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Note

Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the β-glucosyl Yariv reagent

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Abstract

An arabinogalactan-protein (AGP) from pressed juice of *Echinacea purpurea* herb was isolated from a high molecular weight fraction by precipitation with the β-glucosyl Yariv reagent, followed by gel-permeation chromatography. It revealed characteristic features of other AGPs: i.e., a high amount of polysaccharide (83%) with a ratio of galactose to arabinose of 1.8:1, some uronic acids (4–5%), and a low protein content (7%) with high levels of serine, alanine and hydroxyproline. The molecular weight was estimated to be 1.2×10^6 Da. Linkage and ^{13}C NMR analyses showed that the AGP is composed of a highly branched core polysaccharide of 3-, 6-, and 3,6-linked Gal*p* residues with terminal Ara*f*, GlcA*p* and terminal units of Ara*f*-(1→5)-Ara*f*-(1→. Partial acid hydrolysis resulted in loss of Ara*f* residues at the periphery of the molecule. Complete loss of reactivity toward the β-glucosyl Yariv antigen was then noticed.

Keywords: Arabinogalactan-proteins; *Echinacea purpurea*; Structural analysis; β-Glucosyl Yariv reagent

1. Introduction

Extracts and pressed juices from *Echinacea purpurea* are used as unspecific immunostimulants. Pressed juice from the herb has been reported to give good clinical effects in treatment of the common cold [1]. The putative active compounds may be found among the caffeic acid derivatives (cichoric acid, echinacoside), alkamides, polyacetylenes and polysaccharides occurring in this plant. Pharmacological investigations have shown immunomodulatory activities of cichoric acid, alkamides and polysaccharides from *E. purpurea* (for review see Ref. [2]). A range of different polysaccharides have been found in herb, root and cell culture [3–8]. The pressed juice has yielded inulin-type fructans (M_w 6 kDa, [9]), a heterogeneous pectic polysaccharide fraction (M_w 10–50 kDa, [6]) and an acidic, highly-branched arabinogalactan (M_w 70 kDa, [9]).

Macromolecules containing mainly Ara and Gal may occur in polysaccharides or in covalent association with varying amounts of protein. These proteoglycans are called arabinogalactan-proteins (AGPs) and have been

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isolated from many tissues of lower and higher plants and their exudates (for reviews see Refs. [10–13]), as well as from media of suspension-cultured cells [14–20]. Little is known about their function (for review see Ref. [21]), but besides their adhesive and water holding properties, they seem to be important for response to wounding or infections, determination of cellular identity and specific interactions as, e.g., receptors for β-glucan elicitors [11,13,22–25]. Other investigations have shown their role in cell and tissue differentiation [26–31] and control of somatic embryogenesis [32–36].

Their carbohydrate portion (usually > 90%) consists mainly of 3-, 6-, and 3,6-linked β-D-Gal*p* residues, substituted with α-L-Ara*f* residues, and the protein core of AGPs (usually < 10%) is typically rich in hydroxyproline, serine and alanine [10–13].

In this paper, we report the isolation and structural characterization of an AGP from pressed juice from the aerial parts of *E. purpurea* collected at flowering, which might be at least one of the immunomodulating components of this herbal medicine.

2. Results and discussion

Isolation and molecular-weight determination of AGP from pressed juice of E. purpurea.—Free proteins were eliminated by centrifugation after a short boiling procedure. After removal of most of the fructans and other components of molecular weight < 50,000 Da by tangential cross flow filtration, precipitation with Yariv's reagent was very effective in yielding a purified AGP (AGP_1). This AGP_1 accounted for ~0.4% of dry weight of pressed juice. In size-exclusion chromatography on Sepharose CL-4B, AGP_1 eluted as a narrow, nearly symmetrical peak (Fig. 1), which was collected (40–65 mL) and pooled (AGP_2). The apparent molecular weight of AGP_2 was estimated to be 1.2×10^6 Da using pullulans with known molecular masses as reference polysaccharides. The behaviour of macromolecules in gel-filtration is determined by their hydrodynamic volume in solution, so that the estimated molecular mass will be different from that obtained by absolute methods of molecular-weight determination. The apparent molecular weight of the isolated AGPs is comparable with those of some arabinogalactan-protein fractions from Acacia gums, which are in the range $1–4 \times 10^6$ Da [37–40].

