Structural Elucidation and Antigenicity of a Novel Phenolic Glycolipid Antigen from *Mycobacterium haemophilum*†

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ABSTRACT: The structure of a novel antigenic glycolipid that distinguishes the opportunistic pathogen *Mycobacterium haemophilum* from all other mycobacteria was established by a series of degradation reactions leading to products that were analyzed by gas/liquid chromatography–mass spectrometry. The complete structure of the oligosaccharide unit was determined as 2,3-di-*O*-CH_3-α-L-Rha*p*(1→2)3-*O*-CH_3-α-L-Rhap(1→4)-2,3-di-*O*-CH_3-α-L-Rha*p*(1→. The lipid portion of the phenolic glycolipid was composed of two component phenolphthiocerols differing by two methylene groups, as determined by analysis of their per-O-trideuteriomethylated derivatives. The diol unit of the phenolphthiocerols has a threo relative configuration. The absolute stereochemistry of the asymmetric centers of the phenolphthiocerols is uncertain, but the centers are probably 3*R*, 4*S*, 9*R*, and 11*R* as found for phthiocerol A from *Mycobacterium tuberculosis*. The hydroxyl functions of the branched glycolic chain are esterified to a complex mixture of multi-methyl branched mycocerosic acids, C_{27}, C_{30}, C_{32}, C_{34}, and C_{37} with molecular weights (as methyl esters) of 424, 466, 494, 522, and 564, respectively. The stereochemistry of the methyl branches of the mycocerosates have *R* absolute configuration. The glycolipid is highly antigenic and appears to be specific for *M. haemophilum*. There are intriguing similarities between the product from *M. haemophilum* and the well-known phenolic glycolipid I of *Mycobacterium leprae*, a matter that is discussed.

Phenolic glycolipids were first observed some years ago in pathogenic mycobacteria such as *Mycobacterium bovis* (Demarteau-Ginsburg & Lederer, 1963), *Mycobacterium kansasii* (MacLennan et al., 1961), and *Mycobacterium marinum* (Ville & Gastambide-Odier, 1970). Although their aglycon structures are similar, mainly formed by a dimycocerosate of phenolphthiocerol A, the oligosaccharide units and, hence, the inherent antigenicity are species specific (Minnikin, 1982; Brennan, 1988; Daffe, 1989; Dobson et al., 1990). The discovery by Hunter et al. (1982) that the species-specific antigen of the leprosy bacillus, *Mycobacterium leprae*, is a phenolic glycolipid endowed with its own immunoreactive triglycosyl unit has renewed interest in this kind of molecule as potential antigens for serodiagnosis of mycobacterial diseases. Recently, we reported on the detection of a new characteristic phenolic glycolipid antigen from *M. haemophilum* (Besra et al., 1990a), an opportunistic pathogen first described by Sompolinsky et al. (1978) and occurring in immunosuppressed patients. In this paper, we report on the structural elucidation of the unique oligosaccharide unit of this glycolipid and features of its common phenolphthiocerol and fatty acid functions, thereby substantiating its distinctiveness and antigenicity.

Experimental Procedures

Growth of M. haemophilum. *M. haemophilum* strains isolated from human skin lesions were kindly provided by D. J. Dawson (Brisbane, Australia). Cultivation was for 1 month at 30 °C in 1-L Roux flasks containing 250 mL of Dubois broth medium supplemented with 1.5 mg/mL ferric ammonium citrate. Organisms were harvested, washed twice with water, killed by autoclaving, and freeze-dried.

Purification and Antigenicity of PGL. Apolar lipids were extracted from freeze-dried cells as previously described (Minnikin et al., 1987). The PGL,[1] the one distinctive lipid in *M. haemophilum* extracts, was purified by preparative TLC in toluene/acetone (60:40; v/v) to afford 15 mg of pure glycolipid from 2.5 g of freeze-dried cells. Rabbit serum against whole cells of *M. haemophilum* were raised as described previously (Minnikin et al., 1989, 1990). Lipid antigenicity was assessed by ELISA (Minnikin et al., 1989, 1990) where the glycolipid and serum concentrations were 1 μg/mL and 1/1000, respectively. Absorption values were read at 405 nm after 10 and 20 min.

NMR Analysis. Routine proton–proton decoupling and 2D COSY NMR spectra were recorded on a Bruker 600-MHz NMR instrument at the Chemistry Department of Edinburgh University. ^{13}C spectra were obtained on a Bruker ACE-300 NMR at the Colorado State University Department of Chemistry Central Instrument Facility. Spectra were recorded

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[1] Abbreviations: PGL, phenolic glycolipid; ELISA, enzyme-linked immunosorbent assay; 2D COSY, two-dimensional chemical shift correlated spectroscopy; GC/MS, gas chromatography–mass spectrometry; R_f, retardation factor; R_T, retention time; TLC, thin-layer chromatography; TFA, trifluoroacetic acid; Rha*p*, rhamnopyranose, Rha*f*, rhamnofuranose; Rha, rhamnose; atomic mass units

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at a concentration of 10 mg/0.5 mL of C^2HCl_3.

