Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Research 342 (2007) 2599–2603
Carbohydrate RESEARCH

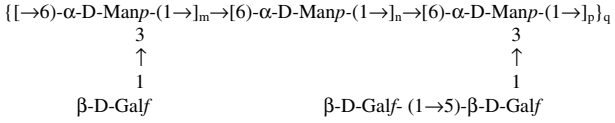
Structure of a galactomannan isolated from the cell wall of the fungus *Lineolata rhizophorae*

M. Inmaculada Giménez-Abián,[a] Manuel Bernabé,[b] J. Antonio Leal,[a] Jesús Jiménez-Barbero[a] and Alicia Prieto[a,*]

[a]*Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain*
[b]*Departamento de Química Orgánica Biológica, Instituto de Química Orgánica, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*

Received 23 May 2007; received in revised form 24 July 2007; accepted 19 August 2007
Available online 23 August 2007

Abstract—The structure of an alkali-extracted water-soluble polysaccharide isolated from the cell wall of the marine fungus *Lineolata rhizophorae* has been elucidated by chemical and spectroscopic means. The idealized repeating unit of this novel structure is



being $m \approx 41$, $n \approx 2$, and $p \approx 5$.

Keywords: Loculoascomycetes; *Lineolata*; Polysaccharide F1SS; Cell wall galactomannan; NMR spectroscopy

1. Introduction

The Loculoascomycetes comprise a group of ascomycetes mainly characterized by the ascolocular origin of their fruit bodies and by their bitunicate asci. Their species are currently placed in two classes: *Chaetothyriomycetes* and *Dothideomycetes*. The subdivisions of the latter and its relationships to other classes are uncertain. Traditional taxonomic classifications and several phylogenetic analysis of this group have been based on morphological characters, giving unsatisfactory results. Molecular data support *Lineolata* as a single-species genus whose taxonomy is unclear, being included among the group of *Dothideomycetes* of *incertae sedis*.

Polysaccharides have been used in the classification of fungi,[1–3] yeasts,[4,5] and lichens.[6] We have found that the alkali-extractable and water-soluble polysaccharides F1SS, which are minor components of the fungal cell wall (2–8%), differ in composition and structure among genera and, in certain cases, among groups of species of a genus.[7–9] They consist of a mannan skeleton or *core*, which is branched by single residues or chains of different residues. These chains may be formed by a unique residue or by different ones. Since the number of polysaccharides, which may be formed by a few different sugars, is almost unlimited[10] a large number of polysaccharides F1SS may be obtained from different groups of fungi. Therefore, fungal cell wall is a source of new polysaccharides. Due to their diversity, they have been proposed as chemotaxonomic characters at the genus or subgenus level.[3,7–9] Comparison of the polysaccharide structures from different fungal groups revealed an evolutionary pattern,[11] which allowed the proposal of evolutionary theories.[3] These polysaccharides are antigenically relevant[12–19] and are probably involved in cell–cell and/or parasite–host recognition mechanisms.[10,20] We report here on the structure of a new

* Corresponding author. Tel./fax: +34 91 8373112; e-mail: aliprieto@cib.csic.es

0008-6215/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2007.08.011

polysaccharide obtained from the cell wall of the fungus *Lineolata rhizophorae*.

