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SIMILARITY OF MONOSACCHARIDE, OLIGOSACCHARIDE AND POLYSACCHARIDE STRUCTURES IN GUM EXUDATE OF *ANACARDIUM OCCIDENTALE*

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Key Word Index—*Anacardium occidentale*; Anacardiaceae; cashew-nut tree; gum; polysaccharide; monosaccharides; oligosaccharides.

Abstract—The gum exudate from the Brazilian cashew-nut tree (*Anacardium occidentale*) contained traces of the reducing sugars, rhamnose (0.005%), arabinose (0.03%), mannose (0.007%), galactose (0.03%), glucose (0.02%), β-D-Gal*p*-(1→6)-αβ-D-Gal (0.05%), α-L-Rha*p*-(1→4)-αβ-D-GlcA (0.008%) and α-L-Rha*p*-(1→4)-β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-αβ-D-Gal (0.008%). Rhamnose, arabinose, glucose and the three oligosaccharides are components of the side-chains of the gum polysaccharide, which has a main chain of (1→3)-linked β-D-Gal*p* units. The structure of this polysaccharide was determined and found to differ from that previously reported for the gum of a tree growing in India, lacking units of 4-*O*-methylglucuronic acid. Other new side-chain structures were characterized, particularly -α-D-Gal*p*-(1→6)-D-Gal*p*- and α-L-Ara*f*-(1→6)-D-Gal*p*-.

INTRODUCTION

We recently detected free, reducing monosaccharides and oligosaccharides in the gum exudate of the tree, *Anadenanthera colubrina*, and compared them structurally with the accompanying polysaccharide in order to suggest mechanisms for their formation [1–3]. There were similarities with the monosaccharides and one oligosaccharide which were isolated and identified, but difficulties were encountered in the purification of the others. The gum of the Brazilian cashew-nut tree (*Anacardium occidentale*) was also found to contain monosaccharides and oligosaccharides, albeit in smaller quantities [4], and the structures of the monosaccharides and more readily purified oligosaccharides are now determined and their structures also compared with those present in the component polysaccharide.

However, prior to carrying out this comparison, it was necessary to re-determine the complex structure of the gum polysaccharide. It had been previously examined in 1974 and 1975 using samples collected in India and Papua (the cashew tree is native to America) [5, 6]. Rhamnose, arabinose, xylose, mannose, galactose, glucose and glucuronic acid were found in molar ratios of 7:14:2:2:61:8:6 and 7:15:0:1:63:9:6, respectively. The more detailed analysis of the Indian gum polysaccharide showed a highly branched structure, with a (1→3)-linked β-D-Gal*p* main chain [6] and that 4-*O*-methyl-glucuronic acid units were present. However, these data differed from those found in our examination of our polysaccharide, isolated from a Brazilian sample, which contained less arabinose with a 2:4:1:82:6:5 molar ratio of rhamnose, arabinose, xylose, galactose, glucose and glucuronic acid, and without 4-*O*-methylglucuronic acid, as evidenced by the absence of -OCH_3 signals in ^1H and ^{13}C NMR spectra. We now report a detailed analysis of the polysaccharide.

RESULTS AND DISCUSSION

The polysaccharide of the Brazilian gum was isolated *via* ethanol precipitation of an aqueous solution and had $[\alpha]_D+21°$, M_r 1.1×10^5 (light-scattering), and gave rise to a well-defined ^1H [H-1 portion, Fig. 1(A)] and ^{13}C NMR spectra [Fig. 1(B)]. Methylation analysis and GC-mass spectrometric examination of the resulting partially *O*-methylated alditol acetates showed non-reducing end-units of Ara*f* (2%), Rha*p* (2%), Glc*p* (< 10%) and Gal*p* (19%), and other Gal*p* residues that were 3-*O*- (6%), 4-*O*- (10%), 6-*O*- (19%) and 3,6-di-*O*-substituted (32%) (Table 1). When the per-*O*-methylated polysaccharide was reduced with

