Chemical structure of the cell-wall mannan of *Candida albicans* serotype A and its difference in yeast and hyphal forms

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The structure of the cell-wall mannan from the J-1012 (serotype A) strain of the polymorphic yeast *Candida albicans* was determined by acetolysis under mild conditions followed by HPLC and sequential NMR experiments. The serotype A mannan contained β -1,2-linked mannose residues attached to α -1,3-linked mannose residues and α -1,6-linked branching mannose residues. Using a β -1,2-mannosyltransferase, we synthesized a three- β -1,2-linkage-containing mannoheptaose and used it as a reference oligosaccharide for ¹H-NMR assignment. On the basis of the results obtained, we derived an additivity rule for the ¹H-NMR chemical shifts of the β -1,2-linked mannose residues. The morphological transformation of *Candida* cells from the yeast

form to the hyphal form induced a significant decrease in the phosphodiesterified acid-labile β -1,2-linked manno-oligosaccharides, whereas the amount of acid-stable β -1,2 linkage-containing side chains did not change. These results suggest that the *Candida* mannan in candidiasis patients contains β -1,2-linked mannose residues and that they behave as a target of the immune system.

Key words: *Candida albicans*, cell-wall mannan, mannosyltransferase, nuclear-magnetic-resonance spectrum (NMR spectrum), yeast hyphal form, β -1,2-linked mannose.

INTRODUCTION

Candida albicans, a polymorphic yeast, has become one of the most common agents of nosocomial infection in immunocompromised patients and those undergoing long-term treatment with antibiotics or other immunosuppressive therapies [1]. In these patients, *C. albicans* invades the deeper tissues and can cause life-threatening systemic infections. The outermost layer of the cell wall of *Candida* consists of mannoproteins with O-glycosylated oligosaccharide and N-glycosylated polysaccharide moieties. Both carbohydrate moieties have been shown to be important in host–fungal interactions and virulence [2–4]. The N-glycosylated polysaccharide has a comb-like structure with an α -1,6-linked backbone moiety and an oligomannose side chain mainly containing α -1,2-, α -1,3-, and β -1,2-linked mannose residues with a small number of phosphate groups. The mannan has three types of β -1,2-linkage-containing manno-oligosaccharides. One of these is located in a phosphodiesterified oligosaccharide moiety and serves as a common epitope of *C. albicans* serotypes A and B. The β -1,2-linked oligosaccharides can be selectively released from these mannans by treatment with weak acid (e.g. 10 mM HCl). The resulting acid-modified mannans of *C. albicans* serotype A still contain β -1,2-linked mannose residues attached to the α -1,2-linked oligomannose side chains. This type of β -1,2-linkage-containing manno-oligosaccharide corresponds to the serotype A-specific epitope of *C. albicans*. The first and second types of β -1,2-linkage-containing side chains correspond to antigenic factors 5 and 6 respectively of the antigenic formula of the *Candida* species [5]. Furthermore, some *Candida* mannans contain a third type of β -1,2-linked mannose residue attached to the α -1,3-linked mannose residues in the side chains.

The β -1,2-linked oligomannose moieties have been proposed to have a number of biological roles. They behave as strong antigens and are involved in cell adhesion [6,7] and stimulate tumour necrosis factor- α production [8–10]. Furthermore, monoclonal

antibodies against the β -1,2-linked oligomannose side chains protect against disseminated and vaginal candidiasis [11]. Thus determination of the chemical structure of the mannan side chains is important for identifying the mechanism of the *C. albicans* pathogenicity and for the prevention and treatment of candidiasis.

In the present paper we propose a revised overall structure of the mannan [12] from the NMR assignments of side-chain oligosaccharides of *C. albicans* J-1012 (serotype A) mannan obtained by mild acetolysis and HPLC separation. Because the ability of *C. albicans* to change between the spherical yeast and long filamentous hyphal forms (dimorphism) is important for virulence [13,14], we also determined the differences between the structures of the mannans in the two cell types.

