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Chemical and Biochemical Studies on Carbohydrate Esters. VI.[1] Further Examinations on Antitumor Activities of Stearoyl Esters of Sucrose[2]

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A series of commercially available, non-ionic surfactants produced by partial acylation of sucrose with the hydrogenated beef-tallow fatty acids, that is, the eight kinds of DK-esters containing the mono-, di-, tri-, and polyesters of stearic and palmitic acids at different ratios were tested for their antitumor effect against Ehrlich ascites carcinoma by total packed cell volume (TPCV) method. The anti-leukemia activity of these DK-esters, as well as of the sucrose–monostearate preparation (SS-C), was also examined by tissue culture method, using a mouse leukemia cell line L-5178Y. Thus, the monoester constituents contained in them proved to exert inhibiting effects against both the tumors, in spite of the negative activities of the co-existing higher esters. In addition, it has been indicated that the preparation SS-C tended to exhibit antitumor effect upon the ascites sarcoma 180 implanted in mice (TPCV method and survival method), though it showed no activity against the same tumor in solid form (so-called host-mediated bioassay).

Keywords——sucrose-monoester of stearic acid; antitumor effect; Ehrlich ascites carcinoma; L-5178Y; Sarcoma 180; DK-esters

Recently, we have revealed that a variety of sucrose- or trehalose-monoester preparations derived from stearic acid and some other fatty acids possessing appropriate chain-lengths could exhibit the remarkable antitumor effect upon Ehrlich ascites carcinoma implanted in mice, when they were administered intraperitoneally.[1,4] Throughout those investigations, the monoester-specimens to be tested were prepared by the Osipow's method,[5] and their antitumor effect was evaluated with the total packed cell volume (TPCV) ratios on the 7th day after the tumor implantation. So far, however, comparison of the antitumor activities between a monoester preparation and the analogous higher ester specimens has not been reported as yet. The antitumor spectral data of this type of carbohydrate esters are also still unknown.

The present work was undertaken in an attempt to provide the preliminary information concerning tthe influence of the degree of acyl-substitution on the antitumor effect. In addition, this paper deals with the inhibitory activities of the stearoyl esters of sucrose against the tumors other than Ehrlich ascites carcinoma, such as a mouse leukemia cell line, L–5178Y, and sarcoma 180 in both ascites and solid forms.

As test-samples, we chose the sucrose-monostearate preparation (SS–C) obtained according to the same manner as described previously,[1] and the industrial products called DK-

1) Part V: Y. Nishikawa, K. Yoshimoto, M. Okabe, T. Ikekawa, N. Abiko, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **25**, 1717 (1977).
2) A part of this work was presented at the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1977.
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5) *a*) L. Osipow, F.D. Snell, W.C. York, and A. Finchler, *Ind. Eng. Chem.*, **48**, 1459 (1956); *b*) For additional references, see our previous paper.[1,4]

esters.[6] The former preparation did not contain the higher esters, but consisted solely of monoester isomers, among which the 6-ester (a monoester carrying its acyl function at the C_6-position of the glucose residue of sucrose) was suggested to be predominant. The DK-esters are produced by partial acylation of sucrose with the hydrogenated beef-tallow, fatty acids employing the modified Osipow's method (Nebraska-DKS process),[7] and they are now widely used as the non-toxic, non-ionic surface active agents, especially in the field of food-additives. At present, eight kinds of DK-esters numbered as F—160, —140, —110, —90, —70, —50, —20, and —10 are commercially available. All of them are composed of the complex mixtures of mono-, di-, tri-, and polyesters, but they differ mutually in the ratios of the monoester constituents to the higher ester components: the number of a DK-ester corresponds increasingly to the higher monoester content (Table I). In the preceding papers, we have indicated that the compositions of the disaccharide-monoesters occurring in the reaction products obtained by the Osipow's method were analysable conveniently by means of gas–liquid chromatography (GLC).[1,4] The GLC behaviors were examined by employment of the trimethylsilyl (TMS) derivatives, using OV–1 (or OV–17) as a stationary phase. Thus, the sucrose-monoester preparations having a series of fattyacyl moieties were found to show, commonly, the characteristic chromatograms each consisting of one large peak, which was tentatively assigned to the 6-ester, and several much smaller peaks presumably attributable to the co-existing minor positional isomers: the higher esters failed to give peaks, due to the insufficient volatilities of their TMS-derivatives. We now

