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Structural studies of fungal cell-wall polysaccharides from two strains of *Talaromyces flavus*

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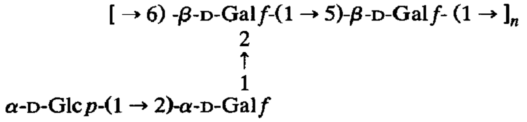
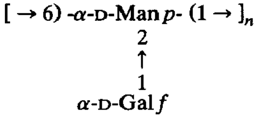
ABSTRACT

The water-soluble cell-wall polysaccharides isolated from strains CBS 352.72 and 310.38 of *Talaromyces flavus* have been investigated by chemical analyses and NMR studies. Two different skeletons coexist, having the structures:



$$\begin{array}{l} [\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow]_n \\ \qquad\qquad\qquad 2 \\ \qquad\qquad\qquad \uparrow \\ \qquad\qquad\qquad 1 \\ \alpha\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Gal}f \end{array}$$

and



$$\begin{array}{l} [\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow]_n \\ \qquad\quad 2 \\ \qquad\quad \uparrow \\ \qquad\quad 1 \\ \quad \alpha\text{-D-Gal}f \end{array}$$

The small differences between the polysaccharides isolated from both strains are probably due to slight diminution of branching in strain 352.72, as compared with strain 310.38.

INTRODUCTION

The fungal cell wall is mainly built up of polysaccharides. Some of them, such as the α- and β-(1 → 3)-glucans or the β-glucan–chitin complex, are widely distributed and can be found in species of different genera, but it is increasingly clear that other polysaccharides appearing in minor amounts could be characteristic of a limited group of fungi such as the genus. Wall polysaccharides have been used for identification of yeasts, and they may be useful in the classification of filamentous fungi[1]. Previous studies have shown that polysaccharides isolated from an alkali- and water-soluble fraction (F1S) are especially interesting in this context. A β-(1 → 5)-galactofuranan has been purified from fifteen species of *Eupenicillium*[2] and from *E. crustaceum*, the type species of the genus[3]. In *Talaromyces helicus*, a

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polysaccharide rich in galactofuranose (1 → 2)- and (1 → 5)-linked has been described[4]. In the genus *Penicillium*, which comprises species having teleomorphs in the former genera, both types of polymers have been found[5,6], in agreement with the existing relationship among them. In *Aphanoascus*, both an α-(1 → 2)-(1 → 6)-mannan having a comb-like structure and an α-(1 → 4)-glucan purified from fraction F1S have been proposed as chemotaxonomic markers[7].

The cell-wall composition of *Talaromyces flavus*, the type species of the genus, has been examined[8], comparing the neutral sugars and linkage types of its alkali-soluble fraction with the same fraction from *E. crustaceum*. A more detailed investigation of these polymers seemed worthwhile in order to find a polysaccharide characteristic of the genus.

The present study describes the structural features of a polysaccharide, obtained from fraction F1S of two strains of *T. flavus*, by means of NMR and methylation analysis.

MATERIALS AND METHODS

Microorganisms and culture media.—Two isolates of *T. flavus* (Klöcker) Stolk & Samson (CBS 352.72 and 310.38) were used for this study. The organisms were maintained on slants of Bacto potato-dextrose agar. The basal medium and culture conditions for mycelium production have been described[8].

Wall material preparation and fractionation.—Cell walls were prepared from 5-day-old mycelium as previously described[9]. Wall material (6 g) was treated with 1 M NaOH at 25°C for 20 h followed by centrifugation, and the precipitate washed with 1 M NaOH and centrifuged again. The two supernatant solutions were pooled, precipitated with an equal volume of EtOH, and dialysed against running tap water. Centrifugation of the dialysed material gave a supernatant solution (alkali- and water-soluble) and a precipitate (alkali-soluble and water-insoluble). Both fractions were lyophilised to give F1S and F1I, respectively.

Purification of polysaccharides from F1S.—Fractions F1S were filtered through a column (40 × 2.6 cm) of Sepharose CL-6B with 0.3 M NaOH as eluent. Eluates were collected and monitored for carbohydrate content by the phenol–H_2SO_4 method[10], and the appropriate fractions were pooled, dialysed, and lyophilised, giving a small fraction F1S-A and a main one F1S-B.

Chemical analysis.—Neutral sugars were released by hydrolysis with 2 or 0.05 M H_2SO_4 at 100°C for 5 h and then converted into the corresponding alditol acetates[11]. Identification and quantification were carried out by gas–liquid chromatography (GLC) using 3% SP-2340 on 100–120 Supelcoport[12]. The absolute configuration of the neutral sugars was established by reported methodology[4].

