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STRUCTURE OF THE K74 ANTIGEN FROM *Escherichia coli* O44:K74:H18, A CAPSULAR POLYSACCHARIDE CONTAINING FURANOSIDIC β-KDO RESIDUES*

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ABSTRACT

The structure of the capsular K74 antigen of *E. coli* H702c (O44:K74:H18) was elucidated by determination of the composition, ^{1}H- and ^{13}C-n.m.r. and c.d. spectroscopy, periodate oxidation, and methylation analysis of the polysaccharide and of a trisaccharide obtained by mild acid hydrolysis. The K74 antigen has the repeating unit →3)-β-D-Rib*f*-(1→2)-β-D-Rib*f*-(1→6)-β-KDO*f*-(2→. Of the repeating units, ~65% are *O*-acetylated, most probably at C-2 of the 3-linked ribose.

INTRODUCTION

Uropathogenic *Escherichia coli* belong to a group of invasive strains which have capsules composed of low-molecular-weight acidic polysaccharides[1–3]. Several of these capsular polysaccharides (K antigens) contain 3-deoxy-D-*manno*-2-octulosonic acid (KDO)[4–11], originally discovered as a constituent of the lipopolysaccharides of Gram-negative bacteria[11]. Most KDO-containing polysaccharides consist of disaccharide repeating-units with 3-linked ribose as the second constituent[4–8], the KDO is β, and, with one exception (K95)[7], it is in the pyranoid form. Until now, two KDO-containing polysaccharides with a trisaccharide repeating-unit have been reported[9,10], one of which[9] has the repeating unit →2)-β-Rib-(1→3)-β-Rib-(1→7)-α-KDO*p*-(2→. In contrast to the above-mentioned strains, *E. coli* O44:K74:H18 is not uropathogenic but enteropathogenic, provoking infantile diarrhoea. We now report on the capsular K74 polysaccharide, which has a trisaccharide repeating-unit containing ribose and β-KDO*f*.

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RESULTS AND DISCUSSION

Isolation and characterisation. — The capsular (K74) polysaccharide, $[\alpha]_D^{22}$ $-50°$ (*c* 0.17, water), was isolated from liquid cultures of *E. coli* H702c (O44:K74:H18) by a sequence[3,12] of 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 polysaccharide was obtained in a yield of 90 mg/L of culture medium and consisted of ribose, KDO, and *O*-acetyl in the molar ratios 2:1:0.65 The D configurations of the ribose and KDO, isolated from the K74 polysaccharide, were indicated by the $[\alpha]_D^{22}$ values ($-28°$ for ribose, $+42°$ for KDO[24]).

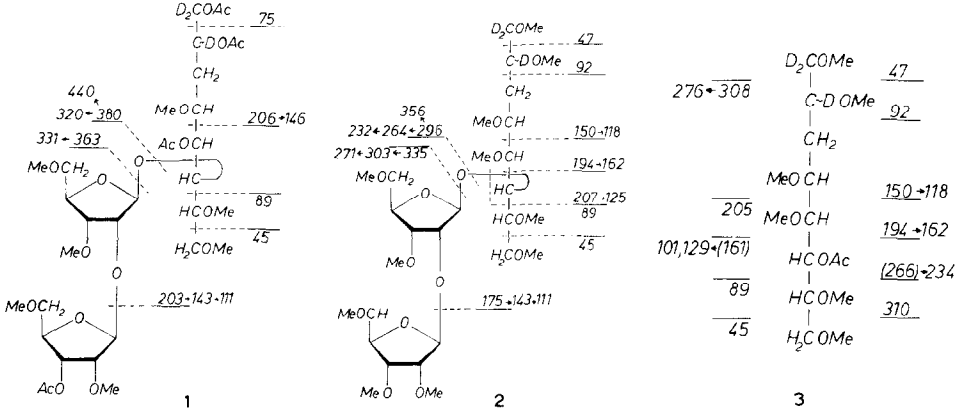
With an anti-K74 serum, the polysaccharide gave a precipitation line in immunoelectrophoresis and exhibited a titer of 10^5 in an enzyme-linked immunosorbent assay (ELISA), indicating K74 specificity.

