Chemistry of the Lyxose-Containing Mycobacteriophage Receptors of *Mycobacterium phlei*/*Mycobacterium smegmatis*†

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ABSTRACT: *Mycobacterium phlei* (strain Timothy) (*Mycobacterium smegmatis* ATCC 19249) is characterized by the presence of a family of alkali-labile glycolipids, reminiscent of the trehalose-containing lipooligosaccharide class of antigens but lacking the nonreducing trehalose core. Through a combination of methylation analyses, ^{1}H and ^{13}C NMR, two-dimensional $^{1}H/^{1}H$ and $^{1}H/^{13}C$ NMR, fast atom bombardment-mass spectrometry, gas chromatography−mass spectrometry, and other analytical techniques, these new structures were shown to possess three distinct features. Firstly, they contained the pentose D-lyxose (Lyx), rarely found in biology, but an epimer of D-arabinose, a key component of the mycobacterial cell wall arabinogalactan and lipoarabinomannnan. Thus, it was apparent that these glycolipids are the same as those described by Bisso et al. and attributed with phage receptor properties [Bisso, G., Castelnuovo, G., Nardelli, M.-G., Orefici, G., Arancia, G., Lanéelle, G., Asselineau, C., & Asselineau, J. (1976) *Biochemie 58*, 87−97]. Secondly, the complex oligosaccharides within the glycolipids contain the repeating units $Lyx_n(6\text{-}O\text{-}CH_3\text{-}Glc)_m$ and $Lyx_n(6\text{-}O\text{-}CH_3\text{-}Glc)_mMan_1$, where $n + m$ equal to approximately 16 glycosyl residues. Thirdly, the *M. phlei* glycolipids were found to be heavily *O*-acylated, such that every D-Lyx residue invariably possesses an acyl function at position −2 and, in some instances, at both positions −2 and −4. The chemical characterization of these glycolipids, not feasible 20 years ago, clearly demonstrates that they are distinct from the type- and species-specific glycopeptidolipids, lipooligosaccharides, phenolic glycolipids, and the genus-specific phosphatidylinositol-based lipoglycans of mycobacteria. This present and previous studies begin to define the precise structural requirements responsible for the attachment of mycobacteriophage to the host cell wall.

Historically, mycobacteriophages have been used mostly in the pragmatic context of phage typing of isolates of *Mycobacterium tuberculosis* (Jones & White, 1978) and, to a lesser extent, other *Mycobacterium* sp. such as *Mycobacterium kansasii* (Engel et al., 1980) and the *Mycobacterium avium*/*Mycobacterium intracellulare* complex (Crawford et al., 1981). More recently, mycobacteriophages themselves or in combination with plasmids (phasmids) have been instrumental in the rapid evolution of mycobacterial genetics (Jacobs et al., 1991). For example, a mycobacteriophage that we once induced from a strain of *M. avium* (Timme & Brennan, 1984) has provided the basis of a generation of shuttle vectors capable of inserting foreign DNA into mycobacteria (Jacobs et al., 1991). Throughout these efforts, mycobacteriophages have been employed as tools, either genetic or for identification/classification purposes. Until recently, little was known of mycobacteriophage structure other than the facts of gross morphology and the presence of 11−14% lipid in highly purified particulate preparations (Mizuguchi, 1984). In this respect, the elucidation of the complete sequence of the DNA of the L5 mycobacteriophage (Hatfull & Jacobs, 1994; Hatfull & Sarkis, 1993) was a pioneering event in that important information of complete structure and assembly of mycobacteriophage and integration and regulation of lysogeny was derived.

However, the physical identity of mycobacteriophage receptors, which are most likely to be present on the mycobacterial cell surface, probably beyond the mycolylarabinogalactan−peptidoglycan complex, has received little attention with the exception of a few isolated reports (Furuchi & Tokunaga, 1972; Goren et al., 1972; Imaeda & San Blas, 1969; Castelnuovo et al., 1970). For instance, Tokunaga et al. (1970) found that ethanol−ether lipid extracts from *Mycobacterium smegmatis* dramatically inactivated mycobacteriophages D4 and D29, both virulent for *M. smegmatis*, presumably by adsorption to the mycobacteriophage tail, and these lipid extracts were found not to affect mycobacteriophages that were specific for other strains of mycobacteria. Later, in two independent studies, Furuchi and Tokunaga (1972) and Goren et al. (1972) recognized that the receptor-site substance for mycobacteriophage D4 was the well-characterized C-mycoside class of antigens, now termed the glycopeptidolipids (GPLs).[1] At the same time, Castelnuovo et al. (1970) and later Bisso et al. (1976) reported that a crude organic soluble fraction from the cell walls of

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Mycobacterium phlei strain Timothy (now regarded as a *M. smegmatis*) was found to be highly specific for the inactivation of the mycobacteriophage phlei. Their conclusion was based on the kinetics, specificity, and general characteristics of phage inactivation studies supported by electron microscopy and a requirement for calcium ions, properties which are characteristic of phage−host cell interaction, suggesting that those fractions contained the receptor sites of the mycobacteriophage *M. phlei*. More recently, we (Besra et al., 1994b) investigated the structural basis of phage resistance in two spontaneous phage-resistant mutants of *M. smegmatis* and implicated the pyruvylated, acylated trehaloses (Saadat & Ballou, 1983) in phage resistance and presumably phage attachment. In this present communication, we have returned to the work of Bisso et al. (1976) in order to address the nature of the phage inactivation material partially characterized by them with the intent of expanding the study of phage−host interactions in *Mycobacterium*.

MATERIALS AND METHODS

Growth of M. phlei and Purification of Glycolipids

M. phlei strain Timothy (now classified as *M. smegmatis* ATCC 19249) was grown in a medium containing glycerol, alanine, and salts (Takayama et al., 1975) for 7−10 days at 37 °C, and the entire suspension was dried at 50 °C in crystallizing dishes. The dried material from 20 L of culture was extracted twice with 800 mL of acetone at room temperature, overnight. The dry acetone-soluble lipid preparations were resuspended in $CHCl_3$−CH_3OH (19:1) and applied to a column of Sephadex LH-20 (2 × 30 cm; Sigma Chemical Co., St. Louis, MO) which was irrigated with 200 mL of $CHCl_3$−CH_3OH (19:1) followed by 200 mL of $CHCl_3$−CH_3OH (1:1). The $CHCl_3$−CH_3OH fractions were evaporated to dryness and both redissolved and partitioned within the aqueous and organic phases arising from a mixture of equal parts of petroleum−ether and 87% aqueous ethanol (Bisso et al., 1976). The aqueous ethanol phase was dried to afford 90 mg of partially purified glycolipids.

