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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE (K6-ANTIGEN) FROM Escherichia coli LP 1092

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SUMMARY

The linkage pattern of the K6-antigen was investigated using material from the urinary pathogen, Escherichia coli LP 1092. The polysaccharide consists of ribose and 3-deoxy-D-manno-2-octulosonate (KDO) in a ratio of 2:1. Colorimetric procedures, Smith degradation, methylation analysis, and nuclear magnetic resonance spectroscopy were applied to the whole polysaccharide and to a trisaccharide "repeating unit" obtained by mild-acid catalyzed hydrolysis. Together, the data are compatible only with a branched chain structure ...3Ribfß1→7KDOpß2→3Ribfß...
2↑ 2↑
1↑ 1↑
Ribfß Ribfß

INTRODUCTION

The capsular polysaccharide from Escherichia coli LP 1092 was the first K-antigen reported to contain (1) 3-deoxy-D-manno-2-octulosonate (KDO), normally a constituent of the lipopolysaccharide (LPS) of Gram-negative bacteria (2,3). Within our efforts (4-6) to design inhibitors of the incorporation of KDO into cell surface structures, we have initiated a study of the biosynthesis of this capsular antigen (7). Previous results from this laborat-

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Abbreviations: Cetavlon®, N-cetyl-N,N,N-trimethylammoniumbromide; g.l.p.c., gas-liquid phase chromatography; h.p.l.c. high-performance liquid chromatography; KDO, 3-deoxy-D-manno-2-octulosonic acid; LPS, lipopolysaccharide; n.m.r., nuclear magnetic resonance; TBA, thiobarbituric acid; TSB, trypticase soy broth; TMS, trimethylsilyl.

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ory (7,8) have demonstrated that the polysaccharide consists of ribose (as ß-furanosyl residues) and KDO (as ß-[pyranosyl]ono residues) in a ratio of 2:1. Presently, we report on experimental evidence which suggests a linkage pattern of this capsular antigen. Structure elucidations of other, KDO-containing capsular polysaccharides have previously been reported by other workers (9-11).

MATERIALS AND METHODS

Bacterial strain. Escherichia coli LP 1092 (1,7,8) was kindly supplied by Dr. P.W. Taylor, School of Medicine, The University of Leeds, England.

Purification of the K6 polysaccharide. The K6 polysaccharide was purified from turbidostatically grown (OD_{600}~1.5, TSB medium) E. coli LP1092 cells, following essentially "procedure III" of Westphal and Jann (12). Details of the purification procedure, including its monitoring by ^{1}H-n.m.r., will be reported elsewhere (13).

Colorimetric methods. KDO contents were determined by the TBA assay of Warren (14), measuring at λmax = 550 nm (ε~80.000). The presence of KDO residues as free reducing end-groups was verified by their reactivity in the TBA assay (λmax = 532 nm, ε~15.000) following reduction with sodium borohydride (20 min, 0°) as described by Unger (6). The TBA assay at pH 5.0 was performed according to "method B" of Dröge, et al. (15). The orcinol assay for pentoses was carried out as described by Dische (16).

Gas-liquid phase chromatography. G.l.p.c. measurements were performed on a Hewlett Packard 5840 A instrument. Alditol acetates were assayed on a packed column of 3 % ECNSS-M, isothermally at 165°, using 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (17) as the standard. A standard of 5-O-methylribose was prepared through methylation (18) of commercial 2',3'-O-isopropylidene-uridine and subsequent acid-catalyzed hydrolysis. TMS-diethyl-dithioacetal derivatives (19) were analyzed on an OV-101 capillary with temperature programming (200-250°, 2° per min).

^{13}C-Nuclear magnetic resonance spectra were recorded on a Bruker Spectrospin WH90 instrument, at 22.63 MHz in the Fourier transform mode with complete proton decoupling, in deuterium oxide with dioxane as the internal standard.

Preparation of the K6 "repeating unit" trisaccharide. The K6 polysaccharide (160 mg) was subjected to hydrolysis as described by Osborn (3) for LPS. The product was fractionated on a Biogel P2 column (2.6 x 100 cm) previously equilibrated with 0.1 M ammonium acetate. The fractions (5 ml) were assayed for KDO and ribose by the TBA and orcinol tests (see colorimetric methods) and desalted on Sephadex G-10.

Smith degradation. Samples of the K6 polysaccharide or trisaccharide "repeating unit" (2.5 to 4.5 mg/20 ml) were subjected to periodate oxidation at pH 5.0 (15). Following reduction with sodium borohydride (60 mg/ml 0.001 N sodium hydroxide) at 20° for 30 min and desalting, the samples were utilized both for the determination of unaltered ribose by the orcinol test (16) and for g.l.p.c. analysis subsequent to hydrolysis and conversion to the respective TMS-diethyldithioacetal derivatives (19).

