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CHEMICAL MODIFICATION AND ANTITUMOR ACTIVITY OF A D-MANNO-D-GLUCAN FROM *Microellobosporia grisea*

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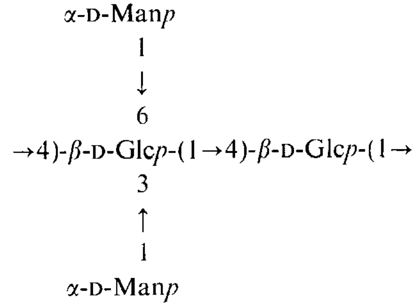
ABSTRACT

An antitumor D-manno-D-glucan from *Microellobosporia grisea*, an actinomycete, has a unique, tetrasaccharide repeating-unit structure, in that a single α-D-mannosyl group is located at both O-3 and O-6 of every other β-D-(1→4)-glucosyl residue. The mannoglucan was chemically modified, and tested for antitumor activities against Ehrlich carcinoma and MM46 adenocarcinoma solid tumors in mice. Acid degradation decreased the antitumor activity, with increase in both the depolymerization and the elimination of the D-mannosyl groups. Various derivatives, such as sulfated, phosphorylated, and stearoylated mannoglucans, showed a decrease in, or loss of, the activity. From these results, it is suggested that both the molecular size and the free hydroxyl groups of the D-mannosyl groups (present as side chains and susceptible to esterification) are responsible for the antitumor activity of the mannoglucan.

INTRODUCTION

Previous work[1,2] has shown that a water-soluble, extracellular polysaccharide from *Microellobosporia grisea* exhibits potent antitumor activity[1], and that it has a unique, tetrasaccharide, repeating-unit structure[2] as follows.



$$\rightarrow 4)\text{-}\underset{\substack{3\\ \uparrow\\ 1\\ \alpha\text{-D-Man}p}}{\overset{\substack{\alpha\text{-D-Man}p\\ 1\\ \downarrow\\ 6}}{\beta\text{-D-Glc}p}}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow$$

It was suggested that the D-mannosyl groups present as side chains should play an important role in the water solubility and antitumor activity of the mannoglucan (MG). In continuation of studies on MG, it was chemically modified, and the antitumor activities of its various derivatives were tested. This paper concerns its degradation with acid, its esterification (sulfation, phosphorylation, and stearoylation), and the antitumor activities of these esters

RESULTS AND DISCUSSION

Chemical properties of MG derivatives. — The conditions required for degradation of MG (mol.wt. ~1 × 10^6) to materials having molecular weights of ~30 × 10^4, 10 × 10^4, and 3 × 10^4 by acid hydrolysis were examined, and a correlation between the conditions of acid hydrolysis and the molecular weight was obtained, as shown in Fig. 1. On the basis of the results, MG was degraded with 0.1M HCl for 4.0 and 7.5 h at 80°, and with 0.2M HCl for 7.0 h, to yield DMG-1, DMG-2, and DMG-3 having molecular weights of 31 × 10^4, 9.8 × 10^4, and 2.8 × 10^4, respectively, in agreement with the values expected. The elution profiles of these acid-degraded mannoglucans in gel-permeation chromatography (g.p.c.) on G4000 SW are shown in Fig. 2.

Sugar analyses showed that MG, DMG-1, DMG-2, and DMG-3 consisted of D-glucose and D-mannose in the molar ratios of 1.14 : 1.00, 1.18 : 1.00, 1.28 : 1.00, and 1.48 : 1.00, respectively, indicating that partial elimination of D-mannosyl groups located as side chains occurred in proportion to the intensity of the conditions of hydrolysis, together with depolymerization by cleavage of the main chain. The

