ELSEVIER

Carbohydrate Research 271 (1995) 43–54

CARBOHYDRATE RESEARCH

^{1}H- and ^{13}C-NMR characterization of the digalactosylmannopentaose liberated from legume seed galactomannan by β-mannanase action

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Received 18 October 1994; accepted 23 December 1994

Abstract

Incubation of Locust bean gum with an *Aspergillus niger* β-D-mannanase released a wide variety of galactomannan oligomers. A single heptasaccharide, digalactosylmannopentaose, was obtained from fractionation of the mixture by size exclusion chromatography. The purity and chemical composition of the sample was demonstrated using mass spectrometry, high performance anion-exchange chromatography and monosaccharide composition analysis. The primary structure of this heptasaccharide was unambiguously identified using 2D ^{1}H and ^{13}C homonuclear and heteronuclear NMR. A complete assignment of the ^{1}H and ^{13}C signals of this oligomer was achieved, producing an NMR dataset that will be of importance in the primary structure elucidation of larger and more complex galactomannan oligomers.

Keywords: Locust bean gum; *Aspergillus niger*; β-D-Mannanase; Galactomannan

1. Introduction

Galactomannans are the seed energy reserve polysaccharides of all endospermic leguminous plants [1,2]. They consist of a linear 1 → 4 β-D-mannopyranose backbone substituted with single 1 → 6 linked α-D-galactopyranose units [3]. The proportion of mannose to galactose units in the polysaccharide varies widely throughout the Legume family, from 3 to 5:1 in the primitive *Leguminoea-caesalpinioideae* species to around 1.1:1 in the more advanced *Leguminoea-faboideae* species [4,5]. These variations are not due to inter-species differences in the biosynthetic pathway for the synthesis of the

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SSDI 0008-6215(95)00038-0

primary galactomannan, but have been attributed instead to the presence of differing ratios of mannosyl to galactosyl transferases between species and to post-deposition modifications [6,7]. The galactose content of the galactomannan polymer has been shown to have a profound effect on its rheological properties as indicated by the examples of Locust bean gum (Man/Gal = 5.5) and Guar gum (Man/Gal = 1.6), where the former is known to have greatly superior properties of self-interaction and gelation in mixed polysaccharide systems [8,9]. Extensive studies of the fine structure of galactomannans from different sources have been made, analysing both qualitatively and quantitatively the oligosaccharides released from the galactomannan polymers by highly purified β-D-mannanase enzyme obtained from either *Aspergillus niger* or Guar seed. Although such studies indicate the galactose distribution in the galactomannans to be non-regular [5,10], the *Aspergillus niger* β-D-mannanase degradation always yields the same oligomers (though in different relative amounts) regardless of the source of the original polymer [5,10]. In order to be able to describe the distribution of the galactosyl side chains along the mannan backbone, detailed knowledge about the relative quantities and the structures of the different oligomers released by β-D-mannanase is of utmost importance.

Considerable work has been done to identify these oligosaccharides using a combination of enzymic, NMR, and chemical procedures: currently, structures containing up to 9 sugar residues (Dp 9) have been characterized [3,11]. These studies have produced ^{1}H-NMR assignments for galactomannan oligomers up to Dp 3 and ^{13}C assignments for oligomers up to Dp 7 [3,11]; however, the assignments were only achieved by extrapolation from comparative NMR data for the monomeric building blocks. Earlier work [11] has shown that a single digalactosylmannopentaose is released by the action of *Aspergillus niger* β-D-mannanase. We now report the results of a 1D and 2D ^{1}H- and ^{13}C-NMR study of this heptasaccharide which gives a complete and reliable assignment of the ^{1}H- and ^{13}C-NMR signals of this molecule and an unambiguous primary structure determination. These data allow identification of potential reporter groups in the NMR spectra characteristic of specific structural features of the oligomer, and may thus be of assistance in the characterization by NMR of larger oligosaccharides and also in subsequent structural studies of galactomannan polymers [12,13].

