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Segetalins B, C and D, Three New Cyclic Peptides from *Vaccaria segetalis* 1)

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Abstract: Three new cyclic peptides, segetalins B, C and D, were isolated from *Vaccaria segetalis* (seeds) and their structures were elucidated by 2D NMR and chemical degradations. Conformational analysis of segetalin B, having an estrogen-like effect, was made by using high field NMR and computational methods. Segetalin B was suggested to possess a solution conformation similar to that of segetalin A in the region of Trp-Ala-Gly-Val. The sequence and conformation of Trp-Ala-Gly-Val may play an important role to show the activity.

Introduction

We have recently studied a series of novel bioactive cyclic peptides from higher plants.[2] Research about bioactive cyclic peptides is widely recognized for its importance to various regions. In our continuous investigation of bioactive cyclic peptides from higher plants, a novel cyclic peptide, segetalin A, cyclo(Gly-Val-Pro-Val-Trp-Ala), showing an estrogen-like activity, has been isolated from the seeds of *V. segetalis* (Caryophyllaceae), and its chemical structure and the conformation in [$^2H_6$]DMSO were reported in the previous papers.[1,3] The seeds of *V. segetalis* have been used to activate blood flow and promote milk secretion, and also to treat amenorrhea and breast infections in China.[4] As a result of our further fractionation efforts, we isolated from the seeds, three novel cyclic peptides, named segetalins B - D (**1** - **3**), as minor components. We report in this paper

isolation and structure elucidation of cyclic peptides (**1** - **3**), and an estrogen-like activity of segetalin B. In addition, solution conformation of segetalin B and conformational similarity between segetalins A and B with estrogen-like activity were also described.

Results and Discussion

Structure determination of segetalins B, C and D

A methanolic extract of seeds of *V. segetalis* was partitioned between ethyl acetate and water. The ethyl acetate soluble material was chromatographed on a silica gel column, followed by HPLC on ODS to give three peptidic compounds as colorless needles, named segetalins B, C and D (**1** - **3**).

Segetalin B (**1**), colourless needles, mp. 153 - 155 ℃, $[\alpha]_D$ +32.4° (c 0.41, pyridine), gave a quasimolecular ion peak in high-resolution FAB-MS spectrum at *m/z* 485.2519 ($(M+H)^+$, Δ +0.7 mmu), which corresponded to the molecular formula, $C_{24}H_{32}N_6O_5$. The strong IR absorptions at 3340 and 1673 cm^{-1}, corresponding to amino and amide carbonyl groups, respectively, suggested **1** to be a peptide. In order to elucidate the amino acid components, **1** was hydrolyed with 6N HCl including 0.6% thioglycolic acid by heating at 110 ℃ for 24 h in a sealed tube. HPLC of the hydrolysate showed that **1** consisted of one glycine (Gly), two alanine (Ala), one valine (Val) and one tryptophan (Trp) per molecule. Each of these amino acid was shown to be of L-configuration by derivatization of the acid hydrolysate with Marfey's reagent,[5] followed by HPLC analysis. The molecular formula of **1** corresponds well to the above amino acid composition, if **1** is a cyclic peptide. The cyclic nature was also deduced from the lack of terminal amino group protons in the ^1H NMR spectrum and the relatively high intensity of the molecular ion in FAB-MS spectrum. In the ^1H and ^{13}C NMR spectra, five amide NH and five amide carbonyl carbons, which corresponds to the cyclic pentapeptide, were observed. Applying the combination of ^1H-^1H COSY, HMQC[6] for direct $^1J_{H-C}$ connectivities and HMBC[7] for long range $^2J_{H-C}$ and $^3J_{H-C}$ ones, we assigned all ^1H and ^{13}C-NMR resonances, shown in Table 1.

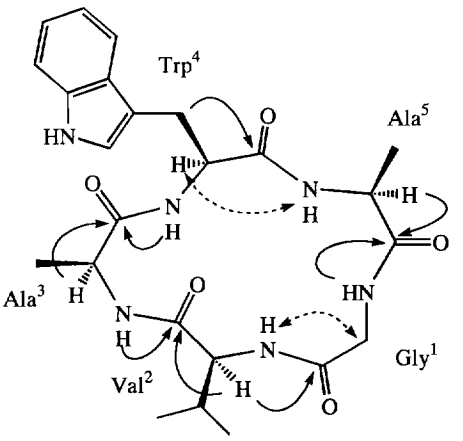

Fig. 1 Structure of Segetalin B (**1**). Arrows show some important HMBC correlations and dashed arrows show ROE enhancements.

