A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

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SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

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To be cited as: *ChemBioChem* 10.1002/cbic.201800150

Link to VoR: http://dx.doi.org/10.1002/cbic.201800150

A Journal of ChemPubSoc Europe

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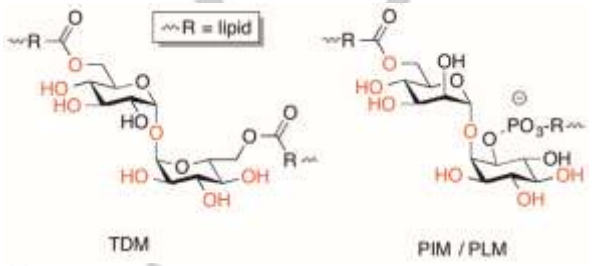
Simple Glycolipid Mimic of Phosphatidylinositol Mannoside Core from *Mycobacterium tuberculosis* Inhibits Macrophage Cytokine Production

Tamim Mosaiab,[a] Sandra Boiteux, [a] Abu Hasanat Md Zulfiker, [b,c] Ming Wei, [b] Milton J. Kiefel,*[a] and Todd A. Houston*[a]

Abstract: Glycolipids from *Mycobacterium tuberculosis* have a profound impact on the innate immune response of the host. Macrophage inducible C-type Lectin (Mincle) is a pattern recognition receptor that has been shown to bind trehalose dimycolate (TDM) from the mycobacteria and instigate intracellular signaling in the immune cell. There are structural similarities between TDM and phosphatidyl inositol mannoside (PIM) structures. We thus hypothesized that these latter structures might also modulate an immune response in a similar manner. To test this, we have synthesized a series of novel mannose derivatives modified with fatty esters at the 6-position and assessed the release of inflammatory cytokines in human U937 macrophages under induction of lipopolysaccharides (LPS) after glycolipid treatment. The results showed that the amount of two major cytokines such as tumor necrosis factor (TNF)-*α* and interleukin (IL)-6 released from LPS stimulated U937 cells decreased significantly when compared to control upon treatment with the prepared glycolipids indicating the reduction of cytokines production by macrophages.

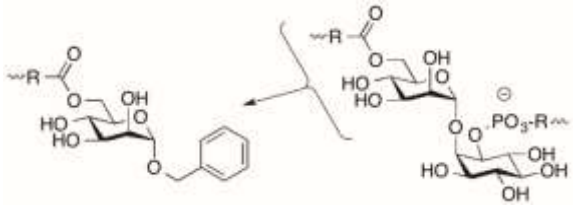
1. Introduction

Glycolipids from *Mycobacterium tuberculosis* (MTB) have a profound impact on the immune response of a human host allowing the microorganism to survive intracellularly upon capture by macrophages. An important core structure common to several complex glycolipids is the phosphatidylinositol mannoside (PIM) (Scheme 1). Importantly, it contains a mannose at the 2-position of inositol, a position that is unsubstituted in human phosphatidylinositol (PI) species. It is likely that such substitution would identify it as "non-self" to pattern recognition receptors (PPRs) of the innate immune system. A second mannose is added to the 6-position of inositol and this PIM2 core can be further decorated with additional mannose (PIM3-PIM6).[1] This core is also the starting point for biosynthesis of the large polysaccharide derivatives, the lipoarabinomannans (LAMs).[2] Both PIMs and LAMs contain additional lipid modifications at various positions including the 6-position of mannose attached directly to the PI anchor.



Scheme 1. Structural similarity between core structures of trehalose dimycolate (TDM) and phosphatidylinositol mannoside (PIM) from Mycobacteria and phospholipomannan (PLM) from Candida [Note: PLM contains a phosphodiester in place of the ester at C-6 of mannose in PIM].

These two families possess different immunomodulatory activity; whereas PIM facilitates early endosome-phagosome fusion, LAM inhibits the phagosome's maturation and late endosome-lysosome fusion.[3] This is in one sense a holding pattern that may allow access to nutrients during MTB latency while avoiding lysosome destruction. LAM from MTB also plays a role in intracellular survival by promoting phosphorylation of the proapoptotic protein Bad from the Bcl-2 family.[4] Based on the diverse structural features and varied immune modulation profile of these glycolipids, it is certain that they interact with multiple targets. For example, the mannose trisaccharide caps found on LAM from pathogenic mycobacteria bind the lectin DC-SIGN and dampen IL-12 production in macrophages through a TLR2-dependent mechanism.[5] To investigate the role of the PIM core on macrophage cytokine production, we have created a simple monosaccharide mimic of this structure, shown in Scheme 2.



Scheme 2. Fatty acylated mannose derivatives as mimics of the PIM structure.

