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A MINOR, PROTEIN-CONTAINING GALACTOMANNAN FROM A SODIUM CARBONATE EXTRACT OF *Cordyceps sinensis**

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ABSTRACT

A water-soluble, minor, protein-containing galactomannan (CT-4N), $[\alpha]_D$ −29.6°, isolated from a 5% sodium carbonate extract of *Cordyceps sinensis*, showed a homogeneous pattern in gel filtration and one spot in glass-fiber paper-electrophoresis. The molecular weight was estimated by gel filtration to be ~23,000. It was mainly composed of D-mannose and D-galactose in the molar ratio of 3:5, and contained a small proportion of protein. From the results of methylation analysis, Smith degradation, stepwise hydrolysis, and ^{13}C-n.m.r. spectroscopy, it was concluded that the polysaccharide has a highly branched structure, and is composed of (1→6)- and (1→2)-linked α-D-mannopyranosyl residues in the main chain. Some of the residues are present as branching points of (1→2,6) and (1→4,6) linkages, and the branches contain short chains having a large proportion of (1→5)-linked β-D-galactofuranosyl residues and a small proportion of (1→6)-linked α-D-galactopyranosyl residues, and the (nonreducing) terminal groups consist of a large proportion of β-D-galactofuranosyl and a small proportion of α-D-mannopyranosyl groups.

INTRODUCTION

Cordyceps sinensis (Ascomycetes) is a fungus parasitic on a larva of Lepidoptera. In China, the fungus has been used as a drug and as a food. Miyazaki *et al.*[2] isolated a galactomannan ($[\alpha]_D$ −45.3°) from a hot-water extract of the ascocarps of the fungus. In a series of studies on the polysaccharides in fungi, we have already reported a galactomannan[3] from *C. cicadae*, and have now isolated a minor, protein-containing galactomannan (CT-4N) from a 5% sodium carbonate extract of the residue recovered after hot-water extraction of *C. sinensis*. The

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present article deals with the purification, characterization, and structural analysis of CT-4N from the fungus.

RESULTS AND DISCUSSION

The crude drug from *C. sinensis* was successively extracted with acetone, hot methanol, hot 70% ethanol, and hot water. The residue, thoroughly extracted with hot water, was then extracted with 5% sodium carbonate at room temperature. The alkaline extract was made neutral, and dialyzed. The non-dialyzable solution was mixed with 3 volumes of ethanol, and the precipitate was dissolved in water, and then deproteinized by the Sevag procedure[4]. The aqueous solution was purified by chromatography on DEAE-Sephadex A-25 and Sephacryl S-300, and the solution was lyophilized, to yield a minor, protein-containing polysaccharide (CT-4N) in 0.08% yield. CT-4N showed one spot in glass-fiber paper-electrophoresis and a homogeneous pattern in gel filtration on Sephacryl S-300 (see Fig. 1).

The polymer (CT-4N) had $[\alpha]_D$ −29.6° (*c* 0.36, water), and its component sugars consisted of D-mannose and D-galactose in the molar ratio of 3:5, and a trace of D-glucose. The absolute configurations of the sugars were determined by the method of Leontein *et al.*[5]. The molecular weight of CT-4N was estimated to be ~23,000 by use of the calibration curve (see Fig. 2) obtained by gel chromatography of standard dextrans on Sephacryl S-300.

The galactomannan was fully methylated by the method of Hakomori[6], and

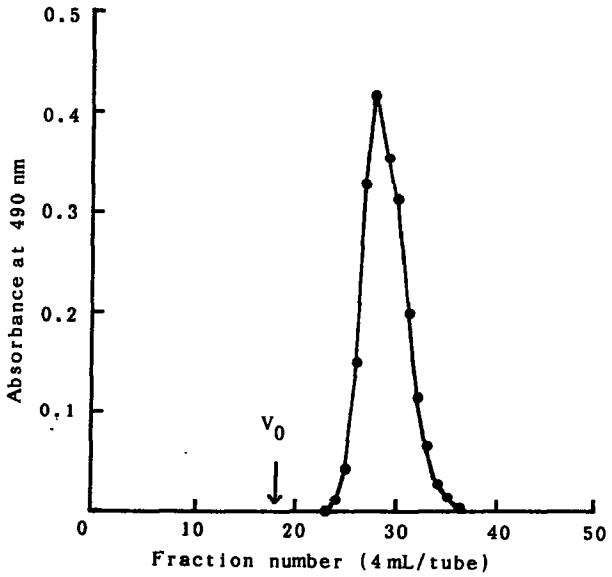

Fig. 1. Chromatogram of CT-4N on Sephacryl S-300. [The column (1.5 × 95 cm) was eluted with 0.1M sodium chloride.]

