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A Glucomannan from the Tubers of *Dioscorea japonica* THUNB.[1)]

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A polysaccharide, $[\alpha]_D^{20}$ −35.8° in water, was isolated from the tubers of *Dioscorea japonica* THUNB. The polysaccharide was homogeneous as judged by ultracentrifugal analysis and gel chromatography. It was mainly composed of D-mannose and D-glucose in a molar ratio of 6:1, and contained 8.8% *O*-acetyl groups. Its molecular weight was estimated to be 3.7×10^5 by gel chromatography. The *O*-acetyl groups were located at position 2, at position 6, at position 3, and at positions 2 and 6, of some of the mannopyranosyl residues. From the results of methylation analysis, periodate oxidation, Smith degradation, and carbon-13 nuclear magnetic resonance spectroscopy, it was concluded that the polysaccharide, a linear glucomannan, was composed of β-1→4 linked D-aldohexopyranosyl units.

Keywords——glucomannan; *Dioscorea japonica*; *O*-acetyl groups; molecular weight; methylation analysis; Smith degradation; ^{13}C-NMR; polysaccharide structure

Dioscorea japonica THUNB. (Japanese name: Yamanoimo or Jinenjo) (Dioscoreaceae) grows wild in forests in Japan, and the tubers are used as a food and medicine. In studies on polysaccharide fractions from the mucilage of plants belonging to Dioscoreaceae, Sato *et al.*[2)] reported a mannan fraction from the mucilage of *Dioscorea batatas* forma *Icho*, and Misaki *et al.*[3)] isolated a β-1→4 linked D-mannan from the tubers of *D. batatas* forma *Tsukune*. Recently, Tomoda *et al.*[4)] isolated "Dioscorea-mucilage B" from the tuberous rhizophores of *D. batatas*, and showed that the polysaccharide moiety is composed mostly of branched β-1→4 linked D-mannopyranosyl residues, with *O*-acetyl groups located at positions 6, and 2, 3, 6 of some of the mannose units. However, the precise structure of a pure polysaccharide from the tubers of *D. japonica* has not yet been reported. We have now isolated a pure polysaccharide fraction from the tubers of the plant. The present article deals with the purification, characterization, and structural analysis of the polysaccharide.

The fresh tubers were sliced, homogenized, and extracted with cold water. In order to remove proteins in the extract, it was treated with Pronase, followed by dialysis. A small amount of insoluble material in the non-dialyzable fraction was removed by centrifugation and filtration. The solution thus obtained was precipitated with ethanol, and the precipitate was dissolved in water, then purified by the Sevag procedure.[5)] The aqueous solution was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25, and the neutral fraction was lyophilized to afford a polysaccharide (Y-N: Yamanoimo-polysaccharide of the neutral fraction) as colorless flakes in *ca.* 0.15% yield.

The polysaccharide, which had a negative specific rotation ($[\alpha]_D^{20}$ −35.8° in water, $c=0.5$), was homogeneous as judged by ultracentrifugal analysis (Fig. 1) and gel chromatography (Fig. 2). The total sugar content of Y-N was found to be 88.9% (as hexosyl residues) by the phenol–sulfuric acid method.[6)] The calibration curve shown in Fig. 3 was obtained by gel chromatography of standard dextrans on Sephacryl S-300; the molecular weight of Y-N thus estimated was 3.7×10^5. Y-N contained mannose and glucose in a molar ratio of 6:1, as

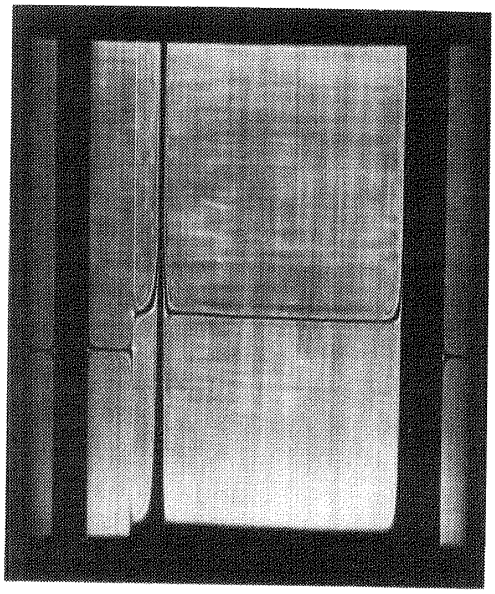

Fig. 1. Ultracentrifugal Pattern of Y-N

Y-N (0.5% in H_2O) after 45 min at 60000 rpm at 20 °C.

