Novel Type-Specific Lipooligosaccharides from *Mycobacterium tuberculosis*[†,‡]

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ABSTRACT: *Mycobacterium tuberculosis* (strain Canetti) is characterized by the presence of two novel glycolipids of the alkali-labile, trehalose-containing lipooligosaccharide class. Their structures were established by permethylation, partial acid hydrolysis, infrared and high-field NMR spectroscopy, and electron-impact and fast atom bombardment mass spectrometry of the native glycolipids and hydrolysis products. The trehalose substituent is unique in that it is methylated at the 6′-position. The structure of the simpler of the two glycolipids is 2-*O*-Me-α-L-Fuc*p*(1→3)-β-D-Glc*p*(1→3)-2-*O*-Me-α-L-Rha*p*(1→3)-2-*O*-Me-α-L-Rha*p*(1→3)-β-D-Glc*p*(1→3)-4-*O*-Me-α-L-Rha*p*(1→3)-6-*O*-Me-α-D-Glc(1↔1)-tri-*O*-acyl-α-D-Glc. Further glycosylation of the octaglycosyl unit of this nonantigenic glycolipid by an incompletely defined N-acyl derivative of a 4-amino-4,6-dideoxy-Gal*p* residue results in the second, highly antigenic nonasaccharide-containing glycolipid. Application of two-dimensional proton correlation spectroscopy demonstrated that the fatty acyl substituents are located on the 2,3,6 and 3,4,6 hydroxyl groups of the terminal glucosyl unit in the proportions of 2:3. Gas chromatography/mass spectrometry and optical rotation measurement allowed identification of the fatty acyl esters as primarily 2*L*-,4*L*-dimethylhexadecanoate, 2*L*-,4*L*-,6*L*-,8*L*-tetramethyloctadecanoate, and 2-methyl-3-hydroxyeicosanoate. The relationship of these glycolipids to different morphological forms of *M. tuberculosis* and to virulence is discussed.

Tuberculosis remains as one of the most common infectious diseases of mankind with an estimated 3 million deaths per annum worldwide, 16 million active cases, and about 1 billion infected persons (Snider, 1989). Consequently, a serologic test that could presage active infection and obviate sputum culture would be of immense value for tuberculosis control programs. Precedent demonstrates that most of the nontuberculous mycobacterial species are endowed with large quantities of a variety of glycolipids, which contain within them small oligosaccharides of such exquisite specificity as to allow unequivocal identification of species and subspecies (Brennan, 1984). In addition, these haptenic oligosaccharides are of sufficient antigenicity as to evoke corresponding specific antibodies and thereby to allow serodiagnosis of some individual mycobacterioses (Gaylord & Brennan, 1987). There have also been several quests for similarly specific substances in the tubercle bacilli (Brennan, 1984). The most notable of these studies (Reggiardo et al., 1980) revealed a collection of glycolipids that are highly immunoreactive but, on account of considerable cross-reactivity, are not likely candidates for the differential diagnosis of human tuberculosis. Daffe et al. (1987) reported the presence of large amounts of a major specific phenolic glycolipid in *Mycobacterium tuberculosis* Canetti and related strains. In addition, another uncharacterized, highly immunoreactive glycolipid was present in this strain of *M. tuberculosis* (Papa et al., 1989). The present communication demonstrates that this last product belongs to the newest class of immunoreactive mycobacterial glycolipids, namely, the trehalose-containing alkali-labile lipooligosaccharides (LOS)[1] (Hunter et al., 1983; Brennan, 1988), but is distinguished by unique structural features at both the reducing and nonreducing ends.

Experimental Procedures

Mycobacteria. M. tuberculosis Canetti was grown in Sauton's medium for 4–6 weeks at 37 °C and harvested by centrifugation (Daffe et al., 1987). *M. tuberculosis* ATCC 27294 (H37Rv), ATCC 25177 (H37Ra), and ATCC 35801 (Erdman), *Mycobacterium bovis* ATCC 35734 (BCG-Pasteur) and ATCC 35733 (BCG-Danish), and a large variety of nontuberculous mycobacteria were grown on 7H11 broth for 2–6 weeks at 37 °C and autoclaved, and the cells were recovered by centrifugation, washed, and lyophilized (Brennan & Goren, 1979). *Mycobacterium leprae* was purified from experimentally infected armadillo tissues (Hunter et al., 1982).

Purification of Lipooligosaccharides. Cells were extracted 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 used. In the case of *M. tuberculosis* Canetti, 50 g of wet cells yielded 2.6 g of total lipids. Washed lipids of *M. tuberculosis* Canetti were dissolved in $CHCl_3$ and applied to a column (3 × 40 cm) of silica gel

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[1] Abbreviations: LOS, lipooligosaccharides; PIM, phosphatidylinositol mannosides; Rha, rhamnose; Fuc, fucose; Glc, glucose; Ose, oligosaccharide; per-Me-Ose, permethylated Ose; BCG, Bacille Calmette-Guerin; TLC, thin-layer chromatography; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; EI-MS, electron-impact mass spectrometry; DMSO, dimethyl sulfoxide; FAB-MS, fast atom bombardment mass spectrometry; amu, atom mass unit; SIM, selective ion monitoring; TMS, trimethylsilyl; 2D-COSY, two-dimensional chemical shift correlated spectroscopy; PBS, phosphate-buffered solution; PBST, PBS with Tween; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; NGS, normal goat serum.

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(60–200 mesh) (Baker Chemical Co., Phillipsburgh, NJ) equilibrated in $CHCl_3$. The column was eluted stepwise with 750 mL each of $CHCl_3$ and 2% CH_3OH in $CHCl_3$ and then with 500 mL each of 5%, 10%, 20%, and 50% CH_3OH in $CHCl_3$. Fractions (250 mL) were collected in bulk and examined by TLC. The 20% CH_3OH in $CHCl_3$ fractions were dried, resuspended in $CHCl_3$, and rechromatographed on columns (2 × 30 cm) of silica gel that were irrigated with $CHCl_3$ and with 5%, 10%, 20%, and 50% CH_3OH in $CHCl_3$. Fractions (10 mL) were again collected and monitored for glycolipid content by TLC.

Degradations and Derivatizations of Lipooligosaccharides. Pure glycolipids (ca. 15 mg) were deacylated as described by Hunter et al. (1983, 1988). The purity of the resulting water-soluble oligosaccharides was established by TLC (Hunter et al., 1988). The resulting organic phases served as a source of fatty acids; these were converted to their methyl esters with 3 M HCl in CH_3OH for 18 h 75 °C.

The oligosaccharides arising from the corresponding lipooligosaccharides (ca. 7 mg) were per-O-methylated with methyl sulfinyl carbanion and C^2H_3I (Hakomori, 1964) and purified on cartridges of Sep-Pak C_{18} (Waters Associates, Inc., Milford, PA) (Waeghe et al., 1983).

Per-O-methylated oligosaccharides were partially hydrolyzed with 2 M CF_3COOH at 85 °C for 1 h to generate smaller, partially methylated oligosaccharide fragments. The hydrolysates were dried, reduced with NaB^2H_4, and remethylated with C^2H_3I, and the products were recovered by chromatography on a Sep-Pak C_{18} cartridge.

In addition, partially deglycosylated LOS-II was obtained by exposing purified intact LOS-II (4 mg) to 4 M HCl at room temperature overnight. The reaction mixture was extracted with $CHCl_3$, the organic phase was dried and redissolved in $CHCl_3$, and the resulting solution was chromatographed on a Sep-Pak silica gel cartridge. The cartridge was irrigated with $CHCl_3$ and with 10% and 20% CH_3OH in $CHCl_3$, and the fractions were analyzed by TLC to identify the partially degraded LOS-II. The upper aqueous phase of the biphasic mixture was in turn applied to a column of mixed-bed resin (Amberlite MB-3, Sigma, St. Louis, MO), and the product was eluted with water, dried, reduced, acetylated, and analyzed by GC/MS in order to identify the sugar component released during hydrolysis.

Other Analytical and Chromatographic Procedures. Intact glycolipids, their O-methylated or deacylated derivatives, and fragments were hydrolyzed under a variety of conditions [2 M CF_3COOH at 95 or 120 °C for 1–3 h; 4 M HCl at 25 or 65 °C for 0.5–18 h; anhydrous HF (1 mL) at 4 or 25 °C for 2 or 5 h; 1 M HCl in CH_3OH at 80 °C for 1 or 3 h] in order to arrive at optimal conditions for the generation of monosaccharides. Hydrolysates were reduced with NaB^2H_4 or NaB^1H_4 at 25 °C for 18 h and acetylated in acetic anhydride at 120 °C for 1 h in tubes fitted with Teflon-lined screw caps.

Intact glycolipids were analyzed by TLC on silica gel aluminum sheets (Merck, Darmstadt, FRG) in $CHCl_3$–CH_3OH–H_2O (90:10:1), $CHCl_3$–CH_3OH–H_2O (30:8:1), $CHCl_3$–CH_3OH–H_2O (60:12:1), or $CHCl_3$–CH_3OH–H_2O (65:25:4). For analysis of oligosaccharides or per-O-methylated oligosaccharides, 1-butanol–pyridine–H_2O (6:4:3) or 1-butanol–pyridine–H_2O (10:3:3) was used. Fatty methyl esters were analyzed by TLC with petroleum ether–dimethyl ether (8:2) as solvent.

