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Studies on the Water-soluble Constituents of Lichens. III.[1] Changes in Antitumor Effect caused by Modifications of Pustulan- and Lichenan-type Glucans[2]

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Two lichen polysaccharides with antitumor activity, a partially O-acetylated pustulan (GE-3) and a lichenan-type glucan (UR-1-1), isolated from *Gyrophora esculenta* Miyoshi and *Usnea rubescens* Stirt., respectively, were subjected to various modifications, and the resulting products were tested for noncytotoxic, host-mediated antitumor action against sarcoma 180 solid tumor in mice by intraperitoneal administration. Treatment of GE-3 with urea afforded products with the same antitumor effect as the original glucan. Injection of GE-3 or UR-1-1 together with urea also caused no marked decrease in the antitumor activity. On O-carboxymethylation, both glucans yielded products with greatly reduced antitumor activity. Ten lots of the lauroyl derivative with different degrees of substitution were prepared from GE-3. Among them, three with lauroyl contents less than 3.3% exhibited strong antitumor activity, while the more highly esterified products were all ineffective. Introduction of both carboxymethyl and lauroyl groups into the GE-3 molecule gave a product with moderate antitumor activity. The ten lots of the lauroyl derivative of GE-3 had no direct antitumor action against Ehrlich ascites carcinoma implanted in mice, as determined by the total packed cell volume method.

Keywords——lichen; *Gyrophora esculenta* Miyoshi; *Usnea rubescens* Stirt.; antitumor effect: sarcoma 180 solid tumor; Ehrlich ascites carcinoma; partially O-acetylated pustulan; lichenan-type glucan; modifications of polysaccharides

In our previous investigations, various lichen polysaccharides have been tested for noncytotoxic antitumor activity.[1,4,5] It was found that two sorts of linear β-D-glucans, pustulan-type and lichenan-type polysaccharides, which occur in some species of lichens, are particularly effective against sarocoma 180 solid tumor in mice. This work was undertaken to investigate

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[1] Part II: Y. Nishikawa, K. Ohki, K. Takahashi, G. Kurono, F. Fukuoka, and M. Emori, *Chem. Pharm. Bull.* (Tokyo), **22**, 2642 (1974).

[2] This paper includes work presented at the 45th and 47th General Meetings of the Japanese Biochemical Society, Hiyoshi and Okayama, Nov. 1972 and Oct. 1974, respectively.

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[4] R.L. Whistler, A.A. Bushway, P.P. Singh, W. Nakahara, and R. Tokuzen, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 32, ed. by R.S. Tipson and D. Horton, Academic Press, New York, 1976, p. 235.

[5] *a*) S. Shibata, Y. Nishikawa, M. Tanaka, F. Fukuoka, and M. Nakanishi, *Zeitschrift für Krebsforsch.*, **71**, 102 (1968); *b*) S. Shibata, Y. Nishikawa, T. Takeda, M. Tanaka, F. Fukuoka, and M. Nakanishi, *Chem. Pharm. Bull.* (Tokyo), **16**, 1639 (1968); *c*) S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *ibid.*, **16**, 2362 (1968); *d*) F. Fukuoka, M. Nakanishi, S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *Gann*, **59**, 421 (1968); *e*) Y. Nishikawa, T. Takeda, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **17**, 1910 (1969); *f*) Y. Nishikawa, *Jap. J. Clinic. Med.*, **27**, 1744 (1969); *g*) T. Takeda, Y. Nishikawa, and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **18**, 1074 (1970); *h*) Y. Nishikawa, M. Tanaka, S. Shibata, and F. Fukuoka, *ibid.*, **18**, 1431 (1970); *i*) R. Tokuzen, W. Nakahara, F. Fukuoka, S. Shibata, and Y. Nishikawa, *Toxicl. Appl. Pharmacol.*, **17**, 529 (1970); *j*) R. Tokuzen and W. Nakahara, *Arzneim. Forsch.*, **21**, 269 (1971); *k*) T. Takeda, M. Funatsu, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **20**, 2445 (1972); *l*) K. Takahashi, T. Takeda, S. Shibata, M. Inomata, and F. Fukuoka, *ibid.*, **22**, 404 (1974).

