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Structural studies of the enterotoxigenic *Escherichia coli* (ETEC) O153 O-antigenic polysaccharide

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Abstract

The O-specific side-chain of the lipopolysaccharide from *Escherichia coli* O153 has been investigated using methylation analysis, Smith degradation, partial hydrolysis, FABMS, and NMR spectroscopy as the principal methods. It is concluded that the polysaccharide is composed of pentasaccharide repeating-units having the following structure.

→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Rib*f*-(1→

Keywords: *Escherichia coli*; Lipopolysaccharide; Enterotoxigenic

1. Introduction

Pathogenic *Escherichia coli* are often isolated from patients with gastroenteritis [1]. Enterotoxigenic *E. coli* (ETEC) strains produce diarrhoeal disease by colonising the mucosa of the small intestine followed by elaboration of the toxin(s). ETEC produce noninvasive, plasmid-mediated heat-labile (LT) or/and heat-stable (ST) enterotoxins. The toxins have different biological activities and immunological properties. In addition, the ETEC strains produce fimbrial adhesins that are of importance for the virulence of these bacteria [2]. Several different serogroups have been identified within the ETEC group and those that belong to serogroups O6, O8, O78, and O153 comprise more than 30% of all ETEC strains [2]. Recently it has been shown that *E. coli* O153 could be isolated from children with diarrhoea in Spain [3] and Chile [4]. The structures of the O-antigenic polysaccharides

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Table 1
Sugar analysis of O153 and oligosaccharides thereof [a]

Sugar	Detector response				
	A	B	C	D	E
Threitol		54			
D-Rib	8	21			
Ribitol			15	13	11
D-Glc	5				
D-Gal	48		37	54	56
D-GlcNAc	37	25	48	33	33
Heptose	2				

[a] Key: A, lipid-free polysaccharide; B, product from Smith degradation; C, tetrasaccharide-alditol from partial hydrolysis; D, pentasaccharide-alditol from partial hydrolysis; E, decasaccharide-alditol from partial hydrolysis.

from *E. coli* O6 [5], O8 [6], and O78 [7] have been published. We hereby report on the structure of the *E. coli* O153 O-antigenic polysaccharide.

2. Results and discussion

The lipopolysaccharide (LPS) from the *E. coli* O153 was obtained by phenol–water extraction [8]. The LPS was delipidated with acid under mild conditions to give the polysaccharide (O153 PS). An acid hydrolysate of O153 PS contained ribose, glucose, galactose, 2-amino-2-deoxyglucose, and heptose (Table 1, column A). Methylation analysis of the O153 PS revealed 2-substituted ribose, 4-substituted galactose, 4-substituted 2-amino-2-deoxyglucose, and 3-substituted 2-amino-2-deoxyglucose (Table 2, column A). It should be noted that the detected amount of 3-substituted glucosamine is less than half of that of 4-substituted glucosamine in this analysis. The ^{1}H NMR spectrum (Fig. 1) and ^{13}C NMR spectrum of the O153 PS (Fig. 2) both showed five signals in the region for

Table 2
Methylation analysis of O153 and some degradation products [a]

Sugar [b]	Detector response				
	A	B	C	D	E
1,3,4,5-Ribitol			20	12	4
3,5-Rib	8	33			6
2,3,4,6-Gal			10	34	14
2,3,6-Gal	64		40	31	51
2,3,4,6-GlcNAc		67	21		3
2,3,6-GlcNAc	21		5	18	18
2,4,6-GlcNAc	8		4	5	5

[a] Key: A, methylated polysaccharide; B, methylated product from Smith degradation; C, methylated tetrasaccharide-alditol; D, methylated pentasaccharide-alditol; E, methylated decasaccharide-alditol. [b] 3,5-Rib = 3,5-di-*O*-methyl-D-ribose, etc.