$(CH_3)_3Si$ derivatives were prepared as described (Sweeley et al., 1963). Permethylated LOS-II was demethylated (Hough & Theobald, 1963) and hydrolyzed with 2 M CF_3COOH at 120 °C for 1 h, and the butyl glycosides were prepared (Gerwig et al., 1978).

GC of alditol acetates and of partially O-acetylated, partially O-methylated alditols was routinely conducted on a DB-23 capillary column as described (Bozic et al., 1988). Samples were injected in the splitless mode. The alditol derivatives were separated by using a temperature gradient of 50–160 °C (30 °C/min) followed by 160–180 °C (8 °C/min) and 180–240 °C (10 °C/min). GC/MS analyses were performed on a Hewlett-Packard 5890A gas chromatograph connected to a Hewlett-Packard 5970 mass selector detector using a 12-m HP-1 Hewlett-Packard column. Samples were injected in the splitless mode and the mass spectrometer scanned from 50 to 800 amu. FAB-MS was performed on a VG 7070 extra-high-frequency mass spectrometer using an ion tech saddle field gun operating at 7–8 kV and 1 mA with xenon gas; a matrix of glycol or of thioglycerol–glycerol (1:1) was used for the intact or peralkylated oligosaccharides, respectively. The mass spectrometer was controlled by a VG Series 2000 data system and scanning was from 2000–500 amu at 20 s/decade.

NMR was performed on a Bruker AM-500. Lipooligosaccharides were dissolved in C^2HCl_3–$C^2H_3O^2H$ (1:1) before and after exchanging protons in CH_3O^2H; oligosaccharides were dissolved in ^2H_2O. Acetone was added to the samples and its signal was assigned to δ 2.225 for ^1H NMR and δ 31.4 for ^{13}C NMR, which corresponds to δ 0 for tetramethylsilane. 2D-COSY was conducted by using the Bruker double-quantum-filter phase-sensitive COSY software (SI1 = 1024, SI2 = 2048, SW1 = 1373, SW2 = 2747). The spectra were recorded at 25 °C. IR spectra were recorded on a Nicolet MX-1 FTIR spectrometer (Nicolet Instrument Co., Madison, WI) and were measured as a thin film on NaCl.

Immunological Procedures. Mice (6–8 weeks old) were injected intravenously six times, 3 days apart, with 250 μg of whole cells of *M. tuberculosis* Canetti in 0.1 mL of PBS. Similarly, a second group of mice received 200 μg of total extracted lipids and 50 μg of whole cells in 0.1 mL of PBS; the suspension was probe-sonicated prior to administration. Blood samples were taken 3 days following the last boost and tested.

Antigens for plate ELISA were dissolved in C_2H_5OH at 20 μg/mL and antibodies were assayed exactly as described (Cho et al., 1983).

Immunoassays were also performed on silica gel GF (Fisher Scientific, Pittsburgh, PA) TLC plates (Minnikin et al., 1985). Crude lipids (150 μg) and pure glycolipids (12.5 μg) were dissolved in $CHCl_3$–CH_3OH (2:1) and chromatographed by using $CHCl_3$–CH_3OH–H_2O (90:10:1, 30:8:1, or 65:25:4) as solvent. TLC plates were dried and blocked by immersion of the plates in 0.25% BSA in PBS for 1 h with rocking at room temperature. Mouse serum raised against *M. tuberculosis* Canetti lipids was diluted (1/150) in a solution of 10% NGS in PBST, and the TLC plates were incubated with this primary antibody for 2 h with rocking at room temperature. Plates were washed four times with PBS, followed by immersion in a second antibody of horseradish peroxidase conjugated goat anti-mouse immunoglobulins G, M, and A at 1/1000 dilution in a solution of 10% NGS in PBST. A final wash was performed, and plates were covered with 8 mL of PBS containing 6 mg of 4-chloro-1-naphthol color reagent (Sigma) in 2 mL of CH_3OH and 30 μL of 30% H_2O_2. The development of dark purple spots was stopped by rinsing plates with H_2O.

Results

Isolation of Major Antigenic Lipooligosaccharides from M. tuberculosis. When total extractable lipids (2.6 g) from

Table I: Glycosyl Composition of LOS-I and LOS-II As Revealed by GC/MS Analysis of Alditol Acetates

O-Ac-*O*-CH_3-alditol identified	corresponding glycosyl group	retention time (min)	mol/mol of LOS		
			LOS-I	LOS-II	LOS-II'[a]
1,3,4,5-tetra-*O*-Ac-2-*O*-CH_3-rhamnitol	2-*O*-CH_3-Rha	12.15	2.0	2.0	2.0
1,3,4,5-tetra-*O*-Ac-2-*O*-CH_3-fucitol	2-*O*-CH_3-Fuc	12.42	1.0	1.0	nd[b]
1,2,3,5-tetra-*O*-Ac-4-*O*-CH_3-rhamnitol	4-*O*-CH_3-Rha	12.83	1.0	1.0	1.0
1,2,3,4,5-penta-*O*-Ac-6-*O*-CH_3-glucitol	6-*O*-CH_3-Glc	18.66	1.0	1.0	1.0
1,2,3,4,5,6-hexa-*O*-Ac-glucitol	Glc	21.06	3.0	3.0	3.0

[a] LOS-II' arose from partial hydrolysis (4 M HCl, 18 h at room temperature) of LOS-II. [b] nd = not detected.

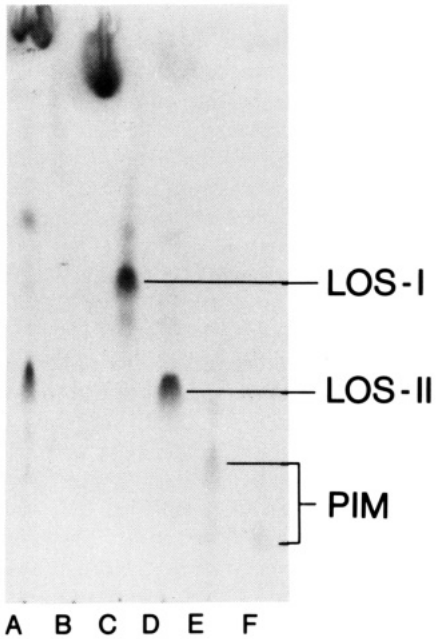

FIGURE 1: TLC of glycolipid fractions from *M. tuberculosis* Canetti in $CHCl_3$–CH_3OH–H_2O (60:12:1). Lane A, total unfractionated lipid; lane B, the major phenolic glycolipid from this strain; lanes C–F, 10%–50% CH_3OH in $CHCl_3$ eluate from the silicic acid column. PIM, phosphatidylinositol mannosides.

M. tuberculosis Canetti were applied to a silica gel column that was irrigated with $CHCl_3$ followed by sequential steps of increasing concentrations of CH_3OH in $CHCl_3$, several carbohydrate-containing lipids were resolved. The polyphthienoyl trehaloses (Daffe et al., 1988a) were eluted with $CHCl_3$, the phenolic glycolipid (Daffe et al., 1987) with 5% CH_3OH in $CHCl_3$, followed by the sulfolipids and the dimycolyl trehaloses (Goren & Brennan, 1979; Asselineau & Asselineau, 1978) once 10% CH_3OH in $CHCl_3$ was applied. The spectrum of lipids eluted with CH_3OH in $CHCl_3$ is shown in Figure 1. Two major classes of glycolipids were readily differentiated on the basis of reactivity to a phosphorus-reactive spray (Kates, 1972). The phosphorus-containing compounds were identified as the phosphomannoinositides (Brennan, 1988). The phosphorus-negative glycolipids were unique in that their presence had not previously been observed in the previous extensive analyses of the lipid population of *M. tuberculosis* (Brennan, 1984, 1988). The two major phosphorus-free glycolipids were identified as LOS-I and LOS-II; 16 and 42 mg of LOS-I and -II, respectively, were recovered from 2.6 g of total extractable lipid, which in turn was obtained from 50 g of bacteria.

Application of a form of TLC–"Western blotting" (Figure 2) and conventional plate ELISA using antiserum raised against the whole homologous bacterium or its lipid population showed that only one of the neutral polar glycolipids, LOS-II, was highly antigenic. When the lipids from a variety of other strains of *M. tuberculosis*, including the prototype strains of virulent and avirulent *M. tuberculosis* and other nontuberculous mycobacteria were similarly analyzed, there was no chemical or serological evidence for any such glycolipids in any of them. Thus, these two novel glycolipids, LOS-I and LOS-II, are apparently confined to the Canetti strains of *M. tuberculosis*, and only one of these, the more polar LOS-II, is antigenic.

