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Studies on Fungal Polysaccharides. XXVII.[1] Structural Examination of a Water-soluble, Antitumor Polysaccharide of *Ganoderma lucidum*[2]

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A water-soluble, antitumor polysaccharide GL-1, $[\alpha]_D^{20}$ +26° (c=1, water), M.W. 40000, was isolated from the fruit bodies of *Ganoderma lucidum* (Rokkakushi, a kind of Reishi). GL-1 consists of glucose, xylose and arabinose in the molar ratio of 18.8: 1.5: 1.0. Structural examination was carried out by periodate oxidation, Smith degradation, methylation analysis, partial acid hydrolysis and α-amylase treatment.

It is concluded that (1) GL-1 is a branched arabinoxyloglucan, (2) GL-1 contains backbone and side-chains involving D-glucopyranosyl (1→4)-α- and -β-, (1→6)-β- and (1→3)-β-linkages, (3) arabinose is present as a part of the non-reducing terminal residues, and (4) xylose may be present as a part of the side-chain. GL-1 strongly inhibited the growth of Sarcoma 180 solid-type tumor (inhibition ratio, 95.6—98.5%) when injected *i.p.* (20 mg/kg) for 10 days. Mild acid hydrolysis and α-amylase treatment of GL-1 had no effect on the antitumor activity. These results suggest that the essential structure for the antitumor activity of GL-1 is a branched glucan core (GL-3) involving (1→3)-β-, (1→4)-β- and (1→6)-β-linkages.

Keywords——*Ganoderma lucidum*; Reishi; Rokkakushi; partial hydrolysis; methylation analysis; α-amylase treatment; branched arabinoxyloglucan; antitumor effect

Fruit bodies of a fungus, *Ganoderma lucidum* (Leyss. ex Fr.) Karst, (Polypolaceae) are well known as a crude drug "Reishi" (Chinese name "Ling zhi cao") used to treat hepato-pathy, chronic hepatitis, nephritis, gastric ulcer, hypertension, arthritis, neurasthenia, insomnia, bronchitis, asthma and poisoning.[3] Nowadays, in China, it is also used for leukopenia.[4]

As a part of structural and biological studies on the polysaccharides in fungal crude drugs, a water-soluble polysaccharide component of the fungus was investigated.

An ethanol precipitate obtained from the non-dialyzable fraction of hot water extract of the dried fruit bodies contained 64.5% neutral sugar[5] (as glucose) and 21.3% protein[6] (as bovine serum albumin). In order to remove the protein portion, the ethanol precipitate was treated with pronase, and further deproteinized by the Sevag method,[7] followed by stepwise precipitation with ethanol. The major fraction, precipitated with 60% ethanol, was further purified by DEAE-celluolse column chromatography with sodium hydrogen carbonate. The purified major fraction, GL-1, gave a single spot on glass-fiber paper electrophoresis and was determined to be homogeneous by ultracentrifugal analysis (Fig. 1). Furthermore, this fraction gave a single peak in gel filtration on columns of Sephadex G-75 (Fig. 2) and Sepharose 6B eluted with water as the eluent. GL-1 contained 98% sugar[5] (as glucose) and was free from nitrogen and phosphorus.[8] The component sugars of GL-1 were identified as glucose, xylose and arabinose by paper and gas liquid chromatographies (PC and GLC) of the acid hydrolysate, and their molar ratio was estimated to be approximately 18.8: 1.5: 1.0 by GLC. In the infrared (IR) spectrum of GL-1, absorbance at 890 cm^{-1}, due to β-glycosidic linkages,[9] was observed. In the ^{13}C nuclear magnetic resonance (^{13}C NMR) spectrum of GL-1, as shown in Fig. 3, signals at 75.3 and 71.5 ppm were observed and this result suggests the presence[10] of C-6 branched β-D-(1→3)-linkages. Signals due to β-D-(1→6)-linkages were also observed, but there were no clear signals ascribable to α-D-(1→4)-linkages. The

