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Note

Effect of sulfation on the biological activity of β-(1→3)-glucans from the tree fungus *Cyttaria harioti* Fischer

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Glucans containing β-(1→3)- and -(1→6)-linkages, present in virtually all fungi, are of interest because of their potential antitumor activity[1]. Investigations on the chemistry of *Cyttaria harioti* Fischer [one of the most abundant species among the Argentine Cyttariales (Discomycetes), which grow as obligate parasites on *Nothofagus,* producing characteristic tumors that infect the tree and finally cause its death[2]] showed that β-(1→3)-D-glucans account for ~89% of the cell walls[3]. The primary structure of the major component (H-G) is similar to that of schyzophyllan[4], scleroglucan[5], or grifolan[6], all of which have been reported to be antitumor active. Antitumor activity against Sarcoma 180 in mice has been reported for a fructoglucan isolated from *Cyttaria johowii*[7]. This paper is concerned with the effect of sulfation on the antitumor activity of the alkali-soluble glucan (H-G) and its periodate oxidation–Smith-degradation product (H-L). The mitogenic responses of murine spleen cells to the sulfated glucans were also examined.

RESULTS AND DISCUSSION

Glucan H-G consists of a (1→3)-linked β-D-glucose backbone, every second or third residue of which is 6-*O*-substituted by short branches (one or two glucose residues)[8]. This primary structure was further confirmed by the ^{13}C-n.m.r. spectrum, which was recorded in alkaline solution because this glucan separates as a uniform gel on neutralization. H-G is the major component of *Cyttaria harioti*

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TABLE I

ANTITUMOR ACTIVITY OF GLUCANS OBTAINED FROM *Cyttaria* SPECIES AGAINST THE SOLID FORM OF SARCOMA 180 IN ICR MICE[a]

Sample	*Dose × 5 (μg/mouse)*	*Tumor weight (g, mean ±SD)*[b]	*Inhibition ratio*	*Cure/total (N° mice)*
H-A	20	2.54 ±3.86	77.5	3/10
	100	0.95 1.29	91.6	1/10
	500	0.44 1.14	96.1	4/10
H-G	20	1.35 2.34	88.0	2/10
	100	0.06 0.08	99.5	6/10
	500	0.52 0.51	95.4	1/10
H-L	20	0.09 0.12	99.2	5/10
	100	0.97 1.68	91.4	0/10
	500	1.47 1.10	87.0	0/10
D-A	20	7.63 6.48	32.4	2/10
	100	0.42 1.06	96.3	4/10
	500	0.17 0.33	98.5	3/10
D-G	20	0.95 1.15	91.6	2/10
	100	0.05 0.12	99.6	6/10
	500	1.11 3.22	90.2	5/10
CM-curdlan	20	0.02 0.06	99.8	8/10
Nil		11.28 5.20	0	0/10

[a]Ref. 24 and references therein. [b]Standard deviation.

(~51% of fungus dry weight), and this, together with a structurally related, alkali-insoluble glucan (H-R), accounts for 89% of the cell walls[3]. It shows potent antitumor activity against the solid form of Sarcoma 180 in ICR mice (Table I). The ^{13}C-n.m.r. spectrum of H-L (the product of periodate oxidation–Smith degradation of H-G), exhibits characteristic signals (*ca.* δ 64.5–62.5 p.p.m.) of several alcoholic groups, and the spectrum resembles that reported for the polyalcohol obtained by periodate oxidation–borohydride reduction of the structurally related β-(1→3)D-glucan, grifolan[9]. Methylation analysis of H-L gave 2,4,6-tri- and 2,4-di-*O*-methyl-D-glucopyranose in the molar ratio 3.3:1, indicating that not all of the oxidized lateral chains were removed under the mild conditions of the hydrolytic step. The antitumor activiy of H-G was not lowered by periodate oxidation (Table I), suggesting that, as previously observed for grifolan[9], the cleavage of some glucose units at C-6 did not influence the antitumor activity.

The absorption maximum of Congo Red in the presence of H-G and H-L was shifted to longer wavelength (Table II) and returned reversibility to 483 nm at higher alkali concentration. This behaviour has been observed for such other β-D-glucans as curdlan, which is known to form a helix structure in dilute NaOH solutions (<0.25M), whereas it behaves practically as a random coil at higher concentration of alkali. This observed phenomenon has been attributed[10] to the formation of a complex between Congo Red and the ordered structure of the glucan.

