Phytochemistry 69 (2008) 2731–2736

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

A 3-*O*-methylated mannogalactan from *Pleurotus pulmonarius*: Structure and antinociceptive effect

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ARTICLE INFO

Article history:
Received 25 February 2008
Received in revised form 13 June 2008
Available online 1 October 2008

Keywords:
Pleurotus pulmonarius
Basidiomycete
Polysaccharide
Mannogalactan
Antinociceptive effect

ABSTRACT

A polysaccharide (M_w 2.39 × 10^4 g/mol) was extracted with cold water from the basidiomycete *Pleurotus pulmonarius*, and its antinociceptive and anti-inflammatory properties were evaluated. It was a mannogalactan (MG), whose structure was characterized using mono- and two-dimensional NMR spectroscopy, methylation analysis, and a controlled Smith degradation. It had a main chain of (1 → 6)-linked α-D-galactopyranosyl and 3-*O*-methyl-α-D-galactopyranosyl units, both of which are partially substituted at O-2 by β-D-mannopyranosyl non-reducing ends. The MG was tested for its effects on the acetic acid-induced writhing reaction in mice, a typical model for inflammatory pain, causing a marked and dose-dependent inhibition of the nociceptive response, with ID_{50} of 16.2 (14.7–17.7) mg/kg and inhibition of 93 ± 3% at a dose of 30 mg/kg. An inflammatory response was not inhibited.

1. Introduction

Mushrooms have long been used either as a food or food-flavoring material, due to their unique and subtle flavors (Chiang et al., 2006). Their consumption is widespread in China, Japan, Korea, Taiwan, Italy, Spain, among others (Chang, 2005; Moreno-Rojas et al., 2004; Manzi et al., 1999). It has been shown that mushrooms are a source of proteins, carbohydrates, vitamins, minerals and dietary fibers (Manzi et al., 2001). Polysaccharides have also been isolated and tested as to their therapeutic properties (Zhang et al., 2007a).

Among the polysaccharides extracted from basidiomycetes, β-glucans (Manzi and Pizzoferrato, 2000) are known to have antitumor, anti-inflammatory, antiradiative, antiviral, hepatoprotective, hyperglycemia, and immunomodulating activities (Zhang et al., 2007a). Although β-glucans are the most studied and characterized fungal polysaccharides, and their biological effects are well known, another class of polysaccharides having the same activities has stimulated interest (Moradali et al., 2007). It includes heteropolysaccharides, including mannogalactans (Jakovljevic et al., 1998), xylomannans (Smiderle et al., 2006), fucomannogalactans (Alquini et al., 2004), and fucoglucogalactans (Zhang et al., 2007b), among others. Therapeutic responses, such as immunomodulating and antitumor effects, have been demonstrated for some of these heteropolymers. Galactoglucomannans (*Lentinus edodes*), xyloglucans (*Pleurotus pulmonarius*), mannogalactoglucans (*Ganoderma lucidum*) are examples of such bioactive molecules (Moradali et al., 2007).

The genus *Pleurotus* include edible and medicinal species, with most of them being currently commercialized in China. The edible mushroom *P. pulmonarius* has been studied and its polysaccharides have different levels of antitumor activities (Wasser, 2002). Two water-soluble fractions containing protein and polysaccharides showed, after three weeks, 74–85% of antitumor activity against sarcoma 180 implanted in mice. Water-insoluble fractions were extracted too, which almost completely inhibited the tumor at the same model. These fractions contained xylose and glucose as their main monosaccharide units. Although, these studies were carried out with crude fractions, the specific structural features that caused this inhibitory effect remained unknown.

Other biological experiments were carried out on a variety of mushroom extracts. It is known that aqueous and methanol extracts of *Geastrum saccatum*, *Ganoderma tsugae* and *Inonotus obliquus* give rise to anti-inflammatory activities, even though there is a lack of information concerning the mechanism that alleviates the inflammation (Park et al., 2005; Lin et al., 2006; Dore et al., 2007).

Consequently, we have now purified and characterized a partially *O*-methylated mannogalactan from *P. pulmonarius*, as well

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doi:10.1016/j.phytochem.2008.08.006

as investigating its antinociceptive and potential anti-inflammatory properties, using a model of inflammatory pain in mice.

