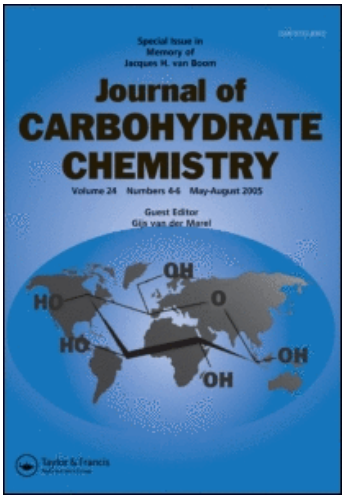

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An Acidic Heteropolysaccharide from the Flowers of *Malva Mauritiana* L.

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AN ACIDIC HETEROPOLYSACCHARIDE FROM THE FLOWERS OF *MALVA MAURITIANA* L.

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ABSTRACT

An acidic heteropolysaccharide composed of L-rhamnose, D-galactose, L-arabinose, D-xylose, and uronic acids in 1.0:0.5:0.1:0.1:1.8 mole proportion was isolated from the flowers of *M. mauritiana* L. Structural studies of the polysaccharide involved compositional and methylation analyses of the intact and carboxyl-reduced polymers, graded acid hydrolysis as well as ^{1}H and ^{13}C NMR spectroscopy. The results of chemical and spectroscopic analyses indicated a branched structure with the backbone made up of alternating sequences of 2- and 4-linked rhamnogalacturonan-type units with linear segments (DP = 2, 4, 12), segments bearing on O-3 of D-GalA monomeric D-GlcA randomly distributed along the chain (DP = 7, 10, 19, 31), and segments having each D-GalA substituted (DP = 15). Moreover, the results of methylation analysis indicated that about 28% of L-rhamnose residues were branched through O-4.

INTRODUCTION

The flowers and leaves of *Malva mauritiana* L. (*Malvaceae*) have been used pharmaceutically for a long time as astringent, emollient, and mucolytic. Though the curing effects have often been ascribed to the mucous material, contained in the herb in relatively large amounts, its chemical composition has not been investigated thus far. Only the monosaccharide composition of and some analytical data on the mucilage from the flowers of this medicinal plant were reported.[2] A similar investigation[3,4] was performed with the related herb *Malva silvestris* L.

Table 1. Sugar composition (mole ratio), M_n and DP of the intact (P), carboxyl-reduced (RP) and degraded (P1, P2, P3) polysaccharides.

Polysaccharide	M_n	DP	Gal	Glc	Ara	Xyl	Rha	Uronic acids
P	23 833	148	0.5	-	0.1	0.1	1.0	1.8
RP	a	a	1.3	0.9	b	b	1.0	-
P1	11 172	69	0.4	-	b	b	1.0	1.8
P2	5 118	31	b	-	-	-	1.0	1.4
P3	3 303	19	-	-	-	-	1.0	1.7

a. Not determined. b. Traces.

In the framework of our research program directed towards isolation and characterization of active polysaccharides of plant origin we examined two neutral polysaccharides, an α-1,6-glucan and an α-1,5-arabino-3,6-ß-galactan, isolated from the flowers of the medicinal plant *Malva mauritiana* L.[5,6] The present paper provides results on the purification and structure determination of an acidic polysaccharide from the same source, which was shown to exhibit high antitussive activity.[7]

RESULTS AND DISCUSSION

Isolation of the acidic heteropolysaccharide. Water extraction of the flowers of *M. mauritiana,* followed by ethanol precipitation of the filtrate, yielded a dark brown product (3.5% per mass of dry flowers), containing carbohydrate material and 22.8% protein.[5] Ion-exchange chromatography of the mucilage, effected by stepwise elution with water and ammonium carbonate solutions of increasing concentration, gave two major fractions: the water eluate, already described,[4,5] and the 0.5 M carbonate eluate (**P**) fraction which was proved to be homogeneous on free-boundary electrophoresis and had Mn = 23,833 and optical rotation $[\alpha]_D^{20} = +40.5°$. This material was composed of L-Rha, D-Gal, L-Ara, D-Xyl, and uronic acids in the mole ratio presented in Table 1.

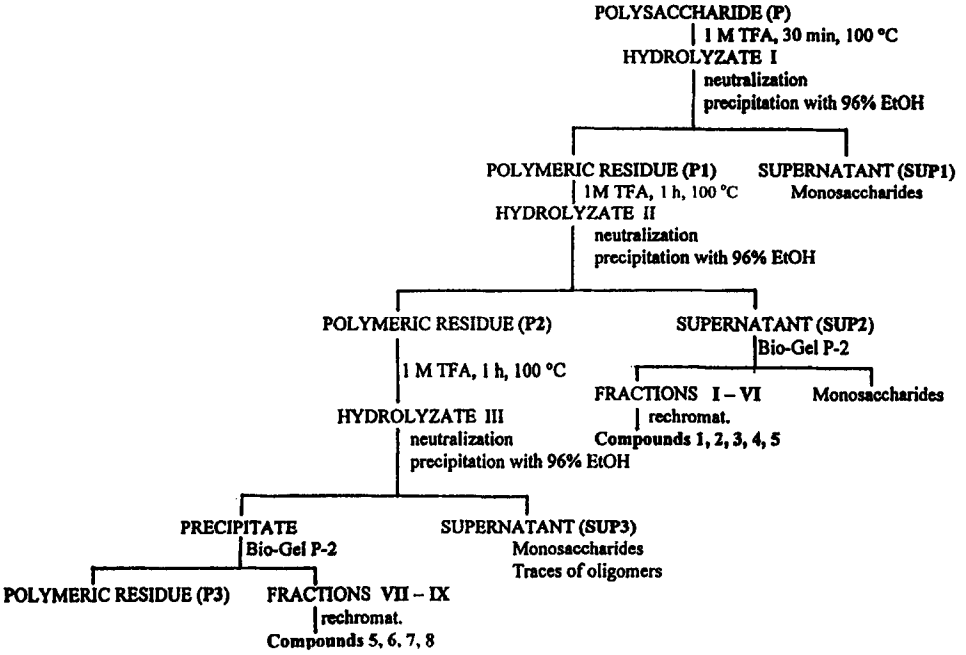
Methylation analysis. The linkage pattern of the sugar components was deduced from the results of methylation analysis of the native (**P**) and carboxyl-reduced (**RP**)

Table 2. Methylation analysis data of the intact P (neutral portion) and carboxyl-reduced (RP) polysaccharides.

