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Structure of an acidic microcapsular glycan from the reference strain (C.D.C. 866-57) for *Serratia marcescens* serogroup O1

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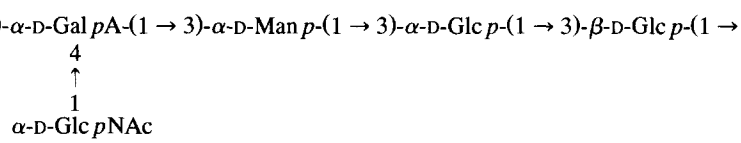
Abstract

The structure of the acidic polysaccharide from *Serratia marcescens* serogroup O1 has been investigated. NMR spectroscopy together with sugar and methylation analysis have been used as well as a uronic acid degradation. The polysaccharide consists of pentasaccharide repeating units having the following structure.



The polysaccharide also contains one equivalent of *O*-acetyl groups per repeating unit present on, inter alia, a hydroxymethyl group.

Keywords: Serratia marcescens; Acidic microcapsular glycan

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1. Introduction

Extensive studies [1] of the surface polysaccharides elaborated by strains of *Serratia marcescens* have revealed that the proposed typing scheme of 26 O-serogroups is actually a hybrid of O types [defined by neutral glycans constituting the side chains of lipopolysaccharides (LPSs)], and K types (defined by acidic glycans, presumed to be of microcapsular origin). When both types of polymer are present, it is the acidic polymer (co-extracted from isolated cell walls with LPS) which is serodominant, as indicated by the fact that different neutral polymers can occur in the same serogroup. For example, at least four distinct neutral polymers occur [2] in separate strains from serogroup O14, which is defined by an acidic glucomannan [3]. The same neutral polymers also occur in strains from other serogroups (O6, O8, O12, O21), and such distributions obviously contribute to the extensive cross-reactions between the different serogroups of *Serratia marcescens*. The reference strain for serogroup O1 is another which contains both neutral and acidic glycans [4]. The repeating unit of the former polymer has structure **1**, which is also a minor unit in the corresponding polymers from the O17 and O19 reference strains [5], and clearly accounts for the cross-reactions with these serogroups. In the preliminary study of the O1 polymers [4], we also identified the components of the acidic glycan as Glc, Man, GlcNAc, and GalA (molar ratios ~ 2 : 1 : 1 : 1), together with an *O*-acetyl group. Here we report the results of a structural study of this polymer.

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1 \rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow$$

1

2. Results and discussion

The acidic glycan was isolated by ion-exchange chromatography from the polymeric fraction obtained from a hydrolysis under mild conditions of the LPS (yield, ~ 57% of the total polymeric material). No evidence for or against covalent incorporation into LPS was obtained, but the glycan was clearly released in soluble form either by aqueous phenol extraction of the cell walls or by the subsequent mild hydrolysis of the LPS.

Hydrolysis of the acidic polysaccharide from *Serratia marcescens* serogroup O1 (SmO1) with 0.5 M trifluoroacetic acid yielded mannose, glucose, and glucosamine in the relative proportions 19 : 48 : 33. Neutral sugars were also identified by paper chromatography. The results of enzymatic assays for Glc (34%) and Man (18%), autoanalysis for GlcN (12%), and colorimetric assay for GalA (12%) indicated a pentasaccharide repeating unit. Galacturonic acid was furthermore identified as its acetylated (+)-2-butyl glycosides and had the D-configuration, which also was the case for the other constituent sugars of the polysaccharide determined essentially as devised by Leontein et al. [6]. Methylation analysis of the native glycan (Table 1, column A) showed the presence of terminal glucosamine, 3-substituted mannose, and 3-substituted glucose. Reduction of methylated SmO1 with lithium borohydride yielded, after subsequent hydrolysis, a methyl ether corresponding to 2,4-disubstituted galacturonic acid in the native polymer in addition to the residues mentioned above (Table 1, column B).

