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Structure of an extracellular polysaccharide produced by *Erwinia chrysanthemi*

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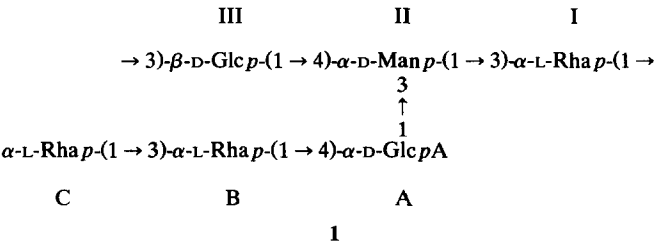
ABSTRACT

Erwinia chrysanthemi pv zeae strain SR260, a phytopathogen of corn, produced from lactose an acidic extracellular polysaccharide which was purified and found to consist of L-rhamnose, D-mannose, D-glucose, and D-glucuronic acid in the ratio of 3:1:1:1. A combination of chemical (carboxyl-group reduction, methylation analysis, periodate oxidation, Smith degradation, and lithium–ethylenediamine degradation) and physical (1 and 2D NMR spectroscopy) methods revealed that the polysaccharide is composed of a hexasaccharide repeating unit **1**:



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INTRODUCTION

Erwinia chrysanthemi is a Gram-negative bacterial phytopathogen that causes soft-rot in a number of tropical and sub-tropical plants[1–8]. *E. chrysanthemi* produces copious amounts of an extracellular polysaccharide (EPS) when grown on lactose. At present, little is known about the polysaccharides produced by phytopathogens. Neither the factors that distinguish the surface carbohydrates of

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virulent and avirulent cells nor their involvement in pathogenicity are known. The structure of the extracellular polysaccharide (EPS, **1**) produced by *E. chrysanthemi pv. zeae* strain SR260, a strain pathogenic to corn, is reported here.

EXPERIMENTAL

Production of polysaccharide.—*E. chrysanthemi pv zeae* strain SR260 was grown on a modified Scott's medium[9] solidified with 1.5% Difco agar (Difco Laboratories, Detroit, MI). Before use, the yeast extract was ultrafiltered (Millipore PSAC 1000, 1000 molecular weight cutoff, Millipore Corporation, Bedford, MA) to remove high-molecular-weight polysaccharides, and the ultrafiltrate was used to make up the medium.

The surface of the medium, which was allowed to dry for 48 h at room temperature, was densely streaked in a cross-pattern with bacterial cells and the plates were incubated first for 24 h at 30°C, and for a further 4 days at room temperature (20–22°C). The dense, mucoid growth was carefully scraped from the plates with a bent glass "hockey stick", diluted with water to decrease the viscosity, and centrifuged (10 000*g*, 90 min, 4°C) to remove the bacterial cells. The supernatant was decanted and the bacterial cells were suspended in water and pelleted again by centrifugation. The supernatants were combined and the crude polysaccharide was recovered by lyophilization. The yield of crude polysaccharide was ~ 0.67 g per L of medium.

Purification of polysaccharide.—Crude polysaccharide in water, 5 mg mL^{-1}, pH 8.5, was precipitated by the slow addition of 3 vol of 95% (w/v) EtOH. The EtOH precipitation step was repeated twice more.

Residual water was removed from the polysaccharide by solvent exchange, first with abs EtOH, and finally with anhyd Et_2O. Recovery of the EPS ranged from 36 to 50% of the crude polysaccharide, depending on the batch.

The polysaccharide was converted to the free acid either by electrodialysis or by passage over a cation-exchange resin (Bio-Rad AG50-X8, H^+) which had previously been extensively washed with deionized water.

Analytical methods.—Descending paper chromatography was carried out on Whatman No. 1 chromatography paper using 12:5:4 EtOAc—pyridine–H_2O as solvent. Thin-layer chromatography was carried out on 0.1-mm cellulose-coated plates (Merck, Darmstadt, Germany) using 15:30:40:15 HCO_2H–butanone–*tert*-butanol–H_2O as solvent. Standards containing D-glucose, D-mannose, D-glucuronic acid, and L-rhamnose were chromatographed together with hydrolyzates of the EPS on these media. Sugars were visualized with the alkaline silver reagent[10]. Total carbohydrate was determined by the phenol–H_2SO_4 procedure[11]. Uronic acid was measured either by a modified carbazole reaction[12] or by the biphenyl procedure[13]. Kdo was determined as described by Karkhanis et al.[14]. Protein assays were carried out by the Coomassie Blue method as described by Bradford[15] using a commercial kit (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Richmond,

CA) and by absorbance at 280 nm. Solutions of the EPS were also scanned in the UV to detect nucleic acids. A Beckman (Palo Alto, CA) model 121MB amino acid analyzer using a standard protein hydrolyzate protocol was used to analyze for amino sugars; galactosamine, glucosamine, and mannosamine were used as standards.

All GLC analyses were performed on either a Hewlett–Packard 5890 (Hewlett–Packard, Avondale, PA) or a Varian 3700 (Walnut Creek, CA) gas chromatograph equipped with a FID detector. Helium was used as a carrier gas (20 cm s^{-1}) in all the analyses. GLC–MS was performed on a Hewlett–Packard 5890 GC coupled to a Hewlett–Packard 5970 mass selective detector (MSD).

Monosaccharides were analyzed, as their alditol acetates, by GLC after hydrolysis (2 M CF_3CO_2H, 121°C, 1 h) and derivatization, essentially as described by York et al.[16]. The alditol acetates were analyzed isothermally at 220°C on a J&W DB 225 fused silica capillary column (30 m × 0.25 mm, J&W Scientific, Folsom, CA).

Methylation was performed as described by York et al.[16] and the resulting per-*O*-methylated alditol acetates were analyzed as already described for the alditol acetates except that a temperature program (180°C for 3 min increased to 220°C at 2°C min^{-1}, and held at 220°C for 37 min) was used. GLC–MS was used to confirm the identity of the partially methylated alditol acetates using conditions similar to those just described. Standards prepared by the methylation of glycogen and dextran (NRRL β-1355 Fraction S) were used to confirm the identities of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, respectively.

