NMR study of the galactomannans of *Trichophyton mentagrophytes* and *Trichophyton rubrum*

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Around 90% of chronic dermatophyte infections are caused by the fungi *Trichophyton mentagrophytes* and *Trichophyton rubrum*. One of the causes of the chronic infection resides in the immunosuppressive effects of the cell-wall components of these organisms. Therefore we have attempted to identify the chemical structure of galactomannan, one of the major cell-wall components. The cell-wall polysaccharides secreted by *T. mentagrophytes* and *T. rubrum* were isolated from the culture medium and fractionated into three subfractions by DEAE-Sephadex chromatography. Analysis of each subfraction by NMR indicated that there are two kinds of polysaccharides present, i.e. mannan and galactomannan. The mannan has a linear backbone consisting of α1,6-linked mannose units, with α1,2-linked mannose units as side chains. The core mannan moiety of the galactomannan was analysed by a sequential NMR assignment method after removing the galactofuranose units by acid treatment. The result indicates that the mannan moiety has a linear repeating structure of α1,2-linked mannotetraose units connected by an α1,6 linkage. The H-1 signals of the two intermediary α1,2-linked mannoses of the tetraose unit showed a significant upfield shift ($\Delta\delta = 0.05$–0.08 p.p.m.), due to the steric effect of an α1,6-linked mannose unit. The attachment point of the galactofuranose units was determined at C-3 of the core mannan by the assignment of the downfield-shifted ^{13}C signals of the galactomannan compared with those of the acid-modified product. In these galactomannans there were no polygalactofuranosyl chains which have been found in *Penicillium charlesii* and *Aspergillus fumigatus*.

INTRODUCTION

The anthropophilic dermatophytes *Trichophyton mentagrophytes* and *Trichophyton rubrum* cause chronic, relatively uninflamed, skin infections of the feet (tinea pedis) groin (tinea cruris) and body (tinea corporis), often associated with infection of the nails (onychomycosis). About 90% of chronic dermatophyte infections are caused by *T. mentagrophytes* and *T. rubrum* [1], in part because these organisms can suppress inflammation and cell-mediated immunity [2]. Green and Balish [3] and Sherwin et al. [4] reported that serum from patients or animals infected with *T. mentagrophytes* or *T. rubrum* inhibited mitogen-stimulated lymphoproliferation, and that this inhibitory activity disappeared from the serum after the infection had cleared. Calderon and Hay [5] reported reduced proliferation of lymphocytes from mice infected with *Trichophyton quincheanum* that correlated with fungal antigenaemia.

The cell-wall polysaccharides of these fungi are known to be the major immunologically active substances. Holden et al. [6] suggested that the cell-wall antigen is sloughed off or secreted by the pathogen, and diffuses into the underlying epidermis or dermis. Based on the hypothesis that the ability of *T. rubrum* to cause chronic infection may be due to an immunosuppressive agent released by this dermatophyte species, Blake et al. [7] examined the ability of a cell-wall mannan isolated from *T. rubrum* to suppress cell-mediated immune reactions, and found that the *T. rubrum* mannan inhibits the lymphoproliferative response of human mononuclear leucocytes to a variety of antigenic and mitogenic stimuli *in vitro*. Furthermore, Grando et al. [8,9] demonstrated that the target cells for this effect of the mannan are monocytes and keratinocytes, by monitoring the binding and uptake of FITC–mannan using flow cytometry and fluorescence microscopy.

Bishop et al. [10–12] studied the structures of the cell-wall polysaccharides of *Trichophyton* species, and isolated and partially characterized galactomannans from *T. rubrum* and several related species. Two galactomannans were separated; galactomannan I was precipitated by Fehling's solution, whereas galactomannan II was not. These galactomannans contain the same side chains, with α1,2-linked mannopyranose and galactofuranose units, except that galactomannan I of *T. rubrum* does not contain galactofuranose and has a different backbone structure. The backbone of galactomannan I consists of α1,6-linked mannopyranose and mannofuranose units, and that of galactomannan II consists of α1,2- and α1,6-linked mannopyranose and mannofuranose units. Kawarasaki et al. [13] analysed the structure of the cell-wall polysaccharide of *T. mentagrophytes* that was precipitated by Fehling's solution. From the results of methylation analysis and acetolysis, they reported that the polysaccharide was mannan with a comb-like structure. Jimenez-Barbero et al. [14] recently reported that the structure of the mannan of *Microsporum* and *Trichophyton*, based on ^{1}H and ^{13}C NMR analyses, is:

$$[\rightarrow 6\text{Man}\alpha 1 \rightarrow 6\text{Man}\alpha 1 \rightarrow 6\text{Man}\alpha 1 \rightarrow]_n$$
$$\uparrow 2$$
$$\text{Man}\alpha 1$$

Abbreviations used: HOHAHA, homonuclear Hartmann–Hahn spectroscopy; HMBC, two-dimensional heteronuclear multiple-bond connectivity.

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However, there are no reports describing the fine chemical structure of galactomannans. Therefore we have examined the structure of the galactomannans of *T. mentagrophytes* and *T. rubrum* using one- and two-dimensional NMR techniques.

MATERIALS AND METHODS

Materials

T. mentagrophytes IFO 5466 and *T. rubrum* IFO 5467 were obtained from the Institute for Fermentation, Osaka (IFO), Japan. An α1,2-linked mannotetraose, Manα1-2Manα1-2Manα1-2Man, was prepared from the cell-wall mannan of *Candida albicans* by acetolysis.

Preparation of cell-wall polysaccharide

Cells were cultivated in a 500 ml flask containing Sabouraud liquid medium supplemented with 0.5 % yeast extract dialysate for 2 weeks at 28 °C using a rotary shaker. The cells were washed and dehydrated with acetone, and crude polysaccharide was extracted with water at 135 °C for 3 h. The crude polysaccharide in the culture filtrate and in the hot-water extract were separately dialysed against water, evaporated and lyophilized.

DEAE-Sephadex chromatography of crude polysaccharide

An aqueous solution of the crude polysaccharide was applied to a column of DEAE-Sephadex A-50 (acetate form; 5 cm × 30 cm). Elution was effected in a stepwise manner using water, 0.05 M and 0.1 M NaCl solutions. Samples of 20 μl of the eluates were assayed for their carbohydrate content using the phenol/sulphuric acid method [15]. The representative elution profiles are shown in Figure 1. Eluates corresponding to each peak were combined, concentrated *in vacuo* to a small volume, dialysed against water and lyophilized. The subfractions were designated fractions I, II, and III.

