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Chemical synthesis and antigenic activity of a phosphatidylinositol mannoside epitope from *Mycobacterium tuberculosis*†

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Phosphatidylinositol mannosides (PIMs) have been investigated as lipidic antigens for a new subunit tuberculosis vaccine. A non-natural diacylated phosphatidylinositol mannoside (Ac_2PIM_2) was designed and synthesized by mimicking the natural PIM_6 processing procedure in dentritic cells. This synthetic Ac_2PIM_2 was achieved from α-methyl D-glucopyranoside 1 in 17 steps in 2.5% overall yield. A key feature of the strategy was extending the use of the chiral *myo*-inositol building block A to the *O*-2 and *O*-6 positions of the inositol unit to allow for introducing the mannose building blocks B1 and B2, and to the *O*-1 position for the phosphoglycerol building block C. Building block A, being a flexible core unit, may facilitate future access to other higher-order PIM analogues. A preliminary antigenic study showed that the synthetic PIM epitope (Ac_2PIM_2) was significantly more active than natural Ac_2PIM_2, which indicated that the synthetic Ac_2PIM_2 can be strongly immunoactive and may be developed as a potential vaccine.

Tuberculosis (TB) is a mycobacterial disease caused by *Mycobacterium tuberculosis* (*Mtb*), which ranks as the second-leading infectious cause of mortality, after the human immunodeficiency virus (HIV).[1] Despite the discovery of the Bacillus Calmette-Guérin (BCG) vaccine in the early 20th century[2] and the development of a new drug treatment for TB, this disease is still not under control.[3] The envelope of *Mtb* is the causative agent of human tuberculosis, and contains a variety of lipids with unique structures that can serve as antigens for the immune system.[4] Among the vital cell envelope components, phosphatidylinositol mannosides (PIMs) and their multiglycosylated counterparts, lipomannans (LMs) and lipoarabinomannans (LAMs), are involved in the modulation of host immune responses and play important roles in the pathogenesis of *Mtb*.[5] In particular, PIMs can regulate cytokines and stimulate early endosomal fusion by acting as ligands to receptors (MR, DC-SIGN and CR-3).[6] In addition, a PIM was identified as a natural antigen for CD1d-restricted T cells,[7] and synthetic PIMs and their analogues can also modulate cell-mediated immunity,[8] indicating their potential as vaccine candidates.[9]

Structurally, PIMs are each composed of a *myo*-inositol unit with a diacylated glycerophospholipid moiety at position *O*-1 and α-mannosylation sites at *O*-2 and *O*-6. The *O*-6 mannose unit of the *myo*-inositol unit may be further substituted at position *O*-6 by four mannosyl units, leading to **PIM_6**. Additional lipid chains may be linked at the *O*-6 position of the 2-*O*-mannosyl unit and the *O*-3 position of *myo*-inositol to form tetraacylated PIMs (**Ac_4PIM_6**),[10] as shown in Fig. 1. Complex native antigen **PIM_6** requires processing within antigen-presenting cells to **PIM_2** to become immunogenic.[11] Lipid antigen **PIM_6** is recognized by surface receptors (MR, DC-SIGN and CR3) and in this way can enter antigen-presenting cells. The oligomannosidic moiety of **PIM_6** was shown to be processed by α-mannosidase in the

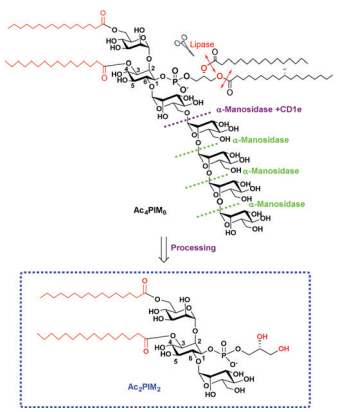

Fig. 1 Structures of natural **Ac_4PIM_6** and the non-natural **Ac_2PIM_2**.

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presence of CD1e,[12] and the acyl chains from the glycerol unit of **PIM$_6$** were artificially cleaved by recombinant lipases (LPLA2 and PLRP2).[4] The resulting non-natural diacylated **PIM$_2$** (**Ac$_2$PIM$_2$**, Fig. 1) that contains only two α-D-mannose units is the antigenic form, which is presented by the CD1b protein to stimulate CD1b-restricted T cells through the T-cell receptor (TCR).