MATERIALS AND METHODS

Materials

C. albicans strains J-1012 (serotype A) and NIH A-207 (serotype A) were kindly supplied by Dr Takako Shinoda, Department of Microbiology, Meiji Pharmaceutical University, Tokyo, Japan. Jack-bean (*Canavalia ensiformis*) α -mannosidase (EC 3.2.1.24) was obtained from Sigma–Aldrich (St Louis, MO, U.S.A.). Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man was prepared from the mannan of *C. albicans* NIH B-792 by treatment with weak acid.

Preparation of mannan

Yeast-form cells were grown at 28 °C for 48 h with shaking in YPD medium containing 0.5 % yeast extract, 1 % peptone and 2 % (w/v) glucose (dextrose). Hyphal-form cells were grown in a synthetic liquid medium (1.5 % soluble starch, 0.5 % saccharose, 0.25 % K₂HPO₄, 0.05 % NaNO₃, 0.1 % L-methionine, 0.05 % L-phenylalanine, 0.05 % N-acetyl-D-glucosamine and 0.5 ng/ml D-biotin, pH 7.5) at 37 °C for 48 h without shaking. The mannan

Abbreviations used: 2D, two-dimensional; ROESY, rotating frame Overhauser enhancement spectroscopy.

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was extracted from the cells with water at 135 °C in an autoclave for 3 h.

Acetolysis of the mannan

Prior to the acetolysis, the mannan was treated with 10 mM HCl at 100 °C for 1 h to release the phosphodiesterified oligosaccharides. The acid-treated mannan was then acetylated, dissolved in acetic anhydride/acetic acid/H₂SO₄ (100:100:1, by vol.) and heated at 40 °C for 36 h. After deacetylation of the reaction product using sodium methoxide, the mixture was deionized and freeze-dried. This treatment selectively cleaves the backbone α -1,6 linkages and yields an oligosaccharide mixture that originates from the oligomannose side-chain moieties.

HPLC of oligosaccharides

HPLC was carried out using a column (10 mm $\times$ 500 mm) of YMC-Pack PA-25 (YMC, Kyoto, Japan). Elution was carried out with acetonitrile/water (52:48, v/v) and the eluate was monitored using a differential refractometer. The eluate corresponding to each peak was collected and rechromatographed on the same column.

NMR spectroscopy

Samples were exchanged twice in ²H₂O with intermediate freeze-drying, then dissolved at 1% (w/v) in ²H₂O (99.97 atom% ²H). The NMR spectra were recorded on JNM-LA400 and LA600 spectrometers (JEOL, Tokyo, Japan) at 45 °C. The ¹H-NMR chemical shifts were referenced relative to the internal acetone [chemical shift (δ) 2.217 p.p.m.]. The TOCSY experiments were performed in the phase-sensitive mode with an MLEV-17 mixing time of 130 ms. The spectral width was 6000 Hz, and 32 scans/*t*₁ increment were recorded. The ROESY (rotating-frame nuclear Overhauser enhancement spectroscopy) experiments were performed in the phase-sensitive mode. The mixing time was 400 ms, the spectral width was 6000 Hz, and 32 scans/*t*₁ increment were recorded. Each 2D (two-dimensional) NMR experiment consisted of a 512 $\times$ 1024 data matrix, which was zero-filled to give a final matrix of 1024 $\times$ 1024 points and was resolution-enhanced by a shifted sine-bell function before Fourier transformation.

Enzymatic synthesis of three- β -1,2-linkage-containing manno-oligosaccharide

The mid-exponential-growth-phase cells of *C. albicans* J-1012 were harvested and homogenized with glass beads using a Bead Beater (Biospec Products, Bartlesville, OK, U.S.A.). The homogenate was centrifuged for 20 min at 15 000 *g*, and the supernatant was further centrifuged for 1 h at 105 000 *g*. The pellet was resuspended and used as the β -1,2-mannosyltransferase fraction [15]. The enzyme reaction was carried out in a total volume of 500 μ l containing 5 mg of the Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man, enzyme fraction ($\sim$ 2 mg of protein), 50 mM Tris/maleate buffer (pH 7.0), 10 mM MnCl₂, 20 mM GDP-mannose and 0.3% Triton X-100. After a 48 h incubation at 30 °C, the reaction mixture was fractionated by HPLC, and the enzyme reaction products were freeze-dried.

