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COMMUNICATION

Inhibition of Mincle signaling by chemically synthesized disaccharide-type 6-*O*-acylated steryl β-glucosides (βASGs) and their analogues derived from plants

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Chemically synthesized disaccharide βASGs from *Dioscorea cayenensis* and analogues effectively block Mincle-mediated signaling. This Mincle signaling inhibition requires a disaccharide backbone with sterol and fatty acid moieties, suggesting these glycolipids as key structural templates for developing novel anti-inflammatory therapeutics targeting this C-type lectin receptor.

Mincle (macrophage-inducible C-type lectin), a C-type lectin receptor (CLR) expressed on the surface of myeloid cells, plays an important role in innate immunity.[1,2] Mincle initiates signaling by forming a complex with the ITAM-bearing adaptor Fc receptor γ chain (FcRγ),[3] which leads to Syk activation and CARD9-dependent signaling, resulting in the production of inflammatory cytokines and chemokines. A defining feature of Mincle is its broad ligand recognition profile, although most ligands reported to date function as agonists. Mincle recognizes a wide range of ligands, including glycan structures as well as molecules bearing diverse lipophilic hydrophobic moieties.[4–7] To date, structurally and biosynthetically diverse molecules have been reported as Mincle ligands, including trehalose dimycolate (TDM), a major glycolipid of the mycobacterial cell wall,[2] the endogenous lipid β-GlcCer,[8,9] and the fungal-derived ligand "44-2".[10–12] Monosaccharide 6-*O*-acylated steryl β-glucosides (βASGs), predominantly found in plants and recently identified as an abnormal fungal metabolite, exhibit Mincle-dependent signaling activity (Figure 1). Ito and co-workers discovered that βASGs act as agonists of Mincle.[13] Subsequently,

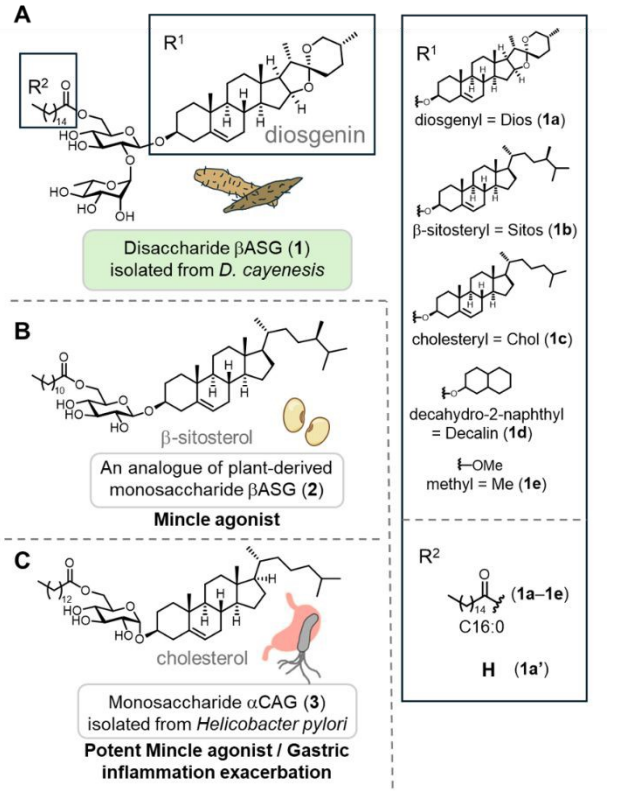

Fig. 1. A) Plant derived disaccharide βASGs (**1a**) and its analogues (**1b**-**1e**) as our target compounds of this study. B) An analogue of plant-derived monosaccharide βASG, which shows potent Mincle-mediated signaling activity.[13] C) Monosaccharide αCAG from *Helicobacter pylori*, exhibits potent Mincle agonistic activity and gastric inflammation exacerbation.[15]

we examined the structure-activity relationships more broadly and identified a fatty acid modified analogue (**2**) as a potent agonist.[14] Through this broad ligand recognition capacity, Mincle is thought to contribute to the maintenance of homeostasis and the regulation of inflammatory responses. Among these diverse ligands, cholesteryl 6-*O*-acyl-α-glucoside (αCAG, **3**), a glycolipid derived from *Helicobacter pylori*, has recently attracted particular attention.[15–17] αCAG is recognized by Mincle and has been shown to induce inflammatory signaling.

