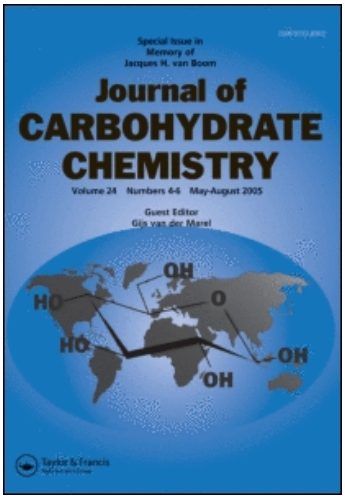

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STRUCTURAL STUDIES ON GALACTOMANNANS FROM BRAZILIAN SEEDS

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ABSTRACT

Chemical structures of galactomannans from the native Brazilian species, *M. scabrella*, *S. barbatiman* and *S. parahybum* were studied. Their seeds, on aqueous extraction, furnished high yields of viscous galactomannans, whose mannose to galactose ratios were 1.1:1, 1.5:1, and 3.0:1, respectively. The polysaccharides were analysed by methylation, periodate oxidation and chromium trioxide oxidation. The results, confirmed by ^{13}C NMR spectroscopy, indicated expected structures of legume galactomannans, namely a polymeric main chain of (1→4)-linked ß-D-mannopyranosyl residues substituted at O-6 by single unit α-D-galactopyranosyl side-chains. ^{13}C NMR spectra showed clear splitting of the O-substituted resonances of C-4 of the ß-D-mannosyl residues depending on the nearest-neighbour probabilities, indicating a random arrangement of the D-galactosyl groups in all of the three galactomannans.

INTRODUCTION

Seed galactomannans are mostly found as endosperm cell wall storage components in leguminous plants. They have industrial application in foods, paints, cosmetics and pharmaceuticals, due to their viscosity and thickening properties. Galac-

tomannans consist of a main chain of (1→4)-linked ß-D-mannopyranosyl residues substituted at O-6 by α-D-galactopyranosyl units.[1] Deviations from this basic structure are not frequent.[2]

The ratio of mannose to galactose residues can range from 1:1 in polysaccharides with a completely substituted main chain to 5.6:1. For galactomannan applications involving aqueous viscosity enhancement, the mannose to galactose ratio is not significant. However, for applications involving co-gelation with other polysaccharides the level and distribution of galactosyl substitution along the mannose main-chain play important roles. The side-chain distribution has been studied using various techniques; chemically,[3,4] by degradation using purified ß-D-mananases,[5,6] by theoretical analysis of periodate oxidation kinetic studies,[7] and by ^{13}C NMR spectroscopy.[8,9,10]

Seed galactomannans have been investigated in many leguminous species. However, the only two galactomannans commercialy utilized in large quantities are guar gum from guar (*Cyamnopsis tetragonolobus*) and locust bean gum from carob (*Ceratonia siliqua*) seeds. In view of the utility of galactomannans and their derivatives, those of native Brazilian species are now under investigation.

Mimosa scabrella (bracatinga) is a fast-growing leguminous tree abundant in Southern Brazil. Its timber is used as fuel and for plywood and charcoal generation. Its main stem hemicellulose is now well characterized as an O-acetyl-4-O-methyl-glucuronoxylan.[11,12] Low molecular weight carbohydrates from *M. scabrella* seeds have also been analysed showing the presence of monosaccharides, cyclitols, polyols, oligosaccharides and a newly discovered trigalactosyl pinitol.[13] Whole seeds of *M. scabrella* contain 30% of galactomannan with a comparatively low mannose to galactose ratio. The rheological properties of this galactomannan have been studied.[14]

Stryphnodendron barbatiman (barbatimão) grows in Central Brazil and its seeds furnish 30% of galactomannan with a high intrinsic viscosity. Its viscosity is related to pretreatment with organic solvent.[15,16]

TABLE 1. Extraction of Galactomannan from the Seeds.

Galactomannan source	Water °C	Yield g %	Monosaccharide (mol%) Man	Gal	Ara	Xyl
M. scabrella	4	30.0	53	47	-	-
	25	0.8	47	35	19	19
	50	0.1	19	19	42	19
S. barbatiman	4	16.4	58.9	38.7	1.5	0.9
	25	8.4	57.7	37.5	3.9	1.5
	50	4.5	52.9	34.5	8.4	3.5
S. parahybum	4	50.0	75	25	-	-
	25	0.5	77	13.6	9.4	-
	50	7.5	82	13.5	4.5	-

Schizolobium parahybum (guapuruvu) is an abundant species in our Atlantic Coastal region. A linear seed arabinofuranan, with a structure different from the highly branched arabinans from other sources, was characterized.[17]

We now describe a comparative structural analysis of the galactomannans from these three species.

