Solution Forms of an Antitumor Cyclic Hexapeptide, RA-VII in Dimethyl Sulfoxide-d_6 from Nuclear Magnetic Resonance Studies

Hideji ITOKAWA,* Hiroshi MORITA and Koichi TAKEYA

Department of Pharmacognosy, Tokyo College of Pharmacy, Horinouchi 1432–1, Hachioji, Tokyo 192–03, Japan. *Received September 24, 1991*

Using high-resolution proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) experiments, we have assigned three discernible configurational isomers observed in dimethyl sulfoxide-d_6 (DMSO-d_6) for an antitumor cyclic hexapeptide, RA-VII isolated from *Rubia cordifolia*. The largest isomer, amounting to 64%, has been assigned as conformer A with only a *cis* configuration between Tyr-5 and Tyr-6. The second configurational isomer, accounting for 32%, has adopted *cis* configurations between both Tyr-5 and Tyr-6 and between Ala-2 and Tyr-3. The third isomer, amounting to 4%, was determined to have *cis* configurations for all of the three *N*-methyl amide bonds.

Keywords RA-VII; conformational analysis; *Rubia cordifolia*; antitumor agent; cyclic hexapeptide

Introduction

Cyclic hexapeptides, RA series, isolated from *Rubia cordifolia* and *R. akane*, are potent antitumor agents. We have already disclosed the structures of RA-I—RA-X,[1)] RAI-III and RAI-VI[2)] and their antitumor activities.[3)] As part of our ongoing program to study their structure–activity relationship, we have undertaken conformational analysis of RAs using the spectroscopic and computational methods.[1b,4,5)]

In an apolar solvent, for example, in $CDCl_3$, the presence of two stable conformational states, *i.e.* conformers A and B, were observed.[4)] These conformers could result from isomerization about one N-methyl amide bond between Ala-2 and Tyr-3 with an isomerization rate slow enough to give separate, signals in the nuclear magnetic resonance (NMR) spectra. In a polar solvent such as dimethyl sulfoxide-d_6 (DMSO-d_6), on the other hand, three different conformers A, B and C were observed in the NMR spectra.[4)]

A detailed knowledge of the conformations of RA-VII under a polar solvent such as DMSO-d_6 is considered to be the basis for structure–activity relationships allowing the design of new derivatives with higher activity. In this paper, by use of various two-dimentional proton and carbon-13 NMR (^{1}H and ^{13}C-NMR) experiments, the structural elucidation of three different conformers of RA-VII in DMSO-d_6 is reported.

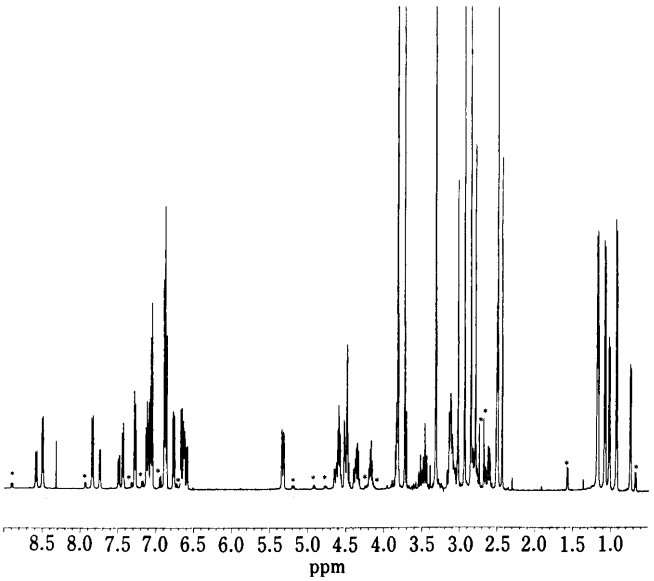


Fig. 1. 500-MHz ^{1}H-NMR Spectrum of RA-VII in DMSO-d_6

Asterisks mark the peaks ascribable to conformer C. Some of the tops of the methoxyl and N-methyl proton signals ascribable to conformers A and B were cut off. Tetramethylsilane was used as an internal standard.

