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Effects of branch distribution and chemical modifications of antitumor (1 → 3)-β-D-glucans

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A potent antitumor-active branched (1 → 3)-β-D-glucan (VVG) purified from fruiting body of *Volvariella volvacea* and some other glucans were chemically modified to study the enhancement of inhibitory activity on the growth of mouse-transplanted tumors. Conversion of the glucosyl groups substituted at *O*-6 atoms of the (1 → 3)-linked D-glucose residues into the corresponding polyhydroxyl groups gave significant enhancement of the original activities both on allogeneic and syngeneic tumors, whereas deletion of the polyhydroxyl groups by mild acid treatment resulted in a great reduction of the activity. When D-glucose residues of the branches were modified to the 3,6-anhydro D-glucose residues by partial sulfation and then alkali treatment, the resulting modified VVG showed essentially no antitumor activity. In connection of the modifications of the branches, a linear (1 → 3)-β-D-glucan was modified to epoxylated glucan (degree of substitution, 0·14), which lost its original activity. On the contrary, conversion of the epoxy groups to hydrophilic glycerol groups remarkably enhanced the original antitumor activity. These results confirmed the previous findings that, besides the conformation of (1 → 3)-β-glucan backbone, the molecular shape and the distribution pattern of the substituted groups located outside the backbone chains, must also play an important role in exhibiting antitumor action.

INTRODUCTION

For the last 15 years, extensive studies have been made for various types of immunomodulating polysaccharides from microbial sources, and their structural correlation to the biological activities has been elucidated. Among the microbial polysaccharides, recent immunopharmacological studies showed that fungal (1 → 3)-β-D-glucans, especially those having β-D-glucosyl side chains, e.g. lentinan (Sasaki & Takasuka, 1976) and schizophyllan (Tabata *et al.*, 1981) exhibit strong host-mediated action of tumor-growth inhibition, by stimulation of the host immune system. Some of these antitumor glucans are currently used as cancer immunotherapeutic drugs in combination with other chemotherapeutic drugs. Furthermore, conformational investigations on lentinan and schizophyllan indicated that their antitumor activities are closely related to their triple helical structure of the (1 → 3)-β-D-glucan backbone chain.

In a series of our studies on the structural diversities of antitumor fungal β-D-glucans and their antitumor effects, we isolated many types of branched (1 → 3)-β-D-glucans with regard to the distribution of branches, having different antitumor activities (Misaki *et al.*, 1981; 1984; Sone *et al.*, 1985). We also found that modification of the D-glucopyranosyl side chains of highly branched glucans, e.g. the alkali-insoluble glucan of *Auricularia auricula-judae* and pestalotan of *Pestalotia* sp., to the corresponding polyhydroxyl groups resulted in enhancement of the original activities.

As reported previously, fruiting body of *Volvariella volvacea*, 'tso ru' in Chinese and 'fukurotake' in Japanese, contains α-linked D-mannogalactan, glycogen, and three kinds of branched (1 → 3)-β-D-glucans. Among these, the cold-alkali extracted, a (1 → 3)-β-D-glucan branched *O*-6 substitution having a degree of branching (db) of 1/5 is expressed as the ratio of branched

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(1 → 3)-linked D-glucosyl residues to all the (1 → 3)-linked D-glucosyl residues), exhibited strong growth inhibitory activity against mouse-transplanted allogeneic tumor (Misaki *et al.*, 1986). This glucan was further purified and its fine structure was elucidated (Kishida *et al.*, 1989*a*).

The present study has been primarily concerned with alteration of the antitumor activities by chemical modification of D-glucosyl groups of the branched (1 → 3)-β-D-glucan of *V. volvacea* (designated VVG). In connection with the chemical modification of VVG, our interest has also been drawn towards the effect of substitution and derivatization of a linear (1 → 3)-β-D-glucan on the antitumor activity.

