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PREPARATIVE-SCALE ISOLATION AND CHARACTERISATION OF 6^1-α-D-GALACTOSYL-(1→4)-β-D-MANNOBIOSE AND 6^2-α-D-GALACTOSYL-(1→4)-β-D-MANNOBIOSE

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ABSTRACT

N.m.r., enzymic, and chemical techniques have been used to characterise the D-galactose-containing tri- and tetra-saccharides produced on hydrolysis of carob and *L. leucocephala* D-galacto-D-mannans by Driselase β-D-mannanase. These oligosaccharides were shown to be exclusively 6^1-α-D-galactosyl-β-D-mannobiose and 6^1-α-D-galactosyl-β-D-mannotriose. Furthermore, these were the only D-galactose-containing tri- and tetra-saccharides produced on hydrolysis of carob D-galacto-D-mannan by β-D-mannanases from other sources, including *Bacillus subtilis*, *Aspergillus niger*, *Helix pomatia* gut solution, and germinated legumes. Acid hydrolysis of lucerne galactomannan yielded 6^1-α-D-galactosyl-β-D-mannobiose and 6^2-α-D-galactosyl-β-D-mannobiose.

INTRODUCTION

Galactomannan polysaccharides from guar and carob seeds consist of a (1→4)-β-D-mannan backbone to which single (1→6)-α-D-galactopyranosyl groups are attached[1]. This basic structure has been confirmed by chemical and enzymic techniques, but there is still considerable disagreement over the arrangement of the D-galactosyl groups along the D-mannan backbone, *i.e.*, the "fine-structure"[2–5].

The value of enzymic techniques in the analysis of the fine structures of polysaccharides is well established. Such studies require the use of well-characterised, highly purified enzymes (devoid of interfering activities) and complete characterisation of the hydrolysis products. Numerous studies of the hydrolysis of galactomannans by endo-β-D-mannanase (EC 3.2.1.78) have been performed[4–7], but, in most cases, these have involved the use of partially purified enzymes. In several cases,

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the β-D-mannanase was obviously contaminated with other enzymes, including α-D-galactosidase and β-D-mannoside mannohydrolase (*i.e.*, exo-β-D-mannanase or β-D-mannosidase, EC 3.2.1.25)[7,8]; in other instances, critical assays to demonstrate the purity of the enzymes apparently were not performed[9].

Oligosaccharides produced on hydrolysis of galactomannans by β-D-mannanase preparations include the β-D-manno-oligosaccharides of degree of polymerisation (d.p.) 2–6, as well as a range of mixed oligosaccharides containing both D-galactose and D-mannose, *i.e.*, *O*-α-D-Gal*p*-(1→6)-D-Man*p*, *O*-α-D-Gal*p*-(1→6)-*O*-β-D-Man*p*-(1→4)-D-Man*p*, *O*-α-D-Gal*p*-(1→6)-*O*-β-D-Man*p*-(1→4)-D-Man*p*-(1→4)-D-Man*p*, a pentasaccharide (Man/Gal = 4:1), and a hexa- and a hepta-saccharide (Man/Gal = 4:2 and 4:3, respectively)[10–12]. Reese and Shibata[13] suggested that the galactose-containing tetrasaccharide released on hydrolysis of carob galactomannan by *Bacillus subtilis* β-D-mannanase contained the D-galactosyl branch on the central D-mannosyl residue of β-D-mannotriose. However, subsequent methylation analysis[10] of this tetrasaccharide and a galactose-containing trisaccharide (also released on hydrolysis of carob galactomannan by *B. subtilis* β-D-mannanase) indicated that, in both cases, the galactosyl branch-unit was exclusively located on the terminal, non-reducing D-mannosyl residue.

The potential use of highly purified β-D-mannanases in the analysis of the "fine-structures" of galactomannans has been demonstrated[4], and enzymes with quite different action patterns have been identified[14]. However, the effective application of β-D-mannanases to such studies requires a detailed knowledge of their action patterns and substrate requirements. Part of this information will be afforded by characterisation of the resistant oligosaccharides produced on hydrolysis of galactomannans by the enzymes.

We have used chemical, physical, and enzymic techniques to characterise the mixed trisaccharide and tetrasaccharide produced on hydrolysis of carob and *Leucaena leucocephala* galactomannans by β-D-mannanase from Driselase preparation. From these studies, techniques have been developed for the preparation, in reasonable yields, of 6-*O*-α-D-galactopyranosyl-4-*O*-β-D-mannopyranosyl-α,β-D-mannopyranose and *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-α,β-D-mannopyranose, *i.e.*, 6^1-α-D-galactopyranosyl-β-D-mannobiose and 6^2-α-D-galactopyranosyl-β-D-mannobiose*, respectively.

