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Plant Mucilages. XVII.[1] Partial Hydrolysis and a Possible Structure of Paniculatan[1a]

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Partial acid hydrolysis of paniculatan, the mucous polysaccharide isolated from the inner barks of *Hydrangea paniculata* SIEB., has led to the isolation of five oligosaccharides. Analysis of components, reduction and methylation, and partial degradation studies provided the evidences that they are *O*-α-(4-*O*-methyl-D-glucopyranosyluronic acid)-(1→4)-D-galactopyranose, *O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose, *O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→2)-L-rhamnopyranose, *O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose, and *O*-α-(D-glucopyranosyluronic acid)-(1→3)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose. From the accumulated evidence, a possible structure of the polysaccharide was proposed.

Keywords——mucous polysaccharide from *Hydrangea paniculata*; partial acid hydrolysis; isolation of five oligosaccharides; reduction and methylation analysis; *O*-α-(4-*O*-methyl-D-glucopyranosyluronic acid)-(1→4)-D-galactopyranose; *O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose; *O*-α-(D-glucopyranosyluronic acid)-(1→3)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose; *O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→2)-L-rhamnopyranose; *O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose; a possible structure of the polysaccharide

The mucous polysaccharide from the inner bark of *Hydrangea paniculata* SIEB., named paniculatan, has been isolated and investigated in this laboratory.[3,4] The substance is composed of L-rhamnose, D-galactose, D-galacturonic acid, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid in the approximate molar ratio of 4: 4: 3: 2: 5. The reduction of carboxyl groups, controlled Smith degradation and methylation studies[4] revealed that its backbone chain was 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. In addition, it was confirmed that all D-glucuronic acid and all 4-*O*-methyl-D-glucuronic acid units were located on the terminals of the molecule, and that all D-galactose and about one third of D-galacturonic acid moieties formed the intermediates in the branching chains.

In this paper, the isolations and characterizations of five oligosaccharides as partial acid hydrolyzates of the mucilage are described, and a possible structure of paniculatan is proposed.

Paniculatan was hydrolyzed with 0.5 N sulfuric acid for 2 hr, and the residue was filtered off. The filtrate was neutralized and applied to a column of Sephadex G-15. The polysaccharide fraction obtained by this chromatography and the insoluble material at the first hydrolysis were combined and hydrolyzed again with 1 N sulfuric acid for 2 hr. The products and the low molecular weight fraction obtained by the first hydrolysis were applied individu-

1) Part XVI: M. Tomoda, S. Kaneko, M. Ebashi, and T. Nagakura, *Chem. Pharm. Bull.* (Tokyo), **25**, 1357 (1977). *a*) This work was reported at VIII International Symposium on Carbohydrate Chemistry, Kyoto, August 1976.
2) Location: *1-5-30, Shibakōen, Minato-ku, Tokyo, 105, Japan.*
3) M. Tomoda and N. Satoh, *Chem. Pharm. Bull.* (Tokyo), **24**, 230 (1976).
4) M. Tomoda, N. Satoh, and Y. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **25**, 968 (1977).

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ally to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (formate form), and when necessary, the fractions obtained by the stepwise elution with dilute formic acid were further purified by paper partition chromatography (PPC). Five oligosaccharides (I to V) were isolated. The outline of the preparation of the partial hydrolyzates is shown on Chart 1.

