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Polysaccharides in Fungi. V.[1] Isolation and Characterization of a Mannan from Aqueous Ethanol Extract of *Dictyophora indusiata* Fisch.[2]

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A new type of mannan (T-2-HN) has been isolated as a neutral homoglycan from the aqueous 70% ethanol extract of the fruit bodies of *Dictyophora indusiata* Fisch., which grows in China. T-2-HN was homogeneous as judged by gel filtration (Sepharose 2B) and Tiselius-type electrophoresis. It was composed of D-mannose and a trace of glucose in a molar ratio of 100: 1, and contained *O*-acetyl groups in the proportion of about one acetyl group per two sugar residues. Its highly positive specific rotation, $[\alpha]_D^{20}$ +116° (c=0.1, H_2O), and the presence of an absorption band at 820 cm^{-1} (type 2a) in the infrared spectrum suggested that most of the glycosidic linkages in this mannan are of α-D configuration. Structural analysis indicated that T-2-HN has a linear chain composed largely of α-1→3 linked D-mannosyl residues, but the linear chain also contains traces of 1→6 linked D-mannopyranosyl (and/or glucosyl) residues. In addition, trace amounts of branching points are present at position 2 or 4 on each of the 1→3 linked D-mannose residues.

Keywords——*Dictyophora indusiata* Fisch.; polysaccharide; neutral homoglycan; α-1→3 linked D-mannan; partially *O*-acetylated α-D-mannan; methylation analysis; Smith degradation

The fruit bodies of *Dictyophora indusiata* Fisch. (Phallaceae)[4] have been used as a food and drug in China. While examining the nutritive value and biological activities of this fungus, we have isolated a new type of partially acetylated mannan (T-2-HN) as a neutral homoglycan from the aqueous ethanol extract of the fruit bodies of this fungus. The present paper deals with the purification, characterization and structural analysis of T-2-HN.

The fruit bodies were washed with hot methanol, and the residue was extracted with hot 70% aqueous ethanol solution.[5] After the aqueous ethanol extract had been concentrated and dialyzed, proteins in the extract were removed by Pronase treatment and by the Sevag procedure,[6] followed by dialysis. The crude polysaccharide fraction (T-2) thus obtained was divided into high and low molecular weight fractions by gel chromatography on a Sephadex G-200 column equilibrated with 0.1 M sodium chloride solution. The high molecular weight fraction (T-2-H) was further purified by column chromatography on diethylaminoethyl (DEAE)-Sephadex A-25 with 0.01 N sodium acetate buffer at pH 6.0 to remove a small amount of acidic fraction. The eluted neutral fraction was lyophilized to afford the polysaccharide (T-2-HN) as colorless flakes in about 0.4% yield. The overall process is outlined in Chart 1.

1) Part IV: S. Ukai, T. Kiho, and C. Hara, *Chem. Pharm. Bull.*, **26**, 3871 (1978).
2) Presented in part at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August, 1979.
3) Location: *6-1, Higashi-5-chome, Mitahora, Gifu, 502, Japan.*
4) Japanese name: Kinugasatake; Chinese name: Tsu Sun (竹篠).
5) The treatment with hot aqueous ethanol solution was carried out in order to inactivate enzymes, and to remove ethanol-soluble proteins and low molecular materials such as amino acids and sugars. As another advantage of this treatment, we found that *O*-acetylated mannan could be efficiently isolated from the mixture extracted with the aqueous ethanol. Direct extraction with hot water gave the mannan together with a large quantity of other polysaccharides, but isolation of the mannan from these polysaccharides was difficult. Details of these procedures will be reported in the near future.
6) M.G. Sevag, *Biochem. Z.*, **273**, 419 (1934).

the fruit bodies of *Dictyophora indusiata* Fisch.

pulverized and dried
extracted with MeOH

extract 1 | residue

dried
extracted with 70% EtOH
on a boiling water bath
(4 hr, 5 times)

extract 2 | residue

dialyzed
deproteinized by Pronase treatment
and by the Sevag method
dialyzed and lyophilized

