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Chemotyping glucans from lichens of the genus *Cladonia*

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Abstract

α-D-Glucans were isolated from the *Cladonia* spp., *C. clathrata, C. connexa, C. crispatula, C. furcata, C. ibitipocae, C. imperialis, C. penicillata*, and *C. signata*, to evaluate their possible significance in chemotyping. Each was isolated in water-insoluble form via successive alkaline extraction and freeze-thawing. They were then individually investigated using ^{13}C and ^{1}H-NMR spectroscopy (including COSY, TOCSY and HMQC techniques), methylation analysis, and Smith degradation, and their specific rotations and monosaccharide compositions determined. Each α-D-glucan contained alternating (1 → 3) and (1 → 4)-linkages (1:1 ratio), as determined by the relative areas of their respective H-1 signals at δ 5.14 and 5.23. Also, the respective relative areas of C-1 signals at δ 99.2 and 100.1, and those of C-3 of 3-*O*-(δ 82.6) and C-4 of 4-*O*-substituted (δ 78.9) units, were similar for each glucan. The presence of α-D-glucans of the nigeran type appears to be typical of *Cladonia* spp.

Keywords: Lichens; *Cladonia* spp; α-D-Glucan; Chemotyping

1. Introduction

Structurally different, species-specific, mannose-containing polysaccharides have been used for the classification and identification of yeasts (Gorin & Spencer, 1970). This led to investigations of polysaccharides isolated from different species of ascomycetous lichens (Teixeira, Iacomini & Gorin, 1995), especially those obtained via Fehling precipitation. In order to study a number of species from the same genus, different lichens from the genus *Cladonia* (Iacomini, Schneider & Gorin, 1985; Woranovicz, Gorin, Marcelli, Torri & Iacomini, 1997) were examined in terms of their heteropolysaccharides, which also proved to serve as 'fingerprints' for each species. On the other hand, lichen homopolymers, namely glucans, have been investigated as an approach to chemotaxonomy (Yokota, Shibata & Saitô, 1978) of different families and genera using ^{13}C-NMR spectroscopy. Further studies were carried out on glucans of several ascomycetous lichens (Gorin, Baron, Corradi da Silva, Teixeira & Iacomini, 1993), which have a linear structure with an α or β-glycosidic configuration. The α linkages are (1 → 3) and (1 → 4) in each one, and their ratio showed considerable variation (Gorin, Baron & Iacomini, 1988), varying from 1:1 to 4:1, being soluble or insoluble in cold water, depending on their proportion. The water-insoluble α-D-glucans that contain alternate (1 → 3)- and (1 → 4)-linkages are similar to nigeran (mycodextran). So far six of these glucans have been isolated from two species of the genus *Cladonia*, sect. *Perviae*, viz *Cladonia crispata* (Ach.) Flot. and *C. squamosa* Hoffm. (Nishikawa et al., 1974), and from four species of the genus *Cladina*, viz *Cladina mitis* (Sandst.) Hustich, *C. rangiferina* subsp. *grisea* (Ahti) Ahti and M. J. Lai, *C. stellaris* (Opiz) Brodo (as *Cladonia alpestris*), and *Cladina confusa* (R. Sant.) Follman and Ahti (as *Cladonia confusa*) (Iacomini et al., 1985; Nishikawa et al., 1974). In other

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glucan studies, this structure has only been detected in the foliose lichen *Flavoparmelia caperata* (L.) Hale (family Parmeliaceae) (Takeda, Nishikawa & Shibata, 1970) (as *Parmelia caperata*). These structures are distinct from a highly branched β-D-glucan isolated from the basidiomycetous lichen *Dictyonema glabratum*, (Iacomini, Zanin, Fontana, Hogge & Gorin, 1987), (as *Cora pavonia*) and different from those of free ascomycetes, which have α or β-structures with less than 10% branching (Gorin & Barreto-Bergter, 1983).

2. Results and discussion

2.1. Glucans of eight Cladonia spp. obtained via alkaline extraction

Lichens were extracted with organic solvents to remove low-molecular weight compounds. The residues were then extracted with hot aqueous potassium hydroxide and the polysaccharide obtained from each species was resuspended in water, frozen, and then thawed gently, resulting in the formation of a precipitate and soluble material (Scheme 1). Depending on the species, each fraction was isolated in varying yields and the mannose-, galactose-, and glucose-containing components were present in different molar ratios (data not shown). In the following investigations, only the purified freeze-thawed precipitates were further analysed.

