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Polysaccharides in Fungi. IX.[1] A *β*-D-Glucan from Alkaline Extract of *Dictyophora indusiata* FISCH.[2]

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A water-soluble *β*-D-glucan (T-5-N) has been isolated from 1 N sodium hydroxide extract of the fruit bodies of *Dictyophora indusiata* FISCH. T-5-N, $[\alpha]_D^{18}$ +28.7° (c=0.5, H_2O), IR ν_{max}^{KBr} cm^{-1}: 890, was homogeneous as judged by ultracentrifugal analysis and Tiselius-type electrophoresis. Structural analyses indicated that T-5-N has a main chain composed of *β*-1→3 linked D-glucopyranosyl residues, and two single *β*-1→6 linked D-glucopyranosyl units are attached as side chains, on average, to every seventh sugar residue of the main chain. In addition, a few internal 1→6 linkages and branching points at position 2 of the *β*-1→3 linked main chain are also present in the molecule.

Keywords——*Dictyophora indusiata*; polysaccharide; *β*-D-glucan; methylation analysis; Smith degradation; partial acetolysis; enzymatic hydrolysis

In our previous paper,[3] we reported structural investigations of partially *O*-acetylated *α*-1→3 linked D-mannan prepared from the hot aqueous ethanol extract of the fruit bodies of *Dictyophora indusiata* FISCH., which grows in China. Now we have isolated a *β*-D-glucan (T-5-N) from alkaline extract of the fruit bodies of this fungus. The present paper deals with the purification, characterization and structural analysis of T-5-N.

The fruit bodies extracted with hot 70% aqueous ethanol solution, as reported in our previous paper,[3] were treated successively with hot water and 2% sodium carbonate solution. The residue was then extracted with 1 N sodium hydroxide solution at room temperature

