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Extracellular Heteroglycan of *Cladosporium tricoides*.[1] Studies on Fungal Polysaccarides. XVI[2]

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Major water-soluble extracellular polysaccharide *C. tricoides* obtained by diethylaminoethyl-cellulose column fractionation is a heteroglycan, $[\alpha]_D^{20}-34°$ ($c=1$, H_2O), which was composed of D-galactose, D-glucose, D-mannose, and L-rhamnose (7.3 : 1.0 : 8.7 : 2.2). The results of periodate oxidation, Smith-type degradation, methylation studies, and GC-mass spectra showed that the polysaccharide has a highly branched structure and contains 1→6 linked mannopyranosyl, 1→4 linked galactopyranosyl and 1→2 linked galactofuranosyl residues as main units, and that the glycan is branched at C2 and C3 or C6 positions of mannose residue. The terminal groups are rhamnopyranosyl, galactofuranosyl, and mannopyranosyl residues. A probable structure was proposed.

The fungi belonging to the genus *Cladosporium* are one of the most common groups in nature. In a previous work, we isolated extracellular and intracellular polysaccharides from *C. herbarum* and their chemical structures were investigated.[2,4] In the present work, chemical structure of the main extracellular polysaccharide of *C. tricoides* was examined. *C. tricoides*

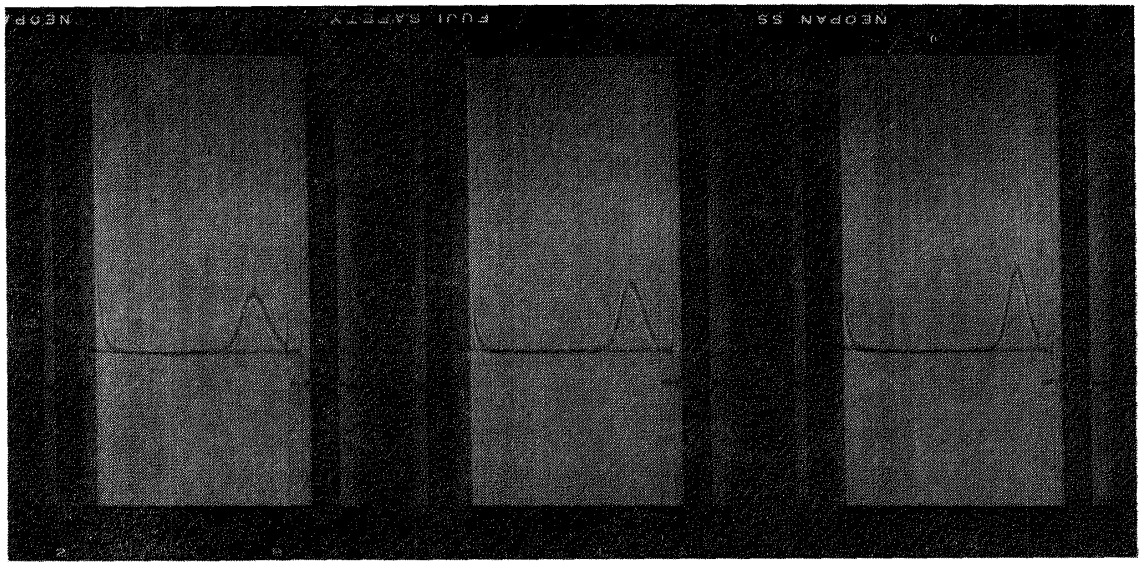


Fig. 1. Ultracentrifugal Sedimentation Patterns of CTP-1

apparatus: Hitachi ultra centrifuge Model 282
solvent: 0.1M NaCl
concentration: 0.5%.
speed: 60000 rpm
bar angle: 70°

1) A part of this work was presented at the 93rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1973.
2) Part XV: T. Miyazaki and Y. Naoi, *Chem. Pharm. Bull.* (Tokyo), **22**, 2058 (1974).
3) Location: *a) 20-1, Kitashinjuku, 3-chome, Shinjuku-ku, Tokyo, 160, Japan; b) 24-1, Hyakunin-cho, 3-chome, Shinjuku-ku, Tokyo, 160, Japan.*
4) Part XIII: T. Miyazaki and Y. Naoi, *Chem. Pharm. Bull.* (Tokyo), **22**, 1360 (1974).

is well known as a kind of pathogenic fungus which causes chromoblastmycosis, and gleyish cream colonies are formed rapidly, but they grow slowly and often show yeast form.