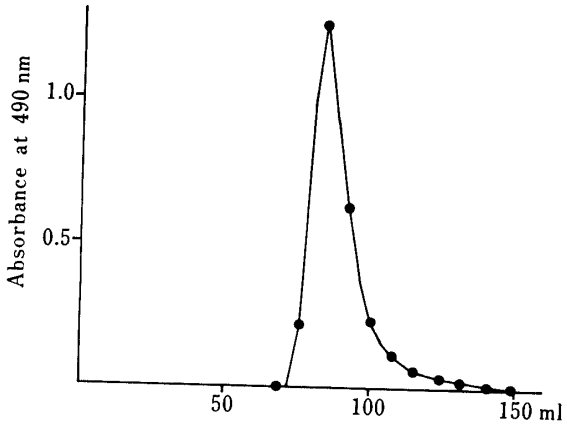

Fig. 2. Chromatogram of Y-N on Sephacryl S-300

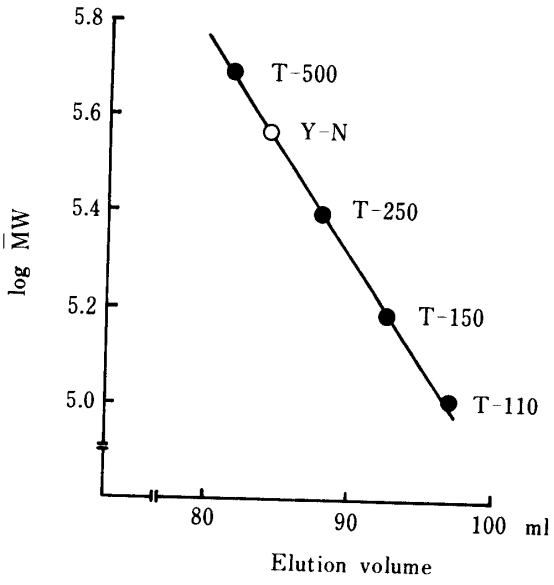

Fig. 3. Determination of the Molecular Weight of Y-N by Gel Chromatography on Sephacryl S-300

determined by gas-liquid chromatography (GLC) of the derived alditol acetates, and the absolute configuration of sugars was identified as D by GLC analysis of the acetylated 2-octyl glycosides.[7] Y-N contained 0.86% nitrogen (by elemental analysis).

The glucomannan was methylated by the method of Hakomori,[8] and the fully *O*-methylated product was hydrolyzed with acid. The hydrolysate was analyzed as the alditol acetates by GLC and GLC-mass spectrometry (GLC-MS). The partially *O*-methylated alditol acetates were identified by comparing the retention times in GLC and the peaks in the mass spectra with the values in the literature.[9] 2,3,6-Tri-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-glucose were identified in a molar ratio of 6.2 : 1.0, in addition to a trace amount of 2,3,4,6-tetra-*O*-methyl-D-glucose (-mannose). The results indicate that Y-N is composed of 1→4 linked D-mannopyranosyl and 1→4 linked D-glucopyranosyl residues in a molar ratio of 6 : 1.

The infrared (IR) spectrum of Y-N showed characteristic absorption bands at 1730 and 1250 cm^{-1}, and the two bands disappeared after alkali treatment, suggesting the presence of *O*-acyl groups in the molecule. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of Y-N showed *O*-acetyl signals at 174.2—174.7 ppm (carbonyl carbon), and both 21.1 and 21.5 ppm (methyl carbon). The split signals also suggest the presence of *O*-acetyl groups at different positions on the sugar residues. Furthermore, the presence of *O*-acetyl groups was confirmed by the detection of acetic acid on GLC of the hydrolysate of Y-N, and the total *O*-acetyl content was colorimetrically determined to be 8.8% by the ferric hydroxamate method.[10] For determination of the location of the *O*-acetyl groups, Y-N was treated with methyl vinyl ether in the presence of *p*-toluenesulfonic acid in dimethyl sulfoxide (DMSO), according to the method of DeBelder and Norrman.[11] The resulting product was then de-*O*-acetylated and methylated by the method of Hakomori.[8] The partially *O*-methylated, *O*-(1-methoxyethyl)ated polysaccharide thus obtained was hydrolyzed, and the hydrolysate was analyzed as the alditol acetate derivatives by GLC and GLC-MS as described in the section on methylation analysis. The sugars identified were as follows: 2,6-di-*O*-methyl-D-mannose, 6-*O*-methyl-D-mannose, 2-*O*-methyl-D-mannose, 3-*O*-methyl-D-mannose, mannose, and glucose (molar ratio, 0.2 : 0.6 : 0.9 : 0.5 : 3.2 : 1.0). The result indicates that *O*-acetyl groups are located at positions 2 and 6, at position 6, at position 2, and at position 3, of some of mannosyl residues.

