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RAI-III and VI, Conformational Isomers of Antitumor Cyclic Hexapeptides, RA-III and VI from *Rubia cordifolia*

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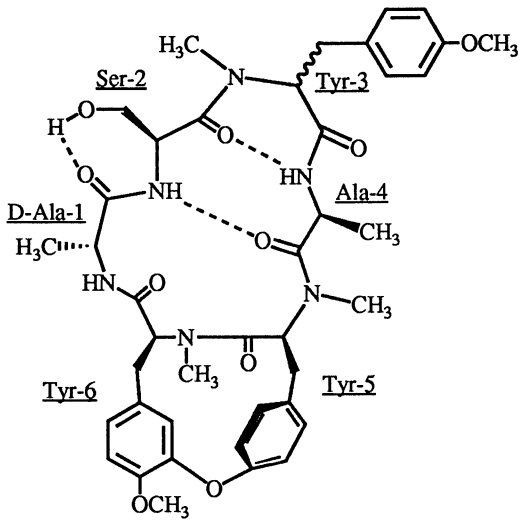
RAI-III and VI, conformational isomers of antitumor bicyclic hexapeptides, RA-III and VI, respectively, were isolated from *Rubia cordifolia*. By the conformational analysis of them using spectroscopic and computational chemical methods, they were shown to have γ-turn structures at residues 2, 3, and 4, which were stabilized by a hydrogen bond between Ser-2-OH and D-Ala-1-CO.

Bicyclic hexapeptides, RAs isolated from *Rubia cordifolia* and *R. akane* are potent antitumor agents. We have already reported about their structures[1] and antitumor activities.[2] As part of our program to study the structure-activity relationship of RAs, we have undertaken conformational analysis of RAs in the previous paper.[3] Chemical examination on minor antitumor principles from *R. cordifolia* led us to isolate two novel cyclic hexapeptides, named as RAI-III and VI, which were disclosed to be conformational isomers of RA-III and VI, respectively. In this communication, structure determination and conformational analysis of RAI-III and VI by chemical, spectroscopic and computational chemical evidences are reported.

Purification of MeOH extract from *R. cordifolia* was followed as cited in the previous paper[1] to give RA-III rich fraction. Further, chromatographic purification by ODS-HPLC (60% MeOH) gave RAI-III and VI.

RAI-III, colorless needles, mp 209-211 °C, $[\alpha]_D$ -38.3° (c 0.12, $CHCl_3$) showed the molecular formula, $C_{41}H_{50}N_6O_{10}$ (786.3597). After acid hydrolysis of RAI-III by 6 mol dm^{-3} HCl, the amino acid composition was determined as D-Ala : L-Ala : L-Ser (1:1:1) similar to those of RA-III.[4] Therefore, structural distinction in comparison to RA-III is considered to be in some of three N-methyl tyrosine units and/or conformational state. The NMR spectra in $CDCl_3$ showed two stable conformational states (Conformers A : B= 62 : 38) at equilibrium. The complete assignments[5] of all proton and carbon signals in RAI-III were made by a combination of 2D-NMR techniques (H-H COSY, C-H



RAI-III : L-Tyr-3
RAI-VI : D-Tyr-3

Fig. 1. Structures of RAI-III and VI.

COSY, and HMBC[6] spectra). The α carbon signal of Tyr-3 characteristic of RAs, which showed unusual lower field chemical shift in comparison to those of other comprising amino acids, was maintained in major conformer A, suggesting that Tyr-3 in RAI-III is L-form. Further, NOE relationships, which were almost the same as those of RA-VII,[3] showed that the two conformational states were based on the cis/trans isomerization of the amide bond at residues 2 and 3. However, unexpected NOE enhancement between Ser-2-NH and Ala-4-NH was observed. This suggested that RAI-III did not take the stable antiparallel conformation with hydrogen bond between Ala-4-NH and Ala-1-CO. This unexpected conformation was considered to contain a γ-turn structure at residues 2, 3, and 4, which was stabilized by the seven-membered ring with a hydrogen bond between Ser-2-OH and Ala-1-CO. The presence of this hydrogen bond was supported by the lower chemical shift of carbonyl carbon (δ172.24) in D-Ala-1, compared with those (δ171.57) of RA-III, and the unequivalent chemical shifts of Hβ protons (δ3.63 and 4.06) in Ser-2. The temperature coefficients[7] on NH protons showed that Ala-4-NH was strongly shielded from the solvent and Ser-2-NH weakly shielded, whereas D-Ala-1-NH was exposed to the solvent. This suggests the presence of a γ-turn which features a seven-membered ring formed via a hydrogen bond between Ala-4-NH and Ser-2-CO.

