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Conformational Analysis of a Cyclic Hexapeptide, Segetalin A from *Vaccaria segetalis*[1)]

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Abstract: To establish a pharmacophore model, conformation in solid and solution states of a cyclic hexapeptide, segetalin A, having an estrogen-like activity, was investigated by a combination of X-ray analysis, high field NMR and computational chemical evidence. The three-dimensional structure of crystalline segetalin A was characterized by two β-turn structures, one being of type I and the other of type VI, fixed by the two trans-annular hydrogen bonds formed between the 1st Gly and the 4th Val. On the other hand, in solution, the molecule was shown to have two β-turn structures, one being of type II and the other of type VI, by NMR and MD calculation, demonstrating that segetalin A takes different backbone conformations in solid and solution states.

Introduction

Recently, a number of naturally occurring cyclic peptides with unique structures and biological activities have been isolated. However, despite their importance, surprisingly few studies of higher plants occurring cyclic peptides exist in the literature.[2)] In our investigation of bioactive cyclic peptides from higher plants,[3)] a novel cyclic hexapeptide, called segetalin A, isolated from the seeds of *Vaccaria segetalis* (Caryophyllaceae) was found to have a potent estrogen-like activity.[4)] Some cyclic peptides such as oxytocin and vasopressin, show hormone activities, however, none is known to have an estrogen-like activity. It is interesting that a peptide having an estrogen-like activity

was isolated from the seeds of *V. segetalis*, which are used to activate blood flow and promote milk secretion, and also to treat amenorrhea and breast infections in China.

Cyclic peptides have come to represent a very important group of compounds having interesting biological functions. For example, oxytocin and vasopressin are regarded as hormones in the endocrine system. Knowledge of the conformation of these compounds is prerequisite to understanding of their biological activities and may provide useful to drug design.[5)]

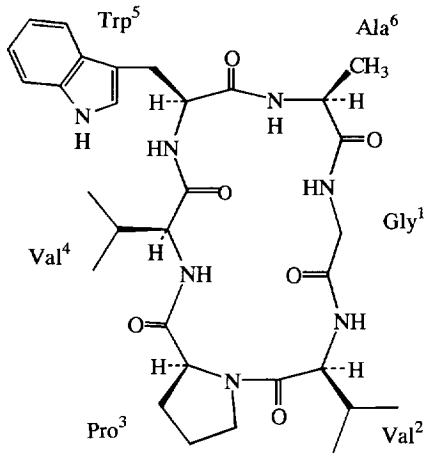


Fig. 1 Structure of Segetalin A (**1**); Gly was provisionally numbered as a first amino acid.

In order to elucidate the mechanisms of biological action of segetalin A and/or the biologically active conformation and orientation within the hormone binding site, detailed knowledge of its conformational characteristics is required. In addition, on the basis of detailed and exact knowledge of the structure of segetalin A in both crystal and solution under different environmental conditions, the structure-activity relationships may be assuredly discussed. Therefore, in the present report, we analyzed the conformation of segetalin A (**1**), whose structure is cyclo(Gly-Val-Pro-Val-Trp-Ala) (Fig. 1), in both solid and solution states, by using X-ray analysis, high field NMR and computational chemical methods with the aim of developing a pharmacophore model of estrogen-like cyclic hexapeptide.

Results and discussion

Solid state conformation of segetalin A

Segetalin A was crystallized from a CH_3OH - H_2O mixture to give orthorhombic crystals of space group $P2_12_12_1$ as pentahydrates. The lattice constants and intensity data collection were obtained on a Rigaku AFC7R automated diffractometer. Because the crystals deteriorated rapidly upon drying, they were sealed in a thin-walled glass capillary containing the mother liquor. Crystallographic data are given in Table 1. Figure 2 shows a ORTEP perspective view of the backbone of compound **1**. It is a cyclic hexapeptide consisting solely of L-amino acid residues. Table 5 shows the backbone dihedral angles in compound **1**. In Figure 3, the ϕ and ψ-angles along the main chain of segetalin A are summarized in a Ramachandran plot.

Cyclic peptide backbone rings are constrained with turns, which have been implicated in the bioactivity of several of naturally occurring peptides, and these turns are often stabilized by intramolecular hydrogen bonds. Such structures may provide good models for the studies of various possible types of turns containing intramolecular hydrogen bonds. The crystal structure of **1** contains two intramolecular NH···O hydrogen bonds between Gly^1-NH and Val^4-CO, and between Val^4-NH and Gly^1-CO [HN1---O4 of 2.12 Å {N1---O4 3.01(1) Å} and HN4---O1 of 1.93 Å {N4---O1 2.85(1) Å}]. These hydrogen bonds make an antiparallel β-sheet structure. As a consequence, in the molecule two β-turns are formed by the Trp^5 and Ala^6 at two corners, and Val^2 and Pro^3 at two corners. The $Trp^5 \rightarrow Ala^6$ turn is type I β-turn formed by the intramolecular hydrogen bond between Gly^1-NH and Val^4-CO. The other turn, $Val^2 \rightarrow Pro^3$ serve a type VI β-turn involving a cis proline amide bond.

