Carbohydrate Research, 217 (1991) 117–125
Elsevier Science Publishers B.V., Amsterdam

Structure of the O-specific polysaccharide chain from *Klebsiella pneumoniae* O1K2 (NCTC 5055) lipopolysaccharide*

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(Received Januari 17th, 1990; accepted for publication, in revised form, August 15th, 1990)

ABSTRACT

The structure of the O-specific polysaccharide of *Klebsiella pneumoniae* O1K2 lipopolysaccharide was investigated by use of methylation, periodate oxidation, partial hydrolysis, and ^{1}H- and ^{13}C-n.m.r. spectroscopy. It was shown to consist of a linear chain composed of two disaccharide repeating units, [→3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→] and [→3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→].

INTRODUCTION

In general O-specific polysaccharides of lipopolysaccharides consist of repeating units of oligosaccharides. In rare cases, the O-specific chain is a homopolysaccharide. The structures of an increasing number of O-specific polysaccharides have been elucidated, among which only a few different O serogroups of *Klebsiella* have been described, mainly by the group of Lindberg (see ref. 1). For serogroups O2, O6, O8, and O9, the O-specific chains are constituted only of D-galactose units in the pyranose and furanose ring structure that are mainly (1→3)-linked[1]. For *Klebsiella pneumoniae* serogroup 1, Kenne and Lindberg[1] showed also the presence of (1→3)-linked α-D-galactopyranosyl and β-D-galactofuranosyl residues, but did not describe a detailed structure. We report herein the structure of the O-specific polysaccharide chain obtained, by h.p.l.c. on an anion-exchange column[2], from the *Klebsiella pneumoniae* O1K2 (NCTC 5055) lipopolysaccharide.

EXPERIMENTAL

Bacterial culture and isolation of lipopolysaccharide. — Bacterial culture of *Klebsiella pneumoniae* O1K2 (NCTC 5055) was carried out as described previously[3]. The

* This work was supported by the Centre National de la Recherche Scientifique (C.N.R.S., UM No. 111), the Université des Sciences et Techniques de Lille Flandres-Artois and by grants from the Laboratoires Cassenne.

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0008-6215/91/$03.50

lipopolysaccharide (LPS) was extracted by the hot phenol–water procedure[4] and purified by ultracentrifugation.

Isolation of the O-specific polysaccharide chain. — The LPS in aqueous 1% acetic acid was heated at 100° for 90 min. The precipitated lipid A was removed by centrifugation. The water-soluble materials were fractionated by h.p.l.c. with an anion-exchange column into six fractions named[2] S1–S6. The O-specific polysaccharide chain was located in the neutral S1 fraction.

N.m.r. spectroscopy. — The 400-MHz ^{1}H-n.m.r. experiments were performed with a Bruker AM-400 WB spectrometer, equipped with a 5-mm ^{1}H–^{13}C mixed probehead, operating in the pulse F.t. mode and controlled by an Aspect 3000 computer. After three exchanges with ^2H_2O (99.96 atom% ^{2}H, Aldrich, Milwaukee, IL, U.S.A.) and intermediate lyophilization, the products were analyzed with a spectral width of 3000 Hz for 16 K frequency-domain points and time-domain data points giving a final digital resolution of 0.365 Hz/point. The 100-MHz ^{13}C-n.m.r. experiments were carried out with the standard Bruker pulse-program POWGATE with ^{1}H-broad-band composite-pulse decoupling. A 90° pulse (6 μs) and a 0.5-s recycle delay were used. The chemical shifts are given relative to sodium 4,4-dimethyl-4-sila(2,3-$^2H_4$)pentanoate. The 2D-homonuclear COSY 45 experiments were performed with the standard Bruker pulse-program COSY. The 2D-heteronuclear-correlated experiments were performed with simultaneous ^{1}H-decoupling by use of the standard Bruker pulse-program XHCORRD.

Analytical methods. — The 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) content was estimed according to Karkhanis *et al.*[5]. The total phosphorus content was determined by the method of Lowry *et al.*[6] with NaH_2PO_4 as standard. The total neutral carbohydrate content was determined by the orcinol–H_2SO_4 method[7].

