ELSEVIER

Carbohydrate Research 304 (1997) 281–291

CARBOHYDRATE RESEARCH

Structure of complex cell wall polysaccharides isolated from *Trichoderma* and *Hypocrea* species

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

[a] *Grupo de carbohidratos, Instituto de Química Orgánica, CSIC, Juan de la Cierva 3, 28006-Madrid, Spain*

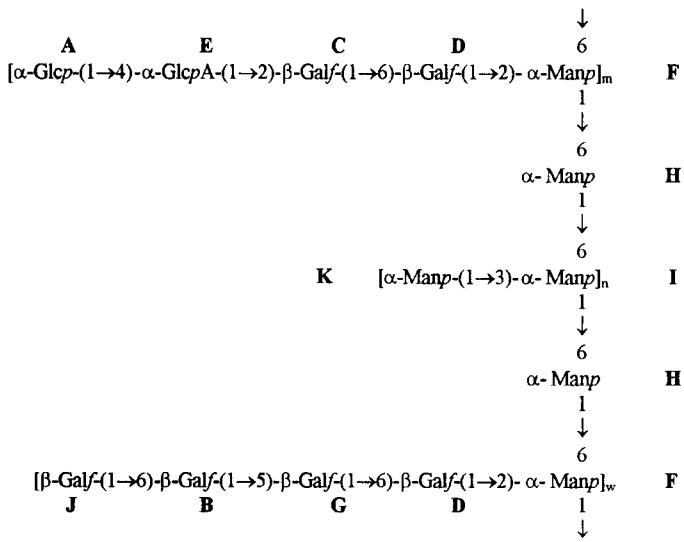
[b] *Centro de Investigaciones Biológica, CSIC, Velázquez 144, 28006-Madrid, Spain*

[c] *SIDI, Universidad Autónoma de Madrid, Cantoblanco, 28049-Madrid, Spain*

Received 13 May 1997; accepted 23 July 1997

Abstract

The structure of fungal polysaccharides isolated from the cell wall of *Trichoderma reesei*, *T. koningii*, and *Hypocrea psychrophila*, have been investigated by means of chemical analyses and 1D and 2D NMR spectroscopy. The polysaccharides have an irregular structure, idealized as follows:



* Corresponding author. Fax: +34-1-5644853; e-mail: iqomb04@cc.csic.es.

0008-6215/97/$17.00 © 1997 Elsevier Science Ltd. All rights reserved.
PII S0008-6215(97)00239-5

The proportions of the different side chains vary from a species to another, being n above some three times larger in *H. psychrophila* than in *T. reesei* or *T. koningii*. © 1997 Elsevier Science Ltd. All rights reserved

Keywords: Fungi; *T. reesei*; *T. koningii*; *H. psychrophila*; Polysaccharides; NMR spectroscopy

1. Introduction

Trichoderma [1] is a genus of hyphomycetous fungi, especially found in soils, which contains species of economical importance which are producers of cellulases [2,3], effective agents for biocontrol of plant pathogenic fungi [4], or a potential threat to the mushroom industry [5]. Some of these species have also been identified as causal agents of infections in immunosuppressed humans.

Rifai [6] defined the genus *Trichoderma* as generally accepted today. *Trichoderma* teleomorphs (perfect states) are found in *Hypocrea* and related *Hypocreaceae* genera [7–9]. The *Trichoderma* growth and morphological characteristics seem unmistakable since only four taxonomic synonyms were mentionated by Rifai. Nevertheless the inclusion of *Gliocladium virens* in *Trichoderma* [10], which has been accepted following DNA sequence analysis [11,12], implies that the *Trichoderma* description has to be modified to include the morphology of *Gliocladium* [1].

Polysaccharides are used as chemotaxonomic markers for the classification of fungi [13], yeasts [14], and lichens [15]. In the last years, different water soluble polysaccharides have been obtained from alkali extracts of fungal cell walls [16–21]. These polysaccharides, which probably are constituents of glycoproteins or peptido–polysaccharides, are antigenically relevant, may be involved in cell–cell and cell–host recognition mecanisms and have been proposed as markers at the genus or infrageneric level and they could also be used to establish the relatedness of the imperfect genera (anamorphs) with their perfect genera (teleomorphs).

The present study describes the structural features of the polysaccharides obtained from two species of *Trichoderma* and one of *Hypocrea*, by means of NMR and chemical analyses.

2. Results and discussion

Analysis for neutral sugars of alkali-extractable water-soluble polysaccharides (fractions F1S-S, see experimental) gave Glc, Gal, and Man, as shown by GLC of the alditol acetates. The absolute configuration was shown to be D for all of them. Methylation and reductive-cleavage analyses gave terminal Glc*p*, Man*p*, and Gal*f*; 2-O-substituted, 5-O-substituted, and 6-O-substituted Gal*f*; and 6-O-substituted, 2,6-di-O-substituted and 3,6-di-O-substituted Man*p*.

The conventional ^{1}H-NMR spectra for the polysaccharides F1S-S of the three species are shown in Fig. 1c–e. The spectra of both species of *Trichoderma* (Fig. 1d, e) were almost identical which demonstrated that both polysaccharides have very similar structures. The polysaccharide from *T. reesei* (Fig. 1e) was then chosen for further studies. The spectrum of *H. psychrophila* (Fig. 1c) was very similar, but the proportion of the components, as deduced from the relationships among the anomeric protons, varied to some extent. The ^{1}H-NMR spectrum of *T. reesei* contained eight major signals and a partially overlapped small peak. The ^{13}C-NMR spectra of both *H. psychrophila* and *T. reesei* (Fig. 1a, b) contained two types of signals: sharp and broad, which seemed indicative of the presence of a low mobility backbone structure with side chains of higher flexibility.

