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Chemical modifications of the (1→3)-α-D-glucan from spores of *Ganoderma lucidum* and investigation of their physicochemical properties and immunological activity

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Abstract

A linear (1→3)-α-D-glucan was isolated from the spores of *Ganoderma lucidum* (Fr.) Karst. Six different functionalized derivatives of the (1→3)-α-D-glucan—aminopropylated, hydroxyethylated, sulfated, carboxymethylated, carboxymethylated and sulfated, and benzylamidated–carboxymethylated—with varying degrees of substitution were synthesized. The structural features and physicochemical properties of all derivatives were investigated by means of chemical and spectral analyses, and their effects on lymphocyte proliferation and antibody production were tested in vitro and in vivo. In general, the structural and physicochemical properties, and lymphocyte proliferation activity of all samples varied with the functionalized groups and the degree of substitution. The results of immunological assays indicated that some modified derivatives had potent stimulating effects on lymphocyte proliferation and antibody production and the introduction of carboxymethyl group with low degree of substitution (DS < 0.28) was the best choice on the improvement of the immunostimulating activity.

Keywords: *Ganoderma lucidum*; (1→3)-α-D-Glucan; Chemical modification; Immunological activity

1. Introduction

Ganoderma lucidum is a well-known Chinese medicinal fungus, which has been clinically used in East Asia and is given considerable attention as a home remedy. Its medicinal effects on cancer, hypertension, hepatitis, and hypercholesterolemia have been demonstrated by pharmacological studies in the last two or three decades.[1–4] Concerning the polysaccharides, several glycans have been isolated from the fruit bodies and the mycelium of *G. lucidum*, some of which have shown antitumor or hypoglycemic activities.[5,6] More recently, we reported the isolation, structural characterization and conformational behavior of three neutral polysaccharides from the spores of this mushroom.[7–9] Of the polysaccharides isolated, a highly branched glucan SP was found to have an immunostimulating activity.[9] As part of our continuing research to find pharmacological active polysaccharides from *G. lucidum*, a linear (1→3)-α-D-glucan has been obtained from the alkali-soluble fraction of the same source.

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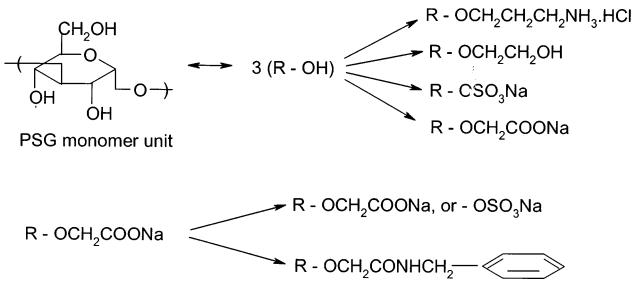
It is generally accepted that some (1→3)-β-D-glucans obtained from fungi or plants are immunomodulators and are beneficial for the treatment of cancers. Compared with the intensive investigation of (1→3)-β-D-glucan, the biological activity of (1→3)-α-D-glucan, which is often isolated from the cell wall of fungi, has been scarcely reported. Chemical modification of this glucan has also hardly been explored. To our knowledge, only a few derivatives of (1→3)-α-D-glucan have been prepared. Sulfated glucan was obtained by sulfation with the sulfur trioxide–pyridine complex, and its antitumor activity against Ehrlich ascites carcinoma has been investigated.[10] In addition, a carboxymethylated derivative with antitumor activity against Sarcoma 180 has been synthesized by substitution with chloroacetic acid.[11] New derivatives showed interesting properties and biological activities, and it might be useful to understand the structure–function relationship of polysaccharide.

In the present paper, we report on the synthesis of a series of ionic and nonionic derivatives of the (1→3)-α-D-glucan obtained from the spores of *G. lucidum* by means of aminopropylation, hydroxyethylation, sulfation, carboxymethylation, carboxymethylation and sulfation, and carboxymethyl–benzylamine coupling. The relationship between the nature of functionalized groups and physicochemical properties of the chemically modified derivatives and their immunomodulating activity is discussed.



Scheme 1. Preparation of the derivatives of the parent (1→3)-α-D-glucan PSG.

2. Results

Isolation and characterization.—The parent polysaccharide, named PSG, was obtained as water-insoluble pale-yellow powder (yield: 3.62%) from the sporoderm-broken spores of *G. lucidum* by alkaline solution extraction. It was eluted as a single symmetrical peak corresponding to an average molecular weight (M_w) of 1.26×10^5 as measured by the SEC method. This indicated that the polysaccharide was homogeneous, and no absorbance at 280 nm suggested that the polysaccharide did not contain any protein.

Sugar compositional analysis of PSG indicated that it was only composed of D-glucose. The high positive value of the specific rotation, $[\alpha]_D^{20} + 243.71°$ (*c* 0.684, 0.5 mol/L NaOH), and the characteristic absorption at ca. 840 cm^{-1} in the IR spectrum (Fig. 1A) were indicative of α-D-glucosidic linkages.[12] The ^{13}C NMR spectrum of PSG (Fig. 2A) showed 6 signals of approximately equal intensity, among which the signal at δ 102.6 was ascribed to the C-1 of glucosyl residues, and the signal at δ 84.7 was assigned to the O-substituted C-3 of glucosyl residues. Other peaks in the spectrum at δ 75.1, 73.4, 72.9 and 63.5 were respectively from the non-substituted C-2, C-5, C-4 and C-6 of glucosyl residues. All these data suggested that this polysaccharide was a linear (1→3)-α-D-glucan. Methylation analysis of PSG revealed two components corresponding to (1→3)-linked (98.5%) and terminal (1.4%) glucopyranosyl residues, which gave a support for the deduction from the ^{13}C NMR spectrum.

Chemical modification.—The goal of the present investigation is to synthesize and structurally analyze the various ionic or nonionic derivatives of the (1→3)-α-D-glucan described above. The synthetic plan is shown in Scheme 1. Especially, some of the basic physicochemical properties of these analogues are to be compared.