The molar composition of monosaccharides was determined by g.l.c. of methyl per-*O*-trifluoroacetyl glycosides according to Zanetta *et al.*[8] or of methyl per-*O*-trimethylsilyl glycosides according to Kamerling *et al.*[9] as modified by Montreuil *et al.*[10]. The anomeric configuration of the D-galactose residues was determined by measuring the optical rotation of the hydrolyzate of the O-specific polysaccharide (4M trifluoroacetic acid, 4 h, 100°) with a Perkin–Elmer polarimeter Model 241.

Methylation analysis. — Methylation analysis was performed as described by Paz Parente *et al.*[11]. The methylated products were methanolyzed with 0.5M HCl in anhydrous methanol at 80° for 24 h. The partially methylated methyl glycosides were acetylated with acetic anhydride and pyridine at room temperature overnight, and then analyzed by g.l.c.–m.s.[12].

Periodate oxidation. — The S1 fraction (5 mg) was treated with 0.05M $NaIO_4$ (2 mL) in the dark for 8 days at 4°. The excess of periodate was reduced by 1,2-ethanediol (100 μL), and the product was reduced with $NaBH_4$ (5 mg) for 6 h. The base was neutralized with acetic acid and the periodate-resistant oligosaccharide was purified by gel filtration on a column of Bio-Gel P-4 (1 cm i.d. x 50 cm).

Partial hydrolysis. — The S1 fraction (100 mg) was partially hydrolyzed with 0.1M trifluoroacetic acid (4 mL) at 80° for 90 min. The acid was neutralized with aqueous NH_4OH and the hydrolyzate fractionated by gel filtration on a Sephadex G-75 column

(3 cm i.d. x 50 cm), eluted with water at a flow rate of 15 mL/h; fractions (3 mL) were collected and a 100-μL sample of each fraction was analyzed with the phenol–H_2SO_4 reagent[13].

RESULTS

LPS was isolated from *Klebsiella pneumoniae* O1K2 by the Westphal and Jann procedure[4]. The content of LPS was about 2% of dry bacterial cells. Partial hydrolysis of the LPS (1 g) with hot aqueous acetic acid gave insoluble lipid A (257 mg) and a water-soluble fraction (597 mg). The materials contained in the water-soluble fraction were separated[2] by h.p.l.c., in an anion-exchange column, into neutral and negatively charged fractions named S1 – S6.

The neutral fraction S1 was readily soluble in water and was found to be composed only of galactose residues identified as the *O*-trifluoroacetyl or *O*-trimethylsilyl derivatives by g.l.c. Kdo and phosphorus were not detected in this fraction. The S1 fraction was hydrolyzed with 4M trifluoroacetic acid solution at 100° for 4 h. After elimination of the acid, the hydrolyzate had $[\alpha]_D$ +64° (*c* 0.95, water). Comparison with the optical rotation of D-galactose, $[\alpha]_D$ +70° (*c* 0.91, water), suggested that the S1 fraction was composed of D-galactose. On the basis of results of Kenne and Lindberg[1], the S1 fraction was identified as the O-specific polysaccharide chain of *Klebsiella pneumoniae* serogroup O1 LPS. Methylation analysis of the polysaccharides yielded 2,4,6- and 2,5,6-tri-*O*-methyl-D-galactose, and a small proportion of 2,3,4,6-tetra-*O*-methyl-D-galactose identified by g.l.c.–m.s. in a molar ratio of 52:9:2; 2,3,5,6-tetra-*O*-methyl-D-galactose was not observed. These data showed that the O-specific chain was composed of a linear, (1→3)-linked sequence of D-galactopyranosyl and D-galactofuranosyl residues with a D-galactopyranosyl nonreducing end-group. Evidence for the presence of D-galactofuranosyl residues was also obtained from periodate-oxidation studies. Thus, the periodate-resistant polysaccharides were shown to contain galactose and arabinose in a molar ratio of 6:1. The structure of the S1 fraction was also determined by n.m.r. spectroscopy. Assignments of the proton resonances were made from a homonuclear COSY experiment (Fig. 1). The proton resonances were assigned on the basis of the cross-peaks and the chemical shifts are summarized in Table I. The ^{1}H-n.m.r. spectrum of the S1 fraction contained four resonances for H-1 protons, at δ 5.253 ($J_{1,2}$ 1.5 Hz), 5.181 ($J_{1,2}$ 4 Hz), 5.088 ($J_{1,2}$ 4 Hz), and 4.701 ($J_{1,2}$ 8 Hz) corresponding to H-1 of β-D-galactofuranosyl (Unit a), α-D-galactopyranosyl (Unit b), α-D-galactopyranosyl (Unit c), and β-D-galactopyranosyl (Unit d) residues in the molar ratio of 1:2:1:2.

