New Antitumour Bicyclic Hexapeptides, RA-IX and -X from *Rubia cordifolia*. Part 3.[1] Conformation–Antitumour Activity Relationship

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RA-IX and -X, new antitumour bicyclic hexapeptides were isolated from *Rubia cordifolia* and their structures were elucidated by spectroscopic and chemical evidences. RA-X containing a glutamic acid at residue 2 showed strong antitumour activity. However, RA-IX with conformation restricted to a type II β-turn at residues 2 and 3 showed almost no antitumour activity. Their conformation–antitumour activity relationships are discussed.

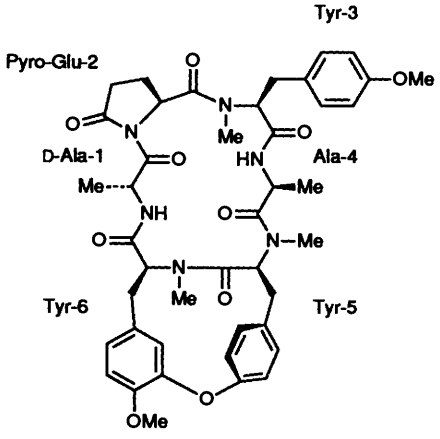
Antitumour bicyclic hexapeptides of the RA series (RA-I-VIII,[1,2] RAI-III and -VI[3]) have been isolated from *Rubia cordifolia* (Rubiaceae) and their structures and activities[4] have already been reported. From their conformational properties,[1,5] the main active principle, RA-VII has two stable conformational states in an apolar solvent, such as $CDCl_3$, in which each conformer has a stable antiparallel conformation with two intramolecular hydrogen bonds between Ala-4 and D-Ala-1. These two conformational states could result from isomerization about the *N*-methyl amide bond between Ala-2 and Tyr-3. The main conformer A with a type II β-turn structure involving residues 2 and 3 was deduced to be the active one and the aromatic side chain of Tyr-3 over this turn, which does not rotate freely, was considered to play a very important role in its antitumour activity.

As a part of our program to relate conformations to antitumour activities, further examination of chemical constituents from *R. cordifolia* led us to isolate two new antitumour bicyclic hexapeptides, named as RA-IX and -X (Fig. 1). It is shown that RA-IX contains pyro-glutamic acid at the second position taking a stable antiparallel conformation in solution and RA-X contains glutamic acid at the same position. In this paper, the structure determination and conformational analysis of RA-IX and -X isolated from *R. cordifolia* and their conformation–activity relationships are discussed.

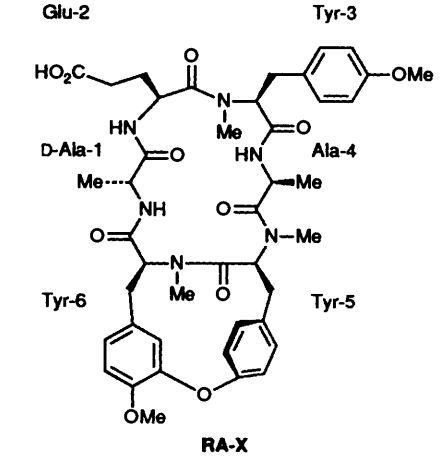
Results and Discussion

Structure Determination.—RA-IX, colourless needles, m.p. 242–243 °C, $[\alpha]_D^{20}$ −158.1° (*c* 0.94, $CHCl_3$), had the molecular formula, $C_{43}H_{51}N_6O_{10}$. The amino acid analysis of RA-IX by separation of optical isomers of Dns derivatives using the mixed chelate complex L-His-Cu^{II} showed that it contained D-Ala:L-Ala in the ratio of 1:1.[6] The COLOC spectrum providing proton–carbon long range couplings show that the composition and position of the constituent amino acids was the same as those of RA-VII except for the residue 2. Further, considering the following results, disappearance of one NH proton and appearance of one more carbonyl carbon in comparison to RA-VII, the presence of a pyro-glutamic acid residue at the second position was suggested. The NMR spectroscopic data indicate that RA-IX has a single stable conformational state in solution, *i.e.*, antiparallel β-pleated structure stabilized by 4–1 hydrogen bond. This conformation is suggested to be the same one as the main conformer in RA-VII.