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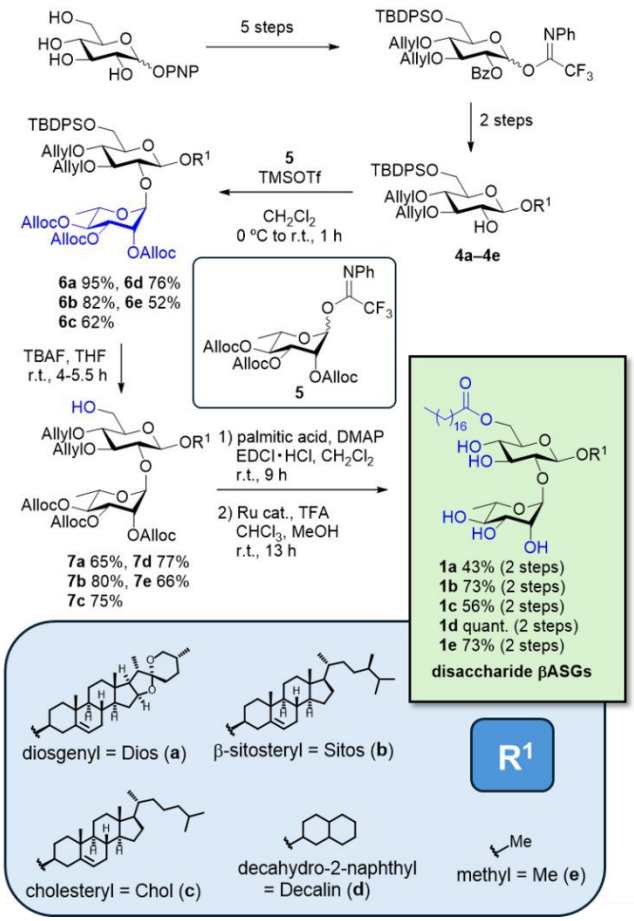
Given the close association of *H. pylori* infection with gastritis, gastric ulcer, and gastric cancer, the αCAG-Mincle interaction is considered to represent an important molecular basis for understanding the inflammatory responses involved in the pathogenesis of these diseases.

In contrast, previous structure–activity relationship (SAR) studies of Mincle ligands have focused primarily on receptor agonism,[4–7] whereas reports addressing inhibition of Mincle-mediated signaling responses remain limited.[18,19] It has been reported that diglucosyl-diacylglycerol (DGDG) derived from Group A *Streptococcus* (GAS) inhibits Mincle activation induced by monoglucosyl-diacylglycerol (MGDG), another glycolipid from GAS.[18] Whereas MGDG contains a single α-glucopyranose linked to a glycerol scaffold, DGDG bears a disaccharide head group in which a second glucose is connected to the first through an α-1,2-linkage. This finding suggests that the glycan moiety can play an important role in the inhibition of Mincle responses. However, the detailed SAR governing inhibitory activity has not been sufficiently examined, and the molecular basis underlying the functional switch from receptor activation to inhibition remains unclear.

On the basis of these observations, disaccharide glycolipids bearing an α-1,2-linkage were hypothesized to act as inhibitors of Mincle-mediated signaling responses. Attention was therefore directed to a disaccharide βASG (**1a**) reported from *Dioscorea cayenensis* and known as yellow yam, which contains an α-L-rhamnose at the C2 position of the glucose unit, a palmitate group at the C6 position of glucose, and diosgenin as the sterol moiety (Figure 1).[20] By applying a previously established modular synthetic strategy for monosaccharide βASGs (**2**), the natural product structure **1a** and a series of related analogues were targeted for synthesis. The resulting disaccharide βASG library was then planned for evaluation with respect to Mincle-mediated signaling activity, inhibitory activity against Mincle responses induced by an analogue of plant-derived monosaccharide βASG (**2**) and *H. pylori*-derived αCAG (**3**), and Mincle binding activity.[21]

The target structures for the chemical synthesis of disaccharide βASGs are shown in Figure 1. In addition to the natural product **1a**, **1b**, bearing β-sitosterol, a sterol commonly found in monosaccharide βASGs, and **1c**, containing cholesterol, were designed. Compounds **1d**, bearing a decahydro-2-naphthyl group, and **1e**, possessing a methyl glycoside structure, were also included as target molecules. In addition, disaccharide βSG **1a′**, which lacks the fatty acid moiety, was selected as a synthetic target. For the decalin-containing analogue, commercially available decahydro-2-naphthol was used as a stereoisomeric mixture, as in the previous synthesis of monosaccharide βASGs.

