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IDENTIFICATION OF PARTIALLY METHYLATED METHYL GLYCOSIDES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF TRIMETHYLSILYL DERIVATIVES

APPLICATION TO MYCOBACTERIAL GLYCOLIPID ANTIGEN ANALYSIS

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SUMMARY

Partially methylated glycosides play an important role in the stereospecificity of glycolipid antigen–antibody binding reactions. A method for the structural determination of partially methylated methyl glycosides is described. The proposed method, which is an alternative to that using alditol acetates, consists in the analysis of trimethylsilyl glycoside derivatives by gas chromatography–mass spectrometry in the electron impact (EI) mode. Seven partially methylated methyl rhamnopyranosides were synthesized, then purified by high-performance liquid chromatography. After trimethylsilylation, their EI mass spectra showed characteristic fragmentation patterns allowing the determination of the position and the number of methoxy groups in the sugar ring. This method was successfully applied to the structural elucidation of partially methylated sugars contained by a quantitatively minor phenolic glycolipid antigen isolated from *Mycobacterium kansasii*. Moreover, the carbons involved in the glycosidic linkages of the glycolipid tetrasaccharide moiety were also identified by this method after permethylation, methanolysis and trimethylsilylation.

INTRODUCTION

Pathogenic mycobacteria are the etiologic agents of certain human[1] and animal[2] infections. These bacteria are characterized by abundant and complex lipid-containing cell walls[3]. Their specific immunoreactive glycolipids have been classified into three families: the polar C-mycoside glycopeptidolipids, the phenolic glycolipids and the

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trehalose-containing oligosaccharides[4]. Their terminal disaccharide, located at the non-reducing end of the oligosaccharide part, constitutes the epitope[4]. It is mainly composed of unique combinations of partially O-methylated mannose, glucose, rhamnose and fucose residues. Also, particular O-methylated sugars such as 2,6-dideoxy-4-O-methyl-α-D-arabinohexopyranoside[5,6] and 4,6-dideoxy-2-O-methyl-3-C-methyl-4-(2-methoxypropionamido)-α-L-mannohexopyranose have been described[7].

It is well known that a minor structural modification of the epitope, such as a change in the O-methylation site, decreases the antigen linkage to the immune serum[4]. So, therefore, when attempting artificial antigen synthesis, the glycolipid epitope structure must be determined exactly[8].

One of the problems with this kind of structural elucidation of glycolipids is the location of the methoxy groups on the sugar skeleton. The method currently applied to resolve this problem consists in the alditol acetate derivatization of the sugars, after glycolipid hydrolysis, followed by gas chromatographic–electron impact mass spectrometric (GC–EI-MS) analysis[9]. However, Matsubara and Hagashi[10] noticed that the structures of partially di-O-methylated methyl glucosides can be determined by GC–EI-MS analysis of their trimethylsilyl (TMS) ether derivatives.

This paper shows that this strategy can be extended to methyl deoxyhexopyranosides such as mono- and di-O-methylated fucoside and rhamnoside and to mono-O-methyl glycosides. This method was successfully applied to the determination of the sugar type after methanolysis of phenolic glycolipids isolated from *M. kansasii*. Moreover, after glycolipid permethylation, followed by methanolysis, analysis by GC–EI-MS of the methyl glycoside TMS derivatives allowed the position of glycosidic bond to be determined.

EXPERIMENTAL

Synthesis and purification of partially O-methylated methyl α-L-rhamnopyranosides

The O-methylation, by Purdie and Truine's method[11], of methyl α-L-rhamnopyranoside mainly gives mono- and di-O-methylated compounds and the unmethylated compound. By silicic acid chromatography, this mixture was resolved into three fractions using an eluent gradient of methanol in chloroform up to 10% chloroform.

From the mono-O-methylated fraction the production of pure 2-O-methyl, 3-O-methyl and 4-O-methyl sugars required the use of two different high-performance liquid chromatographic (HPLC) semi-preparative columns. The first system was a silica Spherisorb (5 μm) column (300 mm × 7.5 mm I.D.) with light petroleum (b.p. 50°C)–acetone (3:5) as mobile phase at a flow-rate of 2 ml/min and a sample amount of 4 mg per 50 μl. Using these conditions, only the methyl 2-O-methyl-rhamnopyranoside compound was separated. The second step, allowing the separation of the 3-O-methyl from the 4-O-methyl compound, utilized a reversed-phase Spherisorb C_{18} (5 μm) column (300 mm × 7.5 mm I.D.) with water–ethanol (96:4) as mobile phase at a flow-rate of 2 ml/min and a sample amount of 4 mg per 50 μl.

