

Structures and Conformations of Metabolites of Antitumor Cyclic Hexapeptides, RA-VII and RA-X

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Metabolites of antitumor cyclic hexapeptides, RA-VII and -X which were isolated from *Rubia cordifolia* were studied by hepatic microsomal biotransformation in rats and in bile juice of rabbits to which these drugs were administered intravascularly. Their structures and conformations were elucidated by two-dimensional nuclear magnetic resonance techniques, temperature effect on NH protons and nuclear Overhauser effect experiments. Specific N-demethylation of Tyr-3, O-demethylation and hydroxylation at aromatic rings of Tyr-3 and -5 were observed. Compared with metabolites of RA-VII, most of RA-X was excreted unchanged in the bile juice. Relationship among their structures, conformations and antitumor activities is also discussed.

Keywords cyclic hexapeptide; RA-VII; RA-X; metabolite; *Rubia cordifolia*; biotransformation; cytotoxic activity; antitumor activity; conformation analysis

Introduction

Bicyclic hexapeptides, called the RA series (RA-I—RA-XIV, RAs),¹⁻³⁾ which have been isolated from *Rubia cordifolia*, showed potent antitumor activities against various experimental murine tumors *in vivo* and cultured cells *in vitro*. We have earlier already reported about their structures and antitumor activities,¹⁾ and RA-VII is now undergoing clinical trials as a new antitumor agent.

The main active principle, RA-VII is soluble in chloroform and neutral cyclic peptides consisting of six amino acids, three of which are N-methylated amino acids as shown in Fig. 1. Sodium salt of RA-X consisting of glutamic acid at residue 2 showed water solubility and was recently nominated as an antitumor principle with less side effect.²⁾

We recently worked on the conformations of RAs in solution and solid states, using the latest techniques in nuclear magnetic resonance (NMR) and computer simulations,^{3,4)} and showed that the main active principle, RA-VII was present in two stable conformational states in apolar

solvents such as CDCl₃.⁴⁾ It was also shown that each conformer takes a stable antiparallel conformation with two intramolecular hydrogen bondings between Ala-4 and D-Ala-1. These conformational phenomena might have resulted from the isomerization-rate about the N-methyl amide bond between Ala-2 and Tyr-3 being sufficient to give the separated signals in the NMR. We are interested in whether the active principle was the major conformer with a type II β -turn or the minor conformer with a type VI β -turn at the residues Ala-2 and Tyr-3⁴⁾ and also, in studying the relationship between the metabolic disposition of RAs and the antitumor activity of the metabolites. Therefore, we attempted to understand the RA metabolism by clarifying the structures of the metabolites *in vitro* and *in vivo*.

In this paper, we describe the hepatic microsomal biotransformation of RA-VII and the biliary metabolites of RA-VII and sodium salt of RA-X (RA-X-Na) in rabbits. Further, the structure determination and conformational analysis of the metabolites are reported in detail with the conformation-activity relationship.

Results and Discussion

First, to isolate the metabolites of RA-VII, the biotransformation was examined by means of rat hepatic microsomes. RA-VII was incubated aerobically with rat liver microsomes in the presence of an NADPH-generating system. After removal of protein with CH₃CN, the chloroform extract of the incubation mixture showed the presence of at least two metabolites in high performance liquid chromatography (HPLC) performed with 35% aqueous MeOH using ODS column. One of them was identified as RA-V,¹⁾ which was biotransformed by O-demethylation of methoxyl group at Tyr-6. The other metabolite (**1**) was isolated as colorless needles, mp >300 °C, by chromatographic purification with the above reversed phase HPLC (35% aqueous MeOH) and silica gel medium pressure liquid chromatography (MPLC; CHCl₃-MeOH 20:1).

The molecular formula C₄₀H₄₈N₆O₉ of **1** was established by the high resolution mass (HRMS) spectrum which gave a molecular ion peak at *m/z* 756.3561 (Calcd 756.3561). In the NMR spectra, the single conformational state was indicated even in a polar solvent such as dimethylsulfoxide-*d*₆ (DMSO-*d*₆), and then the presence of two N-methyl

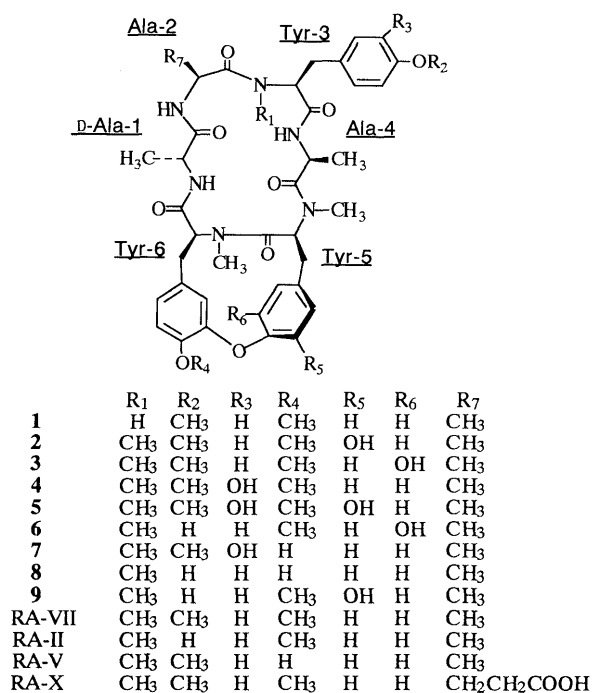


Fig. 1. Structures of RA-VII and Its Metabolites

TABLE I. $^1\text{H-NMR}$ Chemical Shifts at 303 K (δ)

