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COMPARATIVE STRUCTURAL ELUCIDATION OF THE K18, K22, AND K100 ANTIGENS OF *Escherichia coli* AS RELATED RIBOSYL-RIBITOL PHOSPHATES

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ABSTRACT

The structures of the capsular K18, K22, and K100 antigens of *E. coli* O23:K18:H15, O23:K22:H15, and O75:K100:H5, respectively, were elucidated by determination of composition, ^{1}H-, ^{13}C-, and ^{31}P-n.m.r. spectroscopy, periodate oxidation, alkaline hydrolysis followed by incubation with alkaline phosphatase, and methylation analysis of the polymers and their neutral fragmentation products. The polymers are poly(ribosyl-ribitol phosphates) related to the capsular antigen of *H. influenzae* (*Hi*b). The K22 antigen has the repeating unit -P-2)-β-Rib-(1→2)-RibOH-(5-, and the K18 antigen has the same polymer chain with partial 3-*O*-acetylation of the ribose moiety. The K100 antigen consists of repeating units of -P-3)-β-Rib-(1→2)-RibOH-(5- and seems to have a secondary structure different from that of the other antigens. Together with the *Hi*b capsular antigens, the structure of which was reported as -P-3)-β-Rib-(1→1)-RibOH-(5-, these capsular antigens represent a structurally related group of capsular polymers.

INTRODUCTION

The capsules of *Escherichia coli* consist of acidic (K) polysaccharides, which, on the basis of molecular weight, nature of the acidic constituent, or mode of surface expression, can be divided into two groups[1–3]. Many low-molecular-weight (group II[3]) capsular antigens contain phosphate diester bridges in the polymer chain. Thus, they resemble the teichoic acids of Gram-positive bacteria. The K51 antigen is a poly(2-acetamido-2-deoxy-D-glucose phosphate)[4], the K2a antigen is a poly(α-galactosyl-glycerol phosphate) and the K2ab antigen is an *O*-acetylated derivative of the K2a antigen[5,6]. The K100 antigen was reported[7] to be a poly[3-β-ribosyl-(1→2)-ribitol 5-phosphate], although details of the structural elucidation do not seem to be available in the literature. This antigen is related to poly[3-β-(1→1)-ribitol 5-phosphate], the capsular antigen of *H. influenzae* type b (*Hi*b)[8]. We now report on the K18, K22, and K100 antigens, which are related poly(ribosyl-ribitol

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phosphates), and compare these structures with that of the *Hib* capsular antigens.

RESULTS AND DISCUSSION

Isolation and characterisation. — The capsular K18, K22, and K100 antigens were isolated[9–11] from dialysable cultures of *E. coli* strains E39a, H67, and F174, respectively. The yields were 189 (K22), 136 (K18), and 71 mg (K100) per L of liquid culture. Each polymer consisted of ribose, ribitol, and phosphate in equimolar amounts. The K18 antigen also contained 0.25 equiv. of acetyl groups (based on ribose or ribitol).

In immunoelectrophoresis, passive haemagglutination tests, and ELISA[12], the polymers reacted with OK- and K- but not with O-sera, indicating serological K18-, K22-, and K100-specificity, respectively.

The $[\alpha]_D^{22}$ values (*c* 1, water) were −30.5° (K22), −22° (K18), and −41° (K100). The i.r. spectra each contained a band at 1220 cm^{-1} (phosphate, P=O) and that of the K18 antigen showed an additional band at 1720 cm^{-1} (ester, C=O). The ^{13}C-n.m.r. spectrum of the K18 antigen showed, *inter alia*, signals indicative of *O*-acetyl groups [δ 173.9 (CH_3CO) and 21.1 (CH_3CO)], which were absent from the spectra of the K22 and K100 polymers.

Treatment with 0.1M HCl (10 min, 100°) or 0.5M NaOH (4 h, room temp.) degraded each polymer to small fragments (see below). Treatment of the K18 antigen with dilute ammonia (pH 11, 16 h, 4°) removed the acetyl groups to give a polymer that was identical by chemical and ^{13}C-n.m.r. spectroscopic analysis with the K22 antigen. These results together with the n.m.r. data (see below) indicated that the K18 and K22 antigens contain phosphodiester bridges and that the K18 antigen is an *O*-acetylated K22 antigen. Comparative serological studies (data not shown) indicated the K22 antigen to be identical with the *O*-deacetylated K18 antigen.