RESULTS AND DISCUSSION

Seeds of *M. scabrella* and *S. barbatiman* and isolated endosperms of *S. parahybum* were subjected to aqueous extractions at progressively increasing temperatures (Table 1).

Most of the soluble galactomannans present were liberated on cold aqueous extraction and these were used for structural studies, after removal of noncovalently-associated proteins. However, this fraction from each species contained small proportions of residual protein. Their intrinsic viscosities were determined in the Newtonian regime and the values are normal for legume galactomannans (Table 2). The fraction from *S. barbatiman*, which contained arabinose and

TABLE 2. Analysis of the Galactomannans.

Galactomannan source	$[\alpha]_D^{25}$	$[\eta]$ mL/g	Man:Gal ratio	Protein[a]	Protein[b]
M. scabrella	+76.9	900	1.1:1	6.3	3.0
S. barbatiman	+54.7	1400	1.5:1	7.0	4.0
S. parahybum	+32.3	850	3.0:1	8.0	3.8

a. Before purification.
b. After purification.

xylose as minor components, was freed of these pentoses by formation of an insoluble copper complex. These pentoses were probably from contaminating hemicelluloses (Table 1).

The specific rotations (Table 2) were, as expected, strongly influenced by the galactose content, as shown by Noble et al.[18]

The galactomannans were oxidized with sodium periodate and the products reduced with sodium borohydride. GLC analysis of the resulting alditol acetates showed the presence of glycerol, erythritol and mannitol. The mannose units, which were probably protected by hemiacetal formation, were completely oxidized when the polyols were twice reoxidized. Sen et al.[19] showed that when the galactomannan from *Indigofera tinctoria*, which has the conventional structure of legume seed galactomannans, was oxidized with periodate and the polyol once reoxidized, 90% of the total mannose was affected.

Methylation analyses of the three galactomannans were performed by different methods in order to obtain fully O-methylated derivatives. Galactomannans from *M. scabrella* and *S. barbatiman* were soluble in DMSO and were methylated by the methods of Hakomori,[20] and Ciucanu and Kerek,[21] respectively. The galactomannan from *S. parahybum*, with a lower galactose content, was not soluble in DMSO, and was acetylated and then

TABLE 3. Methylation Analysis of the Galactomannans.

O-METHYL COMPONENT (mol %)	POLYSACCHARIDE SOURCE		
	M. scabrella	*S. barbatiman*	*S. parahybum*
2,3,4,6-Me_4-Man	-	1.6	1.8
2,3,4,6-Me_4-Gal	48.2	39.0	25.4
2,3,6-Me_3-Man	6.5	23.0	48.2
2,3-Me_2-Man	46.3	36.4	24.6

methylated successively by the methods of Ciucanu and Kerek, and Hakomori. Hydrolysis of the per-O-methylated derivatives produced mixtures of partially methylated monosaccharides shown in Table 3. The results, which agree with the periodate data, are consistent with a main chain of (1→4)-linked manno-pyranosyl units substituted at O-6 with galactopyranosyl units.

The anomeric configurations of the sugar residues were determined by chromium trioxide oxidation.[22] Oxidation of the fully acetylated polysaccharides and subsequent monosaccharide analysis indicated that the D-mannopyranosyl residues are ß-linked (oxidized more rapidly) and that the D-galacto-pyranosyl residues are α-linked (Table 4).

The galactomannans were also analysed by ^{13}C NMR spectroscopy. As galactomannans generally form viscous solutions, successful spectroscopy was only possible after limited acid depolymerization, which diminished the viscosity.[8,10] ^{13}C NMR examination of galactomannans of *M. scabrella* was perfomed after treatment with cold concentrated orthophosphoric acid[23] or after sonication. Better resolved spectra were obtained using sonication, and this was the technique chosen for the present study[24] **(Figure 1)**.

The resonances in the ^{13}C NMR spectra are in close agreement with those reported for other galactomannans.[8,10] Three

TABLE 4. Chromium Trioxide Oxidation of Galactomannans.

