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The free reducing oligosaccharides of gum arabic: aids for structural assignments in the polysaccharide

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Abstract

Gum arabic contains in small quantities, the free reducing sugars, arabinose, ribose, glucose, mannose, galactose, rhamnose, α-D-Gal*p*-(1 → 3)-αβ-L-Ara, α-L-Ara*f*-(1 → 4)-β-D-Gal*p*-(1 → 6)-αβ-D-Gal, β-D-Glc*p*A-(1 → 6)-αβ-D-Gal*p*, α-L-Rha*p*-(1 → 4)-β-D-Glc*p*A-(1 → 6)-αβ-D-Gal*p*, α-L-Rha*p*-(1 → 4)-β-D-Glc*p*A-(1 → 6)-β-D-Gal*p*-(1 → 6)-αβ-D-Gal*p*, a branched pentasaccharide with a chain of α-D-Gal*p*-(1 → 3)-α-L-Ara*f*-(1 → 3)-β-D-Gal*p*-(1 → 6)-αβ-D-Gal*p* substituted at O-4 of Gal*p*′ with α-L-Ara*f* units, and a doubly branched heptasaccharide with a chain of α-D-Gal*p*-(1 → 3)-α-L-Ara*f*-(1 → 3)-β-D-Gal*p*-(1 → 6)-αβ-D-Gal*p*, disubstituted, respectively, at O-4 and O-6 of Gal*p*′ with α-L-Ara*f* and α-L-Rha*p*-(1 → 4)-β-D-Glc*p*A groups. A small amount of a related heptasaccharide was also present, with non-reducing end units of β-Ara*p*, in place of those of α-Gal*p*. These oligosaccharides were characterized by 1D and 2D NMR spectroscopy, ESI-MS, and in three cases, methylation analysis for final confirmation. The oligosaccharides should represent, with the probable exception of the galactosyl reducing ends, side-chain structures present in the gum arabic polysaccharide.

Keywords: Gum acacia; Polysaccharide; Free, reducing oligosaccharides; Structural similarity

1. Introduction

Recently, complex structures of the high-arabinose polysaccharide, isolated from the gum exudate of the tree angico branco (*Anadenanthera colubrina*), were determined with the aid of accompanying, free reducing mono and oligosaccharides, which were more readily characterized by NMR spectroscopy (Delgobo, Gorin, Tischer & Iacomini, 1999). These were presumed to have similar structures to those of the polysaccharide, since they can be formed as byproducts in its biosynthesis of the polysaccharide, by enzymatic degradation or by autohydrolysis in situ. However, the presence of the acid-labile arabinofuranosyl glycosidic linkages, together with the inferred cleavage of units of the (1 → 3)-linked β-galactopyranosyl main-chain, rendered the latter possibility less likely. We now investigate the oligosaccharides from gum arabic (*Acacia senegal*), which has as its main component, a non-viscous, high-arabinose polysaccharide, whose arabinofuranose-containing sequences cannot be completely characterized by conventional means, although some advances were achieved using two-dimensional (2D) NMR spectroscopy (McIntyre, Cer & Vogel, 1996).[1] This report, in terms of description of structures **6b** and **7b** in gum arabic, has been published in a preliminary form (Tischer, Gorin & Iacomini, 2000).

2. Materials and methods

2.1. Source of gum arabic

The gum was obtained from Sigma-Aldrich Co.

2.2. Assay of reducing monosaccharides in the gum

The gum (2.0 g) was dissolved in H_2O (150 ml) and the solution added to EtOH (500 ml). The resulting precipitate was removed by centrifugation and the pellet was suspended in EtOH using a blendor and the suspension centrifuged. The combined supernatants were evaporated to 10 ml, which was added to EtOH (100 ml) and the precipitate

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[1] More sequential structures might be obtained by oxidation of the primary hydroxyls of Ara*f* units to carboxyl groups with the reagent TEMPO, as carried out on galactomannans (Sierakowski, Milas, Desbrières & Rinaudo, 2000). This could be followed by partial hydrolysis to give aldobiouronic acids.

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filtered off. The filtrate was evaporated to a residue (31.2 mg), of which one half was dissolved in H_2O (3.0 ml), to which $NaBH_4$ (40 mg) was added, followed by allitol internal standard (2.0 mg). The product was acetylated with Ac_2O-pyridine and the resulting alditol acetates examined on a capillary column of DB-225 (30 m × 0.25 cm i.d.), programmed from 50°C (1 min) at 40°C min^{-1} to 220°C (constant temperature).

2.3. Isolation of oligosaccharides

Gum arabic (371 g) was dissolved in H_2O (1.3 l) and the solution added to EtOH (4.5 l), which gave a precipitate, which became more copious on addition of conc. HCl (0.5 ml). This was removed manually and the supernatant evaporated to a smaller volume (600 ml), which had pH 4.0 and was applied to a charcoal-Celite column (Whistler & Durso, 1950). This was eluted with water to give a mixture (2.0 g) of reducing monosaccharides and *myo*-inositol (R_{Lact} 0.83 on PC in *n*-BuOH–pyridine–H_2O, 1:1:1) and then with 40% aqueous EtOH. The latter eluate contained a mixture of rhamnose and oligosaccharides (0.64 g), which was applied to Whatman 3Mm filter papers, run for six days in *n*-BuOH–pyridine–H_2O (5:3:3). Isolated were fractions with the following R_{Lact} values and yields using *n*-BuOH-pyridine-H_2O (1:1:1) as solvent: R_{Lact} 1.32 (109 mg), 0.93 (14 mg), 0.83 (tr.), 0.58 (16 mg), 0.54 (5 mg), 0.41 (18 mg), 0.34 (21 mg), 0.27 (25 mg), and 0.16 (20 mg).