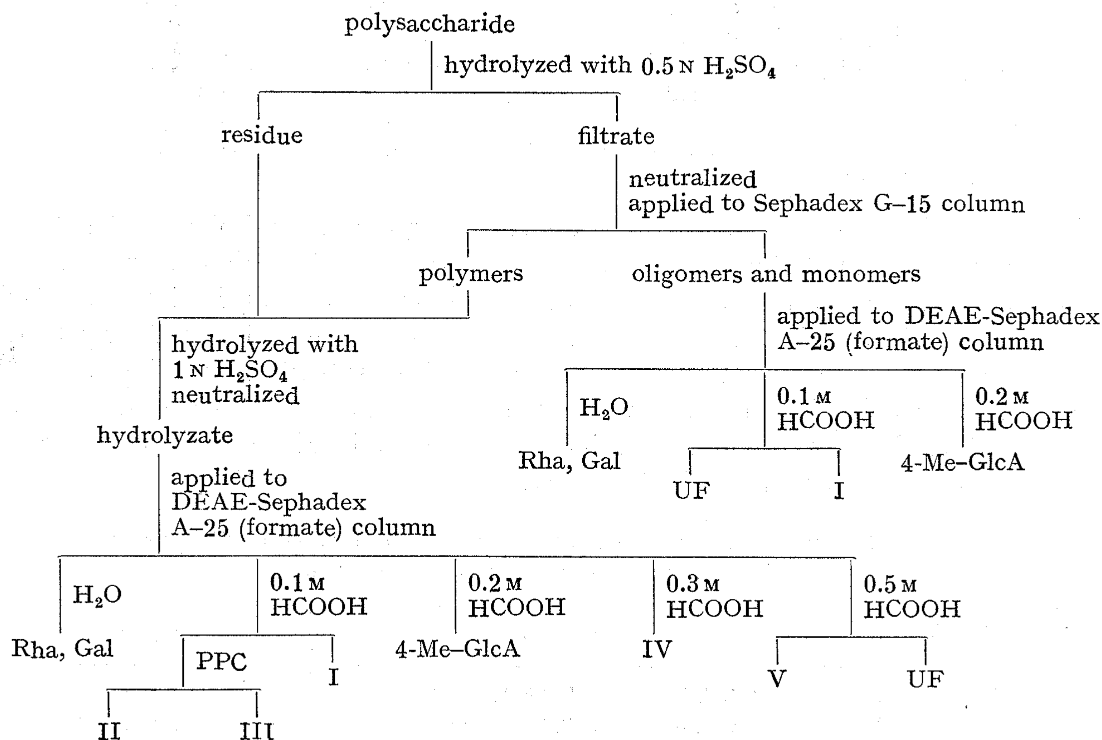

Chart 1. Isolation of Oligosaccharides

Abbreviations: Rha=rhamnose; Gal=galactose; 4-Me-GlcA=4-*O*-methyl-glucuronic acid; UF=unidentified fractions.

The homogeneity of each oligosaccharide was checked by cellulose thin-layer chromatography (TLC) and by paper electrophoresis. Table I gives the R_{GAL} values in TLC and the mobilities in paper electrophoresis.

TABLE I. R_{Gal} Values and Mobilities of Oligosaccharides

Oligosaccharides	Cellulose TLC (R_{Gal})			Paper electrophoresis [distance (cm) from the origin]	
	Solvent A	Solvent B	Solvent C	Buffer A	Buffer B
I	0.36	0.53	0.23	+3.6	+1.2
II	0.44	0.83	0.29	+4.4	+1.8
III	0.31	0.52	0.07	+4.6	+2.1
IV	0.17	0.16	0.10	+5.9	+2.4
V	0.22	0.23	0.13	+6.6	+3.2

For the solvents and buffers, see "Experimental."

The TLC of the hydrolyzates of the oligosaccharides showed their component sugars. Quantitative determinations of the component sugars were carried out by gas-liquid chromatography (GLC) of alditol acetates derived from the hydrolyzate and by a colorimetric method. The results and specific rotations of the oligosaccharides in water are shown in Table II.

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Table II. Specific Rotations and Component Sugars of Oligosaccharides

Oligosaccharides	Specific rotations in water (final values)	Component sugars	Molar ratios
I	$[\alpha]_D^{20}$+140.9° (c=1.0)	4-Me-GlcA: Gal	1.1 : 1.0
II	$[\alpha]_D^{20}$ +96.1° (c=0.4)	GalA: Rha	1.0 : 1.0
III	$[\alpha]_D^{20}$ +80.3° (c=0.3)	GalA: Rha	1.1 : 2.0
IV	$[\alpha]_D^{20}$+109.4° (c=0.3)	GalA: Rha	1.1 : 1.0
V	$[\alpha]_D^{20}$ +79.4° (c=1.2)	GlcA: GalA: Rha	1.1 : 1.1 : 1.0

Abbreviations: Me=methyl; GlcA=D-glucuronic acid; GalA=D-galacturonic acid; Gal=D-galactose; Rha=L-rhamnose.