Y-N and the de-*O*-acetylated polysaccharide (Y-N-D) were subjected to periodate oxidation. The amounts of periodate consumption per anhydrohexose units were 0.5 mol for Y-N, and 1.2 mol for Y-N-D. Complete Smith degradation products[12] of the reduced periodate-oxidized Y-N and Y-N-D were analyzed by GLC after conversion into the alditol acetates. The main Smith degradation products of Y-N were erythritol and mannose (molar ratio, 1.8 : 1.0), but in the case of Y-N-D only erythritol was obtained. The results support the presence of the 1→4 linkage in Y-N, and the presence of the *O*-acetyl groups on the mannosyl residues.

The ^{13}C-NMR spectrum of Y-N is shown in Fig. 4. The signals were assigned by comparison with the data in the literature.[13,14] The chemical shifts of the C-1—C-6 carbons were observed in the region of 61.3—113.3 ppm, with those of *O*-acetyl signals. The signals at 113.3 and 79.5 ppm were assigned to the C-1 and C-4 carbons of β-1→4 linked D-glucopyranosyl chain, and those at 101.0 and 77.3 ppm to the C-1 and C-4 carbons of β-1→4

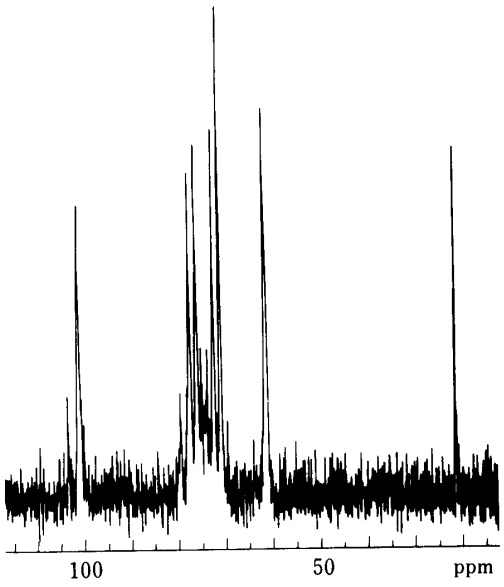

Fig. 4. ^{13}C-NMR Spectrum of Y-N

linked D-mannopyranosyl chain. The configurations of the anomeric carbons were supported by the values of $^{13}C_1$–H_1 coupling constants,[15] 162 (the glucopyranosyl residues) and 164 (the mannopyranosyl residues) Hz. The other prominent signals at 75.9, 72.4, 70.9, and both 61.4 and 61.3 ppm could be assigned to the C-5, C-3, C-2, and C-6 carbons of the mannosyl residues, respectively. Weak signals due to the C-5, C-3, and C-2 carbons of glucosyl residues were observed at 75.6, 74.9, and 73.8 ppm, whereas the signal of the C-6 carbon presumably overlaps with that of the mannosyl residues.

From this investigation, it can be concluded that Y-N, a linear glucomannan, is mostly composed of β-1→4 linked D-mannopyranosyl and β-1→4 linked D-glucopyranosyl residues in a molar ratio of 6 : 1, and the *O*-acetyl groups are located at positions 2 and 6, at position 6, at position 2, and at position 3, of some of the mannosyl residues.

Misaki *et al.*[3] and Tomoda *et al.*[4] isolated partially *O*-acetylated β-1→4 linked D-mannan from the mucilage of *D. batatas* in the Dioscoreaceae family. Their polysaccharides significantly differ from Y-N in the content of glucosyl residues. The structure of the glucomannan, Y-N may be similar to that of "Lilium-A-glucomannan" from the bulbs of *Lilium auratum*,[14] although the molar ratio of the component sugars is different. These results might be useful from a chemotaxonomic point of view. Studies on biological activities of Y-N such as antitumor activity[16] and anti-inflammatory activity,[17] as have been carried out on polysaccharides from various fungi, are in progress.