2. Results and discussion

A dried sample of *P. pulmonarius*, after defatting, was submitted to aqueous extraction and the polysaccharides were recovered by ethanol precipitation (Fig. 1). It was dissolved in water and the solution was frozen and then thawed. A precipitate appeared which was removed by centrifugation, and the soluble fraction remained (SW; 6% yield) was treated with Fehling solution. The insoluble copper complex was converted to polysaccharide (FP; 0.8% yield), which was heterogeneous when analyzed by HPSEC (refractive index detector).

FP was composed of fucose (2%), xylose (1%), mannose (27%), 3-*O*-methyl-galactose (15%), galactose (47%), and glucose (8%). The presence of 3-*O*-Me-galactose was confirmed by GC–MS ions at *m/z* 130 and 190 after reduction with NaB^2H_4 and acetylation. FP was purified by ultrafiltration with membranes of different M_r cut-offs. Eluted material (MG; 0.4% yield) showed an homogeneous profile when analyzed by HPSEC, having a molar mass of 2.39×10^4 g/mol. MG contained mannose (32%), 3-*O*-methyl-galactose (13%) and galactose (55%), while methylation analysis (Table 1) was consistent with a polysaccharide with a main chain composed of Gal*p* and 3-*O*-Me-Gal*p* units (1 → 6)-linked highly substituted, at O-2, by non-reducing of Man*p* end units.

This was confirmed by a partial hydrolysis of MG that gave a ^{13}C NMR spectrum with more intense signals corresponding to α-D-Gal*p* and 3-*O*-Me-α-D-Gal*p* units and minor ones from β-D-Man*p* units, showing that non-reducing ends were removed from main chain (Fig. 2).

NMR spectra are shown in Figs. 2 and 3. The HSQC spectrum (Fig. 3B) contained signals, in the anomeric region, corresponding to C1/H1 of mannopyranosyl, galactopyranosyl 2,6-di-*O*- and 6-*O*-substituted Gal*p* units (103.9/4.84; 101.0/5.18 and 100.6/5.19; 100.4/5.03, respectively). The inverted signal at δ 69.1 in DEPT experiment confirmed the presence of 6-*O*-substituted units.

Glycosidic configurations were confirmed by values of ^1H, ^{13}C and coupling constants $J_{C\text{-}1,H\text{-}1}$ observed in the coupled HSQC. The β-configuration of the Man*p* units is indicated by a high-field H-1 signal at δ 4.84, which is in agreement with the value of 160 Hz (Perlin and Casu, 1969). The low-field H-1 resonances at δ 5.19; 5.18 and 5.03 showed a $J_{C\text{-}1,H\text{-}1}$ = 170 Hz, indicating an α-configuration of Gal*p* and 3-*O*-Me-Gal*p* units, in accordance with the C-1 high-field signals in its ^{13}C-NMR spectrum (Fig. 2A).

The resonance at δ 79.4 in the ^{13}C spectrum is from C-2 of 2,6-di-*O*-substituted Gal*p* units, confirming the O-2 substitution by non-reducing end units of Man*p*. The presence of *O*-methyl groups

Table 1
Partially *O*-methylalditol acetates formed on methylation analysis of mannogalactan isolated from *P. pulmonarius*

Partially *O*-methylated alditol acetates[a]	Mol%		Linkage Type[c]
	MG	SM-MG[b]	
2,3,4,6-Me_4-Man	36	–	Man*p*-(1→
2,3,4-Me_3-Gal	29	54	6→)-Gal*p*-(1→
3,4-Me_2-Gal	35	46	2,6→)-Gal*p*-(1→

[a] Analyzed by GC–MS, after methylation, total acid hydrolysis, reduction with $Na_2B^2H_4$ and acetylation.
[b] MG after oxidation ($NaIO_4$) and reduction ($NaBH_4$) treatment.
[c] Based on derived *O*-methylalditol acetates.