RA-X, colourless needles, m.p. 254.5–255.5 °C, $[\alpha]_D^{20}$ −205.4° (*c* 1.43, $CHCl_3$–MeOH, 1:1), with molecular formula, $C_{43}H_{53}N_6O_{11}$ exhibited similar spectral properties to RA-IX. The main difference from RA-IX is that it has two conformational states in solution (conformers A:B = 85:15 in $CDCl_3$). In the NMR spectra, the presence of a broad carbon signal appeared at 175.00 ppm and three NH protons were observed. Methylation with CH_2N_2 gave a mono methyl ester derivative of RA-X (RA-X-OMe, $C_{44}H_{55}N_6O_{11}$) and treatment with $NaHCO_3$ gave sodium salt of RA-X (RA-X-Na, $C_{43}H_{52}N_6O_{11}Na$), which was soluble in water. From the above results, glutamic acid is thought to be the residue at the second position. This deduced structure was corroborated by the ^1H–^{13}C COSY and COLOC spectra.



RA-IX



RA-X

Fig. 1 Structures of RA-IX and -X; Amino-acid residues are abbreviated by their conventional three-letter notations, i.e. Ala = alanine, Tyr = N-methyl-*O*-methyl-tyrosine, Glu = glutamic acid, Pyro-Glu = pyroglutamic acid

Table 1 ^1H and ^{13}C NMR chemical shifts in $CDCl_3$ at 303K (^1H: 400MHz, ^{13}C: 125MHz)[a]