Experimental

Solutions were concentrated at or below 40 °C with a rotary evaporator under reduced pressure. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. IR spectra were recorded with a JASCO IRA-1 spectrometer. GLC was carried out on a Shimadzu GC-4CM apparatus for sugar analysis, a Shimadzu 8A apparatus for analysis of the absolute configuration, and a JEOL JGC-1100 apparatus equipped with a hydrogen flame ionization detector for analysis of organic acids. GLC-MS was performed with a JEOL JMS-D 300 gas chromatograph-mass spectrometer. Ultracentrifugal analysis was conducted with a MOM 3170/b analytical ultracentrifuge.

Materials——The tubers of *Dioscorea japonica* THUNB. were harvested in November 1982 in forests near Gifu, Japan. Pronase (70000 PUK/g) was purchased from Kaken Chemical Ind. DEAE-Sephadex A-25, Sephacryl S-300, Sephadex LH-20, and standard dextrans (T-500, T-250, T-150, and T-110) were purchased from Pharmacia Fine Chemicals.

Isolation and Purification——The fresh tubers (500 g) were freed from the epidermis, sliced, and homogenized, then allowed to stand in H_2O (3 l) at 5 °C for 20 h. The mixture was centrifuged at 5000 rpm for 30 min, and the supernatant was filtered. The filtrate was treated with Pronase (20 mg) at 38 °C for 1 d, and the procedure was repeated 3 times. The final product was dialyzed against deionized water for 2 d. The inner solution was centrifuged at 9000 rpm for 40 min, and the supernatant was filtered. The filtrate was concentrated to a small volume, and 4 vol. of EtOH was added. The precipitate was collected by centrifugation at 5000 rpm for 20 min and dissolved in H_2O, and the solution was deproteinized by the Sevag procedure.[5] The aqueous phase was concentrated to a small volume, and applied to a column (2.6 × 30 cm) of DEAE-Sephadex A-25 previously equilibrated with 0.1 M phosphate buffer (pH 6.1). The neutral fraction eluted with the buffer was dialyzed and lyophilized to afford a neutral polysaccharide (Y-N) as colorless flakes in *ca.* 0.15% yield.

Gel Chromatography——Each sample (2 mg) was dissolved in 0.1 M NaCl (1 ml), and applied to a column (1.5 × 90 cm) of Sephacryl S-300. The column was eluted with 0.1 M NaCl. Fractions of 4 ml were collected, and an aliquot of each fraction was analyzed by the phenol–H_2SO_4 method at 490 nm.[6] The calibration curve was constructed by the use of dextrans T-500 ($\bar{M}W$, 495000), T-250 ($\bar{M}W$, 253000), T-150 ($\bar{M}W$, 154000), and T-110 ($\bar{M}W$, 105000). The result is shown in Fig. 3.

^{13}C-NMR Spectroscopy——The ^{13}C-NMR spectrum was obtained by using a JEOL-FX 270 spectrometer with Fourier transform for a solution in D_2O (16.4 mg/ml) at 70 °C. The spectral width was 14 kHz, the pulse angle 45°, the repetition time 1.0 s, the pulse width 9 μs, the number of data points 16000, and the number of pulses 45000. The chemical shifts were obtained by the use of tetramethylsilane as an external standard.

Component Sugars——The polysaccharide (5 mg) was hydrolyzed with 2 N H_2SO_4 (2 ml) in a sealed tube at 100 °C for 8 h. The hydrolysate was made neutral with $BaCO_3$, the suspension was filtered, the filtrate was passed through a column of Amberlite CG-120 (H^+) resin, and the eluate was evaporated to a syrup. The hydrolysate was subjected to paper-partition chromatography using Toyo No. 51 filter paper by the double ascending method with the

solvent system of *n*-BuOH : pyridine : H_2O (6 : 4 : 3), and sugars were detected with alkaline $AgNO_3$ reagent.[18] Mannose (R_{glc}, 1.10) and glucose were detected. An aqueous solution of the hydrolysate was reduced with $NaBH_4$, the alditols were acetylated,[19] and the resulting alditol acetates were analyzed by GLC. GLC was carried out with a glass column (1.5 m × 3 mm) packed with 3% ECNSS-M on Gaschrom Q (100 to 120 mesh) (column A) at a flow rate of 50 ml per min of N_2 at 175 °C. The retention time of the acetates of mannitol and glucitol were 31.4 and 42.2 min, respectively, and the molar ratio was 6 : 1.