Derivative	Mole %		Mode of linkage
	P	RP	
2,3,4-Me_3-Rha[a]	3.1	1.6	Rhap-(1→
3,4-Me_2-Rha	47.1	25.3	→2)-Rhap-(1→
3-Me-Rha	15.1	10.5	→2,4)-Rhap-(1→
2,3,4-Me_3-Ara	2.8	0.7	Arap-(1→
2,3-Me_2-Ara	1.4	c	→5)-Araf-(1→
			or →4)-Arap-(1→
2,3,4-Me_3-Xyl	3.5	0.8	Xylp-(1→
2,3-Me_2-Xyl	1.2	c	→4)-Xylp-(1→
2,3,4,6-Me_4-Gal	12.0	4.9	Galp-(1→
6,6-d_2-2,3,4,6-Me_4-Gal[b]	-	1.7	GalpA-(1→
2,3,6-Me_3-Gal	13.8	5.0	→4)-Galp-(1→
6,6-d_2-2,3,6-Me_3-Gal	-	11.6	→4)-GalpA-(1→
6,6-d_2-2,6-Me_2-Gal	-	17.3	→3,4)-GalpA-(1→
6,6-d_2-2,3,4,6-Me_4-Glc	-	20.6	GlcpA-(1→

a. 2,3,4-Me_3-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol-1-d, etc. b. 6,6-d_2-2,3,4,6-Me_4-Gal = 6,6-dideuterio-1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol-1-d, etc. c. Traces.

polysaccharides presented in Table 2. The derivatives in the table demonstrate that the ring form of all sugar components, except arabinose, was pyranose. The derivatives originating from the native polysaccharide indicated the prevalence of 2- and 2,4-linked Rhap residues as well as of nonreducing terminal and 4-linked Galp units. A minor portion of Rha was found to occupy nonreducing terminal positions. Arabinose was detected mainly at nonreducing ends, but was found also to be linked through O-5 (or O-4). The xylose derivatives pointed to a nonreducing end position of this sugar and to units linked through O-4. As shown later, both sugars are located at the periphery of the molecule, because they were detached in the first step of graded acid hydrolysis of the polysaccharide. The carboxyl-reduced polymer afforded, in addition to the above



Scheme 1. Graded acid depolymerization of the acidic heteropolysaccharide.

mentioned derivatives, 6,6-d_2-2,3,4,6-tetra-*O*-methyl-, 6,6-d_2-2,3,6-tri-*O*-methyl- and 6,6-d_2-2,6-di-*O*-methyl-galactopyranose, derived from terminal, 4- and 3,4-linked GalA residues, as well as 6,6-d_2-2,3,4,6-tetra-*O*-methyl-glucopyranose, originating from terminal GlcA units. The proportions of terminal and 4-linked GalA residues were determined from the mass spectra on the basis of the ion intensities at *m/z* 205 and 207, and 233 and 235, respectively, derived from nondeuterated and deuterated C-6 atoms in 2,3,4,6-tetra-*O*-methyl- and 2,3,6-tri-*O*-methylgalactose derivatives, respectively. The portions of L-Ara, D-Xyl and D-Gal in **RP** indicated loss of neutral side chains in the process of reduction.

Graded acid hydrolysis. The stepwise degradation of the heteropolysaccharide with 1 M trifluoroacetic acid yielded three polymeric products **P1-P3** (Table 1) and a series of oligomeric fragments, eight of which were purified to homogeneous form (Scheme 1). The first hydrolysis generated, besides the polymeric residue **P1**, the monomers L-Ara, D-Xyl, and D-Gal. Compositional analysis of **P1** revealed the presence of L-Rha, D-Gal, D-GalA, and D-GlcA as well as trace amounts of L-Ara and D-Xyl. The molecular mass of the polymer was reduced to about half of its original value.

In the next hydrolysis step **P1** afforded a polymeric product **P2** of M_n 5,118, composed of L-Rha, uronic acids and traces of D-Gal, and a mixture of oligo- and monosaccharides (**SUP2**). The mixture **SUP2** was resolved by gel filtration to six oligosaccharide fractions (**I-VI**) and a mixture of monomers, comprising mainly D-Gal, and lesser amounts of L-Ara, D-Xyl, L-Rha and uronic acids. The preferential release of D-Gal monomers suggests that these residues are located in the side chains of the polysaccharide.

The homogeneity of the individual oligosaccharide fractions (**I-VI**) was checked using paper chromatography (S2), thin-layer chromatography (TLC, S3), and ^{1}H NMR spectroscopy.

The fraction **VI,** shown to be a mixture of two components, was separated by preparative paper chromatography (S2) to give compounds **1** and **2.** The sugar components of **1** were D-GalA and L-Rha (equimolar ratio) and **2** consisted of D-GlcA and D-Gal (equimolar ratio). The hydrolytic products of the the borohydride-reduced disaccharides proved the reducing end positions of rhamnose in **1** and galactose in **2.**

Rechromatography of **V** on Bio-Gel P-2 yielded one homogeneous compound **3.** It was composed of D-GalA, L-Rha, and D-Gal, the last component forming the reducing end.

A similar procedure with fraction **III** afforded compound **4,** consisting of D-GalA and L-Rha as the reducing unit.

Compound **5** was obtained by purification of fraction **II** and shown to be made up of D-GalA, D-GlcA, and L-Rha as the reducing unit.

The compounds **1-5** were subjected to structure analysis by ^{1}H and ^{13}C NMR spectroscopy (the results are presented in the following section).

The third degradation step, i.e., hydrolysis of **P2,** produced a polymeric residue **P3** of M_n 3,030, composed of L-Rha, D-GalA, and D-GlcA, three fractions of higher oligosaccharides (**VII-IX**), and minute amounts of D-Gal, L-Rha, and uronic acids.

As shown by TLC, fractions **VII, VIII,** and **IX** were mixtures of four, three, and two components, respectively. All fractions were repeatedly fractionated on Bio-Gel P-4. Rechromatography of **IX** afforded one homogeneous oligomer identical with compound **5.** Compound **6,** recovered from **VIII,** was composed of D-GalA, D-GlcA, and L-Rha, which was proved to be located at the reducing end of the molecule.

From fraction **VII** two oligomers (**7** and **8**) were separated. Compound **7** afforded on hydrolysis L-Rha and D-GalA, while **8** produced D-GalA, D-GlcA, and L-Rha. The reducing unit in both compounds was rhamnose.

