Glycopeptidolipids from *Mycobacterium fortuitum*: A Variant in the Structure of C-Mycoside†

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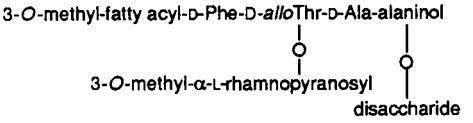
Received May 31, 1991; Revised Manuscript Received August 8, 1991

ABSTRACT: Strains from the *Mycobacterium fortuitum* complex contain surface species-specific lipids allowing their precise identification. In *M. fortuitum* biovar. *peregrinum* two major glycopeptidolipids, of the C-mycoside type, were characterized by a combination of chemical analyses, NMR, and FAB mass spectrometry. Important information was obtained by mass spectrometry both on their molecular weight and on the peptide and saccharide sequences without any derivatization. The basic structure of the two compounds was shown to be



The disaccharide part linked O-glycosidically to alaninol was either 3,4-di-*O*-methyl-α-L-rhamnopyranosyl (1→2) 3,4-di-*O*-methyl-α-L-rhamnopyranoside (mycoside I) or 3-*O*-methyl-α-L-rhamnopyranosyl (1→2) 3,4-di-*O*-methyl-α-L-rhamnopyranoside (mycoside II). This is an unusual structure of a C-mycoside since neither 6-deoxytalose nor its derivatives are present. Moreover, the oligosaccharide part is linked to the alaninol residue instead of the *allo*-threonine.

Among the many fast-growing mycobacteria that are widely distributed in nature, the main species causing human diseases are *Mycobacterium fortuitum* and *Mycobacterium chelonae* (Wallace, 1983). They are considered to be the etiological agents of skin and soft tissue infections, of postsurgical infections, and of some pulmonary diseases. They are known to be resistant to most antituberculous drugs, and their response to other antimicrobials depends on the species and biovariant or subspecies (Good, 1985). Thus, a precise identification of clinical isolates is needed.

In *M. fortuitum–chelonae* the distinction between the different species is easily made by examining their mycolic acid composition (Lévy-Frébault et al., 1983). However, this method fails to characterize the recognized subspecies or biovariants, so other specific compounds from the envelope must be examined. Surface glycolipid antigens have been extensively studied for purposes of identification of species and subspecies in the *Mycobacterium avium* complex (Brennan, 1988). Tsang et al. (1984), using both thin-layer chromatography of the glycolipid fractions and seroagglutination, concluded that *M. fortuitum* biovar. *fortuitum* and *M. fortuitum* biovar. *peregrinum* did contain chemically different glycolipids. The biovar. *peregrinum* contained an alkali-stable C-mycoside antigen, whereas the biovar. *fortuitum* contained several alkali-labile lipids tentatively assigned to the lipo-oligosaccharide-type antigens.

We describe herein the structures of the major C-mycosides from *M. fortuitum* biovar. *peregrinum*. These compounds share, with the already described C-mycosides, a peptidolipid core linked to saccharide units, but they differ in the distribution and the nature of sugar residues, leading to previously undescribed C-mycoside structures.

The present work describes an approach using fast atom bombardment mass spectrometry (FAB-MS)[1] combined with metastable ion spectrometry (mass-analyzed ion kinetic energy spectra) to obtain both the molecular weight of these complex molecules and their sequence. NMR spectroscopy supported the proposed assignments and indicated the anomeric configurations of the sugars.

Materials and Methods

Strain and Growth Conditions. The strain IP111 used in this study, characterized as *M. fortuitum* biovar. *peregrinum*, was obtained from the Institut Pasteur Culture Collection, Paris. Two other strains of *M. peregrinum* ATCC 14467 and IP507 (Lévy-Frébault et al., 1983; Labidi et al., 1984) were tested for the presence of C-mycosides. *M. chelonae–chelonae* NCTC 946 and *Mycobacterium sp.* 1217 (Lanéelle & Asselineau, 1968) were used as references. All bacteria were grown on Sauton medium (Sauton, 1912) at 33 °C for 1 or 2 weeks and harvested by pouring off the medium.

Lipid Extraction. Wet cells were extracted first in 1:2 chloroform/methanol for a few days, then in 2:1 chloroform/methanol (v/v). Pooled extracts were concentrated, washed with water, and evaporated to dryness.

Purification of Glycopeptidolipids. Crude lipids were separated on a Florisil column (60–100 mesh) with increasing

† Supported by a grant from Fondation pour la Recherche Médicale.
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‡ L.M.L.M. is supported by a fellowship from the Consejo Nacional de Ciencia y Tecnologia (Mexico).

[1] Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; IP, Institut Pasteur; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures (London); TFA, trifluoroacetic acid; TLC, thin-layer chromatography; GC, gas chromatography; TMS, trimethylsilyl; GC/MS, gas chromatography coupled to mass spectrometry; EI-MS, electron impact mass spectrometry; MIKE, mass-analyzed ion kinetic energy.

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concentrations of methanol in chloroform. Fractionations were monitored by thin-layer chromatography on silica-coated plates (G60, 0.25-mm thickness; E. Merck, Darmstadt, Germany) developed with chloroform/methanol (90:10 v/v) or with chloroform/methanol/water (65:25:4 v/v/v). Sugar-containing compounds were visualized by spraying plates with 0.2% anthrone in concentrated sulfuric acid, followed by heating.

