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STRUCTURE OF THE K16 ANTIGEN FROM *Escherichia coli* O7:K16:H$^-$, A Kdo-CONTAINING CAPSULAR POLYSACCHARIDE

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ABSTRACT

The K16-antigen from *E. coli* Rk 21510 (O7:K16:H$^-$) is shown to consist of the repeating unit →2)-β-D-Rib*f*-(1→3)-β-D-Rib*f*-(1→5)-α-Kdo*p*-(2→ of which ~33% is *O*-acetylated at position 3 of the 2-linked ribose.

INTRODUCTION

Uropathogenic *E. coli* belong to a group of invasive strains that have capsules composed of acidic polysaccharides[1–3], several of which (K antigens) contain 3-deoxy-D-*manno*-2-octulosonic acid (Kdo)[4–12], originally discovered as a constituent of the lipopolysaccharides of Gram-negative bacteria[11]. Most Kdo-containing polysaccharides consist of di- or tri-saccharide repeating-units with ribose as the second constituent[4–8], the Kdo is mostly β and, with two exceptions (K95 and K74)[7,12], it is pyranoid.

RESULTS AND DISCUSSION

Isolation and characterisation. — The K16 polysaccharide was isolated from liquid cultures of *E. coli* 21510 (O7:K16:H$^-$) by a sequence[3,13] that involved precipitation with cetyltrimethylammonium bromide (Cetavlon), extraction with aqueous calcium chloride, precipitation with ethanol, and removal of contaminating protein by extraction with cold phenol (pH 6.5). The K16 polysaccharide contains Kdo, ribose, and *O*-acetyl in the molar ratios 2:1:0.33. The D configurations of the ribose and Kdo were indicated by the $[\alpha]_D^{20}$ values (−25° and +40°, respectively).

Periodate oxidation. — Oxidation of the native and *O*-deacetylated (aqueous ammonia, pH 11, 16 h, 4°) K16 polysaccharide with sodium metaperiodate, followed by reduction with sodium borohydride, gave a polymer consisting of ribose and a component which was reactive in the thiobarbituric acid assay[14]. The latter had the same electrophoretic mobility (M_{Kdo} 1.1) as 3-deoxy-D-*lyxo*-heptulosonic acid derived from Kdo by cleavage of the C-7–C-8 bond.

These results indicate that the ribose residues of the K16 polysaccharide must

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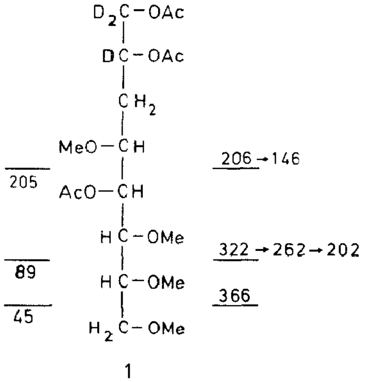

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be present as 2- or 3-linked furanosides or as 3-linked pyranosides, and the Kdo residue must be present as 4- or 5-linked pyranoside or as 6-linked furanoside.

Methylation analysis. — The polysaccharide was methylated[15–17] with KH/Me_2SO and MeI in Me_2SO. The purified (Sep-Pak C_{18}-cartridges) product was hydrolysed, and the products were reduced with sodium borodeuteride, then *O*-acetylated. G.l.c. (ECNSS-M; 3 min, 140°, 5°/min to 200°) gave two products which were shown by e.i.-m.s. to be 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methylribitol-*1-d* and 1,2,4-tri-*O*-acetyl-3,5-di-*O*-methylribitol-*1-d*.

