Detection of Trehalose Monomycolate in *Mycobacterium leprae* Grown in Armadillo Tissues

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Trehalose-6-monomycolate (TMM) was isolated from the lipids of armadillo-derived *Mycobacterium leprae*. Only meagre amounts of this glycolipid were recovered, but its structure was unequivocally established. Only α-mycolates were detected in the TMM by 252Cf plasma desorption mass spectrometry. Electron impact mass spectrometry showed the alpha branch to be principally C20. Trehalose dimycolate (cord factor) was not detectable. Since we have also found TMM in *M. lepraemurium* and in every *Mycobacterium* species so far examined, we suggest that this glycolipid is truly ubiquitous amongst mycobacteria.

INTRODUCTION

Mycolate esters of trehalose should be ubiquitous amongst *Mycobacterium* species. The thesis has its origin in studies by Walker *et al.* (1973) on the biosynthesis of corynomycolic acid by *Corynebacterium diphtheriae*. This low-molecular-mass (32-carbon) α-branched β-hydroxy acid has been a useful model (Gastambide-Odier & Lederer, 1964) for studying late steps in the biosynthesis of the much more complex mycolic acids of mycobacteria (60 to 90 carbons), which are also α-branched, β-hydroxy acids (for reviews see Goren & Brennan, 1979; Minnikin, 1982; Takayama & Qureshi, 1984). According to Walker *et al.* (1973), a cell-free extract from *C. diphtheriae* condensed two molecules of [1-14C]palmitate via a Claisen-type condensation to produce 2-tetradecyl-3-keto-octadecanoic acid, which they reduced chemically to a mixture of erythro and threo corynomycolic acids:

<table>
<thead>
<tr>
<th>C15H31COOH</th>
<th>O</th>
<th>C</th>
<th>CH</th>
<th>C-OH</th>
<th>NaBH4</th>
<th>C15H31-OH(CH)C-CH-C-OH</th>
<th>C14H29</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C15H31</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>erythro/threo corynomycolic acid</td>
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</table>

On the other hand, when *C. diphtheriae* in culture condensed the labelled palmitate, the intermediate keto acid was immediately incorporated into a 6-monoester of trehalose, which was subsequently reduced biologically to trehalose-6-corynomycolate (Promé *et al.*, 1974; Ahibo-Coffy *et al.*, 1978). It seems likely that this sequence is also valid for mycobacteria.

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Abbreviations: MAT, trehalose-6-monooacetate-6'-monomycolate; TDM, trehalose-6,6'-dimycolate (cord factor); TMM, trehalose-6-monomycolate; TMS, O-trimethylsilyl.
Accordingly the completely assembled carbon skeleton of mycobacterial mycolic acid might also be expected to appear immediately as a trehalose derivative, potentially the precursor of trehalose-6-mycolate (TMM) (Kato et al., 1969; Bruneteau & Michel, 1968; Vilkas & Markovits, 1968). One may speculate from the studies just reviewed that the principal function of trehalose mycolates is to serve, either as such or as intermediates, to carry newly synthesized mycolic acid for transfer to growing cell wall.

Indeed, important evidence in support of a trehalose mycolate 'carrier', potentially implicating trehalose-6-monoacetate-6-monomycolate ('MAT') in this function was described by Takayama & Armstrong (1976). Mycobacterium tuberculosis H37Ra incorporated $^{14}$C-acetate to produce MAT labelled in the mycolate moiety. After a chase with unlabelled acetate the radiolabel of this glycolipid declined with time and appeared instead in cell-wall-associated mycolate. It seems reasonable to anticipate, therefore, that all mycobacteria synthesize mycolic acid and incorporate it into cell wall via trehalose mycolate intermediates.

TMM was recently shown to be a direct precursor of TDM (Kilburn et al., 1982), and TDM has already been found in Mycobacterium leprae from bacteria grown in vivo (Goren et al., 1979) as well as from those cultured in cell-free medium (Nakamura et al., 1984). In anticipation that TMM might be the central substance leading from newly synthesized mycolic acid to cell wall and to TDM, our efforts targeted on finding and identifying these extractable glycolipids in Mycobacterium leprae. TMM had thus far evidently not been sought, whilst TDM had heretofore eluded detection in only this Mycobacterium species (Minnikin et al., 1985). These glycolipids therefore represented a 'missing link', whose discovery could nevertheless be confidently expected based on the premises developed above.

