Extracellular Phosphogalactomannan of *Candida mucifera* CCY 29-170-1 Strain

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An extracellular polysaccharide has been recovered from the culture filtrate of the yeast *Candida mucifera* CCY 29-170-1 strain. Gel filtration of the crude polysaccharide yielded the representative galactomannan composed of (*x*/%) D-mannose (1.00), D-galactose (0.14), and covalently bound phosphate (7.6 mole %). On the basis of the results of methylation analysis, acetolysis, and ^{13}C, ^{1}H, and ^{31}P spectral measurements it was concluded that the phosphogalactomannan had an α-(1→6)-D-mannopyranosyl backbone partially substituted on O-2 with side chains of one, two, or three D-mannosyl units connected by α-(1→2) linkages. A small portion of the (1→3) linkages arised from the phosphorylated part of the polymer. D-Galactose was located exclusively at the nonreducing end of side chains. The phosphate was proved to be esterified to primary hydroxyl of side-chain D-mannosyl residues.

It is now recognized that extracellular and cell-wall phosphomannans of yeasts include a variety of structural species containing different amounts of phosphate ester that have in common the D-mannose 6-phosphate [1]. A structural identity among the *Candida* mannans has been demonstrated in that they possess an α-(1→6)-linked D-mannopyranose backbone having α-(1→2)-linked mannosyl branches attached to the backbone by an α-(1→2) linkage [2]. The length and the proportion of side chains as well as the occurrence of α-(1→3) linkages and β-(1→2)-linked units in side chains vary greatly among the yeast species. It is also clear that mannans have the potential to influence multiple biological functions, including both mannan-specific and mannan-nonspecific activities [3]. Resolution of the specific mechanisms involved in immunoregulations induced by mannans will require further homogeneous representatives of this type of polysaccharides.

In the previous paper [4] we reported on isolation and general characterization of extracellular polysaccharides excreted by a new strain of *Candida mucifera* [5] into media containing various carbon sources. In the present study we have attempted the structural determination of the extracellular polysaccharide recovered from the liquid medium with D-glucose as the carbon source.

EXPERIMENTAL

All evaporations were conducted under diminished pressure at 40—45 °C. Analytical paper chromatography was performed by the descending method on Whatman No. 1 paper and the preparative one on Whatman No. 3 paper with the following systems: S_1, ethyl acetate—pyridine—water (φ_r = 8 : 2 : 1) and S_2, ethyl acetate—acetic acid—water (φ_r = 18 : 7 : 8). TLC of oligosaccharides and methylated saccharides was effected on Silufol plates (Kavalier, Sázava) in systems S_3, n-propanol—methanol—water (φ_r = 2 : 1 : 1) and S_4, chloroform—methanol (φ_r = 10 : 1), respectively. Reducing sugars were detected by spraying with anilinium hydrogen phthalate, and alditols with the alkaline silver nitrate reagent. Reducing power was determined by the *Somogyi* method [6]. Determination of the protein content was carried out by the method of *Lowry et al.* [7] using bovine serum albumine as the standard. Total phosphate was determined by the method of *Ames* and *Dubin*, using KH_2PO_4 as the standard [8]. Optical rotations were measured with a Perkin—Elmer Model 141 polarimeter in 0.5 % aqueous solution at 22 °C. Free-boundary electrophoresis was effected with a Zeiss 35 apparatus on a polysaccharide (ρ = 10 mg cm^{-3}) in 0.05 M sodium borate buffer at 10 V and 6 mA.

The number average molecular mass ($\overline{M}_{r,N}$) was determined osmometrically at 30 °C, using a Knauer Vapour Pressure Osmometer. Infrared spectra of the methylated poly- and oligosaccharides were recorded with a Perkin—Elmer Model 9836 spectrometer. GLC was conducted with a Hewlett—Packard Model 5711 chromatograph and (*A*) a column (200 cm × 0.3 cm) of 3 % OV-225 on 150—170 μm Chromosorb W (AW-DMCS), programmed to hold a temperature of 120 °C for 4 min, then to increase it to 170 °C at 2 °C min^{-1}; and (*B*) a column (200 cm × 0.3 cm) of 3 % SP-2340 on 125—150 μm Supelcoport at 110 °C for 2 min, then increasing the temperature to

210 °C at 4 °C min^{-1}. Column *A* was used for quantitative analysis of sugar trifluoroacetates. GLC—MS of alditol acetates of methylated saccharides was carried out with a JGC-20 K gas chromatograph fitted with column *B*, and with helium (inlet pressure 101.3 kPa) as the carrier gas. Mass spectra were obtained at the ionization potential of 23 eV and an emission current of 300 μA, using a JMS-D 100 (Jeol) spectrometer. The inlet temperature was 220 °C and that of the ionization chamber 200 °C.

