

p-Coumaroylamino Acids from Yeast-Elicited *Ephedra distachya* Cultures

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(Received January 3, 1994)

Three *p*-coumaroylamino acids (*p*-CAAs) were isolated from the yeast-elicited *Ephedra distachya* cultures by consecutive purification using XAD-2, silicagel and RP-HPLC. Retention times on HPLC as well as their UV, IR, NMR and MS spectral data indicated that the yeast-induced *p*-CAAs were *p*-coumaroyl-D-valine, *p*-coumaroyl-D-serine and *p*-coumaroyl-D-threonine, respectively. The structures of *p*-CAAs were confirmed by the comparison of their physico-chemical properties with those of synthetic ones. They were isolated and identified for the first time from natural products and supposed to be accumulated as phytoalexins of *Ephedra*.

Key words: *Ephedra distachya*, Ephedraceae, Cell suspension cultures, *p*-Coumaroyl-D-valine, *p*-Coumaroyl-D-serine, *p*-Coumaroyl-D-threonine, Yeast elicitor

INTRODUCTION

Plants accumulate antibiotic low molecular weight substances as a result of plant pathogen interactions (Müller *et al.*, 1940). They are known as phytoalexins and the signaling compounds which induce the phytoalexins are termed as elicitors (Brooks *et al.*, 1984). Phytoalexins have been reported at least in 17 plant families and most of them are concentrated on Angiospermae but rarely on Gymnospermae (Dixon *et al.*, 1986). Recently, the model study using *Ephedra distachya* cultures and yeast elicitor had been carried out in order to understand the fundamental knowledge about plant-pathogen interaction mechanisms in Gymnospermous plant. As a results, it was found that yeast-elicited *E. distachya* cultures produced *p*-coumaroylglycine and *p*-coumaroyl-D-alanine along with some unidentified minor ones (Song *et al.*, 1992). The produced *p*-coumaroylamino acids were supposed to be phytoalexins of the *Ephedra* (Song *et al.*, 1992, 1994a, b). Thus, as one of our serial works (Song *et al.*, 1994a, b, c) on 'plant-pathogen interaction mechanisms in *Ephedra*' we tried to purify the elicitor-induced minor compounds and elucidate the chemical structures of them.

MATERIALS AND METHODS

General

¹H NMR was recorded on 400 MHz (JEOL GSX 400). ¹³C NMR was recorded on 100 MHz. Chemical shifts were given in δ (ppm) from TMS. HPLC and HPLC-UV was carried out by TOSOH SC8010 UV-HPLC system. Merck Art. 5715 pre-coated plate was used for TLC analysis. EI-MS was measured by JEOL JMS-DX303. IR was measured on JEOL JIR-RFX-3001.

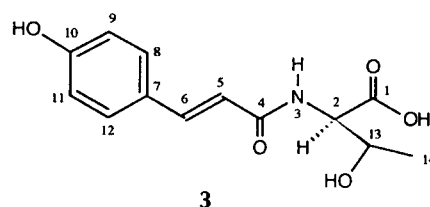
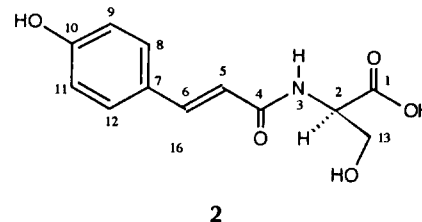
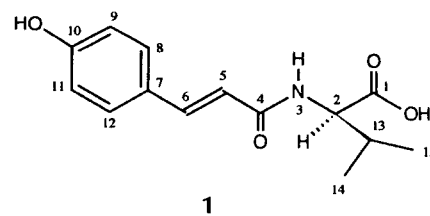
Extraction and Purification

660 g fr. wt. *E. distachya* culture was extracted with 4 L MeOH in water bath. After removal of the solvent, ethylacetate (EtOAc) soluble acidic fr was obtained by routine method (1.5 g). This fr was applied on XAD-2 column (40 mm \times 340 mm) and eluted with aqueous acetone with gradual increase of acetone concentration up to 100%. Seven frs (Fr I to VII) were obtained and re-chromatography of Fr III (220 mg) using silicagel [Merck Art. 9385 (30 mm \times 300 mm), CHCl₃-CH₃OH-HCO₂H (60:7:1)] and RP-HPLC (Merck Linchroprep. RP-18, 25 mm \times 310 mm, flow rate; 2.5 ml min⁻¹, mobile phase; 1% HOAc in 45% MeOH) afforded 2 and 3 in an amount of less than 1 mg each. 1 (2 mg) was purified from Fr V (80 mg) by HPLC under the same conditions with above except for mobile phase (1% HOAc in 90% MeOH).