Methylation analyses were performed according to a modification[13] of the Hakomori method[14]. The permethylated polysaccharides, which showed negligible IR absorption for hydroxyl groups, were analysed both by hydrolysis[9] and the reductive-cleavage method. This was carried out in two steps, with trimethylsilyl triflate and catalyst[15], but the reactions were effected under N_2 and the time during the reductive-cleavage step was shortened to 5 h, to minimise unwanted byproducts.

NMR analysis.—NMR spectra for fraction F1S-B were recorded at 40°C on a Varian Unity 500 spectrometer. Proton chemical shifts were referenced to residual HDO at δ 4.61 ppm. Carbon chemical shifts were referenced to external dioxane at 67.4 ppm.

The double quantum filtered DQF-COSY experiment was performed in the phase sensitive mode using the method of States et al.[16]. A data matrix of $256 \times 2K$ points was used to digitise a spectral width of 1500 Hz; 16 scans were used per increment with a relaxation delay of 2 s. The 90° pulse width was 7.5 μs. Prior to Fourier transformation, zero-filling was used in F1 to expand the data to $1K \times 2K$.

The triple quantum filtered TQF-COSY experiment was collected in the phase sensitive mode. A data matrix of $256 \times 1K$ points was used to digitise a spectral width of 1500 Hz; 24 scans were used per increment with a relaxation delay of 2 s. Zero-filling was used to expand the data to $1K \times 2K$.

The clean 2D-TOCSY experiment[17] was carried out in the phase sensitive mode using MLEV-17 for isotropic mixing. The mixing time was set to 150 ms. A data matrix of $256 \times 1K$ points was used to digitise a spectral width of 1500 Hz; 16 scans were used per increment with a relaxation delay of 2 s. The 90° pulse width during the mixing period was 22.5 μs. Squared cosine-bell functions were applied in both dimensions and zero-filling was used to expand the data to $2K \times 2K$.

The 2D rotating frame NOE (ROESY, CAMELSPIN)[18] experiment was recorded in the phase sensitive mode. The spin-lock period consisted of a train of 30° pulses (2.5 μs), separated by delays of 50 μs. The total mixing time was set at 300 ms. The rf carrier was set at δ 6.0 ppm, 325 Hz downfield from the most deshielded anomeric proton to minimise spurious Hartmann–Hahn effects. A data matrix of $256 \times 2K$ points was used to resolve a spectral width of 2500 Hz; 16 scans were used per increment with a relaxation delay of 2 s. Prior to Fourier transformation, squared sine-bell functions shifted by $\pi/3$ were applied in both dimensions and zero-filling was used in F1 to expand the data to $2K \times 2K$.

The pure absorption 2D NOESY experiments were carried out with mixing times of 200 and 300 ms. A data matrix of $256 \times 2K$ points was used to resolve a spectral width of 1500 Hz; 16 scans were used per increment with a relaxation delay of 2 s. Prior to Fourier transformation, squared sine-bell functions shifted by $\pi/3$ were applied in both dimensions and zero-filling was used in F1 to expand the data to $2K \times 2K$.

The pure absorption one-bond proton–carbon correlation experiment was collected in the ^{1}H-detection mode using the HMQC pulse sequence[19] and a reverse probe. A data matrix of $256 \times 2K$ points was used to resolve spectral widths of 8000 and 1500 Hz in F1 and F2; 16 scans were used per increment with a relaxation delay of 2 s and a delay corresponding to a J value of 145 Hz. A BIRD-pulse was used to minimise the proton signals bonded to ^{12}C. ^{13}C-decoupling was achieved by the WALTZ scheme. Prior to Fourier transformation, squared cosine-bell functions were applied in both dimensions and zero-filling was used in F1 to expand the data to 2K × 2K.

The multiple-bond proton–carbon correlation experiment was carried out in the ^{1}H-detection mode using the HMBC pulse sequence[20] and a reverse probe. A data matrix of 256 × 2K points was used to resolve spectral widths of 8000 and 1500 Hz in F1 and F2; 64 scans were used per increment with a relaxation delay of 2 s and a pulse delay of 60 ms. The processing was performed in the absolute mode after zero-filling in F1 to a 2K × 2K data matrix.

