Pergamon 0031-9422(95)00911-6 *Phytochemistry*, Vol. 42, No. 2, pp. 439–441, 1996

0031-9422/96 $15.00 + 0.00

# A CYCLIC HEPTAPEPTIDE FROM *VACCARIA SEGETALIS*

HIROSHI MORITA, YOUNG SOOK YUN, KOICHI TAKEYA, HIDEJI ITOKAWA* and OSAMU SHIROTA†

Department of Pharmacognosy, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan; †Division of Pharmacognosy and Phytochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan

(*Received in revised form* 6 *November* 1995)

**Key Word Index**—*Vaccaria segetalis*; Caryophyllaceae; segetalin E; cyclic heptapeptide.

**Abstract**—A new cyclic heptapeptide, segetalin E, *cyclo*(-Gly-Tyr-Val-Pro-Leu-Trp-Pro-), has been isolated from the seeds of *Vaccaria segetalis* and the structure elucidated by extensive two-dimensional NMR methods and chemical degradation.

## INTRODUCTION

As part of our continuing study of cyclic peptides from higher plants [1–2], we have investigated several cyclic peptides from *Vaccaria segetalis*, named segetalins which have oestrogen-like activity [3–5]. In addition, a conformational form of segetalin A analysed by NMR and computational methods have also been reported [6]. The seeds of *Vaccaria segetalis* have been used to activate blood flow and promote milk secretion. Furthermore, it is commonly used to treat amenorrhea and breast infections in China [7]. Further chromatographic purification of an ethyl acetate-soluble fraction of *V. segetalis* led us to isolate a new cyclic heptapeptide, named segetalin E (**1**). This paper deals with the isolation and structural elucidation of **1**.

## RESULTS AND DISCUSSION

The concentrated methanol extract of the seed of *Vaccaria segetalis* was extracted with ethyl acetate, followed by 1-butanol. The ethyl acetate phase was successively separated by silica gel column chromatography with a gradient system ($CH_2Cl_2$–methanol) and was subjected to HPLC on an ODS column with 37% $CH_3CN$ containing 0.05% TFA to furnish a new cyclic heptapeptide, segetalin E (**1**, 0.004% yield), together with segetalins A–D (segetalin A: *cyclo*(-Gly-Val-Pro-Val-Trp-Ala-); segetalin B: *cyclo* (-Gly-Val-Ala-Trp-Ala-); segetalin C: *cyclo* (-Gly-Leu-His-Phe-Ala-Phe-Pro-); segetalin D: *cyclo* (-Gly-Leu-Ser-Phe-Ala-Phe-Pro-)] [3, 5].

The FAB-mass spectrum of **1** gave an $[M + 1]^+$ at 813 and the molecular formula, $C_{43}H_{56}N_8O_8$, which was permitted by HR-FAB mass spectrum, indicating 20 degrees of unsaturation, was established. Because the IR absorption bands (3310 and 1635 $cm^{-1}$) characteristics of amino and amide carbonyl groups indicated **1** to be a peptide, it was subjected to the standard amino acid analysis, which implied the presence of 1 mol each of Gly, Tyr, Leu, Val, and 2 mol of Pro. The $^1H$ NMR spectrum in pyridine-$d_5$ indicated five amide NH signals between $\delta$ 8.42 and $\delta$ 9.95, and an indole NH signal at $\delta$ 12.12. The $^{13}C$ NMR spectrum revealed seven carbonyl signals, which were involved in amide linkage. Analyses of the aromatic region of the $^1H$ and $^{13}C$ NMR spectra provided evidence for an indole ring system, indicating Trp. From the molecular formula and from $^1H$ and $^{13}C$ NMR data, it became evident that **1** was a cyclic peptide. Complete assignments for the $^1H$ and $^{13}C$ NMR resonances in pyridine-$d_5$ (Table 1), were accomplished using combination of two-dimensional NMR experiments such as HMQC [8] and PFG-HMBC [9] spectra. The stereochemistry of each amino acid was confirmed to be all L-configuration by Marfey's derivation, followed by HPLC analysis [10].

