



Published in final edited form as:

*J Med Chem.* 2011 October 27; 54(20): 7268–7279. doi:10.1021/jm2008419.

## Synthesis and Toll-Like Receptor 4 (TLR4) Activity of Phosphatidylinositol Dimannoside Analogues

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### Abstract

A series of five PIM<sub>2</sub> analogues were synthesized and tested for their ability to activate primary macrophages and modulate LPS signaling. Structural changes included replacement of the fatty acid esters of the phosphatidyl moiety of PIM<sub>2</sub> with the corresponding ether or amide. An AcPIM<sub>2</sub> analogue possessing an ether linkage was also prepared. The synthetic methodology utilized an orthogonally protected chiral *myo*-inositol starting material that was conveniently prepared from *myo*-inositol in just two steps. Important steps in the synthetic protocols included the regio- and  $\alpha$ -selective glycosylation of inositol O-6 and introduction of the phosphodiester utilizing phosphoramidite chemistry. Replacement of the inositol core with a glycerol moiety gave compounds described as phosphatidylglycerol dimannosides (PGM<sub>2</sub>). Biological testing of these PIM compounds indicated that the agonist activity was TLR4 dependent. An ether linkage increased agonist activity, removal of the inositol ring enhanced antagonist activity and the presence of an additional lipid chain enhanced LPS-induced cytokine production in primary macrophages. Furthermore, the interruption of the LPS-induced TLR4/MD-2 2:2 signaling complex formation by PIM<sub>2</sub> represents a previously unidentified mechanism involved in the bioactivity of PIM molecules.

### Introduction

The cell wall of mycobacteria is a rich source of immuno-modulatory molecules that includes lipids, glycolipids, phosphoglycolipids, lipoproteins and mycolyl-arabinogalactan-peptidoglycan motifs.<sup>1</sup> Included among these is the family of mannosylated phosphatidylinositols, commonly known as phosphatidylinositol mannosides or PIMs,<sup>2</sup> that are of great interest due to their biological activity and synthetic accessibility. Biological activity includes the ability to affect T-cell proliferation,<sup>3,4</sup> recruit natural killer T (NKT) cells,<sup>5,6,7</sup> activate innate receptors<sup>6,8</sup> and function as immune adjuvants.<sup>9,10,11</sup> In contrast PIMs have also been shown to suppress allergic airway disease,<sup>12,13</sup> negatively regulate lipopolysaccharide (LPS) signaling in macrophages<sup>14</sup> and suppress human T-cell proliferation.<sup>15</sup>

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In a previous study<sup>16</sup> we synthesized a PIM<sub>2</sub> mono-ether analogue, PIM<sub>2</sub>ME (**1**), where the glyceryl *sn*-2 acyl group (COC<sub>15</sub>H<sub>31</sub>) had been replaced by an alkyl group (C<sub>16</sub>H<sub>33</sub>). Our motivation for this change was to overcome the inherent lability of this acyl group towards hydrolysis giving rise to *lyso*-PIM species. This analogue, **1**, enhanced cytokine production by bone marrow-derived dendritic cells compared to the natural analogous PIM<sub>2</sub> compound (**2**). Here, we report the syntheses and activity of five novel PIM analogues; **3**, **4**, **5**, **6**, and **7** where the functionality on the glyceryl moiety and the inositol ring has been varied to probe SAR for this class of compound (Figure 1). The targets were specifically designed to gain as much structural information as possible: target **3** probes amide versus ether or ester functionality at the *sn*-2 position; **4** is a positional isomer of **1** (*sn*-1 vs *sn*-2); analogue **5** investigates the effect of introducing a third lipid functionality; and targets **6** and **7** probe the importance of the inositol ring. The previously reported di-ether **8**<sup>16</sup> was also tested to investigate the impact of two ether linkages. These compounds were then assayed and compared for their ability to induce cytokine production and suppress LPS-induced cytokine production in primary macrophages. We also investigated the interactions of PIMs with the TLR4 signaling pathway.

## Results and Discussion

Previous syntheses of PIM compounds have utilized 1-O-allyl-3,4,5-tri-O-benzyl-D-*myo*-inositol as a convenient starting material.<sup>9,17,18,19</sup> This compound is generally obtained from  $\alpha$ -methyl D-glucopyranoside by a series of transformations that, although often produce high yields, suffers from the use of stoichiometric amount of a mercuric trifluoroacetate in a carbocyclisation reaction.<sup>20</sup> Also, this reaction and the subsequent reduction steps both suffer from the unwanted formation of isomers that are difficult to separate resulting in a relatively un-scalable process. For these reasons, we decided to utilize a different approach starting from the known diol **9**<sup>21</sup> that can be prepared from *myo*-inositol via the camphanilidene acetal in a scalable two-step process.<sup>22</sup>

### Synthesis of 6-mannosylated-*myo*-inositol 15

Glycosylation of diol **9** is known to be favored at the C-6 hydroxyl on inositol. Moreover, Martin-Lomas has reported<sup>21</sup> the selective glycosylation of **9** with an 2-azido donor in the synthesis of inositol phosphoglycans (IPG's) and Watanabe demonstrated the synthesis of distearoyl PIM<sub>2</sub> using the analogous racemic cyclohexylidene acetal.<sup>23,24</sup> In the current work, glycosylation of **9** with mannosyl donor **10**<sup>25</sup> gave the desired glycoside **11** as the major product along with smaller amounts of the di-glycosylated product. Lowering the temperature of the glycosylation inhibited the formation of the over glycosylated product resulting in the clean formation of **11**. Routine functional group manipulation *via* **12** through **14** afforded diol **15**<sup>26</sup> that was regioselectively allylated using a minor modification of the literature transformation to **16**.<sup>26</sup>

### Synthesis of phosphoramidites 17 and 18

The phosphoramidites **17** and **18** were prepared for the synthesis of targets **3** and **4** respectively. With this in mind, the known mono-ether **19**<sup>27</sup> was esterified and the *para*-methoxybenzyl group removed by hydrogenolysis to afford alcohol **21**<sup>28</sup> in good yield. The phosphoramidite **17** was prepared by reaction with benzyloxy-bis-(diisopropylamino)phosphine and 1*H*-tetrazole.

The commercially available (*R*)-(+)-2-amino-3-benzyloxy-1-propanol (**22**) was used as a starting material for the synthesis of the 2-amido containing phosphoramidite **18**. Diacylation with palmitoyl chloride and DMAP in chloroform<sup>29</sup> afforded **23** (Scheme 2). Hydrogenolysis of **23** over Pearlman's catalyst proceeded smoothly to give the desired

alcohol **24**. 1*H*-Tetrazole activated reaction of alcohol with benzyloxy-bis-(diisopropylamino)phosphine gave the required phosphoramidite **18** in high yield over the three steps.

### Synthesis of **3** and **4**

Glycosylation with of **16** with **10** afforded an inseparable mixture of the anomeric glycosides. This result again demonstrates the propensity of the O-2 inositol hydroxyl to form unexpected  $\beta$ -anomers.<sup>30</sup> Removal of the benzoate directing group allowed separation of the anomers and access to the desired pseudo-trisaccharide **26**. Subsequent benzylation followed by deallylation provided the known inositol dimannoside head group **28**.<sup>31</sup> The spectroscopic data collected for compound **28** prepared in this way were identical to those reported previously. Inositol dimannoside **28** was then coupled with phosphoramidite **18** to afford the benzyl protected target molecule **30** in 62% yield (Scheme 3). Hydrogenolysis over Pearlman's catalyst gave the target PIM<sub>2</sub>-mono-amide analogue that was redissolved in MeOH/H<sub>2</sub>O with the aid of triethylamine, and then passed through a short column of silica gel. Evaporation and lyophilisation of the residue gave **3**, as the partial triethylammonium salt in 75% yield. Coupling of **28** with phosphoramidite **17** followed by oxidation afforded the fully protected **29** that was subsequently deprotected to afford the PIM *sn*-1 ether analogue **4** in good purity.

For the synthesis of AcPIM<sub>2</sub> mono-ether (**5**), the thioglycoside donor **31** was employed (Scheme 4). This compound was prepared from diol **32**<sup>32</sup> by regioselective acylation of O-6 with palmitoyl chloride followed by acylation of O-2 with 2-azidomethylbenzoyl chloride (AZMBCl).<sup>33</sup> The AZMB protecting group was chosen to provide  $\alpha$ -selectivity in the glycosylation, together with an orthogonal mode of deprotection.

Coupling of **16** and **31** using standard thioglycosylation conditions afforded the  $\alpha,\alpha$ -product **34** in 34% yield (Scheme 5). The two anomeric <sup>13</sup>C NMR signals of **34** were coincident; however, <sup>1</sup>J<sub>CH</sub> values for these signals (175 Hz, 175 Hz) could be extracted from a HSQC experiment run without <sup>13</sup>C decoupling during acquisition. As observed previously (*vide infra*), some  $\beta$ -coupled product was also formed which could be removed by column chromatography.<sup>30</sup> Deallylation followed by installation of the phosphodiester linkage using the known phosphoramidite **36**<sup>16</sup> and deprotection afforded the target compound **5** in high yield and purity.

To probe the effect of the inositol ring in combination with the above changes to the phosphatidyl group, phosphatidylglycerol dimannoside mono-ether analogue **6** (PGM<sub>2</sub>ME), where the inositol ring is replaced by a glycerol unit, was prepared. Preparation of the dimannosylated glycerol derivative was effected by DDQ deprotection of the known PMB-ether **39**<sup>12</sup> to give the desired head group **40** in 72% yield. Reaction of the dimannosylated glycerol alcohol **40** and phosphoramidite **36** provided the protected lipid **41** (Scheme 6). Hydrogenolytic debenylation to **41** and selective hydrolysis of the acetates gave the *sn*-2 ether linked PGM<sub>2</sub> analogue **6**. In a similar manner the bis-acylated congener **7** was prepared from dimannosylated glycerol **40** and phosphoramidite **43**.

### PIM Analogues induce interleukin-6 (IL-6) production and negatively regulate LPS signaling in primary peritoneal macrophages

TLRs play a key role in the innate immune response of macrophages to exogenous pathogens via recognition of pathogen-associated molecular patterns.<sup>34,35,36</sup> Whether any specific interaction(s) occur between PIMs and TLRs is currently not clear, but PIM molecules have been reported to act both as agonists of TLR2 and TLR4,<sup>6,8,37,38</sup> and antagonists of LPS<sup>14</sup> signaling in macrophages. For these reasons, we assessed the ability of

the synthetic PIM analogues to both directly activate primary macrophages and modulate LPS-induced macrophage activation.

Primary peritoneal macrophages were isolated from mice and the cells treated with synthetic PIM analogues *in vitro*. Consistent with previous reports of agonist activity of PIM molecules<sup>8,38</sup>, all of the PIM analogues tested induced macrophage IL-6 production (Figure 2B, Table 1). In all cases, peak IL-6 production was observed between 0.6–3.1  $\mu\text{M}$  PIM and decreased at higher concentrations without the appearance of cytotoxicity<sup>39</sup> (Figure 2B and supplementary information). Self regulation of TLR agonist activity has been previously reported for both TLR4 and TLR2.<sup>40</sup> Therefore it is likely that the observed pattern of cytokine production for the PIM analogues also results from the engagement of negative feedback mechanisms designed to regulate PIM agonist activity via TLRs.

Consistent with our earlier study of dendritic cell responses<sup>16</sup>, the inclusion of an ether rather than an ester group on the *sn*-2 position of glycerol in PIMs increased the induction of IL-6 (Table 1, **1** vs **2** or **6** vs **7**). Moving the ether to the *sn*-1 position resulted in a decrease in IL-6 production (Table 1, **1** vs **4**); however, compound **4** still enhanced IL-6 secretion compared to **2**. In contrast, inclusion of two ether groups, as with compound **8**, or exchanging the ether for an amide group, as with compound **3**, resulted in decreased cytokine production compared to synthetic PIM<sub>2</sub> (**2**). Interestingly, removal of the inositol ring, but retention of the natural glycerol di-ester (**2** vs **7**), resulted in a significant loss of agonist activity that was recovered by introducing the *sn*-2 mono-ether (**6** vs **7**). Agonist activity was also lost with the addition of a third lipid group, as exemplified by AcPIM<sub>2</sub>ME (**5**). Taken together, these results indicate the inositol ring contributes to the agonist activity and that agonist activity is enhanced with a combination of ester and ether linkages on the phosphatidyl group.