Preliminary analysis of the freeze-thawed precipitates showed that mannose- and glucose-containing components were present, as well as glucan. The material from each species was therefore submitted to the purification process described in Section 3, which was essential to obtain purified glucans. The percentage of glucose was between 19 and 63% in the first freeze-thawing step (Precipitate I, Scheme 1), increasing up to 98.9% in the purified fractions (Precipitate III). The content of glucose in the glucan preparation for each species was as follows: *C. signata*: 98.6%; *C. connexa*: 95.0%; *C. ibitipocae*: 94.8%; *C. crispatula*: 98.9%; *C. penicillata*: 96.5%; *C. furcata*: 96.5%; *C. imperialis*: 97.0%; *C. clathrata*: 97.9%.

Examination of pure freeze-thawed precipitated polymers by column gel filtration indicated that they were homogeneous, with those from different species having M_r's of between 5.4×10^4 and 7.6×10^4. The purity of the glucans was confirmed by their by ^{13}C-NMR spectra, which were near identical and did not contain minor signals of mannosyl or galactosyl units (Fig. 1).

The assignment of each ^{13}C resonance of the (1 → 3)-, (1 → 4)-α-D-glucan (total 12 signals) was achieved by examination of the relative peak intensities, and of the ^{13}C-NMR spectra of other D-glucans (Yokota et al., 1978). In order to establish ^{13}C and ^{1}H chemical shifts, 1D and 2D (COSY, TOCSY and HMQC) NMR techniques were used (Table 1). ^{1}H and ^{13}C assignments and the respective molar ratio of linkages (see Section 3) are similar to the results obtained for *C. penicillata*. The α-configurations of the glucans were confirmed by their highly positive specific rotations, which were similar to that of nigeran (Gorin et al., 1993).

Table 1
Proton and ^{13}C chemical shifts (δ) obtained from the glucan of *Cladonia penicillata*[a]

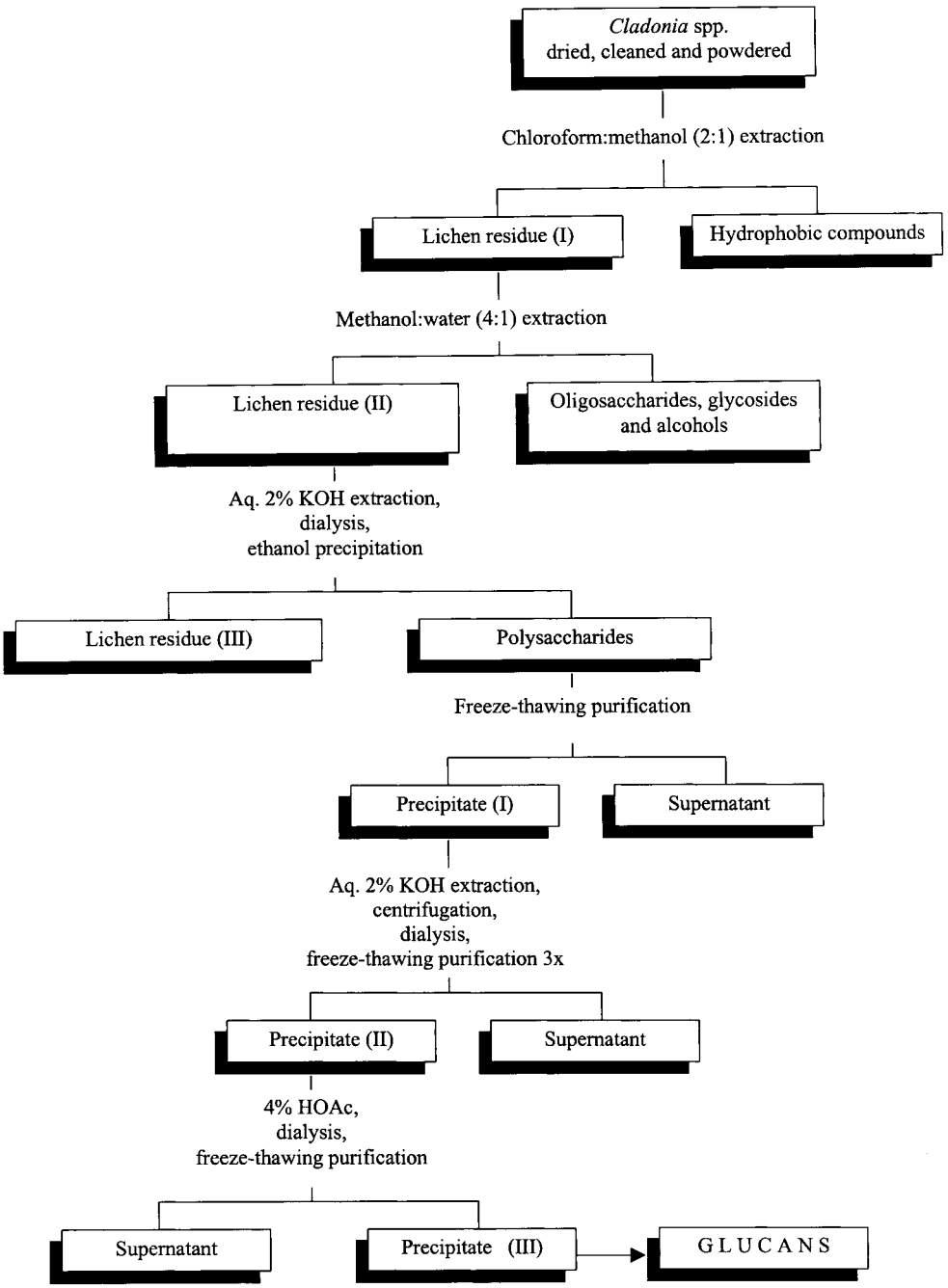
	α (1 → 4)		α (1 → 3)	
H and C units	^{1}H (δ)	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)
H-1/C-1	5.23	100.1	5.14	99.2
H-2/C-2	3.55	70.6	3.48	71.9
H-3/C-3	3.73	72.6	3.70	82.6
H-4/C-4	3.57	78.9	3.52	69.5
H-5/C-5	3.88	73.1	4.03	70.5
H-6/C-6	3.63	60.5	3.76	60.1

