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Antitumor Activity of Shikonin and Its Derivatives

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Shikonin, a naphthoquinone contained in the root of *Lithospermum officinalis* var. *erythrorhizon* Max. and *Macrotomia euchroma* (Royle) Pauls (Boraginaceae), showed a high antitumor activity against the ascites cells of Sarcoma 180. Shikonin completely inhibited the tumor growth at a dose of 5—10(mg/kg/day). At a higher dose (>15 mg/kg/day) shikonin showed toxicity, whereas at a lower dose (1 mg/kg/day) it was inactive.

Four shikonin derivatives and the chromatographic fractions of the plant extracts were also tested and they showed similar antitumor activities against S-180.

Keywords——*Macrotomia euchroma*; *Lithospermum officinalis*; Boraginaceae; shikonin; naphthoquinone; antitumor activity; Sarcoma-180

In the course of our screening tests to find antitumor active natural products, shikonin (**1**), a naphthoquinone contained in a crude drug, "Shikon (紫根)", showed a remarkably high antitumor activity (卅) against the ascites cells of Sarcoma 180 using ICR mice. In Japan "Shikon" has been mainly used as a material to prepare an ointment called "Shiunko (紫雲膏)",[2] which is frequently used for the treatment of wounds and burns. The crude drug "Shikon" is initially the root of *Lithospermum officinalis* var. *erythrorhizon* Max. (Boraginaceae) which was widely distributed in Japan, but now it becomes very difficult to find in wild. Although it is supplied partly by the cultivation of this plant, almost all that available in the market is the root of *Macrotomia euchroma* (Royle) Pauls, "Nanshikon (軟紫根)", imported from China.[3] Concerning to the physiological activities of shikonin, bacteriocidal action and an effect to promote the growth of granulation tissue have been reported.[2,4] Following to the finding of antitumor activities of shikonin, several derivatives were prepared for the test. This paper deals with the antitumor activity of shikonin (**1**), its derivatives and the chromatographic fractions of benzene extracts of "Shikon".

Shikonin (**1**) was isolated from "Nanshikon" by the hydrolysis of the benzene extracts and was purified by column chromatography. When shikonin (**1**) was catalytically hydrogenated under the condition described by Brockmann,[5] the reaction mixture gave several spots on thin-layer chromatography (TLC) along with the spot of alkannane (**2**). Upon chromatography, alkannane (**2**) and a compound giving strong blue fluorescence were obtained from the reaction mixture. Spectral investigation clarified that the latter compound should be represented by the structure **3**. In the case of juglone (**4**), dihydro-compound named β-juglone (**5**) was reported to be formed by hydrogenation using a homogeneous catalyst.[6]

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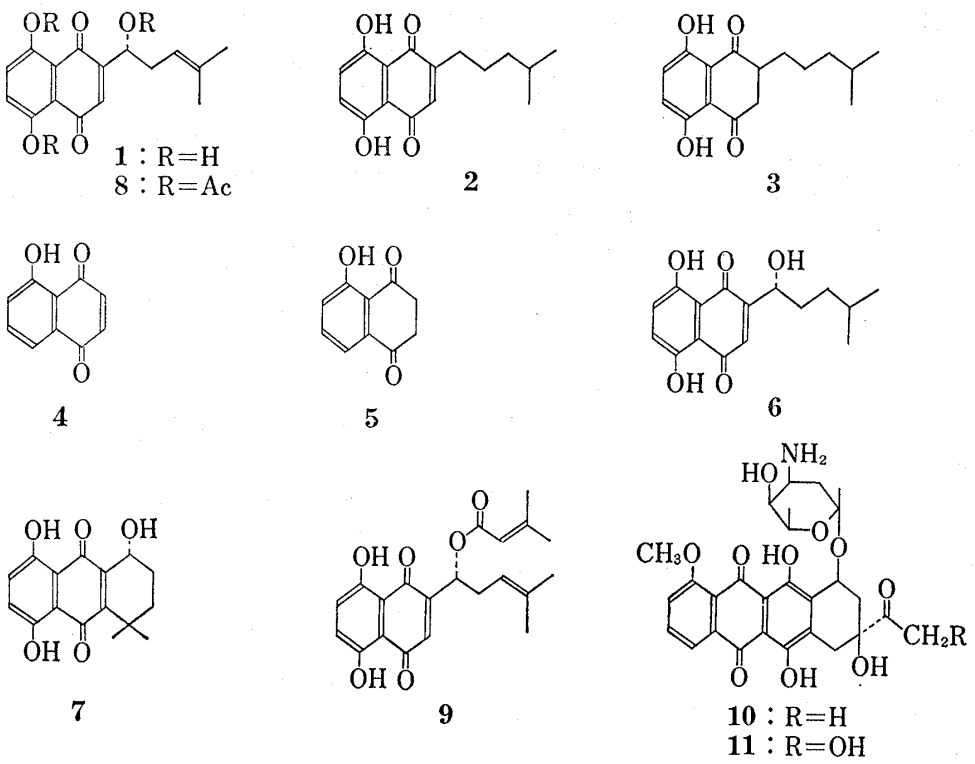

