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Stereochemistry, lipid length and branching influences Mincle agonist activity of monoacylglycerides†

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Herein, we report on the synthesis of a series of enantiomerically pure linear, iso-branched, and α-branched monoacyl glycerides (MAGs) in 63–72% overall yield. The ability of the MAGs to signal through human macrophage inducible C-type lectin (hMincle) using NFAT-GFP reporter cells was explored, as was the ability of the compounds to activate human monocytes. From these studies, MAGs with an acyl chain length ≥C22 were required for Mincle activation and the production of interleukin-8 (IL-8) by human monocytes. Moreover, the iso-branched MAGs led to a more pronounced immune response compared to linear MAGs, while an α-branched MAG containing a C-32 acyl chain activated cells to a higher degree than trehalose dibehenate (TDB), the prototypical Mincle agonist. Across the compound classes, the activity of the *sn*-1 substituted isomers was greater than the *sn*-3 counterparts. None of the representative compounds were cytotoxic, thus mitigating cytotoxicity as a potential mediator of cellular activity. Taken together, **6h** (*sn*-1, iC26+1), **8a** (*sn*-1, C32) and **8b** (*sn*-3, C32) exhibited the best immunostimulatory properties and thus, have potential as vaccine adjuvants.

Introduction

Glycerides are membrane constituents found in most organisms.[1] These compounds, which are fatty acid esters of glycerol, have many fundamental physiological functions including acting as anhydrous reservoirs for the efficient storage of caloric reserves and as intracellular membrane messengers in signal transduction and molecular recognition processes.[2,3] Several glycerides also have antifungal, antibacterial and/or immunomodulatory activities. For example, iso-branched monoacylglycerides (iMAGs, **1**) (Fig. 1), which have been isolated from *Streptomyces* sp. and the marine sponge *Stelletta* sp.,[4,5] possess antifungal and antibacterial properties along with platelet aggregation inhibitory effects,[4,6,7] while monomycoloylglycerol (MMG, **2**), which was first isolated from *Mycobacterium bovis* Bacillus Calmette-Guérin, both as *sn*-1 and *sn*-3 isomers, downregulates the T-helper (Th)-1 immune response when formulated in liposomes and is thought to contribute to the long-term survival of dormant mycobacteria in the host cell.[8,9] Liposomal formulations of synthetically prepared C32 monoacylglycerol (MAG-C32, **3a**) also induce potent Th-1 activity, and thus show promise as vaccine adjuvants,[10] while analogues of momocorynomycoloylglycerol MCMG (**4a** and **4b**) activate dendritic cells (DCs), with lipid length and stereochemistry, but not the configuration of the glycerol moiety, affecting the immune response.[11] In this latter work, the inability of short chain MCMGs to activate DCs was attributed to their cytotoxicity.

To explain how MMGs exert their immunomodulatory profile, Yamasaki and co-workers recently determined that MMG **2** was a ligand for the human ortholog of the macrophage inducible C-type lectin (hMincle), with hMincle signalling leading to the production of inflammatory cytokines.[12] Monobehenoyl-*rac*-glycerol (MBG, **3b**), a synthetic analogue of MMG, failed to signal through mouse Mincle (mMincle), while strong hMincle activity was observed, thus demonstrating the species-specific activity of select classes of Mincle ligands.[12] These findings sparked much interest in the Mincle agonist activity of other glycerides, with Van der Peet *et al.* preparing the (*R*,*R*)-meromycolate isomers of both *sn*-1 (**4a**) and *sn*-3 (**4b**) substituted MCMG and, using a nuclear factor of activated T cell-green fluorescent protein (NFAT-GFP) assay,[13,14] demonstrating that Mincle agonist activity predominantly resides with *sn*-1-isomer **4a**.[15] In 2019, it was also determined that linear racemic MAGs **3b** and **5** containing C22–C30 acyl chains