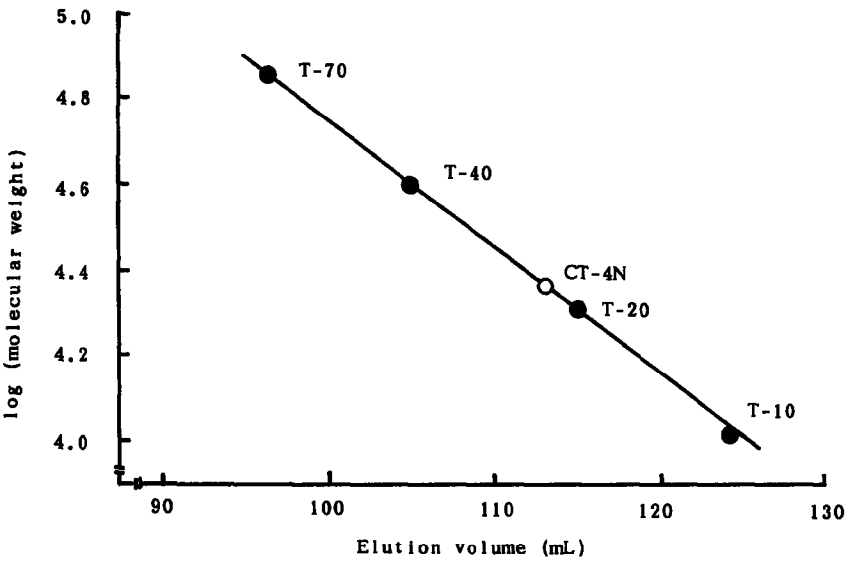

Fig. 2. Determination of molecular weight of CT-4N. The elution volume is plotted against the logarithm of the molecular weight for dextrans T-70, T-40, T-20, and T-10.

the product hydrolyzed; the products were analyzed as the alditol acetates by gas–liquid chromatography (g.l.c.) and g.l.c.–mass spectrometry (g.l.c.–m.s.). Table I shows the results of the methylation analysis, in which the peak having a retention time of 2.47 was found (by the mass spectra) to be composed of a large proportion of 2,3,6-tri-*O*-methylgalactose and a small proportion of 2,3,4-tri-*O*-methyl-mannose derivatives. Galactosyl residues were detected as 2,3,5,6- (a large percentage) and 2,3,4,6-tetra- (a trace), and 2,3,6- (a large percentage) and 2,3,4-tri-*O*-methyl derivatives, which indicated that a large proportion (89%) of non-

TABLE I

G.L.C. AND G.L.C.–M.S. OF ALDITOL ACETATES DERIVED FROM METHYLATED PRODUCT

Methylated sugar (as alditol acetate)	T[a]	*Primary mass fragments* (m/z)	*Molar ratio*	*Mode of linkage*
2,3,4,6-Me_4-Man	1.00	45, 117, 161, 205	1.0	Man*p*-(1→
2,3,5,6-Me_4-Gal	1.15	45, 89, 117, 205	10.8	Gal*f*-(1→
2,3,4,6-Me_4-Gal	1.25	45, 117, 161, 205	0.4	Gal*p*-(1→
3,4,6-Me_3-Man	1.97	45, 161, 189	1.4	→2)-Man*p*-(1→
2,3,6-Me_3-Gal	2.47[b]	45, 117, 233	13.7[c]	→5)-Gal*f*-(1→
2,3,4-Me_3-Man		117, 161, 189, 233		→6)-Man*p*-(1→
2,3,4-Me_3-Gal	3.41	117, 161, 189, 233	3.0	→6)-Gal*p*-(1→
2,3-Me_2-Man	4.89	117, 261	1.8	→4,6)-Man*p*-(1→
3,4-Me_2-Man	5.41	189	6.0	→2,6)-Man*p*-(1→

[a]Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]Overlapping peaks. [c]2,3,6-Me-Gal: a large proportion; 2,3,4-Me-Man: a small proportion.

reducing end-groups are galactofuranosyl groups, and that the tri-*O*-methyl derivatives originated from residues that are engaged in a large proportion of (1→5)-furanosyl or (1→4)-pyranosyl linkages, and some (1→6)-pyranosyl linkages. The results for the methyl derivatives of mannose show the presence also of 3,4-(77%) and 2,3-di-*O*-methyl-D-mannose (23%) as branching sugars, and that of a small percentage of 2,3,4,6-tetra-*O*-methyl-D-mannose as (nonreducing) terminal mannosyl. The presence of (1→2)- and (1→6)-linked D-mannopyranosyl residues was respectively indicated by the detection of 3,4,6- and 2,3,4-tri-*O*-methyl-D-mannose derivatives.