A DQCOSY spectrum showed H-1/H-2 cross-peaks for eleven distinct residues which were labelled **A–K**, according to their anomeric protons (see Figs. 2–4). Partial overlapping or coincidence of these were observed for residues **K–D**, **F–J**, and also **H–I**. The resolution of the spectrum, however, was good enough to allow a 2D-TOCSY (HOHAHA) experiment to be performed in a satisfactory way (Fig. 2b). Despite the small values of $J_{1,2}$ for most of the residues, the TOCSY subspectra through the anomeric signals showed clear connectivities to the rest of the protons for residues **A**, **B**, **C**, **E**, and **G**. For units **H** and **I**, the information obtained was very poor. The heavy overlapping of **F–J** and the low proportion of **K** precluded any discrimination and asignation of cross peaks to those units. The signals corresponding to residue **A** readily identified it as an α-Glc*p*, as deduced from the observed coupling constants ($J_{1,2}$ = 3.7; $J_{2,3}$ = 9.7; $J_{3,4}$ = 9.4; $J_{4,5}$ = 9.6 Hz). The

chemical shifts of H-2, H-3, and H-4 corresponding to residues **B** and **G** were very similar ($\Delta\delta < 0.2$ ppm) and appeared at relatively low field, indicating the presence of Gal*f* moieties. In addition, a five-proton system was observed for unit **E** which, together with a carbonyl peak appearing at 176.4 ppm in the ^{13}C-NMR spectrum, indicated the presence of an uronic acid. In addition, the vicinal coupling constants, measured on a 1D-subspectrum of the TOCSY experiment, showed values similar to those found for residue **A** ($J_{1,2} = 3.7$; $J_{3,4} = 9.7$; $J_{4,5} = 9.5$ Hz), which led to identify **E** as an α-Glc*p*A. The polysaccharide was therefore carboxyl reduced as devised by Taylor and Conrad [22]. Reductive cleavage analysis of the reduced polysaccharide revealed the presence of terminal Glc*p*, Man*p*, and Gal*f*; 2-O-substituted, 5-O-substituted, and 6-O-substituted Gal*f*; 6-O-substituted, 2,6-di-O-substituted and 3,6-di-O-substituted Man*p*, and 4-O-substituted Glc*p*, (the last being produced by reduction of 4-O-substituted Glc*p*A), suggesting a highly branched polysaccharide. As only ten different units were found and the ^{1}H-NMR spectrum detected eleven distinct residues, it seemed obvious that one residue was repeated, although located in two different neighborhoods.

In order to identify further residues and trying to confirm the glycosylation sites, we used an HMQC experiment, which maps the connectivities between carbon atoms and their directly bonded protons. The crosspeaks in the anomeric region corroborated the presence of eleven residues, with overlapping of **F–J** and **K–D**. The severe crowding of non anomeric atoms, for both protons and carbons (Fig. 2a), did not allow unambiguous assignment of all signals. A par-

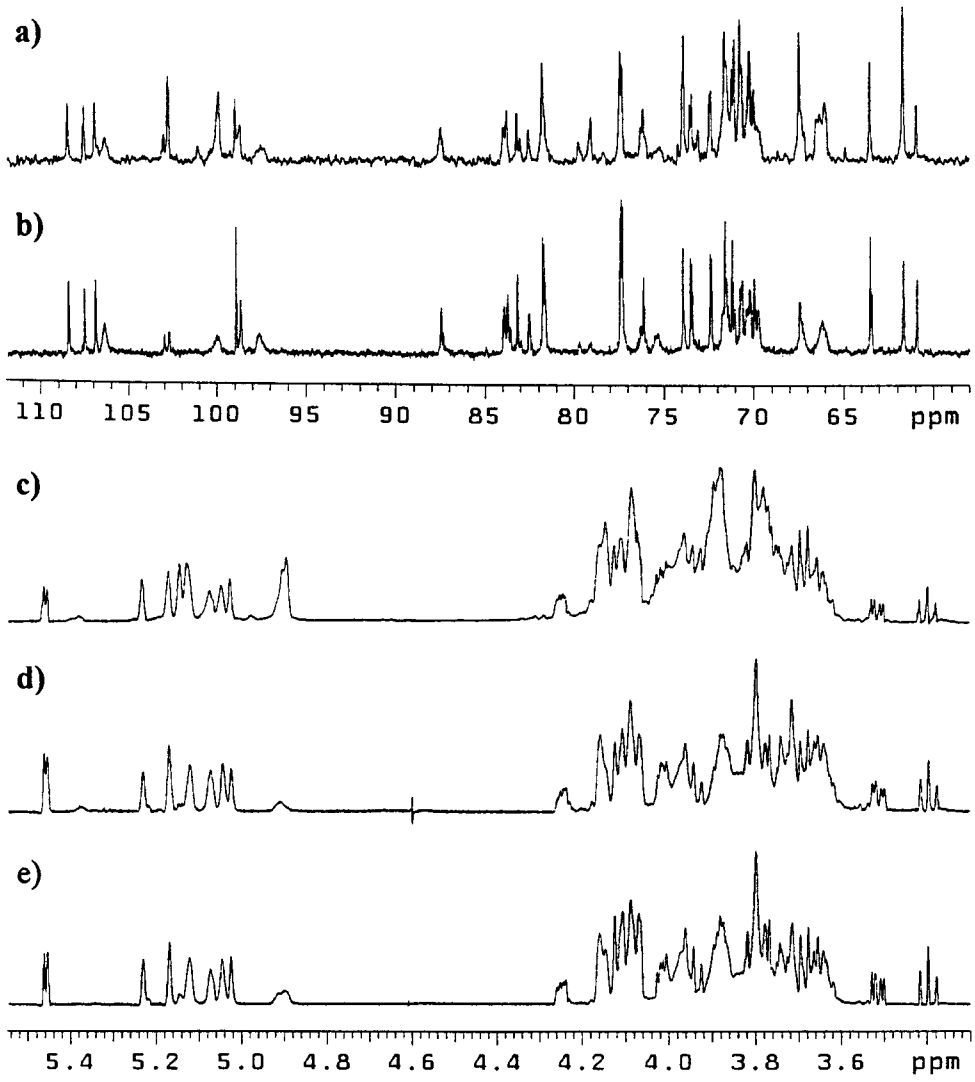


Fig. 1. ^{13}C-NMR spectra (40 °C, 125 MHz) of the cell-wall F1S-S polysaccharides isolated from: *H. psychrophila* (a) and *T. reesei* (b), and ^{1}H-NMR spectra (40 °C, 500 MHz) of *H. psychrophila* (c), *T. koningii* (d), and *T. reesei* (e).

tial assignment, however, led to determine carbon chemical shifts for units **A**, **B**, **C**, and **E**. Comparison of the values obtained (Table 1) with those of model compounds [18,23] confirmed the presence of terminal α-Glc*p* (**A**), 4-O-substituted α-Glc*p*A (**E**), 6-O-substituted Gal*f* (**B**), and 2-O-substituted Gal*f* (**C**).