2.4. Controlled Smith degradation of polysaccharide

The polysaccharide (6.8 g) in H_2O (150 ml) was treated with 2.0 moles $mole^{-1}$ of $NaIO_4$ (18.0 g). After three days, a period necessary to oxidize completely any less susceptible α-Ara*f* units, 1,2-ethanediol (5 ml) was added. The solution was dialyzed, $NaBH_4$ (2.0 g) added and after 1 h, treated with excess HOAc, and then dialyzed against tap followed by distilled water. It was then evaporated to 100 ml, adjusted to pH 2.0 with dilute aqueous H_2SO_4, maintained at 100°C for 30 min (Gorin, Horitsu & Spencer, 1965), dialyzed against tap and then distilled water, evaporated to 100 ml, and the solution added to EtOH (400 ml). The precipitate was isolated (yield 1.72 g) and a portion (1.46 g) was dissolved in water (50 ml), $NaIO_4$ (4.5 g) added and the product submitted to a second controlled Smith treatment to give a galactan (0.56 g). A portion (150 mg) was subjected to a further Smith degradation, but with oxidation for one day and substitution of the EtOH precipitation with a dialysis step. Yield 125 mg. A fourth Smith degradation of the product (90 mg) gave 72 mg of polysaccharide.

2.5. Carbodiimide reduction of acidic polysaccharide

Following the procedure of Taylor and Conrad (1972), oligosaccharide **7b** (see below; 2 mg in 0.5 ml H_2O) was added to 0.2 M 2-*N*-morpholine-ethanesulfhonic acid (5.0 ml), previously adjusted to pH 4.75 with dilute aqueous NaOH, followed by *N*-cyclohexyl-*N*′-[β-(*N*-methylmorpholine)ethyl]-carbodiimide *p*-toluenesulfhonate (5 mg). After 1 h, the pH of the solution was adjusted to 7.0 with 2 M Tris-buffer (2 ml) and small quantities of $NaBD_4$ were gradually added, the bubbling being controlled with a drop of *n*-octanol. After 1 h, the solution was deionized, filtered and the filtrate evaporated to dryness to give a neutral oligosaccharide.

2.6. Methylation analysis

This was carried out using the method of Ciucanu and Kerek (1984) by dissolving each oligosaccharide (**1** and **6b**; ~1 mg) in one drop of water, followed by DMSO (1 ml) and then MeI (1 ml). Powdered NaOH (~0.3 g) was added and the mixture agitated vigorously with a vortex for 30 min and then left overnight. This procedure was adopted to solubilize the oligosaccharide in the reaction medium and then to cause minimum alkaline degradation. After acidification with HOAc, water was added and the per-O-methylated product extracted with $CHCl_3$, which was washed three times with water. After evaporation, the residue was dissolved in H_2SO_4–H_2O (1:1, v/v) for 1 h at 0°C and the solution then diluted to 2 M H_2SO_4, which was heated at 100°C for 18 h. The resulting mixture of partly *O*-methylated aldoses was reduced with $NaBD_4$ and then acetylated to give *O*-methylalditol acetates, which were examined by GC-MS on a column of DB-23 (30 m × 0.25 cm i.d.) programmed from 50°C (1 min) at 40°C min^{-1} to 210°C (const. temp.). This procedure resolved acetates of 2,3,4-Me_3Rha (458), 2,3,5-Me_3Ara (462), 2,3,4-Me_3-Ara (487), 2,5-Me_2-Ara (543), 2,3,4,6-Me_4-Gal (565), 2,4-Me_2-Ara (576), 2,3,6-Me_3-Glc (701), 2,3,4-Me_3-Gal (768), 2,6-Me_2-Gal (787), and 2-Me-Gal (1106), the numerical value for each being its R_t in s.

In the case of the *O*-methylated disaccharide, it was hydrolyzed, the H_2SO_4–H_2O step was omitted, since it was soluble in the following hydrolysis medium.

For the acidic heptasaccharide **7b**, the methylation mixture was acidified with dilute H_2SO_4, prior to $CHCl_3$ extraction. The methylated product was converted to *O*-methylalditol acetates and examined by GC-MS on DB-23. The residue, obtained by carbodiimide reduction, was also subjected to methylation analysis.

2.7. Determination of oligosaccharide structures by NMR spectroscopy

Preliminary examination were carried out by ^{13}C and ^{1}H NMR (^{1}H, ^{13}C HMQC when only small quantities were available) spectroscopy in D_2O at 30°C, unless otherwise stated, using a Bruker 400 MHz DRX spectrometer (shifts expressed as δ ppm, relative to external Me_4Si, $\delta = 0$). COSY, TOCSY, HMBC, ROESY and DEPT spectra were obtained, according to the Bruker manual.

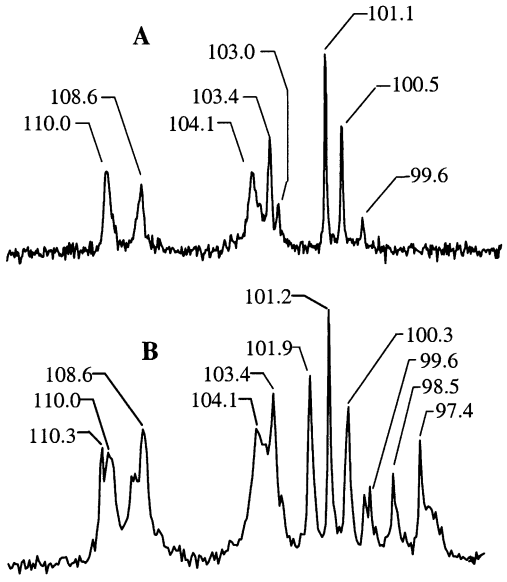


Fig. 1. C-1 portions of ^{13}C NMR spectra of gum arabic polysaccharide (A) and angico branco polysaccharide (B) (D_2O, 33°C).

2.8. ESI-MS molecular-weight determinations on oligosaccharides

The negative-ion mode was used especially for examination of oligosaccharides containing uronic acid and these gave more complex spectra than the positive-ion mode, with more emphasis on daughter ions. Analyses were carried out using a Micromass Quattro LC. The samples (~100 μg) were applied in AcCN–H_2O (2 ml; 1:1 v/v).

2.9. NMR data of oligosaccharides

Disaccharide **1** (R_{Lact} 1.32) — α-Gal*p* non-reducing end (δ values of ^1H and ^{13}C, respectively; N = nucleus): N-1 (5.14, $J = 0$ Hz; 95.8 > 96.1), C-6 (61.11 > 61.45). α-Ara*p* reducing end: N-1 (4.52, $J = 7.3$ Hz; 97.0), N-3 (3.73, 77.6), C-5 (66.40). β-Ara*p* reducing end: N-1: (5.24, $J = 0$ Hz; 92.9), N-3 (3.92, 74.4), C-5 (62.57).

