Carbohydrate Research, 231 (1992) 197–204
Elsevier Science Publishers B.V., Amsterdam

Structure of the O-specific polysaccharide chain of the lipopolysaccharide of *Enterobacter agglomerans*

Yannis Karamanos [a,1], Ossarath Kol [a], Jean-Michel Wieruszeski [a], Gérard Strecker [a], Bernard Fournet [a] and René Zalisz [b]

[a] *Laboratoire de Chimie Biologique de l'Université des Sciences et Techniques de Lille-Flandres Artois et Unité Mixte du C.N.R.S. No. 111, 59655 Villeneuve d'Ascq (France)*
[b] *Laboratoires Cassenne, Centre de Recherches Roussel-Uclaf, B.P. 31, 95521 Cergy-Pontoise (France)*

(Received October 3rd, 1991; accepted January 27th, 1992)

ABSTRACT

The O-specific polysaccharide isolated from the lipopolysaccharide of *Enterobacter agglomerans* was found, by methylation analysis, periodate oxidation, oxidative deamination, and NMR spectroscopy, to have the pentasaccharide repeating-unit {→ 3)-α-L-Fuc*p*NAc-(1 → 3)-α-L-Fuc*p*NAc-(1 → 3)-[α-D-Glc*p*-(1 → 2)-α-L-Rha*p*-(1 → 6)]-β-D-Glc*p*NAc-(1 →}.

INTRODUCTION

A typical lipopolysaccharide (LPS) contains lipid A, a core oligosaccharide, and an O-specific polysaccharide. The structures of O-antigens from different genera of the Enterobacteriaceae have been established[1,2]. The O-antigens usually contain hexoses, but 6-deoxy- and 3,6-dideoxy-hexoses[2] have been found. 2-Acetamido-2,6-dideoxy-L-galactose (L-FucNAc), mainly found in bacterial capsular polysaccharides, has also been identified in some O-specific polysaccharides[3]. We now report the structure of an L-FucNAc-containing O-specific polysaccharide from *Enterobacter agglomerans*.

EXPERIMENTAL

Bacteria and growth conditions.—E. agglomerans was obtained from the Institut Pasteur (CIP 55.49) and cultured in Trypticase Soy Broth (TSB) at 37°, aerated by

[1] Present address: Institut de Biotechnologie, Université de Limoges, 123 avenue Albert Thomas, 87060 Limoges, France.
Correspondence to: Professor B. Fournet, Laboratoire de Chimie Biologique de l'Université des Sciences et Techniques de Lille-Flandres Artois et Unité Mixte du C.N.R.S. No. 111, 59655 Villeneuve d'Ascq, France.

rotation at 120 rpm in an orbital incubator (Gallenkamp) until the end of the logarithmic phase, then harvested by centrifugation (7500*g*, 20 min), and washed in saline.

Isolation of the lipopolysaccharide (LPS).—The LPS was extracted by the hot phenol–water procedure[4]. The aqueous phase was dialysed exhaustively against distilled water. A precipitate appearing during dialysis was removed by centrifugation (4000*g*, 10 min). The opalescent supernatant solution (crude extract) was concentrated under vacuum and lyophilised. To a solution of the crude extract was added aq 3% cetyltrimethylammonium bromide (CTAB, 1 vol) with mild agitation. After storage for 18 h at room temperature, the precipitate (P_{CTAB}) was removed by centrifugation (4000*g*, 20 min) and the supernatant solution (S_{CTAB}) was made M with respect to NaCl and then diluted with EtOH (6 vol). The resulting precipitate, which contained the LPS, was resuspended in water, exhaustively dialysed against water, and lyophilised.

Isolation of the O-specific polysaccharide.—S_{CTAB} was treated with aq 2% acetic acid at 100° for 90 min, the lipid fraction was removed by centrifugation, and the water-soluble material was eluted from a column (1.5 × 90 cm) of Sephadex G-50 with pyridinium acetate (0.05 M, pH 5.5) at 12 mL/h. The fractions were monitored for carbohydrate with phenol–H_2SO_4 reagent[5].