Periodate oxidation. — In phosphate-buffered saline (PBS, pH 7.2), each of the K antigens consumed 1 mol of periodate per mol of ribose/ribitol. *O*-Deacetylation of the K18 antigen prior to oxidation did not change the periodate consumption, indicating that not more than two vicinal hydroxyl groups were present in the repeating unit and that the acetyl groups did not protect from oxidation. The periodate-oxidised and borohydride-reduced products of the K22, *O*-deacetylated K18, and K100 antigens each contained ribose, glycerol, and phosphate in the ratios 1:1:1, and their ^{31}P-n.m.r. spectra (see below) indicated the presence of phosphodiester bridges.

Methylation. — The K antigens were methylated (Hakomori[13,14]) and then hydrolysed, and the products were treated with sodium borohydride followed by acetylation. G.l.c. (CB CP SIL 5, 25 m × 0.25 mm) showed two major peaks in the region of di- and tri-*O*-methylated ribitols, the retention times and the mass-spectral data of which are shown in Table I. The results showed that the K18 and K22 antigens contained 2-linked ribose and 2,5-linked ribitol, and that the K100 antigen contained 3-linked ribose and 2,5-linked ribitol.

TABLE I

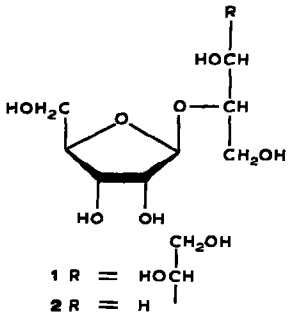
METHYLATION ANALYSIS (G.l.c./m.s.) OF THE K18, K22, AND K100 CAPSULAR ANTIGENS

Alditol	T[c]	*Mass-spectral fragments* (m/z)													
		68	*71*	*86*	*87*	*98*	*101*	*117*	*118*	*128*	*129*	*145*	*161*	*187*	*189*
1,2,4-Tri-*O*-acetyl-3,5-di-*O*-methylribitol[a]	16.15	−	+	−	+	−	+	−	−	−	+	+	+	−	+
1,3,4-Tri-*O*-acetyl-2,5-di-*O*-methylribitol[b]	16.03	+	−	+	−	+	−	−	+	+	+	−	−	+	−
2,5-Di-*O*-acetyl-1,3,4-tri-*O*-methylribitol[a,b]	11.35	−	+	−	+	−	+	+	−	−	+	−	−	+	−

[a]From K18 (5) and K22 (6) antigens. [b]From K100 antigen (7). [c]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

Fragmentation with sodium hydroxide. — Treatment with 0.5M NaOH (4 h, room temp.) depolymerised the K antigens and, on chromatography of the neutralised (HCl) hydrolysates on Biogel P-2, the main products were eluted in the region of di- to tri-saccharides. These products consisted of ribose, ribitol, and phosphate in the ratios 1:1:1 (major products) and 1:1:2 (minor products), and their mobilities relative to that of inorganic phosphate in paper electrophoresis were 0.42 and 0.76, respectively.

Dephosphorylation of the fragments with alkaline phosphatase gave non-reducing neutral **1**, which was eluted from Biogel P-2 with Kd 0.45.



When the periodate-oxidised and borohydride-reduced K antigens were subjected to the same treatments, the non-reducing dephosphorylated product **2** (Kd 0.41) was obtained by chromatography on Biogel P-2. The $[\alpha]_D^{23}$ values [**1**, −21°; **2**, −22° (*c* 1, water)] were the same when the products were obtained from K22, *O*-deacetylated K18, or K100 polymer. The $[\alpha]_D$ value of periodate-oxidised and reduced **1** was ~0°.

Methylation of the dephosphorylated fragments. — The methylated (Hakomori[13,14]) products **3** (from **1**) and **4** (from **2**) had retention times in g.l.c. (CB CP SIL, 5.25 × 0.25 mm; 2°/min from 140°) of 7.51 and 7.35 min, respectively. Their mass spectra and patterns are shown in Fig. 1. The results indicate **1** to be 2-ribosylribitol and **2** to be 2-ribosylglycerol with the glycerol unit representing C-1,2,3 of the ribitol unit.

N.m.r. data. — Table II shows the signal assignments of the ^{13}C-n.m.r. spectra of the K22, *O*-deacetylated K18, and K100 antigens, the products of periodate oxidation, and **1** and **2**. For comparison, the signals of the *Hib* capsular antigen, ribitol, and methyl α- and β-D-ribofuranoside are included. Assignments were facilitated by comparison with data for the *Hib* polymer, the structure of which is known[8], and other data[15]. The assignments of Table II accord with the proposed structure of the K100 antigen[7].

