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(4-*O*-Methyl-α-D-glucurono)-D-xylan from *Rudbeckia fulgida*, var. *sullivantii* (Boynton et Beadle)

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Abstract

From the medicinal plant *Rudbeckia fulgida*, var. *sullivantii* (Boynton et Beadle) a low-molecular-mass (4-*O*-methyl-α-D-glucurono)-D-xylan was isolated by alkaline extraction, followed by ethanol precipitation, ion-exchange chromatography, and gel filtration. The results of compositional and linkage analyses, supported by those of ^{1}H and ^{13}C NMR measurements of oligomers generated on partial acid hydrolysis, showed the (1→4)-linked β-D-xylopyranosyl backbone with about 18% of 4-*O*-methyl-D-glucuronic acid attached to O-2 of the xylose residues. From the mean distance of adjacent carboxyl groups, obtained from experimentally determined single-ion activity coefficients of calcium counterions, it followed that the uronic acid units are separated and distributed regularly along the xylan chain, i.e. approximately each sixth D-xylose unit bears a 4-*O*-methyl-D-glucuronic acid residue.

Keywords: *Rudbeckia fulgida*; (4-*O*-Methyl-α-D-glucurono)-D-xylan; Structure determination; ^{1}H and ^{13}C NMR spectroscopy

1. Introduction

Isolation of compounds from medicinal plants which might be the active principle of a drug is not an easy task. In order to find an explanation for the medicinal use of various extracts from herbs, a great number of chemical and pharmacological studies have been undertaken.

Our continuing effort is to isolate homogeneous polysaccharides from active, crude polysaccharide mixtures of plant origin and characterize their structures as exactly as possible. In previous work [1] we found that the water-extractable polysaccharide complex from the aerial parts of the medicinal plant *Rudbeckia fulgida*, var. *sullivantii* (Boynton et Beadle) showed high antitussive activity, exceeding even the activity of some synthetic non-narcotic drugs used in clinical practice to treat coughing. The hemicellulose fraction obtained from the same source also showed high cough-suppressing activity in our preliminary studies. Therefore, we started a thorough investigation of this active fraction and in the present paper we report on structure analysis of its dominant polysaccharide component, a (4-*O*-methyl-α-D-glucurono)-D-xylan.

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2. Experimental

Materials.—The medicinal plant was collected in 1991 in the garden of the Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic. DEAE-Sephadex A-50 was obtained from Pharmacia (Sweden), Bio-Gel P-4 and P-2 from Bio-Rad (USA), and Sep-pak C_{18} cartridges from Waters Associates (USA).

General.—Concentrations were performed under diminished pressure at a bath temperature not exceeding 45 °C. Optical rotation (1 mL cells) was measured at 20 ± 1 °C with a Perkin-Elmer Model 141 polarimeter. Free-boundary electrophoresis of polysaccharide solution (10 mg/mL) was performed in 0.05 M sodium tetraborate with a Zeiss 35 apparatus at 150 V and 8 mA for 30 min. The number-average molecular mass M_n was determined osmometrically at 30 °C using a Knauer pressure osmometer. The infrared spectrum of the methylated polysaccharide was recorded with a Nicolet Magna 750 spectrometer. High-pressure gel-permeation chromatography (HPGPC) was performed using a commercial instrument (Laboratorní přístroje, Prague, Czech Republic) equipped with two Tessek Separon HEMA BIO-100 exclusion columns (8 mm×250 mm) and aqueous 0.1 M $NaNO_3$ as solvent (0.4 mL/min). The eluate was monitored by IR and UV detectors.

Descending paper chromatography (PC) was performed on Whatman No. 1 paper in the following solvent systems: S1, 8:2:1 EtOAc–pyridine–water; S2, 18:3:1:4 EtOAc–AcOH acid–formic acid–water, the sugars being detected with anilinium hydrogenphthalate. TLC was effected on Silica Gel 60 plates (Merck, Darmstadt, Germany) with 2:1:1 1-propanol–methanol–water. After spraying the plates with 20% $(NH_4)_2SO_4$, the spots were visualized by charring. Carbohydrates were determined by the phenol–H_2SO_4 assay [2] and the uronic acid content potentiometrically and by the method of Blumenkrantz [3].

