Trafficking pathways of mycolic acids: structures, origin, mechanism of formation, and storage form of mycobacteric acids

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Abstract **Mycolic acids, the hallmark of mycobacteria and related bacteria, are major and specific components of their cell envelope and essential for the mycobacterial survival. Mycobacteria contain structurally related long-chain lipids, but the metabolic relationships between these various classes of compounds remain obscure. To address this question a series of C_{35} to C_{54} nonhydroxylated fatty acids (mycobacteric acids), ketones, and alcohols structurally related to the $C_{70–80}$ dicyclopropanated or diethylenic mycolic acids were characterized in three mycobacterial strains and their structures compared. The relationships between these long-chain acids and mycolic acids were established by following the in vivo traffic of ^{14}C labeled α-mycolic acids purified from the same mycobacterial species. The labeling was exclusively found in mycobacteric acids. The mechanism of this degradation was established by incorporation of $^{18}O_2$ into long-chain lipids and shown to consist in the rupture of mycolic acids between carbon 3 and 4 by a Baeyer-Villiger-like reaction. We also demonstrated that mycobacteric acids occur exclusively in the triacylglycerol (TAG) fraction where one molecule of these acids esterifies one of the three hydroxyl groups of glycerol. Altogether, these data suggest that these compounds represent a pathway of metabolic energy that would be used by mycobacteria in particular circumstances.**—Rafidinarivo, E., M-A. Lanéelle, H. Montrozier, P. Valero-Guillén, J. Astola, M. Luquin, J-C. Promé, and M. Daffé. **Trafficking pathways of mycolic acids: structures, origin, mechanism of formation, and storage form of mycobacteric acids. *J. Lipid Res.* 2009.** 50: **477–490.**

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Mycolic acids, 2-alkyl-branched, 3-hydroxylated long-chain fatty acids, represent major (up to 40% of cell wall dry mass) and specific constituents of the envelope of members of the genus *Mycobacterium* (*M.*) that includes several pathogens such as *M. tuberculosis* and *M. leprae*, the causative agents of tuberculosis and leprosy, respectively. These fatty acids play a crucial structural role in the envelope architecture (1–4) and are essential for the mycobacterial growth (5–7). Mycolic acids covalently linked to the cell wall arabinogalactan are organized with other lipids to form an outer barrier with an extremely low fluidity that confers an exceptional low permeability to mycobacteria (8). Trehalose mycolates, found noncovalently attached to the cell wall core, are known to be important for the physiology and virulence of *M. tuberculosis* (3). In addition, the biosynthesis of mycolic acids is the target of several antituberculous drugs, notably isoniazid (2, 3, 9).

Mycolic acids are found in mycobacterial species as a mixture of structurally related molecules that differ from one another by the nature of the chemical groups at the so-called "proximal" and "distal" positions (relative to the carboxyl group) of their main "meromycolic" chain (**Fig. 1**). The least polar α-mycolates is ubiquitous in mycobacteria and consist of $C_{74–82}$ fatty acids that contain two *cis* cyclopropyl groups or *cis*/*trans* double bonds at the proximal and distal positions (Fig. 1); shorter apolar structural analogs ($C_{60–68}$), called α′-mycolates, may occur in some

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Abbreviations: amu, atomic mass unit; BVMO, Baeyer-Villiger monooxygenases; EI, electron impact; MALDI-TOF, MALDI-time-of-flight; MS, mass spectrometry; TAG, triacylglycerol.

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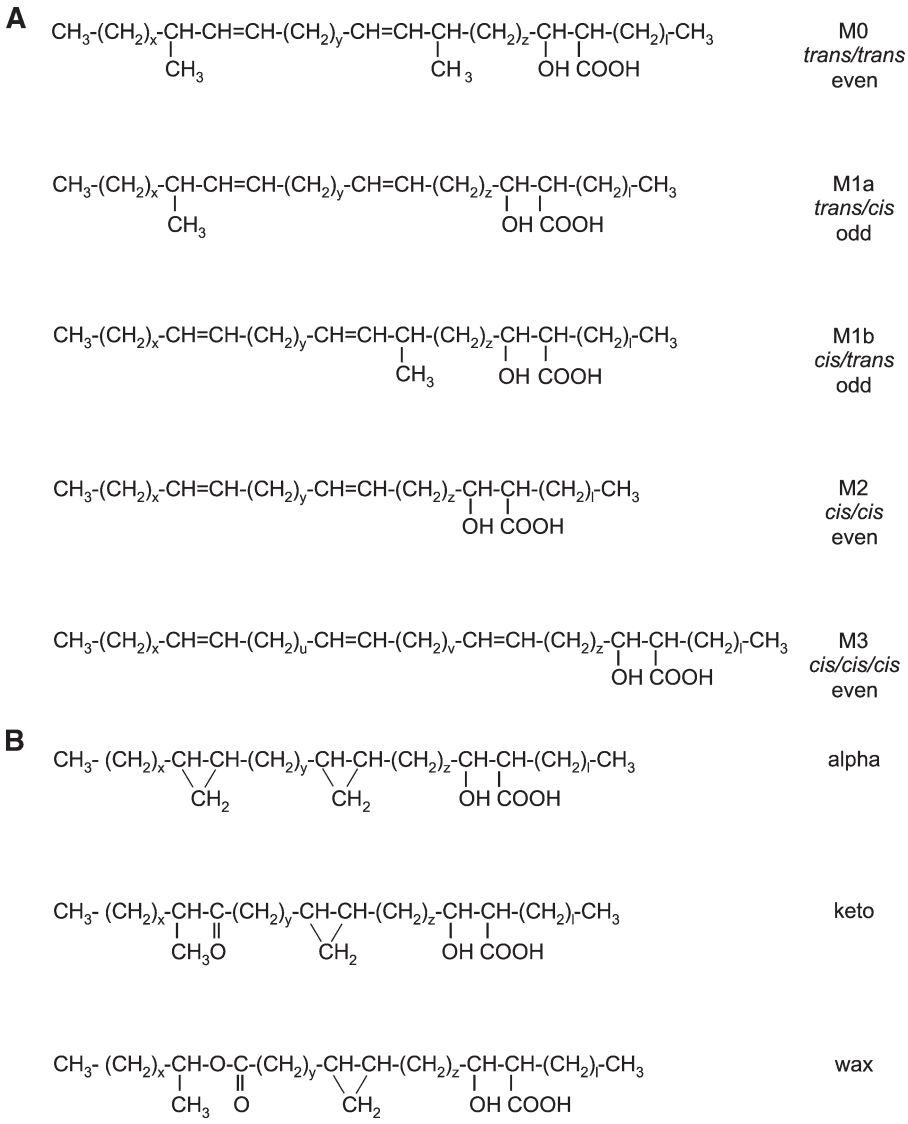

Fig. 1. Structures of the major mycolic acids from *Mycobacterium* (*M.*) *brumae* and *M. fallax* (A) and *M. aurum* (B). *M. brumae* contains four different types of diethylenic mycolates, namely M0 (x = 15; y = 12; z = 17; l = 19), M1a (x = 15; y = 13; z = 17, 19; l = 19), M1b (x = 15; y = 13; z = 15, 17, 19; l = 19), and M2 (x = 13, 15; y = 10, 12, 14; z = 15, 17, 19; l = 19) (28). *M. fallax* also contains four main types of diethylenic mycolates; however, this strain contains M1a (x = 15, 17; y = 15, 17; z = 15, 17, 19; l = 19, 21), M1b (x = 15, 17; y = 15, 17; z = 17; l = 19, 21), and M2 (x = 15,17; y = 14, 16, 18; z = 15, 17, 19; l = 19, 21), and is devoid of M0 but contains M3 (x = 13,15; u = 4; v = 12, 14; z = 15, 17, 19; l = 19, 21). In addition, small amounts of tetraethylenic homologs occur in this species (31). *M. aurum* contains α- (x = 13, 15, 17; y = 12, 14, 16; z = 17, 19; l = 19, 21), keto- and wax-mycolates (x = 13, 15, 17; y + z = 27, 31, 33; l = 19, 21). *cis* (*E*) and *trans* (*Z*) are relative to the configuration of the double bonds. "Odd" and "even" refer to the chain lengths of the mycolates.

rapid-growing species such as *M. smegmatis*. More polar mycolates are also produced by most mycobacteria. These contain an oxygenated function at the distal position, with a methyl group on the vicinal carbon atom, and a *cis* or *trans* (with a methyl group on the vicinal carbon atom) cyclopropyl group or double bond at the proximal position. Depending on the mycobacterial species examined, the oxygenated function may be a methoxy-, a keto-, a hydroxyl-group (in slow-growing species), or an epoxy-ring (in rapid-growing species); in some species, the keto-mycolic acids may be oxidized through a Baeyer-Villiger reaction to yield a wax ester (2, 10). An illustration of the structural diversity of mycolic acids is given by the composition of mycolic acids of the strains under study (i.e., *M. brumae*, *M. aurum*, and *M. fallax*) (Fig. 1).