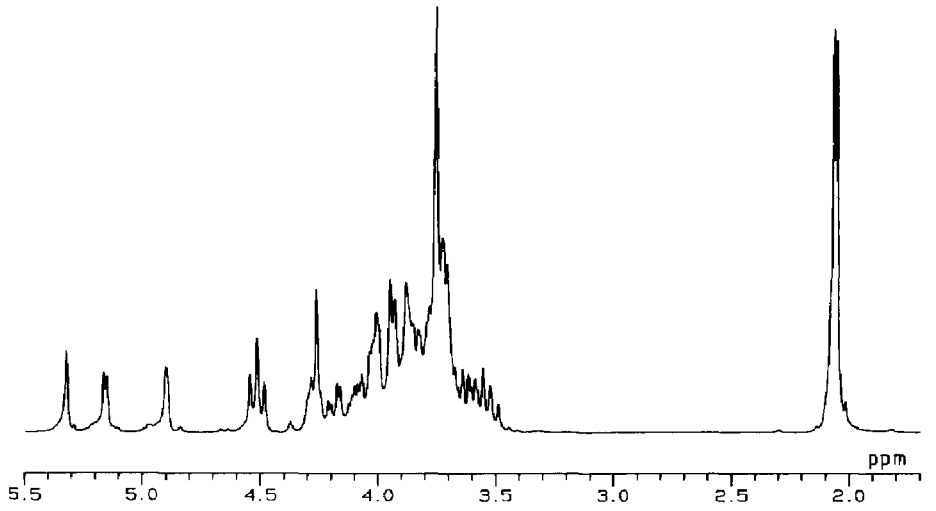

Fig. 1. The ^{1}H NMR spectrum at 270 MHz of the *E. coli* O153 O-polysaccharide.

anomeric protons and anomeric carbons, respectively. The O153 PS should consequently have pentasaccharide repeating-units. The stoichiometry of the sugar and methylation analyses indicates that there should be two 4-substituted galactose residues in the repeating unit. Determination of the absolute configuration of the sugars was performed by a modification of the method developed by Leontein et al. [9], using GLC of their acetylated (+)-2-butyl glycosides, and showed D-ribose, D-galactose, and 2-amino-2-deoxy-D-glucose. The other sugars from the acid hydrolysate are attributed to the core of the LPS.

The ^{1}H NMR spectrum of the O153 PS showed, *inter alia*, signals at δ 5.32 ($J_{H\text{-}1,H\text{-}2}$ not resolved), 5.16 ($J_{H\text{-}1,H\text{-}2}$ 3.7 Hz), 4.90 ($J_{H\text{-}1,H\text{-}2}$ not resolved), 4.53 ($J_{H\text{-}1,H\text{-}2}$ 8.1 Hz), 4.50 ($J_{H\text{-}1,H\text{-}2}$ 8.4 Hz), 2.07 (3 H), and 2.06 (3 H). The ^{13}C NMR spectrum of the same material showed, *inter alia*, signals for anomeric carbons at δ 107.5, 104.3, 103.7, 98.6, and 96.6. Signals for *N*-acetyl groups were observed at δ 175.0, 174.8, 22.9, and 22.7 showing that the amino sugars are *N*-acetylated. The signal at δ 107.5 is derived from the ribose as a furanoside and should, from the chemical shift for its C-1, be β-linked [10]; the other sugar residues are concluded, from NMR data, to be pyranoid. The ^{13}C NMR glycosylation shift for C-2 of the ribofuranose residue is 5.6 ppm in agreement with a 2-substituted ribofuranose residue as determined from methylation analysis. The two residues with δ_H 5.16 and 4.90 are shown to be derived from 2-acetamido-2-deoxy-D-glucose residues by the chemical

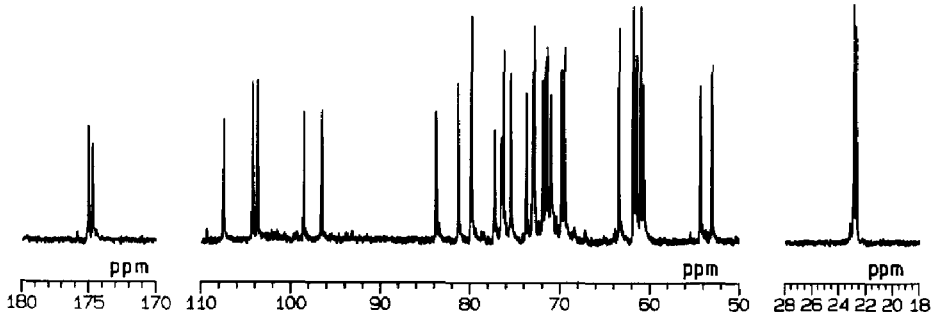

Fig. 2. The ^{13}C NMR spectrum at 67 MHz of the *E. coli* O153 O-polysaccharide.