Amino acid	Proton	1 (DMSO- d_6)	2 (CDCl $_3$)	3 (CDCl $_3$)	4 (CDCl $_3$)	5 (CDCl $_3$)	6 (CD $_3$ OD)	7 (CDCl $_3$)
D-Ala-1	H $_{\alpha}$	4.28 $J_{\alpha\beta}$ = 7.9	4.45 $J_{\alpha\beta}$ = 6.9	4.46 $J_{\alpha\beta}$ = 7.2	4.34 $J_{\alpha\beta}$ = 7.0	4.43 $J_{\alpha\beta}$ = 6.7	4.54 $J_{\alpha\beta}$ = 7.0	4.36 $J_{\alpha\beta}$ = 6.9
	H $_{\beta}$	1.11 $J_{\alpha\text{N}}$ = 7.8	1.35 $J_{\alpha\text{N}}$ = 7.9	1.37 $J_{\alpha\text{N}}$ = 6.9	1.31 $J_{\alpha\text{N}}$ = 6.5	1.35 $J_{\alpha\text{N}}$ = 7.4	1.29	1.31 $J_{\alpha\text{N}}$ = 6.6
	HN	7.80	6.74	6.69	6.44	6.72	b)	6.46
Ala-2	H $_{\alpha}$	4.24 $J_{\alpha\beta}$ = 7.1	4.74 $J_{\alpha\beta}$ = 6.8	4.70 $J_{\alpha\beta}$ = 7.6	4.88 $J_{\alpha\beta}$ = 6.9	4.76 $J_{\alpha\beta}$ = 6.5	4.66 $J_{\alpha\beta}$ = 6.7	4.85 $J_{\alpha\beta}$ = 6.9
	H $_{\beta}$	1.13 $J_{\alpha\text{N}}$ = 8.5	1.35 $J_{\alpha\text{N}}$ = 6.5	1.35 $J_{\alpha\text{N}}$ = 7.9	1.38 $J_{\alpha\text{N}}$ = 8.6	1.37 $J_{\alpha\text{N}}$ = 7.8	1.3	1.37 $J_{\alpha\text{N}}$ = 7.9
	HN	8.21	6.49	6.72	6.17	6.38	b)	6.30
Tyr-3	H $_{\alpha}$	3.92 $J_{\alpha\beta_1}$ = 10.4	3.57 $J_{\alpha\beta_1}$ = 11.0	3.71 $J_{\alpha\beta_1}$ = 10.9	3.58 $J_{\alpha\beta_1}$ = 10.5	3.57 $J_{\alpha\beta_1}$ = 11.1	3.85 $J_{\alpha\beta_1}$ = 9.8	3.59 $J_{\alpha\beta_1}$ = 10.5
	H $_{\beta_1}$ (pro-R)	2.97 $J_{\alpha\beta_2}$ = 4.0	3.30 $J_{\alpha\beta_2}$ = 4.6	3.32 $J_{\alpha\beta_2}$ = 5.2	3.30 $J_{\alpha\beta_2}$ = 5.1	3.27 $J_{\alpha\beta_2}$ = 4.8	3.22 $J_{\alpha\beta_2}$ = 6.5	3.30 $J_{\alpha\beta_2}$ = 4.2
	H $_{\beta_2}$ (pro-S)	3.16 $J_{\beta_1\beta_2}$ = 13.7	3.38 $J_{\beta_1\beta_2}$ = 14.2	3.38 $J_{\beta_1\beta_2}$ = 14.0	3.36 $J_{\beta_1\beta_2}$ = 14.1	3.34 $J_{\beta_1\beta_2}$ = 13.9	3.23 $J_{\beta_1\beta_2}$ = ^{a)}	3.35 $J_{\beta_1\beta_2}$ = 12.9
	H $_{\delta_1}$	7.06 $J_{\delta\epsilon}$ = 8.6 (2H)	7.03 $J_{\delta\epsilon}$ = 7.0 (2H)	7.05 $J_{\delta\epsilon}$ = 8.6	6.70 $J_{\delta_1\delta_2}$ = 2.0	6.68 $J_{\delta_1\delta_2}$ = 2.0	6.98 $J_{\delta\epsilon}$ = 8.2	6.70 $J_{\delta_1\delta_2}$ = 1.9
	H $_{\delta_2}$			7.05	6.62 $J_{\delta_2\epsilon_1}$ = 8.2	6.61 $J_{\delta_2\epsilon_2}$ = 8.2	6.98	6.62 $J_{\delta_2\epsilon_2}$ = 8.2
	H $_{\epsilon_1}$	6.83 $J_{\alpha\text{N}}$ = 7.9 (2H)	6.38 (2H)	6.82			6.73	
	H $_{\epsilon_2}$			6.82	6.78	6.77	6.73	6.77
	MeN	8.36 (HN)	2.84	2.85	2.89	2.87	2.90	2.89
	MeO	3.72	3.79	3.79	3.88	3.87		3.88
	Ala-4	H $_{\alpha}$	4.61 $J_{\alpha\beta}$ = 6.7	4.76 $J_{\alpha\beta}$ = 6.7	4.88 $J_{\alpha\beta}$ = 6.7	4.73 $J_{\alpha\beta}$ = 6.7	4.75 $J_{\alpha\beta}$ = 6.7	4.77 $J_{\alpha\beta}$ = 6.8
H $_{\beta}$		1.04 $J_{\alpha\text{N}}$ = 8.0	1.03 $J_{\alpha\text{N}}$ = 6.7	1.01 $J_{\alpha\text{N}}$ = 8.3	1.13 $J_{\alpha\text{N}}$ = 7.5	1.04 $J_{\alpha\text{N}}$ = 8.1	1.04	1.11 $J_{\alpha\text{N}}$ = 7.7
HN		6.98	6.70	6.75	6.71	6.70	b)	6.71
