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THE LOCATION OF THE *O*-ACETYL GROUPS IN THE (1→3)-α-D-MANNAN FROM *Dictyophora indusiata* FISCH.*

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ABSTRACT

A linear (1→3)-α-D-mannan (T-2-HN) isolated from the hot, 70% aqueous ethanol extract of the fruit bodies of *Dictyophora indusiata* Fisch. contained about one *O*-acetyl group per two D-mannosyl residues in the molecule. The locations of the *O*-acetyl groups were elucidated by the methyl-replacement method involving the use of (1-methoxyethyl) protecting groups. The results indicated that most of the acetyl groups (~88%) are located solely at O-6 of the α-(1→3)-linked D-mannopyranosyl residues in T-2-HN. In addition, small proportions of acetyl group are located on O-2,6, O-4,6, O-2, and O-4 of the D-mannosyl residues, and this conclusion was well supported by the results of a ^{13}C-n.m.r.-spectral study. The molecular weights ($\overline{M}w$) of T-2-HN and its deacetylated product (water-insoluble) were, by gel chromatography on Sepharose CL-2B with 0.1M sodium chloride and 2M sodium hydroxide as the eluants, respectively determined to be 620,000 and 550,000.

INTRODUCTION

Previously[2], we have studied the structural features of a new type of partially *O*-acetylated α-D-mannan (T-2-HN) isolated from the hot, 70% aqueous ethanol extract of the fruit bodies of *Dictyophora indusiata* Fisch. The earlier work indicated that T-2-HN is composed of a linear chain of α-(1→3)-linked D-mannopyranosyl residues (>97%), and contains *O*-acetyl groups (11.4%) in the polysaccharide molecule. The molar ratio of D-mannose (as hexosyl residue) to acetyl group (as $-COCH_3$) in T-2-HN was 1.00:0.51, based on colorimetric determination of each. Furthermore, we reported that, on deacetylation of the native mannan (T-2-HN), the product was insoluble in water, presumably because of molecular association of the linear chains through hydrogen bonds. Therefore, it is reasonable to consider that the significant difference in the solubility of T-2-HN and its deacetylation

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product in water is attributable to the *O*-acetyl groups existing in the native, D-mannan molecule. The present article deals with the locations of the *O*-acetyl groups, and gives some physical properties, *e.g.*, molecular weight, of T-2-HN.

RESULTS AND DISCUSSION

The native mannan (T-2-HN) dissolved slowly in water, to give a slightly viscous solution (intrinsic viscosity $[\eta]$: 3.88 dL/g). In contrast, the *O*-deacetylated polysaccharide prepared by treatment of T-2-HN with alkali was insoluble in water or dimethyl sulfoxide, but it could be dissolved in >2M sodium hydroxide. The molecular weight of T-2-HN was determined by gel filtration on Sepharose CL-2B with 0.1M sodium chloride as the eluant; T-2-HN showed a single peak, as already reported[2]. The calibration curve shown in Fig. 1 (solid line) was made by use of standard dextrans; the molecular weight ($\overline{M}w$) of T-2-HN thus estimated was ~620,000. Furthermore, in gel filtration of the deacetylated T-2-HN on Sepharose CL-2B with 2M sodium hydroxide as the eluant, it also gave a single peak (see Fig. 2), and its molecular weight ($\overline{M}w$) was estimated to be ~550,000, as shown in Fig. 1