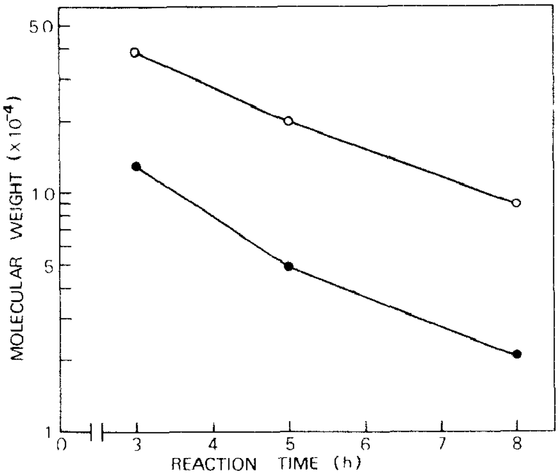


Fig. 1. Correlation between the conditions of acid hydrolysis and the molecular weight. [MG (20 mg) was dissolved in 1.0 mL of 0.1M HCl (— ---) or 0.2M HCl (●—), and heated at 80° for the times indicated. After neutralization of the acid with 0.1M Na_2CO_3, the molecular weights of the acid-degraded products were estimated by g.p.c. on G4000 SW, using dextran T-500, T-70, T-40, and T-10 as standards]

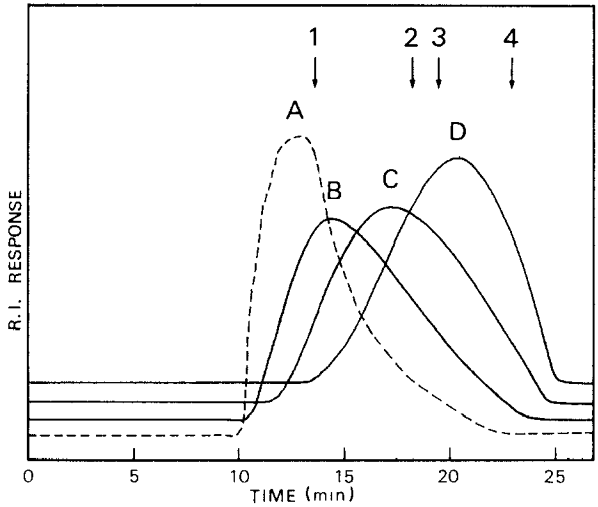


Fig. 2. Gel-permeation chromatography of MG (A), DMG-1 (B), DMG-2 (C), and DMG-3 (D) on G4000 SW, with 0.1M NaCl as the carrier (1.0 mL/min). [Arrows indicate the elution positions of the following standard dextrans: 1 (T-500), 2 (T-70), 3 (T-40), and 4 (T-10).]

position of elimination for DMG-1 and DMG-2 was found by methylation analyses to be O-3 of doubly branched D-glucosyl residues, whereas DMG-3, the most degraded of the three mannoglucans, additionally lost a trace of D-mannosyl groups at O-6 of the doubly branched D-glucosyl residues (see Table I). These results were

TABLE I

METHYLATION ANALYSES OF MG AND ITS ACID-DEGRADED MANNOGLUCANS

O-Methylated sugar (as alditol acetate)	*Molar ratio*			*Mode of linkage*
	MG[a]	*DMG-1*	*DMG-3*	
2,3,4,6-Man[b]	**1.00**	**1.00**	**1.00**	Man*p*-(1→
2,3,6-Glc	0.50	0.54	0.70	→4)-Glc*p*-(1→
2-Glc	0.47	0.43	0.34	↓ 6 →4)-Glc*p*-(1→ 3 ↑
2,3-Glc	0.00	0.09	0.26	↓ 6 →4)-Glc*p*-(1→
2,6-Glc	0.00	tr[c]	0.04	→4)-Glc*p*-(1→ 3 ↑

[a] From ref. 2. [b] 2,3,4,6-Man = 2,3,4,6-tetra-*O*-methyl-D-mannose, etc. [c] Trace.