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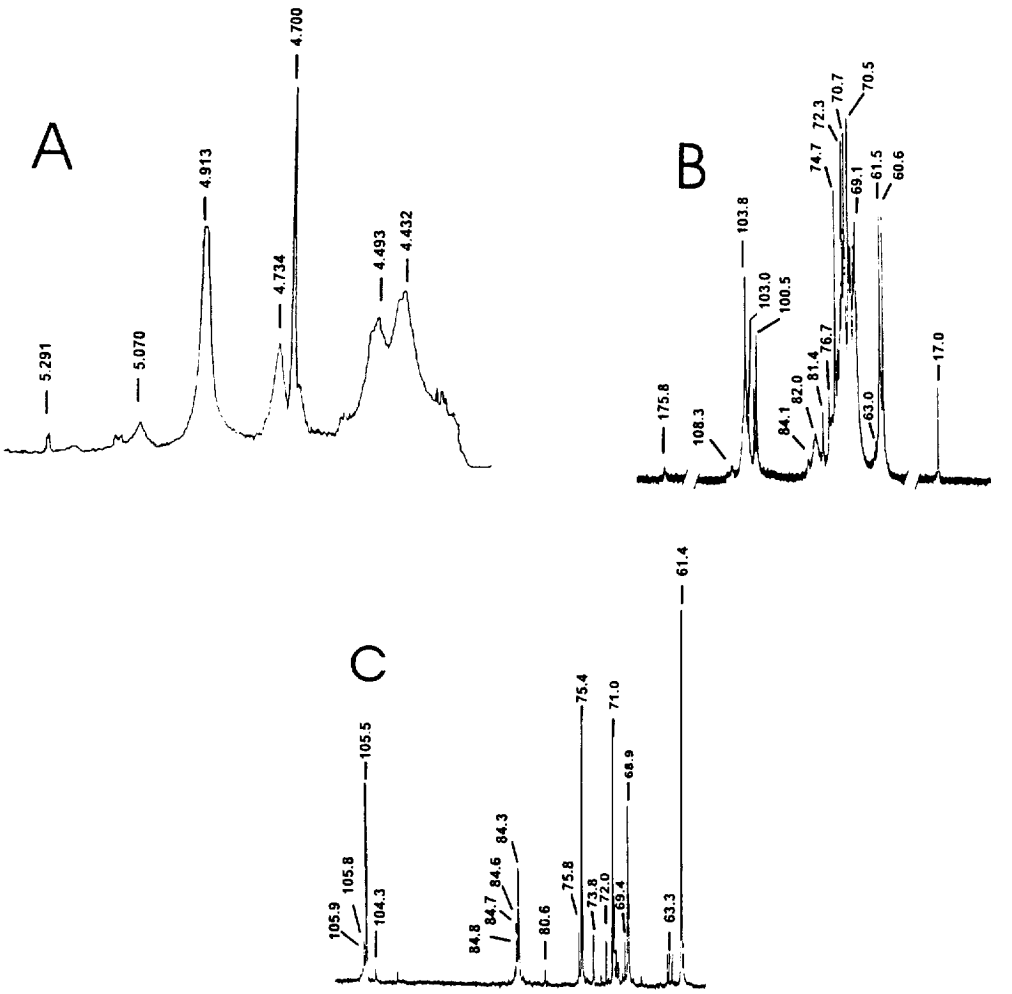


Fig. 1. Cashew polysaccharide: H-1 portion of its ^1H NMR (A) and ^{13}C NMR spectrum (B). ^{13}C NMR spectrum of polysaccharide submitted to one controlled Smith degradation (C).

lithium aluminum deuteride, and the product converted to partially *O*-methylated alditol acetates, non-reducing end- and 4-*O*-substituted-Glc*p*A units were found to be present.

A controlled Smith degradation of the polysaccharide resulted in removal of units of rhamnose and glucuronic acid, giving the ethanol-insoluble polysaccharide core with $[\alpha]_D + 43°$, containing arabinose, xylose, mannose and galactose in a molar ratio of 8:1:1:90. The principal signals of its ^{13}C NMR spectrum [Fig. 1(C)] are typical of a (1→3)-linked β-Gal*p* polysaccharide [7], but with no α-Ara*f* residues. Methylation data (Table 1) show non-reducing end units of Glc*p* (3%) and Gal*p* (11%), with 3-*O*- (67%), 4-*O*- (2%), 6-*O*- (17%) and 3,6-di-*O*-substituted Gal*p* units (14%). A second Smith degradation provided polysaccharide with $[\alpha]_D + 40°$ and an arabinose to galactose ratio of 2:23, while a third one reduced the arabinose content to 3%. Their ^{13}C NMR spectra contained the same minor signals as that of the product of the first Smith degradation, but in progressively diminishing amounts.

Little ethanol-soluble material (4% yield) was obtained on the first Smith degradation. It contained glycerol and components with R_{Gal} 1.20 and 0.31 and, although the latter was not completely identified, it gave rise to ^{13}C NMR signals at δ 100.5 > 103.3, showing the presence of unoxidized α- and β-pyranosyl units.

The native polysaccharide was partially hydrolysed at pH 1 for 5 hr at 100°, which removed Rha and most of the Ara units, to give a polymer with arabinose, xylose, mannose, galactose, glucose and glucuronic acid in a 1:6:5:80:3:5 molar ratio, $[\alpha]_D + 20°$, and a ^{13}C NMR spectrum with complex C-1 and *O*-substituted C-3 regions. Methylation analysis data on the per-*O*-methylated polysaccharide and its $LiAlH_4$-reduced product (Table 1) showed non-reducing end units of Glc*p* (6%) and Gal*p* (35%), and 3-*O*- (9%), 4-*O*- (9%), 6-*O*- (17%) and 3,6-di-*O*-substituted Gal*p*

Table 1. Structures and percentage values of partially *O*-methylated alditol acetates formed from per-*O*-methylated polysaccharides from native cashew polysaccharide (NP), partially hydrolysed NP (PHP), Smith-Degraded NP (SDP) and $LiAl^2H_4$-reduced-NP and -PHP

O-Methylated alditol acetate	R_t (s) DB-210	% of *O*-methylated alditol acetate formed from methylated				
		NP	PHP	SDP	Reduced NP†	Reduced PHP
2,3,5-Me_3-Ara	454	2	—	—	—	—
2,3,4-Me_3-Rha	461	2	—	—	—	—
2,3,4,6-Me_4-Glc*	518	10	6	3	7	5
2,3,4,6-Me_4-Gal	532	19	35	11	17	26
2,4,6-Me_3-Gal	605	6	9	67	10	13
2,3,6-Me_3-Gal	645	10	5	2	6	5
2,3,4-Me_3-Glc	666	—	—	—	tr.	tr.
2,3,4-Me_3-Gal	707	19	17	3	26	22
2,3-Me_2-Glc	836	—	—	—	1	—
2,4-Me_2-Gal	864	32	28	14	33	29

* Confirmed by GC-MS on OV-225.