Degradation of galactomannans by treatment with 100 mM HCl

To remove the galactofuranose units, 100 mg of each mannan was dissolved in 10 ml of 100 mM HCl, and the resultant solution was heated at 100 °C for 30 min [16]. After cooling, the solution was neutralized with 1 M NaOH, concentrated and applied to a column (2.5 cm × 100 cm) of Bio-Gel P-2.

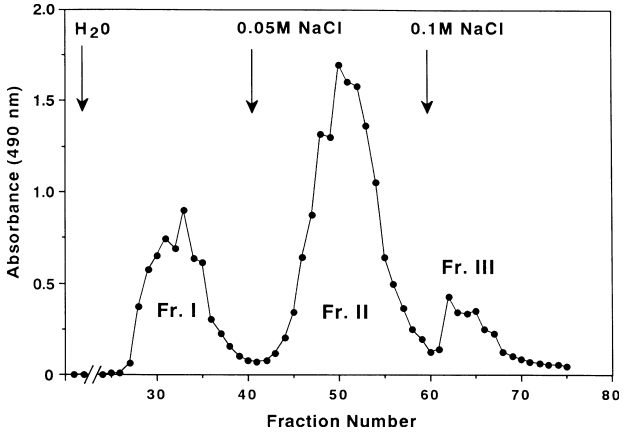

Figure 1 Elution pattern of the polysaccharide from *T. mentagrophytes* IFO 5466 strain

DEAE-Sephadex A-50 column (5 cm × 30 cm) chromatography was performed by stepwise elution with water, 0.05 M NaCl and 0.1 M NaCl. Fr., fraction.

NMR spectroscopy

All NMR experiments were performed with a JEOL JNM-GSX 400 spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C. The spectra were recorded using a 1 % (w/v) solution of each mannan or oligosaccharide in 0.7 ml of ^2H_2O at 45 °C. Acetone (2.217 p.p.m.) [17] and $C^2H_3O^2H$ (49.00 p.p.m.) were used as the internal standards for ^1H and ^{13}C NMR respectively.

Other methods

Total carbohydrate was determined by the phenol/sulphuric acid method of Dubois et al. [15], with D-mannose as standard. Total phosphate was determined by the method of Ames and Dubin [18], using KH_2PO_4 as standard. Total protein was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co.) [19], with BSA as standard. Sugar components were determined by converting the sugars of each acid-hydrolysed specimen into the corresponding alditol acetates. Alditol acetates were chromatographed on a glass column (5 mm × 150 cm) containing column packing, 3 % ECNSS-M on Gas-Chrom Q (80–100 mesh) (Applied Science Laboratories), at 190 °C with N_2 at a flow rate of 60 ml/min.

RESULTS

Fractionation of polysaccharide by DEAE-Sephadex chromatography

The cell-wall extracts and the culture filtrates of *T. mentagrophytes* and *T. rubrum* were each fractionated into three subfractions in a stepwise manner with water, 0.05 M NaCl and 0.1 M NaCl (Figure 1). The chemical compositions and yields of the three subfractions, designated fractions I, II and III, are shown in Table 1. Since these subfractions do not contain phosphate groups, the negative charges of fractions II and III seem to originate from protein moieties. Sugar composition analysis of the subfractions prepared from the culture filtrates of the two strains indicated that fractions I and II contained galactose and mannose, whereas fraction III contained only mannose. On the other hand, fraction I of the cell-wall extracts of both strains contained glucose as well as mannose and galactose. The glucose seems to be derived from cell-wall glucan,

Table 1 Chemical composition of polysaccharide subfractions obtained from the culture filtrates of *T. mentagrophytes*

Yield was determined as a percentage by weight of total polysaccharide in the crude culture filtrate. Sugar composition was determined by GLC, after converting the component sugars of each acid-hydrolysed specimen into the corresponding alditol acetates; the amount of each sugar is expressed as a percentage of the total sugar in each fraction (on a molar basis). Protein content was determined using a BCA protein assay kit [19]. The values shown are percentages of the total (on a mass basis).

Polysaccharide fraction	Yield (%)	Sugar composition (%)			Protein (%)
		Man	Gal	Glc	
I	21.3	79.2	20.8	–	20.0
II	50.7	87.3	12.7	–	17.2
III	27.9	100.0	–	–	36.7

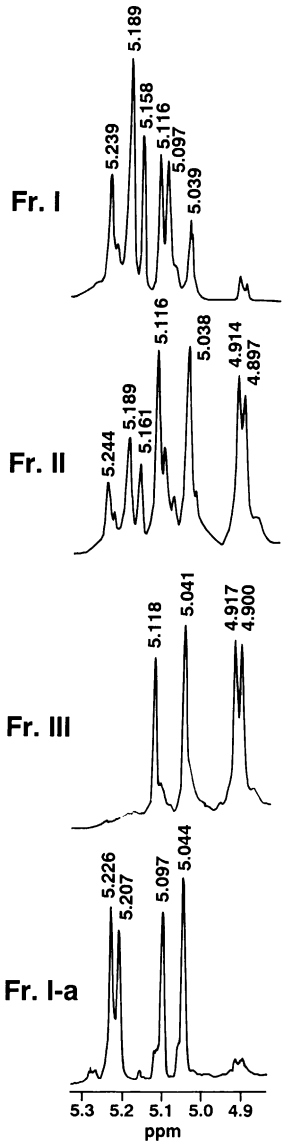

Figure 2 Anomeric region of the ^{1}H NMR spectra of fractions I, II and III and of the acid-modification product of fraction I (fraction Ia)

Spectra were recorded using a JEOL JNM-GSX 400 spectrometer in ^2H_2O solution at 45 °C using acetone as standard (2.217 p.p.m.). Fr., fraction.

as indicated by the presence of broad ^{1}H NMR signals (results not shown). The ^{1}H NMR spectra of these subfractions suggest that fraction II is a mixture of two polysaccharides, fractions I and III (Figure 2). The ^{1}H NMR spectra of fraction III obtained from four crude polysaccharides gave exactly the same signals as those reported by Jimenez-Barbero et al. [14]. Therefore it is clear that the structure of fraction III is as follows:

$$[\rightarrow 6\text{Man}\alpha 1 \rightarrow 6\text{Man}\alpha 1 \rightarrow 6\text{Man}\alpha 1 \rightarrow]_n$$
$$\uparrow 2$$
$$\text{Man}\alpha 1$$

Since the ^{1}H NMR signals of fraction I obtained from the culture media and cell-wall extracts of the two strains were fundamentally the same, a structural study of galactomannan was performed using fraction I obtained from the culture filtrate of *T. mentagrophytes*.

