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Structural Studies on Pectin from Marsh Cinquefoil *Comarum palustre* L.

R. G. Ovodova[1], O. A. Bushneva[1], A. S. Shashkov[2], A. O. Chizhov[2], and Yu. S. Ovodov[1]*

[1]Institute of Physiology, Komi Science Center, The Urals Branch of the Russian Academy of Sciences, ul. Pervomaiskaya 50, 167982 Syktyvkar, Russia; fax: (7-8212) 241-001; E-mail: ovoys@physiol.komisc.ru
[2]Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, 119991 Moscow, Russia; fax: (7-095) 135-5328; E-mail: shash@ioc.ac.ru; chizhov@ioc.ac.ru

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Abstract—Pectin with $[\alpha]_D^{20}$ +192° (*c* 0.1; water), named comaruman, was isolated from marsh cinquefoil *Comarum palustre* L., which is widespread in the European North. The sugar chain of comaruman contains residues of D-galacturonic acid (64%), D-galactose (13%), L-rhamnose (12%), L-arabinose (6%), and trace amounts of xylose and glucose. Partial acid hydrolysis and digestion with pectinase demonstrated that comaruman composed of the backbone comprised regions of linear α-1,4-D-galactopyranosyl uronan interconnected by numerous residues of α-1,2-L-rhamnopyranose. In addition to the backbone (core of the macromolecule), ramified regions are involved in comaruman and comprise α-2,4-L-rhamno-α-4-D-galacturonan with side chains consisting mainly of β-1,4-linked residues of D-galactopyranose. The ramified region contains additionally residues of 5-O-substituted arabinofuranose and 3- and 6-O-substituted galactopyranose. The present 3,4- and 4,6-di-O-substituted residues of galactopyranose appear to be branching points of the side chains. Some galactopyranose residues were found to occupy the terminal positions of the side chains or appeared to be single sugar residues attached to the side chains. Methylation analysis data indicated that comaruman contains residues of terminal, 3- and 3,4-di-O-substituted galactopyranosyl uronic acid, which appeared to be constituents of the side chains, and the latter represented additionally branching points of the backbone.

Key words: plant polysaccharides, pectin, marsh cinquefoil, *Comarum palustre* L., comaruman, enzymic hydrolysis, NMR spectroscopy of polysaccharides, methylation analysis, GLC-MS of methylated sugars

Pectins are well known to be one of the most widely distributed groups of plant polysaccharides. They occur in virtually all the higher plants and in some marine and freshwater phanerogams and show great structural diversity [1]. At present, pectins are considered to be the most complicated polysaccharides in relation to the structural features. They have irregular block sugar chain and contain various macromolecular segments of the linear and ramified regions. The linear region consists of α-1,4-D-galacturonan chain as the backbone of all pectins. The ramified regions contain various heteroglycanogalacturonan segments such as rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), xylogalacturonan, and apiogalacturonan [2].

The available structural information indicates that all pectins are characterized by a certain type of the sugar chain: galacturonan is the main constituent of the macromolecule of bergenan (pectin of *Bergenia crassifolia*); silenan and tanacetan, pectins of *Silene vulgaris* and *Tanacetum vulgare*, respectively, are branched rhamnogalacturonans [3, 4]; lemnan (pectin of *Lemna minor*) contains an apiogalacturonan segment as the main constituent of the macromolecule [5]. Each type of pectic polysaccharide possesses substantial peculiarities of fine structural features of the ramified regions of the macromolecule, these causing differences in physiological activity and physicochemical properties [1]. A search for new pectins possessing higher physiological activity and useful physicochemical properties, especially the gel-forming ability important for industrial use, is of great interest.

The present study is devoted to isolation and elucidation of the structure of comaruman, pectin of the medicinal plant *C. palustre* L. Physiological bioassays have demonstrated that comaruman possesses anti-inflammatory action [6], produces exceptionally viscous aqueous solutions, and is of interest for structural investigation.

* To whom correspondence should be addressed.

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MATERIALS AND METHODS

Isolation of comaruman. Comaruman (CP) was extracted from the fresh aerial part of *C. palustre* with aqueous ammonium oxalate as described previously [7]. The plant material was harvested in Visinga (Komi Republic, Russia) at the period of budding. The fresh collected material was treated with 0.5% formalin for binding polyphenolic compounds and enzyme deactivation. Protopectin was destroyed by adding dilute hydrochloric acid (pH 4.0), and the mixture was stirred at pH 4.0 at 50°C for 3 h. The extract obtained was concentrated and dialyzed using an ultrafiltration column with hollow fibers bearing pores for molecules with molecular mass up to 100 kD. The pectic polysaccharide was precipitated from the purified concentrated solution with four volumes of 96% ethanol, and the material obtained was dissolved in distilled water and lyophilized. As a result, comaruman was obtained with yield of 4% of the air-dried raw material.

General analytical procedures. Glycuronic acid contents were determined using interaction of polysaccharide fractions with 3,5-dimethylphenol in the presence of concentrated sulfuric acid [8] in comparison with a standard curve for D-galacturonic acid. The total protein contents were estimated according to the Lowry method [9] using a standard curve for BSA. The contents of methoxy groups were calculated as in [10] using a standard curve for methanol. All the spectrophotometric measurements were run on an Ultrospec 3000 instrument (England). The specific optical rotations were determined using a Polatronic MHZ polarimeter (Germany).

Monosaccharides were identified using descending paper chromatography on Filtrak FN-12 and Filtrak FN-13 papers in 6 : 4 : 3 (v/v) butan-1-ol–pyridine–H_2O developing system. The sugars were detected by spraying with a solution of aniline hydrogen phthalate following by heating at 105°C.

Neutral monosaccharides as the corresponding alditol acetates were qualitatively and quantitatively analyzed using GLC performed on a Hewlett-Packard 4890A instrument (USA) using a flame-ionization detector and a HP 3395A integrator on an RTX-1 capillary column (0.25 mm × 30 m) (Restek) with argon as carrier gas using the following temperature program: isothermal at 175°C for 1 min, then 3°C/min gradient up to and isothermal at 250°C for 2 min. The percentage of sugars in the total sample was calculated from the peak areas using response factors of the detector [11].

The absolute configurations of monosaccharides from comaruman were determined using GLC of acetylated (+)- and (–)-2-octyl glycosides as described earlier [12] in comparison with the corresponding glycosides of D-galactose, L-rhamnose, and L-arabinose as authentic samples.

GLC–MS of the partially methylated alditol acetates was performed on a chromato-mass-spectrometer Carlo Erba, Finnigan 4200 with an Ultra-1 capillary column (0.2 mm × 30 m) (Hewlett-Packard, USA); the carrier gas was helium; 5°C/min gradient from 150 to 280°C. Mass spectra were obtained with a Finnigan MAT ITD-700 ion trap (England), mass range from m/z 44 to 500. Energy of ionizing electrons was ~70 eV. The temperature of the interface was 220°C, scanning frequency was 1 scan/sec, and acquisition delay was 250 sec.