Deacylation of M. phlei Glycolipids

The preparation of glycolipids (70 mg) was dissolved in $CHCl_3$−CH_3OH (2:1) (7 mL), treated with an equal volume of 0.2 N NaOH in CH_3OH at 37 °C for 1 h (Hunter et al., 1983), neutralized with acetic acid, evaporated to dryness, and partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$ phase was back-washed twice with H_2O. The combined aqueous phases which contained the neutral oligosaccharides (OSE) arising from the glycolipid were subsequently desalted using a column of Bio-Gel P-2 (1 × 100 cm, 200−400 mesh; Bio-Rad Laboratories, Richmond, CA) in H_2O. Fractions (1 mL) were collected and assayed for carbohydrate (Dubois et al., 1956). The organic phase from the above separation served as a source of fatty acids which were methylated and examined by GC-MS (Besra et al., 1992).

Glycosyl Composition and Linkage Analysis

(a) Alditol Acetates. The intact glycolipid, OSE, or prereduced OSE were hydrolyzed in 2 M TFA at 120 °C for 2 h as described (Besra et al., 1992). Glycosyl residues were reduced with NaB^2H_4 and the resultant alditols per-*O*-acetylated and examined by GC and GC−MS (Besra et al., 1992). The absolute configurations of the individual glycosyl residues were determined by GC−MS analysis of trimethylsilyl derivatives of their (*R*)-(−)- and (*S*)-(+)-octyl glycosides which were compared to authentic standards (McNeil et al., 1987). The OSE preparation (10 mg) was initially prereduced using NaB^2H_4 to prevent possible β-elimination before *O*-trideuteriomethylation using the Hakomori procedure as described below. The prereduced OSE was suspended in 0.5 mL of dimethyl sulfoxide (Pierce Chemical Co., Rockford, IL) and 100 μL of 4.8 M dimethyl sulfinyl carbanion added (Stellner et al., 1973; Hakomori, 1964). The reaction mixture was stirred for 1 h, C^2H_3I (50 μL) slowly added, the suspension stirred overnight, and the resulting product applied to a C_{18} Sep-Pak cartridge as described (York et al., 1986; Hakomori, 1964). The resultant per-*O*-CH_3-, per-*O*-C^2H_3-OSEol was hydrolyzed in 250 μL of 2 M TFA at 120 °C for 1 h (Besra et al., 1992). The resulting hydrolysate was reduced with NaB^2H_4, per-*O*-acetylated, and examined by GC−MS. In some cases, the per-*O*-CH_3-, per-*O*-C^2H_3-OSEol (3−5 mg) was partially hydrolyzed with 2 M TFA at 70 °C for 1 h to generate smaller, partially per-*O*-CH_3-, per-*O*-C^2H_3-OSEol fragments. The hydrolysates were dried, reduced with NaB^2H_4, and alkylated with C_2H_5I (Hakomori, 1964) and the products recovered by chromatography on a C_{18} Sep-Pak cartridge as described (York et al., 1986). The mixture of per-*O*-alkylated oligoglycosyl alditols was dissolved in aqueous 30% CH_3CN and injected onto a 0.4 × 25 cm Hibar RP-18 reversed phase column (EM Science, Gibbstown, NJ) connected to a Beckman Model 110A HPLC column (Beckman, San Raman, CA). The solvent gradient was as follows: 1 min with 30% aqueous CH_3CN followed by a gradient to 65% aqueous CH_3CN over 40 min and, finally, a gradient from 65 to 85% aqueous CH_3CN in a 10 min period. The flow rate was 1 mL/min, and 1 ml fractions were collected. Fractions were dried and dissolved in 100 μL of acetone and injected directly onto the GC−MS.

(b) Aldononitrile Derivatives. The purified glycolipid or the OSE arising from it were hydrolyzed in 2 M TFA at 120 °C for 2 h (Besra et al., 1992). The resultant reaction mixture was dried and redissolved in 200 μL of anhydrous methanol containing 1 mg of dry hydroxylamine hydrochloride and 2.5 mg of dry sodium acetate. The mixture was subsequently heated at 60 °C for 1 h, dried, and per-*O*-acetylated, and the resultant aldononitrile acetates were examined by GC and GC−MS (Pfaffenberger et al., 1976; Besra et al., 1992).

Location of Acyl Residues on Intact Glycolipid

In order to establish the location of acyl functions on the oligosaccharide backbone, the native glycolipid was subjected to the neutral alkylating conditions of Prehm (1980) as follows. The glycolipid (2 mg) was resuspended in 200

[1] Abbreviations: LOS, lipooligosaccharide; GPL, glycopeptidolipid; OSE, oligosaccharide(s) derived from the glycolipids; OSEol, the reduced OSE, i.e., oligosaccharide alditol; Glc, glucose; Man, mannose; Lyx, lyxose; Lyxol, lyxitol; *p*, pyranosyl; GC, gas chromatography; GC−MS, gas chromatography−mass spectrometry; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; 2D COSY, two-dimensional chemical shift correlated spectroscopy; J_1, coupling constant; FAB/MS, fast atom bombardment-mass spectrometry; TFA, trifluoroacetic acid; t_R, retention time.

μL of trimethyl phosphate, under N_2, and 30 μL of 2,6-di-*tert*-butylpyridine and 25 μL of methyl trifluoromethanesulfonate were added. The mixture was stirred at room temperature for 5 h, following which 1 mL of water was added. The mixture was applied to a C_{18} Sep-Pak cartridge which was eluted successively with H_2O, CH_3CN, and ethanol. The ethanol eluate was dried to yield the naturally acylated, *O*-methylated glycolipid which was subjected to acid hydrolysis using 250 μL of 2 M TFA at 120 °C for 2 h (Besra et al., 1992). The resulting hydrolysate was reduced with NaB^2H_4, per-*O*-acetylated, and examined by GC−MS.

Fast Atom Bombardment-Mass Spectrometry (FAB-MS) Analyses

The OSE was partially hydrolyzed with 2 M TFA for 30 min at 80 °C and then per-*O*-deuterioacetylated with pyridine-Ac_2O-d_6 (1:1, v:v) at 80 °C for 2 h for FAB-MS analysis. Time-course methanolysis/FAB-MS on per-*O*-deuteriomethylated OSE was performed as described by Dell et al. (1994). Aliquots were taken prior to heating and after 1 min of incubation at 60 °C. FAB-MS spectra were acquired using a VG Analytical High-Field ZAB-HF mass spectrometer fitted with an M-scan gun operated at 10 kV or a ZAB-2SE FPD mass spectrometer fitted with a cesium gun operated at 20−25 kV. Spectra obtained on the former instrument were recorded on oscillographic chart paper and manually counted. Data acquisition and processing on the latter instrument were performed using the VG Analytical Opus software. The matrix used was thioglycerol, and samples were dissolved in methanol for loading onto the target.