Gas Chromatography–Mass Spectrometry Analysis. GC/MS of alditol acetates was performed on a Hewlett-Packard 5890 gas chromtograph connected to a Hewlett-Packard 5970 mass selective detector. Samples were injected in the splitless mode. The injection port temperature was 290 °C and the transfer line 280 °C. The column used was a 12-m HP-1 (Hewlett-Packard, Avondale, PA). The oven was programmed to hold at 50 °C for 1 min followed by a 30 °C/min rise to 200 °C, an 8 °C/min rise to 320 °C, and an 8 min hold at this temperature (program 1). The temperature program that was applied to all trimethylsilyl derivatives of butyl glycosides involved a 50 °C hold for 1 min followed by a 30 °C/min rise to 140 °C, a 6 °C/min rise to 260 °C, a 30 °C/min rise to 280 °C, and a final 2-min hold at this final temperature (program 2). A modified version of program 2 was used in the lipid analysis, where the temperature was 325 °C and held at this temperature for 20 min. The mass spectrometer was set to scan from 50 to 800 amu with 0.81 scans per second.

Glycosyl Composition by Alditol Acetates. Purified PGL was hydrolyzed in 250 μL of 2 M TFA at 120 °C for 1 h as described (McNeil et al., 1987). Glycosyl residues were reduced with NaB^2H_4 and the resultant alditols per-O-acetylated as described (McNeil et al., 1987). The alditol acetates obtained were examined by GC/MS using program 1.

Absolute Configuration of Glycosyl Residues. The PGL was hydrolyzed in 1 M HCl in (*R*)-(−)-2-butanol (Aldrich Chemical Co., Milwaukee, WI), trimethylsilylated with TRI-SIL (Pierce Chemical Co., Rockford, IL) (Gerwig et al., 1978), and the trimethylsilyl (*R*)-(−)-2-butylglycosides were examined by GC/MS using program 2. PGL-I from *M. leprae* (Hunter et al., 1982) was also hydrolyzed in 1 M HCl in (*R*)-(−)-2-butanol and (*S*)-(+)-2-butanol (Aldrich Chemical Co., Milwaukee, WI), and the resulting trimethylsilylated products were compared to those from *M. haemophilum* by GC/MS.

Methylation of PGL. The PGL (2 mg) was suspended in 0.5 mL of dimethyl sulfoxide (Pierce Chemical Co., Rockford, IL), and 50 μL of 4.8 M dimethyl sulfinyl carbanion was added (Stellner et al., 1973; York et al., 1986). The reaction mixture was stirred for 1 h. C^2H_3I (Aldrich Chemical Co., Milwaukee, WI) (20 μL) was slowly added and the suspension again stirred for 1 h. The addition of base and C^2H_3I was repeated twice. An excess of C^2H_3I (45 μL) was added at the end of the third cycle. After 1 h of stirring, the reaction mixture was diluted with 0.5 mL of water and the resulting product applied to a C_{18} Sep-Pak cartridge (Waters, Milford, MA), as described (York et al., 1986). The per-O-trideuteriomethylated PGL appeared in the ethanol eluant as determined by TLC in toluene/acetone (80:20; v/v) (R_f 0.5).

Complete and Partial Hydrolysis of Per-O-trideuteriomethylated PGL. Complete hydrolysis of the PGL was accomplished in 2 M TFA at 120 °C for 1 h. Partial hydrolysis was achieved by using 250 μL of 2 M TFA at 80 °C for 30 min (Hunter et al., 1982). The resulting products from both hydrolyses were reduced with NaB^2H_4, per-*O*-acetylated, and examined by GC/MS.

Analysis of the Lipid Moiety. The PGL (1 mg) was hydrolyzed with 500 μL of 1 M HCl in methanol at 80 °C, overnight. A 100-μL aliquot of the sample was removed and the acid evaporated under a stream of filtered air. The resulting fatty acid methyl esters were examined by GC/MS under the program 2 conditions. The remaining sample was treated wtih 30% potassium hydroxide in methanol (1 mL) and toluene (1 mL) at 100 °C, overnight (Dobson et al., 1990). The reaction mixture was then acidified with dilute HCl (3 mL) and the products extracted with diethyl ether (3 × 1 mL). The organic extracts were combined, washed with water (1 mL), dried over anhydrous magnesium sulfate, filtered, and evaporated under a stream of filtered air. The products obtained were methylated by the modified Hakomori procedure (Stellner et al., 1973; York et al., 1986). The per-O-trideuteriomethylated products were examined by GC/MS using program 2.

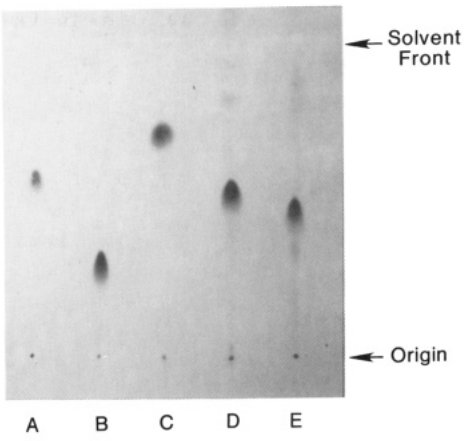

FIGURE 1: Thin-layer chromatogram of phenolic glycolipids isolated from various mycobacteria: Lane A, *M. kansasii*; Lane B, *M. leprae*; Lane C, *M. bovis*; Lane D, *M. haemophilum*; Lane E, *M. tuberculosis* Canetti. Solvent: toluene/acetone (60:40).

