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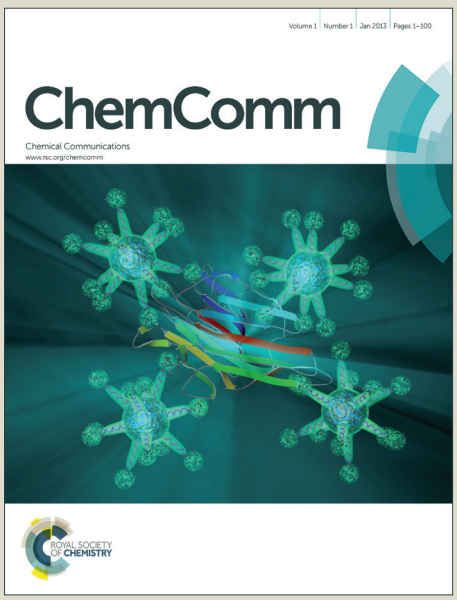

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Mycobacterium tuberculosis β-gentiobiosyl diacylglycerides signal through the pattern recognition receptor Mincle: Total synthesis and structure activity relationships

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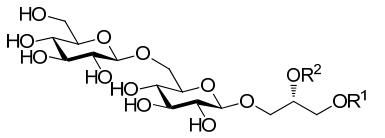
***Mycobacterium tuberculosis* H37Ra produces a range of immunogenic β-gentiobiosyl diacylglycerides. We report the total synthesis of several candidate structures and show that these compounds signal weakly through mouse, but not human, Mincle. Structure-activity relationships reveal a striking dependence upon acyl chain length for gentiobiosyl diacylglyceride signalling through Mincle. Significantly, a truncated β-glucosyl diglyceride was shown to provide potent signalling through both human and mouse Mincle and could activate murine bone marrow derived dendritic cells.**

The human facultative pathogen *Mycobacterium tuberculosis* possesses a complex, lipid-rich cell wall.[1] The components of the cell wall include a range of potently immunogenic species such as glucose[2] and trehalose mycolates (eg trehalose dimycolate (TDM); cord factor),[3,4] glycophospholipids (phosphatidylinositolmannosides, lipomannan and lipoarabinomannan),[5] β-mannosylphosphomycoketide,[6] and didehydromycobactin.[7] Roles for these and related lipid-like species have been demonstrated in modulating host-pathogen interactions and include processes mediated through Toll-like receptors,[8] cluster of differentiation 1 proteins/T cell receptors,[9] and the C-type lectin receptors DC-SIGN,[10] and MCL and macrophage inducible C-type lectin (Mincle).[4,11]

Mincle has emerged as a significant sensing protein for damaged self and pathogen-associated molecular patterns. The archetypal ligand is TDM, and its binding to Mincle leads to phosphorylation of the immunoreceptor tyrosine-based activation motif of the Fc receptor γ (FcγR)-chain molecule and activation of nuclear factor kappa-B (NFκB).[12] NFκB is a rapid-acting transcription factor[13] that induces production of cytokines that shape the activation of naïve T cells. Emerging structure-activity relationships for trehalose-based glycolipids as ligands for Mincle have revealed that trehalose dibehenate (TDB) possesses activity similar to TDM,[3,4,14] and that monoacyl trehalose derivatives also possess the capacity to activate through Mincle,[15] with longer chain lengths generally providing greater potency of activation in Mincle reporter assays,[16] which correlates with direct binding affinities for Mincle.[17] Trehalose corynomycolates also effectively signal through Mincle.[18] Notable differences have been identified between the specificity of mouse and human forms of Mincle; human Mincle is much more sensitive to signalling induced by glycerol monocorynomycolate[19] (and in particular the 2'*S*-isomer)[18] and glucose monocorynomycolate.[18]