The native K18 antigen exhibited ^{13}C signals at δ 105.3, 77.3, 72.1, and 81.6, which were absent from the spectrum of *O*-deacetylated K18, indicating partial 3-acetylation of ribose (α-shift of the C-3 signal, β-shifts of the C-2 and C-4 signals). The identity of the K22 and *O*-deacetylated K18 antigens; as well as of their

TABLE II

^{13}C-N.M.R. DATA (CHEMICAL SHIFTS, P.P.M.)

Atom		*K22*	*-AcK18*[a]	*K100*	Hib[b]	*K18ox*[c]	*K22ox*[c]	*K100ox*[c]	**1**	**2**	*β-MeRib*[d]	*α-MeRib*[d]	*Ribitol*
Ribose	C-1	105.3	105.5	106.9	107.5	106.2	106.2	107.2	107.5	107.5	108.0	103.1	
	C-2	79.1[e]	79.1[e]	74.9[e]	75.4[e]	79.3[e]	79.3[e]	74.6[e]	75.9	75.6	74.3	70.1	
	C-3	70.1[e]	70.1[e]	74.3[e]	74.9[e]	70.6[e]	70.6[e]	75.0[e]	71.8	71.1	70.9	69.8	
	C-4	83.7	83.2	82.5[e]	83.0[e]	83.5	83.5	82.6[e]	83.7	83.5	83.0	84.6	
	C-5	61.9	61.9	61.9	63.3	61.6	61.6	61.5	61.6	61.6	62.9	61.9	
Ribitol	C-1′	61.0	61.1	61.1	69.8	62.2	62.2	62.5	62.6	62.2			63.8
	C-2′	80.4	80.5	80.5	72.6	80.5	80.5	80.3	81.0	80.2			73.5
	C-3′	70.7	70.8	70.9	71.3	62.6[f]	62.6[f]	62.2[f]	71.0	62.8			73.6
	C-4′	71.1[e]	71.2[e]	70.7[e]	72.0[e]	63.5[e,f]	63.3[e,f]	62.1[e,f]	72.7				73.5
	C-5′	68.1[e]	68.1[e]	67.8[e]	67.8[e]	68.2[e]	68.2[e]	68.0[e]	63.8				63.8

[a]*O*-Deacetylated K18. [b]*Hib* capsular polysaccharide[8]. [c]Periodate-oxidised and borohydride-reduced antigen. [d]Methyl β- or α-D-ribofuranoside. [e]C–P coupling (4–7 Hz). [f]Signals of primary hydroxyl groups derived from C-3′ and C-4′ of ribitol by periodate oxidation/borohydride reduction.

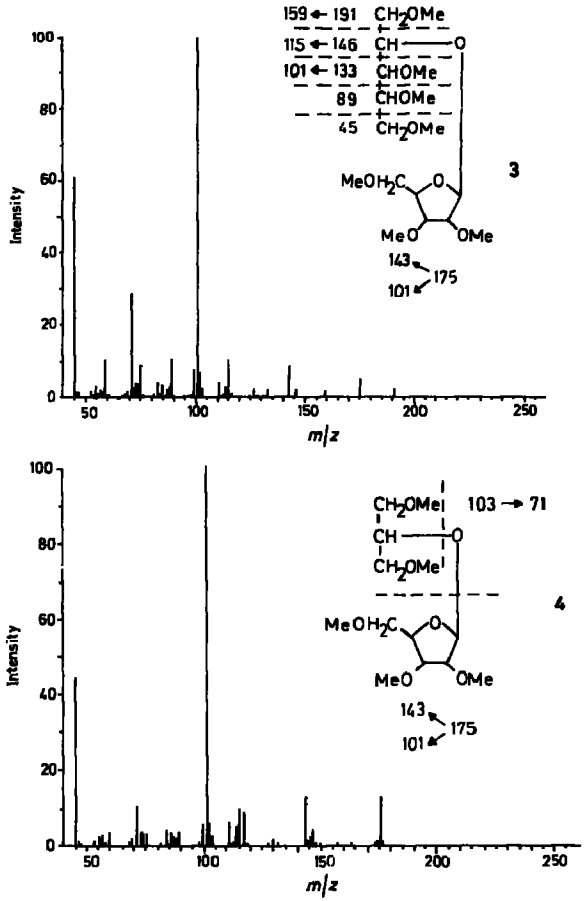


Fig. 1. Mass spectra and fragmentation patterns of **3** and **4**.

periodate-oxidised forms, is clear from the data in Table II. The periodate-oxidised polymers gave the same number of ^{13}C signals as the parent antigens, indicating that oxidative removal of carbon atoms had not occurred.