Trisaccharide **2** (R_{Lact} 0.93) — α-Ara*f* non-reducing end: N-1 (5.26, $J = 0$ Hz; 109.4), N-2 (4.20, 81.7), N-3 (3.94, 77.0), N-4 (4.08, 84.5), C-5 (61.7). Internal β-Gal*p*: N-1 (4.50, $J = 7.0$ Hz; 103.52 > 103.47), N-2 (3.59, 71.4), N-3 (3.79, 75.3), C-6 (61.6). α-Gal*p* reducing end: N-1 (5.22, $J = 0$ Hz; 92.8), N-2 (3.81, 68.8), N-3 (3.66, 73.2), C-6 (69.40). β-Gal*p* reducing end: N-1 (4.58, $J = 7.0$ Hz; 96.8), N-2 (3.50, 72.3), N-3 (3.66, 73.2), C-6 (69.60).

Trisaccharide **3** (R_{Lact} 0.41) — α-Rha*p* non-reducing end: N-1 (4.75, $J = 0$ Hz, 101.2), N-2 (3.98, 70.7), N-3 (3.78, 70.6), N-4 (3.48, 72.4), N-5 (4.07, 69.5), N-6 (1.37, $J = 6.5$ Hz, 16.9). Internal β-Glc*p*A: N-1 (4.52, $J = 7.1$ Hz; 102.89 > 102.86), N-2 (3.39, 73.9), N-3 (3.59, 74.8), N-4 (3.60, 79.6), N-5 (3.76, 76.6), C-6 (175.7). α-Gal*p* reducing end: N-1 (5.27, $J = 1.5$ Hz; 92.8), N-2 (3.81, 68.8), N-3 (3.88, 69.3), N-4 (4.07, 69.7), C-6 (69.6). β-Gal*p* reducing end: N-1 (4.52, $J = 7.1$ Hz; 96.9), N-2 (3.53, 73.2), N-3 (3.68, 73.2), C-6 (69.9).

Tetrasaccharide **4** (R_{Lact} 0.27) — α-Rha*p* non-reducing end: N-1 (4.75, $J = 0$ Hz; 101.25), N-6 (1.35, $J = 6.5$ Hz; 16.9). Internal β-Glc*p*A: N-1 (4.51, $J = 8.0$ Hz; 103.10), C-4 (3.60, 79.6). Internal β-Gal*p*s: N-1 (4.57, 103.38, 103.74). α-Gal*p* reducing end: N-1 (5.27, $J = 1.0$ Hz; 92.9). β-Gal*p* reducing end: (4.52, $J = 8.0$ Hz; 96.9), 70.01 (C-6).

Disaccharide **5** (R_{Lact} 0.54) — β-Glc*p*A nonreducing end: N-1 (4.50, 102.9). α-Gal*p* reducing end: N-1 (5.28, 92.8). β-Gal*p* reducing end: N-1 (4.59, 96.8).

Pentasaccharide **6b** (R_{Lact} 0.58) — α-Gal*p* non-reducing end: N-1 (5.02, $J = 3.0$ Hz; 100.5), H-2 (3.80). α-Ara*f* non-reducing end unit: N-1 (5.40, $J = 0$ Hz; 108.7), N-2 (4.16, 81.8), N-3 (3.91, 77.2). 3-*O*-substituted α-Ara*f*: N-1 (5.27, $J = 0$ Hz; 110.0), N-2 (4.39, 80.6), N-3 (3.93, 85.2). 3,4-Di-*O*-substituted β-Gal*p*: N-1 (4.51, $J = 7.1$ Hz; 103.5), N-2 (3.74, 71.0), N-3 (3.86, 80.4), N-4 (4.17, 74.2). α-Gal*p* reducing end: N-1: (5.26, $J = 0$ Hz; 92.9). β-Gal*p* reducing end: (4.58, $J = 7.1$ Hz; 96.9).

Heptasaccharide **7b** (R_{Lact} 0.16) — α-Rha*p* non-reducing end: N-1 (4.74, $J = 0$ Hz; 101.2), N-2 (3.92), N-3 (3.76), N-4 (3.42), N-5 (4.01), N-6 (1.35, $J = 6.5$ Hz; 17.0). α-Gal*p* non-reducing end: N-1 (5.02, $J = 3.0$ Hz; 100.6), N-2 (3.80), N-3 (3.84). α-Ara*f* non-reducing end: N-1 (5.39, $J = 0$ Hz; 108.6), N-2 (4.15), N-3 (3.90). 3-*O*-substituted α-Ara*f*: N-1 (5.27, $J = 0$ Hz; 110.1), N-2 (4.38), N-3 (3.95, 85.3). 4-*O*-substituted β-Glc*p*A: N-1 (4.49, 103.4), N-2 (3.36), N-3 (3.57), N-4 (3.58, 79.7). 3,4,6-Tri-*O*-substituted β-Gal*p*: N-1 (4.51, 103.4), N-2 (3.71), N-3 (3.86, 80.2), N-4 (4.18, 74.1), C-6 (70.4). β-Gal*p* reducing end: N-1 (5.25, $J = 7.0$ Hz; 96.9), C-6 (70.13). α-Gal*p* reducing end: N-1 (5.27, $J = 0$ Hz, 92.9), C-6 (70.01).

3. Results

The physical and chemical structures of the heteropolysaccharide of gum arabic have been extensively investigated. The ^{13}C NMR spectrum of our sample was examined (Fig. 1A) and was authenticated by comparison with that of samples of *Acacia senegal* (Anderson, Millar & Weiping, 1991; Defaye & Wong, 1986). Although our spectrum was less complex and better defined than that of the high-arabinose, low viscosity, polysaccharide from the gum of angico branco (Delgobo et al., 1999), many C-1 signals were present with identical chemical shifts, showing structural similarities (Fig. 1A and B).