T-2 yield: *ca.* 1%

chromatography on Sephadex G-200

High molecular weight fraction

dialyzed and lyophilized

T-2-H

chromatography on DEAE–Sephadex A-25

Neutral fraction

dialyzed and lyophilized

T-2-HN yield: *ca.* 0.4%

Chart 1. Isolation and Purification of the Polysaccharide

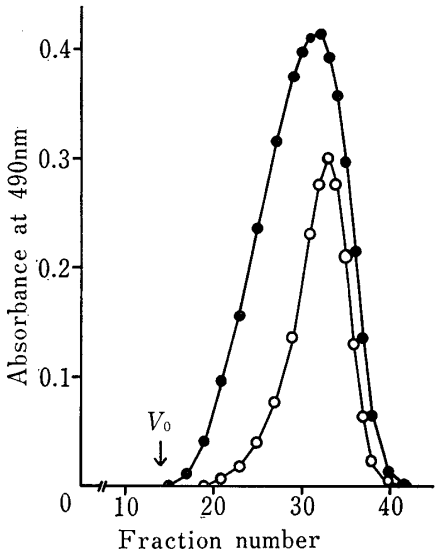

Fig. 1. Gel Chromatography of T-2-HN on Sepharose 2B

—●—: T-2-HN,
—○—: Dextran T-500.

The polysaccharide (T-2-HN) was homogeneous as determined by gel chromatography on a Sepharose 2B column as shown in Fig. 1 and by Tiselius-type electrophoresis in 0.05 M sodium tetraborate buffer at pH 9.3. Figure 1 shows that T-2-HN has a slightly higher molecular weight than standard Dextran T-500 ($\bar{M}w$: 495000).

The infrared (IR) spectrum of T-2-HN showed characteristic absorption bands at 1730, 1260 and 820 cm^{-1}. Two bands (1730 and 1260 cm^{-1}) disappeared on treatment of the polysaccharide with sodium hydroxide. This finding suggests the presence of acyl groups in the molecule. The acidic compound arising from the acyl groups was identified as acetic acid by gas–liquid chromatography (GLC) using a Chromosorb 101 column and paper partition chromatography (PPC) as the hydroxamate derivative,[7] thus confirming the presence of *O*-acetyl groups in the polysaccharide molecule. The total acetyl content of the polysaccharide was determined colorimetrically to be 11.4% by the ferric hydroxamate method of Hestrin.[8]

The component sugar of T-2-HN was identified as mannose, with a trace of glucose, by PPC of the hydrolysate and by GLC of the alditol acetates prepared from the hydrolysate. The approximate molar ratio of mannose and glucose was 100: 1, based on GLC analysis. The total sugar content was found to be 84.8% as anhydromannose by the anthrone-sulfuric acid method.[9] T-2-HN contained a trace of nitrogen (0.49%) on elemental analysis.

The native polysaccharide (T-2-HN) slowly dissolved in water to give a slightly viscous solution. In contrast, the deacetylated polysaccharide prepared by alkali treatment of T-2-HN was insoluble in water and was resistant to acid hydrolysis.

7) E.R. Stadtman and H.A. Barker, *J. Biol. Chem.*, **184**, 769 (1950).
8) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).
9) W.E. Trevelyan and J.S. Harrison, *Biochem. J.*, **50**, 298 (1952).