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p-Coumaroyl-D-valine (1)

1 was obtained as an oily compound.; UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 226 (4.01), 294 KBr(4.30), 301 (4.30), 310 (4.20); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400 (N-H), 1730 (OC=O), 1695 (HNC=O); EI-MS m/z (rel. int.): 263 (27.3) $[M]^+$, 147 (100) $[M-116]^+$; ^1H NMR was measured in DMSO- d_6 at 35°C: 8.00 (1H, d, $J=8.4$ Hz, N-H), 7.39 (2H, d, $J=8.8$ Hz, H-8, H-12), 7.32 (1H, d, $J=16.0$ Hz, H-6), 6.91 (2H, d, $J=8.8$ Hz, H-9, H-11), 6.64 (1H, d, $J=16.0$, H-5), 4.27 (1H, dd, $J_{2,3}=8.4$ Hz, $J_{2,13}=5.6$ Hz, H-2), 2.08 (1H, m, H-13), 0.92 (3H, d, $J=2.8$ Hz, H-14), 0.90 (3H, d, $J=2.0$ Hz, H-15).



p-Coumaroyl-D-serine (2)

2 was purified as colorless powder. Mp of **2** was 186-188°C (decomposed). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 226 (4.00), 295 (4.30), 301 (4.30), 311 (4.18); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400 (N-H), 1732 (OC=O), 1695 (HNC=O); EI-MS m/z (rel. int.): 251 (2.8) $[M]^+$, 147 (100) $[M-104]^+$; ^1H NMR was recorded in DMSO- d_6 at 35°C: δ 8.04 (1H, d, $J=8.0$ Hz, N-H), 7.40 (2H, d, $J=8.4$ Hz, H-8, H-12), 7.33 (1H, d, $J=16.0$ Hz, H-6), 6.80 (2H, d, $J=8.4$ Hz, H-9, H-11), 6.62 (1H, d, $J=16.0$, H-5), 4.41 (1H, dt, $J_{2,3}=8.0$ Hz, $J_{2,13}=4.4$ Hz, H-2), 3.74 (1H, dd, $J_{2,13a}=4.4$ Hz, $J_{13a,13b}=10.4$ Hz, H-13a), 3.67(1H, dd, $J_{2,13b}=4.4$ Hz, $J_{13a,13b}=10.8$ Hz, H-13b).

p-Coumaroyl-D-threonine (3)

3 was isolated as colorless powder. Mp of **3** was 104-106°C (decomposed). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 226 (4.00), 294 (4.28), 301 (4.28), 309 (4.20); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400 (N-H), 1730 (OC=O), 1695 (HNC=O); EI-MS m/z (rel. int.): 265 (10.87) $[M]^+$, 147 (100) $[M-118]^+$; ^1H NMR was recorded in DMSO- d_6 at 35°C: 7.81 (1H, d, $J=9.2$ Hz, N-H), 7.40 (2H, d, $J=8.8$ Hz, H-8, H-12), 7.33 (1H, d, $J=15.2$ Hz, H-6), 6.79 (2H, d, $J=8.8$ Hz, H-9, H-11), 6.73 (1H, d, $J=15.2$, H-5), 4.35 (1H, dd, $J_{2,3}=9.2$ Hz, $J_{2,13}=3.2$ Hz, H-2), 4.17 (1H, dq, $J_{2,13}=3.2$ Hz, $J_{13,14}=6.0$ Hz, H-13), 1.09(3H, d, $J_{13,14}=6.0$ Hz, H-14).

HPLC Analysis

HPLC or HPLC-UV of *p*-coumaroylamino acids were carried out according to the reported method (Song *et al.*, 1992). R_t 's: *p*-coumaroyl-D-serine; 10.47 min, *p*-coumaroyl-D-threonine; 13.20 min, *p*-coumaroyl-D-valine; 22.67 min. The configuration of each *p*-coumaroylamino acids were verified by HPLC equipped with enantiomer-discriminating column. HPLC conditions were as follows: column; TOSOH ENANTIO L1 (4.6 mm x 550 mm), column temp; 50°C, mobile phase; 0.5 mM CuSO_4 , detection; UV 254 nm; flow rate; 0.5 ml min^{-1} . R_t 's: *p*-coumaroyl-D-serine; 25.67 min (L-iso-

mer; 26.17), *p*-coumaroyl-D-threonine; 27.50 min (L-isomer; 28.03), *p*-coumaroyl-D-valine; 33.87 min (L-isomer; 34.53).

