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Differences among the cell wall galactomannans from *Aspergillus wentii* and *Chaetosartorya chrysella* and that of *Aspergillus fumigatus*

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The alkali extractable and water-soluble cell wall polysaccharides F1SS from *Aspergillus wentii* and *Chaetosartorya chrysella* have been studied by methylation analysis, 1D- and 2D-NMR, and MALDI-TOF analysis. Their structures are almost identical, corresponding to the following repeating unit:

[→ 3)-β-D-Gal*f*-(1 → 5)-β-D-Gal*f*-(1 →]$_n$ → mannan core.

The structure of this galactofuranose side chain differs from that found in the pathogenic fungus *Aspergillus fumigatus*, in other Aspergillii and members of Trichocomaceae:

[→ 5)-β-D-Gal*f*-(1 →]$_n$ → mannan core.

The mannan cores have also been investigated, and are constituted by a (1 → 6)-α-mannan backbone, substituted at positions 2 by chains from 1 to 7 residues of (1 → 2) linked α-mannopyranoses.
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Introduction

The genus *Aspergillus* is widely distributed and its members have been found in many different environments from the arctic region to the tropics, and probably are the most frequently encountered fungi in the human environment. Some 20 species are human and animal pathogens which cause a variety of allergic and invasive diseases [1]. Among them, the pathogen most commonly found is *A. fumigatus*, which is involved in diseases such as bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis, the most severe form which has been reported. Nowadays, it is an increasing cause of mortality among immunocompromised patients which have suffered organ transplants, AIDS or cancer. The pathobiology, structure and biosynthesis of the cell wall of *Aspergillus fumigatus* and the utilisation of the pathways involved in the biosynthetic process of this structure as specific drug targets have been recently reviewed [2–4].

The alkali-extractable and water-soluble fungal polysaccharides F1SS, which are minor components of the cell-wall (2–8%), are forming part of peptido-polysaccharides, differ in composition and structure among genera and, in certain cases, among groups of species of a genus [5]. These polysaccharides are antigenically relevant [6–11] and are probably involved in cell-cell and/or cell-host recognition mechanisms.

The alkali-extractable galactomannans from some pathogenic Aspergillii (*A. niger*, *A. terreus*, *A. flavus*, *A. nidulans*, and *A. flavipes*) have been shown to have interrelated chemical structures to that of *A. fumigatus* [7,12].

The galactomannan obtained by alkaline extraction of hyphae of *A. fumigatus* has been described by Barreto-Bergter *et al.* [8] who found that it was constituted by a main chain of (1→6)-α-mannopyranosyl residues, substituted at *O*-2 by small chains of (1→2) interlinked α-mannopyranoses, to which (1→5)-β-galactofuranosyl units are (1→6) linked to the mannan core. Antigenic extracellular polysaccharides of Aspergillii

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have also been described [13–15]. The structures of all these polysaccharides are of interest for specific detection of those fungi by immunoassays.

In this paper we present the study of a different structure of alkali-extractable water-soluble cell-wall galactomannans isolated from *A. wentii* and *Chaetosartorya chrysella*, which are compared with the structure obtained from *Aspergillus fumigatus*.

Materials and methods

Microorganisms and culture media

The species used were: *Aspergillus wentii* (CECT 2887, CBS 121.32, and CBS 118.34), *A. fumigatus* (CBS 120.53) and *Chaetosartorya chrysella* (CBS 472.65). The microorganisms were maintained on slants of Bacto potato dextrose agar supplemented with 1 g L^{-1} of Bacto yeast extract (Difco). The basal medium and growth conditions have been previously described [16].

Wall material preparation and fractionation

Wall material was obtained as reported elsewhere [17]. Polysaccharides F1SS were obtained according to Ahrazem *et al.* [18].

Chemical analysis

For analysis of neutral sugars, the polysaccharides were hydrolyzed with 3 M trifluoroacetic acid (1 h at 121°C). The resulting monosaccharides were converted into their corresponding alditol acetates [19] and identified and quantified by gas-liquid chromatography (GLC) using an SP-2380 fused silica column (30 m × 0.25 mm I.D. × 0.2 μm film thickness) with a temperature program (210°C to 240°C, initial time 3 min, ramp rate 15°C min^{-1}, final time 7 min) and a flame ionization detector.

