesis

Formation of this 5-membered ring assures nucleophilic attack from the top face of the molecule thus directing formation of only the α-anomer **(87)**.

3.2.1.2 Acceptor Synthesis

The acceptor **(94)** had been synthesised[17] using a five step procedure which began with the known[18] conversion of *N*-acetyl glucosamine **(90)** to 2-acetemido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride **(91)**. Octanol was then coupled with the anomeric chloride **(91)** using a standard Koenigs-Knorr procedure[19] with mercuric cyanide and mercuric chloride as the promoters. A similar anchimeric effect to that detailed in Scheme 21 led to complete β-selectivity in the formation of the octyl glycoside **(92)**.

References are on page 90



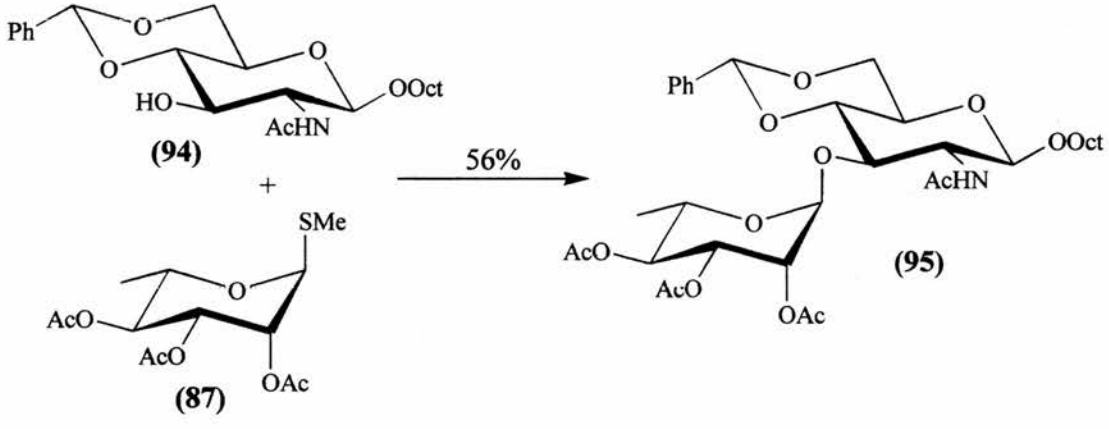
Reagents: (i) AcCl, $HCl_{(g)}$; (ii) $CH_3(CH_2)_7OH$, $HgCN_2$, $HgBr_2$, toluene; (iii) NaOMe, MeOH; (iv) $PhCH(OMe)_2$, CH_3CN, *p*-TsOH

Scheme 22: Synthesis of Acceptor

Deacetylation of **(92)** was carried out using sodium methoxide in methanol. Selective protection of **(93)** was achieved using α,α-dimethoxytoluene which reacts exclusively with the 4- and 6- hydroxyls to form the 4,6-*O*-benzylidene acetal **(94)**. The 3-OH was then free for glycosylation with rhamnose donor **(87)**.

3.2.1.3 Glycosylation and De protection

Compounds **(94)** and **(87)** were coupled using NIS and triflic acid as activators in identical conditions to those employed by Auzanneau *et al*[15]. This reaction proceeded with a yield of 56% and produced only the α-anomer for reasons outlined in Scheme 21.



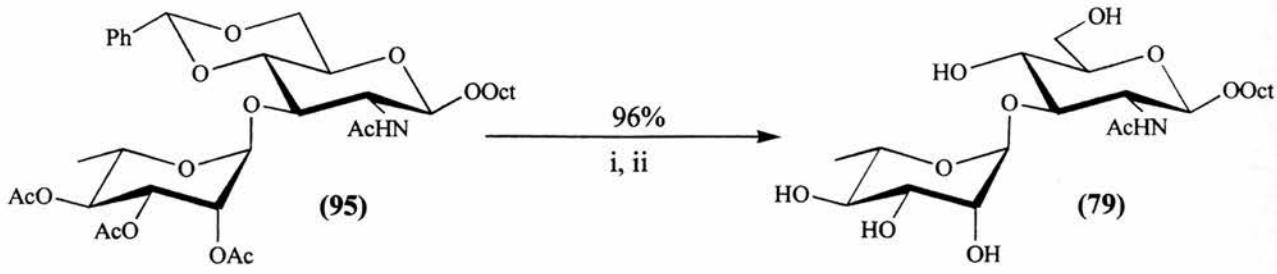
Reagents: NIS, TfOH, CH_2Cl_2

Scheme 23: Glycosylation Reaction to Produce Protected Disaccharide

Deprotection of compound **(95)** was achieved by firstly removing the 4,6-*O*-benzylidine acetal using hydrogenolysis over 10% palladium on carbon and then

References are on page 90

deacetylation was carried out using ammonia in methanol. This produced the fully deprotected disaccharide **(80)** in a yield of 96%.

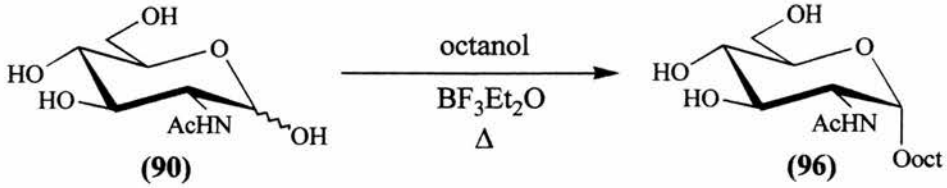


Reagents: (i) 10% Pd on C, 80% HOAc; (ii) 2M NH_3 in MeOH

Scheme 24: Deprotection of Protected Disaccharide

3.2.2 Synthesis of the α-Octyl-Acceptor Analogue

The first step of the GlcNAc acceptor procedure was the only difference between the synthesis of the α-octyl disaccharide analogue and the synthesis of the β-octyl analogue. In order to overcome the anchimeric effect imposed by the *N*-acetyl group, higher glycosylation temperatures were used. This produced the thermodynamically stable α-glycoside **(96)**.



Scheme 25: Synthesis of the α-Octyl Glycoside

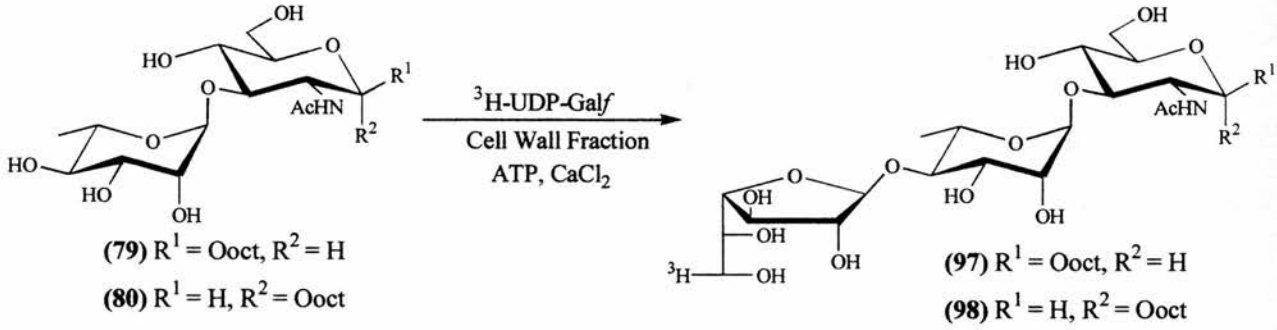
After glycosylation, the synthetic methods used for compound **(79)** were followed for the synthesis of the α-disaccharide **(80)**. This particular piece of work was performed by Christopher Flaherty in the laboratories of G. S. Besra.

References are on page 90

3.2.3 Biological Testing

The testing of these compounds was carried out by Caroline Morehouse in the laboratories of G.S. Besra and *M. smegmatis* was used as the source of cell walls.

The first set of reactions examined acceptors **(79)** and **(80)**, using three different cell wall fractions: membranes[3], p60[3] and an equal mixture of both. The negative controls contained no acceptor and the positive control contained the Gal*f* disaccharide **(76)**. Amphomycin has been shown[14] to inhibit uptake of radioactive donor by endogenous substrates and was therefore used in 50% of the assays. The microcentrifuge tubes (0.6mL capacity) used contained ATP (1mM, 10µl) and $CaCl_2$ (0.1M, 32µl) as well as the contents shown in Table 1 on page 88.



Scheme 26: Hypothetical Incorporation of Radioactive Gal*f* onto Acceptors

As can be seen in Figure 28, the level of radioactive Gal*f* incorporated into both disaccharides **(79)** and **(80)** was comparable to the negative controls. It was clear from these assays that the disaccharides had no biological activity at 2mM and they are clearly distinguished from the Gal*f* disaccharide which had a much higher level of radioactive incorporation.

References are on page 90

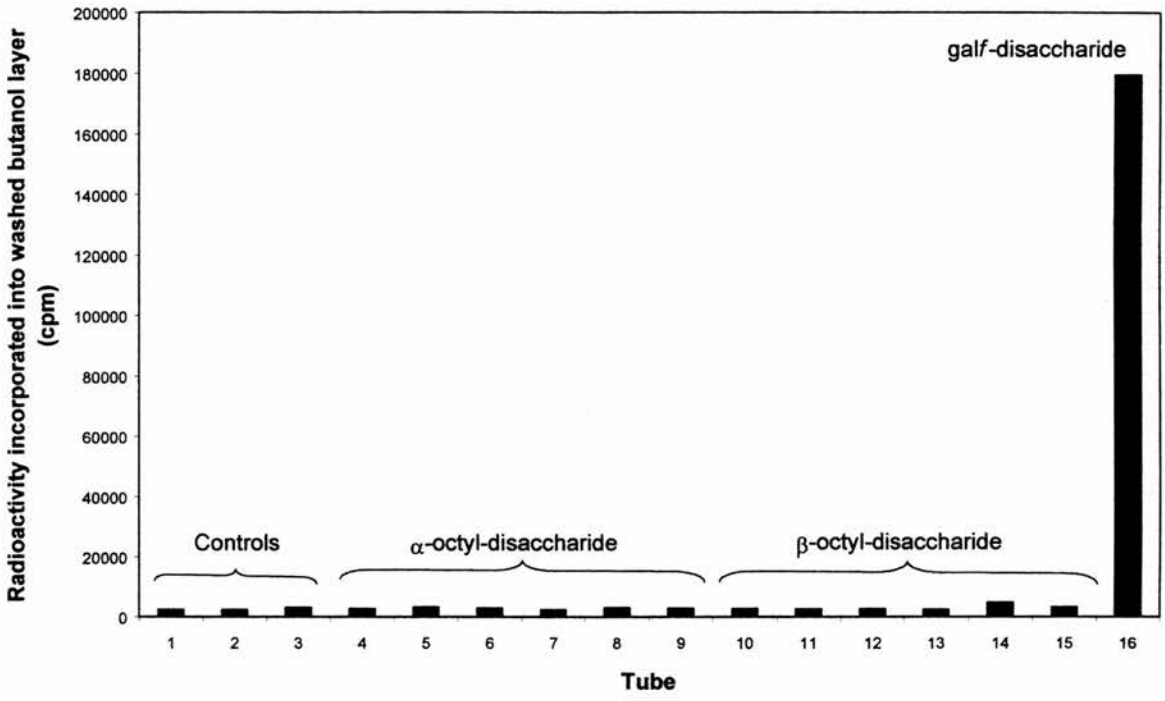


Figure 28: Graph showing incorporation of ^{3}H-Gal*f* onto acceptors (76), (79) and (80) from incubation of acceptors with ^{3}H-UDP-Gal*p* and *M. smegmatis* cell wall fractions

It was therefore decided to test the acceptors at a variety of concentrations (0.1-10.0mM) to ascertain whether this factor was important. The conditions for these reactions are shown in Table 2 on page 89 and the results are illustrated graphically in Figure 29. These results again show the synthetic acceptor analogues **(79)** and **(80)** to be incorporating only similar amounts of radioactivity to the control irrespective of the concentration.

References are on page 90

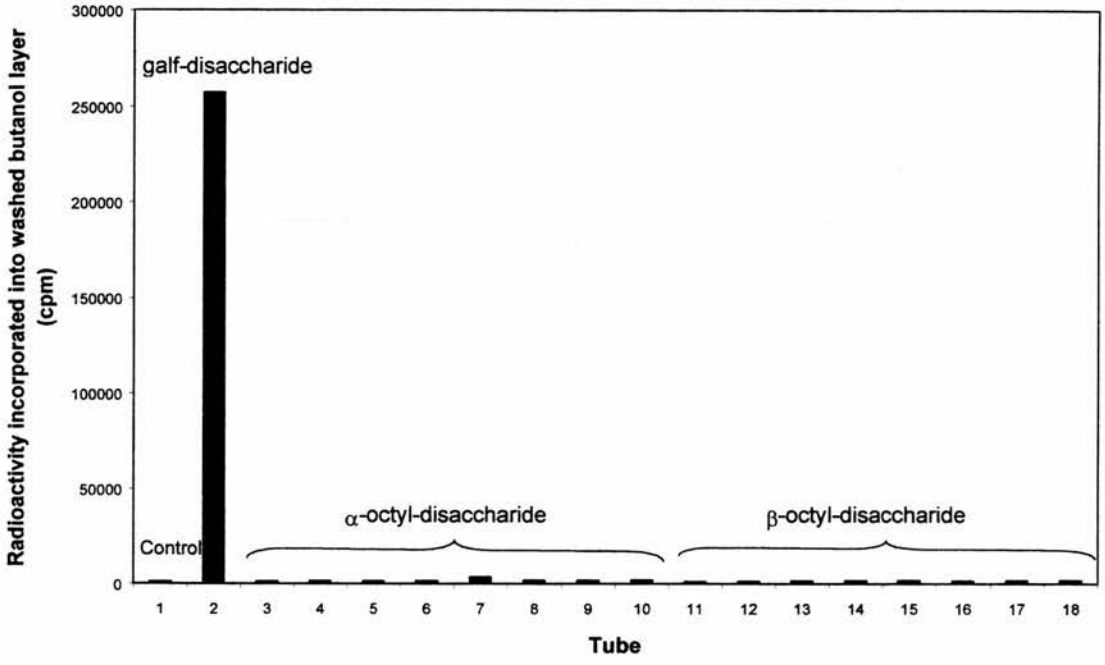


Figure 29: Graph showing relationship between concentration of acceptors (76), (79) and (80) and incorporation of ^{3}H-Galf

References are on page 90

3.2.4 Conclusions

In the planning of this project it was thought that substitution of polyprenyl groups with an octyl group would not affect acceptor activity and that the phosphodiester group of **(63)** would not be important in the recognition of the acceptor. These suppositions were based on the fact that in the case of the galactofuranoside acceptors **(76-78)**, both the allyl and dec-9-enyl glycoside worked very well as acceptor analogues. Since the galactan chain is biosynthesised on the polyprenyl carrier, presumably there is a point where recognition of the rhamnose, GlcNAc and phosphodiester becomes less important. It is unlikely that the complete growing chain enters and interacts with the transferase enzyme active site. That said, it is not unreasonable to propose that a different Gal*f* transferase is involved in the transfer of Gal*f* onto rhamnose. Perhaps in this enzyme, the phosphodiester is crucial for binding in the active site.

It has also recently been discovered[13] that Gal*f*(β1-4)Rha-α1-Soctyl **(99)** is a biologically active acceptor analogue for Gal*f* transferase. In this compound not only is the phosphodiester absent but GlcNAc, a complete sugar unit, is also missing, further bolstering the hypothesis that an exclusive and very particular galactofuranosyltransferase is involved in the transfer of the first Gal*f* unit.

Possible future directions in this project would be to make the phosphodiester linked octyl disaccharide. Should this prove to be biologically active, then replacement of the anomeric phosphodiester with a phosphonate would create a stable and hopefully biologically active acceptor analogue.

References are on page 90

3.3 EXPERIMENTAL

3.3.1 Synthesis

Methyl 2,3,4-tri-O-acetyl-α-L-thiorhamnoside **(87)**

L-Rhamnose (5g, 27.5mmol) was dissolved in pyridine (40ml) and cooled to 0°C whereupon acetic anhydride (25.9cm^3, 275mmol) and a few crystals of DMAP were added. The reaction was allowed to warm to room temperature and was stirred overnight. Upon completion, a standard aqueous work-up was performed on the solution resulting in a light yellow foam which was the α-tetraacetate[20]; δ_H 0.91 (3H, d, $J_{5,6}$ 6.2, H-6), 1.68 (3H, s, *Ac*), 1.76 (3H, s, *Ac*), 1.86 (6H, s, 2 x *Ac*), 3.65 (1H, m, $J_{4,5}$, 10.2, H-5), 4.77 (1H, t, $J_{4,5}$ 10.2, H-4), 4.92 (1H, dd, $J_{2,3}$ 3.3, $J_{1,2}$ 1.8, H-2), 4.97 (1H, $J_{3,4}$ 10.0, $J_{2,3}$ 3.4, H-3), 5.69 (1H, d, $J_{1,2}$ 1.5, H-1), δ_C 17.1 (C-6), 20.2 ($\underline{C}H_3CO$), 20.3 ($\underline{C}H_3CO$ x 2), 20.4 ($\underline{C}H_3CO$), 68.3 (C-3, C-5), 68.5 (C-2), 70.1 (C-4), 90.3 (C-1, $J_{C\text{-}H}$ 176.7). The yellow foam, (2.94g, 8.86mmol) was dissolved in freshly distilled DCE (180ml), ZnI_2 (20.4g, 63.8mmol) was added and the mixture was stirred. TMS-SMe was added dropwise and the temperature was then increased to 50°C and the reaction mixture was stirred for 45 min. The mixture was filtered and the filtrate was diluted with dichloromethane and washed with 10% Na_2CO_3, sat. NaCl, dried over Na_2SO_4, filtered and evaporated to a mobile yellow liquid. Flash column chromatography (EtOAc in toluene 0-17%) resulted in a clear, light yellow oil (2.72g, 96%). (Calcd. for $C_{13}H_{20}O_7S$: C, 48.74; H, 6.29. Found: C, 48.82; H, 6.53); $[\alpha]_D$ -116 (c 1.0, $CHCl_3$) (Lit.,[15] –117, c 1.0, $CHCl_3$); δ_H 1.16 (3H, d, $J_{5,6}$ 6.3, H-6), 1.90 (3H, s, *Ac*), 1.97 (3H, s, *Ac*), 2.07 (3H, s, *Ac*), 2.08 (3H, s, $SC\underline{H}_3$), 4.12 (1H, m, H-5), 5.00 (2H, m, H-4, H-1), 5.15 (1H, dd, $J_{3,4}$ 10.0, $J_{2,3}$ 3.4, H-3), 5.26 (1H, dd, $J_{1,2}$ 1.5, H-2), δ_C 13.4 ($S\underline{Me}$), 17.2 (C-6), 20.3 (*Ac*), 20.5 (*Ac*), 20.6 (*Ac*), 66.7 (C-5), 69.2 (C-3), 70.9 (C-4, C-2), 83.0 (C-1, undec., d, $J_{C\text{-}H}$ 166), 169.4 (C=O, *Ac*), 169.5 (2 x C=O, *Ac*).

References are on page 90

Octyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-(2,3,4-tri-O-acetyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside **(95)**

Compound **(87)** (1.05g, 3.29mmol) and octyl 4,6-O-benzylidene-2-deoxy-2-acetamido-β-D-glucopyranoside (1.20g, 2.74mmol) were dissolved in CH_2Cl_2 ($160cm^3$), 4Å molecular sieves (4.8g) were added and the suspension was stirred overnight at room temperature under N_2. N-Iodosuccinimide (801mg, 3.56mmol) was added, followed by a saturated solution of triflic acid in CH_2Cl_2 ($2.2cm^3$, 0.12mol equiv) which was added dropwise and the reaction was stirred in the dark. After 30 minutes, another 0.12mol equiv of the triflic acid solution was added dropwise and stirring was continued. The colour of the reaction changed from light pink to a darker pink followed by purple and finally a deep brown. After 4h, the reaction was quenched with triethylamine (430μl), filtered, and diluted with CH_2Cl_2, and the solution was washed sequentially with 5% sodium thiosulfate in 0.5M NaOH, 1M HCl, sat. $NaHCO_3$, sat. NaCl, distilled H_2O and was dried over sodium sulfate followed by evaporation to a brown foam. The foam was loaded onto a dry-packed silica gel column using the minimum of CH_2Cl_2 and one column volume of CH_2Cl_2 was eluted followed by a gradient elution of ethyl acetate (10-33%) in toluene. The relevant fractions were combined and evaporated *in vacuo* to give a white powder which, upon crystallisation from hot aqueous ethanol, yielded the title compound as soft, white needles (1.09g, 56%), (Calcd for $C_{35}H_{51}NO_{13}$: C, 60.59; H, 7.41; N, 2.02; Found: C, 60.61; H, 7.69; N, 2.04); $[\alpha]_D$ -62 (c 1.0, $CHCl_3$); m.p. 167-168°C, δ_H 0.59 (3H, d, $J_{5,6}$ 6.2, H-6'), 0.85 (3H, t, J 6.9, Oct C$\underline{H}_3$), 1.24 (10H, bs, Oct C$\underline{H}_2$x5), 1.51 (2H, m, Oct β-C$\underline{H}_2$), 1.97-1.94 (9H, 3s, 3 x Ac), 2.07 (3H, s, NH$\underline{Ac}$), 3.09 (1H, dd, $J_{2,3}$ 9.3, $J_{1,2}$, H-2), 3.55-3.43 (3H, m, H-5, H-4, Oct α-C$\underline{H}_2$ Ha), 3.82-3.74 (2H, m, H-6a, Oct α-C$\underline{H}_2$ Hb), 4.00 (1H,m, H-5'), 4.30 (1H, dd, $J_{6a,6b}$ 11.0, $J_{5,6b}$ 4.9, H-6b), 4.53 (1H, t,$J_{2,3}$ H-3), 4.79 (1H, d, $J_{1',2'}$ 1.4, H-1'), 4.91 (1H, dd, $J_{3',4'}$ 10.0, H-4'), 5.05 (1H, d, $J_{1,2}$ 8.2, H-1), 5.08 (1H, dd, $J_{2',3'}$ 3.3, $J_{1',2'}$ H-2'), 5.25 (1H, dd, $J_{2',3'}$, $J_{3',4'}$, H-3'), 5.49 (1H, s, C$\underline{H}$Ph), 5.93 (1H, d, $J_{2,NH}$ 7.4, N$\underline{H}$), 7.3 (3H, m, Ph), 7.45 (2H, m, Ph), δ_C 14.1 (Oct-$\underline{C}H_3$), 16.5 (C-6'), 20.7 (Ac), 20.8 (Ac), 20.9 (NH-$\underline{Ac}$), 22.6 (Oct-$\underline{C}H_2$), 23.4 (Ac), 25.9 (Oct-$\underline{C}H_2$), 29.3 (Oct-$\underline{C}H_2$), 29.5 (Oct β-CH_2), 31.8 (Oct-CH_2), 59.5 (C-2), 66.1 (C-5), 66.3 (C-5'), 68.8 (C-3', C-6), 70.3 (C-2'), 70.4 (Oct α-$\underline{C}H_2$), 71.1 (C-4'), 75.1 (C-3), 80.4 (C-4), 97.5 (C-1'), 99.6 (C-1), 102.0 ($\underline{C}$HPh), 126.5 (2C, Ar), 128.1

 References are on page 90

(2C,Ar), 129.1 (1C, Ar), 137.2 (quaternary Ar), 169.7 (C=O, Ac), 170.2 (C=O, Ac), 170.4 (C=O, Ac), 171.3 (C=O, NHAc).

Octyl 2-acetamido-2-deoxy-3-(α-L-rhamnopyranosyl)-β-D-glucopyranoside **(79)**

Hydrogenolysis of **(95)** (500mg, 705μmol) was performed in 80% HOAc (8ml) using Pd on carbon (10%, 100mg) at room temperature. The reaction was stirred overnight and TLC indicated completion of the reaction therefore the solution was filtered through Celite and the filtrate was co-evaporated with toluene until no more HOAc could be detected by smell. The intermediate amino disaccharide was dissolved in NH_3 (2M in MeOH, $12cm^3$) and stirred at room temperature overnight whereupon completion of the reaction was observed by TLC. The solution was evaporated to a free flowing white powder (300mg, 96%), $[\alpha]_D$ -74 (c 1.0, $CHCl_3$); m.p. 157-159°C, δ_H 0.86 (3H, m, Oct CH_3), 1.27 (13-14H, 5x Oct CH_2, H-6'a, H-6'b), 1.53 (2H, bs, Oct β-CH_2), 2.03 (3H, s, NH*Ac*), 3.62-3.37 (5H, m, H-3,4,5,4', Oct α-CH_2 Ha), 3.93-3.83 (6H, m, H-2, 6a, 2', 3', 6b Oct α-CH_2 Hb), 4.02-3.94 (1H, m, H-5'), 4.54 (1H, d, $J_{1,2}$ 7.4, H-1), 4.80 (2H, s), 4.83 (1H), δ_C 14.0 (Oct-CH_3), 16.2 (C-6'), 20.9 (NH-Ac), 22.5 (Oct-CH_2), 25.2 (Oct-CH_2), 29.1 (Oct-CH_2), 29.5 (Oct β-CH_2), 31.5 (Oct-CH_2), 58.2 (C-2), 64.2 (C-5'), 64.9 (C-5), 66.9 C-3', 67.6 C-6, 68.2 (C-2'), 70.1 (Oct α-CH_2), 70.3 (C-4'), 73.3 (C-3), 79.2 (C-4), 95.2 (C-1'), 100.3 (C-1), 171.7 (C=O, NHAc), Calcd. for $C_{22}H_{41}NO_{10}$: $M^{\cdot+}$ 479.2730, M+H 480.2809 ; Found: CI+, M+H 480.2817.

References are on page 90

3.3.2 Biological Testing

Purification of Assay Material

The reaction mixtures detailed in Table 1 and Table 2 were incubated at 37°C for 1 hour, followed by the addition of 2133 μL of chloroform/methanol (1:1) to make a solution of chloroform/methanol/water (10:10:3). The tubes were vortexed, centrifuged (14,000 r.p.m.) and the supernatants were removed to 13mm X 100mm tubes and dried under a stream of nitrogen. The residues were resuspended in $1cm^3$ of 50% aqueous ethanol, loaded onto Whatman strong anion exchange (SAX) columns ($1cm^3$), washed with 50% aqueous ethanol (3ml) and then eluted with absolute ethanol (3ml). The tubes were dried under a stream of nitrogen and the residues were dissolved in water (3ml) and *n*-butanol (3ml), mixed at room temperature for 5 hours, centrifuged, and the butanol layers were removed to a 16mm X 100mm tube. The water layers were extracted twice with *n*-butanol (saturated with water) and the combined butanol phases were backwashed once with water (saturated with *n*-butanol) and dried using a stream of air. The residues were resuspended in 200μl of *n*-butanol and 20μl (10%) of each was counted. Analysis of the products by TLC was also carried out.