2. Results and discussion

The proportion of F1SS polysaccharide amounted 4.5% of the cell wall. Gel permeation chromatography gave a single polydisperse peak, with an average molecular mass around 30 kDa. The analysis for neutral sugars gave galactose and mannose in 49.7% and 50.3%, respectively, as shown by GLC of their alditol acetates. The analysis for absolute configuration gave D-configuration for both of them. Methylation analysis gave methyl alditol acetates corresponding to 6-O-substituted and 3,6-di-O-substituted mannopyranose, terminal, and 4- or 5-O-substituted galactose (see Table 1). The identity of the last residue was revealed by reductive cleavage of the permethylated polysaccharide, which gave 5-*O*-acetyl-2,3,6-tetra-*O*-methyl-1,4-anhydrogalactitol, revealing the presence of 5-O-substituted galactofuranose and the absence of 4-O-substituted galactopyranose. The high-resolution ^{1}H NMR spectrum contained two major and two minor signals in the anomeric region, in the proportion 1:8:1:9, labeled **A**–**D** in the order of increasing field (Fig. 1a). The ^{13}C NMR spectrum (Fig. 1b) showed one minor and two major anomeric singlets (108.1, 105.6, and 100.4 ppm, respectively). In order to find the position of the different protons and carbons of the residues we carried out a series of ^{1}H–^{1}H correlation experiments (DQCOSY, TOCSY) and proton-detected ^{1}H–^{13}C heteronuclear correlation experiments (HMQC, HMBC, and HSQC–TOCSY), which led to unambiguous determination of the chemical shifts of the ^{1}H and ^{13}C of the main residues and some of the vicinal coupling constant values of the rings. The values obtained have been gathered in Table 2. The magnitude of the carbon shifts in unit **B**, as compared to those reported for α- and β-galactofuranosides[21] and the relatively low field chemical shift values for C-3 and C-6 in unit **D**, as compared with α- and β-mannopyranosides,[21,22] indicated that units **B** and **D** are, respectively, terminal galactofuranoside and 3,6-di-O-substituted mannopyranoside. This is in accordance with the data found in the methylation analysis.

The information obtained from NMR experiments for units **A** and **C** was poor due to overlapping of

Table 1. Percentages of the linkage types deduced from methylation analysis of the polysaccharides F1SS isolated from four strains of *Lineolata rhizophorae*

t_R[a] (min)	Linkage type	Characteristic fragments (*m*/*z*)	Relative abundance (%)
8.59	Gal*f*-(1→	89, 102, 118, 162, 205	47
10.87	→5)-Gal*f*-(1→[b]	87, 102, 113, 118, 131, 162, 173, 233	2
11.71	→6)-Man*p*-(1→	87, 88, 99, 102, 118, 129, 162, 189	3
14.96	→3,6)-Man*p*-(1→	118, 129, 189, 174, 234	46

[a] Retention time.
[b] The identity of →5)-Gal*f*-(1→ was established by reductive cleavage of the partially methylated polysaccharide.

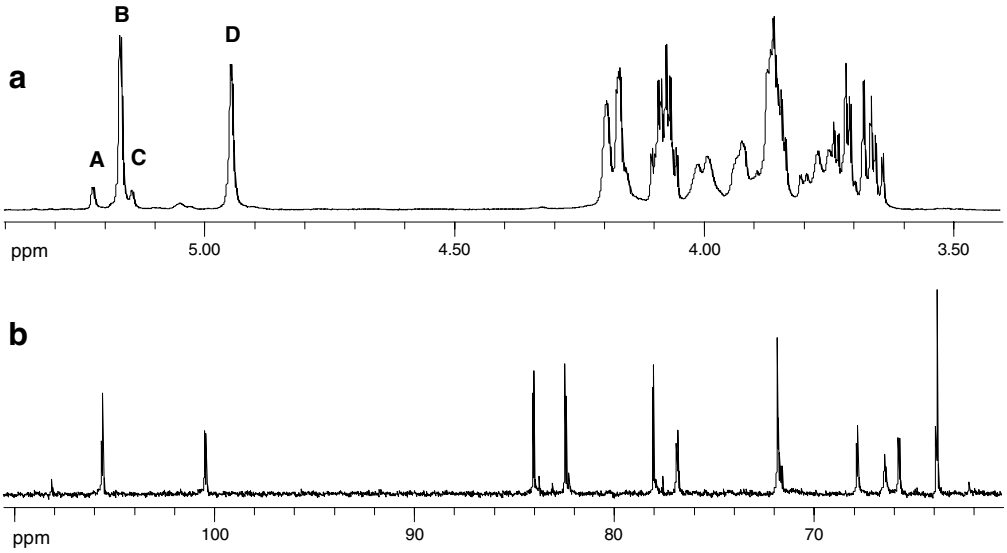


Figure 1. NMR spectra (500 MHz for ^{1}H, D_2O, 35 °C) of polysaccharide F1SS of *L. rhizophorae* CBS 641.66. (a) ^{1}H NMR spectrum showing the four distinct anomeric protons, which have been labeled **A**–**D**; (b) ^{13}C NMR spectrum at 125 MHz.