Quantitative monosaccharide analysis was also performed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) on a Dionex BioLC (Sunnyvale, CA) fitted with a Carbopak PA1 anion-exchange column. The eluents used were as follows: *A* = 100 mM NaOH; *B* = 100 mM NaOH–1000 mM NaOAc; *C* = deionized water. The following elution program was employed to detect both the mono- and oligo-saccharides (up to a glucan 40-mer): T_0, 20% *A*–80% *C* (20 mM NaOH); T_{20}, 20% A–80% *C*; T_{25}, 100% *A* (100 mM NaOH); T_{55}, 50% *A*–50% *B* (100 mM NaOH–500 mM NaOAc); T_{65}, 50% A–50% *B*. This gradient program allowed the separation of a variety of mono- and oligo-saccharides in a single 65-min run.

The absolute configuration of the monosaccharides was determined by gas chromatography of the trimethylsilyl (Me_3Si) derivatives of their *R*-(−)-butan-2-ol glycosides as described by Gerwig et al.[17]. After butanolysis, the mixture was treated as described by Chaplin[18] and not neutralized with $AgNO_3$ as described by Gerwig et al.[17]. The Me_3Si derivatives of the *R*-(−)-butan-2-ol glycosides were prepared according to Sweeley et al.[19] and separated by gas chromatography on a HP OV-101 (Hewlett–Packard) fused silica column (25 m × 0.25 mm) using the following temperature program; 180°C for 3 min increased to 220°C at 2°C min^{-1} and held at this temperature for 20 min. Standard Me_3Si derivatives were prepared by butanolysis of pure D sugars (except for L-rhamnose) with *R*-(−)-butan-2-

ol or *RS*-(±)-butan-2-ol to obtain the retention times corresponding to the L sugars (or D-rhamnose).

Ultracentrifugal analysis of the EPS was performed on a Beckman Model E analytical centrifuge. The EPS (1 mg mL^{-1} in phosphate buffered saline, pH 7.3) was analyzed by ultracentrifugation at 54 800 rpm in an AN-H head on a Beckman Model E Analytical Ultracentrifuge fitted with Schlieren optics.

Reduction of glycosyluronic residues.—Reduction of the D-glycosyluronic residues in the EPS was carried out by reaction with a water-soluble carbodiimide [1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, EDC] as described by Taylor and Conrad[20]. The extent of reduction, which was repeated if necessary, was examined by uronic acid analysis or by methanolysis and analysis of the Me_3Si ethers as described above.

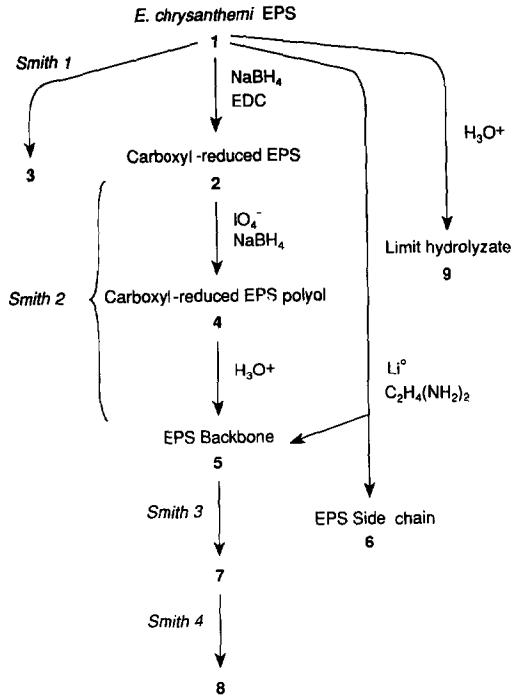
Low-pressure gel-permeation and anion-exchange chromatography.—The following gel-permeation chromatography columns and elution conditions were used in this study: Bio-Gel P-2 (−400 mesh, 1.6 × 80 cm) eluted with water at a flow rate of 18 mL h^{-1}; Bio-Gel A-1.5m (200–400 mesh, 1.6 × 80 cm) eluted with water at a flow rate of 20 mL h^{-1}; Sepharose CL6 (200–400 mesh, 1.5 × 90 cm) eluted with water at a flow rate of 18 mL h^{-1}; Spectra/Gel™ (200–400 mesh, 1.5 × 88 cm) eluted with water at a flow rate of 20 mL h^{-1}.

All columns were operated at room temperature and were calibrated with a series of maltodextrins (maltose to maltoheptose obtained from Boehringer Mannheim) or a series of dextrans obtained from Sigma (St. Louis, MO).

Anion-exchange chromatography of the EPS was performed on a column (2.5 × 33 cm) of DEAE-cellulose, which was equilibrated in 10 mM KCl. The column was eluted initially with two bed-volumes of 10 mM KCl followed by a linear gradient of 10–500 mM KCl (500 ml). Column fractions were analyzed for neutral carbohydrate and uronic acid.

Lithium–ethylenediamine degradation.—Degradation of the EPS by Li–ethylenediamine was performed by the method of Lau et al.[21] and the products fractionated by chromatography on Bio-Gel P-2. Fractions across the low molecular-mass peak were analyzed by HPAE-PAD chromatography and fractions containing a single component were pooled for NMR analysis.

Periodate oxidation and Smith degradation.—Initial Smith degradations of the native- (**1**, Scheme 1) and carboxyl-reduced EPS (**2**) were performed as described by Smith and Montgomery[22]. Briefly, the deionized EPS was oxidized for 200 h in 0.05 M $NaIO_4$ (calculated so that there was a five-fold excess of periodate) at 4°C in the dark. The excess periodate was decomposed by the addition of a five-fold excess of ethylene glycol and the solution was kept at room temperature for 30 min. Thereafter, the sample was extensively dialyzed against deionized water and freeze-dried. The polyaldehyde was taken up in water, and reduced with a five-fold excess of $NaBH_4$ for 16 h at room temperature. The excess $NaBH_4$ was decomposed by the addition of Bio-Rad AG50-X8 (H^+) ion-exchange resin during which time the pH was not allowed to fall below 4.5. The resin was filtered through a



Scheme 1. Chemical degradations of *E. chrysanthemi* extracellular polysaccharide (**1**).

glass wool-plugged funnel and the polyalcohol was freeze-dried. Borate was removed from the lyophilized material by repeated evaporation with MeOH at diminished pressure and temperature ($< 40°C$) and the residue taken up in 1 M CF_3CO_2H and maintained at room temperature for 24 h. After diluting the sample two-fold, the CF_3CO_2H was removed by lyophilization. The products were then fractionated by chromatography on Bio-Gel P-2 yielding a high-molecular-weight fragment, **3**.

