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Structural investigation of two cell-wall polysaccharides of *Penicillium expansum* strains

Enrique Parra [a], Jesús Jiménez-Barbero [a], Manuel Bernabe [a,*], Juan Antonio Leal [b], Alicia Prieto [b], Begoña Gómez-Miranda [b]

[a] *Grupo de carbohidratos, Instituto de Química Orgánica, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*
[b] *Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain*

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Abstract

The structure of two cell-wall polysaccharides isolated from three different strains of *Penicillium expansum*, the type species of the genus, have been established by 1D and 2D NMR spectroscopy, and also by methylation analyses. The water-soluble polysaccharide F1S-B consisted of a linear tetrasaccharide repeating unit with the following structure:

$[\rightarrow 6)$-β-D-Gal$f$-$(1\rightarrow 5)$-β-D-Gal$f$-$(1\rightarrow 5)$-β-D-Gal$f$-$(1\rightarrow 5)$-β-D-Gal$f$-$(1\rightarrow]_n$

The alkali-soluble polysaccharide F1I is a $(1\rightarrow 3)$-α-D-glucan.

1. Introduction

Most studies of *Penicillium* cell walls have been concerned with the chemical analysis of either complete or fractionated hyphal cell-wall material. The cell walls of *P. notatum* [1], *P. chrysogenum* [2,3], *P. roquefortii* [4], *P. italicum*, *P. digitatum* [5], and *P. rubrum* [6] are made up of glucans (with traces of galactose and mannose), chitin, and protein. Leal et al. [7] found two different types of cell-wall polysaccharides in the alkali-soluble fraction of several species of *Penicillium*. In *P. notatum*, *P. oxalicum*, *P. chrysogenum*, and *P. expansum*, the alkali-soluble material was partially characterised as an α-glucan with minor quantities of mannose and galactose. In *P. allahabadense*, *P. erythromellis*, *P. islandicum*, and *P. zacinthae*, the alkali-soluble fraction contained mainly a galactofuranan. Galactofuranose residues are present in the cell wall and extracellular polysaccharides of *P. charlesii* [8–10] and in the cell wall of *P. ochro-chloron* [11] and *P. erythromellis* [12], and they seem to be antigenic determinants of some *Penicillium* species [13].

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From the cell wall of certain species of *Penicillium* [14], an alkali-soluble (1 → 3)-α-D-glucan and an alkali- and water-soluble (1 → 5)-β-D-galactofuranan have been isolated.

Since different polysaccharides have been found in the alkali-soluble fraction of the cell wall of some species of *Penicillium*, our main objective was to determine the composition and structure of the polysaccharides of the cell wall of *P. expansum*, the type species of the genus, and to ascertain whether the polysaccharides had the same structure in different strains.

2. Experimental

Microorganisms and culture media.—The following strains of *Penicillium expansum* Link. were used: CBS 325.48; CECT 2275, and CECT 2278. 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 [15].

Wall material preparation and fractionation.—The preparation of wall material [16] and the fractionation procedure [14] to give fractions F1S, F1I, F3, and F4 were performed as previously described. Fractions F1S were re-fractionated by treatment with a small portion of water (~ 50 mg/mL), followed by centrifugation (10000*g*, 30 min), giving a solution (F1S-B) and a precipitate (F1S-A).

Chemical analysis.—Neutral sugars were released by hydrolysis with 2 M H_2SO_4 at 100°C for 5 h and then converted into their corresponding alditol acetates [17]. Identification and quantification were carried out by gas–liquid chromatography (GLC) using 3% SP-2340 on 100–120 Supelcoport [18]. Similar hydrolysis of fractions F1S-A and B, followed by lyophilisation, gave two clean samples from which the absolute configuration of the neutral sugars was established.

Methylation analyses were performed by the usual methodology [19]. Analyses by the reductive-cleavage method were carried out in two steps, as described [20], with trimethylsilyl triflate as catalyst, but the reactions were effected under N_2 and the time during the reductive cleavage step was shortened to 5–6 h, to minimise unwanted byproducts.

NMR analysis.—Polysaccharides F1S-B (~ 20 mg) were dissolved in D_2O (1 mL) followed by centrifugation (10000*g*, 20 min) and lyophilisation. This process was repeated twice for further deuterium exchange. The final sample was dissolved in 0.7 mL of D_2O (99.98% D) and degassed in the NMR tube under Ar.