NMR spectroscopy.—The 400-MHz ^{1}H-NMR spectra were obtained with a Bruker AM-400 WB spectrometer, equipped with a 5-mm ^{1}H–^{13}C mixed-probe head, operating in the pulsed-FT mode, and controlled by an Aspect 3000 computer. After three exchanges with D_2O (99.96 atom% D) and intermediate lyophilisation, the products were analysed with a spectral width of 3000 Hz for 16 K frequency-domain and time-domain points, giving a final digital resolution of 0.365 Hz/point. The 100-MHz ^{13}C-NMR experiments were carried out with the standard Bruker pulse program POWGATE [^{1}H broad-band composite-pulse decoupling, $D_1 = 0.1$ s, pulse width = 90° (6 μs), $S_1 = S_2 = 1$ W]. A 0.5-s recycle delay was used. The chemical shifts are given relative to that of 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.—Monosaccharides were characterised and determined after methanolysis and trifluoroacetylation[6], or after acid hydrolysis (4 M trifluoroacetic acid, 4 h, 100°) as their alditol acetate derivatives[7]. The unknown monosaccharide was isolated, after acid hydrolysis as above, by ion-exchange chromatography[8] on a column of Dowex 50-X2 (H^+) resin (200–400 mesh). Elution with distilled water gave the neutral sugars (assayed with phenol–H_2SO_4 reagent[5]) and elution with 0.55 M HCl gave the amines (assayed with ninhydrin). The neutral monosaccharides and *N*-acetylated amines[9] were purified by HPLC on a column of Micropak AX-10 by elution with 3:1 acetonitrile–H_2O. The

absolute configuration of each monosaccharide was determined on the basis of the $[\alpha]_D$ value (H_2O) determined with a Perkin–Elmer 241 polarimeter and comparison with standards (D-Glc, $[\alpha]_D + 50°$; D-GlcN, $+72°$; L-Rha, $+5.8°$) or with reference data (L-FucN, $-79°$)[10].

Methylation analysis.—Methylation was carried out according to Hakomori[11], as described by Finne et al.[12]. The methylated products were purified by chromatography on a column (2×30 cm) of Sephadex LH-20 by elution with 1:1 EtOH–$CHCl_3$. Fractions were assayed for carbohydrate with phenol–H_2SO_4 reagent[5]. Methyl glycoside derivatives obtained after methanolysis of the methylated polysaccharide were analysed, after *O*-acetylation, by GLC–MS and identified by comparison of retention times and mass spectra with those of standard compounds[13].

Periodate oxidation and Smith degradation[14].—The polysaccharide (50 mg) was oxidised with 0.03 M $NaIO_4$ (35 mL) for 8 days at 4°. Excess of periodate was destroyed with ethylene glycol, the pH of the mixture was adjusted to 7.0 with 0.1 M NaOH, and the oxidised polysaccharide was reduced conventionally with $NaBH_4$ overnight. The solution was neutralised with acetic acid, and borate was removed by several co-concentrations with MeOH in 1% acetic acid. The residue was dissolved in water, the pH was adjusted to 1 with H_2SO_4, the mixture was stored overnight, then passed through a column of Dowex 1-X2 resin, and desalted on a column of Bio-Gel P2, and the products were reduced with $NaBH_4$.

Oxidative deamination[15] *of the hexosamine.*—Pyridine (500 μL) and ninhydrin (30 mg) were added to the hexosamine (5 mg). The mixture was heated in a sealed tube at 100° for 30 min. The resulting 5-deoxyhexose was purified, using columns of Dowex 50-X8 and 1-X8 resins by elution with water, and was identical with 5-deoxy-L-lyxose obtained by oxidation of L-fucose with bromine[16] and Ruff degradation[17] of the resulting L-fuconic acid.

