Solution Conformation of Ginseng Tetrapeptide H–L-Val–γ-D-Glu–D-Arg–Gly–OH

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The ginseng tetrapeptide H–L-Val–γ-D-Glu–D-Arg–Gly–OH (1) was synthesized using a solution-phase methodology, and its solution conformation in dimethyl sulfoxide (DMSO)-d_6 was determined by a combination of NMR and computational techniques with the DADAS90 program. This compound has a rigid backbone based on three intramolecular hydrogen bonds between D-Arg NH and L-Val CO, between Gly NH and D-Glu CO, and between Gly NH and Gly CO.

Key words ginseng tetrapeptide; conformation; NMR; intramolecular hydrogen bond

Panax ginseng is one of the most widely used medicinal plants, particularly by older people, and is of interest to gerontologists.[1] The water extract of ginseng has been found to delay the degeneration of amnion cells in culture to a greater extent than hydrocortisone.[2] In screening of the water extract for proliferative activity towards baby hamster kidney (BHK)-21 cells, we isolated a heat-stable alkaline tetrapeptide, H–L-Val–γ-D-Glu–D-Arg–Gly–OH (**1**),[3] which has a unique structure containing two D-amino acid residues and a γ peptide bond. This tetrapeptide (**1**) caused 20% enhancement of proliferation of BHK-21 cells at a concentration of 3.4 μM.

This paper deals with conformational analysis of **1** in dimethyl sulfoxide (DMSO)-d_6 by a combination of NMR and computational techniques with the DADAS90 program.[4] Precise knowledge of the conformation of **1** in a polar solvent such as DMSO-d_6 is essential for studies of the structure–activity relationships and for the design of new derivatives with higher activity.

Results and Discussion

It is necessary for conformational analysis to make a complete assignment of ^{1}H- and ^{13}C-NMR signals in DMSO-d_6 solution. These signal assignments were performed by a combination of two dimensional (2D) NMR techniques such as correlation spectroscopy (COSY), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC)[5] and heteronuclear multiple bond connectivity (HMBC)[6] spectra, and are shown in Table 1. The possibility of peptide aggregation of **1** was examined by recording ^{1}H- and ^{13}C-NMR spectra of solutions containing 5—50 mM (**1**) in CD_3OD and DMSO-d_6. No significant changes in chemical shifts or line widths were observed over this concentration range.

The amino proton and carbonyl groups which participate in intramolecular hydrogen bonding were identified as follows. D_2O (10 μl) was added to a solution of **1** (2.3 mg) in DMSO-d_6 (500 μl), and the signals of the amino protons were monitored by ^{1}H-NMR. The signals of D-Glu NH and D-Arg ε and η NH disappeared immediately, and those of Gly and D-Arg NH did so after 120 min. These observations indicated that the latter amino protons are strongly shielded from the solvent and are involved in hydrogen bonding.

Figure 1 shows the solvent dependence of chemical shifts of carbonyl carbons in the ^{13}C-NMR spectra. On addition of D_2O to a solution of **1** (4.6 mg) in DMSO-d_6 (500 μl), the signals of L-Val CO, Gly CO, and D-Glu CO were shifted less than other carbonyl signals. In such D_2O titration, a peptide carbonyl group which is hydrogen-bonded should exhibit little downfield shift.[7] Therefore, these three carbonyl groups were concluded to participate in hydrogen bonding, whereas D-Glu δ CO and D-Arg CO did not. These results were not dependent on the concentration of **1**, indicating that these hydrogen bonds were formed intramolecularly. Thus, the six intramolecular hydrogen-bonding patterns in Chart 1 should be considered.