		δ_H			δ_C		
						RA-X	
Amino acid	Proton	RA-IX	RA-X	Carbon	RA-IX	major(A)	minor(B)
D-Ala-1	H_α	5.51 $J_{\alpha\beta}$ 6.8	4.40 $J_{\alpha\beta}$ 6.8	C_α	48.93	47.90	47.90
	H_β	1.32 $J_{\alpha N}$ 7.8	1.28 $J_{\alpha N}$ 7.2	C_β	19.76	20.69	20.56
	H_N	6.47	6.63	$C_{C=O}$	173.71	172.77	171.68
Glu-2	H_α	4.95 $J_{\alpha\beta1}$ 0.0	4.86 $J_{\alpha\beta}$ 5.2	C_α	54.33	48.27	48.17
	$H_{\beta1}$	2.11 $J_{\alpha\beta2}$ 7.20	2.02 $J_{\beta\gamma}$ 4.7	C_β	21.56	25.60	27.34
	$H_{\beta2}$	2.14 $J_{\beta1\beta2}$ 10.3	2.02 $J_{\alpha N}$ 8.4	C_γ	32.06	29.75	29.61
	$H_{\gamma1}$	3.31 $J_{\beta1\gamma1}$ 1.8	2.37	C_δ	174.36	175.20	175.20
	$H_{\gamma2}$	2.55 $J_{\beta2\gamma1}$ 10.3	2.37	$C_{C=O}$	170.84	172.08	172.77
	H_N	$J_{\beta2\gamma2}$ 9.4	7.58				
		$J_{\gamma1\gamma2}$ 18.2					
Tyr-3	H_α	3.62 $J_{\alpha\beta1}$ 11.2	3.60 $J_{\alpha\beta1}$ 11.4	C_α	68.02	68.50	62.44
	$H_{\beta1}$(pro-*R*)	3.25 $J_{\alpha\beta2}$ 4.4	3.29 $J_{\alpha\beta2}$ 4.4	C_β	32.85	32.78	32.55
	$H_{\beta2}$(pro-*S*)	3.41 $J_{\beta1\beta2}$ 14.3	3.40 $J_{\beta1\beta2}$ 13.9	C_γ	130.53	130.40	130.40
	$2H_\delta$	7.07 $J_{\delta\varepsilon}$ 8.5	7.05 $J_{\delta\varepsilon}$ 8.4	C_δ	130.07	130.11	129.98
	$2H_\varepsilon$	6.85	6.82	C_ε	114.09	114.17	114.42
	Me_N	2.90	2.90	C_ζ	158.39	158.42	158.76
	Me_O	3.79	3.76	$C_{C=O}$	167.95	167.91	168.55
				C_N	39.56	39.87	29.91
				C_O	55.19	55.21	55.20
Ala-4	H_α	4.80 $J_{\alpha\beta}$ 6.7	4.72 $J_{\alpha\beta}$ 6.6	C_α	46.41	46.38	47.13
	H_β	0.94 $J_{\alpha N}$ 8.0	1.11 $J_{\alpha N}$ 7.4	C_β	17.92	18.17	17.44
	H_N	6.58	6.69	$C_{C=O}$	171.75	171.68	170.68
Tyr-5	H_α	5.45 $J_{\alpha\beta1}$ 11.3	5.38 $J_{\alpha\beta1}$ 11.6	C_α	54.62	54.21	54.63
	$H_{\beta1}$(pro-*S*)	3.69 $J_{\alpha\beta2}$ 2.5	3.64 $J_{\alpha\beta2}$ 3.6	C_β	36.70	36.84	36.49
	$H_{\beta2}$(pro-*R*)	2.62 $J_{\beta1\beta2}$ 11.3	2.63 $J_{\beta1\beta2}$ 11.2	C_γ	135.42	134.92	134.92
	$H_{\delta1}$	7.27 $J_{\delta1\delta2}$ 2.2	7.25 $J_{\delta1\delta2}$ 2.1	$C_{\delta1}$	132.72	132.80	132.80
	$H_{\delta2}$	7.40 $J_{\delta1\varepsilon1}$ 7.5	7.37 $J_{\delta1\varepsilon1}$ 8.4	$C_{\delta2}$	130.70	130.72	130.72
	$H_{\varepsilon1}$	6.86 $J_{\delta2\varepsilon2}$ 8.4	6.87 $J_{\delta2\varepsilon2}$ 8.4	$C_{\varepsilon1}$	124.04	124.23	124.23
	$H_{\varepsilon2}$	7.19 $J_{\varepsilon1\varepsilon2}$ 2.4	7.22 $J_{\varepsilon1\varepsilon2}$ 2.4	$C_{\varepsilon2}$	125.79	125.91	125.91
	Me_N	3.06	3.12	C_ζ	158.12	158.28	158.28
				$C_{C=O}$	168.68	169.42	169.42
				C_N	30.53	30.47	30.47
Tyr-6	H_α	4.54 $J_{\alpha\beta1}$ 11.9	4.58 $J_{\alpha\beta1}$ 10.0	C_α	57.13	57.35	57.77
	$H_{\beta1}$(pro-*R*)	3.15 $J_{\alpha\beta2}$ 3.8	3.04 $J_{\alpha\beta2}$ 4.0	C_β	35.52	35.32	35.55
	$H_{\beta2}$(pro-*S*)	2.88 $J_{\beta1\beta2}$ 18.0	2.98 $J_{\beta1\beta2}$ 12.0	C_γ	128.14	128.26	128.44
	$H_{\delta1}$	6.57 $J_{\delta1\delta2}$ 1.9	6.58 $J_{\delta1\delta2}$ 1.4	$C_{\delta1}$	121.07	120.92	120.92
	$H_{\delta2}$	4.36 $J_{\delta1\varepsilon1}$ 8.4	4.39 $J_{\delta1\varepsilon1}$ 8.4	$C_{\delta2}$	113.36	113.57	113.57
	$H_{\varepsilon1}$	6.79	6.78	$C_{\varepsilon1}$	112.35	112.40	112.40
	Me_N	2.74	2.65	$C_{\varepsilon2}$	153.02	153.05	153.05
	Me_O	3.92	3.92	C_ζ	146.51	146.48	146.48
				$C_{C=O}$	170.19	170.67	169.99
				C_N	29.45	29.26	29.26
				C_O	56.10	56.13	56.13

[a] *J* values are given in Hz.