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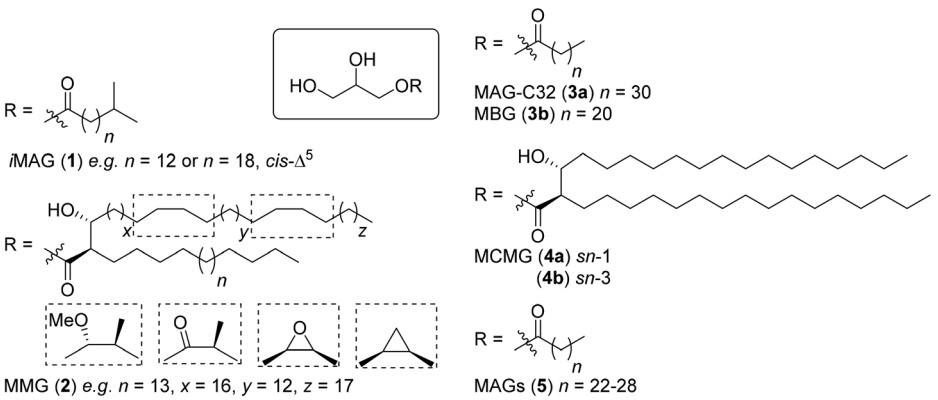


Fig. 1 Structure of representative glycerides.

activate human, but not murine, Mincle.[16] Here, the activity of the MAGs was dependent on the length of the acyl chains.[16]

With a long-standing interest in determining how the structure of Mincle ligands influences their biological response,[17–22] we sought to better understand how glyceride structure affects the ability of the compound to activate murine and human Mincle. Previous investigations into the immunostimulatory profile of Mincle ligands,[23] including trehalose diesters,[20] 6′-acylated mannose and glucose,[24] β-gentiobiosyl diacylglycerides,[25] and lipidated brartemicin analogues,[18] demonstrated that an increase in lipid length generally enhances the Mincle-dependent immune response. The inclusion of iso-branching on the lipid backbone has also been correlated to an enhanced immune response, both for trehalose diesters,[22] and for C17–C20 iMAGs,[4,6] whereby the latter exhibited better antimicrobial activity than their shorter chain or linear counterparts. In view of these findings, as well as the observation that the stereochemistry of the glycerol moiety can influence Mincle activation,[15] we sought to prepare a series of monoacylglycerides with different stereochemistry in the glycerol moiety and different iso-branched **6a–i**, linear **7a–g**, and α-branched **8a,b** lipid chains (Fig. 2). These derivatives would then be assessed for their ability to activate hMincle reporter cells, as well as primary human cells.

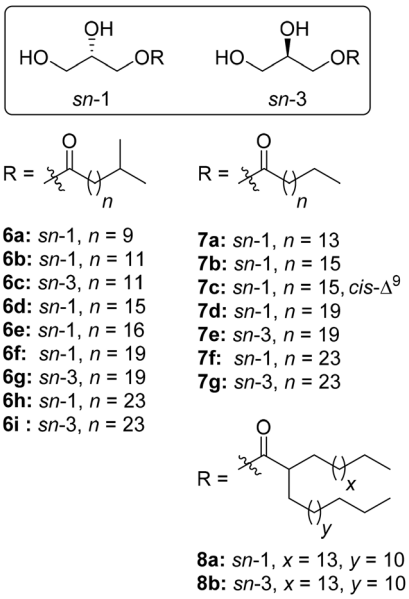
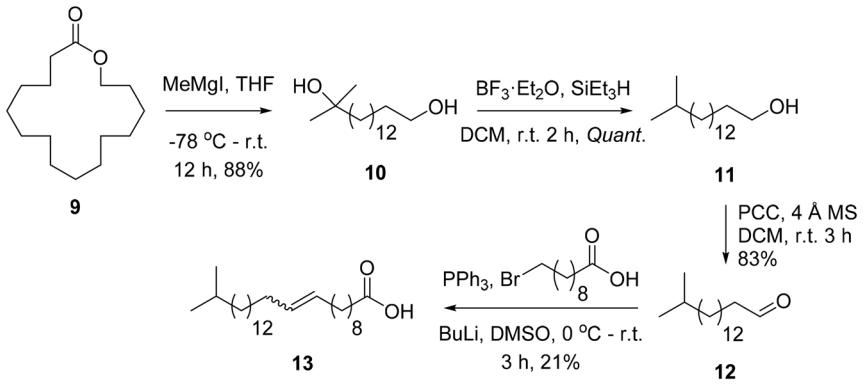


Fig. 2 *sn*-1 and *sn*-3 MAGs to be synthesised.