The number-average degree of polymerization ($\overline{\text{d.p.}}_n$) of H-G, determined by

TABLE II

ABSORPTION MAXIMUM OF CONGO RED (C.R.) IN SODIUM HYDROXIDE SOLUTION IN THE PRESENCE AND ABSENCE OF POLYSACCHARIDES

Sample	λ_{max} *(nm)*			
	0.1M	0.2M	0.3M	0.5M
C.r. only	484	482	483	479
C.r. + curdlan	497	488	484	479
C.r. + laminaran	484	482	483	481
C.r. + grifolan	507	483	485	480
C.r. + H-G	507	483	483	479
C.r. + H-L	491	483	483	n.d.
C.r. + H-A	484	482	483	n.d.
C.r. + G-SO_4 (6)	508	506	506	507
C.r. + R-SO_4 (3)	507	503	505	508
C.r. + L-SO_4 (3)	502	503	501	506

the method of Unrau and Smith[11], was 220, in agreement with the previous observations of Ogawa *et al.*[12]. A $\overline{d.p.}_n$ of ~200 should be the lower limit of chain length for gel formation. On the other hand, gel-permeation experiments performed with H-G indicate molecular aggregation, as the compound eluted as a single peak immediately after the void volume.

The water-soluble heteropolysaccharide of *Cyttaria harioti* (H-A) showed less activity than H-G and H-L (Table I). H-A consists of a β-(1→3)-linked D-glucose backbone with branches at O-6 formed by chains of α-(1→6)-linked D-glucose units[13,14]. Some of the side-chains may start with a D-*arabino*-hexulosonic acid residue, probably attached to other glucose units through O-4. The β-(1→3)-D-glucan moiety should play a major role in the antitumor activity of both neutral (H-G) and acidic (H-A) glucans. The absorption maximum (λ_{max}) of H-A in the presence of Congo Red was not shifted to longer wavelength at any of the NaOH concentrations assayed (Table II), suggesting a different conformational behavior than either H-G and H-L, and which may be explained on the basis of primary structure. Branches occurring at every fourth residue of the main chain in H-A should be formed by at least four units. If a uniform distribution of lateral chains is considered, their length should influence the conformational properties normally assumed for β-D-(1→3)-glucopyranosyl chains.

Antitumor activity has also been determined for two polysaccharides (D-A and D-G) isolated from the related species *C. darwinii*[15]. Structural similarities between H-A and D-A, as well as between H-G and D-G, were also reflected in their biological activities (Table I).

Sulfation was performed wih chlorosulfonic acid–pyridine[16] on glucans H-G, H-L, and H-R, the minor cell-wall component of *C. harioti*, which is also a β-(1→3)-glucan having β-(1→6)-single glucosyl chains every ninth residue of backbone[3], in order to analyze the effect of sulfate substitution on β-D-glucans with

TABLE III

SULFATION OF H-G, H-R, AND H-L

Sample	*CSA/py*[a] *(v/v)*	*CSA/AGU*[b] *(molar)*	*Reaction conditions*[c]	*Yield (%)*	*%S*	*D.s.*
G-SO_4 (1)	1:1	2.5:1	A	21.2	3.6	0.21
(2)	2:1	5:1	A	57.2	8.9	0.63
(3)	2:1	7:1	A	24.9	5.7	0.35
(4)	2:1	10:1	A	67.3	6.8	0.44
(5)	3:1	5:1	B	81.4	9.0	0.64
(6)	2:1	5:1	B	63.3	13.2	1.16
R-SO_4 (1)	2:1	5:1	A	7.2	6.2	0.39
(2)	3:1	5:1	B	39.2	7.2	0.47
(3)	2:1	5:1	B	63.6	11.3	0.90
L-SO_4 (1)	2:1	5:1	A	55.0	6.4	0.41
(2)	2:1	5:1	A[d]	60.9	6.8	0.44
(3)	2:1	5:1	B	70.7	9.1	0.65

[a]CSA, chlorosulfonic acid; py, pyridine. [b]AGU, anhydroglucose unit. [c]See Experimental. [d]Reaction mixture stored 2 d at −4°.

different degree of branching. The reaction was performed under different experimental conditions, and the results are shown in Table III. The sulfating reagent was stable for 2 weeks at −4°. Longer periods of storage of the reagent resulted in poor yields and lower d.s. The more highly substituted samples for each glucan (G-SO_4(6), d.s. 1.16; R-SO_4 (3) d.s. 0.90; and L-SO_4 (3), d.s. 0.65) were obtained when the mixtures were sonicated to achieve greater homogeneity. This last factor appears to have a greater influence than steric hindrance on the degree of sulfation obtained, as evidenced in the fact that H-G is sulfated to a greater extent than the less-substituted structures H-R and H-L. No degradation of the sulfated derivatives was observed on gel-permeation chromatography. The presence of sulfate groups was confirmed by i.r. spectroscopy through the absorption bands at 860–800 cm^{-1}. No appreciable signal below δ 68.5 p.p.m. attributable to *O*-unsubstituted C-6 could be observed in the ^{13}C-n.m.r. spectrum of G-SO_4 (6), indicating a preference for the sulfation of primary hydroxyl groups, as could be expected considering the relative reactivities of primary *vs.* secondary hydroxyl groups.

