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Isolation and Structural Features of Two Glucans from the Rhizomes of *Crinum latifolium*

MASASHI TOMODA,* NORIKO SHIMIZU, KAZUYO SHIMADA, and MIE SUGA

Kyoritsu College of Pharmacy, Shibakōen, Minato-ku, Tokyo 105, Japan

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Two water-soluble glucans (A and B) were isolated from the rhizomes of *Crinum latifolium*. The final preparations were each homogeneous as determined by glass-fiber electrophoresis, gel chromatography, and thin-layer chromatography. Glucan A was composed of 12 glucose units, while glucan B had about 110 glucose residues. Methylation, periodate oxidation, nuclear magnetic resonance, and enzymic degradation studies showed that both glucans are mainly composed of α-1→4 linked D-glucopyranose residues having branches linked through position 6. Glucan A had one side chain composed of a hexasaccharide. The average length of the unit chain in glucan B was about 11, and glucan B had various polymerized side chains. *O*-Acetyl groups were identified in glucan A, and they were located at positions 2 and 3 of a glucose unit.

Keywords——*Crinum latifolium*; rhizome; α-D-glucan; structure; methylation analysis; enzymic degradation; α-1→4 linked chain; α-1→6 branching; acetylated dodecasaccharide; 2,3-di-*O*-acetyl group

In our previous papers,[1-3] the isolation and the structural features of three highly *O*-acetylated glucomannans from plants in the Amaryllidaceae family, namely, the bulbs of *Narcissus tazetta* L. var. *chinensis* ROEMER, *Lycoris radiata* HERBERT, and *Lycoris squamigera* MAXIM., have been reported. These glucomannans are characteristic polysaccharides because of their particularly high acetyl contents. For chemotaxonomical reasons, we have studied on polysaccharides of other genus plants in the Amaryllidaceae family. The present paper is concerned with the isolation and the structures of two pure glucans from the fresh rhizomes of *Crinum latifolium* L. The isolation of four alkaloids from this plant has been reported,[4,5] but no study on the carbohydrates has been published so far.

The rhizomes were sliced and extracted with cold water. After precipitation by addition of ethanol, the crude extract obtained was applied to a column of diethylaminoethyl (DEAE)-cellulose (acetate form). A neutral polysaccharide fraction was obtained from the eluate with water, then the fraction was applied to a column of Sephadex G-50. Elution with water yielded two carbohydrate-containing fractions designated as peaks I and II (Fig. 1). Peak I was subjected to rechromatography on the same column and peak II was rechromatographed on a column of Sephadex G-15. Thus, glucans A and B were obtained from peaks II and I, respectively. Glucan B was the only substance in peak I, while the presence of six minor components other than glucan A was observed in the eluates obtained by rechromatography of peak II.

The glucans A and B each gave a single spot on glass-fiber paper electrophoresis in both pyridine-acetic acid buffer and alkaline borate buffer. In addition, glucan A gave a single spot on thin-layer chromatography (TLC). Furthermore, each glucan gave a single peak on Sephacryl S-200 gel chromatography. Gel chromatography gave molecular weight values of 2000 for A and 18000 for B. Both substances were readily soluble in water and they showed

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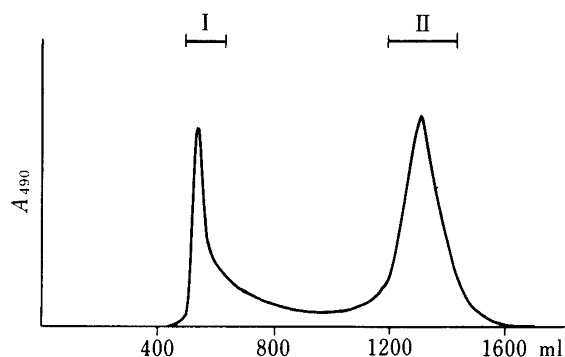

