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Studies on the Structure of a Minor Polysaccharide from the Bark of *Melia azadirachta*

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A water-soluble polysaccharide, designated as CSP-I, was isolated from the bark of *Melia azadirachta* (Meliaceae) and its inhibiting effect on the growth of subcutaneously inoculated Sarcoma-180 was tested in mice. CSP-I gave a single peak on high-performance liquid chromatography and gel filtration. Chemical and spectroscopic studies indicated that the structure is composed of a β-D-(1→3)-linked galactopyranosyl backbone possessing branching points at position *O*-6 to which α-L-arabinofuranose, β-D-galactopyranose and β-D-glucopyranose side chains are attached, on average to three of five galactosyl units.

Keywords——*Melia azadirachta*; arabinogalactan; heteropolysaccharide; ^{13}C-NMR; Sarcoma-180; antitumor activity

We have previously reported[1,2] an investigation of the structures of the water-soluble polysaccharides, designated as GIa,[1] GIb,[1] GIIa[2] and GIIIa,[2] isolated from the bark of *Melia azadirachta* (Meliaceae). The crude polysaccharide fraction, which was obtained by adding ethanol to a non-dialyzable fraction of the hot water extract, was chromatographed on a column of Sephadex G-100 to give three fractions (GI, GII, GIII). Each fraction was further purified by gel filtration. GIa is a α-1→4-D-glucan with one α-1→6-L-arabinofuranosyl group for every five glucose residues, and GIb is a branched arabinofucoglucan. GIa and GIb showed strong antitumor effects against Sarcoma-180. GIIa and GIIIa exhibited significant anti-inflammatory effects on carrageenan-induced edema.

On treatment with α-amylase and glucoamylase, GI fraction gave a large amount of glucose. However, in analytical gel-filtration of the hydrolyzate, a peak of an unhydrolyzed substance was still observed (GI-E, 25%). GI-E was chromatographed on Toyopearl HW-55F. The 0.06 M KH_2PO_4 eluate was dialyzed and lyophilized. The non-dialyzable polysaccharide fraction (GI-K, 13%) was dissolved in water and treated with a solution of 8% cetyltrimethylammonium bromide (Cetavlon). The precipitate was collected by centrifugation (CP). The supernatant was added to 1% boric acid,[3] and the precipitate was collected (CSP, 5%). To the supernatant, acetic acid and 3 volumes of ethanol were added. The precipitate was collected by centrifugation (CSSP, 6%). A flow diagram of the fractionation of GI is shown in Fig. 1.

In the present work, we examined the structure of the polysaccharide designated as CSP and its antitumor activity. The CSP fraction was further purified by Toyopearl HW-60F column chromatography to give an elution pattern consisting of three fractions (CSP-I, II, III) as shown in Fig. 2. Each fraction gave a single peak on high-performance liquid chromatography (HPLC) and sedimentation analysis. CSP-I showed $[\alpha]_D^{16}$ +28.8° (H_2O, $c=0.1$). The component sugars of CSP-I were identified as galactose, arabinose and glucose