Tube	Membrane	P60	Ampho (2mg/mL)	NaOAc (2mg/mL)	Buffer	Acceptor			^{3}H-UDP Gal	Total cpm
						79	80	76		
1	40	0	0	8	72	0	0	0	0	2410
2	0	40	0	8	72	0	0	0	0	2386
3	40	40	0	8	32	0	0	0	0	3124
4	40	0	0	8	40	**32**	0	0	15	2792
5	40	0	8	0	40	**32**	0	0	15	3318
6	0	40	0	8	40	**32**	0	0	15	2940
7	0	40	8	0	40	**32**	0	0	15	2464
8	40	40	0	8	0	**32**	0	0	15	3116
9	40	40	8	0	0	**32**	0	0	15	2856
10	40	0	0	8	40	0	**32**	0	15	2784
11	40	0	8	0	40	0	**32**	0	15	2574
12	0	40	0	8	40	0	**32**	0	15	2678
13	0	40	8	0	40	0	**32**	0	32	2514
14	40	40	0	8	0	0	**32**	0	32	4788
15	40	40	8	0	0	0	**32**	0	32	3314
16	40	0	0	8	40	0	0	**32**	32	179492

Table 1: Incubation Conditions for Acceptors (76), (79) and (80). All values in μL except where indicated.

References are on page 90

Tube	membrane	P60	Buffer	Acceptor (mM)			^{3}H-UDP Gal	UDP Gal	Total cpm
				79	80	76			
1	50	50	32	0	0	0	5	8	1160
2	50	50	0	0	0	**2**	15	8	257430
3	50	50	16	**0.1**	0	0	15	8	1275
4	50	50	24	**0.5**	0	0	15	8	1610
5	50	50	16	**1**	0	0	15	8	1485
6	50	50	0	**2**	0	0	15	8	1635
7	50	50	19.2	**4**	0	0	15	8	3515
8	50	50	12.8	**6**	0	0	15	8	1875
9	50	50	6.4	**8**	0	0	15	8	1900
10	50	50	0	10	0	0	15	8	2035
11	50	50	16	0	**0.1**	0	15	8	1220
12	50	50	24	0	**0.5**	0	15	8	1285
13	50	50	16	0	**1**	0	15	8	1595
14	50	50	0	0	**2**	0	15	8	1815
15	50	50	19.2	0	**4**	0	15	8	1885
16	50	50	12.8	0	**6**	0	15	8	1570
17	50	50	6.4	0	**8**	0	15	8	1690
18	50	50	0	0	**10**	0	15	8	1850

Table 2: Concentration Optimisation Conditions for Acceptors (76), (79) and (80).

References are on page 90

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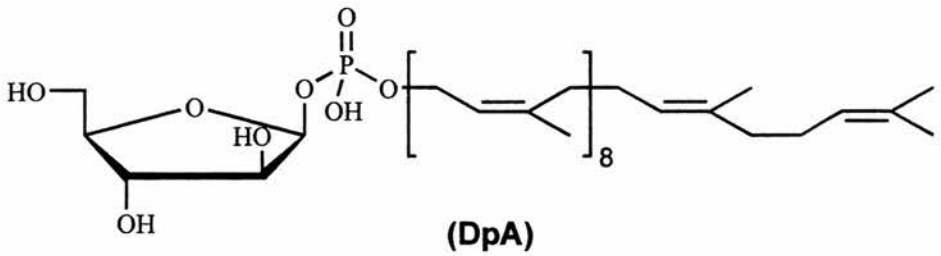
CHAPTER 4: Attempted Synthesis of pApp

4.1 INTRODUCTION

4.1.1 Biosynthesis of Arabinan

As previously discussed in section 1.2.2.3, there are three different linkages between the arabinofuranose residues in arabinan: (α1-5), (α1-3) and (β1-2). It has been discovered that there is a single donor for the arabinose residues in at least two of these linkages: decaprenylphosphoarabinose (DpA).[1-3]



(DpA)

Figure 30: Decaprenylphosphoarabinose, a Donor of Arabinosyl Residues in Arabinan

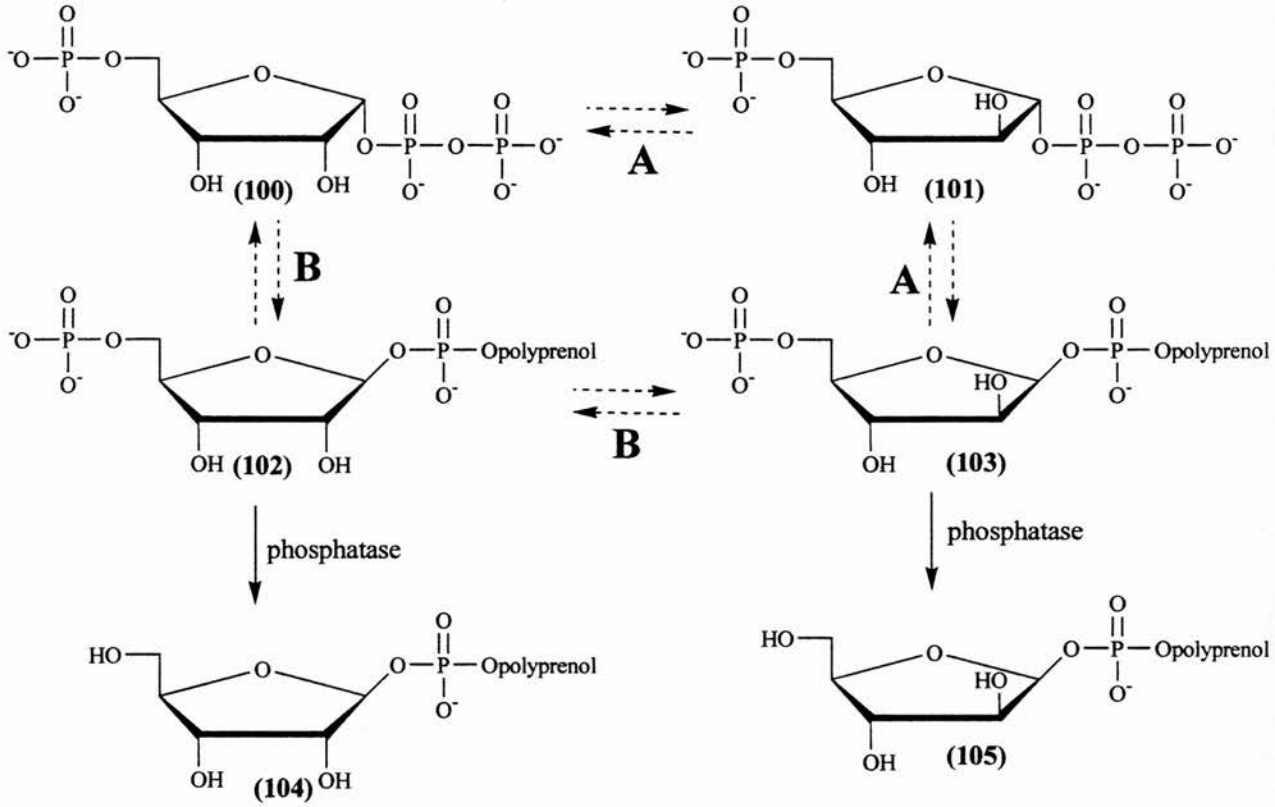
Experiments using synthetic disaccharide acceptors and radioactive DpA established the activity of DpA as a donor of both Ara(β1-2) and Ara(α1-5) residues but no Ara(α1-3) transferase activity was observed.[2] In these experiments it was also shown that ethambutol, a known inhibitor of arabinan biosynthesis[1,4] did not inhibit the action of these two transferases. However, ethambutol was observed to inhibit incorporation of radioactive DpA into endogenous arabinose acceptors in *M. smeg.* cell walls.[1] Therefore, whether DpA is the donor for Ara(α1-3) residues or not has yet to be established.

Since it is now known that DpA is the donor for most of the arabinofuranosyl residues in arabinan it follows that inhibition of the biosynthesis of this molecule should prove deleterious to the organism.

The biosynthesis of this molecule has been shown to originate from 5-phosphoribose-1-pyrophosphate (pRpp) and might occur via one of two primary routes.[5] These routes **A** and **B** are outlined in Scheme 27. Route **A** involves the epimerisation of the 2-hydroxyl which would result in the synthesis of intermediate pApp **(101)** which would be converted by a phosphoarabinosyltransferase resulting in 5-phosphoarabinose-1-phosphopolyprenol **(103)**. However, if route **B** was followed, a phosphoribosyltransferase would convert pRpp **(100)** into intermediate 5-phosphoribose-

References are on page 110

1-phosphopolyprenol **(102)** which would then be epimerised at the 2-hydroxyl to produce **(103)**.



Scheme 27: Possible Biosynthetic Pathways from pRpp (100) to DpA (105)

The intermediates **(102)** and **(103)** have been observed by HPLC and autoradiography as have products **(104)** and **(105)**. However, experiments using radioactive **(102)** and enzymatically active *M. smeg.* cell walls failed to produce any **(103)** or **(105)** but instead polyprenylphosphoribose **(104)** was the only observed product.[5] This suggested a lack of 5-phosphoribose-1-phosphopolyprenol epimerase activity.

A common biosynthetic presursor to polyprenylphosphate sugars are nucleotide diphosphate sugars (NDP-sug) which also serve as donors for many sugar units.[6] Experiments have shown that radioactive DpA cannot be converted to any NDP-Ara using *M. smeg.* cell wall extracts and various nucleotide diphosphates although similar experiments were successful using polyprenylphosphomannose.[5] Therefore it appeared that NDP-Ara donors were unlikely as DpA precursors.

The most likely biosynthetic pathway, therefore, was route **A**, although there was no conclusive evidence to prove this. In order to prove whether or not pApp was an

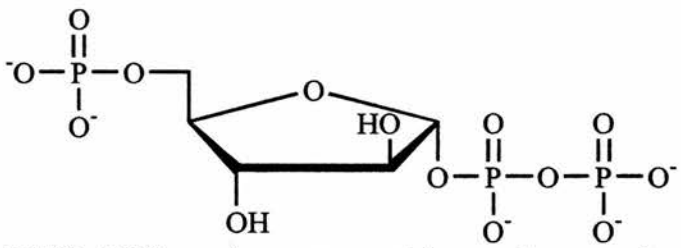
 References are on page 110

intermediate in the biosynthesis of DpA, it was considered necessary to chemically synthesise it.

4.1.2 Aims and Objectives

In the design of potential drug candidates to inhibit specific enzymatic reactions, it is more useful to have the biosynthetic pathway fully elucidated. Such conditions allow the rational synthesis of potential inhibitors based on the structure of known intermediates. In the biosynthesis of DpA, there remains only one step which has not been fully elucidated: the epimerisation step.

Inhibition of the biosynthesis of D-arabinose should be relatively non-toxic to humans because it does not exist in the mammalian system. As it is likely that there are only three steps between pRpp and DpA, elucidation of one of these is a matter of importance.



(101) 5-Phospho-α-D-arabinose-1-pyrophosphate

Figure 31: Target Compound

It was decided to attempt the synthesis of the putative biosynthetic intermediate 5-phospho-α-D-arabinose-1-pyrophosphate **(101)** and subsequently incubate the compound with mycobacterial cell wall extracts. It was also hoped that during synthesis of this compound, a viable route to radioactive **(101)** would be elucidated as this would help detection in the biochemical assays.

References are on page 110

4.2 RESULTS AND DISCUSSION

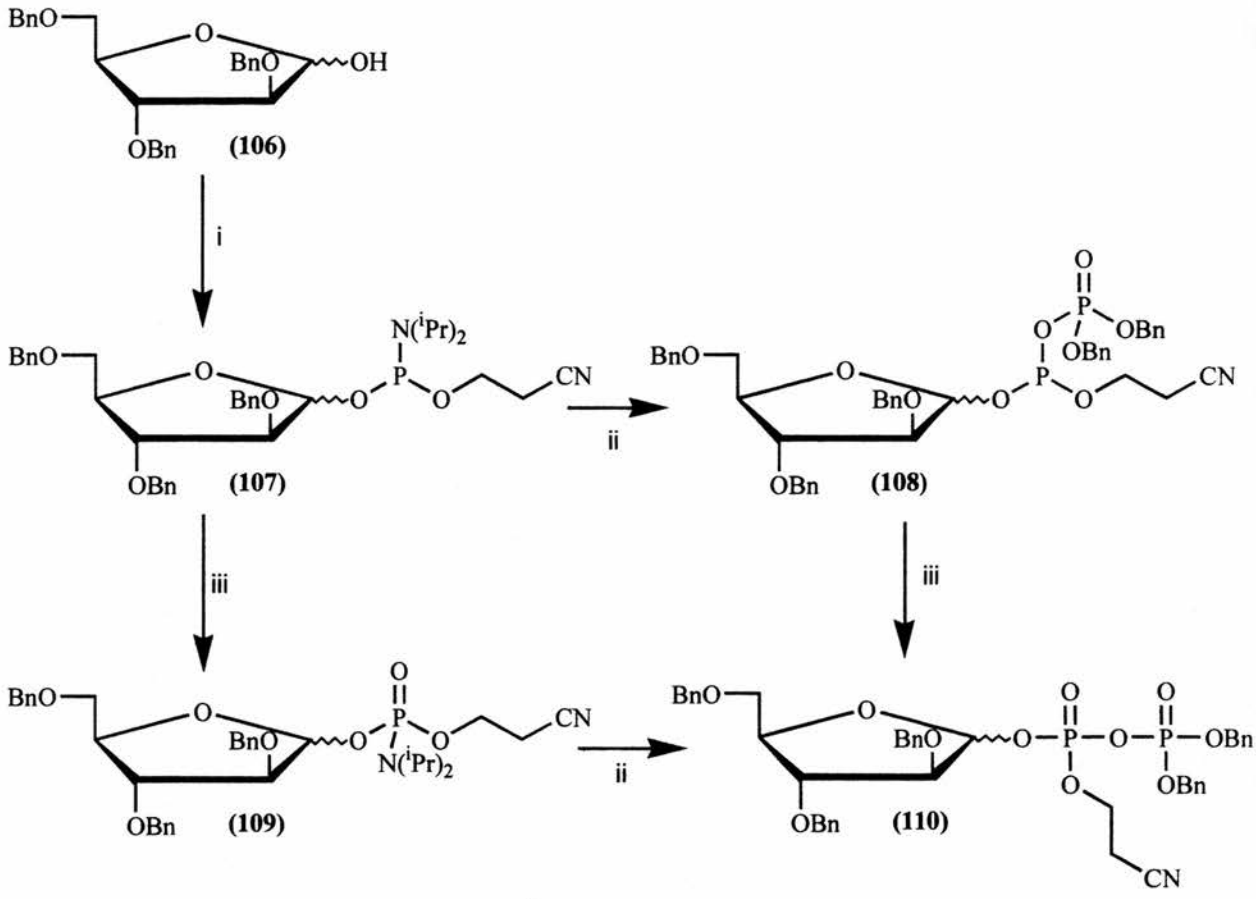
4.2.1 Attempted Synthesis of Anomeric Pyrophosphate using Phosphoramidite Methodology

Phosphoramidite reagents have been employed[1] to synthesise DpA, an arabinosyl phosphodiester and therefore, it was thought that replacement of the prenol moiety for a phosphate would produce the desired anomeric pyrophosphate. At this point anomeric selectivity was considered unimportant and the main target was to produce an anomeric pyrophosphate.

It was decided to use tribenzyl-D-arabinose as a model compound for the pyrophosphorylation attempts. Since an anomeric pyrophosphate is so labile under acidic and basic conditions it would be desirable to have the pyrophosphorylation step as late as possible in the synthesis, therefore the 5-phosphate would need to be in place.

Tribenzylarabinose **(106)** was treated with a commonly used phosphoramidite reagent under basic conditions and a clear change in the reaction mixture was observed by TLC. Since this compound was too unstable to characterise, the disappearance of the starting material spot by TLC was taken as a clear indication that the reaction had proceeded to the intermediate phosphite **(107)**. Activation of the P-N bond using tetrazole, followed by treatment with dibenzyl phosphate was expected to result in phosphorophosphite **(108)** which, again would be too unstable to characterise. Oxidation of **(108)** using mCPBA should have produced the desired anomeric pyrophosphate **(110)**, however, attempts to elucidate this structure by NMR led us to the conclusion that the desired compound had certainly not been made, but it was difficult to characterise exactly what had been made. Since this clearly was not the desired compound, and after observing the same result over repeated reactions, it was decided to attempt another method.

References are on page 110



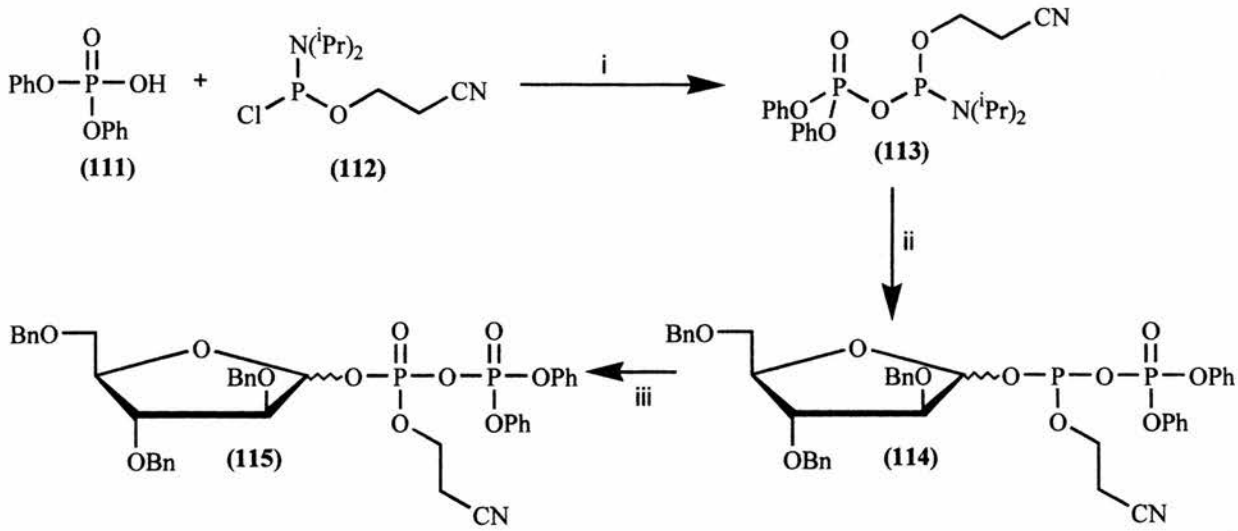
Reagents: (i) $CN(CH_2)_2OP(Cl)N(^iPr)_2$, DIEA, CH_2Cl_2; (ii) tetrazole, DIEA, $(BnO)_2P(O)OH$; (iii) mCPBA, CH_3CN

Scheme 28: Attempts at Anomeric Pyrophosphate Synthesis via an Anomeric Phosphite

It was decided to oxidise rather than activate **(107)** in the hope that the more stable product **(109)** would not degrade and would remain a reactive enough electrophile for dibenzyl phosphate. However, although a new spot which was assumed to be **(109)** was observed by TLC, there was no consumption of starting materials when this product was reacted with dibenzyl phosphate, tetrazole and DIEA. Again, this reaction was repeated several times and the same result was seen.

The final attempt using phosphoramidite reagents used a different approach. Since it was thought that anomeric phosphite instability may have been the main problem in creating an anomeric pyrophosphate, it was decided to attempt a preformation of the phosphorophosphite which could be reacted with the hemi-acetal.

References are on page 110



Reagents: (i) DIEA, CH_2Cl_2; (ii) **(106)**, tetrazole, DIEA, CH_2Cl_2; (iii) mCPBA, CH_3CN

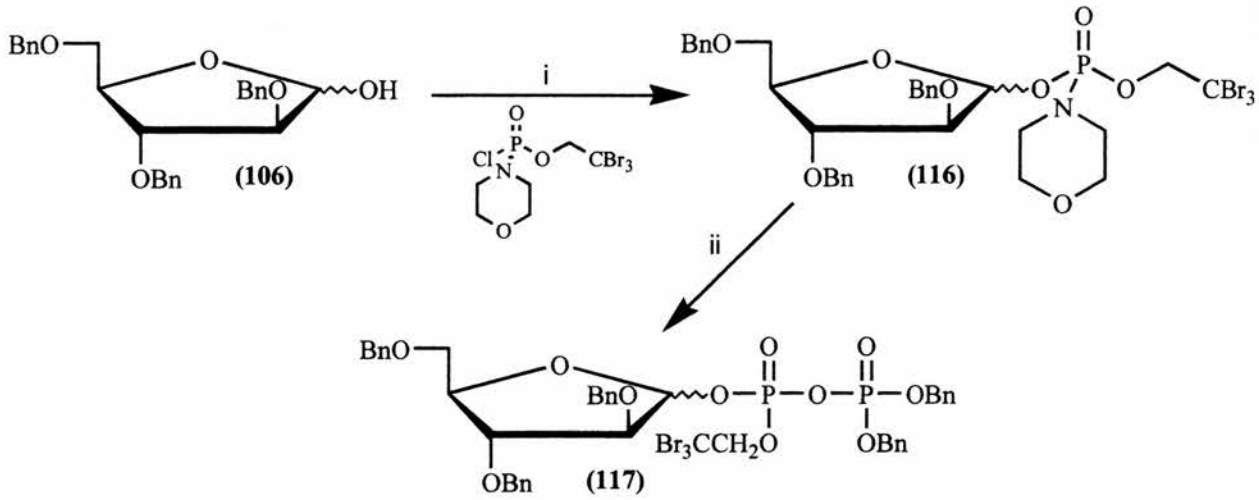
Scheme 29: Attempted Synthesis of Anomeric Pyrophosphate via a Preformed Phosphorophosphite

Diphenylphosphate **(111)** was reacted with the standard phosphoramidite reagent **(112)** in order to create the preformed phosphorophosphite. Under the standard conditions for activation of the P-N bond, however, when mixed with tribenzyl arabinose **(106)**, very little consumption of starting materials was observed.

4.2.2 Attempted Synthesis of Anomeric Pyrophosphate using Phosphoramidate Methodology

In a literature synthesis of carbocyclic pRpp, phosphoramidate chemistry was used to make a pyrophosphate group at the analogous anomeric position.[7] Although there was no ring oxygen in this molecule and thus the anomeric pyrophosphate would be much more stable to acid, the methodology was thought to be reasonably applicable to our problem.

References are on page 110



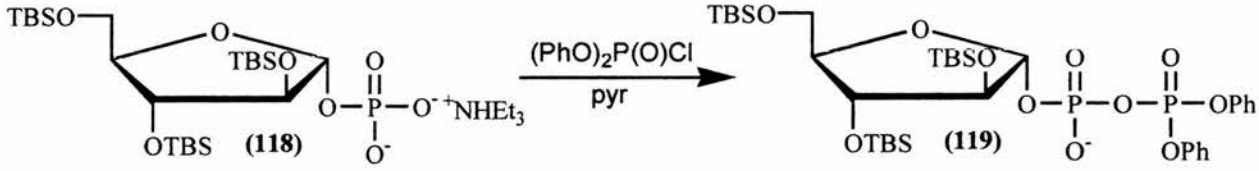
Reagents: (i) tetrazole, pyridine; (ii) $(BnO)_2P(O)OH$, tetrazole, DIEA

Scheme 30: Attempted Pyrophosphate Synthesis using Phosphoramidate Chemistry

However, when tribenzyl arabinose was reacted with the phosphoramidate compound (Scheme 30), no reaction was observed at temperatures up to 45°C. Therefore, this approach was discontinued.

4.2.3 Phosphate Coupling Approach

Due to the availability of considerable amounts of arabinosyl phosphate **(118)** in the Besra group, it was decided to attempt a coupling of this compound with diphenyl chlorophosphate in the hope of producing the pyrophosphate **(119)** (Scheme 31).



Scheme 31: Attempted Pyrophosphate Synthesis using Phosphate Coupling

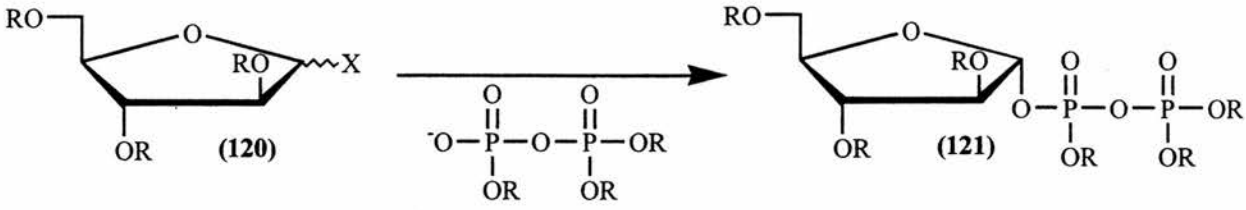
It was observed by TLC that two new products had formed, however, after purification and analysis by NMR, it was clear that neither of these products was the desired product. It was difficult to discern from the spectra exactly what had been formed but due to the lack of both aromatic and butyl protons in the same spectrum as well as the lack of a pair of doublets on the ^{31}P spectrum it was obvious that no pyrophosphorylation had occurred.

References are on page 110

4.2.4 Direct Attack of Pyrophosphate

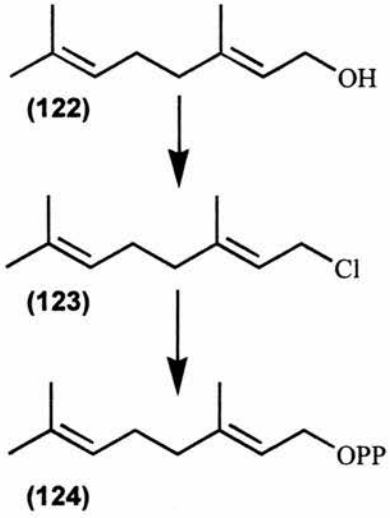
4.2.4.1 Strategy

Since little progress had been attained by the reaction of different phosphate/phosphite with each other, it was decided to adopt a different approach, the direct attack of a pyrophosphate molecule with a suitably protected arabinosyl donor.



Scheme 32: Strategy for the Direct Phosphorylation of an Arabinosyl Donor

The synthesis of various isoprenoid pyrophosphates was carried out in the laboratories of Poulter[8,9] using similar methodology to the aforementioned strategy.



Scheme 33: Pyrophosphorylation of Geranyl Alcohol[9]

Although isoprenoid pyrophosphates are very different from sugar pyrophosphates, the possible allylic substitution was considered similar to the possible substitution via formation of an oxocarbonium ion in carbohydrate structures as illustrated in Figure 32.