Table 2. ^{1}H and ^{13}C NMR chemical shifts (δ) and proton coupling constants (J; Hz) for the alkali-extractable and water-soluble cell wall polysaccharide isolated from *Lineolata rhizophorae*

Residue		1	2	3	4	5	6a	6b
A	H	5.22	4.16	4.07	n.d.	ca. 3.87	3.73	3.68
	J	1.9						
	C	108.1	82.4	77.9	83.7	71.6	63.8	
B	H	5.16	4.17	4.09	4.07	3.85	3.72	3.66
	J	1.7	3.3	6.4	3.9	4.5; 7.2	11.7	
	C	105.6	82.4	78.0	84.0	71.8	63.8	
C	H	5.15	4.16	ca. 4.12	n.d.	ca. 3.98	3.82	3.82
	J	ca. 1.7						
	C	105.6	82.2	77.6	83.1	***76.7***	62.2	
D	H	4.95	4.19	3.93	3.87	ca. 3.85	4.00	3.76
	J	1.5						
	C	100.4	67.8	***76.8***	65.8	71.7	***66.5***	

n.d.: not determined.
Bold italic numbers represent glycosylation sites.

signals, both for proton and carbon spectra. However, from the methylation and reductive cleavage results and after comparison of the obtained ^{13}C chemical shifts with those of model compounds, we were able to establish most of the values of these minor residues and to deduce that **A** was also terminal Gal*f* while **C** was identified as 5-O-substituted Gal*f*. Concerning the configuration of the anomeric centers, the homonuclear coupling constant $J_{1,2}$ values deduced for fragments **A** and **B** (<2 Hz) together with their C-1 ^{13}C NMR chemical shifts (>105 ppm) indicated that all Gal*f* residues have β-configuration (compare with $J_{1,2}$ around 4 Hz and chemical shifts <104 for α-Gal*f*).[21,23] For the mannopyranose fragments, a proton-coupled HMQC experiment allowed the measurement of the value for $^{1}J_{C\text{-}1,H\text{-}1}$ in unit **D** (173.9 Hz), which, in accordance with the low-field chemical shift values, are in favor of the anomeric configuration being α-.[21,24] The measurement of $^{1}J_{C\text{-}1,H\text{-}1}$ of residues **A** and **B** gave 174.2 and 174.1, respectively.

To discriminate between the two possibilities of arrangement of fragment **D**, namely a backbone of α-(1→6)-Man*p* with Gal*f* moieties at C-3, or either a backbone of α-(1→3)-Man*p* with Gal*f* moieties at C-6, we carried out a 2D NOESY experiment, which gave, among others, cross-peaks H-1**A**/ H-5**C**, H-1**B**/ H-3**D** (strong), H-1**B**/H-4**D** (weak), H-1**D**/ H-6a**D**′, H-1**D**/H-6b**D**′, and H-1**D**/H-4**D**′, being **D**′ a second unit of **D**, in support of unit **B** being (1→3) linked to unit **D**, which is, in turn, (1→6) linked to a second unit of **D** (**D**′). Furthermore, a long-range proton–carbon correlation HMBC experiment (Fig. 2), in addition to intra-residue signals, showed cross-peaks for H-1**A**/C-4**A**, H-1**B**/C-4**B**, H-1**C**/C-4**C**, and H-1**D**/C-5**D**, which corroborated the furanoid character of residues **A**, **B**, and **C**, and the pyranoid character of **D**, and crosspeaks H-1**B**/C-3**D**, and H-1**D**/C-6 **D**′, unequivocally demonstrating the above statements. In addition, two small crosspeaks H-1**A**/C-5**C** and H-1**C**/C-3**D** could also be observed in