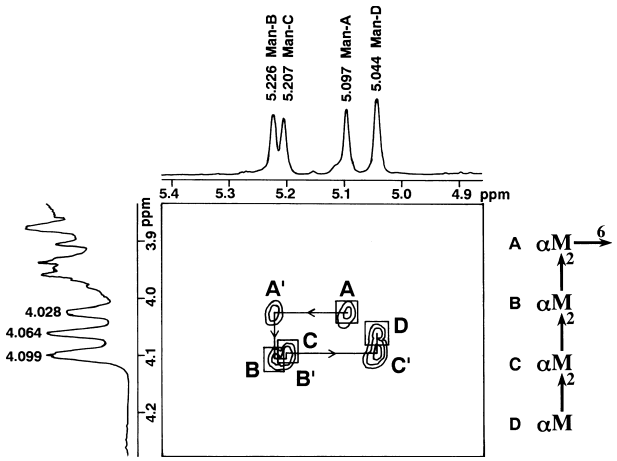

Figure 3 Partial NOESY spectra of fraction Ia

Letters labelled with primes indicate inter-residue H-1–H-2 ' NOE cross-peaks, and non-primed letters indicate H-1–H-2-correlated cross-peaks due to *J*-coupling. Arrows indicate the direction of sequential connectivity from Man-A. M, mannose.

Acid treatment of fraction I

To determine the structure of the mannan moiety of the galactomannan, fraction I from the culture filtrate of *T. mentagrophytes* was treated with 100 mM HCl at 100 °C for 30 min. Bio-Gel P-2 column chromatography of the reaction mixture gave an acid-modified polysaccharide and monosaccharide, which were identified by ^{1}H NMR as mannan and galactose respectively. The mannan obtained by the acid treatment of fraction I was designated fraction Ia.

^{1}H NMR analysis of fraction Ia

To assign the signals and to determine the linkage sequence, a sequential assignment study of the H-1 and H-2 signals of fraction Ia was performed using the method described by Hernandez et al. [20], with slight modifications [21–23]. Figure 3 shows the NOESY spectra of fraction Ia. The boxed cross-peaks labelled with non-primed letters indicate intra-residue H-1–H-2 connectivities, which were confirmed by COSY, and the cross-peaks labelled with primed letters indicate inter-residue H-1–H-2′ NOE connectivities. By using this procedure, the H-1 and H-2 signals were sequentially assigned from H-1 of Man-A as A–A′–B–B′–C–C′–D. This indicates that fraction Ia has a repeating structure of α1,2-linked mannotetraose units.

The upfield shifts ($\Delta\delta = 0.05$–0.08 p.p.m.) of the H-1 signals of the α1,2-linked mannose units, from 5.27–5.28 p.p.m. to 5.20–5.22 p.p.m., seem to be due to the same effect of the α1,6-linked mannose unit as is found for the mannans of *Saccharomyces kluyveri* [24], *C. albicans* [22] and *Candida guilliermondii* [23]. The chemical shifts at 5.097 and 5.044 p.p.m. are almost identical to those of an α1,6-linked mannose unit substituted by an α1,2-linked mannose unit and a non-reducing terminal α1,2-linked mannose unit respectively.

To determine the attachment point of the α1,6-linked mannose unit, a comparison of the two-dimensional HOHAHA (homonuclear Hartmann–Hahn spectroscopy) spectra of fraction Ia, an

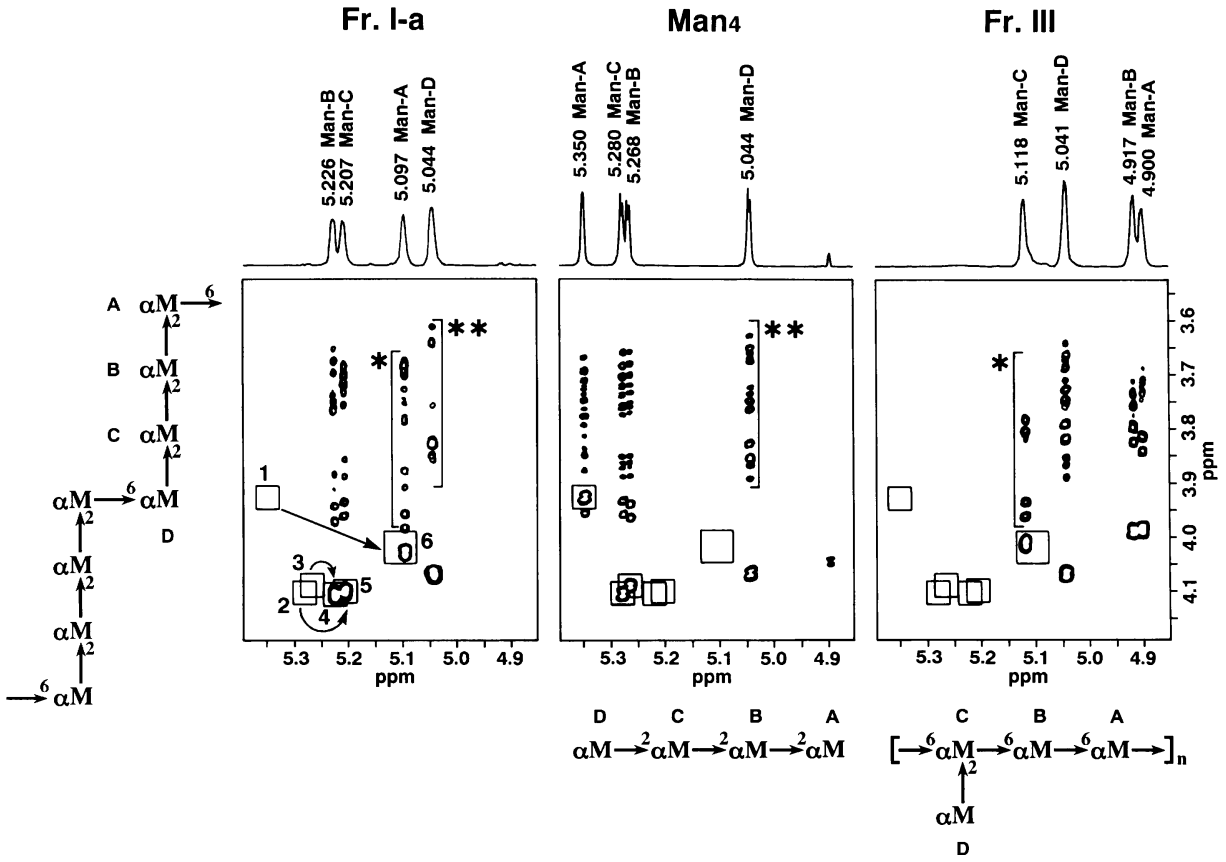

Figure 4 Partial two-dimensional HOHAHA spectra of fraction Ia, an α1,2-linked mannotetraose and fraction III

The asterisks on each panel indicate the signal shift caused by attachment of an α1,6-linked mannose unit. Fr., fraction; M, mannose.