The relationships among the rotating frame nuclear overhauser effect (ROE) enhancements in **1** are listed in Table 2, and the cross-peak intensities were quantified in terms of the volume integral of each cross-peak into strong (s), medium (m), and weak (w). Three important correlations were strong ROEs between L-Val Hα and D-Glu NH, between D-Glu Hα and D-Glu Hγ, and between

Table 1. ^{1}H- and ^{13}C-NMR Chemical Shifts (ppm) of H–L-Val–γ-D-Elu–D-Arg–Gly–OH (**1**)

	Proton	Carbon		Proton	Caron
L-Val			D-Arg		
α	3.69 (d, 5.1)	57.19	α	4.28 (ddd, 5.4, 8.1)	51.80
β	2.09 (m)	29.83	β	1.69, 1.53 (m)	29.05
γ	0.95, 0.92 (d, 6.9)	18.26,	γ	1.50 (m)	24.81
		17.29	δ	3.08 (m)	40.26
CO		167.79	ε	7.95 (m)	
			ζ		156.87
D-Glu			η	7.1—7.6 (m)	
α	4.24 (ddd, 5.6, 7.8)	52.05	NH	8.10 (d, 8.0)	
β	1.98, 1.84 (m)	27.15	CO		171.75
γ	2.23 (m)	31.30			
NH	8.72 (d, 7.5)		Gly		
CO		172.91	α	3.77, 3.71	40.68
CδO		171.21		(dd, 17.3)	
			NH	8.24 (dd, 5.6, 5.7)	
			CO		171.05

A solution containing **1** (8.5 mg) in DMSO-d_6 (500 μl) was used for ^{1}H- and ^{13}C-NMR measurements at 25 °C.

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D-Glu Hγ and D-Arg NH, indicating the presence of a γ-turn-like conformation formed by the three residues, L-Val, D-Glu, and D-Arg, and that D-Arg NH is hydrogen-bonded with L-Val CO, as shown in a, b, and f of Chart 1.

Information about the structural flexibility of **1** can be experimentally obtained from the T_1 relaxation times of the carbon resonances. The NT_1 values[8] (N=number of attached protons, T_1=longitudinal relaxation time) correlate directly with the molecular mobility. All the α carbons gave very similar NT_1 values [214 (L-Val), 271 (D-Glu), 259 (D-Arg), 224 ms (Gly)]. Although linear peptides are generally flexible, these findings suggested that **1** has a rigid backbone due to the formation of the three intramolecular hydrogen bonds. On the other hand, the larger values of the side chain of D-Arg [3780 (Cβ), 3460 (Cγ), 3080 ms (Cδ)] reflect high mobility.

The intramolecular hydrogen-bonding patterns of b and f are more plausible than that of a, because the former two may form a backbone rigid enough to afford similar NT_1 values of α carbons, whereas the latter can not. Futhermore strong and medium ROE correlations between D-Arg Hα and Gly NH, and between D-Glu Hα and Gly NH support the presence of an intramolecular hydrogen bond between Gly NH and D-Glu CO (Table 2).

The similar chemical shift values of the two Hα protons of Gly in the ^{1}H-NMR spectrum, 3.71 and 3.77 ppm (Table 1), implied that the distances between these two protons and the carbonyl group of D-Arg which produced a strong shielding effect were almost the same. These observations indicated that **1** contains the moiety shown in Chart 2, that is, an intra-residue hydrogen bond was formed between Gly NH and Gly CO, as shown in f in Chart 1.

We applied computational procedures using NMR data to elucidate the solution conformation of **1**. The distance geometry calculations were performed with the DADAS90[4] program implemented in the MolSkop system (JEOL Ltd., Akishima, Japan). The program adopted the variable target function method in the dihedral angle space.[9] The target function is only composed of square well functions of ROE constraints, angle constraints (from J spin–spin coupling constants), hydrogen bonding constraints, and Van der Waals soft repulsion, but not constraints of any energy functions. The calculation method used here was developed from the DISMAN program, which is effective for the determination of protein conformations.[10,11]

In the tetrapeptide (**1**) the 27 ROEs shown in Table 2 were classified as s, m, and w, corresponding to upper