To facilitate the purifications, anthrone-positive fractions were deacylated by mild alkaline methanolysis since no change in the chromatographic behavior of the glycopeptidolipids resulted from this treatment. Alkaline deacylation was performed according to Brennan and Goren (1979): lipid fractions were suspended in chloroform/methanol (2:1 v/v) and incubated at 37 °C for 60 min with an equal volume of 0.2 M NaOH in methanol. The mixture was neutralized with glacial acetic acid, concentrated, and extracted with chloroform. The chloroform phase was washed with water, dried, and chromatographed as described above.

Miscellaneous Analytical Techniques. (*a*) *Alkaline Borohydride Reductive Cleavage* (*Brennan & Goren, 1979*). The glycopeptidolipids were heated to 65 °C for 18 h with a mixture of 0.5 M NaOH and 1 M $NaBH_4$ in a total volume of 3 mL of ethanol/water (1:1 v/v). Excess borohydride was destroyed with acetic acid, the solvents were evaporated, and boric acid was eliminated as methyl borate by codistillation with methanol. The residue was dissolved in chloroform/methanol (2:1 v/v), and an equal volume of water was added. The lower phase was washed with a small amount of methanol/water (1:1 v/v), evaporated to dryness, and purified by TLC. The upper phase was concentrated, passed through a small column of Amberlite MB3 to eliminate inorganic salts, and then evaporated to dryness.

(*b*) *Sugar Characterization.* Samples were routinely hydrolyzed with 1 M TFA solution at 110 °C for 1 h (no additional compound was obtained with a 2 M TFA treatment for 4 h). The hydrolysates were then partitioned between chloroform and water. The aqueous phase was dried under N_2 and analyzed both on TLC silica-coated plates with chloroform/methanol (7:3 v/v) as solvent and by GC of their trimethylsilyl derivatives (Sweeley et al., 1963). Authentic standards of 3-*O*-methylrhamnose and 3,4-di-*O*-methylrhamnose were obtained from the hydrolysis of the already described $C_{12:17}$ mycosides (Lanéelle & Asselineau, 1968).

The D or L configuration of the monosaccharides was determined after their demethylation by boron trichloride (Bonner et al., 1960). The retention times on GC analysis of the trimethylsilyl derivatives of their (−)2-butylglycosides were compared to that of (−)2-butyl-L-rhamnoside and (±)2-butyl-L-rhamnosides (Gerwig et al., 1978; Sharp et al., 1984).

Gas chromatography was performed on a Girdel G30 apparatus equipped with a fused silica capillary column (25-m length × 32-mm i.d.) coated with OV-I (0.3-μm film thickness). A temperature gradient of 100–280 °C (2 deg/min) was used for the separation of silylated monosaccharides, whereas isothermal conditions (150 °C) were chosen for butylglycosides.

(*c*) *Identification of the Amino Compounds.* Amino compounds were identified after treatment of the glycopeptidolipids with 6 M HCl at 110 °C for 16 h. The hydrolysates were partitioned between chloroform and water. The aqueous phase was concentrated and analyzed by TLC on precoated cellulose plates (Merck) with 1-butanol/ethanol/water (4:1:1 v/v/v) as solvent. Threonine was differentiated from *allo*-threonine using the upper phase from a mixture of 1-butanol/water/acetone/ammonium hydroxide (8:6:1:1 v/v/v/v) as the developing solvent (Shaw & Fox, 1953).

The amino acid configuration was determined on chiral HPTLC plates (Merck 14285) developed with acetone/methanol/water (10:2:2 v/v/v) according to Günther (1988). Spots were detected by spraying with 0.2% ninhydrin in acetone followed by heating.

(*d*) *Lipid Constituents.* The chloroform extracts from the acid hydrolysis were further treated according to Crombie (1955) with concentrated HCl/ethanol (1:4 v/v) in order to cleave the very stable fatty acyl–amide linkage and determine the fatty acid composition. The ethyl esters obtained were refluxed with a 5% KOH solution in methanol/benzene (8:2 v/v) for 8 h. After acidification and extraction with ether, the free fatty acids were analyzed by GC–MS as trimethylsilyl derivatives.

(*e*) *Spectroscopy.* Infrared spectra of film samples were recorded on a Perkin-Elmer FTIR 1600 spectrophotometer. ^1H NMR spectra were obtained in $CDCl_3$ or $CDCl_3/CD_3OD$ (98:2 v/v) on a Brucker AM 300 WB instrument at 25 °C. ^{13}C NMR spectra were obtained in $CDCl_3/CD_3OD$ on a 62.9-MHz Brucker WM250 apparatus using a Brucker J-Mod sequence.

Mass spectrometry was performed on a ZAB-HS reverse-geometry mass spectrometer (VG Analytical, Manchester, U.K.). FAB spectra were generated by an 8-keV xenon atom beam. Samples were dissolved in methanol/chloroform (1:1 v/v); 1 μL of this solution was mixed on the probe tip with 1 μL of *m*-nitrobenzyl alcohol and 1 μL of a 10% solution of sodium iodide in water. Ten scans of 10 s/decade were accumulated to obtain a spectrum. The resolution of the instrument was set to 1500. MIKE spectra were measured by electrostatic voltage scanning (800 eV/s), while the parent ion resolution was kept to 1500. Between 20 and 40 scans were stored and accumulated for each experiment.

Gas-liquid chromatography–mass spectrometry was performed on the same instrument fitted with an EI ion source. The electron energy was 70 eV. Chromatography separations were carried out on an HT5 capillary column (SGE; Victoria, Australia). The film thickness of this column (12-m length, 0.32-mm i.d.) was 0.1 μm.