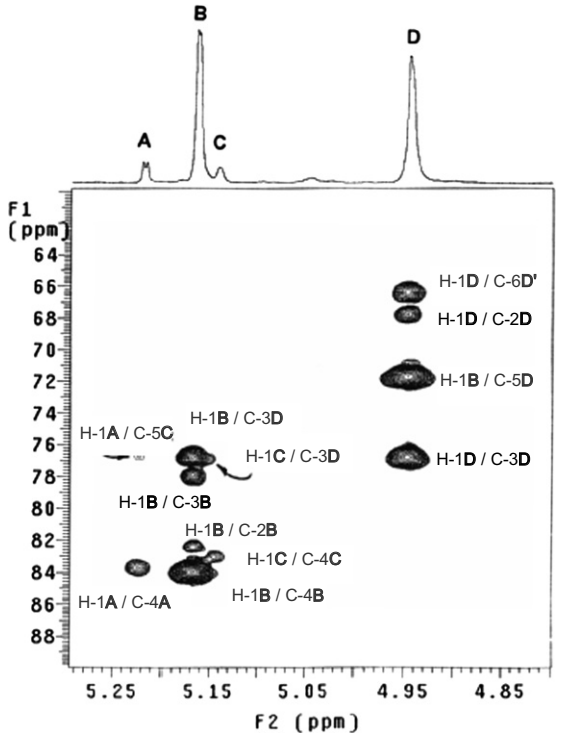
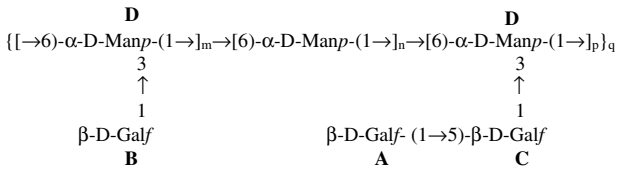


Figure 2. Selected region of the 2D-HMBC spectrum of polysaccharide F1SS of *L. rhizophorae* CBS 641.66. Crosspeaks have been labeled.

the HMBC. Having in mind the integration of the ^{1}H NMR spectrum and the proportion found in the methylation and reductive cleavage results, this fact supports **A** as being connected to C-5 of unit **C**, which is, in turn, linked to C-3 of Man*p* **D**. Thus, around 12% of the Gal*f* side moieties are substituted at C-5 by terminal Gal*f* residues. Finally, the small percentage of 6-O-substituted Man*p* is most probably due to some backbone Man*p* units lacking substituents at C-3. From all the combined data, we propose that the idealized structure for the alkali-extracted water-soluble polysaccharide of *L. rhizophorae* corresponds to



being $m \approx 41$, $n \approx 2$, and $p \approx 5$.

The possibility of establishing the real distribution that the side residues hold along the mannan backbone is still unfortunately beyond our reach.

This structure differs from that found in the polysaccharide F1SS from other fungi. The polysaccharide F1SS described for several species from the order Sordariales, as *Neurospora crassa*, has monosaccharidic β-Gal*f* side chains attached to the C-2 of the mannan backbone instead of to the C-3.[25,26] The structure of the polysaccharide F1SS from *Apodus deciduus* is also different, since its (1→6)-mannose chain is branched at C-3 by an α-D-Galf-α-(1→2)-Galf disaccharide.[27] In the structure described herein, the galactofuranose residues of the side chains display the β-configuration. In fungal polysaccharides F1SS, concretely among the Ascomycetes, the β-galactofuranose side chains are the most frequently found linked to the mannan core.

Our results add a new polysaccharide to those already described for different fungal groups. This kind of molecules has shown to reflect taxonomic and phylogenetic relationships in other Ascomycetes and then should be used to establish the relationships of *L. rhizophorae* with other fungi.

3. Experimental

3.1. Microorganisms and culture media

The strain of *L. rhizophorae* used was CBS 641.66. The microorganism was 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 for mycelium production have been previously described.[28]

3.2. Wall material preparation and fractionation

The preparation of wall material[29] and the fractionation procedure[30] 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). Fraction F1S was re-fractionated by treatment with a small portion of water (about 50 mg mL^{-1}), followed by centrifugation (10,000*g*, 30 min), giving a solution, which was freeze-dried (fraction F1SS), and a precipitate (fraction F1SI). F1I and F1SI were not further investigated. F1SS was treated with 50% ethanol in water. The precipitate was discarded, and the supernatant was dried to eliminate the ethanol, resuspended in water and freeze-dried. The resulting material constituted polysaccharide F1SS. Purity of the polysaccharide was confirmed by GPC on Sepharose CL6B under conditions previously described.[30]

