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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* K-TYPE 63

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ABSTRACT

Structural investigation of the capsular polysaccharide from *Klebsiella* K type 63 by methylation analysis, periodate oxidation, and uronic acid degradation showed the repeating unit to consist of →3)-α-D-Gal*p*-(1→3)-α-D-Gal*p*A-(1→3)-α-L-Fuc*p*(1→. This structure is identical to that of *Escherichia coli* serotype K-42 capsular polysaccharide. The ^{1}H- and ^{13}C-n.m.r. spectra of the original and modified polysaccharide are consistent with the foregoing structure.

INTRODUCTION

In a recent paper, Heidelberger and Nimmich[1] reported data on the cross-reactivities of more than 60 capsular (K)-type specific polysaccharides of *Klebsiella* with 26 types of specific antipneumococcal sera. A knowledge of the primary structure of the antigenic material facilitates interpretation of the relation between chemical structure and immunological specificity. In the present communication, the structure of the capsular polysaccharide from *Klebsiella* K-type 63 is established and its identity with that of *E. coli* K-type 42 is confirmed[2].

RESULTS AND DISCUSSION

In the data provided by Nimmich[3,4] on qualitative analysis of the capsular polysaccharide of *Klebsiella*, serotype K-63 was found to contain galactose, rhamnose, fucose, and galacturonic acid, and formed in itself a unique chemotype.

The capsular polysaccharide, purified by Cetavlon precipitation, had $[\alpha]_{589}$ +133°. No acetal-linked pyruvic acid could be detected in the n.m.r. spectra. Complete acid hydrolysis of the polysaccharide gave fucose, galactose, and an aldobiouronic acid, and acid hydrolysis of the carboxyl-reduced K-63 gave only fucose and galactose, in the approximate ratio of 1:2.

These figures, and those obtained after methylation analysis, indicate that K-63 contains fucose, galactose, and galacturonic acid in the proportion 1:1:1. In contrast to the analytical data of Nimmich[3,4], the capsular polysaccharide K-63

does not contain rhamnose. The presence of only one type of 6-deoxyhexose was confirmed by paper chromatography and g.l.c. of the hydrolysate, and by the presence of only one signal, corresponding to a CH_3 group in the ^1H- and ^{13}C-n.m.r. spectra, and having the chemical shift characteristic of fucose. The ^1H-n.m.r. spectrum recorded in D_2O at 95° with the sodium salt[6] indicated the presence of one 6-deoxy residue and three anomeric protons; the latter were observed in two groups in the proportion 1:2. The presence of *O*-acetate could also be detected; it amounted to <0.2 residue. This latter result agreed with an estimation of acetate carried out by enzymic titration of the sodium acetate released by saponification.

Fucose and galactose were isolated from the hydrolysate of the original polysaccharide, and their optical rotations indicated that they belonged to the L and D series, respectively. Galacturonic acid had previously been shown by immunochemical cross-reactions to belong to the D series[1].

In the course of the sugar analysis with K-63, it was noted that the glycosidic linkage of the aldobiouronic acid was cleaved to the extent of ~50% during acid hydrolysis. This behaviour seems to be frequent with galacturonic acid-containing polysaccharides[8,9].

Methylation analysis of the original capsular polysaccharide gave the neutral sugars shown in Table I (column A), which were identified by g.l.c.–m.s. of their alditol acetates. Here again, partial hydrolysis of the aldobiouronic acid was at first confusing as it gave the methylated fucose and galactose in 1:2 ratio. Methylation, followed by carboxyl reduction with lithium aluminium hydride and subsequent hydrolysis, gave the results summarised in Table I (column B). These results show that the polysaccharide consists of a trisaccharide repeating-unit composed of one L-fucose, one D-galactose, and one D-galacturonic acid residue, the three sugar residues being linked through O-3.

