Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

COMMUNICATION

View Article Online
View Journal | View Issue

Published on 21 January 2021. Downloaded by University of Prince Edward Island on 5/15/2021 7:49:57 PM.

Check for updates

Cite this: *Org. Biomol. Chem.*, 2021, **19**, 2198

Received 25th November 2020,
Accepted 21st January 2021

DOI: 10.1039/d0ob02342f

rsc.li/obc

Cholesteryl glucosides signal through the carbohydrate recognition domain of the macrophage inducible C-type lectin (mincle)†

Mattie S. M. Timmer, *[a] Thomas J. Teunissen,[a] Kristel Kodar,[a] Amy J. Foster,[a] Sho Yamasaki[b,c,d,e] and Bridget L. Stocker*[a]

Cholesteryl α-D-glucosides (αGCs) are unique metabolic products of the cancer-causing human pathogen *Helicobacter pylori*. *Via* signalling through the Macrophage inducible C-type lectin (Mincle) and the induction of a pro-inflammatory response, they are thought to play a role in the development of gastric atrophy. Herein, we prepared the first library of steryl D-glucosides and determined that they preferentially signal through the carbohydrate recognition domain of human Mincle, rather than the amino acid consensus motif. Lipidated steryl D-glucosides exhibited enhanced Mincle agonist activity, with C18 cholesteryl 6-*O*-acyl-α-D-glucoside (2c) being the most potent activator of human monocytes. Despite exhibiting strong Mincle signalling, sito- (5b) and stigmasterol glycosides (6b) led to a poor inflammatory response in primary cells, suggesting that Mincle is a potential therapeutic target for preventing *H. pylori*-mediated inflammation and cancer.

Helicobacter pylori is a bacterial pathogen that colonises the stomach and is the leading cause of gastric ulcers, carcinoma, and lymphoma.[1,2] It is estimated that over 50% of the world's population is infected with *H. pylori*,[3] and as such, there has been much interest in deciphering how *H. pylori* promotes the growth and development of gastric cancers. *H. pylori* senses cholesterol rich regions in host epithelial tissue and, *via* the agency of cholesterol-α-glucosyltransferases (αCgT), converts cholesterol into cholesteryl α-D-glucoside (αCG, **1**), cholesteryl 6-*O*-acyl-α-D-glucosides (αCAGs, *e.g.* **2a**), cholesteryl 6-*O*-phosphatidyl-α-D-glucosides (αCPGs, **3**), and monoacylated derivatives of αCPGs (**4**) (Fig. 1), which constitute *ca.* 25% of the lipid content of the bacteria.[4–6] Collectively, αCG (**1**) and the αCAGs **2** account for over 80% of the cholesteryl-α-D-glucosides found in *H. pylori*.[4] Notwithstanding, how *H. pylori* cholesteryl glucosides influence the cancer-promoting capacity of the bacteria is a topic of much debate.[7–14]

To provide further insight into the mechanisms by which glucosylated cholesterol metabolites influence the immune response, Yamasaki and co-workers isolated metabolites from *H. pylori* and determined that αCAGs **2a** and **2b** signalled through the Macrophage inducible C-type lectin (Mincle), thereby promoting an inflammatory immune response.[9] In these studies, a decrease in the inflammatory response to *H. pylori* was observed in murine strains lacking αCgT. Further-more, while the antibody-mediated neutralisation of Mincle did not lead to an increase in bacterial burden in murine models, inhibition of Mincle led to a reduction in symptoms associated with gastritis.[9] Insomuch, inflammation mediated by glucosylated cholesterol metabolites may play a role in the development of gastric cancer, an observation that is in agreement with clinical studies where the activity of αCgTs from *H. pylori* isolates was positively correlated with the degree of gastric atrophy in patients.[8] However, in the clinical studies, increased αCgT activity resulted in fewer surviving bacteria.[8] In contrast, the phosphorylated cholesteryl α-D-glucosides (*e.g.* **3**) did not signal through Mincle, but rather the dendritic cell immunoactivating receptor (DCAR).[9]

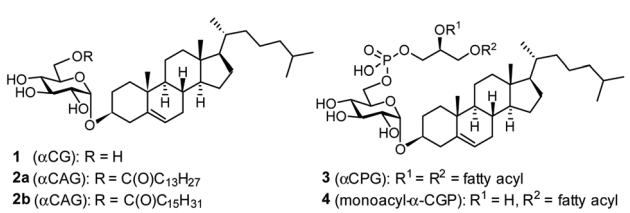


Fig. 1 α-Linked cholesteryl glucosides.