METHODS

Lipids. A preparation designated 'crude M. leprae lipids' was kindly supplied to us by Dr P. J. Brennan. It was obtained from purified M. leprae recovered from irradiated infected armadillo livers (Draper, 1979; Hunter & Brennan, 1981). Lyophilized bacilli were extracted with 2:1 (v/v) CHCl$_3$/CH$_3$OH at 50$^\circ$C for 15–18 h. Partitioning according to Folch et al. (1957) and recovery of the material from the CHCl$_3$ phase afforded the 'crude' product. Dr Philip Draper (National Institute for Medical Research, Mill Hill, London, UK) also kindly gave us 9.5 mg of 'wall lipids' isolated by 1% (v/v) CHCl$_3$/CH$_3$OH extraction of purified cell walls from M. leprae (Draper, 1976).

Column chromatography and TLC. The 'crude M. leprae lipids' (see above) were subjected to column chromatography to seek enrichment of TMM or TDM; this was successful only with this sample. Short columns packed with Whatman DE23 DEAE-cellulose and CF1 long-fibre cellulose (1:2, w/w) were used in the neutralized acetate form (Goren, 1970a). As summarized in Fig. 1, 81 mg of the lipids were loaded in and washed with diethyl ether, and were eluted with diethyl ether/methanol (70:30, v/v). Fractions were monitored by TLC, and TMM-enriched fractions were combined for further enrichment. Sybron-Brinkmann Sil G-25 plates were used for analytical TLC. Plates were sprayed with 60% (w/w) H$_2$SO$_4$ containing 0.01% orcinol and heated at 130$^\circ$C for 4–5 min. TMM and TDM spots assume a characteristic clean blue-grey colour in this treatment (Dhariwal et al., 1984). Samples of synthetized TMM (Liav & Goren, 1984) and TDM (Liav & Goren, 1980) were used as markers.

Preparative TLC of the considerably enriched material obtained from the DEAE-cellulose chromatographies was done on Sybron-Brinkmann 5 cm plates, developed successively in three solvent systems as described by P. Draper (personal communication): chloroform/methanol (90:10, v/v) 15 cm; chloroform/methanol/H$_2$O (14:6:1, by vol.) 10 cm; and hexane/ether/acetic acid (80:18:2, by vol.) to the top of the plate (air dried after each development). The region containing TMM, visualized with iodine, was recovered and extracted with chloroform/methanol/H$_2$O (80:20:0.5, by vol.) and the recovered lipids were re-extracted into wet diethyl ether. As judged from its IR spectrum (see below) the recovered TMM still contained some impurity, partially removed by extracting with hexane and then with CH$_3$OH. The methanol-insoluble lipids (about 400 mg) appeared to be almost pure TMM. For IR examination, neat samples of lipids were deposited on a NaCl microplate, and examined in a Beckman IR-9 spectrophotometer with beam condenser. The IR spectra of TMM and TDM are...
Very similar and possess unique features described below (Noll & Bloch, 1955; Noll et al., 1956; Goren & Brokl, 1974).

**HPLC.** To remove stubbornly persistent contaminants from the isolated TMM, HPLC separation was kindly done for us by Dr K. Takayama and colleagues, who had earlier established the conditions for separating and isolating small amounts of TMM and TDM from cell-free reaction systems. The apparatus and conditions for further purifying about 500 pg of our nearly purified TMM were exactly as described before (Kilburn et al., 1982); semi-synthetic TMM was used to calibrate the system.

**Mass spectrometry.** \(^{252}\text{Cf}\) plasma desorption mass spectra were obtained on an instrument (see Macfarlane, 1983) which was built for the National Institutes of Health by Professor R. Macfarlane of Texas A&M, and subsequently modified by L. Pannell to change samples automatically. Samples dissolved in polar solvents were applied to the conductive surface of an aluminized Mylar film in a sample holder either by evaporation or electrospraying (see above reference). The sample holder was then placed (through a vacuum lock) in front of a \(^{252}\text{Cf}\) source [about 10 \(\mu\text{Ci}\) (370 kBq)]. The chamber is maintained at about 10 \(^{-6}\) Torr (~1 mPa). Very high energy particles from the Cf source (100 MeV) impinge on the sample to generate a plasma containing desorbed molecules with attached sodium cations and these are accelerated down a 42 cm flight tube by a 10 kV grid. Flight times, which are proportional to the square root of the mass are stored in a computer and converted into masses. Each such ion is collected and added to others in the same channel to provide the mass spectrum. This mode of operation is useful principally to determine the molecular masses of the compounds under investigation.

In contrast, electron impact mass spectrometry with high temperature of the probe to volatilize the sample is useful for studying much less polar substances, i.e. lipid esters and permethylation products. It gives rise to fragments whose composition provides data about the intimate structure of the parent molecule (see, for example, Goren & Brennan, 1979).

