CHEMICAL AND ^{13}C NMR STUDIES OF A RHAMNOARABINOGALACTAN FROM THE LEAVES OF *Plantago lanceolata* L. var. *libor*

Alzbeta KARDOSOVA and Peter CAPEK

Institute of Chemistry,
Slovak Academy of Sciences, 842 38 Bratislava, The Slovak Republic

Received August 10, 1994
Accepted October 31, 1994

A rhamnoarabinogalactan isolated from the leaves of *Plantago lanceolata* L. var. *libor* has been investigated by methylation analysis, partial hydrolysis, and ^{13}C NMR spectroscopy. The structural conclusions obtained by ^{13}C NMR measurements were consistent with the data from methylation analysis. The polysaccharide has a β-(1→6)-D-galactan core branched on O-3 by side chains of (1→5)-linked α-L-arabinofuranosyl units, some of which bear in position O-5 terminal β-D-galactopyranosyl residues. L-Rhamnose occurs as nonreducing ends.

Searching for biologically active polysaccharides, we have succeeded in isolation and structure elucidation of polysaccharides from *Althaea officinalis* L. var. *robusta* which were found to be effective in suppressing purposely induced cough reflex in cats[1,2]. *Plantago lanceolata* L. is a medicinal plant with therapeutic effects comparable to those of *A. officinalis*. The only work dealing so far with isolation of polysaccharides from the leaves of this plant was published by Brautigam and Franz[3]. The authors separated from the crude mucilage three distinct polysaccharides, i.e. a glucomannan, an arabinogalactan and a rhamnogalacturonan. Their gross structural features were derived from the results of methylation analysis.

We found that the mucilage isolated from the leaves of a new variety of *P. lanceolata* was effective in the antitussive activity tests. In order to have homogeneous, defined compounds of plant origin for the tests mentioned above, from the mucilage we have so far isolated a linear (1→6)-α-D-glucan[4] and now, we report on isolation and structure determination of a water-soluble rhamnoarabinogalactan.

EXPERIMENTAL

Material and Methods

The medicinal plant *Plantago lanceolata* L. var. *libor* was improved in the Czech Republic at the Cultivation Station, Libochovice. Concentrations were performed under reduced pressure at bath temperature not exceeding 45 °C. Optical rotations (1 ml cells) were measured at 20 ± 1 °C with a Perkin–Elmer Model 141 polarimeter. Free-boundary electrophoresis of polysaccharide solutions

(10 mg ml^{-1}) was performed in 0.05 M sodium tetraborate buffer (pH 9.3) with a Zeiss 35 apparatus at 150 V and 8 mA for 30 min. The number average molecular mass $\overline{M}_n$ was determined osmometrically at 30 °C, using a Knauer Pressure Osmometer. Infrared spectra of the methylated products were recorded with a Perkin–Elmer 9836 spectrometer. HPLC analysis of the polysaccharide was performed with a Separon HEMA-BIO 100 column (8 × 250 mm; 2 columns arranged successively) in a liquid chromatograph (Laboratorni pristroje, Prague) equipped with a differential refractometer. The eluent 0.1 M $NaNO_3$ was pumped at 0.4 ml min^{-1}.

GLC was conducted on a Hewlett–Packard Model 5711 A instrument with (i) a column I (0.3 × 200 cm) of 3% OV-225 on Chromosorb WAW-DMCS (80 – 100 mesh) at 120 °C (4 min) → 180 °C (2 °C min^{-1}), flow rate 30 ml min^{-1}; (ii) a column II (0.3 × 200 cm) of 3% SP-2340 on Supelcoport (100 – 120 mesh) at 110 °C (2 min) → 210 °C (4 °C min^{-1}), flow rate 30 ml min^{-1}. Column I was used for quantitative analysis of sugar trifluoroacetates[5].