[a]*School of Chemical and Physical Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand. E-mail: mattie.timmer@vuw.ac.nz*
[b]*Department of Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, SuitaOsaka 565-0871, Japan*
[c]*Laboratory of Molecular Immunology, Immunology Frontier Research Center (IFReC), Osaka University, SuitaOsaka 565-0871, Japan*
[d]*Division of Molecular Design, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Fukuoka 812-8582, Japan*
[e]*Division of Molecular Immunology, Medical Mycology Research Center, Chiba University, Chiba 260-8673, Japan*

† Electronic supplementary information (ESI) available: Experimental procedures and characterization data for all compounds. See DOI: 10.1039/d0ob02342f

This journal is © The Royal Society of Chemistry 2021

Published on 21 January 2021. Downloaded by University of Prince Edward Island on 5/15/2021 7:49:57 PM.

In view of the recent finding that αCAGs **2a** and **2b** are Mincle agonists,[9] we became interested in determining how modifications to the cholesteryl glucoside scaffold influence Mincle signalling and moreover, where such motifs bind human Mincle. Mincle contains a carbohydrate recognition domain (CRD),[15,16] which recognises pathogen-associated molecular patterns,[17] as well as a cholesterol recognition amino acid consensus (CRAC) motif[18] that is found in hMincle but not murine Mincle,[19] and which binds cholesterol metabolites.[18] Thus, we sought to synthesise the two major cholesteryl α-D-glucosides of *H. pylori*, αCG (**1**) and αCAG (**2a**),[8,10] along with derivatives that contain different steroid backbones or acyl chains (**2c**, **5a,b** and **6a,b**, Fig. 2). As *H. pylori* containing cholesteryl β-D-glucoside (βCG, **7**) induced macrophage phagocytosis yet bacteria containing αCG (**1**) did not,[7] we were also interested in assessing the immune stimulating properties of βCG (**7**). The ability of the derivatives to signal through Mincle and CRAC- or CRD-mutants thereof would be determined using the nuclear factor of activated T cells (NFAT)-green fluorescent protein (GFP) reporter cells coupled to FcRγ,[20] while human-derived monocytes would provide an indication of the compound's functional immune response.[21]

To prepare the steroid-based α-glucosides, an elegant procedure employing the use of a TMS-protected glucosyl iodide,[22] as developed by Gervay-Hague,[23,24] was employed (Scheme 1A). To this end, D-glucose (**8**) was treated with TMSCl in DMF to give per silylated glucose **9** in 70% yield, followed by the *in situ* generation of the glucosyl iodide and subsequent coupling to the steroid of choice. The *in situ* generated TMS-protected cholesteryl α-D-glucoside was then subject to Dowex-H^+ in MeOH to give αCG (**1**). Rather than precipitation and filtration to remove TBAI from the reaction mixture,[23] we obtained higher product yields and purity by employing silica gel flash column chromatography (95 : 5, CH_2Cl_2/MeOH, *v*/*v*) followed by size exclusion chromatography (Sephadex LH-20, CH_2Cl_2/MeOH, 1 : 1). In this way, αCG (**1**) was prepared in 80% yield (over three-steps). The same methodology was then applied to the synthesis of the sterol analogues, with the glucosyl iodide being coupled to β-sitosterol (**10b**) or stigmasterol (**10c**) to yield the TMS-protected steryl glucosides. Global deprotection then gave β-sitosteryl α-D-glucoside (αSiG, **5a**) in 78%, and stigmasteryl α-D-glucoside (αStG, **6a**) in 85% overall yields. In all instances, the α-selectively of the glycosylation reaction was determined *via* ^{1}H and 2D NMR ($J_{1,2}$ = 3.8 Hz for H-1α), with generally excellent α-selectivity being observed (*ca.* 9 : 1, α : β).