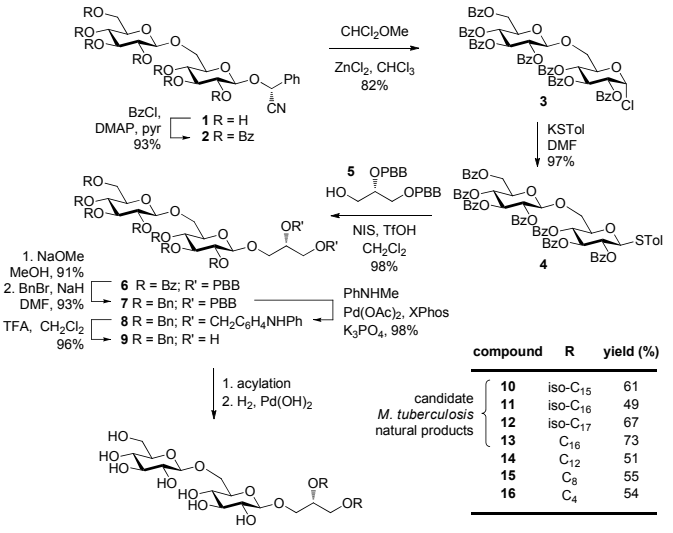
R1, R2	
iso-C_{15}	anteiso-C_{15}
iso-C_{16}	C_{16}
iso-C_{17}	anteiso-C_{17}

Fig. 1 β-Gentiobiosyl diglycerides from *Mycobacterium tuberculosis* H37Ra.[20]

Beyond the trehalose, glucose and glycerol mycolates and corynomycolates, only a limited repertoire of pathogen-derived Mincle ligands are known. These include an unusual mannosyl-lipid from the fungus *Malassesiza pachydermatis*;[21] and various β-gentiobiosyl diglycerides (four lipoforms sn-1/sn-2: anteiso-C_{20}/anteiso-C_{15}, anteiso-C_{19}/anteiso-C_{15}, anteiso-C_{17}/anteiso-C_{15}, anteiso-C_{19}/anteiso-C_{17})[21] isolated from the same organism. In this context, our attention was drawn to an unusual series of β-gentiobiosyl diacylglycerides (Fig 1) isolated by the Brennan group from the H37Ra strain of *M. tuberculosis*, and which were cross-reactive to polyclonal rabbit antibodies raised against whole *M. tuberculosis* bacteria.[20] These compounds contain a range of straight-chain, iso- and *anteiso*-fatty acid groups, although the precise composition of the individual components was not determined.[20] In this work we report the synthesis of candidate β-gentiobiosyl diglycerides from *M. tuberculosis* and various analogues and investigation of their ability to signal through mouse and human Mincle.

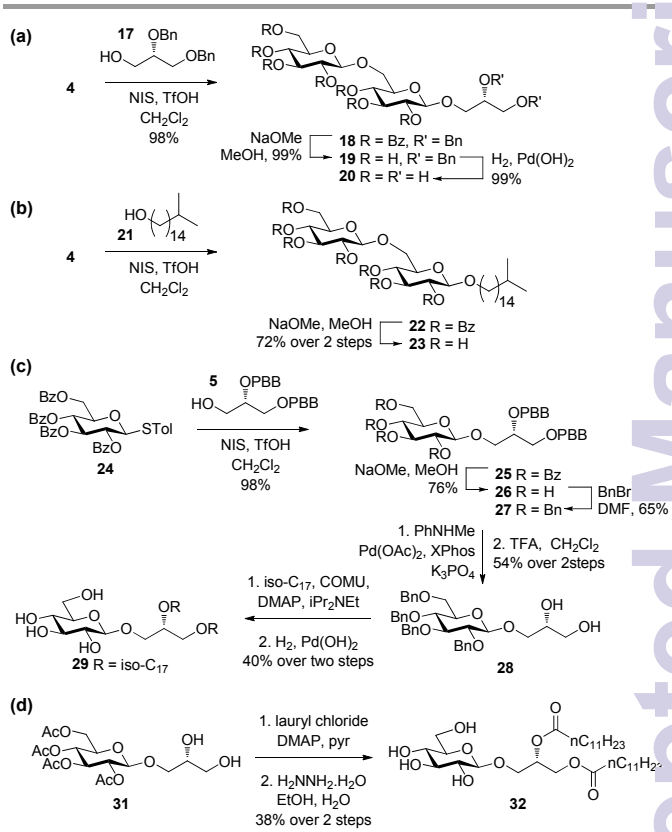
Apricot-kernel derived amygdalin **1** provides a large scale source of the gentiobiose fragment (Scheme 1). Benzoylation of amygdalin afforded heptabenzoate **2**,[22] but while hydrogenolysis of the cyanobenzyl group has been reported we had difficulty in effecting its reliable transformation.[22] Instead, treatment of **2** with 5 eq dichloromethyl methyl ether (DCCME) and $ZnCl_2$ afforded the

