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Terpenoids. L.[1] Antitumor Activity of Diterpenoids from *Rabdosia shikokiana* var. *occidentalis*

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In vitro antitumor activity of diterpenoids isolated from *Rabdosia shikokiana* var. *occidentalis* was determined against HeLa cells. Some of these compounds possess considerable activity against Ehrlich ascites carcinoma inoculated into mice. In particular, the epoxyketone **7** derived from shikoccin (**1**) showed potent activity. The *in vivo* activity of shikoccin (**1**) against six tumor lines was evaluated.

Keywords——antitumor activity; diterpenoid; shikoccin; shikoccidin; Ehrlich ascites carcinoma; *Rabdosia shikokiana*

Roughly a hundred diterpenoids have been isolated from the genus *Rabdosia* (Labiatae). Some of these diterpenoids have been claimed to possess marked cytotoxicity *in vitro* against HeLa cells,[2] KB cells,[3-6] mammary cancer FM 3A/B cells,[7,8] and Ehrlich carcinoma cells.[9] *In vivo* activity of these diterpenoids against Ehrlich ascites carcinoma,[10,11] Walker intramuscular carcinosarcoma,[5] and P388 lymphocytic leukemia,[12,13] has been reported. Very recently, potent *in vivo* activity has been reported for trichorabdals A-C and related compounds against Ehrlich ascites carcinoma inoculated into mice, and the enhanced antitumor activity of these compounds has been attributed to the intramolecular synergism of plural active sites in the molecule.[14] Preliminary results of a clinical trial with oridonin and ponicidin were reported recently.[15] Here we report the *in vitro* and *in vivo* antitumor activity of diterpenoids isolated from *Rabdosia shikokiana* (MAKINO) HARA var. *occidentalis* (MURATA) HARA and related compounds.

Experimental

Materials——The natural products, shikoccin (**1**), O-methylshikoccin (**2**), epoxyshikoccin (**3**), O-methylepoxyshikoccin (**4**), shikoccidin (**5**), leukamenin E (**6**) have already been described.[16] The epoxyketone **7** was obtained from shikoccin (**1**) as described in the preceding paper.[1]

Assay of *in Vitro* Activity Using HeLa Cells——HeLa cells were cultured as monolayers in Eagle's minimal essential medium (Flow Co., U.S.A.) supplemented with 10% calf serum (Flow Co., U.S.A.) at 37 °C in a CO_2-incubator. HeLa cells in the stage of logarithmic growth were recovered by trypsinization, washed and suspended in multi-dish culture trays (Nunc Co., U.S.A.) at 3×10^3 cells/ml on day 0. Compounds were dissolved with dimethyl sulfoxide (DMSO) and added to each well to give final concentrations of 10, 1.0, 0.1, and 0.01 μg/ml on day 1. The amount of DMSO was adjusted to give a final concentration of 0.1% in all cases, including the control cultures. After cultivation for 7 d under the conditions described above, HeLa cells were recovered by trypsinization and the number of cells was counted. In every experiment, the number of cells in the control under these conditions was usually roughly a hundred times as many as that on day 1. The concentrations of drug required to inhibit 50% of

TABLE I. Experimental Conditions for Testing Antitumor Activity of Shikoccin (1) against Various Tumors *in Vivo*

Tumor	L1210 lymphoid leukemia	Colon 38	B16 melano-carcinoma	Lewis lung carcinoma	$CD8F_1$ mammary tumor	CX-1 Colon xenograft
Host (Strain)	$CD_2F_1(CDF_1)$	$B_6D_2F(BFD_1)$	$B_6C_3F_1$	$B_6C_3F_1$	$CD8F_1$	NU/NU Swiss (athymic)
Inoculum	Scitic fluid	Fragment, tumor	Homogenate (or brei) tumor	Homogenate (or brei) tumor	Homobenate (or brei) tumor	Fragment, tumor
Level	10^5 cells	—	Dilution 1—10	10^5 cells	Dilution 1—20	Tumor diameter 9 and 120 OMU
Site[a]	*i.p.*	*s.c.*	*i.p.*	*i.v.*	*s.c.*	*i.r.* or *s.c.r.*
Dose (mg/kg/d)	3.75, 7.5, 15, 30, 60, 120	7.5, 15, 30, 60, 120, 240	1.88, 3.75, 7.5, 15, 30, 60, 120	3.75, 7.5, 15, 30, 60, 120	35.5, 75, 150, 300, 600	30, 60, 120, 240
Route	*i.p.*	*i.p.*	*i.p.*	*i.p.*	*i.p.*	*s.c.*
Frequency of administration	Once a day for 9 d	Twice a week	Once a day for 9 d	Once a day for 9 d	Once a day	Once a day for 4 d
Test animal No./dose	10	10	10	10	10	6
Day of evaluation	30	20	60	60	36	15
Method of evaluation[b]	B	C	A	A	D	E

a) *i.p.*, intraperitoneal; *s.c.*, subcutaneous; *i.v.*, intravenous; *i.r.*, internal inoculation; *s.r.c.*, subrenal capsule. *b*) A, median survival time (treated)/median survival time (control) × 100; B, mean survival time (treated)/mean survial time (control) × 100; C, mean treated tumor weight (diameter)/mean control tumor weight (diameter) × 100; D, group median final tumor weight/group median initial tumor weight × 100; E, group mean final tumor weight/group mean initial weight × 100.