Galactomannan source	Time (hour)	Monosaccharide Composition[a]		
		Xylitol	Mannose	Galactose
M. scabrella	0	1	0.37	0.33
	1	1	0.16	0.25
	2	1	0.13	0.24
	3	1	0.02	0.20
S. barbatiman	0	1	1.30	0.90
	1	1	0.05	0.88
	2	1	0.02	0.40
	3	1	0.01	0.35
S. parahybum	0	1	2.30	0.80
	1	1	1.10	0.50
	2	1	0.70	0.50
	3	1	0.40	0.50

a. Mannose and galactose molar concentrations are related to xylitol used as internal standard during the CrO_3 oxidation process.

types of structural units were identified, namely α-D-galactopyranosyl units, unsubstituted (1→4)-linked ß-D-mannopyranosyl units of the mannan main-chain and the O-6 substituted (1→4)-linked ß-D-mannopyranosyl units. Chemical shifts are recorded in Table 5.

Peak areas of C-1 (substituted Man) and C-1 (Gal) were determined and are in accord with the chemical analyses.

Signals showed clear splitting of the O-substituted resonances of C-4, three of them being observed. This was also found in the spectra of the other galactomannans.[8,10] This can be attributed to sequence related heterogeneity, assuming that the C-4 (Man) resonance is sensitive to whether or not the residue linked to O-4 is branched. The peak at the lowest field (I) originates from groups of two contiguos substitued D-mannopyranosyl residues (**Figure 2**), the intermediate peak (II) represents the superimposition of signals originating from diads in which only one of the two mannose residues is