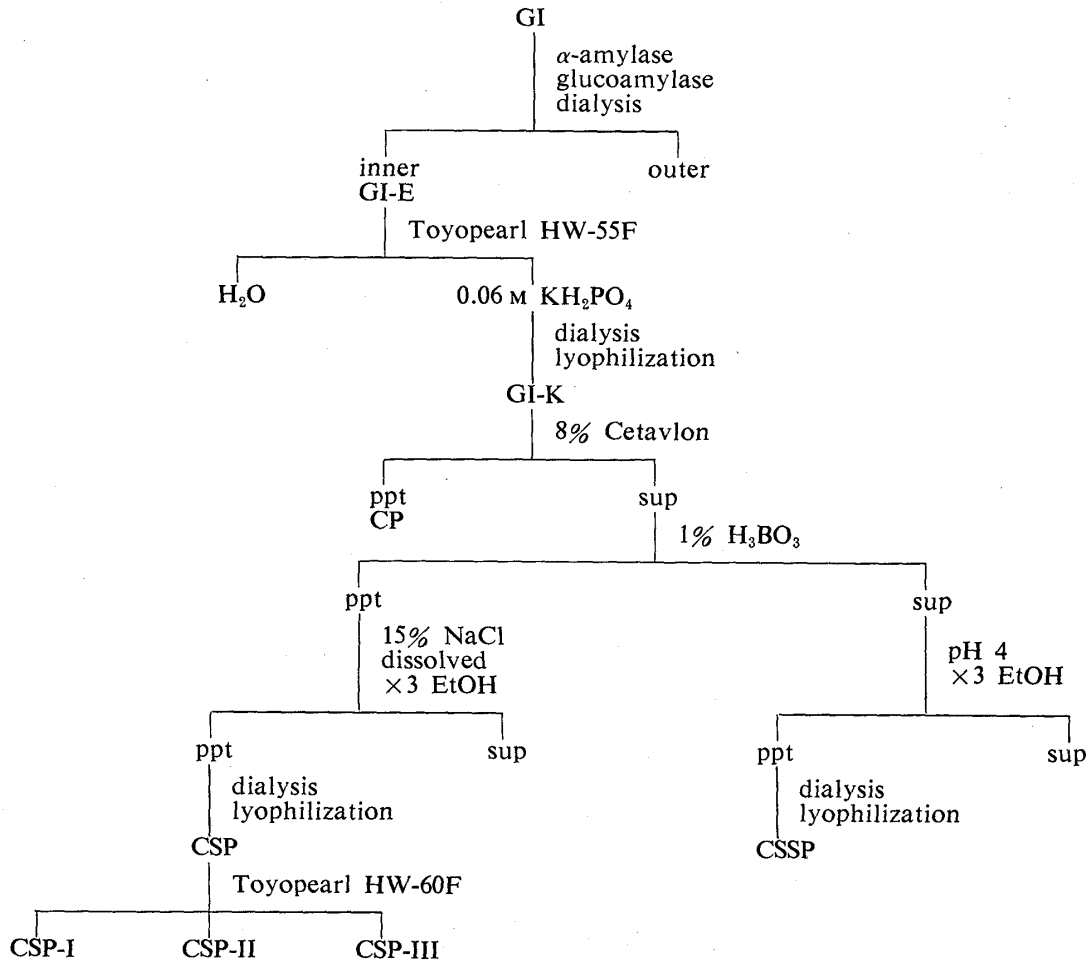

Fig. 1. Flow Diagram of the Fractionation of GI

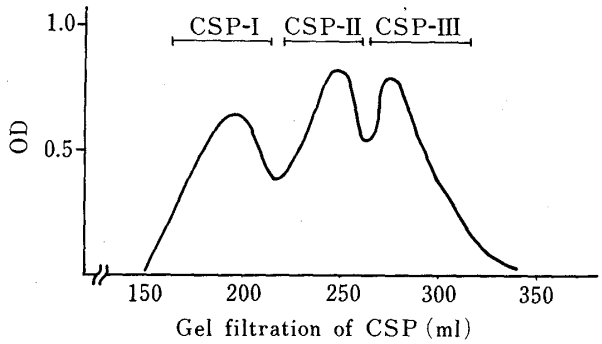

Fig. 2

by gas liquid chromatography (GLC) of the acid hydrolyzate, and quantitative analysis showed that CSP-I contained 23.0% L-arabinose, 69.2% D-galactose, and 7.6% D-glucose, and that their molar ratio was 9 : 3 : 1, while CSP-II and III were found to consist of galactose and arabinose in the ratio of 3 : 1 and 2 : 1, respectively. No nitrogen was found in these polysaccharides.

The carbon-13 nuclear magnetic resonance (^{13}C-NMR) data for CSP-I are summarized in Table I. By comparison of the CSP-I spectrum with those of arabinogalactan,[4] methyl β-D-galactopyranoside[5] and methyl α-L-arabinofuranoside,[5] a complete assignment of CSP-I resonances could be made. As the chemical shift of C-1 in methyl β-D-galactopyranoside is 104.9 ppm, the signal at 105.1 ppm might be regarded as due to C-1 linked β-1→3 or β-1→6.