EXPERIMENTAL

Preparation of galactomannans. — Guar (*Cyamopsis tetragonolobus*) and carob (*Ceratonia siliqua*) galactomannans were prepared from the commercial flours as previously described[16]. Lucerne (*Medicago sativa*) and *Leucaena leucocephala* galactomannans were prepared from milled, whole seeds.

Preparation of enzymes. — α-D-Galactosidase II from germinated guar-seeds

*Named by the short-hand notation introduced by Whelan[15] for branched gluco-oligosaccharides.

was prepared by a technique similar to that used[17] for preparation of lucerne-seed α-D-galactosidase A. The purified enzyme has a specific activity of 870 nkat/mg on *p*-nitrophenyl α-D-galactopyranoside (at pH 5.0 and 40°), appeared as a single protein band in SDS-electrophoresis and iso-electric focusing, and was completely devoid of the interfering enzymes β-D-mannanase and β-D-mannosidase. Driselase β-D-mannanase and Cellulase-preparation β-D-mannanase, prepared as previously described[14], were completely devoid of interfering activities. The last traces of α-D-galactosidase present in Cellulase-preparation β-D-mannanase were removed by chromatography on *N*-ε-aminocaproyl-α-D-galactopyranosylamine–Sepharose 4B. Exo-β-D-mannanase, prepared from germinating guar-seeds[18], gave a single protein band in SDS-electrophoresis, had a specific activity of 967 nkat/mg on mannopentaitol (pH 5.5, 40°), and was completely devoid of α-D-galactosidase and β-D-mannanase activities. β-D-Mannosidase, prepared from crude *Helix pomatia* snail-gut juice (Sigma, G 0876)[19], gave a single protein band in SDS-electrophoresis, had a specific activity of 1590 nkat/mg on *p*-nitrophenyl β-D-mannopyranoside (pH 5.0, 40°), and was completely devoid of α-D-galactosidase and β-D-mannanase activities.

Chromatography. — T.l.c. was performed on DC-Alufolien, Kieselgel 60 (0.2 mm), prepared plates which were developed twice with 7:1:2 1-propanol–ethanol–water. Spots were detected by spraying with 5% sulphuric acid in ethanol and heating to 110°.

Gel-permeation chromatography was performed on a column (3.5 × 80 cm) of Bio-Gel P-2 (<400 mesh) at 60° in distilled water[20].

Preparation of oligosaccharides by enzymic hydrolysis. — To solutions of carob or *Leucaena leucocephala* galactomannan (1 L, 0.5%, pH 5 unbuffered) was added Driselase β-D-mannanase (2 μkat on carob galactomannan), and the solutions were incubated at 40° for 20 h. Each solution was incubated at 100° to denature β-D-mannanase, concentrated to a syrup, and adjusted to 100 mL, and ethanol (200 mL) was added with stirring. After storing at 4° for 20 h, the mixture was centrifuged, the supernatant was concentrated to dryness, and the residue was dissolved in water (30 mL). Aliquots (3–5 mL) of this solution were fractionated by chromatography on Bio-Gel P-2. The trisaccharide fraction contained α-D-galactosyl-β-D-mannobiose and a trace of β-D-mannotriose. The β-D-mannotriose was removed by treatment of a solution of the oligosaccharide with exo-β-D-mannanase from guar seeds (10 nkat on β-D-mannopentaitol/100 mg) for 6 h at 40° and pH 5.5. The hydrolysis products were chromatographed on Bio-Gel P-2, to give pure α-D-galactosyl-β-D-mannobiose (160 mg/g of galactomannan)[4].

α-D-Galactosyl-β-D-mannobiose and α-D-galactosyl-β-D-mannotriose were produced by hydrolysis of carob galactomannan by Driselase β-D-mannanase, under conditions identical to those used for the hydrolysis of *Leucaena leucocephala* galactomannan. The trisaccharide fraction contained ~70% of β-D-mannotriose and 30% of α-D-galactosyl-β-D-mannobiose. The β-D-mannotriose was hydrolysed by incubation with guar-seed exo-β-D-mannanase (20 nkat/100 mg of trisaccharide fraction) at 40° and pH 5.5 for 6 h, and removed by Bio-Gel P-2 chromatography[20]. The

tetrasaccharide fraction recovered from Bio-Gel P-2 chromatography of the galactomannan hydrolysate contained only α-D-galactosyl-β-D-mannotriose. The tri- and tetra-saccharide fractions produced on hydrolysis of carob galactomannan by β-D-mannanases from *Bacillus subtilis*, from lucerne and honey-locust seeds, and from a commercial Cellulase preparation, under conditions identical to those described, were also purified and characterised.

