4-*O*-Methyl-D-glucurono-D-xylan from the leaves of the marsh mallow (*Althaea officinalis* L., var. *Rhobusta*)

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A water-soluble 4-*O*-methyl-D-glucurono-D-xylan of $\bar{M}_n = 21.563$ has been isolated from the leaves of *A. officinalis* L., var. *Rhobusta*. On the basis of the results of methylation analysis and ^{13}C NMR spectral data it was concluded that the polysaccharide was built of (1 → 4)-linked β-D-xylopyranosyl residues, ≈ 70 % being unsubstituted, ≈ 5 % carrying a single substitution at O-2 and/or O-3, and ≈ 11 % being doubly branched at O-2 and O-3. 4-*O*-Methyl-D-glucuronic acid was attached to O-2 as a single terminal unit (1 unit per 7 units of xylose).

Из листьев *A. officinalis* L., var. *Rhobusta* был выделен водорастворимый 4-*O*-метил-D-глюкуроно-D-ксилан с $\bar{M}_n = 21{,}563$. На основе результатов анализа с помощью метилирования и ^{13}C ЯМР спектральных данных был сделан вывод, что данный полисахарид состоит из (1 → 4)-связанных β-D-ксилопиранозных остатков, из которых ≈ 70 % не замещено, ≈ 5 % имеет один заместитель на O-2 и/или O-3, а ≈ 11 % дважды замещены в положениях O-2 и O-3. 4-*O*-Метил-D-глюкуроновая кислота была присоединена к O-2 как одиночная концовая единица (1 единица на 7 ксилозных единиц).

It has long been known that the medicinal plant marsh mallow (*Althaea officinalis* L.) contains relatively large amounts of mucilage which, as some other plant polysaccharides, may function as reserve carbohydrate, protective colloid, and water reservoir. The pharmaceutical effects of mucilages depend on covering the mucous membranes with a layer that protects against irritations. In this capacity they are used externally as skin creams, and internally in cases of bronchitis and enteritis, or as laxatives.

Mucous polysaccharides from *A. officinalis* L. and other related genera and species were studied by *Tomoda et al.* [1—4]. They described beside glucans and pectic substances acidic heteropolysaccharides composed of L-rhamnose, D-galactose, D-glucuronic acid, and D-galacturonic acid.

We studied the mucilageneous material isolated from *A. officinalis* L., var. *Rhobusta*, systematically in order to broaden our knowledge of the chemical structures of its individual polysaccharide components and throw light on the possible relationship between structure and therapeutic effects. In our previous reports we described a highly-branched L-arabinan [5], glucans [6], and an acidic

heteropolysaccharide [7] isolated from the roots, and a glucan [8], isolated from the leaves of this plant. The present work was undertaken to separate and characterize a further component of the mucilage isolated from the leaves, an acidic polysaccharide.

Experimental

Material and methods

Leaves were obtained from plants cultivated at the Centre for Cultivation of Medicinal Plants, Faculty of Medicine of J. E. Purkyně University, Brno (Czechoslovakia) in 1983.

Solutions were concentrated under diminished pressure at temperature below 40 °C. Paper chromatography was performed by the descending method on Whatman No. 1 paper with the following systems: S_1, ethyl acetate—pyridine—water ($\varphi_r = 8:2:1$); S_2, ethyl acetate—acetic acid—formic acid—water ($\varphi_r = 18:3:1:4$). Reducing sugars were detected by spraying with anilinium hydrogen phthalate and heating the papers for 5 min at 105 °C, and alditols with alkaline silver nitrate reagent. Quantitative determination of neutral sugar components of the crude mucilage (as alditol trifluoroacetates) was carried out by GLC on column A. The uronic acid content of the crude mucilage was determined by the carbazole method [9] and that of 4-*O*-methyl-D-glucurono-D-xylan by potentiometric titration. Determination of protein content was carried out by the method of *Lowry et al.* [10]. The amino acid composition was established with an Automatic amino analyzer, type 6020 (Mikrotechna, Prague), after hydrolysis of the polysaccharide with 6 M-HCl for 20 h at 100 °C. The methoxy content was determined by the method of *Viebock* and *Brecher* [11].

Optical rotation was measured with a Perkin—Elmer Model 141 polarimeter in an 0.5 % aqueous solution at 22 °C. Free-boundary electrophoresis was effected with a Zeiss 35 apparatus (Jena), on a polysaccharide ($\varrho = 10\ \mathrm{mg\ cm^{-3}}$) in 0.05 M sodium borate buffer of pH = 9.2, for 30 min at $10\ \mathrm{V\ cm^{-1}}$ and 6 mA. The number average molecular mass ($\bar{M}_n$) was determined osmometrically at 30 °C after equilibration with 0.1 M-NaCl solution, using a Knauer membrane osmometer fitted with a Zweischicht-Membrane (Knauer). Infrared spectrum of the methylated polysaccharide was recorded with a Perkin—Elmer spectrometer, model 9836.