Table 3
Chemical shifts (ppm) of the signals in the ^1H and ^{13}C NMR spectra [a] of *E. coli* O153 PS

Sugar residue	H/C				
	1	2	3	4	5
→2)-β-D-Rib*f*(1→	5.32 107.5	4.25 79.9	4.27 [a] 71.5	4.09 [a] 83.8	n.a. [b] 63.4
→3)-α-D-Glc*p*NAc-(1→	5.16 96.6	4.18 53.1	4.03 81.3	3.64 69.5	3.93 [a] 72.9
→4)-α-D-Glc*p*NAc-(1→	4.90 98.6	3.94 54.4	3.93 [a] 69.9	3.73 [a] 79.9	4.27 [a] 71.0
→4)-β-D-Gal*p*-(1→	4.53 104.3	3.58 71.6	3.72 73.0 [a]	4.00 77.3	3.75 [a] 76.4
→4)-β-D-Gal*p*-(1→	4.50 103.7	3.52 71.9	3.77 73.7 [a]	4.01 76.6	3.75 [a] 75.5

[a] Tentative assignments. [b] n.a., Not assigned.

shifts of their C-2s, δ 53.1 and 54.4, respectively, and are consequently α-linked. The two remaining galactose residues should therefore be β-linked. The residue with δ_H 5.16 is 3-substituted because of the chemical shift of its C-3, δ 81.3, having a glycosylation shift of 9.6 ppm. Assignments of ^1H and ^{13}C NMR signals are given in Table 3.

The sequence of the sugars present in the polysaccharide was obtained by a Smith degradation and by partial acid hydrolysis. The Smith degradation [11], i.e., oxidation by periodate, reduction by sodium borohydride, and partial acid hydrolysis under mild conditions, of the O153 PS yielded, after gel chromatography on a column of Bio-Gel P-2, a product in the oligosaccharide region. The product was shown to be an acetal of a trisaccharide-alditol containing threitol, ribose, and glucosamine (Table 1, column B). The methylation analysis (Table 2, column B) showed terminal glucosamine and 2-substituted ribose. The ^1H NMR spectrum showed, *inter alia*, signals at δ 5.18 ($J_{H\text{-}1,H\text{-}2}$ 1.5 Hz), 5.15 ($J_{H\text{-}1,H\text{-}2}$ 3.7 Hz), 4.85 ($^3J_{H,H}$ 4.4 Hz, *t*, 1 H) and 2.07 (3 H). The FABMS spectrum of the underivatised compound [12] obtained in the positive-ion mode showed a peak at *m/z* 500 attributed to $[M+H]^+$; upon addition of sodium ions, a peak was observed at *m/z* 522 attributed to $[M+Na]^+$. The ^1H NMR signal at δ 4.85 indicates that a 5-membered cyclic acetal [13,14] has been formed in which a 2-hydroxyethylidene group is linked to the 3- and 4-positions of the 2-*O*-substituted D-threitol residue. The molecular weight of this compound determined by FABMS is in agreement with such a structure. The product obtained from the Smith degradation and the above data define the following structural element **1** in the O153 PS.

α-D-Glc*p*NAc-(1→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→

1

Treatment of O153 PS with 0.1 M trifluoroacetic acid for 2 h followed by gel filtration yielded products in the oligosaccharide region. These oligosaccharides were subsequently reduced with sodium borodeuteride and rechromatographed. Tetra-, penta-, and deca-saccharide-alditols were isolated and are discussed below. The tetrasaccharide-alditol contained ribitol, galactose and glucosamine (Table 1, column C). The ^1H NMR spectrum of the tetrasaccharide-alditol showed, *inter alia*, signals at δ 5.13 ($J_{H\text{-}1,H\text{-}2}$ 3.7 Hz), 4.90 ($J_{H\text{-}1,H\text{-}2}$ 3.7 Hz), 4.53 ($J_{H\text{-}1,H\text{-}2}$ 7.7 Hz), 2.08 (3 H), and 2.04 (3 H). The ^{13}C NMR spectrum showed, *inter alia*, signals at δ 175.2, 175.0, 104.5, 99.0, 98.4, 81.8, 80.5, 63.7, 54.8, 53.4,