HPLC analysis of the acetolysis product was performed with an SGX-NH_2 (particle size 5 μm) column (0.4 cm × 25 cm) in a Hewlett—Packard 1050 liquid chromatograph equipped with a differential refractometer. The solvent system was water—acetonitrile (φ_r = 45 : 55) pumped at 1.0 cm^3 min^{-1}.

Proton-decoupled ^{13}C-F.T., ^{31}P, and ^1H NMR spectra were measured with a Bruker AM-300 spectrometer at 75.46 MHz, 121.49 MHz, and 300.13 MHz, respectively, for solutions in D_2O at 25 °C (^{13}C chemical shifts referenced to internal MeOH, δ = 50.15, ^{31}P chemical shifts to external 85 % orthophosphoric acid, δ = 0.00, and ^1H chemical shifts to HOD at δ = 4.80). The other parameters for ^{13}C, ^{31}P, and ^1H measurements were as follows: spectral width 17 kHz, 29 kHz, and 5 kHz, pulse width 12 μs, 20 μs, and 8 μs, the number of data points 16 K, 32 K, and 32 K, and digital resolution 2.10 Hz/PT, 1.7 Hz/PT, and 0.31 Hz/PT, respectively.

Complete hydrolysis (H1) of poly- and oligosaccharides was achieved with 2 M trifluoroacetic acid in a sealed tube at 120 °C for 1 h. Hydrolysis of the polysaccharide either with 2 M-TFA at 105 °C for 20 h (H2), or with 0.5 M-H_2SO_4 at 105 °C for 24 h (H3) gave besides the neutral compounds also an acid product which was isolated from the hydrolyzate by preparative paper chromatography in system S_2.

Periodate oxidation of the acid product of hydrolysis (10 mg) was effected with 30 mM-$NaIO_4$ (4 cm^3) at 4 °C in the dark. The consumption of periodate was monitored by measuring the absorbance of the solution at λ = 225 nm.

Conventional acetolysis was conducted on the polysaccharide essentially as described by *Kocourek* and *Ballou* [9], and mild acetolysis according to the method described by *Shibata et al.* [10, 11]. Gel filtration of the deacetylated acetolyzate was carried out on a column (200 cm × 2.5 cm) of Sephadex G-25 by irrigation with water at 0.5 cm^3 min^{-1}. The fractions were analyzed by the phenol—sulfuric acid assay [12].

Cultivation Conditions and Isolation of the Polysaccharide

The type strain *Candida mucifera* CCY 29-170-1 was maintained on malt agar in the Slovak Collection of Yeasts at the Institute of Chemistry, Slovak Academy of Sciences, Bratislava. The cells grew in a medium consisting of 0.1 mass % KH_2PO_4, 1 mass % $(NH_4)_2SO_4$, 0.05 mass % $MgSO_4 \cdot 7H_2O$, 0.035 mass % $CaCl_2$, 0.3 mass % yeast extract, and 2 mass % D-glucose. Incubation in 1 dm^3 growth flasks containing 500 cm^3 of medium proceeded on a rotary shaker at the frequency of revolution 160 min^{-1} and 28 °C for 7 d.

Cells were removed by centrifugation at 1500 *g* for 10 min. The supernatant was reduced thrice in volume by evaporation, exhaustively dialyzed against distilled water, and precipitated with 96 vol. % ethanol (containing 1 vol. % acetic acid) in the volume ratio of 1 : 3. The precipitate was separated by centrifugation, dissolved in distilled water, and lyophilized to yield the crude polysaccharide P (4.56 mass % based on carbon source).

The product P (500 mg) was dissolved in water and applied to a column (150 cm × 4 cm) of Sephadex G-75. The column was irrigated with water and fractions of 15 cm^3 were collected and analyzed by the phenol—sulfuric acid assay [12]. The elution profile is illustrated in Fig. 1.