On oxidation of the polysaccharide with sodium periodate, CT-4N consumed 1.39 mol of periodate per hexosyl residue. The periodate-oxidized polysaccharide was reduced with sodium borohydride, and the products were hydrolyzed with acid. The hydrolyzate was analyzed by g.l.c., as the trimethylsilyl derivatives of the oxime derivatives, and as the alditol acetate derivatives. Glycolaldehyde, glycerol, glyceraldehyde, threitol, and erythritol were revealed by g.l.c. The identification of glyceraldehyde supported the presence of (1→2)- and (1→2,6)-linked mannosyl residues. The formation of threitol and erythritol also supported the presence of (1→5)- or (1→4)-linked galactosyl residues, and of (1→4,6)-linked mannosyl residues, respectively.

CT-4N was hydrolyzed stepwise with 5mM sulfuric acid for 4 h at 100°, and with 50mM sulfuric acid for 4 h at 100°. Only galactose in the dialyzable fractions was released by both hydrolyses. The acid-degraded polysaccharide as the second nondialyzable fraction was composed only of mannose. These results suggest the presence of a core composed of mannopyranosyl residues having galactofuranosyl residues as side chains.

The ^{13}C-n.m.r. spectrum of CT-4N is shown in Fig. 3. The anomeric configurations were assigned by comparing this spectrum with data in the literature[7–9]. The signal at lowest field, at 110.7 p.p.m., probably represents the nonreducing (terminal) β-D-galactofuranosyl groups[10]. The two lower signals, at 109.7 and 109.0 p.p.m., were attributed to C-1 of (1→5)-linked β-D-galactofuranosyl residues attached to β-D-galactofuranosyl residues and to α-D-mannopyranosyl residues[11]. The data indicate that 2,3,6-tri-*O*-methyl-D-galactose in the aforementioned methylation analysis is derived from (1→5)-linked galactofuranosyl residues. As with ^{13}C-n.m.r. data[3] of a galactomannan from *C. sinensis*, the signals at 103.5, 102.4, and 101.1 p.p.m. are compared with the values of nonreducing terminal, (1→2)-linked, and (1→6)-linked α-D-mannopyranosyl units, respectively. The resonances at 100.2 and 98.7 p.p.m., at high field, would correspond to C-1 of (1→2,6)- and (1→4,6)-linked α-D-mannopyranosyl residues, and the other resonance, at 100.6 p.p.m., was assigned to C-1 of (1→6)-linked α-D-galactopyranosyl residues. The configurations of the anomeric carbon atoms of mannopyranosyl residues were also supported by $^1J_{CH}$ values of 178–179 Hz (see ref. 12).

