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The first unique synthetic mycobacterial cord factors

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ABSTRACT

Synthetic mycolic acids matching the overall structure of the major α- and methoxy-mycolic acids of *Mycobacterium tuberculosis* are coupled to trehalose to generate the corresponding synthetic trehalose dimycolate (TDM; cord factor) and trehalose monomycolate (TMM).

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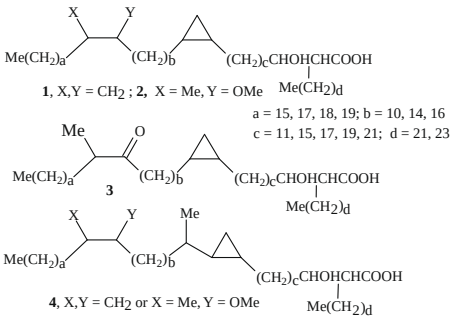
Mycobacteria are unusual in that they contain extremely long-chain β-hydroxy acids, the mycolic acids (MAs). MAs are present in a membrane-bound form, but also as sugar esters including trehalose-6,6′-dimycolates (TDMs, 'cord factors') and trehalose monomycolates (TMMs),[1,2] and as free MAs.[3] Each mycobacterium normally contains several homologues of a number of the classes of MA (Scheme 1), such as α-MA (**1**), methoxy-MA (**2**) and keto-MA (**3**) containing a *cis*-cyclopropane or an α-methyl-*trans*-cyclopropane, for example, **4**.[4] An early Letter leading to an understanding of the chemical structure of these classes was published in this journal in 1966.[5]

Analysis of TMM by MALDI mass spectrometry has been reported.[6] TMM from *Mycobacterium tuberculosis* showed odd-carbon-numbered monocyclopropanoic (or monoenoic) α-mycolates, dicyclopropanoic mycolates, ranging from C-75 to C-85, odd- and even-carbon-numbered methoxymycolates ranging from C-83 to C-94 and even- and odd-carbon-numbered keto-mycolates ranging from C-83 to C-90. In contrast, TMM from *Mycobacterium bovis* (wild strain and Bacillus Calmette-Guerin (BCG) sub-strains) possessed even-carbon-numbered dicyclopropanoic α-mycolates while the BCG Connaught strain lacked methoxymycolates almost completely.[6] The analysis of TDM is more complex, where various combinations of the two MAs bonded to trehalose can lead to a large number of individual structures.[7]

MA-containing glycolipids, in particular TDM, show a number of immunomodifying effects.[8] They stimulate innate, early adaptive and both humoral and cellular adaptive immunity. Most functions can be associated with their ability to induce a range of chemokines (MCP-1, MIP-1α and IL-8) and cytokines (e.g., IL-12, interferon-γ (IFN-γ), TNF-α, IL-4, IL-6 and IL-10).[8] TDM mediates trafficking events during myco-bacterial infection of murine macrophages,[9] and induces cytokine message and protein expression during lung granuloma formation.[10–13] It enhances neovascularisation through growth factor production by neutrophils and macrophages.[14] Intraperitoneal TDM treatment of mice inoculated with encephalomyocarditis virus restricts viral growth, which is correlated to IFN production prior to infection, pointing to the role of IFN-a/3 production prior to infection in the antiviral activities of TDM and, more generally, in the outcome of viral infection.[15] Besides these innate immune activities, TDM, but not a similar glycolipid without MA, also indirectly promotes adaptive immune responses. A strong release of IFN-γ and expansion of natural killer cells lead to macrophages activated for antigen presentation to T lymphocytes.[16]

The diverse immune activities of TDM and TMM indicate multiple biomedical applications. Thus they show positive effects against a range of cancers,[17,18] and may be of relevance for wound healing and hair growth.[19] A Japanese Patent describes the use of a keratinocyte growth-regulating agent which is a sugar ester of MA extracted from cultured microbial cells.[20] Sugar esters of simpler long-chain fatty acids have been claimed to reduce skin changes during ageing.[21] TDM enhances non-specific resistance to influenza virus infection,[22,23] and against infection with *Salmonella typhi* and *Salmonella typhimurium* in mice.[24] Finally, cord factors enriched in particular classes of MA show differential antigenic



Scheme 1. Typical mycolic acids.