**Permethylation analysis.** A portion of almost pure TMM from \(M.\ leprae\) was used for permethylation analysis to establish the position of the mycolate ester. In this technique, the free hydroxyl groups in the acylated trehalose are methylated, the product is hydrolysed with base to free the esterified hydroxyl(s) and the resulting methylated sugar is separated from the waxy mycolic acid and identified (see Noll et al., 1956; Goren, 1970b). For the permethylation about 200 \(\mu\)g lipid was dissolved in 0.3 ml methylene chloride; 50 \(\mu\)l 2,6-di-tert-butyl pyridine and 50 \(\mu\)l methyl trifluoromethanesulphonate were added and the mixture was held overnight in a bath at 60 °C, under reflux. The mixture was evaporated under \(N_2\), dissolved in ethyl acetate, washed with dilute HCl, aqueous
bicarbonate and H$_2$O, and the product was recovered. Our recent experiences with this elegant method described by Arnarp & Lööngren (1978) prove it to give high levels of methylation without inducing either hydrolysis or acyl migration (Liav & Goren, 1986).

Identification of the permethylated carbohydrate. (a) Alkaline hydrolysis of the permethylated TMM and separation of the methylated sugar from the K-mycolate were done as described by Noll et al. (1956). The methylated trehalose, dissolved in aqueous methanol, was then deionized with Bio-Rad mixed-bed resins and recovered.

(b) Acid hydrolysis. A portion of the (hepta-0-methyl) trehalose was dissolved in about 0.3 ml 2 M-HCl, sealed in a small tube and heated at 95 °C for 18 h to hydrolyse the methylated sugar. The aqueous HCl was gently evaporated at about 40–50 °C with a stream of N$_2$; the residue was deionized after dissolving in water and taken to dryness again.

(a) GLC was used to identify both the methylated trehalose and the scission products obtained from it by acid hydrolysis. The methylated trehalose was examined without derivatization by GLC (2 mm × 183 cm column of SE30 at 200 °C, N$_2$ carrier gas at 30 ml min$^{-1}$) and compared with an authentic sample of 2,3,4,6,2',3',4'-hepta-O-methyl trehalose (see Kato & Maeda, 1974; Goren et al., 1976). After acid hydrolysis (as above) the products [as the O-trimethylsilyl (TMS) derivatives] were also examined by GLC (SE30, 125 °C) using authentic TMS derivatives of 2,3,4-tri-O-methyl glucose and of 2,3,4,6-tetra-O-methyl glucose for comparison (Goren et al., 1976; Liav & Goren, 1986).

RESULTS

Preliminary examination of P. Draper's 'wall lipids' (see Methods) by TLC gave no evidence that these contained either TDM or TMM. [We did not search for MAT, but having recently synthesized this rare diester (Liav & Goren, 1986) we plan in the future to search for evidence of MAT amongst various lipid extracts.] The more abundant 'crude M. leprae lipids' contained detectable amounts of what appeared to be TMM, but not of TDM.

Fig. 1 summarizes the procedures detailed in Methods for isolation of the 'enriched TMM'. Its IR spectrum is given in Fig. 2, in which the presumed TMM from _M. leprae_ is compared with a like material obtained from crude lipids (kindly given to us by Dr P. Draper; see Draper & Rees, 1973, for their isolation) of _in vivo_ grown (mice) _M. lepraemurium_ (K. Dhariwal & M. Goren, unpublished results) and with semi-synthetic TMM. Clearly all of the absorption frequencies characteristic of trehalose monomycolate (arrowed) are found in the two products from the _in vivo_-derived bacteria: 3080 cm$^{-1}$ [cyclopropane of the 'alpha-type' mycolic acids (Gastambide-Odier et al., 1964)]; 808 cm$^{-1}$ [a weak band specifically associated with trehalose (Goren,
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Fig. 3. TLC of TMM samples in CHCl₃/CH₃OH/H₂O (75:25:4, by vol.): (a) semi-synthetic, (b) from M. leprae, (c) from M. lepraemurium and (d) from M. microti. The spots were visualized by spraying the plate with 60% (w/w) H₂SO₄ containing 0.01% orcinol and heating at 130 °C for 4–5 min. TMM assumes a characteristic blue-grey colour after this treatment.