NMR spectra were recorded with a Bruker DRX-500 spectrometer (Bruker, Germany) using 3-5% oligo- and polysaccharide solutions in D_2O at 313°K (the internal standard was acetone, δ_H 2.225 ppm, δ_C 31.45 ppm). The signal assignments in NMR spectra were made on the basis of two-dimensional (2D) spectral data (COSY, TOCSY, and $^1H/^{13}C$ HSQC with proton detection) as described previously [3-5]. 2D spectra were performed using the standard Bruker procedures. The ROESY spectra were run using mixing time 200 msec. For TOCSY experiments 60-msec duration of the MLEV 17 spin-lock was used. The points of substitution and sequence of sugars were estimated using 2D experiments in determination of Overhauser effects (ROESY or NOESY).

All aqueous solutions were concentrated in vacuum at 40-45°C followed by centrifugation at 7000-8000*g* for 10-20 min. The sugars obtained were subjected to lyophilization.

Determination of rheological characteristics. The specific viscosity of comaruman and its derivatives was estimated as described previously [13]. A sample was dissolved in 0.01 M NaCl, the solution was filtered, and the specific viscosity ([η], dl/g) was measured using a VPG-4 viscometer (outflow time of solute is 90.0 sec) at 25°C.

Molecular masses of comaruman and its derivatives were determined as described previously [14]. A sample (4 mg) was dissolved in 0.15 M NaCl (2 ml) and the mixture was filtered. An Agilent 1100 chromatograph (Shimadzu, Japan) equipped with a Shodex GS-620 HQ column (Asahipak) (7.6 mm × 30 cm) and Shodex GS-26 7B subcolumn (7.6 mm × 5 cm) and a RID G 136 A refractometer were used for analyses. Elution was carried out with 0.15 M NaCl at 37°C with a flow rate of 0.5 ml/min. The column was calibrated using dextran sulfates with molecular masses in the range of 36-50, 400-600, and 1400 kD (Sigma, USA).

Ash contents in the comaruman samples were estimated as described previously [15].

Mild acid hydrolysis. Trifluoroacetic acid (TFA) (0.05 M, 20 ml) was added to comaruman (100 mg), and the mixture obtained was kept at 80°C for 4 h and then centrifuged. The supernatant was evaporated repeatedly with methanol in vacuum to remove acid, the solution obtained was concentrated, and 96% ethanol was added. The resulting precipitate was dissolved in water and lyophilized to yield fraction CPH-1 (30 mg).

Rigorous acid hydrolysis. TFA (2 M, 20 ml) was poured onto the polysaccharide (100 mg). The resulting mixture was heated at 100°C for 5 h. The unreacted residue of galacturonan was separated by centrifugation and dissolved in water, the pH of the solution was adjusted to 4-5 with aqueous ammonia under rigorous mixing, and then the solution was dialyzed and lyophilized. Galacturonan (20 mg) (fraction CPH-2) was obtained.

Saponification of comaruman. Metallic sodium (75 mg) was added to absolute methanol (50 ml) under vigorous stirring followed by addition of comaruman (115 mg), and the mixture was vigorously stirred at 20°C for 4 h. The resulting precipitate was separated by centrifugation and dissolved in distilled water, and the solution obtained was dialyzed and lyophilized to yield demethoxylated derivative CPS (89 mg).

Digestion of CPS derivative. CPS derivative (50 mg) was dissolved in water (5 ml), 4 mg of pectinase (Fluka, Germany; activity 500 U/mg) was added, and the solution was digested at 37°C. The enzymic hydrolysis was tested by increasing quantities of the reducing sugars using the Nelson and Somogyi procedure [16]. The pectinase was deactivated by boiling for 5 min at 100°C, and a precipitate obtained was removed by centrifugation. The resulting solution was concentrated, and saccharides were precipitated with four volumes of 96% ethanol. The precipitate was separated by centrifugation, dissolved in distilled water, and lyophilized to yield fraction CPSE (14 mg).

Methyl esterification of comaruman. Comaruman was preliminary dried in vacuum over P_2O_5 at 60°C, and the resulting sample (94.8 mg) was suspended in absolute methanol (100 ml); acetyl chloride (0.2 ml) was thrice added to the mixture followed by a mixing for 40 h. A precipitate was separated by a filtration, washed repeatedly with methanol, dissolved in distilled water, and lyophilized to yield methyl-esterified derivative CPM (89 mg).

Preparation of a triethylammonium salt of comaruman. Comaruman (50 mg) was dissolved in distilled water and dialyzed against 1% aqueous triethylamine hydrochloride for two days changing repeatedly the solution of triethylamine hydrochloride. The solution of comaruman triethylammonium salt obtained was dialyzed and lyophilized followed by permethylation.

Methylation analysis of comaruman and its derivatives. Permethylation of comaruman salt, fraction CPH-1, and derivative CPM was carried out in accord with Hakomori [11]. The permethylated comaruman obtained was dissolved in tetrahydrofuran (1 ml), and $LiAlD_4$ (5 mg) was added. The resulting mixture was heated at 70°C for 1 h, neutralized with 10% acetic acid in methanol (200 μl), dialyzed, and lyophilized [17]. The material obtained was hydrolyzed with 2 M TFA (0.5-1 ml) at 100°C for 5 h. Acid was removed by evaporation with methanol in vacuum at 40°C, the methylated sugars obtained were converted into the corresponding alditol acetates, and the derivatives obtained were analyzed using GLC and GLC–MS.

RESULTS AND DISCUSSION

Isolation of comaruman. Comaruman (CP) was isolated from the aerial part of the cinquefoil *Comarum palustre* L. as described previously [7]. A comparatively high content (4% air-dried raw material) of comaruman in the plant must be noted. A high positive specific rotation of comaruman, $[\alpha]_D^{20}$ +192°(*c* 0.1; water), indicated the α-configuration of glycosidic bonds in the backbone of galacturonan. The peculiarity of comaruman is very high viscosity of its aqueous solutions. The specific viscosity of comaruman, $[\eta]^{25}$ 6.78 dl/g, was estimated using viscometry. The weight average molecular mass of comaruman was found to be more 300 kD.

Comaruman contains 3.9% methoxy groups, which appears to influence the viscosity of its aqueous solutions. The sample of comaruman contains insignificant amounts (0.8%) of ash in a comparison to other pectic polysaccharides [18].

