PREPARATION OF [Cys^2]-RA-VII; THE FIRST SULFUR CONTAINING ANALOGUE OF RAS POSSESSING ANTITUMOR ACTIVITY[1]

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Abstract - RA-III (**2**), a potent antitumor cyclic hexapeptide, has been converted to [Cys^2]-RA-VII (**5**) *via* thiolacetate (**3**). Derivatives (**3**) and (**5**) showed antitumor activities against P388 leukemia cells *in vitro* and *in vivo*.

RA-VII (**1**), a bicyclic hexapeptide isolated from *Rubia akane* and *R. cordifolia*, has been attracted much attention by its unique structure and potent antitumor activities.[2] Recently the RA-VII congeners in which Ala^2 (alanine at position 2) is replaced by other amino acids such as serine (RA-III, **2**),[2] threonine,[3] glutamic acid or pyroglutamic acid,[4] have been isolated as minor constituents from the latter plant. The observation that these compounds show different cytotoxicities against tumor cells suggests that the side chain of the 2 nd amino acid has some influence on expressing those activities. These findings prompted us to prepare the cysteine containing RA derivative since such sulfur containing RAs had not been observed in natural resources. Also some changes in biological properties were expected by the incorporation of cysteine in place of Ala^2, as -SH group of the cysteine residue played critical roles especially in enzymatic processes in living organs.

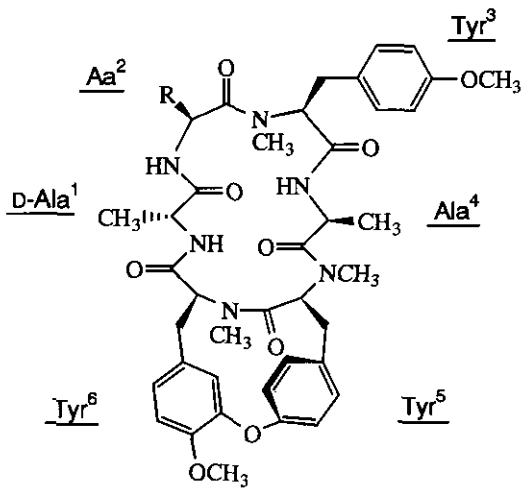
To prepare the cysteinyl derivative, [Cys^2]-RA-VII (**5**), we selected RA-III (**2**) as a starting material. The hydroxyl group of the serine residue of **2** was replaced by acetothiol group under the modified Mitsunobu condition[5] using thiolacetic acid (2 equiv.), triphenylphosphine (3 equiv.) and di-

Table 1. ^{13}C-Nmr Chmical Shifts and Assignments of **2**, **3**, **4** and **5** in $CDCl_3$ at 303 K (major conformer,100 MHz, δ)

Amino acid	carbon	**2**	**3**	**4**[a)]	**5**
D-Ala[1]	C_α	47.72	47.96	48.26	48.02
	C_β	20.85	20.63	21.13	21.01
Aa[2]	C_α	49.31	49.09	48.39	50.79
	C_β	61.65	29.95	39.03	25.40
	Cc=o		195.47		
	C_{Me}		30.49		
Tyr[3]	C_α	68.61	68.68	69.39	68.96
	C_β	32.66	32.19	33.21	32.97
	C_γ	130.44	130.71	131.09	130.72
	C_δ	130.10	130.30	130.84	130.34
	C_ε	114.08	114.14	114.63	114.11
	C_ζ	158.38	158.50	159.01	158.44
	C_N	40.16	39.93	40.43	40.21
	C_O	55.19	55.28	55.53	55.27
Ala[4]	C_α	46.26	46.38	46.86	46.31
	C_β	18.54	18.60	18.47	18.67
Tyr[5]	C_α	54.29	54.23	54.92	54.16
	C_β	36.85	37.04	37.25	37.03
	C_γ	135.06	135.08	135.65	135.02
	$C_{\delta1}$	132.72	132.78	133.02	132.78
	$C_{\delta2}$	130.84	130.97	131.28	130.97
	$C_{\varepsilon1}$	124.19	124.28	124.66	124.29
	$C_{\varepsilon2}$	125.87	125.93	126.53	125.92
	C_ζ	158.23	158.30	158.85	158.26
	C_N	30.47	30.55	30.71	30.48
Tyr[6]	C_α	57.33	57.48	58.00	57.47
	C_β	35.46	35.40	35.25	35.28
	C_g	128.12	128.18	128.97	128.09
	$C_{\delta1}$	120.92	120.91	121.62	120.89
	$C_{\delta2}$	113.41	113.50	114.20	113.43
	$C_{\varepsilon1}$	112.38	112.42	113.15	112.36
	$C_{\varepsilon2}$	153.09	153.18	153.55	153.14
	C_ζ	146.50	146.57	147.03	146.53
	C_N	29.30	29.24	29.71	29.18
	C_O	56.13	56.21	56.52	56.18
	Cc=o	167.88	167.64	169.11	167.60
		169.22	169.46	170.50	169.50
		170.80	170.40	171.17	170.55
		171.59	170.63	171.39	170.83
		171.63	171.72	172.16	171.68
		172.55	172.42	172.75	172.12