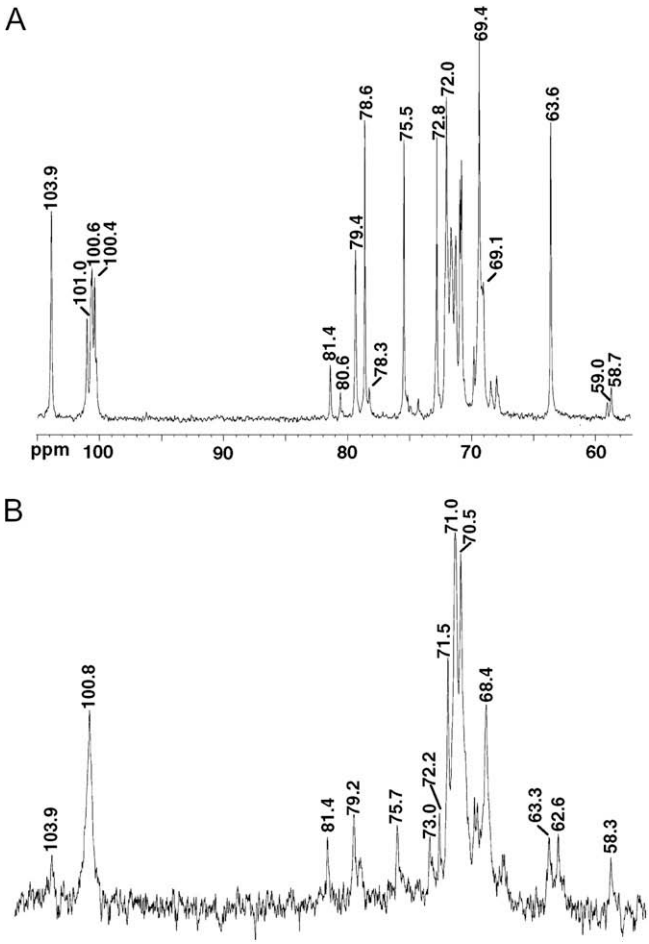

Fig. 2. ^{13}C NMR spectra of native (A) and partially degraded (B) mannogalactan, in D_2O at 40 °C, chemical shifts are expressed in ppm.

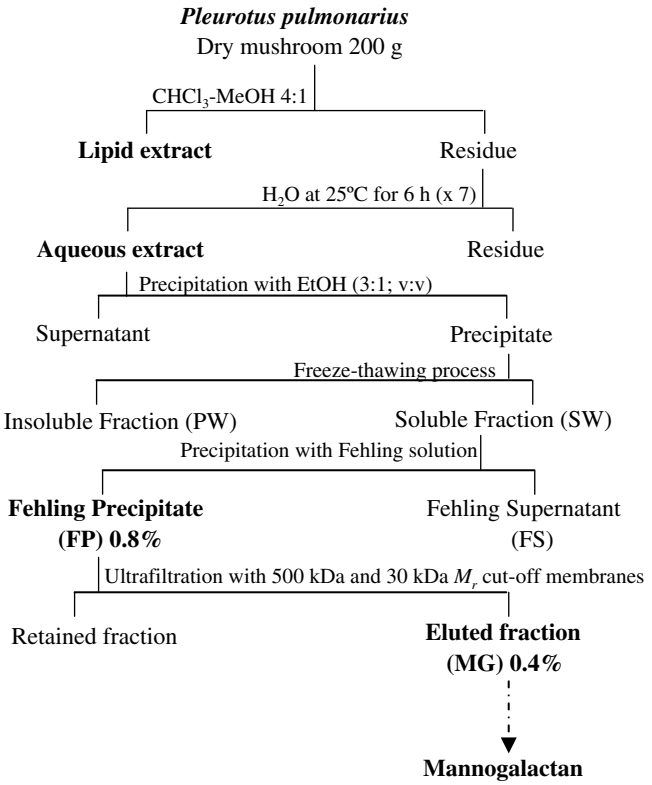

Fig. 1. Extraction and purification of mannogalactan (MG).

