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Structure of the capsular polysaccharide and the O-side-chain of the lipopolysaccharide from *Acetobacter methanolicus* MB 58/4 (IMET 10945), and of oligosaccharides resulting from their degradation by the bacteriophage *Acm1**

H. Dieter Grimmecke[†,‡], Uwe Mamat[‡], Wolfgang Lauk,
Institute of Biotechnology, Permoser Street 15, Leipzig O-7050 (Germany)

Alexander S. Shashkov, Yuriy A. Knirel, Evgeny V. Vinogradov, and Nikolay K. Kochetkov
N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of the U.S.S.R., Moscow B-334 (U.S.S.R.)

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ABSTRACT

The capsular polysaccharide (CPS) and the O-side-chain of the lipopolysaccharide (LPS) of *Acetobacter methanolicus* MB 58/4 (IMET 10945) have been shown to contain the same disaccharide repeating unit, namely, →2)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→. Degradation of the CPS and the LPS with the bacteriophage *Acm1* gave fragments built up of 1–5 repeating units; the octasaccharide preponderated. The phage-associated depolymerase proved to be a β-D-galactofuranoside hydrolase.

INTRODUCTION

Acetobacter methanolicus is a new species of acidophilic Gram-negative bacteria[1] that can utilise methanol as a carbon source[2]. The cell-surface carbohydrates of this micro-organism are being investigated in order to determine phylogenetic relationships between genera, identify strains, and elucidate the structure of the cell envelope and the mechanism of its interaction with bacteriophages. Capsular polysaccharide (CPS) and a lipopolysaccharide (LPS), isolated[3] from *A. methanolicus*, strain MB 58/4 (IMET 10945), can be cleaved by an enzyme associated with the phage *Acm1*. We now report the structures of the CPS, the O-side-chain of the LPS, and the oligosaccharides that resulted from their degradation.

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† Author for correspondence.
‡ Present address: Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, Parkallee 22, D-2061 Borstel, Germany.

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RESULTS AND DISCUSSION

The LPS and CPS were obtained using the phenol–water extraction method[4]. The LPS was purified by digestion with DNase and RNase, and the composition of the CPS was verified by sugar analyses, after hydrolysis, by g.l.c. of the products as the derived alditol acetates. Ion-exchange chromatography of the crude CPS on DEAE-Sephacel gave one hexose-containing peak (CPS) that was eluted without retardation by 10mM Tris/HCl buffer (pH 8.2). Gradient elution with NaCl then gave heterogeneous broad peaks that contained mainly ribose and phosphate, and which were discarded.

The CPS had $[\alpha]_D^{20}$ −53° (*c* 0.5, water) and, on acid hydrolysis, it gave galactose only, which was dextrorotatory and, therefore, D.

The ^{1}H-n.m.r. spectrum of the CPS (Fig. 1) contained signals for anomeric protons at 5.46 (bs) and 4.61 p.p.m. (d, $J_{1,2}$ 7.91 Hz) and, hence, is a regular polymer built up of disaccharide repeating units. The other ^{1}H resonances were assigned by sequential, selective spin-decoupling experiments (Table I). The ^3J values indicated that one of the galactose residues was β-pyranosidic[5] and the other was β-furanosidic[6].

The ^{13}C-n.m.r. spectrum of the CPS (Fig. 2) contained signals for anomeric carbons at 108.8 ($J_{C\text{-}1,H\text{-}1}$ 178.2 Hz) and 103.3 p.p.m. ($J_{C\text{-}1,H\text{-}1}$ 161.1 Hz) indicative[7–9] of β-D-Gal*f* and β-D-Gal*p* units, respectively.

The ^{13}C-n.m.r. spectrum of the CPS was interpreted by using selective heteronuclear $^{13}C\{^1H\}$ double resonance (Table II). The low-field positions of the signals for C-2 of the Gal*f* residue at 90.1 p.p.m. and C-3 of the Gal*p* residue at 81.1 p.p.m. are accounted for by the α-effect of glycosylation[10], and, hence, the Gal*f* is 2-substituted and the Gal*p* is 3-substituted. Thus, the CPS has the following repeating unit.

