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Note

Structure of an antitumor, water-soluble D-glucan from the carpophores of *Tylopilus felleus**

JACQUES DEFAYE†, STANISLAW KOHLMÜNZER‡, KRYSTIAN SODZAWICZNY‡,§ AND EMILE WONG†

Centre de Recherches sur les Macromolécules Végétales, C.N.R.S.-Grenoble, B.P. 68, F-38402 Saint-Martin d'Hères (France), and Department of Pharmaceutical Botany, Nicolaus Copernicus Academy of Medicine, Kraków (Poland)

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Tylopilus felleus is a wildgrowing fungus belonging to the *Boletaceae* family (*Basidiomycetes*), commonly found in European forests. Extraction with hot water of its carpophores was reported to yield a material which exerted an inhibitory effect on the growth of *Neurospora crassa* and *Saccharomyces cerevisiae* strains[3], as well as an antimitotic effect in seedling roots of *Triticum vulgare*[4]. In addition, the extract showed a marked inhibition on the growth of human brain, neoplastic-glioma cells cultivated *in vitro*[5], as well as on Ehrlich carcinoma (ascites form) and Sarcoma 180 cells implanted in mice[6]. The carcinostatic effect in mice was correlated with an increase in the macrophage concentration in the neighborhood of the neoplastic cells[6]. This water-soluble extract showed furthermore an anti-inflammatory effect in the carrageenan-induced edema test[7,8]. Purification of the crude extract by means of a fractional precipitation allowed to ascribe the biological activity to a D-glucan component that was shown to exert a distinct cytotoxic effect on meristematic plant cells[9]. This purified glucan showed an enhanced inhibitory effect (98% of inhibition) on Sarcoma 180 cells implanted in mice[10]. We now report on the structure of this polysaccharide for which we propose the name tylopilan.

RESULTS AND DISCUSSION

Tylopilan showed a single and symmetrical peak on elution from Sephadex

* For preliminary reports, see refs. 1 and 2.
† Centre de Recherches sur les Macromolécules Végétales, C.N.R.S.-Grenoble.
‡ Department of Pharmaceutical Botany, Nicolaus Copernicus Academy of Medicine.
§ Visiting research associate, fellow of the French Government (C.I.E.S. Paris), November 1984–May 1985, at the C.N.R.S.-Grenoble, under the auspices of the exchange program France–Poland.

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G-100 in water. The K_{Av} value indicated an apparent molecular weight of 1.3 10^6, in agreement with the value obtained from light-scattering measurements. As previously reported[9-10], D-glucose was the only identifiable product which could be detected by l.c. after acid hydrolysis.

Methylation analysis[11] of the glucan, with g.l.c.-m.s. characterization of the resulting alditol acetates, led to the identification of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in the ratio of 1:2:1, indicating a branched structure. Furthermore, a single Smith degradation, followed by methylation analysis of the resulting polysaccharide, led to the identification of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol as the sole component. Accordingly, the glucan is assumed to have a (1→3)-linked D-glucopyranan backbone with D-glucopyranosyl groups attached at O-6, on one out of every three backbone residues.

