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Isolation and structural determination of a unique polysaccharide containing mannofuranose from the cell wall of the fungus *Acrospermum compressum*

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Abstract The alkali-extractable and water-soluble fungal polysaccharide F1SS isolated from the cell wall of *Acrospermum compressum* has been studied by methylation analyses, reductive cleavage and 1D- and 2D-NMR spectroscopy. The polysaccharide consists of a regular disaccharide repeating unit with the structure:

$$\begin{array}{c}[\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow]_n \rightarrow \text{Mannan core}\\ \quad\quad 2\\ \quad\quad\uparrow\\ \quad\quad 1\\ \quad\beta\text{-D-Man}f\end{array}$$

The mannan core was obtained by mild hydrolysis of the polysaccharide F1SS and its structure was deduced to be composed of a skeleton of α-(1→6)-mannopyranan, with around 1 out of 11 residues substituted at position 2 by short chains (one to six units) of 2-substituted mannopyranoses. DOSY experiments provided molecular sizes of 60 kDa and 2.5 kDa for the polysaccharide F1SS and the mannan core, respectively. This is the first report of a fungal mannofuranose-containing cell wall polysaccharide.

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Abbreviations

CBS	Centraalbureau voor Schimmelcultures
DEPT	distortionless enhancement by polarization transfer
DOSY	diffusion ordered NMR spectroscopy
DQF-COSY	double quantum filtered correlated spectroscopy
F1SS	alkali extractable and water soluble polysaccharide
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
NOESY	nuclear Overhauser enhancement spectroscopy
TFA	trifluoroacetic acid
TMSi	trimethylsilyl
TOCSY	totally correlated spectroscopy

Introduction

The alkali-extractable and water-soluble fungal polysaccharides F1SS, which are usually minor components of the cell-wall (2–8%), are forming part of peptido-polysaccharides [1]. We have shown that they are in close contact with the external environment [2, 3] and consequently they are antigenically relevant [3–9] and are involved in cell–cell and/or cell–host recognition mechanisms [10]. These polysaccharides differ in composition and structure among genera and, in certain cases, among groups of species of a

Table 1 Effect of hydrolysis conditions on the release of neutral sugars (%) from the polysaccharide F1SS of *A. compressum*

Hydrolysis conditions	Unknown	Mannose	Galactose	Glucose	Recovery (%)
0.038 M TFA 1 h, 100°C	0.0	14.0	10.5	2.3	26.8
0.075 M TFA 1 h, 100°C	0.0	23.1	22.0	2.6	47.7
0.15 M TFA 1 h, 100°C	4.9	17.1	31.8	0.3	54.1
0.15 M TFA 1 h, 120°C	13.0	26.7	52.9	0.3	92.9
2.5 M H_2SO_4 1 H, 100°C	28.8	26.1	39.7	5.0	99.6

genus. Therefore, they have been proposed as chemotaxonomic and evolutionary characters for fungi [11].

In the last years, we have been engaged in the study of the properties and structural determination of these polysaccharides. The subject has been reviewed [11, 12]. Here we report on the unique structure of a polysaccharide F1SS, isolated from the cell wall of *Acrospermum compressum*, a species of family Acrospermaceae in the Ascomycetes.

Materials and methods

Microorganism and culture media

The species used was *Acrospermum compressum* (CBS 102.057). The microorganism was maintained on slants of Bacto potato dextrose agar supplemented with 1 gl^{-1} of Bacto yeast extract (Difco). The basal medium and growth conditions for mycelium production have been previously described [13].

Wall material preparation and fractionation

Wall material was obtained as reported elsewhere [14]. Polysaccharide F1SS was obtained and purified according to Ahrazem *et al*. [15].

Chemical analysis

For analysis of neutral sugars, the polysaccharide was hydrolyzed with TFA at different concentrations under different conditions and with sulfuric acid as indicated in Table 1. The resulting monosaccharides were converted into their corresponding alditol acetates [16] and identified and quantified by 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 same sample was further analysed on a SPB-1 column and detected by GC-MS.