RESULTS AND DISCUSSION

Acid hydrolysates of the polymers from strains CBS 352.72 and 310.38 contained D-galactose, D-glucose, and D-mannose in the relative proportions 63 : 22 : 15 and 52 : 36 : 11, respectively.

The usual methylation analyses and the reductive cleavage procedure gave similar results. The products obtained by the last method are gathered at Table I.

A comparison between the anomeric region of the ^{1}H NMR spectra of 40°C of fractions F1S-B from both strains is given in Fig. 1. As deduced from both spectral and analytical data, the simplest structure corresponds to strain 310.38, so structural studies were carried out on this one.

The high-resolution ^{1}H NMR spectrum and proton-decoupled ^{13}C NMR spectra of F1S-B in D_2O solution (Fig. 2) showed four anomeric signals with the same relative intensity, plus two signals with half relative intensity. The resolution of the ^{1}H NMR spectrum at 40°C was good enough to allow DQF-COSY, TQF-COSY, and TOCSY experiments to be performed in a satisfactory way. Almost all of the resonances in the proton spectrum were assigned by combining these techniques. Thus, TQF-COSY (Fig. 3) permitted assignment of H-5 and the methylene protons of the different residues. Coherence transfer to all the protons of the constituent

TABLE I

Reductive cleavage analyses of F1S-B from *T. flavus* strains

Methylated sugar [a]	Molar ratios	
	CBS 352.72	CBS 310.38
2,3,4,6-Me-1,5-Glcol	1.2	1.2
2,3,5,6-Me-1,4-Galol	0.3	0.6
3,5,6-Me-1,4-Galol	1.0	1.1
2,3,6-Me-1,4-Galol	1.0	1.1
2,3,5-Me-1,4-Galol	0.1	tr. [b]
2,3,4-Me-1,5-Manol	0.1	tr.
3,5-Me-1,4-Galol	1.2	1.0
3,4-Me-1,5-Manol	0.3	0.5

[a] 2,3,4,6-Me-1,5-Glcol, 2,3,4,6-tetra-*O*-methyl-1,5-anhydroglucitol; 3,5,6-Me-1,4-Galol, 2-*O*-acetyl-3,5,6-tri-*O*-methyl-1,4-anhydrogalactitol, etc. [b] tr., Trace.

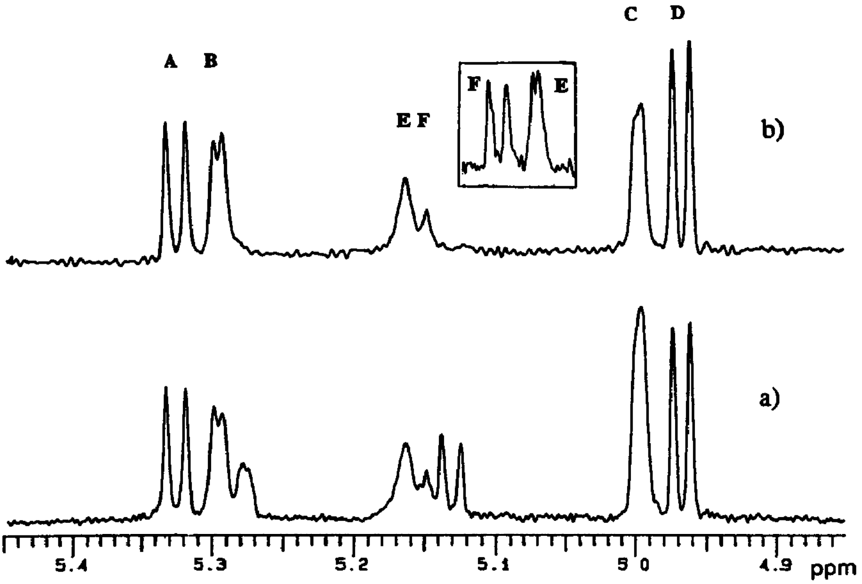


Fig. 1. Anomeric region of the ^{1}H NMR spectra (300 MHz, D_2O, 40°C) of the cell-wall polysaccharides isolated from *T. flavus*: (a) strain CBS 352.72; (b) strain CBS 310.38. Inset: central anomeric signals of the latter, registered at 75°C, showing the separation of anomeric protons of residues **E** and **F** (see text).

