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Cell wall polysaccharides isolated from the fungus *Neotestudina rosatii*, one of the etiologic agents of mycetoma in man

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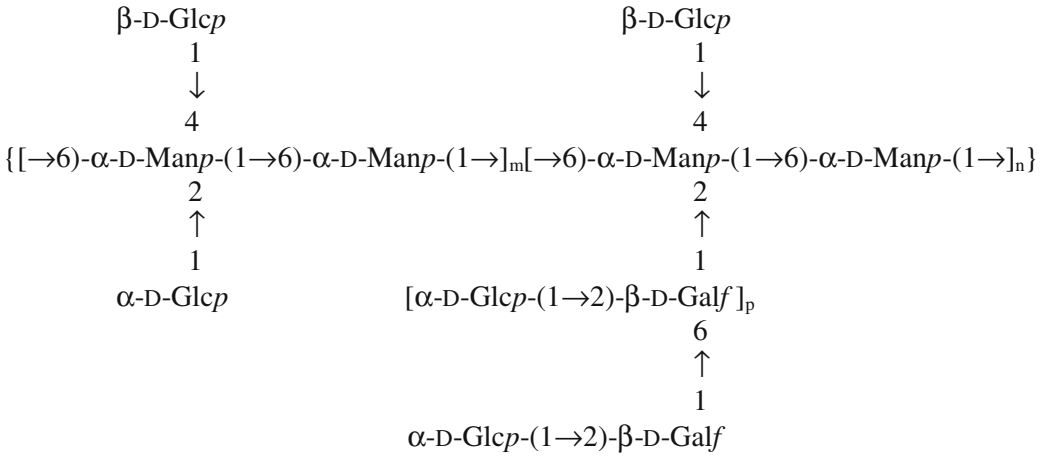
Abstract The alkali-extractable water-soluble polysaccharides F1SS isolated from the cell wall of two isolates of the pathogen *Neotestudina rosatii* and one of *Pseudophaeotrichum sudanense*, which is now considered as a synonym of the former, have been studied by methylation analysis, GC–MS and NMR spectroscopy. The three polysaccharides differ mainly in their content in galactofuranose, and have the following idealized repeating unit:



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with m≈19, and p≈6 in all cases, but being n≈1 for *N. rosatii* CBS 271.75, n≈0.5 for *N. rosatii* CBS 331.78, and n≈0.15 for *P. sudanense*.

Keywords Fungi · Polysaccharides · Mycetoma · NMR spectroscopy

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
SEC	Size exclusion chromatography
TFA	Trifluoroacetic acid
TMSi	Trimethylsilyl
TOCSY	Totally correlated spectroscopy

Introduction

Mycetomas are chronic subcutaneous infections produced by several causal agents (fungi and actinomycetes), who usually penetrate, *via* transcutaneous inoculation, through injuries caused by traumatisms with contaminated splinters, thorns, *etc*. This eventually leads to abscess formation, which may drain onto the skin surface, later involving the muscles and the surrounding bones leading to osteomyelitis [1]. One of the most accepted hypotheses on the etiology of human mycetoma is that infection is due to an unspecific response to the accidental inoculation of certain microorganisms, which mainly live as saprotrophs [2].

Most of the mycetomas are located in the feet and, in order of frequency, ankles, knees and hands. In Sudan people use to carry woods on their head and shoulders, leading to apparition of mycetomas on scalp and nape. The infection does not disseminate to distant organs. However, chronic infection may gradually produce severe deformities and disabilities of the involved region. Administration of antifungal therapy for several years may be required and surgical interventions are frequently needed for correction of the deformities. Most cases of this infection have been described in Africa (Senegal, Somalia, Sudan, *etc*), India and Australia. Typical black-grain mycetomas are caused only by fungi but the etiologic agents are diverse, being the fungus *Neotestudina rosatii* one of them [3].

It is well known that surface components of the fungal cell wall participate in cell-cell interactions, and that preliminary events leading to infection include a close contact of external components, as cell wall glycoproteins, with receptors from the host cell [4–7]. The fungal alkali-extractable water-soluble cell wall polysaccharides (F1SS), are minor components of the fungal cell wall (around 2–8%), and constitute the glycidic moiety of peptido-polysaccharides or glycoproteins [8–10]. These polysaccharides are in close contact with the external environment [11, 12], are antigenically relevant [13–18] and may serve for different biological functions, as its participation in cell–cell and/or cell–host recognition phenomena [7, 19]. Then, a first step in the understanding of the biochemical events occurring during infection should be the determination of the structure of these polysaccharides.