The ^{13}C-n.m.r. spectrum of L-SO_4 (3) showed a signal at δ 62.0 p.p.m., suggesting that not all of the primary hydroxyl groups had been sulfated. The signal attributed to *O*-substituted C-6 (C-6′) is shifted downfield (δ 6.3 p.p.m.) by the sulfate groups, whereas C-4′ and C-5′ are shifted upfield by −0.3 and −1.0 p.p.m., respectively[17]. The spectrum is consistent with a linear β-(1→3)-D-glucopyranosyl chain partially sulfated on O-6, indicating that the periodate-oxidized glucose residues remaining attached to the main chain in H-L were cleaved under the acidic conditions of sulfation.

Sulfated glucans G-SO_4 (6); R-SO_4 (3), and L-SO_4 (3) were assayed for anti-

TABLE IV

ANTITUMOR ACTIVITY OF SULFATED POLYSACCHARIDES ON THE SOLID FORM OF SARCOMA 180[a]

Sample	*D.s.*	*Dose × 5 (μg/mouse)*	*Tumor weight (g, mean ±SD)[b]*	*Inhibition ratio (%)*	*Complete regression*
G-SO_4	1.16	20	7.41 ±5.67	−6.93	0/10
		100	7.74 3.44	−11.69	0/10
		500	7.18 3.30	−3.61	0/9
R-SO_4	0.90	20	5.63 3.17	18.76	0/10
		100	5.88 4.33	15.15	0/10
		500	7.56 2.99	−9.09	0/10
L-SO_4	0.65	20	9.84 4.39	−41.99	0/10
		100	8.10 2.21	−16.88	0/10
		500	8.24 2.90	−18.90	0/10
CM-curdlan		20	0.05 0.07	99.28	5/10
Nil			6.93 3.61		0/18

[a]Ref. 24 and references therein. [b]SD = standard deviation.

tumor activity on a solid form of Sarcoma 180, and the results are shown in Table IV. Interestingly, the antitumor activity of H-G and H-L completely disappeared on sulfation. Similar results were obtained for schyzophyllan, which was sulfated with a SO_3·pyridine complex both in pyridine suspension and dimethyl sulfoxide solution[18]. The authors reported that sulfated schyzophyllan lost its original antitumor activity, regardless of the apparent conformational structure of the sulfated product, based on the absorption maxima of the pyridine suspension and the Me_2SO solutions in the presence of Congo Red in 0.14M NaOH. The suspension showed λ_{max} 510 nm, whereas the Me_2SO solution showed λ_{max} 484 nm. These facts were attributed to triple-helix and random-coil conformations, respectively. Sulfated *Cyttaria* glucans showed an irreversible shift to longer wavelength (Table II), even at 0.5M NaOH concentration, suggesting a different conformational behavior than that observed for other triple-helix-forming polysaccharides. An irreversible shift has also been observed for completed debranched grifolan[9], which shows no antitumor activity.

The sulfur content for both schyzophyllan preparations (suspension and solution) was higher than that achieved for R-SO_4 (3) and L-SO_4 (3) and comparable to that of G-SO_4 (6). However, suppresion of antitumor activity seems to be more related to the nature of the sulfate group than to the degree of substitution. Loss of activity on MMHG adenocarcinoma has been reported for sulfated mannoglucans[16].

The mitogenic activity of murine spleen cells to sulfated *Cyttaria* glucans is shown in Table V. Both G-SO_4 (6) and R-SO_4 (3) enhanced mitosis, and their activity was comparable to that reported for sulfated schyzophyllan[18]. There was no mitogenic activity for L-SO_4 (3) a result better explained from the low sulfur content of the compound than from its linear structure.