The ^{13}C-n.m.r. spectrum of the S1 fraction contained 24 signals which were assigned *via* heteronuclear ^{1}H–^{13}C chemical-shift correlations (Fig. 2, Table II). The data confirmed the presence of both β-D-galactofuranosyl, and α- and β-D-galactopyranosyl residues in the O-specific polysaccharide chain. In agreement with the results of the methylation analysis, significant deshielding was observed for the resonances of C-3 of all the D-galactosyl residues, thus indicating that they are 3-O-substituted. The acid lability of the D-galactofuranosyl residues was used in order to study the sequence of the

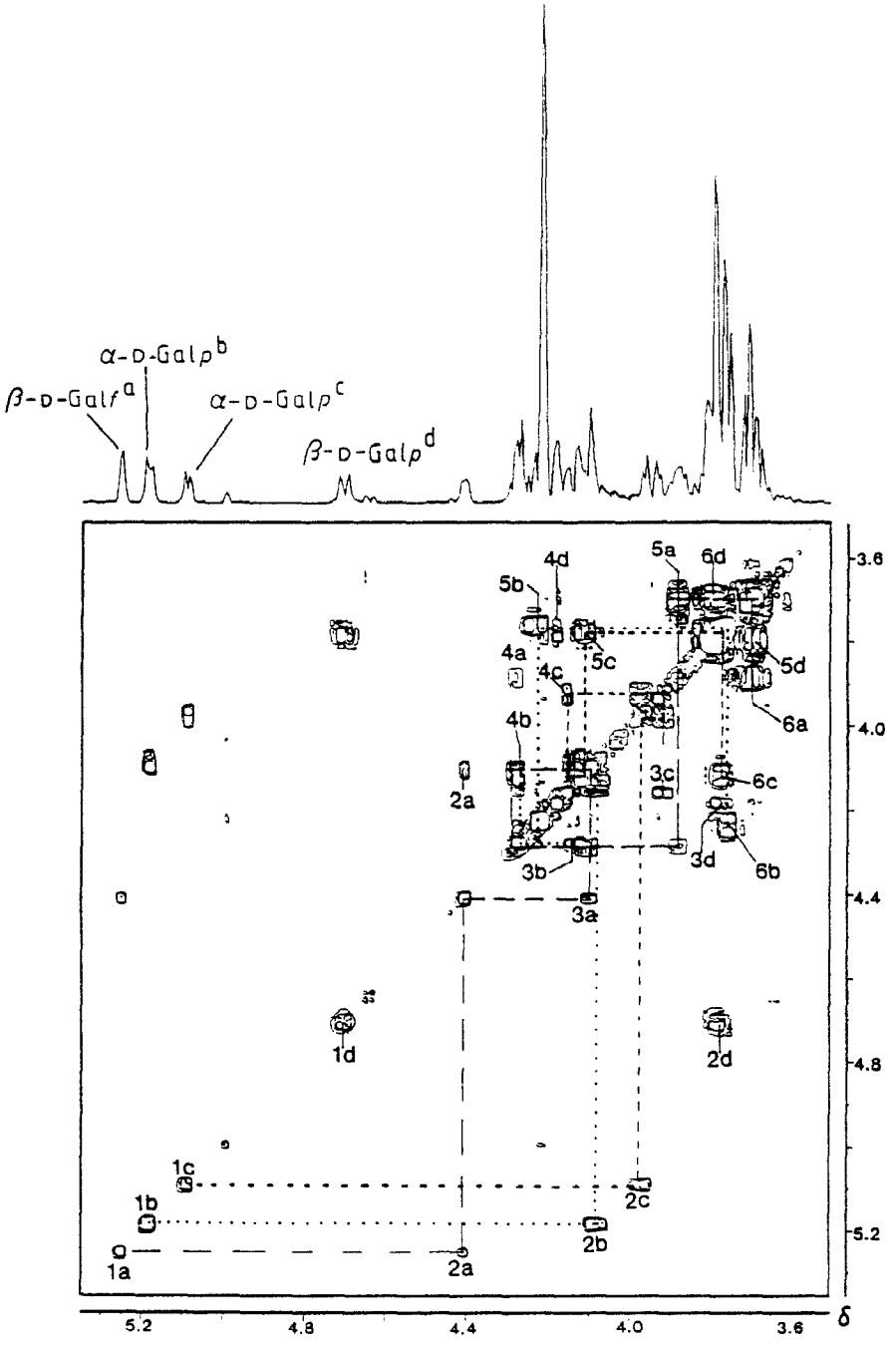

Fig. 1. Contour plot of the COSY spectrum of the S1 fraction isolated from *Klebsiella pneumoniae* O1K2 lipopolysaccharide: a, b, c, and d correspond to units in Table I.

TABLE I

^{1}H-N.m.r. chemical shifts (δ) for the O-specific polysaccharide[a] of *Klebsiella pneumoniae* O1K2 LPS and for the G-75-Fraction 1 obtained by mild acid hydrolysis[b]

Units	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6,6'*[c]
O-Specific polysaccharide						
(a) →3)-β-D-Gal*f*-(1→	5.253	4.408	4.100	4.278	3.695	3.80
(b) →3)-α-D-Gal*p*-(1→	5.181	4.077	4.110	4.270	4.234	3.75
(c) →3)-α-D-Gal*p*-(1→	5.088	3.976	3.919	4.165	4.110	3.77
(d) →3)-β-D-Gal*p*-(1→	4.701	3.772	3.785	4.183	3.695	3.80
G-75-Fraction 1						
(b) →3)-α-D-Gal*p*-(1→	5.193	4.064	4.164	4.300	4.248	3.75
(d) →3)-β-D-Gal*p*-(1→	4.698	3.774	3.816	4.202	3.70	3.80

[a] Measured at 60° relative to internal sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate (δ 0.0). [b] Measured at 27° relative to the same standard (δ 0.0). [c] Values obtained from ^{1}H–^{13}C correlation 2D n.m.r. experiment.