† Reduced NP on OV-225 gave 2,3,4,6-Me_4-Glc (511 s; 4%), 2,3,4,6-Me_4-Gal (543 s, 8%), 2,4,6-Me_3-Gal (623 s, 7%), 2,3,6-Me_3-Gal (631 s, 2%), 2,3,4-Me_3-Glc (643 s, ~2%, dideuterated peak), 2,3,4-Me_3-Gal (696 s, 20%) and 2,4-Me_2-Gal (869 s, 23%). Standards tested on DB-210 were 2,3-Me_2-Glc (836 s) and 2,3,4-Me_3-Glc (658 s). Using DB-210, only 2,3-Me_2-Glc was found with red. NP: only 2,3,4-Me_3-Glc with red. PHP. With red. NP, a peak at 666 s did not correspond to 2,3,4-Me_3-Glc (maybe superimposition occurred). Standards tested with OV-225 were 2,3-Me_2-Glc (650 s) and 2,3,4-Me_3-Glc (829 s). GC-MS on OV-225 gave peaks less resolved than those on DB-210, but qualitatively reduced NP gave a shoulder at 642 s, corresponding to 2,3,4-Me_3-Glc, but nothing attributable to 2,3-Me_2-Glc. Red. PHP showed a shoulder peak at 648 s, corresponding to 2,3,4-Me_3-Glc.

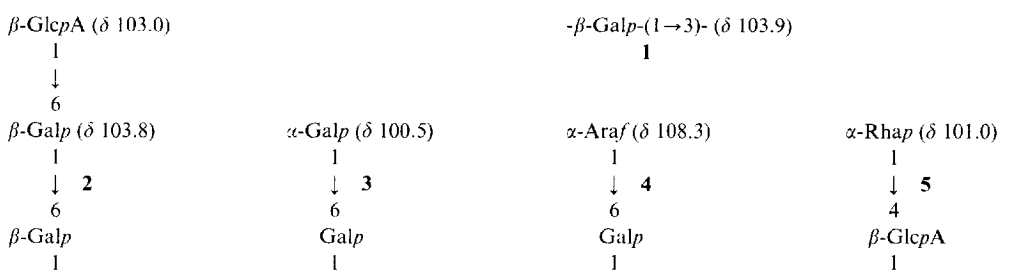
units (28%). Glucuronic acid were present only as non-reducing end units, likely by removal of Rha*p* and/or Ara*f* units from O-4 on partial hydrolysis (a controlled Smith degradation of the polysaccharide provided a polymer with $[\alpha]_D + 34°$).

The latter possibility was eliminated following partial hydrolysis of the native polysaccharide with M acid at 100° for a short time. This cleaved some of the pyranosyl linkages, with the surprising maintenance of some furanosyl linkages, resulting in the formation of α-Ara*f*-(1→6)-αβ-Gal. Other products were β-Gal*p*-(1→3)-αβ-Gal, α-Gal*p*-(1→6)-αβ-Gal, β-Gal*p*-(1→6)-αβ-Gal, β-Glc*p*A-(1→6)-αβ-Gal and β-Glc*p*A-(1→6)-β-Gal*p*-(1→6)-αβ-Gal, each oligosaccharide being characterized by its monosaccharide components and 1D and 2D NMR spectra.

The supernatant obtained during the ethanol precipitation of the native polysaccharide from an aqueous solution, contained a mixture of monosaccharides and oligosaccharides. The monosaccharide contents were quantified as follows: (1) with the intention of preventing their formation by any possible hydrolase activity, the gum was dissolved in water containing sodium borohydride in the presence of allitol as internal standard, and the resulting acetates analysed by GC-mass spectrometry; and (2) the gum together with allitol were first dissolved in water and then reduced with sodium borohydride, prior to conversion to alditol acetates. The oligosaccharides were isolated *via* successive charcoal and cellulose column chromatography of another ethanol supernatant, followed by preparative paper chromatography and were characterized by their monosaccharide compositions and 1D ^1H and ^{13}C NMR spectroscopy, the latter including a DEPT program. The utilized 2D programs were COSY and HMQC.

The structure of the native Brazilian polysaccharide is highly branched consisting of a (1→3)-linked β-D-Gal*p* main chain (**1**), substituted at O-6 by various side-chains. Characterized components were: -β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*- (**2**), -α-D-Gal*p*-(1→6)-D-Gal*p*- (**3**) and α-L-Ara*f*-(1→6)-D-Gal*p*- (**4**). α-L-Rha*p*-(1→4)-β-D-Glc*p*A- groups (**5**), linked (1→6) to D-Gal*p*, are likely to be present. The arabinose-containing side-chains are long, since even three controlled Smith degradations were insufficient to remove them all (these were not α-Ara*f* structures). Based on the ^{13}C NMR data for the oligosaccharides liberated on acid hydrolysis, the partial C-1 NMR spectrum of the native polysaccharide [in Fig. 1(B)] can be assigned as indicated in structures **1–5** (δ values for D_2O solutions).