TABLE II

CHEMICAL PROPERTIES OF MG AND ITS DERIVATIVES

Sample	$[\alpha]_D$[a]	*Anal.*[b] (%)		*S*		*P*		*Stearoyl*	
		C	*H*	%	*d.s.*	%	*d.s.*[c]	%	*d.s.*
MG	+65	42.5	6.4						
DMG-1	64	42.1	6.3						
DMG-2	62	42.3	6.4						
DMG-3	53	41.9	6.2						
MG-SL	+51	32.9	5.0	5.8	0.36				
MG-SH	32	21.0	3.6	14.2	1.32				
DMG-3-SL	+38	29.6	4.7	6.5	0.42				
DMG-3-SH	20	19.8	3.4	16.5	1.78				
DMG-1-PL	+59	39.5	6.2			1.7	0.10		
DMG-1-PH	+26	24.5	4.5			10.5	0.95		
DMG-1-STL	n.d.[d]	45.1	6.4					5.3	0.03
DMG-1-STH	n.d.	60.6	9.2					36.0	0.34
DMG-1-STL-PL	n.d.	44.2	6.6			1.1		6.2	

[a] In degrees (c 0.5, water). [b] Each sample had a nitrogen content of less than 0.4%. [c] As the disodium salt. [d] Not determined, due to insolubility.

supported by the results of a periodate-oxidation study: DMG-1 and DMG-3 consumed 1.20 and 1.18 mol of periodate per mol. with production of 0.44 and 0.41 mol of formic acid per glycosyl residue, respectively, in agreement with the respective values (1.26 and 1.27 mol of periodate, and 0.48 and 0.43 mol of formic acid) expected from the results obtained by methylation analyses (see Table I).

MG and DMG-3 were sulfated with various molar ratios of chlorosulfonic acid to sugar residue, to yield MG-SL and DMG-3-SL with low degrees of substitution (d.s.), and MG-SH and DMG-3-SH with high d.s. The sulfur contents of DMG-3-SL and DMG-3-SH were higher than those of MG-SL and MG-SH, respectively, despite the fact that the same conditions were employed for the respective sulfation of MG and DMG-3 (see Table II). This difference is probably due to a decrease in steric hindrance in DMG-3, based on the depolymerization and the elimination of D-mannosyl groups, both of which occurred in DMG-3, as already described.

Phosphorylated mannoglucans (DMG-1-PL and DMG-1-PH), and *O*-stearoyl derivatives (DMG-1-STL and DMG-1-STH) were obtained by esterification of DMG-1, with varying of the reaction conditions. DMG-1 was also esterified with stearoyl chloride and polyphosphoric acid, to give *O*-stearoyl-DMG-1 phosphate (DMG-1-STL-PL). The chemical properties of these derivatives are summarized in Table II.

Introduction of these substituent groups was confirmed by i.r. spectroscopy; the sulfated, phosphorylated, and stearoylated derivatives showed absorption bands, ascribed to their ester groups, at 1240 and 860–800, 1230 and 970, and 1720 cm^{-1}, respectively. The positions of introduction into MG and its acid-degraded mannoglucans were not established, but the D-mannosyl groups (located as side chains) appear to be much more susceptible to esterification than the D-glucosyl residues comprising their main chains, considering their complicated structures.

Antitumor activities of the MG derivatives. — The derivatives of MG thus obtained were tested for antitumor activities against Ehrlich carcinoma and MM46 adenocarcinoma in mice. As shown in Table III, DMG-1 of the acid-degraded mannoglucans exhibited potent antitumor activity against Ehrlich carcinoma by i.p. administration at a dose of 10 mg/kg, comparable to that of the original mannoglucan MG. On the other hand, DMG-2 and DMG-3, which were of smaller molecular size, with a smaller number of D-mannosyl groups than DMG-1, showed a slightly decreased antitumor activity, compared with MG and DMG-1. In contrast to i.p. administration, oral administration of these degraded mannoglucans resulted in no effect on Ehrlich carcinoma, as MG had no effect (see Table III). As shown in Table IV, DMG-1 and DMG-2 showed almost the same antitumor activity (more potent than that of DMG-3) against MM46 adenocarcinoma by i.p. administration at a dose of 100 mg/kg, whereas the antitumor effect of the three was similar at a dose of 10 mg/kg. Thus, the antitumor activity of MG decreased with increased depolymerization and elimination of D-mannosyl groups, caused by treatment with acid.