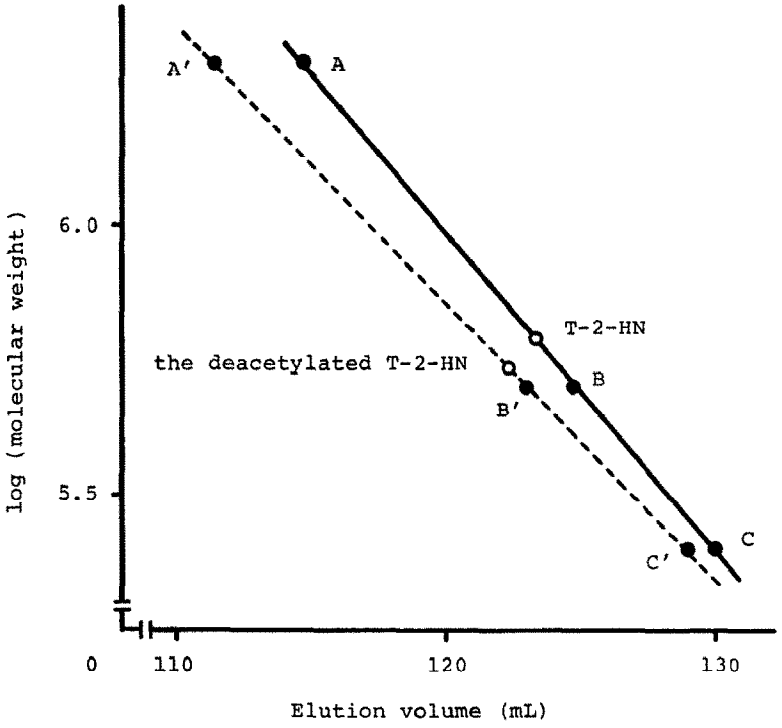


Fig. 1. Determination of molecular weight of T-2-HN and the deacetylated T-2-HN by gel filtration on Sepharose CL-2B. [The elution volume was plotted against the logarithm of the molecular weight of dextrans T-2,000 (AA'; mol.wt. 2,000,000), T-500 (BB'; 495,000), and T-250 (CC'; 253,000). Key: ———, 0.1M sodium chloride as the eluant; – – – –, 2M sodium hydroxide as the eluant.]

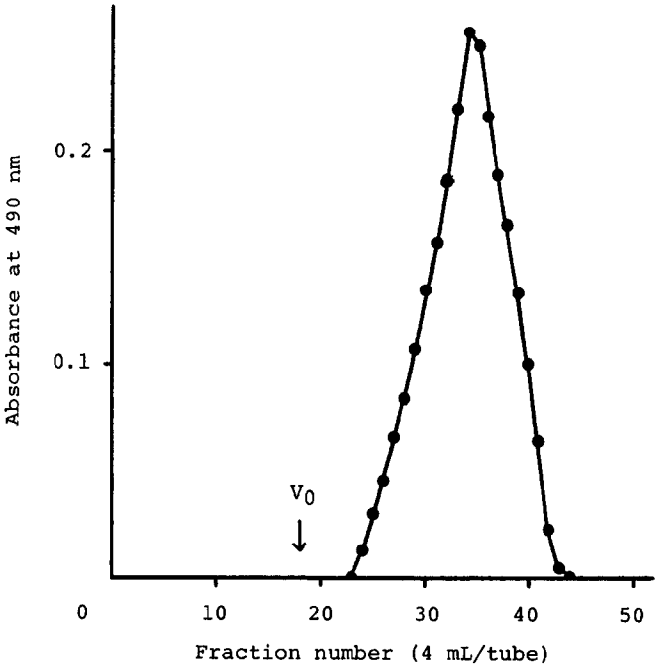


Fig. 2. Chromatogram of the deacetylated T-2-HN on Sepharose CL-2B. [The column (1.5 × 98 cm) was eluted with 2M sodium hydroxide.]

(dotted line). The difference in the molecular weight of T-2-HN and its deacetylated product was compatible with the total amount of acetyl groups contained in the native T-2-HN.

The presence of *O*-acetyl groups in mannans and mannoheteroglycans found in natural sources has already been reported in the literature[3-11], and the locations have been elucidated in the mannan from *Streptomyces griseus*[3], glucomannans from *Bletilla striata*[4] and Liliaceae[7,8], and glucuronoxylomannans from *Tremella fuciformis*[11]. For determination of the locations of *O*-acetyl groups, the native, *O*-acetylated D-mannan (T-2-HN) was treated with methyl vinyl ether, as a protective reagent for the free hydroxyl groups, in the presence of *p*-toluenesulfonic acid as the catalyst in dimethyl sulfoxide, according to the method of de Belder and Norrman[12]. The resulting, partially *O*-acetylated *O*-(1-methoxyethyl)ated polysaccharide was then methylated by the method of Hakomori[13], whereupon the *O*-acetyl groups were replaced by *O*-methyl groups. The partially *O*-methylated, *O*-(1-methoxyethyl)ated polysaccharide thus obtained was subjected to acid hydrolysis. The hydrolyzate was analyzed as the alditol acetate derivatives[14] by gas–liquid chromatography (g.l.c.) and g.l.c.–mass spectrometry (g.l.c.–m.s.).