the changes in antitumor effect caused by modifications of these two types of glucans. We employed two representative preparations, GE-3 and UR-1-1, isolated from *Gyrophora esculenta* Miyoshi and *Usnea rubescens* Stirt., respectively, as starting materials. As has already been demonstrated, the glucan termed GE-3 is composed solely of β-D-(1→6)-linkages, but it differs from pustulan in containing about 2% of acetyl groups at O-3, while the latter preparation, UR-1-1, consists of a lichenan-type glucan, possessing β-D-(1→3)- and β-D-(1→4)-linkages in the ratio 3: 7.[1,5a-f,h,i]

The modifications of both preparations were conducted by treatment with urea, and also by introducing O-carboxymethyl groups into the molecules. In addition, lauroyl derivatives of GE-3 were used as test samples. Throughout the present study, the host-mediated antitumor effect was evaluated under the same experimental conditions as before. Sarcoma 180 ascites tumor was transplanted subcutaneously into mice, and each test sample was administered intraperitoneally once a day for 10 consecutive days starting 24 hr after tumor implantation. At the end of the 5th week, the average tumor weights in the treated (T) and control (C) groups were compared to obtain the inhibition ratios: complete regression of the tumors was also recorded.

The following four specimens were used to test the effect of urea treatment upon the antitumor effect of GE-3 and UR-1-1: i) U-GE-3 (1). This was obtained by stirring GE-3 in 8 M urea solution at 70° for 4 hr, followed by precipitation and washing with ethanol; it contained a small amount of urea. ii) U-GE-3 (2). GE-3 was treated as above, and then subjected to dialysis to afford a nitrogen-free product. iii) GE-3+U and iv) UR-1-1+U. Equal weights of GE-3 or UR-1-1 and urea were mixed together, and, just prior to injection, sterilization was carried out by heating at 130° for 10 min to give clear homogeneous solutions. As shown in Table I, the four products showed marked antitumor effects, though they had slightly lower inhibition ratios than the corresponding parent glucans. It is also noteworthy that these specimens produced pathological liver changes similar to those observed previously in mice receiving both types of polysaccarides.[5i] Based on these findings, it appears that the biological activities of these glucans are not affected significantly by treatment with urea.

In contrast with our present results, certain fungal polysaccharides have been reported to undergo significant alteration in their host-mediated antitumor effect against sarcoma 180 solid tumor upon modification with urea.[6] According to Chihara and co-workers, a product,

Table I. Antitumor Effects against Sarcoma 180 Solid Tumor of the Products obtained by Modification of GE-3 and UR-1-1 with Urea

Sample	Dose[a] (mg/kg × day)	Inhibition ratio[b] (%)	Evaluation of activity[c]	Complete regression	Mortality (died/total)	Average body wt. change (g)
U-GE-3 (1)	150 × 10	84	⧻	5/8	0/8	+9.3
U-GE-3 (2)	150 × 10	96	⧻	3/8	1/8	+2.4
GE-3+U	(150+150) × 10	91	⧻	4/8	0/8	+5.5
Control				0/8	0/8	+4.8
UR-1-1+U	(150+150) × 10	92	⧻	0/6	0/6	+6.1
Control				0/6	0/6	+1.4

a) Test samples were dissolved in H_2O.
b) *cf.* Inhibition ratios obtained by administration of GE-3 and UR-1-1 at a dose of 150 mg/kg × 10 days were 97% and 100%, respectively.[1,5d]
c) For criteria, see "Experimental."

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6) *a*) J. Hamuro, Y.Y. Maeda, Y. Arai, F. Fukuoka, and G. Chihara, *Chem.-Biol. Interactions*, 3, 69 (1971); *b*) Y.Y. Maeda, J. Hamuro, Y.O. Yamada, K. Ishimura, and G. Chihara, "Immunopotentiation," (Ciba Foundation Symposium 18 (new series)), ed. by G.E.W. Wolstenholme and J. Knight, Elsevier, Excerpta Medica, North-Holland, Amsterdam, London, and New York, 1973, p. 259.