We have studied the F1SS polysaccharides of two isolates of *Neotestudina rosatii* and one of its synonyms, *Pseudophaeotrichum sudanense*. The taxonomic position of these species is uncertain, as occurs with other fungal species causing eumycetoma [2]. We herein report on a novel structure for the polysaccharides isolated from these species.

Materials and methods

Microorganisms and culture media

The strains analysed were *Neotestudina rosatii* CBS 271.75 and CBS 331.78 and *Pseudophaeotrichum sudanense* CBS 512.69. Nowadays this last is considered an obsolete name of *N. rosatii* [20], however, the CBS keeps the name of *P. sudanense* in their files, and we have also done so for identification purposes. 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 were as previously described [21].

Wall material preparation and fractionation

Wall material was obtained as reported elsewhere [22]. Polysaccharides F1SS were obtained according to Ahrazem *et al*. [23].

Size exclusion chromatography

Fraction F1SS (100 mg) was dissolved in 1.5 mL of water and centrifuged at 13,000×*g* for 15 min to eliminate insoluble material. The supernatant was added to a column (90×2.6 cm) of Sepharose CL-6B and eluted with water at a flow of 22 mL h^{-1}. Fractions (3.2 mL) were collected and monitored for carbohydrate by the phenol sulfuric acid method [24]. The fractions that tested positive for

Table 1 Percentages of neutral sugars released from polysaccharides F1SS after acid hydrolysis

Strain	Microorganism	Mannose	Galactose	Glucose
CBS 331.78	*N. rosatii*	46.4	3.8	49.7
CBS 271.75	*N. rosatii*	44.0	6.8	50.2
CBS 512.69	*P. sudanense*	48.7	1.4	49.9

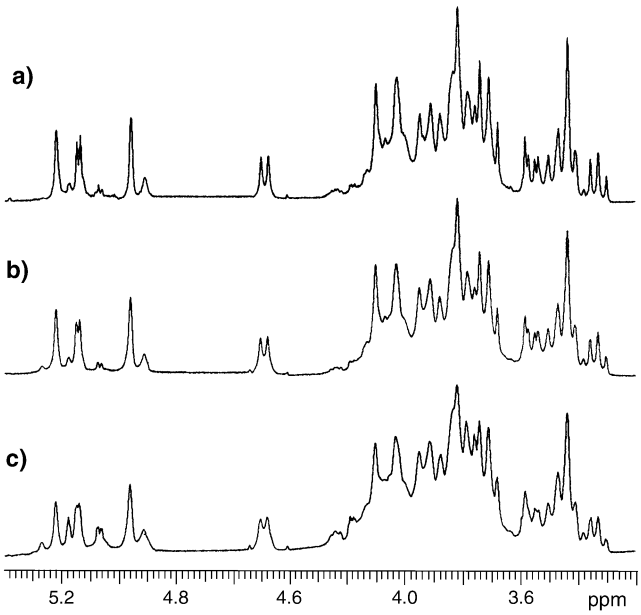


Fig 1 ^{1}H-NMR spectra (D_2O, 35°C, 300 MHz) of the F1SS polysaccharides of *a P. sudanense* (CBS 512.69); *b N. rosatii* (CBS 331.78), and *c N. rosatii* (CBS 271.75)

carbohydrates were combined, concentrated to a small volume, and freeze-dried. The column was previously calibrated with a mixture of standards: T500, T70 and T10 dextrans (Pharmacia).

Chemical analysis

For analysis of neutral sugars the polysaccharides were hydrolyzed with TFA 0.15 M (121°C, 1 h) to release furanoses, the products reduced with $NaBH_4$ and acetylated. In order to get a complete depolymerization of the polysaccharides, the samples were hydrolyzed sequentially two more times, with 3 and 5 M TFA, respectively, reduced with $NaBH_4$ and acetylated. The resulting monosaccharides were converted into their corresponding alditol acetates [25] and identified and quantified by gas–liquid chromatography (GLC) using a 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 according to Gerwig *et al.* [26] and their absolute configuration determined by GC–MS of the tetra-*O*-TMSi-(+)-2-butylglycosides obtained.