GLC was conducted with a Hewlett—Packard Model 5711 chromatograph and (A) a column (200 cm × 0.3 cm) of 3 % OV-225 on 150—175 μm Chromosorb W (AW-DMSC), programmed to hold a temperature of 120 °C for 4 min, then to increase it to 170 °C at $2\ \mathrm{°C\ min^{-1}}$; and (B) a column (200 cm × 0.3 cm) of 3 % SP 2340 on 125—150 μm Supelcoport at 110 °C for 2 min, then increasing the temperature to 210 °C at $4\ \mathrm{°C\ min^{-1}}$. GLC—MS of alditol acetates [12] of methylated saccharides was performed on the same column as (B). The inlet helium pressure was 101.3 kPa, temperature programmed from 160 °C to 240 °C at $6\ \mathrm{°C\ min^{-1}}$. The spectra were determined at the ionization potential of 23 eV.

The ^{13}C NMR spectrum of the glucuronoxylan solution (3 % in D_2O) was obtained with a Bruker AM-300 (75 MHz) spectrometer at 30 °C with suppression of NOE effect. The spectral width was 16.380 Hz; acquisition time 0.5 s; relaxation delay 3 s; data points 16 K with additional 16 K for zero-filling; pulse width 19 μs (90°). Chemical shifts were measured relative to internal methanol ($\delta = 50.15$ ppm).

*Isolation of 4-O-methyl-*D*-glucurono-*D*-xylan*

Dry leaves (500 g) were macerated in cold water (20 dm^3) for 48 h. The extract was filtered, concentrated to 6 dm^3, and poured into 96 % ethanol (36 dm^3) containing 1 vol. % acetic acid. The precipitate was washed with 60 % aqueous ethanol, suspended in water, and dialyzed against distilled water for 3 d. The water-insoluble portion, which separated, was removed by centrifugation and the supernatant was lyophilized to give the crude mucilage (21 g, 4.2 %). The mucilage was suspended in 70 % aqueous ethanol (250 cm^3) and stirred for 6 h at 40 °C. The insoluble portion was centrifuged, dissolved in water, and loaded onto a column (100 cm × 4.5 cm) of DEAE-cellulose (DEAE 23-Cellulose SN Servacel) in carbonate form. The individual components were eluted with water and ammonium carbonate solutions of increasing concentration ($c = 0.1$, 0.25, and 0.5 mol dm^{-3}). The carbonate eluates were dialyzed against distilled water and deionized on a column of Dowex 50W × 4 (H^+). Each fraction was hydrolyzed and analyzed for sugar composition. The water eluate (18 mg) was composed of D-glucose and D-mannose, the 0.1 M and 0.25 M carbonate eluates (12 mg and 15 mg, respectively) contained similar sugars, namely, D-galactose, D-glucose, D-mannose, D-xylose, L-rhamnose, and traces of uronic acid. The product obtained by elution with 0.5 M ammonium carbonate (24 mg) was a homogeneous polysaccharide composed of D-xylose and 4-*O*-methyl-D--glucuronic acid.

Hydrolysis

The crude mucilage was hydrolyzed with 0.5 M sulfuric acid for 20 h and the other polysaccharide fractions with 2 M trifluoroacetic acid for 6 h at 100 °C in sealed tubes.

Methylation analysis

A modified procedure of the method of *Ciucanu* and *Kerek* [13] was applied to methylation of glucuronoxylan.

The polysaccharide (20 mg) was solubilized in dry dimethyl sulfoxide (2 cm^3) at room temperature under stirring. Crushed sodium hydroxide (80 mg) was added and after 1 h reaction methyl iodide (0.5 cm^3) was added dropwise. Methylation proceeded under stirring of the reaction mixture for 1 h. Then water (4 cm^3) was added and the solution was dialyzed against distilled water for 48 h. The solution was extracted with chloroform (5 cm^3), the chloroform layer was washed with water and dried with anhydrous sodium

sulfate. Concentration of the chloroform extract gave the fully methylated product (15.6 mg, 78 %), not showing any IR absorption for hydroxyl.