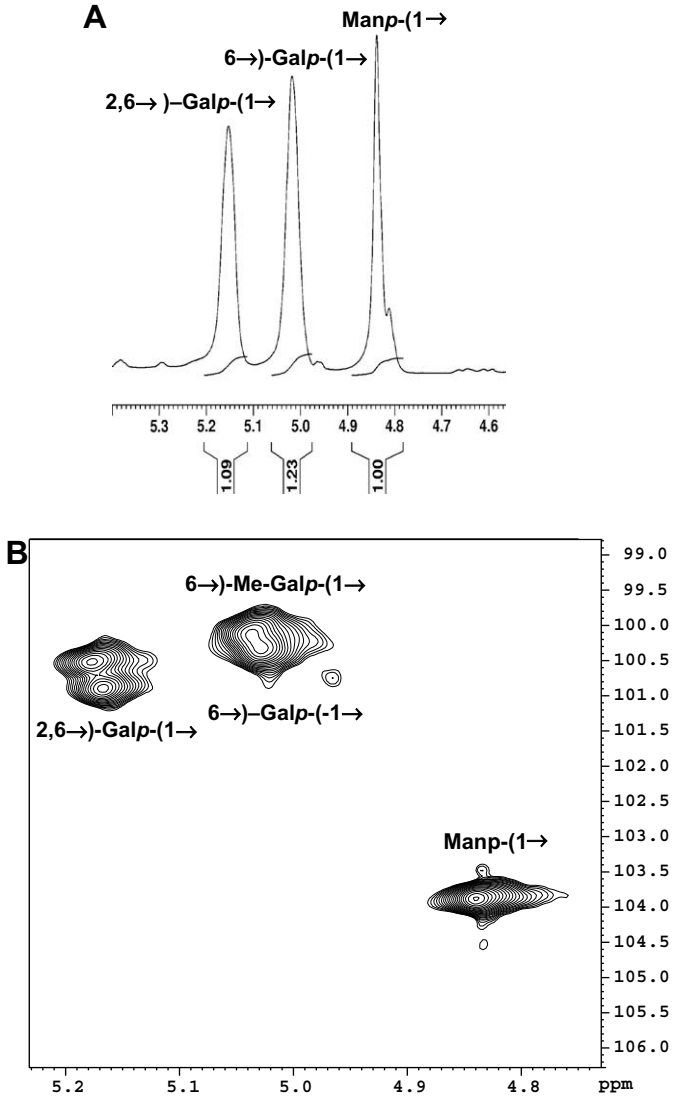

Fig. 3. Anomeric region of ^{1}H NMR (A) and coupled HSQC (B) spectra of mannogalactan, in D_2O at 40 °C, chemical shifts are expressed in ppm.

is confirmed by signals at δ 58.7/3.48 and 59.0/3.51, and a resonance at δ 81.4/3.58 is from CH_3 and C3/H3 of 6-*O*-substituted *O*-Me-Gal*p* units, respectively. Also assigned were those of C-2 (72.8), C-3 (75.5), C-4 (69.4), C-5 (78.6) and C-6 (63.6) of β-D-Man*p* units.

From the above data, this polysaccharide has a main chain composed of (1 → 6)-linked α-D-Gal*p* and *O*-Me-α-D-Gal*p*, some of them substituted at O-2 by β-D-Man*p* non-reducing end units (Fig. 4). To determine in which α-D-Gal*p* units (with or without CH_3- groups) the β-D-Man*p* residues were linked, the fraction MG (150 mg) was submitted to oxidation with $NaIO_4$, followed by dialysis and reduction with $NaBH_4$. After this process, an aliquot (20 mg) was methylated and its partially *O*-methylated alditol acetates derivatives (SM-MG) were analyzed by GC–MS (Table 1). Using this strategy, only the Me-Gal*p* units (13%), present in the main chain, were resistant to periodate oxidation followed by hydrolysis. Considering this, at the final process, 46% of these units were found as 3,4-Me_2-Gal*p* (46%), corresponding to 3-*O*-Me-Gal*p* of the main chain and 2-*O*-substituted by β-D-Man*p* units. The remaining units (54%) were found to be 2,3,4-Me_3-Gal*p*, indicating (1 → 6)-linked 3-*O*-Me-Gal*p* units of the main-chain. Therefore, of the total mannose (32%), only 6% were linked at Me-Gal*p*, while 26% were substituting Gal*p* units.