The partially methylated alditol acetates were identified by comparing their retention times in g.l.c., and their mass spectra, with those of authentic samples, or with the values in the literature[15]. Table I shows the results of the methylation

TABLE I

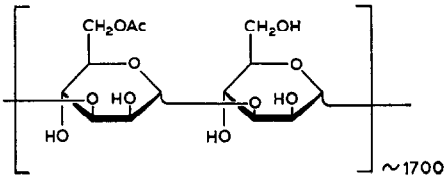
G.L.C. AND G.L.C.–M.S. OF METHYLATED ALDITOL ACETATES DERIVED FROM THE PARTIALLY *O*-ACETYLATED (1→3)-α-D-MANNAN

Peak number	*Methylated sugar (as alditol acetate)*	T[a]	*Main mass-fragments*	*Molar ratio*	*Location of acetyl group*
I	2,6- and 4,6-Me_2-Man[b,c]	3.24	43, 45, 87, 101, 117, 129, 161, 261	0.08	O-2,6, O-4,6
II	6-Me-Man	4.38	43, 45, 87, 115, 129	1.00	O-6
III	2-Me-Man	6.91	43, 117, 139	trace	O-2
IV	4-Me-Man	8.69	43, 85, 87, 99, 127, 129, 189, 261	0.06	O-4
V	Man	9.46	43, 85, 97, 103, 115, 128, 139, 145, 157, 170, 187, 217, 259, 289, 361	1.10	—

[a]Relative retention-time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol (1.00). [b]4,6-Me_2-Man = 4,6-di-*O*-methyl-D-mannose, etc. [c]Overlapping peaks.

analysis. Among the peaks (I–V) observed, peak I indicated the presence of both 2,6- and 4,6-di-*O*-methylmannitol acetate, which have almost the same retention time in g.l.c. (3% ECNSS-M column[16]), and these gave their characteristic mass fragments (*m/z* 43, 45, 87, 101, 117, 129, 161, and 261) in g.l.c.–m.s. Consequently, the identification of 2,6- and 4,6-di-*O*-methylmannose indicated that O-2,6 and O-4,6 of the α-(1→3)-linked D-mannosyl residues in the native polymer were *O*-acetylated. Similarly, the identification of 6-*O*-methylmannose (II), 2-*O*-methylmannose (III), and 4-*O*-methylmannose (IV) indicated that acetyl groups were respectively located on O-6, O-2, and O-4 of the D-mannosyl residues in T-2-HN. The molar ratios of these methylated D-mannose derivatives (I–IV) were ~0.08 : 1.00 : trace : 0.06, as shown in Table I. This result indicated that the substitution of acetyl groups solely at O-6 of the D-mannosyl residues was predominant (~88%), and that ~95% of the total acetyl groups was located on O-6 of the residues. On the other hand, peak V was identified as hexa-*O*-acetylmannitol. The identification of non-*O*-methylated mannose indicated that part of the D-mannosyl residues in T-2-HN are unacetylated. From the results, the molar ratio of acetyl group to D-mannose in T-2-HN was estimated to be 0.54 : 1.00 (see Table I). This ratio is in good agreement with that obtained from individual, colorimetric determination of them, namely, 0.51 : 1.00.

On the basis of these results, it was concluded that T-2-HN contains *O*-acetyl groups, corresponding to approximately one acetyl group per two D-mannosyl residues in the α-(1→3)-linked D-mannopyranosyl chains, and that most of the acetyl groups (~88%) are situated only on O-6 of the D-mannosyl residues. However, small proportions of acetyl groups located on O-2,6, O-4,6, O-2, and O-4 of the D-mannosyl residues were also present. From the structural features previously described[2], and the present results, a possible repeating-unit in T-2-HN is that illustrated in **1**.