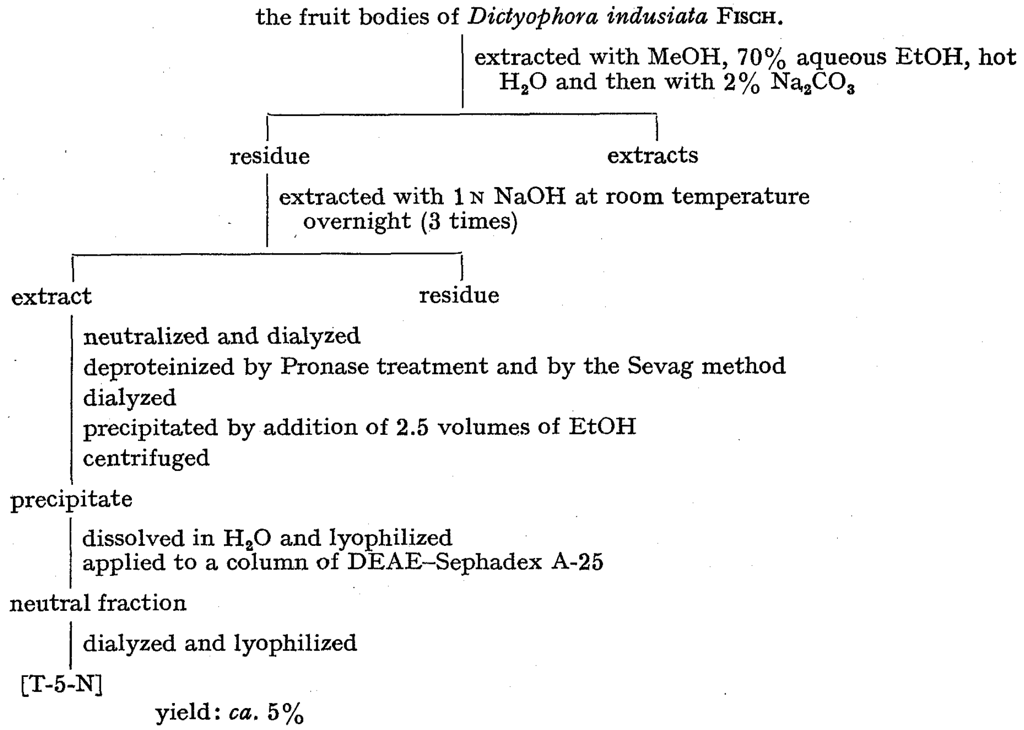


Chart 1. Isolation and Purification of the Polysaccharide

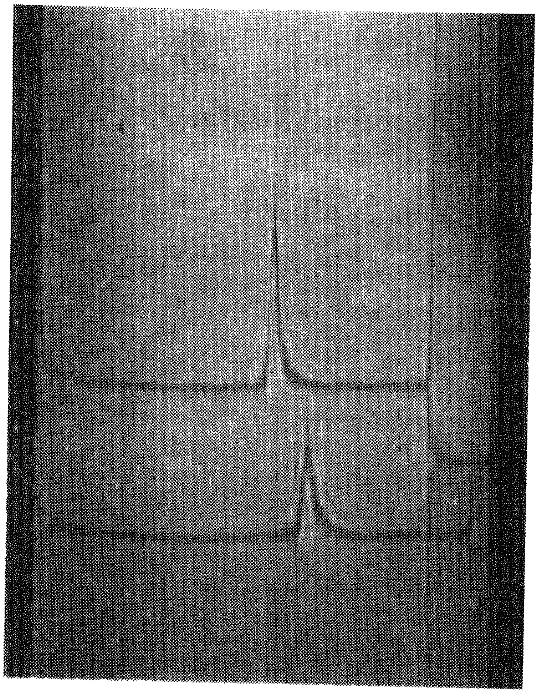


Fig. 1. Ultracentrifugal Pattern of T-5-N

The photograph was taken at 81 min after reaching full speed (50700 rpm).
upper: 0.246% lower: 0.198%.

overnight. After neutralization and dialysis of the alkaline extract, proteins in the extract were removed by Pronase treatment and by the Sevag procedure,[4] followed by dialysis. The non-dialyzable solution was fractionated by addition of ethanol. The crude polysaccharide fraction (T-5) obtained was purified by column chromatography on diethylaminoethyl(DEAE)-Sephadex A-25 with 0.01 M sodium acetate buffer at pH 5.9 to remove a small amount of acidic fraction. The eluted neutral fraction was lyophilized to afford the polysaccharide (T-5-N) as colorless flakes in about 5 % yield. It had $[\alpha]_D^{18}$ +28.7° in water ($c=0.5$). The overall process is outlined in Chart 1.

The polysaccharide (T-5-N) was homogeneous as determined by Tiselius-type electrophoresis, and by ultracentrifugal analysis (Fig. 1).

T-5-N slowly dissolved in water to give a highly viscous solution, and was resistant to acid hydrolysis. When the polysaccharide solution at over 0.2% concentration was cooled below 5°C, a soft gel was formed. The component sugar of T-5-N was identified as glucose by paper partition chromatography (PPC) of the hydrolysate and by gas-liquid chromatography (GLC) of the alditol acetate prepared from the hydrolysate. The total sugar content was found to be 99.3% as anhydroglucose by the phenol-sulfuric acid method.[5] T-5-N contained neither nitrogen nor ash on elementary analysis.

The crystalline glucose (mp 146—148°C, $[\alpha]_D^{25}$ +52.2°C equil., in water ($c=0.092$)) obtained by treating the hydrolysate of T-5-N with ethanol was identified as D-type by mixed melting point determination, analysis of specific rotation and comparison of its infrared (IR) spectrum with those of authentic D- and L-glucose. T-5-N had a characteristic absorption band at 890 cm^{-1} (type 2b) in the IR spectrum, indicating the presence of β-glycosidic linkages.[6] The proton magnetic resonance (^{1}H-NMR) spectrum of the *O*-methylated T-5-N in $CDCl_3$ showed a signal at 4.75 ppm (δ) due to anomeric protons. The signals corresponded to the anomeric proton signal at 4.7 ppm of *O*-methylated β-1→3-D-glucan, *e.g.* laminaran,[7] rather than those of other *O*-methylated α- or β-D-glucans (5.43 ppm: α-1→3[7]; 5.68 ppm: α-1→4[8]; 5.05 ppm: α-1→6[9]; 4.47 ppm: β-1→4[8]). These results indicated that most of the glycosidic linkages in this glucan had the β-D configuration.