TABLE I. ^{13}C-NMR Data for CSP-I (in D_2O)

Carbon assignment	Chemical shifts (δ: ppm) CSP-I	References
Galactose		Me β-D-galactopyranoside
(1→3) C-1	105.1	104.9
C-2	72.0	71.8
C-3	83.0	73.9
C-4	71.0	69.8
C-5	75.1	76.2
C-6	62.8	62.1
(1→6) C-1	105.1	
C-2	72.6	
C-3	74.5	
C-4	70.5	
C-5	75.5	
C-6	70.2	
(1→3, 1→6) C-1	105.1	
C-3	83.0	
C-6	70.7	
Arabinose		Me α-L-arabinofuranoside
C-1	109.2	108.7
C-2	82.8	82.5
C-3	78.5	77.8
C-4	85.8	85.2
C-5	63.1	62.3
(1→5) C-1	110.8	
C-5	68.7	
Glucose		Cellulose
(1→4) C-1	104.4	103.4
C-2	74.7	74.3
C-3	76.8	76.1
C-4	77.4	79.9
C-5	76.2	75.4
C-6	62.4	61.5

TABLE II. GLC and GC-MS of Partially Methylated Alditol Acetates

Methylated sugar (as alditol acetate)	Relative retention time[a)]	Main mass fragments (m/z)	Molar ratio
2,3,5-Me_3-Ara	0.52	43, 45, 71, 87, 101, 117, 129, 161	1.8
2,3,4,6-Me_4-Gal	1.19	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	1.0
2,3-Me_2-Ara	1.29	43, 87, 101, 117, 129, 189	1.0
2,4,6-Me_3-Gal	2.11	43, 45, 87, 101, 117, 129, 161	1.7
2,3,6-Me_3-Glc	2.25	43, 45, 87, 99, 101, 113, 117, 233	1.2
2,3,4-Me_3-Gal	3.10	43, 87, 99, 101, 117, 129, 161, 189, 205	2.6
2,4-Me_2-Gal	5.64	43, 87, 117, 129, 189	3.1

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Accordingly, the peaks at 83.0 and 70.7 ppm can be attributed to C-3 in a β-1→3 link and C-6 in a β-1→6 link, respectively, located in glycosidic bonds. The resonances at 109.2, 82.8, 78.5, 85.8 and 63.1 ppm are attributable to an arabinofuranose residue by comparison with the values for methyl α-L-arabinofuranoside.[4)] Accordingly, the peak at 68.7 ppm is attributed to

C-5 in an α-1→5 link. The remaining resonances in the intermediate field range can be assigned by comparison with the data for cellulose.[4] The resonances at 104.4 and 77.4 ppm are clearly due to C-1 and C-4 of a β-1→4 glucose residue, respectively. CSP-I was methylated[6,7] and then converted into alditol acetates.[8] Each partially methylated alditol acetate was identified from its retention time on gas-liquid chromatography and its fragmentation pattern in a mass spectrometer[9] (Table II). The predominant peaks of CSP-I were assigned to 2,3,4-tri-*O*-methyl-, 2,4,-di-*O*-methyl-, 2,4,6-tri-*O*-methyl- and 2,3,4,6-tetra-*O*-methylgalactitol derivatives in a 3 : 3 : 2 : 1 ratio. 2,3,5-Tri-*O*-methyl- and 2,3-di-*O*-methylarabinitol, and 2,3,6-tri-*O*-methylglucitol derivatives were also detected in a 2 : 1 : 1 ratio.

These results suggested that the minimal repeating unit of the polysaccharide moiety of CSP-I is composed of seven kinds of component sugar units, as shown in Chart 1.

$$[\text{Gal}p\ \beta1\rightarrow]_1\ [\rightarrow3\ \text{Gal}p\ \beta1\rightarrow]_2\ [\rightarrow6\ \text{Gal}p\ \beta1\rightarrow]_3$$
$$[\rightarrow3\ \text{Gal}p\ \beta1\rightarrow]_3\ [\text{Ara}f\ \alpha1\rightarrow]_2\ [\rightarrow5\ \text{Ara}f\ \alpha1\rightarrow]_1$$
$$6\uparrow\qquad[\rightarrow4\ \text{Glc}p\ \beta1\rightarrow]_1$$

Chart 1

It is not yet known whether the backbone of the arabino-3,6-galactose moiety of CSP-I is a (1→3)- and/or (1→6)-linked galactan.