[a] Data obtained from ^{1}H (sample dissolved in NaOD–D_2O), ^{13}C, COSY, TOCSY and HMQC (sample dissolved in DMSO-d_6) experiments.

The ratio of (1 → 3)- to (1 → 4)-linkages was determined by comparing the integrated peak intensity of H-1 [(1 → 3 linkage)] with that of H-1′ [(1 → 4 linkage)], as no overlapping of peaks appear in this region. Moreover, the proportion of 2,3,6- and 2,4,6-tri-*O*-methylglucose obtained from the glucan of *C. penicillata*, as determined by GC-MS examination of derived acetates of 2,3,6- and 2,4,6-tri-*O*-methylglucitol, was 1:1, in agreement with the results observed in the ^{1}H-NMR analysis of each species.

The sequence of linkages in a glucan can be determined by the controlled Smith degradation procedure (Fleming & Manners, 1966) involving the following sequence of reactions: periodate oxidation, borohydride reduction and mild acid hydrolysis. With *C. penicillata* glucan, the procedure gave exclusively 2-*O*-α-D-glucopyranosyl-D-erythritol (Charlson & Perlin, 1956), as determined by paper chromatography (PC), with no other oligosaccharides being observed. This indicates that the majority of the 3-*O*-substituted glucosyl residues are situated between 4-*O*-substituted residues, which is in accord with the proposed structure.

The data show that the freeze-thawing process gave glucans resembling nigeran in different yields from each *Cladonia* sp. This result was found earlier for *Cladina stellaris* (0.12% yield, as *Cladonia alpestris*) and *Cladina confusa* (0.3% yield, as *Cladonia confusa*), except that the latter had a linkage ratio of 53:47 of (1 → 3)- and (1 → 4) linkages respectively, i.e., somewhat similar to that of *Cladonia* spp. Moreover, as cited above, identical glucans were obtained by the



Scheme 1. Extraction and purification of glucans from *Cladonia* spp.

freeze-thawing process from two other species of the genus *Cladonia* and two of the genus *Cladina* (Nishikawa et al., 1974). Many other glucans isolated using the freeze-thawing process have been studied. Except for that of *Flavoparmelia caperata*, all of them showed different (1 → 3)- and (1 → 4)-linkage ratios, varying between 2:1 and 3:1 (Gorin et al., 1988). We thus suggest that the 1:1 ratio and others could be used as a marker to aid lichen taxonomy. To check whether this polysaccharide is a 'fingerprint' in *Flavoparmelia* spp., other species should be investigated, considering that only one species has thus far been studied.