Methylation Analysis

The polysaccharide (70 mg) was methylated twice by the *Hakomori* method [13], and once by the Purdie method [14] to give a fully methylated product (65 mg), as checked by IR absorption for hydroxyl. The methylated polysaccharide (10 mg) was treated with aqueous 90 % formic acid (2 cm^3) at 100 °C for 1 h, the hydrolyzate was concentrated to dryness, and the residue was hydrolyzed with 2 M trifluoroacetic acid (2 cm^3) at 100 °C for 5 h. The hydrolytic products were checked by TLC in system S_4, then converted into their alditol acetates and analyzed by GLC—MS [15]. Methylation of oligosaccharides was conducted in a similar way, except that the Hakomori procedure was applied only once.

RESULTS AND DISCUSSION

The crude, carbohydrate-containing liquid culture medium after centrifugation and exhaustive dialysis was treated with ethanol to yield an off-white, water-soluble product P. Gel chromatography of this product on a Sephadex G-75 column resulted in three fractions (Fig. 1). The fraction P1, eluted at the lowest elution volume, was considered the representative polysaccharide, since it amounted to 68 % and was shown to be homogeneous upon free-boundary electrophoresis. This was subjected to structural study, while the fractions P2 and P3 were

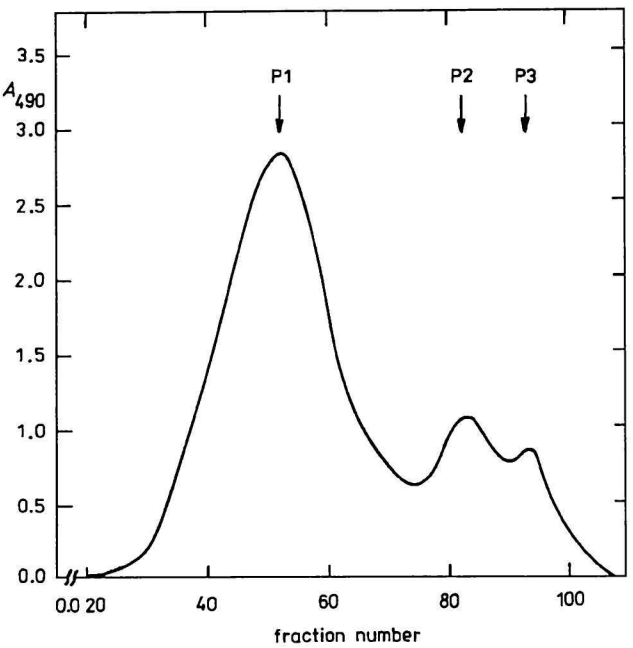

Fig. 1. Gel-filtration pattern of the crude polysaccharide on Sephadex G-75.

not dealt with further. Characterization of the crude (P) and purified (P1) polysaccharides is presented in Table 1.

The component sugars of P1 were identified by paper chromatography and by gas chromatography as their hexitol trifluoroacetates indentical with D-mannose and D-galactose. The polysaccharide contained 2.44 mass % phosphorus (7.6 mass % phosphate). Considering the $\bar{M}_{r,N}$ = 15 889 (DP = 90), it means that approximately each seventh hexose unit was phosphorylated. Hydrolysis H2 and/or H3 afforded besides the two neutral sugars also an acid product, the position of which upon paper chromatography (S_2) corresponded to that of the authentic D-mannose 6-phosphate. After isolation from the hydrolyzate it gave upon complete hydrolysis D-mannose as the only sugar. Determination of its reducing power revealed a phosphorus—D-mannose mole ratio of 0.95. The ^{1}H-decoupled ^{31}P NMR spectrum of this compound displayed a signal at δ = 1.832 which is the region characteristic of phosphomonoesters [16, 17]. In the proton-coupled spectrum this signal was split into a triplet having $J_{H\text{-}6,P} = J_{H\text{-}6',P}$ = 6.0 Hz, which indicated that the phosphate was esterified to the primary hydroxyl [18]. Though the acid stability of the phosphate ester immediately suggested the presence of a hexose 6-phosphate, the position of the phosphate residue was unambiguously established by periodate oxidation and by the ^{13}C NMR spectrum of the sugar phosphate. The periodate consumption per 1 mol of the phosphate ester found after 20 h oxidation (3.9 mol) was nearly theoretical (4.0 mol) for a hexose unit having the O-6 substituted. It is known that phosphorylation of a sugar unit results in a downfield shift of δ = 1.7—4.8 of the resonance of the carbon atom to which the phosphate group is attached [16]. The signal for the C-6 atom at δ = 65.5 was the only signal in the ^{13}C NMR spectrum of the compound under discussion that showed a significant downfield shift of δ = 3.4 compared with that of the C-6 carbon of D-mannopyranose [19].