monosaccharides was achieved by isotropic mixing using 2D-TOCSY (Fig. 4a) with a mixing time of 150 ms. The assignment of each cross-peak was based also on the information obtained from the DQF- and TQF-COSY experiments. The proton chemical shifts are listed in Table I. The six residues were labelled **A**, **B**, **C**, **D**, **E**, and **F** according to their anomeric protons. The signals corresponding to residue **D** readily identified it as an α-glucose residue, once combined with the information deduced from the observed coupling constants. Despite the small values of $J_{1,2}$ for the other residues, the TOCSY subspectra through the anomeric signals showed clear connectivities to the rest of the protons. The fairly large values of J-couplings associated to H-3 of residue **A** (8.3 and 7.0 Hz) suggested the presence of a glucopyranose residue. However, according to the compositional analysis, this conclusion had to be discarded, since the glucose residue was identified as **D**. The chemical shift values of H-2, H-3, and H-4 were very similar $\Delta\delta_{\max} < 0.2$ ppm, and appeared at relatively low field, $\delta > 4$ ppm, indicating the presence of galactofuranoses. Also, galactose **A** was thought to be in the furanose form, since $J_{3,4}$ values for galactopyranoses are much smaller than that observed.

At this point, no sequence information was deduced and, also, the anomeric configurations of the Gal linkages remained to be determined.

Some information on the glycosyl sequence was obtained from the 2D-CAMELSPIN (ROESY) experiment (Fig. 4b). Although the spurious Hartmann–Hahn effects in ROESY spectra of oligosaccharides can never be completely discarded[21], the existence of cross-peaks between signals of different residues

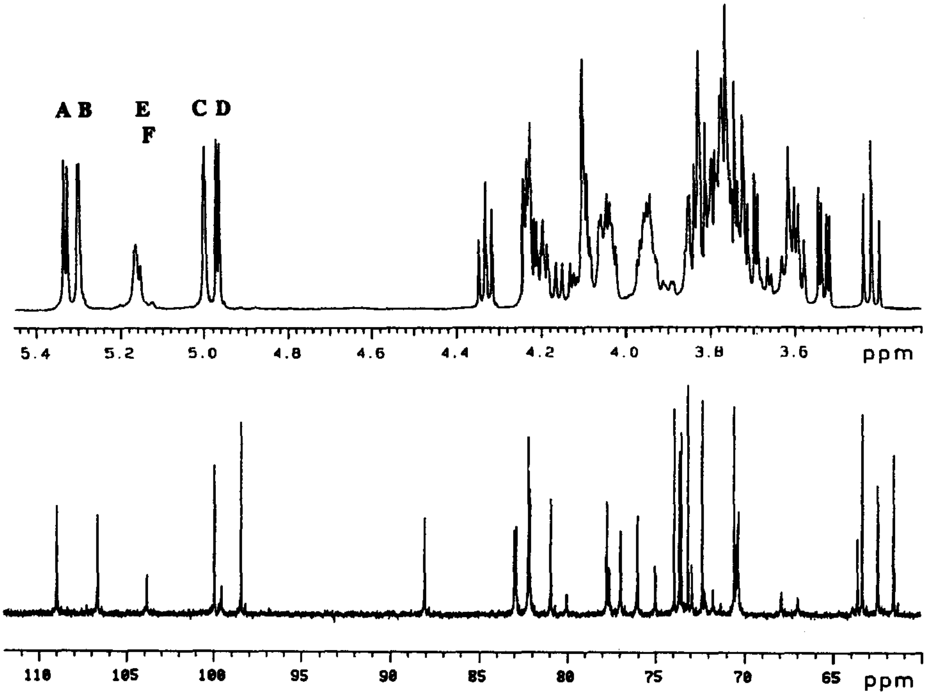


Fig. 2. ^{1}H and ^{13}C NMR spectra (500 and 125 MHz, respectively; D_2O, 40°C) of the polysaccharide F1S-B from *T. flavus*. Anomeric signals are labelled A–D.

indicates their proximity in space and, probably, their connection. Thus, cross-peaks between the anomeric proton of the α-glucopyranosyl residue **D** and H-2 and H-3 of residue **A** could be observed, along with others connecting H-1 of residue **A** with H-2 of unit **B**, H-1 of galactofuranose **B** with H-5 and H-6 of residue **C**, and H-1 of unit **C** with H-6a and H-6b of **B**. Since NOE cross-peaks are dependent on the conformation around the glycosidic bonds, the existence of these cross-peaks does not guarantee the knowledge of the exact position of the linkage although, at this stage, it can be assumed that α-Glc*p* **D** is (1 → 2)- or (1 → 3)-linked to Gal*f* **B**, which is connected via a (1 → 5) or (1 → 6) linkage to Gal*f* **C**, which is in turn (1 → 5)- or (1 → 6)-linked to Gal*f* **B**.