Table 1. Crystal data of segetalin A (**1**)

Empirical Formula	$C_{31}H_{53}N_7O_{11}$(699.80)
Color, Habit	colorless, prismatic
Dimensions	0.45 × 0.30 × 0.10 mm
System	orthorhombic (mmm)
Lattice Type	Primitive
Dcalc	1.214 g/cm^3
Lattice Parameters (Å)	a=13.570(6) b=24.248(5) c=11.635(7) V=3828(2)Å^3
Space Group	$P2_12_12_1$
Z value	4
Final R value (R_W)	0.064 (0.090)

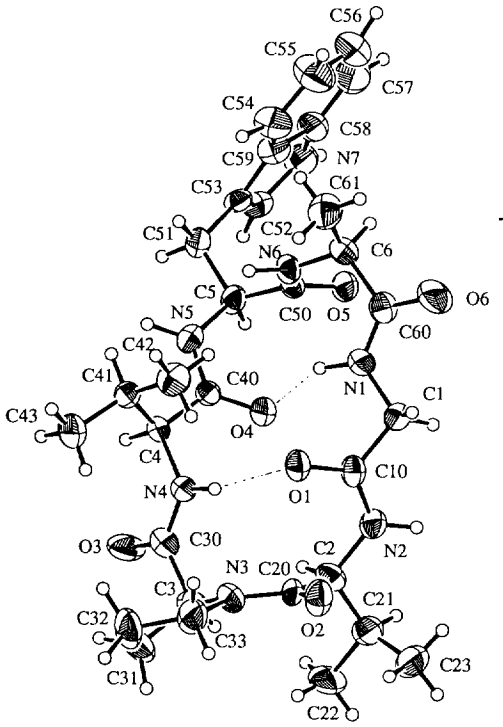


Fig. 2 A ORTEP perspective view of the crystal structure of **1**

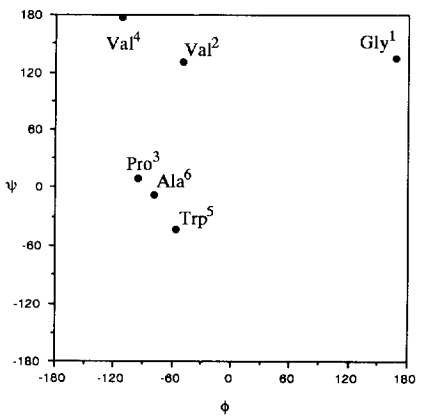


Fig. 3 Rhamachandran plot of the crystal structure of **1**

The backbone dihedral angles in the region of Gly^1 are unique. Though Gly^1 lies almost outside the permissible region for the usual amino acid,[6] Gly which has no Cβ atom and Pro which often

takes a cis amide configuration, sometimes can adopt outside the outer limit regions. Then, more than 10 degrees distortions in ω angles between Gly^1 and Val^2, and between Val^2 and Pro^3, was observed.

Solution state conformation of Segetalin A

Complete assignments of ^1H and ^{13}C NMR signals in [$^2H_6$]DMSO

The conformation or dynamic structure of **1** in a polar solvent such as [$^2H_6$]DMSO, which is essential for the studies of the structure-activity relationships, was obtained by the complete assignments of the signals in various NMR measurements such as ^1H-^1H COSY, HMQC[7] for direct $^1J_{H-C}$ connectivities and HMBC[8] for long range $^2J_{H-C}$ and $^3J_{H-C}$ ones. The results of the ^1H and ^{13}C-NMR resonances are shown in Table 2. In spite of the presence of a proline residue, the presence of a single stable conformer in [$^2H_6$]DMSO on the NMR time scale was displayed by the occurrence of well-resolved sharp signals, and the ^{13}C chemical shifts (δ 31.24 and 21.39) of β and γ positions in Pro^3 residue suggested that the geometry of the proline amide bond was fixed to be *cis*.[9]

Table 2. ^1H and ^{13}C NMR Signal Assignments of Segetalin A (**1**) in [$^2H_6$]DMSO.

assignment	δ_H (int. mult, J(Hz))	δ_C	assignment	δ_H	δ_C
Gly^1			Val^4		
α	3.67 (1H, m)	42.74	α	4.13 (1H, dd 10.0, 19.7)	59.80
	3.37 (1H, dd, 5.5, 11.3)		β	2.04 (1H, m)	29.91
NH	7.45 (1H, t, 5.5)		γ	0.77 (3H, d, 6.3)	18.60*
C=O		169.11		0.75 (3H, d, 6.2)	19.14*
Val^2			NH	7.31 (1H, d, 10.0)	
α	4.52 (1H, dd, 5.0, 9.3)	55.09	C=O		172.64
β	2.11 (1H, m)	29.62	Trp^5		
γ	0.95 (3H, d, 6.7)	17.28	α	4.26 (1H, dt, 6.0, 7.7)	55.47
	0.82 (3H, d, 6.9)	19.48	β	3.08 (1H, dd, 7.7, 14.2)	25.94
NH	7.42 (1H, d, 9.3)			3.14 (1H, dd, 7.7, 14.2)	
C=O		170.73	NH	8.49 (1H, d, 6.0)	
Pro^3			1(NH)	10.89 (1H, s)	
α	4.35 (1H, d, 8.1)	60.27	2	7.14 (1H, d, 2.2)	123.34
β	2.04 (1H, m)	31.24	3		109.13
	1.93 (1H, m)		4	7.55 (1H, d, 7.8)	118.39
γ	1.89 (1H, m)	21.39	5	7.06 (1H, t, 7.8)	121.04
	1.68 (1H, m)		6	6.97 (1H, t, 7.8)	118.30
δ	3.56 (1H, m)	46.80	7	7.33 (1H, d, 7.8)	111.27
	3.58 (1H, m)		8		136.03
C=O		171.46	9		126.99
			C=O		172.95
			Ala^6		
			α	3.67 (1H, m)	49.41
			β	1.21 (3H, d, 7.0)	15.38
			NH	8.93 (1H, d, 6.9)	
			C=O		171.04