It was reported that substitution with amino-containing electrophiles would change the functionality, while maintaining most of the other properties of the polysaccharide intact.[13] We tried to obtain products with higher degree of substitution with 3-chloropropyl-

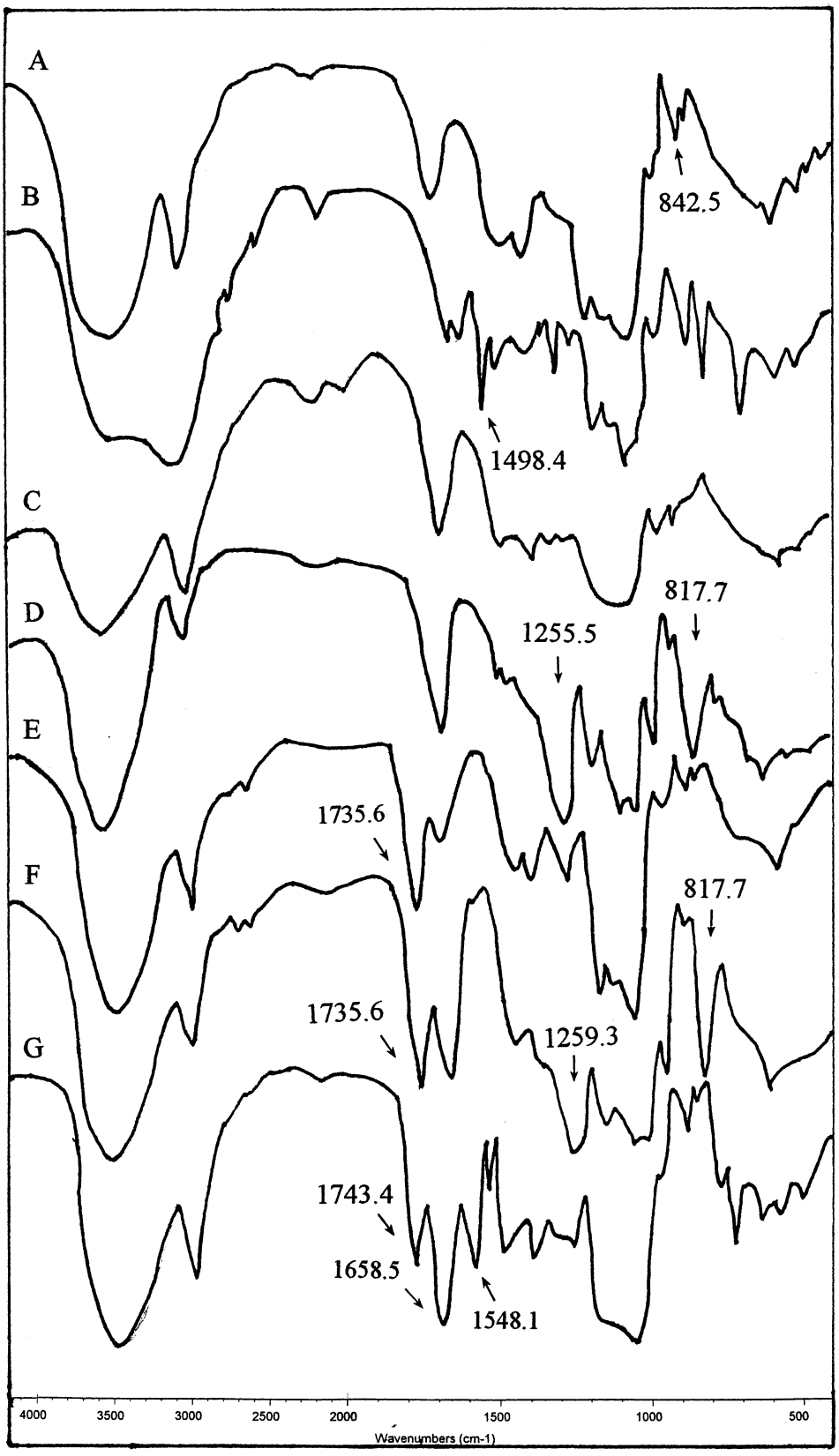


Fig. 1. Comparison of the IR spectra of (A) parent glucan PSG, (B) aminopropylated PSG (DS 0.35), (C) hydroxyethylated PSG (DS 1.2), (D) sulfated PSG (DS 1.1), (E) carboxymethylated PSG (DS 0.69), (F) carboxymethylated and sulfated PSG (DS 0.69 for CM and 0.49 for SA) and (G) carboxymethyl PSG benzylamide (DS 1.12 for CM and 0.68 for benzylamide).