Tne permethylated polysaccharide was subjected to uronic acid degradation by treatment with methylsulphinyl carbanion, followed by mild acid hydrolysis. Hydrolysis of the resulting product gave 2,4-di-*O*-methyl-L-fucose and 2,4,6-tri-*O*-methyl-D-galactose in the ratio ~1:1 (Table I, column D). A second portion of the

TABLE I

METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED *Klebsiella* TYPE 63 POLYSACCHARIDE

Methylated sugars (as alditol acetates)[a]	*T*[b]	*Mole %*[c]			
		A	*B*	*C*	*D*
2,4-Fuc	1.17	35	33	34	42
2,4,6-Gal	2.35	65	35	66	58
2,4-Gal	6.91		31		

[a]2,4-Fuc. = 2,4-di-*O*-methyl-L-fucose, etc. [b]Retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c]Polysaccharide: *A*, original; *B*, carboxyl-reduced after permethylation; *C*, carboxyl-reduced before permethylation; *D*, uronic acid degradation.

degraded material was remethylated by using trideuteriomethyl iodide, and the product hydrolysed and analysed by g.l.c.–m.s. as the alditol acetates 2,3,4-Tri-*O*-methyl-L-fucose trideuteriomethylated at *O*-3 was identified, thus indicating that the specific, uronic acid degradation led to an oligosaccharide wherein a fucose residue occupied a terminal, non-reducing end. This result confirms the position of the linkage between the galacturonic acid and the fucose residue.

The existence of only (1→3) linkages in this capsular polysaccharide was confirmed by the fact that it did not consume periodate.

Partial hydrolysis of the original K-63 polysaccharide gave a mixture of oligomers that was resolved on ion-exchange resin into neutral and acidic fractions. Paper chromatography of the neutral fraction showed essentially three spots, having mobilities corresponding to fucose and galactose, and to an unknown, slower-moving compound **1** having g.l.c. properties corresponding to a disaccharide. This compound was isolated on a column of Sephadex G-15. Its ^{13}C-n.m.r. spectrum showed *inter alia* the characteristic peak corresponding to C-6 of fucose, and three signals of equal intensities in the anomeric region, but no peak corresponding to the carbon atom of a free primary alcohol group of a galactose residue. Additional information was obtained by methylation of **1**, followed by examination by g.l.c.–m.s. The mass spectrum showed clearly a fragment at *m/e* 249 corresponding to an abJ ion[10] and indicating the presence of a 6-deoxyhexoside occupying the terminal reducing position of a permethylated disaccharide (Chart I). Additional evidence was provided by fragments bA, at *m/e* 189, and bA_2 at *m/e* 157. No fragment corresponding to galactose or fucose in a terminal, non-reducing position could be found in the mass spectrum of **1**. On the other hand, the spectrum showed all of the peaks characteristic of a permethylated galacturonic acid group occupying the terminal, non-reducing position. The