Smith degradations on **1** and **2** were also performed in the presence of 0.5 M NaCl as described by Aalmo et al.[23], and in the presence of $NaClO_4$ as described by Dudman[24]. Briefly, deionized EPS (1 mg mL^{-1}) in 0.05 M $NaIO_4$–0.2 M $NaClO_4$ was oxidized in the dark for 96 h at 4°C. The sample was quenched by the addition of ethylene glycol (1 mL) followed 30 min later by $NaBH_4$ (0.5 g). Reduction was allowed to proceed overnight (16 h) after which the excess borohydride was destroyed by the addition of glacial AcOH. The pH of the solution was not allowed to fall below 4.5. After extensive dialysis of the solution against distilled water and lyophilization, the oxidation and reduction was repeated. The resulting polyalcohol (**4**), a portion of which (100 μg) was subjected to methylation analysis, was hydrolyzed by 1 M CF_3CO_2H for 24 h at room temperature and after two-fold dilution, the CF_3CO_2H was removed by lyophilization. The resulting products were purified by chromatography on Bio-Gel P-2 (−400 mesh). The

fraction eluting in the void volume (**5** in the case of the Smith degradation of **2**) of the Bio-Gel P-2 column was further analyzed by chromatography on Spectra/Gel™ HW-75F (Spectrum Industries, Inc., Los Angeles, CA) (200–400 mesh) and found to elute as a single peak. The high-molecular-mass polymer was subjected to a second Smith degradation, and the products purified by chromatography on Bio-Gel P-2. Fractions containing the low-molecular-mass fragments were examined by HPAE-PAD chromatography, and those exhibiting a single component (**7**) were pooled for NMR spectroscopic analysis.

The low-molecular-weight fragment (**7**) from the EPS backbone (derived by Smith degradation of either the carboxyl-reduced EPS or the Li–ethylenediamine degradation of the native EPS) was subjected to a third Smith degradation as just described, except that periodate oxidation was carried out in the absence of $NaClO_4$ for 24 h at room temperature. The excess $NaBH_4$ was decomposed by the addition of Bio-Rad AG50-X8 (H^+) and the dialysis step was omitted. After removal of the excess borate by repeated evaporation from 1% AcOH in MeOH (4 times) and MeOH (4 times), the fragments (**8**) were hydrolyzed in 2 M CF_3CO_2H (121°C, 1 h) and the monosaccharide composition determined by GLC of the alditol acetates.

Production of an aldobiouronic acid (**9**).—Electrodialyzed EPS (175 mg) was hydrolyzed (1 M CF_3CO_2H, 100°C, 6 h), and the CF_3CO_2H evaporated under diminished pressure. The residual syrup was chromatographed on a column (1 × 11 cm) of Bio-Rad AG1-X8 (acetate form) with a linear gradient of 0 to 3 M AcOH. The uronic acid containing peak was pooled as four fractions, and freeze dried. HPAE-PAD analysis of the pooled fractions showed that the first three fractions (**9**) were > 95% pure, and were used for subsequent NMR spectroscopy.

^1H NMR analysis.—Samples for NMR analysis (2–5 mg) were exchanged three times D_2O (Aldrich, Milwaukee, WI) by lyophilization, before being dissolved in D_2O (0.5 mL), containing a trace of acetone as internal standard whose resonance was set equal to 2.225 ppm. All spectra were recorded on a Bruker AMX 600 spectrometer. Standard programs, supplied by Bruker, were used to record the COSY-45 and NOESY-45 spectra.

RESULTS AND DISCUSSION

Production of EPS by E. chrysanthemi.—The Lac^+ strain of *E. chrysanthemi* used in this study produced an extracellular polysaccharide from glucose, galactose, lactose, fructose, and sucrose. The structure of the polysaccharide (**1**) produced on lactose is reported here.

Purity of the EPS.—The polysaccharide (**1**), after purification by ethanol precipitation, migrated as a single component when the EPS was either analyzed by analytical ultracentrifugation or chromatographed over DEAE-cellulose, Bio-Gel A 1.5, Sepharose CL6, or Spectra/Gel™ HW-75F. Qualitative analysis of the monosaccharide composition of fractions across the single peak of polysaccharide

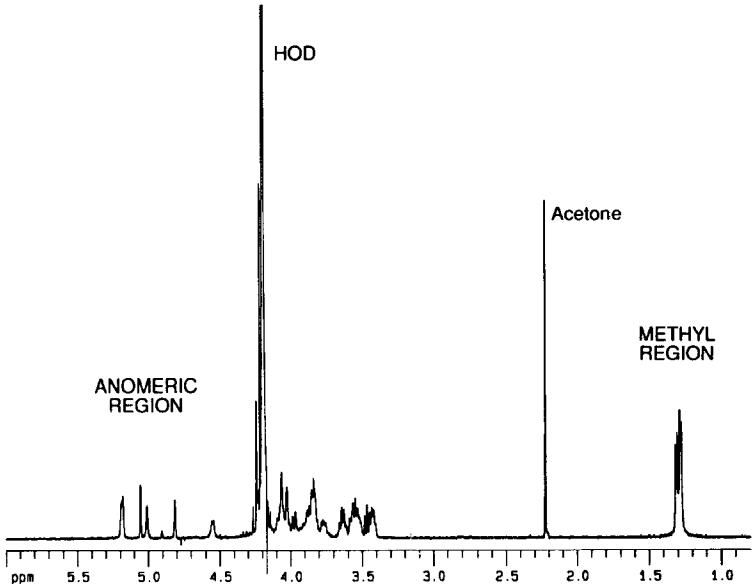


Fig. 1. ^{1}H NMR spectrum of EPS (1) obtained at 600 MHz in D_2O at 350 K.

that eluted from all of these columns, revealed a similar pattern, thus supporting the conclusion that the EPS was homogeneous.