* Assignment may be interchanged.

Hydrogen bonding

Temperature dependence of the amide proton chemical shift give valuable information about the existence of hydrogen bondings or solvent shielded groups.[10] Variable temperature experiments were carried out to assess the solvent accessibilities of the amide protons in ten intervals over the range 300 - 380K in [$^2H_6$]DMSO, using a linear regression analysis. For the purpose of investigating whether the molecules were associated or not, concentration studies were performed at concentrations of 1.0 - 10 mM. The ^1H NMR spectra showed no appreciable change in the chemical shifts. It is, therefore, concluded that aggregation does not occur under these conditions.

Temperature coefficients calculated are reported in Table 3. The amide protons in Trp^5 and Ala^6 shifted significantly upfield as the temperature increased, demonstrating that they were exposed to the solvent and not involved in hydrogen bonding. Whereas the other three NH protons in Gly^1, Val^2 and Val^4 did not change significantly when the temperature was increased, indicating that they were shielded from the solvent or involved in intramolecular hydrogen bondings. The negative temperature coefficient of Val^2-NH may suggest a strong intramolecular hydrogen bonding and/or conformational change around the Val^2-NH proton.

The rate of hydrogen - deuterium exchange also indicates whether NH protons are exposed to or shielded from the solvent either sterically or through hydrogen bonding. The exchange half-life times ($t_{1/2}$) for these 5 NH protons (Table 3) also showed that those of Gly^1, Val^2 and Val^4 are involved in intramolecular hydrogen bonds or shielded from the solvent whereas those of Trp^5 and Ala^6 are not.

However, Val^2-NH is not involved in hydrogen bonding in the crystal state of **1**. Because the crystal of **1** was grown from methanolic solution, the temperature coefficients in [$^2H_3$]MeOH were calculated over the range from 243 - 303K and shown in Table 3. Segetalin A can take several conformers, a major one (90%) and several minor ones (10%), when the crystal was dissolved in [$^2H_3$]MeOH at 243K. The amide protons of the major conformer tend to have larger temperature dependence in [$^2H_3$]MeOH than in [$^2H_6$]DMSO. However, the major solution conformer in [$^2H_3$]MeOH and that in [$^2H_6$]DMSO was considered to possess similar solution conformation because similar solvent dependence pattern in both solvents was shown, which was also supported by the similar ROE relationships. The crystals of **1** may be considered to be derived from one of the minor conformers.

In [$^2H_3$]MeOH over the range of 243 - 303K, the Hα proton shifts do not vary much except for those of the Val^2-Hα and Ala^6-Hα, which give 6.4×10^{-3} and 1.1×10^{-3} ppm/K, respectively. The other Hα protons were in the range of $\pm 0.4 \times 10^{-3}$ ppm/K. This chemical shift change is presumed to result from their average position of some different conformers. This assumption was also suggested by T1 relaxation and MD calculation data as shown below.

These results, however, do not give information about the hydrogen bond pairings.

Table 3 A: Temperature coefficients ($-d\delta/dT \times 10^3$ ppm/K) of the amide protons in [$^2H_6$]DMSO, B: those in [$^2H_3$]MeOH, and C: rate of hydrogen - deuterium exchange ($t_{1/2}$: hr) in [$^2H_6$]DMSO on addition of trace amounts of D_2O in segetalin A (**1**)

	solvent	Gly^1	Val^2	Val^4	Trp^5	Ala^6
A	[$^2H_6$]DMSO	2.84	-1.48	0.33	4.98	9.41
B	[$^2H_3$]MeOH	3.27	3.50	1.47	9.51	11.3
C	[$^2H_6$]DMSO + D_2O	6.28	7.50	5.23	0.75	0.73

Vicinal coupling

The allowed dihedral angles may be calculated from the vicinal NH-CαH coupling constants obtained by ^1H NMR spectrum. The J values and the corresponding dihedral angles, ϕ, calculated by the Karplus type equation proposed by Donzel *et al.*,[11] (Table 5) resemble those calculated by energy minimizations (See MD and energy minimization section).

The populations of side chain rotamers were quantitatively assayed from the homonuclear coupling constants. Though the assignment of each Hβ proton in the side chain of Trp^5 was not definite, the same vicinal coupling constants of 7.7 Hz between Hα and two Hβ were observed (Table 2). The conformation around the Cα-Cβ bond of Trp^5 may constitute three types of rotamers, denoted gauche$^-$, trans, and gauche$^+$. Using the treatment of Pachler,[12] we calculated the relative populations of these three side-chain rotamers (gauche$^-$=I, trans=II, gauche$^+$=III), the results being shown in Table 4. Thus, the populations of rotamers I and II are almost the same in preference to rotamer III.

