Characterization of phthiocerol and phthiodiolone dimycocerosate esters of \textit{M. tuberculosis} by multiple-stage linear ion-trap MS$^\text{\upshape a, g}$

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Abstract Both phthiocerol/phthiodiolone dimycocerosate (PDIM) and phenolic glycolipids are abundant virulent lipids in the cell wall of various pathogenic mycobacteria, which can synthesize a wide range of complex high-molecular-mass lipids. In this article, we describe linear ion-trap MS$^\text{\upshape n}$ mass spectrometric approach for structural study of PDIMs, which were desorbed as the [M + Li]$^+$ and [M + NH$_4$]$^+$ ions by ESI. We also applied charge-switch strategy to convert the mycocerosic acid substituents to their N-(4-aminomethylphenyl) pyridinium (AMPP) derivatives and analyzed them as M$^+$ ions, following alkaline hydrolysis of the PDIM to release mycocerosic acids.$^\text{24}$ The structural information from MS$^\text{\upshape n}$ on the [M + Li]$^+$ and [M + NH$_4$]$^+$ molecular species and on the M$^+$ ions of the mycocerosic acid-AMPP derivative affords realization of the complex structures of PDIMs in \textit{Mycobacterium tuberculosis} biofilm, differentiation of phthiocerol and phthiodiolone lipid families and complete structure identification, including the phthiocerol and phthiodiolone backbonees, and the mycocerosic acid substituents, including the locations of their multiple methyl side chains, can be achieved.$^\text{24}$—Flentie, K. N., C. L. Stallings, J. Turk, A. J. Minnaard, and F-F. Hsu. Characterization of phthiocerol and phthiodiolone dimycocerosate esters of \textit{M. tuberculosis} by multiple-stage linear ion-trap MS. \textit{J. Lipid Res.} \textit{2016}, \textit{57}: 142–155.

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Both phthiocerol/phthiodiolone dimycocerosate (PDIM) esters and phenolic glycolipids (PGLs) are dimycocerosate esters (DIMs) produced by pathogenic mycobacteria. PDIMs were originally isolated from \textit{Mycobacterium tuberculosis} (1–4) and are specific tuberculosis biomarkers (5, 6). They are among the most abundant lipids in the cell wall of various pathogenic mycobacteria, including \textit{M. tuberculosis}, \textit{M. bovis}, \textit{M. leprae}, \textit{M. kansasii}, \textit{M. microti}, and \textit{M. marinum} (7–10), which are known to synthesize a range of complex high-molecular-mass lipids (7). PGLs are produced by the same set of pathogenic mycobacteria species, except that in \textit{M. tuberculosis} only a subset of clinical isolates belonging to the W-Beijing family (11) produces PGLs.

Phthiocerol and phenolphthiocerol and other lipids such as mycolic acids and methyl-branched FAs in cell wall are among those that have been most extensively studied in terms of their biosynthesis and the role in \textit{M. tuberculosis} virulence in vivo (12). In pathogenesis, the role of PDIMs of \textit{M. tuberculosis} was recognized by the studies that identified that mutants of \textit{M. tuberculosis} were unable to either produce or properly localize PDIMs to the cell envelope—and that demonstrated that PDIM-deficient strains were attenuated in animal models of infection (8, 11, 13–17). Recently, \textit{M. tuberculosis} and its close pathogenic relative \textit{M. marinum} were reported to manipulate macrophage recruitment through coordinated use of membrane PDIM and PGLs to initiate infections (18). However, the precise role of these molecules in the course of infection remains largely unknown, and their role in the multiplication of mycobacteria from the tuberculosis complex in organs other than the lungs is also unclear.

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Abbreviations: AMPP, N-(4-aminomethylphenyl) pyridinium; CID, collision-induced dissociation; LIT, linear ion trap; PDIM, phthiocerol/phthiodiolone dimycocerosate; PGL, phenolic glycolipid.$^\text{1}$

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PDIM (Fig. 1) and PGL respectively consist of a long-chain 3-methoxy, 4-methyl, 9,11-dihydroxy glycol (phthiocerol) and a p-glycosylated phenylglycol (glycosyl phenolphthiocerol) backbone diesterified with di-, tri-, and tetra-methyl-branched long-chain mycocerosic (mycoceranic) acids (10, 19, 20). Dependent on the species, the long-chain diol backbone ranges in size from C_{25} to C_{36}, and the mycocerosic acid chain ranges from C_{25} or C_{24} to C_{32} (3, 4, 10, 21, 22). Other variants with 3-keto or 3-hydroxy diols also exist. The phthiocerol family consists of phthiocerol A, phthiocerol B, phthiodiolone A, and phthiotoiol A, of which phthiocerol A and phthiodiolone A are the most commonly present. In M. marinum, a major analogous PGL family consisting of the long-chain β-diol backbone modified with a phenolic group at the terminal, to which a 3-O-methylhamnose is β-O-linked to the phenol ring, is also present (9, 23).