RESULTS

Isolation of the Major C-Mycosides from M. fortuitum biovar. peregrinum. When the crude extractable lipids were fractionated by chromatography on Florisil, several carbohydrate-containing lipids were resolved. Two major glycolipids, named I and II, were eluted by 5% and 10% methanol in chloroform, respectively. Both were phosphorus-free glycolipids presenting the characteristic infrared spectrum of C-mycosides: absorption bands at 3300, 1640, and 1540 cm^{-1}, attributed to peptide linkages. The native compounds were not acylated, as shown by the absence of an infrared absorption band corresponding to carboxyl esters and their insensitivity to alkaline deacylation (Figure 1).

Fatty Acid, Amino Acid, and Sugar Composition of Compounds I and II. The fatty acids were identified, after acid ethanolysis of the C-mycosides, by GC–EI-MS of the TMS derivatives. They consisted mainly of 3-methoxylated long-chain fatty acids containing 26 and 28 carbon atoms as demonstrated by the presence of both a characteristic fragment ion at m/z 175 [$^+CH(OCH_3)$-CH_2-COOTMS] and the [M − CH_3]$^+$ ions. A small amount of 3-hydroxylated fatty acids with the same chain length was also detected.

Strong acid hydrolysis (6 M HCl) released several amino compounds which were identified, on cellulose and chiral TLC

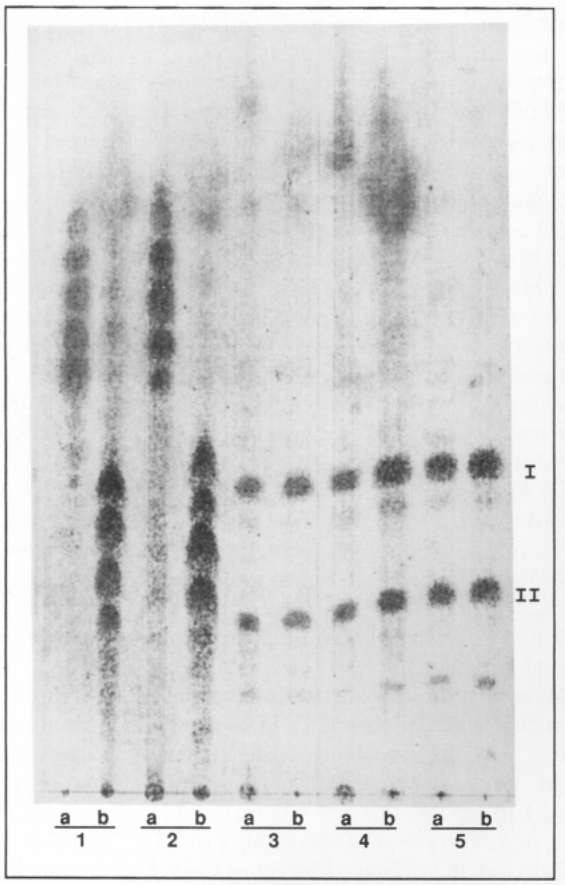

FIGURE 1: TLC (solvent, chloroform/methanol 9:1 v/v) of the intact (a) and the deacylated (b) total lipids from (1) *Mycobacterium sp.* 1217, (2) *M. chelonae* biovar. *chelonae* NCTC 946, (3) *M. fortuitum* biovar. *peregrinum* IP111, (4) *M. fortuitum* biovar. *peregrinum* ATCC 14467, and (5) *M. fortuitum* biovar. *peregrinum* IP507. I and II represent the two major glycopeptidolipids under study.

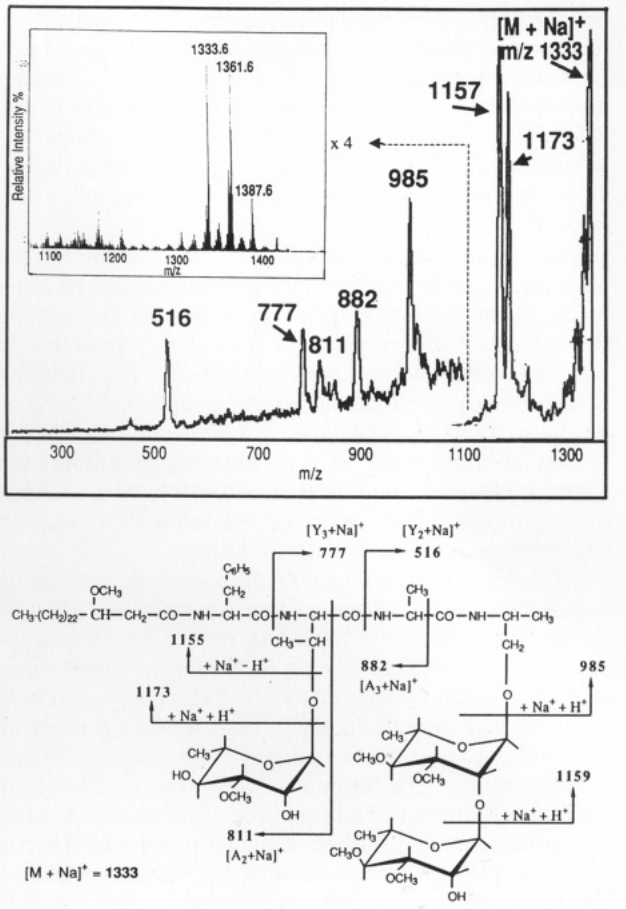

FIGURE 2: FAB mass spectrum of mycoside I (inset) and FAB MIKE spectrum from the sodium-cationized ion at m/z 1333 with the corresponding structure.

plates, as D-phenylalanine, D-*allo*-threonine, D-alanine, and alaninol (assumed to be L as in known C-mycosides; Voiland et al., 1971) by comparison to authentic standards and well-characterized components obtained by hydrolysis of the C-mycoside from *Mycobacterium sp.* 1217 (Lanēelle & Asselineau, 1968).