Other Analytical Procedures

GC of aldononitrile acetates, alditol acetates, and partially per-*O*-alkylated, partially per-*O*-acetylated alditols was conducted on a DB-23 capillary column as described (Besra et al., 1992). 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 (Besra et al., 1992). Routine ^1H, ^{13}C, 2D $^1H/^{13}C$, and 2D COSEY NMR spectra were recorded on a Bruker ACE-300 instrument at the Colorado State University Department of Chemistry Instrument Facility. Spectra for the OSE were obtained in ^2H_2O at a concentration of 15 mg per 0.5 mL of ^2H_2O, before and after exchanging protons with ^2H_2O.

RESULTS

Isolation of the M. phlei Glycolipid. M. phlei strain Timothy (ATCC 19249) was grown under the conditions in everyday use for *M. smegmatis* growth (Takayama et al., 1975). When approximately 180 g of dried cells was extracted with acetone, they yielded 3.6 g of acetone-soluble lipids which was further subjected to size-exclusion chromatography on columns of Sephadex LH-20 irrigated with $CHCl_3$−CH_3OH (19:1) followed by $CHCl_3$−CH_3OH (1:1) (Besra et al., 1992, 1994a). Fractions were collected and samples were subjected to TLC in $CHCl_3$−CH_3OH−H_2O (65:25:4) followed by spraying with α-naphthol-H_2SO_4 (Besra et al., 1992, 1994a) to identify glycolipid-containing fractions. Positive fractions were combined, dried, and resuspended in equal parts of petroleum−ether and 87% aqueous ethanol. The resulting aqueous ethanol phase was dried according to the published procedures of Bisso et al. (1976) to afford *ca*. 90 mg of purified *M. phlei* glycolipid. However, when subjected to TLC, the products resulted in a streaked, nondescript profile, suggesting that these glycolipids were extremely labile and degraded upon silica gel TLC. Treatment of the partially purified glycolipids with 0.2 N NaOH and subsequent TLC demonstrated that they were degraded and, hence, were alkali-labile, similar in this respect to the LOS class of antigens from mycobacteria (Hunter et al., 1983; Besra et al., 1994a). The water-soluble OSE produced by deacylation were desalted using a column of Bio-Gel P-2 to afford approximately 15 mg of OSE from 70 mg of the purified glycolipids.

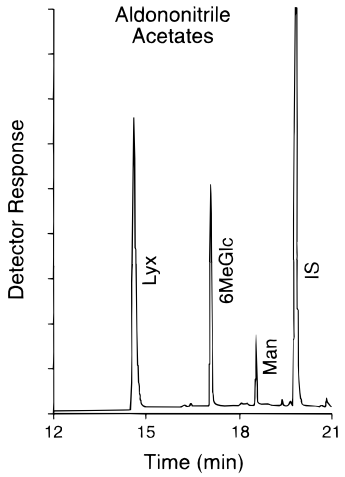


FIGURE 1: GC profile of the aldononitrile acetates derived from the OSE of the *M. phlei* glycolipid. The identities of the glycosyl residues were established by cochromatography with authentic standards. Experimental conditions are described in the text.

Glycosyl Composition. Sugar analysis was conducted primarily on the aldononitrile acetates (Pfaffenberger et al., 1976), since reduction of D-arabinose and D-lyxose yields the same pentitol; i.e., D-arabinitol is the same compound as D-lyxitol.

The glycolipid preparation and the prereduced OSE were hydrolyzed with 2 M TFA and the aldononitrile acetates prepared and subjected to GC with authentic standards. The sugars were identified as Lyx (t_R 14.7 min), 6MeGlc (t_R 17.2 min), and Man (t_R 18.7 min) in the approximate molar ratio of 5.7:2.4:1 (Figure 1). The observation of nonstoichmetric amounts of the above glycosyl residues suggested, firstly, that the *M. phlei* glycolipid preparation consisted of acylated oligosacccharides and, secondly, that in the absence of monomeric glucose, they are probably not based on the well-established trehalose-containing LOS antigens of mycobacteria (Besra et al., 1994a).

Confirmation of the identities of these glycosyl residues, in particular, 6MeGlc (*m/z* 259, 184, 139, and 115) and Man, was obtained by GC and GC-MS of alditol acetates. The enantiomeric configurations of all of the glycosyl residues was established by comparative GC−MS analyses with the standard trimethylsilylated (*S*)-(+)- and (*R*)-(−)-octyl glycosides of D-Lyx, 6Me-D-Glc, and D-Man (McNeil et al., 1989). The latter derivatives cochromatographed exclusively with the trimethylsilyl derivatives of the (*R*)-(−)-octylglycosides prepared from the *M. phlei* glycolipid (results not shown). Thus, it was established that this unique glycolipid from *M. phlei* contained D-Lyx, 6Me-D-Glc, and D-Man.

Table 1: Glycosyl Linkage Analysis of Per-*O*-CH_3-OSE from the *M. phlei*[b] Glycolipids[a]

structure of the *O*-Ac-*O*-C^2H_3-*O*-CH_3 alditol identified by GC−MS	t_R (min)[c]	abbreviated name of the glycosyl residue and linkage pattern[d]	% composition
1,5-di-*O*-Ac-2,3,4-tri-*O*-C^2H_3-lyxitol	5.79	t-D-Lyx*p*	5.4
1,3,5-tri-*O*-Ac-2,4-di-*O*-C^2H_3-lyxitol	6.38	→3)-D-Lyx*p*	61.4
1,5-di-*O*-Ac-2,3,4-tri-*O*-C^2H_3-6-*O*-CH_3-glucitol	6.60	t-6-*O*-CH_3-D-Glc*p*	3.0
1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-C^2H_3-mannitol	6.60	t-D-Man*p*	
1,3,5-tri-*O*-Ac-2,4-di-*O*-C^2H_3-6-*O*-CH_3-glucitol	7.21	→3)-6-*O*-CH_3-D-Glc*p*	19.5
1,3,5-tri-*O*-Ac-2,4,6-tri-*O*-C^2H_3-mannitol	7.28	→3)-D-Man*p*	10.7

[a] See also Figure 2. [b] The prereduced OSE mixture was per-*O*-trideuteriomethylated with C^2H_3I (Hakomori, 1964). The resulting per-*O*-C^2H_3, per-*O*-CH_3-OSEs were hydrolyzed, reduced with NaB^2H_4, per-*O*-acetylated, and examined by GC−MS. [c] GC−MS analyses were performed as described in Materials and Methods. [d] t = terminal; the →3) designation means that the OHs at the C-3 positions of the corresponding glycosyl residues were originally linked to another glycosyl unit.

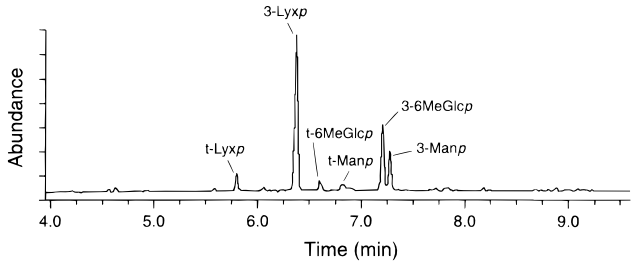


FIGURE 2: GC of the alditol acetates derived from the application of base-catalyzed per-*O*-methylation to the OSE from the *M. phlei* glycolipid. t = terminal, and the 3- designation means that the OHs at the C-3 positions of the corresponding glycosyl residues were originally linked to another glycosyl unit (see Table 1).