[a)] In $CDCl_3$:CD_3OD=1:1

isopropyl azodicarboxylate (3 equiv.) in CH_2Cl_2 (room temperature, 66 h) to afford the thiolacetate (**3**) in 80% yield. Attempt to deacetylate the thiolacetate (**3**) by alkaline hydrolysis (2N KOH in tetrahydrofuran-H_2O) or by $LiBH_4$ reduction in tetrahydrofuran was unsuccessful. However, treatment of **3** with concentrated NH_4OH in MeOH in a sealed tube under argon at 60°C for 7.5 h afforded **4** in 46% yield, possibly formed *via* cysteine derivative (**5**) which was susceptible to oxidation under the basic conditions. The structure of **4** was suggested by the observations of a molecular ion peak $(M+K)^+$ of 1641 in FAB mass spectrum and the Cβ resonance at δ39.03 ppm, a typical value for cystin derivatives,[6] in the ^{13}C-nmr spectrum of **4** (Table 1). Also the observation of three conformers in ^1H and ^{13}C-nmr spectra in $CDCl_3$ solution in a ratio of 12:11:1 supported the dimeric structure for **4**. Since in $CDCl_3$ solution, natural RAs exist in two stable conformations, conformers A and B, characterized by trans and cis amide bond between Aa^2 (amino acid at position 2) and Tyr^3, respectively,[7] this phenomenon can be explained by assuming three conformational pairs (conformers A-A, A-B, and B-B) for observed conformers linked by a disulfide bond. The disulfide bond of **4** was reductively cleaved with $NaBH_4$ (2.5 mol equiv.) in dioxane-H_2O (1:1) at room temperature for 6 h to afford **5** in 41% yield. *The spectral data obtained for* **5**



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1 : CH_3 (RA-VII)
2 : CH_2OH (RA-III)
3 : CH_2SAc
4 : $CH_2S\text{-})_2$
5 : CH_2SH

Table 2. Cytotoxicity of **2**, **3**, **4** and **5** Against P388 Leukemia and KB Cells.

Compound	IC_{50}(μg/ml) P388	KB
2 (RA-III)	0.011	0.024
3	0.0083	0.0060
4	0.52	0.37
5	0.29	0.31

Table 3. Antitumor Activity of **2**, **3** and **5** Against P388 Leukemia in Mice.

	T/C(%) Dose (mg/Kg)			
Compound	0.4	1.6	6.25	12.5
2 (RA-III)	149	156	160	159
3	131	147	150	N.T.
5	129	147	157	35

Dose i.p. on days 1-5. N.T. = not tested.

were satisfactorily assignable to its structure.

[Cys^2]-RA-VII (**5**), its intermediates (**3**) and (**4**), and the parent compound RA-III (**2**) were evaluated by *in vitro* cytotoxicities against P388 leukemia and KB cells (Table 2), and partly by *in vivo* antitumor activities against P388 leukemia (Table 3). The most cytotoxic of the new compounds presented here is thiolacetate (**3**), which is 4 times as toxic as parent RA-III (**2**) against KB cells. On the contrary, cystin derivative (**4**) and [Cys^2]-RA-VII (**5**) are about 15-50 times less toxic than **3** against P388 and KB cells. Interestingly, in spite of a large difference in cytotoxicity, **3** and **5** show similar antitumor activities in *in vivo* experiments.

