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NEW ANTITUMOR BICYCLIC HEXAPEPTIDES, RA-VI AND -VIII FROM *RUBIA CORDIFOLIA* ; CONFORMATION-ACTIVITY RELATIONSHIP II[1)]

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Abstract The structures of new antitumor bicyclic hexapeptides, RA-VI and -VIII from *Rubia cordifolia* were elucidated by the spectroscopic and chemical methods A combination of two-dimentional NMR techniques and NOE relationships showed that the amino acids constituting the β-turn of RA-VI are Ser-2 and D-Tyr-3 and those of RA-VIII, Thr-2 and Tyr-3 By the conformational analysis of RA-VI in its crystalline state using the X-ray diffractometric technique, RA-VI was shown to have, in its solid state, a type V β-turn structure at the residues Ser-2 and D-Tyr-3, while other RAs have type II β-turns Further, by 2D-NMR techniques, temperature effects on NH protons and NOE experiments, in solution of $CDCl_3$, RA-VI was shown to exist only as conformer A and RA-VIII as conformers A, B and C The difference between the solid state and solution state conformations of RA-VI was also shown by the refinement of the restrained molecular dynamics calculations using AMBER program RA-VIII, having a smaller population of conformer A with type II β-turn than other RAs, showed a reduced biological activity, and the N-methyl derivative of RA-VIII, whose conformer A content is further reduced, gave a further reduced activity, suggesting that conformer A contributes to the activity However, RA-VI, existing in solution 100% as conformer A, showed a very low activity and N-methylation increased the activity This shows that the stereochemistry and molecular mobility of the aromatic side chain of Tyr-3 over this turn, as elucidated by the ^{13}C spin lattice relaxation times, plays a more important role in the antitumor activity of the compounds of this series in addition to the type II β-turn structures

Introduction

A series of bicyclic hexapeptides, named RAs, i e RA-I, II, III, IV, V and VII, are potent antitumor peptides isolated from *Rubia cordifolia* and *R akane* [2)] Their structures,[3)] physiological activities[4)] and the total synthesis of RA-VII[5)] have been reported These related compounds are said to inhibit protein synthesis by binding to the eukaryotic 80S ribosome and subsequently inhibiting EF1-dependent binding of aminoacyl-tRNA and EF2-dependent translocation of peptidyl-tRNA [6)] In the previous paper,[1)] the conformational analysis of the main

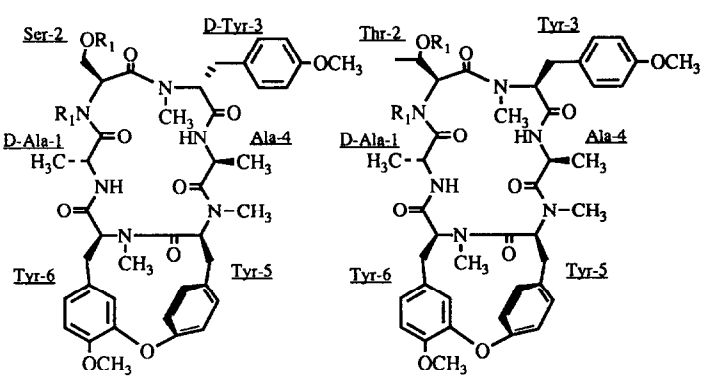


Figure 1
Structures of RA-VI, VIII and their derivatives, The amino-acid residures are shown by their conventional three-letter abbreviations, i e Ala=alanine, Tyr=N-methyl-O-methyl-tyrosine, D-Ala=D-alanine, D-Tyr=N-methyl-O-methyl-D-tyrosine, Ser=serine, Thr=threonine

active principle, RA-VII and its N-methylated derivative (RA-VII-NMe) was conducted by the spectroscopic and computational chemical evidences From the results, the most important site for its antitumor activity was considered to be around the β-turn structure

In our further chemical studies on the minor antitumor principles of the plant, we isolated two new bicyclic hexapeptides, RA-VI and VIII, having different amino acid compositions from those of other RAs and different backbone conformations The amino acids constituting known RAs are all L-form excepting for D-Ala-1 The new hexapeptides isolated this time were found to contain D-Tyrosine at the third position of RA-VI and L-Threonine at the second position of RA-VIII

Cyclic oligopeptides are often used in experimental studies on the structure-biological activity relationships, because their cyclic structures limit the conformational flexibility of the peptide backbones As part of our program to study the structure-activity relationship of RAs, we have undertaken conformational analysis of these newly obtained RA-VI and VIII by the spectroscopic examinations RAs are neutral, cyclic peptides consisting of six amino acids which are all lipophilic amino acids with three of them being N-methylated amino acids On the basis of detailed and exact knowledge of the structures of RAs in solid and solution under different environmental conditions, the structure-activity relationships may be assuredly discussed and new derivatives of these compounds with higher activity and less side effect may possibly be designed

Conformational analysis of RA-VI in crystal form was made by X-ray crystallographic analysis Further, studies of conformations of RA-VI in solution conducted by 2D-NMR techniques, the temperature effects on NH protons, NOE experiments and the refinements of the restrained molecular dynamics calculations are important steps for the precise understanding of the structure-activity relationship of RAs

The ^{1}H-NMR spectrum of RA-VIII suggested the presence of three stable conformational states (conformers A, B and C) in $CDCl_3$ and DMSO-d_6 RA-VIII-NMe, a derivative of RA-VIII giving a conformer composition different from that of RA-VIII, showed a reduced effect on P-388 and KB cells However, RA-VI, suggesting to be in only one stable conformational state (conformer A) in both $CDCl_3$ and DMSO-d_6, showed a considerably reduced activity

In this paper, we report on the structure determination and conformational analysis of the new antitumor bicyclic hexapeptides, RA-VI and VIII, by spectroscopic (NMR and X-ray analysis) and computational chemical methods (molecular dynamics and molecular mechanics calculations), and discuss further on the conformation-biological activity relationship of these compounds

Isolation and structure determination of RA-VI and VIII

The crude RA-III and V fractions prepared from a $CHCl_3$-MeOH extract of Rubiae Radix (roots of *Rubia cordifolia*) in the same way as that described in literature[3b] were subjected to ODS-HPLC column chromatography From the crude RA-III fraction, pure RA-VI was obtained after recrystallization and from the crude RA-V fraction, RA-VIII was obtained