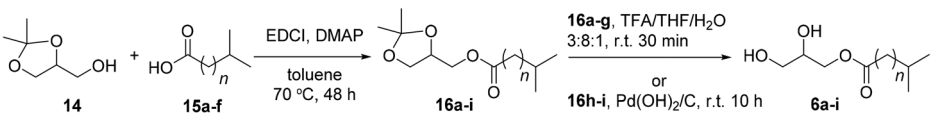
Results and discussion

To synthesise the iso-branched MAGs, commercially available iso-branched fatty acids were used except for iso-methyl-cerotic acid (C26+1), which was prepared from cyclopentadecanolide **9** (Scheme 1). Here, Grignard reaction of cyclopentadecanolide **9** with methyl magnesiumiodide, followed by deoxygenation of diol **10** at the tertiary position using boron trifluoride etherate and triethylsilane,[26] gave primary alcohol **11** in excellent overall yield. Oxidation of **11** using PCC then gave aldehyde **12** in 83% yield.[27] Finally, Wittig olefination was carried out using the triphenylphosphonium salt derived from 10-bromodecanoic acid and PPh_3, and aldehyde **12**, to yield iso-branched lipids **13**.[28] Although the yield of this reaction was modest, acids **13** were the only products observed by TLC [R_f = 0.8 (PE)], and were isolated as a mixture of *E*- and *Z*-isomers in 15 : 1 ratio. The instability of the ylide synthesised *in situ* could be a contributing factor to the low yield of the Wittig olefination. Separation of the *E*- and *Z*-isomers was not undertaken, as hydrogenation of the alkene moiety was to be undertaken in a subsequent step.

With the lipids in hand, iso-branched *sn*-1 and *sn*-3 MAGs **6a–i** were prepared *via* esterification of commercially available (*S*)- or (*R*)-1,2-*O*-isopropylidene glycerol **14** with the appropriate fatty acid (**15a–e** or **13**) in the presence of coupling reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylaminopyridine (DMAP) (Scheme 2). Here, the reaction mixture was stirred for 48 h at 70 °C to yield the corresponding isopropylidene protected iso-branched glycerides **16a–i** in good yields (65–78%). For each product, a Heteronuclear Multiple Bond Correlation (HMBC) between the



Scheme 1 Synthesis of iso-branched fatty acids **13**.



15a: *n* = 9
15b: *n* = 11
15c: *n* = 15
15d: *n* = 16
15e: *n* = 19
13 : *n* = 23, Δ^{10}

16a: *sn*-1, *n* = 9 , 64%
16b: *sn*-1, *n* = 11, 78%
16c: *sn*-3, *n* = 11, 77%
16d: *sn*-1, *n* = 15, 73%
16e: *sn*-1, *n* = 16, 75%
16f : *sn*-1, *n* = 19, 73%
16g: *sn*-3, *n* = 19, 74%
16h: *sn*-1, *n* = 23, 71%, Δ^{10}
16i : *sn*-3, *n* = 23, 75%, Δ^{10}

6a: *sn*-1, *n* = 9, 85%
6b: *sn*-1, *n* = 11, 91%
6c: *sn*-3, *n* = 11, 92%
6d: *sn*-1, *n* = 15, 89%
6e: *sn*-1, *n* = 16, 93%
6f : *sn*-1, *n* = 19, 91%
6g: *sn*-3, *n* = 19, 92%
6h: *sn*-1, *n* = 23, 89%
6i : *sn*-3, *n* = 23, 87%

