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Structural studies of acetan, an exopolysaccharide elaborated by *Acetobacter xylinum*

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ABSTRACT

The exopolysaccharide acetan, elaborated by *Acetobacter xylinum*, has been investigated. The polysaccharide and a heptasaccharide, obtained on enzymic hydrolysis, corresponding to the repeating unit were characterised by sugar and methylation analysis and by NMR spectroscopy and MS. It is concluded that the polysaccharide is composed of repeating units with the following structure.

$$\begin{array}{r}
\rightarrow 4)\text{-}\beta\text{-}\mathrm{D}\text{-Glc}p(1 \rightarrow 4)\text{-}\beta\text{-}\mathrm{D}\text{-Glc}p\text{-}(1 \rightarrow \\
3 \qquad\qquad\qquad\qquad\quad\; \\
\uparrow \qquad\qquad\qquad\qquad\quad\; \\
1 \qquad\qquad\qquad\qquad\quad\; \\
\alpha\text{-}\mathrm{L}\text{-Rha}p\text{-}(1 \rightarrow 6)\text{-}\beta\text{-}\mathrm{D}\text{-Glc}p\text{-}(1 \rightarrow 6)\text{-}\alpha\text{-}\mathrm{D}\text{-Glc}p\text{-}(1 \rightarrow 4)\text{-}\beta\text{-}\mathrm{D}\text{-Glc}p\text{A-}(1 \rightarrow 2)\text{-}\alpha\text{-}\mathrm{D}\text{-Man}p \qquad\qquad\qquad\qquad\qquad\qquad
\end{array}$$

The polysaccharide further contains approximately two *O*-acetyl groups per repeating unit, which have not been assigned, but it appears that they are on primary locations.

INTRODUCTION

Acetobacter xylinum elaborates extracellular cellulose and is used as a model system for studies of cellulose biosynthesis. There are, however, cellulose-positive and cellulose-negative strains of this organism which produce another extracellular polysaccharide, sometimes called acetan. Structural studies of acetan[1–3] indicated that it is composed of D-glucose, D-mannose, L-rhamnose, and D-glucuronic acid in the relative proportions 3–4:1:1:1, and that it contains a backbone of cellulose, to which a tetra- or penta-saccharide residue is linked to the 3-position of every

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other β-D-glucopyranosyl residue. Different preliminary structures have been published, but it seems likely that the various groups have studied the same polysaccharide, acetan. Other extracellular polysaccharides, elaborated by different strains of *A. xylinum*, also have a cellulose backbone but different and only partially characterised structures[4,5].

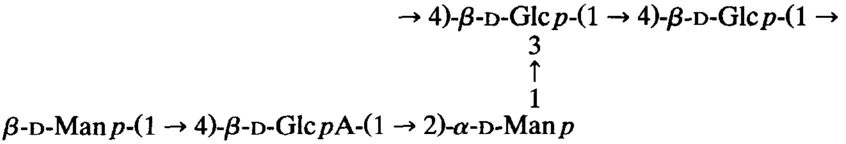
During studies of the biosynthesis of acetan, Couso et al.[3] isolated a heptasaccharide diphosphate prenol. Studies of its structure, by isolating and characterising smaller oligosaccharide prenols that were assumed to be intermediates in the biosynthesis, indicated the tentative structure **1**.

Rha-(1 → 6)-β-Glc-(1 → 6)-α-Glc-(1 → 4)-β-GlcA-(1 → 6)-β-Man-(1 → 3)-β-Glc(1 → 4)-α-Glc(1 → diphosphate-prenol

1

Studies of the biosynthesis of xanthan[6,7], which has structure **2**[8], showed that it followed the same scheme as proposed for acetan. These results prompted a study of the acetan structure based on a comparison with the well characterised xanthan constitution. This study showed that the mannose in **1** is 2-linked and in the α configuration, and that the two structures are closely related[9].

