Carbohydrate Research, 208 (1990) 139–144
Elsevier Science Publishers B.V., Amsterdam

Structure of the K24 antigen of *E. coli* O83:K24:H, a polymer that consists of α-Kdo*p* and glycerol phosphate

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(Received February 23rd, 1990; accepted for publication, April 11th, 1990)

ABSTRACT

The structure of the K24 antigen of *Escherichia coli* O83:K24:H31 was elucidated by determination of composition and by ^{1}H-, ^{13}C-, and ^{31}P-n.m.r. spectroscopy of the polymer and of a Kdo-glycerol (Gro) glycoside, obtained by mild alkaline hydrolysis and subsequent incubation with alkaline phosphatase. The K24 antigen has the repeating unit →7)-α-Kdo*p*-(2→1)-Gro-(3-P. In the polymer, 56% of the repeating units are *O*-acetylated at C-4 of Kdo, ~28% at C-5 of Kdo, and ~16% are not acetylated.

INTRODUCTION

Invasive *E. coli* are surrounded frequently by capsules which enable the bacteria to overcome unspecific host defence, thus acting as virulence determinants[1,2]. The capsules consist of acidic polysaccharide (K antigens) which are subdivided into two groups on the basis of several properties, one of them being the nature of their acidic components[3–5]. The capsular polysaccharides of several uropathogenic *E. coli* contain 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), either in combination with ribose (*e.g.*, K13, K19, and K74)[6–8], rhamnose (K12)[9], 2-acetamido-2-deoxyglucose (K15)[5], or 2-acetamido-2-deoxygalactose (K14)[10]. Most of these polysaccharides are *O*-acetylated, and the acetyl groups are (part of) serological epitopes. We now report on the primary structure of the K24 antigen, which consists of Kdo and glycerol phosphate. This unusual combination relates the K24 antigen to the complex teichoic acids of Gram-positive bacteria.

RESULTS AND DISCUSSION

Isolation and characterisation of the antigen. — The capsular K24 antigen, isolated[6,11] from the dialysable culture of *E. coli* strain H45 (O83:K24:H31) (Freiburg collection No. 21742) in a yield of 190 mg/L, consisted of Kdo, glycerol, phosphate, and acetate in the molar ratios 1:1:1:0.85. The ^{31}P-n.m.r. spectrum of the K24 antigen indicated the presence of phosphodiester linkages (+0.91 p.p.m.; reference H_3PO_4).

Periodate oxidation. — Treatment of the K24 antigen with sodium metaperiodate destroyed ~20% of the Kdo residues, whereas treatment of the *O*-deacetylated (hy-

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0008-6215/90/$ 03.50

droxylamine–ethanol) polymer destroyed all of the Kdo. When the product of the latter reaction was reduced with sodium borohydride and then hydrolysed (30mM acetic acid, 1 h, 100°), a compound was obtained which reacted with the thiobarbituric acid reagent[12] and had an electrophoretic mobility (M_{Kdo} 1.64) the same as that of 2-keto-4-hydroxybutyrate. This product, which represented C-1/4 of Kdo, indicated that oxidative cleavage had occurred at the C-4–C-5 bond of Kdo, which was present as a 7- or 8-linked pyranoside.

Isolation of a Kdo-glycerol glycoside (Kdo-Gro). — Treatment with 0.5M NaOH (5 h, room temperature) degraded the K24 antigen. After incubation with alkaline phosphatase, the product, purified by chromatography on a column (2 × 60 cm) of Trisacryl GF O5M by elution with water, had M_{Kdo} 0.8, consisted of equimolar amounts of Kdo and glycerol, and was non-reducing.

