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The key entity of a DCAR agonist, phosphatidylinositol mannoside Ac_1PIM_1: its synthesis and immunomodulatory function†

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Ac_1PIM_1 is a potential biosynthetic intermediate for phosphatidylinositol mannosides (PIMs) from *Mycobacterium tuberculosis*. We achieved the first synthesis of Ac_1PIM_1 by utilizing an allyl-type protecting group strategy and regioselective phosphorylation of inositol. A very potent agonist of an innate immune receptor DCAR, which is better than previously known agonists, is demonstrated.

Mycobacterium, particularly *Mycobacterium tuberculosis* complex, is a causative agent of tuberculosis (TB).[1] Its unique cell envelope structure and composition with thick lipophilic layers protect it from the environment.[2] One of its cellular components is phosphatidylinositol mannosides (PIMs), and the molecules contain phosphatidylinositol (PI) possessing mono- to hexa-mannosides (PIM_{1-6}) acylated to different degrees (Fig. 1).[3–6] PIMs are recognized by various immune receptors such as innate immune receptor Toll-like receptor (TLR) 2 [7] and C-type lectin receptors (CLRs) like DCAR (dendritic cell immunoactivating receptor)[8] and DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin).[9] It has also been reported that non-polymorphic antigen-presenting glycoprotein CD1d recognizes PIMs to activate NKT cells.[10,11]

Although multiple functions of PIM molecules have been reported, the molecular basis of the receptor recognition is not well understood. Our research focuses on a relatively small PIM molecule with tri-acyl groups, Ac_1PIM_1, which are potential biosynthetic intermediates for complex PIMs.[12,13] Herein we report the first chemical synthesis of Ac_1PIM_1 (**1**). The partial structures, PIs, were also synthesized with or without polar functional groups targeting hydrophilic amino acid residues in the receptor. The effects of targeting the polar functional group in lipid moieties have been previously reported for TLR2 [14] and CD1d.[15] We also analyzed the ability to activate innate immune receptors of these relatively smaller synthesized compounds as the basis to understand complex immunomodulatory functions.

Although Ac_1PIM_1 has not been synthesized, several reports have been published on the synthesis of PIMs. However, many of these syntheses were more focused on the glycan part rather than the phosphatidyl inositol moiety. The first synthesis of PIM_1 and PIM_2 has been reported by Van Boom *et al.* in 1989 and 1992, respectively.[16,17] Liu *et al.* reported the synthesis for PIM_6 using glycosylation between the protected PIM_2 moiety and tetramannosides.[18] Patil *et al.* recently developed regioselective mannosylation of the protected inositol and reported the synthesis of PIM_6 *via* a shorter synthetic route.[19] However, the synthesis of **1** has yet to be reported.

In this research, we first developed synthetic methods for PIMs, namely **1** and PIs (**2a–c**), by modifying the synthesis for protozoan lyso-phosphatidyl inositol compounds, EhLPPG.[20] PI derivatives **2b** and **2c**, which contain a polar group in the lipid moiety, are designed to allow the polar residue of the receptor and the ligand to interact. In this case, the polar func-

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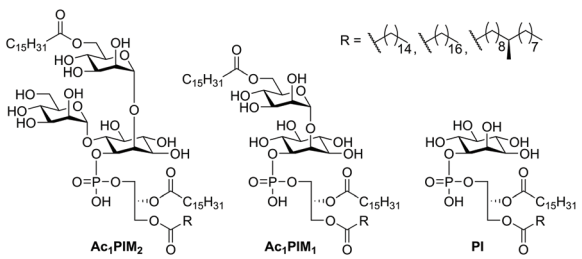


Fig. 1 Structures of the phosphatidyl inositol mannosides (PIMs) derived from *Mycobacterium tuberculosis* and phosphatidyl inositol (PI).