Long-chain compounds structurally related to mycolic acids are also found in more or less significant amounts in mycobacteria and related genera (e.g., *Nocardia*, *Corynebacterium*); these include long-chain acids, ketones, and alcohols whose general structures are given in **Table 1**. Long-chain C_{40-56} fatty acids that exhibit the structural features of the main chains of mycolic acids have been

TABLE 1. General formulae of long-chain lipids structurally related to mycolic acids and comparison of odd/even ratios of their chain lengths in *Mycobacterium* (*M.*) *fallax*

		Odd/even ratios of compounds		
		M1	M2	M3
Mycolic acids (e)	**R_1-CH_2-CH(OH)**-CH(COOH)R2	0.74	0.30	0.16
Mycolones (a)	**R_1-CH_2-CO**-R_2	0.78	0.25	0.26
Mycolols (d)	**R_1-CH_2-CH(OH)**-R_2	0.75	0.20	0.20
Meroaldehydes	**R_1-CH_2-CHO**	0.74	0.36	0.19
Primary alcohols (f)	**R_1-CH_2OH**	0.23	0.82	0.79
Mycobacteric acids (b)	**R_1-COOH**	0.20	0.73	0.81

M1, M2, and M3 refer to the structural features (numbers and configuration of double bonds) of compounds relative to their putative mycolic acid parents: M1 (two double bonds, mixture of M1a and M1b), M2 (two *cis* double bonds), and M3 (three double bonds) that occur in *M. fallax* (see Fig. 1A); compound M1 is a mixture of 1a (*trans/cis*) and 1b (*cis/trans*). Note that *M. fallax* is devoid of M0. The various types of mycolates from *M. fallax* were separated by argentation TLC as **M1a** and **M1b**, **M2** and **M3** (Fig. 1A). Each class of mycolic acids was then pyrolysed to yield the corresponding meroaldehydes. The same argentation TLC strategy was applied to the minor long-chain compounds (i.e., mycolols, primary alcohols, and mycobacteric acids). After purification, mycolic acids and their structurally related lipids were analyzed and their structures were characterized by mass spectrometry. The calculation of the odd/even (relative to the chain lengths of the molecules) ratios reported was based on the relative abundance of the mass peaks determined for the different homologs. The structural features shared by the various classes of compounds are in bold. Letters into brackets refer to compounds presented in TLC (Fig. 2).

characterized in various mycobacteria and are known as mycobacteric acids (11–15). Their structures have been shown to be related to those of mycolic acids from the same species. For instance, keto and methoxyl groups were shown to occur in mycobacteric acids and mycolic acids isolated from *M. tuberculosis* (11); similarly, keto and wax functions were found in both types of fatty acids isolated from *M. aurum* (16). A series of shorter homologous fatty acids have been also isolated from triacylglycerols (TAGs) of a *Nocardia* species; these consist in C_{35-45} fatty acids containing 3 and 4 double bonds as observed in nocardomycolic acids (17). In addition, long-chain ketones and secondary alcohols parented to mycolic acids have been also found in mycobacteria (e.g., the so-called C_{80} mycolones and mycolols) (18, 19), in nocardia (C_{50-60} nocardones, nocardols) (20–23), and in corynebacteria (16-hentriacontanone or palmitone) (24, 25). Interestingly, shorter-chain secondary alcohols and ketones (hentriacontan-16-ol and palmitone) have also been characterized in nocardia (26, 27). Because of the above-mentioned similar structural features, it is tempting to postulate biosynthetic or catabolic links between mycolic acids and the other long-chain compounds. To address this question, mycolic acids and their related lipids from *M. brumae* and *M. fallax*, two species that contain only one type of mycolates, the so called α-mycolates (Fig. 1) (28, 29), were isolated, purified, and comparatively analyzed by various complementary analytical methods. In addition, the in vivo trafficking of labeled mycolates in *M. aurum*, a species known to metabolize lipids, was investigated to validate the conclusions drawn from the structural findings. Importantly, because the elongation unit in the biosynthesis of mycolates is acetate (C_2), the major homologs contain an even number carbon atoms. The introduction of a methyl branch or a cyclopropyl group results in the production of mycolates possessing an odd number of carbon atoms whereas that of two cyclopropyl groups or two methyl branches does not change the parity. The number of double bonds in mycolates, usually one in oxygenated compounds and two in α-mycolates with the notable exception of *M. fallax*, does not impact on the even carbon number of the chains (Fig. 1). Therefore, the even versus odd ratio of the chain lengths of the structurally related lipids was determinant in the definition of the biosynthetic relationships between the long-chain lipids and the mechanism that governs their catabolism.

EXPERIMENTAL PROCEDURES

Strains and media

The type strain of *M. brumae* Collection Institut Pasteur (CIP) 103465^T (28) was grown in Sauton's medium as surface pellicles at 37°C for 3 weeks. That of *M. fallax* CIP 8139^T (29) was grown in Middlebrook 7H9 medium supplemented with 0.5% casitone and 1% glucose (Difco, Detroit, MI) at 30°C under agitation. *M. aurum* strain A^+ was obtained from Pr H. David (Institut Pasteur, Paris) and was grown on Middlebrook 7H9 medium (Difco) supplemented with 0.5% casitone and glucose 1% at 37°C under agitation.

Lipid extraction and purification from *M. brumae*

Complex lipids were extracted from wet cells with $CHCl_3$/CH_3OH 1:2, 1:1, and 2:1 (v/v), successively. The crude lipid extracts were pooled and then fractionated by column chromatography on Florisil 100–200 mesh equilibrated in petroleum ether, using a gradient of diethyl ether in petroleum ether as eluent. TAGs were eluted with petroleum ether/ether 90:10 (v/v). Free fatty acids were eluted from the column with diethyl ether containing 0.1% acetic acid. The separation was monitored by TLC and infrared spectroscopy for the detection of *trans* double bonds at 965 cm^{-1}.

Saponification of complex lipids

Lipids were saponified using a 5% KOH solution in methanol/benzene 8:2 (v/v) for 7 h under reflux. After acidification of the mixture and extraction of lipids with diethyl ether, the organic phase was dried and treated with diazomethane. The resulting long-chain lipids (i.e., ketones, esters, and alcohols) were

fractionated by column chromatography on Florisil as previously described.

Isolation of lipids from *M. fallax*

Cells (17.5 g dry weight obtained from 10l of cultures) were extracted with $CHCl_3/CH_3OH$ as previously indicated to yield 3.1 g crude lipid extract. After saponification, acidification, and methylation, lipids were fractionated on a Florisil column as previously described, and the separation was monitored by TLC. Mycolones (20 mg), mycobacteric esters (20 mg), mycolols (20 mg), and long-chain primary alcohols (10 mg) were obtained, in addition to α-mycolates (1.7 g) and fatty esters that were the main lipids of the mixture.

TLC

TLC was performed on silica gel coated plates (G-60 0.25 mm thickness, Merck, Darmstadt, Germany) developed with either CH_2Cl_2 or with an appropriate combination of petroleum ether/diethyl ether. Lipids (fatty esters, ketones alcohols, and TAGs) were located by spraying either Rhodamine B (0.01% in sodium dihydrogen phosphate 0.25 M) or molybdophosphoric acid (10% solution in ethanol), followed by charring. Glycolipids were detected with anthrone after elution with $CHCl_3/CH_3OH/H_2O$ 65:25/4 (v/v/v). For preparative TLC, the same solvents were used but the detection was obtained by spraying Rhodamine B and the visualization was performed under the UV light. The compounds were then scraped off the plates and extracted with diethyl ether and the ethereal solution was evaporated to dryness. The stain was eliminated by passing through a Florisil Pasteur pipette (1 cm length) that was irrigated with diethyl ether. Fatty acid methyl esters were further fractionated according to their double bond content on $AgNO_3$-impregnated silica gel TLC plates developed with CH_2Cl_2; visualization was performed by spraying a 0.2% ethanolic dichlorofluorescein solution and the compounds were scraped off the plates and eluted with diethyl ether.

Oxidative cleavage of double bonds

Ethylenic lipids were cleaved by permanganate-periodate oxidation (30, 31). Briefly, lipids (around 20 mg) dissolved in benzene (0.6 ml) were added to a mixture of *t*-butanol (8 ml), aqueous 2.10^{-2} M sodium carbonate (2.4 ml), distilled water (1.2 ml) and periodate permanganate (5.4 ml). This last solution contained 0.1 M sodium periodate and potassium permanganate 2.5 10^{-2} M in water. The oxidation was carried out at 30°C for 2 days with constant shaking. The reaction was stopped by additing sodium metabisulphite until discoloration. The solution was acidified with 20% sulfuric acid, and the resulting oxidation products were extracted with diethyl ether and the solvent evaporated. The crude extract was methylated with diazomethane and purified on preparative TLC with CH_2Cl_2 as developing solvent. Compounds were identified by GC-MS analysis according to their retention times and their fragmentation patterns (32).

Preparation of anilide derivatives of mycobacteric acids

Anilide derivatives were prepared as follows: the carbonyl group was first activated by adding 0.5 ml of a 20% solution of oxalyl chloride (Fluka) in anhydrous benzene to fatty acids (5 mg). The reaction was left at room temperature for 2 h, followed by heating at 50°C for 1 h. After evaporation of the solvent under nitrogen, 0.35 ml of a 20% solution of aniline (Aldrich) in anhydrous benzene (20-fold excess) was added to the acyl chloride. After 1 h at room temperature, the reaction mixture was heated at 70°C for 30 min. The solvent was evaporated, and the residue was dissolved in diethyl ether and the excess of aniline was removed by washing the diethyl ether phase with 1 M HCl. Anilides were analyzed by TLC with hexane/dioxane 9:1 (v/v) as the running solvent and detected with Rhodamine B before high-pressure liquid chromatography.

Preparation of pyrrolidide derivatives of mycobacteric acids

Fatty acid methyl esters (1 mg) were dissolved in 1 ml of freshly distilled pyrrolidine (Aldrich chemicals Co., WI) and 0.1ml acetic acid. The mixture was heated at 100°C for 30 min and cooled to room temperature. The amide so-formed was taken up in $CHCl_3$, and the organic phase was washed with 1 M HCl and water (33). After evaporation, the purity of the derivatives was checked by TLC.

Pyrolysis of mycolic acid methyl esters

The pyrolytic reaction was realized at 300°C under vacuum and the oxidation of the yielded meroaldehydes was performed as previously described (34). The resulting meromycolic acids were methylated and purified by preparative TLC as described for long-chain lipids.

