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HIGH RESOLUTION ^{13}C-N.M.R. SPECTROSCOPY OF LEGUME-SEED GALACTOMANNANS†

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ABSTRACT

Five galactomannans obtained by aqueous extraction at different temperatures from the endosperm of the seed of *Gleditsia triacanthos*, and having different Gal:Man ratios, were submitted to a preliminary degradation, and the products analyzed by high resolution ^{13}C-n.m.r. spectroscopy. In these spectra all carbon atoms yield several lines. C-6 (substituted Man) shows, for the first time, a clear splitting, which is explained by considering that this carbon atom is sensitive to whether or not its neighbors are branched; this provides a basis for determining the next-nearest-neighbor probabilities in the galactomannans ("triad frequencies"). Although diad frequencies are roughly consistent with a random arrangement, the values obtained for the triad frequencies indicate a more complex kind of arrangement of the lateral chains.

INTRODUCTION

Analysis of the distribution of single, (1→6)-linked α-D-galactopyranosyl side-groups along the (1→4)-linked β-D-mannan backbone[1] in legume-seed galactomannans has been attempted by different methods, chemically[2,3] and enzymically[4–7]. However, agreement between different groups of workers, using different methods, has been poor[2–12]. Grasdalen and Painter[13] attempted this analysis by 25- and 50-MHz ^{13}C-n.m.r. spectroscopy of depolymerized samples. The resonances of all of the carbon atoms were fully resolved and readily identified, and the Gal:Man ratios calculated from the measurement of the ratios of corresponding peak-areas were in good agreement with those of chemical analysis. The spectra showed a clear splitting of the resonance from C-4 of the D-mannosyl residues, in evident dependence upon the nearest-neighbor probabilities of D-galactosyl groups along the D-mannan chains. Therefore, "diad frequencies" could be determined by taking

†Dedicated to Prof. L. F. Leloir in honour of his 80th birthday.
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into account the peak-area ratios. Analysis of depolymerized D-galactomannans ($\overline{d.p._n}$ ~20–40) from guar, carob, lucerne, and clover seeds indicated that these probabilities take values that are roughly consistent with a random arrangement of D-galactosyl groups, but do not exclude the possibility of more complex kinds of arrangements.

By 50-MHz ^{13}C-n.m.r. spectroscopy, Boziek *et al.*[14] analyzed the galactomannans from locust bean, guar, and fenugreek, that had been depolymerized by employing a different method. They confirmed the results of Grasdalen and Painter[13], with the exception of the assignments of the C-2 and C-3 D-galactosyl resonances, which they inferred from the spectrum of monomeric methyl α-D-galactopyranoside[15].

There are no previous reports on the side-chain-distribution analysis of the D-galactomannans from *Gleditsia triacanthos*. We decided to subject D-galactomannans having different Gal:Man ratios, obtained from the endosperm of the seeds of *Gleditsia triacanthos*, to high resolution ^{13}C-n.m.r. spectroscopy, in order to determine possible differences in the side-chain distribution between them.

EXPERIMENTAL

General methods. — Monosaccharide analysis was performed by the gas–liquid-chromatographic method of Albersheim *et al.*[16] as already described[17]. Methylations were conducted by a combined Haworth–Hakomori procedure, as described[17]. Neutral and methylated products were analyzed as alditol acetates, and identified as described[17]. The $\overline{d.p._n}$ values were determined, before and after the depolymerization step, by measurement of the formaldehyde produced by periodate oxidation, as described[17], or by determination of the reducing power as described by Somogyi[18], with D-mannose as the standard.

Homogeneity of the degraded samples was analyzed by chromatography on a column (1.2 × 60 cm) of Sephadex G-15, using water as the eluant. Solutions (20 mg/mL, 0.5 mL) of the samples were applied at the top of the column, and 2-mL fractions were collected. The eluates were monitored for carbohydrates as described[17]; the fractions corresponding to the same peak were pooled, and freeze-dried.