It was possible to discriminate among some of these alternatives and to ascertain all the configurations of the residues by using an HMQC experiment, which maps the connectivities between carbon atoms and their directly bonded protons. The assignment of most of the signals in the ^{13}C NMR spectrum was straightforward, since almost all the ^{1}H NMR chemical shifts were already known. Carbon chemical shifts are gathered in Table II. The chemical shifts of the anomeric carbons indicate that **A** is an α-Gal*f* residue, while **B** and **C** are β-Gal*f* residues. Moreover, C-2 and C-6 of Gal*f* **B** appeared clearly deshielded, as expected for substitution at those positions; therefore, **B** is a 2,6-di-*O*-substituted β-Gal*f* residue. The relatively low field observed for C-5 of residue **C**, and the

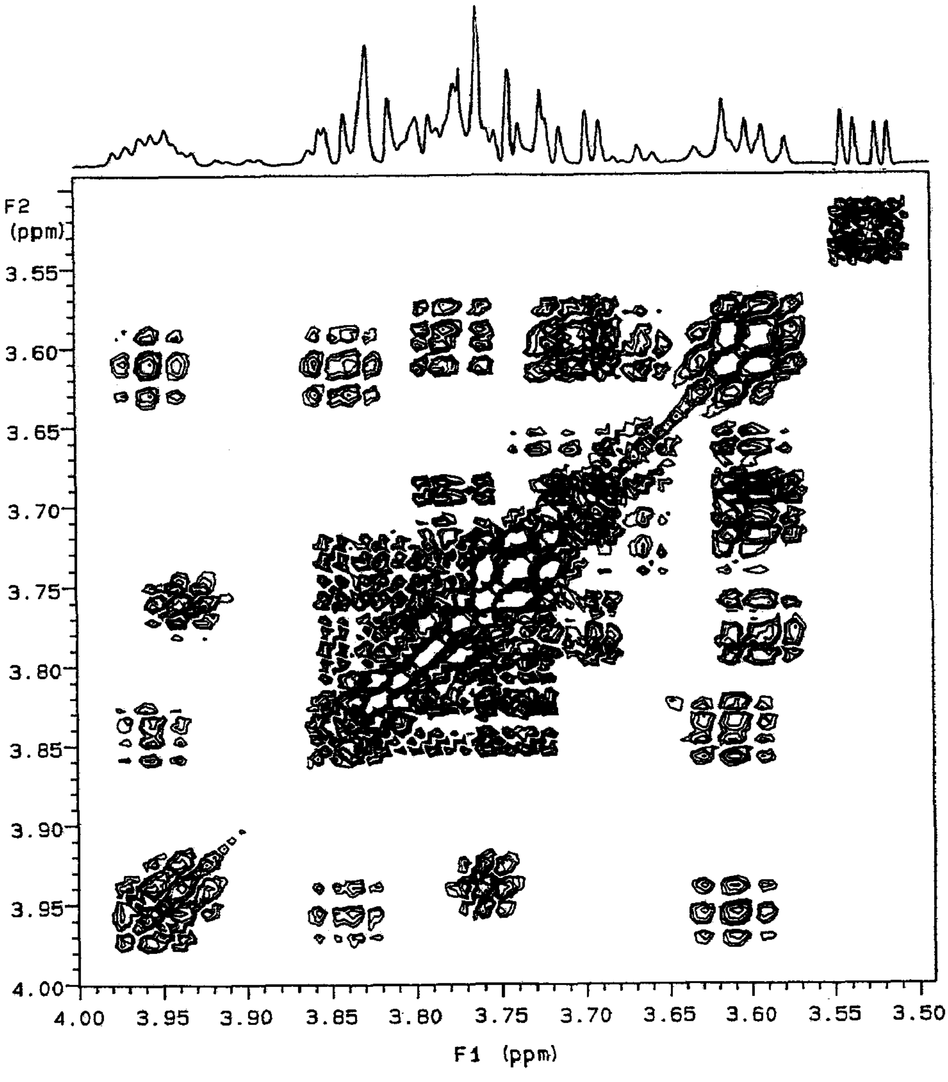


Fig. 3. TQF-COSY spectrum of the polysaccharide F1S-B from *T. flavus*.

lack of deshielding of C-6 of this residue allowed assignment of **C** as a 5-*O*-substituted β-Gal*f* residue. In addition, the values obtained from a coupled HMQC experiment for the $^1J_{\text{C-1,H-1}}$ coupling constants of the residues were as follows: **A** = 182.0; **B** = 179.1; **C** = 179.1; and **D** = 176.1 Hz, which corroborate[22] the ring size and anomeric configurations deduced from vicinal proton coupling constants and ^{1}H and ^{13}C chemical shifts. However, no important glycosylation shift was observed for residue **A**; only C-2 is somehow deshielded.