Solutions of samples from fractions F1I were obtained by dissolving the polysaccharides (~ 15 mg) in 0.3 M NaOD (0.8 mL), followed by centrifugation as above.

NMR spectra were recorded at 40°C with a Varian Unity 500 spectrometer (F1S-B) or at 60°C with a Varian XL-300 spectrometer (F1I). Proton chemical shifts were referred to residual HDO at δ 4.61 and 4.41, respectively, and carbon chemical shifts to internal acetone at δ 31.45.

^{1}H NMR spectra for fractions F1S-B from all strains had been previously recorded and appeared practically identical. The 2D NMR studies were carried

out on polysaccharide F1S-B from strain CECT 2278. The studies of polysaccharides F1I were effected on the strain CECT 2275.

The double-quantum filtered DQF-COSY experiment was performed in the phase sensitive mode using the method of States et al. [21]. A data matrix of 256 × 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 2K × 2K. The clean 2D TOCSY experiment [22] 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 × 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 × 2K. The 2D rotating frame NOE [23] (ROESY, CAMELSPIN) 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 [24]. The total mixing time was set to 300 ms. The rf carrier was set at δ 6.0 ppm, downfield from the most deshielded anomeric proton in order to minimise spurious Hartmann–Hahn effects [25]. A data matrix of 256 × 2K points was used to resolve a spectral width of 2600 Hz; 16 scans were used per increment with a relaxation delay of 2 s. 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 × 2K. The pure absorption 2D NOESY experiments were carried out with mixing times of 300 ms. A data matrix of 256 × 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. A similar processing protocol was employed. The pure absorption one-bond proton–carbon correlation experiment was collected in the ^{1}H-detection mode using the HMQC pulse sequence [26] and a reverse probe. A data matrix of 256 × 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 and a delay corresponding to a J value of 145 Hz. A BIRD-pulse was use to minimise the proton signals bonded to ^{12}C. ^{13}C-Decoupling was achieved by the WALTZ scheme. Squared cosine-bell functions were applied in both dimensions and zero-filling was used in F1 to expand the data to 2K × 2K.

3. Results and discussion

Proportion of the fractions.—The proportions of the fractions obtained from the dry cell-wall material of the different strains of *P. expansum* are shown in Table 1. The polysaccharide material extracted with 1 M NaOH at 20°C contained water-soluble polysaccharides (Fraction F1S) (2.2 to 3.2%), and water-insoluble polysaccharides (Fraction F1I) (26.4 to 42.7%). The material extracted with 1 M NaOH at 70°C was Fraction F3 (2.5 to 4.6%) and the insoluble residue was Fraction F4 (34.4 to 45.3%). On re-fractionation of F1S, a soluble (F1S-B, 60–70%) and a relatively insoluble fraction (F1S-A, 30–40%) were obtained.

Table 1
Percentages of cell-wall fractions from different strains of *P. expansum*

Strain	F1S	F1I	F3	F4
P. expansum CECT 2275	2.6	42.7	2.5	39.1
P. expansum CECT 2278	3.2	39.2	4.6	34.4
P. expansum CBS 325.48	2.2	26.4	3.9	45.3

Monosaccharide composition of F1S and F1I.—The neutral sugars liberated in the hydrolysis of Fractions F1S and F1I are presented in Table 2. Galactose was the main component of Fractions F1S which also contained glucose and mannose. From these, a galactan (F1S-B) and a glucan with small quantities of mannose (F1S-A) were characterised. Fraction F1I was also a glucan with small proportions of mannose and galactose.

Methylation analysis of F1S-B gave 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol and 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methylgalactitol in a molar ratio 2.5 : 1. Since, on this evidence, 4-substituted galactopyranosyl and/or 5-substituted galactofuranosyl residues could be involved in the structure, further methylation analyses were carried out by the reductive-cleavage procedure [20]. A 3.1 : 1 molar ratio of 5-*O*-acetyl-1,4-anhydro-2,3,6-tri-*O*-methylgalactitol and 6-*O*-acetyl-1,4-anhydro-2,3,5-tri-*O*-methylgalactitol was obtained, showing that the polysaccharide contained three 5-linked galactofuranosyl residues for each 6-linked galactofuranosyl residue.