With the exception of nigerans, the majority of α-D-glucans are soluble in cold water, due to the relatively high molar proportion (1:1) of (1 → 4)-linkages (amylose, with exclusive α-(1 → 4)-linkages, is also insoluble in cold water). Other α-D-glucans [isolichenans with (1 → 3)- and (1 → 4)-linkages in 2:1, 3:1, 4:1 ratios] are not usually found in the precipitate freeze-thawing fraction but rather in the Fehling supernatant (Woranovicz et al., 1997). Otherwise, lichenans (β-D-

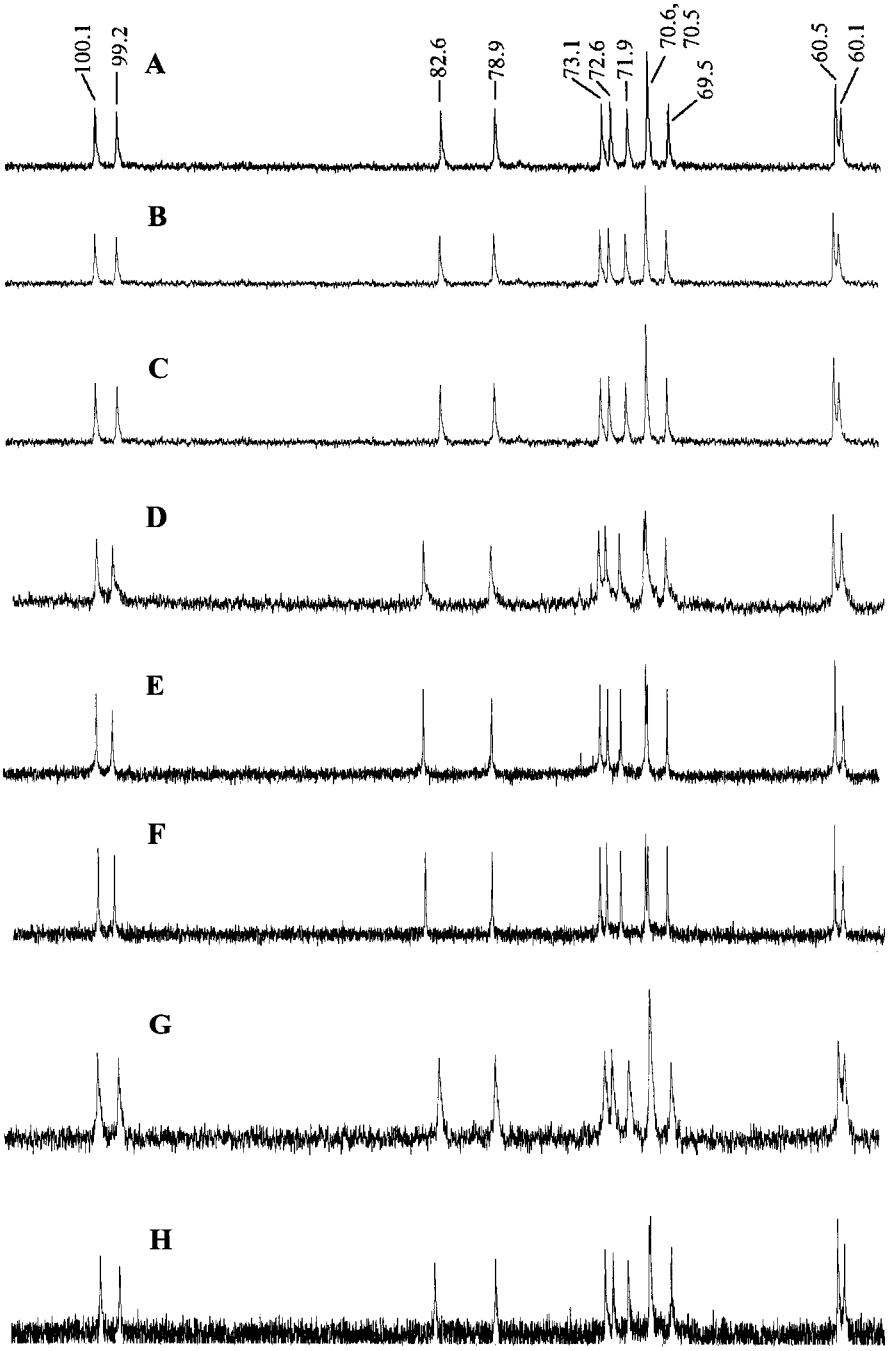


Fig. 1. ^{13}C-NMR spectra (chemical shifts in δ, ppm) of glucans obtained from *C. penicillata* (A), *C. crispatula* (B), *C. clathrata* (C), *C. ibitipocae* (D), *C. furcata* (E) *C. signata* (F), *C. imperialis* (G), and *C. connexa* (H).

glucans) are polysaccharides insoluble in cold water and can be found as a precipitate in the freeze-thawing process, but these did not appear in the present cases.

