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Structure of the O-antigen of *Francisella tularensis* strain 15

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ABSTRACT

The O-specific polysaccharide, obtained by mild acid degradation of the lipopolysaccharide of *Francisella tularensis* strain 15, contained 2-acetamido-2,6-dideoxy-D-glucose (D-QuiNAc), 4,6-dideoxy-4-formamido-D-glucose (D-Qui4NFm), and 2-acetamido-2-deoxy-D-galacturonamide (D-GalNAcAN) in the ratios 1:1:2. Tri- and tetra-saccharide fragments were obtained on treatment of the polysaccharide with anhydrous hydrogen fluoride and partial hydrolysis with 0.1M hydrochloric acid, respectively. On the basis of ^{1}H- and ^{13}C-n.m.r. spectroscopy of the polysaccharide and the saccharides, it was concluded that the O-antigen had the structure:
→4)-α-D-Gal*p*NAcAN-(1→4)-α-D-Gal*p*NAcAN-(1→3)-β-D-Qui*p*NAc-(1→2)-β-D-Qui*p*4NFm-(1→.
This O-antigen is related in structure to those of *Pseudomonas aeruginosa* O6, immunotype 1, and IID 1008, and *Shigella dysenteriae* type 7.

INTRODUCTION

Francisella tularensis is a small Gram-negative coccobacillus which causes tularemia, a zoonotic disease that occurs endemically in latitudes 30–70° North[1]. Two main biovars of *F. tularensis* (*tularensis*, type A, and *palaearctica*, type B), that differ only in a few biochemical characteristics, are known[2,3]. No antigenic difference between these two biovars and no serologically distinct types among strains of *F. tularensis* have been found, and no detailed chemical study of their O-antigens (lipopolysaccharides), which are widely used for diagnostics of tularemia, has been carried out.

We now report the structure of the O-antigenic polysaccharide of the lipopolysaccharide of *F. tularensis* biovar *palaearctica* strain 15 used for the production of vaccine.

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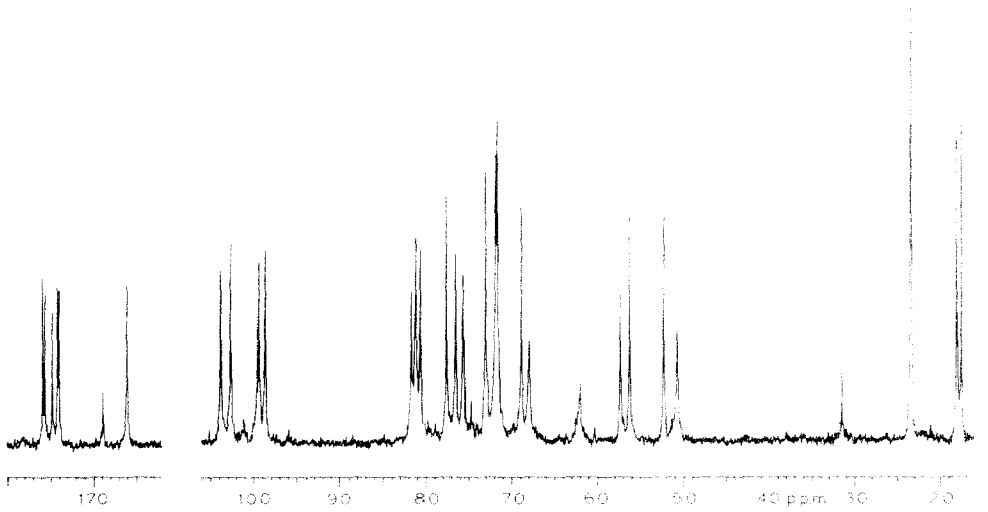


Fig. 1. ^{13}C-N.m.r. spectrum of *F. tularensis* O-specific polysaccharide.

RESULTS AND DISCUSSION

The lipopolysaccharide, isolated from dry bacterial cells by the Westphal procedure[4], was cleaved with dilute acetic acid to give the O-specific polysaccharide, which was isolated by gel-permeation chromatography on Sephadex G-50.

