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Structure of Tanacetan, a Pectic Polysaccharide from Tansy *Tanacetum vulgare* L.

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

[1]*Institute of Physiology, Komi Science Center, Ural Branch of the Russian Academy of Sciences, ul. Pervomaiskaya 50, Syktyvkar, 167982 Russia; fax: (8212) 241-001; E-mail: ovoys@physiol.komisc.ru; polle@physiol.komisc.ru*

[2]*Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, Moscow, 119991 Russia; fax: (095) 135-5328; E-mail: shash@ioc.ac.ru; chizhov@ioc.ac.ru*

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Abstract—Tanacetan TVF was found to have a branched structure with a backbone of linear α-1,4-D-galacturonan. The ramified regions consist of linear α-1,2-L-rhamno-α-1,4-D-galacturonan as the core. The side chains appear to attach to the 4-position of the L-rhamnopyranose residues. They are present as single β-galactopyranose residues or a branching β-1,4-galactopyranan bearing 4,6-substituted β-D-galactopyranose residues as branched points. In addition, the ramified regions contain side chains of a branched α-1,5-arabinofuranan possessing 2,5- and 3,5-substituted α-L-arabinofuranose residues as branching points. Some side chains of rhamnogalacturonan appear to be arabinogalactan which contains branched sugar chains of α-1,5-arabinofuranan attached to the linear chains of β-1,4-galactopyranan by 1,3- and 1,6-linkages. The residues of α-L-arabinofuranose seem to occupy the terminal positions of the arabinogalactan side chains.

Key words: *Tanacetum vulgare* L., pectins, tanacetan, structure of plant polysaccharides, NMR spectroscopy of polysaccharides, GLC-MS of methylated sugars

A pectic polysaccharide named tanacetan TVF was isolated earlier from floscules of tansy *T. vulgare* L., a plant that is widespread in the European North of Russia, using extraction of the fresh plant material with aqueous ammonium oxalate. Residues of D-galacturonic acid, arabinose, galactose, and rhamnose were shown to be the main constituents of the sugar chain of tanacetan TVF in addition to small numbers of the residues of xylose, glucose, mannose, apiose, and 2-*O*-methyl xylose [1].

Like other pectic polysaccharides [2], the backbone of the tanacetan macromolecule has been shown to be a linear chain of α-1,4-D-galacturonan [1, 3].

A fragment contained in a highly branched region of α-L-arabinose residues was obtained earlier [1, 4] on enzymic digestion of tanacetan.

In addition, tanacetan has been found to possess ramified regions consisting of α-1,4-linked D-galacturonic acid residues and α-1,2-linked rhamnopyranose residues involved in the linear sugar chain. Some of the rhamnopyranose residues are 4-*O*-substituted by the terminal and β-1,4-linked galactopyranose residues [3, 4]. The side chains of tanacetan have been shown to contain also β-1,6-linked and 4,6-substituted galactopyranose residues.

The present paper presents our studies of the fine chemical structure of tanacetan TVF using acid hydrolysis, enzymic digestion, and degradation with lithium followed by structural analysis of the fragments obtained by NMR spectroscopy and by methylation studies.

MATERIALS AND METHODS

1. Isolation of tanacetan TVF. Tanacetan TVF was isolated from floscules of tansy *T. vulgare* L. as described earlier [5]. The yield was 2.0% of the fresh plant material. The plant material was harvested near Syktyvkar (Komi Republic, Russia) in the period of tansy flowering.

2. General analytical methods. *Total content of glycuronic acids* was determined using a reaction with 3,5-dimethyl phenol in the presence of concentrated H_2SO_4 [6] and according to a standard curve for D-galacturonic acid. *Protein content* was calculated using Lowry's procedure [7] and a standard curve for BSA. *Spectrophotometric measurements* were made with an Ultrospec-3000 spectrophotometer (England).

* To whom correspondence should be addressed.

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Specific optical rotation was determined in 1-cm cells in H_2O at 20°C on a Polatronic MHZ polarimeter (Germany).