Table 4. ^1H-NMR parameters for Trp^5 side chain of **1** and % of rotamers in [$^2H_6$]DMSO.

Coupling constants (Hz)			Rotamer populations (%)		
J_{AB}	J_{AX}	J_{BX}	I	II	III
14.2	7.7	7.7	46.5	46.5	7.0

A, B=Hβ, X=Hα, I=gauche$^-$, II=trans, III=gauche$^+$

ROE enhancements

The relationship of ROE enhancements in **1** observed by ROESY spectrum[13] is indicated by arrows in Fig. 4, implying the presence of two interesting β-turns. The strong ROEs between Trp^5-Hα and Ala^6-NH, and between Ala^6-NH and Ala^6-Hα indicated the presence of a type II β-turn between Trp^5 and Ala^6 at two corners. The β-turn between Trp^5 and Ala^6 at two corners in the crystal was of type I. As expected from steric repulsion between the carbonyl moiety of i + 1 and side chain of i + 2, type II β-turns generally occurr when i + 2 residue is a D residue or glycine.[14] However, there are some exceptions in small constrained cyclic peptide.[5,15] The strong ROE interaction between Ala^6-NH and Gly^1-NH in **1** is indicative of the type II β-turn between Trp^5 and Ala^6 at two corners.[16] Furthermore, the ROE between Trp^5-NH and Ala^6-NH was not observed, indicating that the β-turn between Trp^5 and Ala^6 at two corners was of type II.

The ROE beteween the Hα protons of Val^2 and Pro^3, indicated the presence of a type VI β-turn between Val^2 and Pro^3 with a cis Pro amide bond. The presence of the cis proline amide bond was found to be most compatible with the X-ray conformation.

Molecular mobility

Information about the structural flexibility of this compound can be experimentally obtained from the T1 relaxation times of the carbon resonances. The NT1 values (N=number of attached protons, T1=longitudinal relaxation time) correlate directly with the molecular mobility. This is due to the fact that the ^{13}C relaxation of these carbons is mainly dominated by the single relaxation mechanism of ^{13}C-^{1}H dipolar interaction with directly bonded hydrogens.[17]

The experimental data in Fig. 5 give information about the rotational motion of the backbone of **1** and about the internal rotations of the side chains. Interestingly, the NT1 value of the α carbon of Val^2 was larger than those of the other α carbons. The strong temperature dependence of Val^2-Hα shown by NMR and the large NT1 value of the Val^2-Cα suggested the fluctuation around Val^2 residue. In practice, ROE correlation involving the protons of Val^2 was rarely observed in this experiment, indicating the fluctuation around Val^2 residue.

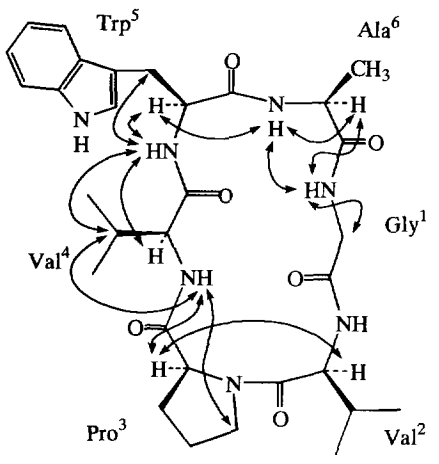


Fig. 4 ROE enhancements of Segetalin A (**1**); The arrows show the ROE relationships confirmed by ROESY experiments in [$^2H_6$]DMSO at 303K.

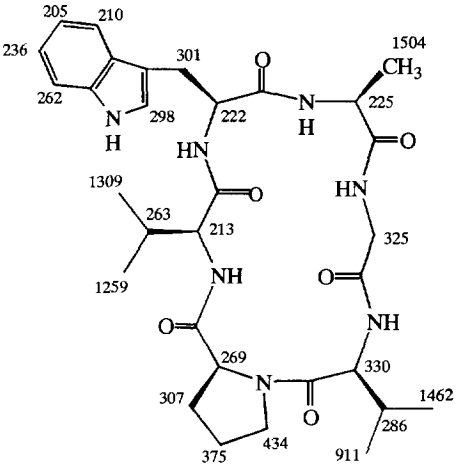


Fig. 5 NT1 values in milliseconds of the carbon atoms of **1** (N=number of attached protons, T1= longitudinal relaxation time).

Then, the aromatic carbon atoms in Trp^5 gave about the same values as those of other methine carbons, suggesting that the rotation about Trp^5 caused little increase in the mobility. A similar observation is reported on the aromatic side chain of Tyr^3 of RA-VII.[5] In addition, the fact that one of the Val^2-Me signals has a lower NT1 value than those of the other methyl groups in Val^2, Val^4 and Ala^6, indicates that this rotates more slowly than the other methyl residues.