T-5-N was methylated by the methods of Hakomori[10] and Purdie *et al.*,[11] and then the fully *O*-methylated polysaccharide was hydrolyzed with 90% formic acid and 0.5 N sulfuric acid. The resulting partially *O*-methylated monosaccharides were analyzed by GLC and gas-liquid chromatography-mass spectrometry (GLC-MS) after conversion into corresponding *O*-acetyl-*O*-methyl glucitols. These glucitol derivatives were identified by comparing the retention times on GLC and the mass spectra with those of authentic samples or with the values in the literature.[12] As shown in Table I, the methylation analysis indicated the presence of 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, 4,6-di- and 2,4-di-*O*-methyl-D-glucose in the molar ratio of 1.00: 2.28: 0.06: 0.07: 0.92. The molar ratio of the *O*-methylated derivatives indicates an average chain length of about 4.3 D-glucose residues per non-reducing end group. The results of methylation analysis suggested that this glucan has many non-reducing end groups (23.1%) and a backbone chain of 1→3 linked D-glucopyranosyl residues, and contains

Table I. Relative Retention Times on GLC and Prominent Peaks (*m/e*) in MS of partially Methylated Alditol Acetates

O-Methylated sugar (as D-glucitol acetate)	Relative retention times[b] Condition A[c]	B[d]	Prominent peaks (*m/e*)	Molar ratio	Mode of linkage
1,5-Ac$_2$-2,3,4,6-Me$_4$-Glc[a]	1.00	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	1.00	Glc 1→
1,3,5-Ac$_3$-2,4,6-Me$_3$-Glc	1.75	1.97	43, 45, 87, 101, 117, 129, 161, 233	2.28	→3 Glc 1→
1,5,6-Ac$_3$-2,3,4-Me$_3$-Glc	2.13	2.42	43, 87, 99, 101, 117, 129, 161, 189	0.06	→6 Glc 1→
1,2,3,5-Ac$_4$-4,6-Me$_2$-Glc	3.22	4.00	43, 45, 87, 101, 129, 161, 261	0.07	→2 →3 Glc 1→
1,3,5,6-Ac$_4$-2,4-Me$_2$-Glc	3.85	5.04	43, 87, 117, 129, 189	0.92	→3 →6 Glc 1→

a) Abbreviations: Ac=acetyl; Me=methyl; Glc=glucitol or glucose.
b) Relative to authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
c) 3% Silicone OV-225, 184°C.
d) 3% ECNSS-M, 170°C.

many branching points (21.3%) at position 6 and a few branching points (1.6%) at position 2 of the 1→3 linked D-glucosyl residues. In addition, a few internal 1→6 linked D-glucopyranosyl residues (1.4%) are present in the polysaccharide.

On partial acetolysis of T-5-N, a series of oligosaccharides was detected by PPC. As shown in Fig. 2, a linear relationship existed between the presumed degree of polymerization of oligosaccharides detected and their log (*Rf*/1-*Rf*) values, as proposed by French *et al.*[13] The relationship was in accord with the observations obtained with other β-1→3 linked D-glucans,[14] *e.g.* laminaran.[15] Moreover, the disaccharide fraction recovered from paper chromatograms was analyzed by GLC as the trifluoroacetylated derivative, after reduction,[16] and only laminarabiitol trifluoroacetate was detected. These results indicated that a homologous series of β-1→3 linked gluco-oligosaccharides was formed by partial acetolysis of T-5-N.

On periodate oxidation of T-5-N, the periodate consumption and the production of formic acid were determined by the spectrophotometric method of Ikenaka[17] and by titration with a standard sodium hydroxide solution,[18] respectively. The amounts of periodate consumed and formic acid produced per anhydroglucose unit were 0.50 and 0.22 mol, respectively. These values were in good agreement with those obtained from the methylation analysis, 0.49 and 0.24 mol, respectively.

Complete Smith degradation products[19] of the reduced periodate-oxidized polysaccharide (polyalcohol T-5-NO, $[\alpha]_D^{25}$ $+7.8°$ in 1.5 N NaOH, $c=0.129$) were analyzed by GLC as their acetyl derivatives, and glycerol and glucose were detected in a molar ratio of 1.00: 2.9 (51.00: 3.09, as evaluated from the methylation analysis of T-5-N). Glycerol must arise from the non-reducing end groups and 1→6 linked sugar residues. The detection of glucose should be due to the presence of oxidation-resistant glucose residues such as 1→3 linked pyranosyl units.