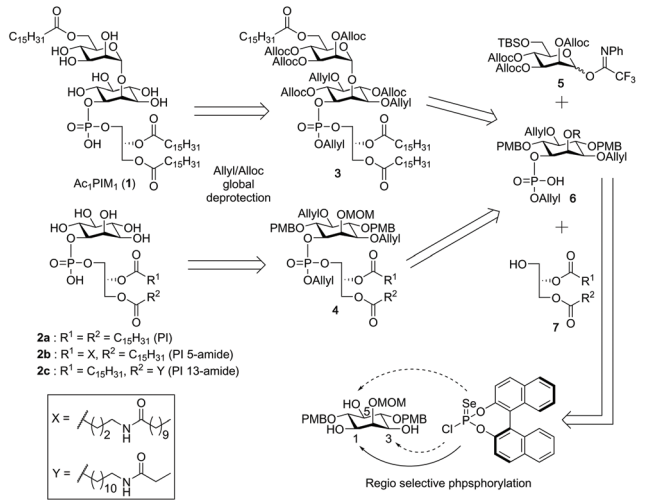
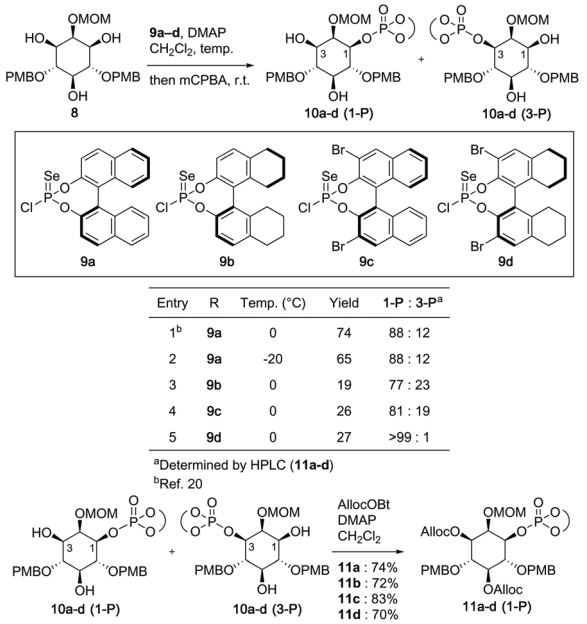


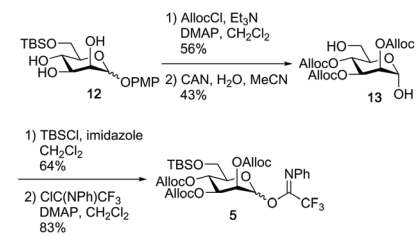
Fig. 2 Retrosynthesis analysis of Ac_1PIM_1 **1** and PI **2a** and derivatives **2b** and **2c**.



Entry	R	Temp. (°C)	Yield	1-P : 3-P[a]
1[b]	**9a**	0	74	88 : 12
2	**9a**	-20	65	88 : 12
3	**9b**	0	19	77 : 23
4	**9c**	0	26	81 : 19
5	**9d**	0	27	>99 : 1

[a]Determined by HPLC (**11a-d**)
[b]Ref. 20

Scheme 1 Regioselective phosphorylation of inositol derivative **8**.



Scheme 2 Synthesis of mannose moiety **5**.