The oligosaccharides were converted to the corresponding neutral oligosaccharides by the reductions of the methyl esters of the methyl glycosides with sodium borohydride. The methylations of the carboxyl-reduced oligosaccharides 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] The products obtained by hydrolysis of the methylated carboxyl-reduced oligosaccharides are shown in Table III.

Table III. Products in Hydrolyzates obtained from Methylated Carboxyl-reduced Oligosaccharides and Molar Ratios of Them

Oligosaccharides	Products	Molar ratios
Carboxyl-reduced I	2,3,4,6-Me-Glc: 2,3,6-Me-Gal	1.0 : 0.9
Carboxyl-reduced II	2,3,4,6-Me-Gal: 3,4-Me-Rha	1.0 : 1.2
Carboxyl-reduced III	2,3,4,6-Me-Gal: 3,4-Me-Rha	1.0 : 2.1
Carboxyl-reduced IV	2,3,4,6-Me-Gal: 2,3,6-Me-Gal: 3,4-Me-Rha	0.8 : 1.0 : 1.9
Carboxyl-reduced V	2,3,4,6-Me-Glc: 2,4,6-Me-Gal: 3,4-Me-Rha	1.0 : 0.8 : 0.7

Abbreviations: Me=methyl; Glc=D-glucose; Gal=D-galactose; Rha=L-rhamnose (*e. g.*, 2,3,4,6-Me-Glc= 2,3 4 6-tetra-*O*-methyl-D-glucose).

From the results of the methylation analysis the values of specific rotation,[7,8] it can be concluded that I, II, and V are *O*-α-(4-*O*-methyl-D-glucopyranosyluronic acid)-(1→4)-D-galactopyranose, *O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose, and *O*-α-(D-glucopyranosyluronic acid)-(1→3)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose.

Partial hydrolysis of III and IV, and of the controlled Smith degradation product with 1 N sulfuric acid gave II in addition to their component monosaccharides. No other oligosaccharide was detected. Consequently, III and IV are *O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→2)-L-rhamnopyranose and *O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose,[9,10] and no adjacent unit of galacturonic acid exist in the backbone chain of paniculatan. On the basis of the principle of optical superposition, the low value[4] of specific rotation of the controlled Smith degradation product led us to express the anomeric configuration in L-rhamnose moiety as α-glycosidic and that in D-galactose moiety as β-glycosidic. The controlled Smith degradation product showed three anomeric proton signals at

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δ 4.80 (1H, d, J=7 Hz), δ 5.14 (4H, d, J=2 Hz), and δ 5.32 (2H, d, J=3.5 Hz) in its proton magnetic resonance (PMR) spectrum. This result indicates that D-galactose residues are β-linked, and that both L-rhamnose and D-galacturonic acid residues are α-linked.[11,12]

These results indicate that D-glucuronic acid residues combine to position 3 of D-galacturonic acid moieties in the backbone chain[4] of the mucilage. Therefore, the chain is composed of 1→2 linked L-rhamnose units having branches at position 4 and 1→4 linked D-galacturonic acid units having D-glucuronic acid residues at position 3 in the approximate molar ratio of 2:1. It can be presumed that the side chains composed of 4-*O*-α-(4-*O*-methyl-D-glucuronic acid)-D-galactose attach to position 4 of L-rhamnose units in the backbone chain. Owing to the results of methylation analysis and Smith degradation of the whole polysaccharide,[4] it seems most probable, although not actually proved, that the branches composed of 4-*O*-α-(4-*O*-methyl-D-glucuronic acid)-D-galacturonic acid attach to position 3 of about one fourth of D-galactose moieties in the side chains. The present study also demonstrates that the residues of L-rhamnopyranose, D-galactopyranosyluronic acid, D-glucopyranosyluronic acid and 4-*O*-methyl-D-glucopyranosyluronic acid are α-linked, and that D-galactopyranosyl residue is β-linked. From the accumulated evidence, a possible structure of paniculatan can be shown in Chart 2.

