ELSEVIER

Carbohydrate Research 303 (1997) 67–72

CARBOHYDRATE
RESEARCH

Structural elucidation of acidic fungal polysaccharides isolated from the cell-wall of genera *Cylindrocladium* and *Calonectria*

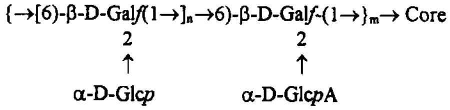
Oussama Ahrazem [a], Alicia Prieto [a], Juan Antonio Leal [a,*],
Begoña Gómez-Miranda [a], Jezabel Domenech [a],
Jesús Jiménez-Barbero [b], Manuel Bernabé [b,*]

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

Received 13 January 1997; accepted 11 May 1997

Abstract

The structure of acidic fungal polysaccharides, isolated from the cell wall of *Cylindrocladium* and *Calonectria* species, has been investigated by chemical analysis, methylation and reductive cleavage analyses, and 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. The polysaccharides have an idealized repeating unit:



$$\{\rightarrow[6)\text{-}\underset{\substack{2\\ \uparrow\\ \alpha\text{-D-Glc}p}}{\beta\text{-D-Gal}f}(1\rightarrow]_n\rightarrow 6)\text{-}\underset{\substack{2\\ \uparrow\\ \alpha\text{-D-Glc}p\text{A}}}{\beta\text{-D-Gal}f}\text{-}(1\rightarrow\}_m\rightarrow \text{Core}$$

linked to a small mannan core (< 5%), with $n = 3$ for *Cylindrocladium penicilloides* and *C. quinqueseptatum* and $n = 5$ for *Calonectria theae, C. crotalariae*, and *C. colhounii*. © 1997 Elsevier Science Ltd.

Keywords: Fungi; *Cylindrocladium*; *Calonectria*; Polysaccharides; NMR spectroscopy

1. Introduction

Polysaccharides have been used in the classification of fungi [1], yeasts [2], and lichens [3]. We have found that the alkali-extractable and water-soluble polysaccharides obtained from the cell-wall of fungi from various genera differ in composition and structure [4–8]. These polysaccharides are minor components of the cell wall (2–8%), where they are probably forming part of glycoproteins or peptido-polysaccharides. They are antigenically relevant and may

---

[*] Corresponding authors.

PII S0008-6215(97)00145-6

also be involved in cell–cell and/or cell–host recognition mecanisms. Due to their diversity, they have been proposed as chemotaxonomic markers at the genus or subgenus level [7], as well as a means to establish the relationship of teleomorphic genera (perfect state) with their anamorphs (imperfect state).

Continuing the search for cell wall polysaccharides which may be useful either as possible immunorelevant compounds and as chemotaxonomic markers, we are studying the complex group of the nectroid fungi. In this paper, we report on the structure of the acidic glucogalactan obtained from the cell walls of species ot the genera *Calonectria* and its anamorph *Cylindrocladium*.

2. Results and discussion

Analysis for neutral sugars gave Gal, Glc, and Man as shown by GLC of the alditol acetates. Uronic acid was also detected (see Table 1).