22.9, and 22.7. The FABMS spectrum of the underivatised tetrasaccharide-alditol obtained in the positive-ion mode showed a peak at m/z 722 attributed to $[M+H]^+$; upon addition of sodium ions, the peak moved to m/z 744 attributed to $[M+Na]^+$. A B/E-linked scan experiment [15], by which daughter ions of a selected parent ion can be monitored, gave in the positive mode on m/z 722, *inter alia*, the fragments with m/z 366 (abA), 569 (abcA), 357 (cdB), and 519 (bcdB) [12]. The methylation analysis showed a 2-substituted ribitol, 4-substituted galactose, a terminal glucosamine, a 3-substituted glucosamine, and two additional components (Table 2, column C), which probably derive from small amounts of the pentasaccharide-alditol (*vide infra*) although this was not evident from NMR spectra. The sequence of the sugars in the tetrasaccharide-alditol is then HexNAc–Hex–HexNAc–Pentitol.

The pentasaccharide-alditol contained the same sugars as the tetrasaccharide-alditol but the relative amount of galactose was increased (Table 1, column D). The ^{1}H NMR spectrum of the pentasaccharide-alditol showed, *inter alia*, signals at δ 5.13 ($J_{H\text{-}1,H\text{-}2}$ 3.7 Hz), 4.91 ($\nu_{1/2}$ 5.0 Hz), 4.53 ($J_{H\text{-}1,H\text{-}2}$ 7.3 Hz), 4.50 ($J_{H\text{-}1,H\text{-}2}$ 7.3 Hz), 2.07 (3 H), and 2.04 (3 H). The ^{13}C NMR spectrum showed, *inter alia*, signals at δ 104.4, 103.8, 98.6, 98.3, 81.6, 80.5, 79.8, 63.7, 54.5, 53.4, 22.9, and 22.7. The FABMS spectrum of the underivatised pentasaccharide-alditol showed a peak at m/z 884 attributed to $[M+H]^+$; upon addition of sodium ions, the peak moved to m/z 906 attributed to $[M+Na]^+$. A B/E-linked scan experiment in the positive-ion mode on m/z 884 gave, *inter alia*, the fragments with m/z 366 (abA), 528 (abcA), 731 (abcdA), and 357 (cdB). The methylation analysis showed a large increase in the amount of terminal galactose as well as in 4-substituted glucosamine (Table 2, column D) compared to the methylation analysis of the tetrasaccharide-alditol. No terminal glucosamine was observed in this analysis. The sequence of the sugars in the pentasaccharide-alditol is then Hex–HexNAc–Hex–HexNAc–Pentitol.

Permethylated HexNAc-containing oligosaccharides show especially intense ions due to primary cleavage through A_1 fragmentation pathways [16]. The secondary fragments formed arise preferentially by elimination of the 3-substituent of the HexNAc residue [16]. Thus, monosaccharide sequence and some linkage positions may be determined using permethylated samples. The permethylated tetrasaccharide-alditol showed in the FABMS spectrum a peak at m/z 918 attributed to $[M+H]^+$. A B/E-linked scan experiment on m/z 918 gave, *inter alia*, the fragments with m/z 709 (abcA), 260 (aA), and 228 (A_2^2) [17]. No fragment was observed at m/z 677 by elimination of methanol from m/z 709. The A_2^2 fragment at m/z 228 can derive from both m/z 709 and 260. The HexNAc residue in the tetrasaccharide-alditol should thus be 3-substituted. The permethylated pentasaccharide-alditol showed in the FABMS spectrum a peak at m/z 1122 attributed to $[M+H]^+$. A B/E-linked scan experiment on m/z 1122 gave, *inter alia*, the fragments m/z 913 (abcdA), 464 (abA), 432 (abA_2^2), and 228 ($abcdA_2^2$). No fragment was observed at m/z 881 by elimination of methanol from m/z 913 whereas the fragment at m/z 432 should derive from elimination of methanol from m/z 464. From the above data, the structures of the tetrasaccharide-alditol, **2**, and the pentasaccharide-alditol, **3**, can be defined.