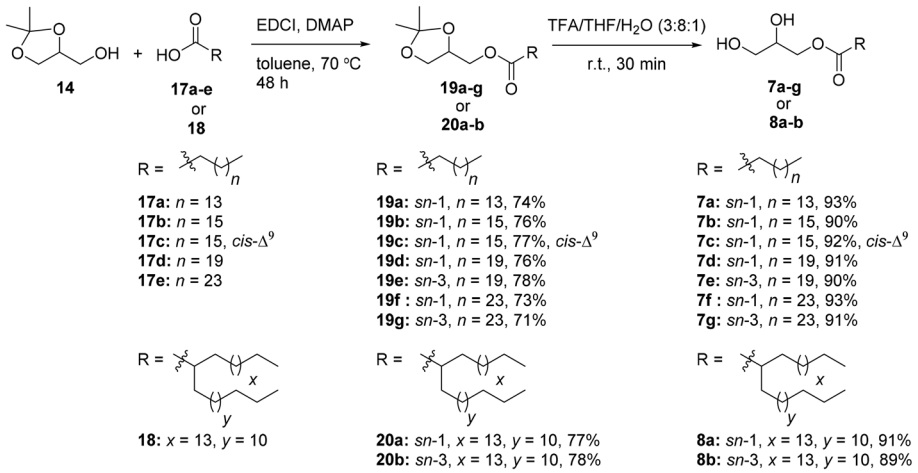
Scheme 2 Synthesis of iMAGs **6a–i**.

methylene protons of the glycerol moiety and the carbonyl carbon of the lipid confirmed the successful installation of the lipid moiety. Deprotection of **16a–g** was then achieved under the agency of trifluoroacetic acid in tetrahydrofuran/water (TFA/THF/H_2O, 3 : 8 : 1),[15] with stirring of the reaction mixture at r.t. for 30 min. This resulted in the desired products **6a–g** being isolated in excellent yields (85–93%) following purification by silica gel flash column chromatography. To prepare glycerides **6h** and **6i**, alkene **16h** and **16i** were subjected to hydrogenation in the presence of Pearlman's catalyst which led to both double bond reduction and isopropylidene deprotection to yield the target compounds in excellent yields (87–89%).

The *sn*-1 or *sn*-3 linear chain MAGs **7a–g** and α-branched MAGs **8a,b** were then prepared in an analogous manner to the iMAGs (Scheme 3). Isopropylidine protected glycerol **14** was esterified using carboxylic acids **17a–e** or **18**,[24] to yield the isopropylidene protected linear and α-branched glycerides **19a–g** and **20a,b**, respectively. Deprotection of **19a–g** and **20a,b** gave the desired products **7a–g** and **8a,b** in excellent yields. The characterisation data of all MAGs was in accordance with previously reported racemic iso-branched[29] and short chain[12,30,31] MAGs.

With the synthesised MAGs in hand, their ability to signal through Mincle was first assessed using NFAT-GFP cell lines expressing hMincle and FcRγ or FcRγ-only.[13,14] In accordance with recently reported findings,[16] MAGs incorporating short acyl chains (iMAGs **6a–e** and MAGs **7a–c**) did not activate hMincle expressing reporter cells (Fig. 3). The iMAGs **6f** (*sn*-1, iC22+1) and **6g** (*sn*-3, iC22+1) showed better activity than their linear counterparts **7d** (*sn*-1, C22) and **7e** (*sn*-3, C22), and a similar trend was observed for iMAG **6h** (*sn*-1, iC26+1) and **6i** (*sn*-1, iC26+1) when compared to **7f** (*sn*-1, C26) and **7g** (*sn*-3, iC26). Moreover, iMAG **6h** (*sn*-3, iC26 + 1) showed hMincle agonist activity equivalent to the positive control, trehalose dibehenate (TDB), which is the prototypical Mincle agonist.[23] In addition, the αMAGs **8a** (*sn*-1, C32) and **8b** (*sn*-3, C32), showed the highest degree of reporter cell activation and were found to induce more GFP production than TDB. These results clearly demonstrate the importance of lipid length and branching on the ability of MAGs to signal through Mincle.

The stereochemistry of the glycerol moiety also affected Mincle signalling, as demonstrated by all three subsets of MAGs with the *sn*-1 (*S*) isomers leading to greater reporter cell activation as compared to their *sn*-3 (*R*) isomeric counterparts at both concentrations of glycolipid tested. For example, **6f** (*sn*-1, iC22+1) led to greater activity compared to **6g** (*sn*-3, iC22+1), as did **7d** (*sn*-1, C22) compared to **7e** (*sn*-3, C22), **7f** (*sn*-1, C26) compared to **7g** (*sn*-3, C26), **6h** (*sn*-1, iC26+1) compared to **6i** (*sn*-3, iC26+1) and **8a** (*sn*-1, C32) compared to **8b**



Scheme 3 Synthesis of linear MAG **7a–g** and αMAG **8a,b**.

