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Structure of the Cell Wall Mannans of the Pathogenic Yeasts of *Candida* Species—A Complex Insight

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ABSTRACT

Cell wall D*-mannans were isolated from all eight known pathogenic strains of genus* Candida. *Their structural characterization was carried out by means of acetolysis and methylation analysis, as well as NMR spectroscopy. The results point out some common features and peculiarities of the structures of individual* D*-mannans. Differences observed in the structures of investigated mannans may be responsible for the differences in immunological properties of the corresponding yeast strains.*

INTRODUCTION

Of the 81 known species of *Candida* only seven are recognized to be regularly pathogenic (Hector *et al.*, 1981). These are *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida pseudotropicalis*, *Candida parapsilosis*, *Candida guilliermondii* and *Candida krusei*, which are the most commonly isolated species of *Candida* from clinical materials. An outburst of severe infections caused by pathogenic *Candida* strains especially in patients with impaired immune defence systems led to the increased interest to investigate these microorganisms including studying their cell wall components. *C. albicans* is the most virulent strain and has been subdivided into two primary serotypes, A and B, the classification based on the structure of cellular mannan (Hasenclever & Mitchel, 1961).

Cell wall α-D-mannan is one of the major components (together with glucan and protein) of the yeast cell wall and is believed to be its surface

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antigen determining immunological properties of the yeasts (Cohen & Ballou, 1980; Suzuki & Fukazawa, 1982). Nevertheless, the comparative structural study of the cell wall mannans of the pathogenic *Candida* species has not been carried out as yet. Lyon & Domer (1985) investigated the monosaccharide composition of cell walls of pathogenic *Candida* spp. by studying the cell wall susceptibility to different enzymes, but have not found any significant dissimilarity and their studies were unrelated to antigenicity. Yu *et al.* (1967) made a limited structural investigation of the mannans of *C. albicans* (both serotypes), *C. parapsilosis*, *C. stellatoidea* and *C. tropicalis*. However, their results were marked by errors caused by the inadequate identification methods of that time.

In this paper we investigated the structure of the cell wall mannans of the mentioned pathogenic *Candida* spp. and tried to correlate it with known immunological relationships among the species.

MATERIALS AND METHODS

Microorganisms

C. albicans CCY 29-3-109 (serotype A), *C. albicans* CCY 29-3-102 (serotype B), *C. tropicalis* CCY 29-7-6, *C. pseudotropicalis* CCY 29-8-4, *C. parapsilosis* CCY 29-20-6, *C. guilliermondii* CCY 29-4-20, *C. stellatoidea* CCY 29-64-3 and *C. krusei* CCY 29-9-8 from the Czechoslovak Collection of Yeasts and Yeast-like Microorganisms (CCY) (Institute of Chemistry, Slovak Academy of Sciences) were used. Yeast cells were grown on a semi-synthetic liquid medium containing 2% D-glucose for 4 days at 28°C (Masler *et al.*, 1966).

Isolation of mannans and the methods of structural analysis

Cellular D-mannans were isolated and purified from the yeast biomass using Fehling's reagent according to the procedure described previously (Kogan *et al.*, 1988a).

Acetylation and acetolysis of mannans were performed as described by Kocourek & Ballou (1969). 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^{-1} at room temperature. Carbohydrate content was detected with a differential refractometer RIDK 101 (Laboratory Equipments, Prague).

Methylation of the mannans was performed according to Hakomori

(1964) and the analysis of the products was carried out as we have previously described (Kogan *et al.*, 1988*a*, *b*).

NMR Spectroscopy

^{1}[H]-NMR spectra of oligosaccharides were recorded with a Bruker AM-300 FT-spectrometer at 300 MHz field frequency and room temperature. Solutions in deuterium oxide were used with acetone as internal standard (2·2 ppm in relation to Me_4Si).