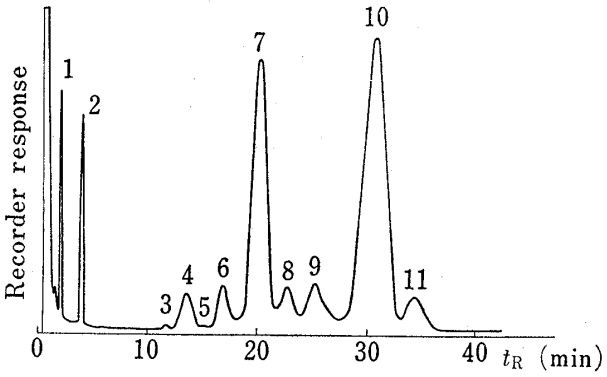

Fig. 1. Gas Chromatogram of DK-Ester F–160 (as TMS-Derivative)[a]

1, sucrose; 2, cholesterol (internal standard); 3, 4,[b] 5, monoesters of myristic acid; 6, 7,[b] 8, monoesters of palmitic acid; 9, 10,[b] 11, monoesters of stearic acid.
a) 1.5% OV-1 on Shimalite W (2m×3mm I.D.), at 295°.
b) the peak tentatively identified as that of the 6-Ester.

Table I. Analytical Data on Compositions of DK-Esters[a]

DK-ester	HLB[b]	Ester-composition[b,c] (%)		Monoester-composition (mg in 100 mg of DK-ester)			
		Mono-	Di-, Tri-, and poly-	Stearate	Palmitate	Myristate	Total
F-10	*ca.* 1	*ca.* 0	*ca.* 100	Trace	Trace	Trace	0
F-20	2	10	90	8.1	2.2	0.1	10.4
F-50	6	30	70	24.7	6.2	0.4	31.3
F-70	8	40	60	26.6	9.5	0.7	36.8
F-90	9.5	45	55	28.7	11.2	0.9	40.8
F-110	11	50	50	34.9	15.0	1.0	50.9
F-140	13	60	40	43.5	20.4	1.2	65.1
F-160	15	70	30	45.7	28.1	1.4	75.2

a) All the DK-esters contained the recovered sucrose at the the concentrations less than 10%.
b) The data were cited from ref. 7*d*.
c) The values were determined by means of preparative TLC.[11]

6) Dai-ichi Kogyo Seiyaku Co., Ltd., Kyoto, Japan.
7) *a*) T. Ishizuka, *Yukagaku*, **21**, 408 (1972); *b*) F. Yamagishi, F. Endo, H. Ooi, and Y. Kozuka, U.S. Patent 3792041 (1974); *c*) N. Mizutani, I. Sasaki, T. Ito, H. Ueno, S. Nishizaki, and T. Ishizuka, U.S. Patent 3748324 (1973); *d*) See also the monographs published by the company manufacturing the DK-esters.[6]

subjected the DK-esters to the similar GLC examinations to determine the approximate total contents and compositions of their monoester components. Cholesterol was adopted as an internal standard. All the DK-esters, with one exception of F–10 in which the mono-substituted esters were virtually absent, resulted in the complex peak-patterns qualitatively resembling to each other. The representative chromatogram is illustrated in Fig. 1. By comparing the relative retention times (rt_R) of the peaks observed with those of the authentic specimens of the previously synthesized sucrose-monoesters, it has been confirmed that the 6-stearate was contained in each DK-ester as a major monoester, followed by the 6-palmitate, together with some other positional isomers of both the monoesters: besides them, the myristoyl monoesters, as well as the recovered sucrose, were also detectable generally, albeit in small quantities. The quantitative results obtained are shown in Table I.