Isolation and methylation of a trisaccharide. — Mild acid hydrolysis of the K16 polysaccharide (30mM acetic acid, 1 h, 100°) gave a Rib–Rib–Kdo trisaccharide, which was eluted from Biogel P2 (electrophoretic mobility M_{Kdo} 0.54). The trisaccharide was methylated and the methyl ester was carboxyl-reduced with sodium borodeuteride in aqueous methanol[18]. Hydrolysis (aq. 90% formic acid, 2 h, 100°, then 0.125M sulfuric acid, 2 h, 100°) of the products, followed by reduction and acetylation, gave a mixture which g.l.c. (160°, ECNSS-M) and e.i.-m.s. showed to contain 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylribitol-*1-d*, 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methylribitol-*1-d*, and 1,2,5-tri-*O*-acetyl-3-deoxy-4,6,7,8-tetra-*O*-methyloctitol-*1,1,2-*d_3 (**1**). This result indicates that the ribose linked to Kdo was 3-substituted and that the Kdo was pyranoid and 5-substituted. Therefore, the sequence in the polymer must be →2)-Rib-(1→3)-Rib-(1→5)-Kdo-(2→.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra of the *O*-deacetylated K16 polysaccharide, before (K16d) and after (K16do) periodate oxidation, and of the Rib–Rib–Kdo trisaccharide (Tri-K16) were compared with those of the methyl α- and β-glycopyranosides of Kdo*p* methyl esters[19], methyl 2- and 3-*O*-methyl-α- and -β-ribofuranoside, and methyl β-ribofuranoside[20,21]. Since the K12 and K14 polysaccharides of *E. coli* contain 5-linked Kdo[10,22], they were also used for the assignments of the Kdo signals. The assignments and the results of the attached proton test (APT)[23,24] with the K16 polysaccharide are shown in Table I.

The signals at δ 35.3 (K16d and K16do) and 34.7 (Tri-K16) are characteristic of C-3 of Kdo*p* and indicated the pyranoid form. The signal of C-5 of Kdo shows

TABLE I

^{13}C-N.M.R. DATA (δ) AND TENTATIVE ASSIGNMENTS[a]

Residue	*Atom*	*K16d*	*K16do*	*Tri-K16*	*2-O-Me-Kdo*		*K12*	*K14*			
					α	β					
Kdo	C-1 (—)[b]	174.9(−)	175.2	—	175.1	173.5	173.5	174.5			
	C-2 (3)	100.3(−)	99.7	97.1	101.0	101.8	102.8	102.7			
	C-3 (17)	35.3(−)	35.3	34.7	35.2	35.8	35.3	36.4			
	C-4 (13)	67.1(+)	67.3	67.2	67.2	68.3	69.0	65.2			
	C-5 (9)	74.0(+)	74.5	74.9	66.7	66.3	73.0	75.0			
	C-6 (10)	71.5(+)	72.8	70.1	72.2	74.3	70.1	74.7			
	C-7 (12)	69.6(+)	63.2	69.7	70.2	69.9	69.7	70.4			
	C-8 (14)	63.5(−)	—	63.5	63.9	64.9	64.1	65.0			
									3-O-Me-Ribf-OMe		
									α	β	
Rib(II)[c]	C-1 (1)	109.2(+)	109.1	109.2					104.1	109.2	
	C-2 (8)	74.9(+)	75.0	74.9					71.8	72.8	
	C-3 (6)	78.0(+)	78.8	78.6					80.1	81.3	
	C-4 (5)	81.5(+)	81.7	81.3					83.6	82.3	
	C-5[d] (15)	62.1(−)	62.6	62.5					62.8	64.0	
									2-O-Me-Ribf-OMe		*Ribf-OMe*
									α	β	
Rib(I)[c]	C-1 (2)	106.8(+)	106.8	108.6					103.1	106.6	109.0
	C-2 (7)	76.5(+)	76.2	75.4					81.0	83.5	75.3
	C-3 (11)	69.8(+)	69.7	71.4					69.4	71.1	71.9
	C-4 (4)	83.5(+)	83.5	83.4					86.3	84.5	83.9
	C-5[d] (16)	61.4(−)	61.4	61.4					62.8	63.6	63.9