Compared with the polysaccharide of gum collected in India, there are similar structures, especially a main chain of (1→3)-linked β-D-Gal*p* units, some of which were substituted at O-6 by side-chains. These were concluded to be long, containing (1→3)- and (1→6)-linked β-D-Gal*p* units, those of (1→2)-linked Ara*f* up to 5 units long, needing five controlled Smith degradiations for their complete removal, and end units of Ara*p*, Ara*f*, Glc*p*, Rha*p*, Man*p*, Xyl*p* and β-D-Glc*p*A-(1→6)-D-Gal*p* [6]. We now report the absence of 4-Me-Glc*p*A units in the Brazilian polysaccharide. The other differences could be quantitative and seem to



be due to the current availability of more modern techniques, especially those of methylation analysis and NMR spectroscopy. Examples are the characterization of Rhap-(1→4)-β-GlcpA-(1→6)-β-Galp- and α-Araf-(1→6)-Gal- non-reducing end-structures.

The gum contained free rhamnose (0.005%), arabinose (0.03%), mannose (0.007%), galactose (0.03%) and glucose (0.02%), and the isolated oligosaccharides were characterized as β-Galp-(1→6)-αβ-Gal (0.05%), α-Rhap-(1→4)-αβ-GlcA (0.008%) and α-Rhap-(1→4)-β-GlcpA-(1→6)-β-Galp-(1→6)-αβ-Gal (0.008%). They were not formed by *in situ* acid hydrolysis of the weakly acidic gum because of the resistance of the pyranosyl glycosidic linkages at ambient temperatures. Other more likely possibilities, because of their structural similarity to the side-chains of the polysaccharide, are that they are formed as by-products from biosynthetic intermediates or by the action of *exo*-hydrolases.

EXPERIMENTAL

Preparation of cashew-nut tree polysaccharide. This was pptd from an aq. soln (250 g in 1250 ml) with EtOH (3 l), the polysaccharide was redissolved in H_2O and then freeze-dried to remove residual EtOH.

Specific rotations. These were determined at 25° in H_2O at 0.3–0.6%, except for controlled Smith degradation products, whose solvent was H_2O containing 3% NaOH.

Determination of M_r *of polysaccharide.* This was carried out using a light-scattering apparatus with a 0.1% aq. soln of polysaccharide with GPC columns in series of OH pak B-804 and OH pak B-805 (Shodex), eluted at 1 ml min^{-1} with 0.1 M $NaNO_3$–0.02% NaN_3 (λ 633 nm).

Monosaccharide composition. Polysaccharides and oligosaccharides were hydrolysed with M H_2SO_4 for 18 hr at 100°. After neutralisation ($BaCO_3$), part of the solns were evapd and examined by PC (solvent: *n*-BuOH–pyridine–H_2O, 5:3:3; spray *p*-anisidine HCl). The other part was reduced with NaB^2H_4, acetylated with Me_2CO-pyridine at 100° and the products examined by GC-MS using an OV-225 capillary column (30 m × 0.25 mm i.d.), held at 50° during injection, then programmed at 40° min^{-1} to 220° (isothermal). Glucitol hexa-acetate gave peaks in the order *m/z* 140 > 139 > 141, showing that some of it arose from glucose, as well as glucuronolactone. The polysaccharide was hydrolysed with 2 M TFA for 8 hr at 100° to give a residue which gave crystalline α-galactose [from MeOH–EtOH (1:1)], $[\alpha]_D$ + 134° (initial value, 80 s) → + 79° (*c*, 0.5 H_2O; constant value). The ^{13}C NMR spectrum of resulting αβ-galactose [8] and its $[\alpha]_D$ [9] indicated the D-enantiometer. Recovered from the mother liquor *via* cellulose CC (eluant: Me_2CO, then Me_2CO–H_2O, 10:1) were L-arabinose, L-rhamnose and D-glucuronolactone (R_{Rham} 1.04), which were identified by their ^{13}C NMR spectra and whose enantiomeric forms were determined by conversion to the acetates of their (−)-2-octyl glycosides and examination by GC-MS using a Durowax-4 capillary column (30 m × 0.25 mm i.d.) programmed from 50° (40° min^{-1}) → 230° (isothermal) [10].

Uronic acid contents. These were determined by the *m*-hydroxy-biphenyl method [11].

Methylation analysis of polysaccharides. Polysaccharides (≈ 50 mg) were methylated by the method of ref. [12], which was necessary in order to render the products soluble in the reaction medium of Kerek and Ciacunu [13]. Methylation was completed using the procedure of ref. [14]. The *O*-methylated products were refluxed with 3% MeOH–HCl for 3 hr and then hydrolysed with M H_2SO_4 at 100° for 18 hr. After NaB^2H_4 reduction and acetylation, the resulting mixts were examined by GC-MS on OV-225 and DB-210, using the conditions described above. Two columns were necessary to resolve the possible isomers of Rha, Ara and Gal. On OV-225, there is superimposition of 2,3,5-Me_3-Ara (447 *s*) and 2,3,4-Me_3-Rha (448 *s*). Also of 2,4-Me_2-Ara (545 *s*), 3,4-Me_2-Ara (542 *s*), and 2,3,4,6-Me_4-Gal (542 *s*). Resolved are 2,5-Me_2-Ara (509 *s*), 3,5-Me_2-Ara (499 *s*), and 2,3-Me_2-Ara (537 *s*). On DB-210, 2,5-Me_2-Ara (533 *s*) and 2,3,4,6-Me_4-Gal (533 *s*) are superimposed. Resolved are 3,5-Me_2-Ara (519 *s*), 2,3-Me_2-Ara (577 *s*), 3,4-Me_2-Ara (571 *s*), 2,4-Me_2-Ara (558 *s*), 2,3,4,6-Me_4-Gal (533 *s*), 2,3,5-Me_3-Ara (462 *s*), and 2,3,4-Me_3-Rha (469 *s*).