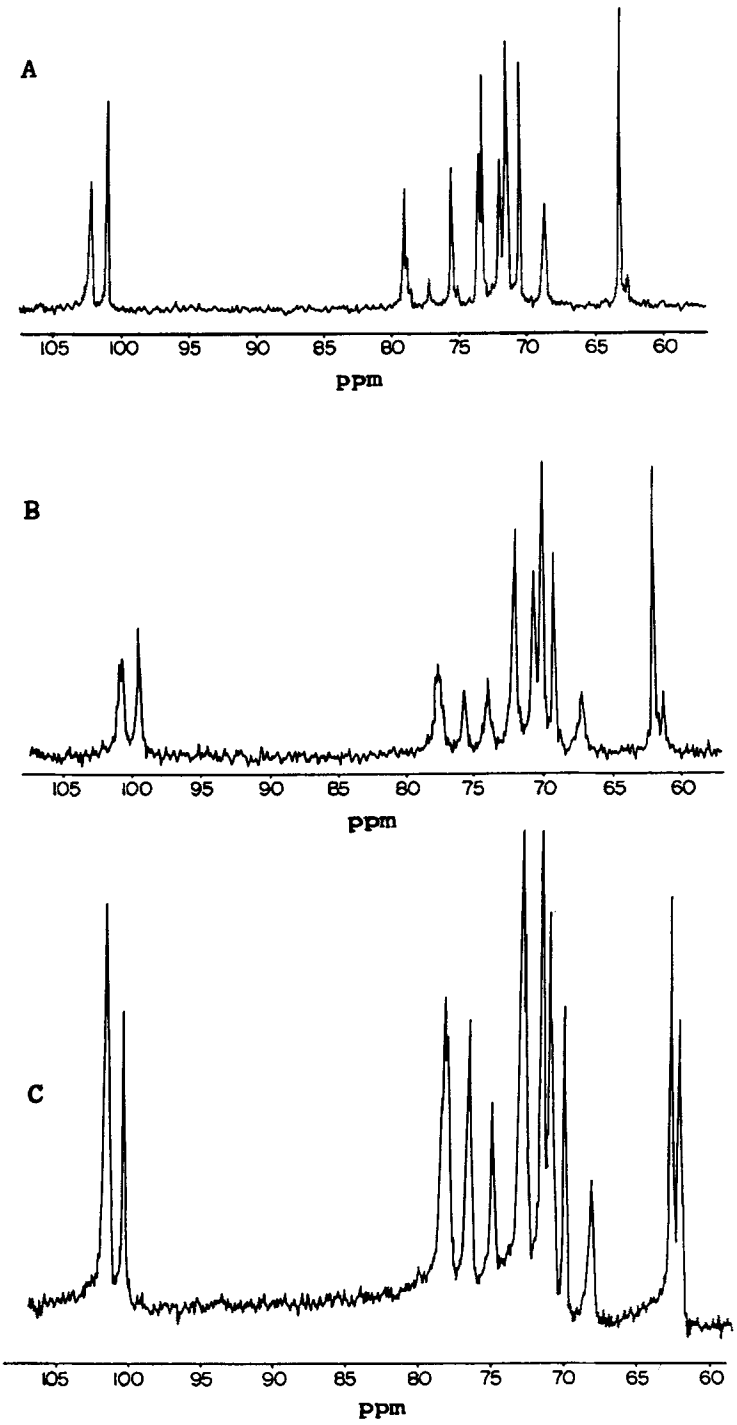


FIG. 1 ^{13}C NMR spectra (75 MHz) in D_2O of galactomannans from A, *M. scabrella*; B, *S. barbatiman*; C, *S. parahybum*.

TABLE 5. ^{13}C NMR Data[a] for the Galactomannans from *M. scabrella* (M.s.), *S. barbatiman* (S.b.) and *S. parahybum* (S.p.).

Carbon	Species	α-D-Galp	ß-D-Manp unbranched[d]	ß-D-Manp branched[d]
C-1	M.s.	100.97	102.16	102.16
	S.b.	99.57	100.74	100.82
	S.p.	100.31	101.57	101.57
C-2	M.s.	70.60	72.05	72.05
	S.b.	69.29	70.73	70.73
	S.p.	69.97	71.50	71.50
C-3	M.s.	71.63	73.56	73.56
	S.b.	70.22	72.12	72.12
	S.p.	70.99	72.96	72.96
C-4	M.s.	70.28	78.60	79.10[b]
	S.b.	70.40	77.43	77.70[c]
	S.p.	70.90	77.90	78.20[c]
C-5	M.s.	73.31	77.17	75.55
	S.b.	72.12	75.81	74.07
	S.p.	72.90	76.54	74.93
C-6	M.s.	63.29	62.75	66.73
	S.b.	62.11	61.38	67.25
	S.p.	62.65	62.12	68.08

a. Shifts (δ in ppm); internal DDS reference.
b. When the preceeding D-mannosyl residue is branched.
c. When the preceeding D-mannosyl residue is unbranched.
d. Refers to branched and unbranched at O-6.

substituted and the peak at high field (III), can be assigned to unsubstituted D-mannosyl units that are adjacent to another residue of the same kind. The peak I dominates in the spectrum of the sample from *M. scabrella*, which is highly substituted with D-galactosyl residues. The intermediate peak II is more intense in the spectra of galactomannans from *S. barbatiman* and *S. parahybum*. Although the galactomannan from *S. parahybum* is less substituted, peak II is the result of superimposition of signals originating from two different diads and the

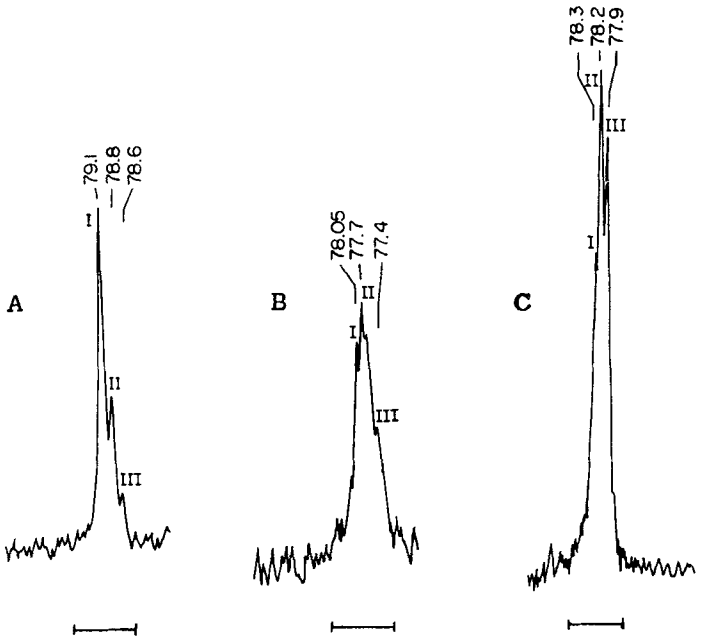
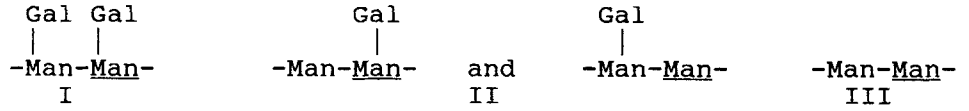