References are on page 110

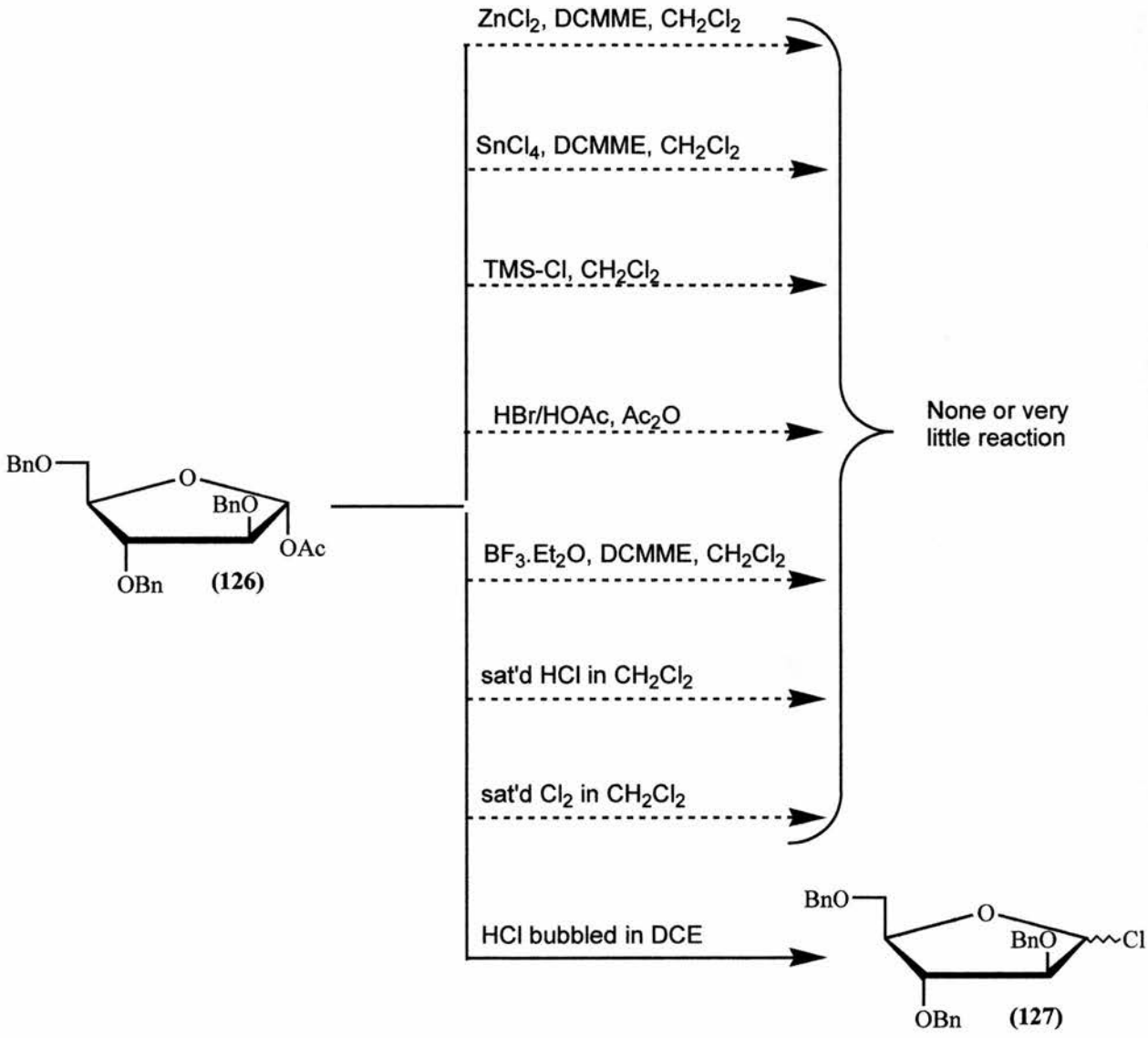
Figure 32: Similar Elimination Possibilities in Isoprenoid and Arabinosyl Pyrophosphates

4.2.4.2 Synthesis of the Arabinosyl Donor

In the design of the arabinosyl donor, the unstable nature of the desired anomeric pyrophosphate had to be considered. Therefore, it was necessary to have a donor which would require reasonably mild activation conditions for the pyrophosphorylation reaction. Evidence[10] that α-D-ribose-1-phosphate is several hundred times more labile than α-D-glucose-1-phosphate suggested that a similar reactivity difference may also apply to anomeric halides between the five- and six-membered ring carbohydrates. Therefore, it was decided to pursue the synthesis of a suitably protected arabinosyl halide.

Although it would be preferable to have the pyrophosphorylation step as the penultimate in the reaction sequence, it was decided to attempt a variety of halogenation reactions on 1-*O*-acetyl-tri-*O*-benzyl-α-D-arabinose **(126)**. This model compound would provide a good indication of anomeric reactivity. Of the many reactions listed in Scheme 34 only the bubbling of HCl gas through a solution of **(126)** in DCE produced the desired anomeric halide. Since bubbling was required for 20 minutes and a concentrated solution of the substrate was better, DCE, as a higher boiling solvent than CH_2Cl_2, proved more useful. This reaction was considered very useful for the pyrophosphorylation reaction because the only required purification was evaporation of the DCE. It was hoped that the chloride could then be reacted with the acceptor in the absence of promoters.

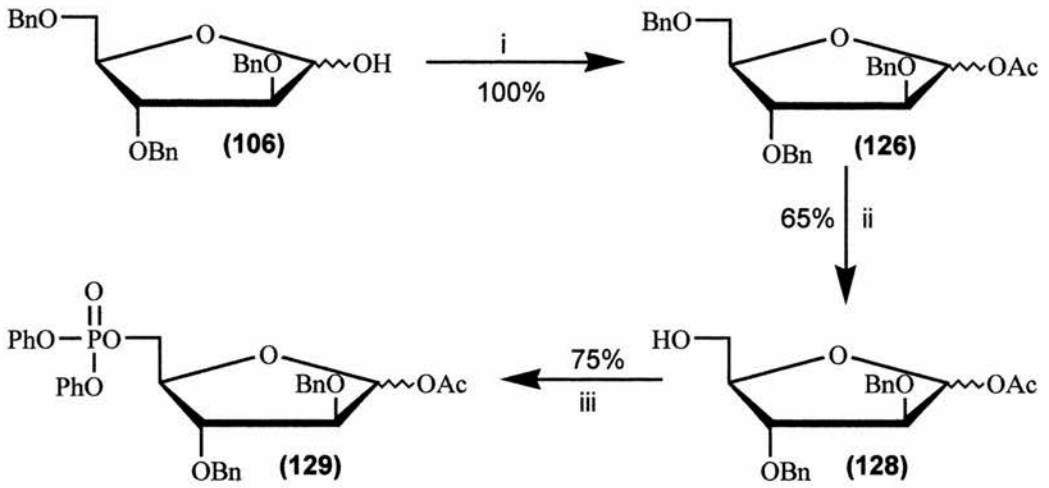
References are on page 110



Scheme 34: Various Attempts at the Halogenation of Protected Arabinose

After the successful chlorination of **(126)**, it was decided to synthesise a precursor to pApp and carry out chlorination and pyrophosphorylation experiments to gain access to the desired final compound. Tri-*O*-benzyl-D-arabinose was acetylated and then selectively debenzylated using the method of Maryanoff[11] which involved palladium catalysed hydrogenation with partial pyridine poisoning of the catalyst.

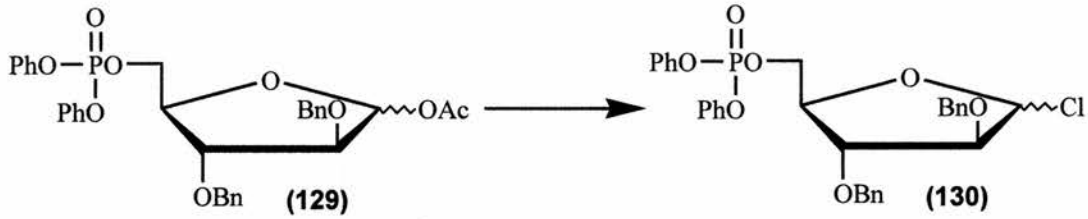
 References are on page 110



Reagents: (i) Ac_2O, pyridine; (ii) 10% Pd on C, HOAc/MeOH, pyridine (0.4%w/w); (iii) $(PhO)_2P(O)Cl$, pyridine

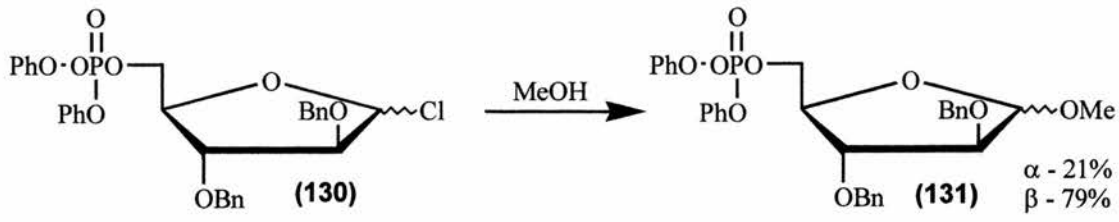
Scheme 35: Synthesis of Precursor to pApp

Phosphorylation of the free hydroxyl was then carried out using diphenyl chlorophosphate in pyridine, a straightforward procedure which produced the phosphorylated precursor **(129)** in an overall yield of 48%. This product was then used in experiments to determine the viability of chlorination and the possibility of pyrophosphorylation.



Scheme 36: Chlorination of a 5-Phosphorylated Arabinosyl Acetate

It was found that compound **(129)** could be chlorinated using the same method as for **(126)** but due to the lower activity of the substrate, the temperature had to be increased to 45°C. It was important to react for less than 20 minutes as degradation of the phosphate began at this point.



Scheme 37: Conversion of Anomeric Chloride into Methyl Glycoside

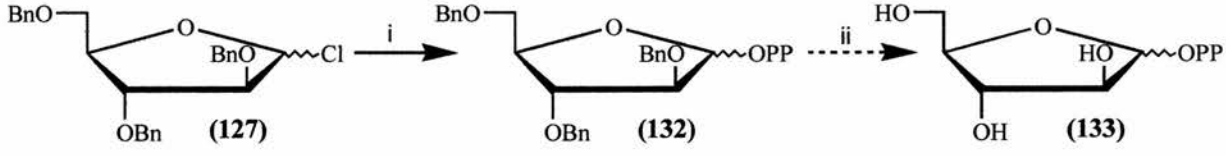
References are on page 110

In order to confirm that reaction had actually taken place, it was decided to evaporate the DCE and add anhydrous MeOH to the donor. Within minutes, the TLC spot indicating the chloride had completely disappeared and a new, slightly more polar spot indicating the methyl glycoside (as confirmed by ^{1}H NMR; the acetyl peak at δ 2.24 had disappeared and new peaks at δ 3.43 and 3.44, corresponding to methyl groups, had appeared) was observed.

4.2.4.3 Attempted Pyrophosphorylation

With both the phosphorylated **(129)** and non-phosphorylated **(126)** compounds readily convertible into chloride donors which could then be efficiently used in the next step by the evaporation of solvent, it was decided to attempt the pyrophosphorylation procedure mentioned in section 4.2.4.1.

Reaction of the chloride **(127)** in the presence of tris(tetra-n-butylammonium) pyrophosphate resulted in the formation of a more polar spot on the TLC plate which contained phosphorous as indicated by the Dittmer-Lester dip.[12] This was considered very positive and purification via a number of ion-exchange and a cellulose column yielded a compound the structure of which was difficult to elucidate by ^{1}H and ^{13}C NMR but which clearly showed two coupled doublets by ^{31}P NMR indicating the presence of a pyrophosphate.



Reagents: (i) tris(tetra-n-butylammonium) pyrophosphate, DCE; (i) 10% Pd on C, H_2, MeOH

Scheme 38: Attempted Synthesis of Arabinose-1-pyrophosphate

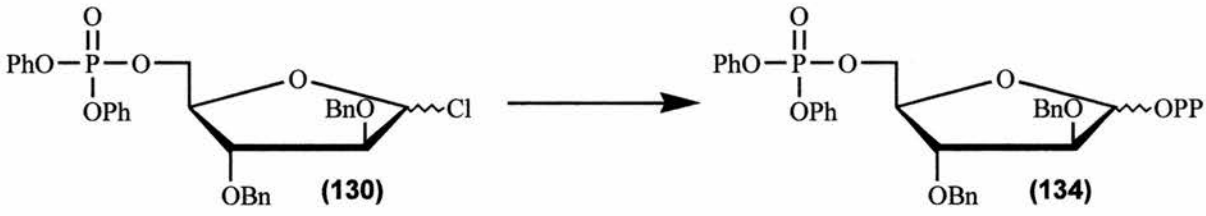
Attempts to deprotect compound **(132)** using Pd on carbon in MeOH resulted in the disappearance of the starting material spot by TLC. However resolution and charring of the new spot was extremely difficult. NMR of the crude product reflected that the desired product had definitely not been made. Neither the ^{1}H, ^{13}C or ^{31}P NMR spectra showed definitively resolved peaks and the mass spec showed no peaks at the relevant masses. Repeated attempts of this reaction failed to produce the desired product.

References are on page 110

Although success was not achieved using the non-phosphorylated donor **(127)**, it was decided to attempt pyrophosphorylarion of the phosphorylated donor **(130)**. The conditions were identical as for **(127)**. After chlorination, pyrophosphoryation and purification of the suspected protected pyrophosphate **(134)**, analysis by ES-MS and FAB-MS showed a predominance of the desired compound. Although the data was not exact, the peaks in both the ES-MS and the FAB-MS were very close to the prospective masses. Figure 33 shows the spectra and the peak assignment table.

As was often the case when working with these compounds, attempts to get clear, well resolved NMR spectra were unsuccessful, even with ^{31}P NMR, therefore the mass spectrometry data was the only piece of analytical data to show the presence of the desired compound. As with the previous pyrophosphorylation attempt, the compound degraded under hydrogenolysis conditions.

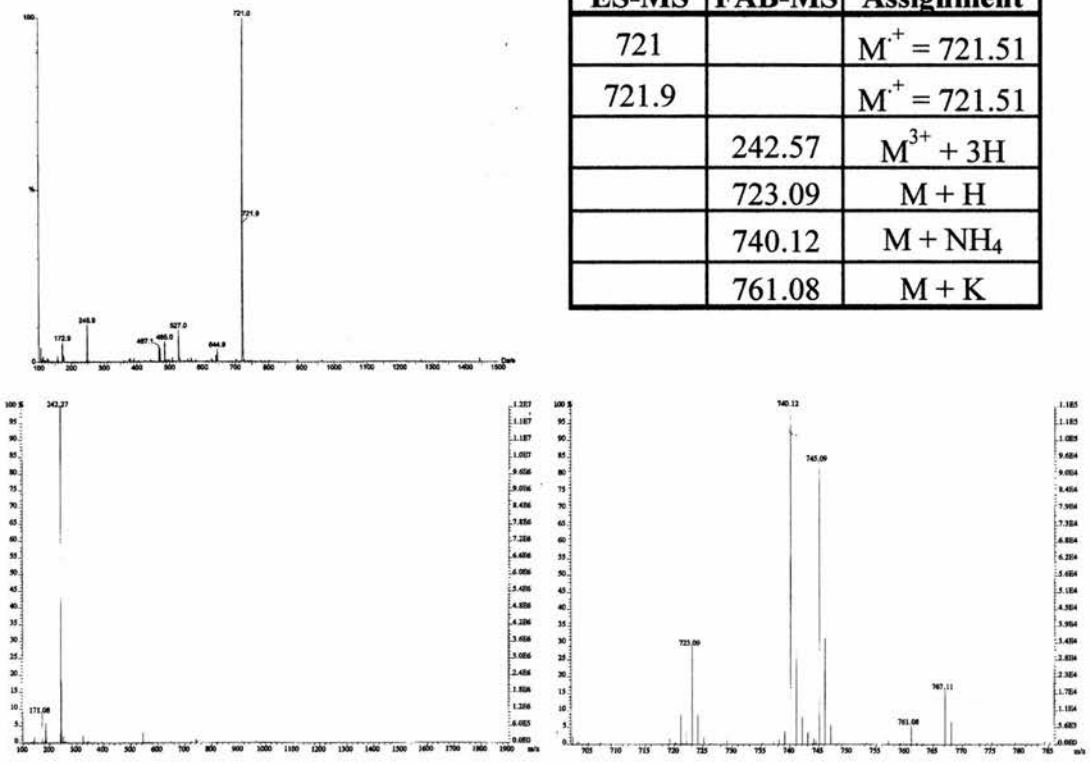
The overall reaction was attempted several times but the mass spectrometry result showing the existence of the protected pApp could not be reproduced.



Reagents: tris(tetra-n-butylammonium) pyrophosphate, DCE

Scheme 39: Synthesis of 2,3-*O*-Benzyl-5-Diphenylphospho-arabinose-1-pyrophosphate

References are on page 110



ES-MS	FAB-MS	Assignment
721		$M^{\cdot+} = 721.51$
721.9		$M^{\cdot+} = 721.51$
	242.57	$M^{3+} + 3H$
	723.09	$M + H$
	740.12	$M + NH_4$
	761.08	$M + K$

Figure 33: Mass Spectrum of (134) and Peak Assignment. Clockwise from top left: ES-MS Spectrum; Peak Assignment Table; FAB-MS Spectrum; FAB-MS Expansion

References are on page 110

4.2.5 Conclusions

A number of attempts were made to synthesise the putative biosynthetic intermediate pApp, however, on no occasion was this compound produced. The most promising of these methods was the direct pyrophosphorylation of an arabinosyl donor using tris(tetrabutylammonium) pyrophosphate. This method is fraught with difficulty due to the necessity of several rounds of column purification and ultimately, the hydrogenolysis conditions necessary for deprotection, proved to be too harsh for the anomeric pyrophosphate.

4.3 EXPERIMENTAL

1-O-Acetyl-2,3,5-tribenzyl-α/β-D-arabinofuranose **(126)**

Tribenzyl-D-arabinose (10g, 23.8mmol) was dissolved in pyridine (40 cm^3) and Ac_2O (5 cm^3) was added. The reaction was allowed to stir at room temperature overnight. The mixture was extracted into CH_2Cl_2 and was subjected to a standard aqueous work-up, dried over Na_2SO_4 and evaporated to dryness. Further purification was performed with flash column chromatography using toluene/EtOAc 9:1 as the eluant. Combination and evaporation of the relevant fractions yielded the target compound as a colourless oil (10.45g, 95%), δ_H 1.99 (3Hβ, s, *Ac*), 2.09 (3Hα, s, *Ac*), 3.58 (2Hβ, d, H-5,5'β), 3.63 (2Hα, d, H-5,5'α), 3.99 (1Hα, dd, $J_{3,4}$ 5.7, $J_{2,3}$ 2.3, H-3α), 4.09 (1Hα, dd, $J_{2,3}$, J 0.5, H-2α), 4.21 (3Hβ, m, H-2,3,4β), 4.37 (1Hα, m, H-4α), 4.50 (2Hαβ, m, PhC$\underline{H_2}$), 4.53 (2Hα, bs, PhC$\underline{H_2}$), 4.56 (2Hα, bs, PhC$\underline{H_2}$), 4.60 (2Hβ, m, PhC$\underline{H_2}$), 4.63 (1Hα, bs, PhC$\underline{H_2}$), 4.67 (3Hβ, m, PhC$\underline{H_2}$), 6.25 (1Hα, s, H-1α), 6.29 (1Hβ, d, $J_{1,2}$ 3.4, H-1β), 7.20-7.39 (15Hαβ, m, *Ar*).

1-O-Acetyl-2,3-O-benzyl-α/β-D-arabinofuranose **(128)**

Following the procedure of Maryanoff[11], compound **(126)** (10.35g, 22.4mmol) was dissolved in a mixture of MeOH/HOAc 1:1 (50 cm^3) and pyridine (42μL, 0.4% w/w) was added. Palladium on carbon (10%, 1g) was then added and the reaction mixture was stirred under a hydrogen atmosphere for 24 h at which point optimal reduction was

 References are on page 110

observed by TLC. The reaction mixture was filtered through Celite, evaporated to dryness and coevaporated several times with toluene. The residue was then loaded onto a flash chromatography column which was eluted with toluene/EtOac 4:1 until separation was observed by TLC. The relevant fractions were pooled and evaporated to a colourless oil (5.42g, 65%), *Anal.* Calcd for $C_{21}H_{24}O_6.0.5H_2O$: C, 66.13; H, 6.61. Found C, 65.98; H, 6.63; δ_H 1.95 (1H, bs, $CH_2O\underline{H}$), 2.08 (3H, s, *Ac*) 3.66 (1H, dd, $J_{5,5'}$ 12.09, $J_{4,5'}$ 4.12, H-5'), 3.85 (1H, dd, $J_{5,5'}$, $J_{4,5}$ 3.02, H-5), 4.06 (1H, dd, $J_{2,3}$ 2.27, $J_{3,4}$ 5.77, H-3), 4.10 (1H, dd, $J_{1,2}$ 0.82, $J_{2,3}$, H-2), 4.27 (1H, ddd, $J_{3,4}$, $J_{4,5}$, $J_{4,5'}$, H-4), 4.66-4.50 (4H, m, 2 x $BnC\underline{H}_2$), 7.39-7.25 (10H, m, 2 x *Ar*), δ_C 21.1 (*Ac*), 64.6 (C-5), 72.1 ($BnC\underline{H}_2$), 72.4 ($BnC\underline{H}_2$), 82.4 (C-4), 84.5 (C-3), 86.2 (C-2), 100.1 (C-1), 127.3-128.9 (*Ar*), 135.4 (quat. *Ar*), 136.7 (quat. *Ar*), 169.6 ($\underline{C}$=O)

1-O-acetyl-2,3-O-benzyl-α/β-D-arabinofuranose 5-(diphenylphosphate) **(129)**

Compound **(128)** (453mg, 1.22mmol) was dissolved in pyridine (4.0cm^3) and diphenyl chlorophosphate (378μL, 1.82mmol) was added. The reaction mixture was diluted with Et_2O (20cm^3), washed several times with H_2O (10cm^3), dried over Na_2SO_4 and evaporated *in vacuo*. The residue was co-evaporated with toluene until no more pyridine could be detected and this was purified using flash column chromatography (eluant: toluene/EtOAc 4:1) which yielded, after combination and evaporation of the relevant fractions, a white solid (554mg, 75%). δ_H 2.24 (3H, s, *Ac*), 3.97 (1H, dd, $J_{3,4}$, $J_{2,3}$, H-3), 4.07 (1H, d, $J_{2,3}$ 1.76, H-2), 4.11-4.18 (2H, m, H-5,5'), 4.34 (1H, dd, $J_{4,5}$ 9.96, $J_{3,4}$ 5.27, H-4), 4.43-4.62 (4H, m, 2 x $PhC\underline{H}_2$), 6.21 (1H, s, H-1), 7.42-7.58 (20H, m, *Ar*), δ_C 21.3 (*Ac*), 66.4 (C-5), 72.3 ($BnC\underline{H}_2$), 72.1 ($BnC\underline{H}_2$), 82.7 (C-4), 84.7 (C-3), 86.6 (C-2), 100.4 (C-1), 127.7-128.7 (*Ar*), 135.7 (quat. *Ar*), 135.8 (phenylphospho-quat. *Ar*), 137.1 (phenylphospho-quat. *Ar*), 137.3 (quat. *Ar*), 169.9 ($\underline{C}$=O).

Methyl 2,3-O-benzyl-5-diphenylphospho-α/β-D-arabinofuranoside **(131)**

Chlorination of **(129)** (100mg, 165μmol) was achieved in DCE (1 cm^3) by bubbling HCl gas directly into the solution at 45°C. After 20 min, the reaction was ceased and the DCE was evaporated *in vacuo*. Anhydrous MeOH was then added (1 cm^3) and the solution was stirred for 5 min at which point the mixture was evaporated to dryness. The crude product was then analysed by NMR and found to be the title compound. *Anal.*

Calcd for $C_{32}H_{33}O_8P.0.5H_2O$: C, 65.64; H, 5.85. Found C, 65.15; H, 5.87; δ_H 3.43 (3Hβ, s, *Me*), 3.44 (3Hα, s, *Me*), 3.99 (1Hα, dd, $J_{3,4}$ 5.5, $J_{2,3}$ 2.1, H-3α), 4.09 (1Hα, m, H-2α), 4.14 (2Hβ, d, H-5,5'β), 4.17 (2Hα, d, H-5,5'α), 4.21 (3Hβ, m, H-2,3,4β), 4.37 (1Hα, m, H-4α), 4.50 (2Hβ, m, PhC$\underline{H}_2$), 4.53 (2Hα, bs, PhC$\underline{H}_2$), 4.56 (2Hα, bs, PhC$\underline{H}_2$), 4.60 (2Hβ, m, PhC$\underline{H}_2$), 4.63 (1Hα, bs, PhC$\underline{H}_2$), 4.67 (3Hαβ, m, PhC$\underline{H}_2$), 4.85 (1Hα, s, H-1α), 4.91 (1Hβ, d, $J_{1,2}$ 3.3, H-1β), 5.02 (4H, t, *J* 8.1, PhC$\underline{H}_2$-OP x 2), 7.19-7.35 (15Hαβ, m, *Ar*).

Attempted Synthesis of 2,3-O-benzyl-5-diphenylphospho-α/β-D-arabinofuranose-1-pyrophosphate **(134)**

Chlorination of **(129)** (200mg, 330μmol) was carried out as before and the residue was dissolved in anhydrous CH_3CN and evaporated to dryness. This was repeated twice and the resultant residue was redissolved in anhydrous CH_3CN (200μL). Tris(tetra-n-butylammonium) pyrophosphate[8] (595mg, 660μmol) was added to anhydrous CH_3CN (1 cm^3) and undissolved material was removed by centrifugation. The supernatant was removed, evaporated to dryness, redissolved in anhydrous CH_3CN and evaporated to dryness. This was repeated twice and the residue was dissolved in anhydrous CH_3CN_3 (700μL) and cooled to 0°C. To this was added, dropwise, the solution of the chloride in CH_3CN and the reaction was stirred at 0°C. After 1.5 h, the reaction mixture had turned red and was concentrated *in vacuo* to a brown gum which was subjected to high-vacuum conditions for 1 h. The gum was then dissolved in the eluting solution $^iPrOH/NH_4HCO_3$ 1:49 (1 cm^3) and the solution was applied to a column of DOWEX 50WX8[NH_4^+] (50-100 mesh) which was pre-equilibrated with the eluant. The column was then eluted and the phosphate containing material came through in about 1.5 column volumes. The compound containing fractions were then pooled and freeze-dried almost to the point of dryness. Care had to be taken not to lyophilise the ammonia from the pyrophosphate and leave a more labile free acid. The ion exchange process was repeated at an eluting speed of 1 column volume every 15 min and the resultant eluate was freeze-dried as before. The resultant residue was dissolved in NH_4CO_3 (0.1M, 1.5 cm^3), transferred to a centrifuge tube and precipitated using $CH_3CN/^iPrOH$ 1:1. The solution was centrifuged and the supernatant was removed and the process was repeated on the residue. The combined supernatants were combined, concentrated *in vacuo* and then freeze-dried. The residue was then dissolved in $^iPrOH/CH_3CN/0.1M\ NH_4HCO_3$ (9:5:2) and applied to

 References are on page 110

a cellulose column which was then eluted using the same solution. The fractions which contained the same product as that observed from the reaction by TLC were combined, evaporated to dryness, redissolved in ^{i}PrOH/25mM NH_4HCO_3 1:49 and lyophilised to a white powder, (40mg) (Calcd. for $C_{31}H_{32}O_{14}P_3$: $M^{\cdot+}$ 721.50; Found: ES^- (721.0, 721.9), $M^{\cdot+}$ = 721.51, FAB-MS (242.57, 723.09, 740.12, 745.09, 761.08).