Next, we investigated whether PIM treatment enhanced or suppressed LPS induced IL-6 production by primary macrophages. Previous work on bone marrow-derived macrophages showed that PIM molecules are able to inhibit LPS-induced cytokine production.<sup>14</sup> Consistent with this finding, the majority of the PIM analogues inhibited LPS-induced macrophage IL-6 production in a dose-dependent manner (Figure 2C and Table 2). In general, suppressive activity fell in the same concentration range as that observed for shutdown of agonist activity. Whether the same regulatory mechanisms are involved in both the inhibition of PIM agonist activity and PIM-dependent inhibition of LPS-induced IL-6 production has yet to be determined.

Removal of the inositol moiety in **7** (IC<sub>50</sub> 12.9  $\mu\text{M}$ ) resulted in increased inhibitory activity and decreased agonist activity compared to **2** (IC<sub>50</sub> 40.2  $\mu\text{M}$ ). Therefore, it appeared that the presence of an inositol ring abrogated inhibitory activity.

Interestingly, only **5** enhanced LPS-induced IL-6 production (Figure 2D). The difference between **1**, which exhibited inhibitory activity, and **5** is the addition of C-16 (palmitic acid) fatty acyl chain to one of the mannosyl moieties. As such, the observed switch from inhibition to enhancement of LPS-induced IL-6 production may be associated with the greater lipophilicity of **5**.

### PIM analogue activities act via TLR4

To determine whether PIMs and their analogues may induce cytokine production via TLRs, macrophages from wild type C57 and TLR4-deficient mice were treated with **2**. IL-6 production was abrogated in TLR4-deficient macrophages indicating that **2** activates macrophages via the TLR4 signaling pathway (Figure 3).

A key event required for LPS signaling via the TLR4 pathway is LPS binding to a heterodimeric 1:1 complex between TLR4 and its co-receptor, myeloid differentiation factor 2 (MD-2), which then induces homodimerization of the 1:1 complex to form a 2:2 complex that leads to activation of the intracellular signaling cascade.<sup>41,42,43</sup> To reveal the molecular basis whereby **2** negatively regulates LPS signaling, we examined the effect of **2** on LPS-mediated formation of the active 2:2 TLR4/MD-2 homodimer. As shown by native PAGE, LPS induces the homodimerization of a purified protein complex between the TLR4 ectodomain (sTLR4) and MD-2 (Figure 4A). This LPS-mediated sTLR4/MD-2 homodimerization was partially inhibited by **2** (Figure 4A).

Given that, for TLR4 activation, LPS is deeply inserted into the hydrophobic cavity of MD-2, we speculated that **2** would inhibit 2:2 TLR4/MD-2 complex formation by competing with LPS for the MD-2 cavity. However, the direct interaction of MD-2 with LPS or **2** could not be addressed due to technical difficulty in recombinant expression of MD-2 alone. Instead, an MD-2 homolog, MD-1, was used as an MD-2 model system. MD-1 has been implicated in the regulation of LPS-induced immune responses and, like MD-2, houses a large hydrophobic cavity that is able to accommodate LPS and presumably other lipoglycan structures such as PIM.<sup>44,45</sup> In native gels, **2** was shown to associate with MD-1 in a dose-dependent manner and to compete with LPS for MD-1 binding (Figure 4B), supporting that **2** directly interacts with MD-2. Taken together, these data suggest that PIM molecules, like MPL,<sup>43</sup> act as partial agonists of TLR4 and likely inhibit LPS signaling by interfering with LPS binding to the TLR4/MD-2 complex.

In summary, we present concise syntheses of PIM analogues **3**, **4** and **5** utilizing the readily available mannosylated chiral inositol **16**, and **6** and **7** from a dimannosylated glycerol intermediate. In general, the PIMs and their analogues exhibit partial agonist activity, which is associated with the inositol ring. Furthermore, this activity is enhanced by replacement of a fatty acid ester moiety with that of the corresponding ether on the phosphatidyl group. SAR for the inhibition of LPS signaling resulted in two notable observations. Firstly, loss of the inositol ring enhanced the antagonist activity, and somewhat surprisingly, addition of a third lipid moiety may synergise with LPS to enhance pro-inflammatory activation of macrophages. For the first time, we also show that the activities of PIMs involve regulation of TLR4 signaling, potentially via interactions with TLR4/MD-2. Together these findings identify key structural features that could be used to direct the synthesis of lipoglycans with optimal agonist, antagonist or adjuvant activities and identify an important role for TLR4 in the bioactivity of PIMs.

## Experimental Section

### General Experimental

NMR spectra are referenced to tetramethylsilane (TMS) (0.0 ppm) or the residual solvent peak (<sup>1</sup>H CHCl<sub>3</sub> δ7.26); <sup>13</sup>C CDCl<sub>3</sub> δ77.0) or an external reference (<sup>31</sup>P H<sub>3</sub>PO<sub>4</sub> δ0.0). Anhydrous solvents were sourced commercially and used without further treatment unless stated. Powdered molecular sieves were flame dried under vacuum immediately prior to use. Flash column chromatography was carried out using 40–63 μm silica gel unless otherwise stated. All flash chromatography solvents were AR-grade. Petroleum ether used was bp 60–80° range. All compounds were isolated after silica-gel column chromatography and fractions collected were one spot by TLC. Thin layer chromatography (TLC) plates were visualised under an UV lamp and/or with a spray consisting of 5% w/v dodecamolybdophosphoric acid in ethanol with subsequent heating. See supplementary information for HPLC conditions. HPLC purity of target compounds, **1** (97%), **2** (95%), **3** (94%), **4** (96%), **5** (95%), **6** (96%), **7** (95%) and **8** (97%).

**6-(2-O-Benzoyl-3,4,5-tri-O-benzyl- $\alpha$ -D-mannopyranosyl)-3,4-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-yl)-1,2-O-((1S,4S)-1,7,7-trimethyl-[2.2.1]bicyclohept-6-ylidene)-D-myo-inositol (11)**—TMSOTf (0.20 mL, 1.1 mmol) was added to a mixture of diol 9 (6.00 g, 10.8 mmol), trichloroacetamide 10 (7.53 g, 10.77 mmol) and 4Å molecular sieves in dry Et<sub>2</sub>O (100 mL) at -70 °C. After stirring for 45 min, the reaction was quenched with saturated NaHCO<sub>3</sub> (100 mL) and extracted with Et<sub>2</sub>O (3×60 mL) and washed with water (100 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified on silica gel (EtOAc/petroleum ether = 1:9) to afford compound 11 (8.95 g, 76%) as a white foam.  $[\alpha]^{20}_{\text{D}} -1.5$  (c = 0.4, CH<sub>2</sub>Cl<sub>2</sub>);  $[\alpha]^{20}_{365} -26$  (c 0.4, CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$ 8.11–8.05 (m, 2H), 7.57–7.50 (m, 1H), 7.41–7.15 (m, 17H), 5.71 (dd, *J* = 1.8, 2.7 Hz, 1H), 5.40 (d, *J* = 1.8 Hz, 1H), 4.87–4.74 (m, 3H), 4.60–4.49 (m, 3H), 4.37–4.30 (m, 1H), 4.20 (dd, *J* = 3.9, 5.4 Hz, 1H), 4.16–4.05 (m, 2H), 4.01–3.85 (m, 4H), 3.84–3.70 (2H, m), 3.36 (ddd, *J* = 1.8, 8.7, 10.1 Hz, 1H), 2.57 (d, *J* = 1.8 Hz, 1H), 2.00–1.80 (m, 2H), 1.74–1.57 (m, 2H), 1.46 (d, *J* = 12.9 Hz, 1H), 1.40–1.13 (m, 3H), 1.12–0.94 (m, 30H), 0.82 (s, 3H), 0.86 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$ 165.7, 138.9, 138.7, 138.3, 132.9, 130.0, 128.3, 128.24, 128.19, 128.1, 128.0, 127.9, 127.5, 127.4, 127.3, 117.7, 96.8, 79.9, 78.6, 77.4, 77.0, 76.9, 76.6, 76.3, 75.7, 75.1, 74.5, 73.3, 72.9, 72.6, 71.6, 71.5, 69.4, 69.2, 51.5, 48.0, 45.2, 45.1, 29.4, 27.1, 20.4, 20.2, 17.6, 17.4, 17.31, 17.26, 17.2, 17.1, 17.0, 13.0, 12.7, 12.5, 12.3, 12.1, 11.7, 9.7. HRMS-ESI [M+Na]<sup>+</sup> calculated for C<sub>62</sub>H<sub>84</sub>O<sub>13</sub>Si<sub>2</sub>Na: 1115.5348. Found 1115.5365.

**6-(3,4,5-Tri-O-benzyl- $\alpha$ -D-mannopyranosyl)-3,4-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-yl)-1,2-O-((1S,4S)-1,7,7-trimethyl-[2.2.1]bicyclohept-6-ylidene)-D-myo-inositol (12)**—Sodium methoxide in MeOH (30% solution, 0.58 mL) was added dropwise to a stirred solution of benzoate 11 (3.91 g, 3.58 mmol) in CH<sub>2</sub>Cl<sub>2</sub>/MeOH (1:1, 50 mL). After 22 h, the mixture was partitioned between Et<sub>2</sub>O (100 mL) and saturated NH<sub>4</sub>Cl (100 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified on silica gel (EtOAc/petroleum ether = 1:9) to afford the title compound 12 (2.75 g, 78%) as a pale yellow oil.  $[\alpha]^{20}_{\text{D}} +33$  (c 0.5, CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$ 7.38–7.15 (m, 15H), 5.38 (d, *J* = 1.2 Hz, 1H), 4.82 (d, *J* = 11.0 Hz, 1H), 4.75–4.61 (m, 3H), 4.59–4.45 (m, 2H), 4.31–4.21 (m, 1H), 4.21–4.15 (m, 1H), 4.15–4.08 (m, 1H), 3.97–3.83 (m, 5H), 3.83–3.71 (m, 2H), 3.71–3.61 (m, 1H), 3.31 (ddd, *J* = 1.5, 8.3, 9.8 Hz, 1H), 2.61–2.56 (m, 1H), 2.50–2.42 (m, 1H), 2.02–1.87 (m, 2H), 1.78–1.64 (m, 2H), 1.49–1.34 (m, 2H), 1.28–1.14 (m, 1H), 1.13–0.82 (m, 37H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$ 138.7, 138.6, 138.2, 128.4, 128.2, 128.0, 127.9, 127.82, 127.76, 127.4, 127.3, 117.7, 98.2, 80.3, 79.7, 77.4, 77.2, 77.0, 76.9, 76.6, 76.4, 75.8, 74.9, 74.5, 73.2, 73.0, 72.4, 72.0, 70.9, 68.9, 68.8, 51.5, 48.0, 45.4, 45.1, 29.6, 27.1, 20.5, 20.2, 17.6, 17.4, 17.34, 17.28, 17.25, 17.22, 17.1, 17.0, 13.0, 12.7, 12.3, 12.1, 9.7. HRMS-ESI [M+NH<sub>4</sub>]<sup>+</sup> calculated for C<sub>55</sub>H<sub>80</sub>O<sub>12</sub>Si<sub>2</sub>NH<sub>4</sub>: 1006.5532. Found 1006.5491.

