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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*

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ABSTRACT

Antitumor activities of two (1→6)-branched (1→3)-β-D-glucans, isolated from the fruiting body of *Auricularia auricula-judae* ("kikurage", an edible mushroom), and other branched polysaccharides containing a backbone chain of (1→3)-α-D-glucosidic or (1→3)-α-D-mannosidic linkages [and their corresponding (1→3)-D-glycans, derived by mild, Smith degradation] were compared. Among these polysaccharides, a water-soluble, branched (1→3)-β-D-glucan (glucan I) of *A. auricula-judae* exhibited potent, inhibitory activity against implanted Sarcoma 180 solid tumor in mice. The alkali-insoluble, branched (1→3)-β-D-glucan (glucan II), a major constituent of the fruiting body, showed essentially no inhibitory activity. When the latter glucan, having numerous branches attached, was modified by controlled, periodate oxidation, borohydride reduction, and mild, acid hydrolysis, the resulting, water-soluble, degraded glucan, having covalently linked polyhydroxy groups attached at O-6 of the (1→3)-linked D-glucosyl residues, exhibited potent antitumor activity. Further investigations using the glucan-polyalcohol indicated that the attachment of the polyhydroxy groups to the (1→3)-β-D-glucan backbone may enhance the antitumor potency of the glucan. On the other hand, partial introduction

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of carboxymethyl groups into glucan II (d.s., 0.47–0.86), which altered the insolubility property, failed to enhance the antitumor activity. The interrelation between the antitumor activity and the structure of the branched (1→3)-β-D-glucan is discussed, on the basis of methylation and ^{13}C-n.m.r. studies of the periodate-modified glucans.

INTRODUCTION

Since 1963, when Johnson *et al.*[1] established the structure of a branched (1→3)-β-D-glucan—having side chains of single D-glucosyl groups at the O-6 atoms—that is elaborated by *Fungi imperfecti* (*Sclerotum glucanicum*), similar branched D-glucans have been found in fruiting bodies and in the culture broth of many fungi, such as *Lentinus edodes* (lentinan)[2,3], *Sclerotinia libertina*[4], and *Schizophyllum commune* (schizophyllan)[5,6]. Harada *et al.* also reported production of a water-insoluble, linear (1→3)-β-D-glucan (curdlan) by a bacterium, *Alcaligenes faecalis*[7,8].

In a previous study[9], two highly branched (1→3)-β-D-glucans (glucans I and II), in addition to an acidic heteropolysaccharide, were isolated from "kikurage", the fruiting body of *Auricularia auricula-judae*, one of the common edible mushrooms belonging to the *Heterobasidiomycetes*.

In recent years, some (1→3)-β-D-glucans have attracted much attention[10] in view of their inhibitory action on the growth of certain tumors in animals; however, structural correlation to the antitumor effects of these polysaccharides is not yet fully understood, except for the molecular-weight dependence of some glucans[11,12]. Our preliminary study showed that the water-soluble glucan I of *A. auricula-judae* exhibits potent antitumor activity against the growth of Sarcoma 180 solid tumor, implanted in mice, whereas the other (alkali-insoluble) glucan II, which is a major constituent of the fruiting body, has essentially no such activity. This has prompted us to investigate the relationship of the structure to the antitumor effect of the branched (1→3)-β-D-glucans and other plant and microbial polysaccharides containing a backbone of (1→3)-D-glycosidic residues.

We report, first, the dependence of the antitumor effects of some polysaccharides containing a (1→3)-D-glycosidically linked backbone on the structure, and, second, a comparison of the antitumor activities of chemically modified glucan II (altered by controlled periodate oxidation and borohydride reduction, and also by partial carboxymethylation).

RESULTS AND DISCUSSION

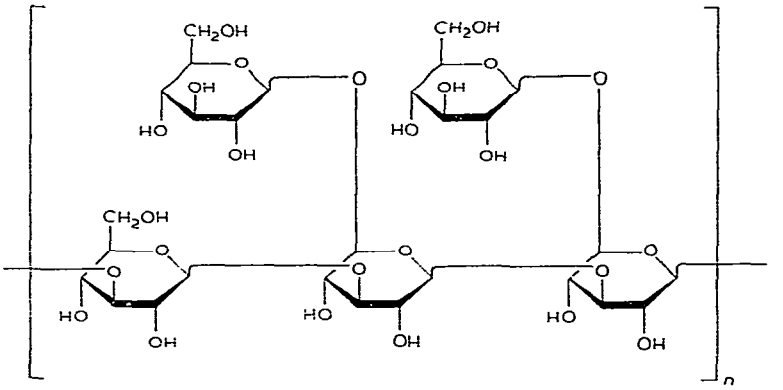
Fractionation of the polysaccharides of kikurage by successive extraction with water and cold and hot M sodium hydroxide afforded an acidic polysaccharide composed of D-glucuronic acid, D-xylose, D-glucose, and D-mannose (molar ratios, 1.3:1.0:1.3:4.3), and two types of β-D-glucan distinguished from each other by their physical properties. The yields and molecular sizes of these polysaccharides are listed in Table I. Both glucans, glucan I, which is water-soluble, and glucan II, which is

TABLE I

POLYSACCHARIDES OF THE FRUITING BODY OF KIKURAGE (*Auricularia auricula-judae*)

Polysaccharide	*Yield* (%)	$[\alpha]_D^{25}$ *(degrees)* (0.5M *NaOH*)	*Solubility*	*Molecular weight*
D-Glucurono-D-xylo-D-gluco-D-mannan	48.7	−20.3	water-soluble	[a]5×10^5
D-Glucan I	2.3	−10.1	water-soluble	[a]1.4×10^6
D-Glucan II	49.0	—	insoluble in 2M NaOH, and in Me_2SO	(d.p., $\sim 6 \times 10^3$)[b]

[a]Determined by high-performance, liquid chromatography (column, Toyosoda PW 5000; carrier, dimethyl sulfoxide). [b]Determined by the D-glucitol dehydrogenase method.