Materials. — The seeds of *Gleditsia triacanthos* were obtained from ripe pods collected at the Ciudad Universitaria (Buenos Aires). The milled endosperm was exhaustively extracted with water at room temperature, 50°, and 95°, successively. Each extract was precipitated by stepwise addition of ethanol; fourteen galactomannan fractions were obtained, distributed between four groups, as previously described[17]. We also analyzed a sample (GmT), from an aqueous extract obtained at 95°, precipitated with ethanol up to 85% concentration.

Preliminary degradation. — (*A*) Galactomannans (1.0–0.1 g) were treated with syrupy orthophosphoric acid as described by Grasdalen and Painter[13]. Yield of degraded samples: GmT, 68; Gm 3, 54; Gm 7, 25; Gm 12, 22; and Gm 14, 39%.

(*B*) Gm 3 (0.22 g) was also depolymerized by employing dilute hydrochloric acid as reported by Boziek *et al.*[14], but, after dialysis, the product was recovered by freeze-drying; yield: 67%.

N.m.r. spectroscopy. — The samples were dissolved in D_2O at pD 7 (10–60 mg for ^{13}C; 40 mg for ^{1}H). ^{1}H-N.m.r. spectra were recorded with a Varian XL-100-15 spectrometer and a 620-L/100 computer. The natural-abundance, ^{13}C-n.m.r. spectra were recorded at 75 MHz with a Varian XL-300 spectrometer. All experiments were performed in the Fourier-transform mode. Free-induction decays were accumulated with a 60° pulse and a repetition time of 0.97 s. A probe temperature of 90° was used, to diminish tumbling-rate. Under the experimental conditions chosen, the peak areas were not significantly influenced by the nuclear Overhauser enhancements. An accumulation of 1,300–54,000 transients was required, according the concentration of the solution. The chemical shifts were measured by using reference settings derived from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) in D_2O at room temperature. Assignment of C-6 (substituted and non-substituted Man) and C-6 (Gal) were confirmed by employing an APT sequence. Peak areas were determined on expanded spectra.

RESULTS AND DISCUSSION

We chose five galactomannans from the endosperm of the seed of *Gleditsia triacanthos* called[17]: Gm 3, Gm 7, Gm 12, Gm 14, and Gm T. All of them were depolymerized by orthophosphoric acid, and Gm 3 was also depolymerized by hydrochloric acid. The $\overline{d.p.}_n$ value of the samples were decreased from 60–80 to 28–38 by employing the first method without any detectable change in the monomeric composition. Methylation analysis of depolymerized Gm T indicated no change in the primary structure of the sample. Gm 3 degraded by hydrochloric acid showed a $\overline{d.p.}_n$ of 23, closely similar to that obtained by the orthophosphoric acid method (namely, 28).

In the ^{1}H-n.m.r. spectra, the resonances of the anomeric protons are well separated, and their identification is self-evident from the known monomeric composition of the samples.

The 75-MHz, ^{13}C-n.m.r. spectra of galactomannans depolymerized with orthophosphoric acid agreed well with those at 25 and 50 MHz reported by Grasdalen and Painter[13]in 1980 and more recently by Boziek *et al.*[14], but the high resolution equipment allows extension of the usefulness of the method. An example is given in Fig. 1. All of the different carbon lines are resolved, and their chemical shifts are recorded in Table I. The assignments of the lines are those reported by Grasdalen and Painter[13], with the exception of the C-2 and C-3 galactosyl resonances, for which we consider that the assignments inferred from the reported spectra of methyl α-D-galactopyranoside[15] are the correct ones.

The ^{13}C-n.m.r. spectra of Gm 3 depolymerized by the two aforementioned methods indicated that this step is critical. The sample hydrolyzed by dilute hydrochloric acid shows very broad resonances, and several lines which are not resolved.

