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# Isolation and Antitumor Activity of Cyclic Hexapeptides Isolated from Rubiae Radix[1)]

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The details of the isolation and antitumor activity of cyclic hexapeptides (RA-VII, RA-V, RA-IV and RA-III) isolated from *Rubia cordifolia* are reported. Studies on a spectrum of experimental tumors in mice revealed that the peptides exhibited significant activity against leukemias and ascites tumors, P-388, L1210, B-16 melanoma and solid tumors, colon 38, Lewis lung carcinoma and Ehrlich carcinoma. RA-V had an especially potent effect on MM2 mammary carcinoma in mice. The effective dose range of RA-IV against P-388 leukemia was different from those of the other cyclic hexapeptides.

**Keywords**—*Rubia cordifolia*; isolation of antitumor substance; antitumor activity; murine tumor; cyclic hexapeptide

In the course of screening studies[2,3)] for antitumor activity of crude drugs and collected plants by means of the total packed cell volume method using Sarcoma 180 ascites in mice,[4)] we found that the methanolic extract prepared from the dried roots of *Rubia cordifolia* L. (Rubiaceae) showed significant antineoplastic activity against Sarcoma 180 ascites and P-388 leukemia in mice. In the previous paper,[5)] we reported on the structures of antitumor cyclic hexapeptides, named RA-VII, RA-V, RA-IV and RA-III, which were isolated from the methanolic extracts of *R. cordifolia* and *R. akane*. In this paper, we describe the isolation of the antitumor substances and the activities of these substances against various experimental tumors in mice.

When the methanolic extract of *R. cordifolia* was fractionated as shown in Chart 1 on the basis of activity against P-388 leukemia in mice, the antitumor activity was concentrated in both the benzene and ethyl acetate extracts. Column chromatography of the benzene extract on silica gel was carried out, but *n*-hexane–ethyl acetate, ethyl acetate or methanol did not elute the antitumor active fraction. The active fraction was eluted with methanol–water (1 : 1). Thus, the benzene extract was twice subjected to column chromatography on Amberlite XAD-2, but in this purification the active fraction was not enriched as much as expected. Next, the chloroform eluate from the Amberlite XAD-2 column chromatography was subjected to droplet counter-current chromatography (DCC) and then to silica gel column chromatography. From the relationship between the behavior of the spots in preparative thin layer chromatography (TLC) on silica gel with various kinds of solvent systems and the antitumor activity against P-388 leukemia in mice, it became apparent that the active fraction could be developed with chloroform–methanol, but not with the other organic solvent systems. Therefore, the active fraction purified by DCC was eluted by employing the solvents shown in Chart 1. The antitumor active fraction eluted with chloroform–methanol (1 : 1) from the silica gel column was subjected to Sephadex LH-20 column chromatography, and the final purification was carried out with an RP-18 Lobar column using the solvent system of methanol–water (4 : 1). The isolation furnished two antitumor active compounds RA-VII and RA-V. The antitumor constituents of the ethyl acetate extract coincided with those of the