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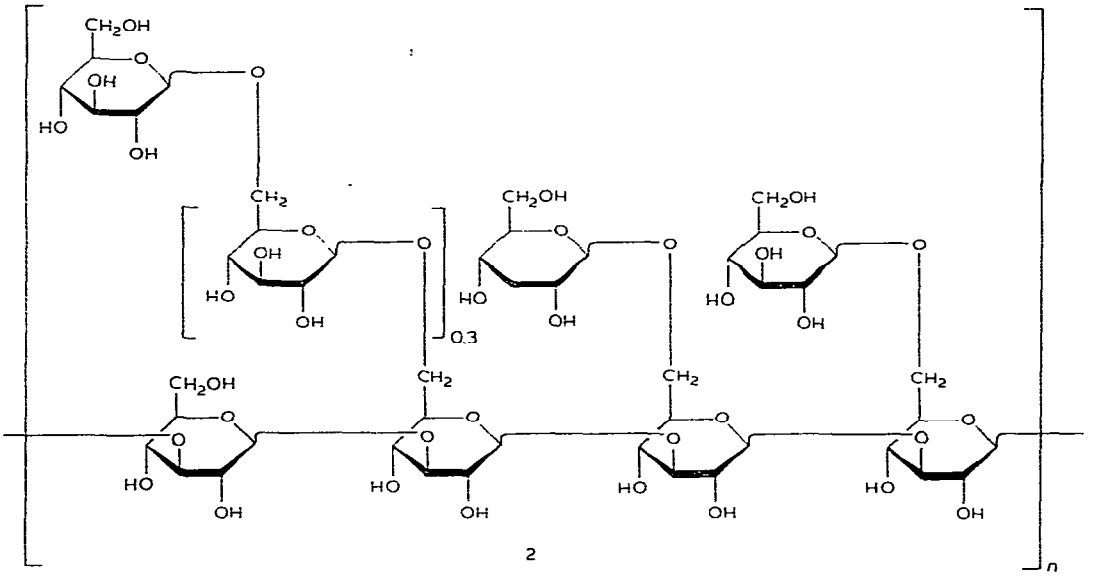
Water-soluble D-glucan



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Alkali-insoluble D-glucan

TABLE II

COMPARISON OF ANTITUMOR ACTIVITIES, ON SARCOMA 180 SOLID TUMOR, OF SOME POLYSACCHARIDES CONTAINING (1→3)-GLYCOSIDIC LINKAGES

Polysaccharide	*Glycosidic linkage*		*Dose*[a] *(mg/kg × days)*	*Average tumor weight (g)*	*Inhibition ratio (%)*	*Complete regression*[b]
	Back bone	*Side chain*				
Schizophyllum commune						
Schizophyllan[c]	β-(1→3)-Glc	β-(1→6)-Glc	1 × 10	0.25	95.1	6/10
Control				4.11		0/10
Auricularia auricula-judae (kikurage)						
β-D-Glucan I (soluble)	β-(1→3)-Glc	β-(1→6)-Glc	8 × 10	0.20	96.6	3/4
β-D-Glucan II (insoluble)	β-(1→3)-Glc	β-(1→6)-Glc	10 × 10	4.74	18.9	0/4
Acidic polysaccharide	α-(1→3)-Man	β-(1→2)-Xyl, β-GlcA-(1→	10 × 10	4.75	18.5	0/6
Periodate-modified	α-(1→3)-Man		10 × 10	3.65	37.4	0/6
Control				5.83		0/6
Streptococcus salivarius						
α-D-Glucan	α-(1→3)-Glc	α-(1→6)-Glc, α-(1→4)-Glc	30 × 10	3.84	18.8	0/4
Control				4.73		0/4
Periodate-modified	α-(1→3)-Glc		10 × 10	3.70	36.5	0/4
Control				5.98		0/4
Brasenia schreberi						
Acidic polysaccharide	β-(1→3)-Gal, β-(1→3)-Man	(1→3)-Rha, (1→3)-Fuc, (1→4)-GlcA	30 × 10	5.98	−26.4	0/6
Control				5.83		0/6
Periodate-modified	β-(1→3)-Gal, β-(1→3)-Man	(1→3)-Fuc	5 × 10	10.78	1.1	0/6
Control				10.90		0/6

[a]Administered intraperitoneally. [b]Number of tumor-free mice to number of mice tested. [c]Data of Tabata *et al.*[6].

soluble in neither alkali nor dimethyl sulfoxide, had been shown by methylation and Smith-degradation studies to contain a backbone of (1→3)-linked D-glucosyl residues[9]. They have many branches of single D-glucosyl groups attached at O-6 of the (1→3)-linked D-glucosyl residues of the backbone chain, many more than in the hitherto-known, branched (1→3)-β-D-glucans[1-5]; in glucan I, two out of three, and in glucan II, three out of four, (1→3)-linked D-glucosyl residues are branched. In addition, glucan II contains a very few, short side-chains of β-(1→6)-linked D-glucosyl residues. These branched structures were confirmed by the present re-investigation. The structures of the repeating unit of these two glucans are shown in **1** and **2**. Glucan I had a molecular weight of 1.4×10^6, as estimated by h.p.l.c., using dimethyl sulfoxide as the carrier. Glucan II, which was insoluble in dimethyl sulfoxide, had an average d.p. of 6×10^3, as measured by an enzymic method according to the procedure of Manners *et al.*[13].