Preparation of oligosaccharides by acid hydrolysis[21]. — Finely milled, lucerne galactomannan (10 g) was added to conc. HCl (100 mL) and stirred for 2 min at room temperature. Ice-cold fuming HCl (100 mL) was then added and the solution was stirred in a sealed flask at room temperature for 4 h. The solution was then added to ice-cold water (1 L), neutralised by careful addition of sodium hydrogencarbonate, and concentrated to dryness, the residue was redissolved in water (250 mL), and ethanol (1 L) was added. After storage at 4° for 24 h and filtration, the solution was concentrated to dryness (<40°) and the residue was dissolved in water (40 mL). Aliquots (5 mL) of this solution were chromatographed[20] on Bio-Gel P-2. The trisaccharide fraction was recovered and treated with guar-seed exo-β-D-mannanase to remove β-D-mannotriose. The remaining trisaccharide consisted of 6^1-α-D-galactosyl-β-D-mannobiose and 6^2-α-D-galactosyl-β-D-mannobiose (see Results and Discussion) in the ratio ~30:70. 6^1-α-D-Galactosyl-β-D-mannobiose could be removed by treatment of the trisaccharide fraction with β-D-mannosidase (500 nkat/50 mg) at pH 5 and 40° for 20 h, followed by Bio-Gel P-2 chromatography. The yield of 6^2-α-D-galactosyl-β-D-mannobiose was 1.5% of the original polysaccharide.

Enzymic hydrolysis of the α-D-galactosyl-β-D-mannobiose and α-D-galactosyl-β-D-mannotriose preparations. — Solutions of oligosaccharide (20 μL, 10 mg/mL) were incubated with guar-seed α-D-galactosidase[19] II (20 μL, 10 nkat on *p*-nitrophenyl α-D-galactopyranoside) plus acetate buffer (5 μL, 0.1M, pH 5); or snail-juice β-D-mannosidase (20 μL, 20 nkat on *p*-nitrophenyl β-D-mannopyranoside) plus acetate buffer (5 μL, 0.1M, pH 5); or guar-seed exo-β-D-mannanase (20 μL, 40 nkat on β-D-mannopentaitol) plus acetate buffer (5 μL, 0.1M, pH 5.5); or Cellulase preparation β-D-mannanase (20 μL, 6 nkat on carob galactomannan) plus acetate buffer (5 μL, 0.1M, pH 4.5). Incubations were performed at 40° for 20 h and aliquots (15 μL) applied to t.l.c. plates.

Methylation analyses[22]. — (*a*) Reduced oligosaccharide (5 mg) was dissolved in freshly distilled dimethyl sulphoxide (3 mL), and a solution of sodium hydride in dimethyl sulphoxide (1.7 mL) was added. The solution was stirred for 5 h under nitrogen. Methyl iodide (2 mL) was then added during 1 h, the solution was extracted with chloroform, and the extract was concentrated. One-fifth of the resulting product was totally hydrolysed using 90% formic acid (1 mL) at 100° for 1 h. After evaporation, the residue was treated with 2M trifluoroacetic acid at 100° for 3 h, and the solution was then concentrated. The resulting monosaccharides were reduced with sodium borohydride and then acetylated, giving alditol acetates that were subjected to g.l.c.–m.s.[23].

G.l.c. of the alditol acetates of methylated sugars was performed on a column

of 3% of SP 23 40 on Chromosorb W-AW (DMCS 100–200 mesh). The alditol acetates of methylated sugars were analysed by coupled g.l.c.–m.s. Mass spectra were recorded with an A.E.I. MS30 spectrometer coupled with a Girdel 3000 instrument, using a 25-m capillary column (SP 23 40); column temperature 180→220° with a temperature gradient of 2° per min, 2 min after application of the sample. The operating conditions for the electron-impact m.s. were: ionisation energy, 70 eV; ionisation current, 100 μA; accelerating voltage, 4 kV; ion-source temperature, 150°.

(*b*) Methylation of α-D-galactosyl-β-D-mannobiose (20 mg) from the enzymic hydrolysis of *L. leucocephala* galactomannan was carried out in *N*,*N*-dimethylformamide by the method of Brimacombe *et al.*[24]. Hydrolysis of the methylated product (15 mg) was effected in 10% hydrochloric acid at 95° for 1 h. After neutralisation (barium carbonate) and deionisation (Zerolit DM-F), the hydrolysate was analysed by h.p.l.c. and mass spectrometry.

A Waters Associates analytical h.p.l.c. system was used, comprising a M6000 pump, U6K injector, R401 refractive-index detector, radial compression module RCM 100, and Dextropak plastic column (10 × 0.8 cm) which, for use, was compressed in the RCM 100. The solvent employed was water–ethanol (9 : 1) at 2 mL/min. The following methylated derivatives were identified by comparison of their retention times with those of standards: 2,3-di-*O*-methyl-D-mannose (2.5 min), 2,3,4,6-tetra-*O*-methyl-D-galactose (6.0 and 6.5 min, α and β anomers), 2,3,4,6-tetra-*O*-methyl-D-mannose (7.1 and 7.8 min), and 2,3,6-tri-*O*-methyl-D-mannose (trace, 4.6 min). Samples of the methylated sugars were also collected from the h.p.l.c. and subjected to chemical-ionisation m.s. in order to confirm their identities. Chemical ionisation mass spectra were run on an AEI MS 902 double-focusing mass spectrometer modified with an SRI CI S2 Chemspec source: source temperature, 180°; accelerating voltage, 8000 V; ionising voltage, 450 V; electron emission, 0.5 mA. The ionising gas was ammonia.