The synthesis of disaccharide-type βASG (**1a**) and its analogues (**1b**–**e** and **1a**') is outlined in Scheme 1. The glycosyl donor (glucosyl *N*-(phenyl)trifluoroacetimidate donor) was prepared in five steps from the starting material (1-PNP-glucose) used in the synthesis of the monosaccharide-type βASG, in which the anomeric position of Glu was protected with a PNP group.[22] This donor was then coupled with various sterols, decahydro-2-naphthol, or methanol, followed by selective removal of the Bz groups to afford **4a**–**e**.[14] Glycosylation of compounds **4a**–**e** with glycosyl donor **5**, in which the 2, 3, and 4 positions of rhamnose were protected with Alloc groups (the synthesis of compound **5** is described in Scheme S1), furnished **6a**–**e** in 52–95% yields. The anomeric stereochemistry of the rhamnose moiety in **6a** was confirmed from the J_{CH} coupling constant (Figure S2). Subsequent removal of the TBDPS group at the C6 position of glucose with TBAF afforded **7a**–**e**. Installation of palmitic acid at the resulting C6 hydroxyl group of glucose, followed by global deprotection of the Allyl and Alloc groups, completed the synthesis of disaccharide-type βASGs **1a**–**e**. The synthetic description of **1a'** is also provided in the Supporting Information. Together, these results show that the synthetic strategy established for monosaccharide-type βASGs can be extended to the synthesis of disaccharide-type βASGs and SG. They further demonstrate that the previously developed approach is broadly applicable as a general platform for the synthesis of diverse βASG analogues.



Scheme. 1. Synthesis of disaccharide βASGs (**1a**) and their analogues (**1b–e**).

The activity and binding properties of disaccharide-type βASGs and their analogues (**1a**–**1e**, **1a**'), are shown in Figure 2 and Figure S1. Their ability to induce Mincle-dependent signaling was assessed using an 2B4 NFAT-GFP reporter cell assay.[3] This method has also been applied for evaluating the inhibition of Mincle activation.[18,19]-Under these conditions, none of the compounds exhibited appreciable agonist activity (Figure

2A, Figure S1). The analysis was therefore extended to determine whether these disaccharide-type glycolipids inhibit Mincle signaling induced by the monosaccharide-type βASG (Figure 2B). In this assay, monosaccharide-type βASG **2** (0.1 nmol/well),[14] which functions as a Mincle agonist, was co-incubated with **1a**–**1e** or **1a**', and the resulting Mincle-mediated signaling response was evaluated. Disaccharide-type βASGs **1a**–**1c**, which bear sterol moieties, strongly inhibited signaling at 0.1 and 1.0 nmol/well in both human and mouse Mincle reporter cells. By contrast, **1d**, which contains a decalin scaffold, methyl glycoside **1e**, and disaccharide-type βSG **1a**' suppressed signaling in human Mincle cells at 0.1 and 1.0 nmol/well, whereas their inhibitory effects were minimal in mouse Mincle cells. It was next examined whether the disaccharide-type βASGs also inhibit Mincle signaling induced by αCAG **3**, a *Helicobacter pylori*-derived ligand. As in Figure 2B, **1a**–**1c** markedly suppressed the signaling response induced by αCAG **3** (0.1 nmol/well) at 0.1 and 1.0 nmol/well, whereas **1d** showed no detectable inhibitory activity (Figure 2C). In contrast to the results shown in Figure 2B, however, **1e** displayed inhibitory activity not only toward human Mincle but also strong inhibition toward mouse Mincle at 0.1 and 1.0 nmol/well. Finally, compound **1b** was evaluated in an ELISA-based binding assay for Mincle,[21] which revealed measurable binding of **1b** to the receptor (Figure 2D).