The uncertainty was removed by using an HMBC experiment which shows long-range connectivities between carbon atoms and their coupled protons through two or three bonds. Using this technique, couplings across the glycosidic linkage

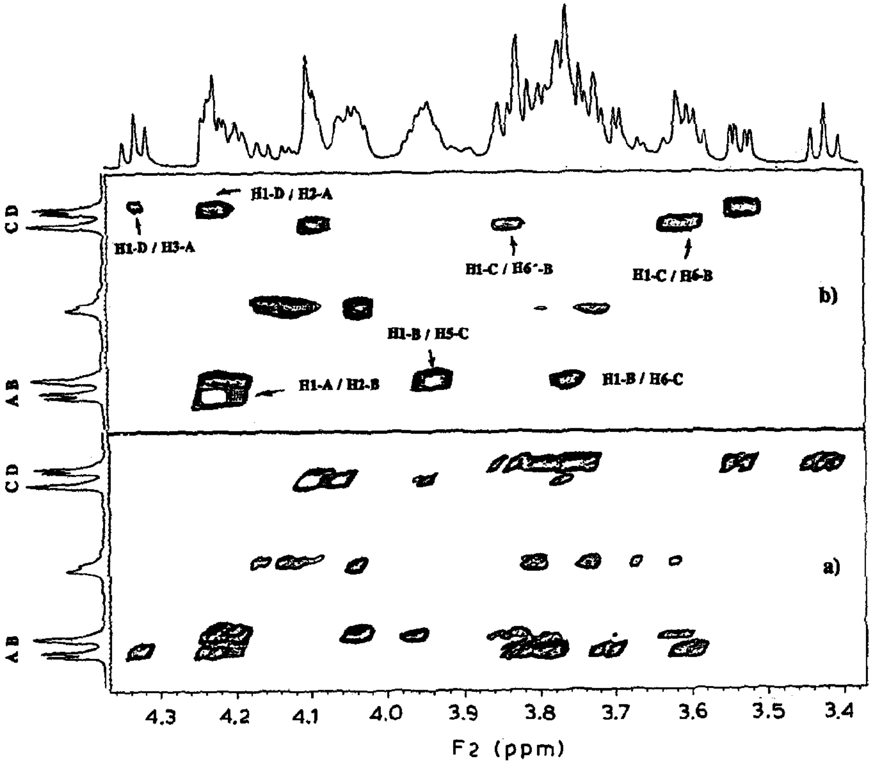
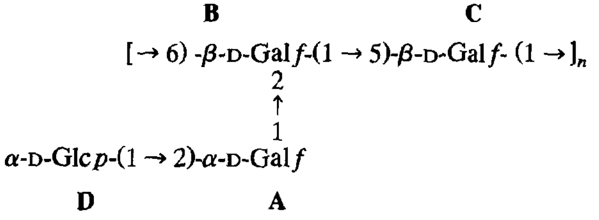


Fig. 4. Anomeric region of the 2D-NMR spectra for the polysaccharide F1S-B from *T. flavus*: (a) TOCSY (HOHAHA) spectrum; (b) ROESY (CAMELSPIN) spectrum. Significant cross-peaks are labelled.

can be unequivocally detected (Fig. 5). The above discussed connectivities for residues **B** and **C** were confirmed through the observation of cross-peaks between C-1 of Gal*f* **A** and H-2 of Gal*f* **B**, C-1 of Gal*f* **B** and H-5 of Gal*f* **C**, C-1 of Gal*f* **C** and H-6 of Gal*f* **B**, C-6 of Gal*f* **B** and H-1 of Gal*f* **C**, C-2 of Gal*f* **B** and H-1 of Gal*f* **A**, and C-5 of Gal*f* **C** and H-1 of Gal*f* **B**. Moreover, the presence of cross-peaks between H-1 of Glc*p* **D** and C-2 of Gal*f* **A**, and H-2 of Gal*f* **A** and C-1 of Glc*p* **D** indicated rigorously that **A** is an O-2 substituted α-galactofuranose residue. Several intraresidue connectivities were also detected.