Initial examination of **1** by high-field proton NMR (Fig. 1) revealed four well-resolved and two overlapped 1-proton resonances in the anomeric region and three 3-proton doublets in the methyl group region. Thus **1** could be considered tentatively a polymer of a repeating unit of six monosaccharide residues, probably three of which were 6-deoxyhexoses. The complex and extensively overlapped ring-proton region (3.4–4.3 ppm), however, precluded the extraction of any further structural information from the polysaccharide **1**, even using two-dimensional NMR analysis. The structure of **1** was then determined through a combination of physical and classical chemical methods (Scheme 1).

Composition.—Quantitative data could not be obtained initially because of the difficulty in completely hydrolyzing the glucosyluronic linkages of the EPS. After the GlcA residues of **1** had been reduced to form the carboxyl-reduced EPS (**2**), however, analysis was carried out by three different methods, which yielded quantitative data for all monosaccharide components (Table I). EPS did not contain GlcN, GalN, ManN, Fuc, Xyl, Ara, Fru, pyruvate, acetate, succinate and propanoate, and Kdo, or protein and nucleic acid. After the stereochemistry of the component monosaccharides had been established by GLC of the Me_3Si derivatives of their R-(−)-butan-2-ol glycosides, it was concluded that the composition of EPS (**1**) was 3 : 1 : 1 : 1 L-Rha : D-Man : D-Glc : D-GlcA.

Linkage analysis.—Methylation analysis of the native **1** (Table II) revealed the presence of terminal nonreducing and 1,3-linked rhamnose residues in a ratio of

TABLE I

Monosaccharide composition of carboxyl-reduced EPS (2)

Method	Derivative	Apparent molar ratio [a]				
		Rha	Man	Glc	(Glc + GlcA) [b]	GlcA
GC	Alditol acetates	3.00	0.97		1.95	
GC [c]	Partially methylated Alditol acetates	3.00	0.91	1.07		1.07 [d]
HPAE-PAD	None	3.00	1.02		2.29	

[a] Results relative to Rha = 3. [b] Glucose derived from both glucose and reduced GlcA residues. [c] Data obtained by summing the values for each partially methylated alditol acetate from the cleavage of the fully methylated EPS followed by reduction and acetylation (see Experimental section). [d] Value obtained for 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol, which arises from the carboxyl-reduced GlcA.

~ 1:2. The value for the 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol was lower than expected, probably because of the volatility of its methylated methyl glycoside obtained from the methanolysis of the methylated EPS. The amount of 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylmannitol recovered was lower than expected, and is probably due to the resistance of the uronic acid linkage to acid hydrolysis. The incompletely hydrolyzed per-*O*-methylated aldobiuronic acid is not detected by GLC of its alditol acetate derivative.

Methylation analysis of **2** (Table II) confirmed the presence of the partially methylated alditol acetates detected in the native EPS (**1**) and, in addition, revealed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, which arises from the reduction of glucuronic acid.

The molar ratios of the partially methylated alditol acetates suggests that **1** has a repeating unit of six monosaccharides in which only the terminal rhamnose residue and the glucuronic acid residue (or the glucose residue derived from the glucuronic acid) contain unmodified vicinal hydroxyl groups and are therefore susceptible to periodate oxidation.

TABLE II

Methylation analysis of *E. chrysanthemi* EPS and its derivatives

Me sugar [b]	EPS or derivative [a]					
	1 (Mol%)	**2**	**3**	**4**	**5**	**5** [c]
2,3,4 Me_3Rha	16.8	15.3	27.0	< 1.0	3.0	4.2
2,4 Me_2Rha	41.2	34.2	36.2	48.8	30.4	29.8
2,4,6 Me_3Glc	24.2	17.8	39.6	27.5	33.5	30.4
2,3,6 Me_3Glc		17.8		< 1.5		4.6
2,3,6-Me_3Man			12.7		30.7	26.4
2,6 Me_2Man	17.9	15.0	22.2	23.7	2.4	4.6

[a] See Scheme 1. [b] 2,3,4 Me_3Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol, etc. [c] Derived by Li–ethylenediamine degradation of **1**.

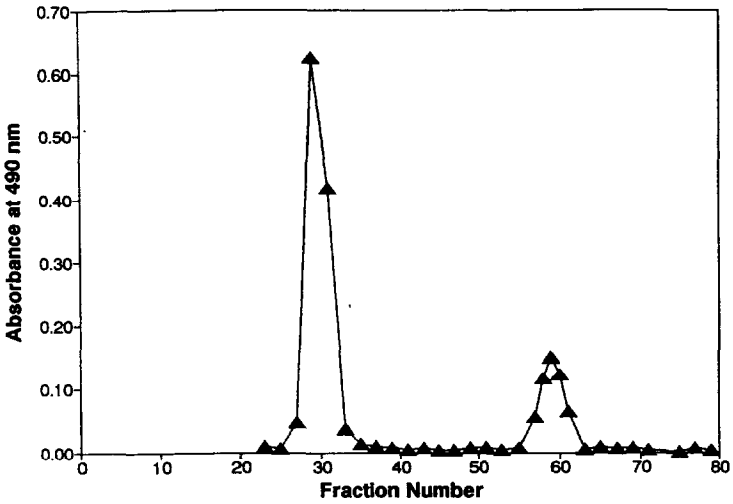


Fig. 2. Chromatography of the products from Smith degradation of the carboxyl-reduced EPS (**2**) on Bio-Gel P-2.

Sequence analysis.—In order to elucidate the sequence of the sugar residues, and to further characterize the branching pattern, Smith degradations were performed on **1** and its derivatives as summarized in Scheme 1. The native EPS (**1**) proved difficult to oxidize completely (Smith-1), even in the presence of sodium chloride or sodium perchlorate, which have been found to facilitate the oxidation of acidic polysaccharides[23,25]. Different polysaccharides are oxidized by periodate at varying rates[23] and this is particularly noticeable in the case of the acidic polysaccharides, where ionic repulsion[25] and the facile formation of inter- and intra-residue hemiacetal linkages[26,27] hinder the completeness of oxidation. Methylation analysis of the Smith-degraded native EPS (**3**), as shown in Table II, revealed that only ~ 30% of the side-chain had been removed.