Chart 1

Since these diketo compounds (**3** and **5**) can be obtained in stable crystalline forms, the diketo forms of dihydronaphthoquinone are more stable than hydroquinone forms. Dihydroshikonin (**6**), which had not been reported previously, was prepared by the catalytic reduction

Table I. Antitumor Activity of Shikonin (**1**) and Its Derivatives

Compound	Dose mg/kg/day	Antitumor activity	Growth ratio[a] (T/C, %)	Mortality[a] (died/used)
Shikonin (**1**)	30	Toxic		6/6
	15	###	0	5/6
	10	###	2	0/6
	10	###	0	0/6
	10	###	3	0/6
	5	###	0	0/6
	1	—	130	0/6
Alkannane (**2**)	30	Toxic		6/6
	10	###	2	0/6
Dihydroshikonin (**6**)	10	###	0	4/6
	5	###	1	0/6
	1	—	89	1/6
Cycloshikonin (**7**)	10	###	0	0/6
	10	###	2	0/6
	3	—	113	1/6
	1	—	103	0/6
Shikonin triacetate (**8**)	15	###	3	0/6
	8	###	3	0/6
	2	##	39	1/6
Fr-4	10	##	30	0/6
Fr-3	10	+	51	2/6
Fr-2	10	###	3	0/6
Fr-1	10	##	39	1/6

a) Growth ratios and mortarities were determined at 7th day.

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of shikonin (**1**) in ethyl acetate using platinum dioxide as a catalyst. The reaction condition for preparing dihydroshikonin (**6**) is very strict. The reduction in polar solvents or the use of palladium carbon resulted in the formation of a complex mixture of unidentified compounds along with dihydroshikonin (**6**) and alkannane (**2**). Cycloshikonin (**7**) and shikonin triacetate (**8**) were prepared by the method reported by Brockmann[5] and gave satisfactory ^{1}H-nuclear magnetic resonance (NMR) spectra. Antitumor activities data of shikonin (**1**) and its derivatives are shown in Table I. Shikonin (**1**) showed complete tumor growth inhibition at a dose of 5—10 mg/kg/day against ascites cells of Sarcoma-180. At a higher dose (>15 mg/kg/day) toxicity accompanied by the death of experimental animals was observed and four out of six mice died at a dose of 15 mg/kg/day. At a higher dose of 30 mg/kg/day all the animals died within 2 days from toxicity, whereas at a lower dose (1 mg/kg/day) shikonin was inactive. The life span prolongation effect of shikonin on mice bearing S–180 by repeated injections is shown in Table II. Shikonin exhibited 92.5% prolongation effect at a dose of 10 mg/kg/day compared with control. Dihydroshikonin (**6**) and alkannane (**2**) exhibited an activity as high as shikonin (**1**) at a dose of 10 mg/kg/day. Dihydroshikonin (**6**), however, showed a considerable toxicity at this dose as manifested by the death (4/6) of animals. Cycloshikonin (**7**) and shikonin triacetate (**8**) showed effects not markedly different from other compounds. Shikonin triacetate (**8**) seems to be less toxic than shikonin (**1**) as far as the present data concerns and further test at a higher dose would be required to estimate the toxicity. Of the chromatographic fraction tested, Fr. 4 showed the highest activity. ^{1}H-NMR spectrum revealed that the main constituent of Fr. 4 is β,β-dimethylacryl shikonin (**9**). In the experiments using different tumor system, shikonin was inactive against L–1210 (Table III) and the solid tumor of Sarcoma-180 (Table IV). Recently, the survey of the antitumor activity of 1500 quinone derivatives has been reported by the group of National Cancer Institute (NCI),

