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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella ozaenae* SEROTYPE K4 CONTAINING 3-DEOXY-D-*glycero*-D-*galacto*-NONULOSONIC ACID*

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ABSTRACT

The acidic capsular polysaccharide from the museum strain 2211 of *Klebsiella ozaenae* serotype K4 is built up of pentasaccharide repeating-units that contain residues of D-glucose, D-mannose, D-glucuronic acid, and 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (Kdn) in the ratios 2:1:1:1, as well as an *O*-acetyl group. The last-named sugar, which is reported in bacterial polysaccharides for the first time, was identified as the methyl (methyl 3-deoxynonulopyranosid)onate obtained by methanolysis of the polysaccharide. On the basis of the results of partial acid hydrolysis, Smith degradation, and computer-assisted ^{13}C-n.m.r. analysis, it was concluded that the capsular polysaccharide has the following structure:

$$\begin{array}{c} \alpha\text{-Kdn} \\ 2 \\ \downarrow \\ 4 \\ \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow \\ 3 \\ | \\ \text{OAc} \end{array}$$

INTRODUCTION

Klebsiella ozaenae serotype K4 is a widespread pathogen that causes respira-

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tory diseases in humans[1]. A capsular polysaccharide, which determines the antigenic specificity of this serotype, has been obtained from the strain isolated from a patient having a chronic lung-infection, and the structure of the tetrasaccharide repeating-unit involving residues of D-glucose, D-mannose, and D-glucuronic acid in the ratios 2:1:1, as well as an *O*-acetyl group, has been established[2]. A 3-deoxynonulosonic acid has been found[3] as a constituent of the capsular polysaccharide produced by the museum strain 2211, which also belongs to the *K. ozaenae* serotype K4. We now describe studies of this capsular polysaccharide which led to a structure differing from that of the polysaccharide from the strain used in previous work[2]. The preliminary results have been reported[4].

RESULTS AND DISCUSSION

The acidic capsular polysaccharide was prepared by mechanical disintegration of cells in a synthetic medium and purified by precipitation with Cetavlon and ultracentrifugation. As judged by ion-exchange chromatography on DEAE-cellulose, the polysaccharide was homogeneous and possessed specific serological activity in the inhibition of the passive haemagglutination reaction.

The ^{13}C-n.m.r. spectrum of the polysaccharide (Fig. 1) contained signals for one *O*-acetyl group (CH_3 at 22.3 p.p.m.), the CH_2 group of a deoxy sugar (41.7 p.p.m.), four CH_2OH groups (61.6–64.3 p.p.m.), five anomeric carbons (100.6–105.2 p.p.m.), twenty-one other sugar carbons (67.2–84.6 p.p.m.), and three CO groups (CH_3COO and COOH; 174.2–176.4 p.p.m.). Four signals of the anomeric carbons were doublets in the gated-decoupling spectrum and thus belonged to aldose residues, whereas the fifth signal (100.6 p.p.m.) was a singlet and belonged to a ketose residue.

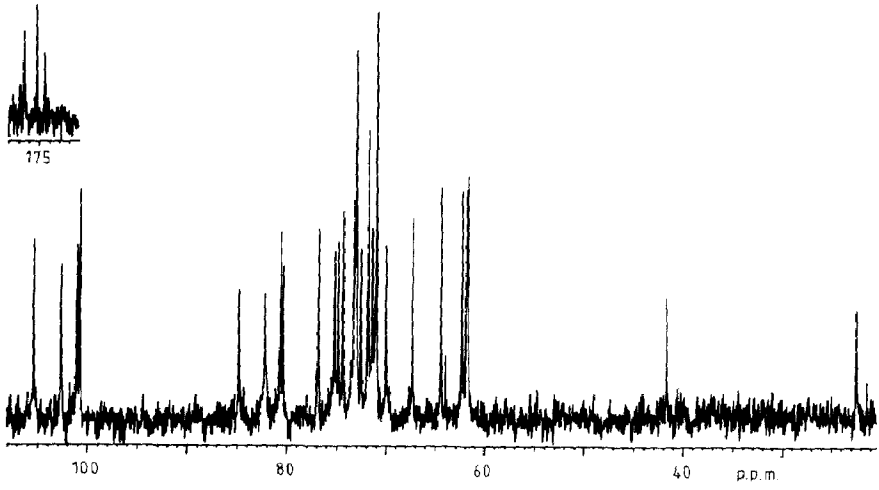


Fig. 1. ^{13}C-N.m.r. spectrum of the *K. ozaenae* capsular polysaccharide.