Controlled Smith degradation of the polyalcohol T-5-NO was carried out by hydrolyzing

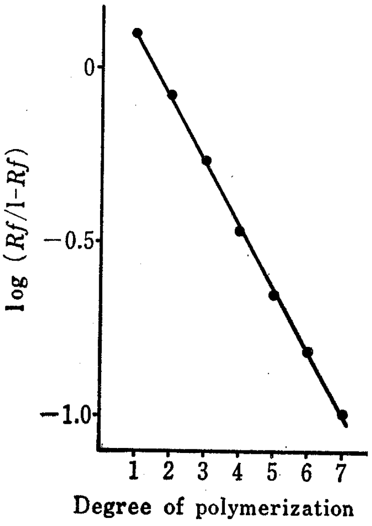


Fig. 2. Relationship between the Degree of Polymerization and log (*Rf*/1-*Rf*) Values of Acetolysis Products on PPC

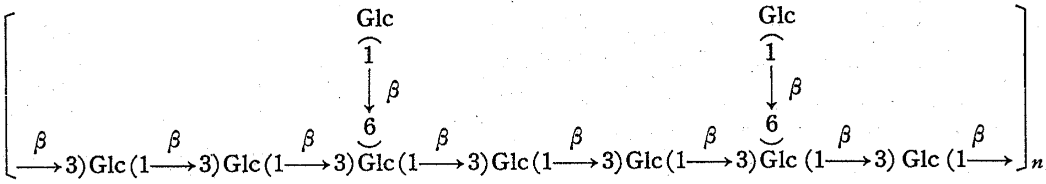
n-propyl alcohol–ethyl acetate–water (6: 1: 3, v/v), triple ascending method.

it with 0.1 N sulfuric acid at 25°C for 24 h. Insoluble material (T-5-NOI, $[\alpha]^{25}_{D}$ $-3.9°$ in 12.5 N NaOH, $c=0.077$) formed in the reaction mixture was separated by centrifugation. In the supernatant, only glycerol was detected by GLC as its acetyl derivative. The controlled Smith degradation product (T-5-NOI) was methylated and analyzed by GLC as *O*-methylated alditol acetates, as described above. Thus, the *O*-methylated T-5-NOI gave predominantly 2,4,6-tri-*O*-methyl-D-glucose, with traces of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-glucose.

When T-5-N was treated with β-D-glucanase (Lysing Enzymes, Sigma Chemical Company, containing *exo*-β-1→3-D-glucanase from Basidiomycetes), it was about 44% degraded as glucose after 10 h with the enzyme, and glucose and gentiobiose were detected by PPC of the dialyzable fraction in a molar ratio of 1.00: 0.37 (1.00: 0.40, as evaluated from the methylation analysis of T-5-N). Gentiobiose produced was independently identified by GLC as the trifluoroacetylated derivatives, as described above. On the other hand, T-5-NOI liberated only glucose in a similar enzymatic degradation.

The results of both the methylation analysis and enzymatic degradation of T-5-NOI strongly indicated that T-5-NOI was composed of a linear chain of β-1→3 linked D-glucopyranosyl residues, and consequently, that the principal part of the side chains joined through position 6 of β-1→3 linked D-glucosyl residues in the native T-5-N was removed by periodate oxidation followed by reduction and controlled Smith degradation. In addition to these results, the fact that T-5-N produced gentiobiose together with glucose upon enzymatic treatment with *exo*-β-1→3-D-glucanase further indicated that the side chains were attached to their branching points by β-1→6 linkage as a single D-glucosyl residue.

The conclusions to be drawn from these results are as follows: A glucan (T-5-N) isolated from 1 N sodium hydroxide extract of the fruit bodies of *Dictyophora indusiata* has a main chain composed of β-1→3 linked D-glucopyranosyl residues and has many side chains of two single β-1→6 linked D-glucopyranosyl units attached on average to every seventh sugar residue of the main chain. A possible repeating unit of T-5-N is illustrated in Fig. 3. However, a few internal 1→6 linkages and branching points at position 2 of the β-1→3 linked D-glucosyl residues of the main chain were observed in the methylation analysis of T-5-N, but the latter was not detected in the case of T-5-NOI. The internal 1→6 linkages may be present in the side chains rather than in the 1→3 linked main chain. If the internal 1→6 likages were contained in the main chain of T-5-N, the main chain should have been depolymerized by controlled Smith degradation to give small fragments. Such small fragments consisting of β-1→3 linked D-glucan chain should be soluble in water and dilute alkali,[20] but our controlled Smith degradation product (T-5-NOI) was a rigid gel and still insoluble in water, dilute alkali and dimethylsulfoxide, though it was soluble in concentrated alkali (about 12 N sodium hydroxide). These findings suggested that the internal 1→6 linkages are not present in the main chain. The minor internal 1→6 linkages may be present in the side chains at positions 6 and/or 2.