Table II. Life Span Prolongation Effect of Shikonin

Sample	Dose (mg/kg/day)	Survival (days)	LSI[a] (%)
Control	—	10.7	—
Shikonin	10×5	20.6	92.5
Endoxane[b]	50×5	>21.5	>100.9

a) Life span increase.
b) Positive control.

Table III. Antitumor Activity against L-1210

Sample	Dose (mg/kg/day)	ILS[a] (%)	Remark
Shikonin (1)	10×5	−31	Toxic
Shikonin (1)	5×5	3	—

a) Increase of life span.

Table IV. Antitumor Activity against Solid Tumor of S–180

Sample	Dose (mg/kg/day)	Inhibition ratio (%)	Death	Complete regerssion	Remark
Shikonin	5×5	6.7[a]	0/6	0/6	—
Shikonin	5×10	15.2[b]	1/9	1/10	±

a) Inhibition ratio was determined at 10th day.
b) Inhibition ratio was determined at 30th day.

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where alkannin, an enantiomer of shikonin (**1**), was recorded to be active against Walker carcinoma 256 and sarcoma 180, but inactive against L-1210. Three fatty acid esters of shikonin have also been tested for cytotoxicity against KB cells in culture and are listed in the compounds that should be tested *in vivo*. The data reported by NCI group are well in accord with those obtained in our experiments. Although marked improvement in the activity has not been attained, the structural similarity of shikonin to daunomycin (**10**) and adriamycin (**11**) suggests that there is a possibility to prepare more active compounds by a further functionalization.

Experimental

TLC was carried out with 1/2 N oxalic acid impregnated silica gel plate using benzene: EtoAc (9: 1) as a solvent. Silica gel used for column chromatography was washed with 1/2 N oxalic acid and reactivated.

Isolation of Shikonin (1)——Shikonin (**1**) was isolated from "Nanshikon" according to the procedure described by Brockmann up to the point to obtain crude shikonin,[5] which was further purified by column chromatography to give shikonin (**1**) mp 139—141° (lit.[6] 146—147°).

Alkannane (2) and 8-Hydroxy-2-isohexyl-4-oxo-tetralone (3) from Shikonin (1)——1 (1 g) in AcOH (100 ml) was catalytically hydrogenated over PtO_2 as a catalyst. The reaction was stopped when 3 mol H_2 had been absorbed. The product were chromatographed over silica gel. The first violet band eluted with *n*-hexane–benzene (1: 1) to give alkannan (280 mg), mp 92—94° (lit.[5] mp 91—92°). NMR ($CDCl_3$): δ 0.89 (6H, d, $J=6$, Me×2), 1.1—1.7 (5H, m, CH, CH_2×2), 2.52 (2H, t, $J=7$, CH_2), 6.69 (1H, br. s, arom. H), 7.03 (2H, s, arom. H×2), 12.12, 12.26 (1H×2, s×2, OH×2).[7] The compound (**3**) showing a strong blue fluorescence was eluted with benzene and chromatographed again on silica gel to give **3** (32 mg) as green leaflets, mp 95°. UV λ_{max}^{EtOH} nm (ε): 258 (12200), 261 (12300), 263 (12300), 270 (8000), 396 (7600), 416 (5750). NMR ($CDCl_3$) δ 0.93 (6H, d, $J=6$, Me×2), 1.02—2.10 (7H, m, CH_2, CH), 2.85—3.25 (3H, m, CH_2, CH), 7.30 (2H, s, arom. H), 11.84, 11.94 (1H×2, s×2, OH×2). MS *m/e*: 276 (M^+), 261, 258, 243. *Anal.* Calcd. for $C_{16}H_{20}O_4$: C, 69.54; H, 7.48; Found: C, 69.68; H, 7.48.