Due to the congested HMQC spectrum, it seemed advisable to run an HMQC–TOCSY experiment which, starting from each HMQC cross-peak of the 2D spectrum, provides additional signals in the same row, caused by TOCSY transfer, thus allowing to find carbon chemical shifts pertaining to each residue (Fig. 3). In this way, most of the remaining carbons and protons were ascribed to their corresponding units (Table 1). Again, comparison of the observed values with those found in the literature for analogous compounds [17,18,23] led to identification of **D**, **F**, and **G** as being 6-Gal*f*, 2,6-di-O-substituted Man *p*,

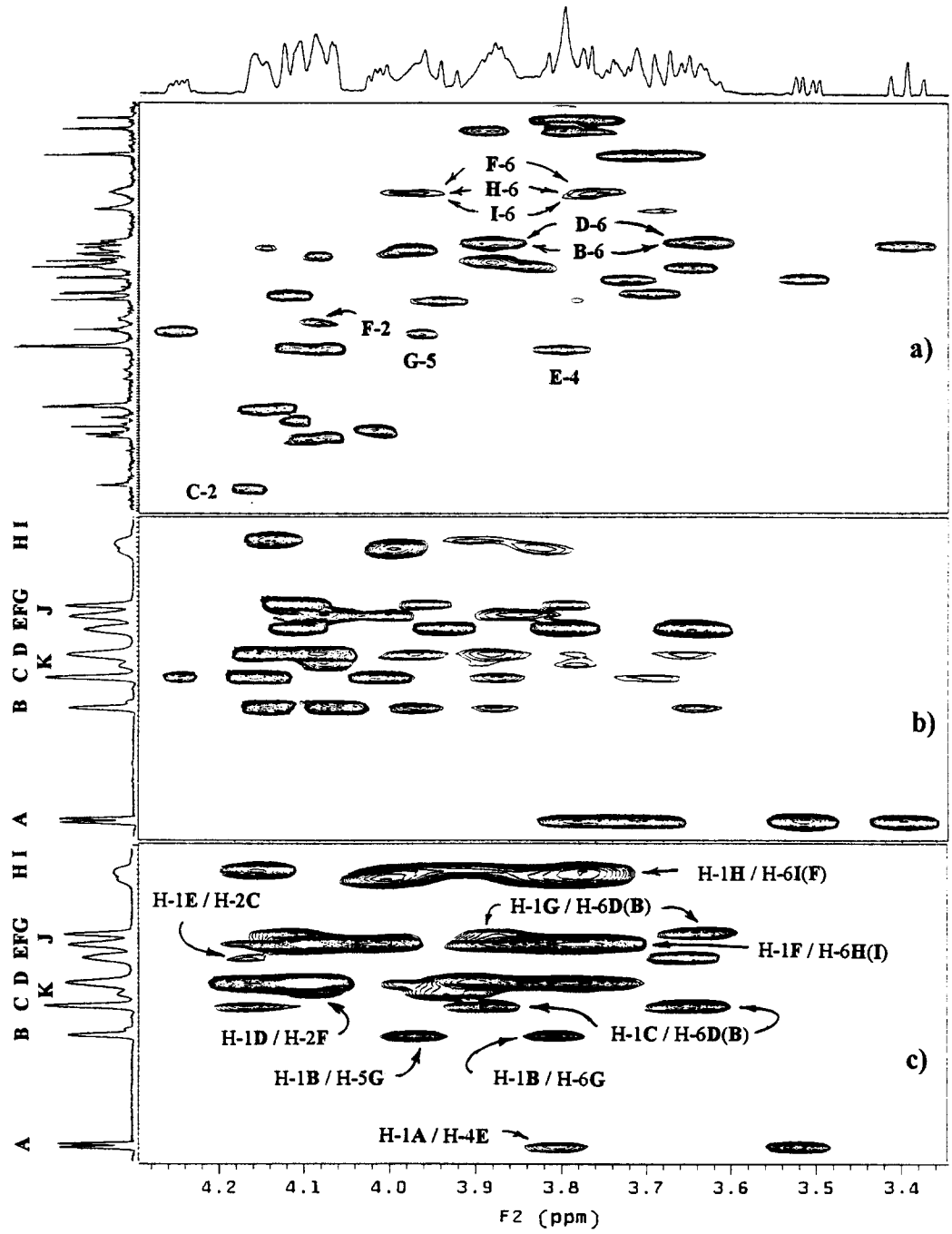


Fig. 2. 2D-NMR spectra (40 °C, 500 MHz) for selected regions of the F1S-S polysaccharide from *T. reesei*: (a) HMQC, (b) TOCSY (HOHAHA), and (c) NOESY subspectra. The anomeric protons have been labelled **A** to **K**.

and 5-O-substituted Gal*f*, respectively. As expected, one of the residues (6-O-substituted galactofuranose) appeared as two different signals in the anomeric region (**B** and **D**). The low field chemical shifts observed for H-2 and C-1 of unit **J**, made us to assign it to terminal Gal*f*.

Concerning the anomeric configuration of the different residues, the vicinal $J_{1,2}$ coupling constants of the Gal*f* moieties **B**, **C**, **D**, **G**, and **J** ($J_{1,2} < 2$ Hz) suggest all have β-configuration (compare with $J_{1,2} > 4$ Hz for α-configuration [19]). The chemical shifts of the anomeric carbons ($\delta > 106$ ppm) are in support of this findings. In addition, a carbon coupled HMQC experiment gave the following values for hetero-one bond anomeric coupling constants ($^1J_{C-1,H-1}$): **A**, 172.4; **B**, and **C**, 175.9; **D**, 174.2; **E**, 170.6; **F**, 170.7; **G**, 174.2; **H**, 176; **J**, 175.0, and **K**, 168.9, which unambiguously confirm β-configura-

