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Studies on Antitumor Cyclic Hexapeptides RA Obtained from Rubiae Radix, Rubiaceae. III. On Derivatives of RA-V and Their *in Vivo* Activities[1)]

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With the aim of obtaining compounds with strong antitumor activity and weak toxicity, we have synthesized a series of alkylethers, fatty acid esters, benzoates and other derivatives of RA-V, an antitumor cyclic hexapeptide discovered in Rubiae Radix, and examined their antitumor activities against P-388 lymphocytic leukemia. It was found that the introduction of C_1 and C_6 chains (hydrophobic coefficient $\log P$ = about 3.2 and 5.8, respectively) on the phenol moiety of RA-V gave the most desirable compounds in terms of antitumor activity and toxicity. The caproic ester of RA-V, the *n*-hexylether of RA-V and RA-VII (C_1) also exhibited strong antitumor activity against other experimental tumors (L-1210 lymphocytic leukemia, B-16 melanoma and MM2 mammary carcinoma).

Keywords——Rubiae Radix; Rubiaceae; cyclic hexapeptides; derivatives synthesis; structure–activity relationship; antitumor activity; P-388 lymphocytic leukemia; B-16 melanoma; MM2 mammary carcinoma; toxicity

We have previously reported on the isolation, chemical structures[2)] and physiological activities[3)] of antitumor cyclic hexapeptides provisionally named RA-VII, RA-V, RA-IV and RA-III, which were originally discovered during a search for antitumor substances of *Rubia cordifolia* and *R. akane* (Rubiaceae) guided by bio-assay against P-388 lymphocytic leukemia (Fig. 1).

These natural compounds, however, showed differences of both pharmacological and toxicological activity which prompted us to try to obtain derivatives with higher pharmacological and lower toxicological activities by substituting the phenol moiety of RA-V with various groups. Derivatives dealt with here are mainly alkylethers, fatty acid esters and benzoates of RA-V.

Synthesis of ether derivatives of RA-V was done by reacting RA-V with alkyl halides in the presence of potassium carbonate in tetrahydrofuran (THF)–MeOH–H_2O under reflux. The structures of the ether derivatives obtained were confirmed by their ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectra (MS), as well as elementary analysis, *etc.* The physical properties are shown in Table I. Table II gives the antitumor activities against P-388 lymphocytic leukemia as well as the toxicities. With the linear ether derivatives, it was found that the antitumor activity increased with increasing number of carbon numbers and reached the maximum with C_3–C_4 derivatives, then declined. Among these derivatives the branched C_3 ether (isopropylether) derivative exhibited the highest activity. The two cyclohexylether and cyclopentylether derivatives had slightly higher activity than the corresponding linear ether compounds. The toxicity showed the same

TABLE I. Physical Properties of Ether Derivatives of RA-V

Comp. No.	R	Formula	mp (°C) (dec.)	$[\alpha]_D^{20-25}$ ($CHCl_3$)	log *P*	Analytical (%) Calcd (Found)		
						C	H	N
1	H (RA-V)	$C_{40}H_{48}N_6O_9 \cdot 5/4H_2O$	>300	−220	2.72	61.64 (61.66	6.35 6.28	10.78 10.73)
2	CH_3 (RA-VII)	$C_{41}H_{50}N_6O_9 \cdot H_2O$	>280	−224	3.17	62.42 (62.39	6.64 6.56	10.65 10.98)
3	CH_2CH_3	$C_{42}H_{52}N_6O_9 \cdot H_2O$	219—225	−194	3.7	62.83 (62.75	6.78 6.73	10.47 10.48)
4	$(CH_2)_2CH_3$	$C_{43}H_{54}N_6O_9 \cdot H_2O$	212—217	−193	4.3	63.22 (63.51	6.91 6.73	10.29 10.28)
5	$CH(CH_3)_2$	$C_{43}H_{54}N_6O_9 \cdot 1/2H_2O$	213—220	−194	4.1	63.93 (63.56	6.86 6.79	10.40 10.37)
6	$(CH_2)_3CH_3$	$C_{44}H_{56}N_6O_9 \cdot 3/2H_2O$	210—216	−192	4.8	62.92 (63.19	7.08 6.81	10.01 10.04)
7	$(CH_2)_4CH_3$	$C_{45}H_{58}N_6O_9 \cdot H_2O$	214—221	−191	5.4	63.96 (64.28	7.16 7.00	9.95 10.02)
8	$(CH_2)_5CH_3$	$C_{46}H_{60}N_6O_9 \cdot 3/2H_2O$	261—268	−178	5.9	63.65 (63.96	7.32 7.06	9.68 9.79)
9	$(CH_2)_6CH_3$	$C_{47}H_{62}N_6O_9 \cdot H_2O$	245—250	−189	6.4	64.66 (64.35	7.39 7.40	9.63 9.57)
10	$(CH_2)_7CH_3$	$C_{48}H_{64}N_6O_9 \cdot 3/2H_2O$	243—249	−178	7.0	64.34 (64.20	7.54 7.36	9.38 9.34)
11	$(CH_2)_8CH_3$	$C_{49}H_{66}N_6O_9 \cdot 3/2H_2O$	245—248	−169	7.5	64.67 (64.93	7.64 7.48	9.23 8.76)
12	$(CH_2)_9CH_3$	$C_{50}H_{68}N_6O_9 \cdot 1/2H_2O$	242—247	−198	8.1	66.28 (65.99	7.68 7.68	9.28 9.18)
13	$(CH_2)_{10}CH_3$	$C_{51}H_{70}N_6O_9 \cdot 1/2H_2O$	228—235	−179	8.6	66.57 (66.64	7.78 7.69	9.13 9.22)
14	$(CH_2)_{11}CH_3$	$C_{52}H_{72}N_6O_9 \cdot 1/2H_2O$	240—242	−189	9.1	66.86 (66.91	7.88 7.71	9.00 9.04)
15	$(CH_2)_{12}CH_3$	$C_{53}H_{74}N_6O_9 \cdot 3/2H_2O$	238—242	−186	9.7	65.88 (66.15	8.03 7.82	8.70 8.85)
16	$(CH_2)_{17}CH_3$	$C_{58}H_{84}N_6O_9 \cdot 2H_2O$	228—235	−153	12.4	66.64 (66.58	8.49 8.10	8.04 8.05)
17		$C_{46}H_{58}N_6O_9 \cdot 1/2H_2O$[a]	215—222	−171	5.2	65.11 (64.93	7.01 6.37	9.91 9.35)
18		$C_{45}H_{56}N_6O_9 \cdot H_2O$	229—233	−155	4.8	64.12 (64.07	6.94 6.75	9.97 9.93)