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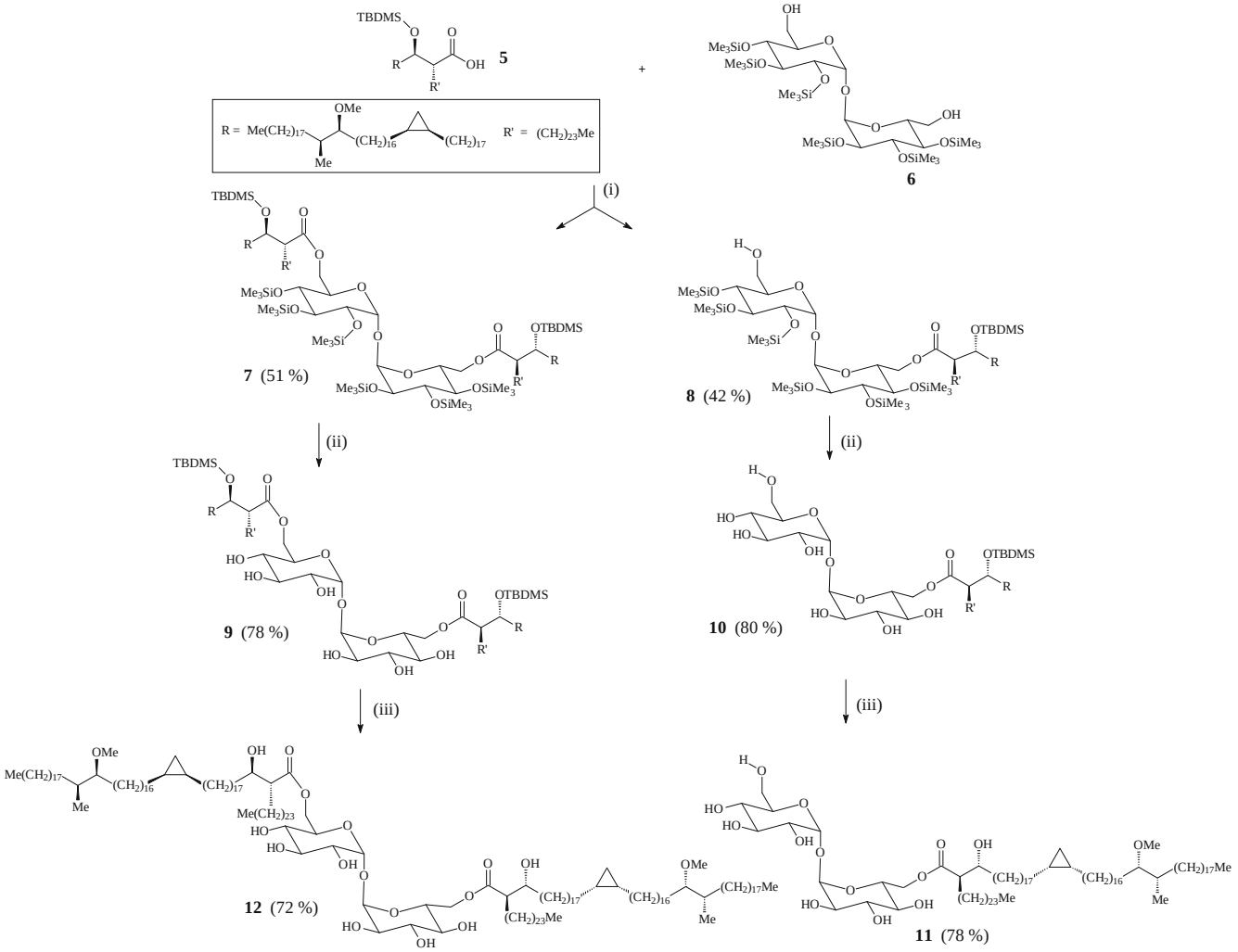
activity towards antibodies of *M. tuberculosis* and *Mycobacterium avium* and have found use in diagnostic assays for the respective diseases.[25–27]

Several methods have been reported by which 'semi-synthetic' cord factors can be reconstituted from mixtures of naturally derived MA, re-forming the sugar esters in a number of ways.[28–31] The combination of a cationic surfactant and a model synthetic 'TDM' made from trehalose and behenic acid proved to be an efficient adjuvant for tuberculosis subunit vaccines.[32] Recently the synthesis of a stereo-defined trehalose ester of a model corynomycolic acid has been reported.[33] However, it is known that the detailed structure of the MAs in TDM affects their biological properties; thus, *trans*-cyclopropanated MAs suppress *M. tuberculosis*-induced inflammation and virulence.[34,35] The synthesis of unique molecules of TDM containing complete MAs matching those present in Nature therefore offers a unique possibility to identify whether the various biological effects described above are selectively caused by specific TDM molecules.