The $J_{C\text{-}1,H\text{-}1}$ values for the anomeric carbons of three of the aldose residues were 170.9, 173.3, and 173.3 Hz (corresponding to the signals at 100.6, 100.8, and 102.1 p.p.m., respectively), whereas that for the anomeric carbon of the fourth aldose residue was 161.1 Hz (corresponding to the signal at 105.2 p.p.m.). These data showed[5] that each residue was pyranosidic, three were α, and one was β.

The ^{1}H-n.m.r. spectrum of the polysaccharide contained signals for an *O*-acetyl group (2.10 p.p.m., s, 3 H), the CH_2 group of a deoxy sugar [1.54 (t, $^2J \approx {}^3J \approx 11.5$ Hz) and 2.59 p.p.m. (dd, ^3J ~5.5 Hz)], four anomeric protons [4.49 (d, $J_{1,2}$ ~7.8 Hz), 5.10 (bs), 5.21 (d, $J_{1,2}$ ~4 Hz), and 5.37 p.p.m. (d, $J_{1,2}$ ~3.5 Hz)], one *H*COAc (5.22 p.p.m., t, ^3J ~9.5 Hz), and other protons (3.2–4.4 p.p.m.).

The n.m.r. data indicated the polysaccharide to contain a pentasaccharide repeating-unit involving three hexoses, a hexuronic acid, and a 3-deoxyulosonic acid which, on the basis of the number of ^{13}C signals, is a 3-deoxynonulosonic acid. The conclusion about the higher sugar is consistent with mass-spectral data[3] of the acetylated methyl (methyl 3-deoxynonulopyranosid)onate, prepared after methanolysis of the polysaccharide.

Hydrolysis of the polysaccharide with 2M trifluoroacetic acid (100°, 6 h) gave glucose and mannose in the ratio ~3:1 as well as glucuronic acid, which were identified by using p.c. and a sugar analyzer. The ulosonic acid was destroyed during hydrolysis.

The glucose–mannose ratio of ~3:1, instead of the expected 2:1, reflects the retention of part of the mannose in an aldobiouronic acid. Partial hydrolysis of the polysaccharide followed by gel chromatography on TSK HW 40 gave a disaccharide (**1**) composed of mannose and glucuronic acid.

The ^{1}H-n.m.r. spectrum of **1** contained signals for H-1 of an α-glucuronic acid residue at 5.30 p.p.m. (d, $J_{1,2}$ ~4.0 Hz) and for H-1α and H-1β of a mannose residue at 5.17 (d, $J_{1,2}$ 1.9 Hz) and 4.91 p.p.m. (bs), respectively. The signals of the α-series in the ^{1}H-n.m.r. spectrum of **1** were interpreted (see Experimental) by using sequential, selective spin-decoupling experiments. Pre-irradiation of H-1 of the glucuronic acid residue at 5.30 p.p.m. caused a n.O.e. on H-3 of the mannose residue at 3.89 p.p.m., and, hence, these monosaccharides are (1→3)-linked. Thus, **1** has the structure α-D-Glc*p*A-(1→3)-D-Man.

A repeating unit thus includes two residues of glucose and a residue of mannose. This conclusion is consistent with the presence in the ^{1}H-n.m.r. spectrum of the polysaccharide of signals for H-1 of two glucose residues (2 d at 4.49 and 5.21 p.p.m.) and one glucuronic acid residue (d at 5.37 p.p.m.) and one mannose residue (bs at 5.10 p.p.m.).