U-pachyman, with considerable antitumor activity can be obtained from inactive, natural pachyman by the procedure used for preparation of U-GE-3 (2). In addition, these authors have found that, when injected together with urea, lentinan[7] and pachymaran[8] lost their antitumor effects, while schizophyllan[9] showed no loss of activity.[6a,10] On the basis of these observations, Chihara claimed that a higher order structure or a micelle structure of a polysaccharide, rather than the primary structure, plays an important role in the antitumor activity. The presence of higher structures in lentinan and pachymaran was suggested by the observation that their optical rotations decreased in urea solution or in dimethyl sulfoxide (DMSO). Thus, we also examined the effect of denaturing reagents on the optical rotations of the present specimens. As shown in Table II, it was found that the specific rotations of the preparations GE-3 and U-GE-3 (2) were similar in water, and the values fell only slightly in 7 M urea solution, though they fell markedly in DMSO. Accordingly, no significant alteration in the polysaccharide conformation appears to take place when GE-3-type glucan is treated with urea.[11] This view is compatible with the theoretical conformation of β-(1→6)-linked glucan proposed by Rees based on computer model building studies: (1→6)-linkages have been suggested to form loosely joined chains with high flexibility.[12] In view of Chihara's earlier studies, the small variation of polysaccharide conformation may be correlated with the unchanged antitumor behavior of the products derived from GE-3. However, another preparation UR-1-1 gave a different result: upon addition of urea, its optical rotation decreased considerably, though on injection with urea its antitumor effect seemed unimpaired. This suggests that the connection of the conformational change with the antitumor activity should be further investigated.

In order to study the effect of O-carboxymethylation on the antitumor effect of GE-3-type and lichenan-type glucans, three lots of carboxymethyl derivative, CM-GE-3, possessing D.S. (degree of substitution) values of 0.51, 0.85, and 0.97, and one carboxymethyl preparation, CM-UR-1-1, with a D.S. of 0.59 were synthesized from GE-3 and UR-1-1, respectively, in the usual manner. As shown in Table III, these products proved to be largely or completely

TABLE II. Changes in Optical Rotation[a]

Sample	$[\alpha]_D^T$ Value ($c=0.5$)		
	In aqueous solvent	In 7 M urea solution	In DMSO
GE-3	−34.4° (T, 24°; H_2O)	−38.3° (T, 28°)	−56.3° (T, 25°)
U-GE-3 (2)	−32.9° (T, 24°; H_2O)	−37.5° (T, 28°)	−59.1° (T, 25°)
UR-1-1	+18.7° (T, 18°; 1 N NaOH)	−19.5° (T, 18°)	−28.7° (T, 18°)

a) *cf.* $[\alpha]_D$ values reported for lentinan[6a]: +19.5—+21.5° ($c=1$; T, 25°; 10% NaOH); +9° (T, 20°; 6 M urea solution); −13° ($c=0.3$; T, 18°).

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7) *a*) G. Chihara, J. Hamuro, Y.Y. Maeda, Y. Arai, and F. Fukuoka, *Cancer Res.*, **30**, 2776 (1970); *b*) T. Sasaki and N. Takasuka, *Carbohydr. Res.*, **47**, 99 (1976).

8) G. Chihara, J. Hamuro, Y.Y. Maeda, Y. Arai, and F. Fukuoka, *Nature*, **225**, 943 (1970).

9) N. Komatsu, S. Okubo, S. Kikumoto, K. Kimura, G. Saito, and S. Sasaki, *Gann*, **60**, 137 (1969).

10) Chihara and co-workers assumed that the differences in the denaturation behavior with urea between lentinan or pachymaran and schizophyllan could be attributed to the abundance of β-(1→6)-linked branches in the β-(1→3)-linked backbone chains of the latter glucan, since, at that time, the former compounds were both regarded as essentially linear β-(1→3)-glucans. Subsequently, however, Sasaki showed that lentinan also contains branch points (2 for every 5 D-glucose residues) at C-6, and that the side chains of lentinan consist of β-D-(1→6)-linked and β-D-(1→3)-linked glucose units attached to the β-D-(1→3)-glucan constituting the main chain; a few internal β-D-(1→6)-linkages might also be present.[7b] Therefore, Chihara's assumption, is questionable.

11) Recently, Hirano has established a method to analyze polysaccharide conformations by comparison of the optical rotation of a test sample in water with that in 7 M urea: *a*) S. Hirano, *Life Sci.*, **10**, 151 (1971); *b*) *Idem*, *Kagaku No Ryoiki*, **28**, 52 (1974).

12) *a*) D.A. Rees and W.E. Scott, *J. Chem. Soc.* (*B*), **1971**, 469; *b*) *Idem*, *Chem. Comm.*, **1969**, 1037.