References are on page 110

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CHAPTER 5:Synthesis of pAcpp and Biological Evaluation of pAcpp and Several Commercially Available Phosphosugars

References are on page 145

5.1 INTRODUCTION

5.1.1 Phosphonates as Isosteres for Biologically Active Phosphates

5.1.1.1 Introduction

As organic phosphates are found in all biological systems and are among the most common biomolecules, it is not surprising that a significant effort has been made to synthesise analogues of these molecules for therapeutic use. The most common method used to produce analogues of phosphates is the replacement of an oxygen, often by a nitrogen, sulfur, or a methylene functionality.[1] There are two basic types of substitution, internal and external, and as outlined in Figure 34 that depends on which oxygen is replaced.

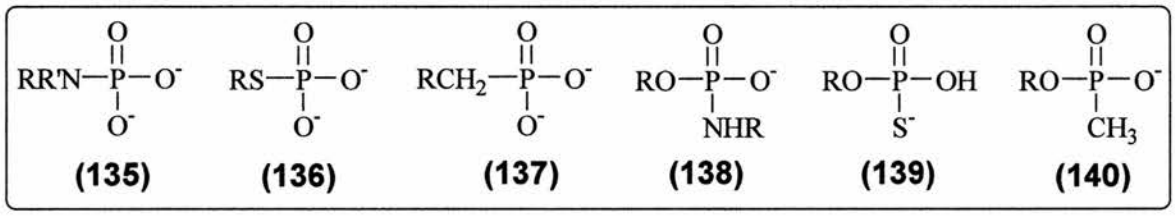

Figure 34: Different Types of Oxygen Substitution on a Phosphate

Internal substitution leads to phosphoramidates **(135)**, thiolphosphates **(136)** and phosphonates **(137)** preserving the overall ionisation character of the parent phosphate. External substitution yields phosphoramidic acids **(138)**, phosphorothioates **(139)** and methyl phosphonic acids **(140)** producing compounds with varying ionisation and optical activity.

The benefit of producing a phosphonate analogue ($X=CH_2$) is the increased stability of the C-P bond and the isosteric nature of the analogue compared with the parent compound. Since phosphates are often leaving groups in the parent biomolecules, a phosphonate should allow binding to a target enzyme whilst preventing catalysis thus, theoretically, making a good inhibitor.

5.1.1.2 Physicochemical Comparisons of Phosphonate Isosteres and Parent Molecules

In order to achieve the highest possible similarity to the parent compound, a phosphate analogue should possess near identical orientation of atoms for binding, bond lengths and ionisation potential of each acidic proton.

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5.1.1.2.1 Ionisation Potential

Substitution of oxygen with carbon results in a net lowering of electronegativity thus increasing the electron density around phosphorus. The result of this is an increase in the pK_a of each ionisable proton in the phosphinic acid. It has been found[2-4] that the second pK_a is the most affected; since this value occurs around physiological pH, it is also the most important for the analogue to mimic. An early study[3] of phosphonic acids found the second pK_a's to be in the range of 7.7-8.2 when attached to a primary alkyl group as compared with values close to 7.0 for the parent phosphates.[5] A more recent comparison[2] (Table 3) showed an increase of the second pK_a from 6.67 to 8.19 upon creating a phosphinic acid.

Compound	1^{st} pK_a	2^{nd} pK_a
$CH_3(CH_2)_2OPO_3H_2$	1.88	6.67
$CH_3(CH_2)_3PO_3H_2$	2.59	8.19
$BrCH_2CH_2PO_3H_2$	2.25	7.3
$BrCH_2PO_3H_2$	1.14	6.52

Table 3: Comparison of pK_a's of Phosphonic Acids and Phosphates

This pK_a increase is important because at most physiological pH's the second hydroxyl of the phosphonic acid would not be ionised thus reducing the likelihood of interactions with cations in the enzyme. As can also be seen from Table 3, halogenation of the α or β carbon results in a lowering of the second pK_a and in the case of α-halogenation the value is lowered to the range of parent phosphates. The argument for α-halogenated phosphonates as good phosphate mimics is strong, and although there has been biological data to support the hypothesis, many display only limited biological activity.[6-8]

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5.1.1.2.2 Atom Orientations

A general hypothesis[4] of bond angles and lengths was made using crystallographic data comparisons of 2-aminoethyl phosphate[9,10] and 2-aminoethylphosphonic acid[11] which yielded the model presented in Figure 35. The bond length between C-P in the phosphonic acid **(142)** was 13% longer than the corresponding O-P bond in the phosphate **(141)** but the comparative distances between positions a and b in the phosphate and a' and b' in the phosphonic acid varied by only 0.8%, with the phosphonic acid distance being greater. As stated previously, this is due to a decrease in electronegativity associated with the carbon

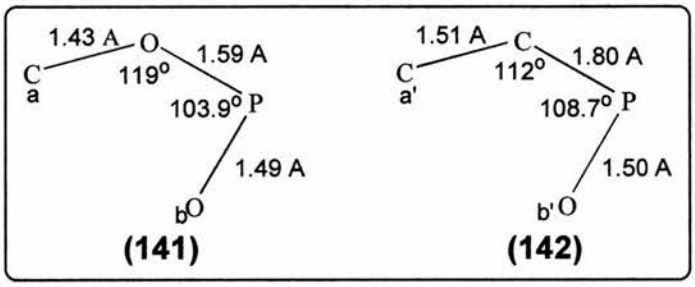


Figure 35: Bond Length and Angle Differences Between Phosphates and Phosphonates

This data lends credence to the theory that phosphonates are good isosteric analogues of phosphates.

5.1.1.3 Glycosyl Phosphonates

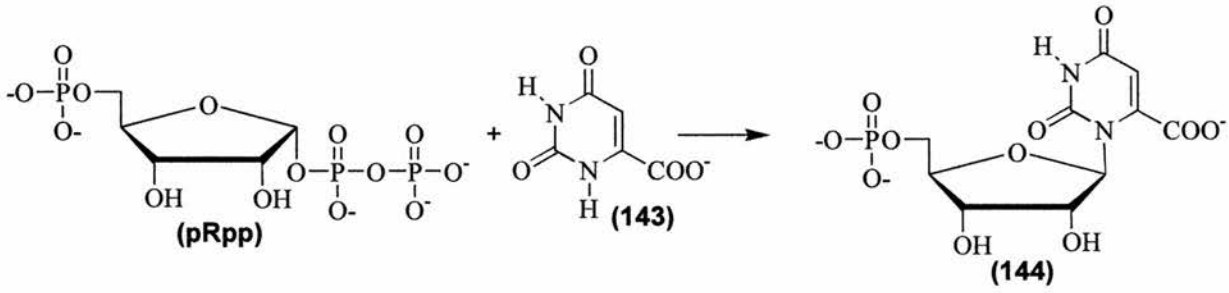
Glycosyl phosphates are very common and important intermediates in the metabolism of carbohydrates. They act as intermediates for the conversion of many sugars to sugar nucleotides, the donors for oligo- and poly-saccharide and glycoconjugate synthesis. Sugar nucleotides also play an important role in the formation and modification of sugars such as glucose, galactose, mannose, fucose and rhamnose.[12] A common problem in the synthesis of any glycosyl compound is the attainment of stereoselectivity or the ability to separate the anomeric mixtures in a facile manner. The synthesis of glycosyl phosphonates has provided similar challenges and various syntheses have been performed under stereoselective and non-stereoselective conditions.[13-16]

References are on page 145

5.1.2 Phosphoribosyltransferases: Known Binding Interactions

5.1.2.1 Introduction

The conversion of pRpp into DpA, an arabinose donor, by mycobacterial enzymes necessitates the presence of a pRpp binding protein. Metabolism of pRpp is a key step in the biosynthesis of pyridine, purine and pyrimidine nucleotides and also in the biosynthesis of histidine and tryptophan. Phosphoribosyltransferases, the group of enzymes which catalyse these reactions, transfer the ribose-5-phosphate moiety from pRpp onto a nitrogenous nucleophile such as orotate **(143)** (which leads to pyrimidine nucleotides), as can be seen in Scheme 40.



Scheme 40: Conversion of pRpp to UMP precursor by Orotate Phosphoribosyltransferase

Since phosphoribosyltranferases are the predominant enzymes involved in binding pRpp, it may be useful to investigate the structures and binding interactions of these proteins to aid in the design of compounds that inhibit the biosynthesis of DpA.

5.1.2.2 General Structures of the Enzyme Class

There are two classes of phosphoribosyltransferases (PRTases), type I and type II. The first of these is the most characterised class of PRTases, as only one structure is known from the type II class (quinolinate PRTase[17] from *Salmonella typhimurium*).

Class I PRTases have a conserved pRpp binding site which contains two adjacent carboxylate residues preceded by 4-5 hydrophobic residues and followed by 4 small polar residues. Interestingly, this conserved binding site is also found in a pRpp synthetase[18] but not in the aforementioned[17] type II PRTase.

5.1.2.3 Putative Mechanism of Catalysis

The mechanism of these PRTases has been reported to proceed via an oxocarbonium ion intermediate **(146)** as shown in Scheme 41.[19-23]

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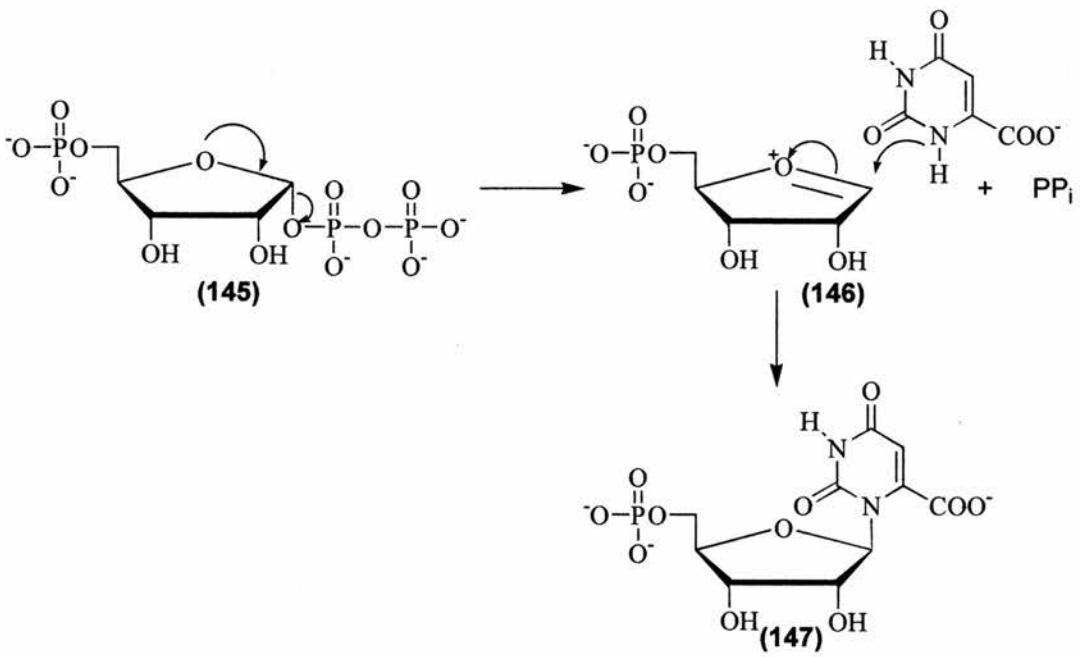

Scheme 41: Mechanism of Phosphoribosyl Transfer

Carbocyclic pRpp (cpRpp[24], **148**), a stable and practically useful analogue of pRpp which has no ring oxygen (Figure 36), has proved to be useful as a stable substrate analogue in crystal structure studies of two PRTases.[20,25] This structure was found to behave in a similar but non-catalytic fashion as compared with a crystal structure containing pRpp.[22]

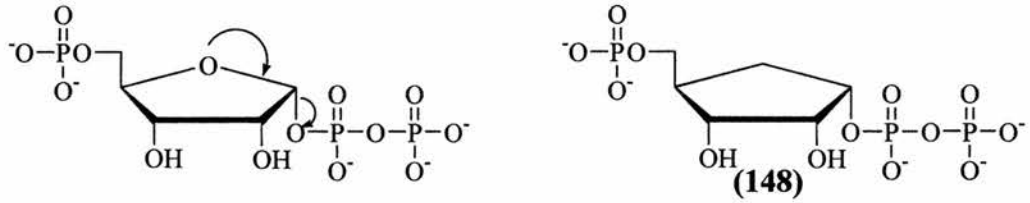

Figure 36: Stable but Structurally Similar Analogue of pRpp

Further support for an oxocarbonium ion transition state came from kinetic isotope studies[19] and isotope exchange studies[23] and it has become the broadly accepted mechanism for type I PRTases.

5.1.2.4 Important Specific Interactions Between pRpp and PRTases

Phosphoribosyltransferases anchor pRpp into the active site via the β-phosphate of the pyrophosphate tail and the 5-phosphate. Interactions with other functionalities of the substrate connect it, via hydrogen bonding, to a magnesium ion. The Mg^{2+} is

 References are on page 145

essential for binding and the electron withdrawing effect of the ion is thought to promote the formation of an oxocarbonium ion which is stabilised by the negative charge on the pyrophosphate tail. Figure 37 illustrates the anchoring of the two terminal phosphates by various protein residues and the interaction of Mg^{2+} with the internal functionalities of cpRpp.[20]

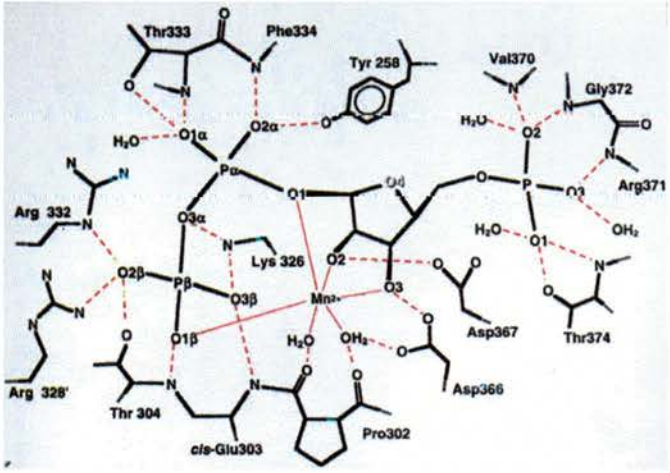

Figure 37: Important Binding Interactions of cpRpp With Glutamine pRpp Amidotransferase. The Mg^{2+} co-ordinates with O1,2 & 3 whereas the protein residues anchor the substrate via the terminal phosphates. O4 is drawn for illustrative purposes only as the ring is carbocyclic.[20]

5.1.2.5 Structural Changes in PRTases during Catalysis

The most dramatic change in the conformation of the PRTase occurs when a flexible loop moves to enclose pRpp, shielding it completely from excess solvent. The size of the site was perfect in shape and charge to the bound $Mn^{2+}(cpRpp)(H_2O)_2$. This substrate complex is buried within the protein through a total of 21 hydrogen bonds and salt bridges, which although remarkable for such a charged molecule, is thought to be necessary to protect it from hydrolysis when activated.[20]

5.1.2.6 Other Substrates Used in PRTases

Compounds which have exhibited inhibitory activity towards various PRTases include *ribo*-methylenebisphosphonates[26,27] **(149)**, *ribo*-phosphorothioates[28] **(150, 151)**, *ribo*-glycosylphosphonophosphates[29,30] **(152, 153)**, and *arabino*-glycosylphosphonophosphates[29] **(154)**. The relative inhibitory activity of these compounds with regards to pRpp is detailed in Table 4. Clearly, the most potent compound was methylenebisphosphonate **(149)** which was synthesised enzymatically from ribose-5-phosphate using β,γ-methylene ATP and *Salmonella typhimurium* pRpp

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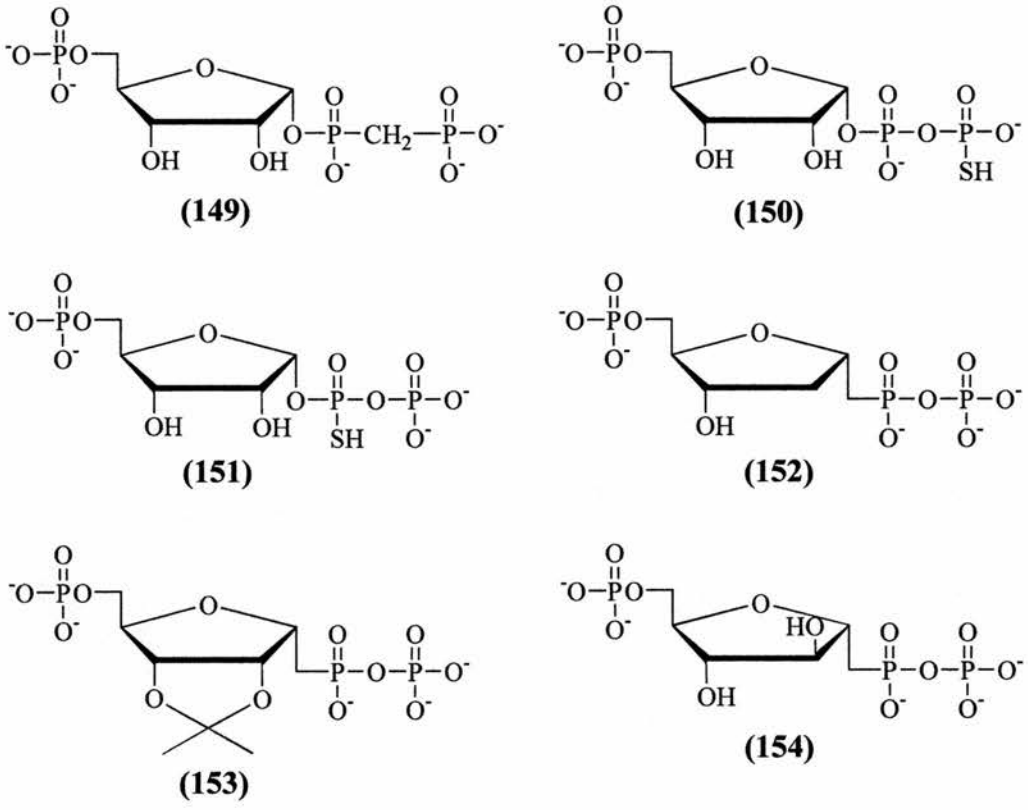
synthetase. Of the phosphorothioates, substitution at the β-phosphate **(11)** resulted in greater inhibition than substitution at the α-phosphate **(151)**.



(149) **(150)** **(151)** **(152)** **(153)** **(154)**

Figure 38: Compounds Which Have Shown Activity Towards PRTases

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Compound	Activity (pRpp=1)	Enzyme	Ref
149	5.88	Adenine PRTase (Ehrlich ascites-tumour cells)	27
149	5.26	Hypoxanthine PRTase (Ehrlich ascites-tumour)	27
149	0.16	Nicotinamide PRTase (rat liver)	27
149	0	Orotate PRTase (yeast)	27
149	2.66	Orotate PRTase (human erythrocyte)	26
149	0	Amido PRTase	27
150	~0.30	Hypoxanthine PRTase (human brain)	28
150	~0.30	Orotate PRTase (yeast)	28
151	~0.08	Hypoxanthine PRTase (human brain)	28
151	~0.08	Orotate PRTase (yeast)	28
152	0.04	Orotate PRTase (yeast)	30
152	0.06	Hypoxanthine-guanine PRTase (human)	30
152	0.33	Glutamine:amido PRTase (*E. coli*)	30
153	0.06	Orotate PRTase (yeast)	29
153	0.02	Orotate PRTase (yeast)	29

Table 4: Relative Activities of PRTase Inhibitors. Higher number reflects greater inhibitory activity with respect to pRpp.

Although substitution of the anomeric oxygen with a methylene bridge as in **(152)**, **(153)**, and **(154)** reduced activity somewhat, these compounds still exhibited competitive inhibition against pRpp in yeast orotate PRTase and **(152)** showed a higher activity towards hypoxanthine-guanine PRTase.

It is obvious from Table 4 that activity varies enormously depending on which enzyme is used (e.g. the most potent compound **(149)** for adenine PRTase showed no activity towards yeast orotate PRTase), therefore it is difficult to construct general structure-activity relationships for this class of enzyme.

5.1.3 Aims and Objectives

In light of difficulties encountered in the attempted synthesis of the putative biosynthetic intermediate pApp it was decided to make an analogue which was closely related in terms of orientation and size but which would be more stable to hydrolytic conditions. It was hoped that this analogue would display inhibitory properties by having a binding affinity for a *phosphoribosyl-* or *phosphoarabinosyl*-transferase involved in the biosynthesis of DpA but having no functionality that allows catalysis.

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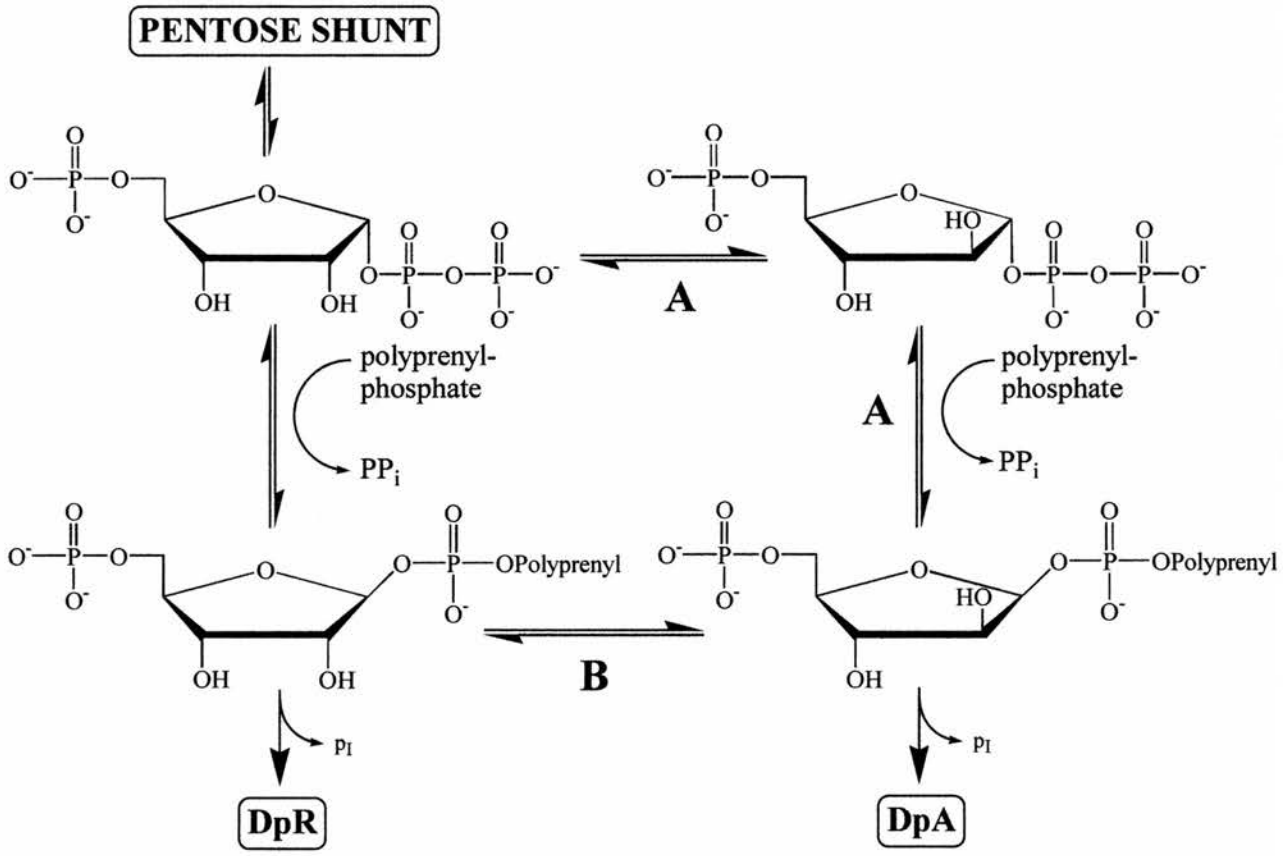
There are two obvious possible routes in the biosynthesis of DpA from pRpp (Scheme 42).[31] Path 'A' would firstly involve a C2 pRpp epimerase and subsequently a phosphoarabinosyltransferase (PATase) whereas path 'B' would initially require a phosphoribosyltransferase (PRTase) and then a 5P-DpR C2 epimerase.



Scheme 42: Biosynthetic Route From pRpp to DpA/DpR

The enzymes involved in the first step of both pathways would require a pRpp binding site. An enzyme catalysing the second step of path 'A' would also require a pApp binding site. It has been demonstrated that a typical binding site of pRpp in many PRTases does not involve O1, O2 or O3 in protein residue binding (see section 5.1.2.4 on page 116). It has also been shown that compounds with *arabino*-, *ribo*- and 2-deoxy-sugars display competitive inhibition at similar concentrations to each other (see section 5.1.2.6 on page 117).

It was therefore decided to synthesise the arabino-configured phosphonate **(154)** because it is a promising candidate for PRTase inhibition and has the correct configuration to fit a putative active site for a PATase. If a PATase did exist then the orientation of the hydroxyl at C2 would presumably be of great importance as it would

be the only basis on which to discriminate between pRpp and pApp. This compound was also desirable because of the stability gained through replacement of the labile anomeric pyrophosphate, a functionality which caused problems in the attempted synthesis of pApp.