α1,2-linked mannotetraose and fraction III was carried out using the methods of Hernandez et al. [20,25] (Figure 4). The ring proton cross-peaks of Man-D correlated with the signal at 5.044 p.p.m. of fraction Ia are apparently shifted downfield compared with those of the non-reducing terminal mannose unit, Man-D (5.044 p.p.m.), of an α1,2-linked mannotetraose. In contrast, the ring proton cross-peaks of Man-A correlated with the signal at 5.097 p.p.m. of fraction Ia are shifted upfield compared with those of the backbone α1,6-linked mannose unit, Man-C (5.118 p.p.m.), substituted by an α1,2-linked mannose unit of fraction III. Boxes 2 and 3 correspond to the normal regions of the H-1–H-2 correlated cross-peaks of intermediary α1,2-linked mannose units. In the spectrum of fraction Ia, however, the cross-peaks were shifted to boxes 4 and 5, concomitant with the shift of the H-1–H-2 correlated cross-peak of the reducing terminal mannose unit, Man-A, from box 1 to box 6, which corresponds to an α1,6-linked mannose unit substituted by an α1,2-linked mannose unit [23,24]. These results indicate that Man-D of fraction Ia is substituted with Man-A of another α1,2-linked mannotetraose unit by an α1,6 linkage. Furthermore, we can state that the Man-A is not substituted by any other α1,6-linked mannose unit. Thus the above results indicate that fraction Ia has the following structure:

D C B A

$[\rightarrow 6\text{Man}\alpha1 \rightarrow 2\text{Man}\alpha1 \rightarrow 2\text{Man}\alpha1 \rightarrow 2\text{Man}\alpha1 \rightarrow]_n$

Sequential NMR assigment of fraction I

The linkage sequence of fraction I was analysed by the same assignment method as used for fraction Ia. In Figure 5, cross-peaks labelled with primed letters indicate through-space inter-residue H-1–H-2′ or H-1–H-3′ dipolar coupling (NOE) between two adjacent carbohydrate units. On the other hand, cross-peaks labelled with non-primed letters indicate intra-residue H-1–H-2- or H-1–H-3-correlated cross-peaks caused by scalar coupling. The numbers of cross-peaks indicate the coupling protons. By using this procedure, the H-1 and H-2 signals were sequentially assigned from H-1 of Man-A as A2–A2′–B2–B2′–C2–C2′–D3–D3′–F2 and A2–A2′–B2–B2′–C3–C3′–E2. Usually an α1,3 linkage gives an H-1–H-2′ NOE cross-peak in addition to an H-1–H-3′ NOE cross-peak. From the results of the ^{1}H–^{13}C COSY and ^{1}H–^{1}H COSY of fraction I (Figure 6), it is apparent that the signals at 5.158 and 5.185 p.p.m. ($J_{1,2} < 2$ Hz) correspond to H-1 of β-galactofuranose units. This suggests that Gal-F is attached to Man-D by a β1,2 or β1,3 linkage, and that Gal-E is attached to Man-C by a β1,3 linkage.

Assignment of ^{13}C NMR signals of fraction I

To determine the attachment point of the galactofuranose units on the core mannan (fraction Ia), assignment of the ^{13}C NMR signals of fraction I was carried out using a combination of two-

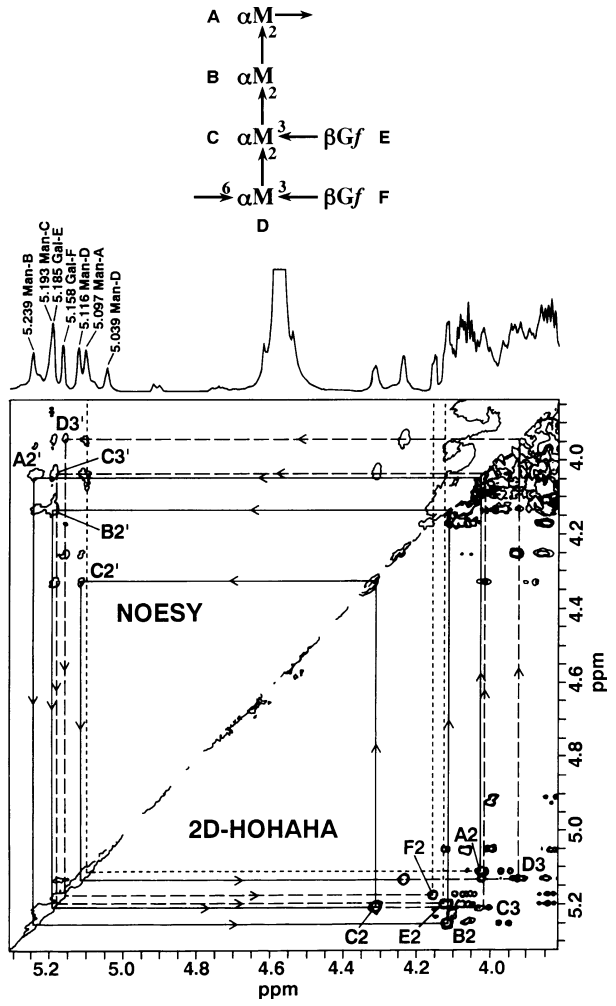

Figure 5 Sequential connectivities of mannose (M) and galactose (G) units of fraction I

The right side of the diagonal shows two-dimensional (2D) HOHAHA, and the left side of the diagonal shows NOESY. Primed letters indicate inter-residue H-1–H-2′ or H-1–H-3′ NOE cross-peaks, and unprimed letters indicate H-1–H-2- or H-1–H-3-correlated cross-peaks. The numbers of the cross-peaks indicate the coupling protons; e.g. A2 indicates the H-1–H-2-correlated cross-peak of Man-A, and A2′ indicates the inter-residue NOE cross-peak between H-2 of Man-A and H-1 of an adjacent mannose unit, Man-B. Arrows indicate the direction of sequential connectivity from Man-A to the two galactofuranose units.