The high-resolution ^{1}H NMR spectrum and the proton-decoupled ^{13}C NMR spectra of the polysaccharide in D_2O solution showed three anomeric signals, one having double the intensity of the other two (Fig. 1), indicating that the polymer consists of a tetrasaccharide repeating unit. The four residues were labelled **A**, **B**, **C**, and **D**, according to their anomeric protons from low to high field. The resolution of the ^{1}H NMR spectrum at 40°C was good enough to allow DQF-COSY and TOCSY experiments to be performed in a satisfactory way. Coherence transfer to all the protons of the four constituent monosaccharides was achieved by

Table 2
Neutral sugars (%) released from fractions F1S and F1I of different strains of *P. expansum*, after hydrolysis with 2 M H_2SO_4 at 100°C for 5 h, determined as alditol acetates by gas–liquid chromatography

Strain	Man	Gal	Glc	Recovery (%)
F1S				
P. expansum CECT 2275	4.9	24.5	21.4	50.8
P. expansum CECT 2278	7.5	44.9	13.4	65.8
P. expansum CBS 325.48	10.4	32.5	15.8	58.7
F1I				
P. expansum CECT 2275	7.1	9.5	71.9	88.5
P. expansum CECT 2278	3.5	1.1	74.6	79.2
P. expansum CBS 325.48	1.4	1.0	76.0	78.4

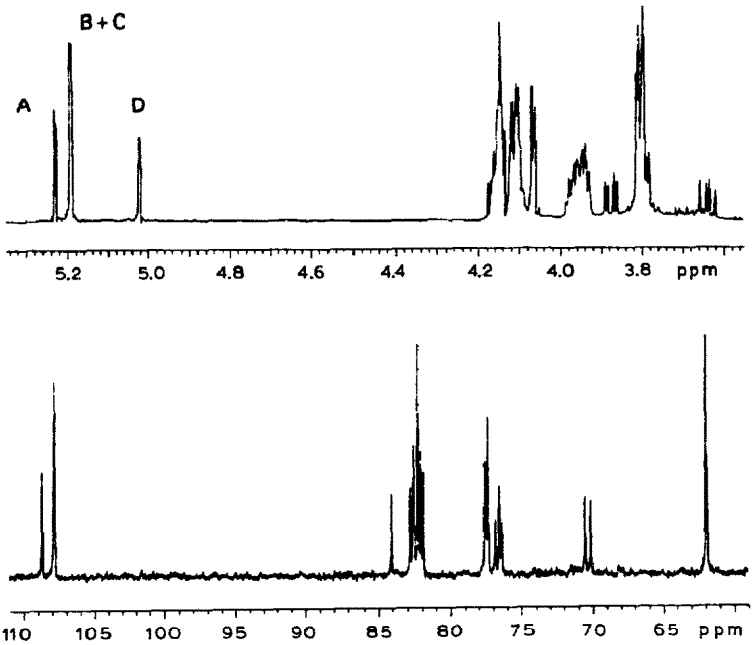

Fig. 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra in D_2O at 40°C for the cell-wall polysaccharide F1S-B from *P. expansum*. The anomeric protons have been labelled A–D.

isotropic mixing using 2D TOCSY with a mixing time of 150 ms (Fig. 2, **a**). It can be observed that two out of the four residues (**B** and **C**) have almost identical proton and carbon chemical shifts, while a third one (**D**) presents slight differences in some of the resonances. The assignment of each cross-peak was based also on the information obtained from the DQF-COSY experiments. The ^{1}H NMR chemical shifts are listed in Table 3. Despite the small values of $J_{1,2}$ for all the four residues ($J \sim 2.0$ Hz), the TOCSY subspectra through the anomeric signals showed clear connectivities to the rest of the protons. The chemical shift values of H-2, H-3, and H-4 were very similar ($\Delta\delta_{max} < 0.2$), and appeared at relatively low field, $\delta > 4$, indicating the presence of galactofuranose residues, as also deduced from the methylation analysis. In addition, according to the the $J_{1,2}$ values, it can now be concluded that all the galactofuranose residues have the β configuration [27].