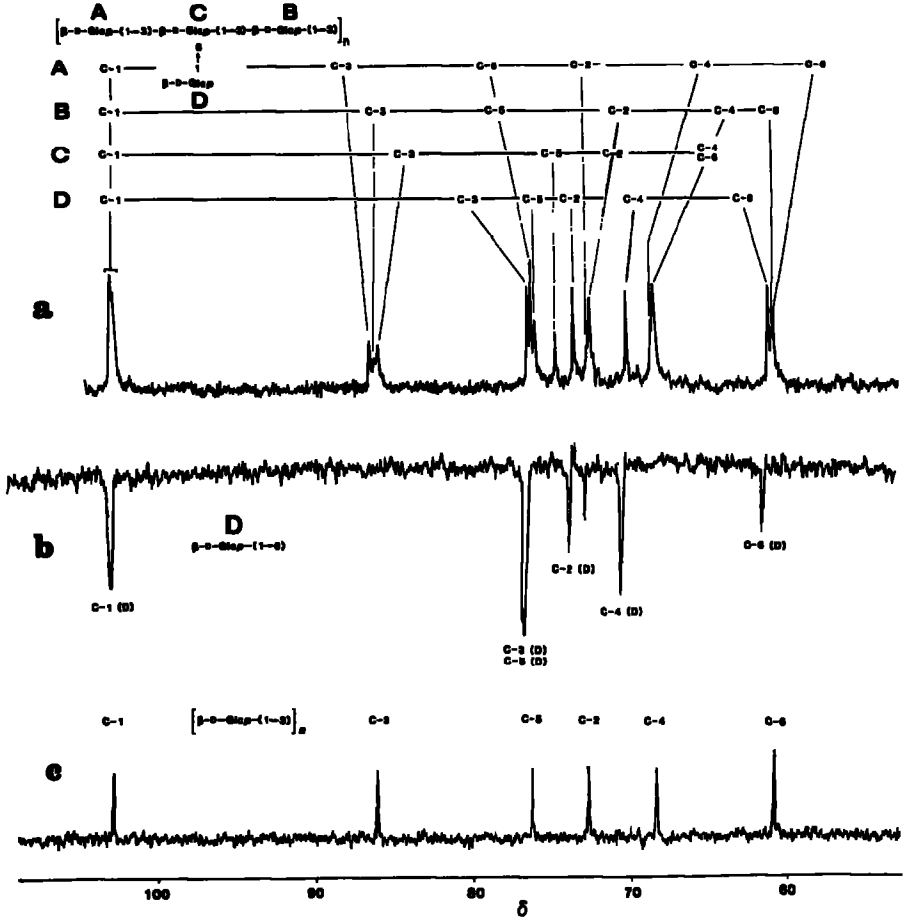


Fig. 1. Comparative ^{13}C-n.m.r. spectra of: (a) tylopilan; (b) partially-relaxed spectrum (τ 0.5 s); and (c) Smith-degraded tylopilan.

Tylopilan gave a well resolved ^{13}C-n.m.r. spectrum, when dissolved in di($^2H_3$)-methyl sulfoxide (Fig. 1a) after a pretreatment of the polysaccharide with the binary cellulose solvent system dimethyl sulfoxide–paraformaldehyde. Such a process has been previously shown to result, with cellulose and amylose, in the formation of oxymethylene hemiacetals at positions usually involved in hydrogen bond associations, thus enhancing their solubilization in dimethyl sulfoxide[12]. Recovery of the starting polysaccharide can result from oxymethylene groups hydrolysis following addition of water, which leads to precipitation of the polysaccharide, or heat decomposition which proceeds through formaldehyde evolution from the solution. When applied to tylopilan, the later pretreatment led to an homogeneous solution, freed from formaldehyde (^{13}C-n.m.r.), which was used as such for the n.m.r. measurements. This solubilization process was also applied to Smith-degraded tylopilan.