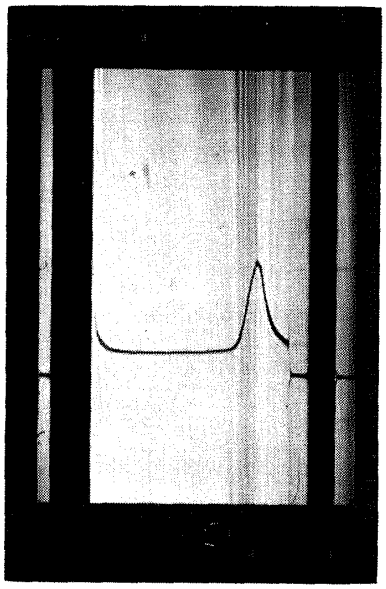

Fig. 1. Ultracentrifugal Sedimentation Pattern of GL-1

Apparatus; Hitachi ultracentrifuge, Model 282,
solvent; 0.1 M NaCl,
concentration; 1%,
speed; 60000 rpm.

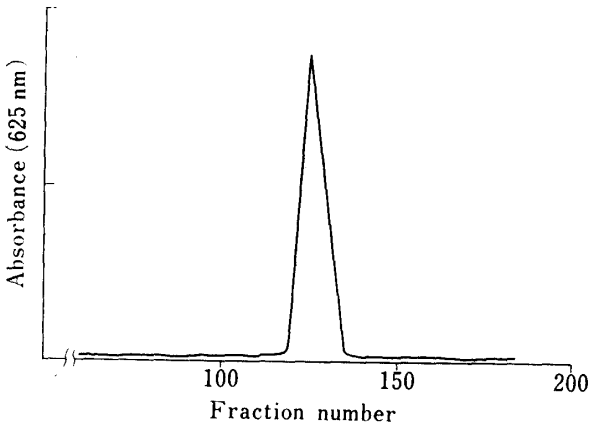

Fig. 2. Chromatogram of GL-1 on Sephadex G-75

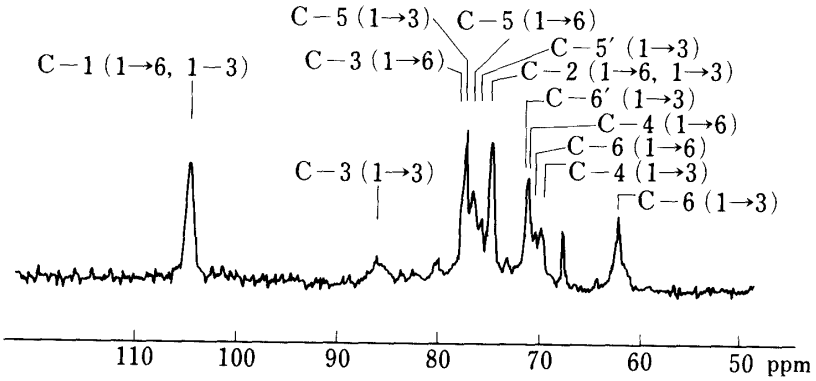

Fig. 3. ^{13}C NMR Spectrum of GL-1

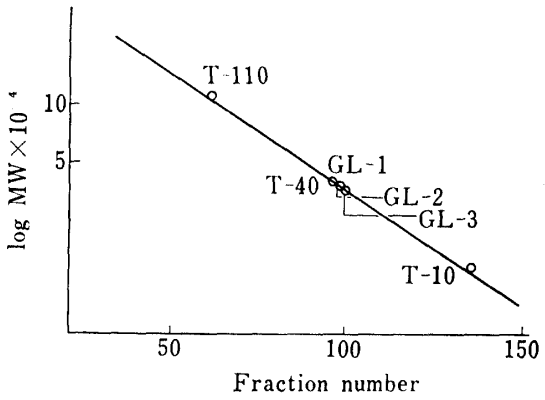

Fig. 4. Determination of the Molecular Weights of GL-1, GL-2 and GL-3

T-110: MW, 110000, T-40: MW, 40000,
T-10: MW, 10000.

molecular weight was estimated to be approximately 40000 by gel filtration (Fig. 4).