TABLE III. Antitumor Effects of the O-Carboxymethyl Derivatives of GE-3 and UR-1-1 against Sarcoma 180 Solid Tumor[a)]

Sample	Inhibition ratio (%)	Evaluation of activity[b)]	Complete regression	Mortality (died/total)	Average body wt. change (g)
CM-GE-3					
Lot 1 (D.S., 0.51)	30	+	0/8	0/8	+6.8
Lot 2 (D.S., 0.85)	36	+	0/8	0/8	+7.4
Lot 3 (D.S., 0.97)	24	±	0/8	0/8	+9.9
CM-UR-1-1 (D.S., 0.59)	6	−	0/8	0/8	+5.8
cf)					
GE-5 (deacetylated GE-3)[c)]	86	卅	1/8	0/8	+6.6

a) Dose: 150 mg/kg × 10 days. Vehicle: H_2O.
b) For criteria, see "Experimental".
c) The data are cited from ref. 5*e*).

ineffective against sarcoma 180 solid tumor. During the course of the carboxymethylation reaction of GE-3, incidental deacetylation occurred, as demonstrated by the infrared (IR) spectra of the resulting products. Although it is known that deacetylation of GE-3 results in partial loss of activity,[5e)] the marked inactivation caused by carboxymethylation of GE-3 should be chiefly due to the introduction of carboxymethyl functions into the GE-3 molecule.

Chihara and co-workers have shown that a carboxymethyl derivative (D.S. 0.95) of pachymaran was active against sarcoma 180, Ehrlich carcinoma, MM-102 carcinoma, and CCM adenocarcinoma. The product was useful, owing to its high solubility in water, for studying the mechanisms involved in the antitumor action of polysaccharide.[13)] By carboxymethylation of a mannan preparation obtained from *Candida utilis*, Oka and co-workers have succeeded in removing the acute toxicity without loss of antitumor activity.[14)] Osswald reported an antitumor effect of carboxymethyl cellulose against Ehrlich ascites carcinoma.[15)]

Recently, Whistler *et al.* have reviewed the antitumor polysaccharides hitherto reported, and pointed out that water-soluble D-glucans are, in general, effective agents, particularly if they are mainly linear, without excessively long branches.[4)] However, our present results indicate that improvement of water-solubility by converting an essentially linear, antitumor glucan into its carboxymethyl derivative may not be accompanied by retention of the original high activity. In addition, it should be mentioned that pathological liver changes were not prevented by the carboxymethylation of the glucans used in the present study.

In another series of papers, we have reported that various monoesters synthesized from non-reducing disaccharides (sucrose and trehalose) and certain long-chain fatty acids by Osipow's procedure can exert direct antitumor action upon Ehrlich ascites carcinoma, sarcoma 180 ascites tumor, and a mouse leukemia cell line, L-5178Y, though they are devoid of host-mediated antitumor activity.[16)]

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13) *a*) J. Hamuro, Y. Yamashita, Y. Ohsaka, Y.Y. Maeda, and G. Chihara, *Nature*, **233**, 486 (1971); *b*) J. Hamuro and G. Chihara, *ibid.*, **245**, 40 (1973); *c*) Y.Y. Maeda and G. Chihara, *Int. J. Cancer*, **11**, 153 (1973); *d*) T. Okuda, Y. Yoshioka, T. Ikekawa, G. Chihara, and K. Nishioka, *Nature New Biology*, **238**, 59 (1972).

14) *a*) S. Oka, N. Kumano, and K. Kurita, *Gann*, **63**, 365 (1972); *b*) N. Kumano, K. Kurita, and S. Oka, *ibid.*, **63**, 675 (1972); *c*) S. Oka, N. Kumano, K. Kurita, K. Sato, K. Tamari, K. Matsuda, H. Hirai, K. Ogawa, S. Kiyooka, T. Ogawa, and K. Miyao, Proc. Intern. Congr. Chemother, VIIth, Prague, 1971, p. 167.

15) H. Osswald, *Arzneim.-Forsch.*, **18**, 1495 (1968).

16) *a*) T. Ikekawa, S. Yanoma, M. Umeji, K. Yoshimoto, and Y. Nishikawa, *Chem. Pharm. Bull.* (Tokyo), in press. *b*) Y. Nishikawa, K. Yoshimoto, T. Manabe, and T. Ikekawa, *ibid.*, **25**, 2378 (1977); *c*) Y. Nishikawa, K. Yoshimoto, M. Okada, T. Ikekawa, N. Abiko, and F. Fukuoka, *ibid.*, **25**, 1717 (1977); *d*) Y. Nishikawa, K. Yoshimoto, M. Okabe, and F. Fukuoka, *ibid.*, **24**, 756 (1976); *e*) Y. Nishikawa, M. Okabe, K. Yoshimoto, G. Kurono, and F. Fukuoka, *ibid.*, **24**, 387 (1976).