On milder acid hydrolysis (1 M TFA), C-mycosides I and II both liberated 3-*O*-methylrhamnose and 3,4-di-*O*-methylrhamnose. It must be pointed out that no deoxytalose was detected. The absolute configuration of the sugars was determined after O-demethylation by boron trichloride (Bonner et al., 1960) according to the method of Gerwig et al. (1978): rhamnose resulting from the demethylation of the two sugars was converted into its (−)2-butylglycoside. Capillary GC of the TMS derivative showed a peak identified as (−)2-butyl-L-rhamnoside, indicating that both the partially methylated rhamnoses belonged to the L-series.

A reductive β-elimination at the threonine site would liberate the O-linked sugars as their corresponding alditols (Anderson et al., 1964). From both mycosides, 3-*O*-methylrhamnitol was produced after this treatment and identified by GC–MS of its trimethylsilyl derivative. Acid hydrolysis of the resulting glycopeptidolipid moiety afforded alanine, phenylalanine, and alaninol, but *allo*-threonine was no longer detected. Instead, an amino acid migrating as 2-aminobutyric acid was liberated. Thus, 3-*O*-methylrhamnose was linked to the *allo*-threonine side chain in both mycosides.

FAB Mass Spectrometry of I and II C-Mycosides. Although all attempts to measure the molecular weight of C-mycosides by fast atom bombardment had failed up to now (Jardine et al., 1989), we succeeded using alkali ion cationization in a *m*-nitrobenzyl alcohol/sodium iodide matrix. Very abundant sodium-cationized molecular ions were observed with an ionic signal lasting for more than half an hour. The use of potassium iodide, instead of sodium iodide, induced a 16-mass-unit shift, which indicated the presence of a single alkali ion per cationized molecular ion.

The FAB spectrum of mycoside I showed two main sodium cationized ions at m/z 1333.6 and 1361.6 (Figure 2 inset), while that of mycoside II exhibited two abundant ions at m/z 1319.7 and 1347.7 (Figure 3 inset). The mass measurement accuracy was estimated as ±0.2 mass unit. Additional minor ions, two mass units lower, indicated the presence of a compound with one ethylenic bond in the acyl chain, a result supported by ^{1}H NMR spectroscopy.

Using the same sodium-spiked matrix, the FAB spectra of the product resulting from reductive β-elimination exhibited a more complex pattern (inset of Figure 4). The expected cationized molecular ions observed at m/z 1143.8 and 1171.7 could result from the loss of a 3-*O*-methylrhamnosyl unit (178 Da) and the gain of two hydrogen atoms in the threonine residue. Incomplete reduction of the conjugated double bond gave additional compounds whose cationized molecular ions appeared at m/z 1141.7 and 1169.7. Finally, another ion pair resulted from an additional loss of methanol from the β-methoxylated fatty acyl chain with a partial reduction of the conjugated double bond (m/z 1111.8 and 1109.8, respectively).

Sequencing of the Constituents by MIKE Spectrometry. Useful structural data were obtained by the examination of the metastable ion spectra (MIKE spectrometry) from selected cationized ions of the intact and β-eliminated molecules.

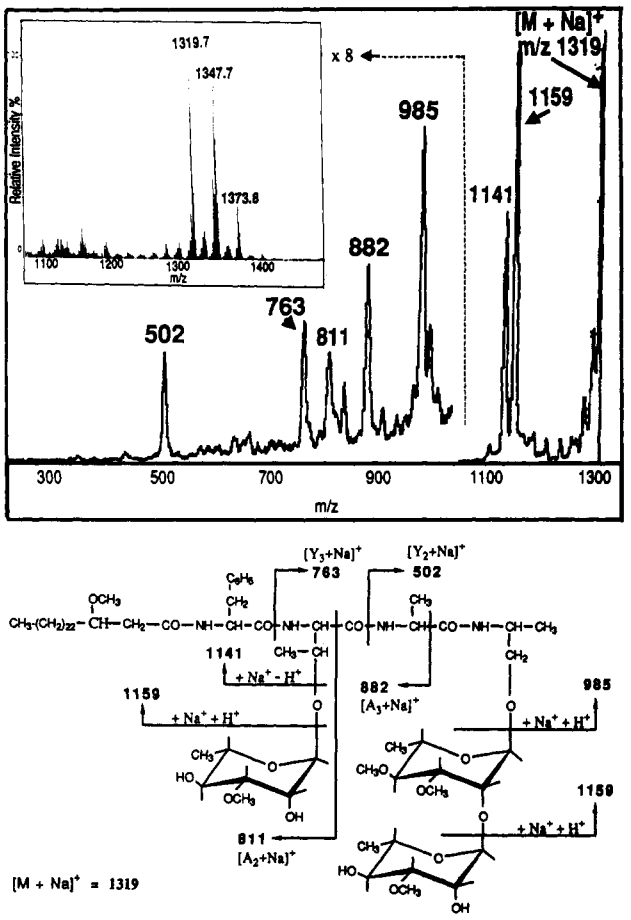

FIGURE 3: FAB mass spectrum of mycoside II (inset) and FAB MIKE spectrum from the sodium-cationized ion at m/z 1319 with the corresponding structure.