Fig. 4. HPLC elution curves of TMM samples: (a) semi-synthetic and (b) from M. leprae. M. leprae TMM prepared as outlined in Fig. 1 was purified further on a Waters Associates Radial-pak C₁₈ cartridge using a linear gradient of 0–60% hexane/2-propanol (2:1, v/v) in 2-propanol/water (9:1, v/v) at a flow rate of 2 ml min⁻¹. Fractions were collected at 1 min intervals after 6 min. The peaks at 25.2 and 26.3 min were the major peaks and nearly correspond to peaks obtained from the semi-synthetic TMM.

1970a); and a succession of about seven peaks between 1000 and 1200 cm⁻¹ highly characteristic of trehalose 6-esters (Goren & Brokol, 1974). The spurious peaks in the region 1200–1400 cm⁻¹ found in the spectrum of the product from M. lepraemurium indicate that it requires further purification. Fig. 3 shows a thin-layer chromatogram which compares the semi-synthetic TMM with the samples derived from M. lepraemurium and M. leprae and with a quite pure TMM that we isolated in abundance from M. microti (K. R. Dhariwal et al., unpublished results).

To obtain a still purer sample, HPLC was carried out on about 400 µg of the M. leprae glycolipid (Fig. 4). The peaks at 25.2 and 26.3 min were very close to the principal peaks obtained for the semi-synthetic TMM sample. The effluents corresponding to the two peaks were combined and used for studying the composition of the mycolate components by electron impact mass spectrometry (see below).

²⁵²Cf plasma desorption mass spectrometry

Application of this technique to the semi-synthetic TMM (Fig. 5) allowed us to establish favourable conditions for obtaining informative spectra. Yet the quality of the spectra depends on variables that are often difficult to control or to recognize: in our interpretation the nature of
the film sample produced by spraying was of critical importance, but was not consistently controllable. For example, successive samples formed from the semi-synthetic TMM in solution in acetone/CH₃OH or in CHCl₃/CH₃OH gave recognizable but nevertheless rather poor spectra during runs lasting 800 min each. In contrast, the spectrum in Fig. 5(a) was obtained during a 30 min scan from a (presumably identical) film also deposited from CHCl₃/CH₃OH solution. With the much scarcer sample from \textit{M. leprae} (also sprayed onto the Mylar from a CHCl₃/CH₃OH solution) the spectrum in Fig. 5(b) was obtained during 5435 min. Nevertheless, it is an unequivocal spectrum. It was the only one successfully obtained with our limited sample.

The scan in Fig. 5(a) shows that the semi-synthetic TMM is characterized by two prominent series of mycolate substituents that are identified as types I and IIa according to the scheme of D. E. Minnikin (Davidson \textit{et al.}, 1982). The series of type I are of the (cis,cis) dicyclopropane types, also referred to as ‘alpha’ mycolates (see below and reviews mentioned earlier). The IIa series (shaded peaks) contain a methoxyl group with an adjacent methyl branch as depicted by the structure

\[
\text{CH₃ OCH₃} \quad \text{CH}_2 \quad \text{OH} \quad \text{O} \\
\text{CH}_3(CH_2)_x-\text{CH}-\text{CH}-(CH_2)_y-\text{CH}-\text{CH}-(CH_2)_z-\text{CH}-\text{CH}-\text{C} \\
\text{C}_{24}\text{H}_{49}
\]

For the peaks at \(m/z\) 1601 and 1629, \(x + y + z = 50, 52\).

The spectrum of the \textit{M. leprae} lipid (Fig. 5b) shows essentially only one principal series of peaks (derived from the sodium-cationized molecules) at \(M + 23 = 1457, 1485, 1513, 1541\) that correspond to trehalose esterified with a type I (‘alpha’) mycolate of the structure

\[
\text{CH}_3-(CH_2)_x-\text{CH}-\text{CH}-(CH_2)_y-\text{CH}-\text{CH}-(CH_2)_z-\text{CH}-\text{CH}-\text{C} \\
\text{C}_{20}\text{H}_{41}
\]
in which $x + y + z = 46, 48, 50, 52$ (principal components 48, 50). The alpha branch is $C_{20}H_{41}$ as described below. These results are in accord with those obtained by Draper et al. (1982) for the type I mycolic acids recovered from defatted cells of (armadillo-grown) *M. leprae*, with principal $x + y + z = 48$. No other peaks definable with confidence can be recognized in our spectrum. *M. leprae* elaborates ketomycolates as well (Draper et al., 1982) but we did not detect them in our TMM.