^{13}[C]-NMR spectra of the mannans were recorded at 75·468 MHz with the same instrument at the same conditions. Methanol was used as internal standard (50·15 ppm in relation to Me_4Si).

RESULTS AND DISCUSSION

Many yeast cell wall mannans possess a backbone of 6-linked α-D-mannosyl units with 2- and 3-linked residues in the side chains (Peat *et al.*, 1961; Gorin *et al.*, 1969; Reiss *et al.*, 1981; Suzuki & Fukazawa, 1982). Our previous works confirmed such comb-like structure for the mannans of *C. albicans* and *C. parapsilosis* (Kogan *et al.*, 1988*a*) but opposed it for mannan of *C. krusei* (Kogan *et al.*, 1988*b*).

Acetolysis, which selectively cleaves (1→6)-linkages (Kocourek & Ballou, 1969), when applied to the mannans led to emergence of the series of oligosaccharides representing the side chains of the mannans. Figure 1 presents the gel-filtration profiles of the deacetylated products of the acetolysis of the mannans after separation on Bio-Gel P-2 column.

Most of the mannans gave rise to a mixture of mannose to mannohexaose (M_1–M_6) that is in accord with earlier investigations (Sunayama & Suzuki, 1970; Suzuki & Fukazawa, 1982; Funayama *et al.*, 1983). Mannose was formed from the unsubstituted units of the backbone, while the branching units turned into the reducing ends of the corresponding oligosaccharides.

In the cases only of *C. krusei* and *C. pseudotropicalis*, the highest obtained oligosaccharides were mannopentaose and mannotetraose, respectively. Although the relative ratio of mannose and individual oligosaccharides varied for different strains, the similar picture obtained upon acetolysis implied a similar structure of the mannans.

In order to obtain more detailed information on the structure of the mannans and the nature of glycosidic linkages in the side chains, we carried out methylation analysis of the mannans. The results obtained are presented in Table 1.