Paper chromatography (PC) was conducted by the descending method on Whatman No. 1 and 3MM papers with the following solvent systems: S1, ethyl acetate–pyridine–water (8 : 2 : 1); S2, ethyl acetate–acetic acid–water (18 : 7 : 8). Sugars were detected with anilinium hydrogen phthalate and alkaline silver nitrate. The mobilities of oligosaccharides (R_{Ara}, R_{Gal}) are expressed relative to those of L-arabinose and D-galactose. Uronic acids were determined by potentiometric titration and carbohydrates by the phenol–sulfuric acid assay[6]. Protein was determined by the method of Lowry et al.[7], using bovine serum albumin as standard.

GLC-MS was carried out with a JGC-20 K gas chromatograph fitted with column II and with helium (inlet pressure 101.3 kPa) as the carrier gas. Mass spectra were obtained at 23 eV and an emission current of 300 μA, using a JMS-D 100 (JEOL) spectrometer. The inlet temperature was 220 °C and that of the ionizing chamber 200 °C.

FT ^{13}C NMR spectra were measured at 35 °C for solutions in D_2O (internal standard MeOH, 50.15 ppm) on a Bruker AM-300 (75 MHz) spectrometer with inverse-gated decoupling. The spectral width was 16.38 kHz; acquisition time 0.5 s; data points 16 K; pulse width 19 μs (90°).

Isolation of Rhamnoarabinogalactan

The air-dried, methanol-preextracted leaves (60 g) were macerated in cold water (2.5 l) for 24 h at room temperature. The extract after filtration and concentration (600 ml) was poured into ethanol (3.6 l). The precipitate was collected by centrifugation, washed with aqueous ethanol (70 vol.%), suspended in water, exhaustively dialyzed against distilled water, and freeze-dried. The brownish crude product (0.9 g; 1.5% based on dry leaves) containing 5.6% protein yielded on hydrolysis: D-Gal, D-Glc, D-Man, L-Rha, L-Ara, D-Xyl, and uronic acids.

In order to separated the neutral polysaccharides from the acid ones, the product (1 g) in water (6 ml) was applied to a column (5 × 100 cm) of DEAE-Sephadex A-50 (carbonate form) and irrigated with water, then with 0.25 M and 0.5 M ammonium carbonate solutions. The water eluate was concentrated to small volume and freeze-dried. In this step, most of the accompanying colour material was removed and the protein content was reduced to 0.8%. The yellowish product *P* (380 mg; 38%) yielded on hydrolysis: D-Gal (41.4%), D-Glc (15.8%), D-Man (1.8%), L-Rha (4.2%), L-Ara (29.1%), and D-Xyl (7.4%).

A solution of *P* (200 mg) in water (5 ml) was applied to a column (4 × 150 cm) of Sephadex G-75 (40 – 120 μm) and irrigated with water. Assay for total carbohydrates (Fig. 1) revealed 2 polysaccharide fractions (*1*, *2*). Fraction *1* (40.5 mg; 20.2%) was confirmed by free-boundary electrophoresis and HPLC analysis to be homogeneous and was used for further investigation. Fraction *2* was identified as a (1→6)-α-D-glucan[4].

Methylation Analysis

The polysaccharide (60 mg) was methylated once by the Ciucanu method[8] and twice by the Purdie method[9] to give a fully methylated product (55 mg), as evidenced by absence of IR absorption for hydroxyl. The methylated polysaccharide (10 mg) was treated with aqueous 90% formic acid (2 ml) at 100 °C for 1 h, the hydrolyzate was concentrated to dryness, and the residue was hydrolyzed with 2 M TFA (2 ml) at 100 °C for 6 h. PC then revealed di-, tri-, and tetra-*O*-methyl saccharides, which were converted into their alditol acetates and analyzed[10] by GLC-MS (column II).

The disaccharide (7 mg) was methylated with methyl iodide (2 ml) and sodium hydride (20 mg) in *N*,*N*-dimethylformamide (2 ml).