3.3. Chemical analyses

Neutral sugars were released 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 were acetylated with pyridine–Ac_2O (1:1) for 1 h at 100 °C. Identification and quantification were carried out by gas-liquid chromatography (GLC) using an SP-2380 (30 m × 0.25 mm, 0.2 μm film thickness) and a temperature program (210–240 °C, 3 min initial hold, 15 °C/min ramp rate and 7 min final time). The absolute configuration of the sugars was determined as devised by Gerwig et al.[31]

3.4. Methylation analyses

The polysaccharide was methylated following Ciucanu and Kerek's method[32] and hydrolyzed with 1.5 M TFA at 121 °C for 1 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 an SPB-1 column (30 m × 0.25 mm, 0.2 μm film thickness), a temperature program (160–210 °C, 1 min initial hold, 2 °C/min ramp rate) and a mass detector Q-mass from Perkin–Elmer. Quantifications were made according to peak area.

3.5. Reductive cleavage analyses

Analyses by the reductive-cleavage method were performed in two steps with trimethylsilyl triflate as catalyst,[33] but the reactions were carried out under Ar and the time during the reductive cleavage was successively shortened to 2 h, to minimize unwanted byproducts or artifacts. The partially methylated anhydroalditol acetates obtained were analyzed by GLC–MS using a fused silica SPB-1 column (30 m × 0.25 mm, 0.2 μm film thickness) and a temperature range of 150–200 °C (3 min initial hold and ramp rate 3 °C min^{-1}).

3.6. NMR analysis

Polysaccharide F1SS (~20 mg) was dissolved in D_2O (0.8 mL) followed by centrifugation (10,000*g*, 20 min). The supernatant (ca. 0.7 mL) was used for recording ^1H NMR spectra. The column-purified sample for 2D

experiments was lyophilized, 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). 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 chemical shifts refer to residual HDO at δ 4.66 and carbon chemical shifts to internal acetone at δ 31.07. The 2D NMR experiments (DQF-COSY, 2D-TOCSY, HMQC) were performed as described previously,[34] using the standard Varian Software.

Acknowledgments

The authors thank Mr. J. López for technical assistance. This work was supported by Grant DGI-CTQ2006-10874-C02-01 and 02 from Ministerio de Educación y Ciencia of Spain.