MG sulfates (MG-SL and MG-SH) showed a moderately potent antitumor effect, less than that of MG, on MM46 adenocarcinoma by i.p. administration at a dose of 100 mg/kg, but had little effect at 10 mg/kg, as shown in Table V. On the other hand, DMG-3 sulfates (DMG-3-SL and DMG-3-SH) showed almost complete

TABLE III

EFFECT OF MG AND ITS ACID-DEGRADED MANNOGLUCANS ON EHRLICH CARCINOMA[a]

Sample[b]	*Route*	*Dose (mg/kg · days)*	*Tumor weight*[c] *(mean ± se) (g)*	*T/C*[d] *(%)*	*Complete regression*[e]
MG	i.p.	10 × 10	0.17 ±0.10[f]	4.5	6/10
DMG-1	i.p.	10 × 10	0.19 ±0.08[f]	5.0	4/10
DMG-2	i.p.	10 × 10	0.74 ±0.30[f]	19.4	4/10
DMG-3	i.p.	10 × 10	1.04 ±0.31[f]	27.2	1/10
Control			3.82 ±0.36	100	0/10
MG	p.o.	100 × 17	2.88 ±0.79	92.6	0/5
DMG-1	p.o.	100 × 17	2.38 ±0.69	76.5	0/5
DMG-2	p.o.	100 × 17	2.24 ±0.71	72.0	0/5
DMG-3	p.o.	100 × 17	2.77 ±1.04	89.1	0/5
Control			3.11 ±0.17	100	0/5

[a] Female ICR mice were inoculated s.c. with Ehrlich tumor cells (3×10^6) on day 0. [b] Administered i.p. daily from day 1, or p.o. daily from day 1. [c] Weighed on day 30 (i.p.), or day 24 (p.o.). [d] Average tumor-weight of treated group/that of control group. [e] No. of tumor-free mice/no. of mice tested. [f] Significant difference from control group ($p < 0.001$).

TABLE IV

EFFECT OF ACID-DEGRADED MANNOGLUCANS ON MM46 ADENOCARCINOMA[a]

Sample[b]	*Dose (mg/kg)*	*Tumor weight*[c] *(mean ± se) (g)*	*T/C*[d] *(%)*	*Complete regression*[e]
DMG-1	10	1.19 ± 0.33[f]	39.1	2/7
	100	0.18 ± 0.09[g]	5.7	3/6
DMG-2	10	1.24 ± 0.11[h]	40.5	0/7
	100	0.17 ± 0.14[g]	5.4	3/6
DMG-3	10	1.48 ± 0.45[h]	48.6	1/7
	100	0.77 ± 0.08[f]	25.1	0/7
Control		3.05 ± 0.45	100	0/7

[a] Male C3H/He mice were inoculated s.c. with MM46 tumor cells (4×10^6) on day 0. [b] Administered i.p. twice, on days 12 and 17. [c] Weighed on day 30. [d] Average tumor weight of treated group/that of control group. [e] No. of tumor-free mice/no. of mice tested. [f] Significant difference from control group ($p < 0.01$). [g] $p < 0.001$. [h] $p < 0.05$.