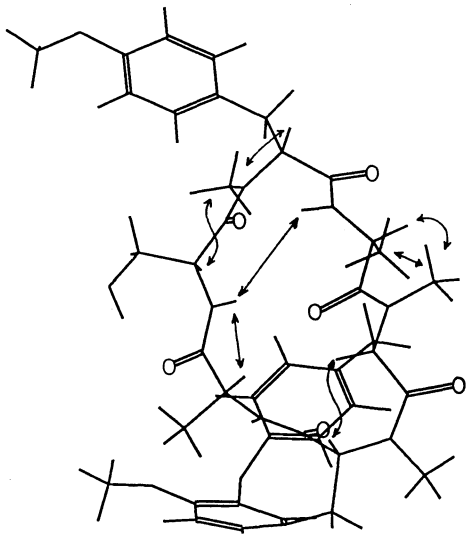


Fig. 2. The arrows show the NOE relationships of main conformer in RAI-III confirmed by NOESYPH experiments in $CDCl_3$ at 300 K.

Table 1. Calculated distances (Å) in major conformer of RAI-III between hydrogen bonds and between some protons observed NOEs

H-bonds	Ser-2-NH	Ala-4-O	2.33
	Ala-4-NH	Ser-2-O	1.89
	Ser-2-OH	Ala-1-O	1.91
NOEs	Ser-2-NH	Ala-4-NH	3.11
	Ser-2-Hα	Tyr-3-NMe(c)	2.53
	Tyr-3-NMe(c)	Tyr-3-Hα	2.54
	Tyr-5-Hα	Tyr-6-Hα	2.34

RAI-VI,[8] colorless needles, mp 200-202 °C, $[\alpha]_D$ -129.4° (c 0.17, $CHCl_3$) showed the same molecular formula to RAI-III (786.3590) and one conformational state in $CDCl_3$, and found out to be conformational isomer of RA-VI by the same method and the same NOE relationships as described above.

In order to obtain a more detailed structure and conformations, we performed the molecular dynamics calculations applying the distance constraints obtained from the NOE enhancements.[9] The resulting structures characterized in terms of relative energies were found to be identical to the solution conformations analyzed by spectroscopic evidences, as shown in Table 1 and Fig. 3.

The mobility of Tyr-3 rotation in RAI-III was suggested to increase by measuring ^{13}C relaxation times (T1).[10] The cytotoxic activities[11] were consistent with the results in the previous paper,[3] that is, the cytotoxic activities of RAs were influenced by the conformational population and the increased mobility of Tyr-3.

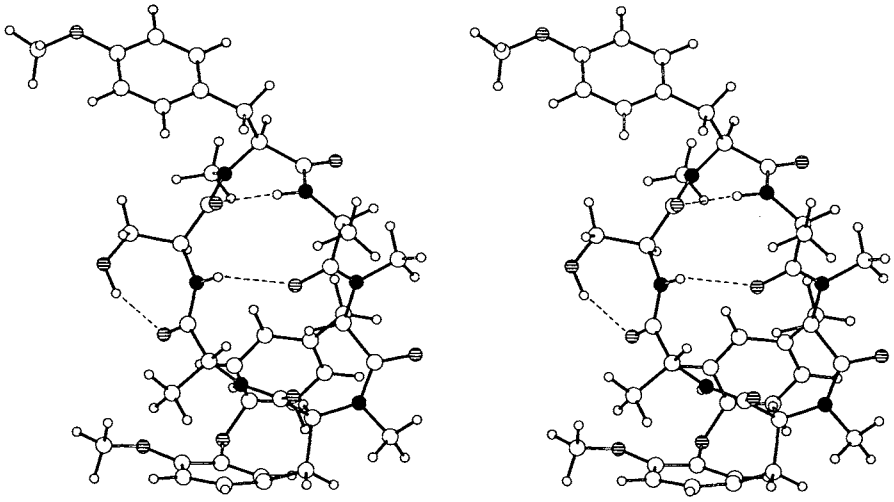


Fig. 3. Stereo drawing of major conformer in RAI-III.