Results

Purification and Antigenicity of the PGL. Earlier studies (Besra et al., 1990a) revealed the presence of a major novel PGL in *M. haemophilum* based on phenophthiocerol, which was chromatographically more polar than other PGLs from *M. kansasii* and *M. marinum* (Minnikin et al., 1987). The glycolipid was purified from other nonpolar lipids by preparative TLC in toluene/acetone (60:40; v/v) as eluant to afford 6 mg of PGL/g of freeze-dried cells. A thin-layer chromatogram of this new phenolic glycolipid against those already characterized from other mycobacteria is shown in Figure 1. The purified PGL was shown to display avid reactivity in ELISA against homologous rabbit serum. With serum against *M. kansasii* ATCC 12478, a weak reaction, about one-tenth of the homologous reaction was seen. No reaction was observed against sera raised to *M. bovis* BCG (Danish), *M. marinum* ATCC 927, *Mycobacterium tuberculosis* H37Rv, *M. tuberculosis* C1PT 140010059 (MNC 1485), and *Mycobacterium scrofulaceum* ATCC 19275.

Chemical Characteristics of the PGL. The ^1H NMR spectrum (Figure 2) of the glycolipid was typical for a phenolic glycolipid (Hunter et al., 1982; Dobson et al., 1990). Three distinct anomeric proton resonances at 5.50 ($J_{1,2}$ = 1.5 Hz), 5.26 ($J_{1,2}$ = 1.7 Hz), and 5.15 ($J_{1,2}$ = 1.8 Hz) ppm (Figure 2, inset) pointed to a triglycosyl unit as part of the PGL, which, in view of the resonances at 3.55, 3.52, 3.50, and 3.48 (2) ppm, seemed to also bear five *O*-methyl groups. A sharp singlet at 3.32 ppm was characteristic of the methoxyl group of phenolphthiocerol A, and the resonance at 4.84 ppm was indicative of >C<u>H</u>OOC–hydrogens (Minnikin et al., 1989). Also a distinct tented double doublet at 7.04 ppm, with J_o ~8 Hz, was indicative of a *para*-1,4-disubstituted aromatic ring. The ^{13}C NMR spectrum of the PGL also showed distinctive resonances at 101.1, 98.4, and 96.3 ppm for the anomeric carbons (results not shown). Thus, the initial results from the $^1H/^{13}C$

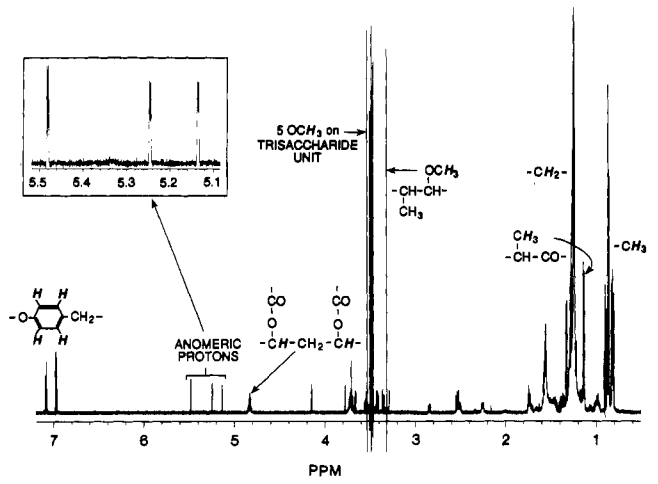

FIGURE 2: Proton NMR of PGL at 600 MHz in C^2HCl_3. The PGL was purified by preparative TLC and dissolved in C^2HCl_3 for analysis at a concentration of 10 mg of PGL/0.5 mL of C^2HCl_3. See the text for an explanation of the resonances.

spectra suggested a phenolic glycolipid based on a triglycosyl diacylated phenolphthiocerol A.

Glycosyl Composition of the PGL. The nature of the individual glycosyl units in the PGL was established through hydrolysis, reduction with NaB^2H_4, by per-O-acetylation and GC/MS of the alditol acetates. The presence of just two sugar derivatives was established, identified as 3-*O*-CH_3-6-deoxyhexitol (R_T 7.20 min; *m/z* 130, 143, 190, 203) and 2,3-di-*O*-CH_3-6-deoxyhexitol (R_T 6.58 min; *m/z* 102, 118, 143, 162, 203) (Figure 3). In view of the earlier ^1H NMR evidence for three anomeric protons and five *O*-CH_3 groups, it thus appeared that only two sugars, a 3-*O*-CH_3-6-deoxyhexose and a 2,3-di-*O*-CH_3-6-deoxyhexose, comprised the trisaccharide. Moreover, the coupling constants of the anomeric protons at <2 Hz suggested that the 6-deoxyhexoses are in the manno configuration and C-2 and thus are likely to be rhamnoses. GC of a mixture of the *O*-CH_3-rhamnitol acetates prepared from *M. leprae* PGL-I showed R_T values of 7.20 min for 3-*O*-CH_3-rhamnitol and 6.58 for 2,3-di-*O*-CH_3-rhamnitol; in comparison, equivalent products derived from partially methylated 6-deoxytalose showed an R_T value of 7.34 min for 3-*O*-CH_3-6-deoxytalitol and 6.87 for 2,3-di-*O*-CH_3-6-deoxytalitol. Thus, this form of comparative GC indicated that the two sugars comprising the PGL from *M. haemophilum* were 3-*O*-CH_3-rhamnose and 2,3-di-*O*-CH_3-rhamnose. Indeed, cochromatography of the rhamnosyl derivatives from the PGL-I of *M. leprae* and that of *M. haemophilum*, under a variety of GC conditions, showed that they were coincident and thus probably identical.