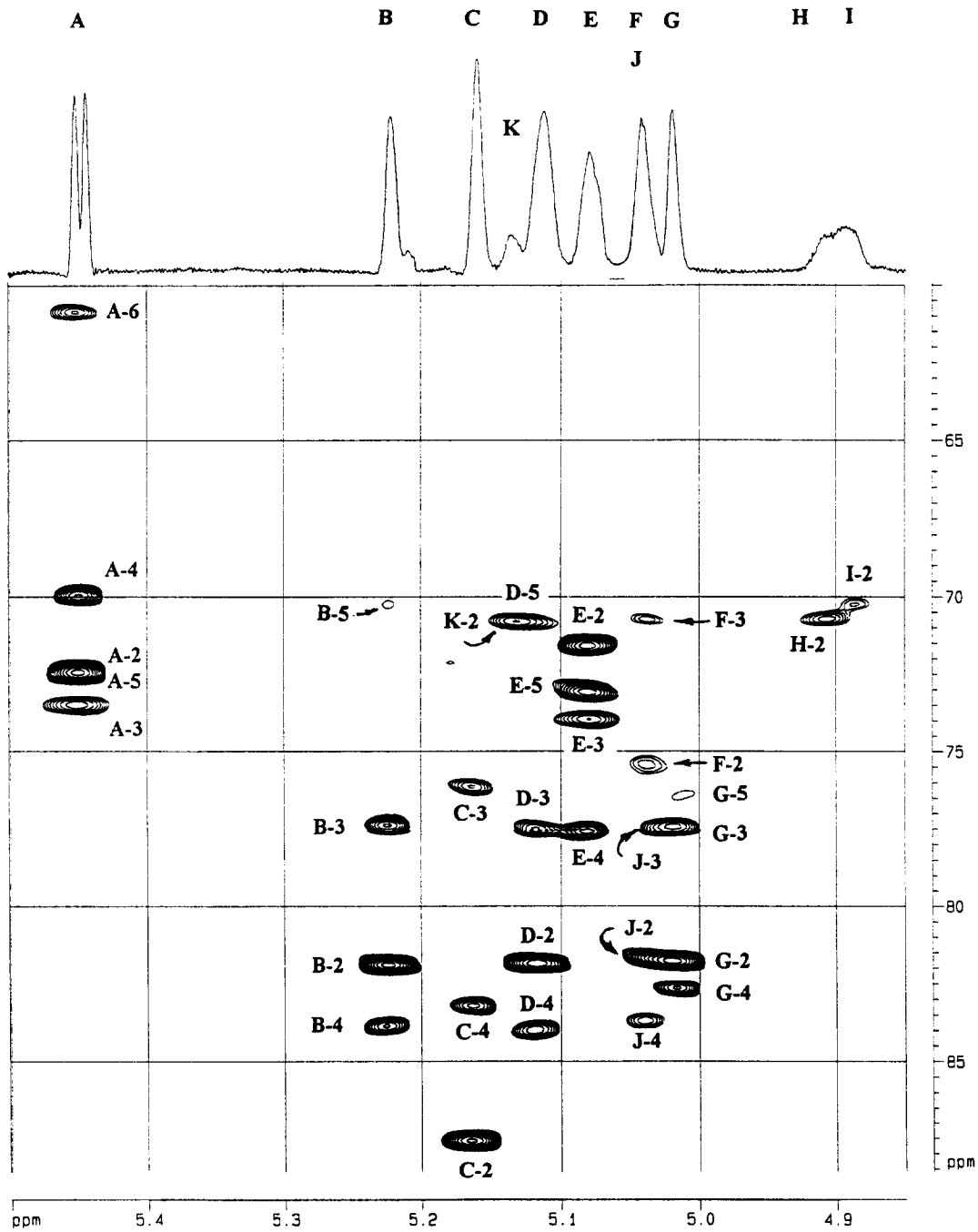


Fig. 3. 2D-HMQC–TOCSY spectrum for relevant region of the F1S-S polysaccharide from *T. reesei*. The crosspeaks have been labelled.

tion for all the Gal*f* units (**B**, **C**, **D**, **G**, and **J**) and α-configuration for Glc*p* (**A**), Glc*p*A (**E**), and all Man*p* residues (**F**, **H**, **I**, and **K**).

A 2D-NOESY spectrum, in addition to intraresidue trivial NOE's, allowed the observation of clear connections between H-1**A**/H-4**E**; H-1**C**/H-6a and H-6b**D** (**B**), and H-1**E**/H-2**C**. Possible connections H-1**D**/H-2**F** and H-1**G**/H-6a + H-6b**B** (**D**) were also detected (Fig. 2c). A 2D HMQC–NOESY experiment (not shown) confirmed the relationships described above.

In order to find additional connections among residues, an HMBC experiment was carried out. In addition to the expected intra-ring connections, peaks corresponding to H-1**A**/C-4**E**, H-1**B**/C-5**G**, H-1**C**/C-6**D**(6**B**), H-1**D**/C-2**F**, H-1**E**/C-2**C**, H-1**F**/C-6**H**(6**I**, 6**F**), H-1**J**/C-6**B**(6**D**), H-1**G**/C-6**B**(6**D**), and H-1**H**/C-6**F**(6**H**, 6**I**) could be observed (Fig. 4). This suggests the following possible sequence for the branching chains: **A** → **E** → **C** → **D** → **F** and **J** → **B** → **G** → **D** → **F**. Not much information could be drawn from the data so far for the mannan core,