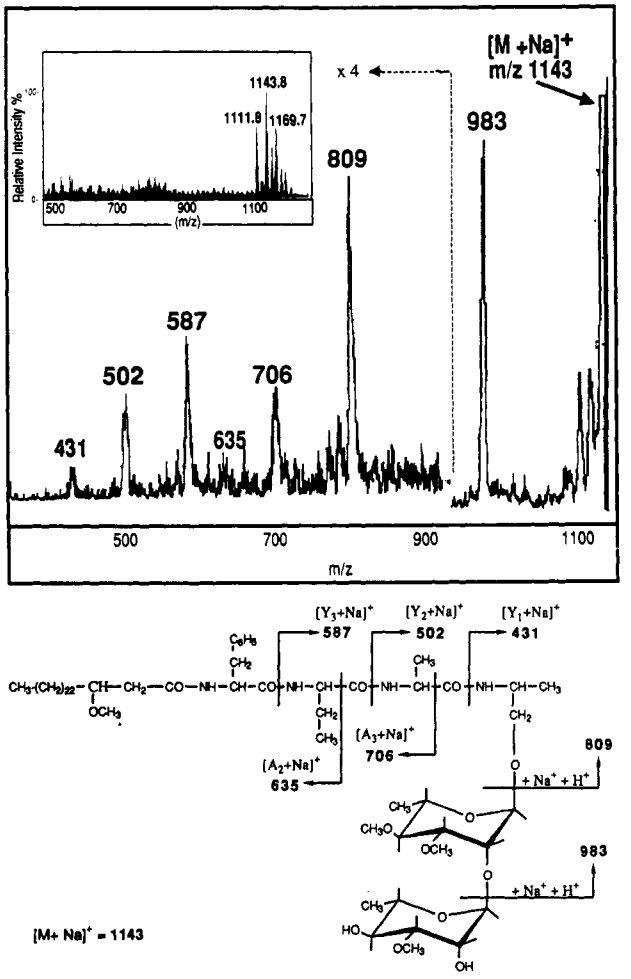

FIGURE 4: Structure and FAB MIKE spectrum from the ion at m/z 1143 of the alkali β-eliminated mycoside II. The inset shows the corresponding FAB mass spectrum.

Figure 3 shows the spectrum from the sodium-cationized ion of intact mycoside II at m/z 1319. It exhibits a very abundant loss of 160 mass units, giving m/z 1159, which was attributed to the loss of an anhydro-3-*O*-methylrhamnose unit. The peak at m/z 985 corresponded to the loss of an anhydrodisaccharide unit containing mono- and di-*O*-methylrhamnosyl moieties. The peak at m/z 502 was due to the cleavage at the threonine–alanine amide linkage, with charge retention on the disaccharide containing part of the cationized molecule: $[Y_2 + Na]^+$ according to the Roepstorff nomenclature (Roepstorff, 1984). Similarly, cleavage between the phenylalanine and threonine residues gave m/z 763 $[Y_3 + Na]^+$. Moreover, peaks at m/z 811 and 882 were attributed to cleavages between amino acid residues with charge retention on the fatty acyl containing moiety ($[A_2 + Na]^+$ and $[A_3 + Na]^+$, respectively). These assignments were confirmed by the MIKE spectrum of the homologous ion at 28 mass units higher (m/z 1347), in which only the $[A_2 + Na]^+$ and $[A_3 + Na]^+$ ions were shifted 28 mass units up (m/z 839 and 910).

A similar fragmentation pattern was observed in the MIKE spectra from the sodium-cationized species of the β- eliminated mycosides. Figure 4 shows the spectrum from m/z 1143.7 of the β-eliminated mycoside II. The loss of anhydro-3-*O*-methylrhamnose and of the anhydrodisaccharidyl moiety was still observed (m/z 983 and 809, respectively), and the cleavage between alanine and aminobutyric residues $[Y_2 + Na]^+$ still gave m/z 502. However, the $[Y_3 + Na]^+$ ion was shifted down by 176 mass units (m/z 587), which confirmed the loss of a 3-*O*-methylrhamnose residue from the threonyl side chain. Similarly, the $[A_2 + Na]^+$ and $[A_3 + Na]^+$ ions were shifted down by the same number of mass units (m/z 635 and 706).

By comparing the MIKE spectra of both the intact and the β-eliminated mycosides, it was observed that the sugar located on the threonine residue was lost both as an intact molecule and as an anhydrosugar, whereas the sugar residue at the nonreducing end of the disaccharide unit located on the alaninol residue was lost only as an anhydrosugar. Thus, the daughter ion spectrum from m/z 1319 of mycoside II (Figure 3) showed an ion at m/z 1141 (loss of 178 mass units corresponding to an intact 3-*O*-methylrhamnose), whereas the corresponding fragment was absent in the spectrum from the β-eliminated mycoside (Figure 4). This observation allowed the two sugar residues to be discriminated.

In summary, the mass spectrum data from mycoside II gave the following information: (i) the peptide sequence is identical to that of other already described C-mycosides, namely *N*-acyl-Phe-*allo*Thr-Ala-alaninol; (ii) 3-*O*-methylrhamnose is O-linked to the *allo*-threonine side chain; (iii) the disaccharide unit is O-linked to alaninol as 3-*O*-methylrhamnosyl 3,4-di-*O*-methylrhamnoside; (iiii) two homologous β-methoxylated fatty acids, differing in their chain length, are N-linked to the phenylalanine residue.