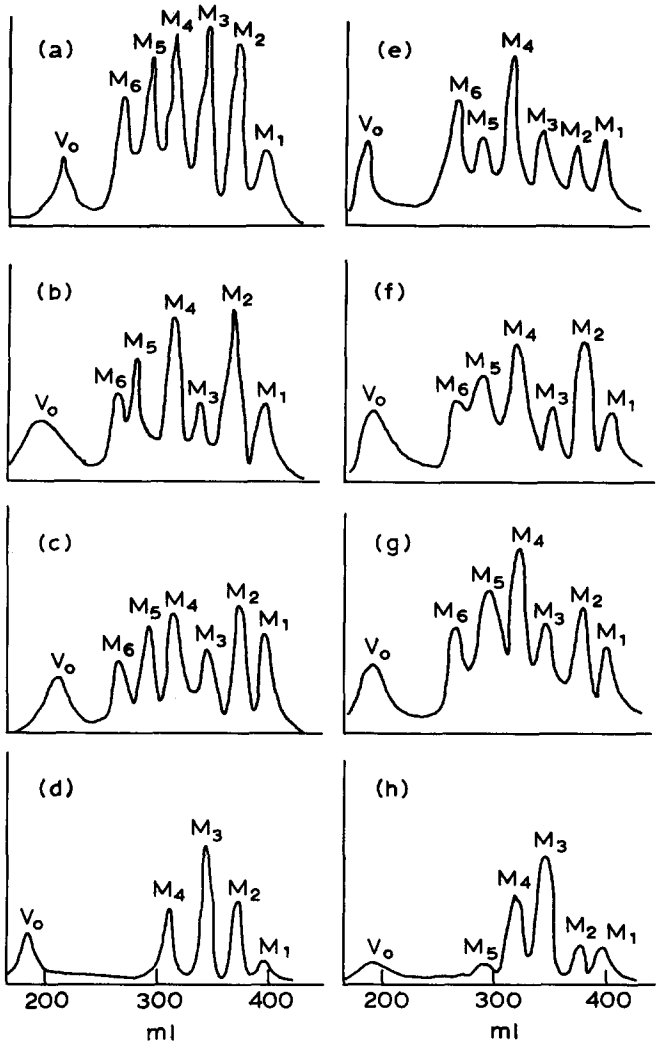

Fig. 1. Gel-filtration profiles of the acetolysis products of the mannans: (a), *C. albicans* serotype A; (b), *C. albicans* serotype B; (c), *C. tropicalis*; (d), *C. pseudotropicals*; (e), *C. parapsilosis*; (f), *C. guilliermondii*; (g), *C. stellatoidea*; and (h), *C. krusei*. M_1, mannose; M_2–M_6, mannobiose to mannohexaose; V_0, void volume.

Evident differences in the amount of non-reducing end-units (represented by 2,3,4,6-tetra-*O*-methylated derivatives) ranging from 3·8% for *C. krusei* mannan to 37·9% for *C. pseudotropicalis* mannan, imply that structures of the mannans can vary significantly. Should the aforementioned comb-like structure be considered as common for all

TABLE 1
Methylation Analysis of *Candida* spp. Mannans

Mannan	*Methyl ether (relative mol%)*					
	2,3,4,6-tetra-	*3,4,6-tri-*	*2,3,4-tri-*	*2,4,6-tri-*	*3,4-di-*	*4,6-di-*
C. albicans serotype A	19·2	38·4	16·6	4·2	20·0	1·6
C. albicans serotype B	20·2	35·7	14·7	6·6	18·3	4·5
C. tropicalis	25·6	35·9	12·8	traces	24·4	1·3
C. pseudotropicalis	37·9	17·4	2·0	—	42·7	—
C. parapsilosis	27·1	35·9	10·5	traces	26·5	—
C. guilliermondii	31·4	29·8	5·2	traces	32·6	1·0
C. stellatoidea	23·4	40·0	11·3	—	25·3	—
C. krusei	3·8	68·1	24·8	—	3·3	—

mannans, the branching frequency of the 6-linked backbone could be calculated from the amounts of 2,3,4-tri-*O*-methylated and 3,4-di-*O*-methylated derivatives. The amount of the former stands for the number of unsubstituted backbone units and that of the latter reflects the number of the backbone units substituted in position 2 with a side chain. Adding these two numbers one can obtain the total percentage of the backbone units and consequently the share of the substituted units among them; therefore, the branching frequency of the backbone can be calculated. The obtained values are presented in Table 2.

It can be seen that in *C. pseudotropicalis* mannan almost every backbone unit is branched, while in *C. krusei* it is only one unit out of nine.

The amount of 3,4,6-tri-*O*-methylated derivative together with that of 2,4,6-tri-*O*-methylated derivative represent the content of 2- and

TABLE 2
Branching Frequency of the Backbone of *Candida* spp. Mannans

Mannan	*Branching frequency (%)*
C. albicans serotype A	54·6
C. albicans serotype B	55·5
C. tropicalis	70·4
C. pseudotropicalis	95·5
C. parapsilosis	71·5
C. guilliermondii	86·2
C. stellatoidea	69·2
C. krusei	11·7

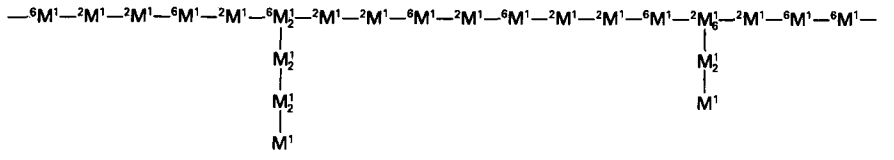

Fig. 2. Fragment of the structure of *C. krusei* cell wall mannan.

