# Further Structural Definition of a New Family of Glycopeptidolipids from *Mycobacterium xenopi*†

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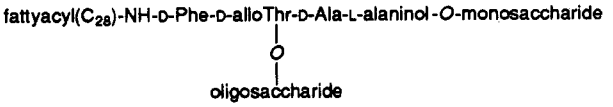
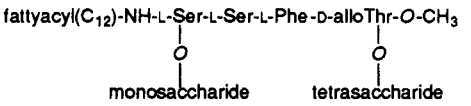
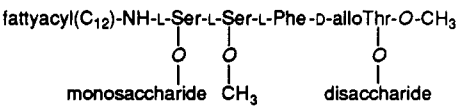
ABSTRACT: The highly antigenic surface glycolipids of serovars of the *Mycobacterium avium* complex are glycopeptidolipids of the general structure




However, it has recently been shown [Rivière, M., & Puzo, G. (1991) *J. Biol. Chem. 266*, 9057–9063; Rivière, M., & Puzo, G. (1992) *Biochemistry 31*, 3575–3580] that the characteristic glycopeptidolipid of a strain of *Mycobacterium xenopi* is of the structure




[monosaccharide, 3-*O*-$CH_3$-6-deoxy-α-L-talopyranose; tetrasaccharide, 2,3,4-tri-*O*-$CH_3$-α-L-rhamnopyranose(1→3)2-*O*-lauryl-α-L-rhamnopyranose(1→3)α-L-rhamnopyranose(1→3)2,4-di-*O*-acetyl/lauryl-6-deoxy-α-L-glucopyranose]. The type of analyses previously applied to the glycopeptidolipids of *M. avium* combined with fast atom bombardment mass spectrometry allowed the recognition of other variants of this structure in other strains of *M. xenopi*:




in which the monosaccharide is 2-*O*-acetyl-3-*O*-$CH_3$-6-deoxy-α-L-talopyranose or 3-*O*-$CH_3$-6-deoxy-α-L-talopyranose, and the disaccharide is 4-*O*-octanoyl(or decanoyl)-α-L-rhamnopyranose(1→3)2-*O*-lauryl-α-L-rhamnopyranose or α-L-rhamnopyranose(1→3)2-*O*-lauryl-α-L-rhamnopyranose. Recognition of serine-containing glycopeptidolipids substituted in one case with a tetrasaccharide and in another case with a disaccharide unit implies that *M. xenopi*, an important opportunistic pathogen, exists as a serocomplex in nature.

The resurgence of interest in mycobacteria and their constituents stems from the resurgence of tuberculosis itself (Kochi, 1991), from the involvement of "atypical", environmental mycobacteria as opportunistic pathogens in individuals with underlying immune dysfunctions (Horsburg & Selik, 1989), and, also, from positive developments in leprosy control such that estimates of worldwide prevalence have now been drastically reduced (Noordeen, 1991). The various members of the *Mycobacterium* genus responsible for these assorted diseases may contain within them representatives of several families of glycolipid antigens with remarkable structural features (Brennan, 1988). These glycolipids, and the more complex lipopolysaccharides that accompany them, have been implicated in diverse aspects of disease pathogenesis, such as interaction of bacterium and macrophage and persistence of bacteria within the intracellular environment (Chan et al., 1991). These glycolipids with their unique sugar constituents also provide precise determinants by which to identify individual pathogen or saprophyte (McNeil et al., 1989), and, in the case of leprosy, a good means for specific serodiagnosis (Gaylord & Brennan, 1987).

Three major classes of glycolipids have been identified in mycobacteria. The trehalose-containing lipooligosaccharides characterize such as *Mycobacterium kansasii*, *Mycobacterium szulgai*, and many others (Hunter et al., 1985, 1988). An acyltrehalose residue invariably occupies the reducing terminus, whereas the rest of the oligosaccharide, which will be variable and may be up to 13 units in length, is always distinguished at the nonreducing end by sugars characteristic of the particular species. The second glycolipid class, namely, the phenolic glycolipids, is based on a phenylphthiocerol and

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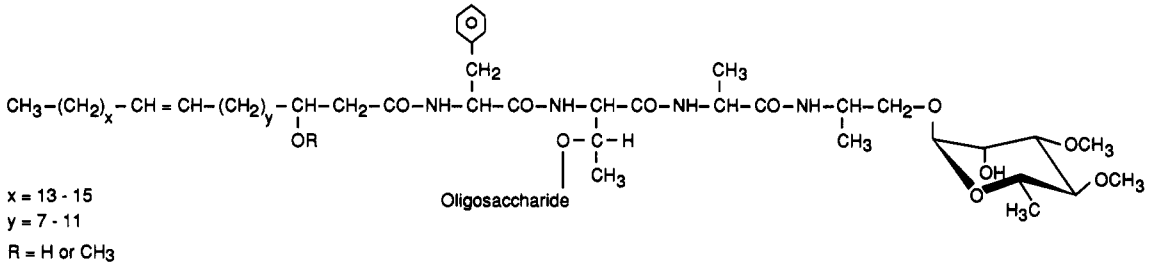
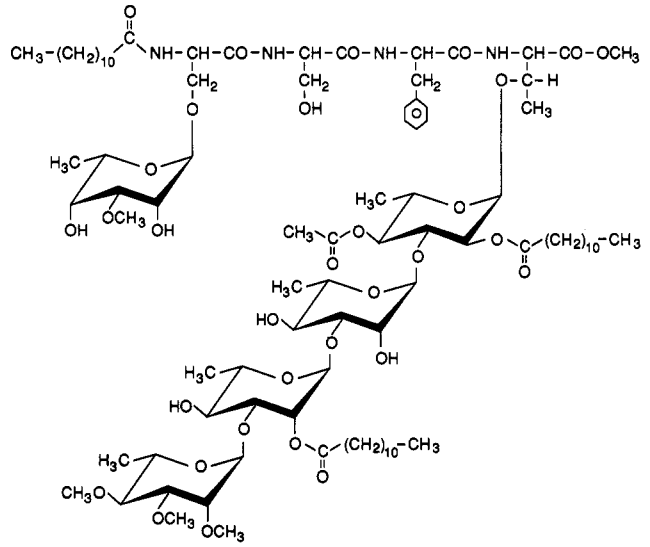
Chart I: General Structure of the Antigenic Polar C-Mycoside Glycopeptidolipids of Members of the *M. avium* complex[a]




[a] Simpler structures (the apolar GPLs), with only one glycosyl residue linked to the alloThr, are also present in *M. avium* serotypes.

nearly always accompanies the characteristic lipooligosaccharide in a given organism (Belisle & Brennan, 1989). Of the three major families of glycolipids, the C-mycoside GPLs[1] (Chart I) have been most extensively examined, to such an extent that the characteristic sugar combination that marks the nonreducing ends of the oligosaccharide unit of the GPL from each serovar of the well-characterized *Mycobacterium avium* complex is now known, and the detailed oligosaccharide structures of 12 of these antigens have been completely defined (Bozic et al., 1988; McNeil et al., 1989). In addition, during the course of less thorough studies, we concluded that the general features of the GPL structure extended to *Mycobacterium simiae* and *Mycobacterium chelonae* (subsp. *chelonae*) and to *Mycobacterium fortuitum* biovar. *peregrinum* (Tsang et al., 1992); Lopez-Marin et al. (1991) recently fully defined the structure of the GPL from *M. fortuitum, peregrinum*. On the basis of the observation of Jenkins et al. (1972) that *Mycobacterium xenopi* should be assimilated into the *M. avium* complex and that its characteristic lipid showed chromatographic features similar to those within *M. avium*, we had concluded that it also contained a characteristic GPL. While we examined the complete structure of the product from this clinically important organism, Rivière and Puzo (1991, 1992) reported that a strain of *M. xenopi* contained an unexpected, unique variant of the GPL structure (a non-C-mycoside GPL), in which the core consisted of fattyacyl-NH-L-Ser-L-Ser-L-Phe-allo-D-Thr-$OCH_3$ (Chart II) rather than fattyacyl-NH-D-Phe-D-allo-Thr-D-Ala-L-alaninol. We have now completed our analysis of the characteristic GPLs of *M. xenopi* (ATCC 19250) and support the essence of the structure described by Rivière and Puzo (1991) but with important modification in both the lipopeptide core and sugar appendages, which, in turn, suggest that *M. xenopi*, like *M. avium*, exists as a serocomplex in nature.