On the basis of the observation in the HMBC experiment (Figure 1), the presence of two structural units, Val-Ala-Trp and Ala-Gly, was established. These units could be linked by ROE enhancements

observed between Ala^5-NH and Trp^4-Hα, and between Val^2-NH and Gly^1-Hα in a phase-sensitive ROESY spectrum.[8] Therefore, the whole structure was determined to be cyclo(Gly-Val-Ala-Trp-Ala).

Table 1 ^{1}H and ^{13}C NMR Signals of Segetalin B (**1**) in [$^2H_5$]pyridine.

	^{1}H NMR	^{13}C NMR			
assignment	δ_H (int. mult, J(Hz))	δ_C		δ_H	δ_C
Gly^1			Trp^4		
α	3.30 (1H, dd, 5.7, 14.8)	43.78	α	4.23 (1H, m)	56.13
	4.10 (1H, d, 5.7, 14.8)		β	3.18 (1H, dd, 6.4, 14.7)	26.38
NH	8.42 (1H, t, 5.7)			3.24 (1H, dd, 8.2, 14.7)	
C=O		169.54	NH	7.96 (1H, d, 8.7)	
Val^2			1(NH)	10.84 (1H, s)	
α	3.92 (1H, dd, 6.9, 8.2)	59.49	2	7.12 (1H, d, 2.2)	120.92
β	1.97 (1H, m)	29.64	3		109.94
γ	0.88 (3H, d, 6.7)	19.09	4	7.34 (1H, d, 7.9)	118.24
	0.87 (3H, d, 6.7)	18.05	5	7.07 (1H, t, 7.9)	123.50
NH	7.74 (1H, d, 8.2)		6	6.98 (1H, t, 7.9)	118.27
C=O		170.26	7	7.56 (1H, d, 7.9)	111.34
Ala^3			8		136.04
α	4.07 (1H, dq, 8.3, 6.9)	49.67	9		127.20
β	1.23 (3H, d, 6.9)	16.98	C=O		170.90
NH	7.99 (1H, d, 8.3)		Ala^5		
C=O		171.34	α	4.23 (1H, m)	48.40
			β	1.21 (1H, d, 7.1)	17.11
			NH	7.93 (1H, d, 8.6)	
			C=O		172.09

Segetalins C and D (**2** and **3**) were composed of the similar amino acid units arranged in similar sequences. Segetalin C (**2**), colourless needles, mp. 172 - 175 °C, $[\alpha]_D$ -23.2° (c 0.42, MeOH), had a molecular formula, $C_{40}H_{51}N_9O_7$, which was deduced from HR FAB-MS spectrum, possessing 20 degrees of unsaturation. Amino acid analysis of **2** showed that it consisted of Pro, Phe × 2, Gly, Ala, Leu and His, all of L-configuration as revealed by the Marfey's derivatization, followed by HPLC analysis. The ^{1}H NMR spectrum, showing no terminal amino proton signal and MS spectrum, showing the relatively high intensity of the molecular ion, implied that **2** was a cyclic peptide. The HMQC data were used to assign the carbon resonances to the individual amino acids in **2** (Table 2). The amino acid sequence was determined by an analysis of HMBC correlations as shown in Fig. 2. The connectivities between neighboring amino acid residues except for that between Phe^6 and Pro^7 were unequivocally determined by the long range $^2J_{H\text{-}C}$ and $^3J_{H\text{-}C}$ correlations. The remaining connectivity between Phe^6 and Pro^7 was supported by the NOE enhancement observed between Phe^2-Hα and Pro-Hδ in a phase-sensitive NOESY spectrum.[9] From this spectroscopic evidence, the structure of segetalin C was elucidated as cyclo(Gly-Leu-His-Phe-Ala-Phe-Pro).