A parallel study was made on mycoside I (Figure 2). The MIKE spectrum from the cationized molecular ions presented the same fragmentation pattern as that from mycoside II. Comparison of the spectra from m/z 1333 (mycoside I) and m/z 1319 (mycoside II) enabled an easy interpretation. Both the $[Y_3 + Na]^+$ and $[Y_2 + Na]^+$ ions (m/z 777 and 516, respectively) were shifted up by 14 mass units. This indicates that 3-*O*-methylrhamnose is still linked to the threonine residue

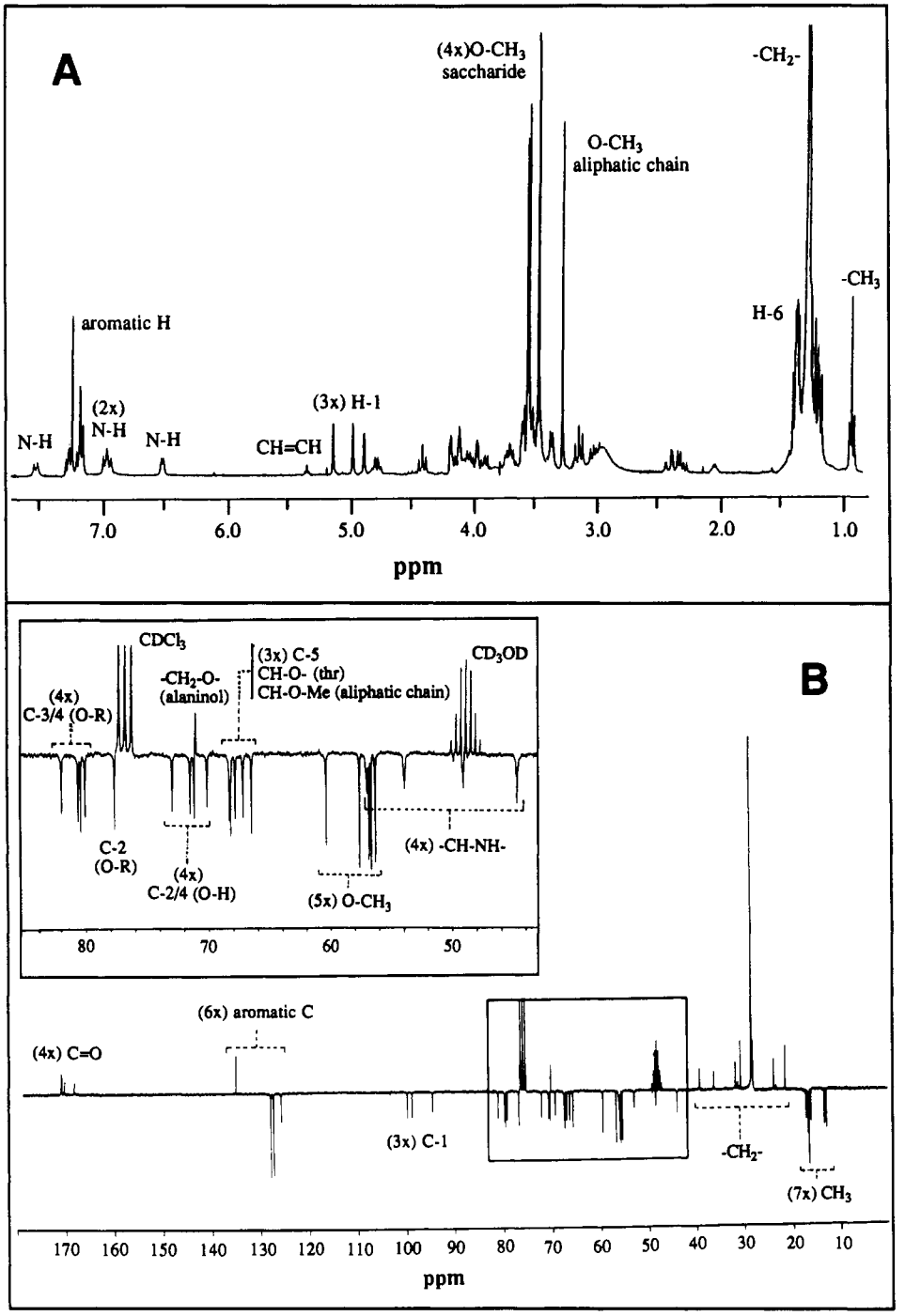

FIGURE 5: NMR spectra of mycoside II. (A) ^1H NMR spectrum in $CDCl_3$; (B) J-modulated ^{13}C spectrum in $CDCl_3/CD_3OD$.

and that the additional methyl group is not borne on the fatty acyl moiety. This was corroborated by the $[A_2 + Na]^+$ and $[A_3 + Na]^+$ ions, which were present at the same m/z value, namely, m/z 811 and 882. Similarly, the ion resulting from the loss of the disaccharide unit linked to alaninol was left unchanged at m/z 985. Thus, the additional methyl group was located on the disaccharide moiety, which is a 3,4-di-*O*-methylrhamnosyl 3,4-di-*O*-methylrhamnoside.

