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STRUCTURAL STUDIES OF MANNANS FROM THE CELL WALLS OF THE PATHOGENIC YEASTS *Candida albicans* SEROTYPES A AND B AND *Candida parapsilosis*

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ABSTRACT

A comparative study of three cell-wall mannans, of *Candida albicans* serotypes A and B and *Candida parapsilosis,* by means of methylation analysis supports a model of yeast mannans as having an α-(1→6)-linked backbone with some units (depending on the origin of the mannan) being substituted at O-2 with oligosaccharides joined by α-(1→2) and, to a lesser extent, by α-(1→3) glycosidic bonds. Branching points in the side chains of *Candida albicans* mannans were found in substantial proportions for the first time, and the corresponding branched hexasaccharides were isolated by means of acetolysis and subsequent gel filtration. ^{13}C-N.m.r. spectroscopy of the mannans, as well as a ^{1}H-n.m.r. spectroscopic study of the oligosaccharides obtained on acetolysis of the mannans, led to results that agreed with those of methylation analysis.

INTRODUCTION

Mannans are peptidopolysaccharide constituents of yeast cell-walls. They are considered to have important physiological functions. Besides physical protection of the cell wall, they may serve to hold such enzymes as invertase and acid phosphatase to the cell wall[1], and may participate in cell–cell recognition. The structure of yeast mannans has been studied by many investigators[2–7]. It was found that yeast mannans are composed mainly of D-mannose, and sometimes also contain such minor components as D-glucose, D-galactose, D-xylose, and phosphate. The antigenically important outer chains of yeast mannans[3] have a highly branched shape consisting of an α-(1→6)-linked backbone substituted by side chains of various length containing mainly α-(1→2) and, to a lesser extent α-(1→3) linkages.

In the past ten years, increased attention has been paid to investigation of the mannan component of medicinally important years of the genus *Candida*.

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Infections by these organisms occur in patients whose immune responses have been damaged by cancer, autoimmune disease, organ transplantations, or antibiotics application. The serious character of this disease and the discovery of mannan antigenemia, have led to increased interest in structural and immunochemical characterization of *Candida* sp. mannans.

We now report the results of a structural investigation of the mannans from two pathogenic yeasts of the *Candida* sp. by means of methylation analysis and acetolysis, as well as ^{13}C- and ^{1}H-n.m.r. spectroscopy.

EXPERIMENTAL

Organisms. — *C. albicans* CCY 29-3-109 (serotype A), *C. albicans* CCY 29-3-102 (serotype B), and *C. parapsilosis* CCY 29-20-6 from the Czechoslovak Collection of Yeasts, Institute of Chemistry, Slovak Academy of Science were used. Yeast cells were grown on a semisynthetic, liquid medium containing 2% of D-glucose[8] for 4 days at 28°.

Extraction and purification of polysaccharides. — After centrifugation of disintegrated yeast cells, glycoproteins were extracted from cell walls by autoclaving at 140° with[9] 0.2M NaCl. Lyophilized glycoproteins were suspended in 2% KOH and heated for 2 h at 100°. Insoluble residue was separated by centrifugation, and mannan was precipitated from the supernatant liquor with Fehling reagent[10]. Sedimented mannan–copper complex was dissolved in 3M HCl, and added dropwise to 8:1 (v/v) methanol–acetic acid. The procedures of dissolution and precipitation were repeated until the methanol–acetic acid mixture ceased to turn green. Finally, the white sediment was separated, dissolved in distilled water, the solution dialyzed against distilled water for 24 h, and the dialyzate lyophilized. Mannan so purified contained no nitrogen, as determined by elementary analysis, and was considered to be completely deproteinized.

Acetylation and acetolysis of mannans. — These were performed as described by Kocourek and Ballou[11]. Samples (~90 mg) were used. The deacetylated acetolysis products were applied to a column (2 × 150 cm) of Bio-Gel P-2, and eluted with distilled water at the rate of 12 mL/h at room temperature, 2- or 2.5-mL fractions were collected. The total carbohydrate content in the effluent was determined by the phenol–sulfuric acid method[12].