The results obtained with sulfated *Cyttaria* glucans should reinforce the

TABLE V

MITOGENIC ACTIVITY OF MURINE SPLEEN TO SULFATED GLUCAN

Sample	*Dose* ($\mu g/mL$)	*CPM* (mean ±SD)[a]	*Stimulation index*
G-SO_4 (6)	100	26035 ±1997	3.7
	500	14417 795	2.1
R-SO_4 (3)	100	17683 2741	2.5
	500	9012 755	1.3
L-SO_4 (3)	100	7270 958	1.0
	500	4604 388	0.7
GRN-LE	12.5	6393 662	0.9
	25	5044 751	0.7
LPS	50	38710 2593	5.6
	100	26957 2270	3.9
Nil		6964 428	

[a]SD = standard deviation.

suggestion previously made for grifolan[9] that not only a small amount of branching, but also an aggregated macromolecular structure (gel formation), are necessary for antitumor activity. The presence of such charged groups as sulfate would discourage aggregated macromolecular structures, thus causing a loss of antitumor activity. Mitogenic response seems to be more closely associated with the presence of sulfate groups than with the primary structure of the parent polysaccharide.

EXPERIMENTAL

General. — ^{13}C-N.m.r. spectra of H-G (pD 14) and R-SO_4 (3) (pD 7) were recorded at 25.2 MHz with a Varian XL-100-15 spectrometer (concentration 100 mg/mL) at room temperature; 1,4-dioxane was used as external standard (δ 67.4 p.p.m. downfield from the signal of Me_4Si). ^{13}C-N.m.r. spectra of H-L (pD 14), G-SO_4 (6) (pD 7), and L-SO_4 (3) (pD 7) were recorded at 90.5 MHz with a Bruker AM-360 instrument (concentration 100 mg/mL), using internal sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (δ 1.6 downfield from the signal of Me_4Si). I.r. spectra were recorded with a Perkin–Elmer Model 710 B spectrometer. G.l.c. was performed with a Hewlett–Packard 5830 gas chromatograph, equipped with a flame-ionization detector, on glass columns packed with 3% ECNSS-M on Gas Chrom Q (0.2 × 180 cm) with T_i 210°, T_d 210°, T_c 170°, flow 28 mL of N_2/min.

Material. — Glucans H-G and H-R were isolated from fruit bodies of *Cyttaria harioti* Fischer as previously described[3]. Periodate oxidation of H-G (0.05M $NaIO_4$, 20 days, room temperature) followed by $NaBH_4$ reduction and Smith degradation (0.05M H_2SO_4, 24 h, room temperature) rendered, after neutralization and dialysis, glucan H-L; ^{13}C-n.m.r. of H-G (pD 14), δ (p.p.m.) 104.3, 103.9 (C-1), 74.5 (C-2), 87.9, 87.2 (C-3), 69.4 (C-4), 77.6, 77.3 (C-5); 62.1 (C-6), and 71.2 (*O*-substituted

C-6). ICR male mice, 6 weeks old and weighing 27–30 g, were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals. Curdlan was purchased from Wako Pure Chemical Industries, Ltd. and lipopolysaccharide (cat. no. L-3129) from Sigma Chemical Co.

Analytical procedures. — Sulfur content of the polysaccharides was determined by the procedure described by Terho and Hartiala[19], and the degree of substitution (d.s.) was calculated from the following approximate equation[20]:

$$\text{d.s.} = \%S \times 162 \times [3200 - \%S \times 103]^{-1}.$$

Methylation of H-G and H-L was effected with butyllithium–methyl iodide[21]. The fully methylated products were hydrolyzed with 72% H_2SO_4 (0.5 mL, 2 h, 25°) and then (4 h) at reflux with 12% H_2SO_4. The partially methylated sugars were converted into the corresponding alditol acetates[22], and these were analyzed by g.l.c. The tri-*O*-methyl- to di-*O*-methylglucose molar ratio was 1.2:1 for H-G and 3.3:1 for H-L.