The number of mannosyl residues and number of the acyl chains present in the PIM molecules determine their antigenic activities.[13] As a result, elegant synthetic strategies have been developed for native PIMs (**PIM$_1$**, **PIM$_2$**, **PIM$_4$**, **PIM$_5$**, **PIM$_6$**)[14] and PIM analogues.[15] However, most of these works have focused on syntheses of higher PIMs, which often display poor immunogenicity, although the structure–activity relationships of PIMs with different acylated forms have yet to be reported. The precise acylation state of the PIM molecule appears to be crucial in the immune responses.[16] The relatively small synthetic **PIM$_2$** was demonstrated by Painter *et al.* to be more active than natural **PIM$_2$**.[17] Recently, Gilleron *et al.* reported that the lipase-cut **PIM$_2$** showed better antigenic activity than did natural **PIM$_2$**,[11] which contains two fatty acids at the *sn*-2 positions of the glycerol unit.

In the current work, we mimicked the natural **PIM$_6$** processing procedure, and designed and synthesized an artificial PIM epitope **Ac$_2$PIM$_2$** (Fig. 1). The preliminary immunomodulatory activity of the synthetic **Ac$_2$PIM$_2$** was also evaluated.

Synthetic strategy: The developed retrosynthetic analysis of **Ac$_2$PIM$_2$** is shown in Fig. 2. If this analysis were to be followed, **Ac$_2$PIM$_2$** would be assembled from the building blocks **A**, **B1**, **B2** and **C**. Specifically, **Ac$_2$PIM$_2$** would be obtained *via* sequential regioselective glycosylations, first of the inositol block **A** at the *O*-6 position with mannose block **B1**, and then at the *O*-2 position with **B2**. Subsequent introduction of the phosphoglycerol moiety to the *O*-1 position of the inositol unit would be carried out using **C** by applying the *H*-phosphonate method. Further palmitoylations would be carried out, at the *O*-3 position of **A** and the *O*-6 position of **B2**. Final deprotection by catalytic hydrogenolysis would afford **Ac$_2$PIM$_2$**. The stereoselectivity of each glycosydic bond formed would be ensured by neighboring *C*-2 acyl participating groups.

In this study, we employed an Fmoc group as a selectively removable protecting group for the mannose building blocks **B**. We chose the Fmoc group—and not an acetyl group—for **B**, because the inositol building block **A** already has an acetyl group at its *O*-1 position and this acetyl group was needed for later introduction of the phosphoglycerol moiety. Thus, the *O*-2 acetate of the mannose building blocks **B** had to be replaced with another participating group, *i.e.*, the Fmoc group, to ensure the α-glycosylation. This method proved advantageous when compared to previous PIM syntheses. Examining the structure of **Ac$_2$PIM$_2$** indicated the key step to be the synthesis of *myo*-inositol **A**, a structural unit with different groups substituted at four of its positions (1, 2, 3, 6). The flexibility of the chiral inositol **A** core unit is expected to facilitate future access to other higher-order PIM analogues.

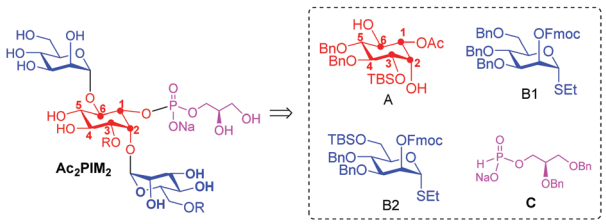
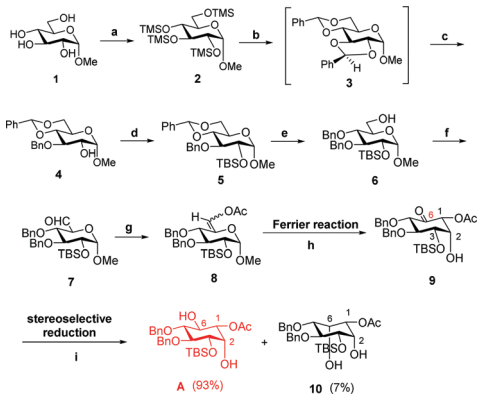


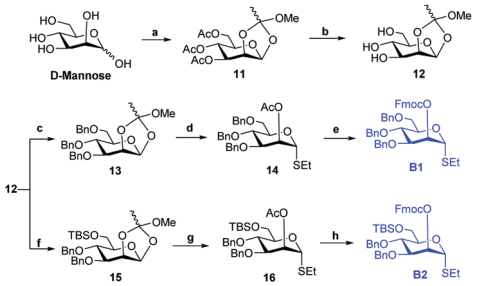
Fig. 2 Retrosynthetic analysis of **Ac$_2$PIM$_2$**.