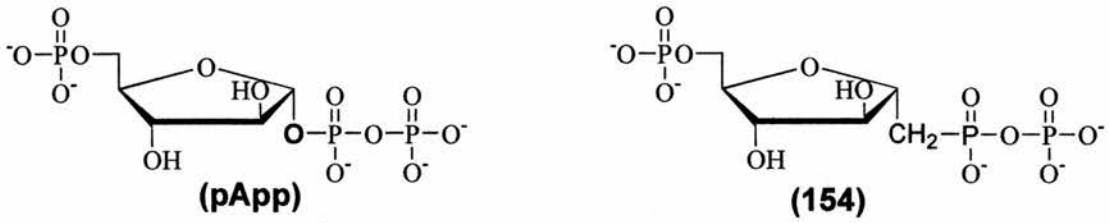


Figure 39: Putative Biochemical Intermediate (pApp) and Synthetic Target Compound (154).

References are on page 145

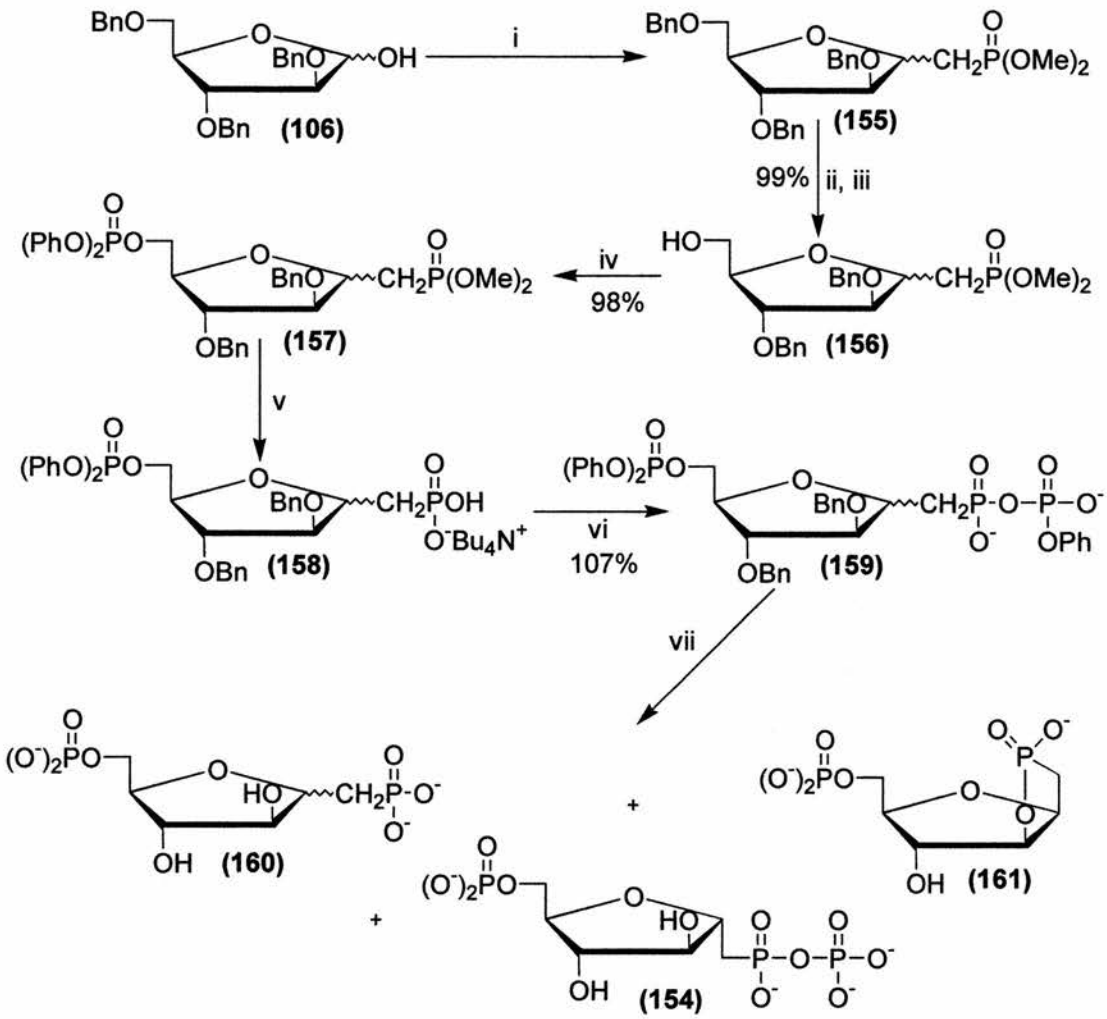
5.2 RESULTS AND DISCUSSION

5.2.1 Synthesis of pApp Phosphonate Analogue

5.2.1.1 Previous Synthesis

A previous synthesis of the pAcpp by McClard and co-workers[29] (Scheme 43) carried through a diastereomeric mixture of compounds which prevented detailed analysis of all but the separated final compounds. We note that McClard's publication relies almost exclusively on ^{1}H and ^{31}P NMR data to confirm structures of both synthetic intermediates and final compounds. No credible microanalyses were reported, and mass spectral analysis was not reported either. Commercially available tribenzylarabinose **(106)** was subjected to a Horner-Wadsworth-Emmons reaction with tetramethyl methylenebisphosphonate giving an inseparable mixture of epimers **(155)**.[26,32] Subsequent acetolysis and transesterification using the methods of Eby[33] yielded the unmasked 6-OH **(156)**. The free alcohol was phosphorylated using diphenylchlorophosphate, the dimethylphosphonate was dealkylated using bromotrimethylsilane and converted to the monotetrabutylammonium salt **(158)**. Synthesis of phosphonophosphate **(154)** was carried out using phenyl imidazolylphosphate (prepared *in situ* from the corresponding sodium salt of phenylphosphate and carbonyl diimidazole) and after deprotection, a separable mixture of three compounds **(154)**, **(160)** and **(161)** was obtained. The desired phosphonate **(154)** was obtained as its lithium salt, contaminated with lithium chloride.

References are on page 145



Reagents: (i) NaH, glyme, $H_2C[P(O)(OMe)_2]_2$; (ii) conc. H_2SO_4 , Ac_2O; (iii) Na, MeOH; (iv) $(PhO)_2(O)PCl$, Pyr; (v) TMSBr, CH_2Cl_2; (vi) carbonyldiimidazole, DMF, $(PhO)(O)P(O^-)_2Na^+_2$; (vii) 10% Pd on C, 10% Pt on C, 10% PtO_2, AcOH, H_2

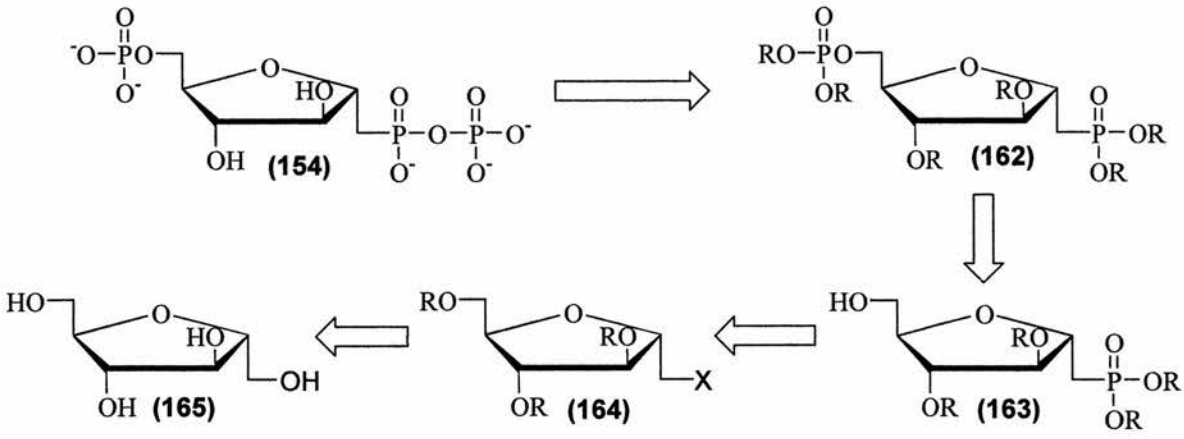
Scheme 43: McLard's synthesis of 2,5-Anhydro-5-phospho-1-phosphonophospho-D-mannitol.[29]

5.2.1.2 Retrosynthetic Analysis

In contrast to McLard's synthesis, it was decided to create a procedure which would allow the analysis of intermediates and that would be stereospecific, therefore eliminating the need for troublesome separations. A retrosynthetic analysis relating to our proposed route is shown in Scheme 44.

The first disconnection shows the need for a phosphate coupling to produce the phosphonophosphate. Since this step would introduce potentially the most labile bonds, it would be desirable to minimise the number of reactions that would follow this step. The introduction of a phosphate at the 6-position prior to phosphonophosphate coupling

would help minimise the later steps but would require an orthogonoal protecting group strategy to subsequently permit selective deprotection of the 1-phosphonate group.

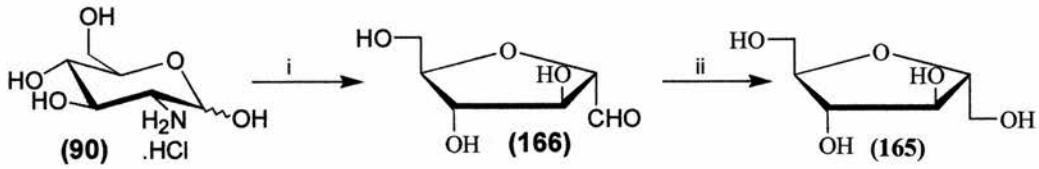


Scheme 44: Retrosynthetic analysis of the target compound.

Introduction of the phosphonate functionality can be achieved in a straightforward manner by an Arbuzov reaction on the appropriate primary halide. This leads us back to a readily available starting material 2,5-anhydro-D-mannitol, which has the necessary functionalities and stereochemistry already in place.

5.2.1.3 Protecting Group Manipulations

The starting material, 2,5-anhydromannitol **(165)**, an expensive commercially available product, was prepared from glucosamine hydrochloride using literature methods.[34,35]

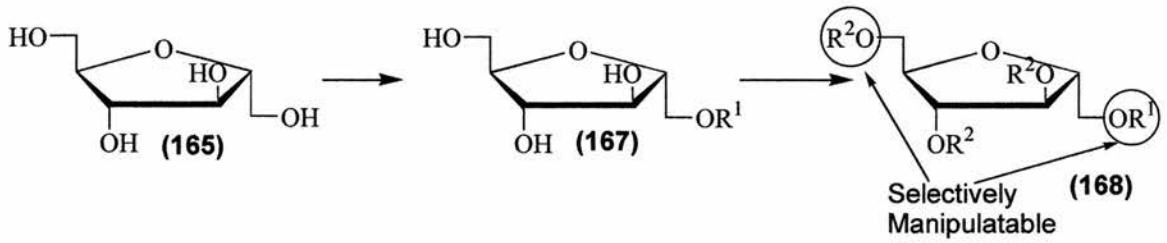


Reagents: (i) $NaNO_2$, H_2O, Amberlite IR120(H^+); (ii) $NaBH_4$, H_2O

Scheme 45: Synthesis of 2,5-Anhydro-D-mannitol.

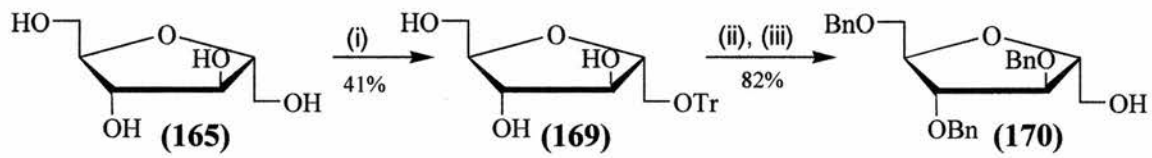
This procedure was easy to scale up, allowing the synthesis of quantities greater than 20g of **(165)**. The aim was then to make available only one of the primary hydroxyl groups of **(165)** for iodination whilst protecting the remaining three in a fashion that allowed selective manipulation of the other primary hydroxyl group.

 References are on page 145



Scheme 46: Protecting Group Strategy.

The first problem was to selectively protect only one of the primary hydroxyl groups of **(165)**. Since the starting material is a C2 symmetrical molecule there is no selectivity between the two primary hydroxyls. Therefore it was decided to use a small excess of a bulky protecting group to effect selectivity between primary and secondary hydroxyls. Of the many bulky protecting groups available, TBDPS and trityl were chosen as candidates due to their frequent use and the many methods for removal.[36] Of the two, tritylation was selected as, in our hands, it gave the higher yield of monoprotected material **(169)** and was thought to be more stable to benzylation conditions since TBDPS has been reported to lyse in the presence of hydride.[37]



Reagents: (i) TrCl, Pyr, 50°C; (ii) NaH, BnBr, DMF; (iii) TsOH, CH_2Cl_2/MeOH 5:1.

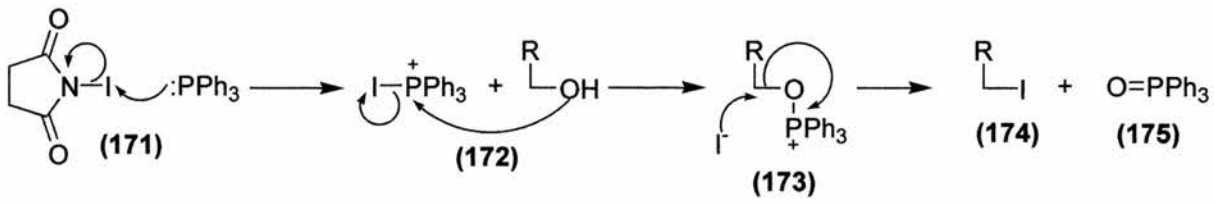
Scheme 47: Protecting Group Manipulations.

Selective tritylation of 2,5-anhydromannitol gave the primary mono-trityl derivative **(169)** in 41% yield. Subsequent benzylation and de-tritylation without purification of the intermediate, gave tri-protected derivative **(170)** in good overall yield. There was evidence[29] that the primary benzyl group of such compounds could be selectively manipulated as would be needed for later introduction of the 6-phosphate group.

5.2.1.4 Synthesis of the pApp Phosphonate Analogue

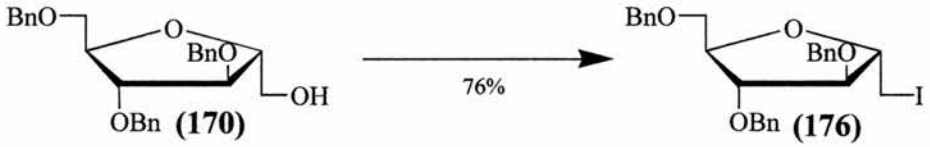
It was decided to convert the unmasked hydroxyl of **(170)** to a phosphonate using an Arbuzov reaction with an alkyl iodide as an intermediate. There are a number of examples[38-40] in the literature of direct iodination of a primary alcohol. The Palcic procedure using PPh_3, NIS and DMF was chosen for its mild conditions.[39]

 References are on page 145



Scheme 48: Mechanism for Direct Iodination of a Hydroxyl Group.

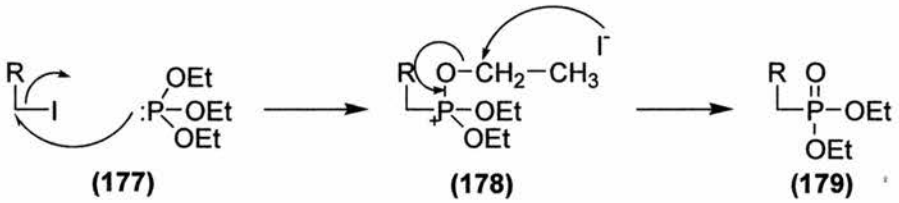
The large upfield shift of the ^{13}C signal attributed to C-1 [from 62.7ppm in **(170)** to 6.9ppm in **(176)**], a characteristic of alkyl iodides, reflected that the reaction had proceeded successfully.



Reagents: NIS, Ph_3P, DMF

Scheme 49: Synthesis of Alkyl Iodide.

The construction of a carbon-phosphorus bond can be undertaken in a variety of ways including the use of stabilised ylides and the Arbuzov reaction. Stabilised ylides have been used in the synthesis of sugar-1-phosphonate analogues.[41-43] As illustrated in McClard's work, the problem with this approach is that the resultant stereochemical mixture of α and β-isomers obviates detailed analysis of intermediates and necessitates difficult separations.[29] Therefore it was decided to proceed with an Arbuzov reaction to synthesise the phosphonate intermediate in a stereochemically pure fashion.

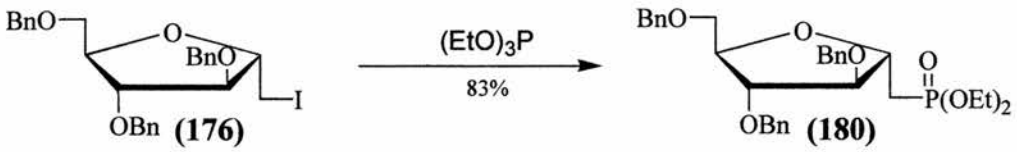


Scheme 50: Mechanism of the Arbuzov Reaction.

In a first attempt using trimethyl phosphite, no conversion of the iodide was observed. This was unfortunate since it would have allowed us to directly compare spectral characteristics of further compounds in the sequence with McClard's data.[29] Upon switching to triethyl phosphite as the phosphonylating agent, the reaction proceeded in a good yield. The identity of the product **(180)** was confirmed by the appearance of a characteristic signal at 28.2ppm in the ^{31}P spectrum and the observed

 References are on page 145

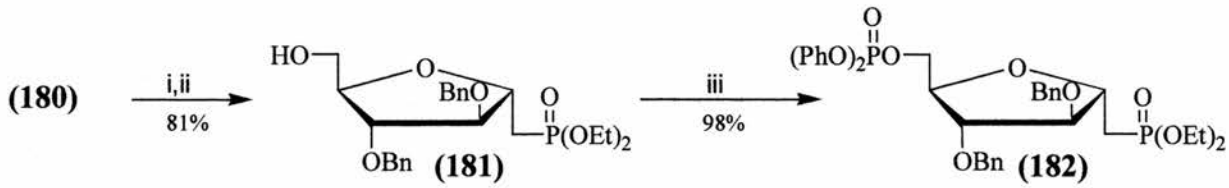
couplings of H-1 & 1'(18.9 & 18.4Hz), C-1 (137.5Hz) and C-2 (7.47Hz) with phosphorus.[29,44] It was postulated that the higher reflux temperature of triethyl phosphite was the reason for the difference in reactivity.



Scheme 51: Synthesis of Phosphonate

Previous work indicated that simultaneous removal of the 6-benzyl group and the ethyl protecting groups on the phosphate was possible using iodotrimethylsilane in CH_2Cl_2.[39] However, attempts to use this procedure on **(180)** resulted in a mixture of many compounds. Selective acetolysis of the benzyl group of the 6-position was therefore carried out using identical conditions to those reported by McClard[29] for the corresponding dimethyl phosphonate. Subsequent deacetylation resulted in the unmasking of the 6-OH giving **(181)** in a very acceptable yield.

References are on page 145

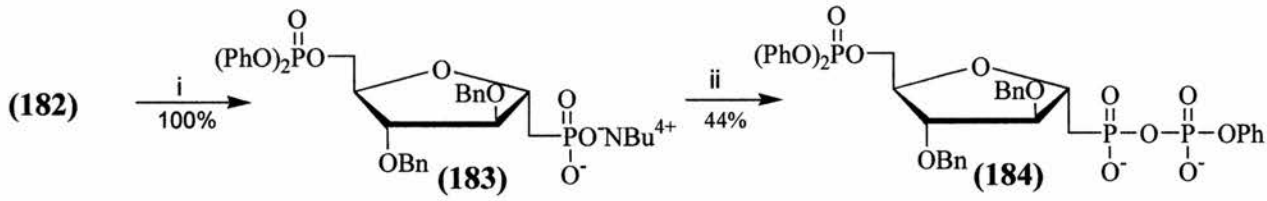


Reagents: (i) conc. H_2SO_4, Ac_2O; (ii) Na, MeOH; (iii) $(PhO)_2(O)PCl$, Pyr.

Scheme 52: Formation of Bisphosphate Analogue.

Phosphorylation of the primary hydroxyl group using diphenyl chlorophosphate, a standard phosphorylating agent,[45] proceeded without difficulty in an almost quantitative fashion to yield **(182)**. The appearance of a new signal at -12.97ppm in the ^{31}P spectrum, characteristic of a phosphate ester,[44] confirmed the identity of the product.

Dealkylation of the phosphonate using bromotrimethylsilane, and subsequent formation of the phosphonophosphate via activation of the phosphonate using carbonyldiimidazole was performed using conditions reported by McClard (Scheme 53).

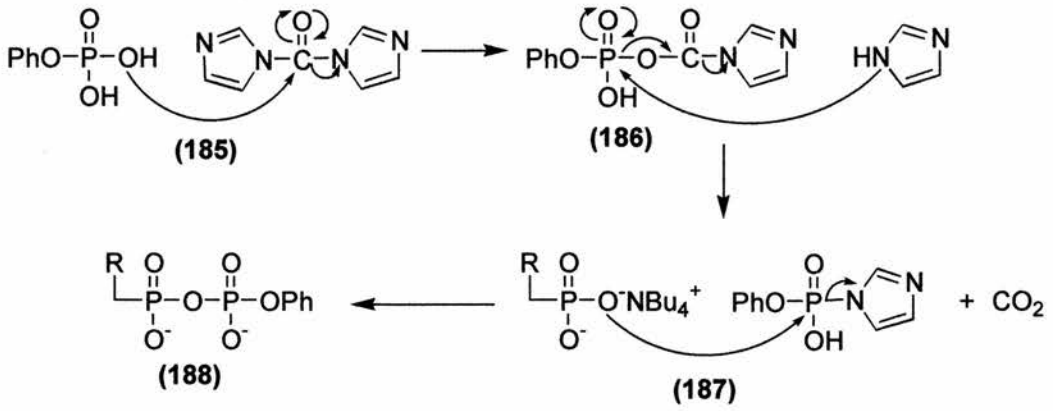


Reagents: (i) TMSBr, CH_2Cl_2; (ii) carbonyldiimidazole, DMF, $(PhO)(O)P(O^-)_2Na^+_2$.

Scheme 53: Phosphate Coupling

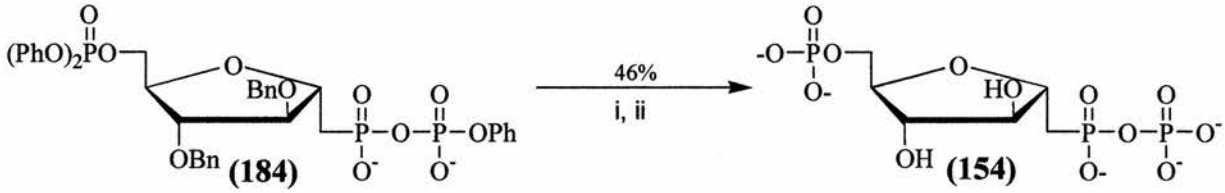
Purification yielded a compound that was pure by ^{31}P NMR and two doublets at 13.39ppm and -16.05ppm both with a coupling of 24.5Hz, indicating P-P coupling confirmed the presence of a phosphonophosphate. Attempts were made to further characterise compound **(184)** using MALDI and FAB mass spectrometry, however it was not possible to get a molecular ion or interpretable fragmentation patterns. The compound was also too hygroscopic for elemental analysis.

References are on page 145



Scheme 54: Mechanism of phosphate coupling.

The phenyl groups on **(184)** were removed from the phosphates using hydrogenation firstly with Adam's catalyst and then platinum on carbon to yield the final compound **(154)**. Attempts to remove the phenyl groups with only Adam's catalyst under hydrogenation conditions yielded an undesirable mixture of compounds. Characterisation of this compound in McClard's[29] synthesis was by NMR and elemental analysis, however, in the elemental analysis, large amounts of associated LiCl (crystallisation solution) were quoted which reflected the difficulty in obtaining accurate analytical data for this compound. The many possible ionisation states makes it difficult to obtain good mass spectrometry data, therefore there is a heavy reliance on NMR data with particular emphasis on H-P coupling constants and ^{31}P NMR data. The data quoted in the experimental section are in good agreement with the literature[29] values.



Reagents: (i) 10% Pd on C, AcOH, H_2; (ii) 10% Pt on C, 10% PtO_2, AcOH, H_2.

Scheme 55: Formation of Final Compound

5.2.1.5 Conclusion

Synthesis of the phosphonate analogue **(154)** was successfully completed over 12 steps from commercially available 2,5-anhydromannitol **(165)** in an overall yield of 3.4%. McClard's synthesis[29] was completed in 7 steps but since no yield is given for the final deprotection step (which necessitated a difficult separation of 3 compounds) a

 References are on page 145

direct comparison of overall yields is not possible. Working with single compounds is generally easier than working with mixtures due to better resolved TLC spots and NMR spectra that are more straightforward to elucidate. Therefore, although our synthesis is 5 steps longer than McClard's, it was possible to analyse the intermediates and the final deprotection step yielded only one compound.

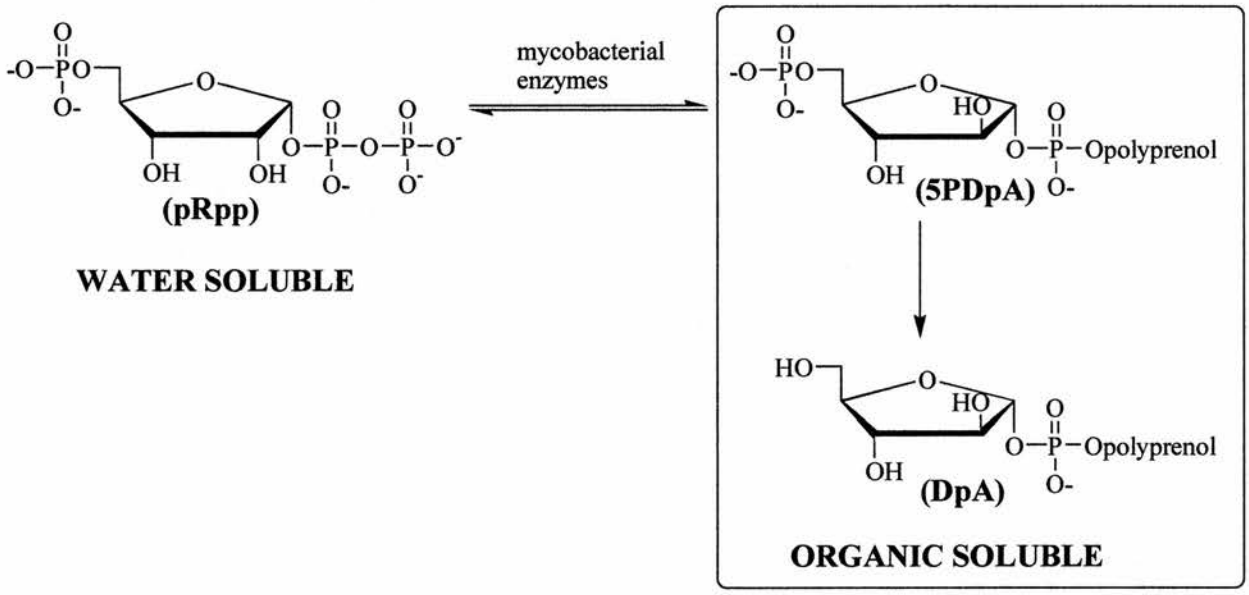
It was hoped that in undertaking this alternative route to the product, more detailed analysis of the intermediate compounds could be undertaken. This was successful with respect to detailed NMR spectra elucidation of the intermediates, however, many of these compounds were too hygroscopic to analyse by combustion analysis and it proved impossible to obtain molecular ions for several compounds by mass spectrometry techniques available in house (CI, ES, FAB, MALDI). Since the previous literature sythesis[29] provided only very brief analytical details on the intermediates, this synthesis has greatly improved what was previously known about these compounds but it still falls some way short in terms of high resolution analytical data.