Tyr-5	H $_{\alpha}$	5.32 $J_{\alpha\beta_1}$ = 11.4	5.64 $J_{\alpha\beta_1}$ = 9.1	5.66 $J_{\alpha\beta_1}$ = ^{a)}	5.42 $J_{\alpha\beta_1}$ = 11.3	5.65 $J_{\alpha\beta_1}$ = 9.42	5.63 $J_{\alpha\beta_1}$ = 9.9	5.41 $J_{\alpha\beta_1}$ = 11.3
	H $_{\beta_1}$ (pro-S)	3.45 $J_{\alpha\beta_2}$ = 3.3	3.93 $J_{\alpha\beta_2}$ = 0	3.66 $J_{\alpha\beta_2}$ = ^{a)}	3.68 $J_{\alpha\beta_2}$ = 3.2	3.94 $J_{\alpha\beta_2}$ = 0	3.51 $J_{\alpha\beta_2}$ = 0	3.69 $J_{\alpha\beta_2}$ = 2.9
	H $_{\beta_2}$ (pro-R)	2.66 $J_{\beta_1\beta_2}$ = 11.4	2.22 $J_{\beta_1\beta_2}$ = 13.1	2.74 $J_{\beta_1\beta_2}$ = ^{a)}	2.64 $J_{\beta_1\beta_2}$ = 11.3	2.23 $J_{\beta_1\beta_2}$ = 13.2	2.85 $J_{\beta_1\beta_2}$ = 13.6	2.64 $J_{\beta_1\beta_2}$ = 11.3
	H $_{\delta_1}$	7.12 $J_{\delta_1\delta_2}$ = 2.2	6.53 $J_{\delta_1\delta_2}$ = 1.8	6.96 $J_{\delta_1\delta_2}$ = 2.9	7.27 $J_{\delta_1\delta_2}$ = 2.2	6.53 $J_{\delta_1\delta_2}$ = 1.8	^{a)} $J_{\delta_1\delta_2}$ = 2.9	7.28 $J_{\delta_1\delta_2}$ = 2.2
	H $_{\delta_2}$	7.43 $J_{\delta_1\delta_2}$ = 8.5	6.74 $J_{\delta_2\epsilon_2}$ = 8.2	6.59 $J_{\delta_1\epsilon_1}$ = 8.7	7.42 $J_{\delta_1\epsilon_1}$ = 8.4	6.74 $J_{\delta_2\epsilon_2}$ = 8.2	6.40 $J_{\delta_1\epsilon_1}$ = 8.6	7.43 $J_{\delta_1\epsilon_1}$ = 8.4
	H $_{\epsilon_1}$	6.77 $J_{\delta_2\epsilon_2}$ = 8.4		6.82	6.88 $J_{\delta_2\epsilon_2}$ = 8.3		6.79	6.84 $J_{\delta_2\epsilon_2}$ = 8.4
	H $_{\epsilon_2}$	7.25 $J_{\epsilon_1\epsilon_2}$ = 2.4	6.90		7.21 $J_{\epsilon_1\epsilon_2}$ = 2.4	6.91		7.20 $J_{\epsilon_1\epsilon_2}$ = 2.4
	MeN	3.00	2.94	3.01	3.13	2.94	2.93	3.11
Tyr-6	H $_{\alpha}$	4.87 $J_{\alpha\beta_1}$ = 11.9	4.85 $J_{\alpha\beta_1}$ = 11.3	4.87 $J_{\alpha\beta_1}$ = ^{a)}	4.55 $J_{\alpha\beta_1}$ = 11.8	4.86 $J_{\alpha\beta_1}$ = 11.4	5.04 $J_{\alpha\beta_1}$ = 11.7	4.55 $J_{\alpha\beta_1}$ = 11.9
	H $_{\beta_1}$ (pro-R)	2.84 $J_{\alpha\beta_2}$ = 3.5	3.03 $J_{\alpha\beta_2}$ = 5.5	3.04 $J_{\alpha\beta_2}$ = ^{a)}	3.11 $J_{\alpha\beta_2}$ = 3.8	3.04 $J_{\alpha\beta_2}$ = 5.3	2.98 $J_{\alpha\beta_2}$ = 5.5	3.07 $J_{\alpha\beta_2}$ = 3.9
	H $_{\beta_2}$ (pro-S)	3.13 $J_{\beta_1\beta_2}$ = 17.6	3.13 $J_{\beta_1\beta_2}$ = 15.6	3.04 $J_{\beta_1\beta_2}$ = ^{a)}	2.96 $J_{\beta_1\beta_2}$ = 14.5	3.13 $J_{\beta_1\beta_2}$ = 15.4	3.27 $J_{\beta_1\beta_2}$ = 14.8	2.93 $J_{\beta_1\beta_2}$ = 17.9
	H $_{\delta_1}$	6.63 $J_{\delta_1\delta_2}$ = 1.7	6.83 $J_{\delta_1\delta_2}$ = 1.6	6.76 $J_{\delta_1\delta_2}$ = 1.8	6.58 $J_{\delta_1\delta_2}$ = 2.0	6.83 $J_{\delta_1\delta_2}$ = 1.5	^{a)} $J_{\delta_1\delta_2}$ = ^{a)}	6.52 $J_{\delta_1\delta_2}$ = 1.8
	H $_{\delta_2}$	4.50 $J_{\delta_1\epsilon_1}$ = 8.4	6.65 $J_{\delta_1\epsilon_1}$ = 8.3	6.47 $J_{\delta_1\epsilon_1}$ = 8.2	4.35 $J_{\delta_1\epsilon_1}$ = 8.3	6.65 $J_{\delta_1\epsilon_1}$ = 8.3	6.73 $J_{\delta_1\epsilon_1}$ = 7.8	4.35 $J_{\delta_1\epsilon_1}$ = 8.2
	H $_{\epsilon_1}$	6.88	6.87	6.84	6.80	6.88	6.96	6.81
	MeN	2.51	2.83	2.86	2.69	2.83	2.76	2.69
	MeO	3.83	3.90	3.91	3.94	3.91	3.86	