[a] Key: K16d, *O*-deacetylated K16 polysaccharide; K16do, periodate-oxidised K16d; Tri-K16, *O*-deacetylated K16 trisaccharide; 2-*O*-Me-Kdo, methyl α- and β-glycopyranosides of Kdo*p* methyl esters; K12, →3)-α-Rha-(1→2)-α-Rha-(1→5)-β-Kdo*p*-(2→; and K14, →6)-β-GalNAc*p*-(1→5)-β-Kdo*p*-(2→. The chemical shifts and sign of the signals in the attached proton test (APT) are given. [b] Numbers in parentheses refer to signal assignments in Fig. 2. [c] Sequence of the sugars in the polysaccharide = —Rib(I)–Rib(II)–Kdo—. [d] The respective δ values may be exchangeable between Rib(I) and Rib(II).

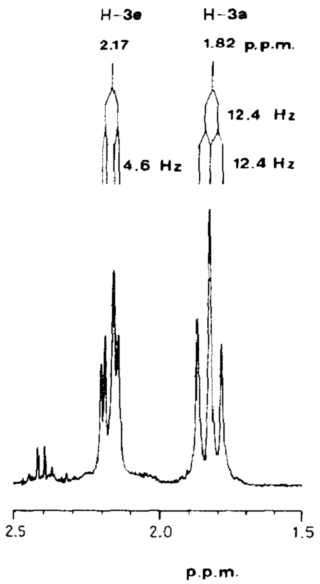

Fig. 1. ^{1}H-N.m.r. spectrum (δ 1.5–2.5) of the *O*-deacetylated K16 polysaccharide with the multiplets for H-3*e*,3*a* of Kdo.

TABLE II

CHEMICAL SHIFTS (δ) AND COUPLING CONSTANTS (*J*, Hz) OF THE RESONANCES OF H-3*e*,3*a* FOR Kdo RESIDUES

Compound	δ *H-3*e	δ *H-3*a	Δδ	$J_{3a,3e}$	$J_{3e,4}$	$J_{3a,4}$	*Configuration*
2-*O*-Me-β-Kdo*p*[25] [a]	2.38	1.74	0.64	12.2	4.3	12.2	β
2-*O*-Me-α-Kdo*p*[26] [a]	2.06	1.79	0.27	15.0	6.0	14.0	α
K12 antigen[27]	2.45	1.99	0.46	11.5	4.0	11.5	β
K19 antigen[28]	2.56	1.90	0.66	12.3	5.0	12.3	β
K16 antigen (-Ac)	2.17	1.82	0.35	12.4	4.6	12.4	α

[a] Methyl esters of methyl α- and β-glycopyranosides of Kdo*p*.

an α-shift of 7–8 p.p.m. (compared to the Kdo*p* methyl glycosides) and indicated 5-substitution (see the shift of the resonance of C-5 of Kdo of K12 and K14).

A comparison of the ribose signals in K16d (and K16do) with those of the methyl β-glycofuranosides of ribose, 2-*O*-methylribose, and 3-*O*-methylribose indicated ribose I to be 2-linked (α-shift of C-2, 1.2 p.p.m.) and ribose II to be 3-linked (α-shift of C-3, 6.1 p.p.m.). The periodate-oxidised polysaccharide (K16do) gave one less signal than K16d, with the appearance of a signal for -CH_2OH at δ 63.2, assigned (APT) to C-7 of the 3-deoxy-2-heptulosonic acid derived from Kdo. The differences of the ribose signals in the spectra of K16d and Tri-K16 indicate the sequence of the ribose residues in the trisaccharide. Both K16d