Glycosyl Linkage Analysis and Location of Acyl Functions. The positions of glycosyl linkages and fatty acyl substituents were determined by analyzing the alditol acetate patterns after various forms of alkylations. The base-solubilized OSE mixture was prereduced and further *O*-trideuteriomethylated using the base-catalyzed method of Hakomori (Hakomori, 1964; Stellner et al., 1973), firstly, to determine the ring form and, secondly, the glycosyl linkage pattern. The resultant per-*O*-CH_3, per-*O*-C^2H_3-OSEs were hydrolyzed with 2 M TFA, reduced with NaB^2H_4, and per-*O*-acetylated (Besra et al., 1992). GC−MS of the resulting alditol acetates (Table 1 and Figure 2) revealed the presence of three terminal glycosyl residues, t-Lyx*p*, i.e., 1,5-di-*O*-CH_3CO-2,3,4-tri-*O*-C^2H_3Lyx[1-^{2}H]ol (*m*/*z* 215, 214, 168, 167, and108), t-6MeGlc*p*, i.e., 1,5-di-*O*-CH_3CO-2,3,4-tri-*O*-C^2H_3-6-*O*-CH_3-Glc[1-^{2}H]ol (*m*/*z* 215, 211, 168, 164, 132, 130, and 108), and t-Man*p*, with the latter two residues coeluting and the t-Man*p* residue identified by the characteristic ion *m*/*z* 167. In addition, three other glycosyl residues were clearly present, 3-linked Lyx*p*, i.e., 1,3,5-tri-*O*-CH_3CO-2,4-di-*O*-C^2H_3Lyx-[1-^{2}H]ol (*m*/*z* 240, 239, 180, 179, 121, and 120), 3-linked 6MeGlc*p*, i.e., 1,3,5-tri-*O*-CH_3CO-2,4-di-*O*-C^2H_3-6-*O*-CH_3-Glc[1-^{2}H]ol, and 3-linked Man*p*, i.e., 1,3,5-tri-*O*-CH_3CO-2,4,6-tri-*O*-C^2H_3Man[1-^{2}H]ol (*m*/*z* 285, 240, 220, 167, 132, 240, and 121). Thus, the glycosyl linkage analysis suggests that the OSE backbone of the glycolipids is 3-linked with the heterogeneity in size and linkage, implicated from the earlier glycosyl compositional analysis, being further substantiated.

The fatty acids derived from the deacylation of the glycolipids were methylated and examined by GC−MS (Ryhage & Stenhagen, 1963) as described previously (Hunter et al., 1983). Cochromatography (Ryhage & Stenhagen, 1963) helped identify the fatty acids as follows: $C_{16:0}$, 4%; $C_{16:1}$, 36%; $C_{18:0}$, 18%; $C_{18:1}$, 20%; and tuberculostearic acid, 22%. The location of these acyl functions on the OSE backbone were determined by the mild (neutral) methylation procedure of Prehm (1980), a modification of that of Arnarp et al. (1975). The resulting naturally *O*-acylated, per-*O*-methylated glycolipids were hydrolyzed with 2 M TFA, reduced with NaB^2H_4, and per-*O*-acetylated and subjected to GC−MS (Figure 3). Accordingly, the existence of the following glycosyl residues (Table 2) was clearly established: t-Lyx*p* with an acyl substituent at position 2, nonsubstituted t-hexose (either 6MeGlc*p* or Man*p*), 3-linked Lyx*p* with an acyl substituent at position 2, 3-linked hexose (either 6MeGlc*p* or Man*p*), 3-linked Lyx*p* acylated at positions −2 and −4, and 3-linked hexose (either 6MeGlc*p* or Man*p*) with an acyl substituent at either position −2 or −4. The approach, however, failed to establish clearly which of the two hexoses, 6MeGlc*p* or Man*p*, was *O*-acylated. In general, the above detailed chemical analysis did demonstrate that the glycolipids are heavily *O*-acylated, illustrated by the fact that every single Lyx residue within the polymer is substituted at position −2 and, in some instances, at both positions −2 and −4.

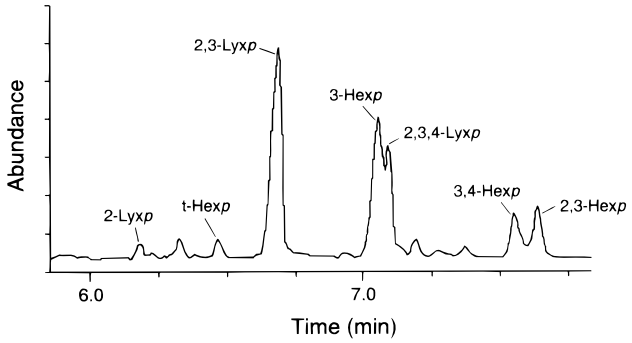


FIGURE 3: GC of the alditol acetates derived from the application of neutral methylation to the intact *M. phlei* glycolipid. Experimental conditions are described in the text. t = terminal, and the 2-, 3-, and 4- designations mean that the OHs at those positions of the glycosyl residue were originally linked to another glycosyl unit or, in fact, *O*-acylated (see Table 2).

High-Resolution NMR Analysis. The ^{1}H NMR spectrum (Table 3) of the deacylated glycolipid reflected the expected complexity indicated earlier by the glycosyl and linkage analyses. Specifically, the anomeric region between δ 4.4 and 5.2 contained five resonances with an integral value of eight protons. The two upfield resonances, each integrating for one proton and appearing as a pair of doublets centered at δ 4.50 with a $J_{1,2}$ coupling constant of 7.9 Hz, were readily assigned to the two 6-*O*-Me-β-D-Glc*p* residues. Additionally, the singlet (6H) at δ 3.29 was assigned to the *O*-CH_3 group of these residues. The ^{13}C NMR (Table 3) of the OSE mixture revealed six anomeric carbon resonances with two of these signals overlapping. Subsequently, 2D ^{1}H/^{13}C