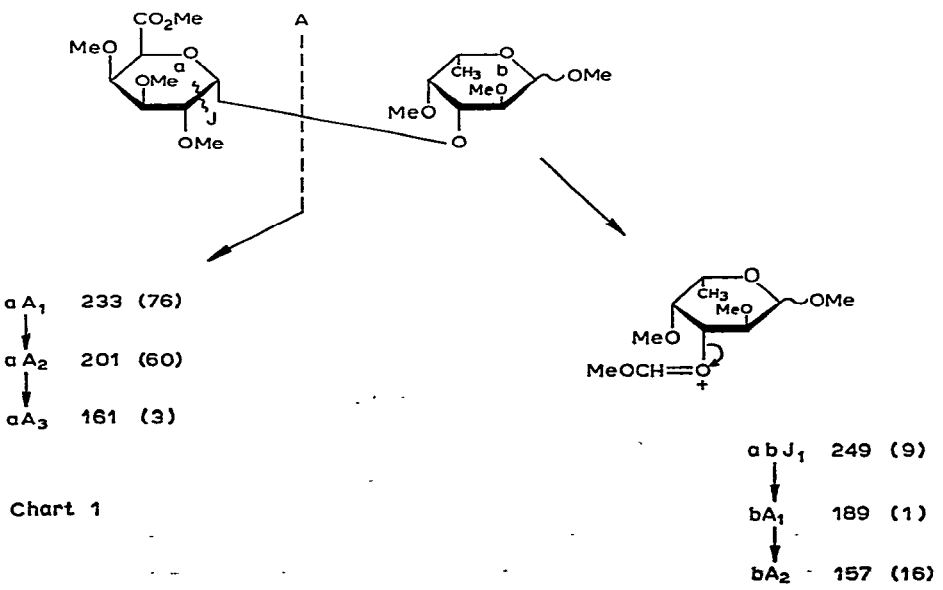

Chart 1

TABLE II

^{1}H-N.M.R. DATA ON *Klebsiella* K-63 CAPSULAR POLYSACCHARIDE

Proton assignment	*δ p.p.m.*[a] *(coupling constant, Hz)*	*Ratio of integral*
H-1 (GalA)	5.35 (≈3.0)	1
H-1 (Fuc + Gal)	5.24 (≈3.0)	2
CH_3 (Fuc)	1.22 (6.5)	3
H-5, H-6a and H-6b (Gal)	3.78	3
(-O-C-CH_3)	2.16	≤0.2

[a]Spectrum recorded in Me_2SO-d_6; δ values are given relative to T.S.P. as internal reference, with a difference of −0.05 p.p.m. relative to Me_4Si as external reference.

TABLE III

^{13}C-N.M.R. DATA ON *Klebsiella* K-63 CAPSULAR POLYSACCHARIDE AND ITS DERIVATIVES

Carbon assignments	*Chemical shifts*[a]		
	Original K-63		*Carboxyl-reduced*
	Me_2SO-d_6	*D_2O*	*Me_2SO-d_6*
CO_2H (GalA)	170	172.40	
C-1 (GalA)	101	101.75	100.75
C-1 (Gal)	100.6		
C-1 (Fuc)	96.4	96.80	96.05
C-3 (GalA)	79.15	79.35	
C-3 (Gal)	77.90	78.60	78.55 78.15
C-3 (Fuc)	75.70	75.20	76.15
C-6 (Gal)	59.90	62.25	60.71 60.11
C-6 (Fuc)	16.30	16.50[b]	16.30
C-2, C-4 and C-5 (Gal, Fuc, GalA)	71.30 70.90 69.90 68.30 67.70 67.60 67.10 61.20	73.05 72.25 71.95 70.65 68.95 68.85 68.30 68.05 67.95	71.0 70.80 70.70 68.40 67.65 67.40 66.15 65.45

[a]Spectra recorded with Me_4Si as external reference. [b]δ recalculated according to Gorin and Mazurek[12].

disaccharide was thus identified as the aldobiouronic acid: methyl 2,4-di-*O*-methyl-3-*O*-(methyl 2,3,4-tri-*O*-methyl-α-D-galactopyranosyluronate) - α-L-fucopyranoside. The most prominent peaks were as follows: a A_1, *m/e* 233, a A_2 *m/e* 201, and A_3, *m/e* 161 (Chart I).

The presence of this acidic oligomer among the neutral sugars may be explained by the fact that a part of the aldobiouronic acid obtained during partial hydrolysis had lactonised, and thus the lactone behaved as a neutral substance[11] during ion-exchange separation by paper chromatography. The lactone was then hydrolysed back to the acid during the methylation step, giving rise to the permethylated aldobiouronate **1**.

It may thus be concluded that the sequence of K-63 is built up of a trisaccharide wherein galacturonic acid is linked through *O*-3 of an L-fucopyranosyl residue, itself linked to *O*-3 of a D-galactopyranosyl residue. Evidence for the configuration at the anomeric linkages was obtained by n.m.r. spectroscopy on the polysaccharide. Because of the very high viscosity of K-63, the spectra were obtained on slightly depolymerized material in order to enhance the solubility of the samples. From the ^{1}H- and ^{13}C-n.m.r. spectra, it appeared that all linkages were α (Tables II and III).