The native and deacetylated polysaccharides showed highly positive specific rotations as follows: $[\alpha]_D^{20}$ +116° in water (c=0.1) for the native form, $[\alpha]_D^{27}$ +119° in 2 N sodium hydroxide (c=0.1) for the deacetylated form. The acid hydrolysate of the deacetylated polysaccharide showed $[\alpha]_D^{16}$ +13.3° in 1 N sulfuric acid, c=0.060 as mannose. On the other hand, authentic D- and L-mannose gave the following values: $[\alpha]_D^{16}$ +14.0° equil., in 1 N sulfuric acid (c=0.139) for D-mannose, $[\alpha]_D^{21}$ −13.9° equil., in 1 N sulfuric acid (c=0.215) for L-mannose. The hydrolysate of the deacetylated polysaccharide, after neutralization, was treated with a mixture of methanol and *iso*-propyl alcohol to give colorless crystals, mp 128—130°. The crystalline substance was identified as D-mannose by mixed melting point determination and by IR spectral comparison with an authentic sample. The highly positive specific rotation in the D configuration[10] and the presence of the IR absorption band at 820 cm^{-1} [11a,b] indicate that most of the glycosidic linkages in the polysaccharide have the α-D configuration.

T-2-HN was methylated twice by the method of Hakomori.[12] The fully methylated polysaccharide was treated with 90% formic acid, and then hydrolyzed with 0.5 N sulfuric acid. The resulting partially *O*-methylated monosaccharides were analyzed by GLC and gas-liquid chromatography–mass spectrometry (GLC–MS) as *O*-methylated alditol acetates. The *O*-methylated alditol acetates were identified by comparing the retention times in GLC and the mass spectra with those of authentic samples or with the values in the literature.[13] The results of the methylation analysis are given in Table I.

Table I. Relative Retention Times and Composition of Methylated Products

Peak number	*O*-Methylated D-mannitol acetates	Relative retention times[b]		Molar percentage	Modes of linkage
		Condition A[c]	Condition B[d]		
I	1,5-Ac_2-2,3,4,6-Me_4–Man[a]	0.99	1.00	0.7	Man 1→
II	1,3,5-Ac_3-2,4,6-Me_3–Man	1.80	2.08	97.2	→3 Man 1→
III	1,5,6-Ac_3-2,3,4-Me_3–Man (and/or-Glc)	2.11	2.39	1.0	→6 Man 1→ (→6 Glc 1→)
IV	1,2,3,5-Ac_4-4,6-Me_2–Man 1,3,4,5-Ac_4-2,6-Me_2–Man	2.75	3.33	1.1	→2, →3 Man 1→ →4, →5 Man 1→

a) Abbreviations: Ac=acetyl; Me=methyl; Man=mannitol or mannose; Glc=glucitol or glucose.
b) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
c) 3% Silicone OV-225, 185°.
d) 3% ECNSS-M, 172°.

Among the peaks (I—IV) observed, peak II showed the greatest molar percentage of *O*-methylated alditol acetates. Peaks I, II and III were identified from their characteristic fragments as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl mannitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl mannitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl mannitol (and/or glucitol), respectively. The mass spectrum of peak IV (*m/e*: 43, 45, 87, 101, 117, 129, 161, 261) revealed fragments characteristic of 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl mannitol, except for the peak at *m/e* 117. The presence of the fragment at *m/e* 117 led us to deduce the coexistence

10) C.S. Hudson, *J. Am. Chem. Soc.*, **31**, 66 (1909); *idem*, *ibid.*, **52**, 1680 (1930); *idem*, *ibid.*, **52**, 1707 (1930).
11) *a*) S.A. Barker, E.J. Bourne, and D.H. Whiffen, *Methods of Biochemical Analysis*, **3**, 213 (1956); *b*) The IR spectrum and specific rotation of the present deacetylated mannan were identical with those of α-1→3 mannan AC-DS ($[\alpha]_D^{23}$ +113° in 1 N NaOH, c=1, IR ν_{max}^{KBr}: 820 cm^{-1}) prepared by controlled Smith degradation of deacetylated acidic heteroglycan from *Tremella fuciformis* Berk. in our laboratory.: *Chem. Pharm. Bull.*, **25**, 338 (1977).
12) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
13) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).

of 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl mannitol (characteristic fragments *m/e*: 43, 45, 87, 117, 129), which showed nearly the same retention time as 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl mannitol on GLC.