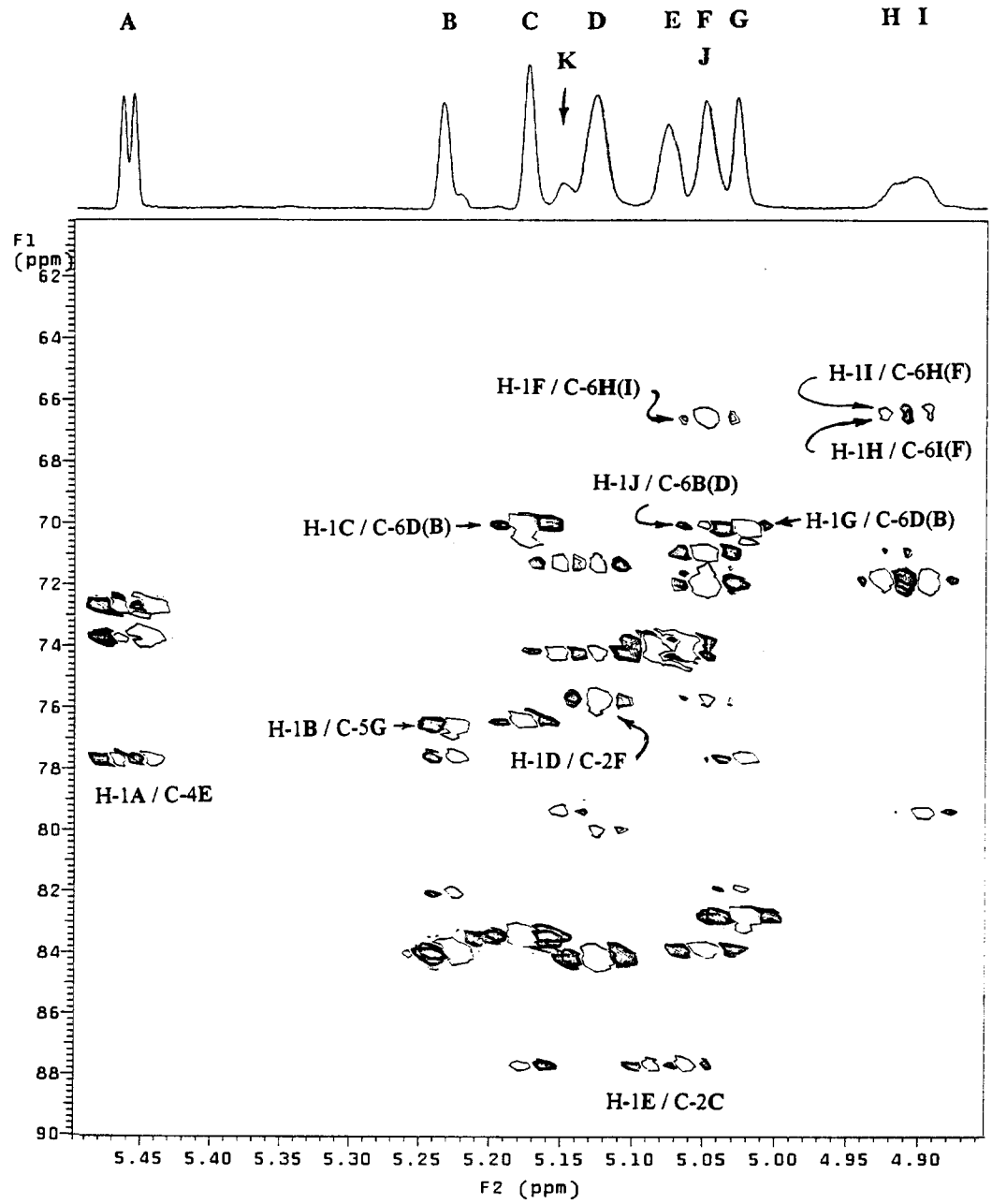


Fig. 4. Selected region of the HMBC spectrum for the F1S-S polysaccharide from *T. reesei*. Relevant crosspeaks have been labelled.

Table 1
^{1}H- and ^{13}C-NMR Chemical shifts (δ) for the alkali-extractable water-soluble polysaccharide isolated from *T. reesei*

Proton or carbon [*]								
Units		1	2	3	4	5	6a	6b
A	H	5.46	3.52	3.70	3.40	3.73	3.76	3.82
	C	99.2	72.7	73.7	70.2	72.6	61.1	
B	H	5.23	4.14	4.08	4.07	3.97	3.64	3.88
	C	107.8	82.0 [a]	77.6	83.8 [b]	70.5 [c]	**69.8**	
C	H	5.17	4.17	4.25	4.02	3.88	3.67	3.73
	C	107.2	**87.7**	76.4	83.4	71.4	63.7	
D	H	5.12	4.16	4.09	4.08	3.97	3.65	3.89
	C	106.7	82.0 [a]	77.6	84.0 [b]	70.9 [c]	**70.0** [d]	
E	H	5.08	3.65	3.94	3.80	4.12	–	–
	C	99.0	71.6	74.2	**77.7**	73.8	176.4	
F	H	5.05	4.09	ca. 3.87	ca. 3.80	ca. 3.81	3.76	3.99
	C	97.9	**75.7**	ca. 70.9	67.7	71.8	ca. **66.4**	
G	H	5.03	4.13	4.12	4.11	3.96	3.80	3.80
	C	108.7	81.9 [a]	77.6	82.6	76.5	61.7	
H	H	4.92	4.00					
	C	100.3	70.9				ca. **66.4**	
I [e]	H	4.90	4.14	3.93	n.d.	n.d.	3.94	3.76
	C	100.3	70.4	**79.4**	n.d.	n.d.	**66.4**	
J	H	5.05	ca. 4.14	ca. 4.08	ca. 4.08		3.68	3.72
	C	108.7	82.0 [a]	77.6	ca. 83.6		63.6	
K [e]	H	5.15	4.08	3.92	3.68	3.78	3.90	3.77
	C	103.0	71.0	71.5	67.6	74.0	61.8	

[*] Underlined bold numbers represent glycosylation sites.
[a,b,c,d] These values may have to be interchanged.
[e] The data for these residues were deduced from *H. psychrophila*, with the exception of H-1, H-2, and C-1.

except that it seemed to be constituted by a linear backbone of α-(1 → 6)-Man*p*. The information obtained for unit **K**, due to its low concentration, was also very poor although, taking into account the values of chemical shifts of the anomeric proton and carbon and also the methylation results, it was assigned to terminal Man*p*.

Investigation of the cell-wall polysaccharide of *H. psychrophila*, a teleomorph of the genus *Trichoderma*, gave a proton spectra analogous to that of *T.*

Table 2
^{1}H- and ^{13}C-NMR chemical shifts (δ) for the mannan core of the polysaccharide from *H. psychrophila*

Proton or carbon [*]								
Units		1	2	3	4	5	6a	6b
H	H	4.91	4.00	3.84 [a]	3.74	3.85 [a]	3.79	3.94
	C	100.2	71.0	71.6 [b]	67.5	71.7 [b]	**66.5**	
I	H	ca. 4.90	4.14	3.93	3.88	n.d.	3.94	3.76
	C	100.3	70.4	**79.4**	66.3	n.d.	**66.7**	
K	H	5.14	4.08	3.94	ca. 3.68	3.85	3.76	3.90
	C	103.0	70.9	71.5	67.6	74.0	61.8	

[*] Underlined bold numbers represent glycosylation sites.
[a,b] These values may have to be interchanged.