TABLE V

EFFECT OF SULFATED MANNOGLUCANS ON MM46 ADENOCARCINOMA[a]

Sample[b]	*Dose (mg/kg)*	*Tumor weight*[c] *(mean ± se) (g)*	*T/C*[d] *(%)*	*Complete regression*[e]
MG-SL	10	1.32 ± 0.36	68.5	1/5
	100	0.50 ± 0.38[f]	26.2	1/5
MG-SH	10	1.59 ± 0.42	82.4	0/5
	100	0.79 ± 0.25[f]	41.0	0/3[g]
DMG-3-SL	10	1.57 ± 0.39	81.6	0/5
	100	1.83 ± 0.28	94.8	0/5
DMG-3-SH	10	1.49 ± 0.20	77.2	0/5
	100	2.75 ± 0.48	143	0/3[g]
MG	10	0.46 ± 0.32[f]	24.0	1/5
	100	0.34 ± 0.23[h]	17.7	2/5
Control		1.93 ± 0.15	100	0/7

[a] Male C3H/He mice were inoculated s.c. with MM46 tumor cells (3×10^6) on day 0. [b] Administered i.p. twice, on days 12 and 17. [c] Weighed on day 28. [d] Average tumor-weight of treated group/that of control group. [e] No. of tumor-free mice/no. of mice tested. [f] Significant difference from control group ($p < 0.01$). [g] Two mice died due to hemorrhage. [h] $p < 0.001$

loss of activity at doses of both 10 and 100 mg/kg (see Table V), whereas DMG-3 significantly inhibited the tumor growth at both dose levels (see Table IV). The administration of MG-SH or DMG-3-SH at 100 mg/kg caused, in test mice, a marked hemorrhage, which must be due to their strong antithrombin activities: the sulfuric

TABLE VI

EFFECT OF DMG-1 DERIVATIVES ON EHRLICH CARCINOMA[a]

Sample[b]	*Dose (mg/kg)*	*Tumor weight*[c] *(mean ±se) (g)*	*T/C*[d] *(%)*	*Complete regression*[e]
DMG-1-PL	10	0.81 ±0.29[f]	29.2	1/6
	100	0.48 ±0.27[f]	17.3	3/6
DMG-1-PH	10	2.55 ±0.64	92.1	0/6
	100	2.25 ±0.32	81.2	0/6
DMG-1-STL	10	1.63 ±0.51	58.8	0/3[g]
	100	1.77 ±0.39	63.9	0/6
DMG-1-STH	10	3.58 ±0.76	129	0/6
	100	2.07 ±0.37	74.7	0/6
DMG-1-STL-PL	10	2.64 ±0.56	95.3	0/6
	100	1.84 ±0.49	66.4	0/6
DMG-1	10	1.25 ±0.53[h]	45.1	1/6
	100	0.34 ±0.22[i]	12.3	2/6
Control		2.77 ±0.40	100	0/14

[a]Female ICR mice were inoculated s.c. with Ehrlich tumor cells (3×10^6) on day 0. [b]Administered i.p. twice, on days 12 and 17. [c]Weighed on day 30. [d]Average tumor-weight of treated group/that of control group. [e]No. of tumor-free mice/no. of mice tested. [f]Significant difference from control group ($p < 0.01$). [g]Accidental death. [h]$p < 0.05$. [i]$p < 0.001$.

esters of high d.s. (MG-SH and DMG-3-SH) gave ID_{50} values of 0.1 and 0.2 μg/mL, respectively.

Of the various derivatives of DMG-1, water-soluble DMG-1-PL (with a low d.s.) showed potent antitumor activity against Ehrlich carcinoma by i.p. administration at doses of both 10 and 100 mg/kg, comparable to that of DMG-1, but water-soluble DMG-1-PH (with a high d.s.) and water-insoluble derivatives (DMG-1-STL and DMG-1-STH) lost most of the activity (see Table VI). Thus, introduction of sulfuric ester, phosphoric ester, or stearoyl groups resulted in decrease in, or loss of, antitumor activity in most cases. DMG-1-STL-PL, a water-insoluble derivative of DMG-1 that was both stearoylated and phosphorylated, showed no effect on the tumor, whereas introduction both of stearoyl and phosphoric ester groups into yeast mannan[3], and both of palmitoyl and phosphoric ester groups into dextran[4] have been reported to give derivatives having antitumor properties.

These results suggest that molecular size, and free hydroxyl groups of D-mannosyl groups located as side chains, are important for the appearance of antitumor activity of the original mannoglucan and its acid-degraded products.