pyranose and furanose forms in the O-specific polysaccharide chain in more details. Partial hydrolysis (of 100 mg), followed by neutralization, and fractionation on Sephadex G-75 gave two fractions (G-75-Fraction 1, 59 mg; and G-75-Fraction 2, 32 mg). A minor fraction corresponding to nondegraded polysaccharides was also observed (Fig. 3). As shown in Fig. 4 and Table I, the ^{1}H-n.m.r. spectrum of the G-75-Fraction 1 indicated that it was constituted of α-D-galactopyranose (Unit b) and β-D-galactopyranose (Unit d) units in an equimolar ratio. This result was confirmed by the ^{13}C-n.m.r. analysis (Fig. 5B, Table II). G.l.c. analysis of the reduced G-75-Fraction 1 gave galactose and galactitol in the molar ratio of 19:1, indicating that the average molecular size of this fraction was ~20 units of galactose. The G-75-Fraction 2 contained a

TABLE II

^{13}C-N.m.r. chemical shifts (δ) for the O-specific polysaccharide[a] of *Klebsiella Pneumoniae* O1K2 LPS and for the G-75-Fraction 1 obtained by mild acid hydrolysis[b]

Units	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
O-Specific polysaccharide						
(a) →3)-β-D-Gal*f*-(1→	111.80	82.33	87.08	84.84	73.63	65.53
(b) →3)-α-D-Gal*p*-(1→	98.27	70.04	81.79	71.84	73.34	63.74
(c) →3)-α-D-Gal*p*-(1→	102.05	69.93	79.68	71.92	73.87	63.88
(d) →3)-β-D-Gal*p*-(1→	106.71	72.46	80.39	67.84	77.48	63.70
G-75-Fraction 1						
(b) →3)-α-D-Gal*p*-(1→	98.00	70.12	81.93	71.93	73.37	63.78
(d) →3)-β-D-Gal*p*-(1→	107.06	72.46	79.87	67.58	77.61	63.74

[a] Measured at 60° relative to internal sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate (δ 0.0). [b] Measured at 27° relative to the same standard (δ 0.0).