**3,4,5-Tri-O-benzyl-6-(2,3,4,5-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-1,2-O-((1S,4S)-1,7,7-trimethyl-[2.2.1]bicyclohept-6-ylidene)-D-myo-inositol (14)**—TBAF (1 M, 7.8 mL) was added to a solution of 12 (3.10 g, 3.13 mmol) in THF (50 mL). After stirring for 1 h, the solvent was removed the residue was purified by flash chromatography on silica gel (MeOH/CH<sub>2</sub>Cl<sub>2</sub> = 3:97 to 1:24) to afford partially purified 13 (2.8 g); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) *inter alia*  $\delta$ 7.38–7.16 (m, 15H), 5.18 (d, *J* = 1.5 Hz, 1H), 4.81 (d, *J* = 11.0 Hz, 1H), 4.72 (d, *J* = 11.4 Hz, 1H), 4.67 (d, *J* = 11.4 Hz, 1H), 4.61–4.45 (m, 3H), 4.28 (dd, *J* = 4.4, 5.4 Hz, 1H), 3.94–3.84 (m, 2H), 3.82–3.50 (m, 7H), 3.21 (dd, *J* = 3.0, 9.4 Hz, 1H), 3.06 (br s, 1H), 2.89 (br s, 1H), 2.67 (d, *J* = 2.3 Hz, 1H), 2.55 (d, *J* = 7.0 Hz, 1H), 2.06–1.85 (m, 2H), 1.80–1.64 (m, 1H), 1.53–1.33 (m, 2H), 1.28–1.14 (m, 1H), 0.98 (s, 3H), 0.86 (s, 3H), 0.82 (s, 3H). HRMS-ESI [M+NH<sub>4</sub>]<sup>+</sup> calculated for C<sub>43</sub>H<sub>54</sub>O<sub>11</sub>NH<sub>4</sub>: 764.4010. Found 764.4013. BnBr (3.7 mL, 31 mmol) was added dropwise to a stirred solution of the

crude tetraol **13** and NaH (0.94 g, 60% in mineral oil, 23.5 mmol) in dry DMF (75 mL) at 0 °C. The reaction was left to slowly warm to rt over 80 min before being diluted with Et<sub>2</sub>O (300 mL) and quenched by the slow addition of water (150 mL). The aqueous phase was re-extracted with Et<sub>2</sub>O (2×100 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified on silica gel (EtOAc/petroleum ether = 3:7) to afford the title compound **14** (3.12 g, 90%). [ $\alpha$ ]<sub>D</sub><sup>20</sup> +19 (c 1.7, CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.45–7.00 (m, 35H), 5.53 (d, *J* = 1.4 Hz, 1H), 4.89 (d, *J* = 11.0 Hz, 1H), 4.82–4.50 (m, 12H), 4.35 (d, *J* = 12.1 Hz, 1H), 4.25 (dd, *J* = 3.4, 6.4 Hz, 1H), 4.09 (dd, *J* = 8.8, 18.4 Hz, 1H), 4.03–3.95 (m, 2H), 3.90 (dd, *J* = 3.1, 9.4 Hz, 1H), 3.86–3.71 (m, 4H), 3.63 (dd, *J* = 3.7, 11.3 Hz, 1H), 3.50 (dd, *J* = 1.3, 11.3 Hz, 1H), 3.27 (dd, *J* = 7.1, 9.7 Hz, 1H), 2.00–1.87 (m, 2H), 1.80–1.65 (m, 2H), 1.49–1.33 (m, 2H), 1.29–1.12 (m, 1H), 1.06 (s, 3H), 0.88 (s, 3H), 0.86 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  139.1, 138.8, 138.6, 138.5, 138.1, 128.4, 128.3, 128.23, 128.16, 128.1, 127.9, 127.81, 127.77, 127.6, 127.5, 127.4, 127.2, 117.9, 96.1, 81.1, 80.7, 79.6, 77.9, 76.8, 76.4, 75.0, 74.91, 74.85, 74.7, 74.5, 73.8, 73.1, 72.5, 72.1, 71.8, 68.9, 51.6, 47.9, 45.1, 44.9, 29.9, 27.2, 20.6, 20.4, 9.8. HRMS-ESI [M+NH<sub>4</sub>]<sup>+</sup> calculated for C<sub>71</sub>H<sub>78</sub>O<sub>11</sub>NH<sub>4</sub>: 1124.5888. Found 1124.5896.

**3,4,5-Tri-O-benzyl-6-(2,3,4,5-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-D-myoinositol (15)**—HCl (37%, 2 mL) was added to a stirred solution of **9** (322 mg, 0.291 mmol) in dioxane/methanol (2:5, 35 mL) at rt. After 48 h, the reaction was diluted with Et<sub>2</sub>O (100 mL), washed with water (100 mL), the aqueous layer was re-extracted with Et<sub>2</sub>O, (50 mL) and the combined organic fractions were washed with saturated NaHCO<sub>3</sub> (100 mL), brine (100 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified on silica gel (EtOAc/petroleum ether = 1:3 to 2:3) to afford the title compound **15** (245 mg, 86%). Analytical data were consistent with those previously reported.<sup>26</sup>

**2-O-Hexadecanoyl-1-O-hexadecyl-3-O-(4-methoxybenzyl)-sn-glycerol (20)**—Palmitoyl chloride (0.300 mL, 0.982 mmol) was added dropwise to a stirred solution of alcohol **19** (270 mg, 0.618 mmol) and dry pyridine (0.300 mL, 3.71 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (15 mL) at 0 °C. The reaction mixture was allowed to warm to rt over 17 h before being quenched with water (100 mL). The mixture was extracted with Et<sub>2</sub>O (2×150 mL) and the ethereal extract washed with a 0.5 M HCl (100 mL), saturated NaHCO<sub>3</sub> (100 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 0:1 to 1:9) to afford the title compound **20** (384 mg, 92%) as a colourless oil. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +1.0 (c 7.7, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.27–7.21 (m, 2H), 6.90–6.83 (m, 2H), 5.16 (quintet, *J* = 5.1 Hz, 1H), 4.49–4.43 (m, 2H), 3.79 (s, 3H), 3.60–3.55 (m, 4H), 3.44–3.35 (m, 2H), 2.32 (t, *J* = 7.5 Hz, 2H), 1.64–1.49 (m, 4H), 1.40–1.20 (m, 50H), 0.92–0.83 (m, 6H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  173.4, 159.3, 130.2, 129.3, 113.8, 73.0, 71.7, 71.4, 69.3, 68.6, 55.3, 34.5, 32.0, 29.8, 29.6, 29.4, 29.2, 26.1, 25.1, 22.8, 14.2. HRMS-ESI [M+Na]<sup>+</sup> calculated for C<sub>43</sub>H<sub>78</sub>O<sub>5</sub>Na: 697.5747. Found 697.5734.

**2-O-Hexadecanoyl-1-O-hexadecyl-sn-glycerol (21)**—Pd(OH)<sub>2</sub>/C (20%, 88 mg) was added to a mixture of **20** (380 mg, 0.563 mmol) in AcOH/EtOH (1:10, 16.5 mL). The mixture was stirred under an H<sub>2</sub> atmosphere for 4 h at rt, then filtered through Celite and the solvent removed. The residue was purified by column chromatography on silica gel (EtOAc/CH<sub>2</sub>Cl<sub>2</sub> = 1:49 to 1:19) to afford the title compound **21** (207 mg, 66%) as an oil. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = –2.8 (c 0.72, CHCl<sub>3</sub>); lit<sup>46</sup> = –2.6 (c 2.1, CHCl<sub>3</sub>); lit<sup>28</sup> = –1.2. Analytical data were consistent with those previously reported.<sup>28,46</sup>

**Benzyl (2-O-hexadecanoyl-1-O-hexadecyl-sn-glycero)-diisopropylphosphoramidite (17)**—1*H*-Tetrazole (35 mg, 0.50 mmol) was added to a

stirred solution of alcohol **21** (205 mg, 0.369 mmol) and benzyloxy-bis-(diisopropylamino)phosphine (262 mg, 0.775 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (20 mL) at rt for 90 min. The solvent was removed and the residue purified by column chromatography on silica gel (Et<sub>3</sub>N/EtOAc/petroleum ether = 3:10:90) to afford the title compound **17** (267 mg, 91%) as an oil. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +4.9 (c 0.72, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.37–7.23 (m, 5H), 5.13 (quintet, *J* = 5.2 Hz, 1H), 4.74–4.62 (m, 2H), 3.83–3.35 (m, 8H), 2.30 (t, *J* = 7.3 Hz, 2H), 1.65–1.48 (m, 4H), 1.32–1.16 (m, 62H), 0.90–0.83 (m, 6H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  173.3, 139.6, 128.3, 127.3, 127.0, 72.2, 71.7, 69.2, 65.5, 65.3, 62.3, 62.1, 61.9, 43.2, 43.1, 34.6, 32.0, 29.8, 29.7, 29.6, 29.4, 29.2, 26.2, 25.1, 24.8, 24.7, 24.6, 22.8, 14.2. <sup>31</sup>P NMR (121.5 MHz, CDCl<sub>3</sub>)  $\delta$  149.5, 149.2. HRMS-ESI [M+H]<sup>+</sup> calculated for C<sub>48</sub>H<sub>91</sub>NO<sub>5</sub>P: 729.6635. Found 792.6638.

### **3-O-Benzyl-2-deoxy-1-O-hexadeconyl-2-hexadeconylamino-*sn*-glycerol (23)**—

Palmitoyl chloride (1.54 mL, 5.08 mmol) was added dropwise to a stirred solution of (*R*)-(+)-2-amino-3-benzyloxy-1-propanol (**22**) (230 mg, 1.27 mmol) and DMAP (621 mg, 5.08 mmol) in dry CHCl<sub>3</sub> (25 mL) at 0 °C. After warming to rt over 6 h, the reaction was diluted with CHCl<sub>3</sub> (30 mL) and washed with H<sub>2</sub>O (30 mL) then 0.5 M HCl (30 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified by column chromatography (EtOAc/petroleum ether = 1:4) to afford the title compound **23** (705 mg, 84%) as a gummy solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.38–7.26 (m, 5H), 4.79 (d, *J* = 8.6 Hz, 1H), 4.51 (br s, 2H), 4.42–4.31 (m, 1H), 4.25 (dd, *J* = 6.1, 10.9 Hz, 1H), 4.13 (dd, *J* = 6.0, 10.9 Hz, 1H), 3.59 (dd, *J* = 3.4, 9.6 Hz, 1H), 3.48 (dd, *J* = 4.7, 9.6 Hz, 1H), 2.26 (dd, *J* = 7.4, 7.6 Hz, 2H), 2.14 (dd, *J* = 7.4, 7.8 Hz, 2H), 1.65–1.53 (m, 4H), 1.30–1.24 (m, 48H), 0.91–0.85 (m, 6H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  173.8, 172.9, 137.8, 128.5, 128.0, 127.8, 73.4, 68.8, 63.1, 48.0, 36.9, 34.3, 32.0, 29.9, 29.2, 25.8, 25.0, 22.8, 14.2. HRMS-ESI [M+Na]<sup>+</sup> calculated for C<sub>42</sub>H<sub>75</sub>NO<sub>4</sub>Na: 680.5594. Found 680.5588.

**2-Deoxy-1-O-hexadeconyl-2-*N*-hexadeconylamino-*sn*-glycerol (24)<sup>29</sup>**—Pd(OH)<sub>2</sub>/C (20%, 200 mg) was added to a mixture of **23** (663 mg, 1.01 mmol) in AcOH/EtOH (1:10, 33 mL). The mixture was stirred under an H<sub>2</sub> atmosphere for 16 h at rt, then filtered through Celite and the solvent removed. The residue was purified by column chromatography on silica gel (EtOAc/CH<sub>2</sub>Cl<sub>2</sub> = 3:7) to afford the title compound **24** (555 mg, 0.98 mmol, 97%) as a white solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  6.02 (d, *J* = 6.6 Hz, 1H), 4.27–4.12 (m, 3H), 3.72–3.56 (m, 2H), 2.96 (br s, 1H), 2.33 (dd, *J* = 7.6, 7.6 Hz, 2H), 2.19 (dd, *J* = 7.6, 7.6 Hz, 2H), 1.67–1.55 (m, 4H), 1.35–1.19 (m, 48H), 0.91–0.84 (m, 6H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  177.6, 173.8, 62.5, 62.0, 50.5, 36.8, 34.3, 32.0, 30.0, 29.2, 25.8, 25.0, 22.8. HRMS-ESI [M+Na]<sup>+</sup> calculated for C<sub>35</sub>H<sub>69</sub>NO<sub>4</sub>Na: 590.5124. Found 590.5118

### **2-Deoxy-1-O-hexadeconyl-2-*N*-hexadeconylamino-*sn*-glycero-3-O-benzyl-(*N*,*N*-diisopropyl)-phosphoramidite (18)**—

*1H*-Tetrazole (28 mg, 0.397 mmol) was added to a stirred solution of alcohol **24** (205 mg, 0.361 mmol) and benzyloxy-bis-(diisopropylamino)phosphine (244 mg, 0.722 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (10 mL) at rt for 60 min. The solvent was removed and the residue purified by column chromatography on silica gel (Et<sub>3</sub>N/EtOAc/petroleum ether = 3:10:90) to afford the title compound **18** (280 mg, 96%) as a clear oil that solidified on standing. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.37–7.25 (m, 5H), 4.89–4.58 (m, 2H), 4.38–4.25 (m, 1H), 4.22–4.04 (m, 2H), 3.87–3.56 (m, 2H), 2.28 (dd, *J* = 7.6, 7.6 Hz, 2H), 2.12–2.04 (m, 1H), 1.94–1.87 (m, 1H), 1.64–1.45 (m, 4H), 1.33–1.17 (m, 60H), 0.91–0.85 (m, 6H); <sup>31</sup>P NMR (121.5 MHz, CDCl<sub>3</sub>)  $\delta$  150.3, 149.9. HRMS-ESI [M+Na] calculated for C<sub>48</sub>H<sub>89</sub>N<sub>2</sub>O<sub>5</sub>Na: 827.6407. Found 827.6407.