As some β-D-glucans, such as scleroglucan, lentinan, and schizophyllan, which contain a backbone of (1→3)-β-linked D-glucosyl residues, have been found to inhibit the growth of Sarcoma 180 solid tumor implanted in mice[10], the antitumor activities of the kikurage glucans were compared with those of schizophyllan and other polysaccharides containing a backbone of (1→3)-linked glycosyl residues, *i.e.*, the mainly (1→3)-linked α-D-glucan of *Streptococcus salivarius*[14], a D-glucurono-D-xylo-D-gluco-D-mannan containing a (1→3)-α-D-mannosidic back bone, which was isolated from kikurage[9] (see Table I), and an acidic heteropolysaccharide of the mucilage of a water-plant, *Brasenia schreberi*, having an extremely highly branched structure consisting of (1→3)-linked β-D-galactosyl, β-D-mannosyl, L-fucosyl, and L-rhamnosyl residues[15]. The (1→3)-linked D-glycans that constitute the backbone chains of these polysaccharides were prepared from the corresponding, parent polysaccharides by mild, Smith degradation (periodate oxidation, borohydride reduction, and mild, acid hydrolysis). The tumor-inhibitory activities of these polysaccharides were compared by successive, intraperitoneal administration of each polysaccharide as an aqueous solution or suspension, at a dosage of 8–30 mg per kg of ICR-JCL mouse per day, for 10 days, starting 24 h after subcutaneous implantation of Sarcoma 180 cells. After 5 weeks, tumor-inhibition ratios, and the number of treated mice whose tumors had completely regressed, were examined. The results are listed in Table II.

It is apparent that the water-soluble, branched (1→3)-β-D-glucan of kikurage (glucan I) gave the highest antitumor activity, as in the case of schizophyllan; however, glucan II of kikurage, which is water-insoluble, showed no antitumor activity. Other polysaccharides containing (1→3)-α-linked D-glucosyl or D-mannosyl residues as their backbones also showed no antitumor activity, although their Smith-degraded polysaccharides, consisting solely of (1→3)-α-D-glycosidic linkages, showed some slight activity. The acidic heteropolysaccharide of the water-plant *B. schreberi*, and its Smith-degraded polysaccharide, which may still have a branched structure containing a backbone of (1→3)-β-D-galactosidic and -D-mannosidic linkages[16], also had no antitumor activity. As regards the antitumor effects of (1→3)-linked D-glucans,

TABLE III

ANTITUMOR ACTIVITIES, AGAINST SARCOMA 180 SOLID TUMOR, OF PERIODATE-MODIFIED, KIKURAGE GLUCAN II

Modified glucan	*Oxidation conditions (molar ratio of periodate to glucosyl residue)*	*Yield (%)*	*Dose (mg/kg × days)*	*Inhibition ratio (%)*	*Complete regression*[a]
Native glucan			10 × 10	18.9	0/6
CSD-25-S (soluble)	0.55	68.8	5 × 10	86.3	3/4
CSD-25-I (insoluble)	0.55	31.2	5 × 10	82.9	5/6
CSD-50-S (soluble)	1.10	83.4	5 × 10	100.0	6/6
CSD-50-I (insoluble)	1.10	12.6	5 × 10	40.7	0/6
CSD-50-SA (insoluble)[b]			5 × 10	62.1	4/6
CSD-75-S (soluble)	1.50	83.0	5 × 10	99.4	5/6
CSD-75-SA (insoluble)[b]			5 × 10	4.8	0/6
Control					0/6

[a]No. of tumor-free mice/no. of mice tested. [b]Treated with 0.2M sulfuric acid for 2 h at 100°.

it should be noted that the β configuration of the D-glucosyl residues, which affords a helical chain-conformation, is essential, as far as the growth inhibition of Sarcoma 180 tumor is concerned. X-Ray analysis and viscosity studies on the hitherto-known (1→3)-β-D-glucans, *e.g.*, lentinan, schizophyllan, and curdlan, indicated that most of them may have triple-strand helices, by hydrogen bonding between (1→3)-linked chains of D-glucosyl residues[17–19], whereas the molecules of (1→3)-linked α-D-glucans may have a ribbon-like, single-chain conformation, extended along the fiber axis[20,21].