Acid hydrolysis of comaruman. Using a rigorous acid hydrolysis (2 M TFA, 100°C, 5 h) of comaruman, the main constituents of its carbohydrate chain were shown to be the residues of D-galacturonic acid, L-rhamnose, L-arabinose, and D-galactose. The residues of glucose and xylose are present in minor quantities (Table 1). A high content of rhamnose is a peculiarity of comaruman in comparison with other pectins [1, 2].

Mild acid hydrolysis of comaruman with 0.001-0.05 M TFA afforded some polysaccharide fractions and low molecular weight compounds. Hydrolysis with 0.01 M TFA led to releasing arabinose only, thus appearing to demonstrate the terminal localization of the arabinofuranose residues or the presence of side chains consisting of arabinofuranose residues only.

A substantial amount of the liberated arabinose was detected in the hydrolyzate of pectin with 0.05 M TFA. Fragment CPH-1 was precipitated from the concentrated hydrolyzate with four volumes of ethanol. Complete acid hydrolysis of CPH-1 afforded galacturonic acid as well as rhamnose and galactose in approximately equal ratios. Residues of arabinose are present in CPH-1 in trace amounts (Table 1).

Hydrolysis of comaruman with 2 M TFA at 100°C for 5 h afforded, in addition to monosaccharides, galacturonan (fragment CPH-2) containing 98% galacturonic acid, with specific viscosity $[\eta]^{25}$ 0.83 dl/g. In addition, L-rhamnose and D-galactose were present as traces (Table 1). The high positive specific rotation of galacturonan, $[\alpha]_D^{20}$ +246° (*c* 0.1; water), indicates D-configuration of the galacturonic acid residues. A peculiarity of galacturonan CPH-2 is the absence of methyl esterified

Table 1. Yields and sugar compositions of comaruman and its derivatives

Comaruman and its derivatives	Yield, %	Content, %****					
		D-Gal*p*A	Neutral monosaccharides				
			L-Rha	D-Gal	L-Ara	Xyl	Glc
CP	4.0*	64.0	12.0	11.3	6.0	tr.	2.1
CPH-1	30.0**	39.8	27.1	20.1	2.4	—	4.6
CPH-2	20.0**	98.0	0.5	1.8	—	—	—
CPM	78.0**	65.0	11.5	13.2	2.8	tr.	3.1
CPS	76.6**	50.0	7.0	10.9	3.8	—	2.1
CPSE	33.6***	55.1	13.5	13.1	3.0	—	2.4

* Of air-dried raw material.
** Of the parent comaruman CP.
*** Of fraction CPS.
**** Contents of D-galacturonic acid and neutral sugars are determined in wt. %.
Note: tr., trace.

carboxyl groups on the galacturonic acid residues (Table 1). Thus, galacturonan CPH-2 appears to represent the pectic acid.

The ^{13}C-NMR spectral data confirmed the occurrence of galacturonan segments consisting of α-1,4-linked D-galactopyranosyl uronic acid residues showing resonance of anomeric C-atom at 101.2 ppm (Fig. 1) as constituents of the CPH-2 carbohydrate chain. The position of signals of other atoms of the D-galacturonic acid residues (C2 70.1, C3 70.5, C4 79.9, C5 72.9,

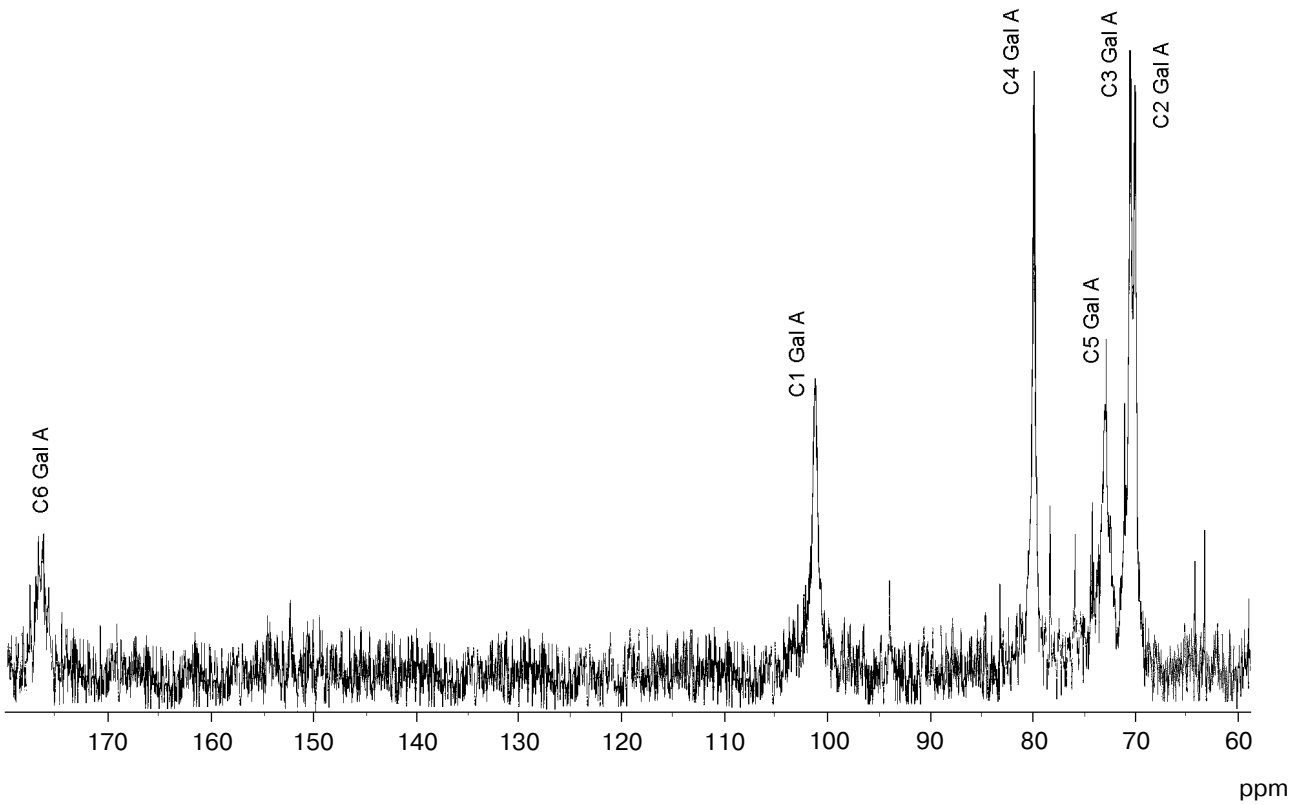

Fig. 1. ^{13}C-NMR spectrum of CPH-2.

