Isolation and Characterization of an *O*-Methylglucose-containing Lipopolysaccharide Produced by *Nocardia otitidis-caviarum*

By MARIE-THÉRÈSE POMMIER* AND GEORGES MICHEL

Laboratoire de Biochimie Microbienne, Université Claude Bernard, Lyon 1, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

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The low- M_r lipopolysaccharide produced by *Nocardia otitidis-caviarum* is composed of 6-*O*-methyl-D-glucose (11 mol), D-glucose (8 mol) and 3-*O*-methyl-D-glucose (1 mol). Glyceric acid was also found as a constituent. Methylation and periodate oxidation analyses suggested that the backbone was formed by (1→4)-linked glucose and *O*-methylglucose residues. Glucose (1 mol) and 6-*O*-methylglucose (1 mol) served as branching points. Acetic acid was the major acyl substituent esterifying glucose residues. The lipopolysaccharide was isolated from the cytoplasmic fraction. Several other *Nocardia* strains were examined; all possessed the same lipopolysaccharide in their extracts.

INTRODUCTION

Polysaccharides containing *O*-methylhexoses have been found in various members of the Actinomycetales. 3-*O*-Methylmannose polysaccharides were isolated from mycobacteria (Gray & Ballou, 1971; Maitra & Ballou, 1977) and from *Streptomyces griseus* (Candy & Baddiley, 1966; Harris & Gray, 1977). Ballou (1968) reported the presence of a 6-*O*-methyl-D-glucose-containing lipopolysaccharide in different strains of mycobacteria and undertook to determine the structure of this compound (Keller & Ballou, 1968; Saier & Ballou, 1968*a, b, c*; Gray & Ballou, 1972; Forsberg *et al.*, 1982). Lornitzo & Goldman (1968) and Kobatake *et al.* (1981) gave a description of similar lipopolysaccharides produced by other mycobacterial strains. These molecules seem to have an important role in the regulation of fatty acid synthesis (Ilton *et al.*, 1971) by their ability to form tight complexes with long-chain acyl-CoA derivatives (Machida & Bloch, 1973; Knoche *et al.*, 1973; Yabusaki & Ballou, 1978).

The reducing end of the 6-*O*-methylglucose-containing lipopolysaccharide consists of a D-glucosyl-(1'→2)-glyceric acid residue (Saier & Ballou, 1968*a*). A 2',3'-di-*O*-acyl derivative of the same glucoside has also been isolated as a specific glycolipid from strains of *Nocardia otitidis-caviarum* (Pommier & Michel, 1981, 1985). This paper describes the presence of the 6-*O*-methylglucose-containing lipopolysaccharide in *N. otitidis-caviarum* strains and in other *Nocardia* species and discusses a possible relationship between glyceric acid-containing glycolipid and lipopolysaccharide in the genus *Nocardia*.

METHODS

Strains and cultivation. *Nocardia otitidis-caviarum* IM 1381, *N. farcinica* IM 1377, *N. kirovani* IM 1374 and *N. brasiliensis* IM 1019 were obtained from Institut Mérieux, Marcy l'Etoile, France. *N. asteroides* ATCC 9970 was supplied by Dr M. Tsukamura, National Chubu Hospital, Aichi-Ken, Japan, and *N. otitidis-caviarum* N 393 by Dr M. Goodfellow, University of Newcastle upon Tyne, UK. Strains were grown on Sauton medium (Sauton, 1912) for 3 weeks at 37 °C. '*Corynebacterium adiphtheriae*' and *C. pseudodiphtheriticum* provided by Laboratoires de Thérapeutique Moderne, Châtillon sur Chalaronne, France, were cultured in a liquid medium, containing 0.75% glycerol, 1% yeast extract, 0.1% NaCl (w/v), for 8 d at 37 °C.

Lipopolysaccharide extraction. Lipopolysaccharide was extracted from lyophilized bacteria with chloroform/methanol (2:1, v/v), followed by ethanol/water (7:3, v/v) under reflux. Extracts were concentrated to dryness and

Abbreviation: FAB/MS, fast-atom-bombardment mass spectrometry.

partitioned in two layers with the Folch solvent (Folch *et al.*, 1957): chloroform/methanol/water (20:10:7.5, by vol.). The upper aqueous layer was dialysed against distilled water, lyophilized, dissolved in 0.1 M-acetic acid and then purified by gel filtration on a Sephadex G-50 column (2 × 50 cm) eluted with 0.1 M-acetic acid followed by ion-exchange chromatography on DEAE-cellulose equilibrated with saturated Na₂CO₃. The products were eluted first with 60 ml water and then with 100 ml of a linear gradient of 0 to 0.1M-NaHCO₃. Fractions of 2 ml were collected and their sugar content was measured by the anthrone method (Scott & Melvin, 1953). The purity of lipopolysaccharide-containing fractions was tested by chromatography on a DEAE-Sephadex A-25 column (borate form). The lipopolysaccharide was eluted in 2 ml fractions, first with water and then in a linear gradient of 0 to 0.075 M-sodium borate.