The ^{13}C spectra showed 17 distinct signals, one of which corresponded to two carbon atoms. It must be noted, however, that one difficulty in the assignment of the signals arises from the fact that K-63 is made up of three different sugar residues having clearly related configurations. Comparison between the ^{1}H- and ^{13}C-n.m.r. spectra of the original and the carboxyl-reduced K-63 allowed the assignment of a certain number of signals. (Table III). It may be seen that carboxyl reduction affected only slightly the chemical shifts of the corresponding carbon atoms, and thus, the signal at δ 96–94 must arise from the L-fucosyl residue, although this value differs somewhat from that found for methyl α-L-fucoside[12]. In the spectrum of the carboxyl-reduced polysaccharide, the C-1, C-3, and C-6 signals of the original galactose residue and of that arising from the reduction of the galacturonic acid exhibited slightly different chemical shifts. This is attributable to the fact that the two galactoses are substituted by a fucosyl or a galactosyl residue, respectively.

The three glycosidic linkages of the repeating unit were shown to have the α configuration, as demonstrated by the $^1J_{C\text{-}1,H\text{-}1}$ coupling constants (168–171 Hz) measured by the gated-decoupling technique. This assignment was confirmed by examination of the ^{1}H-n.m.r. spectrum (Table II).

The ^{13}C-n.m.r. spectra of both polysaccharides showed the same pattern, but the insufficient sample of K-42 did not allow a detailed comparison of the spectra.

Interestingly, X-ray fibre diffraction studies of *Klebsiella* K-63 were performed[15] before the chemical structure was established, and helped in the determination of the length of the repeat unit. Here again, the agreement between the conformations by X-ray diffraction of both *Klebsiella* K-63 and *E. coli* K-42 polysaccharide determined by E.D.T. Atkins* and S. Arnott were in complete agreement.

*Personal communication.

EXPERIMENTAL

General methods.[16] — Paper chromatography was performed on Whatman No. 1 paper for analytical purposes and on Whatman no. 3MM for preparative purposes, and the following solvent systems (v/v) were used: (*A*) 8:2:1 ethyl acetate–pyridine–water; (*B*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; and (*C*) 10:4:3 ethyl acetate–pyridine–water. Chromatograms were developed with silver nitrate. G.l.c. analyses were performed on a Packard–Becker 417 instrument fitted with dual flame-ionisation detectors. Peak areas were measured with a Hewlett–Packard 3380 A digital integrator. Glass columns (1/8 in. o.d.) were used, with a carrier-gas flow rate of 60 mL/min.

Columns were (*A*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 180° (for alditol acetates) or 150° (for partially methylated alditol acetates); (*B*) 3% of OV-225 on Chromosorb WAW-DMCS (100–120 mesh) at 150° (for partially methylated alditol acetates); (*C*) 2% of XE-60 on the same support (for oligosaccharide derivatives). G.l.c.–m.s. was performed on a Girdel 3000 chromatograph coupled to an AEI MS-30 mass spectrometer. Spectra were recorded at 70 eV with an ionisation current of 100 μA and an ion-source temperature of 100°.

Isolation of the polysaccharide from Klebsiella K-63. — The capsular polysaccharide was collected and purified as described previously[16]. The purified polysaccharide showed $[\alpha]_D$ +133° (*c* 2.0, water) and its equivalent weight by sodium hydroxide titration was ~560.

