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Plant Mucilages. XV.[1] The Main Structural Features of the Backbone Chain of Paniculatan

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Paniculatan, the mucous polysaccharide isolated from the inner barks of *Hydrangea paniculata* SIEB., was found to be composed of L-rhamnose: D-galactose: D-galacturonic acid: D-glucuronic acid: 4-O-methyl-D-glucuronic acid in the approximate molar ratio of 4: 4: 3: 2: 5. The mucilage has been subjected to the reduction of carboxyl groups and to controlled Smith degradation. As the results of methylation analysis of these products and the original polysaccharide, possible structures of the backbone chain were proposed. The chain is composed of 1→2 linked L-rhamnopyranose residues having branches at position 4 and 1→4 linked D-galactopyranosyluronic acid residues having branches at position 3 in the approximate molar ratio of 2: 1.

Keywords——mucous substance from *Hydrangea paniculata*; reduction of the polysaccharide; methylation analysis; gas–liquid chromatography–mass spectrometry; controlled Smith degradation; possible backbone structures

The representative mucous substance obtained from the inner bark of *Hydrangea paniculata* SIEB., named paniculatan, has been investigated in this laboratory.[3] The substance is an acidic polysaccharide having a high branching structure. All rhamnose units form the "backbone" chain, whereas all 4-O-methyl-glucuronic acid residues occupy the terminals in it.

The present work was undertaken to perform the reduction and the methylation analysis of paniculatan, and the controlled Smith degradation product of the original polysaccharide was also studied by the reduction and the methylation analysis. In the previous work,[3] we overlooked the presence of glucuronic acid as one of the component sugars. This failure of analysis caused the erroneous conclusion regarding a part of the terminals of the molecule. The previous structural assumption is corrected and the features of the backbone chain in the polysaccharide are discussed in this paper.

The carboxyl groups of uronic acid residues in the polysaccharide were reacted with a carbodiimide reagent, then reduced with sodium borohydride to the corresponding neutral sugar units.[4] Quantitative determination of the component sugars of the carboxyl-reduced polysaccharide was carried out by gas–liquid chromatography (GLC) of alditol acetates derived from the hydrolyzate. The results showed that the sample contained rhamnose, galactose, glucose, and 4-O-methyl-glucose in the molar ratio of 4.0: 7.6: 2.3: 5.0. The analysis of the component sugars of the original polysaccharide showed that the proportion of rhamnose: galactose: hexuronic acids was 4.0: 4.1: 10.3. The component sugars were also analyzed by cellulose thin-layer chromatography (TLC). From these results, it can be concluded that paniculatan is composed of L-rhamnose: D-galactose: D-galacturonic acid: D-glucuronic acid: 4-O-methyl-D-glucuronic acid in the approximate molar ratio of 4: 4: 3: 2: 5. The component sugars were isolated and elucidated to have the configurations given

1) Part XIV: M. Tomoda, S. Kaneko, C. Ohmori, and T. Shiozaki, *Chem. Pharm. Bull.* (Tokyo), **24**, 2744 (1976).
2) Location: *1-5-30, Shibakôen, Minato-ku, Tokyo, 105, Japan.*
3) M. Tomoda and N. Satoh, *Chem. Pharm. Bull.* (Tokyo), **24**, 230 (1976).
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above as described in a previous paper.[3] O-Acetyl groups are present in a part of the component sugar residues also as described in the previous paper.[3]

The methylations of the original and the carboxyl-reduced polysaccharide were performed with methylsulfinylmethyl sodium and methyl iodide in dimethyl sulfoxide.[5] The fully methylated products were successively hydrolyzed with formic acid and dilute sulfuric acid. The hydrolyzates were analyzed by gas–liquid chromatography–mass spectrometry (GLC–MS) after conversion to alditol acetates.[6,7] As shown in Fig. 1, the hydrolysis products of the fully methylated carboxyl-reduced polysaccharide were identified as 3-mono-O-methyl-L-rhamnopyranose, 2,3,6-tri-O-methyl-D-galactopyranose, 2,6-di-O-methyl-D-galactopyranose, and 2,3,4,6-tetra-O-methyl-D-glucopyranose, and they were obtained in the molar ratio of 4.0: 4.3: 3.0: 6.5. Methyl ethers of the hexuronic acids were removed from the hydrolyzate of methylated paniculatan by treatment with an anion-exchange resin, and as shown in Fig. 2, the residual products were identified as 3-mono-O-methyl-L-rhamnopyranose, 2,3,6-tri-O-methyl-D-galactopyranose, and 2,6-di-O-methyl-D-galactopyranose. They were obtained in the molar ratio of 4.0: 3.2: 1.2.