Molecular dynamics and energy minimization

In order to analyze conformational features of segetalin A, it is necessary to use a computational method. Restrained molecular dynamics simulation using structural constraints derived from the NMR data was conducted to define the conformation in the same solvent as the NMR experiments. Simulated annealing calculation method,[18] which we have already reported about several cyclic peptides such as surfactin,[19] astin B[20] and pseudostellarin A,[21] was applied to analyzing this problem. All model building, energy minimization and molecular dynamics simulations were performed in vacuo, using SYBYL[22] molecular modeling package. The starting coordinates were taken from the X-ray structure of segetalin A. The distance constraints derived from the ROE experiments were classified into three ranges, 1.8-2.5, 1.8-3.5 and 3.0-5.0 Å, corresponding to strong, medium and weak ROEs, respectively. No hydrogen bonding restraints were used. The J-derived ϕ angles were not included in the constraints due to the fact that a coupling constant can correspond to four dihedral angles and the coupling constant could be the result of motional averaging. Each system was equilibrated for 5400 fs with a thermal bath at 500K and thereafter successively for 900 fs with a thermal bath 10K lower in temperature until a final temperature of 50K was obtained. This temperature was chosen after several trial- and error tests, judging from the arrival at equilibrium between possible conformers. Twenty cycles are performed, and each frozen conformation was sampled from the minimum temperature at 50K. Each low energy conformation was finally minimized by the use of molecular mechanics calculation of AMBER all-atom force field.[23] The calculation was done without solvent.

The type I β-turn structure constructed by Trp^5 and Ala^6, as found in the solid-state conformation was readily changed to type II β-turn structure. Calculated low energy conformers were analyzed in detail and were found to fall into two conformational families. Both groups possess the type II β-turn between Trp^5 and Ala^6 at two corners and the type VI β-turn between Val^2 and Pro^3 at two corners, characterized by a cis amide bond. However, they differ in the orientation of the amide bond between Gly^1 and Val^2. One structural form (conformer A) possesses stable antiparallel β-sheet backbone conformation with the intramolecular hydrogen bonds between Gly^1 and Val^4 (Gly^1-NH...Val^4-CO 1.91 Å and Val^4-NH...Gly^1-CO 1.87 Å). In the other conformer (conformer B), it adopts the same two well-defined β-turn conformation as in conformer A consisting of Trp^5-Ala^6 and Val^2-Pro^3. However, the amide proton of Val^2 was in a different orientation from that in conformer A. The amide proton of Val^2 orients inside to the backbone and an intramolecular hydrogen bond between Val^2-NH and Val^4-CO (1.86 Å) together with that between Gly^1-NH and Val^4-CO (1.90 Å) was formed. Nine of these 20 calculated conformations belong to the conformer A and have backbone atom RMSDs of less than 0.23 Å (STD 0.11), relative to the lowest energy conformation. The range of energies for the nine lowest energy conformations is only 2.18 kcal/mol. A snapshot

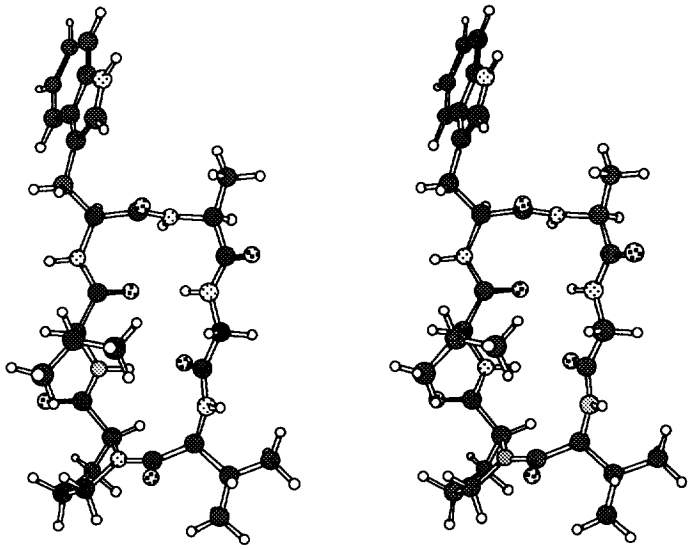
with the lowest energy of each group was selected as an relevant conformation and their stereoscopic view of the lowest energy conformation (conformers A and B) were depicted in Fig. 6. The energies of these two conformers are comparable; conformer B has a slightly higher energy (conformer A: 66.094 kcal/mol; conformer B: 68.527 kcal/mol). It is clear from the relevant dihedrals provided in Table 5 and the Rhamachandran plot shown in Fig. 7 that the conformer A is consistent with the observed J values.

Table 5. Backbone dihedrals in **1** calculated from vicinal NH-CαH coupling constants, X-ray analysis and energy calculations.