In the present work, acetan is analysed from a more rigorous chemical viewpoint.



$$\begin{array}{rl} & \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow \\ & \qquad\qquad\quad 3 \\ & \qquad\qquad\quad \uparrow \\ & \qquad\qquad\quad 1 \\ \beta\text{-D-Man}p\text{-}(1 \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1 \rightarrow 2)\text{-}\alpha\text{-D-Man}p & \end{array}$$

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RESULTS AND DISCUSSION

Crude acetan was purified by anion-exchange chromatography on a column of DEAE-trisacryl, and eluted at a sodium chloride concentration of ~ 0.4 M. On hydrolysis of acetan, using CF_3CO_2H for 18 h at 120°C, D-glucose, D-mannose, and L-rhamnose were obtained, as shown by GLC analysis of the corresponding alditol acetates. Determination of the absolute configuration was made using the trimethylsilylated (−)-2-butyl glycosides[10,11]. The acidic component of acetan was identified as D-glucuronic acid. Five derivatives corresponding to terminal L-rhamnose, 4-linked D-glucose, 6-linked D-glucose, 2-linked D-mannose, and 3,4-linked D-glucose were detected in the methylation analysis. When the permethylated polysaccharide was reduced with lithium borohydride, a 2,3-di-*O*-methylhexose was also obtained, demonstrating that the D-glucuronic acid was 4-linked.

Native acetan gave viscous solutions and consequently poor NMR spectra. The ^1H NMR spectrum of *O*-deacetylated acetan was better resolved (Fig. 1) and showed, *inter alia*, signals for seven anomeric protons. Four of these, at δ 4.48

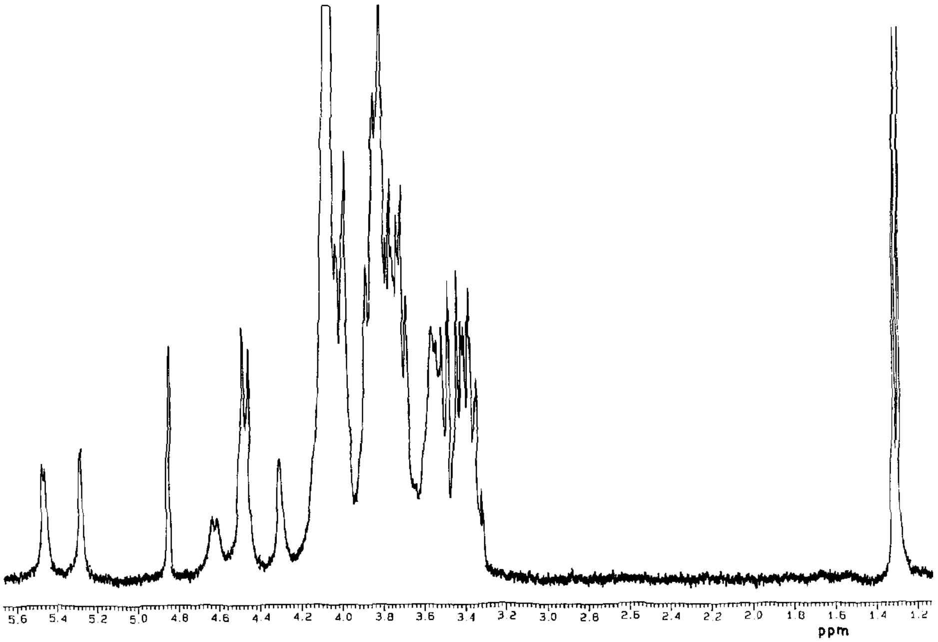


Fig. 1. ^{1}H NMR spectrum of the *O*-deacetylated acetan.

($J_{1,2}$ ~ 7 Hz, 3 H) and 4.62 ($J_{1,2}$ ~ 7 Hz, 1 H), correspond to pyranosyl residues with the *β-gluco* configuration, and that at δ 5.48 ($J_{1,2}$ ~ 3.3 Hz, 1 H) to a pyranosyl residue with the *α-gluco* configuration. A signal at δ 5.29 ($J_{1,2}$ ~ 1 Hz, 1 H) could be assigned to an α-D-mannopyranosyl residue and another at δ 4.86 (not resolved, 1 H) to a second residue with the *manno* configuration, the anomeric configuration of which could not be assigned at the present stage of the investigation.