Composition of AGP.—A characteristic feature of AGPs is a high proportion of carbohydrate. According to the monosaccharide analysis, the isolated AGP_2 also contains a high proportion of polysaccharide (83% w/w). Gal (59.1%) and Ara (33.2%) comprised over 90% of the monosaccharide constituents of the purified AGP_2 with minor amounts of GlcA (4%), Man (2.6%) and Rha (1.1%). Uronic acid residues were detected after reduction to the corresponding neutral monosaccharide. The Ara:Gal ratio was 1:1.8.

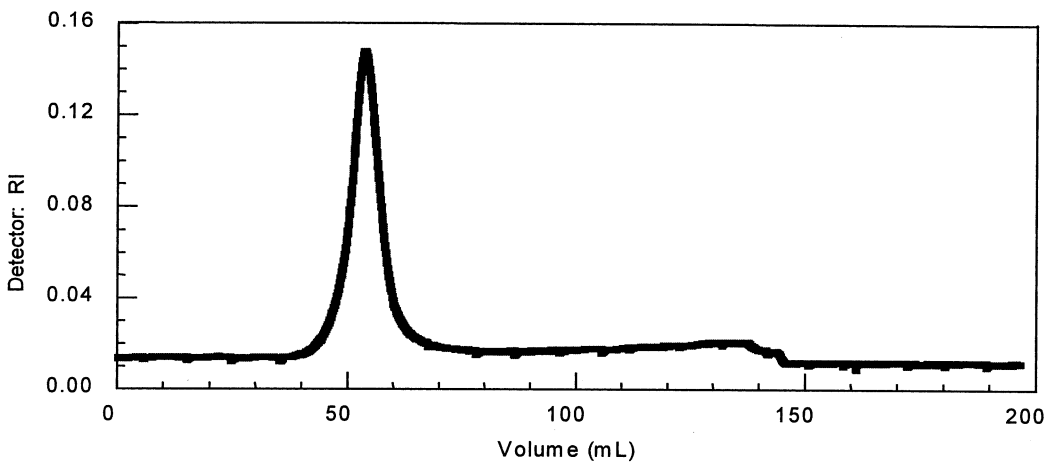

Fig. 1. Gel filtration of Yariv precipitate (AGP_1) on Sepharose CL-4B.

Table 1
Neutral monosaccharide composition of AGP_1 and its degradation products after partial acid hydrolysis

Neutral monosaccharide	AGP_1 (mol%) (Yariv precipitate)	GP_1 (mol%) (80% ethanol-insoluble fraction after partial acid hydrolysis of AGP_1)	A_1 (mol%) (80% ethanol-soluble fraction after partial acid hydrolysis of AGP_1)	A_1 (mol%) without TFA hydrolysis
Ara	35.2	6.8	84.5	93.9
Gal	61.8	90.6	11.4	2.0
Others	3.0	2.6	4.1	4.1

The protein content of AGP_1 was 7.1% (w/w), calculated from the amino acid analyses. The amino acid composition of the protein component of the AGP was characteristic for AGPs and typically rich in Ser (16.0%), Ala (12.5%), Hyp (11.5%), Asx (10.6%), Thr (10.5%), and Glx (9.4%). Minor amounts of Arg (7.5%), Gly (4.6%), Val (4.1%), His (3.7%), Lys (3.3%), Leu (2.5%), Ile (2.2%) and Phe (1.6%) were also detected.

Partial acid hydrolysis.—After hydrolysis of AGP_1 with 12.5 mM oxalic acid and precipitation with ethanol, the precipitate and supernatant were freeze-dried and analyzed for their neutral sugar composition. The results are given in Table 1.

Monosaccharide analysis of the residual polysaccharide remaining after partial acid hydrolysis (GP_1) showed that the major part of the galactose content of the starting material was recovered in this fraction. Most of the arabinose of the initial sample was recovered in the 80% ethanol-soluble fraction (A_1), indicating that arabinose is located at the periphery of the molecule. Monosaccharide analysis of A_1 without any hydrolysis revealed 2% of Gal. After TFA-hydrolysis of A_1 the amount of Gal increased to 11.4%, suggesting that some galactose di- or oligosaccharides have been present in this fraction.