Hydrolysis of native polysaccharide, and sugar analysis. — Different conditions of hydrolysis were used: (*a*) M trifluoroacetic acid at 100° for 0.5 to 3 h; the excess of acid was then evaporated in a rotary evaporator and the sugars were analysed as their alditol acetates; (*b*) 72% sulphuric acid at room temperature then diluted to 0.5M and for 6 h at 100°, as described earlier[16]. Hydrolysates were examined by paper chromatography in solvents *A* and *B* and isolated by preparative, paper chromatography. The fucose showed $[\alpha]_D^{20}$ −30° (*c* 2, water), and galactose $[\alpha]_D^{20}$ +61° (*c* 1.3, water).

Carboxyl reduction of the native polysaccharide. — This was performed according to Taylor and Conrad[5]. The treatment was repeated twice to achieve complete reduction, and checked by g.l.c. after hydrolysis of the reduced sample.

Methylation analysis. — Because of the low solubility of K-63 in dimethyl sulfoxide it was necessary to acetylate the polysaccharide before submitting it to the usual Hakomori conditions[17]. Acetylation was effected in 2:1:1 formamide–pyridine–acetic anhydride for 24 h and the mixture was then evaporated. The residue was dialysed against distilled water and freeze-dried. The acetylated K-63 dissolved in dimethyl sulphoxide and was methylated by one Hakomori treatment followed by two consecutive treatments[18] to yield a product that showed no hydroxyl absorption in the i.r. spectrum. A part of the methylated, original polysaccharide was hydrolysed with formic acid (90%; 1 h at 100°) and then with trifluoroacetic acid (2M; 3 h at 100°). The resulting, partially methylated sugars were analysed as their alditol acetate

derivatives by g.l.c.–m.s.[20]. (Table I, column A). A second part of the methylated K-63 was reduced with lithium aluminium hydride (4 h reflux); after isolation, the product was hydrolysed and analysed as before (Table I, column B).

Uronic acid degradation[21]. — Permethylated K-63 (83 mg) was dissolved in a 19:1 mixture of dimethyl sulphoxide–2,2-dimethoxypropane (15 mL) and the solution was kept in an ultrasonic bath for 30 min, under nitrogen. Methylsulphinyl anion in dimethyl sulphoxide (2M, 9 mL) was added and the mixture kept overnight at room temperature. After neutralization with 50% acetic acid and then diluted with water, the mixture was extracted with chloroform. The chloroform extracts were washed with water and evaporated. A part of the degraded material was hydrolysed with 0.25M sulphuric acid (8 h, 100°), neutralized with barium carbonate, and then analysed by g.l.c.–m.s. as before (Table I, column D). Another portion of the degraded polysaccharide was subjected to mild acid hydrolysis with 50% acetic acid (15 mL, 1 h, 100°). The mixture was evaporated to dryness and sodium borodeuteride (100 mg) was added in a mixture (11 mL) of 1,4-dioxane and ethanol (8:3)[21]. The solution was stirred overnight, and then treated with Dowex-50 (H^+) resin. The mixture was evaporated to dryness and the borate ions removed by distillation of methanol from the residue. The residue was remethylated using trideuteriomethyl iodide as already described, and examined by g.l.c.–m.s.

Partial acid hydrolysis and characterization of oligosaccharide 1. — Native polysaccharide (150 mg) was hydrolysed with 0.5M trifluoroacetic acid for 45 min at 100° and then evaporated to dryness. The syrup was dissolved in water and applied to the top of a column of Dowex-1 × 4 resin (acetate). Successive elution with water and then with 10% acetic acid afforded the neutral and acidic sugars, respectively. Examination of the neutral sugars by paper chromatography in the solvent system 2:1:1 butanol–acetic acid–water showed the presence of fucose, galactose, and a compound having R_{Glc} 0.78. This compound (**1**) was isolated on a column (90 × 3 cm) of Sephadex G-15 eluted with the system 10:4:1000 pyridine–acetic acid–water at a flow rate of 10 mL/h. Compound **1** was methylated and characterised by g.l.c.–m.s. Its mass spectrum displayed, *inter alia*, the following fragments: *m/e* 75 (35), 85 (12), 88 (42), 101 (100), 125 (3), 129 (67), 141 (15), 145 (23), 157 (16), 161 (3), 169 (71), 201 (60), 233 (76), 249 (9), and 319 (1), and it was thus shown to be an aldobiouronic acid (see text).