Catalytic hydrogenation of double bonds in long-chain alcohols

Around 5 mg of lipids were dissolved in 1ml of hexane in the presence of 2 mg of platinum oxide. Hydrogenation was carried out under shaking for 2 h at room temperature. The mixture was then filtered off to remove the catalyst. The solvent was evaporated under vacuum. Electron impact (EI)-MS analysis showed that the hydrogenation was complete in these conditions.

Incorporation of ^{14}C acetate in long-chain lipids of *M. brumae*

To a growing culture of *M. brumae*, 1 μCi ml^{-1} of 1-^{14}C acetate (2GBq $mmole^{-1}$) were added. After 90 min, the medium was discarded and 30 ml of fresh Sauton's medium was added. At various time intervals (0, 24, 48, 72, 96, and 120 h) a loopful of cells was collected and saponified. Fatty acids were methylated and the profile of long-chain compounds (alcohols, ketones, and fatty esters) was analyzed by TLC with CH_2Cl_2 as developing solvent. The TLC plates were read using a PhosphoImager after a 48-h exposure.

Preparation of labeled 1-^{14}C mycolic acids of *M. aurum*

One μCi ml^{-1} of [1,2-^{14}C] sodium acetate (50–60 mCi $mmol^{-1}$) was added to a 72-h grown 100-ml culture. After a 90-min growth under shaking, bacteria were harvested by centrifugation and saponified.

Biotransformation of ^{14}C-labeled mycolic acids by *M. aurum*

An ethereal solution of ^{14}C-labeled mycolic acids (0.5 ml, specific radioactivity of 4.10^6 cpm mg^{-1}) was added to a 100 ml culture medium. Bacteria were then inoculated as a 1% suspension after the removal of diethyl ether and collected by centrifugation after a 72-h growth.

Growth under ^{18}O atmosphere

A 4-day culture of *M. fallax* was added to 250 ml medium in a vacuum flask to obtain a 1% cell suspension. Two vacuum 500 ml flasks were used in parallel: one for the assay and the second for the control. Each flask was filled with 250 ml culture medium and inoculated to obtain a 1% cell suspension. After a 5-day growth, the air was partly removed from each flask under a slight vacuum. Approximately 150 ml of $^{18}O_2$ (99% purity) was introduced in the assay flask and the same amount of $^{16}O_2$ was filled in the control flask. Both flasks were hermetically closed and the cultures were allowed to grow at 30°C for 48 h. Bacteria were then collected by

centrifugation and saponified. In order to estimate the amount of ^{18}O uptake, the relative abundances between *m/z* 803 and 805 were more precisely measured by multichannel detection.

Instrumentation

Infrared spectra were recorded using a Perkin-Elmer model FTIR 1600 apparatus. Samples were analyzed as a film between two NaCl discs.

The optical rotations of purified molecules at 589 nm ($[\alpha]_D$) were determined with a Perkin-Elmer model 141 polarimeter. Samples were dissolved in $CHCl_3$ (5 mg ml^{-1}).

Nuclear magnetic resonance (NMR) spectra of the purified samples dissolved in $CDCl_3$ (100% D) were recorded using a Brucker Avance 600 MHz spectrometer equipped with a 5 mm TCi cryoprobe at 298 K. Chemical shifts values were relative to the internal $CHCl_3$ resonance (at 7.27 ppm).

High-pressure liquid chromatography was carried out on a Waters 6000A apparatus equipped with a μ Porasil (0.39 × 30 cm) column for adsorption chromatography; the elution was performed with hexane/dioxane 9/1 (v/v). The flow rate was 0.5 ml min^{-1}, and the detection was followed using an UV detector Waters 450 at 242 nm for chromophoric derivatives. All solvents were degassed by filtration under vacuum.

GC-MS was performed on a Hewlett-Packard 5989A mass selective detector connected to a Hewlett-Packard 5890 gas chromatograph. The temperature of the direct inlet probe was set at 270°C, the source temperature at 300°C, and the electron energy was 70 eV.

EI-MS was performed using a VG AutoSpec (Fison) instrument fitted with an EI ion source. The accelerating voltage was 8 kV, the electron energy was 70 eV, the source temperature was set at 250°C. The temperature of the solid probe was ramped from 100°C to 300°C in 10 min (35). The same instrument (provided with a caesium gun, 25 kV) was employed for fast-atom bombardment-MS in positive and negative modes; the accelerating voltage was 8 kV and the matrix used was *m*-nitrobenzyl alcohol.

MALDI-time of flight (MALDI-TOF) mass spectrometry (MS) was performed on a 4700 Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a Nd:YAG laser YAG (355 nm wave length) operating by pulses of 500 ps with a frequency of 200 Hz. Twenty-five hundred shots were accumulated in positive ion mode, and MS data were acquired using the instrument default calibration. Lipid samples were dissolved in $CHCl_3$ at a concentration of 1 mM, and were directly spotted onto the target plate as 0.5 μl droplets, followed by the addition of 0.5 μl of 2-5-dihydroxybenzoic acid (10 mg ml^{-1}) in $CHCl_3$/CH_3OH (1:1, v/v) (36). Samples were allowed to crystallize at room temperature.

The radioactivity was located using an automatic TLC linear analyzer Berthold LB 2832 or a PhosphoImager.

RESULTS

Mycobacteria contain long-chain lipids whose structures are related to those of mycolic acids. They consist on long-chain fatty acids, primary and secondary alcohols, and ketones (**Fig. 2**) (Table 1). As they are present in small amounts, usually less than 1% of saponified lipids, their detection on TLC is not easy. Accordingly and in order to visualize them and to follow their relationships with mycolic acids, exponentially grown cells of mycobacterial species were labeled with ^{14}C-acetate for 90 min. The medium was then discarded and replaced by a fresh medium and the labeling was followed for various times (0 h, 24 h, 48 h, 72 h, 96 h, and 10 days) by taking off a loopfull of bacteria. After saponification of cells, the resulting lipid extracts were methylated and analyzed by TLC using CH_2Cl_2 as developing solvent. Besides mycolates (compounds **e**, **Fig. 3**) and C_{16-24} fatty esters (compounds **c**), which are the expected major constituents of the mixtures, mycobacteria accumulate substantiate amounts of a variety of long-chain compounds (Fig. 3). Compound **b**, which corresponds to long-chain fatty esters, was also detected after methylation of *M. brumae* native lipids extracted from cells by organic solvents, indicating that these substances exist also as free acids.

Structural features of the long-chain fatty acids from *M. brumae*

To determine the structure of compounds **b**, free fatty acids were isolated by column chromatography of the extractable lipids from *M. brumae*. Following methylation with diazomethane, the main component of this fraction exhibited a mobility higher than that of nonhydroxylated fatty acid methyl esters (compound **c**) as observed for compound **b** on TLC using CH_2Cl_2 as developing solvent. Infrared spectroscopy of the methyl ester derivatives showed the characteristic bands of nonhydroxylated fatty esters: absorption bands assigned to C-H stretching at 2,921 and 2,845 cm^{-1}, to C = O stretching of carboxylic esters at 1,745 cm^{-1} and to long-chain deformations at 722 cm^{-1}. The presence of an intense band at 965 cm^{-1} was indicative of the occurrence of *trans* double bond(s) and suggestive of a methyl branch at their allylic position and, as a consequence, of negative optical rotation values, as found in mycolic acids (37). Consistently, a negative molecular rotation ($[M]_D = -42°$) was measured for the purified ester.

^1H-NMR analysis of the methyl esters of the purified free fatty acids from *M. brumae* (compound **b**) showed the occurrence of characteristic signal resonances of long-chain fatty acids (**Table 2**); these include signals attributable to terminal C**H$_3$**- (at 0.89 ppm, triplet, 3H), to -C**H$_2$**- (at 1.28 ppm, broad signal), to -C**H$_2$**-CH_2-COO- (at 1.65 ppm, multiplet), and to -C**H$_2$**-COO- (at 2.38 ppm, multiplet). More specific resonances were observed at 5.35 ppm (assigned to ethylenic protons, two multiplets, 2H), at 2.04 ppm (-C**H** - CH = CH-) and 0.96 and 0.99 ppm (attributed to C**H$_3$**- adjacent to double bonds). Successive uncouplings of vicinal CH protons allowed the identification of direct neighboring groups, whereas the analysis of the ^1H homonuclear 2D correlated spectroscopy spectrum further extended the attributions (data not shown). For instance, a cross-peak was observed between the proton resonance at 5.35 ppm (C**H** = C**H**-) and that at 2.04 ppm (-C**H**-CH = CH-); the latter resonance correlated with and that at 0.99 ppm (C**H$_3$**- adjacent to double bonds). This allowed the location of a methyl branch at the α-position of a *trans* double bond. The main data deduced from the analysis of the ^1H-NMR and ^{13}C-NMR spectra are consigned in Table 2 and consistent with the compounds being a mixture of long-chain ethylenic fatty esters with methyl branch(es) adjacent to *trans* double bond(s), as observed in the merochains of mycolic acids (28).