Dihydroshikonin (6)——1 (1.0 g) in EtOAc (100 ml) was shaken with PtO_2 in the atmosphere of H_2 until 2.3 mol H_2 was absorbed. Reaction products were chromatographed over silica gel. Dihydroshikonin was eluted with benzene and recrystallized from benzene to give dark purple powder (685 mg) mp 100—107°. High resolution MS: Calcd. for $C_{16}H_{18}O_5$; *m/e* 290.1070; Found *m/e* 290.1111. NMR ($CDCl_3$); 0.90 (6H, d, $J=5$, Me×2), 1.02—1.95 (5H, m, CH_2, CH), 4.85 (1H, t, $J=5$, C<u>H</u>–OH), 7.10 (1H, s, arom. H), 7.15 (2H, s, arom. H), 12.40, 12.55 (1H×2, s×2, OH×2).

Cycloshikonin (7)[5]——Anhydrous $SnCl_4$ (0.5 ml) was added dropwise to a solution of 1 (250 mg) in dry benzene (250 ml) under stirring. Stirring was continued further 30 min, and the reaction mixture was washed with dil. AcOH to remove $SnCl_4$. The products were chromatographed on silica gel. Cycloshikonin was eluted with benzene and recrystallized from MeOH to give dark purple rods (162.2 mg), mp 94—96° (lit. 79—80°). NMR ($CDCl_3$): δ 1.35, 1.37 (3H×2, s×2, Me×2), 1.6—1.9, 2.66 (4H, m×2, CH_2×2), 5.09 (1H, m, C<u>H</u>–OH), 7.14 (2H, s, arom. H×2), 12.36 (2H, s×2, OH×2).

Shikonin Triacetate (8)[5]——A mixture of 1 (1 g), AcONa (5 g) and Ac_2O (5 g) was heated at 60°. After usual work up the reaction products were chromatographed on silica gel. Fraction eluted with benzene–acetone (10: 1) gave **8** (200 mg), mp 102—105° (from MeOH). NMR ($CDCl_3$): δ 1.56, 1.67 (3H×2, br.s×2, Me×2), 2.08 (3H, a, Acetyl), 2.41 (6H, s, Acetyl×2), 5.04 (1H, m, vinyl H), 5.83 (1H, m, C<u>H</u>–OH), 6.62 (1H, br. s. arom. H), 7.20, 7.32 (1H×2, s×2, arom. H×2).

Fraction 1—Fraction 4——The benzene extracts were chromatographed over silica gel using *n*-hexane, benzene and acetone, and divided into 4 fractions by monitoring with TLC. Fr. 1—4 contain mainly the compounds showing *Rf* 0.28, 0.50, 0.66 and 0.73 respectively.

Evaluation of Antitumor Activity——The antitumor bioassay was carried out by Total Packed Cell Volume Method (TPCV) reported by Hoshi, *et al.*[8] The ascites cell of sarcoma 180 and female ICR mice were used for TPCV assay. The samples were dissolved in *n*-saline containing 0.5% CMC and administered intraperitoneally. Antitumor activity was evaluated as −: 100—66, +: 65—41, ++: 40—11, +++: 10—0 of *T/C*%. Life span prolongation effects were calculated after daily injections for 5 days. BDF_1 mice were used for the experiment with L-1210. Antitumor activity test against solid tumor was tested by the method of Nakahara.[9]

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