α-D-Glc*p*NAc-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→2)-D-Ribitol-1-*d*

2

β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→2)-D-Ribitol-1-*d*

3

One additional oligosaccharide was isolated from the partial hydrolysis, viz., a decasaccharide. The sugar analysis showed almost identical relative proportions as the pentasaccharide (Table 1, column E). The methylation analysis showed components in agreement with a structure of two repeating-units from the polysaccharide (Table 2, column E). The ^{1}H NMR spectrum of the decasacchride-alditol showed, *inter alia*, signals at δ 5.34 ($\nu_{1/2}$ 3.1 Hz), 5.16 ($J_{H\text{-}1,H\text{-}2}$ 3.7 Hz), 5.13 ($J_{H\text{-}1,H\text{-}2}$ 3.7 Hz), 4.91 (2 H), 4.53 (2 H), 4.50 (2 H), 2.07 (6 H), 2.06 (3 H), and 2.04 (3 H). The FABMS spectrum of the underivatised decasaccharide-alditol obtained in the positive-ion mode showed a peak at *m/z* 1747 attributed to $[M+H]^+$; upon addition of sodium ions, the peak moved to *m/z* 1769 attributed to $[M+Na]^+$.

The above data define the pentasaccharide repeating-unit of the O153 PS as **4**.

→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Rib*f*-(1→

4

The structure is further supported by the measurement of nuclear Overhauser effects between anomeric protons and protons on glycosylated carbons in a NOESY experiment. Interresidue NOEs were observed, *inter alia*, from H-1, δ 5.32, of the ribofuranose residue to H-4, δ 4.01, of the galactose residue having its anomeric proton at δ 4.50; from H-1, δ 5.16, of the 3-substituted glucosamine residue to H-2, δ 4.25, of the ribofuranose residue; and from H-1 of the galactose residue having its anomeric proton at δ 4.53 to H-3, δ 4.03, of the 3-substituted glucosamine residue.

Ribofuranose has been reported to be present in both capsular polysaccharides [18,19] and in lipopolysaccharides [20]; however, it has not been reported to be present in the O-antigenic polysaccharides of enterotoxigenic *E. coli*.

3. Experimental

General methods.—Evaporations were performed under diminshed pressure at <40°C (bath) or by flushing with air. For GLC, a Hewlett–Packard 5890A instrument, fitted with a flame-ionisation detector, was used. Separations were performed on an HP5 fused-silica capillary column, using a temperature program 180°C (1 min) →250°C at 3°C/min. GLC–MS was performed on a Hewlett–Packard 5890-5970 instrument, using the same phase. Hydrolysis of underivatised material was performed with 2 M CF_3CO_2H 120°C for 2 h. Methylation analyses were performed as previously described [21,22]. The absolute configurations of ribose, galactose, and 2-acetamido-2-deoxyglucose were determined essentially as described by Leontein et al. [9] by GLC of their acetylated (+)-2-butyl glycosides. A differential refractometer was used for monitoring the gel chromatography effluents. FABMS spectra in the positive mode were recorded on a Jeol SX 102 instrument, using Xe atoms (6kV) and a matrix of glycerol, at a resolution of 1000. The B/E-linked scan experiment used He as collision gas.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 70°C using a Jeol GSX-270 instrument. Chemical shifts are reported in ppm relative to sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and acetone (δ_C 31.00) as internal references. COSY, relayed COSY, and C,H-COSY experiments were used to assign signals and performed according to standard pulse sequences. The NOESY experiment used a mixing time of 300 ms.

Smith degradation.—A solution of O153 PS (25 mg) and $NaIO_4$ (85 mg) in 0.1 M acetate buffer, pH 3.9 (10 mL), was kept in the dark for 90 h at 4°C. Ethylene glycol (0.1 mL) was then added and the samples dialysed extensively against deionised water. The sample (10 mg) in water (10 mL) was reduced with $NaBH_4$ (400 mg) for 16 h at room temperature, and excess of borohydride was decomposed with AcOH. The polymeric fraction was recovered by gel filtration on a Bio-Gel P-2 column (2.5 × 70 cm). The product (6 mg) was treated with 0.5 M CF_3CO_2H (5 mL) at room temperature for 24 h and the hydrolysate was concentrated to dryness. Gel filtration of the product yielded an oligosaccharide (1 mg).