Fig. 1. Chromatogram of Polysaccharides on Sephadex G-50

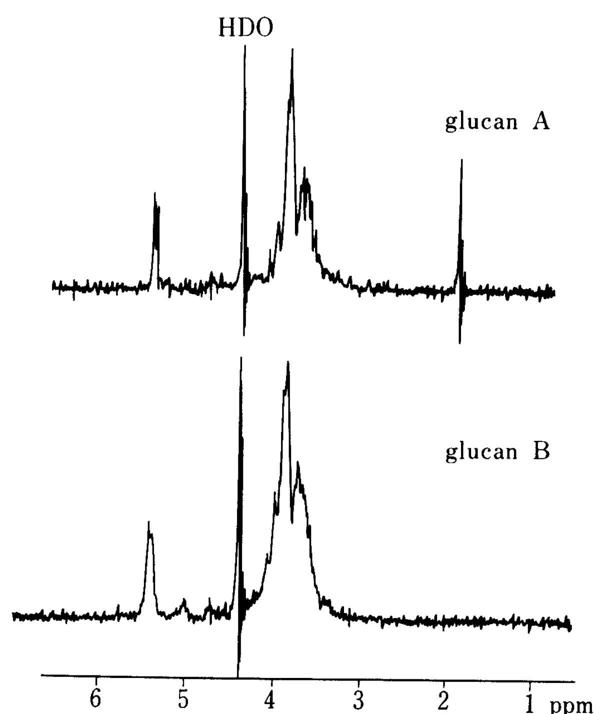

Fig. 2. ^{1}H-NMR Spectra of Glucans A and B

high positive specific rotations (A, $[\alpha]_D^{24}$ $+160.6°$ in H_2O, $c=1.2$; B, $[\alpha]_D^{24}$ $+181.9°$ in H_2O, $c=0.6$). Glucose was identified as the only component sugar by cellulose TLC of the hydrolysate and by gas-liquid chromatography (GLC) of the trimethylsilyl derivative from both substances. They contained no nitrogen.

As shown in Fig. 2, the proton magnetic resonance (^{1}H-NMR) spectra of the glucans showed an acetyl signal at δ 1.92 in A, while no acetyl signal was observed in B. The ^{1}H-NMR spectra also showed anomeric proton signals at δ 5.42 (d, $J=3.5$ Hz) in A and δ 5.40 (d, $J=3.5$ Hz) in B. These data suggest that the glucose residues in A and B are α-linked.

From the results of molecular weight determination, it can be concluded that glucan A possesses 12 glucose units, and that glucan B has about 110 glucose residues. These conclusions were also supported by the results of the determination of reducing terminals. The ratio of integrals of acetyl and anomeric proton signals in the ^{1}H-NMR of glucan A was 6 : 12. The presence of acetyl groups in glucan A was also confirmed by GLC of the acid hydrolysate,[6] and the acetyl content was determined to be 4.2%. Thus, close agreement of acetyl values as determined by the ^{1}H-NMR and GLC methods was obtained.

Each glucan was methylated with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[7] The product was hydrolyzed, reduced, and acetylated. The partially methylated glucitol acetates thus obtained were analyzed by gas-liquid chromatography-mass spectrometry (GLC-MS).[8] Three peaks were detected on the gas chromatogram in each case. These were identified as 2,3,4,6-tetra-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and 2,3-di-*O*-methyl-D-glucitol acetates in molar ratios of 2.0 : 9.6 : 1.0 from A and 1.0 : 8.9 : 1.0 from B.

The glucans A and B were oxidized with periodate. It was found that 1.12 and 0.92 mol of periodate per mol of component anhydrohexose unit were consumed with liberation of 0.36 and 0.06 mol of formic acid by glucans A and B, respectively.

In order to elucidate the locations of the *O*-acetyl groups, glucan A was exhaustively treated with methyl vinyl ether, as a protective reagent for the free hydroxyl groups, in the presence of *p*-toluenesulfonic acid as a catalyst in dimethyl sulfoxide.[9] After conversion of the

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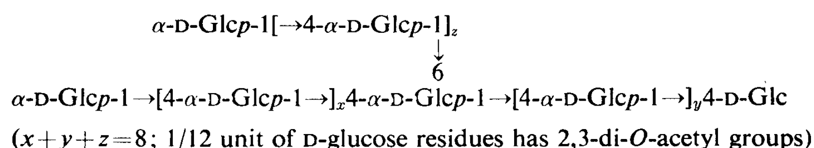
free hydroxyl groups to 1-methoxyethyl ethers, the derivative was deacetylated, then methylated as described above. The product was hydrolyzed and analyzed by GLC-MS after conversion to alditol acetates. A glucose methyl ether was detected and identified as 2,3-di-*O*-methyl-D-glucose. This result indicates that glucan A is composed of a 2,3-di-*O*-acetyl-D-glucose and eleven D-glucose units.