Some information concerning the assignment of signals to the glycosyl residues **A–D** was obtained from the 2D NOESY and the 2D CAMELSPIN (ROESY) experiments. Although spurious Hartmann–Hahn effects in ROESY spectra of oligosaccharides can never be completely ruled out [28], the existence of cross-peaks between signals of different residues indicates their proximity in space and, probably, their connection (Fig. 2, **b**). Thus, cross-peaks for the anomeric proton of the residue **D** and the H-6's of residue **A** could be observed, along with others connecting H-1 of residue **A** with H-5 and H-6 of unit **B** or **C**. The NOE cross-peaks for H-1 of galactofuranoses **B** and **C** virtually coincide with the connectivities in the TOCSY spectrum (Fig. 2), which seems to indicate that both

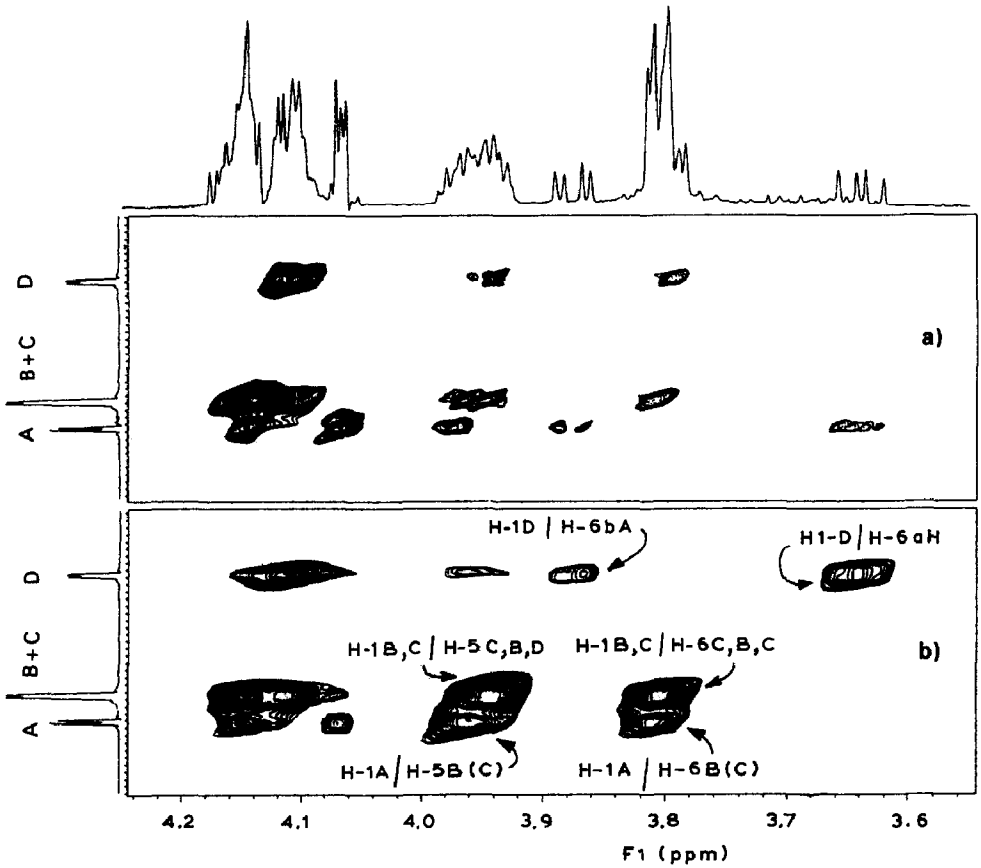

Fig. 2. Anomeric region of the 2D TOCSY (**a**) and 2D CAMELSPIN (ROESY) (**b**) spectra for fraction F1S-B, showing the connectivities with the rest of the protons. The anomeric protons (A–D) and relevant cross-peaks have been labelled.

residues are neighbours. Since NOE cross-peaks are dependent on the conformation around the glycosidic bonds, the existence of these cross-peaks does not guarantee knowledge of the exact position of the linkage, although at this stage it can be assumed that β-Gal*f*-**D** is (1 → 5)- or (1 → 6)-linked to Gal*f*-**A**, which is connected probably via a (1 → 5) or (1 → 6) linkage to Gal*f*-**C/B**, which is in turn (1 → 5)- or (1 → 6)-linked to Gal*f*-**B/C**.