Gas chromatography of alditol trifluoroacetates was conducted on a Hewlett–Packard 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm×25 m) at a temperature program of 110–125 °C (2 °C/min) to 165 °C (20 °C/min) and a flow rate of hydrogen of 20 mL/min.

GC–MS analysis of partially methylated alditol acetates was effected on a Finnigan Mat SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm×30 m) at 80–240 °C (6 °C/min), 70 eV, 200 μA, and ion-source temperature 150 °C.

Total hydrolysis of polysaccharides was effected with 2 M CF_3CO_2H (TFA) at 120 °C for 2 h. Potentiometric titrations were performed using a Radiometer PHM 64 (Denmark) equipped with a GK 2401 C combined electrode. Spectrophotometric measurements were carried out on a Specol 11 (Zeiss, Jena, Germany) spectrometer. The CD spectra both of potassium and calcium salts of the polysaccharide were measured in 1.00 mmol/L solutions with a Jobin Yvon Mark III (France) dichrograph. Reagents used in the determination of activity coefficient of Ca^{2+} were: 0.058 mol/L KOH, 0.021 mol/L $Ca(OH)_2$, 0.005 mol/L $CaCl_2$, redistilled water freed from carbon(IV) oxide, and tetramethylmurexide synthesized according to the method described previously [4].

NMR spectra were recorded at 25 °C on an FT NMR Avance DPX 300 spectrometer (^1H at 300.13 MHz and ^{13}C at 75.46 MHz) equipped with a selective excitation unit and gradient enhanced spectroscopy kit (GRASP) for generation of z-gradients up to 50 Gauss/cm in a 5-mm inverse probe. The samples in Na^+ form were dissolved in D_2O and chemical shifts of signals were referenced to internal acetone (2.225 and 31.07 ppm for ^1H and ^{13}C, respectively). 2D phase-sensitive TOCSY (States-TPP1) [5] and 1D TOCSY with z-filter [5,6] experiments were performed using MLEV 17 sequence for mixing. Two power levels for excitation and spinlock were utilized in 2D TOCSY, while selective excitation with Gaussian shaped pulses was used in 1D TOCSY and 1D transient NOESY [6] experiments. In the TOCSY experiments mixing times up to 61.6 ms were applied. In the ^1H detected heterocorrelated HSQC experiments [7] with composite GARP sequence decoupling as well as in 2D TOCSY the signal of water was suppressed using 3–9–19 pulse sequence [8] with pulse field gradients. For measurement of 2D DQF COSY spectra a pulse sequence of Davis [9] with pulse field gradients was used.

Methylation analysis.—The polysaccharide (50 mg), pretreated with $NaBH_4$, was methylated according to the method of Ciucanu and Kerek [10], followed by the Purdie method [11] to give a fully methylated product, as evidenced by the absence of IR absorption for hydroxyl. The methylated sample was recovered using a Sep-pak C_{18} cartridge by the procedure of Waeghe et al.

[12]. The product was then converted into partially methylated alditol acetates by hydrolysis, reduction with $NaBD_4$, and acetylation, and was subjected to linkage analysis by GC–MS.

Determination of activity of Ca^{2+} in the solution of the calcium salt of 4-O-methyl-D-glucurono-D-xylan.—The polysaccharide was converted into the H^+ form by percolating the polysaccharide solution through Amberlite IR 120 (H^+) and then it was neutralized to the point of equivalence with saturated $Ca(OH)_2$ solution. The activity of Ca^{2+} ions was determined in the solution of the calcium salt of the polysaccharide (3.00 mmol $COOCa_{0.5}$/L) by a spectrophotometric method using tetramethylmurexide ($c = 4\times10^{-5}$ mol/L) as the metallochromic indicator [13,14]; the solution did not contain any additional inert electrolyte. The calibration curve was constructed from the data obtained with $CaCl_2$ solution. The single-ion activity coefficient $\gamma_{Ca^{2+}}$ was calculated from the Ca^{2+} activity and total concentration of Ca^{2+} in the solution (1.5 mmol/L).