The complete assignments of the ^1H and ^{13}C NMR resonances of RA-IX and X in $CDCl_3$ (Table 1) were carried out with a combination of 2D-NMR experiments (^1H–^1H COSY, ^1H–^{13}C COSY, and COLOC spectra). According to the NMR spectra of RA-IX, it exists in a single stable conformational state in apolar solvents such as $CDCl_3$ and also polar solvents such as [$^2H_6$]-DMSO. This is considered to be due to the constrained structure due to the five membered ring of the Pyro-Glu-2 residue. RA-X apparently exists in two conformational states in $CDCl_3$ (conformers A:B = 85:15) and [$^2H_6$]-DMSO (conformers A:B = 65:35), the minor conformer could not be unambiguously assigned since it is present in a small amount with overlapping and poorly resolved resonances. However, RA-X is clearly assigned as *cis* and *trans* configurations about the *N*-methyl amide bond between Glu-2 and Tyr-3 by the ^{13}C NMR resonances around Tyr-3 in comparison with those of RA-VII.[5] The remarkable sensitivity of *cis*/*trans* equilibria to the solvent environment and the heavy preponderance of *trans* isomers regarless of the polarity of the surroundings accord with the overwhelming preference of peptide bonds for the *trans* configuration.

Solution Conformation of RA-IX and -X.—NOE studies were undertaken to provide further support to the proposed type II β-turn conformation in RA-IX, which can be recognized by the observation of NOEs between the Pyro-Glu-Hα and Tyr-3-NCH_3, and between Tyr-3-Hα and Tyr-3-NCH_3. Both sets of NOEs are diagnostic of a type II β-turn structure. The observed intraresidue NOEs are also observed in [$^2H_6$]-DMSO. The observed intraresidue NOEs between Tyr-5-Hα and Tyr-6-Hα provide evidence in favour of the type VI β-turn structure between Tyr-5 and Tyr-6. In addition, we also observed a through-space interaction between the Tyr-5-NCH_3 and Ala-4-CH_3/Ala-4-Hα, suggesting a *trans* configuration of the *N*-

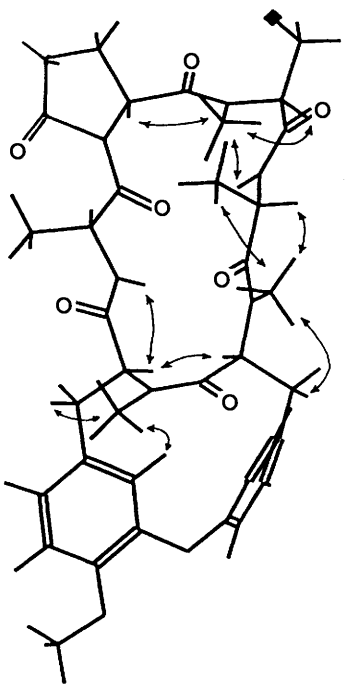


Fig. 2 NOE enhancements of RA-IX. The arrows show the NOE relationships confirmed by NOESYPH experiment in $CDCl_3$ at 303K.

Table 2 Effect of temperatures on the NH chemical shifts of RA-IX and X-OMe, $-\Delta\delta/\Delta T$ (10^3 ppmK^{-1})

Compounds	Solvent	D-Ala-1	Glu-2	Ala-4
RA-IX	$CDCl_3$	0.56	—	−0.21
	$[^2H_6]$-DMSO	4.89	—	−0.81
RA-X-OMe	$CDCl_3$	1.48	10.73	−0.17
	$[^2H_6]$-DMSO	4.17	5.53	−0.39

Table 3 NT1 values (ms) of the δ, ε and methoxy carbon atoms of Tyr-3 in RA-IX, X and X-OMe

	C_δ	C_ε	C_{OMe}
RA-IX	379	396	5658
RA-X	300	328	5359
RA-X-OMe	282	294	5072

Table 4 Cytotoxic activity (IC_{50} μg cm^{-3}) against KB and P-388 cell lines

	KB	P-388
RA-IX	0.30	0.37
RA-X	0.15	0.18
RA-X-OMe	0.02	0.05
RA-X-Na	0.09	0.08

methyl amide bond between Ala-4 and Tyr-5 in both compounds. The relationship in the NOE enhancements of RA-IX is shown in Fig. 2, and the same relationship for the main conformer A in RA-X is also obtained.