The ^{13}C-n.m.r. spectrum (Fig. 1) showed that the polysaccharide had a tetrasaccharide repeating-unit; it contained signals for four anomeric carbons (98.6–103.8 p.p.m.), four carbons attached to nitrogen (50.7–57.2 p.p.m.), two HC*Me* groups (17.5 and 18.0 p.p.m.), two $CONH_2$ groups (174.0 and 174.1 p.p.m.), three NAc groups (3 Me at 23.3–23.4 p.p.m. and 3 C=O at 174.8–175.8 p.p.m.), one NCHO group (166.0 and 168.8 p.p.m. corresponding to the *E* and *Z* forms due to hindered rotation of the C–N bond[5]), and 12 signals for other sugar ring carbons (67.9–81.6 p.p.m.) (Table I).

There was no significant yield of monosaccharides on acid hydrolysis of the polysaccharide, which reflected the resistance of the glycosidic linkages. Deamination of the product in the hydrolysate with nitrous acid gave 2,5-anhydro-6-deoxymannose, formed from 2-amino-2,6-dideoxyglucose (quinovosamine)[6] and identified by g.l.c. of the derived alditol acetate.

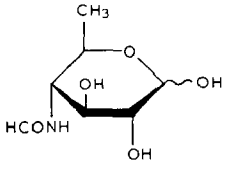
Solvolysis of the polysaccharide with anhydrous hydrogen fluoride afforded a mono- (**1**) and a tri-saccharide (**2**) which were separated by gel-permeation chromatography on TSK HW 40. After purification by p.c., **1** was converted into the alditol acetate **3**, the mass spectrum of which contained peaks for the fragments C-1,2,3,4 (*m/z* 274) and C-4,5,6 (*m/z* 144) and indicated **1** to be a 4,6-dideoxy-4-formamidohexose. This conclusion was confirmed by ^{1}H- and ^{13}C-n.m.r. spectra of **1**, which contained signals for one 6-deoxy group [1.17 (d, $J_{5,6}$ 6 Hz, H-6,6,6α), 1.20 (d, $J_{5,6}$ 6 Hz, H-6,6,6β),

TABLE I

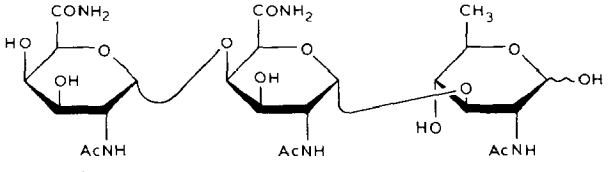
^{13}C-N.m.r. data[a] (δ in p.p.m.)

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
2-Acetamido-2-deoxy-D-galacturonamide (unit A)						
4	99.1	50.5	68.2	70.0	72.5	
5[b]	99.8	50.5	68.3	70.0	72.6	
6	99.2	51.1	69.0	79.9	72.0	
7	99.2	51.0	68.9	79.8	71.9	
8	98.6	52.2	68.8	80.5	71.8	174.0
2-Acylamino-2-deoxy-D-galact-uronic acid or -uronamide (unit B)						
4	100.2	50.5	67.5	76.5	71.9	
5[b]	99.0	49.3	67.7	76.5	72.1	
6	100.2	50.6	67.6	76.6	72.0	
7	100.2	50.6	67.5	76.6	71.9	
8	99.4	50.7	67.9	76.4	71.8	174.1
2-Acetamido-2,6-dideoxy-D-glucose or -glucitol (unit C)						
4	61.6	55.2	75.4	77.9	67.5	20.2
5[b]	61.7	54.7	75.6	77.7	67.9	19.6
6	61.7	55.2	75.5	78.0	67.5	20.2
7	61.6	55.2	75.4	78.0	67.4	20.2
8	102.6	56.2	81.1	77.5	73.0	17.5
4,6-Dideoxy-4-formamido- or 4-amino-4,6-dideoxy-D-glucose (unit D)						
1 α	93.2	73.2	71.4	56.8	67.5	18.1
β	97.0	75.9	74.6	56.7	72.0	18.1
6	105.1	75.5	74.4	56.7	72.0	18.1
7	105.0	75.3	72.9	58.1	69.9	18.0
8	103.8	81.6	75.6	57.3	71.8	18.0

[a] Additional signals: NAc at 23.1–23.3 (Me) and 174.8–175.8 p.p.m. (C=O), NCHO at 165.9–166.0 and 168.8–168.9 p.p.m. (*E* and *Z* forms). [b] Data from ref. 8.