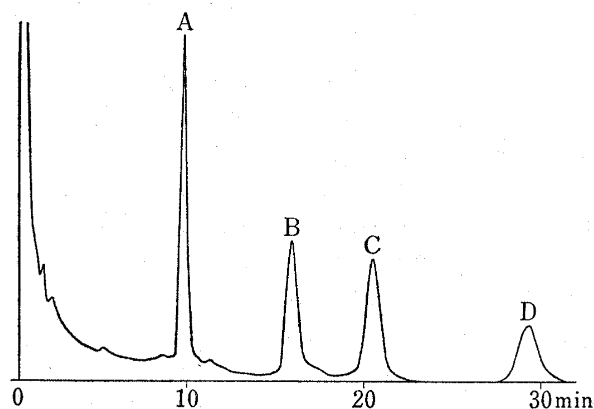

Fig. 1. Gas–Liquid Chromatogram of O-Methylated Alditol Acetates obtained from the Methylated Carboxyl-reduced Polysaccharide

The conditions are described in the Experimental.
peak A: 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol
peak B: 1,2,4,5-tetra-O-acetyl-3-mono-O-methyl-L-rhamnitol
peak C: 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-galactitol
peak D: 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-galactitol

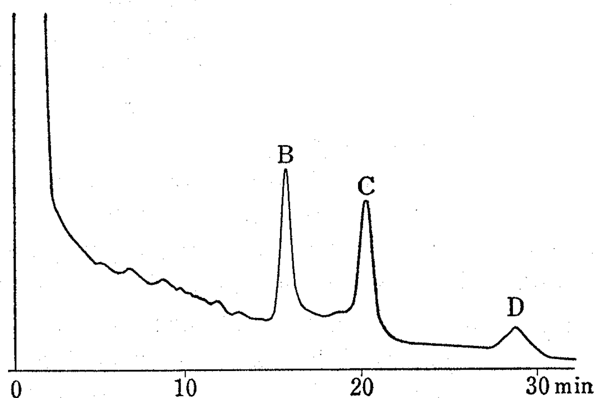

Fig. 2. Gas–Liquid Chromatogram of O-Methylated Alditol Acetates obtained from the Methylated Original Polysaccharide

The conditions and peaks B to D are the same as those of Fig. 1.

These results suggested that the minimal repeating unit of paniculatan was composed of seven kinds of the component sugar units as shown in Chart 1.

Paniculatan was subjected to periodate oxidation, and after stopping the reaction by addition of ethylene glycol, the product was reduced with sodium borohydride.[8] The selective cleavage of acetal linkages was achieved by mild hydrolysis with dilute sulfuric acid. The controlled Smith degradation product was isolated by the gel chromatography using Sephadex G–15. None of the component sugars was detected in the low molecular weight fraction. The controlled Smith degradation product gave single spot on glass-fiber paper electrophoresis in alkaline borate buffer, and it showed a positive specific rotation ($[\alpha]_D^{23}+3.7°$ in H_2O, $c=2.4$). The value of 10000 as the molecular weight of it was estimated from the calibration curve[9] obtained by the gel chromatography using Sephadex G–200.

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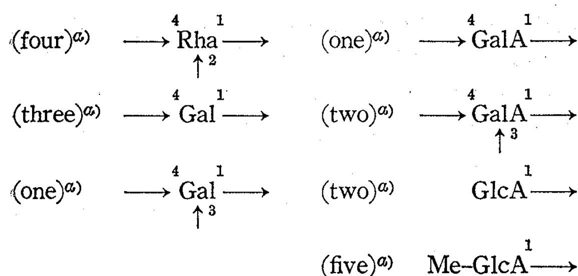

Chart 1. Component Sugar Residues in the Minimal Repeating Unit in the Structure of Paniculatan

a) number of residues
Rha: L-rhamnopyranose
Gal: D-galactopyranose
GalA: D-galactopyranosyluronic acid
GlcA: D-glucopyranosyluronic acid
Me-GlcA: 4-O-methyl-D-glucopyranosyluronic acid