MS has played important roles in the elucidation of the structures of PDIMs and PGLs. For example, Minnikin et al. (10, 24–26) and Daffe et al. (9, 27–29) used GC/MS analysis together with NMR spectroscopy for complete characterization of DIMs, following extraction steps, chromatographic separations, and chemical reactions. Recently, MALDI-TOF (16, 29, 30) and ESI-Fourier transform ion cyclotron resonance (31) MS have also been applied for profiling PDIM and PGLs. However, the ESI/MS/MS method useful for direct structural identification of these complex lipids has not been established. Here, we describe MS linear ion trap (LIT) with high-resolution MS toward characterization of the structures of PDIMs, which were desorbed as the [M + Alk]^+ (Alk = Li, Na, NH₄) ions by ESI. This mass spectrometric approach affords realization of the structures of this lipid family isolated from the biofilm formed by M. tuberculosis, including the identities of the mycocerosic acid side chains and the phthiocerol backbone.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals and solvents were purchased from Fisher Scientific (Waltham, MA). Standard C_{26}/C_{30}-mC_{26:0} PDIM (refer to Nomenclature) was synthesized as previously described (32).

**Bacterial strains and growth conditions**

*M. tuberculosis* Erdman was cultured at 37°C in Middlebrook 7H9 or Middlebrook 7H10 agar plates supplemented with 60 μl/1 oleic acid, 5 g/1 BSA, 2 g/1 dextrose, 0.005 g/l catalase, 0.5% glycerol, and 0.05% Tween 80 (broth) or in Sauton’s liquid medium unless otherwise indicated. Bacterial biofilms were inoculated with stationary phase planktonic cultures into Sauton’s medium unless otherwise indicated. Bacterial biofilms were harvested at the indicated time, pelleted, and boiled for 30 min. Samples were then extracted twice by adding chloroform-methanol (2:1), sonicating for 5 min, incubating 1 h, and centrifuging, and the organic phase from two extractions (twice) was pooled and dried under nitrogen gas. Lipids were dissolved in chloroform-methanol (2:1) before analysis.

**LC/MS fractionation of PDIMs**

Preparative HPLC experiments were carried out using a Thermo Scientific (San Jose, CA) TSQ Vantage mass spectrometer with Thermo Accela UPLC operated by Xcalibur software. Separation of lipid was achieved by a Supelco 100 × 2.1 mm (2.7 μm particle size) Ascentis C-8 column at a flow rate of 260 μl/min. The mobile phase contained 10 mM ammonium formate (pH 5.0) in solvent A-acetonitrile-water (60:40, v/v), solvent B-2-propanol-acetonitrile (90:10, v/v); a gradient elution in the following manner was applied: 68% A, 0–1.5 min; 68–55% A, 1.5–4 min; 55–48% A, 4–5 min; 48–42% A, 5–8 min; 42–34% A, 8–11 min; 34–30% A, 11–14 min; 30–25% A, 14–18 min; 25–3% A, 18–23 min; 3–0% A, 25–30 min; 0% A, 30–35 min; 68% A, 35–40 min. During fractionation, ~95% of the lipid was sent to a fraction collector, and a small percentage (5%) of the lipid was sent to the mass spectrometer via a tee to identify the structure. The PDIM fraction was eluted at 30.5–34.5 min, and family of phthiocerol dimycocerosates eluted earlier than that of phthiodiolone dimycocerosate (see supplementary Fig. 1B). PDIM fractions from several injections were collected and pooled, dried under a stream of nitrogen, and subjected to further structural analyses as described subsequently.

**Alkaline hydrolysis and preparation of N-(4-aminomethylphenyl) pyridiniumderivative with reagent**

Alkaline hydrolysis to yield mycoceric acids from PDIM was carried out following the protocol as previously described (34) with modification. To the tube containing dried PDIM, 400 μl methanol, 200 μl diethylether, and 200 μl tetrahydroammonium hydroxide (40 wt% solution in water) were added. The tube was capped and heated at 100°C overnight and cooled to room temperature, and 50 μl HCl (37%) was added. Following addition of 1 ml water and 1 ml chloroform, vortexing for 1 min, and centrifuging at 1,200 g for 2 min, the organic layer was transferred to another tube and was washed twice with 1 ml water. The final organic layer containing FFAs was dried under nitrogen, and subjected to further structural analyses as described subsequently.
collision energy ranging from 30% to 45%, an activation in the Orbitrap mass analyzer. Mass spectra were accumulated in the ion trap or high-resolution accurate mass detection of monoisotopic ion to the ion trap for CID for unit resolution. Residual abundance of precursor ion ( Δm/z = 0.25), and the activation time at 10 ms to leave a minimal residue of the precursor ion ( Δm/z = 0.20). During the mass selection window for the precursor ion, was set at 1 Da wide to admit the monoisotopic ion to the ion trap for CID for unit resolution detection in the ion trap or high-resolution accurate mass detection in the Orbitrap mass analyzer. 