Chart II: Structure of the Ser-Containing Glycopeptidolipid of *M. xenopi* (Strain CIPT 140 35004) As Described by Rivière and Puzo (1991)



## EXPERIMENTAL PROCEDURES

*Growth and Extraction of M. xenopi.* *M. xenopi* (ATCC 19250) was grown in a medium containing glycerol, alanine, and salts (Takayama et al., 1975) for 8–10 weeks at 37 °C. Both cells and medium were autoclaved together and evaporated to dryness at 50 °C. The resulting solids were extracted twice with $CHCl_3$–$CH_3OH$ (2:1) and partitioned between the aqueous and organic phases arising from a mixture of $CHCl_3$–$CH_3OH$–$H_2O$ (8:4:2) (Folch et al., 1957). The contents of the lower organic phase were dried, and 4.7 g of lipid, from 89 g of dry cells, was solubilized in 250 mL of hot acetone and allowed to precipitate overnight at 4 °C. The precipitate was removed by centrifugation at 10000*g* for 10 min, and the supernatant was evaporated to yield 1.67 g of acetone-soluble lipids.

*Purification of the Species-Specific Glycolipids.* All of the acetone-soluble lipid preparations were applied to a column (2.5 × 30 cm) of Florisil (100–200 mesh) (Fisher Scientific Co., Pittsburgh, PA) which was irrigated with 500 mL each of $CHCl_3$ followed by 2%, 5%, 10%, and 15% $CH_3OH$ in $CHCl_3$, as described (Hunter et al., 1985). Eluates (10 mL) were collected, subjected to TLC in $CHCl_3$–$CH_3OH$–$H_2O$ (90:10:1) and sprayed with 10% sulfuric acid in ethanol followed by heating at 110 °C for 5 min. The GPL-containing fractions were pooled and further purified by preparative TLC on silica gel (Merck 5735 silica gel $60F_{254}$; Darmstadt, Germany) in $CHCl_3$–$CH_3OH$–$H_2O$ (90:10:1) to afford highly purified GPL-I ($R_f$ 0.21), GPL-II ($R_f$ 0.36), GPL-III ($R_f$ 0.41), and GPL-IV ($R_f$ 0.57).

*Glycosyl Group Analysis.* Purified GPLs, the per-O-methylated/per-O-trideuteriomethylated GPLs, and the per-O-methylated oligoglycosyl alditols were hydrolyzed in 2 M $CF_3COOH$ at 120 °C for 1 h. Glycosyl residues were reduced with $NaB^2H_4$, and the resultant alditols were per-O-acetylated and examined by GC and GC/MS (McNeil et al., 1987). The absolute configurations of glycosyl residues were determined by GC/MS analysis of $(CH_3)_3Si$ derivatives of (*R*)-(−)- and (*S*)-(+)-2-butanol and by comparison with authentic standards (Gerwig et al., 1978). Glycosyl linkage analysis and the location of acyl functions were achieved through the application of a combination of the neutral alkylating conditions of Prehm (1980) followed by a modified Hakomori methylation (York

[1] Abbreviations: amu, atomic mass units; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; FAB/MS, fast atom bombardment mass spectrometry; GC/MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography; $R_f$, retardation factor; GPL, glycopeptidolipid; Rha, rhamnose; 6-dTal*p*, 6-deoxytalopyranose; alloThr, allothreonine; 3-*O*-$CH_3$-Ser, 3-*O*-methylserine; *N*(*O*)-HFB, *N*(*O*)-heptafluorobutyryl.

et al., 1986) as described previously (Camphausen et al., 1987). Release of the oligosaccharide hapten was achieved by reductive β-elimination as described (Chatterjee et al., 1987).

*Amino Acid Analysis.* Amino acids were analyzed as their *N*(*O*)-HFB butyl esters (MacKenzie & Tenaschuk, 1975). GPLs (0.5 mg) were completely hydrolyzed in 200 μL of 6 N HCl (Pierce Chemical Co., Rockford, IL) at 110 °C for 12 h. Upon evaporation to dryness, the resulting reaction mixture was treated with 100 μL of 3 M HCl in butanol [isobutyl alcohol or to determine the absolute configuration (*R*)-(−)-2-butanol)] at 120 °C for 20 min. Upon careful drying, 100 μL of anhydrous ethyl acetate and 40 μL of heptafluorobutyric anhydride (Aldrich Chemical Co., Milwaukee, WI) were added. The reaction mixture was heated at 150 °C for 5 min and subsequently analyzed by GC/MS as described (Hirschfield et al., 1990) and by comparative GC; the 3-*O*-methylserine was obtained from Sigma Chemical Co. (St. Louis, MO).

*NMR Analysis.* Routine $^1$H-NMR spectra were recorded on a Bruker ACE-300 at the Colorado State University Department of Chemistry Central Instrument Facility. Spectra were obtained for the native GPL at a concentration of 8–24 mg/0.5 mL of $C^2HCl_3$–$C^2H_3O^2H$ (4:1) before and after exchanging protons with $C^2HCl_3$–$C^2H_3O^2H$ (4:1).

*Gas Chromatography/Mass Spectrometry Analysis.* GC/MS of alditol acetates was performed on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5790 mass selective detector as decribed (Besra et al., 1991). The temperature program that was applied to all $(CH_3)_3Si$ derivatives of butyl glycosides involved a 50 °C hold 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. In the case of the *N*(*O*)-HFB butyl esters of the amino acids, the oven was programmed to hold at 50 °C for 1 min followed by a 30 °C/min rise to 100 °C, an 8 °C/min rise to 250 °C, a 30 °C/min rise to 320 °C, and a 7-min hold at this final temperature. The mass spectrometer was set to scan from 50 to 800 amu with 0.81 scan per second. GC of alditol acetates was routinely conducted on a fused silica capillary column of Durabond-1 (J&W Scientific, Rancho Cordova, CA) as described (McNeil et al., 1987). For this analysis, the oven was programmed to hold at 80 °C for 2 min followed by an 8 °C rise to 280 °C and an 8-min hold at this final temperature.

*Fast Atom Bombardment Mass Spectrometry.* All spectra were acquired with *m*-nitrobenzyl alcohol as matrix. FAB/MS was carried out using a VG Analytical ZAB-HF mass spectrometer fitted with an M-Scan FAB gun operating at 10 kV (Dell, 1990); an exception was made in the case of the methanolysis experiments which were carried out on a ZAB-2SE FPD mass spectrometer fitted with a cesium ion gun operating at 25 kV. Deuterioacetylation was conducted in pyridine–hexadeuterioacetic anhydride (1:1) at 80 °C for 2 h. Methanolysis was conducted in methanolic HCl (prepared by bubbling HCl gas into anhydrous methanol until the solution was very hot; approximately 1 M) at room temperature for 30 min, after which time the samples were dried and redissolved in $CH_3OH$ for FAB/MS analysis (Dell, 1990).

*Antibody Generation and Serology.* In an attempt to prepare rabbit antisera specific to the *M. xenopi* GPLs, a 7-lb New Zealand white rabbit was immunized intramuscularly with a mixture of 250 μg each of GPL-I–IV suspended by brief sonication in 300 μL of phosphate-buffered saline (PBS) and emulsified in 200 μL of Titermax adjuvant (Cythx Corp., Norcross, GA); this adjuvant has been shown by the manufacturer to stimulate a higher B-cell immune response than Fruend's incomplete adjuvant, as used in our standard protocol (Brennan & Goren, 1979). Second and third identical boosts were administered at 2- and 3-month post-initial injection. Antiserum was taken one week following each boost and tested in plate ELISA against 50 μg/mL *M. xenopi* whole cells coated in 0.1 M carbonate buffer, pH 9.6, at 37 °C or against the purified GPL-I, GPL-II, GPL-III, and GPL-IV at 20 μg/mL of ethanol as described (Rivoire et al., 1989).

Antisera were also raised against whole *M. xenopi* cells; control preimmune sera were collected prior to immunization. Wells were blocked with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO) in PBS containing 0.05% Tween 80 (Sigma) (PBST) and incubated for 1 h at 37 °C in a humid chamber. After the blocking reagent was removed, dilutions of antisera in 0.1% BSA in PBST were added to their apropriate wells and ELISA plates were incubated for 1 h at 37 °C. Wells were washed with PBS, followed by the addition of a 1 in 3000 dilution in 0.1% BSA in PBST of horseradish peroxidase conjugated to goat anti-rabbit IgG, IgM, and IgA (Organon Teknika Corp., West Chester, PA) and incubated for 1 h at 37 °C. Immunoreactivity was detected by the addition of tetramethylbenzene in citrate–acetate buffer and 0.013% $H_2O_2$. The reaction was then stopped with 2.5 N sulfuric acid, and optical densities were read at 450 nm.