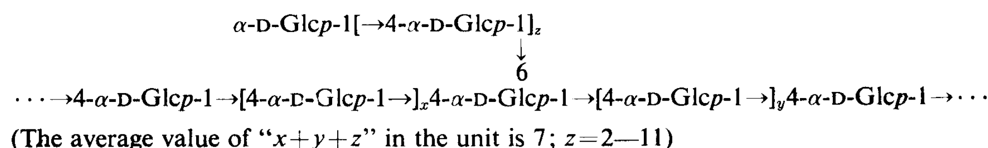
Glucan B was treated with isoamylase (glycogen 6-glucanohydrolase [3.2.1.68] from *Pseudomonas amyloderamosa*), and the reaction products were analyzed by TLC. Ten kinds of oligosaccharides in the range of disaccharide to undecasaccharide were detected. This result indicates that glucan B has various polymerized side chains. Glucan A resisted the action of isoamylase; therefore the degradation of the deacetylation product of glucan A with the same enzyme was attempted, but a good result could not be obtained. Further studies on the enzymic degradation of glucan A are in progress.

Based on the accumulated evidence described above, the structural features of glucan A are shown in Chart 1, and the average structural unit of glucan B is given in Chart 2.



($x+y+z=8$; 1/12 unit of D-glucose residues has 2,3-di-*O*-acetyl groups)

Chart 1. Structural Features of Glucan A



(The average value of "$x+y+z$" in the unit is 7; $z=2$—11)

Chart 2. Structural Unit of Glucan B

Thus, glucans A and B obtained from the rhizomes of *Crinum latifolium* are structurally similar to amylopectin and glycogen. No glucomannan was found in the water extract obtained from the rhizomes of this plant. Amylopectins are widely distributed in the seeds, fruits, leaves, rhizomes, bulbs, and tubers of higher plants. However, there are large differences in the extents of branching between amylopectins and the glucans obtained by us. Glucan B is an example of so-called phytoglycogen-type polysaccharides. A few examples of phytoglycogens isolated from *Zea mays*[10,11] and *Salix alba*[12] have been reported so far, while glucans A and B from *Crinum latifolium* both possess characteristic low molecular weights. Very recently, a glucan having an amylopectin-like structure and relatively low molecular weight has been obtained from the roots of *Angelica acutiloba*.[13] However, its value of specific rotation was very low. Neither glucan A nor B gave the iodine color reaction.

Among the carbohydrates of higher plants, glucan A, the main water-soluble neutral polysaccharide from the rhizomes of *Crinum latifolium*, is a unique dodecasaccharide having acetyl groups.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Optical rotation was measured with a JASCO DIP-140 automatic polarimeter. Infrared (IR) spectra were recorded on a JASCO IRA-2 infrared spectrophotometer. ^{1}H-NMR spectra were recorded on a JEOL MH-100 NMR spectrophotometer in heavy water containing 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 70 °C. GLC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector. GLC-MS was

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performed with a JEOL JGC-20K gas chromatograph and a JEOL JMS-D100 mass spectrometer.