Other methods

The α -mannosidase treatment of the manno-oligosaccharide mixture (200 mg) was carried out in 50 mM sodium acetate buffer, pH 4.6, containing 20 units of α -mannosidase at 37 °C for 48 h.

Figure 1 Elution patterns of oligosaccharides obtained from *C. albicans* J-1012 mannan by acetolysis

Oligosaccharides obtained from the mannan by acetolysis were subjected to HPLC before (A) and after (B) treatment with α -mannosidase. AM₁–AM₇ indicate the mannose and α -linked manno-oligosaccharides from biose to heptaose. BM₅–BM₈ indicate the β -linkage-containing manno-oligosaccharides from pentaose to octaose.

The total carbohydrate content was determined by the phenol/H₂SO₄ method of Dubois et al. [16], with D-mannose as the standard.

RESULTS

Acetolysis of *C. albicans* J-1012 mannan

To demonstrate the presence of the α -1,6-branched mannose residue and the third type of β -1,2-linked mannose residue, we subjected the mannan to mild acetolysis and then isolated the α - and β -linked manno-oligosaccharides. Figure 1(A) shows the HPLC profile of the acetolysate. The α - and β -linkage-containing oligosaccharide isomers were effectively separated by HPLC. A part of the acetolysate was digested with α -mannosidase, and the resultant enzyme-resistant oligosaccharides that contain β -1,2-linked mannose residue(s) were separated by HPLC (Figure 1B), rechromatographed, then freeze-dried. The α -linked manno-oligosaccharides from biose to hexaose were designated AM₂–AM₇, and the β -1,2-linkage-containing manno-oligosaccharides from pentaose to octaose were designated BM₅–BM₈ respectively.

¹H-NMR analysis of oligosaccharides

Figure 2 shows the H-1 region of the ¹H-NMR spectra from pentaose to octaose obtained by acetolysis. The absence of a signal at 4.91 p.p.m. in AM₅ and AM₆₋₁ indicates that they have no α -1,6-linked mannose residue. In contrast, AM₆₋₂ and AM₇ showed signals at 4.91 p.p.m. and 5.22–5.24 p.p.m, which correspond to a 3,6-di-*O*-substituted mannose residue [17]. This indicates that the α -1,6-linked mannose residues form the following branched structures:

Figure 2 Anomeric region of the ^1H NMR spectra of oligosaccharides obtained by acetolysis

AM₅–AM₇ (A) and BM₅–BM₈ (B) are as indicated in Figure 1.

BM₅ and BM₆ showed pure signals corresponding to one- and two- β -1,2-linkage-containing oligosaccharides respectively. On the other hand, signals of BM₇ and BM₈ suggest that they are mixtures of isomers with various numbers of β -1,2-linked mannose residues.

Sequential NMR assignment

The sequential assignment of the H-1 and H-2 signals of BM₇ and BM₈ was performed using TOCSY and ROESY, as shown in Figure 3, and the assignment results are shown in Table 1. In the TOCSY spectra of BM₇ (Figure 3A), cross-peak 5 indicates an α -1,2-linked mannose residue, Man-C. Therefore the ^1H NMR signals of BM₇ were sequentially assigned from Man-C. The spectrum in Figure 3(B) shows the inter-residue H-1–H-2' connectivities between Man-C of the H-1 signal at 5.26 p.p.m. and an α -mannose residue with the H-1 signal at 5.14 p.p.m. (C2–C2'–D2). Similarly, we can trace the connectivities to the non-reducing terminal β -mannose residue, Man-G (D2–D2'–E2–E2'–F2–E2'–G2), indicating that the major isomer in BM₇ has the following structure: Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man. The presence of cross-peak 25 in Figure 3(A) indicates that BM₇ contains a two- β -1,2-linkage-containing isomer. ^1H -NMR signals of BM₈ were also sequentially assigned from Man-B to Man-H (Figure 3D). This result indicates that BM₈ has the following structure: Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man.