Each steroid-glucoside was then acylated using Novozym 435 and the appropriate vinyl ester.[23,24] To this end, αCG (**1**) was treated with Novozym 435 and vinyl myristate[25] to provide C14-αCAG (**2a**) in excellent (90%) yield following purification by silica gel flash column chromatography and size exclusion chromatography (Scheme 1A). Similarly, C18-αCAG (**2c**) was prepared *via* the enzyme-catalysed acylation of αCG (**1**) using vinyl stearate,[25] while the acylated β-sitosterol and stigmasterol glycosides, αSiAG (**5b**) and αStAG (**6b**), respectively, were prepared *via* enzyme-mediated coupling with vinyl myristate. Next, βCG **7** was prepared *via* a TMSOTf-mediated glycosylation reaction between cholesterol (**10a**) and imidate **11**[26] to provide the β-product exclusively, with methanolysis furnishing glucoside **7** in 70% yield in 2 steps (Scheme 1B).

The ability of the steryl glucosides to signal through mMincle or hMincle was then determined using 2B4 NFAT-GFP reporter cells expressing mMincle + FcRγ, hMincle + FcRγ, FcRγ (negative control), with trehalose dibehenate (TDB) serving as the positive control (Fig. 3).[27] The 6-*O*-acyl steroid derivatives [αCAG (**2a** and **2c**), αSiAG (**5b**), and αStAG (**6b**)] showed good mMincle agonism at both 0.1 and 1 nmol per well. Of the non-acylated derivatives, αCG (**1**) exhibited good mMincle agonist activity at 1 nmol per well, αStG (**6a**) led to modest mMincle activity, while αSiG (**5a**), βCG (**7**) and cholesterol (**10a**) did not signal through mMincle (Fig. 3A).

With the exception of cholesterol (**10a**), all derivatives activated hMincle reporter cells at both ligand concentrations (Fig. 3B), with the non-acylated derivatives αCG (**1**), αSiG (**5a**) and αStG (**6a**) eliciting slightly less activity than their acylated counterparts [αCAG (**2a**), αSiAG (**5b**) and αStAG (**6b**), respectively]. However, this difference was not as pronounced as that observed when using mMincle reporter cells, which might be attributed to the presence of both the CRD and the CRAC motif in hMincle. Changes to the steroid backbone appeared to have little effect on the ability of the steryl glucosides to signal through hMincle. Cholesterol (**10a**) only led to a significant increase in GFP production by hMincle reporter cells at a

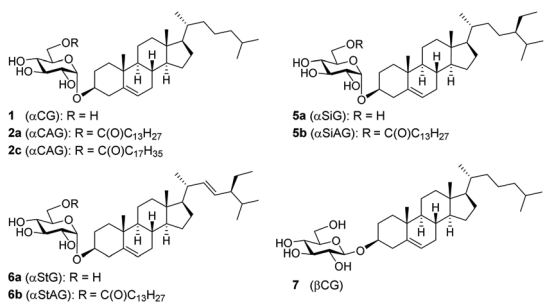
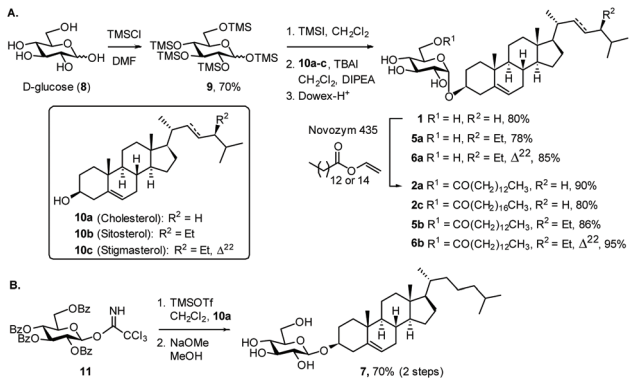


Fig. 2 Target steryl D-glucosides.



Scheme 1 Synthesis of steryl D-glucosides.

Published on 21 January 2021. Downloaded by University of Prince Edward Island on 5/15/2021 7:49:57 PM.