The assignments of the ^{13}C-n.m.r. signals were based on comparisons with literature data for branched β-(1→3) and β-(1→6)-linked D-glucans (Table I), and linear β-(1→3)-linked D-glucans (Table II), and by taking into account the data from the methylation analysis and Smith degradation. At least three poorly resolved signals could be detected in the range for the anomeric carbon resonances, $\delta \sim 103.6$; this was indicative of a β-D anomeric configuration at all anomeric positions. Such poor signal dispersion is usually found for anomeric signals of diversely substituted β-glucans[13]. Furthermore, three signals were seen distinctly at $\delta \sim 61.5$, in the region of hydroxymethyl carbon resonances, and the same signal multiplicity appeared at $\delta \sim 87$–88, in the C-3 resonance range. Based on these observations, together with the excellent resolution of the ^{13}C-n.m.r. spectrum, the D-glucan could comprise a preponderant repeating unit of at least three β-(1→3)-linked D-glucopyranosyl residues in a different magnetic surrounding. Since methylation analysis indicated that one in every three D-glucopyranosyl residues is substituted at O-6, the resonance of such a hydroxymethyl carbon atom would be expected to be found, according to the O-substitution shift rule, at ~8–11 p.p.m. downfield, the vicinal C-5 resonance being shifted correlatively 1–2 p.p.m. higher than the other C-5 resonances. The same argument was applied to the assignment of the C-2, C-3, and C-4 resonances of the same unit, as compared to the β-(1→3)-linked D-glucan components. In agreement with these considerations, the ^{13}C-n.m.r. spectrum of Smith-degraded tylopilan displayed a simple pattern (Fig. 1c) with six signals that could be assigned unambiguously to a β-(1→3)-linked D-glucan by reference with literature data for various β-(1→3)-linked homopolysaccharides (Table II). Thus, the assignment of the entire spectrum (Fig. 1a) became clear and was further confirmed by an examination of a partially relaxed spectrum of the untreated polysaccharide. Such a technique gives information on the molecular motion of complex molecules and has been applied to the identification of side chains in oligo- and poly-saccharides[19]. As expected from such branched structure, an appropriate inversion–recovery pulse-sequence, applied to the entire polysaccharide in dimethyl sulfoxide solution, gave the inverted spectrum (Fig. 1b) which was assigned, in agreement with literature data[14–16,18], to the more mobile (1→6)-linked D-glucopyranosyl side-groups of tylopilan. Based on these data, a branched

TABLE I

COMPARATIVE ^{13}C-N.M.R. DATA (δ) FOR TYLOPILAN AND VARIOUS β-(1→3)-, AND β-(1→6)-BRANCHED D-GLUCANS[a]

Polysaccharides	*Structural units*[b]	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Tylopilan	A	103.63	73.68	87.59	69.46	77.32	61.56
	B	103.63	73.6	87.38	69.33	77.32	61.7
	C	103.63	73.6	87.02	69.33	77.06	69.33
	D	103.63	74.61	77.54	71.11	75.75	61.79
Scleroglucan[14]	A	103.9	73.8	87.65	69.45	77.7	61.85
	B	103.9	73.65	87.15	69.4	77.05[c]	61.90
	C	103.9	73.65	86.9	69.4	77.3[c]	69.4
	D	103.9	74.6	77.55	71.15	77.3	62.05
HA β-Glucan (*Pleurotus ostreatus*)[15]	A,B	103.7	73.8	87–87.7	69.3	76.7–77	61.8
	C	103.7	73.6	86.4	69.3	75.4	69.3
	D	103.7	74.5	77.4	70.9	76.7	61.8
Schizophyllan[16]	[d]	104.5	74.3	87.9	70.2	77.8–75.4[e]	62.5–71.6[e]
Lentinan[16]	[d]	104.6	74.3	87.7	70.2	77.9–75.3[e]	62.5–71.5[e]

[a]Solutions in $(^2H_3)Me_2SO$. [b]See scheme 1. [c]These data have been erroneously inverted (ref. 17). [d]Not given. [e]Corresponding to unit B in Scheme 1.

TABLE II

COMPARATIVE ^{13}C-N.M.R. DATA (δ) FOR THE POLYSACCHARIDE RESULTING FROM A SINGLE SMITH DEGRADATION OF TYLOPILAN AND VARIOUS β-(1→3)-LINKED D-GLUCANS

Glucan	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Smith-degraded tylopilan[a]	103.83	73.72	87.1	69.36	77.27	61.84
PS 13140 polysaccharide[a,16]	104.5	74.3	87.5	69.9	77.7	62.4
Curdlan[a,14]	103.7	73.6	86.9	69.9	77.1	61.7
Laminaran[b,18]	103.8	74.4	85.5	69.3	76.8	61.9

