Cyclic Peptides from Higher Plants. 34.[1] Segetalins G and H, Structures and Estrogen-like Activity of Cyclic Pentapeptides from *Vaccaria segetalis*

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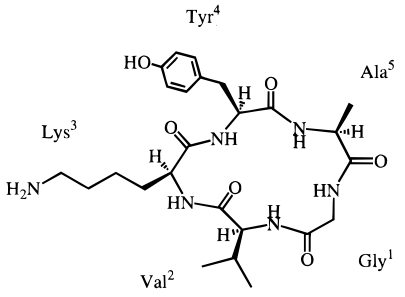
Segetalins G and H (**1**−**2**) possessing estrogen-like activity are cyclic pentapeptides from the seeds of *Vaccaria segetalis*. Their structures, *cyclo*(-Gly-Ala-Lys-Tyr-Val) (**1**) and *cyclo*(-Gly-Phe-Ser-Tyr-Arg-) (**2**), were determined by interpretation of spectral data.

Cyclic peptides are frequently encountered natural products exhibiting a wide variety of essential biological functions. Large numbers of cyclic peptides with unique structures and with various pharmacological activities are reported from marine and microorganisms.[2] However, few examples are known from higher plants.[3−7]

As part of our continuing investigation of new biologically active cyclic peptides from higher plants, we have previously reported five cyclic peptides, named segetalins from the EtOAc-soluble fraction of the seeds of *Vaccaria segetalis* (NECK.) GARCKE (Caryophyllaceae).[8,9] Segetalins A and B with a common sequence, Trp-Ala-Gly-Val, showed estrogen-like activity assayed by the increment of uterus against ovariectomized rats.[10] By the guidance of this assay-measured increment of uterus, purification of the *n*-BuOH soluble fraction led to the isolation of two new cyclic peptides, named segetalins G (**1**) and H (**2**), containing basic amino acids such as Arg and Lys. In this paper, we report the isolation and structural elucidation of **1** and **2** and their estrogen-like activity.



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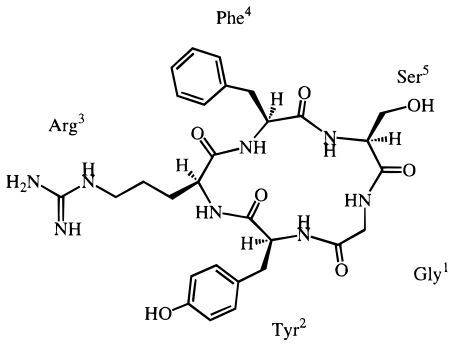

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Results and Discussion

The methanolic extract of the seeds of *V. segetalis* was partitioned between H_2O and EtOAc and then *n*-BuOH, successively. The *n*-BuOH-soluble material was subjected to a Diaion HP-20 column (H_2O−MeOH). The 80% MeOH-eluted fraction was chromatographed on ODS-MPLC and finally purified by ODS-HPLC to isolate two peptides, named Segetalins G (**1**, 0.001%) and H (**2**, 0.002%).

Segetalin G (**1**), colorless powder, $[\alpha]_D$ −89.0° (*c* 0.40, MeOH), showed a high-resolution FAB-MS spectral quasimolecular ion peak at *m*/*z* 519.2931 ($M^+ + H$, Δ +0.02 mmu) corresponding to the molecular formula $C_{25}H_{38}O_6N_6$. The IR absorption bands at 3310 and 1674 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The pentapeptide nature of **1** was evident from its ^1H and ^{13}C NMR spectra, which showed five amide NH signals and five carbonyl signals, respectively (Table 1). Amino acid analysis of the acid hydrolysate of **1** revealed the presence of Gly, Val, Lys, Tyr, and Ala. These amino acids in **1** were confirmed to have the L-configuration by derivation of the acid hydrolysate with Marfey's reagent, followed by HPLC analysis.[11] The ^1H and ^{13}C NMR signals for individual amino acids obtained above were readily assigned by extensive analysis of $^1H-^1H$ COSY and HMQC spectra. The gross structure including the sequence was determined on the basis of connectivity observed in the HMBC experimental results (Figure 1). Therefore, the sequence was identified as *cyclo*(-Gly-Val-Lys-Tyr-Ala-). The deduced structure of segetalin G (**1**) was also in good agreement with the results of the NOE correlation in a phase-sensitive ROESY spectrum.