References

1. Bartnicki-García, S. *Annu. Rev. Microbiol.* **1968**, *22*, 87–108.
2. Bartnicki-García, S. Evolutionary biology of the fungi. In *The Cell Wall: A Crucial Structure in Fungal Evolution*; Rayner, A. D. M., Brasier, C. M., Moore, D., Eds.; Cambridge University Press: New York, 1987; pp 389–403.
3. Prieto, A.; Ahrazem, O.; Bernabé, M.; Leal, J. A. Pathogenic fungi: structural biology and taxonomy. In *Polysaccharides F1SS: Taxonomic and Evolutionary Characters for Ascomycetes*; San-Blas, G., Calderone, R., Eds.; Caister Academic Press: Wymondham, Norfolk, 2004; Vol. 10, pp 319–360.
4. Gorin, P. A. J.; Spencer, J. F. T. *Adv. Appl. Microbiol.* **1970**, *13*, 25–89.
5. Weijman, A. C. M.; Golubev, W. I. The expanding realm of yeast-like fungi. In *Carbohydrate Patterns and Taxonomy of Yeasts and Yeast-like Fungi*; de Hoog, G. S., Smith, M. T. H., Weijman, A. C. M., Eds.; Elsevier Science: Amsterdam, 1987; pp 361–371.
6. Teixeira, A. Z. A.; Iacomini, M.; Gorin, P. A. J. *Carbohydr. Res.* **1995**, *266*, 309–314.
7. Leal, J. A. Microorganisms in ruminant nutrition. In *Water-soluble Polysaccharides of Fungal Cell Walls*; Prins, R. A., Stewart, C. S., Eds.; Nottingham University Press, 1994.
8. Leal, J. A.; Bernabé, M. Chemical fungal taxonomy. In *Taxonomic Applications of Polysaccharides*; Frisvad, J. C., Bridge, P. D., Arora, D. K., Eds.; Marcel Dekker: NY Basel Hong Kong, 1998; Vol. 7, pp 153–181.
9. Leal, J. A.; Prieto, A.; Ahrazem, O.; Pereyra, T.; Bernabé, M. *Rec. Res. Devel. Microbiol.* **2001**, *5*, 235–248.
10. Albersheim, P.; Darvill, A. G.; Davis, K. R.; Lau, J. M.; McNeil, M.; Sharp, J. K.; York, W. S. Structure, function, and biosynthesis of plant cell walls. In *Why Study the Structures of Biological Molecules? The Importance of Studying the Structures of Complex Carbohydrates*; Dugger, W. M., Bartnicki-García, S., Eds.; American Society of Plant Physiologists: Rockville, Maryland, 1984; pp 19–51.
11. Bernabé, M.; Ahrazem, O.; Prieto, A.; Leal, J. A. *EJEAFChe* **2002**, *1* http://ejeafche.uvigo.es.
12. Suzuki, S. *Curr. Top. Med. Microbiol.* **1997**, *8*, 57–70.
13. Ahrazem, O.; Gómez-Miranda, B.; Prieto, A.; Barasoaín, I.; Bernabé, M.; Leal, J. A. *Mycol. Res.* **2000**, *104*, 603–610.
14. Bennett, J. E.; Bhattacharjee, A. K.; Glaudemans, C. P. J. *Mol. Immunol.* **1985**, *22*, 251–254.
15. Domenech, J.; Barasoaín, I.; Prieto, A.; Gómez-Miranda, B.; Bernabé, M.; Leal, J. A. *Microbiology* **1996**, *142*, 3497–3503.
16. Domenech, J.; Prieto, A.; Barasoaín, I.; Gómez-Miranda, B.; Bernabé, M.; Leal, J. A. *Microbiology* **1999**, *145*, 2789–2796.
17. Leitao, E. A.; Bittencourt, V. C. B.; Haido, R. M. T.; Valente, A. P.; Peter-Katalinic, J.; Letzel, M.; de Souza, L. M.; Barreto-Bergter, E. *Glycobiology* **2003**, *13*, 681–692.
18. Pinto, M. R.; Mulloy, B.; Haido, R. M.; Travassos, L. R.; Barreto Bergter, E. *Microbiology* **2001**, *147*, 1499–1506.
19. Latgé, J. P.; Debeaupuis, J. P.; Sarfati, J.; Diaquin, M.; Paris, S. *Arch. Med. Res.* **1993**, *24*, 269–274.
20. Bernardo, D.; Cabo, A. P.; Novaes-Ledieu, M.; Mendoza, C. G. *Can. J. Microbiol.* **2004**, *50*, 729–735.
21. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
22. Ahrazem, O.; Blázquez, G.; Prieto, A.; Bernabé, M.; Leal, J. A. *Mycol. Res.* **2002**, *106*, 1187–1192.
23. Cyr, N.; Perlin, A. S. *Can. J. Chem.* **1979**, *57*, 2504–2511.
24. Bock, K.; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 293–297.
25. Leal, J. A.; Jiménez-Barbero, J.; Gómez-Miranda, B.; Prieto, A.; Domenech, J.; Bernabé, M. *Carbohydr. Res.* **1996**, *283*, 215–222.
26. Nakajima, T.; Yoshida, M.; Hiura, N.; Matsuda, K. *J. Biochem.* **1984**, *96*, 1013–1020.
27. Ahrazem, O.; Prieto, A.; Leal, J. A.; Jiménez-Barbero, J.; Bernabé, M. *Carbohydr. Res.* **2002**, *337*, 1503.
28. Gómez-Miranda, B.; Moya, A.; Leal, J. A. *Exp. Mycol.* **1988**, *12*, 258–263.
29. Prieto, A.; Rupérez, P.; Hernández-Barranco, A.; Leal, J. A. *Carbohydr. Res.* **1988**, *177*, 265–272.
30. Ahrazem, O.; Gómez-Miranda, B.; Prieto, A.; Bernabé, M.; Leal, J. A. *Arch. Microbiol.* **2000**, *173*, 296–302.
31. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1979**, *77*, 10–17.
32. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
33. Gray, G. R. *Methods Enzymol.* **1987**, *138*, 26–38.
34. Jiménez-Barbero, J.; Bernabé, M.; Leal, J. A.; Prieto, A.; Gómez-Miranda, B. *Carbohydr. Res.* **1993**, *250*, 289–299.