Carbohydrate Research, 178 (1988) 191–201
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF THE CAPSULAR K3 ANTIGEN OF *Escherichia coli* O4:K3:H4, A POLYSACCHARIDE CONTAINING A 4-DEOXY-2-HEXULOSONIC ACID*

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(Received July 17th, 1986; accepted for publication, October 7th, 1986)

ABSTRACT

The K3-antigenic capsular polysaccharide (K3 antigen) of *Escherichia coli* contains L-rhamnose, a 4-deoxy-2-hexulosonic acid, and an *O*-acetyl group in the molar ratio of 3:1:1. The backbone consists of a →2)-*O*-α-L-rhamnopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→ repeating unit. Either one of the 3-linked L-rhamnopyranosyl residues of each repeating unit may be substituted at O-2 with a 4-deoxy-2-hexulosonic acid, an isomer of the furanosyl form of KDO, about 90% of which is acetylated at O-6. The 4-deoxy-2-hexulosonic acid residue is linked to the L-rhamnan backbone in a very labile linkage which is split by 1% acetic acid (30 min, 100°). The K3 polysaccharide has a molecular weight of ~38 000, corresponding to ~60 repeating units.

INTRODUCTION

Invasive *Escherichia coli* develop capsules which enable the bacteria to overcome unspecific host defenses, thus acting as virulence determinants[1]. The capsules (K antigens) consist of acidic polysaccharides which may be subdivided on the basis of chemical composition, physical properties, and mode of expression[1,2]. 3-Deoxy-D-*manno*-2-octulosonic acid (KDO) was found to be a major constituent of the capsular polysaccharides of many uropathogenic *E. coli*. It is most commonly present in the β configuration and the pyranose form, either linked at O-5 (in the K12, K14, and K15 antigens[3–5]), or linked at O-7 (in the K6, K13, K20, and K23 antigens[6–9]). More recently 8-linked KDO in the furanose form was found in the K95 antigen[10]. With the exception of one report[7], KDO in the pyranose form was found in the β configuration. When the structure of the K95 polysaccharide was reported, the anomeric configuration of KDO*f* could not be determined because of a lack of reference compounds. A recent comparative n.m.r. study[11] of glycosides of KDO*p* and KDO*f* now allowed to formulate β-KDO*f* as a constituent of the K95

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0008-6215/88/$ 03.50

antigen. The variety of ketodeoxy compounds in the capsular polysaccharides of *E. coli* is amplified by our finding of a 4-deoxy-2-hexulosonic acid as a constituent of the K3 antigen from *E. coli* O4:K3:H5.