Periodate oxidation of T-2-HN was carried out, monitoring by the spectrophotometric method of Ikenaka.[14] The periodate consumption per anhydrohexose residue was 0.06 mol. The low consumption of periodate was in good agreement with the value of 0.03 mol obtained from the methylation analysis. Smith degradation products[15] of the reduced periodate-oxidized polysaccharide were analyzed by GLC as the acetyl derivatives, and glycerol and mannose were detected in a molar ratio of about 1.0: 64.9 (1.0: 57.8, from the methylation analysis). The glycerol must arise from the non-reducing terminals and 1→6 linked sugar residues. The detection of mannose should be due to the presence of oxidation-resistant mannose residues such as 1→3 linked pyranose units.

The main conclusion to be drawn from these results is as follows. The mannan (T-2-HN) is a linear chain composed largely of α-1→3 linked D-mannopyranosyl residues, but the linear chain also contains traces of 1→6 linked D-mannopyranosyl (and/or glucosyl) residues. Furthermore, trace amounts of branching points are present at position 2 or 4 on each of the 1→3 linked D-mannose residues. Acetyl groups are present in the molecule in the proportion of about one acetyl group per two mannose residues.

Polysaccharides possessing a backbone of α-1→3 linked D-mannopyranose residues that have been studied include heteroglycans such as glucuronoxylomannan from *Tremella fuciformis*,[1] and xylomannan from *Polyporus tumulosus*[16] and *Armillaria mellea*,[17] *etc.* However, the existence of an α-1→3 linked mannan that is homoglycan in nature has not previously been reported. Therefore, this α-1→3 linked D-mannan, a homoglycan, from *Dictyophora indusiata* Fisch. is a novel polysaccharide.

The presence of *O*-acetyl groups in mannan molecules has been demonstrated in β-1→4 linked mannan[18] from "Yamanoimo" and glucomannans from Lilium genus.[19] These studies suggested that such *O*-acetyl groups might affect the solubility of native polysaccharides in water, *i.e.* the deacetylated mannans would no longer be soluble because of molecular association of the linear chains through hydrogen bonds. Our native mannan turned out to be insoluble in water on deacetylation. This observation is similar to those with other acetylated mannans.[18,19] In connection with the influence of deacetylation on the physical properties of our native mannan, it would be of interest to elucidate the exact location of the *O*-acetyl groups in the molecule.

Further investigations on the nature of this mannan are in progress.

Experimental

Solutions were concentrated at or below 40° with a rotary evaporator under reduced pressure. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. Infrared (IR) spectra were analyzed with a JASCO IRA-I spectrometer. Gas-liquid chromatography (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. Gas–liquid chromatography-mass spectrometry (GLC–MS) was carried out with a Shimadzu LKB-9000 gas chromatograph and mass spectrometer.

Materials——The dried fruit bodies of *Dictyophora indusiata* Fisch. are commercially available in Hong Kong. Partially methylated D-mannitol acetates used as reference compounds for GLC and GLC-MS were newly prepared by the modified method of Haworth.[20] Pronase (45000 PUK/g) was purchased from Kaken Chemical Ind., Ltd. DEAE-Sephadex A-25, Sephadex G-200, Sepharose 2B and standard dextrans (Dextran

14) T. Ikenaka, *J. Biochem.* (Tokyo), **54**, 328 (1963).
15) J.K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **78**, 5907 (1956).
16) S.J. Angyal, V.J. Bender, and B.J. Ralph, *Biochim. Biophys. Acta*, **362**, 175 (1974).
17) H.O. Bouveng, R.N. Fraser, and B. Lindberg, *Carbohydr. Res.*, 4, 20 (1967).
18) A. Misaki, T. Ito, and T. Harada, *Agric. Biol. Chem.* (Tokyo), **36**, 761 (1972).
19) M. Tomoda and N. Satoh, *Chem. Pharm. Bull.*, **27**, 468 (1979).
20) N. Handa and R. Montgomery, *Carbohydr. Res.*, **11**, 467 (1969).