From the di-O-methylated fraction the 2,4-, 3,4- and 2,3-isomers were purified in one step (Fig. 1) using the following separation column system: silica Spherisorb (5 μm) column (300 mm × 7.5 mm I.D.) with light petroleum–acetone (5:2) as mobile

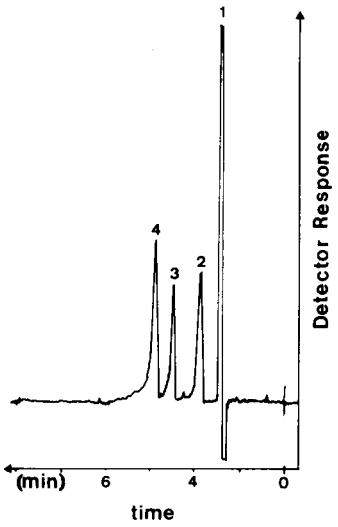

Fig. 1. Separation of methyl di-O-methyl-α-L-rhamnopyranosides. See text for details. 1 = Solvent; 2 = methyl 2,4-di-O-methyl-α-L-rhamnopyranoside; 3 = methyl 3,4-di-O-methyl-α-L-rhamnopyranoside; 4 = methyl 2,3-di-O-methyl-α-L-rhamnopyranoside.

phase at a flow-rate of 1.3 ml/min and a sample amount of 3 mg per 50 μl. These compounds were identified by EI-MS of their alditol acetate derivatives.

Synthesis and purification of the various mono-O-methyl isomers of methyl α-D-glucopyranosides

A monomethylated fraction of methyl α-D-glucopyranoside was obtained by partial methylation using Purdie and Truine's method[11] followed by chromatography on a silicic acid column. The 2-O-methyl, 3-O-methyl and 4-O-methyl derivatives were purified from this fraction in one step using HPLC with an NH_2-bonded analytical column (Fig. 2). The conditions were as follows: NH_2-Spherisorb (5 μm) column (250 mm × 4.6 mm I.D.); mobile phase, acetonitrile–water (95:5); flow-rate, 1 ml/min; sample size, 1 mg per 20 μl.

The compounds were identified by direct probe EI-MS of their alditol acetates. The 6-O-methyl glucoside was not detected in this mixture, and it was specifically synthesized[12].

Gas chromatography–mass spectrometry

GC–MS was performed using a 2-m column of 1% OV-1 and a Nermag Model R 10/10 quadrupole mass spectrometer connected to a PDP-8M computer and graphic output. The GC–MS conditions were as follows: injection temperature, 180°C; oven temperature, increased from 100 to 200°C at 5°C/min; separator temperature, 250°C; ion source temperature, 200°C; electron impact energy, 70 eV.

Direct EI-MS analyses were performed on a Finnigan-MAT 311 A instrument.

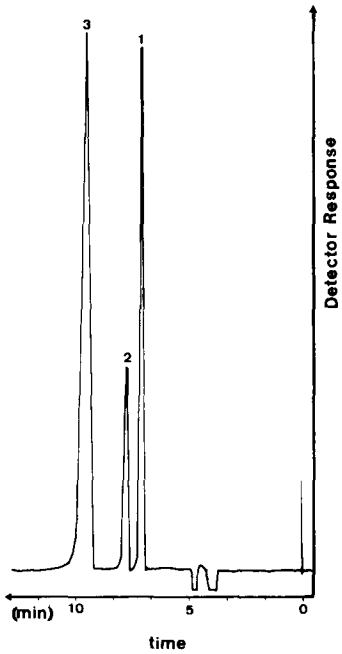

Fig. 2. Separation of methyl O-methyl-α-D-glucopyranosides. See text for details. 1 = Methyl 3-O-methyl-α-D-glucopyranoside; 2 = methyl 4-O-methyl-α-D-glucopyranoside; 3 = methyl 2-O-methyl-α-D-glucopyranoside.