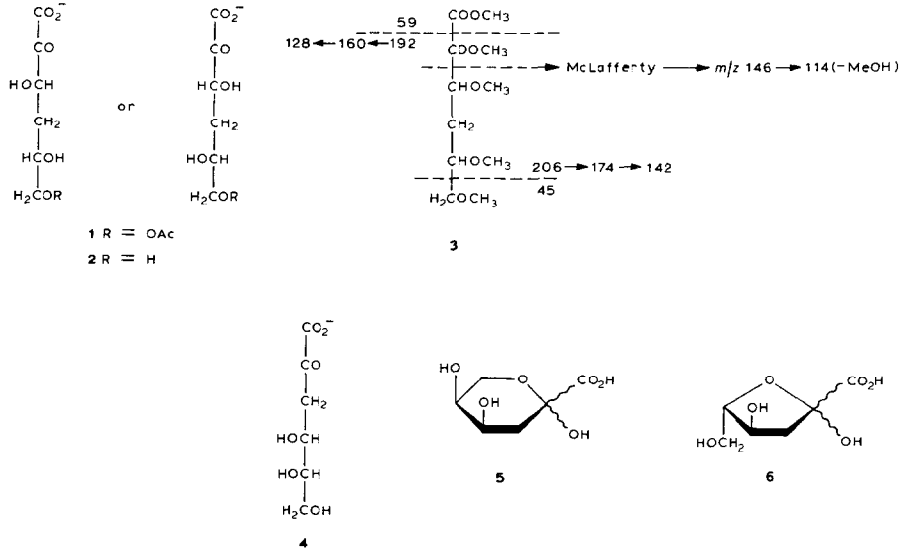
RESULTS AND DISCUSSION

In pilot experiments, it was found that the capsular K3 polysaccharide (K3PS) of *E. coli* is well expressed by agar-grown bacteria and much less by bacteria grown in liquid medium. *E. coli* U-4-41 (O4:K3:H5) was grown at the fimbrial restrictive temperature[12] of 20°. Growth on agar at that temperature suppressed fimbrial expression but, in contrast to many other K polysaccharides of invasive *E. coli*, permitted extensive formation of K3PS. After 16 h at 20°, the bacteria were harvested and extracted with phosphate-buffered saline solution (PBS). After removal of the bacteria by centrifugation, the supernatant was subjected to the isolation procedure[10,13] usually applied with low-molecular-weight K polysaccharides of invasive *E. coli*. Owing to the extreme sensitivity of K3PS to acid (*vide infra*), the phenol-extraction step was omitted from the purification procedure. K3PS was obtained in a yield of ~2.5 mg per agar plate (14 cm), corresponding to ~2–3% based on bacterial dry-weight.

The polysaccharide had an average molecular weight of ~38 000, as determined by the method of Yphantis[14]. It consisted of rhamnose, an acidic component **2**, and *O*-acetyl groups. On the basis of chemical and n.m.r. data (*vide infra*), an approximate molar ratio of Rha:**2**:OAc of 3:1:1 was obtained. The L configuration of the rhamnose units was established by g.l.c. of the derived (+)-2-octyl 2,3,4-tri-*O*-acetylrhamnosides, which exhibited the same pattern of peaks as the corresponding derivatives of authentic L-rhamnose (*T* 1.725, 1.960, 2.375, and 2.590, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol).

In immunoelectrophoresis, K3PS exhibited a precipitation line with Cetavlon[15] and with an anti-K3 antiserum, but not with an anti-O4 antiserum, indicating serological K3 specificity. When K3PS was kept for several hours at room temperature in aqueous solution (pH 5–6), its electrophoretic mobility, as well as reactivity with Cetavlon and anti-K3 antiserum, was gradually diminished. This showed that a charged constituent in an acid-labile linkage was (part of) an epitope of the polysaccharide. Treatment of the polysaccharide with 1% acetic acid (30 min, 100°) resulted in the complete liberation of **1** with the formation of a neutral polysaccharide, consisting only of L-rhamnose. The latter (K3PS $- H^+$) showed only a very weak reactivity against an anti-K3 antiserum.

K3PS was methylated[16,17] and then hydrolyzed, and the products were converted into alditol acetates. G.l.c. (170° on ECNSS-M) showed three components in approximate equimolar ratio (*T*, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 0.89, 1.00, and 1.65) the m.s. of which showed them to be 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol, and 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylrhamnitol. No derivatives of **1** were detected.



When K3PS − H^+ was subjected to methylation analysis, only two products were found in the ratio of 1:2; they were identified by m.s. as 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol and 1,3,5-di-*O*-acetyl-2,4-di-*O*-methylrhamnitol. These results indicated that K3PS is a L-rhamnan consisting of a sequence of three L-rhamnose units, one linked at O-2 and two linked at O-3, in which one of the rhamnose units is substituted by **1**.

Both polysaccharides were oxidized with sodium periodate subsequent to incubation at pH 11 (16 h, 4°) for the removal of *O*-acetyl groups, and then treated with $NaBH_4$. Analysis of the products excluded from Bio-Gel P-2 indicated that in both polysaccharides ~30% of the rhamnose units had been destroyed. Paper electrophoresis and n.m.r. spectrometry showed that the acidic component (**2**, *vide infra*) was present in the oxidized K3PS in apparently unaltered amounts. These results indicated that **1** is linked to one of L-rhamnose units linked at O-3 in K3PS, and that it is periodate resistant when bound as glycoside.

The ^{13}C-n.m.r. signals of K3PS − H^+, which constitutes the backbone of the native polysaccharide, were tentatively assigned by comparisons with the reported values for L-rhamnose[18] and with the values calculated for 2-*O*- and 3-*O*-linked α-L-rhamnopyranosyl residues; in this case[19], the increments were +7 p.p.m. for a glycosidic linkage at C-1, +9 p.p.m. for a substitution at any of the other C atoms, and −1 p.p.m. for the C atoms adjacent to substitutions (see Table I). The data obtained are in agreement with a trisaccharide repeating-unit consisting of one 2-*O*-linked L-rhamnopyranosyl residue and two 3-*O*-linked L-rhamnopyranosyl residues. A poly-D-rhamnan having the same linkage sequence as that of K3PS − H^+ has recently been described[20] as the O-specific polysaccharide of *Pseudomonas syringae* pv. *morsprunorum* C28. Its ^{13}C-n.m.r. data, which agree with those of K3PS − H^+, are included in Table I.