We have already reported about the structures of RA series with a different kind of amino acid at residue 2.[1,12] In a view point of biosynthetic pathway, cyclization process may be occurred around residue 2 and these conformational isomers, RAI-III and VI may be produced during this cyclization process.[13]

References

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2) H. Itokawa, K. Takeya, N. Mori, S. Kidokoro, and H. Yamamoto, *Plant Med.*, **50**, 313 (1984); H. Itokawa, K. Takeya, N. Mori, M. Takanashi, H. Yamamoto, T. Sonobe, and S. Kidokoro, *Gann*, **75**, 929 (1984); H. Itokawa, K. Takeya, N. Mori, T. Sonobe, T. Hamanaka, S. Mihashi, M. Takanashi, and H. Yamamoto, *J. Pharmacobio-Dyn.*, **8**, s-63 (1985).

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4) S. Lam, F. Chow, and A. Karmer, *J. Chromatogr.*, **199**, 295 (1980).

5) ^{1}H-NMR δppm in $CDCl_3$; (Conformers A:B); D-Ala-1 (4.62 : 4.66, Hα), (1.28 : 1.33, Hβ), (6.64 : 6.70, HN); Ser-2 (4.90 : 4.33, Hα), (3.63 : 3.32, Hβ1), (4.06 : 3.43, Hβ2), (9.09 : 7.30, HN); Tyr-3 (3.63 : 4.49, Hα), (3.49 : 3.25, Hβ1), (3.41 : 2.89, Hβ2), (7.10 : 7.09, 2Hδ), (6.84 : 6.84, 2Hε), (2.74 : 2.86, MeN), (3.79 : 3.76, MeO); Ala-4 (4.91 : 4.47, Hα), (1.26 : 1.42, Hβ), (6.25 : 7.43, HN); Tyr-5 (5.42 : 5.27, Hα), (3.60 : 3.69, Hβ1), (2.84 : 2.73, Hβ2), (7.28 : 7.28,Hδ1), (7.33 : 7.39, Hδ2), (6.91 : 6.89, Hε1), (7.18 : 7.18, Hε2), (3.23 : 3.14, MeN); Tyr-6 (4.56 : 4.58, Hα), (3.03 : 2.93, Hβ1), (2.91 : 3.15, Hβ2), (6.58 : 6.60, Hδ1), (4.45 : 4.46, Hδ2), (6.81 : 6.81, Hε1), (2.68 : 2.53, MeN), (3.94 : 3.94, MeO); ^{13}C-NMR; (Conformers A:B); D-Ala-1 (48.20 : 49.25, Cα), (18.71 : 19.89, Cβ), (172.24 : 172.21, CC=O); Ser-2 (49.13 : 48.53, Cα), (63.01 : 63.10, Cβ), (172.44 : 172.37, CC=O); Tyr-3 (69.53

: 63.62, Cα), (33.27 : 32.32, Cβ), (130.46 : 128.88, Cγ), (130.02 : 129.91, Cδ), (114.19 : 114.37, Cε), (158.76 : 158.76, Cζ), (166.93 : 167.99, CC=O), (55.27 : 55.33, CO), (39.82 : 29.62, CN); Ala-4 (45.27 : 47.17, Cα), (18.07 : 17.03, Cβ), (170.92 : 171.71, CC=O); Tyr-5 (53.74 : 55.04, Cα), (36.03 : 36.62, Cβ), (134.66 : 135.00, Cγ), (133.17 : 132.87, Cδ1), (130.36 : 130.60, Cδ2), (124.82 : 124.44, Cε1), (126.03 : 126.00, Cε2), (158.50 : 158.32, Cζ), (170.23 : 170.54, CC=O), (30.58 : 30.76, CN); Tyr-6 (57.90 : 58.14, Cα), (35.54 : 33.80, Cβ), (128.32 : 128.00, Cγ), (120.79 : 120.96, Cδ1), (113.87 : 113.87, Cδ2), (112.47 : 112.38, Cε1), (153.29 : 153.15, Cε2), (146.71 : 146.64, Cζ), (170.47 : 171.23, CC=O), (29.58 : 28.90, CN), (56.26 : 56.22, CO).