One PRR that may bind the PIM core is macrophage inducible C-type lectin (Mincle), an important element of the innate immune response to both mycobacterial and fungal infections.[6] It was initially discovered to play a role in identifying damaged or dead cells by responding to spliceosome-associated protein 130.[7] Several

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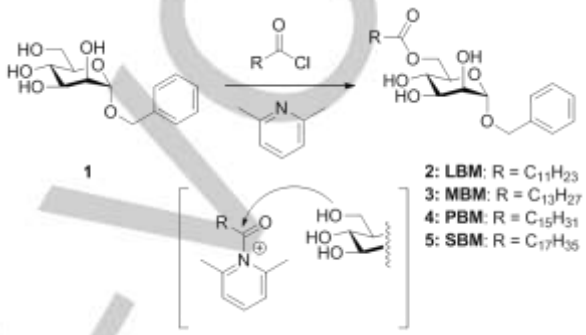
carbohydrate ligands have been identified—trehalose dimycolate (TDM) from mycobacteria [8] and glycolipids from *Malessezia pachydermatis* [9] —and undoubtedly many more will be discovered for this critical component of immune surveillance. We have noted the close structural similarity between TDM and both phosphatidylinositol mannosides (PIMs) [10] from mycobacteria and phospholipomannan (PLM) [11] from *Candida albicans* and believe that this motif (2-mannosylinositol) is likely to be recognized by Mincle. Since inositol modified at the 2-position has not been identified in mammalian cell-surface components, it is reasonable to assume that Mincle, as a pattern-recognition receptor scouting for pathogen-associated molecular patterns, might have evolved to identify this motif in macrophage-resident microorganisms such as *M. tuberculosis* and *C. albicans*. In fact, Lee and colleagues have shown that Mincle has a higher affinity for simple mannosides than the corresponding glucosides. [12] Thus, it is possible that TDM may not be the only cell-surface glycoconjugate on mycobacteria recognized by this lectin as *M. tuberculosis* is replete with mannose conjugates on its exterior. The structures of trehalose and 2-mannosylated inositol are quite similar as shown in Figure 1, particularly along the sides of both rings *opposite* the lipid anchors of TDM and PIM/PLM. It is important to note that these structures have identical stereochemistry in eight out of nine contiguous stereocentres as highlighted by the red oxygen atoms in Figure 1. The difference between PIM and PLM occurs at C-6 of mannose where some PIM derivatives contain fatty esters and PLM extends a large oligosaccharide (mannan) at this site.

We are interested in novel methods of drug delivery to treat macrophage infections [13] and are seeking to identify targeting ligands for this purpose. As Mincle appears to be up-regulated in response to these infections, it could be a target for selective drug delivery to infected cells. Ideally, we require a Mincle ligand that binds the lectin with some avidity but does not activate an inflammatory response in uninfected macrophages. It is important to note that the dimeric structure of TDM is important to both Mincle affinity and its subsequent activation and glucose monoesters do not activate the Mincle signalling cascade.[8] Here, we report the direct synthesis of 6-acyl and -amido mannosides with varying lipid lengths to determine the optimal chain length to balance maximal affinity and minimal activation. Ainge, et al., have reported evidence the inositol is responsible for TLR agonist activity, [10b] whereas more complex, natural PIM_6 structures negatively regulate TLR-4.[10a] Thus, we have targeted benzyl mannoside derivatives as the aromatic ring is expected to mimic the hydrophobic face of inositol that contains three axial hydrogens (Scheme 2). In addition, inositol groups often bind aromatic groups on proteins or synthetic receptors [14] and the benzyl group may also interact with cationic side chains via π-cation interaction. Moreover, mannose and its derivatives are widely used to target the macrophage mannose receptor (MMR), a pattern recognition receptor involved in host defense, innate immunity, triggering cytokine production, and modulating cell surface receptors.

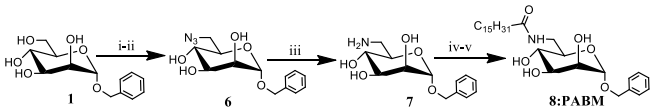
2. Results and Discussion

Several methods are available for the selective introduction of an ester linkage at the C-6 primary hydroxyl group of a hexopyranoside. [15] Previously, we have investigated esterification under Mitsunobu activation to facilitate similar transformations. [16] However, alkanoic acids tend to be less reactive partners relative to aryl carboxylic acids in these reactions. Yamamoto and co-workers have shown the C-6 primary alcohol of hexopyranosides can be selectively esterified using sym-collidine activation of acid chlorides. [17] 2,6-lutidine also contains methyl substituents on the carbons adjacent to the pyridine nitrogen. The pyridinium intermediate shown in Figure 3 is more reactive toward primary over secondary alcohols due to steric shielding of the carbonyl carbon by the ortho-type methyl groups on collidine. We chose to compare sym-collidine and 2,6-lutidine for selective esterification of benzyl *α*-D-mannoside (**1**).



