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Note

Antitumor active β-D-glucans from *Phytophthora parasitica*

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In recent studies, several glucans have been isolated by Fabre *et al.*[1] from cell walls of *Phytophthora parasitica,* a phytopathogenic fungus of carnation. These polysaccharides were characterized as a mixture of linear (1→3)-β-D-glucans with various (1→6)-linked mono-, di- and tri-saccharide branches having 0, 1, or 2 (1→3)-linked β-D-glucose residues (**1**). The structures of purified polysaccharides were determined by such chemical methods as methylation, periodate oxidation, and acetolysis. Several polysaccharides isolated from bacteria, fungi, yeasts, lichens, and higher plants have been found to exhibit a potent antitumor activity[2–9]; this activity could be correlated to their (1→3)-β-D-glucan structure. Assays of antitumor activity of glucans from *Phytophthora parasitica* were found to be positive, and acetolysis. Several polysaccharides isolated from bacteria, fungi, yeasts, lichens, and higher plants have been found to exhibit a potent antitumor activity[2–9]; this on the antitumor activity.

EXPERIMENTAL

Materials. — The β-D-glucans were prepared as described previously[1].

^{13}C-N.m.r. spectra. — The spectra were recorded with a Brucker AM 300 spectrometer at 75.46 MHz for solutions in D_2O, with acetone as internal reference taken at δ 31.07 relative to the signal of Me_4Si.

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Smith degradations. — Samples (25 mg) were oxidized with 0.05M $NaIO_4$ (10 mL) at 20° in the dark during 48 h. The oxidation was stopped by addition of 1,2-ethanediol and the solution dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h, neutralized with 50% acetic acid, dialyzed, and partially hydrolyzed in 0.5M trifluoroacetic acid for 15 h at 20°. Acid was removed after repeated addition and evaporation of water, and the residue (12 mg) freeze-dried. A second and third sequence of the Smith degradation were performed under the same conditions.

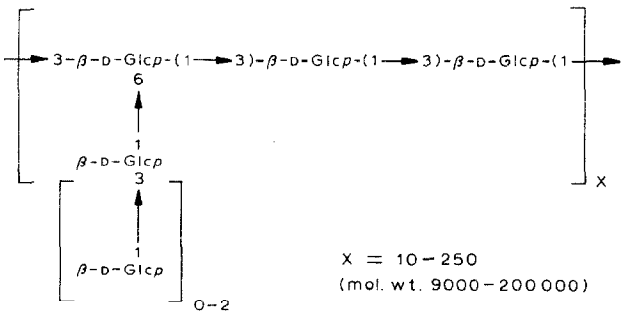
Selective hydrolysis of the branched glucans. — The native glucans were hydrolyzed with M trifluoroacetic acid for 1 h at 100°. Excess acid was evaporated and the process repeated once.

Assay of antitumor activity. — Seven-day-old Sarcoma-180 ascites (0.1 mL, ~5 × 10^6 cells) were transplanted subcutaneously into the right side of female CD1 mice (weighing ~23 g). The test samples, dissolved in saline solution, were intraperitoneally injected daily for 10 days, starting 24 h after tumor implantation. At days 10, 20, and 30, the tumor diameter was determined with a caliber square. At day 30, the mice were killed, and the tumors extirpated and weighed. The inhibition ratio, expressed in percent, was calculated by comparing the average weight of the tumors of treated mice with that of untreated controls.

RESULTS AND DISCUSSION

Fabre *et al.*[1] have described the isolation and the structure determination of neutral polysaccharides of *Phytophthora parasitica*. Briefly, mycelial walls of *Phytophthora parasitica* were extracted with hot water, and the extract was fractionated by sequential chromatography on columns of DEAE-cellulose, Sephadex G-25, and concanavalin A–Sepharose. Neutral polysaccharides were studied by methylation, Smith degradation, and acetolysis; it was concluded[1] that these polysaccharides have a main chain of (1→3)-linked β-D-glucopyranosyl residues with (1→6)-linked branched saccharide residues (**1**).