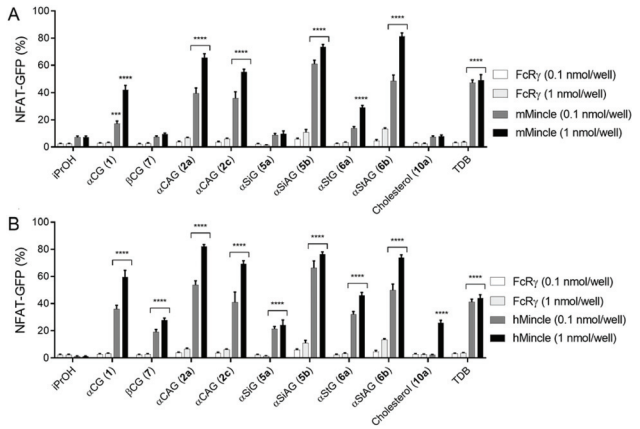


Fig. 3 Ability of steryl glucosides to signal through mMincle and hMincle. (A) mMincle + FcRγ, (B) hMincle + FcRγ, and FcRγ only expressing 2B4 reporter cells were incubated in plates coated with the prepared ligands (0.1 or 1 nmol per well), TDB (0.1 or 1 nmol per well), or iPrOH only. After 20 hours, the cells were harvested and analysed for GFP expression by flow cytometry. Data represents the mean (±SEM) of three independent experiments performed in duplicate. Statistical significance was calculated in comparison to iPrOH only using 2-way ANOVA (Sidak's multiple comparisons test), ***$P \leq 0.001$; ****$P \leq 0.0001$.

concentration of 1 nmol per well.[18] Taken together, hMincle appears to better accommodate changes to the steryl α-glucoside scaffold than mMincle, though for both Mincle orthologues incorporation of a lipid chain at the 6-position of glucose enhanced Mincle signalling. Moreover, βCG (7) only signalled through human but not murine Mincle.

Given the relevance of *H. pylori* as a human pathogen, we then sought to determine how the steryl glucosides signalled through hMincle. To this end, NFAT-GFP reporter cells with mutations to the CRAC motif (hMincle R135 → L135)[18] (Fig. 4A) or the carbohydrate-binding EPN motif [hMincle EPN → QPD][15] (Fig. 4B) were treated with the steryl glucosides. It is known that a mutation in the hMincle CRAC motif from Arg135 to Leu prevents cholesterol binding,[18] however, further insight into the exact binding mode remains to be determined.

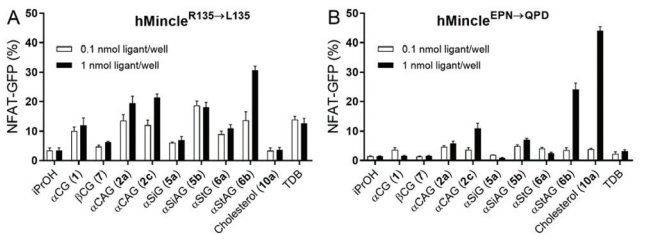


Fig. 4 Binding site for steryl α-glycoside. 2B4 reporter cells expressing hMincle with mutation in (A) CRAC motif, hMincle(R135L) + FcRγ, and in (B) CRD, hMincle(EPN-QPD) + FcRγ, were incubated with plate coated ligand; reporter cells expressing WT hMincle + FcRγ and FcRγ only were used as controls. After 20 hours, the cells were harvested and analysed for GFP expression by Flow cytometry. Data represents the mean (±SEM) of three independent experiments performed in duplicate.

In contrast, more is known about the CRD in hMincle. This contains a Ca^{2+} binding site that includes an EPN motif involved in the binding of the 3- and 4-hydroxyls of one glucose moiety of α,α′-trehalose.[15] Mutation of this site into glutamine-proline-aspartic acid (QPD), a putative galactose-recognition sequence, abrogates the binding of α,α′-trehalose containing glycolipids (*i.e.* trehalose dimycolate, TDM) to Mincle.[15]

For our steryl glucosides, signalling through the EPN motif appeared to dominate over the CRAC motif, although for some steryl glucosides, this preference was less pronounced. For example, βCG (7) could not signal through either mutated Mincle receptor, which might reflect the lower binding preference of this ligand for hMincle, while stigmasterol glycoside (αStAG, **6b**) signalled through both Mincle domains at higher ligand concentrations. As has been previously reported, cholesterol (**10a**) was unable to activate hMincle with a mutated CRAC motif[18] but retained activity when using hMincle EPN → QPD, while TDB signalled through the carbohydrate-binding EPN motif[27] but not to the CRAC motif.