Hydroxyproline content of GP_1 was also determined and could be shown to be higher compared with AGP_1, due to the fact that the polysaccharide moiety decreases during mild acid hydrolysis. This indicates that the linkages between the protein and polysaccharide moiety are stable under these hydrolytic conditions.

GP_1 was not precipitated with Yariv's reagent. The interaction of AGPs with this reagent is still not understood in molecular terms. The result that terminal Ara*f* units are required for interaction is an important step for understanding the molecular mechanism of this reaction, and in agreement with previous observations of Komalavilas et al. [18].

High-performance thin-layer chromatography (HPTLC) analyses.—High-performance thin-layer chromatography (HPTLC) analyses confirmed the results of GC analyses and additionally revealed the presence of GlcA in AGP_1, AGP_2 and GP_1. Thus, partial acid hydrolysis with 12.5 mM oxalic acid resulted in loss of Ara and a small amount of Gal residues, but no detectable loss of GlcA.

Linkage analyses of AGPs.—AGP_2 was subjected to linkage analysis by methylation after reduction of carboxyl groups. AGP_1 and GP_1 were investigated directly by methylation analysis (Table 2 and Fig. 2(a) and (b)).

Ara was present in the furanose form in the three polysaccharides AGP_2, AGP_1 and GP_1. The major part of the Ara*f* was terminally-linked, but small amounts of 5-linked Ara*f* were also detected. Gal was present in the pyranose form and was mostly 3,6-linked in the original AGPs from pressed juice (AGP_1, AGP_2). In addition, appreciable amounts of 3- and 6-linked Gal*p* were detected and also small amounts of terminally-linked Gal*p* residues. Methylation analysis of reduced AGP_2 revealed the presence of terminal GlcA in the pyranose form. Because of shortage of material, AGP_1 was not reduced before methylation, so that GlcA could not be detected; however, the presence of GlcA was shown by HPTLC. The molar ratio of Ara:Gal:GlcA in AGP_2, determined by GLCMS, corresponded well with results from GC analyses of this polysaccharide. The molar

recovery of branched residues (3,6-linked Gal*p*) was approximately equal to the molar recovery of non-reducing terminal residues (Ara*f* and Gal*p*), indicating complete methylation.

Partial acid hydrolysis of AGP_1 with oxalic acid leads to loss of $\sim 75\%$ of Ara residues to give GP_1. The loss of terminal Ara*f* and units of Ara*f*-(1→5)-Ara*f*-(1→ leads to an increase of terminal linked Gal*p*, originating from 6- or 3,6-linked Gal*p*. The amount of 6-linked Gal*p* is much higher in GP_1 compared with AGP_1, due to loss of terminal groups from 3,6-linked Gal*p*. This leads to a smaller amount of 3,6-linked Gal*p* residues in GP_1 compared with AGP_1. There was only a slight increase in the proportion of 3-linked Gal*p* units in GP_1 compared with AGP_1, suggesting that no Ara residues were linked to the 6-position of 3,6-linked Gal*p*. Normally, 3-linked Gal*p* is part of the polysaccharide backbone in AGPs, which is not susceptible to mild acid hydrolysis. The slight increase might be due to loss of shorter side chains from 3,6-linked Gal*p* residues of the backbone.

GP_1 had not been reduced before methylation; thus GlcA residues could not be detected by methylation analysis. The presence of GlcA in GP_1, however, was shown by HPTLC.

^{13}C NMR spectroscopy.—In the ^{13}C NMR spectrum of AGP_2, two sharp signals at 110.9 and 109.0 ppm were assigned to C-1 of terminal α-L-Ara*f* and C-1 of 5-linked Ara*f*. Characteristic responses of C-4 and C-5 of terminal α-L-Ara*f* could be seen at 85.5 and 62.9 ppm, respectively.

Resonances corresponding to C-1 of terminal β-D-Gal*p* and 3-, 6- and 3,6-linked Gal*p* appeared as a cluster of signals from 104.3 to 105.1 ppm. There were no signals attributable to α-glycosidic linkages of Gal*p*. A small signal at 175 ppm was assigned to the carboxyl group of GlcA.