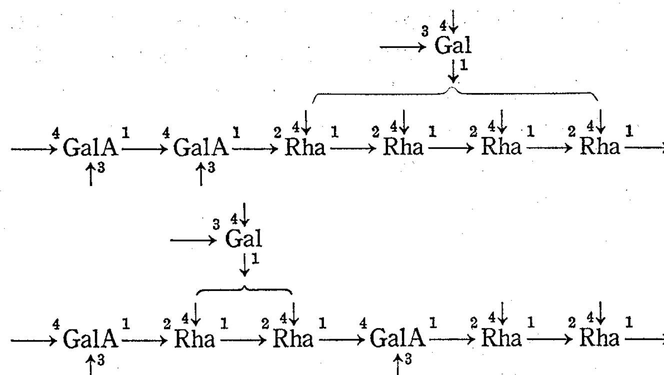

Chart 2. Two Possible Backbone Structures of Paniculatan

Analysis of its hydrolyzate showed that the product was composed of rhamnose: galactose: galacturonic acid in the molar ratio of 2.0: 0.6: 1.0.

In addition, the product was reduced with sodium borohydride after treatment with methanol and Dowex 50W (H^+), and the carboxyl-reduced derivative was obtained. The controlled Smith degradation product and its carboxyl-reduced derivative were fully methylated[5] and the hydrolyzates of them were analyzed by GLC–MS as described above. As the hydrolyzates of the carboxyl-reduced product, 3,4-di-O-methyl-L-rhamnopyranose, 3-mono-O-methyl-L-rhamnopyranose, 2,3,4,6-tetra-O-methyl-D-galactopyranose, 2,3,6-tri-O-methyl-D-galactopyranose, and 2,6-di-O-methyl-D-galactopyranose were identified and obtained in the molar ratio of 2.8: 1.0: 1.1: 1.4: 0.2. It is conceivable that the incomplete cleavage of acetal linkages at controlled Smith degradation caused the appearance of 2,6-dimethyl-galactose. The methyl ethers of the neutral sugar components of the methylated controlled Smith degradation product were also identified by GLC–MS to be 3,4-di-O-methyl-L-rhamnopyranose, 3-mono-O-methyl-L-rhamnopyranose, and 2,3,4,6-tetra-O-methyl-D-galactopyranose in the molar ratio of 2.7: 1.0: 1.2.

Owing to these results, it can be presumed that the backbone chain in the mucous polysaccharide is composed of rhamnose and galacturonic acid in the approximate molar ratio of 2: 1. The chain must be composed of 1→2 linked L-rhamnopyranose residues and 1→4 linked D-galactopyranosyluronic acid residues, and it has branches at position 4 of each rhamnose and at position 3 of each galacturonic acid. On the other hand, all glucuronic acid and all 4-O-methyl-glucuronic acid units are located on the terminals of the molecule. All galactose and about one third of galacturonic acid units form the intermediates in the branching chains. About one fourth of galactose units must form the other branched points by 1→3 and 1→4 linkages, and these galactose units are present in part of the controlled Smith degradation product in addition to rhamnose and galacturonic acid units in the backbone chain. Thus possible backbone structures of paniculatan can be shown in Chart 2.

The presence of the component unit composed of 1→2 linked L-rhamnopyranose and 1→4 linked D-galactopyranosyluronic acid in the main parts of mucous polysaccharides has been reported in the cases of the gums from *Khaya grandifolia*,[10] *Khaya senegalensis*,[11] and *Sterculia urens*,[12] and of the mucilages from cotyledon meals,[13] lemon-peels,[14] inner barks

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of *Ulmus fulva*,[15] and rapeseed hulls.[16] In the majority of these examples, though several oligosaccharides were obtained as the partial hydrolyzates, the whole structures of the polysaccharides have not been elucidated yet. Paniculatan should be added to the similar group as in these acidic heteroglycans, and this mucilage occupies a relatively unique position for its very high branching structure and for the presences of three kinds of hexuronic acids among the rest. The whole structure of the polysaccharide will be reported in a following paper.

Experimental

Solutions were concentrated at or below 40° with rotary evaporators under reduced pressure. Optical rotation was measured with JASCO model DIP-SL automatic polarimeter. GLC was carried out by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ionization detector. GLC–MS was performed by the use of JEOL model JGC-20K gas chromatograph and JEOL model JMS-D100 mass spectrometer.

Reduction of Paniculatan——Ammonium salt of paniculatan (100 mg) was dissolved in water (30 ml), then 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-*p*-toluenesulfonate (1 g) was added into this solution. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1N hydrochloric acid under stirring for 2 hr. Then 2M sodium borohydride (10 ml) was added slowly into the reaction mixture during 4 hr and the pH was maintained at 7.0 by titration with 4N hydrochloric acid under stirring at room temperature. The solution was dialyzed against running water overnight, then the non-dialyzable fraction was concentrated up to 30 ml. The product was reduced again under the same conditions. The final non-dialyzable fraction was applied to a column (5×53 cm) of Sephadex G-15 (Pharmacia Co.). The column was eluted with water, and fractions were collected at 20 ml. The eluates obtained from tubes 18 to 27 were combined, concentrated and lyophilized. Yield, 79.2%.