The monosaccharides released after hydrolysis were derivatised as devised by Gerwig *et al.* [20] and their absolute configuration was determined by GC-MS of the tetra-*O*-TMSi-(+)-2-butylglycosides using an SPB-1 fused silica column (30 m × 0.25 mm I.D. × 0.2 μm film thickness) with a temperature program (150°C to 250°C, initial time 3 min, ramp rate 2°C min^{-1}, final time 30 min). The components of the sample were identified on the basis of their retention times and mass spectra.

Methylation and reductive cleavage analyses

The polysaccharide (1–5 mg) was methylated according to the method of Ciucanu and Kerek [21]. The methylated material was treated and analysed according to Ahrazem *et al.* [22].

Reductive cleavage analyses were carried out in two steps, as described by Bennek *et al.* [23], with trimethylsilyl triflate as catalyst, but the reactions were carried out under argon and the time during the reductive cleavage step was shortened to 5–6 h to minimise unwanted by-products.

Partial hydrolysis of the polysaccharides F1SS

A sample of the corresponding polysaccharide (50 mg) was hydrolysed with 5 mL of 0.05 M H_2SO_4 for 5 h at 100°C. The degraded polysaccharide (mannan core) was recovered by dialysis (molecular weight cutoff ca. 3 kDa) and lyophilization.

Acetolysis of the mannan core of the polysaccharide of C. chrysella

The polysaccharide (50 mg) was treated according to the method of Stewart and Ballou [24]. The mixture of peracetylated oligosaccharides obtained was subjected to deacetylation using the Zemplén reaction [25].

MALDI-TOF analysis

The oligomers obtained by acetolysis of the sample were analyzed on a Biflex time-of-flight instrument (Bruker-Franzen Analytik, Bremen, Germany) operated in the reflectron mode. The matrix used was 2,5-dihydroxybenzoic acid (10 mg mL^{-1} in water). Equal volumes of the sample (in water) and the matrix solution were mixed for a few seconds and centrifuged. The resulting solution (1 μL) was deposited onto the target and allowed to dry. The mass of the oligosaccharides were observed as the $[M + Na]^+$ ions, and their relative amounts were calculated according to peak area.

NMR analysis

The polysaccharides F1SS and the mannan cores (ca. 20 mg) were dissolved in D_2O (1 mL) followed by centrifugation (10000 g, 20 min) and lyophilization. The process was repeated twice and the final sample was dissolved in D_2O (0.7 mL, 99.98 D).

1D- and 2D- ^1H- and ^{13}C-NMR experiments were carried out at 40°C on a Varian INOVA (300 MHz) or a Varian Unity 500 (500 MHz) spectrometer with a reverse probe and a gradient unit. Proton chemical shifts refer to residual HDO at δ 4.61 ppm. Carbon chemical shifts refer to internal acetone at δ 31.07 ppm. The 2D-NMR experiments (DQF-COSY, TOCSY, NOESY, HMQC, HSQC-TOCSY, and HMBC) were performed by using the standard Varian software.

Results and discussion

Chemical analysis of the alkali-extractable water soluble (F1SS) polysaccharides gave galactose and mannose in a proportion ca. 5:1 in the strains of *A. wentii*, ca. 3:1 in *C. chrysella*, and ca. 5:1 in *A. fumigatus*, as shown by GLC of their alditol acetates. Absolute configuration was shown to be D for both sugars.