→2)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→

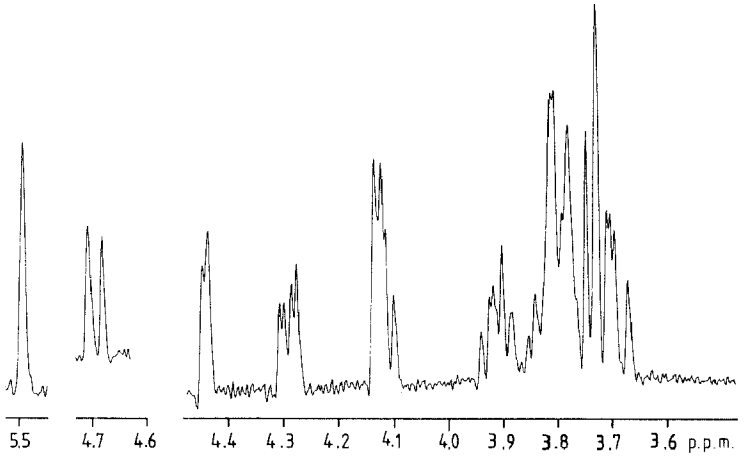


Fig. 1. ^{1}H-N.m.r. spectrum (300 MHz) of the capsular polysaccharide (CPS) of *A. methanolicus* MB 58/4 (IMET 10945).

TABLE I

^{1}H-N.m.r. data for the *A. methanolicus* (IMET 10945) capsular polysaccharide

Unit	*Proton*	*Chemical shift (p.p.m.)*	*Multiplicity*	*Coupling constant (Hz)*	
β-D-Gal*p*	H-1	4.69	d	$J_{1,2}$	7.6
	H-2	3.71	dd	$J_{2,3}$	~9
	H-3	3.76	dd	$J_{3,4}$	4.2
	H-4	4.13	d	$J_{4,5}$	<1
	H-5,6a,6b (3 H)	3.81	m		
β-D-Gal*f*	H-1	5.49	b	$J_{1,2}$	<1
	H-2	4.44	dd	$J_{2,3}$	3.0
	H-3	4.29	dd	$J_{3,4}$	6.5
	H-4	4.11	dd	$J_{4,5}$	4.2
	H-5	3.91	ddd	$J_{5,6a}$	4.6
	H-6a	3.76	dd	$J_{6a,6b}$	~12
	H-6b	3.70	dd	$J_{5,6b}$	7.0

Acid hydrolysis of the LPS gave[3] galactose, rhamnose, glucose, and mannose in the ratios 3.9:2.8:1.4:1, together with heptose, 2-amino-2-deoxyglucose, 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), and phosphate. Attempts to split off the lipid part of the LPS by hydrolysis with aqueous 2% acetic acid (2 h, 100°) were unsuccessful and the use of dilute hydrochloric acid depolymerised the polysaccharide chain. Therefore, the LPS was treated with alkali to cleave the *O*-acyl groups and give a water-soluble *O*-deacylated polysaccharide (PS) that was isolated by gel-permeation chromatography on Sephadex G-50. Acid hydrolysis of the PS revealed galactose, rhamnose, glucose, and mannose in the ratios 6.1:3.8:2:1, *i.e.*, the same sugars present in the LPS, but with the relative proportion of galactose increased to some extent.