The presence of cross-peaks 19 and 22 in Figure 3(C) indicates that BM₈ contains a three- β -1,2-linkage-containing isomer.

Furthermore, the H-1 signal at 5.22 p.p.m. of BM₇ and BM₈ gave cross-peak 14, and the presence of this characteristic cross-peak indicates that these oligosaccharides possess a β -1,2-linked mannose residue attached to an α -1,3-linked mannose residue [18]. This result indicates that BM₇ and BM₈ contain the third type of β -1,2 linkage-containing isomers: Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man and Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man (about 25 and 40% of each oligosaccharide respectively, estimated from the signal dimension).

Enzymatic synthesis of the pure three- β -1,2-linkage-containing oligosaccharides using a β -1,2-mannosyltransferase

To confirm the structure and assignment of the three- β -1,2-linkage-containing oligosaccharide in BM₇, we conducted the enzymatic synthesis of oligosaccharides from the pure two- β -1,2-linkage-containing hexaose (BM₆): Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man, using a β -1,2-mannosyltransferase of *C. albicans*. HPLC revealed that approx. 60% of the hexaose was converted into heptaose, which was designated eM₇ (Figure 4A). As shown in Figure 4(B), the β -mannose signals of eM₇ were different from those for BM₇ obtained by acetolysis. Because eM₇ did not contain isomers, we could unambiguously assign all of the H-1 and H-2 signals and confirmed the assignments for BM₇. The three β -1,2-linked mannose residues (Man-E, Man-F, and Man-G) of eM₇ gave almost the same H-1 and H-2 chemical shifts as those of the

Figure 3 600-MHz TOCSY and ROESY spectra of BM₇ and BM₈

Sequential assignment of the H-1 and H-2 signals was performed using the TOCSY (A, C) and ROESY spectra (B, D) for BM₇ (A, B) and BM₈ (C, D). In this Figure, the upper panels indicate the TOCSY spectra of each oligosaccharide. The boxed cross-peaks indicate the intra-residue H-1–H-2 correlation due to J-coupling, and the numerical symbols of the boxed cross-peaks are the same as those shown in Figure 6. In the lower panels, which show the ROESY spectra, the cross-peaks labelled with the primed designations (e.g. C2') indicate the through-space inter-residue H-1–H-2' connectivities between two adjacent mannose residues.

Table 1 Chemical shifts (δ) of the oligosaccharides obtained by acetolysis followed by α-mannosidase treatment

Oligosaccharide	Sugar residue and structure							δ (p.p.m.)						
	H	G	F	E	D	C	A	H	G	F	E	D	C	A
BM ₅	Mβ1-2Mα1-2Mα1-2Mα1-2Mα							H-1			4.77	5.16	5.27	5.26
								H-2			4.03	4.27	4.11	4.09
BM ₆	Mβ1-2Mβ1-2Mα1-2Mα1-2Mα1-2Mα							H-1		4.84	4.85	5.14	5.26	5.35
								H-2		4.15	4.26	4.25	4.11	3.92
BM ₇	Mβ1-2Mβ1-2Mβ1-2Mα1-2Mα1-2Mα1-2Mα							H-1	4.91	4.91	4.84	5.14	5.26	5.35
								H-2	4.14	4.40	4.24	4.26	4.1	3.92
	Mβ1-2Mβ1-2Mα1-2Mα1-2Mα1-2Mα1-2Mα							H-1	4.84	4.85	5.14	5.26	5.26	5.35
								H-2	4.15	4.26	4.25	4.1	4.1	3.92
BM ₈	Mβ1-2Mβ1-2Mα1-3Mα1-2Mα1-2Mα1-2Mα							H-1	4.84	4.84	5.22	5.03	5.26	5.35
								H-2	4.15	4.27	4.25	4.21	4.1	3.92
	Mβ1-2Mβ1-2Mβ1-2Mβ1-2Mα1-2Mα1-2Mα1-2Mα							H-1	4.93	5.01	4.90	4.84	5.14	5.35
								H-2	4.14	4.37	4.38	4.24	4.26	3.92
	Mβ1-2Mβ1-2Mβ1-2Mα1-2Mα1-2Mα1-2Mα1-2Mα							H-1	4.92	4.91	4.84	5.14	5.26	5.35
								H-2	4.14	4.39	4.24	4.26	4.1	3.92

