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STRUCTURAL STUDIES OF THE O-ANTIGEN POLYSACCHARIDES OF *Klebsiella* O5 AND *Escherichia coli* O8

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ABSTRACT

The O-antigen polysaccharides of *Klebsiella* serotype O5 and *Escherichia coli* serotype O8 are serologically very similar or identical. The structures of these two polysaccharides have now been re-investigated. N.m.r. spectroscopy, chromium trioxide oxidation, hydrolysis with a specific phage enzyme, and f.a.b. mass spectrometry were the principal methods used. It is concluded that the O-antigen has the following structure, in which D-Man3Me is 3-*O*-methyl-D-mannose and *n* is ~10.

$$\alpha\text{-D-Man}p3\text{Me-}(1\rightarrow3)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow)_n$$

Biosynthetic studies[1] indicate that these antigens are synthesised by addition of D-mannopyranosyl groups to the "non-reducing" end of the mannan chain, and it seems possible that addition of a 3-*O*-methyl-D-mannopyranosyl group involves termination.

INTRODUCTION

The *Klebsiella* O5 and *E. coli* O8 O-antigens, which are serologically very similar or identical[2], are composed of mannose and 3-*O*-methylmannose[3]. Structural studies of the *Klebsiella* O5 antigen[4] demonstrated that it was linear and composed of 2- and 3-linked D-mannopyranosyl residues, and that most or all of the chains were terminated by 3-*O*-methyl-D-mannopyranosyl groups[4]. It was

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further assumed that all of the D-mannopyranosyl residues were α-linked and that the polysaccharide was composed of pentasaccharide repeating-units containing three 2-linked and two 3-linked, non-adjacent residues. ^{1}H-N.m.r. evidence, however, indicated that it was composed of trisaccharide repeating-units containing one β- and two α-linked residues[5].

Similar studies of the *E. coli* O8 antigen[6] indicated that it was composed of trisaccharide repeating-units containing one 3-linked and two 2-linked α-D-mannopyranosyl residues. No 3-*O*-methyl-D-mannose was observed in these studies.

In the biosynthesis of most bacterial O-antigens, an oligosaccharide, representing the biological repeating-unit, linked to the pyrophosphate of an isoprenoid alcohol (often a C_{55} derivative) is formed first. This repeating unit is then transferred to the polymer, which grows from its "reducing" end. The biosynthesis of the *E. coli* O8 and *E. coli* O9 O-antigens, however, has been reported to follow a different course[1]. The mannan chain is built by transfer of D-mannopyranosyl groups from GDP-D-mannose to undecaprenol diphosphate D-glucose, and the polysaccharide chain consequently grows from the non-reducing end. This unusual mode of biosynthesis makes these antigens of especial interest and we have now re-investigated the structure of the *Klebsiella* O5 and *E. coli* O8 O-antigens.

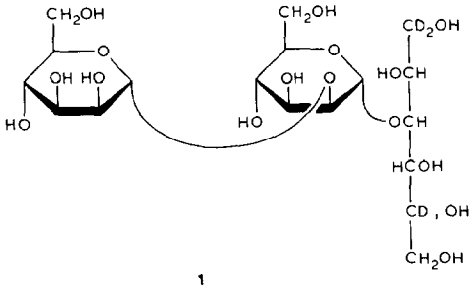
RESULTS AND DISCUSSION

The polysaccharides were prepared from the lipopolysaccharides of *Klebsiella* O5, strain K 57, and *E. coli* O8, strain F 492, by hydrolysis with acid under mild conditions. Hydrolysates of these polysaccharides contained 3-*O*-methylmannose, mannose, glucose, galactose, 2-acetamido-2-deoxyglucose, and heptose in the relative proportions 3:87:4:2:2:2 (*K* O5) and 3:74:5:4:8:6 (*E.c.* O8), respectively. Mannose was shown to have the D configuration by using the method of Leontein *et al.*[7], and 3-*O*-methylmannose was assumed also to have the D configuration. Both O-antigen polysaccharides were investigated by sugar and methylation analyses and by ^{1}H- and ^{13}C-n.m.r. spectroscopy. As the results were virtually identical, only the *E. coli* O8 antigen was used for further studies.