It is generally admitted that any NH group not exposed to an intermolecular interaction is likely to be involved in an intramolecular hydrogen bond.[7] For this reason, we examined the temperature effect on NH chemical shifts in $CDCl_3$ and $[^2H_6]$-DMSO solutions. The temperature coefficient ($-\Delta\delta/\Delta T$) values of RA-IX and X-OMe in Table 2 clearly show that Ala-4-NH is strongly shielded from the solvents, which is characteristic of a proton engaged in a strong hydrogen bond. Dependence of Ala-1-NH in $CDCl_3$ is stronger than in $[^2H_6]$-DMSO. This is due to association effects[8] occurring in a non-hydrogen bonding solvent like $CDCl_3$. In contrast, the Glu-2-NH of RA-X-OMe has a high $\Delta\delta/\Delta T$ value, suggesting that this group is exposed to solvent. This pattern of temperature effects is similar to those of RA-VII.[5] However, RA-IX, in particular, adopts an intramolecular antiparallel β-sheet structure in solution state and this structure is significantly populated in solvents such as $CDCl_3$ and $[^2H_6]$-DMSO. The results emphasize that the introduction of Pyro-Glu-2 fixes a type II β-turn with a definite stereochemical constraint between the carbonyl oxygen at Pyro-Glu-2 and the side chain of Tyr-3 (See below) and stabilized the antiparallel β-sheet conformation.

^{13}C spin-lattice relaxation times of protonated carbons are relatively easy to relate to dynamic properties of the molecules in solution, because the ^{13}C relaxation is mainly dominated by the ^{13}C–^1H dipolar interaction with directly bonded hydrogens.[9] In addition, to deduce the correlation between structure and biological activity, it is important to know about molecular flexibility. We have already pointed out that the NT1 values[10] of the δ, ε and methoxy carbon atoms of Tyr-3 in RA-VI, which are influenced by the tyrosyl ring rotation, are higher than the other RAs and this increased mobility may closely be related to their biological activities.[1] Those values observed in RA-IX, X, and X-OMe are listed in Table 3. The NT1 values of Tyr-3 increases in the order of RA-X-OMe, RA-X and RA-IX. This is in accord with the fact that the mobility due to rotation about Tyr-3 increases in this order. Furthermore, the molecular mobility elucidated by the NT1 values of the aromatic side chain is closely correlated with the cytotoxic activities as shown in Table 4.

Antitumour Activities.—The antitumour activities against P-388 are shown in Table 5. The present study indicated that pyro-glutamic acid is stereochemically constrained to restrict the conformational freedom of the peptide backbone in RA-IX. The most striking aspect of the present results is that no *in vivo* activity of RA-IX was observed. That is to say, RA-IX showed a weak cytotoxic activity but no antitumour activity. It is considered that the steric effect of the five membered-ring in the Pyro-Glu-2 residue causes the reducing of binding ability against ribosome 80s[11] or otherwise the minor conformer in RA-X may play an important role in the antitumour activity. On the other hand, RA-X, RA-X-OMe and RA-X-Na show the potent antitumour activity *in vivo* at the higher dose than the main active principle, RA-VII from *R. cordifolia*. We newly nominate the RA-X as an antitumour principle with less side effects.

Experimental

General Details.—M.p.s were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 spectrometer, $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Mass spectra were taken with a Hitachi M-80 spectrometer. UV and IR spectra were recorded on a Hitachi 557 spectrophotometer and a Perkin Elmer 1710 spectrophotometer, respectively. Medium-pressure liquid chromatography (MPLC) was performed with a CIG column system (22 mm i.d. × 100 mm, Kusano Scientific Co., Tokyo) packed with 10 μm silica gel. High-pressure liquid chromatography (HPLC) was performed with a Shim-pack PREP-SIL(L) (50 mm i.d. × 250 mm, Shimadzu Co., Kyoto) packed with 15 μm silica gel. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck). Spots on TLC were

