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Note

# Immunostimulant (1→3)-D-glucans from the cell wall of *Cryphonectria parasitica* (Murr.) Barr strain 263

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**Abstract**

A (1→3)-β-D-glucan with approximately 30% of the residues having a β-D-Glc-(1→6) branch is the main water-soluble component of the cell wall polysaccharide of *Cryphonectria parasitica* (Murr.) Barr strain 263. A (1→3)-glucan with both α and β anomeric linkages was found in the water-insoluble polysaccharide fraction. Both fractions possess immunological activity, being able to induce the production of either tumour necrosis factor α (TNF-α) or nitrite ($NO_2^-$). © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Glucan; *Cryphonectria parasitica*; Immunological activity; Cell wall; Fungi

*Cryphonectria parasitica* (Murr.) Barr is the causal agent of chestnut blight [1], which produces in vitro in the stationary growth a large amount of an exopolysaccharide fraction. The major component of this fraction is pullulan, a linear α-glucan built up of (1→4)-(1→6)-substituted glucose units in the ratio 2:1. The minor components were found to be a β-D-galactan and a galactomannan, which showed phytotoxic activity higher than that of the crude exopolysaccharide fraction [2].

Cell walls of fungi are mainly constituted of (1→3)-β-D-glucans, which are known to exhibit a significant antitumor activity as a result of activation of the host's immune system, rather than by direct cytotoxicity [3]. Two immunomodulating (1→3) - β - D - glucans, lentinan, from the fruiting body of *Lentinus edodes*, and schizophyllan, an extracellular polysaccharide from *Schizophyllum commune*, are used as immunotherapeutic agents [4]. This paper reports the isolation, the chemical characterisation and data on the immunostimulant activity of the two main (1→3)-D-glucans isolated from the cell wall of *C. parasitica* strain CP263. Polysaccharides were extracted from *C. parasitica* mycelium with sodium hy-

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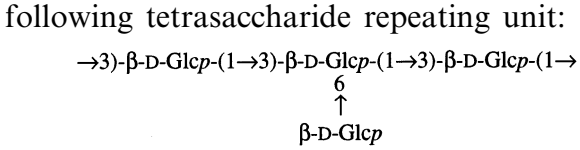
droxide obtaining a water-soluble (S-263) and a water-insoluble (I-263) fraction. The protein content was 15 and 6% for S-263 and I-263, respectively. GLC analysis of alditol acetate derivatives showed that I-263 and S-263 were constituted by glucose and by glucose (58%), galactose, mannose and rhamnose, respectively. The D configuration of glucose was obtained by GLC analysis of the 2-octyl glycoside derivatives. Methylation analysis indicates the presence of terminal 3-linked and 3,6-disubstituted glucopyranose units in both I-263 and S-263. Fraction S-263 was eluted in the void volume when chromatographed on both Bio-Gel P-100 and on Bio-Gel A 0.5m, but was resolved into two peaks by Bio Gel A 5m chromatography. The excluded A fraction contained only glucose, whereas the retained B peak contained Rha, Man, Gal and Glc in 1.8:3.5:3.8:0.8 ratios.

The $^{1}$H NMR spectrum of fraction A displayed two broad anomeric signals at $\delta$ 4.60 and 4.39 in a 2:1 ratio, the chemical shifts of which indicated a β-glucopyranoside structure. Anomeric signals were present at $\delta$ 104.0, 104.2, and 103.6 in the $^{13}$C NMR spectrum. Two shifted low field signals at $\delta$ 88.5 and 87.7 (carbinolic methines) and one at $\delta$ 69.6 (hydroxymethyl) suggested the presence of both 3-linked and 3,6-linked residues. Further signals were the carbinolic methines at $\delta$ 78.3, 77.7, 76.3, 74.7 and 71.6 and a hydroxymethyl at $\delta$ 62.2. These data strongly supported a branched $(1\rightarrow3)$-β-D-glucan structure, which was confirmed by methylation analysis indicating the presence of *t*-Glc*p*, 3-Glc*p* and 3,6-Glc*p* residues in 1:2:1 ratios.

Smith degradation of fraction A afforded a linear glucan consisting, from methylation analysis, of only 3-Glc*p* units. NMR data were identical with those of curdlan, a linear 3-β-glucan [5]. The lack of D-glucosylglycerol units among Smith degradation products confirmed that the side chains consisted of a single glucosyl residue.

These results allowed us to define the complete structure of β-glucan consisting of the following tetrasaccharide repeating unit:




Spectroscopic analysis of the water-insoluble I-263 fraction was performed on the crude product. The $^{13}$C NMR spectrum, recorded in 0.1 M NaOD, showed two anomeric signals in a 4:1 ratio at $\delta$ 104.4, assignable to β-glucan, and at $\delta$ 101.2, respectively. The latter signal was assigned to a α-glucan on the basis of its chemical shift value and because it was correlated to a proton signal at $\delta$ 4.99 that appeared as a broad singlet. In addition, the presence of a shifted methine carbinolic signal at $\delta$ 84.8, besides the signal of 3-β-glycosylate carbon at $\delta$ 87.7, indicated a substitution at C-3 of α-glucopyranose units [6].