Periodate oxidation. — Oxidation of the native and *O*-deacetylated (pH 11, 16 h, 4°) K74 polysaccharide with sodium metaperiodate, followed by reduction with sodium borohydride, gave a polymer consisting of ribose and a constituent which was reactive in the thiobarbituric acid assay[13]. The latter had the same electrophoretic mobility (M_{KDO} 1.1) as the 3-deoxy-D-*lyxo*-heptulosonic acid derived[5,10] from KDO by oxidation between C-7 and C-8. Neither KDO nor 3-deoxyhexulosonic acid[7] could be detected. These results indicated that the ribose must be present as 2- or 3-linked furanosides or as 3-linked pyranosides and that KDO must be present as 4- or 5-linked pyranoside or as 6-linked furanoside.

Methylation analysis of the K74 polysaccharide. — The polysaccharide was methylated[14–16] with KH/Me_2SO and MeI in Me_2SO. The purified (Sephadex LH20) product was hydrolysed[14] and the products in the neutralised hydrolysate were reduced with sodium borodeuteride and *O*-acetylated with acetic anhydride–pyridine. G.l.c. (180°, isothermal, ECNSS-M) gave two products which c.i.(ammonia)-m.s. showed to have a mass of 307 ($[M + NH_4]^+$ 325). E.i.-m.s. showed these products to be 1,3,4-tri-*O*-acetyl-1-deuterio-2,5-di-*O*-methylribitol and 1,2,4-tri-*O*-acetyl-1-deuterio-3,5-di-*O*-methylribitol.

In order to analyse the substitution of KDO, the K74 polysaccharide was methylated and the methyl ester was reduced with sodium borodeuteride[17]. Subsequent mild acid hydrolysis[18] (30mM acetic acid, 1 h 100°) preferentially cleaved the ketosidic linkages. Reduction of the products in the neutralised hydrolysate followed by *O*-acetylation with acetic anhydride–pyridine gave **1**, which c.i.-m.s. showed to have a mass of 759 ($[M + NH_4]^+$ 777). The e.i.-m.s. fragmentation pattern is shown in **1**. The data are in accord with the formulation of **1** as methylated 1,2,5-tri-*O*-acetyl-6-*O*-[2-*O*-(3-*O*-acetyl-β-D-ribofuranosyl)-β-D-ribofuranosyl]-3-deoxy -1,1,2-trideuterio-octitol. For further analysis, **1** was *O*-deacetylated and remethylated to give **2**, which c.i.-m.s. showed to have a mass of 647 $[M + NH_4]^+$ 665). The fragmentation pattern indicated **2** to be methylated 1,1,2-trideuterio-6-*O*-(2-*O*-D-ribofuranosyl-β-D-ribofuranosyl)-3-deoxy-octitol.

Hydrolysis and acetylation[18] converted **2** into 6-*O*-acetyl-3-deoxy-1,1,2-trideuterio-1,2,4,5,7,8-hexa-*O*-methyl-octitol (**3**), the fragmentation pattern of which is shown in **3**. Thus, the methylation analysis showed that the K74 polysaccharide contains one 2-linked ribose, one 3-linked ribose, and one 6-linked KDO*f*.