A similar 3-*O*-methyl mannogalactan has been isolated from *P. ostreatus* (Jakovljevic et al., 1998), and an *O*-methyl galactan, having the main chain of the present *P. pulmonaris* polysaccharide, has been extracted from *P. eryngii* and *P. ostreatoroseus* (Carbonero et al., 2008). These findings show that such polysaccharides could be characteristic of the genus *Pleurotus*.

In order to evaluate the antinociceptive and anti-inflammatory properties of the mannogalactan MG, it was administrated (3–30 mg/kg, i.p.), 0.5 h prior to irritant injection, and caused a marked and dose-dependent inhibition of the acetic acid-induced nociceptive response, with ID_{50} of 16.2 (14.7–17.7) mg/kg and inhibition of 93 ± 3% at a dose of 30 mg/kg. While the dexamethasone (2 mg/kg, s.c.) and indomethacin (10 mg/kg, i.p.), used in positive controls, inhibited the number of writhes by 30 ± 8 and 92 ± 5%, respectively (Fig. 5A). However, MG (3–30 mg/kg) and indomethacin (10 mg/kg, i.p.) did not inhibit leukocyte infiltration (total cell migration) induced by acetic acid, but dexamethasone (2 mg/kg, s.c.) reduced it by 92 ± 2% (Fig. 5B). MG (3–30 mg/kg) and dexamethasone (2 mg/kg, s.c.) also did not reduce the peritoneal capillary permeability (Evans blue dye exudation) induced by acetic acid, in contrast with indomethacin that reduced it by 82 ± 2% (Fig. 5C).

These results demonstrated that intraperitoneal treatment with mannogalactan promotes a markedly and a dose-dependent inhibition of the acetic acid-induced nociceptive response. However, administration of MG did not modify either peritoneal capillary permeability or leukocyte infiltration caused by acetic acid.

The acetic acid-induced writhing reaction in mice is commonly employed as a screening method because of its simplicity and sensitivity, besides it is described as a typical model for inflammatory pain (Vinegar et al., 1979; Tjølsen and Hole, 1997). The major transmission pathway for inflammatory pain has been documented as that comprising peripheral polymodal receptors around small vessels that signal to the CNS via sensory afferent C-fibers entering the dorsal horn (Kumazawa et al., 1996). C-fibers are a type of nociceptor that, when stimulated by algiogenic molecules, causes painful sensations. Some of these molecules are bradykinin, prostaglandin and cytokines, such as TNF-α and interleukin-1β. They were

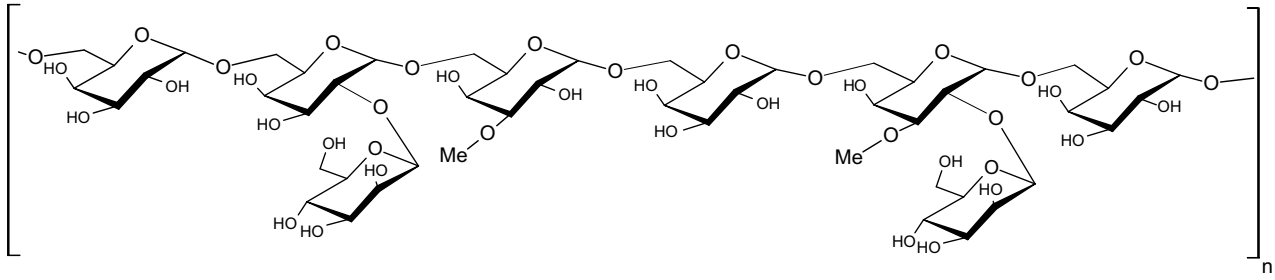

Fig. 4. Fragment of the chemical structure of the mannogalactan isolated.