Isolation of Polysaccharides——The materials was obtained in September 1982 from plants cultivated in Saitama prefecture. The fresh rhizomes (740 g), which contained 85.2% water, were sliced and extracted with water (3700 ml) under stirring at room temperature for 1 h. After suction filtration, the filtrate was poured into two volumes of ethanol. The precipitate was obtained by centrifugation, then dried *in vacuo* (yield, 5.73 g). A half of the crude extract was dissolved in water (50 ml) and the solution was centrifuged. The supernatant was applied to a column (4 × 26 cm) of DEAE-cellulose (acetate form). The column was eluted with water, and fractions of 100 ml were collected and analyzed by the phenol–sulfuric acid method.[14] The eluates obtained from tubes 4 to 8 were combined, concentrated and lyophilized (yield, 0.70 g). A part of this eluate (100 mg) was dissolved in water and applied to a column (5 × 70 cm) of Sephadex G-50. The column was eluted with water, and fractions of 20 ml were collected and analyzed as described above. The eluates obtained from tubes 25 to 32 were combined, concentrated and lyophilized. Glucan B (25 mg) was obtained as a white powder after rechromatography of this fraction (peak I) under the same conditions. The eluates obtained from tubes 60 to 72 were combined, concentrated and lyophilized. This fraction (peak II) was dissolved in water and applied to a column (5 × 77 cm) of Sephadex G-15. The column was eluted with water, and fractions of 20 ml were collected and analyzed as described above. The eluates obtained from tubes 34 and 35 were combined, concentrated and lyophilized. Glucan A (27 mg) was obtained as a white powder.

Glass-Fiber Paper Electrophoresis——Electrophoresis was carried out with Whatman GF 83 glass-fiber papers in the manner described in a previous report,[15] with the following buffers and conditions: A, 0.08 M pyridine–0.04 M acetic acid (pH 5.4) at 570 V for 90 min; B, 0.025 M borax: 0.1 N sodium hydroxide (10 : 1, pH 9.3) at 570 V for 45 min. Each sample gave a single spot at distances of 5.6 cm (glucan A) and 5.4 cm (glucan B) in A and of 8.4 cm (glucan A) and 8.2 cm (glucan B) in B from the center toward the cathode.

TLC of Glucan A——TLC was performed on Merck precoated Kieselgel 60 plates. As a developing solvent, the following mixture was used: *n*-butanol–acetic acid–water (2 : 1 : 1, v/v). The detection of spots on TLC plates was done by spraying 0.2% orcinol in 20% sulfuric acid followed by heating at 110 °C for 5 min. R_{Glc} value of glucan A was 0.25, while glucan B did not move from the origin.

Gel Chromatography——The sample (3 mg) was dissolved in water and applied to a column (2.6 × 94 cm) of Sephacryl S-200. Elution was carried out by the descending method with 0.1 M Tris–HCl buffer (pH 7.0) as an eluant. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans having known molecular weights (Shodex standard P-5, P-10, P-20, and P-50) were run on the column and gave the calibration curve shown in Fig. 3.

Qualitative and Quantitative Analyses of Component Sugars——Qualitative analysis was carried out under the same conditions as in a previous report.[16] However, GLC of trimethylsilyl derivatives was carried out as follows (condition A): on a column (0.3 × 200 cm long spiral glass) packed with 2% OV 101 on Uniport HP (80 to 100 mesh) and with a programmed temperature increase of 4 °C per min from 180 to 240 °C at a helium flow of 50 ml per min; t_R (min), glucose trimethylsilyl ether 9.4, 11.1. Glucose was also determined by the phenol–sulfuric acid method. The results revealed that glucan A was composed of 96.9% glucose in addition to acetyl groups and that glucan B was composed of 98.8% glucose.

Determination of *O*-Acetyl Groups——The sample (3 mg) was hydrolyzed with 0.2 N hydrochloric acid (0.05 ml) in a sealed tube at 100 °C for 2 h. The hydrolysate was directly applied to GLC with propionic acid as an internal standard. GLC was carried out as follows (condition B): on a column (0.3 × 200 cm long spiral glass) packed with 5% Thermon-1000–0.5% phosphoric acid on Chromosorb W (80 to 100 mesh) at 120 °C with a helium flow of 30 ml per

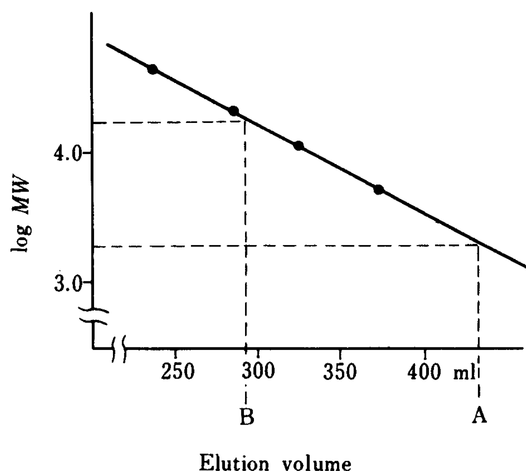

Fig. 3. Plot of Elution Volume against log MW for Standard Pullulans on Sephacryl S-200