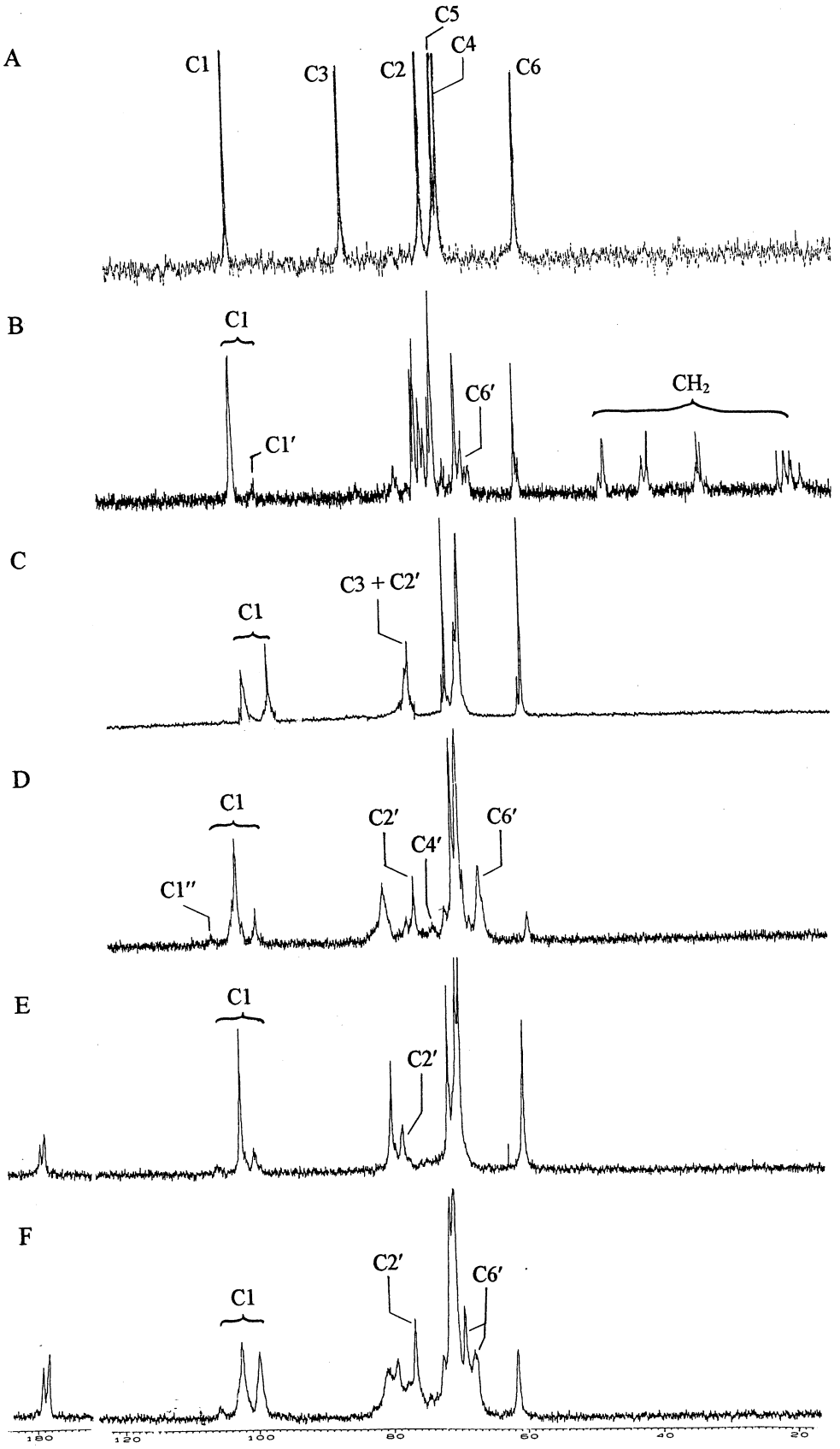


Fig. 2. Comparison of the ^{13}C NMR spectra of (A) parent glucan (D_2O and 2% NaOD), (B) aminopropylated PSG (DS 0.15, D_2O), (C) hydroxyethylated PSG (DS 1.2, D_2O), (D) sulfated PSG (DS 1.1, D_2O), (E) carboxymethylated PSG (DS 0.69, D_2O) and (F) carboxymethylated and sulfated PSG (DS 0.69 for CM and 0.49 for SA, D_2O).

amine as the etherifying agent under similar conditions, as previously described for the aminoethylation of dextran.[14] Since we used the HCl salt of the amino compounds, two equivalents of sodium hydroxide were required. In general, a small excess of NaOH was used. From Table 1, it is clear that highly substituted products can be obtained using a molar excess of amino compounds under relatively harsh conditions. However, these products were highly degraded as indicated by the yield, molecular weight and [α]. Clearly, under these conditions side reactions became important, which were not obvious under milder conditions. For example, in several cases a conglomeration of the reactants occurred, and the color of the mixture became deeper. Possibly, a polymerization of the amine occurred as suggested by de Nooy et al.,[15] which was not investigated further. To improve the yield of the product, several experiments were carried out at low reaction temperature or short reaction time with a lower ratio of amine/PSG. The degree of substitution obtained was lower, but yields were much better. On the other hand, the low-substituted products were water-insoluble or poorly soluble in water, whereas the high-substituted products were readily soluble in cold water.

As a result of substitution, the absorbance peak for the N–H stretching vibration appeared in the IR spectrum of aminopropylated PSG at ca. 1490 cm^{-1}, and there was also a gradual increased intensity of this peak, indicating an increased substitution of aminopropylation (Fig. 1B).[13] Moreover, as shown in Fig. 2B, the signals of the ^{13}C NMR spectrum of the derivative (DS 0.15) became more crowed and complicated in comparison with those of the intact glucan, among which the characteristic signal of anomeric carbon split into two peaks, and the upfield signal was ascribed to the anomeric carbon of the glucosyl unit aminopropylated at O-2. The signals at δ 20–55 confirmed the presence of the aminopropyl group. Due to the complexity and poor resolution, other signals could not be all assigned. In addition, Table 1 shows that the M_w and [α] of the derivatives decrease sharply with the introduction of the aminopropyl group. It should be noted that the M_w was determined by HPSEC[9] using the neutral T-dextran as the standard so that the apparent value was not the absolute molecular weight of the tested samples. Since the tested samples had the different electrical charge from the standards, the apparent values should be considered as relative.

Hydroxyethylated PSGs were prepared by the use of ethylene oxide in NaOH solution.[16] To prevent oxidative degradation, it was advisable to work under inert conditions. Three samples with different degree of substitution (DS from 0.5 to 1.2) were obtained by varying the ratio of ethylene oxide/PSG. The DS of them was estimated from the ^1H NMR signals belonging to the anomeric protons of the anhydroglucose units as described by Wulff et al.[17] The IR spectrum (Fig. 1C) of hydroxyethylated derivative showed a more broader and intensive peak of ca. 3450 cm^{-1} related to stretching vibration of the O–H in comparison with that of the PSG, which resulted from the substitution of hydroxyethyl groups. Regarding the ^{13}C NMR spectrum of hydroxyethylated PSG (DS 1.2) as shown in Fig. 2C, the signal of the anomeric carbon was also split into two peaks with similar intensities, indicating the C-2 of an anhydroglucose unit was mainly substituted. The high-intensity peaks at δ 76.6 and 61.6 were assigned to the CH_2 signals of the hydroxyethyl groups. In principle, all three hydroxyl groups at C-2, C-3 and C-6 of the anhydroglucose units could react, but the major substitution was observed at O-2. From Table 2, it can be seen that the apparent M_w increases with the increased degree of substitution and the [α] values show an opposite tendency. Additionally, the water-solubility of the hydroxyethylated derivatives is strongly improved compared with that of the aminopropylated derivatives of PSG.

Sulfated samples of PSG were prepared according to the procedures of Yoshida et al.[18] A series of sulfated samples with different degree of substitution were obtained by varying the molar ratio of chlorosulfonic acid/PSG, reaction temperature, and time of reaction (Table 3). As shown in Table 3, all the resulting sulfated products had good water solubility, and the apparent M_w decreased sharply with the increased degree of substitu-

Table 1
Influence of the reaction conditions on the aminopropylation of parent glucan PSG.