MATERIALS AND METHODS

Materials

An air-dried sample of fruiting body of *V. volvacea* was obtained from a commercial source in T'aichung, Taiwan. The cold-alkali extracted and hot-alkali extracted (1 → 3)-β-D-glucans were prepared as reported in previous paper (Misaki *et al.*, 1986). The other fungal glucans used in this study, pestalotan of *Pestalotia* sp. 815 and the glucan of *A. auricula-judae*, were obtained from our previous works (Misaki *et al.*, 1981; 1984). Curdlan (MW, 81 000) was purchased from Wako Pure Chemical Industries, Ltd, Osaka. Other chemicals were commercially available.

Mice and tumor

Female ICR-JCL mice (*c.* 23 g) were obtained from CLEA Japan, Inc., Osaka, and female BALB/c mice (*c.* 20 g) were from Charles River Co. Inc., Kanagawa, Japan.

Sarcoma 180, initially provided by Dr T. Sasaki, National Cancer Center Research Institute, was maintained in ICR-JCL mice by intraperitoneal inoculation of ascites cells at weekly intervals. Meth-A ascites tumor cells, also donated by Dr T. Sasaki, were maintained in BALB/c mice by serial intraperitoneal passage.

Assay of antitumor activity

Antitumor activity of a polysaccharide sample was assayed by the method described previously (Misaki *et al.*, 1981). Sarcoma 180 ascite cells (0·05 ml, 6×10^6 cells) were transplanted subcutaneously into the right groin of the ICR-JCL mice. Meth-A cells (1×10^5 cells) were similarly transplanted into the BALB/c mice. The test samples, suspended in 0·01 M phosphate buffered saline (PBS, pH 7·0) in adequate concentrations, were injected intraperitoneally daily for 10 days into mice, starting 24 h after tumor transplantation. The growth of tumors was observed for 5 weeks, and then the mice were killed and tumors extirpated.

The tumor volume at each week was estimated by the following equation:

$$\text{Tumor volume (ml)} = 3{\cdot}14 \times d_1 d_2^2/6,$$

where d_1 is the length (cm) of major axis and d_2 is that of minor axis of the solid tumor. On an average, solid tumor of 1·0 ml calculated by the above equation corresponded to 1·0 g.

Tumor-growth inhibition ratio was calculated by the following equation:

$$\text{Inhibition ratio (\%)} = (A - B) \times 100/A,$$

where A is the average tumor weight of the control group and B is that of the treated group.

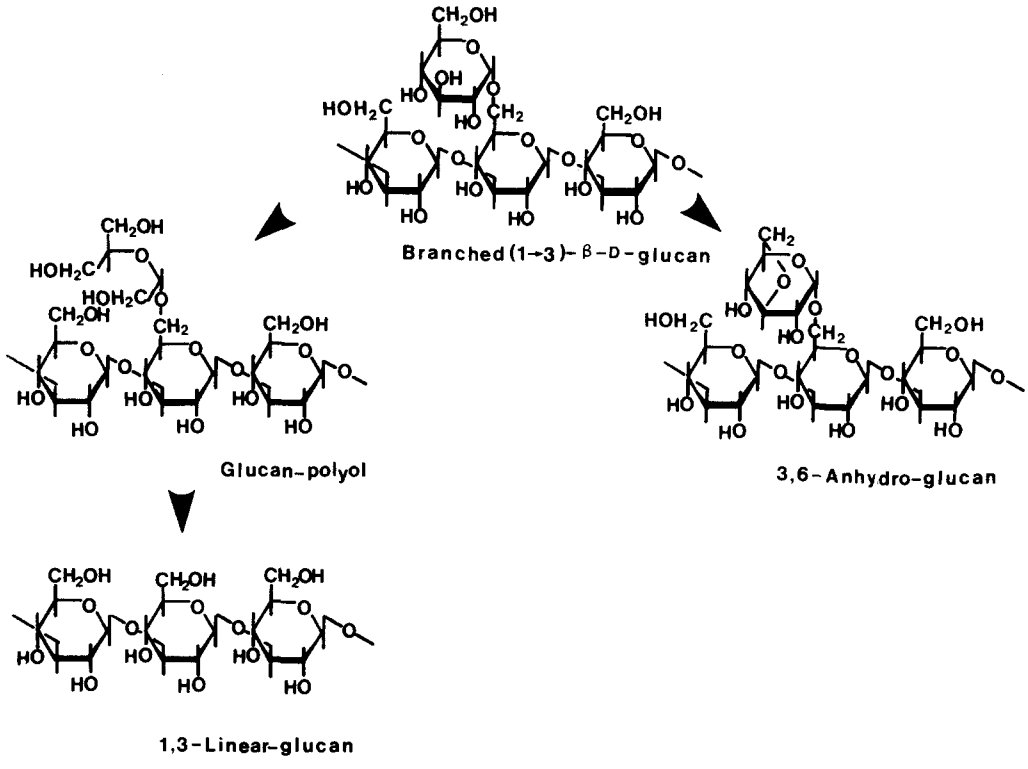
Complete regression was expressed as the ratio of the number of tumor-free mice to the number of mice tested after 5 weeks.