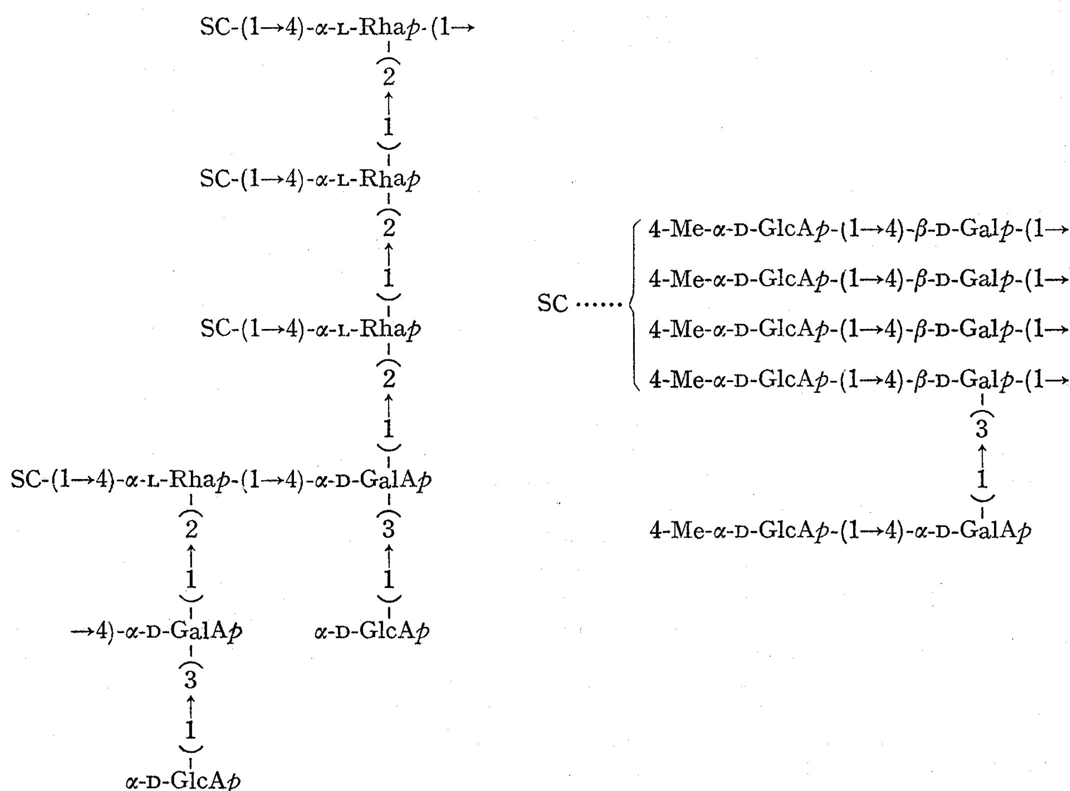

Chart 2. A Possible Structural Fragment of Paniculatan

Abbreviation: SC=side chains.