It was possible to discriminate among 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 also listed in Table 3. As may be seen, some uncertainties remain, but all are within 0.5 ppm and therefore are irrelevant. The chemical shifts of the anomeric carbons indicate unequivocally [29] that all of the monosaccha-

Table 3
^{1}H and ^{13}C NMR chemical shifts (δ) and coupling constants (J, Hz, in brackets) for the cell-wall polysaccharides F1S-B and F1I from *P. expansum*

Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
F1S-B							
A	5.23 (2.0)	4.15	4.07	4.07	3.97	3.88	3.64
B	5.19 (2.0)	4.13	~ 4.14	4.17 [a]	3.84 [b]	3.81	3.81
C	5.19 (2.0)	4.13	~ 4.14	4.16 [a]	3.86 [b]	3.81	3.81
D	5.02 (2.0)	4.10	4.11	4.11	3.93	3.79	3.79
F1I							
→ 3)-α-D-Glc*p*-(1 →	5.14 (4.0)	3.46 (9.3)	3.69 (9.2)	3.36 (9.5)	3.82 (5.9) (2.6)	3.52 (−12.2)	3.71

Unit	C-1	C-2	C-3	C-4	C-5	C-6
F1S-B						
A	108.3	82.4 [a]	78.0 [b]	84.4	70.9	70.5
B	108.2	82.6	77.7	83.0 [c]	76.8 [d]	62.3
C	108.2	82.6	77.7	82.9 [c]	77.2 [d]	62.3
D	109.0	82.3 [a]	77.9 [b]	83.2	76.7	62.2
F1I						
→ 3)-α-D-Glc*p*-(1 →	101.2	71.9	83.9	71.3	73.6	62.0

[a,b,c,d] These values may have to be interchanged.

rides are β-Gal*f* residues. On the other hand, the observed deshielding of C-6 of residue **A** allows us to assign it as a 6-*O*-substituted β-Gal*f* unit, while the values for the C-5 chemical shifts show that residues **B**–**D** are 5-*O*-substituted galactofuranosyl units. Additional evidence was obtained by using an HMBC experiment which shows long-range connectivities between carbon atoms and their coupled protons through two or three bonds. Applying this technique, couplings across the glycosidic linkage can be detected with certainty (Fig. 3). The above glycosidic connectivities were confirmed through the observation of cross-peaks between C-1 of Gal*f*-**D** with H-6a and H-6b of Gal*f*-**A**, C-5 of **B**(**C**) with H-1 of **A**, C-5 of **B**(**C**,**D**) with H-1 of **B**(**C**), and C-6 of **A** with H-1 of **D**. Overlapping cross-peaks of C-1 of **A**, **B**, and **C** with H-5 of **B**, **C**, and **D** can also be seen.

The above connectivities and substitutions are in agreement with the analytical results and allow us to propose the following structure for the tetrasaccharide repeating unit of the *P. expansum* polysaccharide:

$$[\rightarrow 6]\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow)_n$$

A **B** **C** **D**

Concerning the alkali-soluble polysaccharide from fraction F1I, the analysis of the ^{1}H NMR simple spectrum (Fig. 4) allowed straightforward assignment of all