N.m.r. spectroscopy. — Samples of β-D-mannobiose and α-D-galactosyl-β-D-mannobiose (from β-D-mannanase hydrolysis of *Leucaena leucocephala* galactomannan and from acid hydrolysis of lucerne galactomannan) were dissolved in D_2O (80 mg/mL). The deuterium resonance was used as the field frequency lock, and acetone as the internal reference (5%). Experiments were performed at 25° and 250 MHz for ^1H spectra, and at 62.86 MHz for ^{13}C spectra, with a Cameca spectrometer.

^1H Spectra were acquired in the continuous wave (c.w.) mode and partial assignments were allowed by the INDOR technique. Acetone [δ 2.23 p.p.m. downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.)] was used as internal standard.

^{13}C Spectra were obtained in the Fourier-transform mode, using 8192 data points and a spectral width of 5000 Hz. Free induction decays were accumulated with a 13-μs pulse and an acquisition time of 1.64 s (resolution, 0.61 Hz per point). Unequivocal ^{13}C assignments were made by using the selective, heteronuclear, spin-decoupling technique[25–27]. Chemical shifts were expressed in p.p.m. downfield from D.S.S., with acetone as internal standard (31.07 p.p.m. from D.S.S.).

6^2-α-D-Galactosyl-β-D-mannobiose, prepared as described in the text, was analysed using the same conditions as for the other oligosaccharides, with the following exceptions: sample concentration in D_2O, 30 mg/mL; ^1H spectra were acquired in the c.w. and F.t. mode; ^{13}C spectra were obtained with a spectral width of 12,500 Hz, an acquisition time of 0.65 s, and a digital resolution of 1.52 Hz per point.

RESULTS AND DISCUSSION

Methylation studies. — G.l.c. of the methylation products (method *a*) of the D-galactosyl-β-D-mannobiose obtained on β-D-mannanase hydrolysis of *Leucaena leucocephala* galactomannan showed three peaks. Two of these corresponded to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, and gave mass spectra in agreement with the known fragmentations[23]. The third peak corresponded to 4,6-di-*O*-acetyl-1,2,3,5-tetra-*O*-methyl-D-mannitol and was identified by mass spectrometry (primary fragments at *m/z*: 45, 89, 117, 233, and 277). Peaks corresponding to 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methyl-D-mannitol and to 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol were absent. These results indicate that this oligosaccharide is pure and is 6-*O*-α-D-galactopyranosyl-4-*O*-β-D-mannopyranosyl-α,β-D-mannopyranose, *i.e.*, 6^1-α-D-galactosyl-β-D-mannobiose. This conclusion was also reached by using the second methylation procedure (see Experimental). In this case, the ratios of dimethylmannose/tetramethylmannose/tetramethylgalactose were close to 1 : 1 : 1. The only other peak detected in the h.p.l.c. was due to 2,3,6-tri-*O*-methyl-D-mannose (~2.5%). As noted previously, the α and β anomers of certain methylated sugars are resolved by this chromatography system[28].

The D-galactosyl-β-D-mannobiose produced on acid hydrolysis of lucerne galactomannan showed five peaks in g.l.c. of the alditol acetates of the methylated sugars. Three of these peaks corresponded to those obtained on analysis of the enzymically produced D-galactosyl-β-D-mannobiose. The others were identified as belonging to 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methyl-D-mannitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol, and were characterised by their mass spectra. The ratio of these fragments indicated that this compound is a mixture of 6^2-α-D-galactopyranosyl-(1→4)-β-D-mannobiose and 6^1-α-D-galactopyranosyl-(1→4)-β-D-mannobiose in the ratio 2 : 1.