phosphodiesterified β-1,2-linked mannotetraose (Figure 4C). On the basis of these results, we propose one possible chemical structure for the cell-wall mannan of *C. albicans* J-1012 (serotype A) (Figure 5).

Additivity rule for β-1,2-linked mannose residues

Figure 6 shows the TOCSY spectrum of *C. albicans* J-1012 mannan. As shown by the broken arrow in the spectrum, the

Figure 4 Enzymatic synthesis of oligosaccharide

A mannoheptaose was enzymatically generated from BM₆ and separated by HPLC (A). The H-1 and H-2 regions of ¹H-NMR spectra of the reaction products eM₇ (B) and the standard β -1,2-linked mannotetraose (β -1,2 M₄) (C) are shown.

addition of one β -1,2-linked mannose residue to the α -1,2-linked non-reducing terminal mannose residue caused a downfield shift of the latter's cross-peak from 10 to 15 ($\Delta\delta$ of H-1/H-2, +0.11/+0.21 p.p.m.), and the addition of one more β -1,2-linked

Figure 6 600 MHz TOCSY spectrum of *C. albicans* J-1012 mannan

The boxed regions in the spectrum indicate the H-1–H-2-correlated cross-peaks of the α - and β -mannose residues in the acid-stable polysaccharide moieties. The circled cross-peaks correspond to the β -1,2-linked mannose residues of the phosphodiesterified acid-labile oligosaccharide side chains. Assignments of these cross-peaks are shown in Table 2. The broken- and continuous-line arrows indicate the shifts in the cross-peaks caused by the addition of β -1,2-linked mannose residues and α -1,2-linked mannose residues respectively.

mannose residue caused an upfield shift of the cross-peak of the α -1,2-linked mannose residue from 15 to 16 ($\Delta\delta$ of H-1/H-2, –0.01/–0.02 p.p.m.) (Table 2). Similar shifts were also observed for the cross-peak of the non-reducing terminal α -1,3-linked mannose residue from 7 to 13 ($\Delta\delta$ of H-1/H-2, +0.11/+0.20 p.p.m.) and from 13 to 14 ($\Delta\delta$ of H-1/H-2, –0.03/–0.02 p.p.m.). These shifts were found not only for the α -mannose residues, but also for the β -mannose residues of the side-chain oligosaccharides. The shift caused by the addition

Figure 5 One of the possible structures of *C. albicans* J-1012 mannan

'M' denotes a D-mannopyranose residue. This structure is one of the possibilities out of a statistical ensemble. It seems that there is no specified side-chain order. The molar ratio of the side chains in the mannan was calculated from the dimensions of the ¹H-NMR signals shown in Figure 6 and is expressed as a percentage of the total side chains.

Table 2 Assignments and additivity rule of the NMR signals of *C. albicans* J-1012 mannanChemical shifts are for the mannose residues shown in bold typeface. $n > 0$.