References are on page 145

5.2.2 Biological testing of pApp Phosphonate Analogue

5.2.2.1 Testing **(154)** using *M. bovis* BCG strain

Testing of the compound **(154)** was firstly carried out using *M. bovis* BCG strain due to its similarities to *M. tuberculosis* and its lack of pathogenicity. Incubation of p[^{14}C]Rpp with the cell walls of the aforementioned organism at 37°C for 30 min in the absence and presence of **(154)** was carried out and the conversion to polyprenylphosphatearabinose organic soluble products 5-P-DpA and DpA is detailed in Figure 40.



Scheme 56: Conversion of pRpp into Organic Soluble Products

References are on page 145

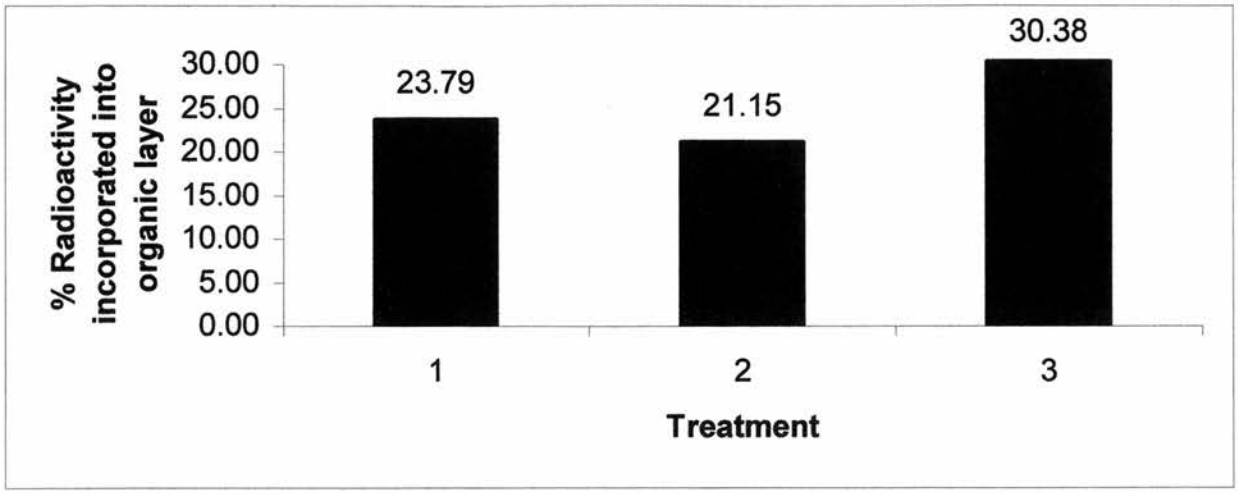


Figure 40: Percentage of radioactive organic soluble products derived from treatment of *M. bovis* BCG cell walls with radioactive pRpp in the absence (control) and presence of (154) (NH_3^+). 1: Control, 2: **(154)** (NH_3^+) 5mM, 3: **(154)** (NH_3^+) 5mM + 1% CHAPS.

The high conversion of p[^{14}C]Rpp to organic soluble material by the mycobacterial enzymes illustrated the lack of inhibitory activity associated with **(154)**. Since the analogous compound resembled the putative biochemical intermediate so closely, this came as a surprise. Experiments have shown that the addition of detergent to *M. smegmatis* cell walls increases the amount of pRpp converted into organic soluble product but that these products are exclusively ribose based and no arabinose based products were observed.[31] As an additional experiment it was decided to test **(154)** in the presence of detergent to ascertain if there was any difference in enzyme activity. This experiment (Figure 40, treatment 3) showed an increase in the level of enzyme activity and, as in previous experiments[31], the organic soluble products were ribose based only.

5.2.2.2 Testing **(154)** using *M. smegmatis*

In the presence of *M. smegmatis* cell walls, p[^{14}C]Rpp has been converted into a mixture of organic soluble products that are both ribose and arabinose based.[31] However, HPLC analysis of the neutral sugars derived[31] from the BCG cell wall mediated conversion of p[^{14}C]Rpp showed only arabinose to be present. Since the organisms display differences in enzyme activities, it was decided to test **(154)** using *M. smegmatis* cell walls as the source of mycobacterial enzymes to ascertain whether the compound would display inhibitory activity in a different organism. These experiments

 References are on page 145

were conducted using Na^+ and tetrabutylammonium salt forms of **(154)** in the presence and absence of detergent.

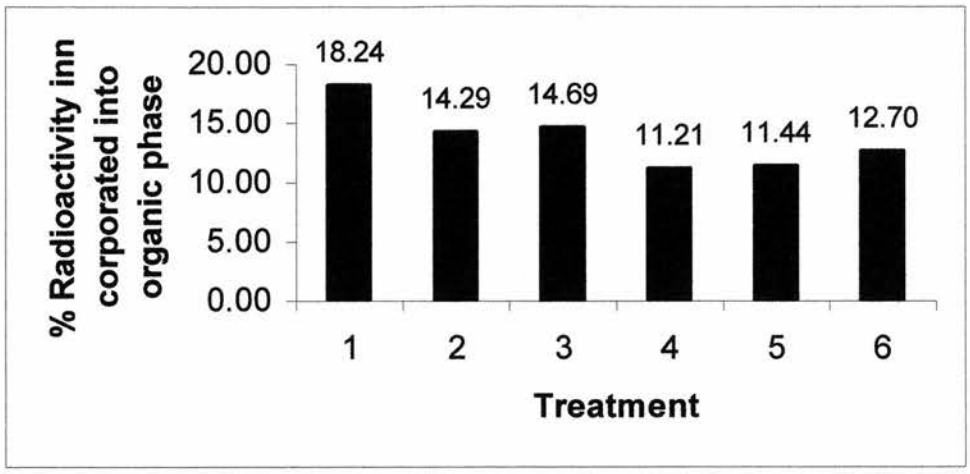


Figure 41: Graph showing percentage of radioactive organic soluble products derived from treatment of *M. smegmatis* cell walls with radioactive pRpp in the presence of various added materials. 1: Control, 2: **(154)** (Na^+) 5mM, 3: **(154)** (BuN_4^+) 5mM, 4: 1%CHAPS, 5: **(154)** (Na^+) 5mM + 1%CHAPS, 6: **(154)** (BuN_4^+) 5mM + 1%CHAPS.

The results, indicated in Figure 41 show that **(154)** inhibited radioactive incorporation into the organic layer approx. 20% at 5mM concentration with both salt forms in the absence of detergent (treatments 2 & 3) and in the presence of detergent (treatments 5 & 6) showed little difference from the detergent control (treatment 4).

5.2.3 Biological Testing of some Sugar 5- and 6- Phosphates

Since substitution of the anomeric pyrophosphate in pApp resulted in an analogue which possessed no inhibitory activity, it was decided to test a range of commercially available phosphosugars in order to ascertain how important ring size and 2-hydroxyl orientation was.

References are on page 145

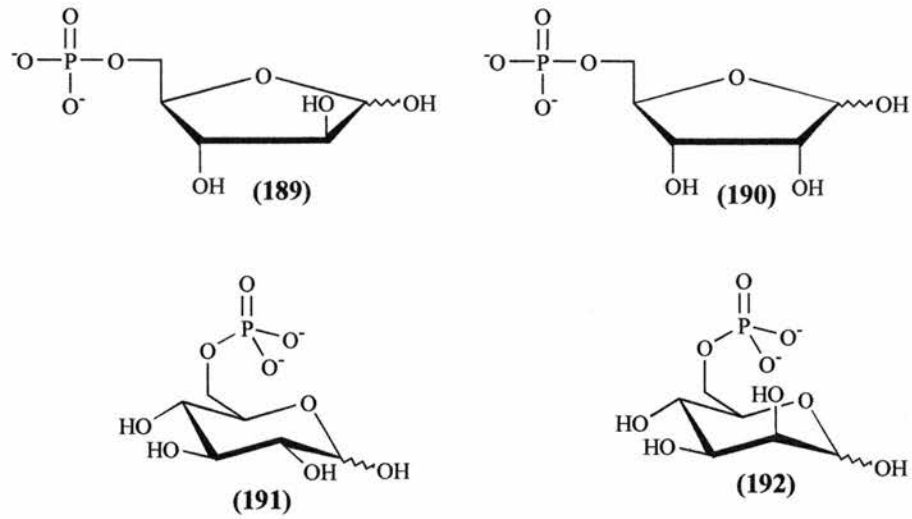


Figure 42: Commercially Available Phosphosugars to be Tested for Biological Activity

The compounds chosen were ribose-5-phosphate **(190)**, arabinose-5-phosphate **(189)**, glucose-6-phosphate **(191)** and mannose-6-phosphate **(192)**. The furanoses were selected due to their analogy to pRpp and pApp and the pyranoses were selected because they represented a six-membered ring analogy of ribose and arabinose due to the orientation of the 2-hydroxyls. It was decided to test them in both BCG and *M. smeg*.

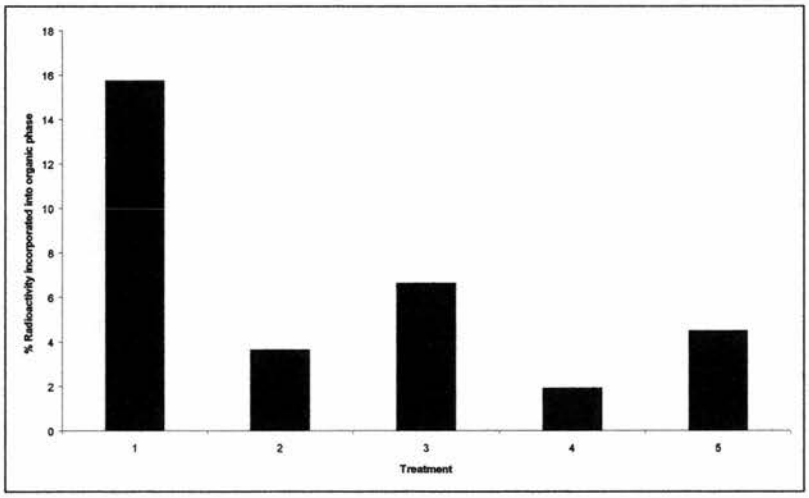


Figure 43: Percentage of radioactive organic soluble products derived from treatment of *M. bovis* BCG cell walls with radioactive pRpp in the presence of various phospho- sugars. 1: Control, 2: Rib-5-P 10mM, 3: Ara-5-P 10mM, 4: Glc-6-P 10mM, 5: Man-6-P 10mM.

In BCG at 10mM final concentration, the compounds all exhibited marked inhibitory activity ranging from 58-88%. The most active, surprisingly, was the Glc-6-P followed by Rib-5-P, Man-6-P and then Ara-5-P. It appeared that the most important

 References are on page 145

factor that the 2-hydroxyl was in the *ribo-* configuration and the results also showed that the pyranose configuration was favoured over the furanose configuration.

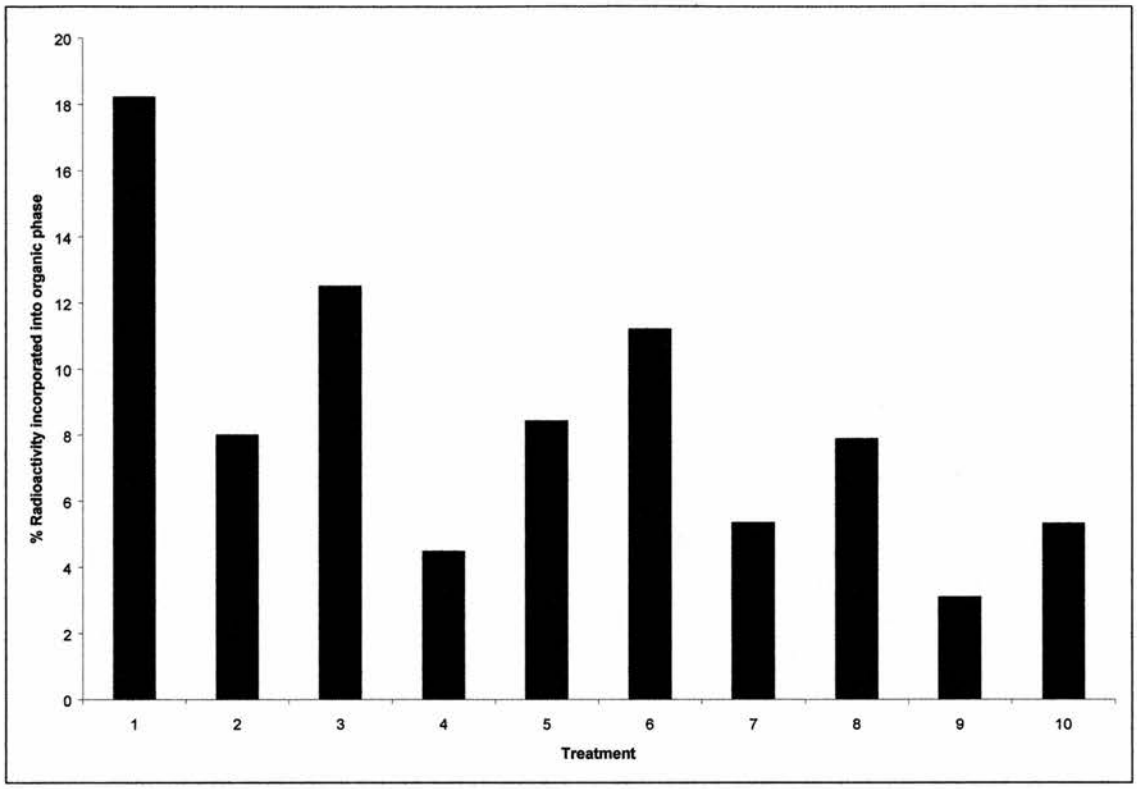


Figure 44: Graph showing percentage of radioactive organic soluble products derived from treatment of *M. smegmatis* cell walls with radioactive pRpp in the presence of various phospho- sugars. 1: Control, 2: Rib-5-P 5mM, 3: Ara-5-P 5mM, 4: Glc-6-P 5mM, 5: Man-6-P 5mM , 6: 1%CHAPS, 7: Rib-5-P 5mM + 1%CHAPS, 8: Ara-5-P 5mM + 1%CHAPS, 9: Glc-5-P 5mM + 1%CHAPS, 10: Man-5-P 5mM + 1%CHAPS.

In *M. smeg* at the lower final concentration of 5mM and in the presence and absence of detergent, the results followed a similar pattern. Inhibition levels ranged from 30-75% with reference to the respective controls (with and without detergent) and highest inhibitory level was obtained by Glc-6-P followed by Rib-5-P, Man-6-P and Ara-5-P.

References are on page 145

5.2.4 Conclusion

Although **(154)** is a closely related analogue of a putative intermediate in the conversion of pRpp into DpA, the above experiments show disappointingly that the compound shows no *in vitro* inhibitory activity against DpA formation at 5mM concentration. There are a number of possible explanations for this.

As was shown in Table 4 on page 119 a compound (**(149)** in this example) which displays high inhibitory activity in one enzyme system (mammalian orotate PRTase) may exhibit no activity in the same enzyme system of another organism (yeast orotate PRTase). In our work, it was not even known if a PRTase or a PATase was the target enzyme or if the putative PRTase was even of the type I variety.

It is also possible that the pH conditions in which the compound was tested were not high enough to cause the second ionisation **(154)** since phosphonates generally have higher pK_a's than their corresponding phosphates (see Table 3 on page 113). A third possibility is that precomplexation of **(154)** with a divalent metal ion may have been necessary for binding of the substrate as has been discovered in many PRTases (see section 5.1.2.4 on page 116).

Given that the most inhibitory compounds against PRTases contained methylene bridges in the pyrophosphate moiety (P-C-P), the synthesis of such an analogue of pApp ie. pApcp, probably represents the next logical step for future work in this area.

Testing of the four phosphosugars (Glc-6-P, Rib-5-P, MAn-6-P, Ara-5-P) showed that the biosynthesis of DpA could be inhibited without the use of detergent in two different organisms at concentrations of 5-10mM. Although these concentrations were too high for the commercially available compounds to be considered for biotherapeutic evaluation, this was the first report of the specific inhibition of DpA biosynthesis without the use of detergent. Exactly which enzyme in the biosynthetic pathway between pRpp and DpA these phosphosugars were inhibiting is a matter of conjecture.

References are on page 145

5.3 EXPERIMENTAL

5.3.1 Synthesis

2,5-Anhydro-1-O-trityl-D-mannitol **(169)**

2,5-Anhydro-D-mannitol (3g, 18.3mmol) was dissolved in pyridine (180cm^3) and trityl chloride was added (6.38g, 22.9mmol). The reaction temperature was increased to 50°C and the mixture was stirred for 2h at which point the pyridine was evaporated *in vacuo* and the residue was coevaporated several times with toluene. The residue was purified by flash column chromatography and the relevant fractions were combined and evaporated to the title compound as a sticky white solid (3.04g, 41%); δ_H 3.22 (1H, dd, $J_{6,6'}$ 10.3, $J_{5,6}$ 3.7, H-6), 3.38 (1H, bs, 6-OH), 3.40 (1H, dd, $J_{6,6'}$, $J_{5,6'}$ 3.3, H-6'), 3.71 (1H, bd, $J_{1,1'}$ 11.4, H-1), 3.82 (1H, dd, $J_{1,1'}$, $J_{1',2}$ 2.4, H-1'), 4.02-4.17 (6H, m, H-2,3,4,5, 3-OH, 4-OH), 7.11-7.45 (15H, m, 3x*Ar*) δ_C 62.2 (C-1), 64.9 (C-6), 78.0, 79.4 (C-3 and C-4), 83.8 (C-5), 84.4 (C-2), 87.5 (CPh$_3$), 125.3-129.0 (*Ar*), 143.4 (quat. *Ar*). This material was used immediately in the next step.

2,5-Anhydro-3,4,6-tri-O-benzyl-D-mannitol **(170)**

A suspension of NaH (782mg, 32.5mmol) in DMF (65cm^3) was carefully added to a solution of compound **(169)** (2.94g, 7.24mmol) in DMF (65cm^3) at 0°C and the mixture was stirred at this temperature until bubbling ceased. To this mixture was slowly added BnBr (2.84cm^3, 23.9mmol) and the reaction was allowed to warm to room temperature whilst stirring was continued overnight. Quenching was achieved by the careful dropwise addition of MeOH (25cm^3) and the mixture was extracted with CH_2Cl_2 which was washed several times with a dilute brine solution, dried over Na_2SO_4 and evaporated under vacuum. Residual DMF was discovered, therefore the syrup was dissolved in Et_2O, washed, dried and evaporated as above to a thin, light yellow oil. The oil was dissolved in CH_2Cl_2/MeOH (5:1) (350 cm^3), TsOH (2.06g, 10.86mmol) was added and the reaction was stirred for 1 h. The reaction mixture was washed several times with saturated $NaHCO_3$, dried over Na_2SO_4 and evaporated *in vacuo*. Further purification was carried out using flash column chromatography to yield the title compound as a white powder (2.57g, 82%); $[\alpha_D]$ –10.1 (*c* 0.25, $CHCl_3$), m.p. 139-141°C, δ_H 3.60 (1H, dd, $J_{6,6'}$ 10.0, $J_{5,6}$ 5.6, H-6), 3.64 (1H, dd, $J_{6,6'}$, $J_{5,6'}$ 6.1, H-6'), 3.78 (1H, dd, $J_{1,1'}$ 11.8, $J_{1,2}$ 3.6, H-1), 3.72 (1H, dd, $J_{1,1'}$, $J_{1',2}$ 5.3, H-1'), 4.11 (2H, m, H-3,4), 4.18 (1H, m, H-2),

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4.30 (1H, bdt, $J_{4,5}$ 3.9, $J_{5,6}$ & $J_{5,6'}$ 5.8, H-5), 4.57 (4H, s, 2 x BnC$\underline{H}_2$), 4.60 (2H, s, BnC$\underline{H}_2$), 7.28-7.41 (15H, Ar), δ_C 62.7 (C-1), 70.0 (C-6), 71.8 (Bn$\underline{C}H_2$), 72.0 (Bn$\underline{C}H_2$), 73.4 (Bn$\underline{C}H_2$), 81.9 (C-5), 83.3 (C-2), 84.2 (C-3), 84.6 (C-4), 127.7-128.4 (*Ar*), 137.6 (quat. *Ar*), 137.7 (quat. *Ar*), 138.0 (quat. *Ar*). Calcd. for $C_{27}H_{30}O_5$: $M^{\cdot+}$ 434.2093, $M^{\cdot}$+H 435.2171; Found: CI, $M^{\cdot}$+H 435.2181.

2,5-Anhydro-3,4,6-tri-O-benzyl-1-diethylphosphono-D-mannitol **(180)**

To a solution of **(170)** (2.46g, 5.67mmol), in DMF (86cm^3) was slowly added NIS (2.55g, 11.30mmol), followed by Ph_3P (2.96g, 11.3mmol) with stirring and the reaction mixture turned dark red. After 0.5 h, the reaction was quenched by the addition of MeOH and the solvent mixture was evaporated to a thin syrup which was dissolved in Et_2O. The resultant emulsion was washed several times with ice cold water followed by 5% aqueous sodium thiosulfate solution, dried over Na_2SO_4 and evaporated to a white solid. The solid was dissolved in the minimum volume of CH_2Cl_2 and applied to a silica gel column which was then eluted with 100% hexane followed by hexane/EtOAc 6:1 whereupon the compound containing fractions were combined and evaporated to give 2,5-Anhydro-3,4,6-tri-O-benzyl-1-iodo-D-mannitol **(176)** as a yellow oil (2.35g, 76%); δ_H 3.30 (1H, dd, $J_{1,1'}$ 10.1, $J_{1,2}$ 5.6, H-1), 3.36 (1H, dd, $J_{1,1'}$, $J_{1',2}$ 8.0, H-1'), 3.54 (1H, dd, $J_{6,6'}$ 10.0, $J_{5,6}$ 6.2, H-6), 3.61 (1H, dd, $J_{6,6'}$, $J_{5,6'}$ 5.9, H-6'), 4.10 (1H, m, H-3,4), 4.24 (1H, ddd, $J_{1',2}$, $J_{1,2}$, $J_{2,3}$ 2.9, H-2), 4.35 (1H, bdt, $J_{5,6}$ & $J_{5,6'}$ 6.0, $J_{4,5}$ 3.2, H-5), 4.52 (2H, s, BnC$\underline{H}_2$), 4.57 (2H, s, BnC$\underline{H}_2$), 4.58 (2H, s, BnC$\underline{H}_2$), 7.26-7.42 (15H, m, *Ar*), δ_C 6.9 (C-1), 70.3 (C-6), 71.7 (Bn$\underline{C}H_2$), 72.0 (Bn$\underline{C}H_2$), 73.4 (Bn$\underline{C}H_2$), 82.9 (C-5), 83.0 (C-2), 84.9 (C-3), 86.1 (C-4), 127.7-128.5 (*Ar*), 137.6 (quat. *Ar*), 138.0 (2 x quat. *Ar*). Compound **(176)** (2.13g, 3.91mmol) was dissolved in triethyl phosphite (40cm^3) and the solution was refluxed overnight at which point the reaction had proceeded to completion. Excess triethyl phosphite was evaporated under a high vacuum and the resulting residue was purified by flash column chromatography. Combination and evaporation of the phosphonate containing fractions yielded the title compound as a hygroscopic yellow oil (1.80g, 83%); δ_H 1.25 (3H, t, *J* 7.1, C$\underline{H}_3$CH$_2$OP), 1.26 (3H, t, *J* 7.1, C$\underline{H}_3$CH$_2$OP), 2.15 (1H, ddd, $J_{1,P}$ 18.9, $J_{1,1'}$ 15.2, $J_{1,2}$ 6.3, H-1), 2.26 (1H, ddd, $J_{1',P}$ 18.4, $J_{1,1'}$, $J_{1',2}$ 8.0, H-1'), 3.53 (1H, dd, $J_{6,6'}$ 9.9, $J_{5,6}$ 6.4, H-6), 3.59 (1H, dd, $J_{6,6'}$, $J_{5,6'}$ 6.0, H-6'), 3.88-4.08 (6H, m, 2 x CH$_3$C$\underline{H}_2$OP, H-3,4), 4.23 (1H, bdt, $J_{5,6}$ & $J_{5,6'}$ 6.2, $J_{4,5}$ 3.0, H-5), 4.41-4.60 (7H, m, 3 x BnC$\underline{H}_2$, H-2), 7.30 (15H, m, *Ar*), δ_C 16.5 ($\underline{C}H_3CH_2OP$),

16.6 ($\underline{C}H_3CH_2OP$),30.1 (C-1, $J_{C,P}$ 137.5), 61.7 ($CH_3\underline{C}H_2OP$, $J_{C,P}$ 6.59), 61.9 ($CH_3\underline{C}H_2OP$, $J_{C,P}$ 6.56), 70.7 (C-6), 71.7 (Bn$\underline{C}H_2$), 71.9 (Bn$\underline{C}H_2$), 73.5 (Bn$\underline{C}H_2$), 78.4 , 82.5, 85.3 (C-3, C-4 and C-5), 86.7 (C-2, $J_{C,P}$ 7.47), 127.8-128.6 (*Ar*), 137.8 (quat. *Ar*), 138.0 (quat. *Ar*), 138.3 (quat. *Ar*), δ_P 28.2 ($RCH_2\underline{P}(O)OR$). No molecular ion could be obtained by CI, ES, MALDI and FAB MS.