Methylation analysis. Polysaccharide samples (5-10 mg) were methylated by the Hakomori procedure (20) as described by Reske and Jann (21). Deprotonation of the polysaccharide was for 6 h. The methylation had to be repeated for completion, whereby a certain amount of destruction of the polysaccharide occurred. Hydrolysis was carried out as described by Lindberg (22) and, following neutralization with Amberlite IRA 410 (HCO_3), the partially methylated samples were converted to the alditol acetates as described by Björndal, et al. (23). Trisaccharide samples (∿ 45 mg) were analogously methylated, except that one course of methylation was sufficient for completion. The methylated trisaccharide was hydrolyzed (6 h, 100°, 0.25 N sulfuric acid), the products reduced with sodium borohydride, and acetylated (23). The ribitol acetates were separated by h.p.l.c. (LiChroSorb SI 60, petroleum ether:acetone, 5:1) and their substitution patterns analyzed by ^{1}H-n.m.r. (90.02 MHz, chloroform-d-solutions).

RESULTS AND DISCUSSION

Measurements performed with the whole polysaccharide. Previous results from this laboratory (7,8) have indicated that the K-6 polysaccharide consists of ß-ribofuranosyl and 3-deoxy-D-manno-2-(octulopyranosyl)ono residues in a ratio of 2:1. This is consistent (Table I) with the color yields obtained from the whole polysaccharide in the orcinol (16) and TBA (14) assays. The color yield obtained in the TBA reaction at pH 5.0 (15) is of the same magnitude as that obtained (14) at strongly acidic pH and indicates that the cis-diol grouping at C-4/C-5 of the KDO residues is unsubstituted. The formation of erythritol, apparently from C-5-C-8 of the KDO residues (9), during Smith degradation provides further evidence in this regard, and demands that O-7 or O-8 (or both) be involved in (a)glycosidic linkage(s). Three signals (at 61.2, 64.0, and 64.8 p.p.m. from tetramethylsilane) attributable to unsubstituted hydroxymethyl carbon atoms, are observed in the ^{13}C-n.m.r. spectrum, suggesting that O-7 rather than O-8 of the KDO moieties is part of a glycosidic linkage. In addition, this feature of the ^{13}C-n.m.r. spectrum indicates that both C-5' and C-5" (of the ribofuranosyl residues)are unsubstituted (Fig. 1).

Table I. Colorimetric analysis of the K6-polysaccharide (each value represents the average of 4-6 determinations)

constituent sugars (μmoles per mg of polysaccharide)		
ribose	KDO	molar ratio (ribose:KDO)
3.21[a]	1.70[b] 1.98[c]	1.89 : 1

[a] Orcinol assay (16). [b] TBA assay of Warren (14). [c] TBA assay of Dröge, et al. (15).

When the K-6 polysaccharide is oxidised with periodate and the product reduced with sodium borohydride, the color yield obtained in the orcinol test of the reduced material is ca. half of that obtained from the untreated polysaccharide (Table II). This indicates that one of the ribofuranosyl residues contains an unsubstituted 2,3-diol grouping whereas, in the other, O-2 or O-3 (or both) are substituted. Together with the ^{13}C-n.m.r. evidence, this finding suggests that one ribofuranosyl moiety is present as a "side branch" of the polysaccharide. This is

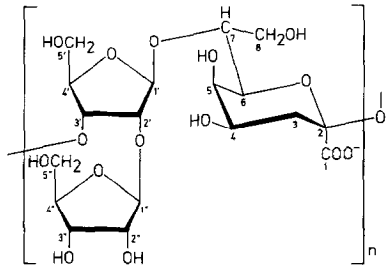

Fig. 1. Structure of the K6-polysaccharide antigen, with the numbering of individual carbon atoms as used in the text. The "repeating unit" trisaccharide contains hydrogen in the place of the glycosyl residue attached to O-3', with the KDO residue present as one of its tautomeric hemiketal forms (13). The K13-antigen (11) contains hydrogen in the place of the ribofuranosyl residues attached to O-2', and acetyl substituents at O-4 or O-5 of the KDO residues.