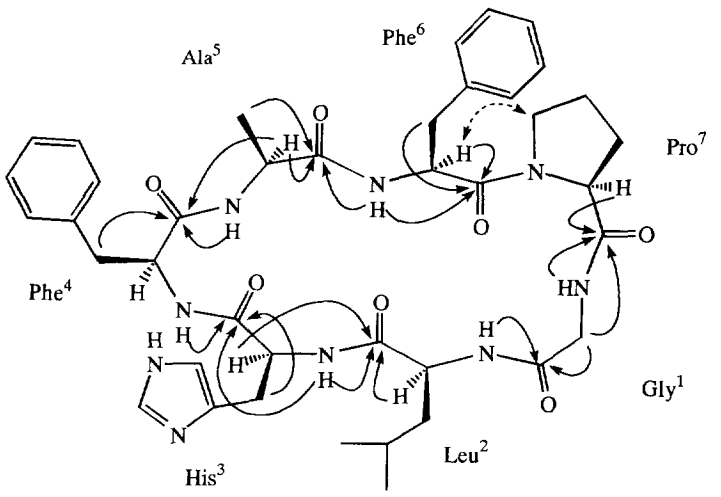

Fig. 2 Structure of Segetalin C (**2**). Arrows show some important HMBC correlations and a dashed arrow show NOE enhancement between Phe^6-Hα and Pro^7-Hδ.

Table 2 ^{1}H and ^{13}C NMR Signals of Segetalin C (**2**) in [$^2H_5$]pyridine.

assignment		^{1}H NMR δ_H (int. mult, J(Hz))	^{13}C NMR δ_C	assignment		δ_H	δ_C
Gly^1				Ala^5			
	α	3.87 (1H, dd, 3.6, 16.9)	37.01		α	5.39 (1H, dq, 9.2, 7.4)	49.14
		4.93 (1H, dd, 8.6, 16.9)			β	1.80 (3H, d, 7.4)	18.26
	NH	10.21 (1H, dd, 3.6, 8.6)			NH	10.14 (1H, d, 9.2)	
	C=O		169.82		C=O		172.86
Leu^2							
	α	5.35 (1H, dd, 8.2, 16.2)	53.91				
	β	1.54 and 1.88 (each 1H,m)	43.98	Phe^6			
	γ	1.88 (1H, m)	24.83		α	5.12 (1H, ddd, 3.7,7.8,10.3)	54.15
	δ	0.78 (3H, d, 6.5)	21.88		β	3.00 (1H, dd, 10.3, 12.7)	38.93
		0.86 (3H, d, 6.5)	22.72			3.14 (1H, dd, 3.7, 12.7)	
	NH	8.74 (1H, d, 8.2)			γ		137.48
	C=O		172.21		δ	7.52 (2H, d, 7.5)	130.01
His^3					ε	7.33 (2H, t, 7.5)	128.82
	α	4.97 (1H, m)	58.56		ξ	7.25 (1H, t, 7.5)	127.00
	β	3.73 (1H, dd, 2.7, 15.0)	28.97		NH	8.01 (1H, d, 7.8)	
		3.95 (1H, dd, 6.0, 15.0)			C=O		169.86
	2	8.11 (1H, s)	136.33				
	4	7.19 (1H, s)	116.75				
	5		135.70				
	NH	8.66 (1H, d, 5.3)		Pro^7			
	C=O		173.39		α	4.43 (1H, t, 7.8)	62.11
Phe^4					β	1.28 (1H, m)	29.49
	α	4.98 (1H, dd, 4.7, 8.7)	49.10			1.85 (1H, m)	
	β	3.38 (1H, dd, 8.7, 14.7)	37.01		γ	1.28 (1H, m)	24.69
		3.58 (1H, dd, 4.7, 14.7)				1.54 (1H, m)	
	γ		137.93		δ	2.70 (1H, dd, 8.5, 14.3)	47.96
	δ	7.42 (2H, d, 7.0)	129.43			3.14 (1H, ddd, 3.9,9.9,14.3)	
	ε	7.39 (2H, t, 7.0)	128.85		C=O		172.04
	ξ	7.29 (1H, t, 7.0)	127.10				
	NH	9.32 (1H, br s)					
	C=O		171.61				