Table IV. Antitumor Effects of the Lauroyl Derivatives of GE-3 against Sarcoma 180 Solid Tumor and Ehrlich Ascites Carcinoma

Sample		Effect against sarcoma 180 solid tumor								
No.	Lauroyl content (%)	Dose[a)]	Vehicle[b)]	Inhibition ratio (%)	Evaluation of activity[c)]	Complete regression	Mortality (died/total) C	Mortality (died/total) T	Average body wt. change (g) C	Average body wt. change (g) T
GE-3-L										
Lot 1	1.3	150	A	95	+++	2/10	0/8	0/10	+7.7	+8.9
Lot 2	2.4	100	B	98	+++	4/8	1/8	0/8	+6.9	+6.5
Lot 3	3.3	150	A	82	+++	1/10	0/8	0/10	+7.7	+7.4
Lot 4	12.0	150	A	23	±	0/10	0/8	0/10	+7.7	+7.9
Lot 5	18.9	100	B	−22	−	0/8	1/8	0/8	+6.9	+7.9
Lot 6	24.5	150	A	−13	−	0/8	0/8	0/8	+8.0	+7.9
Lot 7	42.2	150	A	−8	−	0/8	0/8	0/8	+8.0	+8.0
Lot 8	50.1	100	B	−31	−	0/8	0/8	0/8	+7.1	+7.2
Lot 9	58.1	100	B	−50	−	0/8	1/8	0/8	+6.9	+8.3
Lot 10	70.6	100	B	−14	−	0/8	0/8	0/8	+7.1	+4.9
CM-GE-3-L[d)]	40.2	150	C	51	++	0/6	1/6	0/6	+1.4	+3.2

Sample		Effect against Ehrlich ascites carcinoma[a)]						
No.	Lauroyl content (%)	Vehicle[b)]	TPCV ratio (% T/C)	Evaluation of activity[c)]	Mortality (died/total) C	Mortality (died/total) T	Average body wt. change (g) C	Average body wt. change (g) T
GE-3-L								
Lot 1	1.3	A	97	−	1/6	0/6	−0.1	−0.7
Lot 2	2.4	B	125	−	0/6	0/6	−0.2	−0.6
Lot 3	3.3	A	91	−	1/6	0/6	−0.1	−2.0
Lot 4	12.0	A	92	−	1/6	0/6	−0.1	−1.6
Lot 5	18.9	B	88	−	0/6	0/6	−0.2	−3.3
Lot 6	24.5	B	122	−	0/6	0/6	−0.2	+1.1
Lot 7	42.2	B	81	−	1/6	0/6	+0.5	−1.7
Lot 8	50.1	B	92	−	1/6	2/6	+0.5	−2.3
Lot 9	58.1	B	118	−	0/6	1/6	−0.2	−1.6
Lot 10	70.6	B	100	−	1/6	0/6	+0.5	−2.7
GE-3		B	90	−	1/6	0/6	+0.5	−0.03

a) 100: 100 mg/kg × 10 days. 150: 150 mg/kg × 10 days. All experiments on the antitumor effect against Ehrlich ascites carcinoma were conducted at a dose of 100 mg/kg × 5 days.
b) A: 0.9% saline. B: 0.9% saline with 0.2% Tween 80. C: H_2O.
c) For criteria, see "Experimental".
d) Degree of carboxymethyl substitution, 0.30.

Generally, the antitumor polysaccharides so far reported are effective only against restricted types of implanted solid tumors, but they do not have direct cytotoxicity towards ascites tumor cells,[4)] and are ineffective against autochthonous or spontaneous tumors.[5j)] In one attempt to enhance the antitumor activity of polysaccharides, we performed fatty acylation of GE-3, expecting to obtain products with both direct and indirect antitumor abilities. GE-3 was lauroylated according to Westphal to various extents to give ten products termed GE-3-L.[17)] Deacetylation did not take place during the reaction as indicated by the IR spectra of the products. When tested for host-mediated antitumor action as described above,

