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STRUCTURAL STUDIES ON THE *Klebsiella* O GROUP 12 LIPOPOLYSACCHARIDE

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ABSTRACT

The structure of the O-specific side-chains in the *Klebsiella* O group 12 lipopolysaccharide has been investigated. Methylation analysis, and *N*-deacetylation followed by deamination or acid hydrolysis, with subsequent isolation and characterisation of the resulting oligosaccharides, were the principal methods used. From these studies, it is concluded that the O-specific side-chains are composed of disaccharide repeating-units having the structure: →3)-β-D-GlcNAc*p*-(1→3)-α-L-Rha*p*-(1→.

INTRODUCTION

Immunological studies of *Klebsiella* species have indicated the existence of twelve different O-groups[1], and the monosaccharide components of the corresponding lipopolysaccharides (LPS) have been determined by Nimmich and Korten[2]. Structural studies of O 1[3] (identical with O 6), O 3[4], O 4[5] (later proved immunologically to be identical to O 11[6]), O 5[7], O 7[8], O 8[9], O 9[10], and O 10[11] LPS have been reported. Preliminary studies of different O-antigens belonging to O group 2 indicated that their structures were similar or identical to those of either O 8 or O 9[12]. The number of different O-groups should therefore probably be reduced to nine. Of these, that designated as O 12 contains L-rhamnose and 2-acetamido-2-deoxy-D-glucose as principal sugar components. We now report structural studies on this O-antigen.

RESULTS AND DISCUSSION

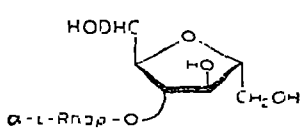
The LPS was isolated by using phenol–water extraction as previously described[2]. The polysaccharide (PS) was prepared from the LPS by mild hydrolysis with acid, and purified by using gel-filtration. The PS showed $[\alpha]_{578}$ −31°. A hydrolysate of the PS contained rhamnose (45%) and 2-acetamido-2-deoxyglucose (43%), together with trace amounts of glucose and heptoses, analysed by g.l.c.[13]–m.s.[14] of their

alditol acetates. However, these percentages are somewhat uncertain due to the difficulty in obtaining reproducible response factors in g.l.c. of acetamido sugar derivatives. Another hydrolysate was subjected to diazotisation, reduction with sodium borohydride, and acetylation, as devised by Dmitriev *et al.*[15]. G.l.c.–m.s. analysis then showed rhamnose and 2,5-anhydromannitol in the ratio 1:0.85. Rhamnose and 2-amino-2-deoxyglucose hydrochloride were isolated from a hydrolysate and shown to have the L and D configurations, respectively. The n.m.r. spectrum of the PS contained, *inter alia*, signals at δ 1.05–1.35 (3 H, broad doublet, methyl group of L-rhamnose), 2.04 (s, 3 H, *N*-acetyl group), 4.72 (1 H, $J_{1,2}$ 8 Hz, anomeric proton of 2-acetamido-2-deoxy-D-glucose residue), and 4.88 (1 H, $J_{1,2}$ ~2 Hz, anomeric proton of L-rhamnose residue). These data corroborate the results of the sugar analyses; *i.e.*, the PS contains equimolecular amounts of L-rhamnose and 2-acetamido-2-deoxy-D-glucose. Inasmuch as the anomeric protons of the L-rhamnose residues cannot give rise to high coupling constants (J ~7–10 Hz), the n.m.r. spectrum further indicates that the 2-acetamido-2-deoxy-D-glucose residues are β-linked. The results also strongly suggest that the PS is composed of disaccharide repeating-units.

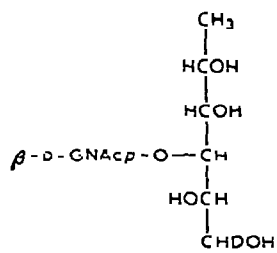
Methylation analysis[16–19] of the PS yielded 2,4-di-*O*-methyl-L-rhamnose and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, which were identified by g.l.c.–m.s. of their alditol acetates. Again, difficulties in obtaining reproducible response factors on g.l.c. for the latter compound prevented its accurate quantification.

The LPS was *N*-deacetylated by treatment with sodium hydroxide and sodium thiophenolate in aqueous methyl sulfoxide[21]. Deamination of this product with nitrous acid and subsequent borodeuteride reduction yielded a disaccharide (**1**) which was purified by gel filtration.