GLC–MS.—GLC of trifluoroacetylated methyl glycosides was performed on a column (300×0.3 cm) of 5% OV-210 and partially methylated and acetylated methyl glycosides on a capillary column (25 m $\times$ 0.32 mm) coated with OV-101 silicone. EI-MS was carried out with a Riber 10-10 Quadripole spectrometer at 70 eV and an ionisation current of 0.2 mA.

RESULTS AND DISCUSSION

The lipopolysaccharide (LPS, 1.3% of the dry bacteria) obtained by hot phenol–water extraction was purified by precipitation with cetyltrimethylammonium bromide (CTAB) and then with ethanol. Mild hydrolysis of the LPS with dilute acetic acid gave insoluble lipid A and a water-soluble fraction. The O-specific polysaccharide in the latter fraction was separated from the core oligosaccharide and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) by fractionation on Sephadex G-50. The mass of the polysaccharide was < 10 kDa as determined by gel-filtration chromatography on Sephacryl S-300 and comparison with standard dextrans (data

TABLE I

GLC (see Experimental) of partially methylated and acetylated methyl glycosides obtained from methylated O-specific polysaccharide (A) and the Smith-degradation product (B)

Derivatives	Molar ratio	
	A	B
2,3,4,6-Me_4-Glc	1.05	
3,4-Me_2-Rha	0.96	
3,4,6-Me_3-GlcNAc	trace	0.14
4,6-Me_2-GlcNAc		0.98
4-Me-GlcNAc	1.04	
4-Me-FucNAc	2	2

not shown). An acid hydrolysate of the O-specific polysaccharide contained rhamnose, glucose, 2-amino-2-deoxyglucose, and 2-amino-2,6-dideoxygalactose (0.92:1.00:1.17:2.2) together with heptose (0.3) and Kdo (trace). The 2-amino-2,6-dideoxygalactose was identified as follows. The foregoing acid hydrolysate was fractionated on Dowex 50-X2 resin to give neutral (FnI and FnII) and amine-containing fractions (FbI and FbII). Each fraction was purified by HPLC on Micropak AX-10. FnI was enriched in heptose and Kdo, FnII contained L-Rha ($[\alpha]_D$ + 5°) and D-Glc ($[\alpha]_D$ + 49°) and FbI contained D-GlcN ($[\alpha]_D$ + 71°). CI (ammonia)-MS of FbII gave two major ions at m/z 332 $(M + H)^+$ and 349 $(M + NH_4)^+$, as well as the A_1 ion at m/z 272 indicative of a 2-amino-2,6-dideoxyhexopyranose. Oxidative deamination of FbII gave a 5-deoxypentose having the same retention time in GLC as that of 5-deoxy-L-lyxose synthesised from L-fucose by bromine oxidation[16] and Ruff degradation[17]. Therefore, FbII had the *galacto* or *talo* configuration. FbII was shown to be 2-amino-2,6-dideoxy-L-galactose by comparison {GLC, MS, $[\alpha]_D$ value (−61°)} with L-FucNac isolated[18] from pneumococcal capsular polysaccharide serotype IV.

Methylation analysis of the native polysaccharide yielded derivatives of 2,3,4,6-Me_4-Glc, 3,4-Me_2-Rha, 4-Me-FucNAc, and 4-Me-GlcNAc in the molar ratios 1:1:2:1 as determined by GLC–MS (Table I, column A). Thus, the O-specific polysaccharide has a pentasaccharide repeating unit. The formation of the 4-Me-GlcNAc derivative indicated that the repeating unit is branched with 3,6-disubstituted GlcNAc at the branch point.

On the basis of these results, only the Rha and Glc residues should be oxidised by periodate. The product of Smith degradation (periodate oxidation, borohydride reduction, mild hydrolysis with acid) of the O-polysaccharide had almost the same molecular size as the parent, indicating that the backbone was intact and hence did not contain the Rha and Glc residues. As expected, the Smith-degradation product contained GlcNAc and FucNAc in the molar ratio 1:2 and methylation analysis gave 4,6-Me_2-GlcNAc and 4-Me-FucNAc, together with a small proportion of 3,4,6-Me_3-GlcNAc which resulted from the non-reducing end of the polysaccharide backbone (Table I, column B).