The crude polysaccharide isolated from the culture liquid was treated with Pronase E and by the Sevag method, followed by diethylamisoethyl (DEAE)-cellulose column chromatography using sodium hydrogen carbonate for elution and then rechromatography over a column of DEAE-cellulose (borate form).

The purified main polysaccharide (CTP-1), $[\alpha]_D^{20}-34°$ ($c=1$, H_2O), gave a single spot on paper electrophoresis using a borate buffer (0.026M, pH 10.0), and contained 94% of sugar (as glucose, by the procedure of Dubois, *et al.*[5]). Neither nitrogen nor phosphorus was detected by elemental analysis. CTP-1 was found to be pure by the ultracentrifugal analysis (Fig. 1).

Component sugars of CTP-1 were identified as galactose, glucose, mannose, and rhamnose by paper chromatography (PPC) of the acid hydrolyzate, and their molar ratio was estimated as approximately 7.3: 1.0: 8.7: 2.2 by the procedure of Dubois, *et al.*[5]

On periodate oxidation of CTP-1, the amount of periodate consumption, and formic acid and formaldehyde liberated per sugar unit were 1.35, 0.29, and 0.18 moles, respectively.

Smith–type degradation[6] products were arabinose, threitol, glycerol, 1,2-propanediol, and oxidation-resistant component sugars. By this treatment, the molar ratio changed to 1.0: 1.0: 1.5: 1.0: 2.0: 2.1: 6.1 (Gal -Glc -Man -Rha -Ara -Thr -Gly) and a small amount of 1,2-propanediol was also produced. These results suggested, at least the presence of 1,2- or 1,3-linked galactofuranosyl (source of arabinose), 1,4-linked galactopyranosyl, 1,5- or 1,6-linked galactofuranosyl (source of threitol), 1,2-linked or terminal rhamnopyranosyl (source of propanediol), 1,2-, 1,6-, or terminal linked hexopyranosyl (source of glycerol), and oxidation-resistant linkages such as 1,3-linked or branching point of each component sugar residues.

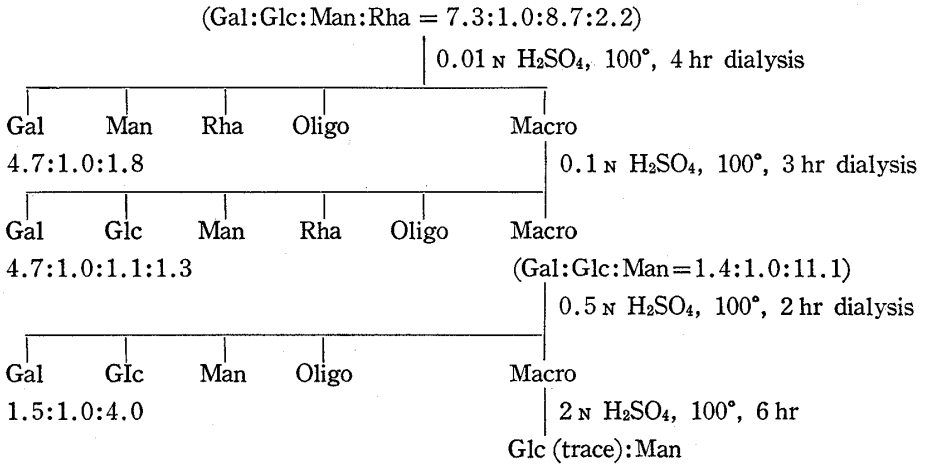


Chart 1. Partial Acid Hydrolysis of the Polysaccharide
Macro: non dialyzable material