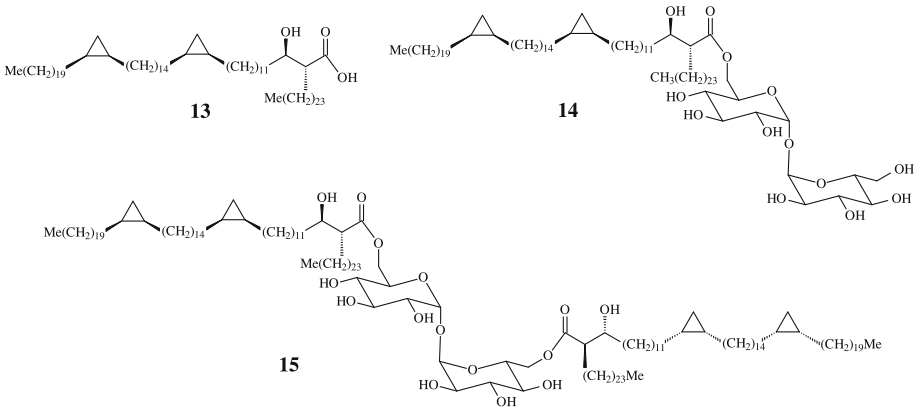
We have recently reported the synthesis of a number of MAs that either are identical to the major isomers of α-, methoxy-, keto- and hydroxy-MAs of *M. tuberculosis*, or differ only in their stereochemistry (in some cases this remains to be determined for the natural materials).[36–40] We now report the conversions of two of these synthetic MAs into the corresponding TDM and TMM.

The protected methoxy-MA **5** (Scheme 2) was prepared from the corresponding hydroxy acid by reaction with an excess of *tert*-butyldimethylsilyl chloride and imidazole in the presence of 4-dimethylaminopyridine for 24 h at 70 °C, followed by hydrolysis of the TBDMS ester on the acid group using potassium carbonate in THF–water–methanol (10:1:1) and then acidification with $KHSO_4$.[28]

Compound **5** was coupled to hexatrimethylsilyl trehalose (**6**) using 1-(3-dimethylaminopropyl-3-ethylcarbodiimide hydrochloride, 4-dimethylaminopyridine and 4 Å molecular sieves in dichloromethane for six days at ambient temperature. This gave the protected TDM **7** (51%) and the protected TMM **8** (42%). Deprotection of **7** and **8**, respectively, using tetrabutylammonium fluoride for 1 h at 5 °C in dry tetrahydrofuran removed all the trimethylsilyl groups and led to the TBDMS-protected TDM **9** and TMM **10**; these showed one acetal carbon signal and two acetal carbon signals, respectively, by ^{13}C NMR. Removal of the TBDMS groups from each of these released the corresponding unprotected molecules **11** and **12** (Scheme 2). The TMM **11** was characterised by MALDI-MS and by proton and ^{13}C NMR;[41] in the former, two signals were seen for the acetal hydrogens (δ 5.07, 5.02).[41] The TDM **12** also showed the correct mass ion,[42] but in this case there was a single acetal signal in the proton NMR, and a single acetal signal and only eight other signals for the carbons adjacent to oxygen in the ^{13}C NMR.



Scheme 2. Reagents and conditions: (i) EDCI, 4-DMAP, CH_2Cl_2, 4 Å MS, 6 d, rt; (ii) TBAF, THF, 5 °C, 1 h; (iii) pyridine, THF, HF–pyridine complex, 43 °C, 17 h, then neutralise with aq $NaHCO_3$.



Scheme 3. TMM **14** and TDM **15** from synthetic α-mycolic acid **13**.