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectrum of the native glucans



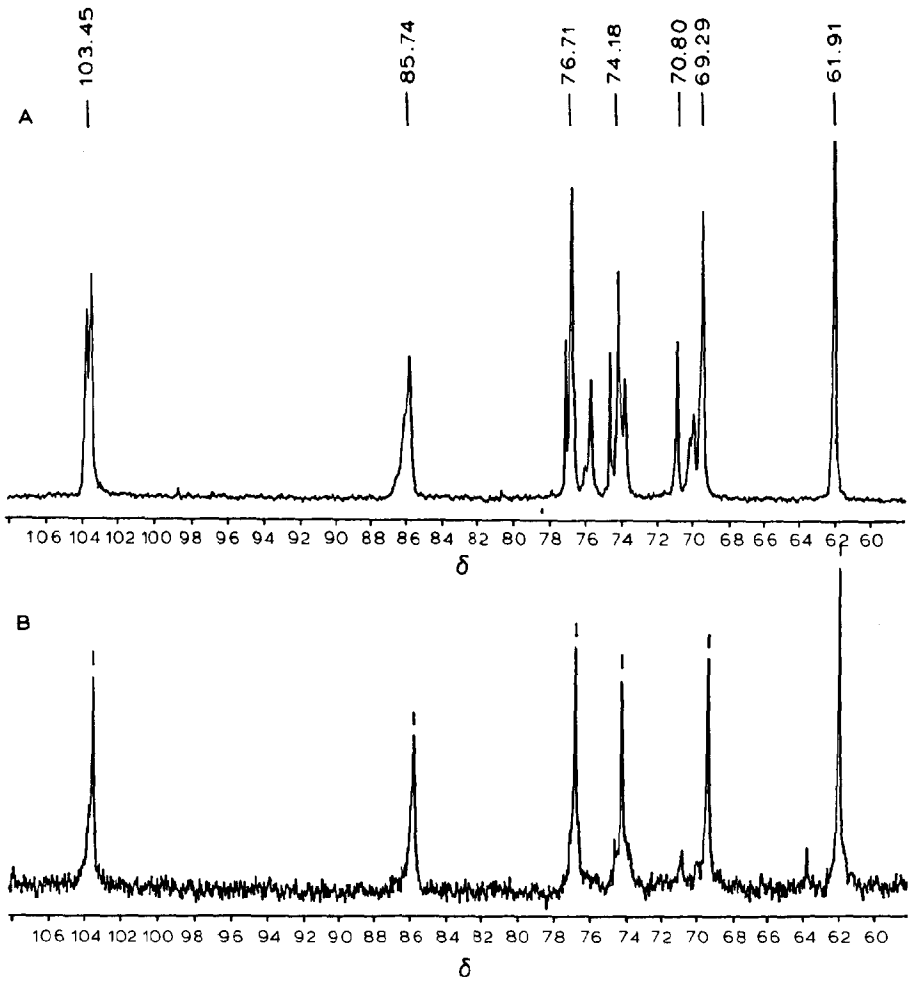


Fig. 1. ^{13}C-N.m.r. spectra of: (A) Native D-glucans from *Phytophthora parasitica*. (B) Smith-degraded derivatives.

from *Phytophthora parasitica* showed multiple resonances (Fig. 1) which agree with their branched (1→3)-β-D-glucan structure[1]. The β configuration of D-glucosyl residues was clearly evidenced by the presence of two anomeric peaks in the region δ 103.5–104, and branchings at C-6 were shown by signals of O-substituted C-6 at δ 70.8 and of unsubstituted C-6 at δ 61.9. The predominance of the latter, together with the typical signal of O-substituted C-3 at δ 85.7 supported the high proportion of β-D-(1→3) linkages in a linear arrangement that was previously demonstrated by chemical analysis. The multiplicity of the signals and the broad C-3 signal at δ 85.7 could be ascribed to the presence, in the glucans, of linear β-D-(1→3), branched β-D-(1→3, 1→6), and terminal β-D-glucopyranosyl residues. Since the number of terminal residues equals the number of branched points, it was not