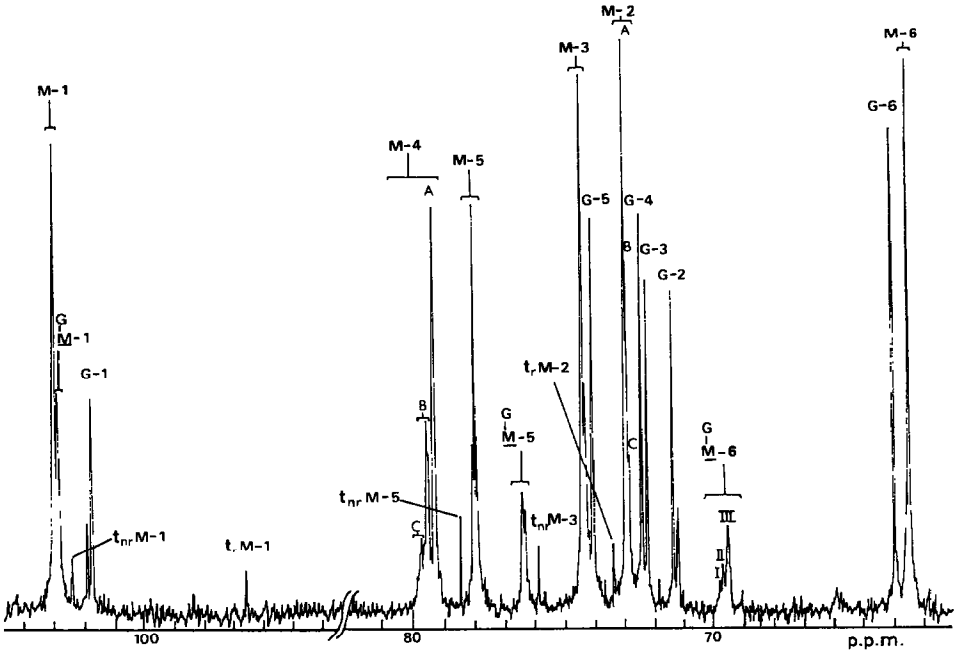


Fig. 1. ^{13}C-N.m.r. spectrum (75 MHz) of a solution (25 mg/mL) in D_2O (at pD 7 and 90°, with 57 k transients) of depolymerized galactomannan Gm T-HP. (M = mannose; G = galactose; t_r = reducing-end residue; t_{nr} = nonreducing-end residue).

TABLE I

ASSIGNMENTS OF PEAKS IN THE ^{13}C-N.M.R. SPECTRA OF D-GALACTOMANNANS

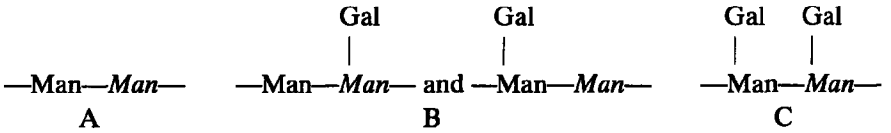
Type of unit	*Chemical shifts*[a]					
	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Galactopyranosyl	101.70	71.33	72.18	72.36	74.02	63.99
β-D-Mannopyranosyl residue, unbranched at O-6	102.93	72.89[b] 72.84[f]	74.34[c] 74.25[c]	79.26[b] 79.51[f]	77.98[d] 77.92[d] 77.85[d]	63.48[e]
β-D-Mannopyranosyl residue, branched at O-6	102.80	72.84[b] 72.78[f]	74.25[c]	79.51[b] 79.73[f]	76.34[g] 76.27[g]	69.80[h] 69.68[i] 69.51[j]
β-D-Mannopyranosyl (nonreducing) end-chain group	102.35	72.89[k]	75.80	69.51[k]	78.40	63.48[k]
β-D-Mannopyranose (reducing) end-chain residue	96.55	73.29	74.34[k]	79.26[k]	77.92[k]	63.48[k]

[a]Shifts (p.p.m.) downfield from external DSS. [b]When the preceding D-mannosyl residue is unbranched. [c]From some one of the diads A, B, or C. [d]From some one of the triads in which the intermediate D-mannosyl unit is unbranched (IV, V, or VI). [e]Superposition of the triads IV, V, and VI. [f]When the preceding D-mannosyl residue is branched. [g]From some one of the triads in which the intermediate D-mannosyl unit is branched (I, II, or III). [h]Intermediate unit from triad III. [i]Intermediate unit from triad II. [j]Intermediate unit from triad II. [k]Superimposed on the corresponding peak of the β-D-mannopyranosyl residues of the central chain.