				$\Delta\delta$ of H-1/H-2		
				Cross-peak shifted by the addition of:		
Cross-peak	δ (p.p.m.)		Residue	Man β 1 $\rightarrow$ 2	Man β 1 $\rightarrow$ 2(Man β 1 $\rightarrow$ 2) $_n$	
	H-1	H-2				
1	5.55	4.21	Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ phosphate	2 (−0.01/−0.02)		
2	5.54	4.19	Man β 1 $\rightarrow$ 2(Man β 1 $\rightarrow$ 2) $_n$ Man α 1 $\rightarrow$ phosphate			
3	5.38	4.10	α 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 2			
4	5.29	4.10	Man α 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2			
5	5.26	4.10	Man α 1 $\rightarrow$ 2(Man α 1 $\rightarrow$ 2) $_n$ Man α 1 $\rightarrow$ 2			
6	5.24	4.10	α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2			
			$\uparrow$ 6 Man α 1			
7	5.14	4.07	Man α 1 $\rightarrow$ 3	13 (+0.11/+0.20)	14 (+0.08/+0.18)	
8	5.10	4.00	α 1 $\rightarrow$ 6 Man α 1 $\rightarrow$ 6 Man α 1 $\rightarrow$ 6			
			$\uparrow$ 2 Man α 1($\rightarrow$ 2 Man α 1) $_n$			
9	5.08	4.01	($\rightarrow$ 6 Man α 1 $\rightarrow$) $_n$			
			$\uparrow$ 2 Man α 1($\rightarrow$ 2 Man α 1) $_n$			
10	5.05	4.07	Man α 1 $\rightarrow$ 2	15 (+0.11/+0.21)	16 (+0.10/+0.19)	
11	4.92	4.01	Man α 1 $\rightarrow$ 6			
12	4.91	4.00	Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 6) $_n$ Man α 1 $\rightarrow$ 6			
13	5.25	4.27	Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 3	14 (−0.03/−0.02)		
14	5.22	4.25	Man β 1 $\rightarrow$ 2(Man β 1 $\rightarrow$ 2) $_n$ Man α 1 $\rightarrow$ 3			
15	5.16	4.28	Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2	16 (−0.01/−0.02)		
16	5.15	4.26	Man β 1 $\rightarrow$ 2(Man β 1 $\rightarrow$ 2) $_n$ Man α 1 $\rightarrow$ 2			
17	5.03	4.21	α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 2			
18	5.01	4.37	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2			
19	4.91	4.40	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2	20 (−0.01/−0.02)		
20	4.90	4.38	Man β 1 $\rightarrow$ 2(Man β 1 $\rightarrow$ 2) $_n$ Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2			
21	4.93	4.14	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2			
22	4.91	4.14	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2	18 (+0.10/+0.23)		
23	4.85	4.26	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2	24 (−0.01/−0.02)		
24	4.84	4.24	Man β 1 $\rightarrow$ 2(Man β 1 $\rightarrow$ 2) $_n$ Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2			
25	4.84	4.15	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2	19 (+0.07/+0.25)	20 (+0.06/+0.23)	
26	4.78	4.04	Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ 2	23 (+0.07/+0.22)	24 (+0.06/+0.20)	
a	4.83	4.07	Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ phosphate	d (+0.08/+0.23)	c (+0.06/+0.21)	
b	4.85	4.17	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ phosphate	e (+0.08/+0.24)		
c	4.89	4.28	Man β 1 $\rightarrow$ 2(Man β 1 $\rightarrow$ 2) $_n$ Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ phosphate			
d	4.91	4.30	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ phosphate			
e	4.93	4.41	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ phosphate	e (−0.02/−0.01)		
f	4.93	4.16	Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man β 1 $\rightarrow$ 2 Man α 1 $\rightarrow$ phosphate			

of one β -1,2-linked mannose residue was significantly different from that caused by the addition of one α -1,2-linked mannose residue, as shown by the continuous-line arrows ($\Delta\delta$ of H-1/H-2, +0.24/+0.03 p.p.m.) (Figure 6). Using this additivity rule, we can predict the location of the other cross-peaks. For example, the cross-peak of a phosphodiesterified α -mannose residue, **Man** α 1 $\rightarrow$ phosphate-, must appear at the starting point of the arrow that terminates at cross-peak 1 in Figure 6.