Unlike for mycoside II, the loss of the terminal monosaccharide units could not be observed in the MIKE spectrum because of the insufficient mass resolution. Thus, although the ion at m/z 1173 (Figure 2) might be attributed to the loss of an anhydromono-*O*-methylrhamnose from the threonine residue, the very abundant ion at m/z 1157 did not correspond exactly either to the loss of anhydro-di-*O*-methylrhamnose (which would give m/z 1159) or to that of an intact mono-*O*-methylrhamnose (which would give m/z 1155). In fact, it corresponded to the average mass of the two ions due to the partial superimposition of their peaks. This was also supported by the enhanced abundance of the observed peak.

In conclusion to this mass spectrum study, C-mycosides I and II have a very similar structure, comprising a common acylpeptide core, 3-*O*-methylrhamnose linked to the *allo*-threonine side chain, and a disaccharide unit glycosidically linked to alaninol. The two glycolipids differ in the nature of the sugar at the nonreducing end, i.e., 3-*O*-methylrhamnose in mycoside II and 3,4-di-*O*-methylrhamnose in mycoside I.

NMR Analyses of C-Mycosides I and II. (*a*) *The Number of Sugar and Amino Acid Units.* High-resolution ^{13}C and ^1H NMR spectra of both native compounds showed the presence of three sugar units per molecule in agreement with the mass spectrum assignments. They exhibited their anomeric reso-

nances at δ_H 4.7–5.3 and δ_c 95.6–100.9 (Figure 5). Additionally, the spectra showed four amide groups exhibiting exchangeable NH proton signals between δ_H 6.5 and 7.6, the corresponding CH–NH resonances at δ_c 45–58 and their four carbonyl signals at δ_c 168–172. Moreover, the ^1H and ^{13}C spectra of compound I showed the presence of six OCH_3 signals (δ_H 3.2–3.5), whereas those of compound II (Figure 5) contained only five such resonances. One of these signals, the most shielded one in the ^1H NMR spectra, has been assigned to the resonance of a methoxyl group located on the fatty acyl moiety (Daffé et al., 1988). The data obtained here confirm that compound I contains two residues of 3,4-di-*O*-methylrhamnose and one residue of 3-*O*-methylrhamnose and that compound II contains two residues of the latter sugar and one of the former.

(*b*) *Anomeric Configuration of the Rhamnosides.* The anomeric configuration of the three rhamnosyl units could not be established by ^1H NMR because some of the observed chemical shifts (δ_H 4.7–5.0) were consistent with either α or β configurations (Kasai et al., 1979). Moreover, the $J_{1,2}$ values are of no use in the determination of the anomeric configuration in the *manno* series since for both anomers the anomeric proton signals appear as a slightly broadened singlet with similar coupling constant values. In contrast, the J_{C-H} value was shown to be useful in differentiating the α and the β anomers (Kasai et al., 1979). For both mycosides, the J_{C-H} coupling constant values were found equal to 171 ± 1 Hz establishing an α configuration for the three rhamnosides. The anomeric configuration of the carbohydrate residue directly linked to *allo*-threonine in other C-mycosides had not been previously determined.

(*c*) *Nature of the Linkage in the Dirhamnoside.* From the sugar composition of the diglycoside linked to the peptidolipid, it was obvious that in both compounds the terminal sugar residue was attached to position 2 of a 3,4-di-*O*-methylrhamnose unit which in turn was linked to alaninol. This conclusion was also supported by the analysis of the *J*-modulated ^{13}C NMR spectra, which showed, for both mycosides, a signal at δ 77.9, corresponding to the expected resonance of the O-substituted C-2 of a rhamnosyl unit (Pozsgay et al., 1981). The two unsubstituted C-2 in both compounds had their resonances at δ 70.9 and 71.6 (Figure 5). The spectra of compounds I and II differed, however, in the number of signals assignable to the resonances of methoxylated C-3 and C-4: five signals were observed in the ^{13}C NMR spectrum of compound I instead of four for compound II, in agreement with the proposed structure.

Conclusion

The present results substantiate evidence that the glycopeptidolipids of *M. fortuitum* biovar. *peregrinum* differ from all previously described C-mycosides: although the same basic structure composed of an invariant core, fatty acyl-D-Phe-D-*allo*Thr-D-Ala-alaninol-*O*-(3,4-di-*O*-methylrhamnosyl), is present, neither 6-deoxytalosyl nor its methylated derivatives are found. Instead a 3-*O*-methylrhamnosyl is directly bound to the *allo*-threonine residue. Moreover, the disaccharide unit is linked to the alaninol residue of the core. This novel distribution of saccharides in C-mycosides, as well as in a recently described glycopeptidolipid (which is not a C-mycoside) from *Mycobacterium xenopi* (Rivière & Puzo, 1991), points out the diversity of the glycosylation systems occurring in mycobacteria.

This unusual structure seems to be characteristic of *M. fortuitum* biovar. *peregrinum* since it was present in the three examined strains. Our results agree with the proposal that this biovariant is a distinct species of *M. fortuitum* (Baess, 1982; Tsang et al., 1984).

M. fortuitum peregrinum present serological cross-reactions with the two subspecies of *M. chelonae* (Tsang et al., 1984), suggesting that while their C-mycosides have a different structure (dissimilar TLC profiles), they share a common epitope. Until now, the epitope in C-mycosides has been assumed to be in the specific oligosaccharide linked to 6-deoxytalose (Brennan, 1988). As the C-mycosides described herein differ from this point of view, the recognized determinant in the antigenic reactions associated to them remains to be determined.