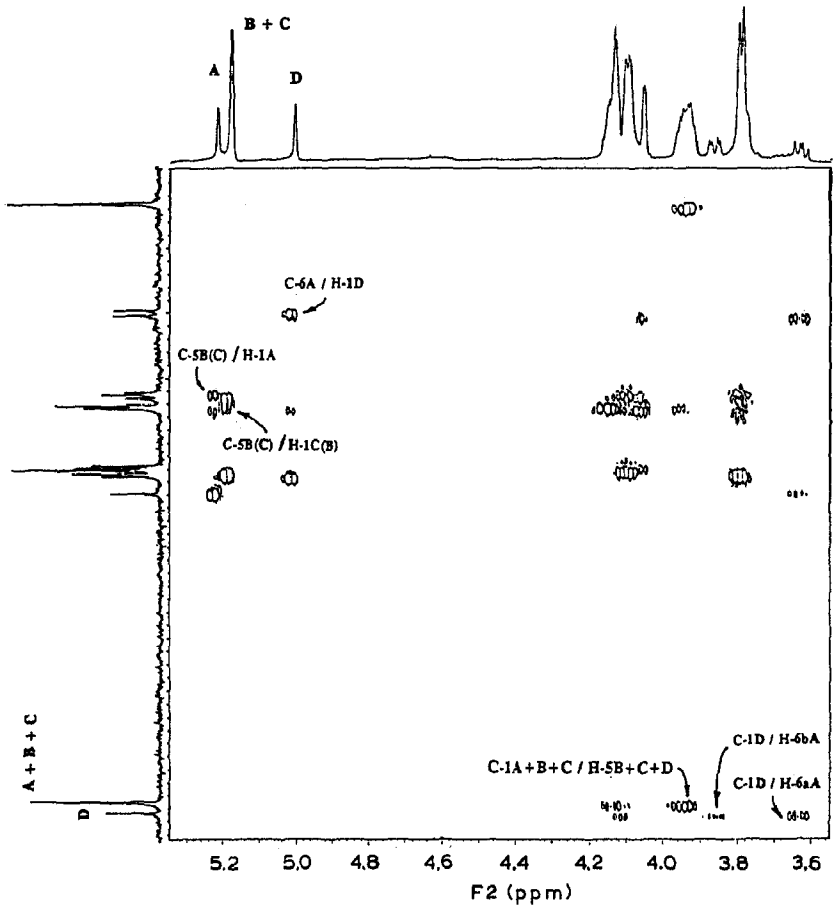

Fig. 3. HMBC spectra for fraction F1S-B, showing ^{1}H–^{13}C long-range connectivities. The anomeric protons, carbons (A–D), and significant cross-peaks have been labelled.

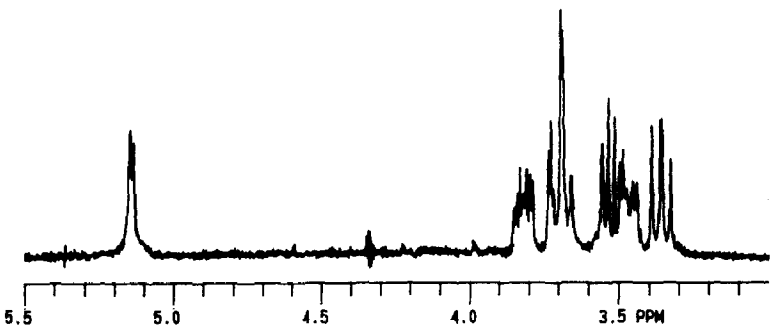

Fig. 4. ^{1}H NMR spectrum (300 MHz) in 0.3 M NaOD at 60°C for the cell-wall polysaccharide F1I from *P. expansum*.

the signals of the single unit (Table 3). The values of the coupling constants correspond to a glucan with the α configuration. A $^{1}H-^{13}C$ heterocorrelation experiment allowed unambiguous assignment of the ^{13}C NMR spectrum (see Table 3). The observed deshielded value of C-3 (δ 83.9) reveals that the repeating unit is a 3-*O*-substituted glucose, in agreement with chemical analysis, which showed that fractions F1I were resistant to oxidation with periodate. These results clearly demonstrate that the alkali-soluble polysaccharides from fractions F1I mainly comprise linear chains of (1 → 3)-linked α-D-glucopyranosyl residues.

The cell-wall F1S-B polysaccharide is characteristic of *P. expansum* since, to the best of our knowledge, it has not been found in other species of *Penicillium* [12,14]. The structural differences of this polysaccharide with the (1 → 5)-β-D-galactan isolated from species of *Eupenicillium* [14,30,31] or with the (1 → 2)-(1 → 5)-β-D-galactan obtained from species of *Talaromyces* (ref 32 and unpublished results from this group) might indicate that these genera are not teleomorphic states of *P. expansum*.

The study of a large number of species of *Penicillium* and related genera may lead to the isolation of new galactans that could be used as chemotaxonomic markers and allow the establishment of filogenetic relationships among them.

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