On periodate oxidation of GL-1, the consumption of periodate was 1.1 mol per anhydro sugar unit, and the amount of formic acid liberated from the unit was 0.42 mol. Formaldehyde was not produced. Smith degradation of GL-1 gave glycerol, erythritol, glucose and xylose as major products. Glucose and xylose were identified as their reduced derivatives.

Methylation analysis of GL-1 was carried out after full methylation by the methods of Hakomori and Purdie.[11,12] The methylated GL-1 was hydrolyzed with acid, converted into alditol acetates, and analyzed by GLC–mass spectroscopy (GLC–MS). 1,4-Di-*O*-acetyl-2,3,5-

tri-*O*-methyl arabinitol, 1,2,4-tri-*O*-acetyl-3,5-di-*O*-methyl xylitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-, and 1,3,5,6-tetra-*O*-acetyl 2,4-di-*O*-methyl and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucitols were identified. The molar ratio of the above 2,3,5,-2,3,4,6- and 3,5-, 2,4,6-, 2,3,4-, 2,3,6-, 2,4-, and 2,3-*O*-methylated alditol acetates was 0.5: 5.0: 4.3: 6.3: 6.5: 3.3: 1.0 (Table I).

Table I. GLC of the Methylated Alditol Acetates derived from GL-1, GL-2, GL-3 and GL-4

Sugar (as alditol acetate)	Mol. ratio				Relative retention time[b]
	GL-1	GL-2	GL-3	GL-4	
2,3,5-Tri-*O*-Me Ara	0.5	—	—	—	0.48
2,3,4,6-Tetra-*O*-Me Glc	5.0[a]	3.2	3.6	3.8	1.00
3,5-Di-*O*-Me Xyl		—	—	—	1.08
2,4,6-Tri-*O*-Me Glc	4.3	2.0	1.8	2.9	1.95
2,3,4-Tri-*O*-Me Glc	6.3	4.0	4.6	5.3	2.49
2,3,6-Tri-*O*-Me Glc	6.5	3.5	2.7	3.7	2.50
2,4-Di-*O*-Me Glc	3.3	1.7	2.2	2.4	5.10
2,3-Di-*O*-Me Glc	1.0	1.0	1.0	1.0	5.39

a) Under these conditions, the two compounds almost overlapped.
b) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Mild acid hydrolysis of GL-1 with 0.1 N sulfuric acid at 100°C for 2 h gave glucose, arabinose, xylose, gentiobiose and unidentified substances as dialyzable fragments. The non-dialyzable fraction, GL-2, M.W. *ca.* 38000, $[\alpha]_D^{20}$ +36° (c=1, water), gave only glucose on complete acid hydrolysis. GLC of the methylated alditol acetates derived from GL-2 revealed the presence of 2,3,4,6-tetra-*O*-methyl-, 2,4,6-, 2,3,4- and 2,3,6-tri-*O*-methyl-, and 2,4- and 2,3-di-*O*-methyl derivatives in the molar ratio of 3.2: 2.0: 4.0: 3.5: 1.7: 1.0.

Enzymic treatment of GL-2 with α-amylase (saccharifying type, from *Bacillus subtilis*) gave glucose, maltose and unidentified substances as dialyzable fragments, and the non-dialyzable fraction, GL-3 (M.W. *ca.* 37000), showed $[\alpha]_D^{20}$ −12° (c=1, water). GLC of the methylated alditol acetates derived from GL-3 revealed the presence of 2,3,4,6-tetra-*O*-methyl-, 2,4,6-, 2,3,4- and 2,3,6-tri-*O*-methyl-, and 2,4- and 2,3-di-*O*-methyl glucitol derivatives in the molar ratio of 3.6: 1.8: 4.6: 2.7: 2.2: 1.0.