Determination of distribution of 4-O-methyl-D-glucuronic acid in the polysaccharide.—The mean distance b (nm) between two adjacent free carboxyl groups in their perpendicular projection on the axis of the D-xylan chain was estimated from the analytical curve $\gamma_{Ca^{2+}} = f(b)$. This function was introduced in earlier work [15] using the activity coefficient values determined in model solutions of variously esterified pectins ($E > 43\%$) [16].

Isolation of (4-O-methyl-α-D-glucurono)-D-xylan.—The methanol-pretreated, water-extracted, and air-dried aerial part of the plant (1 kg) was extracted with 0.5 M NaOH (20 L) for 24 h at room temperature. The residue was then separated from the supernatant by filtration. The supernatant after centrifugation was poured into EtOH (1:4, v/v) and the precipitate was collected by centrifugation, suspended in water, exhaustively dialyzed, and freeze-dried to give a brownish product (HC, 45 g). This product was purified by washing with 80% aqueous EtOH acidified with HCl (5 vol%), loaded onto a column (5×100 cm) of DEAE-Sephadex A-50 and irrigated successively with water and 0.25 M and 0.5 M carbonate solutions. The respective fractions were dialyzed and freeze-dried. The dominant, non-dialyzable 0.25 M carbonate fraction (37.8 g) consisted of xylose, 4-*O*-methyl-D-glucuronic acid, and trace amounts of galactose, glucose, and rhamnose. Gel filtration of this fraction on a column (4×150 cm) of Bio-Gel P-2 yielded a polysaccharide (35.1 g) homogeneous by free-boundary electrophoresis and HPGPC and consisting of xylose and 4-*O*-methyl-D-glucuronic acid only.

Partial acid hydrolysis of glucuronoxylan.—The polysaccharide (300 mg) was hydrolysed with 1 M TFA (200 mL) for 90 min at 100 °C. After cooling, the solution was made neutral, concentrated to a smaller volume, and added slowly into 4 vol. EtOH to precipitate the high-molecular-weight portion. The supernatant was concentrated, desalted on a Sephadex G-10 column, and then the mixture of hydrolytic products was fractionated on a column (2.5×150 cm) of Bio-Gel P-2 and/or P-4, respectively, by elution with water. The separation process was monitored by the phenol–H_2SO_4 assay and the purity of the individual fractions was checked by thin layer chromatography. Purification of the oligomers was performed by rechromatography on the same columns.

3. Results and discussion

From the methanol-pretreated and water-extracted plant material a crude hemicellulose fraction was obtained in 4.5% yield by alkaline extraction, followed by ethanol precipitation. The product was freed from the accompanying UV-absorbing compounds (most probably lignins) by washing with acidified aqueous ethanol. The non-dialyzable portion showed, after hydrolysis, xylose as the major spot on PC, smaller amounts of uronic acid, and four other neutral sugars, suggesting heterogeneity of the product (Table 1). Therefore, ion-exchange chromatography was employed to separate the accompanying polysaccharide species from the dominant xylan. The 0.25 M carbonate

Table 1
Characterization of the crude hemicellulose fraction (HC)

Yield [a]	4.5%
Ash	5.8%
Uronic acid [b]	11.6%
Composition of neutral sugars (mol ratio)	
Xylose	8.8
Glucose	0.5
Galactose	0.4
Rhamnose	0.1
Arabinose	0.1

[a] On dry herb basis.
[b] Potentiometric titration.

Table 2
Characterization of (4-*O*-methyl-α-D-glucurono)-D-xylan

D-Xylose	81.7%
4-*O*-Methyl-D-glucuronic acid	18.3%
OCH_3	3.2%
M_n	17 440
$[\alpha]_D$ (*c* 0.5, water)	−36°
Mol ratio D-Xyl:4-OMe-D-GlcA	6.4:1

Table 3
Methylated derivatives from the hydrolyzate of the methylated glucuronoxylan

Derivative [a]	Mol%	Linkage indicated
2,3,4-Me_3-Xy	2.6	Xyl*p*-(1→
2,3-Me_2-Xyl	78.1	→4)-Xyl*p*-(1→
3-Me-Xyl	18.8	→2,4)-Xyl*p*-(1→
Xylose	0.4	→2,3,4)-Xyl*p*-(1→

[a] 2,3,4-Me_3-Xyl = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-D-xylitol, etc.

eluate, comprising about 84% of all fractions, and still containing traces of galactose, glucose, and rhamnose, was further purified by gel filtration on Bio-Gel P-2 to give a polysaccharide homogeneous by free-boundary electrophoresis and HPGPC. As determined by the *m*-hydroxydiphenyl method and potentiometric titration, the polysaccharide contained 18.3% uronic acid, which was proved in the hydrolyzate to be 4-*O*-methyl-D-glucuronic acid, confirmed also by the methoxyl content (Table 2) and ^{13}C NMR spectral data (Table 4).