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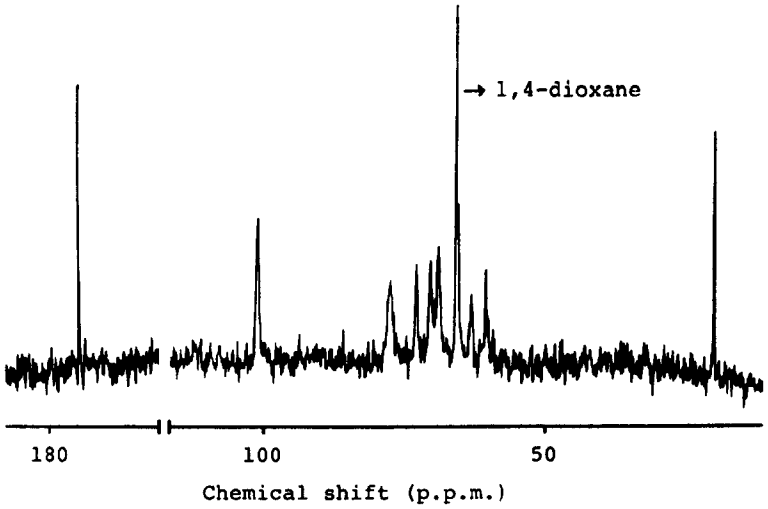

Fig. 3. ^{13}C-N.m.r. spectrum of T-2-HN.

TABLE II

ASSIGNMENT OF SIGNALS IN THE ^{13}C-N.M.R. SPECTRUM OF T-2-HN

Chemical shifts[a]	*Assignment*
102.8 (+0.9)[b]	C-1 of α-(1→3)-D-Man[c], and C-1 of 6-*O*-acetylated α-(1→3)-D-Man
70.6 (−0.6)	C-2 of α-(1→3)-D-Man, and C-2 of 6-*O*-acetylated α-(1→3)-D-Man
79.0 (+7.2)	C-3 of α-(1→3)-D-Man, and C-3 of 6-*O*-acetylated α-(1→3)-D-Man
67.1 (−0.9)	C-4 of α-(1→3)-D-Man, and C-4 of 6-*O*-acetylated α-(1→3)-D-Man
74.4 (+0.7)	C-5 of α-(1→3)-D-Man
72.0 (−1.7)	C-5 of 6-*O*-acetylated α-(1→3)-D-Man
64.6 (+2.5)	C-6 of 6-*O*-acetylated α-(1→3)-D-Man
62.1 (0)	C-6 of α-(1→3)-D-Man
21.3	methyl carbon in acetyl group
174.6	carbonyl carbon in acetyl group

[a]Chemical shifts are given in p.p.m. from internal 1,4-dioxane (67.4 p.p.m.). [b]Values in parentheses represent the difference of the shift from that of the corresponding carbon in methyl α-D-manno-pyranoside; see ref. 17. [c]α-(1→3)-D-Man = α-(1→3)-linked D-mannopyranosyl residue.

Furthermore, confirmation of the disaccharide repeating-unit (**1**) of T-2-HN, and evidence for the location of the acetyl substituents, were provided by the results of carbon-13 nuclear magnetic resonance (^{13}C-n.m.r.) spectroscopy. The assignment of each signal in the spectrum (see Fig. 3) was based on the data with respect to those for mono-*O*-methylated methyl α-D-mannopyranosides[17] and methyl α-D-manno-oligosaccharides[18]. The results are given in Table II. The presence of acetyl groups was confirmed by sharp signals at 21.3 (methyl carbon) and 174.6 p.p.m. (carbonyl carbon) in the spectrum. The signals at 102.8, 70.6, 79.0, and 67.1 p.p.m. could be assigned to C-1, C-2, C-3, and C-4 of both α-(1→3)-linked D-mannopyranosyl and 6-*O*-substituted (6-*O*-acetylated) α-(1→3)-linked D-mannopyranosyl residues in T-2-HN, as signals for C-1, C-2, C-3, and C-4 of α-(1→3)-linked D-mannosyl residues are uninfluenced by 6-*O*-substitution[17,18]. In contrast, chemical shifts for C-5 and C-6 are affected by 6-*O*-substitution[17,18]. Accordingly, two resonances, at 74.4 and 72.0 p.p.m. (upfield shift) would respectively correspond to C-5 of α-(1→3)-linked D-mannose and its 6-*O*-substituted residue. Similarly, the resonances at 62.1 and 64.6 p.p.m. (downfield shift) could be respectively assigned to C-6 of α-(1→3)-linked D-mannosyl and 6-*O*-substituted D-mannosyl residues. The two peaks for C-5 and C-6 gave almost the same relative areas; these arise as a result of the presence of one acetyl group per two D-mannosyl residues of the repeating-unit in T-2-HN.