Partial acid-hydrolysis of CTP-1 using sulfuric acid was examined under four different conditions (treatment (a), 0.01N 100°, 4 hr; treatment (b), 0.1N 100°, 3 hr; treatment (c), 0.5N 100°, 2 hr; treatment (d), 2N 100°, 6 hr), and the fragments released were separated by dialysis. Results of partial hydrolysis are summarized in Chart 1. The dialyzable fragments were submitted to PPC, an aliquot of the non-dialyzable material was hydrolyzed to determine their molar ratio, and the residue was submitted to the next treatment. The dialyzable fragments liberated by treatment (a) were galactose, mannose, and rhamnose in approximate molar ratio of 4.7: 1.0: 1.8, and the second dialyzable fragments released by treatment (b)

5) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
6) J.K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **78**, 5907 (1956).

of the non-dialyzable material were galactose, glucose, mannose, and rhamnose in approximate molar ratio of 4.7: 1.0: 1.1: 1.3. After treatment (b), component of the non-dialyzable fragment changed to galactose, glucose, and mannose (molar ratio, 1.4: 1.0: 11.1). The rhamnose content liberated by treatment (a) and (b) corresponded to 61% and 38% of the total rhamnose, respectively. Similarly, the released galactose content corresponded to 47% and 38% of the total galactose, respectively. Thus, all of rhamnose and a large amount of galactose were released by the treatment with 0.01N and 0.1N sulfuric acid. The dialyzable fragments released by treatment (c) were galactose, glucose, and mannose in approximate molar ratio of 1.5: 1.0: 4.0, and the final treatment (d) of the non-dialyzable fragment gave glucose (trace) and mannose. The results of partial hydrolysis suggested that this gluco-rhamnogalactomannan has an acid-resistant core consisting of mannose, and it has branching moieties including acid-labile galactosyl and rhamnosyl residues.

After CTP-1 was methylated by the methods of Hakomori[7] and of Purdie,[8] methanolysis and hydrolysis were carried out, and *O*-methyl-monosaccharides formed were examined by PPC, thin-layer chromatography (TLC), paper electrophoresis (PE), and gas-liquid chromatography (GLC).

TABLE I. Relative Retention Time of Acetyl Alditol of the Methylated CTP-1 Relative to 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol

	CTP-1 component (ECNSS-M column)	Authentic *O*-methyl-alditol[9]	CTP-1 component (OV-225 column)	Authentic *O*-methyl-alditol[9]	Molar ratio
1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol	0.47	0.47	0.35	0.35	2
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl mannitol	1.00	1.00	0.99	0.99	0.4
1,4-Di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl galactitol	1.15	1.15	1.10	1.10	1.5
1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methyl rhamnitol	1.00	0.99	0.94	0.94	0.4
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol	1.95	1.95	1.81	1.82	1
1,2,4-Tri-*O*-acetyl-3,5,6-tri-*O*-methyl galactitol	1.95	—	1.81	—	2
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol	2.28	2.28	2.02	2.03	0.2
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol	2.47	2.42	2.20	2.22	2
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-Methyl mannitol	2.47	2.48	2.20	2.19	4
1,4,6-Tri-*O*-acetyl-2,3,5-tri-*O*-methyl galactitol	3.28	3.28	2.76	2.76	1
1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methyl mannitol	3.28	3.28	2.92	2.92	1
1,2,5,6-Tetra-*O*-acetyl-3,4-di-*O*-methyl mannitol	5.35	5.37	4.32	4.36	3

From the results of PPC and TLC, di-*O*-methyl-, tri-*O*-methyl-, and tetra-*O*-methyl-monosaccharides were detected in the ratio of 1: 2.9: 1. Di-*O*-methyl-monosaccharide fraction was assumed to be 4,6-di-*O*-methyl- and 3,4-di-*O*-methyl-mannose (MG values, 0.43 and 0.50) by PE using 1% borate. The methylated CTP-1 was converted into alditol acetates