The ^{13}C NMR data of *E. purpurea* AGP were in close agreement with data reported for AGPs from *Nicotiana alata* [41] and data for AGPs from grape [42,43]. There is little information in the literature about chemical shifts typical for 5-linked Ara*f*, but resonances corresponding to C-1 (109.0), C-2 (81.8), C-3 (77.1), C-4 (82.9) and C-5 (68.3) of 5-linked Ara*f* obtained in this study correspond with data for AGPs from grape berries [42].

Proposed structure of E. purpurea.—The structure of the carbohydrate moiety is consistent with models of the arabino-3,6-galactans [11], classified by Aspinall [44] as type II arabinogalactans.

The molecule is composed of a backbone of 3-linked β-D-Gal*p* residues, which is highly branched at O-6 to (1→6)-β-D-galactopyranosyl side chains. These 6-linked Gal*p* residues are substituted mainly with terminal α-L-Ara*f* and some terminal Gal*p* residues.

The structure of the isolated Echinacea AGP is a little more complicated compared with other AGPs, for example those from *Nicotiana alata* [41], *Lolium multiflorum* [14], *Gladiolus gandavensis* [45] or *Phaseolus vulgaris* [16]. Echinacea AGP additionally contains 5-linked Ara and *t*-GlcA residues. It shows similarities to structures of AGPs from gum arabic [37,38,46–48]; but in these AGPs

Table 2
Linkage analyses of Echinacea AGPs and the degradation product after partial acid hydrolysis

Glycosyl residue	Linkage	AGP_2 (mol%) (Yariv precipitate/GPC)	AGP_1 (mol%) (Yariv precipitate)	GP_1 (mol%) (80% ethanol-insoluble fraction after partial acid hydrolysis of AGP_1)
Ara*f*	terminal	23.7	24.8	7.2
	5-	8.7	11.7	2.2
Gal*p*	terminal	1.9	2.3	13.9
	3-	10.9	12.7	15.0
	6-	14.7	15.4	35.9
	3,6-	30.0	31.8	20.4
GlcA*p*	terminal	5.3	present [a]	present [a]
others		4.8	1.3	5.4

[a] Determined by HPTLC.