2. Materials and methods

Preparation of Locust bean gum (LBG) galactomannan oligomers.—Preparation of galactomannan oligomers was performed essentially as described by McCleary et al. [11]. To 25 mL of a 0.4% solution of galactomannan (80% ethanol precipitate from commercial LBG; Meyhall fleur M225) in water was added 250 U of *Aspergillus niger* β-D-mannanase (E.C. 3.2.1.25; MegaZyme, Australia. 1 U = the amount of enzyme required to liberate 1 μmol of reducing power per minute at 40° C, pH 4.0, using carob galactomannan as substrate) and the resulting solution incubated at 40° C for 20 h. After heating at 100° C for 10 min to deactivate the enzyme, the solution was centrifuged (2000 g, 10 min) and the supernatant freeze-dried. This material, consisting of the galactomannan oligomers, was dissolved in 3 mL of water and fractionated on a Bio-Gel P-2 size exclusion column (2.6 × 100 cm) thermostatically controlled at 60° C: elution

was monitored by RI. Fractions were collected, the relevant ones pooled and then concentrated by freeze-drying.

Mannan oligomers were prepared from the relevant galactomannan liberated oligomers by the action of α-D-galactosidase (from guar seed; E.C. 3.2.1.22; MegaZyme, Australia).

High performance anion-exchange chromatography (HPAEC).—10 μL of a 1% solution of sample was injected onto a Dionex DX-300 IC system fitted with an Eluent Degas Module and a Model PED-2 detector working in the pulsed amperometric mode. Separation was performed at ambient temperature on an analytical CarboPac PA-100 column (4 × 250 mm) equipped with a CarboPac PA-100 guard column (4 × 50 mm). The mobile phase employed consisted of a linear gradient (0 to 25 mM over 40 min) of sodium acetate in 35 mM NaOH at a flow rate of 1 mL/min. The PED-2 detector pulse potential and durations applied were: $E_1 = 0.05$ V, 500 ms; $E_2 = 0.60$ V, 80 ms; $E_3 = -0.60$ V, 60ms. Data was collected using Dionex AI-450 software (version 3.31).

Monosaccharide analysis.—The sample (0.1 mg) was subjected to methanolysis (2.0 M methanolic HCl, 20 h, 85° C) followed by GLC of the trimethylsilylated methyl glycosides [14] on a CpSil 5CB fused-silica capillary column (0.32 mm × 25 m, Chrompack) using a Carlo Erba HRGC 5300 gas chromatograph with a split/splitless injector.

Molecular weight determination.—The sample (1 μL of a 1000:1 w/w dilution in H_2O) was added to 1 μL of a solution of 2,5 dihydroxybenzoic acid and examined by matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF) on a Fisons Instruments TofSpec spectrometer. An accumulation of spectra from 21 laser shots was acquired.

^{1}H- and ^{13}C-NMR spectroscopy.—NMR spectra were measured on a Bruker AMX400 spectrometer operating at a probe temperature of 329 K. The sample was repeatedly exchanged against D_2O (99.9 atom %D, Fluorochem Ltd) with intermediate freeze-drying, finally using 99.96% D_2O (Fluorochem Ltd). Chemical shifts were measured relative to internal acetone ($\delta = 2.225$ ppm for ^{1}H and 31.55 ppm for ^{13}C).

The 2D TOCSY experiment [15] was recorded using the sequence 90°-t_1-SL-acq where SL is a multiple of the MLEV-17 sequence flanked by two 2.5 ms trim pulses. The spin-lock field strength was 10.2 kHz and the spin-locking time was 120 ms. 512 experiments of 4K data points were recorded covering a spectral width of 969 Hz in each dimension. A relaxation delay of 1.5 s between scans was allowed. The data matrix was zero-filled to 1K data points in F1 and multiplied by a cosine window function in each dimension prior to Fourier transformation.

The 2D ROESY experiment [16] was recorded using the sequence 90°-t_1-SL-acq where SL is a continuous spin-lock pulse of 220 ms duration at a field strength of 2.2 kHz. The carrier frequency was placed to the left of the spectrum in order to minimize TOCSY type magnetization transfer. 512 experiments of 4K data points were recorded covering a spectral width of 1785 Hz in each dimension. A relaxation delay of 1.5 s between scans was allowed. The data matrix was zero-filled to 1K data points in F1 and multiplied by a $\pi/3$ shifted sine-bell window function in each dimension prior to Fourier transformation.

^{13}C–^{1}H correlation experiments [17] were recorded on an inverse probe. The HMQC

experiment was acquired with a 282 ms delay after the BIRD pulse, a dephasing/refocusing delay of 3.571 ms and a relaxation delay of 0.5 s between scans. GARP decoupling was applied during acquisition. The HMBC sequence was recorded with a 3.571 ms delay for the low-pass J filter, a 90 ms delay for evolution of long range couplings and a relaxation delay of 0.7 s. In each experiment 1024 increments of 2K data points were collected covering a spectral width of 969 Hz in F2 and 4424 Hz in F1. Before Fourier transformation the data matrix was zero-filled in F1 and/or F2 as necessary and multiplied by a cosine squared window function in F1 and an appropriate Gaussian window function in F2.