These results demonstrated that the polysaccharide has a backbone comprising the trisaccharide repeating unit [→ 3)-L-FucNAc-(1 → 3)-L-FucNAc-(1 → 3)-D-GlcNAc-(1 →] with a branch of [D-Glc-(1 → 2)-L-Rha-(1 →] 6-linked to the GlcNAc residue.

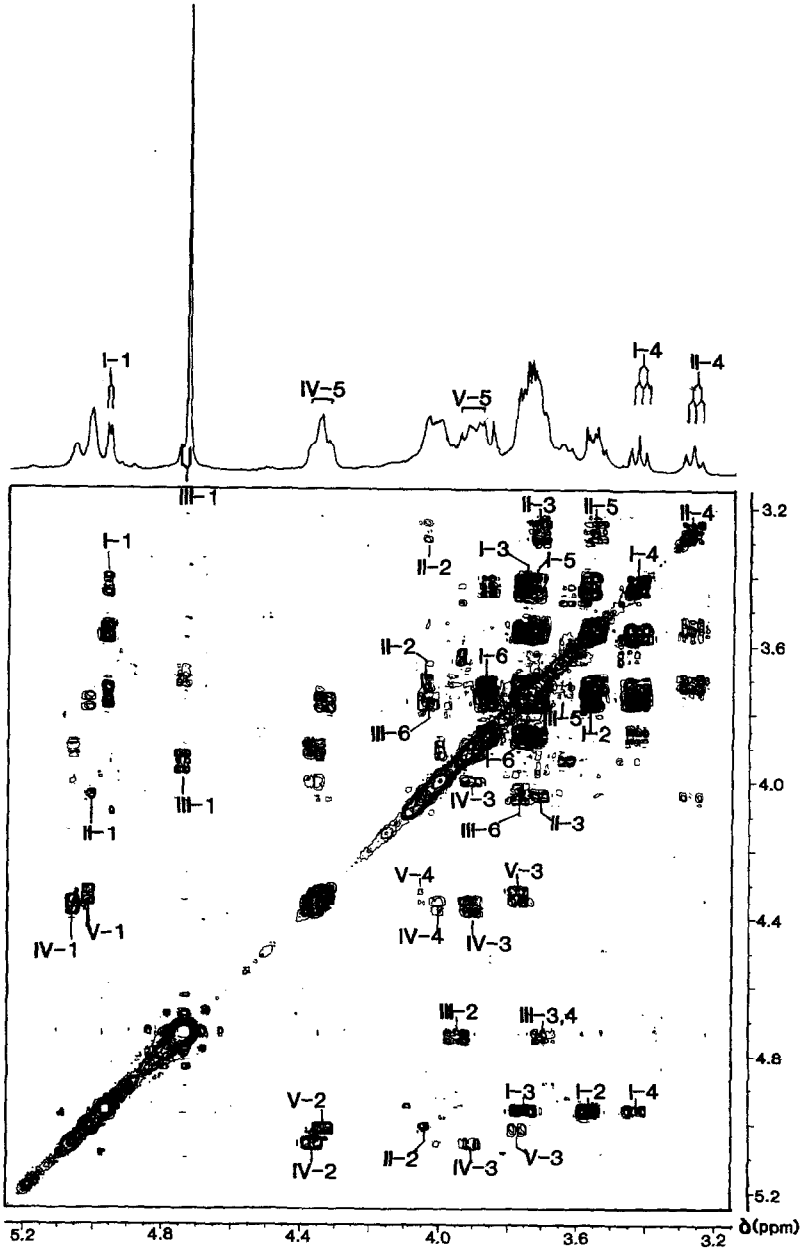


Fig. 1. Double-relayed COSY spectrum of the O-specific polysaccharide (for identification of I–V, see 1).