Sulfation. — Glucans H-G, H-R and H-L were sulfated with chlorosulfonic acid–pyridine[16]. The sulfating reagent was prepared by dropping different amounts of $ClSO_3H$ (1, 2 or 3 mL) into pyridine (1 mL), under vigorous stirring, below 0°. The reagent was used within two days after preparation. A suspension of the glucan sample (1 g) in anhydrous formamide (50 mL) was cooled at 0°, and varying amounts of the reagent were added (Table III). The mixture was either stirred for 2.5 h at 25° and 4 h at 40° (conditions *A*), or sonicated for 3 h at room temperature and then heated for 4 h at 60°, without further stirring (conditions *B*). The pyridinium salt of the product, obtained by precipitation with MeOH was separated from the unreacted water-insoluble glucan by redissolution and centrifugation after neutralization of the acid solution with NaOAc. After reprecipitation with EtOH, the pyridinium salt was converted into the sodium salt by successive treatment with Dowex 50W [H] and 2M NaOH. The sodium salt was recovered by dialysis and lyophilization; ^{13}C-n.m.r. of G-SO_4 (6) (pD 7), δ (p.p.m.) 103.8, 102.5 (C-1), 74.4 (C-2); 85.1 (C-3), 69.2 (C-4), 75.7, 75.4 (C-5), 68.5, 69.0 (*O*-substituted C-6); ^{13}C-n.m.r. of R-SO_4 (3) (pD 7), δ (p.p.m.) 103.5 (C-1), 74.1 (C-2), 85.0 (C-3), 69.4, 69.2, 68.9 (C-4), 75.3, 75.0, 76.4 (C-5), 61.7 (C-6), 68.6, 69.9 (*O*-substituted C-6). ^{13}C-n.m.r. of L-SO_4 (3) (pD 7), δ (p.p.m.) 103.7 (C-1), 74.5 (C-2), 85.3 (C-3), 69.3, 69.0 (C-4), 76.8 (C-5), 62.0 (C-6), 68.3 (*O*-substituted C-6).

Gel-permeation chomatography was performed on columns of Sephacryl S-200 or Sephacryl S-300 (1.5 × 100 cm) in M NaCl. The columns were previously calibrated with standard sulfated dextrans (Sigma Chemical Co.); MW 5,000; 8,000; 18,100 and 80,700. Fractions (1 mL) were analyzed by the phenol–sulfuric acid method[23].

Complex formation with Congo Red. — The change of absorption maximum of Congo Red (Merck) in the presence of glucans H-G, H-L and H-A and sulfated derivatives (G-SO_4 (6), R-SO_4 (3) and L-SO_4 (3) was monitored by the procedure

of Ogawa *et al.*[10]. Glucans or sulfated glucans in NaOH solution (1 mg/mL) and Congo Red in NaOH solution (4 mg/100 mL) were mixed in equal volumes, and the λ_{max} values were measured using a Beckman DK-2A spectrophotometer.

Preparation of CM-curdlan. — Curdlan (100 mg) was dissolved in 7 mL of 0.5M NaOH, and the mixture was stirred for 30 min at room temperature. Sodium monochloroacetate (1 g) was added to the sample solution with vigorous stirring in a water bath for 4 h at 60°. The reaction was stopped by adding AcOH, and the mixture was dialyzed and lyophilised to give *O*-(carboxymethyl)curdlan (CM-curdlan).

Antitumor activity. — The antitumor activity was evaluated against the solid form of Sarcoma 180 tumor. Tumor cells (5×10^6 cells/mouse) were inoculated subcutaneously into the right groin of mice. Each sample was administered 5 times (days 7, 9, 11, 13, and 15) after the tumor inoculation (day 0). Five weeks after tumor inoculation, the mice were sacrificed, and the tumor weights were compared. The inhibition ratio was calculated as follows: (1 − average tumor weight of the treated group/average tumor weight of the control group) × 100%. The significance was evaluated according to Student's *t*-test.

Mitogenic activity. — Mice were killed by cervical dislocation, and cell suspensions from the spleens were prepared by teasing in cold RPMI 1640 medium containing *N*-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES) and antibiotics. The cell suspensions were passed through a 200-gauge stainless-steel sieve and then allowed to stand to precipitate tissue fragments. The cell suspensions were centrifuged (600*g*, 5 min), resuspended gently in a 0.83% ammonium chloride solution to lyse red cells, and washed with fresh medium. Viability was assessed with the Trypan Blue dye-exclusion test. The cell suspensions were adjusted to 5×10^6 viable cells per mL. Each sample solution (50 μL) was placed in a flat-bottomed, 96-well tissue-culture plate, 2-fold enriched medium (50 μL) was added for osmotic stabilization, and then 100 μL of the cell suspension prepared above was added to each well. The plates were incubated for 48 h at 37° in a CO_2 incubator. Twenty h before harvesting, 0.5 μCi of tritiated thymidine (^{3}H-TdR), specific activity 15.1 Ci/mmol (New England Nuclear, Boston, Mass.) was added to the culture in a volume of 20 μL. At the end of the culture period, the cells were harvested, and radioactivity was measured in a liquid scintillation counter. Results were measured in a liquid scintillation counter. Results were expressed as the arithmetic mean with standard deviation of triplicate cultures.

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