Compounds were crystallized from MeOH as colorless needles, except for *a*) (colorless amorphous solid).

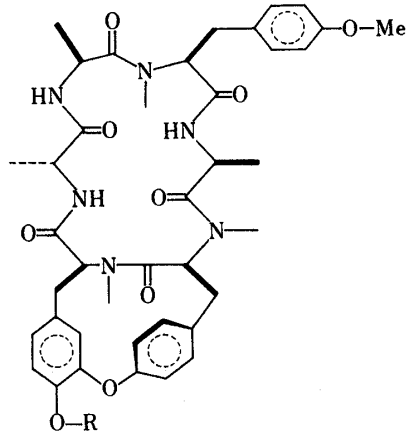
tendency as the antitumor activity and was found to be strong with the C_2–C_5 derivatives. As can be seen from Table II, it was considered that RA-VII and the *n*-hexylether of RA-V were the most desirable compounds from the viewpoint of both activity and toxicity.

From the above findings, we hypothesized that both antitumor activity and toxicity were closely related to the hydrophobicity of the derivatives. Thus, the hydrophobic coefficient *P* values of the derivatives were determined by actual measurement using high performance liquid chromatography (HPLC) or by calculation using parameters (π values) described in the literature.[4] When a linear dose–response relation was observed for antitumor activity, $\log(1/D)$ was calculated at doses that would provide increases of life span (ILS, T/C) of 150

TABLE II. Antitumor Activities on P-388 Lymphocytic Leukemia and Toxicities of Ether Derivatives of RA-V

Comp. No.	R	T/C (%)				Toxicity[f] Dose (mg/kg)						
		0.05 mg/kg	0.5 mg/kg	2.0 mg/kg	4.0 mg/kg							
1	H (RA-V)	131.1[c]	152.5[c]	164.2[c]	165.3[c]				20	30	40	50
									0/7	2/7	5/7	5/5
2	CH_3 (RA-VII)	138.6[c]	156.7[c]	164.2[c]	173.6[b]		10	15	20	30		
							0/3	3/3	3/3	3/3		
3	CH_2CH_3	137.3[c]	165.4[c]	162.2	Toxic	5	10					
						1/3	3/3					
4	$(CH_2)_2CH_3$	138.4[c]	146.0[a]	93.7	Toxic	5	10					
						1/3	3/3					
5	$CH(CH_3)_2$	142.2[b]	175.1[c-e]	105.4	Toxic	5	10					
						3/3	3/3					
6	$(CH_2)_3CH_3$	133.0[c]	144.9[a]	Toxic	Toxic	5	10					
						0/3	3/3					
7	$(CH_2)_4CH_3$	122.2[c]	142.7[c]	165.4[c]	Toxic	5	10		20			
						0/3	0/3		3/3			
8	$(CH_2)_5CH_3$	110.3[b]	137.3[c]	153.5[c]	173.0		10		20	30		
							0/3		1/3	3/3		
9	$(CH_2)_6CH_3$	115.8[c]	144.7[c]	150.1[c]	164.0[b]							
10	$(CH_2)_7CH_3$	136.1	146.8[c]	162.9[c]	152.2[b]							
11	$(CH_2)_8CH_3$	112.5[b]	141.5[b]	150.1[c]	155.4[b]							
12	$(CH_2)_9CH_3$	101.0	120.2[b]	132.7[c]	137.5[a]							
13	$(CH_2)_{10}CH_3$	115.4	108.7	121.2[b]	123.1[c]							
14	$(CH_2)_{11}CH_3$	93.0	101.0	105.8	112.5[a]							
15	$(CH_2)_{12}CH_3$	93.0	99.0	115.4	125.0[c]							
16	$(CH_2)_{17}CH_3$	101.0	98.1	101.0	108.7							
17		126.5	162.2	164.3[b]	Toxic							
18		127.6	140.5[a]	149.2[c]	143.8							

Significantly different from control at *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.
from RA-V at *d*) $p<0.05$, from RA-VII at *e*) $p<0.05$.
f) Toxicity: number dead/number tested.