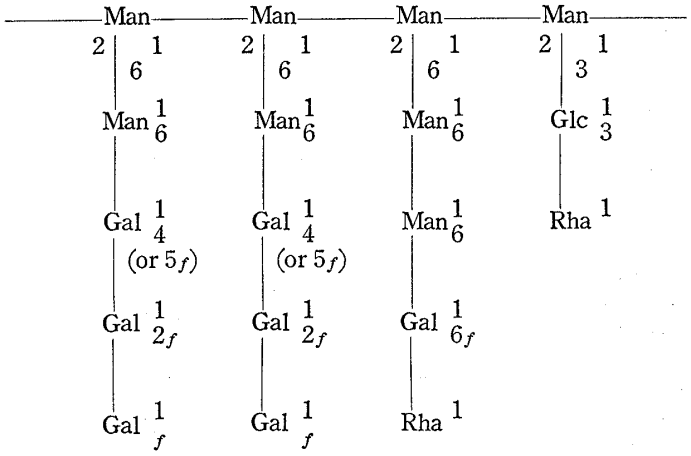
7) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
8) T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).
9) G. Victor, *Methods Enzymol.*, **28**, 182 (1972).

by the procedure described in our previous paper,[4] and then the products were analyzed by GLC. Their relative retention time was consistent with the values in literature.[9] As shown in Table I, the products detected were 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl-D-galactitol, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol, 1,2,4-tri-*O*-acetyl-3,5,6-tri-*O*-methyl-D-galactitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol, 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methyl-D-galactitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol, 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-D-mannitol, and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-mannitol.

The methylated CTP-1 was converted into methylglycosides by methanolysis, the products were further analyzed by GLC under a different condition, and results on the methylglycosides obtained by GLC were quite consistent with those of the alditol acetates of CTP-1.

The results of gas–mass spectrometry (GC-mass) of the peaks corresponding to 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methyl-D-galactitol, 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-D-mannitol, and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-mannitol were consistent with those in the literature.[10] The peak corresponding to 1,2,4-tri-*O*-acetyl-3,5,6-tri-*O*-methyl-D-galactitol was identified by GC-mass (*m/e*: 43, 45, 87, 89, 101, 117, 129, 161, 189). Therefore, the source of arabinose in the Smith degradation product should be 1,2-linked galactofuranosyl residue, and similarly, source of threitol should be 1,4-linked galactopyranosyl or either 1,5- or 1,6-linked galactofuranosyl residue. In the periodate oxidation, rapid formation of formaldehyde suggests the presence of furanosyl residues.

From these results, it is concluded that CTP-1 has a highly branched structure and a probable structure of main portion in the polysaccharide will be as follows:



In 1970, Lloyd[11] reported the presence of a peptide-phospho-galactomannan in the cell wall of *C. werneckii*. This heteroglycan contains both acid- and alkali-sensitive linkages, and it is constructed with 1,2-, 1,3-, and 1,6-linked mannosyl and terminal galactosyl residues. On the other hand, as described in our previous paper,[2,4] cell wall of *C. herbarum* contains a water-soluble 1,3- and 1,4-linked linear glycan including a few branches, and the main extracellular polysaccharide of *C. herbarum* has a highly branched structure which contains 1,2,4-linked mannan core, 1,4-linked galactofuranosyl, 1,6-linked mannopyranosyl, and terminal galactofuranosyl and mannopyranosyl residues. In the case of CTP-1, as described above, it has a highly branched structure consisting of 1,2,6- and 1,2,3-linked mannan core and heterogenous branches including, 1,2-linked galactofuranosyl residue. Therefore, it is certain that

10) H. Bjorndal, B. Lindberg, and S. Svensson, *Carbohyd. Res.*, **5**, 433 (1967).
11) K.O. Lloyd, *Biochemistry*, **9**, 3446 (1970); *idem*, *FEBS Lett.*, **11**, 91 (1970).

both extracellular polysaccharides of *Cladosporium* have evidently different structures. Particularly, presence of rhamnose and different mannan core is taxonomically quite interesting.

Minor extracellular polysaccharide CTP-2, consisting of galactose, glucose, and mannose was isolated from the sodium hydrogen carbonate eluted fraction, but could not examine in detail.

Experimental

Isolation of Crude Extracellular Polysaccharide——The organism used in this study, *Cladosporium tricoides* OUT 4294, was kindly supplied by the Faculty of Engineering, Osaka University. Incubation was carried out at 25° for 20 days in a 300 ml Erlenmeyer flask containing 4% glucose and 1% dialyzable peptone. After the mycelium was removed by filtration, the culture fluid (10 liters) was dialyzed in a Visking cellulose tubing against running water for 3 days. Non-dialyzable solution was concentrated to a small volume in vacuum below 40°. A precipitate, formed by the addition of 5 volumes of EtOH, was collected by centrifugation, washed with EtOH and $(CH_3)_2CO$, and dried *in vacuo*.