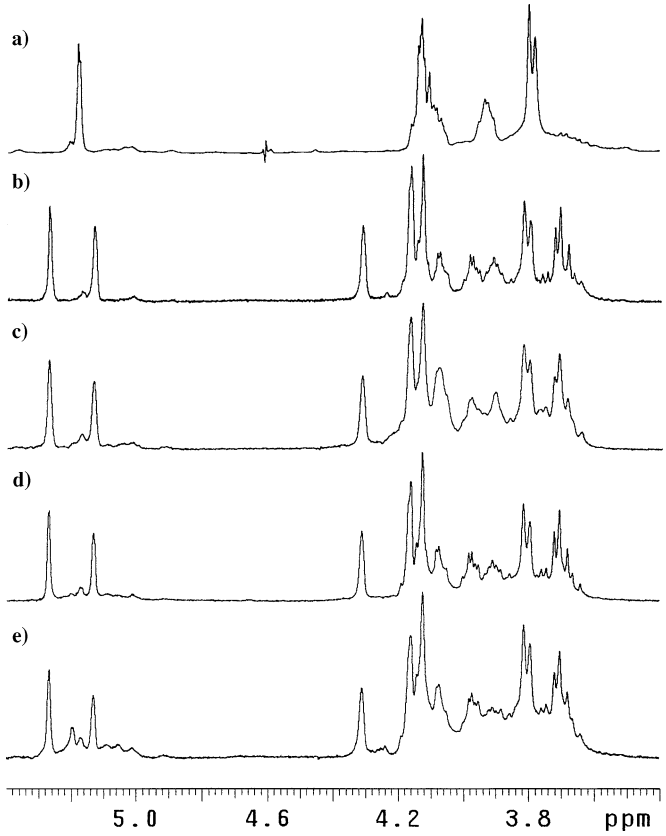


Figure 1. ^{1}H-NMR spectra (D_2O, 40°C, 300 MHz) of the alkali-extracted water-soluble cell wall F1SS polysaccharides of: (a) *Aspergillus fumigatus* (CBS 120.53), (b) *A. wentii* (CBS 121.32), (c) *Ibid.* (CECT 2887), (d) *Ibid.* (CBS 118.34) and (e) *Chaetosartorya chrysella* (CBS 472.65).

^{1}H-NMR spectra (Figure 1) of the F1SS polysaccharides from the microorganisms were recorded. The spectrum of the of F1SS from *A. fumigatus* shows a main anomeric signal at 5.18 ppm in the anomeric region. Methylation analysis and reductive cleavage of this polysaccharide indicated that it was mainly composed of (1→5) galactofuranose, although traces of 2-*O*-substituted mannopyranose were also detected. The spectra of the polysaccharides from *C. chrysella* and the three strains of *A. wentii* were practically identical. Then, only that from strain CBS 121.32 was purified by column chromatography and used for further studies. Methylation analysis of this strain reveals the presence of 6-*O*-substituted and 2,6-di-*O*-substituted Man*p*, terminal Gal*f*, 3-*O*-substituted Gal*f*, and either 5-*O*-substituted Gal*f*, 4-*O*-substituted Gal*p*, and 5,6-di-*O*-substituted Gal*f*, 4,6-di-*O*-substituted Gal*p* or both, in a proportion close to (0.3:0.3:0.1:1:1.1:0.1). The presence of 5-*O*-substituted Gal*f* and 5,6-di-*O*-substituted Gal*f*, and the absence of galactopyranose residues was demonstrated after reductive cleavage of the permethylated polysaccharide.

The ^{1}H-NMR spectrum (Figure 2a) contained, *inter alia*, two main anomeric signals at 5.27 and 5.13 ppm, and a small signal at 5.17 ppm, in the proportion 1:1:0.1. The residues

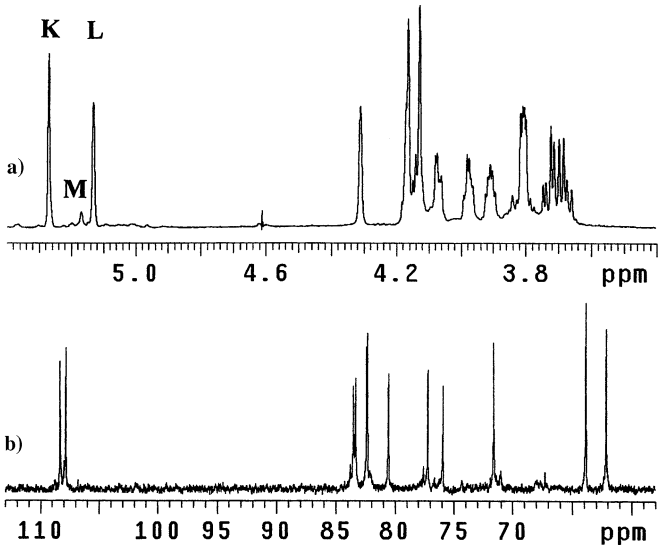


Figure 2. (a) ^{1}H- (D_2O, 40°C, 500 MHz) and (b) ^{13}C-(D_2O, 40°C, 125 MHz) NMR spectra of the alkali-extracted water-soluble (F1SS) polysaccharide from *A. wentii*. The anomeric proton peaks have been labelled.

were labelled **K**-**L**-**M**, respectively, according to their anomeric protons. The anomeric coupling constants for the residues were $J_{1,2} \approx 2$ Hz, which suggested galactofuranose residues with β-configuration for them (compare with α-anomeric coupling constants around 4 Hz) [26]. The ^{13}C-NMR spectrum (Figure 2b) showed also two main and small anomeric singlets at 108.4, 107.9, and 108.0 ppm, respectively, which are also indicative of β-Gal*f* units [27]. 2D homo- (COSY, TOCSY) and hetero- (HMQC) NMR experiments led to the complete assignment of the proton and carbon chemical shifts of residues **K** and **L**, and partial assignment of residue **M** (see Table 1).