Scheme 3. Synthesis of C-6 esterified benzyl *α*-D-mannosides. Reagents and conditions: **1**, corresponding fatty acid chloride, 2,6-lutidine, 0°C, 3 h, rt, 1h.

Progenitor benzyl *α*-D-mannoside **1** was synthesized using the method of Zunk, et al., in 98% yield.[18] Selective esterification of this monosaccharide was attempted using a range of fatty acid chlorides and either sym-collidine or 2,6-lutidine as promoter. The 2,6-lutidine required higher temperatures than sym-collidine (0-20˚C vs. -40˚C) and did lead to small amounts of diesters being formed. However, these undesired compounds were easily removed by flash column chromatography. The higher reaction temperature used with 2,6-lutidine allowed conversion to the desired monoesters in a shorter time frame and thus was the preferred method. Four novel compounds namely benzyl 6-lauroyl-*α*-D-mannoside (LBM, **2**), benzyl 6-myristoyl-*α*-D-mannoside (MBM, **3**), benzyl 6-palmitoyl-*α*-D-mannoside (PBM, **4**), benzyl 6-stearoyl-*α*-D-mannoside (SBM, **5**), were synthesized by this protocol in reasonable yields (65-75%). Of particular note is compound **4** derived from palmitoyl chloride as its lipid matches the chain length common in some PIM structures. [10]



Scheme 4. Synthesis of benzyl palmitoylamido-*α*-D-mannoside from **1**. Reagents and conditions: (i) p-TsCl, pyridine, 0°C, 12 h; (ii) NaN_3, DMF, 100°C, 24 h; (iii) Zn/NH_4Cl, Methanol/Water (9:1), rt, 1 h; (iv) Palmitoyl chloride, pyridine, 0°C, 12 h; (v) Na, Methanol, rt, 24 h.

Since these esters may be subject to cleavage by lipases, we synthesized an amide derivative as a control. To synthesize benzyl palmitoylamido-*α*-D-mannoside (PABM), benzyl 6-azido-6-deoxy-*α*-D-mannoside (**6**) was obtained following published protocol of Wang and co-workers, omitting the acetylation/deacetylation sequence

(Scheme 4).[19] The azido group was reduced to the benzyl 6-amino-6-deoxy-α-D-mannoside (**7**) with zinc and ammonium chloride under mild conditions.[20] The resulting primary amine was then reacted with palmitoyl chloride to give benzyl 6-palmitoylamido-α-D-mannoside with small amounts of acylation at C3 and C4 positions (confirmed via NMR, data not shown). Deacylation of the esters using sodium in methanol provide benzyl 6-palmitoylamido-α-D-mannoside (PABM, **8**).

Having successfully prepared the acyl and amido benzyl α-D-mannosides, the toxicity of these compounds was investigated against human U937 macrophages at different concentrations by MTT assay, to allow exclusion of a nonspecific cellular toxicity as a credible explanation for any altered cytokine output. As shown in Figure 1, the glycolipids displayed little cytotoxicity on the differentiated U937 cells up to 40 µM. The results show that these mannosides are relatively non-toxic even at fairly high concentrations and are suitable for interrogating macrophage cytokine output.

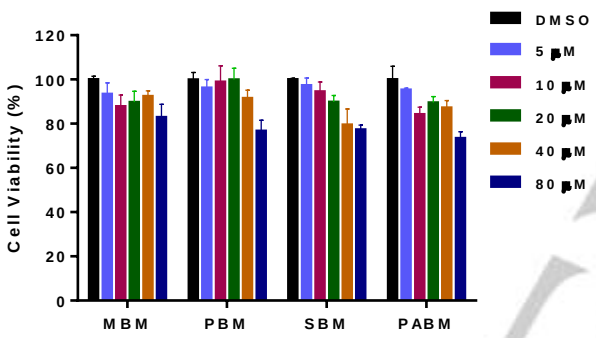


Figure 1. Effects of glycolipids on U937 cell viability. A MTT assay was performed to detect In vitro cytotoxic effects of MBM, PBM, SBM, and PABM against U937 cells after 24 h incubation. Cells were treated with 0-80 µM of corresponding glycolipids. Results were expressed as mean ± S.D (n = 6) of three independent experiments.

Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation. Stimulated by bacterial endotoxins, e.g., lipopolysaccharide (LPS), macrophages can secrete large amounts of inflammatory cytokines, such as TNF- α and IL-6, and these inflammatory cytokines further activate macrophages, creating a vicious feedback cycle increasing the inflammatory response.[21] To explore the abilities of these glycolipids to interact with macrophages, we set out to determine the proinflammatory cytokine (TNF-α and IL-6) secretion by ELISA after incubation with the synthesized glycolipids. Interestingly, all the glycolipids significantly inhibited cytokine production compared to control in a dose dependent manner in LPS primed macrophages, and this inhibition was greater for compounds with longer lipid chains (C16/C18). For instance, the secretions of IL-6 and TNF-α were significantly lower at higher concentration (20 µM) compared to untreated macrophages (PBM, Fig. 2 a and b). Similar results were observed with extended steroyl group on the 6-OH position of the mannose (SBM, Fig. 2 c and d). The levels of TNF-α were not significantly altered upon treatment with PBM and SBM in the LPS untreated cells. To explore the variation of cytokine production by the amide-linked chain at 6-OH position of mannose, 6-palmitoylamido-benzyl-α-D-mannoside (PABM) was tested in a similar manner. Upon treatment with PABM, both IL-6 and TNF-α secretions decreased in the LPS treated cells (Fig. 2 e and f). However, the amount of IL-6 was enhanced at 40 µM of PABM concentration (Fig. 2 e), opposite to that observed with the related ester PBM.

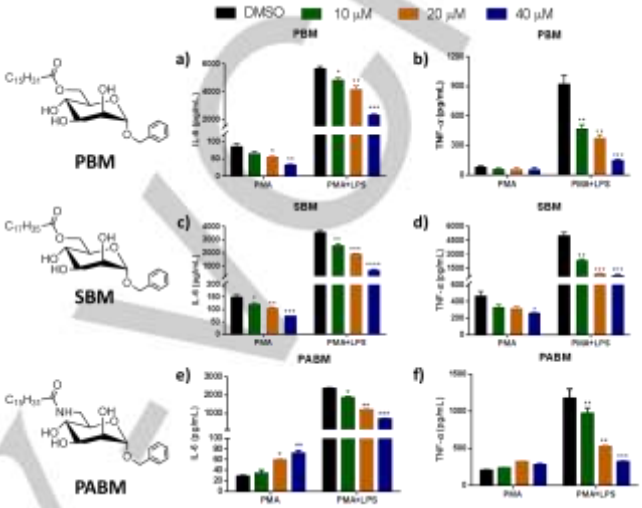


Figure 2. Production of proinflammatory (IL-6 and TNF-α) cytokines upon 24 h treatment with PBM (2a and 2b), SBM (2c and 2d) and PABM (2e and 2f) against LPS treated (10 nm) and untreated U937 cells. The supernatants were analysed for IL-6 and TNF-α production at 24 h by ELISA. For all graphs, the means and S.D. of triplicate samples from representative experiment performed in duplicate are shown. * p <0.05, ** p <0.01, *** p <0.001 vs. blank control group.

Macrophage phagocytosis of virulent MTB involves the MMR, and our results are consistent with previously published results that simple mannose can inhibit the secretion of proinflammatory cytokines by upregulated expression of MMR.[22] The MMR inhibitor mannan (1 mg/ml) was used to block mannose receptors by treating the differentiated U937 macrophages for an hour. The cells were then treated with the synthesized glycolipids to determine the cytokine production after 24 h at a fixed concentration of 20 µM. We still observed significant inhibition of IL-6 and TNF-α secretion in LPS treated cells (Fig. 3a and b); however, there was no considerable amount of cytokines secreted from the LPS untreated cells. This indicates these glycolipids exert their anti-inflammatory potential through receptors other that the MMR.

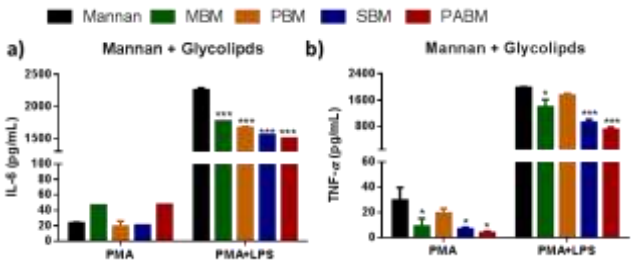


Figure 3. In Figure 3a and 3b, LPS treated (10 nm) and untreated cells were incubated 24 h with 20 μM of corresponding glycolipids following treatment with Mannan (1mg/ml) for 1 hour. The supernatants were analysed for IL-6 and TNF-*α* production at 24 h by ELISA. For all graphs, the means and S.D of triplicate samples from representative experiment performed in duplicate are shown. * p <0.05, ** p <0.01, *** p <0.001 *vs*. blank control group.