In order to establish the absolute configuration of the rhamnosyl residues, the pure PGL was butanolyzed with optically pure (*R*)-(−)-2-butanol. GC/MS of the major trimethylsilyl derivatives showed R_T values of 7.02 min for the 3-*O*-CH_3-Rha derivative (*m/z* 117, 146, 217, 305) and 6.60 min for the 2,3-di-*O*-CH_3-Rha derivative (*m/z* 88, 117, 175, 247). These products coeluted with the corresponding L-Rha derivatives prepared from *M. leprae* PGL-I and were different from the trimethylsilyl (*S*)-(+)-2-butylglycosides of PGL-I. Thus, these results clearly demonstrated that the glycosides from *M. haemophilum* are rhamnosides in the L absolute configuration.

Ring Form and Linkage of Glycosyl Residues. The PGL from *M. haemophilum* was per-O-trideuteriomethylated, hydrolyzed, reduced with NaB^2H_4, and per-O-acetylated, and the sugar derivatives were analyzed by GC/MS, as described

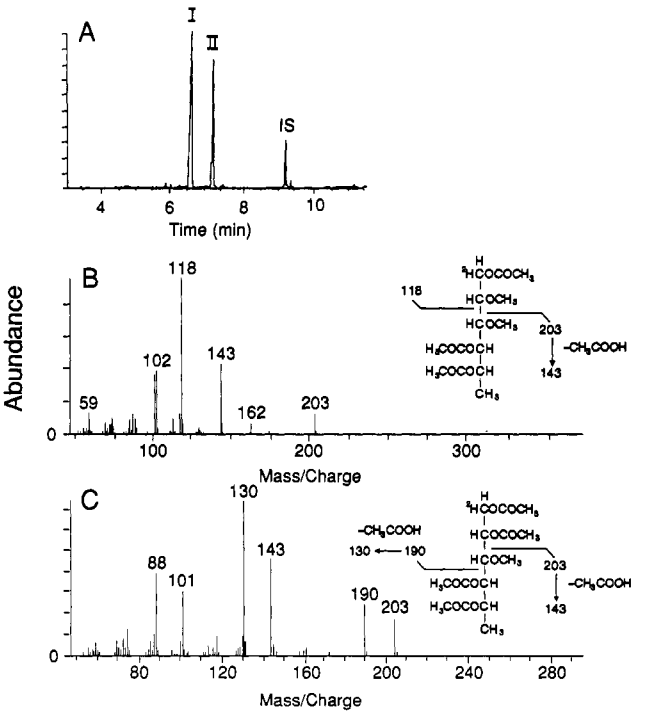

FIGURE 3: GC/MS profile of the alditol acetates derived from the PGL of *M. haemophilum*. (A) Total ion chromatogram; I, 2,3-di-*O*-CH_3-rhamnitol (R_T 6.58 min); II, 3-*O*-CH_3-rhamnitol (R_T 7.20 min); IS, inositol. (B) Mass spectrum of (I) 2,3-di-*O*-CH_3-rhamnitol showing characteristic fragment ions. (C) Mass spectrum of (II) 3-*O*-CH_3-rhamnitol showing characteristic fragment ions.

(McNeil et al., 1987). Three substituted alditols were identified as 1,5-di-*O*-CH_3CO-4-*O*-C^2H_3-2,3-*O*-CH_3-rhamnitol (R_T 6.21 min; *m/z* 102, 118, 134, 162, 209), 1,2,5,-tri-*O*-CH_3CO-4-*O*-C^2H_3-3-*O*-CH_3-rhamnitol (R_T 6.84 min; *m/z* 88, 130, 134, 143, 190, 203), and 1,4,5-tri-*O*-CH_3CO-2,3-di-*O*-CH_3-rhamnitol (R_T 6.88 min; *m/z* 102, 118, 143, 162, 203). The 2,3-di-*O*-CH_3-Rha was consequently recognized as the nonreducing terminus and to be in the pyranose ring form. Likewise, from this information it can be concluded that the 3-*O*-CH_3-Rha residue (R_T 6.84 min) is also in the pyranose ring form and linked at the C-2 position. The remaining 2,3-di-*O*-CH_3-Rha glycosyl residue could be either a 4-linked Rha*p* or a 5-linked Rha*f*, an uncertainty that was resolved by further inspection of the $^1H/^{13}C$ NMR spectra of the PGL. Since resonances associated with a *trans*-furanose, ~108 ppm (^{13}C), and a *cis*-furanose ring, $J_{1,2}$ > 2 Hz (^1H), were absent (Joseleau et al., 1977; Mizutani et al., 1989), the 2,3-di-*O*-CH_3-Rha glycosyl residue (R_T 6.88 min; ^1H, $J_{1,2}$ = 1.5 Hz; ^{13}C, 101.1 ppm) must be in the pyranose ring form. The chemical shifts of the anomeric protons were all >5 ppm, indicating that all of the Rha*p* sugars are in the α form.