The foregoing data indicated that the polysaccharide, a highly branched

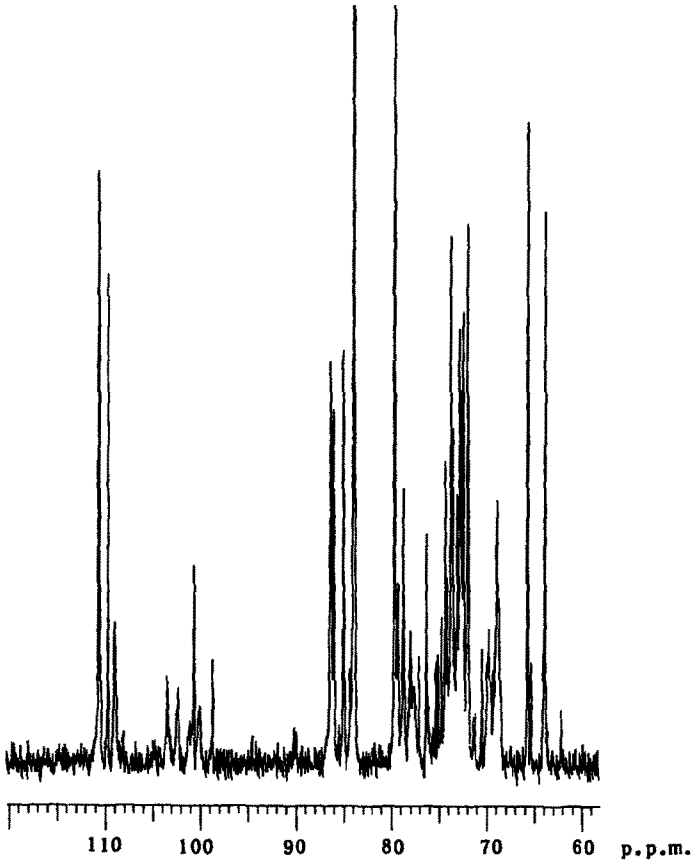

Fig. 3. ^{13}C-N.m.r. spectrum of CT-4N in deuterium oxide at 70°.

galactomannan, is composed of a core of (1→2)- and (1→6)-linked α-D-mannopyranosyl residues, some of which are substituted at O-2 or -6 and at O-4 or -6 with short chains of a large proportion of (1→5)-linked β-D-galactofuranosyl residues and a small percentage of (1→6)-linked α-D-galactopyranosyl residues. CT-4N also has a large percentage of β-D-galactofuranosyl residues and a small percentage of α-D-mannopyranosyl groups as (nonreducing) terminal groups.

CT-4N contained small proportions of protein (1.55% of nitrogen by elementary analysis) which could not be removed by the Sevag procedure, by gel filtration on Sephacryl S-300, or by anion-exchange chromatography on DEAE-Sephadex A-25. The amino acid composition of CT-4N is shown in Table II, but it remains obscure as to whether a moiety of protein is associated with, or linked to, the galactomannan of CT-4N. Gander *et al.*[13,14] reported on the 5-*O*-β-D-galactofuranosyl-containing, exocellular glycopeptide (peptidophosphogalactomannan) of *Penicillium charlesii*. However, differences between CT-4N and the glycopeptide were observed in the mode of branching points of the mannopyranosyl residues, in

TABLE II

AMINO ACID COMPOSITION OF CT-4N

Amino acid	*Molar composition (%)*
Alanine	14.2
Glutamic acid	13.3
Glycine	12.9
Serine	11.8
Threonine	10.7
Proline	10.5
Valine	8.4
Aspartic acid	6.3
Leucine	2.6
Isoleucine	2.5
Cysteine	1.5
Lysine	1.4
Phenylalanine	1.4
Arginine	1.2
Histidine	0.8
Tyrosine	0.5

the proportions in the amino acid composition, and in the content of phosphate (CT-4N: negligible).

Miyazaki *et al.*[2] reported structural features of a galactomannan termed CS-I (mannose:galactose, 1:1), $[\alpha]_D$ −45.3°, from the hot-water extract of the fungus. Although the molecular weight of CS-I was not reported, both our and their galactomannan contain (1→2)-linked D-mannopyranosyl main-chains and (1→5)-linked D-galactofuranosyl side-chains. However, significant differences between CT-4N and CS-I were observed, in which CS-I contained (1→2,3) linkages of D-mannopyranose residues, and (1→3) and (1→6) linkages of galactofuranose, but did not have (1→4,6) linkages of mannopyranose and (1→6) linkages of galactopyranose. On the other hand, we reported[3] on the galactomannan termed C-3 (mannose:galactose, 4:3), $[\alpha]_D$ +30°, from *C. cicadae*. Comparison between CT-4N and C-3 shows a similarity of structural features consisting of (1→6)- and (1→2)-linked α-D-mannopyranosyl main-chains and of β-D-galactofuranosyl side-chains. However, C-3 has (1→2)-linked galactofuranosyl residues instead of (1→5)-linked galactofuranosyl residues, and does not have (1→4,6)-linked mannopyranosyl residues. Thus, the galactomannans from Cordyceps species have a core of D-mannopyranosyl residues and D-galactofuranosyl side-chains, and some differences in linkage mode are observed.

EXPERIMENTAL

Materials. — The dried, crude drug from *Cordyceps sinensis* (Berk.) Sacc. is commercially available in Hong Kong. DEAE-Sephadex A-25, Sephacryl S-300, and standard dextrans (dextran T-70, T-40, T-20, and T-10) were purchased from Pharmacia Fine Chemicals.