RA-VI, showing a molecular ion peak at *m/z* 786 in the MS spectrum, has the molecular formula, $C_{41}H_{50}N_6O_{10}$ according the high resolution MS spectrum, and was considered to have an exocyclic oxygen atom as in the case of RA-III because it gave a dehydration peak at *m/z* 768 (M^+-18) The amino acid analysis of RA-VI by separation of optical isomers of Dns derivatives using mixed chelate complex (L-His-Cu(II)) showed that it contained D-Ala L-Ala L-Ser in the ratio of 1 1 1 as in RA-III [7] Therefore, the structural difference

between RA-VI and RA-III was considered to be in the three N-methyl tyrosine units The ^{1}H and ^{13}C NMR spectra of RA-VI showed quite different features from those of RA-III In RAs, two or three conformational states are produced by the isomerization about one or more N-methyl amide bonds, which are characteristic of other RAs But such plural conformational states were not observed in RA-VI, showing that it exists in only one conformational state The presence of NOE between Tyr-5-Hα and Tyr-6-Hα suggests that these two tyrosines are L-form However, Tyr-3 may be D-form because NOE was not observed between Tyr-3-NMe and Tyr-3-Hα NOE should be observed, if a type II β-turn structure similar to that in RA-VII is present The complete structure of RA-VI was determined by the X-ray analysis (See crystal conformation section) to be an epimer of RA-III with D-tyrosine at the third position

RA-VIII, $C_{42}H_{52}N_6O_{10}$, which was shown to contain D-Ala L-Ala L-Thr in the ratio of 1 1 1 [7] exists, in solution, like other RAs, in three stable conformational states at equilibrium However, the ratio of the three conformers of RA-VIII at equilibrium was different from those of other RAs

A relatively large proportion of minor conformers of this compound may help to analyze the effect of various conformers on the activity The position of the new component amino acid, L-threonine, was determined to be at the second position by the 2D-NMR techniques such as HMBC spectrum (See solution conformation Section)

Crystalline Structure of RA-VI

The X-ray diffraction method was used to determine the exact structure and to obtain detailed information on the conformation of the molecule

RA-VI crystallizes from MeOH-AcOEt solution in orthorhombic crystals of space group $P2_12_12_1$ with lattice constants a=14 970(8), b=33 007(20), c=9 413(6)Å and Z=4 [8] The final R value was 0 083 for the 2556 reflections observed The molecule consists of four L-amino acids and two D-amino acids linked together by peptide linkages A characteristic feature of this molecule is that it has, in addition to the 18-membered peptide ring, another 14-membered ring formed by the oxidative coupling of the phenolic oxygen of one tyrosine with a carbon ortho to the phenolic OH group of the adjacent tyrosine The N-methyltyrosine residue on the 18-membered peptide ring extend outwardly from the ring Five of the peptide bonds are in trans conformation and the sixth peptide bond between the residues 5 and 6 is in cis conformation which serves to fold the peptide chain to form a cyclic structure The cis conformation and the three

Table 1 X-ray- and MD-calculated backbone dihedrals in RA-VI and RA-V-p-bromobenzoate

Residue	Dihedral angle	RA-VI		RA-V-p-bromobenzoate
		MD	X-ray	X-ray[a]
D-Ala-1	φ	167 3	137 9(6)	137 8(17)
	ψ	-168 7	-142 2(6)	-169 7(13)
	ω	-178 7	-177 4(5)	-175 3(15)
Ser-2(Ala-2)[b]	φ	-73 7	-115 8(7)	-83 1(19)
	ψ	113 2	90 7(9)	120 9(16)
	ω	-169 4	-174 0(5)	-178 3(14)
D-Tyr-3	φ	56 5	102 3(9)	53 8(23)
(Tyr-3)[b]	ψ	43 8	-51 4(11)	38 5(23)
	ω	-179 6	172 8(6)	-167 9(13)
Ala-4	φ	-161 1	-75 5(10)	-158 5(12)
	ψ	156 5	162 9(6)	170 5(14)
	ω	179 3	170 8(6)	174 1(12)
Tyr-5	φ	-115 3	-116 9(8)	-136 9(14)
	ψ	90 9	111 3(9)	101 7(17)
	ω	5 3	-1 0(13)	1 8(25)
Tyr-6	φ	-101 6	-86 0(10)	-91 6(17)
	ψ	133 1	161 2(6)	163 2(13)
	ω	165 9	171 6(5)	171 4(13)

a) Data given in reference 1

b) Ala-2 and Tyr-3 in parenthesis are those of RA-V-p-bromobenzoate

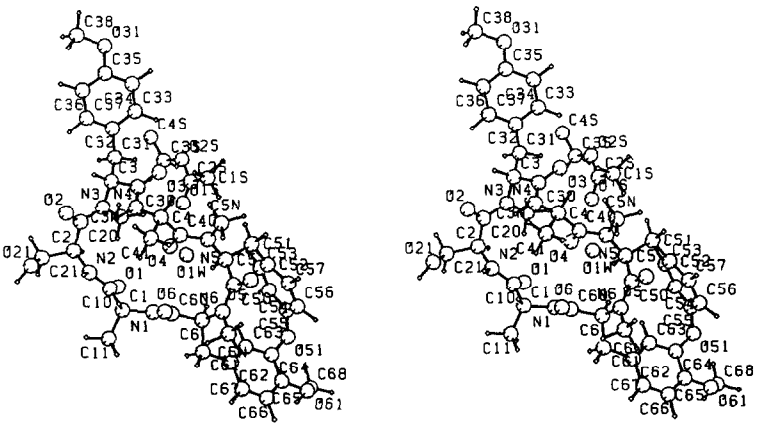


Figure 2 Stereoscopic view with each atom numbered of the crystal structure of RA-VI by PLUTO drawing

Table 2 Lengths (Å) of hydrogen bonding in X-ray structure of RA-VI

Hydrogen bridge atom			
From	To	Symmetry	Lengths (Å)
N1	O21	(ii)	2 972(11)
HN1	O21	(ii)	1 99(11)
N2	O1	(iii)	2 865(10)
HN2	O1	(iii)	1 81(11)
O21	O1W	(iii)	2 709(12)
HO21	O1W	(iii)	2 02(11)
O1W	O4	(i)	2 772(12)
O1W	O1S	(i)	2 809(18)

Symmetry code

(i) x, y, z

(ii) 1/2-x, -y, 1/2+z

(iii) 1/2-x, -y, -1/2+z

W=H_2O, S=AcOEt

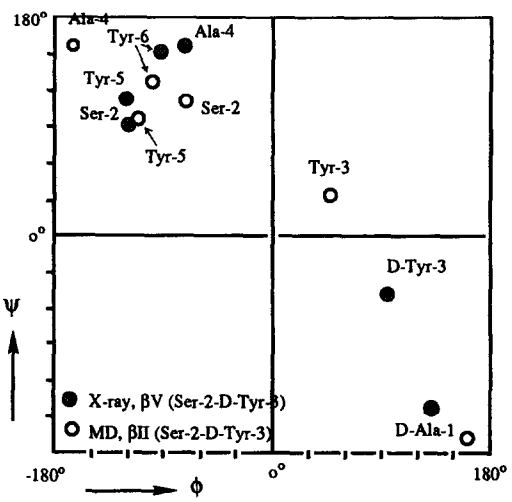


Figure 3 Ramachandran plot of RA-VI, Φ and ψ angles calculated by X-ray and MD

dimentional molecular form are probably retained by the additional 14-membered ring produced by the oxidative coupling