All spectra were acquired with presaturation of the HOD signal during the relaxation delay, in the HMQC experiment presaturation was also applied during the delay following the BIRD pulse. Where appropriate (i.e. with the exception of the HMBC experiment) spectra were recorded phase sensitive using TPPI for t_1 amplitude modulation [18].

3. Results and discussions

Oligosaccharides liberated from LBG galactomannan by *Aspergillus niger* β-D-mannanase digestion were separated by size exclusion chromatography on a Bio-Gel P-2 column. The elution pattern obtained (not shown) was identical to the one published by McCleary et al. [11] and the fraction believed to contain the digalactosylmannopentaose (Gal_2Man_5) was analysed further. The molecular weight of this fraction was determined by MALDI-TOF mass spectrometry which confirmed the presence of a heptasaccharide $(M + Na)^+ = 1174$ Da); small quantities of octasaccharides were also detected ($M + Na^+ = 1334$ Da). Impurities were also detectable in the HPAEC-PED chromatogram (Fig. 1) which contains additional peaks responsible for approximately 17.8% of the total PED response. The Man/Gal ratio of 2.3, as determined by monosaccharide composition analysis, is consistent with the expected structure of Gal_2Man_5.

The primary structure of Gal_2Man_5 was unambiguously determined using high-field ^{1}H- and ^{13}C-NMR. All NMR measurements were made at 329 K for two reasons: to shift the HOD resonance out of the spectral regions of interest in the ^{1}H dimension and also to ensure that the acquired chemical shift data could subsequently be compared to that obtained from polymeric systems (where it is usual to measure spectra at elevated temperatures in order to reduce solution viscosity). The resolution enhanced 1D ^{1}H-NMR spectrum of Gal_2Man_5 is shown in Fig. 2. Tentative assignment of the anomeric protons ($\delta = 4.7$ to 5.2; see Fig. 2) of this molecule was achieved by comparison to the spectra of the analogous mannotriose and mannopentaose oligomers. Assignment of the H-1 resonance of mannose residue **1** as being due to either the α or β anomer was done by comparison to data for mannose [19]. The H-1 resonances of the galactose residues ($\mathbf{G}_A$ and $\mathbf{G}_B$) overlap and can only be resolved using very severe resolution enhancement.

The positions of most of the Gal_2Man_5 ^{1}H-resonances were determined from a 2D TOCSY spectrum (not shown) acquired with a mixing time of 120 ms. The H-2 resonances from residues **5**, **4′**, **3′**, and **2** were immediately assignable from this spectrum (see Fig. 2 for assignments) and since the experiment was acquired with a long

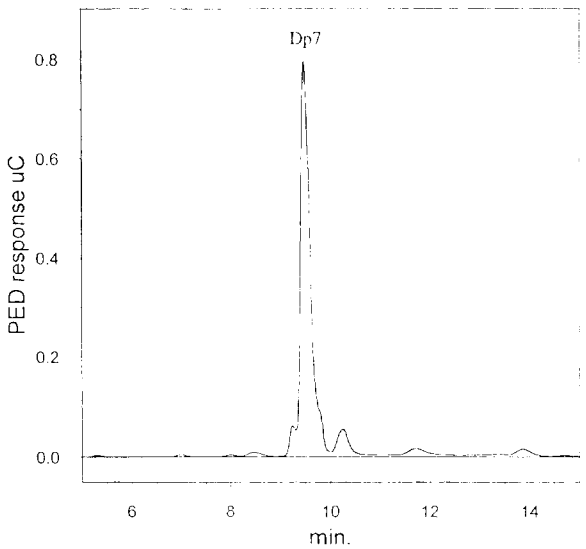


Fig. 1. HPAEC-PED elution profile of the BioGel P-2 galactomannan heptasaccharide fraction on a CarboPac PA-100 column.