Although it appears that the triple-helix chains of (1→3)-β-D-glucans are responsible for the antitumor activity, the fact that the glucan II of kikurage (which has many more branches than glucan I), and also schizophyllan-type glucans, showed essentially no antitumor effect prompted us to remove, partially, side chains in glucan II, whereby its structural features might become close to those of the antitumor active glucan I. For this purpose, glucan II was subjected to controlled, periodate oxidation, using a limited ratio of sodium periodate to glucan (0.5–2.0 mol of periodate per D-glucosyl residue) for 8 days at 10°, and the partially oxidized glucan resulting was reduced with borohydride. The resulting glucan-polyalcohol was then hydrolyzed with 0.1M acid for 24 h at 25°, according to the customary procedure of the Smith degradation. This reaction yielded a water-soluble, modified glucan fraction (CSD-S glucan) and a still water-insoluble glucan (CSD-I glucan). The proportions of water-soluble, and insoluble, modified glucan fractions obtained under different conditions of oxidation, and their antitumor activities against Sarcoma 180

are shown in Table III. It is evident that all of the modified glucan fractions exhibit antitumor effects significantly higher than that of the native glucan II; in particular, the soluble, modified glucans, such as CSD-50-S and CSD-75-S, showed nearly 100% tumor-inhibition ratios, and a high rate of complete regression with small dosages (5 mg/kg/day), although the water-insoluble fractions gave lower activities. However, when these highly active, periodate-modified glucan fractions (for instance, CSD-50-S) were further hydrolyzed by heating with 0.2M acid for 2 h at 100°, the high-molecular-weight product (CSD-50-SA) became water-insoluble, and the antitumor activity was markedly decreased, compared to that of the original, modified glucan.

In connection with such biological activities of these periodate-modified glucans, the distributions of branches were examined by methylation analysis. The results are shown in Table IV. It was surprising that all of the antitumor, modified glucans, particularly the water-soluble fractions, which contained many more (1→3)-interresidue linkages than did glucan II, were found to have much higher degrees of O-6 substitution of (1→3)-linked D-glucose residues than the corresponding, (nonreducing) terminal D-glucosyl group, as indicated by the ratios of 2,4-di- to 2,3,4,6-tetra-*O*-methyl-D-glucose. For instance, the molar ratios of the nonreducing terminal, (1→3)-linked interresidue and disubstituted D-glucosyl residues in CSD-50-S and CSD-75-S are 1:25.25:16.53, and 1:25.5:19.45, respectively. These results strongly indicate that, in addition to the increase in the linear portion of the (1→3)-linked β-D-glucan chain, some of the (1→3)-linked D-glucose residues have certain groups other than D-glucosyl residues attached at O-6.

This was confirmed by methylation analysis of the high-molecular, degraded

TABLE IV

MOLAR RATIOS OF ACID-HYDROLYSIS PRODUCTS OF PERIODATE-MODIFIED GLUCAN II, AND OF METHYLATED D-GLUCOSES FROM THE METHYLATED POLYSACCHARIDES

Modified glucan II	*Molar ratio of acid hydrolysis products*[a]		*Molar ratios of O-methyl-D-glucoses from methylated, modified glucan*		
	Glycerol	*Glucose*	*2,3,4,6-tetra-*	*2,4,6-tri-*	*2,4-di-*
CSD-25-S	1.0	1.35	1.0	4.42	3.30
CSD-25-I	1.0	2.12	1.0	2.90	3.40
CSD-50-S	1.0	0.75	1.0	25.25	16.53
CSD-50-I	1.0	1.30	1.0	4.39	18.75
CSD-50-SA			1.0	48.90	0.47
CSD-75-S	1.0	2.43	1.0	25.47	19.45
CSD-75-SA			1.0	113.70	1.06
Native glucan			1.0	0.3	0.9

[a]Modified glucans were first hydrolyzed with 18:7 sulfuric acid–water at 10°, and then with M sulfuric acid for 6 h at 100°. The hydrolyzates were analyzed by h.p.l.c.

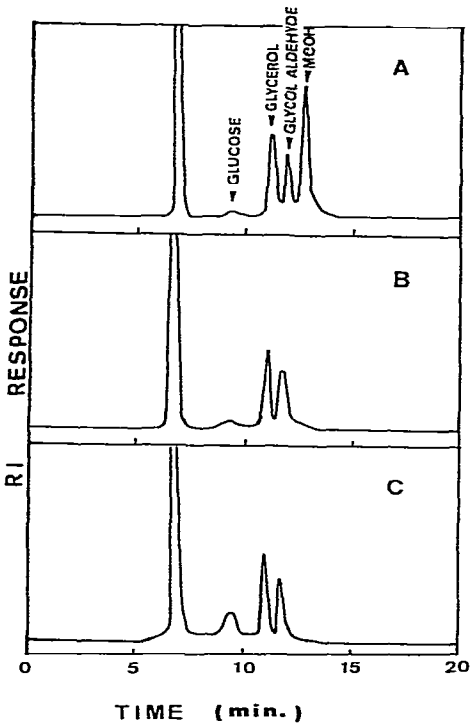

Fig. 1. Release of glycerol and glycolaldehyde during hydrolysis (0.2M sulfuric acid at 100°) of periodate-modified glucan II (CSD-50-S), monitored by h.p.l.c. [Conditions: a column (8 × 500 mm) of Hitachi, with water as the carrier (30 kg/cm²), MeOH; methanol used as the external standard. A, After 1 h; B, 2 h; and C, 4 h of hydrolysis.]