EXPERIMENTAL

Material. — The D-manno-D-glucan (MG) was prepared as described previously[1].

Analytical methods. — Periodate oxidation and methylation analysis were performed as described previously[2]. Sulfur and phosphorus contents were determined by the methods of Antonopoulos[5] and Chen *et al.*[6], respectively. The content of stearoyl group was determined by g.l.c. after methanolysis of a sample with 5% methanolic HCl, with heptadecanoic acid as the internal standard; g.l.c. was performed in a column (0.3 × 200 cm) of Unisole 3000 on Uniport C at 200° with a Hitachi model 163 chromatograph equipped with a flame-ionization detector. Molecular weight was estimated by gel-permeation chromatography (g.p.c.) with dextran T-500, T-70, T-40, and T-10 (Pharmacia Fine Chem.) as standards; the g.p.c. was conducted at 40° in a column (7.5 × 600 mm) of G4000 SW with a Toyo Soda model 803A liquid chromatograph equipped with a refractive-index detector, using 0.1M NaCl as the carrier at a flow rate of 1.0 mL/min. Other analytical methods used in this work have been described[1,2].

Acid degradation. — A solution of MG (50.0 g) in 0.1M HCl (2.0 L) was heated for 4.0 h at 80°, cooled in an ice–water bath, made neutral with 5M NaOH, and poured into ethanol (6.0 L). The resulting precipitate was collected by centrifugation, washed with ethanol, and dissolved in water (3.0 L). The solution was passed through two columns (4.0 × 20 cm each) of Dowex-1 X-2 (Cl^-) and Dowex-50 X-2 (H^+) resin, concentrated to 2 L *in vacuo*, centrifuged to remove a trace of insoluble material, and the acid-degraded product (DMG-1) precipitated from the supernatant liquor with ethanol (6.0 L). DMG-1 was collected by centrifugation, washed successively with ethanol and acetone, and dried over P_2O_5 *in vacuo* (yield, 44.0 g).

Other acid-degraded mannoglucans, DMG-2 (43.2 g), and DMG-3 (37.5 g), were prepared from MG (50.0 g) as just described, except that MG was heated with 0.1M HCl for 7.5 h at 80°, and with 0.2M HCl for 7.0 h, for the preparation of DMG-2 and DMG-3, respectively.

Sulfation. — MG and DMG-3 were sulfated with chlorosulfonic acid and pyridine[7]. To a solution of MG (12.0 g) in formamide (600 mL) was added a sulfating reagent prepared by dropping chlorosulfonic acid (4.9 mL), while vigorously stirring, into pyridine (24 mL) below 0°, and stirring was continued for 8 h at room temperature. The mixture was poured into methanol (4.5 L) at 0°, and the product was precipitated as the pyridinium salt. The precipitate was collected by filtration, washed with methanol, and dissolved in water (650 mL) at 0°. The pH of the solution was adjusted to ~8 with sodium acetate, the suspension was centrifuged, and the resulting sodium salt was precipitated with ethanol (4.5 L) from the supernatant liquor. Reprecipitation from ethanol, followed by drying over P_2O_5 *in vacuo*, gave 12.7 g of MG-SL. DMG-3 (12.0 g) in formamide (240 mL) was sulfated as just described, to give DMG-3-SL (15.3 g).

For preparations of mannoglucan sulfates with high d.s. (MG-SH and DMG-3-SH) more-vigorous conditions for sulfation were used. MG (8.0 g) in formamide (400 mL) was sequentially sulfated with the reagent ($ClSO_3H$, 16.4 mL; pyridine, 8.0 mL) for 2.5 h at room temperature and then for 4 h at 45°. On pouring the mixture into methanol (4.0 L) at 0°, the product (pyridinium salt) was precipitated as a syrupy substance which was washed with methanol, and dissolved in water (550 mL) at 0°. The solution was made neutral with sodium acetate, centrifuged, and the supernatant liquor poured into ethanol (4.0 L). The resulting precipitate was collected by filtration, and dissolved in water (600 mL), and the solution was passed through a column (4.0 × 30 cm) of Dowex-50 X-2 (H^+) resin at 5°, to convert the salt into free acid form. The effluent and washing were combined, made neutral with 2M NaOH, concentrated to ~400 mL, and poured into ethanol (4.0 L). The precipitate (sodium salt) was collected by filtration, and dried over P_2O_5 *in vacuo*, to give MG-SH (12.7 g). DMG-3 (8.0 g) in formamide (160 mL) was sulfated under the conditions used for the preparation of MG-SH, to give DMG-3-SH (16.8 g).