When CSP-I was subjected to periodate oxidation, 0.92 mol of periodate per anhydro sugar unit was consumed. Periodate oxidation followed by reduction and acid hydrolysis gave 0.53 mol of glycerol and 0.08 mol of erythritol. The reaction solution was dialyzed against running water. The non-dialysate was lyophilized to give polysaccharide [CSP-I-S], which consisted of D-galactose exclusively. The methylation analysis of this mild Smith's degradation[10] product revealed the presence of terminal galactopyranosyl and 3-linked galactopyranosyl residues in the ratio of 1 : 37. This polysaccharide shows ^{13}C-NMR signals at 105.1, 72.1, 83.0, 71.0, 75.1, and 62.8 ppm, indicating that it is a β-1→3-galactan.[11]

CSP-I was suggested to contain a backbone of (1→3)-linked galactopyranose with (1→6)-linked galactopyranose, (1→5)-linked L-arabinofuranose and (1→4)-linked glucopyranose attached to position 6 of galactopyranose residues of the backbone, on average to three of five galactosyl units. The molecular weight of CSP-I, based on estimation of reducing end groups by the Park–Johnson method[12] and gel filtration, was determined to be about 53000. The arabinogalactans can be grouped into three main structural types, namely, the arabino-4-galactans (Type I of Aspinall[13]), the arabino-3,6-galactans (Type II), and polysaccharides with arabinogalactan side chains. CSP-I is thought to belong to the second type. Yamada *et al.*[14] reported the isolation of an arabinogalactan with anti-complementary activity from roots of *Angelica acutiloba* KITAGAWA. This polysaccharide consists of a β-D-(1→6)-linked galactopyranosyl backbone with branching points at position *O*-3. Recently, Tanaka *et al.*[11] reported the isolation of an arabinogalactan with a reticuloendothelial system-potentiating activity from the roots of *Panax notoginseng* and showed that it consisted of β-D-(1→3)-linked galactan possessing branching points at position *O*-6.

The antitumor activities of fractions CSP-I, II and III are listed in Tables III and IV. Hitherto, certain polysaccharides derived from other natural sources, such as higher plants, fungi, yeasts, and lichens, have been shown to inhibit the growth of transplanted tumors.[15] Tables III and IV show the curative effects of the polysaccharide preparations. According to the method described by Hamuro and Akiyama,[16] the test samples were intraperitoneally injected once daily for 10 d, starting 6 d after subcutaneous inoculation of Sarcoma-180 cells. At lower doses (2 mg/kg), the antitumor activity of CSP-I seems to be higher than that of CSP-II. The mechanisms of action of these polysaccharides are not clear yet, but the results show that CSP-I and/or CSP-II have no direct cytocidal effect on Sarcoma-180 cells *in vitro*.

TABLE III. Antitumor Activities of the Polysaccharides against Sarcoma-180

Sample	Dose (mg/kg)	Mean tumor wt. ±S.E.[a] (g)	Inhibition ratio (%)
Control		2.04±1.04	
GI	2	2.28±0.78	−11.9
	10	0.23±0.22[e]	88.6
	50	0.16±0.33[e]	91.9
GI-E	2	1.64±0.88	19.8
	10	0.98±1.38	51.9
	50	0.20±0.28[e]	90.0
GI-K	2	1.89±1.39	7.5
	10	0.47±0.32[e]	76.8
	50	0.55±0.48[e]	72.8
CSP	2	0.99±0.63[c]	51.5
	10	0.30±0.36[e]	85.4
	50	0.26±0.24[e]	87.5
CSSP	2	2.69±1.08	−32.1
	10	2.68±1.08	−31.4
	50	0.97±0.59[c]	52.5
Endoxan	10	1.06±0.60[b]	47.8

a) Each value is the mean±S.E. for 7 animals. Significant differences from the control: *b*) $p<0.05$, *c*) $p<0.02$, *d*) $p<0.01$, *e*) $p<0.001$.