Structural differences between the mannans from yeast- and hyphal-form cells

The TOCSY spectra of the mannans of the yeast (Fr. Y) and hyphal (Fr. H) forms of *C. albicans* NIH A-207 are shown in Figure 7. Cross-peaks 1 and 2, which correspond to the phosphodiesterified β -1,2-linked manno-oligosaccharides, were absent in Fr. H, but cross-peak 27, which corresponds to the phosphodiesterified mannose residue, appeared at the position predicted by the additivity rule and was enhanced in Fr. H. This result indicates that the β -1,2-linked mannose residues in

the phosphodiesterified oligosaccharides, which correspond to factor 5, were significantly decreased in the hyphal-form cells. Cross-peaks 11 and 6, which correspond to the α -1,6-linked branching mannose residue and to the upfield-shifted α -1,2-linked mannose residue caused by the presence of the 3,6-di-O-substituted mannose residue respectively, were reduced in Fr. H. On the other hand, there was an increase in cross-peaks 8 and 12 in Fr. H, indicating that the α -1,6-linked backbone mannose residues without side-chain substitutions were increased in the hyphal-form cells. Furthermore, cross-peaks 23 and 25, which correspond to the acid-stable β -1,2-linked mannose residues, factor 6, were increased in the mannan of the hyphal-form cells.

DISCUSSION

We demonstrated the presence of α -1,6-linked branching mannose residues and the third type of β -1,2-linked mannose residues in the side chain of the *C. albicans* serotype A mannan. In a previous study [12] we did not detect these structures, because the separation and purification of the side-chain oligosaccharides

Figure 7 600 MHz TOCSY spectra of mannans from yeast (A) and hyphal (B) forms of *C. albicans* NIH A-207

The numerical symbols of the boxed cross-peaks are the same as those used in Figure 6.

and NMR resolution were insufficient. It is known that the connection of one β -1,2-linked mannose residue to a β -1,2-linked oligosaccharide affects the H-1 and H-2 chemical shifts of all of the other mannose residues. Although the β -1,2-linked mannose residue has such unusual properties, which may be due to its unique compact helical conformation [19], the additivity rule was applicable to all three types of β -1,2-linkage-containing manno-oligosaccharide side chains. In the present study we assigned all of the H-1–H-2-correlated cross-peaks on the TOCSY spectrum of the mannan. As shown in Figure 5, *C. albicans* serotype A mannan has the most complicated structure among the *Candida* species that we have analysed to date. Therefore, we seem to be able to use the additivity rule as an effective tool for the structural determination of the other mannans.

Recently, we showed that the mannans of the hyphal form cells grown in BACTEC™ [BD (Becton–Dickinson), Franklin Lakes, NJ, U.S.A.] fungal medium at 37°C have few or no β -1,2-linked mannose residues [20]. The present results, however, indicate that the acid-stable β -1,2-linked mannose residues are present in the mannan of the hyphal-form cells grown at 37°C, even though the culture medium is different from that employed in a previous study. Although the structural analysis of *Candida* mannans has mostly been conducted using yeast-form cells grown at 28°C, the *Candida* cells invade tissue and cause pathogenicity at 37°C in the hyphal form [14]. Thus it is important that the mannan of the hyphal-form cells grown at 37°C retains the acid-stable β -1,2 linkage-containing side chains which participate in the adhesion of the *Candida* cells to organs and induce cytokine production. Trinel et al. [21] reported that the β -1,2-mannosylation of phospholipomannan was not affected by raising the growth temperature from 28 to 37°C. The ability of an anti- $(\beta$ -1,2-linked mannose) monoclonal antibody to protect against *Candida* infection, demonstrated by Han and Cutler [11], seems to be closely related to the presence of the β -1,2 linkage in the

mannan and phospholipomannan of the infectious hyphal-form cells in the host at 37°C. Thus it is reasonable to predict that invasive candidiasis can be diagnosed by detecting the circulating mannan in the patients' sera using an antibody to the β -1,2-linked mannose residue.

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Received 15 January 2007/27 February 2007; accepted 1 March 2007

Published as BJ Immediate Publication 1 March 2007, doi:10.1042/BJ20070081