The solid state conformation of the molecule with each atom numbered is shown in Figure 2 by a PLUTO stereoscopic drawing [9] The torsion angles along the peptide chain of RA-VI in comparison with those of RA-V-p-bromobenzoate and the lengths of hydrogen bondings are listed in Tables 1 and 2, respectively The ϕ and ψ values are plotted on a Ramachandran plot in Figure 3

It can be seen from the Figure, the residues 2 and 3 form one turn of the cyclic structure and the residues 5 and 6 another turn The amide bond between the residues 5 and 6 is cis, as shown by the ω angle value, -1 0° It is interesting that cis configuration occurs at the methyl-substituted amide bond. The cis amide bond is unusual for

peptide units,[10] though it is in some cases observed, especially in peptide linkages involving the imino nitrogen atoms of proline, hydroxyprolin and the other amino acid residues whose imino hydrogen atom is replaced by a methyl group [10,11]

The ω angle for cis amido bond does not deviate much from 0°. In the case of trans amido bonds, the ω angles fall in the range 180±10°, and the deviations from the ideal value are of the same order of magnitude as in the cis bonds.

The most significant point to be noted is that the ϕ and ψ values at the residues 2, 3 and 4 of RA-VI are different from those of RA-V-p-bromobenzoate, probably because of the solvent, i.e. ethyl acetate cocrystallized with the peptide and that the Tyr-3 side chain is not bent over the backbone. Instead of a type II β-turn observed in other RAs, type V β-turn is formed at the residues 2 and 3 probably due to D-form Tyr-3.

Although the lengths and angles obtained agreed with the proposed chemical structure and generally with those found in other peptides, some significant deviations were observed in the angles of the bonds involving cis amide group. The angles Cα-C'-N and C'-N-Cα (C' is a carbonyl carbon atom) were significantly larger, while O-C'-N and C'-N-C (N-methyl) were smaller than those involving trans amide groups. This may be caused by the repulsive forces between the two α-carbon atoms which are cis to each other. Indeed, the distance between the two Cα atoms across the cis amide bonds were found to be C5-C6=3.018Å.

Another point to be noted is the absence of intramolecular hydrogen bonding within the peptide ring (See Table 2). It was shown that transannular hydrogen bonds are not necessarily essential to the conformational stability of the cyclic peptide rings.

Although the X-ray analysis of single crystals gives the most reliable three dimentional structural form, it provides information only about its solid-state conformation. The solid state structure is often determined by intermolecular hydrogen bonds, whereas the conformation in solution is determined mainly by intramolecular hydrogen bonds. Hence, the results of an X-ray analysis must be used with care when the results are used for the conformational structure in solution.

Solution forms of RA-VI and VIII

Complete assignments of ^{1}H and ^{13}C NMR signals

According to the NMR spectrum of RA-VI, RA-VI exists in a single stable conformational state in not only apolar solvents such as $CDCl_3$ but also polar solvents such as DMSO-d_6, which is considered to be due to the lack of repulsion between the carbonyl carbon at Ser-2 and the aromatic side chain at D-Tyr-3. The other RAs including RA-VIII apparently exist in two or three conformational states in solution.

The complete assignments of the signals in various NMR measurements may provide more reliable information about the dynamic structures in solution. The assignments of ^{1}H and ^{13}C-NMR signals of RA-VI and VIII, shown in Table 3, were made by the combination of ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and HMBC spectra. The HMBC,[12] which provides ^{1}H-^{13}C long-range couplings, was proved to be extremely valuable for the assignment. For the assignments of the signals of the minor component, conformer B of RA-VIII, HOHAHA[13] spectrum was quite useful.

The conformational determination of RA-VI and VIII in solution was made on the basis of the results of the following experiments.

Table 3 ^{1}H and ^{13}C-NMR Chemical Shifts in $CDCl_3$ at 303K (^{1}H: 500MHz, ^{13}C: 125MHz)

Amino acid	RA-VI	RA-VIII			RA-VI	RA-VIII	
	proton	major (A)	minor (B)	carbon		major (A)	minor (B)
D-Ala-1	Hα	4 34 Jαβ=6 9	4 48 Jαβ=6 9	4 47 Jαβ=7 0	Cα	48 48	47 85	47 76
	Hβ	1 29 JαN=6 9	1 33 JαN=7 0	1 36 JαN=6 8	Cβ	20 81	21 05	21 13
	HN	6 42	6 40	6 45	CC=O	172 40	172.27	172 58
Ser-2	Hα	5 10 Jαβ1=2 8	4 45 Jαβ=1 5	4 23 Jαβ=0 0	Cα	48 40	51 34	50.88
(Thr-2)	Hβ1	3 84 Jαβ2= **	4 08 Jβγ=6 4	3 54 Jβγ=6 4	Cβ	62 24	66 14	67 05
	Hβ2	3 65 Jβ1β2=11 8			Cγ		19 32	17 90
	Hγ1		1 20 JαN=7 9	0 81 JαN=7 9	CC=O	173 26	172 86	172 70
	HN	7 37 JαN=9 9	6 71	6 56				
	HOH	3 96						
Tyr-3	Hα	5 54 Jαβ1=10 8	3 63 Jαβ1=10 8	4 61 Jαβ1=11 1	Cα	57 39	68 55	62 27
	Hβ1(pro-R)	2 82 Jαβ2=5 5	3 34 Jαβ2=5 0	** Jαβ2=3 9	Cβ	32 08	32 77	33 67
	Hβ2(pro-S)	3 50 Jβ1β2=15 5	3 39 Jβ1β2=14 1	** Jβ1β2=**	Cγ	128 82	130 43	130 43
	2Hδ	7 12 Jδε=8 6	7 06 Jδε=8 6	7 11 Jδε=8 6	Cδ	129 42	130 11	129 99
	2Hε	6 82	6 85	6 85	Cε	114 02	114 22	114 30
	MeN	3 12	2 98	2 99	Cζ	158 47	158 51	158 85
	MeO	3 78	3 80	3 78	CC=O	168 75	167 56	168 11
					CN	31 32	40 31	29 67
					CO	55 19	55 30	55 36
Ala-4	Hα	4 70 Jαβ=7 0	4 73 Jαβ=6 7	4 54 Jαβ=7 1	Cα	45 85	46 24	46 14
	Hβ	1 22 JαN=7 9	1 10 JαN=7 4	1 19 JαN=7 3	Cβ	18 12	18 60	18.24
	HN	6 36	6 72	6 61	CC=O	171 66	171 63	171 74
Tyr-5	Hα	5 30 Jαβ1=11 6	5 41 Jαβ1=11 4	5 42 Jαβ1=10 8	Cα	53 15	55 39	54 95
	Hβ1(pro-S)	3 60 Jαβ2=3 7	3 68 Jαβ2=3 0	3 70 Jαβ2=3 0	Cβ	36 61	37 01	36 58
	Hβ2(pro-R)	2 74 Jβ1β2=11 6	2 63 Jβ1β2=11 1	2 75 Jβ1β2=11 3	Cρ	134 39	135 15	135 08
	Hδ1	7 27 Jδ1δ2=2 2	7 27 Jδ1δ2=2 2	7 27 Jδ1δ2=2 2	Cδ1	132 93	132 78	132 78
	Hδ2	7 44 Jδ1ε1=8 4	7 40 Jδ1ε1=8 4	7 46 Jδ1ε1=8 4	Cδ2	130 80	130 92	130 87
	Hε1	6 91 Jδ2ε2=8 4	6 87 Jδ2ε2=8 4	6 89 Jδ2ε2=8 4	Cε1	124 50	124 27	124 39
	Hε2	7 24 Jε1ε2=2 4	7 21 Jε1ε2=2 4	7 24 Jε1ε2=2 4	Cε2	125 97	125 93	125 93
	MeN	3 22	3 12	3 11	Cζ	158.32	158 28	158 44
					CC=O	170.42	169 30	169 86
					CN	30.35	30 52	30 67
Tyr-6	Hα	4 42 Jαβ1=11 6	4 57 Jαβ1=12 4	4 66 Jαβ1=11 7	Cα	57 74	57 41	57 92
	Hβ1(pro-R)	3 09 Jαβ2=4 0	** Jαβ2=4 2	** Jαβ2=4 0	Cβ	35 19	35 49	35 80
	HB2(pro-S)	3 01 Jβ1β2=20 0	** Jβ1β2=**	** Jβ1β2=**	Cγ	128 20	128 16	128 61
	Hδ1	6 57 Jδ1δ2=1 9	6 58 Jδ1δ2=1 8	6 58 Jδ1δ2=1 9	Cδ1	120 63	120 97	120.97
	Hδ2	4 33 Jδ1ε1=8 4	4 35 Jδ1ε1=8 3	4 38 Jδ1ε1=8 3	Cδ2	113 72	113 44	113 52
	Hε1	6 79	6 80	6 80	Cε1	112 36	112 35	112 35
	MeN	2 63	2 69	2 68	Cε2	153 10	153 13	153 13
	MeO	3 94	3 93	3 94	Cζ	146 38	146 56	146 56
					CC=O	170 57	170 75	170 48
					CN	29 03	29 32	29 39
					CO	56 12	56 18	56 18