### **1-O-Allyl-3,4,5-tri-O-benzyl-6-(2,3,4,5-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-2-O-(3,4,5-tri-O-benzyl- $\alpha$ -D-mannopyranosyl)-D-myo-inositol (26)**—TMSOTf (12



$\mu\text{L}$ , 0.066 mmol) was added to a mixture of alcohol 16<sup>26</sup> (0.339 g, 0.39 mmol) and trichloroacetamide 10 (0.46 g, 0.66 mmol) and 4Å molecular sieves in dry Et<sub>2</sub>O (50 mL) at -40 °C. After stirring for 1 h, the reaction was quenched with Et<sub>3</sub>N, filtered through Celite and evaporated to dryness. The crude residue was purified on silica gel (EtOAc/petroleum ether = 1:9 to 1:4) afford the intermediate compound 25 (0.473 g) as an inseparable mixture of anomers. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) *inter alia*  $\delta$ 5.92 (d,  $J$  = 2.9 Hz, 0.2H), 5.79–5.60 (m, 2H), 5.58–5.53 (m, 0.8H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) *inter alia*  $\delta$ 99.2 (<sup>1</sup> $J_{\text{CH}}$  = 175 Hz), 98.9 (<sup>1</sup> $J_{\text{CH}}$  = 173 Hz). HRMS-ESI [M+Na]<sup>+</sup> calculated for C<sub>98</sub>H<sub>100</sub>O<sub>17</sub>Na: 1571.6858. Found 1571.6818. Sodium methoxide in MeOH (30% solution, 0.1 mL) was added dropwise to a stirred solution of 25 (323 mg) in CH<sub>2</sub>Cl<sub>2</sub>/MeOH (1:1, 20 mL). After 24 h, the mixture was quenched by the careful addition of saturated NH<sub>4</sub>Cl (50 mL) and extracted with Et<sub>2</sub>O (2×50 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified on silica gel (EtOAc/petroleum ether = 1:9 to 3:7) to afford the title compound 26 (208 mg, 63%) as a colourless oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$ 5.77 (ddd,  $J$  = 5.5, 5.5, 10.4, 17.2 Hz, 1H), 4.50 (d,  $J$  = 1.4 Hz, 1H), 5.22 (dd,  $J$  = 1.4, 17.2 Hz, 1H), 5.21 (d,  $J$  = 1.1 Hz, 1H), 5.14 (dd,  $J$  = 1.2, 10.4 Hz, 1H), 4.95–4.50 (m, 16H), 4.49–4.41 (m, 2H), 4.38–4.28 (m, 2H), 4.24–3.75 (m, 13H), 3.52–3.20 (m, 6H), 3.16 (dd,  $J$  = 1.6, 9.7 Hz, 1H), 2.36 (d,  $J$  = 1.9 Hz, 1H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$ 139.1, 138.8, 138.71, 138.68, 138.6, 138.5, 138.2, 138.1, 138.0, 137.9, 133.9, 128.6, 128.3, 128.23, 128.17, 128.13, 128.05, 128.02, 127.96, 127.91, 127.87, 127.8, 127.6, 127.5, 127.4, 127.32, 127.27, 127.2, 117.7, 100.1, 98.6, 81.6, 81.5, 81.4, 80.1, 79.5, 78.9, 77.4, 77.2, 77.0, 76.6, 76.1, 75.9, 75.6, 75.3, 75.0, 74.9, 74.8, 74.1, 73.4, 73.1, 72.3, 72.2, 72.1, 71.8, 71.0, 70.84, 70.77, 68.7, 68.2. HRMS-ESI [M+Na]<sup>+</sup> calculated for C<sub>91</sub>H<sub>96</sub>O<sub>16</sub>Na: 1467.6596. Found 1467.6563.

**1-O-Allyl-3,4,5-tri-O-benzyl-2,6-di-O-(2,3,4,6-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-D-myo-inositol (27)**—NaH (0.054 g, 60% dispersion in mineral oil, 1.4 mmol) was added to a stirred solution of **26** (0.98 g, 0.68 mmol) and benzyl bromide (0.20 mL, 1.7 mmol) in dry DMF (30 mL) at 0 °C. The mixture was allowed to warm to rt over 18 h before being diluted with Et<sub>2</sub>O (50 mL) and quenched by the slow addition of water (50 mL). The aqueous phase was re-extracted with Et<sub>2</sub>O (2×25 mL), dried (MgSO<sub>4</sub>) filtered and the solvent removed. The crude residue was purified on silica gel (EtOAc/petroleum ether = 1:9 to 3:7) to afford the title compound **27** (1.01 g, 97%). Analytical data were consistent with those previously reported.<sup>17,31</sup>

**3,4,5-Tri-O-benzyl-2,6-di-O-(2,3,4,6-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-1-O-(2-O-hexadecanoyl-1-O-hexadecyl-*sn*-glycero-3-benzylphosphoryl)-D-myo-inositol (29)**—1H-Tetrazole (21 mg, 0.30 mmol) was added to a stirred solution of alcohol **28** (90 mg, 0.060 mmol) and phosphoramidite **17** (135 mg, 0.170 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (7 mL) at 0 °C under argon. After 2 h the reaction mixture was cooled to -40 °C and a dried (MgSO<sub>4</sub>) solution of *m*-CPBA (~55%, 90 mg, 0.29 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added to the reaction. After warming to rt over 1 h, the reaction was quenched by addition of Na<sub>2</sub>SO<sub>3</sub> (10%, 50 mL) and extracted with Et<sub>2</sub>O (50 mL). The organic phase was washed with saturated NaHCO<sub>3</sub> (50 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 1:9 to 3:7) followed by further purification on silica gel (EtOAc/CH<sub>2</sub>Cl<sub>2</sub> = 1:49 to 1:19) to afford the title compound **29** (68 mg, 52%) as an oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) mixture of isomers  $\delta$  7.38–7.00 (m, 60H), 5.53–5.51 (m, 1H), 5.33–5.32 (m, 1H), 5.05–4.40 (m, 23H), 4.30–3.78 (m, 17H), 3.56–3.20 (m, 9H), 2.23–2.10 (m, 2H), 1.58–1.41 (m, 4H), 1.31–1.15 (m, 50H), 0.89–0.82 (m, 6H). <sup>13</sup>C (75 MHz, CDCl<sub>3</sub>) selected signals  $\delta$  173.3, 99.9, 98.9. <sup>31</sup>P NMR (121.5 MHz, CDCl<sub>3</sub>)  $\delta$  0.26, 0.00. HRMS-ESI [M+Na]<sup>+</sup> calculated for C<sub>137</sub>H<sub>173</sub>O<sub>22</sub>PNa: 2224.2054. Found 2224.2051.

**2,6-(Di-O- $\alpha$ -D-mannopyranosyl)-1-O-(2-O-hexadecanoyl-1-O-hexadecyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol (4)**—Pd(OH)<sub>2</sub>/C (20%, 25 mg) was added to a stirred solution of **29** (68 mg, 0.031 mmol) in THF/MeOH (2:3, 5 mL). The mixture was stirred under an H<sub>2</sub> atmosphere for 4 h at rt, then filtered through Celite and the solvent removed. The residue was purified by column chromatography on silica gel (CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O = 70:40:0 to 70:60:6) to afford the title compound **4** (26 mg, 74%) as a white powder. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +39 (*c* 0.40, CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O = 70:60:6). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O 70:40:6)  $\delta$  5.28–5.20 (m, 1H), 5.15 (br s, 1H), 5.12 (br s, 1H), 4.33 (m, 1H), 4.12–3.96 (m, 7H), 3.87–3.60 (m, 12H), 3.57–3.42m, 3H), 3.30 (t, *J* = 7.0 Hz, 1H), 2.41 (t, *J* = 7.2 Hz, 2H), 1.65–1.55 (m, 4H), 1.33–1.22 (m, 50H), 0.92–0.85 (m, 6H); <sup>13</sup>C (125 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O 70:40:6)  $\delta$  176.0, 103.0, 102.9, 79.9, 79.8, 78.2, 78.1, 74.8, 74.4, 74.4, 74.2, 73.5, 73.5, 73.0, 72.1, 72.1, 71.8, 71.6, 70.7, 68.4, 68.4, 65.4, 65.4, 62.8, 62.6, 35.7, 33.2, 31.0, 30.9, 30.7, 30.6, 30.4, 27.4, 26.4, 23.9, 15.0, 10.0; <sup>31</sup>P NMR (121.5 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O 70:40:6)  $\delta$  0.63. HRMS-ESI [*M*–H]<sup>–</sup> calculated for C<sub>53</sub>H<sub>100</sub>O<sub>22</sub>P: 1119.6444. Found 1119.6456.

**3,4,5-tri-O-benzyl-2,6-di-O-(2,3,4,6-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-1-O-(2-deoxy-3-benzylphosphoryl-1-O-hexadecanoyl-2-O-hexadecanoylamino-*sn*-glycero)-D-*myo*-inositol (30)**—*H*-Tetrazole (10 mg, 0.135 mmol) was added to a stirred solution of alcohol **28** (68 mg, 0.045 mmol) and phosphoramidite **18** (109 mg, 0.135 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (10 mL) at 0 °C under argon. After 1 h, the reaction mixture was cooled to –40 °C and a dried (MgSO<sub>4</sub>) solution of *m*-CPBA (~50%, 47 mg, 0.135 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was added to the reaction. After warming to rt over 2 h the reaction was quenched by addition of Na<sub>2</sub>SO<sub>3</sub> (10%, 50 mL) and extracted with Et<sub>2</sub>O (50 mL). The organic phase was washed with saturated NaHCO<sub>3</sub> (50 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 1:9 to 1:4) to afford the title compound **30** (61 mg, 62%) as an oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) mixture of isomers  $\delta$  7.48–7.03 (m, 60H), 6.54 (d, *J* = 7.5 Hz, 3H), 6.40 (d, *J* = 8.6 Hz, 7H), 5.48 (br s, 3H), 5.44 (br s, 7H), 5.10–5.42 (m, 21H), 4.51–4.27 (m, 5H), 4.26–3.66 (m, 15H), 3.52–3.12 (m, 6H), 2.37–2.27 (m, 1H), 2.17–2.09 (m, 1H), 2.03–1.95 (m, 2H), 1.71–1.37 (m, 4H), 1.35–1.12 (m, 48H), 0.91–0.84 (m, 6H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) selected signals  $\delta$  173.8, 173.5, 173.2, 173.1, 98.8, 98.7, 98.4, 98.1; <sup>31</sup>P NMR (121.5 MHz, CDCl<sub>3</sub>)  $\delta$  0.94, –0.45. HRMS-ESI [*M*+Na]<sup>+</sup> calculated for C<sub>137</sub>H<sub>172</sub>NO<sub>5</sub>PNa: 2237.2006. Found 2237.2007.