Table II. Colorimetric analysis of the K6-polysaccharide or its trisaccharide "repeating unit", following periodate oxidation and reduction with sodium borohydride.

	constituent sugars prior to periodate oxidation (μmoles per mg of starting material)		time of oxidation	ribose[a] protected against oxidation by periodate	
	ribose[a]	KDO[b]	(h)	μmoles per mg of starting material	%
polysaccharide	3.32	1.59	70	1.64	49
trisaccharide	3.19	1.69	48	1.51	47

[a] Orcinol assay (16). [b] TBA assay of Warren (14).

confirmed by the formation, during methylation analysis, of 1,4-di-O-acetyl-2,3,5-tri-O-methylribitol (Table III). The presence of 5-O-methyl-1,2,3,4-tetra-O-acetyl-ribitol as the other product found in the methylation analysis indicates that, in the polysaccharide, one ribofuranosyl moiety is present which

Table III. G.l.p.c. peaks of partially methylated ribitol acetates following Hakomori methylation (20) of the K6 polysaccharide or its "repeating unit" trisaccharide.

	peak	relative areas	retention time		interpretation
			found[a]	Ref. (17)	
polysaccharide	A	0.76	0.39	0.40	2,3,5-O-methylribitol[b]
	C	1.00	1.49	-	5-O-methylribitol[c]
trisaccharide	A	1.16	0.39	0.40	2,3,5-O-methylribitol[b]
	B	1.00	0.77	0.77	3,5-O-methylribitol[b,d]

[a] Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (17). [b] Ref. (17). [c] Retention time of 1,2,3,4-tetra-O-acetyl-5-O-methylribitol. See "Materials and Methods" for details. [d] Confirmed by ^{1}H-n.m.r.-spectroscopic analysis following the isolation of the ribitol acetate by preparative h.p.l.c. See "Results and Discussion".

carries substituents (KDO and the ribofuranose "side branch") both in positions 2' and 3' (Table III and Fig.1).

Measurements performed with the "repeating unit" trisaccharide.

The presence of the highly acid-labile, ketosidic linkages of KDO in the K6-polysaccharide allowed for the preparation of a "repeating unit" trisaccharide in 90 % yield by hydrolysis in 1 % acetic acid (3). By ^{1}H-n.m.r. (8,13), the glycosidic linkages of the ribofuranosyl moieties are seen to be unaffected by the conditions of hydrolysis (data not shown).

The color yield obtained in the orcinol reaction (16) of the trisaccharide, following its oxidation with periodate and reduction of the product with sodium borohydride, is ca. half of that obtained from the intact trisaccharide. This finding indicates that one ribofuranosyl residue of the trisaccharide is protected from oxidation by periodate (Table II). The result is to be expected if both the 2' and 3'-hydroxyl groups (Fig. 1) are blocked in the polysaccharide, since only one of them is liberated by the hydrolytic cleavage of the ketosidic bonds to KDO.

The question of the respective linkage positions of KDO and the ribofuranose side branch on the "middle" ribofuranose residue was decided on the basis of the following experiment. Preparative methylation of the trisaccharide (20,21), followed by derivatization (23) and separation of the partially methylated ribitol acetates by h.p.l.c., gave 2 compounds which were identified (Table III) as 3,5-di-O-methyl-1,2,4-tri-O-acetylribitol and 1,4-di-O-acetyl-2,3,5-tri-O-methylribitol [by g.l.p.c. (17) and by ^{1}H-n.m.r.]. The 3,5-di-O-methyl derivative, apparently originating from the "middle" ribofuranose residue (Fig. 1), was confirmed by the analysis of its ^{1}H-n.m.r. spectrum (chemical shifts and couplings of the protons at C-1 and C-2): H-1, δ 4.43 (dd, $J_{1,2} \sim 3.5$ Hz,

J1,1'∿12 Hz); H-1', 4.15 (dd, J1',2∿6.7 Hz, J1',1∿12 Hz); H-2, 5.26 p.p.m. (ddd, J2,1∿3.5 Hz, J2,1'∿6.7 Hz, J2,3∿4.8 Hz). These assignments were confirmed by a double irradiation experiment (13) and, together with the g.l.p.c. retention time (Table III), prove that 3,5-di-O-methyl-1,2,4-tri-C-acetylribitol rather than the 2,5-di-O-methyl-1,3,4-tri-O-acetyl derivative, is obtained during methylation analysis of the "repeating unit" trisaccharide. Hence, the linkage to KDO, in the polysaccharide, is located at O-3' of the "middle" ribofuranosyl residue (Fig.1). Finally, the assignment of 1,4-di-O-acetyl-2,3,5-tri-O-methylribitol, the other product obtained by preparative methylation of the trisaccharide, is made as originating from the side branch ribofuranosyl residue, whose substitution pattern is expected not to change during mild-acid catalyzed hydrolysis (Table III).

Together, the data presented are compatible only with the structure of the K6-polysaccharide as drawn in Fig. 1. Except for the presence of the ribofuranosyl side branch, the structure is analogous to that of the K13-antigen as elucidated by Vann and Jann (11). A forthcoming, detailed report will include an attempt to interpret the highly complex, ^{13}C-n.m.r. spectrum of the K6 polysaccharide with the aid of model compounds (13).

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