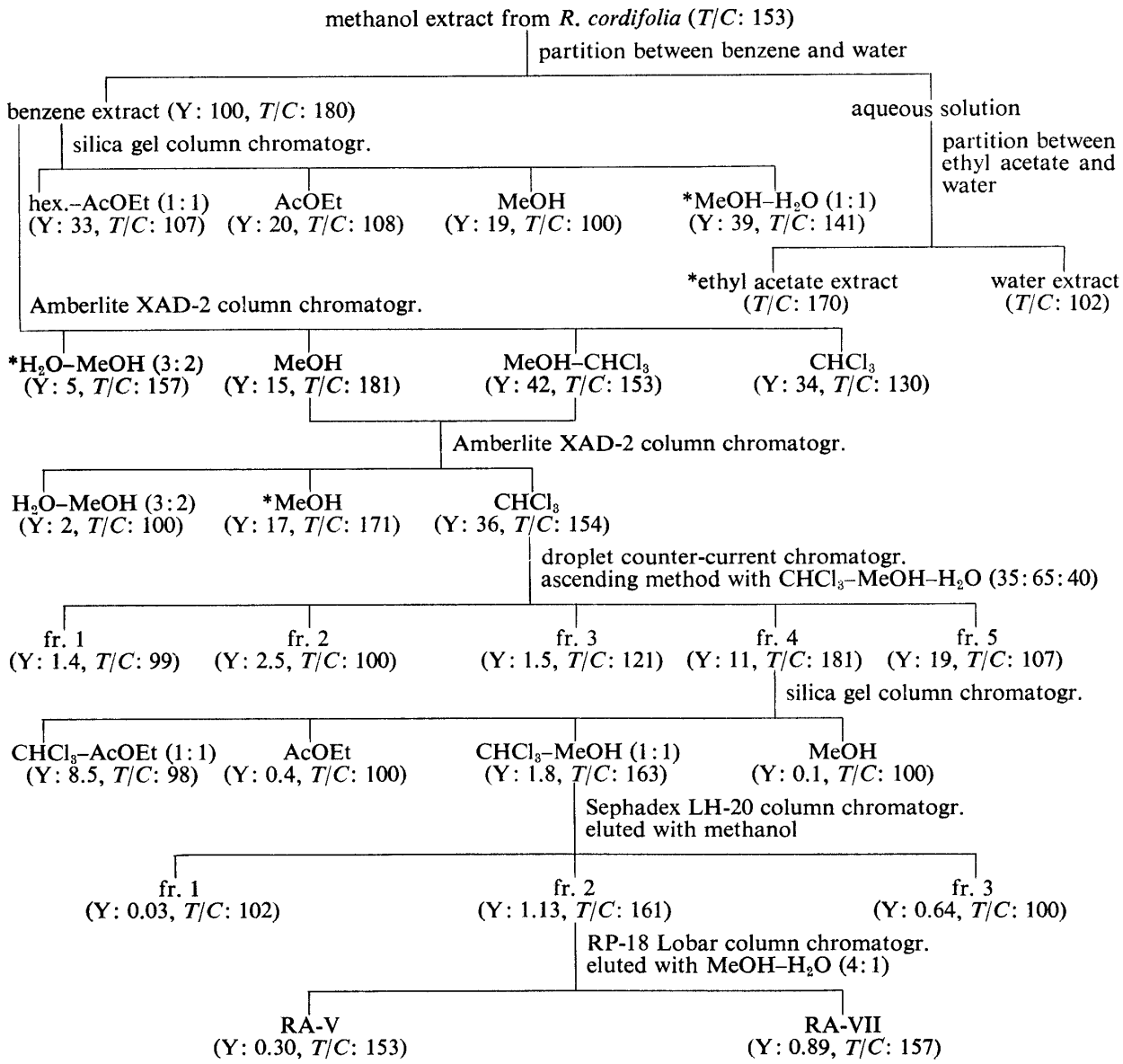
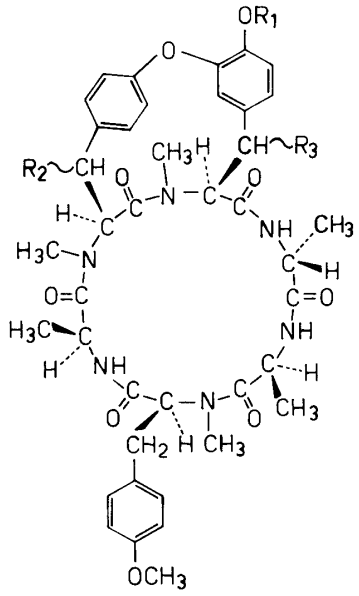



Chart 1. Antitumor Activity of Fractions Isolated from *R. cordifolia*

1) Antitumor activity was determined against P-388 leukemia in mice. P-388 was implanted *i.p.* ($1 \times 10^6$ cells/0.1 ml) in CDF1 mice ($n=6$) on day 0.
2) Y means yield (%) against the benzene extract. *T/C* means survival effect as a percentage. *T/C* (%) $>125$ is considered to be active.
3) Methanol, benzene, ethyl acetate and water extracts were administered at 200 mg/kg dose. The dose of each fraction was decided according to the following formula, $200 \times (Y/100)$ mg/kg. Drugs were given daily at the indicated doses (*i.p.*) for 5 consecutive days (1—5 d).
4) It was confirmed by TLC (silica gel and RP-18) that the antitumor constituents of the asterisked fraction coincided with RA-V and RA-VII.

benzene extract. On the other hand, when RA-VII and RA-V were isolated from a large amount of the methanolic extract in order to obtain samples for various kinds of bio-assay, compounds RA-IV and RA-III, the chromatographic properties of which were similar to those of RA-VII and RA-V, were also isolated as antitumor-active minor products. The structures of these cyclic hexapeptides (Fig. 1) were established by chemical and spectroscopic evidence.[5]

