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Structural determination of the complex exopolysaccharide from the virulent strain of *Cryphonectria parasitica*

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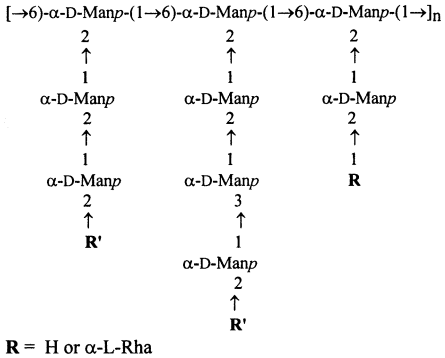
This paper is dedicated to Professor Lorenzo Mangoni on occasion of his 70th birthday

Abstract

The structure of a new exopolysaccharide from the virulent strain of *Cryphonectria parasitica* was elucidated by means of 2D NMR spectroscopy and selective degradations (mild hydrolysis and acetolysis). The polysaccharide is built up of mannose, galactose and rhamnose and has a rather complex non-repetitive structure that can be idealised as follows:



R = H or α-L-Rha
R' = H or [→6)-β-D- Gal*f*-(1→5)-β-D-Gal*f*-(1→]n

Keywords: Exopolysaccharide; *Cryphonectria parasitica*; Fungi; NMR spectroscopy

1. Introduction

Exopolysaccharides (EPSs) of fungi are thought to be agents of phytotoxicity and although the mechanism by which fungi invade plants is not known, EPSs can play a role in plant–fungal interactions.[1] *Cryphonectria parasitica* (Murr.) Barr is the causal agent of chestnut blight,[2] which is characterised by the formation of a 'gelatinous zone' beyond the advancing edge of the mycelium. It is well known that in a *C. parasitica* population it is easily possible to isolate forms that appear different and that have reduced virulence.[2,3] It has been found to produce a large quantity of pullulan as EPS together with a galactan built up of galactofuranose residues.[3] Furthermore, only the virulent strain CP159 produces also a small amount of a polymer containing rhamnose, galactofuranose and mannose, the structure of which is the purpose of this structural elucidation.

2. Results and discussion

The crude exopolysaccharide material was analysed by compositional analysis and found to contain D-glu-

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cose and traces (lower than 5%) of D-galactose, D-mannose and L-rhamnose. The methylation data were in agreement with the presence of a gluco-polymer already detected as pullulan.[3] This was removed by precipitation with ethanol and the supernatant, free of glucose, still contained galactose mannose and rhamnose in a proportion of 4:4:1. After lyophylisation it was subjected to several gel permeation chromatographies but it was constantly eluted as a single peak. The methylation analysis showed the presence of terminal Gal*f*, 6-substituted Gal*f*, 5-substituted Gal*f* (otherwise 4-substituted Gal*p*), terminal Rha*p*, terminal Man*p*, 2-substituted Man*p*, 3-substituted Man*p* and 2,6-di-substituted Man*p*.

The ^{1}H NMR spectrum showed a complex pattern of signals in the anomeric region and a broad signal in the shielded region which could be attributed to the methyl group of rhamnose residue. The ^{13}C NMR spectrum showed several signals in the anomeric region, some of them deshielded around 108 ppm validating the hypothesis of the presence of furanose residues, most likely in a β-configuration. The use of several 2D NMR experiments (DQF-COSY, TOCSY, NOESY, HSQC, HSQC-TOCSY and HMBC) allowed the identification of ^{1}H and ^{13}C resonances of ten different pyranose spin systems (Table 1).

From the HSQC (Fig. 1) experiment it was possible to assign all the anomeric protons (**A**–**L**) to their correlated carbons, thus the signals **A** and **L** at δ 5.24 and 5.03 ($^{3}J_{\mathrm{H\text{-}1,H\text{-}2}}$ 1.9 Hz) were correlated to the signals at 107.9 and 108.3 ppm, respectively, thus indicating the β-galactofuranose residues[4] ($^{1}J_{\mathrm{C,H}}$ 181 Hz). Going through all the measured spectra, **A** and **L** residues were identified as 6-substituted Gal*f* and 5-substituted Gal*f*, respectively, in accordance with the downfield shifts of the C-6 of residue **A** and C-5 of the residue **L**. The residues **D** and **F** were both endorsed as terminal non reducing β-galactofuranose units possessing all typical chemical shifts and without any carbon shifted by glycosylation. The finding of two different resonances suggests a different chemical environments for the terminal β-galactofuranose.