Linkage analysis.—The main product of methylation of the polysaccharide was 2,3-di-*O*-methyl-D-xylopyranose (Table 3), arising from the (1→4)-linked xylose chain units, while the 3-*O*-methyl derivative indicated O-2 substitution of these units. The sum of the branch points was 19.6 mol% (18.8 single substitution + 0.4×2 double substitution) and the amount of non-reducing xylose residues, indicated by the 2,3,4-tri-*O*-methyl derivative, was only 2.6 mol%. Consequently, the remaining 17 mol% branch points evidently carried the 4-*O*-methyl-D-glucuronic acid units. This value fits well the amount of uronic acid found by the previous methods (Table 2).

The ^{13}C NMR spectral data of the polysaccharide were in good agreement with the results of the linkage analysis. The C-4 signal of the internal xylose units was downfield shifted to 77.2 ppm, thus confirming the involvement of this carbon in (1→4) linkages. For substituted xylose units, besides the C-4 signal at 76.9 ppm, the C-2 signal was also downfield shifted to 77.7 ppm due to O-2 substitution with 4-OMe-D-GlcA. The signals generated by the resonance of anomeric carbons of chain xylose units appeared at 102.5 ppm and reflected, together with the vicinal coupling constant $^3J_{1,2}$ 7.3 Hz, the β-type of the xylose interunit bonds. The α-linkage of 4-OMe-D-GlcA was evidenced by the vicinal coupling constant $^3J_{1,2}$ 3.4 Hz.

*Distribution pattern of 4-*O*-methyl-D-glucuronic acid*.—The distribution pattern of uronic acid in the xylan molecule was elucidated on the basis of the determined single-ion activity coefficient of calcium counterions in the solution of the calcium

Table 4
^1H and ^{13}C NMR data for sugar residues of glucuronoxylan and oligomers generated by mild acid hydrolysis

Sugar residue	Chemical shift (ppm)						
	H/C						OMe
	1	2	3	4	5	6	
Xylose							
α	5.16	3.52	3.74	na	na		
	92.8	72.3	71.8	77.3	59.8		
β	4.57	3.24	3.57	3.76	4.04, 3.35		
	97.4	74.8	74.7	77.5	63.9		
Int	4.45	3.26	3.56	3.77	4.09, 3.37		
	102.6	73.6	74.6	77.2	63.9		
Int-s	4.61	3.45	3.61	3.79	4.13, 3.42		3.46
	102.3	77.7	72.1	76.9	na		
Nr	4.44	3.26	3.42	3.62	3.96, 3.29		
	102.8	73.2	76.5	70.1	66.1		
Uronic acid	5.27	3.56	3.74	3.20	4.31		
	98.5	73.1	na	83.3	72.9	177.5	60.8

na, Not assigned; Int, internal xylose unit; Int-s, internal substituted xylose unit; Nr, non-reducing xylose unit.

salt of the polysaccharide. The activity coefficient of counterions bound by electrostatic bonds to the polysaccharide is a function of linear-charge density of the macromolecule, characterized by the mean distance (b, nm) between two adjacent carboxyl groups [16]. The electrostatic bond between calcium and carboxyl groups in the studied polysaccharide was confirmed by identical circular dichroic spectra both of calcium and potassium salts, revealing the maximum in the negative ellipticity range around 210 nn (Fig. 1). The distance between two vicinal carboxyl groups in the studied glucuronoxylan, $b = 3.2$ nm, corresponding to the activity coefficient of 0.66, was obtained from the linearized form of the function $\gamma_{Ca^{2+}} = f(b)$ [16]. Although this relationship was proved to be valid for linear acid polysaccharides, i.e. wherein the uronic acid units are built in the linear chain, we consider this dependence applicable to the glucuronoxylan because the uronic acid is attached to the linear xylan chain exclusively as monomeric terminal units.