In the case of α-amylase treatment of GL-1, almost the same dialyzable fragments were detected, and the non-dialyzable fraction yielded GL-4, which showed $[\alpha]_D^{20}$ −11° (c=1, water). GLC of the methylated alditol acetates derived from GL-4 revealed the presence of 2,3,4,6-tetra-*O*-methyl-, 2,4,6-, 2,3,4- and 2,3,6-tri-*O*-methyl-, and 2,4- and 2,3-di-*O*-methyl glucitol derivatives in the molar ratio of 3.8: 2.9: 5.3: 3.7: 2.4: 1.0 (Table I).

From these results, it is concluded that GL-1 is an arabinoxyloglucan, and has a highly branched structure possessing (1→4)-α- and -β-, (1→6)-β-, (1→3)-β-, (1→3, 1→6)- and (1→4, 1→6)-linked D-glucopyranosyl residues. Arabinose may be present as a part of the non-reducing terminal residues because of its disappearance upon Smith degradation and the production of the tri-*O*-methyl derivative. Another minor component, xylose, may be present as a part of the side-chains because only the di-*O*-methyl derivative was found by methylation analysis and, further, the acid-resistant core portion (GL-2) was found to be free from xylose.

The α-amylase treatment indicated that approximately 20% of the (1→4)-glucosidic linkages in GL-1 and approximately 30% of those in GL-2 are in an enzyme-sensitive form, such as (1→4)-α-D-linked side-chain residues. The major anomeric configuration of the acid-resistant and α-amylase-resistant core portion, GL-3, may be β form in view of the negative

Table II. Antitumor Effects of GL-1, GL-2 and GL-3

Sample	Dose (mg/kg × day)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression
PS-K[a)]	10×10	0.02	99.7	6/7
GL-1	5×10	3.40	42.0	1/5
GL-1	10×10	1.20	81.0	2/6
GL-1	20×10	0.09	98.5	4/5
Control	—	5.90	—	0/7
GL-1	20×10	0.38	96.4	5/9
GL-2	10×10	2.30	76.9	1/9
GL-2	20×10	0.53	94.6	4/8
Control	—	9.90	—	0/9
GL-1	20×10	0.42	95.6	5/9
GL-3	20×10	0.31	96.7	6/9
Control	—	9.50	—	0/9

a) PS-K: a commercial product.

specific rotation. As described above, the ^{13}C NMR spectrum of GL-1 suggests the presence of β-D-(1→3), and β-D-(1→6) linkages and branching sequences.

In the assay of antitumor activity, as described in the experimental part, similar inhibition ratios were observed among GL-1, GL-2 and GL-3. This result suggests that the branched glucan portions in GL-1 and GL-3, consisting of (1→3)-, (1→4)- and (1→6)-β-D-linkages, participate in the antitumor activity (Table II). Previously, Miyazaki *et al.*[20)] reported that the presence of a trisaccharide unit containing a (1→3)-β-linkage with a branch at position 6 is of primary importance for the antitumor activity of the glucans from *Grifola umbellata*. Furthermore, the degree of antitumor activity is influenced by a number of factors. Therefore, further work is required in order to elucidate the relationship between the structure and antitumor activity of the glycans obtained from this fungus.

An alkali-soluble polysaccharide fraction, which was obtained from water-extracted fruit bodies, was also antitumor-active. Details of this polysaccharide fraction will be presented in the following paper.