The antitumor effect of the DK-esters was first tested with Ehrlich ascites carcinoma by the TPCV method, and the results are listed in Table II. As has already been reported, the sucrose-monoester preparations derived from stearic and palmitic acids proved to exhibit the marked antitumor activity under these bioassay conditions.[1] Therefore, it was not surprising that, among eight DK-esters employed, the five specimens, from F–160 to F–70, which contained the monoesters of both the fatty acids at higher concentrations, were found to be remarkably effective against the tumor. On the other hand, F–50 and F–20 whose monoester contents were poorer than those of the formers tended to exert somewhat inferior antitumor effect, and F–10 which consisted exclusively of the higher esters was indicated to be nearly ineffective. From these findings, it has been suggested that the degree of acyl-substitution would give significant influence upon the antitumor effect of this type carbohydrate esters: the mono-substituted esters appeared to be more favorable than the corresponding higher esters. Accordingly, it was also presumable that the value of hydrophile-lipophile-balance (HLB) of a test sample might have some correlation with its antitumor intensity. (*cf.* Table I).

Using the tissue culture method, we next examined the anti-leukemia activity of the preparation SS–C, stearic acid, methyl stearate, and the DK-esters against L–5178Y. As

Table II. Antitumor Effect of DK-Esters against Ehrlich Ascites Carcinoma (TPCV Method)[a]

Exptl. No.	DK-Ester	TPCV ratio (% *T/C*)	Evaluation of activity[b]	Body wt. change (g)	Deaths/Total
1	F-10	56	+	−0.5	0/6
	F-20	25	++	−1.0	0/6
	F-50	14	++	−2.8	2/6
	F-70	3	+++	−2.9	0/6
	F-90	1	+++	−2.9	0/6
	F-110	5	+++	−0.9	0/6
	F-140	3	+++	−2.3	1/6
	Control[c]			−0.6	0/6
2[d]	F-160	0	+++	−8.6	1/6
	Control[c]			+3.1	0/6
cf.[e]	Prepn. SS–C[f]	2	+++	−2.4	0/6
	Control[c]			+2.0	0/5

a) Unless otherwise stated, each agent was administered into female ddY mice at the dose of 250 mg/kg/day × 5 days.

b) Criteria employed were as follows:

Criterion	+++	++	+	−
% *T/C*	0—10	11—40	41—65	66—100

c) N-saline.

d) Female ICR mice were used.

e) The data were cited from our previous paper.[1]

f) With the dose of 50 mg/kg/day × 5 days, the preparation has been reported to give the TPCV ratios around 0—30%.[1]

can be seen in Table III, the preparation SS–C inhibited the tumor-growth completely, when it was administered at relatively higher concentrations; treatment with less doses was, however, nearly ineffective. In contrast, stearic acid and the methyl ester were both revealed to possess negative activity. Similar relationship among the three specimens has been observed previously in their screeinig tests upon Ehrlich ascites carcinoma.[4a] The anti-leukemia effect of the DK-esters is shown in Table IV. The five DK-esters from F–160 to F–70, which, as described above, proved to be effective against the Ehrlich ascites tumor, exhibited the anti-leukemia activity at the dose of 500 γ/ml, though they were ineffective at the concentration of 100 γ/ml. The rest of the DK-esters, that is, F—50, —20, and —10, whose anti-Ehrlich tumor effect was demonstrated to be inferiour to that of the formers, did not manifest the activity against the leukemia cells at both the dosages. From these results, it was considered that the anti-leukemia activity should be attributable to the monoester constituents contained in the DK-esters, but not to the co-existing higher esters.