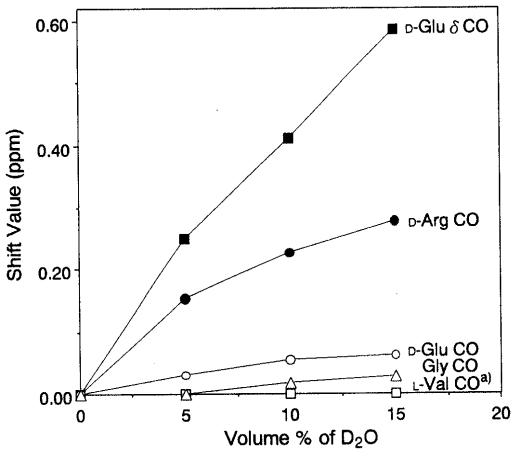

Fig. 1. Shifts of Carbonyl Signals of **1** in ^{13}C-NMR Spectra upon Addition of D_2O

a) L-Val CO is taken as the internal reference.

Table 2. Observed ROESY[a] Cross-Peaks and Their Intensities

NH	HCα	HCβ	HCγ	HCδ	Others	Intensity[b]
	Val	Val				s
	Val				Val Me	s
		Val			Val Me	s
Glu	Val					s
Glu		Val				w
Glu					Val Me	w
Glu	Glu					m
Glu		Glu				w
Glu			Glu			m
	Glu	Glu				s
	Glu		Glu			s
		Glu/Glu				s
		Glu	Glu			s
Arg			Glu			s
Arg	Arg					s
Arg			Arg			w
	Arg	Arg				m
	Arg		Arg			m
	Arg			Arg		m
		Arg	Arg			s
		Arg		Arg		s
			Arg	Arg		s
			Arg		Arg εNH	w
				Arg	Arg ε NH	s
Gly	Glu					m
Gly	Arg					s
Gly	Gly					s

a) A solution containing **1** (8.5 mg) in DMSO-d_6 (500 μl) was used for ROESY measurement at 25 °C. *b*) s, m, and w mean strong, medium, and weak cross-peaks, respectively.