mixing time, a section through the appropriate H-2 resonance allowed the total scalar coupled network of each of these residues to be observed (it is not possible to achieve this end by sectioning through the H-1 resonances in the TOCSY spectrum since the small $^{3}J_{H1,H2}$ coupling constant in mannose sugars renders homonuclear Hartmann-Hahn transfer from H-1 inefficient). Residue **5** was thus unambiguously assigned from the multiplet patterns of its resonances. Complete assignment of residues **4′**, **3′** and **2** was rendered difficult because of strong coupling between H-3 and H-4; however, in each case H-5 and one of the H-6 protons could be located in addition to H-1 and H-2. At the reducing end of the oligosaccharide the entire coupling network of residue **1** of the β anomer was available from the H-5 track of the TOCSY (at ca. 3.5 ppm); once again, overlap and strong coupling allowed the assignment of H-1, H-2, H-5 and one of the H-6's only. From the α anomer of residue **1** only H-2 was assignable in the TOCSY spectrum, other signals were present on the H-1 track but could not be interpreted at this point in the analysis. Signals from the individual galactose residues $\mathbf{G}_A$ and $\mathbf{G}_B$ were not separately resolved in the 2D spectrum: assignments for H-2, H-3 and H-4 were obtainable from any row through H-1, a sum of several rows revealed the much weaker H-5 and H-6 signals. The complete ^{1}H-NMR data set is collated in Table 1.

Because of overlap of signals and second-order effects in the ^{1}H-dimension, the assignment was continued using ^{13}C–^{1}H correlation spectroscopy. The 1D ^{13}C-{^{1}H} NMR spectrum is shown in Fig. 3 and the accumulated ^{13}C data are given in Table 2. The ^{13}C signals derived from mannose residue **5** were immediately assignable from the HMQC spectrum (not shown). For mannose residues **4′**, **3′** and **2** it was possible to assign C-1, C-2, C-5 and C-6 from the HMQC and to obtain the approximate positions of the C-3 and C-4 signals; however, the resolution was not good enough to distinguish