General. — All evaporations were conducted under diminished pressure at a bath temperature not exceeding 40°. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. Infrared (i.r.) spectra were recorded with a JASCO IR A-1 spectrometer. Glass-fiber paper-electrophoresis was conducted on Whatman GF-81 glass-fiber paper with 0.05M sodium tetraborate buffer (pH 9.5) as described previously[3]. Paper chromatography (p.c.) was performed on Toyo No. 51 filter paper with a solvent system of 6:4:3 1-butanol–pyridine–water by the double-ascending method, and sugars were detected with an alkaline silver nitrate reagent[15]. The alditol acetate and partially methylated alditol acetate derivatives were separated on a glass column (2 m × 3 mm) packed with 3% of ECNSS-M on Gaschrom Q (100–120 mesh), and the trimethylsilyl derivatives on a glass column (2 m × 3 mm) packed with 5% of Silicone GE SE-30 on Chromosorb W (60–80 mesh). These columns were fitted to a Shimadzu 4CM apparatus equipped with a flame-ionization detector. The acetylated (+)-2-octyl glycosides were applied to a capillary column fitted to a Shimadzu 8A apparatus equipped with a flame-ionization detector. G.l.c.–m.s. was achieved with a JEOL JMS-D 300 apparatus equipped with a glass column (1 m × 2 mm) packed with 3% of ECNSS-M as previously described[18]. Amino acids were determined with a Hitachi 835-50 amino acid analyzer. Phosphorus was analyzed by the method of Chen *et al.*[17].

Isolation of the polysaccharide. — The crude drug (265 g) of *C. sinensis* was pulverized, and successively extracted with acetone, hot methanol and hot 70% aqueous ethanol, and the residue was exhaustively extracted with hot water. The residual material was suspended in 5% sodium carbonate (3 L) for 12 h at room temperature. The alkaline suspension was filtered, and the filtrate was made neutral with acetic acid, and then dialyzed against distilled water. The non-dialyzable solution was mixed with 3 volumes of ethanol, the precipitate was dissolved in water, and the solution was deproteinized by the Sevag procedure[4]. The aqueous solution was further purified by column chromatography on DEAE-Sephadex A-25 (phosphate form). The neutral fraction was dialyzed, and lyophilized, to afford, in 0.1% yield, crude polysaccharides. The crude polysaccharides were separated on a column of Sephacryl S-300 to remove a small proportion of high-molecular-weight polysaccharide. The minor, protein-containing polysaccharide (CT-4N) was thus obtained as colorless flakes, in 0.08% yield.

Gel filtration. — A solution of CT-4N (1 mg) in 0.1M sodium chloride (1 mL) was applied to a column (95 × 1.5 cm) of Sephacryl S-300. The column was eluted with 0.1M sodium chloride at a flow rate of 8 mL/h. Fractions (4 mL each) were collected, and an aliquot of each fraction was analyzed by the phenol–sulfuric acid method[16]. The calibration curve was constructed by use of dextran T-70 (mol. wt. 70,000), T-40 (39,500), T-20 (22,300), and T-10 (10,400) as shown in Fig. 2, and the molecular weight was then estimated.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra were recorded, with a JEOL-FX 270 spectrometer in the Fourier-transform mode with complete proton-

decoupling, for a solution in D_2O (~50 mg/mL) in a 10-mm tube at 70°. The chemical shifts were obtained by the use of tetramethylsilane as an external standard.

Analysis of constituent sugars. — CT-4N was hydrolyzed with M sulfuric acid for 8 h at 100°, and the hydrolyzate was analyzed by p.c. and by g.l.c. as alditol acetates, as previously described[18]. Determination of absolute configurations was achieved as follows. The sugars obtained by lyophilization of the hydrolyzate (3 mg) were heated with (+)-2-octanol (0.5 mL) and a drop of trifluoroacetic acid, in a sealed tube with stirring, for 24 h at 130°. The reaction mixture was evaporated to dryness at 55°, and the residue acetylated with 1:1 acetic anhydride–pyridine for 30 min at 100°. The products were separated in an FS-WCOT capillary column (25 m × 0.25 mm) packed with SP-1000, at 210°, at a pressure of 49 kPa (0.5 kg/cm^2) of nitrogen, with a split ratio of 1:13. The relative retention-times with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol were 7.68 and 11.07 for D-mannose derivatives, and 8.51, 9.95, and 10.55 for D-galactose derivatives.