Table 5 Antitumour activities[a] of RA-IX, -X, -X-OMe and -X-Na on P388 leukemia

Compound	Dose ($mgkg^{-1}$)	Route	Survival time (mean ± S.D.)	T/C (%)
Control	0.0	i.p.	8.6 ± 0.9	—
RA-IX	15.0	i.p.	8.8 ± 0.4	105.3
RA-X	1.5	i.p.	10.6 ± 1.1	126.3
	4.5	i.p.	12.0 ± 1.0	142.1
	15.0	i.p.	14.2 ± 2.5	159.7
	45.0	i.p.	3.8 ± 2.5	—
RA-X-OMe	1.5	i.p.	11.4 ± 0.5	131.6
	4.5	i.p.	12.8 ± 0.8	147.4
	15.0	i.p.	14.2 ± 0.4	160.5
	45.0	i.p.	2.0 ± 0.0	—
RA-X-Na	1.5	i.p.	10.2 ± 0.4	118.4
	4.5	i.p.	13.2 ± 2.8	142.1
	15.0	i.p.	14.2 ± 0.8	163.2
	45.0	i.p.	2.0 ± 0.0	—

[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 consecutive 9 d from day 1 to 9.

detected by their absorption under UV light. NMR coupling constants (J) are given in Hz.

Materials.—Commercial Rubiae Radix (220 kg, roots of *Rubia cordifolia*) purchased in China was extracted three times with $CHCl_3$–MeOH (1:1, 500 dm^3). The combined extracts were washed with water and evaporated to dryness under reduced pressure. The syrup obtained (7 kg) was purified in the same way as that reported in the previous paper [2] to give a crude RA fraction. This crude fraction was subjected to silica gel HPLC column chromatography using CH_2Cl_2–AcOEt–MeOH (20 : 2 : 1) as the mobil phase to give RA-IX (450 mg) and -X (1.1 g). R_f values were 0.51 for RA-IX and 0.11 for RA-X, for TLC performed with $CHCl_3$–MeOH (100:7). RA-IX: Colourless needles, m.p. 242–243 °C (from MeOH), $[\alpha]_D^{20}$ −158.1 (*c* 0.94, $CHCl_3$); *m/z* 811 (Found: 811.3659. Calc. for $C_{43}H_{51}N_6O_{10}$, $M^+ + 1$: 811.3667); λ_{max}(EtOH)/nm 279 (ε 4860); ν_{max}(KBr)/cm^{-1} 3394 (NH) and 1647 (amide).

RA-X; Colourless needles, m.p. 254.5–255.5 °C (from MeOH), $[\alpha]_D^{20}$ −205.4 (*c* 1.43, $CHCl_3$–MeOH, 1:1); *m/z* 829 (Found 829.3806 Calc. for $C_{43}H_{53}N_6O_{11}$, $M^+ + 1$: 829.3773); λ_{max}(EtOH)/nm 279 (ε 6600); ν_{max}(KBr)/cm^{-1} 3387 (NH) and 1661 (amide). The ^1H and ^{13}C NMR spectroscopic data of RA-IX and -X in $CDCl_3$ are shown in Table 1.

NMR Spectra.—The proton and carbon spectra were recorded on Bruker spectrometers (AM400 and AM500) and processed on a Bruker data station with an Aspect 3000 computer. Samples (10 mg) of RA-IX, -X, its methyl ester, and its sodium salt each dissolved in $CDCl_3$ or D_2O (0.5 cm^3) in a 5 mm tube were used for the homonuclear measurement and samples (30 mg) in $CDCl_3$ or D_2O (0.5 cm^3) in a 5 mm tube for the heteronuclear measurement. The spectra were recorded at 303 K. NOESYPH experiments were made with a mixing time of 0.6s. The measurements of $T1$ values were made by the inversion recovery method (180–t–90° pulse sequence) with 32 K data points at 303 K, allowing t to vary in the range 0.05–10.0s. $T1$ values were determined from the relaxation data by using the regression analysis that was incorporated in the $T1$ routine of the Bruker acquisition and processing program.