dimensional HOHAHA and ^{1}H–^{13}C COSY spectra (Figure 6). At first, cross-peaks corresponding to the H-1 to H-4 protons in the two-dimensional HOHAHA spectrum were assigned from the results of ^{1}H–^{1}H COSY and relayed COSY (results not shown). As shown in the two-dimensional HOHAHA spectrum of Figure 6, the H-2 protons of Man-C and Man-D appeared in a downfield region, at 4.311 p.p.m. ($\Delta\delta = 0.212$ p.p.m.) and 4.235 p.p.m. ($\Delta\delta = 0.171$ p.p.m.) respectively, compared with those of fraction Ia. ^{1}H–^{13}C COSY indicates that the C-2 and C-4 signals of Man-C of fraction I were shifted upfield, to 75.08 p.p.m. ($\Delta\delta = 3.81$ p.p.m.) and 66.32 p.p.m. ($\Delta\delta = 1.48$ p.p.m.) respectively, and the C-2 and C-4 signals of Man-D of fraction I were also shifted upfield, to 67.70 p.p.m. ($\Delta\delta = 3.50$ p.p.m.) and 65.34 p.p.m. ($\Delta\delta = 2.46$ p.p.m.) respectively, compared with those of fraction Ia. On the other hand, the C-3 signals of Man-C and Man-D were shifted downfield, to 76.57 p.p.m. ($\Delta\delta = 5.68$ p.p.m.) and 76.37 p.p.m. ($\Delta\delta = 5.48$ p.p.m.) respectively, compared with those of fraction Ia. The assignments of the ^{1}H and ^{13}C signals of fractions I and Ia are shown in Table 2. It is known that the signals of the glycosylated carbon atom and of its neighbouring carbon atoms show significant downfield and upfield shifts in comparison with those of the corresponding unglycosylated atoms [27]. Therefore the above results indicate that the galactofuranose units in fraction I are attached to Man-C and Man-D via a β1,3 linkage.

Two-dimensional heteronuclear multiple-bond connectivity (HMBC) spectrum of fraction I

The linkage sequence was confirmed by a HMBC experiment. As shown in Figure 7, the presence of inter-residue connectivities between Man-A (C-2 = 79.46 p.p.m.) and Man-B (H-1 = 5.239 p.p.m.), Man-B (C-2 = 79.19 p.p.m.) and Man-C (H-1 = 5.193 p.p.m.), Man-C (C-2 = 75.08 p.p.m.) and Man-D (H-1 = 5.116 p.p.m.), and Man-D (C-6 = 66.17 p.p.m.) and Man-A (H-1 = 5.097 p.p.m.) through the cross-peaks A′, B′, C′ and D′ respectively is in good agreement with the results of the sequential assignment of fraction I (Figure 5). The H-1–C-3′-correlated cross-peaks c′ and d′ between Gal-E (H-1 = 5.185 p.p.m.) and Man-C (C-3 = 76.57 p.p.m.) and between Gal-F (H-1 = 5.158 p.p.m.) and Man-D (C-3 = 76.37 p.p.m.) respectively indicate that these galactofuranose units are connected to C-3 of these mannose units.

Overall structures of the galactomannans

The H-1 signal dimensions of fraction I obtained from *T. mentagrophytes* and *T. rubrum* were calculated in order to estimate the molar ratios of the carbohydrate units, using the method described previously [23,24]. Although, as shown in Table 1, the molar ratio of mannose to galactose was 8:2, the ratio of the H-1 signal dimensions of Man-D substituted and unsubstituted by galactofuranose was 1:0.75. Therefore we depicted the representative structure as having two repeating units. In one unit, Man-C and Man-D are substituted by galactofuranoses; in the other unit, Man-C is substituted by galactofuranose but Man-D is not. As shown in Figure 8, the structures of the galactomannans of the two strains are almost the same. Only the ratio of the galactofuranose units attached to the core mannan of the galactomannan differs; that of *T. mentagrophytes* galactomannan is a little larger than that of *T. rubrum* galactomannan.

DISCUSSION

For analysis of the cell-wall mannans of the genus *Candida*, we were able to use acetolysis to fragment the polysaccharide [28–30]. However, this method was not useful for the galactomannans of *Trichophyton* species because the galactofuranose linkage is labile towards acetolysis, and we could not obtain galactofuranose-containing oligosaccharides [16,31–33]. On the other hand, covalent structural determination of polysaccharides by non-empirical multinuclear NMR methods utilizing through-bond correlations offers advantages over chemical methods. Here we have demonstrated the successful application of several two-dimensional NMR methods to obtain the chemical structures of the galactomannans of *T. mentagrophytes* and *T. rubrum*. Although Bishop et al. [11,12] reported the structure of the galactomannan of *Trichophyton* from the results of methylation analysis, it is impossible to determine the linkage sequence from these data. Therefore it was informative to apply the sequential assignment method using NOESY and/or HMBC, as used by