polysaccharide after hydrolysis with hot acid. When CSD-50-S and CSD-75-S glucan were treated with 0.2M sulfuric acid for 2 h at 100°, the resulting, water-insoluble polysaccharides had essentially linear, or slightly branched, structures, indicated by the ratios of the nonreducing terminal ends and branch points (see Table IV). Moreover, as shown in Fig. 1, glycerol and glycolaldehyde were gradually released during acid hydrolysis; after 48 h, glucose was released. The molar ratios of glycerol to D-glucose on complete hydrolysis with M sulfuric acid during 6 h at 100° are listed in Table IV. The results indicated that the polyhydroxy groups originating from β-D-glucosyl branches in glucan II are rather stable to acid hydrolysis, and are still attached to the backbone of (1→3)-β-linked D-glucosyl residues. Thus, the hydrolysis conditions employed in the ordinary procedure for mild Smith degradation, *i.e.*, acid hydrolysis[22] with 0.1M acid for 15–20 h at 25°, are apparently insufficient for cleavage of the acetal linkages of the polyhydroxy groups to the (1→3)-β-linked D-glucosyl residues in such water-insoluble, branched β-D-glucans as the glucan II of kikurage.

The aforementioned changes in the structural features of branched (1→3)-β-D-glucans of kikurage during Smith degradation were supported by their ^{13}C-n.m.r.-spectral data, shown in Fig. 2. The ^{13}C-n.m.r. spectra of the native glucan (dimethyl

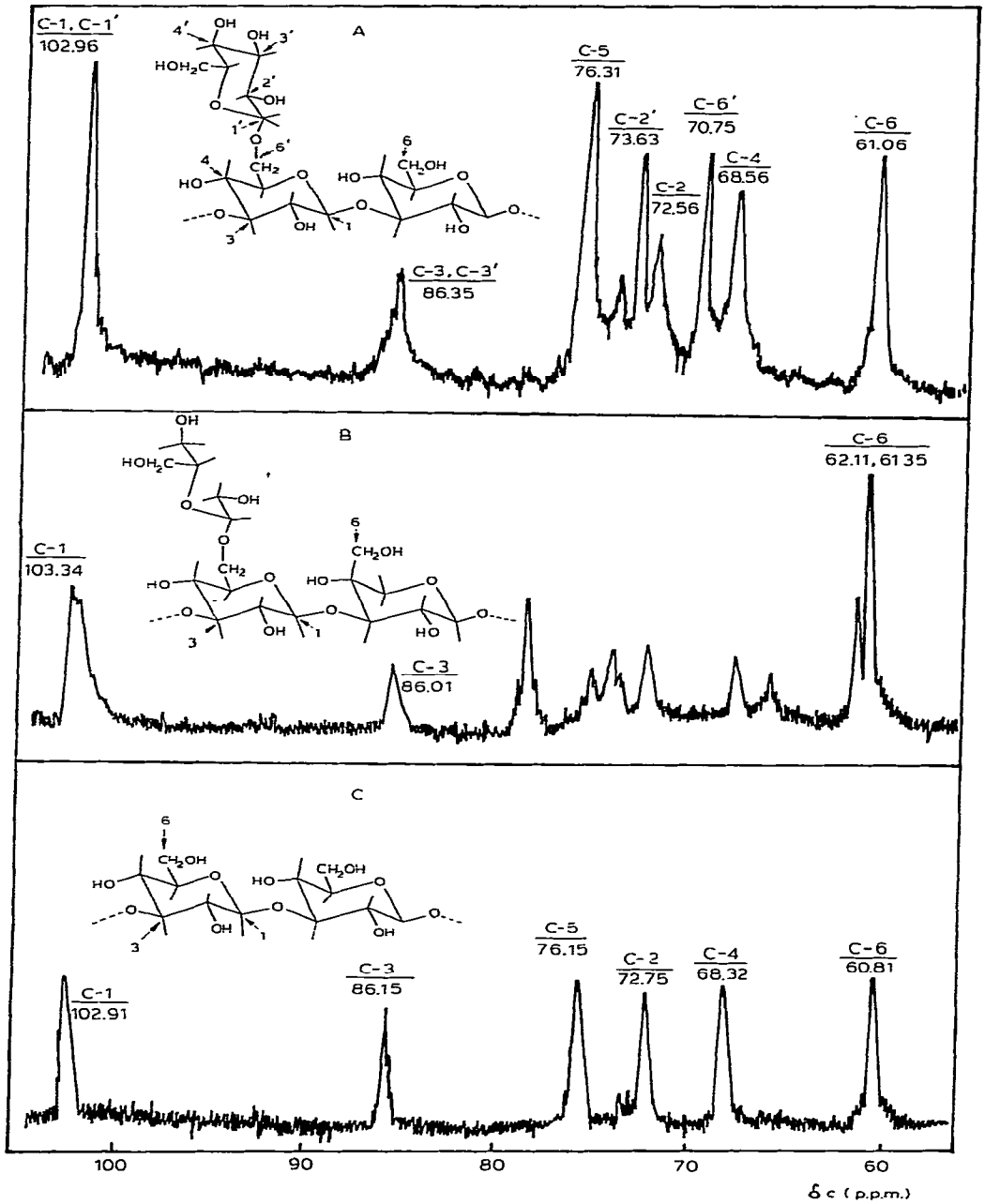

Fig. 2. Comparison of the ^{13}C-n.m.r. spectra of (A) glucan I of kikurage, (B) periodate-modified glucan II, CSD-50-S, and (C) debranched glucan II, CSD-50-SA, measured for a solution in Me_2SO-d_6 at 55°.

