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Lipid length and iso-branching of trehalose diesters influences Mincle agonist activity

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ABSTRACT

We report on the efficient synthesis of linear trehalose diesters (TDEs) and iso-branched TDEs (maradolipids or iso-TDEs) and their ability to activate bone marrow-derived macrophages (BMDMs) as determined by cytokine (IL-1β, IL-12, IL-6, IL-10) and chemokine (MIP-2) production. Both classes of TDEs were found to activate BMDMs in a Mincle-dependent manner, with longer-chain (≥C18) lipids leading to a robust inflammatory response. On the whole, the iso-branched TDEs led to greater cytokine production and a faster immune response when compared to their linear counterparts. Moreover, C12-TDE and iso-C12-TDE elicited the production of MIP-2 by BMDMs, thereby providing the first example of TDEs with a chain length of ≤ C12 leading to a Mincle-dependent immune response and one that is less inflammatory in nature.

1. Introduction

Since the identification of the Macrophage inducible C-type lectin (Mincle, Clec4e, or Clecf9)[1,2] and the knowledge that the mycobacterial glycolipid, trehalose dimycolate (TDM, **1**, Fig. 1), binds and activates this receptor,[3] there has been much interest in the potential of Mincle agonists as immunostimulators and in understanding how changes to the ligand structure influences the ensuing immune response.[4] Notably, trehalose dibehenate (TDB, **2**, $n = 20$), which is a simplified trehalose diester (TDE), activates Mincle in a manner similar to TDM, with induction of the FcRγ-Syk-Card9-Bcl10-Malt1 signalling axis and a T helper (Th)-1-polarised immune response.[5–7] When formulated in dimethyldioctadecyl ammonium (DDA) liposomes,[8] TDB has found wide application as a vaccine adjuvant in a number of pre-clinical and clinical vaccination studies.[9,10] Other synthetic Mincle agonists, such as 6′-acylated mannose and 6′-acylated glucose sugars,[11] homogeneous TDMs, trehalose monomycolate and glucose mycolate,[12] also exhibit promising adjuvant activity, while β-GlcCer[13] and cholesterol derivatives[14,15] were recently identified as endogenous Mincle ligands, thereby highlighting the breadth of compounds found to activate this receptor.

To better understand the scope and specificity of the Mincle-binding site and how ligand binding correlates to a functional immune response, a number of potential Mincle ligands have been synthesised and their immunomodulatory properties assessed.[11,12,16–23] Early studies by our group revealed that TDEs with a carbon chain of ≥ C18 (but not ≤ C12) elicited a pro-inflammatory response by bone marrow derived macrophages (BMDMs),[16] and that trehalose monoesters activate macrophages in a Mincle-dependent manner.[17] The immunomodulatory profile of the Mincle ligands β-gentiobiosyl diacylglyceride,[18] glucose monocorynomycolate,[19] and 6′-acylated glucose and mannose monoesters[11] were also found to be influenced by lipid length. To a lesser extent, changing the mycolic acid structure altered the cytokine response of macrophages and dendritic cells (DCs) to TDMs[12,20] and trehalose monomycolates,[12] with modifications to stereochemistry also affecting Mincle agonist activity for both mycolic acid-containing glucolipids[12] and glycerol monocorynomycolates.[21]

Given the potential of TDEs to bind and activate Mincle, we became interested in exploring the ability of maradolipids to act as Mincle agonists. Maradolipids consist of a mixture of symmetrical

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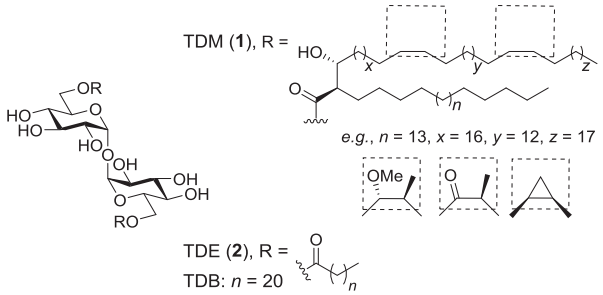


Fig. 1. TDM (**1**) and TDE (**2**).

and asymmetrical glycolipids containing mainly iso-branched and straight chain fatty acids (Fig. 2), and are produced by the dauer larva of the nematode *Caenorhabditis elegans*.[24,25] While maradolipids are structurally very similar to TDEs, structure-activity studies from previous Mincle-agonist work indicates that the incorporation of the iso-branch might be sufficient to give compounds with distinct immunomodulatory properties. Accordingly, we sought to prepare symmetrical iso-branched maradolipids **3a-f** (*i*Cn+1) and the linear TDEs (**2a**, $n = 9$; **2b**, $n = 11$; **2c**, $n = 15$) to assess their immunomodulatory profiles by measuring cytokine production by wild-type and Mincle$^{-/-}$ BMDMs. Herein, we determine that lipid length, as well as the presence or absence of the iso-branch, has a remarkable effect on the response of macrophages to TDEs.