Thus, it is suggested that the presence of a 6-*O*-acetyl group on the D-mannosyl residues might affect the solubility of the native polysaccharide (T-2-HN) in water or dimethyl sulfoxide; that is, the loss of such acetyl groups from T-2-HN probably causes molecular association by accelerating the formation of hydrogen bonds between linear, α-(1→3)-linked D-mannopyranosyl chains. This presumption is similar to those made for other, *O*-acetylated mannans[5-8]. In addition, in the course of investigations on the biological properties of this D-mannan, we have found that T-2-HN exhibits antitumor and anti-inflammatory activities.

EXPERIMENTAL

Materials. — The purified (1→3)-α-D-mannan (T-2-HN) used in this study was prepared as reported previously[2]. The *O*-deacetylated polymer was obtained, as a water-insoluble material, by treatment of T-2-HN with 0.2M sodium hydroxide for 2 h at room temperature. Sepharose CL-2B, Sephadex LH-20, and standard dextrans (dextran T-2,000, T-500, and T-250) were purchased from Pharmacia Fine Chemicals.

General. — All evaporations were conducted under diminished pressure at bath temperatures not exceeding 40°. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. Infrared (i.r.) spectra were recorded with a JASCO IRA-1 spectrometer. G.l.c. was performed in a JEOL JGC-1100 apparatus equipped with a flame-ionization detector. A glass column (0.3 × 200 cm) packed with 3% of ECNSS-M on Gaschrom Q (100–120 mesh) was used at 178°, with nitrogen as the carrier gas at a pressure of 2.2 kg/cm^2. G.l.c.–m.s. was conducted with a JEOL

JMS-300 apparatus equipped with a glass column (0.2 × 100 cm) packed with 3% of ECNSS-M, at 182°, at a pressure of helium of 1.2 kg/cm^2. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μA, and a temperature of the ion source of 220°.

The ^{13}C-n.m.r. spectrum was obtained by using a JEOL-FX 100 spectrometer with Fourier transform, for a solution in deuterium oxide (40 mg/mL) in a 10-mm tube at 68°. The spectral width was 6024 Hz, the pulse angle 45°, the repetition time 3.0 s, the pulse width 7 μs, the number of data points 8,192, and the number of pulses 28,000. The chemical shifts were obtained by use of 1,4-dioxane as the internal standard (at 67.4 p.p.m.).

Gel filtration and estimation of molecular weight. — Gel filtration of the native polymer (T-2-HN) was conducted with 0.1M sodium chloride as the eluant. The sample (1.4 mg) was dissolved in the eluant (0.5 mL), and applied to a column (1.5 × 98 cm) of Sepharose CL-2B. The column was eluted with 0.1M sodium chloride at a flow rate of 5 mL/h. Fractions (4 mL each) were collected, and an aliquot of each fraction was analyzed by the phenol–sulfuric acid method[19].

Gel filtration of the deacetylated T-2-HN was performed with 2M sodium hydroxide as the eluant, by a procedure similar to that just described. In both experiments, calibration curves were constructed by use of dextran T-2,000 (mol. wt. 2,000,000), T-500 (495,000), and T-250 (253,000), and the molecular weight was estimated. The results are shown in Figs. 1 and 2.