TABLE V

ANTITUMOR ACTIVITIES, ON SARCOMA 180 SOLID TUMOR, OF THE KIKURAGE GLUCAN-POLYALCOHOL AND OF THE CARBOXYMETHYLATED GLUCAN

Polysaccharide	*Dose*[a] *(mg/kg × days)*	*Average tumor weight (g)*	*Inhibition ratio (%)*	*Complete regression*[b]
Glucan II	10 × 10	8.93	18.9	0/6
Glucan II polyalcohol	1 × 10	2.31	79.0	2/8
(water soluble)	5 × 10	0.29	97.4	6/8
	10 × 10	0.34	96.9	6/8
Glucan II polyalcohol (water insoluble)	5 × 10	1.75	84.1	1/6
Glucan II polyalcohol (water soluble)	500 × 10[c]	9.53	17.1	0/8
Control		11.01		0/8
Debranched glucan II[d]	5 × 10	6.81	4.8	0/6
Control		7.15		0/8
CM-glucan II (DS, 0.47)	5 × 10	4.57	43.7	0/6
CM-glucan II (DS, 0.86)	5 × 10	5.00	38.4	0/6
Control		11.01		0/8

[a]Intraperitoneally administered. [b]No. of tumor-free mice/no. of mice tested. [c]Orally administered. [d]Prepared by hydrolysis of water-soluble glucan polyalcohol with 0.2M sulfuric acid for 2 h at 100°.

sulfoxide-soluble glucan I, instead of the insoluble glucan II), periodate-modified glucan II (fraction CSD-50-S), and completely debranched glucan (fraction CSD-50-SA) in dimethyl sulfoxide-d_6 were recorded at 55°. The native glucan gives nine distinct, ^{13}C-resonance peaks (see Fig. 2, A), characteristic of (1→6)-branched (1→3)-β-D-glucan, as reported for lentinan[23] and schizophyllan[6,23], whereas CSD-50-SA, which is presumed to be a linear (1→3)-β-D-glucan, gives six such peaks (see Fig. 2, C), such as those of curdlan, a linear (1→3)-β-D-glucan of *Alcaligenes faecalis*[24]. On the other hand, CSD-50-S, which contains numerous polyhydroxy groups attached to the O-6 atoms of the (1→3)-linked β-D-glucan backbone, shows a somewhat intermediate state compared to glucan I and CSD-50-SA (see Fig. 2, B), and there appears to be no peak corresponding to C-6′. It gives two characteristic peaks around the C-6 area (61.35 and 62.11 p.p.m.), one of which may be due to many primary alcohol groups present in the branches.

Because these polyhydroxy groups attached to the (1→3)-β-linked glucan backbone appeared to play an important role in the antitumor activity, either through alteration of the insolubility property or the presence of many (hydrophilic) hydroxyl groups, the glucan-polyalcohol [derived from glucan II by limited periodate oxidation (1.0 molecule of periodate per D-glucosyl residue) followed by reduction with borohydride] was tested against Sarcoma 180 solid tumor by intraperitoneal administration of 1–20 mg/kg for 10 days. As shown in Table V, both the water-soluble and -insoluble fractions of the glucan II polyalcohol exhibit potent antitumor activity,

TABLE VI

ANTITUMOR EFFECT OF KIKURAGE GLUCAN POLYALCOHOL ON EHRLICH CARCINOMA SOLID TUMOR

Polysaccharide	*Dose (mg/kg × days)*	*Average tumor weight (g)*	*Inhibition ratio (%)*	*Complete regression*[a]
Glucan II polyalcohol	1 × 10	6.07	59.6	0/6
(water soluble)	5 × 10	2.70	82.0	2/6
	20 × 10	0.34	97.7	5/7
Control		15.01		0/7

[a]No. of tumor-free mice/no. of mice tested.

although the former has a higher antitumor activity than the latter at the same dosage (5 mg/kg). The water-soluble glucan-polyalcohol had a very high antitumor activity at dosages of 5–10 mg/kg for 10 days, with a significant inhibition ratio (97%) and a high rate of complete regression (6/8). In contrast to intraperitoneal administration, however, its oral administration showed no effect on Sarcoma 180 solid tumor.

As regards the effect of chemical modification of the (1→3)-β-D-glucan, Sasaki *et al.*[25] recently found that the antitumor activity of water-insoluble curdlan, a linear (1→3)-β-D-glucan, is remarkably enhanced by partial introduction of carboxymethyl groups (d.s., 0.47), probably because of its providing water solubility, but carboxymethylated curdlan having a d.s. above 1.0 was less active. In order to ascertain whether alteration of the insolubility of glucan II would effect enhancement of its antitumor activity, this glucan was partially carboxymethylated by reaction with monochloroacetic acid and sodium hydroxide. The resulting *O*-(carboxymethyl)-D-glucans (d.s., 0.47 and 0.86), which were water-soluble, were tested for antitumor activity against Sarcoma 180 solid tumor. As indicated in Table V, these partially carboxymethylated derivatives of glucan II show higher antitumor activities than the parent, insoluble glucan II, but are much less active than the glucan-polyalcohol.