The polysaccharide was analyzed for its glycosidic linkages after converting it into the corresponding *O*-acetyl-*O*-methyl-hexitols by subsequently adopting the methods of *Hakomori* [13] and *Jansson et al.* [15]. The results are summarized in Table 2. As seen from the table, D-galactose is represented by one derivative, *i.e.* by 2,3,4,6-tetra-*O*-methyl-D-galactose, the amount of which is proportional to that of D-galactose in the polysaccharide, showing that this saccharide occupies exclusively a terminal position as nonreducing ends. D-Mannose occurs in the form of terminal nonreducing ends, (1→6)-linked nonbranching backbone units, (1→2)-linked nonbranching side-chain units (confirmed by acetolysis), and as 1,2,6-tri-*O*-substituted residues. The small portion of (1→3)-linked units originates from the side chains bearing the phosphate groups, since in the neutral portion of the acetolysis products we have not found any oligomer containing (1→3) linkages.

The foregoing results were supported by the ^{13}C NMR spectrum of the polysaccharide, the anomeric region of which is presented in Fig. 2. The assignment of signals was effected on the basis of the chemical shifts of oligomers obtained on acetolysis, and literature data [20]. The seven signals observable in this region indicated a branched structure of the polymer. The resonance at δ = 104.3 can be assigned to C-1 of terminal nonreducing β-D-galactopyranose units. The other six signals originated from resonances of C-1 of α-D-mannopyranosyl units in-

Table 1. Characterization of the Crude (P) and the Purified (P1) Polysaccharides

Polysaccharide	Yield[a]	$[\alpha]_D^{ob}$	*w*(Phosphorus)	*w*(Protein)	$\bar{M}_{r,N}$	Mole ratio of sugar components			
	%		%	%		D-Gal	D-Glc	D-Man	L-Ara
P	4.56	+ 37.5	1.09	10.5		0.08	0.10	1.00	Traces
P1	3.10	+ 41.0	2.44	2.7	15 889	0.14	–	1.00	–

a) Based on the carbon source; *b*) (D, 22 °C, ρ = 5 g cm^{-3}, water).

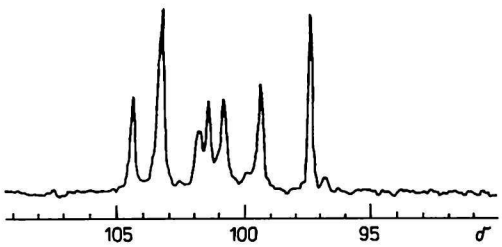

Fig. 2. ^{13}C NMR spectrum (anomeric region) of phosphogalactomannan.

volved in various linkages. The chemical shift of the resonance at δ = 103.17 is consistent with the assignment to α-D-mannopyranosyl residues which are unglycosylated and the anomeric carbons of which are linked to C-2 or C-3 of other α-D-mannopyranosyl residues. The chemical shifts at δ = 101.68 and 101.35 may be assigned to side-chain α-D-mannopyranosyl units involved in (1→2) and (1→3) linkages. It is not a one-to-one assignment but it refers to the group as a whole. The signal at δ = 100.76 is attributed to the unsubstituted (1→6)-linked backbone α-D-mannopyranosyl residues, while that at δ = 99.30 can be assigned to 1,2,6-tri-*O*-substituted α-D-mannopyranosyl units. The last signal in the anomeric region was observed at δ = 97.31. According to [21], this shift originates from the resonance of C-1 atom of a D-mannopyranose unit bearing a phosphate group.