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS (δ) OF K3PS – H^+, COMPARED WITH THE REPORTED VALUES FOR THE L-RHAMNAN FROM THE LPS OF *P. syringae* PV *morsprunorum* C28 (Psm-C28), THE CALCULATED VALUES FOR 2-*O* AND 3-*O*-LINKED α-L-RHAMNOPYRANOSYL RESIDUES, AND REFERENCE α-L-RHAMNOSE

Polysaccharide or sugar	*Residue*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
K3PS – H^+	→2)-L-Rha	101.7	78.7	70.8	73.1	70.2	17.5
	→3)-L-Rha′	102.8	70.8	78.9	72.2	70.1	17.5
	→3)-L-Rha″	103.0	70.8	79.0	72.5	70.1	17.5
Psm-C28[a]	→2)-D-Rha(C)	101.1	78.4	70.1	72.8	69.7	17.0
	→3)-D-Rha(A)	102.2	70.4	78.2	71.8	69.7	17.0
	→3)-D-Rha(B)	102.2	70.4	78.2	72.0	69.4	17.0
α-L-Rhamp[b]	→2)-L-Rha	101.1	78.9	71.1	73.3	69.4	18.0
	→3)-L-Rha	102.0	69.9	80.0	72.3	69.4	18.0
α-L-Rhamnose[c]		95.0	71.9	71.1	73.3	69.4	18.0

[a]Ref. 20. [b]Calculated values[19]. [c]Ref. 18.

The ^{13}C-n.m.r. spectrum of native K3PS (containing **2**) exhibited additional signals, the most conspicous ones being at δ 175.6, 108.6, and 48.1. Similar signals (at δ 176.4, 110.2, and 45.5) had previously been detected for the 3-deoxy-*manno*-oct-2-ulofuranosylonic acid residue of the K95 antigen[10]. They indicated that **1** has a chemical structure similar to that of the latter compound. The spectrum of K3PS was too complex for determining which of the L-rhamnopyranosyl residues was substituted by **1**.

In a gated-decoupling experiment[18,21,22], the signal for C-1 of the L-rhamnosyl residues was found to have $J_{1,2}$ 170–175 Hz, indicating the α configuration. This was in agreement with the chemical shift of C-5 (δ 70.1–70.2) for all L-rhamnosyl residues (Table I). The signal for C-5 of a β-L-rhamnosyl residue is at δ 73.4. In the gated-decoupling experiment, the signal at δ 108.6, due to C-1 of **1**, was a singlet, indicating that **1** is a ketose derivative.

The ^{1}H-n.m.r. spectra of K3PS – H^+ (Fig. 1) exhibited three anomeric signals (at δ 5.14, 4.99, and 4.91), due to the L-rhamnosyl residues of the trisaccharide repeating unit. The strong signals at δ 1.20–1.30 are due to the CH_3-6 groups of these residues. The spectrum of K3PS (Fig. 1) contains five poorly resolved anomeric signals (at δ 5.14, 5.05, 5.03, 4.99, and 4.91); they correspond to unsubstituted L-rhamnosyl residues and L-rhamnosyl residues substituted with **1**. No distinct substitution pattern could be derived from the spectrum. In contrast to the spectrum of K3PS, that of K3PS – H^+ contained only very weak signals due to *O*-$COCH_3$ and to H-4 of **1** (*vide infra*), which indicated that OAc is part of **1**.

K3PS was treated with 1% acetic acid (100°, 30 min), and liberated **1** was separated from the residual polysaccharide (K3PS – H^+) by chromatography on a