The monosaccharides released after hydrolysis were derivatised as devised by Gerwig *et al*. [17] 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 [18]. The methylated material was treated and analysed according to Ahrazem *et al*. [19].

Reductive cleavage analyses were carried out in two steps, as described by Gray [20], with trimethylsilyl triflate as catalyst, but the reactions were carried out under Ar and the time during the reductive cleavage step was shortened to 5–6 h to minimize unwanted by-products.

Partial hydrolysis of the polysaccharide

A sample of the polysaccharide F1SS (155 mg) was hydrolysed with 10 ml of 0.15 M TFA for 5 h at 100°C. The resistant polysaccharidic material was recovered by dialysis (molecular weight cutoff *ca.* 3 kDa) and lyophilization. This material constitutes the mannan core of the polysaccharide F1SS [21], which amounted to 6 mg (3.8% of the native polysaccharide).

NMR spectroscopy

The polysaccharide F1SS (*ca.* 25 mg) and the mannan core (*ca*. 5 mg) were dissolved in D_2O (1 ml) followed by centrifugation (10,000 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- ^{1}H- and ^{13}C-NMR experiments were carried out at 40°C on a Varian Unity 500 (500/125 MHz $^{1}H/^{13}C$) 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. 1D- and 2D-NMR experiments (DQF-COSY, TOCSY, NOESY, HMQC, HSQC-TOCSY, and HMBC) were performed by using the standard Varian

Table 2 GC-MS analysis of the partially methylated alditol acetates obtained from the polysaccharide F1SS of *A. compressum*

Retention time (min)	Assignment	Characteristic fragments (*m/z*)	Relative abundance (%)
8.90	Gal*p*-(1→	88, 102,118, 129, 145, 161, 162, 205	3.5
12.36	→6)-Gal*f*-(1→	102, 117, 118, 130, 162, 173, 233	3.0
15.53	→2,6)-Gal*f*-(1→	88, 101, 117, 130, 173, 190, 233	43.7
7.90	Man*f*-(1→	89, 102, 118, 162, 205	46.5
10.20	→2)-Man*f*-(1→	129, 130, 161, 190	tr.
11.12	→6)-Man*f*-(1→	102, 117, 118, 130, 162, 173, 233	3.2
11.30	→2)-Glc*p*-(1→	87, 88, 101, 129, 130, 161, 190	tr.

software. The standard Bruker DOSY protocol was used at 298 K on an Advance 500 MHz spectrometer. Thirty-two 1D ^{1}H spectra were collected with a gradient duration of δ= 4 ms and an echo delay of Δ=400 ms. Samples of commercially available dextrans with different molecular weights were used to build the calibration curve. The ledbpg2s pulse sequence, with stimulated echo, longitudinal eddy current compensation, bipolar gradient pulses, and two spoil gradients, was run with a linear gradient (53.55 G cm^1) stepped between 2 and 95%. The 1D ^{1}H spectra were processed and automatically baseline corrected. The diffusion dimension, zero filled to 1 K, was exponentially fitted according to preset windows for the diffusion dimension ($-9.6<\log D<-10.5$).

Results and discussion

The polysaccharide F1SS amounted to *ca*. 2.5% of the dry cell wall material of *A. compressum*. It was purified by gel filtration chromatography. GC analysis of the alditol acetates obtained after hydrolysis of the polysaccharide under mild conditions (0.15 M TFA, 120°C, 1 h) revealed the presence of mannose, galactose, a third component with retention time close to that of arabinose, and a minor amount of glucose (Table 1). Further analysis of these derivatives by GC-MS demonstrated that the third component had retention time and mass spectrum (*m/z* 43, 69, 81, 98, 102, 112, 115, 140, 157, and 186) incompatible with arabinose, and was a probable degradation product of the polysaccharide.