a) Could not be determined in the present study. b) The signals disappeared because of hydrogen-deuterium (H-D) exchange.

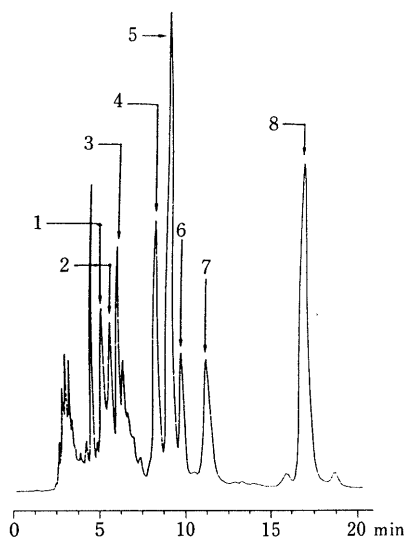


Fig. 2. Chromatogram of the Chloroform Extract from Bile in Administration of RA-VII to Rabbits Intravascularly

HPLC conditions: column, TSKgel ODS-80T $_M$ (21.5 mm i.d. \times 300 mm); solvent, 35% aqueous MeOH; flow rate, 6.5 ml/min; wavelength, 280 nm.

Peak 1, 8, 9; 2, 7, 5; 3, 6; 4, RA-II; 5, 4, 2; 6, RA-V; 7, 1, 3; 8, RA-VII.

groups and four NH protons that resulted from N-demethylation in one of three N-methyl tyrosines was observed. The ^1H - ^1H correlated spectroscopy (COSY) spectrum of **1** suggested that the α proton of Tyr-3 was coupled with one of four NH protons, *i.e.* that the N-methyl group of Tyr-3 was specifically demethylated. Two

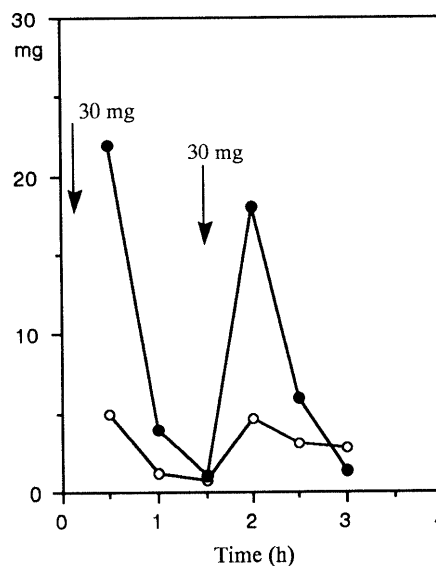


Fig. 3. Time Course of Metabolites of RA-VII and RA-X-Na

—○—, RA-VII; —●—, RA-X-Na.

dimensional (2-D) NMR techniques including heteronuclear multiple quantum coherence (HMQC)⁵⁾ and heteronuclear multiple bond connectivity (HMBC)⁶⁾ enabled us to give the complete assignments of ^1H and ^{13}C signals (Tables I and II) and to corroborate this structure. The structure of **1** was thus determined to be [*N*-demethyl-Tyr-3]RA-VII.⁷⁾

TABLE II. ^{13}C -NMR Chemical Shifts at 303 K (δ)

Amino acid	Carbon	1 (DMSO- d_6)	2 (CDCl $_3$)	3 (CDCl $_3$)	4 (CDCl $_3$)	5 (CDCl $_3$)	6 (CD $_3$ OD)	7 (CDCl $_3$)	8 (CD $_3$ OD)	9 (CD $_3$ OD)	
D-Ala-1	C $_{\alpha}$	47.09	47.72	47.52	47.90	47.71	48.49	47.90	a)	48.36	
	C $_{\beta}$	20.51	20.58	20.68	20.66	20.57	20.83	20.66	20.97	20.78	
	C $_{\text{C=O}}$	170.85	172.61	172.64	172.30	172.64	173.84	172.33	173.01	173.83	
Ala-2	C $_{\alpha}$	48.56	44.97	44.97	44.61	44.99	45.94	44.69	45.60	45.98	
	C $_{\beta}$	16.65	16.39	16.37	16.56	16.35	16.28	16.58	16.38	16.27	
Tyr-3	C $_{\text{C=O}}$	172.01	172.65	172.76	172.79	172.68	174.68	172.62	173.43	174.68	
	C $_{\alpha}$	54.58	68.48	68.05	68.32	68.37	68.84	68.29	68.87	68.90	
	C $_{\beta}$	33.72	32.74	32.73	32.91	32.95	33.71	32.98	33.74	33.76	
Tyr-3	C $_{\gamma}$	130.67	130.77	130.59	131.93	131.94	130.35	132.00	130.39	130.38	
	C $_{\delta_1}$	130.16	130.26	130.35	115.42	115.45	131.47	115.49	131.45	131.46	
	C $_{\delta_2}$	130.16	130.26	130.35	120.67	120.66	131.47	120.70	131.45	131.46	
	C $_{\epsilon_1}$	113.41	114.08	114.03	145.75	145.73	116.37	145.80	116.40	116.40	
	C $_{\epsilon_2}$	113.41	114.08	114.03	110.75	110.75	116.37	110.81	116.40	116.40	
	C $_{\zeta}$	157.61	158.46	158.47	145.38	145.36	157.33	145.43	157.36	157.34	
	C $_{\text{C=O}}$	168.77	168.13	168.67	167.91	168.06	171.08	168.06	171.04	171.05 ^{b)}	
	C $_{\text{N}}$		39.87	39.89	39.87	39.92	40.40	39.81	40.31	40.39	
	C $_{\text{O}}$	54.87	55.29	55.30	56.00	56.00		56.03			
	Ala-4	C $_{\alpha}$	45.50	46.28	46.35	46.45	46.26	47.58	46.47	47.73	47.60
		C $_{\beta}$	18.49	18.34	18.11	18.46	18.30	18.68	18.52	18.83	18.68
		C $_{\text{C=O}}$	170.31	171.27	171.15	171.76	171.23	172.30	171.80	172.42	172.66
	Tyr-5	C $_{\alpha}$	53.05	53.18	52.97	54.25	53.13	54.45	54.32	55.82	54.96
C $_{\beta}$		35.72	35.50	27.80	37.00	35.49	28.37	36.93	36.70	36.70	
C $_{\gamma}$		135.13	130.58	126.11	135.15	130.57	127.47	135.70	136.68	131.79	
C $_{\delta_1}$		132.73	118.74	119.04	132.81	118.69	119.08	131.08	133.97	120.21	
C $_{\delta_2}$		130.37	124.89	120.94	131.00	124.92	120.74	133.05	131.71	125.68	
C $_{\epsilon_1}$		123.78	143.94	117.84	124.26	143.84	117.19	125.90	125.16	146.31	
C $_{\epsilon_2}$		125.71	116.75	150.64	125.92	116.70	151.26	124.18	127.25	118.67	
C $_{\zeta}$		157.98	145.98	149.46	158.24	145.94	151.51	157.94	160.21	148.14	
C $_{\text{C=O}}$		169.12	170.05	170.17	169.36	170.02	172.76	169.18	171.32	172.48 ^{b)}	
C $_{\text{N}}$		29.99	30.38	30.44	30.54	30.37	30.89	30.54	31.06	30.89	
Tyr-6		C $_{\alpha}$	56.67	59.44	60.18	57.39	59.40	60.93	57.51	58.89	61.04
		C $_{\beta}$	34.83	37.54	37.49	35.50	37.52	38.16	35.66	37.40	38.16
		C $_{\gamma}$	129.53	127.90	128.31	128.18	127.84	129.86	127.70	128.92	130.06
	C $_{\delta_1}$	120.95	124.77	123.42	120.93	124.77	125.22	121.68	122.66	125.41	
	C $_{\delta_2}$	114.34	117.96	117.20	113.41	117.87	119.20	115.73	117.60	119.49	
	C $_{\epsilon_1}$	112.60	112.95	112.67	112.28	112.86	114.23	113.00	115.56	114.47	
	C $_{\epsilon_2}$	152.28	149.22	148.72	153.12	146.85	149.35	151.08	a)	150.62	
	C $_{\zeta}$	145.79	146.93	148.89	146.52	149.17	150.24	143.00	145.09	148.85	
	C $_{\text{C=O}}$	169.83	170.05	170.17	170.72	170.02	171.63	170.66	172.30	171.62 ^{b)}	
	C $_{\text{N}}$	28.97	31.59	31.60	29.30	31.60	32.58	29.37	30.11	32.55	
	C $_{\text{O}}$	55.73	56.13	56.16	56.17	56.10	56.71			56.78	

a) Could not be determined in the present study. b) Assignments may be interchangeable.