6) A. Bax and M. F. Summers, *J. Am. Chem. Soc.*, **108**, 2094 (1986).

7) Temperature effect on NH protons in $CDCl_3$ ($-\Delta\delta/\Delta T$, 10^3 ppm/K): RAI-III, (Conformer A), D-Ala-1: 17.3, Ser-2: 4.8, Ala-4: -1.8; (Conformer B), D-Ala-1: 15.2, Ser-2: 4.0, Ala-4: 2.1; RAI-VI, D-Ala-1: 20.0, Ser-2: 4.0, Ala-4: -0.5; Some examples of γ-turn: M. A. Khaled, D. W. Urry, and K. Okamoto, *Biochem. Biophys. Res. Commun.*, **72**, 162 (1976); R. Kishore and P. Balaram, *Biopolymers*, **24**, 2041 (1985); A. F. Spatola, M. K. Anwer, A. L. Rockwell, and L. M. Gierasch, *J. Am. Chem. Soc.*, **108**, 825 (1986).

8) ^{1}H-NMR δppm in $CDCl_3$; D-Ala-1 (4.66, Hα), (1.32, Hβ), (6.45, HN); Ser-2 (5.02, Hα), (3.62 and 4.01, Hβ), (7.86, HN); D-Tyr-3 (5.63, Hα), (2.86, Hβ1), (3.44, Hβ2), (7.14, Hδ), (6.83, Hε), (2.75, MeN), (3.78, MeO); Ala-4 (4.59, Hα), (1.29, Hβ), (6.94, HN); Tyr-5 (5.36, Hα), (3.64, Hβ1), (2.73, Hβ2), (7.29, Hδ1), (7.42, Hδ2), (6.91, Hε1), (7.24, Hε2), (3.19, MeN); Tyr-6 (4.61, Hα), (2.98, Hβ1), (3.06, Hβ2), (6.60, Hδ1), (4.46, Hδ2), (6.82, Hε1), (2.60, MeN), (3.95, MeO); 13C-NMR; D-Ala-1 (48.58, Cα), (18.50, Cβ), (171.37, CC=O); Ser-2 (49.05, Cα), (63.18, Cβ), (172.85, CC=O); D-Tyr-3 (56.97, Cα), (31.02, Cβ), (128.90, Cγ), (129.50, Cδ), (114.19, Cε), (158.47, Cζ), (168.07, CC=O), (30.46, CN), (55.27, CO); Ala-4 (46.64, Cα), (18.03, Cβ), (172.29, CC=O); Tyr-5 (53.99, Cα), (36.74, Cβ), (134.70, Cγ), (133.16, Cδ1), (130.53, Cδ2), (124.55, Cε1), (126.13, Cε2), (158.47, Cζ), (170.03, CC=O), (29.94, CN); Tyr-6 (57.74, Cα), (34.52, Cβ), (127.77, Cγ), (120.89, Cδ1), (113.90, Cδ2), (112.52, Cε1), (153.25, Cε2), (146.78, Cζ), (170.75, CC=O), (29.04, CN), (56.25, CO).

9) The initial calculations were started with RA-III to obtain a major conformer A. Molecular dynamics calculations were made at 298 K for 100 ps with the time step 1fs and the structures were sampled every 0.1 ps (AMBER 3.0 Rev. A package, ε=1r). The NOE between Ser-2-NH and Ala-4-NH was taken into consideration, and were calculated with an extra square well potential of the form $E=\Sigma 5$ kcal $Å^{-2}(r-r_{max})^2$ for $r>r_{max}$ (r_{max}=3.0Å). All the snapshots were then energy minimized and one with the lowest energy (6.27kcal/mol) was selected as the relevant conformation.

10) The T1 values (msec.) of Cδ and Cε in Tyr-3 of RAI-3, conformer A: 435(Cδ), 550(Cε); conformer B: 525(Cδ), 449(Cε).

11) Cytotoxic activities (IC_{50} μg/ml) of RAI-III against P388 and KB cells: 0.14 (P388), 0.74 (KB).

12) The 38th Annual Meeting of the Japanese Society of Pharmacognosy, Kobe, 1991, Abstract papers, pp.183-184; H. Itokawa, T. Yamamiya, H. Morita, and K. Takeya, in preparation.

13) We did not succeed in thermodynamic conversion from RA-III to RAI-III.

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