Methylation analysis

The polysaccharides (1–5 mg) were methylated according to the method of Ciucanu and Kerek [27]. The partially methylated polysaccharides were sequentially hydrolyzed with TFA 0.15, 3 and 5 M TFA (121°C, 1 h) as described above, but the products were reduced with $NaBD_4$, acetylated and analyzed by GC–MS according to Ahrazem *et al*. [23].

NMR spectroscopy

Routine ^{1}H-NMR spectra were recorded at 35° on a Varian Avance 300 spectrometer. 1D- and 2D-^{1}H- and ^{13}C-NMR experiments were carried out at 35°C on a Varian Unity 500 spectrometer with a reverse probe and a gradient unit. Proton

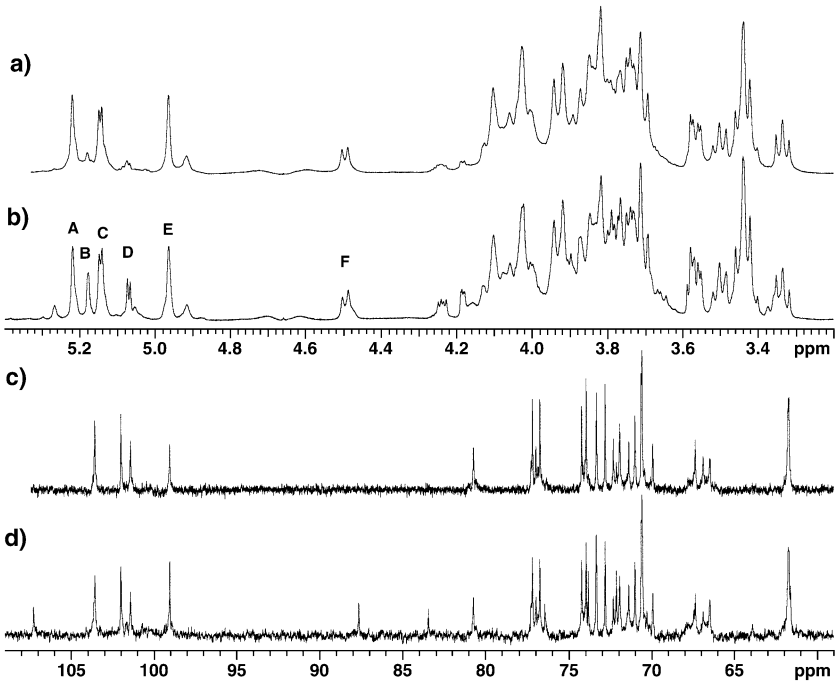


Fig 2 ^{1}H- and ^{13}C-NMR spectra (D_2O, 35°C, H/C: 500/125 MHz) of the F1SS polysaccharides of *a* and *c*: *P. sudanense* (CBS 512.69) and *b* and *d*: *N. rosatii* (CBS 271.75). Anomeric protons have been labelled

chemical shifts refer to residual HDO at δ 4.66 ppm. Carbon chemical shifts refer to internal acetone at δ 31.07 ppm. The polysaccharide F1SS (*ca*. 20 mg) was 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). 2D-NMR experiments (DGF-COSY, TOCSY, NOESY, HMQC, HSQC-TOCSY and HMBC) were performed by using the standard Varian software. The standard Bruker DOSY protocol was used at 298 K on an Avance 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

Polysaccharides F1SS amounted around 3% of the cell wall in the three isolates. They were purified by SEC, giving a single polydisperse peak.

Acid hydrolysis revealed the presence of similar amounts of glucose (49.7–50.2%) and mannose (46–50%) and a small percentage of galactose (1.5–6.8%) in the three strains (Table 1). The absolute configuration analysis showed D-configuration for all three sugars. Methylation analysis gave partially methylated alditol acetates corresponding to terminal Glc*p*, 6-*O*-substituted and 2,4,6-tri-*O*-substituted Man*p*, and 2,6-di-*O*-substituted Gal*f*, although the amount of this last residue in *P. sudanense* was at the traces level.