Methylation was conducted by treatment of mannans with methylsulfinyl carbanion–methyl iodide in dimethyl sulfoxide according to the method of Hakomori[13] as described by Lindberg[14].

After the dialysis, and extraction into chloroform, the extract was evaporated, and the residue was re-methylated by refluxing in methyl iodide with silver oxide. The oligosaccharides were methylated by the same procedure, but the dialysis was omitted, and the reaction mixture was directly extracted with chloroform after having been poured into water.

The partially methylated alditol acetates were analyzed by use of a JGC-20K

gas chromatograph equipped with a column (200 × 0.3 cm) of SP-2340 on Chromosorb WAW-DMCS (80–100 mesh) at 180° (4 min) → 210° (2° per min) with helium (inlet pressure, 101.3 kPa) as the carrier gas (flow rate 30 mL/min). The molar composition of the mixture was calculated from the peak areas by using the response factors for individual methylated derivatives[15]. Mass spectra were recorded at 23 eV and an emission current of 300 μA, using a JMS D 100 (JEOL) spectrometer. The inlet temeprature was 220° and that of the ionizing chamber, 200°. The fragmentation patterns were compared with those reported by Jansson *et al.*[16].

N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra of oligosaccharides were recorded with a Bruker AM-300 FT-spectrometer at 300-MHz field frequency and room temperature for solutions in deuterium oxide, using acetone as internal standard (2.2 p.p.m. in relation to Me_4Si). ^{13}C-N.m.r. spectra of mannans were recorded at 75.468 MHz with the same instrument for solutions in deuterium oxide at room temperature. Methanol was used as internal standard (50.15 p.p.m. in relation to Me_4Si).

RESULTS AND DISCUSSION

The results of methylation analysis of the mannans of the three types of *Candida* genus yeasts are presented in Table I. The 2,3,4,6-tetra-*O*-methylalditol acetate is derived from the terminal, nonreducing mannosyl group and is present in almost equal proportions in both *C. alibicans* mannans and in somewhat higher proportions in the mannan of *C. parapsilosis*. The most abundant 3,4,6-tri-methylated derivative corresponds to (1→2)-linked, internal side-chain mannosyl units and is present in almost equivalent proportions in all three species studied. *C. albicans* serotype A mannan afforded relatively the largest amount of this derivative, and that indicates longer side-chains in serotype A mannan in comparison with the two other species. This result is in accord with those of Sunayama and Suzuki[17] and Reiss *et al.*[4], but disagrees with the results of Yu *et al.*[18], who found the lowest proportion of this derivative in *C. albicans* serotype A mannan

TABLE I

RELATIVE MOL% OF METHYLATED ALDITOL ACETATES OF THE MANNANS INVESTIGATED

Methyl ether	C. albicans *(serotype A)*	C. albicans *(serotype B)*	Candida parapsilosis
2,3,4,6-tetra-	19.2	20.2	27.1
3,4,6-tri-	38.4	35.7	35.9
2,3,4-tri-	16.6	14.7	10.5
2,4,6-tri-	4.2	6.6	trace
4,6-di-	1.6	4.5	
3,4-di-	20.0	18.3	26.4

TABLE II

METHYLATION ANALYSES OF *Candida* SP. MANNANS BY DIFFERENT AUTHORS (RELATIVE MOL%)

Methyl ether	C. albicans *serotype A*			C. albicans *serotype B*			C. parapsilosis
	Reiss[4]	*Sunayama*[17]	*Yu*[18]	*Reiss*[4]	*Sunayama*[17]	*Yu*[18]	*Yu*[18]
2,3,4,6-tetra-	23.0	12.6	35.7	28.6	16.3	25.6	21.0
3,4,6-tri-	46.2	36.5	22.3	37.1	26.0	41.0	48.0
2,3,4-tri-	7.6	1.0	3.4	7.9	3.7	5.1	6.3
2,4,6-tri-	4.7	1.2	2.5	3.5	2.6	5.1	4.2
4,6-di-							
3,4-di-	11.0	11.0	19.9	11.4	16.2	12.8	10.4
2,4-di-	5.3	1.8	16.2[a]	9.2	2.6	10.3[a]	10.4[a]

[a]This derivative was incorrectly characterized in original work as the 3,5-di-*O*-methyl derivative, but was later found[19,20] to be 2,4-di-*O*-methyl.