Next, functional immune responses elicited by the steryl glucosides were investigated. Human monocytes, which have previously been shown to express Mincle,[21] were purified from whole blood and stimulated with 40 or 80 μM of the steryl α-glucosides or TDB (positive control), or untreated (negative control). The secretion of IL-8, a chemokine involved in neutrophil chemotaxis, as well as proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α, were then determined by ELISA (Fig. 5). All ligands, except for the non-lipidated αStG (**5a**) and cholesterol (**10a**), led to IL-8 production by human monocytes (Fig. 5A), with C18-αCAG (**2c**) eliciting the highest levels of IL-8 at both concentrations of ligand tested. The strongest pro-inflammatory response was elicited by monocytes stimulated with αCAG (**2c**), which contained a stearic acid ester at the 6-*O*-position of glucose, with this ligand leading to the production of IL-1β and TNF-α at both ligand concentrations (Fig. 5B and C). The non-acylated glucoside αCG (**1**), and to a lesser extent the stigmasterol derivative αStG (**6a**), also led to TNF-α production by human monocytes when higher concentrations of ligand were used, however neither ligand resulted in the production of statistically significant levels of IL-1β. While we cannot conclusively say that cytokine production by human monocytes in response to the steryl glucosides is solely dependent on Mincle, the Mincle signalling observed in the reporter assays indicates that monocyte activation is, at least in part, due to the engagement of the steryl glucosides with hMincle.

Taken as a whole, it appears that hMincle is better able to tolerate a wider variety of steryl glucosides than mMincle. This may be due to the presence of two ligand-binding sites in hMincle, and indeed, some species-specific differences were observed, most notably with regard to βCG (7), which was a ligand for hMincle but not mMincle. This highlights the care that needs to be taken when translating observations from one species to another.[17] Notwithstanding, the incorporation of a lipid chain at the 6-position of glucose generally enhanced

 This journal is © The Royal Society of Chemistry 2021

Published on 21 January 2021. Downloaded by University of Prince Edward Island on 5/15/2021 7:49:57 PM.

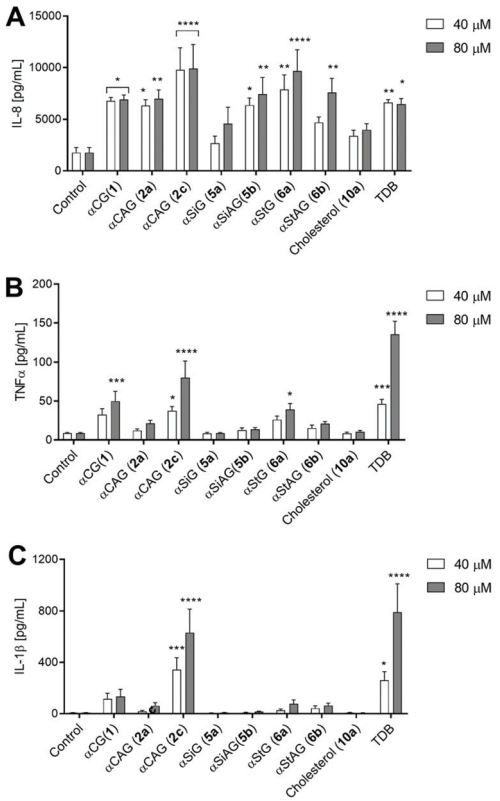


Fig. 5 Activation of human monocytes by steryl α-glucosides. Monocytes were purified from whole blood by negative selection, seeded onto 96-well plate (2×10^5 cell per well) and stimulated with 40 or 80 μM of ligand, TDB was used as a positive control. The expression of IL-8 (A), TNF-α (B), and IL-1β (C) was measured from supernatants at 24 h using ELISA. Data represents the mean (±SEM) of three independent experiments performed in triplicates. Statistical significance was calculated in comparison to untreated control using 2-way ANOVA (Sidak's multiple comparisons test); *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

both mMincle and hMincle signalling. Using hMincle mutants with modifications to the carbohydrate binding EPN motif or the CRAC motif the steryl glucosides were found to preferentially bind at the EPN motif. However, subtle changes to ligand structure can influence Mincle-ligand interactions, as illustrated by stigmasterol glycoside **6b**, which can signal through both the CRAC motif and the CRD.