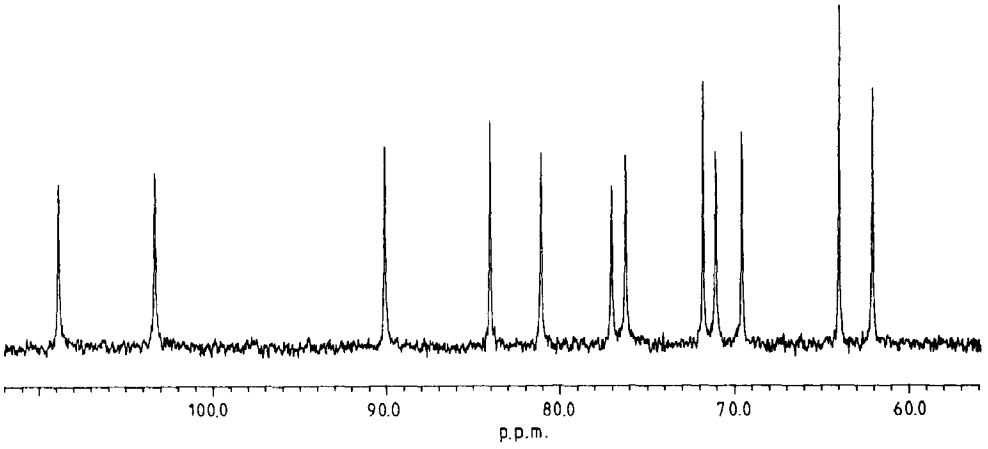


Fig. 2. ^{13}C-N.m.r. spectrum (62.89 MHz) of the capsular polysaccharide (CPS) of *A. methanolicus* MB 58/4 (IMET 10945).

TABLE II

Chemical shifts (p.p.m.) in the ^{13}C-n.m.r. spectra

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
	Unit A					
4	103.5	71.9	73.9	69.9	76.4	62.1
3	103.5	72.0	73.9	69.8	76.5	62.1
2	103.5	71.9	73.8	69.7	76.4	62.1
1[a]	105.6	72.4	73.9	69.9	76.2	62.1
	104.9	72.6	73.9	69.9	76.5	62.1
Methyl β-D-galactopyranoside	104.9	71.8	73.9	69.8	76.2	62.1
	Unit B					
CPS	108.8	90.1	77.0	84.0	71.8	64.0
4	108.8	90.1	77.0	84.0	71.8	64.0
3	109.1	90.1	77.2	83.9	71.8	64.0
2	109.0	90.0	77.1	83.8	71.7	64.0
	Unit C					
CPS	103.3	71.1	81.1	69.6	76.2	62.1
4	103.3	71.1	81.1	69.6	76.2	62.1
3	103.3	71.2	81.1	69.7	76.3	62.1
	Unit D					
4[a]	105.5	71.5	81.2	69.7	76.0	62.1
	104.5	71.5	81.2	69.7	76.3	62.1
3[a]	105.6	71.6	81.2	69.8	76.1	62.1
	104.5	71.6	81.2	69.8	76.5	62.1
2[a]	105.5	71.5	81.1	69.7	76.0	62.1
	104.5	71.5	81.1	69.7	76.4	62.1
	Unit E					
α-**4**	93.3	79.3	69.5	70.6	71.8	62.2
β-**4**	96.3	81.1	74.0	70.1	76.2	62.1
α-**3**	93.4	79.4	69.6	70.6	71.8	62.4
β-**3**	96.4	81.1	74.1	69.9	76.3	62.1
α-**2**	93.3	79.4	69.5	70.6	71.7	62.3
β-**2**	96.3	81.2	74.1	69.9	76.4	62.1
α-**1**	93.3	79.2	69.5	70.6	71.5	62.1
β-**1**	96.4	81.5	73.9	70.0	76.3	62.1
D-Galactopyranose[5]						
α	93.6	69.8	70.5	70.6	71.7	62.5
β	97.7	73.3	74.2	70.1	76.3	62.3

[a] The chemical shifts of the signals for the α form of unit E are listed first.

The ^{13}C-n.m.r. spectrum of the PS was identical with the spectrum of CPS (Fig. 2), and, hence, the O-side-chain of the LPS and the CPS have the same structure. The absence from the spectra of the PS and CPS of even minor signals for terminal non-reducing sugar residues reflects a d.p. of >20 repeating units. Also, there were no signals in the spectrum of the PS for monosaccharides other than those of galactose, and their absence may be accounted for by the micelle-like structure of the PS in which the aggregation of molecules due to hydrophobic interaction of *N*-linked fatty acids makes only the O-side-chain amenable to n.m.r. analysis.