Next, we examined the antitumor activity of these cyclic hexapeptides against various experimental tumors in mice. The dose-dependence of the effects of RA-VII, RA-V, RA-V-23



RA-VII : $R_1=CH_3$, $R_2=R_3=H$
RA-V : $R_1=H$, $R_2=R_3=H$
RA-V-23 : $R_1=Ac$, $R_2=R_3=H$
RA-IV : $R_1=CH_3$, $R_2=H$, $R_3=OH$
RA-III : $R_1=CH_3$, $R_2=OH$, $R_3=H$

Fig. 1. Structures of Cyclic Peptides Isolated from *Rubia cordifolia*

*RA-V-23 was obtained by acetylation of RA-V.

TABLE I. Antitumor Effects of Cyclic Peptides on P-388 Leukemia

| | Dose (mg/kg) | Survival time (d, mean ± S.E.) | $T/C$ (%) | B.W.C. (g) |
|---|---|---|---|---|
| RA-VII | 0.002 | 10.30 ± 0.33 | 109.8 | +4.7 |
| | 0.01 | 12.20 ± 0.17 | 130.1 | +3.4 |
| | 1.0 | 15.00 ± 0.31 | 159.9 | +2.0 |
| | 4.0 | 16.20 ± 1.02 | 173.6 | −1.0 |
| | 5.0 | 9.20 ± 2.40 | 96.8 | −3.2 |
| RA-V | 0.01 | 10.20 ± 0.40 | 108.7 | +4.1 |
| | 0.05 | 12.30 ± 0.21 | 131.1 | +3.4 |
| | 2.0 | 15.40 ± 0.43 | 164.2 | +2.8 |
| | 10.0 | 17.80 ± 0.58 | 187.4 | −0.3 |
| | 20.0 | 2.83 ± 0.48 | 29.8 | — |
| RA-V-23 | 0.01 | 10.30 ± 0.21 | 114.4 | +5.2 |
| | 0.05 | 12.00 ± 0.37 | 133.3 | +3.6 |
| | 2.0 | 15.20 ± 0.17 | 168.9 | +2.9 |
| | 10.0 | 18.50 ± 0.56 | 194.7 | −1.2 |
| | 20.0 | 2.17 ± 0.17 | 22.8 | — |
| RA-IV | 0.05 | 9.17 ± 0.17 | 101.9 | +5.7 |
| | 0.5 | 9.60 ± 0.25 | 106.7 | +6.2 |
| | 2.0 | 10.20 ± 0.31 | 113.3 | +5.0 |
| | 10.0 | 11.40 ± 0.20 | 126.7 | +4.9 |
| RA-III | 0.05 | 11.30 ± 0.33 | 121.1 | +4.3 |
| | 0.5 | 13.00 ± 0.52 | 139.3 | +2.9 |
| | 2.0 | 15.00 ± 0.63 | 160.8 | +2.8 |
| | 10.0 | 18.30 ± 0.52 | 196.1 | +0.7 |

P-388 was implanted *i.p.* ($1 \times 10^6$ cells/0.1 ml) in CDF1 mice ($n=6$) on day 0. Drugs were given daily at the indicated doses (*i.p.*) for 5 consecutive days (1—5 d).

(mono-acetate of RA-V), RA-IV and RA-III against P-388 leukemia in mice was tested and the results are shown in Table I. As can be seen from Table I, the effective dose ranges of these cyclic hexapeptides (five days of *i.p.* administration) were 0.01—4.0 mg/kg in RA-VII, and