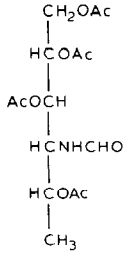


1



A B C

2



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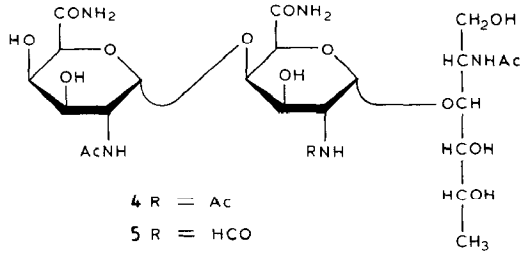


4 R = Ac

5 R = HCO

TABLE II

^{1}H-N.m.r. data[a] (δ in p.p.m., J in Hz)

H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*
4,6-Dideoxy-4-formamido-D-glucose (**1**)					
α Anomer					
5.21 (d)	3.60 (dd)	3.73 (t)	3.64 (t)	4.00 (q)	1.17 (d)
$J_{1,2}$ 3.5	$J_{2,3}$ 9.5	$J_{3,4}$ 9.5	$J_{4,5}$ 9.5	$J_{5,6}$ 6	
β Anomer					
4.62 (d)	3.30 (dd)	3.52 (t)	3.66 (t)	3.62 (q)	1.20 (d)
$J_{1,2}$ 8	$J_{2,3}$ 8.5	$J_{3,4}$ 8.5	$J_{4,5}$ 9.5	$J_{5,6}$ 6	
Trisaccharide-alditol **4**					
2-Acetamido-2-deoxy-D-galacturonamide (unit A)					
5.07 (d)	4.18 (dd)	4.05 (dd)	4.29 (dd)	4.85 (d)	
$J_{1,2}$ 4	$J_{2,3}$ 11	$J_{3,4}$ 3	$J_{4,5}$ 1.5		
2-Acetamido-2-deoxy-D-galacturonamide (unit B)					
5.28 (d)	4.32 (dd)	4.09 (dd)	4.48 (d)	4.40 (bs)	
$J_{1,2}$ 4	$J_{2,3}$ 11	$J_{3,4}$ 2.5			
2-Acetamido-2,6-dideoxy-D-glucitol (unit C)					
		4.11 (bs)	3.42 (d)	3.67 (dq)	1.25
		$J_{3,4}$ < 2	$J_{4,5}$ 8	$J_{5,6}$ 6	
Tetrasaccharide-alditol **7**					
2-Acetamido-2-deoxy-D-galacturonamide (unit A)					
5.07 (d)	4.26 (dd)	4.14 (dd)	4.50 (d)	4.85 (bs)	
$J_{1,2}$ 4	$J_{2,3}$ 8	$J_{3,4}$ 3			
2-Acetamido-2-deoxy-D-galacturonamide (unit B)					
5.28 (d)	4.31 (dd)	4.09 (dd)	4.48 (d)	4.40 (bs)	
$J_{1,2}$ 4	$J_{2,3}$ 8	$J_{3,4}$ 2.5			
2-Acetamido-2,6-dideoxy-D-glucitol (unit C)					
		4.11 (bs)	3.41 (d)	3.67 (dq)	1.22 (d)
		$J_{3,4}$ < 2	$J_{4,5}$ 8	$J_{5,6}$ 6	
4-Amino-4,6-dideoxy-D-glucose (unit D)					
4.56 (d)	3.38 (t)	3.60 (t)	2.92 (t)	3.67 (dq)	1.34 (d)
$J_{1,2}$ 8	$J_{2,3}$ 8	$J_{3,4}$ 8	$J_{4,5}$ 8	$J_{5,6}$ 6	

[a] Additional signals: NAc (s) for **4** and **7** at 1.97–2.05 p.p.m., NCHO (2 s, *E* and *Z* forms) for **1** at 8.01 and 8.19 p.p.m.

and 18.1 p.p.m. (C-6α,β)] and one NCHO group [*E* and *Z* forms; 8.01 and 8.19 (2 s, C*H*), 165.9 and 168.9 p.p.m. (*C*H)].