Digestion of Crude Polysaccharide with Pronase E——About 10 g of the crude polysaccharide was dissolved in 100 ml of distilled water, the solution was adjusted to pH 7.8 with Na_2CO_3, and 0.5 g of Pronase E (Kaken Kagaku Co. Ltd.,) was added, and the mixture was incubated for 4 days at 30°. Then the mixture was dialyzed in a Visking cellulose tubing against running water for 2 days. The solution in the tube was concentrated to 100 ml and shaken vigorously for 0.5 hr with 20 ml of $CHCl_3$–BuOH (4: 1). After repeated centrifugation until a gelatinous substance was no longer formed in the mixture, the supernatant was concentrated to a small volume *in vacuo* below 40°, and 5 volumes of EtOH was added to this concentrate. The precipitate was collected by centrifugation, washed with EtOH, $(CH_3)_2CO$, and $(C_2H_5)_2O$, and dried *in vacuo*. The same procedure was repeated 3 times. Yield, 0.28 g per liter of cultivated fluid.

Fractionation of Crude Polysaccharide by Ion-exchange Chromatography——The crude polysaccharide (2 g) was applied to a column (4.5×50 cm) of DEAE-cellulose (OH^-), and treated as described in our previous paper.[4] Yields were as follows: H_2O eluate, 1.47 g (73.5%); total of each fraction of $NaHCO_3$ eluate, 0.23 g (11.5%); 0.1M NaOH eluate, trace. The H_2O fraction (1.47 g) in H_2O (20 ml) was further submitted to a column (3.5×45 cm) of DEAE-cellulose (borate form). Stepwise elutions with H_2O and 0.01M, 0.05M, and 0.1M $Na_2B_4O_7$, and finally with 0.1M NaOH were carried out in the same way as above, and yields were as follows: H_2O eluate (CTP-1) 1.41 g (70.5%); 0.01M, 0.05M, and 0.1M $Na_2B_4O_7$ eluates trace, and 0.1M NaOH eluate nil. The $NaHCO_3$ fraction (0.23 g) dissolved in 15 ml of H_2O was further treated similarly, and yields were as follows: H_2O eluate nil, 0.01M $Na_2B_4O_7$ eluate 0.14 g (7%) (CTP-2), 0.05M $Na_2B_4O_7$ eluate trace, 0.1M $Na_2B_4O_7$ eluate 0.08 g (4%), 0.1M NaOH eluate nil.

Properties of CTP-1——Paper electrophoresis of CTP-1 using 0.026M borate buffer (pH 10.0) showed a single spot (detected with the periodate-Schiff reagent[12]). CTP-1, $[\alpha]_D^{20}$ −34° (c=1, H_2O), contained 94% of sugar (as glucose, by the method of Dubois, *et al.*[5]) and free from P and N (elemental analysis).

Sedimentation Analysis——Sedimentation analysis of CTP-1 was performed with a Hitachi ultracentrifuge Model 282. Measurement was made at 60000 rpm at a concentration of 10 mg/ml of CTP-1 in 0.1M NaCl at 18° and photographed at 38, 50, 62, 74, 86, and 98 min after reaching full speed.

Component Sugars of CTP-1——A solution of 20 mg of CTP-1 dissolved in 3 ml of 1N H_2SO_4 in a sealed tube was heated in a boiling water bath for 6 hr. After neutralization ($BaCO_3$) and filtration, a portion of the hydrolyzate was concentrated and applied to Whatman No. 1 filter paper. PPC was carried out by the ascending method, using AcOEt–pyridine–H_2O (10: 4: 3) (solvent system A). Sugars were detected by spraying a solution of alkaline $AgNO_3$[13] and *p*-anisidine hydrochloride.[14] Galactose, glucose, mannose, and rhamnose were identified with a molar ratio of 7.3: 1.0: 8.7: 2.2 (by the method of Dubois, *et al.*[5]).