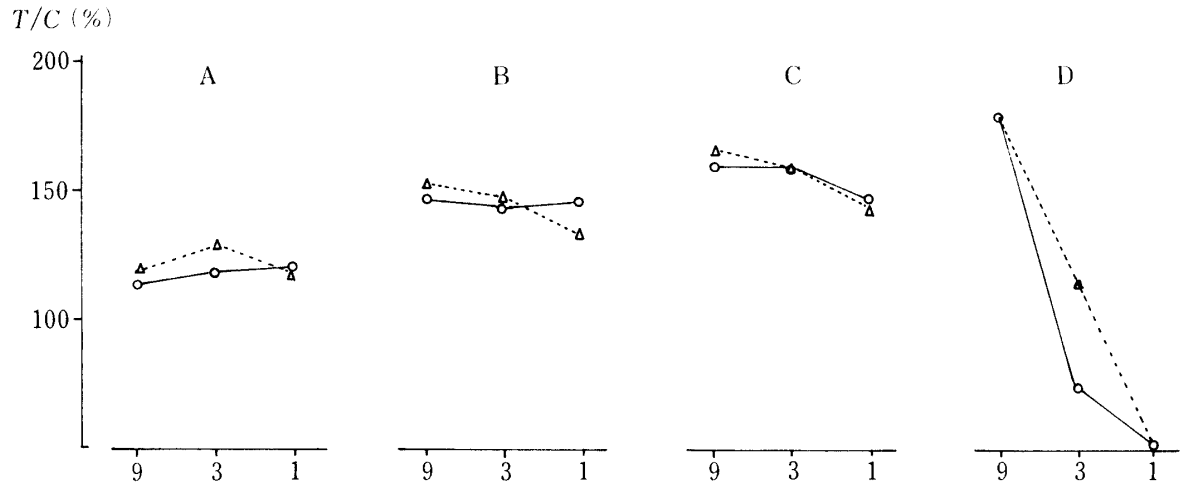



Fig. 2. Schedule Dependency of RA-V-23 and RA-VII Effect on P-388 Leukemia

△---△, RA-V-23; ○—○, RA-VII.
Administration route: *i.p.–i.p.*
Administration schedule: 9, 1—9 d; 3, 1, 5 and 9 d; 1, 1 d.
Total dose (mg/kg) RA-V-23, A, 0.25; B, 2.5; C, 10.0; D, 50.0; RA-VII, A, 0.10; B, 1.0; C, 5.0; D, 20.0.

TABLE II. Antitumor Activity of RA-V-23 and RA-VII Administered by Various Routes on P-388 Leukemia in Mice

| Compound | System | Optimal dose (mg/kg)[a] | ILS max (%)[b] | MED (mg/kg)[c] | CR[d] |
|---|---|---|---|---|---|
| RA-V-23 | *i.p.–i.p.* | 10.0 | 94.7 | 0.036 | 278 |
| | *i.p.–i.v.* | 5.6 | 71.5 | — | — |
| | *i.p.–p.o.* | 30.0 | −5.1 | — | — |
| RA-VII | *i.p.–i.p.* | 4.0 | 73.6 | 0.01 | 400 |
| | *i.p.–i.v.* | 2.2 | 50.1 | — | — |
| | *i.p.–p.o.* | 8.0 | 20.4 | — | — |

*a*) The dose that produces ILS max. *b*) Maximum increase in life span.
*c*) The dose that gives $ILS_{30}$. *d*) Chemotherapeutic ratio (=optimal dose/MED).

0.05—10.0 mg/kg in RA-V and RA-V-23. The maximal effective doses of RA-IV and RA-III could not be examined because of the very small amounts of samples obtained, but their minimum effective doses were 10 mg/kg in RA-IV and 0.05—0.5 mg/kg in RA-III. The effective dose range of RA-III was similar to those of RA-V and RA-V-23. The significant deviation of the effective dose ranges of RA-V from those of the other cyclic hexapeptides appears to indicate that the conformation of their diphenyl ether moieties, having a *cis* peptide-linkage,[5] influences the antitumor activity. The effect of administration schedule of RA-V-23 and RA-VII was also studied on P-388 leukemia in mice (Fig. 2). In both cases, several administrations were preferable to one at the same total dose in mice. Table II shows the antitumor activity against P-388 leukemia of RA-VII and RA-V-23 administered by various routes in mice. When RA-VII and RA-V-23 were given *i.p.*, *i.v.* and *p.o.*, *i.p.* administrations gave good effects in both cases and the chemotherapeutic ratios were significant; however, *i.v.* and *p.o.* administrations did not have much effect (except for *i.v.* administration of RA-VII).

Next, RA-V, RA-VII and RA-V-23 were investigated on a spectrum of experimental murine tumors, such as leukemias and ascites tumors (L1210 leukemia, C1498 leukemia,