Periodate oxidation of the carboxyl-reduced EP2 (**2**) and chromatography of the product on Bio-Gel P-2 revealed two components (Fig. 2), one of high (**4**) and one of low molecular weight. Methylation analysis of the high-molecular-weight polyalcohol, both before (**4**) and after (**5**) mild acid hydrolysis, demonstrated complete oxidation (Table II).

Side-chain sequences.—The observation that a high molecular polysaccharide (**5**) remained after complete Smith degradation indicated that the two periodate-susceptible residues of **2** must be in the side chain. This finding was confirmed when the results of the methylation analysis were considered. As observed in Table II, there is the loss of the terminal nonreducing Rha and the 4-linked Glc residues in the Smith degradation of **2** to **4**. In addition, there is the loss of a third residue (3-linked Rha) after the mild acid hydrolysis of **4** to form **5**. Since a 3-linked Rha residue cannot be oxidized by periodate, its loss indicates that it is present *between* the nonreducing terminal Rha and the 4-linked Glc residues of the side chain.

TABLE III

^{1}H NMR data [a] and residue assignments for oligosaccharides derived from **1** (see also Fig. 5).

Cmpd [b]	Residue		Chemical shifts (ppm) and coupling constants (Hz)					
	Symbol [c]	Assignment [d]	H-1($J_{1,2}$)	H-2($J_{2,3}$)	H-3($J_{3,4}$)	H-4($J_{4,5}$)	H-5($J_{5,6}$)	H-6($J_{6,6'}$)
5	I	α-L-Rha*p* [e]	5.175 (< 2.0)	4.277 (< 2.0)	3.885 (9.7)	3.535 (9.5)	4.058 (6.2)	1.269 [f] (< 0.1)
	II	α-D-Man*p* [e]	5.043 (< 2.0)	4.058 (3.0)	4.009 (9.4)	3.893 (9.6)	3.506 (4.9)	3.840 (−13.5)
							(2.4)	4.027
	III	β-D-Glc*p*	4.537 (8.3)	3.434 (8.8)	3.621 (8.9)	3.478 (9.3)	3.524 (5.5)	3.737 (−11.5)
							(< 2.0)	3.938
6	B′	L-Rhamnitol	3.642 (7.2)	3.892 (5.6)	3.946 (2.0)	3.606 (8.3)	3.736 (6.3)	1.273 [f] (< 0.1)
			3.823 (3.6)					
			($J_{1,1'} = -11.7$)					
	C	α-L-Rha*p* [e]	4.952 (1.6)	4.029 (3.3)	3.805 (9.6)	3.464 (9.6)	3.861 (6.3)	1.287 [f] (< 0.1)
7	I	α-L-Rha*p* [e]	5.140 (1.7)	4.051 (3.5)	3.788 (9.8)	3.457 (9.7)	4.016 (6.3)	1.252 [f] (< 0.1)
	II′	Erythritol	3.763 (5.3)	3.815 (5.6)	3.796 (6.5)	3.672		
			3.849 (2.9)		(3.5)	3.862		
			($J_{1,1'} = -12.0$)			($J_{4,4'} = -12.3$)		
	III	β-D-Glc*p*	4.567 (8.1)	3.430 (9.2)	3.606 (9.1)	3.472 (10.8)	3.470 (4.1)	3.724 (−12.2)
							(1.3)	3.909
9	II	α-D-Man*p* [g]	5.148 (1.9)	4.056 (3.2)	3.939 (9.0)	3.858 (11.0)	3.764 (6.1)	3.858 (< 0.1)
							(6.1)	3.858
		β-D-Man*p* [g]	4.906 (< 0.1)	4.070 (2.5)	3.775 (11.1)	3.764 (9.7)	3.420 (6.3)	3.737 (−12.3)
							(2.1)	3.903
	A	α-D-Glc*p*A	5.286 (3.9)	3.619 (9.7)	3.817 (9.6)	3.570 (9.8)	4.528	

[a] Obtained at 600 MHz in D_2O at 298 K. [b] See Scheme 1. [c] Symbols as used in Fig. 6. Residue sequence designators were chosen to be consistent throughout the entire series of derivatives. [d] Assigned according to procedures of Koerner et al.[28] using 600-MHz 2D COSY-45 spectra and integrated 1D spectra for each compound. [e] Anomeric form assigned from 2D NOESY data presented in Table IV. [f] Three-proton singlet. [g] Based on the integration of well-resolved resonances the equilibrium anomeric composition was 72% α anomer and 28% β anomer; no furanose forms were observed.

TABLE IV

Cross-peaks [a] observed for anomeric protons in the NOESY spectra of oligosaccharides derived from 1

Compound [b]	Anomeric signal (ppm)	Upfield signal (ppm)	Interpretation [c]
5	5.175	4.277	I-1 → I-2 (1,2-*eq* / *eq*)
	5.043	4.277	II-1 → I-2 (glycosidic)
	5.043	4.058	II-1 → II-2 (1,2-*eq* / *eq*)
6	4.952	4.029	C-1 → C-2 (1,2-*eq* / *eq*)
7	5.140	4.051	I-1 → I-2 (1,2 *eq* / *eq*)
		3.606	I-1 → III-3 (glycosidic)
	4.567	3.470	III-1 → III-5 (1,5-*ax* / *ax*)
9	5.148	4.056	II-1 → II-2 (1,2 *eq* / *eq*)
	5.286	3.619	A-1 → A-2 (1,2-*eq* / *ax*)

[a] Obtained at 600 MHz; mixing time 185 ms. [b] See Scheme 1. [c] Symbols as used in Fig. 6. Abbreviations used: *eq*, equatorial; *ax*, axial. Assigned according to the procedure of Koerner et al.[28]

Finally, the observation that mild acid hydrolysis of **4** results in the exposure of the 3 hydroxyl group of the mannose residue in **5** to methylation shows that the side chain is linked to the 3 position of the branch point residue and that the side chain is composed of no more than the three residues already noted.