**2,6-(Di-O- $\alpha$ -D-mannopyranosyl)-1-O-(2-deoxy-1-O-hexadecanoyl-2-*N*-hexadecanoylamino-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol (3)**—Pd(OH)<sub>2</sub>/C (20%, 40 mg) was added to a stirred solution of **28** (28 mg, 24.8 mmol) in THF/MeOH (2:3, 5 mL). The mixture was stirred under an H<sub>2</sub> atmosphere for 16 h at rt, then filtered through Celite and the solvent removed. The residue was purified by column chromatography on silica gel (CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O = 70:40:4) to afford the title compound **3** (21 mg, 75%) as a white powder. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +35 (*c* 0.15, CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O = 40:40:10). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O 40:40:10)  $\delta$  5.15 (d, *J* = 1.6 Hz, 1H), 5.10 (d, *J* = 1.5 Hz, 1H), 4.32–4.28 (m, 2H), 4.15 (dd, *J* = 8.0, 11.4 Hz, 1H), 4.08–4.03 (m, 3H), 4.01–3.93 (m, 4H), 3.85–3.78 (m, 5H), 3.77–3.59 (m, 5H), 3.48 (dd, *J* = 7.6, 10.1 Hz, 1H), 3.30 (dd, *J* = 9.2, 9.3 Hz, 1H), 3.16 (q, *J* = 7.3 Hz, 6H), 2.33 (dd, *J* = 8.3, 6.9 Hz, 2H), 2.24 (dd, *J* = 7.5, 7.5 Hz, 2H), 1.65–1.55 (m, 4H), 1.31 (t, *J* = 7.3 Hz, 9H), 1.29–1.26 (m, 48H), 0.91–0.87 (m, 6H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O 40:40:10)  $\delta$  176.3, 175.5, 102.4, 102.2, 79.3, 77.55, 77.50, 74.1, 73.8, 73.7, 73.6, 71.5, 71.4, 71.11, 71.07, 70.9, 67.8, 67.7, 65.2, 65.1, 64.2, 62.2, 62.0, 47.4, 37.0, 34.8, 32.6, 30.5, 30.42, 30.37, 30.35, 30.31, 30.30, 30.26, 30.1, 30.01, 30.00, 29.9, 26.7, 25.5, 23.3, 14.5. <sup>31</sup>P NMR (121.5 MHz, CDCl<sub>3</sub>)  $\delta$  4.63. HRMS-ESI [*M*–H]<sup>–</sup> calculated for C<sub>53</sub>H<sub>99</sub>NO<sub>22</sub>P: 1132.6396. Found 1132.6382.

**Phenyl 3,4-di-O-benzyl-6-O-hexadecanoyl-1-thio- $\alpha$ -D-mannopyranoside (33)**—Hexadecanoyl chloride (2.6 mL, 8.5 mmol) was added dropwise to a stirred solution of diol **32**<sup>32</sup> (3.50 g, 7.73 mmol) and pyridine (6.25 mL, 77 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (30 mL) at 0 °C and the reaction was allowed to warm to rt overnight. The reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (70 mL), washed with 1 M aq HCl (100 mL), saturated aq NaHCO<sub>3</sub>, dried (MgSO<sub>4</sub>), filtered and the solvent removed. The residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 1:9 to 3:7) to afford the title compound **33** (4.82 g, 90%) as a yellow oil. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +148 (c 1.3, CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.47–7.43 (m, 2H), 7.39–7.21 (m, 13H), 5.57 (d, *J* = 1.5 Hz, 1H), 4.87 (d, *J* = 10.8 Hz, 1H), 4.71 (s, 2H), 4.58 (d, *J* = 10.8 Hz, 1H), 4.38–4.30 (m, 3H), 4.26–4.23 (m, 1H), 3.90 (dd, *J* = 3.1, 9.0 Hz, 1H), 3.81 (app t, *J* = 9.2 Hz, 1H), 2.65 (d, *J* = 2.5 Hz, 1H), 2.27–2.22 (m, 2H), 1.62–1.52 (m, 2H), 1.33–1.24 (m, 24H), 0.90–0.86 (m, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  173.5, 137.8, 137.4, 133.6, 131.6, 129.0, 128.6, 128.5, 128.2, 128.01, 127.99, 127.9, 127.5, 87.1, 80.3, 75.2, 74.4, 72.2, 70.5, 69.7, 63.1, 34.1, 31.9, 29.65, 29.62, 29.57, 29.4, 29.3, 29.2, 29.1, 24.8, 22.7, 14.1; HRMS-ESI [M+Na]<sup>+</sup> Calculated for C<sub>42</sub>H<sub>58</sub>O<sub>6</sub>SNa 713.3852. Found 713.3881.

**Phenyl 2-O-(2-azidomethylbenzoyl)-3,4-di-O-benzyl-6-O-hexadecanoyl-1-thio- $\alpha$ -D-mannopyranoside (31)**—2-Azidomethylbenzoyl chloride (AZMBCl) was prepared as described<sup>47</sup> and used without purification. A solution of **32** (1.35 g, 1.95 mmol) in pyridine (10 mL) was added to ice-cooled AZMBCl (0.49 g, 2.5 mmol) and the stirred mixture was allowed to warm to rt overnight. Excess pyridine was concentrated under reduced pressure, and the residue was taken up in EtOAc (150 mL), washed with 1 M aq HCl (2 x 50 mL), saturated aq NaHCO<sub>3</sub> (2 x 50 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 7.5:92.5) to afford the title compound **31** (1.016 g, 61%) as an oil. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +55 (c 0.9, CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.04 (dd, *J* = 1.2, 7.8 Hz, 1H), 7.58 (dt, *J* = 1.2, 7.4 Hz, 1H), 7.52–7.48 (m, 3H), 7.42–7.24 (m, 14H), 5.83 (dd, *J* = 1.6, 3.0 Hz, 1H), 5.60 (d, *J* = 1.6 Hz, 1H), 4.92 (d, *J* = 10.9 Hz, 1H), 4.80 (d, *J* = 11.3 Hz, 1H), 4.75 (s, 2H), 4.64 (d, *J* = 11.3 Hz, 1H), 4.61 (d, *J* = 10.9 Hz, 1H), 4.48–4.32 (m, 3H), 4.07 (dd, *J* = 3.0, 9.2 Hz, 1H), 3.92 (app t, *J* = 9.3 Hz, 1H), 2.29–2.24 (m, 2H), 1.63–1.53 (m, 2H), 1.32–1.23 (m, 24H), 0.90–0.86 (m, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  173.4, 165.6, 137.8, 137.6, 137.4, 133.2, 133.0, 132.1, 131.4, 129.7, 129.1, 128.4, 128.2, 128.1, 127.95, 127.1, 86.1, 78.5, 75.3, 74.3, 71.9, 70.9, 70.8, 63.1, 52.9, 34.1, 31.9, 29.7, 29.63, 29.58, 29.5, 29.3, 29.23, 29.20, 24.8, 22.7, 14.1; HRMS-ESI [M+Na]<sup>+</sup> Calculated for C<sub>50</sub>H<sub>63</sub>N<sub>3</sub>O<sub>7</sub>SNa: 872.4284. Found 872.4283.

**1-O-Allyl-2-O-(2-O-[2-azidomethylbenzoyl]-3,4-di-O-benzyl-6-O-hexadecanoyl- $\alpha$ -D-mannopyranosyl)-6-O-(2,3,4,6-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-3,4,5-tri-O-benzyl-D-myo-inositol (34)**—Acceptor **16**<sup>26</sup> (160 mg, 0.16 mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and cooled to –60 °C. In a separate flask, a mixture of donor **31** (125 mg, 0.147 mmol), diphenyl sulfoxide (83 mg, 0.412 mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP, 91 mg, 0.441 mmol) was co-evaporated from dry CH<sub>2</sub>Cl<sub>2</sub> (10 mL). The reagents were redissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and cooled to –60 °C with stirring, before the addition of Tf<sub>2</sub>O (35  $\mu$ L, 0.21 mmol). After 5 min, the solution of acceptor was transferred to the reaction vessel (cannula), rinsing with CH<sub>2</sub>Cl<sub>2</sub> (2 mL), and the reaction mixture was allowed to warm to 0 °C over 3 h. The reaction was quenched with saturated aq NaHCO<sub>3</sub> and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic extracts were washed with saturated aq NaHCO<sub>3</sub>, dried (MgSO<sub>4</sub>), filtered and the solvent removed. The residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 15:85) followed by a second column (CH<sub>2</sub>Cl<sub>2</sub>/petroleum ether/diethyl ether = 30:20:1) to afford the title compound **34** (88 mg, 34%). [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +19 (c 0.96, CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$

8.03, (dd,  $J = 1.1, 7.8$  Hz, 1H), 7.59–7.50 (m, 2H), 7.41–7.08 (m, 46H), 5.82–5.68 (m, 2H), 5.57 (d,  $J = 1.6$  Hz, 1H), 5.26–5.19 (m, 2H), 5.08–5.03 (m, 1H), 4.94–4.45 (m, 19H), 4.31–3.82 (m, 15H), 3.40 (dd,  $J = 3.1, 11.3$  Hz, 1H), 3.33–3.25 (m, 3H), 3.17 (dd,  $J = 1.7, 9.7$  Hz, 1H), 2.24–2.19 (m, 2H), 1.59–1.51 (m, 2H), 1.33–1.21 (m, 24H), 0.90–0.86 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  173.3, 165.1, 139.1, 138.85, 138.77, 138.6, 138.4, 138.2, 138.1, 137.7, 137.6, 133.8, 132.7, 131.3, 129.4, 128.7, 128.5, 128.4, 128.32, 128.29, 128.2, 128.1, 128.04, 127.95, 127.82, 127.78, 127.7, 127.5, 127.40, 127.35, 127.21, 127.15, 117.4, 98.9, 81.5, 81.4, 80.9, 80.0, 78.5, 75.9, 75.7, 75.3, 75.1, 74.9, 74.8, 73.6, 73.2, 72.55, 72.50, 72.1, 72.0, 71.5, 70.8, 69.8, 69.1, 68.6, 62.8, 52.9, 34.1, 31.9, 29.7, 29.63, 29.59, 29.5, 29.3, 29.23, 29.20, 24.8, 22.7, 14.1; HRMS-ESI  $[\text{M}+\text{Na}]^+$  Calculated for  $\text{C}_{108}\text{H}_{125}\text{N}_3\text{O}_{18}\text{Na}$ : 1774.8856. Found 1774.8870.

**2-O-(2-O-[2-Azidomethylbenzoyl]-3,4-di-O-benzyl-6-O-hexadecanoyl- $\alpha$ -D-mannopyranosyl)-6-O-(2,3,4,6-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-3,4,5-tri-O-benzyl-D-*myo*-inositol (35)**—(1,5-

Cyclooctadiene)bis(methyldiphenylphosphine)iridium(I) hexafluorophosphate (29 mg, 34  $\mu\text{mol}$ ) was stirred with a solution of 34 (301 mg, 172  $\mu\text{mol}$ ) in THF (10 mL) under Ar at rt for 10 min. The reaction was exposed to an atmosphere of  $\text{H}_2$  for ~30 s, during which time the colour changed from red to pale yellow, then stirred under Ar for 45 min, at which point TLC (20% EtOAc/petroleum ether) indicated complete consumption of the starting material. The mixture was concentrated under reduced pressure and dissolved in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (2:1, 12 mL) containing 0.45 mL AcCl. After 1 h at rt, solid  $\text{NaHCO}_3$  was added and the mixture stirred for a further 5 min. The reaction mixture was diluted with water, extracted with  $\text{CH}_2\text{Cl}_2$ , dried ( $\text{MgSO}_4$ ), filtered and the solvent removed. The crude residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 1:4) to afford the title compound 35 (240 mg, 82%).  $[\alpha]_D^{20} +32$  ( $c$  1.1,  $\text{CH}_2\text{Cl}_2$ );  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  8.07, (d,  $J = 7.9$  Hz, 1H), 7.59–7.50 (m, 2H), 7.39–7.14 (m, 46H), 5.70–5.69 (m, 1H), 5.40 (d,  $J = 1.6$  Hz, 1H), 5.30 (d,  $J = 2.0$  Hz, 1H), 4.91–4.26 (m, 20H), 4.19–3.76 (m, 11H), 3.64–3.56 (m, 2H), 3.47 (dd,  $J = 6.6, 10.3$  Hz, 1H), 3.36–3.27 (m, 3H), 2.26–2.21 (m, 2H), 1.61–1.52 (m, 2H), 1.33–1.21 (m, 24H), 0.90–0.86 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  173.4, 165.3, 138.53, 138.49, 138.4, 138.2, 138.1, 137.80, 137.76, 137.7, 132.8, 131.4, 129.3, 128.6, 128.44, 128.41, 128.38, 128.30, 128.26, 128.2, 128.0, 127.9, 127.8, 127.7, 127.63, 127.59, 127.54, 127.46, 127.4, 99.0, 96.0, 81.2, 80.4, 80.1, 79.4, 78.2, 76.1, 75.6, 75.3, 75.1, 75.02, 74.97, 74.5, 73.7, 73.3, 72.5, 72.2, 72.14, 72.09, 71.6, 71.1, 69.7, 69.3, 69.2, 62.8, 52.9, 34.1, 31.9, 29.7, 29.62, 29.58, 29.5, 29.3, 29.23, 29.20, 24.8, 22.7, 14.1; HRMS-ESI  $[\text{M}+\text{Na}]^+$  Calculated for  $\text{C}_{105}\text{H}_{121}\text{N}_3\text{O}_{18}\text{Na}$ : 1734.8543. Found 1734.8586.