Hydrolysis methods. Acid hydrolysis was done with 0.1 M-HCl at 100 °C for 48 h, and alkaline hydrolysis with 0.1 M-NaOH at 25 °C for 16 h.

Analytical methods. The following quantitative colorimetric methods were used: anthrone method for hexoses and *O*-methylhexoses (Scott & Melvin, 1953), orcinol method for pentoses (Brown, 1946), D-glucose oxidase method for D-glucose and hydroxamate method for *O*-acyl determination (Lee, 1966). The ratio of aldoses to *O*-methylaldoses was determined by GLC of their alditol acetate derivatives or by a phthalic acid/aniline method after paper chromatographic separation (Wilson, 1959).

Periodate oxidation was followed by spectrometry at 224 nm (Dixon & Lipkin, 1954). Proteins were estimated by the Lowry method.

Chromatographic methods. Paper chromatography was done on Whatman no. 1 paper with the following solvent systems: (1) butanol/pyridine/water (10:3:3, by vol.); (2) butanol/pyridine/water/toluene (upper phase) (5:3:3:4, by vol.); (3) ethyl acetate/acetic acid/formic acid/water (18:4:1:3, by vol.); (4) propanol/ethyl acetate/butanol/acetic acid/water (12:20:7:7:6, by vol.).

High-voltage paper electrophoresis was done in buffer system (5): pyridine/acetic acid/water (25:170:2000, by vol.) at pH 3.6.

Chromatograms and electrophoregrams were stained with periodate/benzidine reagent (Gordon *et al.*, 1956) or with silver nitrate reagent (Trevelyan *et al.*, 1950).

GLC was done on Intersmat IGC 120 FL or IGC 121 C gas-chromatographs equipped with a flame ionization detector, using the following columns: (A) a glass column (6 mm × 200 cm) packed with 3% ECNSS-M on 100–120 mesh Gas Chrom Q at 180 °C or 150 °C; (B) a glass column (6 mm × 150 cm) packed with 3% OV-225 on 100–120 mesh Gas Chrom Q at 190 °C or 170 °C; (C) a fused silica capillary column (0.2 mm × 50 m) coated with CP-Sil 5, temperature programming from 220 °C, delay 5 min, to 250 °C at 0.5 °C min⁻¹; (D) a fused silica capillary column (0.2 mm × 25 m) coated with OV-101, temperature programming from 160 °C, delay 3 min, to 260 °C at 2 °C min⁻¹; (E) a glass column (6 mm × 200 cm) packed with 8% Carbowax 20M and 2% terephthalic acid on 80–100 mesh Chromosorb WAW at 110 °C; (F) a stainless steel column (3 mm × 150 cm) packed with Porapak Q 100–120 mesh at 200 °C.

Methylation methods. The polysaccharide was methylated as described by Hakomori (1964). Methylated polysaccharide was purified on a Sephadex LH-20 column and eluted with chloroform/ethanol (1:2, v/v). For the localization of fatty acid residues, the lipopolysaccharide was methylated by the method of Promé *et al.* (1976), then dialysed and freeze-dried. Methylated samples were hydrolysed with 0.1 M-HCl at 100 °C for 48 h and analysed as alditol acetate derivatives.

Periodate oxidation. Periodate oxidation of polysaccharide or lipopolysaccharide was done for 72 h, in the dark, with 0.05 M-NaIO₄ solution in 0.02 M-sodium acetate buffer pH 5. Unreacted reagent was destroyed by the addition of an excess of ethylene glycol and the reaction mixture was treated with NaB²H₄. The solution was neutralized with 17 M-acetic acid, dialysed against distilled water, concentrated and hydrolysed before analysis by paper chromatography or GLC.

Demethylation. The following procedure, based on those of Allen *et al.* (1958) and Brennan *et al.* (1981), was used. Dried sugar (100 µg) was suspended by sonication in 1 ml BCl₃ in dichloromethane and placed in a closed dry desiccator for 18 to 24 h. Boron was removed by evaporation with chloroform and the residue was passed over a small column of Amberlite MB₃. The demethylated sugar was identified by paper chromatography and as the alditol acetate by GLC.