RA-V : R=H
RA-VII : R=Me

Fig. 1. Structure of Cyclic Peptides from *Rubia cordifolia*

and 160%. The minimum lethal dose was taken as the lowest dose which killed all 3 animals in one group. Doses used were 5, 10, 15, 20, 30, 40 and 50 mg/kg.

The relationships among the ILS (150 and 160%), the minimum lethal dose (MLD) and the hydrophobic coefficient of the ether series of RA-V were analyzed according to both the Hansch–Fujita model,[4,5] and the bilinear model of Kubinyi.[6,7] When the parabolic model obtained from the Hansch–Fujita equation was applied to the ILS and MLD, significant results could not be obtained. However, the calculated data from the bilinear model Eqs. 1, 2 and 3 furnished satisfactory results.

Ethers T/C 150% activity:

$$\log 1/D = 1.71\ (\pm 1.70)^{b)} \log P - 2.08\ (\pm 1.82)^{b)} \log(BtP+1) - 3.94\ (\pm 3.65)^{a)} \quad (1)$$

$$\log Bt = -2.91 \qquad \log P_{opt} = 3.6$$

$$n=9 \quad r=0.93 \quad \text{S.D.}=0.19 \quad F=19.5$$

Ethers T/C 160% activity:

$$\log 1/D = 1.40\ (\pm 1.14)^{b)} \log P - 1.81\ (\pm 1.31)^{b)} \log(BtP+1) - 3.87\ (\pm 3.36)^{b)} \quad (2)$$

$$\log Bt = -3.30 \qquad \log P_{opt} = 3.8$$

$$n=8 \quad r=0.91 \quad \text{S.D.}=0.20 \quad F=12.6$$

Ethers minimum lethal dose:

$$\log 1/D = 1.12\ (\pm 0.41)^{b)} \log P - 1.59\ (\pm 0.57)^{b)} \log(BtP+1) - 4.66\ (\pm 1.29)^{b)} \quad (3)$$

$$\log Bt = -3.68 \qquad \log P_{opt} = 4.0$$

$$n=8 \quad r=0.95 \quad \text{S.D.}=0.10 \quad F=25.6$$

a) 90%, *b*) 95% confidential interval.

Therefore, the coefficients among the hydrophobicity $\log P$ and $\log 1/D$ values were calculated from the bilinear model equations. All Eqs. 1, 2 and 3 gave an optimum $\log P$ value from 3.6 to 4.0. However, since the optimum $\log P$ values of ILS 150 and 160% differed from that of MLD, it was considered that the most suitable ether derivative of RA-V for antitumor activity might be selected from the region away from the optimum $\log P$ of MLD and approximating the $\log 1/D$ value in the optimum $\log P$ of ILS (Fig. 2). Thus, RA-VII and the *n*-hexylether of RA-V should be useful compounds on this basis.

Synthesis of ester derivatives was carried out by two methods. In most cases, the common method of treating an acid anhydride in absolute pyridine solution at room temperature was

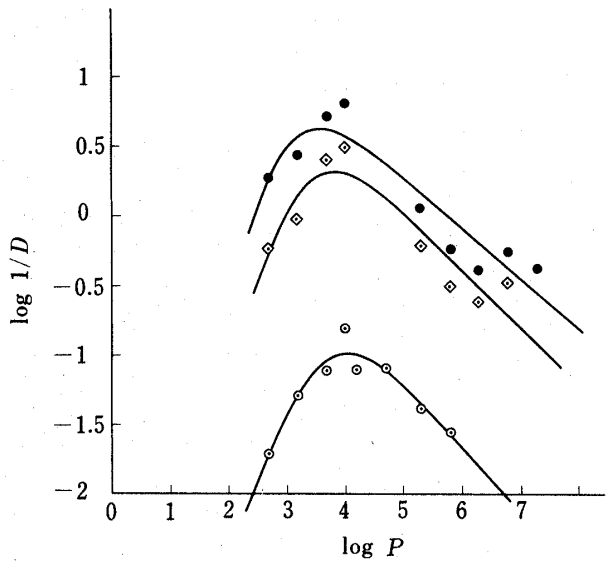


Fig. 2. Structure–Antitumor Activity and Toxicity Relationships of Alkyl Ethers of RA-V on P-388 Leukemia in Mice

These results were analyzed on the basis of the bilinear model (see the text).
●, T/C 150%; ◇, T/C 160%; ⊙, toxicity.

used. When the desired compounds (**33**—**37**) could not be obtained, the Taylor method[11] was employed (RA-V was treated with thallium ethoxide in absolute THF–ethanol at room temperature) and the resultant thallium salt of RA-V was reacted with RCOCl in absolute THF at room temperature. Physical properties of the ester derivatives are shown in Table III and their antitumor activities in Table IV.