Products[a]	[Amine]/[PSG][b]	[NaOH]/[PSG][b]	Temp. (°C)	Time (h)	Yield (g)	N (%)	DS[c]	M_w ($\times 10^{-4}$)	$[\alpha]_D^{20}$ (°)	Water solubility[d]
PSA-AP-1	2.0	5.0	50	24	0.91	0.48	0.06	nd[e]	+215.6	insol.
PSA-AP-2	2.0	5.0	80	24	0.85	0.52	0.06	nd[e]	+208.0	insol.
PSA-AP-3	3.5	8.0	80	24	0.63	0.99	0.13	1.04	+164.6	warm
PSA-AP-4	3.5	8.0	80	36	0.52	1.16	0.15	1.02	+136.7	warm
PSA-AP-5	5.0	11.0	80	24	0.34	2.48	0.35	0.63	+120.9	cold
PSA-AP-6	5.0	11.0	95	36	0.26	4.48	0.74	0.55	+97.4	cold

[a] From the parent glucan PSG 1.0 g.
[b] Initial ratios of 3-chloropropylamine or NaOH to PSG monomer units.
[c] Degree of substitution with respect to the monomer units of PSG.
[d] Warm or cold means that the derivative can be dissolved in water at the concentration of 2% (w/v) by heating or at room temperature, respectively.
[e] Not determined.

Table 2
Composition and physicochemical properties of the hydroxyethyl, carboxymethyl and sulfate, and carboxymethyl–benzylamine coupled derivatives of the parent glucan PSG.

Derivatives	Composition analyses (DS)[a]				Yield[b] (%)	M_w ($\times 10^{-4}$)	$[\alpha]_D^{20}$ (°)	Water solubility
	HE	CM	SA	B				
PSG-HE-1	0.5	0	0	0	154	2.60	+169.7	cold
PSG-HE-2	0.7	0	0	0	128	3.16	+150.3	cold
PSG-HE-3	1.2	0	0	0	143	3.41	+122.5	cold
PSG-CMS-1	0	0.28	0.62	0	98.4	8.04	+162.3	cold
PSG-CMS-2	0	0.28	1.15	0	145	7.53	+147.5	cold
PSG-CMS-3	0	0.69	0.49	0	133	1.60	+139.9	cold
PSG-CMS-4	0	0.69	0.95	0	151	1.38	+123.8	cold
PSG-CMB-1	0	0.45	0	0.34	117	nd[c]	nd[c]	insol.
PSG-CMB-2	0	1.12	0	0.68	128	nd[c]	nd[c]	warm

[a] Degree of substitution with respect to the monomer units of PSG. HE, CM, SA and B mean hydroxyethyl group, carboxymethyl group, sulfuric acid group and benzylamide groups, respectively.
[b] The mass of the resulting derivatives compared with the starting materials.
[c] Not done.

Table 3
Influence of the reaction conditions on the sulfation of parent glucan PSG.

Products[a]	$[ClSO_3H]$/[PSG][b]	Temp. (°C)	Time (h)	Yield (g)	S (%)	DS	M_w ($\times 10^{-5}$)	$[\alpha]_D^{20}$ (°)	Water solubility
PSG-SA-1	3.0	25	2	0.61	5.8	0.4	5.58	+205.1	warm
PSG-SA-2	3.0	35	4	0.75	12.6	1.1	4.16	+152.0	cold
PSG-SA-3	3.0	55	8	0.92	14.6	1.4	2.94	+129.8	cold
PSG-SA-4	4.5	55	8	1.02	15.7	1.6	2.06	+118.3	cold
PSG-SA-5	6.0	75	4	0.97	17.1	1.9	1.72	+116.0	cold
PSG-SA-6	8.0	95	2	0.89	18.5	2.3	1.02	+86.9	cold

[a] From the parent glucan PSG 0.50 g.
[b] The initial ratio of $ClSO_3H$ to PSG monomer units.

tion. And the [α] values showed the same tendencies. In the IR spectrum of sulfated PSG (Fig. 1D), compared with that of nonsulfated glucan, two new absorption peaks at ca. 820 cm^{-1} and 1260 cm^{-1} appeared due to the presence of the bonds of C–S–O and S=O, respectively, indicating the sulfation reaction had actually occurred. The ^{13}C NMR spectrum of sulfated PSG (DS 1.1) is shown in Fig. 2D. Compared with the ^{13}C NMR spectrum of PSG, five signals appeared resulting from the sulfation of the hydroxyl groups, and the splitting of the anomeric carbon signals was also a result of sulfation. The peaks at δ 69.6, 76.2 and 78.3 were assigned to the signals of the O-6, O-4 and O-2 substituted carbons, respectively. The position at δ 99.5 was ascribed to the signal of C-1' with sulfate substitution at C-6 and C-2 in the glucose residue, and the relatively low-intensity anomeric signal at δ 105.1 was due to one sulfate substitution at C-6 in the glucosyl residues.[18] In addition, from the intensity of the signals of the O-substituted carbons, it was found that the substitution at O-6 was the major reaction compared with other two positions at C-2 or C-4 in a glucosyl residue.

Carboxymethylation of polysaccharides, most notably cellulose, with chloroacetic acid is a well-documented reaction.[19] In previous reports, this nucleophilic substitution was generally performed in aqueous solution at such a high pH (> 13) with heating that at least part of the hydroxyl groups of the polysaccharide were dissociated. However, the polysaccharide would invariably be decomposed, and β-elimi-

nation reactions and hydrolysis of the etherifying agent were unavoidable under such harsh reaction conditions. So we used the 2-propanol as the medium to perform the substitution.[16] In order to obtain ideal degree of substitution, several experiments were carried out under different conditions (Table 4). The water-solubility and apparent M_w of carboxymethylated glucan increased with the increased degree of substitution, while the $[\alpha]$ values of these derivatives showed an opposite tendency (Table 4).