The presence of the side chain composed of 4-*O*-α-(4-*O*-methyl-D-glucuronic acid)-D-galactose has been reported in the cases of the gums from *Khaya grandifolia*,[13] *Khaya senegalensis*,[7,14] and *Acacia drepanolobium*.[15] Regarding to the manner of the main chain,

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several examples of the unit of 2-*O*-α-(D-galacturonic acid)-L-rhamnose in the chains have been reported in the cases of the gums from *Khaya grandifolia*,[13] *Khaya senegalensis*,[14] and *Sterculia urens*,[8] and the mucilages from cotyledon meals,[9] lemon-peels,[10] leaves and stems of *Medicago sativa*,[16] inner barks of *Ulmus fulva*,[17] and rapeseed hulls.[18] Most of them[9,10,13,14,16,18] also possess the unit of 4-*O*-α-(D-galacturonic acid)-D-galacturonic acid. But in this study, any adjacent galacturonic acid unit was not found. As already reported partly in a previous paper,[4] paniculatan is a relatively unique polysaccharide for its high branching structure and for the possession of three kinds of hexuronic acids.

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 eqipped 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. PMR spectrum was recorded with JEOL model MH-100 NMR spectrometer in heavy water at 70°.

Partial Hydrolysis and Isolation of Oligosaccharides——The polysaccharide (2.94 g) was suspended in 0.5 N sulfuric acid (600 ml) and heated under reflux at 100° for 2 hr. An insoluble material was filtered off, washed with water, then the filtrate and washings were combined and neutralized with barium carbonate. The solution was passed through a column (2×12 cm) of Dowex 50W-X8 (H^+). The eluate was concentrated and applied to a column (5×84 cm) of Sephadex G-15 (Pharmacia Co.). Fractions of 50 ml were collected and analyzed by phenol–sulfuric acid method.[19] The eluates obtained from tubes 10 to 13 were combined, concentrated and added to the insoluble material described above. The mixture was used as the sample for the secondary partial hydrolysis. The eluates obtained from tubes 15 to 21 were combined, concentrated and applied to a column (1.5×40 cm) of DEAE-Sephadex A-25 (formate form, Pharmacia Co.). The column was eluted successively with water (100 ml), 0.05 M formic acid (100 ml), 0.1 M formic acid (360 ml), and 0.2 M formic acid (300 ml). Fractions of 10 ml were collected and analyzed by phenol-sulfuric acid method. The eluates obtained from the column were divided into four groups: Frac. 1a, tubes 6 to 10; Frac. 2a, tubes 33 to 41; Frac. 3a, tubes 42 to 53; Frac. 4a, tubes 72 to 82. The yields were 173.8 mg in Frac. 1a, 36.9 mg in Frac. 2a, 642.5 mg in Frac. 3a, and 41.0 mg in Frac. 4a. I was obtained directly from Frac. 3a. Fracs. 1a and 4a contain rhamnose, galactose, and 4-O-methyl-glucuronic acid. Frac. 2a was a mixture of unidentified substances.

On the other hand, the sample for the secondary partial hydrolysis was suspended in 1 N sulfuric acid (420 ml) and heated under reflux at 100° for 2 hr. After neutralization with barium carbonate, the filtrate was passed through a column (2×8 cm) of Dowex 50W-X8 (H^+). The eluate was concentrated and applied to a column (1.5×34 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (100 ml), 0.05 M formic acid (100 ml), 0.1 M formic acid (300 ml), 0.2 M formic acid (230 ml), 0.3 M formic acid (140 ml), 0.4 M formic acid (200 ml), 0.5 M formic acid (220 ml), and 1 M formic acid (220 ml). Fractions of 10 ml were collected and analyzed as described above. The eluates obtained from the column were divided into seven groups: Frac. 1b, tubes 4 to 10; Frac. 2b, tubes 38 to 42; Frac. 3b, tubes 43 to 48; Frac. 4b, tubes 63 to 73; Frac. 5b, tubes 74 to 83; Frac. 6b, tubes 111 to 121; Frac. 7b, tubes 122 to 129. The yields were 45.8 mg in Frac. 1b, 19.9 mg in Frac. 3b, 17.9 mg in Frac. 4b, 13.1 mg in Frac. 5b, 37.6 mg in Frac. 6b, and 11.5 mg in Frac. 7b. Frac. 1b and 4b contain rhamnose, galactose, and 4-*O*-methyl-glucuronic acid. Frac. 2b was further applied to PPC. PPC was carried out by ascending method using Tôyô-Roshi No. 50 and with solvent A, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3). II was obtained from a part showing *Rf* value of 0.54 and III was obtained from a part showing *Rf* value of 0.44. The yields were 14.0 mg in II and 13.2 mg in III. I, IV, and V were obtained directly from Fracs. 3b, 5b, and 6b. Frac. 7b was a mixture of unidentified substances.