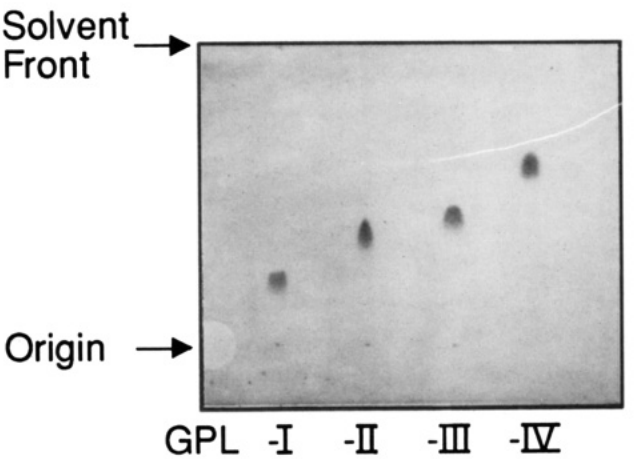



FIGURE 1: Thin-layer chromatogram of the purified GPLs-I–IV isolated from *M. xenopi* ATCC 19250. The solvent was $CHCl_3$–$CH_3OH$–$H_2O$ (90:10:1). The plate was sprayed with 10% $H_2SO_4$ in ethanol and heated at 110 °C for 5 min, to yield a yellow to yellow-gold color characteristic of all GPLs.

Table I: Results of the Application of ELISA to the Purified GPL I-IV from *M. xenopi*

| | | $A_{450}$[a] for the antigens | | | | |
|---|---|---|---|---|---|---|
| rabbit antiserum | serum dilution | GPL-I | GPL-II | GPL-III | GPL-IV | GPL from *M. avium* serovar 6[b] |
| preimmune | 1:100 | 0.021 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 1:800 | 0.007 | 0.000 | 0.000 | 0.000 | 0.000 |
| anti-*M. xenopi* | 1:100 | 1.132 | 1.576 | 1.290 | 0.198 | 0.000 |
| | 1:1000 | 0.460 | 0.975 | 0.624 | 0.041 | 0.000 |
| anti-*M. xenopi* | 1:100 | 0.028 | 0.000 | 0.000 | 0.000 | 0.031 |
| GPL-I–IV | 1:800 | 0.000 | 0.000 | 0.000 | 0.000 | 0.021 |

[a] Background signals from wells coated with buffer were subtracted. [b] The C-mycoside GPL from *M. avium* serovar 6 was used as an antigen control. It was also coated at a concentration of 20 μg/mL of ethanol as described for the *M. xenopi* GPLs.

## RESULTS

*Purification and Antigenicity of the GPLs.* A total washed lipid fraction was obtained from *M. xenopi* (ATCC 19250) according to the procedure applied to many members of the *M. avium* complex (McNeil et al., 1989). Trituration with

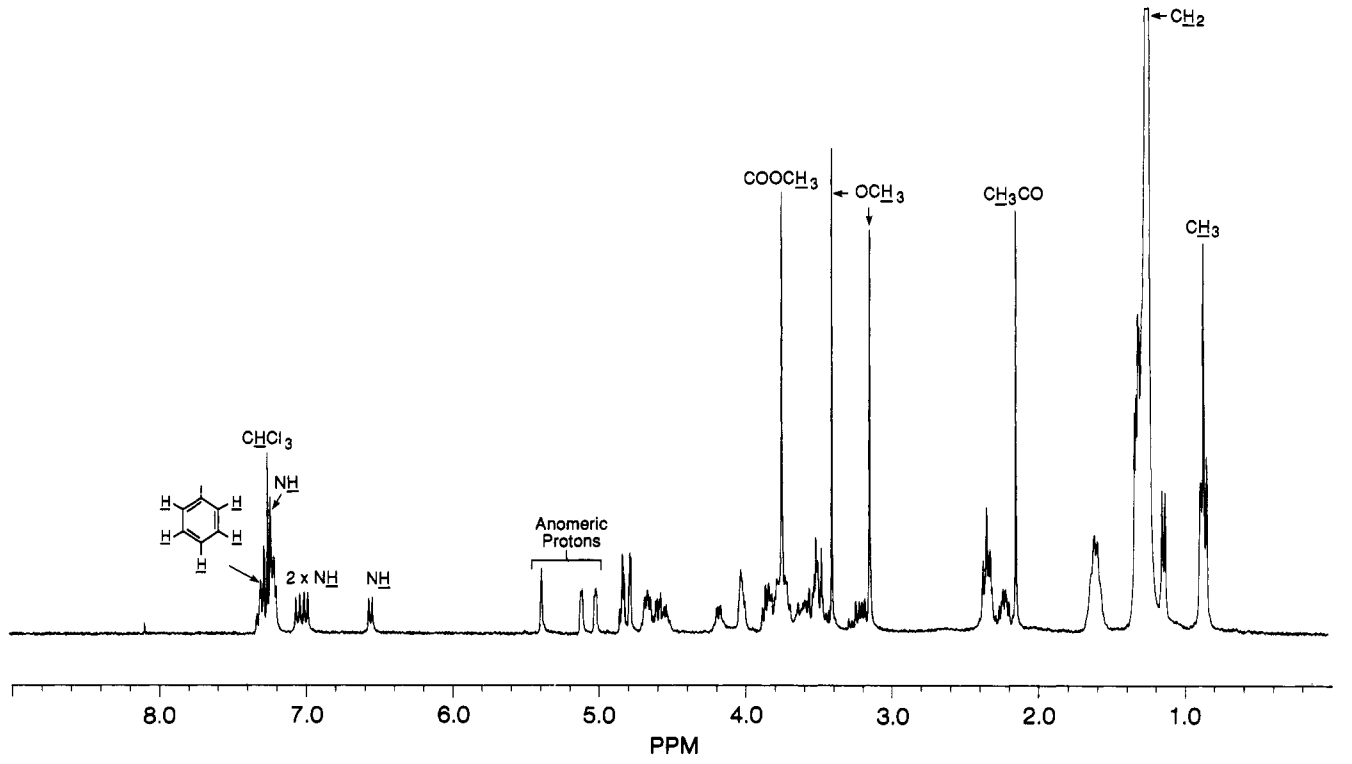



FIGURE 2: $^1$H-NMR (300 MHz) of GPL-IV at a concentration of 24 mg in 0.5 mL of $C^2HCl_3$–$C^2H_3O^2H$ (4:1).

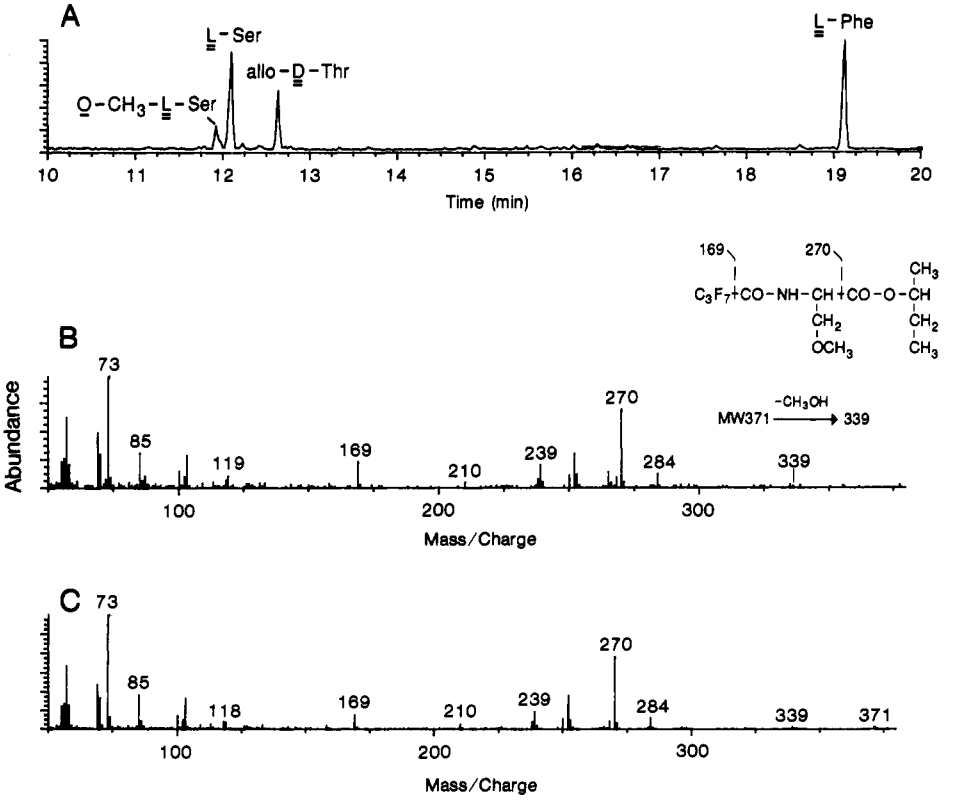



FIGURE 3: GC/MS profile of the amino acid *N*(*O*)-heptafluorobutyryl (*R*)-2-butyl esters derived from GPL-IV: (A) Total ion chromatogram; (B) Mass spectrum of the *O*-$CH_3$-Ser from GPL-IV; (C) Mass spectrum of the standard 3-*O*-$CH_3$-Ser.