The structures of these oligomers were elucidated on the basis of ^{1}H and ^{13}C spectral measurements, as in the case of compounds **1-5.**

NMR spectroscopy of oligosaccharides. In the ^{1}H NMR spectra of the compounds **1, 4** and **7** (Fig. 1) the signals could be easily assigned on the basis of comparison with the spectrum of beet rhamnogalacturonan published recently.[8] The H4 signal of the unbranched terminal D-GalA residue was at δ 4.29, while that of internal unbranched D-GalA units was located at δ 4.42 in agreement with reference 8. Their intensity ratio in the spectra of compounds **4** and **7** (Fig. 1) indicated n = 2 and n = 6, respectively (Scheme 2). The spectrum of **1** (Fig. 1) displayed only one GalA H4 signal at δ 4.29 and two signals in the anomeric region at δ 5.23 and 4.94, arising from the resonances of H1 L-Rha α and β, respectively. Their corresponding D-GalA H1 signals were at δ 5.06, and 5.17. The chemical shifts of other Rha α, β proton signals were in accordance with those found for compound **4** (Table 3). The presented data gave evidence about a disaccharide structure of compound **1**, i.e., n = 1 in a structure repeating unit **A** (Scheme 2). Also, the ^{13}C NMR spectra of **4** and **7** showed spectral patterns similar to that presented in reference 8. In the ^{13}C NMR spectrum of **4** the following signals were identified - C1: Rha internal 99.1, Rha α and β 92.3 and 94.7, respectively, GalA 98.4, 98.1; C2: Rha internal 76.7, Rha α 77.4 and C4: GalA 77.9. The ^{13}C NMR data from **7** were in agreement with those presented in Table 4.

In comparing the spectra of compounds **1, 4** and **7** with those of compounds **5, 8** (Fig. 1) and **6,** remarkable changes due to the presence of βGlcA were observed. The well resolved GalA H4 signals were among the most important structural feature signals, suggesting GalA O3 substitution. The H4 signal of unsubstituted internal GalA as well as that of the substituted terminal GalA appears at δ 4.42, while that of unsubstituted terminal GalA was observed at δ 4.29. In compound **5** two new H4 signals were observed at δ 4.60 and 4.54. They were assigned on the basis of comparison with the ^{1}H NMR data (manuscript in preparation) of the rhamnogalacturonan tri- and hexasaccharides isolated from *Althaeae officinalis* L.,[8] regularly branched at GalA O3 with βGlcA. The H4 signal at δ 4.60 was assigned to the substituted internal GalA, while the one at δ 4.54 to the substituted GalA unit next to the reducing Rha residue.

In compounds **5, 6** and **8**, due to substitution of GalA at O3 with βGlcA, the H1 signal of the Rha unit linked to O4 of this GalA residue was shifted downfield to δ 5.43. In the spectrum of compound **5** the intensities of the Rha and GalA anomeric signals indicated n = 3 (repeating structure unit B, Scheme 2). However, the intensity of the downfield shifted H1 Rha signal at δ 5.43, suggesting the presence of one GlcA residue (m = 1), was not in agreement with the number and the intensities of H4 GalA signals. The ratio of the H4 signal intensities at δ 4.60, 4.54 and 4.42 was 1:1:2 (the H4 signal at δ 4.29 was found to be overlapped). Moreover, for some of the H1 GalA signals the spectral

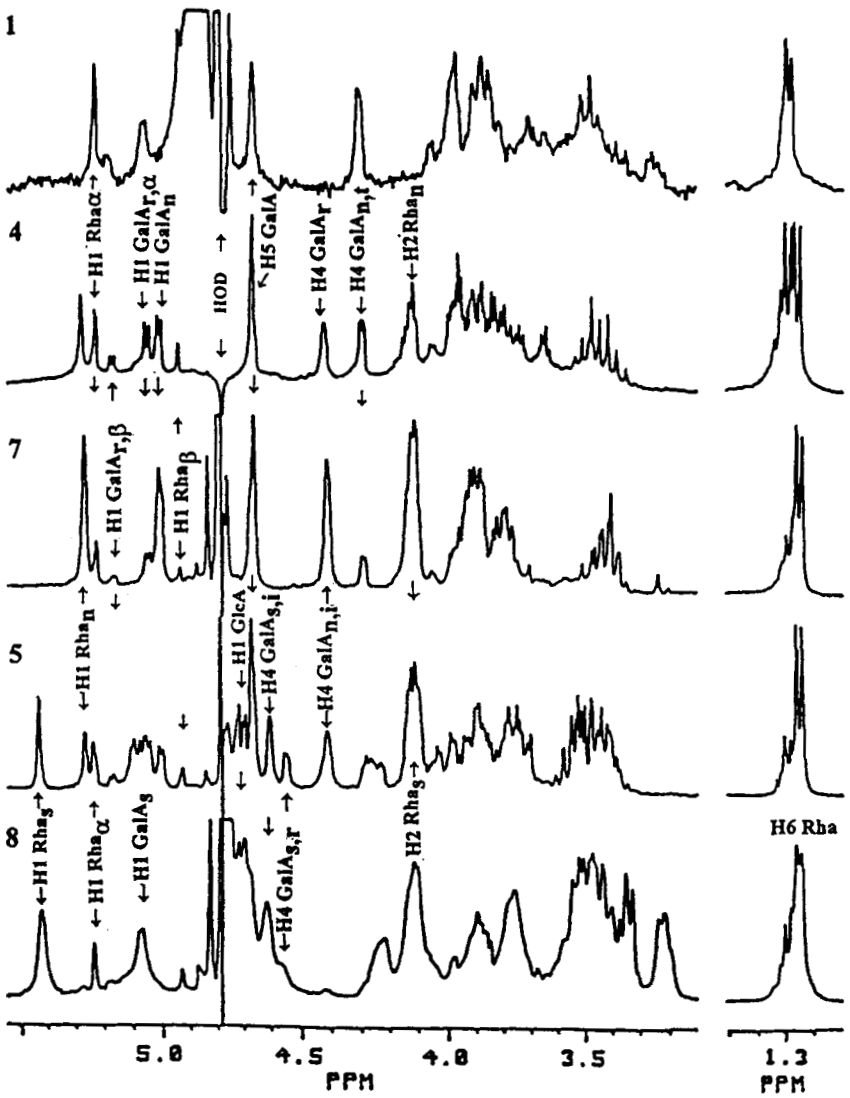


Fig. 1. ^{1}H NMR spectra (300 MHz) of oligomers **1, 4, 5, 7, 8**. Abbreviations: $GalA_s$ - GalA unit substituted with GlcA; $GalA_n$ - GalA not substituted; Rha_s - Rha unit neighbouring $GalA_s$ (on the left side); Rha_n - Rha unit next to $GalA_n$; $GalA_{s,i}$ - internal GalA unit substituted with GlcA; $GalA_{n,i}$ - internal GalA unit not substituted with GlcA; $GalA_s$, - GalA substituted with GlcA, next to α,β Rha; $GalA_r$ - GalA next to α,β Rha; $GalA_t$ - terminal GalA. The intensities of the H6 Rha signals are reduced.