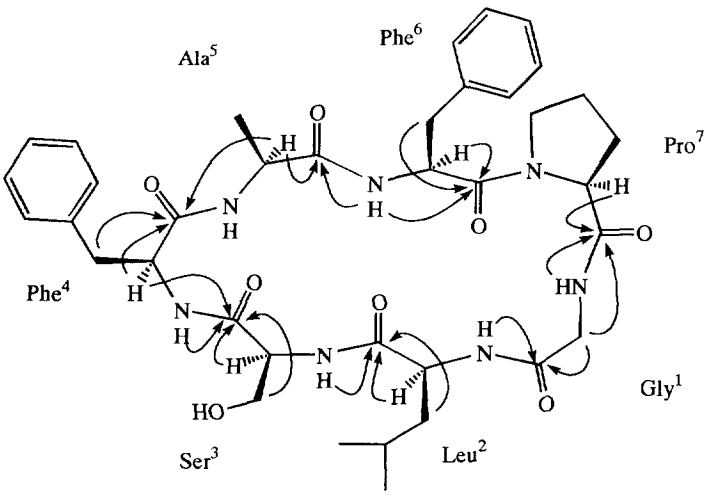

Fig. 3 Structure of Segetalin D (**3**). Arrows show some important HMBC correlations.

Table 3 ^{1}H and ^{13}C NMR Signals of Segetalin D (**3**) in [$^{2}H_5$]pyridine.

assignment	^{1}H NMR δ_H (int. mult, J(Hz))	^{13}C NMR δ_C	assignment	δ_H	δ_C
Gly[1]			Ala[5]		
α	3.89 (1H, dd, 3.8, 16.8)	44.20	α	5.10 (1H, dq, 8.7, 7.5)	50.10
	4.95 (1H, dd, 8.5, 16.8)		β	1.57 (3H, d, 7.5)	18.86
NH	10.27 (1H, dd, 3.8, 8.5)		NH	8.64(1H, d, 8.7)	
C=O		169.90	C=O		172.77
Leu[2]					
α	5.42 (1H, td, 5.6, 10.0)	53.80	Phe[6]		
β	1.93 (2H, dd, 5.6, 12.6)	43.89	α	5.25 (1H, ddd, 4.5,8.7,12.8)	53.59
γ	2.07 (1H, m)	29.58	β	3.20 (1H, dd, 4.5, 12.8)	36.62
δ	0.83 (3H, d, 6.3)	21.89		3.46 (1H, dd, 8.7, 12.8)	
	0.92 (3H, d, 6.3)	23.13	γ		137.33
NH	8.83 (1H, d, 10.0)		δ	7.40 (2H, d, 7.5)	130.14
C=O		172.56	ε	7.34 (2H, t, 7.5)	128.90
Ser[3]			ξ	7.25 (1H, t, 7.5)	127.13
α	4.99 (1H, dd, 2.5, 5.8)	57.79	NH	7.87 (1H, d, 8.7)	
β	4.66 (1H, dd, 2.5, 10.9)	63.40	C=O		170.17
	4.56 (1H, dd, 2.5, 10.9)				
NH	9.15 (1H, d, 5.8)		Pro[7]		
C=O		171.11	α	4.50 (1H, t, 7.7)	62.32
Phe[4]			β	1.94 (1H, m)	29.58
α	5.03 (1H, ddd,4.0,4.6,9.6)	55.61		2.04 (1H, m)	
β	3.31 (1H, dd, 9.6, 14.6)	40.14	γ	1.31 (1H, m)	24.82
	3.59 (1H, dd, 4.0, 14.6)			1.57 (1H, m)	
γ		138.02	δ	2.74 (1H, dd, 8.2, 16.8)	48.05
δ	7.86 (2H, d, 7.0)	129.28		3.28 (1H, dd, 4.5, 8.2)	
ε	7.32 (2H, t, 7.0)	128.90	C=O		171.14
ξ	7.28 (1H, t, 7.0)	127.13			
NH	9.35 (1H, d, 4.6)				
C=O		171.25			

Segetalin D (**3**), colourless needles, mp. 165 - 167 °C, $[\alpha]_D$ +13.7° (c 0.41, MeOH), was shown to have the molecular formula, $C_{37}H_{49}N_7O_8$, by HR FAB-MS spectrum, indicating 17 degrees of unsaturation. Amino acid analysis showed that **3** consisted of Pro, Phe × 2, Ser, Gly, Ala and Leu, all of which were of L-configuration as shown by Marfey's derivatization, followed by HPLC analysis. Amino acid composition of **3** was identical with that of segetalin C except that **3** contained Ser instead of His in **2**.