The per-O-trideuteriomethylated PGL was partially hydrolyzed with 2 M CF_3COOH for 30 min at 80 °C, reduced with NaB^2H_4, and per-O-acetylated, and the products were analyzed by GC/MS. Three products were obtained whose structures were deciphered by their mass spectra. These were the holistic trisaccharide identified as 4-*O*-C^2H_3-2,3-di-*O*-CH_3-Rha*p*-(1→2)-4-*O*-C^2H_3-3-*O*-CH_3-Rha*p*-(1→4)-1,5-di-*O*-CH_3CO-2,3-di-*O*-CH_3-rhamnitol (Figure 4) and two disaccharides (not shown in Figure 4), 4-*O*-C^2H_3-2,3-di-*O*-CH_3-Rha*p*(1→2)1,5-di-*O*-CH_3CO-4-*O*-C^2H_3-3-*O*-CH_3-α-L-rhamnitol (R_T 11.67 min; *m/z* 88, 192, 265, 325) and 2-*O*-CH_3CO-4-*O*-C^2H_3-3-*O*-CH_3-α-L-Rha*p*(1→4)1,5-di-*O*-CH_3CO-2,3-di-*O*-CH_3-α-L-rhamnitol (R_T 12.14 min; *m/z* 118, 162, 188, 220, 262, 322). In summary, the structure of the

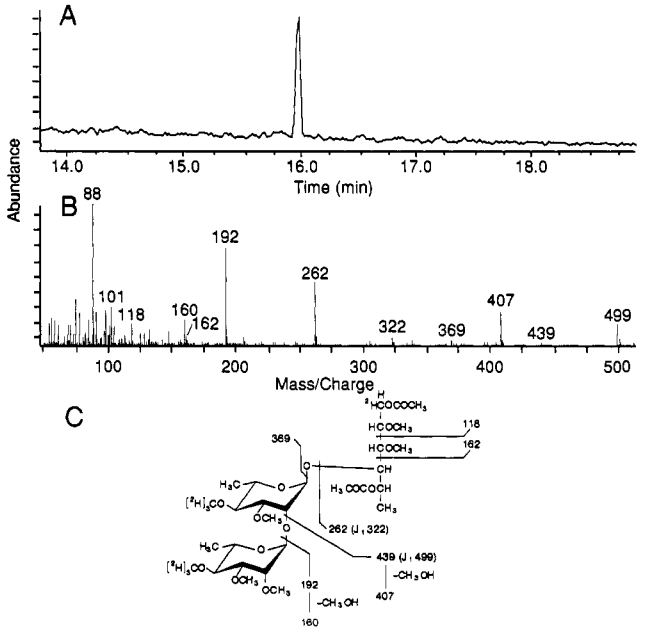

FIGURE 4: GC/MS and structure of the trisaccharide unit of PGL. Per-O-trideuteriomethylation was followed by partial hydrolysis with 2 M TFA at 80 °C for 30 min and per-*O*-acetylation. The resulting partially methylated, partially trideuteriomethylated, partially acetylated diglycosyl alditol acetates were subjected to GC/MS analysis. (A) Total ion chromatogram; (B) mass spectrum of major component; (C) an illustration of the formation of the major fragment ions.

Table I: Analysis of the PGL from *M. haemophilum* for Mycocerosates, as the Methyl Esters

$CH_3(CH_2)_n[CH_2CH(CH_3)]_pCOOCH_3$, with n = 16–20 and p = 2–5

carbons in parent acid	R_T (min)	percent	M^+
27	17.51	2.4	424
30	18.98	2.4	466
32	20.29	27.5	494
34	21.54	59.0	522
37	22.77	8.7	564

trisaccharide unit of the PGL from *M. haemophilum* (see Figure 6A) is 2,3-di-*O*-CH_3-α-L-Rha*p*(1→2)-3-*O*-CH_3-α-L-Rha*p*(1→4)2,3-di-*O*-CH_3-α-L-Rha*p*→.

Structures of the Fatty Acids and Phenolic Phthiocerol Core of PGL. The PGL from *M. haemophilum* was initially treated with 1 M HCl/CH_3OH and an aliquot examined by GC/MS for fatty acid methyl esters (Table I). Five fatty acid methyl esters were present that gave molecular ion peaks, M^+, of 424, 466, 494, 522, and 564, corresponding to C_{27} (R_T 17.51 min; 2.4%), C_{30} (R_T 18.98 min; 2.4%), C_{32} (R_T 20.29 min; 27.5%), C_{34} (R_T 21.54 min; 59.0%), and C_{37} (R_T 22.77 min; 8.7%) mycocerosate methyl esters. The mass spectra of all five fatty acid methyl esters showed intense peaks at m/z 88 and 101 indicative of α-methyl branched fatty acids. The C_{30}, C_{32}, and C_{34} mycocerosate methyl esters coeluted with the corresponding derivatives from *M. leprae* PGL-I (Hunter et al., 1982), which suggests that these fatty acid methyl esters are in fact 2,4,6,8-tetramethyl branched mycocerosate methyl esters. The >C<u>H</u>OOC–hydrogens appeared at 4.84 ppm in the ^1H NMR spectrum of the intact PGL, similar to those in the products from *M. leprae* and *M. kansasii*,[2] which suggested that the methyl branches have an *R* absolute configuration (Daffe et al., 1988).