Addressing the structure of the branch point, the NMR spectrum of aldobiouronic acid (**9**), composed of equimolar Glc*p*A and Man*p* (Table III), the small vicinal coupling constant ($J_{1,2}$ 3.9 Hz) manifest by the anomeric proton of the Glc*p*A residue (A), as well as its single 1,2 equatorial–axial and lack of 1,3 or 1,5 diaxial cross peaks (Table IV), indicated that this proton was equatorial and that the acid moiety was α-D-Glc*p*A. Although there are no *trans*-glycosidic cross-peaks detected in the 2D NOESY spectrum of **9**, the sequence of this disaccharide can be deduced from its 1D spectrum. The Man*p* residue is observed to be in an anomeric equilibrium (Table III), so that the Man*p* residue must be at the reducing end of the disaccharide and the sequence of **9** must be A → II (the residue symbolism is shown in Fig. 6).

The degradation of the native EPS (**1**) by Li–ethylenediamine, as described by Lau et al.[21], produced two fractions, which were separated by chromatography on Bio-Gel P-2 (Fig. 3, panel A). Monosaccharide and methylation analysis of the high-molecular-weight fragment gave data (Table II) identical to that obtained for the backbone (**5**) generated by the Smith degradation of the carboxyl-reduced EPS (**2**). Rechromatography of fractions from the overlapping region of the low-molecular-weight peak on Bio-Gel P-2 gave rise to two incompletely resolved peaks of these components (Fig. 3, panel B). The major component (**6**), which constituted more than 80% of the material, eluted in the leading edge of the peak and was composed of rhamnose and rhamnitol; the minor component (< 20%) eluted in the trailing edge of the peak was composed of only rhamnose. The production of a mixture of an aldose and an alditol was a similar finding to that obtained by Lau et

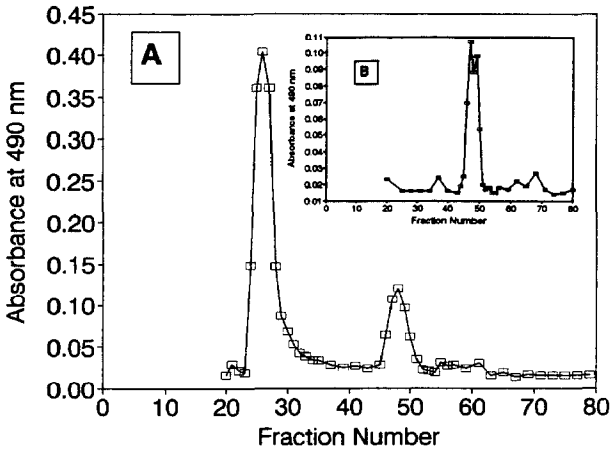


Fig. 3. (A) Chromatography of the products produced by degradation of the native EPS (**1**) by Li–ethylenediamine on Bio-Gel P-2. (B) Rechromatography of the trailing edge of the low-molecular peak in chromatogram A (fractions 49 to 51) on Bio-Gel P-2.

al.[21] in their studies on the application of the Li–ethylenediamine degradation of uronic acid-containing polysaccharides.

Examination of **6** by NMR (Table III) showed that the anomeric proton at the Rha*p* residue (C-1) manifests a 1,2-equatorial–equatorial, but no 1,3 or 1,5 diaxial cross-peaks, indicating its α-L-linkage. No *trans*-glycosidic cross-peaks were detected between the rhamnitol (B′) and Rha*p* (C) residues of **6**, possibly due to the conformational flexibility of the rhamnitol residue. However, it may be deduced from the 1D data (Table III) that the rhamnosyl residue (C) must be the terminal and nonreducing residue and the sequence of the last two residues of the side chain must be C → B. The anomeric form of the 3-linked Rha residue (B) in the side chain could not be assigned from the data available. However, in light of the findings that Rha residues I and C were α-L-linked and that L-Rha residues are usually found to be α-L-linked in bacterial polysaccharides[30], it is reasonable to assume that residues B is α-L-linked. Thus it can be concluded that the structure of the side chain of the carboxyl-reduced **2** is:

α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 4)-α-D-Glc*p*-(1 → 3)-R

and that of the parent structure **1** is:

α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 4)-α-D-Glc*p*A-(1 → 3)-R,

where R is the Man in the backbone of the polysaccharide.

Backbone sequence.—Monosaccharide analysis of the high-molecular-weight fragment (**5**), obtained either by Smith degradation (No. 2) or by reductive cleavage, revealed that it was composed of glucose, rhamnose, and mannose in a 1 : 1 : 1 ratio. From the methylation analysis of **5** (Table II, column 5) the backbone polysaccharide **5** was composed of three residues: 3-linked Rha, 3-linked Glc, and 4-linked Man. For such a composition there are theoretically six possible trisaccha-

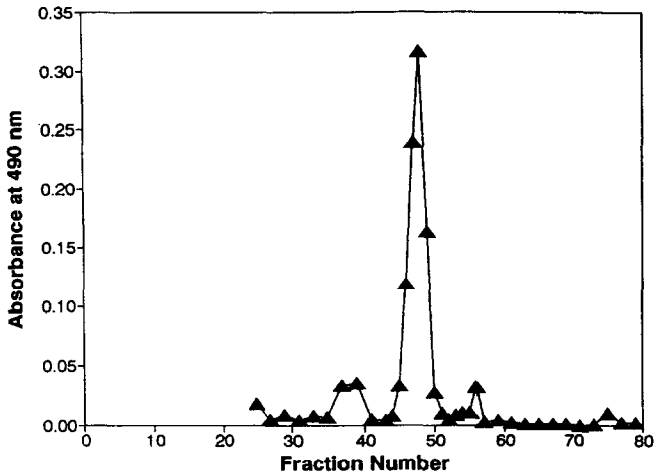


Fig. 4. Chromatography of the products from Smith degradation of the EPS backbone (**5**) on Bio-Gel P-2.

ride sequences for the structure of the backbone; however, the degeneracy resulting from polymer formation allows for only two unique sequences for the repeating unit of **5**. These two possible sequences are:

5a: → 3)-L-Rha*p*-(1 → 4)-D-Man*p*-(1 → 3)-D-Glc*p*-(1 →

and

5b: → 3)-D-Glc*p*-(1 → 4)-D-Man*p*-(1 → 3)-D-Rha*p*-(1 → .

The resolution of these two structures was achieved by carrying out two more Smith degradations (No. 3 and 4, Scheme 1), first on **5** to form **7** (Fig. 4), then on **7** to form **8**. Since only Glc was detected in **8** the two possibilities are reduced to **5b**.