Isolation and methylation of a trisaccharide. — Mild acid hydrolysis of K74PS (aqueous 1% acetic acid, 1 h, 100°) gave **4**, which was eluted from Biogel P-2 with Kd 0.8, M_{KDO} 0.55, and which analysis before and after reduction with sodium borohydride showed to be the trisaccharide Rib–Rib–KDO. Methylation[14–16] of **4**, followed by hydrolysis, reduction,and peracetylation, gave a product which g.l.c. (180°, ECNSS-M) showed to contain two major components. By e.i.-m.s., they were found to be 1,4-di-*O*-acetyl-1-deuterio-2,3,5-tri-*O*-methylribitol and 1,2,4-tri-*O*-acetyl-1-deuterio-3,5-di-*O*-methylribitol. This result indicated that the ribose linked to KDO is 2-substituted and that the sequence in the polymer must be →3)-Rib-(1→2)-Rib-(1→6)-KDO-(2→. An analysis of the ribosyl-KDO linkage by g.l.c.-m.s. of **4** was hampered by the formation of its anhydro derivative[19]. This compound was obtained by g.l.c. (SE-54; 110° for 3 min, 3°/min to 250°) and had $[M + NH_4]^+$ 614, corresponding to a mass of 596, as opposed to an expected value of 642. It was not investigated further.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra of the *O*-deacetylated (pH 11, 16 h, 4°) polysaccharide before and after periodate oxidation (K74d and K74do) were compared with those of the *O*-deacetylated K95 polysaccharide[7], the methyl α- and β-glycosides (**5** and **6**) of KDO*f* methyl esters[20], methyl β- and α-Rib*f*[8] (**7** and **8**), and the methyl α- and β-glycosides (**9** and **10**) of β-Rib-(1→2)-β-Rib-(1→7)-KDO*p*[21]. The signal assignments and the results of the attached proton test (APT)[22,23] with K74d and K74do are shown in Table I. The latter method uses a spin echo technique (SEFT) in which signals due of CH_3 and CH= are positive and those due to CH_2– and C≡ are negative. The signals at δ 45.1 (K74d and K95d) and 45.3 (K74do) are characteristic of C-3 of KDO*f*[7,20]. The C-3 signal of KDO*p* is at δ

TABLE I

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

Atom		*K74d* δ *APT*	*K74do* δ *APT*	*K95d* δ	**5** δ	**6** δ	**7** δ	**8** δ	**9** δ	**10** δ
KDO	C-1	175.1(−)	175.4(−)	176.5	172.3	171.2			176.1	176.4
	C-2	109.0(−)	109.5(−)	110.0	106.7	107.1			101.4	102.2
	C-3	45.1(−)	45.3(−)	45.1	44.1	44.9			35.1	35.2
	C-4	72.7(+)	72.5(+)	73.3	72.2	72.1			66.8	68.3
	C-5	87.6(+)	88.2(+)	87.2	87.3	87.2			67.0	66.1
	C-6	77.9(+)	78.2(+)	71.7	70.7	71.0			70.7	73.0
	C-7	71.7(+)	61.4(−)	71.6	70.9	72.0			75.6	75.8
	C-8	63.1(−)		70.1	63.7	63.7			60.0	61.1
Rib	C-1	106.5(+)	106.1(+)				108.0	103.1	108.8	108.7
	C-2	81.5(+)	82.2(+)				74.3	71.1	75.4	75.4
	C-3	70.6(+)	71.0(+)				70.9	69.8	71.6	71.7
	C-4	83.9(+)	83.9(+)				83.0	84.6	83.9	83.9
	C-5	63.1(−)	63.3(−)				62.9	61.9	63.7	63.7
Rib	C-1	108.5(+)	109.1(+)	108.6					104.8	104.8
	C-2	75.2(+)	75.4(+)	75.7					82.3	82.0
	C-3	75.2(+)	75.4(+)	75.7					70.5	70.7
	C-4	82.5(+)	82.7(+)	82.5					83.6	83.8
	C-5	64.0(−)	64.3(−)	63.9					62.6	63.1

[a]For the *O*-Deacetylated K74 polysaccharide (K74d), its periodate-oxidised form (K74do), and the *O*-deacetylated K95 polysaccharide (K95d)[7]; reference compounds: methyl α- and β-glycosides of KDO*f* methyl esters (**5** and **6**), methyl β- and α-Rib*f* (**7** and **8**), and methyl α- and β-glycosides of β-Rib-(1→2)-β-Rib-(1→7)-KDO*p* (**9** and **10**). The chemical shifts (δ) and sign of the signals in the attached proton test (APT)[22,23] are given.