The glucan-polyalcohol, particularly the water-soluble fraction, was found to exhibit growth-inhibitory activity against other transplantable tumors, such as Ehrlich carcinoma, in mice at dosages of 1–20 mg/kg, as shown in Table VI. These modified glucans also significantly inhibit the growth of certain syngenic tumors, such as subcutaneously transplanted Meth/A in Balb/C mice (unpublished result).

The mechanism of the antitumor effects of (1→3)-β-D-glucans has not yet been elucidated, but the results of several previous studies suggested that augmentation of the defence of the host to the tumor growth is the most likely explanation, probably through stimulation of cell-mediated response. It is not yet known whether there is a definite effect of the polyhydroxy groups located as branches of the periodate-modified glucan, such as in glucan II polyalcohol. It is conceivable, however, that the presence of numerous (hydrophilic) polyhydroxy groups, probably arranged outside the triple-helix chains formed by the (1→3)-β-D-glucan backbone, would alter the water insolubility of the highly branched glucan II of kikurage. In addition, the present

discovery that the partial introduction of carboxymethyl groups into glucan II, which also altered the water insolubility, failed to produce much enhancement of the antitumor activity, suggests another biological role of the polyhydroxy groups in the tumor-inhibitory effect of the periodate-modified glucan. In this connection, we have recently observed that periodate modification of another branched (1→3)-β-D-glucan, produced extracellularly by *Pestalotia* sp., also provided high growth-inhibitory effects on various types of implanted tumors in mice (unpublished results).

EXPERIMENTAL

Materials. — The fruiting body of *Auricularia auricula-judae* (kikurage) used in this study was a commercial product, cultivated in the Ohita Prefecture, Japan. A sample of the water-insoluble α-D-glucan containing a (1→3)-α-D-glucan backbone was isolated from a culture of *Streptococcus salivarius* TTL-LPI in 5% sucrose medium. A linear (1→3)-α-D-glucan was obtained by prolonged periodate oxidation, followed by borohydride reduction, and hydrolysis with 0.1M sulfuric acid for 20 h at 25°, as previously described[14]. A sample of the acidic heteropolysaccharide of a water plant, *Brasenia schreberi* J. F. Gmel, isolated from the mucilaginous, water-shield material of the plant in a previous study[15], was from our laboratory stock. Its Smith-degraded polysaccharide was obtained by a one-cycle, mild Smith-degradation, resulting in elimination of the peripheral side-chains[16]. D-Glucitol dehydrogenase was purchased from Sigma Chemical Company.

General methods. — Evaporations were conducted under diminished pressure at temperatures not exceeding 37°, unless otherwise stated.

Paper chromatography was performed with Toyo-Roshi No. 50 paper, developed with (*a*) 6:4:3 1-butanol–pyridine–water or (*b*) 1-butanol–ethanol–water (4:1:5; upper layer). Sugars on paper chromatograms were detected with alkaline silver nitrate.

High-performance liquid chromatography (h.p.l.c.) of the product from acid hydrolysis of the branched (1→3)-β-D-glucan polyalcohol was performed with a Hitachi Liquid Chromatograph Model 635, in a column (8 × 500 mm) of Hitachi 2618, with water at 25° as the carrier, under a pressure of 30 kg/cm^2, and the sugars were monitored with a refractive-index indicator.

Gas–liquid chromatography (g.l.c.) of neutral or methylated sugars, after conversion into the corresponding alditol acetates, was usually performed with a Hitachi Gas Chromatograph Model 663 (fitted with a flame-ionization detector), in a glass column (0.4 × 200 cm) packed with 3% of ECNSS-M on Gas Chrom Q, at 190° (neutral sugars) or 175° (methylated sugars).

Methylation analysis. — Methylation of the periodate-modified glucans was performed by the method of Hakomori[26]. The polysaccharide (20 mg) was dissolved in dimethyl sulfoxide (2 mL) by ultrasonication in a nitrogen atmosphere. The solution was treated with methylsulfinyl carbanion (0.5 mL) for 3 h at room temperature, and then with methyl iodide (1.5 mL) for 1.5 h at 20°. The methylation procedure

was repeated twice, and the permethylated polysaccharide was hydrolyzed with 90% formic acid (0.5 mL) for 8 h at 100°, and then with 2M trifluoroacetic acid (0.5 mL) for 3 h. The methylated sugars were reduced with sodium borohydride, the resulting alditols were acetylated by heating with 1 : 1 acetic anhydride–pyridine (0.2 mL) for 2 h at 100°, and the products were analyzed by g.l.c.

The alkali-insoluble glucan II, which was insoluble in dimethyl sulfoxide, was, after dispersion in liquid ammonia at −60°, as previously described[9], first methylated with sodium and methyl iodide, and the partially methylated polysaccharide was then permethylated by the method of Hakomori.

Determination of molecular weight. — The average molecular weights of water-soluble polysaccharides, *e.g.*, glucan I and the acidic heteropolysaccharide of kikurage, were determined by h.p.l.c. in a modified column of Toyo Soda G-5000 PW (provided by Toyo Soda Co., Ltd.), with dimethyl sulfoxide as the carrier at 20 ±2°, at 120 kg/cm^2, by using purified dextran fractions having definite molecular weights (provided by Meito Sangyo Co., Ltd.) as standards. The average d.p. of glucan II, which was insoluble in dimethyl sulfoxide, was estimated by enzymic determination of the acid hydrolyzate of borohydride-reduced glucan, using dextrans of different molecular weights as standards, according to the method of Manners *et al.*[13].