** not determined in the present experiment

NOE enhancements

The relationship in the NOE enhancements of RA-VI and the two conformers A and B of RA-VIII are shown in Figure 4 In the field of studies on the peptide structure-activity relationships, the occurrence of β-turns in peptide have come to attract more attention From extensive studies on peptides by NMR, β-turns have been shown to be produced by the stabilization of a 4→1 type hydrogen bond In solid state, RA-VI takes a type V β-turn conformation which is not stabilized by 4→1 hydrogen bond The conformation of type V noted by Lewis et al [14] is such that a 7-membered ring (including H) is formed by a hydrogen bond (in the case of the 4→1 hydrogen bond, a 10-membered ring is formed) If a type II β-turn occurs in RA-VI, NOE is expected to be observed between Ser-2-Hα and D-Tyr-3-NCH3, whose β-turn is stabilized by 4→1 hydrogen bond

The intensity of the Ser-2-Hα signal is increased when N-methylated proton at D-Tyr-3 is saturated In this case, it is difficult to distinguish between type II and type V β-turns simply by the NOE enhancement observed between Ser-2-Hα and D-Tyr-3-NCH3 However, the NOE observed between D-Tyr-3-NCH3 and Ala-4-NH provides an evidence in favor of the proposed type II β-turn in solution On the other hand, the NOE relationship of two conformers A and B in RA-VIII shown in Figure 4 is similar to that of RA-VII described in the previous paper [1] In the main component, conformer A, the NOEs between Thr-2-Hα and Tyr-3-NCH3, and Tyr-3-NCH3 and Tyr-3-Hα showed the presence of a type II β-turn at the residues 2 and 3, whereas, in the minor component, conformer B, the intensive NOE enhancement between Thr-2-Hα and Tyr-3-Hα suggested that the N-methylated amide bond between Thr-2 and Tyr-3 has a cis-trans isomerized conformation The N-methyl amide bond between Tyr-5 and Tyr-6 was considered to be a cis bond by the NOE enhancements between Tyr-5-Hα and Tyr-6-Hα in both RA-VI and VIII The NOE enhancements between the Tyr-5-NCH3 protons and Ala-4-CH3/Ala-4-Hα showed that trans configuration is maintained in the N-methyl amide bond between Ala-4 and Tyr-5 in both compounds Further, the cross peak between the Tyr-3-Hδ, one of the aromatic protons, and Thr-2-Hβ in the phase sensitive NOESY (NOESYPH)[15] spectrum suggested that the Tyr-3 aromatic side chain, which is considered not to rotate freely, is over the N-methyl amide bond

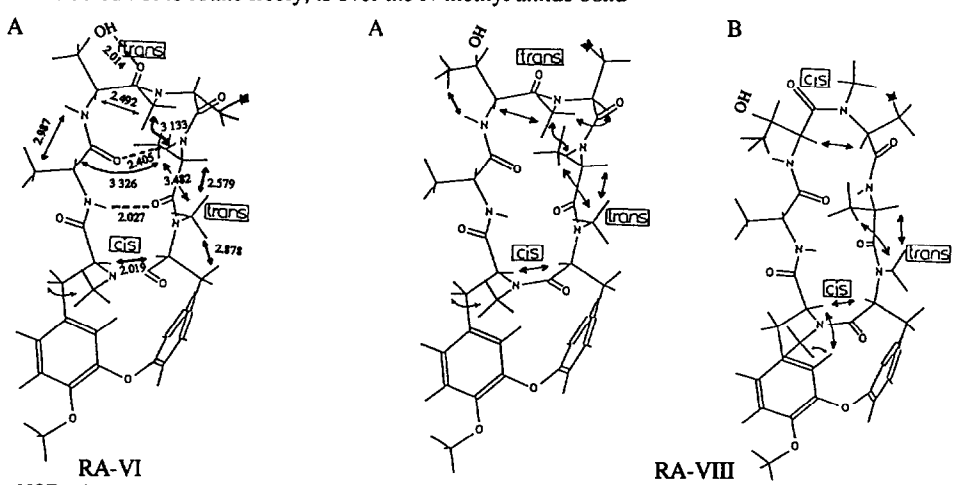


Figure 4 NOE enhancements in conformers A and B of RA-VI and VIII The arrows show the NOE relationships confirmed by 1D-NOE and NOESYPH experiments in CDCl3 at 303K The values(Å) in RA-VI indicate the distances, which were obtained by energy calculations (See quenched molecular dynamics section), between the hydrogen bondings and between the protons(the carbons for methyl groups) related to NOE enhancements NOE enhancements of RA-VI-DiMe showed the similar relationship to RA-VI about backbone conformation

Hydrogen bonding

The first step in the procedures of 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 purpose is to determine the temperature effects on the NH protons[16] the NH protons exposed to solvents will show a higher temperature dependence