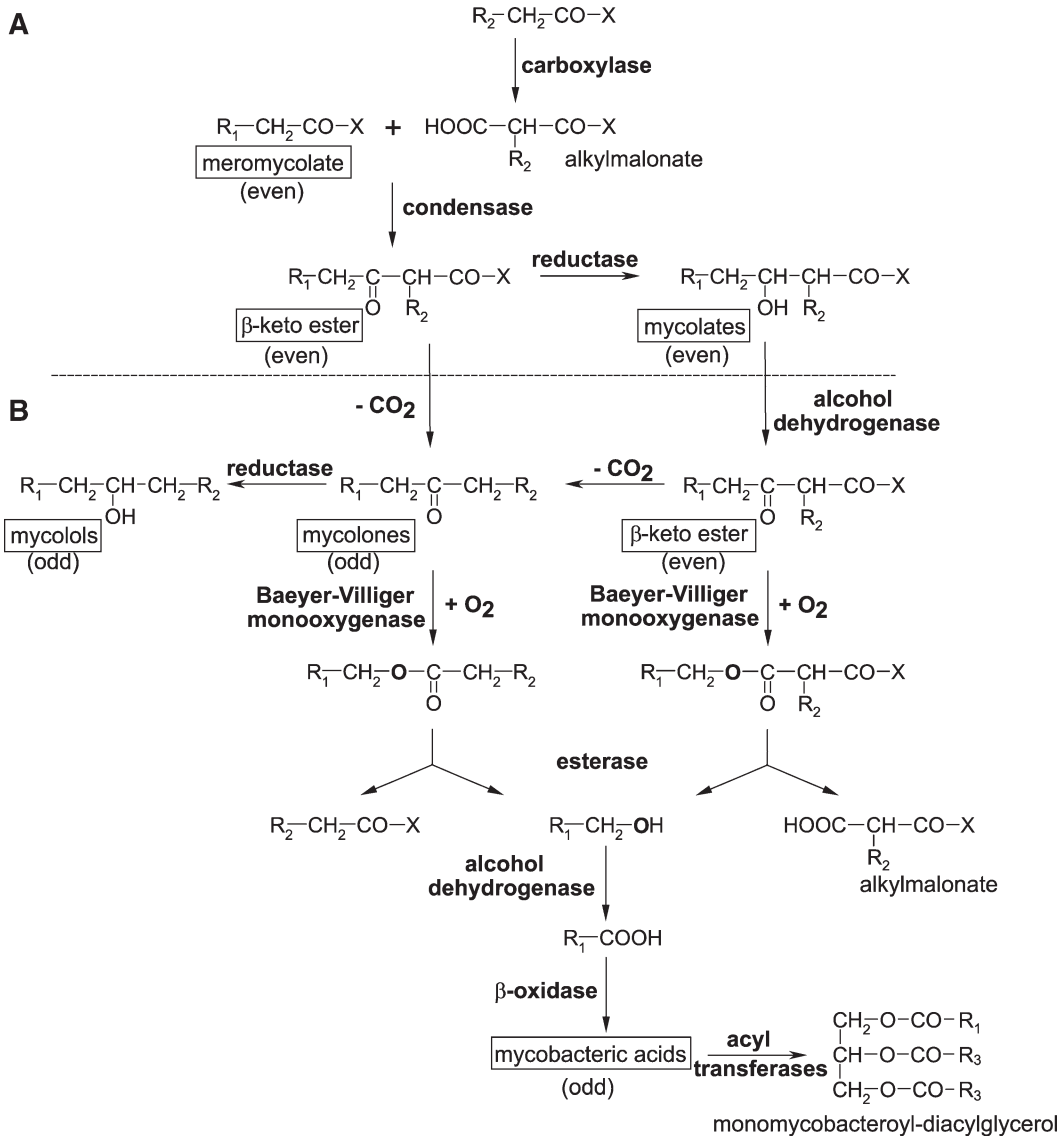

Fig. 2. Trafficking in mycolic acids pathways. A: Biosynthesis of mycolic acids. The long-chain acid at the origin of the meromycolic chain (R_1-CH_2-COOH with an even number of carbon atoms) is first activated (X), in the present case as an acyl AMP derivative. The R_2-CO-X (X = CoA) with 20, 22, or 24 carbon atoms at the origin of the α-branch chain in mycolates, is carboxylated as alkylmalonate. The condensation between the two activated derivatives gives the β-keto ester, the reduction of which leads to mature mycolic acids (with an even number of carbon atoms) that exist either as esters (X = trehalose or cell arabinogalactan). B: Degradation of mycolic acids. The β-keto ester intermediate in the synthesis of mycolates (with an even number of carbon atoms) and/or derived from the oxidation of mycolates would be decarboxylated (either as a consequence of the chemical hydrolysis of lipids or by a decarboxylase) to yield mycolones (with an odd number of carbon atoms). The β-keto esters, and possibly mycolones would be then oxidized, through the addition of an oxygen atom near the keto group by a putative Baeyer-Villiger monoxygenase, to yield a wax. The cleavage of the ester linkage would give primary alcohols (with an odd number of carbon atoms) whose oxidation would be at the origin of the $C_{44–56}$ and their probably β-oxidation $C_{35–44}$ series of mycobacteric acids. These latter substances were isolated both as free fatty acids and triacyl glycerol containing one mycobacteroyl residue (R_1-CO) and two moles of conventional ($C_{16–18}$) fatty acyl substituents (R_3-CO). The positions of the fatty acyl residues in the triacyl glycerol are arbitrary. Bold character **O** labels correspond to ^{18}O incorporated in lipids. "Even" and "odd" refer to the number of carbon atoms of lipid chains.

Analyses of the fatty acids, as free acids and as methyl ester derivatives by MALDI-TOF MS were unsuccessful. Molecular mass data were obtained by fast-atom bombardment and EI-MS of the free acids and were consistent with the occurrence of a family of $C_{37–45}$ fatty acids of odd and even chain lengths (i.e., mycobacteric acids); the mass peaks of the major homologs were seen at 546, 560, 574 *m/z* and corresponded to fatty acids containing C_{37}, C_{38}, C_{39} with two unsaturations.

Localization of the double bonds in mycobacteric acids of *M. brumae*

Fatty acids methyl esters were submitted to oxidative cleavage and the resulting fragments, monocarboxylic and dicarboxylic acids, were methylated, purified by preparative TLC, and analyzed by GC-MS. The major monocarboxylic methyl ester was identified as a 2-methyl-octadecanoate (molecular mass peak at 312 *m/z* and base peak at 88 *m/z*); minor linear and branched homologs containing 15, 17,

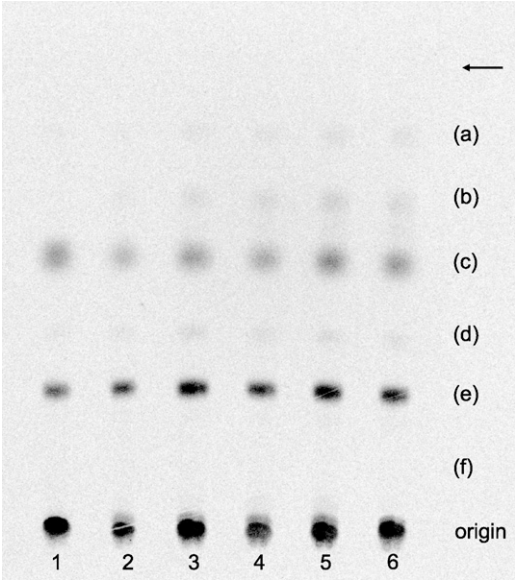

Fig. 3. Radio-TLC of the saponification products from *M. brumae*. Cells were labeled with ^{14}C acetate for 90 min, then the medium was discarded and a fresh Sauton's medium was added. At various time points (i.e., 0 h, 24 h, 48 h, 72 h, 96 h, and 10 days (lanes 1, 2, 3, 4, 5, and 6, respectively) aliquots were taken up and saponified. Lipids were extracted, methylated, and analyzed by TLC using dichloromethane as eluent. (a) mycolones, (b) mycobacteric acid methyl esters, (c) C_{16-24} fatty acid methyl esters, (d) mycolols, (e) methyl mycolates. (f) indicates the Rf of primary long-chain alcohols that are not detected in significant amounts in *M. brumae* but present in *M. fallax*. See Table 1 for general formula of the compounds. The arrow indicates the solvent front.

19, and 21 carbon atoms were also found. This result allowed us to localize the double bond at the ω-18 position of the molecules, as found in *M. brumae* mycolic acids (Fig. 1A) and in agreement with the occurrence of a methyl branch at ω-17 position and adjacent to a (*trans*) double bond, as revealed by NMR and infrared spectroscopies.

The dicarboxylic methyl esters arising from the oxidation of the double bonds consisted of a complex mixture of linear molecules with 12, 13, and 14 carbons and compounds with shorter chain lengths that contained branched and linear molecules. As these substances corresponded to the chemical entities between the two double bonds and those located between the proximal double bond and the carboxyl group, it was not possible to precisely assign the identified dicarboxylic acids to one of the two classes. To circumvent this problem and to confirm the molecular masses of the dicarboxylic acids, the native purified fatty acid mixture was derivatized as pyrrolidides and analyzed by EI-MS (33). A regular series of peaks with 14 atomic mass units (amu) interval corresponded to the primary fragmentation of the polymethylenic chain. All ions retained the pyrrolidide group. Interestingly, when a double bond is present in the molecule, a gap of 12 amu was observed between the two consecutive mass peaks. The position of the double bond can be deduced from a rule defined for mono- and polyenoic acid pyrrolidides (33).