According to these results, the primary structure proposed for the tetrasaccharide repeating unit of the polysaccharide is:



$$\begin{array}{l}
\qquad\qquad\qquad\quad \mathbf{B} \qquad\qquad\qquad\qquad\quad \mathbf{C} \\
\qquad\quad [\to 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\to 5)\text{-}\beta\text{-D-Gal}f\text{-}(1\to]_n \\
\qquad\qquad\qquad\qquad\quad 2 \\
\qquad\qquad\qquad\qquad\quad \uparrow \\
\qquad\qquad\qquad\qquad\quad 1 \\
\alpha\text{-D-Glc}p\text{-}(1\to 2)\text{-}\alpha\text{-D-Gal}f \\
\quad \mathbf{D} \qquad\qquad\qquad\qquad \mathbf{A}
\end{array}$$

TABLE II

^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and coupling constants (Hz, in brackets) for the cell-wall polysaccharide F1S-B from *T. flavus*

Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A	5.33	4.23	4.33	3.82	3.78	3.71	3.59
	(4.3)	(8.5)	(7.1)	(6.2)			
B	5.30	4.23	4.20	4.04	3.96	3.62	3.84
	(2.0)						
C	5.00	4.11	4.06	4.08	3.94	3.76	3.75
	(< 1)						
D	4.97	3.53	3.74	3.43	3.80	3.84	3.74
	(3.8)	(9.9)	(9.4)	(9.6)			
E	5.17	4.13			3.84 [a]	3.74 [a]	3.78 [a]
	(2.1)						
F	5.16	4.12			3.72	3.62	3.66
	(4.9)						
Unit	C-1	C-2	C-3	C-4	C-5	C-6	
A	100.0	81.3	73.6	82.2	73.2	63.3	
B	106.2	88.0	76.0	83.3	70.3	70.2	
C	108.8	81.9	77.5	82.9	76.8	62.2	
D	98.4	72.2	73.9	70.6	73.1	61.6	
E	99.6	80.0	72.2	67.9	71.7	67.0	
F	103.8	77.6 [a]	75.0	82.2	72.9 [a]	63.6	

[a] Most probable values.

The small glycosylation shift observed for C-2 of Gal*f* **A** may arise from the neighboring effect of the nearby Gal*f* **B** or through-space effects with residues **C** or **D**. Also, it is worth mentioning that care should be taken when inferring a type of residue based solely on coupling constant data, since, as mentioned above, the ^3J values found for the 2-*O*-substituted residue **A** are strikingly close to those expected for an α-Glc*p* residue. The couplings found for **A** indicate a fairly rigid conformation for the five-membered ring, with an almost pure N-type conformation, in contrast to the results usually found for furanose rings[23].

A similar structure has been assigned to varianose, an extracellular polysaccharide elaborated by *Penicillium varians*[24], for which the tetrasaccharide repeating unit was described by Jansson and Lindberg who, in addition, found polymeric material rich in mannose, a glucosylgalactose, and galactose.

With respect to the two minor units, **E** and **F**, additional ^{1}H NMR data were obtained from spectra recorded either at 25 or 75°C, which in both cases led to a clean separation of the minor signals into two doublets (Fig. 1, inset), with coupling constants $J_{1,2}$ 2.1 and 4.9 Hz, respectively (Table II). The DQF-COSY, TQF-COSY, and TOCSY spectra allowed only partial discrimination of the signals corresponding to **E** and **F** in the ^{1}H NMR spectrum. However, in accord with the chemical composition and the results of the reductive cleavage analysis, residue **E** should be 2,6-disubstituted mannose, and unit **F** terminal galactofuranose. A minute exami-