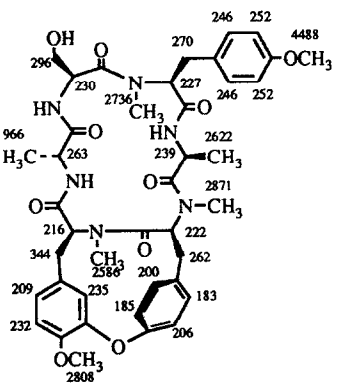
Table 4 Effect of temperatures on the NH chemical shifts of RA-VI, RA-VI-DiMe and VIII, $-\Delta\delta/\Delta T$ (10^3 ppm/K)

Conformers		Solvent	D-Ala-1	Ser-2 (Thr-2)	Ala-4
RA-VI	A	DMSO-d_6	5 0	3 7	1 0
	A	$CDCl_3$	2 7	16 7	1 0
RA-VI-DiMe	A	DMSO-d_6	5 0	---	1 7
	A	$CDCl_3$	2 0	---	0 3
RA-VIII	A	DMSO-d_6	5 3	6 3	0
	B	DMSO-d_6	4 3	2 7	-1 0
	A	$CDCl_3$	0 8	6 2	0
	B	$CDCl_3$	0 3	7 0	-2 7

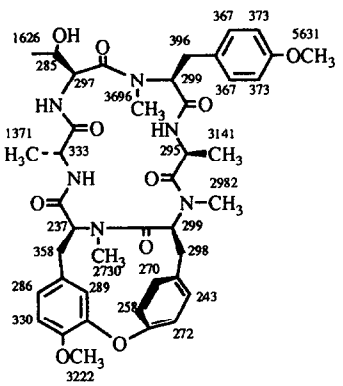
The temperature coefficients ($d\delta/dT$) of RA-VI and VIII given in Table 4 clearly show that Ala-4-NH is strongly shielded from the solvent, whereas Ser-2-NH, Thr-2-NH and D-Ala-1-NH are exposed to the solvent as shown by the temperature effects D-Ala-1-NH showed a higher temperature dependence in DMSO-d_6 than in $CDCl_3$, while Ser-2-NH and Thr-2-NH showed a higher value in $CDCl_3$ than in DMSO-d_6. The strong intramolecular hydrogen bonding between Ala-4-NH and D-Ala-1-CO is considered to be necessary to stabilize the type II β-turn structure which in formed by Ser-2 and D-Tyr-3, or Thr-2 and Tyr-3 in RA-VI or RA-VIII, respectively

Molecular mobility (T1) and side chain conformation

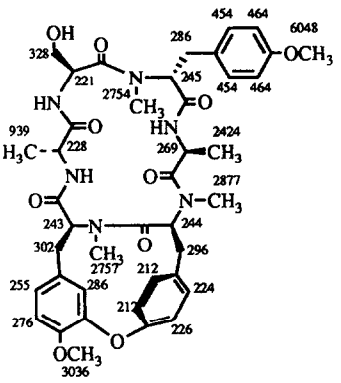
The molecular mobility of molecules is closely related to their energy levels It does, of course, affect the conformational structure and accordingly, affect the structure-biological activity relations Therefore, it is important to know the exact molecular mobility One of the most effective techniques for the measurment of the molecular mobility is NMR spectroscopy The effect of ^{13}C-spin-lattice relaxation times (T1) of protonated carbons of peptides on the dynamic properties in solution was studied, because the ^{13}C relaxation of such carbons is mainly dominated by the ^{13}C-^{1}H dipolar interaction with the direct bonded hydrogens[17] The experimental data of the conformer As of RA-III and VIII, and RA-VI are given in Figure 5



RA-III



RA-VIII



RA-VI

Figure 5 NT1 values (N times T1 values in which N=number of attached protons and T1=longitudinal relaxation time), of RA-III (main conformer A), RA-VIII (main conformer A) and RA-VI

The dynamic properties of the protonated backbone carbons of the three compounds, RA-III, VIII and VI were very similar to those of RA-VII In the case of RA-VI, the NT1 values of the α and β carbons of the D-Tyr-3 residue are similar to the NT1 values of the corresponding carbons of Tyr-5 and Tyr-6 However, flexibility is certainly observed in the side chain of D-Tyr-3 and not in those of Tyr-5 and Tyr-6 In RA-VI, the NT1 values of the ortho and meta carbon atoms, which are influenced by the tyrosyl ring rotation, are, however, twice the NT1 values of the corresponding carbons of the Tyr-5 and 6 residues in the rigid 14-membered ring Furthermore, the same results were obtained with the NT1 values of the Tyr-3-OCH_3 carbons the -OCH_3 NT1 value of more freely moving Tyr-3 of RA-VI was higher than those of RA-III and VIII The NT1 value of Tyr-3-OCH_3 increases in the order of RA-III, RA-VIII and RA-VI This is in accord with the fact that the mobility due to rotation about Tyr-3 increases in this order the anisotropic effect of aromatic ring of D-Tyr-3 decreases in this order However, the NT1 value of Tyr-3-NCH3 of RA-VIII, showing intermediary mobility of Tyr-3, is quite large This may be due to the much increased fluctuation of the β-turn involving Thr-2 and Tyr-3 The Tyr-3 mobility of RAs was considered to closely related to their biological activities (See conformational-activity relationship section) Further, the fact that the NT1 values of Ala-4-CH_3 are very high compared with other alanine residues, 2-3 times, which is characteristic of RAs, may mean that it is not fixed in one rotamer D-Tyr-3 in RA-VI has a mobility higher than that of RA-VIII and extends into a slightly different direction to produce more influence on the ^{1}H and ^{13}C signals around there When compared with the ^{1}H-NMR data of RA-VIII, the N-methyl signal of D-Tyr-3 of RA-VI is down field shifted to 3 12ppm due to the lack of anisotropic effect of the aromatic ring of D-Tyr-3, while the α proton signal of D-Tyr-3 showed the normal chemical shift value of 5 54ppm [18] In the ^{13}C-NMR spectrum, the chemical shift of D-Tyr-3-NCH_3 and that of D-Tyr-3-Cα showed high field shifted to 31 32 and 57 39ppm, respectively, whose values are normal chemical shift [18] These changes in chemical shifts, are considered to be mainly caused by the lack of steric repulsion between the carbonyl moiety of Ser-2 and the side chain of D-Tyr-3, and also by the lack of anisotropic effect of the aromatic ring in D-Tyr-3

Conformational analysis by quenched molecular dynamics[19]

We applied computational procedures using the NMR data to the elucidation of the solution conformation of RA-VI and further to the disclosure of the difference between the conformation in the solid and solution states. We performed the quenched molecular dynamics calculations starting with the X-ray structure Three distance constraints about hydrogen bondings derived from the NMR experiments were used to show that this solution structure of RA-VI is consistent with the experimental data including NOE relationship (See Figure 4) The program used for the MD calculations and the analysis of RA-VI were obtained from the AMBER 3 0 Rev A program package [20] All calculations were performed on IRIS 4-D computer The resulting structures were characterized in terms of relative energies and conformational properties The starting structure with a type V β-turn used in the calculation leads to an immediate flip of the amide bond to take the type II β-turn conformation