The IR spectra (Fig. 1E) of carboxymethylated PSG showed a characteristic peak at ca. 1743 cm^{-1}, indicating that the carboxymethylation reaction occurred. As shown in Fig. 2E, the signal at δ 179.9–180.6 in the ^{13}C NMR spectrum of the derivative (DS 0.69) confirmed the presence of a carboxyl group. As described above, the anomeric carbon signal was also split, and the upfield peak was due to the O-2 substitution. By comparison with the ^{13}C NMR spectrum of the parent glucan, a characteristic signal at δ 81.2 was assigned to an O-2 substituted carbon. And the signals of the O-4 and O-6 substituted carbons were not very distinct, indicating that the substitution at O-4 or O-6 was a minor reaction for carboxymethylation. Thus, these data suggest that the substitution with carboxymethyl groups at position-2 was the dominant reaction, which was different from that of the sulfation reaction in which the position-6 was the major target as described above. These results are in a good accord with those of carboxymethylated cellulose and curdlan.[20,21]

After substitution with a carboxymethyl group with an appropriate degree of substitution, the carboxymethylated PSG (DS 0.28 or 0.69) was further sulfated with piperidine-*N*-sulfonic acid as the esterifying reagent, which is a milder sulfating agent than the complex of pyridine–chlorosulfonic acid, to obtain highly negatively charged materials. The resulting products were somewhat similar to heparin with two negatively charged ionic groups. As shown in Table 2, the degree of substitution with a carboxymethyl group did not decrease after sulfation, indicating that the presence of a carboxymethyl group was not affected by the sulfation reaction. With the substitution of sulfate after carboxymethylation, the water solubility of the resulting products was further improved and $[\alpha]$ decreased. Compared with the apparent M_w of the corresponding carboxymethylated derivatives, the value of M_w decreased after sulfation, and the degree of decrease increased with the degree of substitution with sulfate. This result was in accordance with that of the sulfated PSGs without carboxymethylation, suggesting the sulfation would lead to a decrease of the apparent M_w of the resulting materials. From the IR spectrum of carboxymethylated and sulfated PSG (Fig. 1F), we can see the characteristic absorptions at ca. 820 cm^{-1} and 1260 cm^{-1} for the sulfate group and at ca. 1743 cm^{-1} for carboxymethyl group. In comparison with Fig. 2D, in Fig. 2E the intensity of the signal at δ 99.3 that was formed by substitution with a carboxymethyl group or a sulfate group at O-2 increased considerably, and the signals at

Table 4
Influence of the reaction conditions on the carboxymethylation of parent glucan PSG.

Derivatives[a]	[CA]/[PSG][b]	[NaOH]/[PSG][b]	Temp. (°C)	Time (h)	Yield (g)	DS	M_w ($\times 10^{-5}$)	$[\alpha]_D^{20}$ (°)	Water solubility
PSA-CM-1	1.0	2.5	20	1.5	0.98	0.17	1.38	+226.4	warm
PSA-CM-2	1.0	2.5	20	3.0	1.01	0.25	1.49	+219.8	warm
PSA-CM-3	2.0	3.5	30	3.0	1.07	0.28	1.63	+211.1	warm
PSA-CM-4	2.0	3.5	50	3.0	1.25	0.45	3.64	+198.1	cold
PSA-CM-5	3.0	4.5	70	3.0	1.32	0.69	4.33	+185.0	cold
PSA-CM-6	3.0	4.5	50	3.0×2[c]	1.45	1.12	6.29	+128.2	cold
PSA-CM-7	3.0	4.5	70	3.0×2[c]	1.51	1.44	8.10	+121.0	cold

[a] From the parent glucan PSG 1.00 g.
[b] Initial ratios of chloroacetic acid or sodium hydroxide to PSG monomer units.
[c] Two successive experiments were performed.

Table 5
Effects of the derivatives of the parent glucan PSG on ConA- or LPS-induced lymphocyte proliferation in mouse splenocytes in vitro.

Samples	Concentration (μg/mL)	Lymphocyte		Samples	Concentration (μg/mL)	Lymphocyte	
		T cell (A_{570})	B cell (A_{570})			T cell (A_{570})	B cell (A_{570})
Control		0.55 ± 0.03	0.41 ± 0.05	Control		0.55 ± 0.03	0.41 ± 0.05
PSG-AP-4	1	0.57 ± 0.04	0.43 ± 0.04	PSG-CM-1	1	0.65 ± 0.02**	0.54 ± 0.02**
	100	0.58 ± 0.06	0.45 ± 0.05		100	0.75 ± 0.03***	0.65 ± 0.03***
PSG-AP-5	1	0.52 ± 0.03	0.42 ± 0.07	PSG-CM-2	1	0.62 ± 0.03*	0.49 ± 0.02*
	100	0.56 ± 0.05	0.48 ± 0.05		100	0.66 ± 0.02**	0.54 ± 0.01**
PSG-AP-6	1	0.49 ± 0.02	0.39 ± 0.04	PSG-CM-3	1	0.57 ± 0.04	0.44 ± 0.05
	100	0.55 ± 0.04	0.40 ± 0.05		100	0.61 ± 0.03*	0.50 ± 0.02*
PSG-HE-1	1	0.55 ± 0.05	0.51 ± 0.03*	PSG-CM-4	1	0.54 ± 0.05 0.40 ± 0.07	
	100	0.54 ± 0.05	0.52 ± 0.02*		100	0.58 ± 0.03	0.45 ± 0.05
PSG-HE-2	1	0.58 ± 0.03	0.49 ± 0.07	PSG-CM-5	1	0.52 ± 0.03	0.41 ± 0.02
	100	0.61 ± 0.02*	0.53 ± 0.03*		100	0.46 ± 0.01*	0.33 ± 0.03*
PSG-HE-3	1	0.58 ± 0.04	0.50 ± 0.06	PSG-CM-7	1	0.50 ± 0.05	0.34 ± 0.02*
	100	0.67 ± 0.02**	0.52 ± 0.02*		100	0.43 ± 0.02**	0.30 ± 0.02**
PSG-SA-1	1	0.51 ± 0.07	0.43 ± 0.05	PSG-CMS-1	1	0.56 ± 0.04	0.42 ± 0.06
	100	0.56 ± 0.04	0.41 ± 0.03		100	0.58 ± 0.02	0.40 ± 0.05
PSG-SA-2	1	0.59 ± 0.08	0.38 ± 0.03	PSG-CMS-2	1	0.53 ± 0.04	0.38 ± 0.03
	100	0.52 ± 0.03	0.40 ± 0.06		100	0.59 ± 0.07	0.41 ± 0.05
PSG-SA-4	1	0.52 ± 0.01	0.41 ± 0.03	PSG-CMS-3	1	0.52 ± 0.05	0.37 ± 0.04
	100	0.50 ± 0.04	0.45 ± 0.04		100	0.48 ± 0.03	0.39 ± 0.07
PSG-SA-6	1	0.47 ± 0.05	0.40 ± 0.07	PSG-CMS-4	1	0.48 ± 0.06	0.34 ± 0.03*
	100	0.54 ± 0.03	0.39 ± 0.08		100	0.57 ± 0.08	0.33 ± 0.02*
PSG-CMB-1	1	0.55 ± 0.02	0.44 ± 0.05	PSG-CMB-2	1	0.58 ± 0.07	0.38 ± 0.02
	100	0.58 ± 0.03	0.42 ± 0.03		100	0.55 ± 0.03	0.35 ± 0.08