Chemical modifications of (1 → 3)-β-D-glucans of *V. volvacea*

The purified glucan from the cold-alkali extracted glucan fraction of *V. volvacea* was designated VVG (Kishida *et al.*, 1989*a*), and used for the chemical modification. VVG polyol having substitution at *O*-6 atoms with 1-(1,3-dihydroxy-2-propyroxy)-2-hydroxyethyl groups was prepared by periodate oxidation of VVG (0·05 M sodium periodate, 5 °C, 7 days) followed by borohydride reduction (yield, 52%) (Misaki *et al.*, 1986). The methylated polyol gave, on acid hydrolysis, 2,4,6-tri-*O*-methyl D-glucose and 2,4-di-*O*-methyl D-glucose (ratio, 4·6 : 1·0) but no 2,3,4,6-tetra-*O*-methyl D-glucose was detected, confirming that all D-glucosyl groups in side chains were converted into polyhydroxyl groups. VVG polyol (100 mg) was treated with 0·05 M sulfuric acid at 90 °C for 60 min (Misaki *et al.*, 1981), and the resulting insoluble, branch-deleted glucan was collected by centrifugation and washed thoroughly with water (yield, 39·5 mg). It was proved to be a β-(1 → 3)-linked linear D-glucan by methylation analysis.

The β-D-glucan having 3,6-anhydro glucosyl branches (3,6-anhydro-VVG) was prepared by chemical modification involving sulfation and the alkali-treatment in the manner used in our previous work (Misaki & Tsumuraya, 1980). VVG (400 mg) was first sulfated by the reaction with sulfur trioxide-pyridine complex in dimethyl sulfoxide (DMSO) (Miyaji & Misaki, 1973). The sulfated glucan so obtained (623 mg) was treated at 80 °C for 48 h with 2 M NaOH containing sodium borohydride (20 mg) to give 3,6-anhydro-D-glucan (yield, 147 mg).

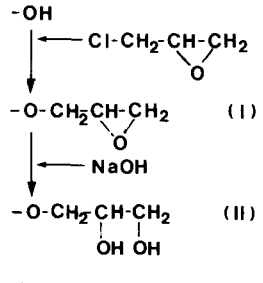
The procedures for chemical modification of VVG are shown in Scheme 1.



Scheme 1 Chemical modification of VVG.

Chemical modification of a linear (1 → 3)-β-D-glucan

To introduce epoxy groups into a (1 → 3)-β-D-glucan, curdlan (200 mg) was dissolved in 0·5 M NaOH (100 ml), and epichlorohydrin (4·2 ml) was added to the solution, the mixture being stirred at 40 °C for 2 h, according to the procedure used for epoxy-Sepharose (Matsumoto *et al.*, 1979). The solution was dialyzed against water and the non-dialyzable fraction was lyophilized to give curdlan having epoxy groups (epoxy-curdlan, 185 mg), (I) in Scheme 2. The content of epoxy groups was estimated by the method of Sundberg and Porath (1974). The epoxy-curdlan (150 mg), (I), was dissolved in 0·1 M NaOH (40 ml), and the solution was stirred at 55 °C for 6 h. After cooling, the reaction mixture was dialyzed, and the non-dialyzable fraction was lyophilized to give glyceryl-curdlan (138 mg), (II). Chemical substitutions of hydroxyl groups in the (1 → 3)-β-D-glucan are summarized in Scheme 2.