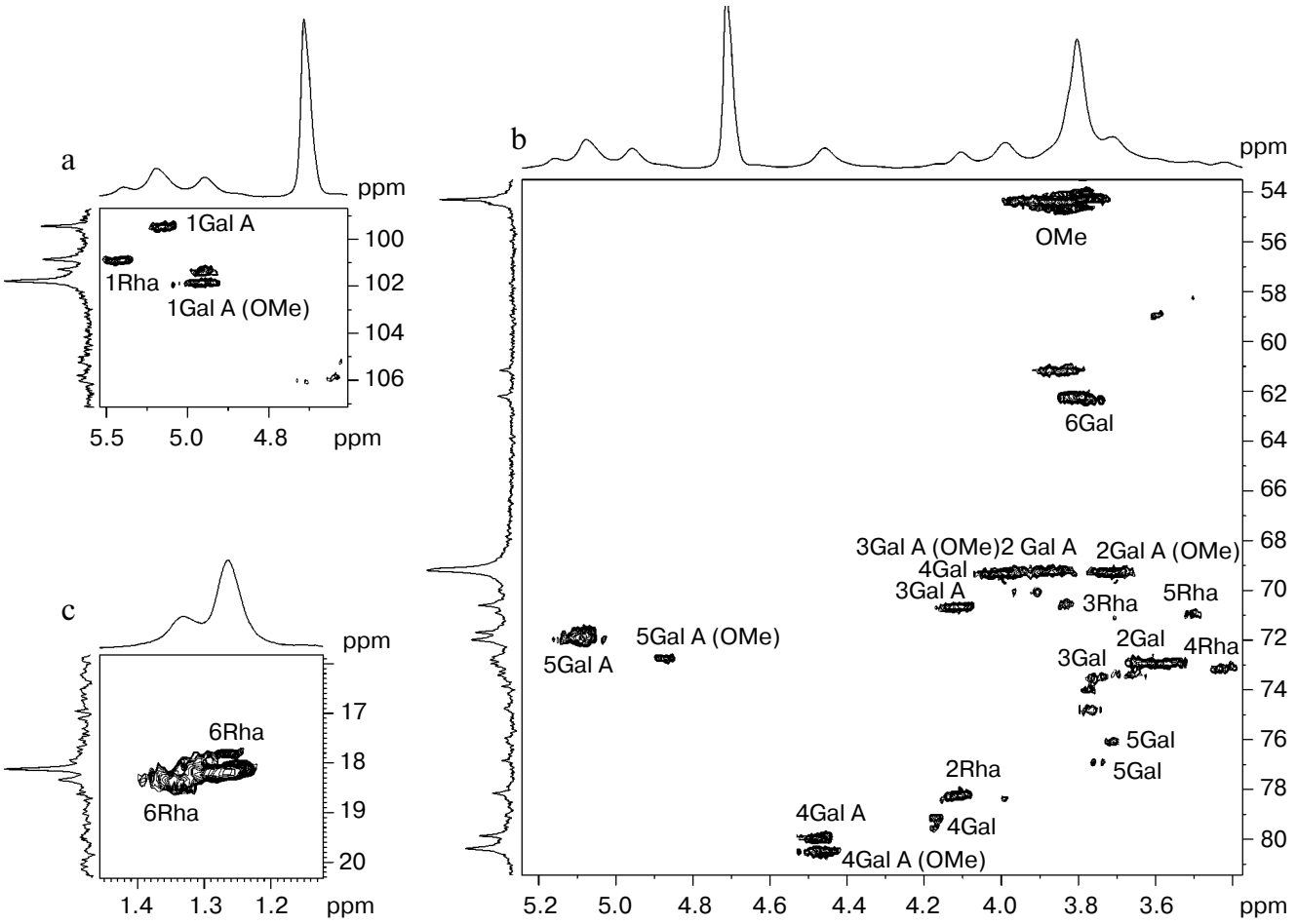

Fig. 2. $^{1}H/^{13}C$ HSQC spectrum of CPM.

C6 176.3 ppm) corresponded to that for ^{13}C-NMR spectra of authentic α-1,4-D-galactopyranosyl uronan [19].

Enzymatic hydrolysis. Digestion of comaruman with pectinase caused the cleavage of polysaccharide into some fragments, but free galacturonic acid was not formed. Comaruman appeared to be a highly methyl esterified and branched pectic polysaccharide.

A cleavage of the sugar chain is observed after preliminary saponification of comaruman with sodium methylate in methanol followed by a digestion of the saponified comaruman (CPS) with pectinase. Precipitation of the pectinase cleaved material with four volumes of ethanol yielded fraction CPSE having a sugar composition similar to the parent comaruman (Table 1). Using paper chromatography, a substantial amount of free D-galacturonic acid was detected in the supernatant. The data indicate that pectin of *C. palustre* contains considerable segments of linear galacturonan and ramified region.

Methyl esterification of comaruman (CP) and NMR spectroscopy of CPM derivative. Comaruman was esterified with absolute methanol in the presence of hydrogen chloride using acetyl chloride in absolute methanol for decreasing the viscosity of the pectin and increasing its solubility to obtain samples for NMR spectroscopy. As a result, derivative CPM with a high content of methyl esterified carboxyl groups of the D-galacturonic acid residues (Table 1) was obtained. Complete acid hydrolysis demonstrated that CPM consisted of the rhamnose and galactose residues and small amounts of glucose as the neutral monosaccharides. Residues of L-arabinose are virtually completely released.

Positions of signals in the $^{1}H,^{13}C$ HSQC spectrum (Fig. 2, a and b) of CPM (Tables 2 and 3) indicate the occurrence of α-1,4-linked D-galactopyranosyl uronic acid residues in the sugar chain of CPM. The resonances of C-atoms of the D-galacturonic acid residues of CPM and those of galacturonan CPH-2 in ^{13}C-NMR spectrum (Fig. 1) are identical. The presence of considerable amounts of esterified carboxyl groups in the galacturonic acid residues of CPM is confirmed by signals of C/H atoms of CH_3O-groups at 54.3/3.82 ppm in the $^{1}H/^{13}C$ HSQC spectrum (Fig. 2b).

Table 2. Chemical shifts for the resonances of protons of CPM in the ^{1}H-NMR spectrum

Fragment	Chemical shifts (δ), ppm (acetone, δ_H 2.225 ppm)						
	H1	H2	H3	H4	H5	H6	OMe
→4)-α-Gal*p*A(OMe)-(1→	4.96	3.71	3.99	4.46	4.96	n.d.	3.82
→4)-α-Gal*p*A-(1→	5.08	3.87	4.10	4.46	5.08	n.d.	
→2)-α-Rha*p*-(1→	5.16	4.10	3.82	3.42	3.48	1.28	
→2,4)-α-Rha*p*-(1→	5.16	4.07	3.90	3.68	3.58	1.33	
β-Gal*p*-(1→	4.70	3.65	3.70	4.01	3.77	3.78	
→4)-β-Gal*p*-(1→	4.64	3.67	3.75	4.18	3.72	3.78	

Note: n.d., not determined.