Results are shown as mean ± S.D. based on three independent experiments.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$, significantly different from the control.

δ 78.4 and 69.3–69.7 that arose from the O-2 or O-6 substitution, respectively, also became more intense. These observations indicated that a carboxymethyl group and a sulfate group could be introduced into the (1→3)-α-D-glucan by steps, and they could coexist in a sugar ring.

It was reported that the introduction of benzylamine into the carboxymethylated dextran not only was helpful in enabling the derivatives to penetrate the cell, but also brought about other biological activities, such as inhibitory activity against mammary tumor cell growth.[22] So, the benzylamine was incorporated with the carboxymethylated PSG with the help of EEDQ as the coupling reagent.[23] In order to obtain products with an appropriate coupling degree of benzylamine, we chose the DS of 0.45 and 1.12 of carboxymethylated PSG as the starting materials, and three successive coupling reactions were performed. The structural analyses of the final products are summarized in Table 2, and their apparent M_w and [α] have been not measured because of their poor water solubility. From the IR spectrum (Fig. 1G) of the coupled product, the absorption peak at ca. 1743 cm^{-1} is related to the free carboxyl group without coupling with benzylamine, and the peaks at ca. 1658 cm^{-1} and 1548 cm^{-1} resulted from the stretching vibration of the carbonyl bond of the amide group and the bending vibration of the N–H bond, respectively. These results indicated the carboxymethyl groups were partially coupled with benzylamine.

Immunomodulating assays.—The effects of the synthesized derivatives of PSG as described above on mitogenicity of ConA or LPS induced lymphocytes were tested in vitro (Table 5). The carboxymethylated derivatives

with a low degree of substitution showed an excellent activity of enhancing the T and B lymphocyte proliferation, and this effect decreased with the increased degree of substitution at the experimental concentrations of 1 or 100 μg/mL. Moreover, when the degree of substitution was more than 0.6, the carboxymethylated PSG began to exhibit a lymphocyte suppressive activity. In addition, the hydroxyethylated PSGs with DS values from 0.5 to 1.2 also showed such an enhancing effect, but their effect was less pronounced than that of the carboxymetylated derivatives of PSG with low degrees of substitution. Other samples, including aminopropylated, sulfated, carboxymethylated and sulfated, and benzylamidated carboxymethyl derivatives, did not show a marked effect on lymphocyte proliferation at the range of tested concentrations. There was no observed toxicity of any of the samples as measured by cell viability at the experimental concentration range.

The immunomodulating activity of the carboxymethylated PSG with a low degree of substitution was further investigated in mice. The results are shown in Table 6. Clearly, the carboxymethylated (1→3)-α-D-glucan with a low degree of substitution (<0.28) significantly enhanced the T and B lymphocyte proliferation and antibody production and increased the mass amount of spleen tissue in the tested mice, but did not significantly influence the serum IgG and C-3 levels at a dose of 25 or 50 mg/kg. The lower DS compounds seemed more active than the higher ones, which was in accordance with the in vitro study.

3. Discussion

(1→3)-β-D-Glucans have been shown to exert immunomodulating and antitumor activities, and various chemical modification studies have been carried out to elucidate structure–function relationships. Although, up-to-now, there is no accepted mechanism and not even any agreement on the parameters which influence the activities, it is obvious that the introduction of ionic or nonionic groups can significantly affect the physicochemical properties and immunomodulating activities of the parent glucan. Therefore, we prepared a series of ionic and nonionic derivatives of the (1→3)-α-D-glucan to examine whether the nature of functionalized groups and degree of substitution would cause the changes of physicochemical properties and affect the immunomodulating activity of the parent PSG. In order to assess the basic physicochemical properties of the derivatives, M_w, [α] and water solubility were measured. Lymphocyte proliferation response was mainly used to evaluate the immunological activity.

Table 6
Effects of intraperitoneal injections of carboxymethylated derivatives of the parent glucan PSG with low degree of substitution on the spleen mass, lymphocyte production, antibody production, serum IgG and C-3 levels in mice.

Samples	Dose (mg/kg)	Mass of spleen (mg/g)	Antibody (A_{520})	IgG (mg/mL)	C-3 (mg/mL)	Lymphocyte	
						T cell (A_{570})	B cell (A_{570})
Control		6.79 ± 0.90	0.83 ± 0.02	12.9 ± 3.7	1.4 ± 0.7	0.68 ± 0.01	0.82 ± 0.01
PSG-CM-1	25	8.89 ± 2.36*	1.38 ± 0.02**	12.1 ± 1.9	1.9 ± 0.4	0.99 ± 0.01***	0.99 ± 0.02***
	50	9.67 ± 1.53**	1.44 ± 0.02**	14.5 ± 2.3	1.6 ± 0.3	0.87 ± 0.01**	1.01 ± 0.01***
PSG-CM-2	25	8.27 ± 0.61	1.24 ± 0.01*	12.6 ± 2.2	1.8 ± 0.6	0.97 ± 0.03**	0.83 ± 0.01
	50	8.69 ± 1.50*	1.23 ± 0.02*	13.6 ± 3.4	1.3 ± 0.4	0.97 ± 0.01**	0.88 ± 0.03*
PSG-CM-3	25	6.64 ± 0.92	1.07 ± 0.05	15.6 ± 1.1	1.2 ± 0.3	0.71 ± 0.02	0.80 ± 0.04
	50	7.41 ± 1.72	1.22 ± 0.03*	13.0 ± 1.3	1.6 ± 0.2	0.84 ± 0.01*	0.82 ± 0.01

Each value represents the mean ± S.D. from seven mice in each group.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$, significantly different from the control.

