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# Chemical Characterization of the Ester-Linked 3-Hydroxy Fatty Acyl-Containing Lipids in *Mycobacterium tuberculosis*

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**In a previous study (S. Alugupalli, F. Portaels, and L. Larsson, J. Bacteriol. 176:2962–2969, 1994), we reported the occurrence of 21 3-hydroxy fatty acids (3-OH-FAs) in the methanolysis products of different mycobacterial species. The present study was undertaken in order to chemically characterize the ester-linked complex native forms of these acids in *Mycobacterium tuberculosis*. Three 3-OH-FA-containing lipids were purified by chromatography and analyzed by one- and two-dimensional nuclear magnetic resonance spectroscopy, by fast atom bombardment-mass spectrometry, and by various conventional chemical analysis techniques. 3-OH-2,4,6-trimethyl-FAs were found in 2,3-diacyl trehalose and 2,3,6,6 -tetra-acyl-2 -sulfate trehalose (sulfatide I), two specific glycolipids of the tubercle bacillus, explaining the specific occurrence of these fatty acids in the methanolysis products of virulent strains of *M. tuberculosis*. Straight-chain 3-OH-FAs were localized in phosphatidyl ethanolamine but not in the other phospholipids, suggesting a possible role of this class of phospholipids in the metabolism of fatty acids in actinomycetes.**

Tuberculosis remains the leading cause of death in the world from a single pathogen (7, 18). The slow growth of the tubercle bacillus and the lack of easy and reliable tests for detecting the disease at an early stage also limit the program of control and eradication of tuberculosis. In this context, recent efforts have been devoted to more knowledge of the disease and its pathogen.

Most of the unique features of mycobacteria, such as their mold-like growth pattern and their resistance to chemical agents, as well as many of their biologic activities have been attributed to their high lipid content (17). Detailed chemical studies demonstrated the diversity and the complexity of the mycobacterial fatty acids, which have been used in chemotaxonomy in order to discriminate among mycobacterial species (4, 8, 24). These compounds include 2-alkyl branched, 3-hydroxylated long chain fatty acids (up to 90 carbon atoms), the so-called mycolic acids, multi-methyl (Me) branched-chain fatty acids having chiral centers with an L or D configuration, and α,β-unsaturated compounds. We recently characterized 21 3-hydroxy fatty acids (3-OH-FAs), i.e., straight-chain and 2-Me and 2,4,6-tri-Me branched-chain fatty acids, in 27 strains belonging to 12 mycobacterial species (2). According to their 3-OH-FA patterns, the mycobacterial species could be subdivided into six groups, suggesting a taxonomic interest for these fatty acids. Virulent strains of *Mycobacterium tuberculosis* exhibited an unique pattern of 3-OH-FAs in that they contain multi-Me branched long chain hydroxy fatty acids, the potential precursors of fatty acids found in the biologically important mycobacterial lipids (4, 24). The present study was undertaken in order to chemically characterize the ester-linked native forms of straight- and Me branched-chain 3-OH-FAs in *M. tuberculosis*.

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*M. tuberculosis* H37Rv was grown for 4 weeks on Sauton's medium (26) as surface pellicles at 37°C, and cells were harvested by pouring off the medium while the pellicles remained attached to the flasks. Wet cells were extracted first with $CHCl_3$-$CH_3OH$ (1:2 [vol/vol]) for 5 days and then with $CHCl_3$-$CH_3OH$ (2:1 [vol/vol]). Pooled extracts were concentrated, washed with water, and evaporated to dryness to yield the crude lipid extract. This material (approximately 1 g) was dissolved in a few milliliters of $CHCl_3$ and applied on a florisil column (60/100 mesh), which was irrigated successively with 200 ml of $CHCl_3$, with 200 ml of increasing concentrations of $CH_3OH$ in $CHCl_3$ (5, 10, 20, 30, and 50% [vol/vol]), and finally with 200 ml of a mixture of $CHCl_3$, $CH_3OH$, and $H_2O$ (65:25:4 [vol/vol/vol]). Eluates were analyzed by thin-layer chromatography on silica gel-60 precoated plates (0.25-mm thickness; E. Merck, Darmstadt, Germany) developed with $CHCl_3$-$CH_3OH$-$H_2O$ (30:8:1 and 65:25:4 [vol/vol/vol]). Sugar-containing compounds were visualized by spraying the plates with 0.2% anthrone in concentrated sulfuric acid, followed by heating at 110°C. The Dittmer-Lester reagent (14) was used for revealing phosphorus-containing substances. A ninhydrin reagent was used to reveal the presence of an amino group. Each fraction was analyzed for its fatty acid content. Final purification of neutral lipids was achieved by reapplying the hydroxylated fatty acid-rich fractions on a silicic acid column; sulfate-containing lipids were purified by refractionating the 20% $CH_3OH$-$CHCl_3$ elution on a DEAE-cellulose column, whereas phospholipid purification was done by reapplying the $CHCl_3$-$CH_3OH$-$H_2O$ (65:25:4 [vol/vol/vol]) fraction on a high-performance liquid chromatography (HPLC) system as described elsewhere (27), using a Waters (Milford, Mass.) model 600-MS HPLC pump equipped with a variable-volume Waters model U6k injector.