When considering the analogy with the macromolecule of D-mannuronan, which has the same type of glycosidic bonds [β-(1→4)] of the saccharide units in $^4C_1$ conformation, the length of one xylose unit in the D-xylan chain is 0.52 nm [17]. Then from the b value of 3.2 nm it follows that approximately each sixth D-xylose residue in the macromolecule is substituted by one unit of 4-*O*-methyl-D-glucuronic acid, which is consistent with the results presented above.

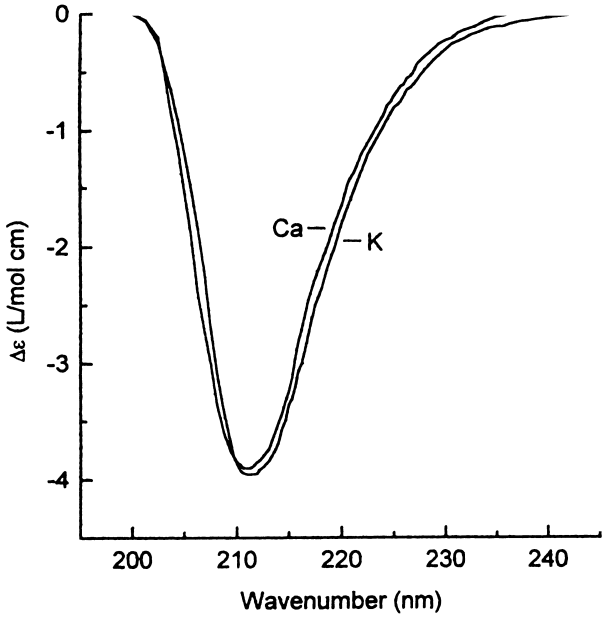


Fig. 1. CD spectra of potassium and calcium salts of (4-*O*-methyl-α-D-glucurono)-D-xylan. Δ = molar ellipticity.

^1H and ^{13}C NMR spectral analysis of oligosaccharides.—Partial acid hydrolysis of the polysaccharide also generated, besides di-, tri-, and tetramers of (1→4)-linked xyloses, a series of acidic fragments of dp = 4–11 and 14 which were characterized by ^1H and ^{13}C NMR spectroscopy. The degree of polymerization (dp) was estimated on the basis of integral intensities of the signals appearing in the range 5.3–3.9 ppm in the ^1H NMR spectra. The spectral data of the three neutral xylooligomers were identical with those already described [18,19]. Comparison of the integrals of anomeric signals of acidic oligosaccharides showed that in the compounds with dp < 11 only one 4-OMe-D-GlcA unit was present, while in that of dp = 14 two units were found. Using homonuclear 2D COSY and 1D and 2D TOCSY as well as heterocorrelated HSQC experiments, a set of data was obtained (Table 4) which is in agreement with the already published data for xylobiose [20] and xylotriose [21] with one 4-OMe-D-GlcA attached to the non-reducing xylose unit and to the unit next to the reducing end, respectively.

In the ^1H NMR spectra the H-1, H-2, and H-3 signals of the substituted xylose unit showed a significant downfield shift in comparison with the data of the unsubstituted residues, thus confirming O-2 substitution. However, there was no secondary effect of the 4-OMe-D-GlcA substituent on the chemical shift of the signals arising from the adjacent unsubstituted xylose units (Table 4). Consequently, it was not possible to identify which internal xylose unit bears the 4-OMe-D-GlcA residue. The ^1H and ^{13}C NMR spectral pattern of the samples with lower dp suggested a mixture of oligosaccharides substituted at different xylose units. This was especially remarkable in the spectra (A, B in Figs 2 and 3) of samples with dp = 5. Substitution of the reducing-end xylose with 4-OMe-D-GlcA was excluded because neither the ^{13}C nor the ^1H NMR spectra showed additional anomeric C-1α, C-1β and H-1α, H-1β signals, respectively. Substitution of terminal non-reducing xylose was revealed on the basis of additional C-4* and C-5* signals at 70.2 and 65.8 ppm due to the terminal non-reducing xylose (spectra A, B, C in Fig. 3). Chemical shifts of these signals corresponded with those of the respective carbon signals due to the teriminal non-reducing-end xylose unit substituted with 4-OMe-D-GlcA of the xylobiose described