Fractions F1S-S from the different species were dissolved in D_2O and their NMR spectra were compared. *Cylindrocladium penicilloides* was selected for further studies and purified by column chromatography. Methylation analysis gave the acetates of 2,3,4,6-tetra-*O*-methyl glucitol, 3,5-di-*O*-methyl galactitol, and small amounts (< 5%) of 2,3,4-tri-*O*-methyl mannitol, and 3,4-di-*O*-methyl-mannitol. The high resolution ^{1}H NMR spectrum of the polysaccharide from *C. penicilloides* (pD = 6.0, Fig. 1a) contained two signals at 5.17 and 5.07 ppm which were labelled **A** and **B**, respectively. Assignment of the non-anomeric protons of the residues was achieved from DQCOSY and 2D-TOCSY (HOHAHA) experiments, which demonstrated that three different residues were actually present, one of them in less than half the proportion (labelled **C**) of the other two. The anomeric signal of **C** overlapped with that of residue **B**. The chemical shifts values of H-2, H-3, and H-4 corresponding to residue **A** were very similar (< 0.2 ppm), and appeared at relatively low field (> 4 ppm), indicating the presence of galactofuranose. It was possible to measure most of the coupling constants. The large values observed for the *J*-couplings of proton H-4 associated with residue **B** ($J_{3,4} = J_{4,5} = 9.4$ Hz) suggested a glucopyranose residue with α-configuration, as deduced from the anomeric coupling ($J_{1,2}$ 3.8 Hz). In addition, the minor residue **C** also showed two fairly large coupling constants for H-4 ($J_{3,4}$ 9.6, $J_{4,5}$ 9.4 Hz), and was assigned as glucopyranuronic acid, as inferred from its five-proton system.

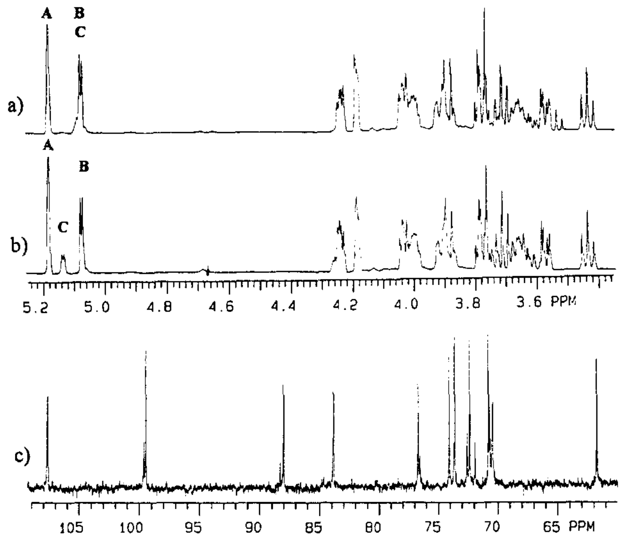


Fig. 1. ^{1}H and ^{13}C NMR spectra at 500 MHz of *C. penicilloides* (D_2O, 40 °C). (a) ^{1}H NMR spectrum at pD = 6. (b) ^{1}H NMR spectrum at pD = 3, showing the three distinct anomeric protons, which have been labelled **A**–**C**. (c) ^{13}C NMR spectrum at 125 MHz (D_2O; pD = 3; 40 °C). The C = O peak at 172.0 ppm is not shown.

Table 1
Percentage of the neutral and acidic sugars detected by GLC and the carbazole procedure, respectively, on the fraction F1S-S. Due to the presence of glucuronic acid, the galactofuranose gave lower proportion than that deduced from ^{1}H NMR analysis

	Glc	Gal	Man	GlcA	Recovery %
Cylindrocladium penicilloides	28.7	27.1	2.5	14.0	72.3
C. quinqueseptatum	25.4	25.1	2.0	12.6	65.1
Calonectria colhounii	30.9	30.0	3.1	7.8	71.8
C. crotalariae	32.5	32.9	4.3	8.1	77.8
C. theae	30.3	35.6	4.1	7.3	77.3

The ^{13}C NMR spectrum at pD = 6 also showed two peaks in the anomeric region, one of them at 107.2 ppm, suggesting the presence of a β-galactofuranose residue, and a carbonyl signal at 176.2 ppm, which supported the presence of uronic acid. The polysaccharide was then carbonyl-reduced according to Taylor and Conrad's method [17] and subjected to methylation and reductive cleavage analysis. Methylation analysis gave the same alditol acetate derivatives than before reduction, but the proportion of the acetates of 2,3,4,6-tetra-*O*-methyl glucitol and 3,5-di-*O*-methyl galactitol increased substantially. The reductive-cleavage method led to the same results.