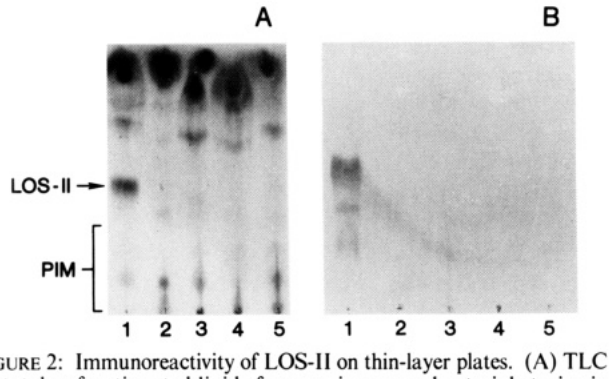

FIGURE 2: Immunoreactivity of LOS-II on thin-layer plates. (A) TLC of total unfractionated lipids from various mycobacterial strains in $CHCl_3$–CH_3OH–H_2O (30:8:1) and reaction with H_2SO_4. (B) TLC of lipid extracts and subsequent immunoreaction with the mouse antiserum raised against lipid extracts of *M. tuberculosis* Canetti. Lane 1, *M. tuberculosis* Canetti; lane 2, *M. leprae*; lane 3, *M. tuberculosis* (H37Rv); lane 4, *M. tuberculosis* (H37Ra); lane 5, *M. tuberculosis* (Erdman). Some 21 other mycobacterial strains gave profiles similar to those illustrated in lanes 2–5. PIM, phosphatidylinositol mannosides.

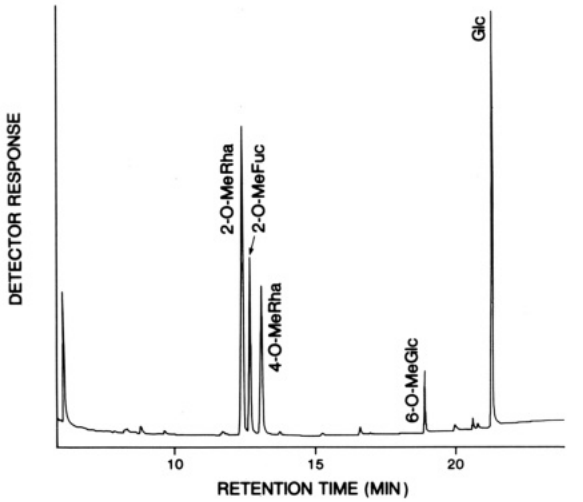

FIGURE 3: GC profile of the alditol acetates derived from acid hydrolysis (2 M CF_3COOH, 120 °C, 1 h) of LOS-II.

Glycosyl Composition and Anomeric Configuration of LOS-I and LOS-II. Upon treatment of fractions containing LOS-I and -II with 0.2 N NaOH in CH_3OH and subsequent TLC of the proceeds, all semblances of the two glycolipids disappeared. Thus, in this respect, they conformed to the specification of acylated oligosaccharides (Hunter et al., 1983; Brennan, 1984, 1988). Hydrolysis of LOS-I and II with 2 M CF_3COOH and capillary GC of the alditol acetates on the

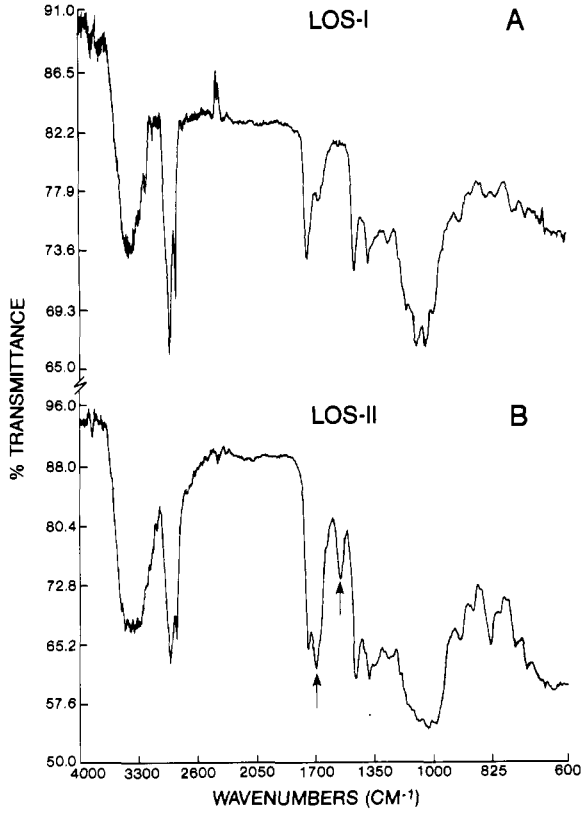

FIGURE 4: Infrared spectra of LOS-I (A) and LOS-II (B) measured as a thin film on NaCl. The absorption bands indicating the occurrence of an amide group are indicated by an arrow.

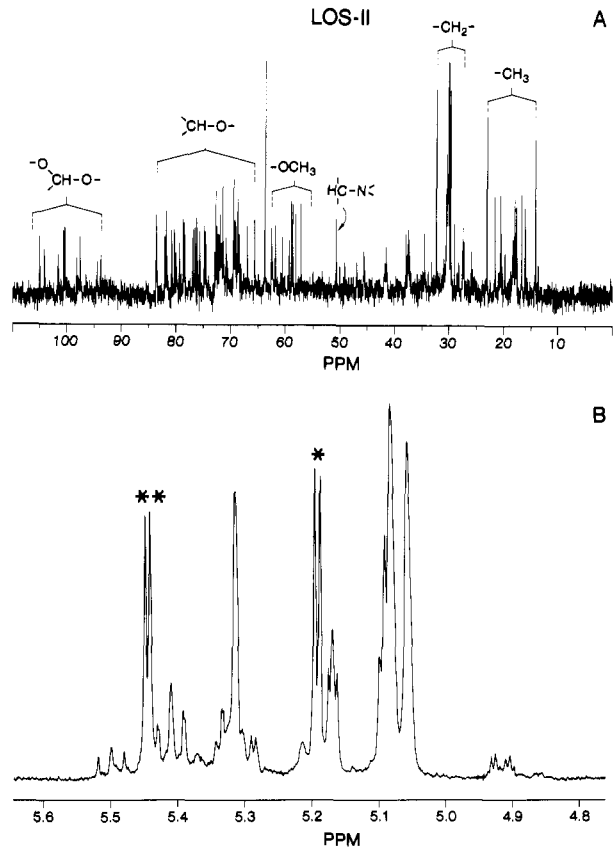

FIGURE 5: ^{13}C NMR spectrum of LOS-II [$CDCl_3$–CD_3OD (1:1), concentration 15 mg/mL] (A) and 500-MHz ^1H NMR spectrum of LOS-II [4.8–5.6 ppm region, $CDCl_3CD_3OD$ (1:1), concentration 15 mg/mL] (B). The doublet marked with an asterisk (*) is absent from the spectrum of LOS-I; the doublet marked with two asterisks (**) is absent from the spectrum of partially deglycosylated LOS-II.

DB-23 column showed five sugar components (Figure 3 and Table I). GC/MS of the alditol acetates and cochromatography with authentic standards established the identities. Both LOS-I and LOS-II contained 2-*O*-Me-Rha (2 mol), 2-*O*-Me-Fuc (1 mol), 4-*O*-Me-Rha (1 mol), 6-*O*-Me-Glc (1 mol) and Glc (3 mol), suggesting an acylated octasaccharide. It was immediately suspected, on the basis of precedent (Hunter et al., 1988) and relative TLC mobility, that LOS-II may contain additional glycosyl residues over and above those in LOS-I. Indeed, upon mild acid hydrolysis (4 M HCl, 25 °C), LOS-II, but not LOS-I, yielded two fragments, a partially deglycosylated lipooligosaccharide (LOS-II′) containing seven sugar residues (Table I) and a disaccharide that on analysis yielded 2-*O*-Me-Fuc and a novel sugar.[2] IR spectroscopy (Figure 4) of LOS-I and LOS-II showed the expected evidence for hydroxyl (3250–3500 cm^{-1}) and ester (1740 cm^{-1}) functions as well as CH, CH_2, and CH_3 stretching bands (2900–2800, 1460, and 1380 cm^{-1}). In addition, the existence of IR absorption bands at 1550 and 1690 cm^{-1} in the spectrum of LOS-II, but not of LOS-I, suggested the presence of an amino sugar in the more polar antigenic glycolipid, presumably part of the antigen determinant. High-resolution ^{13}C and ^1H NMR of LOS-II (Figure 5) showed the existence of an exchangeable proton (at δ 7.4; not shown in Figure 5B) and of a CH–NH carbon signal (Bundle et al., 1973; Sheinblatt et al., 1982) at 51.0 ppm (Figure 5A), again supporting the evidence for the presence of an amino sugar in LOS-II over and above the sugars common to LOS-I. Moreover, ^{13}C NMR indicated the presence of nine anomeric carbons (δ 93-105) in LOS-II (Figure 5A), whereas LOS-I showed only eight such anomeric carbon signals (spectrum not shown). The ^1H NMR spectrum at 500 MHz of LOS-II showed a doublet ($J_{1,2}$ = 3.5 Hz) at δ 51.9 present in LOS-II but absent in the corresponding spectrum of LOS-I, again supporting the evidence for the existence of an additional sugar residue in LOS-II; this last signal was assigned to the H-1 of an amino sugar containing axial–equatorial substituents (in respect to C-1 and C-2). Another signal, the most deshielded doublet at δ 5.45 ($J_{1,2}$ = 3.7 Hz), present in the spectra of both LOS-I and LOS-II (Figure 5B), was absent from the partially deglycosylated LOS-II fragment, i.e., LOS-II′. Since glycosyl composition analysis of this degradative product demonstrated the same array of sugar residues present in the native LOS-II (Table I) except for 2-*O*-Me-Fuc, then the most deshielded signal must belong to this residue. The three broad singlets at δ 5.05, 5.09, and 5.32 (Figure 5B) were observed in the spectra of both LOS-I and LOS-II; these were assigned to the three α-rhamnosides (see Table I), in accordance with data in the literature (Hunter et al., 1982). However, integration of proton signals between δ 5.00 and 5.10 showed the presence of additional deshielded protons. These latter resonances, as well as the other deshielded, quantitatively minor multiplets (at δ 5.50, 5.40, 5.30, 5.18, and 4.92), were assigned to the resonances of the H-1 of 6-*O*-Me-Glc (δ 5.10) and to protons linked to carbons bearing acylated hydroxy groups (see results of 2D-COSY, below). The signals of the β-glucosyl residues observed at δ 104.0 and 104.8 by ^{13}C NMR (Figure 5A) gave a multiplet centered at δ 4.50 in the ^1H NMR spectrum (results not