Comparison of the data obtained with standard values [27] led to the conclusion that **K** was 3-*O*-substituted-β-Gal*f* and **L** 5-*O*-substituted-β-Gal*f*. The low proportion of **M** did not allow to obtain all the chemical shifts of the residue but, according to

Table 1. ^{1}H- and ^{13}C-NMR chemical shifts (δ) for the alkali-extractable water-soluble polysaccharide F1SS isolated from *A. wentii*

		*Proton or carbon**						
Units		*1*	*2*	*3*	*4*	*5*	*6a*	*6b*
K	H	5.27	4.31	4.16	4.17	3.91	3.73	3.68
	C	108.4	80.6	**83.6**	83.4	71.7	63.9	
L	H	5.13	4.13	4.13	4.07	3.97	3.81	3.79
	C	107.9	82.4	77.3	82.6	**76.1**	62.2	
M	H	5.17	4.15					
	C	108.0	82.1	77.7	83.9	71.5		

*Underlined bold numbers represent glycosylation sites.

the methylation results and the values observed in model compounds [28,29], should be assigned to terminal galactofuranose. The anomeric peak of the 5,6-disubstituted galactofuranose was not detected in the ^{1}H-NMR nor in the HMQC spectra, probably due to its low proportion.

A NOESY experiment allowed to observe, among others, crosspeaks H-1**K**/H-5**L** and H-1**L**/H-3**K**.

From all the combined data, the structure of the polysaccharide of *A. wentii* was deduced to consist of long chains of $[\rightarrow 3)$-*O*-β-D-Gal*f*-$(1\rightarrow 5)$-*O*-β-D-Gal*f*-$(1\rightarrow]_n$, with 2–5% of terminal Gal*f* linked to the 6-*O*-position of some of the 5-*O*-substituted furanosidic residues, as deduced from the presence of 5,6-disubstituted galactofuranose found in the methylation analysis. These chains are attached to a mannan core. Small proportions of terminal and 5,6-disubstituted Gal*f* residues have been reported for the polysaccharides isolated from *A. fumigatus* and *Penicillium digitatum* [14], *P. erubescens* and *P. corylophilum* [30], and *Geosmithia putterillii* [31].

Investigation of the cell wall polysaccharide of *C. chrysella* gave proton spectra analogous to those of *A. wentii*, but the broad irregular anomeric signals corresponding to the mannan core appeared here in a larger proportion (Figure 1e).

The polysaccharides F1SS were treated with dilute acid, which selectively hydrolyzed the furanosidic side chains, releasing the mannan core. Methylation analysis of these materials demonstrated the presence of terminal Man*p*, 2-*O*-substituted, 6-*O*-substituted, and 2,6-di-*O*-substituted Man*p*.