In order to determine the proportion and the glycosidic sequence of the three residues, the solution (pD = 6.0) was stirred with Amberlite IR-120 to convert the sodium salt of uronic acids into the free carboxylic acids. The resulting acidic solution (pD = 3.0) was freeze-dried and dissolved in D_2O (99.98%). The ^1H NMR spectrum (Fig. 1b) now contained three distinct signals in the anomeric region, which were labelled **A**, **B**, and **C**, as above, in the proportions 25: 19: 8, respectively. **C** appeared as a doublet, $J_{1,2}$ 3.7 Hz, which demonstrated that the GlcA moiety had the α-configuration. The assignment of the non anomeric protons was accomplished from a 2D-TOCSY experiment (Fig. 2a), although at this pD, some non anomeric proton resonances overlapped.

The ^{13}C NMR spectrum (pD = 3, Fig. 1c) showed the signal centred at 99 ppm split into two, one peak being some 1/3 the height of the other, and only two methylene signals, one of them at 70.2 ppm, this last indicative of a 6-linked hexosyl residue. Assignment of most of the signals for the three residues was elucidated from HMQC experiments, which map the connectivities between carbon atoms and their directly bonded protons (Fig. 2b). The signals corresponding to C-2 and C-3 of residue **A** were also split into two, in each case one being 1/3 the height of its pair (Table 2).

Comparison of the chemical shifts values with those reported by Bock et al. [9] allowed the glycosylation positions to be deduced. Thus, terminal α-Glc*p* and α-Glc*p*A acid, and 2,6-di-*O*-substituted-β-