3-linked D-mannosyl units of the side chains. It could be expected that the greater is branching frequency, the larger should be the amount of the side chain units. Since this amount depends also on the length of the side chains, correlation of the branching frequency value (Table 2) with the amount of side chain units (Table 1) may imply that *C. pseudotropicalis* mannan is extremely highly branched; but the side chains are very short, whereas *C. krusei* mannan is sparsely branched but possesses very long side chains. The latter statement, however, contradicts the results of acetolysis of *C. krusei* mannan (Fig. 1) that afforded pentasaccharide as the longest side chain, while the main portion comprised tetra- and trisaccharide. On the basis of the detailed study of this mannan, we proposed a structure different from the common comb-like one (Kogan *et al.*, 1988*b*) (Fig. 2). The presented fragment contains 1,2-, 1,6- and 1,2,6-linked mannosyl units according to the results of the methylation analysis. Its structure also reflects the results of the acetolysis of *C. krusei* mannan, especially the ratio of mannobiose and mannose formed from two contiguous 1,6-linked mannosyl units — Fig. 1(h). The possibility of occurrence of two different types of branching points giving rise to the same methylation product was also taken into account at the construction of the scheme of this fragment. This unique structure fully explains the low antigenicity of *C. krusei* and the negligible cross-reactivity of *C. krusei* mannan with antisera prepared against other *Candida* spp. (Shinoda, 1972; Paulovičová & Šandula, 1986; Pavliak & Šandula, 1988; Kogan *et al.*, 1988*b*).

Methylation results of other than *C. krusei* mannans did not contradict the comb-like structure. The largest amount of 3,4,6-tri-*O*-methylated alditol acetate found for *C. stellatoidea* mannan implies that it possesses the longest 2-linked side chains. (1 → 3) Glycosidic linkages were detected in considerable amount only in the mannans of *C. albicans* of both serotypes and it was shown in our previous paper (Kogan *et al.*, 1988a) that they were located most probably at the non-reducing ends of the side chains of these mannans. In the mannans of *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii*, this type of linkage was detected only in trace amounts. Lack of 2,4-di-*O*-methylalditol acetate among the

methylation products implies that the side chains are linked to the backbone exclusively by (1→2) glycosidic bonds, and 3,4-di-*O*-methylated derivative represents this branching point.

The 4,6-di-*O*-methylated derivative was detected in the mannans of *C. albicans*, *C. tropicalis* and *C. guilliermondii*. This compound is derived from a 2-linked side chain mannosyl unit branched at C-3. Previously, we demonstrated that this branching point was located mainly in the pentasaccharide side chains of *C. albicans* mannans (Kogan *et al.*, 1988*a*). We suggested that significant quantitative difference in the content of these branching points (Table 1) might be responsible for the clear serological difference observed between two serotypes of *C. albicans*. Formerly, such a branching point was found only in traces in *C. albicans* serotype B mannan (Fukazawa *et al.*, 1980), *C. parapsilosis* mannan (Funayama *et al.*, 1983) and *C. tropicalis* mannan (Šimkovicová, 1983). Fukazawa *et al.* (1980) have proposed that such branched pentasaccharide represented an antigenic factor 13b of *C. albicans* serotype B.

It was confirmed by Sunayama & Suzuki (1970) and by Reiss *et al.* (1981) that serotype A mannan contained all determinants present in serotype B mannan and, in addition, some A-specific determinant. Reiss *et al.* (1981) suggested that this unknown A-specific hapten could be a branched mannopentaose side chain. However, this oligosaccharide was proposed by Fukazawa *et al.* (1980) as a determinant of serotype B. The question of the structure of this additional A-specific hapten seems to be unclear as yet.

Previous work (Sunayama & Suzuki, 1970; Shinoda, 1972; Pavliak & Šandula, 1987) showed that the mannans we found to contain branched side chains strongly cross-react with each other. This confirms that respective yeasts are immunochemically related.