The presence of Gal*f* residues in the O-side-chain is probably responsible for the depolymerisation during mild hydrolysis of the LPS with hydrochloric acid. Support for this view was obtained when the alkali-degraded LPS was hydrolysed with aqueous 2% acetic acid (20 h, 105°). Gel-permeation chromatography on Fractogel TSK HW-50(S) of the products revealed several galacto-oligosaccharides which were not investigated further. The opalescent solution eluted in the void volume of the column contained fragment(s) built up of rhamnose, glucose, mannose, and galactose in the ratios ~10:2.5:1:0.6. Therefore, it seems likely that rhamnose and glucose are the main sugar constituents of the core region of the LPS and are not amenable to n.m.r. analysis without removing the *N*-linked fatty acids.

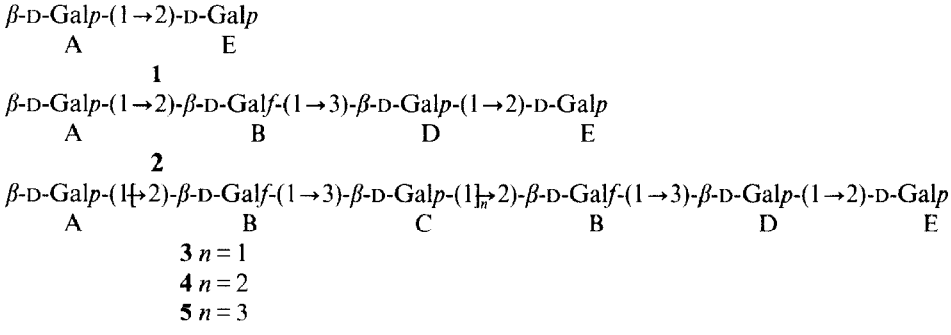
The oligosaccharides **1**–**5** derived from the CPS and LPS by treatment with bacteriophage *Acm1* were isolated and purified by gel-permeation chromatography on BioGel P-2 and preparative p.c.[3] The chromatographic data indicated that they constituted an homologous series of di- to deca-saccharides with the octasaccharide preponderating[3]. On acid hydrolysis, **1**–**5** each gave galactose only, and ^{13}C-n.m.r. spectroscopy confirmed each d.p. and the structure.

Thus, **1** was a disaccharide composed of two Gal*p* residues (A and E, see formula). The reducing unit E gave two series of signals consistent with an α,β-ratio of ~2:1. The signals of the non-reducing unit A were assigned by comparison with the data for methyl β-D-galactopyranoside[7] (Table II). C-1,2,5 of unit A gave two series of signals due to α and β forms of unit E. The ^{13}C-n.m.r. data for **1** proved unit E to be 2-substituted. In the spectra[11,12] of D-galactopyranose[7] and of oligosaccharides that contained Gal*p* residues substituted at position 2, the difference between chemical shifts of signals for C-1α and C-1β was >4 p.p.m., whereas it was 3.1 p.p.m. for unit E (*cf.* 3.5 p.p.m. for 2-*O*-L-rhamnopyranosyl-D-galactose[11]). Such a decrease in the difference is caused by unequal β-effects of glycosylation on C-1α and C-1β. The chemical shifts of the signals for C-4,5,6 of unit E and D-galactopyranose[7] were similar (Table II), whereas those for C-2 and C-3 were shifted by the α- and β-effects of 2-glycosylation[10]. Thus, **1** had the structure shown.

For **2**, the ^{13}C signals for the reducing unit E and the non-reducing unit A were identified easily. Unit A gave only one series of signals, whereas unit E, as in **1**, gave two series of signals consistent with an α,β-ratio of ~2:1. In addition, there were signals for a substituted β-Gal*f* residue (unit B) and a substituted non-reducing β-Gal*p* residue (unit D). The chemical shifts of all signals in unit B and those of C-2/6 in unit D were similar to those of the corresponding signals for the CPS (units B and C, respectively). However,

C-1 of unit D in **2** gave two lines, thus indicating its attachment to reducing unit E. The chemical shifts of both lines (105.5, and 104.5 p.p.m.) differed from that (103.3 p.p.m.) of the signal for C-1 of unit C of the CPS, which is accounted for by the different (pyranosidic or furanosidic) forms of the aglycon for this unit in **2** and CPS. Thus, **2** was a tetrasaccharide composed of one Gal*f* and three Gal*p* residues, with the former located between two non-reducing Gal*p* residues.