Table 3. ^{1}H NMR data of oligosaccharides 1-4.

Compound	Chemical shift (δ / ppm) / Coupling constants (Hz)						
	H1	H2	H3	H4	H5	H6a	H6b
1							
αGalA(1→	5.06	a	a	4.29	4.67		
→2)Rha α	5.23	3.97	3.89	3.48	3.83	1.28	
αGalA(1→	5.17	a	a	4.29	4.67		
→2)Rha β	4.94	4.05	3.68	3.38	a	1.30	
2							
αGlcA(→	4.90(3.4)	3.61(10.1)	3.79 (9.8)	3.22 (10.0)	4.44		
→4)Gal β	4.64(7.8)	3.53(10.4)	3.70 (3.0)	3.99 (<1)	a	a	a
αGlcA(→	4.91(3.4)	3.61(10.1)	3.79 (9.8)	3.22 (10.0)	4.42		
→4)Gal α	5.30(3.7)	3.86(10.6)	3.93 (2.8)	4.05 (<1)	a	a	a
3							
αGalA(→	5.03(3.7)	3.83(10.3)	3.98 (3.4)	4.29 (<1)	4.66		
→2)αRha(1→	5.20(1.8)	4.18 (3.5)	3.90 (10.0)	3.50 (8.7)	a	a	a
→4)Gal β	4.60(7.8)	3.54(10.0)	3.79 (3.2)	4.02 (<1)	a	a	a
→4)Gal α	5.26(3.7)	3.86(10.6)	3.98 (3.2)	4.29 (<1)	a	a	a
4							
αGalA(→	5.01 3.7)	3.82(10.6)	3.96 (3.2)	4.29 (<1)	4.67		
→2)αRha(1→	5.28(1.8)	4.12 (3.7)	3.90 (10.1)	3.40 (9.8)	3.90 (6.3)	1.28	
→4)αGalA(→	5.05(3.7)	3.96(10.6)	4.12 (2.9)	4.42 (<1)	4.67		
→2)Rhaα	5.23(1.8)	3.97 (3.9)	3.89 (10.1)	3.47 (9.8)	3.82 (6.3)	1.26	
αGalA(→	5.01 3.7)	3.82(10.6)	3.96 (3.2)	4.29 (<1)	4.67		
→2)αRha(1→	5.28(1.8)	4.12 (3.7)	3.90 (10.1)	3.40 (9.8)	3.90 (6.3)	1.28	
→4)αGalA(1→	5.17(3.7)	3.97(10.6)	4.14 (2.8)	4.42 (<1)	4.67		
→2)Rhaβ	4.94(1.5)	4.05 (3.2)	3.67 (9.6)	3.41 (9.8)	3.41 (6.3)	1.28	

a. Not assigned.

pattern of the 2D COSY spectrum revealed two H2 GalA crosspeaks. All these findings, together with the unsuccessful attempt to obtain NOE responses for the individual H1 signals, suggested the presence of a mixture of heptasaccharides in compound **5**, differing in position of the branched GalA unit.

In the ^{1}H NMR spectrum of **6** the number of anomeric signals was in agreement with n = 4 and the intensity of the downfield shifted Rha H1 signal at δ 5.43 indicated the presence of two GlcA units (m = 2 in the repeating structure unit **B**, Scheme 2). The spectral pattern of its 2D spectra was similar to that of the compound **5**. The intensity ratio of the H4 signals at δ 4.61, 4.54, and 4.42, was found to be ca. 2:1:3. The intensity of the

Main chain

Repeating structure unit **A**

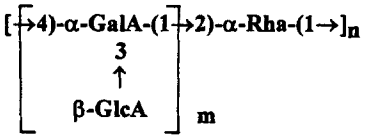
$$[\rightarrow 4)\text{-}\alpha\text{-GalA-}(1\rightarrow 2)\text{-}\alpha\text{-Rha-}(1\rightarrow]_n$$

compound **1** n = 1

compound **4** n = 2

compound **7** n = 6

Repeating structure unit **B**



compound **5** n = 3 m = 1

compound **6** n = 4 m = 2

compound **8** n = 5 m = 5

P3 n = 7 m = 5

P2 n = 12 m = 7

P1 n,m not identified

Side chains

compound **2** α-GlcA-(1→4)-Gal

compound **3** α-GalA-(1→2)-α-Rha-(1→4)-Gal

Scheme 2. Repeating units and oligomers in the *Malva* polysaccharide.

Table 4. ^{13}C and ^{1}H NMR data of repeating structure unit A (regardless of α, β equlibrium at the reducing end).

Repeating unit	Chemical shift δ / ppm					
	C1/H1	C2/H2	C3/H3	C4/H4	C5H5	C6/H6
[→4)-α-GalA-(1→	98.2	68.6	71.0	77.9	72.2	175.4
	5.00	3.92	4.12	4.40	4.67	
→2)-α-Rha-(1→]$_n$	99.2	76.7	70.1	72.7	69.7	17.3
	5.27	4.12	3.90	3.40	3.78	1.26

GalA H4 signal at δ 4.29 could not be estimated as it was overlapped. NOESY experiments failed also in this case in sequencing of the sugar units. Employing the same type of analysis, compound **6** was identified as a mixture of decasaccharides branched with two monomeric βGlcA residues, located at different GalA units in each decasaccharide.

Compound **8** was identified as an oligosaccharide with n = 5 fully branched at every GalA O3 with a monomeric βGlcA residue (Fig. 1). The H1 signals of Rha at δ 5.43 and that of the GalA units branched on O3 with GlcA at δ 5.08 were dominant in the ^{1}H NMR spectrum. The H1 signals of Rha α and β were observed at 5.23 and 4.94 ppm, respectively. In agreement with the previous findings, the H4 signal due to the internal GalA residues was detected at δ 4.63 and those of GalA next to the reducing Rha and of terminal GalA at 4.54 and 4.42 ppm, respectively.