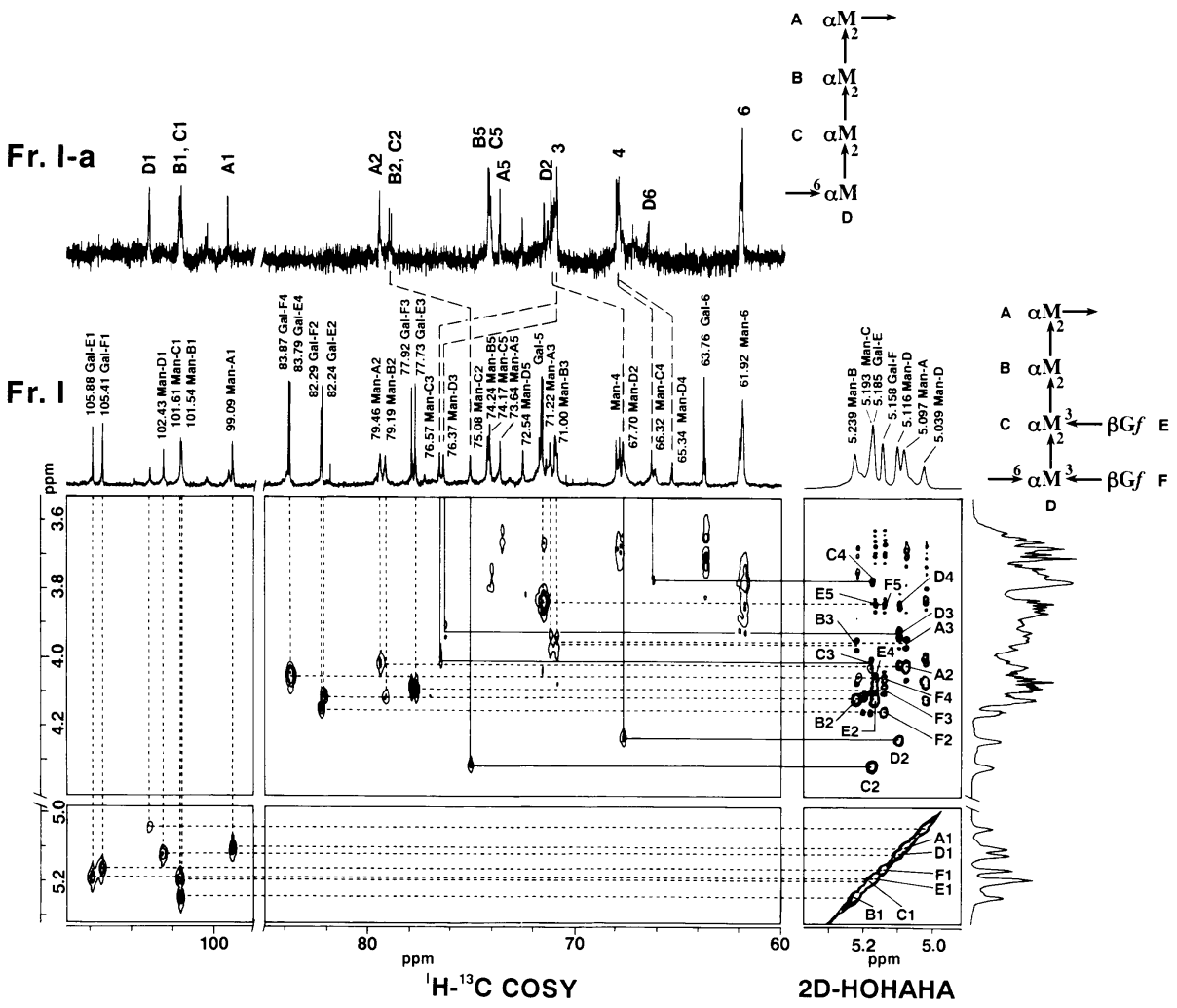

Figure 6 Assignment of ^{13}C signals by two-dimensional (2D) HOHAHA and ^{1}H–^{13}C COSY

The ring proton signals of each mannose (M) and galactose (G) unit were assigned by ^{1}H–^{1}H COSY and relayed COSY (results not shown). Then the corresponding ring carbon signals were assigned from the H–C-correlated cross-peaks of ^{1}H–^{13}C COSY. Broken lines between the two ^{13}C NMR spectra indicate the upfield or downfield shifts of mannose carbon signals caused by glycosylation with the galactofuranose units. Fr., fraction.

Gasa et al. [34], Hernandez et al. [20], Alvarado et al. [35], Abeygunawardana et al. [36] and Shibata et al. [21–24,37].

Although the repeating structure of the backbone mannan of fraction I, i.e. α1,2-linked mannotetraose units, was the same as in the galactomannans of *Penicillium charlesii* [26,31], *Aspergillus niger* [32] and *A. fumigatus* [38], this is the first report of the assignment of the ^{1}H NMR spectrum of this structure. The H-1 proton chemical shift of the α1,2-linked mannose units appeared in an upfield region ($\Delta\delta = 0.05$–0.08 p.p.m.) compared with that of linear α1,2-linked manno-oligosaccharides [17,22]. The reason for the upfield shift of the H-1 signals seems to be the same as for those observed in the mannans of *S. kluyveri* [24], *C. albicans* [22] and *C. guilliermondii* [23]. Namely, all of these mannans contain an α1,6-linked mannose unit attached to an α1,2-linked unit, and the H-1 signal of the neighbouring α1,2-linked mannose unit of the 6-*O*-substituted unit was shifted from 5.28 p.p.m. to 5.22 p.p.m. ($\Delta\delta = 0.06$ p.p.m.) due to the steric effect of the α1,6-linked mannose unit. We are currently attempting to determine the three-dimensional structure of the α1,6-linkage-containing oligosaccharide in aqueous solution in order to interpret this upfield shift effect.

Once the backbone structure of the galactomannan has been assigned, interpretation of the complicated ^{13}C NMR spectrum of the native polysaccharide can provide the exact positions of the galactofuranose units. The two galactofuranose units were identified to be linked to Man-C and Man-D by a β1,3 linkage, based on the results of ^{1}H–^{13}C COSY and HMBC. The C-1 chemical shifts of the galactofuranose units of fraction I (105.41 and 105.88 p.p.m.) were similar to those of the galactofuranose unit of a synthesized oligosaccharide, Gal*f*β1-3Manα1-Me (106.5 p.p.m.) [39], and were different from those of the galactofuranose units of Gal*f*β1-2Manα1-Me (107.7 p.p.m.) and Gal*f*β1-6Manα1-Me (109.7 p.p.m.). Furthermore, mild Smith degradation of fraction I gave biose as the main product (results not shown). These findings also strongly support the above conclusions.

T. rubrum [2] and *C. albicans* [40] mannans were shown to inhibit cell-mediated immunity. However, one problem needs to

Table 2 NMR chemical shifts of fractions I (galactomannan) and Ia obtained from *T. mentagrophytes* IFO 5466 strain

Values marked with the same superscript letter ([a], [b] or [c]) may have to be interchanged.

(a) Fraction Ia	Chemical shift (p.p.m.)			
Assignment	→ 6Manα1 → D	→ 2Manα1 → C	→ 2Manα1 → B	→ 2Manα1 → A
^{1}H				
H-1	5.044	5.207	5.226	5.097
H-2	4.064	4.097	4.103	4.028
H-3	–	3.947	3.955	3.968

(b) Fraction I	Chemical shift (p.p.m.)					
Assignment	Galfβ1 → F	Galfβ1 → E	→ 6Manα1 → (3↑) D	→ 2Manα1 → (3↑) C	→ 2Manα1 → B	→ 2Manα1 → A
^{1}H						
H-1	5.158	5.185	5.116	5.193	5.239	5.097
H-2	4.145	4.117	4.235	4.311	4.112	4.017
H-3	4.091	4.090	3.929	4.013	3.952	3.948
H-4	4.052	4.056	3.844	3.777	3.676	–
H-5	3.846	3.841	–	–	3.749	–
H-6	3.651	3.714	–	–	–	–
H-6′	3.710	3.656	–	–	–	–
^{13}C						
C-1	105.41	105.88	102.43	101.61	101.54	99.09
C-2	82.29	82.24	67.70	75.08	79.19	79.46
C-3	77.92	77.73	76.37	76.57	71.00	71.22
C-4	83.87	83.79	65.34	66.32	67.87[a]	68.03[a]
C-5	71.60	71.65	72.54	74.17	74.24	73.64
C-6	63.67[b]	63.76[b]	66.17	61.92[c]	61.92[c]	62.05[c]