35–35.5, as seen in the spectra of **9** and **10**. Correspondingly, in the spectra of K74d, K74do, K95d, **5**, and **6**, the signal of C-5 of KDO experiences an α-shift (20–22 p.p.m., as compared to **10**) due to the furanosidic ring linkage.

Comparison of the ribose signals in K74d and K74do with those of **7** indicates that one of the riboses is 2-linked (α-shift of C-2 of 7.4 p.p.m.) and the other is 3-linked (α-shift of C-3 of 4.3 p.p.m.). The periodate-oxidised polysaccharide (K75do) exhibits one signal less than K74d, with the appearance of a signal for $-CH_2OH$ at δ 61.4. The latter is assigned to C-7 of the 3-deoxy-2-heptulosonic acid derived from KDO[5,10]. The interpretations of the n.m.r. data are in accord with the results of the methylation analysis.

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

In order to determine the anomeric configuration of KDO*f*, the chemical shifts and coupling constants of the H-3 and H-3′ resonances[14,24] in the ^{1}H-n.m.r. spectra of the K74 polysaccharide were analysed. The values, together with those from the ^{1}H-n.m.r. spectra of the K95 polysaccharide[7] as well as **5** and **6**[14], are shown in Table II. From the $J_{3,4}$ values, it is evident that both polysaccharides and **6** contain β-KDO*f*, whereas **5** contains α-KDO*f*.

An independent proof of the β configuration of KDO*f* in the K74 polysaccharide was obtained by c.d. spectroscopy[26], applying the planar rule with a two-fold spatial division[27] and with the assumption[28] that the carboxyl group of KDO is eclipsed with the sugar ring. On this basis, the carboxyl group of β-KDO should exhibit a positive, and that of α-KDO a negative, Cotton effect ($\Delta\epsilon$, $mol^{-1}.cm^{-1}$) at 217 nm. For the K74 polysaccharide, this value was +0.038, as compared to +0.49 for the β-KDO-containing K13 polysaccharide[3,29], +0.13 for 2-*O*-methyl-β-KDO[29], and −0.66 for 2-*O*-methyl-α-KDO.

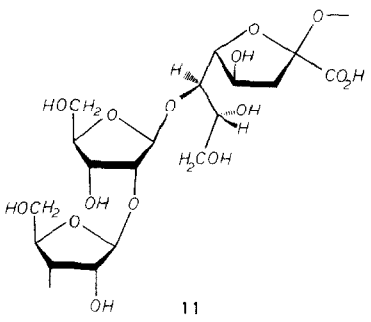
The results show that the K74 polysaccharide contains two β-riboses and one β-KDO*f*.

Structure of the K74 polysaccharide. — From the above results, the repeating unit of the K74 polysaccharide can be formulated as **11**. The position(s) of *O*-acetylation can be defined with some certainty from the ^{13}C-n.m.r. spectra. As indicated by the chemical shifts for their carbon atoms (C-5 of the ribose and C-8 of the

TABLE II

CHEMICAL SHIFTS (δ) AND COUPLING CONSTANTS *(J)* OF THE RESONANCES OF H-3 AND H-3′ OF K74PS, K95PS[7], AND THE METHYL ESTERS OF METHYL α-KDO*f* (mαK) AND METHYL β-KDO*f* (mβK)

Compound	*H-3′*	*H-3*	$J_{3,3'}$	$J_{3,4}$	$J_{3',4}$
mαK	2.35	2.53	13.9	6.5	6.7
mβK	2.19	2.49	14.5	2.5	7.7
K95	2.19	2.32	14.6	1.7	6.3
K74	2.24	2.40	14.5	1.5	6.0