All the fractions eluted from the florisil column were subjected to both mild and strong acid methanolysis (2). Fatty acid methyl esters were subjected to trimethylsilyl derivatization and analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (2). For sugar analyses, an aliquot (1 mg) of the chromatographic fractions and purified lipids was

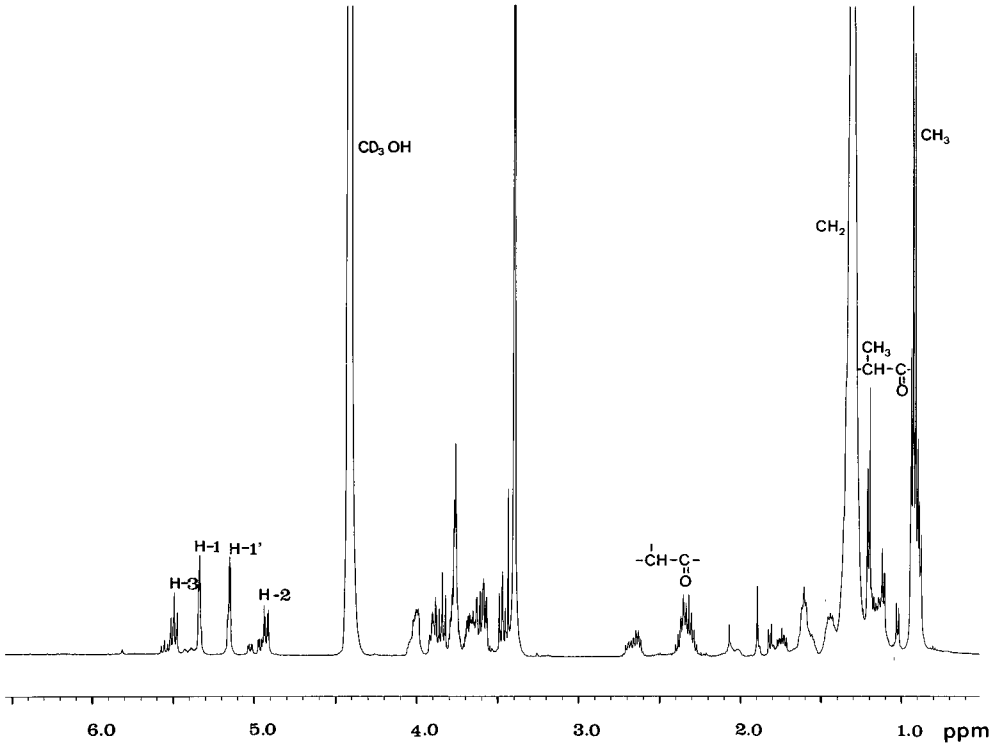


FIG. 1. $^1$H-NMR spectrum (10 mg of lipid in 0.5 ml of $CDCl_3$-$CD_3OD$ [2/1 {vol/vol}]) of diacyltrehaloses of *M. tuberculosis*.

routinely hydrolyzed with a 1 M aqueous trifluoroacetic acid (Sigma, St. Louis, Mo.) solution at 110°C for 1 h. The hydrolysates were then partitioned between $CHCl_3$ and $H_2O$. The aqueous phase was dried under nitrogen and subjected to trimethylsilyl derivatization and analyzed by GC with a Girdel G-30 apparatus equipped with a fused silica column (25 m by 0.22 mm [internal diameter]) coated with OV-1 (0.3-mm film thickness). A temperature gradient of 100 to 280°C (2° $min^{-1}$) was used. Purified sulfatide was subjected to desulfation (16) by the method of Daffé et al. (11).