In the NMR spectra of **3** (Table 3), six amide protons and seven amide carbonyl carbons were observed, corresponding to seven amino acids with one proline as indicated above. The sequence analysis was conducted, in a similar way to those in **1** and **2**, by the 2D NMR analysis using PFG-HMBC spectrum.[10] From the results of the important long range $^2J_{H\text{-}C}$ and $^3J_{H\text{-}C}$ correlations as shown in Figure 3, the sequence of the seven amino acids was unequivocally determined to be Pro-Gly-Leu-Ser-Phe-Ala-Phe, and accordingly, the whole structure of **3** was established to be cyclo(Gly-Leu-Ser-Phe-Ala-Phe-Pro), being also satisfied by 17 degrees of unsaturation. A difference between the structures of **2** and **3** was found only in the His (**2**) and Ser (**3**) parts. Then, the β and γ carbon chemical shifts (δ 29.49 and 24.69 in **2**; δ 29.58 and 24.82 in **3**) of Pro residues in both **2** and **3** are diagnostic of a trans proline peptide bond between Phe^6 and Pro^7,[11] which is also supported by the the occurrence of a triplet signal of Hα in Pro^7, correlating with the trans proline peptide bond.[12]

Estrogen-like activity estimated by uterine weight in ovariectomized rats

In our previous communication,[3] the estrogen-like activity of segetalin A on accessory reproductive organs in ovariectomized female rats was reported. The result of estrogen-like activity was shown in Table 4, when segetalins A - D were administered to ovariectomized rats. When 2.5 mg/kg of segetalin B was administered to rats, uterine weight was increased to 75.6 ± 8.87 mg and segetalin A (2.5 mg/kg) also had an effect on the increase of uterine weight. However, segetalins C and D were ineffective on uterine weight. As indicated above, only segetalins A and B showed the estrogen-like effects. Segetalins A and B possess the same sequence unit, Trp-Ala-Gly-Val. This common sequence may be important structural unit to show estrogen-like activity for segetalins.

Table 4. Effects of segetalins on uterine weight of ovariectomized rats

	concentration	uterine weight (mg)
control	-	45.0 ± 7.90
Segetalin A	2.5 mg/kg	65.4 ± 6.12
Segetalin B	2.5 mg/kg	75.6 ± 8.87
Segetalin C	2.5 mg/kg	57.4 ± 6.15
Segetalin D	2.5 mg/kg	50.8 ± 12.56

All values are the mean ± S.E. from 5 rats each group. Differences between the control group and other group were examined using student's t-test. The structure of segetalin A is cyclo(Gly-Val-Pro-Val-Trp-Ala).

Table 5 Temperature coefficients ($-d\delta/dT \times 10^3$ ppm/K) of the amide protons in segetalin B (**1**)

solvent	Gly^1	Val^2	Ala^3	Trp^4	Ala^5
[$^2H_6$]DMSO	4.6	5.0	3.3	3.3	5.0

Table 6 Calculated backbone ϕ angles in segetalin B by vicinal NH-CαH coupling constants*

Residue	NH-CαH coupling constant (Hz)	Calculated ϕ angles
Gly^1	5.7	120, 75
Val^2	8.2	80, 41, 214, -94
Ala^3	8.3	78, 42, 216, -96
Trp^4	8.7	75, 45, 218, -98
Ala^5	8.6	76, 44, 217, -97

* Calculated using the Donzel equation: $^3J_{HN\alpha}=9.7\cos^2|60-\phi|-0.4\cos|60-\phi|+0.1\sin^2|60-\phi|$[14] for non-Gly residues and $\Sigma^3J_{HN\alpha 2}=6.0\cos^2\phi-1.5\cos\phi+12.5\sin^2\phi$ for Gly residue.[15]

Conformational analysis of segetalin B

In our previous report,[1] solution form of segetalin A in [$^2H_6$]DMSO, was shown to take a characteristic type II and VI β-turn structures with Trp and Ala at two corners, and with Val and Pro at two corners, respectively. In order to analyze the conformational similarity of segetalins A and B and relations between their biological activities, conformational analysis of segetalin B in solution was conducted by using high field NMR and computational chemical methods as follows.