NMR. ^1H soln spectra were obtained at 400 MHz at 70° in D_2O using presatn of the DOH resonance. For ^{13}C spectra, the solvent was D_2O at 30°, except in the case of Smith-degraded polysaccharides, when the solvent was 5% NaOD in D_2O. Small OCH_3 signals were not detected in the ^1H (δ ~ 3.2) and ^{13}C spectra [δ ~ 59; Fig. 1(B)]. Chemical shifts are expressed in δ, based on a standard of TMS. The ^{13}C NMR spectrum

of the polysaccharide in D_2O contained signals that were assigned on the basis of those of oligosaccharides, obtained *via* partial hydrolysis and described below. C-1 signals were at δ 175.8 (very small; CO_2H), 108.3 (α-Ara*f*-(1→6)-Gal*p*, very small), the largest one at δ 103.8 (β-Gal*p*-(1→6)-Gal*p*) > 100.5 (α-Gal*p*-(1→6)-Gal*p*) > 101.0 (α-Rha*p*) > 103.0 (β-Glc*p*A-(1→6)-Gal*p*) = 103.9 (β-Gal*p*-(1→3)-Gal*p*). There was a very small signal at δ 84.1 (C-4, α-Ara*f*) and a larger broader complex of three signals from δ 81.4 to 82.0 (3-*O*-substituted β-Gal*p*'s), 17.0 (C-6, Rha). Negative ^{13}C DEPT signals of C-6 of Gal*p* units were not detectable because of superimposition on positive ones with the same shift. The ^1H NMR spectrum contained broad H-1 signals at δ 4.432, 4.493, 4.700, 4.734, 4.913, 5.070 and 5.291.

Successive controlled Smith degradations. To cashew polysaccharide (6.50 g) dissolved in H_2O (200 ml), was added $NaIO_4$ (20 g), and after 2 days it was followed by ethylene glycol (5 ml) and the solution then dialysed. $NaBH_4$ (2 g) was added and after 3 hr the soln was acidified with HOAc and dialysed, first against tap (20 hr) and then distilled H_2O (20 hr). The resulting polyalcohol contained glycerol, arabinose and galactose in a 75:3:22 molar ratio. The soln was evapd to 100 ml, adjusted to pH 2.0 with aq. H_2SO_4, heated at 100° for 1 hr, neutralized with $BaCO_3$, and then filtered. The filtrate was evapd to 20 ml and added to EtOH (200 ml), giving a pp., which was isolated (Ara, Xyl, Man, Gal; 8:1:1:90 molar ratio), yield 1.76 g. It was only sparingly soluble in cold H_2O. Its ^{13}C NMR spectrum in D_2O-5% NaOH contained [Fig. 1(C)] predominant signals at δ 61.4 (C-6; inverted in DEPT spectrum), 68.9 (C-4), 71.0 (C-2), 75.4 (C-5), 84.3 (C-3) and 105.55 (C-1), corresponding to a (1→3)-linked β-galactopyranan. Respective values obtained for internal units of β-Gal*p*-[(1→3)-β-Gal*p*]$_4$-(1→4)-αβ-Glc in D_2O were 61.7, 69.4, 71.1, 75.9, 82.8 and 104.8 [7]. The mother liquor contained, following chromatography on a cellulose column (Me_2CO–H_2O, 10:1 then 3:1), material (260 mg) which gave rise to (PC) spots with R_f 0.31 and 1.20, as well as one of glycerol.

The polysaccharide (1.56 g) mostly dissolved in H_2O (100 ml) at 100° after 1 hr and, on addition of $NaIO_4$ (3 g), the remaining insol. material dissolved. This was converted to the polyalcohol, which contained glycerol, arabinose and galactose in a 69:4:27 ratio. The controlled Smith degradation procedure was repeated. Obtained were polysaccharide, pptd with Me_2CO–EtOH (1:1) (0.74 g) containing Ara (R_t 657 s) and Gal in a 2:23 molar ratio and soluble material (0.21 g). The ^{13}C NMR spectrum of the polysaccharide (5% NaOH in D_2O) also corresponding to a (1→3)-linked β-galactopyranan.

A portion (0.5 g) of the polysaccharide was submitted to a further controlled Smith degradation, first giving the polyalcohol which contained glycerol, arabinose and galactose in a 2:1:47 ratio. The resulting polysaccharide (224 mg) contained Ara and Gal in a 3:97 ratio and gave a ^{13}C NMR spectrum similar to those obtained from polysaccharide after one and two degradations, with the six main signals described above, but minor ones at δ 101.3, 104.3, 105.8 and 105.9 (C-1's) and 3-*O*-substituted β-C-3's at δ 84.6, and 84.7, and another at δ 80.6, possibly from *O*-substituted α-C-3 or C-4. EtOH-sol. material was also formed (240 mg).