(see Table II). The 2,3,4-tri-*O*-methyl derivative corresponds to an unbranched, (1→6)-bound, backbone mannosyl unit. Its proportion is the largest in *C. albicans* serotype A mannan, and the lowest in *C. parapsilosis* mannan. The values found by us are twice those presented by Reiss *et al.*[4], and almost four times those reported by Yu *et al.*[18]. Sunayama and Suzuki[17] found the lowest proportion of this derivative in comparison with other authors' data. The third 2,4,6-tri-*O*-methylalditol acetate is derived from (1→3)-linked, internal, side-chain mannosyl units, and was found in greater proportion in *C. albicans* serotype B mannan, in agreement with the data of Yu *et al.*[18] and Sunayama and Suzuki[17], but contradicting those of Reiss *et al.*[4]. In *C. parapsilosis* mannan, this compound was detected only in traces, and that testifies to a small number of (1→3)-bound sugar units in its structure, and contradicts the (only available) data of Yu *et al.*[18].

The 3,4-di-*O*-methyl derivative is formed from the (1→6)-linked, backbone mannosyl unit branched at C-2. The second 4,6-dimethylated derivative was found for the first time in *C. albicans* mannans in considerable proportion. Formerly, it had been detected only in traces in *C. albicans* serotype B mannan[21], *C. parapsilosis* mannan[6], and *C. tropicalis* mannan[22]. This compound may be derived from a (1→2)-bound side-chain mannosyl unit branched at C-3. *C. albicans* serotype B mannan contains almost three times as much of this derivative as serotype A mannan. This may be responsible for the clear serological difference observed between these two strains. In general, this type of branching appears to be common to several kinds of yeasts, a fact to which little attention has as yet been paid. It may occur in different antigenic factors (*e.g.*, 13b, as proposed by Fukazawa *et al.*[21]) and in this way determine immunochemical properties of the yeast mannans.

In *C. parapsilosis* mannan, no such branching point in its side chains was detected. We have not found any trace of the 2,4-di-*O*-methyl derivative, although it was reported to be present among the methylation products in the aforementioned papers[4,16,17]; this means that the side chains are linked to the backbone mannosyl units exclusively at their C-2 atoms.

The data obtained by methylation analysis support a model of *C. albicans* mannans as having a (1→6)-linked backbone in which 55% of all units (~4 of every 7 units) are substituted at O-2 with oligosaccharides joined by (1→2) linkages and, occasionally, by (1→3) linkages. The ratio of (1→2) to (1→3) linkages in serotype A mannan was 9.1:1 and in serotype B, 5.4:1, in perfect accord with the data of Reiss *et al.*[4]. Some branching at C-3 is present in the side-chain mannosyl units. *Candida parapsilosis* mannan may be regarded as having the same (1→6)-linked backbone, with almost 72% of all units (~3 of every 4) being substituted at O-2 with mainly (1→2)-linked oligosaccharides. Only a negligible number of (1→3) linkages were found and no branching in the side chains was detected.

The results of methylation analysis are consistent with the results of ^{13}C-n.m.r.-spectral measuring of the mannans studied. In order to facilitate interpretation of the spectra, the partial structure of the mannans studied, containing all possible types of substituted mannosyl units and their designation is presented (see Fig. 1).

The assignment of signals was based on the data of Allerhand and Berman[23] and Kočiš *et al.*[24]. ^{13}C-N.m.r. spectra of the mannans investigated are presented in Figs. 2–4.