Table 1
Methylation analysis of SmO1 and derivatives thereof [a]

Sugar [b]	Detector response		
	A	B	C
2,3,4,6-Man			12 [c]
2,4,6-Glc	62	51	62
2,4,6-Man	24	33	26
2,3,4,6-GlcNAc	14	9	
3-Gal		7	

[a] Key: A, methylated polysaccharide; B, methylated and carboxyl-reduced polysaccharide; C, uronic acid-degraded polysaccharide.
[b] 2,4,6-Glc = 2,4,6-Tri-*O*-methyl-D-glucose, 2,3,4,6-GlcNAc = 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, etc.
[c] Labelled with trideuteriomethyl at O-3.

The ^{1}H NMR spectrum of native SmO1 showed, inter alia, signals for *O*-acetyl and *N*-acetyl groups at δ 2.14 (3 H) and 2.08 (3 H), respectively. A signal at δ 2.09 (3 H) remained in this region of the spectrum after *O*-deacetylation. For native SmO1, in the region for anomeric protons, signals were observed at δ 5.51 (1 H); 5.31 (1 H); 5.17 and 5.14 (total 1 H); 5.01, 4.98 and 4.95 (total 1 H); and 4.68 (1 H) showing heterogeneity of the material, which could be due to different locations of the *O*-acetyl substituent. The ^{13}C NMR spectrum of native SmO1 showed, inter alia, signals at δ 21.0 and 64.2, both of which disappeared on *O*-deacetylation. The latter chemical shift is indicative of acetylation at a primary position.

The ^{1}H NMR spectrum of *O*-deacetylated SmO1 (Fig. 1) showed, inter alia, signals for five anomeric protons (Table 2) and the corresponding residues are labelled **A–E** with respect to decreasing chemical shift of the signals from anomeric protons. The ^{13}C

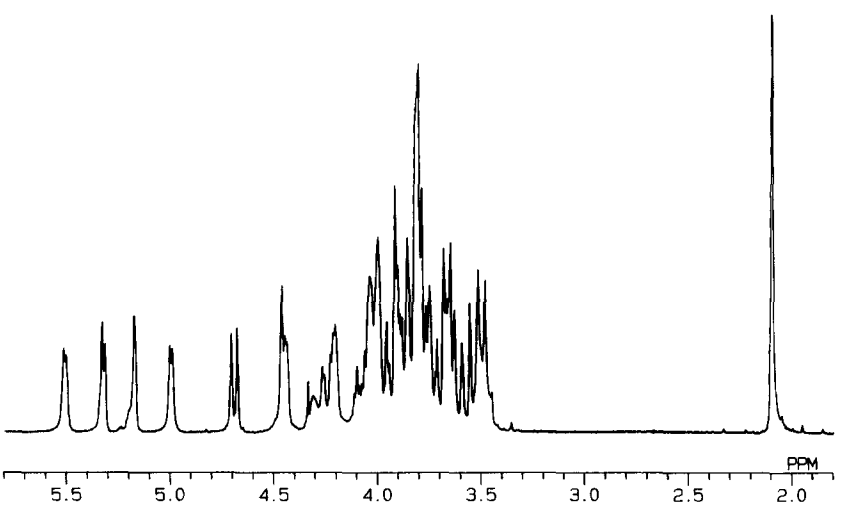


Fig. 1. The ^{1}H NMR spectrum at 270 MHz of the *O*-deacetylated *Serratia marcescens* serogroup O1 *O*-polysaccharide.

Table 2
Chemical shifts (ppm) of selected signals in the ^{1}H and ^{13}C NMR spectra [a] of *O*-deacetylated SmO1 *O*-polysaccharide

Sugar residue	H/C				
	1	2	3	4	5
→ 3)-α-D-Gal*p*A-(1 →	5.50	4.02	4.24	4.45	4.47
A 4 ↑	100.8	79.2	68.9	80.5	72.0
	(3.2) [174]				
→)-α-D-Glc*p*-(1 →	5.31	3.66	3.85	3.58	4.02
B	100.1	71.4	81.5	70.5	72.7 [b]
	(3.8) [173]				
→ 3)-α-D-Man*p*-(1 →	5.17	4.20	4.02	3.89 [b]	4.02 [b]
C	102.0	70.8	80.5 [b]	66.7	73.8
	(n.r.) [174]				
α-D-Glc*p*NAc-(1 →	4.99	3.93	3.74	3.52	4.08
D	99.7	54.5	72.0	70.8	73.3
	(3.0) [173]				
→ 3)-β-D-Glc*p*-(1 →	4.69	3.48	~ 3.66	~ 3.71 [b]	3.50 [b]
E	104.8	73.1	83.7	70.5	76.6
	(8.1) [163]				

[a] $J_{H\text{-}1,H\text{-}2}$ values are given in parentheses and $J_{C\text{-}1,H\text{-}1}$ in square brackets; n.r. = not resolved.
[b] Tentative assignment.