Mass spectrometry (MS). GLC/MS was done with a Perkin-Elmer Sigma 3B chromatograph coupled with a VG 70-70F or a VG ZAB HF spectrometer. FAB/MS was done with a VG ZAB HF spectrometer. The compound was dissolved in 5% (v/v) acetic acid and the sample was loaded into a drop of glycerol. Negative ion spectra were recorded at 7 kV accelerating voltage and masses were determined by counting the spectral lines.

RESULTS

Isolation of the O-methylglucose polymer

The extraction procedure is summarized in Fig. 1. The crude extract had the following composition: 57% (w/w) protein, 34.5% (w/w) hexose, 0.4% (w/w) pentose. About 40% of this

Fig. 1. Procedure for the extraction of the crude lipopolysaccharide.

extract was dissolved in 0.1 M-acetic acid and contained 40% (w/w) protein, 47% (w/w) hexose and 1% (w/w) pentose.

The crude lipopolysaccharide was purified by chromatography on Sephadex G-50; it was eluted from the column as a major peak with $V_e/V_0 = 4.2$ which contained 21% (w/w) protein and 55% (w/w) hexose. A sample was further purified by ion-exchange chromatography on DEAE-cellulose (Fig. 2). The *O*-methylglucose polymer (fraction B) was eluted with a linear gradient of 0 to 0.1 M- NaHCO_3 . Fraction A, eluted with water, contained mannan and arabinomannan polysaccharides. The homogeneity of fraction B was checked by passing it through a DEAE-Sephadex A-25 column (borate form). A single peak was eluted between 0.01 M and 0.04 M-sodium borate.

Composition of the lipopolysaccharide

The lipopolysaccharide obtained by DEAE-Sephadex A-25 chromatography accounted for 1% of the dry weight of cells. Hexose content (80 to 82%) was analysed by paper chromatography (solvents 1 and 2) and by GLC of alditol acetate derivatives (columns A, C and D). D-Glucose and two *O*-methylhexoses (3-*O*-methylglucose and 6-*O*-methylglucose) were identified by their chromatographic behaviour and by comparison of their mass spectra with standards. Demethylation products of both *O*-methylhexoses gave only D-glucose; the configuration was determined by the D-glucose oxidase method.

Fig. 2. DEAE-cellulose chromatography of the major fraction from Sephadex G-50 chromatography containing 6-*O*-methylglucose lipopolysaccharide. The sample was applied to a column (2×5 cm) of DEAE-cellulose equilibrated with saturated Na_2CO_3 and eluted first with H_2O and then with a linear gradient of 0 to 0.1 M- NaHCO_3 (---). All the fractions (2 ml) were analysed for carbohydrates by the anthrone method (●).

D-Glucose, 6-*O*-methyl-D-glucose and 3-*O*-methyl-D-glucose were present in the molar ratio 8:10.5:1. The content of acyl groups was determined by the hydroxamate method; the molar ratio of glucose to acyl groups was 10:7. Glyceric acid was also identified by paper chromatography (solvents 1, 2 and 3) and by electrophoresis in pyridine/acetic acid/water (solvent 5) at pH 3.6. The presence of 1 mole of glyceric acid was deduced from the M_r obtained by FAB/MS.

The M_r of the deacylated lipopolysaccharide was determined by FAB/MS. The negative ion spectrum showed a major signal at m/z 3513, in accordance with a formula $\text{C}_{135}\text{H}_{230}\text{O}_{104}$ for the polysaccharide, corresponding to the molar ratio D-glucose:6-*O*-methyl-D-glucose:3-*O*-methyl-D-glucose:glyceric acid 8:11:1:1. The spectrum was quite similar to that of the polysaccharide isolated from *Mycobacterium smegmatis* (Dell & Ballou, 1983a).

Methylation analyses

Two methylation cycles by the method of Hakomori (1964) were required for the total methylation of the deacylated polysaccharide. GLC/MS of derived alditol acetates revealed three compounds: 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methylglucitol, 1,4,5-tri-*O*-acetyl 2,3,6-tri-*O*-methylglucitol and 1,3,4,5-tetra-*O*-acetyl 2,6-di-*O*-methylglucitol.