[2] The released disaccharide was reduced, acetylated, and analyzed by IR and GC/MS. The IR spectrum showed absorption bands at 1640 and 1550 cm^{-1} (amide I and II), indicating the presence of the amino sugar. Chemical ionization (NH_3) MS indicated a molecular weight of 534. Direct probe EI-MS of the diglycosyl alditol produced an A ion at m/z 228, arising from the terminal amino sugar, and an alditol J_2 ion at m/z 290 from the 2-*O*-Me-1,4,5-tri-*O*-Ac-fucitol.

Table II: Glycosyl Linkage Analysis of Per-Me-Ose-I, -II, and -II′[a]

O-Ac-*O*-CH_3-*O*-C^2H_3 alditol	deduced glycosyl linkage[b]	mol/mol of Ose		
		per-Ose-I	per-Ose-II′	per-Ose-II
1,5-di-*O*-Ac-2-*O*-CH_3-3,4-di-*O*-C^2H_3-fucitol	*t*-2-*O*-Me-Fuc	1.0	1.0	nd[c]
1,3,5-tri-*O*-Ac-2-*O*-CH_3-4-*O*-C^2H_3-rhamnitol	→3)2-*O*-Me-Rha	2.0	2.0	2.0
1,3,5-tri-*O*-Ac-4-*O*-CH_3-2-*O*-C^2H_3-rhamnitol	→3)4-*O*-Me-Rha	1.0	1.0	1.0
1,3,5-tri-*O*-Ac-2-*O*-CH_3-4-*O*-C^2H_3 fucitol	→3)2-*O*-Me-Fuc	nd	nd	1.0
1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-C^2H_3-glucitol	*t*-Glc	1.0	1.0	1.0
1,3,5-tri-*O*-Ac-2,4,6-tri-*O*-C^2H_3-glucitol	→3)Glc	2.0	2.0	2.0
1,3,5-tri-*O*-Ac-6-*O*-CH_3-2,4-di-*O*-C^2H_3-glucitol	→3)6-*O*-Me-Glc	1.0	1.0	1.0

[a] Each oligosaccharide (1 mg) deriving from the intact LOS-I or LOS-II was methylated with C^2H_3I. The resulting mixture of per-Ose-II and -II′ was resolved. Per-Ose-I, -II, and -II′ were hydrolyzed, reduced with NaB^2H_4, acetylated, and analyzed by GC/MS. [b] *t* = terminal; the →3) designation means that the OH-3 of the glycosyl residue was originally linked to another glycosyl unit. [c] nd = not detected.

shown). It follows then that the major antigenic LOS-II contains two β-linked glycosyl ($\delta > 104$) and seven α-linked glycosyl units (Bradbury & Jenkins, 1984). On the basis of this interpretation of the NMR spectra (Figure 5), the heteronuclear ($^{13}C/^1H$) COSY, and other data from the literature (Bradbury & Jenkins, 1984; Gorin, 1975; Pozsgay et al., 1981), it was possible to assign the ^{13}C NMR anomeric signals (Figure 5A) to α,α′ trehalose (δ 93.8 and 94.5), 2-*O*-Me-α-Fuc, the two residues of 2-*O*-Me-α-Rha, and one each of the α-amino sugar and 4-*O*-Me-α-Rha (δ 97.6, 98.3, 100.3, 100.6, and 101.6, respectively).

GC of the $(CH_3)_3Si$-R-(−)-2-butyl glycosides conducted on fully demethylated LOS-I and -II demonstrated that the two deoxy sugars (Rha and Fuc) were in the L configuration, whereas Glc was in the D configuration (results not shown). Accordingly, the 6-*O*-Me-Glc must be based on D-Glc.

Glycosyl Linkage Analysis of LOS-I and -II. Methylation of LOS-II produced two permethylated oligosaccharides (per-Ose-II and II′, Table II), which were readily purified on columns of silica gel by using $CHCl_3$ and 5% CH_3OH in $CHCl_3$. Analysis of the partially O-deuteriomethylated, partially O-methylated and partially O-acetylated alditol derivatives indicated that the least polar of these (per-Ose-II′) was identical with the Ose derived from LOS-I (per-Ose-I) and was a permethylated octasaccharide (Table II), a fact that was confirmed by NMR, FAB-MS, and TLC (see below). Thus, the results suggested that the amino sugar of LOS-II was lost during the permethylation process. The most polar of this pair of permethylated Ose (Per-Ose-II) did contain the amino sugar residue, according to the data in Table II. From this information, it is clear that both per-Ose-I and -II′ contain two nonreducing terminal glycosyl residues (Glc and Fuc) and that all of the other sugar residues are 1→3 linked. However, glycosyl linkage composition analysis of per-Ose-II (Table II) indicates that the 2-*O*-Me-Fuc unit is not terminal but is substituted at the 3-position, presumably by the terminal amino sugar.

Evidence for the Presence of a 6-O-Me-Trehalose Unit. The presence of the basic trehalose unit in LOS was supported by both ^{13} NMR and glycosyl linkage analysis (Table II). In addition, the presence of 6-*O*-Me-Glc among the hydrolysis products of both LOS-I and LOS-II raised the possibility of the existence of a unique 6-*O*-Me-trehalose unit therein. Accordingly, LOS-I and -II were subjected to a variety of partial acid hydrolysis conditions (see Experimental Procedures). GC/MS of the products arising from either LOS-I or LOS-II through the action of 2 M CF_3COOH at 95 °C for 1 h (or 4 M HCl at 25 °C for 18 h), as their alditol acetates, showed evidence of 2-*O*-Me-Rha, 2-*O*-*Me*-Fuc, 4-*O*-Me-Rha, 6-*O*-Me-Glc, Glc, and small quantities of a residual *O*-acetyl oligosaccharide (Figure 6A). EI-MS of this per-*O*-acetyl oligosaccharide showed strong aA_1 (*m/z* 331),

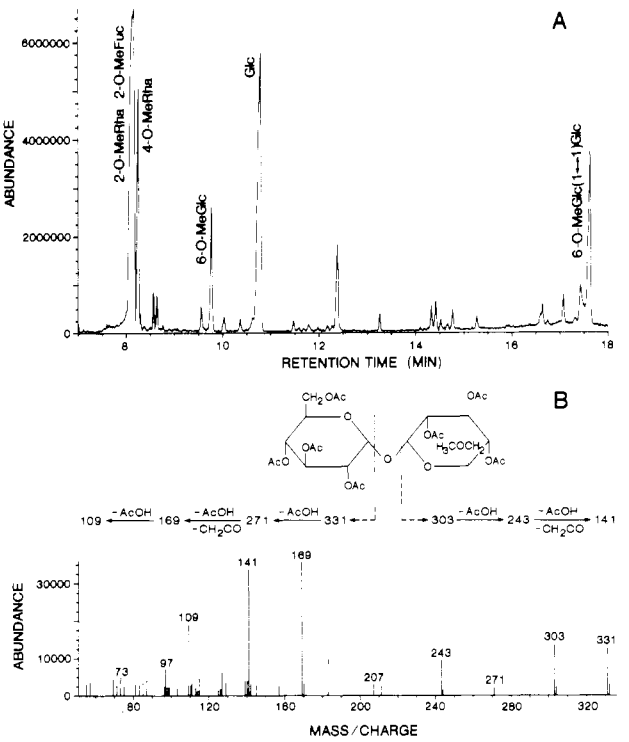

FIGURE 6: Total ion chromatogram produced during GC/MS analysis of the alditol acetates derived from the partial acid hydrolysis (2 M CF_3COOH, 95 °C, 1 h) of LOSs (A). Electron-impact mass spectrum of the peracetylated derivative of the 6′-*O*-Me-trehalose isolated from LOSs (B).

aA_2 (*m/z* 271), and aA_3 (*m/z* 109) ions (Figure 6B) characteristic of a peracylated glucosyl unit (Kochetkov & Chizhov, 1966) and a second set of aA_1 (*m/z* 303), aA_2 (*m/z* 243), and aA_3 (*m/z* 141) ions, attributable to an O-methylated, O-acetylated glucosyl residue within an O-methylated trehalose, obviously a 6-*O*-Me Glc residue. Accordingly, it was concluded that the two LOS were based on a 6-*O*-Me-trehalose-containing oligosaccharide.