NMR spectra were obtained on a Bruker DRX-500 instrument (Germany) for 3-5% solutions of oligo- and polysaccharides in D_2O at 313 K (the internal standard was acetone, δ_H 2.225 ppm, δ_C 31.45 ppm). Two-dimensional spectra were run using the standard Bruker procedures.

Gel permeation chromatography was carried out using an Uvicord SII chromatographic apparatus (Sweden) at 206 nm on columns as follows:

a) Sephacryl S-500 (2.4 × 60 cm; eluent H_2O; void volume 88 ml; rate of eluent flow 28 ml/h). Fractions (6.8 ml) were collected;

b) BioGel P-2 (200 mesh; 1.6 × 56 cm; eluent H_2O; void volume 25.5 ml; rate of eluent flow 16 ml/h). Fractions (1.6 ml) were collected.

The elution curve was tested using the reaction of an eluate aliquot with phenol in the presence of concentrated H_2SO_4 [8].

HPLC was used for isolation of neutral monosaccharides on a preparative scale. HPLC was run using a DYNAMAX plunger reciprocating pump (USA) and a preparative column (24 × 250 mm) with Diasorb-130-Amine sorbent (particle size 11 μm (BioKhimMak ST, Russia). The eluent was a mixture of acetonitrile–H_2O (84 : 16 v/v), and isocratic elution was used with a rate of 10 ml/min. Sugars were detected using a RIDK-101 differential refractometer (Czechoslovakia). Authentic samples of rhamnose, xylose, arabinose, mannose, glucose, and galactose were used as standards.

Qualitative and quantitative analysis of neutral monosaccharides [9] as the corresponding alditol acetates were carried out using GLC on a Hewlett-Packard model 4890A chromatograph (USA) equipped with a flame-ionization detector and HP 3395A integrator. GLC was run on an RTX-1 capillary column (USA, 0.25 mm × 30 m) using the temperature program of 175°C (1 min) → 250°C (2 min) (rate Δ3°C/min), with argon as carrier gas.

Combined gas–liquid chromatography–mass spectrometry (GLC-MS) of the methylated sugars as the corresponding methyl ethers of alditol acetates was performed using a Carbo Erba Fractovap 4200 instrument (Italy) with an HP Ultra-1 capillary column (USA, 0.2 mm × 25 m), carrier gas helium, the temperature program of 140°C (1 min) → 280°C (1 min) (rate Δ5°C/min). MS: ion trap Finnigan MAT ITD-700 (Germany), mass range from m/z 44 to m/z 500, electron impact energy ≈ 70 eV. The temperature of the interface was 190°C, multiplier voltage 1700 V, scanning frequency 1 scan/sec, acquisition delay 250 sec.

All the aqueous solutions were evaporated in vacuum at 40-45°C, centrifuged at 7000-8000*g* for 10-20 min. The samples were then lyophilized.

3. Hydrolysis of tanacetan TVF and isolation of monosaccharides on preparative scale. Tanacetan TVF (800 mg) was heated with 2 M trifluoroacetic acid (TFA, 100 ml) for 6 h at 100°C. The precipitate was separated by centrifugation, washed with methanol, and the solution obtained was combined with a supernatant followed by removing TFA using repeated evaporation with methanol. The residual aqueous solution was filtered using Diapack Amine concentrating chips (BioKhimMak) packed with the same sorbent as used in a column for a preparative HPLC.

The mixture of monosaccharides obtained (255 mg) was dissolved in a mixture (1 ml) of CH_3CN–H_2O (84 : 16 v/v) and separated using HPLC followed by estimation of the specific optical rotations for the individual monosaccharides.

As a result, mono-*O*-methyl-D-xylose (2.2 mg), L-rhamnose (6.8 mg), D-xylose (1.5 mg), L-arabinose (16.0 mg), and D-galactose (11.6 mg) were obtained. Mannose and glucose failed to separate under these conditions and were eluted altogether.