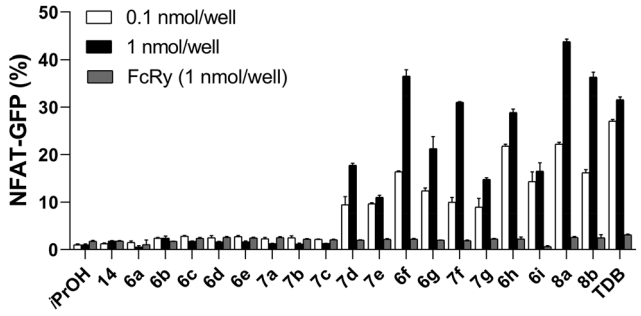


Fig. 3 NFAT-GFP 2B4 reporter cells expressing hMincle + FcRγ, or FcRγ-only were stimulated using MAG-coated plates (0.1 or 1 nmol per well) for 18 h. The cells were then harvested and examined for NFAT-GFP expression. Data reported is representative of two independent experiments performed in duplicate (mean ± SEM).

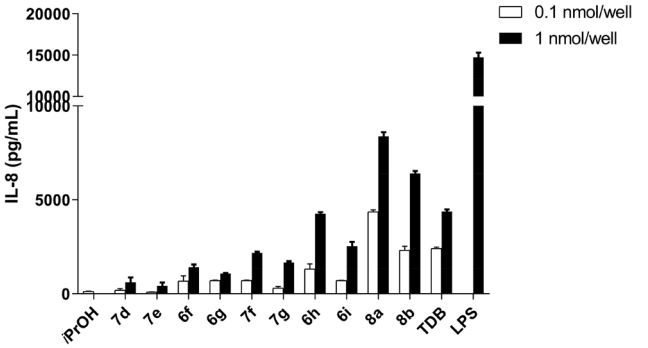


Fig. 4 IL-8 production of human monocytes by treatment with plate-coated MAGs or TDB (0.1 or 1 nmol per well) or solubilised LPS (100 ng mL^{-1}). Cytokine production was measured by ELISA from the supernatant collected after 24 hours. Mean ± SEM of triplicate samples from representative experiment of three performed are shown.

(*sn*-1, C32). This result corroborates the earlier findings of Van der Peet *et al.* and further demonstrates that the Mincle agonist activity predominantly resides with the *sn*-1-isomer.[15] Overall, **8a** (α-branched C32, *sn*-1) was the most potent agonist of those tested. Moreover, iMAGs **6a–e** and MAGs **7a–e** were also tested for their ability to activate murine Mincle using the NFAT-GFP mMincle reporter cell assay.[13,14] However, as anticipated,[12,15] these compounds did not signal through Mince (ESI Fig. 1†).

In addition to the reporter cell assays, the proficiency of MAGs to activate human monocytes, which have previously been shown to express Mincle,[32–34] was explored. After isolation of the monocytes from peripheral blood mononuclear cells (PMBCs), the production of the proinflammatory cytokine IL-8[35,36] by the monocytes was assessed following stimulation with the C22 (**6f**, **6g**, **7d**, **7e**), C26 (**6h**, **6i**, **7f**, **7g**) and C32 (**8a**, **8b**) MAGs at 0.1 or 1 nmol per well (Fig. 4). Once again, lipid length played an important role in the immunostimulatory activity of the compounds, with increasing lipid length corresponding to increased IL-8 production. Moreover, the production of IL-8 in response to the iso-branched MAGs was greater than that elicited by the corresponding linear MAGs (*i.e.* compare **6f** to **7d**, **6g** to **7e**, **6h** to **7f** and **6i** to **7g**). Relative cytokine production by the monocytes following stimulation with the MAGs also followed the trends observed when using the NFAT-GFP reporter assay, whereby monocytes stimulated with 1 nmol per well of iMAG **6h** (*sn*-1, iC-26+1) led to IL-8 production that was comparable to TDB, while αMAG **8a** (*sn*-1, C32) and **8b** (*sn*-3, C32) led to significantly higher levels of IL-8 compared to TDB. The stereochemistry of the glycerol backbone also affected IL-8 production by the monocytes, with the *sn*-1 analogues primarily leading to higher levels of IL-8 cytokine production than the corresponding *sn*-3 isomer. While we cannot conclusively say that the production of IL-8 by human monocytes in response to the monoacylglycerides is solely dependent on Mincle signalling, our results from the reporter cell assay where Mincle-dependence was observed indicates that IL-8 production, at least in part, is due to the engagement of the monoacylglycerides with hMincle.