Glycosyl Sequence of Ose-I and Ose-II. To arrive at the complete structure of the oligosaccharide moiety of LOS-II, OSE-II was analyzed by positive-ion FAB-MS (Figure 7). The $(M + Na)^+$ ion observed at *m/z* 1673 was shifted to *m/z* 1657 $(M + Li)^+$ with the addition of LiCl (results not shown), clearly demonstrating a molecular weight of 1650 for Ose-II. This molecular weight is in accord with the presence of the eight known glycosyl residues (Tables I and II) plus an additional residue of mass 330 amu. This residue is expected to be at the nontrehalose terminus, attached to the penultimate 2-*O*-Me-fucosyl; indeed, a strong A ion at *m/z* 331, conforming to the weight of this residue, was observed (Figure 7, inset). The sequence of the remainder of the oligosaccharide

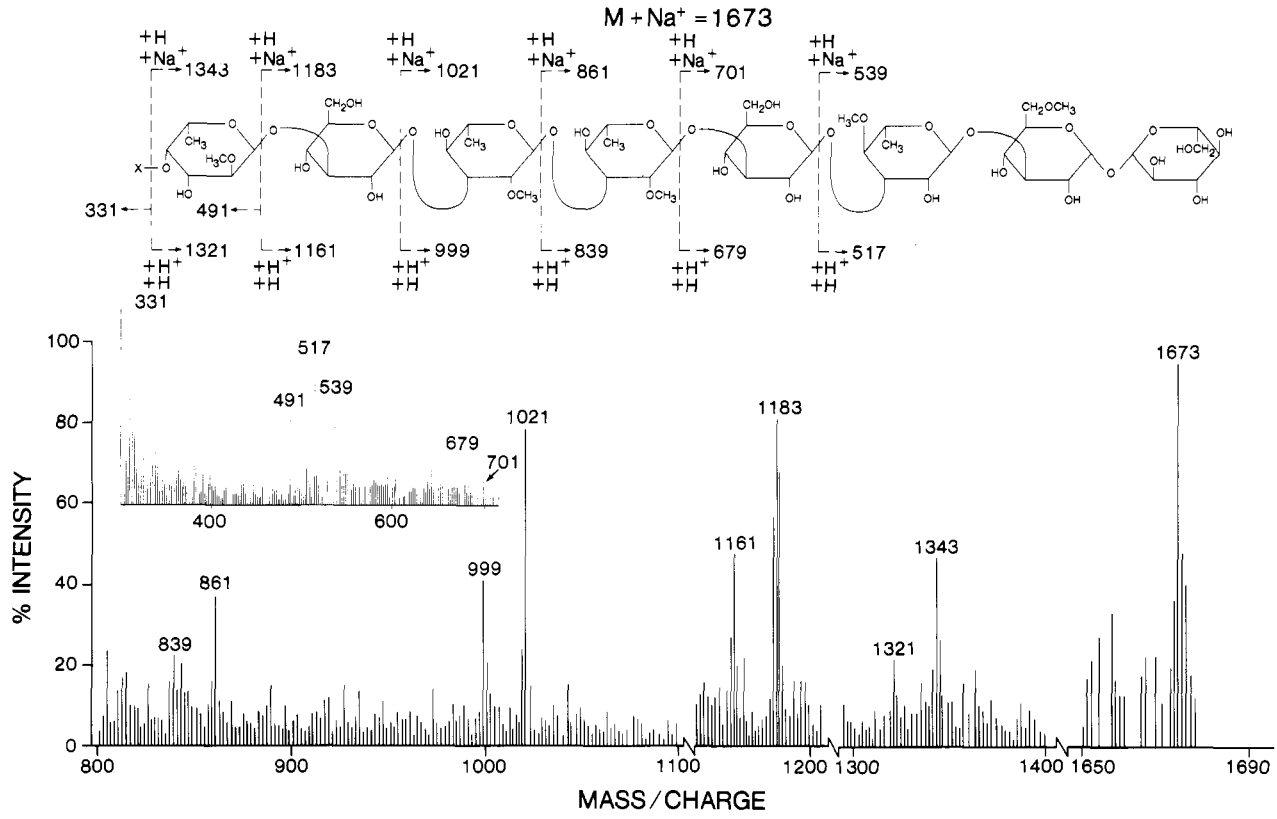

FIGURE 7: FAB-MS of the nonasaccharide (Ose-II) derived from LOS-II. x = amino sugar residue.

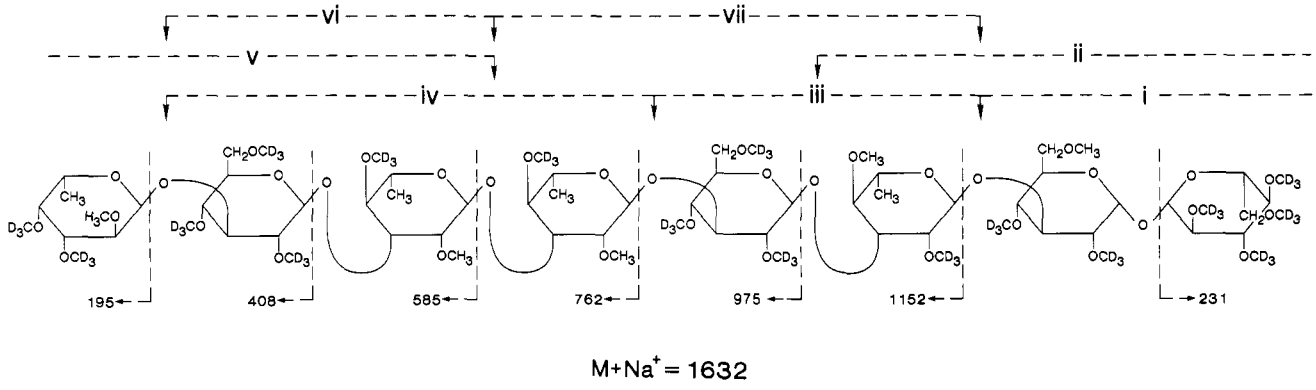

FIGURE 8: Sequence of sugar in per-Ose-I and II′, the origins of the individual fragments that had been characterized as permethylated derivatives, and the interpretation of the FAB-MS of the peralkylated oligosaccharides.

was deduced as demonstrated in Figure 7. The J_2 trehalose-containing ions were found in two forms, $J_2 + OH + Na^+$ and $J_2 + OH + H^+$ (Dell & Thomas-Oates 1989). The smallest of this series of ions, those at m/z 517 ($J_2 + OH + H^+$) and m/z 539 ($J_2 + OH + Na^+$), corresponded to a trisaccharide containing a mono-*O*-Me-6-deoxyhexosyl residue linked to 6′-*O*-Me-trehalose. Note that mass increments of 162 correspond to hexosyl residues, which must be Glc (Table I), and the increments of 160 correspond to mono-*O*-methyl-6-deoxyhexosyl units. The 2-*O*-Me-Fuc was identified as the nonreducing terminal residue by glycosyl linkage analysis (Table II) as discussed above, and thus, the remaining 6-deoxyhexosyls must be Rha. This analysis could not establish which of the three remaining *O*-Me-Rha are 4-*O*-Me-Rha and which are 2-*O*-Me-Rha.

In order to confirm the sequence of the glycosyl residues in Ose-II and to obtain glycosyl sequence data for Ose-I, each was perdeuteriomethylated. Previously, we had concluded that, during the course of methylation of Ose-II, the non-reducing-end amino sugar was eliminated, resulting in an oligosaccharide fragment (per-Ose-II′) identical with per-Ose-I. This result was confirmed by the fact that the FAB mass spectra of per-Ose-I and per-Ose-II′ were identical. Molecular ions at m/z 1627 $(M + NH_4)^+$ and m/z 1632 $(M + Na)^+$ tallied with the evidence for an octasaccharide derivative. Glycosyl cleavage via the A ion route was weak, but A ions corresponding to the cleavages shown in Figure 8 could be seen. These ions confirmed the sequence shown in Figure 8 for both per-Ose-I and per-Ose-II′.