Results and Discussion

In the previous paper,[4)] we were unable to clearly elucidate the three conformers, named conformers A, B and C, of RA-VII in DMSO-d_6 solution. Figure 1 showed a one-dimensional (1-D) ^{1}H-NMR spectrum of RA-VII in DMSO-d_6. The population of three conformers, A, B and C, was in the ratio of 64:32:4, and a very complicated

TABLE I. ^{1}H-NMR Chemical Shifts of RA-VII, δ (ppm) from Tetramethylsilane in DMSO-d_6 at 303 K (J/Hz, 500 MHz)

Amino acid	Proton	RA-VII		
		Conformer A	B	C
D-Ala-1	H_α	4.35 $J_{\alpha\beta}=6.9$	4.17 $J_{\alpha\beta}=7.0$	3.89 $J_{\alpha\beta}=7.0$
	H_β	1.08 $J_{\alpha N}=8.5$	1.02 $J_{\alpha N}=6.9$	1.05 $J_{\alpha N}=5.5$
	H_N	7.81	7.72	7.91
Ala-2	H_α	4.60 $J_{\alpha\beta}=6.8$	4.20 $J_{\alpha\beta}=6.4$	4.15 $J_{\alpha\beta}=6.5$
	H_β	1.17 $J_{\alpha N}=7.3$	0.74 $J_{\alpha N}=9.0$	0.67 $J_{\alpha N}=9.6$
	H_N	8.47	8.56	8.87
Tyr-3	H_α	3.83 $J_{\alpha\beta1}=$ a)	4.38 $J_{\alpha\beta1}=10.8$	5.19 $J_{\alpha\beta1}=11.0$
	$H_{\beta1(pro\text{-}R)}$	3.12 $J_{\alpha\beta2}=$ a)	2.82 $J_{\alpha\beta2}=3.7$	2.76 $J_{\alpha\beta2}=2.6$
	$H_{\beta2(pro\text{-}S)}$	3.12 $J_{\beta1\beta2}=$ a)	3.05 $J_{\beta1\beta2}=14.6$	3.26 $J_{\beta1\beta2}=$ a)
	$2H_\delta$	7.05 $J_{\delta\varepsilon}=8.6$	7.08 $J_{\delta\varepsilon}=8.6$	7.08 $J_{\delta\varepsilon}=8.6$
	$2H_\varepsilon$	6.88	6.86	6.86
	MeN	2.85	2.79	2.68
	MeO	3.73	3.73	3.70
Ala-4	H_α	4.60 $J_{\alpha\beta}=6.7$	4.47 $J_{\alpha\beta}=6.8$	4.91 $J_{\alpha\beta}=6.3$
	H_β	0.92 $J_{\alpha N}=8.2$	1.16 $J_{\alpha N}=7.3$	1.57 $J_{\alpha N}=9.2$
	H_N	6.66	6.59	6.73
Tyr-5	H_α	5.32 $J_{\alpha\beta1}=11.4$	5.32 $J_{\alpha\beta1}=11.4$	4.23 $J_{\alpha\beta1}=$ a)
	$H_{\beta1(pro\text{-}S)}$	3.46 $J_{\alpha\beta2}=2.9$	3.52 $J_{\alpha\beta2}=2.9$	3.60 $J_{\alpha\beta2}=$ a)
	$H_{\beta2(pro\text{-}R)}$	2.61 $J_{\beta1\beta2}=11.4$	2.67 $J_{\beta1\beta2}=11.4$	3.60 $J_{\beta1\beta2}=$ a)
	$H_{\delta1}$	7.27 $J_{\delta1\delta2}=2.1$	7.28 $J_{\delta1\delta2}=2.2$	7.31 $J_{\delta1\delta2}=2.6$
	$H_{\delta2}$	7.43 $J_{\delta1\varepsilon1}=8.5$	7.48 $J_{\delta1\varepsilon1}=8.5$	7.42 $J_{\delta1\varepsilon1}=8.1$
	$H_{\varepsilon1}$	6.76 $J_{\delta2\varepsilon2}=8.4$	6.76 $J_{\delta2\varepsilon2}=8.4$	6.85 $J_{\delta2\varepsilon2}=8.5$
	$H_{\varepsilon2}$	7.12 $J_{\varepsilon1\varepsilon2}=2.2$	7.11 $J_{\varepsilon1\varepsilon2}=2.3$	7.17 $J_{\varepsilon1\varepsilon2}=2.2$
	MeN	2.93	3.02	2.74
Tyr-6	H_α	4.50 $J_{\alpha\beta1}=$ a)	4.64 $J_{\alpha\beta1}=12.0$	4.77 $J_{\alpha\beta1}=7.0$
	$H_{\beta1(pro\text{-}R)}$	3.10 $J_{\alpha\beta2}=$ a)	3.10 $J_{\alpha\beta2}=3.7$	2.91 $J_{\alpha\beta2}=7.0$
	$H_{\beta2(pro\text{-}S)}$	2.82 $J_{\beta1\beta2}=$ a)	2.82 $J_{\beta1\beta2}=$ a)	2.91 $J_{\beta1\beta2}=$ a)
	$H_{\delta1}$	6.62 $J_{\delta1\delta2}=1.4$	6.62 $J_{\delta1\delta2}=1.7$	6.72 $J_{\delta1\delta2}=1.4$
	$H_{\delta2}$	4.48 $J_{\delta1\varepsilon1}=8.3$	4.52 $J_{\delta1\varepsilon1}=8.3$	4.48 $J_{\delta1\varepsilon1}=8.5$
	$H_{\varepsilon1}$	6.88	6.88	6.94
	MeN	2.50	2.43	2.52
	MeO	3.81	3.82	3.83