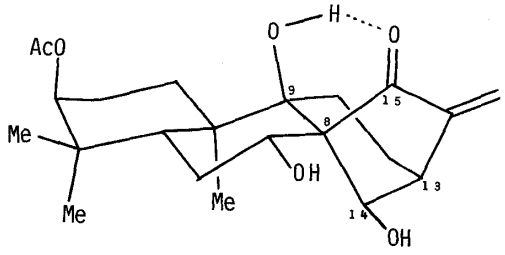
cellular growth (IC_{50}, μg/ml) were obtained by comparing the mean number of cells of three treated groups with that of the control groups.

Test for Antitumor Activity against the Ascites Form of Ehrlich Carcinoma——Groups of 7 5-week-old male ddY mice with a body weight of 25—29 g were inoculated intraperitoneally (*i.p.*) with 2×10^6 Ehrlich carcinoma cells on day 0. A given amount of test compound was administered *i.p.* once a day from day 1 to day 7 at a volume of 0.1 ml per 10 g mouse body weight. Antitumor effects were evaluated by comparison of the mean survival time (d) with that of the control groups.

Test for Antitumor Activity of Shikoccin (1)[17]——The tumors were inoculated into mice and shikoccin (**1**) dissolved or suspended in hydroxypropyl cellulose was injected intraperitoneally or subcutaneously into the mice 24 h after inoculation. The conditions are summarized in Table I.

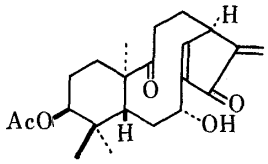
Results and Discussion

The *in vitro* activity of the test compounds against HeLa cells was compared with that of oridonin (**8**), whose *in vivo* activity against Ehrlich ascites carcinoma has been confirmed.[10,11] The results are given in Table II. All the compounds tested showed stronger activity than oridonin (**8**). Shikoccin (**1**), shikoccidin (**5**), and epoxyketone **7** all possess a potent *in vitro*

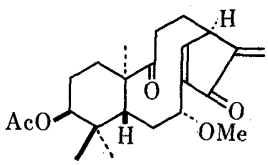


shikoccidin

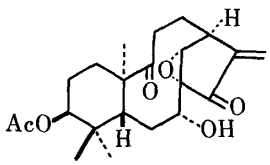
Fig. 1



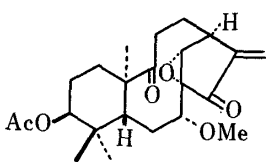
shikoccin (1)



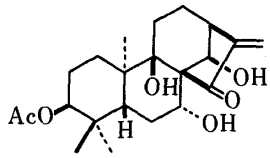
O-methylshikoccin (2)



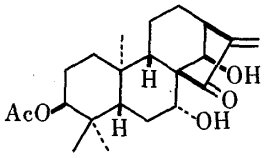
exoxyshikoccin (3)



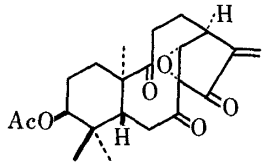
O-methylepoxyshikoccin (4)



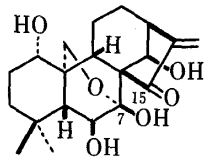
shikoccidin (5)



leukamenin E (6)



7



oridonin (8)

TABLE II. Growth-Inhibiting Effect on HeLa Cells in Culture

Compound	1	2	3	4	5	6	7	8
IC_{50} (μg/ml)	0.08	0.30	0.25	0.13	0.10	0.21	0.10	0.50

TABLE III. Effect on Ehrlich Ascites Carcinoma in Mice (*i.p.-i.p.*)