These 75-MHz spectra show splitting of all of the D-mannose lines. C-1 (Man) appeared as two (almost completely resolved) signals, which were respectively assigned to non-substituted and substituted residues, according to the monomeric composition. In addition, peak areas of C-1 (substituted Man) and C-1 (Gal) show a 1.0:1.0 ratio.

Both C-2 (Man) and C-3 (Man) show two well-resolved lines and a third incipient one. Relative peak-areas of the first two lines do not correspond to the Gal:Man ratio. It is suggested that these splittings, as well as that of C-4 (Man), are due to structures A, B, and C (see later).

The C-4 (Man) pattern of lines is similar to that previously reported[13,14], although, in the 75-MHz spectra, several shoulders may be observed on the three peaks. It is possible that the resonances of C-4 of the substituted and non-substituted D-mannosyl units are sensitive, not only to the nearest neighbor but also to other structural details. The nearest-neighbor probabilities calculated on these absorptions are shown in Table II.



(The unit involved is italicized.)

The C-5 (non-substituted Man) signal also shows a splitting into three, poorly resolved, lines, and C-5 (substituted Man) appears as two well-resolved signals and a third incipient one (which is clearly resolved in the spectra of Gm 12). In addition, C-6 (non-substituted Man) appears as a broad line with a shoulder, and C-6 (substituted Man) shows three, almost completely resolved, absorptions. These can be explained by assuming that the resonances are sensitive to whether or not both D-mannosyl units linked to it are branched. C-5 and C-6 (substituted Man) resonances would correspond, therefore, to triads in which the intermediate residue is substituted and C-5 and C-6 (non-substituted Man) absorptions would correspond to those in which the intermediate unit is not substituted (see later).

Comparison of the C-6 (substituted Man) resonances, which are clearly resolved in the spectra of the different, depolymerized galactomannans from *Gleditsia triacanthos* (see Fig. 2) having different Gal:Man ratios, and in the 25-MHz spectrum of the galactomannan-like oligosaccharides ($\overline{d.p.}_n$ 15) from the same endosperm[19] allows assignment of each line. The reasoning of Grasdalen and Painter[13] applied to these patterns of lines suggested that the peak at the lowest field (69.90 p.p.m.) originates from the C-6 resonance of the intermediate unit from groups of three contiguous, substituted D-mannosyl residues (I); that the absorption at higher field (69.51 p.p.m.) is due to blocks of three contiguous D-mannosyl units, where only the intermediate residue is substituted (III); and that

TABLE II

SEQUENTIAL STRUCTURE OF D-GALACTOMANNANS FROM THE ENDOSPERM OF THE SEED OF *Gleditsia triacanthos*, IN TERMS OF DOUBLET AND TRIPLET FREQUENCIES[a]

Galactomannan	*Gm 3-HP*	*Gm 7-HP*	*Gm 12-HP*	*Gm 14-HP*	*Gm T-HP*
Gal:Man ratio	0.45[b]	0.33[b]	0.24[b]	0.31[b]	0.35[b]
	0.49[c]	n.d.	n.d.	n.d.	0.40[c]
	0.43[d]	0.34[d]	0.26[d]	0.30[d]	0.35[d]
Doublet frequencies[e]					
F_{11}	0.16(0.18)	0.14(0.11)	0.12(0.07)	0.13(0.09)	0.13(0.12)
$F_{12} = F_{21}$	0.24(0.25)	0.23(0.23)	0.20(0.19)	0.19(0.21)	0.19(0.23)
F_{22}	0.35(0.32)	0.40(0.43)	0.47(0.55)	0.49(0.49)	0.49(0.42)
Triplet frequencies[e]					
F_{111}	0.02(0.08)	0.06(0.04)	0.07(0.02)	0.02(0.03)	0.04(0.04)
$F_{112} = F_{211}$	0.05(0.11)	0.04(0.08)	0.04(0.05)	0.08(0.06)	0.04(0.08)
F_{212}	0.26(0.14)	0.21(0.15)	0.17(0.15)	0.18(0.15)	0.17(0.15)
F_{121}	(0.11)	(0.08)	(0.05)	(0.06)	(0.08)
$F_{221} = F_{122}$	0.63(0.14)	0.64(0.15)	0.68(0.15)	0.64(0.15)	0.72(0.15)
F_{222}	(0.18)	(0.28)	(0.40)	(0.34)	(0.27)