RESULTS AND DISCUSSION

The EI-MS fragmentation patterns of methyl per-O-TMS-α-D-glucopyranoside were established by Dejongh *et al.*[13]. The EI mass spectrum mainly shows three intense peaks at *m/z* 133 $(TMS{-}O{-}CH{=}O{-}CH_3)^+$, *m/z* 204 $(TMS{-}O{-}CH{=}CH{-}O{-}TMS)^{+\cdot}$ and *m/z* 217 $(TMS{-}O{-}CH{=}CH{-}CH{=}O{-}TMS)^+$. The fragment ion of *m/z* 204 mainly contained C-2 and C-3 carbon atoms while that of *m/z* 217 mainly arose from cleavage of the C-1–C-2 and C-4–C-5 bonds. These fragmentation patterns were preserved when the TMS derivatives of methyl deoxypyranosides were analysed by EI-MS. Hence from these results we can use the ions of *m/z* 133, 204 and 217 and also their partially methylated ions shifted down by 58 or 116 a.m.u. as reported ions for the location of the methoxy group on the sugar ring.

EI-MS of partially O-methylated methyl-6-deoxypyranosides

Mass spectra of methyl TMS ethers of 2-O-methyl-, 3-O-methyl- and 4-O-methyl-rhamnopyranosides are shown in Fig. 3. The 2-O-methyl compound is characterized by a base peak at *m/z* 146 (*m/z* 204 → *m/z* 146) whereas the 4-O-methyl compound is distinguishable from the latter by the presence of a base peak at *m/z* 204. The 3-O-methyl compound is unambigously identified by the presence of two intense signals at *m/z* 146 (100%) and *m/z* 217. This assignment is also supported by the signal at *m/z* 75, which occurs from the shift of the fragment ion of *m/z* 133, confirming that the ion of *m/z* 133 resulted from the transfer of the C-3 trimethylsiloxy group to the anomeric carbon.

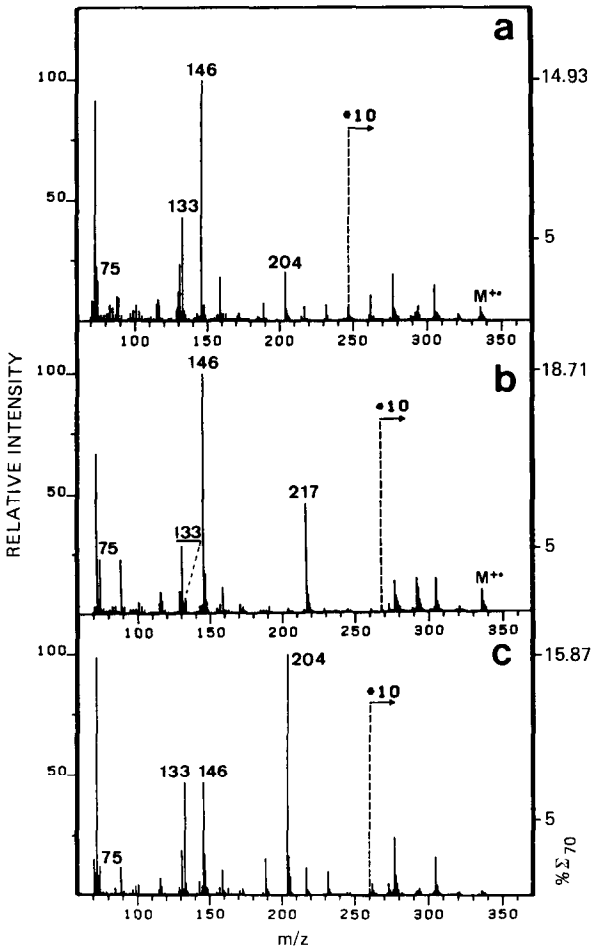

Fig. 3. Direct probe EI mass spectra of (a) methyl 2-O-methyl-, (b) 3-O-methyl- and (c) 4-O-methyl-pertrimethylsilyl-α-L-rhamnopyranosides. Molecular ions were not observed when the sample was analysed by GC–MS.

The mass spectra of the methyl TMS ethers of 2,3-, 2,4- and 3,4-di-O-methyl-α-L-rhamnopyranosides, presented in Fig. 4, are drastically different. The mass spectrum of the 2,3-di-O-methyl compound is characterized, as expected, by an intense signal at *m/z* 88 (base peak), whereas in the 2,4-di-O-methyl sugar the base peak corresponds to the signal at *m/z* 146. This signal is also the base peak in the mass spectrum of the 3,4-di-O-methyl compound. However, the latter compound can be distinguished from the 2,4-di-O-methyl compound by the presence of two other intense peak at *m/z* 159 (55%) and *m/z* 75 (50%).