Qualitative Analysis of Component Sugars——Each sample was hydrolyzed with 2N sulfuric acid in a sealed tube at 100° for 6 hr followed by neutralization with barium carbonate. The solutions of the hydrolyzates containing hexuronic acids were finally passed through a small column of Dowex 50W-X8 (H^+). The hydrolyzate was applied to TLC using Avicel SF cellulose and following two solvent systems: A, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3); B, C_6H_5OH: 1% NH_4OH (4: 1). Component sugars were revealed with *p*-anisidine hydrochloride reagent[17] and silver nitrate reagent.[18] *Rf* values on TLC are shown in Table I. Specific rotation of D-glucuronic acid (lactone) in water at 21° was +21.5°. Those of the other component sugars have already been reported in the previous paper.[3]

TABLE I. *Rf* Values of Components on TLC and Retention Times (min) of Their Derivatives on GLC

	Cellulose TLC (*Rf*)		GLC (t_R) Condition A
	Solvent A	Solvent B	
Rhamnose	0.77	0.60	
4-O-Methyl-glucose	0.74	0.69	
Glucose	0.52	0.29	
Galactose	0.47	0.36	
4-O-Methylglucuronic acid	0.35	0.19	
Glucuronolactone	0.86	0.58	
Glucuronic acid	0.23	0.09	
Galacturonic acid	0.18	0.10	
Rhamnitol acetate			5.2
4-O-Methylglucitol acetate			18.0
Galactitol acetate			19.0
Glucitol acetate			21.2

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18) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature*, **166**, 444 (1950).

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Determination of Component Sugars——The sample was hydrolyzed with 2N sulfuric acid at 100° for 6 hr followed by neutralization with Dowex 2 (OH^-). The filtrate was reduced with sodium borohydride for 1 hr. After neutralization with Dowex 50W-X8 (H^+), the filtrate was evaporated and boric acid was removed by repeated addition and evaporation of methanol. Then the product was acetylated with acetic anhydride–pyridine mixture (1: 1) at 100° for 20 min. After evaporation of the solution, the residue was dissolved in chloroform–methanol mixture (1: 1) and applied to GLC. GLC was carried out under condition A, a column (0.3 cm × 2 m long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 210° with a flow of 30 ml per min of nitrogen. Xylose was used as an internal standard. Retention times of alditol acetates are given in Table I. Hexuronic acids were determined by modified carbazole method[19] and the value was calculated on the assumption that the molar ratio of galacturonic acid: glucuronic acid: 4-O-methyl-glucuronic acid is 3: 2: 5. The results of determination are shown in Table II.

Table II. Sugar Compositions (%) of the Polysaccharide and Its Derivatives

	Original polysaccharide	Carboxyl-reduced polysaccharide	Smith degradation product
Rhamnose	18.7	18.9	50.6
Galactose	20.3	39.7	18.4
Glucose	—	12.0	—
4-O-Methyl-glucose	—	29.3	—
Galacturonic acid	59.0[a]	—	31.0
Glucuronic acid		—	—
4-O-Methylglucuronic acid		—	—

a) total hexuronic acid value

Methylation——Sodium hydride (100 mg) was mixed with dimethyl sulfoxide (10 ml) and the mixture was stirred at 70° for 1 hr. The sample (50 mg) was dissolved in dimethyl sulfoxide (35 ml) and the solution of methylsulfinylmethyl sodium was added into this mixture. After stirring at room temperature for 5 hr, methyl iodide (5 ml) was added and the mixture was stirred overnight at room temperature. All procedures were carried out in nitrogen atmosphere. After dilution with water (150 ml), the mixture was extracted with chloroform (30 ml each) four times. The combined extract was washed with water (150 ml each) five times, then dried over sodium sulfate and the filtrate was evaporated. The residue was methylated three more times under the same condition. The IR spectrum of the final product had no absorption near 3400 cm^{-1}.