The degraded lipid was recovered and treated with alkali to completely deacylate the PGL. The crude phenolic phthiocerol core was then per-O-trideuteriomethylated and analyzed by GC/MS (Figure 5A), revealing the presence of two components, R_T 30.67 min (68.5%) and R_T 34.95 min (31.5%), which gave molecular ion peaks, M^+, of 599 and 627. Closer examination of both mass spectra showed peaks at mass numbers 73 and 124, which are characteristic of the two termini of the methyl ether of phenolphthiocerol A. The major component (R_T 30.67 min, 68.5%; Figure 5B) contained a strong fragment ion at m/z 381, indicating the presence of 16 methylene groups on one segment of the backbone, and the m/z 204 fragment ion confirms the presence of 4 methylene groups on the other segment of the phenophthiocerol A backbone. The minor component (R_T 34.95 min, 31.5%) contained similar fragments at m/z 409 and 204, indicating the presence of clusters of 18 and 4 methylene groups on the phenolphthiocerol A backbone. It therefore appears that a pair of homologues of phenolphthiocerol A is present in the PGL, with the minor homologue coeluting with the phenolphthiocerol A from *M. leprae* PGL-I (Hunter et al., 1982). The ^1H NMR spectrum of the PGL showed that the >C<u>H</u>OOC–hydrogens were at 4.84 ppm, indicating that the diol unit of phenolphthiocerol A has threo relative configuration, as found for *M. kansasii* (Besra et al., 1990b) and *M. leprae*.[2] In contrast, phenolphthiocerols from *M. marinum* and *M. ulcerans* have erthyro relative stereochemistry for their diol units (Besra et al., 1989, 1990b). Since phthiocerol A from *M. tuberculosis*, a close relative of phenolphthiocerol A, has the same threo relative configuration (Maskens & Polgar, 1966) and an absolute stereochemistry of 9*R*,11*R* (Welby-Gieusse & Tocanne, 1970), it therefore may be assumed that the diol unit of phenolphthiocerol A from *M. haemophilum* has the same absolute stereochemistry. Similarly the remaining chiral centers of phenolphthiocerol A from *M. haemophilum* at C-3 and C-4 may have 3*R*,4*S* absolute stereochemistry as found for phthiocerol A from *M. tuberculosis* (Maskens & Polgar, 1973a–c).

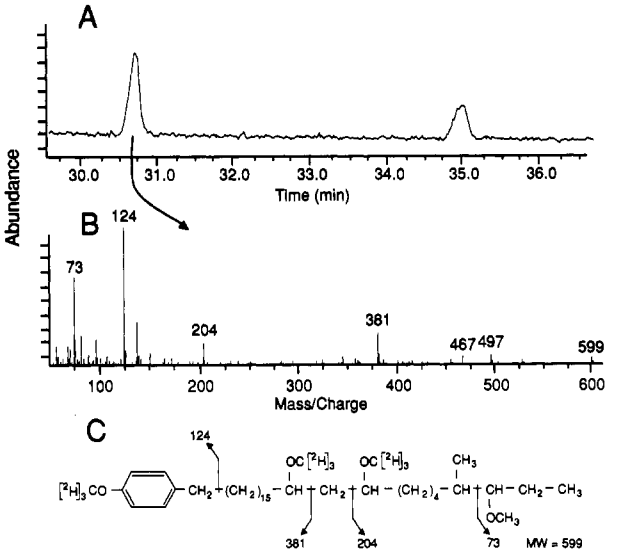

FIGURE 5: GC/MS of the phenolic phthiocerol "core" from the PGL. The deacylated phenolphthiocerol A moiety was per-O-trideuteriomethylated and examined by GC/MS. (A) Total ion chromatogram; (B) mass spectrum of major component; (C) an illustration of the formation of the major fragment ion.

Discussion

The present work describes a specific phenolic glycolipid marker attributable solely to *M. haemophilum*, an organism long associated with immunosuppression (Ryan & Dwyer,

[2] G. S. Besra and D. E. Minnikin, unpublished results.

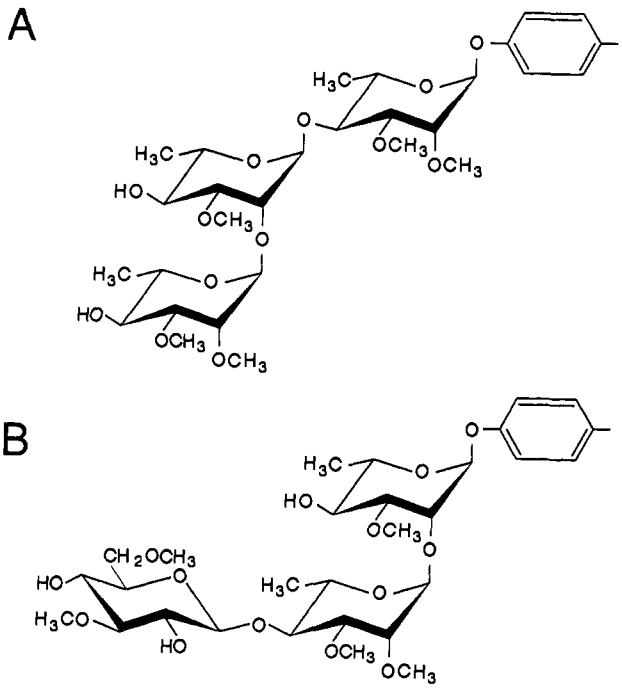

FIGURE 6: Comparison of structures of the specific triglycosyl segments of the PGL from (A) *M. haemophilum* and (B) *M. leprae*.