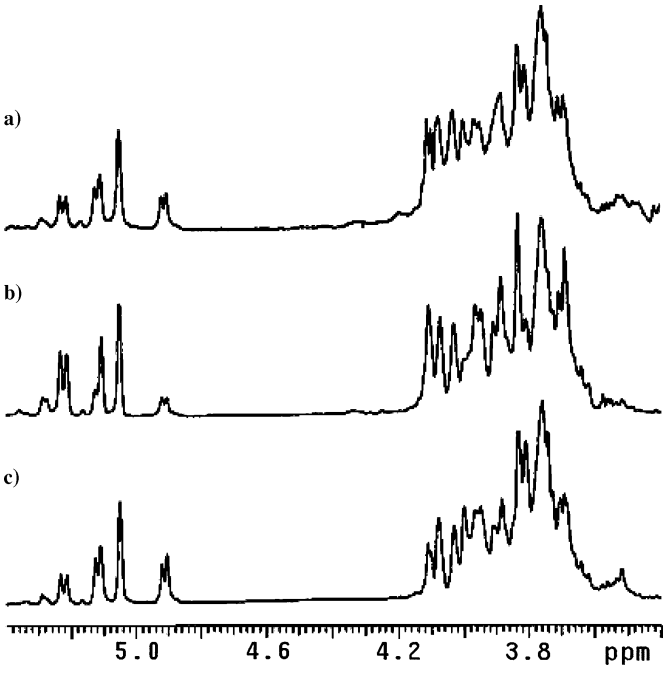


Figure 3. ^{1}H-NMR spectra (D_2O, 40°C, 500 MHz) of the mannan core polysaccharides obtained by mild acid hydrolysis of the F1SS polysaccharides from: (a) *Aspergillus wentii*, (b) *Chaetosartorya chrysella* and (c) *A. fumigatus*.

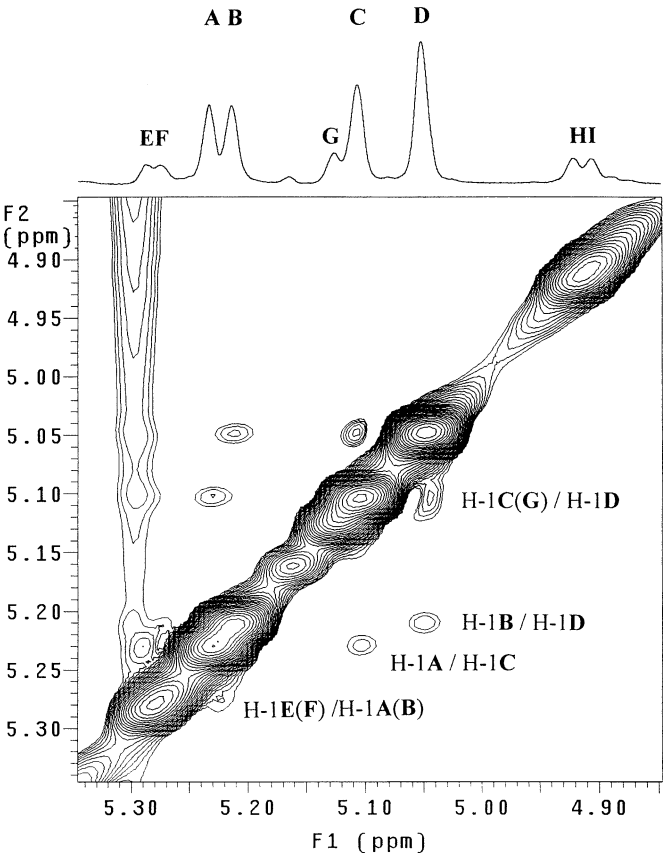


Figure 4. Partial 2D-NOESY spectrum of the mannan core of the polysaccharide from *C. chrysella*. Anomeric peaks and relevant cross peaks have been labelled.

The ^{1}H-NMR spectra (Figure 3) contained four main anomeric signals, labelled **A**–**D** from low to high field, and five minor anomeric signals, labelled **E**–**I** (see anomeric expansion on top of Figures 4 and 5). As only four different residues were detected in the methylation analysis, it is obvious that some of these anomeric signals correspond to identical residues in different neighbourhoods or with different conformational features.

A series of homo-(COSY, TOCSY, NOESY) and hetero-(HMQC, HMBC, HSQC-TOCSY) 2D-NMR experiments allowed almost complete assignment of residues **A**–**D**, and partial assignment of residues **E**–**I** (Table 2). Particularly useful was the study of the HSQC-TOCSY spectra, which allowed the identification of the carbon chemical shifts of the main residues, although not all the protons could be unambiguously assigned. Concerning the minor residues, only a few carbons from the chain were recognised, although they were significant enough for their identification. Comparison of the chemical shifts with those of known values [27] led to conclude that **A**, **B**, **E**, and **F** were 2-*O*-substituted Man*p*; **C** and **G**, 2,6-di-*O*-substituted Man*p*; **D**, terminal Man*p*, and **H** and **I**, 6-*O*-substituted Man*p*.A coupled HMQC experiment allowed the measurement of one bond