The signals in the lowest field belong to C-1 atoms of various units. The weak signal at ~103.54 p.p.m. corresponds to the C-1 atom of unit E, and it is the strongest in the spectrum of *C. albicans* serotype B mannan and almost negligible in that of *C. parapsilosis*. The next signal at ~103.4 p.p.m. belongs to C-1 of unit

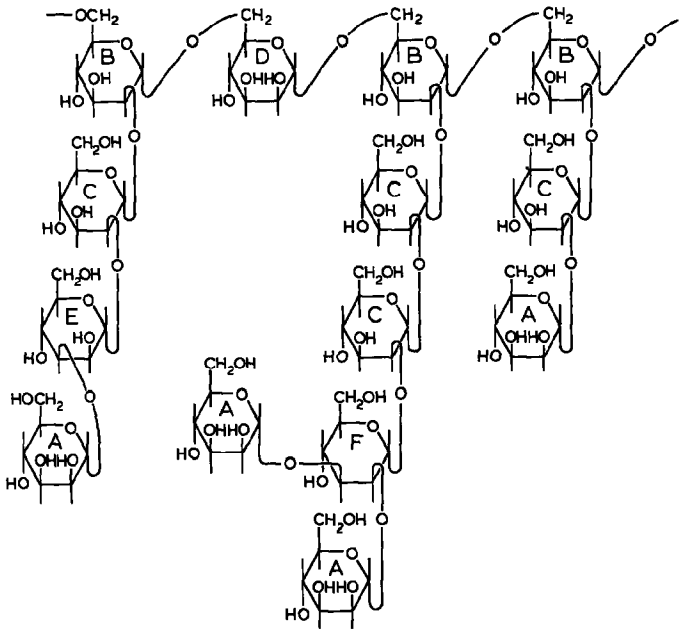

Fig. 1. Possible types of mannopyranosyl units present in the mannan structure.

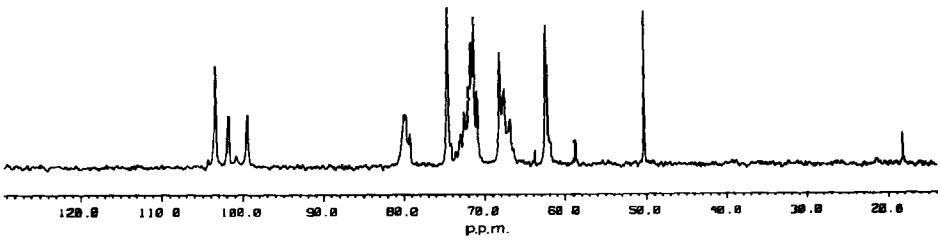

Fig. 2. ^{13}C-N.m.r. spectrum of *C. albicans* serotype A mannan.

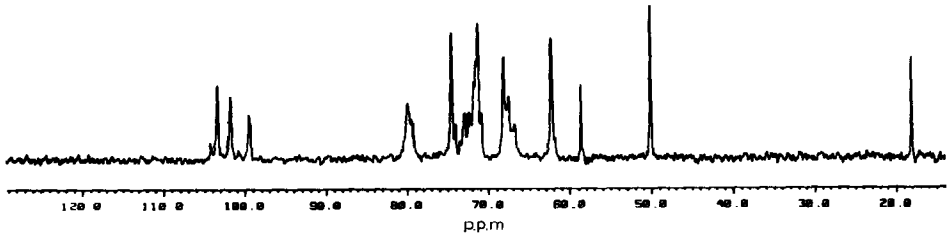

Fig. 3. ^{13}C-N.m.r. spectrum of *C. albicans* serotype B mannan.

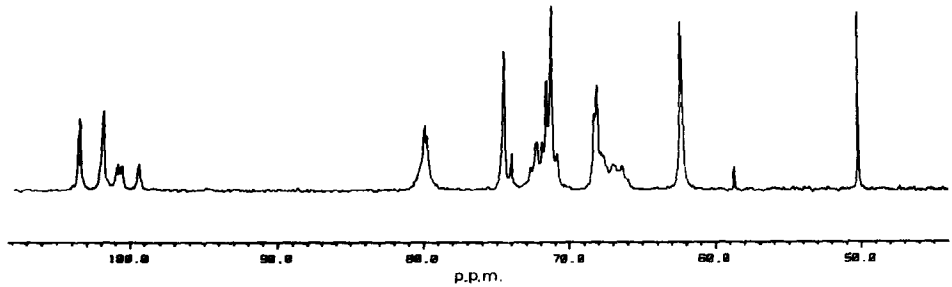

Fig. 4. ^{13}C-N.m.r. spectrum of *C. parapsilosis* mannan.