From the immunological data, it was obvious that the carboxymethylated derivatives with relatively lower degree of substitution (< 0.28) had a strong effect on enhancing lymphocyte proliferation, whereas the effect decreased and even an opposite effect appeared with the increased degree of substitution. Disappearance of the mitogenic activity of the carboxymethylated glucan was not due to extensive degradation of the polymer chain during the derivatization because the apparent M_w of the products increased in comparison with the parent glucan. Apart from the carboxymethylated derivatives, other positive or negative charged derivatives of PSG did not significantly influence the lymphocyte proliferation response, which suggested that the nature of ionic groups in the derivatives was important for the mitogenic activity of the lymphocytes. The introduction of suitable kinds of ionic groups with appropriate degrees of substitution would not only enhance the water solubility of the intact (1→3)-α-D-glucan, but would also change the intramolecular and intermolecular hydrogen bonding and strengthen the effect of electrostatic repulsion, which enabled the polymer chain to adopt a certain structure in aqueous solution.[10,16] The effect of chain conformation of ionic glucan in aqueous solution on immunomodulating activity could not be neglected.[24] On the other hand, the water-soluble neutral derivatives from hydroxyethylation also showed a mitogenic activity of lymphocytes to some extent, indicating that the presence of ionic groups was not essential, but that suitable water solubility might be an important factor for this activity, too.

In conclusion, present results regarding the different effects of PSG derivatives on the immunomodulating activity suggest that suitable water solubility and low degree of substitution with a carboxymethyl group are the best choices for the improvement of the lymphocyte proliferation response of the intact (1→3)-α-D-glucan. For this activity, the β configuration of D-glucan may be not necessary. To gain deeper insight into this important topic, it would be useful to investigate the derivatives of the glucan with more defined structures and test these derivatives to determine if they stimulate other immunological parameters.

4. Experimental

Materials.—The spores of *G. lucidum* were collected in Shanxi province, P.R. China. They were identified by Professor Xiulan Huang and stored as a voucher specimen (No.: 99064) in the Herbarium of the Phytochemistry Department of Shanghai Institute of Materia Medica. Sephacryl S-300 HR and dialysis bags were purchased from Pharmacia Biotech. T-dextrans in a series of different molecular weights were from Pharmacia. 2-Ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) and $NaBH_4$ were obtained from Aldrich Chemical Co. TFA and Me_2SO were from E. Merck, and Me_2SO was dried over 4 Å molecular sieves before use. ConA and LPS were from Sigma Chemical Co., and MTT was from Fluka Chemical Co.. Medium RPMI-1640 was purchased from Gibco Laboratories, supplied with HEPES buffer 10 μmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, L-glutamine 2 mmol/L, 2-mercaptoethanol 50 μmol/L and 10% new-born bovine serum, pH 7.2. All other chemicals were commercially available reagents of the highest grade produced by Chinese chemical companies.

General.—The sugar content was determined by the phenol–sulfuric acid method as described before.[25] IR spectra (KBr or Nujol pellets) were obtained with a Perkin–Elmer 599B FTIR spectrophotometer. Nitrogen content was measured with a Carlo–Erba 1108 elemental analyzer. Specific rotations were measured with an automatic polarimeter WZZ-1S in water or sodium hydroxide solution (0.5 mol/L) at 20 ± 1 °C. GLC was carried out with a Shimadzu GC-14B apparatus equipped with a 5% OV225/AW–DMCS–Chromosorb W (80–100 mesh) column (2.5 × 3 mm). HPSEC was performed with a Waters instrument fitted with GPC software, using a Waters 2410 RID as detector. GLC–MS spectrum was obtained with a Finnigan MD 800 instrument equipped with a HP-1 column. The ^1H and ^{13}C NMR spectra were obtained at

400 and 100 MHz, respectively, with a Bruker AM 400 spectrometer with a dual probe in the FT mode at room temperature. The DEPT experiments were carried out using a polarization transfer pulse of 135°. For NMR measurements, the parent polysaccharide was dissolved in $D_2O + NaOD$ (2%), and other samples were dissolved in D_2O.

Isolation and purification.—The sporoderm-broken spores of *G. lucidum* (1.0 kg) were alcohol-defatted and then extracted with 20 L of boiling water, and the resulting residue was solubilized in 10 L of 0.5 mol/L sodium hydroxide at 4 °C for about 12 h. The aqueous fraction obtained from centrifugation was neutralized with 2 mol/L HCl to pH ≈ 7, and the resulting mixture was extensively dialyzed against running water for 3 days, and then against distilled water for 1 day. After centrifugation, a water-insoluble material was obtained from the nondialyzable fraction. The crude product was dissolved in 0.5 mol/L NaOH and precipitated by the slow addition of 1 mol/L HCl. The residue was recovered by centrifugation. This treatment was repeated five times to remove the water-soluble substances, giving a water-insoluble polysaccharide (yield: 3.62% from the dried crude material), the chemical homogeneity of which was tested by the SEC method.

Homogeneity and molecular weight.—Measurements were carried out on a Sephacryl S-300 HR (1.6 × 100 cm) column, eluting with 0.30 mol/L NaOH at a flow rate of 0.4 mL/min. The eluates were monitored by a refractive index detector and by UV at 280 nm. The column was pre-calibrated using a T-dextran series of different molecular weights. All samples were prepared as 1% (w/v) solution, and 1 mL of solution was subjected in each run.