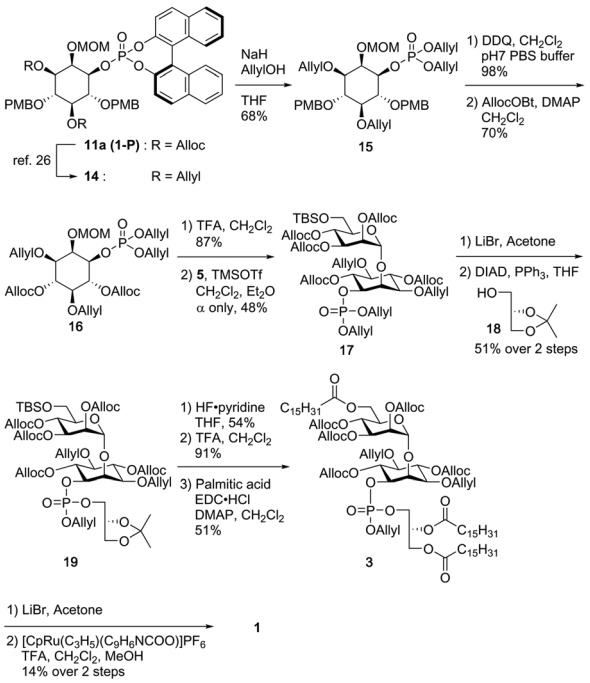
tional groups in **2b** and **2c** should link to the polar amino acids in mTLR2 (Ser346)[14] and mCD1d (Ser28),[15] respectively. Then the structure–activity relationships are investigated using the synthesized compounds.

Fig. 2 shows the synthetic strategy for **1** and **2a–c**. An allyl-type protecting group with either an allyl- or Alloc-group is used as the permanent protecting group of the hydroxyl groups, because the allyl-type protecting group can be cleaved with a Ru complex[21] under mild and near-neutral conditions in polar solvents at the end of the synthesis.[22] Compounds **3** and **4** are delivered from mannose donor **5**, inositol phosphate moiety **6**, and glycerol moiety **7**. To synthesize inositol phosphate moiety **6**, we investigated the regioselective phosphorylation utilizing BINOL-derived selenophosphoryl chlorides[23] with modified BINOL structures.

Firstly, the regioselective phosphorylation of the inositol was examined (Scheme 1). That is, we investigated the regioselective phosphorylation of compound **8** prepared from *myo*-inositol[24] utilizing BINOL-derived phosphoryl reagents **9a–d**. Compared to entry 1, which is the previously developed condition, a lower reaction temperature (entry 2) did not improve the regioselectivity of the 1-position *vs.* 3-position. To investigate the phosphoryl reaction and to improve the regioselectivity, the BINOL moiety was modified to prepare **9b–d**. Although reagent **9d** gave a very high selectivity, the yield was unsatisfactory because of its low reactivity (entry 5), even though the conditions of higher temperature and a higher reagent amount were examined. Other reagents **9b–c** gave lower yields and selectivities. In order to separate diastereomers and to analyze the regioselectivity in silica gel column chromatography, phosphorylated compounds **10a–d** were converted to **11a–d**.

The mannose moiety was also prepared as shown in Scheme 2. Mannose derivative **12** was initially prepared from D-mannose *via* previously reported methods.[25] After Alloc protection of the hydroxyl groups, the selective removal of the PMP group was attempted with CAN, but the TBS group was also cleaved to produce **13**. We then performed the silylation of the 6-hydroxyl group of **13** with TBSCl and imidazole, and *N*-phenyl-2,2,2-trifluoroacetoimidate was introduced to an anomeric position to give mannosyl donor **5**.

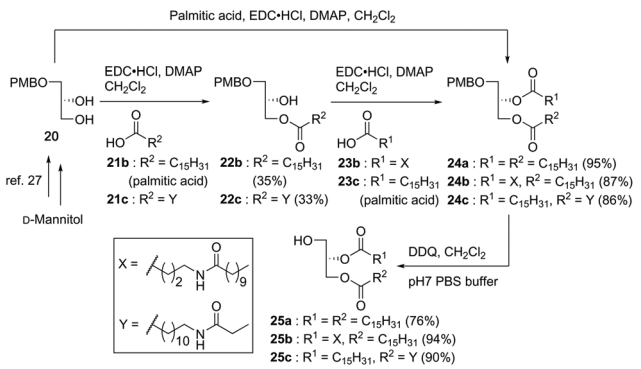
Scheme 3 shows the synthesis of **1**. The Alloc group of compound **11a** was converted to the allyl group using a Ru catalyst to give **14**.[26] Transesterification of the phosphate in **14**, from BINOL to allyl ester with an allyl alcohol and NaH, gave inositol derivative **15**. The exchange of the protecting group from PMB to the Alloc group yielded compound **16**. After the removal of the MOM group by TFA, mannosylation of **16** was achieved using mannosyl donor **5** and TMSOTf to give mannosyl inositol phosphate **17** in an α-selective manner. Although we examined the equivalent of the Lewis acid (TMSOTf) for optimizing the reaction, at a lower equivalent such as 0.1 eq., the reaction did not proceed, and at a higher equivalent (1.0 eq.), the TBS group was cleaved and dimannose compounds were obtained as byproducts. So, we used 0.5 eq. as the optimal condition. Introducing glycerol moiety **18** to the mannosylated inositol phosphate by the Mitsunobu reaction gave