Table 2: Location of Acyl Functions within the *M. phlei*[b] Glycolipid[a]

structure of the *O*-Ac-*O*-CH_3 alditol identified by GC−MS[c]	t_R (min)	*m*/*z* values	abbreviated name of the glycosyl residue and linkage pattern[d]	location of the acyl group[e]
1,2,5-tri-*O*-Ac-3,4-di-*O*-CH_3-lyxitol	6.17	190, 130, 117	→2)-D-Lyx*p*	2
1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-CH_3-glucitol and/or	6.46	205, 162, 145, 118, 102	t-6-*O*-CH_3-D-Glc*p*	−
1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-CH_3-mannitol	6.46		t-D-Man*p*	−
1,2,3,5-tetra-*O*-Ac-4-*O*-CH_3-lyxitol	6.69	262, 202, 117	→2,→3)-D-Lyx*p*	2
1,3,5-tri-*O*-Ac-2,4,6-tri-*O*-CH_3-glucitol and/or	7.06	234, 202, 161, 129, 118	→3)-D-6-*O*-CH_3-Glc*p*	−
1,3,5-tri-*O*-Ac-2,4,6-tri-*O*-CH_3-mannitol	7.06		→3)-D-Man*p*	−
1,2,3,4,5-penta-*O*-Ac-lyxitol	7.10	290, 289, 218, 217, 188, 187, 146, 145, 116,115	→2,→3,→4)-D-Lyx*p*	2 and 4
1,3,4,5-tetra-*O*-Ac-2,6-di-*O*-CH_3-glucitol and/or	7.56	305, 185, 118	→3,→4)-6-*O*-CH_3-D-Glc*p*	4
1,3,4,5-tetra-*O*-Ac-2,6-di-*O*-CH_3-mannitol	7.56		→3,→4)-D-Man*p*	4
1,2,3,5-tetra-*O*-Ac-4,6-di-*O*-CH_3-glucitol and/or	7.64	262, 161, 129	→2,→3)-6-*O*-CH_3-D-Glc*p*	2
1,2,3,5-tetra-*O*-Ac-2,4-di-*O*-CH_3-mannitol	7.64		→2,→3)-D-Man*p*	2

[a] See Figure 3. [b] The *M. phlei* OSE was per-*O*-methylated according to the procedure of Prehm (1980), and the resulting per-*O*-CH_3-OSE was hydrolyzed, reduced with NaB^2H_4, per-*O*-acetylated, and examined by GC−MS. [c] GC−MS analyses were performed as described in Materials and Methods. [d] t = terminal; the →2,→3,→4) designation means that the OHs at those positions of the glycosyl residue were originally linked to another glycosyl unit or, in fact, *O*-acylated. [e] A comparison of the linkage pattern obtained through Prehm methylation (Table 2) and Hakomori methylation (Table 1) allowed the location of acyl groups within the *M. phlei* LOS to be deduced.

Table 3: Assignment of Anomeric ^1H and ^{13}C Resonances and $^1J[^{13}CH]$Coupling Constants of the *M. phlei* OSE

^{13}C (ppm)	^1H (δ)	$^1J[^{13}CH]$ (Hz)	assignment
104.2	4.50[a]	162	6-*O*-CH_3-β-D-Glc*p*
104.1[b]	5.05	170	α-D-Lyx*p*[c]
103.6	4.50[a]	162	6-*O*-CH_3-β-D-Glc*p*
101.3[b]	4.70	162	β-D-Lyx/β-D-Man*p*[d]
101.0	4.72	162	β-D-Lyx/β-D-Man*p*[d]
100.8	4.71	160	β-D-Lyx/β-D-Man*p*[d]

[a] $^3J_{1,2}$ coupling constant = 7.9 Hz. [b] These resonances were about twice the area of the others and probably resulted from two residues. [c] The ^{13}C and ^1H NMR analyses applied on the intact OSE were consistent with this resonance being assigned to either α-D-Lyx*p* or α-D-Man*p*, but further analysis of fragmented oligoglycosyl alditols (see below) revealed that the α-residue was D-Lxy*p*. [d] The exact assignment to β-D-Lxy*p* versus β-D-Man*p* was not deduced.

correlation in the C/H coupled mode allowed the $^1J[^{13}CH]$ coupling constants of all resonances to be measured (Table 3). The anomeric protons of the two 6-*O*-Me-β-D-Glc*p* described earlier were found to correlate to the anomeric carbons at 104.2 and 103.6 ppm with an expected $^1J[^{13}CH]$ coupling constant of 162 Hz. The remaining proton and carbon anomeric resonances were only tentatively assigned after 2D $^1H/^{13}C$ NMR (Table 3). In summary, the NMR data revealed a complex mixture of OSEs consisting of at least eight chemically distinct glycosyl residues. The 6MeGlc*p* and all but two of the Man*p*/Lyx*p* residues were in the β-configuration, while the remaining two Man*p*/Lyx*p* residues were α. The degradative chemical analyses discussed later, in fact, demonstrated that the Lyx*p* residues were α and the Man*p* residues were indeed β. In addition, information provided through FAB-MS analyses clearly demonstrated that the glycolipid preparation is composed of a mixture of complex oligosaccharides.

Partial Depolymerization of the OSE. In order to determine the sequence of sugars in the OSE, the product was *O*-trideuteriomethylated, partially hydrolyzed, reduced, and *O*-ethylated, and the resulting mixture of compounds was separated by HPLC and analyzed by GC−MS. This series of reactions is illustrated in the context of the formation of the per-*O*-alkylated diglycosyl alditol "trimer" 6MeGlc-

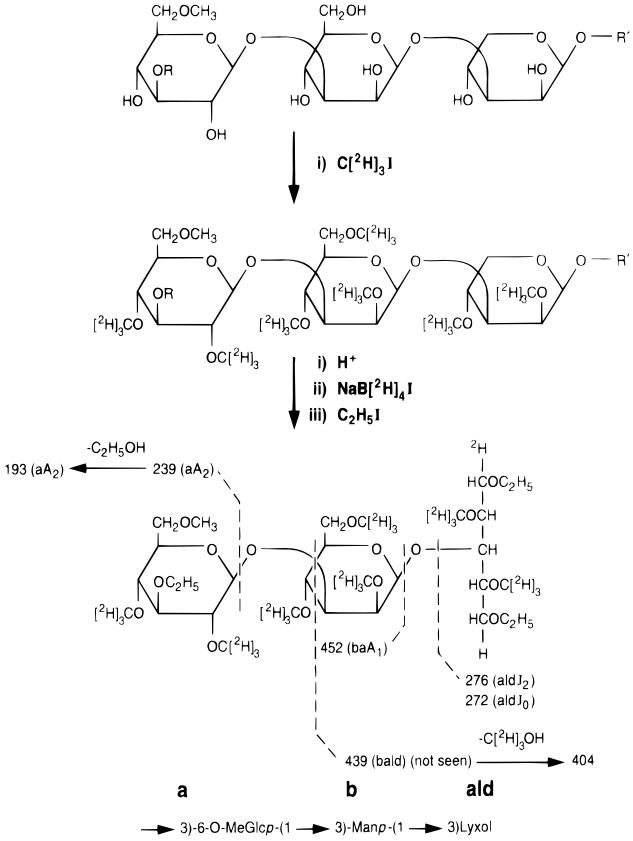


FIGURE 4: Illustration of the sequence of reactions used to produce compound 6 (Table 4). The hydrolytic conditions resulted in random cleavage of the per-*O*-trideuteriomethylated polymer, resulting in the production of all of the products, plus others, described in Table 4. Also illustrated are the mass spectral fragment ions and the nomenclature (Kochetkov & Chizhov, 1966) used in Table 4. R and R′ are other glycosyl residues.