The mass spectrum of *M. brumae* pyrrolidide derivatives of mycobacteric acids exhibited molecular ion peaks at 585, 599, 613, 627, and 641 *m/z*, which corresponded to the molecular masses of diethylenic C_{36} to C_{41} compounds (**Table 3**); the major mass peak was seen at 627 *m/z* and corresponded to a C_{39} diunsaturated pyrrolidide. However, due to the presence of carbon chain homologs and the possible presence of double bond isomers possessing or not methyl branch, no clear conclusion could be drawn from the analysis of the mixture. Consequently, the pyrrolidide derivatives were fractionated by argentation TLC, a technique that separates substances according to the number and configuration of the double bonds (28, 31), isolated and analyzed as above. The EI mass spectrum of the major purified compound showed an intense molecular ion at 627 *m/z*, the expected value for the $C_{39:2}$ pyrrolidide, with a base peak at 113 *m/z* that is originated from a McLafferty rearrangement (**Fig. 4A**). The presence of an intense peak at 402 *m/z*, attributable to the fragment bearing the methyl branch, indicated the occurrence of a methyl branch at the adjacent position of a (*trans*) double bond. Oxidative cleavage of this fraction, followed by methylation, yielded three compounds (Fig. 4A). GC-MS analysis of these molecules led to the identification of methyl esters of: *i*) 2-methyl octadecanoate (molecular ion at 312 *m/z* and base

TABLE 2. Assignments of the main resonance signals of the NMR spectra of the mycobacteric acids from *M. brumae*

	^{13}C (ppm)	^{1}H (ppm)
-$(CH_2)_n$-CH_2-**CH_3**	14.11	0.89**
-$(CH_2)_n$-**CH_2**-CH_3	22.68–24.67	1.28**
-(**CH_2**)$_n$-CH_2-CH_3	29.06–29.77	1.28
-$(CH_2)_n$-CH = CH-CH(**CH_3**)-$(CH_2)_n$-	20.90–22.60	0.96*
-$(CH_2)_n$-CH = CH-**CH**(CH_3)-$(CH_2)_n$-	36.69–37.23	2.04*
-$(CH_2)_n$-CH = CH-CH(CH_3)-**CH_2**-$(CH_2)_n$-		1.28
-$(CH_2)_n$-CH = CH-**CH_2**-$(CH_2)_n$-	31.92–32.58	1.99***
-$(CH_2)_n$-CH = CH-CH_2-**CH_2**-$(CH_2)_n$-		1.35***
-$(CH_2)_n$-CH = CH-CH_2-CH_2-(**CH_2**)$_n$-	29.06–29.77	1.28
-$(CH_2)_n$-CH = **CH**-CH(CH_3)-$(CH_2)_n$-	136.45	5.25*–5.35*
-$(CH_2)_n$-**CH**=CH-CH(CH_3)-$(CH_2)_n$-	128.41–130.19	5.25*–5.35*
-$(CH_2)_n$-CH_2-**CH_2**-COOH	37.23	2.38″
-$(CH_2)_n$-**CH_2**-CH_2-COOH	22.68–24.67	1.65″
-(**CH_2**)$_n$-CH_2-CH_2-COOH	29.06–29.77	1.28″
-$(CH_2)_n$-CH = CH-CH(**CH_3**)-CH_2-COOH	20.90–22.60	0.99*

Bold characters indicate the proton and carbon atoms of interest. *, **, ***, ″ Cross-peaks observed in homonuclear COSY spectrum.

TABLE 3. Chain lengths of *M. brumae* mycobacteric acid pyrrolidide derivatives

m/z	Carbon numbers	Relative abundance
697	44:2	0.05
683	43:2	0.10
669	42:2	0.10
655	431:2	0.35
641	**40:2**	**0.55**
627	**39:2**	**1.00**
613	**38:2**	**0.50**
599	**37:2**	**0.60**
585	36:2	0.40
571	35:2	0.05

Abundance refers to the proportion of the molecular mass of a given homolog in the mass spectrum of the mycobacteric acid derivatives relative to the major mass peak whose abundance was arbitrary fixed at 1. The main homologs are indicated in bold.

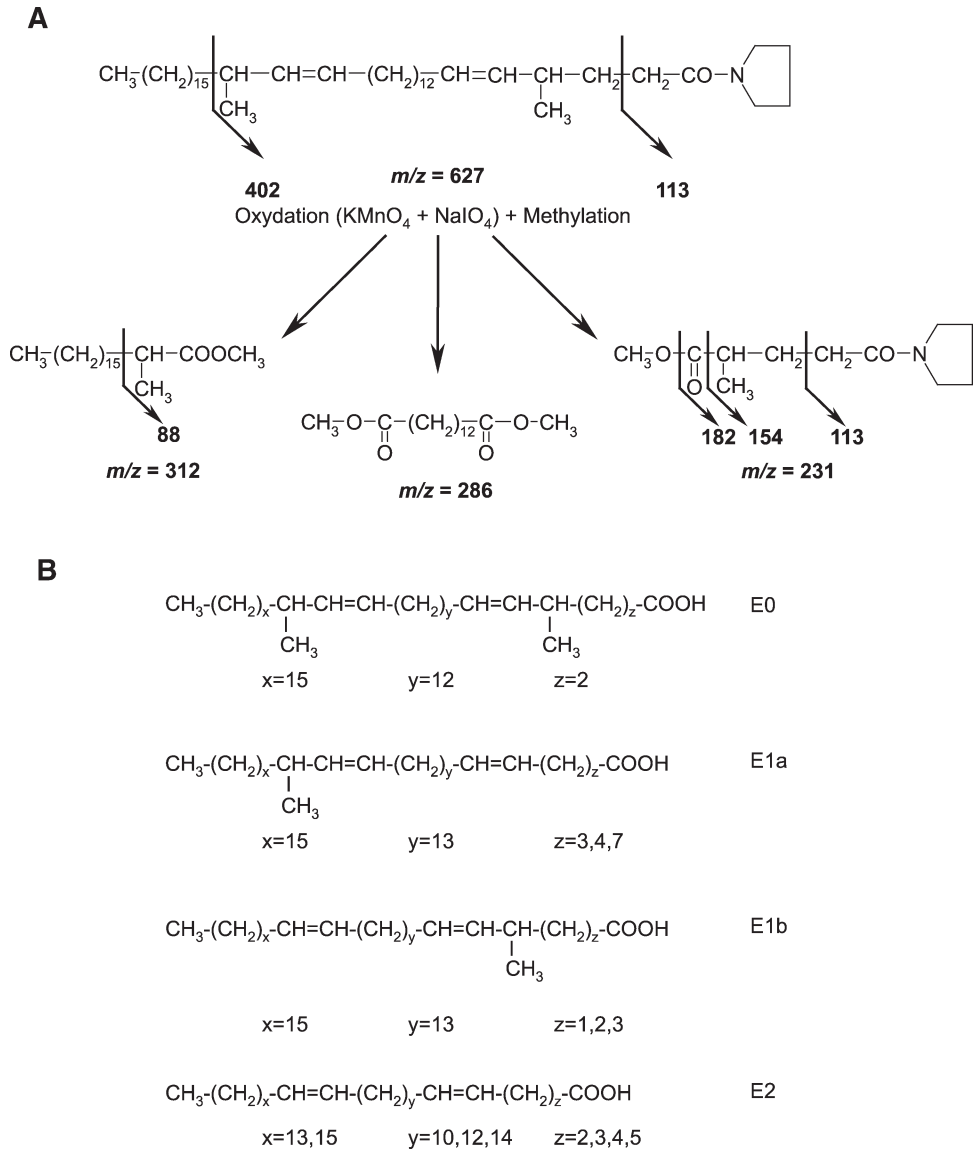

Fig. 4. Structures of the major homologs of mycobacteric acids from *M. brumae*. A: Illustration of the strategy used to establish the structures of mycobacteric acid derivatives deduced from the data from the electron impact (EI)-mass spectra of the pyrrolidide derivatives and from the characterization by GC-MS analyses of the fragments obtained after oxidative cleavage. The C_{39} H_{74} O_2 acid (E0) is given as an example. B: Structures of the major homologs of the mycobacteric acids: E0 refers to acids with 2 *trans* double bonds, E1a to molecules with *trans* and *cis* double bonds, E1b to substances with *cis* and *trans* double bonds and E2 to compounds with two *cis* double bonds.

peak at 88 *m/z*); *ii*) tetradecane-1,14- dioate (base peak at 98 *m/z*, due to a double cleavage (38), and molecular ion at 286 *m/z*); and *iii*) a C6 pyrrolidide (molecular mass at 213 *m/z* and base peak at 113 *m/z*) with additional peaks at 182 *m/z* (M-31) and 154 (Fig. 4A). Altogether these results established the structure of the major homolog of free mycobacteric acids of *M. brumae* as a 4,21-dimethyl-5,19-di-(*trans*)-enoyl-heptatriacontanoic acid (compound E0, Fig. 4B). Among the other homologs separated by argentation TLC, compounds E1a (Fig. 4B) related to M1a mycolic acids, were identified. Compounds containing one *cis* double bound in the distal part of the molecule and one *trans* double bond in the proximal portion of the molecule were also characterized (compound E1b, Fig. 4B). Detailed NMR analyses led us to propose for some of them the occurrence of a methyl group at the proximal part of the molecule, namely at C3, in agreement with both ^{1}H-NMR prediction (Table 2, data not shown). The most polar compound on argentation TLC was identified as a mixture of 5,21- and 7,23-di-enoyl pyrrolidides. Indeed, the configuration of the two double bonds was shown to be *cis*, and no methyl branch was detected by EI-MS and NMR analyses of the pyrrolidide derivatives (compounds E2, Fig. 4B). It is important to note, however, that although all the structures established for the mycobacteric acids E0, E1a, E1b, and E2 of *M. brumae* (Fig. 4B) are related, respectively, to mycolic acids M0, M1a, M1b, and M2 (Fig. 1), the fact that the major homolog E0 has an odd chain length

(C_{39}) was unexpected because mycolic acids (and their main merochains) that contain two methyl branches or two cyclopropyl rings have been observed to possess an even chain length in accordance with the proposed mechanism of synthesis of *trans* double bonds (39).

Identification of the natural forms of mycobacteric acids of *M. brumae*

The linked forms of the long-chain acids were investigated by fractionating the crude lipid extract by chromatography on a Florisil column and seeking for compounds with *trans* double bonds by infrared spectroscopy. The 965 cm^{-1} absorption band that indicates the occurrence of *trans* double bonds was exclusively found associated with fractions eluted with petroleum ether/ether. Analysis of these fractions by TLC showed that they were composed of two compounds migrating in the region of TAGs: one of these, TAG2, exhibited an Rf similar to that of the standard tripalmitin, whereas the other, TAG1, was less polar. The two TAGs were purified by preparative TLC, and their infrared spectra were similar to that of tripalmitin except for the presence of an intense band at 965 cm^{-1} in the spectrum of TAG1. Expectedly, the molecular rotation ($[M]_D$) of this triglyceride was negative (−40°), consistent with the contribution of the chiral methyl group adjacent to *trans* double bonds (28).