The ability of the steryl α-glucosides to elicit a functional immune response by human monocytes typically paralleled studies using the NFAT-GFP reporter cells. All steryl α-glucosides, except for the non-lipidated sitosterol derivative **5a**, led to the production of IL-8 by human monocytes, with **5a** also eliciting only modest hMincle activation in the reporter cell assay. Similarly, cholesterol-coated plates did not lead to a significant increase in cytokine production by human monocytes and exhibited only weak hMincle agonist activity in the reporter cell assay. This may be due to the presentation of the ligand[28] and indeed, cholesterol crystals at concentrations of 100–300 μg mL^{-1} were used to elicit an immune response in studies using hMincle expressing RAW-Blue cells, or human monocyte-derived dendritic cells.[18] The most marked difference in functional response to steryl α-glucosides was the ability of the ligands to stimulate human monocytes to produce IL-1β and TNF-α. Here, αCAG (**2c**), containing the C18 lipid, led to a significant production of IL-1β and TNF-α by human monocytes, while αCG (**1**) induced a less notable, but nonetheless, pro-inflammatory response. This result parallels our earlier observation that long lipids enhance the agonist activities of Mincle ligands.[29] Our SAR data also revealed that acylated sitosteryl and stigmasteryl glucosides **5b** and **6b** exhibited strong Mincle signalling but led to a poor inflammatory response in primary cells.

Given the complex interaction of *H. pylori* with a number of different human antigen presenting cell (APC) subsets,[30] understanding how to best mitigate the detrimental effects of *H. pylori* is a complex task. Notwithstanding, we have, for the first time, revealed that the CRD is the preferred Mincle binding site of steryl α-D-glucosides, thereby providing a framework for the future design of Mincle agonists or antagonists. These findings support the earlier observation that cholesteryl α-D-glucosides signal through Mincle and lead to an inflammatory response which has been implicated in *H. pylori*-mediated gastric cancer progression.[9] Moreover, we identified α-D-glucosides (*e.g.*, αSiAG **5b** and αStAG **6b**) that signal through Mincle but do not lead to the production of key proinflammatory cytokines by primary human cells. Taken together, it is thus possible that the synthesis and application of Mincle antagonists that counter the pro-inflammatory effects of *H. pylori* metabolites might assist in the development of better therapies for the treatment of gastric cancer.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors would like to thank the NZ Health Research Council for financial support (Hercus Fellowship, BLS). The use of human leukocyte from healthy donors with written informed consent was approved by NZ Northern A Health and Disability Ethics Committee (approval no. 15/NTA/178).

Notes and references

1 J. G. Kusters, A. H. M. van Vliet and E. J. Kuipers, *Clin. Microbiol. Rev.*, 2006, **19**, 449–490.
2 J. Khatoon, R. P. Rai and K. N. Prasad, *World J. Gastrointest. Oncol.*, 2016, **8**, 147–158.
3 H. M. S. Algood and T. L. Cover, *Clin. Microbiol. Rev.*, 2006, **19**, 597–613.
4 Y. Hirai, M. Haque, T. Yoshida, K. Yokota, T. Yasuda and K. Oguma, *J. Bacteriol.*, 1995, **177**, 5327–5333.

Published on 21 January 2021. Downloaded by University of Prince Edward Island on 5/15/2021 7:49:57 PM.