Since this component was not detected in any other of the analyses performed to characterize the polysaccharide (see below), several hydrolytic conditions were investigated in order to ascertain which residue was degraded. Hydrolysis with TFA at concentrations up to 0.075 M at 100°C for 1h did not produce the degradation compound, while reaction with 0.15 M TFA led to a significant decrease of mannose and the appearance of the degradation compound (see Table 1) which demonstrated that it were mannofuranosidic moieties that underwent partial decomposition. The hydrolysis with 0.15 M TFA at 120°C gave the best results, since galactose was completely liberated (around 50%), and mannose reached 26.7%, but the degradation product amounted to 13%. Since the degradation of mannose hampers its determination, the results of the methylation analysis and the integration of the anomeric signals of the ^{1}H-NMR spectrum were also used for its quantification. Neither methanolysis nor aqueous hydrolysis, after permethylation of the polysaccharide, produced degradation of the mannosidic components.

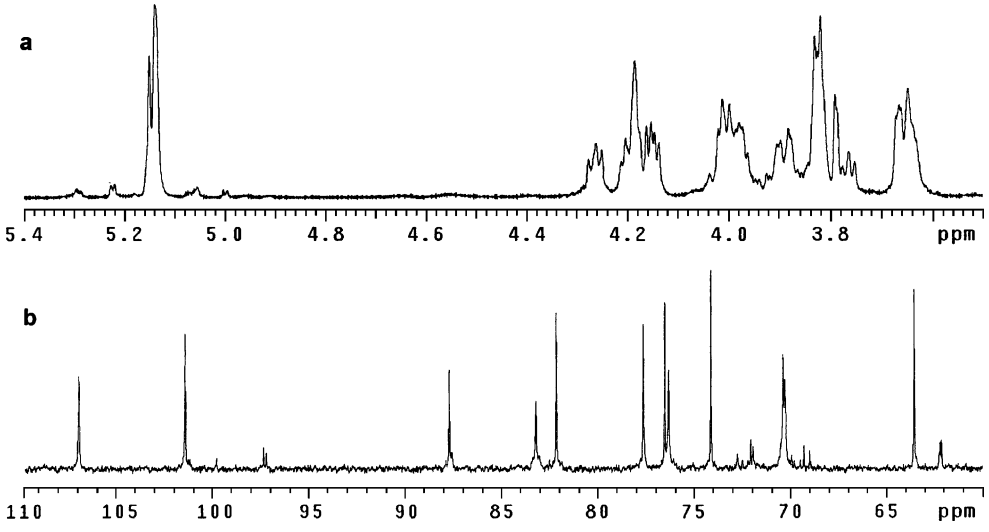


Fig. 1 **a** ^{1}H- (500 MHz) and **b** ^{13}C-NMR (125 MHz) spectra in D_2O at 40°C for the cell-wall F1SS polysaccharide isolated from *Acrospermum compressum*