MS

Both high-resolution (R = 100,000 at m/z 400) higher energy collision activation dissociation and low-energy collision-induced dissociation (CID) MS/MS experiments were conducted on a Thermo Scientific LTQ Orbitrap Velos mass spectrometer with Xcalibur operating system. Lipid extracts in chloroform-methanol (2:1) were infused (1.5 µl/min) to the ESI source, where the skimmer was set at ground potential, the electrospray needle was set at 4.0 kV, and the temperature of the heated capillary was 300°C. The automatic gain control of the ion trap was set to 5 × 10^4, with skimmer was set at ground potential, the electrospray needle was set at 4.0 kV, and temperature of the heated capillary was 300°C. The organic layer was transferred to another vial, dried under a stream of nitrogen, and stored at −20°C until use.

Nomenclature

For simplicity, the terms phthiocerol dimycocerosate and phthioglycol, abbreviated as PDIM, will be used for all the families of the parent waxes without implying one particular stereochemistry for the component of the multimethyl-branched acids. The term phthioglycol, a modification of Sten dal’s original nomenclature (35), will be used to refer to the family of compounds, and the term phthiocerol will be reserved for the original 3-methoxy congener. Thus, the abbreviation of the phthiocerol backbone of a PDIM, for example, the 3-methoxy congener. The observation of the ions at m/z 519 (loss of 32:0-FA substituent, and at 487 (519 – CH_3OH) corresponding to loss of CH_3OH residue, supports the notion that the phthiocerol possesses a methoxy side chain, and the molecule belongs to the 3-methoxy 4-methyl PDIM family. It appeared that the 32:0-FA substituent at C11 was first cleaved from the phthiocerol backbone via a charge-remote fragmentation process to yield a lithiated ion of m/z 1,006.1 (Fig. 2A), which possesses a double bond at C-11 (Scheme 1). This is followed by elimination of the remaining 32:0-FA at C9 to form the lithiated ion of m/z 525 that consists of a conjugated double bond at C9 and C11. This assumption is based on the findings that the MS^3 spectrum of the ion of m/z 525 (1,486 → 1,006–525; Fig. 2E) contained the major ion at m/z 968, arising from further loss of CH_3OH, along with the ions at m/z 519 from additional loss of the 32:0-FA substituent, and at m/z 487 (519 – CH_3OH) arising from further loss of the methoxy group. The losses of the FA substituents and NH_3, and methanol were supported by high-resolution MS (data not shown). The observation of the ions at m/z 968 (1,000 – CH_3OH) and at 487 (519 – CH_3OH) corresponding to loss of CH_3OH residue, supports the notion that the phthiocerol possesses a methoxy side chain, and the molecule belongs to the 3-methoxy 4-methyl PDIM family.

RESULTS

CID MS^n on the 3-methoxy, 4-methyl, 9,11-di(3,5,7,9-tetramethyloctaicosanoyl) tetraatriacontanediol (C_{32a}/C_{32a}mC_{35a}PDIM) standard

PDIMs readily form [M + Alk]^+ ions (Alk = Li, Na, NH_3) when subjected to ESI in the presence of alkaline ion in positive-ion mode. Corresponding ions in the fashion of [M + X]^+ (X = Cl, HCO_2) were also observed in the negative-ion mode; however, CID MS^n on these latter ions failed to provide sufficient information for structural identification and will not be discussed further. To gain insight into the fragmentation processes, we first performed MS^n on the [M + Li]^+ and [M + Na]^+ adduct ions of C_{32a}/C_{32a}mC_{35a}PDIM standard to explore their utilities toward structural determination of PDIMs. As shown in Fig. 2A, the MS^n spectrum of the [M + Li]^+ ion at m/z 1,486.5 contained ions at m/z 1,006.0 arising from loss of 3,5,7,9-tetramethyloctaicosanoic acid residue (32:0) and at m/z 525 (1,006.0–480.5) arising from further loss of the remaining 32:0-FA substituent (480.5 Da), along with ion at m/z 487, representing a lithiated 32:0-FA cation. The consecutive loss of the FA substituent was further supported by the MS^2 spectrum of the ions of m/z 1,006 (1,486 → 1,006; Fig. 2C), which contained major ions at m/z 525 and 487. Similarly, the MS^2 spectrum of the corresponding [M+ Na]^+ ions at m/z 1,502.5 (Fig. 2B) contained ions at m/z 1,022.1, 541, and 503 that are 16 Da heavier, and the MS^3 spectrum of the ions of m/z 1,022 (1,502 → 1,022; data not shown) also contains the ions of m/z 541 and 503, which are also 16 Da heavier, consistent with the losses of the 32:0-FA moieties.