Segetalin H (**2**), colorless powder, $[\alpha]_D$ −79.0° (*c* 0.40, MeOH), showed a molecular formula of $C_{29}H_{38}O_7N_8$ by HR-FABMS, indicating 15 degrees of unsaturation. Amino acid analysis of **2** showed the presence of Gly, Tyr, Arg, Phe, and Ser, which were confirmed to be all of the L-configuration by Marfey's derivatization, followed by HPLC analysis. In the NMR spectra of **2** (Table 2), five amide protons and carbonyl carbons were observed, corresponding to five amino acids. The sequence analysis was conducted, in a similar way to those in **1**, by 2D NMR analysis, *e.g.*, HMBC and ROESY spectra. From the results of the useful HMBC correlations as shown in Figure 2, the structure was established to be *cyclo*(-Gly-Tyr-Arg-Phe-Ser-), which was also confirmed by NOE correlations observed in the ROESY spectrum.

When the Segetalins G and H were administrated at

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Table 1. ^{1}H and ^{13}C NMR Assignments for Segetalin G (**1**) in DMSO-d_6

	position	δ_H (int; mult; *J* (Hz))	δ_C
Gly[1]			
	α	3.38 (1H, dd, 5.6, 14.8)	43.10
		4.07 (1H, dd, 5.6, 14.8)	
	NH	8.39 (1H, t, 5.6)	
	C=O		169.33
Val[2]			
	α	3.91 (1H, t, 8.2)	60.15
	β	1.97 (1H, m)	29.60
	γ	0.89 (3H, d, 6.1)	19.18
		0.87 (3H, d, 6.3)	18.32
	NH	7.73 (1H, d, 8.2)	
	C=O		170.35
Lys[3]			
	α	4.03 (1H, m)	54.10
	β	1.50 (2H, m)	26.51
	γ	1.03 (1H, m)	22.27
		1.14 (1H, m)	
	δ	1.58 (2H, m)	30.98
	ϵ	2.70 (2H, m)	38.95
	NH_2	7.72 (2H, br s)	
	NH	7.86 (1H, d, 8.6)	
	C=O		170.51
Tyr[4]			
	α	4.19 (1H, m)	56.27
	β	2.91 (2H, m)	35.95
	γ		127.49
	δ	6.97 (2H, d, 8.4)	114.95
	ϵ	6.66 (2H, d, 8.4)	129.94
	ζ		155.88
	NH	8.06 (1H, d, 8.6)	
	C=O		170.54
Ala[5]			
	α	4.20 (1H, m)	48.44
	β	1.22 (3H, d, 6.9)	16.63
	NH	7.73 (1H, d, 8.2)	
	C=O		171.70

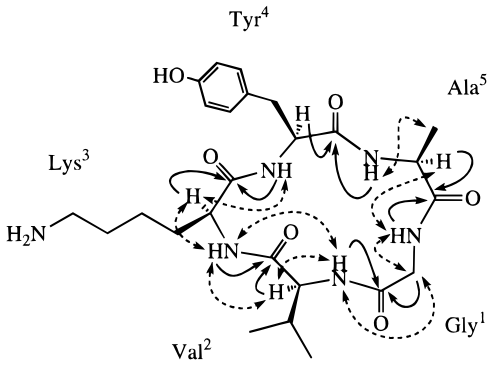

Figure 1. HMBC (arrows) and NOE correlations (dashed arrows) for segetalin G (**1**) in DMSO-d_6.

a dosage of 2.5 mg/kg to ovariectomized rats daily for 14 days, the weight of uterus significantly increased compared to the control (Table 3).