3. Conclusions

We have successfully synthesized novel mannose derivatives and further tested against human macrophages for pro-inflammatory cytokines measurement. The results showed significant reduction of cytokine production in the LPS-stimulated macrophages. These novel mannose derivatives follow the same trend of activity as monoacylated trehalose derivatives where decreasing affinity to Mincle based on fatty ester lipid chain length has been observed. [23] Further work is necessary to verify the immune cell target(s) of the compounds presented here. These derivatives may be used in liposome preparations or as components in nanoparticle drug delivery systems for the treatment of macrophage-resident infections. In addition, a recent report suggests that compounds of this type (e.g. the methyl mannoside analogous to **2**) may have antimicrobial activity as well. [24]

4. Experimental Section

4.1 Preparation of acylated benzyl *α*-D-mannopyranosides: General Procedure

A solution of benzyl *α*-D-mannoside (0.2g, 0.75 mmol) in 2,6-lutidine (5 ml, 43.2 mmol) at 0˚C under nitrogen was prepared and a solution of desired fatty acid chloride (~1.0 mmol) in 3 ml dichloromethane was added dropwise. The resulting mixture was stirred vigorously at that temperature for 3 hours, and at room temperature (20˚C) for 1 h. The reaction was monitored by TLC and visualization of TLC was performed by UV fluorescence and by charring plates with a mixture of 5% H_2SO_4 in ethanol. Following reaction time, 2 mL of methanol was poured into the suspension, and the mixture was concentrated under reduced pressure. The solid residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 10:1) to yield desired fatty acylated benzyl *α*-D-mannosides (~ 65-70%) as colorless oils.

Benzyl 6-lauroyl-*α*-D-mannopyranoside (LBM, 2):

Benzyl 6-lauroyl-*α*-D-mannopyranoside (**2**) was obtain from benzyl *α*-D-mannopyranoside in 70% yield.

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.81 (t, 3 H, J = 6.9 Hz, RCH_3), 1.13-1.24 (m, 16 H, RCH_2R), 1.54-1.61 (m, 2 H, RCH_2R), 2.31-2.35 (m, 2 H, -$COCH_2R$), 3.51 (t, 1 H, J = 9.6 Hz, H-4), 3.66-3.70 (m, 1 H, H-5), 3.83 (dd, 1 H, J = 9.3, 3.4 Hz, H-3), 3.93 (d, 1 H, J = 1.9 Hz, H-2), 4.07 (dd, 1 H, J = 12.3, 2.2 Hz, H-6a), 4.46 (d, 1 H, J = 11.9 Hz, -OCH_2Ph), 4.54 (dd, 1 H, J = 12.3, 4.0 Hz, H-6b), 4.64 (d, 1 H, J = 11.9 Hz, -OCH_2Ph), 4.88 (d, 1 H, J = 1.1 Hz, H-1), 7.24-7.29 (m, 5 H, Ar-H); ^{13}C NMR ($CDCl_3$) δ: 14.13 (RCH_3), 22.69 (RCH_2R), 24.77 (RCH_2R), 24.96 (RCH_2R), 29.18 (RCH_2R), 29.31 (RCH_2R), 29.35 (RCH_2R), 29.49 (RCH_2R), 29.62 (RCH_2R), 29.63 (RCH_2R), 31.92 (RCH_2R), 34.24 (-$COCH_2R$), 63.53 (C-6), 67.72 (C-4), 69.9 (OCH_2Ph), 70.54 (C-2), 70.72 (C-5), 71.44 (C-3), 99.01 (C-1), 128.02, 2 x 128.05, 2 x 128.52 (Ar-H), 136.93 (ipso-Ar), 175.0 (-$COCH_2R$) ; ES-MS: m/z = 475.3 $[M + Na]^+$, HRMS (ESI): m/z calcd for $[C_{25}H_{40}O_7+Na]^+$: 475.2666; found: 475.2676.

Benzyl 6-myristoyl-*α*-D-mannopyranoside (MBM, 3):

6-myristoyl-benzyl-*α*-D-mannopyranoside (**3**) was obtain from benzyl-*α*-D-mannopyranoside in 72% yield.