The ^{1}H-n.m.r. spectrum of the antigen showed, *inter alia*, signals of equal intensity in the region for anomeric protons at δ 4.75 (not resolved), 5.12 (not resolved), and 5.30 (not resolved), indicating that it is composed of trisaccharide repeating-units containing one β- and two α-linked D-mannopyranosyl residues. The ^{13}C-n.m.r. spectrum showed, *inter alia*, three equally strong signals for anomeric carbons at δ 99.1 ($J_{C,H}$ 162 Hz), 100.8 ($J_{C,H}$ 171 Hz), and 101.5 ($J_{C,H}$ 171 Hz). From the coupling constants[8], it is evident that one of the signals is given by a β-pyranosyl residue and two by α-pyranosyl residues. Signals for primary carbons were found at δ 61.4 and 61.8 (2 C), and for ring carbons at δ 67.0, 67.8 (2 C), 70.5, 70.8, 71.4, 74.1 (2 C), 76.9, 77.9, 79.4, and 81.1. Two weak signals in the spectrum, at δ 103.1 and 57.1, were assigned to the anomeric carbon of the 3-*O*-methyl-D-mannopyranosyl group and to its methoxyl carbon, respectively. Methylation

analysis of the O-antigen confirmed the presence of 2- and 3-linked D-mannopyranosyl residues in the ratio ~2:1.

The fully acetylated O-antigen was treated with chromium trioxide in acetic anhydride[9]. During this treatment, α-linked D-mannopyranosyl residues should be inert but β-linked residues should be oxidised to D-*lyxo*-5-hexulosonate residues. On subsequent treatment with sodium borodeuteride, the latter should be deacetylated and transformed into a mixture of D-mannitol and L-gulitol residues, deuterated at C-1 and C-5. Methylation analysis of the oxidised and reduced product (Table I, column A) demonstrated that the main components were the alditols **1**. The polysaccharide is consequently composed of trisaccharide repeating-units having the structure **2**.



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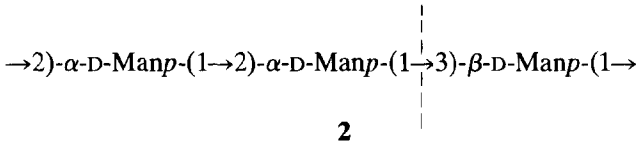

2

TABLE I

METHYLATION ANALYSIS OF VARIOUS *E. coli* O8 DERIVATIVES[a]

Sugar[b]	T[c]	*Mole %*			
		A	*B*	*C*	*D*
1,3,4,5,6-Mannitol	0.48		33	14	15
1,2,4,5,6-Mannitol[d]	0.48	21			
2,3,4,6-Man	1.02	43	39	24	17
3,4,6-Man	1.61	32	28	47	44
2,4,6-Man	1.73	3		15	24

[a]Key: A, Product after chromium trioxide degradation; B, trisaccharide after phage degradation; C, hexasaccharide; D, heptasaccharide. [b]Man = 2,3,4,6-tetra-*O*-methyl-D-mannose, *etc.* [c]Retention time of the derived alditol acetate on an SE-54 column at 150°, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [d]A mixture of D-mannitol and L-gulitol derivatives, dideuterated at C-1 and monodeuterated at C-5.

Bacterial polysaccharides are often hydrolysed in a highly specific manner by the action of bacteriophage-associated endo-glycanases[10,11]. The phage Ω 8 thus hydrolysed the O-antigen into a mixture of oligosaccharides that were fractionated by gel-permeation chromatography and characterised by n.m.r. spectroscopy. The ^{1}H-n.m.r. spectrum of the smallest oligosaccharide, which was a trisaccharide, showed, *inter alia*, signals at δ 4.73 (H), 5.12 (H), and 5.31 (H). The ^{13}C-n.m.r. spectrum showed three equally strong signals at δ 93.4 ($J_{C,H}$ 172 Hz), 99.4 ($J_{C,H}$ 161 Hz), and 100.8 ($J_{C,H}$ 174 Hz). Chemical-shift correlation spectroscopy was then used to assign nuclei coupled to each other *via* one-bond spin–spin coupling. The ^{13}C–^{1}H shift correlation map[12] showed correlation between signals at δ 99.4 and 4.73 and between those at δ 100.8 and 5.12, thus confirming the presence of one β- and one α-linkage in the trisaccharide. Methylation analysis of the reduced trisaccharide (Table I, column B) showed that both the alditol and the central D-mannopyranosyl residue were linked through O-2. From these results, and those discussed above, it was concluded that the trisaccharide has structure **3**.