Experimental

Analytical Procedures——Total carbohydrate was determined by the method of Dubois *et al.*,[5)] protein by the method of Lowry *et al.*[6)] with bovine serum albumin as a standard, phosphate by the method of Fiske and SubbaRow,[8)] and nitrogen by elemental analysis. Acid hydrolysis was carried out by heating with sulfuric acid. The hydrolysate was neutralized with barium carbonate, then filtered, and the filtrate was evaporated to a syrup *in vacuo*. GLC was carried out with a Shimadzu GC-5A gas chromatograph, fitted with a flame-ionization detector, by using a glass column (0.3×200 cm) packed with 3% ECNSS-M on Chromosorb W (aw-dmcs, 60—80 mesh). Nitrogen was used as a carrier gas at a flow rate of 40 ml/min or 20 ml/min (methylated sugars). Neutral sugars and their methyl derivatives were converted into the corresponding alditol acetates by heating with a mixture of pyridine and acetic anhydride (1: 1) at 60°C for 6 h, and separated on a GLC column of 3% ECNSS-M at 190°C (neutral sugars), or at 130°C increasing to 180°C at a rate of 2°C/min (Smith degradation products), at 140°C 160 min, followed by an increase to 180°C at a rate of 1°C/min, or at 180°C (methylated sugars). GLC–MS spectra were recorded with a Shimadzu LKB-9000 mass spectrometer equipped with a glass column packed with 3% (w/w) silicone OV-225 on Chromosorb W (AW-DMCS, 80 to 100 mesh) at 170°C and operated at an ionization voltage of 70 eV, a trap current of 60 μA and an ion source at 310°C. IR spectra were recorded with a Hitachi 215 spectrometer. Optical rotations were determined with a JASCO automatic polarimeter, model DIP-SL. Thin layer chromatography (TLC) was carried out on cellulose plates (Merck, Art. 5716) with solvent system A (ethyl acetate–pyridine–water 10: 4: 3) or B (ethyl acetate–pyridine–acetic acid–water 5: 5: 1: 3). Electrophoresis (EP) on glass paper (Whatman paper chromedia GF 83 glass fiber paper) was carried out in borate buffer 0.026 M (pH 9.2) at a constant current of 5 mA/cm for 30 min, and the sugar component was detected with α-naphthol–sulfuric acid reagent. The sedimentation pattern was observed at 60000 rpm by using a Hitachi Model 282 analytical ultracentrifuge

equipped with a schlieren optical system. ^{13}C NMR spectra were measured with a JEOL spectrometer operating at 25.03 MHz in the pulsed Fourier transform mode. Free induction decays were accumulated with a 90°C pulse (22 μs) and a repetition time of 0.6 ms. A phase-alternating pulse sequence was used to minimize base-line aberrations. The spectrum was recorded by using 4K data points and a spectral width of 4 KHz. ^{13}C-Chemical shifts are expressed in parts per million downfield from external tetramethylsilane. NMR measurements were carried out at ambient temperature (28°C). GL-1 was dissolved in D_2O in 10 mm o.d. sample tubes (20 mg/ml). Signals (p.p.m.): C-1; 104.6, 104.9, C-2; 74.5, 75.0, C-3; 78.0, 86.3, C-4; 69.9, 71.4, C-5; 77.1, 77.7, C-6; 62.5, 70.0, the carbons of C-6 branched β-D-(1→3)-linkages; 75.3 (C-5′), 71.5 (C-6′).

Isolation of Water-soluble Polysaccharide from *G. lucidum*——The dried fruit bodies of *G. lucidum* (Rokkakushi, a kind of Reishi, cultured in Wakayama Pref. Japan) (100 g) were cut into small pieces and extracted with hot water (7×5 vol). The extract was dialyzed against running water through Visking cellophane tubing, and the internal solution was concentrated to a small volume. Addition of ethanol gave a precipitate that was collected by centrifugation and dried by washing with acetone and ether. The yield was about 3.4% of the dry weight of the fruit bodies. Neutral sugar[5] content was 64.5% and protein[6] content was 21.3%. In order to remove the protein portion, the precipitate (3 g) was dissolved in 50 ml of water and pronase was added to the solution (100 mg, Kaken Kagaku Co. Ltd.,). The mixture was treated by the same procedure as described in a previous paper.[1] The yield of the de-proteinized fraction was *ca.* 2.5% of the dried fruit bodies.