T-500 and Blue Dextran T-2000) were purchased from Pharmacia Fine Chemicals.

Extraction and Isolation of Polysaccharide——The fruit bodies (100 g) were pulverized and washed with MeOH. The residue (60 g) was extracted with aqueous 70% EtOH (1 l) for 4 hr in a boiling water bath (5 times). The supernatant obtained by centrifugation at 5000 rpm for 30 min was concentrated and then dialyzed against distilled water for 5 days. The internal solution, after removal of a small amount of insoluble material by centrifugation at 8000 rpm for 30 min, was concentrated and adjusted to pH 7.7 with 1 N NaOH. The mixture was then treated with Pronase (30 mg) at 37° for 48 hr. The reaction mixture was further deproteinized by the Sevag method.[6] The aqueous phase was dialyzed against distilled water for 4 days. The non-dialyzable material (T-2) recovered was dissolved in 0.1 M NaCl and applied to a column (2.6×95 cm) of Sephadex G-200. The column was eluted with 0.1 M NaCl at a flow rate of 12 ml per hr. Fractions (10 ml) were collected and an aliquot of each fraction was analyzed for carbohydrate by the phenol–H_2SO_4 method.[21] The high molecular weight fraction (T-2-H) thus obtained was further purified by column chromatography on DEAE-Sephadex A-25 (2×23 cm), previously equilibrated with 0.01 N sodium acetate buffer at pH 6.0. The neutral fraction eluted with the buffer was dialyzed and lyophilized to afford T-2-HN as colorless flakes (yield: about 0.4% from the starting material).

The purified polysaccharide (T-2-HN) was soluble in H_2O and gave a slightly viscous solution. $[\alpha]_D^{20}$ +116° (c=0.1, H_2O). IR ν_{max}^{KBr} cm^{-1}: 1730, 1260 (ester), 820 (type 2a, α-glycosidic linkage), 880 (type 2c), 793, 915, 975. Elemental analysis (%): C, 43.60; H, 6.06; N, 0.49; ash, nil.

Gel Chromatography on Sepharose 2B——T-2-HN (1.9 mg) and standard Dextran T-500 (1.3 mg; $\bar{M}w$, 495000) were dissolved in 0.1 M NaCl (0.4 ml) and applied to a column (1.5×97.5 cm) of Sepharose 2B. The column was eluted with 0.1 M NaCl at a flow rate of 8 ml per hr. Fractions of 4 ml were collected and analyzed by the phenol–H_2SO_4 method.[21]

Free-boundary Electrophoresis——Tiselius-type electrophoresis of T-2-HN was carried out with a Hitachi HID-1 boundary electrophoresis apparatus in 0.05 M sodium tetraborate buffer at pH 9.3 for 80 min. Electrophoretic mobility: $u=0.26\times10^{-5}$ $cm^2/V\cdot sec$.

Identification of *O*-Acetyl Group——1) T-2-HN (7 mg) was suspended in MeOH (2 ml) containing 0.2 M sodium methoxide, and the mixture was allowed to stand at room temperature for 5 hr. The reaction mixture was centrifuged at 5000 rpm for 15 min, and the supernatant was evaporated to dryness. The residue was dissolved in H_2O (0.4 ml), and a small amount of Amberlite CG-120 (H^+ form) resin was added. After thorough mixing, the supernatant was analyzed by GLC. Gas–liquid chromatography was carried out under the following conditions: a glass column packed with Chromosorb 101 (60 to 80 mesh) at 165° at a flow rate of 41 ml per min of N_2. Only one peak was observed, and the retention time (2.5 min) was identical with that of acetic acid.

2) T-2-HN (3 mg) and penta-*O*-acetyl-β-D-glucose as a standard were treated with hydroxylamine according to the procedure of Stadtman *et al.*[7] Paper partition chromatography of the hydroxamate derivative thus obtained was carried out on Toyo Roshi No. 51A filter paper by the ascending method with *n*-BuOH–AcOH–H_2O (4: 1: 1, v/v). Ferric chloride solution was used as spraying reagent for detection. Only one spot was detected and the *Rf* value (0.50) of the hydroxamate derivative from T-2-HN was the same as that of acetylhydroxamate from penta-*O*-acetyl-β-D-glucose.