The $J_{H,H}$ values for **1** (Table II) were characteristic for α and β anomers of a hexopyranose that had the *gluco* configuration. The ^{13}C-n.m.r. data (Table I) for the ring carbons of **1** were similar to those of 4,6-dideoxy-4-glycylamino-D-glucose isolated[7] from the O-specific polysaccharide of *Shigella dysenteriae* type 7. The respective $[\alpha]_D$ values [+57° and +125° (water)] of **1** and 4,6-dideoxy-4-glycylamino-D-glucose indicated **1** to be a D sugar.

Therefore, one of the components of the polysaccharide was 4,6-dideoxy-4-formamido-D-glucose, apparently not found hitherto in Nature. From the identification of **1**, it followed that the other amino sugar constituents of the polysaccharide were *N*-acetylated.

Reduction of **2** with sodium borohydride gave the trisaccharide-alditol **4**. The ^{13}C-(Table I) and ^{1}H-n.m.r. (Table II) data showed that **4** contained 3 NAc groups. The ^{1}H-n.m.r. spectrum of **4** was assigned with the help of sequential, selective spin-decoupling experiments. The $J_{H,H}$ values of the hexopyranose residues (units A and B) were characteristic for α-galactopyranose. The signals for H-5 (d with $J_{4,5}$ 1.5 Hz and a bs) showed the absence of protons at C-6, and indicated units A and B to be derivatives of galacturonic acid.

The use of selective heteronuclear ^{13}C–^{1}H double resonance demonstrated that C-2 of units A and B resonated at 50.5 p.p.m. Hence, they carried nitrogen and, therefore, were residues of 2-acetamido-2-deoxygalacturonic acid. The alditol residue (unit C) of **4** was derived from 2-acetamido-2,6-dideoxyglucose (see above).

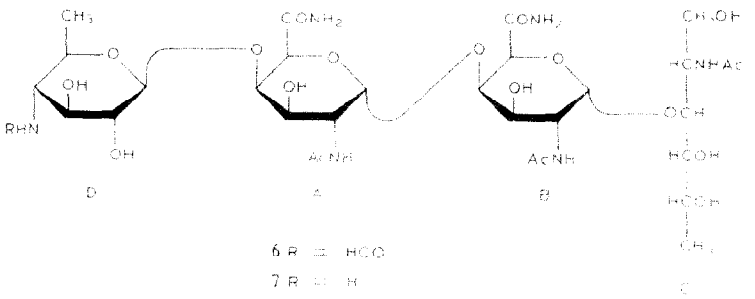
On irradiation of H-1 of unit A at 5.07 p.p.m., an n.O.e. was observed on H-4 of unit B at 4.48 p.p.m., and irradiation of H-1 of unit B at 5.28 p.p.m. caused an n.O.e. on H-3 of unit C at 4.11 p.p.m. These data proved that unit B was 4-substituted by unit A and that unit C was 3-substituted by unit B.

The ^{13}C-n.m.r. spectrum (Table I) showed that **4** resembled the trisaccharide-alditol **5**, prepared[8] by solvolysis with anhydrous hydrogen fluoride of the O-specific polysaccharides of *Pseudomonas aeruginosa* immunotype 1 followed by borohydride reduction, with the exception that the *N*-formyl group in **5** was replaced by an *N*-acetyl group in **4**. Compound **4** was neutral in anion-exchange chromatography and, therefore, both uronic acid residues were in the amide form. This conclusion was confirmed by reversed-phase h.p.l.c., where **4** had a retention time that was the same as that of an authentic sample prepared from **5** by *N*-deformylation followed by *N*-acetylation. The $[\alpha]_D$ values of **4** and **5** were similar [respectively, $+140°$ and $+131.6°$ (water)] as were the ^{13}C-n.m.r. spectra, which are sensitive to the relative absolute configuration of the monosaccharide components of oligosaccharides[9]. Thus, as in **5**, each constituent sugar in **4** was D and its structure, and consequently that of **2**, are as depicted.