NMR spectrum of *O*-deacetylated SmO1 (Fig. 2) showed signals for five anomeric carbons (Table 2), and in addition to the data in Table 2 signals were observed at δ 23.1, 61.2, 61.4 (2 C), 61.8, 174.9, and 175.4. From these data it is concluded that SmO1 contains pentasaccharide repeating units carrying one equivalent of *O*-acetyl groups per repeating unit. From the results of methylation analysis there are two 3-substituted glucose residues in the repeating unit. In order to determine the anomeric

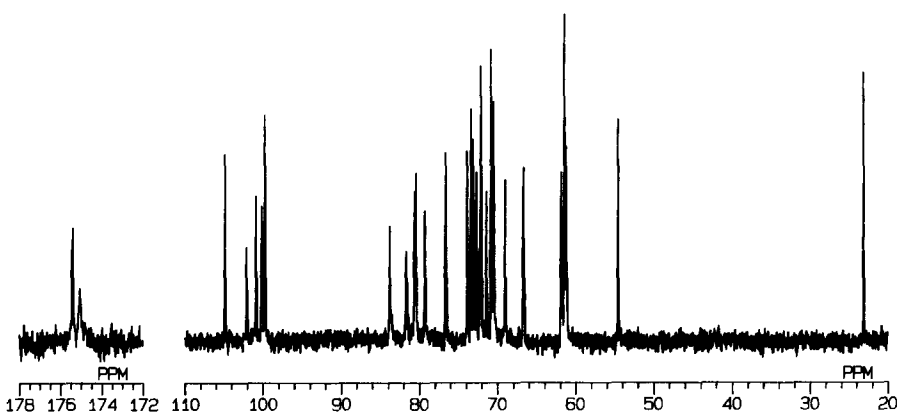
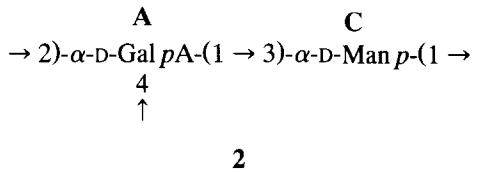


Fig. 2. The ^{13}C NMR spectrum at 67 MHz of the *O*-deacetylated *Serratia marcescens* serogroup O1 *O*-polysaccharide.

configuration of the sugar residues, the ^{1}H and ^{13}C resonances of H-1 to H-5 and C-1 to C-5 of the *O*-deacetylated SmO1 were assigned via ^{1}H, ^{1}H- and ^{1}H, ^{13}C-correlated NMR spectra (Table 2) and together with data from methylation analysis it is evident that all the residues are pyranoid. Residue **A** was assigned to the 2,4-disubstituted α-galacturonic acid residue since the chemical shifts of H-2 to H-5 of the spin-system are all > 4.0 ppm and the $J_{H\text{-}1,H\text{-}2}$ value is 3.2 Hz. The high values are only to be expected from α-galacturonic acid since the corresponding chemical shifts for the monomer are either near to or higher than 4 ppm. Residue **D** was assigned to the terminal 2-acetamido-2-deoxyglucose group from the chemical shift of its C-2 signal, δ 54.5, and, from the $J_{H\text{-}1,H\text{-}2}$ value, is α-linked. Residue **C** has a low $J_{H\text{-}1,H\text{-}2}$ value and is the 3-substituted mannose residue and is α-linked since the $J_{C\text{-}1,H\text{-}1}$ value is 174 Hz. The remaining two residues, **B** and **E**, thus correspond to an α- and a β-linked 3-substituted glucose residue, respectively, as evident from their $J_{H\text{-}1,H\text{-}2}$ values. Further assignments were corroborated from intra-residue correlations via $^{2}J_{C,H}$ and $^{3}J_{C,H}$ couplings from an HMBC experiment [7]. These were, inter alia, H-4 in **A** to its C-2 and C-3, H-1 in **B** to its C-5, and H-1 in **C** to its C-3 and C-5.