Mild partial hydrolysis. Polysaccharide (5.34 g) was dissolved in H_2O (200 ml), which was adjusted to pH 1 with dilute aq. H_2SO_4 and kept at 100° for 5 hr. The soln was neutralized with $BaCO_3$, filtered, the filtrate treated with cation-exchange resin, filtered, evapd to a small vol. and the polysaccharide pptd with excess EtOH; yield 2.35 g. It contained Ara, Xyl, Man, Gal and Glc in a 1:6:5:85:3 molar ratio. The ^{13}C NMR spectrum contained very broad signals in the C-1β (δ 103.7 > 104.5 > 103.9 > 104.0 > 104.2 > 103.1) and 3-*O*-substituted regions (broad and continuous from δ 81.25 to 82.8). There were DEPT inverted signals at δ 61.46 (large) and δ 60.54 (small) and, possibly, obscured ones at δ 69.5 and 69.9. Smaller C-1 signals were present at δ 92.7, 96.6, 96.9 and 100.4.

Controlled Smith degradation of partially hydrolysed polysaccharide. Degraded polysaccharide (0.6 g) was successively oxidized with $NaIO_4$ (3 g) in H_2O (50 ml) for 24 hr and reduced with $NaBH_4$, as described above, to give a polyalcohol which contained glycerol, threitol, arabinitol and galactose in a 76:2:1:21 molar ratio. The polyalcohol was partially hydrolysed at pH 2 (adjusted with H_2SO_4) for 1 hr at 100°, giving rise to EtOH-insol. material (0.18 g).

Stronger partial hydrolysis of polysaccharide: characterisation of resulting oligosaccharides. Polysaccharide (4.12 g) in H_2O (150 ml) containing MTFA (12.5 ml) was kept at 100° for 1 hr. The product was fractionated on a column of charcoal-diatomaceous earth (40 g: 1:1): first with H_2O (3 l) and then with 30% aq. EtOH (2 l), the latter giving a residue of 0.53 g. This gave on PC (*n*-BuOH–pyridine–H_2O, 1:1:1), apart from galactose (R_{Lact} 1.59), spots with R_{Lact} 0.32 > 0.86 > 1.15, along with smaller quantities of those with R_{Lact} 0.14, 1.00, 1.15, 1.30 and 1.44. The mixt. was fractionated on Whatman No. 3 filter paper and each component isolated. Some of the fractions were pure as determined by ^{13}C NMR and monosaccharide composition. In the case of frs containing glucuronic acid, traces of glucitol hexa-acetate, labelled with $^2H_2$ at C-6, were formed. Structures of pure frs were determined, principally by ^{13}C NMR spectroscopy, including ^{13}C DEPT, which served for the identification of 6-*O*-substituted Gal*p* signals, which were inverted in the δ 67–70 region. R_{Lact} 0.14·29 mg, giving Ara, Gal and Glc (2:95:3). It had the ^{13}C NMR spectrum of *O*-β-D-Glc*p*A-(1→6)-*O*-β-D-Gal*p*-(1→6)-αβ-D-Gal with signals at δ 103.1 (C-1″), 103.7 and 103.6 (C-1′, α- and β-isomer, resp.), 96.9 (C-1β), 92.8 (C-1α), and 176.2 (CO_2H). No unsubstituted C-6 signals of Gal or those of *O*-substituted C-2, C-3, or C-4 were detected and with DEPT, inverted signals