Partial hydrolysis of the polysaccharide with 0.1M hydrochloric acid yielded two main tetrasaccharides, which were isolated by gel-permeation chromatography on TSK HW 40 and reduced with sodium borohydride to give the alditol derivatives **6** (minor) and **7** (major).

The ^{13}C-n.m.r. spectra (Table I) showed **6** and **7** to be tetrasaccharide-alditols which differed only in the presence or absence of the *N*-formyl group (signals at 165.9 and 168.9 p.p.m. for **6**) on the 4-amino-4,6-dideoxyglucose residue (unit D). The ^{1}H-n.m.r. spectrum of **7** was interpreted completely by using sequential, selective spin-decoupling experiments (Table II). The $J_{1,2}$ value of 8 Hz proved unit D to be β. The n.O.e. on H-4 of unit A at 4.50 p.p.m., on irradiation of H-1 of unit D at 4.56 p.p.m., indicated that the latter was 4-linked to the former. This conclusion accorded with the displacement of the signal for C-4 of unit A of **4** from 70.0 p.p.m. to 79.8–79.9 p.p.m. for **6** and **7** caused by 4-glycosylation[9].

Therefore, **6** and **7** differed from **4** by the presence at the non-reducing end of a 4,6-dideoxy-4-formamido- or 4-amino-4,6-dideoxy-β-D-glucopyranose residue. Thus, **6** was the alditol derivative of the repeating unit of the polysaccharide.



The ^{13}C-n.m.r. spectra of **6** and **7** were interpreted by comparison with those of **1** and **4** (Table I). Comparison of the ^{13}C-n.m.r. spectrum of **6** with that of the polysaccharide revealed the signal for C-1 of unit C to be at 102.9 p.p.m. in the latter and indicated[9] it to be β. The displacement of the signal for C-1 of unit D from 105.1 p.p.m. for **6** to 103.8 p.p.m. for the polysaccharide was due to the β-effect of 2-substitution of this unit[9] and, hence, units C and D were connected in the polysaccharide by a β-(1→2) linkage.

The configurations of the glycosidic linkages were confirmed by the $J_{C-1,H-1}$ values determined from the gated-decoupling ^{13}C-n.m.r. spectrum of the polysaccharide. They were ~175 Hz for the signals of C-1 of units A and B at 98.6 and 99.4 p.p.m. and ~163 Hz for the signals of C-1 of units C and D at 102.6 and 103.8 p.p.m., respectively. These data showed[10] units A and B to be α, and units C and D to be β.

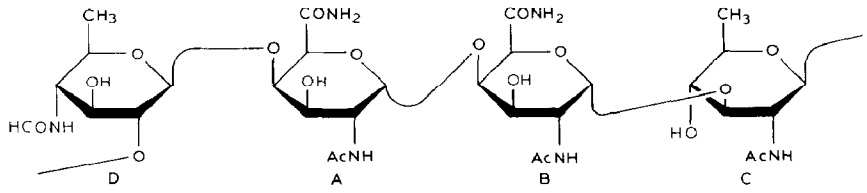
Thus, it was concluded that the O-specific polysaccharide has the structure **8**.