In order to determine the absolute configurations of glucose and glucuronic acid, the polysaccharide was subjected to methanolysis, carboxyl reduction with sodium borodeuteride, and hydrolysis, and the products were converted into the alditol acetates. G.l.c.–m.s. then showed that one-third of the glucose derivative contained two deuterium atoms at position 6, thus indicating complete reduction of glucuronic acid. All of the glucose in the hydrolysate was oxidised by D-glucose

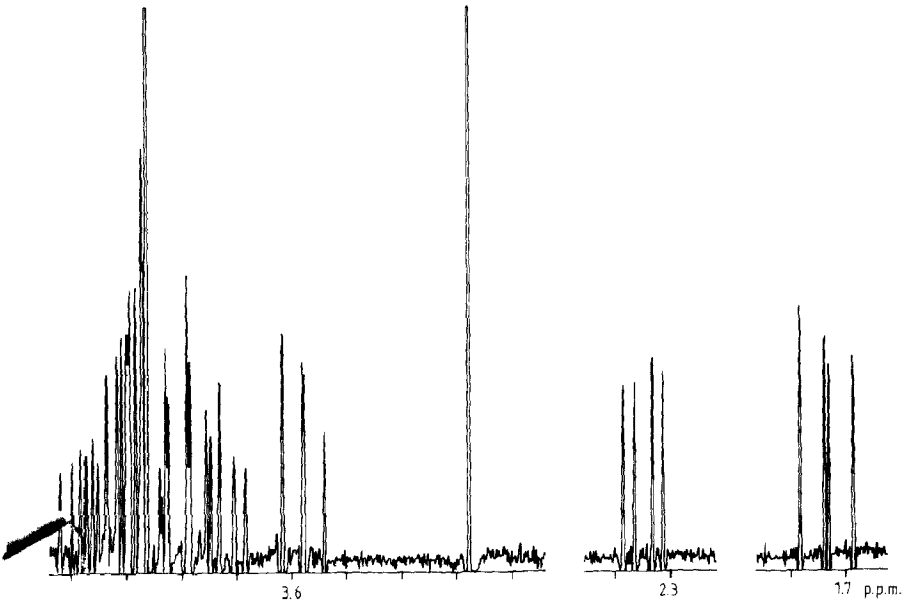


Fig. 2. ^{1}H-N.m.r. spectrum of methyl (methyl 3-deoxy-D-*glycero*-D-*galacto*-nonulopyranosid)onate **2**.

oxidase and, hence, was D. The $[\alpha]_D$ value of the mannose, isolated from the hydrolysate by preparative p.c., indicated it to be D.

In order to determine the configuration of the 3-deoxynonulosonic acid, the polysaccharide was methanolysed to yield the derivative **2**, isolated by gel chromatography on TSK HW 40 and purified by h.p.l.c. on reversed-phase C_{18}.

According to the ^{1}H- (Fig. 2, Table I) and ^{13}C-n.m.r. spectra (see Experimental), **2** is a methyl (methyl 3-deoxynonulopyranosid)onate. The $^3J_{H,H}$ values were

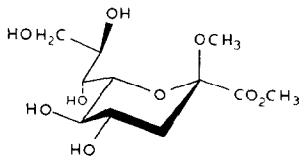
TABLE I

^{1}H-N.M.R. DATA FOR **2**

Proton	*Chemical shift (δ)*	*Multiplicity*	*Coupling constant[a] (Hz)*	
H-3*a*	1.74	dd	$J_{3a,3e}$	13.3 (13.2)
			$J_{3a,4}$	11.4 (11.8)
H-3*e*	2.35	dd	$J_{3e,4}$	5.1 (5.0)
H-4	3.97	ddd	$J_{4,5}$	9.0 (10.4)
H-5	3.58	dd	$J_{5,6}$	9.9 (10.7)
H-6	3.81	dd	$J_{4,7}$	1.3 (1.2)
H-7	3.92	dd	$J_{7,8}$	9.0 (9.4)
H-8,9	3.84–3.92	m		
H-9′	3.68–3.76	m		

[a]The values in parentheses are for *N*-acetyl-β-neuraminic acid[5].

similar to those of *N*-acetyl-β-neuraminic acid (Table I), suggesting that **2** has the D-*glycero*-D-*galacto* (or L-*glycero*-L-*galacto*) configuration. On the basis of the chemical shifts of the signals for H-3*a* and H-3*e* (1.74 and 2.35 p.p.m., respectively) and the known regularity for glycosides of sialic acids[6], it is concluded that **2** has the carboxyl group equatorial and is thus the β anomer.