Scheme 1 Synthesis of inositol building block **A**. *Reagents and conditions*: (a) TMSCl, Py, 0 °C; (b) (i) PhCHO, $FeCl_3{\cdot}6H_2O$; (ii) CH_2Cl_2/CH_3CN, 0 °C to rt; (c) (i) Et_3SiH; (ii) TBAF, 65%, 3 steps; (d) TBDMSCl, DMF, 45 °C, quant.; (e) $BH_3{\cdot}THF$, $CoCl_2$, 0 °C, quant.; (f) $SO_3{\cdot}Py$, DIPEA, DMSO, 0 °C; (g) K_2CO_3, Ac_2O, 62%, 2 steps; (h) $Hg(AcO)_2$, 64%; (i) $NaBH(AcO)_3$, 93%.

*Synthesis of myo-inositol building block **A***: The generation of optically pure *myo*-inositol derivatives with appropriate protecting groups is a challenging work. Inspired by the work of Bender *et al.*[18] on the conversion of methyl α-D-glucopyranoside to an enantiomerically pure *myo*-inositol using a Ferrier reaction, we developed a new method for the synthesis of chiral *myo*-inositol building block **A**, as shown in Scheme 1. First, α-methyl D-glucopyranoside **1** was converted to tetra-*O*-silylated glucopyranoside **2**, which was treated with benzaldehyde and $FeCl_3{\cdot}6H_2O$ to give the bis-benzylidene acetal **3** as a single diastereomer.[19] This one-pot tandem reaction proceeded well at room temperature and gave **4** in 65% yield over three steps. Silylation of **4** with *tert*-butyldimethylsilyl chloride (TBDMSCl) gave the 4,6-*O*-benzylidene hexopyranoside **5** in quantitative yield. Regioselective reductive ring-opening of **5** was performed by using $CoCl_2$ and $BH_3{\cdot}THF$, and gave **6** in quantitative yield.[20] Swern oxidation of **6** provided the air-sensitive aldehyde **7**. Without any purification, **7** was immediately treated with acetic anhydride and K_2CO_3 to afford enol acetate **8** (62% yield, over two steps) as a mixture of *Z*- and *E*-isomers. It was not necessary to isolate the two isomers because the geometry of the enol acetate **8** would not affect the stereochemical outcome of the Ferrier reaction.[21] The Ferrier rearrangement of **8** gave **9** in 64% yield as a single product. Subsequent reduction of **9** with $NaBH(AcO)_3$ provided building block **A** in 93% yield with a good diastereoselectivity and its diastereomer **10** in 7% yield.

*Preparation of the mannose building blocks **B1** and **B2***: The procedure for preparing the building blocks **B1** and **B2** is shown in Scheme 2. First, **11** was produced from D-mannose by following the methodology developed by Iadonisi.[22]



Scheme 2 Syntheses of mannose building blocks **B1** and **B2**. *Reagents and conditions*: (a) (i) Ac_2O, Py; (ii) I_2, Et_3SiH, CH_2Cl_2; (iii) 2,6-lutidine, MeOH; (b) NaOMe, MeOH; (c) NaH, BnBr, 70%, 4 steps; (d) $HgBr_2$, EtSH, 60%; (e) (i) NaOMe, MeOH; (ii) Fmoc-Cl, Py, 75%, 2 steps; (f) (i) TBDMSCl; (ii) BnBr, NaH, 46%, 2 steps; (g) $HgBr_2$, EtSH, 72%; (h) (i) NaOMe, MeOH, quant.; (ii) Fmoc-Cl, Py, 86%.