EXPERIMENTAL

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Infrared (ir) spectra were taken on a Perkin Elmer 1710 spectrophotometer. Optical rotations were measured with a JASCO DIP-4 polarimeter. ^{1}H and ^{13}C-Nmr spectra were recorded on a Bruker AM-400 spectrometer. Chemical shifts were expressed in ppm with tetramethylsilane as an internal standard. The mass spectra (ms) were taken with a Hitachi M-80 and VG AutoSpec spectrometer. The ultraviolet (uv) and visible absorption spectra were recorded on a Shimadzu UV-240 spectrophotometer.

[S-Acetyl-Cys^2]-RA-VII (3). To a solution of **2** (78.7 mg, 0.10 mmol) and thiolacetic acid (14 µl, 0.20 mmol) in CH_2Cl_2 (5 ml) was added a solution of triphenylphosphine (79.1 mg, 0.30 mmol) and diisopropyl azodicarboxylate (59 µl, 0.30 mmol) in CH_2Cl_2 (5 ml) at 0°C. The mixture was stirred at room temperature for 66 h. The mixture was diluted with CH_2Cl_2 and washed successively with 1N HCl, sat. $NaHCO_3$ and brine, and dried over $MgSO_4$. The solvent was removed *in vacuo* to leave a residue which was chromatographed on silica gel eluting with CH_2Cl_2-AcOEt-MeOH (12:2:1), then CH_2Cl_2-acetone (4:1), followed by recrystallization from MeOH to give **3** (67.9 mg, 80%) as a colorless powder, mp 218-219°C, $[\alpha]_D$ −100.7°(c=0.15, $CHCl_3$). Ir ν($CHCl_3$): 3406, 1678, 1636, 1096 cm^{-1}. Uv λ_{max}(EtOH) nm(log ε): 220(4.55), 277(3.67), 283(3.66). High-resolution FAB-ms Calcd for $C_{43}H_{53}N_6O_{10}S$: 845.3544 $[M+H]^+$, Found: 845.3526. FAB-ms m/z (%): 844(10, M^+), 121(100). ^{1}H-Nmr(400 MHz, $CDCl_3$, major conformer, δ): 1.12(3H, d, J=6.6 Hz, Ala^4-Hβ), 1.28(3H, d, J=6.9 Hz, Ala^1-Hb), 2.36(3H, s, Cys^2-SCOMe), 2.63(1H, dd, J=11.3, 3.2 Hz,

Tyr^5-Hβa), 2.67(3H, s, Tyr^6-NMe), 2.92(3H, s, Tyr^3-NMe), 2.98-3.08(2H, m, Tyr^6-Hβ), 3.12(3H, s, Tyr^5-NMe), 3.32(1H, dd, J=14.3, 11.1 Hz, Tyr^3-Hβa), 3.43(1H, dd, J=14.3, 4.7 Hz, Tyr^3-Hβb), 3.61(1H, dd, J=11.1, 4.7 Hz, Tyr^3-Hα), 3.66(1H, t, J=11.3 Hz, Tyr^5-Hβb), 3.79(3H, s, Tyr^3-OMe), 3.93(3H, s, Tyr^6-OMe), 4.35(1H, d, J=1.9 Hz, Tyr^6-Hδa), 4.39(1H, m, Cys^2-Hβa), 4.40(1H, quintet, J=6.9 Hz, Ala^1-Hα), 4.54(1H, dd, J=11.8, 3.6 Hz, Tyr^6-Hα), 4.71(1H, dd, J=6.8, 2.3 Hz, Cys^2-Hβb), 4.72(1H, dq, J=9.1, 6.6 Hz, Ala^4-Hα), 4.88(1H, m, Cys^2-Hα), 5.38(1H, dd, J=11.3, 3.2 Hz, Tyr^5-Hα), 6.38(1H, d, J=6.9 Hz, Ala^1-NH), 6.58(2H, m, Cys^2-NH and Tyr^6-Hδb), 6.71(1H, d, J=9.1 Hz, Ala^4-NH), 6.80(1H, d, J=8.9 Hz, Tyr^6-Hε), 6.85(2H, d, J=8.4 Hz, Tyr^3-Hε), 6.88(1H, dd, J=8.4, 2.2 Hz, Tyr^5-Hεa), 7.11(2H, d, J=8.4 Hz, Tyr^3-Hδ), 7.20(1H, dd, J=8.4, 2.2 Hz, Tyr^5-Hεb), 7.26(1H, dd, J=8.4, 2.0 Hz, Tyr^5-Hδa), 7.41(1H, dd, J=8.4, 2.0 Hz, Tyr^5-Hδb).