The methylated product (8 mg) was hydrolyzed with 70 % sulfuric acid (0.3 cm^3) for 45 min at room temperature. Then the solution was diluted with water (1.6 cm^3) and refluxed for 6 h. The solution was neutralized with barium carbonate and filtered. The excess barium ions were removed on a column of Dowex 50W × 4 (H^+). The hydrolyzate of the methylated polysaccharide was separated on a column of Dowex 1 × 8 (acetate form) into neutral and acidic fractions. The neutral partially methylated derivatives were eluted with distilled water, reduced with sodium borodeuteride, acetylated, and analyzed by GLC—MS.

Results and discussion

In the framework of a detailed investigation of mucilages of the roots and leaves of *A. officinalis* L., var. *Rhobusta* (a hybrid of *A. officinalis* L. and *A. armeniaca* TEN, improved and cultivated in Czechoslovakia), from the leaves of the plant we have so far isolated and structurally characterized the dominant component of the mucilage, a glucan. Now, we have undertaken structural studies of an acidic carbohydrate polymer, the presence of which in the crude mucilage was indicative by the uronic acid content (Table 1).

Table 1

Characterization of the crude mucilage isolated from the leaves of the marsh mallow (*Althaea officinalis* L., var. *Rhobusta*)

Yield[a]	4.20 %
w(Protein)	1.70 %
Sugar composition (mole ratio)	
D-Glc	1.00
D-Gal	0.16
D-Man	0.12
L-Rha	0.92
L-Ara	0.08
D-Xyl	0.22
Uronic acid	0.11

a) Relative to dry mass of the leaves.

The crude mucilage was isolated from dry leaves by extraction with cold water, precipitation of the extract with ethanol, and subsequent dialysis of the precipitate against distilled water to remove the accompanying low-molecular glucans. The sugar composition is presented in Table 1 and the amino acid composition of the protein moiety of the mucilage is listed in Table 2. After

Table 2

Amino acid composition of the crude mucilage from the leaves of the marsh mallow (*Althaea officinalis* L., var. *Rhobusta*)

Amino acid	x/mole %
Lysine	6.00
Histidine	1.96
Arginine	3.61
Aspartic acid	9.95
Threonine	6.82
Serine	7.73
Glutamic acid	9.68
Proline	7.10
Glycine	10.56
Alanine	9.27
Valine	8.56
Methionine	1.50
Isoleucine	3.97
Leucine	7.07
Tyrosine	2.71
Phenylalanine	3.51

treatment of the mucilage with 70 % aqueous ethanol at 40 °C, the ethanol-insoluble portion was loaded onto a DEAE-cellulose column in carbonate form. The fractions eluted with water, 0.1 M and 0.25 M ammonium carbonate contained heterogeneous polymers differing in qualitative composition of constituent sugars. The water eluate did not contain any uronic acid and the uronic acid content of the 0.1 M and 0.25 M eluates was negligible.

The 0.5 M carbonate eluate afforded a water-soluble polysaccharide homogeneous upon electrophoresis which did not contain any protein. Its characterization is presented in Table 3. The products of acid hydrolysis of the polysaccharide were identified by paper chromatography as D-xylose, 4-*O*-methyl-D-glucuronic acid, and an aldobiuronic acid identical with the authentic reference, 2-*O*-(4-*O*-methyl-α-D-glucopyranuronosyl)-D-xylose. The amount of

Table 3

Characterization of 4-*O*-methyl-D-glucurono-D-xylan

w(D-Xylose)	83.10 %
w(4-*O*-Methyl-D-glucuronic acid)	16.90 %
w(OCH_3)	2.72 %
$\bar{M}_n$	21 563
[α] (D, 22 °C, $\varrho = 5.0$ g dm^{-3}, water)	−77.3°
n(D-Xylose) : *n*(4-*O*-methyl-D-glucuronic acid)	7.09 : 1.00

4-*O*-methyl-D-glucuronic acid in the polysaccharide, determined by potentiometric titration, was 16.9 % which agrees with the methoxyl content 2.72 %.

The polysaccharide was methylated by a modification of the method of Ciucanu and Kerek and the efficiency of methylation was checked by the absence of IR absorption for hydroxyl. The product (78 %), achieved in one-step methylation, was hydrolyzed, the neutral components were separated on a column of Dowex 1 × 8 (acetate form), converted into alditol acetates, and analyzed by GLC and GLC—MS. The results are shown in Table 4.