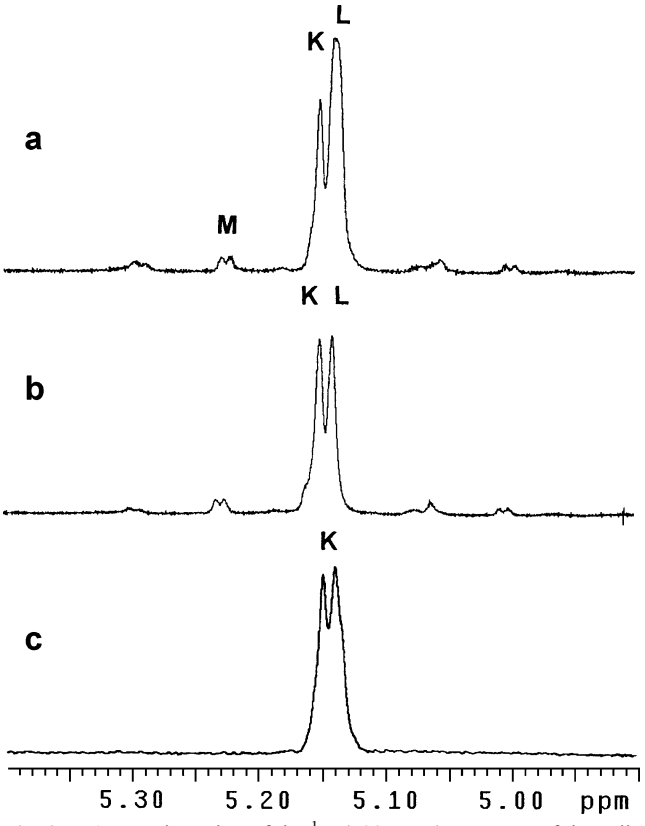


Fig. 2 **a** Anomeric region of the ^{1}H (500 MHz) spectrum of the cell-wall F1SS polysaccharide isolated from *A. compressum*; **b** Spectrum obtained on irradiation of the H-2 protons of units **K** and **L**, showing the proportion of the corresponding anomeric protons; **c** 1D-TOCSY spectrum of the anomeric proton of unit **K**, obtained by selective excitation of proton H-3 followed by a spin-lock mixing of 60 ms. Anomeric protons have been labelled

Methylation analysis (Table 2) and reductive cleavage of this polysaccharide indicated that it was mainly composed (>90%) of 2,6-di-*O*-substituted galactofuranose and terminal mannofuranose in a molar ratio 1:1. Traces to small amounts of 2-*O*-substituted mannofuranose, 6-*O*-substituted mannofuranose, 2-*O*-substituted glucopyranose, 6-*O*-substituted galactofuranose, and terminal galactopyranose were also detected.

The ^{1}H-NMR spectrum (Fig. 1a) contained, *inter allia*, two main signals in the anomeric region: a sharp signal at 5.15 ppm and a major somewhat broad signal at 5.14 ppm. On irradiation of their vicinal protons, the anomeric signals (Fig. 2a) were converted into two equal singlets (Fig. 2b), revealing that the anomeric pattern derived from a doublet overlapped with a non-resolved doublet ($J_{1,2}$<2 Hz). According to the anomeric protons, the corresponding residues were labelled **K** and **L**, respectively. In addition, a small doublet (**M**) at 5.23 ppm ($J_{1,2}$=3.4 Hz) could be observed in the anomeric region, along with a few minor signals which in many other examples of fungal polysaccharides are usually due to mannopyranosidic components of a small mannan core. The measurement of the anomeric coupling constant of **K** was achieved through a 1D-TOCSY experiment, by selectively exciting its H-3 proton, followed by a spin-lock mixing time of 60 ms (Fig. 2c). From this, a $J_{1,2}$=4.7 Hz was obtained.

The anomeric coupling constants are in agreement with the presence of mannofuranose and galactofuranose residues with β-configurations (compare with coupling constants around 3 Hz for α-mannofuranose, and near 4 Hz for α-galactofuranose) [22]. The ^{13}C- NMR spectrum (Fig. 1b) showed two major and a small anomeric singlets at 107.1, 101.4, and 97.2 ppm which, in agreement with the methyl-