Scheme 2 Chemical modification of hydroxy groups of (1 → 3)-β-D-glucan. (I), Epoxy-derivative; (II), glyceryl-derivative.

RESULTS AND DISCUSSION

Comparison of antitumor activities of glucans of *V. volvacea* and other fungal sources

Antitumor activities of different (1 → 3)-β-D-glucan fractions from the cold and hot alkali extracts of 'fukurotake', the fruiting body of *V. volvacea* were examined in Sarcoma 180-ICR mice system by intraperitoneal administration. In Table 1, the tumor-growth inhibition activities of *V. volvacea* glucans are compared with those of other fungal (1 → 3)-β-D-glucans having different degrees of branching, previously obtained from *A. auricula-judae* (Misaki *et al.*, 1981), *Ganoderma lucidum* (Sone *et al.*, 1985), and pestalotan extracellularly produced by *Pestalotia* sp. 815 (Misaki *et al.*, 1984). Of three kinds of *V. volvacea* glucans, the moderately branched glucan, db 1/5, obtained from the cold-alkali extract exhibited significantly high antitumor activity, i.e. 81·7% at a dose of 10 mg/kg, 97·7% at 5 mg/kg, and 73·2% at 1 mg/kg, respectively. As regards the host-mediated antitumor action, *V. volvacea* glucan may have an optimal dosage, as observed with lentinan (Chihara, 1983) and other antitumor polysaccharides. This highly active glucan fraction was purified. The purified glucan, designated VVG, has *O*-6 substitutions with dominantly single D-glucosyl groups, on average

Table 1. Antitumor activities of glucans of *V. volvacea* and other fungal sources

Glucan	db	Dose[a] (mg/kg)	Average tumor weight (g)		Tumor growth inhibition (%)	Complete regression
			Treated	Control		
Against Sarcoma 180 solid tumor in ICR-JCL mice						
V. volvacea						
Cold alkali-extracted	1/5	1	2·5	9·5	73·2	3/6
(VVG)		5	0·3	10·2	97·0	4/5
		10	2·0	10·9	81·7	1/5
Hot alkali-extracted	1/2	10	7·2	10·9	33·9	0/5
Schizophyllum commune[b]						
Schizophyllan	1/3	1	0·3	4·1	95·1	6/10
A. auricula-judae[c]						
Alkali-insoluble glucan	3/4	10	4·7	5·8	18·9	0/4
G. lucidum[d]						
Water extracted	1/3	10	0·2	9·4	97·7	4/5
Cold alkali-extracted	1/17	10	3·8	9·4	59·6	2/6
Pestalotia sp. 815[e]						
Pestalotan	2/3	5	4·2	9·8	57·3	0/6
Against Methylcholanthrene-induced fibrosarcoma in DBA/2 mouse[f]						
VVG		5	0	2·04	100	6/6

[a] Injected intraperitoneally daily for 10 days.
[b] Tabata *et al.* (1981).
[c] Misaki *et al.* (1981).
[d] Sone *et al.* (1985).
[e] Misaki *et al.* (1984).
[f] Tested by Dr T. Sasaki, National Cancer Center Research Institute, Japan.

one out of five (1 ⟶ 3)-D-glucose residues. It has structural heterogeneity with regard to the distribution of branches (Kishida *et al.*, 1989*a*).

In addition to *V. volvacea* glucans, tumor growth inhibition activities exhibited by other branched glucans indicate that the antitumor activity is affected by the mode of distribution of glucosyl side chains attached to the (1 ⟶ 3)-β-linked backbone chains. Thus, the glucans having db 1/3–5 appear to exhibit most potent activity as shown in Table 1.