2,5-Anhydro-3,4-di-O-benzyl-1-diethylphosphono-D-mannitol **(181)**

A solution of conc. H_2SO_4 (2 drops/cm^3) in Ac_2O (0.5cm^3) was added to a solution of **(180)** (500mg, 0.90mmol) in Ac_2O (2.0cm^3) and the reaction was stirred for 2 h. The mixture was poured onto crushed ice, stirred for 1 h, extracted into CH_2Cl_2 and the layers were separated. The organic extract was washed twice with Na_2CO_3 (10% aqueous solution), dried over Na_2SO_4 and evaporated to a sweet-smelling yellow oil. The oil was then dissolved in anhydrous MeOH (25cm^3), a small piece of sodium was added at 0°C and the reaction was left stirring overnight. Amberlite IR120(H^+) resin was added until the solution reached neutrality and the mixture was then filtered and evaporated. Flash column chromatography was utilised to purify the product further and this resulted in a hygroscopic off-white solid (342mg, 81%); δ_H 1.28 (6H, t, *J* 7.1, 2 x $C\underline{H}_3CH_2OP$), 2.17 (1H, ddd, $J_{1,P}$ 18.9, $J_{1,1'}$ 15.2, $J_{1,2}$ 6.7, H-1), 2.26 (1H, ddd, $J_{1',P}$ 18.5, $J_{1,1'}$, $J_{1',2}$ 7.8, H-1'), 3.70 (2H, m, H-6,6'), 4.01-4.14 (6H, m, 2 x $CH_3C\underline{H}_2OP$, H-3,4), 4.17 (1H, bdt, $J_{5,6}$, $J_{5,6'}$ 4.8, $J_{4,5}$ 3.5, H-5), 4.47-4.63 (5H, m, 2 x Bn$C\underline{H}_2$, H-2), 7.25-7.35 (10H, m, *Ar*), δ_C 16.5 ($\underline{C}H_3CH_2OP$), 16.6 ($\underline{C}H_3CH_2OP$), 29.8 (C-1, $J_{C,P}$ 137.5), 61.8 ($CH_3\underline{C}H_2OP$, $J_{C,P}$ 6.44), 62.0 ($CH_3\underline{C}H_2OP$, $J_{C,P}$ 6.24), 63.1 (C-6), 71.7 (Bn$\underline{C}H_2$), 72.2 (Bn$\underline{C}H_2$), 78.4 (C-5), 84.2, 84.6 (C-3 and C-4), 86.1 (C-2, $J_{C,P}$ 8.50), 127.9-128.7 (*Ar*), 137.6 (quat. *Ar*), 137.7 (quat. *Ar*), δ_P 28.2 ($RCH_2\underline{P}(O)OR$). No molecular ion could be obtained by CI, ES, MALDI and FAB MS.

2,5-Anhydro-3,4-di-O-benzyl-1-diethylphosphono-6-diphenylphospho-D-mannitol **(182)**

Diphenyl chlorophosphate (208μL, 1.0mmol) was added to a solution of **(181)** (312mg, 669μmol) in pyridine (6cm^3) at 0°C and the reaction mixture was allowed to warm to room temperature overnight with stirring. The reaction mixture was evaporated *in vacuo* and was coevaporated several times with toluene at which point minimal $CHCl_3$ was added until dissolution occurred. The solution was applied to a silica gel column pre-packed in toluene and one column volume of toluene was eluted followed by an isocratic

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elution using EtOAc/toluene 6:1. Combination and evaporation of the relevant fractions yielded the desired compound as a white solid (458mg, 98%); *Anal.* Calcd for $C_{36}H_{42}O_{10}P_2$: C, 62.07; H, 6.08. Found C, 61.80; H, 6.35, δ_H 1.27 (6H, dt, *J* 7.06, *J* 1.54, 2 x $C\underline{H}_3CH_2OP$), 2.15 (1H, dd, $J_{H,P}$ 18.9, $J_{1,2}$ 6.4, H-1), 2.20 (1H, dd, $J_{H,P}$ 18.4, $J_{1',2}$ 7.85, H-1'), 3.97-4.61 (14H, m, 2 x $CH_3C\underline{H}_2OP$, 2 x $BnC\underline{H}_2$, H-2,3,4,5,6,6'), 7.12-7.38 (20H, *Ar*), δ_C 16.3 ($\underline{C}H_3CH_2OP$), 16.4 ($\underline{C}H_3CH_2OP$), 61.6 ($CH_3\underline{C}H_2OP$, $J_{C,P}$ 6.54), 61.9 ($\underline{C}H_3CH_2OP$, $J_{C,P}$ 6.54), 68.1 (C-6, $J_{C,P}$ 6.60), 71.6 ($Bn\underline{C}H_2$), 71.8 ($BnCH_2$), 79.0 (C-3 or 4), 81.7 (C-5, $J_{C,P}$ 8.40), 84.3 (C-3 or 4), 85.8 (C-2, $J_{C,P}$ 7.95), 120.0-129.7 (*Ar*), 137.3 (quat. Bn *Ar*), 137.5 (quat. Bn *Ar*), 150.4 (quat. Ph *Ar*), 150.5 (quat. Ph *Ar*), δ_P 26.07 ($RCH_2\underline{P}$-), -12.97 (RCH_2OP-).

Lit.,[29] δ_H 2.03 (2H, m, H-1,1'), 3.72 (6H, m, $-OCH_3$), 4.17 (10H, br m, sugar envelope and $-C\underline{H}_2Ph$), 7.34 (20H, *Ar*), δ_P 30.22 ($RCH_2\underline{P}$-), -11.63 (RCH_2OP-).

2,5-Anhydro-3,4-di-O-benzyl-1-phosphono-6-diphenylphospho-D-mannitol **(183)**

To a solution of **(182)** (420mg, 603μmol) in CH_2Cl_2 (3.5cm^3) was added bromotrimethylsilane (224μL, 1.70mmol) and the reaction was stirred for 16 h. Excess solvent was evaporated *in vacuo* and MeOH was evaporated off the residue several times to afford a thick brown syrup (422mg, >100%), δ_H 2.20-2.32 (2H, m, H-1,1'); 3.91-4.93 (10H, 2 x $BnC\underline{H}_2$, H-2,3,4,5,6,6'), 7.13-7.31 (*Ar*), 10.48 (2-3H, s, $RCH_2P(O)(O\underline{H})_2$, δ_C 29.5 (C-1, $J_{C,P}$ 140.0), 68.4 (C-6), 71.9 ($Bn\underline{C}H_2$), 72.0 ($Bn\underline{C}H_2$), 80.9 (C-5, $J_{C,P}$ 5.59), 84.0 (C-3 or 4), 86.9 (C-2, $J_{C,P}$ 13.59), 120.0-129.9 (*Ar*), 137.2 (quat. Bn *Ar*), 137.4 (quat. Bn *Ar*), 150.2 (quat. Ph *Ar*, $J_{C,P}$ 4.83), 150.3 (quat. Bn *Ar*), δ_P 28.57 ($RCH_2\underline{P}$-), -13.66 (RCH_2OP-). This compound was used directly in the next step.

2,5-Anhydro-3,4-di-O-benzyl-1-(phenylphospho(oxy))phosphono-6-diphenylphospho-D-mannitol **(184)**

Disodium phenyl phosphate (198mg, 779μmol) was dissolved in minimal H_2O and applied to a column of Amberlite 118 (H^+) and the acidic eluate was collected. The eluate was evaporated *in vacuo*, coevaporated several times with anhydrous CH_3CN and the resultant off-white solid was dissolved in anhydrous DMF (3.5cm^3). Carbonyldiimidazole (127mg, 779μmol) was added to the solution and the reaction temperature was elevated to 45°C for 45 min. Compound **(183)** was dissolved in 0.391M tetrabutylammonium hydroxide (1.41cm^3), diluted with EtOH and the solution

was evaporated *in vacuo* then coevaporated several times with anhydrous CH_3CN. The residue was dissolved in anhydrous DMF (3.5mL) and this solution was added dropwise to the phenylphosphoimidazolate solution above. The mixture was stirred at 45°C for 18 h. The reaction mixture was evaporated under high vacuum, coevaporated several times with toluene followed by CH_3CN and was put on a vacuum manifold for 10 min. The resultant residue was dissolved in H_2O (10cm^3) with 2 drops of NH_4OH (29.5%) and was applied to a column of Amberlite 118 (NH_4^+). Collection and evaporation of the alkaline fraction resulted in a residue that was dissolved in CH_3CN/H_2O/29.5% NH_4OH (100:2:1), and loaded onto a flash column (200x30mm). The column was then eluted with a gradient of CH_3CN/H_2O/29.5% NH_4OH (100:2:1; 175cm^3, 93:7:1; 200cm^3, 88:12:1; 500cm^3) and 8cm^3 fractions were taken. Fractions 122-137 were combined and evaporated to yield the title compound as a white solid (196mg, 44%); δ_H 1.58-2.15 (2H, m, H-1,1'), 3.85-4.45 (10H, m 2 x BnC$\underline{H}_2$, H-2,3,4,5,6,6'), 6.85-7.30 (*Ar*), δ_C 67.4 (C-6, $J_{C,P}$ 5.2), 71.2 (Bn$\underline{C}H_2$), 71.53 (Bn$\underline{C}H_2$), 79.4 (C-3 or 4), 80.3 (C-5, $J_{C,P}$ 6.7), 83.4 (C-3 or 4), 87.4 (C-2, $J_{C,P}$ 13.1) δ_P 13.39 (RCH$_2\underline{P}$(O)(OH)OP, $J_{P,P}$ 24.5), -12.83 (RCH_2OP-), -16.05 (PhO(OH)$\underline{P}$(O)OP, $J_{P,P}$ 24.5). (Calcd. for $C_{38}H_{39}O_{13}P_3$: M$^+$+H 797.64, M$^+$+Na 819.62; Found MALDI MS M$^+$+H 797.2050, M$^+$+Na 819.1490. No molecular ion could be obtained by ES, CI and FAB MS.

Lit.,[29] δ_H 2.05 (2H, m, H-1,1'), 4.01 (10H, m 2 x BnC$\underline{H}_2$, H-2,3,4,5,6,6'), 7.15 (*Ar*), δ_P 13.76 (RCH$_2\underline{P}$(O)(OH)OP, $J_{P,P}$ 27), -12.34 (RCH_2OP-), -16.30 (PhO(OH)$\underline{P}$(O)OP, $J_{P,P}$ 27).

2,5-Anhydro-1-phospho(oxy)phosphono-6-phospho-D-mannitol **(154)**

Compound **(184)** (150mg, 194μmol) was dissolved in AcOH (2cm^3), 10% Pd on carbon (40mg) was added and the reaction was stirred under H_2 (1 atm) for 4 h. The reaction mixture was filtered through Celite and to the filtrate was added 10% PtO_2 (40mg) and 10% Pt on carbon (40mg). The mixture was stirred under H_2 (1 atm) for 4 h at which point the reaction had proceeded to completion. Evaporation *in vacuo* removed the AcOH and the residue was purified on Dowex 1-X8 using a gradient of NH_4HCO_3 (0.1-0.8M). The relevant fractions were combined, evaporated and coevaporated many times with EtOH to remove residual NH_4HCO_3 to yield the title compound as a white solid (37mg, 46%), δ_H 2.25 (2H, dd, $J_{1\&1',P}$ 18.7, $J_{1,1'}$ 7.0, H-1,1'), 3.98 (2H, m, H-6,6'), 4.00-4.21 (4H, m, H-2,3,4,5), δ_C 28.9 (C1), 60.9 (C6), 74.0 (C4), 75.6 (C2), 77.3 (C3 or C5),

78.1 (C3 or C5), δ_P 14.31 ($RCH_2\underline{P}(O)(OH)OP$, broad unresolved peak), 0.72 ($RCH_2O\underline{P}$), -10.57 ($O\underline{P}(O)(OH)OPCH_2$). Lit.,[29] δ_H 2.19 (2H, dd, $J_{1\&1',P}$ 18.0, $J_{1,1'}$ 6.4, H-1,1'), 3.95 (2H, m, H-6,6'), 4.00-4.22 (4H, m, H-2,3,4,5), δ_C 29.85 (C1, $J_{C,P}$ 136.0), 61.70 (d, C6, J_{POC} 5.1), 73.96 (probably C4), 76.07 (C2), 78.16 (d, C3 or C5, J 7.2), 78.60 (d, C3 or C5, J 7.9), δ_P 11.80 (d, J_{POP} 25.64, $RCH_2\underline{P}(O)(OH)OP$), 0.97 ($RCH_2O\underline{P}$), -10.24 (d, J_{POP} 25.64,$O\underline{P}(O)(OH)OPCH_2$). (Calcd. for $C_6H_{15}O_{13}P_3$: $M^{\cdot}$ 387.9725, $M^{\cdot}$-H 386.9647; Found, ESMS (-ve ion), $M^{\cdot}$-H 386.9650.

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5.3.2 Biological Testing

M. bovis BCG Cell Wall Enzyme Preparation

M. bovis BCG was grown to mid-log phase in Sautons broth,[46] harvested by centrifugation, and transferred into buffer A, which consisted of 50mM MOPS and 5mM $MgCl_2$ at a pH of 7.6. The suspended cells were then subjected to probe sonication at 4°C for a total time of 20 min in twenty 60 s pulses with 90 s cooling periods. If unbroken cells were observed to collect as an aggregate at the bottom of the tube after allowing the sonicate to stand for 5 min then the suspension was sonicated further using the above methodology until complete homogeneity. The suspension was then centrifuged at 14,000g and 4°C for 20 min, the supernatant was decanted and the pellet was resuspended in buffer A. This was repeated twice and the resultant pellet was resuspended in a minimal amount of buffer B, which consisted of 50mM HEPES, 5mM $MgCl_2$ and 0.5mM MnCl2 at a pH of 7.6. Protein concentration was determined using the Bradford assay[47] and the suspension was adjusted to a concentration of 5 mg/mL using buffer B and was then used as the "BCG cell walls."

Treatment of p[^{14}C]Rpp with BCG Cell Walls

Pentadecaprenol phosphate (1μL) was added to a microcentrifuge tube and was dried by centrifugal evaporation. To this material was added BCG cell walls (200μL), ATP (40mM, 4μL), and β-NAD^+ (40mM, 4μL), the mixture was sonicated in a standard ultrasound cleaning bath for 30 s and pre-incubated at 37°C for 5 min at which point pRpp (186,000 cpm, 5μL in 0.7 M sodium acetate) was added. The mixture was then incubated for 30 min at 37°C, $CHCl_3$, MeOH and H_2O were added to give a 8:4:3 Folch mixture which was vortexed and briefly centrifuged at 2000 x g. The polyprenylphosphate-[^{14}C]-pentoses were contained in the organic (bottom) layer and were analysed by scintillation counting, TLC and autoradiography analysis and HPLC using the methods described in the literature[31].

Treatment of p[^{14}C]Rpp with BCG Cell Walls in the Presence of **(154)**

The conditions were identical as for the treatment of p[^{14}C]Rpp with BCG cell walls but **(154)** (5mM final conc.) was added after the β-NAD^+.

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M. smegmatis Experiments

The cell walls of *M. smegmatis* were prepared by the conditions described in the literature.[31] The experimental conditions for the treatment of p[^{14}C]Rpp with *M. smegmatis* cell walls in the presence and absence of **(154)** was identical to the experiments above with the substitution of BCG cell walls with *M. smegmatis* cell walls.

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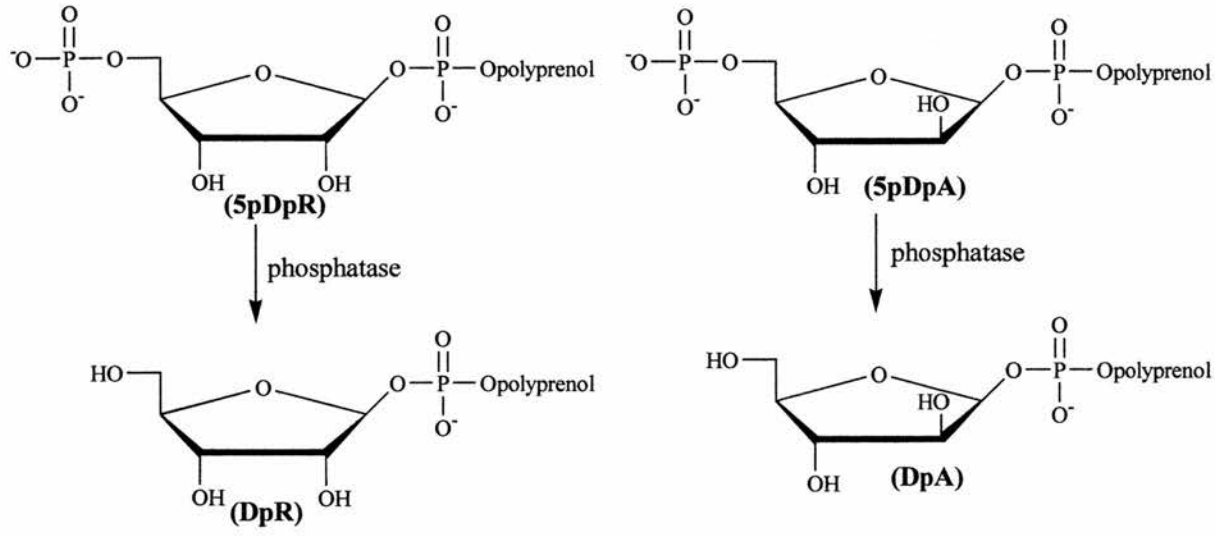
CHAPTER 6:Synthesis and Biological Evaluation of Methoxycarbonyloctyl Ara/Rib-5-phosphate

References are on page 162

6.1 INTRODUCTION

6.1.1 Dephosphorylation of 5-P-DpA and 5-P-DpR

It has been ascertained that 5-phospho-DpA is dephosphorylated in mycobacteria to produce the arabinose donor DpA.[1] It has also been elucidated that the same fate is accorded 5-phospho-DpR to produce DpR for which there is no concluded function.[1,2]



Scheme 57: Dephosphorylation of 5-P-DpA and 5-P-DpR

Experiments have shown that 5pDpR can be converted back to pRpp by treatment with PP_i and cell wall extracts.[1] The degree of conversion was dependent on PP_i concentration. However, no experiments have shown direct conversion of 5pDpR or DpR into arabinose based products. These experiments appear to support the argument that there is no epimerisation of 5pDpR to give a direct route to DpA.

It has been made clear that phosphatases must catalyse the transformation of 5pDpR and 5pDpA into DpR and DpA respectively. However there remains the possibility that a single phosphatase is capable of catalysing both reactions.

6.1.2 Analogue Strategies

Inhibition of the phosphatase responsible for producing DpA would have the desirable effect of preventing arabinan formation since DpA has been elucidated as the donor of at least two out of three of the arabinan linkages.[3] Isolation of this enzyme would also be useful for the construction of *in vitro* assays testing possible inhibitors on a medium or high throughput basis.

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For an analogue to be useful as a lead compound and a tool for enzyme purification it must be stable and it is advantageous if the compound is straightforward to synthesise. With these guidelines in mind, it was decided that shortening of the anomeric prenol to a more manageable chain length and removal of the labile anomeric phosphodiester linkage would result in a stable compound that was fairly straightforward to synthesise.

With enzyme purification being a possibility, it was also decided to make analogues that could be attached to a solid support for the purposes of creating an affinity column. A hydrophobic aglycone which has previously been used for this purpose[4] is the 8-methoxycarbonyloctyl group. Saponification of the methyl ester produces a carboxylic acid which can the be attached to a solid support via a variety of linkages.

There is little evidence in the literature of phosphatases acting on polyprenylphosphosugar-5 or 6-phosphates, however, there was evidence[5,6] that many phosphatases can act on both inositol polyphosphates and phosphatidylinositol polyphosphates. From this it seemed reasonable to hope that the lipid component of DpA would not be the most crucial moiety for binding the phosphatase.

6.1.3 Aims and Objectives

It was decided to synthesise analogues **(193)** and **(194)** in order to test them for inhibitory properties against the phosphatases or phosphatase that produces DpA and DpR. Should either of these compounds display inhibitory activity, then the compound would be saponified and attached to a solid phase resin with the aim of purifying the phosphatase or phosphatases from cell free extracts.

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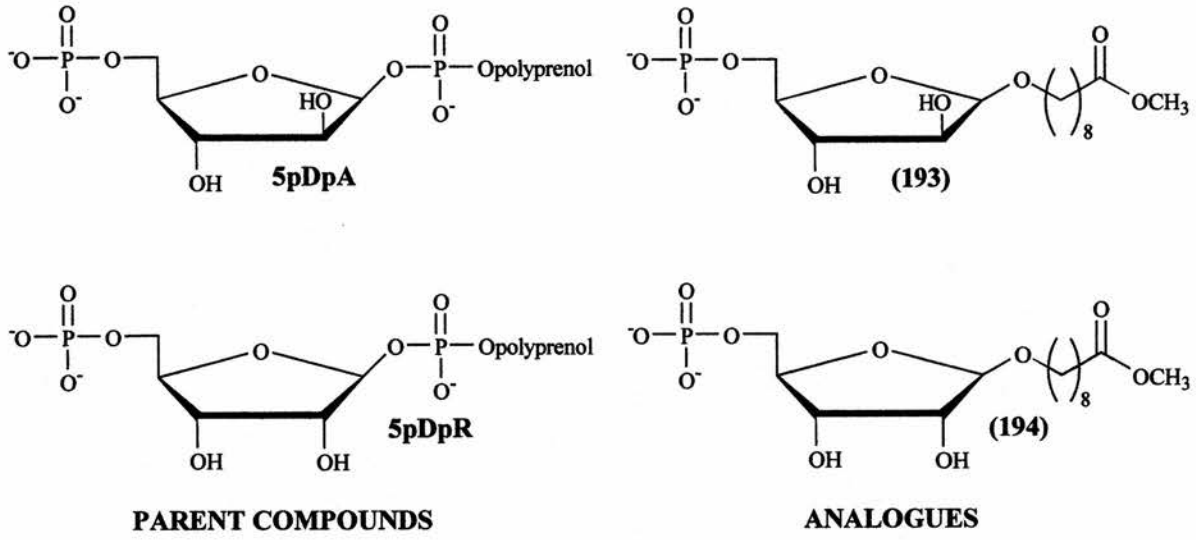


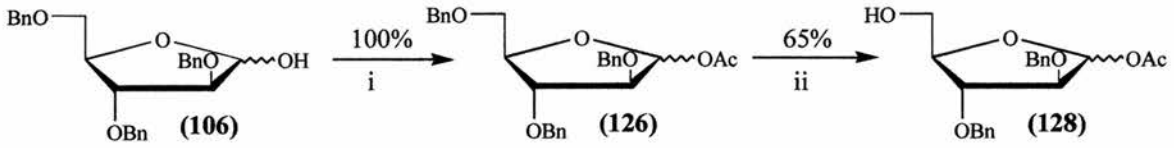
Figure 45: Proposed 5pDpA and 5pDpR Analogues

References are on page 162

6.2 RESULTS AND DISCUSSION

6.2.1 Synthesis of the *arabino*-configured Analogue

It has previously been outlined that the acetylation of commercially available tribenzyl arabinose **(106)** produces predominantly the α anomer. Following the procedure of Maryanoff[7], the primary benzyl group of **(126)** was selectively hydrogenated using a palladium catalyst which had been partially poisoned with pyridine. This produced primary alcohol **(128)** in 65% yield which could then be phosphorylated.



Reagents: (i) Ac_2O, pyr; (ii) HOAc/MeOH 1:1, 0.4%w/w pyr, Pd on C (10%)

Scheme 58: Acetylation and Selective Hydrogenation of Tribenzyl Arabinose

It was thought desirable to use a phosphorylation reagent that would ultimately allow a single deprotection step and thus a benzyl protected compound was looked for. There were two commercially available reagents that were considered. Firstly, dibenzyl *N,N*-diisopropylphosphoramidite, a commonly used compound in the synthesis of oligonucleotides which would provide, after oxidation, a dibenzyl protected phosphate. Secondly, tetrabenzyl pyrophosphate **(196)**, which has been used in inositol phosphorylation, would also produce the same product. It was decided to use the former reagent **(195)** as it was cheaper.

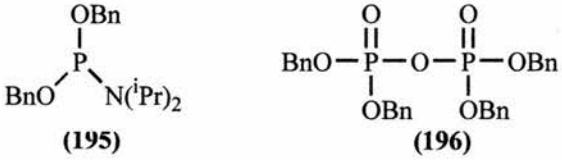


Figure 46: Phosphorylating Reagents

The phosphitylation reaction followed by oxidation using mCPBA proceeded without incident and yielded the desired compound **(197)** in 85% yield.

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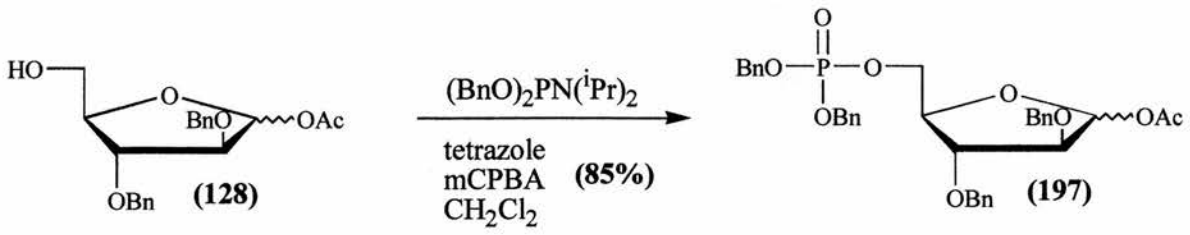
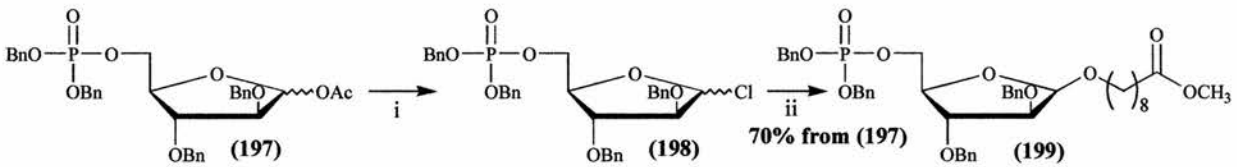


Scheme 59: Phosphorylation of Protected Arabinose

Activation of the anomeric centre was now required in order to glycosylate using 8-methoxycarbonyoctanol. In section 4.2.4.2 on page 100, a number of anomeric activation reactions were attempted for 5-phosphorylated-1-*O*-actyl-2,3-*O*-benzylarabinose and chlorination by directly bubbling HCl gas through a solution of **(126)** in DCE was found to be the most effective. This was utilised for the glycosylation of **(197)** with 8-methoxycarbonylalcohol and the reaction proceeded in a 70% overall yield.