In contrast, the MS^2 spectrum of the [M+ NH_4]^+ ion at m/z 1,497.5 (Fig. 2D) is dominated by the ion at m/z 1,000 arising from losses of the 3,5,7,9-tetramethyloctaicosanoic acid and NH_3, similar to that observed for the [M + NH_4]^+ ion of triacylglycerol (TAG) (36). The spectrum also contained the ion at m/z 968, arising from further loss of CH_3OH, along with the ions at m/z 519 from additional loss of the 32:0-FA substituent, and at m/z 487 (519 – CH_3OH) arising from further loss of the methoxy group. The losses of the FA substituents and NH_3, and methanol were supported by high-resolution MS (data not shown). The observation of the ions at m/z 968 (1,000 – CH_3OH) and at 487 (519 – CH_3OH) corresponding to loss of CH_3OH residue, supports the notion that the phthiocerol possesses a methoxy side chain, and the molecule belongs to the 3-methoxy 4-methyl PDIM family.
Fig. 2. The MS² spectra of the [M + Li]⁺ ion of C₃₂₀/C₃₂₋₃nC₄₄₀ PDIM at m/z 1,486.5 (A), of the corresponding [M + Na]⁺ ions at m/z 1,502.5 (B), and the consecutive MS³ spectrum of the ion of m/z 1,006.1 (1,486.5 → 1,006.1) (C); the MS³ spectrum of the ion of 525 (1,486.5 → 1,006.1 → 525) (E) from the [M + Li]⁺ ion. Panel D shows the MS² spectrum of the corresponding [M + NH₄⁺]⁺ ion at m/z 1,497.5, and the MS³ spectrum of the ion of m/z 1,000.1 (1,497.5 → 1,000.1) (F), MS⁴ spectrum of the ion of m/z 487 (1,497.5 → 1,000.1 → 519 → 487) (G), MS⁵ spectrum of the ions of m/z 417 (1,497.5 → 1,000.1 → 521 → 487 → 417) (H) support the fragmentation processes depicted in Scheme 2. Please also note that the mass discrepancy of the labeling in all the figures is due to mass defect.
of the ion at \( m/z \) 481, representing a protonated 32:0-FA ion, in Fig. 2F.

The dissociation of the ion of \( m/z \) 1,000 (1,497 → 1,000; Fig. 2F) eliminates the 32:0-FA substituent at C9 to give rise to the major ion of \( m/z \) 519, which may represent a protonated phthiocerol ion possessing a conjugated double bond with proton relocated at the methoxy side chain. This is followed by loss of a methanol molecule to yield the ion of \( m/z \) 487, in which the charge site may situate at C7 via hydrogen shift. These fragmentation processes leading to the ions of \( m/z \) 487 were supported by the MS\(^1\) spectrum of the ion of \( m/z \) 519 (1,497 → 1,000 → 519; data not shown). The loss of the 32:0-FA substituent is also consistent with the observation of the ion at \( m/z \) 481, representing a protonated 32:0-FA ion, in Fig. 2F.

The dissociation of the ion of \( m/z \) 487 (1,497 → 1,000 → 521 → 487; Fig. 2G) gave rise to the ion series of \( m/z \) 445, 431, 417, 403, 389, 375, and so forth, along with the ion series of \( m/z \) 151, 165, 179, 193, 207, 221, and so forth (intensity in the descending order), arising from cleavages of the C-C bonds of phthiocerol chain. The observation of these two ion series is consistent with the notion that the charge may primarily reside at C7, and cleavages of these C-C bonds may be similar to the “mobile proton” model (37, 38), in which more than one charge sites are energetically and/or kinetically favored due to proton rearrangement.

Scheme 1. The fragmentation processes proposed for the [M + Li\(^+\)] ion of 32:0/32:0-m35:0-PDIM at \( m/z \) 1,486.

Scheme 2. The fragmentation process proposed for the [M + NH\(_4\)]\(^+\) ion of 32:0/32:0-m35:0-PDIM at \( m/z \) 1,497.
along the phthiocerol backbone. For example, C-C bond cleavages distal to the methyl side chain terminal from precursor ions possessing charge located at C7 gave rise to ions at m/z 195, 207, 221, 235, and so forth; while ions at m/z 445, 431, 417, 403, 389, and so forth arose from C-C cleavages from the methyl side chain terminal. Further dissociations of the ions of m/z 431, 417, and 403 also gave rise to ions of m/z 389, 375, and 361 by loss of propylene residue (39); while ions of m/z 151 (loss of C19-alkene), 137 (loss of C20-alkene), and 123 (loss of 21:0-alkene) can also arise from, for example, m/z 417 by consecutive fragmentation processes that eliminate an alkene residue (Scheme 2). These fragmentation processes are supported by MS\(^5\) spectra of the ions of m/z 431 (1,497 → 1,000 → 521 → 487 → 417; Fig. 2H), and 403 (1,497 → 1,000 → 521 → 487 → 403; not shown), which contained the whole array of ions arising from cleavages of C-C bonds. Similarly, fragmentations resulting from precursor ion holding charge... 