Compound	Dose (mg/kg/d)	Body wt. change (g, day 8 − day 0)	Survival time (d, mean ± SD)	T/C (%)[a]
Control	—	+2.6	10.00 ± 1.94	—
1	20	+0.9	12.29 ± 3.15	123
	10	+1.3	13.29 ± 2.43[b]	133
	5	+2.0	13.00 ± 2.71[c]	130
2	20	+1.3	10.29 ± 2.56	103
	10	+1.7	10.71 ± 2.43	107
	5	+2.0	10.57 ± 1.90	106
3	20	−2.1	15.71 ± 4.15[b]	157
	10	+0.9	15.00 ± 3.21[b]	150
	5	+2.0	12.14 ± 1.86[c]	121
4	20	−0.4	13.00 ± 2.58[c]	130
	10	+2.3	12.00 ± 2.00	120
	5	+2.4	11.14 ± 2.41	111
5	20	+0.7	13.86 ± 2.19[b]	139
	10	+1.0	13.57 ± 2.82[b]	136
	5	+2.6	12.57 ± 2.07[c]	126
6	20	+0.3	17.86 ± 3.63[d]	179
	10	+1.4	13.00 ± 1.53[b]	130
	5	+2.9	12.00 ± 2.00	120
7	20	—	4.00 ± 0.58	Toxic
	10	−2.7	> 23.57 ± 4.83[d]	> 236[e]
	5	−0.9	19.14 ± 4.30[d]	191

a) T, mean survival days for treated mice; C, mean survival days for untreated mice.
b) $p < 0.01$. *c*) $p < 0.05$. *d*) $p < 0.001$. *e*) Two out of seven mice survived more than 30 d.

activity.

The results of *in vivo* activity against Ehrlich ascites carcinoma inoculated into mice are listed in Table III. A Michael acceptor such as the α,β-unsaturated carbonyl group has been

TABLE IV. Antitumor Activity of Shikoccin (1) against Various Tumors in Mice

Tumor	Sex	Dose (mg/kg/d)	Evaluation T/C (%)	Method of evaluation[a)]
L-1210 lymphocytic leukemia	F	120	89	B
		60	96	
		30	101	
		15	98	
		7.5	100	
		3.75	97	
	M	120	Toxic	B
		60	95	
		30	98	
		15	95	
		7.5	98	
		3.75	97	
B16 melanocarcinoma	M	120	Toxic	A
		60	Toxic	
		30	118	
		15	100	
		7.5	89	
		3.75	106	
	F	60	—	A
		30	104	
		15	114	
		7.5	111	
		3.75	104	
		1.88	96	
Colon 38	M	240	Toxic	C
		120	Toxic	
		60	81	
		30	89	
		15	111	
		7.5	90	
Lewis lung carcinoma	F	120	Toxic	A
		60	93	
		30	104	
		15	100	
		7.5	100	
		3.75	105	
	M	120	Toxic	A
		60	93	
		30	104	
		15	100	
		7.5	100	
		3.75	105	
$CD8F_1$ mammary tumor	F	600	Toxic	D
		300	Toxic	
		150	30	
		75	88	
		35.5	83	
CX-1 Colon xenograft	M	240	Toxic	E
		120	116	
		60	91	
		30	139	

a) See Table I.

claimed to be an important moiety to exert biological activities, including antitumor activity, by reacting with biologically important sulfhydryl groups.[18] The α-methylene cyclopentanone system in *Rabdosia* diterpenoids has been suggested to be responsible for the antitumor activity of these compounds. The enhanced activity of oridonin (**8**) against Ehrlich ascites carcinoma as compared with other *Rabdosia* diterpenoids may be ascribed to hydrogen bonding between the hydroxyl group at C(7) and the C(15)-carbonyl group, so that the electrophilicity at C(17) is increased. An X-ray crystallographic analysis of shikoccidin (**5**)[19] revealed the existence of the same type of hydrogen bonding between the hydroxyl group at C(9) and the carbonyl oxygen at C(15), as shown in Fig. 1. On the basis of the reasoning applied to oridonin (**8**), shikoccidin (**5**) was expected to show the antitumor activity higher than that of leukamenin E (**6**), which has a similar structure but no hydrogen bonding. Contary to our expectation, however, the *in vivo* activity of shikoccidin (**5**) was on the same level as that of leukamenin E (**6**) at doses of 5 mg/kg and 10 mg/kg, or rather lower at a dose of 20 mg/kg indicating non-importance of the particular hydrogen bonding in this case. Methylation of the hydroxyl group at C(7) seems to lower the activity (compare **1** and **3** with **2** and **4**, respectively), whereas introduction of the epoxide in the cyclopentenone system increases the activity (compare **1** and **2** with **3** and **4**, respectively).

The high level of activity observed with the epoxyketone **7** could be attributed to synergism[14] of the two active sites, *i.e.*, the *exo*-methyleneketone and α,β-epoxyketone moieties, in the same molecule.

The *in vivo* activity of shikoccin (**1**), the major diterpenoid from *R. shikokiana*, against six tumor lines was tested and the results are summarized in Table IV. Moderate activity was observed towards $CD8F_1$ mammary tumor at doses of 35.5 mg/kg and 75 mg/kg, at a dose of 150 mg/kg, the tumor weight of the treated mice was decreased to one third of that of the untreated groups.

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