The HMBC analyses suggested the seven amino acid sequence presented in structure **1**. Two segments, Pro-Gly-Tyr-Val and Pro-Leu-Trp were assigned by two bond $^1H$–$^{13}C$ correlations as follows; NH (Gly1)/CO (Pro7), NH (Tyr2)/CO (Gly1), NH (Val3)/CO(Tyr2), and NH (Leu5)/CO (Pro4), NH (Trp6)/CO (Leu5) (Fig. 1). Two structural units analysed by the HMBC correlations could be combined by ROE enhancements around two Pro residues in a phase-sensitive ROESY spectrum [11]. The ROE cross-peaks between Val3-H$\alpha$ and Pro4-H$\alpha$, and between Trp6-H$\alpha$ and Pro7-H$\delta$ supported connectivities across Val3–Pro4 and Trp6–Pro7, respectively. Additional ROE correlations such as Tyr2-H$\alpha$/Val3-NH, Leu5-NH/Trp6-NH, and Pro7-H$\alpha$/Gly1-NH supported the above sequence and the structure of **1** was determined to be *cyclo*(-Gly-Tyr-Val-Pro-Leu-Trp-Pro-). A through-space interaction between

*Author to whom correspondence should be addressed.

Table 1. $^1$H and $^{13}$C NMR signal assignments of segetalin E (**1**) in pyridine-$d_5$

| Assignment | $^1$H NMR $\delta_H$ (int. mult, $J$(Hz)) | $^{13}$C NMR $\delta_C$ | Assignment | $^1$H NMR $\delta_H$ | $^{13}$C NMR $\delta_C$ |
|---|---|---|---|---|---|
| Gly1 | | | Leu5 | | |
| $\alpha$ | 4.00 (1H, *dd*, 6.3, 16.8) | 40.65 | $\alpha$ | 5.03 (1H, *dd*, 4.4, 8.2) | 55.07 |
| | 4.15 (1H, *dd*, 6.3, 16.8) | | $\beta$ | 2.00 (1H, *m*) | 40.05 |
| NH | 8.44 (1H, *t*, 6.3) | | | 2.17 (1H, *m*) | |
| C=O | | 168.21 | $\gamma$ | 1.83 (1H, *m*) | 24.92 |
| Tyr2 | | | $\delta$ | 0.90 (3H, *s*) | 20.60 |
| $\alpha$ | 5.12 (1H, *ddd*, 3.9, 7.5, 10.1) | 54.70 | | 0.96 (3H, *d*, 6.5) | 22.39 |
| $\beta$ | 3.09 (1H, *dd*, 3.9, 13.6) | 35.98 | NH | 8.96 (1H, *d*, 8.2) | |
| | 3.34 (1*H*, *dd*, 10.1, 13.6) | | C=O | | 172.07 |
| $\gamma$ | | 128.73 | Trp6 | | |
| $\delta$ | 7.24 (2H, *d*, 8.5) | 130.19 | $\alpha$ | 5.29 (1H, *ddd*, 4.2, 6.3, 6.5) | 52.76 |
| $\varepsilon$ | 7.01 (2H, *d*, 8.5) | 115.19 | $\beta$ | 3.66 (1H, *dd*, 4.2, 15.0) | 26.94 |
| $\zeta$ | | 156.37 | | 3.93 (1H, *dd*, 6.3, 15.0) | |
| NH | 8.67 (1H, *d*, 7.5) | | NH | 8.42 (1H, *d*, 6.5) | |
| C=O | | 172.53 | 1(NH) | 12.12 (1H, *s*) | |
| Val3 | | | 2 | 8.01 (1H, *d*, 2.2) | 125.35 |
| $\alpha$ | 4.46(1H, *dd*, 5.0, 8.0) | 58.77 | 3 | | 108.66 |
| $\beta$ | 2.17 (1H, *m*) | 29.68 | 4 | 8.22 (1H, *d*, 7.5) | 118.40 |
| $\gamma$ | 0.96 (3H, *s*) | 18.47 | 5 | 7.23 (1H, *t*, 7.5) | 121.07 |
| | 1.09 (3H, *d*, 6.9) | 18.51 | 6 | 7.21 (1H, *t*, 7.5) | 118.74 |
| NH | 9.95 (1H, *d*, 5.0) | | 7 | 7.51 (1H, *d*, 7.5) | 111.39 |
| C=O | | 170.60 | 8 | | 136.30 |
| Pro4 | | | 9 | | 128.31 |
| $\alpha$ | 4.95 (1H, *d*, 6.4) | 60.98 | C=O | | 171.03 |
| $\beta$ | 1.83 (1H, *m*) | 30.12 | Pro7 | | |
| | 2.81 (1H, *dd*, 6.4, 11.8) | | $\alpha$ | 4.42 (1H, *dd*, 7.2, 9.3) | 58.77 |
| $\gamma$ | 1.75 (2H, *m*) | 21.54 | $\beta$ | 2.07 (1H, *m*) | 28.37 |
| $\delta$ | 3.49 (1H, *m*) | 45.57 | | 1.88 (1H, *m*) | |
| | 3.78 (1H, *m*) | | $\gamma$ | 1.88 (1H, *m*) | 25.20 |
| C=O | | 170.43 | | 1.59 (1H, *m*) | |
| | | | $\delta$ | 3.28 (2H, *m*) | 47.12 |
| | | | C=O | | 171.54 |