Methylation with IC^2H_3 followed by 0.1 M-HCl hydrolysis, NaB^2H_4 reduction and GLC/MS of the acetate derivatives gave the position of the 3-*O*-methylglucose residue in the molecule. In the spectrum of the 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methylglucitol, the peaks at m/z 211, 214 derive from the fission between C-2 and C-3 in *O*-methylhexose and glucose derivatives, respectively. Other characteristic fragments are (C-1,C-3) and (C-1,C-5) moieties at m/z 165 and m/z 284; they indicated that 3-*O*-methylglucose is located at the non-reducing end. Characteristic fragments m/z 45, 236, 261 and 308 obtained with 6-*O*-methylglucose derivatives were also found in spectra of 1,4,5-tri-*O*-acetyl 2,3,6-tri-*O*-methylglucitol and 1,3,4,5-tetra-*O*-acetyl 2,6-di-*O*-methylglucitol (Fig. 3, Table 1).

Periodate oxidation of the polysaccharide from the deacylated lipopolysaccharide

After periodate oxidation of the polysaccharide 16% (w/w) of the total hexose was recovered and 2.6 mol NaIO_4 per glucose residue were consumed. The oxidized polysaccharide was hydrolysed and the products were treated with NaB^2H_4 ; three compounds were identified by

Fig. 3. Primary fragments observed by MS analyses of hexose derivatives obtained after methylation of lipopolysaccharide. (a) Tetra-*O*-methylglucose derivatives; (b) tri-*O*-methylglucose derivatives; (c) di-*O*-methylglucose derivatives.

paper chromatography (solvents 2, 3 and 4) and by GLC/MS of their acetate derivatives: glycerol, 4-*O*-methylerythritol and erythritol in the molar ratio 2:9.4:5. Glucose, 3-*O*-methylglucose and 6-*O*-methylglucose (0.8:1.3:1) were also detected. These results show that the polysaccharide was composed of five (1→4)-linked glucose, one (1→4, 1→3)-linked glucose, ten (1→4)-linked 6-*O*-methylglucose, one (1→4, 1→3)-linked 6-*O*-methylglucose and two non-reducing terminal D-glucose residues.

Identification and localization of acyl groups

Acyl groups of lipopolysaccharide was analysed by GLC in columns E and F. Acetic acid was found as the major compound. Small amounts of propionic acid and isobutyric acid were also detected. Methylation according to Promé *et al.* (1976) gave the position of acyl residues in the lipopolysaccharide. The water-soluble fraction obtained by acid hydrolysis of methylated lipopolysaccharide was reduced with NaBH₄ and acetylated. The derivatives were analysed by GLC: the derivatives of 6-*O*-methylglucose and of glucose were identified in the molar ratio 2:1. Moreover periodate oxidation of the native lipopolysaccharide consumed 2.6 mol NaIO₄ per glucose residue. Thus acyl groups were located on the O-6 position in glucose residues.

Localization of the lipopolysaccharide in *N. otitidis-caviarum*

Cells of *N. otitidis-caviarum* (10 g) were suspended in water and disrupted ultrasonically (5 × 20 min). Several fractions were separated by centrifugation (Pommier & Michel, 1981). After hydrolysis with 0.1 M-HCl for 16 h, the presence of 6-*O*-methyl-D-glucose in hydrolysates was determined by paper chromatography or by GLC. The lipopolysaccharide was found only in the soluble cytoplasmic fraction: after purification it was found to be identical to that obtained from total non-disrupted bacteria.

Characterization of the lipopolysaccharide in the culture medium

The medium (2 litres) recovered from 3-week-old cultures of *N. otitidis-caviarum* was treated with 14% (w/v) trichloroacetic acid. Insoluble material was removed by centrifugation and the supernatant solution was dialysed and concentrated. The fraction was filtered through a Sephadex G-50 column (2 × 50 cm) and eluted with 0.1 M-acetic acid (Fig. 4). Fraction II contained 6-*O*-methylglucose polymer. This fraction was purified on a column of DEAE-cellulose as described above. The purified compounds from the culture medium and from the bacteria had the same composition; the yield was about 0.2 mg per litre of culture. This lipopolysaccharide may possibly have resulted from partial lysis of bacterial cells.

Table 1. *Methylation analyses of the deacylated lipopolysaccharide*

O-Methyl-D-glucitol acetate derivative	Type of linkage	T_r^*		Primary fragments†			Parent sugar
		Column: A	B	Molar ratio	ICH ₃	Methylation with: IC ² H ₃	
2,3,4,6-tetra-O-	Non-reducing end group	1-0	1-0	1-1	45, 118, 161, 162, 205, 206	48, 121, 165, 167, 211, 212, 284	3-O-Me-Glc
						48, 121, 167, 168, 214, 215, 287	Glc
2,3,6-tri-O-	(1-4)	2-5	2-0	7-5	45, 118, 162, 189, 233	45, 121, 168, 236	6-O-Me-Glc
						48, 121, 168, 239	Glc
2,6-di-O-	Branch point C-1, C-3, C-4	3-8	3-3	1-0	45, 118, 261, 305, 334	45, 121, 261, 308, 337	6-O-Me-Glc
						48, 121, 264, 311, 337	Glc

* Retention time relative to that of 1,5-di-O-acetyl 2,3,4,6-tetra-O-methylglucitol.