2

The ^{1}H-n.m.r. spectrum of **2** was similar to that of the methyl (methyl 3-deoxynonulopyranosid)onate obtained on methanolysis[7] of a glycoprotein from the egg of the rainbow trout *Salmo gairdneri*. Since the ^{1}H-n.m.r. spectrum of the latter sugar was identical to that of a product prepared by deamination[8] of the corresponding dimethyl derivative of neuraminic acid, the D-*glycero*-D-*galacto* configuration was assigned[7], although the absolute configuration was not proved unambiguously. The product obtained by deamination of methyl (methyl 3-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosid)onate and **2** had identical retention times in h.p.l.c. (reversed-phase C_{18}), and ^{1}H-n.m.r. spectra, and similar $[\alpha]_D$ values (−53° and −46°, respectively).

Thus, the higher sugar in the polysaccharide is 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid, which has not been reported hitherto as a constituent of a bacterial polysaccharide.

The position of the signals for H-3*a* and H-3*e* of the 3-deoxyaldulosonic acid residue in the ^{1}H-n.m.r. spectrum of the polysaccharide at 1.54 and 2.59 p.p.m., respectively, proved[6] that the carboxyl group is axial and, hence, the sugar is α (the difference between the chemical shifts of these protons in 3-deoxy-β-aldulopyranosides[6] is <0.8 p.p.m., *e.g.*, 0.61 p.p.m. for the β-anomer **2**, Table I).

Methylation analysis of the polysaccharide gave 2,4,6-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylmannose in the ratio ~3:1, and, hence, the glucose and mannose were 3-substituted. Methylation followed by carboxyl reduction of the polysaccharide revealed glucose and mannose in the ratio ~2:1 together with 3-*O*-methylglucose derived from glucuronic acid. Therefore, the residues of glucuronic acid are 2,4-disubstituted and, hence, the polysaccharide is branched. No derivative of the 3-deoxynonulosonic acid was detected in the methylation analysis, but its terminal position in a side chain was indicated by further studies.

Mild hydrolysis of the polysaccharide with dilute acetic acid released the higher sugar, isolated as its ammonium salt, and left a modified polysaccharide (PS-I). G.l.c.–m.s. of the trimethylsilylated higher sugar[9] revealed it to be an anhydro derivative, probably formed during the hydrolysis (the mass-spectrum

peaks at *m/z* 595 and 493 correspond to the fragments M − Me and M − $COOSiMe_3$ respectively). The 3-deoxynonulosonic acid was also present as a minor component, and its mass spectrum was characterised by peaks at *m/z* 757 and 655.

The ^{13}C-n.m.r. spectrum showed PS-I to contain the same components as the parent polysaccharide except for the 3-deoxynonulosonic acid and *O*-acetyl groups. Methylation analysis of PS-I followed by carboxyl reduction gave 2,4,6-tri-*O*-methylglucose, 2,4,6-tri-*O*-methylmannose, and 3,4-di-*O*-methylglucose. Thus, PS-I is linear and represents the backbone of the parent polysaccharide. The formation of 3,4-di-*O*-methylglucose showed that the original glucuronic acid was 2-substituted and, consequently, the 3-deoxynonulosonic acid was linked at position 4.

A computer-assisted ^{13}C-n.m.r. analysis[10] of PS-I was carried out using the cited[10] data on chemical shifts and glycosylation effects for glucose and mannose. For the resonances of C-1,2,3,4,5 of isolated glucuronic acid the chemical shifts in Table II were used. The chemical shift of the resonance for C-6 (the carboxyl group) was excluded from the calculation, and the glycosylation effects used were the same as for glucose.

The evaluation showed that only two structures, namely, **3** and **4**, characterised by the S values 0.9 and 1.1, respectively (where S is the sum of the squared deviations for the chemical shifts of the corresponding signals in the observed and evaluated spectra), are consistent with both methylation (see above) and ^{13}C-n.m.r. data (Table II).