In the next step, the metabolites from biliary juice in rabbits were researched, because the yield of these metabolites *in vitro* was very low (**1**: 0.001%; RA-V: 0.112%) and most of the mother compound was recovered.

A typical HPLC chromatogram of bile collected for 3 consecutive hours after the intravenous administration of RA-VII to rabbits is shown in Fig. 2. RA-VII (30 mg/body) was administered two times at 1.5 h intervals because its excretion was observed immediately after the administration. The time course of the recovery every 30 min is shown in Fig. 3. Total recovery of the metabolites of RA-VII was slightly 25%. Purification by HPLC and MPLC led to the isolation of eleven metabolites (**1**–**9**, RA-II and RA-V). Two of them were identified as RA-II and RA-V,¹⁾ which were biotransformed by the O-demethylation process.

Compounds **2** and **3**, showing the same molecular formula, C $_{41}$ H $_{50}$ N $_6$ O $_{10}$, by fast atom bombardment mass spectra (FAB-MS), were isolated as colorless needles, mp >300 °C (**2**), >300 °C (**3**), respectively. Coupling pattern of the aromatic proton region based on ^1H – ^1H COSY

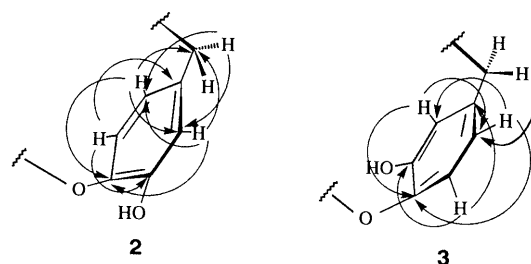


Fig. 4. HMBC Correlations of Metabolites **2** and **3**

spectra suggested the presence of two 1,3,4-substituted benzene rings and one 1,4-substituted benzene ring. This finding indicated that the aromatic ring of Tyr-3 or Tyr-5 was mon-hydroxylated. The ^1H and ^{13}C assignments were made by the HMQC and HMBC spectra (Table I), and the additional hydroxyl group of **2** was determined to be linked to the C $_{\epsilon_1}$ of Tyr-5 on the basis of the ^1H – ^{13}C long range correlations between Tyr-5-H $_{\delta}$ and Tyr-5-C $_{\beta}$, Tyr-5-H $_{\epsilon_2}$ and Tyr-5-C $_{\gamma}$, and Tyr-5-H $_{\epsilon_2}$ and Tyr-5-C $_{\epsilon_1}$ by the

HMBC spectrum (Fig. 4). That of **3** was also determined to be linked to the $C_{\epsilon 2}$ of Tyr-5 by the HMBC correlations around Tyr-5 (Fig. 4). From these findings, compounds **2** and **3** were characterized as [$\epsilon 1$ -hydroxy-Tyr-5]RA-VII and [$\epsilon 2$ -hydroxy-Tyr-5]RA-VII, respectively.

Compound **4** was obtained as a major metabolite and showed the molecular formula, $C_{41}H_{50}N_6O_{10}$ from the electron impact mass spectra (EI-MS) (m/z 786, M^+) and ^{13}C -NMR spectra. Occurrence of mono-hydroxylation to the aromatic ring of Tyr-3 was speculated, because the 1H and ^{13}C signals of the aromatic region in Tyr-3 were non-equivalently observed as the 1,3,4-substituted benzene ring in the NMR spectra. The hydroxyl group was elucidated to be linked the C_{ϵ} of Tyr-3 by the HMBC correlations, and the structure of **4** was characterized to be [ϵ -hydroxy-Tyr-3]RA-VII.

Compound **5** was considered to possess the molecular formula, $C_{41}H_{50}N_6O_{11}$, by FAB-MS (m/z 825, $M^+ + Na$) and ^{13}C -NMR spectra. The NMR spectra suggested the presence of three non-equivalent aromatic rings. Because the NMR chemical shifts of the aromatic rings of Tyr-3 and Tyr-5 were respectively similar to those of Tyr-3 in compound **4** and Tyr-5 in compound **2**, the dihydroxylation was thought to occur at the C_{ϵ} of Tyr-3 and the $C_{\epsilon 1}$ of Tyr-5. These substituents were established by the HMBC correlations and the structure of **5** was characterized as [ϵ -hydroxy-Tyr-3- $\epsilon 1$ -hydroxy-Tyr-5]RA-VII.

Compounds **6** and **7**, having the same molecular formula, $C_{40}H_{48}N_6O_{10}$ by FAB-MS (m/z 773, $M^+ + 1$) and ^{13}C -NMR spectra, were presumed to be biotransformed by hydroxylation and O-demethylation processes as follows. The aromatic signal pattern of Tyr-5 in **6** and that of Tyr-3 in **7** were similar to those of **3** and **4**. Only one methoxyl proton signal was observed in each. The position of methoxyl groups and hydroxyl groups of **6** and **7** were elucidated by the HMBC correlations and their structures were characterized as [O -demethyl-Tyr-3- $\epsilon 2$ -hydroxy-Tyr-5]RA-VII and [ϵ -hydroxy-Tyr-3]RA-V, respectively.

Compounds **8** and **9** were determined to be [O -demethyl-Tyr-3]RA-V and [O -demethyl-Tyr-3- $\epsilon 1$ -hydroxy-Tyr-5]RA-VII, respectively, by comparing the NMR signals with those of RA-VII.⁴⁾

We recently reported on the sodium salt of a new antitumor cyclic hexapeptide, RA-X with water solubility.²⁾ We were interested in a comparison of metabolites between the lipophilic peptide, RA-VII and the hydrophilic one, RA-X-Na. Collection of the biliary juice after administration of RA-X-Na was thus performed in a similar method to the case of RA-VII. The bile was extracted with 1-butanol three times, because RA-X-Na was not soluble in chloroform, and the extract was subjected to HPLC analysis. A large portion of RA-X-Na was found to be excreted to biliary juice as the mother compound, differing from the case of RA-VII (Fig. 3). The total recovery of RA-X-Na was 75%. RA-X-Na did not show antitumor activity against P-388 leukemia in mice by i.v. administration. This fact was also presumed to be attributable to the fast metabolic turnover indicated above.