The ^{1}H NMR spectrum of native acetan showed a signal at δ 2.14 (6 H) indicating the presence of two *O*-acetyl groups, and the corresponding peaks at δ 21.1 and 21.2 were observed in the ^{13}C NMR spectrum. A comparison between the ^{1}H NMR spectra of native and *O*-deacetylated polysaccharide showed no extra signals downfield of 4.40 ppm in the ^{1}H NMR spectrum of the native polysaccharide, indicating that the *O*-acetyl groups were located on primary positions. In agreement with this, a signal at 64.5 ppm was observed in the ^{13}C NMR spectrum of native acetan.

Treatment of *O*-deacetylated acetan with a cellulase from *Aspergillus niger* caused partial hydrolysis. Gel filtration on a column of Bio-Gel P4 yielded a low molecular weight fraction together with a mixture of larger molecules. The mixture was not further investigated. The FAB mass spectrum (Fig. 2), obtained in the negative mode using triethanolamine as matrix, gave the pseudomolecular ion, $(M-H)^-$, at *m/z* 1149. This corresponds to a heptasaccharide composed of one

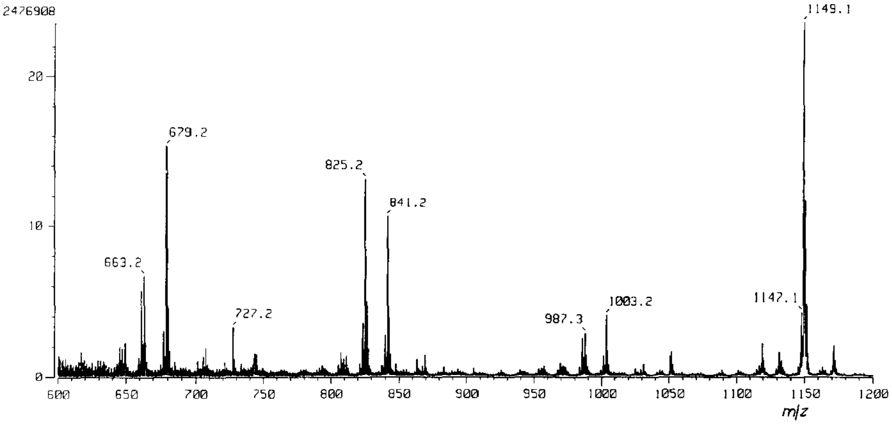
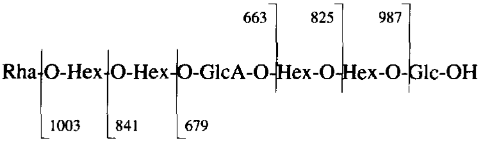


Fig. 2. FAB mass spectrum of the heptasaccharide-alditol obtained in the negative mode using triethanolamine as matrix.

D-glucuronic acid residue, one L-rhamnose residue, and five hexose residues. The spectrum further contained two series of fragments corresponding to the B and C fragmentation pathways[12]. These fragmentations involve cleavage on either side of the glycosidic oxygen and a hydrogen transfer, and the charge may be retained on either the reducing (B) or the nonreducing (C) end. The m/z values for the main fragments are indicated in formula 3.



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From these values, it may be concluded that the heptasaccharide is linear, and that rhamnose occupies a terminal position and glucuronic acid a central position, as the latter is present in both fragment series. All the strongest fragments, three found in pathway B and three in pathway C cleavage, all contain the glucuronic acid residue. No such predominance of GlcA-containing fragments was observed in FAB-MS in the positive mode.

Methylation analysis of the heptasaccharide-alditol confirmed that it was linear. The absence of 2,3,6-tri-*O*-methyl-D-glucose demonstrated that the reducing end was a 4-linked D-glucose residue. 2,6-Di-*O*-methyl-D-glucose, obtained from the *O*-deacetylated acetan, had been replaced by 2,4,6-tri-*O*-methyl-D-glucose, demonstrating that, as expected, the main chain was linked (1 → 4) and consequently that the side chain was linked (1 → 3) to the main chain, and that the glycosidic linkage of the → 4)-D-Glc-(1 → had been cleaved by the enzymic treatment.