4. Enzymic digestion. Tanacetan TVF (1 g) was dissolved in water (100 ml), an aqueous solution of pectinase (20 mg, Ferbak, Germany) (protein 6.6 mg, activity 56.7 IU) was added, and the mixture obtained was incubated in a dialysis sac with simultaneous dialysis against distilled water for 3 h at 37°C [1]. The pectinase was inactivated by heating at 100°C. Denatured protein was removed by centrifugation. The solution obtained was concentrated and precipitated by adding 96% ethanol (4 volumes). The precipitate obtained was separated by centrifugation followed by washing with methanol until the disappearance of free galacturonic acid in washing solutes (testing by paper chromatography). The precipitate was dissolved in water and then lyophilized. The yield of TVF-E was 291.6 mg.

5. Degradation with lithium. Tanacetan TVF (100 mg) dried previously was stirred with ethylene diamine (20 ml) for 24 h up to complete solubilization. Particles of Li (2-5 mm) were added to the solution and the mixture was stirred until permanent blue color was attained (after 90 min) and stirring was continued for 60 min. The reaction was stopped by adding distilled water (25 ml) on cooling (ice bath), the solution obtained was evaporated after adding toluene. The residual material was dissolved in distilled water (5 ml) and the solution obtained was chromatographed on a column (8.5 × 4 cm) with Amberlite 50W × 12-200 (H^+) using elution with distilled water. The elution curve was followed using the reaction of aliquots with phenol in the presence of concentrated H_2SO_4 [8]. The main fraction was purified using gel filtration on BioGel P-2 followed by lyophilization. As a result, the fragment TVF-L1 (K_{AV} = 0), yield 8.5 mg, was obtained.

Dried TVF-E (100 mg) was dissolved in ethylene diamine (10 ml) with stirring for 4 h. Particles of Li (2-5 mm) were added to the solution until a permanent blue color appeared (after 3 h), and then stirring was continued for 2 h. The reaction mixture was treated as described

above. As a result, the fraction TVF-L2 (K_{AV} = 0), yield 39.8 mg, was obtained.

6. Methylation analysis. The dried oligosaccharide was permethylated following Hakomori [10]. TVF-L1 (2.8 mg) was dissolved in dry dimethyl sulfoxide (DMSO, 1 ml) in a stream of nitrogen for 2 h. Methyl sulfinyl carbanion (2 M, 0.5 ml) was added to the solution and the mixture was stirred at room temperature for 5 h. The material was frozen and methyl iodide (0.25 ml) was added. The mixture was stirred for 1 h, CH_3I was removed in a stream of nitrogen and the residual material was diluted with an equal volume of water; the solution was dialyzed against distilled water for 24 h and the permethylated TVF-L1 was lyophilized.

Permethylated TVF-L1 (2.3 mg) was hydrolyzed with 2 M TFA (1 ml) for 5 h at 100°C, and the acid was removed by repeated evaporation with methanol. The resulting mixture of methylated sugars was reduced in 1 M aqueous ammonia by adding sodium borohydride (3-5 mg) and keeping the reaction mixture overnight (for 16 h) in the dark. Adding some drops of glacial acetic acid to pH 4-5 destroyed the excess of sodium borohydride and the solution was then evaporated with methanol. Dry pyridine and acetic anhydride (0.2 ml each) were added to the dry residue and the mixture was kept overnight in the dark or heated for 1 h at 80°C. The solution was evaporated to dryness with toluene (0.2 ml) and then repeatedly with methanol (1 ml) for removing pyridine and acetic anhydride. As a result, alditol acetates of the corresponding partially methylated sugars were obtained and identified using GLC and GLC-MS.

RESULTS AND DISCUSSION

1. Isolation of tanacetan TVF. Tanacetan TVF was isolated from the floscules of tansy *T. vulgare* L. as described earlier [1]. The data of qualitative and quantitative analysis of sugar composition of tanacetan TVF are listed in Table 1.