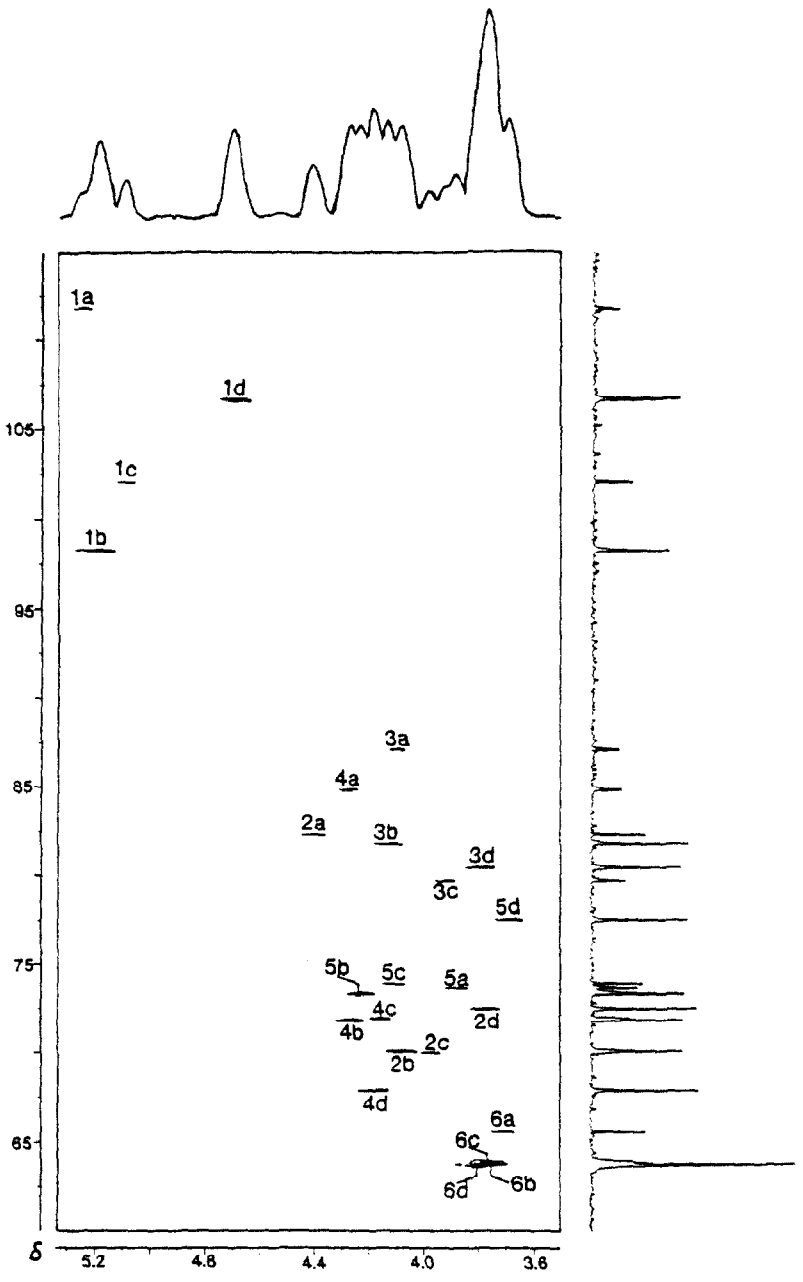

Fig. 2. Contour map of the ^{1}H–^{13}C shift correlation of the S1 fraction isolated from *Klebsiella pneumoniae* O1K2 lipopolysaccharide: a, b, c, and d correspond to units in Table II.

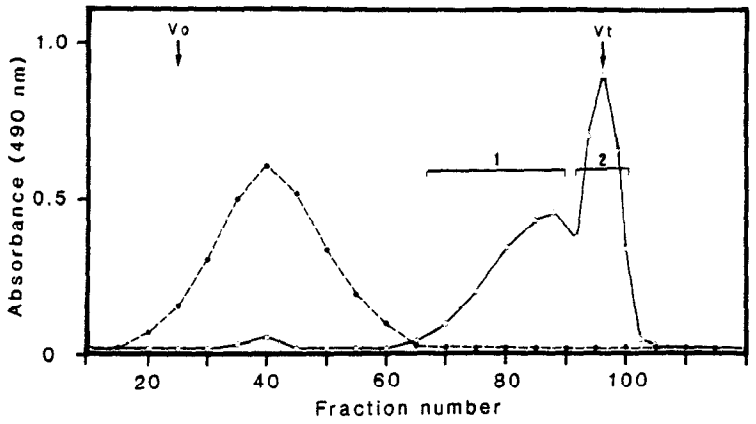

Fig. 3. Sephadex G-75 chromatography of native (--●--) and mild-acid treated (-○-) S1 fraction. Samples were applied to a column (3 x 50 cm) and eluted with water. Fractions (3 mL) were collected and 100-μL samples were analyzed with the phenol–sulfuric acid reagent[13]. Neutral sugar-containing fractions were pooled as indicated: (1) G-75-Fraction 1, and (2) G-75-Fraction 2.