N.m.r. data for the α-D-galactosyl-β-D-mannobiose produced by β-D-mannanase hydrolysis of L. leucocephala *galactomannan.* — In the 250-MHz, ^1H-n.m.r. spectra of β-D-mannobiose, and of the β-D-mannanase-produced α-D-galactosyl-β-D-mannobiose, the resonances of the anomeric protons were well separated and their identification was consistent with the literature data[29–32] for monomeric α- and β-D-mannopyranose and for α-D-galactopyranosyl and β-D-mannopyranosyl residues (Tables I and II). Furthermore, the signal ratios for H-1α and H-1β were 70:30 for both compounds. A partial assignment of the other protons was made by using the INDOR technique. The chemical shifts and coupling constants obtained for the non-reducing D-mannose protons of β-D-mannobiose and α-D-galactosyl-β-D-mannobiose

TABLE I

CHEMICAL SHIFTS[a] (p.p.m.) AND COUPLING CONSTANTS (Hz) FOR THE ANOMERIC PROTONS OF β-D-MANNOBIOSE AND α-D-GALACTOSYL-β-D-MANNOBIOSE[b]

	H-1α	*H-1β*	*H-1'[c]*	*H-1 Gal*
Man_2	5.12	4.85	4.69	
Gal-Man_2	5.13	4.87	4.7	4.96
	$J_{1,2}$			
Man_2	1.6	1.0	0.9	
Gal-Man_2	1.6	1.0	0.9	3.0

[a]Relative to D.S.S. with acetone as internal standard (2.23 p.p.m. from D.S.S.). [b]The galactose-containing trisaccharide released on hydrolysis of *L. leucocephala* galactomannan by Driselase β-D-mannanase. [c]Refers to the non-reducing mannosyl residue.

TABLE II

CHEMICAL SHIFTS (p.p.m.) AND COUPLING CONSTANTS (Hz) FOR SOME PROTONS OF β-D-MANNOBIOSE AND α-D-GALACTOSYL-β-D-MANNOBIOSE[a]

	H-1'[b]	*H-2'*	*H-3'*	*H-4'*	*H-5'*	*H-6A'*	*H-6B'*	*H-2 (Gal)*	*H-2α,β*
Man_2	4.69	4.02	3.61	3.52	3.38	~3.89	~3.68		3.93
Gal-Man_2	4.7	4.04	3.59	3.52	3.37	~3.90	~3.66	3.81	—
	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{5',6A'}$	$J_{5',6B'}$			
Man_2	0.9	2.7	9.5	9.5	1.9	6.8			
Gal-Man_2	0.9	2.7	9.0	9.0	1.6	6.8			

[a]The galactose-containing trisaccharide released on hydrolysis of *L. leucocephala* galactomannan by Driselase β-D-mannanase. [b]The primed numbers refer to the non-reducing mannosyl residue.

are shown in Table II. The closeness of the respective chemical shifts for both compounds indicates that their electronic environments are very similar. Thus, for α-D-galactosyl-β-D-mannobiose, the non-reducing D-mannose is not perturbed by the α-D-galactopyranosyl group, supporting the results obtained on methylation analysis of this oligosaccharide, *i.e.*, that the D-galactosyl residue is attached to the reducing D-mannose. Further evidence that this is the case is given by the ^{13}C-n.m.r. data.

The proton-decoupled, ^{13}C-n.m.r. chemical shifts for α-D-galactosyl-β-D-mannobiose produced on β-D-mannanase hydrolysis of *Leucaena leucocephala* galactomannan, and for β-D-mannobiose, are presented in Table III. Assignments for the non-reducing D-mannose group were facilitated by selective, heteronuclear, spin-decoupling experiments[25–27]. The resonances associated with the reducing

TABLE III

^{13}C-N.M.R. CHEMICAL SHIFTS[a] (p.p.m.) FOR β-D-MANNOBIOSE, 6^1-α-D-GALACTOSYL-β-D-MANNOBIOSE, AND 6^2-α-D-GALACTOSYL-β-D-MANNOBIOSE

		Man$_2$	6^1-α-D-*Galactosyl*-β-D-*mannobiose*[b]	6^2-α-D-*Galactosyl*-β-D-*mannobiose*[c]
Reducing D-mannose residue	C-1 α	94.6	94.65	94.60
	β	94.5	94.60	94.45
	C-2 α	71.0	70.95	70.90
	β	71.4	71.35	71.25
	C-3 α	69.8	69.7	69.75
	β	72.5	72.45	72.50
	C-4 α	77.6	77.85	78.10
	β	77.3	77.5	77.85
	C-5 α	71.7	70.25	71.55
	β	75.6	74.05	75.45
	C-6 α	61.3	67.4	61.35
	β		67.3	
Non-reducing D-mannosyl residue	C-1′	101	100.7(α) and 100.8(β)[d]	101.15[d]
	C-2′	71.4	71.35	71.25
	C-3′	73.65	73.65	73.65
	C-4′	67.5	67.5	67.40 or 67.10
	C-5′	77.2	77.3	75.30
	C-6′	61.85	61.8	67.10 or 67.40
D-Galactosyl residue	C-1		99.65(α) and 99.55(β)[d]	99.15
	C-2		69.25	69.25
	C-3		70.30	70.20
	C-4		70.05	70.05
	C-5		72.1	71.75
	C-6		62.0	61.90

[a]Chemical shifts are expressed downfield from D.S.S. with acetone as internal standard (31.07 p.p.m. from D.S.S.). [b]The galactose-containing trisaccharide released on hydrolysis of *L. leucocephala* galactomannan by Driselase β-D-mannanase. [c]The galactose-containing trisaccharide from acid hydrolysis of lucerne galactomannan which is resistant to hydrolysis by snail β-D-mannosidase. [d]These signals are sensitive to the anomeric configuration of the reducing D-mannose residue.