The O-antigen of *F. tularensis* is related in structure to the O-antigens of *Pseudomonas aeruginosa* O6 (Lányi and Bergan classification), immunotype 1 (refs. 6, 8, and 11), and IID 1008 (ref. 12), and *Shigella dysenteriae* type 7 (ref. 7) (structures **9**–**11**). Thus, **8** differs from **9**–**11** only in one constituent monosaccharide, namely, 4-amino-4,6-dideoxy-D-glucose instead of L-rhamnose in **9** and **10** or 2-amino-2,6-dideoxy-D-glucose instead of 2-amino-2-deoxy-D-glucose in **11**, so that these O-antigens possess a common trisaccharide block. Other differences are associated with the nature of *N*-acyl substituents (the *N*-acetyl group at the residues of the 2-amino-2-deoxy-D-galacturonic acid or 4-amino-4,6-dideoxy-D-glucose residue may be replaced by an *N*-formyl group), the presence or absence of a 3-*O*-acetyl group on a 2-amino-2-deoxy-D-galacturonic acid residue, and the presence of one or both of these residues in the amide form.

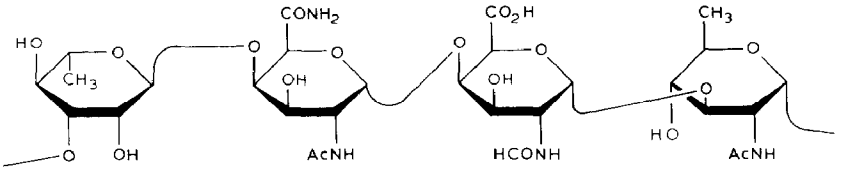
P. aeruginosa O6 and *S. dysenteriae* type 7 cross-react serologically[13], probably due to the similarity of their O-antigens[11]. Nothing is known about the serological relationship of these two species and *F. tularensis*.

EXPERIMENTAL

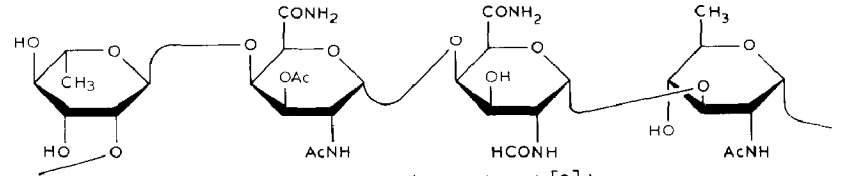
General. — ^{1}H-N.m.r. spectra were recorded with a Bruker WM-250 spectrometer for solutions in D_2O at 30°. Sequential, selective spin-decoupling experiments were carried out as described[14] and modified[15]. ^{13}C-N.m.r. spectra were recorded with a Bruker AM-300 instrument for solutions in D_2O at 60° for the polysaccharide or 30° for



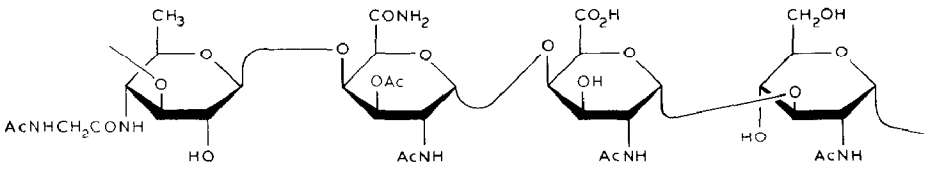
8 (*F. tularensis*)



9 (*P. aeruginosa* O6a,6c [8])



10 (*P. aeruginosa* immunotype 1 [8])



11 (*S. dysenteriae* type 7 [7])

the saccharides (internal acetone, ^{1}H 2.24, ^{13}C 31.45 p.p.m.). Optical rotations were measured with a JASCO DIP 300 polarimeter for solutions in water at 25°.

G.l.c. was performed with a Hewlett–Packard 5890 instrument equipped with a flame-ionisation detector, using a glass capillary column (0.2 mm × 25 m) coated with OV-1. Gel-permeation chromatography was performed (*a*) on a column (3.5 × 70 cm) of Sephadex G-50, using a pyridine acetate buffer (pH 5.5), with monitoring by the orcinol–sulfuric acid reaction using a Technicon sugar analyser; or (*b*) by elution from a column (80 × 1.6 cm) of TSK HW 40 (S) with water and monitoring with a Knauer differential refractometer. H.p.l.c. was performed on a column (30 × 0.8 cm) of Silasorb SPH C18, using 0.05% trifluoroacetic acid in aqueous 2–4% methanol and monitoring by a Knauer variable wavelength monitor at 220 nm. Analysis of amino sugars was performed with a BC-200 amino acid analyser in 0.35M sodium citrate buffer (pH 5.28) under standard conditions. P.c. was carried out on FN-11 paper, using pyridine–1-butanol–water (6:4:3) with detection by alkaline silver nitrate.