TABLE IV. Antitumor Activities of the Polysaccharides against Sarcoma-180

Sample	Dose (mg/kg)	Mean tumor wt. ±S.E.[a] (g)	Inhibition ratio (%)
Control		1.79±0.17	
GI	2	1.44±0.26	19.7
	10	0.14±0.08[c]	92.3
CSP	0.4	1.49±0.47	16.7
	2	0.18±0.08[c]	90.2
	10	0.11±0.06[c]	93.8
CSP-I	0.4	1.85±0.38	−3.3
	2	0.38±0.11[c]	78.9
	10	0.13±0.06[c]	92.7
CSP-II	0.4	1.25±0.27	30.3
	2	0.59±0.11[c]	66.8
	10	0.19±0.13[c]	89.5
CSP-III	0.4	2.00±0.53	−11.8
	2	1.75±0.47	2.3
	10	0.67±0.45[b]	62.3
CSSP	0.4	2.29±0.36	−27.7
	2	2.39±0.63	−33.3
	10	2.22±0.41	−23.8
Endoxan	10	2.08±0.31	−16.3

a) Each value is the mean±S.E. for 7 animals. Significant differences from the control: *b*) $p<0.01$, *c*) $p<0.001$.

This is the first time that an arabinogalactan has been reported to have an antitumor activity.

Experimental

General Methods——Optical rotations were measured with a JASCO DIP-4 digital polarimeter. ^{13}C-NMR spectra were recorded with a JEOL FX-100 spectrometer operating at 25.0 MHz in the pulsed Fourier-transform

mode. Free induction decays were accumulated with a 45° pulse. All spectra were recorded in D_2O at 70 °C by using 8000 data points and a spectral width of 5 kHz. The ^{13}C-chemical shifts are expressed in ppm downfield from external tetramethylsilane. Gas liquid chromatographic analyses were carried out with a Shimadzu GC-6A gas chromatograph equipped with a hydrogen flame detector. Sedimentation analysis was performed at 58000 rpm with a Beckman Spinco model E ultracentrifuge equipped with a schlieren optical system. HPLC was carried out on a Shimadzu LC-5A instrument equipped with a refractive index (RI) detector. The eluent (H_2O) flow rate was 1 ml/min at 40 °C on a column of G 2000 SW.

Isolation and Purification——Finely powdered bark of *Melia azadirachta* (7.5 kg) was extracted with benzene and then with methanol in order to remove soluble components, and the residue was extracted further with distilled water on a boiling water bath. Ethanol was added to the hot filtered extracts to form precipitates (476 g). The crude polysaccharide was dialyzed. The non-dialyzable fraction (143 g) was chromatographed on a column of Sephadex G-100, and elution with water provided three fractions (GI 57 g, GII 28 g, GIII 57 g). A solution of GI (50 g) in 1200 ml of distilled water (pH 7.0, adjusted with NaOH) was incubated with α-amylase (255000 units, from *Bacillus* species, type II, Seikagaku Co., Ltd.) at 40 °C for 24 h. The reaction mixture was heated at 100 °C and then dialyzed with Spectrapor 3. The non-dialyzable fraction was further treated with glucoamylase (1600 units, from *Rhizopus niveus*, Seikagaku Co., Ltd.) at 40 °C for 24 h and then dialyzed. The non-dialyzable fraction, obtained as a brown powder (GI-E, 12.5 g) after lyophilization, was chromatographed on a column of Toyopearl HW-55F (3.2 × 45 cm) with 0.06 M KH_2PO_4. The 0.06 M KH_2PO_4 eluate was lyophilized to give GI-K as a white powder (6.5 g). GI-K (6.5 g) was dissolved in water (25 ml) and treated with 8% cetyltrimethylammonium bromide solution. After standing for 24 h at 20 °C, the precipitate was collected by centrifugation and redissolved in 15% NaCl. This solution was dialyzed against water for 3 d. The non-dialyzable fraction was obtained as the lyophilizate (CP). The supernatant was added to 25 ml of 1% boric acid. The solution was stirred and the pH was adjusted to 8.5 by the addition of 2 N NaOH. The precipitate was redissolved in 15% NaCl. Acetic acid was added to the solution, followed by 3 volumes of ethanol. The precipitate was dissolved in water and this solution was dialyzed against water for 3 d. The non-dialyzable fraction was obtained as the lyophilizate (CSP 2.5 g). Acetic acid was added to the supernatant, followed by 3 volumes of ethanol. The precipitate was washed with acetone, and dried with ether, yielding CSSP (3 g).

CSP was fractionated by Toyopearl HW-60F gel chromatography, affording CSP-I (0.78 g), CSP-II (0.83 g) and CSP-III (0.78 g).