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17) U. Hämmerling and O. Westphal, *Europ. J. Biochem.*, 1, 46 (1967).

lots 1 and 2 containing 1.3% and 2.4% lauroyl groups, respectively, exhibited very strong activity, and lot 3 with 3.3% lauroyl content also showed rather strong action, whereas lots 4—10, having 12% to 70.6% lauroyl groups, were all ineffective (Table IV). The results indicate that extensive lauroylation of GE-3 causes loss of noncytotoxic antitumor effect. Although it seems probable that limited substitution does not significantly affect the antitumor activity, this requires confirmation. The marked effect shown by lots 1, 2, and 3 may be partially attributable to the possible presence of unreacted GE-3; since these experiments were preliminary, no attempt was made to remove unesterified GE-3 from the products. The active lots were soluble in water, while the inactive lots with higher degrees of lauroyl substitution were insoluble in water. Accordingly, we investigated whether increased antitumor activity could be obtained by converting the highly lauroylated GE-3 into a derivative with improved water solubility. For this purpose we prepared a specimen, CM-GE-3-L, by carboxymethylation and subsequent lauroylation of GE-3.[18] The degree of carboxymethyl substitution and the lauroyl content of the product were determined to be 0.30 and 40.2%, respectively. The product was soluble in water, and, as shown in Table IV, it exhibited moderate antitumor action. This result suggests that there may be some correlation between the inactivation caused by extensive substitution of GE-3 with lauroyl groups and the sparing water solubility of the resulting products. It is also of interest that CM-GE-3-L did not produce liver changes of the type encountered in mice treated with GE-3, CM-GE-3, and GE-3-L.

The direct antitumor actions of GE-3 and the ten lots of GE-3-L were evaluated by the total packed cell volume (TPCV) method, using Ehrlich ascites carcinoma. The bioassay conditions were similar to those adopted for fatty acyl derivatives of disaccharides.[16] The tumor cells were implanted intraperitoneally into mice, and the test sample was administered by intraperitoneal injection once daily for 5 consecutive days, starting 24 hr after tumor transplantation. On the 7th day after tumor implantation, the antitumor activity was evaluated by comparing the TPCV ratio of the treated mice with that of the control. As shown in Table IV, all the samples tested were completely ineffective. On the basis of this finding, it was concluded that lauroylation of GE-3 to any extent did not afford products with direct antitumor action upon Ehrlich ascites carcinoma.

Some promising antitumor results have been reported with natural and artificial lipopolysaccharides by earlier workers.[4,19,20] Mizuno *et al.* found that the lipopolysaccharide of *Proteus vulgaris* caused marked stimulation of the reticuloendothelial system, in addition to a slight cytocidal effect.[19] According to Suzuki and co-workers, modification of a yeast mannan by stearoylation followed by phosphorylation yields a product which is effective against sarcoma 180 and Ehrlich ascites carcinoma *in vivo*.[20a] Later, palmitoyl dextran phosphates were also demonstrated to possess similar antitumor action.[20b] In addition to polysaccharide esters, certain synthetic, fatty acyl derivatives of mono- and disaccharides are known to show antitumor action and related immunological activities.[16,21] Thus, further investigations on the antitumor activities of various carbohydrate esters are now under way in our laboratory. The results will be reported shortly.

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18) Attempts to obtain the carboxymethyl ether of GE-3 laurate by lauroylation and subsequent carboxymethylation of GE-3 were unsuccessful, as complete deacylation took place during the course of the carboxymethylation reaction.

19) D. Mizuno, O. Yoshioka, M. Akamatsu, and T. Kataoka, *Cancer Res.*, **28**, 1531 (1968).

20) *a*) M. Suzuki, T. Matsumoto, T. Mikami, and S. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **24**, 1100 (1976); *b*) M. Suzuki, T. Mikami, M. Kadowaki, T. Matsumoto, and S. Suzuki, *Cancer Res.*, **37**, 3448 (1977).

21) *a*) V.N. Nigam, C.A. Brailovski, and C. Chopra, *Cancer Res.*, **38**, 3315 (1978); *b*) U.H. Behling, B. Campbell, C.M. Chang, C. Rumpf, and A. Nowortony, *J. Immunol.*, **117**, 847 (1976); *c*) D.L. Rosenstreich, J. Asselineau, S.E. Mergenhagen, and A. Nowortony, *J. Exptl. Med.*, **140**, 1404 (1974); *e*) I. Azuma, K. Sugimura, T. Taniyama, M. Yamawaki, Y. Yamamura, S. Kusumoto, S. Okada, and T. Shiba, *Infection and Immunity*, **14**, 18 (1976).