In the case of the ester derivatives, the maximum dose used was raised to 10 mg/kg; toxicity (seen with ether compounds at 4 mg/kg) was not noticed. The highest values for antitumor activity of the ether derivatives were 197.2% with RA-V caproic ester (C_6 derivative), 170—190% with C_2–C_5 derivatives, and 150—170% with C_7–C_{10} and C_{18} derivatives.

A comparison at a dose of 10 mg/kg among the saturated linear chain (**21**), the branched chain (**22**) and the double-bonded linear chain (**23**) derivatives of C_4, revealed a slight increase in activity in the latter two compounds (Table IV). RA-V benzoate exhibited a rather strong activity (185.6%), but the enhancing effect expected in the phenylacetyl (158.8%) and cinnamic (150.9%) ester derivatives was not observed.

The above results prompted us to introduce a branched chain and a double-bonded chain

TABLE III. Physical Properties of Ester Derivatives of RA-V

Comp. No.	R	Formula	mp (°C) (dec.)	$[\alpha]_D^{20-25}$ ($CHCl_3$)	log *P*	Analysis (%) Calcd (Found)		
						C	H	N
1	H (RA-V)	$C_{40}H_{48}N_6O_9 \cdot 5/4H_2O$	>300	−220	2.72	61.64 (61.66	6.35 6.28	10.78 10.73)
19	$COCH_3$	$C_{42}H_{50}N_6O_{10} \cdot 1/2H_2O$	233—240	−190	3.19	62.44 (62.71	6.36 6.38	10.40 10.38)
20	$COCH_2CH_3$	$C_{43}H_{52}N_6O_{10} \cdot 1/2H_2O$	222—228	−171	3.7	62.84 (62.93	6.50 6.36	10.23 10.21)
21	$CO(CH_2)_2CH_3$	$C_{44}H_{54}N_6O_{10} \cdot 3/2H_2O$	196—201	−165	4.2	61.89 (61.82	6.73 6.37	9.84 9.75)
22	$COCH(CH_3)_2$	$C_{44}H_{54}N_6O_{10} \cdot 1/2H_2O$	225—232	−140	4.1	63.22 (62.89	6.63 6.49	10.05 9.93)
23	$COCH=CHCH_3$	$C_{44}H_{52}N_6O_{10} \cdot 3/2H_2O$	215—223	−88	4.0	62.03 (62.01	6.51 6.32	9.86 9.88)
24	$CO(CH_2)_3CH_3$	$C_{45}H_{56}N_6O_{10} \cdot H_2O$	203—208	−178	4.8	62.92 (62.97	6.81 6.62	9.78 9.75)
25	$CO(CH_2)_4CH_3$	$C_{46}H_{58}N_6O_{10} \cdot H_2O$	205—210	−178	5.3	63.29 (63.35	6.93 6.72	9.63 9.65)
26	$CO(CH_2)_5CH_3$	$C_{47}H_{60}N_6O_{10} \cdot 1/2H_2O$	195—200	−182	5.9	64.29 (64.33	7.00 6.95	9.57 9.61)
27	$CO(CH_2)_6CH_3$	$C_{48}H_{62}N_6O_{10}$	190—195	−173	6.4	63.98 (63.97	7.16 6.94	9.33 9.32)
28	$CO(CH_2)_8CH_3$	$C_{50}H_{64}N_6O_{10}$	174—180	−174	7.5	64.64 (64.89	7.38 7.13	9.05 9.12)
29	$CO(CH_2)_{16}CH_3$	$C_{58}H_{82}N_6O_{10}$	105—110	−131	11.8	68.08 (68.35	8.08 7.98	8.21 8.20)
30	CO– 	$C_{47}H_{52}N_6O_{10} \cdot H_2O$	240—245	−188	4.8	64.22 (64.29	6.19 6.07	9.56 9.63)
31	$COCH_2$– 	$C_{48}H_{54}N_6O_{10} \cdot 1/2H_2O$[a]	205—212	−179	5.2	65.22 (65.00	6.27 6.78	9.51 9.49)
32	$COCH=CH$– 	$C_{49}H_{54}N_6O_{10} \cdot 1/2H_2O$	>300	−150	5.9	63.15 (62.97	6.38 6.13	9.02 8.74)

Compounds were crystallized from EtOH as colorless needles, except for *a*) (colorless powder).