**Fig. 3.** The MS\(^5\) spectra of the [M + Li]\(^+\) ion of C\(_{29:0}/C\(_{29:0} - kC\(_{35:0}\) PDIM at m/z 1,386.4 (A), MS\(^3\) spectra of the ions of m/z 509 (1,386 → 509) (C), and of m/z 481 (1,400.5 → 481) (from C\(_{29:0}/C\(_{29:0} - kC\(_{32:0}\) PDIM at m/z 1,397.5 (B), its MS\(^5\) spectrum of the ion of m/z 942 (1,397 → 942) (D), and MS\(^3\) spectrum of the ion of m/z 485 (1,397 → 942 → 503 → 485) (F).
ions of m/z 948 arising from loss of the 29:0-FA substituent at C11, and of m/z 509 arising from further loss of the remaining 29:0-FA at C9 (Scheme 3). The speculation of the preferential loss of FA substituent at C11 is consistent with the observation of the ion of m/z 215 in the MS3 spectrum of the ion of m/z 509 (1,386 → 509; Fig. 3C). This ion is equivalent to the ion of m/z 231 in Fig. 2E, arising from cleavage of allylic bond (Scheme 3) similar to that observed for the methoxy-PDIM family (Scheme 1). The spectrum (Fig. 3C) also contained the ions of m/z 385 (another allylic cleavage), and of m/z 399, 481 (cleavage of C2-CO bond), 491 (509 – H2O), and 423 (β-cleavage with γ-H rearrangement) (Scheme 3), indicating that the ion of m/z 509 may represent a lithiated Δ9,11 k35:0 phthiocerol. Similar fragmentation processes (Scheme 3) were also seen in the MS2 spectrum of the analogous ion of m/z 481 (Fig. 3E), representing a lithiated Δ9,11 k33:0 phthiocerol arising from the [M + Li]+ ion of m/z 1,400 consisting of the 29:0/32:0-k33:0 major isomer together with 29:0/30:0-k35:0 and 27:0/32:0-k35:0 minor isomers (equivalent to the NH4+ adduct ion of m/z 1,411 in Table 1; the structural assignments of the 29:0/32:0-k33:0, 29:0/30:0-k35:0, and 27:0/32:0-k35:0 isomers can be found in supplementary Fig. 2).

The MS2 spectrum of the [M+ NH4]+ ion at m/z 1,397 (Fig. 3B), again, contained the prominent ion of m/z 941.9, arising from expulsion of 29:0-FA and NH3, and further loss of the remaining 29:0-FA gave rise to the ion of m/z 503 (Fig. 3D), in which the charge site is relocated at the carbonyl group (Scheme 4). The ion at m/z 485 arose from additional loss of a water molecule (Fig. 3D), probably involving a prior 1,6-H shift to yield a carbonium ion with 3-OH side chain, whose charge may reside at C-7. This is followed by loss of a water molecule to form a stable allylic carbonium ion of m/z 485 with the participation of the adjacent hydrogen at C-4 (Scheme 4). These fragmentation processes are supported by the MS3 spectrum of the ion of m/z 941.9 (1,397 → 942; Fig. 3D), and the MS4 spectrum of the ion of m/z 503 (not shown).

Further dissociation of the ion of m/z 485 (1,397 → 941 → 503 → 485; Fig. 3F) gave rise to the ion series of m/z 457, 443, 429, 415, and 401, … , and so forth, in which the ion of m/z 457 is likely arising from loss of an ethane residue, in contrast to loss of a C3-alkane observed for the ion of m/z 487 originated from a methoxy-PDIM compound as shown in Fig. 2G. The spectrum also contained the ion series of m/z 403, 389, 375, 361, and so forth, consistent with the notion that elimination of a water molecule involves the participation of the hydrogen at C-4.

**Scheme 3.** The fragmentation processes proposed for the [M + Li]+ ion of 29:0/29:0-k35:0-PDIM at m/z 1,386.
with m/z 459 (491 – CH₂OH) and 897.9 (929.9 – CH₂OH) arising from further loss of CH₂OH. The results indicate that the molecule belongs to the PDIM family consisting of a major 29:0/29:0-m33:0 species. The spectrum also contains the 519/487 ion pair, signifying the presence of 29:0-k33:0-PDIM. The results indicate that the ions m/z 1,385 also represent 30:0/26:0-m35:0; 30:0/27:0-m34:0; 30:0/28:0-m33:0; and 30:0/29:0-m32:0 minor isomers. The assignment of 30:0/26:0-m35:0 isomer is consistent with the observation of the ion of m/z 972.1 arising from loss of 26:0-FA in Fig. 4A. The MS³ spectrum of m/z 972 (1,385 → 972; Fig. 4E) contained the m/z 519/487 ion pairs indicating the presence of 29:0/27:0-m35:0 PDIM isomer. In Fig. 4A, ions at m/z 915.9, arising from loss of 30:0-FA acid substituent, were also observed. MS³ on the ions of m/z 915.9 (1,385 → 915; Fig. 4D) yielded ion pairs of 519/487, 505/473, 491/459, 477/445, reflecting the presence of 26:0/m35:0, 27:0/m34:0, 28:0/m33:0, 29:0/m32:0 substituents, respectively. The results indicate that the ions of m/z 1,385 also represent 30:0/26:0-m35:0; 30:0/27:0-m34:0; 30:0/28:0-m33:0; and 30:0/29:0-m32:0 minor isomers. The assignment of 30:0/26:0-m35:0 isomer is consistent with the earlier identification