Experimental

Evaluation of Host-mediated Antitumor Effect upon Sarcoma 180 Solid Tumor——Sarcoma 180 ascites tumor was transplanted subcutaneously into the right groin of ICR female mice, weighing 22±2 g. The sample to be tested was injected intraperitoneally once a day for 10 consecutive days starting 24 hr after transplantation. The mice were killed at the end of the 5th week, and the tumors were extirpated and weighed to determine the inhibition ratios. The criteria used were as follows:

inhibition ratio (%)	0—10	11—30	31—50	51—70	71—100
evaluation	−	±	+	++	+++

Evaluation of Direct Antitumor Action against Ehrlich Ascites Carcinoma——Ehrlich ascites tumor cells (*ca.* 7×10^6 cells/mouse) were implanted intraperitoneally in ddY female mice, weighing 23±2 g. The agent to be tested was dissolved or suspended in 0.9% saline or in 0.9% saline with 0.2% Tween 80. Treatment was initiated 24 hr after tumor implantation, the agent being given by intraperitoneal injection once daily for 5 consecutive days. On the 7th day after tumor transplantation, the antitumor activity was evaluated in terms of TPCV (TPCV (ml) = volume of ascites (ml) × ascitocrit) ratio (% T/C). The criteria employed were as follows:

TPCV ratio (% T/C)	0—10	11—40	41—65	66—100
evaluation	+++	++	+	−

Starting Materials used for Modifications——The preparations GE-3 (partially O-acetylated, pustulan-like glucan)[5a−f] and UR-1-1 (lichenan-type glucan),[1] obtained from *Gyrophora esculenta* Miyoshi and *Usnea rubescens* Stirt., respectively, in our previous studies, were used.

Treatment with Urea——GE-3 (3 g) was stirred in 8 M urea solution (200 ml) at 70° for 4 hr. A half of the mixture was poured into EtOH, and the precipitate formed was collected by centrifugation, washed repeatedly with EtOH, and then dried *in vacuo* to afford a slightly brownish white powder (U-GE-3 (1)). It contained 2.2% N (elemental analysis), owing to uneliminated urea. The other half was dialyzed against distilled water (three times) using Visking tubing, and the undialysable portion was treated as above to afford a nitrogen-free product (U-GE-3 (2)). The preparations obtained by mixing equal weights of GE-3 or UR-1-1 and urea are referred to as GE-3+U and UR-1-1+U, respectively. Before use, each mixture was sterilized by heating at 130° for 10 min to give a clear, homogeneous solution. The host-mediated antitumor effects of these preparations are shown in Table I. The mice treated with these preparations showed pathological liver changes.[22] Changes in optical rotation caused by the addition of denaturing reagents to GE-3 and UR-1-1 are presented in Table II.

O-Carboxymethylation——The reaction was performed according to the procedure described by Green with slight modifications.[23] A slurry of GE-3 (or UR-1-1) in 2-propanol was stirred vigorously while 30% NaOH was added dropwise at room temperature. Stirring was continued for 30 min, and then chloroacetic acid was added slowly. The resulting mixture was stirred for 2 hr at 50° on a water-bath. The product was collected by filtration, washed with 70% MeOH–AcOH solution, 80% aqueous MeOH, acetone and ether successively, then dried *in vacuo*. The D.S. value was determined by the acid-wash method.[23] By using different amounts of chloroacetic acid, three lots of sodium carboxymethyl derivative, CM-GE-3 (lots 1, 2, and 3), possessing D.S. values of 0.51, 0.85, and 0.97, respectively, were obtained from GE-3, while one product, CM-UR-1-1, with a D.S. of 0.59 was obtained from UR-1-1. These products were all readily soluble in water. Their IR spectra (KBr) showed broad absorption bands around 1590 cm^{-1} (–COO$^-$). The three lots of CM-GE-3 had no absorption bands around 1730 cm^{-1} in their IR spectra. The antitumor activities of the carboxymethyl derivatives prepared are shown in Table III. Injections of these preparations into mice produced pathological liver changes.[22]