1983). The lipid portion of the phenolic glycolipid from *M. haemophilum* showed showed striking similarities to the analogous components from *M. leprae* PGL-I (Hunter et al., 1982). It was apparent from GC/MS studies that the core of the phenolic glycolipid from both species conforms in broad structural principles to the aromatic analogue of phthiocerol A acylated at the hydroxyls on the main chain by mycocerosic acids; however, in the case of the *M. haemophilum* glycolipid, an additional homologue differing by two methylene groups was present as the major component. ^{1}H NMR of the intact phenolic glycolipid suggested that the diol unit of phenolphthiocerol A moiety has a threo relative configuration, as found for *M. kansasii* (Besra et al., 1990b) and *M. leprae*,[2] which is in contrast to *M. marinum* and *M. ulcerans*, which have an erythro relative stereochemistry (Besra et al., 1989, 1990b). The absolute stereochemistry of the asymmetric centers of the phenolphthiocerol A moiety are uncertain but are likely to be the same as those of phthiocerol A from *M. tuberculosis* (Maskens & Polgar, 1966, 1973a–c; Welby-Gieusse & Tocanne, 1970) and represented by 3*R*, 4*S*, 9*R*, and 11*R*.

Until now the C_{34} tetramethyl branched mycocerosic acid has been thought to be characteristic of *M. leprae* PGL-I (Hunter et al., 1982). However, this present report clearly demonstrates that this complex methyl branched fatty acid is also a major constituent of the phenolic glycolipid of *M. haemophilum*. The stereochemistry of the methyl branches of the mycocerosates were found to have an *R* absolute configuration, as found for similar substances in *M. kansasii*, *M. leprae*, and *M. tuberculosis*[2] (Daffe & Laneelle, 1988). In contrast, mycocerosates from *M. marinum* and *M. ulcerans*[2] have an *S* absolute configuration for their methyl branches (Daffe & Laneelle, 1988). Such differences in the phthiocerol and fatty acyl constituents are generally regarded as of minor import compared to those that may distinguish the oligoglycosyl appendage. Within the oligosaccharide lies much of the antigenic uniqueness and specificity of mycobacterial species and subspecies (Brennan, 1988; McNeil et al., 1989) and the promise of a specific antigenic probe for the detection of *M. haemophilum* infections. The key steps in the structural elucidation of the oligoglycosyl residue was partial acid hydrolysis coupled with a series of other degradative reactions. The structure and sequence of the trisaccharide unit is unique to the phenolic glycolipid from *M. haemophilum* and has not been encountered in any other mycobacterial species. Interestingly, however, the two internal residues, the 2-linked 3-*O*-CH_3-α-L-Rha*p* and 4-linked 2,3-di-*O*-CH_3-α-L-Rha, are identical with the two internal glycosyl residues of *M. leprae* PGL-I (Hunter et al., 1982) but attached in the opposite sequence (Figure 6). Thus, on the basis of this key dominant phenotype, intriguing relationships exist between the opportunistic but relatively nonpathogenic *M. haemophilum* and *M. leprae*, an explanation of which may provide clues to the noncultivability and pathogenicity of the latter.

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REFERENCES

Besra, G. S., Mallet, A. I., Minnikin, D. E., & Ridell, M. (1989) *Chem. Comm.* 1451–1452.

Besra, G. S., Minnikin, D. E., Rigouts, L., Portaels, F., & Ridell, M. (1990a) *Lett. Appl. Microbiol. 11*, 202–204.

Besra, G. S., Minnikin, D. E., Sharif, A., & Stanford, J. L. (1990b) *FEMS Microbiol. Lett. 66*, 11–14.

Brennan, P. J. (1988) in *Microbial Lipids*, (Ratledge, C., & Wilkinson, S. G., Eds.) Vol. 1, pp 203–298, Academic Press, London.

Daffe, M. (1989) *Biochim. Biophys. Acta 1002*, 257–260.

Daffe, M., & Laneelle (1988) *J. Gen. Microbiol. 134*, 2049–2055.

Demarteau-Ginsburg, H. G., & Lederer, E. (1963) *Biochim. Biophys. Acta 70*, 424–453.

Dobson, G., Minnikin, D. E., Besra, G. S., Mallet, A. I., & Magnusson, M. (1990) *Biochim. Biophys. Acta 1042*, 176–181.

Gerwig, G. J., Kamerling, J. P., & Vliegenthart, J. F. G. (1978) *Carbohydr. Res. 62*, 349–357.

Hunter, S. W., Fujiwara, T., & Brennan, P. J. (1982) *J. Biol. Chem. 257*, 15072–15078.

Joseleau, J.-P., Chambat, G., Vignon, M., & Barnoud, F. (1977) *Carbohyrd. Res. 58*, 165–175.

MacLennan, A. P., Randall, H. M., & Smith, D. W. (1961) *Biochem. J. 80*, 309–318.

McNeil, M., Tsang, A. Y., & Brennan, P. J. (1987) *J. Biol. Chem. 262*, 2630–2635.

McNeil, M., Chatterjee, D., Hunter, S. W., & Brennan, P. J. (1989) in *Methods in Enzymology* (Ginsburg, V., Ed.) Vol. 179, pp 215–242, Academic Press, San Diego.

Maskens, K., & Polgar, N. (1973a) *J. Chem. Soc., Perkin Trans. 1*, 109–115.

Maskens, K., & Polgar, N. (1973b) *J. Chem. Soc., Perkin Trans. 1*, 1117–1121.

Maskens, K., & Polgar, N. (1973c) *J. Chem. Soc., Perkin Trans. 1*, 1909–1912.