Table III. Antitumor Effect of Sucrose-monostearate Preparation (SS–C) and Related Compounds against L–5178Y (Tissue Culture Method)

Sample	Cell number ratio (% T/C) Concentration of agent (γ/ml)			
	500	100	10	1
Prepn. SS–C (lot. 1)[a]	0	0	65	76
Prepn. SS–C (lot. 2)[a]	0	0	64	76
Stearic acid	102	113	—[b]	—
Methyl stearate	104	112	—	—

a) Lots. 1 and 2 differentiate the products obtained in the two experiments which were performed independently under the similar conditions.
b) —: undetermined.

Table IV. Antitumor Effect of DK-Esters against L-5178Y (Tissue Culture Method)

DK-Ester	Cell number ratio (% T/C) Concentration of agent (γ/ml)	
	500	100
F-10	121	124
F-20	102	113
F-50	85	117
F-70	4	115
F-90	14	110
F-110	0	86
F-140	0	81
F-160	0	86

Table V. Antitumor Effect of Sucrose-monostearate Preparation (SS–C) against Sarcoma 180 in Ascites Form (TPCV Method)

Dose (mg/kg/day × days)	TPCV ratio (% T/C)	Evaluation of activity[a]	Body wt. change (g)	Deaths/Total
50×5	44	+	+1.5	0/6
50×5	58	+	+1.5	0/6
Control[b]			+1.8	0/6

a) For criterion, see foot-note b) in Table II.
b) N-saline.

Finally, the antitumor activities of the preparation SS–C upon sarcoma 180 in either ascites or solid form were evaluated by three different methods. The agent showed a tendency to exhibit some effect, when assayed by TPCV method at the doses of 50 and 25 mg/kg/day×5 days (Table V).[8] Survival test up to 30 days was also carried out with the ascites tumor, using the conditions as described in Experimental. All the mice receiving the sample at the dose of 2 mg/mouse survived well for the whole period: treatment with 100 γ/mouse of the specimen resulted in slight prolongation of the life-span, too (Fig. 2). The ILS (increase in life-span) values were calculated to be over 74.4% and 17.4%, respectively. Effect against the solid tumor was estimated by employment of the so-called "host-mediated bioassay," which was originally developed for screening the antitumor polysaccharides.[9] As shown in Table VI, the preparation SS–C was observed to be completely ineffective against the solid sarcoma 180 in contrast with its activity upon the ascites of the same tumor. The result may suggest that the monoester preparation is devoid of the indirect, host-mediated antitumor effect. At present, we are not able to discuss the exact mechanism involved in the antitumor activities of this type of disaccharide-monoesters. However, it is conceivable that these agents would act directly on the tumor cell membrane, and subsequently alter or destroy it.[10]

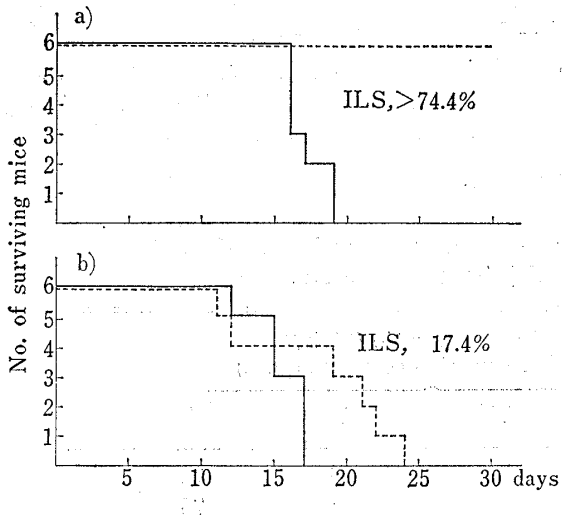

Fig. 2. Antitumor Effect of Sucrose-monostearate Preparation (SS–C) against Sarcoma 180 in Ascites Form (Survival Method)

——, treated group; ——, control.
a) Dose: 2 mg/mouse.
b) Dose: 100 γ/mouse.