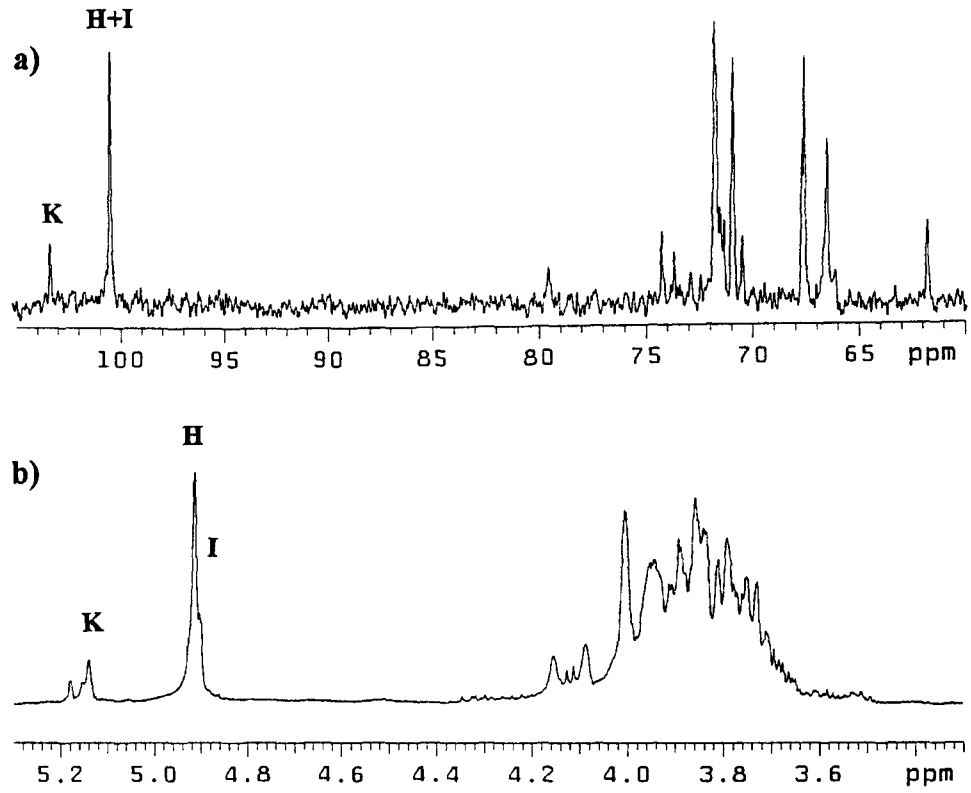
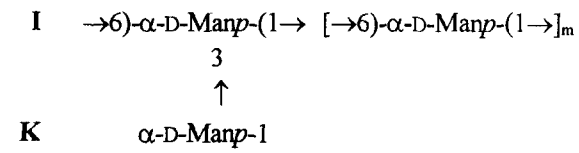


Fig. 5. ^{13}C- (a) and ^{1}H-NMR (b) spectra of the polysaccharide obtained after smooth hydrolysis of the polysaccharide isolated from *H. psychrophila*. Anomeric peaks have been labelled according to the corresponding residues in the intact polysaccharide.

reesei, but residues **K** and **I** appeared here in a greater proportion (Fig. 1c). Hence, we partially studied this polysaccharide, using an identical methodology, which allowed comparison of spectral data of both polysaccharides. It was thus established that **K** was terminal α-Man*p*, and **I**, 3,6-di-O-substituted α-Man*p*. However, the information on the core backbone was still very poor.

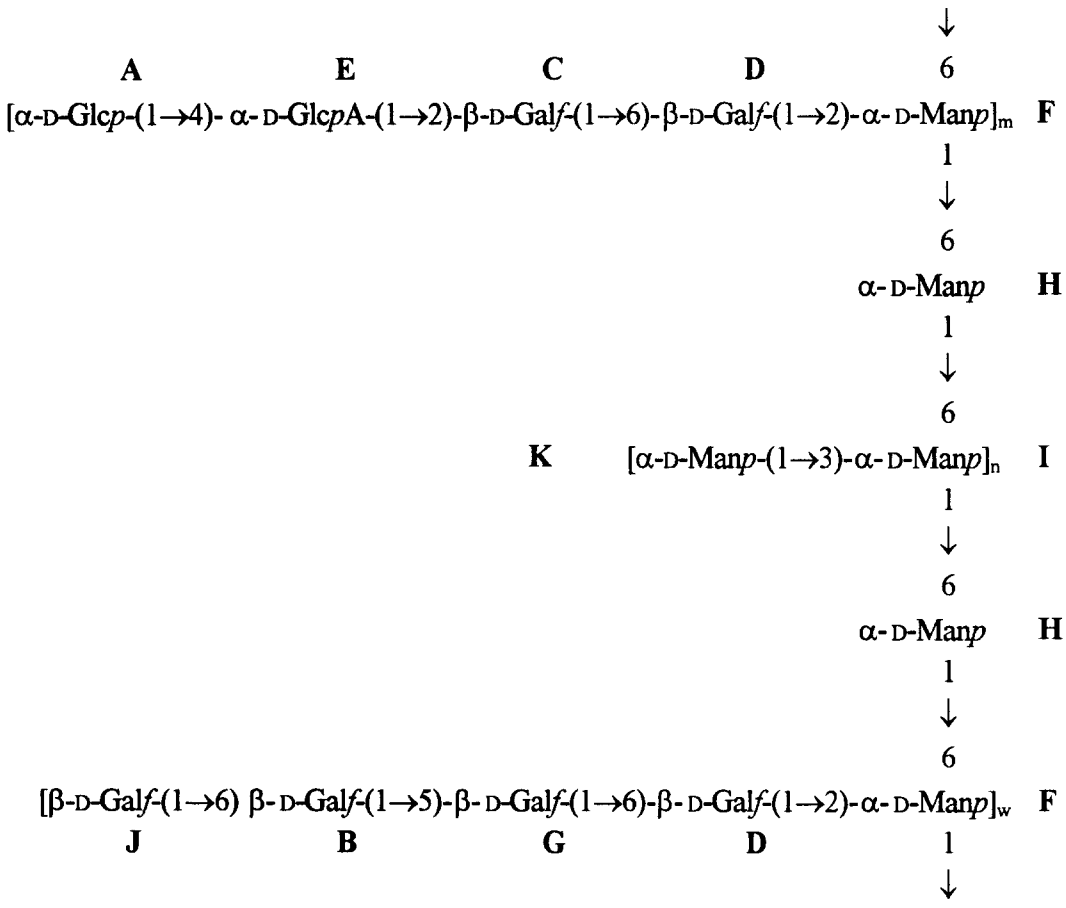
In order to further investigate the mannan core, we selectively hydrolysed the side chains of the polysaccharide of *H. psychrophila*, taking advantage of the lability of the glycosydic linkages of the furanoid rings, as compared with those of the mannan pyranoid rings. Treatment of this polysaccharide with 0.05 M H_2SO_4 for 5 h at 100 °C gave a new polysaccharide, composed exclusively of Man. Methylation analysis revealed the presence of terminal Man*p*, 6-O-substituted, and 3,6-di-O-substituted Man*p*, in the proportion 1:4:1. The ^{1}H-NMR spectrum contained three main anomeric signals (Fig. 5a), which were labelled **I**, **H**, and **K**, according to the corresponding anomeric protons of the intact polysaccharide, in a molar proportion 1:5:1. The ^{13}C-NMR spectrum (Fig. 5b), gave only three anomeric carbons. An HMQC spectrum led to assignment of most of the crosspeaks, for both the major and the minor units. The resulting chemical shifts are shown in Table 2. Once more, comparison of the values of carbon chemical shifts with those published for similar mannans [24–26] led to the identification of **H** as 6-O-linked Man*p*, **K**, as terminal Man*p*, and **I** as 3,6-di-O-substituted Man*p*. Measurement of the $^{1}J_{C-1,H-1}$ values gave 175 Hz for all three residues, which support α-configurations for all of them. Similar hydrolysis of the polysaccharide of *T. reesei* led to very similar results, but the branching mannose was here in about half the proportion that in the case above (1:11:1).