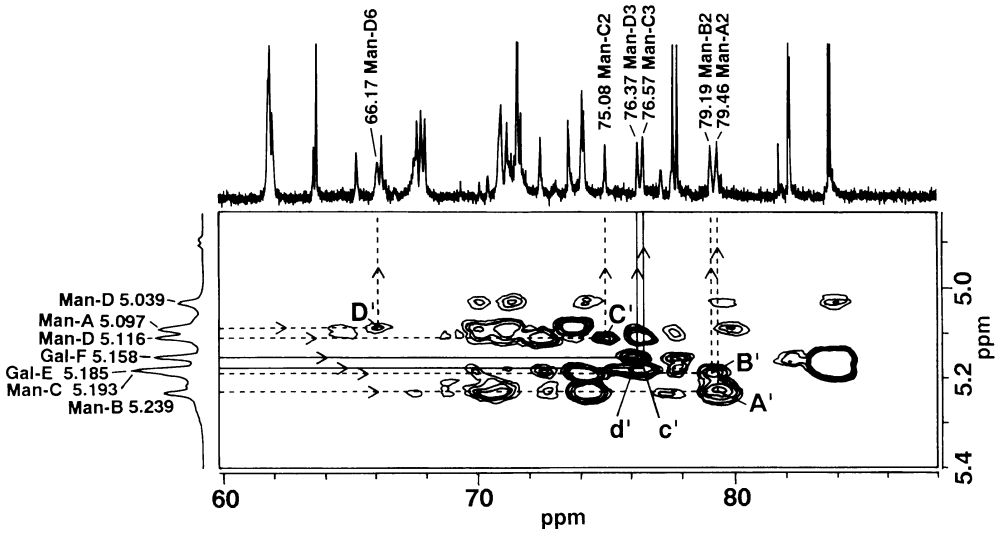

Figure 7 Partial HMBC spectrum of fraction I

Primed upper-case letters indicate the cross-peaks between H-1 of mannose units and C-2 or C-6 of adjacent mannose units. Primed lower-case letters indicate the cross-peaks between H-1 of galactofuranose units and C-3 of adjacent mannose units.

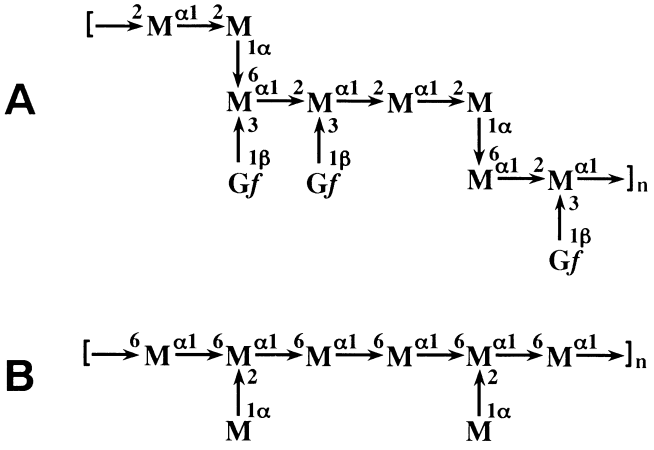

Figure 8 Possible structures of galactomannan (A) and mannan (B) of *T. mentagrophytes* IFO 5466 and *T. rubrum* IFO 5467 strain cells

M and G*f* denote D-mannopyranose and D-galactofuranose units respectively.

be solved before the molecule responsible for this immunoinhibitory activity can be identified. The mannans were isolated by precipitation using cetyltrimethylammonium bromide, which can also provoke similar effects on lymphocytes *in vitro* [7]. Although it was stated that this reagent was not responsible for the inhibitory effect of the mannans, further studies with other preparations of mannans and galactomannans will be necessary in order to establish their biological inhibitory activity on immune function. Precipitation of the crude polysaccharide in the culture filtrate or the cell-wall extract by Fehling's solution [14] gave only mannan corresponding to fraction III (results not shown), while fractionation of the crude polysaccharide using the DEAE-Sephadex column gave galactomannan as well as mannan. Therefore these fractions could be useful for the assay of immunological activity.

The galactomannan peptide of *Trichophyton* species, 'Trichophytin', is known to be an allergen [41–43]. Grappel et al. [44,45] indicated that the galactofuranose units of the galactomannan of *T. mentagrophytes* are the main epitopes. The galactomannans of *Penicillium* and *Aspergillus* species contain β1,5-linked consecutive galactofuranose units [38,46–49]. Stynen et al. [48] prepared a monoclonal antibody against the long β1,5-linked galactofuranosyl oligosaccharide of the galactomannan of *A. fumigatus* for the diagnosis of aspergillosis. In the present study, however, we demonstrated that the galactomannan of *Trichophyton* contains no consecutive galactofuranose units, consistent with the findings of Bishop et al. [11,12]. Notermans et al. [50] reported that the β1,5-linked galactofuranose tetramer and higher oligomers showed the greatest inhibitory effects in an ELISA inhibition assay using antibodies against the cells of *Penicillium* and *Aspergillus* and their galactomannans. Furthermore, van Cutsem et al. [51] reported that a monoclonal antibody against the galactomannan of *A. fumigatus* did not react with the sera of animals suffering from invasive *T. mentagrophytes* infection. These findings indicate that the epitope structure of *T. mentagrophytes* galactomannan is apparently different from that of the galactomannans of *Penicillium* and *Aspergillus*, and might be unique. Monoclonal antibodies specific for *T. mentagrophytes* may be useful for detecting Trichophytin in sera or tissues of subjects.

In summary, we have shown that galactomannans isolated from *T. mentagrophytes* and *T. rubrum* are similar to each other, but different from the galactomannans of other fungi. These variations might account for differences in the ability of these fungi to induce immunity and inflammation, and they may also account for the antigen specificity of these organisms *in vivo* and *in vitro*.