The other anomeric signals (**B**, **C**, **E**, **G**, **H** and **I**) all had α-*manno* configuration ($^{3}J_{\mathrm{H\text{-}2,H\text{-}3}}$ 3 Hz and $^{1}J_{\mathrm{C,H}}$ 171 Hz) and represented the different mannose residues except for the spin system **I** at 5.05/102.7 ppm which was attributed to α-rhamnose due to the cross peaks of the methyl signal at 1.31/17.3 found in the COSY, TOCSY and HSQC.

The location of the Gal*f* residues was deduced by NOESY spectrum, the anomeric signal **A** showed inter-residual contacts with the H-5 proton of **L** while H-1 of

Table 1
^{1}H and ^{13}C resonances of the isolated polymer after precipitation with cold ethanol. The NMR spectra were measured in D_2O at 27 °C and chemical shifts are expressed relative to acetone (^{1}H, 2.225 ppm; ^{13}C, 31.5 ppm). Residues are in the pyranose ring if not specified

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
A	5.24	4.15	4.08	4.08	3.97	3.80/3.60
6-Gal*f*	107.9	81.7	76.8	83.3	70.3	69.9
B	5.24	4.14	3.99	3.79	3.71	3.88/3.72
2-Man	101.2	77.2	70.0	68.0	73.0	61.3
C	5.22	4.14	4.01	3.79	3.72	3.88/3.72
2-Man	101.2	78.0	70.0	68.0	73.0	61.3
D	5.20	4.12	4.09	4.08	3.99	3.85/3.84
t*-Gal*f	105.5	82.0	77.0	84.0	71.0	63.5
E	5.18	4.23	4.00	n.d.	3.75	3.88/3.73
3-Man	102.6	71.1	79.1	n.d.	73.6	61.8
F	5.12	4.09	4.07	4.09	3.99	3.85/3.84
t*-Gal*f	106.5	82.0	77.5	84.3	71.2	63.5
G	5.09	4.09	3.69	3.69	3.85	3.65/4.00
2,6-Man	99.2	79.1	70.7	67.8	73.6	66.5
H	5.07	4.12	3.85	3.67	3.78	3.88/3.73
***t*-Man**	102.6	71.1	71.1	67.7	73.9	61.8
I	5.05	4.07	3.70	n.d.	4.12	1.31
***t*-Rha**	102.7	70.7	73.0	n.d.	68.9	17.3
L	5.03	4.13	4.08	4.08	3.98	3.80
5-Gal*f*	108.3	81.7	76.8	82.9	75.9	62.0

n.d., not determined.