These data demonstrate that an idealised average structure for the core mannan of the polysaccharides isolated from *T. reesei* and *H. psychrophila* is:



where $m = 10$ for *T. reesei*, and $m = 4–5$ for *H. psychrophila*.

From the combined evidence, it is therefore proposed that the cell-wall polysaccharides isolated from *T. reesei* and *H. psycrophila* have an irregular structure like:



where the relationship *m*:*n*:*w* approximates to 5:1:3 for *T. reesei* and *T. koningii* and 5:5:3 for *H. psychrophila*.

A polysaccharide with similar sugar composition, which increased β-glucosidase activity, has also been isolated from *T. reesei* [27]. The structure of the mannan backbone of this polysaccharide [28], that stimulated the β-glucosidase activity by 90%, is similar to the mannan backbone obtained herein, although no branching of the skeleton has been reported in this case.

The heteroglycan obtained from the cell wall of *Trichoderma* and *Hypocrea* differs from polysaccharides obtained in a similar way from *Talaromyces* [19,20], *Neosartorya* [16], *Eupenicillium* [21], *Penicillium expansum* [18], and *Neurospora* [17]. It seems, therefore, that it could be a good chemotaxonomic marker for *Trichoderma* and its teleomorphic state *Hypocrea*.

This constitutes an additional example as the water-soluble material obtained from the alkali extracts of fungal cell walls is a source of new polysaccharides which can be used as chemotaxonomic markers at the genus or subgenus level.

3. Experimental

Growth of organisms, cell - wall preparation, isolation and chemical analyses.—Two species of *Trichoderma* and one species of *Hypocrea* were studied: *T. reesei* (CECT 2414), *T. koningii* (CECT 2412), and *H. psychrophila* (CBS 262.71). Fungi culture, cell wall preparation [29], isolation and purification of polysaccharidic fractions from hyphae, and chemical analyses were carried out as previously reported [30]. The water-soluble polysaccharide extracted from the alkali-soluble fraction of the cell-wall (F1S) was re-fractionated by treatment with distilled water (ca. 50 mg mL^{-1}) followed by centrifugation (10,000 *g*, 30 min), obtaining a solution (F1S-S) and a precipitate (F1S-I).

Reduction of the polysaccharide.—The uronic acids were detected in the NMR experiments and

measured by the carbazole reaction [31]. The polysaccharides were reduced according to the method of Taylor and Conrad [22].

Chemical and methylation analyses.—Neutral sugars were released by treating the samples overnight with methanolic 0.6 M HCl at 80 °C and hydrolysing with 3 M TFA for 1 h at 121 °C. The hydrolysis products were reduced with sodium borodeuteride and the corresponding alditols were 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 [32]. The absolute configuration of the sugars was determined as devised by Gerwig et al. [33].

The reduced samples were methylated following the method of Ciucanu and Kerek [34]. The methylated polysaccharides were hydrolysed and the monosaccharides released converted into partially methylated alditol acetates and analysed as previously described [32].

Reductive cleavage analyses were carried out in two steps, as described by Rolf et al. [35], 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 byproducts.

Partial hydrolysis of the polysaccharides of T. wreesei *and* H. psychrophila.—A sample of the corresponding polysaccharide (50 mg) was hydrolysed with 5 mL of 0.05 M H_2SO_4 for 5 h at 100 °C. The degraded polysaccharide was recovered by dialysis (molecular weight cutoff ca. 3 kDa) and lyophilization.

NMR analysis.—1D- and 2D- ^{1}H- and ^{13}C-NMR experiments were carried out at 40 °C on a Varian Unity 500 spectrometer. Proton chemical shifts refer to residual HDO at δ 4.61 ppm. Carbon chemical shifts refer to internal acetone at δ 31.07 ppm. The polysaccharides (ca. 20 mg) from fractions F1S-S 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).

The parameters used for 2D experiments were as follows: The double quantum filtered DQF–COSY experiment was performed in the phase sensitive mode using the method of States et al. [36]. 256 × 1K data matrix, zero filled to 2K × 2K, 16 scans per increment, recycle delay 2 s, spectral width 1500 Hz, sine bell filtering in both dimensions. The clean 2D-TOCSY experiment [37] was carried out in the phase sensitive mode using MLEV-17 for isotropic mixing, mixing time 145 ms, 512 × 1K data matrix, zero filled to 2K × 2K, squared cosine bell functions were applied in both dimensions. Similar parameters were used for the 2D rotating frame NOE [38] (ROESY, CAMELSPIN) experiment (mixing time 300 ms) and the 2D-NOESY experiment (mixing time 300 ms). The pure absorption one bond proton–carbon correlation experiments were collected in the ^{1}H-detection mode using the HMQC pulse sequence [39] and a reverse probe, 256 × 1K data matrix, spectral widths of 8000 and 1500 Hz in F_1 and F_2, recycling delay 1.5 s; delay corresponding to a J value of 145 Hz, squared cosine bell filtering in F_2 and gaussian filtering in F_1. 16 (^{13}C-decoupled) or 32 (^{13}C-coupled) scans were accumulated per t_1 increment. The HMBC experiment [40] was performed using a delay of 60 ms and 96 scans per increment. HMQC–TOCSY experiments were performed on a Bruker AMX 300 spectrometer. A MLEV-17 module was applied during the 60 ms of isotropic mixing. The HMQC module used a delay of 3.4 ms for evolution of the ^{1}H–^{13}C coupling constant. WALTZ-decoupling was employed during acquisition. 64 scans were used and 256 increments were acquired in the F1 dimension. TPPI was employed for processing. Square cosine bells were applied as window functions. HMQC–NOESY experiments were performed at 500 MHz, using 200 ms mixing times. Processing parameters were similar to those described for the HMQC experiments.