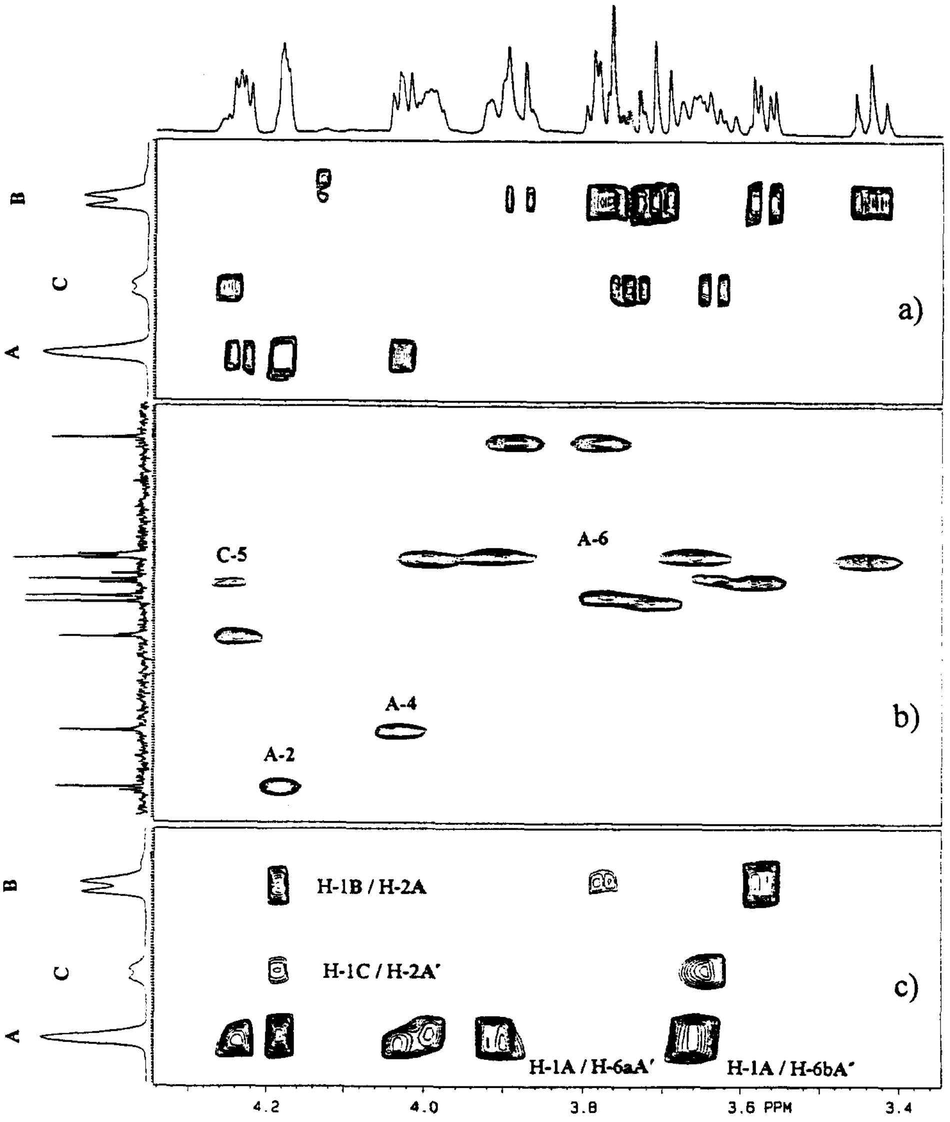


Fig. 2. Selected regions of the 2D-TOCSY (a), HMQC (b), and 2D-NOESY (c) spectra for fraction F1S-S of *C. penicilloides*, showing the connectivities with the rest of the protons and carbons. Anomeric protons and relevant cross-peaks have been labelled.

Table 2
^{1}H and ^{13}C NMR chemical shifts (δ) and proton coupling constants (J, Hz) at pD = 6.0 and pD = 3.0 for the alkali-extractable water-soluble cell-wall polysaccharide isolated from *Cylindrocladium penicilloides*

Residue		1	2	3	4	5	6a	6b
pD = 6.0								
A	H	5.17	4.18	4.23	4.03	3.99	3.91	3.65
	J	1.5	3.7	6.6	4.5			
	C	107.2	87.4	76.3	83.4	70.5	70.2	
B	H	5.06	3.57	3.71	3.44	3.77	3.87	3.76
	J	3.8	9.8	9.4	9.4			
	C	99.0	72.0	73.7	70.5	73.3	61.4	
C	H	5.06	3.61	ca. 3.74	3.54	4.04	–	–
	J	3.7	9.9	9.6	9.4			
	C	99.0	71.9	73.4	72.8	73.5	176.2	
pD = 3.0 [a]								
$\mathbf{A}_1$	H	5.18	4.19	4.24	4.03	4.00	3.92	3.65
	C	107.2	87.7	76.4	83.5	70.5	70.2	
$\mathbf{A}_2$	H	5.18	4.19	4.24	4.03	4.00	3.92	3.65
	C	107.2	87.9	76.3	83.5	70.5	70.2	
B	H	5.07	3.57	3.72	3.44	3.78	3.88	3.78
	C	99.1	72.1	73.8	70.6	73.3	61.6	
C	H	5.13	3.64	ca. 3.74	ca. 3.74	4.25	–	–
	C	99.2	71.7			72.3	172.0	

[a] The coupling constants at this pD were within the experimental error (0.1 Hz) of that observed at pD = 6.0.

Gal*f* moieties were considered to be present in the polysaccharide, in accordance with methylation analyses. However, as no clear signals of mannopyranose residues were detected in the ^{1}H NMR spectrum, we deduced that the mannans were part of the polysaccharide core.

To discriminate between the two possible glycosylation sequences (namely, a backbone of β-(1 → 6)-Gal*f*, substituted at position 2 of each residue by either α-Glc*p* or α-Glc*p*A, or else a skeleton of β-(1 → 2)-Gal*f* substituted at position 6 by terminal moieties) we obtained a 2D-NOESY spectrum (Fig. 2c) which, in addition to expected intraresidue connections, exhibited cross-peaks for H-1**A**/H-6a**A**′, H-1**A**/H-6b**A**′, H-1**B**/H-2**A**, and H-1**C**/H-2**A**′, which strongly supported the first possibility. The splitting of C-2 and C-3 of residue **A** indicated that the neighborhood of position 2 was affected by the different nature of residues **B** and **C**. Hence, we have labelled $\mathbf{A}_1$ the residue to which moiety **B** is attached and $\mathbf{A}_2$ the one linked to **C**. We denote a second molecule of 2,6-di-*O*-substituted Gal*f*, comprising either $\mathbf{A}_1$ or $\mathbf{A}_2$, as **A**′.