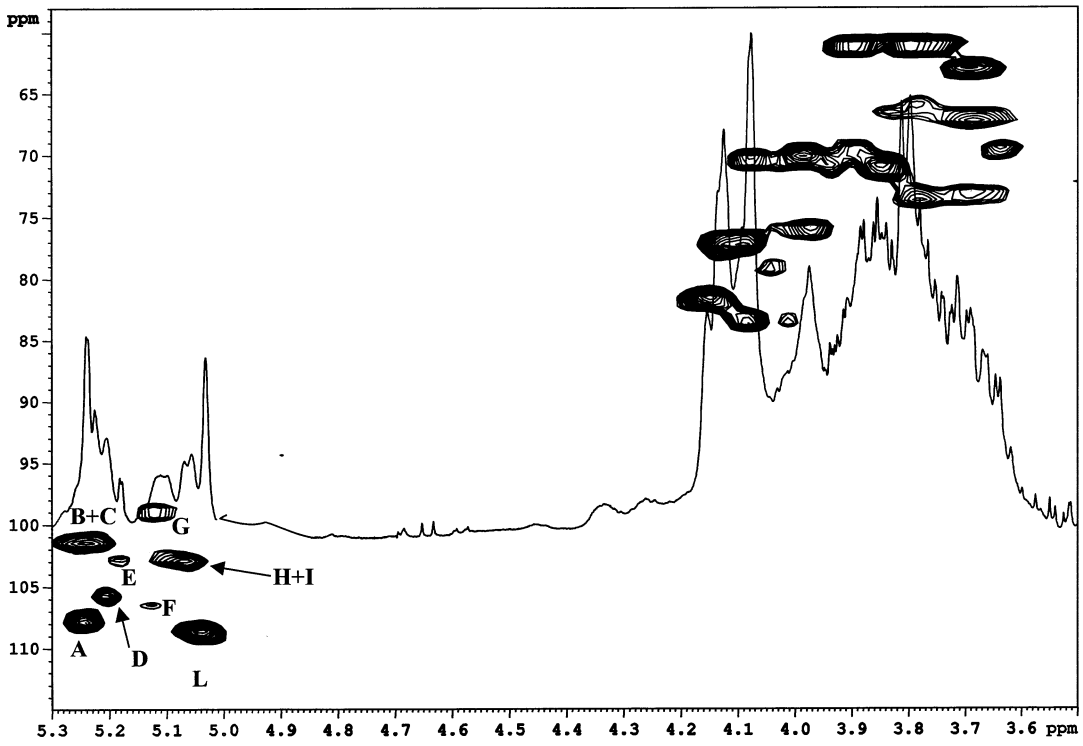


Fig. 1. The HSQC spectra with ^{1}H NMR in the inset of EPS with the anomeric signals assignments.

this last showed NOE contact with H-6a, H-6b protons of residue **A**, therefore allowing the hypothesis of two alternating galactofuranose units in the chain. The HMBC spectrum confirmed the assigned substructure containing the required long-range correlations between C-6 and C-5 of **A** and **L** with the anomeric proton signals of **L** and **A**, respectively. These structural data are very close to those of galactans found in the avirulent strains (CP102 and CP263) of the same fungus.[3] The possibility that this galactan can contaminate the polysaccharide arising from the virulent strain cannot be excluded nevertheless the failed attempts to isolate it. However, chemical and spectroscopical evidences (see below) clearly showed the existence of covalent linkage between the galactan substructure and the rest of polysaccharide, which is a mannan.

In the HMBC spectrum it was possible to identify a long range correlation between the anomeric protons of the 5-Gal*f* residue **L** to C-2 of 2-substituted mannose **C** and from the H-2 of this residue to the anomeric carbon of **L**. In agreement, in the NOESY spectrum **L** showed NOE contact with the proton H-2 of **C**. Likewise, the anomeric proton of rhamnose exhibited a NOE contact with the proton H-2 of 2-substituted Man*p*. Because of the several overlapping signals in the anomeric and H-2 region no defined assumptions were possible regarding the mannose sequence. Thus, exploiting the acid lability of the galactofuranose residues, compared with mannose and rhamnose, the polymer was treated with 0.01 M HCl (100 °C for 24 h) in order to obtain a mannan polymer (**EPS I**). Only galactose was found as free monosaccharide, and the newly obtained polymer was subjected to compositional (0.1:4:1 Gal:Man:Rha) and methylation analyses. The methylation data showed the presence terminal Rha*p*, terminal Man*p*, 2-substituted Man*p*, 3-substituted Man*p*, 2,6-disubstituted Man*p*. In addition, while 6-substituted Gal*f*, 5-substituted Gal*f* residues were lacking, terminal Gal*f* was present supporting the existence of the covalent linkage with the mannan counterpart. The ^{1}H and ^{13}C NMR spectra showed a reduced pattern of anomeric signals. The newly obtained polymer was subjected to 2D NMR analysis. From the HSQC spectrum (Fig. 2) seven anomeric signals were identified for seven different residues (**A**–**G**), and one of these, residue **C** interestingly was still identified as Gal*f* residue, owing to its deshielded carbon chemical shift. The anomeric resonances were correlated to the ring protons by means of the COSY and TOCSY spectra while HSQC and HMBC were effective for the assignment of ^{13}C resonance. All the NMR data (Table 2) were in agreement with the presence of a mannan polymer in which galactofuranose and rhamnose are present in minimal amounts and only as terminal residues.