acetone yielded 1.67 g of acetone-soluble lipids from 4.7 g of total lipid. This material was subjected to low-pressure absorption chromatography on columns of Florisil and irrigated with $CHCl_3$ and gradual increments of $CH_3OH$ in $CHCl_3$. TLC showed the presence of partially purified lipids in the 2%–10% $CH_3OH$ eluates which yielded a distinct yellow coloration when charred with 10% sulfuric acid in ethanol, identical to that produced by the GPLs of *M. avium* (Brennan & Goren, 1979). The partially purified GPLs were pooled and repurified by preparative TLC to yield GPL-I (8 mg); GPL-II (12 mg); GPL-III (9 mg); and GPL-IV (24 mg) (Figure 1). The purified glycolipids, when treated with alkali, were found to be still lipid-soluble, again, a characteristic feature of the alkali-stable GPLs (Brennan & Goren, 1979).

ELISA demonstrated that the rabbit antiserum generated aginst whole *M. xenopi* reacted strongly to purified GPL-I,

GPL-II, and GPL-III, but only weakly to GPL-IV (Table I). However, efforts to generate antibodies to the glycolipids themselves were not successful (Table I), probably due to our inability to emulate the adjuvanticity of the mycobacterial cell wall. Clearly, however, GPL-I–III evoke a strong B-cell response when presented to the immune system in the context of the whole infectious agent, implying that they are among the dominant surface antigens of this species (Jenkins et al., 1972).

*Characterization of the Sugar Substituents of the GPLs.* The $^1$H-NMR spectra of the four glycolipids were similar and revealed four amide and three anomeric resonances, suggesting that this family of GPLs had the same basic structure but differed in the fatty acyl population associated with each member. For example, the $^1$H-NMR spectrum of GPL-IV (Figure 2) revealed the presence of three characteristic anomeric protons centered at 5.46 ppm ($J_{1,2} < 1.5$ Hz), 5.14 ppm ($J_{1,2} < 1.5$ Hz), and 5.04 ppm ($J_{1,2} < 1.5$ Hz). The low-field region of the spectrum between 6 and 8 ppm contained the four amide protons and also a complex multiplet at 7.24 ppm associated with an aromatic ring. Resonances associated with two methoxy groups (3.16 ppm, singlet; 3.40 ppm, singlet) and one methoxy ester (3.78 ppm, singlet) were also apparent. However, a difference among the GPLs was noted in that GPL-II and -IV yielded a singlet at 2.12 ppm (Figure 2) associated with an acetoxy group, whereas GPL-I and -III were devoid of this resonance.

The nature of the individual glycosyl units in all four GPLs was examined through hydrolysis, reduction with $NaB^2H_4$, per-*O*-acetylation, GC, and GC/MS of the alditol acetates as described (McNeil et al., 1989). In this way, the presence of two sugars in the approximate ratio of 2:1 was seen (results not shown), in accordance with the observation of resonances for three anomeric protons (Figure 2). The less abundant sugar component was shown by GC/MS (*m/z* 130, 143, 190, 203) and cochromatography with the authentic standard (Brennan & Goren, 1979) to be 1,2,4,5-tetra-*O*-$CH_3CO$-3-*O*-$CH_3$-6-deoxytalitol. The more abundant sugar product was identified as 1,2,3,4,5-penta-*O*-$CH_3CO$-rhamnitol by GC/MS (*m/z* 115, 129, 171, 201, 231, 290) and cochromatography. Thus, the GPLs were shown to contain a single 3-*O*-$CH_3$-6-dTal and two Rha residues, not at all unlike the sugar composition of the more apolar GPLs of the *M. avium* complex. The $\alpha$-anomeric configuration was assigned to all residues since all of the anomeric hydrogens displayed chemicals shifts greater than 5 ppm. The enantiomeric configurations of the 3-*O*-$CH_3$-6-dTal and Rha residues were determined by comparative GC/MS analyses of the $(CH_3)_3$Si-(*R*)-(−)-2-butyl glycosides. The derivatives prepared from all four GPLs cochromatographed with the $(CH_3)_3$Si-(*R*)-(−)-2-butyl glycosides of 3-*O*-$CH_3$-L-6-dTal and L-Rha and differed from the $(CH_3)_3$Si-(*S*)-(+)-2-butyl glycosides. Thus, all of the glycosyl residues were considered to be in the L absolute configuration.

The positions of glycosyl linkages and fatty acyl substitutions were determined by analyzing the alditol acetate patterns after various forms of alkylations. Application of the mildly acidic methylation conditions of Prehm (1980) as described (Camphausen et al., 1987) to GPL-IV followed by analysis of alditol acetates showed the presence of 1,4,5-tri-*O*-$CH_3$-CO-2,3-di-*O*-$CH_3$-rhamnitol ($R_T$ 7.62 min; *m/z* 118, 143, 162, 203), 1,2,5-tri-*O*-$CH_3CO$-3,4-di-*O*-$CH_3$-6-deoxytalitol ($R_T$ 7.66 min; *m/z* 130, 131, 190), and 1,2,3,5-tetra-*O*-$CH_3$-CO-4-*O*-$CH_3$-rhamnitol ($R_T$ 8.28 min; *m/z* 131, 202, 262). A sample of per-O-methylated GPL-IV, prepared under the milder acidic conditions, was also per-O-trideuteriomethylated

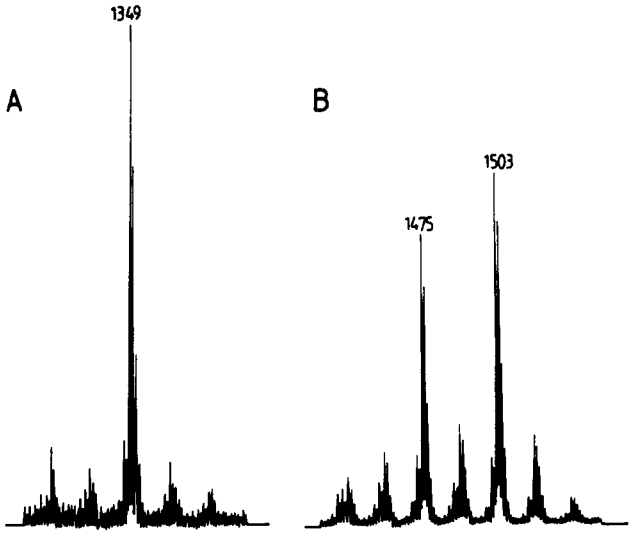



FIGURE 4: Molecular ion region of the FAB/MS spectrum of the native GPL-II (A) and GPL-IV (B).

by the base-catalyzed method (Stellner et al., 1973), in which case the trideuteriomethyl group will mark the position of base-labile acyl functions. Three new alditol acetates resulted from this treatment: 1,5-di-*O*-$CH_3CO$-4-*O*-$C^2H_3$-2,3-di-*O*-$CH_3$-rhamnitol; 1,3,5-tri-*O*-$CH_3CO$-2-*O*-$C^2H_3$-4-*O*-$CH_3$-rhamnitol; and 1,5-di-*O*-$CH_3CO$-2-*O*-$C^2H_3$-3,4-di-*O*-$CH_3$-6-deoxytalitol. Accordingly, the existence of a terminal 3-*O*-$CH_3$-$\alpha$-L-6-dTal*p* with an acyl substitution at position 2 and a terminal $\alpha$-L-Rha*p* with an acyl group at position 4 was established. The third glycosyl residue consisted of a 3-linked $\alpha$-L-Rha*p* unit with an acyl function at the 2-position. At this stage of the analysis, the glycosyl linkage data suggested the existence of one of two possible disaccharides, 4-*O*-acyl-$\alpha$-L-Rha*p*(1→3)2-*O*-acyl-$\alpha$-L-Rha*p* or 2-*O*-acyl-3-*O*-$CH_3$-$\alpha$-L-6-dTal*p*(1→3)2-*O*-acyl-$\alpha$-L-Rha*p*, and also the presence of one of two possible monosaccharide units, either 4-*O*-acyl-$\alpha$-L-Rha*p* or 2-*O*-acyl-3-*O*-$CH_3$-$\alpha$-L-6-dTal*p*. The resolution of these possibilities as well as the determination of where on the peptide the mono- and disaccharides are located was elucidated unequivocally by FAB/MS.