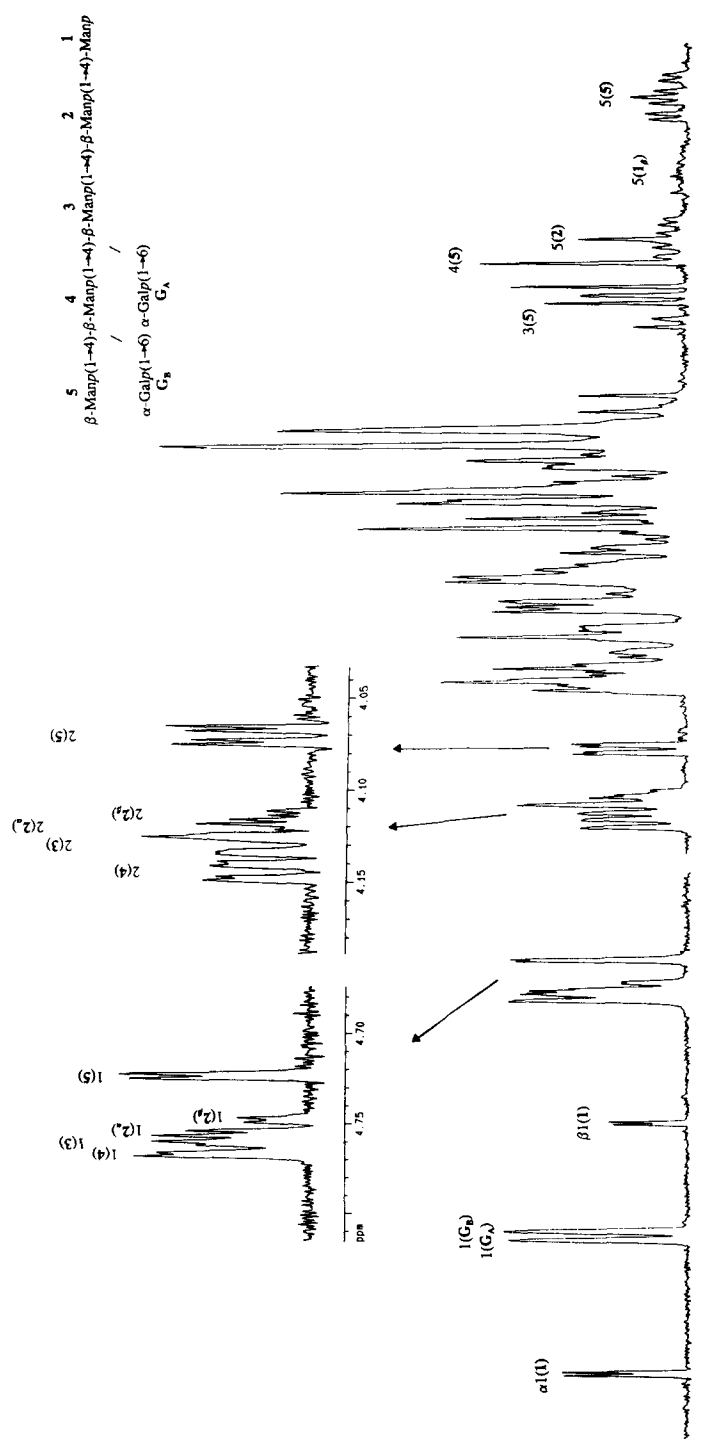


Fig. 2. Resolution enhanced 400 MHz ^{1}H-NMR spectrum of the digalactomannopentasaccharide showing assignments of the easily resolvable signals. The mannose residues are sequentially labelled **1** to **5** with residue **1** at the reducing end; $1(2_\alpha)$ denotes the anomeric proton (H-1) on mannose residue **2** where the reducing end of the sugar has the α configuration. The galactose residues are labelled $\mathbf{G}_A$ and $\mathbf{G}_B$, with the former being attached to residue **3**'.

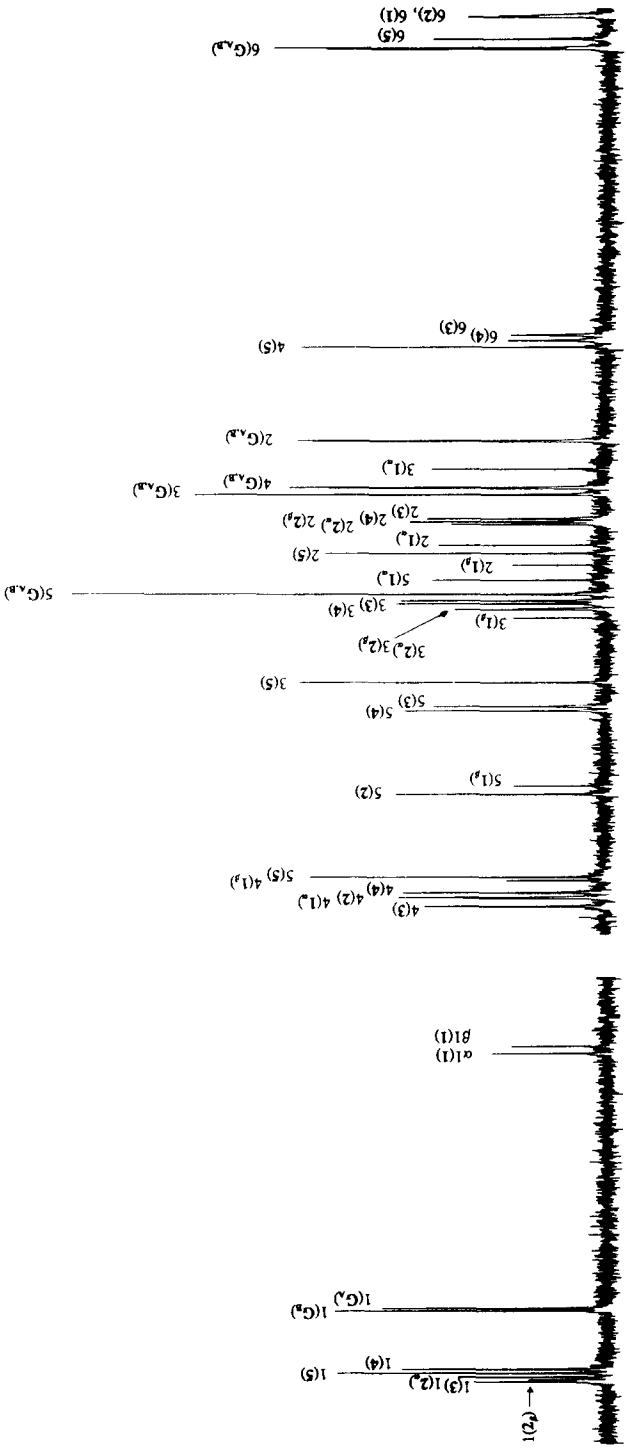


Fig. 3. ^{13}C-{^1H} NMR spectrum of the digalactomannopentasaccharide showing a complete assignment; the labelling scheme is analogous to that given in Fig. 2 for the proton resonances.