→3)-β-D-Glc*p*-(1→2)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→

3

→3)-β-D-Glc*p*-(1→2)-α-D-Glc*p*A-(1→3)-α-D-Glc*p*-(1→3)-α-D-Man*p*-(1→

4

All alternative structures were either precluded by the methylation data or had S values >2, and, hence, were inconsistent with the ^{13}C-n.m.r. data. Structures **3** and **4** differ only in the sequence of the α-D-Glc*p* and α-D-Man*p* residues, but only **3** could afford the aldobiouronic acid **1**. Thus, PS-I has structure **3**. The configurations of the glycosidic linkages in **3**, determined by the computer-assisted analysis, are consistent with the $J_{C\text{-}1,H\text{-}1}$ values measured from the gated-decoupling ^{13}C-n.m.r. spectrum of the parent polysaccharide (see above). That the glucuronic acid residue was α was confirmed also by the structure of disaccharide **1**. The tentative assignment of the ^{13}C signals for PS-I are given in Table II.

In seeking to determine the location of the *O*-acetyl group in the parent polysaccharide, *O*-deacetylation with aqueous triethylamine[11] was attempted but failed, and treatment with aqueous sodium hydroxide destroyed the polymer. Smith degradation of the polysaccharide destroyed all 3-deoxynonulosonic acid residues, and the products were so labile that the glycosidic linkages were cleaved during the periodate oxidation or the work-up so that mild acid hydrolysis was not necessary.

TABLE II

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

Unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
GlcA α	93.6	72.7	73.6	72.5	71.7	
β	97.3	75.0	76.5	72.3	75.7	
PS-I[a]						
→3)-β-Glc*p*-(1→	105.0	73.4	84.1	70.8	76.8	61.7
	(104.7)	(73.2)	(83.8)	(70.8)	(77.2)	(62.1)
→2)-α-Glc*p*A-(1→	101.2	81.5	72.5	72.6	71.8	173.7
	(101.2)	(81.7)	(72.3)	(72.5)	(72.5)	
→3)-α-Man*p*-(1→	102.2	70.8	81.2	67.0	74.2	62.2
	(102.0)	(71.3)	(80.1)	(67.6)	(74.2)	(62.3)
→3)-α-Glc*p*-(1→	100.4	71.1	81.2	71.1	73.1	61.6
	(100.0)	(71.5)	(80.8)	(71.8)	(73.2)	(61.9)
PS-II[b]						
→3)-β-Glc*p*-(1→	105.2	73.2	83.2	70.7	76.4	61.4
→2)-α-Glc*p*A-(1→	101.2	80.2	74.0	71.3	72.7	174.8
→3)-α-Man*p*-(1→	102.2	70.7	81.1	66.8	74.0	62.0
→3)-α-Glc*p*-(1→	100.3	71.0	81.2	71.2	72.7	61.3

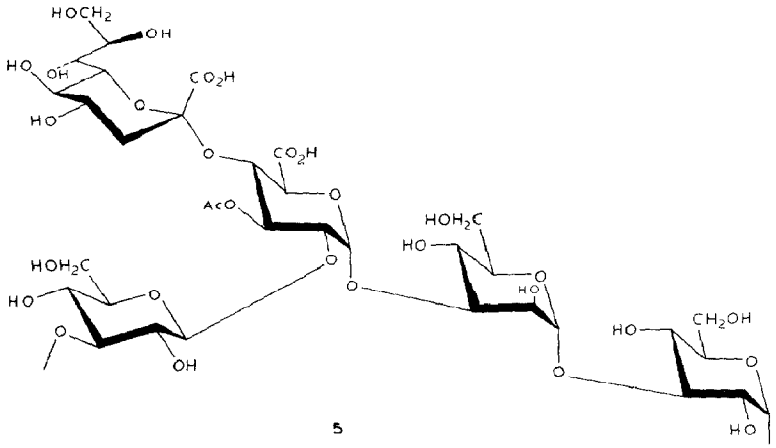
[a]The calculated chemical shifts are given in parentheses. [b]Assignments with differences in chemical shifts of <0.5 p.p.m. may be interchanged.