^{13}C-N.m.r. spectroscopy. — The spectra of *O*-deacetylated K24 antigen (K24d) and Kdo-Gro were compared with those of the methyl α- and β-glycosides of Kdo, *sn*-glycerol 3-phosphate (Gro-3-P, at pH 7), and the K13 capsular polysaccharide of *E. coli* which contained 7-linked Kdo. The tentative assignments and the results of the attached proton test (ATP)[13,14] are shown in Table I. The signals at δ 35.2 (K24d) and 35.0 (Kdo-Gro) are characteristic of C-3 of Kdo*p*. Comparison of the spectrum of the K24 antigen with that of α-Kdo*p*-OMe (see below) showed shifts of +4.6 (from δ 70.2 to 74.8) and −1.1 p.p.m. for the signals assigned to C-7 and C-6, respectively, of Kdo. Each signal showed C,P-coupling, indicative of phosphate substitution. These changes were not observed in the spectrum of Kdo-Gro, in which Kdo is terminal and unsubstituted. One of the secondary carbon resonances (assigned as C-1) of the glycerol residue of *O*-deacetylated K24 antigen exhibited a shift of +2.2 p.p.m. (from δ 63.1 to 65.3), when compared with that of C-1 of the *sn*-Gro-3-P. The lack of C,P-coupling indicated that the substitution was not by phosphate but by Kdo. C,P-Coupling of the signals assigned to C-2,3 and a shift of the C-3 signal showed that, in the K24 polysaccharide, C-7 of Kdo and C-3 of Gro were connected by a phosphodiester bridge. This interpretation accorded with the data of the periodate oxidation and the alkali-mediated fragmentation.

Anomeric configuration. — Table II shows the n.m.r. data for H-3*e* and H-3*a* of Kdo obtained from the spectra of *O*-deacetylated K24 antigen, the K12, K19, and K16 capsular polysaccharides[7,9,15] of *E. coli*, and the methyl esters[16,17] of α- and β-Kdo*p*-OMe. The data showed that, in the K24 antigen, the Kdo was α. This inference was corroborated by a negative Cotton effect[18,19] at 217 nm (data not shown).

Configuration of the glycerol residue. — Attempts to release the glycerol phosphate unit from the K24 polymer without phosphate migration failed and it was not possible to determine the configuration at C-2 of the glycerol phosphate moiety.

O-*Acetyl substitution.* — Whereas the n.m.r. spectrum of *O*-deacetylated K24 antigen contained one signal per C, most signals arising from the Kdo residue in the K24 antigen appeared as three lines with constant relative intensities of 0.16:0.28:0.56, when measured in the inverse-gated mode[20,21] (Fig. 1). In each set of signals, the signal with the lowest intensity had the same chemical shift as the corresponding signal for *O*-deacety-

TABLE I

^{13}C-N.m.r. data (δ) and tentative assignments

	K24d	$J_{C,P}$	*Kdo-Gro*	*K24-1*	*K24-2*	*K24-3*	*Kdo-OMe*		*Gro-3-P*	*K13*
							α	β		
Kdo	C-1 175.8 (−)		176.2	176.0	174.8	174.5	175.1	173.5		174.2
	C-2 101.3(−)		100.8	101.3	101.2	101.1	101.0	101.8		102.8
	C-3 35.2 (−)		35.0	35.2	35.6	32.6	35.2	35.8		35.6
	C-4 67.0 (+)		67.0	67.0	66.3	70.8	67.2	68.3		68.4
	C-5 66.6 (+)		66.8	66.6	69.8	65.0	66.7	66.3		66.4
	C-6 71.1 (+)	5.7	72.0	71.1	69.5	70.9	72.2	74.3		73.5
	C-7 74.8 (+)	3.4	70.1	74.8	74.8	74.6	70.2	69.9		76.1
	C-8 62.2 (−)		63.8	62.2	61.5	62.2	63.9	64.9		63.6
Glycerol (Gro)	C-1 65.3 (−)		65.0	65.3	65.3	65.3			63.1	
	C-2 70.6 (+)	7.9	72.2	70.6	70.6	70.6			72.0	
	C-3 68.2 (−)	3.4	63.6	68.2	68.2	68.2			65.5	
Ac	C-1				175.6	175.6				
	C-2				21.8	21.9				

TABLE II

Chemical shifts (δ, p.p.m.) and coupling constants (J, Hz) of the resonances of H-3*e*,3*a* in Kdo of the K24, K16, K19, and K12 antigens, and the methyl esters of α- and β-Kdo*p*-OMe.