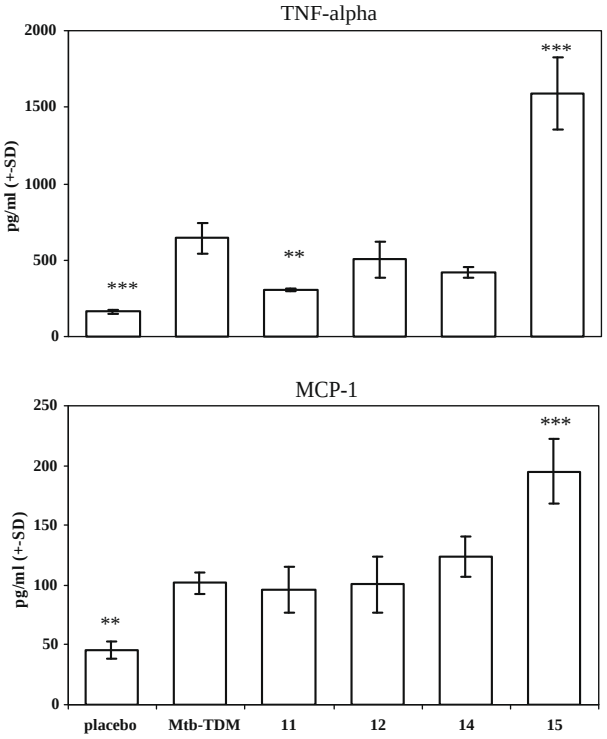


Figure 1. TNF-alpha and MCP-1 assays for commercial TDM (Mtb-TDM) compared to synthetic TMMs (**11**, **14**) and TDMs (**12**, **15**).

In the same way, the synthetic α-MA **13** was converted into the TMM **14**[43] and TDM **15**[44] (Scheme 3).

These, and other synthetic TDM and TMM species, prepared in a similar manner, are currently being tested to determine whether the potent biological effects outlined above for complex natural mixtures can be separated into effects characteristic of individual unique molecular species. Preliminary results show the alpha-TDM **15** to be about three times more effective than a commercial sample of TDM in stimulating the production of the pleiotropic inflammatory cytokine TNF-alpha in mouse RAW264.7 macrophages, while the other three synthetic materials were somewhat less effective (Fig. 1).[45] TDM **15** was also twice as effective in raising the level of the immunoregulatory chemokine MCP-1 as was a commercial TDM sample.[45]