The free reducing sugars present in gum arabic were isolated by addition of an aqueous solution to excess ethanol. They were detected by PC and the monosaccharides identified by GC-MS as their derived alditol acetates and quantitated in the presence of allitol as internal standard. Only arabinose (0.10%) was found in an appreciable quantity, together with glucose (0.0046%), galactose (0.028%), mannose (0.0025%), ribose (0.0025%), and rhamnose

Table 1
NMR and ESI-MS data for free reducing linear oligosaccharides

Oligosaccharide	Key NMR signals; *m/z* values for molecular and daughter ions
1	α-Gal*p*′: 95.8 > 96.1 (C-1); 5.14, $J = 0$ Hz (H-1), 61.11 > 61.45 (C-6, DEPT inverted). αβ-Ara*p*: 97.0 (C-1_α); 4.52, $J = 7.3$ Hz (H-1_α) > 92.9 (C-1_β); 5.24, $J = 0$ Hz(H-1_β), 77.6 (C-3_α); 3.73 (H-3_α) > 74.5 (C-3_β); 3.92 (H-3_β), 66.40 > 62.57 ($C_{\alpha\beta}$-5, DEPT inverted).
2	α-Ara*f*″: 109.4 (C-1); 5.26, $J = 0$ Hz (H-1), 61.7 (C-5, DEPT inverted). β-Gal*p*′: 103.52 > 103.47 (C-1); 4.50, $J = 7.0$ Hz (H-1), 75.3 (C-4), 3.79 (H-4), 61.6 (C-6, DEPT inverted). αβ-Gal*p*: 96.8 (C-1_β); 4.58, $J = 7.0$ Hz (H-1_β) > 92.8 (C-1_α); 5.22, $J = 0$ Hz (H-1_α), 69.60 > 69.40 (C-6, DEPT inverted). ESI-MS Na^+ ions: 497, 365 (-Ara), 335 [tr. -Gal].
3	α-Rha*p*‴: 101.2 (C-1); 4.75, $J = 0$ Hz (H-1), 16.9 (C-6); 1.37, $J = 6.5$ Hz (H-1). β-Glc*p*A′: 102.89 > 102.86 (C-1); 4.52, $J = 7.1$ Hz (H-1), 79.6 (C-4); 3.60 (H-4), 175.7 (C-6). αβ-Gal*p*: 92.8 < 96.9 ($C_{\alpha\beta}$-1), 69.9 (C-6, DEPT inverted). ESI-MS negative ion: 501. Positive ions (Na^+): 525, 379 (-Rha), 203 - (Rha + GlcA).
4	-Rha*p*‴: 101.25 (C-1); 4.75, $J = 0$ Hz (H-1), 16.9 (C-6); 1.35, $J = 6.5$ Hz (H-6). β-Glc*p*A″: 103.10 (C-1); 4.59 (H-1), $J = 8.0$ Hz, 79.6 (C-4), 3.60 (H-4). β-Gal*p*′s: 103.38 and 103.74 (C-1) 70.01 (C-6 DEPT inverted), αβ-Gal*p*: 70.01 (C-6, DEPT inverted), 92.9 < 96.9. ESI-MS negative ions: 663, 517 (-Rha), small peaks at 795 and 928.
5	β-Glc*p*A′: 102.9 (C-1); 4.50 (H-1). αβ-Gal*p*: 92.8 < 96.8 ($C_{\alpha\beta}$-1), 5.28 < 4.59 (H-$1_{\alpha\beta}$). Negative ESI-MS ions: 355, 193 (-Gal).

(0.023%). The trace amounts of oligosaccharides in the supernatant were enriched by charcoal column chromatography, by which monosaccharides and myo-inositol were eluted with water and oligosaccharides plus rhamnose with 40% ethanol (0.21% yield). By this method, the cyclitol was eliminated and did not prejudice PC fractionation of the oligosaccharides.

This fractionation was carried out using *n*-BuOH–pyridine–H_2O (5:3:3), as solvent, to give fractions which were homogeneous on PC. The monosaccharide composition and monosaccharide sequence of oligosaccharides of each fraction were investigated by ESI-MS determination of positive- and negative-mode molecular and daughter ions. However some ambiguity could occur, since for example, a molecular weight of 344 could indicate GalRha or AraGlcA or one of 340 could arise from Ara-4OMeGlcA or RhaGlcA. This was resolved by quantitative and qualitative GC-MS determination of monosaccharides formed on acid hydrolysis. However, oligosaccharides containing glucuronic acid formed glucuronolactone, which when reduced with sodium borodeuteride, gave trideuterated glucitol hexaacetate in a low yield. Each oligosaccharide fraction that was examined for purity, as shown by ^{13}C, ^1H and ^1H, ^{13}C HMQC NMR spectroscopy. Detailed sequential structures were also determined using the (COSY–TOCSY)–HMQC–ROESY approach (Delgobo et al., 1999), which was very informative, despite incomplete connectivity in α-Ara*f*, Ara*p*, and β-Gal*p* units. Inter-unit ROESY proved to be better than the HMBC (H-1 → ^{13}C) approach. When necessary, a methylation analysis was carried out. Eight fractions were examined, and these are denoted as follows by their R_{Lact} values in *n*-BuOH–pyridine–H_2O (1:1:1).

Fraction A with R_{Lact} 1.32 — This consisted of a disaccharide, since it gave arabinose and galactose in a molar ratio of 1:1 (GC-MS of derived alditol acetates) on acid hydrolysis. Methylation analysis (GC-MS) gave rise to acetates of 2,3,4,6-Me_4-galactitol (47%), 2,4-Me_2- (22%), and 2,5-Me_2-arabinitol (31%). Its ^{13}C and ^1H NMR spectra contained low field C-1′ signals at δ 95.8 > 96.1 (both at δ 5.14, $J = 0$ Hz) and C-1_α and C-1_β signals at δ 97.0 (4.52, $J = 7.3$ Hz) >92.9 (5.24, $J = 0$ Hz), respectively, indicating structure **1** exists in solution. In confirmation of 3-O-substitution, COSY and TOCSY spectra revealed an H-3 signal at δ 3.73, that correlated using HMQC to the 3-*O*-substituted C-3 of α-Ara*p* at δ 77.6, while its ROESY spectrum showed enhancement between H-1′ at δ 5.24 and the same H-3 resonance. HMBC coupling between H-1′ and a ^{13}C nucleus of the reducing end was not observed.