The sugars obtained by lyophilization of the hydrolysate (5 mg) solution were heated with two drops of CF_3COOH (TFA), and 2-octanol (0.8 ml) in a sealed tube with stirring at 130 °C for 1 d. The reaction mixture was then concentrated to dryness at 55 °C, and acetylated with Ac_2O–pyridine (1 : 1, 1 ml) at 100 °C for 1 h, then the products were analyzed by GLC. GLC was performed on an FS-WCOT column (25 m × 0.25 mm) (Gaschro Kogyo Co., Ltd.) packed with SP-1000 at 210 °C, at a flow rate of 0.6 ml per min of N_2, with a split ratio of 1 : 13. The peaks of the acetylated 2-octyl glycosides were detected at retention times of 64.8, 72.0, 74.2, 81.2, 86.0, and 88.0 min, and were identified by comparison with the retention times of the acetylated 2-octyl mannoside and glucoside from standard D-sugars.

Methylation Analysis——The polysaccharide (10 mg) was dissolved in DMSO (6 ml) at 50 °C for 6 h, and methylated by the method of Hakomori as described in a previous paper.[20] The methylation reaction was repeated 4 times. The fully *O*-methylated polysaccharide was hydrolyzed with 2 N TFA in a sealed tube at 100 °C for 8 h. After removal of TFA by evaporation, the hydrolysate was reduced with $NaBH_4$ to the corresponding alditols, which were acetylated.[19] The partially *O*-methylated alditol acetates were analyzed by GLC at 170 °C with an N_2 flow of 30 ml per min, using 3% ECNSS-M (column A). GLC-MS was carried out with a glass column (1 m × 0.2 cm) packed with 3% ECNSS-M at 180 °C at 1.0 kg/cm^2 of He. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μA, and an ion source temperature of 220 °C. The relative retention times of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol and -D-glucitol with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol were 2.19 and 2.45, respectively. The prominent fragments in the mass spectra of both products were *m/z*: 43, 87, 99, 101, 113, 117, 233.

***O*-Acetyl Determination**——Y-N (11 mg) was stirred with 0.1 M MeONa in MeOH, and the mixture was filtered, then washed with MeOH. The residue was dried *in vacuo* to give the de-*O*-acetylated product (Y-N-D) (8 mg). Y-N-D did not show the IR absorption bands (ν_{max}^{KBr} cm^{-1}: 1730, 1250) observed in Y-N.

Y-N (5 mg) containing propionic acid as an internal standard was hydrolyzed with 1 N HCl (1 ml) at 100 °C for 3 h in a sealed tube. The hydrolysate was directly applied to a column (2 m × 3 mm) packed with 2% H_3PO_4–Porapack Q (80 to 100 mesh) at 198 °C at a flow rate of 50 ml per min of N_2. One peak in addition to that of an internal standard (8.0 min) was observed, and the retention time was identical with that of AcOH (4.2 min).

The total *O*-acetyl content of Y-N was determined to be 8.8% by the ferric hydroxamate method[10] using penta-*O*-acetyl-β-D-glucose as a standard.

Acetalation with Methyl Vinyl Ether——The polysaccharide (17 mg) was dissolved in DMSO (15 ml), and then *p*-toluenesulfonic acid (15 mg) and methyl vinyl ether (condensed at −10 °C, 2 ml) were added. The mixture was stirred at 15 °C for 4 h. After addition of pyridine (0.5 ml), the excess of methyl vinyl ether was evaporated off by flushing with N_2 gas, and then the solution was dialyzed against running water overnight. The non-dialyzable fraction was concentrated to dryness, and the acetalation procedure was repeated 4 times. The final solution was fractionated on a column (2.4 × 30 cm) of Sephadex LH-20 equilibrated with anhydrous acetone. The eluate corresponding to the first yellow band was collected, and concentrated to dryness. The final product showed no hydroxyl absorption band in the IR spectrum.

Methylation Analysis of the *O*-Acetyl-*O*-(1-methoxyethyl)polysaccharide——A solution of the product (10 mg) in DMSO (5 ml) was treated with methylsulfinyl carbanion (2 ml), and then CH_3I (1 ml) was added, according to the method of Hakomori.[8] The product thus obtained showed no hydroxyl absorption band in its IR spectrum. The *O*-

TABLE I. Relative Retention Times on GLC and Prominent Fragments in MS of Methylated Alditol Acetates

O-Methylated D-mannitol acetate	Relative retention times[a]	Prominent fragments (*m/z*)
1,3,4,5-Ac_4-2,6-Me_2-D-Mannitol[b]	3.49	43, 45, 87, 117, 129
1,2,3,4,5-Ac_5-6-Me-D-Mannitol	4.72	43, 45, 87, 115, 129
1,3,4,5,6-Ac_5-2-Me-D-Mannitol	7.60	43, 117, 139
1,2,4,5,6-Ac_5-3-Me-D-Mannitol	9.67	43, 85, 87, 99, 127, 129, 189, 261

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
b) Abbreviations: Ac = acetyl, Me = methyl.