To determine whether there was a correlation between the cytotoxicity of the glycerides and their ability to activate immune cells, as was previously demonstrated by Bertelsen

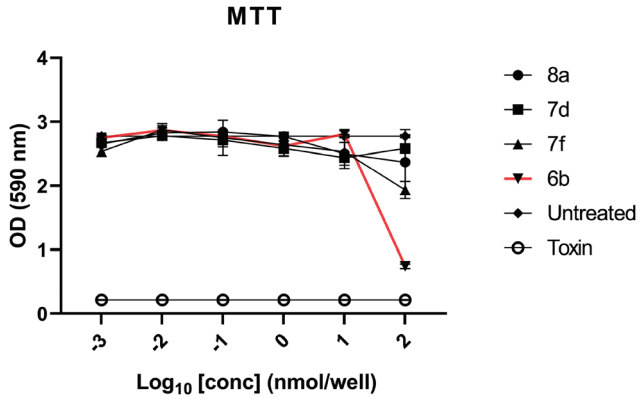


Fig. 5 HL-60 were treated with 0.001, 0.01, 0.1, 1, 10, or 100 nmol per well of plate-coated **6b**, or **7d**, or **7f**, or **8a**. Mean ± SEM of triplicate MTT assay samples from representative experiment of three performed are shown. The toxin cyclohexamide was used as a positive control.

et al. when assessing the ability of MCMGs to active DCs,[11] we determined the cytotoxic effects of representative MAGs on HL-60 cells using the MTT assay.[37] To this end, HL-60 cells were treated with titrated amounts of **6b** (*sn*-1, iC14+1), **7d** (*sn*-1, C22), **7f** (*sn*-1, C26) and **8a** (*sn*-1, C32), and, after 18 h, the percentage of live/dead cells was analysed (Fig. 5). No cytotoxicity was observed for the MAGs with an acyl chain length ≥C22 at any concentration tested, while the short acyl chain length iMAG **6b** (iC14+1) exhibited cytotoxicity only at a very high concentration, *i.e.* 100 nmol per well, which is a concentration that greatly surpasses that used in the NFAT-GFP reporter assay. Accordingly, the inability of **6b** to activate reporter cells does not appear to be due to the cytotoxicity of the compound but is likely due to ineffective binding of this ligand in the hydrophobic groove of Mincle.[17–22,38] Moreover, the pro-inflammatory effects of the longer chain glycerides (**7d**, **7f** and **8a**) were not due to cytotoxicity.

Conclusion

In this study a series of linear, iso-branched, and α-branched MAGs were synthesised with defined stereochemistry and in good overall yields (63–72%). The effect of acyl chain length, iso-branching and stereochemistry on Mincle activation was explored and it was determined that the incorporation of longer lipid chains and branching on the lipid backbone enhanced the immunmodulatory activity of the compounds, with an acyl chain length ≥C22 being required for signalling through hMincle and the production of IL-8 by human monocytes. The stereochemistry of the glycerol moiety also influenced cellular activation with the *sn*-1 isomers leading to more pronounced immune responses compared to the *sn*-3 isomers. The immune response to the MAGs was not due to cytotoxicity. Thus, in summary, key structure–activity relationships with regard to the Mincle-agonist activity of MAGs have been determined, with three MAGs [**6h** (*sn*-1, iC26+1), **8a** (*sn*-1, C32) and **8b** (*sn*-3, C32)] exhibiting immunostimulating properties that were equivalent to, if not better than, TDB. Accordingly, the ease of their syntheses and their immunostimulatory properties makes MAGs particularly promising vaccine adjuvants.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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