Acknowledgements

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41. Compound **11**, a colourless solid $[\alpha]_D^{26}$ +47.8 (*c* 0.49, $CHCl_3$) {Found $[M+Na]^+$: 1601.39; $C_{97}H_{188}O_{14}Na$ requires: 1601.39}; δ_H (500 MHz, $CDCl_3$+a few drops of CD_3OD): 5.07 (1H, br s), 5.02 (1H, br s), 4.62 (1H, br d, *J* 10.7 Hz), 4.17 (1H, br s), 3.99 (1H, br m), 3.87–3.81 (4H, m), 3.64–3.61 (2H, m), 3.54 (1H, br d, *J* 9.5 Hz), 3.48 (1H, br d, *J* 9.75 Hz), 3.32–3.27 (4H, including s (3H) at δ 3.30), 3.23 (1H, br t, *J* 9.45 Hz), 2.93 (1H, m), 2.36 (1H, m), 1.65–1.50 (2H, m), 1.45–1.02 (153H, m), 0.83 (6H, t, *J* 7 Hz), 0.80 (3H, d, *J* 7 Hz), 0.63–0.55 (2H, m), 0.51 (1H, dt, *J* 3.75, 7.85 Hz), −0.38 (1H, br q, *J* 5.1 Hz); δ_C: 175.5, 94.4, 85.5, 72.6 (broad), 72.4, 72.3, 71.4, 71.2, 71.0, 70.9, 70.0, 64.2, 62.1, 57.6, 52.3, 35.3, 34.6, 32.3, 31.8, 30.4, 30.1, 29.85, 29.8, 29.7, 29.6, 29.55, 29.45, 29.35, 29.2, 28.6, 27.4, 27.2, 26.0, 25.1, 22.6, 15.6, 14.7, 13.9, 10.8; ν_{max}: 3349, 2919, 2850, 1719, 1467, 992 cm^{-1}.
42. Compound **12**, a colourless solid, mp 42–44 °C $[\alpha]_D^{26}$ +31.4 (*c* 0.84, $CHCl_3$) {Found $[M+Na]^+$: 2837.6600; $C_{182}H_{354}O_{17}Na$ requires: 2837.7441}; δ_H (500 MHz, $CDCl_3$ CD_3OD): 4.93 (2H, d, *J* 3.15 Hz), 4.56 (2H, br d, *J* 11.05 Hz), 4.15 (2H, br t, *J* 9.15 Hz), 3.93 (2H, br q, *J* 7.25 Hz), 3.71 (2H, t, *J* 9.5 Hz), 3.58 (2H, br, m), 3.42 (2H, dd, *J* 3.2, 9.75 Hz), 3.25 (6H, s), 3.17 (2H, t, *J* 9.45 Hz), 2.89 (2H, br pent, *J* 4.4 Hz), 2.35 (2H, m), 1.57–0.98 (302H, m), 0.78 (12H, t, *J* 7 Hz), 0.76 (6H, d, *J* 7 Hz), 0.6–0.52 (4H, m), 0.47 (2H, dt, *J* 4.1, 8.2 Hz), −0.43 (2H, br q, *J* 5.05 Hz); δ_C: 175.4, 94.7, 85.5, 72.4, 71.15, 71.1, 69.7, 64.1, 57.4, 52.2, 35.2, 34.6, 32.2, 31.7, 30.3, 30.02, 30.01, 29.75, 29.7, 29.6, 29.55, 29.5, 29.48, 29.44, 29.35, 29.25, 29.15, 28.5, 27.3, 27.1, 25.9, 25.1, 22.5, 15.6, 14.6, 13.8, 10.7; ν_{max}: 3362, 2920, 2851, 1722, 1467, 1100 cm^{-1}.
43. Compound **14**, a white solid $\{[\alpha]_D^{26}$ +41.53, (*c* 1.83, $CHCl_3)\}$ {Found $[M+Na]^+$: 1485.14; $C_{90}H_{172}O_{13}Na$ requires: 1485.31}; δ_H (500 MHz, $CDCl_3$ + CD_3OD): 5.11 (1H, br s), 5.07 (1H, br s), 4.62 (1H, br d, *J* 8.2 Hz), 4.13 (1H, br m), 4.06 (1H, br m), 3.92–3.83 (5H, m), 3.58 (1H, br d, *J* 7.5 Hz), 3.53 (1H, br d, *J* 8.85 Hz), 3.36 (2H, t, *J* 6.5 Hz), 2.69 (1H, br, s), 2.41–2.39 (1H, br m), 1.72 (1H, m), 1.61 (3H, m), 1.50–1.11 (143H, m), 0.87–0.84 (6H, t, *J* 7 Hz), 0.62 (1H, m), 0.56–0.51 (1H, dt, *J* 4.1, 8.2 Hz), −0.34–0.37 (1H, q, *J* 5 Hz); δ_C: 175.5, 94.1, 72.6, 72.5, 72.3, 71.4, 70.9, 70.1, 64.1, 62.1, 58.8, 52.1, 31.9, 30.2, 30.2, 30.15, 29.8, 29.6, 29.59, 29.54, 29.4, 29.3, 28.7, 28.6, 23.9, 22.6, 19.6, 15.7, 15.68, 14.0, 13.5, 10.9, 10.8; ν_{max}: 3356, 2919, 2850, 1728, 1468, 1148 cm^{-1}.
44. Compound **15**, a colourless solid, mp 44–46 °C $\{[\alpha]_D^{26}$ +27.9 (*c* 2.2, $CHCl_3)\}$ {Found $[M+Na]^+$: 2604.7600; $C_{168}H_{322}O_{15}Na$ requires: 2605.40}; δ_H (500 MHz, $CDCl_3$ + CD_3OD): 4.98 (2H, br d, *J* 3.75 Hz), 4.60 (2H, br d, *J* 11.35 Hz), 4.16 (2H, br t, *J* 9.45 Hz), 4.00 (2H, br d, *J* 7.6, 11.65 Hz), 3.73 (2H, br t, *J* 9.45), 3.64–3.60 (2H, m), 3.46 (2H, br d, *J* 3.45, 9.75 Hz), 3.21 (2H, br t, *J* 9.75 Hz), 2.39–2.34 (2H, br m), 1.38–1.05 (290H, m), 0.83 (6H, t, *J* 6.65 Hz), 0.62–0.57 (4H, m), 0.52 (2H, dt, *J* 4.1, 8.2 Hz), −0.36–0.39 (2H, br q, *J* 5.1 Hz); δ_C: 175.4, 94.9, 72.5, 72.45, 71.3, 71.2, 69.8, 64.4, 52.2, 34.7, 31.8, 30.1, 30.08, 29.7, 29.6, 29.55, 29.4, 29.3, 29.2, 28.6, 27.2, 25.1, 22.5, 15.65, 13.9, 10.8, 0.8; ν_{max}: 3391, 2918, 2850, 1730, 1467, 1260, 1020, 800 cm^{-1}.
45. The mouse macrophage cell line RAW264.7 was stimulated with 10 μg/well of each compound in quadruplicate in a 24-well cell culture plate (*i*-PrOH method).[35] After 20 h the culture medium was analysed for cytokine and chemokine content with the Mouse Inflammation Cytometric Bead Array kit (BD) on a FACScalibur. The activity was compared to that of a commercial sample of *M. tuberculosis* TDM (Sigma). Asterisks indicate significance of the differences in mean as compared to the Mtb-TDM group, determined by Turkey's multiple comparison test ($^{**}p < 0.01$, $^{***}p < 0.001$).