β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man

3

This structure was also confirmed by n.m.r. studies of the fully acetylated trisaccharide. The ^{13}C-n.m.r. spectrum showed, *inter alia*, three equally strong signals at δ 91.6 ($J_{C,H}$ 178 Hz), 96.3 ($J_{C,H}$ 162 Hz), and 99.0 ($J_{C,H}$ 171 Hz). Two weak signals at δ 90.4 ($J_{C,H}$ 165 Hz) and 97.5 ($J_{C,H}$ 171 Hz) were assigned to the anomeric carbons of the reducing sugar and the middle residue of the trisaccharide in which the reducing sugar has a β-linked *O*-acetyl group. The upfield shift, after acetylation, of ~3 p.p.m. (δ 99.4 to 96.3) for C-1 of the β-linked residue indicated that the corresponding residue was acetylated at O-2. The ^{1}H-n.m.r. spectrum showed, *inter alia*, three equally strong signals for anomeric protons at δ 4.86 ($J_{1,2}$ not resolved), 5.02 ($J_{1,2}$ not resolved), and 6.15 ($J_{1,2}$ 2.2 Hz). Heteronuclear shift correlation was found between the signals at δ 91.6 and 6.15, δ 96.3 and 4.86, and δ 99.0 and 5.02, demonstrating that the signal at δ 4.86 corresponds to a β-linked residue and the other two to α-linked residues. Acetyl substituents induce strong downfield-shifts of α-protons (*i.e.*, on the same carbon) and the signal overlap therefore becomes less severe[13]. The homonuclear shift correlation map showed that the anomeric protons resonating at δ 4.86, 5.02, and 6.15 were coupled to those resonating at δ 5.40, 4.27, and 4.07, respectively. The chemical shifts for H-2 protons of the native trisaccharide were 4.00, 4.24, and 3.92. Thus, HO-2 of the β-linked residue is acetylated, as the signal for H-2 shifts to δ 5.40. Since the signals for the other H-2's are virtually unaffected, the respective residues are consequently 2-linked. Structure **3** is thus confirmed.

The phage enzyme therefore cleaves the α-(1→3) linkages, as indicated by the dashed line in formula **2**. The enzymic hydrolysis was not complete, and a

hexasaccharide and nonasaccharide were also obtained. The ^{13}C-n.m.r. spectrum of the hexasaccharide showed, *inter alia,* signals for anomeric carbons at δ 93.4, 99.2, 99.4, 100.7, 100.9, and 101.5. Methylation analysis of its alditol (Table I, column C) indicated the presence of six component sugars. Another oligosaccharide, which on hydrolysis yielded 3-*O*-methyl-D-mannose in addition to D-mannose, was also obtained. Methylation analysis of its alditol (Table I, column D) indicated that it was a heptasaccharide containing one 2-linked alditol residue, two 3-linked and three 2-linked hexosyl residues, and one hexosyl group. The ^{13}C-n.m.r. spectrum of the heptasaccharide showed, *inter alia,* signals at δ 93.3, 99.1 (2 C), 100.7, 100.8, 101.5, and, in addition to the hexasaccharide, signals at δ 103.1 ($J_{C,H}$ 174 Hz) and 57.1, demonstrating that the additional sugar, assumed to be the 3-*O*-methyl-D-mannopyranosyl group, is α-linked.

The positive fast-atom-bombardment (f.a.b.) mass spectrum of the alditol showed signals at *m/z* 1169 and 1191, corresponding to the $(M + H)^+$ and $(M + Na)^+$ ions of a heptasaccharide-alditol containing one mono-*O*-methylated residue. In addition, weaker ions at *m/z* 1479 and 1501 demonstrated that the heptasaccharide-alditol was contaminated with a nonasaccharide-alditol that did not contain any methylated sugar. Fragmentation of the oligosaccharides in f.a.b.-m.s. (in the positive mode) was insignificant and the sample was acetylated to permit detection of sequence ions. Acetyl derivatives are cleaved at the glycosidic linkages, giving A_1-type ions[14]. The alditol acetate mixture gave two series of fragments, namely, *m/z* 1743, 1455, 1167, 879, 591, 303, and *m/z* 2347, 2059, 1771, 1483, 1195, 907, 619, 331. These represent the two complete series of A_1-type ions from the acetylated hepta- and nona-saccharide-alditol acetates, respectively. The smallest fragment (*m/z* 303) from the acetylated heptasaccharide-alditol is derived from the terminal 3-*O*-methyl-D-mannopyranosyl group, and the others, with $n \times 288$ higher mass numbers, are the corresponding fragments containing this group and 1–5 further mannopyranosyl residues (**5**).

α-D-Man*p*3Me-(1→3)-β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Mannitol

5

As the hydrolysis with the phage enzyme should be highly specific, any oligosaccharide which does not contain 3*n* sugar residues should be derived from the terminal of the O-antigen polysaccharide. The result of the f.a.b.-m.s. experiments therefore confirms that the outermost sugar in the O-polysaccharide is 3-*O*-methyl-D-mannose. From ^{13}C-n.m.r. spectroscopy and sugar analysis, the number of repeating units of the O-specific chain can be determined as ~10.