D-mannose (for both compounds) and the D-galactosyl (for α-D-galactosyl-β-D-mannobiose) residues were distinguished (*a*) by comparison with the resonances of methyl α-D-galactopyranoside[33–37], and of α- and β-D-mannose[38–40]; (*b*) by making use of the anomeric composition of the compounds as defined by ^{1}H-n.m.r. analysis; and (*c*) by comparison between the resonances observed for both compounds.

Considerable data were obtained from the spectral region of the anomeric carbons (94–101 p.p.m.) of α-D-galactosyl-β-D-mannobiose. Each sugar residue gave two C-1 resonances with intensities in the α:β-ratio (defined previously). This

corresponds to a structural arrangement in which each C-1 resonance is sensitive to the anomeric configuration of the reducing D-mannose residue, indicating that the α-D-galactopyranosyl group was linked to the reducing D-mannose and not to the non-reducing D-mannose. In the latter case, only C-1 of each D-mannose residue would show two resonances.

Examination of the C-6 spectral region (60–62 p.p.m.) showed two signals corresponding to two carbons. The third C-6 signal was found at lower field (67.4 and 67.3, as it is also sensitive to the anomeric configuration of the reducing D-mannose residue) in agreement with a downfield shift of 6 p.p.m., which is commonly experienced for a carbon attached to a glycosidic linkage[27,31,41].

Other interesting features were given by the chemical shifts of the signals for C-5α and C-5β (these were assigned unambiguously using peak intensities). These signals were shifted by 1.5 p.p.m. upfield relative to the corresponding resonances of β-D-mannobiose, in agreement with what occurs to a carbon adjacent to a carbon attached to a glycosidic linkage (β-effect). Similar, but much smaller, shifts (0.2 p.p.m.) were shown by C-4α and C-4β. These results provide further evidence that the structure of the trisaccharide is 6-*O*-α-D-galactopyranosyl-4-*O*-β-D-mannopyranosyl-α,β-D-mannopyranose. Similar shifts for anomeric carbons attached to a glycosidic bond and for adjacent carbons (downfield shift by 6–8 p.p.m. and upfield shift by 1–2 p.p.m., respectively) can be detected in going from α-D-galactose to an α-D-galactosyl residue, and from α- or β-mannose to β-D-mannobiose.

N.m.r. data for the α-D-galactosyl-β-D-mannobiose produced by acid hydrolysis of lucerne galactomannan. — The ^{13}C-n.m.r. spectrum of this α-D-galactosyl-β-D-mannobiose is complex and is the sum of the spectra for 6^1-α-D-galactosyl-β-D-mannobiose and 6^2-α-D-galactosyl-β-D-mannobiose. The ratio between 6^1-α-D-galactosyl-β-D-mannobiose and 6^2-α-D-galactosyl-β-D-mannobiose is ~30:70. 6^2-α-D-Galactosyl-β-D-mannobiose was obtained devoid of the other trisaccharide by treatment of the mixture with snail β-D-mannosidase. This enzyme hydrolyses 6^1-α-D-galactosyl-β-D-mannobiose to D-mannose and 6-*O*-α-D-galactosyl-α,β-D-mannose (Fig. 1), which are removed from 6^2-α-D-galactosyl-β-D-mannobiose by Bio-Gel P-2 chromatography. The proton-decoupled, ^{13}C-n.m.r. spectrum of 6^2-α-D-galactosyl-β-D-mannobiose was obtained at 62.86 MHz, and the assignments (Table III) were made as in the case of β-D-mannobiose and 6^1-α-D-galactosyl-β-D-mannobiose, by selective, heteronuclear, spin-decoupling experiments and by comparison between the resonances observed for the different compounds.

The spectral region of the anomeric carbons (94–101 p.p.m.) is in good agreement with the known formula of the compound. The resonance for C-1′ of the non-reducing D-mannosyl group is a wide peak, as this carbon is sensitive to the anomeric configuration of the reducing D-mannose residue. In contrast, C-1 of galactose shows a sharp line, as it is not sensitive to this phenomenon, being much too far from C-1α and C-1β. Examination of the C-6 spectral region (60–62 p.p.m.) shows two signals corresponding to two carbons not attached to a glycosidic linkage. The signal of the third carbon, C-6′, is found at a lower field (67.10 or 67.40 p.p.m.), in agreement with