The sequence of sugars in the repeating unit was determined by using a uronic acid degradation and by 2D NMR spectroscopy where, for the latter, through-space and through-bond connectivities were determined. Subjecting SmO1 to a uronic acid degradation [8], i.e., treatment of the methylated polysaccharide with base followed by the addition of trideuteriomethyl iodide, led to the degradation of the galacturonic acid residue and labelling of the position to which it was linked with a trideuteriomethyl group. Furthermore, the sugar substituting the galacturonic acid residue in position 4 should be eliminated by treatment with base and subsequently degraded. Hydrolysis of the degraded material with trifluoroacetic acid and analysis with GLC-MS of the alditol acetates showed that the galacturonic acid residue had been linked to the 3-position of the α-D-mannose residue although the degradation was not complete (Table 1, column C). The following partial structure **2** can thus be deduced.



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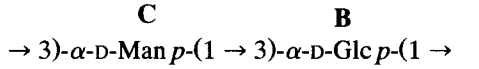
It was found that the 2-acetamido-2-deoxy-α-D-glucose group was completely degraded.

The remaining sequence was obtained from NOESY and HMBC experiments performed on *O*-deacetylated SmO1. The anomeric proton, δ 5.17, in **C** showed, inter alia, an NOE connectivity to H-3, δ 3.85, in **B** thereby establishing structural element **3**, which was further corroborated by long-range correlations in an HMBC experiment where the anomeric carbon, δ 102.0, in **C** showed a correlation to H-3, δ 3.85, in **B** and H-1, δ 5.17, in **C** showed a correlation to C-3, δ 81.5, in **B**.



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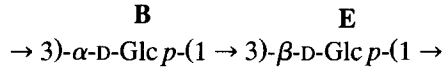
The α-linked glucose residue **B** substitutes the β-linked glucose residue **E** at the 3-position since in the HMBC experiment H-1, δ 5.31, in **B** showed a long-range correlation to C-3, δ 83.7, in **E**, thus defining structural element **4**.



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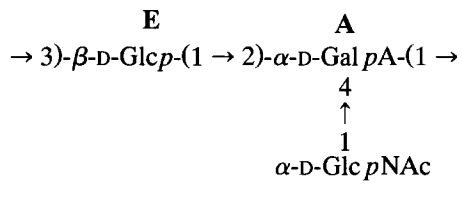
The galacturonic acid residue **A** is substituted in the 2-position by the β-linked residue **E** since a long-range correlation was observed in the HMBC spectra between H-1, δ 4.69, in **E** to C-2, δ 79.2, in **A**. In the NOESY spectrum a cross-peak was observed between H-1, δ 4.69, in **E** and H-2, δ 4.02, in **A**. Consequently, residue **D** substitutes position 4 in **A** and in accord with this a weak correlation was observed between H-1, δ 4.99, in **D** to a carbon signal at δ 80.5, the chemical shift of C-4 in **A**. These data define structural element **5** which is in agreement with the complete loss of the 2-acetamido-2-deoxy-α-D-glucose group in the uronic acid degradation discussed above.



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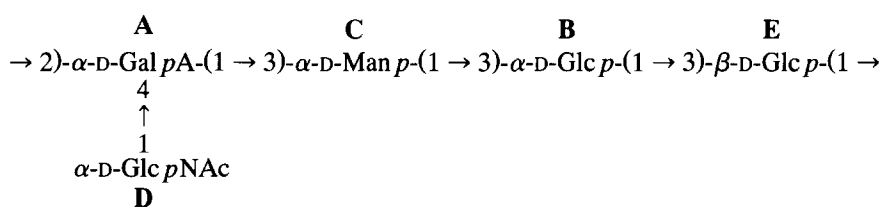
The above data define the structure for the repeating unit of *O*-deacetylated SmO1 as **6**. The polysaccharide also contains one mole of *O*-acetyl groups per repeating unit distributed over at least two positions, one of these being a hydroxymethyl group.