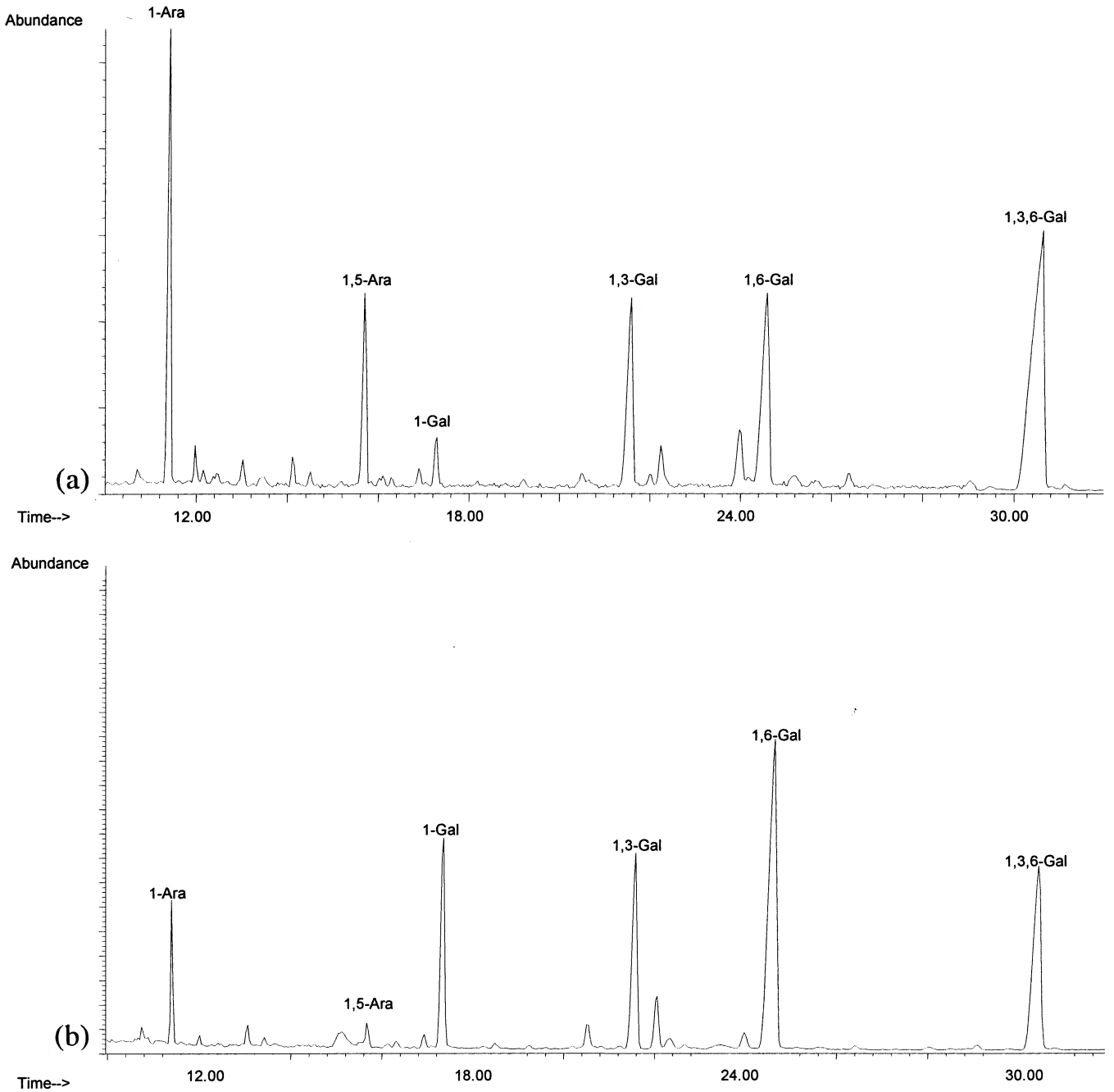

Fig. 2. (a) Total ion chromatogram of AGP_1 after methylation analysis. (b) Total ion chromatogram of GP_1 after methylation analysis.

glucuronic acid content is higher, rhamnose occurs as additional monosaccharide, and the protein content is lower. The Echinacea AGP shows great similarities to AGPs from suspension culture medium of *Rosa sp.* [18], as well as grape juice [43] and apple juice [49] with regard to protein and uronic acid content, Gal:Ara ratios and linkage analyses.

Linkage of arabinogalactan substituents to the polypeptide backbone through an alkali-stable β-D-Gal*p*-Hyp linkage has been proposed by some authors [13,41,43], although linkages through serine or threonine in AGPs might also be possible [50].

The Echinacea AGP contains $\sim 7\%$ protein, as estimated from the amino acid analysis. The molecular weight of the molecule was estimated to be 1.2×10^6 Da. Assuming that the real molecular weight is comparable to the hydrodynamic volume, the

protein portion comprises ~700 amino acids and the polysaccharide moiety ~7000 glycosyl residues. A Gal-Hyp linkage would mean that if all Hyp-residues are glycosylated, every Hyp carries a carbohydrate side chain of approximately 100 sugar residues. This is in good agreement with results of Fincher et al. [13] and Gane et al. [41], who proposed carbohydrate side chains of 95 or 120 sugar residues, respectively.

The isolation of an AGP of high molecular weight from pressed juice of *E. purpurea* by precipitation with Yariv's reagent is an important step in understanding whether it is one of the immunologically active components of this herbal medicine.

3. Experimental

Material.—Pressed juice of *E. purpurea* L. Moench, a kind gift of Madaus AG, Köln, Germany, was prepared from the aerial parts of the plant *E. purpurea* collected while in bloom and expressed immediately at high pressure (300 bar).

Preparation of the β-glucosyl Yariv reagent.—The reagent (1,3,5-tris-[4-β-D-glucopyranosyl-oxyphenylazo]-2,4,6-trihydroxybenzene) was prepared as described by Yariv et al. [51].

Isolation of AGP.—The pressed juice was heated to boiling for 10 min. After centrifugation (5000*g*, 5 min), the supernatant was filtrated by tangential cross flow dialysis using membranes with a molecular weight cut off of 50 kD (Biomax membrane, Millipore, Eschborn, Germany). The freeze-dried, high-molecular-weight fraction (400 mg) was dissolved in 100 mL of distilled water and precipitated by addition of an equal volume of an aq soln of 1 mg/mL β-glucosyl Yariv reagent and NaCl at a final concentration of 0.15 M [34]. The AGP–Yariv complex was precipitated at 4 °C overnight, centrifuged at 10000*g* for 15 min, washed with 0.15 M NaCl and dissolved in water. Sodium hydrosulfite ($Na_2S_2O_4$) was added to a final concentration of 10% (w/v) to decompose the β-glucosyl Yariv reagent [35]. The solution was heated to 50 °C until the red colour disappeared, dialysed extensively against water at 4 °C and freeze-dried (AGP_1).