Conformational analysis of the metabolites in the *in vitro* and *in vivo* experiments was done by nuclear Overhauser effect (NOE) experiments (1D-NOE and phase sensitive NOE spectroscopy (NOESY)) and by estimating the

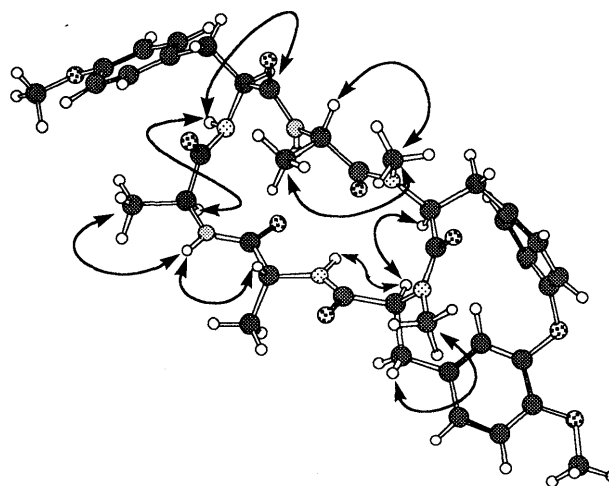


Fig. 5. NOE Relationship of **1**

The arrows show the negative NOE relationships by 1D-NOE and NOESYPH in $DMSO-d_6$ at 303 K.

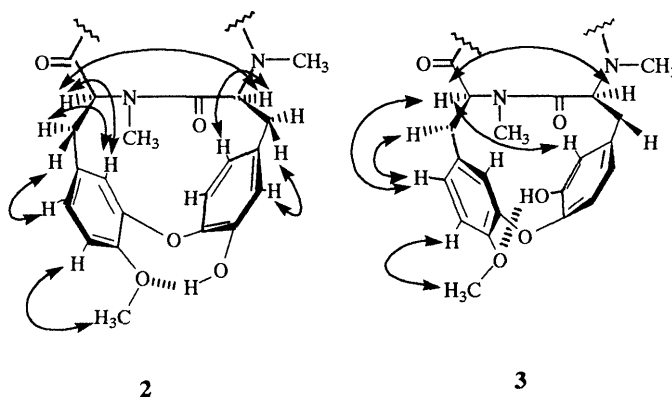


Fig. 6. NOE Relationships of 14-Membered Rings of **2** and **3**

The arrows show the NOE relationships confirmed by NOESYPH experiments in $CDCl_3$ at 303 K.

temperature effects on the NH protons, to clarify the relationship between conformation and antitumor activity.

Compound **1** was indicated to be single conformational state in $DMSO-d_6$ and the relationship of NOE enhancements (all negative NOEs) is shown in Fig. 5.⁷⁾ From these data, N-demethylation of Tyr-3 was considered to be one method locking the type II β -turn conformation of RA-VII in an antiparallel structure stabilized by a 4-1 hydrogen bond.

In 18-membered rings, the major conformer of compounds **2**, **3**, **5**, **6** and **9** has a conformation similar to that of RA-VII with the type II β -turn between Ala-2 and Tyr-3. However, the aromatic proton Tyr-6- $H_{\delta 2}$ found in an extremely high field in RA-VII that is characteristic of the RA series, was not observed in these compounds. This high field shift had been considered to be influenced by the anisotropic effect of Tyr-5 aromatic ring. The NOEs for these compounds (Fig. 6) were different from those in RA-VII,⁴⁾ i.e., NOEs between Tyr-6-NMe and Tyr-6- $H_{\delta 2}$ were not observed in any compound. Furthermore, NOEs between Tyr-6- $H_{\delta 2}$ and Tyr-6- H_{α} /Tyr-6- $H_{\beta 2}$ were present in compounds **2**, **5** and **9**, having a hydroxyl group at $\epsilon 1$ of Tyr-5, suggesting the aromatic ring rotation of Tyr-6. Then, in compounds **3** and **6**, with a hydroxyl group at $\epsilon 2$

TABLE III. Effect of Temperature on the NH Chemical Shifts of RA-VII, and Compounds 1–7 in DMSO-*d*₆, $-\Delta\delta/\Delta T$ (10^{-3} ppm/K)

Conformer		D-Ala-1	Ala-2	Tyr-3	Ala-4
RA-VII	A	6.0	4.7	—	0.0
	B	4.3	4.0	—	0.0
	C	3.7	4.0	—	0.7
Compound 1		6.0	4.8	8.3	0.0
Compound 2	Main	3.0	4.7	—	-0.33
	Minor	2.0	3.0	—	0.0
Compound 3	Main	3.3	4.3	—	-0.33
	Minor	2.3	3.0	—	0.33
Compound 4	Main	5.7	4.3	—	-0.33
	Minor	4.7	3.0	—	^{a)}
Compound 5	Main	3.7	4.7	—	0
	Minor	2.7	3.7	—	0.33
Compound 6	Main	3.3	4.3	—	0
	Minor	2.3	3.0	—	^{a)}
Compound 7	Main	5.7	4.0	—	-0.33
	Minor	4.3	3.0	—	0

^{a)} Could not be determined in the present study.

at Tyr-5, NOEs were observed between Tyr-6-H_{δ1} and Tyr-6-H_α/Tyr-6-H_{β2} (Fig. 6), also suggesting the ring rotation of Tyr-6 in a different direction from those of compounds 2, 5 and 9. These slight ring rotations were considered to be supported by the intramolecular hydrogen bond between Tyr-5-OH and methoxy-oxygen of Tyr-6, which was confirmed by the infrared (IR) absorption band of hydroxyl stretching vibration at 3580 cm⁻¹.

Compounds 4 and 7 were present in the single conformational state in apolar solvent such as CDCl₃, though minor conformers were also slightly present in polar solvent such as DMSO-*d*₆. These conformations arising from the isomerization of N-methyl amide bond between Ala-2 and Tyr-3 were speculated to be influenced by this rotation of the aromatic ring of Tyr-3 in 4 and 7.

The first step in the determination of the secondary structure of peptides in solution by NMR is to distinguish the NH protons exposed to the solvent or shielded from the solvent either sterically or through hydrogen bonding. The most common procedure for that is to determine the temperature effects on the NH protons in a hydrogen-bond accepting solvent such as DMSO-*d*₆.⁸⁾ The temperature coefficients ($d\delta/dT$) of these metabolites in DMSO-*d*₆ are shown in Table III.