Composition and methylation analyses.—The sugar composition was analyzed by GLC after conversion of the hydrolysate into alditol acetates as described previously.[26] The parent polysaccharide was methylated by the method of Needs and Selvandran,[27] and the resulting permethylated product was hydrolyzed, reduced, acetylated and analyzed by GLC–MS as previously reported.[9]

Aminopropylation.—3 - Chloropropylamine was synthesized according to the method of Speziale and Hamm using 3-amino-1-propanol as the starting material (yield: 60.8%).[28] The parent polysaccharide (1.0 g) was dissolved in a solution of NaOH in water (40 mL). The amount of NaOH, 3-chloropropylamine (HCl salt) and the reaction temperature and time were varied (Table 1). After the reaction was finished, the mixture was poured into four volumes of acidified (1% HCl) ethanol, and the precipitate was washed with four volumes of 80% alcohol. The precipitate was then dispersed in distilled water and extensively dialyzed for 3 days. The nondialyzate product was then dried by lyophilization to give aminopropylated polymers. The degree of substitution was measured by elemental analysis.

Hydroxyethylation.—The parent polysaccharide (500 mg) was dissolved in 10 mL of 0.5 mol/L NaOH (50 mg/mL), and a certain amount of 30% NaOH solution (2, 4 or 6 mL) was added at 0 °C. The solution was kept at −10 °C in an ice-salt bath for 15 min and then a varied amount of ethylene oxide (10, 14 or 20 mL) was added. The whole mixture was stirred for another 2 h at −10 °C and further stirred at 4 °C overnight. After the reaction was stopped, the reaction mixture was neutralized with 1 mol/L HCl and isolated by extensive dialysis and lyophilization as described above. The degree of substitution was estimated by the method of Wulff et al.[17]

Sulfation.—The sulfating agent was prepared using dry pyridine and chlorosulfonic acid as described by Yoshida et al.[18] The parent polysaccharide (500 mg) was suspended in 20 mL of dry formamide at room temperature with stirring for 30 min. The amount of sulfating reagent and the reaction temperature and time were varied (Table 3). After the reaction was finished, the mixture was cooled and neutralized. The product was isolated by extensive dialysis against saturated $NaHCO_3$ for 1 day and against distilled water for 3 days and then lyophilized. The degree of substitution was measured by the method proposed by Silvestri et al.[29]

Carboxymethylation.—The parent polysaccharide (1.0 g) was suspended in 2-propanol (30 mL) at room temperature. After stirring for 30 min, a certain amount of 30% NaOH

was slowly added within 15 min, and vigorous stirring was continued for about 90 min (Table 4). Then, a certain amount of chloroacetic acid was added, and the mixture was stirred for another 3 h at a varied reaction temperature (Table 4). After the reaction was stopped, the mixture was subsequently cooled, diluted and neutralized. The product was isolated as described above and the degree of substitution was measured by titration.[13]

Carboxymethyl–sulfation.—The carboxymethylated polysaccharides with degree of substitution of 0.28 or 0.69 were prepared as described above. The sulfating reagent was prepared from piperidine and chlorosulfonic acid according to the method of Nagasawa and Yoshidome.[30] The carboxymethylated polymer (500 mg) was suspended in dry Me_2SO (50 mL), and a certain amount of piperidine-*N*-sulfonic acid (1.22 g or 2.44 g for DS 0.28; 0.74 g or 1.48 g for DS 0.69) was added to the mixture. The mixture was stirred for 60 min at 85 °C, cooled with an ice bath, neutralized with 1.0 mol/L NaOH solution and then isolated by dialysis and lyophilization as described above. The degree of substitution of sulfation was also measured by the method of Silvestri et al.,[29] but blanks were run for different carboxymethylated polysaccharides.

Carboxymethyl–benzylamine coupling.—The coupling between a carboxymethyl group and benzylamine was performed by the procedures of Chaubet et al. using EEDQ as the coupling reagent.[23] Briefly, the carboxymethylated polysaccharide (500 mg) with a degree of substitution (DS 0.45 or 1.12), was reacted with 2.85 g (or 3.60 g) EEDQ at pH 4.0 maintained by the addition of 2 mol/L HCl. After stirring for 30 min, 1.26 mL (or 1.60 mL) of benzylamine was added, and the pH of the mixture was maintained at pH 9.0 with 1.0 mol/L NaOH for 1 h. After completion of the reaction, the reaction mixture was extracted with diethyl ether (3×10 mL) in order to eliminate the excess of benzylamine and the byproducts of the coupling reaction. The resulting aqueous phase was extensively dialyzed and freeze-dried. Three successive coupling reactions were undertaken as described above by calculating the amount of reactants and solvents with reference to the carboxymethyl group and benzylamide contents. After three steps, elemental analysis and acidimetric titration were performed to estimate the number of benzylamide and free carboxymethyl groups.

Immunological assay.—Immunomodulating activity of the chemically modified derivatives of parent glucan was assessed by measuring the ConA- or LPS-induced T and B lymphocyte proliferation in vitro and in vivo. Serum IgG and C-3 levels were also measured in mice.

Inbred ICR (♀) mice, three months old, weighing 22 ± 2 g, were obtained from Shanghai Experimental Animal Laboratory, Chinese Academy of Sciences (certificate No. 133). For the tests in vitro, various dilutions of the functional polysaccharide samples (1 or 100 μg/mL) were incubated with mouse splenocytes in the presence of mitogen ConA (5.0 μg/mL) or LPS (10 μg/mL). After incubation for 44 h at 37 °C in a humidified 5% CO_2 atmosphere, T and B lymphocyte proliferation was assayed by the MTT method.[31,32]

For the in vivo study, 49 mice were divided randomly into seven groups: normal saline (control group), PSG-CM-1 (DS 0.17) 25 or 50 mg/kg, PSG-CM-2 (DS 0.25) 25 or 50 mg/kg and PSG-CM-3 (DS 0.28) 25 or 50 mg/kg. The mice were challenged by intraperitoneal injection (i.p.) of 0.2 mL of 5% SRBC on day 0. Each sample was injected i.p. into the mice from day 1 to day 4. Mice were sacrificed on day 5, and their spleens were removed, minced and passed through a sterilized ion mesh (200 mesh) to obtain a single-cell suspension. Erythrocytes in the cell mixture were destroyed by the rapid addition of H_2O. Finally, the cells were suspended to 5×10^6 cells/mL in RPMI-1640 medium. The lymphocyte proliferation and antibody production of spleen cells were measured by the MTT method or by quantitative hemolysin spectrophotometric assay.[31,32] Serum IgG and complement C-3 level were measured by single immunodiffusion method.[32] The diameters of samples on a rabbit anti-mouse serum plate diffusion ring were measured.

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