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Consistent with these results, only GlcNAc was destroyed during periodate oxidation of the *O*-deacetylated native glycan. As expected, Smith degradation gave a polymer with a tetrasaccharide repeating unit. The ^{1}H NMR spectrum (80°C) of the product contained anomeric signals with δ 5.45 ($J_{H-1,H-2}$ 3.3 Hz), 5.30 ($J_{H-1,H-2}$ 3.7 Hz), 5.16 (unresolved), and 4.67 ($J_{H-1,H-2}$ 7.8 Hz). The ^{13}C NMR spectrum (50°C) contained 22 discrete signals (two corresponding to 2 C each), including anomeric signals at δ 104.0, 101.3, 100.4, and 99.4, a signal for the carbonyl carbon of GalA with δ 174.4, and three signals for unsubstituted hydroxymethyl carbons with δ 61.0, 60.6, and 60.3.

The acidic glycan characterised in this study of the reference strain defines *Serratia marcescens* serogroup O1, but it is probably not the LPS side chain [1,4]. In the necessary [9] fundamental reappraisal of the serotyping scheme, the acidic glycan may be useful to define a different (K-type) serogroup.

3. Experimental

General methods.—Evaporations were performed under diminished pressure at $< 40°C$ or under a stream of air or N_2. For GLC, a Hewlett-Packard 5890 instrument fitted with a flame-ionisation detector was used. GLC-MS (EI) was performed on a Hewlett-Packard 5970 MSD instrument or a Finnigan 1020 B instrument. Alditol acetates and partially methylated alditol acetates were analysed on an HP-5 capillary column (25 m × 0.20 mm × 0.33 μm) using the temperature program 180°C (1 min) → 250°C at 3°/min. Paper chromatography (PC) and high-voltage electrophoresis (HVE) were carried out on Whatman No. 1 paper with solvent A, 5:5:3:1 EtOAc–pyridine–water–AcOH for PC and buffers B, 5:2:43 pyridine–AcOH–water (pH 5.3) or C, 1:10:89 pyridine–AcOH–water (adjusted to pH 2.7 with formic acid) [10] for HVE. Detection reagents used were alkaline $AgNO_3$, aniline hydrogenoxalate, and ninhydrin.

NMR spectroscopy.—NMR spectra of solutions, with the polysaccharide as its sodium salt, in D_2O were recorded at 70°C using either a JEOL GSX-270 or Alpha-400 instrument unless otherwise stated. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropanoate-d_4 (TSP, δ_H 0.00) and acetone (δ_C 31.00) as internal references. ^{1}H, ^{1}H COSY, ^{1}H, ^{1}H-relayed COSY, NOESY, and ^{1}H, ^{13}C-COSY were performed using JEOL standard pulse-sequences. Relayed COSY spectra were obtained using a delay time of 30 or 60 ms. The $^1J_{C\text{-}1,H\text{-}1}$ values were determined by a HMQC inverse-detected experiment, and the ^{1}H, ^{13}C long-range couplings were investigated with a HMBC inverse-detected experiment using a delay time of 60 ms.

Sugar and methylation analysis.—Methylation was carried out essentially according to methods described earlier [11,12]. Carefully dried methylated polysaccharide (1 mg) was dissolved in dry THF (1 mL) and treated with $LiBH_4$ at 80°C for 2 h to obtain the methylated carboxyl-reduced polysaccharide. The native and methylated polysaccharide were each treated with 0.5 M trifluoroacetic acid at 100°C overnight. The sugars in the hydrolysates were converted into the alditol acetates and partially methylated alditol acetates. Additionally, the neutral sugars were identified by PC and enzymatic assays [13], and by GLC of the alditol acetates. 2-Amino-2-deoxyglucose was identified by PC, HVE (buffer B), and autoanalysis (Locarte). Galacturonic acid was identified by HVE (buffer C) and by GLC (analysis of the carboxyl-reduced glycan); quantitative data were obtained by colorimetric assays [14]. The absolute configuration of the sugars, in a hydrolysate obtained by treatment with 2 M CF_3CO_2H at 120°C for 2 h, were determined essentially as devised by Leontein et al. [6] but using (+)-2-butanol instead of (+)-2-octanol.