^{1}H-NMR spectra of the polysaccharides F1SS from the three isolates are shown in Fig. 1. All of them have very similar spectra, with four major peaks and two minor additional signals (at 5.18 and 5.07 ppm), whose relative intensities vary among the three species.

The high resolution ^{1}H-NMR spectrum of the polysaccharide from *N. rosatii* contained, *inter alia*, six main anomeric signals, which were labeled **A**–**F** in order of increasing field (Fig. 2b), with integrated areas 2.2:1:2.2:1:2.3:2.2. The proton spectrum of the polysaccharide from *P. sudanense* contained the same signals, but the intensities of **B** and **D** were almost negligible (Fig. 2a). In order to assign the four main signals of the spectra, we carried out further studies on the polysaccharide from *P. sudanense*, since its spectrum was obviously less complex. 2D shift correlation spectroscopy, *i.e.* DQF-COSY and TOCSY experiments, allowed the assignment of most of the main signals of the proton spectrum. The ^{13}C-NMR spectrum (Fig. 2c) showed four singlets in the anomeric region.

A HMQC experiment, which also exhibited four cross peaks in the anomeric region and twenty cross peaks in the ring zone gave the direct connectivities between the protons of residues **A**, **C**, **E**, and **F** and their corresponding carbons (Fig. 3a) and, together with a HSQC-TOCSY experiment (not shown), which gave chains of carbons pertaining to

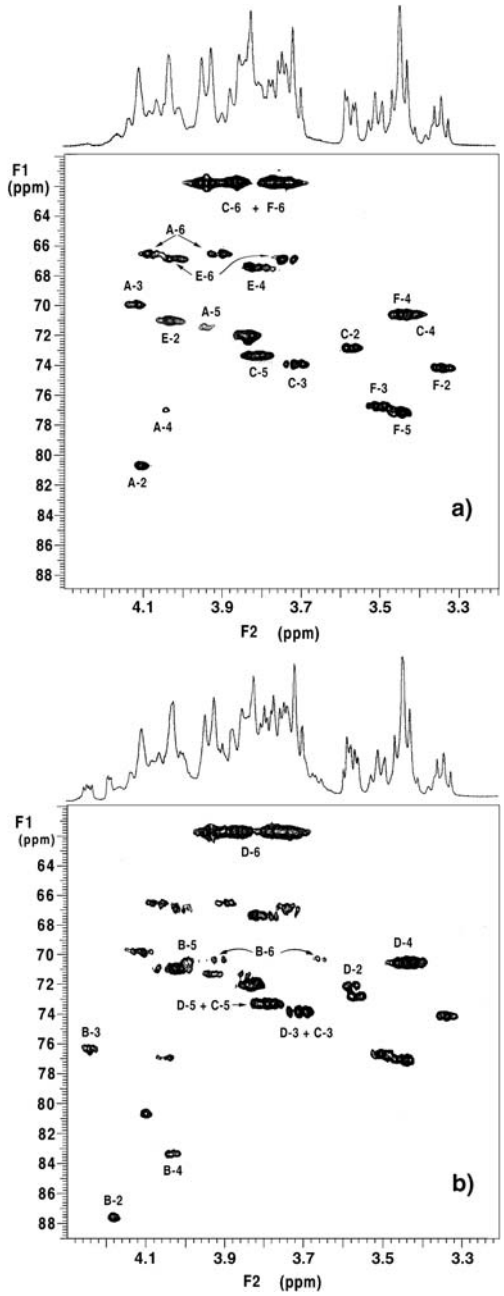


Fig 3 Selected region of the 2D-HMQC spectra of the F1SS polysaccharide of **a** *P. sudanense* and **b** *N. rosatii* (CBS 271.75). Significant crosspeaks have been labelled

Table 2 ^{1}H- and ^{13}C-NMR chemical shifts (δ) for the alkali-extractable water-soluble cell-wall polysaccharide F1SS isolated from *P. sudanense*