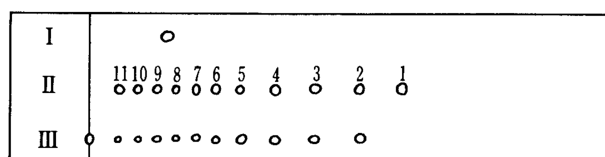

Fig. 4. Thin-Layer Chromatograms of Enzymic Degradation Products

I: Glucan A.
II: α-1→4-Amylodextrins (numbers show the degree of polymerization).
III: Degradation products of glucan B.
Plate, Kieselgel 60. Solvent, BuOH–AcOH–H_2O (2 : 1 : 1).

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

	Relative retention times[a]	Main fragments (m/z)
1,5-Ac-2,3,4,6-Me-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac-2,3,6-Me-D-Glucitol	2.06	43, 45, 87, 99, 101, 113, 117, 233
1,4,5,6-Ac-2,3-Me-D-Glucitol	3.72	43, 101, 117, 261

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

min; t_R (min), acetic acid 3.6; propionic acid (internal standard) 5.1.

Methylation of Each Glucan——The sample (5 mg) was dissolved in dimethyl sulfoxide (1 ml). Sodium hydride (10 mg) was mixed with dimethyl sulfoxide (2 ml) in an ultrasonic bath for 30 min, followed by stirring at 70 °C for 1 h, then the resulting methylsulfinyl carbanion was added to the sample solution. The reaction mixture was stirred at room temperature for 4 h, then methyl iodide (2 ml) was added and the whole was stirred overnight at room temperature. All procedures were carried out under nitrogen. After addition of water (15 ml), the reaction mixture was extracted five times with chloroform (15 ml each). The combined extract was washed five times with water (75 ml each), then dried over sodium sulfate, and the filtrate was concentrated to dryness. The residue was methylated twice under the same conditions. The final residue was dissolved in chloroform–methanol mixture (2 : 1, v/v), then applied to a column (2 × 20 cm) of Sephadex LH-20. The column was eluted with the same solvent, and fractions of 1 ml were collected. The eluates obtained from tubes 7 to 10 were combined and concentrated to dryness. The final product (3.5 mg) was a yellow powder. Its IR spectrum showed no absorption band due to hydroxyl groups.

Analysis of the Methylated Product——A part of the product (1 mg) was hydrolyzed with 90% formic acid (0.2 ml) in a sealed tube at 90 °C for 16 h. After removal of the acid by evaporation, the product was hydrolyzed with 0.5 N sulfuric acid (0.2 ml) in a sealed tube at 100 °C for 3 h. After neutralization with Dowex 2 (OH^-), the hydrolysate was reduced and acetylated as described in a previous report.[17] GLC and GLC-MS were carried out as follows (condition C): on a colum (0.3 × 200 cm long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 200 °C with a helium flow of 60 ml per min. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and their main fragments in the mass spectra, are listed in Table I.

Periodate Oxidation——The sample (10 mg) was dissolved in water (5 ml), and after addition of 0.1 M sodium metaperiodate (5 ml), the reaction mixture was kept at 5 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[18] The oxidation was completed after 3 d, then 2 ml of the solution was used for the measurement of formic acid liberation by titration with 0.01 N sodium hydroxide after addition of one drop of ethylene glycol.

1-Methoxyethylation of Glucan A Followed by De-*O*-acetylation——A sample of 7 mg was used and the procedures were carried out by the methods described in a previous report.[1]

Methylation of the *O*-(1-Methoxyethyl) Derivative and Analysis of the *O*-Methyl Derivative——These were carried out in the manner and under the conditions described above.