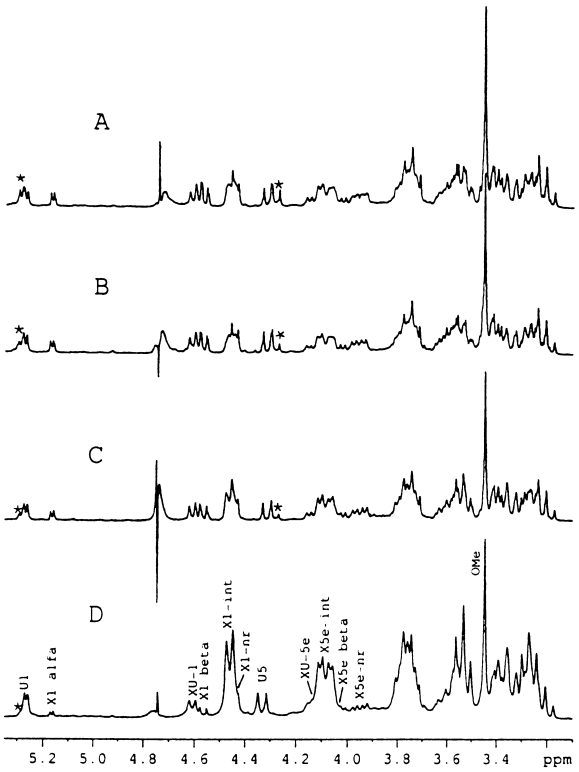


Fig. 2. ^{1}H NMR spectra of acidic xylooligosaccharides: A, dp = 5 (X = 4, U = 1); B, dp = 5 (X = 4, U = 1); C, dp = 7 (X = 6, U = 1); D, dp = 14 (X = 12, U = 2). X = D-xylose, U = 4-*O*-methyl-D-glucuronic acid, XU = xylose unit substituted with U, X-int = xylose unit at the non-reducing end, X-int = internal xylose unit, * denotes H-1 and H-5 signals of U linked to non-reducing xylose residue.