possible to differentiate their signals on the basis of their relative intensity. However the signals of the β-(1→3)-linked linear D-glucan could be assigned in the spectrum of the Smith-degraded glucan. Three sequential periodate oxidations, followed by partial acid hydrolysis, afforded a linear (1→3)-β-D-glucan, as shown by the only six well-defined signals that were left in the spectrum of the degraded polysaccharide. The assignment of the carbon resonances is given in Table I. This confirmed that the linear-extended β-(1→3) side-chains do not exceed three glycosyl residues. By difference between the spectra of the native and Smith-degraded polymer, it was possible to assign a few other signals in the spectrum of the branched glucan (Table I). In particular, the second anomeric signal could be ascribed to branching on the linear glucan, either to the β-(1→6) branch-points or to the terminal residues.

One linear (1→3)-β-D-glucan could also be obtained by mild hydrolysis with trifluoroacetic acid, which is selective for (1→6) linkages. The spectrum of this partially hydrolyzed polysaccharide resembled the spectrum of the Smith-degraded glucans, but still contained numerous extraneous peaks which indicated that the hydrolysis of the side chains was not complete. The uncomplete hydrolysis of the appending chains may explain that the hydrolyzed glucan did not form a gel, as it was the case for the (1→3)-β-D-glucan scleroglucan [10] and for the antitumoral β-D-glucan isolated from the fruiting body of *Volvariella volvacea*[11]. However, l.c. analysis of the dialyzable material obtained during partial hydrolysis showed glucose as the preponderant sugar, with only traces of dimer and trimer, thus confirming[1] that the side chains consisted essentially of single terminal D-glucopyranosyl groups.

TABLE I

^{13}C-N.M.R. DATA FOR THE D-GLUCANS FROM *Phytophthora parasitica*

D-*Glucan*	*Residues and linkages*	*Chemical shifts (δ)*					
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Native	→3)-β-D-Glc*p*-(1→	103.7[a]	74.6[a]	85.9		76.7	69.8
	6	103.4	74.1	85.7	69.3	75.6	70.1
	↑		73.7	76.6[a]	70.8[a]	77.1[a]	61.9
	1						
	β-D-Glc*p*						
	3						
	↑						
	1						
	β-D-Glc*p* $]_{0\text{-}2}$						
Smith degraded	→3)-β-D-Glc*p*-(1→	103.4	74.1	85.7	69.3	76.7	61.9
Partially hydrolyzed	→3)-β-D-Glc*p*-(1→	103.4	74.0	85.3	70.4[a]	76.7	69.6
					69.3	76.6	61.9

[a]Terminal β-D-glucopyranosyl group.

Antitumor activity. — The branched (1→3, 1→6)-β-D-glucans from the cell walls of *Phytophthora parasitica* showed some structural similarities with schizophyllan, a polysaccharide which is already in clinical trial[12–16]. The antitumor activity of *Phytophthora* glucans was tested on the allogenic solid Sarcoma-180 in mice. This tumor model is known to be very useful for testing immunomodulating substances[17]. In all experiments, ~5 × 10^6 Sarcoma-180 tumor cells (ascites form) were subcutaneously transplanted into the right side of female CD1 mice. The evaluation of the antitumor activity was performed by measuring the tumor diameter at 10-day intervals, and by determining the weight of the excised tumors at day 30 after tumor inoculation. It could be shown that the antitumor effect of the *Phytophthora* glucans is dose dependent with an optimum activity at 1 mg/kg (Fig. 2, Table II).