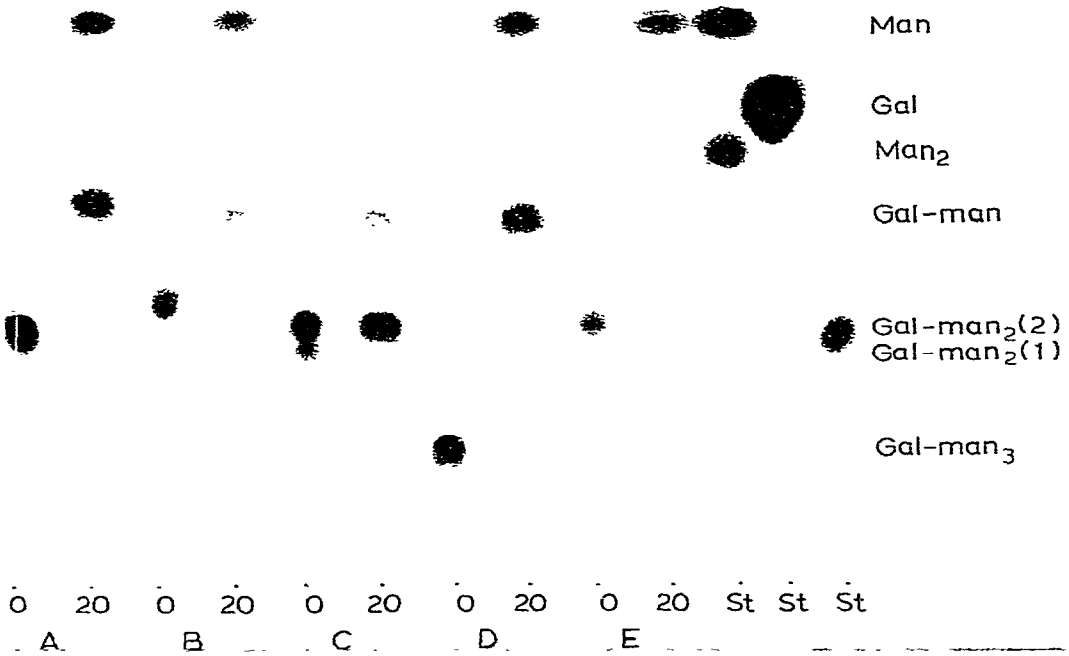

Fig. 1. T.l.c. of the products of hydrolysis of D-galactose-containing β-D-manno-oligosaccharides (20 μL, 10 mg/mL) by snail β-D-mannosidase (20 nkat). Oligosaccharides A–E were incubated with β-D-mannosidase for 0–20 h before t.l.c. Oligosaccharides are: A, α-D-galactosyl-β-D-mannobiose (from β-D-mannanase hydrolysis of *L. leucocephala* galactomannan; B, borohydride-reduced A; C, α-D-galactosyl-β-D-mannobiose (from acid hydrolysis of lucerne galactomannan); D, α-D-galactosyl-β-D-mannotriose (from β-D-mannanase hydrolysis of carob galactomannan); and E, mannotri-itol. St., standard sugars. Gal-Man$_2$(1) is 6^1-α-D-galactosyl-β-D-mannobiose, Gal-Man$_2$(2) is 6^2-α-D-galactosyl-β-D-mannobiose, Gal-Man is 6-*O*-α-D-galactosyl-α,β-D-mannose, and Man$_2$ is (1→4)-β-D-mannobiose.

a downfield shift of 5–6 p.p.m. for a carbon attached to a glycosidic linkage. In addition, relative to the corresponding resonances of β-D-mannobiose, C-5′ gives a line which is shifted by 1.5–2.0 p.p.m. upfield (β-effect). Also, some slight variations can be noticed for the chemical shifts of C-1 of galactose, C-5 of galactose, C-1′, C-4α, and C-4β of 6^2-α-D-galactosyl-β-D-mannobiose compared with those for β-D-mannobiose and 6^1-α-D-galactosyl-β-D-mannobiose.

Susceptibility of the trisaccharides to hydrolysis by β-D-mannosidase. — The chromatographic behaviour, and the susceptibility to hydrolysis by snail β-D-mannosidase, of the α-D-galactosyl-β-D-mannobiose trisaccharides produced on enzymic and acid hydrolysis of galactomannans, are shown in Fig. 1. The α-D-galactosyl-β-D-

mannobiose purified from the β-D-mannanase hydrolysate of *L. leucocephala* galactomannan appeared as a single spot in t.l.c., and was completely hydrolysed to D-mannose and α-D-galactosyl-D-mannose by snail β-mannosidase, and to D-galactose and β-D-mannobiose by α-D-galactosidase. However, α-D-galactosyl-β-D-mannobiose obtained on acid hydrolysis of lucerne galactomannan appeared as two spots in t.l.c. One of these compounds was resistant to hydrolysis by β-D-mannosidase, whereas the other (which co-chromatographed with 6^1-α-D-galactosyl-β-D-mannobiose) was completely hydrolysed to D-mannose and α-D-galactosyl-D-mannose. Both compounds were hydrolysed to D-galactose and β-D-mannobiose by α-D-galactosidase.