Residues **A** and **B** were both identified as 2-substituted mannose since they showed a downfield shift of the C-2, while residue **D** was identified as 2,6-di-substituted mannose owing to its downfield chemical shifts of both C-2 and C-6. Residue **G** was identified as 3-substituted mannose, actually its C-3 chemical shift was

downfield shifted at 79.1 ppm and **F** residue was identified as terminal mannose since its ^{13}C chemical shift values were similar to those reported in literature for unsubstituted mannose.[5,6] Residue **E** was identified as terminal rhamnose, since the ring protons in the COSY and TOCSY correlated to a methyl signal.

The interresidual sequence was established by a NOESY spectrum (Fig. 3) which showed a cross peak of both H-1 of **A** and **B** with H-2 of **D** residue. This confirmed that a mannan side-chain is present as branching to mannan backbone. H-1 of residue **D** showed a cross peaks with H-6a and H-6b of the same spin system, confirming the presence of a linear backbone of α-(1 → 6)-linked mannose residues. In the same spectrum the H-1 proton of terminal rhamnose **E** was correlated to H-2 of **A** and **B**, hence this residue is only

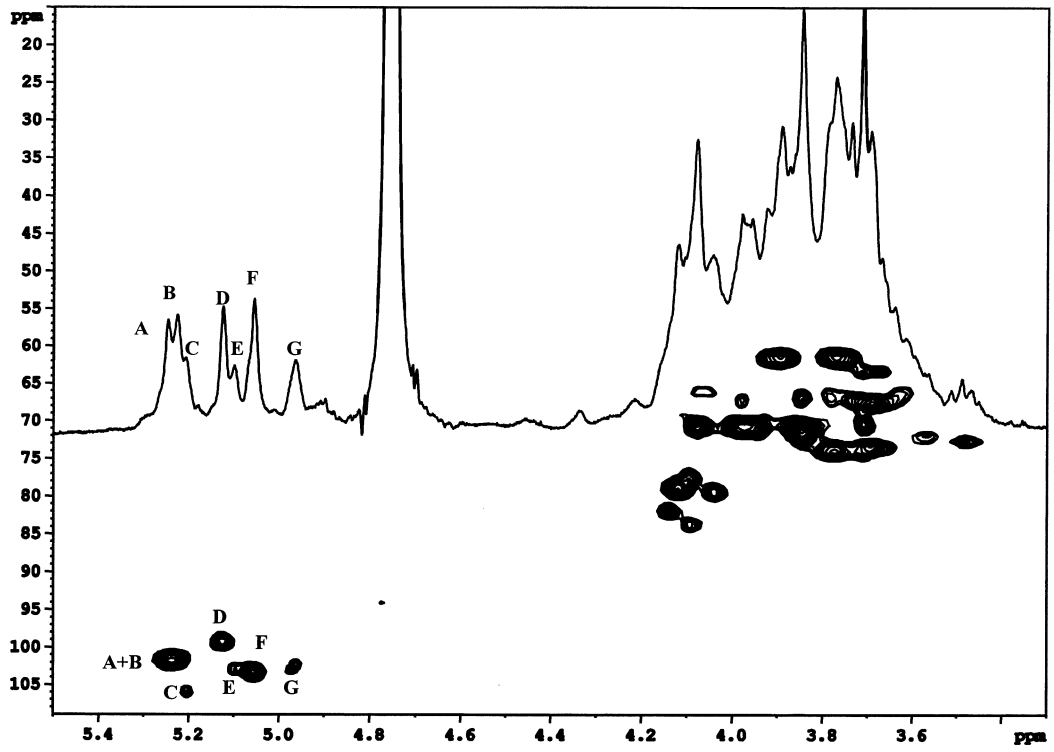


Fig. 2. The HSQC spectrum of **EPS I** with the anomeric signals assignments.