appeared at δ 70.0, 69.83, and 69.76 (*O*-substituted C-6's). The H-1 region of the ^{1}H NMR spectrum contained consistent signals at δ 4.43, J = 7.7 Hz (H-1′, α-anomer), 4.44, J = 7.9 Hz (H-1′, β-anomer), 4.50, J = 7.7 Hz (H-1″), 4.58, J = 7.8 Hz (H-1β) and 5.25, J = 3.7 Hz (H-1α). R_{Lact} 0.32·139 mg, giving Gal and Glc (79:21). It had the ^{13}C NMR spectrum of 6-*O*-β-D-Glc*p*A-$\alpha\beta$-D-Gal with signals at δ 175.6, 103.0, 96.85 > 92.8, no *O*-substituted at δ > 76.2, inverted with DEPT at δ 69.70 > 69.77. The H-1 region of the ^{1}H NMR spectrum contained consistent signals at δ 4.48, J = 7.9 Hz (H-1′, α-anomer), 4.49, J = 8.1 Hz (H-1′, β-anomer), 4.55, J = 7.0 Hz (H-1β) and 5.23, J = 3.7 Hz (H-1α). R_{Lact} 0.86·47 mg, giving Gal. Its ^{13}C NMR signals of C-1 were at δ 103.6 > 103.7, 99.0, 96.88 > 96.92 and 92.8 (smaller than β's). No *O*-substituted signals at δ > 74.27. DEPT inverted signals at 61.4 > 61.6, 67.1 > 67.3 and 69.9. Since the β-C-1 signal is split, it was a disaccharide from its R_{Lact} and a mixture of 6-*O*-α- and 6-*O*-β-D-Gal*p*-$\alpha\beta$-D-Gal. The presence of this mixt. was consistent with the ^{1}H NMR spectrum, which contained H-1 signals of 6-*O*-β-D-Gal*p*-$\alpha\beta$-D-Gal at δ 4.430, J = 7.7 Hz (H-1′; α-anomer); δ 4.44, J = 8.1 Hz (H-1′, β-anomer); 4.58, J = 8.0 Hz (H-1β) and 5.24, J = 4.0 Hz (H-1α) and those of 6-*O*-α-D-Gal*p*-$\alpha\beta$-D-Gal at δ 4.96, J = 3.5 Hz (H-1′); 4.57, J = 7.9 Hz (H-1β) and 5.25, J = 4.0 Hz (H-1α). The assignments of the H-1α signals of each disaccharide are interchangeable. R_{Lact} 1.0·7 mg, giving Ara, Man, Gal and Glc (7:5:82:6). The ^{13}C NMR spectrum showed a mixt. with 14 signals in the C-1 region and the fr. was not further examined. R_{Lact} 1.15·20 mg, giving Ara and Gal (1:24). Its ^{13}C NMR corresponded to 3-*O*-β-D-Gal*p*-$\alpha\beta$-D-Gal with signals of the β-isomer at δ 104.76 (C-1′), 96.6 (C-1) and 79.8 (*O*-substituted C-3) and smaller ones of the α-isomer at δ 104.81 (C-1′), 92.6 (C-1) and 82.9 (*O*-substituted C-3). According to a DEPT determination, there were signals of *O*-substituted C-6β (large) at δ 61.39 and *O*-substituted C-6α (small) at δ 61.57. The H-1 portion of its ^{1}H NMR spectrum, in accord, contained signals at δ 4.59, J = 7.5 Hz (H-1′; α-anomer), 4.60, J = 7.5 Hz (H-1β), 4.62, J = 7.1 Hz (H-1′; β-anomer) and 5.27, J = 2.7 Hz (H-1α). R_{Lact} 1.30·17 mg, pure, giving Ara and Gal (46:54). It gave rise to ^{13}C signals of α-Ara*f* at δ 63.13 (C-5′β) > 63.17 (C-5′α), inverted with DEPT; 77.15 (C-3′), 81.45 (C-2′), 83.3 (C-4′) and 108.2 (C-1′) [13]. Those of Gal were at δ 67.25 (C-6β) > 67.8 (C-6α), both inverted with DEPT, 92.8 (C-1α; smaller) and 96.9 (C-1β). Its ^{1}H NMR spectrum contained signals of H-1′ of α-Ara*f* at δ 4.98 (J = 2.0 Hz) and H-1α (δ 4.55, J = 8.0 Hz), and H-1β (δ 5.24, J = 2.8 Hz) of Gal*p*. The disaccharide had $[\alpha]_D + 5°$, indicating an α-L-configuration. It thus corresponds to 6-*O*-α-L-Ara*f*-$\alpha\beta$-D-Gal and this was confirmed as follows. The HMQC spectrum defined the signals of H-6β (δ 3.74) and H-6α (δ 3.84) by correlation with the C-6 signals and these values were used to define the H-6 signals in a ROESY spectrum, in which they were correlated with H-1′ of α-Ara*f* at δ 4.98. Correlations were not found in the HMBC spectrum. R_{Lact} 1.44·8 mg, giving Ara, Man and Gal (7:16:70). It was impure as the C-1 region of its ^{13}C NMR spectrum contained 12 signals: it was not further investigated.

Estimation of monosaccharides in gum samples. Gum (504 mg) and allitol (5 mg) in H_2O (20 ml) were stirred in the presence of $NaBH_4$ (50 mg). Bubbles on the surface of the gum showed that it was reacting with the reducing agent. It dissolved after 16 hr, but a little more reductant (20 mg) was added and after 3 hr, the soln was adjusted to pH 7 with HOAc. It was then freeze-dried, dissolved in H_2O (5 ml) and added to EtOH (15 ml). The resulting ppt. was removed by filtration and the filtrate evapd to a residue, which was dissolved in MeOH. This was evapd and the process repeated × 2. Acetylation was carried out with Ac_2O–pyridine (2 ml, 1:1) at 100° for 1 hr, the mixt. treated with ice-water for 1 hr and then extracted with $CHCl_3$, which was then evapd. The residue was analysed by GC-MS. In another expt, gum (500 mg) and allitol (5.0 mg) were dissolved in H_2O prior to addition of $NaBH_4$ (50 mg). After 16 hr, the time it took to dissolve the gum, the reductant was added and, after 3 hr, the mixt. was converted to alditol acetates, as described above, which were then analysed by GC-MS.