TABLE III. Screening Systems and Antitumor Activities of RA-V-23 and RA-VII against Transplanted Tumors

| Leukemias and ascites tumors | Host animal | Inoculation size | Criteria | Dose (mg/kg/d) | Survival effect (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | RA-V | RA-V-23 | RA-VII |
| L1210 leukemia (*i.p.–i.p.*) | CDF1 | $10^6$ | MST | 0.2 × 5 | 110.6[a] | 110.0 | 115.7[a] |
| | | | | 1.0 × 5 | 112.1[a] | 116.7[a] | 120.7[c] |
| | | | | 2.0 × 5 | 116.3[b] | 114.4[a] | 129.3[c] |
| | | | | 5.0 × 5 | 120.0[a] | 120.0[b] | 137.3[c] |
| | | | | 10.0 × 5 | 128.9[a] | 128.9[b] | Toxic |
| C1498 leukemia (*i.p.–i.p.*) | BDF1 | $10^6$ | MST | 0.4 × 5 | | 96.2 | 100.8 |
| | | | | 2.0 × 5 | NT | 89.4 | 103.0 |
| | | | | 5.0 × 5 | | NT | 87.1 |
| Ehrlich carcinoma (*i.p.–i.p.*) | ICR | $5 \times 10^6$ | MST | 2.0 × 5 | | 104.3 | 82.0 |
| | | | | 5.0 × 5 | NT | 89.6 | Toxic |
| | | | | 10.0 × 5 | | Toxic | NT |
| MM2 mammary carcinoma (*i.p.–i.p.*) | C3H/He | $10^6$ | MST | 1.0 × 5 | 131.8[b,d] | NT | 103.1 |
| | | | | 2.0 × 5 | 138.7[a,e] | 109.2[b] | 118.4 |
| | | | | 5.0 × 5 | 138.7[c,f] (2/6 > 60) | 117.8[c] | 90.8 |
| | | | | 10.0 × 5 | 145.7[b,g] (1/7 > 60) | 120.9[c] | NT |
| MH134 hepatoma (*i.p.–i.p.*) | C3H/He | $10^6$ | MST | 2.0 × 5 | | 90.8 | 108.0 |
| | | | | 5.0 × 5 | NT | 96.0 | Toxic |
| | | | | 10.0 × 5 | | Toxic | NT |
| B-16 melanoma (*i.p.–i.p.*) | BDF1 | Homogenate | MST | 0.25 × 9 | | NT | 133.8[c] |
| | | | | 0.5 × 9 | | 116.9[a] | NT |
| | | | | 1.0 × 9 | NT | 128.5[c] | 135.3[c] |
| | | | | 2.0 × 9 | | 147.8[c] | 139.1[c] |
| | | | | 4.0 × 9 | | 141.1[b] | 143.0[c] |
| Colon 26 adenocarcinoma (*i.p.–i.p.*) | CDF1 | $5 \times 10^5$ | MST | 2.0 × 9 | | 109.7 | 125.0[b] |
| | | | | 4.0 × 9 | NT | 108.7 | 125.9[b] |
| | | | | 8.0 × 9 | | 106.5 | NT |
| Solid tumors | | | | | | Inhibition (%) RA-V-23 | RA-VII |
| Lewis lung carcinoma (*s.c.–i.p.*) | BDF1 | $5 \times 10^5$ | TWD17 | 1.0 × 11 | | NT | −1.1 |
| | | | | 2.0 × 11 | | 16.3 | 27.4 |
| | | | | 4.0 × 11 | | 38.2 | 51.4 |
| | | | | 8.0 × 11 | | 65.4[a] | NT |
| Colon 38 carcinoma (*s.c.–i.p.*) | BDF1 | Homogenate | TWD17 | 1.0 × 11 | | NT | 13.7 |
| | | | | 2.0 × 11 | | 12.3 | 45.8 |
| | | | | 4.0 × 11 | | 18.6 | 70.1[a] |
| | | | | 8.0 × 11 | | 51.9 | NT |
| Ehrlich carcinoma (*s.c.–i.p.*) | ICR | $5 \times 10^6$ | TWD17 | 1.0 × 11 | | NT | 20.3 |
| | | | | 2.0 × 11 | | 25.0 | 60.5[b] |
| | | | | 4.0 × 11 | | 46.9[a] | ND |

Drugs were administered on the indicated days. MST = mean survival time. TWD = tumor weight on day (tumor weight was calculated from the dimensions determined with callipers). *a*) $p < 0.05$, *b*) $p < 0.01$, *c*) $p < 0.001$ as compared to control groups. NT = not tested. ND = not detected. *d*) 0.56 mg/kg × 9 d, *e*) 2.78 mg/kg × 9 d, *f*) 5.56 mg/kg × 9 d, *g*) 6.94 mg/kg × 9 d. ( ) = 60-day survivors.