The ^{13}C signals of **3** were all present in the spectrum of **2**, but the intensities for the Gal*f* (unit B) were doubled and there were signals for an additional Gal*p* residue (unit C), the chemical shifts of which were similar to those of unit C in the CPS. Thus, **3** is a hexasaccharide homologue of **2**. The ^{13}C-n.m.r. spectra of **3** and **4** were similar, with the exception of the increased relative intensities of signals for units B and C in **4**. As judged by the ratio (~1:2) of intensities of signals for C-1 of units A and C, **4** is an octasaccharide homologue of **3**.



The conclusions based on the ^{13}C-n.m.r. data are in a good agreement with the results of gel-permeation chromatographic analysis[3] of **1**–**4**. The ^{13}C-n.m.r. spectrum of OS **5** was not recorded because of insufficient amount of material; however, taking into account the chromatographic data, **5** appears to be the decasaccharide homologue of **4**.

The above results show that the identical structures of the CPS (K-antigen) and the O-side-chain of the LPS (O-antigen) account for the ability of the bacteriophage *Acm1* to split each polymer, and, hence, it must be classified as both a K- and O-phage. This cleavage gave similar mixtures of oligosaccharides made up of disaccharide repeating units and with a β-Gal*p* residue at the non-reducing terminus. Therefore, the enzyme associated with the phage *Acm1* cleaves specifically β-D-galactofuranosidic linkages, and the formation of the octasaccharide **4** reflects the extended catalytic site of the enzyme acting in an *endo*-mechanism.

The above study shows that ^{13}C-n.m.r. spectroscopy can provide an indirect method of characterisation of bacteriophage-associated enzymes and for understanding phage–host interactions.

EXPERIMENTAL

Bacteria: extraction and purification of the polysaccharide. — The conditions of growth have been described[1,2]. Freeze-dried bacteria were extracted with hot phenol–water according to a standard method[4]. The aqueous phase was dialysed extensively against water and the water was evaporated *in vacuo*. In order to separate the CPS from the LPS, an ultracentrifugation step[4] was performed. The clear supernatant solution contained the crude CPS. The sedimented LPS was suspended in 0.1M Tris/HCl buffer (pH 7.5) that contained 50mM NaCl and 10mM $MgCl_2$, incubated (16 h, 37°) with DNase I (Boehringer; EC 3.1.27.5) and RNase (Calbiochem; EC 3.1.27.5), washed with water by repeated ultracentrifugation, and then lyophilised.

The crude CPS was applied to a column (45 × 3 cm) of DEAE-Sephacel (Cl^- form) equilibrated with 10mM Tris /HCl (pH 8.2). Elution with the buffer gave a neutral polysaccharide, and a subsequent elution with a linear gradient of NaCl (0 → 0.5M) gave acidic material. Fractions were assayed for hexoses[13], uronic acids[14], and phosphate[15]. The fractions that contained the polymers were desalted by dialysis and lyophilised.

General methods. — Descending p.c. was performed on FN 11 paper (Filtrak), using 1-butanol–pyridine–water (6:4:3) and detection with the *p*-anisidine–*o*-phthalic acid reagent.

Gel-permeation chromatography was conducted on columns of Sephadex G-50 (70 × 3 cm) in pyridinium acetate buffer (pH 5.4), Fractogel TSK HW-50 (40 × 2 cm), and BioGel P-2 (−400 mesh) (90 × 2 cm) in water. Elution was monitored with a Technicon sugar analyser and a Knauer differential refractometer. The former instrument was used for identification[16] of monosaccharides in addition to g.l.c. of derived alditol acetates[17].

The ^1H- and ^{13}C-n.m.r. spectra were recorded with Bruker AM-300 and WM-250 instruments on solutions in D_2O at 30° [internal acetone for ^1H (2.23 p.p.m.), and methanol for ^{13}C (50.15 p.p.m.)].

Optical rotations were measured for aqueous solutions with an EPO-1 (U.S.S.R.) polarimeter at 20°.

Polymer solutions were concentrated *in vacuo* at 40° and freeze-dried.