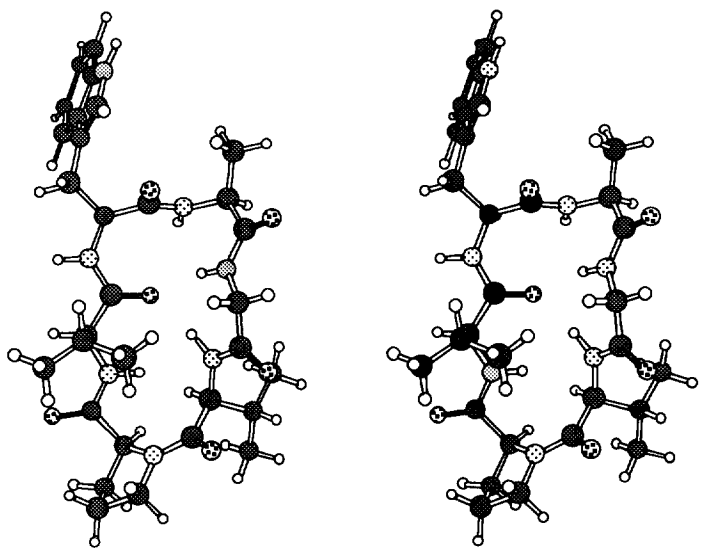
Residue			Calculated dihedral angle of segetalin A(1)			
		NH-CαH Couling constant (Hz)	NMR*	X-ray	Conformer A	B
Gly^1	ϕ	5.5	-77, 21, 99, 197	168	193	-139
	ψ			135	-172	-37
	ω			-170		
Val^2	ϕ	9.3	-104, -136, 60	-50	-101	75
	ψ			130	109	114
	ω			12		
Pro^3	ϕ			-94	-106	-90
	ψ			8	74	58
	ω			-178		
Val^4	ϕ	10.0	-114, -126	-112	-136	-147
	ψ			177	176	160
	ω			-175		
Trp^5	ϕ	6.0	-79, -161, 24, 96	-56	-52	-51
	ψ			-43	94	102
	ω			-176		
	χ^1			176		
	χ^2			80		
Ala^6	ϕ	6.9	-86, -154, 30, 90	-79	69	66
	ψ			-7	37	28
	ω			179		

* Calculated by using the Donzel equation: $^3J_{HN\alpha}=9.7\cos^2|60-\phi|-0.4\cos|60-\phi|+0.1\sin^2|60-\phi|$ 11)

Conformer A shown in Fig. 6 completely satisfied the distance constraints derived from the characteristic ROE relationship (Fig. 4). In addition, the distances between Gly^1-NH and Val^4-CO, and Val^4-NH and Gly^1-CO in conformer A, involving intramolecular hydrogen bondings corresponded to the temperature effects on NH chemical shift as described above. The amide proton of Val^2 showing slow hydrogen-deuterium exchange rate may be shielded from the solvent sterically by Val^2 and/or Val^4 side chains.



Conformer A



Conformer B

Fig. 6 Stereoscopic view of two stable backbone structures of segetalin A (**1**) obtained by MD and energy minimizations, above: conformer A (66.094 kcals/mol); below: conformer B (68.527 kcals/mol)

From the foregoing evidence, it was shown by X-ray diffraction and NMR investigations that segetalin A adopts different backbone conformations in a crystalline environment and in solution. In addition, our MD simulation indicated that the conformer A in solution are highly populated. It is interesting that the conformational energy needed to convert the X-ray backbone into the NMR backbone is relatively small. It is highly probable that the conformational characteristics of **1** in solution is two β-turn structures (type II and VI β-turns). These findings may be related closely to the mode of action of segetalin A which probably interact with specific receptor sites. Studies on the conformation and the biological activity relationship of derived segetalin A are in progress.

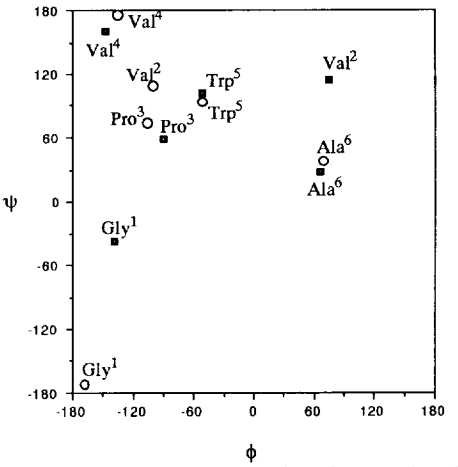


Fig. 7 Ramachandran plot of conformers A and B calculated by energy minimization (○ conformer A; □ conformer B)

Experimental

Proton and carbon NMR spectra were recorded on Bruker spectrometers (AM400 and AM500) and processed on a Bruker data station with an Aspect 3000 computer. 10 mg sample of segetalin A in a 5mm tube (0.5ml [$^2H_6$]DMSO and [$^2H_3$]MeOH, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 303K. ROESY experiments were made with a mixing time of 90 msec, as it causes no secondary effects. The delay to optimize one-bond correlations in the HMQC spectrum and to suppress them in the HMBC spectrum was 3.2 msec and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec.

Materials

The extraction and isolation procedures of **1** from seeds of *V. segetalis* was performed as described in our previous paper.[4)]

T1 relaxation times

All spectra were recorded on a Brucker AM400 spectrometer at 100.6 MHz by using proton broad-band decoupling at 303K. The spectra contained 32K data points over a 24 kHz frequency range. Relaxation data were obtained by using the inversion-recovery 180-τ-90° pulse sequence. Repetition time between two acquisitions was 30 s for segetalin A (**1**) in [$^2H_6$]DMSO. The spin-lattice relaxation times were determined from the relaxation data by using a regression analysis incorporated in the T1 routine of the Bruker 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 12 different values in an appropriate range. Standard deviations were in the range of 0.01 to 0.05s.