TABLE II

^{1}H-NMR data [a] (δ in ppm) for the O-specific polysaccharide

Units (see I)	H-1	H-2	H-3	H-4	H-5	H-6/6′
I α-D-Glc*p*-(1 →	4.959	3.564	3.750	3.426	3.719	3.880/3.740
II → 2)-α-L-Rha*p*-(1 →	5.007	4.031	3.706	3.269	3.544	1.298
III → 3)-β-D-Glc*p*NAc-(1 →	4.743	3.945	3.691	3.738	3.641	4.038/3.745
IV → 3)-α-L-Fuc*p*NAc-(1 →	5.056	4.364	3.906	3.994	4.350	1.197
V → 3)-α-L-Fuc*p*NAc-(1 →	5.012	4.332	3.769	4.011	3.896	1.290

[a] Other signals: δ 2.061, 2.035, 1.996 (NAc).

The structure of the O-specific polysaccharide was confirmed by NMR spectroscopy. Assignments of the ^{1}H resonances were made from a homonuclear relayed-COSY experiment (Fig. 1). The ^{1}H resonances, assigned on the basis of the cross-peaks and the chemical shifts, are summarised in Table II. There were five H-1 signals at δ 5.056 ($J_{1,2}$ 3 Hz), 5.012 ($J_{1,2}$ 3 Hz), 5.007 ($J_{1,2}$ 1 Hz), 4.959 ($J_{1,2}$ 3 Hz), and 4.743 ($J_{1,2}$ 8 Hz), indicating the presence of four α sugars and one β sugar. Signals at δ 2.061, 2.035, and 1.996 corresponded to NAc groups, and those at δ 1.290, 1.197, and 1.298 corresponded to H-6,6,6 of two FucNAc residues and one Rha residue, respectively. The signals related to Rha were identified on the basis of connectivities between H-6,5,4, starting from H-6 (not shown), and H-5,4,3,2, starting from H-4 (Fig. 1). Moreover, the ^3J values ($J_{1,2}$ 1, $J_{2,3} < 5$, $J_{3,4}$ 9, $J_{4,5}$ 9 Hz) confirmed the *manno* configuration. For the FucNAc residues, linked to L-FucNAc (unit V) and D-GlcNAc (unit IV), application of the rules[19,20] allowed the C-1 signals at δ 96.00 and 99.08 to be assigned to α-FucNAc 3-linked to L-FucNAc (unit V) and 3-linked to D-GlcNAc (unit IV), respectively (Table III). These values are shown on the ^{1}H–^{13}C COSY spectrum (Fig. 2) for the assignment of the corresponding H-1 resonances, and on the double-relayed COSY spectrum (Fig. 1) for the complete assignment of the sub-spectra of FucNAc, Rha, Glc, and GlcNAc. Furthermore, the ^{1}H–^{13}C COSY spectrum showed strong downfield shifts for the resonances of C-3 of FucNAc and GlcNAc, C-2 of Rha, and C-6 of

TABLE III

^{13}C-NMR data (δ in ppm) for the O-specific polysaccharide [a,b]

Units (see I)	C-1	C-2	C-3	C-4	C-5	C-6
I α-D-Glc*p*-(1 →	99.63	72.88	74.47	70.89	73.24	61.93
II → 2)-α-L-Rha*p*-(1 →	94.67	79.37	71.19	73.35	70.69	17.02
III → 3)-β-D-Glc*p*NAc-(1 →	103.81	56.97	79.81	69.28	75.50	66.87
IV → 3)-α-L-Fuc*p*NAc-(1 →	99.08	48.54	73.56	68.22	67.92	16.69
V → 3)-α-L-Fuc*p*NAc-(1 →	96.00	48.40	73.20	67.92	68.42	18.17

[a] Measured at 27° relative to sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate. [b] Other signals: δ 23.60, 23.57, 23.39 (CH_3CO), and 175.58, 175.53 (CH_3CO).