α-D-Gal*p*-(1 → 3)-αβ-L-Ara*p* **1**

Fraction B with R_{Lact} 0.93 — This fraction gave, on hydrolysis, Gal and Ara in a molar ratio of 1.8:1 (GC-MS), corresponding to a trisaccharide, a structure which was confirmed by its positive-ion ESI-MS spectrum. This contained a molecular ion (Na^+ form) with *m/z* 497 with two daughter ions, one corresponding to removal of a Gal and the other to an Ara group (Table 1). Its ^{13}C and ^1H NMR spectra were identical to those of structure **2**, previously identified in angico branco gum (Delgobo et al., 1999).

α-L-Ara*f*-(1 → 4)-β-D-Gal*p*-(1 → 6)-αβ-D-Gal*p* **2**

Fraction C with R_{Lact} 0.41 — Its ESI-MS, ^{13}C and ^1H NMR spectra (Table 1) were identical to those of trisaccharide **3**, found in angico branco gum (Delgobo et al., 1999). Acid hydrolysis and derivatization gave monodeuterated Rha and Gal, and trideuterated Glc alditol acetates in a 41:39:11 molar ratio (GC-MS).

α-L-Rha*p*-(1 → 4)-β-D-Glc*p*A-(1 → 6)-αβ-D-Gal*p* **3**

Fraction D with R_{Lact} 0.27 — This contained mainly $RhaGlcAGal_2$, according to its ESI-MS spectrum in the negative-ion mode, with a molecular ion at *m/z* 663 and a daughter ion (-Rha) at *m/z* 517. Small peaks were present at *m/z* 957 ($RhaGlcAGal_3Ara$), 927 ($RhaGlcAGal_2Ara_2$), and 795 ($RhaGlcAGal_2Ara$), showing some impurities. The presence of Ara comtaminants was confirmed by hydrolysis and GC-MS of derived alditol acetates, which showed Rha, Ara, Gal, and Glc in a 17:24:51:8 molar ratio. However, the principal signals in its ^{13}C NMR spectrum were consistent with a predominant structure **4** (Menestrina, Iacomini, Jones

Table 2
NMR and ESI data for free reducing branched oligosaccharides

Oligosaccharide	Key NMR signals; *m/z* values for molecular and daughter ions
6b	α-Gal*p*: 100.5 (C-1); 5.02, $J = 3.0$ Hz (H-1). -(1 → 3)-α-Ara*f*-(1 → 3)-β-Gal*p*-: 110.0 (C-1); 5.27, $J = 0$ Hz (H-1), 85.2 (C-3); 3.93 (H-3). α-Ara*f*-(1 → 4: 108.7 (C-1); 5.40, $J = 0$ Hz (H-1). β-Gal*p*′: 103.5 (C-1); 4.51, $J = 7.1$ Hz (H-1), 80.4 (C-3); 3.86 (H-3), 74.2 (C-4); 4.17 (H-4). αβ-Gal*p*: 92.9 < 96.9 ($C_{\alpha\beta}$-1); 4.58, $J = 7.1$ Hz > 5.26, $J = 0$ Hz ($H_{\alpha\beta}$-1). Positive ESI-MS ions: 791 (Na^+), 659 (Na^+, -Ara), 497 [Na^+, -(Ara + Gal)], 365 [Na^+, -(Ara_2 + Gal)])
7b	α-Rha*p*: 101.2 (C-1); 4.74, $J = 0$ Hz (H-1), 17.0 (C-6); 1.35, $J = 6.5$ Hz (H-6). β-Glc*p*A: 103.4 (C-1), 79.7 (C-4), 3.58 (H-4). α-Gal*p*: 100.6 (C-1); 5.02, $J = 3.0$ Hz (H-1). α-Gal*p*-(1 → 3)-α-Ara*f*-(1 → 3)-β-Gal*p*-: 110.1 (C-1); 5.27, $J = 0$ Hz (H-1), 85.3 (C-3), 3.95 (H-3). α-Ara*f*-(1 → 4)-β-Gal*p*- 108.6 (C-1), 5.39 (H-1). β-Gal*p*′: 103.4 (C-1), 80.2 (C-3), 3.86 (H-3); 74.1 (C-4), 4.18 (H-4); 70.4 (C-6). αβ-Gal*p*: 92.9 < 96.9 ($C_{\alpha\beta}$-1), 5.27, $J = 0$ Hz < 4.59, $J = 7.0$ Hz ($H\text{-}1_{\alpha\beta}$), 70.01 > 70.13 ($C_{\alpha\beta}$-6). ESI-MS negative ions: 1089, 1059[a], 957 (-Ara), 943 (-Rha), 927 (-Gal), 825 (-Ara_2)[b], 811 [-(Ara + Rha)], 795 [-(Gal + Ara)], 663 [-(Gal + Ara_2)]. In the positive mode: 1129 (K^+), 997 (K^+, -Ara), 365 [Na^+, -(Gal + Ara_2 + GlcA + Rha)]

[a] Molecular ion from **7a** component.
[b] Fragment from **7a** component.

& Gorin, 1998), which has a basic structure of **3**, but with an additional 6-*O*-substituted β-Gal*p* unit (Table 1).

α-L-Rha*p*-(1 → 4)-β-D-Glc*p*A-(1 → 6)-β-D-Gal*p*-(1 → 6)-αβ-D-Gal*p*

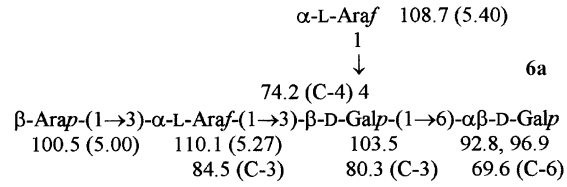
4

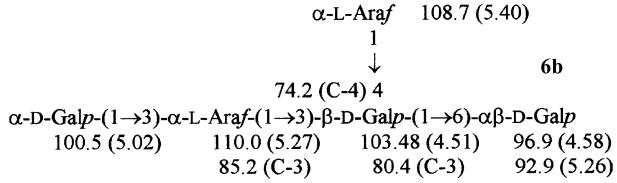
Fraction E with R_{Lact} 0.54 — Its ESI-MS molecular ion (-ve mode) with *m/z* 355 corresponded to GlcAGal (**5**), which gave a daughter ion at *m/z* 193 (-Gal). Only a small quantity was available, but its HMQC spectra corresponded to the ^{13}C NMR spectrum (Table 1) of authentic β-Glc*p*A-(1 → 6)-αβ-Gal (Delgobo et al., 1999). No signal was present in the δ 74–85 range, confirming 6-*O*-substitution.