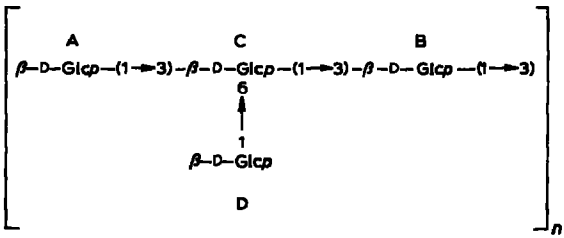
[a]Solutions in ($^2H_3$)Me_2SO. [b]Solutions in D_2O, pD 7.

polysaccharide structure bearing a preponderant repeating unit was assigned to tylopilan (Scheme 1).

Such a structure has previously been found for various fungal polysaccharides like scleroglucan[20], schizophyllan[21], and more recently for grifolan[22] and the HA β-glucan[15] from *Pleurotus ostreatus*. These polysaccharides display ^{13}C-n.m.r. spectra[14,15,16,22] which bear a close similarity to that of Fig. 1a. They all exhibit carcinostatic activity on solid tumors, and this activity is generally believed to be linked with the elicitation of a cell-mediated[23] immune response, in agreement with the results obtained for tylopilan[10]. In addition, tylopilan was shown to exert a cytotoxic effect on meristematic plant cells[9]. This unexpected property is reminiscent of the hypersensitivity effect sometimes found in plant-parasite interaction. Branched β-(1→3), (1→6)-D-glucans, known as elicitors of phytoalexins, have shown such properties in plant[24].

EXPERIMENTAL

Materials. — Carpophores of *Tylopilus felleus* (Bull. ex Fr.) P. Karst. were collected in September 1978 at Lopuszna (southern Poland), dried at 40°, and dis-



Scheme 1. Proposed repetitive structure for tylopilan (capital letters refer to assignments in Fig. 1 and Table I).

integrated in a blender. The dried powder (200 g) was exhaustively extracted with boiling water. The resulting water extracts, after careful filtration, were concentrated under reduced pressure to a volume resulting in the appearance of a slight turbidity. An equal volume of ethanol (96%) was then added, and the mixture was kept for 24 h at 4°. The resulting fibrous precipitate (9 g) was collected by centrifugation, redissolved in water (500 mL), and the solution deproteinized according to the technique of Sevag[25].

A preliminary purification of the crude polysaccharide was achieved by dialysis against tap water for 72 h, followed by an ultrafiltration on Serva membrane, and precipitation by addition of ethanol. The precipitate was recovered by filtration and dissolved in an aqueous NaOH solution (5%, 500 mL). Addition of ethanol (500 mL) led to a precipitate which was recovered by filtration. The procedure was repeated until the supernatant solution became colorless. The resulting purified polysaccharide was dissolved in water (1 000 mL) and dialyzed successively against tap water for 72 h, then de-ionized water (24 h), and finally freeze-dried to give tylopilan (1.87 g) as a white powder; $[\alpha]_D^{20}$ +4.5° (water, *c* 0.2).