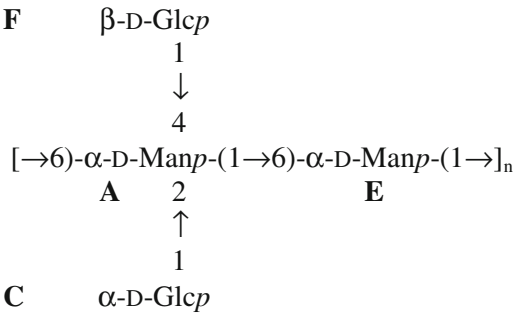
Residue		1	2	3	4	5	6a	6b
A	H	5.22	4.10	4.12	4.05	3.94	4.09	3.91
	C	99.0	*80.7*	69.9	*77.0*	71.4	*66.5*	
C	H	5.14	3.56	3.71	3.44	3.80		
	C	102.0	72.8	74.0	70.6	73.3	61.7	
E	H	4.96	4.03	3.83	3.83	3.85	4.02	3.74
	C	101.4	71.0	71.9	67.4	71.4	*66.9*	
F	H	4.49	3.33	3.50	3.44	3.45	3.93	3.74
	C	103.6	74.2	76.7	70.3	77.2	61.8	

Italicized numbers represent glycosylation sites

each different unit, allowed the assignment of all the proton and carbon chemical shifts of the four residues.

The values deduced have been gathered in Table 2. Comparison of these values with those of reference analogous compounds [28, 29] revealed that **A** was a 2,4,6-tri-*O*-substituted Man*p*, **C** and **F**, terminal Glc*p*, and **E**, a 6-*O*-substituted Man*p* moiety.

Concerning the configuration of the different units, the anomeric proton coupling constants of units **C** (3.7 Hz) and **F** (7.5 Hz) demonstrated α- and β-configurations for them, respectively. Furthermore, a carbon coupled HMQC experiment gave $^{1}J_{\text{C-1, H-1}} \approx 176$ Hz for the mannopyranose units, which demonstrated α-configuration for residues **A** and **E** [30]. With regard to connections of the different residues, they were unequivocally deduced from an HMBC experiment, which showed cross peaks H-1**A**/C-6**E**, H-1**C**/C-2**A**, H-1**E**/C-6**A**, and H-1**F**/C-4**A**. Then, the major residues of the polysaccharide F1SS of *P. sudanense* can be arranged according to the following idealized structure:



In order to ascertain the size of the most complex polysaccharide from *N. rosatii* (Fig. 2b) and also to exclude the possible existence of a mixture of independent compounds, we carried out diffusion ordered NMR spectroscopy (DOSY) [31] 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 [32, 33]. The results demonstrated (Fig. 4) that the polydisperse polysaccharide F1SS was not just a physical mixture, and also that its molecular size was around 60 kDa. This value is in good agreement with the data obtained by SEC analysis of the three polysaccharides.

The characterization of residues **B** and **D** was studied in the polysaccharide F1SS from *N. rosatii* CBS 271.75, which displayed signals with good intensity both in the ^{1}H-NMR and ^{13}C-NMR spectra (Fig. 2b and d). The ^{13}C-NMR spectrum (Fig. 2d) showed five singlets in the anomeric region but, in the 2D HMQC spectrum, six cross peaks appeared in the anomeric zone, two of them (labelled **A** and **D**) at identical carbon chemical shift. The use of 2D DQF-COSY, TOCSY, HSQC-TOCSY, and HMQC experiments showed, in addition to the cross peaks observed in the case of the polysaccharide of *P. sudanense* above described, a set of new signals (Fig. 3b), corresponding to the new residues, and allowed the assignment of most of the carbons and protons directly linked (Table 3). Comparison of the values of the chemical shifts deduced with those of reference compounds [34, 35] revealed, in addition to the four units described above, the presence of 2,6-di-*O*-substituted galactofuranose (**B**) and terminal glucopyranose (**D**). The anomeric carbon of **B** appeared at low field (107.3 ppm). Together with its anomeric proton coupling constant (<2 Hz), this demonstrated β configuration for **B** [34, 36]. The anomeric proton coupling constant for **D** (3.8 Hz) revealed α configuration for it. As it shows different proton and carbon chemical shifts than **C**, we may deduce that **D** must be in a different neighbourhood than **C**, and that it is probably in connection with **B**.