These galactofuranosidic chains are probably connected at position 2 of one of every four or five mannopyranose residues of a (1 → 6) mannan core, which represents about 5% of the total polysaccharide.

We also studied the cell wall polysaccharides isolated from species of closely related genera. Thus, the polysaccharides from *Cylindrocladium quinqueseptatum*, *Calonectria theae*, *C. colhounii*, and *C. crotalariae* were dissolved in D$_2$O and treated with Amberlite IR-120, as above, and the ^{1}H NMR spectra of the samples were compared. All of them were very similar (Fig. 3), hence indicating structural similarity to that of the polysaccharide isolated from *C. penicil-*

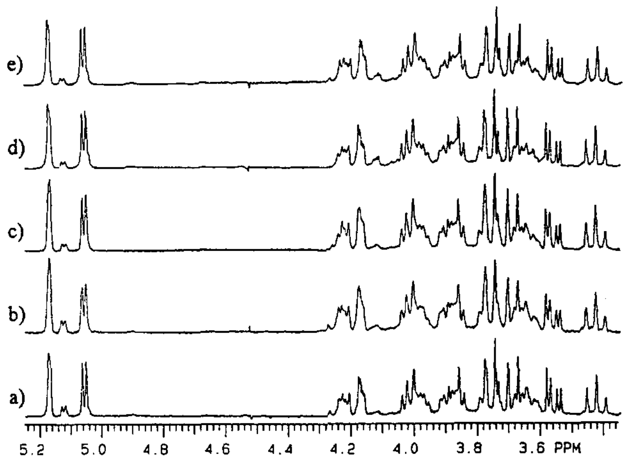
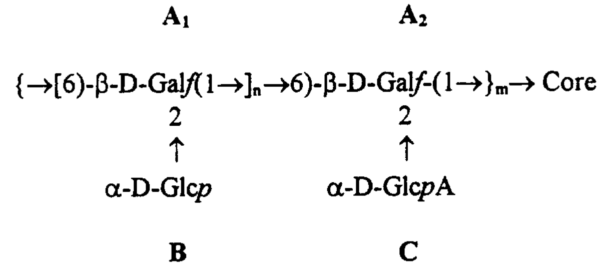


Fig. 3. ^{1}H NMR spectra at 300 MHz (D$_2$O; pD = 3; 50 °C) of fractions F1S-S of the cell wall polysaccharides isolated from: *C. penicilloides* (a), *C. quinqueseptatum* (b), *C. theae* (c), *C. colhounii* (d), and *C. crotalariae* (e).

loides. However, whilst the proportion of glucuronic acid in both species of *Cylindrocladium* was about the same (some 12–13%), in *Calonectria theae*, *C. colhounii* and *C. crotalariae* it was lower (about 8%).

The results from methylation analyses, together with the NMR spectral data, suggest the following idealized structures for the cell-wall polysaccharides from those genera:



$$\begin{array}{ccc} \mathbf{A_1} & & \mathbf{A_2} \\ \{\rightarrow[6)\text{-}\beta\text{-D-Gal}f(1\rightarrow]_n & \rightarrow & 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow\}_m\rightarrow \text{Core} \\ 2 & & 2 \\ \uparrow & & \uparrow \\ \alpha\text{-D-Glc}p & & \alpha\text{-D-Glc}p\text{A} \\ \mathbf{B} & & \mathbf{C} \end{array}$$

being $n = 3$ for both species of *Cylindrocladium*, and $n = 5$ for the three species of *Calonectria*.