Acetalation with methyl vinyl ether. — The dried sample (T-2-HN; 14 mg) was dissolved in dimethyl sulfoxide (4 mL), and then *p*-toluenesulfonic acid (10 mg) and methyl vinyl ether (2 mL; condensed at −10°) were added[12]. The mixture was stirred for 5 h at 15°, to give a clear, orange solution. After addition of pyridine (0.5 mL), the excess of methyl vinyl ether was evaporated under diminished pressure, and the resulting syrup was fractionated in a column (2.1 × 46 cm) of Sephadex LH-20 equilibrated with anhydrous acetone, the specific rotation of fractions (5 mL each) being measured. Fractions from tubes 14 to 19, which were optically active, were combined, and evaporated to dryness. The product thus obtained was re-treated by the method already described. The final product showed no hydroxyl absorption band in its i.r. spectrum.

Methylation analysis. — A solution of the *O*-acetyl-*O*-(1-methoxyethyl)polysaccharide (15 mg) in dimethyl sulfoxide (5 mL) was treated with methylsulfinyl carbanion (2 mL), and then with methyl iodide (1 mL), according to the method of Hakomori[13]. The mixture was then dialyzed against distilled water for 2 days. the nondialyzable fraction extracted with chloroform, and the extract evaporated to dryness. The methylation procedure was repeated twice. The final product showed no hydroxyl absorption band in its i.r. spectrum.

The *O*-(1-methoxyethyl)-*O*-methyl-polysaccharide (11 mg) was heated with 90% formic acid (3 mL) for 3 h at 100° in a sealed tube. After removal of the formic acid by evaporation, the residue was hydrolyzed with 0.25M sulfuric acid (4 mL) for 15 h at 100°. The hydrolyzate was made neutral with barium carbonate, the

suspension filtered, and the filtrate passed through a column of Amberlite CG-120 (H^+) resin. The resulting methylated sugars were reduced with sodium borohydride to the corresponding alditols, and these were acetylated with 1:1 acetic anhydride–pyridine for 90 min at 95°. A mixture of the partially methylated alditol acetates was analyzed by g.l.c. and by g.l.c.–m.s. The results are shown in Table I.

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REFERENCES

1 C. HARA, T. KIHO, Y. TANAKA, AND S. UKAI, *Carbohydr. Res.*, 110 (1982) 77–87.
2 S. UKAI, C. HARA, T. KIHO, AND K. HIROSE, *Chem. Pharm. Bull.*, 28 (1980) 2647–2652.
3 B. E. KARI AND G. R. GRAY, *J. Biol. Chem.*, 254 (1979) 3354–3357.
4 M. TOMODA, S. NAKATSUKA, AND N. SATOH, *Chem. Pharm. Bull.*, 22 (1974) 2710–2713.
5 A. MISAKI, T. ITO, AND T. HARADA, *Agric. Biol. Chem.*, 36 (1972) 761–771.
6 T. SATOH, *Nippon Kagaku Zasshi*, 88 (1967) 982–985.
7 M. TOMODA, S. KANEKO, AND S. NAKATSUKA, *Chem. Pharm. Bull.*, 23 (1975) 430–436.
8 B. S. PAULSEN, E. FAGERHEIM, AND E. ØVERBYE, *Carbohydr. Res.*, 60 (1978) 345–351.
9 S. UKAI, K. HIROSE, T. KIHO, AND C. HARA, *Chem. Pharm. Bull.*, 22 (1974) 1102–1107.
10 S. UKAI, S. MORISAKI, M. GOTO, T. KIHO, C. HARA, AND K. HIROSE, *Chem. Pharm. Bull.*, 30 (1982) 635–643.
11 T. KIHO, C. HARA, AND S. UKAI, *Chem. Pharm. Bull.*, 29 (1981) 225–228.
12 A. N. DE BELDER AND B. NORRMAN, *Carbohydr. Res.*, 8 (1968) 1–6.
13 S.-I. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
14 J. H. SLONEKER, *Methods Carbohydr. Chem.*, 6 (1972) 20–24.
15 H. BJÖRNDAL, J. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem., Int. Ed. Engl.*, 9 (1970) 610–619.
16 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801–1808.
17 P. A. J. GORIN, *Carbohydr. Res.*, 39 (1975) 3–10.
18 T. OGAWA AND K. SASAJIMA, *Carbohydr. Res.*, 97 (1981) 205–227.
19 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.