Analysis of the proton NMR spectra of **5** confirmed its composition of equimolar Glc*p*, Man*p*, and Rha*p*. Assignment of a β-D-anomeric form to the Glc*p* residue follows from the large vicinal coupling of its H-1 proton ($J_{1,2}$ 8.3 Hz). However, the small vicinal coupling of the H-1 resonances of Man*p* and Rha*p* residues, a characteristic of the *manno* configuration of these residues, shed no light on their anomeric configuration, which was however resolved in the 2D NOESY spectra of **5** (Table IV)[28]. In this spectrum, the H-1 resonance of β-D-Man*p* and β-L-Rha*p* residues would have been expected to manifest intense cross-peaks due to strong 1,3 and 1,5 diaxial interactions, as well as strong 1,2 axial–equatorial interaction; whereas, the H-1 resonances of the α-linked anomers of these residues would have been expected to manifest a cross peak due to only a strong 1,2 equatorial–equatorial interaction. In fact, only 1,2 cross-peaks are observed for the H-1 resonances of these residues. Thus, the D-Man*p* and L-Rha*p* residues of **5** must both be α-linked. Also observed in the NOESY spectra of **5** was a strong cross-peak arising from a *trans*-glycosidic interaction between the Man H-1 (II-1) and Rha H-2 (I-2). This finding was useful in confirming that the

sequence of these two residues is II → I but was apparently contradictory with the methylation data (Table II, **5**), since the cross-peak indicated that the glycosidic linkage site on the backbone was Rha O-2; the methylation data indicated that the linkage site on the residue was O-3. The resolution of this dilemma is the realization that NOESY cross-peaks can occasionally occur preferentially to adjacent protons rather than linkage-site protons[29]. In the case of the backbone Rha

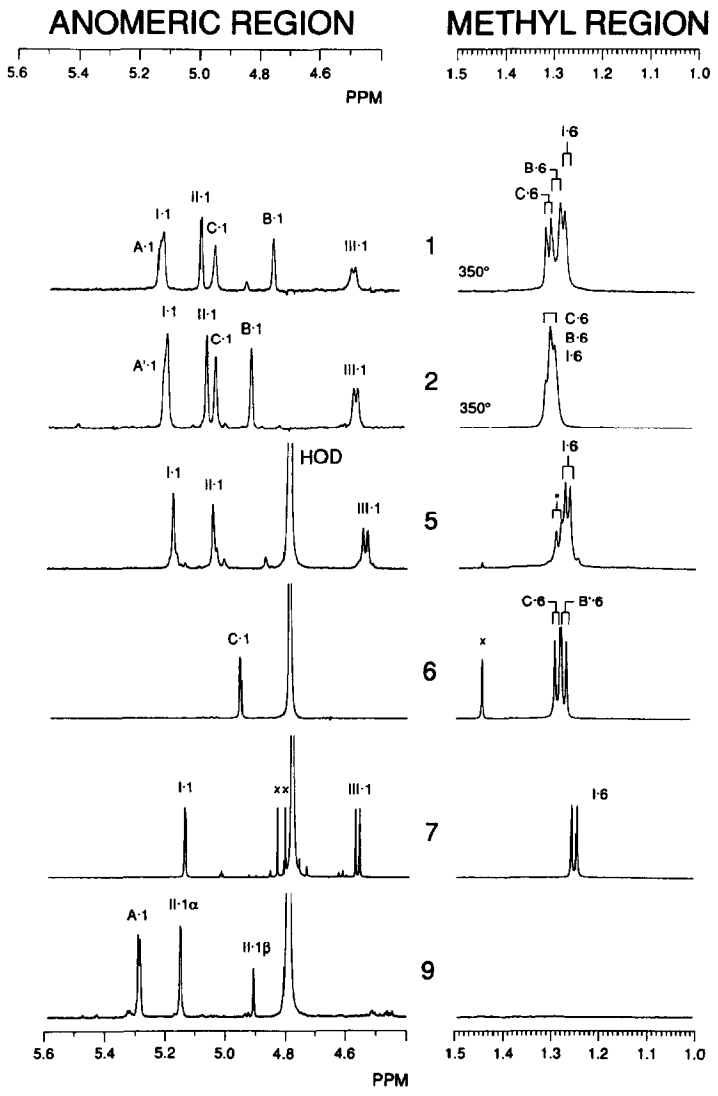


Fig. 5. The anomeric and methyl regions observed in the ^1H NMR spectra of EPS (**1**) and its derivatives (**5–7** and **9**), obtained at 600 MHz in D_2O at 298 K except where noted. Resonance assignments are as defined in Fig. 6. Other symbols are: X, nonsaccharide resonances and *, resonances from trace amounts of side chain rhamnose residues remaining attached to **5**.

(I) residue, this is certainly possible, since both H-2 and H-3 of this residue are on the same side of the pyranose ring.

Analysis of the NMR spectra of **7** presented another opportunity to study the polysaccharide backbone as well as to observe directly the effects of Smith degradation on **5**. As may be seen in Table III, compound **7** was composed of equimolar Rha*p*, Glc*p*, and erythritol. The survival of these two residues confirms the decomposition of the Man*p* residue (II) by the Smith degradation (No. 2). The observation of equimolar erythritol confirms the periodate cleaved between the II-2 and II-3 carbons. As observed for **5**, the large vicinal coupling displayed by the H-1 proton of D-Glc*p* ($J_{1,2}$ 8.1 Hz) indicates that this residue (III) is β-linked. Inspection of the NOESY spectrum of **7** reveals an intense cross peak for H-1 of Rha*p* due to a 1,2 equatorial–equatorial interaction, but no evidence of 1,3- or 1,5-diaxial interaction, which confirms the α-linkage at this residue (I). Also noted