In order to distinguish between the remaining mono-*O*-Me-rhamnosides, 2-*O*-Me-Rha and 4-*O*-Me-Rha, per-O-trideuteriomethylated Ose-I and Ose-II′ were subjected to sequential partial acid hydrolysis, NaB^2H_4 reduction, and pertrideuteriomethylation, as described (McNeil et al., 1982). The resulting complex mixture of partially O-methylated, partially O-deuteriomethylated oligoglycosyl alditol fragments was subjected to GC and GC/MS (the results of this approach are summarized in Figure 8). The mass spectrum and contingent interpretation of the data on the one crucial oligosaccharide used in locating the 4-*O*-Me-Rha residue are shown

Table III: Identification and Interpretation of the Major EI-MS Ions Used in the Analysis of the Fragments Derived from Partial Hydrolysis of Per-Ose-I and -II′[a]

	m/z value for ion[c]													
fragment[b]	$aldJ_2$	$aldJ_1$	$aldJ_0$	aA_2	aA_1	bA_2	bA_1	cA_1	abA_1	$aaldJ_2$	$aaldJ_1$	bcA_1	$baaldJ_2$	$baaldJ_1$
i				196	231	193	228							
ii				196	231			195				405	441	504
iii[d]	215	278		196	231									
iv	215		261			196	231		408	392	455			
v[d]	215		261				195		408	428				
vi[d]	215	278		196	231									
vii[d]	215		261				195		408	428				

[a] See Table II for the explanation of the origins of per-Ose-I and per-Ose-II′. [b] Per-Ose-I and -II′ were partially hydrolyzed (2 M TFA, 1 h at 85 °C), reduced with NaB^2H_4, and methylated with C^2H_3I, and the mixture was analyzed by GC/MS. [c] The nomenclature is that of Kochetkov and Chizhov (1966). [d] Oligosaccharides iii and vi on the one hand and v and vii on the other have the same retention times and share the same major ions in EI-MS. However, each one can be identified by the presence of a weak specific alditol cleavage ion monitored by the selector ion monitoring (SIM) method. For example, in fragments iii and vi, the location of the $-OCH_3$ group on the alditol was deduced from the existence of ions at *m/z* 365 and 368 derived from the ion at *m/z* 400 ($M - CH_3CHOC^2H_3$) through loss of C^2H_3 or CH_3OH from the β position of the fragment ion (Lonngren & Svensson, 1974).

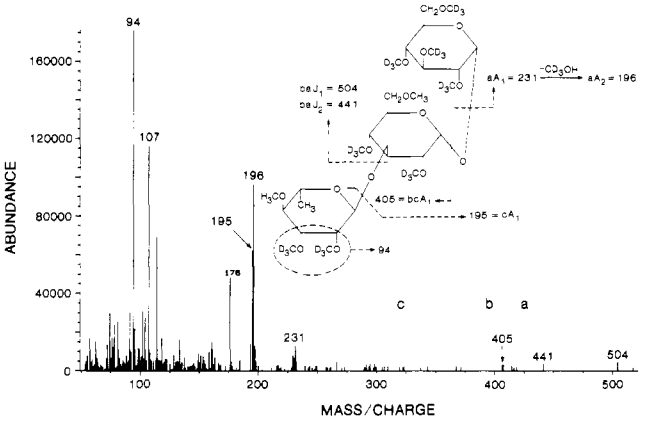

FIGURE 9: Electron-impact mass spectra of the permethylated derivative of compound ii (see Figure 8).

in Figure 9. The absence of alditol (ald) ions (Kochetkov & Chizhov, 1966; Sharp & Albersheim, 1986) in the mass spectrum clearly indicates that the triglycoside contains the 6′-*O*-Me-trehalose unit. Most importantly, the presence of a strong cA_1 ion at *m/z* 195 indicates that a mono-*O*-Me-Rha occupies the left-hand terminus of the triglycoside. The H_1^2 ion (Kochetkov & Chizhov, 1966), which contain C-2, C-3, and their *O*-alkyl substituents at *m/z* 94, demonstrates that both C-2 and C-3 of the mono-*O*-Me-Rha are substituted with trideuteriomethyl groups, and thus the endogenous *O*-CH_3 group must be at C-4. Incidentally, since no ion at *m/z* 94 was seen in the EI-MS spectrum of permethylated trehalose (compound i, Figure 8) the *m/z* 94 ion is not from trehalose. Further confirmation of the origin of the H_1^2 ion at 94 arose from the observation that the EI-MS spectrum of 2-*O*-Me-deoxyhexosyl alditol (compounds v and vii, Figure 8) showed no ion at *m/z* 94 but rather at *m/z* 91, corresponding to the H_1^2 ion containing both *O*-CH_3 and *O*-C^2H_3 groups (data not shown). The aA_1 ion at *m/z* 231 and the baJ_2 ion at *m/z* 441 (Figure 9) confirmed the location of the 6-*O*-methyl group on the internal glucosyl residue of the trehalose. In the same way, the structures of several more per-O-methylated oligoglycosyl alditol fragments were established (Table III), providing further confirmatory evidence for the proposed structures.

Partial Structure of the N-Acyl Sugar of LOS-II. An initial attempt was made to characterize the crucial amino sugar that governs the antigenicity of LOS-II by NMR analysis of Ose-II. The location of the nitrogen-carrying carbon was established by homo- and heteronuclear ($^{13}C/^1H$) COSY. The CH–NH carbon signal at δ 51.0 (Figure 5A) was found to correlate with a proton resonance at δ 4.63 in the heteronuclear COSY spectrum. In the homonuclear COSY spectrum, it was possible to assign this latter resonance to that of H-4 since it had connectivities to H-3 (δ 3.80) and H-5 (δ 4.34). *J*-Connectivity from H-5 could be traced to H-6 at δ 1.18. Likewise, H-3 (δ 3.80) leads to H-2 at δ 3.72. The resonance for the anomeric H-1 was at δ 5.19 (Figure 5B). The coupling constant values of $J_{1,2}$ (3.5 Hz), $J_{2,3}$ (10.8 Hz), $J_{3,4}$ (4.0 Hz), $J_{4,5}$ (1.8 Hz), and $J_{5,6}$ (6.8 Hz) established the galacto configuration for the novel amino sugar. Thus we conclude that this sugar is a derivative of 4-amino-4,6-dideoxygalactopyranose. The amino group is apparently acylated since the molecular weight of Ose-II (1650 Da; Figure 7) requires an acyl function of 186 Da attached to the terminal sugar. This weight for the acyl function is confirmed by the *m/z* value of 331 for the A ion of the acylated 4-amino-4-deoxygalactosyl residue (Figure 7). The structure of this acyl function has not yet been determined, but its weight of 186 Da translates to a molecular weight of 203 Da of the parent fatty acid before its linkage to the sugar amino group.

Structures of the Native LOS-I and LOS-II. TLC of the fatty methyl esters derived from both LOS-I and LOS-II showed two products [R_f 0.57 and 0.72 in petroleum ether–diethyl ether (8:2)], indicative of hydroxylated and nonhydroxylated fatty acid methyl esters (Daffe et al., 1988a). GC/MS revealed that the nonhydroxylated class consisted of straight-chain fatty methyl esters [characterized by a McLafferty base ion at *m/z* 74 (Ryhage & Stenhagen, 1963)] and α-methyl-branched fatty methyl esters (base ion at *m/z* 88). The hydroxylated group of esters was obviously α-methyl-branched and β-hydroxylated (base ion at *m/z* 117 corresponding to C_3–C_4 bond cleavage). From the EI-MS spectrum of the individual esters as fractionated by GC, the structures of the different acids were established on the bases of McLafferty ions, molecular ions, and rearrangement ions (Ryhage & Stenhagen, 1963, 1969; Ryhage et al., 1961). The β position of the hydroxyl group was confirmed by analysis of TMS derivatives of the hydroxylated fatty methyl esters by GC/MS. This latter EI-MS spectrum showed prominent ions resulting from the C_3–C_4 and C_2–C_3 bond cleavages at *m/z* 189 and 341, respectively. The three major components of the mixture were identified as 2,4-dimethylhexadecanoic, 2,4,6,8-tetramethyloctadecanoic, and 2-methyl-3-hydroxy-eicosanoic methyl esters. The hydroxylated compound was previously recognized among the fatty methyl ester mixture from *Mycobacterium gordonae* (Daffe et al., 1983). The optical rotation value of the nonhydroxylated methyl-branched fatty methyl esters ($[\alpha]_D$ = +8.0°, $CHCl_3$, *c* = 0.1) points to the L configuration for the chiral centers bearing the methyl

Table IV: Assignment of the Ring Protons on the Terminal Glc Residue of (Nondeacylated) LOS-II

H position	chemical shift	acylation deduction
Component Present as 60% of Total		
H-1	5.18	glycosidic linkage
H-2	3.73	no acyl group
H-3	5.41	acyl substituent at O-3
H-4	5.08	acyl substituent at O-4
H-5	4.25	acyl substituent at O-6[a]
Component Present as 40% of Total		
H-1	5.29	glycosidic linkage
H-2	4.90	acyl substituent at O-2
H-3	5.50	acyl substituent at O-3
H-4	3.70	no acyl group
H-5	4.20	acyl substituent at O-6[a]

[a] Previous studies (Daffe et al., 1988) have demonstrated that when the chemical shift of H-5 is greater than δ 4, then O-6 is substituted with an acyl function.

groups (Daffe & Laneelle, 1988). Accordingly, these fatty acids belong to the phthioceranic series (Asselineau, 1982). The hydroxylated fatty methyl esters gave a negative optical rotation value ($[\alpha]_D = -1.7°$, $CHCl_3$, $c = 1.0$).