**2-O-(2-O-[2-Azidomethylbenzoyl]-3,4-di-O-benzyl-6-O-hexadecanoyl- $\alpha$ -D-mannopyranosyl)-1-O-(1-O-hexadecanoyl-2-O-hexadecyl-*sn*-glycero-3-benzylphosphoryl)-6-O-(2,3,4,6-tetra-O-benzyl- $\alpha$ -D-mannopyranosyl)-3,4,5-tri-O-benzyl-D-*myo*-inositol (37)**—1H-Tetrazole (13 mg, 0.19 mmol) was added to a stirred solution of alcohol 35 (106 mg, 0.062 mmol) and phosphoramidite 36 (147 mg, 0.186 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (8 mL) under Ar at 0 °C. After stirring at rt for 3 h, the reaction mixture was cooled to –40 °C and a solution of *m*-CPBA (50%, 86 mg, 0.25 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 mL) was transferred by cannula into the reaction mixture. After being stirred at rt for 1 h, the reaction was quenched by addition of 10% aq  $\text{Na}_2\text{SO}_3$  (50 mL) and the combined mixture extracted with  $\text{Et}_2\text{O}$  (100 mL). The ethereal extract was washed with saturated aq  $\text{NaHCO}_3$  (3  $\times$  50 mL), dried ( $\text{MgSO}_4$ ), filtered and the solvent removed. The residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 1:9 to 1:4) followed by a second column (acetone/toluene = 1:50 to 3:97) to afford the title compound 37 (59 mg, 0.024 mmol, 39%) as an oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.04–7.98 (m, 1H), 7.58–7.43 (m, 2H), 7.36–7.03 (m, 51H), 5.66–5.62 (m, 1H), 5.56–5.62 (m,

1H), 5.33–5.29 (m, 1H), 5.11–5.04 (m, 2H), 4.95–4.39 (m, 20H), 4.36–3.82 (m, 17H), 3.60–3.22 (m, 7H), 2.35–2.13 (m, 4H), 1.61–1.48 (m, 4H), 1.42–1.17 (m, 76H), 0.90–0.86 (m, 9H); <sup>13</sup>C (75 MHz, CDCl<sub>3</sub>) selected signals δ 173.2, 99.6, 98.6; <sup>31</sup>P NMR (121 MHz, CDCl<sub>3</sub>) δ 0.1, 0.0; HRMS-ESI [M+Na]<sup>+</sup> calcd for C<sub>147</sub>H<sub>196</sub>N<sub>3</sub>O<sub>24</sub>NaP: 2441.3845. Found 2441.3855.

**2-O-(3,4-Di-O-benzyl-6-O-hexadecanoyl-α-D-mannopyranosyl)-1-O-(1-O-hexadecanoyl-2-O-hexadecyl-sn-glycero-3-benzylphosphoryl)-6-O-(2,3,4,6-tetra-O-benzyl-α-D-mannopyranosyl)-3,4,5-tri-O-benzyl-D-myo-inositol (38)**—

Tri-*n*-butyl phosphine (34 μL, 0.14 mmol) was added to a degassed solution of AZMB ester 37 (58 mg, 0.024 mmol) in THF/H<sub>2</sub>O (9:1, 10 mL) under Ar. After stirring at rt for 3 h, toluene (20 mL) was added and the solvent removed *in vacuo*. The residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 1:4 to 3:7) to afford the title compound 38 (42 mg, 78%) as an oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.40–6.99 (m, 50H), 5.46–5.42 (m, 1H), 5.19 (m, 1H), 5.11–4.39 (m, 20H), 4.19–3.78 (m, 18H), 3.62–3.20 (m, 7H), 2.54–2.49 (m, 1H), 2.28–2.18 (m, 4H), 1.60–1.40 (m, 6H), 1.33–1.22 (m, 74H), 0.90–0.86 (m, 9H); <sup>13</sup>C (75 MHz, CDCl<sub>3</sub>) selected signals δ 173.5, 101.5, 98.4; <sup>31</sup>P NMR (121 MHz, CDCl<sub>3</sub>) δ 0.0, –0.2; HRMS-ESI [M+Na]<sup>+</sup> calcd for C<sub>139</sub>H<sub>191</sub>O<sub>23</sub>PNa: 2282.3412. Found 2282.3401.

**1-O-(1-O-Hexadecanoyl-2-O-hexadecyl-sn-glycero-3-phosphoryl)-2-O-(6-O-hexadecanoyl-α-D-mannopyranosyl)-6-O-(α-D-mannopyranosyl)-D-myo-inositol (5)**—

Pd(OH)<sub>2</sub>/C (20%, 36 mg) was added to a stirred solution of compound 38 (42 mg, 0.019 mmol) in THF/MeOH (2:3, 5 mL). The mixture was stirred under a H<sub>2</sub> atmosphere for 3.5 h at rt, then filtered through Celite and the solvent removed. The residue was purified by column chromatography on silica gel (CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O = 70:40:0 to 70:40:8) afforded the title compound 5 (19 mg, 75%) as a white powder. 95% pure by HPLC system 1 [α]<sub>D</sub><sup>20</sup> = +30 (c 0.10, CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O, 70:40:6); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O 70:40:6) δ 5.15 (br s, 1H), 5.10 (br s, 1H), 4.38–4.08 (m, 8H), 4.05 (dd, *J* = 1.7, 3.3 Hz, 1H), 4.01–3.96 (m, 2H), 3.94–3.89 (m, 1H), 3.84–3.73 (m, 6H), 3.70–3.65 (m, 3H), 3.60 (app t, *J* = 9.5 Hz, 1H), 3.55–3.51 (m, 1H), 3.46 (dd, *J* = 2.5, 10 Hz, 1H), 3.29 (t, *J* = 9.3 Hz, 1H), 2.39–2.33 (m, 4H), 1.65–1.54 (m, 6H), 1.37–1.27 (m, 74H), 0.90–0.87 (m, 9H); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O 70:40:6) δ 175.7, 175.3, 102.3, 102.2, 79.4, (br d), 79.2, 77.5, 77.4, 74.0, 73.8, 73.5, 71.44, 71.35, 71.3, 71.2, 71.0, 70.9, 70.7, 67.8, 67.6, 64.9, 64.7, (br d), 64.3, 61.9, 34.9, 34.5, 32.51, 30.46, 30.40, 30.37, 30.3, 30.23, 30.18, 30.03, 29.97, 29.9, 29.8, 26.7, 25.6, 25.4, 23.2, 14.5; <sup>31</sup>P NMR (202 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O 70:40:6) δ –0.3; HRMS-ESI [M-H]<sup>–</sup> calcd for C<sub>69</sub>H<sub>130</sub>O<sub>23</sub>P: 1357.8741. Found 1357.8729.

**1,3-bis-(2-O-Acetyl-3,4,5-tri-O-benzyl-α-D-mannopyranosyl)-2-hydroxypropane (40)**—

DDQ (35 mg, 0.154 mmol) was added to a stirred solution of **39** (149 mg, 0.128 mmol) in CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O (9:1, 4 mL). After 50 min the reaction was diluted with Et<sub>2</sub>O (80 mL), washed with water (3×20 mL), saturated NaHCO<sub>3</sub> (3×20 mL), dried (MgSO<sub>4</sub>), filtered and the solvent removed. The crude residue was purified by column chromatography on silica gel (EtOAc/petroleum ether = 2:3) to afford the title compound **40** (96 mg, 72%) as waxy solid. [α]<sub>D</sub><sup>20</sup> = +30 (c 0.82, CH<sub>2</sub>Cl<sub>2</sub>). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.35–7.22 (m, 26H), 7.18–7.13 (m, 4H), 5.38–5.35 (m, 2H), 4.87–4.82 (m, 4H), 4.71–4.62 (m, 4H), 4.55–4.50 (m, 2H), 4.50–4.45 (m, 4H), 3.97–3.90 (m, 3H), 3.89–3.78 (m, 4H), 3.78–3.72 (m, 2H), 3.72–3.65 (m, 3H), 3.62 (dd, *J* = 6.2, 10.7 Hz, 1H), 3.56 (dd, *J* = 4.5, 10.7 Hz, 1H), 3.51 (dd, *J* = 6.6, 10.6 Hz, 1H), 2.13 (s, 6H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 170.3, 138.09, 138.07, 137.9, 128.34, 128.28, 128.26, 128.0, 127.8, 127.7, 127.6, 127.5, 98.5 (*J*<sub>CH</sub> = 170 Hz), 98.3 (*J*<sub>CH</sub> = 170 Hz), 78.1, 78.0, 75.11, 75.09, 74.2, 73.4, 71.89, 71.85, 71.7, 69.8, 69.6,

69.2, 68.79, 68.77, 68.7, 68.6, 21.0. HRMS-ESI  $[M+Na]^+$  calculated for  $C_{61}H_{68}O_{15}Na$ : 1063.4456. Found 1063.4445.

**2-O-(Benzyloxy-1-O-hexadecanoyl-2-O-hexadecyl-*sn*-glycero-3-phosphoryl)-1,3-bis-(2-O-acetyl-3,4,5-tri-O-benzyl- $\alpha$ -D-mannopyranosyl)glycerol (41)**—

A mixture of alcohol 40 (86 mg, 0.083 mmol) and phosphoramidite 36 (98 mg, 0.124 mmol) was co-evaporated from dry  $CH_2Cl_2$  (10 mL) then placed under high vacuum for 1 h. The reagents were dissolved in dry  $CH_2Cl_2$  (2 mL) and stirred for 1 h at rt over 4Å molecular sieves. The solution was cooled to 0 °C before the addition of 4,5-dicyanoimidazole (16 mg, 0.14 mmol) then warmed to rt over 18 h. The solution was then cooled to -15 °C before the addition of dried ( $MgSO_4$ ) *m*CPBA (~60%, 50 mg) in  $CH_2Cl_2$ . After warming to rt over 1 h, the reaction mixture was diluted with  $Et_2O$  (30 mL) and washed  $Na_2S_2O_3$  (10%, 30 mL), saturated  $NaHCO_3$  (3 × 20 mL), brine (30 mL), dried ( $MgSO_4$ ), filtered and the solvent removed. The crude residue was purified by column chromatography on silica gel ( $EtOAc$ /petroleum ether = 1:4 to 2:3) to afford the title compound 41 (93 mg, 64%).  $^1H$  NMR (500 MHz,  $CDCl_3$ ) mixture of isomers  $\delta$  7.37–7.19 (m, 31H), 7.17–7.10 (m, 4H), 5.11–5.01 (m, 2H), 4.90–4.79 (m, 4H), 4.70–4.57 (m, 5H), 4.52–4.39 (m, 6H), 4.17–4.10 (m, 1H), 4.09–3.73 (m, 10H), 3.71–3.58 (m, 4H), 3.58–3.52 (m, 1H), 3.50–3.37 (m, 2H), 2.26–2.21 (m, 2H), 2.12 (s, 3H), 2.11 (s, 3H), 1.62–1.51 (m, 2H), 1.50–1.41 (m, 2H), 1.35–1.15 (m, 50H), 0.93–0.80 (m, 6H);  $^{13}C$  NMR (125 MHz,  $CDCl_3$ )  $\delta$  173.3, 170.2, 138.4, 138.2, 137.9, 135.8, 135.75, 135.71, 135.69, 128.6, 128.5, 128.4, 128.32, 128.30, 128.25, 128.2, 128.1, 128.0, 127.81, 127.80, 127.78, 127.7, 127.5, 98.32, 98.31, 98.15, 98.11, 78.20, 78.19, 75.79, 75.77, 75.73, 75.71, 75.4, 75.31, 75.26, 75.10, 75.07, 74.04, 74.00, 73.40, 73.38, 71.92, 71.88, 71.85, 71.83, 70.65, 70.61, 69.42, 69.38, 68.71, 68.66, 68.6, 68.4, 68.1, 66.72, 66.68, 66.42, 66.38, 66.3, 62.5, 34.1, 31.9, 29.9, 29.7, 29.64, 29.62, 29.5, 29.32, 29.28, 29.1, 26.0, 24.8, 22.6, 21.0;  $^{31}P$  NMR (202 MHz,  $CDCl_3$ )  $\delta$  -1.44, -1.48; HRMS-ESI  $[M+Na]^+$  calculated for  $C_{103}H_{143}O_{21}PNa$ : 1769.9757. Found 1769.9751.