Smith degradation afforded a polysaccharide fraction, which was constituted by only $(1\rightarrow3)$-Glc*p* units and appeared as a single peak by Bio-Gel P 100, Bio-Gel 0.5m and Bio-Gel 1.5m chromatographies. Its NMR spectra showed, as diagnostic lines, two anomeric proton signals at $\delta$ 4.53 (d, 7.0 Hz) and 5.09 (brs), which were correlated to anomeric carbon signals at $\delta$ 104.1 and 102.0, respectively, and the resonances of two 3-glycosylated carbons at $\delta$ 87.6 and 85.2 (Fig. 1). The data above suggest for the I-263 fraction a $(1\rightarrow3)$-glucan structure containing both α and β linkages carrying branches constituted by a single Glc*p* unit.

The finding of sharp signals assignable to α-$(1\rightarrow3)$- and β-$(1\rightarrow3)$-glucan structures in the $^{13}$C NMR spectrum of Smith fraction, and the lack of any signals that could be attributed to residues across α and β regions, suggests that the I-263 fraction is an unresolved polysaccharide mixture.

Fractions S-263 (S) and I-263 (Z), fraction A of S-263 (A), and the Smith degradation product from fraction A of S-263 (D) were tested for their immunological activity on macrophages J774.

Treatment of J774 cells with LPS (0.06; 0.25 and 1 μg/mL) for 3 h caused an accumulation of tumour necrosis factor α (TNF-α) ($802\pm261$, $921\pm10$ and $986\pm55$ pg/mL; respectively) as compared with unstimulated cells ($<15$ pg/mL). The results are expressed as pg/mL of TNF-α and represent the mean ± S.E.M. of six experiments run in triplicate. When the cells were incubated for 3 h with the

β-glucans A, D, S and Z at various concentrations (5, 20 and 80 μg/mL) in absence of LPS, a substantial release of TNF-α was induced by the fractions tested (Fig. 2(A)). Fractions D and Z were particularly active at all doses used.

The production of nitrite ($NO_2^-$) (stable metabolites of NO) as a parameter of macrophages activation and iNOS induction was measured. Unstimulated J774 cells generated undetectable ( $< 5$ nmol/mL) amounts of $NO_2^-$. Stimulation of the cells with LPS (0.06, 0.25 and 1 μg/mL) produced a dose-dependent release of $NO_2^-$ ($18 \pm 1$, $27 \pm 1$ and $32 \pm 3$ nmol/mL, respectively) When the cells were incubated with the β-glucans A, D, S and Z at 5, 20 and 80 μg/mL, a substantial release of $NO_2^-$ was observed particularly for fraction D and Z (Fig. 1(B)).

## 1. Experimental

*General.*—The $^1H$ and $^{13}C$ NMR spectra were obtained in $D_2O$ and in 0.1 M NaOD at 400 and 100 MHz, respectively, with a Bruker AM 400 spectrometer equipped with a dual probe, in the FT mode at 30 °C. $^{13}C$ and $^1H$ chemical shifts are expressed in $\delta$ relative to internal 1,4-dioxane (67.4 ppm) and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-$d_4$), respectively. GLC was performed with a Dani instrument equipped with a flame ionisation detector and GLCMS with a Hewlett–Packard 5890 instrument. The sugar composition was obtained by GLC analysis of alditol acetates as reported in Ref. [7]. Polysaccharide samples were methylated [8] and the partially methylated acetylated products were analysed as reported in Ref. [9]. Determination of the absolute configuration was performed accord-