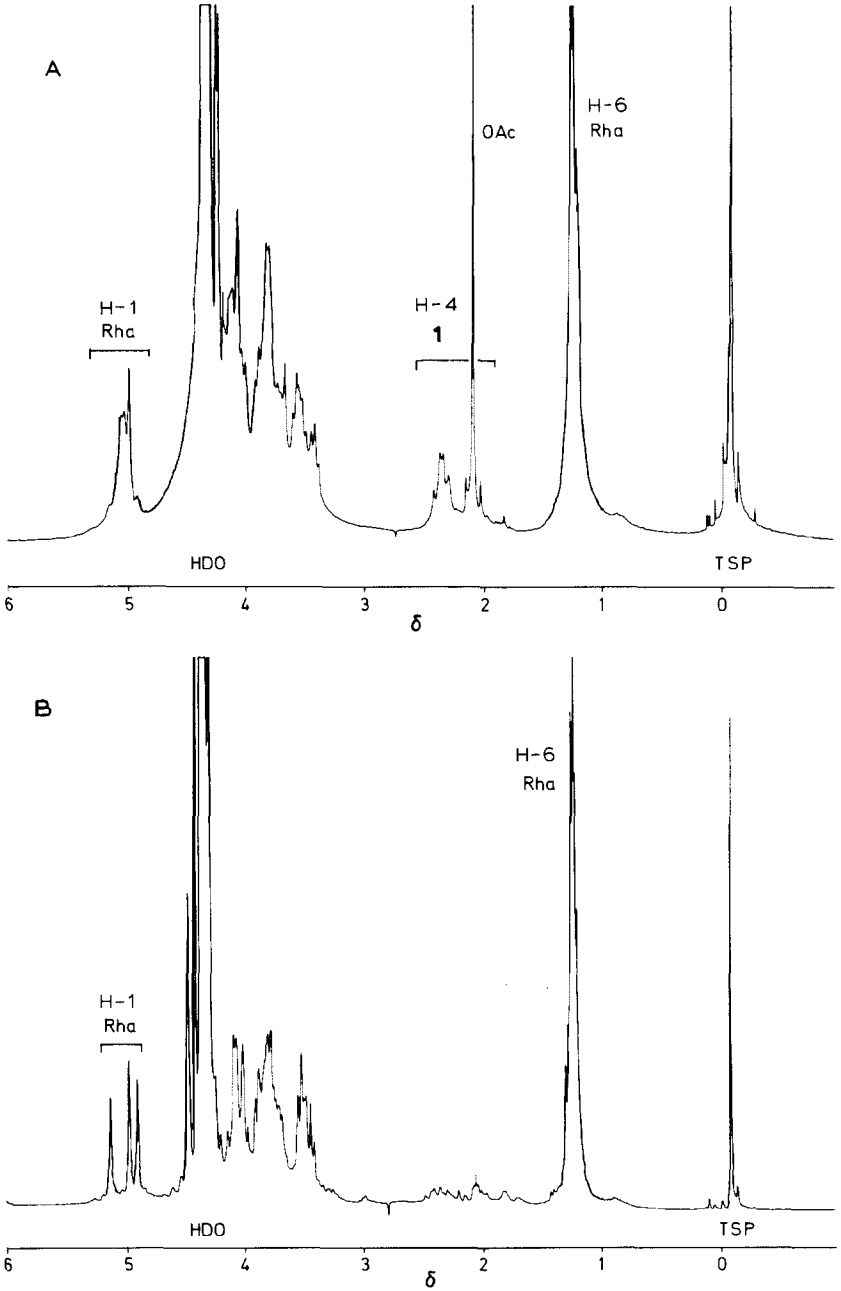


Fig. 1. ^{1}H-N.m.r. spectrum (300 MHz) of: (A) Native K3PS, (B) K3PS after mild acid treatment, K3PS $-$ H^+. Solutions in D_2O, at 70° with external sodium 4,4-dimethyl-4-sila(2,3-$^2H_4$)pentaonate.

Bio-Gel P-2 column. The fraction having K_d 0.5 containing **1** was subjected to paper electrophoresis (40 v/cm, pH 5.4, 60 min). Staining with the alkaline $AgNO_3$ reagent revealed a major compound **1** (M_{KDO} 1.15) and a minor compound **2** (M_{KDO} 1.25). By treatment at pH 11 (16 h, 4°), **1** was converted into **2**. Both compounds did not stain with the thiobarbituric acid reagent.

After elution from preparative paper electropherograms and subsequent purification by chromatography on a Bio-Gel P-2 column, **2** (M_{KDO} 1.25) was reduced with $NaBD_4$, and the product methylated[16,17] to yield **3** which gave one peak in g.l.c. The e.i.–m.s. and the fragmentation pattern of **3** are shown in Fig. 2. The absence of ions at *m/z* 162 and 130, characteristic of a fragmentation between C-4 and C-5 preponderant in KDO and 3-deoxy-L-*erythro*-2-hexulosonic acid[10] (**4**), is in agreement with a 4-deoxy group in **1** and **2**. The fragment at *m/z* 146 appears to be derived from a McLafferty rearrangement[23]. The corresponding signal was missing from the m.s. of **4**. C.i.–m.s. of **3**, with ammonia as the reagent gas, gave a peak for the *quasi*-molecular ion $(M + NH_4)^+$ at *m/z* 269, indicating a mol. wt. of 251, in agreement with structure **3** as the deutero-reduced and methylated derivative of a 4-deoxy-2-hexylosonic acid. The non-reactivity of **1** and **2** with the periodate–thiobarbituric acid reagent[24] agrees also with this structure.