VVG was also capable of inhibiting syngeneic tumors, such as Meth-A fibrosarcoma in BALB/c mice and methylcholanthrene-induced fibrosarcoma in DBA/2 mice, by intraperitoneal injection at a dose of 5 mg/kg for 10 days.

Figure 1 shows the growth curve of Sarcoma 180 solid tumor, subcutaneously inoculated into ICR mice. The growth of tumors without VVG treatment shows linear relation to the day: the average volume, 2·5 ml at day 21, growing exponentially until the average weight reached a half of total body weight by day 35–42. On intraperitoneal administration at a dose of 5 mg/kg from day 1 to day 10, VVG showed a strong antitumor activity, and was found complete elimination of the solid tumor in all mice after 21 days. Intraperitoneal administration of VVG, at a dose of 5 mg/kg, starting from day 14 and 21 for 10 days into tumor-bearing mice also resulted in growth inhibition of Sarcoma 180, and the mice were cured at day 28 and 42, respectively. Administration of VVG into mice at day 28 and 35, when the tumor exponentially grew, also reduced the tumor volume to a great extent, about one fifth and half control mice, respectively.

Effects of chemical modifications of (1 ⟶ 3)-β-D-glucans on their antitumor activities

As reported previously, chemical modification of D-glucosyl groups attached to the (1 ⟶ 3)-β-D-glucan by substitution at *O*-6 atoms to the corresponding polyhydroxyl groups resulted in significant enhancement of the original activities (Table 2), first, shown in the case of the alkali-insoluble glucan of *A. auricula-judae* and, later, pestalotan of *Pestalotia* sp. 805 (Misaki *et al.*, 1981; 1984). Therefore, in the present study, chemical modifications of VVG were attempted, involving conversion of glucosyl groups into the polyhydroxyl groups, deletion of the side chains, and introduction of 3,6-anhydro ring by sulfation followed by alkali treatment, as shown in Scheme 1. Introduction of 3,6-anhydro-ring into D-glucosyl residues of the (1 ⟶ 3)-β-D-glucans was confirmed by detection of methyl-

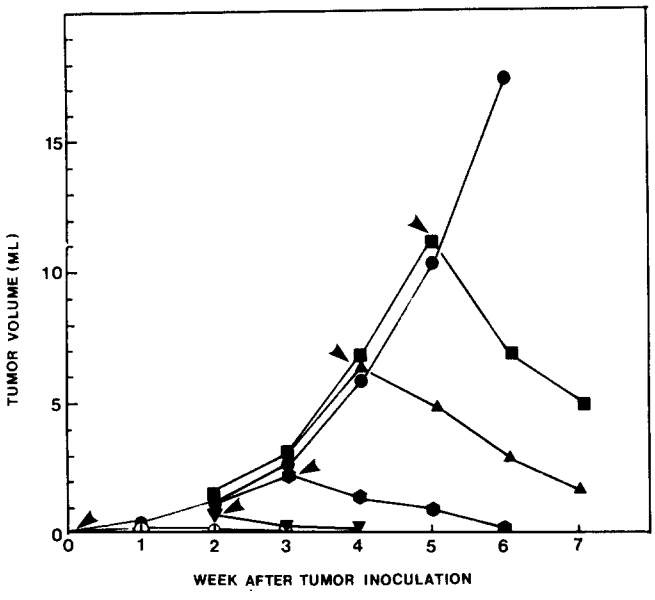


Fig. 1. Growth curves of Sarcoma 180 solid tumor with or without treatment with VVG. (●) without the treatment (control); (○), (▼), (◆), (▲) and (■) with the treatment (5 mg/kg daily for 10 days), arrow (↓) indicating the starting day of VVG injections.

glycoside of 3,6-anhydro-D-glucoside after methanolysis (4% hydrogen chloride methanol solution, 65 °C, 24 h). TLC (solvent, chloroform: methanol, 10 : 1) gave two spots, corresponding to methyl 3,6-anhydro-2,5-diacetyl-D-glucofuranosides (R_f 0·56), and α- and β-methyl-D-glucopyranoside (R_f 0·067), respectively. The mixture of methylglucosides was acetylated by heating with pyridine: acetic anhydride (1 : 1 mixture), and analyzed by GLC (column, 3% ECNSS-M on gas chrom Q, 180 °C), which revealed peaks of acetyl derivatives of α-methyl and β-methyl D-glucofuranoside (R_t, 0·107 and 0·170), relative to that of β-methyl D-glucopyranoside (R_t, 1·00).

