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Corynomycolic acid-containing glycolipids signal through the pattern recognition receptor Mincle

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An enantioselective synthesis of (+)-corynomycolic acid, and its elaboration to esters of trehalose, glucose and glycerol, is described. Trehalose dicyorynomycolate and trehalose monocyorynomycolate activate human and mouse Mincle as effectively as trehalose dimycolate (cord factor). Glucose monomycolate is revealed to be a potent activator of both mouse and human Mincle. Glycerol monomycolate signals through human Mincle, with the activity predominantly residing in the 2'S-isomer.

Macrophage inducible C-type lectin (Mincle) is a germline-encoded pattern recognition receptor that provides immune sensing of bacterial and fungal pathogens. The discovery that the Mycobacterium tuberculosis glycolipid trehalose dimycolate (TDM, cord factor) signals through Mincle has led to a surge of activity to understand how PRR-costimulation through this receptor can alter immune responses (Fig. 1). Ligand binding to Mincle leads to phosphorylation of the immunoreceptor tyrosine-based activation motif of the Fc receptor γ (FcRγ)-chain molecule, and activation of nuclear factor kappa-B (NFκB) via Card9–Bcl10–MALT1 signalosomes. NFκB drives transcription of cytokines that shape the development of naïve T cells into various effector helper T cell (Th) subtypes. In particular, there is growing interest in the development of Mincle ligands as vaccine adjuvants to provide Th1/Th17 biased immune responses.

Emerging structure-activity relationships of trehalose-based glycolipids as ligands for Mincle have revealed that trehalose dibehenate (TDB) possesses activity similar to TDM, and that monoacyl trehalose derivatives also possess the capacity to activate through Mincle, with longer chain lengths generally providing greater potency of activation in Mincle reporter assays, which correlates with direct binding affinities for Mincle. Treatment of TDM with trehalase, which ostensibly cleaves the glycolipid into two molecules of glucose monomycolate, resulted in loss of Mincle activation. Glycerol monomycolate (GroMM) is also a Mincle ligand, but is selective for binding to the human, and not the mouse form of the receptor.

The reported structure-activity relationships for Mincle activation have in some cases used heterogeneous glycolipids isolated from bacterial sources, for which the precise structure(s) of the glycolipid is unclear. On the other hand studies performed using authentic synthetic materials, which represent the 'gold standard' for immunological studies, have utilized much simpler structures bearing straight chain acyl groups, and relating the measured activities to homogeneous full length natural materials has not been possible. Mycolic acids from corynebacteria (termed corynomycolic acids) are shorter in length and simpler in structure
than mycobacterial mycolic acids and constitute synthetically accessible targets whose relationships to the authentic mycobacterial materials is more readily appreciated. In a recent work we have developed a approach for the formation of a range of corynebacterial glycolipids, using homogeneous synthetic (+)-corynomycolic acid. These glycolipids were used to study activation of a reporter cell line by human and mouse Mincle, revealing a comprehensive structure-activity relationship that provides new leads for the development of simpler ligands for Mincle activation.

A range of approaches have previously been reported for the synthesis of racemic and enantipure corynomycolic acids. Our strategy for the synthesis of (+)-2R,3R-corynomycolic acid aimed to set the crucial anti stereochemistry using the boron-mediated aldol reaction of chiral ketones developed by the Paterson group. Paterson reported that aldol reactions of sterically-demanding boron enolates of ketones derived from benzoyl lactate undergo aldol reactions to afford anti-configured products. While this approach has been widely applied using lactate-derived ketones bearing small methoxy α-substituents, it has only seen limited application to larger groups. The ketone 12 was prepared by treatment of (S)-ethyl lactate derived Weinreb amide with pentadecylmagnesium bromide, followed by boron enolate (Scheme 2). The relative and absolute stereochemistry was assumed as 2R,3R, and subsequently confirmed as described below. Completion of the synthesis of (+)-2R,3R-corynomycolic acid required manipulation of 13 to a carboxylic acid. In practice this was best achieved through sequential reduction with NaBH₄ and then debenzoylation (NaOMe/MeOH) to afford triol 14. In this way epimerization at the ketone α-position was avoided. Glycol cleavage (NaOEt/SiO₂) of 14 followed by Lindgren oxidation, afforded (+)-corynomycolic acid (9) in 91% over the two steps. The relative anti-stereochemistry was defined by conversion to the acetonide, which displayed a characteristic large coupling constant ($J = 10.5$ Hz) between the α- and β-protons. The absolute stereochemistry was then defined by comparison of the optical rotation with literature data for (+)-corynomycolic acid ([α]D $^{24}+8.5^\circ$ (c 1.02, CHCl₃)) and [α]D $^{20}+$7.8° (c 1.0, CHCl₃).

While various dehydration coupling approaches have been utilized for the formation of sugar esters with various mycolic acids, an alternative approach utilizes substitution of a leaving group by an alkali salt, preferably cesium, of a mycolic acid. TDCM (3) and TMCM (4) were prepared by substitution of 15 or 17 with 9 in the presence of CsHCO₃ in THF/DMF followed by deprotection (Scheme 2). Reaction of 9 with the known tosylate 19 under the same conditions, followed by deprotection, afforded GMCM (6). To our knowledge this comprises the first complete report of the synthesis of enantiopure GMCM, a compound that appears to have only been synthesized as a mixture of diastereoisomers from racemic corynomycolic acid. The two C2' epimeric forms of GroCMC were prepared through a similar strategy. Thus, known (R)-1,2-isopropyldiene glycol 21 or (S)-1,2-isopropyldiene glycol 23 were coupled with 9 in the presence of CsHCO₃ to afford 22 and 24, respectively. TFA treatment of 22 or 24 afforded the GroCMC epimers 2'R-8 and 2'S-8 in 97% and 94% yields, respectively. It appears that this work represents the first synthesis of the enantiopure GroCMC epimers, with previous syntheses having utilized racemic corynomycolic acid affording these epimers as mixtures of anti-diastereoisomers, or providing no synthetic details.