The ^{1}H-NMR spectra of TAG1 and TAG2 were compared with that of the commercial tripalmitin. They shared characteristic signals of triglycerides, notably those assignable to the proton resonances of the fatty acyl moiety at δ 0.85 (terminal CH_3, 9H, triplet), 1.23 (-CH_2-, broad), 1.58 (-CH_2-CH_2-COO, 6H, distorted triplet), 2.28 (-CH_2-COO, 6H, two overlapping triplets). In addition, proton resonances attributable to those of methylene(s) and methine(s) bearing hydroxyl groups esterified by fatty acyl residues were observed at 4.09, 4.13, 4.25, and 4.28 ppm and assigned to those of positions 1 and 3 of glycerol (-CH_2-OOC); the resonance of the proton at position 2 of glycerol (-CH-OOC) was observed at δ 5.25. The spectrum of TAG1 also contained specific proton resonances of ethylenic long-chain esters observed at δ 5.25−5.35 (-CH = CH-), 2.04 [-CH = CH-CH(CH_3)-CH_2-], 0.96 [-CH = CH-CH(CH_3)-CH_2-] and 0.99 [-CH = CH-CH(CH_3)-CH_2-COO]. All the attributions were established by 2D homo- and heteronuclear correlated spectroscopy, demonstrating that TAG1 corresponded to a TAG that contained one or more fatty acyl residue(s) containing methyl branch(es) adjacent to the double bond(s).

To firmly establish the structure of TAG1, we determined the molecular masses of the triglyceride constituents of *M. brumae* by MALDI-TOF mass spectroscopy (**Fig. 5**). Compared with the spectrum of the commercial tripalmitin (Fig. 5A), which contained a major sodiated molecular ion peak observed at 829 *m/z* expected for a [C_{51} H_{98} O_6 Na], that of the TAG fraction of *M. brumae* was more complex. Two series of peaks were seen in its mass spectrum: one with of molecular ion peaks in the range of 773 to 1,051 *m/z* and the other massif at 1,105 to 1,344 *m/z*. The two massifs corresponded to the masses of TAG2 and TAG1, respectively, as demonstrated by the analysis of the mass spectra of the purified compounds (Fig. 5B, C). The spectrum of TAG2 exhibited a series of intense peudomolecular ion peaks from 801 to 967 *m/z*, differing one another by 28 amu, and corresponding to the mass values of triglycerides composed of fatty acid substituents with C_{14-22}. (Fig. 5B). The mass spectrum of the purified TAG1 contained pseudomolecular ion peaks between 1,105 and 1,344 *m/z* (Fig. 5C). The major peak, seen at 1,147 *m/z* [C_{74} H_{140} 0_6 Na], corresponded to the mass of a TAG molecule bearing one mole of $C_{39:2}$ and two moles of $C_{16:0}$ fatty acyl residues. The specific location of mycobacteric acids in TAG, a storage compound, suggested that they correspond to the degradation products of mycolic acids; consistent with this hypothesis is the observation that they have the same structural features as mycolic acids but with shorter and different ratios of odd versus even chain lengths.

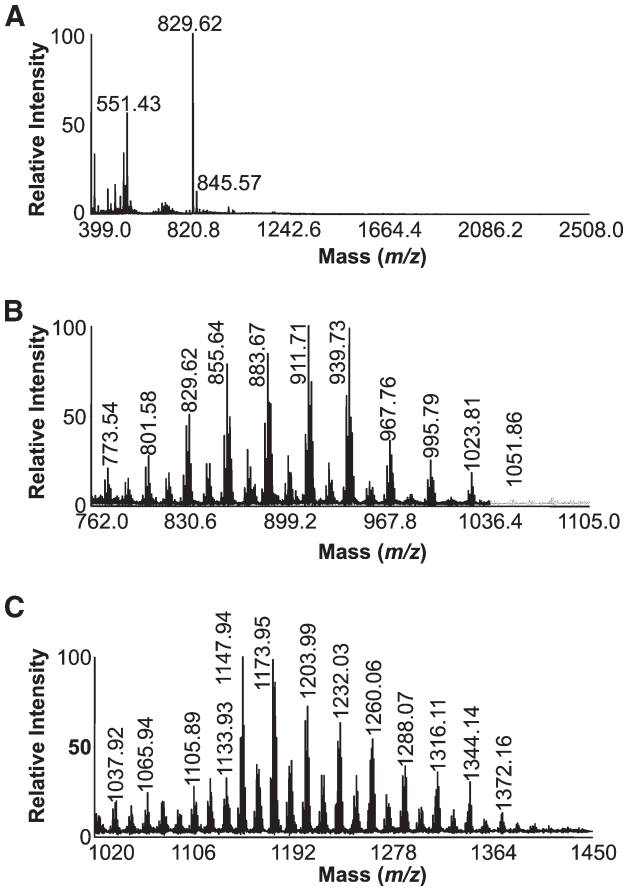

Fig. 5. MALDI-time-of-flight (TOF) spectra of triacylglycerols (TAGs). Mass spectra of the commercial tripalmitin (A), the product isolated from *M. brumae* (TAG2) with a Rf similar to that of tripalmitin (B) and the compound from *M. brumae* with a higher Rf (TAG1) (C). The Rfs of TAG1 and TAG2 from *M. aurum* are shown in Fig. 5A.

Comparative structural analysis of the meromycolic acids and compounds derived from the metabolism of mycolic acids of *M. fallax*

To determine the possible biosynthetic relationships between mycolic acids and the structurally related long-chain

ketones, secondary and primary alcohols (compounds **a**, **d**, and **f**, respectively, Fig. 3) we directly compared their ratios of odd versus even chain lengths, a clear indication of catabolism, to those of the meroaldehyde chains obtained by pyrolitical cleavage of mycolic acids. For this purpose, *M. fallax* was chosen because this species accumulated more ketones and alcohols, notably compound **f**, than did *M. brumae*. The various types of mycolates from *M. fallax* were separated by argentation TLC as **M1a** and **M1b**, **M2**, and **M3** (Fig. 1A). Each class of mycolic acids was then pyrolysed to yield the corresponding meroaldehydes. The same argentation TLC strategy was applied to the long-chain compounds. After purification, mycolic acids and their structurally related minor lipids were analyzed and their structures compared. The results from MS analyses allowed calculating the ratios of odd/even chain lengths for each class of compounds (Fig. 1) (Table 1). Interestingly, the chain lengths of the meroaldehydes from mycolic acid pyrolysis were one carbon longer than those of the long-chain mycobacteric esters. Consistently, the ratio of odd versus even chains was reversed in the mycobacteric acids compared with mycolic acids and the corresponding meroaldehydes, mycolones, and mycolols (Fig. 1) (Table 1). This observation was also valid for long-chain primary alcohols. Furthermore, the meromycolic acids resulting from the reduction of the meroaldehydes exhibited carbon numbers between 50 and 55, whereas mycobacteric acids and long-chain primary alcohols contained a larger pool of homologs with shorter chain lengths, from C_{35-44} and C_{44-56}, respectively. Altogether, these data supported the hypothesis that mycobacteric acids **(b)** and the primary alcohols **(f)** derive from the mero aldehyde chain, whereas mycolones **(a)** and their reduced form **(d)** probably result from the decarboxylation of a β-keto ester.

Degradation of exogenic mycolic acids by *M. aurum*

The structural analogies between mycobacteric acids and the meromycolic chains suggest that the former compounds derive from the degradation of mycolic acids after the cleavage of the mycoloyl chain at the level of the α-branched/β-hydroxylated structure, the so-called mycolic unit. To test this hypothesis, the strain A+ of *M. aurum* that is known to degrade exogenic lipids (40) was used. Accordingly, uniformly ^{14}C labeled α-mycolic acids were prepared by growing strain A+ of *M. aurum* on a medium supplemented with ^{14}C acetate. The labeled α-mycolates were purified and added to the growth medium of the same strain and their degradation was followed. Around 30% to 60% of the labeling was found in the extractable lipids. The labeling was observed in the TAG fraction and most exclusively in TAG1 (**Fig. 6A**), whereas no labeling was detected in phospholipids, trehalose mycolates, and bacterial residues. This labeled fraction was isolated by preparative TLC, saponified, and the resulting fatty acids were converted into their anilides derivatives and separated by normal phase liquid chromatography in two fractions, LC1 and LC2 (Fig. 6B). The anilide derivatives of the two fractions were analyzed by field desorption mass spectrometry, a technique that produces molecular ions without fragmentation of the car-