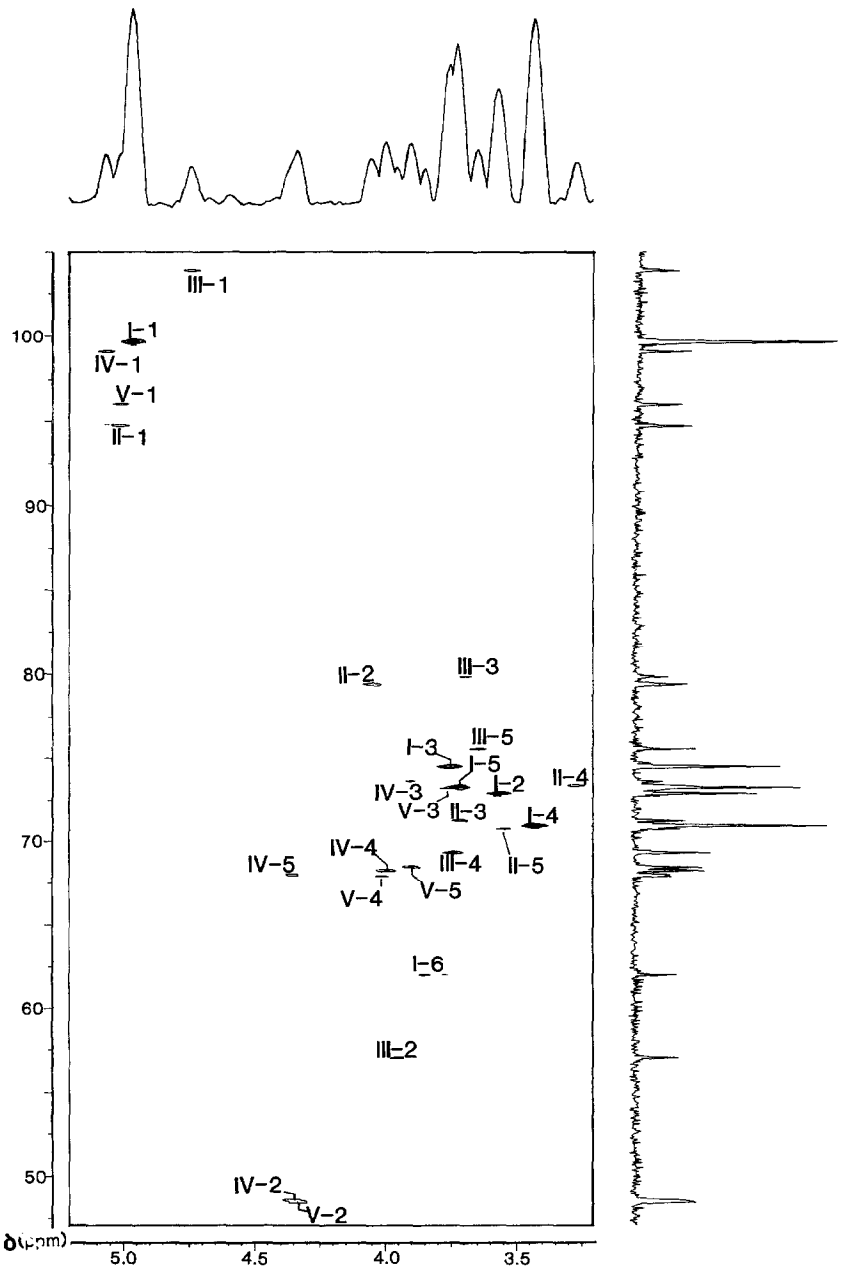
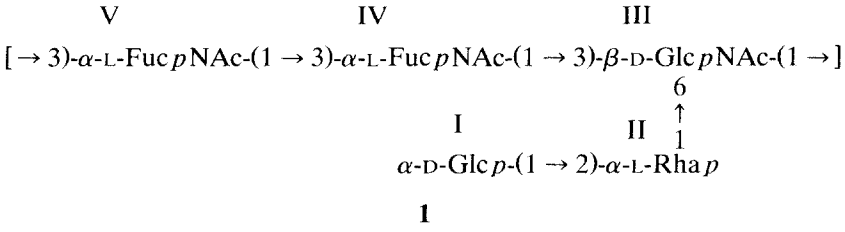


Fig. 2. The $^{1}H-^{13}C$ shift correlation of the O-specific polysaccharide (for identification of I–V, see **1**).