TABLE IV. Antitumor Activities of Ester Derivatives RA-V on P-388 Lymphocytic Leukemia

Comp. No.	R	T/C (%)			
		0.05 mg/kg	0.5 mg/kg	2.0 mg/kg	10.0 mg/kg
1	H (RA-V)	131.1[c]	152.5[c]	164.2[c]	187.4[b]
19	$COCH_3$	133.3[c]	155.6[c]	168.9[c]	194.7[a]
20	$COCH_2CH_3$	129.4[b]	145.3[b]	164.4[c]	175.0[c]
21	$CO(CH_2)_2CH_3$	125.1[a]	144.2[a]	155.9[c]	170.7[c]
22	$COCH(CH_3)_2$	124.1[b]	150.6[a]	155.9[c]	183.5[c]
23	$COCH=CHCH_3$	124.1[b]	148.5[c]	162.3[c]	189.6[c]
24	$CO(CH_2)_3CH_3$	117.9[b]	149.5[c]	157.9[c]	171.6[a]
25	$CO(CH_2)_4CH_3$	133.6[a]	143.2[c]	151.6[c]	197.2[c]
26	$CO(CH_2)_5CH_3$	113.6[b]	144.0[c]	150.1[c]	118.7
27	$CO(CH_2)_6CH_3$	126.7[a]	146.7[c]	166.7[a]	168.9[c]
28	$CO(CH_2)_8CH_3$	122.0	146.3[c]	151.6[c]	150.6[c]
29	$CO(CH_2)_{16}CH_3$	105.6	120.0[c]	136.7[c]	142.2
30	CO– 	127.8[c]	185.6[b]	175.6[b]	183.3
31	$COCH_2$– 	117.9[b]	147.4[c]	158.9[c]	138.9
32	COCH=CH– 	111.2[c]	141.8[c]	150.9[b]	109.1

Significantly different from Control at *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

into the C_6 ester, and Cl and NO_2 into the benzoate. However, none of the derivatives showed higher activity than their mother compounds (Tables V and VI).

Quantitative structure–activity relationship (QSAR) analysis of the ester series was also attempted by application of the Hansch–Fujita and the bilinear models. However, significant results could not be obtained from either model. This result may be attributed to the unstable character of the ester linkage with respect to esterase in mice.

Based on the above screening results, our attention was especially drawn to the C_6 derivatives, namely caproic ester and *n*-hexylether of RA-V. Therefore, their antitumor activities were compared with those of RA-V, RA-V acetate (**19**) and RA-V methylether (RA-VII, **2**) using various experimental tumors (Table VII).

Assays of activity against by P-388 and L-1210 leukemias were carried out with CDF_1 mice. P-388 leukemia (1×10^6 cells/body) was transplanted *i.p.*, as well as L-1210 leukemia (1×10^5 cells/body). Medication was started one day after the transplantation and continued for 5 consecutive days. The evaluations were made in terms of ILS ($T/C\%$) and body weight changes. Assays of activity against MM2 mammary carcinoma were carried out with C_3H/He male mice (5 weeks of age) by transplanting 1×10^6 cells/body *i.p.* and giving medication *i.p.* from the next day for 9 consecutive days. On the other hand, assays of activity against B-16 melanoma were performed with BDF_1 male mice of 6—8 weeks of age by transplanting 0.25 ml/body (20% suspension) *i.p.* and giving medication *i.p.* for 5 consecutive days.

In the P-388 and L-1210 leukemia assays, the *n*-hexylether and caproic ester derivatives showed about the same degree of activity as the lead compound RA-V. In the B-16 melanoma assay, however, the introduction of the C_6 chain enhanced the activity by approximately 10%, as compared with RA-VII. In the case of MM2 assay, the introduction of the C_6 chain greatly enhanced the activity. With the *n*-hexylether derivative, which was given at a dose of 2.78 mg/kg for 9 d consecutively, the T/C ratio was above 324% and there were 60-d survivors (4/7), while with the caproate derivative, the ratio was above 326% and again there were 60-d

TABLE V. Physical Properties of Substituted of Caproate and Benzoate Derivatives of RA-V