The Deacetylated Polysaccharide——The native polysaccharide (T-2-HN, 17.0 mg) was dissolved in 0.5 N NaOH (8 ml) and stirred at room temperature for 3 hr. A flocculent material was liberated by neutralization of the alkaline solution with 0.5 N HCl. The resulting insoluble material was separated by centrifugation at 8000 rpm for 20 min, washed 5 times with H_2O and dried in a vacuum desiccator (yield: 13.5 mg, 81.3%). The alkali-treated polysaccharide thus obtained was insoluble in H_2O. $[\alpha]_D^{27}$ +119° (c=0.1, 2 N NaOH). IR ν_{max}^{KBr} cm^{-1}: 820, 880, 793, 915, 975.

Component Sugars——T-2-HN (1.5 mg) was hydrolyzed with 2 N H_2SO_4 (1 ml) in a sealed tube at 100° for 6 hr. The 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. Paper partition chromatography was carried out on Toyo Roshi No. 51A filter paper by the double ascending method 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 $AgNO_3$[22] and naphthoresorcinol-H_3PO_4[23] reagents. Mannose and a trace of glucose were detected in the hydrolysate. *Rglc*: mannose 1.17, glucose 1.00 (solvent A), mannose 1.10, glucose 1.00 (solvent B).

A residual portion of the hydrolysate was then reduced with $NaBH_4$ for 3 hr at room temperature. The excess borohydride was decomposed by addition of Amberlite CG-120 (H^+ form) resin. After removal of the resin by filtration, the filtrate was concentrated to dryness and freed from boric acid by repeated evaporations with MeOH. The residue was acetylated with Ac_2O and dry pyridine (1: 1, 0.5 ml) at 95° for 90 min. After the mixture had been concentrated with a small amount of toluene, the residue was dissolved

21) M. Dubois, K.A. Gills, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
22) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature* (London), **166**, 444 (1950).
23) V. Prey, H. Berbalk, and M. Kausz, *Mikrochim. Acta*, **1961**, 968.

in $CHCl_3$ and analyzed by GLC. Gas-liquid chromatography was carried out under the following conditions: a glass column packed with 3% ECNSS-M on AW-Gaschrom Q (100 to 120 mesh) at 185° at a flow rate of 43 ml per min of N_2. The retention times of acetates of mannitol and glucitol were 27.2 and 35.6 min, respectively. The molar ratio of Man: Glc was about 100: 1, as determined by GLC analysis of the alditol acetate derivatives.

Total Sugar and Acetyl Content——Total sugar content of T-2-HN was determined as anhydromannose by the anthrone–H_2SO_4 method.[9] Acetyl content was estimated by the method of Hestrin[8] using penta-*O*-acetyl-*β*-D-glucose as a standard.

Recovery of Crystalline D-Mannose from the Hydrolysate of the Deacetylated Polysaccharide——The deacetylated polysaccharide (4.3 mg, $[\alpha]_D^{27}$ +119° in 2 N NaOH, $c=0.1$) was hydrolyzed with 2 N H_2SO_4 (4 ml) in a sealed tube at 100° for 24 hr. After cooling, one volume of H_2O was added to the hydrolysate, and the specific rotation was measured; $[\alpha]_D^{16}$ +13.3° in 1 N H_2SO_4, $c=0.060$ as mannose. Authentic D- and L-mannose gave the following results: D-mannose, $[\alpha]_D^{16}$ +14.0° equil., in 1 N H_2SO_4, $c=0.139$. L-Mannose, $[\alpha]_D^{21}$ −13.9° equil., in 1 N H_2SO_4, $c=0.215$. The hydrolysate was neutralized with $BaCO_3$ and then treated with a column of Amberlite CG-120 (H^+ form) resin as described above. The resulting residue was crystalized from a mixture of MeOH and iso-propyl alcohol by allowing it to stand in a freezer for several days. The crystals (mp 128—130°) thus obtained were identified as D-mannose by mixed melting point determination and by IR spectral comparison with an authentic sample.