Gel-permeation chromatography (GPC).—Freeze-dried AGP_1 was dissolved in distilled water, filtered (0.2 μm), applied to a column of Sepharose CL-4B (XK 16 × 100 cm, Pharmacia, Freiburg, Germany), eluted in water and the eluent monitored by RI-detection (ERC-7515A, Erma, Tokyo, Japan). The molecular weight of the arabinogalactan-protein fraction (AGP_2) was estimated using pullulans (Shodex, Denkko, Japan) with known molecular masses as reference polysaccharides.

Analysis of components.—Neutral monosaccharides were analysed after acid hydrolysis (trifluoroacetic acid: 2 M, 1 h, 121 °C) as their alditol acetates by gas chromatography [52].

For amino acid analyses, AGP samples were hydrolyzed in 6 M HCl at 110 °C for 22 h, dried by evaporation and dissolved in Na-borate buffer (pH 2.2). After centrifugation (2000 *g*, 5 min), the amino acid composition of the solution was determined in an amino acid analyser (LC 3000, Eppendorf, Hamburg, Germany) by ninhydrin detection.

Colorimetric determination of OH-proline was carried out according to 'Lebensmittel- und Bedarfsgegenständegesetz' (LMBG, §35, L06.00/8).

Reduction of carboxyl groups.—Reduction of carboxyl groups in AGP_2 (10 mg) was carried out with 1-cyclohexyl-3-[2-methylmorpholinoethyl]-carbodiimide-metho-4-toluolsulfonate and sodium borodeuteride according to the method of Taylor and Conrad [53].

Methylation analysis.—Methylation was performed with potassium methylsufinyl carbanion and methyl iodide in Me_2SO, as described by Harris et al. [54]. GLCMS of partially methylated alditol acetates was carried out on a fused silica capillary column (0.25 i.d. × 25 m, OV-1701, Macherey & Nagel, Düren, Germany) with the following temperature program: 2 min 170 °C, increase of 1 °C per min up to 210 °C, 10 min 210 °C. Helium flow was 0.7 mL/min. Mass spectra were recorded on a HP MS Engine 5898 A (Hewlett Packard) instrument. Mass spectrometer conditions were: ionization potential 70 eV; source temperature 200 °C.

Partial acid hydrolysis.—AGP (20 mg) was dissolved in 2 mL of 12.5 mM oxalic acid and heated at 100 °C for 5 h [45]. The hydrolysate was cooled to room temperature, precipitated by addition of EtOH (final concentration 80% v/v), left overnight at 4 °C and centrifuged (20,000*g*, 10 min). After two washing steps with 80% ethanol, the precipitated material (GP_1) and the combined supernatants (A_1) were freeze-dried.

High-performance thin-layer chromatography (HPTLC).—AGP_1, AGP_2 and GP_1 were hydrolysed in 2 M trifluoroacetic acid (TFA) for 1 h at 121 °C, evaporated to dryness three times with 5 mL of water, and dissolved in 1:1 MeOH–water. A_1 was dissolved directly in the same solvent. HPTLC was carried out on Silica Gel 60 plates (10 × 10 cm, E. Merck, Germany), which were developed horizontally in 85:14:1 acetonitrile–water–AcOH three times (6 cm) and heated at 80 °C for 10 min after each development. Detection was achieved with 2% aniline and 2% diphenylamine in 10:1 MeOH–85% phosphoric acid, followed by heating at 120 °C for 10 min. Suitable carbohydrate standards were used.

NMR spectroscopy.—AGP samples were dissolved in D_2O (30 mg/mL) and ^{13}C NMR spectra were recorded with ^{1}H composite pulse decoupling on a Bruker ARX-300 spectrometer at 75.5 MHz at 27 °C for 18 h with a 2 s pulse delay, 2.2 s aquisition time and a 90° pulse angle. Chemical shifts were set relative to Me_2SO (39.5 ppm). Peaks were assigned by comparison with published spectra [41–43].

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