The ^{13}C-n.m.r. signals of **2** and the pyranosidic (**5**) and furanosidic (**6**) ring

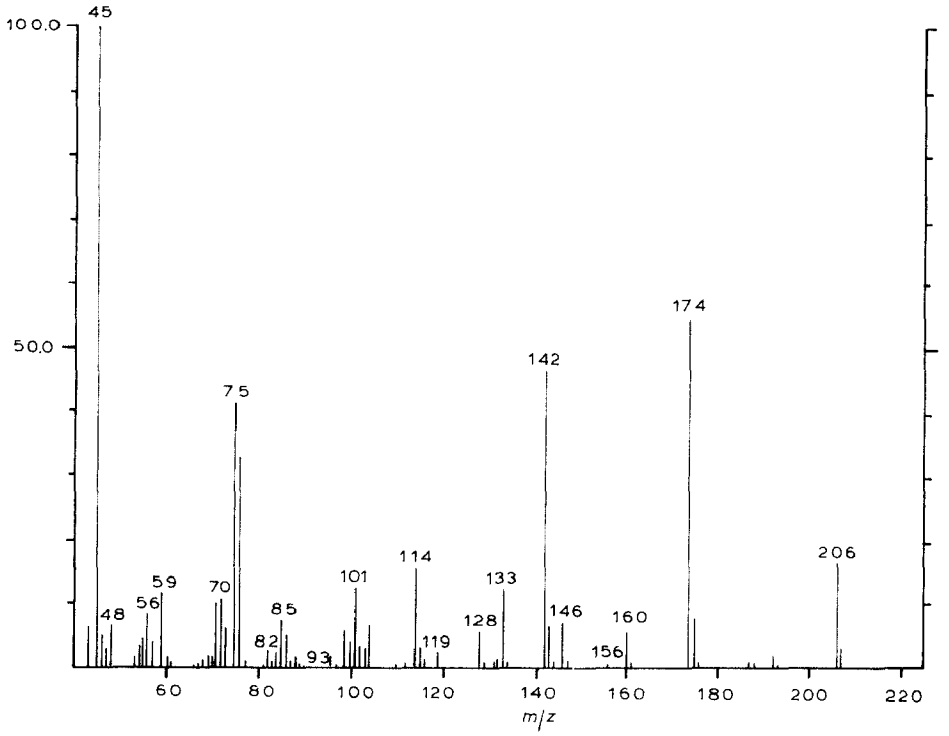


Fig. 2. Mass spectrum of sodium borodeuteride-reduced and methylated **2** (**3**).

TABLE II

^{13}C-N.M.R. CHEMICAL SHIFTS (δ) OF THE MAJOR AND MINOR ANOMERS OF FREE **2** IN THE FURANOSE FORM, **1** BOUND IN NATIVE K3PS, **2** BOUND IN *O*-DEACETYLATED K3PS, AND OF THE MAJOR AND MINOR ANOMERS OF 3-DEOXY-L-*erythro*-2-HEXULOSONIC ACID FROM THE K95 ANTIGEN[a] IN THE FURANOSE (**6**) AND THE PYRANOSE FORM (**5**)

Atom	*Free compound* **2**[b]		*Compound* **1** *bound in K3PS*	*Compound* **2** *bound in O-deacetylated K3PS*	*Compound* **6**		*Compound* **5**	
	Major	*Minor*			*Major*	*Minor*	*Major*	*Minor*
C-1	177.6	177.2	175.6	175.8	177.6	177.5	177.2	176.6
C-2	104.9	105.2	108.6	108.9	104.7	104.2	97.4	97.1
C-3	76.9	76.9	76.4	76.5	44.9	44.1	34.8	36.7
C-4	46.6	46.3	48.1	47.8	72.4	72.8	68.3	n.d.
C-5	82.2	81.5	79.6	81.4	87.5	87.7	65.9	n.d.
C-6	65.8	66.0	69.3	66.6	63.2	62.7	65.3	n.d.