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KDO*f*), the primary hydroxyl groups are unsubstituted. Because of the periodate sensitivity of the C-7–C-8 bond in KDO*f*, the same is true for C-7. Thus, C-3 of the 2-linked ribose, C-2 of the 3-linked ribose, and C-4 of KDO are possible sites of *O*-acetylation. Comparison of an inverse-gated[25,30] ^{13}C-n.m.r. spectrum of the native K74 polysaccharide, the δ 70–80 region of which is given in Fig. 1, with the ^{13}C-n.m.r. spectrum of K74d (Table I) shows that the former spectrum contains two additional signals (δ 77.1 and 73.9). The signal at δ 75.2, which is also present in the spectrum of K74d and which arises from coinciding C-2 and C-3 signals of the 3-linked ribose, is much too small in the quantitative representation of Fig. 1 to be a signal of double intensity. Therefore, it is assumed that the signal at δ 77.1 in Fig. 1 may be due to an α-shift (1.9 p.p.m.) of *O*-acetylated C-2 of the 3-linked ribose (δ 71.1) and that the signal at δ 73.9 may be due to a corresponding β-shift (−1.3 p.p.m.)

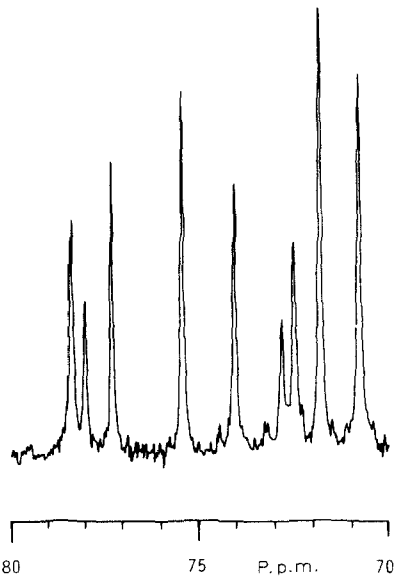

Fig. 1. Partial (δ 70–80) ^{13}C-n.m.r. spectrum (77.47 MHz) of the K74 polysaccharide [D_2O, external sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^2H_4$)pentanoate] run at 33°.

of C-3 of the same ribose (δ 75.2). This situation can be interpreted as indicating partial *O*-acetylation at C-2 of the 3-linked ribose. The small differences in chemical shifs (0.3 p.p.m.) of C-4 (δ 72.7) and C-6 (δ 77.9) of KDO*f* cannot be explained.

The K74 polysaccharide is the second capsular polysaccharide[7] of *E. coli* in which furanosidic β-KDO has been found. The β form of KDO seems to be preferred in capsular polysaccharides, α-KDO*p* being present, other than in LPS[12], only in the capsular LP1092 polysaccharide of *E. coli* [9]. The LP1092 polysaccharide, which has the repeating unit →3)-β-Rib-(1→2)-β-Rib-(1→7)-α-KDO*p*-(2→, differs from the K74 polysaccharide only in the ring form and substition pattern of KDO. This is one more example of the close relationship amongst ribose- and KDO-containing capsular polysaccharides, and it will be of interest to determine the genetic basis of this relatively high structural variability within this group of *E. coli* polysaccharides.

The capsular K74 polysaccharide, although belonging to the group of KDO–ribose polysaccharides, is exhibited by an *E. coli* strain provoking a pathogenic mechanism (enteropathogenic) different from that of all the other strains (uropathogenic) of this group. Moreover, enteropathogenic *E. coli* usually do not exhibit capsules[1,2] and *E. coli* H702c is an exception in this respect.

Immunoelectronmicroscopy[31] indicated that, in cultures of *E. coli* O44:K74:H18, all the bacterial cells are surrounded with the K74 specific polysaccharide which forms a relatively thick capsule. The K74 polysaccharide belongs to a group of *E. coli* capsular polysaccharides which are not expressed at 20° and below[32]. Accordingly, no capsule could be demonstrated by immunoelectronmicroscopy after growth of the bacteria at 18°. This phenomenon will be helpful in further immunochemical and biochemical studies of these capsular polysaccharides.