mixture of low-molecular-weight oligosaccharides which were further fractionated by paper chromatography in 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water for 16 h. A hexasaccharide was obtained in a sufficient quantity (10 mg) for ^{13}C-n.m.r. analysis. As shown in Fig. 5C, it mainly consisted of 3-O-substituted β-D-galactofuranose (Unit a) and 3-O-substituted α-D-galactopyranose (Unit c) units in an equimolar ratio. The same structure was also described for a D-galactan isolated from *Serratia marcescens* O20[14] and for the O-specific polysaccharide of *Pasteurella haemolytica* serotypes T4[15] and T10[16].

DISCUSSION

The results of ^{1}H- and ^{13}C-n.m.r. spectroscopic analysis of the native material indicated the presence of four distinct anomeric H-1 or C-1 that are significant for an homogeneous repeating unit. According to the ^{13}C-n.m.r. spectrum, the ratio pyranose (*p*) to furanose (*f*) forms was 5:1, which allows to propose two alternative sequences: $(ppppp f)_n$ or $(pp)_{2n} \rightarrow (pf)_m$. The former sequence would furnish five different signals for anomeric H-1, whereas the latter sequence would give only four signals. On the other hand, the partial acid hydrolysis provided a polymer (G-75-Fraction 1) devoid of galactofuranose units and giving two anomeric signals (α-D-Gal*p* and β-D-Gal*p*) in the ratio of 1:1 (Fig. 5B). The second hypothesis was preferred on the basis of these results, indicating that the disaccharide repeating units, $[\rightarrow 3)$-α-D-Gal*p*-$(1 \rightarrow 3)$-β-D-Gal*p*-$1 \rightarrow]_n$ and $[\rightarrow 3)$-α-D-Gal*p*-$(1 \rightarrow 3)$-β-D-Gal*f*-$(1 \rightarrow]_m$, are clustered in the O-specific polysaccharide chain. These results, however, did not allow to ascertain which disaccharide repeating-unit cluster is attached to the core of the lipopolysaccharide.