Compounds **2** and **3** were characteristic of the side chain branchings. In the 1D and 2D relayed COSY experiments of **2**, for the H1 signal at δ 4.905 an easy transfer of polarization occurred due to a coupling constant network characteristic of the *gluco* configuration (Table 3). The coupling constants $^3J_{1,2}$ 3.4 Hz and $^3J_{4,5}$ 10.0 Hz indicated the presence of α GlcA residue and the 2D NOESY spectrum confirmed its linkage to O4 of Gal at the reducing end. In the ^{13}C NMR spectrum, the αGlcA C1, and C6 signals were present at δ 100.4, and 177.5, respectively, while those due to Gal C1, C4 and C6 α and β forms at 93.1, 79.1, 61.1 and 97.3, 77.8, 60.6 ppm, respectively. On the basis of the 1D and 2D COSY and relayed COSY as well as NOESY experiments, compound **3** was identified as a trisaccharide α-GalA-(1→2)-α-Rha-(1→4)-Gal (Table 3). We assume that this trisaccharide originates from side chain, as we have not found any oligomer with a Gal unit comprised in the repeating structure units (Scheme 2).

NMR spectroscopy of the intact and degraded polymers. Interpretation of the ^{1}H and ^{13}C NMR spectra (Figs. 2, 3) of the intact (**P**) and degraded (**P1**, **P2**, and **P3**) polysaccharides was performed using the data from oligosaccharides **1-8**. The ^{1}H NMR spectra (Fig. 2) of all polymers revealed several signals characteristic of the polymer chain composition. The monomeric βGlcA branching at GalA O3 was identified by the presence of the GlcA H1 signal at δ 4.71. In the **P2** and **P3** spectra (Fig. 2) a doublet of GlcA H5 ($^3J_{4,5}$ 9.6 Hz) at δ = 3.74 was also recognized. Comparison of the Rha H1 signals at δ 5.43 and 5.27 as well as those of GalA H4 at δ 4.63, 4.54 and 4.42 gave information about the GlcA quantity and location. On the other hand, the region of the GalA H1 signals reflected the distribution of GlcA units. In particular, the intensity of the GalA H4 signals in **P3** spectrum, in agreement with the Rha H1 signal intensities, confirmed that the degree of the GalA branching was higher than in **P2**, **P1**, and **P** (Fig. 2). The intensity of the GalA H4 signals in the **P** spectrum suggested that more than half of the GalA residues were branched. Discrepancies in the Rha H1 signal intensities in **P** and **P1** spectra could be

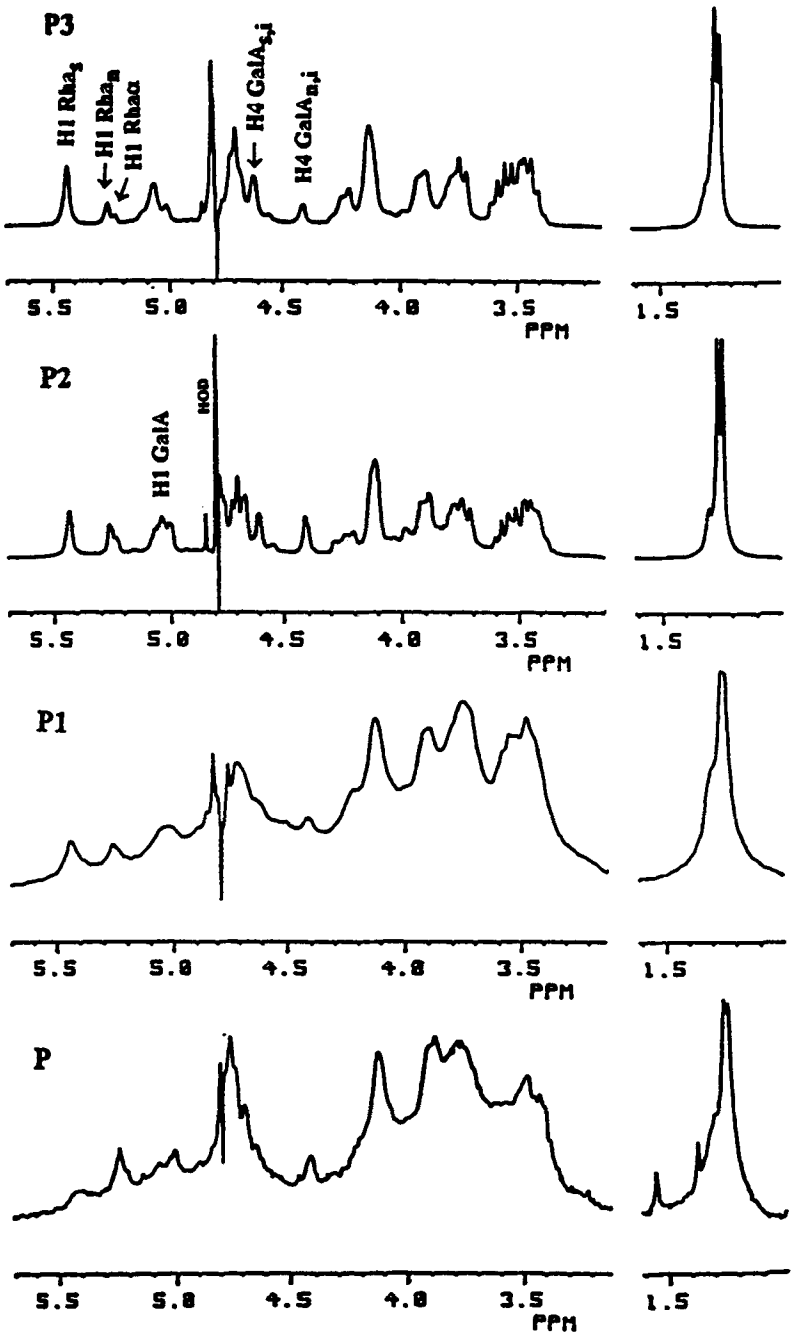


Fig. 2. ^{1}H NMR spectra (300 MHz) of intact P and degraded P1–P3 *Malva* polysaccharides. The abbreviations are the same as in Fig. 1.