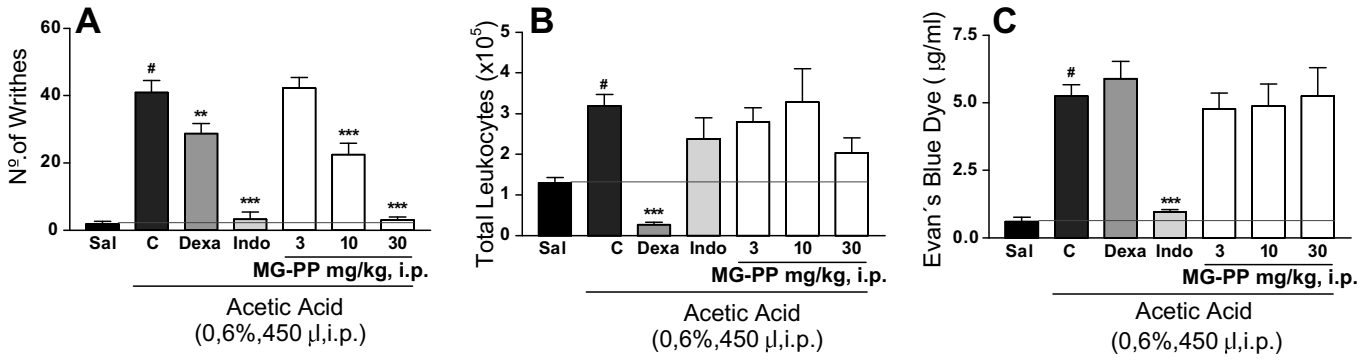

Fig. 5. Effect of intra-peritoneal (i.p.) administration of MG (3–100 mg/kg) on acetic acid-induced abdominal constriction (A), Evans blue leakage (B), and leukocyte infiltration (C) in mice. Data are expressed as means ± S.E.M.; $n = 6–8$ mice per group. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control (C), and $^{\#}P < 0.001$ vs. saline (Sal), ANOVA followed by Newman–Keuls' test; Dexamethasone (Dexa) and indomethacin (Indo) were used as positive controls.

documented as mediators involved in the writhing responses induced by acetic acid (Moncada et al., 1975; Correa et al., 1996; Ribeiro et al., 2000). In addition, glutamate and aspartate are considered, based on several lines of evidence, important for the transmission of nociceptive inputs in a variety of nociceptive models, acting as direct or indirect neurotransmitters (Feng et al., 2003).

The use of steroids to control inflammatory process promotes some benefits such as prevention pro-inflammatory cytokine increase. Predominant effects of corticosteroids, such as dexamethasone, are to switch off multiple genes encoding pro-inflammatory molecules and enzymes, inhibiting cytokine production that have been activated during the inflammatory process (Moura et al., 2001; Barnes, 2006). Also indomethacin, a non-steroidal anti-inflammatory drug, inhibits the production of prostaglandins from arachidonic acid by inhibition of enzyme cyclooxygenase (COX) (Brunton, 2001).

Our data demonstrated that MG and indomethacin gave rise to a similar analgesic effect, while the inflammatory response, characterized by leukocyte infiltration and peritoneal capillary permeability, was inhibited by dexamethasone and indomethacin, but not MG. Thus, our results could indicate that the mannogalactan isolated from *P. pulmonarius* showed an antinociceptive action, independent of an anti-inflammatory activity, in the acetic acid-induced writhing test. The mechanism by which MG produces antinociception still remains unclear, but the inhibition of cytokine pro-inflammatory release or glutamate in the cerebrospinal fluid increase seems to explain, at least in part, its antinociceptive effects.

3. Conclusions

Polysaccharides of mushrooms have been widely studied, especially for their therapeutic properties. As supported by the structure of the mannogalactan from *P. pulmonaris*, the presence of 3-*O*-methyl galactopyranosyl units appears to be common for the genus *Pleurotus*. This polysaccharide was tested by its anti-inflammatory and antinociceptive activity, and using a model of inflammatory pain in mice, it was shown that the polysaccharide has an analgesic effect.

4. Experimental

4.1. General experimental procedures

Gas liquid chromatography–mass spectrometry (GC–MS) was performed using a Varian (model 3300) gas chromatograph linked to a Finnigan Ion-Trap model 810 R-12 mass spectrometer, with He as carrier gas. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection and then programmed at 40 °C min^{-1} to 220 °C or 210 °C (constant temperature), was used for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates, respectively.

Ultrafiltration was performed on a filter holder (Sartorius – Model 16249), with compressed air at 10 psi as carrier gas.