Partial Hydrolysis

The polysaccharide (300 mg) was treated with 0.1 M TFA (2 h, 100 °C), and then freed from low-molecular-weight products, i.e. Ara, Rha, compounds *I* and *II*, by elution from a Biogel P-2 column (2.5 × 130 cm). The polymeric portion was collected, analyzed for chemical composition, and submitted to further hydrolysis with 1 M TFA (40 min, 100 °C) to afford Ara, Gal, compound *III*, and the polymeric portion *DG*. The low-molecular-weight products from both gel filtrations were purified by preparative paper chromatography on Whatman No. 3MM paper in S1.

RESULTS AND DISCUSSION

The cold-water extract of lipid-free leaves of *P. lanceolata* contained polymeric material, accounting for 1.5% of dry leaves, the sugar composition of which suggested a mixture of different polysaccharides. This material was resolved on DEAE-Sephadex A-50 to neutral and acid portions. The neutral portion was subsequently fractionated by gel permeation chromatography on Sephadex G-75 (Fig. 1) to give a polysaccharide (fraction *1*) homogeneous by gel filtration and free-boundary electrophoresis. $\overline{M}_n$ 22 000 (DP 148), $[\alpha]_D$ +80° (*c* 0.5, water). It was composed of D-Gal, L-Ara, and L-Rha in

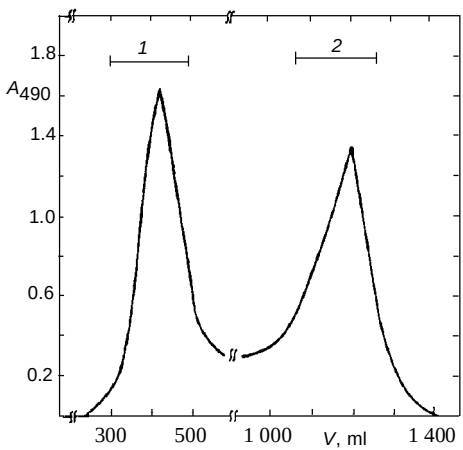

FIG. 1
Gel chromatography elution profile of product *P* on Sephadex G-75; A_{490} absorbance at 490 nm; *V* elution volume; *1*, *2* polysaccharide fractions

the molar ratio 1.00 : 0.66 : 0.21. HPLC analysis of the polysaccharide showed a narrow symmetrical peak, indicating a narrow distribution of molecular masses.

The polysaccharide was subjected to controlled acid hydrolysis under mild conditions. Treatment of the polysaccharide with 0.1 M TFA, followed by gel filtration on Biogel P-2, yielded besides the polymeric material free Ara, Rha as well as compounds *I* and *II*. Hydrolysis of the polymeric material under stronger conditions (1 M TFA) resulted in cleavage of further arabinose units, galactose monomer, compound *III*, and the polymeric residue *DG*.

Compound *I* (2 mg), $[\alpha]_D$ −14° (*c* 0.25, water) gave Ara on hydrolysis. Its mobility on PC R_{Ara} 0.81 (S1) was identical with that of the authentic standard of L-arabinofuranosyl-(1→5)-α-L-arabinofuranose. Due to shortage of the material, further analysis could not have been done.

Compound *II* (11 mg), R_{Gal} 0.89 (S1), $[\alpha]_D$ −0.61° (*c* 0.5, water) gave Ara and Gal on hydrolysis. The borohydride-reduced *II* yielded on hydrolysis arabinitol and galactose, proving the reducing end of the compound to be arabinose. The ^{13}C NMR spectrum (Table I) showed the signal for C-5 of Ara to be shifted downfield to 67.32 ppm due to the α-effect brought about by substitution of this carbon. The other signal shifted to lower field was that for C-1 of the Gal unit at 104.37 ppm, proving the involvement of this carbon in a glycosidic linkage of the β-type. Compound *II* is then a D-Galp-(1→5)-β-L-Araf. Similar disaccharide was found in the polysaccharide isolated[11] from stem of *Avena sativa*.