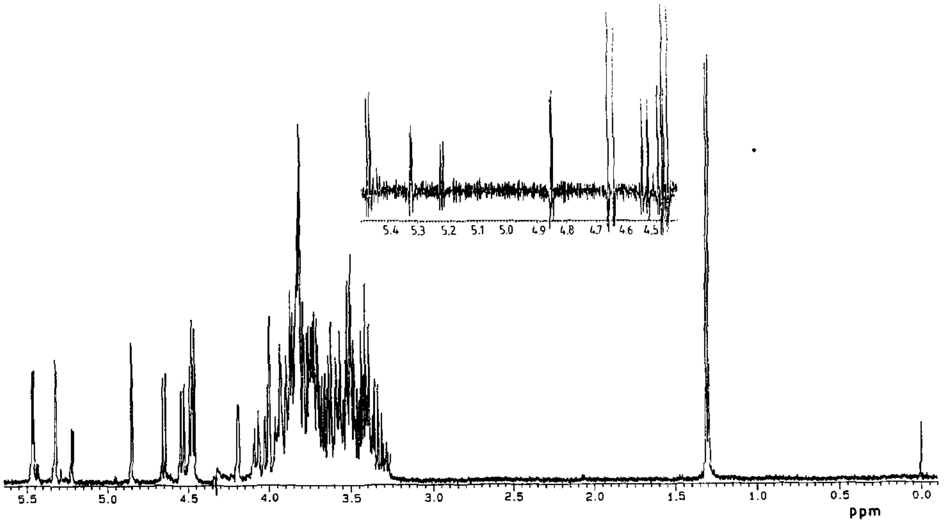


Fig. 3. ^{1}H NMR spectrum of the heptasaccharide obtained on enzymatic degradation of *O*-deacetylated acetan.

From the results discussed above, partial structure **4** can be proposed.

L-Rha*p*-(1 → x)-Hex*p*-(1 → y)-Hex*p*-(1 → 4)-D-Glc*p*A-(1 → z)-Hex*p*-(1 → 3)-D-Glc*p*-(1 → 4)-D-Glc*p*

4

The 1H NMR spectrum of the heptasaccharide (Fig. 3) confirms that the reducing terminal is a D-glucopyranose residue, as it gives signals for the α and the β form at δ 5.22 ($J_{1,2}$ 3.9 Hz) and δ 4.65 ($J_{1,2}$ 7.8 Hz). The anomeric signal at δ 4.54 ($J_{1,2}$ 7.8 Hz) is twinned, indicating that it may be assigned to the residue linked to the reducing residue. This is the D-Glc*p* linked through O-3, which is consequently β-linked.

A phase-sensitive H,H-COSY spectrum of the heptasaccharide was recorded and most of the proton signals were assigned (Table I). The results confirm that all sugar residues are pyranosides. It is seen that the signal from H-3 of residue F is shifted ~ 0.16 ppm downfield compared to the monomer, corroborating that it is linked through O-3 and not through O-6, which would have been the other alternative.

The α-linked residue (A), with the *gluco* configuration, must then either be the 4-linked D-Glc*p*A residue or one of the 6-linked D-Glc*p* residues, as the 3-linked D-glucose residue was assigned the β configuration. The chemical shift, δ 5.45, is compatible only with proximity to a strongly electron-withdrawing group, which has to be the carboxyl group in the D-Glc*p*A residue. The corresponding signal from the anomeric proton in the D-Glc group in the disaccharide α-D-Glc-(1 → 4)-α-D-