Maskens, K., Minnikin, D. E., & Polgar, N. (1966) *J. Chem. Soc. C*, 2113–2215.

Minnikin, D. E. (1982) in *The Biology of the Mycobacteria* (Ratledge, C., & Stanford, J. L., Eds.) pp 95–104, Academic Press, London.

Minnikin, D. E., Dobson, G., Parlett, J. H., Goodfellow, M., & Magnusson, M. (1987) *Eur. J. Clin. Microbiol. 6*, 703–707.
Minnikin, D. E., Ridell, M., Wallerstrom, G., Besra, G. S., Parlett, J. H., Bolton, R. C., & Magnusson, M. (1989) *Acta Leprol. 7 (Suppl. 1)*, 51–54.
Minnikin, D. E., Ridell, M., Bolton, R. C., & Magnusson, M. (1990) *FEMS Microbiol. Lett. 67*, 55–58.
Mizutani, K., Kasai, R., Nakamura, M., Tanaka, O., & Matsuura, H. (1989) *Carbohydr. Res. 185*, 27–38.
Ryan, C. G., & Dwyer, B. W. (1983) *J. Clin. Microbiol. 18*, 976–977.
Sompolinsky, D., Lagziel, A., Naveh, D., & Yankilevitz, T. (1978) *Int. J. Syst. Bacteriol. 28*, 67–75.
Stellner, K., Saito, H., & Hakomori, S. (1973) *Arch. Biochem. Biophys. 262*, 2630–2635.
Ville, V., & Gastambide-Odier, M. (1970) *Carbohydr. Res. 12*, 97–107.
Welby-Gieusse, M., & Tocanne, T. F. (1970) *Tetrahedron 26*, 2875–2882.
York, W. S., Darvill, A. G., McNeil, M., Stevenson, J. T., & Albersheim, P. (1986) *Methods Enzymol. 118*, 3–40.

pH Dependency of the Reactions Catalyzed by Chorismate Mutase–Prephenate Dehydrogenase from *Escherichia coli*†

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ABSTRACT: The variation with pH of the kinetic parameters associated with the mutase and dehydrogenase reactions catalyzed by chorismate mutase–prephenate dehydrogenase has been determined with the aim of elucidating the role that ionizing amino acid residues play in binding and catalysis. The pH dependency of log V for the dehydrogenase reaction shows that the enzyme possesses a single ionizing group with a pK value of 6.5 that must be unprotonated for catalysis. This same group is observed in the $V/K_{prephenate}$, as well as in the V/K_{NAD}, profile. The $V/K_{prephenate}$ profile exhibits a second ionizing residue with a pK value of 8.4 that must be protonated for the binding of prephenate to the enzyme. For the mutase reaction, the $V/K_{chorismate}$ profile indicates the presence of three ionizing residues at the active site. Two of these residues, with similar pK values of about 7, must be protonated, while the third, with a pK value of 6.3, must be unprotonated. It can be concluded that all three groups are concerned with the binding of chorismate to the enzyme since the maximum velocity of the mutase reaction is essentially independent of pH. This conclusion is confirmed by the finding that the K_i profile for the competitive inhibitor, (3-*endo*,8-*exo*)-8-hydroxy-2-oxabicyclo[3.3]non-6-ene-3,5-dicarboxylic acid, shows the same three ionizing groups as observed in the $V/K_{chorismate}$ profile. By contrast, the K_i profile for carboxyethyldihydrobenzoate shows only one residue, with a pK value of 7.3, that must be protonated for binding of the inhibitor. On the basis of the aforementioned data, hypotheses are proposed for the chemical mechanisms of the prephenate dehydrogenase and chorismate mustase reactions. For the mutase reaction, the data suggest that the groups of chorismate required for the binding of substrate at the active site of the enzyme are the 4-hydroxyl, the ring carboxyl, and the oxygen moiety of the enolpyruvyl side chain. The group binding the latter moiety could also be involved with catalysis.

Chorismate mutase–prephenate dehydrogenase from *Escherichia coli* is a bifunctional enzyme that has a molecular weight of 78 000–88 000 and is composed of two identical subunits (SampathKumar & Morrison, 1982a; Hudson et al., 1984; Turnbull et al., 1990). Each subunit catalyzes two sequential reactions along the tyrosine biosynthetic pathway. Chorismate mutase (EC 5.4.99.5) catalyzes the rearrangement of chorismate to prephenate, while the dehydrogenase (EC 1.3.1.12) is responsible, in the presence of NAD, for the oxidative decarboxylation of prephenate to 4-hydroxyphenylpyruvate. Evidence now suggests that the two reactions are catalyzed at separate active sites (Turnbull & Morrison, 1990). It has been established that the kinetic mechanism of the prephenate dehydrogenase reaction conforms to a rapid equilibrium, random mechanism with catalysis as the rate-limiting step (SampathKumar & Morrison, 1982b), while hydride transfer and decarboxylation occur in a concerted manner (Hermes et al., 1984). The isomerization of chorismate to prephenate, as catalyzed by chorismate mutase, occurs through a Claisen rearrangement, and stereochemical studies have shown that this rearrangement proceeds through an isomerization of chair-like geometry (Andrews et al., 1973; Sogo et al., 1984). In addition, the reaction mechanism has been probed further through the use of isotope effects (Addadi et al., 1983; Guilford et al., 1987).

To gain further information about the chemical mechanisms of the mutase and dehydrogenase, studies have been under-

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