β-D-Glc*p*A-(1 → 6)-αβ-D-Gal*p* **5**

Fraction F with R_{Lact} 0.58 — The fraction ($[\alpha]_D$ −7°) was obtained in a small quantity and its positive-ion mode ESI-MS molecular ion corresponded to an Ara_2Gal_3 structure, with daughter Na^+ ions arising by removal of Ara, AraGal, and Ara_2Gal groups (Table 2). Acid hydrolysis and GC-MS of derived alditol acetates showed Ara and Gal in a 1:1.4 ratio. Its ^1H, ^{13}C HMQC NMR spectrum contained ^{13}C resonances similar to those of an Ara_3Gal_2 pentasaccharide (**6a**), isolated from angico branco gum (Delgobo et al., 1999), but there were small shift differences of the H-1 signal at δ 5.02, compared with δ 5.00 and a ^{13}C signal at δ 85.2 instead of δ 84.5. The above data suggested the presence of non-reducing α-Gal*p* units instead of those of β-Ara*p*, as in structure **6b**. (COSY–TOCSY)–HMQC–ROESY showed H-1 (δ 5.02) of the α-Gal*p* unit to be linked to C-3 of an α-Ara*f* unit with resonances of H-3,C-3 (δ 3.93, 85.2). This in turn correlated, via its H-1 signal (δ 5.27) to H-3,C-3 resonances (δ 3.86, 80.4) of the adjacent β-Gal*p* unit. The non-reducing end unit of α-Ara*f* (δ 5.40) was similarly correlated with H-4,C-4 (δ 4.17, 74.2) of the same β-Gal*p* unit (Table 2). Methylation analysis confirmed structure **6b**, since it gave rise to the *O*-methyladitol acetates (GC-MS), corresponding to non-reducing end-units of Gal*p* (23%) and Ara*f* (23%), 3-*O*-substituted Ara*f* (30%), and 6-*O*-(12%) and 3,4-di-*O*-substituted Gal*p* units (12%).

Fraction G with R_{Lact} 0.34 — This had a $RhaGlcAGal_2Ara_2$ structure, according to ESI-MS and acid hydrolysis gave Rha, Ara, Gal, and Glc in a 17:24:51:8 molar ratio (GC-MS of alditol acetates, incorporating sodium borodeuteride reduction). However, its ^{13}C NMR spectrum contained three α-Ara*f* signals at δ 108.79, 109.38, 109.90, showing a mixture, which was not further examined





Fraction H with R_{Lact} 0.16 — The fraction ($[\alpha]_D$ −17°), on ESI-MS examination. (−ve mode) gave a molecular ion at *m/z* 1089, consistent with an $Gal_3Ara_2GlcARha$ structure. A daughter ion at *m/z* 927 was formed, arising from removal of a galactosyl unit along with others formed by removal of Ara, Rha, Ara_2, Ara + Rha, and Gal + Ara_2 groups (Table 2; Fig. 2). Acid hydrolysis gave Rha, Ara, Gal, and Glc alditol acetates in a 19:39:45:5 molar ratio. A heptasaccharide structure was shown by the C-1 portion of its ^{13}C NMR spectrum, which resembled that of heptasaccharide **7a**, isolated from angico branco gum (Delgobo et al., 1999). Perceptible exceptions were an H-1 signal at δ 5.02 instead of δ 5.00 and a ^{13}C signal at δ 85.3 instead of δ 84.6 (compare **7b** with **7a**), again suggesting β-Ara*p* instead of α-Gal*p* units (compare **6b** and **6a**). The (COSY–TOCSY)–HMQC–ROESY sequence showed that the H-1 resonance (δ 5.02) of the α-Gal*p* non-reducing end units correlated with H-3,C-3 (δ 3.95, 85.3) of the adjacent α-Ara*f* units,