Fractionation of the Crude Polysaccharide——The crude polysaccharide solution was fractionated by the stepwise addition of ethanol to 30%, 60% and 90%. Each precipitate was collected by centrifugation and dried *in vacuo*. The yields of these precipitates were 21%, 75% and 4%, respectively. An aqueous solution of the 60% ethanol-precipitated fraction (500 mg) was applied to a column (4×60 cm) of DEAE-cellulose. Stepwise elution was carried out with water, 0.1 M, 0.3 M, 0.5 M sodium hydrogen carbonate and 0.1 N sodium hydroxide at a flow rate of 100 ml/h. An aliquot (0.1 ml) of each fraction was mixed with 2.0 ml of water and 4 ml of 0.2% anthrone reagent,[13] and the optical density was read at 625 nm on a colorimeter. Each fraction was dialyzed against running water for 3 days. The internal solution was concentrated to a small volume *in vacuo* and 3 volumes of ethanol was added to the concentrate. The collected precipitate was washed with ethanol, acetone and ether, and dried *in vacuo*. The yields were as follows: water eluate, 50 mg; 0.1 M eluate (GL-1), 190 mg; 0.3 M and 0.5 M sodium hydrogen carbonate eluates, 70 mg; 0.1 N sodium hydroxide eluate, 175 mg (brown color). Gel chromatographies of GL-1 on columns of Sepharose 6B (2.4×95 cm) and DEAE-Sephadex G-75 (2.6×95 cm) were carried out with water as the eluent and with anthrone reagent[13] for detection (Fig. 2).

Component Sugars of GL-1——GL-1 (*ca.* 5 mg) in 2 ml of 1 N sulfuric acid sealed in a tube was heated in a boiling water bath for 8 h. A portion of the hydrolysate was applied to a Toyo Roshi No. 50 filter paper and developed by the ascending method with solvent system A. Sugars were detected with *p*-anisidine hydrochloride[14] and alkaline silver nitrate.[15] GLC of the hydrolysate was carried out by the method described above. Molecular proportions of the component sugars, glucose, xylose and arabinose, were calculated as 18.8: 1.5: 1.0 from the peaks on the chromatogram.

Periodate Oxidation of GL-1——GL-1 (52 mg) was dissolved in 10 ml of distilled water and the volume was made up to 50 ml with 4 ml of 0.026 M sodium periodate and distilled water. The mixture was allowed to stand in the dark at room temperature and the consumption of periodate, and the amounts of formic acid and formaldehyde produced were determined by the procedures of Malaprade,[16] Whistler[17] and O'Dea and Gibbons.[18] The numbers of mole of sodium periodate consumed per anhydro sugar unit of GL-1 were as follows; 0.19 (0.5 h), 0.34 (1 h), 0.56 (3 h), 0.68 (6 h), 0.82 (10 h), 0.91 (12 h), 0.95 (24 h), 1.02 (48 h), 1.08 (72 h), 1.10 (120 h) and 1.10 (148 h), and the values of formic acid produced were 0.01 (0.5 h), 0.12 (1 h), 0.24 (3 h), 0.30 (6 h), 0.33 (10 h), 0.35 (12 h), 0.37 (24 h), 0.38 (48 h), 0.42 (72 h), 0.42 (120 h) and 0.42 (148 h); no formaldehyde was produced.

Smith Degradation of GL-1——When the oxidation was complete (after 96 h), the oxidized GL-1 was reduced with sodium borohydride, and hydrolyzed with 1 N trifluoroacetic acid, at 110°C for 6 h. The hydrolysate was concentrated and analyzed by GLC as described above. Retention times (min): 9.4, 12.0, 21.8 and 34.4; glycerol 9.4, erythritol 12.0, xylitol 21.8 and glucitol 34.4.