*Amino Acid Composition.* Early on, it was observed that the GPLs of *M. xenopi* yielded an unusual amino acid pattern, different from those of the conventional GPLs of the *M. avium* complex. The report of Rivière and Puzo (1991) provided an explanation. Comparative GC and GC/MS of the *N*(*O*)-HFB butyl esters of the amino acids of GPL-IV showed four amino acids, three of which were readily identified as Ser, alloThr, and Phe (Figure 3). Since FAB/MS analysis (see below) suggested the presence of *O*-methylserine, it was specifically sought. A derivative with a lesser detector response was thus identified as 3-*O*-$CH_3$-Ser by comparative GC/MS (Figure 3). The enantiomeric configuration of the amino acids was established by comparing the GC profile of the *N*(*O*)-HFB (*R*)-2-butyl esters of the amino acids in GPL-I–IV with the corresponding derivatives of authentic standards. The 3-*O*-$CH_3$-Ser, Ser, and Phe are in the L configuration, whereas the alloThr is in the D configuration. The 3.78 ppm singlet in the $^1$H-NMR spectrum of the GPLs (Figure 2) points to the presence of a methyl ester at the COOH terminus of the peptide.

*Structural Elucidation of GPL-II and -IV by FAB/MS.* FAB/MS enabled sequencing of the amino acids in the tetrapeptide core, assignment of individual glycosyl residues to either the disaccharide or monosaccharide appendages, location of the positions of the disaccharide and monosac-

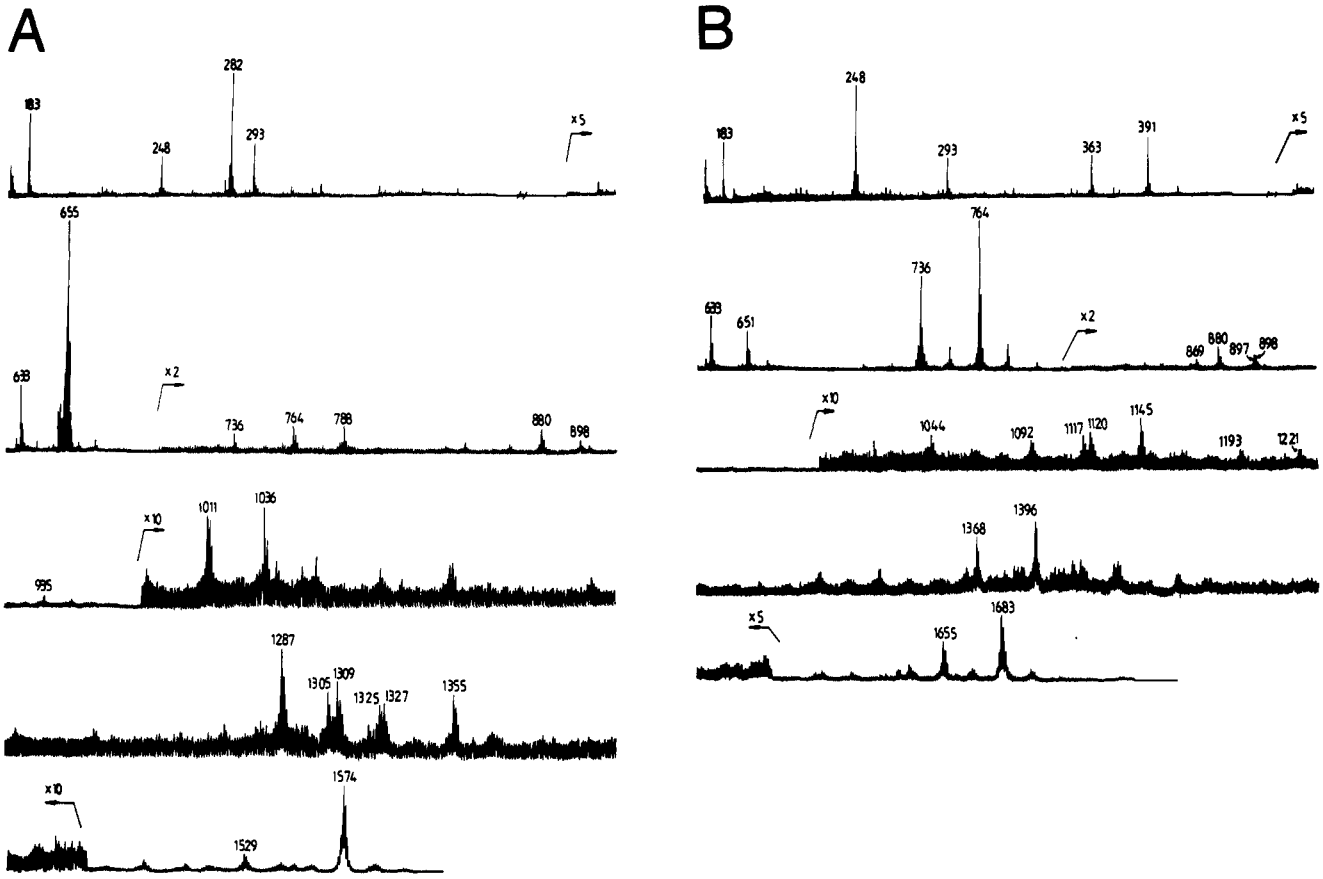



FIGURE 5: FAB/MS spectra of the per-*O*-trideuterioacetylated GPL-II (A) and GPL-IV (B). Major signals were assigned as shown in Figure 6. Signals at *m/z* 1309 and 1327 in (A) are the sodiated analogues of *m/z* 1287 and 1305, respectively, while the signal at *m/z* 1355 (28 amu higher than *m/z* 1327) corresponds to a similarly sodiated fragment ion resulting from ring cleavage of the 6-deoxytalose residue (Dell, 1987). The signals at *m/z* 1011 (A) and 1092/1120 (B) are 25 mass units below *m/z* 1036 (A) and 1117/1145, respectively. It is possible that these signals are sodiated C-terminal fragment ions resulting from cleavage between the amide nitrogen and the $\alpha$-carbon of the methylated serine concomitant with loss of methoxide from the side chain. The small signals at *m/z* 736 and 764 in (A) were probably derived from minor higher molecular weight components. The signals at *m/z* 633 and *m/z* 651 were derived from loss of both saccharide moieties from the peptide core. The same signals were observed in the FAB spectra of the native glycolipids (see text).

charide units on the tetrapeptide backbone, and definition of the fatty acyl functions and their locations. FAB/MS analysis of GPL-II in the positive ion mode, using *m*-nitrobenzyl alcohol as matrix, afforded an intense molecular ion $[M + Na]^+$ of *m/z* 1349 (Figure 4A), whereas the native GPL-IV yielded a pair of molecular ion signals of *m/z* 1475 and 1503 (Figure 4B). The mass difference between GPL-II and GPL-IV (126 and 154 amu) suggested the presence of an additional $C_8$ or $C_{10}$ fatty acyl chain on GPL-IV, which would be in accordance with the relative migration of GPL-II and GPL-IV on TLC (Figure 1).

FAB/MS analysis on the per-*O*-trideuterioacetylated GPL-II (Figure 5A) and GPL-IV (Figure 5B) yielded further valuable molecular weight information and readily interpretable fragmentation patterns. Assignments of the major signals are summarized in Figure 6. The mass shifts of the $[M + Na]^+$ molecular ions of the deuterioacetylated GPLs, relative to the native glycolipids, pointed to the incorporation of five trideuterioacetyl groups into GPL-II and four into GPL-IV. These are the exact numbers of trideuterioacetyl groups required to achieve full substitution of the free hydroxyl functions on the glycosyl residues of the proposed structures for GPL-II and -IV, respectively (Figure 6). Thus, there were no apparent free hydroxyl functions on the lipopeptide core or the other acyl groups. In fact, this FAB/MS analysis first suggested the presence of *O*-$CH_3$-Ser in the GPLs of *M. xenopi*.