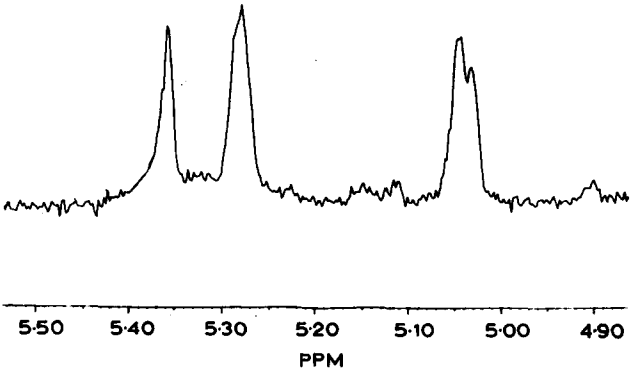

Fig. 3. ^{1}[H]-NMR spectrum of a tetrasaccharide obtained by acetolysis of *C. stellatoidea* mannan.

C. pseudotropicalis mannan has very short side chains linked with (1→2) glycosidic bonds. It showed weak cross-reactivity with antisera prepared against another *Candida* sp. (Fukazawa *et al.*, 1980; Pavliak & Šandula, 1987) and it was suggested that (1→2)-linked mannobiose side chain could be its immunochemical determinant (Fukazawa *et al.*, 1980).

^{1}[H]-NMR spectra of the deacetylated acetolysis products of the majority of mannans contained three signals at 5·36, 5·28 and 5·04 ppm, corresponding to anomeric hydrogen atoms of the reducing end-group, internal (1→2)-linked groups and non-reducing end-group, respectively (Fig. 3). Minor signals at 5·14 ppm were observed in the spectra of the higher oligosaccharides of *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii*. These signals belonged to anomeric protons of (1→3) glycosidically linked mannosyl units (Cohen & Ballou, 1980) and were present in the spectra of all mannooligosaccharides except mannobiose derived from mannans of *C. albicans* (Kogan *et al.*, 1988*a*). Hexasaccharides of *C. albicans* contained also additional signals of small intensities located at 5·24 ppm, which were assigned to the anomeric

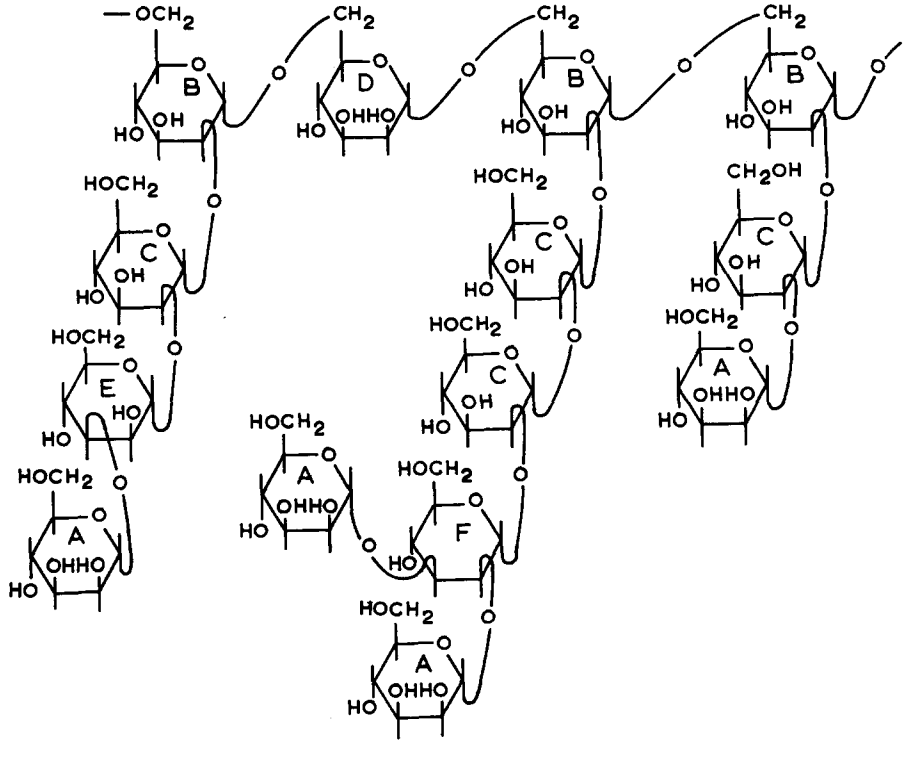

Fig. 4. Possible types of mannopyranosyl units present in the mannan structure.