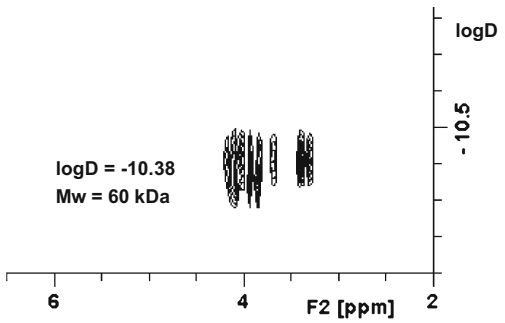


Fig 4 DOSY spectrum of the F1SS polysaccharide of *N. rosatii* (CBS 271.75). The *x*-axis contains the standard ^{1}H dimension, and the *y*-axis contains the diffusion dimension

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

Residue		1	2	3	4	5	6a	6b
A	H	5.22	4.10	4.12	4.05	3.94	4.09	3.91
	C	99.0	*80.7*	69.9	*77.0*	71.4	*66.5*	
B	H	5.18	4.18	4.24	4.03	*ca.* 4.0	3.91	3.65
	C	107.3	*87.6*	76.5	83.5	70.5	*70.3*	
C	H	5.14	3.56	3.71	3.44	3.80		
	C	102.0	72.8	74.0	70.6	73.3	61.7	
D	H	5.07	3.57	3.70	*ca.* 3.44	*ca.* 3.78	*ca.* 3.85	3.74
	C	99.0	72.1	74.0	70.6	73.3	61.7	
E	H	4.96	4.03	3.83	3.83	3.85	4.02	3.74
	C	101.4	71.0	71.9	67.4	71.4	*66.9*	
F	H	4.49	3.33	3.50	3.44	3.45	3.93	3.74
	C	103.6	74.2	76.7	70.3	77.2	61.8	

Italicized numbers represent glycosylation sites

With regard to the geometry and arrangement of the different residues, they were unequivocally deduced from an HMBC experiment (Fig. 5), which showed cross peaks H-1**A**/C-5**A**, H-1**B**/C-4**B**, H-1**C**/C-5**C**, H-1**D**/C-5**D**, and H-1**E**/C-5**E**, revealing both the furanoid character of **B** and the pyranoid structure of **A**, **C**, **D**, and **E**, and also cross peaks H-1**A**/C-6**E**, H-1**D**/C-2**B**, H-1**C**/C-2**A**, H-1**B**/C-6**B′** (being **B′** a second unit of **B)**, H-1**E**/C-6**A**, and H-1**F**/C-4**A**. These results demonstrated the connections of the residues into an array constituted of a main chain of α-(1→6) mannopyranose, in which a linear residue alternates with a branched unit, substituted at positions C-2 by α-Glc*p* and at C-4 by β-Glc*p*. In addition, there exists a small size comb-like chain of β-galactofuranoside, substituted at positions C-2 by units of α-Glc*p*, and linked at C-2 of some of the 2,4,6-tri-*O*-substituted Man*p* residues.

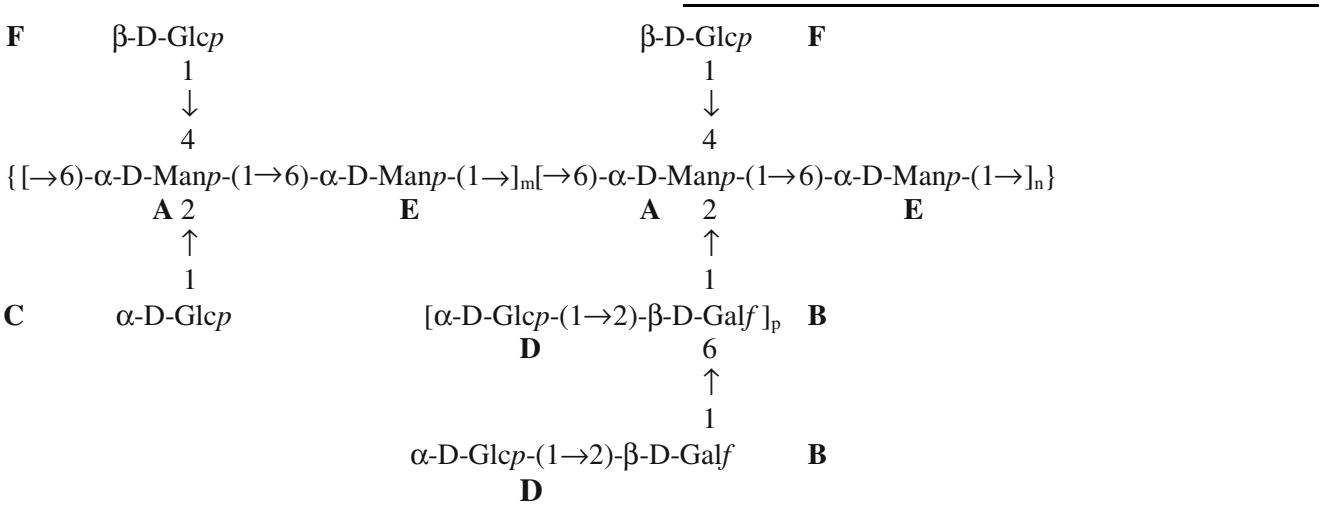
From all the combined data, the idealized structure of the polysaccharide F1SS from *N. rosatii* was deduced to be:

The proportion of the different residues, deduced from integration of the proton spectra and chemical analysis, allowed to deduce that m≈19, and p≈6 in all cases, but being n≈1 for *N. rosatii* CBS 271.75, n≈0.5 for *N. rosatii* CBS 331.78, and n≈0.15 for *P. sudanense*.

In addition, three minor anomeric signals may be seen in the ^{1}H-NMR spectra at 5.26, 5.05 and 4.91 ppm. In our experience, they are usually due to a small mannan core, which appears in most of the spectra of F1SS fungal polysaccharides [37–39]. The signals correspond to 2-substituted-α-Man*p*, terminal α-Man*p*, and residues of 6-*O*-substituted α-Man*p* linked to a second unit of 6-*O*-substituted α-Man*p*, respectively.

Glycosylation is a highly conserved process [40] but gives raise to a big diversity of glycan structures. This data show how these three organisms display a single common general pattern in the structure of this polymer, which can be used as a chemotaxonomic marker for this group even though each individual isolate has particularities concerning the branching degree and the amount of galactofuranose chains.

The specific role of most of these carbohydrates attached to the outer surface of the fungal cells has not been yet documented. It is well known that fungal cell walls, and



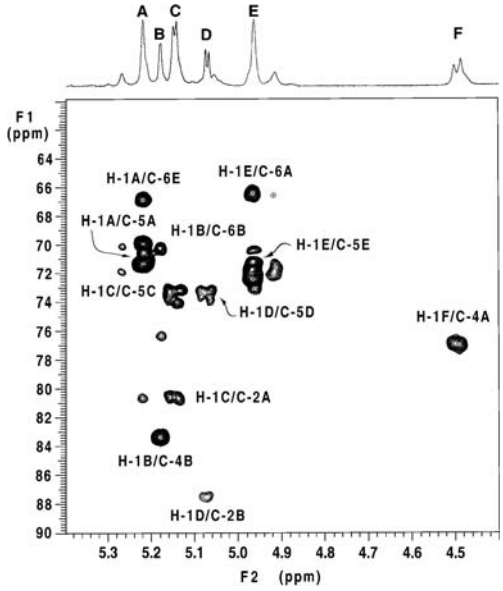


Fig 5 Selected region of the 2D-HMBC spectrum of the F1SS polysaccharide of *N. rosatii* (CBS 271.75). Significant crosspeaks have been labelled

more specifically their glycoconjugates and polysaccharides, are implicated in the regulation of the primary events of contact between the parasite and the host [4, 41]. As an example, it has been described that during the recognition step previous to infection, a lectin of the fungus *Agaricus bisporus* recognizes and binds to a surface glucogalactomannan from the mycopathogen *Verticilllium fungicola* [4]. Concerning to human pathogens, the relevance of fungal cell wall glycans has been put forward, as they make first contact with the immune system. It has recently been demonstrated that *N*-linked and *O*-linked mannosyl groups of glycoproteins and β-glucans of the outer surface of *Candida albicans* cell wall cooperate in the activation of the innate immune response, and that recognition of these components is a multilevel process [42]. Highly specific antibodies have been raised against some of these molecules, which have been used for staining by immunofluorescence techniques, to show mycelia containing the antigens. Further studies, both structural and biochemical, will be necessary to enlighten the role of cell wall carbohydrates and/or glycoconjugates in fungal infection.

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