Compound *III* (12 mg), R_{Gal} 0.49 (S1), $[\alpha]_D$ +24.7° (*c* 0.5, water) gave Gal on hydrolysis. The mass spectrum of methylated *III* contained characteristic peaks of ions aA_1 (*m*/*z* 219), aA_2 (*m*/*z* 187), aA_3 (*m*/*z* 155), abJ_1 (*m*/*z* 279), bA_1 (*m*/*z* 219), bA_2 (*m*/*z* 187), and baD_1 (*m*/*z* 353), proving the permethyl 6-*O*-hexapyranosylhexapyranoside structure; calculated molecular mass M = aA_1 + bA_1 + 16 = 454. The ^{13}C NMR spectrum (Table I) confirmed the results of mass fragmentation. Two signals were shifted downfield against those of the unsubstituted galactose unit, i.e. the signal at 104.27 ppm, indicating the involvement of C-1 in a β-glycosidic linkage, and the signal at 69.72 ppm, proving the substitution in position C-6 of the galactose unit. Compound *III* is then a D-Galp-(1→6)-β-D-Galp.

The polymeric portion *DG*, $\bar{M}_n$ 10 000 (DP 62), $[\alpha]_D$ +14° (*c* 0.5, water) afforded Gal on total hydrolysis. The ^{13}C NMR spectrum (Table I) showed the signal for C-1 at 104.55 ppm, and that for C-6 at 69.82 ppm, proving the β-(1→6) linkage between the D-galactose units.

The partially methylated sugars obtained upon hydrolysis of the fully methylated polysaccharide were analyzed by GLC as their alditol acetates, and their identities were confirmed by GLC-MS (ref.[10]). The proportions of the different methylated derivatives and the corresponding linkage analysis is given in Table II.

Identification of *I – III*, *DG*, and the results of methylation analysis proved the rhamnoarabinogalactan macromolecule to have a branched structure. The methyl derivatives of D-galactose indicated a skeleton of (1→6)-linked β-D-galactopyranosyl residues, almost half of which were substituted at the 3-position. As evidenced by the tetramethyl derivative, a small amount of D-Gal formed nonreducing ends, terminating the L-arabinofuranosyl side chain, as indicated by compound *II*.

TABLE I
^{13}C NMR data for the oligosaccharides and the D-galactan core derived from rhamnoarabinogalactan

Compound	Residue	Chemical shifts, ppm					
		C-1	C-2	C-3	C-4	C-5	C-6
II D-Galp-(1→5)-β-L-Araf	Aα	102.25	82.40	76.93	82.76	67.32	–
B A	Aβ	96.5	76.93	75.50	82.40	67.32	–
	Bβ	104.37	71.78	73.28	70.02	76.93	62.11
III D-Galp-(1→6)-β-D-Galp	Aα	93.46	69.72	69.90	70.43	71.90	69.72
B A	Aβ	97.53	73.76	74.90	70.43	74.90	69.32
	Bβ	104.27	72.89	73.76	70.43	76.28	62.11
DG (1→6)-β-D-galactan	–	104.55	71.90	73.78	70.65	74.91	69.81

TABLE II
Methylated sugars from the hydrolyzate of the methylated rhamnoarabinogalactan

Derivative[a]	*T*[b]	Mole %	Linkage indicated
2,2,4-Me_3-Rha	0.80	6.98	L-Rhap-(1→
2,3,5-Me_3-Ara	0.83	11.42	L-Araf-(1→
2,3,4,6-Me_4-Gal	1.00	3.80	D-Galp-(1→
2,3-Me_2-Ara	1.01	22.92	→5)-L-Araf-(1→
2,3,4-Me_3-Gal	1.14	29.20	→6)–D-Galp-(1→
2,4-Me_2-Gal	1.26	25.65	→3,6)-D-Galp-(1→

[a] 2,3,4-Me_3-Rha: 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol; 2,3,5-Me_3-Ara: 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol; 2,3,4,6-Me_4-Gal: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol; 2,3-Me_2-Ara: 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-L-arabinitol; 2,3,4-Me_3Gal: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol; 2,4-Me_2-Gal: 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol. [b] Retention time of the corresponding alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol.

L-Arabinose was found not only at terminal nonreducing position, but also as α-(1→5)-linked side-chain units, as indicated by the presence of 2,3-di-*O*-methyl-L-arabinose, as the only dimethyl ether of this sugar.