Analysis of the Methylated Products——A part of the product was successively treated with 90% formic acid at 90° for 16 hr and 0.5N sulfuric acid at 100° for 2.5 hr. After neutralization with Dowex 2 (OH^-), the hydrolyzate was reduced with sodium borohydride, then acetylated with acetic anhydride–pyridine mixture as described above. GLC of partially methylated alditol acetates was carried out under condition A′, the same column as condition A but at 180° with a flow of 30 ml per min of nitrogen. GLC–MS was carried out under condition A′ using helium as carrier gas. Relative retention times of the products to 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol and fragments of them in the mass spectra are shown in Table III.

Table III. Relative Retention Times on GLC and Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention times[a] Condition A′	Fragments
2,3,4,6-Me-1,5-Ac-D-glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
2,3,4,6-Me-1,5-Ac-D-galactitol	1.17	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
3-Me-1,2,4,5-Ac-L-rhamnitol		43, 87, 101, 129, 143, 189, 203
2,3,6-Me-1,4,5-Ac-D-galactitol		43, 45, 87, 99, 101, 113, 117, 233
2,6-Me-1,3,4,5-Ac-D-galactitol		43, 45, 87, 117, 129

a) relative to 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol
abbreviations: Me=methyl; Ac=acetyl
(*e.g.*, 2,3,4,6-Me-1,5Ac-=2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl)

19) T. Bitter and H. Muir, *Anal. Biochem.*, 4, 330 (1962).

NII-Electronic Library Service

Controlled Smith Degradation——The mucilage (100 mg) was oxidized with 0.05M sodium metaperiodate (50 ml) at 5° for 30 days in a dark place. The periodate consumption was measured by a spectrophotometric method[20] and the value of 0.95 mole per one mole of the average component anhydro sugar unit of the polysaccharide was obtained. The reaction mixture was neutralized with 0.1N sodium hydroxide after addition of ethylene glycol (1 ml), then filtered and concentrated *in vacuo*. A precipitate was obtained by addition of ethanol, then dissolved in water (12 ml) and reduced with sodium borohydride (500 mg) at 5° for 16 hr followed by addition of acetic acid up to pH 5. The solution was applied to a column (5×83 cm) of Sephadex G-15 (Pharmacia Co.). Fractions of 50 ml were collected and analyzed by phenol–sulfuric acid method.[21] The eluates obtained from tubes 11 to 14 were combined, concentrated up to 10 ml and hydrolyzed with 1N sulfuric acid at room temperature for two days. After neutralization with barium carbonate, the filtrate was passed through a small column of Dowex 50W-X8 (H^+). The eluate was applied to a column (5×83 cm) of Sephadex G-15 as described above. The eluates obtained from tubes 10 to 12 were combined, concentrated and lyophilized. Yield, 36.6 mg.

Glass-Fiber Paper Electrophoresis——Electrophoresis was carried out with Whatman GF 81 glass-fiber and alkaline borate buffer of pH 9.2 (0.1N sodium hydroxide: 0.025M borax, 1: 10) at the condition of 380 volt for 2 hr in the same manner as a former report[9] of this series. The controlled Smith degradation product gave single spot at a distance of 7.9 cm from the origin toward the cathod.

Determination of Molecular Weight——A column (2.6×96 cm) of Sephadex G-200 (Pharmacia Co.) was prepared and the elution was carried out as described in the first report[22] of this series.

Reduction and Methylation of the Controlled Smith Degradation Product——The sample (2 mg) was mixed with methanol (2 ml) and Dowex 50W-X8 (H^+) (20 mg) and heated at 67° for 24 hr in a sealed tube. After filtration and washing with water and methanol followed by evaporation to dryness, the reaction was similarly repeated. The product was dissolved in water (1 ml), then reduced with sodium borohydride (20 mg) at room temperature for 18 hr followed by addition of Dowex 50W-X8 (H^+) up to pH 4. The filtrate was evaporated and boric acid was removed as described above. Then the product was dissolved in dimethyl sulfoxide (1 ml) and the solution of methylsulfinylmethyl sodium was added. The latter reagent was prepared by mixing with sodium hydride (10 mg) and dimethyl sulfoxide (1 ml) at 70° for 1 hr. The reaction mixture was stirred at room temperature for 4 hr, then methyl iodide (1 ml) was added and the mixture was stirred overnight at room temperature. All procedures were carried out in nitrogen atmosphere. After dilution with water (15 ml), the mixture was extracted with chloroform (15ml each) four times. The combined extract was washed with water (15 ml each) four times, then dried over sodium sulfate and the filtrate was evaporated. The residue was methylated two more times under the same condition. The IR spectrum of the final product had no absorption near 3400 cm^{-1}.

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