N.m.r. spectroscopy. — The ^{1}H- and ^{13}C-n.m.r. spectra were recorded with a CAMECA-250 spectrometer, with D_2O or Me_2SO-d_6 as solvent. The polysaccharide, which gave very viscous solutions had to be partially depolymerised by acid treatment (0.5 M trifluoroacetic acid for 30 min at room temperature) in order to reach the desired concentration. The ^{1}H-n.m.r. spectra (250 MHz) were recorded[6] at 90° with 5-mm (o.d.) tubes. The ^{13}C-n.m.r. spectra were recorded in 5-mm tubes for oligosaccharides and 8-mm tubes for the polymer (≈50 mg in 1.5 mL of D_2O) at 70°. Chemical shifts in δ values were measured relative to tetramethylsilane as external reference; normal ^{13}C spectra were recorded with complete proton-decoupling at 62.86 MHz in a spectrometer equipped with Fourier transform (spectral windows

200 p.p.m. and digitalization into 12 K data points); pulse width 10 μs ($\sim$70°) and interval between the pulses 0.6 sec (corresponding to the acquisition time).

Determination of the coupling constants were obtained with a gated, ^{1}H-decoupler sequence to retain nuclear Overhauser enhancements (interval between the pulses: 1.6 sec; coupling time: 1.0 sec).

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REFERENCES

1 M. Heidelberger and W. Nimmich, *Immunochemistry*, 13 (1976) 67–80.
2 H. Neimann, A. K. Chakraborty, H. Friebolin, and S. Stirm, *J. Bacteriol.*, 133 (1978) 390–391.
3 W. Nimmich, *Z. Mikrobiol. Immunol.*, 154 (1968) 117–131.
4 W. Nimmich, *Acta Biol. Med. Ger.*, 26 (1971) 397–403.
5 R. L. Taylor and H. E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.
6 Y. M. Choy and G. G. S. Dutton, *Can. J. Chem.*, 31 (1973) 3021–3026.
7 B. Lindberg, J. Lönngren, and J. L. Thompson, *Carbohydr. Res.*, 28 (1973) 351–357.
8 J. P. Joseleau, to be published.
9 J. P. Kamerling, B. Lindberg, J. Lönngren, and W. Nimmich, *Acta Chem. Scand., Ser. B*, 29 (1975) 593–598.
10 V. Kováčik, Š. Bauer, J. Rosík and P. Kováč, *Carbohydr. Res.*, 8 (1968) 282–290.
11 J. D. Blake and G. N. Richards, *Carbohydr. Res.*, 8 (1968) 275–281.
12 P. A. J. Gorin and M. Mazurek, *Can. J. Chem.*, 53 (1975) 1212–1223.
13 K. Jann, B. Jann, F. Ørskov, I. Ørskov, and O. Westphal, *Biochem. Z.*, 342 (1965) 1–22.
14 H. Niemann, A. K. Chakraborty, H. Friebolin, and S. Stirm, *J. Bacteriol.*, 133 (1978) 390–391.
15 E. D. T. Atkins, personal communication.
16 J. P. Joseleau, M. Lapeyre, M. Vignon, and G. G. S. Dutton, *Carbohydr. Res.*, 67 (1978) 197–212.
17 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
18 J. F. Carson and W. D. Maclay, *J. Am. Chem. Soc.*, 68 (1946) 1015–1017.
19 T. Purdie and J. C. Irvine, *J. Chem. Soc.*, 83 (1903) 1021–1024.
20 J. Lönngren and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.
21 M. Curvall, B. Lindberg, J. Lönngren, and W. Nimmich, *Carbohydr. Res.*, 42 (1975) 73–82.