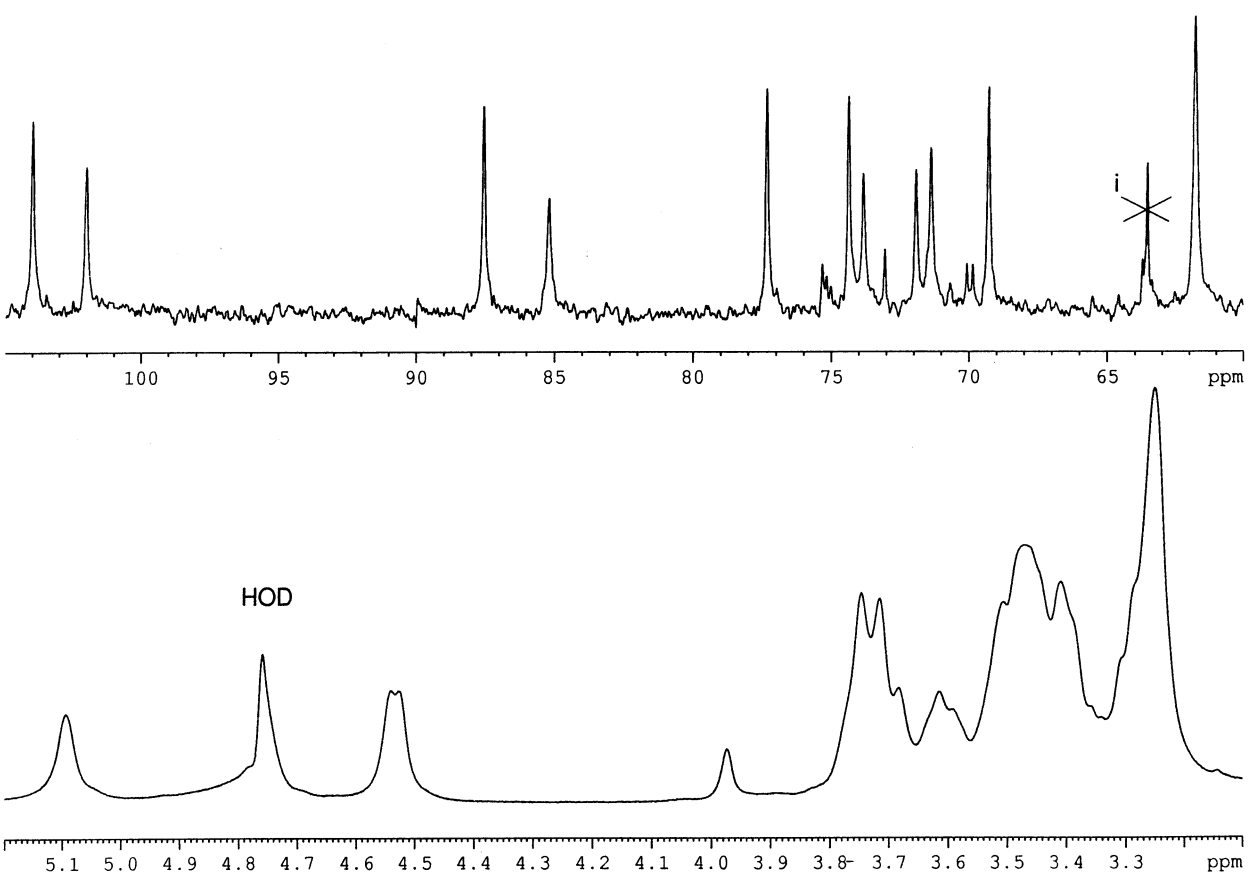



Fig. 1. $^1H$ and $^{13}C$ NMR spectra of I-263 Smith degraded products recorded at 30 °C in NaOD (0.1 M).