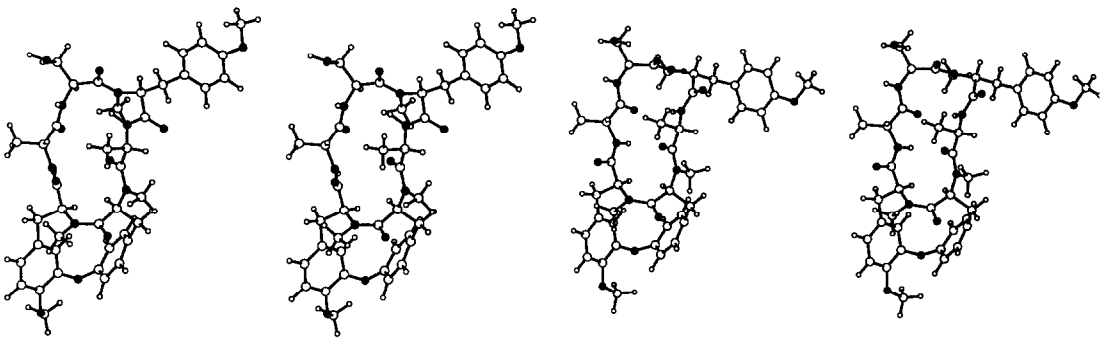


Figure 6 Stereo drawings of low-energy conformers of RA-VI by quenched MD calculations Left starting conformation (12 75kcal/mol) with type V β-turn between Ser-2 and D-Tyr-3 given by X-ray analysis Right. the lowest energy conformation (10 75kcal/mol) with type II β-turn after quenched MD calculation

Conformational-activity relationship

The percent conformer compositions of these compounds and their cytotoxic activities are listed in Table 5 and 6, respectively

Table 5 Percent of various conformers in RA-III, VI, VII, VIII and their derivatives in $CDCl_3$ and DMSO-d_6

Compounds	Conformers A	B ($CDCl_3$)	C	A	B (DMSO-d_6)	C
RA-III	74 4	25 6		77 6	22 4	
RA-III-DiMe	62 9	37 1		45 3	34 5	20 2
RA-VI	100			100		
RA-VI-DiMe	100			100		
RA-VII	88 6	11 4		63 7	31 8	4 5
RA-VII-NMe	77 3	21 2	1 6	57 1	25 3	17 6
RA-VIII	70 7	26 2	3 1	63 2	29 6	7 2
RA-VIII-DiMe	39 6	53 5	6 9	37 4	40 4	22 2

RA-VIII with type II β-turn containing less percentage of conformer A showed a lower biological activity than RA-III, which is an epimer of RA-VI Further, the reduced activities of the methyl derivatives of RA-III, VII, and VIII when compared with those of RA-III, VII and VIII suggest that the reductions were caused by the reduced percentage of confomer A present However, RA-VI with type II β-turn in solution showed a considerably reduced activity in comparison with RA-III This finding suggests the importance of the aromatic side chain of Tyr-3 locating over the β-turn Furthermore, in this regard, the molecular mobility elucidated by the T1 values of the aromatic side chain is considered to be closely correlated with the biological activity In the case of the methyl derivative of RA-VI with hydrophobic methyl function around the β-turn, the effect of temperatures on the NH chemical shifts (Table 4) and NOE enhancements which show the similar relation to those of RA-VI (Figure 4) suggest to take

Table 6 Cytotoxic activities of RA-III, VI, VII, VIII and their derivatives on P388 and KB cells (IC_{50} μg/ml)

Compounds	KB	P388
RA-III	$4\,0\times10^{-3}$	$1\,2\times10^{-2}$
RA-III-DiMe	$5\,4\times10^{-2}$	$5\,9\times10^{-2}$
RA-VI	1 2	3 5
RA-VI-DiMe	0 3	$5\,5\times10^{-2}$
RA-VII	$1\,8\times10^{-3}$	$1\,4\times10^{-3}$
RA-VII-NMe	$2\,3\times10^{-3}$	$1\,6\times10^{-3}$
RA-VIII	$6\,7\times10^{-2}$	$3\,1\times10^{-2}$
RA-VIII-DiMe	$1\,0\times10^{-1}$	$4\,7\times10^{-2}$

the homologous conformation to RA-VI in solution and the striking aspect is that the cytotoxic activity is more emphatically seen than that of RA-VI In our previous paper,[3c] by using a QSAR approach, it was found that some hydrophobicity was needed for RA derivatives from the viewpoints of both antitumor activity and toxicity Petroski et al [21] reported that neither O-desmethylbouvardin nor bouvardin catechol with hydrophilic function such as hydroxyl group over the β-turn produced by microbial transformation of bouvardin was active These results suggest that some hydrophobic function is needed around the β-turn for the compound to show antitumor activity Then, on the basis of this fact that RA-VI-DiMe showed the increased biological activity in comparison to RA-VI, the reduced biological activity of RA-VII-NMe,[1] when compared to that of RA-VII, was considered to be caused by the change in the proportions of the conformers A and B On the other hand, Boger et al[22] reported that synthetic cyclo-(D-Ala-Ala-N-Me-Tyr(OCH3)-Ala) having a conformationally constrained 12-membered cyclic tetrapeptide, which includes the first four amino acids of the 18-membered ring amino acids of RAs showed the activity This agrees with our results that assert the importance of conformational rigidity around the β-turn

On the basis of the above results, we may summarize the confomational-biological activity relationship of these compounds as follows the typical type II β-turn structure and the aromatic side chain of Tyr-3 over this turn were considered to play a very important role in its antitumor activity

Conclusion

This study showed the structures and conformations of the new antitumor bicyclic hexapeptides, RA-VI and VIII RA-VI takes the type V β-turn conformation in solid state, and the type II β-turn conformation in $CDCl_3$ and DMSO-d_6 solutions It seems to indicate that D-Tyr-3 is stereochemically constrained to restrict the conformational freedom of the peptide backbone in RA-VI Further, the conformational analysis of these compounds and their methyl derivatives gave useful information about the conformational-activity relationships of the compounds of this series the type II β-turn structure involving the residues 2 and 3 and the presence of an aromatic side chain over this turn, which does not rotate freely, are considered to play the most important role in their antitumor activity

Experimental

General

Proton and carbon spectra were recorded on Bruker spectrometers (AM400 and AM500) and processed on a Bruker data station with an Aspect 3000 computer 5mg samples of RA-VI, VIII and their methyl derivatives each dissolved in 0 5ml $CDCl_3$ or DMSO-d_6 (degassed) in a 5mm tube were used for the homonuclear measurement and 30mg sample in 0 5ml $CDCl_3$ or DMSO-d_6 (degassed) in a 5mm tube for the heteronuclear measurement The spectra were recorded at 303K NOESYPH experiments were made with a mixing time of 0 6s