Table 4

Methylated xyloses from the hydrolyzate of the methylated 4-*O*-methyl-D-glucurono-D-xylan

Sugar derivative[a]	t^b	Mode of linkage	Mole ratio	*x*/mole %
2,3,4-Me_3—Xyl	0.79 (0.80)[c]	Xylp-(1 →	0.18	14.06
2,3-Me_2—Xyl	1.36 (1.37)	→ 4)-Xylp-(1 →	0.89	69.53
2- and 3-Me—Xyl	1.90 (1.94)	→ 3,4)-Xylp-(1 → and → 2,4)-Xylp-(1 →	0.07	5.46
Xylose	2.40	→ 2,3,4)-Xylp-(1 →	0.14	10.93

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

b) Retention times relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

c) Values in parentheses are from Ref. [14].

The ^{13}C NMR shifts for the polysaccharide are given in Table 5. The assignment of signals was effected on the basis of the results of methylation analysis and literature data [14—18].

The main product of methylation was 2,3-di-*O*-methyl-D-xylopyranose (69.53 %) indicating that the main chain of the polysaccharide was built of (1 → 4)-linked D-xylopyranosyl residues. Characteristic of this bond are the signals in the ^{13}C NMR spectrum for C-1 at $\delta = 102.78$ ppm ((1 → 4)-linked units in the main chain) and at $\delta = 102.44$ ppm ((1 → 4)-linked units in the main chain substituted at O-2), for C-4 at $\delta = 77.49$ ppm, and C-5 at $\delta = 64.10$ ppm. The signals for C-1 of xylopyranosyl residues and the value of specific optical rotation of the polysaccharide indicated the type of the linkage to be β.

As the 2-*O*- and 3-*O*-methyl derivatives have the same retention time on the SP 2340 stationary phase, they could not be distinguished by GLC. However, the mass spectra of the borodeuteride-reduced and acetylated samples showed that both positional isomers were present [12], the 2-*O*-methyl derivative dominating. These derivatives indicated single branching at O-2 and O-3, while the further methylation product, D-xylose, indicated double substitution at positions

Table 5

^{13}C NMR data of 4-*O*-methyl-D-glucurono-D-xylan

Chemical shift δ/ppm[a]		Assignment
177.31	C=O	4-*O*-methyl-α-D-GlcpA
102.94	C-1	Terminal nonreducing β-D-Xylp
102.78	C-1	(1 → 4)-β-D-Xylp, main chain
102.44	C-1	(1 → 4)-β-D-Xylp substituted at O-2
98.73	C-1	4-*O*-methyl-α-D-GlcpA
83.43[c]	C-4	4-*O*-methyl-α-D-GlcpA
77.95	C-2	(1 → 4)-β-D-Xylp substituted at O-2
77.69	n.a.[b]	
77.49	C-4	(1 → 4)-β-D-Xylp, main chain
77.15	n.a.[b]	
76.87	C-3	Terminal nonreducing β-D-Xylp
74.89	C-3	(1 → 4)-β-D-Xylp, main chain
73.92	C-2	(1 → 4)-β-D-Xylp, main chain
73.44	C-2	Terminal nonreducing β-D-Xylp
73.31	n.a.[b]	
72.89	C-3	4-*O*-methyl-α-D-GlcpA
72.43	C-2	4-*O*-methyl-α-D-GlcpA
70.41	C-4	Terminal nonreducing β-D-Xylp
66.37	C-5	Terminal nonreducing β-D-Xylp
64.10	C-5	(1 → 4)-β-D-Xylp, main chain
63.76	n.a.[b]	
61.02	OCH_3	

a) Relative to methanol as the internal reference ($\delta = 50.15$ ppm); *b*) not assigned; *c*) comprises probably the signal for C-3 of (1 → 4)-β-D-Xylp substituted at O-3.

O-2 and O-3. The branching at C-2 of xylopyranosyl units is indicated also by the shift of the signal for substituted C-2 from $\delta = 73.44$ ppm to $\delta = 77.95$ ppm. According to [19], the signals corresponding to C-3 of O-3 substituted β-D-xylopyranosyl residues appear at $\delta = 82$—85 ppm. In our case the only signal appearing in this region of the spectrum was the one at $\delta = 83.43$ ppm, assigned to C-4 of 4-*O*-methyl-D-glucuronic acid units. As the O-3 substitution was proved in methylation analysis and considering the upfield shifts due to substitutions at O-2 and O-4, we assume that the signal mentioned above comprises probably also the signal for C-3 of (1 → 4)-linked xylopyranosyl residues substituted at O-3. Since the sum of branch points in Table 4 (0.07 moles simple substitution + 0.14 × 2 moles double substitution) is 0.35 moles, the polysaccharide should contain theoretically the same amount of terminal nonreducing units attached to the above-mentioned positions of (1 → 4)-linked xylosyl units. 2,3,4-Tri-*O*-methyl-D-xylose as the further methylation product represented