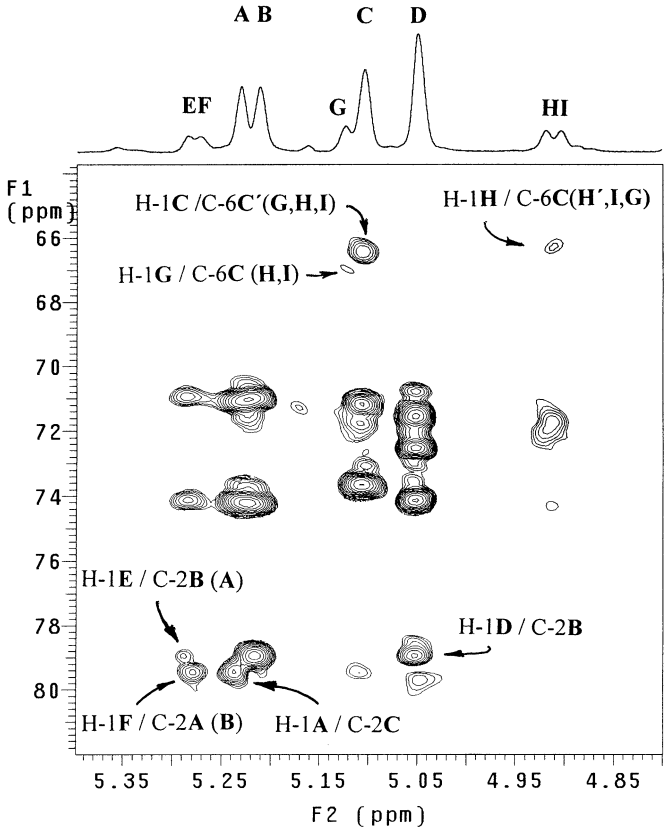


Figure 5. Partial 2D-HMBC spectrum of the mannan core of the polysaccharide from *C. chrysella*. Anomeric peaks and relevant cross peaks have been labelled.

^{1}H-^{13}C anomeric coupling constants. The values obtained for all the residues were in the range $^{1}J_{H\text{-}1\text{-}C\text{-}1} = 173 \pm 0.6$ Hz, which are demonstrative of α-configuration for all of them [32].

With regard to the arrangement of the different linkages, a 2D-NOESY spectrum of the anomeric region of the mannan (Figure 4) showed crosspeaks H-1**A**/H-1**C**, H-1**B**/H-1**D**, H-1**D**/H-1**C** + **G**, **H**-1**E**(**F**)/H-1**A**(**B**). In addition, crosspeaks H-1**C**/H-6a + 6b**C**′ (where **C**′ represent a second unit of **C**) and H-1**G**/H-6a + 6b**H** (**I**) could be observed, which strongly suggest that **A** are 2-substituted Man*p* units directly linked to the inner α-(1→6)-Man*p* backbone, **B** are 2-substituted Man*p* units linked to the terminal Man*p* residues (**D**) at the end of the lateral chains, and **E** and **F**, 2-substituted Man*p* units in between **A** and **B**.

An HMBC spectrum (Figure 5), in addition to intra-ring signals, showed crosspeaks H-1**A**/C-2**C**, H-1**D**/C-2**B**, H-1**E**/C-2**B** (**A**), H-1**F**/C-2**A** (**B**), H-1**C**/C-6**C**′ (**G**, **H**, **I**), H-1**G**/C-6**C** (**H**, **I**), H-1**H**/C-6**C** (**H**′, **I**, **G**), corroborating the NOESY results which, together with the methylation data, suggest a structure consisting of a linear α-(1→6)-mannan mostly substituted at positions 2 either by a single residue of mannose or by chains

Table 2. ^{1}H- and ^{13}C-NMR chemical shifts (δ) for the mannan core of the polysaccharide isolated from *C. chrysella*

		*Proton or carbon**						
Units		*1*	*2*	*3*	*4*	*5*	*6a*	*6b*
A	H	5.23	4.11	3.96			3.90	3.76
	C	101.6	**78.9**	70.9[a]	67.9[c]	74.1[d]	61.9	
B	H	5.21	4.11	3.96			3.90	3.76
	C	101.5	**79.0**	70.8[a]	68.0[c]	74.2[d]	61.9	
C	H	5.10	4.03	3.83	3.82	3.73	4.02	3.72
	C	99.3	**79.4**	71.4[b]	67.9[c]	73.7	**66.5**	
D	H	5.05	4.07	3.84	3.68	3.76	3.90	3.76
	C	103.1	70.9	71.4[b]	67.7	74.1[d]	61.9	
E	H	5.29	4.10				3.89	3.75
	C	101.4	**79.0**	71.5[b]	ca. 68.0	74.0[d]	61.9	
F	H	5.27	4.10				3.89	3.75
	C	101.4	**79.0**	71.5[b]	ca. 68.0	74.0[d]	61.9	
G	H	5.13	4.03					
	C	99.1	**79.4**				ca. **66.7**	
H	H	4.92	4.00				3.95	3.74
	C	100.4	70.9	ca. 71.4	ca. 67.4		**66.7**	
I	H	4.90	4.00				3.97	3.76
	C	100.4	70.9	ca. 71.4	ca. 67.4		**66.1**	

*Underlined bold numbers represent glycosylation sites.
[a,b,c,d] These values may have to be interchanged.