Table 3. Chemical shifts of the resonances of carbon atoms of CPM in the ^{13}C-NMR spectrum

Fragment	Chemical shifts (δ), ppm (acetone, δ_C 31.45 ppm)						
	C1	C2	C3	C4	C5	C6	OMe
→4)-α-Gal*p*A(OMe)-(1→	101.8	69.2	69.3	80.4	72.0	172.1	54.3
→4)-α-Gal*p*A-(1→	99.4	69.2	70.6	79.9	71.7	172.3	
→2)-α-Rha*p*-(1→	100.9	78.2	70.5	72.9	70.9	18.1	
→2,4)-α-Rha*p*-(1→	100.9	78.2	70.5	n.d.	n.d.	18.3	
β-Gal*p*-(1→	106.1	72.8	73.3	70.0	76.8	62.2	
→4)-β-Gal*p*-(1→	105.8	73.3	74.7	79.1	76.0	62.2	

Note: n.d., not determined.

Table 4. Chemical shifts of the resonances of protons of CPH-1 in the ^{1}H-NMR spectrum

Fragment	Chemical shifts (δ), ppm (acetone, δ_H 2.225 ppm)						
	H1	H2	H3	H4	H5	H6	OMe
→4)-α-Gal*p*A(OMe)-(1→	4.97	3.73	4.02	4.47	5.09 5.12	n.d.	3.80
α-Gal*p*A-(1→	4.95	3.92	3.98	4.33	4.75	n.d.	
→4)-α-Gal*p*A-(1→	5.04	3.90	4.14	4.46	4.98	n.d.	
→2)-α-Rha*p*-(1→	5.22	4.12	3.85	3.44	3.66	1.25	
→4)-α-Gal*p*A-(1→	4.95	3.86	4.12	4.46	4.98	n.d.	
→2,4)-α-Rha*p*-(1→	5.22	4.09	3.90	3.78	3.70	1.33	
β-Gal*p*-(1→	4.71	3.66	3.65	4.02	3.77	3.84 3.78	
→4)-β-Gal*p*-(1→	4.65	3.65	3.77	4.18	3.75	3.77	
→6)-β-Gal*p*-(1→	4.50	3.51	3.67	4.17	3.95	3.92 4.12	

Note: n.d., not determined.

Intensive signal (C1/H1 at 100.9/5.16 ppm) of anomeric atoms of the rhamnopyranose is observed in the resonance region of anomeric atoms (Fig. 2a). In addition, the high-field resonances (C6/H6 at 18.1/1.28, 18.3/1.33 ppm) from methyl group of the rhamnopyranose residues are easily identified (Fig. 2c).

The homonuclear two-dimensional TOCSY, COSY, and NOESY spectra and the heteronuclear $^{1}H/^{13}C$ HSQC spectrum indicated the presence in comaruman of rhamnopyranose residues involved in α-1,2-linkages between the linear blocks of galacturonan (Tables 3 and 4) as noted also for silenan and tanacetan [3, 4]. The $^{1}H/^{13}C$ HSQC spectrum (Fig. 2b) demonstrates α-configuration of the rhamnopyranose residues (the resonance of C5/H5 at 70.9/3.48 ppm) and their substitution at the second position (C2/H2 78.2/4.10 ppm).

The *trans*-glycosyl correlation peak of anomeric proton of the L-rhamnopyranose residues with H4 of the D-galacturonic acid residues substituted at the fourth position (H1/H4 5.16/4.46 ppm) is observed in the NOESY spectrum (Fig. 3a). This type of a substitution is confirmed by the comparatively weak correlation peaks of H5 (Fig. 3a) and H6 (Fig. 3b) of the α-L-rhamnopyranose residues with H4 of α-D-galacturonic acid residues (H5/H4 3.48/4.46 ppm, H6/H4 1.28/4.46 ppm, respectively).

The homonuclear TOCSY and COSY spectra demonstrate the occurrence of α-1,2-linked L-rhamnopyranose residues substituted in C-4 position. The low-field shifts of H4, H5, and H6 signals of the 2,4-di-O-substituted α-L-rhamnopyranose residues in a comparison with their positions in 2-O-substituted α-L-rhamnopyranose residues are observed in the proton spectra (Table 2).

The important correlation peak of high intensity of H6 of 2,4-di-O-substituted α-L-rhamnopyranose residues and anomeric protons of the β-D-galactopyranose residues (H6/H1 1.33/4.64 ppm) is present in the NOESY spectrum (Fig. 3b), clearly demonstrating localization of the galactose residues at C4 of the rhamnopyranose residues. The presence of this peak is especially important due to the correlation peak of anomeric protons of the β-D-galactopyranose residue with H4 of 2,4-di-O-substituted α-L-rhamnopyranose residues (H1/H4