### TABLE 1. The molecular species of PDIMs ([M + NH₄⁺]⁺) from M. tuberculosis identified by LIT MS with high-resolution MS

| Measured m/z | Theoretical Mass | Deviation (%) | Relative Intensity | Composition | Major Structures | Minor Structures |
|--------------|------------------|--------------|-------------------|-------------|-----------------|-----------------|
| 1,245.2759   | 1,245.2759       | 0.11         | 0.27              | C₈₂H₁₆⁶O₅N  | 27:0/27:0-m27:0  |                |
| 1,257.2759   | 1,257.2759       | 0.32         | 0.32              | C₈₃H₁₆⁶O₅N  | 27:0/27:0-m29:0  |                |
| 1,273.3074   | 1,273.3072       | 0.23         | 0.41              | C₈₄H₁₇⁶O₅N  | 27:0/27:0-m32:0  |                |
| 1,285.3074   | 1,285.3074       | 0             | 0.67              | C₈₅H₁₇⁶O₅N  | 27:0/27:0-m34:0  |                |
| 1,299.3235   | 1,299.3235       | 0.52         | 0.71              | C₈₆H₁₇⁶O₅N  | 26:0/29:0-m31:0  |                |
| 1,313.3391   | 1,313.3387       | 0.41         | 1.52              | C₈₇H₁⁵⁷O₅N  | 27:0/27:0-m33:0  |                |
| 1,327.3543   | 1,327.3543       | 0.12         | 1.96              | C₈₈H₁⁷⁶O₅N  | 26:0/29:0-m33:0  |                |
| 1,341.3700   | 1,341.3700       | 0.06         | 8                 | C₈₉H₁⁷⁶O₅N  | 27:0/29:0-m33:0  |                |
| 1,355.3856   | 1,355.3856       | 0.03         | 11.12             | C₉₀H₁⁸⁸O₅N  | 29:0/29:0-m32:0  |                |
| 1,357.4038   | 1,357.4013       | 1.73         | 10.95             | C₉₀H₁⁸⁸O₅N  | 27:0/29:0-m33:0  |                |
| 1,369.4012   | 1,369.4013       | 0.04         | 45.27             | C₉₁H₁⁸⁸O₅N  | 29:0/29:0-m33:0  |                |
| 1,371.4176   | 1,371.4169       | 0.69         | 14.47             | C₉₁H₁⁸⁸O₅N  | 29:0/29:0-m33:0  |                |
| 1,383.4370   | 1,383.4369       | 0.07         | 23.97             | C₉₂H₁⁸⁸O₅N  | 29:0/30:0-m33:0  |                |
| 1,385.4327   | 1,385.4326       | 0.12         | 61.93             | C₉₂H₁⁸⁸O₅N  | 29:0/29:0-m33:0  |                |
| 1,397.4322   | 1,397.4326       | 0.37         | 55.45             | C₉₃H₁⁹⁸O₅N  | 29:0/29:0-m33:0  |                |
| 1,409.4488   | 1,409.4482       | 0.63         | 27.54             | C₉₃H₁⁹⁸O₅N  | 29:0/30:0-m33:0  |                |
| 1,413.4614   | 1,413.4612       | 0.57         | 58.04             | C₉₄H₁⁹⁸O₅N  | 29:0/30:0-m33:0  |                |
| 1,425.4647   | 1,425.4639       | 0.84         | 21.92             | C₉₅H₁⁹⁸O₅N  | 29:0/30:0-m33:0  |                |
| 1,457.4806   | 1,457.4795       | 1.13         | 52.4              | C₉₅H₁⁹⁸O₅N  | 29:0/30:0-m33:0  |                |
| 1,463.4957   | 1,463.4952       | 0.53         | 34.67             | C₉₇H₁⁹⁸O₅N  | 32:0/32:0-m33:0  |                |
| 1,477.5114   | 1,477.5108       | 0.6           | 57.3              | C₉₇H₁⁹⁸O₅N  | 29:0/30:0-m33:0  |                |
| 1,483.5108   | 1,483.5104       | 0.49         | 9.6               | C₉₈H₁⁹⁸O₅N  | 32:0/30:0-m33:0  |                |
| 1,499.5273   | 1,499.5265       | 0.86         | 29.83             | C₉₈H₁⁹⁸O₅N  | 30:0/32:0-m33:0  |                |
| 1,505.5268   | 1,505.5254       | 0.32         | 35.06             | C₉₉H₁⁹⁸O₅N  | 32:0/30:0-m33:0  |                |
| 1,455.5273   | 1,455.5265       | 0.6           | 57.3              | C₉₇H₁⁹⁸O₅N  | 29:0/30:0-m33:0  |                |
| 1,505.5268   | 1,505.5254       | 0.32         | 35.06             | C₉₉H₁⁹⁸O₅N  | 32:0/30:0-m33:0  |                |
| 1,499.5273   | 1,499.5265       | 0.86         | 29.83             | C₉₈H₁⁹⁸O₅N  | 30:0/32:0-m33:0  |                |
| 1,455.5273   | 1,455.5265       | 0.6           | 57.3              | C₉₇H₁⁹⁸O₅N  | 32:0/30:0-m33:0  |                |
| 1,467.5268   | 1,467.5264       | 0.49         | 9.6               | C₉₈H₁⁹⁸O₅N  | 32:0/30:0-m33:0  |                |

aAbundances in the descending order.
bMinor isomers not defined.
of 30:0/26:0-m35:0 isomer (in this assignment, fatty acyl groups at C9 and C11 are not specified). The identification of the 26:0/32:0-m33:0 isomer is also consistent with the observation of the ion of m/z 897 in Fig. 4A, arising from loss of 32:0-FA substituent, and is further supported by the MS3 spectrum of the ion of m/z 897 (1,385 → 897; data not shown), which contains the m/z 491/459 and 477/445 ion pairs.