[a]Ref. 10. [b]Furanose form.

forms of **4** are compared in Table II. The aforementioned results of the methylation analysis were used for assignments of the signals from C-3 and C-4. With the exception of C-1, the signals of **2** and **6** appeared at a field lower than those of 3-deoxy-L-*erythro*-2-hexulopyranosonic acid[10] (**5**) (and KDO in the pyranose form, not shown), which indicated that, in aqueous solution, only the furanose form of free **2** could be detected. The signals of **2** bound in K3PS (Table II) indicated that, in the polysaccharide, **2** was present also in the furanose form. Since it was shown that **2** occurs in K3PS as an *O*-acetyl derivative (**1**), the position of the *O*-acetyl group was ascertained by a comparison of the chemical shifts due to **1** and **2** in the spectra of K3PS and of its *O*-deacetylated form (K3PS-dOa). As shown in Table II, the most predominant shifts were those for the signals of C-6 (−2.7 p.p.m.) and C-5 (+1.8 p.p.m.). This indicated that **1** was the 6-*O*-acetyl derivative of **2**. The formulation of **1** and **2** as furanosides was corroborated by the chemical shifts of C-5 of bound (δ 81.4) and free (δ 82.2, 81.5) **2**, when compared to the C-5 signal of **6** (δ 87.5, 87.7), of **5** (δ 69.5) (Table II), and of KDO in the pyranose form[6,25] (δ 66–77). The difference in the chemical shifts for C-5 of **1** and **6** is probably due to the different shielding effect of a neighboring methylene group (C-4 of **1**) *vs.* a tert. hydroxyl group (at C-4 of **6**).

The ^{1}H-n.m.r. spectrum of **2** lacked a signal in the anomeric region (δ 4.5–6.0), indicating a ketose derivative. The region of δ 1.9–2.5 contained eight signals of methylene protons indicating, in agreement with the results shown in Table II, the presence of only two tautomers of **1**. To assign these signals, a 2D–^{1}H-n.m.r. spectrum was performed in the COSY mode[26]. As shown in Fig. 3, signals 1, 2, 7, and 8 were coupled and, according to their intensities, arose from the minor configuration. Correspondingly, signals 3, 4, 5, and 6 constituted a group of coupled signals arising from the major configuration. The signals illustrated in Fig. 3 and reported in Table III together with their coupling constants and assignments arose