Activation through Mincle was assessed using an NFAT-GFP reporter assay that has been previously used to identify Mincle ligands. NFAT-GFP reporter cells transfected with FeRγ alone or together with human or mouse Mincle were stimulated with plate-coated mycobacterial TDM, and the NFAT-dependent activation of cells was monitored by the detection of GFP-positive cells by flow cytometry. As shown in Fig. 3A, a dose-dependent increase in the percentage of GFP-positive cells was observed for human and mouse Mincle/FeRγ-expressing cells when exposed to mycobacterial TDM, whereas the cells expressing FeRγ alone did not show any reactivity (data not shown). While (+)-corynomycolic acid (9) did not activate the reporter cell lines, TDCM (3) resulted in a similar dose-dependent response to TDM, confirming that variation in structure of...
mycobacterial/corynebacterial mycolates has little effect on MinCle
activation (see SI for data replotted against molar quantities). TMCM
was shown to be an effective activator, a result in
accordance with structure-activity relationships that reveal monoaoyl
trehaloses to be effective MinCle ligands.\textsuperscript{3,9,11} Interestingly, GMCM
(6) was revealed to signal through MinCle, albeit less potently than
TDM, TDCM or TMCM, a result at odds with previous work that
studied MinCle activation after trehalase treatment of TDM.\textsuperscript{2} A
possible explanation for the difference is that trehalase
activates through human, but not mouse MinCle.\textsuperscript{11} As both 2'R-
and 2'S-GroMM were detected in lipid extracts of \textit{M. bovis} BCG,\textsuperscript{27} these results show that CD1b-restricted presentation of
GroMM to T cells, and MinCle C-type lectin receptor provide
complementary recognition of the two natural diasteroisomers of
this bacterial glycolipid.

In summary, we have completed the total synthesis of single
diasteroisomers of various corynomycolate esters and established their
ability to signal through MinCle. These results extend early work by
Nishizawa \textit{et al.} who demonstrated that TDCM and TMCM exhibit
pro-inflammatory activity through release of IL-6,\textsuperscript{36} although
signalling through MinCle was not considered. Surprisingly, GMCM
(6) also signals through MinCle, albeit less potently than TDCM, a
result that provides a new lead for the development of simplified
vaccine adjuvants. However, simplifying the lipid chain to form
glucose 6-monobehenate yielded an inactive compound. Further, we
reveal that ability of GroMCM to signal through human but not
mouse MinCle resides predominantly in the 2'S-isomer.

Notes and references
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\textsuperscript{†} This work was supported by the Australian Research Council and the
JSPS Grant-in-Aid for Scientific Research.

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

1 a) M. Wuthrich, G. S. Deepe, Jr. and B. Klein, \textit{Annu. Rev. Immunol.},
2012, 30, 115; b) S. E. Hardison and G. D. Brown, \textit{Nat. Immunol.},
2012, 13, 817; c) M. B. Richardson and S. J. Williams, \textit{Front. Immunol.},
2014, 5, 288.
2 E. Ishikawa, T. Ishikawa, Y. S. Morita, K. Toyonaga, H. Yamada, O.
Takeuchi, T. Kinoshita, S. Akira, Y. Yoshikai and S. Yamasaki, \textit{J. Exp.
Med.}, 2009, 206, 2879.
3 H. Schoenen, B. Bodendorfer, K. Hitchens, S. Manzanero, K.
Werninghaus, F. Nimmerjahn, E. M. Agger, S. Stenger, P. Andersen, J.
Ruland, G. D. Brown, C. Wells and R. Lang, \textit{J. Immunol.}, 2010, 184,
2756.
5 K. Werninghaus, A. Babia, O. Gross, C. Holsch, H. Dietrich, E. M.
Agger, J. Mages, A. Mocsai, H. Schoenen, K. Finger, F. Nimmerjahn, G.
D. Brown, C. Kirschning, A. Heit, P. Andersen, H. Wagner, J. Ruland
6 S. Yamasaki, E. Ishikawa, M. Sakuma, H. Hara, K. Ogata and T. Saito,
7 C. Desel, K. Werninghaus, M. Ritter, K. Jozefowski, J. Wenzel, N.
Russkamp, U. Schleicher, D. Christensen, S. Wirtz, C. Kirschning, E. M.
8 B. L. Stocker, A. Khan, S. H. Chee, F. Kamena and M. S. Timmer,
9 A. Furukawa, J. Kamishikiryo, D. Mori, K. Toyonaga, Y. Okabe, A.
Natl. Acad. Sci. USA}, 2013, 110, 17438.
10 H. Feinberg, S. A. Jegouzo, T. J. Rowntree, Y. Guan, M. A. Brash, M.
Taylor, W. I. Weis and K. Drickamer, \textit{J. Biol. Chem.}, 2013, 288,
28457.
11 Y. Hattori, D. Morita, N. Fujiwara, D. Mori, T. Nakamura, H.
Harashima, S. Yamasaki and M. Sugita, \textit{J. Biol. Chem.}, 2014, 289,
15405.
12 A. A. Khan, S. H. Chee, R. J. McLaughlin, J. L. Harper, F. Kamena, M.
Little is known about the substrate specificity of the porcine trehalase used in the study by Ishikawa et al. (Ref. 2). However, a trehalase of *Mycobacterium smegmatis* was inactive on TDM. See: J. D. Carroll, I. Pastuszak, V. K. Edavanya, Y. T. Pan and A. D. Elbein, *FEBS J.*, 2007, 274, 1701.