Reagents: (i) HCl(g), DCE; (ii) 8-methoxycarbonyloctanol, 4Å MS, $Hg(CN)_2$, $HgBr_2$, DCE

Scheme 60: Synthesis of Protected 8-Methoxycarbonyloctyl 5-phospho arabinose

Deprotection was then executed using palladium on carbon in a hydrogen atmosphere, standard hydrogenolysis conditions. Purification of the final compound was achieved by the use of two short columns. Firstly, a solution of **(193)** in H_2O was loaded onto a Sephadex AG25 strong anion exchange column which was eluted with water to get rid of any non-anionic compounds. The phosphate **(193)** was then eluted in a solution of NaCl onto a Sep-Pak® C-18 column which was then eluted with water to get rid of any compounds which did not contain a hydrophobic group. The purified phosphate **(193)** was then eluted with MeOH, evaporated and freeze dried to a clean, white powder.

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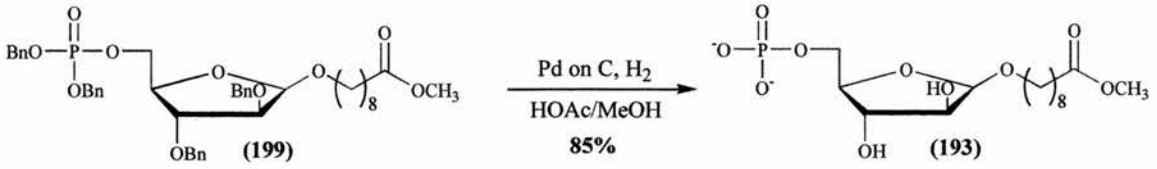
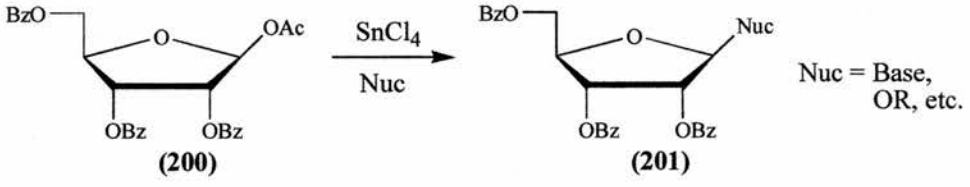


Scheme 61: Deprotection of (199)

The target compound was thus synthesised in six steps with an overall yield of 31% from cheap, commercially available starting materials.

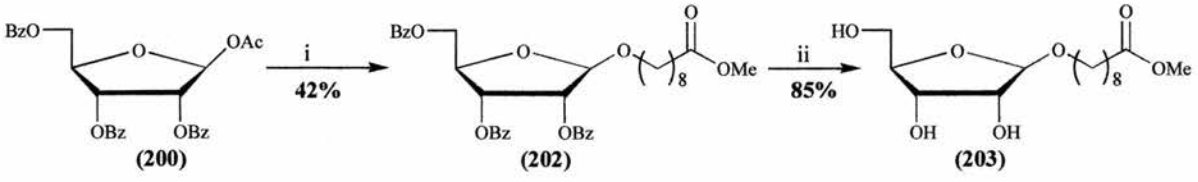
6.2.2 Synthesis of the *ribo*-configured Analogue

In considering the synthesis of the *ribo*-configured acceptor, the chemistry used in the synthesis of nucleoside and nucleotide analogues was thought relevant.[8-11] From these studies, it was thought that glycosylation of a commercially available protected ribose **(200)** would be the best way forward in the synthesis of the desired final product.



Scheme 62: Typical Synthesis of Ribonucleosides

It was decided to glycosylate commercially available **(200)** with 8-methoxycarbonyoctanol using $SnCl_4$ as the activator. This was a fairly straightforward reaction that was easy to carry out and purify thereby making up for the low yield. Transesterification of **(202)** produced the glycosylated riboside **(203)** which was used directly for phosphorylation.



Reagents: (i) $SnCl_4$, 8-methoxycarbonyloctanol, CH_2Cl_2; (ii) Na, MeOH

Scheme 63: Synthesis of 8-Methoxycarbonyloctyl β-D-riboside

It has been shown that a primary alcohol will be phosphorylated using diphenylphosphoryl chloride in pyridine in the presence of unprotected secondary

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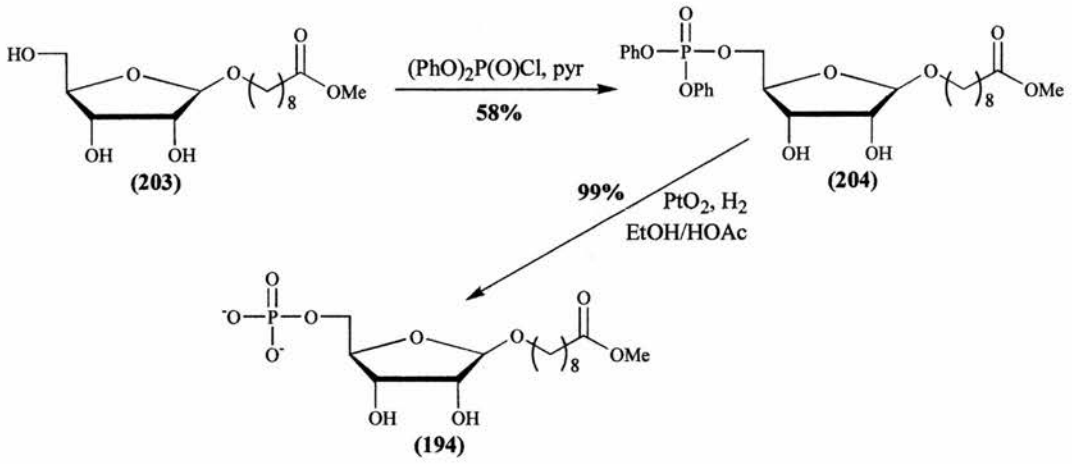
alcohols.[7] Using these conditions, **(203)** was selectively phosphorylated yielding 58% of the protected adduct which was then deprotected by hydrogenolysis over a platinum catalyst.



Scheme 64: Synthesis of 8-Methoxycarbonyloctyl 5-phospho β-D-riboside

The target compound was thus successfully synthesised in four steps with an overall yield of 20%.

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6.2.3 Biological Testing

The testing of these compounds was carried out under identical conditions to those in section 5.2.2.2. The compounds were tested at a final concentration of 5mM and the results are illustrated in Figure 47. Although it appears clear that the lack of inhibition of radioactive uptake into the organic layer shows negligible inhibition of the phosphatase, it must be remembered that 5-P-DpA would also be incorporated into the organic layer.

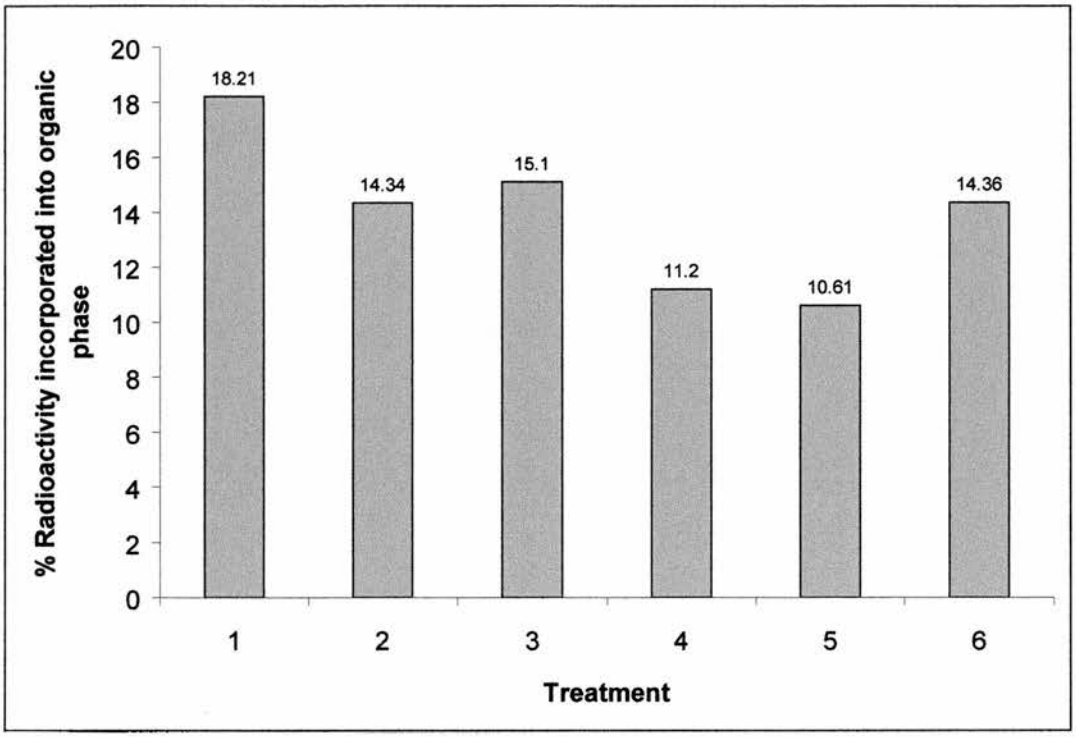

Figure 47: Graph showing percentage of radioactive organic soluble products derived from treatment of *M. smegmatis* cell walls with radioactive pRpp in the presence of (193) and (194) with and without detergent. 1: Control, **2: (193)**(Na^+) 5mM, **3: (194)**(Na^+) 5mM, **4:** 1%CHAPS, **5: (193)**(Na^+) 5mM + 1% CHAPS, **6: (194)**(Na^+) 5mM + 1%CHAPS

Therefore, a sample of each treatment was applied to a TLC, ran, and analysed by autoradiography. The resultant autoradiogram is shown in Figure 48, where the fastest running spot is DpA, closely behind is DpR, and near the bottom of the picture is the spot that represents a mixture of 5-P-DpR and 5-P-DpA. Treatments 1 and 3 are the control and the control with detergent respectively whereas 5 and 2 as well as 4 and 6 contain compounds **(193)** and **(194)** with and without detergent, respectively. It can be immediately ascertained that there is little difference between the controls and the analogue containing assays.

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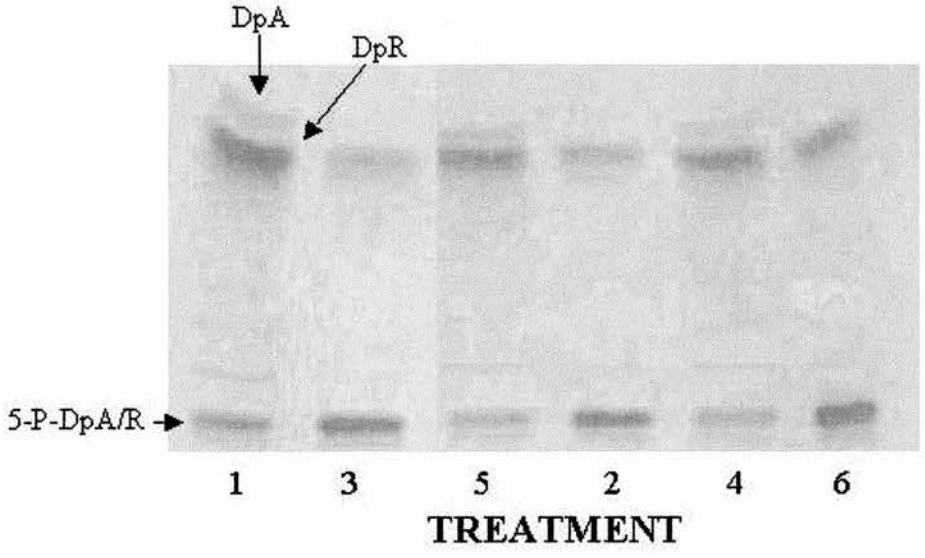

Figure 48: Autoradiogram of Samples from Each Treatment Detailed in Figure 47

The absence of greater 5-P-DpA/R on the baseline indicates no inhibition of the dephosphorylation event catalysed by the phosphatase or phosphatases.

6.2.4 Conclusions

Since the development of these analogues was based on biosynthetic intermediates that were known to exist, it can only be the structure of these compounds that explains the lack of biological activity. It must be assumed that either the lack of an anomeric phosphodiester or the shortening of the hydrophobic chain caused the phosphatase or phosphatases not to recognise either **(193)** or **(194)**.

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6.3 EXPERIMENTAL

1-O-Acetyl-2,3,5-tribenzyl-α-D-arabinofuranose **(126)**,
1-O-Acetyl-2,3-O-benzyl-α-D-arabinofuranose **(128)**,
1-O-acetyl-2,3-O-benzyl-α-D-arabinofuranose 5-(dibenzylphosphate) **(197)**
See section 4.3 on page 106

8-Methoxycarbonyloctyl 2,3-O-benzyl-5-dibenzylphospho-β-D-arabinofuranoside **(199)**

Chlorination of **(197)** (100mg,) was achieved in DCE (1 cm^3) by bubbling HCl gas directly into the solution. After 25 min, the reaction was ceased and the DCE was evaporated *in vacuo*. The residue was then dissolved in DCE (2cm^3) and 4Å molecular sieves (4.5g) were added followed by 8-methoxycarbonyloctanol (80μL, 316μmol) and the mixture was stirred at room temperature for 30 min. $HgCN_2$ (44mg, 174μmol) and $HgBr_2$ (5.7mg, 15.8μmol) were then added and the reaction was stirred for 2 days at room temperature under nitrogen when TLC indicated consumption of the anomeric chloride. The mixture was then diluted with dichloromethane and subjected to an aqueous work-up followed by purification using flash column chromatography. Evaporation of the relevant fractions yielded the desired compound as a mobile yellow liquid (84mg, 70%) α_D –23.6° (*c* 0.25, $CHCl_3$), δ_H 1.25 (8H, m, 4x$C\underline{H}_2$), 1.56 (4H, m, $C\underline{H}_2CH_2O$, $C\underline{H}_2CH_2COOMe$), 2.29 (2H, m, $CH_2C\underline{H}_2COOMe$), 3.27 (1H, dt, ^1J 9.62, ^3J 6.87, O-$C\underline{H}_2CH_2$ x 1), 3.72-3.60 (4H, m, O-$C\underline{H}_2CH_2$ x 1, $C\underline{H}_3OCO$), 4.13-4.01 (5H, m, H-3,4,5,5',2), 4.65-4.53 (4H, m, Ph$C\underline{H}_2$ x 2), 4.87 (1H, d, *J* 4.12, H-1), 5.03 (4H, d, *J* 7.97, Ph$C\underline{H}_2$-OP x 2), 7.44-7.15 (20H, m, *Ar*), δ_C 24.9 (CH_2 x 1), 26.1 (CH_2 x 1), 29.1 (CH_2 x 1), 29.2 (CH_2 x 1), 29.2 (CH_2 x 1), 29.4 (CH_2 x 1), 34.0 (R$\underline{C}H_2COOMe$), 68.2 (R$\underline{C}H_2OC$-1), 69.1 (1C, d, C-5), (69.3, Ph$\underline{C}H_2OP$), 69.4, (Ph$\underline{C}H_2OP$), 72.4 (Ph$\underline{C}H_2O$), 72.5 (Ph$\underline{C}H_2O$), 79.7 (1C, d, C-4), 82.8 (C-3), 84.2 (C-2), 100.8 (C-1), 127.8-128.7 (*Ar*), 135.9 (quat *Ar*), 137.8 (quat *Ar*), 138.1 (quat *Ar*), 174.5 (C=O), (Calcd. for $C_{43}H_{53}O_{10}P$; $M^{\cdot}$ 760.3376, $M^{\cdot}$+Na 783.3274 ; Found, 783.329099 (High Res FAB-MS).

8-Methoxycarbonyloctyl 5-phospho-β-D-arabinofuranoside **(193)**

Compound **(199)** (84mg, 110μmol) was dissolved in MeOH/HOAc 3:2 (5cm^3) and Pd on carbon (10%, 20mg) was added. The solution was stirred at room temperature under H_2 overnight at which point it was filtered through Celite, evaporated *in vacuo* and

coevaporated several times with toluene. When the toluene had been completely removed, the residue was dissolved in the minimum of H_2O and applied to a BIO-RAD column of Sephadex AG25 strong anion exchange resin and the column was washed with several column volumes of water. The column was then eluted with aqueous NaCl (1M) directly onto a SEP-PAK C-18 cartridge which was washed with $8cm^3$ of water and then eluted with MeOH until TLC detected no more compound coming from the column ($10cm^3$). The eluate was evaporated and then freeze-dried to yield the title compound as a white powder (36mg, 85%), FABMS- M-H_2O+Na 405.2453, δ_H 1.23 (8H, bs, C$\underline{H_2}$ x 4), 1.48 (4H, bs, RC$\underline{H_2}$CH$_2$OC-1 & RC$\underline{H_2}$CH$_2$COOMe), 2.27 (2H, t, *J* 7.4, RC$\underline{H_2}$COOMe), 3.25-4.15 (10H, m, H-2,3,4,5,5', C$\underline{H_3}$OCO & RC$\underline{H_2}$OC-1), 4.94 (1H, bs, H-1), δ_C 24.6 (CH_2 x 1), 25.4 (CH_2 x 1), 28.7 (CH_2 x 1), 28.9 (CH_2 x 1), 30.0 (CH_2 x 1), 33.4 (R$\underline{C}$H$_2$COOMe x 1), 50.6 ($\underline{C}$H$_3$OCO x 1) 65.9 (1C, d, C-5), 67.8 (R$\underline{C}$H$_2$OC-1), 78.2 (1C, d, C-4), 81.4 (C-3), 83.7 (C-2), 102.2 (C-1), 174.8 (C=O), (Calcd. for $C_{15}H_{29}O_{10}P$; M$^{\cdot}$ 400.1498, M$^{\cdot}$-H_2O+Na 405.1290; Found, 405.2453 (High Res FAB-MS).

8-Methoxycarbonyloctyl 2,3,5-O-benzoyl-β-D-ribofuranoside **(202)**

1-*O*-Acetyl-2,3,5-*O*-benzoyl-β-D-ribose (100mg, 198μmol), was dissolved in CH_2Cl_2 ($2.6\ cm^3$) and cooled to 0°C. To this was added $SnCl_4$ (140μL, 1.2mmol) and the solution was stirred for 5 min at which point 8-methoxycarbonyloctanol (40μL, 158μmol) was added dropwise. The solution was allowed to warm to room temperature, stirred for 1 h and quenched with ice/water. The mixture was partitioned and the organic layer was washed several times with sat. aqueous $NaHCO_3$, dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash column chromatography using toluene/EtOAc 20:1 as the eluant. Collection and evaporation of the relevant fraction yielded the target compound as a mobile oil (43mg, 42%); (Calcd for $C_{36}H_{40}O_{10}$: C, 68.34; H, 6.37. Found C, 68.31; H, 6.67); α_D +62.8 (*c* 0.25, $CHCl_3$), δ_H 8.08-8.00 (4H, m, *Ar*), 1.27 (9.53H, m, C$\underline{H_2}$ x 4), 1.57 (4.73H, m, C$\underline{H_2}$ x 2), 2.31 (2H, m, RC$\underline{H_2}$COOMe), 3.44 (1H, dt, ^1J 9.34, ^3J 6.87, RC$\underline{H_2}$OC-1 x 1), 3.66 (3H, s, C$\underline{H_3}$OCO), 3.76 (1H, dt, ^1J 9.34, ^3J 6.59, RC$\underline{H_2}$OC-1 x 1), 4.51 (1H, dd, $J_{5,5'}$, $J_{4,5}$, H-5), 4.71 (1H, dd, $J_{5,5'}$ 12.91, $J_{4,5'}$ 4.12, H-5'), 4.74 (1H, d, $J_{2,3}$, H-3), 5.24 (1H, s, H-1), 5.67 (1H, d, $J_{2,3}$, H-2), 5.86 (1H, dd, $J_{4,5}$ 6.6, $J_{4,5'}$ 4.9, H-4), 7.60-7.15 (9H, m, *Ar*), 7.97-7.85 (2H, m, *Ar*), δ_C 24.9 ($\underline{C}$H$_2$ x 1), 25.9 ($\underline{C}$H$_2$ x 1), 29.1 ($\underline{C}$H$_2$ x 2), 29.1 ($\underline{C}$H$_2$ x 1), 29.3 ($\underline{C}$H$_2$ x 1),

 References are on page 162

34.1 (R<u>C</u>H$_2$COOMe), 51.4 (<u>C</u>H$_3$OCO), 65.0 (R<u>C</u>H$_2$OC-1), 68.6 (C-5), 72.7 (C-3), 75.7 (C-2), 78.8 (C-4), 105.6 (C-1), 129.9-128.5 (*Ar*), 133.2 (quat. *Ar*), 133.5 (quat. *Ar*), 133.6 (quat. *Ar*), 165.6 (PhO<u>C</u>=O x 2), 166.4 (PhO<u>C</u>=O x 1), 174.5 (<u>C</u>(O)OMe).

8-Methoxycarbonyloctyl 5-phospho-β-D-ribofuranoside **(194)**

8-Methoxycarbonyloctyl β-D-ribofuranoside **(203)**

To a solution of **(202)** (435mg, 670μmol) in MeOH (5 cm^3) at 0°C was added a small chunk of sodium and the reaction was allowed to warm to room temperature and stir overnight. The reaction mixture was deionised using Amberlite IR120[H$^+$] and evaporated to dryness. The residue was purified by flash column chromatography using EtOAc/toluene 5:1 as the eluant. Combination and evaporation of the relevant fractions yielded 8-Methoxycarbonyloctyl β-D-ribofuranoside **(203)** as a colourless oil (183mg, 85%) δ_H 1.34 (8H, C<u>H</u>$_2$ x 4), 1.60 (4H, m, RC<u>H</u>$_2$CH$_2$OC-1 & RC<u>H</u>$_2$CH$_2$COOMe), 2.34 (2H, t, *J* 7.4, RC<u>H</u>$_2$COOMe), 3.38 (1H, m, RC<u>H</u>$_2$OC-1 x 1), 3.57 (1H, dd, $J_{5,5'}$ 10.4, $J_{4,5}$, H-5), 3.67 (3H, s, C<u>H</u>$_3$OCO), 3.74 (2H, m, H-5', RC<u>H</u>$_2$OC-1 x 1), 3.90 (1H, d, $J_{2,3}$, H-2), 3.95 (1H, m, $J_{4,5}$ 3.37, H-4), 4.05 (1H, dd, $J_{3,4}$ 6.7, $J_{2,3}$ 4.8, H-3), 4.87 (1H, s, H-1), δ_C 26.1 (CH$_2$ x 1), 27.3 (CH$_2$ x 1), 30.2 (CH$_2$ x 1), 30.6 (CH$_2$ x 2), 30.8 (CH$_2$ x 1), 34.9 (R<u>C</u>H$_2$COOMe), 52.1 (<u>C</u>H$_3$OCO), 65.3 (R<u>C</u>H$_2$OC-1), 69.0 (C-5), 73.0 (C-3), 76.4 (C-2), 84.9 (C-4), 108.8 (C-1), 176.1 (C=O).

Compound **(203)** (156mg, 487μmol) was dissolved in pyridine (1.0 cm^3) and cooled to 0°C. Diphenyl chlorophosphate (103μL, 496μmol) was added dropwise and the reaction mixture was stirred for 2 h at 0°C, allowed to warm to room temperature, then stirred at room temperature for 2 h. The reaction mixture was then extracted with CH_2Cl_2 which was then washed with HCl (1M) several times, dried over Na_2SO_4 and evaporated *in vacuo*. Flash column chromatography was used to further purify the compound (eluant toluene/EtOAc 4:1) and the relevant fractions were pooled and evaporated to yield 8-Methoxycarbonyloctyl 5-diphenylphospho-β-D-ribofuranoside **(204)** as colourless oil (156mg, 58%) δ_H 1.27 (8H, C<u>H</u>$_2$ x 4), 1.60-1.47 (4H, m, RC<u>H</u>$_2$CH$_2$OC-1 & RC<u>H</u>$_2$CH$_2$COOMe), 2.27 (2H, t, *J* 7.4, RC<u>H</u>$_2$COOMe), 3.33 (1H, dt, RC<u>H</u>$_2$OC-1 x 1), 3.68-3.60 (4H, s & dt, C<u>H</u>$_3$OCO & RC<u>H</u>$_2$OC-1 x 1), 3.98 (1H, d, $J_{2,3}$ 4.7, H-2), 4.14 (1H, dd, $J_{5,5'}$ 11.5, $J_{4,5}$ 5.5, H-5), 4.39-4.23 (6H, m, H-3,4,5'), 4.92 (1H, s, H-1), 7.36-7.16 (10H, *Ar*), δ_C 24.8 (CH$_2$ x 1), 25.9 (CH$_2$ x 1), 28.9 (CH$_2$ x 1), 29.0 (CH$_2$ x 2), 29.3

(CH_2 x 1), 34.0 (R<u>C</u>H_2COOMe), 51.5 (<u>C</u>H_3OCO), 68.0 (R<u>C</u>H_2OC-1), 69.9 (C-5), 72.1 (C-3), 75.1 (C-2), 81.2 (C-4), 107.2 (C-1), 120.2 (*Ar*), 125.7 (*Ar*), 130.0 (*Ar*), 174.6 (C=O).

Platinum dioxide (55mg) was added to a solution of **(204)** (135mg, 244μmol) in EtOH (4 cm^3) and HOAc (1.5 cm^3) was added. The reaction mixture was stirred at room temperature under H_2 for 6 h at which point the reaction had gone to completion according to TLC observations. The mixture was filtered through Celite, evaporated *in vacuo* and coevaporated sequentially several times with toluene, MeOH, and CH_2Cl_2. The residue was then dissolved in minimal H_2O and further purified using Sephadex and SEP-PAK as for compound **(193)** thus yielding **(194)** as a white solid (98mg, 99%); *Anal.* Calcd for $C_{15}H_{27}O_{10}PNa.1H_2O$: C, 41.01; H, 6.65. Found C, 41.29; H, 6.82, δ_H 1.17 (8H, bs, C<u>H</u>$_2$ x 4), 1.45 (4H, bs, RC<u>H</u>$_2$CH_2OC-1 & RC<u>H</u>$_2$CH_2COOMe), 2.24 (2H, t, *J* 7.4, RC<u>H</u>$_2$COOMe), 3.32-4.17 (10H, m, H-2,3,4,5,5', C<u>H</u>$_3$OCO & RC<u>H</u>$_2$OC-1), 4.88 (1H, bs, H-1), δ_C 24.2 (CH_2 x 1), 25.0 (CH_2 x 1), 28.0 (CH_2 x 2), 28.4 (CH_2 x 1), 33.6 (R<u>C</u>H_2COOMe x 1), 52.0 (<u>C</u>H_3OCO x 1), 66.4 (1C, d, C-5), 68.7 (R<u>C</u>H_2OC-1), 70.9 (C-3), 74.1 (C-2), 81.1 (1C, d, C-4), 106.9 (C-1), δ_P 0.49.

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