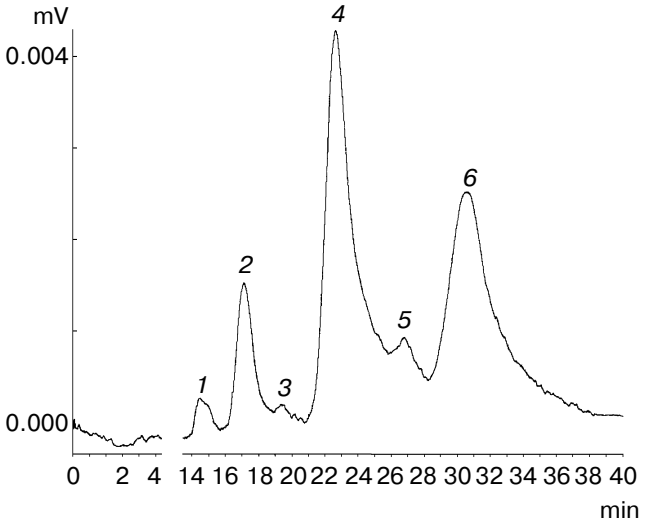

Fig. 1. HPLC of monosaccharides from tanacetan. Peaks: *1*) 2-*O*-MeXyl; *2*) Rha; *3*) Xyl; *4*) Ara; *5*) Man + Glc; *6*) Gal.

2. Determination of monosaccharide configuration. The configuration (D- or L-series) of the sugar residues in the composition of the tanacetan sugar chains was determined using complete acid hydrolysis of tanacetan followed by separation of the sugar mixture obtained and estimation of optical rotations of isolated monosaccharides. Complete acid hydrolysis of tanacetan resulted in 31.9% neutral monosaccharides. Their separation on preparative scale was carried out by HPLC using a mixture of acetonitrile–water (84 : 16 v/v) as the optimal eluent. Most of the sugars were separated (Fig. 1). However, mannose and glucose failed to separate under these conditions and show a single peak on the elution curve. The purified monosaccharides were obtained as follows: mono-*O*-methyl xylose, rhamnose, xylose, arabinose, and galactose. Optical rotations (positive values in all cases) indicate that L-arabinose, D-galactose, and L-rhamnose represent the main sugar constituents of tanacetan.

Table 1. Analytical data for tanacetan and its fragments: TVF-E, -L1, and -L2 (content*, %)

Tanacetan	Yield, %	D-GalA	L-Ara	D-Gal	L-Rha	D-Xyl	Glc	Man	Protein
TVF	2.0**	61.4	14.7	10.2	3.7	0.4	0.5	0.3	4.0
TVF-E	29.2***	42.0	29.0	14.8	8.6	0.7	1.8	1.6	2.2
TVF-L1	8.5***	—	73.7	20.0	2.1	trace	1.6	trace	—
TVF-L2	39.8****	40.0	21.4	20.7	11.3	trace	1.9	0.6	—

* Contents of protein and D-galacturonic acid are calculated as wt. %, quantities of the neutral sugars are given in mole %.
** Of the fresh plant raw material.
*** Of the parent tanacetan.
**** Of the parent TVF-E.

Table 2. GLC-MS analysis of hydrolyzate of the permethylated oligosaccharide fragment -L1

Methylated sugars*	Molar ratio	Linkages
2,3,5-Me3-Ara	27.4	L-Ara*f*-(1→
2,5-Me_2-Ara	1.5	→3)-L-Ara*f*-(1→
2,3-Me_2-Ara	27.5	→5)-L-Ara*f*-(1→
2-Me-Ara	20.1	→3,5)-L-Ara*f*-(1→
3-Me-Ara	1.2	→2,5)-L-Ara*f*-(1→
2,3,4,6-Me_4-Gal	1.3	D-Gal*p*-(1→
2,3,6-Me_3-Gal	6.3	→4)-D-Gal*p*-(1→
2,4,6-Me_3-Gal	1.3	→3)-D-Gal*p*-(1→
2,3,4-Me_3-Gal	2.0	→6)-D-Gal*p*-(1→
2,4-Me_2-Gal	2.4	→3,6)-D-Gal*p*-(1→

* Identified as the corresponding alditol acetates.

3. Enzymic digestion. Digestion of tanacetan with α-1,4-D-polygalacturonase (pectinase) led to the polysaccharide fragment -E, yield 29.2%. Analytical data for -E are given in Table 1. Decreasing contents of D-galacturonic acid to 42% with simultaneous increasing of the neutral sugar quantities demonstrated that the linear α-1,4-D-galacturonan represented the backbone of tanacetan as noted earlier [1].