a) Not determined in the present study.

spectrum was indicated. At first, to assign the ^1H and ^{13}C signals of three different conformers, various two-dimensional (2-D) NMR measurements were carried out. Complete assignments of conformers A and B were possible using a combination of ^1H–^1H correlated spectroscopy (COSY), ^1H–^{13}C COSY and heteronuclear multiple bond correlation spectroscopy (HMBC),[6] which provides ^1H–^{13}C long range couplings. As shown in Fig. 1, we were lucky to resolve the signals around δ 5.0 ascribable to minor conformer C. Though some signals were ambiguous because of overlapping, homonuclear Hartmann–Hahn (HOHAHA)[7] spectrum was quite efficient for interpreting the intra-residue proton connectivities of each amino acid in conformer C. The assignments of ^1H and ^{13}C signals of conformers A, B and C in DMSO-d_6 are shown in Tables I and II.

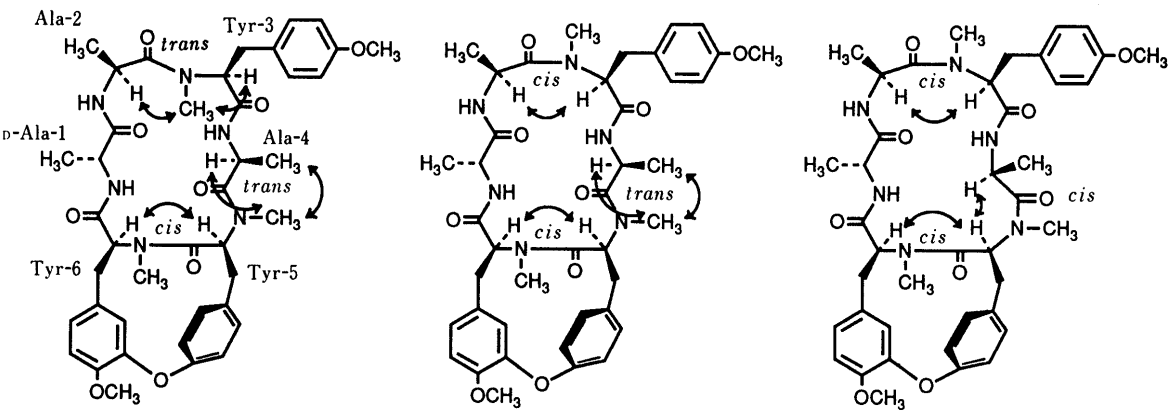
TABLE II. ^{13}C-NMR Chemical Shifts of RA-VII, δ (ppm) from Tetramethylsilane in DMSO-d_6 at 303 K (125 MHz)