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* H702c (O44:K74:H18) was obtained from Drs. I. and F. Ørskov (Copenhagen). The bacteria were grown to the late logarithmic phase in a fermenter in 10-L batches, which contained per L: $K_2HPO_4 \cdot 3H_2O$ (9.7 g), KH_2PO_4 (2 g), sodium citrate·$5H_2O$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), casamino acids (1 g), ammonium sulfate (20 g), D-glucose (2 g), 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 polysaccharide and the bacterial cells were precipitated from the liquid culture by the addition of 1 vol. of aqueous 0.2% hexadecyltrimethylammonium bromide (Cetavlon). All of the following operations were carried out at 4°. The polysaccharide was extracted from the precipitate with M calcium chloride, purified by three cycles of precipitation from aqueous solution with ethanol (to 80% final concentration). Contaminating proteins were removed by repeated extractions with cold phenol (80%, buffered to pH 6.5 with sodium acetate)[3,12]. The combined, final aqueous phases were centrifuged for 4 h at 105,000*g* and the supernatant solution

was lyophilised. The residue was further purified by chromatography on Sephadex G-50.

Analytical methods. — Ribose was determined, after hydrolysis with M sulfuric acid for 2 h at 100°, by g.l.c. of the alditol acetate on ECNSS-M. KDO was determined, after hydrolysis with aqueous M trifluoroacetic acid for 10 min at 100°, by the thiobarbituric acid assay[13]. Determination of acetate[33] was by g.l.c. on Poropak QS. Quantitation of the components was also 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 (50 mg) was hydrolysed with aqueous M trifluoroacetic acid (30 min, 100°). From the hydrolysate, ribose and KDO were isolated by high-voltage paper electrophoresis. The components were eluted with water, their concentration was determined, and their optical rotations were measured with a Perkin-Elmer 141 polarimeter. High-voltage paper electrophoresis was run on Schleicher & Schüll 2043a paper at pH 5.3 for 90 min at 42 V/cm. 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 Hewlett-Packard 5985 and a Finnigan MAT 1020B automatic system at 70eV on a CB CP SIL 5 (25 m × 0.25 mm) column, using helium as the carrier gas. C.i.-m.s. was done with ammonia as the reactant 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 4,4-dimethyl-4-sila-(2,2,3,3-$^{2}H_4$)pentanoate]. The n.m.r. values given are related to the signal for Me_4Si.

Periodate oxidation, reduction with sodium borohydride or sodium borodeuteride, and *O*-deacetylation have been described[3,7,10].

Methylation. — Modification[19] of the Hakomori method[18] has been described[15,16]. In brief, a 35% suspension of KH in mineral oil (Aldrich) (3 mg) was washed with light petroleum (b.p. 40–60°; 5 × 3 mL) in a closed vial, using a syringe. After removal of the solvent, the solid KH was dried *in vacuo*. Dry Me_2SO (6 mL) was added and the hydrogen gas formed was allowed to escape through a hypodermic needle. The reaction was complete after ~10 min, resulting in the formation of a pale green-yellow solution, which could be kept frozen for about a month without loss of reactivity. The deprotonation of the polysaccharide was monitored by adding a small sample of the reaction mixture, with a syringe, to triphenylmethane[32]. A red color indicated the presence of excess of reagent. The addition of MeI at low temperature and the work-up procedure have been described[14–16]. It was useful, especially after the methylation of oligosaccharides, to purify the products by reversed-phase chromatography on a Silica-C18 cartridge (SEP-PAK C18, Waters). Before use, the cartridges were washed with 5 mL each of water, chloroform, methanol, and water. After application of the sample in Me_2SO–water (1:1), methylated monosaccharides were eluted with acetonitrile–water (1:9) and methylated oligosaccharides with an increasing proportion of acetonitrile. After concentration *in vacuo*, the fractions were analysed by g.l.c.-m.s.

In order to fragment the methylated polysaccharide, either methanolysis (methanolic 0.5M HCl for 1 h at 85°) or partial acid hydrolysis (0.03M acetic acid for 1 h at 100°) was used[22].

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