It was shown in the NMR spectra that Ala-4-NH is involved in a strong intramolecular hydrogen bond, as well as being the major conformer of RA-VII. The temperature coefficients of Ala-2-NH including Tyr-3-NH in 1⁷⁾ were revealed to be a larger value than 4×10^{-3} ppm/K. Therefore, it was suggested that these NH protons were not involved in the intramolecular hydrogen bond.⁹⁾ On the other hand, the protons of Ala-1-NH were divided into two groups: compounds 1, 4 and 7 possessing the similar 14-membered conformation to that of RA-VII, and compounds 2, 3, 5 and 6, whose 14-membered conformations differ from that of RA-VII by the ring rotation of Tyr-6. It was thus indicated that D-Ala-1-NH of the former group was not involved in intramolecular hydrogen bond, however, the latter group formed a weak intramolecular hydrogen bond between D-Ala-1-NH and Ala-4-CO. From the above findings, the conformation of 14-membered ring

TABLE IV. Cytotoxic Activity of Metabolites against P388 Cell Line

Compound	IC ₅₀ (μg/ml)
1	0.09
2	4.53
3	3.80
4	0.17
5	12.20
6	7.02
7	> 30

TABLE V. Antitumor Activity^{a)} of Compounds 1 and 4 on P388 Leukemia

Compound	Dose (mg/kg)	Route	T/C (%)
1	3.5	i.p.	113
4	6.3	i.p.	154

^{a)} P388 was implanted i.p. (1×10^6 cells cm⁻³) in CDF₁ mice at day 0. Drugs were given daily at indicated doses for 9 consecutive days from day 1 to 9. T/C, test group/control.

was considered to affect that of 18-membered ring, especially the intramolecular hydrogen bond between D-Ala-1-NH and Ala-4-CO.

Cytotoxic activity of above metabolites from RA-VII *in vitro* and *in vivo* against P-388 cells and the antitumor activity against P-388 leukemia in mice are listed in Tables IV and V. Compounds 1 and 4, in particular, showed strong cytotoxic activity, though weaker than those of RA-VII. Compound 4 also showed significant antitumor activity against P-388 leukemia in mice at the dose of 6.3 mg/kg. However, Petroski *et al.* reported that O-desmethylbouvardin and bouvardin catechol derived from microorganic biotransformation of bouvardin did not show antitumor activity at all.¹⁰⁾ Consequently, it was thought that methoxy-methyl group at Tyr-3 was necessary to show antitumor activity *in vivo*. It was also suggested that the N-methyl group of Tyr-3 was important for the demonstration of antitumor activity because compound 1, which was locked to the type II β-turn conformation between Ala-2 and Tyr-3, did not show this activity. The appearance of antitumor activity may therefore be involved not in type II β-turn but in type VI β-turn with *cis* N-methyl amide bond between Ala-2 and Tyr-3.

Furthermore, comparing the cytotoxic activity of 2 with that of 5, 3 with 6, 4 with 5 and 4 with 7, the additional hydroxylation in metabolism *in vivo* decreased the cytotoxic activity. Therefore, the hydroxylation and demethylation reactions *in vivo* are considered to be a bioinactivation process.

Studies on the synthesis of conformationally constrained derivatives and their antitumor activity are in progress.

Experimental

All melting points were recorded on a Yanagimoto micro melting point apparatus and are uncorrected. Spectral data were obtained on the following instruments: optical rotation on a JASCO DIP-4, IR on a JASCO A-302 and Perkin Elmer 1710, UV on a Hitachi 557, NMR on a Bruker AM-400 and AM-500 and MS on a Hitachi M-80 and VG AutoSpec. MPLC was carried out on a CIG column system (Kusano Scientific Co., Tokyo) with 10 μm silica gel (22 mm i.d. × 100 mm) as the stationary phase. Reversed phase HPLC was carried out on a YMC D-ODS-5 (20 mm i.d. × 250 mm) and TSKgel ODS-80T_M (21.5 mm i.d. × 300 mm) using a JASCO HPLC 880-PU. Thin layer chromatography (TLC) was run on

0.25 mm silica gel (60 F₂₅₄, Merck) or RP-18 (F_{254s}, Merck) plates. Silica gel column chromatography was carried out on Kieselgel 60 (50–100 times the amount of the sample).

Materials RA-VII and RA-X used in this experiment were obtained as reported previously.¹⁾ The purity was confirmed by means of the HPLC and NMR to be over 99.8%. Cofactor for S-9 Mix was purchased from Boehringer Mannheim GmbH, phenobarbital sodium salt and pentobarbital sodium salt from Tokyo Kasei Kogyo Co., sodium carboxymethyl cellulose (CMC) and sodium dodecyl sulfate (SDS) from Kanto Chemical Co., and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma Chemical Co.

Preparation of Liver Homogenate Fraction S-9 We employed the procedure of Ames *et al.*¹¹⁾ Male rats (Sprague-Dawley strain, 5–7 weeks old, Saitama Animal Laboratories Co.) were maintained on Clea Japan Co. CE-2 chow. For a week before they were killed, 0.05% of sodium phenobarbital was given as their drinking water. The rats were killed by a blow to the head, and the liver was removed and washed thoroughly with ice-chilled 1.15% KCl to remove residual blood. All following steps were performed at 0–4°C with cold solutions and glassware. The liver was minced with scissors in three volumes of 1.15% KCl (3 ml/g of wet liver) and homogenized using a tissue grinder with a Teflon pestle. The homogenate was centrifuged (Kubota KR-20000T) for 20 min at 10500 × g, and the supernatant, which we call the S-9 fraction, was decanted and saved. The fresh S-9 fractions were quickly frozen in dry ice, and stored at –80°C in a freezer. As required, sufficient S-9 fraction was thawed at room temperature and kept on ice; the unused portion was discarded at the end of the day.

Incubation Conditions and Extraction of Metabolites RA-VII (5 mg) dissolved in 2.5 ml of dimethylsulfoxide was incubated with 10 ml of the S-9 fraction and 40 ml of cofactor solution for S-9 Mix (MgCl₂ 8 μmol/ml, KCl 33 μmol/ml, glucose-6-phosphate 5 μmol/ml, NADPH 4 μmol/ml, NADH 4 μmol/ml, sodium phosphate buffer pH 7.4 100 μmol/ml) at 37°C for 5 h. After the usual excision of protein with 80 ml of acetonitrile, the reaction mixture was extracted with 70 ml of chloroform three times. The concentrated extract was subjected to HPLC.

Extraction of Biliary Metabolites Male rabbits (2.5–3.0 kg, NZW or JW strains) were used. For four days before they were examined, 0.05% of sodium phenobarbital was given as their drinking water. For the identification of metabolites, bile duct cannulation was performed with a polyethylene tube under anaesthesia with sodium pentobarbital (30 mg/kg i.v.); the rabbits were held in a Bollman cage after the intravenous administration of RA-VII (35 mg) or RA-X-Na (35 mg), which was suspended in 0.1% CMC physiological saline solution. Bile was collected for 3 consecutive hour on ice. Non-hydrolyzed biliary juice was extracted with the same amount of chloroform in the case of RA-VII and with 1-butanol in RA-X-Na three times, respectively. Each concentrated extract was applied to HPLC.