Table VI. Antitumor Effect of Sucrose-monostearate Preparation (SS–C) against Sarcoma 180 in Solid Form (Host-mediated Bioassay)

Dose (mg/kg/day×days)	Inhibition ratio(%)[a]	Evaluation of activity	Body wt. change (g)	Deaths/Total
150×10	0	—	+3.7	0/5
150×10	−2.4	—	+6.0	0/5
50×10	6	—	+4.3	0/6
50×10	−6.0	—	+3.8	0/6
Control[b]			+4.1	0/6

a) Complete regression of the tumor took place in none of the treated mice.
b) Distilled water.

On the basis of the present investigations, it has been revealed with almost certainty that the products obtained by the Osipow's method starting from the appropriate fatty acids are effective not only against the Ehrlich ascites caricnoma, but also upon a leukemia

8) Examination by use of the higher doses was not attainable in the present study, owing to the shortage of the material. Recently, however, the specimen has been indicated to exert significant activity against ascites sarcoma 180 at the dose of 100 mg/kg/day×5 days (private communication from Dr. K. Kuretani).
9) 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, San Francisco, and London, 1976, p. 235.
10) A. Kato, K. Ando, G. Tamura, and K. Arima, *Cancer Res.*, 31, 501 (1971).

cell line (L-5178Y) and sarcoma 180 in the ascites form, and that their antitumor activities are attributable to the monoester constituents contained in them, but not to the co-existing higher esters. In order to provide the additional antitumor spectral data and to establish the more detailed structure-activity relationships, further studies are now under way.

Experimental

Assay Method Used for Ehrlich Ascites Carcinoma (TPCV Method)——The bioassay conditions employed were similar to those described previously.[1,4] Unless otherwise stated, female ddY strain mice weighing 22 ± 2 g were used, and 0.05 ml of seven-day-old Ehrlich ascites carcinoma containing about 7×10^6 cells was inoculated intraperitoneally. The agent to be tested was dissolved or suspended in N-saline, and injected intraperitoneally once daily for five consecutive days, starting 24 hr after the tumor implantation, at the standard dosage of 250 mg/kg/day $\times$ 5 days. The antitumor activity of the agent was evaluated with TPCV ratio (% T/C) on the 7th day after the tumor implantation. The results obtained by the method are shown in Table II.

Assay Method Used for L-5178Y (Tissue Culture Method)——The leukemia cells were cultured in a tube with stopper, using RPMI-1640 medium supplemented with 10% calf serum at 37°. Antitumor activity was determined by the ratio of cell number in treated and control groups (% T/C) after 48 hr incubation of *ca.* 2.0×10^5 cells/ml at various concentrations of the test agent (500, 100, 10, and 1 γ/ml). The results determined by this method are presented in Tables III and IV.

Assay Method Used for Ascites Sarcoma 180 (TPCV Method)——The general experimental conditions were same as employed for Ehrlich ascites carcinoma (see above). Daily doses used were 50 and 25 mg/kg/day. Table V lists the results assayed by this method.

Assay Method Used for Ascites Sarcoma 180 (Survival Method)——A test sample (2 mg or 100 γ/mouse) dissolved in N-saline was mixed with 0.05 ml of ascites containing the tumor cells (*ca.* 7×10^6 cells). Within 5 min, the resulting mixture was injected intraperitoneally into ddY mice, and the life-spans of the treated group up to 30 days were compared with those of the control mice. For each group, six mice were used. The results given by this method are depicted in Fig. 2.

Assay Method Used for Solid Sarcoma 180 (So-called "Host-mediated Bioassay")——Seven-day-old sarcoma 180 ascites (0.05 ml; *ca.* 8×10^6 cells) were transplanted subcutaneously into the right groins of female ICR mice. The test agent dissolved in distilled water was injected intraperitoneally with 150 or 50 mg/kg/day doses for ten consecutive days, starting 24 hr after the tumor implantation. After observing the tumor growth for 4 weeks, the tumor weights of treated mice were compared with those of untreated mice. Inhibition ratio was calculated by the following equation: inhibition ratio (%) $=((C-T)/C)\times100$; where C is the average tumor weight of the control group, and T is that of the treated group. The results observed by this method are exhibited in Table VI.