Chemical studies of lichens investigated to date have shown that all species of the genus *Cladonia* have the same polysaccharide in the glucan fraction studied herein. It appears likely that nigeran is common to all species of the genus *Cladonia*.

3. Experimental

3.1. Lichens

Lichens were collected representing different sections of *Cladonia* as follows: *C. connexa* Vain., *C. furcata* (Huds.) Schrad. and *C. signata* (Eschw.) Vain. belong to sect. *Ascyphiferae*; *C. clathrata* Ahti and L. Xavier Filho, *C. imperialis* Ahti and Marcelli and *C. penicil-*

lata Ahti and Marcelli belong to sect. *Cladonia* (all taxa with verticillate podetia); *C. crispatula* (Nyl.) Ahti belongs to sect. *Perviae*; and *C. ibitipocae* Ahti and Stenroos is indefinite as to the sectional position (either in sect. *Ascyphiferae* or sect. *Unciales*). All of them were collected in the Serra da Mantiqueira, Minas Gerais, Brazil, in 1993–1994 in cooperation with Dr Marcelo P. Marcelli.

3.2. General methods

Specific rotations of 2% aqueous NaOH solutions were obtained with an Autopol III polarimeter at 25°C Voucher specimens are housed in the Herbario Maria Eneyda P. Kauffman Fidalgo, of the Instituto de Botânica de São Paulo. Gas chromatography-mass spectrometry (GC-MS) was performed using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 800 mass spectrometer, with He as carrier gas. Capillary columns (30 m × 0.25 mm id) of OV-225 (a) and DB-210 (b) were used, held at 50°C during injection and then programmed at 40°C/min to 220°C (constant temperature). Columns (a) and (b) were used for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates. Acetylation of alditols was carried out with Ac_2O-pyridine (1:1) for 12 h at room temperature (Woranovicz et al., 1997). Descending PC was carried out on Whatman no. 1 filter paper (solvent: *n*-BuOH–pyridine-H_2O, 5:3:3), sugars being detected by the acetone-$AgNO_3$ dip reagent (Trevelyan, Procter & Harrison, 1950).

3.3. Preparation of glucans

Each sample (50 g) of *Cladonia* spp. studied herein was previously extracted successively with $CHCl_3$–MeOH (2:1 v/v; 300 ml) and 80% aqueous MeOH (250 ml), and then treated with 2% KOH at 100°C for 2 h (Woranovicz et al., 1997). The solutions were neutralised (HOAc), filtered, and the extracted polysaccharides precipitated by addition of excess EtOH. They were isolated, dried, dissolved in hot H_2O, and the solutions were frozen, then thawed at 4°C (Baron, Gorin & Iacomini, 1989). The insoluble material which formed was removed by centrifugation (5000 rpm) and the freezing and thawing process was repeated on the supernatants until precipitates no longer appeared. The combined supernatants were then treated with Fehling solution (Jones & Stoodley, 1965). The resulting precipitates of Cu complexes and supernatants were treated under previously described conditions (Woranovicz et al., 1997).

The insoluble material, obtained by freeze-thawing each alkaline extract, was extracted once more with 2% KOH at 100°C for 2 h. After centrifugation, supernatants were dialysed against tap water to neutral pH, with the freeze-thawing process repeated three more times to remove residual galactomannans. The precipitated fraction was treated with 4% aqueous HOAc at 100°C for 2 h to remove possible proteinaceous aggregates. Dialysis was carried out against tap water for 2 days, when the last freeze-thawing step was performed. The final precipitate of each species consisted of purified glucans.

3.4. Monosaccharide composition analysis

Neutral monosaccharides, liberated by hydrolysis of crude and purified polysaccharides with 1 M TFA (2.0 ml) for 8 h at 100°C, were analysed as their alditol acetates by GC-MS (see Section 3.2) by successive $NaBH_4$ reduction and acetylation with Ac_2O–pyridine (1:1 v/v) at 25°C for 18 h (Woranovicz et al., 1997).