This method has been succesfully applied to the location of the methoxy group in methyl mono-O-methyl- and di-O-methyl-α-L-fucopyranosides synthesized as described for the partially methylated rhamnopyranosides.

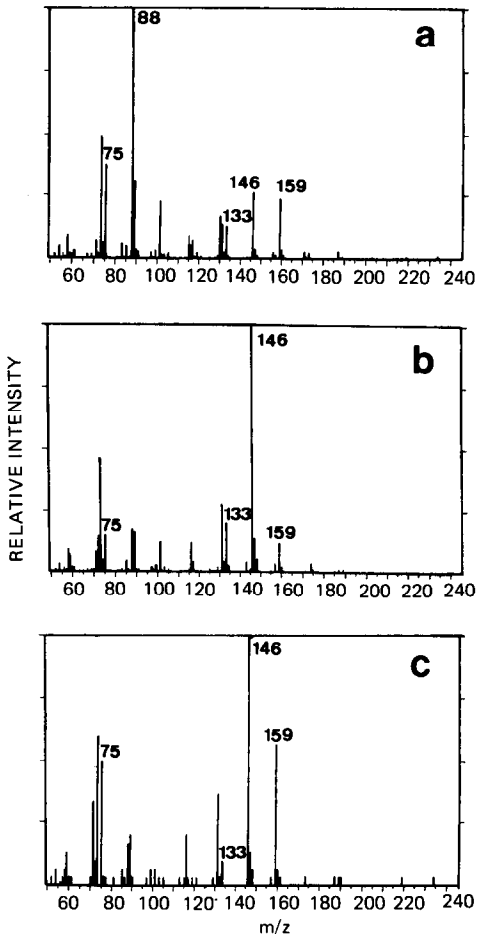

Fig. 4. GC–EI-MS mass spectra of methyl (a) 2,3-, (b) 2,4- and (c) 3,4-di-O-methyl-pertrimethylsilyl-α-L-rhamnopyranosides.

EI-MS of methyl mono-O-methyl glucosides

The EI mass spectra of the TMS ethers of the 2-, 3-, 4- and 6-O-methyl methyl-α-D-glucopyranosides (Fig. 5) show characteristic differences based on the relative abundances of the reported ions of *m/z* 133, 204 and 217 and their O-methylated analogues of *m/z* 75, 146 and 159. In both the 2-O-methyl and 3-O-methyl compounds the base peak corresponds to the signal at *m/z* 146. The 3-O-methyl sugar can be unambigously identified from the 2-O-methyl compound by the presence of an intense signal at *m/z* 217 (90%) and by the signal at *m/z* 75, whose intensity is higher than that of the peak at *m/z* 133. The 6-O-methyl compound is characterized by a base peak at *m/z* 204. For the 4-O-methyl compound the base peak is observed at *m/z* 73 and all the reporter ions are present, the most intense corresponding to *m/z* 204 (60%).