Isolation of RA-VI and VIII

Commercial Rubiae Radix (220kg, roots of *Rubia cordifolia*) purchased in China was extracted three times with $CHCl_3$-MeOH (1 1, 500L) The combined extracts were washed with water and evaporated to dryness in

vacuo The obtained syrup (7kg) was purified in the same way as that reported in the previous paper[3b] to give crude RA-III and V fractions The crude RA-III fraction was subjected to reversed phase column chromatography and then recrystallized from MeOH-AcOEt to give RA-VI (150mg) The crude RA-V fraction was subjected to ODS-HPLC with 65% MeOH to give RA-VIII (100mg), which gave a very broad peak when the eluate was monitered with a UV detector at 254nm The Rf values were 0 27 for RA-VI and 0 26 for RA-VIII, when TLC was performed on 0 25mm silica gel plates (60F$_{254}$, Merck) with CHCl$_3$-MeOH (100 7) The physical and spectral data for RA-VI and VIII were as follows

RA-VI Colorless needles, mp 219-220°C (from MeOH-AcOEt) MS m/z 786 (M^+, Calcd for $C_{41}H_{50}N_6O_{10}$ 786 3588, Found 786 3597), 768 (M^+-H_2O) $[\alpha]_D$ -118 6° (c 0 68, CHCl$_3$)

RA-VIII Colorless needles, mp 267-269°C (from MeOH) MS m/z 800 (M^+, Calcd for $C_{42}H_{52}O_{10}N_6$ 800 3745, Found 800 3817) $[\alpha]_D$ -159 5° (c 0 39, CHCl$_3$)

T1 relaxation times (100MHz)

All spectra were recorded on a Brucker AM400 spectrometer at 100 6MHz using proton broad-band decoupling at 303K The spectra contained 32K datum points over a 24KHz frequency range The relaxation data were obtained by using the inversion-recovery 180-τ-90° pulse sequence The repetition times between two acquisitions were 60s in CDCl$_3$ The spin-lattice relaxation times were determined from the relaxation data by using the regression analysis that was incorporated in the T1 routine of the Brucker acquisition and processing program and given by the expression Y=A3 + A2*exp(-t/T1), in which A3 and A2 are the constants representing the delay times between the 180° and 90° pulses For the calculation of T1, we used the relative intensities of the ^{13}C signals at 15 different values in an appropriate range Standard deviations were in the range of 0 005 to 0 059s

Quenched molecular dynamics calculations

Computer modeling was made with the MOL-GRAPH program system (ver 2 0) on an IRIS 4-D workstation The initial calculations were started with the whole coordinates for the X-ray structure of RA-VI The molecular mechanics and dynamics calculations were performed with the AMBER 3 0 Rev A package[20] with the distance-dependent dielectric, $\varepsilon=R_{ij}$ The data of the measurement of three intramolecular hydrogen bondings of NH4-O1, NH1-O4 and OH2-O2 were taken into consideration, and the constrained dynamics trajectories were calculated with an extra square well potential[23] of the form $E=\Sigma K(r-r_{max})^2$ for $r>r_{max}$ and E=0 0 for $r<r_{max}$ added to the force field (K=5kcal/A^2, r=interproton distances, r_{max}=3 0Å(NH1-O4), 2 5Å(NH4-O1)) The relevant solution-phase conformations were predicted by the general method described below Molecular dynamics calculations were made at 298K for a total of 100psec with the time step 1fsec and the structures were sampled every 0 1psec All the snapshots from the dynamics trajectories were then energy minimized A snapshot with the lowest energy was selected as the relevant conformation

Acid hydrolysis of RA-VI and VIII

Solutions of RA-VI and VIII (each containing 5mg of peptide) in 6NHCl were heated at 100°C for 17h After cooling, each solution was concentrated to dryness The residue was dansylated with 2% NaHCO$_3$ (1ml) and 5mM dansyl chloride in acetone (0 5ml) at 37°C for 1hr At the same time, authentic amino acids DL-Ala, D-Ala, DL-Ser, L-Ser, DL-Thr, L-Thr were also dansylated in the same manner The dansyl amino acids were subjected to HPLC under the following two conditions column, 4mm i d x250mm (Nucleosil 5μm) flow rate, 0 8ml/min, detection, 340nm, (a) solvent, 20%CH$_3$CN (5mM L-His, 5mM CH$_3$COONH$_4$, 25mM CuSO$_4$ 5H$_2$O, PH7 0),

The t_R values were L-Ala 10 5, D-Ala 11 6, L-Ser 7 4, D-Ser 7 1min (b) solvent, 15%CH_3CN (5mM L-Pro, 5mM CH_3COONH_4, 25mM $CuSO_4$ $5H_2O$, PH7 0), The t_R values were L-Thr 17 8 and D-Thr 22 1min

RA-III-DiMe, RA-VI-DiMe and RA-VIII-DiMe

RA-III, VI and VIII were stirred with iodomethane and FK/Al_2O_3[24] in 1,2-dimethoxyethane at room temperature for 24hr Each reaction mixture was filtered and then concentrated to give RA-III-DiMe, RA-VI-DiMe and RA-VIII-DiMe, respectively The yields were about 100%

RA-III-DiMe Colorless needles, mp 188-190°C, MS m/z 814 ($C_{43}H_{54}O_{10}N_6$, M^+, 30)

RA-VIII-DiMe Colorless needles, mp 178-180°C, MS m/z 828 ($C_{44}H_{56}O_{10}N_6$, M^+, 30)

RA-VI-DiMe Colorless needles, mp 168-170°C, MS m/z 814 ($C_{43}H_{54}O_{10}N_6$, M^+, 20) ^{1}H-NMR δppm in $CDCl_3$ 1 20 (3H, d, J=6 9Hz, Ala-1-Hα), 1 26 (3H, d, J=6 7Hz, Ala-4-Hα), 2 66 (3H, s, Tyr-6-NMe), 3 03 (3H, s), 3 13 (3H, s, Tyr-3-NMe), 3 22 (3H, s, Tyr-5-NMe), 3 28 (3H, s), 3 78 (3H, s, Tyr-3-OMe), 3 95 (3H, s, Tyr-6-OMe), 4 35 (1H, d, J=1 8Hz, Tyr-6-Hδ2), 4 45 (1H, dd, J=3 9, 11 6Hz, Tyr-6-Hα), 4 78 (1H, m, Ala-1-Hα), 4 81 (1H, m, Ala-4-Hα), 5 30 (1H, dd, J=3 6, 11 5Hz, Tyr-5-Hα), 5 63 (1H, dd, J=5 1, 11 4Hz, Tyr-3-Hα), 5 87 (1H, dd, J=6 0, 8 8Hz, Ser-2-Hα), 6 31 (1H, d, J=8 6Hz, Ala-1-NH), 6 45 (1H, d, J=7 2, Ala-4-NH), 6 58 (1H, dd, J=2 1, 8 2Hz, Tyr-6-Hε1), 6 82 (2H, d, J=8 6Hz, Tyr-3-Hε), 6 91 (1H, dd, J=2 4, 8 4Hz, Tyr-5-Hε1), 7 14 (2H, d, J=8 6Hz, Tyr-3-Hδ), 7 14 (1H, m, Tyr-5-Hε2), 7 28 (1H, m, Tyr-5-Hδ1), 7 43 (1H, dd, J=2 1, 8 4Hz, Tyr-5-Hδ2)