Proton nuclear magnetic resonance ($^1$H-NMR) spectra were obtained in $CDCl_3$-$CD_3OD$ (2:1 [vol/vol]) on a Brucker AM 500 WB instrument at 25°C. Two-dimensional chemical-shift correlated spectroscopy data were obtained by using the previously described parameters (12). MS was performed on a ZAB-MS reverse geometry mass spectrometer (VG Analytical). Fast atom bombardment was generated by an 8 KeV xenon atom beam as previously described (23). GC-MS was performed on Trio-1 S (VG Mass Lab) connected to a Hewlett-Packard model 5890 gas chromatograph. 3-OH-FA methyl esters were identified and quantified as described previously (2).

The crude lipid extract of *M. tuberculosis* was fractionated on a florisil column, and the 3-OH-FA content of the various fractions was determined (2). Fractions eluted with 20 and 30% $CH_3OH$ in $CHCl_3$ and with $CHCl_3$-$CH_3OH$-$H_2O$ (65:25:4 [vol/vol/vol]) contained significant amounts (>1%) of 3-OH-FAs; these were reapplied on a silicic acid column, and their components were eluted with increasing concentrations of $CH_3OH$ in $CHCl_3$. This resulted in the identification of two carbohydrate-containing, phosphorus-free substances. The two glycolipids were easily purified by anion-exchange chromatography on DEAE-cellulose. One glycolipid was eluted from the column with $CHCl_3$-$CH_3OH$ (9:1 [vol/vol]), whereas the other one was eluted only when the column was irrigated with a $CHCl_3$-$CH_3OH$ solution containing ammonium salts, indicating the presence of an acidic substituent in this compound. The purified neutral glycolipid exhibited the same chromatographic mobility as did diacyltrehalose (DAT) isolated from *M. fortuitum* (3, 15). The occurrence of 3-OH-FAs in this glycolipid was confirmed by GC-MS of the methanolysis products. They represented 10% of the total fatty acyl substituents and were composed mainly of 2,4,6-tri-methyl-3-OH-$C_{24}$; a tiny amount of the $C_{22}$ homolog was also identified. The nonhydroxylated fatty acyl substituents of the glycolipid consisted of $C_{16:0}$ and $C_{18:0}$ (the major components) and 2,4-di-Me-$C_{22}$. Trehalose was the only carbohydrate component present in the water-soluble material resulting from the deacylation of the glycolipid; acid hydrolysis of this latter material released glucose. The $^1$H-NMR spectrum (Fig. 1) was superimposable to those of DATs (3, 6, 15, 21) and informative in that it confirmed the diacyltrehalose nature of the glycolipid and allowed the location of the fatty acyl substituents. The two doublets observed in the low field region at 5.15 and 5.34 ppm ($J_{1,2}$ = 3.5 Hz) were assigned to the H-1 resonances of α,α′-trehalose (9, 11). The two other major signals in this region, integrating 1H each, were assigned to the H-2 and H-3 resonances of 2,3-acylated trehalose. Their resonances were centered at 4.92 and 5.47 ppm, respectively. Less intense signals, assignable to H-2 and H-3 resonances (at 5.01 and 5.50 ppm), were observed and confirmed the heterogeneity commonly occurring in DATs. The other signals in the $^1$H-NMR spectrum were also in agreement with the proposed structure, especially those in the high field region: a broad singlet at 1.36 ppm ($CH_2$ resonance) and a multiplet centered at 0.92 ppm (terminal and multi-Me branched-chain fatty acid proton resonances). The fast atom