Acid Hydrolysis of RA-IX and -X.—Solutions of RA-IX and -X (each containing 5 mg of peptide) in 6 mol dm^{-3} HCl were heated at 110 °C for 14 h. After cooling, each solution was concentrated to dryness. The residue was dansylated with 2% $NaHCO_3$ (1 cm^3) and 5 mmol dm^{-3} dansyl chloride in acetone (0.5 cm^3) at 37 °C for 1 h. At the same time, authentic amino acids of L-Ala and D-Ala were also dansylated in the same manner. The dansyl amino acids were subjected to HPLC under the following condition: 4 mm i.d. × 250 mm (Nucleosil 5 μm) flow rate, 0.8 cm^3 min^{-1}, detection, λ/nm 340 solvent, 20% CH_3CN, 85 mmol dm^{-3} L-His, 5 mmol dm^{-3} $CH_3CO_2NH_4$, 25 mmol dm^{-3} $CuSO_4 \cdot 5H_2O$, PH 7.0. The t_R values were L-Ala 12.3 and D-Ala 13.1 min.

Methylation of RA-X with Diazomethane.—RA-X (15 mg) was methylated with CH_2N_2 in diethyl ether at room temperature. The evaporated reaction mixture was purified by MPLC to give RA-X methyl ester (14 mg) as an amorphous powder, m.p. 199–201 °C, $[\alpha]_D^{20}$ −228.9 (*c* 0.10, $CHCl_3$); δ_H ($CDCl_3$, main conformer) 4.33 (1 H, dq, *J* 7.5, 6.9, D-Ala-1-Hα), 1.30 (3 H, d, *J* 6.9, L-Ala-1-Hβ), 6.39 (1 H, d, *J* 7.5, D-Ala-1-NH), 4.87 (1 H, m, Glu-2-Hα), 2.00 (2 H, m, Glu-2-Hβ), 2.36 (1 H, m, Glu-2-Hγ1), 2.46, (1 H, m, Glu-2-Hγ2), 6.39 (1 H, d, *J* 10.2, Glu-2-NH), 3.68 (3 H, s, Glu-2-OMe), 3.61 (1 H, dd, *J* 11.1, 4.3, Tyr-3-Hα), 3.33 (1 H, dd, *J* 11.1, 14.2, Tyr-3-Hβ1), 3.44 (1 H, dd, *J* 4.3, 14.2, Tyr-3-Hβ2), 7.07 (2 H, d, *J* 8.6, Tyr-3-Hδ), 6.81 (2 H, d, *J* 8.6, Tyr-3-Hε), 2.91 (3 H, s, Tyr-3-NMe), 3.78 (3 H, s, Tyr-3-OMe), 4.73 (1 H, dq, *J* 7.4, 6.7, Ala-4-Hα), 1.14 (3 H, d, *J* 6.7, Ala-4-Hβ), 6.67 (1 H, d, *J* 7.4, Ala-4-NH), 5.40 (1 H, dd, *J* 11.3, 3.1, Tyr-5-Hα), 3.66 (1 H, t, *J* 11.3, Tyr-5-Hβ1), 2.63 (1 H, dd, *J* 3.1, 11.3, Tyr-5-Hβ2), 7.27 (1 H, dd, *J* 2.2, 8.4, Tyr-5-Hδ1), 7.42 (1 H, dd, *J* 2.2, 8.4, Tyr-5-Hδ2), 6.88 (1 H, dd, *J* 2.4, 8.4, Tyr-5-Hε1), 7.20 (1 H, dd, *J* 2.4, 8.4, Tyr-5-Hε2), 3.13 (3 H, s, Tyr-5-NMe), 4.53 (1 H, dd, *J* 11.9, 3.8, Tyr-6-Hα), 3.10 (1 H, dd, *J* 11.9, 18.1, Tyr-6-Hβ1), 2.96 (1 H, dd, *J* 3.8, 18.1, Tyr-6-Hβ2), 6.58 (1 H, dd, *J* 1.9, 8.3, Tyr-6-Hδ1), 4.37 (1 H, d, *J* 1.9, Tyr-6-Hδ2), 6.80 (1 H, d *J* 8.3, Tyr-6-Hε1), 2.68 (3 H, s, Tyr-6-NMe) and 3.94 (3 H, s, Tyr-6-OMe); δ_C ($CDCl_3$, main conformer) 47.72 (D-Ala-1-Cα), 20.62 (D-Ala-1-Cβ), 172.54 (D-Ala-1-CO), 48.07 (Glu-2-Cα), 25.67 (Glu-2-Cβ), 29.52 (Glu-2-Cγ), 172.86 (Glu-2-Cδ), 171.52 (Glu-2-CO), 51.98 (Glu-2-OMe), 68.24 (Tyr-3-Cα), 32.73 (Tyr-3-Cβ), 130.43 (Tyr-3-Cγ), 130.02 (Tyr-3-Cδ), 113.99 (Tyr-3-Cε), 158.27 (Tyr-3-Cζ), 167.87 (Tyr-3-CO), 39.72 (Tyr-3-CN), 55.07 (Tyr-3-OMe), 46.20 (Ala-4-Cα), 18.26 (Ala-4-Cβ), 171.52 (Ala-4-CO), 54.09 (Tyr-5-Cα), 37.78 (Tyr-5-Cβ), 134.97 (Tyr-5-Cγ), 132.60 (Tyr-5-Cδ1), 130.80 (Tyr-5-Cδ2), 124.06 (Tyr-5-Cε1), 125.77 (Tyr-5-Cε2), 158.08 (Tyr-5-Cζ), 169.30 (Tyr-5-CO), 30.33 (Tyr-5-NMe), 57.25 (Tyr-6-Cα), 35.23 (Tyr-6-Cβ), 128.10 (Tyr-6-Cγ), 120.78 (Tyr-6-Cδ1), 113.35 (Tyr-6-Cδ2), 112.30 (Tyr-6-Cε1), 152.96 (Tyr-6-Cε2), 146.35 (Tyr-6Cζ), 170.45 (Tyr-6-CO), 29.11 (Tyr-6-NMe) and 56.04 (Tyr-6-OMe); *m/z* 843 ($M^+ + 1$) (Found: $M^+ + 1$ 843.3916. $C_{44}H_{54}N_6O_{11}$ requires M + 1, 843.3929).