The ^{13}C-n.m.r. spectrum of the Smith degraded polysaccharide (PS-II) showed its repeating unit to contain the *O*-acetyl group (CH_3 at 21.6 p.p.m.). Since PS-II was resistent to periodate, it followed that the *O*-acetyl group was attached to position 3 of the glucuronic acid residue that is 4-substituted by 3-deoxynonulosonic acid. Comparison of the ^{13}C-n.m.r. data for PS-I and PS-II (Table II) confirmed the position of the *O*-acetyl group. Thus, the displacements of the signals for C-2,3,4 of the glucuronic acid residue from 81.5, 72.5, and 72.6 p.p.m. in PS-I to 80.2, 74.0, and 71.3 p.p.m., respectively, in PS-II are typical of the α- and β-effects of 3-*O*-acetylation[12] of this monosaccharide.

Steric hindrance may account for the somewhat unusual behaviour of the 3-*O*-acetyl group of the glucuronic acid residue since it is flanked by substituents. As noted above, the 3-*O*-acetyl group was resistant to aqueous triethylamine, but was readily removed together with the 3-deoxynonulosonic acid residue on hydrolysis with dilute acetic acid, which usually does not affect *O*-acetyl groups. The ^{13}C signal for CH_3CO of the initial polysaccharide was shifted downfield (to 22.3 p.p.m.) in comparison with its usual position at 21.2–21.8 p.p.m. No anomalies of these types were observed for the *O*-acetyl group in PS-II, which was readily *O*-deacetylated with aqueous triethylamine to give PS-I, and the ^{13}C signal for CH_3CO was at 21.6 p.p.m.

Thus, it was concluded that the capsular polysaccharide of *K. ozaenae* serotype K4 strain 2211 has structure **5**, which differs from that[2] for the capsular polysaccharide of another strain of *K. ozaenae* serotype K4 isolated from a patient. The latter polysaccharide was linear with a tetrasaccharide repeating-unit contain-

ing D-glucose, D-mannose, and D-glucuronic acid in the ratios 2:1:1 together with an *O*-acetyl group (position not determined). The structure of the backbone differed from that of the backbone of the polysaccharide of strain 2211 only in the sequence of the α- and β-Glc*p* residues.



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EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded with a Bruker WM-250 instrument for solutions in D_2O at 30° (internal acetone, δ 2.23). N.O.e.'s were measured as described[13]. ^{13}C-N.m.r. spectra were recorded with a Bruker AM-300 instrument for solutions in D_2O at 60° for polysaccharides and 30° for monosaccharides (internal methanol, δ 50.15). U.v. spectra were recorded with a Uvicord instrument. Optical rotations were measured with a Jasco DIP-360 polarimeter for solutions in water at 25°.

Ascending p.c. was performed on FN-11 and FN-15 paper with 1-butanol–pyridine–water (6:4:3) or pyridine–ethyl acetate–acetic acid–water (5:5:1:3) and detection with alkaline silver nitrate. T.l.c. was carried out on Kieselgel (Merck) with pyridine–ethyl acetate–acetic acid–water (5:5:1:3) and detection by charring with sulfuric acid. Neutral sugars were identified[14] by a Technicon analyzer and uronic acids were determined with a Biotronic LC-2000 analyzer, using a column (15 × 0.37 cm) of Dionex DAx8-11 resin in M potassium borate buffer (pH 9.6) at 65°.

G.l.c. was performed with a Pye Unicam 104 instrument, using a column (150 × 0.4 cm) packed with 3% of OV-1 on Diatomite CQ (100–120 mesh) and nitrogen as the carrier gas. G.l.c.–m.s. was performed with a Varian MAT 311 instrument, using a glass-capillary column coated with SE-30 stationary phase; carrier gas, helium.

Gel filtration was carried out on a column (80 × 2.5 cm) of Biogel P-2 with

0.5M ammonium hydrogencarbonate and on a column (80 × 1.7 cm) of TSK HW 40 with aqueous 1% acetic acid. Ion-exchange chromatography was performed on a Whatman column (10 × 3 cm) of DEAE-cellulose with a gradient of sodium chloride from 0.1 to 1.0M in sodium phosphate buffer. H.p.l.c. was performed on an analytical Ultropack column packed with the reversed-phase C_{18} with aqueous 5% methanol using an Altex instrument. Unless otherwise stated, elution profiles were recorded with a Knauer differential refractometer.