(1→3)Man(1→3)Lyx in Figure 4. Since the alkylation sequence resulted in all three residues having a different molecular weight, as determined by MS, the resulting fragments were readily identified through their diagnostic cleavage ions as presented in Table 4. Thus, it was evident that the 6MeGlc was glycosidically linked to either a Man

Table 4: Identification of Partially *O*-Ethylated, *O*-Methylated, Partially *O*-Trideuteriomethylated Oligoglycosyl Alditols

fragment	HPLC t_R (min)	GC t_R (min)	structure	EI/MS[a]
1	15	6.36	→3)Lyx*p*-(1→3)-6MeGlcol[b]	149 (aA_2), 195 (aA_1), 270 ($aldJ_2$), 344 ($aldJ_1$)
2	17	14.18	→3)Lyx*p*-(1→3)-6MeGlc*p*(1→3)-lyxol	149 (aA_2), 195 (aA_1), 405 (baA_1), 226 ($aldJ_2$), 401 ($baldJ_2$-C^2H_3OH)
3	17	14.69	→3)Lyx*p*-(1→3)-6MeGlc*p*(1→3)-Manol	149 (aA_2), 195 (aA_1), 405 (baA_1), 370 (baA_2), 448 ($baldJ_2$-C^2H_3OH), 273 ($aldJ_2$), 319 ($aldJ_0$)
4	18	6.88	→3)6MeGlc*p*(1→3)Lyxol	239 (aA_1), 193 (aA_2), 226 ($aldJ_2$), 300 ($aldJ_1$)
5	18	7.57	→3)6MeGlc*p*(1→3)Manol	239 (aA_1), 193 (aA_2), 273 ($aldJ_2$), 347 ($aldJ_1$)
6	18	13.95	→3)6MeGlc*p*(1→3)Man*p*(1→3)Lyxol	239 (aA_1), 193 (aA_2), 226 ($aldJ_2$), 272 ($aldJ_0$), 452 (baA_1), 404 ($baldJ_2$-C^2H_3OH)
7	18	20.13	→3)Lxy*p*(1→3)6MeGlc*p*(1→3)Man*p*(1→3)Lyxol	149 (aA_2), 195 (aA_1), 405 (baA_1), 617 ($cbaA_1$), 226 ($aldJ_2$), 485 ($caldJ_0$), 439 ($caldJ_2$), 404 ($caldJ_2$-C^2H_3OH), 614 ($bcaldJ_2$-C^2H_3OH)

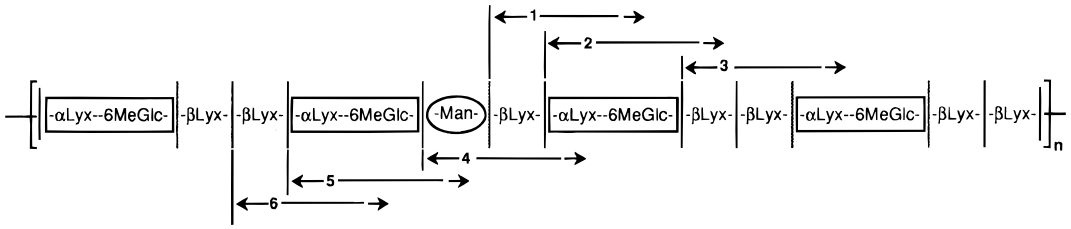
[a] See Kochetkov and Chizhov (1966) and Figure 4 for interpretation and nomenclature used in MS. [b] →3)Lxy*p*(1→3)6MeGlcol = 3−*O*-ethyl-2,4-bis(*O*-trideuteriomethyl)Lyxopyranosyl(1→3)6-*O*-methyl-2,4-bis(*O*-trideuteriomethyl)-1,5-di-*O*-ethylglucitol. Other compounds are identified analogously.

(fragments 3, 5, and 7) or a Lyx (fragments 2 and 4). In either case, a Lyx was attached to the 3 position of the 6MeGlc (see fragments 1−3 and 7 in Table 4). In addition, ^{1}H NMR analyses of fragments 2 and 3 demonstrated that these D-Lyx*p* residues were in the α-configuration. These results also allowed recognition of the fact that the *M. phlei* glycolipid contained a tetrasaccharide, →3)-α-D-Lyx*p*(1→3)-6-*O*-Me-*β*-D-Glc*p*(1→3)-*β*-D-Man*p*(1→3)-*β*-D-Lyx*p*, and a trisaccharide, →3)-α-D-Lyx*p*(1→3)-6-*O*-CH_3-*β*-D-Glc*p*(1→3)-*β*-D-Lyx*p*. To determine how these two oligosaccharides are arranged in the intact OSE required analysis of much larger fragments by FAB-MS.

FAB-MS Analysis of the Partially Depolymerized Glycolipid: Evidence for a Two-Tetrasaccharide Repeat. The occurrence of D-Lyx*p*, 6-*O*-Me-*β*-D-Glc*p*, and Man*p* in an approximate 5.7:2.4:1 ratio in a total of eight distinct chemical environments as defined by NMR studies was indicative of regular repeating sequences in an oligosaccharide larger than eight glycosyl residues. Also, there were, on average, a chemically similar pair of α-D-Lyx*p*, *β*-D-Lyx*p*, and 6-*O*-Me-*β*-D-Glc*p* plus another *β*-D-Man and *β*-D-Lyx in distinctive environments as deduced from the NMR data. The detailed characterization of the partial hydrolysates of the per-*O*-alkylated OSE led to the identification of →3)-Lyx(α1→3)6MeGlc(*β*1→3)Man(*β*1→3)Lyx(*β*1→ and →3)-Lyx(α1→3)6MeGlc(*β*1→3)Lyx(*β*1→ as two distinct sequences in the OSE. With the apparent "shortage" of one *β*-Lyx residue, these two sequences together would otherwise account for all of the eight distinct residues identified. Significantly, the inability to recover Lyx_n oligomers suggested that a *β*-Lyx*p* linkage may be particularly labile to acid hydrolysis, especially when linked to another Lyx.