TABLE I

^{1}H NMR data for the heptasaccharide obtained on enzymic hydrolysis of acetan

Sugar residue	Chemical shift ^{a} (δ)						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→ 6)-α-D-Glc*p*-(1 →	5.46 [4.2]	3.52	3.70	3.50	3.85	3.86	4.08
A	(0.23)	(−0.02)	(−0.02)	(0.07)	(0.01)	(0.02)	(0.24)
→ 2)-α-D-Man*p*-(1 →	5.32 [1.2]	4.20	3.88	3.73	3.93		
B	(0.14)	(0.26)	(0.02)	(0.05)	(0.11)		
→ 4)-α-D-Glc	5.22 [3.9]	3.58	3.83	3.62	3.94		
C	(−0.01)	(0.04)	(0.09)	(0.20)	(0.10)		
α-L-Rha*p*-(1 →	4.85 [1.5]	~ 4.01	3.80	3.44	3.75	1.30	
D	(−0.27)	(0.09)	(−0.01)	(−0.01)	(−0.11)	(0.02)	
→ 4)-β-D-Glc	4.65 [7.8]	3.29	3.63				
E	(0.01)	(0.04)	(0.13)				
→ 3)-β-D-Glc*p*-(1 →	4.54 [7.8]	3.40	3.66	3.57	3.50	3.91	3.73
F	(−0.10)	(0.15)	(0.16)	(0.17)	(0.04)	(0.01)	(0.01)
→ 4)-β-D-Glc*p*A-(1 →	4.48 [7.6]	3.43	3.77	3.79			
G	(−0.17)	(0.13)	(0.25)	(0.25)			
→ 6)-β-D-Glc*p*-(1 →	4.47 [7.8]	3.34	3.51	3.39	3.57	3.72	4.02
	(−0.17)	(0.09)	(0.01)	(−0.02)	(0.11)	(0.00)	(0.12)

^{a} *J*-values (Hz) in square brackets; and chemical shift differences, relative to the corresponding monomers, in parenthesis.

GlcA-OMe[13] is at δ 5.43, which further supports this assumption. The structural elements **5** and **6** have consequently been demonstrated.

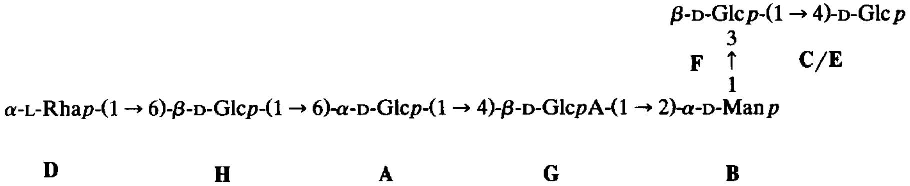
→ 3)-β-D-Glc-(1 → 4)-D-Glc
5

→ 4)-α-D-Glc-(1 → 4)-β-D-GlcA-(1 →
6

The chemical shift of the H-5 signal for the L-Rha*p* residue (**D**), δ 3.75, demonstrates that it has the α configuration. The corresponding chemical shift from α-L-Rha*p* is 3.86 and from β-L-Rha*p* is 3.39. The anomeric configuration of the D-Man*p* residue is consequently α. The only remaining structural problem is now to determine the relative order of → 6)-β-D-Glc-(1 → (**H**) and → 2)-α-D-Man-(1 → (**B**).

The anomeric signal of the L-Rha*p* group, at δ 4.85, is shifted upfield 0.27 ppm compared to the monomer. This is typical of a sugar linked to the 6-position of a hexose, and is due to the shielding from the 6-protons[14]. The α-L-Rha*p* group is consequently linked to → 6)-β-D-Glc-(1 → (**H**), and not to → 2)-α-D-Man-(1 → (**B**).

From the combined evidence, it is concluded that the heptasaccharide has structure **7** and that acetan consequently has structure **8**. The *O*-acetyl groups have not been located.



$$\begin{array}{r} \beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-D-Glc}p \qquad\qquad\qquad \\ 3 \qquad\qquad\qquad\qquad\qquad \\ \mathbf{F}\ \uparrow \qquad \mathbf{C/E} \qquad\qquad\qquad\qquad \\ 1 \qquad\qquad\qquad\qquad\qquad \\ \alpha\text{-L-Rha}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p \\ \mathbf{D} \qquad\qquad \mathbf{H} \qquad\qquad \mathbf{A} \qquad\qquad \mathbf{G} \qquad\qquad \mathbf{B} \qquad \end{array}$$