GlcNAc, which confirmed the results obtained by methylation analysis. The CH_3CO signals at δ 23.60/2.061, 23.57/1.996, and 23.39/2.035 were not assigned.

The NMR and chemical data were in accord and confirmed that the O-specific polysaccharide has the repeating unit shown in structure **1**.



1

ACKNOWLEDGMENTS

We thank the Conseil Régional du Nord-Pas de Calais, the Centre National de la Recherche Scientifique, the Ministère de l'Education Nationale, the Ministère de la Recherche et de l'Enseignement Supérieur, and the Association pour la Recherche sur le Cancer for their contribution to the acquisition of the NMR and MS instruments; Yves Leroy, Guy Ricart, and Bernard Mouchel for their contribution to the MS and NMR analyses; and the Centre National de la Recherche Scientifique (Unité Mixte du C.N.R.S. No. 111, Directeur Professeur A. Verbert), the Université des Sciences et Techniques de Lille-Flandres Artois, and the Laboratoires Cassenne for financial support.

REFERENCES

1 L. Kenne and B. Lindberg, in G.O. Aspinall (Ed.), *The Polysaccharides*, Vol. 2, Academic Press, New York, 1983, pp. 287–363.
2 E.T. Rietschel, *Handbook of Endotoxins*, Vol. 1, Elsevier, Amsterdam, 1984, pp. 138–186.
3 A.J. Griffiths and D.B. Davies, *Carbohydr. Polym.*, 14 (1991) 241–279.
4 O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
5 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
6 J.P. Zanetta, W.C. Breckenridge, and G. Vincendon, *J. Chromatogr.*, 69 (1972) 291–304.
7 J. Montreuil, S. Bouquelet, H. Debray, B. Fournet, G. Spik, and G. Strecker, in M.F. Chaplin and J.F. Kennedy (Eds.), *Carbohydrate Analysis: A practical Approach*, IRL, Oxford, 1986, pp. 143–204.
8 S. Gardell, *Acta Chem. Scand.*, 7 (1953) 207–212.
9 S. Roseman and J. Ludowieg, *J. Am. Chem. Soc.*, 76 (1954) 301–304.
10 M.B. Perry and V. Daoust, *Can. J. Chem.*, 51 (1973) 974–977.
11 S.I. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
12 J. Finne, T. Krusius, and H. Rauvala, *Carbohydr. Res.*, 80 (1980) 336–339.
13 B. Fournet, G. Strecker, Y. Leroy, and J. Montreuil, *Anal. Biochem.*, 116 (1981) 489–502.
14 I.J. Goldstein, G.W. Hay, B.A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
15 S. Gardell, F. Heijkenstold, and A.E. Ochnorlund, *Acta Chem. Scand.*, 4 (1950) 970–976.
16 H.S. Isbell, *Methods Carbohydr. Chem.*, 2 (1963) 13–14.
17 H.G. Fletcher, Jr., *Methods Carbohydr. Chem.*, 1 (1962) 77–79.
18 J.D. Higginbotham and M. Heidelberger, *Carbohydr. Res.*, 23 (1972) 165–173.
19 Yu.A. Knirel, E.V. Vinogradov, A.S. Shashkov, B.A. Dmitriev, N.K. Kochetkov, E.S. Stanislavsky, and G.M. Mashilova, *Eur. J. Biochem.*, 150 (1985) 541–550.
20 Yu.A. Knirel, I.M. Skvortsov, A. Shashkov, B.A. Dmitriev, N.K. Kochetkov, E.S. Stanislavsky, and G.M. Mashilova, *Eur. J. Biochem.*, 150 (1985) 551–557.