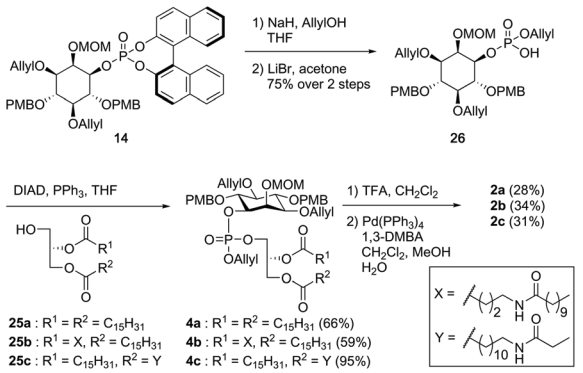
Scheme 3 Synthesis of Ac_1PIM_1 (**1**).

compound **19**. After deprotection of the TBS group and the isopropylidene group, palmitic acid was introduced to afford compound **3**. Cleaving the allyl ester of phosphate in **3** realized the global deprotection of allyl/Alloc groups using a Ru catalyst, and the desired Ac_1PIM_1 **1** was synthesized. The isolated yield of the target product **1** was relatively low, because the purification was difficult due to the low solubility (mixture-solvent of $CHCl_3$, CH_3OH and H_2O can be only used) and also the additional usage of a metal scavenger to remove the Ru catalyst was necessary.

To synthesize PI and its derivatives containing an amide group in the lipid chain, **2a–c**, we used common intermediate **14** (Scheme 5). After converting BINOL to the allyl group and deprotection of the allyl group on the phosphate, phosphoinositol compound **26** was condensed with glycerolipids **25a–c** synthesized from glycerol derivative **20**[27] (Scheme 4) by the



Scheme 4 Synthesis of glycerolipid moieties **25a–c**.



Scheme 5 Synthesis of PI and derivatives **2a–c**.

Mitsunobu reaction to give compounds **4a–c**. The global deprotection of the allyl-type protecting groups of **4a–c** using Pd $(PPh_3)_4$ afforded **2a–c**.

We next evaluated the immunomodulatory functions of the synthesized PIM and PI compounds. Initially, we investigated the DCAR activities of PIM compounds (**1** and **2a–c**) using 2B4 NFAT-GFP reporter cells co-expressing DCAR and FcRγ (Fig. 3).[28] Interestingly, **1** induced a potent reporter activity. In fact, it was higher than that of the known agonist, Ac_1PIM_2. In contrast, **2a–c** showed no or weak activity. The results suggested that Ac_1PIM_1, which was the mannose-truncated structure from Ac_1PIM_2, was the active entity for DCAR recognition.

We also evaluated the compounds for TLR2 and CD1d binding affinities and the activation of the synthesized ligands (Fig. S1–S3†). Not all compounds induced definite activity to both TLR2 and CD1d (Fig. S1 and S2), although the compounds interacted with the receptors (Fig. S3†). That is, we investigated the binding potential of the synthesized ligands to TLR2 and CD1d. All the ligands displayed lower binding affinities compared with the known TLR2 ligand Pam_2CSK_4 and CD1d ligand α-galactosyl ceramide (α-GalCer). However, they interacted weakly with the receptor proteins.