The apparent discrepancy between the proportion of the terminal nonreducing units and the branching points (Table 2) can be explained as follows. We have proved unambiguously the site of esterification at C-6 of the D-mannose unit in the phosphogalactomannan. However, it is known that in many yeast phosphomannans the phosphate group is diesterified to the primary hydroxyl group of the side-chain units and to the anomeric hydroxyl group of a single D-mannosyl residue [1, 22]. We assume that in our phosphogalactomannan the phosphate linkages are also (1→6) phosphoric diester bridges. This

Table 2. Methylated Sugars from the Hydrolyzate of the Methylated Phosphogalactomannan

Derivative[a]	t_r[b]	Mole ratio	*x* mole %	Mode of linkage
2,3,4,6-Me_4-D-Man	1.00	1.00	24.3	1-
2,3,4,6-Me_4-D-Gal	1.03	0.48	11.7	1-
3,4,6-Me_3-D-Man	1.12	0.42	10.2	1,2-
2,4,6-Me_3-D-Man	1.19	0.19	4.6	1,3-
2,3,4-Me_3-D-Man	1.24	0.86	20.9	1,6-
3,4-Me_2-D-Man	1.43	1.16	28.2	1,2,6-

a) 2,3,4,6-Me_4-D-Man = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, *etc.*; *b*) retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol.

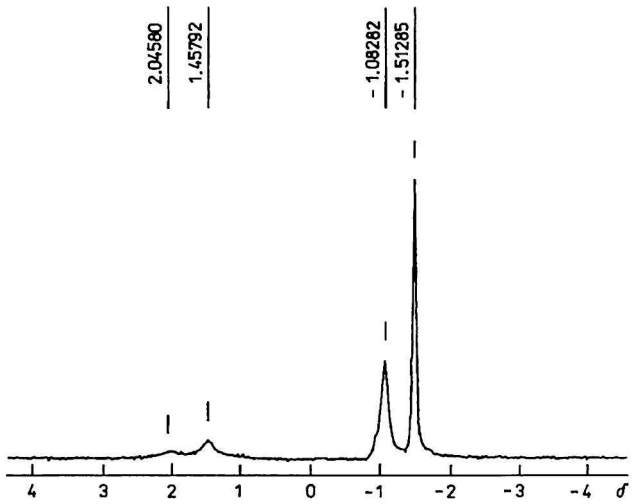

Fig. 3. ^{1}H-Decoupled ^{31}P NMR spectrum of phosphogalactomannan.

assumption may be supported by the following evidence. The ^{1}H-decoupled ^{31}P NMR spectrum of the polysaccharide (Fig. 3) displayed signals at δ = −1.08 and −1.51 which is the region characteristic of diesterified phosphate [17]. The ^{13}C NMR spectrum showed a signal at δ = 97.30 that may be assigned to C-1 of a D-mannose unit bearing a phosphate group [21]. The acetolysis products of the phosphogalactomannan contained by about 7 % higher amount of the D-mannose monomer than it should correspond to the amount of the (1→6)-linked nonbranching backbone D-mannose units, represented in Table 2 by the 2,3,4-tri-*O*-methyl derivative. This extra amount of D-mannose may originate from (partial) cleavage of the hemiacetal phosphate linkage to single mannosyl residues, taking place upon acetolysis. When accepting these evidences and the possibility of (partial) deesterification taking place during methylation of the polysaccharide in alkali medium, then it is clear that the position O-6, originally occupied by the phosphate group, will be also methylated and, consequently, the proportion of the dimethyl derivative will be lower than which should correspond to the tetramethyl analogue.

Conventional acetolysis was employed in order to prepare oligosaccharides composed of non-(1→6)-linked side chains attached to (reducing) D-mannose residues that were formerly part of the (1→6)-linked D-mannosyl backbone. The neutral products of acetolysis were identified by paper and thin-layer chromatography as D-galactose, D-mannose, and three mannose-containing oligosaccharides. As the log (R_f/(1 − R_f)) plotted *vs.* the degree of polymerization of these products gave a linear relationship [23], the peaks *I*—*IV* on the HPLC chromatogram of the acetolysis products (Fig. 4) were considered a homologous series. The eluates corresponding to the void volume region of the elution profile contained

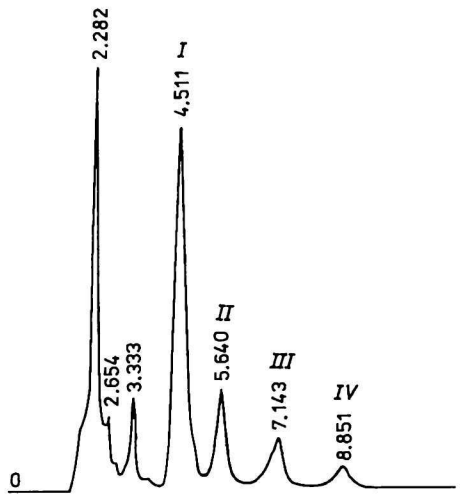

Fig. 4. HPLC chromatogram of acetolysis products of phosphogalactomannan. 0 designates the point of injection.

acid fragments, wherein each third hexose unit was phosphorylated. This part of the acetolyzate has not been studied in the present work as the material was insufficient for a substantial structural study.