These results indicate that the only galactose-containing trisaccharide produced on hydrolysis of *L. leucocephala* galactomannan by Driselase β-D-mannanase is 6-*O*-α-D-galactopyranosyl-4-*O*-β-D-mannopyranosyl-α,β-D-mannopyranose. This finding accords with the conclusions reached on analysis of this trisaccharide by g.l.c. and n.m.r. spectroscopy. In contrast, acid hydrolysis of lucerne galactomannan releases the two possible galactose-containing trisaccharides. The oligosaccharide 6^2-α-D-galactosyl-β-D-mannobiose is completely resistant to hydrolysis by snail β-D-mannosidase, indicating that this enzyme is a typical glycosidase, approaching β-D-manno-oligosaccharide substrates from the non-reducing end, and being unable to cleave beyond a branch point (*i.e.*, a D-galactosyl substituent).

Structures of galactose-containing tri- and tetra-saccharides produced on hydrolysis of carob galactomannan by various β-D-mannanases. — The tri- and tetra-saccharide fractions produced on hydrolysis of carob galactomannan by various highly purified β-D-mannanases were purified by chromatography on Bio-Gel P-2. The β-D-mannanases employed were from a commercial Driselase preparation, *Aspergillus niger* (Sigma C-7502), *Bacillus subtilis* TX1, and *Helix pomatia* gut-solution (Sigma G0876), and from seeds of lucerne and honey locust, and were purified by substrate affinity chromatography[14].

The trisaccharide fractions contained only β-D-mannotriose and α-D-galactosyl-β-D-mannobiose, but the ratio varied markedly. Thus, the ratios of α-D-galactosyl-β-D-mannobiose to β-D-mannotriose, in the trisaccharide fractions produced on hydrolysis of carob galactomannan by lucerne-seed and *A. niger* β-D-mannanases, were 5:95 and 44:56, respectively. The tetrasaccharide fraction contained only α-D-galactosyl-β-D-mannotriose and β-D-mannotetraose and the ratio of these two varied from 34:66 (in lucerne-seed β-D-mannanase hydrolysates) to 100:0 (in *A. niger* β-D-mannanase hydrolysates). Incubation of any of the tri- or tetra-saccharide fractions with snail β-D-mannosidase always gave only D-mannose and α-D-galactosyl-D-mannose. This showed that the only galactose-containing tri- and tetra-saccharides produced on hydrolysis of carob galactomannan by all of these β-D-mannanases were 6^1-α-D-galactosyl-β-D-mannobiose and 6^1-α-D-galactosyl-β-D-mannotriose. In support of this, the galactose-containing trisaccharide co-chromatographed with 6^1-α-D-galactosyl-β-D-mannobiose (Fig. 1). These results contrast with those of Courtois and Le Dizet[10].

In previous studies[12], it was concluded that hydrolysis of carob galactomannan

by honey-locust β-D-mannanase yielded 6^2-α-D-galactosyl-β-D-mannotriose. This conclusion is now known to be incorrect. In those studies, the D-galactose-containing tetrasaccharide was treated with a β-D-mannosidase from lucerne seed and yielded D-mannose and α-D-galactosyl-β-D-mannobiose as the final hydrolysis products. This result was interpreted as indicating that the D-galactosyl group was located on the central D-mannosyl residue of β-D-mannotriose. However, the lucerne β-D-mannosidase has since been shown to be very similar to the exo-β-D-mannanase from guar seeds[18]. Both of these enzymes readily hydrolyse 6^1-α-D-galactosyl-β-D-mannotriose to D-mannose and 6^1-α-D-galactosyl-β-D-mannobiose, but have essentially no action on the latter trisaccharide, *i.e.*, these enzymes, unlike snail β-D-mannosidase, are unable to cleave a D-mannosyl residue adjacent to a D-mannosyl residue substituted by D-galactose.

These results show that, although β-D-mannanases from different sources differ in their ability to hydrolyse galactomannans, particularly those highly substituted with D-galactose[14], the structures of the D-galactose-containing tri- and tetrasaccharides released are the same. This situation contrasts with the results obtained on hydrolysis of α-L-arabinosyl-β-D-xylans by β-D-xylanases of diverse origin. In this case, the two possible α-L-arabinosyl-β-D-xylobioses and the three possible α-L-arabinosyl-β-D-xylotrioses have all been identified in the β-D-xylanase hydrolysates of α-L-arabinosyl-β-D-xylans[11]. Apparently, different β-D-xylanases vary in their ability to cleave directly adjacent to α-L-arabinosyl branch-units in α-L-arabinosyl-β-D-xylans, and this is reflected in the various "fine-structures" of the released oligosaccharides.

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