protons of 2,3-branched mannosyl units that were found in these oligosaccharides by means of methylation analysis (Kogan *et al.*, 1988*a*).

The results of methylation analysis of mannans were consistent with those of ^{13}[C]-NMR investigation. Partial structure of the mannan containing all possible mannosyl units is presented in Fig. 4. This scheme reflects the structure of the mannans of all *Candida* spp. except *C. krusei* mannan, the structure of which was presented in Fig. 2 and contained only A, B, C and D units (according to Fig. 4). The spectra of the mannans resembled one another and Fig. 5 presents those of the mannans of *C. tropicalis*, *C. stellatoidea*, *C. pseudotropicalis* and *C. guilliermondii*. ^{13}[C]-NMR spectra of the mannans of both serotypes of *C. albicans*, *C. parapsilosis* and *C. krusei* were presented in our previous papers (Kogan *et al.*, 1988*a*, *b*). A complete table of signals could be made (Table 3) upon assigning of all signals (Kogan *et al.*, 1988*a*).

Rademacher (1983) carried out a ^{13}[C]-NMR investigation of mannans and their acetolysis products of all pathogenic *Candida* spp. and *Candida utilis*. However, he studied only the anomeric region of the spectra and was able to identify exclusively (1→2) and (1→6) glycosidically linked units in the structures of all mannans. Kočiš *et al.* (1984) investigated a series of yeast mannans by means of ^{13}[C]-NMR spectroscopy. Table 4 presents their results on relative contents of the structural units in the mannans obtained from the quantification of the spectra. It can be seen that the major difference with our results lies in the significant amount of 3-linked units (units C) (10–15%).

It is probable that the results of spectroscopic investigations only, without the confirmation by methylation or any other structural analyses, are insufficient for making quantitative structural conclusions. This could be due to the possible influence of nuclear Overhauser effect on the intensities of integrated signals (Kogan *et al.*, 1988*b*).

TABLE 3
Assignment of the Signals in ^{13}[C]-NMR Spectra of the Mannans

		Chemical shift (ppm)					
Unit	*Carbon atom:*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
A		103·4	71·5	71·5	68·0	74·0	62·4
B		99·4	79·8	71·2	67·9	72·4	66·9
C		101·8	79·6	70·8	68·0	74·5	62·4
D		100·7	71·3	71·8	67·9	72·8	66·4
E		103·5	70·8	80·0	67·5	73·0	62·0
F		101·9	79·2	79·4	67·5	72·9	62·0