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.9 (t, 3 H, J = 6.8 Hz, RCH_3), 1.27-1.31 (m, 20 H, RCH_2R), 1.67 (dt, 2 H, J = 14.8, 7.4 Hz, RCH_2R), 2.34-2.46 (m, 2 H, -$COCH_2R$), 3.61 (t, 1 H, J = 9.6 Hz, H-4), 3.75-3.79 (m, 1 H, H-5), 3.92 (dd, 1 H, J = 9.3, 3.4 Hz, H-3), 4.02 (dd, 1 H, J = 3.3, 1.4 Hz, H-2), 4.17 (dd, 1 H, J = 12.3, 2.2 Hz, H-6a), 4.55 (d, 1 H, J = 11.9 Hz, -OCH_2Ph), 4.62 (dd, 1 H, J = 12.3, 4.0 Hz, H-6b), 4.73 (d, 1 H, J = 11.9 Hz, -OCH_2Ph), 4.97 (s, 1 H, H-1), 7.31-7.40 (m, 5 H, Ar-H); ^{13}C NMR ($CDCl_3$) δ: 14.13 (RCH_3), 22.70 (RCH_2R), 24.77 (RCH_2R), 24.96 (RCH_2R), 29.13 (RCH_2R), 29.20 (RCH_2R), 29.33 (RCH_2R), 29.37 (RCH_2R), 29.51 (RCH_2R), 29.67 (RCH_2R), 29.68 (RCH_2R), 29.71 (RCH_2R), 31.94 (RCH_2R), 34.25 (-$COCH_2R$), 63.69 (C-6), 67.75 (C-4), 69.2 (OCH_2Ph), 70.58 (C-2), 70.72 (C-5), 71.50 (C-3), 98.99 (C-1), 128.01, 2 x 128.06, 2 x 128.52 (Ar-H), 136.93 (ipso-Ar), 174.87 (-$COCH_2R$) ; ES-MS: m/z = 503.3 $[M + Na]^+$, HRMS (ESI): m/z calcd for $[C_{27}H_{44}O_7+Na]^+$: 503.2979; found: 503.2985.

Benzyl 6-palmitoyl-benzyl-*α*-D-mannopyranoside (PBM, 4):

Benzyl 6-palmitoyl-*α*-D-mannopyranoside (**4**) was obtain from *α*-D-benzylmannopyranoside in 68% yield.

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.9 (t, 3 H, J = 6.9 Hz, RCH_3), 1.27-1.31 (m, 20 H, RCH_2R), 1.67 (dt, 2 H, J = 12.3, 2.3 Hz, RCH_2R), 2.37-2.49 (m, 2 H, -$COCH_2R$), 3.60 (t, 1 H, J = 9.6 Hz, H-4), 3.77 (ddd, 1 H, J = 9.8, 3.7, 2.3 Hz, H-3), 3.92 (dd, 1 H, J = 9.2, 3.1 Hz, H-2), 4.02 (dd, 1 H, J = 3.4, 1.5 Hz, H-5), 4.16 (dd, 1 H, J = 12.3, 2.3 Hz, H-6), 4.56 (d, 1 H, J = 11.9 Hz, -OCH_2Ph), 4.64 (dd, 1 H, J = 12.3, 3.9 Hz, H-6a), 4.73 (d, 1 H, J = 11.9 Hz, -OCH_2Ph), 4.97 (d, 1 H, J = 1.1 Hz, H-1), 7.31-7.40 (m, 5 H, Ar-H); ^{13}C NMR ($CDCl_3$) δ: 14.14 (RCH_3), 22.71 (RCH_2R), 24.97 (RCH_2R), 29.19 (RCH_2R), 29.33 (RCH_2R), 29.38 (RCH_2R), 29.51 (RCH_2R), 29.66 (RCH_2R), 29.68 (RCH_2R), 29.72 (RCH_2R), 31.94 (RCH_2R), 34.25 (-$COCH_2R$), 63.62 (C-6), 67.74 (C-4), 69.24 (OCH_2Ph), 70.55 (C-2), 70.73 (C-5), 71.46 (C-3), 99.05 (C-1), 128.02, 2 x 128.06, 2 x 128.52 (Ar-H), 136.93 (ipso-Ar), 174.93 (-$COCH_2R$) ; ES-MS: m/z = 531.1 $[M + Na]^+$, HRMS (ESI): m/z calcd for $[C_{29}H_{48}O_7+Na]^+$: 531.3292; found: 531.3302.

Benzyl 6-stearoyl-*α*-D-mannopyranoside (SBM, 5):

Benzyl 6-stearoyl-*α*-D-mannopyranoside (**5**) was obtain from benzyl *α*-D-mannopyranoside in 65% yield.

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.81 (t, 3 H, J = 6.6 Hz, RCH_3), 1.17-1.18 (m, 28 H, RCH_2R), 1.53 (dd, 2 H, J = 14.0, 6.9 Hz, RCH_2R), 2.27-2.31 (m, 2 H, -$COCH_2R$), 3.56 (t, 1 H, J = 9.4 Hz, H-4), 3.69-3.72 (m, 1 H, H-5), 3.79 (d, 1 H, J = 7.1 Hz, H-3), 3.89 (s, 1 H, H-2), 4.19 (d, 1 H, J = 11.6 Hz, H-6a), 4.36 (d, 1 H, J = 5.5 Hz, -OCH_2Ph), 4.41 (d, 1 H, J = 11.7 Hz, H-6b), 4.62 (d, 1 H, J = 11.8 Hz, -OCH_2Ph), 4.82 (s, 1 H, H-1), 7.21-7.28 (m, 5 H, Ar-H); ^{13}C NMR ($CDCl_3$) δ: 14.13 (RCH_3), 22.71 (RCH_2R), 24.97 (RCH_2R), 29.20 (RCH_2R), 29.33 (RCH_2R), 29.38 (RCH_2R), 29.52 (RCH_2R), 29.68 (RCH_2R), 29.69 (RCH_2R), 29.73 (RCH_2R), 31.94 (RCH_2R), 34.25 (-$COCH_2R$), 63.63 (C-6), 67.74 (C-4), 69.23 (OCH_2Ph), 70.56 (C-2), 70.72 (C-5), 71.47 (C-3), 99.0 (C-1), 128.01, 2 x 128.06, 2 x 128.52 (Ar-H), 136.93 (ipso-Ar), 174.91 (-COCH2R) ; ES-MS: m/z = 559.4 $[M + Na]^+$, HRMS (ESI): m/z calcd for $[C_{31}H_{52}O_7+Na]^+$: 559.3605; found: 559.3618.