Hydrolysis of **1** gave L-rhamnose and 2,5-anhydro-D-mannitol in equimolecular amounts, as indicated by analysis of the derived alditol acetates by g.l.c.[13]–m.s.[14]. Methylation analysis of **1** gave 2,3,4-tri-*O*-methyl-L-rhamnose and 2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol-*1-d* in the ratio 1:1.2, as shown by analysis of their alditol acetates by g.l.c.–m.s. The mass-spectrometric fragmentations of the acetate of the latter compound as well as the related 2,5-anhydro-1,3,6-tri-*O*-methyl-D mannitol-*1-d* have recently been studied[20]. Investigation of the mass spectrum of methylated **1** further confirmed the sequence 6-deoxyhexosyl-(1→3)-2,5-anhydro-



1



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hexitol[17]. The n.m.r. spectrum of **1** showed, *inter alia*, signals at δ 1.28 (3 H, $J_{5,6}$ 6 Hz, methyl group of L-rhamnose residue) and 4.88 (1 H, $J_{1,2}$ 3 Hz, anomeric proton). Disaccharide **1** had $[\alpha]_{578}$ −41°, indicating the L-rhamnosyl residue to be α-linked. From the above results, the structure of **1** is unambiguously defined as shown in the formula.

The glucosaminidic linkages in the *N*-deacetylated PS should be essentially resistant to acid hydrolysis. Accordingly, a disaccharide alditol (**2**) was isolated from the *N*-deacetylated PS by gel filtration after sequential application of acid hydrolysis, sodium borodeuteride reduction, and *N*-acetylation. Sugar analysis involving diazotisation[13–15] of this product yielded L-rhamnitol-*1-d* and 2,5-anhydro-D-mannitol in the ratio 1:0.74. Investigation of the mass spectrum of methylated **2** confirmed the sequence 2-acetamido-2-deoxyhexosyl-(1→3)-6-deoxyhexitol-*1-d* in the disaccharide alditol[17]. Disaccharide **2** showed $[\alpha]_{578}$ −32°, and its n.m.r. spectrum contained signals, *inter alia*, at δ 1.24 (3 H, $J_{5,6}$ 6 Hz, methyl group of L-rhamnitol residue), 2.04 (s, 3 H, *N*-acetyl group of 2-acetamido-2-deoxy-D-glucose residue), and 4.66 (1 H, $J_{1,2}$ 7 Hz, anomeric proton). From the low optical rotation of **2** and the high value of $J_{1,2}$ for the 2-acetamido-2-deoxy-D-glucosyl residue, it is concluded that this residue is β-linked. From the above results, the structure of **2** is fully defined as shown in the formula.

From the combined evidence given above, it is proposed that the O-specific side-chains of the *Klebsiella* O group 12 LPS are composed of disaccharide repeating-units having the structure **3**.

→3)-β-D-GlcNAc*p*-(1→3)-α-L-Rha*p*-(1→

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EXPERIMENTAL

General methods. — For g.l.c., a Perkin–Elmer 990 instrument with flame-ionisation detectors was used. Separations were performed on glass columns (180 × 0.15 cm) containing (*a*) 3% of OV-225 (neutral sugar derivatives) and (*b*) 3% of OV-17 (acetamido sugar derivatives and permethylated oligosaccharide derivatives). For quantitative analyses, a Hewlett–Packard 3370 B integrator was used. G.l.c.–m.s. was performed by using the above-mentioned columns in a Varian MAT 311-SS 111 MS g.l.c.–m.s.–computer system; spectra were recorded at 70 eV. N.m.r. spectra were obtained at 100 MHz on D_2O solutions at 85°. A Varian XL-100 instrument operated in the PFT-mode was used. High-speed liquid chromatography was performed with the equipment described in Ref. 22: two Micro-Bondapak Carbohydrate (Waters) (30 × 0.5 cm) columns were used with acetonitrile–water (9:1) as eluant. Gel filtrations were performed on columns (90 × 2.5 cm or 80 × 0.8 cm) of Sephadex G-15 and monitored by using a Waters R 403 differential refractometer. Optical rotations were measured on a Perkin–Elmer 141 instrument, with 100-mm semi-micro cells.

Isolation of the lipopolysaccharide from Klebsiella *strain O 12:K 80 (708).* — This was performed as earlier described[2]. The PS (for preparation from LPS, see

below) showed $[\alpha]_{578}^{25}$ −31° (*c* 0.3, water). In the i.r. spectrum (KBr), no significant absorptions at ~1735 cm^{-1} (*O*-acyl region) were observed.