2. Results and discussion

To synthesise the iso-branched and linear TDEs we envisioned using the strategy of Toubiana and co-workers to prepare 2,3,4,2′,3′,4′-hexa-*O*-trimethylsilyl-α,α′-trehalose in one step from α,α′-trehalose,[26] with subsequent elaboration to the target glycolipid.[16,27] To this end, bis(trimethylsilyl)acetamide (BSA) and catalytic tetrabutylammonium fluoride (TBAF) were used to persilylate α,α′-D-trehalose (**4**), with the most labile TMS groups at the 6- and 6′-positions being subsequently removed via the agency of K_2CO_3 to afford hexa-TMS protected trehalose **5** in a moderate yield (67% over two-steps, Scheme 1). Esterification with the commercially available iso-branched fatty acids **6a** and **6c-f**, or the C_{14+1} fatty acid **6b**, which can be synthesised via a Wittig reaction using the triphenylphosphonium salt derived from 11-bromoundecanoic acid and Ph_3P, and isobutyraldehyde, followed by hydrogenation,[28] then occurred under the mediation of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylaminopyridene (DMAP) in toluene at 56 °C for 8–10 h to give the corresponding esters **8a-f** in good to excellent yield (71–90%) following purification by silica gel flash column chromatography. For each glycolipid, Heteronuclear Multiple Bond Correlations (HMBCs) between H-6a and H-6b of the trehalose moiety with the carbonyl carbons of the lipids confirmed the successful conjugation of the lipid moieties. The protected linear TDEs (**9a, b** and **d**) were prepared in a similar manner via the conjugation of hexa-TMS protected trehalose **5** with the commercially available carboxylic acids **7a**, ($n = 9$), **7b** ($n = 11$), and **7d** ($n = 15$).

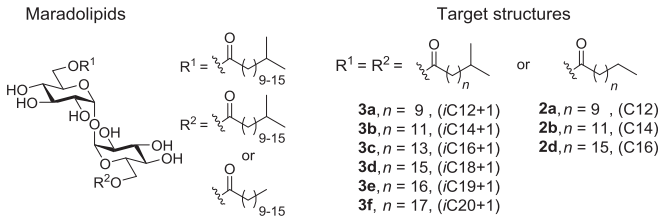
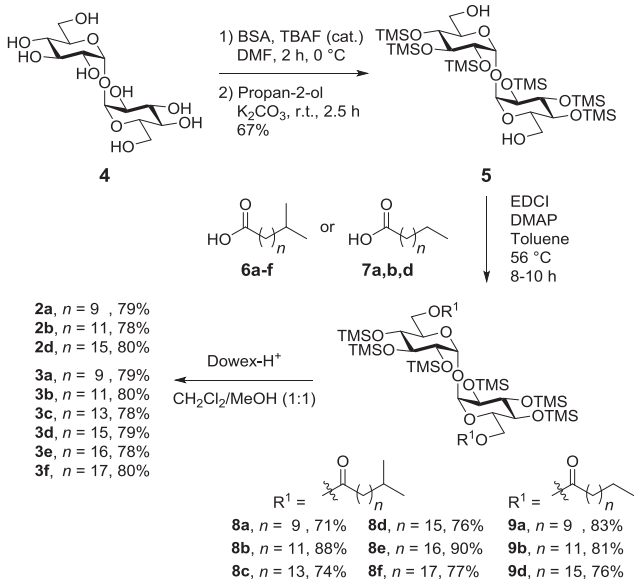


Fig. 2. Maradolipids and target structures.



Scheme 1. Synthesis of iso-branched maradolipids (iso-TDEs) and linear TDEs.

Deprotection of protected iso-TDEs **8a-f** was then achieved by using Dowex-H^+ in DCM and MeOH (1:1), which provided excellent yields of the desired iso-TDEs **3a-f** after purification by silica gel flash column chromatography. Again, 1D- and 2D-NMR techniques were used to analyse the products, and the presence of only one signal for the anomeric centres (at *ca.* $\delta = 5.9$ ppm) confirmed the formation of symmetric products. Formation of the linear TDEs (**2a, 2b** and **2d**) was achieved in an analogous manner, with the spectral data for **2b** and **2d** matching those previously reported.[16,29]

With the iso-branched maradolipids in hand, we then assessed the production of cytokines and chemokines by granulocyte-monocyte colony-stimulating factor (GM-CSF) BMDMs upon stimulation with the ligands. Here, the linear TDEs **2a** (C12), **2b** (C14), and **2d** (C18) could be directly compared to **3a** (*i*C12 + 1), **3b** (*i*C14 + 1) and **3d** (*i*C18 + 1) to ascertain whether the incorporation of a single methyl group affected the type and magnitude of immune response, while the C4 linear TDE, which we had previously synthesised and found not to activate BMDMs,[16,17] served as a negative control. TDB and LPS served as positive controls.

It is known that TDM and TDB lead to a pro-inflammatory Th-1 immune response upon the binding and activation of Mincle.[6,7] Accordingly, we first determined the production of the pro-inflammatory cytokine interleukin (IL)-1β by BMDMs upon stimulation with the glycolipids (Fig. 3A). To this end, the TDEs were assessed for their ability to activate BMDMs at two different concentrations (20 μM and 40 μM), with cytokine production being measured at 24 h and 48 h. Here, maradolipids **3a**,**b** (*i*C12 + 1 and *i*C14 + 1) and the corresponding linear TDEs (C12 and C14) did not result in significant IL-1β production at either time point, however cytokine production was observed by all TDEs with a lipid length ≥ C16. The iso-branched maradolipid **3d** (*i*C18 + 1) led to greater IL-1β production when compared to the analogous linear C18, while cytokine production upon BMDM stimulation with the longest iso-TDE (*i*C20 + 1) was similar to, if not better than, TDB. In all instances, IL-1β production was concentration dependent and

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