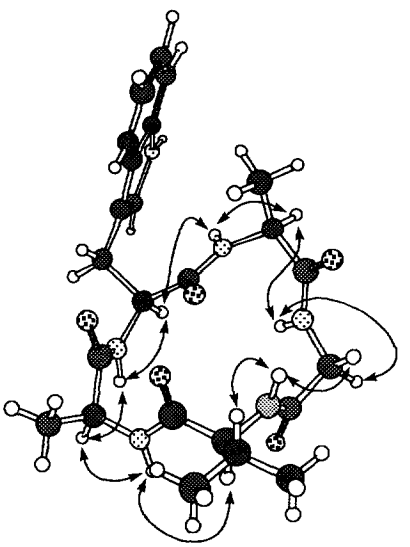

Fig. 4 ROE relationship of segetalin B

First, to clarify the presence of intramolecular hydrogen bonds, the temperature dependence of the amide proton chemical shift was examined by NMR.[13] The temperature coefficients recorded in ten intervals over the range 300 - 330K in [$^2H_6$]DMSO (Table 5) showed that, irrespective of the relatively small backbone ring size of **1**, none of five amide protons was involved in intramolecular hydrogen bondings.

The solution form of segetalin B was shown to be as in Fig. 4 by using ROE relationship in the ROESY spectrum.[8] The strong ROEs between Trp^4-Hα and Ala^5-NH, and between Ala^5-NH and Ala^5-Hα suggested that it seems to take type II β-turn structure between Trp^4 and Ala^5 at two corners as in segetalin A.[1] Furthermore, the ROE between Trp^4-NH and Ala^5-NH was not observed, indicating that the β-turn was of type II.

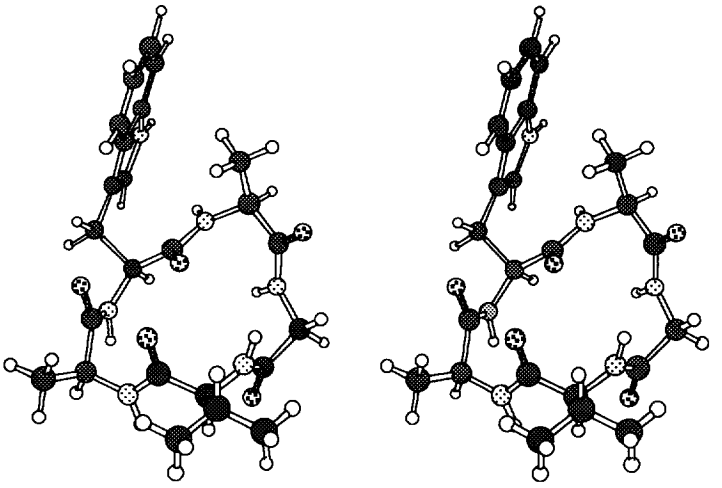

Fig. 5 Stereoscopic view of energetically stable conformer of segetalin B

It is possible to calculate the allowed dihedral angles from the vicinal NH-CαH coupling constants obtained by ^{1}H NMR spectrum. The J values and the corresponding dihedral angles, ϕ, calculated by Karplus type equation proposed by Donzel *et al.*,[14] are shown in Table 6. These dihedral angles gave us useful information to analyze solution conformation by energy minimizations as follows.

In order to clarify the solution form that satisfy the above ROE relationship, the computational chemical method using simulated annealing calculation[16] was carried out. The condition of the MD simulation followed the previous report.[1] The calculation was done without solvent. As the crystal of segetalin B was unsuitable for X-ray analysis, initial calculations started with the coordinates modeled by J-derived ϕ angles restraint energy minimization using AMBER all-atom force field.[17] This starting coordinates with the following ϕ angles (Gly^1: 120°, Val^2: -94°, Ala^3: 42°, Trp^4: 45°, Ala^5: 44°) in Table 6, which is chosen after several trial and error tests, is the only unique example that satisfied the above all ROE relationship and had no intramolecular hydrogen bonds. The distance constraints derived from the ROE experiments were classified into two ranges, 1.8-2.5 and 1.8-3.5 Å, corresponding to strong and medium ROEs, respectively. After MD simulation, each low energy conformation was finally minimized by the use of molecular mechanics calculation of AMBER all-atom force field. A snapshot with the lowest energy was selected as an relevant conformation (Fig. 5). The lowest energy conformer has no intramolecular hydrogen bonds and satisfies the characteristic ROE relationship. So it is considered to be the relevant solution conformer. The 10 lowest energy conformations have averaged backbone RMSD (STD) 0.144 Å (0.055) relative to the lowest energy conformation, for the best fit of each Cα carbon. The range of energies for the 10 lowest energy conformations is only 1.87 kcal/mol. The superposition of this stable conformer of