**Characterization of the mycolate alpha branch: electron impact mass spectrometry**

The *M. leprae* TMM purified by HPLC as described above was directly hydrolysed to provide the free mycolic acid. The methyl ester (obtained with CH$_2$N$_2$) was then examined by electron impact mass spectrometry. A principal spectrum was obtained at a temperature of about 260 °C in which a base (pyrolysis) peak was obtained at $m/z$ 354 with a smaller peak at $m/z$ 382; methylhexacosanoate ($m/z$ 410) was imperceptible. Therefore, the mycolate alpha branch of *M. leprae* TMM is principally a $C_{20}$ constituent, which yields a methyldeicosanoate during the pyrolytic cleavage, with a smaller complement of tetracosanoate generated from a $C_{22}$ branch.

Etemadi (1967) established that under the pyrolytic conditions in the vicinity of the probe holding the sample, methyl mycolates undergo a reverse Claisen reaction in which the alpha branch, the alpha carbon and the carbomethoxy group are eliminated cleanly as a methly ester whose size defines the alpha branch, viz.

$$
\text{OH} \quad \text{O} \\
\text{R-CH-CH-C-OCH}_3 \quad \text{C}_{20}\text{H}_{41}\text{CH}_2\text{-COOCH}_3 \\
\quad \text{methyldeicosanoate ($m/z = 354$)}
$$

Our results are largely in agreement with those reported by Kusaka et al. (1981) (who, however, did not find evidence for a $C_{22}$ α-branch), by Asselineau et al. (1981), and by Draper et al. (1982), in whose studies both the $C_{20}$ and $C_{22}$ α-branches were described.

**Characterization of the sugar–ester bond**

As described in Methods, the TMM of *M. leprae* was permethylated, hydrolysed with alkali and the partially methylated trehalose recovered. By GLC it was found to be identical with authentic 2,3,4,6,2′,3′,4′-hepta-O-methyl trehalose prepared recently from semi-synthetic TMM (Liav & Goren, 1986). Both samples had identical retention times (10.8–10.9 min). Authentic 2,3,4,2′,3′,4′-hexa-O-methyl trehalose (Goren et al., 1976) had a retention time of 15.8 min under the same conditions.

Acid hydrolysis of the hepta-O-methyl trehalose generated the two partially methylated glucose moieties. Their structures were determined by comparing them with authentic 2,3,4-tri- and 2,3,4,6-tetra-O-methyl glucose by GLC of the TMS derivatives. The following data were obtained:

<table>
<thead>
<tr>
<th>Sample (TMS derivatives)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Tetra-O-Me glucose</td>
<td>6.7, 8.2, 8.9*</td>
</tr>
<tr>
<td>2,3,4-Tri-O-Me glucose</td>
<td>6.7, 8.2, 8.9*</td>
</tr>
<tr>
<td>Products from <em>M. leprae</em> TMM</td>
<td>6.7, 8.2, 8.9*</td>
</tr>
</tbody>
</table>

* Anomers resolved.

Taken together with the results of mass spectrometry, these data prove that the glycolipid is a trehalose derivative acylated at a single 6-position with alpha (type I) mycolic acids bearing principally a $C_{20}$ alpha branch, and therefore characteristic of *M. leprae*.

**DISCUSSION**

The sum of our evidence indicates that *M. leprae* synthesizes TMM in vivo. The most convincing evidence derives from the plasma desorption mass spectrum in which nearly exact
molecular masses of the glycolipid are defined, from IR spectrophotometry, from the pattern of the HPLC elution, and from the permethylation studies.

We judge that the maximum amount of TMM that we recovered is probably no more than 400 μg from 1 g (dry wt) of harvested M. leprae. In recent studies of M. microti cultured in vitro we recovered a minimum of 5 mg TMM per g (dry wt) of bacteria and an abundance of TDM as well (unpublished results). The M. leprae lipids, however, contained no trace of TDM that we could detect. It is of course possible that a careful separation of a considerably larger gross lipid sample may allow TDM to be detected and perhaps isolated. We were also unable to detect any sulphatides (‘sulpholipids’) behaving like those of M. tuberculosis (these are also trehalose esters). Minnikin et al. (1985) also reported that their M. leprae sample did not contain either sulpholipids or TDM.

The minuscule quantity of TMM that we were able to recover would suggest that fulfilling of its probable anabolic role in cell wall biosynthesis almost exhausts the amount of this glycolipid that is available to the cell at any given time and, therefore, it may be unavailable for TDM biosynthesis. It is in accord with current observations respecting the precursor/product relationship between TMM and TDM that the cell’s economy hardly allows for any diversion of such an important substance (TMM) into what may be a biosynthetic ‘dead end’. Finally, we suggest that the identification of TMM in M. leprae secures for this glycolipid the distinction of being truly ubiquitous among mycobacteria.

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