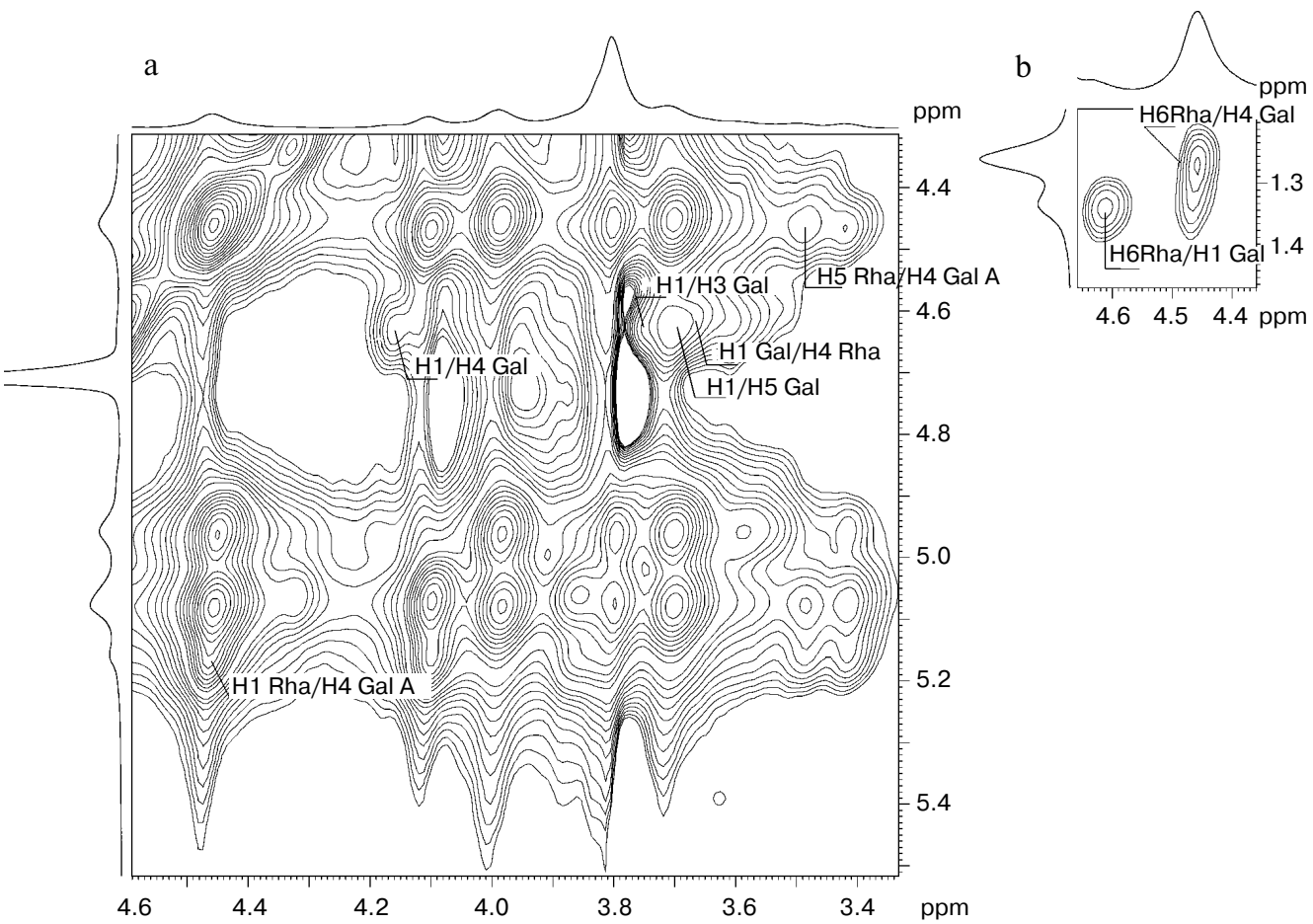

Fig. 3. NOESY spectrum of CPM.

4.64/3.68); this is disguised by the residual peaks of anomeric proton with H3 and H5 of 1,4-linked β-D-galactopyranose residues (H1/H3, H5 4.64/3.75, 3.72 ppm) (Fig. 3a). These data indicate a substitution of the α-L-rhamnopyranose residues of the backbone by the β-1,4-linked D-galactopyranose residues. This statement is confirmed by a low-field shift of the C6 atom resonance of 2,4-di-O-substituted α-L-rhamnopyranose (18.3 ppm) in the $^{1}H/^{13}C$ HSQC spectrum (Fig. 2c) in a comparison with its position (18.1 ppm) in the residue of 2-O-substituted α-L-rhamnopyranose, just as described previously for tanacetan [4].

Assignment of the D-galactopyranose residue resonances in the heteronuclear $^{1}H/^{13}C$ HSQC spectrum (Fig. 2, a and b) just as was made for silenan [3] demonstrates the occurrence of the terminal and 1,4-linked β-D-galactopyranose residues. A low-field shift of signal of C4 atom (79.1 ppm) of the β-D-galactopyranose residues substituted into O-4 position in comparison with its position in unsubstituted residue β-D-galactopyranose (70.0 ppm) is observed in this case. In addition, the presence in the NOESY spectrum of *trans*-glycosidic correlation peaks of anomeric proton with H3, H4, and H5 of the β-D-galactopyranose residues (Fig. 3a) substituted into C4 clearly indicates the occurrence of the following oligosaccharide chain: ...→4)-β-Gal*p*-(1→4)-β-Gal*p*-(1→... .

NMR spectroscopy of fragment CPH-1. NMR spectra of fraction CPH-1 (Tables 4 and 5) confirmed and supplemented the data concerning structure of comaruman obtained by analysis of NMR spectra of the methyl esterified derivative CPM. The resonance of the C-atom of the methyl esterified galacturonic acid residue at 172.1 ppm was detected in the ^{13}C-NMR spectrum of CPH-1. The presence of esterified carboxyl groups in the galacturonic acid residues is confirmed by signals of C/H-atoms of CH_3O-group at 54.1/3.80 ppm in the $^{1}H/^{13}C$ HSQC spectrum (Fig. 4).

NMR spectra of fragment CPH-1 confirmed the occurrence of β-1,4-linked D-galactopyranose residues. In addition to *trans*-glycosidic correlation peak of the anomeric proton with H4 of β-D-galactopyranose residues substituted at C4 (H1/H4 4.65/4.18 ppm), the *trans*-glycosidic correlation peak of the anomeric proton of the terminal β-D-galactopyranose residues with H4 of