As discussed above, the mannan chain in the O-antigen polysaccharides investigated is biosynthesised by transfer of single mannopyranosyl groups from GDP-D-mannose to the "non-reducing" end of the growing chain. It is conceivable

that these O-antigens have a regular, repeating structure, but the term "biological repeating unit" obviously has no significance as the monomers are added one by one. It seems most probable that the introduction of a 3-*O*-methyl-D-mannopyranosyl group at the "non-reducing" end of the antigen should terminate the synthesis of the chain.

The polysaccharide chain in the *Klebsiella* O10 O-antigen is terminated by a 3-*O*-methyl-α-L-rhamnopyranosyl group[15], and it seems possible that it is biosynthesised by a route similar to that of the *E. coli* O8 and O9 antigens.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath) or at room temperature by flushing with air. For g.l.c., a Hewlett–Packard 5830A instrument fitted with a flame-ionisation detector was used. Separation of alditol acetates was performed on an SE-54 fused-silica capillary column, using a temperature program 150→220° at 2°/min. Partially methylated alditol acetates were separated on the same phase at 150°. G.l.c.–m.s. was performed on a Hewlett–Packard 5790 instrument, also using the same phase. All identifications of mass spectra were unambiguous and will not be discussed.

Hydrolysis was performed with 2M trifluoroacetic acid for 2 h at 100°. The absolute configuration of the mannose was confirmed by the method of Leontein *et al.*[7].

Methylation analyses were performed essentially as previously described[16]. Products were recovered by reverse-phase chromatography on Sep-Pak C_{18} cartridges[17]. The sample was diluted with an equal volume of water and applied to the cartridge. This was washed with water and acetonitrile–water (15:85), and the sample was eluted with acetonitrile.

N.m.r. spectra of solutions in deuterium oxide were recorded at 27° (^{13}C) or 70° (^{1}H) with a JEOL GX-270 instrument. Chemical shifts are reported in p.p.m. relative to internal 1,4-dioxane (δ 67.4 downfield from external tetramethylsilane) for ^{13}C, and internal acetone (δ 2.21 downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate) for ^{1}H.

F.a.b.-m.s. was carried out by using a VG Analytical ZAB-HF mass spectrometer fitted with an M-scan f.a.b. gun. Xenon was used as the bombarding gas and the atom gun was operated at 10 kV, 15 μA. The samples were dissolved in aqueous 5% acetic acid (underivatised samples) or methanol (acetylated samples), and 1-μL aliquots were loaded into a matrix composed of a mixture of glycerol and monothioglycerol (1:1). Spectra were recorded on oscillographic paper, using a 300-s mass-controlled linear scan. Signals were assigned by counting the spectra.

Preparation of LPS, PS, and oligosaccharides. — The *E. coli* O8 lipopolysaccharide was isolated by extracting batch-grown cultures of bacteria using the phenol–water method[18].

The lipopolysaccharide in aqueous 2% acetic acid was kept at 100° for 2 h,

and the solution was cooled and centrifuged. The supernatant was neutralised and freeze-dried, and the product was fractionated on a column (90 × 3 cm) of Bio-Gel P10 that was irrigated with water. The polysaccharide was eluted in the void volume.

Oligosaccharides from the *E. coli* O8 O-polysaccharide were obtained after partial degradation of the polysaccharide with the Ω8 bacteriophage-associated endo-mannosidase[19], using the dialysis-bag technique[20]. The dialysable oligosaccharides were separated by repeated gel-permeation chromatography on Bio-Gel P2.

Chromium trioxide oxidation. — *E. coli* O8 (11 mg) PS was dissolved in formamide (2 mL) and treated with acetic anhydride–pyridine (1:1, 1 mL) at room temperature for 16 h. The solution was dialysed against tap water overnight and then against distilled water. The lyophilised material was dissolved in acetic anhydride (5 mL), chromium trioxide (70 mg) was added, and the mixture was sonicated at 50° for 1 h. 2-Propanol (2 mL) was added and the mixture was sonicated for 25 min at 50°. Water (10 mL) was added and the oxidised material extracted with chloroform (3 × 5 mL). The chloroform phase was concentrated and the residue was dried *in vacuo* over phosphorus pentaoxide. The material was dissolved in a mixture of dry 1,4-dioxane (2 mL) and ethanol (2 mL), sodium borodeuteride (30 mg) was added, and the mixture was kept at room temperature overnight. Dowex 50 (H^+) resin and water (10 mL) were added and, when the pH had decreased to 4, the resin was filtered off. The solution was concentrated and the boric acid was removed by codistillation with methanol. The product was subjected to methylation analysis (Table I, column A).

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