**2-O-(1-O-Hexadecanoyl-2-O-hexadecyl-*sn*-glycero-3-phosphoryl)-1,3-bis-(2-O-acetyl- $\alpha$ -D-mannopyranosyl) glycerol (42)**—

A suspension of 41 (90 mg, 51 mmol) and  $Pd(OH)_2$  in 2:3 THF/MeOH (5 mL) were stirred under an atmosphere of  $H_2$  ambient temperature for 4 h. The mixture was filtered through Celite, concentrated and purified by purified by column chromatography (neat  $CHCl_3$  to 2:1  $CHCl_3$ /MeOH) to give 42 (40 mg, 70%) as a white powder.  $[\alpha]_D^{20} = +27$  (c 0.74, 2:1  $CHCl_3$ /MeOH);  $^1H$  NMR (500 MHz, 2:1  $CDCl_3$ / $CD_3OD$ )  $\delta$  5.08–5.03 (m, 2H), 4.83 (d,  $J = 0.9$  Hz, 1H), 4.81 (d,  $J = 1.1$  Hz), 4.54–4.46 (m, 1H), 4.29 (dd,  $J = 2.8, 11.6$  Hz), 4.17–4.11 (m, 1H), 4.06–3.86 (m, 8H), 3.80–3.51 (m, 11H), 2.35 (t,  $J = 7.5$  Hz, 1H), 2.12 (2 × s, 6H), 1.67–1.52 (m, 4H), 1.38–1.22 (m, 50H), 0.92–0.85 (m, 6H);  $^{13}C$  NMR (125 MHz, 2:1  $CDCl_3$ / $CD_3OD$ )  $\delta$  174.8, 171.61, 171.58, 98.1, 98.0, 76.8, 76.7, 74.4, 74.3, 73.7, 73.6, 72.72, 72.69, 71.2, 69.8, 68.7, 68.5, 67.0, 65.43, 65.41, 64.0, 62.5, 62.4, 34.6, 32.3, 30.3, 30.05, 30.00, 29.90, 29.87, 29.7, 29.5, 26.4, 25.3, 23.0, 20.9, 14.2;  $^{31}P$  NMR (202 MHz, 2:1  $CDCl_3$ / $CD_3OD$ )  $\delta$  2.7; HRMS-ESI  $[M-H]^-$  Calculated for  $C_{54}H_{100}O_{21}P$ : 1115.6495. Found 1115.6488.

**2-O-(1-O-Hexadecanoyl-2-O-hexadecyl-*sn*-glycero-3-phosphoryl)-1,3-bis-O-( $\alpha$ -D-mannopyranosyl) glycerol (6)**—

Sodium methoxide in MeOH (0.5 M solution, 50  $\mu$ L) was added dropwise to a stirred solution of 42 (26 mg, 0.023 mmol) in MeOH (5 mL). After 2 h the mixture was quenched with Dowex 50W8-100 ( $H^+$ ) resin, filtered and subsequently made neutral by treatment with Dowex 50W8-100 ( $Na^+$ ) resin. After filtration and concentration, the crude residue was purified on silica gel ( $CHCl_3$ /MeOH/ $H_2O$  = 70:35:1.75 to 70:35:6) to afford the title compound 6 (17 mg, 71%).  $[\alpha]_D^{20} = +39$  (c 0.14,  $CHCl_3$ /MeOH/ $H_2O$  70:40:6);  $^1H$  NMR (500 MHz,  $CDCl_3$ / $CD_3OD$ / $D_2O$  70:35:6)  $\delta$  4.87 (br,

1H), 4.84 (br, 1H), 4.39 (br, 1H), 4.30 (dd,  $J = 2.7, 11.8$  Hz, 1H), 4.11 (dd,  $J = 7.3, 11.7$  Hz, 1H), 3.95–3.83 (m, 8H), 3.77–3.59 (m, 12H), 3.53 (dt,  $J = 7.0, 9.4$  Hz, 1H), 2.35 (t,  $J = 7.6$  Hz, 2H), 1.65–1.60 (m, 2H), 1.59–1.53 (m, 2H), 1.34–1.27 (m, 50H), 0.90–0.87 (m, 6H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{D}_2\text{O}$  70:35:6)  $\delta$  175.2, 100.8, 100.7, 77.3, 77.2, 73.5, 73.4, 71.5, 71.3, 70.94, 70.90, 67.8, 67.7, 67.3, 67.1, 64.9, 64.7, 64.6, 61.9, 61.8, 34.8, 32.4, 30.4, 30.23, 30.19, 30.14, 30.08, 30.05, 29.8, 29.7, 26.5, 25.5, 23.1, 14.4;  $^{31}\text{P}$  NMR (202 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{D}_2\text{O}$  70:35:6)  $\delta$  -0.8; HRMS-ESI  $[\text{M}-\text{H}]^-$  Calculated for  $\text{C}_{50}\text{H}_{96}\text{O}_{19}\text{P}$ : 1031.6283. Found 1031.6288.

**Cytokine release assay**—Thioglycollate-elicited peritoneal murine macrophages were generated as previously described.<sup>48</sup> Macrophages were cultured in 96 well plates  $2 \times 10^5$  cells/well in supplemented RPMI-1640 (100 U/mL penicillin-streptomycin, 10% fetal calf serum). Compounds and 10 ng/ml LPS were added to macrophage cultures to a final culture volume of 200  $\mu\text{l}$ /well and incubated at 37°C, 5%  $\text{CO}_2$  for 18h. Supernatants were collected and IL-6 levels measured by ELISA. Cell viability was measured by trypan blue exclusion.

**Expression and purification of sTLR4/MD-2**—A complex between TLR4 ectodomain (sTLR4) and MD-2 was expressed by a baculovirus expression system using a modified dual expression vector, pAcUW51 (BD Biosciences). sTLR4 and MD-2 are appended to a C-terminal His<sub>6</sub>-tag and a Strep-tag II, respectively. sTLR4/MD-2 expressing baculovirus was generated by co-transfecting SF9 insect cells with the sTLR4/MD-2 expression vector and linearized baculovirus DNA, BaculoGold (BD Biosciences). For sTLR4/MD-2 expression, Hi5 insect cells were infected with the amplified recombinant virus. The secreted sTLR4/MD-2 protein was initially purified by Ni-NTA and Strep-Tactin affinity chromatographies and its C-terminal tags were removed by thrombin. The resultant protein was further purified by size exclusion chromatography.

**Expression and purification of MD-1**—MD-1 was expressed by a baculovirus expression system using a modified pAcGP67 expression vector and purified by three steps including Ni-NTA affinity, anion exchange, and gel filtration chromatography, as described previously.<sup>44</sup>

**Native PAGE**—Purified sTLR4/MD-2 protein was mixed with **2** first and then immediately with LPS in 20 mM Hepes pH 7.4/150 mM NaCl and the mixture was incubated at room temperature for 1 hour. The mixture was analyzed by native PAGE, which was performed at room temperature at pH 8.8 using a 4–20% gradient polyacrylamide gel (Bio-Rad).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The work was supported by the New Zealand Foundation for Research and Technology Contract C08X0808 and NIH grant AI1042266 (IAW) and the Skaggs Institute for Chemical Biology of the Scripps Research Institute. The authors thank Dr Regan Anderson for his very helpful contribution in the preparation of this manuscript.

## Abbreviations used

<b>TLR</b>	Toll-Like Receptor
<b>PIM</b>	phosphatidylinositol mannoside

<b>NKT</b>	natural killer T
<b>LPS</b>	lipopolysaccharide
<b>MD-2</b>	myeloid differentiation factor 2

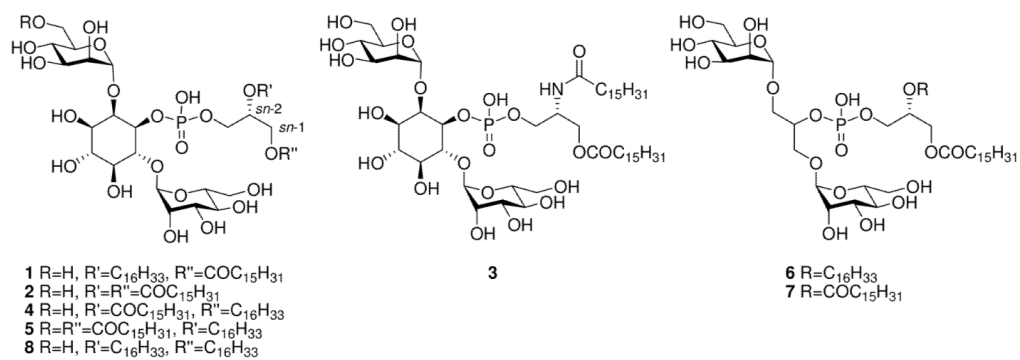
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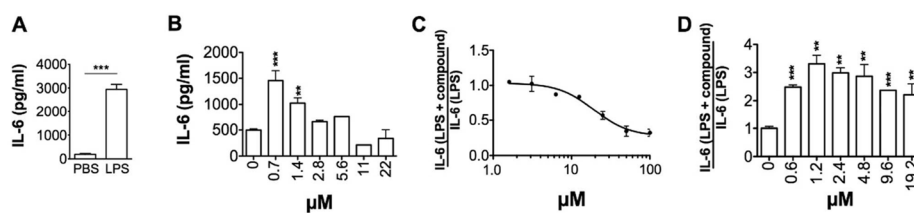


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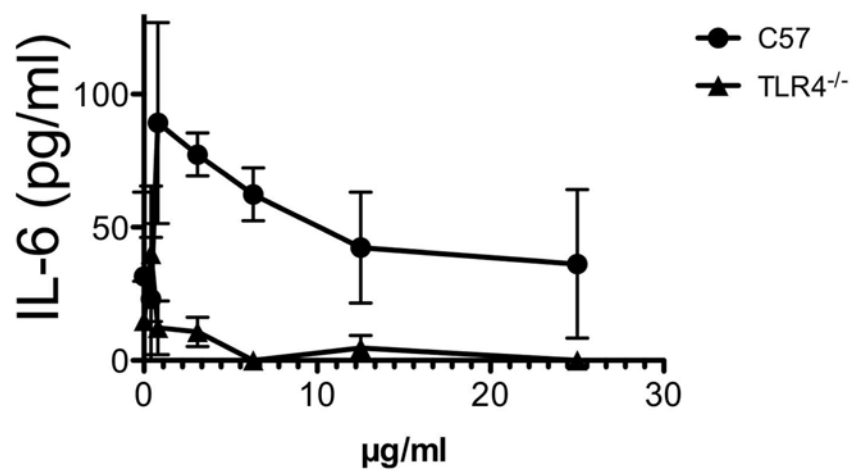