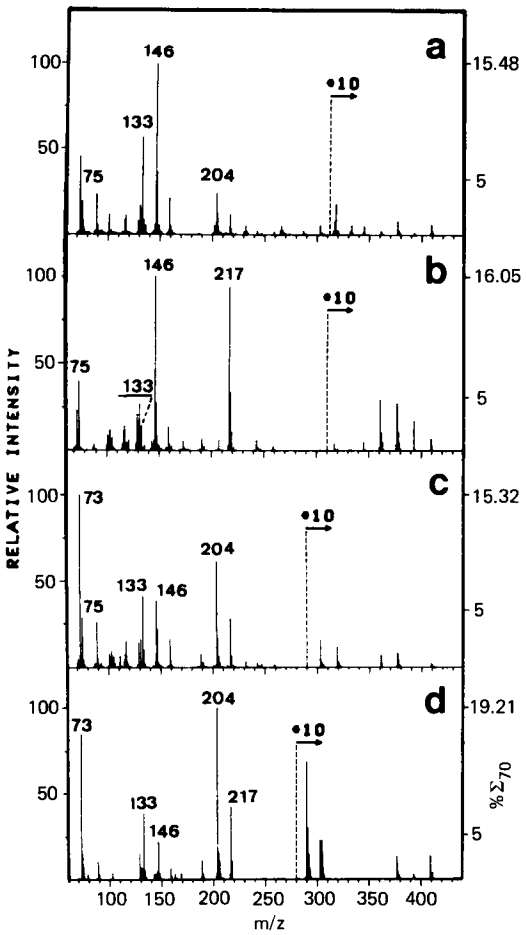

Fig. 5. Direct probe EI mass spectra of methyl (a) 2-, (b) 3-, (c) 4- and (d) 6-O-methyl-pertrimethylsilyl-α-D-glucopyranosides.

Application to the structural elucidation of the sugar in a minor phenolic glycolipid (Phe GL K-II) isolated from Mycobacterium kansasii

In previous work[13] the following Phe GL K-II structure was proposed: 2,4-di-O-methyl-D-Manp(1α → 3)-4-O-acetyl-2-O-methyl-L-Fucp(1α → 3)-2-O-methyl-L-Rhap(1α → 3)-2,4-di-O-methyl-L-Rhap1α → phenolphthiocerol dimycocerosate. However, the determination of the position of the methoxy groups on the sugar ring and the carbons involved in the glycosidic linkage of the Phe GL K-II sugar part based on the EI-MS analysis of the methyl glycosides was not described. This paper reports an alternative to the alditol acetate technique which seems to be more appropriate for the structural elucidation of the oligosaccharidic part of glycolipids.

Phe GL K-II methanolysis (2 *M* hydrochloric acid in methanol, 80°C, 1 h) yielded methyl glycosides and the aglycone. This mixture was separated into two fractions by chromatography on a Sep-Pak C_{18} cartridge with methanol followed by chloroform as the eluent. The methanol fraction contained the methyl glycosides,

TABLE I

RELATIVE ABUNDANCES OF THE REPORTER IONS (*m/z* 75–217) OBSERVED IN THE GC–EI-MS SPECTRA OF THE TMS METHYL GLYCOSIDE DERIVATIVES YIELDED BY THE METHANOLYSIS OF THE PHENOLIC GLYCOLIPID

A dash indicates an intensity lower than 5%. The sugar assignment is also based on the GC retention times and from the fragment ions of higher masses.

Assignment	*Relative abundance (% base peak)*						
	m/z 217	*m/z 204*	*m/z 159*	*m/z 146*	*m/z 133*	*m/z 88*	*m/z 75*
2,4-Di-O-methyl-α-rhamnopyranoside	–	–	12	100	20	18	12
2-O-Methyl-α-fucopyranoside	–	17	15	100	31	7	15
2-O-Methyl-α-rhamnopyranoside	–	20	18	100	42	10	16
2,4-Di-O-methyl-α-mannopyranoside	–	–	17	100	37	18	27

which were trimethylsilylated and analysed by GC–EI-MS. Four kinds of methyl glycoside were observed from the gas chromatogram and their respective EI mass spectra, whose characteristic fragment ions are summarized in Table I, allowed the identification of 2,4-di-O-methyl-deoxypyranoside, 2-O-methyl-deoxypyranoside, 2-O-methyldeoxypyranoside and 2,4-di-O-methyl-hexopyranoside. From their retention times, these partially methylated sugars were assigned to rhamnose, rhamnose, fucose and mannose, respectively.

In order to determine the carbons involved in the glycosidic bonds, Phe GL K-II was permethylated by Ciucanu and Kerech's method[15]. The permethylated glycolipid was methanolysed and the resulting methyl glycosides from the aglycone were purified as described above. After trimethylsilylation and GC–MS only the C-3 hydroxy group of fucoside and rhamnoside were found to be trimethylsilylated, supporting the existence of a unique 1 → 3 glycosidic bond in the Phe GL K-II tetrasaccharide.

From these results, we conclude that the analysis of the fragment ions of *m/z* 75, 88, 133, 146, 159, 204 and 217 unambigously allows the location of methoxy groups in partially methylated sugars. Moreover, this method is a powerful tool for the identification of glycosidic linkages. The proposed strategy, unlike the widely used alditol acetate method, does not require the reduction step and thus salt elimination. Moreover, methanolysis avoids the partial loss of volatile O-methylated sugars which could occur during hydrolysate concentration[16,17].

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