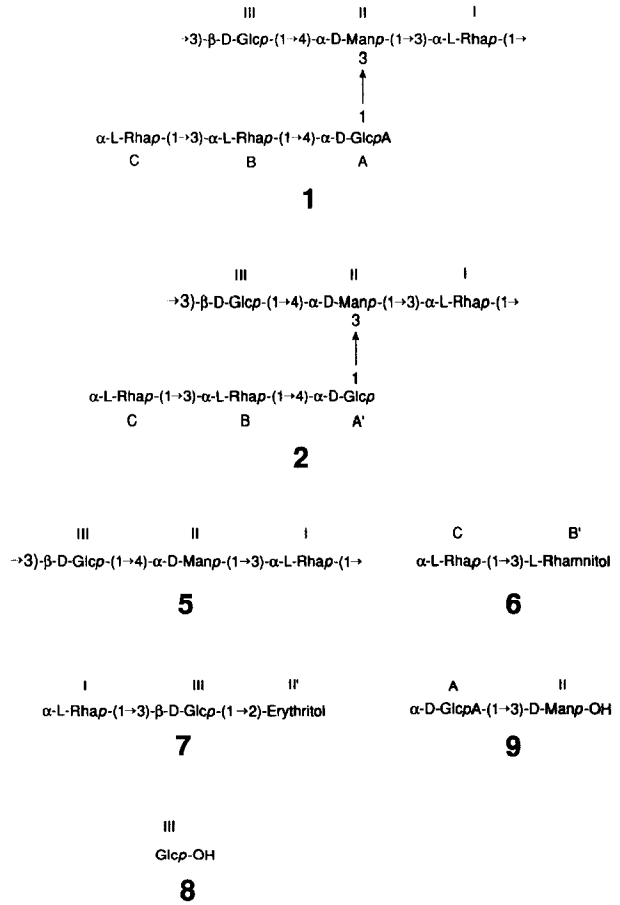


Fig. 6. The structure of EPS (**1**) and its derivatives.

is a cross-peak due to a 1,5 diaxial interaction for the D-Glc*p* residue (III), thus confirming again its β-linkage. Lastly and most importantly, an intense cross-peak is seen between H-1 of Rha*p* and H-3 of Glc*p* due to a *trans*-glycosidic interaction (I-1 → III-3). This observation confirmed both the I → III sequence required by the repeating trisaccharide backbone and the O-3 linkage site on the Glc*p* residue.

Finally, the issue of the nature of the repeating unit of the backbone of the polysaccharide, for example, an $(XYZ)_n$ versus $(XXXYYYZZZ)_m$ structures was further resolved by observing how many microenvironments there are in which a particular sugar residue exists. For the former pattern, the answer would be *one* for each sugar; whereas, for the latter pattern there are *three* for each. A multiplicity of microenvironments would result in a broadening of the resonances for each proton in each sugar. The 1D and 2D proton studies of the polymer backbone (**5** and **7**), as well as the side chain (**6**) and backbone (**9**) fragments, all manifest only sharp and unique resonances for residue protons, when the multiplicity of anomerization in **9** is discounted. Particularly striking in this regard is the finding that Smith degradation (No. 3) of **5** results in a magnetically homogenous product (**7**), as is best seen in Fig. 5. Thus, we can conclude that there is only a single hexasaccharide repeating unit.

Taking all of the chemical and NMR data together, we conclude that the structure of the EPS (**1**) and its derivatives are as shown in Fig. 6.

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REFERENCES

1 A.K. Chatterjee and M.P. Starr, *Annu. Rev. Microbiol.*, 34 (1980) 645–676.
2 R.S. Dickey, *Phytopathology*, 69 (1979) 324–329.
3 R.S. Dickey, *Phytopathology*, 71 (1981) 23–29.
4 R.S. Dickey, C.H. Zumoff, and J.K. Uyemoto, *Phytopathology*, 74 (1984) 1388–1394.
5 R.S. Dickey, L.E. Claflin, and C.H. Zumoff, *Phytopathology*, 77 (1987) 426–430.
6 M.J. Daniels, J.M. Dow, and A.E. Osbourn, *Annu. Rev. Phytopathol.*, 26 (1988) 285–312.
7 E. Schoonejans, D. Expert, and A. Toussaint, *J. Bacteriol.*, 169 (1987) 4011–4017.
8 J.J. Bradshaw-Rouse, L. Sequeira, A. Kelman, and R.S. Dickey, *Phytopathology*, 78 (1988) 996–999.
9 J.S.S. Gray, W.A. Lindner, J.M. Brand, and J.P. Mildenhall, *J. Bacteriol.*, 168 (1986) 886–891.
10 W.E. Trevelyan, D.P. Proctor, and J.S. Harrison, *Nature (London)*, 166 (1950) 444–445.
11 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
12 T. Bitter and H.M. Muir, *Anal. Biochem.*, 4 (1962) 330–334.
13 N. Blumenkranz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
14 Y.D. Karkhanis, J.Y. Zeltner, J.J. Jackson, and D.J. Carlo, *Anal. Biochem.*, 85 (1978) 595–601.

15 M. Bradford, *Anal. Biochem.* , 72 (1976) 248–254.
16 W.S. York, A.G. Darvill, M. McNeil, T.J. Stevenson, and P. Albersheim, *Methods Enzymol.*, 118 (1986) 3–40.
17 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
18 M.F. Chaplin, *Anal. Biochem.*, 123 (1982) 336–341.
19 C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, 85 (1963) 2497–2507.
20 R.L. Taylor and H.E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.
21 J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 168 (1987) 219–243.
22 F. Smith and R. Montgomery, *Methods Biochem. Anal.*, 3 (1956) 153–212.
23 K.M. Aalmo, M.F. Ishak, and T.J. Painter, *Carbohydr. Res.*, 63 (1978) c3–c7.
24 W.F. Dudman, *Carbohydr. Res.*, 66 (1978) 9–23.
25 J.E. Scott and R.J. Harbinson, *Histochemie*, 14 (1968) 215–220.
26 T. Painter and B. Larsen, *Acta Chem. Scand.*, 24 (1970) 813–833.
27 M.F. Ishak and T. Painter, *Acta Chem. Scand.*, 25 (1971) 3875–3877.
28 T.A.W. Koerner, J.H. Prestegard and R.K. Yu, *Methods Enzymol.*, 138 (1987) 38–59.
29 V.D. Dua, B.N.N. Rao, S. Wu, V.E. Dube and C.A. Bush, *J. Biol. Chem.*, 261 (1986) 1599–1610.
30 J.F. Kennedy and C.A. White, in J.F. Kennedy (Ed.), *Carbohydrate Chemistry*, Clarendon Press, Oxford, 1988, pp 245–259.