Comp. No.	R	Formula	mp (°C) (dec.)	$[\alpha]_D^{20-25}$ ($CHCl_3$)	log *P*	Analytical (%) Calcd (Found)		
						C	H	N
25	$CO(CH_2)_4CH_3$	$C_{46}H_{58}N_6O_{10}$						
33	$CO(CH_2)_2CH(CH_3)_2$	$C_{46}H_{58}N_6O_{10}\cdot H_2O$	285—292	−181	5.2	63.95	6.88	9.73
						(64.13	6.82	9.82)
34	$COCH_2CHCH_2CH_3$ CH_3	$C_{46}H_{58}N_6O_{10}\cdot H_2O$	193—198	−164	5.2	63.95	6.88	9.73
						(63.80	6.64	9.75)
35	$COCH(CH_2)_2CH_3$ CH_3	$C_{46}H_{58}N_6O_{10}\cdot 1/2H_2O$	195—201	−178	5.2	63.29	6.93	9.63
						(63.11	6.73	9.54)
36	$COCH=CH(CH_2)_2CH_3$	$C_{46}H_{56}N_6O_{10}\cdot H_2O$	268—274	−164	5.1	64.43	6.71	9.65
						(63.12	6.50	9.65)
37	$COCH_2CH=CHCH_2CH_3$	$C_{46}H_{56}N_6O_{10}\cdot 1/2H_2O$	198—203	−183	5.1	64.09	6.67	9.75
						(63.79	6.49	9.77)
30	CO–C$_6$H$_5$	$C_{47}H_{52}N_6O_{10}\cdot H_2O$	240—245	−188	4.8	64.22	6.19	9.56
						(64.29	6.07	9.63)
38	CO–C$_6$H$_4$(2-Cl)	$C_{47}H_{51}ClN_6O_{10}\cdot 1/2H_2O$	219—224	−171	4.9	62.00	6.42	9.23
						(62.26	6.03	8.78)
39	CO–C$_6$H$_4$(3-Cl)	$C_{47}H_{51}ClN_6O_{10}\cdot H_2O$	221—226	−160	5.6	61.80	5.85	9.20
						(61.95	5.78	9.06)
40	CO–C$_6$H$_4$(4-Cl)	$C_{47}H_{51}ClN_6O_{10}\cdot H_2O$	225—231	−155	5.5	61.80	5.85	9.20
						(61.50	5.64	9.17)
41	CO–C$_6$H$_4$(3-NO$_2$)	$C_{47}H_{51}N_7O_{12}\cdot 1/2H_2O$	224—230	−135	4.8	61.70	5.73	10.72
						(61.43	5.57	10.71)
42	CO–C$_6$H$_4$(4-NO$_2$)	$C_{47}H_{51}N_7O_{12}\cdot 3/2H_2O$	197—202	−140	4.8	60.51	5.83	10.51
						(60.32	5.64	10.29)

survivors (5/6), (Table VII).

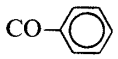
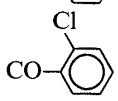
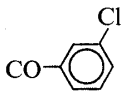
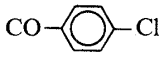
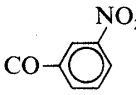
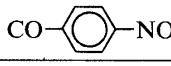
The LD_{50} values (acute toxicity) of the *n*-hexylether, caproic ester and benzoate derivatives were obtained according to Litchfield and Wilcoxon,[8] and the results are shown in Table VIII. These derivatives were compared with the natural compounds RA-V, RA-VII and RA-V acetate. It was found that the *n*-hexylether derivative was less toxic than the methylether and the benzoate was less toxic than the acetate by both *i.p.* and *i.v.* routes. The caproate derivative showed less toxicity than the acetate by the *i.v.* route, but the position was reversed upon *i.p.* administration. For all compounds, the toxicity by the *i.v.* route tended to be lower than that by the *i.p.* route. This is contrary to what is often found.

In recent years, many workers have attempted to design compound with superior properties by using a QSAR approach.[9,10] This approach has been employed in the present study, and it was concluded that the introduction of C_1 and C_6 chains on the phenol moiety of RA-V (hydrophobic coefficient log *P* of 3.2 and 5.8) gave the most desirable compounds from the viewpoints of both antitumor activity and toxicity.

Experimental

Melting points were measured on a Yamato melting point apparatus model MP-21, and are uncorrected. UV, IR, ^{1}H-NMR and ^{13}C-NMR spectra were measured with Hitachi UV-VIS 320, Nihonbunko IRA-I and JEOL FX-200 machines, respectively. MS were run on a JEOL JMS D300 instrument. Thin layer chromatography (TLC) was

TABLE VI. P-388 Antitumor Activities of Substituted Caproate and Benzoate Derivatives of RA-V

Comp. No.	R	T/C (%)			
		0.05 mg/kg	0.5 mg/kg	2.0 mg/kg	10.0 mg/kg
25	$CO(CH_2)_4CH_3$	133.6[a]	143.2[c]	151.6[c]	197.2[c]
33	$CO(CH_2)_2CH(CH_3)_2$	111.6[a]	133.9[c]	152.1[c]	118.7
34	$COCH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}HCH_2CH_3$	114.6[b]	140.0[c]	159.2[c]	Toxic
35	$CO\underset{\displaystyle CH_3}{\underset{\vert}{C}}H(CH_2)_2CH_3$	116.6[c]	142.0[c]	154.2[c]	Toxic
36	$COCH=CH(CH_2)_2CH_3$	116.6[b]	150.1[c]	165.3[c,d]	Toxic
37	$COCH_2CH=CHCH_2CH_3$	121.7	145.0[c]	162.3[c]	Toxic
30		127.8[c]	185.6[b]	175.6[b]	183.3
38		117.8[c]	136.3	160.3[c]	Toxic
39		114.5[c]	143.9[c]	160.3[c]	Toxic
40		114.5[c]	141.8[c]	154.9[b]	Toxic
41		111.2[c]	143.9[c]	152.7[c]	Toxic
42		116.7[c]	145.0[c]	156.3[c]	101.3

Significantly different from Control at *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$. Significantly different from Comp. No. 33 at *d*) $p<0.01$.

performed on silica gel (Kieselgel $60F_{254}$, G. Merck) with a solvent system of $CHCl_3$–MeOH (100:7) and high performance liquid chromatography (HPLC) on a Nucleosil C-18 column with 50% acetonitrile (detector: UV 225 nm).