A, and that at ~101.8 p.p.m. to unit C. In the case of *C. albicans* mannans, this signal is superimposed on a signal of unit F (the difference is ~0.1 p.p.m. towards lower field). A small signal at ~100.7 p.p.m. belongs to unit D, and the last one at ~99.4 p.p.m. to unit B of the polymannosyl chain.

The next group of signals is located at ~80 p.p.m., and consists of those corresponding to C-3 atoms of units E (80.0 p.p.m.) and the C-2 atoms of units B (79.8 p.p.m.), C (79.6 p.p.m.), and F (79.2 p.p.m.). The spectrum of *C. parapsilosis* mannan has only two signals in this region, corresponding to C-2 atoms of B and C units, and that is in agreement with the results of methylation analysis.

Absence of signals in the region of 76–77 p.p.m. indicates the exclusive α configuration of the glycosidic bonds in the mannan molecules. This finding is not

in accord with the observation of Suzuki and Fukazawa[25], who suggested the presence of a β-(1→6) linkage by which a side chain was linked to the backbone of the mannan molecule.

The signals of C-5 atoms of all types of units are located in a region lying between 72 and 75 p.p.m. The prominent signal at ~74.5 p.p.m. belongs to C-5 of unit C, and the weaker signal, at ~74.0 p.p.m., may correspond to units A. Much smaller signals at ~73.0 p.p.m. in the spectra of *C. albicans* mannans evidently correspond to C-5 atoms of E and F units. The spectrum of *C. parapsilosis* mannan does not contain such signals. The signals at 72.8 and 72.4 p.p.m. respectively correspond to C-5 atoms of D and B units. The next group of very closely placed signals consists of those corresponding to C-3 atoms of units D (71.8 p.p.m.) and A (71.5 p.p.m.), C-2 atoms of units A (71.5 p.p.m.) and D (71.3 p.p.m.), the C-3 atoms of units B (71.2 p.p.m.) and C (70.8 p.p.m.), and the C-2 atoms of units E and F (70.8 p.p.m.). The next group of signals at ~68 p.p.m. belongs to C-4 atoms of all types of units. It consists of two subgroups in the case of *C. albicans* mannans, the first located in the lower field at ~68.1 p.p.m., consisting of the signals of units A, B, C, and D, and the second at 67.5 p.p.m., probably corresponding to units E and F. This region in the spectrum of *C. parapsilosis* mannan consists of two almost superimposed signals, one of which stands for A and C units (lower field) and the other for B and D units (higher field). The distance between these two signals is ~0.15 p.p.m.

The signals of C-6 atoms are also subdivided in two groups. The first consists of the signals of B (66.9 p.p.m.) and D units (66.4 p.p.m.), and the second consists of the signals that belong to A and C units (62.35 p.p.m.). A small signal is visible in the spectra of *C. albicans* mannans at 62.3 p.p.m., apparently corresponding to C-6 of E and F units.

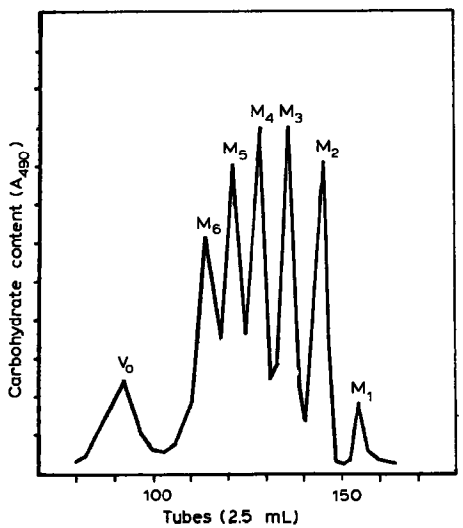

Fig. 5. Gel-filtration profile of acetolysis oligosaccharides of *C. albicans* serotype A mannan.