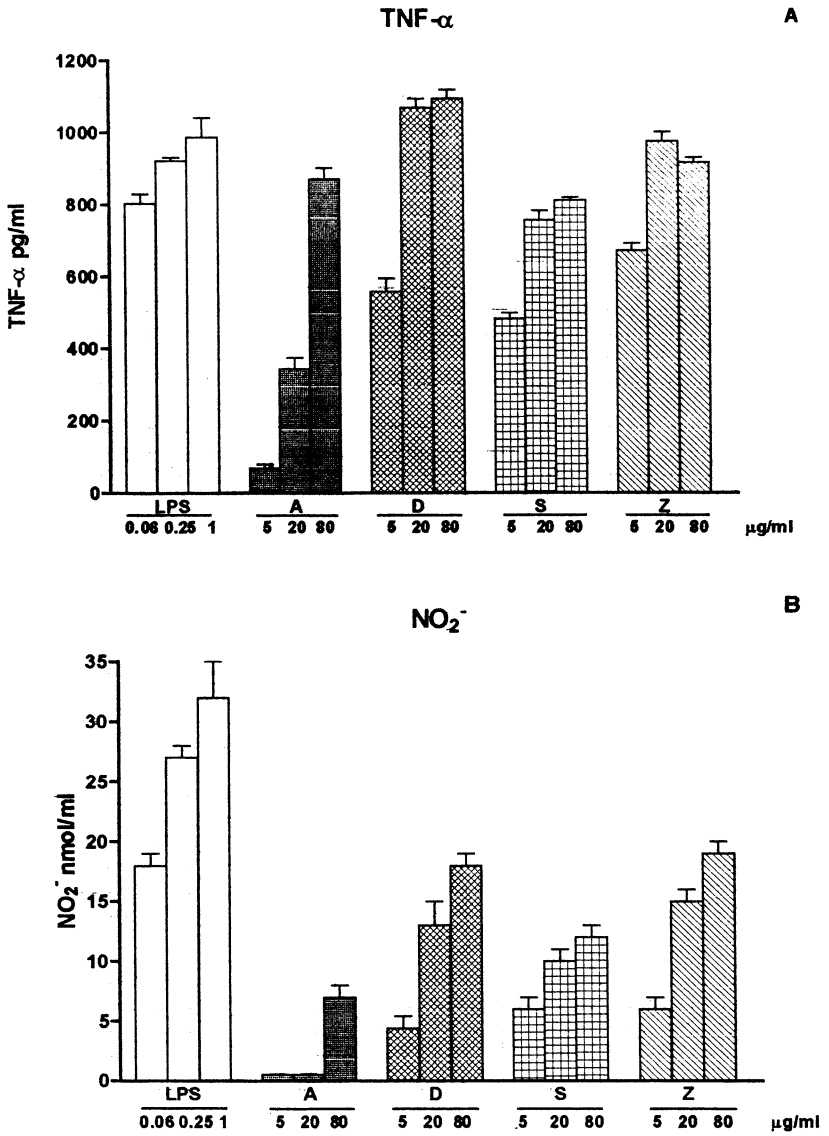



Fig. 2. Release of TNF-α of the macrophage J774 at different concentrations of the assayed glucans (A) and production of $NO_2^-$ (stable metabolite of NO) after the incubation with the glucans from *C. parasitica*. (B). Both assays were compared with LPS.

ing to the procedure described in Ref. [10]. The GLC analysis was performed on a SP-2330 capillary column (30 m × 0.25 mm i.d.), column conditions: 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. Smith degradations were performed as described [11].

*Preparation of cell wall polysaccharides.*— The mycelium material collected from the culture (750 mL) of *C. parasitica* strain 263, grown as described [2], was lyophilised. The dried material (7 g) was stirred overnight with 1 M NaOH (750 mL) at room temperature. The suspension was centrifuged at 10 °C at 7000 rpm for 45 min. The supernatant was neutralised with 12 M HCl at 10 °C and lyophilised. The residue was dissolved in ultrapure Milli-Q water (200 mL), brought to 4 °C, mixed with three volumes of absolute EtOH (600 mL), and left overnight at −20 °C. The resulting precipitate was collected, dissolved in Milli-Q water (150 mL) and re-precipitated with cold EtOH (450 mL), as described above. After 24 h, the precipitate was collected by centrifugation at 10 °C at 7000 rpm for 45 min and suspended in ultrapure Milli-Q water. The suspension was centrifuged in the same conditions as above, obtaining a soluble fraction S-263 (0.758 g) and an insoluble one I-263 (1.867 g).

*Purification of S-263 fraction.*—A sample of S-263 (100 mg) was chromatographed on Bio-Gel P100 and on A 0.5 m (Bio-Rad) column, eluted with 50 mM ammonium bicarbonate buffer and fractions (1.5 mL) were collected. The chromatographic profile, revealed by the phenol test [12], showed only one peak eluted in the void volume. The fraction S-263 (200 mg) was further chromatographed on Bio-Gel A 5m (Bio-Rad) (50 mM ammonium bicarbonate buffer) to give two peaks collected as fraction A (130 mg) and fraction B (50 mg).

*Cell cultures.*—The murine monocyte/macrophages cell line J774 was from EACC. J774 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker) and cultured at 37 °C in humified 5%$CO_2$–95% air. The culture medium was supplemented with 10% foetal bovine serum (FBS; Hyclone), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 25 mM HEPES and 5 mM sodium pyruvate (Biowhittaker). The cells were plated in 24 well culture plates (Falcon) at a density of $2.5 \times 10^6$ cells/mL/well and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated by lipopolysaccharide (LPS 0.06, 0.25, 1 μg/mL) from *E. coli* (Fluka) or by various β-glucans. At different time points (3 or 24 h), according to the cytokine or the metabolite being measured, the culture medium was removed, centrifuged and the supernatant used for the determination of TNF-α and $NO_2^-$ production. Cell viability ($>95\%$) was determined with an MTT assay [13].

*TNF-α assay.*—TNF-α levels in the culture media from J774 cells were measured 3 h after LPS or β-glucans stimulation using a commercially available mouse cytokine enzyme-linked immunosorbent assay kit from Genzyme according to the manufacturers instructions. Results are expressed as pg/mL of TNF-α and represent the means ± S.E.M. of *n* experiments run in triplicates.

*$NO_2^-$ assay.*—$NO_2^-$ levels in culture media from J774 macrophages were measured 24 h after LPS or β-glucan challenge with the Griess reaction, as previously described [14]. Results are expressed as nmol/mL of $NO_2^-$ and represent the means ± S.E.M. of *n* experiments run in triplicates.

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