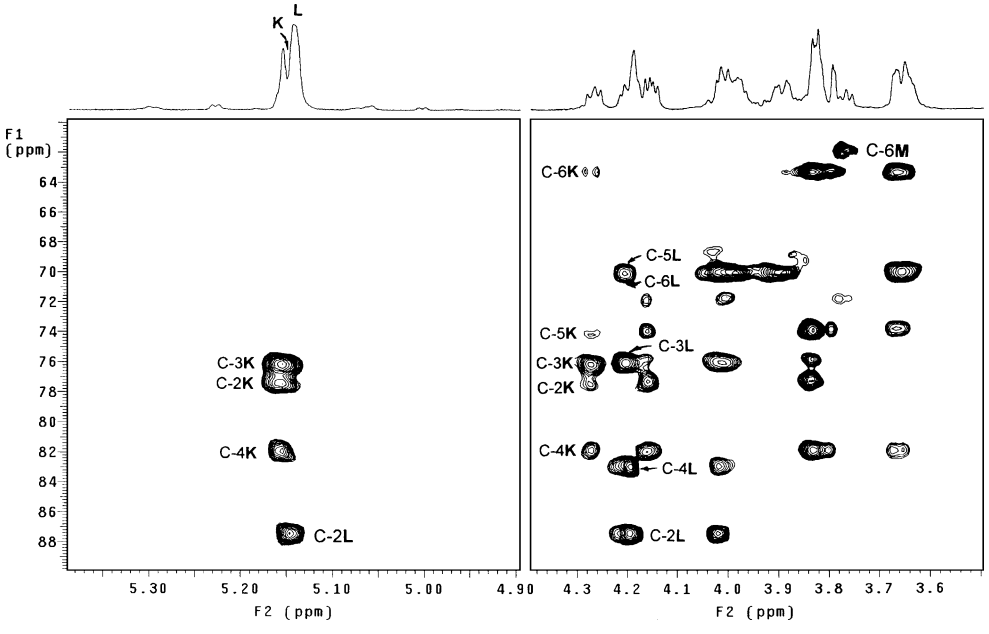


Fig. 3 Partial 2D HSQC-TOCSY spectrum of the F1SS polysaccharide from *A. compressum*. Significant cross peaks have been labelled. *Vertical rows* contain carbons pertaining to the same residue

Table 3 ^{1}H- and ^{13}C-NMR chemical shifts (δ) for the alkali-extractable water-soluble polysaccharide F1SS isolated from *A. compressum*

Units		1	2	3	4	5	6a	6b
K	H	5.15	4.16	4.27	3.84	3.84	3.82	3.67
	C	101.4	77.4	76.3	82.0	73.9	63.4	
L	H	5.14	4.20	4.22	4.02	3.98	3.90	3.66
	C	107.1	*87.6*	76.1	83.0	70.1	*70.0*	
M	H	5.23	3.97				3.78	
	C	97.2	69.3				62.2	

Italicized numbers represent glycosylation sites.

ation results, were also indicative of β-Gal*f*, β-Man*f*, and α-Gal*p* units, respectively [23].

2D homo- (COSY, TOCSY) and hetero-nuclear (HMQC, HSQC-TOCSY, Fig. 3) 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 3).

The analytical results and comparison of the values of proton and carbon NMR data with those of standard compounds [23] led to the conclusion that **K** was terminal β-Man*f*, **L** 2, 6-di-*O*-substituted-β-Gal*f*, and **M** terminal Gal*p*.

To discriminate between the two possibilities of arrangement of the main residues (namely, a chain of Gal*f*-(1→2)–Gal*f* with mannofuranosyl substituents at position 6 of each unit, or else a Gal*f*-(1→6)-Gal*f* chain with Man*f* substituents at positions 2 of each unit), a 2D-NOESY spectrum (mixing time of 250 ms) was carried out which gave, among others, crosspeaks H-1**K**/H-2**L**, H-2**K**/H-3**K**, H-3**K**/H-4**K**, H-1**L**/H-6a**L**′, and H-1**L**/H-6b**L**′. Furthermore, an HMBC experiment (Fig. 4) gave, among others, crosspeaks H-1**K**/C-4**K**, H-1**K**/C-2**L**, H-1**L**/C-4**L**, and H-1**L**/C-6**L**′, where **L**′ is a second molecule of **L**.