4. Degradation with lithium. Selective splitting of regions containing glycosyl uronic acid residues with lithium is now often used for structural analysis of pectins [11].

A treatment of tanacetan with lithium in ethylene diamine led to a substantial degradation of the polysaccharide and yielded oligosaccharide fragment -L1 (yield 8.5%), which was purified using gel filtration on a BioGel P-2 column (Table 1).

Fraction -L1, $[\alpha]_D^{20}$ −80.1° (*c* 0.1; H_2O), was shown to consist of residues of L-arabinose and D-galactose (73.7 and 20.0%, respectively) as the main neutral monosaccharide constituents (Table 1) and did not contain residues of D-galacturonic acid.

Degradation of -E with lithium yielded polysaccharide fraction -L2 (yield 39.8%) that was purified using gel filtration on a BioGel P-2 column (Table 1).

As can be seen from Table 1, the sugar chain of -L2, $[\alpha]_D^{20}$ +140.8° (*c* 0.1; H_2O), consisted of D-galacturonic acid, L-arabinose, D-galactose, and L-rhamnose residues as the main constituents.

5. Methylation studies of -L1. The glycosidic bonds between the sugar residues of the oligosaccharide fragment -L1 were determined using methylation analysis. Fraction -L1 was permethylated by the method of Hakomori [10] followed by complete acid hydrolysis resulting in a mixture of methylated sugars. The methylated sugars were identified using GLC-MS as the corresponding alditol acetates (Table 2). As seen from Table 2, the terminal residues are present mainly as L-arabinose in addition to comparatively small amounts of the terminal

Table 3. The $^1H/^{13}C$-HSQC-NMR spectral data for the fragment -L2

Residue	Chemical shift (δ), ppm					
	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5;H5'	C6/H6;H6'
α-L-Ara*f*-(1→5	108.4/5.17	82.7/4.22	77.8/3.94	85.3/4.12	62.6/3.82;3.69	
α-L-Ara*f*-(1→3	110.3/5.23	82.7/4.22	77.8/3.94	85.3/4.12	62.6/3.82;3.69	
→5)-α-L-Ara*f*-(1→	108.8/5.07	82.2/4.12	78.1/4.02	83.8/4.22	68.2/3.87;3.79	
→2)-α-L-Ara*f*-(1→	108.4/5.16	85.2/4.13	77.8/3.96	85.2/4.05	62.4/3.84;3.73	
→3,5)-α-L-Ara*f*-(1→	108.7/5.11	80.5/4.28	84.3/4.08	83.0/4.30	67.9/3.93;3.83	
→2,5)-α-L-Ara*f*-(1→	108.8/5.09	85.2/4.11	77.9/4.04	83.8/4.22	67.2/3.95;3.83	
β-D-Gal*p*-(1→4	104.6/4.48	72.0/3.55	73.8/3.68	69.9/3.97	76.2/3.68	62.1/3.76
→4)-β-D-Gal*p*-(1→	105.5/4.62	73.8/3.68	74.5/3.77	78.4/4.17	75.7/3.72	62.2/3.87;3.82
→4,6)-β-D-Gal*p*-(1→	104.4/4.47	73.0/3.53	74.0/3.66	77.8/3.93	74.0/3.65	70.5/4.05;3.92
→4)-α-D-Gal*p*A-(1→	100.2/5.07	69.2/3.91	69.9/3.92	78.4/4.42	72.5/4.65	175.8/–
→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	98.8/5.02	69.2/3.91	71.5/4.10	78.4/4.42	73.5/4.72	175.8/–
α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	98.8/5.02	69.2/3.91	69.9/3.92	72.0/4.27	72.5/4.65	175.8/–
→2)-α-L-Rha*p*-(1→	99.7/5.25	77.3/4.12	70.5/3.88	73.2/3.40	70.1/3.77	17.8/1.25
→2,4)-α-L-Rha*p*-(1→	99.7/5.25	77.3/4.12	70.5/3.87	81.8/3.68	69.5/3.76	18.0/1.30
β-D-Gal*p*-(1→4)-α-L-Rha*p*	105.5/4.63	72.5/3.64	75.0/3.91	70.0/3.95	76.2/3.68	62.1/3.76
→4)-β-D-Gal*p*-(1→4)-α-L-Rha*p*	104.4/4.52	73.8/3.66	74.5/3.74	78.8/4.16	75.0/3.92	62.2/3.78
→5)-α-L-Ara*f*-(1→4)-α-L-Rha*p*	110.3/5.24	82.2/4.12	78.1/4.02	83.8/4.22	68.2/3.87;3.79	