Methylation Analysis——Each polysaccharide was fully methylated by the methods of Hakomori[11] and Purdie,[12] and hydrolyzed with 90% formic acid at 100°C for 5 h, and then with 1 N sulfuric acid at 100°C for 5 h. Alditol acetate derivatives of the hydrolysate were analyzed by GLC as described above. Retention values are given relative to the mobility of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (Table I). These data were identical with those of authentic samples.

In the GLC–MS, the following results were observed: 1,4-di-*O*-acetyl(Ac)-2,3,5-tri-*O*-methyl(Me)-arabinitol, *m/e*: 43, 45, 71, 87, 101, 117, 129 and 161, 1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-Me-glucitol, *m/e*: 43, 45, 71, 87, 101, 117, 129, 145, 161 and 205, 1,2,4-tri-*O*-Ac-3,5-di-*O*-Me-xylitol, *m/e*: 43, 45, 71, 87, 101, 129, 161 and 189, 1,3,5-tri-*O*-Ac-2,4,6-tri-*O*-Me-glucitol, *m/e*: 43, 45, 87, 101, 117, 129 and 161, 1,5,6-tri-*O*-Ac-2,3,4-tri-*O*-Me-glucitol, *m/e*: 43, 87, 99, 101, 129, 161 and 189, 1,4,5-tri-*O*-Ac-2,3,6-tri-*O*-Me-glucitol, *m/e*: 43, 45, 87, 99, 101, 113, 117 and 233, 1,3,5,6-tetra-*O*-Ac-2,4-di-*O*-Me-glucitol, *m/e*: 43, 87, 117, 129 and 189, 1,4,5,6-tetra-*O*-Ac-2,3-di-*O*-Me-glucitol, *m/e*: 43, 101, 117 and 261.

Partial Acid Hydrolysis of GL-1——Partial acid hydrolysis of GL-1 (100 mg) was carried out with 0.1 N sulfuric acid (5 ml) at 100°C for 2 h. The solution was dialyzed against distilled water through Visking cellophane tubing. TLC of the dialyzable fraction gave six spots corresponding to arabinose (*Rf* 0.75), xylose (*Rf* 0.68), glucose (*Rf* 0.64), gentiobiose (*Rf* 0.49) and unidentified substances (*Rf* 0.30 and 0.11).

The non-dialyzable solution was concentrated, and the ethanol precipitate yielded GL-2, which was examined by methylation analysis as described above.

α-Amylase-treatment of GL-1 and GL-2——alpha-Amylase from *B. subtilis*, saccharifying type, was purchased from Seikagaku Kogyo Co. Ltd., Japan. Mixtures of 10 mg of enzyme and 100 mg of the glycans dissolved in water were kept for 48 h at 55°C. Each mixture was dialyzed against distilled water after inactivation of the enzyme at 100°C for 5 min. This procedure was repeated twice for the non-dialyzable fraction. Each dialyzable fraction was examined by TLC on a cellulose plate with solvent system B, and the non-dialyzable fractions, GL-3 and GL-4, were examined by methylation and GLC. *Rf* values of the dialyzable fraction: 0.75, 0.68, 0.64, 0.49, 0.30, 0.11; arabinose; 0.75, xylose; 0.68, glucose; 0.46, gentiobiose; 0.49.

Assay of Antitumor Activity——Female ICR mice weighing about 23 g (5 week) were used for the antitumor assay. Seven-day-old Sarcoma-180 ascites (0.05 ml, 6×10^6 cells) were transplanted subcutaneously into the right groin of mice. The test samples dissolved in saline were intraperitoneally injected daily for 10 days, starting 24 h after tumor implantation. At the end of the 5th week, the mice were killed, and the tumors were extirpated and weighed. The inhibition ratios were calculated by the use of the following formula:[19] Inhibition ratio $(\%)=[(A-B)/A]\times100$, where A is the average tumor weight of the control group, and B is that of the treated group.

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