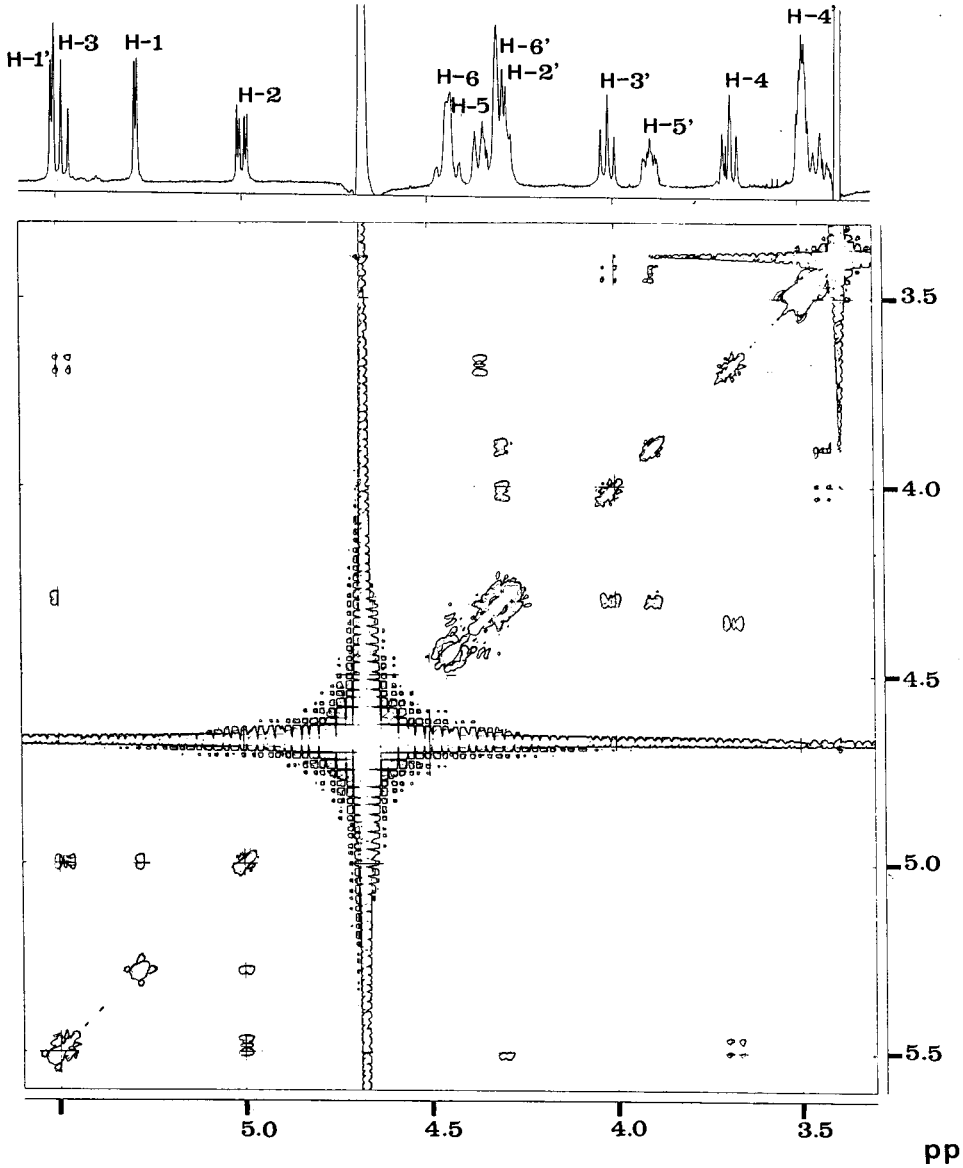


FIG. 2. Contour plot of two-dimensional chemical-shift correlated spectroscopic spectrum (15 mg of lipid in 0.5 ml $CDCl_3$-$CD_3OD$ [2/1 {vol/vol}]) of the purified sulfatide of *M. tuberculosis*.

bombardment-MS spectrum of the glycolipid (data not shown) confirmed the diacyl trehalose structure and the presence of hydroxylated fatty acid substituents (5). It followed then that the major 3-OH-FA-containing compound of *M. tuberculosis* was a family of 2,3-diacyltrehaloses.

The acidic glycolipid was analyzed by thin-layer chromatography and exhibited the same chromatographic mobility as did the major sulfatide (SL-I) of *M. tuberculosis* (16). After a desulfation experiment, the chromatographic mobility of the purified glycolipid shifted from an $R_f$ value of 0.20 to 0.74 with $CHCl_3$-$CH_3OH$ (9:1 [vol/vol]) as the developing solvent, further substantiating that it belonged to the family of mycobacterial sulfatides (16). The purified glycolipid contained 2% 3-OH-FAs. The $^1$H-NMR spectrum of the acidic glycolipid (Fig. 2) showed the occurrence of trehalose and several acylated positions of this sugar in the region of anomeric and other deshielded protons (4.0 to 5.5 ppm). The two doublets at 5.29 and 5.51 ppm ($J_{1,2}$ = 3.5 Hz) were attributed to the resonances of the anomeric protons of α,α′-trehalose (9, 11). The acylated positions, as well as the other ring proton resonances, were identified by a two-dimensional chemical-shift correlated spectroscopy experiment (Fig. 2). The anomeric proton resonance at 5.29 ppm showed a cross peak with the H-2 resonance at 5.00 ppm ($J_{1,2}$ = 3.5 Hz; $J_{2,3}$ = 9.5 Hz), which in turn gave a cross peak with the H-3 resonance at 5.49 ppm ($J_{2,3}$ = $J_{3,4}$ = 9.5 Hz). This latter signal overlapped with the doublet of H-1′ at 5.41. The H-3 resonance showed a cross peak with the H-4 resonance at 3.68 ppm ($J_{3,4}$ = $J_{4,5}$ = 9.5 Hz). Likewise, the resonances of H-5 and H-6 were identified at 4.36 and 4.46 ppm, respectively. The deshielded chemical shift of the H-2, H-3, and H-6 resonances, but not that of H-4, demonstrated that the glucosyl residue was acylated at positions 2, 3, and 6 (9–11). Similarly, the chemical shifts of the proton resonances of the other glucosyl unit were determined.