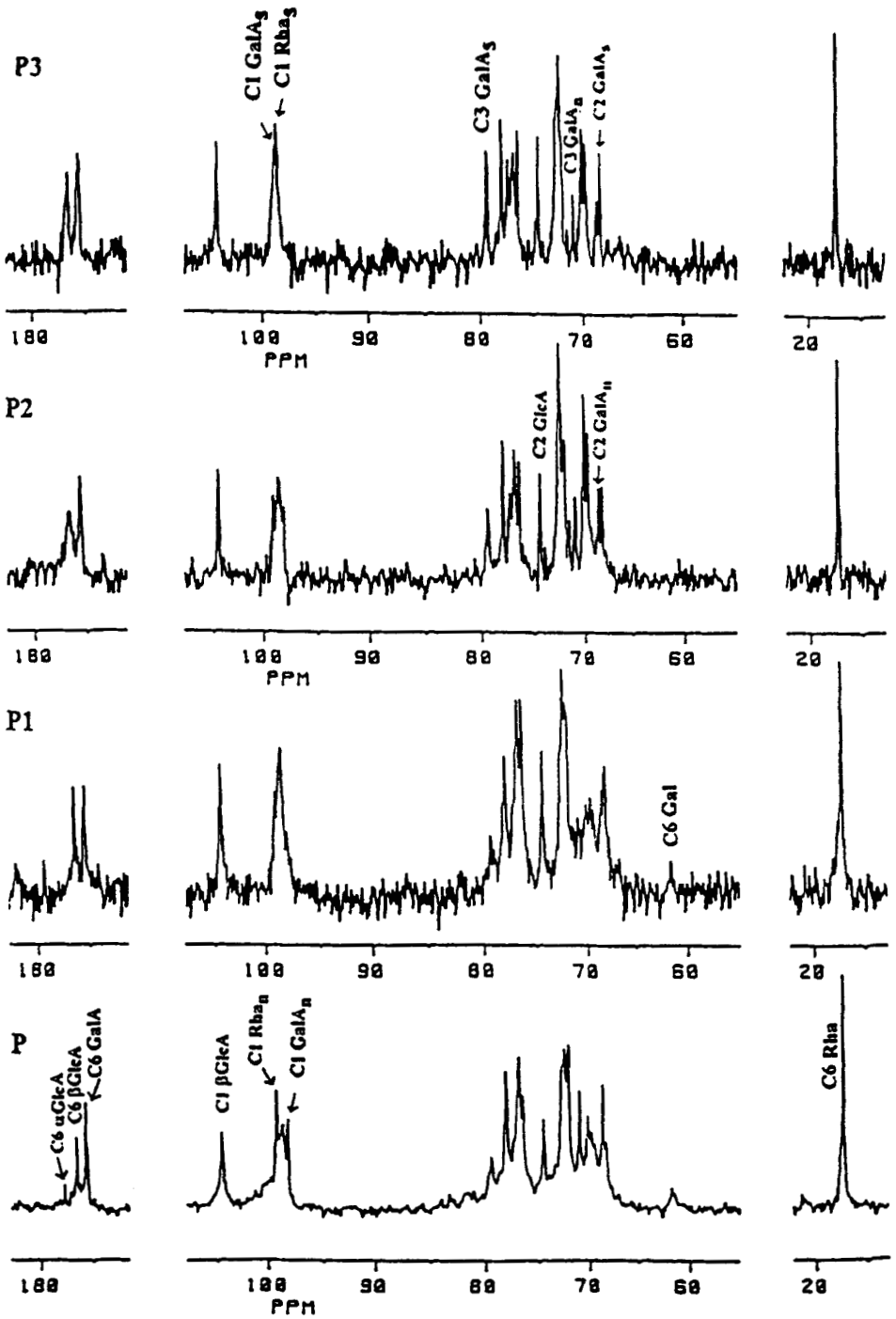


Fig. 3. ^{13}C NMR spectra (300 MHz) of intact P and degraded P1–P3 *Malva* polysaccharides. The abbreviations are the same as in Fig. 1.

Table 5. ^{13}C and ^{1}H NMR data of repeating structure unit B (regardless of α, β equlibrium at the reducing end).

Repeating unit	Chemical shift δ / ppm					
	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
[→4)-α-GalA-(1→	98.9	68.2	79.4	78.0	72.2	175.6
3	5.05	4.12	4.22	4.60	4.67	
↑						
β GlcA	104.5	74.4	76.4	72.4	76.5	176.5
	4.71	3.44	3.51	3.56	3.72	
→2)-α-Rha-(1→]$_n$	98.6	77.3	70.1	72.7	69.7	17.3
	5.43	4.12	3.89	3.40	3.78	1.26

explained by polydispersity of the intact polymer and probably also by the configuration of monosaccharides found in the side chains of **P**.

Generally, random distribution of GlcA in polymers influences significantly the spectral pattern of the GalA H1 signals. In the **P3** spectrum the dominant GalA H1 signal at δ 5.08 indicates regular GlcA distribution in longer segments, contrary to random ones in **P2** (Fig. 2).

The different degree of substitution of the linear rhamnogalacturonan chain with β GlcA in **P3** and **P2** was reflected also in their ^{13}C NMR spectra. This fact enabled the assigment of the carbon signals for both repeating structure units **A** and **B** (Tables 4 and 5). A comparative NMR analysis was reported[10,11] on rhamnogalacturonan-type oligosaccharides isolated from plants of the *Malvaceae* family which consisted of equimolar portions of L-Rha, D-GalA, and D-GlcA. Our data (Table 5), based on H-C heterocorrelated spectra (Fig. 4), include revised assignment of some signals, e.g., those of GalA C4, GlcA C3 and C5.