Sugar Components of CSP-I, -II, and -III——CSP-I, -II, and -III were hydrolyzed with 1 N sulfuric acid for 8 h at 90 °C followed by neutralization with Dowex 2 (OH^-). The filtrate was reduced with sodium borohydride for 1 h. After neutralization with Dowex 50W-X8 (H^+), the filtrate was evaporated and boric acid was removed by repeated addition and evaporation of methanol. Then the products were acetylated with acetic anhydride–pyridine mixture (1 : 1) at 100 °C for 20 min. After evaporation of the solution, the residue was dissolved in chloroform–methanol mixture (1 : 1) and subjected to GLC. GLC was carried out on a column (0.3 cm × 2 m) packed with 3% ECNSS-M 190 °C with a gas flow of 35 ml per min of nitrogen. CSP-I contained galactose, arabinose and glucose in a ratio of 9 : 3 : 1. In the case of CSP-II and -III, galactose and arabinose were identified in ratios of 3 : 1 and 2 : 1, respectively. Relative retention times: arabinose, 0.29 min; galactose, 0.91 min; glucose, 1.00 min.

Absolute Configurations of Component Sugars——By the method of Oshima *et al.*,[17] the configurations of component sugars were determined by GLC of trimethylsilylated α-methylbenzylaminoalditol derivatives. By comparison with the peaks of authentic samples, L-arabinose, D-galactose and D-glucose were detected.

Permethylation of CSP-I——1,1,3,3-Tetramethylurea (2 ml) and methylsulfinylcarbanion solution (2 ml) were added to a solution of CSP-I (13.3 mg) in dimethyl sulfoxide (2 ml), and after 30 min, methyl iodide (0.5 ml) was added dropwise under vigorous stirring, with cooling to keep the reaction temperature below 50 °C. When the methylation was completed, the mixture was diluted with water, and dialyzed against running water to remove the excess of methyl iodide and tetramethylurea, as well as the sodium iodide formed. The dialysate was extracted with chloroform, and the extracts were washed with water, dried, and evaporated (14.7 mg). The product showed no hydroxyl absorption in the infrared spectrum.

Methylation Analysis——The methylated product (6.1 mg) was hydrolyzed with 90% formic acid (0.5 ml) at 80 °C for 24 h, followed by 0.5 N sulfuric acid (1 ml) at 100 °C for 3 h, then reduced with sodium borohydride and acetylated with pyridine–acetic anhydride in a usual manner. GLC and gas chromatography–mass spectrometry (GC-MS) of the partially methylated alditol acetates were performed with a column (3 mm × 2 m) packed with 3% ECNSS-M at 190 °C with a helium flow of 35 ml per min. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GLC and their main fragments in the mass spectra are listed in Table II.

Periodate Oxidation, Mild Smith Degradation and Analysis of Products——CSP-I (50 mg) was added to a solution of 0.02 M sodium periodate (20 ml). Oxidation was carried out in the dark at 7 °C. Aliquots (1 ml) were removed from the solution at intervals for estimation of their iodate content. When the oxidation was complete (after 48 h), the oxidized CSP-I was reduced with sodium borohydride (20 mg) then hydrolyzed with 0.1 N sulfuric acid (5 ml) at room temperature for 3 h. Glycerol, glycolaldehyde and erythritol were identified as their trimethylsilylated derivatives by GLC [3% ECNSS-M on Gaschrom Q; column temperature, 190 °C; N_2 flow rate, 40 ml min^{-1}]. The reaction solution

was dialyzed against running water. The non-dialyzable fraction was lyophilized to give a polysaccharide [CSP-I-S]. Methylation analysis of CSP-I-S by the same procedure as used for CSP-I revealed the formations of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl galactitol in the ratio of 37 : 1.

Assay of Antitumor Activity——ICR mice weighing about 25 g (5 week) were used for the antitumor assay. The ascites tumor cells (2×10^6) of Sarcoma-180 were transplanted subcutaneously into the right groin of mice. The test samples were dissolved in phosphate-buffered saline (pH 7.4) and the solutions were intraperitoneally injected daily for 10 d, starting 6 d after tumor implantation. As the end of the 3 rd week, the mice were sacrificed, and the tumors were extirpated and weighed. The inhibition ratios were calculated by the use of the following formula: inhibition ratio (%) $=[C-T/C] \times 100$, where C is the average tumor weight of the control group, and T is that of the treated group.

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