Val3-H$\alpha$ and Pro4-H$\alpha$ is indicative of a *cis* peptide bond between Val3 and Pro4, which is also supported by the carbon resonances ($\delta$ 30.12 and $\delta$ 21.54) of $\beta$ and $\gamma$ in Pro4 [12], and the occurrence of a doublet signal of H$\alpha$ in Pro4 has also been correlated the *cis* peptide bond [13]. Segetalin E showed moderate cell growth inhibitory activity against P-388 cells ($IC_{50}$ 40 $\mu$g ml$^{-1}$).

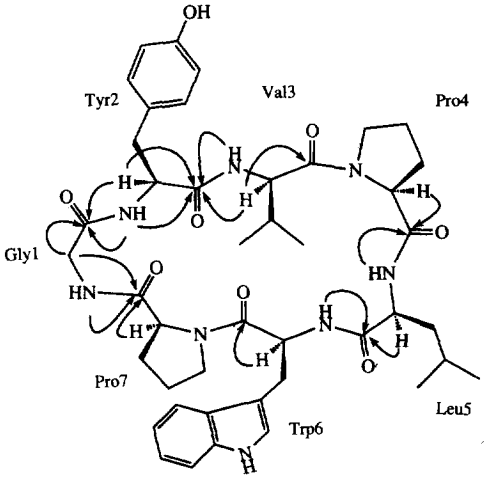


Fig. 1. Structure of segetalin E (**1**); arrows show some important HMBC correlations.

## EXPERIMENTAL

*General.* IR and UV spectra were recorded on JASCO A-302 spectrometer and Hitachi 557 spectrophotometer, respectively. Optical rotation was measured with a JASCO DIP-4 spectrometer and $[\alpha]_D$ values are given in $10^{-1}$ deg. cm$^2$ g$^{-1}$. FABMS and HRMS with a VG Autospec spectrometer. HPLC was performed with an Inertsil PREP-ODS column (20 mm i.d. × 250 mm and 30 mm i.d. × 250 mm, GL Science Inc.) packed with 10 $\mu$m ODS. Peptide-containing fractions were traced by TLC on precoated TLC plated 60 $F_{254}$ (Merk) using a solvent system consisting of $CHCl_3$/MeOH = 8.5:1.5 in a saturated chamber. All NMR spectroscopy was carried out on a Varian Unity 400 spectrometer. The spectra were recorded at 300 K at a concn of 20 mg ml$^{-1}$ in pyridine-$d_5$. The phase-sensitive ROESY experiments were acquired with mixing times of 90 msec. The delay to optimize one-

bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 150 msec and the evolution delay for long-range couplings in the HMBC spectrum was set to 90 msec.

*Plant Material.* The seeds of *V. segetalis* were purchased in Shanghai, the Peoples 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 kg) were extracted with hot MeOH × 3 to give a MeOH extract which was treated with EtOAc, *n*-BuOH and $H_2O$. The EtOAc sol. fraction (47 g), was subjected to silica gel CC using a $CH_2Cl_2$–MeOH gradient system (1:0–0:1). The fraction eluted by 15% MeOH was finally subjected to ODS HPLC with 37% $CH_3CN$ containing 0.05% TFA solvent system to give segetalin E (200 mg) as colourless needles.

*Segetalin E* (**1**). Needles, mp 166–168° (from MeOH), $[\alpha]_D$ − 59° (c 0.40, MeOH); *m/z* 813 (Found: $M^+ + H$, 813.4339. $C_{43}H_{57}N_8O_8$ requires, 813.4299); $\nu_{max}$ (KBr)/$cm^{-1}$ 3310 (NH), 1635 (amide C=O) and 1516; $\lambda_{max}$ (MeOH)/257 nm ($\varepsilon$ 2400).