D-galactose residues. Note the occurrence of 5- and 3,5-substituted L-arabinofuranose residues and 1,4-linked D-galactopyranose residues as the main constituents of -L1 sugar chains.

6. NMR spectroscopy of fragment -L2. The signal in the heteronuclear $^{1}H/^{13}C$-HSQC of the polysaccharide fragment -L2 (Fig. 2) indicated the occurrence of linear α-1,2-L-rhamno-α-1,4-D-galacturonan region that was detected earlier in tanacetan [4]. These data were confirmed by two-dimensional homonuclear TOCSY, COSY, and ROESY spectra and by the heteronuclear HMBC spectrum (Table 3). In addition, the $^{1}H/^{13}C$-HSQC and TOCSY spectra of fragment -L2 demonstrated the presence of regions consisting of α-1,4-linked D-galacturonic acid residues and showed a signal of anomeric atoms C1/H1 at 100.2/5.07 ppm (Table 3). The signals of the other C-atoms of D-galacturonic acid residues in the ^{13}C-NMR spectra of -L2 and tanacetan [1] coincided.

A low-field shift of the resonance from C4/H4 (78.4/4.42 ppm) in the $^{1}H/^{13}C$-HSQC spectrum (Fig. 2) indicated the presence of the following fragment in the sugar chains of -L2:

α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→...

The residues of α-1,2-linked L-rhamnopyranose 4-*O*-substituted by the terminal and β-1,4-linked β-D-galactopyranose residues showed a shift of resonance from C6/H6 (18.0/1.30 ppm) (Table 3) in the $^{1}H/^{13}C$-HSQC spectrum as observed earlier [4]. The signal of the anomeric atom of the terminal β-D-galactopyranose residue β-1,4-linked with α-L-rhamnopyranose residue is characterized by a low-field shift. This type of substitution is confirmed by the ROESY spectrum in which *trans*-glycosyl correlation peaks of the anomeric proton of β-D-galactopyranose with H4 of 2,4-substituted α-L-rhamnopyranose (H1/H4 4.63/3.68 ppm and 4.52/3.68 ppm) occur. The signals of the D-galactopyranose residues β-1,4-linked with α-rhamnopyranose residues are observed also in the heteronuclear $^{1}H/^{13}C$-HSQC spectrum, as noted earlier [4].

A low-field shift in the $^{1}H/^{13}C$-HSQC spectrum of the signal of an anomeric atom (5.24/110.3 ppm) demonstrated the presence of α-1,5-linked L-arabinofuranose residues attached by 1,4-linkage to α-1,2-linked L-