	*H-3*e	*H-3*a	$\Delta\delta$	$J_{3a,3e}$	$J_{3e,4}$	$J_{3a,4}$	*Configuration*
β-Kdo*p*-OMe[16,17]	2.38	1.74	0.64	12.2	4.3	12.2	β
α-Kdo*p*-OMe[16,17]	2.06	1.79	0.27	15.0	6.0	14.0	α
K12 antigen[9]	2.45	1.99	0.46	11.5	4.0	11.5	β
K19 antigen[7]	2.56	1.90	0.66	12.3	5.0	12.3	β
K16 antigen[15] (-Ac)	2.17	1.82	0.35	12.4	4.6	12.4	α
K24 antigen (-Ac)	2.00	1.70	0.30	12.6	4.6	12.6	α

lated K24 antigen. Thus, the spectrum of the K24 antigen was considered as the superposition of spectra due to the non-acetylated repeating unit (K24-1 in Table I, 16% of the total) and two different *O*-acetylated repeating units (K24-2, 28%; K24-3, 56%). Comparison of the chemical shift data for K24-2 and K24-3 with those of K24-1 (identical with those of *O*-deacetylated K24 antigen, K24d in Table I) showed that the most prominent group of signals were those due to C-3 of Kdo (δ 32.6–35.6). The signals at δ 35.2 and 32.6 were assigned to C-3 of Kdo in K24-1 and K24-3, respectively, and reflected the shift due to 4-acetylation. The signal at δ 35.6 was assigned to C-3 of Kdo in K24-2 and reflected a weak shift due to 5-acetylation. Similarly, comparison of the data in Table I revealed shifts of 3.8 and 3.2 p.p.m., respectively, for the resonances of C-4 of Kdo in K24-3 and C-5 of Kdo in K24-2. These results indicated that, in the K24 antigen, 16% of the repeating units were not acetylated, 56% were *O*-acetylated at C-4 of Kdo*p*, and 28% at C-5 of Kdo*p*. Double acetylation at C-4,5 did not occur.

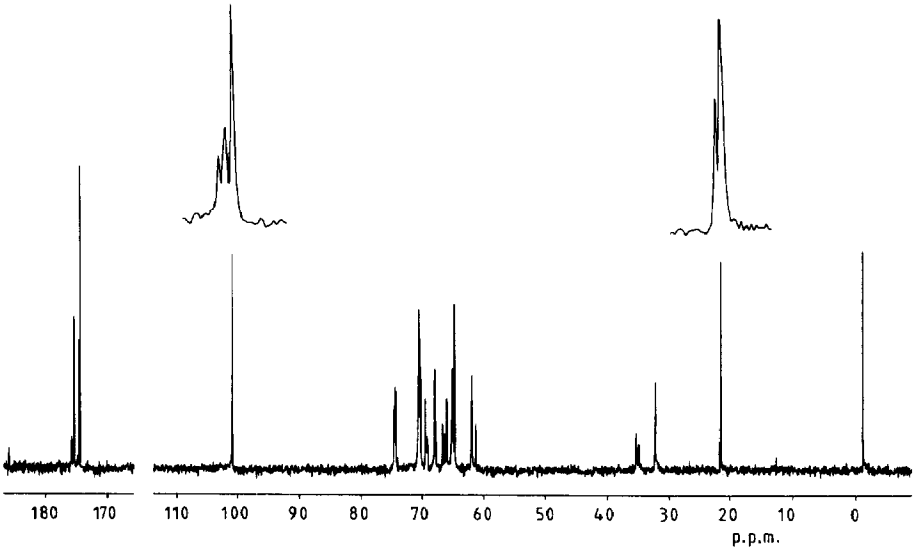
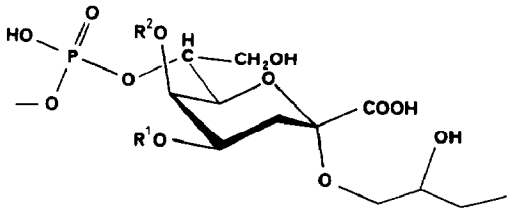