$$\left[\xrightarrow{\beta}3)\mathrm{Glc}(1\xrightarrow{\beta}3)\mathrm{Glc}(1\xrightarrow{\beta}3)\overset{\substack{\mathrm{Glc}\\ 1\\ \downarrow\beta\\ 6}}{\mathrm{Glc}}(1\xrightarrow{\beta}3)\mathrm{Glc}(1\xrightarrow{\beta}3)\mathrm{Glc}(1\xrightarrow{\beta}3)\overset{\substack{\mathrm{Glc}\\ 1\\ \downarrow\beta\\ 6}}{\mathrm{Glc}}(1\xrightarrow{\beta}3)\mathrm{Glc}(1\xrightarrow{\beta}\right]_n$$

Glc: D-glucopyranosyl residue

Fig. 3. A Possible Repeating Unit of T-5-N

Glucans possessing a main chain of β-1→3 linked D-glucosyl residues with branches of a single β-D-glucosyl unit joined through position 6 on every third D-glucosyl residue of the main chain have been isolated from some fungal cell walls[14,21] and the culture broth of some microorganisms.[22-25] Among such β-D-glucans, the structural features of sclerotan isolated from the sclerotia of *Sclerotinia libertiana*[14] and schizophyllan produced by *Schizophyllum com-*

mune[22] seem to be essentially similar to those of the present glucan (T-5-N). However, some significant differences between these β-D-glucans and T-5-N were observed in the chemical structure, that is, internal 1→6 linkages and branching points at position 2 of the β-1→3 linked main chain in T-5-N were not found in these β-D-glucans, although sclerotan had a trace of internal β-1→4 linkages.[26] Further, our glucan differs greatly from other β-D-glucans in molecular weight (T-5-N: 1.0×10^6 [27]; schizophyllan: 4.3×10^6 [22]; sclerotan: degree of polymerization ($\overline{\text{DP}}$) 10[14]).

Since β-D-glucans, *e.g.* sclerotan and schizophyllan, having the basic structural unit mentioned above have been reported to possess antitumour activity against subcutaneously implanted Sarcoma 180 in mice,[28] it would be of interest to examine the biological activity of the present β-D-glucan (T-5-N). Further investigations on the nature and biological activities of this glucan are in progress.

Experimental

Solutions were concentrated at or below 40°C with a rotary evaporator under reduced pressure. IR spectra were recorded with a JASCO IRA-1 spectrometer. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. GLC was carried out with a glass column (2 m length × 0.3 cm inner diameter) on a JEOL JGC-1100 gas chromatograph equipped with a hydrogen flame ionization detector. GLC–MS was carried out with a Shimadzu LKB-9000 gas chromatograph-mass spectrometer.

Materials——The dried fruit bodies of *Dictyophora indusiata* Fisch. are commercially available in Hong Kong. Partially *O*-methylated D-glucitol acetates used as reference compounds for GLC and GLC-MS were newly prepared by the modified method of Haworth.[29] Pronase (45000 PUK/g) and Lysing Enzymes were purchased from Kaken Chemical Ind., and Sigma Chemical Company, respectively. DEAE–Sephadex A-25 was purchased from Pharmacia Fine Chemicals.

Extraction and Isolation of Polysaccharide——The fruit bodies (100 g), after being extracted with hot 70% aqueous EtOH as reported in our previous paper,[3] were treated with hot H_2O and then with 2% Na_2CO_3. Each extract was separated by centrifugation, and the residue was successively extracted 3 times with 1 N NaOH (1 l) at room temperature overnight. The supernatant obtained by centrifugation at 5000 rpm for 30 min was neutralized with 3 N HCl and dialyzed against distilled water for 5 d. The internal solution, after removal of a small amount of insoluble material by filtration, was concentrated and adjusted to pH 7.8 with 1 N NaOH. The solution (*ca.* 700 ml) was treated with Pronase (40 mg) at 38°C for 48 h. The reaction mixture was further deproteinized by the Sevag method.[4] The aqueous phase was then dialyzed against distilled water for 4 d. To the internal solution was added 2.5 volumes of EtOH. The resulting precipitate was collected by centrifugation, dissolved in H_2O and lyophilized. The crude polysaccharide fraction (T-5) thus obtained was further purified by column chromatography on DEAE–Sephadex A-25 (5 × 20 cm) previously equilibrated with 0.01 M sodium acetate buffer at pH 5.9. The neutral fraction eluted with the buffer was dialyzed and lyophilized to afford the polysaccharide (T-5-N) as colorless flakes (yield: about 5% from the starting material). $[\alpha]_D^{18}$ +28.7° (c=0.5, H_2O). IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 890 (type 2b, β-glycosidic linkage). *Anal.* Calcd for $(C_6H_{10}O_5)_n$: C, 44.45; H, 6.22. Found: C, 43.85; H, 6.36; N, nil; ash, nil.