General methods. — Light-scattering measurement was performed on solutions in water with a Chromatix KMX-6 low-angle, light-scattering photometer operated at θ 6–7° and $\lambda_{633\ nm}$, according to ref. 26. The optical rotation was determined with a Perkin–Elmer model 241 polarimeter. ^{13}C-N.m.r. spectra were recorded at 75.468 MHz with a Bruker AM 300 instrument for solutions in dimethyl sulfoxide (Me_2SO) and the central line of Me_2SO as internal lock signal; all spectra were recorded at 80°, by use of a 9-μs pulse angle (90°) and a spectral width of 13 150 Hz; the interval between pulses was 0.5 s, and the number of scans 30 000. The partially relaxed spectrum was measured at 25.182 MHz with a Bruker WP-100 spectrometer and the sequence $[\pi - \tau - \pi/2 - T]$. Gas-liquid chromatography (g.l.c.) of partially methylated alditol acetates was performed with a Girdel 3 000 instrument (Paris), fitted with a flame-ionization detector and a GC^{2}WCOT OV-17 column (20 m) with temperature programming of 2° · min^{-1}, from 150 to 250°. The g.l.c.-m.s. analysis was conducted with a AEI MS-30 double-beam mass spectrometer, directly fitted to the capillary columns, using electron-impact ionisation, with a source temperature of 150°, a trap current of 100 μA, a ionisation potential of 100 eV, and an accelerating energy of 4 kV. Liquid chromatography was performed with a Waters liquid chromatograph Model 201 U/6000, fitted with a UK6 injector, and a differential refractometer detector (R401), connected to a Sefram Servotrace graphic recorder operating at 10 mV full-scale. The column used was a μ-Bondapak-NH_2 (stainless steel, 300 × 3.8 mm, i.d.) and 4:1 acetonitrile–water as eluent. The pressure was 3.5 MPa and the flow rate was 1 mL/min. Acetonitrile was previously distilled and filtered through a Millipore membrane (0.5 μm). Dialysis membranes (Visking, Union Carbide) retained molecular weights higher than 6000–8000.

Total acid hydrolysis of tylopilan. — Tylopilan (50 mg) was refluxed in aqueous M H_2SO_4 (10 mL) overnight. The acid was neutralized by addition of powdered $BaCO_3$ and the neutral solution was filtered on Celite and freeze-dried. Liquid

chromatography of this hydrolyzate gave a single peak corresponding to D-glucose.

Methylation analysis of tylopilan. - Methylation was performed (on 100-mg scale samples) in two successive steps, by use of the method of Hakomori[27], followed by a Purdie[28] methylation of the resulting partially methylated polysaccharide. The fully methylated polysaccharide (68 mg) was then hydrolyzed, first in aqueous formic acid (90%, 10 mL), and after removal of the formic acid, in trifluoroacetic acid (2 M, 10 mL) for 4 h at 100°. After concentration of the solution under reduced pressure, the residue was dissolved in 1:1 water–methanol (v/v, 100 mL) and reduced with $NaBH_4$ (200 mg). After the usual work-up, the resulting partially methylated alditols were acetylated (1:1, v/v, acetic anhydride–pyridine, 1 mL) for 1 h at 100°. G.l.c. of the partially methylated alditol acetate mixture showed three peaks in a molar ratio 1:2:1, and respective retention times of 5.15, 7.53, and 11.15 min, whose identification was confirmed[28,29] by m.s. as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol.

Periodate oxidation and Smith degradation of tylopilan. — Freeze-dried tylopilan (200 mg) was dissolved in water (250 mL), and an aqueous solution of 0.1M $NaIO_4$ (250 mL) was added. The mixture was kept in the dark for 72 h at 5°. 1,2-Ethanediol (5 mL) was added, and the solution dialyzed against water overnight and freeze-dried. $NaBH_4$ (300 mg) in water (500 mL) was added and, after storage for 1 h at room temperature, the solution was dialyzed against tap water overnight at room temperature, and then freeze-dried. A solution of the product in 0.5M trifluoroacetic acid (300 mL) was stored overnight at room temperature, and then dialyzed and freeze-dried to give a powdery residue (135 mg). A sample (20 mg) was converted into methylated alditol acetates according to the above-described procedure, and analyzed by g.l.c. and g.l.c.-m.s. A single peak (r.t. 7.53 min), identified as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, was detected in both experiments. A second Smith degradation did not modify these data.

Sample preparation for n.m.r. measurements. — To facilitate the solubilization of the tylopilan and Smith-degraded tylopilan samples in Me_2SO, the following procedure was followed: Dimethyl sulfoxide (1.2 mL) and paraformaldehyde (100 mg) were added to freeze-dried tylopilan (100 mg), and the suspension was heated (80°) until the polysaccharide dissolved. Further heating (80°C) removed the paraformaldehyde, and the solutions were used as such for the n.m.r. measurements.

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