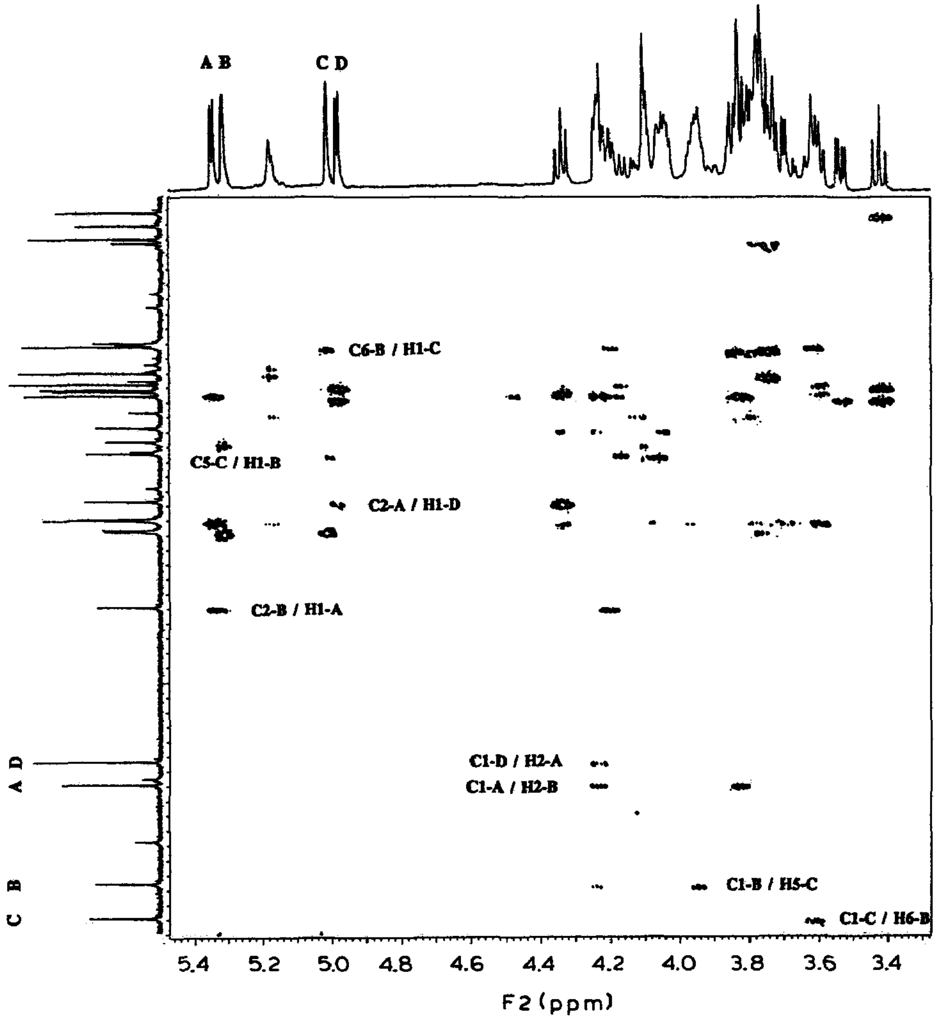
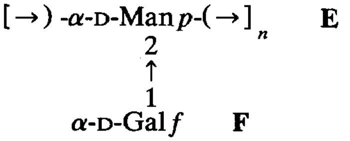


Fig. 5. HMBC spectrum of fraction F1S-B from *T. flavus*. Relevant conectivities are labelled.

nation of the ^{13}C, HMQC, and HMBC spectra led to complete assignation of the ^{13}C NMR spectrum. The measure of $^{1}J_{\text{C-1,H-1}}$ gave the values: E = 176.1, F = 182.0 Hz, which are in agreement with α configurations for both units. Accordingly, units E and F constitute the disaccharide repeating block:



$$\begin{array}{ll} [\rightarrow)\text{-}\alpha\text{-D-Man}p\text{-}(\rightarrow]_n & \text{E} \\ \quad 2 & \\ \quad \uparrow & \\ \quad 1 & \\ \alpha\text{-D-Gal}f & \text{F} \end{array}$$

As no connections could be noticed between the tetrasaccharide and the disaccharide repeating blocks and all attempts to fractionate the polymer by gel filtration column chromatography, HPLC, or copper-complex formation were unfruitful, we have not been able to determine whether we are dealing with a mixture of two distinct polysaccharides or (less likely) with a unique polymer consisting of one or two chains of the tetrasaccharide, plus a chain of the disaccharide repeating blocks, all of them linked to a single monosaccharide (mannose, for instance).

Concerning the composition of fraction F1S-B from strain 352.72, only minor differences were detected with the analogous fraction of strain 310.38, both in the NMR spectra and chemical composition. The reductive cleavage results (Table I) show the occurrence of small amounts of 6-substituted mannopyranose and 6-substituted galactofuranose, together with a decrease in the relative proportions of terminal galactofuranose and 2,6-disubstituted mannopyranose, suggesting that small amounts of Gal*f* **F** and the **D–A** branch have been lost in the chain of the disaccharide and tetrasaccharide repeating blocks, respectively, in the first strain.

ACKNOWLEDGMENT

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