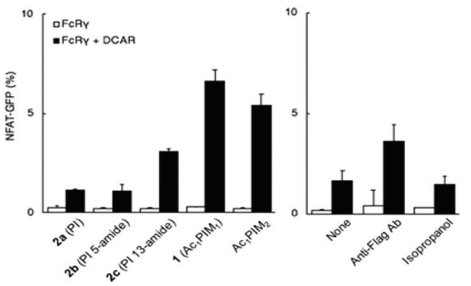


Fig. 3 DCAR recognizes synthetic Ac_1PIM_1 **1**. NFAT-GFP reporter cells expressing FcRγ alone and FcRγ + DCAR were stimulated with phosphatidyl-inositol lipids (0.8 nmol per well), anti-flag tag antibody (Anti-Flag Ab) and trehalose dibehenate (TDB) for 18 h. GFP expression was analyzed by flow cytometry. Ac_1PIM_2 was purified from *M. bovis* BCG as previously described.[8]

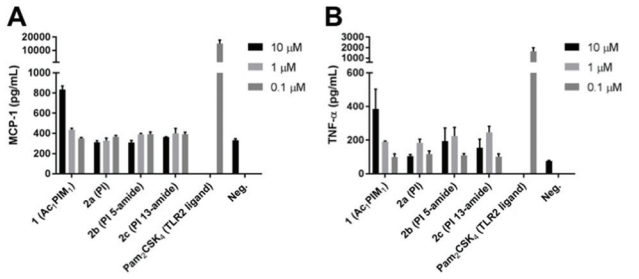


Fig. 4 Proinflammatory cytokine induction by the synthesized compounds in RAW264.7 cells. (A) MCP-1 induction. (B) TNF-α induction. RAW264.7 cells were seeded into 96-well plates (5.0×10^4 cells per well), and ligands were added with the indicated concentrations before incubation at 37 °C for 24 h. Cytokine release was measured using an ELISA kit (Affymetrix).

We also analyzed the proinflammatory cytokines of our synthesized ligands. The dependence of MCP-1 and TNF-α induction on the synthesized compounds was determined in mouse macrophage cell line RAW 264.7 cells (Fig. 4) and mouse bone marrow-derived dendritic cells (BMDCs) (Fig. S4†), which expressed the innate immune receptors, DCAR, TLR2, and lipid-antigen presenting CD1d. As a result of MCP-1 and TNF-α induction in RAW 264.7 cells and BMDCs, **1** showed weak but definite activities. **2a–c** did not exhibit MCP-1 induction activity in both cells. However, these compounds showed a slight activity by measuring TNF-α induction in BMDCs. The proinflammatory cytokine production by the synthesized compounds was consistent with the results of DCAR activation. Presumably, the other TLR2 and CD1d did not contribute to the cytokine induction activities in these cases (Fig. 3 and S1†). The resultant immunomodulatory functions of the synthesized compounds **1** and **2a–c** should be fundamental to understanding the molecular recognition of DCAR and other lipid-recognizing innate immune receptors.

Conclusions

Herein the first synthesis of **1** was achieved by utilizing an allyl-type protecting group strategy and a regioselective phosphorylation reaction with BINOL-derived selenophosphoryl chlorides. **2a–c** were also synthesized with the newly developed synthetic strategy. The immunomodulatory functions of the synthesized compounds were then investigated. **1** exhibited a very potent activity as a DCAR agonist, which is higher than that of the known agonist, Ac_1PIM_2, while **2a–c** showed no or weak activity. Our results suggested that DCAR most strongly recognized the Ac_1PIM_1 moiety in PIMs. We also showed that **1** and **2a–c** weakly interacted with other innate immune receptors such as TLR2 and CD1d, although they did not exhibit obvious activities. The knowledge of these molecular interactions should serve as a basis for further understanding of the immunomodulatory function of lipid molecules, especially on antigen-presenting cells.

Conflicts of interest

There are no conflicts to declare.

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