Bacterium and growth. — Strain 15 of *F. tularensis* biovar *palaearctica* was cultivated for 24 h at 37° on a solid medium (erythritol agar supplemented with cystine, glucose, vitamins, and tryptic hydrolysate of "black albumin", produced in the All-

Union Institute of Applied Microbiology). Deep cultivation was carried out in a 12-L Electrolux fermenter (Sweden) for 16–18 h at 37° on a medium that contained sulphuric acid hydrolysate of fish-bone flour supplemented with salts, vitamins, cystine, and D-glucose (All-Union Institute of Applied Microbiology). Bacterial cells were harvested, centrifuged at 15,000*g* for 40 min at 4°, and dried with acetone.

Isolation of the lipopolysaccharide and the O-specific polysaccharide. — Acetone-dried bacterial cells were extracted with hot aqueous phenol[4], and the extract was dialysed without separation of the phenol and aqueous layers, then centrifuged. The pH of the supernatant solution was adjusted to 2.5 with aqueous 50% trichloroacetic acid in order to precipitate protein and nucleic acid contaminants, and the solution was dialysed and freeze-dried.

The resulting lipopolysaccharide was cleaved with aqueous 1% acetic acid (100°, 2.5 h), the lipid was removed by centrifugation, the supernatant solution was concentrated, and the O-specific polysaccharide was isolated by gel-permeation chromatography on Sephadex G-50.

Monosaccharide composition. — The polysaccharide (2 mg) was hydrolysed with 4M hydrochloric acid (4 h, 100°), the hydrolysate was concentrated, and the residue was analysed using the amino acid analyser and g.l.c. of the derived alditol acetates. Part of the hydrolysate was deaminated with nitrous acid[6], and the products were converted into alditol acetates, and analysed by g.l.c.

Solvolysis with anhydrous hydrogen fluoride. — Anhydrous hydrogen fluoride (~10 mL) was added to the polysaccharide (60 mg), the mixture was stirred for 2 h at room temperature, then poured into cold ether (200 mL). The precipitate was collected on a stainless-steel filter, washed several times with cold ether, and dissolved in water, and the trisaccharide **2** (15 mg) and 4,6-dideoxy-4-formamido-D-glucose (**1**, 4 mg) were isolated by gel-permeation chromatography on TSK HW 40.

After purification by preparative p.c., **1** had $[\alpha]_D$ +57° (*c* 0.5). The ^{1}H- and ^{13}C-n.m.r. data are shown in Tables I and II. The alditol acetate **3** was obtained conventionally. Mass spectrum: *m/z* 43 (100%), 84 (50), 87 (31), 98 (35), 114 (29), 126 (22), 127 (28), 128 (21), 144 (12), 154 (13), 158 (11), 168 (15), 169 (13), 172 (14), 186 (21), 187 (15), 214 (11), 228 (16), 246 (17), 274 (17), 288 (19).

Reduction of **2** with sodium borohydride followed by gel-permeation chromatography on TSK HW 40 gave the trisaccharide-alditol **4**, $[\alpha]_D$ +140° (*c* 1.6). The ^{1}H- and ^{13}C-n.m.r. data are given in Tables I and II.

Partial acid hydrolysis. — The polysaccharide (60 mg) was heated in 0.1M hydrochloric acid (100°, 0.5 h) and the hydrolysate was concentrated. The products were subjected to gel-permeation chromatography on TSK HW 40, reduced with sodium borohydride, and desalted as above, to give **6** (5 mg) and **7** (16 mg). The latter was purified by h.p.l.c. and analysed by ^{1}H- and ^{13}C-n.m.r. spectroscopy (Tables I and II).

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