Table 2
^{1}H and ^{13}C resonances of the degraded polymer **EPS I** obtained by mild acid hydrolysis. The spectra were measured in D_2O at 27 °C and chemical shifts are expressed relative to acetone (^{1}H, 2.225 ppm; ^{13}C, 31.5 ppm). Residues are in the pyranose ring if not specified

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
A	5.24	4.09	4.03	3.78	3.71	3.90/3.78
2-Man	101.6	78.9	70.9	67.1	73.9	61.5
B	5.22	4.09	4.02	3.79	3.72	3.90/3.78
2-Man	101.6	78.9	70.9	68.0	73.9	61.5
C	5.21	4.14	4.11	4.09	3.91	3.71/3.65
t*-Gal*f	105.6	82.0	78.8	84.6	70.8	63.1
D	5.13	4.04	3.70	3.69	3.84	3.63/4.00
2,6-Man	99.0	79.1	70.7	67.7	73.6	66.2
E	5.10	4.07	3.70	n.d	4.11	1.31
***t*-Rha**	102.7	70.7	73.0	n.d.	68.9	17.3
F	5.05	4.08	3.85	3.65	3.78	3.90/3.78
***t*-Man**	102.8	71.1	71.1	67.7	73.9	61.5
G	4.97	4.22	4.04	n.d	3.72	3.90/3.78
3-Man	102.6	69.7	78.0	n.d.	73.9	61.5

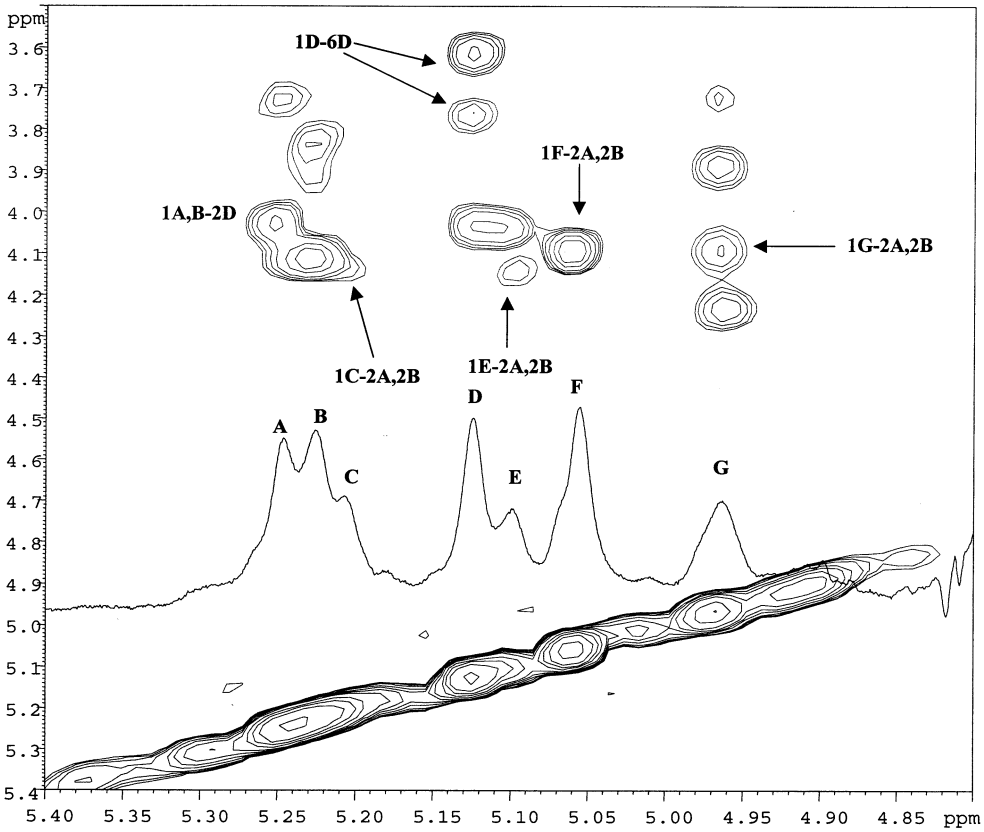


Fig. 3. Section of the NOESY spectrum of **EPS I** showing the significant cross peaks necessary for the interresidual assignment.

present as final residue of the branching chain. Whilst, the anomeric resonance of terminal mannose **F** showed NOE with H-2 of residue **A** and **B**, thus showing that this terminal mannose is linked as terminal to the branching chain. Unfortunately, it was not possible to distinguish between the two 2-mannose residues **A** and **B** which of course should be in different magnetic/chemical environment because of their slight different chemical shifts.