The H-1′ through H-6′ resonances were found at 5.51, 4.30, 4.01, 3.42, 3.88, and 4.31 ppm, respectively. The deshielded resonance of H-2′ indicated the location of the sulfate group (23) and that of H-6 proved the acylation of position 6 of the glucosyl residue (10, 11). It followed then that the 3-OH-FA-containing acidic glycolipid was 2,3,6,6′-tetra-acyl-2′ sulfate trehalose, the so-called sulfatide I of *M. tuberculosis* (16). The 3-OH-FAs consisted mainly of trimethyl branched-chain 3-OH-$C_{24}$. The presence of this type of 3-OH-FA was not previously reported in this family of compounds, probably because of the relatively small quantities of these acyl substituents in sulfatides. Instead, multi-Me- branched-chain 17-OH-FAs (hydroxyphthioceranates) were reported to be major components of sulfatides (16). The identification of multi-Me branched-chain 3-OH-FAs in the methanolysis products of virulent strains of *M. tuberculosis*, and not in those of an avirulent strain of the tubercle bacillus and of representative strains of nontuberculous mycobacteria (2), may now be explained, in view of the data herein presented, by the absence of DAT and sulfatides in the nontuberculous mycobacteria. Sulfatides are known to be specific to virulent tubercle bacilli (16), and DAT has been shown to be absent from the lipid extracts of 39 mycobacterial species (25). Although DAT has been characterized in *M. fortuitum* (3, 15), its acyl substituents are different from those of *M. tuberculosis*. The purified DAT of *M. fortuitum* is devoid of multi-Me branched-chain 3-OH-FAs and contains small amounts (2.5%) of 2-Me-3-OH-FAs (1). In *M. tuberculosis*, the amounts of multi-Me-3-OH-FAs can be expected to vary between individual strains depending on their sulfatides and DAT content, as not all the tubercle bacilli contain DAT (22).

The fraction eluted from the florisil column with $CHCl_3$-$CH_3OH$-$H_2O$ contained mainly compounds which readily reacted with the Dittmer-Lester reagent, demonstrating the presence of phosphorus. HPLC of this fraction (data not shown) gave three major phosphorus-containing lipids. The three phospholipids were identified as phosphatidyl ethanolamine (PE), diphosphatidyl glycerol and phosphatidyl inositol mannosides, respectively, by their chromatographic mobility on thin-layer chromatography and their reactions with anthrone and ninhydrin. PE was the only one containing 3-OH-FAs; the main fatty acyl substituents were identified as $C_{16:0}$ and 10-Me-$C_{18}$ (tuberculostearate). The 3-OH-FAs were composed of 3-OH-$C_{16}$, 3-OH-$C_{18}$ and 3-OH-$C_{20}$; small amounts of 3-OH-$C_{22}$ were also detected. It has to be noted that in the mycobacterial world straight-chain 3-OH-FAs have been previously described only, in an amide-linked form, in ornithine-containing lipids (19) and C-mycoside type glycopeptidolipids of some mycobacterial species (8). To the best of our knowledge, the present study is the first report of the presence of an ester-linked straight-chain 3-OH-FA-containing lipid in mycobacteria. The analysis of the phospholipids of *M. fortuitum* also demonstrated that straight-chain 3-OH-FAs were present only in PE (1). This observation has to be related to the previous finding demonstrating that 2- or 3-OH-FAs were present in PE but not in other phospholipids of members of the orders of *Actinomycetales* (20, 28) and *Amycolatopsis* (29). Finally, the occurrence of 3-OH-FAs in PE of mycobacteria merits further comments. It has to be recalled that these fatty acids are considered to be the precursors of straight-chain fatty acids (4). In this context, the isolation of 3-oxo-2-tetradecyloctadecanoate-containing phospholipid in a cell extract of *Corynebacterium diphtheriae* (13), thought to be the acyl carrier-containing product of a Claisen-type condensation leading to corynomycolic acid, suggests that the 3-OH-FA-containing PE may play a role in the biosynthesis of complex fatty acids in actinomycetes, including mycobacteria.

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