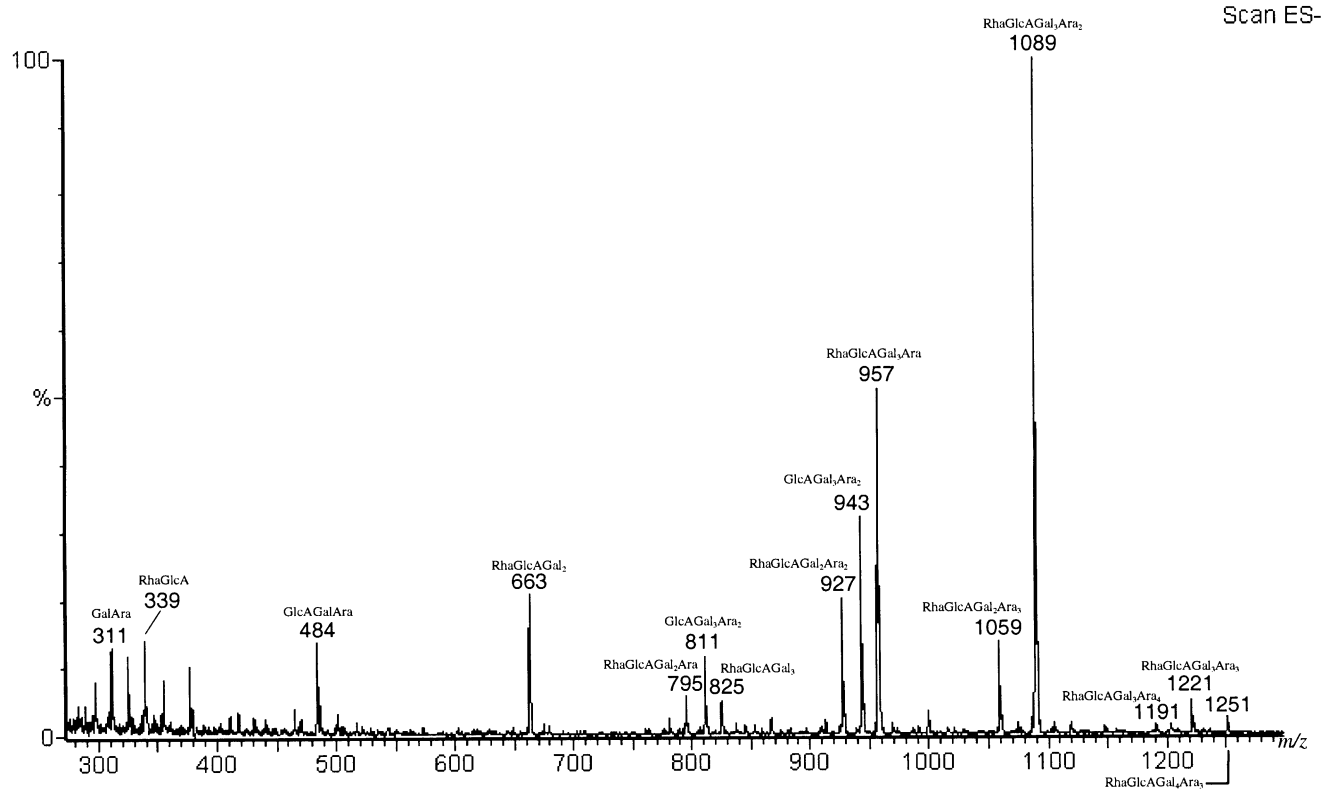
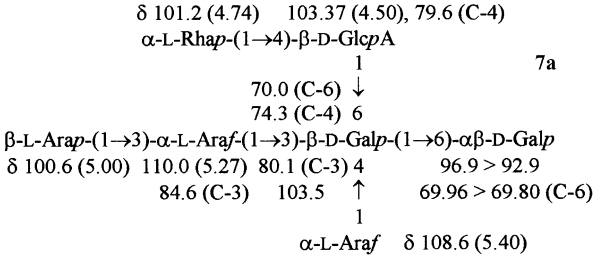
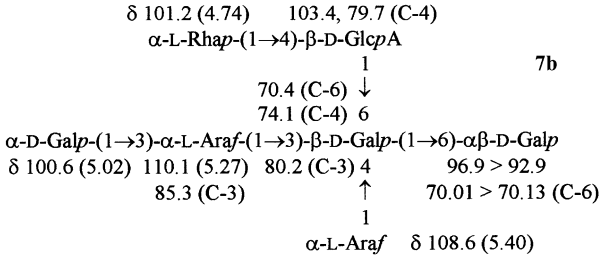


Fig. 2. ESI-MS spectrum, negative-ion mode, of Fraction H (**7a** + **7b**).

whose H-1 (δ 5.27) in turn correlated with H-3,C-3 (δ 3.86, 80.2) of the *O*-substituted β-Gal*p*′ units. The H-1 resonance of the non-reducing end units of α-Ara*f* (δ 5.40) correlated with H-4,C-4 (δ 4.18, 74.1) of the same β-Gal*p*′ unit and H-1 of α-Rha*p* (4.74) correlated with H-4,C-4 (δ 3.58, 79.7) of β-Glc*p*A units (Table 2)





These assignments were in accord with methylation analysis, which gave rise to *O*-methylalditol acetates, indicating (GC-MS) non-reducing end-units of Ara*f* (21%) and Gal*p* (24%), with 3-*O*-substituted Ara*f* (19%), and 6-*O*- (17%) and 3,4,6-tri-*O*-substituted Gal*p* units (13%). Due to β-elimination in the methylation process, only 3% of Rha*p* non-reducing end units were detected, and no peaks corresponding to a Glc*p*A derivative. Non-reducing end-units of Ara*p* (3%) were also present in a ~1:7 ratio when compared with those of Gal*p*, confirmed by comparison of negative ion peaks with *m/z* 1059 and 1089 (Fig. 2). Fraction H thus contained structure **7b** with a small amount of **7a**, not detected by NMR spectroscopy.

A carbodiimide carboxy-reduced heptasaccharide, on acid hydrolysis, gave rise to Rha, Ara, Gal, and Glc in a 1:2:3:1 molar ratio (GC-MS of alditol acetates) and methylation analysis showed non-reducing end units of Rha*p*, Ara*f*, and Gal*p*, with substitution at O-3 of Ara*f*, O-4 of Glc*p*A, and O-3,4,6 of Gal*p*. A trace of Ara*p* was also detected.

Minor negative ESI-MS ions were obtained from Fraction H (Fig. 2) at *m/z* 1221 > 1251 > 1191, possibly from $RhaGlcAGal_3Ara_3$, $RhaGlcAGal_4Ara_2$, and $RhaGlcAGal_2Ara_4$ structures, respectively.

4. Discussion

The structure of the polysaccharide of gum acacia has been extensively investigated in terms of its main chain

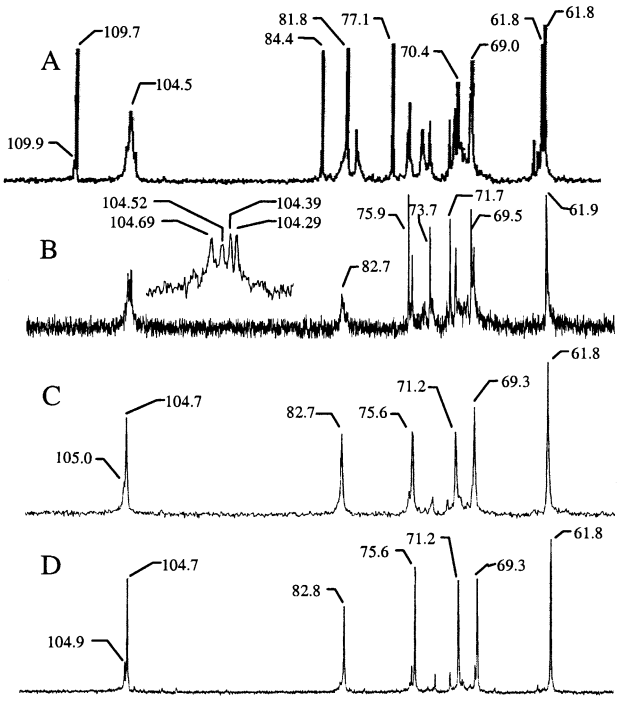


Fig. 3. ^{13}C NMR spectra of gum arabic polysaccharide after (A) one controlled Smith degradation (33°C); (B) two degradations (expansion of H-1 region as insert) (50°C); (C) three degradations (50°C), (D) and four degradations (50°C).

and side chain structures. These essentially started with the studies of Smith (1939, 1940), who characterized non-reducing end units of α-Gal*p*, 3,6-di-*O*-substituted Gal*p* residues, those of Ara*f*, which were non-reducing ends and 3-*O*-substituted, and 4-*O*-substituted and non-reducing end units of Glc*p*A and continued until the 2D NMR determinations of McIntyre et al. (1996).