The presence of intraresidue crosspeaks H-1/C-4 in **K** and **L** confirm the furanosidic character of both monosaccharides, while the interresidue crosspeaks demonstrate the connection of the units according to the second possibility indicated above. Thus, the main structure of the polysaccharide of *A. compressum* is proposed to be:

$$[\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow]_n \rightarrow \text{Mannan core}$$
$$\begin{matrix} 2 \\ \uparrow \\ 1 \end{matrix}$$
$$\beta\text{-D-Man}f$$

The presence of small amounts of 6-*O*-Gal*f* in the methylation analysis must be due to a few residues without Man*f* attachments. Around 3% of Gal*p* and 6-*O*-substituted-Man*f* were also detected in the methylation analysis. The existence of both residues could indicate the presence of small amounts of fragments of Gal*p* linked to the position 6 of some of the Man*f* units in the polysaccharide of *A. compressum*. A ^{13}C-NMR DEPT experiment showed the existence of two major hydroxymethyl peaks at 63.4 and 70.0 ppm (Man*f* and Gal*f*, respectively) and three minor hydroxymetyl signals at 62.2 (C-6 of Gal*p*), 69.8, and 70.2 ppm (probable due to 6-*O*-substituted-Gal*f* and 6-*O*-substituted Man*f*) which support the above assumptions.

In order to get information on the core, the polysaccharide F1SS was subjected to partial hydrolysis with diluted acid, which selectively removed the furanosidic chains. Around 3.8% of a residual polysaccharidic material, composed exclusively of mannopyranose, was obtained. Methylation analysis of this residual material demonstrated the presence

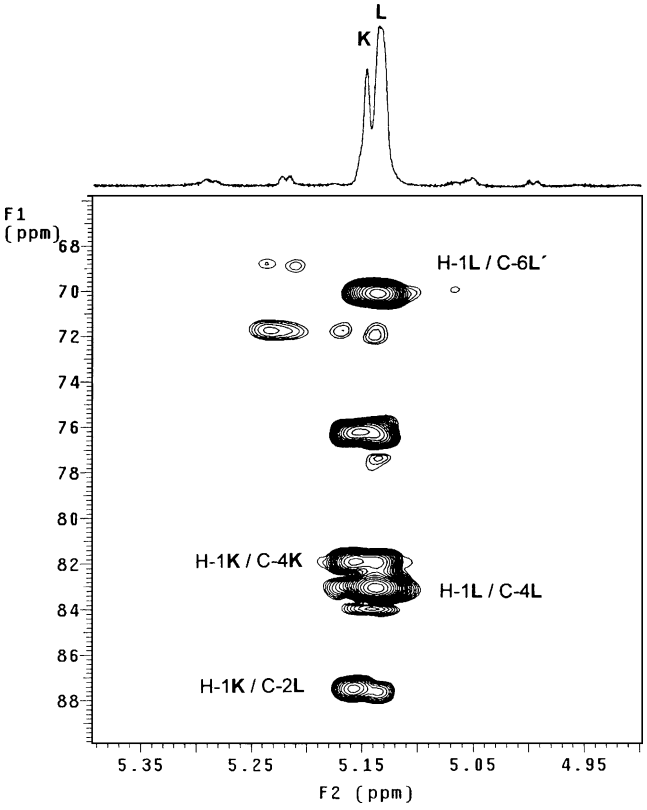


Fig. 4 Partial 2D HMBC spectrum of the cell wall F1SS polysaccharide from *A. compressum*, showing ^{1}H-^{13}C long range connectivities. The anomeric protons and significant cross peaks have been labelled