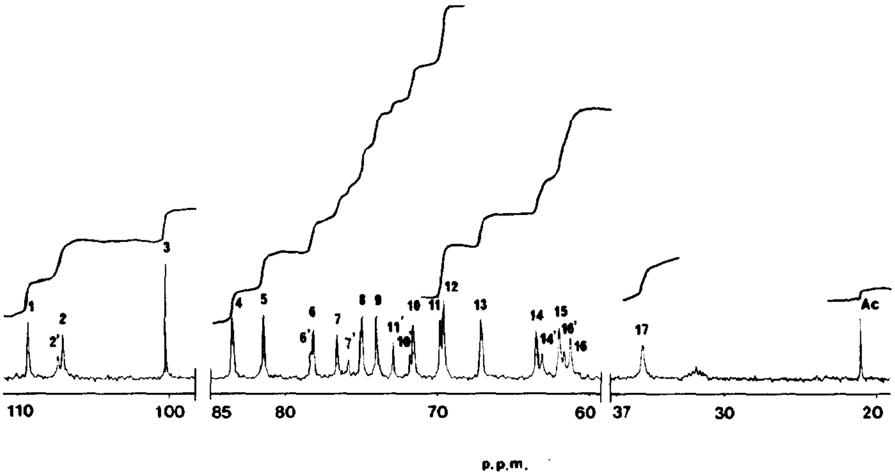

Fig. 2. Inversed gated decoupling ^{13}C-n.m.r. spectrum (110–100, 86–59, and 38–19 p.p.m.) of a solution of the K16 polysaccharide in D_2O.

and Tri-K16 contained 3-linked ribose II, whereas the 2-linked ribose I in K16d corresponded to the non-reducing end of the trisaccharide.

The interpretation of the n.m.r. data accords with the results of the methylation analysis.

Anomeric configurations. — From the chemical shifts of the C-1 signal of the two ribofuranosides, as compared to those of methyl substituted methyl α- and β-ribofuranosides (Table I), it can be concluded that both ribosides are β.

In order to determine the anomeric configuration of Kdo*p*, the chemical shifts and *J* values of the H-3 protons[25,26] in the ^{1}H-n.m.r. spectra (Fig. 1) of the K16 polysaccharide were analysed. The values, together with those from the ^{1}H-n.m.r. spectra of the K12 and K19 polysaccharide[27,28] as well as the methyl esters of methyl α- and β-Kdo*p*, are shown in Table II. These values indicate that the K16 polysaccharide contained α-Kdo*p*. This was substantiated by a negative cotton effect at 210 nm in the CD spectrum of the K16 polysaccharide.

Structure of the K16 polysaccharide. — From the above results, the repeating unit of the K16 polysaccharide can be formulated as →2)-β-D-Rib*f*-(1→3)-β-D-Rib*f*-(1→5)-α-Kdo*p*-(2→ (**2**). The position of *O*-acetylation can be defined from the ^{13}C-n.m.r. spectra. As indicated by the chemical shifts of the resonances for C-5 of the riboses and C-8 of Kdo*p*, the primary hydroxyl groups are unsubstituted. Because of the sensitivity of the C-7–C-8 bond in Kdo*p* to periodate, the same is true for C-7. Thus, C-3 of 2-linked ribose I, C-2 of 3-linked ribose II, and C-4 of Kdo are possible sites of *O*-acetylation. A differentiation was possible by comparing the ^{13}C-n.m.r. spectra of the native and *O*-deacetylated forms of the K16 polysaccharide. As shown in Fig. 2, the inversed gated decoupling ^{13}C-n.m.r. spectrum[29,30] of the native K16 polysaccharide contains, in comparison to that of the *O*-deacetylated K16 polysaccharide (Table I, Fig. 2), an additional group of signals (primed) which are caused by *O*-acetylation. The *O*-acetylation is ~33%

according to the integrated intensities of the signal at δ 21.4 of the *O*-acetyl group and the reference signal (δ 35.3) for C-3 of Kdo (signal 17 in Table I and Fig. 2).

In the spectra of the *O*-deacetylated K16 polysaccharide, signal 11′ at 72.7 p.p.m. was missing and signal 11 at 69.8 p.p.m. was one-third more intense when compared to the reference signal. This situation was taken as an α-shift (2.9 p.p.m.) indicating the partial *O*-acetylation of the K16 polysaccharide at C-3 of ribose I. A corresponding β-shift (0.9 p.p.m.) of signal 7 to 7′, due to C-2 of ribose I, was also observed.