Passive haemagglutination was carried out as described[1].

Isolation of the capsular polysaccharide. — The culture of *K. ozaenae* strain 2211 (serotype K4) was obtained from the Collection of Capsulated Cultures of the Division of Microbiology of the Minsk Institute of Medicine. The culture was grown for 3 days under aerobic conditions at 28° in a synthetic nutrient medium containing K_2HPO_4 (7 g), KH_2PO_4 (2 g), sodium citrate ($Na_3C_6H_5O_7 \cdot 5\ H_2O$, 0.5 g), $MgSO_4 \cdot 7\ H_2O$ (0.1 g), $(NH_4)_2SO_4$ (1 g), and NaCl (5 g) in 1 L of water, with the addition of sterile glucose (10 g); ~20×10^6 microbial cells/mL; final pH, 8.0. The culture was centrifuged at 20,000 r.p.m. for 1 h at 4°, the sediment was suspended in 5 vol. of 0.9% saline, stirred with glass beads for 5–10 min, and centrifuged at 20,000 r.p.m. for 1 h at 4°. The supernatant solutions of the culture medium and saline suspension were combined, concentrated *in vacuo*, dialysed against water for 2 days at 20°, and precipitated with 5 vol. of ethanol. The precipitate was dissolved in water and the precipitation with ethanol was repeated thrice.

The crude capsular polysaccharide was purified by precipitation with Cetavlon[15] followed by ion-exchange chromatography on DEAE-cellulose. The yield of the pure polysaccharide was 1.8–2.6% of the dry weight of the cells. The polysaccharide was eluted from DEAE-cellulose as a single peak characterised by the u.v. absorption, reactions with thiobarbituric acid[16] and phenol–sulfuric acid, and serological activity.

Monosaccharide composition. — (*a*) The polysaccharide (5 mg) was hydrolysed with 2M trifluoroacetic acid (120°, 1 h, in a sealed tube) and the hydrolysate was concentrated. The residue was studied by p.c., the sugar analyzer, and g.l.c. of the derived alditol acetates.

(*b*) The polysaccharide (40 mg) was hydrolysed with 2M trifluoroacetic acid (100°, 6 h) and the hydrolysate was concentrated. Preparative p.c. of the residue afforded D-mannose (5 mg), $[\alpha]_D$ +9° (*c* 0.5); lit.[17] $[\alpha]_D$ +14.2° (water).

(*c*) The polysaccharide (5 mg) was dried *in vacuo* over phosphorus pentaoxide at 50° and heated in a sealed tube with methanolic M hydrogen chloride (2 mL, 85°, 4 h). The solution was concentrated, the residue was reduced with an excess of sodium borodeuteride in aqueous 50% 2-propanol (20°, 16 h), the solution was concentrated, and methanol was evaporated thrice from the residue which was hydrolysed with 2M trifluoroacetic acid (120°, 1 h). The hydrolysate was concentrated, and a portion of the residue was converted into alditol acetates and analysed by g.l.c.–m.s. To a solution of the other portion in water (0.2 mL) was added D-glucose oxidase (0.6 mg/mL, Boeringer), and the solution was incubated for 2 h at 37°. The oxidation was monitored by using the sugar analyzer.

(*d*) The polysaccharide (30 mg) was dried and heated with methanolic 0.5M hydrogen chloride (2 mL, 70°, 4 h). The solution was concentrated and methanol was evaporated thrice from the residue. Preparative h.p.l.c. yielded **2** (3.5 mg), $[\alpha]_D$ −46° (*c* 0.35). ^{13}C-N.m.r. data: δ 171.7 (C-1), 100.5 (C-2), 73.1 (C-6), 71.5 (C-8), 70.9 (C-5), 69.6 (C-7), 69.1 (C-4), 64.7 (C-9), 40.1 (C-3). For the ^{1}H-n.m.r. spectrum, see Table I.