FIG.2. ^{13}C NMR spectral region at 75 MHz, of C-4 of the D-mannosyl residues in galactomannans from **A**, *M. scabrella* (Man:Gal ratio 1,1:1); **B**, *S. barbatiman* (Man:Gal ratio 1,5:1); **C**, *S. parahybum* (Man:Gal ratio 3:1). The unit involved is underlined:

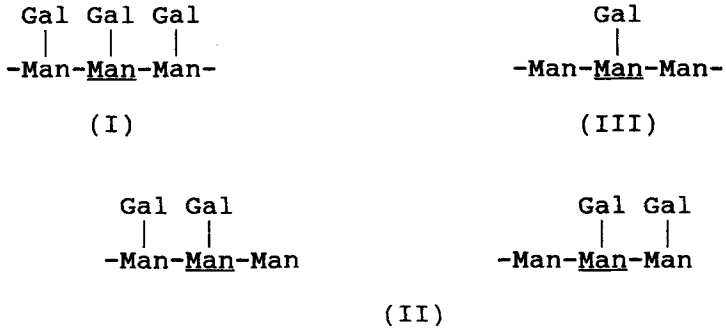


relative intensity of peak III is greater in this spectrum, indicating the presence of non substituted regions (**Figure 2**).

These results are consistent with a random arrangement of D-galactosyl groups in the three galactomannans.

Manzi et al.[9] compared the C-6 (substituted Man) resonances, which were clearly resolved in the spectra of depolymerized galactomannans from *Gleditsia triacanthus* having different Man:Gal ratios. They suggested that the peak at the lowest field originated from the C-6 resonance of the inter-

mediate unit from groups of three contiguous substituted D-mannosyl residues (I), that the signal at higher field is due to blocks of three contiguous D-mannosyl units, where only the intermediate residue is substituted (III) and that the intermediate peak represents the superimposition of signals from triads, where two contiguous units are substituted (II).



In the spectrum of *S. barbatiman* galactomannan, the splitting in the C-6 substituted Man region was resolved and the more intense peak corresponded to the intermediate resonance, i.e., triad II. In the spectra of *M. scabrella* and *S. parahybum* this region was not well resolved and the peaks at the C-6 substituted Man region probably indicate a predominance of triads I and III respectively.

EXPERIMENTAL

General Methods. Polysaccharides were hydrolyzed with 1M trifluoroacetic acid (4 h, 100 °C). Hydrolyzates were reduced with sodium borohydride and acetylated with pyridine-acetic anhydride (1:1 v/v, 16 h, at room temperature). The resulting alditol acetates were analyzed by GLC using a model 2440 Varian chromatograph at 180 °C with a 3% OV-225 or ECNSS column (0.15 id x 200 cm; gas chrom Q support). The carrier gas was nitrogen (40 mL/min). The methylated polysaccharides were hydrolysed[25] with 72% w/w aqueous H_2SO_4 (1h, 0-4 °C), and

then water was added to a final acid concentration of 0.5 M (5 h, 100 °C). The solutions were neutralized ($BaCO_3$) and the products converted into O-methyl alditol acetates that were analysed by GLC-MS with a Model 330 Varian instrument equipped with an OV-225 capilary column (0.25 mm id x 30 m) linked to a Finnigan ion trap, model 410 mass spectrometer unit at 70 eV. Injections were carried out at 50 °C and the column then programmed (40 °C/min) to 220 °C, and held.

^{13}C NMR spectroscopy was performed after samples were submitted to sonication (Branson B 12, 150 W apparatus) with a Bruker AC-300 spectrometer at 75 MHz in the Fourier-transform mode, with complete proton-decoupling at 80 °C, using D_2O as solvent contained in a tube (0.5 cm diam). The spectral width was 200 ppm. Chemical shifts are expressed in δ (ppm) relative to the resonance of DSS (sodium 4,4-dimethyl-4-silopentane-1-sulphonate) as an external standard (δ = 0).

Specific rotation of aqueous solutions (c 0.35) were measured with a Perkin-Elmer Model 141 polarimeter.