This similarity in the structures of their cell-wall polysaccharides strongly support the new interpretation of the genus *Calonectria*, which is now circumscribed to include only nectroid species having a particular suite of ascomatal characteristics and a *Cylindrocladium* anamorph [10].

It is worthwhile pointing out that polysaccharides containing a main chain of Gal*f*, with different types of linkages, have been reported in the genera *Eupenicillium*, *Penicillium*, *Talaromyces*, *Neosartorya*, and *Paecylomyces* [4–8] which are classified in the family *Trichocomaceae* order *Eurotiales*, well differentiated morphologically from the order *Hypocreales* in which are included the nectroid fungi.

These results strongly support the proposition that the water soluble polysaccharides obtained from alkali extracts of fungal cell walls, besides their immunological relevance, are useful as chemotaxonomic markers for delineation of fungal genera. In addition, they can be used to establish the relatedness of anamorphic with their teleomorphic genera. Moreover, the variety of structures found up to the moment demonstrates the possibilities of using fungal cell walls as a source of new polysaccharidic structures.

3. Experimental

Microorganisms and culture media.—The species used were *Cylindrocladium penicilloides* (CBS 174.55), *C. quinqueseptatum* (CBS 582.50), *Calonectria theae* (CBS 279.90), *C. colhounii* (CBS 455.92), and *C. crotalariae* (CBS 463.76). 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 [11].

Cell-wall material preparation and fractionation.—The preparation of wall material [12] and the fractionation procedure [13] were performed as previously described. The crude polysaccharidic preparation extracted from the dry cell wall material with 1 M NaOH at 20 °C contained water soluble polysaccharides (Fraction F1S) and water insoluble polysaccharides (F1I). Fractions F1S were re-fractionated by treatment with a small amount of water (about 50 mg mL^{-1}), followed by centrifugation ($10000 \times g$, 30 min), giving a solution (F1S-S), and a precipitate (F1S-I). F1I and F1S-I were not further investigated.

For preparative chromatography, 100 mg of fraction F1S-S from *C. penicilloides* were processed according to Leal et al. [13].

Chemical analysis.—Neutral sugars were released by treating the samples overnight with methanolic 0.6 M HCl at 80 °C followed by hydrolysis with 3 M trifluoroacetic acid for 1 h at 121 °C. The hydrolysis products were reduced with sodium borohydride and the corresponding alditols acetylated with pyridine-acetic anhydride (1:1) for 1 h at 100 °C. Identification and quantification were carried out by gas–liquid chromatography (GLC) using 3% SP-2340 on 100–120 Supelcoport [14]. The absolute configuration of the sugars was determined as devised by Gerwig et al. [15]. The uronic acid was determined by using the carbazol reaction with D-glucuronic acid as standard [16].

Reduction of the polysaccharide.—As uronic acids were shown to be present, a sample of fraction F1S-S of the polysaccharide was dissolved in water (5 mL), and eluted through a column of Dowex 50W × 4 (H^+) ion-exchange resin to remove Na^+, the eluate was concentrated to a small volume by evaporation and lyophilized. Each sample was reduced according to the method of Taylor and Conrad [17], using $NaBD_4$.

Methylation analyses.—The reduced polysaccharide was methylated following the method of Ciucanu and Kerek [18] and hydrolysed sequentially with 90% formic acid in water at 80 °C for 2 h and 5 M TFA at 100 °C for 5 h. The products were reduced with $NaBD_4$ and then acetylated as above. The corresponding partially methylated alditol acetates were examined by GC–MS using a SPB-1 column (30 m × 0.25 mm, 0.2 μm film thickness), a temperature program (160 to 210 °C, 1 min initial hold, 2 °C/min ramp rate) and a mass detector Q-mass from Perkin–Elmer. Quantification was according to peak area.

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

NMR analysis.—Polysaccharides F1S-S (~ 20 mg) were dissolved in D_2O (0.8 mL) followed by centrifugation (10000 × *g*, 20 min). The supernatants (ca. 0.7 mL) were used for recording ^1H NMR spectra. The sample from *C. penicilloides* for 2D experiments was lyophilised, redissolved in D_2O (1 mL) and the process repeated twice for further deuterium-exchange. The final sample was dissolved in 0.7 mL of D_2O (99.98% D).