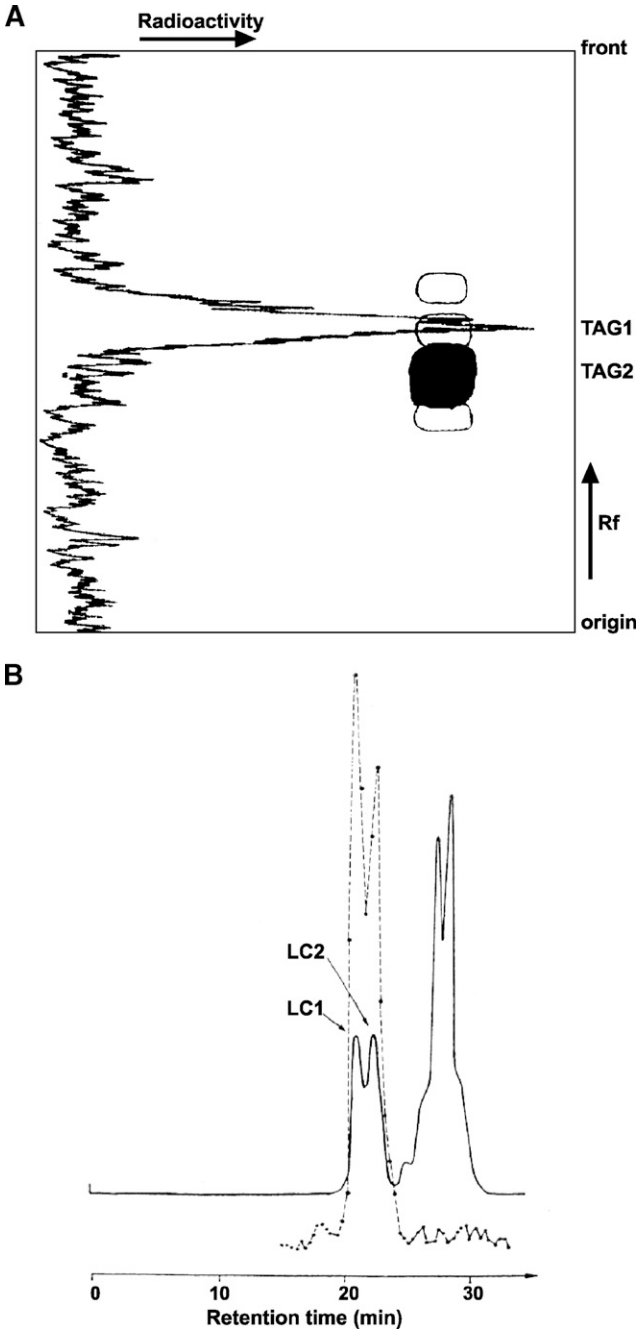

Fig. 6. Radio-TLC and -HPLC of the TAG fraction of *M. aurum*. Bacteria were grown in a medium containing ^{14}C α-mycolate from *M.aurum* for 72 h under agitation. Cells were then extracted with chloroform/methanol and the resulting lipids were separated on a Florisil column. A: Radio-TLC of the TAG fraction eluted from the column with petroleum ether/ether 85:15 (v/v) and analyzed by TLC with the same solvent system. TAG1 and TAG2 correspond, respectively, to TAGs that contained long (C_{34-55}) and moderate (C_{16-24}) fatty acids chains. The mass spectra of the corresponding substances from *M. brumae* are shown in Fig. 4A, B, respectively. B: Labeling (dotted lines) and UV (λ = 242 nm) profiles of anilide derivatives of the fatty acids obtained by saponification of the TAG1 fraction. The acid derivatives were fractionated using a μPorasil HPLC column (0.39 × 30 cm) eluted with hexane/dioxane (9:1, v/v); a flow rate of 1 ml/min was applied. LC1 and LC2 correspond to the two classes of long-chain fatty acid anilide derivatives.

bon chain. Analysis of the mass spectra showed that the compounds consisted in a mixture of long-chain acids composed of both odd and even chain lengths (**Table 4**). LC2 was composed of acyl anilides having C_{35} to C_{44} acyl moieties, the most abundant corresponding to a $C_{39:2}$ acyl group (molecular mass at 649 *m/z*), whereas LC1 consisted of homologs with 44 to 54 carbon atoms, with the main constituents being $C_{49:2}$ and $C_{50:2}$ acyl groups (molecular masses at 789 and 803 *m/z*). The metabolic products under study contained the two cyclopropyl rings found at the proximal and distal positions of the α-mycolates of *M. aurum* (Fig. 1B), consistent with a structural relationship between the latter compounds and the long-chain fatty acids LC1 and LC2 found in TAG1.

Incorporation of ^{18}O in long-chain alcohols of *M. fallax*

Two processes of cleavage of mycolic acids may lead to these labeled mycobacteric acid dead products: *i*) a retro-Claisen reaction, the reverse process of mycolic acid condensation, that would lead to compounds with an even number of carbon atoms, as occurring during the biosynthesis of meromycolates; and *ii*) a Baeyer-Villiger oxidation (i.e., oxidation of a keto group by the addition of molecular oxygen to give a wax that would yield upon hydrolysis long-chain fatty alcohols possessing one carbon less than the meromycolates and an alkymalonate) (Fig. 2). The ratio odd versus even chain lengths of mycobacteric acids characterized from *M. brumae* and *M. fallax* (see previous discussion) did not support the retro-Claisen hypothesis.

TABLE 4. Molecular masses and chain lengths of the mycobacteric acids obtained from the degradation of exogenous ^{14}C-labeled α-mycolic acids from *M. aurum*

	m/z	Carbon numbers	Relative abundance
	859	54:2	0.15
	845	53:2	0.39
	831	**52:2**	**0.60**
	817	**51:2**	**0.94**
	803	**50:2**	**1.00**
LC1	**789**	**49:2**	**1.00**
	775	**48:2**	**0.91**
	761	**47:2**	**0.83**
	747	**46:2**	**0.62**
	733	45:2	0.32
	719	44:2	0.20
	719	44:2	0.12
	705	43:2	0.25
	691	42:2	0.46
	677	**41:2**	**0.67**
	663	**40:2**	**0.72**
LC2	**649**	**39:2**	**1.00**
	635	**38:2**	**0.67**
	621	37:2	0.17
	607	36:2	0.15
	593	35:2	0.05

Triacyl glycerols (TAGs) were fractionated by TLC and the only labeled class of TAGs (TAG1, Fig. 5A) was purified, saponified, and the resulting long-chain fatty acids were transformed into their anilide derivatives, and separated by liquid chromatography into LC1 and LC2 (Fig. 5B). Abundance refers to the content of the homolog (relative to the major mass peak whose abundance was arbitrary fixed at 1) in the mycobacteric acid mixtures from LC1 and LC2. The main homologs are indicated in bold. Solvent: hexane/dioxane (9:1, v/v).

To test the Baeyer-Villiger oxidation hypothesis, *M. fallax* was preferred to *M. brumae* and *M. aurum* for the occurrence and abundance of all the lipids. Accordingly, cells of *M. fallax* were incubated under either $^{18}O_2$ or $^{16}O_2$ atmosphere, and the reaction was stopped by saponification. Lipids were isolated, and mycolic acids and alcohols were purified on $AgNO_3$-impregnated TLC and analyzed by EI-MS. The spectra of alcohols from both conditions were compared. For normal growth conditions, the major molecular ion was seen at 742 *m/z*, the expected mass of a $C_{52:2}$ alcohol. When cells were grown in presence of $^{18}O_2$, a significant increase of the M+2 ion peak was observed at *m/z* 744; the same mass shift was also found for the other homologs (**Fig. 7A**). This increase in the abundance of the M+2 isotope strongly suggested the incorporation of one ^{18}O atom into the long-chain alcohols. To ascertain that this increase was due to the incorporation of ^{18}O isotope, and not to the gain of hydrogen atom during the assay, catalytic hydrogenation was performed and the resulting saturated alcohols were trimethlysilylated to improve the abundance of high mass ions by EI-MS (Fig. 7B). In the high mass range, intense M-15 ions (loss of a methyl group from the trimethylsilyl substituent) of the major homologs of alcohols were obtained. Upon growth in the presence of ^{16}O, the M-15 ion from the C_{52} homolog was seen at 803 *m/z*. Under incubation in an ^{18}O atmosphere, the relative intensity of the ion at 805 *m/z* increased dramatically, indicating that the increase of two amu in the M-15 ion was readily due to the incorporation of an ^{18}O atom into the molecules.

To determine the specificity of the incorporation of the ^{18}O into long-chain compounds, a comparative analysis of structurally related lipids, such as mycolones and mycolic acids, was performed. Accordingly, the oxygenated lipids were purified by argentation TLC and the EI spectra of the secondary alcohols derived from mycolones (i.e., mycolols) and those of mycolic acids were compared. Interestingly, comparable EI-MS profiles were obtained for these compounds grown under either ^{18}O or ^{16}O (data not shown). Consequently, the absence of incorporation of ^{18}O in the hydroxyl groups of mycolols and mycolic acids proved the specificity of incorporation of ^{18}O into the long-chain primary alcohols. It was thus concluded that the long-chain primary alcohols derived from the Baeyer-Villiger oxidation of mycolic acids through a keto intermediate (Fig. 2). Moreover, an alkylmalonate was identified in the GC-MS profile of volatile fatty esters (fragmentation ions *m/z* 132 and 145), in agreement with this mechanism.