4.2 Synthesis of benzyl palmitoylamido-*α*-D-mannopyranoside: General Procedure

Benzyl 6-azido-6-deoxy-*α*-D-mannopyranoside (**6**) was synthesized according to the procedure by Wang et al. [19] Reduction of **6** using zinc and ammonium chloride in methanol/water (9:1) produced the desired product benzyl 6-amino-6-deoxy-*α*-D-mannopyranoside (**7**, 92.5% yield). To a solution of 7 (1g, 3.7 mmol) in 20 mL of dry pyridine at 0˚C under nitrogen environment palmitoyl chloride (4.5 mM) was added dropwise. The reaction was monitored via TLC. and the starting material was consumed completely by 12h. After quenching with methanol, the solvent was evaporated under vacuum. The crude compound was then dissolved in CH_2Cl_2 (50 mL) and washed with 1 M HCl (3x 50 mL), $NaHCO_3$ (50 mL), and brine (50 mL), dried over Na_2SO_4, filtered and concentrated to yield yellowish solid compound. The residues were purified by flash chromatography using hexane/EtOAc 5:1 as eluent and obtain a mixture of acylated benzyl palmitoylamido-*α*-D-mannopyranosides as white solid (confirmed by ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy, data not shown).

For the deacylation of the acylated compound 0.1 mmol was dissolved in dry methanol (50 mL) and sodium metal (0.023g, 1.0 mmol) was added to it slowly at 0^{o}C. The ice bath was removed when the sodium was dissolved completely and the solution was stirred for 24 h at room temperature. After reaction time, the solution was neutralized with ion exchange resin (Dowex 50 WX 8-400 ion exchange resin, Sigma-Aldrich), filtered and concentrated under reduced pressure to give a yellowish solid. The residue was purified by flash chromatography using EtOAc/Hexane 2:3 to 3:2 to obtain the desired benzyl palmitoylamido-*α*-D-mannopyranoside as a white solid with yields between 30 and 55%.

Benzyl 6-palmitoylamido-*α*-D-mannopyranoside (PABM, 9):

Benzyl 6-palmitoylamido-*α*-D-mannopyranoside (**9**) was obtain from benzyl 6-amino-6-deoxy-*α*-D-mannopyranoside (**7**) in 40% yield.

^{1}H-NMR (400 MHz, $CDCl_3$/Methanol; 9:1) δ: 0.81 (t, *J* = 6.8 Hz, 3H, RCH_3), 1.09-1.25 (m, 24 H, RCH_2R), 1.51-1.59 (m, 2 H, RCH_2R), 2.14-2.21 (m, 2 H, -$COCH_2R$), 2.95 (d, 1 H, *J* = 14.8 Hz, H-4), 3.40 (dd, 1 H, *J* = 11.5, 7.7 Hz, H-3), 3.54 (d, 1 H, *J* = 9.7 Hz, H-2), 3.92 (s, 1 H, H-5), 4.44 (d, 1 H, *J* = 11.9, H-6), 4.60 (d, 1 H, *J* = 11.9 Hz, -OCH_2Ph), 4.81 (d, 1 H, *J* = 1.1 Hz, H-1), 7.27 (dt, 5 H, *J* = 13.5, 4.6 Hz, Ar-H); ^{13}C NMR ($CDCl_3$) δ: 13.96 (RCH_3), 22.59 (RCH_2R), 25.78 (RCH_2R), 29.27 (RCH_2R), 29.42 (RCH_2R), 29.56 (RCH_2R), 29.59 (RCH_2R), 29.66 (RCH_2R), 29.68 (RCH_2R), 31.83 (RCH_2R), 36.23 (RCH_2R), 39.65 (-$COCH_2R$), 67.42 (C-6), 69.25 (C-4), 70.37 (OCH_2Ph), 71.21 (C-2), 76.78 (C-5), 77.30 (C-3), 99.26 (C-1), 127.80, 2 x 127.87, 2 x 128.39 (Ar-H), 137.01 (*ipso*-Ar), 175.95 (-$COCH_2R$) ; ES-MS: m/z = 530.4 [M + Na]+.