All L-rhamnopyranose residues represented nonreducing ends, probably terminating the arabinofuranosyl side chains. Similar terminal position of L-Rha units was found in arabinogalactans of various plant origin[12–14].

The ^{13}C NMR spectrum of the rhamnoarabinogalactan supported the ratio of sugar components, the type of glycosidic linkages and their anomeric configuration, derived from chemical analysis and partial hydrolysis. The assignment of signals was based on comparison with the literature data for oligosaccharides[15,16], polysaccharides[12,14] of similar composition, and taking into account the effect of glycosylation.

The very characteristic low-field signals in the anomeric region at 110.35 and 108.53 ppm were consistent with the presence of furanosidic forms of α-L-Ara units. The C-1 signals at 104.25 ppm arose from β-D-Galp residues (nonreducing, chain units, branching points), and the signal at 100.90 ppm represented the resonance of C-1 of L-Rhap units. Under the conditions of spectral measurement (suppressed NOE and complete relaxation of nuclei between the individual pulses), the signal intensities permitted conclusions on the relative proportion of the constituent sugars of the polysaccharide. The relative molar proportions of α-L-Araf, β-D-Galp, and L-Rhap units, detected from the integrated intensities of C-1 signals, were 0.6 : 1.0 : 0.2, which corresponded fairly well with those found in GLC analysis of the component sugars of the polysaccharide.

The resonances of other carbons involved in glycosidic linkages were observed at 81.28 ppm (assigned to C-3 of 1,3,6-linked β-D-Galp chain units), 69.80 – 69.47 ppm (ascribed to C-6 of 1,6- and 1,3,6-linked β-D-Galp chain units), and at 67.40 – 67.02 ppm (arising from C-5 of 1,5-linked α-L-Araf side chains). Due to complexity of the spectrum in the non-anomeric region, calculations of relative proportions of the glycosylated carbons from the integrated intensities of the respective signals were not attempted.

On the basis of the obtained results it may be concluded that the rhamnoarabinogalactan has a D-galactan skeleton, carrying (1→5)-linked α-L-Araf side branchings attached to O-3 of approximately each second (1→6)-linked β-D-Galp unit. Some of these branchings are terminated by β-D-Galp residues. We failed to prove whether the L-Rhap residues terminate the side α-L-Araf branchings only or are attached also to O-3 of the D-galactan core.

REFERENCES

1. Nosalova G., Strapkova A., Capek P., Kardosova A.: Pharm. Pharmacol. Lett. *2*, 195 (1992).
2. Nosalova G., Strapkova A., Kardosova A., Capek P.: J. Carbohydr. Chem. *12*, 589 (1993).
3. Brautigam M., Franz G.: Planta Med. *1985*, 293.
4. Kardosova A.: Chem. Papers *46*, 127 (1992).
5. Shapira J.: Nature *222*, 792 (1969).

6. Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A., Smith F.: Anal. Chem. *28*, 350 (1956).
7. Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
8. Ciucanu I., Kerek F.: Carbohydr. Res. *131*, 209 (1984).
9. Hirst E. L., Percival E.: Methods Carbohydr. Chem. *5*, 287 (1965).
10. Jansson P. E., Kenne L., Liedgren H., Lindberg B., Lonngren J.: Chem. Commun. Univ. Stockholm *8*, 1 (1976).
11. Buchala A. J., Fraser C. G., Wilkie K. C. B.: Phytochemistry *11*, 2803 (1972).
12. Varljen J., Liptak A., Wagner H.: Phytochemistry *28*, 2379 (1989).
13. Akiyama Y., Eda S., Kato K.: Agric. Biol. Chem. *46*, 1395 (1982).
14. Saulnier L., Brillouet J. M., Moutounet M., Herve du Penhoat C., Michon V.: Carbohydr. Res. *224*, 219 (1992).
15. Bradbury J. H., Jenkins G. A.: Carbohydr. Res. *126*, 125 (1984).
16. Agrawal P. K.: Phytochemistry *31*, 3307 (1992).