gentiobiosyl chloride **3**,[22] which underwent a clean substitution with potassium thiocresolate, to afford thioglycoside **4**. NIS/TfOH-promoted glycosylation of (*S*)-1,2-isopropylideneglycerol by **4** was ineffective, affording a pair of glycerols epimeric at the C2' position. Instead, NIS/TfOH-promoted glycosylation of the D-mannitol-derived 4-bromobenzyl-protected glycerol **5** (see SI) led to **6** with no epimerization at the glycerol C2' position. Zemplén deprotection of **6** and benzylation afforded heptabenzyl ether **7**. Selective deprotection of the glycerol moiety was achieved according to Buchwald and Seeberger.[23] Thus, palladium-catalyzed amination of the bromobenzyl groups of **7** with *N*-methylaniline, 20% $Pd(OAc)_2$/XPhos and K_3PO_4 over 40 h, followed by acidic deprotection using TFA/CH_2Cl_2, afforded the diol **9**. We also explored the use of the pre-catalyst, XPhos-Pd-G3,[24] at a 10% Pd-equivalent loading. While this pre-catalyst provided higher rates at a lower loading for formation of the monoaminated intermediate, beyond this point reaction rates lowered significantly and given the higher cost of this pre-catalyst, the marginal improvement of performance was deemed uneconomical compared to $Pd(OAc)_2$/XPhos. We have previously reported preparation of an extensive suite of iso-fatty acids;[25] direct esterification of the diol **9** was achieved using fatty acid and COMU/DMAP/iPr_2NEt[26] or by direct acylation using the corresponding acid chloride in DMAP/pyr. Finally, hydrogenolysis of the benzyl groups at elevated pressure afforded the β-gentiobiosyl diglycerides **10**-**16**.



Scheme 1 Synthesis of β-gentiobiosyl diglycerides **10**-**16** from amygdalin (**1**).

In order to explore structure-activity relationships, a range of analogues were prepared based on the iso-C_{17} gentiobiosyl diglyceride **12**. Glycosylation of dibenzylglycerol **17** with **4** under the agency of NIS/TfOH afforded glycoside **18**. Debenzoylation (NaOMe/MeOH) and debenzylation (H_2, $Pd(OH)_2$) afforded gentiobiosyl glycerol **20** (Scheme 2a). Glycosylation of iso-C_{17} alcohol **21** with **4** under similar conditions afforded the glycoside **22**; debenzoylation (NaOMe/MeOH) provided the gentiobioside **23** (Scheme 2b). A glucosyl analogue was prepared by an equivalent route to that used for the gentiobiosyl diglycerides: NIS/TfOH-promoted glycosylation of glycerol **5** with glucosyl donor **24** afforded β-glucoside **25**. Debenzoylation of **25** (NaOMe/MeOH) and sequential benzylation (BnBr, NaH, DMF) furnished tetrabenzyl ether **27**. Selective deprotection of the glycerol moiety of **27** was achieved by stepwise amination with *N*-methylaniline, 20% $Pd(OAc)_2$/XPhos and K_3PO_4, followed by acidic deprotection using TFA/CH_2Cl_2, affording diol **28**. Direct esterification of **28** using iso-C_{17} acid and COMU/DMAP/iPr_2NEt,[26] and finally hydrogenolysis afforded glycoside **29** (Scheme 2c).



Scheme 2 Synthesis of analogues of β-gentiobiosyl diglycerides.

Compounds **10**-**13** represent candidate structures for the *M. tuberculosis* natural products. Based on their similarity to the *M. pachydermatis* compounds, which are known to signal through mouse Mincle, we explored their signalling through mouse and human Mincle (Fig. 2). Signalling through Mincle was assessed using a nuclear factor of activated T cells (NFAT)-green fluorescent protein (GFP) reporter assay. NFAT-GFP reporter cells transfected with FcRγ alone or FcRγ and hMincle or mMincle were stimulated by plate-bound samples. All four compounds provided measurable but weak stimulation of mouse Mincle. No stimulation of human Mincle was observed. The degree of stimulation was commensurate with that observed for related gentiobiosyl diglycerides from *M. pachydermatis*.[21]