5 M. Haque, Y. Hirai, K. Yokota, N. Mori, I. Jahan, H. Ito, H. Hotta, I. Yano, Y. Kanemasa and K. Oguma, *J. Bacteriol.*, 1996, **178**, 2065–2070.
6 A. H. Lebrun, C. Wunder, J. Hildebrand, Y. Churin, U. Zahringer, B. Lindner, T. F. Meyer, E. Heinz and D. Warncke, *J. Biol. Chem.*, 2006, **281**, 27765–27772.
7 C. Wunder, Y. Churin, F. Winau, D. Warnecke, M. Vieth, B. Lindner, U. Zähringer, H.-J. Mollenkopf, E. Heinz and T. F. Meyer, *Nat. Med.*, 2006, **12**, 1030–1038.
8 Y. Ito, J. L. Vela, F. Matsumura, H. Hoshino, A. Tyznik, H. Lee, E. Girardi, D. M. Zajonc, R. Liddington, M. Kobayashi, X. Bao, J. Bugaytsova, T. Borén, R. Jin, Y. Zong, P. H. Seeberger, J. Nakayama, M. Kronenberg and M. Fukuda, *PLoS One*, 2013, **8**, e78191.
9 M. Nagata, K. Toyonaga, E. Ishikawa, S. Haji, N. Okahashi, M. Takahashi, Y. Izumi, A. Imamura, K. Takato, H. Ishida, S. Nagai, P. Illarionov, B. L. Stocker, M. S. M. Timmer, D. G. M. Smith, S. J. Williams, T. Bamba, T. Miyamoto, M. Arita, B. J. Appelmelk and S. Yamasaki, *J. Exp. Med.*, 2021, **218**, e20200815.
10 H. M. Jan, Y. C. Chen, Y. Y. Shih, Y. C. Huang, Z. Tu, A. B. Ingle, S. W. Liu, M. S. Wu, J. Gervay-Hague, K. K. T. Mong, Y. R. Chen and C. H. Lin, *Chem. Sci.*, 2016, **7**, 6208–6216.
11 S.-Y. Du, H.-J. Wang, H.-H. Cheng, S.-D. Chen, L. H.-C. Wang and W.-C. Wang, *J. Microbiol., Immunol. Infect.*, 2016, **49**, 636e645.
12 M. Beigier-Bompadre, V. Moos, E. Belogolova, K. Allers, T. Schneider, Y. Churin, R. Ignatius, T. F. Meyer and T. Aebischer, *J. Infect. Disease*, 2011, **204**, 1339–1348.
13 P. Morey, L. Pfannkuch, E. Pang, F. Boccellato, M. Sigal, I. Imai-Matsushima, V. Dyer, M. Koch, H.-J. Mollenkopf, P. Schlaermann and T. F. Meyer, *Gastroenterology*, 2018, **154**, 1391–1404.
14 H.-M. Jan, *et al.*, *Commun. Biol.*, 2020, **3**, 120.
15 A. Furukawa, J. Kamishikiryo, D. Mori, K. Toyonaga, Y. Okabe, A. Toji, R. Kanda, Y. Miyake, T. Ose, S. Yamasaki and K. Maenaka, *Proc. Nat. Acad. Sci.*, 2013, **110**, 17438–17443.
16 H. Feinberg, N. D. S. Rambaruth, S. A. F. Jégouzo, K. M. Jacobsen, R. Djurhuus, T. B. Poulsen, W. I. Weis, M. E. Taylor and K. Drickamer, *J. Biol. Chem.*, 2016, **291**, 21222–21233.
17 C. D. Braganza, T. Teunissen, M. S. M. Timmer and B. L. Stocker, *Front. Immunol.*, 2018, **88**, 1940.
18 R. Kiyotake, M. Oh-hora, E. Ishikawa, T. Miyamoto, T. Ishibashi and S. Yamasaki, *J. Biol. Chem.*, 2015, **290**, 25322–25332.
19 H. Li and V. Papadopoulos, *Endocrinology*, 1998, **139**, 4991–4997.
20 S. Yamasaki, E. Ishikawa, M. Sakuma, H. Hara, K. Ogata and T. Saito, *Nat. Immunol.*, 2008, **9**, 1179–1188.
21 K. Kodar, M. J. McConnell, J. L. Harper, M. S. M. Timmer and B. L. Stocker, *Immunol. Cell Biol.*, 2020, **98**, 411–422.
22 J. Gervay-Hague, *Chem. Res.*, 2016, **49**, 35–47.
23 R. A. Davis, J. C. Fettinger and J. Gervay-Hague, *J. Org. Chem.*, 2014, **79**, 8447–8452.
24 R. A. Davis, C.-H. Lin and J. Gervay-Hague, *Chem. Commun.*, 2012, **48**, 9083–9085.
25 D. Swern and E. F. Jordan, *Org. Synth.*, 1950, **30**, 106–107.
26 S. Deng, B. Yu, J. Xie and Y. Hui, *J. Org. Chem.*, 1999, **64**, 7265–7266.
27 E. Ishikawa, T. Ishikawa, Y. S. Morita, K. Toyonaga, H. Yamada, O. Takeuchi, T. Kinoshita, S. Akira, Y. Yoshikai and S. Yamasaki, *J. Exp. Med.*, 2009, **206**, 2879–2888.
28 B. L. Stocker, K. Kodar, K. Wahi, A. J. Foster, J. L. Harper, D. Mori, S. Yamasaki and M. S. M. Timmer, *Glycoconjugate J.*, 2019, **36**, 69–78.
29 A. A. Khan, S. H. Chee, R. J. McLaughlin, J. L. Harper, F. Kamena, M. S. M. Timmer and B. L. Stocker, *ChemBioChem*, 2011, **17**, 2572–2576.
30 M. Fehlings, *et al.*, *Infect. Immun.*, 2012, **80**, 2724–2734.

 This journal is © The Royal Society of Chemistry 2021