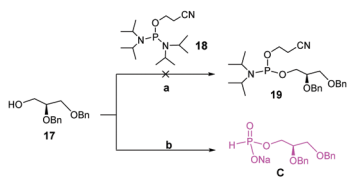
Deacetylation of **11** under Zemplen conditions[23] gave **12**, which was subsequently benzylated with benzyl bromide to provide orthoester **13** in 70% yield over four steps. $HgBr_2$-mediated ring-opening of the orthoester **13** with ethanethiol gave **14** in 60% yield as a mixture of α and β anomers (with the major one being α).[24] Removal of the *O*-2 acetyl group of **14** was carried out and the resulting product was treated with 9-fluorenylmethyl chloroformate (Fmoc-Cl) to give **B1** in 75% yield over two steps.[25] Similarly, regioselective silylation of the *O*-6 position of **12**[26] and subsequent benzylation with benzyl bromide gave **15** in 46% yield over two steps. $HgBr_2$-mediated ring-opening of **15** afforded **16** in 72% yield as a single α-anomer. Deacetylation of **16** and protection of the resulting product with the Fmoc group gave **B2** in 86% yield.

Synthesis of building block C: At first, we set out to use either the phoshphoramidite **19** or the H-phosphonate **C** as the building block for the introduction of the phosphoglycerol moiety, because both of them have been used successfully in previous syntheses of PIM compounds.[27] However, in our case, treatment of **17** with 2-cyanoethyl tetraisopropyl phosphorodiamidite **18** in the same conditions as reported in the ref. 27 failed to yield the phosphoramidite **19**. We suspected that the nearby benzyl groups in **17** sterically hindered the OH group and prevented this group from reacting with the coupling reagent. Fortunately, the building block **C** was readily prepared according to literature procedures (Scheme 3).[28] The yield of building block **C** was 77%.

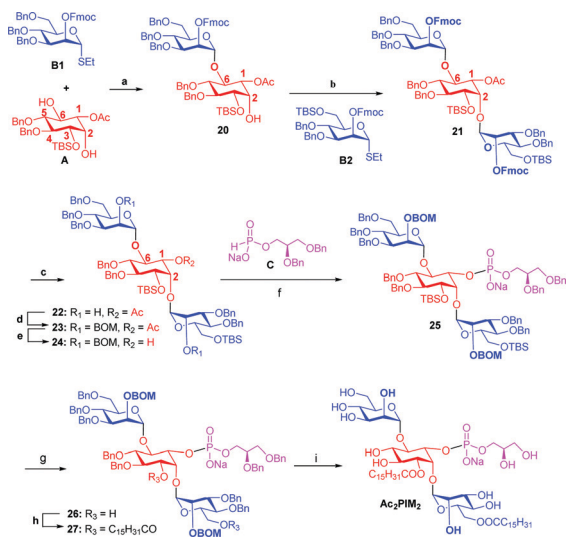
Assembly of $\mathbf{Ac_2PIM_2}$: The synthesis of $\mathbf{Ac_2PIM_2}$ is shown in Scheme 4. Due to the equatorial OH at the 6 position of *myo*-inositol building block **A** being more reactive than the axial OH at the 2 position, selective mannnosylation of the inositol building block **A** at the *O*-6 position with building block **B1** in the presence of *N*-iodosuccinimide (NIS) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) gave pure α-linked disaccharide **20** in 64% yield.[29] Subsequent glycosylation of the *O*-2 position of **20** with building block **B2** afforded the pure α-linked trisaccharide **21** in 82% yield. The Fmoc group needed to be replaced with another protecting group for the later reactions. The benzyloxy-methyl (BOM) group was selected to replace the Fmoc group because the BOM group can be introduced onto the OH group under mild conditions utilizing DIPEA as a basic catalyst and hence without affecting the *O*-1 acetyl group of the inositol unit. The Fmoc group was removed from **21** by treating **21** with Et_3N to give **22** in 72% yield. The resulting two OH groups of **22** were protected with the BOM group to give **23** in 55% yield. Removal of the *O*-1 acetyl group of **23** under Zemplen conditions gave **24** in quantitative yield. Employing the *H*-phosphonate method,[29] compound **24** was coupled with building block **C** in the presence of pivaloyl chloride (PivCl) and then oxidized with a solution of I_2 in aqueous pyridine to successfully produce the desired **25** in 85% yield (Scheme 4). Removal of the two TBS groups of **25** gave **26** in 85% yield, and palmitylations of the two free OH groups of **26** with palmitic acid ($C_{15}H_{31}COOH$) afforded **27** in 89% yield. Finally, global deprotection of **27** achieved by subjecting it to catalytic $(Pd(OH)_2/C)$ hydrogenolysis led to $\mathbf{Ac_2PIM_2}$ in 80% yield (Scheme 4). The total yield of $\mathbf{Ac_2PIM_2}$ based on building block **A** was 11%.