Ehrlich ascitic carcinoma, MM2 mammary carcinoma, MH134 hepatoma, B-16 melanoma, Colon 26 adenocarcinoma) and solid tumors (Lewis lung carcinoma, Colon 38 adenocarcinoma and Ehrlich carcinoma). The results are shown in Table III. As can be seen from Table III, RA-VII and RA-V-23 exhibited remarkable antitumor activity against L1210, B-16

melanoma, Lewis lung carcinoma, Colon 38 and Ehrlich carcinoma. RA-V had an especially good effect on MM2 mammary carcinoma in mice (two of six mice given 5 mg/kg and one of seven mice given 10 mg/kg recovered). On the other hand, in the acute toxicity studies of RA-VII and RA-V-23 against male mice (ICR), the $LD_{50}$ values calculated by the Litchfield–Wilcoxon method[6] were 10.0 mg/kg *i.p.*, 16.5 mg/kg *i.v.* and 63.0 mg/kg *p.o.* for RA-VII and 18.4 mg/kg *i.p.*, 20.0 mg/kg *i.v.* and 229.0 mg/kg *p.o.* for RA-V-23.

Recently, the antitumor activity of RC-18 isolated from *R. cordifolia* was reported,[7] but its structure is still not known. We consider that RC-18 may be a cyclic hexapeptide, because its spectrum of action against experimental murine tumors is similar to those of RA-VII and RA-V-23. The mechanism of action is probably related to that of bouvardin, which is a cyclic hexapeptide from *Bouvardia ternifolia* (Rubiaceae).[8–10]

## Experimental

Silica gel colum chromatography was carried out on Wakogel C-200 (100—200 mesh). In general procedures, supports for column chromatography were employed at amounts equivalent to 50—100 times the sample amount. TLC was carried out on 0.25 mm silica gel plates ($60F_{254}$, Merck) with $CHCl_3$–MeOH (10 : 1) or on RP-18 plates ($F_{254s}$, Merck). Spots were detected under ultraviolet (UV) light (254 nm). The DCC apparatus (hand-build) was equipped with 800 columns (40 cm length and 1.65 mm in diameter).

**Extraction and Isolation**——The roots of *Rubia cordifolia* used in this experiment were purchased in China. The crude drug (8.5 kg) was extracted with methanol (18 l) three times. The concentrated methanolic extract (553 g) was partitioned between benzene (1.5 l) and water (1.5 l) in a separatory funnel three times. The combined benzene layers were concentrated to a dark gum (242 g). Next, the water layer was partitioned with ethyl acetate (1.5 l) three times. The combined ethyl acetate layers were concentrated to yield a dark solid (175 g). The benzene extract was fractionated as shown in Chart 1. Our present method for isolating cyclic hexapeptides from the roots of *R. cordifolia* is as follows. Rubiae Radix (220 kg) was extracted with methanol (500 l) three times. The combined methanolic solution was concentrated to 200 l. The methanolic extract was partitioned with *n*-hexane (200 l) then further concentrated to 100 l, and then 300 l of water was added. The aqueous solution was partitioned with chloroform (400 l) three times. The chloroform extract (6 kg) was subjected to column chromatography on silica gel (30 kg) and was eluted with chloroform (180 l), ethyl acetate (180 l) and chloroform–methanol (3 : 2, 180 l). The residue from the chloroform–methanol eluate (350 g) was applied to an activated charcoal (350 g) column to yield an oily substance (80 g) upon elution with ethyl acetate. Repeated silica gel column chromatography of the oily substance furnished 17.6 g of RA-VII, 10.5 g of RA-V, 42 mg of RA-IV and 60 mg of RA-III. The *Rf* values were 0.31 for RA-VII, 0.21 for RA-V, 0.18 for RA-IV and 0.13 for RA-III, when TLC was carried out on 0.25 mm silica gel plates ($60F_{254}$, Merck) with $CHCl_3$–MeOH (100 : 7).

**Tumor System**——Experimental tumor models were obtained from Jpn. Found. Cancer Res. The procedures for the maintenance of tumors and the experiments were according to the protocols set down by the Developmental Therapeutics Programme, National Cancer Institute.[11] The strain of mice, inoculation size and site, and administration route were shown in Tables I—III, Chart 1 and Fig. 2.

**Drug Treatment**——A 0.5% solution of carboxymethyl cellulose (CMC) in isotonic sodium chloride was used as a vehicle for the injection of cyclic hexapeptides in these experiments. The dose ranges used for treatment are shown in Tables I—III, Chart 1 and Fig. 2. Control group mice received equal volumes of normal saline containing 0.5% CMC. The results were evaluated according to the standard methods recommended in the protocol.[11]

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