Table 1
The ^{1}H chemical shifts of galactomannan oligosaccharide Gal_2Man_5 [a]

Atom	Residue [b]						
	$\mathbf{1}_\alpha$	$\mathbf{1}_\beta$	**2**	**3′**	**4′**	**5**	$\mathbf{G}_{A,B}$
1	5.184	4.907	4.760 ($\mathbf{2}_\alpha$) 4.753 ($\mathbf{2}_\beta$)	4.766	4.772	4.728	5.032 ($\mathbf{G}_A$) 5.031 ($\mathbf{G}_B$)
2	4.00	4.004	4.127 ($\mathbf{2}_\alpha$) 4.122 ($\mathbf{2}_\beta$)	4.136	4.150	4.075	3.838
3	3.99	3.80	3.802	3.825	3.808	3.640	3.944
4	3.90	3.86	3.851	3.898	3.881	3.595	4.014
5	3.90	3.499	3.571	3.767	3.755	3.427	3.905
6	3.842	3.90–3.80	3.921	3.985	3.974	3.945	3.767
6′	3.755	3.746	3.80–3.75	3.801	3.810	3.748	

[a] Measured in ppm at 329 K relative to acetone at 2.225 ppm. [b] The Man residue in reducing position is denoted **1**, etc.: $\mathbf{2}_{\alpha/\beta}$ means reducing Man residue (**1**) has α/β configuration (anomerization effect).

Table 2
^{13}C-NMR chemical shift assignment of galactomannan oligosaccharide Gal_2Man_5 [a]

Atom	Residue						
	$\mathbf{1}_\alpha$	$\mathbf{1}_\beta$	**2**	**3′**	**4′**	**5**	$\mathbf{G}_{A,B}$
1	95.15	95.01	101.46 ($\mathbf{2}_\alpha$) 101.52 ($\mathbf{2}_\beta$)	101.55	101.31	101.38	100.11($\mathbf{G}_A$) 100.16($\mathbf{G}_B$)
2	71.67	72.03	71.29 ($\mathbf{2}_\alpha$) 71.27 ($\mathbf{2}_\beta$)	71.18	71.24	71.82	69.76 69.77
3	70.28	73.00	72.85 ($\mathbf{2}_\alpha$) 72.83 ($\mathbf{2}_\beta$)	72.68	72.74	74.19	70.74
4	78.18	77.86	78.17	78.33	78.08	68.05	70.63 70.61
5	72.31	76.12	76.28	74.64	74.72	77.79	72.57
6	61.93	61.93	61.93	67.83	67.93	62.34	62.52 62.50

[a] Measured in ppm at 329 K relative to acetone at 31.55 ppm.

the C-3 and C-4 signals from each other nor to assign individual resonances to a particular residue. Accurate assignment of these resonances was straightforward from the HMBC spectrum (Fig. 4): intra-residue H-2/C-3 and H-2/C-4 correlations are well enough resolved to assign the individual C-3 and C-4 lines to a given sugar unit. The C-1, C-2 and C-3 signals of residue **2** were split into two components, i.e. these signals show sensitivity to the α or β configuration at the reducing end of the sugar (anomerization effect).

The ^{13}C resonances of the galactose residues ($\mathbf{G}_A$ and $\mathbf{G}_B$) were assignable directly from the HMQC spectrum. The chemical environments of these two residues are apparently very similar since only very small chemical shift differences were discernable between them in the ^{13}C dimension. In the cases of C-1, C-2, C-4 and C-6 it was possible to distinguish between the $\mathbf{G}_A$ and $\mathbf{G}_B$ resonances, but because of the lack of resolution in the ^{1}H dimension only C1 could be unambiguously assigned to either $\mathbf{G}_A$ or $\mathbf{G}_B$. This was achievable because the resolution in the ^{1}H dimension allowed C-1($\mathbf{G}_A$) and C-1($\mathbf{G}_B$) to be distinguished in the HMQC spectrum; H-1($\mathbf{G}_A$) and H-1($\mathbf{G}_B$) are assigned as being linked to mannose residues **3′** and **4′** respectively as described below.