The profiles of the MS4 spectra of the ions of m/z 487 (1,385 → 958 → 487; not shown), 473 (1,385 → 958 → 473; not shown), 459 (1,385 → 930 → 459; Fig. 4F), and 445 (1,385 → 915 → 445; not shown) are similar to that shown in Fig. 2G, supporting the notion that they represent the demethoxylated C35-, C34-, C33-, and C32-phthiocerol ions with conjugated double bond (Scheme 1).

Similar approaches were also applied to reveal the structural complexity of phthiodiolone dimycerosate family of the extract (Table 1). For example, the MS2 spectrum of the [M + NH4]+ ion at m/z 1,411.5 (Fig. 5A) contained major ions at m/z 956 and 914 arising from losses of NH3 and 29:0- and 32:0-mycosanoic acids respectively, indicating the linkage of 29:0- and 32:0-mycosanoic acids to 9- and 11- of the phthiodiolone backbone. Further dissociation of the ion of m/z 956 (1,411 → 956; Fig. 5B) gave rise to ions at m/z 475, arising from loss of the remaining 32:0-acid, consistent with the presence of the protonated 32:0-mycosanoic acid ion at m/z 481. The spectrum also consisted of the ion of m/z 457 arising from further loss of water from m/z 475. This loss of water is supported by the MS3 spectrum of the ion of m/z 475 (1,411 → 956 → 475; data not shown), which is dominated by the ion of m/z 457, indicating that the molecule belongs to the phthiodiolone family. The above structural information led to assignment of the major 29:0/32:0-k33:0 structure. Because the spectrum (Fig. 5B) also contained the ions of m/z 503 (loss of 30:0-FA) and 485 (503 → H2O), a 29:0/30:0-k35:0 isostructural structure can be defined. MS3 on the ion of m/z 914 (1,411 → 914; Fig. 5C) gave rise to the major ions of m/z 475 and 457 (475 → H2O), arising from loss of 29:0-FA substituent, consistent with the structural assignment of the 29:0/32:0-k33:0 isomer. The spectrum also contained the minor ions of m/z 503 and 485 (503 → H2O), arising from loss of 29:0-FA substituent, pointing to the presence of 29:0/30:0-k35:0 minor isomer.

In Fig. 5A, ions at m/z 984 and 942, arising from loss of 27:0- and 30:0-FA substituents, respectively, were also observed. Further dissociation of the ion of m/z 984 (1,411 → 984; Fig. 5D) gave rise to ions of m/z 503 and 485 (503 → H2O), defining the 32:0-FA substituent. These results further support the earlier assignment of the 27:0/32:0-k35:0 isomer. MS3 on the ion of m/z 942 (1,411 → 942; Fig. 5E) also yielded ions of m/z 503 and 485 (503 → H2O), indicating the presence of 29:0-FA substituent, and the k35:0-phthiodiolone chain, and confirming the assigned 29:0/30:0-k35:0 structure as described earlier.

The MS5 spectra of the ions of m/z 485 stemming from m/z 942 (1,411 → 942 → 503 → 485), 984 (1,411 → 984 → 503 → 485), and 956 (1,411 → 956 → 503 → 485) are identical to that shown in Fig. 3D, and the profiles of the MS5 spectra of the ions of m/z 457 originating from m/z 914 (1,411 → 914 → 475 → 457; Fig. 5F) and 956 (1,411 → 956 → 475 → 457; not shown) are also identical. These spectra comprise the ion series defining the k35- and k33:0-phthiodiolone chains, respectively. These results led to
the notion that the ion of m/z 1,411 consists of a major 29:0/32:0-k33:0 isomer together with minor isomers of 27:0/32:0-k35:0 and 29:0/30:0-k35:0 (Table 1).

Characterization of multiple-methyl-branched long-chain mycocerosic (mycoceranic) acid substituents