We next explored variation of the acyl chain length through comparison of **13**-**16** bearing C_{16}, C_{12}, C_8 and C_4 acyl chains (Fig. 3a). Maximal signalling through mouse Mincle was observed for the C_{12} analogue **14**, which for the first time also showed measurable stimulation of human Mincle. The striking dependence upon chain length for signalling through Mincle is reminiscent of that reported for activation of bone marrow macrophages by trehalose diesters.[14] In that work trehalose diesters of C_{18}-lipids or shorter did not result in release of NO or interleukin-6 and -1β, whereas those of C_{20} or longer provided robust activation.[14] Removal of the acyl groups altogether in compound **20**, the simple glycoside **23**, or gentiobiose **30**, did not result in signalling though Mincle, demonstrating the

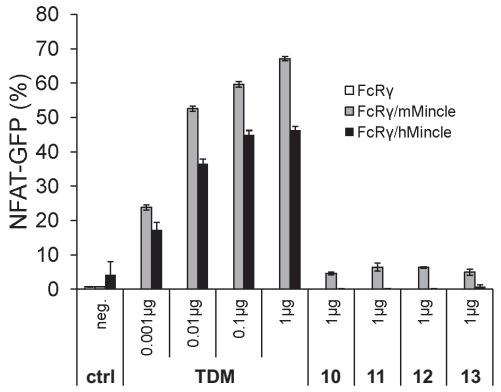


Fig 2 NFAT-GFP reporter cells expressing human Mincle/FcRγ or mouse Mincle/FcRγ as well as those expressing FcRγ alone were tested for their reactivity to trehalose dimycolate (TDM) and candidate *M. tuberculosis* H37Ra gentiobiosyl diglycerides **10**-**13**. Assays were performed in triplicate; bars show the mean values; error bars show standard deviations. Replots of the data using quantities expressed in pmol are available in the ESI.

need for the complete glycosyl diglyceride structure. Next, we examined whether the disaccharide is required for signalling through Mincle (Fig. 3b). Remarkably, iso-C_{17} glucosyl diglyceride **29** displayed significantly enhanced signalling through mouse and human Mincle, greater than that seen for any gentiobiosyl analogue. We therefore undertook the synthesis of C_{12} glucosyl diglyceride **32**, a hybrid of the most potent gentiobioside **14** and glucoside **29**. In this case we developed an alternative route that involved acylation of acetylated diol **31** (see SI), followed by hydrazine-mediated selective deacetylation (Scheme 2d). This alternative route highlighted the challenges in obtaining a high-yielding selective deacetylation, even on a monosaccharide, providing only a 38% yield of the C_{12}-glucoside **32**. C_{12}-glucoside **32**, while maintaining robust signalling through mouse Mincle, signalled only very weakly though human Mincle.

We sought to independently confirm the unusual structure-activity relationship studies identified by the NFAT-GFP Mincle reporter cells. Evidence for direct binding of activating glycolipids was obtained by an enzyme-linked immunosorbent assay (ELISA) using mouse and human Mincle Ig fusions (see Fig. S3 in ESI†). In particular binding to mouse, but not human, Mincle Ig fusions was observed for compounds **14** and **29**. We also examined the ability of activating glycolipids to stimulate murine bone marrow derived dendritic cells (BMDCs) by measuring the production of tumor necrosis factor (TNF) and macrophage inflammatory protein 2 (MIP-2). Consistent with the signaling assays, of the series of gentiobiosides **13**-**16** bearing C_{16}, C_{12}, C_8 and C_4 acyl chains, only compound **14** provided significant stimulation (see Fig. S4 in ESI†). As well, when comparing iso-C_{17} gentiobioside **12** and iso-C_{17} glucoside **29**, only the latter resulted in BMDC stimulation, consistent with its greater potency in the mMincle signalling assay (see Fig. S4 in ESI†).

M. tuberculosis strain H37 was originally isolated from a human donor in 1905 and was noted for its high virulence in guinea pigs but was only moderately so in rabbits, behaviour considered distinctly 'human' in type.[27] In 1934 strain H37 was dissociated into so-called 'virulent' (H37Rv) and 'avirulent' (H37Ra) strains;[28] while H37Rv produces virulent disease, H37Ra maintains an ability to infect and proliferate within animal hosts but does not cause disease.[29] We