Fig. 1. Inverse-gated decoupled ^{13}C-n.m.r. spectrum (185–165 and 115–0 p.p.m.) of a solution of the K24 polysaccharide in D_2O. The insets show the signals of C-2 of Kdo and $COCH_3$ spread with a factor of 1:12.5.

Structure of the K24 antigen. — From the above results, the repeating unit of the K24 antigen can be formulated as 1. With respect to *O*-acetylation, two possibilities can be envisaged, namely (*a*) a random sequence of repeating units with 16% unacetylated, 28% *O*-acetylated at C-5 of Kdo, and 56% *O*-acetylated at C-4 of Kdo; (*b*) a mixture of three polymers with 16% unacetylated, 28% with every Kdo 5-acetylated, and 56% with every Kdo 4-acetylated. If there was acetyl migration between C-4 and C-5 of Kdo, the polymer would be randomly *O*-acetylated.



$R^1 = H, R^2 = H$ (16%)
$R^1 = H, R^2 = Ac$ (28%)
$R^1 = Ac, R^2 = H$ (56%)

1

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* H45 (O83:K24:H31) was obtained from Drs. I and F. Ørskov (WHO Seruminstitute, Copenhagen) and grown to the late logarithmic phase in 10-L batches, which contained per L: $K_2HPO_4 \cdot 3H_2O$ (9.6 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 bacteria were precipitated from the liquid culture by the addition of 1 vol. of aqueous 2% cetyltrimethylammonium bromide[6,11] (Cetavlon). All of the 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 aqueous 80% phenol buffered to pH 6.5 with sodium acetate. The aqueous phases were centrifuged for 4 h at 100 000*g* and the supernatant solution was lyophilised. The residue was eluted from a column of Sephadex G-50 with water.

Isolation and purification of a Kdo-Gro glycoside. — A solution of the K24 antigen (50 mg in 5 mL of 0.5M NaOH) was kept for 5 h at room temperature, neutralised with Dowex 50 (H^+) resin, filtered, and then incubated for 12 h at room temperature with alkaline phosphatase (EC 3.1.3.1, 50 μL containing 35 units) in 50mM sodium carbonate buffer (pH 9.6). The product was purified by repeated elution from a column (2 × 60 cm) of Trisacryl GF05M with water.

Analytical methods. — Kdo was determined, after hydrolysis (0.5M trifluoroacetic acid, 30 min, 100°) by the thiobarbituric acid assay[12], phosphate by the ammonium molybdate assay[22], glycerol by the u.v. method (Boehringer Mannheim, manual), and acetate[23] by g.l.c. on Porapak QS. Components were quantified by ^{1}H- and ^{13}C-n.m.r. spectroscopy. High-voltage paper electrophoresis (pH 5.6, 42 V/cm, 60–90 min) was performed on Schleicher & Schüll 2043a paper. G.l.c. was performed with a Varian Aerograph 1400 instrument, equipped with an autolinear temperature programmer and a Hewlett–Packard 3340 integrator. The c.d. spectra were recorded with a Cary 61 spectropolarimeter at 20° (0.2-cm path length). N.m.r. spectra were recorded with a Bruker WM 300 spectrometer in the F.t. mode at 70° (^{1}H) and 25° (^{13}C) (external sodium 3-trimethylsilyl(2,2,3,3-$^2H_4$)propionate). The δ values are related to Me_4Si.

ACKNOWLEDGMENT

We thank Mrs. Helga Kochanowski for running the n.m.r. spectra.

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