Antitumor activities of the modified glucans were compared with that of the original VVG (Table 2). As anticipated, VVG-polyol showed a high antitumor with complete tumor regression at a dose of 1–5 mg/kg for 10 days, higher than those of native VVG under the similar assay conditions (Table 1). These results confirmed that the introduction of polyol group significantly enhances the antitumor activity of the original VVG.

VVG-polyol also showed remarkable antitumor activity against syngeneic tumors i.e., Meth-A and methylcholanthrene-induced fibrosarcoma transplanted into BALB/c and DBA/2 mice, respectively (Table 2). The inhibition ratio against Meth-A tumor was 97·1% with complete regression, three out of five, when tested at a dose of 5 mg/kg, and also against methylcholanthrene-induced fibrosarcoma. At a dose of 5 mg/kg, it gave the high inhibition ratio of 97·1%, with complete regression, three out of five.

Thus, the present results confirm our previous conclusion that the attachment of polyol groups to (1 → 3)-β-D-glucan backbone enhances the original

Table 2. Comparison of antitumor activities of branched (1→3)-β-D-glucans and their derivatives of fungal sources

Polysaccharide	Dose[a] (mg/kg)	Average tumor weight (g)		Inhibition ratio (%)	Complete regression
		Treated	Control		
Against Sarcoma 180-solid tumor in ICR-mice					
V. volvacea					
VVG-polyol	1	0	9·5	100	6/6
	5	0	9·5	100	6/6
	10	2·8	9·5	70·7	3/6
1,3-Linear-VVG	5	11·6	9·5	−22·5	0/6
3,6-Anhydro-VVG	5	15·6	9·5	−63·8	0/6
A. auricula-judae					
Glucan	10	8·9	11·0	18·9	0/6
Glucan-polyol	5	0·3	11·0	97·4	6/8
Pestalotia					
Pestalotan	5	4·2	9·8	57·3	0/6
Pestalotan-polyol	1	0	10·2	100	6/6
Against Meth-A fibrosarcoma in BALB/c mice					
VVG-polyol	5	0·1	3·5	97·1	3/5
Against Methylcholanthrene-induced fibrosarcoma in DBA/2 mice[b]					
VVG-polyol	5	0·06	2·04	97·1	3/5

[a]Injected intraperitoneally daily for 10 days.
[b]Tested by Dr T. Sasaki, National Cancer Center Research Institute, Japan.

antitumor activity. Although such effect might be partly due to increase in the solubility, it is most feasible that the distribution of numerous polyhydroxyl groups outside of the triple helix chains of (1 → 3)-β-D-glucans may afford augmentation of the immunomodulating potency of the host. In connection with this, our previous study showed that the antitumor activity of pestalotan polyol was correlated to the content of polyol groups (Misaki *et al.*, 1984). It is interesting to note that complete deletion of all polyol groups in VVG-polyol by mild acid hydrolysis resulted in elimination of the original activity, as shown in Table 2. In connection to the molecular shape of branches to the antitumor activity of the *O*-6/branched (1 → 3)-β-D-glucan, the glucose residues in side chains of VVG was modified to the corresponding 3,6-anhydro derivative, by sulfation followed by alkali treatment. The glucan having 3,6-anhydro D-glucosyl branches lost the antitumor activity of the original glucan, or showed rather stimulation of the growth of Sarcoma 180 solid tumor cells (inhibition ratio, −63·8%, at dose 5 mg/kg).