The occurence of the signal for the CH_2 group in the **P1** spectrum (Fig. 3) indicated the presence of a Gal residue, most probably as a building unit of side chains which, as documented by compositional analysis and absence of this signal in the spectra of **P2** and **P3**, were split off during graded acid hydrolysis. The **P** and **P1** spectra (Fig. 3) when compared to those of **P2** and **P3**, displayed also some remarkable differences in the signal intensities of the skeletal carbons. In particular, the C4 and C5 signals of 2-linked Rha, found at δ 72.7 and 69.7, respectively (Table 4, 5), showed reduced intensities. This fact, together with the results of methylation analysis (10.5% of 2,4-linked Rha units), points to

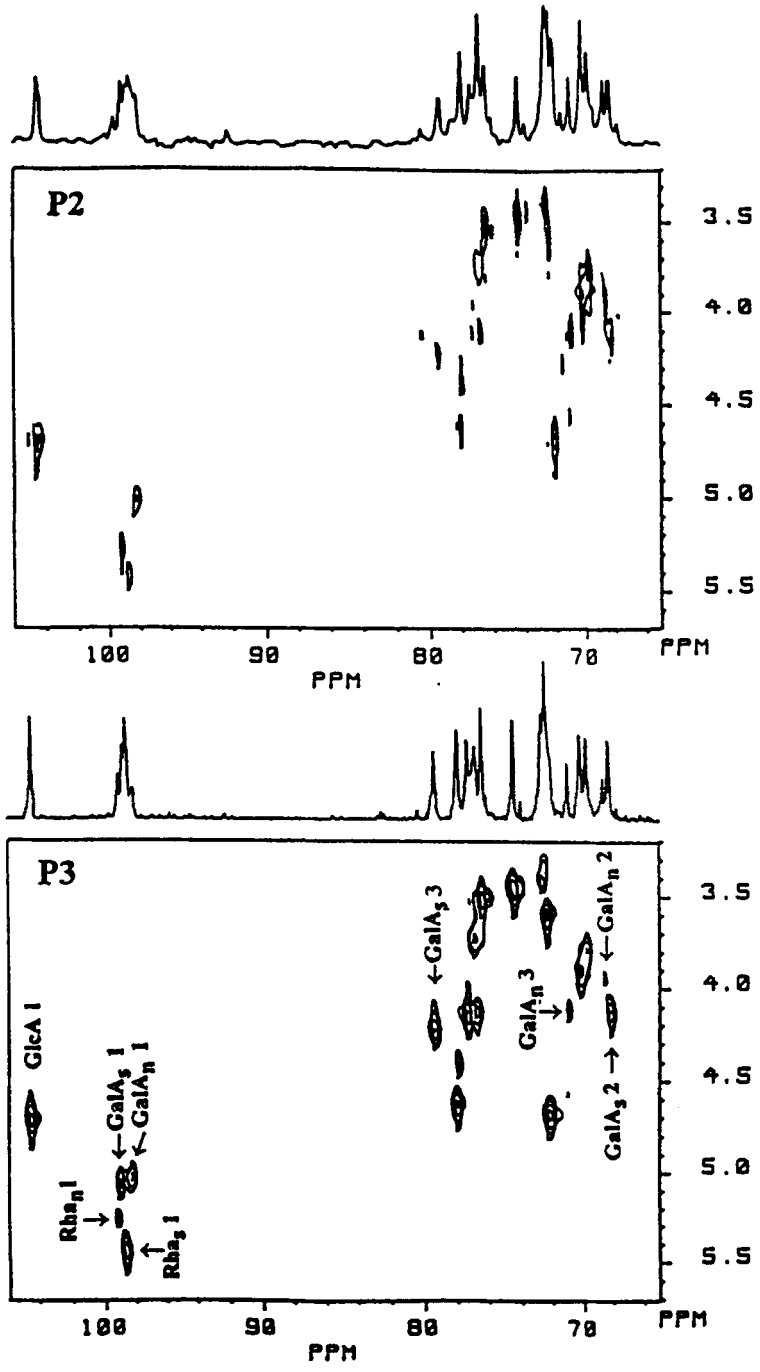


Fig. 4. Heterocorrelated spectra of **P2** and **P3**. Abbreviations of sugar units as in Fig. 1. For the sake of simplicity, the number of the skeletal atom denotes the origin of crosspeaks: for example $GalA_n$ 2 denotes the crosspeak between C2 and H2 of $GalA_n$.

substitution of Rha O4, which decreased during graded acid depolymerization of the native polysaccharide.

The results of chemical and spectroscopic analyses indicated a branched structure of the acidic heteropolysaccharide. Its backbone was formed by alternating sequences of 2- and 4-linked rhamnogalacturonan-type units. The oligomeric fragments obtained on graded degradation demonstrated that the backbone contained linear segments with →4)-α-D-GalA-(1→2)-α-L-Rha-(1→ sequences (DP = 2, 4, 12), segments bearing on O-3 of D-GalA monomeric D-GlcA randomly distributed along the chain (DP = 7, 10, 19, 31), and also segments having each D-GalA substituted (DP = 15). The results of methylation analysis indicated that about 28% of L-Rha residues were branched on O-4. The side chains consisted, besides smaller amounts of arabinose and xylose, mainly of galactose residues. In addition, we assume that the two oligomers α-D-GlcA-(1→4)-D-Gal and α-D-GalA-(1→2)-α-L-Rha-(1→4)-D-Gal, generated on partial acid hydrolysis, originate from side chains as well.

The basic structural features of the *Malva* heteropolysaccharide are similar to those isolated from *Abelmoschus manihot,*[10] *Abelmoschus glutinotextilis,*[13] *Althaea officinalis,*[9,14,15] *and Althaea rosea.*[16] Some differences are evident in the degree of branching of D-GalA as well as in the composition of the side chains. While in the rhamnogalacturonans isolated from the above mentioned plants all D-GalA residues are substituted at the O-3 position by monomeric β-D-GlcA, in our case only 56% are substituted. Additionally, in the *Malva* rhamnogalacturonan, D-GlcA was found not only in β-configuration (linked to O-3 of D-GalA) but also in α-configuration (~50%) linked to O-4 of D-Gal in side chains (α-D-GlcA-(1→4)-D-Gal). The occurence of D-GalA and L-Rha in side chains, indicated by the isolated trisaccharide α-D-GalA-(1→2)-α-L-Rha-(1→4)-D-Gal, wherein the position of D-GalA was supported also by detection of terminal D-GalA units in linkage analysis (Table 2), is a new finding in this type of polysaccharides.

EXPERIMENTAL

Material. The flowers of *M. mauritiana* L. were purchased from Slovakofarma, Medicinal plants (Malacky, Slovak Republic).