Free-boundary Electrophoresis——Tiselius-type electrophoresis of T-5-N was carried out at 60 V with a Hitachi HID-1 boundary electrophoresis apparatus in 0.05 M sodium tetraborate buffer at pH 9.3 for 70 min. One symmetrical peak was observed. Electrophoretic mobility: $u=0.65\times10^{-4}$ cm²/V·s.

Ultracentrifugal Analysis——Ultracentrifugal analysis of T-5-N was carried out with a Hitachi UCA-1 analytical ultracentrifuge with a schlieren optical system. Measurement was made at 50700 rpm at two polysaccharide concentrations (0.246 and 0.198%) in 0.3 M NaCl–0.001 M phosphate buffer (pH 7.0) at 20°C (Fig. 1).

^{1}H-NMR Spectroscopy——T-5-N (20 mg) was methylated by the method of Hakomori[10] as described in our previous paper.[30] The *O*-methylated polysaccharide was dissolved in $CDCl_3$ (1 ml), and the ^{1}H-NMR spectrum was recorded at ambient temperature with a JEOL FX-100 spectrometer, using tetramethylsilane as an internal standard.

Component Sugar——Hydrolysis of T-5-N was carried out in a sealed tube in the following ways: (A) a sample (2 mg) was treated with 2 N H_2SO_4 (2 ml) at 100°C for 24—33 h; (B) a sample (2 mg) was heated with 90% HCOOH (2 ml) at 100°C for 6 h, and then, after removal of HCOOH by evaporation, the residue was treated with 0.5 N H_2SO_4 (2 ml) at 100°C for 15 h. Each hydrolysate was neutralized with $BaCO_3$ and filtered. The filtrate was passed through a column of Amberlite CG-120 (H^+ form) resin, and the eluate was concentrated to a small volume. PPC was carried out on Toyo Roshi No. 51 filter paper with the following solvent systems (V/V): (A) AcOEt–pyridine–H_2O (10: 4: 3), (B) *n*-BuOH–pyridine–H_2O (6: 4: 3). Sugar spots were detected with alkaline $AgNO_3$[31] and naphthoresorcinol–H_3PO_4[32] reagents. Only glucose was detected in each solvent, *Rf*: 0.19 (solvent A), 0.41 (solvent B).

A residual portion of the hydrolysate was reduced with $NaBH_4$ (5 mg) for 2 h at room temperature to the corresponding alditol. The excess borohydride was decomposed by addition of Amberlite CG-120 (H^+) resin. After removal of the resin by filtration, the filtrate was concentrated to dryness and freed from boric acid by repeated evaporation with MeOH. The dried residue was acetylated with a mixture of Ac_2O and pyridine (1: 1, 0.5 ml) at 95°C for 90 min. After the mixture had been evaporated with a small amount of toluene, the residue was dissolved in $CHCl_3$ and analyzed by GLC. GLC was carried out under condition A: a glass column packed with 3% ECNSS-M on AW-Gaschrom Q (100 to 120 mesh) at 182°C at a flow rate of 43 ml per min of N_2. Only one peak was observed, and the retention time (43.7 min) was identical with that of authentic hexa-*O*-acetyl-D-glucitol.

Total Sugar Content——Total sugar content of T-5-N was determined as anhydroglucose by the phenol-H_2SO_4 method[5] using D-glucose as a standard.

Recovery of Crystalline D-Glucose from the Hydrolysate of T-5-N——T-5-N (15 mg) was treated with 90% HCOOH and then with 0.5 N H_2SO_4 as described above, and the hydrolysate was crystallized from EtOH (2 ml) by allowing it to stand in a freezer. The crystals (mp 146—148°C, $[\alpha]^{25}_{D}$ $+52.2°$ equil., in H_2O, $c=0.092$) thus obtained were identified as α-D-glucose by mixed melting point determination and comparison of its IR spectrum with those of authentic D- and L-glucose.