Methylation analysis. — The polysaccharide was methylated three times by the method of Hakomori, as previously described[19]. The final methylation-product showed no hydroxyl absorption-band in the i.r. spectrum. The fully methylated polysaccharide was successively heated in a sealed tube with 90% formic acid for 5 h at 100°, and 0.25M sulfuric acid for 10 h at 100°. After the acid had been neutralized with barium carbonate, the hydrolyzate was converted into alditol acetates. The resulting, partially methylated alditol acetates were analyzed by g.l.c. [column temperature: 170°; carrier gas (nitrogen): 35 mL/min] and g.l.c.–m.s.; the results are shown in Table I.

Periodate oxidation and Smith degradation. — Periodate oxidation (10 mg) was conducted in the dark with 10mM sodium metaperiodate (40 mL) for 8 days at 5°. The periodate consumption was estimated by an arsenite method[20], and the amount per sugar residue was 1.39 mol. A part of the polyalcohol (3 mg) prepared from the periodate-oxidized product with sodium borohydride was analyzed by g.l.c. as alditol acetate derivatives; the column temperature was increased by 5° per min from 100 to 180°. A part of the polyalcohol (3 mg) was treated with 0.5M hydrogen chloride for 30 min at 35°, and then with hydroxylamine hydrochloride for 30 min at 80° in a sealed tube. The reaction mixture was evaporated to dryness, and the residue was dissolved in pyridine containing 2-ethyl-2-(hydroxymethyl)-1,3-propanediol as the internal standard, trimethylsilylated with hexamethyldisilazane and chlorotrimethylsilane, and the resulting mixture subjected to g.l.c. as already described[3].

Stepwise hydrolysis with acid. — CT-4N (15 mg) was heated with 5mM sulfuric acid for 4 h at 100°. The resulting solution was dialyzed against de-ionized water, and the internal solution was treated with 50mM sulfuric acid for 4 h at 100°, and dialyzed. Each external solution was analyzed by p.c. The second non-dialyzable fraction was hydrolyzed with 0.25M sulfuric acid for 5 h at 100°, and analyzed by p.c. and g.l.c. as alditol acetates.

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REFERENCES

1 C. HARA, T. KIHO, AND S. UKAI, *Carbohydr. Res.*, 145 (1986) 237–246.
2 T. MIYAZAKI, N. OIKAWA, AND H. YAMADA, *Chem. Pharm. Bull.*, 25 (1977) 3324–3328.
3 S. UKAI, S. MATSUURA, C. HARA, T. KIHO, AND K. HIROSE, *Carbohydr. Res.*, 101 (1982) 109–116.
4 M. G. SEVAG, *Biochem. Z.*, 273 (1934) 419–429.
5 K. LEONTEIN, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res.*, 62 (1978) 359–362.
6 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1974) 205–208.
7 P. A. J. GORIN, E. M. BARRETO-BERGTER, AND F. S. DA CRUZ, *Carbohydr. Res.*, 88 (1981) 177–188.
8 J. H. BRADBURY AND G. A. JENKINS, *Carbohydr. Res.*, 126 (1984) 125–156.
9 M. B. PERRY AND L. A. BABIUK, *Can. J. Biochem. Cell. Biol.*, 62 (1973) 1037–1040.
10 C. J. UNKEFER AND J. E. GANDER, *J. Biol. Chem.*, 254 (1979) 12,131–12,135.
11 P. A. J. GORIN AND M. MAZUREK, *Carbohydr. Res.*, 48 (1976) 171–186.
12 K. BOCK, I. LUNDT, AND C. PEDERSEN, *Tetrahedron Lett.*, (1973) 1037–1040.
13 J. E. GANDER, N. H. JENTOFT, L. R. DREWES, AND P. D. RICK, *J. Biol. Chem.*, 249 (1974) 2063–2072.
14 P. D. RICK, L. R. DREWES, AND J. E. GANDER, *J. Biol. Chem.*, 249 (1974) 2073–2078.
15 W. E. TREVELYAN, D. F. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1955) 444–445.
16 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
17 P. S. CHEN, T. Y. TORIBARA, AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756–1758.
18 T. KIHO, C. HARA, AND S. UKAI, *Chem. Pharm. Bull.*, 33 (1985) 270–275.
19 S. UKAI, C. HARA, T. KIHO, AND K. HIROSE, *Chem. Pharm. Bull.*, 26 (1978) 1729–1736.
20 P. F. FLEURY AND J. LANGE, *J. Pharm. Chim.*, 17 (1933) 196–206.