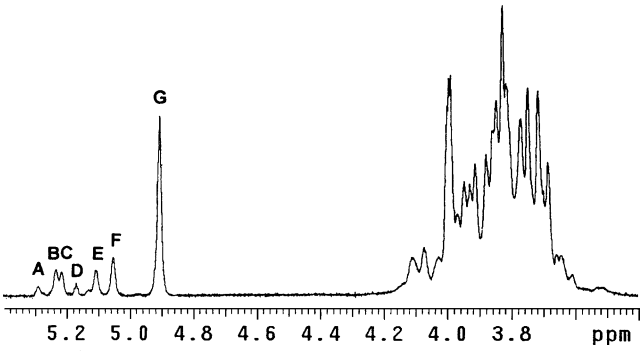
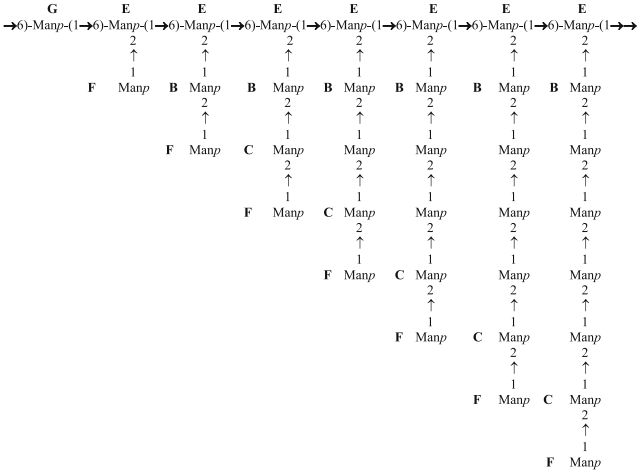


Fig. 5 ^{1}H- (500 MHz) spectrum of the mannan core of the F1SS polysaccharide from *A. compressum*. The anomeric protons have been labelled

of terminal Man*p*, 2-*O*-substituted, 6-*O*-substituted, and 2,6-di-*O*-substituted Man*p*. The ^{1}H-NMR spectrum of this partially hydrolyzed material (Fig. 5) contained in the anomeric region a major signal at 4.91 ppm, and six minor signals between 5.3 and 5.0 ppm, labelled **A–G**, from low to high field. As only four different residues were found in the methylation analysis, it is obvious that some of these anomeric signals correspond to identical residues placed in different neighbourhoods or with different conformational features.

The spectrum was very similar to those of the mannan cores obtained in the partial hydrolysis of several fungal F1SS polysaccharides [21, 24–26]. The constituents of these mannan cores are a backbone of α-(1→6) mannopyranose, substituted at various positions 2 by variable amounts of either a single unit of Man*p* or small chains (two to seven units) of α-(1→2) Man*p* residues. We have reported on the structures deduced for the core of *Chaetosartorya chrysella* and *Aspergillus wentii* [21] which were unambiguously determined by using chemical and NMR analysis. According to the values of the chemical shifts found in the ^{1}H-NMR spectrum of the core of *A. compressum* and, after comparison with that of the mannan core of *A. wentii*, we concluded that **A**–**D** are 2-*O*-substituted Man*p* units, located at different places along the side chains of the core, **E** is the 2,6-di-*O*-substituted Man*p* units forming the backbone of the core, **F**, terminal Man*p*, linked either to C- 2 of some of the backbone residues (**E**) or C-2 of the last units of the side chains, and **G**, 6-*O*-substituted Man*p* residues of the backbone, intercalated among those of **E**. Concerning the location of the 2-*O*-substituted Man*p* residues, a NOESY experiment on the similar mannan core of *C. chrysella* and *A. wentii* [21] led to conclude that **B** is 2-Man*p* linked to the α-(1→6) mannopyranose backbone, **C** is that connected to the terminal position of the side chains, and **A** and **D** are 2-Man*p* located in between **B** and **C**. Integration of the different anomeric protons allowed an estimation of the relative proportion of the residues. Thus, 2-*O*-Man*p*, 2,6-di-*O*-Man*p*, *t*-Man*p*, and 6-*O*-Man*p* amount near 26, 11, 11, and 52%, respectively. A general formula for the mannan core could be represented as follows:



The results of the integration of the proton spectrum above reveals that around 1 out of 11 residues of the (1→6)-mannopyranan skeleton are substituted at position 2 by short chains (one to six residues) of 2-*O*-substituted Man*p*. A carbon-coupled HMQC experiment allowed the measurement of one bond ^{1}H-^{13}C anomeric coupling constants. The values obtained for all of the residues were in the range $^{1}J_{\text{H-1-C-1}}=173\pm0.6$ Hz, which are demonstrative of α-configuration for all [27]. Unfortunately, the possibility of finding the positions that each side chain holds along the mannan backbone is still beyond our reach.