Evaluation of antigenic activity: The IFN-γ-producing activity of synthetic $\mathbf{Ac_2PIM_2}$ compared with those of the natural PIMs ($\mathbf{PIM_2}$, $\mathbf{PIM_6}$, $\mathbf{Ac_4PIM_2}$, $\mathbf{Ac_3PIM_2}$, $\mathbf{Ac_2PIM_2}$, Fig. 3A) are shown in Fig. 3B.

We achieved the chemical synthesis of a non-natural phosphatidylinositol mannoside (PIM) epitope capable of binding the CD1b protein to activate T cells and release the IFN-γ



Scheme 3 Synthesis of building block **C**. *Reagents and conditions*: (a) 1*H*-tetrazole; (b) (i) PCl_3, imidazole; (ii) Et_3N, toluene, 0 °C to rt, 77%.



Scheme 4 Assembly of $\mathbf{Ac_2PIM_2}$. *Reagents and conditions*: (a) NIS, TMSOTf, −20 °C, 64%; (b) NIS, TMSOTf, −20 °C, 82%; (c) Et_3N, THF, rt, 72%; (d) BOMCl, TBAI, DIPEA, rt, 55%; (e) NaOMe, MeOH, quant.; (f) (i) PivCl, Py; (ii) I_2, Py/H_2O, 85%; (g) TBAF/THF, 40 °C, 85%; (h) $C_{15}H_{31}COOH$, DCC, DMAP, toluene,100 °C, 89%; (i) $Pd(OH)_2/C$, H_2, rt, 80%.

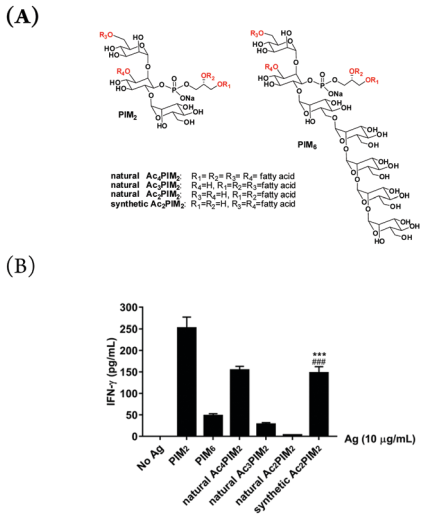


Fig. 3 Use of AcnPIMs loaded onto CD1b to activate T cells. (A) Structures of natural **PIM_2** (left) and **PIM_6** (right). The acylation sites are indicated by R_1–R_4; (B) stimulation of CD1b-restricted T cells with different acyl forms of AcnPIMs, Ag = antigen. ***$P < 0.001$ compared to natural **Ac_3PIM_2** and ###$P < 0.001$ compared to natural **Ac_2PIM_2**.

factor. This synthetic **Ac_2PIM_2** was achieved from **1** in 17 steps in a 2.5% overall yield. A series of efficient synthetic transformations incorporated three building blocks (**A**, **B1**, **B2**) into the pseudo-trisaccharide **24**. The overall efficiency of the assembly process benefited from the use of shared mannose building blocks (**B1**, **B2**) and the carefully chosen mannoside protecting groups (Fmoc, BOM). The key intermediate building block **A** allowed for efficient stereoselective glycosylations of the mannoside buliding blocks **B** and introduction of the phosphoglycerol building block **C**. The synthetic PIM epitope **Ac_2PIM_2** was significantly more active than was natural **Ac_2PIM_2** in inducing the production of IFN-γ, and hence could be developed as a potential vaccine against tuberculosis.

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Conflicts of interest

There are no conflicts to declare.

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