† The nature of primary fragments is explained in Fig. 3.

Fig. 4. Fractionation of medium extracts on a column (2×50 cm) of Sephadex G-50. Fractions (2.5 ml), eluted with 0.1 M-acetic acid, were analysed for carbohydrates by the anthrone method; fractions 22 to 41 were combined for further purification.

Table 2. *Composition of lipopolysaccharide (LPS) isolated from various strains of Nocardia*

Strain	Percentage (w/w) of dry cells		Molar ratios in LPS			
	Crude LPS	LPS	Acyl groups	Glc	6-O-Me-Glc	3-O-Me-Glc
<i>N. otitidis-caviarum</i> IM 1381	1.2	0.1	6	8	11	1
<i>N. otitidis-caviarum</i> N 939	0.6	0.02	4	8	11	1
<i>N. farcinica</i> IM 1377	1.2	0.04	3	7	11	1
<i>N. brasiliensis</i> IM 1019	0.2	0.02	1	9	10	1
<i>N. kirovani</i> IM 1374	0.2	0.01	2	11	14	1

Occurrence of lipopolysaccharide in other nocardiae

Crude polymer was extracted from 19 strains of *Nocardia*; it accounted for 0.2 to 1.7% (w/w) of dry cells. All these extracts contained 6-O-methyl-D-glucose, the characteristic sugar of the lipopolysaccharide. In order to confirm the presence of 6-O-methyl-D-glucose-containing lipopolysaccharide, four strains corresponding to well-established taxa were examined: *N. brasiliensis* IM 1019, *N. otitidis-caviarum* N 939, *N. farcinica* IM 1377 and *N. kirovani* IM 1374. Purified polymer from all these strains contained D-glucose, 6-O-methyl-D-glucose, 3-O-methyl-D-glucose and glyceric acid. The compositions of these lipopolysaccharides are shown in Table 2. The same procedure was applied to two strains of corynebacteria but no lipopolysaccharide was detected.

DISCUSSION

Lipopolysaccharides containing O-methyl-D-glucose have been isolated from mycobacteria (Lee, 1966; Saier & Ballou, 1968c). O-Methyl-D-glucose polysaccharide and its acylated derivatives, the O-methyl-D-glucose lipopolysaccharides, have been analysed by FAB/MS, and

the complete structure of the polysaccharide and partial structures of the lipopolysaccharides have been determined (Dell & Ballou, 1983*a, b*). We have isolated a similar compound from a strain of *N. otitidis-caviarum*. Methylation studies, periodate oxidation and FAB/MS of the polysaccharide gave results which were in agreement with the structure proposed by Dell & Ballou (1983*a*) for the mycobacterial polysaccharide. This polysaccharide possesses a long chain containing 6-*O*-methyl-D-glucose, D-glucose, 3-*O*-methyl-D-glucose and glyceric acid in the molar ratio 11:8:1:1; 3-*O*-methyl-D-glucose is the terminal residue. Two branching points, a 6-*O*-methyl-D-glucose and a D-glucose residue, have a D-glucose substituent in the C-3 and C-4 position, respectively. The reducing D-glucose residue is linked by a (1'→2) osidic bond to glyceric acid. The acyl groups of the lipopolysaccharide consist essentially of acetyl groups and, in smaller amounts, propionyl and butyryl groups substituting 6-hydroxyl groups of D-glucose residues. The lipopolysaccharides of nocardia differ from those of mycobacteria, which have a greater heterogeneity in short-chain acyl group substitution.

A primary aim of our work was to determine if a relationship existed between the different compounds which contained glyceric acid. Indeed, in a previous study a glycolipid was found in all the strains of *Nocardia otitidis-caviarum* and not in other species of nocardia. This glycolipid is a diacyl ester of a D-glucosyl-(1'→2)-D-glyceric acid (Pommier & Michel, 1981). As the same glucoside residue is present in the lipopolysaccharide of nocardiae one could suppose that a metabolic relationship existed between these two compounds. We have demonstrated that the lipopolysaccharide was present in all the species of *Nocardia* examined, while the glycolipid was specific to *N. otitidis-caviarum*. Thus it is doubtful that a metabolic relationship could exist between these two kinds of glyceric acid-containing compounds.

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