Direct FAB-MS analysis of the per-*O*-deuterioacetylated mild acid hydrolysates of the OSE mixture afforded major molecular ion signals corresponding to $Lyx_{1-4}6MeGlc_1$, $Lyx_{1-3}6MeGlc_1Man_1$, and $Lyx_{3,4}6MeGlc_2Man_1$ with the di-, tri-, and tetrasaccharides dominating the spectrum (data not shown). The two tetrasaccharides recovered correspond to $Lyx_3$6MeGlc$_1$ and $Lyx_2$6MeGlc$_1$Man$_1$. On the assumption that (i) the single α-Lyx*p* linkage is more stable than all other *β*-pyranosyl linkages and (ii) the Lyx(*β*1→3)Lyx bond is particularly susceptible to acid hydrolysis and taking into consideration the two complete sequences defined above, it is probable that the OSE mixture is comprised of two dominating sequences of αLyx-6MeGlc-Man-*β*Lyx and αLyx-6MeGlc-*β*Lyx-*β*Lyx, where the residue immediately preceding αLyx is *β*Lyx in both instances. The αLyx-6MeGlc-*β*Lyx-*β*Lyx sequence would readily yield the trisaccharide αLyx-6MeGlc-*β*Lyx upon hydrolysis of the labile *β*Lyx−*β*Lyx bond, whereas hydrolysis of other *β*-linkages would give rise to αLyx-6MeGlc-Man and αLyx-6MeGlc, the latter being the only major disaccharide observed. Further, a linear stretch of (αLyx-6MeGlc-Man-*β*Lyx-αLyx-6MeGlc-*β*Lyx-*β*Lyx)$_n$ or (αLyx-6MeGlc-*β*Lyx-*β*Lyx-αLyx-6MeGlc-Man-*β*Lyx)$_n$ could yield $Lyx_4$6MeGlc but not $Lyx_5$6MeGlc on the basis that the α-Lyx*p* linkage is relatively resistant to mild acid. The larger OSEs observed, i.e., $Lyx_3$6MeGlc$_1$Man$_1$, $Lyx_3$6MeGlc$_2$Man$_1$, and Lyx_4-6MeGlc$_2$Man$_1$, are also consistent with this interpretation. The absence of $Lyx_4$6MeGlc$_1$Man$_1$, which would necessarily contain a *β*Lyx-*β*Lyx sequence, conforms to the argument that *β*Lyx-*β*Lyx is most readily hydrolyzed and will thus not be observed as a major product, just as αLyx-6MeGlc-*β*Lyx is more prominent than αLyx-6MeGlc-*β*Lyx-*β*Lyx.

Further Evidence for a Two-Tetrasaccharide Repeat. To find further data in support of a linear arrangement of two tetrasaccharides, the OSE was per-*O*-trideuteriomethylated, subjected to time-course methanolysis, and monitored by FAB-MS directly. All of the major ions observed after 1 min of heating at 60 °C (Table 5) correspond to $[M + NH_4]^+$ and $[M + Na]^+$ of methyl glycosides containing one free OH group, consistent with a 3-linked linear oligosaccharide with no other branching point. Although the low-mass region was dominated by matrix signals, it is clear that, as with the mild acid hydrolysis, the only disaccharide observed was $Lyx_1$6MeGlc$_1$; no Lyx_n was present. Likewise, the major products were $Lyx_2$6MeGlc$_1$, $Lyx_3$6MeGlc$_1$, and Lyx_2-6MeGlc$_1$Man$_1$. A much smaller amount of $Lyx_1$6MeGlc$_1$-Man$_1$ was also produced. Signals corresponding to methyl glycosides larger than a tetramer may be seen as belonging to two series: Lyx_n6MeGlc$_m$ and Lyx_n6MeGlc$_m$Man$_1$. In the former series, which is in greater abundance than the latter series, the presence of ions such as $Lyx_6$6MeGlc$_2$ and Lyx_7-6MeGlc$_2$ indicated that the OSE cannot be a simple polymer of (αLyx-6MeGlc-Man-*β*Lyx-αLyx-6MeGlc-*β*Lyx-*β*Lyx)$_n$. A possible stretch of linear sequence which would yield both series of ions upon parital methanolysis is drawn in Figure 5, on the basis of the same assumption discussed above; i.e, the single α-Lyx*p* linkage is more stable than all other *β*-pyranosyl linkages.



	$Lyx_n6MeGlc_mMan_1$			$Lyx_n6MeGlc_m$		
	$Lyx_{1\text{-}4}6MeGlc_1Man_1$	$Lyx_{3\text{-}7}6MeGlc_2Man_1$	$Lyx_{6\text{-}10}6MeGlc_3Man_1$		$Lyx_{1\text{-}5}6MeGlc_1$	$Lyx_{4\text{-}8}6MeGlc_2$	$Lyx_{7\text{-}11}6MeGlc_3$
4	$Lyx_{2\text{-}4}6MeGlc_1Man_1$	$Lyx_{5\text{-}7}6MeGlc_2Man_1$	$Lyx_{8\text{-}10}6MeGlc_3Man_1$	**1**	$Lyx_{2\text{-}4}6MeGlc_1$	$Lyx_{5\text{-}7}6MeGlc_2$	$Lyx_{8\text{-}10}6MeGlc_3$
5	$Lyx_{1\text{-}2}6MeGlc_1Man_1$	$Lyx_{3\text{-}5}6MeGlc_2Man_1$	$Lyx_{6\text{-}8}6MeGlc_3Man_1$	**2**	$Lyx_{1\text{-}3}6MeGlc_1$	$Lyx_{4\text{-}6}6MeGlc_2$	$Lyx_{7\text{-}9}6MeGlc_3$
6	$Lyx_{2\text{-}3}6MeGlc_1Man_1$	$Lyx_{4\text{-}6}6MeGlc_2Man_1$	$Lyx_{7\text{-}9}6MeGlc_3Man_1$	**3**	$Lyx_{3\text{-}5}6MeGlc_1$	$Lyx_{6\text{-}8}6MeGlc_2$	$Lyx_{9}6MeGlc_3$

FIGURE 5: Simplest repeating unit sequence of OSE which will accommodate all the data presented. A 16-mer is proposed to comprise the tetrasaccharides αLyx6MeGlcβLyxβLyx and αLyx6MeGlcManβLyx in 3:1 ratio. In this model, the β-linkages are proposed to be more susceptible to partial hydrolysis (indicated by vertical lines) than the αLyx6MeGlc unit (boxed). All possible combinations of Lyx_nMeHex_m and $Lyx_nMeHex_mHex_1$ which would be produced from this repeating 16-mer sequence can be inferred from the cleavages represented by **1**−**6** as listed in the figure. The relative abundance of the ions observed in FAB-MS analysis is in good agreement with the theoretical number of alternative ways in which a particular ion can be produced. Accordingly, ion composition excluded by this model was not observed.