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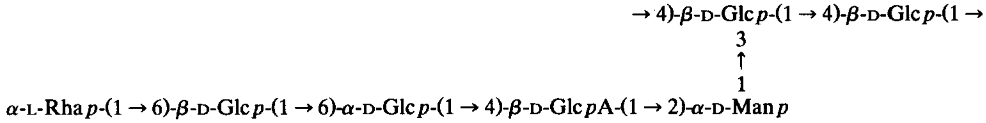

$$\begin{array}{r} \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \\ 3 \qquad\qquad\qquad\qquad\qquad \\ \uparrow \qquad\qquad\qquad\qquad\qquad \\ 1 \qquad\qquad\qquad\qquad\qquad \\ \alpha\text{-L-Rha}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p \qquad\quad \end{array}$$

8

The tentative structures deduced from the work of Tayama et al.[2] and Couso et al.[3,9] have been confirmed, and the anomeric configurations of all the linkages, including the terminal L-Rha*p*, have been clearly established.

EXPERIMENTAL

General methods.—Concentrations were performed under diminished pressure at < 40°C or in a stream of air. 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. Gel filtrations were performed on Bio-Gel P-2, using water that contained 1% of 1-butanol, and monitored using a differential refractometer. The absolute configurations of the sugars were determined according to Gerwig et al.[10,11].

Isolation of crude acetan.—*Acetobacter xylinum*, strain RCGrl, a derivative from strain NRRL B42[15], was grown in liquid medium with rotary shaking (200 rpm) at 28°C for 7 days and freed from cellulose as described[16]. Crude acetan was isolated from the cell supernatant solutions as previously described[9].

Purification of the polysaccharide.—The crude polysaccharide was fractionated on a column (1.6 × 20 cm) of DEAE-Trisacryl, which was irrigated first with 0.01 M NaH_2PO_4 (pH 5.1, 400 mL) and then with a linear gradient of NaCl (0 → 0.5 M) in 0.01 M NaH_2PO_4 (400 mL). The polysaccharide in the eluate was detected by the anthrone test, and the solution was desalted on a column (2.5 × 90 cm) of Bio-Gel P-2, to give the polysaccharide (17 mg, 31%).

Sugar and methylation analysis.—The native and methylated polysaccharides were treated with 2 M CF_3CO_2H at 120°C for 12 h. The sugars in the hydrolysates were then converted into alditol acetates or partially methylated alditol acetates. Alditol acetates were analysed on an HP-5 capillary column (25 m × 0.20 × 0.33 mm), using the temperature programme 200°C (3 min) → 250°C at 2°C min or at 220°C on a DB-225 column. Partially methylated alditol acetates were analysed on the HP-5 column, using the temperature programme 185°C (8 min) → 250°C (5°C/min) or isothermally at 190°C.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 70°C (^{13}C) and 85°C (^{1}H), using a JEOL GX-400 or GSX-270 instrument. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropanoate-d_4 (δ 0.00) for ^{1}H and acetone (δ 31.07) for ^{13}C, as internal references. The H,H-COSY spectra were performed in the phase-sensitive double-quantum-filtered mode using the JEOL standard pulse sequence.

Enzymatic depolymerization.—The *O*-deacetylated polysaccharide (75 mg) was treated with cellulase from *Aspergillus niger* (Sigma, 20 mg) in a 0.05 M NaOAc buffer (pH 5.0, 12 mL) at 30°C for 48 h. After heating and centrifugation, to remove the precipitated enzyme, the supernatant solution was applied to a column (86 × 2.5 cm) of Bio-Gel P4. The fractions between 1.5 and 1.8 void volumes were rechromatographed. A fraction eluting at 1.6 void volumes (4 mg) was homogeneous according to NMR spectroscopy and MS and was used for further studies. Material eluting earlier in the column was indicated by NMR spectroscopy to be a mixture and was not further characterised.

FAB mass spectrometry.—FAB-mass spectra were recorded on a JEOL SX 102 instrument. Ions were produced by a beam of Xe atoms (6 keV). Native oligosaccharides were measured either in the positive mode using glycerol as matrix or in the negative mode using triethanolamine. Sodium acetate in MeOH was added in order to obtain sodium-adduct pseudomolecular ions.

ACKNOWLEDGMENTS

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