Degradative methods.—For *O*-deacetylation, the polymer was treated with 0.1 M NaOH at room temperature for 17 h, followed by passage of the solution down a column of Dowex 50 resin (H^+ form). After rotary evaporation, the product was converted into

the ammonium salt by ion exchange. For periodate oxidation and Smith degradation, the *O*-deacetylated, reduced ($NaBH_4$) glycan was treated with 50 mM $NaIO_4$ at 4°C for 4 days. After consecutive additions of ethylene glycol, $NaBH_4$, and 2 M AcOH, the solution was freeze-dried and the residue fractionated on Sephadex G-50. The polymeric material was treated with 2 M CF_3CO_2H at room temperature for 16 h, the acid was removed by rotary evaporation, and the products were again fractionated on Sephadex G-50.

Uronic acid degradation.—Carefully dried methylated polysaccharide (1 mg) was dissolved in Me_2SO (0.5 mL) and treated with a trace of *p*-toluenesulfonic acid and 2,2-dimethoxypropane. Sodium methylsulfinylmethanide (2 M, 0.25 mL) was then added at room temperature and the solution left overnight. After cooling, CD_3I (0.25 mL) was added and the material was recovered and treated as described for the methylation analysis.

Growth of bacteria and isolation of the acidic glycan.—Two (20 L) batches of cells of the O1 reference strain (C.D.C. 866-57), grown as described [4] (yields of wet cells: batch 1, 140 g; batch 2, 130 g), were used for the preparation of cell walls (yields of dry products: batch 1, 4.4 g; batch 2, 2.9 g). 'LPS' was isolated from the aqueous phase after extraction of the defatted cell walls with hot, aqueous phenol, after exhaustive dialysis and freeze-drying (yields: batch 1, 1.01 g; batch 2, 0.95 g). The water-soluble products obtained after mild hydrolysis of the 'LPS' (aq 1% AcOH, 100°C, 2.25 h) were fractionated first by chromatography on Sephadex G-50. As well as oligomeric and monomeric fractions, two overlapping peaks for polymeric materials were detected (yields of total polymeric materials from 'LPS': batch 1, 50%; batch 2, 37%). The neutral and acidic glycans present in the polymeric fractions were separated [4] by chromatography on DEAE-Sepharose CL-6B (yields of acidic glycan: batch 1, ~ 60%; batch 2, ~ 53%). Both batches of acidic glycan had the same ^1H NMR spectra.

Acknowledgments

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References

[1] S.G. Wilkinson, in A. Nowotny, J.J. Spitzer, and E.J. Ziegler (Eds.), *Cellular and Molecular Aspects of Endotoxin Reactions, Endotoxin Research Series*, Vol. 1, Elsevier, Amsterdam, 1990, pp 95–102.
[2] M.A. Gaston, P.S. Duff, and T.L. Pitt, *Curr. Microbiol.*, 17 (1988) 27–31.
[3] C.J. Brigden and S.G. Wilkinson, *Carbohydr. Res.*, 138 (1985) 267–276.

[4] S. Furn and S.G. Wilkinson, *Carbohydr. Res.*, 139 (1985) 293–297.
[5] D. Oxley and S.G. Wilkinson, *Carbohydr. Res.*, 198 (1990) 168–172.
[6] K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359–362.
[7] A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, 108 (1986) 2093–2094.
[8] G.O. Aspinall and K.-G. Rosell, *Carbohydr. Res.*, 57 (1977) c23–c26.
[9] M.A. Gaston and T.L. Pitt, *J. Clin. Microbiol.*, 27 (1989) 2702–2705.
[10] M. Kosakai and Z. Yosizawa, *Anal. Biochem.*, 69 (1975) 415–419.
[11] P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lönngren, *Chem. Commun., Univ. Stockholm*, 8 (1976) 1–75.
[12] T.J. Waeghe, A.G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
[13] C.J. Brigden and S.G. Wilkinson, *Carbohydr. Res.*, 138 (1985) 267–276.
[14] N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.