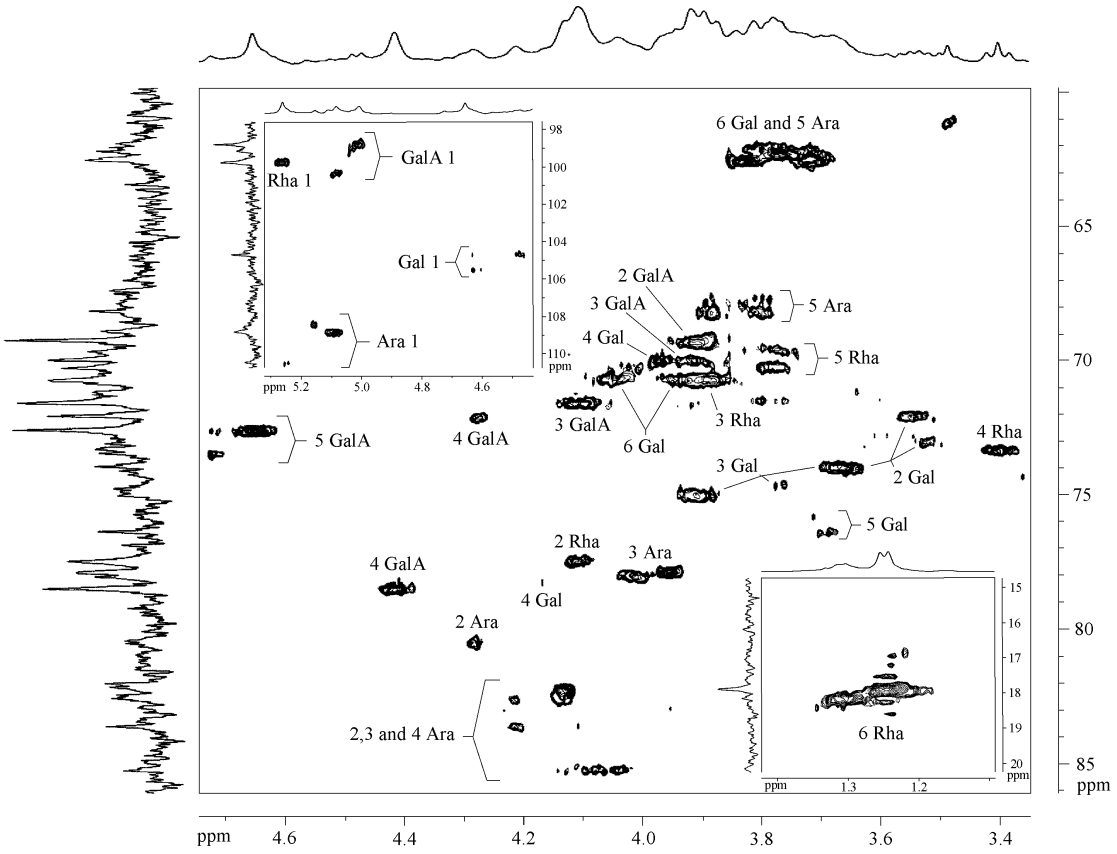

Fig. 2. $^{1}H/^{13}C$-HSQC spectrum of polysaccharide fragment -L2.

rhamnopyranose residues of the backbone. This kind of substitution is confirmed by the presence of *trans*-glycosyl correlation peaks of the anomeric proton of α-L-arabinofuranose with H4 of 2,4-substituted α-L-rhamnopyranose (H1/H4 5.24/3.68 ppm) in the ROESY spectrum.

The signal of C4/H4 at 77.8/3.93 ppm in the $^{1}H/^{13}C$-HSQC spectrum and low-field shift of the resonance from C6/H6; H6′ (70.5/40.5; 3.92 ppm) (Fig. 2) indicated the occurrence of 4,6-substituted β-D-galactopyranose residues. The other signals confirming this kind of substitution was interpreted in accord with the data obtained previously [4] using the heteronuclear $^{1}H/^{13}C$-HSQC and two-dimensional homonuclear TOCSY spectra (Table 3). In addition, the terminal, 2-, 3,5-, and 5-substituted α-L-arabinofuranose residues and β-1,4-linked D-galactopyranose residues were identified (Table 3).

The data indicate that tanacetan is a pectic polysaccharide with backbone of linear α-1,4-D-galacturonan. The ramified regions appear to be rhamnogalacturonan-I with core of α-1,2-L-rhamno-α-1,4-D-galacturonan. The side sugar chains are attached by 1,4-linkages to the L-rhamnopyranose residues of the core and consist of single residues of β-galactopyranose and β-1,4-galactopyranan with branching points of 4,6-substituted β-D-galactopyranose residues. In addition, the branching regions contained the side chains of a branched α-1,5-arabinofuranan bearing 2,5- and 3,5-substituted α-L-arabinofuranose residues as the branching points. Some side chains of rhamnogalacturonan appear to represent arabinogalactan, which contains the branched sugar chains of α-1,5-arabinofuranan attached to the linear chains of β-1,4-galactopyranan by 1,3- and 1,6-linkages. The residues of α-L-arabinofuranose appear to occupy the terminal positions of the arabinogalactan side chains.

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