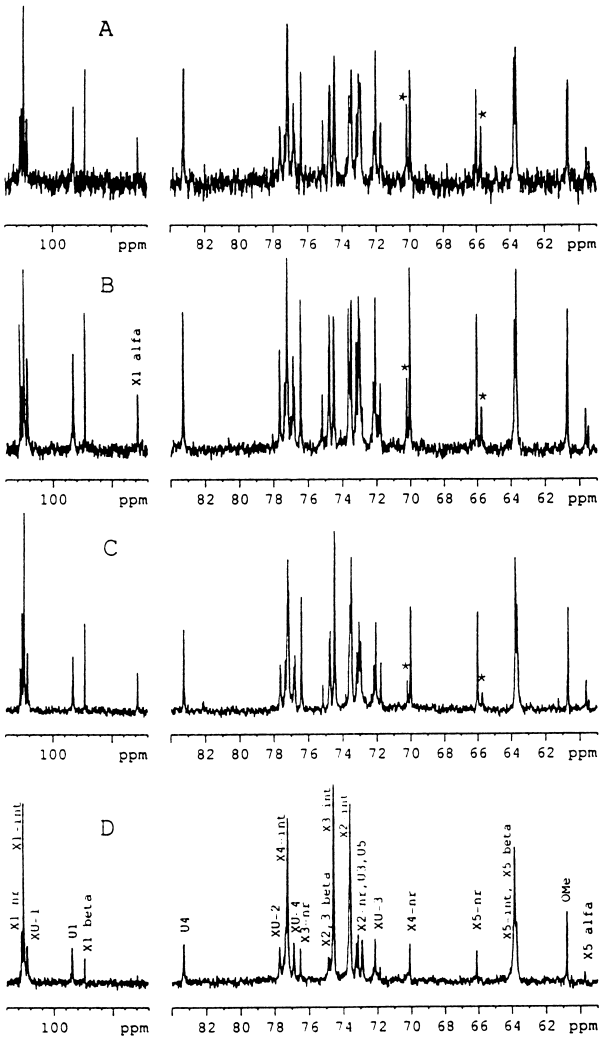


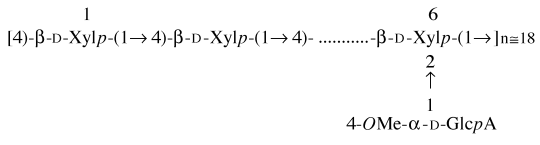
Fig. 3. ^{13}C NMR spectra of acidic xylooligosaccharides: A, dp = 5 (X = 4, U = 1); B, dp = 5 (X = 4, U = 1); C, dp = 7 (X = 6, U = 1); D, dp = 14 (X = 12, U = 2). X = D-xylose, U = 4-*O*-methyl-D-glucuronic acid, XU = xylose unit substituted with U, X-nr = xylose unit at the non-reducing end, X-int = internal xylose unit, * denotes C-4 and C-5 signals of U linked to non-reducing xylose residue.

previously [20]. In the spectra of oligomers with equal dp the intensity of these signals was different (spectra A and B, Fig. 3) and it changed randomly from one spectrum to the other, thus reflecting the content of such substituted oligosaccharide in each oligomeric sample. With increasing dp the intensity of these C-4* and C-5* signals decreased and they could not be detected in the spectra of samples with dp > 9 (spectrum D in Fig. 3). The attachment of the uronic acid substituent to two different xylose units was evidenced also by the presence of two OMe signals found at 60.75 and 60.69 ppm.

In the ^{1}H NMR spectra A and B (Fig. 2), the anomeric signals H-1α and H-1β at 5.16 and 4.57 ppm, respectively, showed additional splitting, indicating changes on the neighbouring xylose unit, and thus suggesting also the presence of an oligosaccharide with a substituted xylose unit next to the reducing end. In the ^{1}H NMR spectra of tri- and tetrasaccharides with 4-OMe-D-GlcA linked to the xylose unit next to the reducing end, a splitting of the H-1 and H-5 4-OMe-D-GlcA signals was observed as a consequence of the influence of the α,β equilibrium at the reducing-end xylose unit [20,21]. In spectra A, B, and C (Fig. 2), besides the H-1 and H-5 4-OMe-D-GleA signals at 5.27 and 4.32 ppm, respectively, also additional H-1* and H-5* signals at 5.29 and 4.99 ppm could be observed. In some spectra the change of their intensities from one spectrum to another was in agreement with the above-mentioned intensity change observed in the ^{13}C NMR spectra for C-4* and C-5* signals due to the substituted non-reducing-end xylose and it was not consonant with the intensity ratio of H-1α and H-1β signals of the reducing-end xylose unit. These

facts were especially remarkable in spectrum A (Fig. 2) and they suggest that the origin of H-1* and H-5* could not be explained only by the presence of an oligosaccharide with substituted xylose next to the reducing-end xylose unit.

It may be concluded that the spectral data of the oligomers confirmed the 4 and 2,4 linkages of xylose units, the β configuration of the internal xylose bonds, and the α type of the 4-OMe-D-GlcA attachment. Although most of the samples studied were shown to be a mixture of oligomers with the same dp but with a different substituted xylose unit, the fact that none of the oligomers with $dp < 11$ contained more than one 4-OMe-D-GlcA residue supported the findings that the uronic acid units are separated and distributed regularly along the xylan chain, i.e. that each sixth xylose unit bears a 4-OMe-D-GlcA monomer. Thus, the structure of the repeating unit in the polysaccharide is the following:



A similar type of glucuronoxylan was isolated from sunflower hulls [22]. The knowledge of the primary structure of the prevalent glucuronoxylan component of the hemicellulose fraction, which was shown in our preliminary tests to be highly active in cough-reflex suppression, is important from the point of view of a structure–activity relationship. We believe that it will contribute to the explanation of the mechanism of peripheral action of plant polysaccharides in the treatment of coughing. Moreover, the results may be utilized in classification of hemicelluloses of different origins.

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