Methylation Analysis——T-5-N (7 mg) was methylated by the methods of Hakomori[10] and Purdie *et al.*[11] as described in our previous paper.[30] The final *O*-methylated product showed no hydroxyl absorption band in the IR spectrum. The fully *O*-methylated polysaccharide was heated with 90% HCOOH (2 ml) at 100°C for 5 h in a sealed tube. After removal of HCOOH by evaporation, the residue was hydrolyzed with 0.5 N H_2SO_4 (3 ml) at 100°C for 15 h followed by neutralization with Amberlite CG-400 (CO_3^{2-}) resin (10 ml). The resulting *O*-methylated sugars were reduced with $NaBH_4$ (35 mg) at 5°C for 41 h to the corresponding alditols, and then acetylated with a mixture of Ac_2O and pyridine as described above. GLC of the partially *O*-methylated alditol acetates was carried out under the following conditions: condition B, a glass column packed with 3% Silicone OV-225 on AW-DMCS-Chromosorb W (80 to 100 mesh) at 184°C at a flow rate of 43 ml per min of N_2; condition C, the same 3% ECNSS-M column at 170°C. Table I shows the relative retention times of the alditol acetates derived from *O*-methylated sugars relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and their molar ratio.

GLC-MS of the partially *O*-methylated alditol acetates was carried out on the same 3% Silicone OV-225 column (0.4 × 100 cm) at 180°C at a flow rate of 30 ml per min of helium. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 60 μA and a temperature of the ion source of 290°C. The result is shown in Table I.

Partial Acetolysis——T-5-N (50 mg) was suspended in Ac_2O (5 ml), and then a mixture of AcOH (3 ml) and H_2SO_4 (0.5 ml) was added to the suspension under cooling. The mixture was allowed to stand at room temperature for 3 d with occasional shaking. The clear reaction mixture was poured into ice-water (50 ml) and neutralized with $NaHCO_3$. The solution was extracted with $CHCl_3$ (30 ml each, 5 times). The extracts were combined, dried over anhydrous Na_2SO_4 and then filtered. The filtrate was evaporated to dryness, and the residue was dissolved in MeOH (15 ml) containing 0.05 M $NaOCH_3$ and deacetylated by allowing the solution to stand at 5°C overnight. After addition of H_2O (5 ml), the solution was concentrated to about 2 ml and passed through columns (1.5 × 5 cm) of Amberlite CG-120 (H^+) resin and Amberlite CG-400 (CO_3^{2-}) resin. The eluate was concentrated to a small volume.

Analyses of Partial Acetolysate——PPC of the partial acetolysate of T-5-N was carried out on Toyo Roshi No. 51 filter paper by the triple ascending method with the following solvent system, (C) *n*-propyl alcohol–AcOEt–H_2O (6: 1: 3, v/v). Sugar spots were detected with alkaline $AgNO_3$ reagent.[31] The relation between the presumed degree of polymerization of oligosaccharides detected and their log (*Rf*/1-*Rf*) values[13] is shown in Fig. 2. The disaccharide fraction, which was eluted with H_2O from paper chromatograms, was reduced with $NaBH_4$ (6 mg) as described above. The reduced product was treated with *N*,*N*-dimethylformamide (70 μl) and trifluoroacetic anhydride (50 μl) at room temperature.[16] GLC of the trifluoroacetylated glycitol prepared was carried out under condition E: a glass column packed with 2% GE-XF 1105 on AW-Chromosorb P (80 to 100 mesh) at 200°C at a flow rate of N_2 (2.5 kg/cm^2). Only one peak was observed, and the retention time (27.8 min) was identical with that of authentic trifluoroacetylated laminarabiitol.

Periodate Oxidation——T-5-N (11.2 mg) and methyl α-D-glucoside (13.5 mg) as a standard were oxidized with 0.01 M $NaIO_4$ (40 ml) under stirring at 2—4°C for 17 d in the dark. After various times, the periodate consumption was measured by the spectrophotometric method (290 nm) used by Ikenaka[17] and the formic acid production was determined by titration with standard 0.005 N NaOH.[18] The amounts of periodate consumption and formic acid production per anhydroglucose unit were 0.50 and 0.22 mol for T-5-N, and 2.01 and 0.99 mol for methyl α-D-glucoside, respectively. Another oxidation of T-5-N (52.2 mg) was carried out with 2.5 mM $NaIO_4$ in 0.0025 M sodium acetate buffer (pH 4.0, 400 ml) as described above. In this experiment, 0.48 mol of periodate was consumed after 23 d.

Reduction of the Periodate-oxidized T-5-N——Each oxidized polysaccharide solution, after addition of ethylene glycol (0.5 ml), was dialyzed against distilled water for 2 d. Then the non-dialyzable material was reduced with $NaBH_4$ for 48 h at 4°C. After acidification with 0.1 N AcOH to pH 5, the mixture was dialyzed and lyophilized to afford the polyalcohol T-5-NO (yield: 47.5 mg). T-5-NO thus obtained was only slightly soluble in H_2O. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 890. $[\alpha]_D^{25}$ $+7.8°$ in 1.5 N NaOH, $c=0.129$.