Total carbohydrates were assayed by the phenol sulfuric acid method[26] and protein by the Folin-Ciocalteau reagent.[27]

Polysaccharide Source. Seeds of *M. scabrella* were collected at the metropolitan region of Curitiba, Paraná; seeds of *S. barbatiman* were collected at the Estação Experimental de Paraguaçu Paulista of the Instituto Florestal de São Paulo; seeds of *S. parahybum* were collected on the Campus of Universidade do Rio dos Sinos, São Leopoldo, Rio Grande do Sul.

Polysaccharide Isolation. The seeds of *M. scabrella* and *S. barbatiman* (50 g) were crushed and extracted with 2:1 (v/v) benzene:ethanol in a Soxhlet apparatus for 16 h, and then 4:1 (v/v) methanol:water for 30 min. The residues were sucessively extracted with water at 4 °C (500 mL),25 °C (250 mL) and 50 °C (125 mL). The extractions at each temperature were repeated three times, the supernatants combined and reduced to a small volume at 40 °C. Two volumes of ethanol were added and the precipitated polysaccharides collected.

The seeds of *S. parahybum* were treated with boiling water for 30 min and then kept at room temperature until swelling

took place. Thereafter, the endosperm, seed coat and embryo were separated manually. The dry endosperm was milled (10 g) and submitted to the same aqueous extraction procedure as used for crushed seeds. The fractions obtained at 4 °C from each species were called, respectively, M.s., S.b. and S.p..

Polysaccharide Purification. Polysaccharide fractions M.s., S.b. and S.p. (aqueous 3% solutions) were submitted to sequential shaking cycles with a 5:1 (v/v) mixture of chloroform-butanol, as indicated by Sevag and described by Staub.[28]

Fraction S.b. was further purified as follows. A 25 mg/mL solution was poured into an excess of Fehling solution.[29] The resulting insoluble complex was centrifuged, decomposed with Dowex 50 W x 8 (H^+) and precipitated with an excess of ethanol.

Periodate Oxidation of Galactomannans. Samples of the purified galactomannans (50 mg) were oxidized with 50 mM sodium metaperiodate in the dark, at 25 °C, for 7 days. Excess reagent was decomposed with a few drops of ethylene glycol and the solutions dialysed against water for 24 h. The products were reduced with $NaBH_4$ for 6 h at room temperature. Excess $NaBH_4$ was destroyed by addition of acetic acid, the mixture dialysed and the solutions concentrated to 10 mL. Aliquots (2 mL) were removed and the remainder treated again (twice) with $NaIO_4$ and reduced with $NaBH_4$ as described above. The aliquots and the reoxidized reduced solutions were hydrolysed and analysed by GLC as alditol acetates.

Methylation Analysis. 1. Galactomannan fraction S.b. was methylated as follows.[21] To a solution of dry polysaccharide (100 mg) in DMSO (7 mL) were added powdered NaOH (100 mg) and methyl iodide (2 mL). The mixture was stirred for 10 min at room temperature, cooled, neutralized with acetic acid and extracted with $CHCl_3$. The process was repeated. The partially methylated polysaccharide was remethylated by the method of Hakomori.[20] Infrared spectroscopy of per-O-methylated polysaccharide showed that OH groups were absent.

2. Galactomannan fraction M.s. was methylated by the method of Hakomori (twice) and then by the method of Purdie.[30]

3. To a solution of dry polysaccharide fraction S.p. (100 mg) in formamide (50 mL), acetic anhydride (10 mL) and pyridine (10 mL) were added. After 24 h water was added and the product extracted with $CHCl_3$. The acetylated polysaccharide was then methylated sequentially according to Ciucanu and Kerek,[21] and Hakomori.[20]

Each methylated polysaccharide was hydrolysed with H_2SO_4 as described above and analysed by GLC-MS of O-methylated alditol acetates.

Oxidation of Galactomannans with Chromium Trioxide.[22] The purified galactomannans were twice acetylated as described above. The peracetylated polysaccharides (75 mg), together with 20 mg of xylitol pentacetate as internal standard were dissolved in 1.5 mL of $CHCl_3$ and treated with 1.89 mL of glacial acetic acid and 189 mg of chromium trioxide, at 50 °C. Aliquots were abstracted at the zero, 1, 2 and 3 h, water was added, and the material recovered by extraction with chloroform, hydrolysed and analysed by GLC of alditol acetates.

Viscosity Measurements. Viscosity analyses were carried out using an Ostwald viscosimeter at 25 °C. Intrinsic viscosities, $[\eta]$, are expressed as mL/g and obtained by extrapolation to zero concentration when plotting the reduced viscosities against polysaccharide concentration.

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