*Amino acid analysis.* Segetalin E (1 mg) was hydrolysed in 1 ml 6 M HCl in a sealed vial at 110° for 24 h. HCl was removed under red. pres., and residue was dissolved in 0.02 M HCl. Amino acids were determined by ion-exchange resin chromatography on a Hitachi L-8500 amino acid analyser with ninhydrin detection.

*Absolute configuration of the amino acids.* A soln of segetalin E in 6 M HCl was heated at 110° for 12 hr. After being cooled, it was concd to dryness. The residue was soluble 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° for 1 hr. After being cooled, 2 M HCl was added and then concd to dryness. This residue was subjected to HPLC (Lichrospher 100, RP-18 (10 $\mu$m), Merck), 1 ml $min^{-1}$, detection 340 nm, solvent: 10–50% $CH_3CN$–50 mM triethylamine phosphate (TEAP) buffer.

*Cytotoxic activity on P388 cells.* The MTT (3 - [4,5 - dimethylthiazol - 2 - yl] - 2, 5 - diphenyltetrazolium bromide) colorimetric assay was performed in a 96-well plate. The blue formazan produced by the mitochondrial dehydrogenase of viable cells was measured spectrophotometrically. RPMI-1640 medium, 100 $\mu$l, supplemented with 5% fetal calf serum and 100 $\mu$g $ml^{-1}$ kanamycin and containing mouse P388 leukaemia cells ($3 \times 10^4$ cells $ml^{-1}$) was added to each well. After overnight incubation (37°, 5% $CO_2$), 100, 30, 10, 3, 1, 0.3, and 0.1 $\mu$g $ml^{-1}$ of sample solns were added to the wells and the plates were incubated for 48 hr. Then, 20 $\mu$l of MTT was added to each well and the plates were incubated for 4 hr. The resulting formazan was dissolved in 100 $\mu$l of 10% SDS containing 0.01 M HCl. Each well was mixed gently with a pipette for 1 or 2 min and the plate was read on a microplate reader (Tosoh MPR-A4i) at 540 nm. The $IC_{50}$ ($\mu$g $ml^{-1}$) value was defined as the concn of sample which achieved 50% reduction of viable cells with respect to the control.

*Acknowledgement*—The authors thank the Ministry of Education, Science and Culture, Japan, for financial support through Grant-in-Aid for General Scientific Research.

## REFERENCES

1. Cyclic Peptide from Higher Plant. Part. 25., Part 24 Morita, H., Kayashita, T., Shimomura, M., Takeya, K. and Itokawa, H., *J. Nat. Prod.*, in press.
2. Morita, H., Kayashita, T., Takeya, K., Itokawa, H. and Shiro, M. (1995) *BioMed. Chem. Lett.* **5**, 2353 (and references cited therein).
3. Morita, H., Yun, Y. S., Takeya, K. and Itokawa, H. (1994) *Tetrahedron Letters* **35**, 9593.
4. Itokawa, H., Yun, Y. S., Takeya, K., Morita, H. and Yamada, K., (1995) *Planta Med.* **61**, 561.
5. Morita, H., Yun, Y. S., Takeya, K. and Itokawa, H. (1995) *Tetrahedron* **51**, 6003.
6. Morita, H., Yun, Y. S., Takeya, K. and Itokawa, H. (1995) *Tetrahedron* **51**, 5987.
7. Kee Chang Huang (1993) *The Pharmacology of Chinese Herbs*, pp. 254–255. CRC Press, Inc., Boca Raton.
8. Bax, A. and Subramanian, S. (1986) *J. Magn. Reson.* **67**, 565.
9. Hurd, R. E. and John, B. K. (1991) *J. Magn. Reson.* **91**, 648.
10. Marfey, P. (1984) *Carlsberg Res. Commun.* **49**, 591.
11. Bother-By, A. A., Stephens, R. L., Lee, J., Warren, C. D. and Jeanloz, R. W. (1984) *J. Am. Chem. Soc.* **106**, 811.
12. Dorman, D. E., and Bovey, F. A. (1973) *J. Org. Chem.* **38**, 2379.
13. Kopple, K. D., Schumper, T. J. and Go, A. (1974) *J. Am. Chem. Soc.* **96**, 2597.