The anomeric proton of 3-substitued mannose **G** showed a NOE effect with H-2 of **A** and **B** residues, thus also residue **G** was part of the branching chain. Finally, the anomeric proton of the single galactofuranose **C** lasted after the hydrolysis presented a NOE contact with H-2 of residue **A** and **B**. This last information confirmed the hypothesis that galactofuranose residues are linked as non reducing end oligosaccharide moieties to the mannan branching chain. The HMBC spectrum confirmed the inter-residue correlations shown by the NOESY spectrum, in particular an interresidual cross peak was present which correlated the anomeric proton of galactofuranose **C** and both C-2 carbons of residue A and B, thus the covalent linkage between galactofuranose and mannose was confirmed.

The close similarity of chemical shifts values with those reported in literature[6–9] supported the hypothesis of a sugar backbone consisting of α-(1→6)-linked mannopyranose units branched at C-2 with 2-substituted mannose residues. The absence of any 6-substituted mannose indicates a fully branched, comb-like structure. The presence of a single galactose in the hydrolysed polymer could be due to its inner position and combined to the high number of branching points it could be hindered to the hydrolysing agent. In order to confirm this and to establish the length of the branches, an acetolysis reaction, whereby 6-linked sugars are preferentially cleaved,[10] was performed on **EPS I**. The crude reaction mixture was separated on Bio-Gel P-2 to give four fractions (**1**–**4**). Fraction **1** consisted of a mannose tetrasaccharide, the structure of which was completely assigned by 2D NMR and methylation data and comparison with literature.[7]

α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man

Fraction **2** was a mixture of two trisaccharides, one of the two containing rhamnose. The methylation data yielded terminal mannose, terminal rhamnose, 2-substituted mannose and terminal reducing mannose. Thus, these data, fully matching with NMR and methylation analyses, suggested the two structure below:

α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man

α-L-Rha*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man

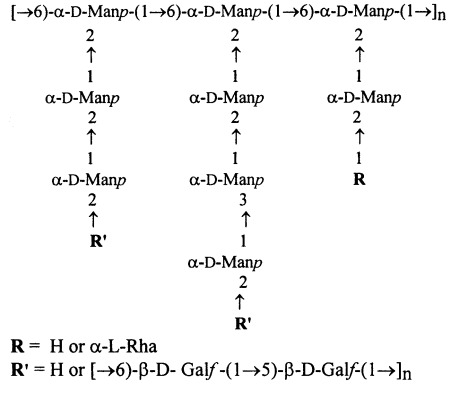
Fraction **3** was univocally determined by NMR as the disaccharide.[6]

D-Man*p*-(1→2)-D-Man

While fraction **4** was composed by terminal reducing mannose and galactose. Mannose was only in little amount and it was not possible to establish if it was an authentic product of the acetolysis, thereby demonstrating the presence of a little amount of unbranched mannose in the mannan backbone. Otherwise it could be a side product of acetolysis. Galactofuranose was unselectively cleaved by acetolysis and thus it was not possible to establish univocally its attachment site.

Thus, from acetolysis it was possible to argue that the side chains of **EPS I** are built up of a minimum terminal mannose residue but they can also be composed by two or three residues. Only in the case of a disaccharide side chain a consistent aliquot is containing terminal rhamnose. The galactofuranose oligosaccharide can be linked to whichever mannose residue of the side chain but certainly not to rhamnose which is present only as terminal residue.

Hence, the structure of this complex polysaccharide can be rationalised as:



3. Conclusions

The evidence that only the virulent strain of this fungus produces this EPS allows the hypothesis that this macromolecule is implicated in its virulence. No traces of this polymer were found in the hypovirulent strains. Furthermore, EPS can be used as taxonomic markers for the classification of fungi and the finding of this new polysaccharide can help in establish the relationships among genera. A similar polysaccharide differing in the galactan side-chain has been found as a cell-wall component of *Neurospora crassa*,[11] while an identical galactan polysaccharide has already been reported in the cell-wall of *Arachniotus verrucolosus*.[12] There are several examples already present in literature[6–9,11,12] of galactofuranose residues in fungi and also an arabinogalactan produced by mycobacteria.[13] In the latter organisms, the pathway by which polysaccharides containing Gal*f* residues are biosynthesised are rapidly being elucidated, because of their importance. After the ring contraction of Gal*p* catalysed by a mutase, a single enzyme, a polyfunctional galactofuranosyl transferase catalyses the addition of the Gal*f* to the growing chain.[14] By analogy, a galactofuranosyl transferase should be present in *C. parasitica*.