Comparison of the chemical shifts of the signals for C-2 and C-3 of ribose from the K22, *O*-deacetylated K18, K100, and *Hi*b polymers indicated that ribose was substituted at C-2 in the first two and at C-3 in the last two. Likewise, a comparison of the C-1′ and C-2′ signals of the compounds in Table I showed that ribitol was substituted at C-1′ in *Hi*b and at C-2′ in K22, *O*-deacetylated K18, and K100. The α-shift exhibited by the C-5′ signal of ribitol in each polymer, as compared to that of **1** and of ribitol, was evidence for substitution at this carbon in the polymers.

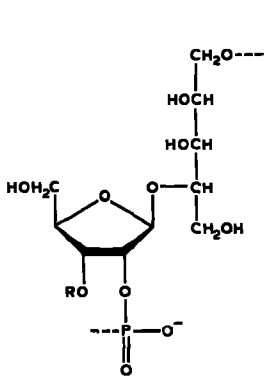
The postulated identity of the products (**1**) of alkaline fragmentation and dephosphorylation from the K22, *O*-deacetylated K18, and K100 polymers was verified by their ^{13}C-n.m.r. spectra. The same is true for the corresponding products (**2**) obtained from the periodate-oxidised forms.

The 2,5-disubstitution of ribose was also borne out by use of the attached

proton test (APT)[16,17]. In spectra recorded with this spin-echo technique, signals due to =CH– and CH_3– groups are positive and those due to ≡C- and $-CH_2-$ groups are negative. The opposite sign of the C-3′ and C-4′ signals in the APT spectra of the K22 and *O*-deacetylated K18 antigens (both positive) and in those of their periodate-oxidised and borohydride-reduced forms (both negative) were in keeping with a splitting of the C-3′–C-4′ bond of ribitol by periodate.

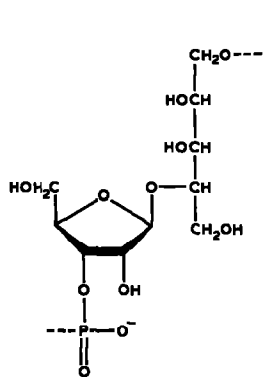
The technique of gated decoupling[15,17,18], which can be used successfully with pyranosides, is not applicable to furanosides. However, from the chemical shifts of the C-1 signal of ribose in the polymers, and especially in **1** and **2**, as compared to those of methyl α- and β-ribofuranoside, it can be concluded that ribose is β in each of these products. This interpretation is in keeping with the $[\alpha]_D$ values and indicates the presence of β-D-ribose. The absolute configuration of the ribitol moiety was indicated by the lack of optical asymmetry in periodate-oxidised **1** (see below), which is a symmetrical compound. Thus, ribitol is present in the polymers as D-ribitol 5-phosphate (or L-ribitol 1-phosphate).

The foregoing results indicated that the K18, K22, and K100 antigens can be formulated as **5**–**7**. Together with the *Hib* antigen[8] (**8**), these antigens form a group of structurally related poly(ribosyl-ribitol phosphates). The K18 and K22 antigens differ only in partial 3-acetylation of the ribose residue. Thus, they have the same relationship as the *E. coli* K2a and K2ab antigens, which are both poly(α-galactosyl-glycerol phosphates), differing only in a partial 4-acetylation of the galactose residue of the K2ab antigen. Therefore, these antigens were renamed K2a (formerly K2) and K2ab (formerly K62). The K18 and K22 antigens should be subjected to a similar change in terminology, indicating the close chemical relationship also in the serological formulae. The only structural difference between the K18/K22 antigens and the K100 antigen is the phosphate substitution of the ribose residue.