Partial acid hydrolysis. — (*a*) The polysaccharide (40 mg) was hydrolysed with M trifluoroacetic acid (1 mL, 100°, 1 h). The hydrolysate was concentrated and gel chromatography of the residue on TSK HW 40 afforded 3-*O*-(D-gluco-pyranosyluronic acid)-D-mannose (**1**, 8 mg). ^{1}H-N.m.r. data: δ 5.30 (d, $J_{1',2'}$ 4.0 Hz, H-1'), 5.17 (d, $J_{1,2}$ 2.0 Hz, H-1), 4.24 (d, $J_{4',5'}$ 0.1 Hz, H-5'), 4.07 (dd, $J_{2,3}$ 3.0 Hz, H-2), 3.95 (m, H-5), 3.89 (dd, $J_{3,4}$ 9.8 Hz, H-3), 3.85 (t, $J_{4,5}$ 10 Hz, H-4), 3.83 (t, $J_{2',3'}$ 10.0 Hz, H-3'), 3.63 (dd, H-2'), 3.58 (t, $J_{3',4'}$ 10 Hz, H-4').

(*b*) The polysaccharide (80 mg) was hydrolysed with aqueous 1% acetic acid (2 mL, 100°, 1.5 h). The hydrolysate was concentrated, and water was evaporated twice from the residue which was then eluted from a column of Biogel P-2. The eluate was monitored by u.v. absorption and reaction with thiobarbituric acid[14] and phenol–sulfuric acid, to give a monosaccharide fraction (6 mg) and PS-I, which was additionally purified by gel filtration on TSK HW 40 (yield, 42 mg).

Smith degradation. — The polysaccharide (40 mg) was oxidised in the dark with 0.2M sodium metaperiodate (2 mL, 20°, 24 h), ethylene glycol (0.2 mL) was then added, the mixture was kept for 30 min, sodium borohydride (100 mg) was added, and, after 30 min, the mixture was dialysed against distilled water. Gel filtration on TSK HW 40 then afforded PS-II (18 mg). Periodate oxidation of PS-II was carried out under the same conditions.

O-*Deacetylation.* — PS-II (18 mg) was treated with aqueous 4% triethylamine (2 mL, 60°, 3 h). The solution was concentrated, and gel filtration of the residue on TSK HW 40 gave PS-I (10 mg).

The initial polysaccharide was unaffected under these conditions.

Methylation analysis. — An aqueous solution of the polysaccharide (10 mg) was treated with KU-1 (H^+) resin and freeze-dried. The residue was dried over phosphorus pentaoxide *in vacuo* at 50° and then methylated (Hakomori[18]). The methylated polysaccharide was isolated[19] using a Sep-Pack C_{18} cartridge. A portion of the product was hydrolysed with 2M trifluoroacetic acid (120°, 1 h), and the products were converted into the alditol acetates, and analysed by g.l.c.–m.s., using data[20] for identification of partially methylated monosaccharides. The other portion was reduced with an excess of lithium borohydride in aqueous 70% 2-propanol (1 mL, 20°, 2 h). The solution was acidified with conc. acetic acid and concentrated; methanol was evaporated thrice from the residue, an aqueous solution of which was desalted with KU-2 (H^+) resin and then analysed as described above.

Methylation analysis of PS-I was carried out similarly.

Synthesis of **2**. — *N*-Acetylneuraminic acid (60 mg) was heated with methanolic 0.5M hydrogen chloride (2 mL, 80°, 24 h, in a sealed tube), and the

resulting dark-brown solution was concentrated to dryness. Methanol was evaporated thrice from the residue, a solution of which in water was extracted with 1-butanol and concentrated. A solution of the residue in water (0.5 mL) was treated with aqueous 33% acetic acid and 5.5M sodium nitrite (0.2 mL) for 40 min at 20°, then with KU-2 (H^+) resin. The resin was collected and washed with aqueous 50% methanol, and the combined filtrate and washings were concentrated. Gel chromatography of the residue on TSK HW 40 was monitored by t.l.c. Further purification by h.p.l.c. then afforded **2** (3 mg), $[\alpha]_D$ −53° (*c* 0.37).

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