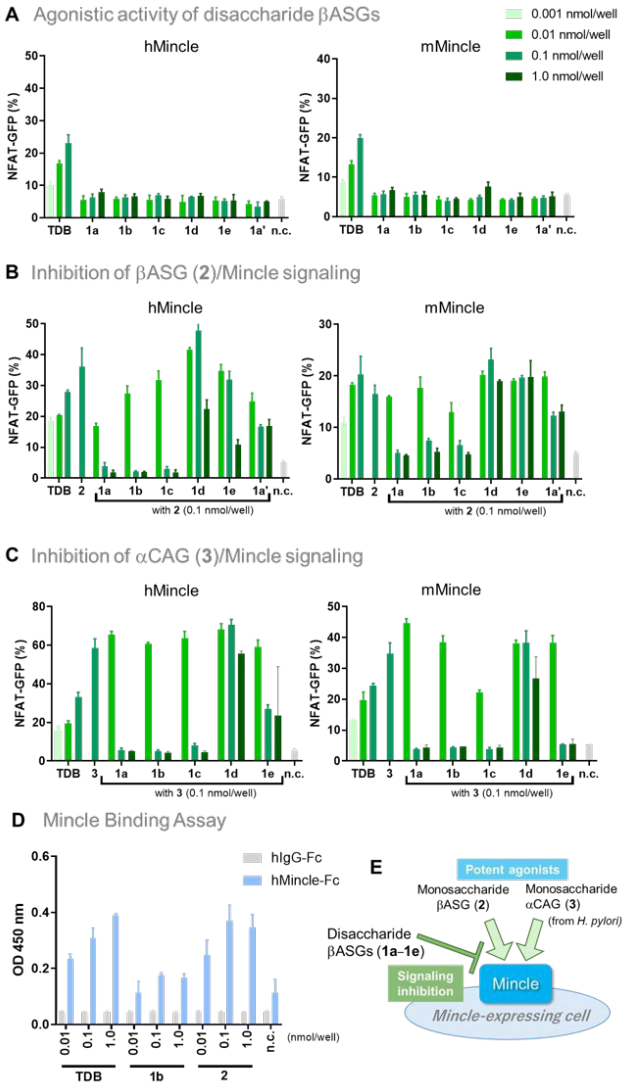


Fig. 2 Mincle-mediated signaling activity of ASGs by using 2B4 NFAT-GFP reporter cells expressing hMincle or mMincle. The cells were incubated on a plate with the ligands, TDB, or ^{i}PrOH as a negative control at 36 ºC for 18h. GFP expression was evaluated by flowcytometry. Agonist activity of disaccharide βASGs (A), inhibition of monosaccharide βASG/Mincle-mediated signaling activity (B), and Inhibition of αCAG/Mincle-mediated signaling activity (C). (D) Affinity of hMincle-Fc or hIgG-Fc for compounds **1b** and **2**. Binding affinity was evaluated using ELISA, measuring absorbance at 450 nm. hMincle-Fc / hIgG-Fc was added at 75 ng/well. n.c.: vehicle (^{i}PrOH). (E) Schematic illustration of the inhibitory effects of **1a–1e** against responses induced by **2** or **3**.

Together, these results identify disaccharide-type βASGs as a new class of inhibitory ligands that exhibit little intrinsic Mincle agonist activity but suppress Mincle signaling induced by monosaccharide-type βASG and αCAG. Notably, the results obtained for **1a**–**1c** indicate that introduction of rhamnose switches the function of monosaccharide-type βASGs from agonism to antagonism. Comparison with **1d**, **1e**, and **1a**' further suggests that, in addition to the disaccharide framework, the structure of the hydrophobic moiety, particularly one containing a sterol scaffold, is important for inhibitory activity. Direct binding assays (Figure 2E) confirmed that these βASGs exhibit affinity for Mincle, with the monosaccharide βASG (**2**) showing higher affinity than the corresponding disaccharide βASG (**1b**). The observed binding of **1b** to Mincle further supports the idea that disaccharide-type βASGs suppress Mincle responses through direct engagement of the receptor. Considering the agonist activity of the monosaccharide βASGs, these findings are consistent with the disaccharide βASGs interacting with the receptor at or near the canonical binding site. At the same time, it should also be considered that the rhamnose moiety attached at the 2-position of glucose may sterically or electronically interfere with the precise conformational changes or receptor clustering required for signal transduction, thereby leading to the observed inhibitory activity. Based on the reported docking model of Mincle with βASG,[13,23] along with other analyses (Figure S2), the rhamnose moiety attached to the C2 position of glucose is expected to protrude toward the solvent-exposed surface. This may sterically prevent Mincle clustering, resulting in antagonistic activity.

In conclusion, in this study, we applied our previously established modular synthetic strategy for βASGs to the synthesis of *D. cayenensis*-derived disaccharide-type βASGs and sterol-modified analogues, thereby constructing a library of disaccharide-type βASGs. Functional evaluation using Mincle-expressing reporter cells identified the 1,2-α-disaccharide motif in disaccharide-type βASGs as a key structural element underlying the functional switch from receptor activation to inhibition. The identification of disaccharide-type βASGs from an edible yam also expands the potential relevance of these molecules beyond glycolipid chemistry, particularly to the fields of food immunology and food-derived lipid mediator research. Notably, the disaccharide-type βASGs effectively suppressed αCAG-induced Mincle signaling, demonstrating that structural modification of plant-derived glycolipids can alter the functional polarity of the Mincle response. These findings provide new

insight into the molecular basis of Mincle-mediated immune responses and establish a foundation for the design of new immunomodulatory molecules. Given the pathophysiological relevance of the αCAG–Mincle interaction, the disaccharide-type βASGs identified in this study may also serve as candidate scaffolds for the development of new Mincle-targeted immunoregulatory strategies.

Author contributions

KY: Data Curation, Formal Analysis, Investigation, Writing–original draft & editing. TM: Conceptualization, Funding Acquisition, Writing–original draft, Writing–review & editing. SY: Resources. YF: Conceptualization, Supervision, Funding Acquisition, Writing–review & editing.

Data availability

The data supporting this article have been included as part of the ESI.† The ESI† includes the experimental section and the ethics approval for the genetic recombination experiments and the animal experiments.

Conflicts of interest

There are no conflicts of interest to declare

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Data availability

The data supporting this article have been included as part of the ESI.† The ESI† includes the experimental section and the ethics approval for the genetic recombination experiments and the animal experiments.