Amino acid	Carbon	RA-VII Conformer A	B	C
D-Ala-1	C_α	46.48	47.30	48.87
	C_β	20.69	20.69	18.68
	$C_{C=O}$	171.27	171.17	170.13
Ala-2	C_α	43.58	43.00	42.05
	C_β	16.08	17.26	16.38
	$C_{C=O}$	172.20	170.94	171.96
Tyr-3	C_α	66.53	61.61	61.72
	C_β	32.29	32.45	31.93
	C_γ	130.72	129.63	a)
	C_δ	130.12	129.93	129.82
	C_ε	113.60	113.82	a)
	C_ζ	157.82	158.09	a)
	$C_{C=O}$	167.92	169.17	167.29
	C_N	39.15	29.49	29.55
	C_O	54.90	55.03	a)
Ala-4	C_α	45.47	47.48	46.23
	C_β	18.17	16.14	20.28
	$C_{C=O}$	170.44	169.68	173.09
Tyr-5	C_α	53.53	53.31	59.67
	C_β	35.90	35.86	37.12
	C_γ	135.50	135.00	135.33
	$C_{\delta1}$	132.62	132.62	133.18
	$C_{\delta2}$	130.45	130.38	a)
	$C_{\varepsilon1}$	123.61	123.82	123.52
	$C_{\varepsilon2}$	125.66	125.66	125.66
	C_ζ	157.68	157.99	157.15
	$C_{C=O}$	168.77	169.68	167.59
	C_N	30.00	29.86	30.42
Tyr-6	C_α	56.50	56.82	55.61
	C_β	34.65	34.82	33.48
	C_γ	129.55	129.07	128.81
	$C_{\delta1}$	121.10	120.90	121.10
	$C_{\delta2}$	114.08	114.66	114.46
	$C_{\varepsilon1}$	112.69	112.69	113.33
	$C_{\varepsilon2}$	152.27	153.32	152.01
	C_ζ	145.82	145.76	145.76
	$C_{C=O}$	169.62	170.74	170.06
	C_N	29.02	28.79	29.20
	C_O	55.74	55.74	a)

a) Not determined in the present study.

The assignments of conformers A and B were almost identical with those observed in $CDCl_3$.[4] In the ^1H assignment of conformer C, the chemical shift of Tyr-3-H_α (δ 5.19) was shifted to a lower field and that of Ala-2-CH_3 (δ 0.67) to a higher field, compared with those of conformer B (δ 4.38 and 0.74, respectively). The unusual low-field chemical shift of Ala-4-CH_3 (δ 1.57) and a high-field chemical shift of Tyr-5-H_α (δ 4.23) were shown. The α proton of D-Ala-1 (δ 3.89) was also shifted to a high field. In the ^{13}C assignment of conformer C, the chemical shift of Tyr-3-C_α (δ 61.72), which was similar to that of conformer B (δ 61.61) with a *cis* configuration between Ala-2 and Tyr-3, was shifted to a higher field than that of conformer A (δ 66.53). The chemical shifts of Ala-4-CH_3 (δ 20.28) and Tyr-5-C_α (δ 59.67) were shown in a lower field than those of conformer B (δ 16.14 and 53.31, respectively). In this manner, conformer C showed a chemical shift similar to that of conformer B around Ala-2 and Tyr-3, but was deduced to possess different configurations and/or conformations around Ala-4 and Tyr-5.

To confirm the above hypothesis, the measurement of intraresidual nuclear Overhauser effects (NOEs) among each conformer was made by a phase sensitive nuclear Overhauser and exchange spectroscopy (NOESYPH) spectrum.[8] The characteristic NOE relationships to determine the configurations of three N-methyl amide bonds were indicated in Fig. 2. In Fig. 3, the expansion plot of 4.0—5.5 ppm in NOESYPH spectrum, which showed signals representative of conformer C, was shown. By the NOE relationship, conformers A and B in DMSO-d_6 are completely identical with those in $CDCl_3$. On the other



conformer A conformer B conformer C

Fig. 2. Molecular Structures of Three Different Conformers A, B and C of RA-VII in DMSO-d_6

The arrows show the NOE relationships confirmed by NOESYPH experiments.

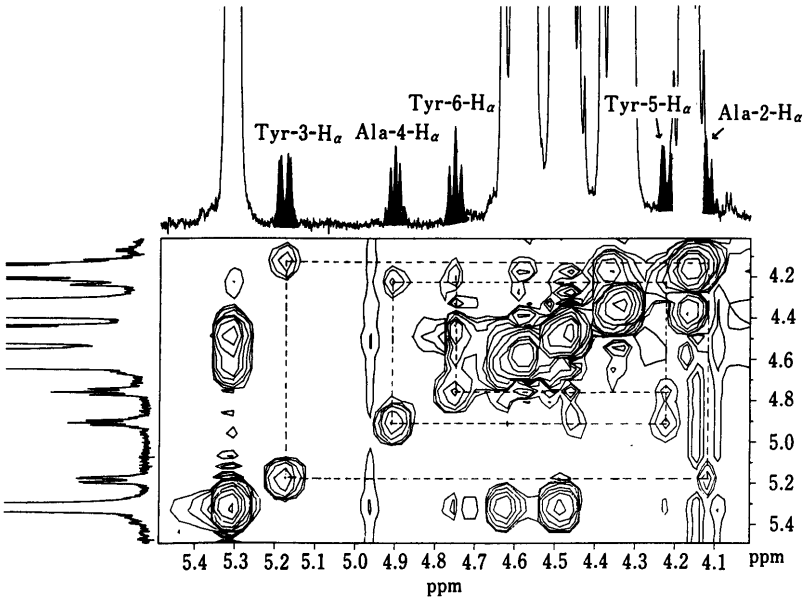


Fig. 3. Expansion Plot in the Range of 4.0 to 5.5 ppm of NOESYPH Spectrum of RA-VII in DMSO-d_6