Bacteriophage-mediated degradation of the CPS. — To a solution of the CPS (100 mg in 20 mL) in 40mM sodium acetate (pH 4.5) that contained 10^{-2}M NaCl, 10^{-3}M $CaCl_2$, and 10^{-3}M $MgCl_2$ was added a suspension of bacteriophage *Acm1* (1 mL, 5 × 10^{11} plaque-forming units), and the mixture was incubated for 24 h at 30°. In order to complete the degradation, another portion of phage was added using the same parameters. The mixture was then centrifuged (4 h, 100 000*g*), deionised with Dowex 50 (H^+) and 1-X2 (AcO^-) resins, concentrated, and eluted from a column of BioGel P-2. The resulting oligosaccharides (**1**–**5**) were purified by preparative p.c. The yields were as follows: **1**, 9 mg; **2**, 10 mg; **3**, 10 mg; **4**, 45 mg; and **5**, 8 mg.

Degradation of LPS with alkali. — A solution of LPS (100 mg) in 0.1M sodium hydroxide (10 mL) was heated for 1 h at 100°, then neutralised with M HCl, centrifuged, and subjected to gel-permeation chromatography on Sephadex G-50 to give PS (70 mg).

Cleavage of degraded LPS with acetic acid. — A solution of the alkali-degraded LPS (60 mg) in aqueous 2% acetic acid (15 mL) was heated for 20 h at 105°, then centrifuged (15 min, 3000*g*), and freeze-dried. The product was eluted from a column of Fractogel TSK HW-50(S). A polymer fraction and three main oligosaccharide fractions were obtained and analysed after acid hydrolyses.

Acid hydrolysis. — Samples (1–2 mg) of CPS, LPS, PS, and **1–5** were hydrolysed with 2M HCl (0.5 mL, 3 h, 105°), the solvent was evaporated, and water was evaporated twice from each residue, which was then analysed by p.c. and the sugar analyser.

In a preparative experiment, CPS (10 mg) was hydrolysed and treated as described above, and the residue was dried *in vacuo* to give D-galactose (10 mg), $[\alpha]_D$ +48° (*c* 0.5, water); lit.[18] $[\alpha]_D$ +80° (water).

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REFERENCES

1 H. Uhlig, K. Karbaum, and A. Steudel, *Int. J. System. Bacteriol.*, 36 (1980) 317–322.
2 M. W. Gründig and W. Babel, *Arch. Microbiol.*, 149 (1987) 149–154.
3 U. Mamat and H. D. Grimmecke, unpublished results.
4 O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
5 C. Altona and C. A. G. Haasnoot, *Org. Magn. Reson.*, 13 (1980) 417–429.
6 S. J. Angyal, *Carbohydr. Res.*, 77 (1979) 37–50.
7 P. A. J. Gorin and M. Mazurek, *Can. J. Chem.*, 53 (1975) 1212–1223.
8 N. Cyr and A. S. Perlin, *Can. J. Chem.*, 57 (1979) 2504–2511.
9 K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293–297.
10 P. A. J. Gorin, *Adv. Carbohydr. Chem. Biochem.*, 38 (1980) 13–104.
11 A. Liptak, Z. Szurmai, P. Nanasi, and A. Neszmelyi, *Carbohydr. Res.*, 99 (1982) 13–21.
12 A. I. Usov, V. V. Barbakadze, S. V. Yarotsky and A. S. Shashkov, *Bioorg. Khim.*, 4 (1978) 1507–1511.
13 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350 356.
14 T. Bitter and H. M. Muir, *Anal. Biochem.*, 4 (1962) 330–334.
15 O. H. Lowry, N. R. Roberts, K. Y. Leiner, M. L. Wu, and A. L. Farr, *J. Biol. Chem.*, 207 (1954) 1–17.
16 B. A. Dmitriev, Y. A. Knirel, N. K. Kochetkov, and I. L. Hofman, *Eur. J. Biochem.*, 66 (1976) 559–566.
17 J. Metz, W. Ebert, and H. Weicker, *Chromatographia*, 4 (1971) 345–350.
18 J. Stanek, M. Cerny, J. Kocourek, and J. Pacak, *The Monosaccharides*, Publishing House of the Czechoslovak Academy of Sciences, Prague, 1963, p. 99.