Sequential homology among segetalins A, B, and G is recognized in Tyr-Ala-Gly-Val of segetalin G and in Trp-Ala-Gly-Val of segetalins A and B. Furthermore, the sequence of Phe-Ser-Gly in segetalin H is partly similar to the above sequence in segetalins A, B, and G. The sequential homology and estrogen-like activity may be related to each other. It was known that isoflavones from higher plants have estrogen-like activity;[12] however, segetalins are unique examples of cyclic peptides that show estrogen-like activity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-4 polarimeter.

Table 2. ^{1}H and ^{13}C NMR Assignments for Segetalin H (**2**) in DMSO-d_6

	position	δ_H (int; mult; *J* (Hz))	δ_C
Gly[1]			
	α	3.41 (1H, dd, 5.7, 14.9)	43.16
		3.85 (1H, dd, 5.7, 14.9)	
	NH	8.42 (1H, t, 5.7)	
	C=O		169.77
Tyr[2]			
	α	4.26 (1H, ddd, 5.5, 8.4, 9.5)	56.27
	β	2.74 (1H, dd, 9.5, 14.0)	36.19
		2.92 (1H, dd, 5.5, 14.0)	
	γ		127.55
	δ	7.03 (2H, d, 8.4)	129.77
	ϵ	6.66 (2H, d, 8.4)	115.43
	ζ		155.92
	NH	8.09 (1H, d, 8.4)	
	C=O		170.89
Arg[3]			
	α	4.01 (1H, dt, 7.9, 8.2)	53.80
	β	1.58 (2H, m)	28.51
	γ	1.16 (1H, m)	24.98
		1.28 (1H, m)	
	δ	3.03 (2H, m)	40.30
	ϵ		156.79
	-NH	7.63 (1H, t, 5.7)	
	NH	7.82 (1H, d, 8.2)	
	C=O		170.87
Phe[4]			
	α	4.18 (1H, m)	57.11
	β	3.09 (2H, d, 7.8)	36.33
	γ		137.54
	δ	7.27 (2H, d, 7.2)	128.33
	ϵ	7.21 (2H, t, 7.2)	129.05
	ζ	7.21 (1H, t, 7.2)	126.43
	NH	8.34 (1H, d, 8.1)	
	C=O		170.94
Ser[5]			
	α	4.21 (1H, m)	55.16
	β	3.59 (1H, dd, 5.6, 10.8)	60.72
		3.66 (1H, dd, 6.3, 10.8)	
	NH	8.03 (1H, d, 8.1)	
	C=O		170.58

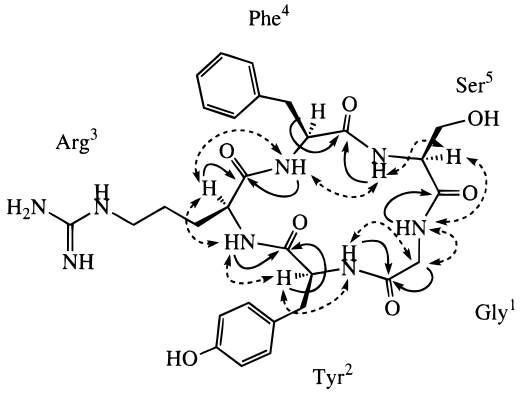

Figure 2. HMBC (arrows) and NOE correlations (dashed arrows) for segetalin H (**2**) in DMSO-d_6.