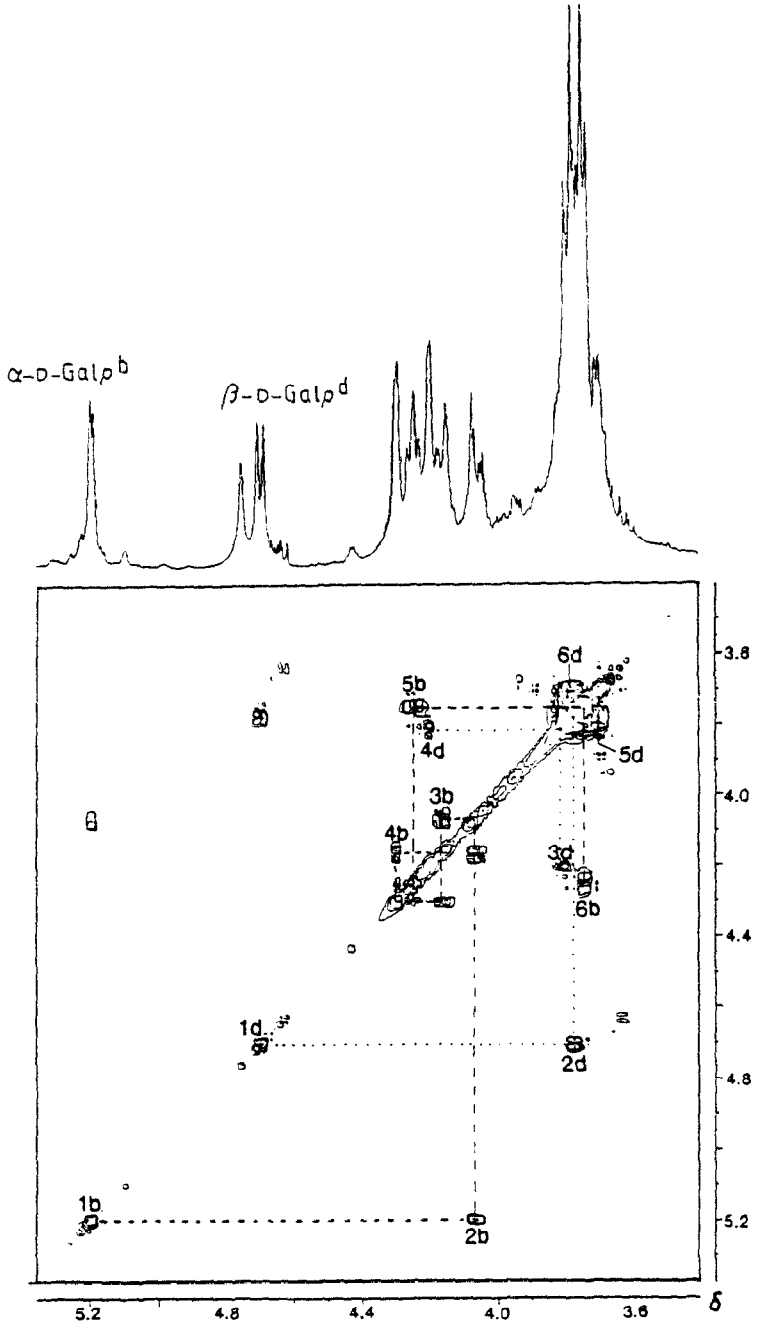

Fig. 4. Contour plot of the COSY spectrum of the G75-Fraction 1 obtained by mild acid hydrolysis of the S1 fraction: b and d correspond to units in Table I.

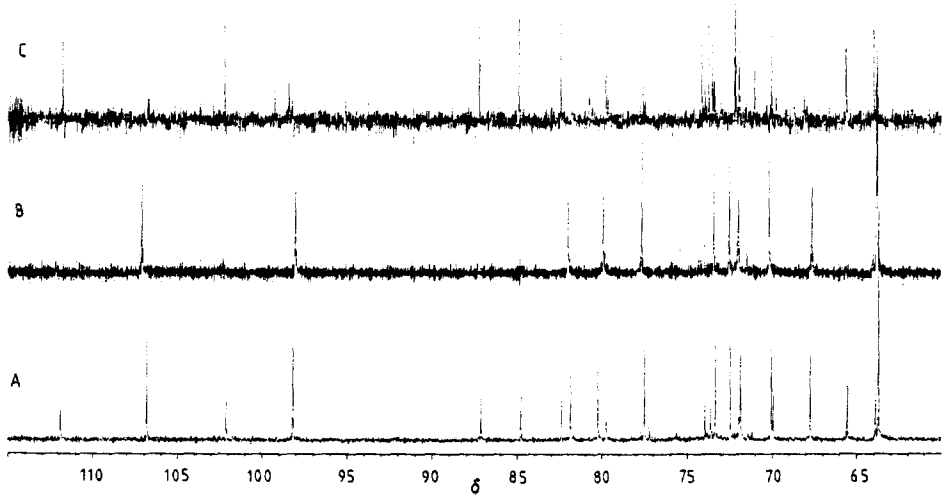

Fig. 5. ^{13}-C-N.m.r. spectra of the S1 fraction and its products of hydrolysis isolated from *Klebsiella pneumoniae* O1K2 lipopolysaccharide: (A) S1 Fraction; (B) G-75-Fraction 1 obtained by mild acid hydrolysis of S1; and (C) hexasaccharide obtained by paper chromatography of G-75-Fraction 2 after mild acid hydrolysis of the S1 fraction.

ACKNOWLEDGMENTS

The authors are grateful to the Conseil Régional du Nord–Pas de Calais, the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de l'Enseignement Supérieur, the Ministère de l'Éducation Nationale, and the Association pour la Recherche sur le Cancer for their contribution to the acquisition of the 400-MHz n.m.r. and m.s. instruments. They are indebted to Mr. Y. Leroy (C.N.R.S. Engineer) for his skilful technical assistance.

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