The A-type ions at *m/z* 248 in the spectra of both the GPL-II and -IV (Figure 5A,B) were considered to arise from a mono-*O*-methyl-, mono-*O*-acetyl-, mono-*O*-trideuterioacetyl-6-deoxyhexosyl residue. Since the only *O*-$CH_3$-sugar present in the GPLs is 3-*O*-$CH_3$-6-dTal and since this is acylated at position 2, the ion at *m/z* 248 points to the presence of an acetyl function. A similar A-type ion at *m/z* 282 in the spectra of GPL-II corresponds to a tri-*O*-deuterioacetyl-Rha residue. In the case of GPL-IV, the ion at *m/z* 282 was replaced by ions at *m/z* 391 and *m/z* 363, consistent with a di-*O*-trideuterioacetyl-mono-*O*-acyl-Rha residue in which the acyl function is either a C-10 (*m/z* 391) or a C-8 (*m/z* 363). The earlier methylation analysis demonstrated that the fatty acyl functions must be on C-4 of the Rha unit. In addition, A-type fragment ions were seen (Figure 5) at *m/z* 655 for GPL-II and *m/z* 764 and 736 for GPL-IV, indicative of the presence of dirhamnosyl units as opposed to a combination of 3-*O*-$CH_3$-6-dTal and Rha. These ions demonstrate that, in both cases, the reducing Rha residue is substituted with a C-12 fatty acid. The methylation data already demonstrated that the fatty acid must be on C-2.

Fragment ions which allowed definition of the peptide sequence and the attachment points of the mono- and disaccharides were also found. Thus, the ions at *m/z* 788 in the case of GPL-II and *m/z* 897 and 869 for GPL-IV indicated that the dirhamnoside was *O*-linked to the carboxymethylated D-alloThr. The additional series of ions at *m/z* 935 and 1036 for GPL-II and *m/z* 1044/1016 and *m/z* 1145/1117 for GPL-IV demonstrated that the glycosylated carboxymethylated D-alloThr unit is attached to L-Phe, which is followed by the

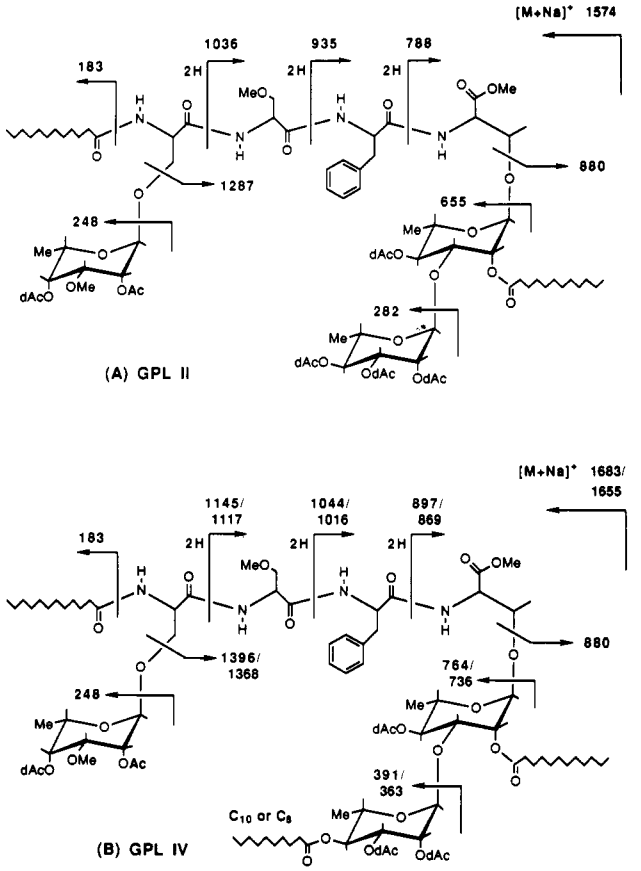



FIGURE 6: Schematic representation of the full structures of the perdeuterioacetylated GPL-II (A) and GPL-IV (B). The major ions observed in their FAB/MS spectra (Figure 5) are indicated. Abbreviations: dAc, deuterioacetyl; Ac, acetyl; Me, methyl.

*O*-$CH_3$-Ser unit (Figure 6). The L-Ser unit must therefore occupy the N-terminal end of the peptide with the 3-*O*-$CH_3$-6-dTal residue attached to its hydroxyl group and the $C_{12}$ fatty acid linked to the amino function. Confirmation of the location of the glycosyl residues was afforded by the ions at *m/z* 880 and 898, attributable to loss of the acylated dirhamnosyl group due to β-elimination and β-cleavage, respectively. A similar loss of the 2-*O*-$CH_3CO$-3-*O*-$CH_3$-6-dTal residue resulted in signals at *m/z* 1287 (due to β-elimination) and 1305 (due to β-cleavage) for GPL-II and *m/z* 1396/1368 (β-elimination) for GPL-IV.

The FAB/MS fragmentation pattern of the native GPLs (Figure 7) helped confirm both the presence of *O*-$CH_3$-Ser and the sequence of amino acids. Thus, ions at *m/z* 382 and 281 corresponded to the C-terminal fragments $^+H_3N$-(*O*-$CH_3$)Ser-Phe-Thr-$COOCH_3$ and $^+H_3N$-Phe-Thr-$COOCH_3$, respectively, and ions at *m/z* 518, 371, and 270 were indicative of the N-terminal fragments (*N*-acyl)Ser-(*O*-$CH_3$)Ser-Phe-$CO^+$, (*N*-acyl)Ser-(*O*-$CH_3$)Ser-$CO^+$, and (*N*-acyl)Ser-$CO^+$, respectively. Loss of the acylium–CO moiety (28 amu) and $CH_3OH$ (32 amu) from *m/z* 518 yielded ions at *m/z* 490 and 486, respectively. In addition, very prominent signals of *m/z* 651 and 633 were present in the spectra of both GPLs (data not shown). The former corresponded in mass to $[M + H]^+$ of the lipopeptide core upon loss of all of the glycosyl residues. Further loss of a water molecule, probably resulting from β–elimination rather than β-cleavage of one of the glycosyl substitutents, gave rise to the latter signal at 18 amu lower (*m/z* 633). In the lower mass region, signals attributable to A-type ions were observed as follows: $Rha^+$ at *m/z* 147 for GPL-II; *O*-$CH_3CO$-*O*-$CH_3$-6-$dTal^+$ at *m/z* 203 for GPL-II and -IV; $C_8$-fattyacyl-$Rha^+$ at *m/z* 273 for GPL-IV; $C_{10}$-fattyacyl-$Rha^+$ at *m/z* 301 for GPL-IV; and $C_{12}$-fattyacyl-Rha-$Rha^+$ at *m/z* 475 for GPL-II (Figure 7).

Much of this information on the nature and positions of the acyl and glycosyl substituents was supported by application of mild methanolysis followed by FAB/MS of the native GPLs. Under the experimental conditions employed (Dell, 1987, 1990), partial losses of labile substituents were observed as new or enhanced molecular ion signals (results not shown). Interestingly, cleavage of acyl and glycosyl residues occurred at similar rates. Loss of an acetyl group from GPL-II gave rise to a signal at *m/z* 1307, and subsequent loss of the 3-*O*-$CH_3$-6-dTal unit gave rise to an ion at *m/z* 1147. These data are consistent with the interpretation of the presence of an acetyl group on the 3-*O*-$CH_3$-6-dTal unit. A similar loss of an acetyl group from GPL-IV afforded $[M + H]^+$ molecular ion pairs at *m/z* 1439 and 1411. Independent loss of the $C_{12}$, $C_{10}$, or $C_8$ fatty acyl functions resulted in signals at *m/z* 1285, 1257, and 1229, indicative of the deacetylated GPL-IV retaining a single fatty acyl group of $C_{12}$, $C_{10}$, and $C_8$, respectively. Significantly, a single signal at *m/z* 1141 (with no further signals of 28 amu higher or lower) corresponded to deacetylated GPL-IV having lost an acyl-Rha concomitant with the heterogeneity. This information supported the evidence that the $C_8/C_{10}$, but not the $C_{12}$, fatty acyl substituents are on the terminal Rha unit. The same peptide-related ions seen in the low mass region during FAB/MS of the native GPLs were considerably enhanced in the methanolysis experiments with concomitant esterification of the N-terminal fragments (data not shown).