Periodate Oxidation of CTP-1——CTP-1 (20 mg) was oxidized with 50 ml of 0.018M $NaIO_4$ at room temperature in the dark. A blank solution containing no glycan was processed similarly. A 3 ml aliquot was set aside at different periods for determination of $NaIO_4$ consumption,[15] and for formation of HCOOH[16] and HCHO.[17] The moles of $NaIO_4$ consumed per anhydro component sugar unit were as follows: 0.22 (1 hr), 0.76 (3 hr), 0.90 (6 hr), 1.10 (12 hr), 1.24 (24 hr), 1.26 (48 hr), 1.35 (72 hr). HCOOH: 0.18 (3 hr), 0.21 (6 hr), 0.24 (12 hr), 0.26 (24 hr), 0.29 (48 hr), 0.29 (72 hr). HCHO: 0.18 (2 hr), 0.08 (12 hr), 0.06 (24 hr), 0.04 (48 hr), 0.05 (72 hr).

12) E. Köiw and A. Grönwall, *Scand. J. Clin. Invest.*, **4**, 244 (1952).
13) L. Malaprade, *Bull. Soc. Chim. France*, [5] **1**, 833 (1934).
14) R.L. Whistler and J.L. Hickson, *J. Am. Chem. Soc.*, **76**, 1671 (1954).
15) J.F. O'Dea and R.A. Gibbons, *Biochem. J.*, **55**, 580 (1953).
16) M. Lambert and A.C. Neish, *Can. J. Res.*, **28B**, 83 (1950).
17) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature*, **166**, 444 (1950).

Smith-type Degradation of Periodate-oxidized CTP-1——After oxidation of CTP-1 as described above, the reaction mixture was submitted to Smith-type degradation as described in our previous paper,[4] and 6 spots corresponding to standard galactose, glucose, mannose, rhamnose, arabinose, threitol, and glycerol were detected (alkaline–$AgNO_3$[13] and *p*-anisidine–HCl[14]) as the main products. The relative molar ratio of hexoses and rhamnose was determined by the method of Dubois, *et al.*,[5] and that of threitol and glycerol by the method of Lambert-Neish.[18] The molar ratio was approximately 1.0: 1.0: 1.5: 1.0: 2.0: 2.1: 6.1 (Gal -Glc -Man -Rha -Ara -Thr -Gly). Propanediol was detected by GLC of trimethylsilyl (TMS) derivative of Smith-type degradation products. The hydrolyzate was neutralized with $BaCO_3$, and after passing through a short column of Amberlite IR-120 (H^+), the clear filtrate was evaporated to dryness and the residue was treated with 5 ml of pyridine–hexamethyldisilazane–trimethylchlorosilane (10: 2: 1). The mixture was extracted by $CHCl_3$, then the $CHCl_3$ layer was treated with $CuSO_4$ solution, $CHCl_3$ layer further evaporated to dryness, and the residue was dissolved in $(CH_3)_2CO$. TMS derivatives was performed in a Shimadzu GC-5A unit, equipped with a flame ionization detector, using a 200×0.3 cm glass column packed with 10% polydiethylene glycol adipate on Neopak 1A (40—60 mesh); column temperature, 75—150°; N_2 flow rate, 50 ml/min. Retention time was quite consistent with that of the control.

Partial Acid Hydrolysis of CTP-1——Partial acid hydrolysis of CTP-1 using H_2SO_4 was examined under four different conditions (treatment (a): 0.01N, 100°, 4 hr; treatment (b): 0.1N, 100°, 3 hr; treatment (c): 0.5N, 100°, 2 hr; treatment (d): 2N, 100°, 6 hr). The fragments released were separated by dialysis and non-dialyzable material was treated by the procedure described in our previous paper.[4] Results of partial hydrolysis of each fraction are summarized in Chart 1.

Methylation of CTP-1——Methylation of CTP-1 (50 mg) was carried out by the procedure described in our previous paper.[2] Completely methylated polysaccharide was extracted with $CHCl_3$ from this reaction mixture which showed no significant absorption band of OH in the 3500 cm^{-1} region in IR spectrum.