To assign the structure of mycoceranic acid substituents, released free acids by alkaline hydrolysis were converted to the AMPP derivatives and subjected to ESI MS\textsuperscript{n} analysis in the positive-ion mode. Three major species were observed at m/z 647, 619, and 605, corresponding to C32-, C30-, and C29-FA AMPP derivatives, respectively. The MS\textsuperscript{2} spectra of m/z 647 (Fig. 6A), 619 (Fig. 6B), and 605 (Fig. 6C) all contained abundant ions at m/z 183 and 169, which are characteristic of FA-AMPP derivatives (40–44). The MS\textsuperscript{2} spectrum of the ion of m/z 647 (Fig. 6A) also contained the ion series at m/z 365, 323, 281, and 239, along with the ion series of m/z 351, 295, and 253, pointing to the position of the methyl side chains at C-2, 4, 6, and 8 of 32:0-mycoceranic acid (see insets for fragmentation pathways), and gave assignment of 2,4,6,8-tetramethyl-octaeicosanoic acid structure. The MS\textsuperscript{2} spectrum of the ion of m/z 619 (Fig. 6B) contained the similar ion series, indicating the presence of 2, 4, 6, 8-tetramethyl-hexaeicosanoic acid (30:0-mycoceranic acid). In contrast, the MS\textsuperscript{2} spectrum of the ion of m/z 605 (Fig. 6C) contained the ion series of...
at C11 than that at C9 upon CID as observed in this study may be applicable for specifically defining the mycoceranic acid substituents on the phthiocerol backbone (at C9 or C11). However, more studies with standard compounds with two different mycoceranic acid chains are required to confirm this finding. In the negative-ion mode, in contrast, the MS\textsuperscript{n} spectra obtained from the [M + X]/H\textsubscript{11002} adduct ions do not provide sufficient informative ions applicable for structure identification, despite that ions in the fashions of [M + X]/H\textsubscript{11002} (X = Cl, HCO\textsubscript{2}) are readily formed, and the elemental compositions derived from high-resolution MS are also distinguishable among the methoxy-PDIM and keto-PDIM families (data not shown).

Fragment ions arising from cleavages of C-C bonds along the phthiocerol backbone following loss of the mycoceranic acid substituents may involve the precursors in various resonance forms differed by the charge sites due to proton delocalization (37, 38). Thus, charge-remote fragmentation processes may not be invoked (44, 45), and other fragmentation processes become available (46).

**DISCUSSION**

PDIM formed alkaline metal adduct ions ([M+ Alk]\textsuperscript{+}; Alk = Li, Na, K) and [M + NH\textsubscript{4}\textsuperscript{+}] ions when subjected to ESI in the presence of Li\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+}, or NH\textsubscript{4}\textsuperscript{+}. ESI LIT MS\textsuperscript{n} with high-resolution MS on these adduct ions affords a near complete structural determination of PDIMs, revealing the presence of many homologous and isomeric structures (Table 1). Elemental compositions derived from high-resolution MS readily distinguish methoxy-PDIM and keto-PDIM molecules, while MS\textsuperscript{n} on the [M + Alk]\textsuperscript{+} adduct ions (Alk = Li, Na) and [M + NH\textsubscript{4}\textsuperscript{+}] provide spectroscopic evidence for unambiguous distinction of this two families via the specific neutral losses (i.e., methanol vs. water loss). The more facile cleavages of the mycoceranic acid substituent

![Fig. 5](image-url)
stark differences between the MS\textsuperscript{5} spectra of m/z 485 (Fig. 3F) and of m/z 487 (Fig. 2G) lies on the notion that the former spectrum contains abundant ions at m/z 191, 177, 163, 149, 135, and so forth; while the analogous ions seen at m/z 193, 179, 165, 151, 137, and so forth in the latter spectrum are less prominent (Fig. 3F). The prominence of the ion series of m/z 191, 177, 163, 149, and so forth, arising from m/z 485 (Fig. 3F) may be attributable to the fact these ions consist of one more double bond than the analogous ions of m/z 193, 179, 165, and so forth, arising from m/z 487 and are more conjugated and more stable. Striking differences were also observed for the MS\textsuperscript{5} spectra of the ions of m/z 459 arising from m34:0 (Fig. 4F) and of 457 arising from k33:0 backbones (Fig. 5F). These differences in the profiles of the MS\textsuperscript{5} spectra between those arising from phthiocerol and from phthiodiolone families also provide useful information for their structural differentiation by MS.

The advantage of characterization of mycoceranic acid substituents using charge-switch formation of the FA-AMPP derivatives is that feature ions of m/z 183 and 169 are
readily recognizable (Fig. 6) (34, 40–43), and ions from charge-remote fragmentations can locate the methyl side chains (34, 43) and the functional groups unambiguously (Fig. 6, inset). By contrast, the traditional GC/MS method for identification of mycocerosic acids is laborious, requiring acid methanolation to form the methyl ester, separated by TLC, followed by reductive degradation to mycocerosic alcohol and another two-dimensional TLC purification, before formation of the final t-butyldimethylsilyl ether derivatives (23–25). The superb sensitivity gained for the acid detected as the AMPP derivative (as compared with its underivatized form) also facilitates structural identification and quantitation (34, 40–43). This aspect may deserve further investigation.

The insight into the detailed structures of the PDIMs (Table 1) in this study also permits the realization of the structure similarities among the methoxy- and keto-PDIM families. For example, the major structures of the ions of m/z 1,369 (29:0/29:0-k30:0) and 1,385 (29:0/29:0-m30:0) all contained 29:0/29:0 substituents; the FA substituents in the species of m/z 1,411 (29:0/32:0-k30:0) of keto family and of m/z 1,427 (29:0/32:0-m30:0) in the methoxy family are all 29:0/32:0. The ESI/MS profiles of these two families are also similar (see supplementary Fig. 2C, D). The results are consistent with the notion that methoxy-PDIM is formed from keto-PDIM (14, 30, 47). It is also notable that keto-PDIMs are more prominent than the methoxy-PDIM species, while phthiodiolone dimycocerosates were the minor components previously reported in the M. tuberculosis cells (30).

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