Complete Smith Degradation of T-5-NO and Analysis of the Products——The polyalcohol T-5-NO (2 mg) was hydrolyzed with 2 N H_2SO_4 (2 ml) at 100°C for 33 h in a sealed tube. The hydrolysate neutralized with $BaCO_3$ was reduced with $NaBH_4$ (10 mg) and acetylated with a mixture of Ac_2O and pyridine as described above. The acetylation mixture of the Smith degradation products was analyzed by GLC (condition D) using dual columns packed with 3% ECNSS-M on AW-Gaschrom Q; the column temperature was increased by 6°C per min from 60°C to 185°C; carrier gas, N_2 (43 ml per min). The retention times of acetylated derivatives of sugar alcohols were as follows: glycerol 16.5 min, glucitol 51.6 min. The molar ratio of glycerol and glucitol was approximately 1.00: 2.95, as determined by GLC analysis of the alditol acetates. When a standard acetylation mixture of authentic glycerol and glucitol (molar ratio, 1: 3) was analyzed by GLC under the same conditions, the molar ratio was 1.00: 2.85.

Controlled Smith Degradation of T-5-NO——T-5-NO (34.2 mg) was suspended in 0.1 N H_2SO_4 (40 ml) and hydrolyzed mildly at 25°C for 24 h with stirring. The insoluble materials formed were separated by centrifugation, washed thoroughly with H_2O and dried in a vacuum desiccator (yield: 25.2 mg, 73.7%). The product (T-5-NOI) thus obtained was insoluble in H_2O, dilute alkali and dimethylsulfoxide. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 890. $[\alpha]_D^{25}$ $-3.9°$ in 12.5 N NaOH, $c=0.077$.

The above supernatant and washings were combined and neutralized with $BaCO_3$. The solution was concentrated to dryness and then acetylated with a mixture of Ac_2O and pyridine as described above. The acetylation mixture of low molecular Smith degradation product was analyzed by GLC (condition D). Only one peak was observed, and the retention time (16.6 min) was identical with that of authentic glycerol acetate.

Methylation Analysis of T-5-NOI——T-5-NOI (12 mg) was suspended in a mixture of Ac_2O and pyridine (1: 1, 5 ml) and acetylated at 90°C for 5 h and then at room temperature overnight. After removal of the reactants by evaporation, the *O*-acetylated product was methylated 4 times by the method of Hakomori.[10] The fully *O*-methylated T-5-NOI was hydrolyzed with 90% HCOOH and 0.5 N H_2SO_4, reduced with $NaBH_4$ and acetylated with a mixture of Ac_2O and pyridine as described above. The partially *O*-methylated alditol acetates thus prepared were analyzed by GLC (conditions B and C).

Enzymatic Degradation with β-D-Glucanase and Analyses of the Products——T-5-N (2.7 mg) was dissolved in 0.03 M McIlvaine buffer (pH 4.9, 9 ml), and enzyme solution (Lysing Enzymes, 1 ml, 1.25 mg) was added. The reaction mixture was incubated at 38°C under stirring. After various times, a portion of the reaction mixture was analyzed for reducing activity by the method of Somogyi and Nelson,[33] using D-glucose as a standard. The extent of the hydrolysis in 10 h was about 44%. The reaction mixture was heated at 100°C for 15 min and then dialyzed against distilled water (1 l). The outer solution was concentrated to about 2 ml and passed through columns (1.5 × 7 cm) of Amberlite CG-4B (OH^-) resin and Amberlite CG-120 (H^+) resin to remove the buffer. Then the eluate was concentrated to a small volume. On PPC with solvent (C), only glucose and gentiobiose were detected. R_{glc}: glucose 1.00, gentiobiose 0.75. The molar ratio of glucose and gentiobiose, which was determined by the phenol–H_2SO_4 method[5] after separation of the products from the paper chromatograms, was 1.00: 0.37. The gentiobiose obtained was also identified by GLC (condition E) as the trifluoroacetylated glycitol derivative[16] (retention time: 29.6 min).

T-5-NOI (3.2 mg) was digested with the enzyme by a similar procedure, and only glucose (*Rf*: 0.54) was detected by PPC (solvent (C)) in the dialyzable fraction.

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