**Figure 1.**  
Structure of PIM analogues

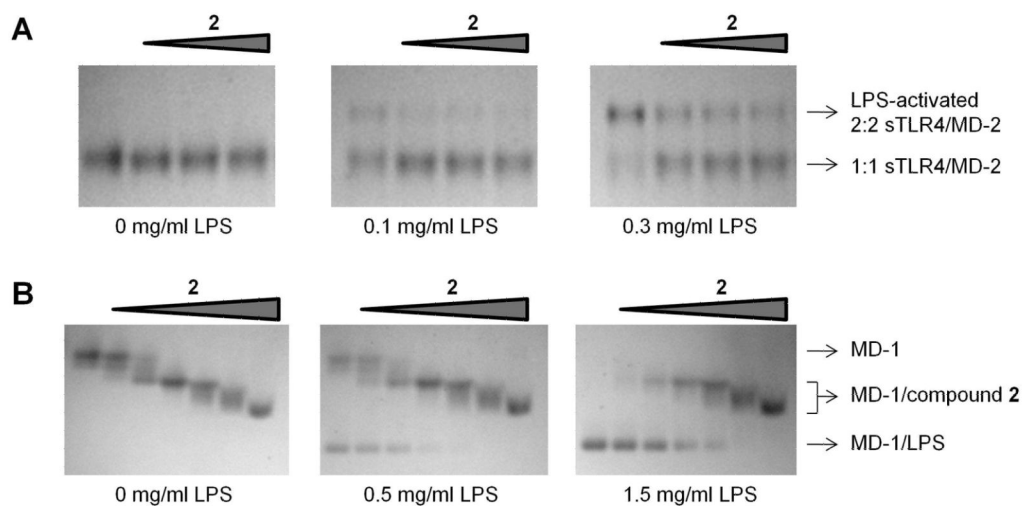


**Figure 2.**

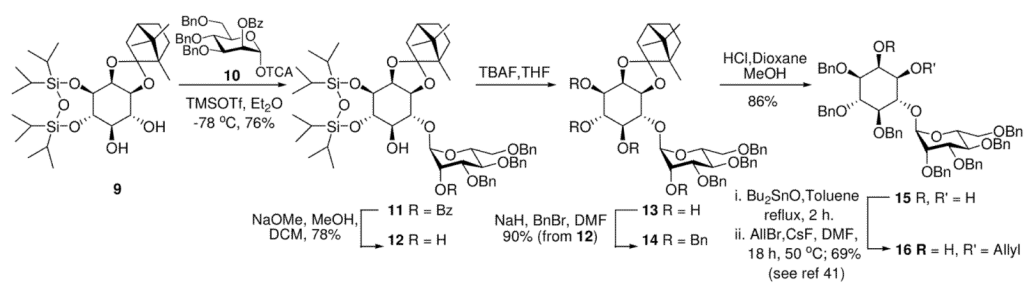
Agonist and antagonist activity of PIM compounds. Thioglycollate-induced peritoneal macrophages were treated with (A) LPS (10 ng/ml) or (B) **1** alone for 18 hours. Peritoneal macrophages were treated with LPS (10 ng/ml) in the presence of (C) **1** or (D) **5** for 18 hours. Supernatants were collected and the levels of IL-6 determined by ELISA. Data is representative of at least two replicate experiments. Statistical significance was calculated by 1-way ANOVA. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to PBS control.



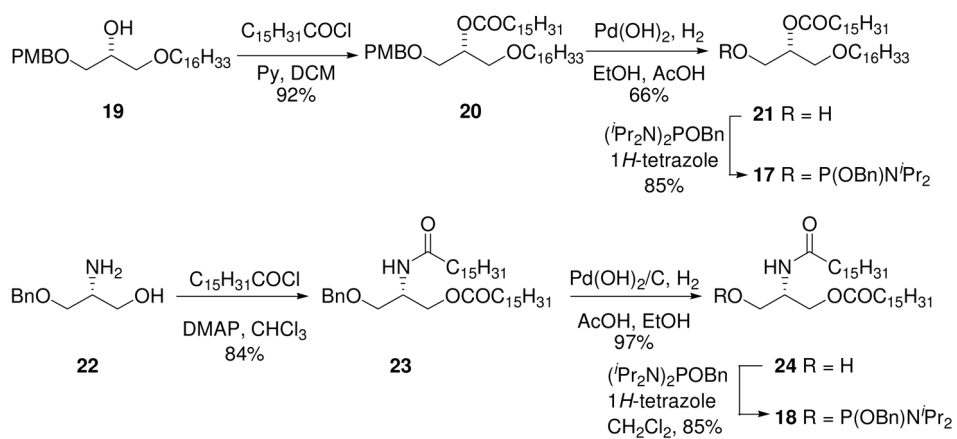
**Figure 3.** Agonist activity of **2** in thioglycollate-induced macrophages is TLR4 dependent. Data is representative of at least two replicate experiments. Data is representative of at least two replicate experiments. Statistical significance between the curves was calculated by 2-way ANOVA.  $p < 0.0007$ .



**Figure 4.** Compound **2** interferes with LPS binding to TLR4/MD-2 (A) and MD-1 (B) as shown by native PAGE. **2** binding to MD-1 results in a band shift (B, left), whereas **2**-induced sTLR4/MD-2 band shift was not observed (A, left) potentially due to a large size of sTLR4/MD-2 (87 kDa), compared to MD-1 (17 kDa).

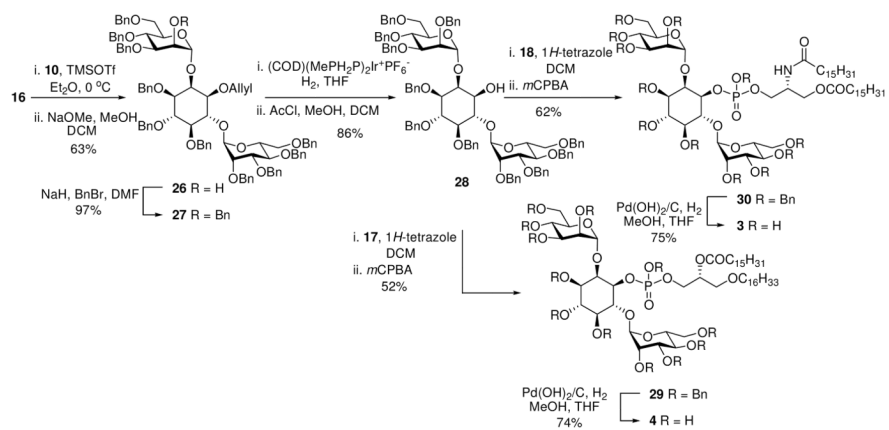


**Scheme 1.**  
Synthesis of common intermediate 16

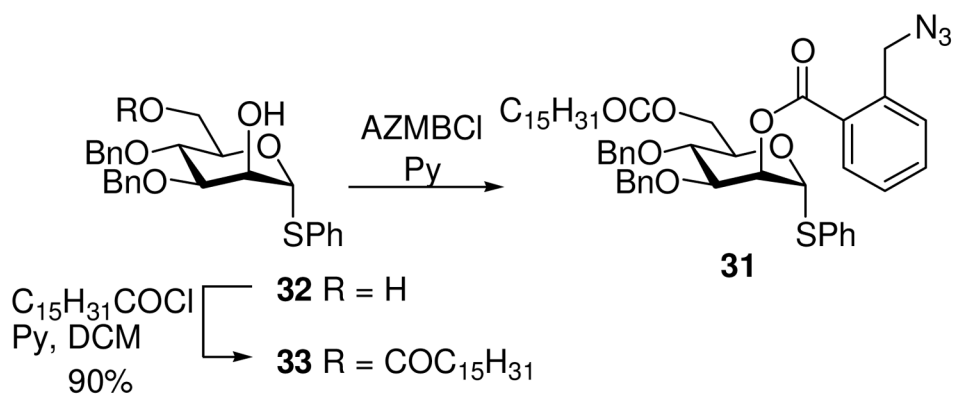


**Scheme 2.**  
Synthesis of phosphoramidites 17 and 18

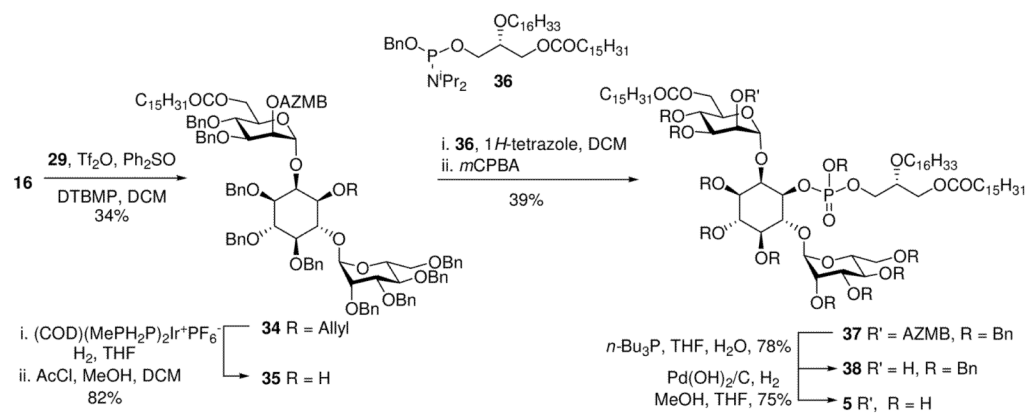




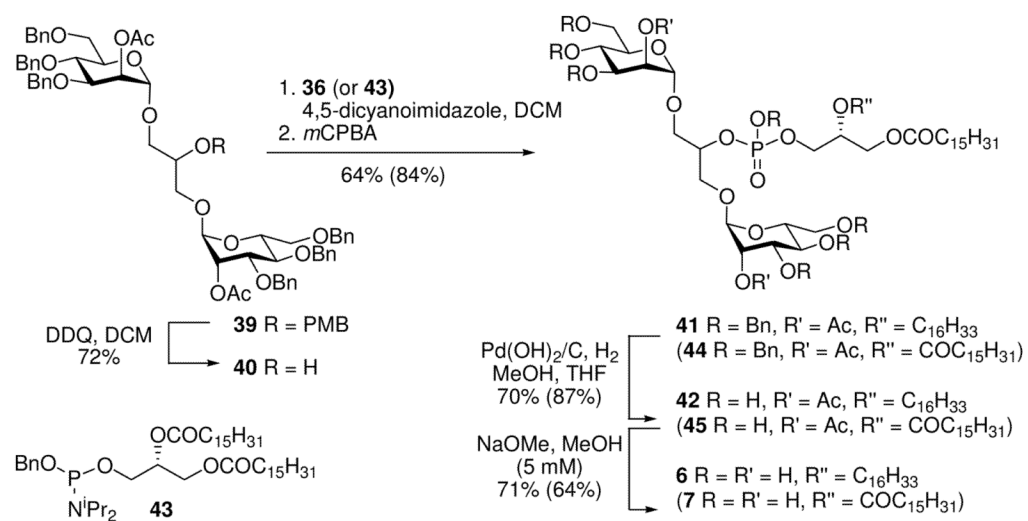
**Scheme 3.**  
 Synthesis of 3 and 4



**Scheme 4.**  
Synthesis of donor 31



**Scheme 5.**  
Synthesis of 5



**Scheme 6.**  
Synthesis of targets 6 and 7

**Table 1**

PIM-induced IL-6 production by thioglycollate-elicited macrophages.

Compound	IL-6 <sub>max</sub> (pg/ml)	SE	Structural variation
1	1456	161	PIM <sub>2</sub> , <i>sn</i> -2 ether, <i>sn</i> -1 ester
2	817	210	PIM <sub>2</sub> , <i>sn</i> -2 and <i>sn</i> -1 both ester
3	664	332	PIM <sub>2</sub> , <i>sn</i> -2 amide, <i>sn</i> -1 ester
4	1130	33	PIM <sub>2</sub> , <i>sn</i> -2 ester, <i>sn</i> -1 ether
5	275	62	AcPIM <sub>2</sub> , <i>sn</i> -2 ether, <i>sn</i> -1 ester
6	923	197	PGM <sub>2</sub> , <i>sn</i> -2 ether, <i>sn</i> -1 ester
7	143	31	PGM <sub>2</sub> , <i>sn</i> -2 and <i>sn</i> -1 both ester
8	494	161	PIM <sub>2</sub> , <i>sn</i> -2 and <i>sn</i> -1 both ether

**Table 2**

Inhibition of IL-6 production by LPS-stimulated thioglycollate-elicited macrophages.

CN	IC <sub>50</sub> (μM)	SE	Structural variation
1	30.3	4.9	PIM <sub>2</sub> , <i>sn</i> -2 ether, <i>sn</i> -1 ester
2	40.2	6.1	PIM <sub>2</sub> , <i>sn</i> -2 and <i>sn</i> -1 both ester
3	22.7	1.8	PIM <sub>2</sub> , <i>sn</i> -2 amide, <i>sn</i> -1 ester
4	28.8	3.8	PIM <sub>2</sub> , <i>sn</i> -2 ester, <i>sn</i> -1 ether
6	42.8	5.1	PGM <sub>2</sub> , <i>sn</i> -2 ether, <i>sn</i> -1 ester
7	12.9	3.0	PGM <sub>2</sub> , <i>sn</i> -2 and <i>sn</i> -1 both ester
8	23.3	5.0	PIM <sub>2</sub> , <i>sn</i> -2 and <i>sn</i> -1 both ether