Isolation and characterisation of free reducing oligosaccharides. The gum (250 g) was dissolved in H_2O (1.25 l) and the soln added to EtOH (3 l). The resulting ppt. was isolated by filtration (yield 80%) and the filtrate evapd to dryness. It was dissolved in H_2O and applied to a column of activated charcoal-diatomaceous earth (≅Celite, 40 g, 1:1), which was eluted with H_2O (4 l), followed by 30% (2 l) and 50% aq. EtOH, followed by evapn. Eluted material was fractionated on Whatman No. 3 filter paper (*n*-BuOH–pyridine–H_2O, 1:1:1). EtOH eluates were each fractionated on a cellulose column, using as eluants mixts of Me_2CO–H_2O of 7:1 (1 l), 4:1 (1 l), 3:1 (2 l) and 5:2 (2 l), followed by further fractionation by PC as before. Further purification was carried out giving fractions with R_fs on cellulose TLC (same solvent) of 0.44, 0.58, 0.67 and 0.92. These were characterised as follows; as bases for ^{13}C assignments for these oligosaccharides, δ values for Me α-Rha*p*, Me α- and β-Glc*p*A and Me α- and β-Gal*p* [8] were used, but with a correction of −0.7 ppm. R_f 0.92. This gave a ^{13}C NMR spectrum identical with that of β-Gal*p*-(1→6)-$\alpha\beta$-Gal [3] with δ 103.43 (C-1′β) > 103.49 (C-1′α), 96.7 (C-1β) > 92.6 (C-1α), 75.5 (C-5′), 74.1 (C-5β), 72.9 (C-3β and C-3′β), 69.4 (C-6β) > 69.6 (C-6α) and 61.3 (C-6). R_f 0.67. Based on the ^{13}C spectra of Me α-Rha*p* and Me α- and β-Glc*p*A, and COSY and HMQC examination, it corresponds to α-Rha*p*-(1→4)-$\alpha\beta$-GlcA with δ 101.2 (C-1′), 96.4 (C-1β) > 92.4 (C-1α), 80.1 (C-4α) < 79.7 (C-4β), 77.0 (C-3β?), 74.89 and 74.81 (? and C-5β), 72.45, 72.1, 70.6, 69.4 (C-5′), 17.0 (C-6′) and 175.7 (C-6). R_f 0.44. For the characterisation of this fr., it was necessary to use as standard, α-Rha*p*-(1→4)-β-Glc*p*A-(1→6)-$\alpha\beta$-Gal of Angico

gum [3], which was characterized using its 1D ^{1}H and ^{13}C (with DEPT) spectra, with COSY and HMQC determinations to make the assignments: δ 102.9 (C-1′), 101.2 (C-1″), 96.9 (C-1β) > 92.9 (C-1α), 79.7 (C-4′), 76.73 (C-5′), 74.78 (C-3′), 74.3 (C-5β), 73.7 (C-2′), 73.15 (C-3β), 72.46 (C-3′), 72.35 (C-2β), [69.7 (C-6α), 69.6 (C-6β); weak DEPT because of overlap], 17.0 (C-6″) and 175.6 (C-6′). This fr. corresponds to α-Rha*p*-(1→4)-β-Glc*p*A-(1→6)-β-Gal*p*-(1→6)-$\alpha\beta$-Gal, with signals at δ 103.73 (C-1′α) < 103.66 (C-1′β), 103.1 (C-1″), 96.9 (C-1β) > 92.9 (C-1α), 79.6 (C-4″), 76.8 (C-5″), 74.8 (C-3″), 74.33 (C-5′), 74.27 (C-5β), 17.0 (C-6‴), 175.7 (C-6″). R_f 0.58. This fraction was a mixt. and not characterised.

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REFERENCES

1. Delgobo, C. L., Gorin, P. A. J. and Iacomini, M., *XXII Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology*, May 1–4, L 37, 1993, p. 113.
2. Delgobo, C. L., Gorin, P. A. J., Torri, G. and Iacomini, M., *XXIII Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology*, May 14–17, L 27, 1994, p. 123.
3. Delgobo, C. L., Gorin, P. A. J. and Iacomini, M., *Phytochemistry* (in press).
4. Menestrina, J. M., Delgobo, C. L., Iacomini, M., Jones, C. and Gorin, P. A. J., *XXV Annual Meeting of The Brazilian Society of Biochemistry and Molecular Biology*, May 4–7, L-55, 1996, p. 116.
5. Anderson, D. M. W., Bell, P. C. and Millar, J. R. A., *Phytochemistry*, 1974, **13**, 2189.
6. Anderson, D. M. W. and Bell, P. C., *Analytica Chimica Acta*, 1975, **79**, 185.
7. Collins, J. G., Bradbury, H., Trofinoff, E. and Messer, M., *Carbohydrate Research*, 1981, **92**, 136.
8. Gorin, P. A. J. and Mazurek, M., *Canadian Journal of Chemistry*, 1975, **53**, 1212.
9. Pigman, W. and Isbell, H. S., *Advances in Carbohydrate Chemistry*, 1968, **23**, 11.
10. Gorin, P. A. J., Iacomini, M., Feijó, M. A. L., Zanin, S. M. W. and Hogge, L., *Arquivos Biologia Tecnologia* (*Paraná*), 1985, **28**, 387.
11. Blumenkrantz, N. and Asboe-Hansen, G., *Analytical Biochemistry*, 1973, **54**, 484.
12. Haworth, W. N., *Journal of the Chemical Society*, 1915, 8.
13. Ciacunu, I. and Kerek, F., *Carbohydrate Research*, 1984, **131**, 209.
14. Kuhn, R., Trischmann, M. and Löw, I., *Angewandte Chemie*, 1955, **67**, 32.