The fact that the major peak **G** was not observed in the intact polysaccharide and appeared after elimination of the long furanosidic chains suggested that those chains were probably connected to C-2 of residues **G**, as have been observed in other fungal polysaccharides [28].

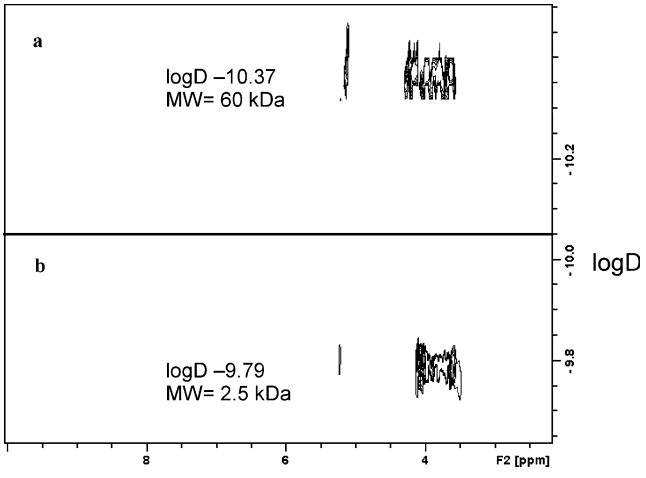


Fig. 6 2D DOSY spectra of: **a** the intact F1SS polysaccharide; and **b** the mannan core polysaccharide. The *x*-axis contains the standard ^{1}H dimension, and the *y*-axis contains the diffusion dimension

In order to ascertain the size of both the intact polysaccharide and the mannan core, and also to exclude the existence of a mixture of both as independent compounds, we carried out diffusion ordered NMR spectroscopy (DOSY) [29] experiments which provide a procedure for molecular size determination through the measurement of diffusion coefficients (log D), and is also useful in resolving chemical mixtures, giving the 1D ^{1}H-NMR spectra and the log D of individual components [30, 31]. The results demonstrated (Fig. 6) that the polydisperse polysaccharide F1SS was not just a physical mixture with the mannan core, and also that the molecular sizes of both polysaccharides were around 60 kDa and 2.5 kDa, respectively.

Galactofuranose residues are well known to form part of fungal polysaccharides. Thus, β-(1→5)-galactofuranans constitute the antigenic determinant of *Aspergillus fumigatus* and other Aspergilli [7, 8, 32]. Chains containing β-(1→6)-galactofuranoses with different degrees of substitution at positions 2 by residues of Glc*p* or Glc*p*A have been found in *Gibberella* and *Nectria* peptidoglycans [1, 2, 33]. From many other fungal genera, polysaccharides containing α- or β-galactofuranoses have been described [11, 34]. Galactofuranoses have also been found in *Leishmania*, *Trypanosoma* and *Mycobacteria* genera [35, 36]. Arabinofuranoses are also known to be constituents of *Mycobacterium*, *Rhodococcus*, and *Nocardia* spp. [37]. On the other hand, although mannofuranosidic fragments, detected in dermatophytes by using chemical degradation [38, 39], have been proposed to be embodied in the main chain of α-(1→6)-mannans, no confirmation of such structures, by employing either chemical means or non destructive additional techniques, have been reported. Instead, chains of α-(1→6)-mannopyranoses, differently substituted by side mannose or galactofuranose residues or by chains of 2-O-substituted mannopyranoses have been described for the polysaccharides isolated from dermatophytes [40, 41].

Thus, to the best of our knowledge, mannofuranose-containing polysaccharides have not been previously reported in fungi. We are now investigating if carbohydrates with similar features are present in the polysaccharides F1SS of related species.

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