From the proton integration data and the structures of the fatty acids, it was estimated that three fatty acyl substituents were present per mole of LOS. In order to locate these substituents on the oligosaccharide backbone, 2D-COSY was applied (Daffe & Servin, 1989). The original ^1H NMR spectrum (Figure 5B) showed well-resolved triplets centered at δ 5.41 and 5.50, suggesting that the H-3 of glucosyl units ($J_{2,3} - J_{3,4} = 9.5$ Hz) have an esterified hydroxyl group located on the C_3 ring carbon (Daffe et al., 1988). These resonances provided convenient starting points for the 2D-COSY analysis. Figure 10 presents a contour plot of a COSY spectrum of the native LOS-II. Beginning at the diagonal, successive vertical and horizontal lines, which correspond to scalar connectivities, can be constructed, allowing the grouping of resonances into subspectra belonging to a single residue (Daffe & Servin, 1989; Yu et al., 1986). Beginning at δ 5.41 (H-3 resonance of a glucosyl residue), it is easy to find cross peaks showing connectivities to H-2 (δ 3.73) and H-4 (δ 5.08). From the H-2 resonance, a connectivity to the H-1 resonance at δ 5.18 can be found and, likewise, from H-4 to the H-5 resonance at δ 4.25. These values are summarized in Table IV. Because the ring protons adjacent to esterified hydroxyl groups have their resonances in the low-field region (δ 4.8–5.6) (Daffe et al., 1988), positions 3 and 4 but not position 2 of a glucosyl residue are acylated (Table IV). Additionally, position 6 of the same glucosyl unit is also acylated because the H-5 resonance of a 6-acyl glucosyl has its resonance at $\delta \geq 4.00$ (Daffe et al., 1988). Furthermore, the three acyl substituents are located on the terminal glucosyl residue because it is the only glucosyl unit with position 3 available for acylation in the native LOS-II (Table II). However, the proton integration data show that this 3,4,6-tri-O-acyl glucosyl represents 60% of the LOS (the H-3 representing only 0.6 protons). This result was consistent with the finding of a resonance at δ 5.50 (Figure 5B), corresponding to that of H-3 of a glucosyl residue ($J_{2,3} = J_{3,4} \sim 9.5$ Hz). Examination of the 2D-COSY spectrum (Figure 10), in the same fashion as described above, allowed the identification of the corresponding H-2, H-4, H-1, and H-5 (Table IV). Consequently, the positions 2, 3, and 6 of this glucosyl unit are acylated. The acylation of position 6 of the glucosyl residue indicates that this latter also has to be the terminal glucosyl unit of the 6′-*O*-Me-trehalose. Consequently, LOS-II must consist of a mixture of 3,4,6- and 2,3,6-tri-O-acyl nonasaccharides. Therefore, the structure

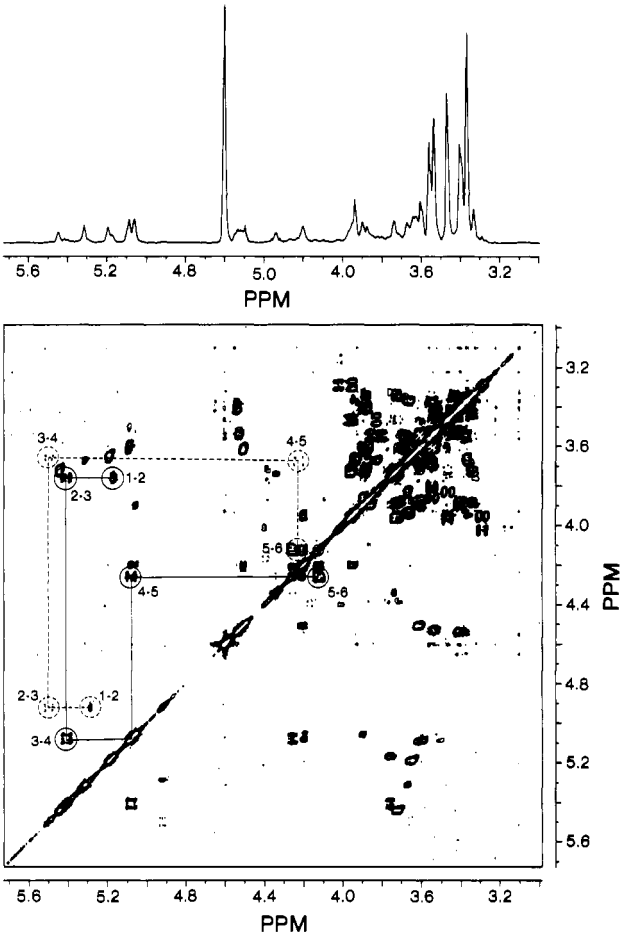

FIGURE 10: Contour plot of the 500-MHz 2D-COSY spectrum of the native LOS-II. The scalar connectivities from H-1 through H-5 are illustrated by the solid lines for the 60% component (see its structure in Figure 11) and by the irregular dashed lines for the 40% component. 1–2, 2–3, etc., correspond to cross peaks resulting from the connectivity between H-1 and H-2, H-2 and H-3, etc.

illustrated in Figure 11 can be proposed for the major constituent of LOS-II.

Discussion

It is now clear from this and previous work (Papa et al., 1989) that LOS-II is the dominant immunoreactive glycolipid of the Canetti strain of *M. tuberculosis* and that it differs significantly from other lipooligosaccharides from other species of *Mycobacterium* (Saadat & Ballou, 1983; Hunter et al., 1983, 1985, 1988; McNeil et al., 1987; Camphausen et al., 1987). Clearly, the immunodominancy of LOS-II is resident in the partially characterized amino sugar; LOS-I, identical with LOS-II in all other respects, is devoid of antigenicity. The amino sugar was shown by NMR to be a 4-amino-4,6-dideoxygalactosyl residue. Its absolute configuration was not determined. The N-acyl function was shown to have a molecular weight of 186, corresponding to a free fatty acid of MW 203, which must therefore be nitrogen-containing. In addition, the right-hand end ("reducing" end) of LOS-II is unique in that it contains a 6′-*O*-Me-trehalose substituent. Previously we have reported that the family of lipooligosaccharides in *Mycobacterium szulgai* are based on a 2-*O*-Me-trehalose unit (Hunter et al., 1988). In addition, unlike other well-characterized members of the LOS family, which are invariably acylated at the 3, 4, and 6 (or 4′ and 6) positions (Brennan, 1988), the product from *M. tuberculosis* Canetti consists of a mixture of 2,3,6- and 3,4,6-tri-O-acylated oligosaccharides. The location of the three acyl substituents on the terminal

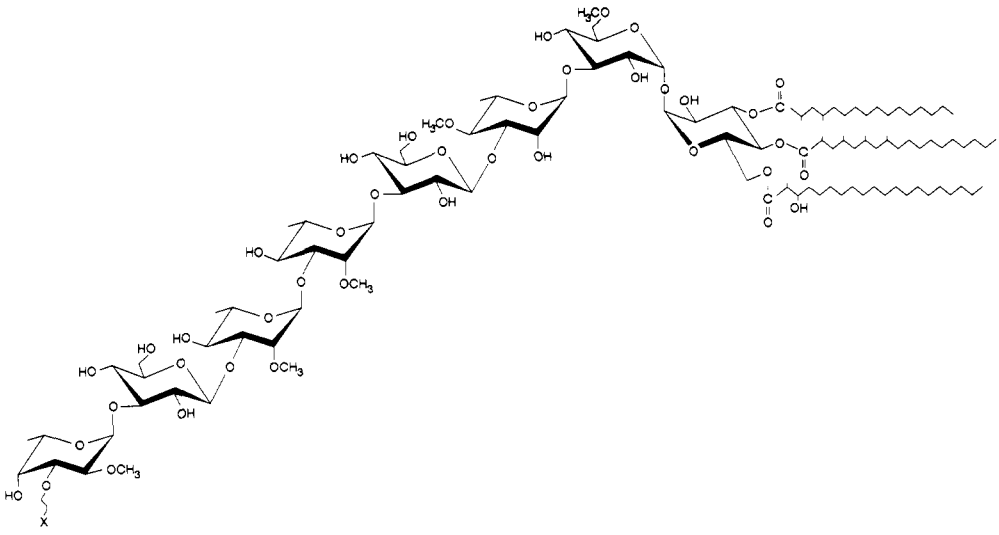

FIGURE 11: Proposed structure for the major component of LOS-II. Location of the three classes of fatty acyl substituents is arbitrary. x = amino sugar residue.

glucosyl residue of the trehalose unit in both subclasses of tri-O-acyl oligosaccharide confers on these glycolipids, as in the case for others described previously (Brennan, 1988), distinct polarity with implications for overt antigenicity and interaction with host macrophage (Brennan, 1988; Camphausen et al., 1987; Hunter et al., 1988). The methyl-branched nature of the fatty acyl substituents of LOS found in the present and previous studies (Hunter et al., 1983, 1988; McNeil et al., 1987; Camphausen et al., 1987; Saadat & Ballou, 1983) raised the pertinent question of the configuration of the chiral centers present in these fatty acids; both the D and L series occur in mycobacterial species (Daffe & Laneelle, 1988; Asselineau, 1982). The present study demonstrates that these fatty acids belong to the phthioceranic series (Daffe & Laneelle, 1988; Asselineau, 1982), whereas the reverse configuration usually occurs in the fatty acyl substituents of phenolic glycolipids (Daffe & Laneelle, 1988).