segetalin B and conformer A of segetalin A reported in our previous paper[1] was depicted in Fig. 6. The RMSD for the best fit of the backbone atoms in the sequence of Trp-Ala-Gly in segetalin B was 0.184 (STD 0.074) Å against conformer A of segetalin A. Furthermore, the direction of the side chain in Trp^4 was roughly identical with that of segetalin A.

From the foregoing evidence, segetalins A and B possess the same sequence units, Trp-Ala-Gly-Val, and also the similar solution conformation each other at this sequence. The estrogen-like activity of segetalins A and B may be responsible for the segment, Trp-Ala-Gly-Val and/or its conformation. Now, examination to clarify the above relationship and mode of action showing estrogen-like activity are in progress.

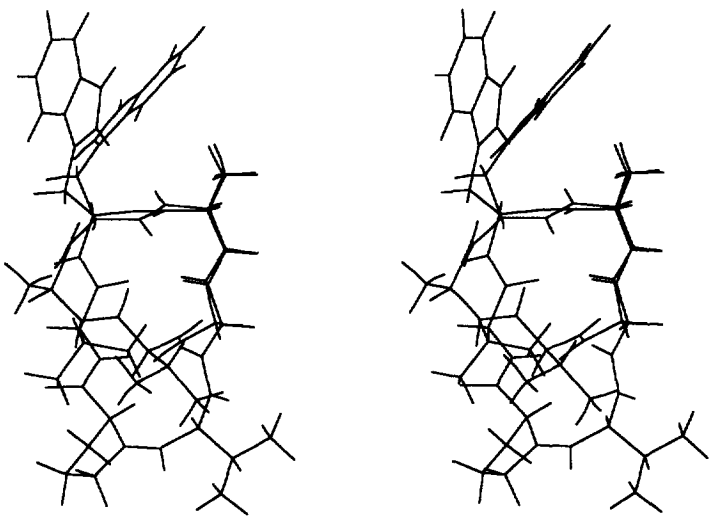

Fig. 6 A stereoview of a superposition of the energy minimized conformers of segetalins A (conformer A) and B

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 spectrometer and the $[\alpha]_D$ values are given in 10^{-1}deg cm^2 g^{-1}. FAB and high resolution mass spectra were taken with a VG Autospec spectrometer. IR spectrum was recorded on a JASCO A-302 spectrophotometer. High-pressure liquid chromatography (HPLC) was performed with an Inertsil PREP-ODS column (20mm i.d.×250mm and 30mm i.d.×250mm, GL Science Inc.) packed with 10μm ODS. TLC was

conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck) and the spots were detected by spraying Dragendorff reagent. Proton and carbon spectra were recorded on Bruker (AM400 and AM500) and Varian Unity 400 spectrometers. The 10 mg each sample of segetalins B, C and D in a 5mm tube (0.5ml [$^2H_5$]pyridine and [$^2H_6$]DMSO, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 303K. Phase-sensitive ROESY experiments were acquired with a mixing time of 90 msec. The value of the delay to optimize one-bond correlations in the HMQC spectrum and 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.

Extraction and Isolation

The seeds of *Vaccaria segetalis* (5.0 Kg) were extracted with hot MeOH three times to give a MeOH extract which was treated with ethyl acetate, *n*-BuOH and H_2O. The ethyl acetate soluble fraction (43 g), showing estrogen-like activity, was subjected to silica gel column chromatography using a CH_2Cl_2 - MeOH gradient system (1:0 - 0:1). The fraction eluted by 10% MeOH was finally subjected to ODS HPLC with an 35% CH_3CN and 65% MeOH solvent system to give segetalin B (200 mg), C(20 mg) and D (200 mg) as colourless needles.