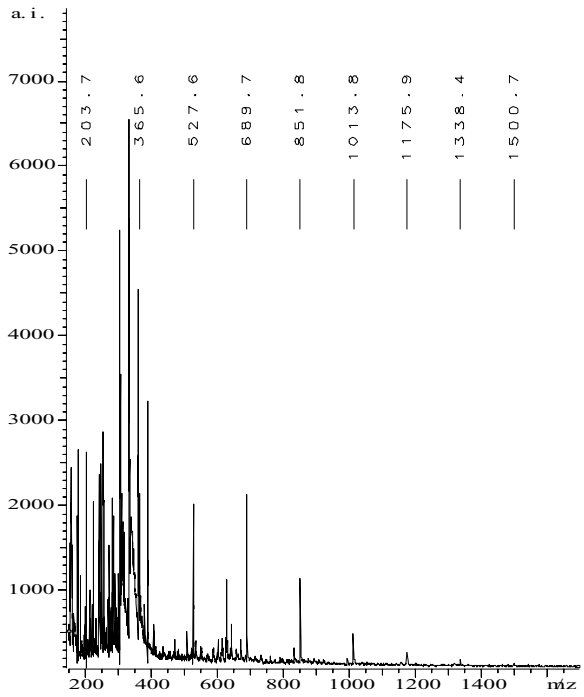
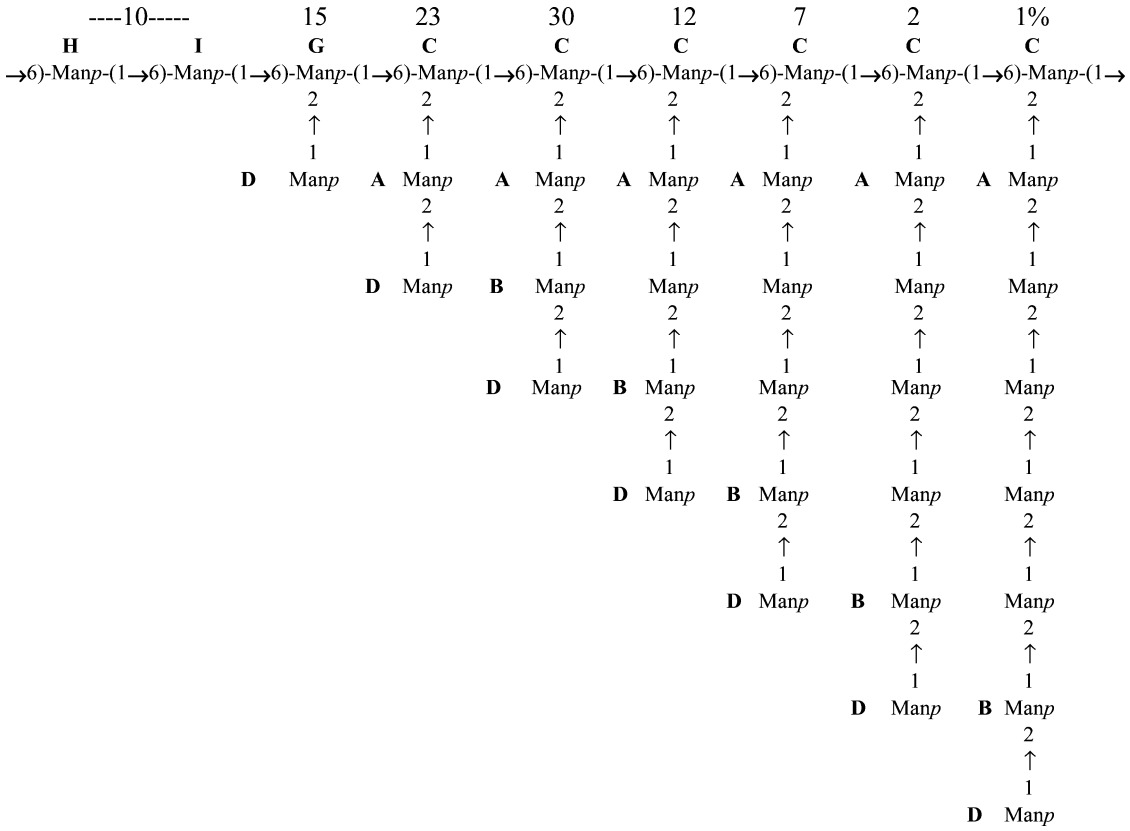