[Cys^2]-RA-VII [Cys^2]-RA-VII (4). To a solution of **3** (65 mg, 0.077 mol) in MeOH (2 ml) was added 29% NH_4OH (2 ml), and the mixture was stirred at 60°C for 7.5 h under an atmosphere of argon in a sealed tube. The solvent was removed *in vacuo* to leave a residue which was chromatographed on silica gel eluting with CH_2Cl_2-AcOEt-MeOH (12:2:1), followed by recrystallization from MeOH to give **4** (28.2 mg, 46%) as a colorless powder, mp 278-280°C, $[\alpha]_D$ −161.4°(c=0.07, $CHCl_3$). Ir ν($CHCl_3$): 3405, 1677, 1634 cm^{-1}. Uv λ_{max}(EtOH) nm(log ε): 220(4.67), 279(3.86), 283(3.86), 293(3.65). FAB-ms m/z (%): 1641(5, M+K$^+$). ^{1}H-Nmr(400 MHz, $CDCl_3$, major conformer, δ): 1.11(3H, d, J=6.5 Hz, Ala^4-Hβ), 1.32(3H, d, J=7.0 Hz, Ala^1-Hβ), 2.63(1H, dd, J=11.2, 2.3 Hz, Tyr^5-Hβa), 2.66(3H, s, Tyr^6-NMe), 2.92(1H, dd, J=18.5, 2.8 Hz, Tyr^6-Hba), 2.99(3H, s, Tyr^3-NMe), 3.00-3.15(3H, m, Cys^2-Hβ, and Tyr^6-Hβb), 3.12(3H, s, Tyr^5-NMe), 3.23(1H, dd, J=14.2, 11.0 Hz, Tyr^3-Hβa), 3.42(1H, dd, J=14.2, 4.2 Hz, Tyr^3-Hβb), 3.58(1H, dd, J=11.0, 4.2 Hz, Tyr^3-Hα), 3.67(1H, t, J=11.2 Hz, Tyr^5-Hβb), 3.76(3H, s, Tyr^3-OMe), 3.94(3H, s, Tyr^6-OMe), 4.32(1H, d, J=2.0 Hz, Tyr^6-Hδa), 4.45(1H, qd, J=7.0, 6.9 Hz, Ala^1-Hα), 4.55(1H, dd, J=9.9, 2.8 Hz, Tyr^6-Hα), 4.70(1H, dq, J=6.8, 6.9 Hz, Ala^4-Hα), 5.07-5.13(1H, m, Cys^2-Hα), 5.37(1H, dd, J=11.2, 2.3 Hz, Tyr^5-Hα), 6.36(1H, d, J=6.9 Hz, Ala^1-NH), 6.57(1H, dd, J=8.3, 2.0 Hz, Tyr^6-Hδb), 6.72(1H, d, J=8.2 Hz, Ala^4-NH), 6.80(1H, d, J=8.6 Hz, Tyr^6-Hε), 6.81(1H, d, J=8.3 Hz, Cys^2-NH), 6.82(2H, d, J=8.6 Hz, Tyr^3-Hε), 6.87(1H, dd, J=8.4, 2.4 Hz, Tyr^5-Hεa), 7.09(2H, d, J=8.6 Hz, Tyr^3-Hd), 7.21(1H, dd, J=8.4, 2.1 Hz, Tyr^5-Hεb), 7.26(1H, dd, J=8.4, 2.1 Hz, Tyr^6-Hδa), 7.40(1H, dd, J=8.3, 2.1 Hz, Tyr^6-Hδb).