5 R = H

6 R = Ac (~25%), H(~75%)



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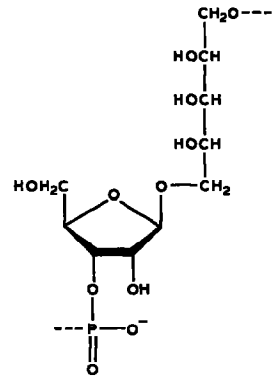

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The ^{31}P-n.m.r. spectrum of each of the polymers showed an intense signal at δ −0.9 to −1.2 due to the phosphodiester bridge[4,19]. No signals at δ −3 (phosphomonoesters) were detected. The spectra of the K22 and *Hi*b antigens each exhibited small signals at δ 18.0 and 20.1 (cyclic monophospho diesters) in addition, whereas no such signals were observed in the spectra of the K100 and K18 antigens. With cAMP as a reference, the signal at δ 20.1 was assigned to ribose 2,3-phosphate. The signal δ 18.0 is due, presumably, to ribitol 4',5'-phosphate[19]. After reaction with 1-ethyl-3-[3-dimethylamino)propyl]carbodiimide (EDAC), which converts monophosphates into cyclic diesters[19], the K22 antigen exhibited a ^{31}P-n.m.r. spectrum in which the signals at δ 18.0 and 20.1 had increased intensities. The EDAC-treated *Hi*b antigen exhibited a ^{31}P-n.m.r. spectrum in which only the signal at δ 20.1 was markedly increased, and the spectra of the EDAC-treated K18 and K100 antigens were unchanged. These results indicated that these preparations contain neither terminal phosphomonoesters nor cyclic phosphodiesters. In contrast, the K22 antigen appeared to contain equal amounts of chains ending with ribitol phosphate and ribose phosphate, respectively. By the same token, the *Hi*b antigen seems to contain mainly chains terminating in ribose phosphate and fewer chains terminating in ribitol phosphate. This finding agrees well with published data[19].

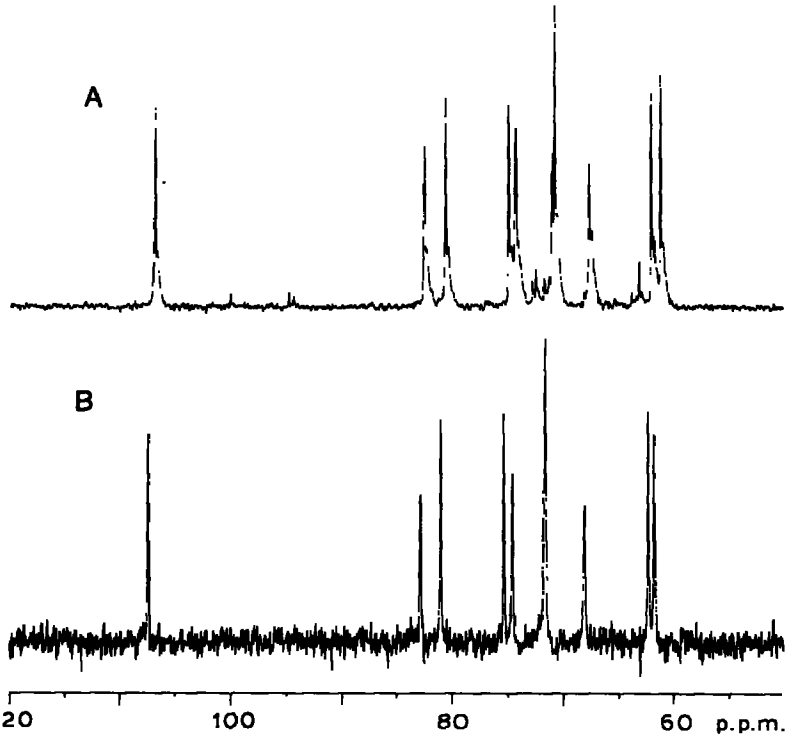

Fig. 2. ^{13}C-N.m.r. spectra [37°, external sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^{2}H_4$)pentanoate] of the K100 antigen in *A*, D_2O; and *B*, D_2O/Me_2SO (1:1).

Comparison of the ^{13}C-n.m.r. spectra recorded at 37° revealed that only that of the K100 antigen contained, exclusively, closely spaced double signals (Fig. 2A), comprising a major sharp component at lower field and a minor broader component at higher field, all with a coupling constant of 17 Hz. As shown in Fig. 2B, the double signals collapsed to sharp single signals when the spectrum was taken in the presence of methyl sulfoxide. The reason for this unusual behaviour is unknown, but it is possible that the spectrum in Fig. 2A indicated rotational restraints in the K100 antigen associated with hydrogen bonding which disappeared in the presence of methyl sulfoxide. The ^{13}C-n.m.r. spectrum of the K100 antigen recorded at 70° consisted of single signals. The above spectral phenomena were not observed with the K22, *O*-deacetylated K18, and *Hib* polymers.