[a]Diad and triad frequencies, calculated for a random distribution, from the Gal:Man ratios measured by ^{13}C-n.m.r. spectroscopy, are indicated between parentheses. Experimental values were obtained for diad frequencies by integrating C-4 (Man) peak-areas, and triad frequencies, by integrating C-6 (Man) peak-areas. HP = hydrolyzed with orthophosphoric acid. [b]Determined by g.l.c. [c]Determined by ^{1}H-n.m.r. spectroscopy. [d]Determined by considering the intensities of C-1 mannose and galactose signals in the ^{13}C-n.m.r. spectra. [e]The subscripts 1 and 2 refer to branched and unbranched D-mannosyl residues, respectively.

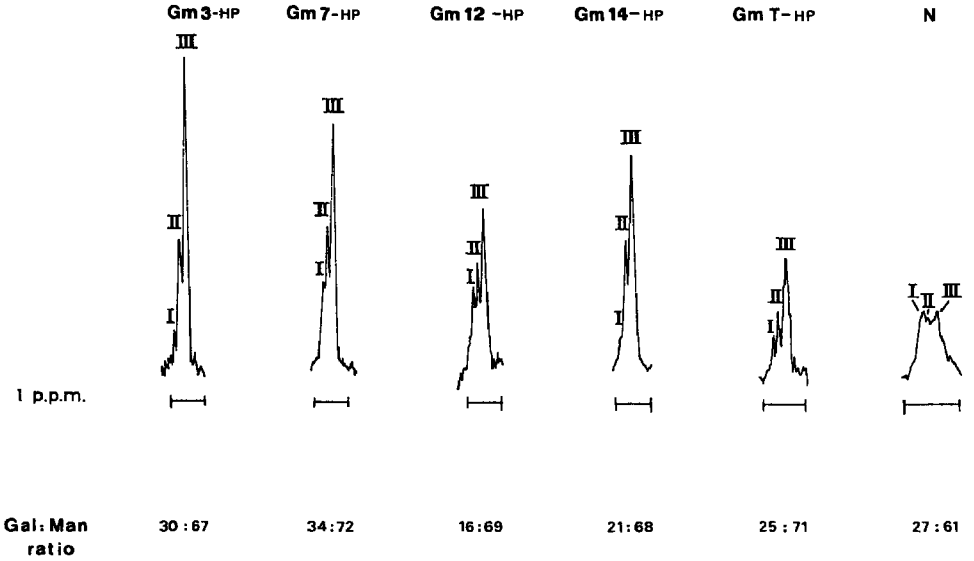
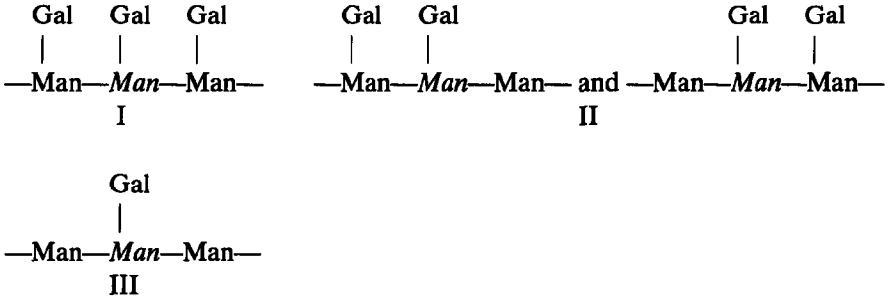
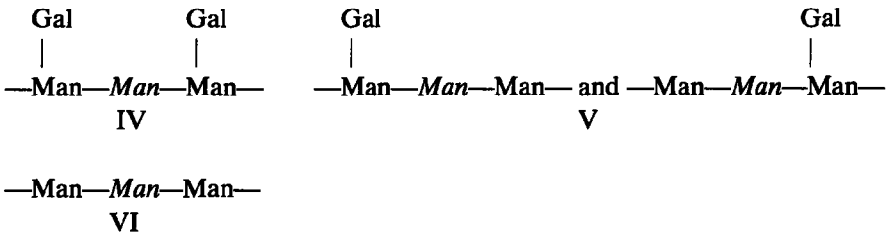