Enzymic Degradation and Analysis of the Products——Glucan B (1 mg) was dissolved in 0.1 ml of 0.5 M acetate buffer (pH 3.4). After addition of isoamylase (Seikagaku Kogyo Co.; 1180 units in 0.1 ml, from the culture fluid of *Pseudomonas* sp.[19]), the solution was incubated at 40 °C for 20 h. After successive treatments with Dowex 50W-X8 (H^+) and Dowex 2 (OH^-) using small columns (0.5 × 2 cm), the eluate with water was concentrated and analyzed by TLC as described above. The results of TLC of the enzymic degradation products and standard samples are shown in Fig. 4.

Determination of Reducing Terminal——The sample (1 mg) was dissolved in water (0.5 ml), then sodium borohydride (0.2 mg) was added and the solution was stirred at room temperarure for 30 min. After neutralization with Dowex 50W-X8 (H^+), the filtrate was evaporated and boric acid was removed by repeated addition and evaporation of methanol. The product was hydrolyzed with 2 M trifluoroacetic acid (0.2 ml) in a sealed tube at 100 °C for 3 h. After evaporation of the acid, the residue was acetylated with acetic anhydride–pyridine mixture (1 : 1) at 100 °C for 1 h. After evaporation of the solution, the residue was dissolved in chloroform–methanol mixture (1 : 1) and subjected to GLC. GLC was carried out under condition D, on a column (0.3 × 200 cm long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 220 °C with a helium flow of 50 ml per min. Retention times of the peracetates of glucose and glucitol were 14.7 and 18.9 min, respectively.

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References

1) M. Tomoda, M. Yokoi, A. Torigoe, and K. Maru, *Chem. Pharm. Bull.*, **28**, 3251 (1980).
2) M. Tomoda and N. Shimizu, *Chem. Pharm. Bull.*, **30**, 3965 (1982).
3) M. Tomoda, N. Shimizu, K. Shimada, T. Ishii, and M. Ogawa, *Chem. Pharm. Bull.*, **31**, 3878 (1983).
4) S. Rangaswami and E. Rao, *Curr. Sci.*, **24**, 25 (1955) [*Chem. Abstr.*, **49**, 9233*c* (1955)].
5) F. A. Kincl, V. Troncoso, and G. Rosenkranz, *J. Org. Chem.*, **22**, 574 (1957).
6) M. Tomoda, N. Satoh, and C. Ohmori, *Chem. Pharm. Bull.*, **26**, 2768 (1978).
7) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
8) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).
9) A. N. DeBelder and B. Norrman, *Carbohydr. Res.*, **8**, 1 (1968).
10) S. Peat, W. J. Whelan, and J. R. Turvey, *J. Chem. Soc.*, **1956**, 2317.
11) C. T. Greenwood and P. C. Das Gupta, *J. Chem. Soc.*, **1958**, 703.
12) S. Karacsonyi and M. Pasteka, *Collect. Czech. Chem. Commun.*, **40**, 1240 (1975) [*Chem. Abstr.*, **83**, 40189*p* (1975)].
13) H. Yamada, H. Kiyohara, and Y. Otsuka, *Phytochemistry*, **23**, 587 (1984).
14) J. E. Hodge and B. T. Hofreiter, "Methods in Carbohydrate Chemistry," Vol. I, ed. by R. L. Whistler and M. L. Wolfrom, Academic Press, New York and London, 1962, pp. 388—389.
15) M. Tomoda, Y. Yoshida, H. Tanaka, and M. Uno, *Chem. Pharm. Bull.*, **19**, 2173 (1971).
16) M. Tomoda, S. Kaneko, C. Ohmori, and T. Shiozaki, *Chem. Pharm. Bull.*, **24**, 2744 (1976).
17) M. Tomoda, K. Shimada, Y. Saito, and M. Sugi, *Chem. Pharm. Bull.*, **28**, 2933 (1980).
18) *a*) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954); *b*) G. O. Aspinall and R. J. Ferrier, *Chem. Ind.* (London), **1957**, 1216.
19) K. Yokobayashi, A. Misaki, and T. Harada, *Biochim. Biophys. Acta*, **212**, 458 (1970).

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