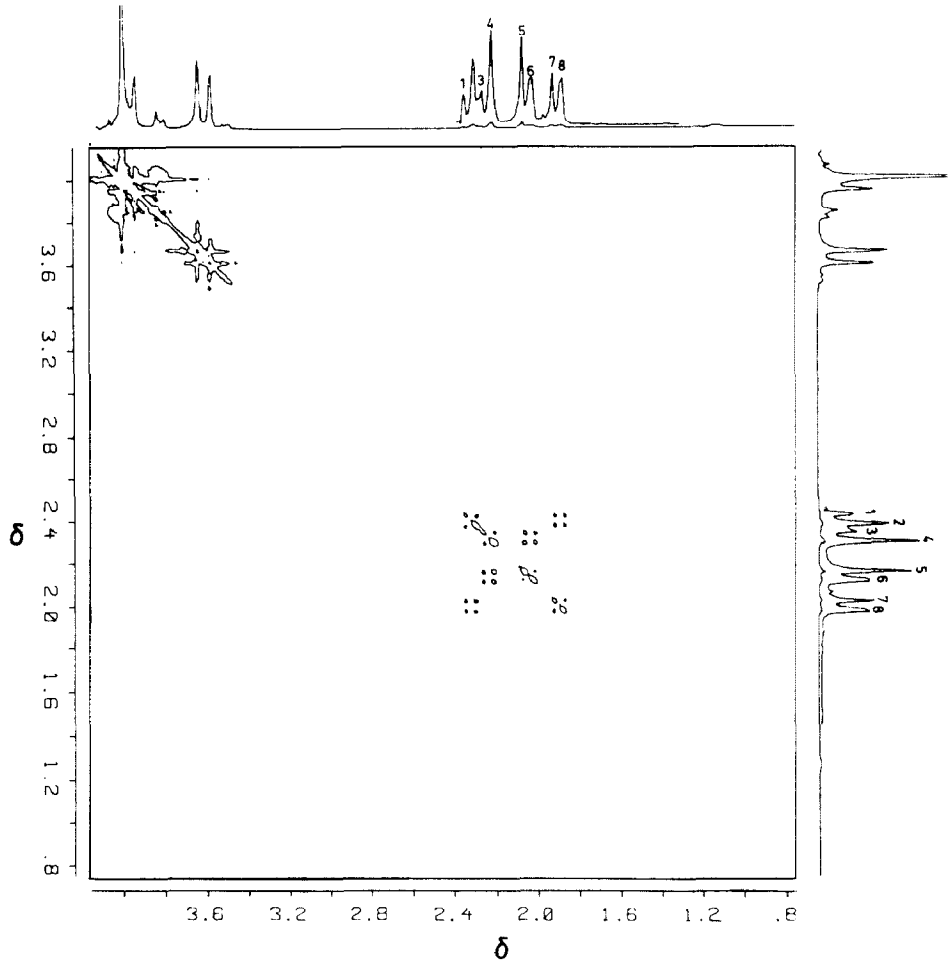


Fig. 3. Contour plot of the 2-D, shift correlated ^{1}H-n.m.r. experiment of **2** [90 – t_1 – 45 pulse sequence ('COSY-45')]. A total of 256 f.i.d. (spectral width 1024 Hz) were acquired in quadrate detection, each with 64 transients. For transformation of the data matrix (1024 × 256 points), Gaussian multiplication was applied in the $F1$, and the sine-bell window function in he $F2$-dimension.

TABLE III

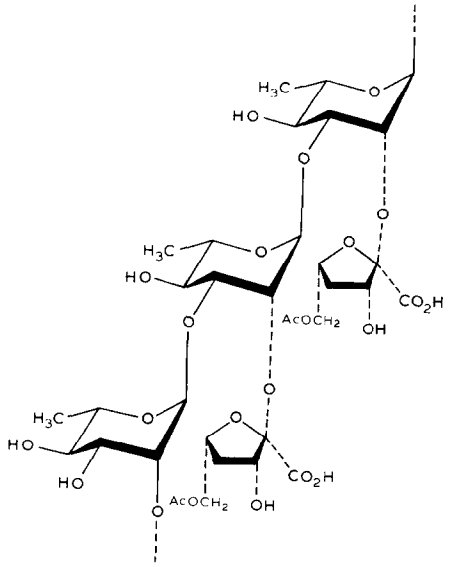
^{1}H-N.M.R. CHEMICAL SHIFTS, SPLITTING, AND GEMINAL COUPLING OF THE SIGNALS FROM THE COSY EXPERIMENT[a] OF **2**

Anomer	*Chemical shift (δ)*	*Splitting*	*Signals*	$J_{4,4}$ *(Hz)*
Major	2.32	d	3,4	14.4
Major	2.13	d	5,6	14.4
Minor	2.40	d	1,2	14.4
Minor	2.00	d	7,8	14.2

[a]In D_2O, at 70° and pH 7.

from the geminal C-4 protons of **2**. No signals due to vicinal coupling of H_2-4 with H-3 and H-5 could be detected. Since in a *threo* configuration H-3 and H-5 of **1** are both *gauche* to H_2-4, a vicinal ($J_{4,3}$ and $J_{4,5}$) coupling would be very small and probably not detectable. The lack of this type of coupling prompts us to suggest that **1** has a *threo* configuration.

On the basis of the foregoing results, the capsular K3 polysaccharide K3PS can be formulated as **7**. The complexity of the ^{1}H-n.m.r. spectrum of K3PS indicated that either one of the two L-rhamnopyranosyl residues linked at O-3 may be substituted by **1**. Since **1** is linked to the L-rhamnan backbone (K3PS $- H^+$) through a very labile linkage and is probably partially split off from K3PS during isolation and purification, the degree of substitution of native K3PS by **1** remains unknown. The relative amounts of **1** and **2** obtained after mild acid treatment of K3PS and paper electrophoresis, suggest more than 90% of the substitution is by **1**.



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To assess the role of **1** and **2** in the serological activity of the capsular K3 polysaccharide, the reaction of K3PS in the ELISA test was inhibited with K3PS itself, *O*-deacetylated K3 containing **2** instead of **1**, and K3PS $- H^+$ lacking both **1** and **2**. Removal of the *O*-acetyl groups reduced the serological K3 activity of K3PS by ~50%, and complete removal of **1** and **2** diminished the reactivity by ~80%. Thus, 6-*O*-acetyl-4-deoxy-2-hexulosonic acid (**1**) is the immunodominant epitope of the capsular K3 polysaccharide (**7**) of *E. coli*.