This leaves only residue **1** to be assigned in the ^{13}C dimension. For the α anomer,

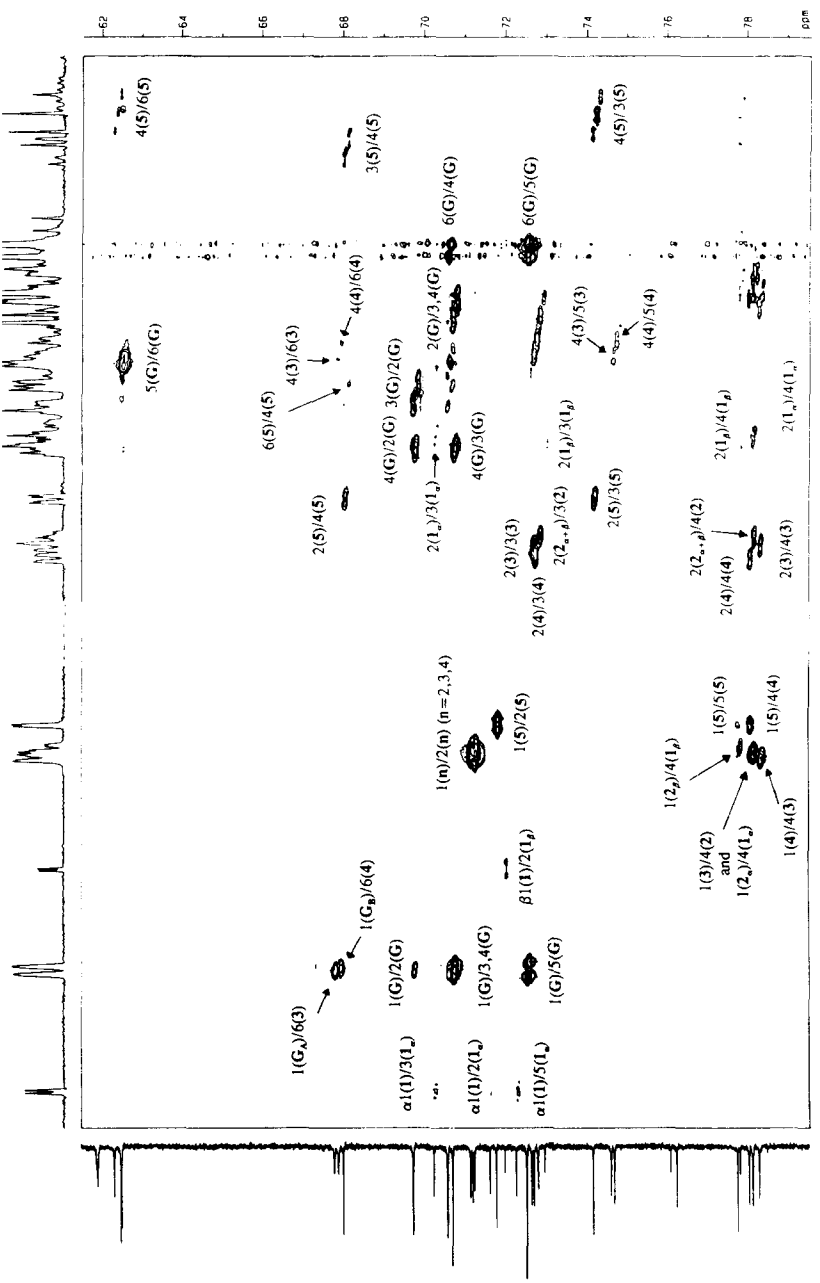


Fig. 4. Partial HMBC spectrum of the digalactomannopentasaccharide with labelled $^{13}C-^{1}H$ correlations. The labelling scheme is as follows: proton no. (residue)/carbon no. (residue).

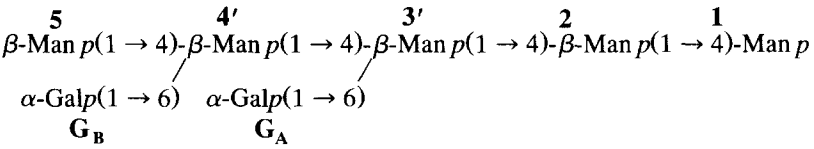
C-1 and C-6 were directly available from the HMQC (the peaks at δ = ca. 62 in the ^{13}C dimension and δ = ca. 3.85 in the ^{1}H dimension do not overlap with the scalar coupled networks of any residue except the one in question). Since H-2 overlapped in the proton dimension with another signal from this sugar unit (subsequently determined to be H-3), C-2 and C-3 were also observable in the HMQC but were not immediately distinguishable. The remaining assignments for this anomer were obtained from the HMBC spectrum where intra-residue H-1/C-2, C-3, C-5 and H-2/C-3, C-4 correlations were observed, thereby assigning C-3 (correlating to both H-1 and H-2) and C-4. In addition, assignment of C-3 allowed C-2 to be unambiguously identified which thus gave C-5 by a process of elimination. Assignment of residue **1** of the β anomer is as follows: C-1, C-2 and C-5 were available from the HMQC and H-2/C-3, C-4 correlations were seen in the HMBC; C-3 and C-4 were distinguished by comparison of their chemical shifts to those of the other mannose residues in the molecule. No $^{1}H/^{13}C$ correlations assigning C-6($\mathbf{1}_{\beta}$) were observed, it is therefore assumed to overlap with the C-6 resonance of the α anomer. Comparative data for β-Man*p*$(1 \rightarrow 4)$-β-Man*p*$(1 \rightarrow 4)$-Man*p* suggests this assumption is reasonable [20].