To confirm the role of hydrophilic side chains to the antitumor action of the (1 → 3)-β-glucan, curdlan, a bacterial linear (1 → 3)-β-D-glucan was, first, partially epoxylated and then glycerylated, as shown in Scheme 2, and their antitumor activities were compared. Under optimal conditions, 0·2% curdlan in 0·5 M sodium hydroxide was reacted with 5% epichlorohydrin, which afforded epoxylated curdlan having degree of substitution (ds) 0·14 (ds is expressed as the degree of substitution with epoxy groups per anhydro glucose unit.), substituted most probably at *O*-6 atoms of β-(1 → 3)-linked D-glucose residues (Scheme 2, (I)). Alkali treatment of (I) yielded glyceryl curdlan (Scheme 2, (II)); no epoxy group was detected in (II)). These derivatives of curdlan so prepared were tested for antitumor action on Sarcoma 180 solid tumor by intraperitoneal administration of 10 mg/kg for 10 days, as shown in Table 3. In this system, glyceryl-curdlan gave complete regression of the tumor cells (inhibition ratio, 100%), higher than the original glucan (inhibition ratio, 72·2%). On the other hand, the epoxy derivative of curdlan had no antitumor effect (inhibition ratio, −8·3%). These results also support that introduction of hydrophilic polyol or glyceryl groups should give enhancement effects, whereas introduction of hydrophobic groups such as epoxy groups may result in the opposite effect. As regards the effect of chemical derivatization of a linear (1 → 3)-β-D-glucan, the antitumor activity of curdlan, the water-insoluble glucan, was enhanced by partial carboxymethylation (ds, <1), probably due to water solubility (Sasaki *et al.*, 1979). However, our investigation showed the water-soluble carboxymethyl derivatives (ds, 0·47 and 0·56) of the highly branched, water-insoluble glucan of *A. auricula-judae* was less active on Sarcoma 180, compared with that executed with its glucan polyol (Misaki *et al.*, 1981).

It has been well established that the antitumor actions of (1 → 3)-β-glucans are closely related to their triple helix conformation, as suggested by recent works on the depolymerized schizophyllan (Tabata *et al.*, 1981; Kojima *et al.*, 1986). In addition to the triple strand conformation of the backbone chain, our previous and present studies have provided ambiguous evidence that the polyhydroxyl groups localized outside of the triple strand backbone by substitution at *O*-6 atoms of the (1 → 3)-linked D-glucose residues should be involved in enhancing effect of antitumour actions. In connection with this, our recent study also showed that chemically synthesized branched (1 → 3)-β-glucans having D-arabinofuranosyl or D-mannosyl side chains exhibited strong antitumor activity, while branched (1 → 4)-β-glucans having similar side chains had no antitumor effect (Matsuzaki *et al.*, 1986). The mechanism of the antitumor actions of these (1 → 3)-β-D-glucans having glucosyl branches has not been elucidated, but stimulation of the immunopotentiation of hosts, involved in activation of macrophages, promoting differentiation of T-cells, augmentation of natural killer (NK) activity and others, may be the most likely explanation, as suggested by several immunopharmacological studies (Chihara, 1983). Recently we obtained specific antibodies to the antitumor VVG and its polyol derivative (Kishida *et al.*, 1989*b*). These antibodies or the corresponding immunoadsorbent column may provide a useful probe for further investigation of the host-mediated antitumor actions of (1 → 3)-β-D-glucans.

Table 3. Antitumor activities of curdlan and curdlan-derivatives against sarcoma 180 solid tumor

Polysaccharides	Dose[a] (mg/kg)	Average tumor weight (g)		Inhibition ratio (%)	Complete regression
		Treated	Control		
Curdlan	10	2·00	7·20	72·2	2/6
Glyceryl-curdlan	10	0	7·20	100	5/5
Epoxy-curdlan	10	10·46	9·66	−8·3	0/5

[a]Injected intraperitoneally daily for 10 days.

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REFERENCES

Chihara, G. (1983). Immunopharmacology of lentinan and the glucans. *J. Immunol. and Immunophamaco.* **IV**, 85–96.

Kishida, E., Sone, Y. & Misaki, A. (1989*a*). Purification of an antitumor-active, branched (1 → 3)-β-D-glucan from *Volvariella volvacea*, and elucidation of its fine structure. *Carbohydr. Res.* **193**, 227–39.