Figure 6. MALDI-TOF spectrum of the mixture of fragments obtained by acetolysis of the mannan core of the polysaccharide F1SS from *C. chrysella*. The numbers indicate the mass of the $[M + Na]^{+}$ ion from each fragment.

of 2 to 7 units of (1→2) linked Man*p*, as follows:



In order to ascertain the proportion of the different chains, we performed an acetolysis of the mannan core, which selectively hydrolysed the (1→6)-Man*p* linkages [24]. The mass of the different fragments obtained (from monomers to octamers) and their proportion was determined by MALDI mass analysis (Figure 6). The proportion of the monomers in the MALDI results were larger than expected, probably due to cleavage of some terminal residues of the lateral chains. For this reason, we adopted the value obtained by integration of the 6-substituted Man*p* residues in the proton spectrum as the monomer proportion, and deduced the rest from the MALDI results. The proportions obtained have been included at the top of the formula. The most abundant side chains contained from one to three residues, and the proportion was decreasing with the length of the chains. Unfortunately, we are still far from being able to find the positions that each side chain holds along the mannan backbone.

The ^{1}H-NMR spectra of the three mannan cores are very similar (Figure 3), hence the structures must be closely related. The differences observed among them may be due to different proportions of the distinct units (Table 3). The core of *C. chrysella* has higher content of 2-*O*-substituted Man*p*, which indicates higher proportions of long chains in this species than in both Aspergillii.

Concerning the linking positions between the Gal*f* chains and the mannan core, they should be linked to the terminal residue of the (1→2)-Man*p* side chains, since terminal Man*p* was not detected in the analyses of the intact polysaccharides while 25–30% of this residue appears in that of the mannan cores (Table 3). In addition, Barreto-Bergter *et al*. [8] reported that the (1→5)-β-Gal*f* chains found in *A. fumigatus* were linked to positions *O*-6 of the terminal Man*p* units of the side mannan chains.

The polysaccharide F1SS from the microorganisms studied have a similar structure in the mannan core. The galactofuranose side chains are also very similar in *A. wentii* (anamorph) and *C. chrysella* (teleomorph), which reveals the relatedness

Table 3. Percentage of the different units in the mannan cores of *A. wentii*, *C. chrysella*, and *A. fumigatus*, as deduced from integration of the anomeric signals in the proton spectra

Species	*2-O*-Man*p*	*2,6-di-O*-Man*p*	*Terminal* Man*p*	*6-O*-Man*p*
A. wentii	23.0	30.0	30.0	17.0
C. chrysella	38.0	26.5	26.5	9.0
A. fumigatus	19.0	29.0	30.0	22.0

of these microorganisms. These results are in agreement with the conclusions obtained from comparison of the nucleotide sequences of the large subunit of rDNA from these fungi [33]. In *A. fumigatus*, the side chains of the polysaccharide F1SS are composed of only (1→5)-β-galactofuranose residues, as previously described by Barreto-Bergter *et al.* [8]. It is interesting to mention that the polysaccharide F1SS from strain CBS 113.26 contains [→6)-β-D-Gal$f$-(1→5)-β-D-Gal$f$-(1→5)-β-D-Gal$f$-(1→]$_n$ side chains [34]. This may be due to a misidentification of some of these strains, since all isolates of a single species should have the same polysaccharide.

The polysaccharides F1SS from species of *Aspergillus* and *Penicillium* and their teleomorphs, which belong to family Trichocomaceae [35], consist of galactofuranose chains with different lengths and linkage types attached to a mannan core [5,12,16,30,31]. The (1→3)(1→5)-β-galactofuranose chains of *A. wentii* and *C. chrysella* have not yet been found in other members of this family.

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