1) Physical and spectral data were shown in a previous paper.⁷⁾

2) Colorless needles, C₄₁H₅₀N₆O₁₀, mp > 300°C, [α]_D –151.1° (c = 0.9, CHCl₃). EI-MS *m/z* (%): 786 (M⁺, 5), 729 (5), 524 (7), 164 (66), 121 (100).

3) Colorless needles, C₄₁H₅₀N₆O₁₀, mp > 300°C, [α]_D –137.5° (c = 0.4, CHCl₃). FAB-MS *m/z* (%): 787 (M⁺ + 1, 7), 327 (6), 224 (12), 164 (52), 134 (100).

4) Colorless powder, C₄₁H₅₀N₆O₁₀, mp 240–247°C, [α]_D –174.4° (c = 0.9, CHCl₃). EI-MS *m/z* (%): 786 (M⁺, 15), 729 (6), 311 (37), 180 (100), 137 (86).

5) Colorless powder, C₄₁H₅₀N₆O₁₁, mp > 300°C, [α]_D –125.0° (c = 0.2, CHCl₃). FAB-MS *m/z* (%): 825 (M⁺ + Na, 4), 803 (M⁺ + 1, 4), 787 (5), 180 (31), 134 (100).

6) Colorless powder, C₄₀H₄₈N₆O₁₀, mp > 300°C, [α]_D –60.5° (c = 0.4, MeOH). FAB-MS *m/z* (%): 795 (M⁺ + Na, 26), 773 (M⁺ + 1, 36), 327 (13), 185 (23), 134 (100).

7) Colorless powder, C₄₀H₄₈N₆O₁₀, mp > 300°C, [α]_D –195.5° (c = 0.4, MeOH). FAB-MS *m/z* (%): 795 (M⁺ + Na, 6), 773 (M⁺ + 1, 27), 297 (26), 207 (14), 180 (100).

8) Colorless powder, C₃₉H₄₆N₆O₉, mp > 300°C, [α]_D –250.0° (c = 0.3, MeOH). FAB-MS *m/z* (%): 743 (M⁺ + 1, 8). ¹H-NMR (CD₃OD) δ: 1.09 (3H, d, J = 6.7 Hz), 1.24 (3H, d, J = 7.0 Hz), 1.30 (3H, d, J = 6.8 Hz), 2.63 (3H, s), 2.68 (1H, dd, J = 2.9, 11.5 Hz), 2.93 (3H, s), 2.96–3.10 (2H, complex), 3.06 (3H, s), 3.20 (1H, dd, J = 4.9, 13.9 Hz), 3.27 (1H, dd, J = 10.8, 14.2 Hz), 3.58 (1H, dd, J = 11.4 Hz), 3.84 (1H, dd, J = 5.2, 10.8 Hz), 4.45 (1H, m), 4.60 (1H, d, J = 2.1 Hz), 4.62 (1H, m), 4.75 (1H, dd, J = 1.9, 6.7 Hz), 4.82 (1H, m), 5.46 (1H, dd, J = 3.2, 11.4 Hz), 6.55 (1H, dd, J = 2.1,

8.2 Hz), 6.71–6.76 (3H, complex), 6.80 (1H, dd, J = 2.4, 8.4 Hz), 7.00 (2H, d, J = 8.5 Hz), 7.21 (1H, dd, J = 2.2, 8.5 Hz), 7.27 (1H, dd, J = 2.4, 8.4), 7.46 (1H, dd, J = 2.2, 8.4 Hz).

9) Colorless powder, C₄₆H₄₈N₆O₁₀, mp > 300°C, [α]_D –179.4° (c = 0.3, MeOH). FAB-MS *m/z* (%): 795 (M⁺ + Na, 7), 773 (M⁺ + 1, 12). ¹H-NMR (CD₃OD) δ: 1.03 (3H, d, J = 6.8 Hz), 1.29 (3H, d, J = 7.0 Hz), 1.30 (3H, d, J = 6.8 Hz), 2.27 (1H, d, J = 13.3 Hz), 2.77 (3H, s), 2.89 (3H, s), 2.90 (3H, s), 2.99 (1H, dd, J = 11.6, 15.2 Hz), 3.21–3.28 (3H, complex), 3.79–3.88 (2H, complex), 3.89 (3H, s), 4.52 (1H, m), 4.66 (1H, m), 4.76 (1H, m), 5.01 (1H, dd, J = 5.3, 11.5 Hz), 5.63 (1H, d, J = 9.7 Hz), 6.41 (1H, d, J = 1.7 Hz), 6.72–6.76 (4H, complex), 6.85 (1H, d, J = 8.2 Hz), 6.92 (1H, dd, J = 1.9, 8.0 Hz), 6.97–7.01 (3H, complex).

Assay of Cytotoxic Activity Using P-388 Cells P-388 cells, supplied by Dr. S. Tsukagoshi of the Japan Foundation for Cancer Research, were maintained in RPMI-1640 medium (Nissui Pharm. Co., Ltd.) supplemented with 5% fetal calf serum (Mitsubishi Chemical Industry Co., Ltd.) and kanamycin (100 μg/ml). The cells (3 × 10³ cells/well) were cultured in Corning disposable 96-well plates containing 100 μl of growth medium per well and were incubated at 37°C in a humidified atmosphere of 5% CO₂. Various drug concentrations (10 μl) were added to the cultures at day 1 after the transplantation. At day 3, 20 μl of MTT solution (5 mg/ml) per well was added to each cultured medium. After a further 4 h of incubation, 100 μl of 10% SDS–0.01 N HCl solution was added to each well and the formazan crystals in each well were dissolved by stirring with a pipette. The optical density measurements were made using a microplate reader (Tohso MPR-A4i) with a two wavelength system (550 and 700 nm). In all these experiments, 3 replicate wells were used to determine each point.¹²⁾

Assay of Activity Against P-388 Lymphocytic Leukemia CDF₁ male mice, 5 weeks old, supplied by Japan Charles River Co., Ltd., were used in groups of 6 animals. P-388 lymphocytic leukemia was implanted i.p. at 1 × 10⁶ cells/mouse. A test drug was given i.p. at day 1 after the implantation and continued for 9 d. The effectiveness was evaluated in terms of the increase of lifespan (ILS, T/C%).

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