General Methods. Solutions were concentrated under diminished pressure below 40 °C. Free-boundary electrophoresis of 1% solutions of polysaccharides was effected with a Zeis 35 apparatus, using 0.05 M sodium tetraborate buffer (pH 9.2) at 150 V/cm and 6 mA for 30 min. The number average molecular mass (M_n) was determined osmometrically at 30 °C, using a Knauer vapour-pressure osmometer. Optical rotations (1

mL cells) were measured at (20 ± 1) °C on a Perkin-Elmer Model 141 polarimeter. Determination of proteins was effected by the method of Lowry *et al.*[17]. Infrared spectra of the methylated products were recorded with a Nicolet Magna 750 spectrometer. Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C. Paper chromatography was performed by the descending method on Whatman No.1 and 3MM papers with the systems S1, 8:2:1 ethyl acetate-pyridine-water and S2, 5:5:1:3 ethyl acetate-acetic acid-formic acid-water. The saccharides were detected with anilinium hydrogen phthalate. TLC was carried out on Kieselgel 60 (Merck, Germany) in the solvent system S3, 2:3:1 1-butanol-formic acid-water. The saccharides were visualized by spraying the plates with 20% aqueous ammonium sulfate and heating at 200 °C. The uronic acid content was determined by potentiometric titration and spectrophotometrically with the 3-hydroxybiphenyl reagent.[18] Quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates[19] by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm x 25 m) using a temperature program of 110-125 (2 °C/min) -165 °C (20 ° C/min) and flow rate of hydrogen 20 mL/min. Gas chromatography-mass spectrometry of partially methylated alditol acetates[20] was effected on a FINNIGAN MAT SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm x 30 m) at 80-240 °C (6 ° C/min), 70 eV, 200 μA, and ion-source temperature 150 °C.

NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were measured in deuterated water at 298K using a Bruker AM 300 spectrometer. Chemical shifts in the ^{1}H NMR spectra were referenced to the HOD signal at 4.78 ppm with respect to external acetone (2.225 ppm) and in the ^{13}C NMR spectra with respect to external acetone at 31.07 ppm. The samples of oligosaccharides (ca 5-8 mg/mL) were lyophilized 2-3 times from D_2O and flushed with argon before measurement. For the assignment of the signals in ^{1}H NMR spectra, 1D and 2D homonuclear chemical shift correlated COSY, multistep relayed COSY as well as C-H chemical shift correlated spectra using DEPT polarization transfer were performed.[21] In the 1D spectra rectangular soft pulses were used for the selective excitation of the signals. ^{13}C NMR spectra for quantitative evaluation were measured with suppression of NOE enhancement.

Isolation of the acidic heteropolysaccharide. The dry flowers of *M. mauritiana* (500 g) were macerated in distilled water (12 L) for 24 h at room temperature. The aqueous extract was centrifuged, concentrated to 1 L and poured into 96% ethanol (4 L) containing 1% (v/v) of acetic acid. The precipitate was washed with 70% aqueous ethanol, suspended in water, dialyzed, and freeze-dried. The dark brown material (17.5 g), containing 22.8% protein, yielded on acidic hydrolysis D-Gal, D-Glc, D-Man, L-Ara, D-

Xyl, L-Rha, and uronic acids in the mole ratio 1.0:0.2:0.2:0.6:0.3:0.4:0.7 as well as trace amount of L-Fuc.[5]

The crude product (4.3 g) was dissolved in water (100 mL) and applied to a column (4 x 40 cm) of DEAE-Sephadex A-50 in carbonate form and eluted successively with water, 0.1 M, 0.25 M, 0.5 M, and 1 M ammonium carbonate solutions. Elution with water gave a mixture (1.2 g) of neutral polysaccharides.[5,6] The ammonium carbonate eluates were deionized (Dowex 50W x 4), concentrated, and freeze-dried. The 0.1 M (0.1 g), 0.25 M (0.4 g), and 1 M (0.2 g) carbonate solutions differed in proportion of neutral components but, due to low amounts of these fractions, they were not studied further. The dominant fraction (**P**; 1.7 g), obtained by elution with 0.5 M ammonium carbonate and composed of L-Rha, D-Gal, L-Ara, D-Xyl, and uronic acids, was subjected to structure analysis.

Reduction of the polysaccharide. The polysaccharide **P** was reduced according to the method of Taylor and Conrad.[22] **P** (100 mg) was dissolved in water (25 mL) and 1-cyclohexyl-3-[2-(4-methylmorpholino)ethyl]carbodiimide *p*-toluenesulfonate (10 mol eq) was added to the solution. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1 M hydrochloric acid under stirring. After the reaction had proceeded for 2 hours, 2 M aqueous sodium borodeuteride was added gradually to the mixture within 2 hours, while the pH was maintained at 7.0 by titration with 1 M hydrochloric acid. A few drops of amyl alcohol were added to prevent foaming. The solution was dialyzed, concentrated and freeze-dried. The procedure was repeated four times to yield a carboxyl-reduced polysaccharide.

Methylation analysis. The dry samples of polysaccharides (3-4 mg) were solubilized in dry dimethyl sulfoxide (1 mL) and methylated by the Hakomori method.[23] The methylated products were isolated by partition with dichloromethane, concentrated, and hydrolyzed with 90% formic acid (1 h, 100 °C) and 2 M trifluoroacetic acid (1 h, 120 ° C). The partially methylated saccharides were reduced with sodium borodeuteride, acetylated and analyzed by GLC-MS (Table 1).

Graded acid degradation of the polysaccharide (Scheme 1). The polysaccharide **P** (1 g) was treated with 1 M trifluoroacetic acid (100 mL) for 30 min at 100 °C. The solution was neutralized with 1 M potassium hydroxide, concentrated (50 mL), and precipitated with 96% ethanol (200 mL). The precipitate was removed by centrifugation, desalted on a column (100 x 2.5 cm) of Sephadex G-10, and freeze-dried (**P1**; ~900 mg). The supernatant after concentration, desalting on the same column and freeze-drying, was shown to contain only monosaccharides (**SUP1**). Similar acid degradation of **P1** (850 mg) yielded a polymeric product (**P2**; ~420 mg) and a mixture of oligo- and monosaccharides

(**SUP2**; ~360 mg). Subsequent depolymerization of **P2** (300 mg) gave an ethanol-precipitable residue (**P3**; ~40 mg), as well as oligo- and monosaccharides (**SUP3**). The low-molecular hydrolysis products were separated on columns (200 x 2.5 cm) and (200 x 1.7 cm) of Bio-Gel P-2 and P-4, respectively. The compounds were eluted with 50 mM acetate buffer of pH 5.0 at a flow rate of 12 mL/h. Fractions of 2.5 mL were collected and analyzed for the carbohydrate content by phenol-sulfuric acid assay.

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