Segetalin B (1). - Colourless needles, mp. 153-155°C (from MeOH), $[\alpha]_D$ -32.4° (c 0.41, pyridine); *m/z* 485 (Found: $(M+H)^+$, 485.2519. $C_{24}H_{33}N_6O_5$ requires, 485.2512); ν_{max} (KBr)/cm^{-1} 3340 (NH), 1673 (amide C=O), 1656 and 1636; λ_{max} (MeOH) / nm 289 (ε 3870) and 281 (4400).

Segetalin C (2).- Colourless needles, mp. 172-175°C (from MeOH), $[\alpha]_D$ -23.2° (c 0.42, MeOH); *m/z* 770 (Found: $(M+H)^+$, 770.3989. $C_{40}H_{52}N_9O_7$ requires, 770.3990); ν_{max} (KBr)/ cm^{-1} 3317 (NH), 1656 (amide C=O) and 1525; λ_{max} (MeOH) / nm 260 (ε 5000).

Segetalin D (3). - Colourless needles, mp. 165-167°C (from MeOH), $[\alpha]_D$ +13.7° (c 0.41, MeOH); *m/z* 720 (Found: $(M+H)^+$, 720.3680. $C_{37}H_{50}N_7O_8$ requires, 720.3720); ν_{max}(KBr)/ cm^{-1} 3324 (NH), 1665 (amide C=O), 1626 and 1522; λ_{max} (MeOH) / nm 257 (ε 2400).

Acid Hydrolysis of 1 - 3

Solutions of **1** - **3** (each containing 1 mg of peptide) in 6N HCl including 0.6% thioglycolic acid were heated at 110°C for 24h. After cooling, each solution was concentrated to dryness. The hydrolysates were soluble in 0.02N HCl and applied to the analysis by an amino acid analyzer.

Absolute Configuration of Amino Acids[5)]

Solutions of **1** - **3** (each containing 1 mg of peptides) in 6N HCl including 0.6% thioglycolic acid were heated at 110°C for 12h. After being cooled, each solution was concentrated to dryness. The residue was soluble in water and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 1M $NaHCO_3$ at 35° for 1h. After being cooled, 2M HCl was added and then concentrated to dryness. This residue was subjected to HPLC (Lichrospher 100, RP-18 (10μm), Merck), flow rate 1 ml/min, detection 340nm, solvent : 10 - 50% CH_3CN / 50mM triethylamine phosphate (TEAP) buffer. The t_R values were L-Ser 20.25, L-Pro 28.04, L-His 15.66, L-Val 33.75, L-Phe 40.79, L-Leu 41.08, L-Ala 25.39 and L-Trp 40.69 min, respectively.

Estrogen-like activity on uterine weight in ovariectomized rats

The ovary of the female SD strain rat weighting 90 - 100 g (4-week-old, Saitama Experimental Animal Co., Ltd.), were excised to have no hormone activity. Ovariectomized rats were maintained in an airconditioned room with lighting from 7 a.m. to 7 p.m. The room temperature (23 ± 2 °C) and humidity (55 ± 5 %) were controlled automatically. The standard rat food (Oriented MF) and water were given freely. Segetalins (each 2.5 mg/kg) were suspended in Tween 80 - saline solution. Commercial estriol (Sigma chemical company) as standard, adjusted to concentration 10 μg/kg, was dissolved in sesame oil including ethanol, and then ethanol was evaporated in water bath. The compounds (0.1 ml/rat) were administered by subcutaneous injection for two weeks. After final administration, each uterus was excised from rats tested and weighted to evaluate the activity. Value were expressed as means ± standard error (Mean ± S.E.). Differences between the control group and other group were examined using student's t-test.

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. The starting coordinates with the following ϕ angles (Gly^1: 120°, Val^2: -94°, Ala^3: 42°, Trp^4: 45°, Ala^5: 44°) in Table 6 was chosen after several trial and error test. Only this coordinates were consistent with all of the ROE relationships. Molecular mechanics and dynamics calculations were performed with the AMBER all-atom force field.[17] The dielectric constant (ε) was assumed to be proportional to the interatomic distances (r) as $\varepsilon=r$. Solvent molecules were not included in the calculations. The ROE relationships were taken into account in the calculations of the constraint 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 snapshot 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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