The shadow peaks are ascribable to conformer C.

hand, the cross peak between Tyr-3-H_α and Ala-2-H_α, in addition to that between Tyr-5-H_α and Tyr-6-H_α in Fig. 3, indicated the *cis* configurations in both of the N-methyl amide bonds. In addition, the configuration of the other N-methyl amide bond between Ala-4 and Tyr-5 was determined to be *cis* by the strong correlated cross peak between Ala-4-H_α and Tyr-5-H_α. The reason for the lower field chemical shift of Ala-4-CH_3 in conformer C as indicated above was considered to be due to the deshielding effect of the aromatic ring in Tyr-5.

Based on the above ^1H and ^{13}C assignments and NOE relationships, the largest isomer has been assigned as conformer A with only a *cis* configuration between Tyr-5 and Tyr-6, and the second isomer has adopted both *cis* configurations between Tyr-5 and Tyr-6 and between Ala-2 and Tyr-3. Then, the third isomer was determined to be *cis* configurations for all three N-methyl amide bonds. The conformational analysis of RAs in a vital system is in progress.

Experimental

Material RA-VII used in this experiment was isolated from *Rubia cordifolia* by the procedure cited in reference 1.

NMR Spectra The proton and carbon spectra were recorded on a Bruker spectrometer (AM500) and processed on a Bruker data station with an Aspect 3000 computer. 10 mg samples of RA-VII dissolved in 0.5 ml DMSO-d_6 in a 5 mm tube were used for the homonuclear measurement and 30 mg samples in 0.5 ml DMSO-d_6 in a 5 mm tube for the heteronuclear measurement. The spectra were recorded at 300 K. The NOESYPH experiment was made with a mixing time of 0.6 s.

References

1) *a*) H. Itokawa, K. Takeya, K. Mihara, N. Mori, T. Hamanaka, T. Sonobe and Y. Iitaka, *Chem. Pharm. Bull.*, **31**, 1424 (1983); H. Itokawa, K. Takeya, N. Mori, T. Hamanaka, T. Sonobe and K. Mihara, *ibid.*, **32**, 284 (1984); H. Itokawa, K. Takeya, N. Mori, T. Sonobe, S. Mihashi and T. Hamanaka, *ibid.*, **34**, 3762 (1986); *b*) H. Itokawa, H. Morita, K. Takeya, N. Tomioka, A. Itai and Y. Iitaka, *Tetrahedron*, **47**, 7007 (1991); H. Itokawa, T. Yamamiya, H. Morita and K. Takeya, *J. Chem. Soc., Perkin Trans. 1*, **1992**, 455.
2) H. Itokawa, H. Morita, K. Takeya, N. Tomioka and A. Itai, *Chem. Lett.*, **1991**, 2217.
3) 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**, 63 (1985); T. Hamanaka, M. Ohgoshi, K. Kawahara, K. Yamakawa, T. Tsuruo and S. Tsukagoshi, *ibid.*, **10**, 616 (1987); T. Kato, Y. Suzumura, F.-Z. Liu, H. Tateno, T. Ogiu and K. Ota, *Jpn. J. Cancer Res. (Gann)*, **80**, 290 (1989).
4) H. Morita, K. Kondo, Y. Hitotsuyanagi, K. Takeya, H. Itokawa, N. Tomioka, A. Itai and Y. Iitaka, *Tetrahedron*, **47**, 2757 (1991).
5) H. Itokawa, K. Saitou, H. Morita and K. Takeya, *Chem. Pharm. Bull.*, **39**, 2161 (1991).
6) A. Bax and M. F. Summers, *J. Am. Chem. Soc.*, **108**, 2094 (1986).
7) A. Bax and D. G. Davis, *J. Magn. Reson.*, **65**, 355 (1985).
8) G. Bodenhauser, H. Koger and R. R. Ernst, *J. Magn. Res.*, **58**, 370 (1984).