Test Samples——Stearic acid and methyl stearate were purchased from the commercial source (Tokyo Kasei Kogyo Co., Ltd.). The DK-esters employed were the generous gifts from the company manufacturing them.[6] All the chemicals mentioned above were used for the antitumor bioassays without further purification. The sucrose-monostearate preparation (SS-C) was obtained according to the same manner as described in our previous paper, and confirmed to consist solely of monoester isomers by means of GLC and TLC (thin-layer chromatography).

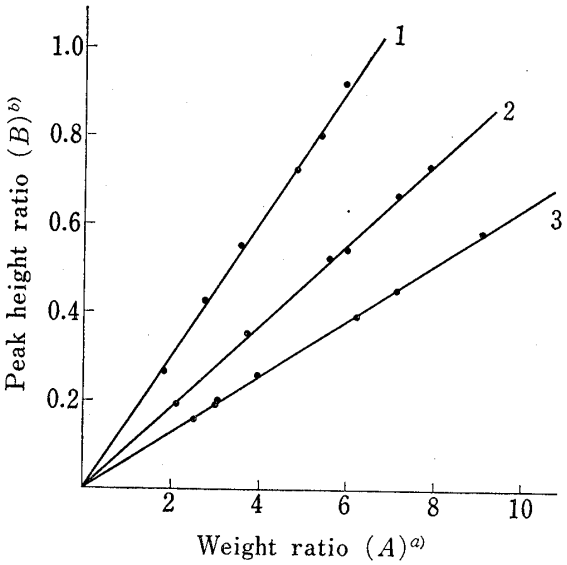

Fig. 3. Calibration Curves

Curve No.	Preparation	Recorder response factor ($=A/B$)
1	Sucrose-monomyristate	6.56
2	Sucrose-monopalmitate	10.83
3	Sucrose-monostearate	16.22

a) $A=\dfrac{\text{weight of monoester-preparation}}{\text{weight of internal standard}}$

b) $B=\dfrac{\text{paek height of the 6-ester}}{\text{peak height of internal standard}}$

GLC Analysis of DK-Esters——Each DK-ester was weighed exactly, and added with a known amount of cholesterol as an internal standard. The mixture was converted into the TMS-derivative in the usual way, and then applied to the Shimadzu Gas Chromatograph GC-4CMPF equipped with a hydrogen flame ionization detector. The operating conditions employed were as follows: 1.5% OV-1 on Shimalite W (80—100

mesh); glass column, 2.0 m×3 mm I.D.; column temp., 295°; carrier gas, N_2 (80 ml/min). The respective DK-esters gave the chromatograms qualitatively identical each other, with one exception of F-10, which, due to the virtual absence of the monoester components, showed no detectable peaks. The representative chromatogram is illustrated in Fig. 1. Presence of the monoesters of stearic, palmitic, and myristic acids in the DK-esters was revealed by comparison of the rt_R values of the peaks observed with those of the corresponding authentic specimens previously prepared. The peak of the recovered sucrose was also detected commonly in all the chromatograms. The rt_R values of the peaks tentatively identified as 6-monoesters and that of the sucrose-peak were as follows: stearate, 7.75; palmitate, 5.10; myristate, 3.40; sucrose, 0.45; cholesterol, 1.00 (4.0 min). To determine the recorder response factors, the calibration curves for the 6-esters were prepared, using the authentic monoester-preparations of stearic, palmitic, and myristic acids (Fig. 3). Taking the recorder response factors obtained into account, the quantitative monoester-composition of each DK-ester was estimated by comparison of the peak-heights of the 6-esters with that of the internal standard. The resulting values are listed in Table I. The monoester-contents found were compatible, though not strictly, with those anticipated from the fatty acid composition of the hydrogenated tallow (stearic acid, 66%; palmitic acid, 32%; myristic acid, 2%).[11]

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11) T. Ishizuka and S. Nakamura, *Eiyo To Syokuryo*, **27**, 71 (1974).