Synthesis of Ether Derivatives of RA-V——RA-V (100 mg) was added to a mixture of 4 ml of THF, 8 ml of MeOH and 2 ml of water, then 20 ml of alkyl bromide and excess anhydrous calcium carbonate were added. The whole was refluxed in an oil bath at 100 °C. If starting material remained unreacted (as detected by TLC), further alkyl bromide was added and refluxing was continued. After the conclusion of the reaction, the reaction mixture was extracted with ethyl acetate. The extract was washed with saturated saline solution and dried over anhydrous magnesium sulfate. The concentrated extract was washed with *n*-hexane and purified by preparative TLC. The product was recrystallized from MeOH.

Synthesis of Ester Derivatives of RA-V——Anhydrous carboxylic acid or carboxylic chloride (15 mg) was added to 5 ml of anhydrous pyridine solution containing 50 mg of RA-V by instillation under stirring. The reaction mixture was left overnight, then anhydrous toluene was added and the solvent was removed by evaporation *in vacuo*. The residue was washed with *n*-hexane and then recrystallized from MeOH.

Preparation from the thallium salt of RA-V was done as follows.[11] Thallium ethoxide (40 mg) in 2 ml of anhydrous EtOH was added to 1 ml of anhydrous THF solution containing 110 mg of RA-V under stirring, and the reaction mixture was stirred for 3 h at room temperature, then evaporated to dryness. Next, 20 mg of carboxylic acid chloride in 1 ml of anhydrous THF was added dropwise to the thallium salt of RA-V thus obtained. The solution was stirred for 2 h and then filtered to remove the solid material. The filtrate was evaporated to dryness, then the residue was washed with *n*-hexane and purified by preparative TLC. The product was recrystallized from MeOH.

Assay of Activity against P-388 Lymphocytic Leukemia[12]——CDF_1 male mice aged 5 weeks, supplied by Japan Charles River Co., Ltd., were used in groups of 6—7 animals. P-388 lymphocytic leukemia, provided by the Cancer Research Institute and maintained in successive generations by us, was implanted *i.p.* at 1×10^6 cells/body. Administration of a test drug was started at one day after the implantation and continued for 5 d in the case of the *i.p.* route and for 9 d in the case of the *i.v.* route. The effectiveness was evaluated in terms of the ILS (*T*/*C*%) and

TABLE VII. Antitumor Activities of Derivatives of RA-V

Compound	Dose mg/kg × d	P-388 (*i.p.*–*i.p.*) *T/C* (%)	L-1210 (*i.p.*–*i.p.*) *T/C* (%)	B-16 (*i.p.*–*i.p.*) *T/C* (%)	Dose mg/kg × d	MM2 (*i.p.*–*i.p.*) *T/C* (%)
RA-V	1.0 × 5	157.9[c]	112.1[a]		0.56 × 9	131.8[b]
	2.0	NT	116.3[b]			
	5.0	165.3[c]	120.0[a]	NT	2.78	138.7[a]
	10.0	187.4	128.9[a]		5.56	231.2< (2/6>60)
	20.0	Toxic	NT		6.94	184.3< (1/7>60)
RA-VII (RA-V-methylether)	0.01 × 5	130.1[c]	105.6	NT		
	0.5	156.7[c]	118.6[c]	133.8[c] (0.25 mg)		
	1.0	159.9[c]	120.7[c]	135.3[c]	0.56 × 9	209.8< (2/6>60)
	2.0	164.2[c]	129.3[c]	139.1[c]	1.11	209.8< (2/6>60)
	4.0	173.6[b]	137.3[c] (5 mg)	143.0[c]	2.78	220.9< (2/6>60)
					5.56	80.6
RA-V-acetate	0.05 × 5	133.3[c]	100.9	NT		
	0.5	155.6[c]	110.0	116.9[a]	0.5 × 5	102.5
	2.0	168.9[c]	114.4[a]	128.5[c]	2.0	109.2[b]
	5.0	175.8[a]	120.0[b]	147.8[c] (4 mg)	5.0	117.8[c]
	10.0	194.7[a]	128.9[b]	141.1[b] (8 mg)	10.0	120.9[c]
RA-V-*n*-hexylether	0.05 × 5	130.4	NT	NT		
	0.5	143.5[c]	115.6[b]	130.5[c]	0.11 × 9	108.6
	2.0	150.0[a]	126.6[c]	158.2[a]	0.56	250.1<[a] (3/7>60)
	4.0	NT	130.9[a]	136.2	2.78	324.4<[b] (4/7>60)
	5.0	176.1[b]	NT	NT	5.56	Toxic
RA-V-caproate	0.01 × 5	133.7[a]	NT	NT		
	0.1	135.9[b]	NT	NT	0.11 × 9	163.3< (1/6>60)
	0.5	NT	115.6[c]	143.5[c]	0.56	145.9[a]
	2.0	152.2[c]	126.6[a]	152.0[c]	2.78	325.5<[b] (5/6>60)
	5.0	NT	Toxic	165.5[c]	5.56	122.4
RA-V-benzoate	0.05 × 5	127.8[c]	NT			
	0.5	185.6[b]	113.7[c]	NT		NT
	2.0	175.6[b]	132.3[b]			
	10.0	183.3[a]	127.7[a] (8 mg)			

Significantly different from Control at *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$. (Dose; mg/kg) or (60-d survivors). NT; Not tested.

body weight changes.