[Cys^2]-RA-VII (5). To a solution of **4** (30.5 mg, 0.019 mmol) in 50% dioxane (2 ml) was added $NaBH_4$ (2.0 mg, 0.053 mmol), and the mixture was stirred at room temperature for 6 h. The mixture was diluted with H_2O and extracted with CH_2Cl_2. The extracts were washed successively with 1N HCl, sat. $NaHCO_3$ and brine, and dried over $MgSO_4$. The solvent was removed *in vacuo* to leave a residue which was chromatographed on silica gel eluting with CH_2Cl_2-AcOEt-MeOH (12:2:1), followed by recrystallization from MeOH to give **5** (12.6 mg, 41%) as a colorless powder, mp 279-280°C, $[\alpha]_D$ −200.7°(c=0.38, $CHCl_3$). Ir ν($CHCl_3$): 3403, 1675, 1636 cm^{-1}. Uv λ_{max}(EtOH) nm(log ε): 220(4.50), 277(3.59), 284(3.52). Anal. Calcd for $C_{41}H_{51}N_6O_9S\cdot 1/2H_2O$: C,60.65; H,6.33; N,10.35. Found: C,60.54; H,6.17; N,10.13. ^{1}H-Nmr(400MHz, $CDCl_3$, major conformer, δ): 1.12(3H, d, J=6.6 Hz, Ala^4-Hβ), 1.30(3H, d, J=6.9 Hz, Ala^1-Hβ), 2.63(1H, dd, J=11.3, 3.2 Hz, Tyr^5-Hβa), 2.66(3H, s, Tyr^6-NMe), 2.70-2.79(1H, m, Cys^2-Hβa), 2.85-2.94(1H, m, Cys^2-Hβb), 3.02(3H, s, Tyr^3-NMe), 3.04-3.11(2H, m, Tyr^6-Hβ), 3.13(3H, s, Tyr^5-NMe), 3.29(1H, dd, J=14.2, 10.9 Hz, Tyr^3-Hβa), 3.45(1H, dd, J=14.2, 4.2 Hz, Tyr^3-Hβb), 3.66(1H, t, J=11.3 Hz, Tyr^5-Hβb), 3.78(3H, s, Tyr^3-OMe), 3.93(3H, s, Tyr^6-OMe), 4.34(1H, d, J=1.8 Hz, Tyr^6-Hδa), 4.40(1H, dd, J=7.0, 6.9 Hz, Ala^1-Hα), 4.53(1H, dd, J=11.6, 3.8 Hz, Tyr^6-Hα), 4.71(1H, dq, J=7.2, 6.6 Hz, Ala^4-Hα), 4.93(1H, td, J=8.7, 6.3 Hz, Cys^2-Hα), 5.38(1H, dq, J=11.3, 3.2 Hz, Tyr^5-Hα), 6.39(1H, d, J=7.0 Hz, Ala^1-NH), 6.57(1H, dd, J=8.4, 1.8 Hz, Tyr^6-Hδb), 6.78(1H, d, J=8.4 Hz, Tyr^6-Hε), 6.82(2H, m, Cys^2-NH and Ala^4-NH), 6.82(2H, d, J=8.6 Hz, Tyr^3-Hε), 6.87(1H, dd, J=8.4, 2.4 Hz, Tyr^5-Hεa), 7.12(2H, d, J=8.6 Hz, Tyr^3-Hδ), 7.20(1H, dd, J=8.4, 2.4 Hz, Tyr^5-Hεb), 7.26(1H, dd, J=8.6, 2.2 Hz, Tyr^5-Hδa), 7.40(1H, dd, J=8.4, 2.2 Hz, Tyr^5-Hεb).

Cell survival by MTT assay. MTT colorimetric assay was performed in a 96-well plate.[8] The assay is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. Human KB oral epidermoid carcinoma cells (1x10^4 cells/ml) or mouse P388 leukemia cells (2x10^4 cells/ml) were inoculated in each well with 100 μl of RPMI1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, UK), 100 units/ml of penicillin and 100 μg/ml of streptomycin. After overnight incubation (37°C, 5% CO_2), 100 μl of sample solution was added to each well and the plates were incubated for 3 days (KB) or 2 days (P388). Then 50 μl of MTT (200 μg/ml PBS) was added to each well and the plates were incubated for a further 4 h. The

resulting formazan was dissolved in 150 μl of DMSO. The plates was placed on a plate shaker for 5 min and read immediately at 540 nm. The IC_{50} (μg/ml) value was defined as that concentration of sample which caused 50% reduction of growth in sample-treated cells, with respect to the controls. The IC_{50} was calculated by using the probit test.

***In vivo* antitumor activity.** P388 murine leukemia cells ($1x10^6$ cells) were inoculated i.p. into female CDF_1 mice (6-7 weeks old, control :n=16; test :n=8) on day 0. Samples, suspended in 0.5% gum arabic-saline solution, were administered i.p. on days 1-5. The antitumor activity was estimated according to the NCI tumor panel screening method.[9]

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