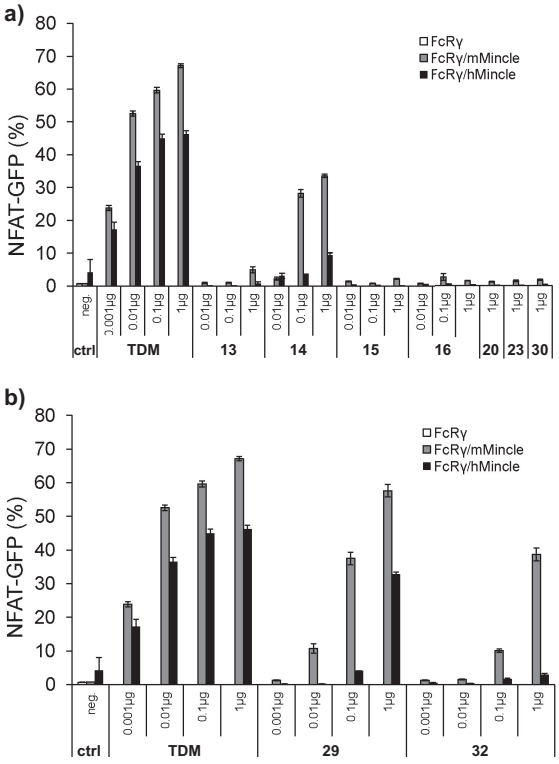


Fig 3 NFAT-GFP reporter cells expressing either human Mincle/FcRγ or mouse Mincle/FcRγ as well as those expressing FcRγ alone were tested for their reactivity to trehalose dimycolate (TDM) and gentiobiosyl diglyceride analogues. a) Structure-activity variations in the diacylglyceride fragment. **30** = gentiobiose. b) Glucosyl diglycerides. Assays were performed in triplicate; bars show the mean values; error bars show standard deviations. Replots of the data using quantities expressed in pmol are available in the ESI.

demonstrate here that a poorly studied group of β-gentiobiosyl diglycerides from *M. tuberculosis* H37Ra possess the ability to signal through mouse, but not human, Mincle. While to our knowledge these compounds have not been found in H37Rv or any other pathogenic *M. tuberculosis* strains, their low abundance (2 mg from 117g of cell pellet)[20] may have complicated their detection.[30] It is possible that the ability of these gentiobiosides to stimulate mouse but not human Mincle may be related to adaptation occurring during long-term culture of H37-derived strains. β-Gentiobiosyl diglycerides are biosynthetic end products in their own right[31,32] but are also precursors for lipoteichoic acids,[34,35] a widespread group of phosphoglycolipids, and thus β-gentiobiosyl diglycerides may represent a widely distributed group of pathogen-associated molecular patterns.

Our structure-activity relationships reveal a sharp dependence on acyl chain length for signalling by gentiobiosyl diglycerides, with the C_{12}-gentiobioside **14** the most potent in the series, and within this series only compound **14** demonstrated direct binding to mouse Mincle and the ability to stimulate cytokine production by murine BMDCs. Truncation of the disaccharide of **12** to the iso-C_{17} β-glucosyldiglyceride **29** resulted in the most potent signalling through mouse and human Mincle of all the compounds studied. Unlike compound **12**, compound **29** was demonstrated to directly bind mouse Mincle and could also stimulate murine BMDCs. In the context of the published structures and analysis of the carbohydra

recognition domain (CRD) of Mincle,[16,17,36] it seems likely that the carbohydrate portion of the gentiobiosyl diglycerides bind in the same region as trehalose in the trehalose–Mincle-CRD complex,[17] with the first glucose residue engaging the Ca^{2+} in the primary site, and with the acyl chains binding into the hydrophobic channel adjacent to sugar binding site. The unusual dependence of activity on acyl chain length suggest that in addition to the precise glycosyl diglyceride structure required for binding to and signalling through the Mincle receptor, a particular amphipathic profile is required for solubility and partitioning of the ligand in cell based assays for optimal exposure to the receptor. Enticingly, β-glucosyl diglycerides are biosynthetic precursors to the β-gentiobiosyl diglycerides,[32,35] and it can be speculated that elaboration to the β-gentiobioside is a strategy employed by *M. tuberculosis* to attenuate its immunological properties in humans and dampen Mincle-mediated immune responses. As well, β-glucosyl diglycerides are produced by assorted commensal (*Staphylococcus epidermidis*[31]) and pathogenic bacteria (*Mycoplasma neurolyticum*[37] and *Mycoplasma genitalium*[32]). Whether these and other glycolipids also signal through Mincle is an open question.

Notes and references

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Electronic supplementary information (ESI) available: Full synthetic procedures, characterisation of novel compounds and immunological methods and data. See DOI: 10.1039/c000000x/

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