Phosphorylation. — DMG-1 was phosphorylated principally by the method of Whistler and Towle[8]. DMG-1 (2.0 g) was dissolved in formamide (100 mL), and tributylamine (10 mL) and polyphosphoric acid (5.0 g) were added to the clear solution. The mixture was stirred for 24 h at room temperature, and then poured into ethanol (600 mL). The resulting precipitate was collected by centrifugation, and dissolved in water (100 mL). The pH of the solution was adjusted to 10 with 2M NaOH, and the solution was evaporated at 37° *in vacuo* (to remove the liberated tributylamine), and dialyzed against de-ionized water. The nondialyzable suspension was concentrated to ~100 mL, and filtered, and sodium acetate (0.2 g) was added to the filtrate. DMG-1-PL, precipitated from the filtrate with ethanol (600 mL), was collected by centrifugation, washed with ethanol, and dried over P_2O_5 *in vacuo* (yield, 2.15 g). For the preparation of DMG-1 phosphate with high d.s. (DMG-1-PH), DMG-1 (1.0 g) in formamide (50 mL) was phosphorylated for 4 h at 120° with tributylamine (10 mL) and polyphosphoric acid (5.0 g) to give DMG-1-PH (1.80 g).

Stearoylation. — Stearoylation was conducted principally by the method of Hämmerling and Westphal[9]. To a solution of DMG-1 (0.50 g) in *N,N*-dimethylformamide (DMF; 50 mL) at 70° were added pyridine (1.0 mL) and stearoyl chloride (0.1 g) in DMF (1.0 mL). The mixture was stirred for 3 h at 50°, filtered, and the filtrate poured into ethanol (300 mL) containing sodium acetate-saturated methanol (1.0 mL). The resulting precipitate was collected by filtration, washed successively with ethanol, acetone, and ethyl ether, and dried over P_2O_5 *in vacuo*, to give DMG-1-STL (0.55 g).

For preparation of a stearoyl derivative with high d.s. (DMG-1-STH), DMG-1 (0.50 g) in DMF (50 mL) was stearoylated for 3 h at 50° with pyridine (10 mL) and stearoyl chloride (1.0 g) in DMF (5.0 mL), to give DMG-1-STH (0.71 g).

Stearoylation and phosphorylation. — Pyridine (1.0 mL) and stearoyl chloride (0.1 g) in DMF (1.0 mL) were added to DMG-1 (0.50 g) in DMF (50 mL), and the mixture was stirred for 3 h at room temperature. Tributylamine (5.0 mL) and polyphosphoric acid (2.5 g) were added, and the mixture was heated for 2 h at 80°, and poured into ethanol (300 mL). The resulting precipitate was collected by centrifugation, washed successively with water, 0.1M HCl, water, 0.2M sodium acetate, water, ethanol, acetone, and ethyl ether, and dried over P_2O_5 *in vacuo*, to give DMG-1-STL-PL (0.38 g).

Assay of antitumor activity. — Antitumor activities of MG and its derivatives against Ehrlich carcinoma and MM46 adenocarcinoma solid tumors in mice were assayed as previously described[1]. The tumors were implanted s.c. into the right groin of mice, and the test samples, dissolved or suspended in physiological saline, were administered i.p., twice on days 12 and 17, or daily for 10 days, starting 24 h after tumor implantation.

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