REFERENCES

1 Hay, R. J. (1982) Br. J. Dermatol. **106**, 1–7
2 Dahl, M. V. (1994) J. Am. Acad. Dermatol. **31**, S34–S41
3 Green, III, F. and Balish, E. (1979) Infect. Immun. **26**, 554–562
4 Sherwin, W. K., Ross, T. H., Rosenthal, C. M. and Petrozzi, J. W. (1979) Arch. Dermatol. **115**, 600–604
5 Calderon, R. A. and Hay, R. J. (1984) Immunology **53**, 457–464
6 Holden, A. J., Hay, R. J. and MacDonald, D. M. (1981) J. Invest. Dermatol. **61**, 207–211
7 Blake, J. S., Dahl, M. V., Herron, M. J. and Nelson, R. D. (1991) J. Invest. Dermatol. **96**, 657–661
8 Grando, S. A., Hostager, B. S., Herron, M. J., Dahl, M. V. and Nelson, R. D. (1992) J. Invest. Dermatol. **98**, 876–880
9 Grando, S. A., Hostager, B. S., Herron, M. J., Dahl, M. V. and Nelson, R. D. (1992) Acta Derm. Venereol. **72**, 273–276
10 Bishop, C. T., Blank, F. and Hranisavljevic-Jakovljevic, M. (1962) Can. J. Chem. **40**, 1816–1825
11 Bishop, C. T., Perry, M. B., Blank, F. and Cooper, F. P. (1965) Can. J. Chem. **43**, 30–39
12 Bishop, C. T., Perry, M. B. and Blank, F. (1966) Can. J. Chem. **44**, 2291–2297
13 Kawarasaki, I., Takeda, T., Ogihara, Y., Shimonaka, H. and Nozawa, Y. (1979) Chem. Pharm. Bull. **27**, 2073–2075
14 Jimenez-Barbero, J., Prieto, A., Gomez-Miranda, B., Leal, J. A. and Bernabe, M. (1995) Carbohydr. Res. **272**, 121–128
15 Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Anal. Chem. **28**, 350–356
16 Nakajima, T., Suzuki, C. and Matsuda, K. (1982) Agric. Biol. Chem. **46**, 869–875
17 Cohen, R. E. and Ballou, C. E. (1980) Biochemistry **19**, 4345–4358
18 Ames, B. N. and Dubin, D. T. (1960) J. Biol. Chem. **235**, 769–775
19 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85
20 Hernandez, L. M., Ballou, L., Alvarado, E., Gillece-Castro, B. L., Burlingame, A. L. and Ballou, C. E. (1989) J. Biol. Chem. **264**, 11849–11856
21 Shibata, N., Hisamichi, K., Kikuchi, T., Kobayashi, H. and Suzuki, S. (1992) Biochemistry **31**, 5680–5686
22 Shibata, N., Ikuta, K., Imai, T., Satoh, Y., Satoh, R., Suzuki, A., Kojima, C., Kobayashi, H., Hisamichi, K. and Suzuki, S. (1995) J. Biol. Chem. **270**, 1113–1122
23 Shibata, N., Akagi, R., Hosoya, T., Kawahara, K., Suzuki, A., Ikuta, K., Kobayashi, H., Hisamichi, K., Okawa, Y. and Suzuki, S. (1996) J. Biol. Chem. **271**, 9259–9266
24 Shibata, N., Kojima, C., Satoh, Y., Satoh, R., Suzuki, A., Kobayashi, H. and Suzuki, S. (1993) Eur. J. Biochem. **217**, 1–12
25 Hernandez, L. M., Ballou, L., Alvarado, E., Tsai, P. K. and Ballou, C. E. (1989) J. Biol. Chem. **264**, 13648–13659
26 Unkefer, C. J. and Gander, J. E. (1990) J. Biol. Chem. **265**, 685–689
27 Pfeffer, P. E., Valentine, K. M. and Parrish, F. W. (1978) J. Am. Chem. Soc. **101**, 1265–1274
28 Kocourek, J. and Ballou, C. E. (1969) J. Bacteriol. **100**, 1175–1181
29 Shibata, N., Kobayashi, H., Tojo, M. and Suzuki, S. (1986) Arch. Biochem. Biophys. **251**, 697–708
30 Kobayashi, H., Shibata, N., Mitobe, H., Ohkubo, Y. and Suzuki, S. (1989) Arch. Biochem. Biophys. **272**, 364–375
31 Gander, J. E., Jentoft, N. H., Drewes, L. R. and Rick, P. D. (1974) J. Biol. Chem. **249**, 2063–2072
32 Bardalaye, P. C. and Nordin, J. H. (1977) J. Biol. Chem. **252**, 2584–2591
33 Nakajima, T., Yoshida, M., Nakamura, M., Hiura, N. and Matsuda, K. (1984) J. Biochem. (Tokyo) **96**, 1013–1020
34 Gasa, S., Nakamura, M., Makita, A., Ikura, M. and Hikichi, K. (1986) Eur. J. Biochem. **155**, 603–611
35 Alvarado, E., Ballou, L., Hernandez, L. M. and Ballou, C. E. (1990) Biochemistry **29**, 2471–2482
36 Abeygunawardana, C., Bush, C. A. and Cisar, J. O. (1991) Biochemistry **30**, 6528–6540
37 Shibata, N., Hisamichi, K., Kobayashi, H. and Suzuki, S. (1993) Arch. Biochem. Biophys. **302**, 113–117

38 Latge, J. P., Kobayashi, H., Debeaupuis, J. P., Diaquin, M., Sarfati, J., Wieruszeski, J. M., Parra, E., Bouchara, J. P. and Fournet, B. (1994) Infect. Immun. **62**, 5424–5433
39 Gorin, P. A. J., Barreto-Bergter, E. M. and da Cruz, F. S. (1981) Carbohydr. Res. **88**, 177–188
40 Nelson, R. D., Shibata, N., Podzorski, R. P. and Herron, M. J. (1991) Clin. Microbiol. Rev. **4**, 1–19
41 Barker, S. A. and Trotter, M. D. (1960) Nature (London) **188**, 232–233
42 Barker, S. A., Cruickshank, C. N. D., Morris, J. H. and Wood, S. R. (1962) Immunology **5**, 627–632
43 Kitazima, Y., Banno, Y., Noguchi, T., Nozawa, Y. and Ito, Y. (1972) Arch. Biochem. Biophys. **152**, 811–820
44 Grappel, S. F., Blank, F. and Bishop, C. T. (1968) J. Bacteriol. **95**, 1238–1242
45 Grappel, S. F., Blank, F. and Bishop, C. T. (1968) J. Bacteriol. **96**, 70–75
46 Barreto-Bergter, E. M., Travassos, L. R. and Gorin, P. A. J. (1980) Carbohydr. Res. **86**, 273–285
47 Barreto-Bergter, E. M., Gorin, P. A. J. and Travassos, L. R. (1981) Carbohydr. Res. **95**, 205–218
48 Stynen, D., Sarfati, J., Goris, A., Prevost, M.-C., Lesourd, M., Kamphuis, H., Darras, V. and Latge, J.-P. (1992) Infect. Immun. **60**, 2237–2245
49 van Bruggen-van der Lugt, A. W., Kamphuis, H. J., de Ruiter, G. A., Mischnick, P., van Boom, J. H. and Rombouts, F. M. (1992) J. Bacteriol. **174**, 6096–6102
50 Notermans, S., Veeneman, G. H., van Zuylen, C. W. E. M., Hoogerhout, P. and van Boom, J. H. (1988) Mol. Immunol. **25**, 975–979
51 van Cutsem, J., Meulemans, L., van Gerven, F. and Stynen, D. (1990) Mycoses **33**, 61–69

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