X-ray analysis of segetalin A

A colorless prismatic crystal of $C_{31}H_{53}N_7O_{11}\cdot 5H_2O$ having approximate dimensions of 0.45 × 0.30 × 0.10 mm was sealed in a glass capillary. All measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated Cu-Kα radiation and a 12kW rotating anode generator. Cell constants and an orientation matrix for data collection, obtained from a least-square refinement using the setting angles of 25 carefully centered reflections in the range $45.97<2\theta<54.99°$ corresponded to a primitive orthorhombic cell with dimensions: a=13.570(6), b=24.248(5), c=11.635(7) Å, V=3828(2) Å^3. For Z=4 and F.W.=699.80, the calculated density is 1.21 g/cm^3. The systematic absences of: h00: h ≠ 2n, 0k0: k ≠ 2n, 00l: l ≠ 2n, uniquely determined the space group to be: $P2_12_12_1$(#19). The data were collected at a temperature of 20 ± 1°C using the ω-2θ scan technique to a maximum 2θ value of 110.2°. Omega scans of several intense reflections, made prior to data collection, had an average width at half-height of 0.25° with a take-off angle of 6.0°. Scans of (1.80 + 0.30 tan θ)° were made at a speed of 4.0° / min (in omega). The weak reflections ($I < 10.0\sigma(I)$) were rescanned (maximum of 9 scans) and the counts were accumulated to ensure good counting statistics. Stationary background counts were recorded on each side of the reflection. The ratio of peak counting time to background counting time was 2:1. The diameter of the incident beam collimator was 1.0 mm, the crystal to detector distance was 235 mm, and the computer controlled detector aperture was set to 3.0 × 9.0 mm (horizontal × vertical).

Of the 2760 reflections which were collected, 2758 were unique (R_{int}=0.115). The intensities of three representative reflection were measured after every 50 reflections. Over the course of data collection, the standards decreased by 5.9 %. A polynomial correction factor was applied to the data to account for this phenomenon. The linear absorption coefficient, μ, for Cu-Kα radiation is 7.7 cm^{-1}. An empirical absorption correction based on azimuthal scans of several reflections was applied which resulted in transmission factors ranging from 0.74 to 1.00. The data were corrected for Lorentz and polarization effects.

Table 6 Atomic coordinates and B*iso*/B*eq* of Segetalin A (**1**)

atom	x	y	z	Beq
O(1)	-0.7554 (6)	-0.1595 (3)	-0.5781 (7)	5.3 (2)
O(2)	-0.6374 (8)	-0.1836 (3)	-0.3608 (8)	6.8 (2)
O(3)	-0.4955 (7)	-0.2517 (5)	-0.8034 (9)	8.2 (3)
O(4)	-0.7045 (6)	-0.1278 (3)	-0.8193 (7)	5.2 (2)
O(5)	-0.8378 (6)	-0.0258 (3)	-0.9417 (7)	5.4 (2)
O(6)	-1.0002 (8)	-0.0273 (5)	-0.6400 (9)	8.9 (3)
O(7)	-0.5022 (7)	-0.2918 (4)	-0.0323 (8)	7.2 (3)
O(8)	-0.6600 (1)	-0.2432 (5)	-0.1550 (8)	10.0 (4)
O(9)	-0.8190 (1)	-0.3057 (9)	-0.1690 (2)	18.8 (8)
O(10)	-0.9260 (3)	-0.3784 (7)	-0.0070 (2)	28.0 (1)
O(11)	-0.8480 (1)	-0.5000 (1)	-0.0020 (2)	25.0 (1)
N(1)	-0.8689 (8)	-0.0799 (4)	-0.6766 (8)	5.4 (2)
N(2)	-0.6549 (8)	-0.0914 (3)	-0.5128 (9)	5.3 (2)
N(3)	-0.5660 (8)	-0.2283 (4)	-0.5074 (9)	6.2 (3)
N(4)	-0.6421 (7)	-0.2251 (3)	-0.7320 (7)	4.6 (2)
N(5)	-0.7646 (8)	-0.1651 (4)	-0.9809 (8)	5.7 (3)
N(6)	-0.9279 (7)	-0.1022 (3)	-0.8971 (8)	4.8 (2)
N(7)	-0.8650 (1)	0.0157 (4)	-1.2848 (10)	6.9 (3)
C(1)	-0.8237 (9)	-0.0685 (4)	-0.5664 (10)	5.2 (3)
C(2)	-0.5657 (10)	-0.1264 (5)	-0.5110 (1)	5.4 (3)
C(3)	-0.5000 (1)	-0.2367 (6)	-0.6040 (1)	6.7 (4)
C(4)	-0.6937 (8)	-0.2267 (4)	-0.8425 (9)	4.4 (2)
C(5)	-0.7895 (10)	-0.1104 (4)	-1.0330 (9)	5.0 (3)
C(6)	-0.9954 (9)	-0.0709 (4)	-0.8240 (1)	5.5 (3)
C(10)	-0.7416 (10)	-0.1093 (4)	-0.5513 (10)	4.9 (3)
C(20)	0.5920 (1)	-0.1826 (4)	-0.4520 (1)	5.2 (3)
C(21)	-0.4850 (1)	-0.0969 (5)	-0.4530 (1)	6.9 (4)
C(22)	-0.3870 (1)	-0.1239 (7)	-0.4720 (2)	9.1 (5)
C(23)	-0.4970 (1)	-0.0862 (6)	-0.3260 (1)	9.5 (5)
C(30)	-0.5480 (1)	-0.2383 (5)	-0.7230 (1)	5.8 (3)
C(31)	-0.4550 (2)	-0.2929 (8)	-0.5800 (2)	10.0 (6)
C(32)	-0.5310 (2)	-0.3236 (6)	-0.5260 (2)	10.4 (6)
C(33)	-0.5840 (1)	-0.2828 (5)	-0.4520 (1)	7.9 (4)
C(40)	-0.7210 (7)	-0.1696 (4)	-0.8785 (10)	4.3 (3)
C(41)	-0.7809 (8)	-0.2674 (4)	-0.8360 (1)	5.3 (3)
C(42)	-0.8570 (1)	-0.2499 (5)	-0.7480 (1)	6.7 (3)
C(43)	-0.7450 (1)	-0.3255 (5)	-0.8140 (1)	7.8 (4)
C(50)	-0.8536 (10)	-0.0773 (4)	-0.9524 (10)	4.9 (3)
C(51)	-0.8335 (10)	-0.1194 (4)	-1.1522 (9)	5.2 (3)
C(52)	-0.8082 (10)	-0.0291 (5)	-1.2600 (1)	5.7 (3)
C(53)	-0.8648 (8)	-0.0683 (4)	-1.2047 (9)	4.5 (3)
C(54)	-1.0490 (1)	-0.0631 (6)	-1.1500 (1)	7.3 (4)
C(55)	-1.1290 (1)	-0.0276 (9)	-1.1550 (2)	10.4 (6)
C(56)	-1.1290 (2)	0.0234 (9)	-1.2030 (2)	11.3 (7)
C(57)	-1.0410 (2)	0.0429 (7)	-1.2490 (2)	9.6 (6)
C(58)	-0.9560 (1)	0.0096 (5)	-1.2450 (1)	6.3 (4)
C(59)	-0.9625 (10)	-0.0434 (5)	-1.1940 (1)	5.7 (3)
C(60)	-0.9515 (10)	-0.0580 (5)	-0.7050 (1)	5.3 (3)
C(61)	-1.0910 (1)	-0.1008 (6)	-0.8140 (1)	7.7 (4)