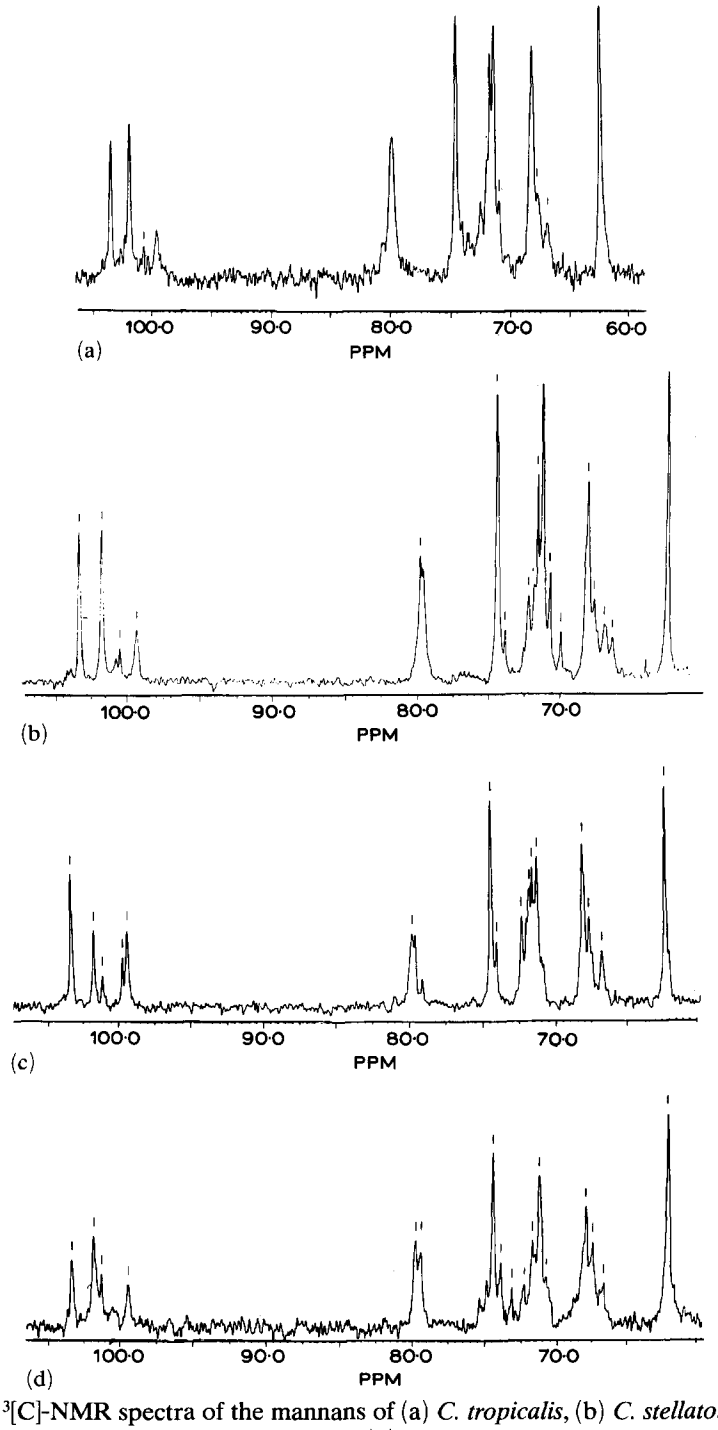

Fig. 5. ^{13}[C]-NMR spectra of the mannans of (a) *C. tropicalis*, (b) *C. stellatoidea*, (c) *C pseudotropicalis* and (d) *C. guilliermondii*.

TABLE 4
Molar Ratio of Different Structural Units in *Candida* spp. Mannans[a]

Mannan	*Unit*				
	A	*B*	*C*	*D*	*E*
C. albicans serotype B	2·3	2·3	2·3	—	1·0
C. pseudotropicalis	4·7	4·7	1·0	—	1·7
C. parapsilosis	1·6	1·6	4·0	1·2	1·0
C. krusei	1·8	1·8	2·0	—	1·0

[a]According to Kočiš *et al.* (1984).

CONCLUSION

The cell wall mannans of the pathogenic yeasts of the genus *Candida* playing a role of surface antigens of the yeast cells and determining their immunological reactivity, with the exception of *C. krusei* mannan, have similar comb-like structures with a backbone consisting of 6-linked units and the side chains built predominantly of 2-linked mannosyl units. The mannans differ in the branching frequency of the backbone, the length of the side chains and the occurrence of (1 → 3)-linkage or branching points in their structures. *C. krusei* mannan has a different structure of a slightly branched polymannosyl chain with (1 → 2)- and (1 → 6)-linkages in the ratio 3:1. All these peculiarities of the mannan structures may cause different immunological properties within the individual strains of *Candida*.

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