0.18 moles (Table 4). The remaining 0.17 moles of branch points should, therefore, carry terminal 4-*O*-methyl-D-glucuronic acid units. Considering the total amount of 4-*O*-methyl-D-glucuronic acid in the polysaccharide, one unit of uronic acid against 7.09 units of D-xylose (Table 3), the theoretical amount of 4-*O*-methyl-D-glucuronic acid 0.18 moles fits quite well this assumption. The assumption is supported by chemical shifts of the signals belonging to carbon atoms of 4-*O*-methyl-D-glucuronic acid units. The signal for C-1 of α-D-glucuronic acid at $\delta = 93.2$ ppm [17] was shifted in the spectrum to $\delta = 98.73$ ppm indicating the involvement of this carbon in a linkage. The only other signal indicating a substitution was that for C-4 at $\delta = 83.43$ ppm (against $\delta = 72.4$ ppm in free uronic acid [17]). This carbon carried the methoxyl group, the signal for which appeared at $\delta = 61.02$ ppm. The aldobiuronic acid, 2-*O*-(4-*O*-methyl-α-D-glucopyranuronosyl)-D-xylose, identified in the hydrolyzate of the original polysaccharide indicates that the uronic acid units are attached to O-2 of the (1 → 4)-linked D-xylopyranosyl residues. This is in agreement with the literature data [20, 21] and general knowledge on glucuronoxylans that the uronic acid units are usually linked to O-2 of the xylan backbone.

On the basis of the results mentioned above it can be concluded that the 4-*O*-methyl-D-glucurono-D-xylan is built of (1 → 4)-linked β-D-xylopyranosyl residues, ≈ 70 % being unsubstituted, ≈ 5 % carrying a single substitution at O-2 and/or O-3, and ≈ 11 % being doubly branched at O-2 and O-3. The 4-*O*-methyl-D-glucopyranuronosyl units are attached to O-2 as single terminal residues (1 unit per 7 xylose units).

References

1. Tomoda, M., Shimizu, N., Suzuki, H., and Takasu, T., *Chem. Pharm. Bull. 29*, 2277 (1981).
2. Tomoda, M., Satoh, N., and Shimada, K., *Chem. Pharm. Bull. 28*, 824 (1980).
3. Shimizu, N., Tomoda, M., and Adachi, M., *Chem. Pharm. Bull. 34*, 4133 (1986).
4. Tomoda, M., Gonda, R., Shimizu, N., Nakanishi, S., and Hikino, H., *Phytochemistry 26*, 2297 (1987).
5. Capek, P., Toman, R., Kardošová, A., and Rosík, J., *Carbohydr. Res. 117*, 133 (1983).
6. Capek, P., Toman, R., Rosík, J., and Kardošová, A., *Collect. Czechoslov. Chem. Commun. 49*, 2674 (1984).
7. Capek, P., Rosík, J., Kardošová, A., and Toman, R., *Carbohydr. Res. 164*, 443 (1987).
8. Kardošová, A., Rosík, J., Toman, R., and Capek, P., *Collect. Czechoslov. Chem. Commun. 48*, 2082 (1983).
9. Bitter, T. and Muir, H., *Anal. Biochem. 4*, 330 (1962).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem. 193*, 265 (1951).
11. Viebock, F. and Brecher, C., *Ber. 63B*, 3207 (1930).
12. Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B., and Lönngren, J., *Chem. Commun.* (Stockholm) *8*, 1—75 (1976).
13. Ciucanu, I. and Kerek, F., *Carbohydr. Res. 131*, 209 (1984).

14. Hromádková, Z., Ebringerová, A., and Petráková, E., *Carbohydr. Res. 163*, 73 (1987).
15. Hirsch, J., Kováč, P., and Petráková, E., *Carbohydr. Res. 106*, 203 (1982).
16. Hirsch, J., Petráková, E., and Schraml, J., *Carbohydr. Res. 131*, 219 (1984).
17. Utille, J. P., Kováč, P., Sauriol, F., and Perlin, A. S., *Carbohydr. Res. 154*, 251 (1986).
18. Brillouet, J. M. and Joseleau, J. P., *Carbohydr. Res. 159*, 109 (1987).
19. Kováč, P., Hirsch, J., Shashkov, A. S., Usov, U. I., and Yarotsky, S. V., *Carbohydr. Res. 85*, 177 (1980).
20. Carpita, N. C. and Whittern, D., *Carbohydr. Res. 146*, 129 (1986).
21. Wilkie, K. C. B., *Adv. Carbohydr. Chem. Biochem. 36*, 215 (1979).

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