The structure was solved and expanded by using Fourier techniques.[24] The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 1551 observed reflection ($I>3.00\sigma(I)$) and 442 variable parameters and converged (largest parameter was 0.06 times its esd) with unweighted and

weighted agreement factors of : R=0.064, R_w=0.090. The standard deviation of an observation of unit weight was 1.20. The weighting scheme was based on counting statistics and included a factor (p=0.146) to downweight the intense reflections. Plots of $\Sigma\omega(|Fo|-|Fc|)^2$ versus $|Fo|$, reflection order in data collection, $\sin\theta/\lambda$ and various classes of indices showed no unusual trends. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.42 and -0.20 $e^-/Å^3$, respectively. Neutral atom scattering factors were taken from Cromer and Waber.[25] Anomalous dispersion effects were included in Fcalc[26]; the values for $\Delta f'$ and $\Delta f''$ were those of Creagh and McAuley.[27] The values for the mass attenuation coefficients are those of Creagh and Hubbel.[28] All calculations were performed using the teXsan[29] crystallographic software package of Molecular Structure Corporation.

The refined fractional atomic coordinates were shown in Table 6. The bond lengths, the bond angles, the hydrogen-atom coordinates and the thermal parameters have been deposited at the Cambridge Crystallographic Data Centre (CCDC).

Simulated annealing calculation.

Computer modeling and all calculations were performed by using the molecular-modeling software SYBYL ver. 6.03 (Tripos Associates, St. Louis, MO) on an IRIS 4-D work station. Initial calculations were started with the coordinates of the crystal structure of segetalin A analyzed by X-ray. Molecular mechanics and dynamics calculations were performed with the AMBER all-atom force field.[23] The dielectric constant (ε) was assumed to be proportional to the interatomic distances (r) as ε=r. Solvent molecules were not included in the calculations. The ROE relationships shown in Fig. 4, were taken into account in the calculations of the constrained minimizations and dynamics with an extra harmonic term 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. A simulation was performed by using a time step of 1 fs, and the structures were sampled every 90 fs. Each system was equilibrated for 5400 fs with a thermal bath at 500K, and thereafter, successively, for 900 fs with a thermal bath 10 K lower in temperature, until a final temperature of 50 K was obtained. Twenty cycles were performed, giving a total simulation time of 126 ps, and each frozen conformation was sampled from the minimum temperature at 50 K. The snapshots from the minimum temperature at 50K were then energy minimized with the AMBER force field. The conformers after minimization were divided into two groups and a snapshot in each group with the lowest energy was selected as an relevant conformation. Each energy minimization was carried out until the derivatives became less than 0.01 $kcal\cdot mol^{-1}\cdot Å^{-1}$.

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