^{13}C-N.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were recorded with a JEOL PET-11 EC-100 spectrometer operating at 25 MHz in the pulsed, Fourier-transform mode. All spectra were recorded for solutions in dimethyl sulfoxide-d_6 at concentrations of 5–7%, at 55 ±2°, by using 8 k data points and a spectral width of 4 kHz. ^{13}C-Chemical shifts are expressed in p.p.m. downfield from external tetramethylsilane.

Assay of antitumor activity. — Seven-day-old ascites Sarcoma 180 tumor (6×10^6 cells), maintained in the National Cancer Center Research Institute, Tokyo, was implanted subcutaneously into the right groin of ICR-JCR mice (each weighing ~23 g). In every case, this produced a solid tumor at the site of injection. The test samples, dissolved or suspended in distilled water, were injected intraperitoneally, daily for 10 days, starting 24 h after the tumor implantation. The mice were kept under observation for 5 weeks, and then killed for final evaluation of the effect of the treatment on the tumor growth.

Inhibition ratios were calculated by the following equation.

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

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

Complete regression is indicated as the ratio of the number of tumor-free mice to the number of mice tested.

In another antitumor experiment, Ehrlich carcinoma tumor (4.8×10^6 cells) was implanted subcutaneously in ICR-JCL mice, and the polysaccharide sample was administered intraperitoneally, successively for 10 days, starting 24 h after tumor

implantation. Inhibition ratios and the number of completely regressed mice were estimated as already described.

Preparation of periodate-modified glucans. — As, on complete oxidation, glucan II consumed 0.92 molecule of periodate per D-glucosyl residue, with concomitant liberation of 0.42 molecule of formic acid[9], the controlled periodate oxidation was conducted by using a limited concentration of sodium periodate. The samples (1.0 g) of glucan II were homogenized in a suitable volume of water, to which were then added different quantities of 0.5M sodium periodate (molar ratio of periodate to D-glucosyl residue, 0.5–1.5), the final volume of each solution being adjusted to 200 mL. The oxidation of each mixture was conducted for 8 days at 10°, with mechanical stirring. After completion of the oxidation, ethylene glycol (0.2–0.6 mL) was added, the suspension was centrifuged, and the insoluble residue (oxidized glucan) was reduced by the addition of sodium borohydride (500 mg in 10 mL water) in the usual way. After decomposition of the excess of borohydride by careful addition of acetic acid (final pH, 6.0), the viscous, still-turbid solution was dialyzed. The non-dialyzable fraction was hydrolyzed with 0.1M sulfuric acid for 24 h at 25°, with mechanical stirring. The hydrolyzate was centrifuged for 30 min at 12,000 r.p.m., to give a water-soluble fraction (CSD-S glucan II) and a water-insoluble fraction (CSD-I glucan II). The aqueous layer, containing CSD-S, was made neutral with M sodium hydroxide, dialyzed against water, and then lyophilized. The yield of periodate-modified glucan II (*i.e.*, CSD-S and CSD-I fractions) was dependent on the conditions used in the periodate oxidation (see Table III).

Glucan II polyalcohol, used for assay of the antitumor activity, was prepared by oxidation with sodium periodate, 1.0 molecule of periodate per D-glucosyl residue in the glucan, as already described. After reduction with sodium borohydride, and decomposition of the excess of borohydride, the water-soluble fraction of glucan II polyalcohol was dialyzed, precipitated with ethanol (2 vol.), dissolved in water, and the solution lyophilized (analysis: C, 40.58; H, 6.29; N, 0.00). Its aqueous solution showed an intrinsic viscosity at 25° of 16.3 dL/g. The proportion of glycerol released on complete hydrolysis with 18:7 sulfuric acid–water for 1.5 h at 10°, and then with M sulfuric acid for 6 h at 100°, corresponded to 26.3 mol% of the glucan polyalcohol. There was no appreciable toxicity on intraperitoneal administration to mice (LD_{50} 2.0 g/kg).

Preparation of partially carboxymethylated glucan. — Each sample (1.5 g) of powdered glucan II was dispersed in isopropyl alcohol (40 mL), and then treated with monochloroacetic acid (0.75, 1.08, or 1.8 g) and 30% sodium hydroxide (2.9, 3.6, or 6.0 mL), the mixture being stirred for 5 h at 60–65°; the insoluble product was successively washed with 7:3 methanol–acetic acid, 4:1 methanol–water, and methanol. The resulting, partially (carboxymethyl)ated glucan (CM-glucan) was dissolved in water (100 mL), and the pH of the solution was adjusted to 8.5. After removal of a small amount of insoluble material by centrifugation, the aqueous solution was concentrated to a small volume, and the CM-glucan was precipitated with acetone (3 vol.), washed with methanol, and dried *in vacuo* (yield, 1.2–1.3 g). The molar

content (d.s.) of carboxymethyl groups in the CM-glucan was found to be linearly related to the monochloroacetic acid added (under the experimental conditions described). Thus, when the glucan reacted with 0.75, 1.20, and 2.10 molecules of monochloroacetic acid per D-glucosyl residue, the d.s. was 0.47, 0.62, and 0.86, respectively.

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