(1-methoxyethyl)-*O*-methyl-polysaccharide was successively heated with 90% HCOOH at 100 °C for 5 h, and with 0.5 N H_2SO_4 at 100 °C for 10 h. The partially *O*-methylated sugars were converted into alditol acetates, followed by GLC and GLC-MS analysis as already described. The relative retention times and mass fragments of the alditol acetate derivatives are listed in Table I.

Periodate Oxidation——Periodate oxidations of Y-N and Y-N-D (each, 20 mg) were carried out in the dark with 0.01 M $NaIO_4$ (50 ml) at 5 °C for 8 d, with stirring. The periodate consumptions were estimated by an arsenite method,[21] and the amounts per anhydrosugar unit were 0.5 mol for Y-N, and 1.2 mol for Y-N-D.

Smith Degradation——Ethylene glycol (1 ml) was added to a solution of each oxidized product obtained as described above, the mixture was dialyzed, and the product was reduced with $NaBH_4$ (10 mg) at room temperature overnight. The solutions were acidified with 1 N AcOH to pH 5, and dialyzed, then the non-dialyzable fractions were hydrolyzed with 1 N H_2SO_4 in a sealed tube at 100 °C for 6 h. The hydrolysates were reduced, and acetylated, as described above, and then the acetates were analyzed by GLC using dual columns packed with 3% ECNSS-M (column A): the column temperature was increased by 5 °C per min from 100 °C to 180 °C; carrier gas, N_2 (50 ml/min). The retention times were as follows: glycerol acetate 7.5 min, erythritol acetate 14.4 min, mannitol acetate 36.6 min, and glucitol acetate 44.1 min.

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References and Notes

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2) T. Sato, J. Mizuguchi, S. Suzuki, and M. Tokura, *Nippon Kagaku Zasshi*, **88**, 216 (1967).
3) A. Misaki, T. Ito, and T. Harada, *Agric. Biol. Chem.*, **36**, 761 (1972).
4) M. Tomoda, K. Ishikawa, and M. Yokoi, *Chem. Pharm. Bull.*, **29**, 3256 (1981).
5) M. G. Sevag, *Biochem. Z.*, **273**, 419 (1934).
6) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
7) K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, **62**, 359 (1978).
8) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
9) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967); H. Björndal, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem. Int. Ed. Engl.*, **9**, 610 (1970).
10) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1947).
11) A. N. De Belder and B. Norrman, *Carbohydr. Res.*, **8**, 1 (1968).
12) J. K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **78**, 5907 (1956).
13) J. H. Bradbury and G. Jenkins, *Carbohydr. Res.*, **126**, 125 (1984).
14) T. Usui, T. Mizuno, K. Kato, M. Tomoda, and G. Miyajima, *Agric. Biol. Chem.*, **43**, 863 (1979).
15) K. Bock, I. Lundt, and C. Perdersen, *Tetrahedron Lett.*, **1973**, 1037.
16) S. Ukai, T. Kiho, C. Hara, M. Morita, A. Goto, N. Imaizumi, and Y. Hasegawa, *Chem. Pharm. Bull.*, **31**, 741 (1983).
17) S. Ukai, T. Kiho, C. Hara, I. Kuruma, and Y. Tanaka, *J. Pharmacobio-Dyn.*, **6**, 983 (1983); C. Hara, T. Kiho, Y. Tanaka, and S. Ukai, *Carbohydr. Res.*, **110**, 77 (1982).
18) W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature* (London), **166**, 444 (1950).
19) J. H. Sloneker, "Methods in Carbohydrate Chemistry," Vol. VI, ed., by R. L. Whistler and J. N. BeMiller, Academic Press, New York and London, 1972, pp. 20—24.
20) S. Ukai, C. Hara, T. Kiho, and K. Hirose, *Chem. Pharm. Bull.*, **26**, 1729 (1978).
21) P. F. Fleury and J. Lange, *J. Pharm. Chim.*, **17**, 196 (1933).