The implication of these glycolipids for tuberculosis as a disease is a moot point. The earlier report of the presence of a specific triglycosyl phenolic phthiocerol in *M. tuberculosis* Canetti (Daffe et al., 1987) raised the hope of a product analogous to the phenolic glycolipid I of *M. leprae* (Hunter et al., 1982) with implications for selective disease serodiagnosis (Gaylord & Brennan, 1987). However, the phenolic glycolipid of the Canetti strain is not a prominent product in the majority of *M. tuberculosis* isolates arising from infected individuals (M. Daffe, S.-N. Cho, P. J. Brennan, and D. Chatterjee, unpublished results). In the present case, it can also be concluded that LOS-I and -II are not prominent in the majority of those *M. tuberculosis* isolates intensively studied for over half a century (Brennan, 1988).

Nevertheless, these present observations and conclusions thereof may have implications for the pathogenesis of tuberculosis. *M. tuberculosis* Canetti is clearly of a smooth, moist texture and of umbonate elevation (Papa et al., 1989; Daffe et al., 1989), unlike the vast majority of field isolates of *M. tuberculosis*, which are of rough, dry texture and flat (Jenkins et al., 1982; Wayne & Kubica, 1986). *Mycobacterium kansasii* also exhibits analogous colony variations (Fregnan & Smith, 1962; Fregnan et al., 1961; Runyon, 1970; Vestal & Kubica, 1966), and, recently, we have demonstrated that all rough variants of *M. kansasii* are devoid of all semblances of a different set of lipooligosaccharides, the *M. kansasii* specific LOS (Belisle & Brennan, 1989). In addition, it has been observed by others that these same rough variants of *M. kansasii* result in a chronic systemic infection in mice, in contrast to the smooth variants, which are rapidly cleared from the organs of the infected animal (Collins & Cunningham, 1981). We have suggested that the key to the relatively greater persistence of the rough LOS$^-$ variants of *M. kansasii* may be not so much the absence of lipooligosaccharide but rather exposure to other underlying moieties such as lipoarabinomannan, already implicated in the pathogenesis of mycobacterioses (Ellner & Spagnuoto, 1979; Kaplan et al., 1987), that are cryptic in the smooth LOS$^+$ variants.

In the case of *M. tuberculosis*, we may be able to extend this relationship one step further. Research long ago led to the revelation of the existence in virulent strains of *M. tuberculosis* of lipids responsible for their cord morphology (Middlebrook et al., 1947), the "cord factors". Two distinct and characteristic properties of the isolated cord factors have been described: their systemic toxicity and their ability to inhibit migration of leukocytes (Block, 1950). Subsequent chemical characterizations showed that the toxicity was due to the dimycolyltrehalose component of the mixture (Noll & Bloch, 1955; Noll et al., 1956), which we found later to be present in all the mycobacterial species analyzed, including saprophyte and nonpathogenic species, as well as in related genera (Brennan, 1988; Asselineau & Asselineau, 1978; Goren & Brennan, 1979). However, substances having both leukotactic and leukotoxic activities were found to consist of phthienoic acids (Goren & Brennan, 1979), and these acids were found only in lipids, including acyltrehaloses, derived from virulent *M. tuberculosis* and *M. bovis* (Daffe et al., 1983, 1988a; Asselineau, 1982; Goren & Brennan, 1979; Minnikin et al., 1985). Consequently, the presence of phthienoic acids in a lipid profile was regarded as an interesting marker for virulent strains of *M. tuberculosis* (Daffe et al., 1988a). In the case of the *M. tuberculosis* Canetti strain, although the phthienoic-containing acyltrehaloses are present (Daffe et al., 1988a), the acyltrehaloses occur largely in the form of multiglycosylated versions, namely LOS-I and LOS-II. We speculate that *M. tuberculosis* Canetti and the other smooth LOS$^+$ variants of the tubercle bacilli may be attenuated forms of virulent *M. tuberculosis* by virtue of their ability to synthesize large amounts of phthioceranic acids (instead of related phthienoic acids). As an alternative, the absence of LOS in the rough variants of *M. tuberculosis* (the most commonly encountered morphotypes) may facilitate the exposure of "virulent" factors, playing an important role in the protection

of the bacteria from the infected host.

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Mitochondrial Metabolism of Valproic Acid†

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ABSTRACT: The β-oxidation of valproic acid (2-propylpentanoic acid), an anticonvulsant drug with hepatotoxic side effects, was studied with subcellular fractions of rat liver and with purified enzymes of β-oxidation. 2-Propyl-2-pentenoyl-CoA, a presumed intermediate in the β-oxidation of valproic acid, was chemically synthesized and used to demonstrate that enoyl-CoA hydratase or crotonase catalyzes its hydration to 3-hydroxy-2-propylpentanoyl-CoA. The latter compound was not acted upon by soluble L-3-hydroxyacyl-CoA dehydrogenases from mitochondria or peroxisomes but was dehydrogenated by an NAD^+-dependent dehydrogenase associated with a mitochondrial membrane fraction. The product of the dehydrogenation, presumably 3-keto-2-propylpentanoyl-CoA, was further characterized by fast bombardment mass spectrometry. 3-Keto-2-propylpentanoyl-CoA was not cleaved thiolytically by 3-ketoacyl-CoA thiolase or a mitochondrial extract but was slowly degraded, most likely by hydrolysis. The availability of 2-propylpentanoyl-CoA (valproyl-CoA) and its β-oxidation metabolites facilitated a study of valproate metabolism in coupled rat liver mitochondria. Mitochondrial metabolites identified by high-performance liquid chromatography were 2-propylpentanoyl-CoA, 3-keto-2-propylpentanoyl-CoA, 2-propyl-2-pentenoyl-CoA, and trace amounts of 3-hydroxy-2-propylpentanoyl-CoA. It is concluded that valproic acid enters mitochondria where it is converted to 2-propylpentanoyl-CoA, dehydrogenated to 2-propyl-2-pentenoyl-CoA by 2-methyl-branched chain acyl-CoA dehydrogenase, and hydrated by enoyl-CoA hydratase to 3-hydroxy-2-propylpentanoyl-CoA. The latter compound is dehydrogenated by a novel NAD^+-specific 3-hydroxyacyl-CoA dehydrogenase to 3-keto-2-propylpentanoyl-CoA which accumulates in the mitochondrial matrix and is slowly degraded, apparently by hydrolysis.

Valproic acid (2-*n*-propylpentanoic acid) is an effective anticonvulsant drug, which is widely used in the treatment of several forms of epilepsy (Browne, 1980). In a small number of patients treatment with valproic acid causes severe liver damage that can be fatal (Nau & Löscher, 1984). This situation has prompted many studies aimed at identifying the metabolites of valproic acid in vivo, at elucidating the metabolism of valproic acid in vitro, and at assessing the effects of valproic acid on hepatic metabolism.

Metabolites of valproic acid with oxygen functions at carbon atoms 5 and 4 including dehydration products thereof are formed by microsomal ω-oxidation and (ω − 1)-oxidation, respectively (Rettie et al., 1987, 1988). Metabolites with oxygen functions at carbon atom 3 and its dehydration product were thought to be formed by β-oxidation (Matsumoto et al., 1976). However, when it was reported that 3-hydroxy-2-propylpentanoic acid can be formed by microsomal (ω − 2)-oxidation (Prickett & Baillie, 1984), the metabolism of valproic acid by β-oxidation was left in doubt. But valproic acid affects mitochondrial functions as evidenced by valproate-induced inhibition of medium-chain and long-chain fatty acid β-oxidation (Becker & Harris, 1983; Turnbull et al., 1983; Coudé et al., 1983; Bjorge & Baillie, 1985). Since valproic acid is converted in mitochondria to 2-propylpentanoyl-CoA (Becker & Harris, 1983; Turnbull et al., 1983) which can be dehydrogenated to 2-propyl-2-pentenoyl-CoA (2-en-valproyl-CoA) by 2-methyl-branched chain acyl-CoA dehydrogenase (Ito et al., 1990), valproic can at least be partially degraded by β-oxidation. It remains unclear, however, how far valproic acid can be metabolized by this pathway and whether its β-oxidation occurs in mitochondria or peroxisomes or both organelles. Also, the possible accumulation of metabolites of valproate β-oxidation in mitochondria and their efflux from mitochondria have not yet been investigated.

This study was initiated with the aim of elucidating the metabolism of valproic acid by β-oxidation in rat liver mitochondria and/or peroxisomes.

Experimental Procedures

Materials. Sigma was the source of octanoyl-CoA, Nycodenz, pigeon breast muscle carnitine acetyltransferase (EC 2.3.1.7), pig heart 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and all standard biochemicals. 2-Octynoic acid and valproic acid (2-*n*-propylpentanoic acid) were obtained from

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