Although the capsular polysaccharides described here differ in primary and probably also secondary structure, they are sufficiently similar to cross-react serologically. Since *H. influenzae* b is pathogenic in man and *E. coli* strains with the K18, K22, or K100 antigen are not particularly pathogenic, the structural relations between these capsular antigens is of interest for the immunologist and the results of the serological studies will be published elsewhere.

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* strains E39a (O23:K18:H15, Freiburg collection No. 21361), H67 (O23:K22:H15, No. 21362), and F174 (O75:K100:H5, No. 21483) were obtained from Drs. I. and F. Ørskov (Copenhagen) and grown to the late log phase (5–7 h) in a fermentor at 37° in 10-L batches containing per L: $K_2HPO_4 \cdot 3\ H_2O$ (9.7 g), KH_2PO_4 (2 g), sodium citrate·5 H_2O (0.5 g), $MgCl_2 \cdot 7\ H_2O$ (0.1 g), casamino acids (20 g), and the dialysable part of yeast (100 mL from 500 g in 5 L of deionised water).

Isolation and purification of the polymers. — The polymers and the bacterial cells were precipitated from the liquid cultures by addition of 1 vol. of aqueous 2% cetyltrimethylammonium bromide (Cetavlon), and the following operations were performed at 4°. The polymers were extracted from the precipitates with M calcium chloride, and purified by three cycles of precipitation from aqueous solutions with ethanol (to 80% final concentration) followed by repeated extractions with cold phenol (aqueous 80%, buffered to pH 6.5 with sodium acetate)[9–11]. The combined aqueous phases were centrifuged for 4 h at 100,000*g* and the supernatant solutions were lyophilised. The residues were further purified by chromatography on Sephadex G-50.

Analytical methods. — Ribose and ribitol were determined by g.l.c. of the acetylated products in neutralised hydrolysates (0.1M HCl, 100 h, 100°) of the polymers before and after borohydride reduction. Ribose was also determined with the orcinol reagent. Phosphate was determined by the method of Ames[20]. The acetate content of the polymers was determined by g.l.c.[21] (ECNSS-M) and by the hydroxamate method[22]. Glycerol was determined by the u.v. method (Boehringer Mannheim).

Optical rotations were measured with a Perkin–Elmer 141 polarimeter, and g.l.c. was performed on an ECNSS-M column (1.5 m × 2 mm) at 190° with a Varian Aerograph Series 1400 instrument equipped with an autolinear temperature programmer and a Hewlett–Packard 3380 integrator. G.l.c.–m.s. was performed with a Finnigan MAT 1020 B automatic system at 70 eV on a column (25 m × 0.25 mm) of CBCP SIL 5 with helium as the carrier gas and with a temperature gradient of 2°/min from 140°. I.r. spectra were recorded with a Gilford spectrometer 240 and n.m.r. spectra were recorded with a Bruker WM 300 spectrometer in the F.t. mode at 70° [external sodium 4,4-dimethyl-4-sila(2,2,3,3-$^2H_4$)pentanoate]. The ^{13}C-n.m.r. values were corrected (−1.31 p.p.m.) by using 1,4-dioxane (δ 67.4 based on Me_4Si).

The procedures for periodate oxidation, *O*-deacetylation, and methylation have been described[23–25].

Preparation of **1**. — Solutions of the K22, *O*-deacetylated K18, or K100 antigen (100 mg of each) in 0.5M NaOH (10 mL) were kept for 4 h at room temperature, then neutralised with Dowex 50 (H^+) resin, and incubated for 12 h at room temperature with alkaline phosphatase (EC 3.1.3.1, 100 μL containing 35 units) in 50mM sodium carbonate buffer (pH 9.6). The products were purified by elution from a column (1.5 × 70 cm) of Biogel P-2 with water.

Preparation of **2**. — Prior to alkaline hydrolysis, the K22, *O*-deacetylated K18, and K100 antigens (100 mg of each) were separately oxidised with 50mM sodium metaperiodate in PBS (pH 7.2) for 70 h at 4°. Excess of periodate was removed as the insoluble barium salt and the oxidised polymers were reduced with sodium borohydride. Each product (40 mg) was dissolved in 4 mL of 0.5M NaOH (4 mL) and treated as described for the preparation of **1**.

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