X-ray analysis of RA-VI

Crystal data $C_{41}H_{50}N_6O_{10}$ H_2O $C_4H_8O_2$, orthorhombic, space group $P2_12_12_1$, Z=4, a=14 970(8), b=33.007(20), c=9 413(6)Å, V=4651Å^3, D_X=1 275gcm^{-3} The intensity data were measured on a Philips fourcircle diffractometer with graphite-monochromated CuKα radiation A total of 2556 reflections were observed as above the 2σ(I) level, within the 2θ range from 6° through 130° The structure was determined by the direct method using the MULTAN program,[25] and the refinement was carried out by the method of block-diagonal-matrix least-squared method The final R value was 0 083 for the 2556 reflections, assuming anisotropic thermal vibrations for 64 atoms and isotropic thermal vibrations for 55 hydrogens

References and Notes

1) 32th SYMPOSIUM on THE CHEMISTRY OF NATURAL PRODUCTS, CHIBA, SYMPOSIUM PAPERS pp 72-78, H Morita, K Kondo, Y Hitotsuyanagi, K Takeya, H Itokawa, N Tomioka, A Itai and Y Iitaka, *Tetrahedron*, **1991**, 47, 2757

2) H Itokawa, K Takeya, K Mihara, N Mori, T Hamanaka, T Sonobe and Y Iitaka, *Chem Pharm Bull*, **1983**, 31, 1424

3) a) H Itokawa, K Takeya, N Mori, T Hamanaka, T Sonobe and K Mihara, *Chem Pharm Bull*, **1984**, 32, 284, b) H Itokawa, K Takeya, N Mori, T Sonobe, S Mihashi and T Hamanaka, *ibid*, **1986**, 34, 3762, c) H Itokawa, K Takeya, N Mori, T Sonobe, N Serisawa, T Hamanaka and S Mihashi, *ibid*, **1984**, 32, 3216

4) H Itokawa, K Takeya, N Mori, S Kidokoro and H Yamamoto, *Plant Med*, **1984**, 50, 313, H Itokawa, K Takeya, N Mori, M Takanashi, H Yamamoto, T Sonobe and S Kidokoro, *Gann*, **1984**,

75, 929, H Itokawa, K Takeya, N Mori, T Sonobe, T Hamanaka, S Mihashi, M Takanashi and H Yamamoto, *J Pharmacobio-Dyn*, **1985**, 8, s-63, T Hamanaka, M Ohgoshi, K Kawahara, K. Yamakawa, T Tsuruo and S Tsukagoshi, *J Pharmacobio-Dyn*, **1987**, 10, 616, T Kato, Y Suzumura, F-Z Liu, H Tateno, T Ogiu and K Ota, *Jpn J Cancer Res*, **1989**, 80, 290

5) T Inaba, I Umezawa, M Yuasa, T Inoue, S Mihashi, H Itokawa and K Ogura, *J Org Chem*, **1987**, 52, 2957, D L Boger and D Yohannes, *J Am Chem Soc*, **1991**, 113, 1427
6) M Zalacain, E Zaera, D Vazquez and A Jimenez, *FEBS Let*, **1982**, 148, 95
7) S Lam, F Chow and A Karmer, *J Chromatogr*, **1980**, 199, 295
8) Final crystallographic coordinates and the structure factor table have been deposited in the Cambridge Crystallographic Data Center
9) W D S Motherwell, (1972), PLUTO A program for drqwing crystal and molecular structures, University Chemical Laboratory, Cambridge, England
10) D E Stewart, A Sarkar and J E Wampler, *J Mol Biol*, **1990**, 214, 253
11) Recently, a few examples about cyclic cis peptides without N-substituted amides have been reported., D F Mierke, T Yamazaki, O E Said-Nejad, E R Felder and M Goodman, *J Am Chem Soc*, **1989**, 111, 6847, H Kessler, U Anders and M Schudok, *ibid*, **1990**, 112, 5908
12) A Bax and M F Summers, *J Am Chem Soc*, **1986**, 108, 2094
13) D G Davis and A Bax, *J Am Chem Soc*, **1985**, 107, 2820
14) P N Lewis, F A Momany and H A Scheraga, *Israel, J Chem*, **1973**, 11, 121
15) G Bodenhauser, H Koger, R R Ernst, *J Magn Res*, **1984**, 58, 370
16) Some examples, a) H Kessler, *Angew Chem*, **1982**, 94, 509, *ibid*, Int Ed, **1982**, 21, 512, b) A Ravi, B V V Prasad and P Balaram, *J Am Chem Soc*, **1983**, 105, 105, c) M Iqbal and P Balaram, *ibid*, **1981**, 103, 5548, d) K D Kopple, M Ohnishi and A Go, *ibid*, **1969**, 91, 4264, e) M Ohnishi and D W Urry, *Biochem Biophys Res Commun*, **1969**, 36, 194
17) A Allerhand, D Doddrell and R Komoroski, J Chem Phys, **1971**, 55, 189, J R Lyerla and G C Levy, Top carbon-13 NMR Spectrosc **1974**, 1, 79-148
18) The significant lowerfield shift of Tyr-3-Cα in conformer A of RA-VIII agrees with those observed in α-helix type peptides, H Saito, R Tabeta, J Ando, T Osaki and A Shoji, *Chem Lett*, **1983**, 1437
19) P Auffinger, G Wipff, *J Comp Chem*, **1990**, 11, 19
20) AMBER 3 0 Rev A G Seibel, U C Singh, P K Weiner, J Caldwell, P Kollman, Univ California, San Francisco (1989)
21) R J Petroski, R B Bates, G S Linz and J P Rosazza, *J Pharmaceutical Sciences*, **1983**, 72, 1291
22) D L Boger and D Yohannes, *J Org Chem*, **1988**, 53, 487
23) G M Clore, A M Gronenborn, A T Brunger, M Karplus, *J Mol Biol*, **1985**, 186, 435
24) T Ando, J Yamawaki, T Kawate, S Sumi and T Hanafusa, *Bull Chem Soc Jpn*, **1982**, 55, 2504, T Ando, S J Brown, J H Clark, D G Cork, T Hanafusa, J Ichihara, J M Miller and M S Robertson, *J Chem Soc Perkin trans II*, **1986**, 1133
25) G Germain, P Main and M M Woolfson, *Acta Crystallogr*, **1971**, A27, 368