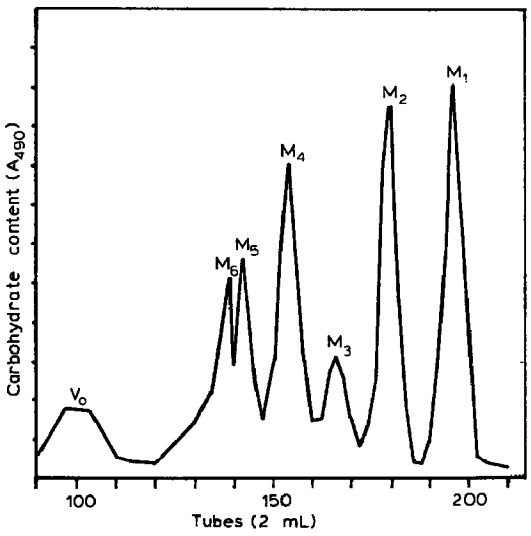

Fig. 6. Gel-filtration profile of acetolysis oligosaccharides of *C. albicans* serotype B mannan.

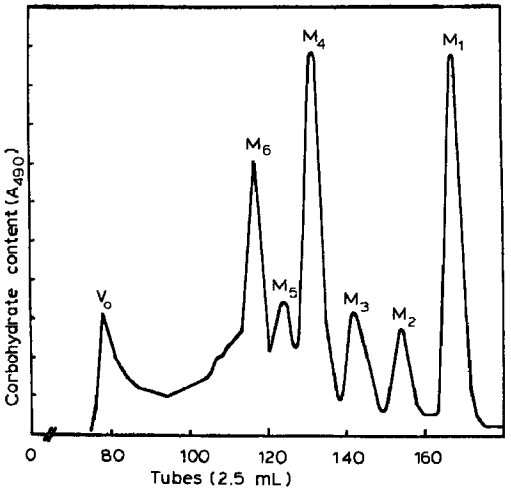

Fig. 7. Gel-filtration profile of acetolysis oligosaccharides of *C. parapsilosis* mannan.

Acetolysis of the mannans investigated, followed by gel filtration, gave 6 peaks in each case. The corresponding acetolysis patterns are presented in Figs. 5–7. The carbohydrate-containing peaks, reading from right to left in each pattern, correspond to mannose (M_1), mannobiose (M_2), mannotriose (M_3), mannotetraose (M_4), mannopentaose (M_5), and mannohexaose (M_6), respectively. It may be seen that the three patterns are almost identical, confirming the similar chemical structure of the mannans studied. In order to investigate the nature of the glycosidic linkages in the individual oligosaccharides, ^{1}H-n.m.r. analyses of isolated fractions were carried out. The results of the investigation of the anomeric region of the

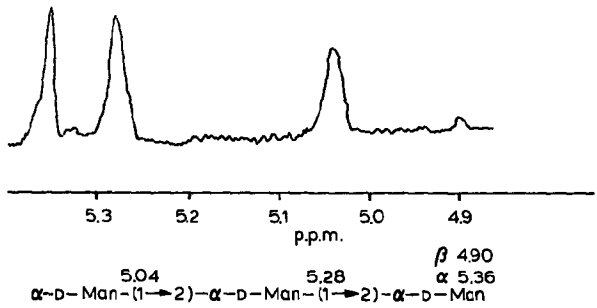

Fig. 8. ^{1}H-N.m.r. spectrum of a trisaccharide obtained by acetolysis of *C. parapsilosis* mannan, and its structure as deduced from signal assignment according to Cohen and Ballou[26].

^{1}H-n.m.r. spectra of the oligosaccharidic fractions obtained were consistent with the results of methylation and ^{13}C-n.m.r. analyses of the mannans. Thus, the spectra of *C. parapsilosis* oligosaccharides indicate almost complete absence of (1→3) glycosidic linkages. They contained signals at 5.36, 5.28, and 5.04 p.p.m., corresponding to anomeric hydrogen atoms of the reducing end-group, internal group, and nonreducing end-group, respectively. Thus, the assignment of the signals may be made as follows (see Fig. 8).