The area under each peak of the neutral portion of acetolyzate mentioned above was, after the correction factors had been applied to the monomer and the disaccharide [24], expressed as a percentage of the total area. This percentage area in each peak was divided by its assigned DP value, to give the relative mole amounts of the oligosaccharide presented in Table 3.

Table 3. Relative Mole Amounts of the Neutral Products Present in the Acetolyzates

Acetolysis	Degree of polymerization			
	1	2	3	4
Conventional	32.10[a]	4.80	2.65	1.00
Mild	12.28	0.91	0.68	Traces

a) Comprising D-mannose (28.6) and D-galactose (3.5).

Mild acetolysis applied to the phosphogalactomannan afforded lower amounts of the same neutral products (Table 3), but no β-(1→2) linkage-containing oligomers.

The acetolysis products were separated by gel chromatography on Sephadex G-25. The individual di- (M2), tri- (M3), and tetrasaccharides (M4) were methylated and their hydrolytic products after conversion to alditol acetates were analyzed for glycosidic linkages by GLC—MS. The results indicate that all oligosaccharides consisted solely of 1,2-linked mannopyranose units. The mole ratios of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl mannitols (for M2, M3, and M4 1.00 : 1.00; 2.04; 2.99, respectively) derived from all mannooligomers agreed with their molecular masses.

The above indicated type of linkages in the oligomers was confirmed by their ^{13}C chemical shifts (Table 4) the assignment of which was conducted by correlation with the data in [25]. The only signals shifted downfield were those for the anomeric C-1 carbon atoms of the nonreducing and inter-mannosidic units, and for the C-2 atoms of the reducing and inter-units, evidencing their involvement in a linkage. The α-configuration at all inter-mannosidic linkages was confirmed by the $^1J_{C,H}$ values observed in the region of 169.43 —171.5 Hz as well as by the optical rotation values and the anomeric-proton signals presented in Table 5.

Table 5. Specific Optical Rotations and Anomeric-Proton Chemical Shifts for D-Mannooligosaccharides

Oligosaccharide	$[\alpha]/^{oa}$	Chemical shift, δ Sugar residue			
		D	C	B	A
M2	+ 43.2			5.049	5.390
M3	+ 49.8		5.060	5.310	5.390
M4	+ 52.6	5.060	5.309	5.310	5.392

a) (D, 22 °C, = 10 g cm^{-3}, water); A refers to the reducing unit.

The data obtained on the phosphogalactomannan suggest that this polymer structurally resembles other phosphomannans of *Candida* species in that it has an α-(1→6)-linked D-mannopyranosyl backbone

Table 4. ^{13}C Chemical Shifts (δ) of D-Mannooligosaccharides (α-Anomers) Obtained on Acetolysis

Carbon	M2		M3			M4			
	Sugar residue								
	B	A	C	B	A	D	C	B	A
C-1	103.26	93.61	103.30	101.63	93.56	103.32	101.62	101.69	93.55
C-2	71.05	80.19	71.05	79.63	80.42	71.47	79.73	79.61	80.03
C-3	71.40	71.05	71.41	71.05	71.05	71.26	71.03	71.03	71.04
C-4	67.93	68.12	67.91	68.12	68.12	67.90	68.05	68.12	68.12
C-5	74.31	73.54	74.30	74.30	73.54	74.30	74.31	74.36	73.55
C-6	62.04	62.14	62.04	62.14	62.14	62.04	62.15	62.14	62.13

A refers to the reducing residue.

substituted on O-2 with neutral side chains of one, two, or three D-mannosyl units connected by α-(1→2) linkage.

A small portion of (1→3) linkages occurs probably in side chains, wherein some D-mannopyranosyl residues have the primary hydroxyl phosphorylated. There is evidence indicating that these units are, in turn, attached in a diester linkage to position 1 of single D-mannose units. D-Galactose occupies exclusively nonreducing positions and is attached by β-glycosidic linkage to either O-2 or O-3 of D-mannose side-chain residues.

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