Sugar and methylation analyses. — These were performed essentially as previously described[13–20]. A response factor of 1.3 against neutral sugars was used for 2-acetamido-2-deoxy-D-glucose derivatives in sugar analyses. Rhamnose and 2-amino-2-deoxy-D-glucose hydrochloride were isolated from the PS (10 mg) by high-speed liquid chromatography of an aqueous hydrochloric acid hydrolysate. The rhamnose had $[\alpha]_{578}^{25}$ +6° (*c* 0.1, water), and the 2-amino-2-deoxy-D-glucose hydrochloride had $[\alpha]_{578}^{25}$ +70° (*c* 0.04, water).

Preparation of PS[23]. — In a typical experiment, the LPS (100 mg) was treated for 1 h at 100° with 1% aqueous acetic acid, precipitated lipid material was removed by centrifugation, and the supernatant was freeze-dried. The resulting material was purified by gel filtration, and the polymeric material (50 mg) was collected and freeze-dried.

N-*Deacetylation of LPS*[21]. — The LPS (180 mg) was treated under nitrogen with sodium hydroxide (0.8 g) and sodium thiophenolate (0.8 g) in a mixture of water–methyl sulfoxide (12 ml, 1:5) for 15 h at 80°. The reaction mixture was neutralised with acetic acid, dialysed, and freeze-dried. Yield: 70 mg. The absence of a signal for *N*-acetyl protons in the n.m.r. spectrum indicated that *N*-deacetylation was complete.

Deamination of N-*deacetylated LPS.* — The *N*-deacetylated LPS (35 mg) was dissolved in water (0.6 ml), and a mixture of 33% aqueous acetic acid (1 ml) and 5% aqueous sodium nitrite (1 ml) was added. The mixture was kept at room temperature for 4 h, and then treated with Dowex 50(H^+) resin and freeze-dried. The resulting material was reduced with sodium borodeuteride (50 mg) in water, and the solution was acidified with Dowex 50(H^+) resin and evaporated, with codistillation of methanol (3 × 10 ml) from the residue. Gel filtration yielded polymeric material (2.1 mg, not further investigated) and the disaccharide **1** (9.4 mg), $[\alpha]_{578}^{26}$ −41° (*c* 0.2, water). Methylated **1** showed T_{Lac} (retention time relative to lactitol nonamethyl ether) 0.45 (OV-17 column at 205°). The mass spectrum showed, *inter alia*, the following fragments (relative intensities in brackets and some assignments[17] of fragments in square brackets): 88 (100), 101 (23), 157 (4) [aA_2], 158 (9) [bA_2], 189 (2) [aA_1], 190 (7) [bA_1], and 250 (8) [abJ_1]. The 2,5-anhydro-1,4,6-tri-*O*-methylmannitol-*l*-*d* obtained in the methylation analysis showed *T* (retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol) = 0.23 (OV-225 column at 165°). The mass spectrum contained, *inter alia*, the following fragments (relative intensities in brackets): 43 (100), 45 (65), 46 (46), 71 (16), 87 (16), 129 (75), 130 (8), 143 (9), 144 (14), 157 (7), 203 (20), and 204 (4). This compound was identical (g.l.c.–m.s.) with an authentic specimen.

Hydrolysis of N-*deacetylated PS.* — The *N*-deacetylated PS (20 mg) was hydrolysed with M hydrochloric acid (10 ml) for 4 h at 100°, and the hydrolysate was then concentrated to dryness. The residue was dissolved in water and treated with sodium borodeuteride (50 mg) for 4 h, and the solution was acidified with acetic acid

and evaporated, with codistillation of methanol (3 × 10 ml) from the residue. The product was acetylated with acetic anhydride–pyridine (2 ml, 1:1) for 30 min at 100°, the mixture was concentrated to dryness, and the residue was *O*-deacetylated with ammonia in methanol (5 ml) overnight at room temperature. Gel filtration yielded the disaccharide alditol **2** (1.5 mg), $[\alpha]_{578}^{25}$ −32° (*c* 0.2, water). Methylated **2** showed T_{Lac} 1.87 (OV-17 column at 240°), and its mass spectrum showed, *inter alia*, the following fragments (relative intensities in brackets, and some assignments[17] of fragments in square brackets): 46 (58), 59 (77), 90 (22), 101 (100), 103 (26), 129 (77), 142 (52), 196 (5) [aA_3], 206 (10) [bA_1], 228 (25) [aA_2], 260 (15) [aA_1], 266 (13) [abJ_1], 276 (22), and 308 (11).

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