In this study, important structural information of the intact glycopeptidolipids of *M. fortuitum* biovar. *peregrinum* was successfully obtained by mass spectrometry. Alkali ion cationization allowed FAB-MS to determine the molecular weight of the C-mycosides without any derivatization. Moreover, MIKE spectra analysis allowed the determination of the complete sequence of the molecule. FAB-MS–MS proved to be a practical and rapid approach for the structural elucidation of mycobacterial glycopeptidolipids.

Acknowledgments

We are grateful to Dr. H. L. David and Dr. V. Lévy-Frébault (Institut Pasteur, Paris) for providing strains of *M. fortuitum-chelonae* complex. P. Winterton revised the manuscript.

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Effect of Calcium on the Dynamic Behavior of Sialylglycerolipids and Phospholipids in Mixed Model Membranes. A ^2H and ^{31}P NMR Study†

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Received May 14, 1991; Revised Manuscript Received August 12, 1991

ABSTRACT: DTSL, a sialic acid bearing glyceroglycolipid, has been deuteriated at the C3 position of the sialic acid headgroup and at the C3 position of the glycerol backbone. The glycolipid was studied as a neat dispersion and in multilamellar dispersions of DMPC (at a concentration of 5–10 mol % relative to phospholipid), using ^2H and ^{31}P NMR. The quadrupolar splittings, $\Delta\nu_Q$, of the headgroup deuterons were found to differ in the neat and mixed dispersion, suggesting different headgroup orientations in the two systems. In DTSL–DMPC liposomes, two quadrupolar splittings were observed, indicating that the axial and equatorial deuterons make different angles with respect to the axis of motional averaging. The splittings originating from the equatorial and axial deuterons were found to increase and decrease with increasing temperature, respectively, indicating a temperature-dependent change in average headgroup orientation. Longitudinal relaxation times, T_{1Z}, were found to be short (3–6 ms). The field dependence of T_{1Z} suggests that more than one motion governs relaxation. At 30.7 MHz a T_{1Z} minimum was observed at approximately 40 °C. At 46.1 MHz the T_{1Z} values were longer and increased with temperature, demonstrating that the *dominant* rigid-body motions of the headgroup at this field are in the rapid motional regime ($>10^8$ s^{-1}). DTSL labeled at the glycerol C3 position was studied in DMPC multilamellar dispersions. Whereas two quadrupolar splittings have been observed for other glycolipids labeled at this position, only a single $\Delta\nu_Q$ was observed. This shows that the orientation of the C2–C3 segment of DTSL relative to the bilayer normal differs from that of other glycolipids. T_{1Z} values were short (3–7 ms) and increased with temperature, demonstrating that motion is in the rapid motional regime. Quadrupolar splittings and T_{1Z} values were also obtained for the headgroup-labeled DTSL in the presence of 5 and 50 mM Ca^{2+}. As the Ca^{2+} concentration was increased, the ratio of outer to inner quadrupolar splittings increased, suggesting a small change in headgroup orientation. From the longitudinal relaxation times the rate of the dominant headgroup motion(s) appeared to decrease. The DTSL–DMPC liposomes were also studied by ^{31}P NMR and by two-dimensional solid-state ^{31}P NMR, the latter technique giving information on the orientational exchange of phospholipid molecules. DTSL appeared to alter the headgroup orientation of DMPC and also to increase the rate of orientational exchange. The latter most likely reflects an increase in the rate of lateral diffusion of the phospholipid. Ca^{2+} was found to reverse both of these effects partially.

Glycolipids are an important class of lipid found in animals, plants, and microorganisms (Gigg, 1980). Glycolipids can be divided into two distinct classes: glycosphingolipids, the carbohydrate-bearing lipids of animal cells, and glycoglycerolipids, which occur in plants (Quinn & Williams, 1978), bacteria (Rogers et al., 1980), and mycoplasma (Wieslander et al., 1978). Glycosphingolipids have been implicated as specific recognition sites for a variety of important cellular processes, including immune recognition (Hakomori, 1984b), cell–cell interaction (Critchly et al., 1979), and binding of viruses and proteins (Fishman & Brady, 1976). Both classes of lipids have the potential to modulate membrane physical properties (Curatolo, 1987a). This is particularly clear for such organisms as *Acholeplasma laidlawii*, where the glycoglycerolipids are the major constituent lipid of the cellular membrane (Rottem, 1980; Razin, 1980). The biological roles of glycolipids depend not only on the structure of the surface carbohydrate but also on its accessibility to external factors. Thus, the spatial organization of the membrane surface, which will be determined by such factors as the orientation, ordering, and dynamics of the carbohydrate residues, is expected to be of critical importance in understanding cell-surface phenomena.

^2H NMR[1] is a powerful technique for studying the anisotropic molecular environment of biological and model mem-

† This work was partially supported by an NSERC research grant (to R.R.).
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[1] Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DTSL, 1,2-di-*O*-tetradecyl-3-*O*-α-D-sialyl-*sn*-glycerol; [$^2H_2$-NeuAc]DTSL, 1,2-di-*O*-tetradecyl-3-*O*-α-D-[3,3-$^2H_2$]sialyl-*sn*-glycerol; [$^2H_1$-3G]DTSL, 1,2-di-*O*-tetradecyl-3-*O*-α-D-sialyl-*sn*-[3-$^2H_1$]glycerol; β-DTGL, 1,2-di-*O*-tetradecyl-3-*O*-β-D-glucopyranosyl-*sn*-glycerol; α-DTML, 1,2-di-*O*-tetradecyl-3-*O*-α-D-mannopyranosyl-*sn*-glycerol; NMR, nuclear magnetic resonance.

0006-2960/91/0430-10542$02.50/0