*Analysis of GPL-I and -III by FAB/MS.* FAB/MS of the native GPL-I afforded a strong molecular ion at *m/z* 1307 (results not shown), 42 amu less than that of GPL-II (Figure 4A). All of the key fragment ions observed for GPL-II (Figure 7A) were also seen except for the ions at *m/z* 203 (A-type ion of 2-*O*-$CH_3CO$-3-*O*-$CH_3$-6-dTal) and *m/z* 171 (*m/z* 203 minus methanol). Upon perdeuterioacetylation, GPL-I afforded a molecular ion at *m/z* 1577 compared to *m/z* 1574 for GPL-II, pointing to the absence of an *O*-acetyl function from GPL-I as first indicated by the $^1$H-NMR analysis. The remainder of the FAB/MS spectrum was similar to that of GPL-II (Figure 5A), exhibiting all of the important sequence ions (including *m/z* 1036, 788, 655, and 282). Significantly, ions at *m/z* 248, 880, and 898 were shifted to *m/z* 251, 883, and 901, respectively. Thus, the FAB/MS data strongly support the proposition that GPL-I is identical to GPL-II except for the absence of the *O*-acetyl substituent on the 3-*O*-$CH_3$-6-dTal residue.

The native GPL-III afforded a pair of molecular ions on FAB/MS at *m/z* 1433 and *m/z* 1461, 42 amu less than those of GPL-IV (results not shown). As expected, the perdeuterioacetylated samples yielded molecular ion signals at *m/z* 1658 and *m/z* 1686, 3 amu greater than the corresponding ions for the perdeuterioacetylated GPL-IV. All of the sequence ions observed in the FAB/MS spectrum of the perdeuterioacetylated GPL-IV (Figure 5B) were present except those *m/z* 248 and *m/z* 880 which were shifted up by 3 amu to *m/z* 258 and *m/z* 883, respectively. Thus, GPL-III is identical to GPL-IV except for the absence of the *O*-acetyl substituent on the 3-*O*-$CH_3$-6-dTal unit. A minor component was observed in the GPL-III preparation by FAB/MS which yielded a series of $[M + Na^+]$ signals at 14 amu intervals, at *m/z* 1801, *m/z* 1815, and *m/z* 1829. Upon methanolysis, this component was observed to readily lose 146 amu, consistent with its containing a terminal nonalkylated, nonacylated, 6-deoxyhexosyl unit.

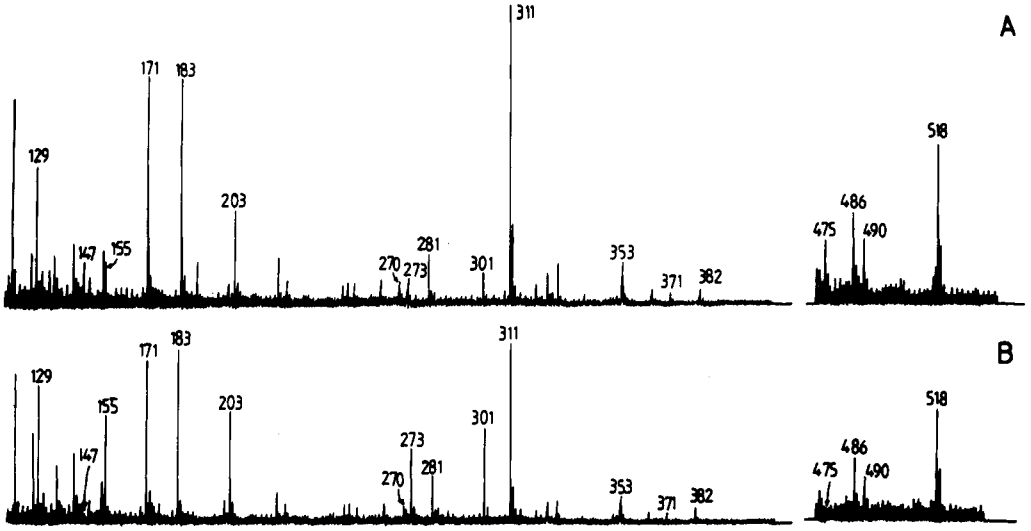
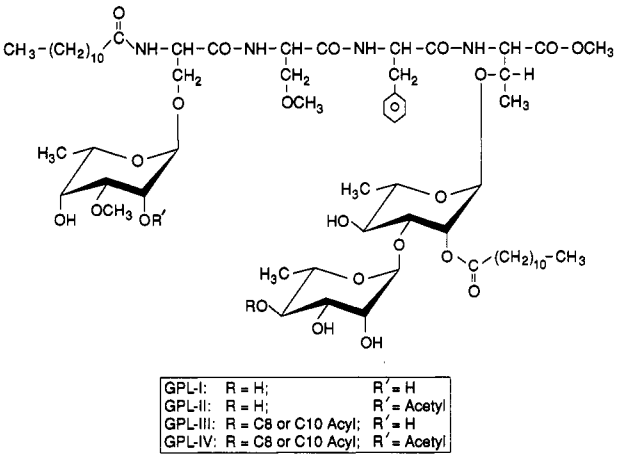



FIGURE 7: Low mass region of the FAB/MS spectra of the native GPL-II (A) and GPL-IV (B). The molecular ion regions of these spectra are shown in Figure 4. In addition to the signals described in the text, there was a major signal at $m/z$ 311 which corresponds to the A-type ion of the inner rhamnosyl residue which had lost its 3-linked rhamnosyl substituent by $\beta$-elimination. Other unassigned signals are identified as follows: $m/z$ 171 (elimination of methanol from the signal at $m/z$ 203; this signal supports the assignment of methylation at the 3-position of the 6-deoxytalose residue), 183 (acylium ion for the $C_{12}$ fatty acyl moiety), 155 (acylium ion for the $C_{10}$ fatty acyl moiety). The small signals at $m/z$ 273 and 301 in (A) are most probably derived from minor components having the same acylated terminal rhamnosyl residue as that present in GPL-IV. Minor signals (approximately 5% of the size of the molecular ions shown in Figure 4A) were present in the region $m/z$ 1750–1950. It is possible that some of these gave rise to $m/z$ 273 and 301 and to their acetylated counterparts in Figure 5.

## DISCUSSION

As the final chapter on the chemistry of the C-mycoside glycopeptidolipids of *M. avium* is being written (Aspinall et al., 1993), another has just begun. Two important variations on the classical polar C-mycoside glycopeptidolipid structure (Chart I) were recently observed by two French groups, for mycobacteria outside the *M. avium* complex. Lopez-Marin et al. (1992) demonstrated that the L-alaninol unit was the site of additional glycosylation in the product from *M. fortuitum* subsp. *peregrinum*. Rivière and Puzo (1991, 1992) showed that a new lipopeptide core structure applied to the major glycolipid from a strain of *M. xenopi* (CIPT 140 35004), a product which contained a simpler fatty acyl function, and they showed the presence of serine rather than alaninol as an additional site of glycosylation, but which in the sugar appendages contained features which are now common among the other classes of mycobacterial glycolipids (Chart I), such as a range of unusual 6-deoxyhexoses, usually O-methylated, and unusual *O*-acyl functions (Brennan, 1988; Puzo, 1990). The present report corroborates much of the earlier proposed structure (Chart II) and amplifies it in major and minor ways. The complete proposed structures of the four simpler, less glycosylated members of the class of serine-containing glycopeptidolipids of *M. xenopi* (ATCC 19250) are shown in Chart III. The unequivocal identification of the presence of *O*-methylserine in all members of this family serves to further establish that the primary point of glycosylation is the N-terminal serine residue. Also, the observation that only the alloThr occurred in the D configuration, whereas Phe, unlike in the C-mycosides, and serine are both L, is important since it points to a biosynthetic pathway simpler than that proposed for the C-mycosides (Brennan, 1984) but does remove a potential drug target in that the biogenesis of the C-mycoside glycopeptidolipids, and, specifically, the racemization of L-alanine to D-alanine, is sensitive to D-cycloserine.[2] Interestingly, in the early days of exploration on the structures of the C-mycoside glycopeptidolipids, other French workers (Vilkas et al., 1965) had observed the presence of *N*-methyl-

Chart III: Proposed Complete Structures of the Four Glycopeptidolipids from *M. xenopi*




*O*-methylserine in the C-mycoside GPL from *Mycobacterium butyricum*. Also, Lanéelle and Asselineau (1968) have reported on the presence of ethanolamine in the product from an unclassified *Mycobacterium* 1217. Reports on the presence of D-leucine and perhaps D-alloisoleucine in these structures have often cropped up (Ikawa & Snell, 1962). Thus, several lipopeptide cores may be in existence among the glycopeptidolipids of *Mycobacterium* spp. However, there should be no ambiguity about the present structure, especially concerning the nature of the unique lipopeptide core. In the 1960s, several versions of the C-mycoside lipopeptide core (Chart I) were advanced, e.g., D-Phe-D-alloThr-D-Ala-D-alloThr-D-Ala-D-alloThr-D-Ala (Chaput et al., 1962), before recognition of the role of aminopropanol/alaninol (Lanéelle, 1966) and advancement of the accepted structure (Lanéelle & Asselineau, 1968; Vilkas & Lederer, 1968). Further unequivocal proof of the accepted structures was provided in the case of the singly glycosylated, apolar C-mycoside glycopeptidolipids by field desorption mass spectrometry (Daffé et al., 1983) and, in the case of the multiglycosylated, polar C-mycoside glycolipids from *M. avium* serovar 4 and serovar 12, by Californium desorption mass sepctrometry (Bozic et al., 1988;

[2] Work conducted with Hee-Joo Park.