Acknowledgements

The authors thank Mr. J. López for technical assistance. This work was supported by Grants PB 93/1027 and PB 95/0078 from Dirección General de Investigación Científica y Técnica.

References

[1] G.J. Samuels, *Mycol. Res.*, 100 (1996) 923–935.
[2] M. Mandels, *Biochem. Soc. Trans.*, 13 (1985) 414–416.
[3] C.P. Kubicek, D.E. Eveleigh, H. Esterbauer, W. Steiner, and E.M. Kubicek-Pranz (Eds.), *Trichoderma reesei Cellulase: Biodiversity, Genetics, Physiology and Applications*. Royal Society of Chemistry, Cambridge, 1990.
[4] G.C. Papavizas, *Ann. Rev. Phyt.*, 23 (1985) 23–54.
[5] S. Muthumeenakshi, P.R. Mills, A.E. Brown, and D.A. Seaby, *Microbiol.*, 140 (1994) 769–777.

[6] M.A. Rifai, *Mycol. Pap.*, 116 (1969) 1–56.
[7] J. Bissett, *Can. J. Bot.*, 69 (1991) 2357–2372.
[8] G.J. Samuels and D.J. Lodge, *Mycologia*, 88 (1996) 301–315.
[9] N. Doi and Y. Doi, *Bull. Natl. Sci. Mus. Ser. B (Bot)*, 5 (1979) 117–123.
[10] J.A. von Arx, *Nova Hedwigia Beihefte*, 87 (1987) 1–288.
[11] S.A. Rehner and G.J. Samuels, *Mycol. Res.*, 98 (1994) 625–634.
[12] S.A. Rehner and G.J. Samuels, *Can. J. Bot.*, 73 (1995) 5816–5823.
[13] S. Bartnicki-García, *Annu. Rev. Microbiol.*, 22 (1968) 87–108.
[14] P.A.J. Gorin and J.F.T. Spencer, *Adv. App. Microbiol.*, 13 (1970) 25–89.
[15] A.Z.A. Teixeira, M. Iacomini, and P.A.J. Gorin, *Carbohydr. Res.*, 266 (1995) 309–314.
[16] J.A. Leal, J. Jiménez-Barbero, B. Gómez-Miranda, E. Parra, A. Prieto, and M. Bernabé, *Carbohydr. Res.*, 273 (1995) 255–262.
[17] J.A. Leal, J. Jiménez-Barbero, B. Gómez-Miranda, A. Prieto, J. Domenech, and M. Bernabé, *Carbohydr. Res.*, 283 (1996) 215–222.
[18] E. Parra, J. Jiménez-Barbero, M. Bernabé, J.A., Leal, A. Prieto, and B. Gómez-Miranda, *Carbohydr. Res.*, 257 (1994) 239–248.
[19] E. Parra, J. Jiménez-Barbero, M. Bernabé, J.A. Leal, A. Prieto, and B. Gómez-Miranda, *Carbohydr. Res.*, 251 (1994) 315–325.
[20] A. Prieto, M. Bernabé, and J.A. Leal, *Mycol. Res.*, 99 (1995) 69–75.
[21] J.A. Leal, B. Gómez-Miranda, A. Prieto, and M. Bernabé, *FEMS Microbiol. Lett.*, 108 (1993) 341–346.
[22] R.L. Taylor and H.E. Conrad, *Biochemistry*, 11 (1972) 1383.
[23] K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–225.
[24] E.V. Bovina, V.V. Deryabin, V.N. Gagloev, and N.G. Serebryakov, *Prikl. Biokhim. Mikrobiol.*, 24 (1988) 218–225.
[25] R.E. Cohen and C.E. Ballou, *Biochemistry*, 19 (1980) 4345–4358.
[26] J. Jiménez-Barbero, A. Prieto, B., Gómez-Miranda, J.A. Leal, and M. Bernabé, *Carbohydr. Res.*, 272 (1995) 121–128.
[27] R. Messner, K. Hagspiel, and C.P. Kubicek, *Arch. Microbiol.*, 154 (1990) 150–155.
[28] J. Rath, R. Messner, P. Kosma, F. Altmann, L. März, and C.P. Kubicek, *Arch. Microbiol.*, 164 (1995) 414–419.
[29] B. Gómez-Miranda, A. Prieto, and J.A. Leal, *FEMS Microbiol. Lett.*, 70 (1990) 331–336.
[30] J.A. Leal, C. Guerrero, B. Gómez-Miranda, A. Prieto, and M. Bernabé, *FEMS Microbiol. Lett.*, 90 (1992) 165–168.
[31] T. Bitter and H.M. Muir, *Anal. Biochem.*, 4 (1962) 339.
[32] B. Gómez-Miranda, P. Rupérez, and J.A. Leal, *Curr. Microbiol.*, 6 (1981) 243–246.
[33] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
[34] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
[35] D. Rolf, J.A. Bennek, and G.R. Gray, *J. Carbohydr. Chem.*, 2 (1983) 373–383.
[36] D.J. States, R.A. Haberkorn, and D.J. Ruben, *J. Magn. Reson.*, 48 (1982) 286–292.
[37] C. Griesinger, G. Otting, K. Wüthrich, and R.R. Ernst, *J. Am. Chem. Soc.*, 110 (1988) 7870–7872.
[38] (a) A.A. Bothner-By, R.L. Stephens, J.-M. Lee, C.D. Warren, and R.W. Jeanloz, *J. Am. Chem. Soc.*, 106 (1984) 811–813. (b) A. Bax and D.G. Davis, *J. Magn. Reson.*, 63 (1985) 207–213.
[39] A. Bax and S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565–569.
[40] A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, 108 (1986) 565–569.