Sodium Salt of RA-X.—RA-X (20 mg) was treated with $NaHCO_3$ in 50% MeOH and concentrated. The mixture was washed with EtOH and the EtOH-soluble fraction was evaporated to give RA-X sodium salt (20 mg) as an amorphous powder, m.p. 260–261 °C, $[\alpha]_D^{20}$ −194.0 (*c* 1.00, MeOH); δ_C (D_2O, main conformer) 46.68 (D-Ala-1-Cα), 18.74 (D-Ala-1-Cβ), 171.46 (D-Ala-1-CO), 47.44 (Glu-2-Cα), 25.72 (Glu-2-Cβ), 31.13 (Glu-2-Cγ), 179.60 (Glu-2-Cδ), 171.20 (Glu-2-CO), 65.71 (Tyr-3-Cα), 32.19 (Tyr-3-Cβ), 129.00 (Tyr-3-Cγ), 129.32 (Tyr-3-Cδ), 113.08 (Tyr-3-Cε), 156.80 (Tyr-3-Cζ), 168.63 (Tyr-3-CO), 38.17 (Tyr-3-NMe), 54.18 (Tyr-3-OMe), 45.22 (Ala-4-Cα), 16.33 (Ala-4-Cβ), 170.40 (Ala-4-CO), 53.03 (Tyr-5-Cα), 34.72 (Tyr-5-Cβ), 133.89 (Tyr-5-Cγ), 131.53 (Tyr-5-Cδ1), 129.71 (Tyr-5-Cδ2), 122.85 (Tyr-5-Cε1), 125.30 (Tyr-5-Cε2), 156.65 (Tyr-5-Cζ), 169.23 (Tyr-5-CO), 29.06 (Tyr-5-NMe), 56.35 (Tyr-6-Cα), 33.34 (Tyr-6-Cβ), 127.47 (Tyr-6-Cγ), 120.51 (Tyr-6-Cδ1), 112.59 (Tyr-6-Cδ2),

111.76 (Tyr-6-Cε1), 151.12 (Tyr-6-Cε2), 144.87 (Tyr-6-Cζ), 169.82 (Tyr-6-CO), 28.20 (Tyr-6-NMe) and 54.81 (Tyr-6-OMe).

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Paper 1/05248I
Received 15*th October* 1991
Accepted 4*th November* 1991