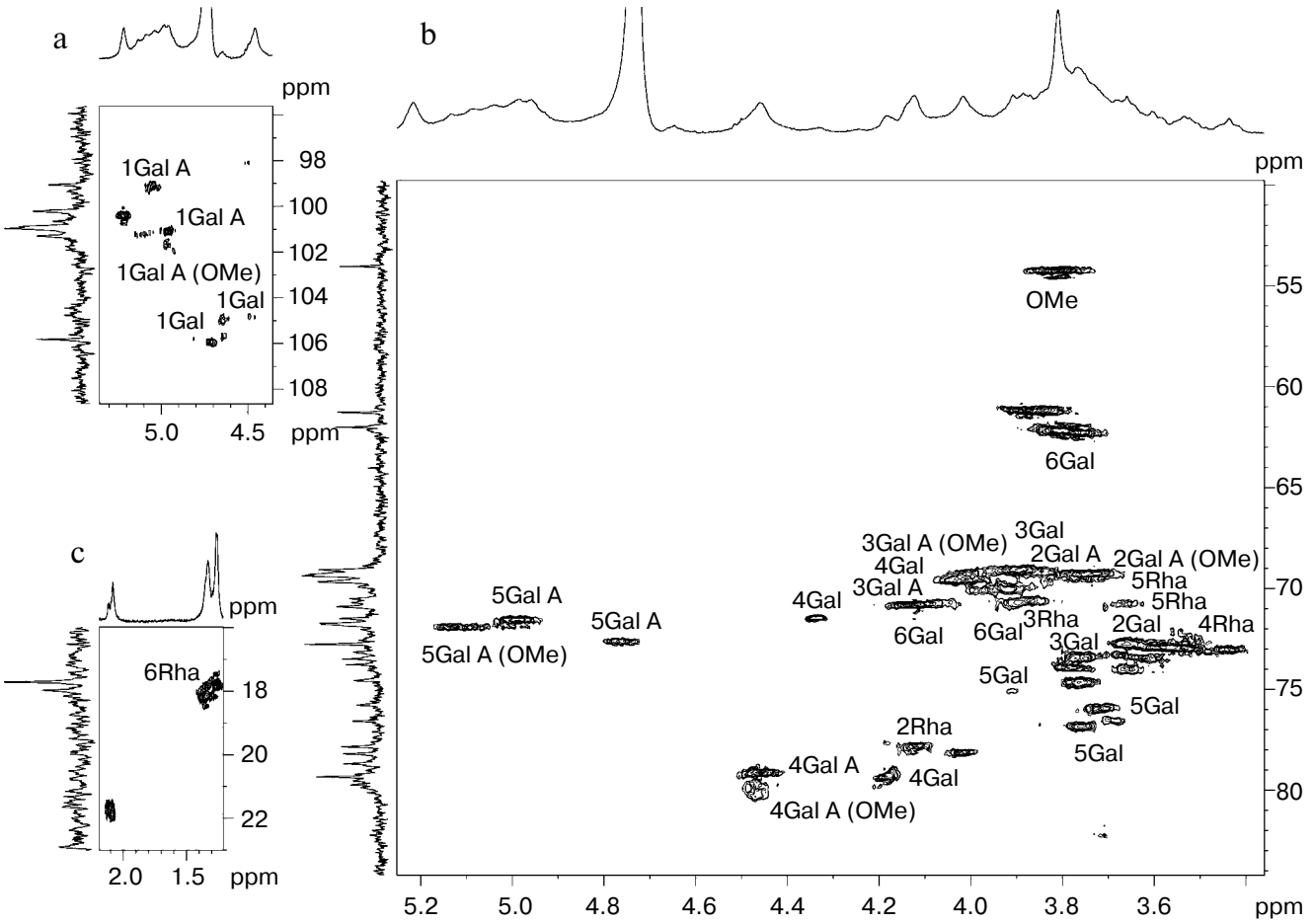

Fig. 4. $^{1}H/^{13}C$ HSQC spectrum of CPH-1.

Table 5. Chemical shifts of the resonances of carbon atoms of CPH-1 in the ^{13}C-NMR spectrum

Fragment	Chemical shifts (δ), ppm (acetone, δ_C 31.45 ppm)						
	C1	C2	C3	C4	C5	C6	OMe
→4)-α-Gal*p*A(OMe)-(1→	101.8	69.3	69.4	79.7	71.7	171.9	54.1
α-Gal*p*A-(1→	—	69.7	69.7	71.2	72.6	173.6	
→4)-α-Gal*p*A-(1→	99.1	69.1	70.9	78.7	71.5	174.4	
→2)-α-Rha*p*-(1→	100.2	77.9	70.6	72.9	70.8	17.7	
→4)-α-Gal*p*A-(1→	101.0	69.1	70.9	78.7	71.5	174.4	
→2,4)-α-Rha*p*-(1→	100.2	77.8	70.5	n.d.	69.4	18.0	
β-Gal*p*-(1→	105.8	72.8	74.6	69.9	76.4	62.1	
→4)-β-Gal*p*-(1→	104.8	72.6	73.5	79.3	75.8	62.0	
→6)-β-Gal*p*-(1→	104.5	72.9	73.8	n.d.	75.0	70.7	

Note: n.d., not determined.

Table 6. Methylation analysis of comaruman and its derivatives CPH-1 and CPM

Sugars*	Fragments	CP	CPH-1	CPM
Methylated				
2,3,4-O-Me_3-Rha*p*	Rha*p*-(1→	—	—	+
3,4-O-Me_2-Rha*p*	→2)-Rha*p*-(1→	++	++	++
3-O-Me-Rha*p*	→2,4)-Rha*p*-(1→	++	++	++
2,3-Me_2-Ara*f*	→5)-Ara*f*-(1→	++	—	—
2,3,4,6-Me_4-Gal*p*	Gal*p*-(1→	++	++	++
2,4,6-Me_3-Gal*p*	→3)-Gal*p*-(1→	—	—	+
2,3,6-Me_3-Gal*p*	→4)-Gal*p*-(1→	++	++	++
2,3,4-Me_3-Gal*p*	→6)-Gal*p*-(1→	—	+	+
2,6-Me_2-Gal*p*	→3,4)-Gal*p*-(1→	+	—	—
2,3-Me_2-Gal*p*	→4,6)-Gal*p*-(1→	—	—	++
2,3,6-Me_3-Glc*p*	→4)-Glc*p*-(1→	—	—	+
2,3,4-Me_3-Glc*p*	→6)-Glc*p*-(1→	—	—	+
2,4-Me_2-Glc*p*	→3,6)-Glc*p*-(1→	—	—	+
Deuterium-labeled				
2,3,4-Me_3-Gal	GalA-(1→	++	—	—
2,4-Me_2-Gal	→3)-GalA-(1→	+	—	—
2,3-Me_2-Gal	→4)-GalA-(1→	++	—	—
2-O-Me-Gal	→3,4)-GalA-(1→	+	—	—

* Identified as the corresponding alditol acetates.

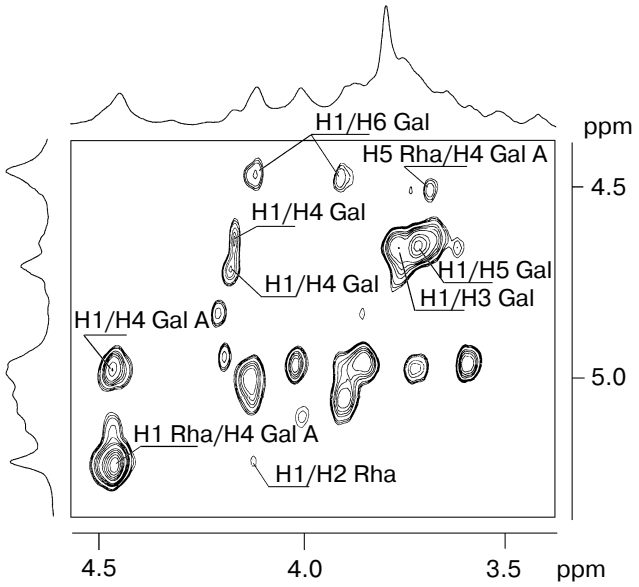

Fig. 5. ROESY spectrum of CPH-1.

β-D-galactopyranose residues substituted at C4 (H1/H4 4.71/4.18 ppm) is observed in the ROESY spectrum (Fig. 5). In addition, the *trans*-glycosidic correlation peak of the anomeric proton with H6 (H1/H6 4.50/3.92, 4.12 ppm) of the β-D-galactopyranose residues substituted at C6 is present in the ROESY spectrum (Fig. 5). The availability of this glycosidic linkage is confirmed by the resonance of an anomeric atom at 104.5 ppm in the heteronuclear $^{1}H/^{13}C$ HSQC spectrum (Fig. 4) and by a low-field shift of the C6 signal (70.7 ppm) of β-1,6-linked D-galactopyranose residues in comparison with its position in the unsubstituted residue of D-galactopyranose (62.1 ppm).