In all experiments, during the first 10 days after tumor inoculation, the tumors of the glucan-treated mice increased at the same rate as the tumors of the control group. After about 15 days, the tumor diameter in the treated group decreased, and, in many cases, the tumors showed complete regression at day 30. This delayed antitumor effect is an indication for the indirect mode of action of the

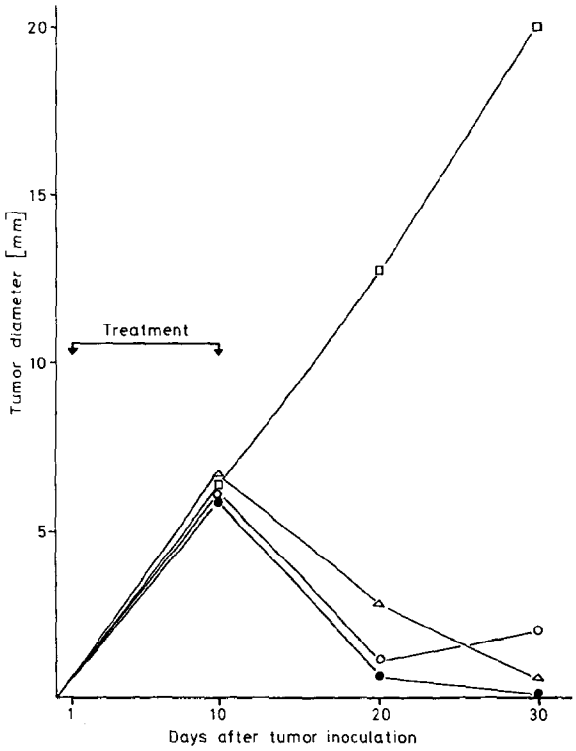


Fig. 2. Dependence of tumor growth on *Phytophthora parasitica* D-glucan concentration. Tumor inoculation at day 0 (5 × 10^6 Sarcoma-180 cells, CD1 mouse, s.c.). Treatment with *Phytophthora* D-glucans daily for 10 days at a dose of: ○, 0.2; ● 1; and △, 5 mg/kg. □, Control.

TABLE II

ANTITUMOR EFFECT OF *Phytophthora* D-GLUCANS ON SOLID SARCOMA-180

Samples	*Dose*[a] *(mg/kg)*	*Average tumor weight (g)*	*Inhibition*[b] *(%)*	*Complete*[c] *regression*	*Significance*[d] (p<)
Dose dependence					
Control		4.52		0/10	
D-Glucans	0.2	0.36	92	8/9	0.02
	1	0.002	99	9/10	0.01
	5	0.01	98	7/8	0.01
Effect of pretreatment					
Control		5.5		0/9	
D-Glucans	1[e]	0.32	95	8/10	0.001

[a]Treatment by i.p. injections of the glucans, dissolved in saline solution, daily for 10 days, starting 24 h after tumor inoculation. [b](C − T)/C · 100. T, Average tumor weight of treated group. C, Average tumor weight of control group. [c]Number of tumor-free mices/number of treated mices. [d]Evaluated according to Student's T-test with $p < 0.05$ as a criterion of a significant difference. [e]Treatment with 1 mg/kg daily for 10 consecutive days, starting 11 days prior to tumor inoculation.

Phytophthora glucans. To confirm an immunomodulating action, we examined the antitumor effect after a pretreatment of the mice with the glucans; this pretreatment started 11 days before tumor inoculation, for 10 consecutive days, by daily i.p. injections of the polysaccharides. The results clearly indicated that a pretreatment has almost the same effectiveness as a treatment after tumor inoculation (Table II). This indirect mode of antitumor action suggests an involvement of the immune system.

Our data are consistent with previous reports[7,8] concerning a possible correlation between antitumor activity and (1→3)-β-D-glucan structure. The glucans of *Phytophthora parasitica* contains two major fractions of different molecular masses, which have been separated by chromatography on Sephadex G-200 column, *i.e.*, Fraction I, M_r ~200 000 and Fraction II, M_r ~10 000. These fractions differ by the presence of (1→6)-branched chains consisting of D-glucose and D-Glc*p*-(1→3)-D-Glc*p* residues for the lowest-molecular-mass polysaccharides or D-Glc*p*-(1→3)-D-Glc*p*-(1→3)-D-Glc*p* for the highest-molecular-mass polysaccharides. Further investigations of the influence of molecular mass and of the nature of branching chains on the antitumor activity are in progress.

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