The IR spectra (KBr) were obtained on a Perkin-Elmer 1710 spectrophotometer. Mass spectra were recorded on a VG Autospec instrument. HPLC was performed on an Inertsil PREP-ODS packed with 10 μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck), and the spots were detected by spraying with Dragendorff's reagent. ^{1}H-NMR and ^{13}C-NMR spectra were run in DMSO-d_6 using a Bruker AM-500 and Varian Unity 400 instruments, with chemical shifts (δ) reported in ppm. The spectra were recorded at 303 K. A phase-sensitive ROESY NMR experiment was acquired with mixing times of 100 ms. The value of the

Table 3. Effect of Test Samples on Uterus Weight[a]

sample	dose (mg/kg)	uterine wt (mg)	*n*
control		36.4 ± 1.85	5
segetalin A	2.5	54.4 ± 6.83[b]	5
segetalin B	2.5	56.3 ± 6.46[b]	5
segetalin G	2.5	51.2 ± 2.42[b]	5
segetalin H	2.5	54.8 ± 3.22[b]	5

[a] The values are mean ± S.E. *n* is the number of experiment animals. [b] Difference significant ($p < 0.01$, 0.05) from control by multiple comparison of Dunnett.

delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 Hz, and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 ms.

Plant Material. The seeds of *V. segetalis* were purchased in Shanghai, the People's Republic of China, in May 1993. The botanical identification was made by Dr. Zhi-Sheng Qiao, Department of Pharmacognosy, College of Pharmacy, Second Military Medical University, Shanghai, China. A voucher specimen has been deposited in the herbarium of the Tokyo University of Pharmacy and Life Science.

Extraction and Isolation. The seeds of *V. segetalis* (5.0 kg) were extracted with hot MeOH four times to give a MeOH extract (180 g) that was partitioned between *n*-BuOH and H_2O. The *n*-BuOH-soluble fraction (86 g) was subjected to Diaion HP-20 column chromatography using a H_2O−MeOH gradient system (1:0−0:1). The fraction eluted with 80% MeOH was further subjected to ODS MPLC with a 35% CH_3CN solvent system, followed by ODS HPLC with a 15% CH_3CN containing 0.05% TFA solvent system to give segetalin G (**1**, 55 mg) and segetalin H (**2**, 90 mg). Segetalin G (**1**): colorless powder; $[\alpha]_D$ −89.0° ($c = 0.40$, MeOH); IR (KBr) ν_{max} 3310 and 1674 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Table 1; FABMS *m/z* $[M + H]^+$ 519; HRFABMS *m/z* found 519.2907, calcd for $C_{25}H_{39}N_6O_6$ 519.2931. Segetalin H (**2**): colorless powder; $[\alpha]_D$ −79.0° ($c = 0.40$, MeOH); IR (KBr) ν_{max} 3348 and 1670 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Table 2; FABMS *m/z* $[M + H]^+$ 611; HRFABMS *m/z* found 611.2951, calcd for $C_{29}H_{39}N_8O_7$ 611.2941.

Absolute Configuration of Amino Acids. Each solution of **1** and **2** (1 mg) in 6 N HCl was heated at 110 °C for 12 h. The solution was concentrated to dryness. The residue was dissolved in H_2O and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 1 M $NaHCO_3$ at 35 °C for 1 h. After cooling, 2 M HCl was added and then concentrated to dryness. The resulting residue was subjected to HPLC [Lichrospher 100, RP-18 (10 μm), Merck], flow rate 1 mL/min, detection 340 nm, solvent 10−50% CH_3CN/50 mM triethylamine phosphate buffer.

Estrogen-Like Activity on Uterine Weight in Ovariectomized Rats. The ovaries of the female SD strain rat weighting 70−80 g (4-week-old, Tokyo Ikagaku Experimental Animal Co., Ltd.) were excised to have no hormone activity. Ovariectomized rats were maintained in an air-conditioned room with lighting from 7 am to 7 pm. 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 Co.) as standard, adjusted to concentration 10 μg/kg, was dissolved in sesame oil including ethanol, and then the ethanol was evaporated in a water bath. The compounds (0.1 mL/rat) were administered by subcutaneous injection for 2 weeks. After final administration, each uterus was excised and weighed to evaluate the activity. Values were expressed as means ± standard error (mean ± S.E.). Differences between the control group and the test group were examined using the multiple comparison of Dunnett.

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References and Notes

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