Fig. 2. ^{13}C-N.m.r.-spectral region of C-6 (substituted Man) residues: Gm 3-HP, Gm 7-HP, Gm 12-HP, Gm 14-HP, and Gm T-HP, at 75 MHz, and N (galactomannan-like oligosaccharide[19]) at 25.2 MHz. [HP = hydrolyzed by orthophosphoric acid. For interpretation, see the text.]

the intermediate peak (II) must represent the superposition of signals from triads wherein two contiguous units are substituted.



The broad line of C-6 (non-substituted Man) would represent superposition of the signals originated by triads IV, V, and VI.



Splitting of the C-6 (substituted Man) resonance provides, therefore, the basis for determining the next-nearest-neighbor probabilities (triad frequencies) along the central chain. C-5 resonances are not clearly resolved, preventing an accurate determination of the triad frequencies.

Diad and triad frequencies, determined from the peak areas of C-4 (Man) and C-6 (Man) resonances, for the five galactomannans are given in Table II, wherein theoretical values calculated for a random distribution are included for comparison. From the spectrum of Gm T, it is also possible to calculate diad frequencies from the peak areas of C-2 (Man) signals (this is not possible for the other spectra, because of the poor resolution of the signals). The results (F_{11} = 0.15, F_{12} = F_{21} = 0.19, and F_{22} = 0.48), which are in close agreement with those obtained from C-4 (Man) signals, allowed assignment of the three peaks observed in this region: absorption at the highest field (72.28 p.p.m.) originates from two contiguous, substituted D-mannosyl residues, the intermediate peak (72.84 p.p.m.) represents the superposition of signals originating from diads in which only one of the two D-mannosyl residues is substituted, and the peak at low field (72.89 p.p.m.) must be due to non-substituted D-mannosyl residues that are adjacent to another residue of the same kind.

Although peak III has the major and peak I the lowest intensity in the

spectra of all of the galactomannan samples, comparison of the C-6 (substituted Man) spectral region of each sample (see Fig. 2) and the figures in Table II, indicated that the distribution of D-galactosyl side-chains is different for the different samples; this explains the difference found[17] between the physical properties of samples having the same Gal:Man ratio and $\overline{d.p.}_n$.

The experimental values for the diad frequencies are roughly consistent with those predicted for a random distribution, but higher deviations from the theoretical values may be observed for the triad frequencies. Both sets of experimental values permit discarding of limit structures having a strictly alternating arrangement of branched and unbranched D-mannosyl units, or that in which a block of branched D-mannosyl residues is contiguous to a block of non-substituted D-mannosyl residues. The data would be consistent with a more complex kind of arrangement, as was found for guaran and locust-bean gum by periodate-oxidation analysis[5].

In addition to the substituted and non-substituted, internal, (1→4)-linked β-D-mannopyranosyl units of the backbone, and the D-galactosyl (nonreducing) end-chains of the lateral chains, these high resolution spectra allow identification of other structural units in the galactomannans, namely, (*a*) the 4-linked β-D-mannopyranose residue forming the reducing end of the D-mannose backbone, whose carbon resonances accord well with those of 4-*O*-methyl-β-D-mannopyranose[20]; and (*b*) the β-D-mannopyranosyl group forming the nonreducing end of the D-mannosyl backbone, whose resonances agree with those of methyl β-D-mannopyranoside[20] (see Table I).

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