Lauroylation——The reaction was carried out by the method of Westphal.[17] GE-3 (810 mg) was stirred in N,N-dimethylformamide (20 ml) at 100° for 6 hr, and then pyridine (1.26 g) and an appropriate amount of lauroyl chloride were added. After stirring at 100° for 24 hr, MeOH (*ca.* 150 ml) was added to the mixture to form a precipitate, which was collected by filtration, washed with MeOH, acetone and ether, and dried *in vacuo*. For preparation of the ten lots of GE-3 laurate (GE-3-L), the following amounts of lauroyl chloride were used (mg): lot 1, 3833; lot 2, 2957, lot 3, 2190; lot 4, 1643; lot 5, 1090; lot 6, 548; lot 7, 438; lot 8, 328; lot 9, 219; lot 10, 109. The IR spectra (KBr) of these products showed strong ester bands around 1740 cm^{-1}. On saponification (0.2% NaOH, 30 min), they liberated acetic acid, which was identified by GLC[24]; column

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22) The liver was markedly enlarged and perceptibly tough in consistency. Histological examinations revealed extensive multifocal mesenchymal cell accumulations in the sinusoids throughout the liver.[5i]

23) J.W. Green, "Methods in Carbohydrate Chemistry,' Vol. 3, ed. by R.L. Whistler, Academic Press, New York and London, 1963, p. 322.

24) Throughout the present work, GLC analysis was carried out with a Shimadzu GC-4BPF gas chromatograph equipped with a hydrogen flame ionization detector, using a glass column (2.0 m × 4 mm I.D.).

packing, Porapak Q (80—100 mesh) (product of Waters Associates Inc.); column temp., 200°; carrier gas, N_2 (50 ml/min). The retention time of AcOH was 2.2 min. The lauroyl contents were estimated by GLC analysis of the methanolysates, which were obtained according to the method of Hyun.[25,26] A mixture of known amounts of GE-3-L and myristic acid (internal standard) was heated with BF_3–MeOH reagent at 100° for 2 hr in a sealed tube.[27] The reaction mixture was treated with H_2O, then extracted with petr. ether. The extract was evaporated to a syrup and injected into the gas chromatograph[24]: column packing, 5% DEGS (diethyleneglycol succinate) on Shimalite W; column temp., 150°; carrier gas, N_2 (50 ml/min). A linear calibration plot was obtained: the recorder response factor (=peak area ratio (GE-3-L/myristic acid)/weight ratio (GE-3-L/myristic acid)) was determined to be 1.0. The lauroyl contents obtained are shown in Table IV. The ten lots of GE-3-L were subjected to both direct and indirect antitumor bioassays and the results are presented in Table IV. Treatment of mice with GE-3-L caused pathological liver changes.[22] Lots 1—3 were soluble in H_2O, while the others were largely or completely insoluble.

Preparation of CM-GE-3-L[18)]——A carboxymethyl derivative with a D.S. of 0.3 was prepared from GE-3 by the procedure described above. The product (810 mg) was stirred in pyridine (27 ml) for 30 min, and then treated with lauroyl chloride (4.0 g) and dioxane (20 ml). After stirring at 100° for 24 hr, 10% MeOH–toluene was added to dissolve the reaction products. MeOH was poured into the solution to afford a precipitate, which was collected by filtration, washed with MeOH, and dried *in vacuo*. The resulting material was dissolved in toluene, and the solution was neutralized with 0.7% NaOH–EtOH. The precipitate formed by adding EtOH to the solution was collected, washed, and dried to yield CM-GE-3-L as a slightly brownish powder; yield, 970 mg. Lauroyl content, 40.2%. IR ν_{max}^{Nujol} cm^{-1}: 1740 (broad: lauroyl), 1600 (broad: $-COO^-$). The preparation was soluble in H_2O and $CHCl_3$, but insoluble in MeOH. The host-mediated antitumor activity of the preparation is shown in Table IV. Injection of this preparation caused no changes in the livers of mice.

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25) S.A. Hyun, G.V. Vohouny, and C.R. Treadwell, *Anal. Chem.*, 10, 193 (1965).

26) Hydrolysis reactions with other reagents. such as 0.5 N NaOH in 95% EtOH, 3 N HCl, and 3% MeOH–HCl, were also performed with some lots of GE-3-L, and it was confirmed that differences in the hydrolysis conditions did not cause significant variations in the observed lauroyl contents of each lot.

27) Preliminary experiments indicated that 2 hr was sufficient for the completion of methanolysis.