The main chain structure was defined by Anderson, Hirst and Stoddart (1966), who found that even after seven controlled Smith degradations, the product gave β-Gal*p*-(1 → 6)-αβ-Gal*p* on partial acid hydrolysis. This showed a long sequence of 3-*O*-substituted β-Gal*p* units in the side chains, finally linked to those of O-6 of main-chain units, also 3-*O*-substituted.

For side chain structures, partial hydrolysis of the polysaccharide gave α-D-Gal*p*-(1 → 3)-αβ-L-Ara (Smith, 1939) and β-L-Ara*p*-(1 → 3)-αβ-L-Ara (Andrews & Jones, 1955). Methylation analysis showed that the latter arose from non-reducing end units of Ara*p* (Aspinall, Charlson, Hirst & Young, 1963). α-L-Rha*p*-(1 → 4)-β-D-Glc*p*A (Aspinall et al., 1963) and α-L-Rha*p*-(1 → 4)-β-D-Glc*p*-(1 → 6)-D-Gal (Aspinall & Young, 1965), which were isolated following partial acetolysis of carboxy-reduced polysaccharide. Aspinall and Rosell, 1977, by base degradation of the methylated polysaccharide, demonstrated that other sugar residues were attached to O-3 of 6-*O*-substituted Gal*p* units. These structures are similar to those contained in the present free, reducing oligosaccharides. None were found containing 4-*O*-methylglucuronic acid or (1 → 3)-linked Gal*p* units. The gum arabic polysaccharide has been reviewed by Stephen (1983).

The only exceptions to polysaccharide structures not found in our oligosaccharides are those containing groups of -β-Gal*p*-(1 → 3)-β-Gal*p*- and α-Ara*f*-(1 → 4)-β-Glc*p*A, the latter being inferred by immunological studies (Pazur, Maskiel, Witham & Marchetti, 1991). Another possible example is the presence of (1 → 5)-linked α-Ara*f* side chains, which occurred in a arabinogalactan-protein, secreted by liquid cultured cells of *A. senegal* (Mollard & Joseleau, 1994).

The C-1 chemical shifts of the free reducing oligosaccharides (Tables 1 and 2) were helpful in accompanying sequential controlled Smith degradations of the gum polysaccharide. The first degradation removed units of α-Rha*p* (δ 101.1), β-Glc*p*A (δ 103.0 and 175.3), α-Gal*p* and β-Ara*p* (δ 100.5), α-Ara*f* (δ 108.6), and β-Ara*f* residues (δ 99.6, minor signal; Figs. 1A and 3A). While this degradation left 3-*O*-substituted α-Ara*f* side-chain units intact, (δ 109.7; Fig. 3A), they were removed by the second degradation (Fig. 3B), in agreement with Defaye and Wong (1986). This result does not completely agree with the interpretation of 2D NMR spectra by McIntyre et al. (1996), who proposed α-Gal*p*, β-Ara*f*, and α-Ara*p*-(1 → 3)-α-Ara*f* residues, each linked (1 → 3) to Ara*f*. Our resulting galactan had a complex structure with four β-Gal*p* C-1 signals at δ 104.29, 104.39, 104.52, and 104.69 (Fig. 3B; insert). After a third degradation, the product had a considerably simplified ^{13}C NMR spectrum (Fig. 3C), the majority of signals corresponding to those of a (1 → 3)-linked β-galactan. However, its C-1 signal (δ 104.72) had a shoulder at δ 104.96 and although DEPT examination did not give a signal corresponding to C-6 of 6-*O*-substituted units, due to overlapping, HMQC showed two CH_2 signals at δ 4.03 and 4.16, which correlated with a ^{13}C signal at δ 69.97 (Fig. 4). After a fourth degradation, the product retained its viscosity viscous and the small signal at δ 104.96 (Fig. 3D; C-1 of 3,6-di-*O*-substituted residues) remained, as did the two CH_2 signals in the HMQC spectrum (Fig. 4). This would be expected from the above-cited investigation of Anderson et al. (1966).

The structures of some of the free reducing oligosaccharides of gum arabic from *A. senegal* resemble those of angico branco (*Anadenanthera colubrina*). This contains a related, but more complex high-arabinose heteropolysaccharide, but lacking side chains containing 3-*O*-substituted β-Gal*p* units (Delgobo et al., 1999; ^{13}C NMR spectrum: Fig. 1B). Although the free, reducing oligosaccharides of the two gums are related structurally, those of gum arabic have a preference for α-Gal*p* over β-Ara*p* non-reducing end units. This is apparent in structure **1**, and is 100% in the case of structure **6b** over **6a**. Both structures **7b** and **7a** are present, but in a ratio of ~7:1. Structures **6b**, **7a**, and **7b** have not been previously suggested as components of gum arabic.

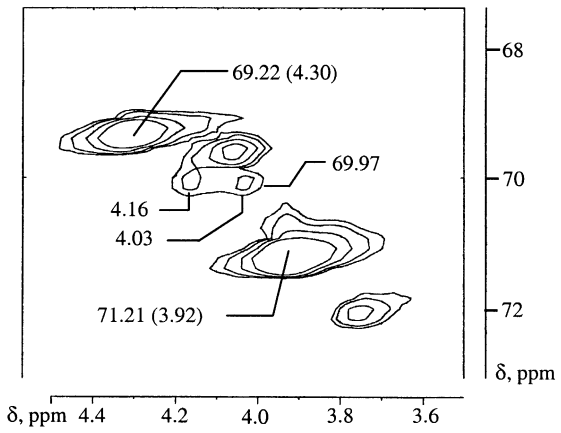


Fig. 4. Partial HMQC spectrum of polysaccharide after three and four controlled Smith degradations of gum arabic polysaccharide (30°C).

Acknowledgements

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