Partial hydrolysis of O153 PS.—The polysaccharide (25 mg) was treated with 0.1 M CF_3CO_2H at 100°C for 2 h. After neutralisation, the sample was freeze-dried. Gel filtration of the sample on a Bio-Gel P-2 column (2.5 × 70 cm) yielded products in the oligosaccharide region. Reduction of these oligosaccharides with sodium borodeuteride followed by conventional work-up and gel filtration yielded a decasaccharide-alditol (3 mg), a pentasaccharide-alditol (2 mg), and a tetrasaccharide-alditol (3 mg).

Bacterial strain.—*E. coli* O153:K?:H7 (CCUG 31997) was obtained from the Culture Collection, University of Göteborg, Sweden.

Isolation and purification of the O-polysaccharide.—*E. coli* O153 bacteria were grown in Ty medium (30-L culture). Bacteria were killed by the addition of formaldehyde (1% final concentration) and harvested by centrifugation. Lipopolysaccharide (LPS) was extracted by the hot phenol–water method [8]. The LPS was treated with aq 2% AcOH at 100°C for 2 h. Liberated lipid A was centrifuged, and the supernatant solution was neutralised, dialysed, and lyophilised. The product was further purified by column chromatography on a column (2.6 × 90 cm) of Bio-Gel P-6.

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References

[1] H.W. Ewing, *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th ed., Elsevier, New York, 1986, Chapter 6.

[2] R.J. Gross and B. Rowe, *J. Hyg.*, 95 (1985) 531–550.

[3] E.A. Gonzales, J. Blanco, I. Garabal, and M. Blanco, *J. Med. Microbiol.*, 34 (1991) 89–95.

[4] M.E. Agüero, L. Reyes, V. Prado, I. Ørskov, F. Ørskov, and F.C. Cabello, *J. Clin. Microbiol.*, 22 (1985) 576–581.
[5] P.-E. Jansson, B. Lindberg, J. Lönngren, C. Ortega, and S.B. Svenson, *Carbohydr. Res.*, 131 (1984) 277–283.
[6] P.-E. Jansson, J. Lönngren, G. Widmalm, K. Leontein, K. Slettengren, S.B. Svenson, G. Wrangsell, A. Dell, and P.R. Tiller, *Carbohydr. Res.*, 145 (1985) 59–66.
[7] P.-E. Jansson, B. Lindberg, G. Widmalm, and K. Leontein, *Carbohydr. Res.*, 165 (1987) 87–92.
[8] O. Westphal and K. Jann, *Methods Carbohydr. Chem. Biochem.*, 5 (1965) 83–91.
[9] K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359–362.
[10] K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.
[11] I.J. Goldstein, G.W. Hay, B.A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
[12] A. Dell, *Adv. Carbohydr. Chem. Biochem.*, 45 (1987) 19–72.
[13] P.A.J. Gorin and J.F.T. Spencer, *Can. J. Chem.*, 43 (1965) 2978–2984.
[14] B. Lindberg, J. Lönngren, and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 185–240.
[15] D.S. Millington and J.A. Smith, *Org. Mass. Spectrom.*, 12 (1977) 264–265.
[16] G. Grönberg, P. Lipniunas, T. Lundgren, K. Erlandsson, F. Lindh, and B. Nilsson, *Carbohydr. Res.*, 191 (1989) 261–278.
[17] N.K. Kochetkov and O.S. Chizhov, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
[18] G.G.S. Dutton and L.A.S. Parolis, in V. Crescenzi, I.C.M. Dea, and S.S. Stivala (Eds.), *Biomedical and Biotechnological Advances in Industrial Polysaccharides*, Gordon and Breach, New York, 1989, pp 223–240.
[19] P.L. Hackland, H. Parolis, and L.A.S. Parolis, *Carbohydr. Res.*, 219 (1991) 193–201.
[20] M. Berst, C.G. Hellerqvist, B. Lindberg, O. Lüderitz, S. Svensson, and O. Westphal, *Eur. J. Biochem.*, 11 (1969) 353–359.
[21] P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lönngren, *Chem. Commun., Univ. Stockholm*, 8 (1976) 1–75.
[22] T.J. Waeghe, A.G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.