Minute signals in the region at δ 5.14, which could be ascribed to (1→3)-glycosidically linked mannose units, were observed only in the spectrum of the pentasaccharide. In contrast, the spectra of all acetolysis fragments, except mannobiose derived from mannans of both serotypes of *Candida albicans*, contained signals at 5.14 p.p.m., indicating the presence of (1→3)-glycosidic linkages between mannosyl units. Each molecule contained only one such linkage, as was found by integrating the spectra. The reducing end-units were exclusively linked with (1→2)-glycosidic bonds. Probably, a (1→3)-glycosidic linkage was located at the nonreducing end of oligosaccharide molecules, but no further detailed investigation was carried out. Thus, a generalized scheme of the *C. albicans* acetolysis-fragmentation oligosaccharides may be presented as follows (see Fig. 9).

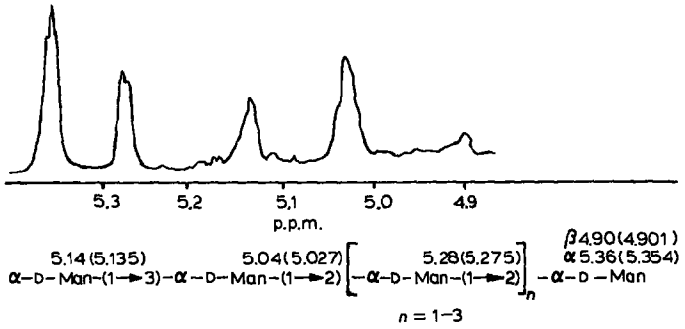

Fig. 9. ^{1}H-N.m.r. spectrum of a pentasaccharide obtained by acetolysis of *C. albicans* serotype A mannan, and a generalized scheme of the *C. albicans* acetolysis fragmentation oligosaccharides.

TABLE III

METHYLATION ANALYSIS OF ACETOLYSIS FRAGMENTS OF *C. albicans* MANNANS (MOL%)

Fragment	*Serotype*	*Methyl ether*			
		2,3,4,6-tetra-	*3,4,6-tri-*	*2,4,6-tri-*	*4,6-di-*
M_5	A	24.5	62.4	13.1	—
	B	22.3	60.8	16.0	trace
M_6	A	20.0	68.2	8.5	3.2
	B	18.1	63.0	10.2	8.2

The ^{1}H-n.m.r. chemical-shift values in parentheses were reported by Cohen and Ballou[26].

The intensity of the signals at 5.04 p.p.m. was in each case greater than that of the signals at 5.14 p.p.m. This fact may be explained by the presence of oligosaccharides having a (1→2)-linked, terminal nonreducing unit; consequently, each fragment studied was a mixture of two types of oligosaccharide. The only exception was the mannobiose, which contained exclusively the (1→2) linkage. In the spectra of hexasaccharides of *Candida albicans,* signals of small intensity were observed at 5.24 p.p.m., which could be ascribed to the branching units of the side chains of the mannans (2,3-disubstituted mannosyl units).

In order to provide further evidence of the location of this kind of branching in the side chains of *Candida albicans* mannans, methylation of M5 and M6 acetolysis fragments of these polysaccharides was performed; the results are presented in Table III.

Our data are in very good accord with those of Fukazawa *et al.*[21], who had suggested that this branching point might be present in antigenic factor 13b, which is characteristic for *C. albicans* serotype B mannan.

It is evident that, should the M6 fraction consist only of the branched hexasaccharide, the molar percentage of 4,6-di-*O*-methyl derivative should be 16.6% (one unit of the six). Hence, it could be roughly estimated that, in serotype B mannan, approximately every second pentasaccharide side-chain is branched, whereas in serotype A mannan, it is every fifth one.

In conclusion, it may be suggested that the difference in immunochemical properties of the mannans studied could be ascribed to variation of glycosidic linkage types and the proportion of branching points in their side-chain structures, rather than only to their lengths.

Some quantitative difference between our results and the data of other authors[4,16,17] could presumably be ascribed to variation in the strains of *Candida albicans,* or differences in cultivation conditions, or both.

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