DISCUSSION

The presence of long-chain fatty acids structurally related to mycolic acids is known for long to occur in mycobacteria. Two size ranges of chain lengths have been generally found, namely $C_{36–47}$ fatty acids for which the name of mycobacteric acids has been proposed, and the other with chains between $C_{50–56}$ (11–15). Both classes of long-chain fatty acids contain the structural features observed in the mycolic acids of the same species (i.e., cyclo-

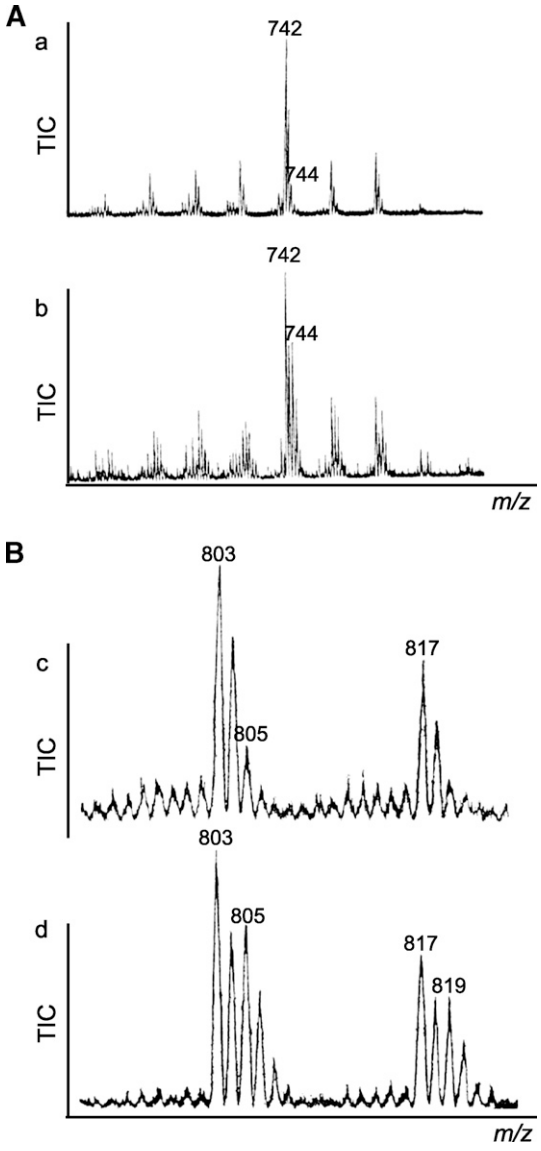

Fig. 7. EI-MS analysis of long-chain primary alcohols from *M. fallax* cultures incubated in presence of ^{18}O. A: Partial EI-mass spectra of *M. fallax* long-chain alcohols. Cells were grown under normal conditions (^{16} O atmosphere) (a) and under ^{18}O atmosphere (b). Ions at *m/z* 742 and 744 correspond to a C_{52} alcohol. B: Partial-EI-mass spectra of *M. fallax* long-chain alcohols after catalytic reduction of double bonds, followed by silylation. (c) normal conditions; (d) ^{18}O atmosphere. Ions at *m/z* 803 and *m/z* 817 correspond to $[M\text{-}15]^+$. Ions at *m/z* 805 and *m/z* 819 correspond to [M-15] + 2 (from ^{18}O).

propyl rings, double bonds, and/or oxygenated functions) (11, 13, 16). They have been isolated from a large variety of mycobacteria [e.g., *M. tuberculosis* (11, 12), *M. smegmatis* (15), *M. kansasii* (41), and from a species of *Nocardia* (17)]. In the present work, a series of C_{35-39} acids with odd and even numbers of carbon atoms and containing *trans* and *cis* double bonds was characterized and shown to be structurally related to the mycolic acids of *M. brumae* (28). Two pools of fatty acids, with C_{39} and C_{51} as the main terms, were also characterized in *M. fallax* and were shown to contain double bonds located at the same positions as in the meromycolic chain of the mycolic acids of this species. The similarities between the structural features of these mycobacteric acids and those of mycolic acids were a priori compatible with the former products being either the precursor or degradation compounds of the latter molecules. In this connection, a role for this type of long-chain fatty acids as mycolic acid precursors in growing cells has been suggested (41, 42). What seems to support this hypothesis is the fact that a cell-free system from *M. tuberculosis* was shown in earlier studies to synthesize saturated and unsaturated C_{30-40} and C_{48-56} fatty acids, the latter exhibiting chain lengths similar to those of the meromycolic chain (14). Similarly, of the two condensing enzymes KasA and KasB, which are components of the fatty acid synthase II system implicated in the biosynthesis of the main (mero) chains of mycolic acids, characterized in *M. tuberculosis*, KasA specifically elongates palmitate into long-chain fatty acids averaging 40 carbons in length; KasB, in the presence of KasA, produces longer fatty acids averaging C_{54} and structurally related to the meromycolic acids (43). However, if mycobacteric acids are implicated in a biosynthetic process, it is puzzling to observe that compounds characterized in the present work and in past studies contain a ratio even versus odd chains different from that of the aldehydes obtained by pyrolytical cleavage of α-mycolic acids.

To arrive to a documented working model about the origin of long-chain fatty acids, we followed the degradation of uniformly ^{14}C labeled dicyclopropanated α-mycolic acids of *M. aurum* by a growing culture of *M. aurum*, a strain known to degrade long-chain compounds (40). Upon saponification of the cells, the labeling was exclusively found in fatty acids with chain averaging C_{39} and C_{51}, as demonstrated by the presence of ^{14}C radiolabeled mycobacteric acids. Interestingly, the two series of labeled fatty acids were shown to derive from the meromycolic chain of the α-mycolic acids, as they contain the two cyclopropyl groups. These results indicated that mycobacteric acids result from the degradation of the mero mycolic chain, implying first the cleavage of the mycolic acid unit to liberate the mero chain, followed by shortening the so-formed long chain by β-oxidation. At least two possibilities of cleavage of the mycolic acid unit would yield the meromycolate chain. One of them implies a mechanism similar to a retro-Claisen reaction, the reverse of the mycolic condensation. Such a reaction has been chemically performed with β-keto acyl trehalose, the product of the mycolic condensation (44, 45). Upon the growth of *M. aurum* cells in the presence of labeled mycolates, however, we were unable to detect radiolabeled acyl trehalose among the complex lipids; instead, the only radiolabeled complex lipid observed was apolar TAGs. Importantly, the retro-Claisen reaction would lead to mycobacteric acids analogous of the biosynthetic precursors, the meromycolic acids, in terms of parity of chain lengths. Because the meromycolic chains of the α-mycolic acids used in this experiment contained an even carbon atom number, it could be postulated that all long-chain acids so-formed and subsequently degraded by β-oxidation would retain an even carbon number. The ratio odd versus even chain lengths of the long-chain labeled molecules we characterized does not support this hypothesis. Alterna-

tively, the rupture of the mycolic acid unit may be realized through a biological process similar to a Baeyer-Villiger reaction. Such a reaction consists in the insertion of one oxygen atom in the C-C bond adjacent to a keto group, yielding an ester function (wax). To perform such a process the reaction substrate would be a β-keto ester or a ketone (Fig. 2). The chain cleavage of the so-formed wax by esterases would occur between carbons 3 and 4 in the case of a keto ester and should produce, on the one hand, a long-chain alcohol that would contain one carbon less than the mero-chain and, on the other hand, an alkylmalonate (Fig. 2). The primary alcohol could be further dehydrogenated into a long-chain fatty acid that would be the substrate of β-oxidation. The chain lengths of mycobacteric acids with the main odd numbered 39 and 51 carbon terms and the characterization of the C_{22}-alkymalonate in the lipids of saponification support this hypothesis (Fig. 2).

An efficient and specific insertion of one oxygen atom into an organic substrate is easily realized by specific enzymes, the flavoprotein-dependent enzymes [e.g., flavin monooxygenases and Baeyer-Villiger monooxygenases (BVMO)]. These enzymes play a role in the removal of toxic ketones and participate to the degradation of hydrocarbons by microorganisms. Flavin monooxygenases and BVMO have almost identical sequences (46), and several monooxygenases have been characterized in *Corynebacterinae*. For example, a putative BVMO has been detected in *M. avium* (47), a species containing keto- and its Baeyer-Villiger oxidation product, wax-mycolic acids (48–50). Interestingly, *M. tuberculosis* contains seven genes with BMVO motif (51). Their roles remain to be established but it was recently shown that a BMVO (Rv3854c) is responsible for the activation and therefore the efficacy of the widely used antituberculous prodrug ethionamide (52, 53). In the present work, the demonstration that the ^{18}O labeling was exclusively found in long-chain primary alcohols parented to the mero mycolic chain, with the expected chain lengths, and not in other structurally related oxygenated long-chain lipids examined, clearly establishes that a key step in the degradation of mycolic acids is catalyzed by a Baeyer-Villiger-like process. The ability of these enzymes to convert relatively hydrophobic substrates and their membrane association fits well with a function in mycolic acid degradation. Future biochemical studies should clarify the role of oxygenases in mycobacterial physiology. Interestingly, because no BVMO have been identified in plants, animals, and humans, the identification of new classes of substrates and the corresponding reactivity as Baeyer-Villiger oxidation open new opportunities for developing chemotherapy for treating mycobacterial infections.

We showed that mycobacteric acids occurred either free or esterifying glycerol, solely as TAG but neither as trehalose lipids nor in phospholipids. MS analysis further demonstrated that the main homologs consisted of TAGs bearing one mycobacteric acyl residue per molecule. Interestingly, structurally related apolar nonhydroxylated fatty acids have been recently characterized in TAGs from various other mycobacteria, which include both slow growers (e.g., *M. kansasii*, *M. gastri*, and *M. bovis* BCG) and rapid growers (e.g., *M. smegmatis* mc^2155 and *M. thermoresistibile*) (41). TAG fractions of *M. smegmatis* and *M. bovis* have been shown to be extremely asymmetric molecules, having a series of very long fatty acids located on position 3 of glycerol, in contrast to phospholipids where C_{20-26} fatty acyl substituents were absent (54). TAGs containing significant amounts of C_{37}, C_{39}, and C_{41} acids with odd chain lengths have been previously found in a *Nocardia* species that contained C_{56-58} linear ethylenic mycolic acids. More polar mycobacteric acids, with additional oxygen functions such as keto and wax, were also characterized in the TAG fractions of mycobacteria producing keto- and wax- mycolates: 2-octadecanol, a secondary alcohol found in wax mycolic acids, has been identified after saponification of TAG from *M. aurum* (16). The proposed storage role of TAGs may be advantageous for mycobacteria in vivo and relevant to the survival in a nonreplicating state. This concept is supported by a report on the upregulation of diacylglycerol transferases involved in TAG biosynthesis in stationary phase of *M. tuberculosis* cultures and by the observation that TAGs accumulate in dormant-like cultures of the bacillus (55). Likewise, *M. kansasii* was reported to accumulate TAGs and a so-called monomeromycoloyl diacylglycerol (similar to TAG1 characterized in the present study) during the late growth phase (41).

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