Reduction and Acetylation of *O*-Methyl-monosaccharide Obtained from Methylated CTP-1——The methylated CTP-1 (50 mg) was heated with 90% HCOOH in a boiling water bath for 4 hr, and treated by the procedure described in our previous paper.[2]

GLC of Methyl *O*-Methylated Glycosides and *O*-Methylated Alditol Acetates Obtained from Methylated CTP-1——Methylated CTP-1 was converted into methylglycosides by the procedure described in our previous paper.[2] GLC of methanolyzate and alditol acetate of methylated CTP-1 was performed in a Shimadzu GC-5A unit equipped with a flame ionization detector under three different conditions. For the methanolyzate of methylated CTP-1: A glass column (200×0.3 cm) packed with 15% polybutane-1,4-diol succinate on Celite 545 (60—80 mesh) was used; N_2 flow rate, 50 ml/min; column temperature, 175°. For the alditol acetate of methylated CTP-1: (1) A glass column (200×0.3 cm) containing 5% (w/w) of ECNSS-M on Chromosorb W (aw-dmcs, 60—80 mesh) was used; N_2 flow rate, 40 ml/min, column temperature, 180°; (2) A glass column (200×0.3 cm) containing 3% (w/w) of Silicone OV-225 on Chromosorb W (aw-dmcs, 60—80 mesh) was used; N_2 flow rate, 20 ml/min; column temperature 170°.

Gas Chromatograph-Mass (GC-mass) Spectra of Partially Methylated Alditol Acetates from CTP-1——Methylated CTP-1 in water was reduced with $NaBH_4$ (100 mg) for 12 hr. After treatment with Amberlite IR-120 (H^+) and codistillation with MeOH in the usual manner, the resulting product was treated with 5 ml of Ac_2O–pyridine (1: 1), at 100° for 15 min. After acetylation, the mixture was treated as described in our previous paper,[2] dissolved in $(CH_3)_2CO$, and subjected to GC-mass spectrometry. The spectra were determined with Shimadzu Model LKB-9000 mass spectrometer, equipped with a glass column packed with 3% Silicone OV-225 on Chromosorb W (aw-dmcs, 80—100 mesh), operated at 170°; electron energy 70 eV, trap current 60 μA; temperature of the ion source, 310°. 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylmannitol: *m/e* 43, 45, 87, 101, 129, 161, and 261; 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylmannitol: 43, 87, 99, 129, and 189; 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methylgalactitol: 43, 101, and 117.

PPC and Paper Electrophoresis of the Hydrolyzate of Methylated CTP-1——The methylated CTP-1 was treated with HCOOH and dil. H_2SO_4 method.[19] PPC of the hydrolyzate of methylated CTP-1 was examined by using AcOEt–AcOH–H_2O (9: 2: 2), and 3 spots corresponding to tetra-*O*-methyl-, tri-*O*-methyl-, and di-*O*-methyl-monosaccharides were detected. Di-*O*-methyl-monosaccharide was suggested from PE using 1% $Na_2B_4O_7$ solution as 4,6-di-*O*-methyl-D-mannose (MG value; 0.43), 3,4-di-*O*-methyl-D-mannose (MG value; 0.50). Reported value[20] for di-*O*-methyl mannoses: 0.49 (3,4-), 0.43 (4,6-), 0.09 (2,6-), 0.14 (2,3-), 0.39 (3,6-).

Component Sugars of Minor Polysaccharide (CTP-2)——A part of the minor fraction was hydrolyzed with 1N H_2SO_4 for 6 hr at 100°, and the resultant sugars were detected by PPC as described above. Galactose, glucose, and mannose were detected.

18) L. Hough, J.K.N. Jones, and W.H. Wadman, *J. Chem. Soc.*, 1702 (1950).
19) H.O. Bouveng, H. Kiessling, B. Lindberg, and J. McKay, *Acta. Chem. Scand.*, **16**, 615 (1962); H.O. Bouveng and B. Lindberg, *Methods Carbohyd. Chem.*, **5**, 297 (1965).
20) T. Miyazaki, *Chem. Pharm. Bull.* (Tokyo), **9**, 831 (1961).