4.3 Cell culture, differentiation and sample collection for ELISA

Human leukemic monocyte lymphoma cells U937 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to the ATCC procedure. Briefly, cells were cultured in RPMI 1640 (Sigma-Aldrich) medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100U/mL of penicillin, and 100 µg/mL of Streptomycin (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 at 37°C and used for experiments between passages 12 and 16. To differentiate the cell line into macrophage-like cells, 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) was added to 5 x 105 cells in 1 mL of RPMI 1640 medium for 2 days in 12 wells plate. After differentiation, the adherent cells were washed with PBS to remove PMA and non-adherent cells. The adherent cells, referred to as U937 macrophages, were treated with various concentration of fatty acylated and amido mannose derivatives with or without further stimulation by the lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich, USA) in fresh culture medium for 6, 12 and 24 h. Then, 500 µL aliquots were taken from the supernatant and stored at -80°C until they were used for measurements by sandwich ELISA assays.

4.4 Cell Viability: MTT Assays

Cell proliferation was evaluated using the MTT assay. U937 cells with 50 ng/mL of PMA in 100 µL RPMI 1640 were seeded into 96-well plate (5 x 10^3 cells/well) for 48 h, washed, and treated with 10, 20, 40 and 80 µM of different glycolipids for 24 h at 37^{0}C. After 24 h treatment, cells were washed with PBS and MTT stock solution (5mg/mL) was then added to each well and incubated for an additional 4 h. At the end of the experiment, the medium was replaced with 100 µL of DMSO to dissolve the formazan crystals, and the absorbance was measured at 540 nm using Polarstar Omega 96-well microplate reader (BMG Labtech GmBH, Germany). The cell viability was calculated using the following formula: Cell viability (%) = $(OD_{exp} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$.

4.5 ELISA: Determination of the TNF-*α* and IL-6 Release

Both cytokines were quantified by enzyme-linked immunosorbent assay (ELISA) obtained from elisakit.com.au, Australia and the ELISA kit was performed according to producer's instructions. Briefly, diluted supernatants (1:5, in RPMI 1640 for U937) were incubated in antibody-coated 96-well plates to facilitate binding of released cytokines. The incubation with a biotinylated primary antibody was performed. The next step was incubation with streptavidin-horseradish peroxidase. All incubation steps were followed by washing, which guarantees the removal of unbound molecules. Horseradish peroxidase is capable of oxidizing its substrate tetramethylbenzidine (TMB), which develops a stable staining complex with sulfuric acid detectable at 450 nm by means of a plate reader. The limit of sensitivity for detection of TNF-*α* and IL-6 was <5 and <1 pg/mL, respectively, with both intra-assay and inter-assay variability < 10%.

4.6 Statistical Analysis

Data from all samples within each group were combined with means ± standard error of mean (S.E.M.). Data were averaged from three independent experiments each containing at least 4 replicates. Statistical significant was determined by one-way analysis of variance (one-way ANOVA) and a Dunnett's multiple comparison was used to compare the effect among groups using GraphPad Prism software (version 6.01, La Jolla, CA). Values with *P<0.05* were considered significant.

Acknowledgements

The authors wish to thank the Institute for Glycomics and Griffith University for financial support through a PhD scholarship to T.M. and infrastructure funding within the Institute.

Keywords: mannose • macrophage • lectin • inflammation • Mincle

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10.1002/cbic.201800150

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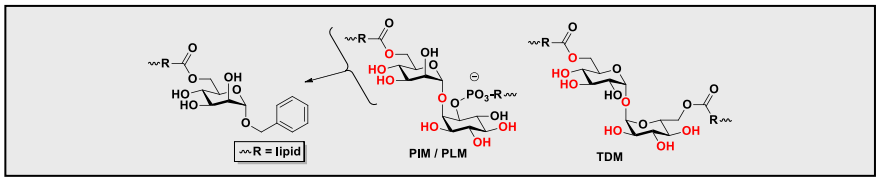

Novel mannose derivatives modified with fatty esters at the C6-position reduce the release of inflammatory cytokines in human U937 macrophages under induction of lipopolysaccharides (LPS) after synthetic glycolipid treatment.

Tamim Mosaiab, Sandra Boiteux, Abu Hasanat Md Zulfiker, Ming Wei, Milton J. Kiefel, and Todd A. Houston**

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Simple Glycolipid Mimic of Phosphatidylinositol Mannoside Core from *Mycobacterium tuberculosis* Inhibits Macrophage Cytokine Production