4. Experimental

Fungal cultures.—The virulent strain (CP 159) of *C. parasitica* from the Collection of the Department of Plant Pathology of the University of Bari, Italy, was used. The microrganisms were grown and the EPS recovered a described.[3]

Purification of EPS.—The native EPS obtained from the virulent strain CP159 (950 mg from 3 L of culture) strain was dissolved in a minimum amount of water and allowed to precipitate with 40% EtOH overnight at −20 °C. The procedure was repeated and the precipitate fractions were collected by centrifugation (3600 rpm, 10 min, yield 890 mg) were pooled together. The precipitate was identified as pullulan by methylation analysis and ^{1}H NMR. The supernatant fraction (45 mg, 5% yield) was analysed by compositional analysis and resulted free from glucose.

All attempts to extra purify the supernatant fraction failed, using both further 2-propanol precipitation and chromatography on Bio-Gel P-10 and P-100.

NMR spectroscopy.—The ^{1}H and ^{13}C NMR spectra were obtained in D_2O at 400 and 100 MHz, respectively, with a Bruker DRX 400 spectrometer equipped with a reverse probe, in the FT mode at 27 °C. ^{1}H and ^{13}C chemical shifts are expressed in δ relative to internal acetone (2.225/34.5 ppm). Two-dimensional spectra (DQF-COSY, TOCSY, NOESY, HSQC, HSQC-TOCSY and HMBC) were measured using standard Bruker software. A mixing time of 200 ms was used in the NOESY experiment, while a mixing time of 80 ms was used in the TOCSY.

Gas chromatography.—GC was performed on an Hewlett–Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco), for compositional and methylation analyses the temperature program was: 150 °C for 5 min, then 5 °C min^{-1} to 300 °C, for absolute configuration analysis was: 150 °C for 8 min, then 2 °C min^{-1} to 200 °C for 0 min, then 6 °C min^{-1} to 260 °C for 5 min.

Compositional and methylation analysis.—The monosaccharides were identified as acetylated *O*-methyl glycosides derivatives: briefly, samples were methanolysed with 2 M HCl/MeOH at 85 °C 20 h, dried under reduced pressure and then acetylated with acetic anhydride in pyridine at 80 °C 30 m. After work-up, the sample was analysed by GLC–MS. Absolute configurations were determined by GLC of acetylated glycosides of (+)-2-octanol according to the published method.[15]

Polysaccharide and oligosaccharide samples were methylated as reported.[16] The crude reaction product was filtered on a C-18 Sep-Pak cartridge (Waters) washed previously with EtOH (20 mL), MeCN (2 mL) and water (10 mL). The fractions were eluted with water (50 mL), 4:1 water:MeCN mixture (8 mL), MeCN (2 mL) and EtOH (4 mL). The last two fractions were pooled and evaporated to give the methylated polysaccharide which was hydrolysed with 2 M TFA. The partially methylated products were reduced with $NaBD_4$, acetylated and analysed by GLC–MS.

Mild hydrolysis.—An aliquot of sample (30 mg) was submitted to mild hydrolysis which was performed in 0.01 M HCl 24 h at 100 °C, then the sample was dried under vacuum and applied to a GPC column, Bio-Gel P2 (96 × 1.5 cm), eluted with distilled water and monitored with a Waters differential refractometer. Two peaks were detected, the first (**EPS I**) eluted in the void volume (17 mg) and the second (6 mg) which contained only galactofuranose reducing oligosaccharides.

*Acetolysis of **EPS I**.*—Acetolysis on 15 mg of product was performed as reported.[10] The deacetylated product was applied to a column (1.5 × 96 cm) of Bio-Gel P-2, and eluted with distilled water at a flow rate of 10 mL h^{-1} at room temperature; and monitored with a Waters differential refractometer and 1.0 mL fractions were collected. Four fractions (**1**–**4**) were collected and analysed by methylation analysis and NMR.

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