Resonances of low intensity of linked D-galactopyranose residues are present in the NMR spectra of both samples.

Thus, from analysis of the NMR spectra of CPM and CPH-1, the occurrence of the following fragments in the carbohydrate chain of comaruman was ascertained:

→4)-α-Gal*p*A-(OMe)-(1→,

→4)-α-Gal*p*A-(1→,

→6)-β-Gal*p*-(1→,

α-Gal*p*A-(1→4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A-(1→,

→4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A-(1→,

β-Gal*p*-(1→4)-β-Gal*p*-(1→4)$_n$-β-Gal*p*-(1↗4).

Methylation analysis of comaruman and its derivatives CPH-1 and CPM. Methylation analysis was used for elucidation of linkage types between sugar residues in comaruman and its derivatives CPH-1 and CPM. The method includes permethylation according to Hakomori [11] followed by reduction of carboxyl groups with $LiAlD_4$ in tetrahydrofuran [17], acid hydrolysis, reduction of methylated sugars with $NaBH_4$, and acetylation. The corresponding alditol acetates of methylated sugars (Table 6) were identified using GLC–MS. Comaruman was previously transferred into its triethylammonium salt to increase the solubility of comaruman in dimethyl sulfoxide, thus enhancing the effectiveness of permethylation of this polysaccharide.

The data of GLC–MS confirmed and supplemented conclusions concerning the structure of sugar chains and localization of the main substituents in comaruman. Approximately equal amounts of 2-O- and 2,4-di-O-substituted rhamnopyranose residues in addition to 1,4-substituted galactopyranosyl uronic acid residues were detected among the main constituents of sugar chains of comaruman and its derivatives CPH-1 and CPM. Therefore, these derivatives contain closely related segments of the linear region having the backbone consisting of α-2-L-rhamno-α-4-D-galacturonan and the ramified region involving fragments of α-2,4-L-rhamno-α-4-D-galacturonan.

The data of methylation analysis indicates that comaruman contains terminal 3- and 3,4-di-O-substituted galactopyranosyl uronic acid residues, which appear to be constituents of the side chains and, additionally, 3,4-di-O-substituted derivatives representing the branching points of the backbone of comaruman thus making up the region of the ramified galacturonan. This assumption corresponds to suppositions suggested previously [20, 21].

The data of GLC–MS confirmed those of NMR spectroscopy that the side chains of the ramified region of comaruman consisted mainly of 4-O-substituted galactopyranose residues. Some terminal galactopyranose residues appear to make up single side branches and/or to terminate the side chains.

In addition, the residues of 5-O-substituted arabinofuranose as well as 3- and 6-O-substituted galactopyranose were found to be constituents of the ramified region of comaruman. The occurrence of 3,4- and 4,6-di-O-substituted galactopyranose residues appears to represent the branching points of the side chains. Insignificant amounts of 4-, 6-, and 3,6-di-O-substituted glucopyranose residues are present in the hydrolyzate of permethylated CPM.

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REFERENCES

1. Ovodov, Yu. S. (1998) *Russ. J. Bioorg. Chem.*, **24**, 423-439.
2. O'Neill, M. A., Albersheim, P., and Darvill, A. G. (1990) in *Methods in Plant Biochemistry* (Dey, P. M., ed.) Academic Press, London, pp. 415-441.
3. Bushneva, O. A., Ovodova, R. G., Shashkov, A. S., Chizhov, A. O., and Ovodov, Yu. S. (2003) *Biochemistry (Moscow)*, **68**, 1360-1368.
4. Polle, A. Ya., Ovodova, R. G., Shashkov, A. S., and Ovodov, Yu. S. (2002) *Carbohydr. Polym.*, **49**, 337-344.
5. Golovchenko, V. V., Ovodova, R. G., Shashkov, A. S., and Ovodov, Yu. S. (2002) *Phytochemistry*, **60**, 89-97.
6. Ovodov, Yu. S., Ovodova, R. G., and Popov, S. V. (2003) *IV All-Russ. Sci. Seminar "Chemistry and Medicine"* [in Russian], Ufa, pp. 17-18.
7. Ovodova, R. G., Vaskovsky, V. E., and Ovodov, Yu. S. (1968) *Carbohydr. Res.*, **6**, 328-332.
8. Usov, A. I., Bilan, M. I., and Klochkova, N. G. (1995) *Bot. Marina*, **38**, 43-51.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
10. Wood, P. J., and Siddiqui, I. R. (1971) *Analyt. Biochem.*, **39**, 418-423.
11. York, W. S., Darvill, A. G., McNeil, M., and Stevenson, T. T. (1985) *Meth. Enzymol.*, **118**, 3-40.
12. Leontein, K., Lindberg, B., and Lönngren, J. (1978) *Carbohydr. Res.*, **62**, 359-362.
13. Rafikov, S. R., Budtov, V. P., and Monakov, Yu. B. (1978) *Introduction to the Physics and Chemistry of Polymers* [in Russian], Nauka, Moscow.
14. Knutsen, S. H. (1992) *Doctoral Thesis*, University of Trondheim, Norway.
15. Golubev, V. N., and Shelukhina, N. P. (1995) in *Pectin: Chemistry*, *Technology*, *Application* [in Russian], ATN RF Publishers, Moscow.
16. Hodge, J. E., and Hofreiter, B. T. (1962) in *Methods in Carbohydrate Chemistry* (Whistler, R. L., Wolfrom, M. L., Bemiller, J. N., and Shafizadeh, F., eds.) Academic Press, New York, pp. 380-394.
17. Perepelov, A. V., Babicka, D., Shashkov, A. S., Arbatsky, N. P., Senchenkova, S. N., Rozalski, A., and Knirel, Y. A. (1999) *Carbohydr. Res.*, **318**, 186-192.
18. Sysoyeva, K. N., Gorin, A. G., and Yakovlev, A. I. (1979) *Rast. Resursy*, **15**, 89-91.
19. Keenan, M. H. J., Belton, P. S., Matthew, J. A., and Howson, S. J. (1985) *Carbohydr. Res.*, **138**, 168-170.
20. Round, A. N., MacDougall, A. J., Ring, S. G., and Moriss, V. J. (1997) *Carbohydr. Res.*, **303**, 251-253.
21. Vincken, J.-P., Schols, H. A., Oomen, R. J. F. J., MeCann, M. E., Holvskov, P., Voragen, A. G. J., and Visser, R. G. F. (2003) *Plant Physiol.*, **132**, 1781-1789.