Jardine et al., 1989). In the present case, FAB/MS provided overwhelming proof of the presence of *O*-methylserine in the peptide core and of the sequence of amino acids in the core and allowed assignment of sugars to either of the glycosylation sites, in addition to providing information on the nature of the acyl functions on either peptide or sugar. Thus, the results from FAB/MS should obviate the type of equivocation that attended the definiton of the core structure of the C-mycoside glycopeptidolipids in former times.

We have been unable to find the precise tetrasaccharide-containing GPL described by Rivière and Puzo (1991) among the lipids of *M. xenopi* ATCC 19250, which may indicate that *M. xenopi*, like *M. avium*, *M. scrofulaceum*, and *M. simiae*, is a serocomplex based on serine-containing glycopeptidolipids rather than on the polar C-mycosides. Clearly, some of the products described here are antigenic. Precedence in the case of the GPLs of *M. avium* (McNeil et al., 1989) would suggest that the active epitope involves the terminal nonacylated, nonalkylated L-Rha unit. The fact that the GPL-IV is not antigentic could be attributable to the acylation of this terminal rhamnosyl unit. However, the fact that GPL-III is immunoreactive is puzzling, considering that the only difference between the two is the presence of an acetyl group on the 3-*O*-$CH_3$-6-deoxytalose residue. A possible explanation is that the active component in the GPL-III preparation is the uncharacterized high molecular weight product for which there was evidence for a terminal nonacylated, nonalkylated rhamnosyl residue. Further study of the serology of these new glycolipids is clearly necessary. Indeed, in view of the clinical importance of *M. xenopi* (Ausina et al., 1988) and in view of the clinical significance of just a few of the *M. avium* serovars (Tsang et al., 1992), the search for a range of *M. xenopi* serovars should begin, on the basis of the definitive chemical principles described here and elsewhere (Rivière & Puzo, 1991).

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## REFERENCES

Aspinall, G. O., Chatterjee, D., & Brennan, P. J. (1993) *Adv. Carbohydr. Chem. Biochem.* (in press).

Ausina, V., Barrio, J., Luquin, M., Gurgui, M., Verger, G., & Prats, G. (1988) *Ann. Intern. Med. 109*, 929–930.

Belisle, J. T., & Brennan, P. J. (1989) *J. Bacteriol. 171*, 3465–3470.

Besra, G. S., McNeil, M., Minnikin, D. E., Portaels, F., Ridell, M., & Brennan, P. J. (1991) *Biochemistry 30*, 7772–7777.

Bozic, C. M., McNeil, M., Chatterjee, D., Jardine, I., & Brennan, P. J. (1988) *J. Biol. Chem. 263*, 14984–14991.

Brennan, P. J. (1984) in *Microbiology 1984* (Leive, L., & Schlessinger, D., Eds.) pp 366–375, American Society for Microbiology, Washington, D.C.

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

Brennan, P. J., & Goren, M. B. (1979) *J. Biol. Chem. 254*, 4205–4211.

Camphausen, R. T., McNeil, M., Jardine, I., & Brennan, P. J. (1987) *J. Bacteriol. 169*, 5473–5480.

Chan, J., Fan, X., Hunter, S. W., Brennan, P. J., & Bloom, B. R. (1991) *Infect. Immun. 59*, 1755–1761.

Chaput, M., Michal, G., & Lederer, E. (1962) *Biochim. Biophys. Acta 63*, 310–326.

Chatterjee, D., Aspinall, G. O., & Brennan, P. J. (1987) *J. Biol. Chem. 262*, 3528–3533.

Daffé, M., Lanéelle, M. A., & Puzo, G. (1983) *Biochim. Biophys. Acta 751*, 439–443.

Dell, A. (1987) *Adv. Carbohydr. Chem. Biochem. 45*, 19–72.

Dell, A. (1990) *Methods Enzymol. 193*, 647–660.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Gaylord, H., & Brennan, P. J. (1987) *Annu. Rev. Microbiol. 41*, 645–675.

Gerwig, G. J., Kamerling, J. P., & Vliegenthart, J. F. G. (1978) *Carbohydr. Res. 58*, 165–175.

Hirschfield, G. R., McNeil, M., & Brennan, P. J. (1990) *J. Bacteriol. 172*, 1005–1013.

Horsburgh, C. R., & Selik, R. M. (1989) *Am. Rev. Respir. Dis. 139*, 4–7.

Hunter, S. W., Jardine, I., Yanagihara, D. L., & Brennan, P. J. (1985) *Biochemistry 24*, 2798–2805.

Hunter, S. W., Barr, V. L., McNeil, M., Jardine, I., & Brennan, P. J. (1988) *Biochemistry 27*, 1549–1556.

Ikawa, M., & Snell, E. E. (1962) *Biochim. Biophys. Acta 60*, 186–188.

Jardine, I., Scanlan, G., McNeil, M., & Brennan, P. J. (1989) *Anal. Chem. 61*, 416–422.

Jenkins, P. A., Marks, J., & Schaefer, W. B. (1972) *Tubercle 53*, 118–127.

Kochi, A. (1991) *Tubercle 72*, 1–6.

Lanéelle, G. (1966) *C. R. Acad. Sci. Paris, Ser. C. 263*, 502–503.

Lanéelle, G., & Asselineau, J. (1968) *Eur. J. Biochem. 5*, 487–491.

Lopez-Marin, L. M., Lanéelle, M.-A., Prome, D., Daffé, M., Lanéelle, G., & Prome, J.-C. (1991) *Biochemistry 30*, 10536–10542.

MacKenzie, S. L., & Tenaschuk, D. (1975) *J. Chromatogr. 111*, 413–415.

McNeil, M., Chatterjee, D., Hunter, S. W., & Brennan, P. J. (1989) *Methods Enzymol. 179*, 215–242.

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

Noordeen, S. K. (1991) *Lepr. Rev. 62*, 72–86.

Prehm, P. (1980) *Carbohydr. Res. 78*, 372–374.

Puzo, G. (1990) *Crit. Rev. Microbiol. 17*, 305–327.

Rivière, M., & Puzo, G. (1991) *J. Biol. Chem. 266*, 9057–9063.

Rivière, M., & Puzo, G. (1992) *Biochemistry 31*, 3575–3580.

Rivoire, B., Ranchoff, B. J., Chatterjee, D., Gaylord, H., Tsang, A. Y., Kolk, A. H. J., Aspinall, G. O., & Brennan, P. J. (1989) *Infect. Immun. 57*, 3147–3158.

Stellner, K., Saito, H., & Hakomori, S. (1973) *Arch. Biochem. Biophys. 262*, 2630–2635.

Takayama, K., Schnoes, H. K., Armstrong, E. L., & Boyle, R. W. (1975) *J. Lipid Res. 16*, 308–317.

Tsang, A. Y., Denner, J. C., Brennan, P. J., & McClatchy, J. K. (1992) *J. Clin. Microbiol. 30*, 479–484.

Vilkas, E., & Lederer, E. (1968) *Tetrahedron Lett. 26*, 3089–3092.

Vilkas, E., Rojas, A., & Lederer, E. (1965) *Compt. Rend. 261*, 4258–4260.

York, W. S., Darvill, A. G., McNeil, M., Stevenson, J. T., & Albersheim, P. (1986) *Methods Enzymol. 118*, 3–40.