RESULTS AND DISCUSSION

Ephedra distachya callus was induced from the aerial part of the plant and the callus was transferred to Murashige-Skoog liquid medium to propagate suspension cultures as described previously (Song *et al.*, 1992). 500 mg yeast ext was added to the 27-28 day old cultures and subsequently cultured for 48 hr. Elicited cells were harvested and extracted with CH_3OH . EtOAc soluble acidic fr was obtained by routine way. Repeated chromatography of this fr afforded an oily (**1**) and two white powdered compounds (**2**, **3**).

UV absorption at 290-310 nm of compound **1-3** suggested the presence of coumaroyl moiety in their structures. IR absorption at 1695 cm^{-1} and the proton resonance at δ 8.00 ppm (d, $J=8.4$ Hz) of compound **1** indicated those of typical amide bond. These facts and the aromatic protons at δ 7.32 and 7.39 ppm, methine protons at δ 2.09 (m, $J=2.8, 5.6$ Hz) and 4.27 ppm (dd, $J=8.4, 5.6$ Hz) and two methyl signals at δ 0.88 and 0.90 ppm (d, $J=2.8$ Hz, each) in ^1H NMR spectra revealed that compound **1** has a valine moiety whose amine group is conjugated to *p*-coumaric acid through amide bond.

2 also exhibited proton resonances of 4-substituted benzene ring at δ 6.80 and 7.40 ppm (2H, d, $J=8.4$ Hz, each). It showed amide stretching band at 1695 cm^{-1} in IR spectrum and exhibited the resonance of

Table 1. ^{13}C NMR spectral data* of **1**, **2** and **3** (δ ppm from TMS)

C No.	1	2	3
1	175.18	173.61	174.02
2	59.27	56.29	59.32
4	169.37	169.22	169.56
5	117.98	117.97	117.92
6	142.59	142.63	142.75
7	127.73	127.65	127.66
8,12	130.72	130.75	130.77
9,11	116.75	116.74	116.75
10	160.48	160.53	160.57
13	31.87	63.10	68.64
14	18.45		20.51
15	19.68		

*Measured at 100 MHz in CD_3OD

an exchangeable proton at δ 8.04 (d, $J=8.4$ Hz) in ^1H NMR, suggesting the presence of amide bond. Two proton resonances at δ 3.74 (dd, $J=4.4$ and 10.4 Hz) and 3.67 ppm (dd, $J=4.4$ and 10.8 Hz) as well as a molecular ion peak at m/z 251 in EI-MS indicated the presence of serine moiety in its structure. From these observations, **2** was supposed to be a *p*-coumaroyl conjugate of serine.

3 showed a doublet methyl proton resonance at δ 1.09 ppm ($J=6.0$ Hz) along with two methine proton signals at δ 4.35 (dd, $J=3.2$ and 9.2 Hz) and 4.17 ppm (dq, $J=3.2$ and 6.0 Hz). These protons were supposed to be originated from threonine moiety. It was tentatively identified as a *p*-coumaroylthreonine from its UV, IR, NMR and MS data in combination with above observations.

The ^{13}C NMR data of compound **1-3** are presented in Table 1. The structures of **1**, **2** and **3** were confirmed by the direct comparison of their ^1H and ^{13}C NMR, retention times on HPLC, UV and IR spectrum with those of synthetic standards. Synthesis was carried out according to the reported method (Song et al., 1992; Van Brussel et al., 1970).

To check the optical purity and configurations of **1**, **2** and **3**, HPLC analysis using enantiomer-discriminating column was carried out. Only D-isomer of each three *p*-coumaroylamino acids was confirmed, therefore, **1**, **2** and **3** were finally identified as *p*-coumaroyl-D-valine, *p*-coumaroyl-D-serine and *p*-coumaroyl-D-threonine, respectively.

N-Acylamino acids such as *N*-benzoyl-L-leucine and *N*-phenylacetyl-L-leucine were reported to inhibit the growth of some phytopathogens (Takano, 1978). This fact and the induction of a series of *p*-coumaroylamino acids by yeast ext elicitation in *E. distachya* cultures

strongly suggested that they might be produced as phytoalexins of *Ephedra*. Compound **1-3** had been once chemically synthesized in order to analyse the metabolites of human urine (Van Brussel et al., 1970). However, it is the first report that these compounds were isolated and identified from natural product.

ACKNOWLEDGEMENT

Dr. K.-S. Song would like to express special thanks to Dr. Seung-Jo Yoo, a professor emeritus of Sung-KyunKwan University, for his valuable advices. We also would like to cerebrate his glorious retire of the professor of College of Pharmacy, SungKyunKwan University.

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