NMR spectra (^1H, ^{13}C, DEPT, and coupled HSQC) were obtained using a 600 MHz Bruker Avance spectrometer incorporating Fourier transform. Analyses were performed at 40 °C on sample dissolved in D_2O. Chemical shifts are expressed in δ relative to acetone at δ 32.77 (^{13}C) 2.21 (^1H), based on DSS (sodium 2,2-dimethyl-2-silapentane-3,3,4,4,5,5-d_6-5-sulfonate) at $\delta = 0.00$ for ^{13}C and ^1H in accordance with IUPAC recommendations.

4.2. Fungal material

Pleurotus pulmonarius (Fr.) Quel. was furnished by Renato A. Yamasita, at Fazenda Bom Jesus das Araucárias, located in the town of Reserva, State of Paraná (PR), Brazil. Voucher specimen was deposited at Museu Botânico Municipal (No. 332755), located in Curitiba-PR, Brazil.

4.3. Polysaccharide extraction and purification

The dried fungus (200 g) was milled and extracted with $CHCl_3$–MeOH (4:1, v/v) at 60 °C for 3 h (×3, 350 ml each), to remove apolar compounds. The residue was submitted to aqueous extraction at 25 °C for 6 h (×7, 3000 ml each).

The aqueous extract was evaporated to a small volume and the polysaccharides were precipitated by addition of excess EtOH (3:1 v/v) and centrifuged at 8500 rpm (13,600) at 10 °C for 20 min. The sediment was dialyzed (12–14 kDa M_r cut off) against tap H_2O for 20 h, concentrated under reduced pressure and freeze-dried. The extract was then dissolved in H_2O, with the solution submitted to freezing followed by mild thawing at 4 °C. The soluble fraction (SW), following centrifugation (8500 rpm at 4 °C for 20 min), was treated with Fehling solution (100 ml) and the resulting insoluble Cu^{2+} complex was isolated. Both the complex (FP) and supernatant (FS) were neutralized with HOAc, dialyzed (12–14 kDa M_r cut off) against tap H_2O and deionized with Dowex 50 × 8 (H^+ form) ion-exchange resin. FP was further purified by ultrafiltration through membranes of 500 kDa and 30 kDa M_r cut-off (Millipore®; polyethersulfone), giving rise to eluted material from both membranes, which was denominated MG (Fig. 1).

4.4. Monosaccharide composition

Each polysaccharide fraction (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The

residue was successively reduced with excess $NaBH_4$ or NaB^2H_4 and acetylated with Ac_2O-pyridine (1:1, v/v) at room temperature for 12 h. (Wolfrom and Thompson, 1963a,b) The resulting alditol acetates were analyzed by GC–MS, as indicated above, identified and quantified by their typical retention times and electron impact profiles.

4.5. Determination of homogeneity of polysaccharides and their molecular weight

The determination of the homogeneity and molar mass (M_w) of the fractions was performed on a Waters high-performance size-exclusion chromatography (HPSEC) apparatus coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). Waters Ultrahydrogel columns (2000, 500, 250 and 120) were connected in series and coupled to multidetection equipment, using a $NaNO_2$ solution (0.1 M) as eluent, containing 0.5 g/l NaN_3. The polysaccharide solutions (1 mg ml^{-1}) were dissolved in the same solvent and filtered through a nitrocellulose Millipore® membrane, with pores of 0.22 or 0.45 μm. HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. The specific refractive index increment (d*n*/d*c*) was determined using a Waters 2410 detector. All experiments were carried out at 25 °C.

4.6. Methylation analysis

Per-*O*-methylation of each isolated polysaccharide (10 mg) was carried out by dissolution in DMSO (1 ml), followed by iodomethane (1 ml), and powdered NaOH (20 mg) (Ciucanu and Kerek, 1984). The sample was submitted to vigorous stirring for 30 min, at 25 °C and maintained overnight. The reaction was interrupted by addition of water, neutralization with HOAc, dialysis (8 kDa M_r cut off) against distilled H_2O and freeze-drying. The products were submitted to one more cycle of methylation, and sequentially isolated by partition between $CHCl_3$ and H_2O. The per-*O*-methylated derivatives from the lower phase, following evaporation, were treated with 45% (v/v) HCO_2H at 100 °C for 10 h, followed by evaporation to dryness (Stortz et al., 1997). The residues were converted into partially *O*-methylated alditol acetates, and analyzed by GC–MS as described above.