EXPERIMENTAL

Methods. — L-Rhamnose was determined with the cysteine reagent[27] and as

alditol acetate by g.l.c. on an ECNSS-M column. Acetyl content was determined by g.l.c.[28] on a Poropak QS column. Component **1** was quantitatively determined by ^{1}H-n.m.r. spectroscopy.

For the determination of the absolute configuration of rhamnose, the polysaccharide (10 mg) was hydrolyzed with 0.5M H_2SO_4 (1 mL) for 2 h at 100°. The hydrolyzate was made neutral and the rhamnose isolated by high-voltage paper electrophoresis (Schleicher and Schüll paper 2043b, 42 V/cm, pH 5.3, 90 min). The sample was heated with (+)-2-octanol (1 mL) and trifluoroacetic acid (1 drop, 15 h, 100°). The reagents were removed by codistillation with water under reduced pressure (55°) and the residue was acetylated with acetic anhydride (0.5 mL) and pyridine (0.7 mL) for 30 min at 100°. After removal of the reagents by codistillation with toluene, the product was analyzed by g.l.c. on columns of ECNSS-M (150°) and 2.5% SE (170°).

G.l.c. was performed with a Varian Aerograph Series 1400 instrument equipped with an autolinear temperature programmer, a Hewlett–Packard 3380 integrator, and a CB CP SIL 5 column (25 m × 0.25 mm), using He as the carrier gas. N.m.r. spectra were recorded with a Bruker WM 300 spectrometer in the F.t. mode at 70° [external standard: sodium 4,4-dimethyl-4-sila(2,3-$^2H_4$)pentanoate]. The ^{13}C values were corrected by −1.3 p.p.m. (based on the signal of 1,4-dioxane, δ 67.4, related to Me_4Si), and the ^{1}H-values by −0.07 p.p.m. (1,4-dioxane signal at δ 3.7).

Bacteria and cultivation. — *E. coli* U-4-41 (O4:K3:H5) was obtained from Drs. I. and F. Ørskov (Copenhagen) and grown for 16 h at 37° on TT agar plates containing (per L) $Na_2HPO_4 \cdot 12\ H_2O$ (2.3 g), NaCl (3 g), D-glucose (3 g), tryptose (7.5 g), tryptone (7.5 g), beef extract (5 g), yeast extract (5 g), and agar (1.5 g).

Isolation and purification of the capsular polysaccharide. — The bacteria from 100 agar plates (14 cm) were suspended in phosphate-buffered saline solution (pH 7.5, 200 mL) and the suspension was agitated (Omnimixer, 5 × 5 min) at 37°. After a further 35 min at 37°, the bacteria were removed by centrifugation (20 min, 16 000 *g*). The supernatant solution was dialysed and lyophilized and the residue dissolved in 0.08M NaCl (160 mL). The capsular polysaccharide (K3PS) was precipitated with 1 g (hexadecyl)trimethylammonium bromide (Cetavlon) in 0.08M NaCl (40 mL). The precipitate was purified by three cycles of precipitation from aqueous solution (mM NaCl) with ethanol (85% final concentration). The final precipitate was dialyzed against de-ionized water and centrifuged for 4 h at 105 000*g*. The supernatant solution was lyophilized. The yield of K3PS from 100 large agar plates was ~250 mg, corresponding to ~2% based on bacterial dry-weight.

O-Deacetylation. — A solution of K3PS (100 mg) in dilute aqueous NH_4OH (5 mL, pH 11) was kept for 16 h at 4°, then made neutral, dialyzed against de-ionized water, lyophilized, and the residue chromatographed on Sephadex G-50. After lyophilization, 75 mg of *O*-deacetylated K3PS were obtained.

Isolation of **1** *and* **2**. — A solution of K3PS (100 mg) in 1% acetic acid (5 mL) was heated for 30 min to 100°. After cooling, **1** was obtained from the neutralized

hydrolyzate by chromatography on a Bio-Gel P-2 column (10 × 900 mm). The material having K_d 0.5 was collected. Paper electrophoresis (Schleicher and Schüll paper 2043b, 42 V/cm, pH 5.3, 90 min) showed **1** as the major spot (M_{KDO} 1.15). Compound **1** was *O*-deacetylated, as described above, to yield **2**. In the purification of **2**, the dialysis step was omitted. On paper electrophoresis, **2** had M_{KDO} 1.25, corresponding to the minor spot observed on paper electrophoresis of the neutralized hydrolyzate (*vide supra*).

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