TLC and Paper Electrophoresis——TLC was carried out using Avicel SF cellulose and the following two solvent systems in addition to solvent A: B, AcOEt: AcOH: HCOOH: H_2O (18: 3: 1: 4); C, BuOH: pyridine: H_2O (6: 4: 3). Electrophoresis was carried out using Tôyô-Roshi No. 51 (12×38 cm long) and the following two buffers: A, 0.025 M borax: 0.1 N sodium hydroxide (10: 1, pH 9.2); B, 1 M acetic acid: pyridine (100: 1, pH 3.7). The condition of 570 volts for 2 hr was used. Samples were applied in line at the center. The inside of the apparatus was cooled with dry ice. Sugars were revealed with *p*-anisidine hydrochloride[20]

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and silver nitrate[21] reagents in both TLC and paper electrophoresis. Table IV gives the *Rf* values of the component sugars in TLC and the mobilities of them in paper electrophoresis.

TABLE IV. *Rf* Values and Mobilities of Component Sugars

	Cellulose TLC (*Rf*)			Paper electrophoresis [distance (cm) from the origin]	
	Solvent A	Solvent B	Solvent C	Buffer A	Buffer B
Rhamnose	0.77	0.54	0.65	−1.1	−0.3
Galactose	0.47	0.19	0.35	−1.2	−0.3
4-*O*-Methyl-glucuronic acid	0.35	0.59	0.20	+5.7	+3.4
(Glucuronolactone)	0.86	0.64	0.74	—	—
Glucuronic acid	0.23	0.24	0.12	+6.3	+3.6
Galacturonic acid	0.18	0.17	0.08	+6.2	+2.7

Determination of Component Sugars——The sample was hydrolyzed with 2 N 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 40 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 (min) of rhamnitol acetate, galactitol acetate, and xylitol acetate were 5.2, 19.0, and 8.9. Hexuronic acids were determined by modified carbazole method,[22] and in the case of V, the values were calculated on the assumption of the presences of equimolar galacturonic acid and glucuronic acid based on the result of methylation analysis.

Reduction and Methylation of Oligosaccharides——Each sample (1 mg) was mixed with methanol (1 ml) and Dowex 50W-X8 (H^+) (10 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 (10 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 (2.5 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 (15 ml 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.

TABLE V. Relative Retention Times on GLC and Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention times[a] Condition A′	Main fragments (*m/e*)
2,3,4,6-Me-1,5-Ac-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
3,4-Me-1,2,5-Ac-L-Rhamnitol	0.87	43, 89, 129, 131, 189
2,3,4,6-Me-1,5-Ac-D-Galactitol	1.17	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
2,4,6-Me-1,3,5-Ac-D-Galactitol	1.95	43, 45, 87, 101, 117, 129, 161
2,3,6-Me-1,4,5-Ac-D-Galactitol	2.09	43, 45, 87, 99, 101, 113, 117, 233

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-1,5-Ac=2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-).

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

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Analysis of Methylated Products——The product was successively treated with 87% formic acid at 100° for 1 hr and 0.5 N sulfuric acid at 100° for 6 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 main fragments of them in the mass spectra are shown in Table V.

Further Partial Hydrolysis and Analysis——Each of III, IV, and the controlled Smith degradation product (1 mg) was dissolved in 1 N sulfuric acid (0.1 ml) and heated in a sealed tube at 100° for 1 hr. After neutralization with barium carbonate followed by treatment with Dowex 50W-X8 (H^+), the hydrolyzate was analyzed by TLC and paper electrophoresis as described above.

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