3.5. Determination of homogeneity of polysaccharides and their molecular weight

Glucans (2.0 mg) were dissolved in 1 M NaOH (0.3 ml), and the solution applied to a column of Sepharose CL-4B (61 × 1.7 cm id). It was eluted with 0.05 M NaOH and resulting fractions of 2 ml were tested for carbohydrates (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The column was calibrated for molecular weight using dextrans of M_r 8.16×10^4, 2.66×10^5, 5.0×10^5, and 2.0×10^6.

3.6. Per-O-methylation of the glucan from C. penicillata

The polysaccharide (40 mg) was partially *O*-methylated thrice using Me_2SO_4–aqueous NaOH (Haworth, 1915) and the process completed with NaOH–Me_2SO–MeI (Ciucanu & Kerek, 1984) until the products did not have an OH absorption in the IR at 3400 cm^{-1}. The methylated glucan was treated with refluxing 3% HCl in MeOH for 3 h, then 2 M H_2SO_4 at 100°C for 14 h, and the resulting mixtures of *O*-methyl aldoses successively reduced with $NaBH_4$ and acetylated. The resulting *O*-acetylated, partially *O*-methylated alditols were analyzed by capillary GC-MS, as described in Section 3.2, and identified by their electron impact breakdown profiles and retention times (Jansson, Kenne, Liedgren, Lindberg & Lönngren, 1976).

3.7. Partial Smith degradation of the glucan from C. penicillata

Glucan (40 mg) was oxidised in 0.05 M $NaIO_4$ (50 ml) for 72 h at 25°C in the dark (Abdel-Akher, Hamilton, Montgomery & Smith, 1952; Hay, Lewis & Smith, 1965). 1,2-Ethanediol was then added, and the solution was dialysed, reduced with $NaBH_4$, neutral-

ised with HOAc, and after 24 h redialysed. It was then concentrated to 20 ml and partial hydrolysis effected by adjustment to pH 2.0 with dil. aqueous H_2SO_4 and heating at 100°C for 30 min. Following deionisation and evaporation of the solution, it was examined by PC (solvent: *n*-BuOH–pyridine–H_2O, 5:3:3 v/v; developer: $AgNO_3$–NaOH–acetone dip), which showed the presence of 2-*O*-α-D-glucopyranosyl-D-erythritol exclusively.

3.8. ^{13}C and ^{1}H-NMR spectroscopy

NMR spectra were recorded using a Bruker DRX 400 spectrometer, with a 5 mm inverse detection probe. ^{13}C-NMR (100.6 MHz) analyses were performed at 70°C, the samples (40 mg) being dissolved in DMSO-d_6 (1 ml) and a small volume of D_2O, number of transients 110,000 and pulse interval 1 s.

For the integration measurements, 1D ^{1}H-NMR (400.13 MHz) spectra were recorded at 30°C and samples were dissolved in D_2O containing 3% NaOH. The integrals of H-1 signals found for each glucan (for details, see Table 1) were: *C. penicillata*: 1.00:1.00; *C. furcata*: 1.00:1.04; *C. signata*: 1.00:1.08; *C. imperialis*: 1.00:1.01; *C. crispatula*: 1.00:1.03; *C. ibitipocae*: 1.00:1.10; *C. connexa*: 1.00:1.05; *C. clathrata*: 1.00:1.03. COSY was used to assign proton signals, by sequential correlation from H-1 to H-6 for each of the (1 → 3)- and (1 → 4)-linked α-Glc*p* units and TOCSY to confirm that each proton series arose from the same glucosyl unit. ^{13}C assignments were then made via HMQC, which correlated proton resonances with those of chemically linked ^{13}C nuclei. For HMQC, TOCSY, and COSY, a matrix of 1 × 1 K was used with 16 scans. 2D NMR experiments were obtained only for *C. penicillata* glucan (COSY (Zähringer et al., 1995), TOCSY (Braunschweiler & Ernst, 1983) and HMQC (Friebolin, 1993)). The sample was dissolved in DMSO-d_6 containing a small amount of D_2O and the spectrum obtained at 70°C. Chemical shifts (δ) are expressed relative to the resonance of TMS ($\delta = 0$) obtained in a separate experiment. Coupling constants and chemical shifts were obtained from a first-order analysis of the spectra.

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