Once complete ^{13}C assignment was achieved remaining uncertainties in the ^{1}H assignment were resolved by examination of the HMQC and HMBC spectra.

Having assigned all of the ^{1}H and ^{13}C resonances, the primary structure is directly obtainable from the HMBC spectrum where three-bond inter-residue $^{1}H/^{13}C$ correlations are detectable. Reference to Fig. 4 demonstrates that the following correlations are clearly seen: H-1(**5**)/C-4(**4′**), H-1(**4′**)/C-4(**3′**) and H-1($\mathbf{2}_{\beta}$)/C-4($\mathbf{1}_{\beta}$). Although the correlation peak H-1(**3′**)/C-4(**2**) is not resolvable from that for H-1($\mathbf{2}_{\alpha}$)/C-4($\mathbf{1}_{\alpha}$) there is no ambiguity in the assignment of these peaks since no alternative assignment is chemically feasible given that the linkage of residues **2** to **1** is already established by H-1($\mathbf{2}_{\beta}$)/C-4($\mathbf{1}_{\beta}$). Therefore the initial assignment of the anomeric protons is confirmed and $(1 \rightarrow 4)$ linked mannan backbone is established. Finally, the H-1($\mathbf{G}_{A}$)/C-6(**3′**) and H-1($\mathbf{G}_{B}$)/C-6(**4′**) correlations establish the $(1 \rightarrow 6)$ attachment of $\mathbf{G}_{A}$ and $\mathbf{G}_{B}$ to mannose residues **3′** and **4′** respectively.

The conformation and anomeric configuration of the individual sugars was confirmed by a ROESY experiment (not shown). The presence of rotating frame NOEs from H-1 to H-2, H-3 and H-5 is consistent with the β-pyranose $^{4}C_{1}$ conformation for the mannose residues. Data for the galactose residues are less clear: NOEs from H-3 to H-1, H-2, H-4, H-5 and from H-4 to H-2, H-3, (H-5), H-6 are indicative of the α-pyranose configuration; however, the ring structure seems to be more flexible, as might be expected for a terminal sugar. Additional evidence for the α-pyranose configuration of the galactose residues comes from the ^{1}H-NMR data given in Table 1 which are more consistent with values published for the terminal α-Gal*p* structure [19] than the terminal α-Gal*f* structure [21]. Due to severe overlap of some ^{1}H signals and low NOE intensities in the ROESY spectrum, caused by the highly increased rotational mobility of the separate monomer units at the temperature used, no attempt was made to measure $^{1}H-^{1}H$ distances enabling the elucidation of the 3D-solution conformation of Gal_2Man_5. Using higher-field NMR and lower temperatures, to limit the conformational freedom of the molecule, might enable us to do so.

The NMR assignment is consistent with the structure:



4. Conclusions

The use of 2D homonuclear and heteronuclear NMR has made possible the unambiguous assignment of the ^{1}H and ^{13}C signals of the sole digalactomannopentaose derived from the *Aspergillus niger* β-D-mannanase digest of LBG. The dataset thus obtained will be of importance in the primary structure elucidation of larger and more complex galactomannan oligomers. In particular the unambiguous assignments of the carbon resonances will aid in the study of structural features in larger oligosaccharides and ultimately in polymers. Combining structure determination with quantitative analyses of the *Aspergillus niger* β-D-mannanase digests of different Legume-seed galactomannans will enable us to refine the second-order Markov model describing the galactose distribution along the galactan backbone in a statistical manner [10]. Such refinement should bring us a step closer to a good description of the structure ↔ function relationship of galactomannans. The NMR techniques described in this report will be used in combination with limited enzymatic degradation to elucidate the primary structure of the galactomannan decasaccharide, isolated from an *Aspergillus niger* β-D-mannanase digest of Tara gum.

Acknowledgements

We want to thank John T. Mitchell for the chemical analysis, John Wilkins for recording the MS spectrum and Rick Peppermans for helpful advice on the set-up of the NMR experiments.

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