Methylation Analysis——T-2-HN (3.8 mg) was methylated twice by the method of Hakomori[12] as described in our previous report.[24] The final methylated product showed no hydroxyl absorption band in the IR spectrum. The fully methylated polysaccharide was heated with 90% HCOOH (1 ml) at 100° for 2 hr in a sealed tube. After removal of HCOOH by evaporation, the residue was hydrolyzed with 0.5 N H_2SO_4 at 100° for 15 hr followed by neutralization with Amberlite CG-400 (CO_3^{2-} form) resin. The resulting methylated sugars were reduced with $NaBH_4$ to the corresponding alditols, and then acetylated with Ac_2O and pyridine as described above. Gas–liquid chromatography (GLC) of the partially methylated alditol acetates was carried out under the following conditions: condition A, a glass column packed with 3% Silicone OV-225 on AW-DMCS-Chromosorb W (80 to 100 mesh) at 185° at a flow rate of 43 ml per min of N_2; condition B, a glass column packed with 3% ECNSS-M on AW-Gaschrom Q (100 to 120 mesh) at 172° at a flow rate of 43 ml per min of N_2. Table I shows the relative retention times of methylated sugars with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Gas–liquid chromatography–mass spectrometry (GLC–MS) of partially methylated alditol acetates was carried out using the above 3% Silicone OV-225 column (0.4×100 cm) at 185° 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°.

Periodate Oxidation of T-2-HN——Periodate oxidation was carried out, monitoring by the method of Ikenaka.[14] T-2-HN (7.4 mg) and methyl α-D-glucoside (3.0 mg) as a standard were oxidized with 2.5 mM $NaIO_4$ in 0.0025 N sodium acetate buffer (pH 4.2, 40 ml) at 3—5° for 16 days in the dark. After various times, the periodate consumption was measured by a spectrophotometric method (290 nm). The periodate consumption per anhydrohexose unit was 0.06 mol. In this experiment, the value of 1.98 mol of periodate consumption per methyl α-D-glucoside was in good agreement with the theoretical value.

Reduction of the Periodate-oxidized T-2-HN——The above oxidized polysaccharide solution, after addition of ethylene glycol (0.1 ml), was dialyzed against distilled water for 3 days. The non-dialyzable material was then reduced with $NaBH_4$ (24 mg) for 48 hr at 5°. After acidification with 0.1 N AcOH to pH 5, the mixture was dialyzed against distilled water for 4 days. The flocculent insoluble material formed in the internal solution was collected by centrifugation at 8000 rpm for 30 min, and dried in a vacuum desiccator (yield: 5.3 mg). The polyalcohol thus obtained showed no absorption band (1730 and 1260 cm^{-1}) due to ester linkages in the IR spectrum.

Smith Degradation and Analysis of the Products——The polyalcohol (1.0 mg) was hydrolyzed with 2 N H_2SO_4 (1.5 ml) at 100° for 24 hr. The hydrolysate was neutralized with $BaCO_3$, reduced with $NaBH_4$ and acetylated with Ac_2O and pyridine as described above. The acetylation mixture of the Smith degradation products was analyzed by GLC using dual columns packed with 3% ECNSS-M on AW-Gaschrom Q; the column temperature was increased by 6° per min from 60° to 185°; carrier gas, N_2 (43 ml per min). Retention times of acetate derivatives of sugar alcohols were as follows: glycerol 17.1 min, mannitol 47.4 min. The molar ratio of glycerol and mannose, as determined by GLC analysis, was about 1.0: 64.9.

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24) S. Ukai, C. Hara, T. Kiho, and K. Hirose, *Chem. Pharm. Bull.*, **26**, 1729 (1978).