4.7. Controlled smith degradation (modified)

Fraction MG (150 mg) was submitted to oxidation with 0.05 M aq. $NaIO_4$ (20 ml) for 72 h at 25 °C in the dark (Abdel-Akher et al., 1952; Hay et al., 1965). The resulting material was then dialyzed (8 kDa M_r cut off) against tap H_2O for 48 h and treated with $NaBH_4$ (pH 9–10) for 20 h. After dialysis, the sample was freeze-dried. An aliquot (1 mg) was analyzed as its monosaccharide components, and another one (10 mg) was submitted to methylation analysis giving the sample denominated SM-MG.

4.8. Partial hydrolysis of mannogalactan

MG (200 mg) was treated with 0.16 M H_2SO_4 (1 ml) at 100 °C, for 3 h. The product was lyophilized and analyzed by ^{13}C NMR spectroscopy.

4.9. Experimental animals

Male Swiss mice (25–35 g) were kept in an automatically controlled temperature room (23 ± 2 °C) in 12-h light-dark cycles, with water and food freely available. Animals were acclimatized to the laboratory for at least 2 h before testing and were used only once for experiments. These were performed after approval of the protocol by the Institutional Ethics Committee and were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

4.10. Abdominal constriction, peritoneal capillary permeability and leukocytes infiltration caused by intraperitoneal injection of 0.6% aqueous acetic acid

Abdominal constrictions were induced according to previously described procedures (Lucena et al., 2007), which resulted in contraction of abdominal muscle together with stretching of the hind limbs, in response to the intraperitoneal injection (i.p.) of 0.6% HOAc. At the beginning of the experiment, mice were pre-treated intravenously with 2.5% Evans blue dye solution (10 ml/kg), used as a peritoneal capillary permeability marker. One hour later, mice received MG (3–30 mg/kg, i.p.) or positive controls, dexamethasone (2 mg/kg, s.c.) or indomethacin (10 mg/kg, i.p.) and 0.5, 4 and 0.5 h before the acetic acid injection, respectively. Control animals received a similar volume of the saline solution (10 ml/kg, i.p.) used to dilute the MG. After the challenge, the mice were placed individually into glass cylinders of 20 cm diameter, and abdominal constrictions were counted cumulatively over a period of 20 min. Antinociceptive activity is expressed as the reduction in the number of abdominal constrictions (i.e., the difference between control mice (mice pre-treated with saline) and animals pre-treated with MG, dexamethasone or indomethacin). Immediately after the test, mice were sacrificed by cervical dislocation and the peritoneal cavity was washed with 1 ml of sterile saline plus heparin (25 IU/ml) and the volume collected with automatic pipettes. Total leukocyte counts were performed using a Neubauer chamber via optical microscopy after diluting a sample of the peritoneal fluid with Türk solution (1:20). A sample of the collected fluid (700 μl) was centrifuged at 1000 rpm for 10 min and the absorbance of the supernatant was read at 550 nm with an ELISA analyzer. The peritoneal capillary permeability induced by HOAc is expressed in terms of dye (μg/ml), which leaked into the peritoneal cavity according to the standard curve of Evans blue dye (Lucena et al., 2007).

4.11. Statistical analysis

The results are expressed as means ± S.E.M., except the ID_{50} values (i.e. the dose of MG reducing the abdominal constriction, peritoneal capillary permeability and leukocyte infiltration responses by 50%, relative to the control value), which are expressed as geometric means accompanied by their respective 95% confidence limits. The ID_{50} values were determined by linear regression from individual experiments using linear regression GraphPad software (Graph Pad software, San Diego, CA, USA). The statistical significance of differences between groups was detected by ANOVA followed by Newman–Keuls' test. $P < 0.05$ is considered to be significant.

Acknowledgements

The authors would like to thank the Brazilian funding agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), FAPESC (Fundação de Apoio à Pesquisa Científica Tecnológica do Estado de Santa Catarina), and Fundação Araucária for financial support, and the Istituto di Ricerche Chimiche e Biochimiche "G. Ronzoni", Milan, Italy, for carrying out 2D NMR experiments.

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