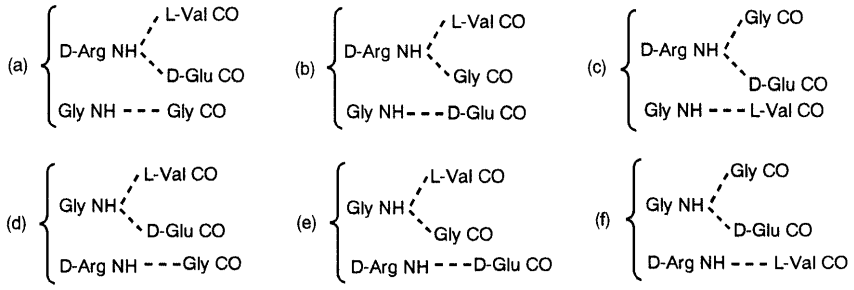

Chart 1

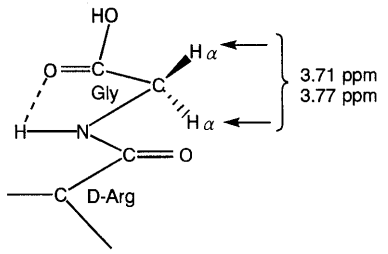

Chart 2

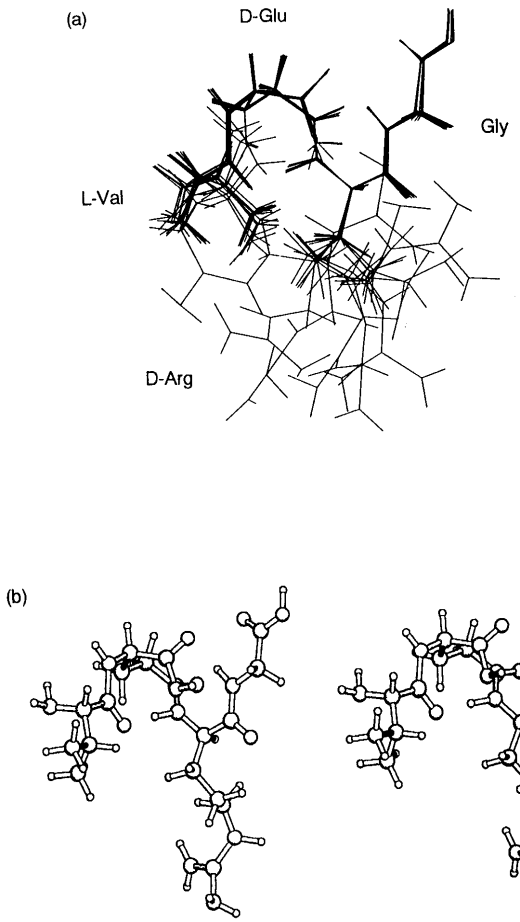

Fig. 2. Superposition of 10 Structures (a) of **1**, and One of the 10 Structures (b)

limits of 3, 4, and 5Å, respectively. Upper limits of distances for methyl protons and non-stereospecifically assigned methylene protons become larger when a correction for pseudo atoms is included.[12] The two angle constraints obtained from $J_{NH-CH\alpha}$ of D-Glu and D-Arg (Table 1) were adopted,[13] and were set from $-170°$ to $-70°$ and $-160°$ to $-80°$, respectively. Hydrogen bond constraints were based on the pattern of f (Chart 1); constraints for O–H distance were set from 1.6 to 2.1 Å and for N–O distance from 2.7 to 3.1 Å.

Starting from 100 randomly generated structures, the target functions were minimized, and then the converged structures were chosen which possessed lower target function values and small pairwise root-mean-square deviations (RMSD) within the ensemble. The superposition of the 10 structures of **1** in Fig. 2a shows that the structures were well determined. The average pairwise RMSD for backbone atoms was 0.11 Å and that for all heavy atoms was 1.54 Å. One of the 10 structures is shown in Fig. 2b.

Experimental

NMR Spectra NMR spectra were obtained on JEOL Lambda 500 spectrometers operating at 500.00 MHz for ^1H and 125.65 MHz for ^{13}C at 25 °C . Chemical shift values were expressed in ppm downfield from tetramethylsilane (TMS) as an internal standard. Samples were dissolved in DMSO-d_6 (99.8% D, Aldrich Chemical Company, Inc.) in a 5 mm ϕ sample tube.

T_1 values were estimated using the standard inversion-recovery sequence to determine the null in signal intensity. Rotating frame nuclear overhauser effect spectroscopy (ROESY) experiments[14] were done using a mixing time of 250 ms in the phase-sensitive mode.

Synthesis of 1 The tetrapeptide (**1**) was synthesized by a solution-phase methodology using *Z*-L-Val, Boc-D-GluOBzl, Boc-D-Arg(NO_2), and Tos·GlyOBzl as starting materials. The peptide chain was extended by use of the mixed anhydride (MA) method, and 4 N HCl–dioxane was used for the removal of the Boc group.

The resulting protected linear tetrapeptide *Z*-L-Val–γ-D-Glu(Bzl)–D-Arg(NO_2)–Gly–OBzl (740 mg, 0.90 mmol) was hydrogenated over 10% Pd–C catalyst in 10% AcOH/MeOH. After 12 h, the catalyst was filtered off and the filtrate was evaporated *in vacuo*. The residue was recrystallized from MeOH to give colorless crystals (**1**). Yield 314 mg (76%), *Rf* (iso-ProOH : water : AcOH = 100 : 50 : 3) 0.16, mp 213—216 °C, $[\alpha]_D^{22}$ +26.0° (c = 0.2, MeOH), FAB-MS m/z: 460 $(M+1)^+$.

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