Kishida, E., Sone, Y., Shibata, S. & Misaki, A. (1989*b*). Preparation and immunochemical characterization of antibody to branched β-(1 → 3)-D-glucan of *Volvariella volvacea*, and its use in studies of antitumor actions. *Agric. Biol. Chem.* **53** (7), 1849–59.

Kojima, T., Tabata, K., Ito, W. & Yanaki, T. (1986). Molecular weight dependence of the antitumor activity of schizophyllan. *Agric. Biol. Chem.* **50** (1), 231–2.

Matsumoto, I., Mizuno, Y. & Seno, N. (1979). Activation of Sepharose with epichlorohydrin and subsequent immobilization of ligand for affinity adsorbent. *J. Biochem.* **85** (4), 1091–8.

Matsuzaki, K., Sato, T., Enomoto, K., Yamamoto, I., Ohshima, R., Hatanaka, K., Uryu, T., Kaku, H., Sone, Y. & Misaki, A. (1986). Synthesis of water-soluble branched polysaccharides having D-mannopyranose, D-arabinofuranose, or oligo-D-arabinofuranose side-chains and their antitumor activity. *Carbohydr. Res.* **157**, 171–82.

Misaki, A. & Tsumuraya, Y. (1980). Structure and enzymatic degradation of elsinan, a new α-D-glucan produced by *Elsinoe leucospila*, In *Fungal Polysaccharides*, ed. P. A. Sandford & K. Matsuda, ACS series 126, American Chemical Society, Washington DC, pp. 197–220.

Misaki, A., Kakuta, M., Sasaki, T., Tanaka, M. & Miyaji, H. (1981). Studies on interrelation of structure and antitumor effects of polysaccharides: antitumor action of periodate-modified, branched (1 → 3)-β-D-glucan of *Auricularia auricula-judae*, and other polysaccharides containing (1 → 3)-glycosidic linkages. *Carbohydr. Res.* **92**, 115–29.

Misaki, A., Kawaguchi, K., Miyaji, H., Nagae, H., Hokkoku, S., Kakuta, M. & Sasaki, T. (1984). Structure of Pestalotan, a highly branched (1 → 3)-β-D-glucan elaborated by *Pestalotia* sp. 815, and the enhancement of its antitumor activity by polyol modification of the side chains. *Carbohydr. Res.* **129**, 209–27.

Misaki, A., Nasu, M., Sone, Y., Kishida, E. & Kinoshita, C. (1986). Comparison of structure and antitumor activity of polysaccharides isolated from *Fukurotake*, the fruiting body of *Volvariella volvacea*. *Agric. Biol. Chem.* **50** (9), 2171–83.

Miyaji, H. & Misaki, A. (1973). Distribution of sulfate groups in the partially sulfated dextrans. *J. Biochem.* **74** (6), 1131–9.

Sasaki, T. & Takasuka, N. (1976). Further study of the structure of lentinan, an anti-tumor polysaccharide from *Lentinus edodes*. *Carbohydr. Res.* **47**, 99–104.

Sasaki, T., Abiko, N., Nitta, K., Takasuka, S. & Sugino, Y. (1979). Antitumor activity of carboxymethylglucans obtained by carboxymethylation of (1 → 3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes* IFO 13140. *Europ. J. Cancer*, **15**, 211–15.

Sone, Y., Okuda, R., Wada, N., Kishida, E. & Misaki, A. (1985). Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing culture of mycelium of *Ganoderma lucidum*. *Agric. Biol. Chem.* **49** (9), 2641–53.

Sundberg, L. & Porath, J. (1974). Preparation of adsorbents for biospecific affinity chromatography. *J. Chromatogr.* **90**, 87–98.

Tabata, K., Ito, W., Kojima, T., Kawabata, S. & Misaki, A. (1981). Ultrasonic degradation of schizophyllan, an antitumor polysaccharide produced by *Schizophyllum commune* fries. *Carbohydr. Res.* **89**, 121–35.