^1H NMR spectra for fractions F1S-S were recorded at 50 °C using a Varian XL-300. 1D and 2D ^1H and ^{13}C NMR experiments for *C. penicilloides* were carried out at 40 °C using a Varian Unity 500 spectrometer. Proton chemical shifts refer to residual HDO at δ 4.51 and 4.61, at 40 °C and 50 °C respectively, and carbon chemical shifts to internal acetone at δ 31.07.

The 2D-NMR experiments (DQF-COSY [20], 2D-TOCSY [21], NOESY and HMQC [22]) were performed as described previously [23].

Acknowledgements

We thank Mr. J. López for technical assistance. This work was supported by Grants PB 95/0078 and PB 93/0127 from Dirección General de Investigación Científica y Técnica.

References

[1] S. Barnicki-García, *Ann. Rev. Microbiol.*, 22 (1968) 87–108.

[2] P.A.J. Gorin and J.F.T. Spencer, *Adv. Appl. Microbiol.*, 13 (1970) 25–89.

[3] A.Z.A. Teixeira, M. Iacomini, and P.A.J. Gorin, *Carbohydr. Res.*, 266 (1995) 309–314.

[4] J.A. Leal, B. Gómez-Miranda, A. Prieto, and M. Bernabé, *FEMS Microbiol. Lett.*, 108 (1993) 341–346.

[5] E. Parra, J. Jiménez-Barbero, M. Bernabé, J.A. Leal, A. Prieto, and B. Gómez-Miranda, *Carbohydr. Res.*, 251 (1994) 315–325.

[6] A. Prieto, M. Bernabé, and J.A. Leal, *Mycol. Res.*, 99 (1995), 69–75.

[7] J.A. Leal, J. Jiménez-Barbero, B. Gómez-Miranda, E. Parra, A. Prieto, and M. Bernabé, *Carbohydr. Res.*, 273 (1995) 255–262.

[8] J. Domenech, A. Prieto, M. Bernabé, and J.A. Leal, *Current Microbiology*, 28 (1994) 169–173.

[9] K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–225.

[10] A.Y. Rossman, *Mycologia*, 88 (1996) 1–19.

[11] B. Gómez-Miranda, A. Moya, and J.A. Leal, *Exp. Mycol.*, 12 (1988) 258–263.

[12] B. Gómez-Miranda, A. Prieto, and J.A. Leal, *FEMS Microbiol. Lett.*, 70 (1990) 331–335.

[13] J.A. Leal, C. Guerrero, B. Gómez-Miranda, A. Prieto, and M. Bernabé, *FEMS Microbiol. Lett.*, 90 (1992) 165–168.

[14] B. Gómez-Miranda, P. Rupérez, and J.A. Leal, *Curr. Microbiol.*, 6 (1981) 243–246.

[15] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.

[16] T. Bitter and H.M. Muir, *Anal. Biochem.*, 4 (1962) 330–334.

[17] R.Y. Taylor and H.E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.

[18] Y. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.

[19] D. Rolf, J.A. Bennek, and G.R. Gray, *J. Carbohydr. Chem.*, 2 (1983) 373–383.

[20] D.J. States, R.A. Haberkorn, and D.J. Ruben, *J. Magn. Reson.*, 48 (1982) 286–292.

[21] C. Griesinger, G. Otting, K. Wütrich, and R.R. Ernst, *J. Am. Chem. Soc.*, 110 (1988) 7870–7872.

[22] A. Bax and S. Subramanian, *J. Magn. Reson.*, 86 (1986) 346–357.

[23] J. Jiménez-Barbero, M. Bernabé, J.A. Leal, A. Prieto, and B. Gómez-Miranda, *Carbohydr. Res.*, 250 (1993) 289–299.