Based on the foregoing results, the primary structure of the K16 polysaccharide is **2** with 33% *O*-acetylation per repeating unit at C-3 of the 2-linked ribose.

Seven signals (primed in Fig. 2) are displaced in the spectrum of the *O*-acetylated K16 antigen and the carbons associated with these signals are located in all residues of the repeating units.

The structure of the K16 polysaccharide furnishes another example of the close relationship among ribose and Kdo-containing polysaccharides (K6, K13, K19, and K74), and it will be of interest to determine the genetic basis of this relatively high structural variability within this group of *E. coli* polysaccharides.

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* RK 21510 (O7:K16:H6) was obtained from W. Nimmich (Rostock). The bacteria were grown to the late logarithmic phase in a fermenter in 10-L batches, which contained per L:$K_2HPO_4 \cdot 3\ H_2O$ (9.6 g), KH_2PO_4 (2 g), sodium citrate·5 H_2O (0.5 g), $MgSO_4 \cdot 7\ H_2O$ (0.1 g), casamino acids (1 g), ammonium sulfate (20 g), D-glucose, and the dialysable part of yeast (100 mL from 500 g in 5 L of deionised water).

Isolation and purification of the capsular polysaccharide. — The acidic capsular polysaccharides and the bacterial cells were precipitated from the liquid culture by the addition of 1 vol. of aq. 2% cetyltrimethylammonium bromide (Cetavlon). All following operations were carried out at 4°. The polysaccharide was extracted from the precipitate with M calcium chloride, and purified by three cycles of precipitation from aqueous solution with ethanol (to 80% final concentration), followed by repeated extractions with cold aq. 80% phenol (buffered to pH 6.7 with sodium acetate)[3,13]. The combined aqueous phases were centrifuged for 4 h (100 000*g*) and the supernatant solution was lyophilised. The residue was further purified on a column of Sephadex G-50 with water as eluant.

Analytical methods. — Kdo was determined, after hydrolysis (0.1M trifluoroacetic acid, 2 h, 100°) by the thiobarbituric assay[14]. Ribose was determined, after hydrolysis (M trifluoroacetic acid, 2 h, 100°), with the orcinol reagent[31]. Acetate was determined[33] by g.l.c. on Porapak QS. Quantitation of the components was done by ^{1}H- and ^{13}C-n.m.r. spectroscopy of the polysaccharide.

For the determination of the absolute configuration of ribose and Kdo, the polysaccharide was hydrolysed with 0.5M trifluoroacetic acid (30 min, 100°). From the hydrolysate, ribose and Kdo were isolated by high-voltage paper electrophoresis (Schleicher & Schüll 2043a paper, pH 5.3, 90 min, 42 V/cm), and their $[\alpha]_D$ values were measured with a Perkin–Elmer 141 polarimeter. G.l.c. was performed with a Varian Aerograph Series 1400 instrument, equipped with an autolinear temperature programmer and a Hewlett–Packard 3380 integrator, and g.l.c.–m.s. was performed with a Finnigan MAT 1020B automatic system at 70 eV on a CB CP SIL 5 column (25 m × 0.25 mm), using helium as carrier gas. N.m.r. spectra were recorded with a Bruker WM 300 spectrometer in the F.t. mode at 70° (^{1}H) and 33° (^{13}C) [external sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)propionate]. The δ values are related to Me_4Si.

Methylation. — The Hakomori method[15] as modified[16,17,32] was used. The methylated, acetylated oligosaccharides were purified by reversed-phase chromatography on silica-C18 cartridges (SEP-PAK C18 Waters)[34]. Before use, the cartridges were washed with 5 mL each of methanol, ethyl acetate, and methanol, and 10 mL of water. After application of the sample in Me_2SO–water (1:1), the methylated monosaccharides and oligosaccharides were eluted with methanol–water (1:1), and methylated polysaccharides were eluted with methanol. The fractions were analysed by g.l.c.–m.s.

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