Table 5: Major Molecular Ion Signals[a] Observed in the Spectrum of the Mild Methanolysates of Per-*O*-trideuteriomethylated OSE[b]

	$Lyx_nMeGlc_{n'}$ series		$Lyx_nMeGlc_nMan_{n''}$ series	
size	*m*/*z*	composition	*m*/*z*	composition
dimer	426	$Lyx_16MeGlc_1$	−	
trimer	592	$Lyx_26MeGlc_1$	639	$Lyx_16MeGlc_1Man_1$
4-mer	758	$Lyx_36MeGlc_1$	805	$Lyx_26MeGlc_1Man_1$
5-mer	924	$Lyx_46MeGlc_1$	971	$Lyx_36MeGlc_1Man_1$
6-mer	−		1181	$Lyx_36MeGlc_2Man_1$
7-mer	1300	$Lyx_56MeGlc_2$	1347	$Lyx_46MeGlc_2Man_1$
8-mer	1466	$Lyx_66MeGlc_2$	1513	$Lyx_56MeGlc_2Man_1$
9-mer	1632	$Lyx_76MeGlc_2$	1679	$Lyx_66MeGlc_2Man_1$
10-mer	−		1889	$Lyx_66MeGlc_3Man_1$
11-mer	2008	$Lyx_86MeGlc_3$	2055	$Lyx_76MeGlc_3Man_1$
12-mer	2174	$Lyx_96MeGlc_3$	2221	$Lyx_86MeGlc_3Man_1$
13-mer	−			
14-mer	−			
15-mer	2716	$Lyx_{11}6MeGlc_4$	2763	$Lyx_{10}6MeGlc_4Man_1$

[a] As illustrated in Figure 5, the 5-, 6-, 9-, 10-, 13-, and 14-mers for both series, which are more restricted in the way they can be produced, were expected to be of low abundance, although some of them could be readily detected as very minor signals except the 13- and 14-mers. [b] All ions tabulated correspond to $[M + NH_4]^+$ of the methyl glycosides of $Lyx_nMeGlc_{n'}Man_{n''}$ containing a free hydroxyl group as a consequence of methanolysis. $[M + Na]^+$ at 5 mass units higher was also observed for most ions.

Although other more complicated, nonrepeating sequences could also yield the ions observed, the proposed 16-mer sequence represents the simplest one which would still accommodate all the data presented here. It is also conceivable that, while this 16-mer constitutes the major portion of the OSE, a minor stretch of a simple octamer repeat of αLyx-6MeGlc-βLyx-βLyx-αLyx-6MeGlc-Man-βLyx or a tetramer repeat of αLyx-6MeGlc-βLyx-βLyx may also be present either within the same molecule or as minor components of the microheterogeneous OSE. It should be noted that, in either way, the ratio of (Lyx+Man):6-*O*-Me-Glc should be approximately 3:1, which is in good agreement with the observed ratio of 6.7:2.4. On the other hand, if the OSE consists of regular repeating unit of the proposed 16-mer, the ratio of Lyx:Man:6MeGlc should be 11:1:4. The glycosyl composition data which gave a ratio of 5.7:1:2.4 thus indicate that the OSE is probably comprised of a mixture of both the octamer and 16-mer units.

DISCUSSION

Some 20 years ago, strong phylogenetic evidence dictated that all strains of *M. phlei* (the Timothy gross bacillus) should be classified as *M. smegmatis* (Hendren, 1975). Indeed, the organism *M. phlei* ATCC 356 that was the source of the pioneering work on the acidic 6-*O*-methyl-D-glucose-containing lipopolysaccharide (MGLP), which bears similarities to the present D-lyxose-containing lipooligosaccharide, was later classified as *M. smegmatis* ATCC 356 (Maitra & Ballou, 1977). Yet we have not found any evidence of the presence of D-Lyx in *M. smegmatis* mc^2155 (derived from ATCC 607), the strain commonly used in modern-day genetic and biochemical work, or in other common strains of *M. smegmatis*. For that reason, we have retained the old species name. Nevertheless, in view of the difficulty of distinguishing D-Lyx from D-Ara (see Results), the possibility of a wider distribution of D-Lyx in *Mycobacterium* should be examined.

The impetus for the present study was the report 20 years ago from the Asselineau laboratory (Bisso et al., 1976) of a glycolipid which inactivated the phage *M. phlei* and was considered the receptor site in *M. phlei* for the phage. In this study, we have not attempted to address the exact molecular structure within the glycolipid molecule responsible for phage attachment; Bisso et al. (1976) thought that acetal-linked oxaloacetates on the molecule were directly responsible for phage attachment, although the evidence was weak. Before addressing this question, we first better defined the molecule itself.

The alkaline lability of the glycolipid mixture and the recovery of a mixture of oligosaccharides point to members of the LOS class of mycobacterial glycolipids, which we first observed in *M. kansasii* (Hunter et al., 1983) and are now known to be widespread among mycobacteria (McNeil et al., 1989; Besra & Brennan, 1994). However, all of these contain a trehalose unit at the "reducing" end (obviously, with a trehalose at the right-hand terminus, there is no true

reducing end), and hence, we are reluctant to classify these latest products as members of the LOS class of surface glycolipid antigens (Besra & Brennan, 1994).

Yet, there is an interesting caveat to this issue. Lyxose is rare in nature. However, L-lyxose is present in flambamycin, an antibiotic from *Streptomyces hygroscopicus*, and in other members of this family of structurally related antibiotics, including curamycin, avilamycin, and the everninomycins (Ollis et al., 1974). What is most striking about the presence of lyxose in these structures is that it is part of a nonreducing oligosaccharide. For example, everninomycin-D on hydrolysis yielded a mixture of products, including everninose (Herzog et al., 1965) which was established to be 2,6-di-*O*-Me-*β*-D-Man*p*(1↔1)-2-*O*-Me-*β*-L-Lyx (Ganguly, 1969). Also, lyxose is a component of flambamycin; specifically, it is the right-hand terminal sugar of the flambabiose, flambatriose, and flambatetrose isolated from this antibiotic, i.e., in the case of the flambatriose 4-*O*-Me-D-Fuc(α1→4)-2,6-di-*O*-Me-D-Man(1↔1)-L-Lyx. Trehalose α-D-Glc*p*(1↔1)α-D-Glc*p* is the only other example of a naturally occurring disaccharide of the nonreducing type, and of course, trehalose or the 6-*O*-Me (Daffe et al., 1991) or the 2-*O*-Me version (Hunter et al., 1988) is the fundamental unit of the LOS group of prominent mycobacterial antigens. Obviously, the striking relationship suggested to us that the Lyx-containing glycolipids share with the trehalose-containing LOSs the property of an (α1↔1α)-linked disaccharide at the reducing end of the oligosaccharide chain. Nevertheless, we have not been able, in this present study, to establish categorically the validity of this hypothesis. Clearly, Lyx[1-^{2}Hol was evident in several products, but these were always the result of hydrolysis. Perhaps the Lyx-containing glycolipids of *M. phlei*/*M. smegmatis* bear greater analogy to the surface lipopolysaccharides of the virulent phase I cells of *Coxiella burnetti*, where Lyx occurs in the form of the 3-*C*-(hydroxymethyl)-L-lyxose and is a terminal sugar of the parent lipopolysaccharides (Schramek et al., 1985; Dahlman et al., 1986). The acid lability and its role as a serological determinant are in accord with the lability of the Lyx-containing glycolipids, their phage binding capacity, and our belief that they are the strain-specific antigens of this particular strain of *Mycobacterium*.

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