Assay of Activity against L-1210 Leukemia[12]——CDF_1 male mice aged 5 weeks, supplied by Japan Charles River Co., Ltd., were used in groups of 6 animals. L-1210 leukemia, provided by the Cancer Research Institute and maintained in successive generations by us, was implanted *i.p.* at 1×10^5 cells/body. A test drug was given *i.p.* at one day after the implantation and continued for 5 d. The effectiveness was evaluated in the same way as above.

Assay of Activity against MM2 Mammary Carcinoma[12]——C_3H/He male mice aged 5 weeks, supplied by Japan Charles River Co., Ltd., were used in groups of 6 animals. MM2 mammary carcinoma, provided by the Sasaki Laboratory and maintained in successive generations by us, was implanted *i.p.* at 1×10^6 cells/body. A test drug was given *i.p.* at one day after the implantation, and continued for 9 d. The evaluation of effectiveness was made in the same way as above.

Assay of Activity against B-16 Melanoma[12]——BDF_1 male mice aged 6—8 weeks, supplied by Japan Charles River Co., Ltd., were used in groups of 6—8 animals. B-16 melanoma, provided by the Cancer Research Institute and maintained in successive generations by us, was implanted *i.p.* at 0.25 ml/body as a 20% suspension. The evaluation of

TABLE VIII. Acute Toxicities of *n*-Hexylether and *n*-Caproic Ester Derivatives of RA-V

Compound	LD_{50} and 95% confidence limits (mg/kg)	
	i.p.	*i.v.*
RA-V	16.0 (13.3—19.3)	34.0 (28.1—41.2)
RA-VII (RA-V-methylether)	10.0 (7.0—14.3)	16.5 (12.6—21.5)
RA-V-acetate	18.4 (16.9—20.0)	20.0 (15.2—26.3)
RA-V-*n*-hexylether	12.0 (10.1—14.3)	23.0 (20.4—26.0)
RA-V-*n*-caproate	14.5 (13.1—16.1)	35.9 (31.8—40.5)
RA-V-benzoate	27.4 (23.5—32.2)	27.1 (20.2—36.3)

The LD_{50} values were calculated by the Litchfield-Wilcoxon method.
Animal; ICR (CD1) mice, male, 4 weeks of age (Japan Charles River).

effectiveness was made in the same way as above.

Acute Toxicity——ICR specific-pathogen-free male mice aged 4 weeks were used in groups of 3—7 animals. A test drug was administered by the *i.v.* or *i.p.* route. The LD_{50} was calculated according to the Litchfield-Wilcoxon method.[9] Further, in order to examine the relationship between toxicity and chemical structure, the lethal dose causing the death of 3 out of 3 animals was determined.

Determination of Hydrophobic Coefficient log *P* with *n*-Octanol–H_2O——The hydrophobic coefficients of RA-V, RA-VII and RA-V acetate were determined by HPLC under the conditions given below, while those of the other compounds were calculated from the values given in the literature.[4] HPLC conditions were as follows: column, Lichrosorb RP-2 (5 cm × 4 mm i.d.); detection, UV 220 nm; sensitivity, 0.02 AUFS; mobile phase, *n*-octanol saturated with water; elution speed, 4 ml/min.

Method of calculation:

Retention time of non retained material	t_O
Retention time of sample	t_R
Retention parameter of sample	$k=(t_R-t_O)/t_O$
	$k(t_O)=t_R-t_O=t_K$

Measured values and calculated values:

$t_O=0.11$ min (1/4 of the value obtained at the eluting speed of 1 ml/min).

Calibration curve:

Standards	t_R	$\log t_K$	$\log P$
Nitrobenzene	2.6 min	0.396	1.85
Methylparaben	2.9	0.446	1.96
Benzene	5.2	0.707	2.13
Ethylparaben	9.1	0.954	2.47
Benzhydrol	14.6	1.16	2.67
Propylparaben	32.8	1.51	3.04
Butylparaben	119.0	2.08	3.57

Thus, $\log P=1.02\log t_K+1.47$ (correlation coefficient $r=0.998$)

Measurement of samples:

Samples	t_R	$\log t_K$	$\log P$
RA-V	17.2 min	1.23	2.72
RA-VII	46.6	1.67	3.17
RA-V acetate	49.4	1.69	3.19

The log P values were also determined by the flask shaking method. The results were similar to those of the HPLC method.

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References and Notes

1) A part of this work was presented at the Annual Meeting of the Pharmaceutical Society of Japan, April 1983.
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