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N-ACYLAMINO ACIDS FROM *EPHEDRA DISTACHYA* CULTURES

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Key Word Index—*Ephedra distachya*; Ephedraceae; suspension culture; *N*-acylamino acid; *Nα*-malonyl-L-tryptophan; *p*-coumaroylglycine; *p*-coumaroyl-D-alanine.

Abstract—Cell suspension cultures of *E. distachya* produced three acylamino acids. *Nα*-Malonyl-L-tryptophan was isolated along with 5′-*S*-methyl-5′-thioadenosine and stigmast-4-ene-3-one from normally cultured cells, while the production of *p*-coumaroylglycine, *p*-coumaroyl-D-alanine and cinnamic acid was induced by the addition of yeast extract to the early stationary phase of cultures.

INTRODUCTION

Several species belonging to the genus *Ephedra* are known to produce a series of clinically important *Ephedra* bases represented by 1-(−)ephedrine [1]. Induction of callus cultures of *E. gerardiana* and *E. foliata* and production of *Ephedra* bases in these cultures have been reported [2, 3]. The production of useful metabolites by callus cultures has been hampered by low yields of the target compounds and various approaches have been attempted in order to improve the yield of product. One promising approach is the use of phytoalexin elicitors to induce enzyme(s) of secondary metabolism [4]. Successful induction of biosynthetic enzymes by yeast extract or active glucan preparation of yeast in cell culture systems [5, 6] led us to test this approach for the production of alkaloids in the cell suspension cultures of *E. distachya*, though the host–pathogen interaction in gymnospermous plants has rarely been documented [7, 8].

RESULTS AND DISCUSSION

Ephedra distachya callus was induced from the aerial part of plant on solid Murashige–Skoog's (MS) medium [9] (see Experimental). Induced callus was transferred to the MS liquid medium. The growth of cell (fr. wt) in liquid medium reached a maximum at *ca* 30 days after transfer. Cells harvested at 32 days were extracted with 70% aqueous acetone. After evaporation of solvent, the extract was partitioned between ethyl acetate and 0.1 M HCl to separate basic and non-basic fractions. HPLC analysis of the basic fraction with RP column revealed the presence of ephedrine and pseudoephedrine [10–50 ng g^{-1} fr. wt; details will be reported elsewhere]. Presence of UV absorbing compounds was noted in the non-basic fraction and they were separated by silica gel and preparative HPLC to isolate three compounds. Two of them were readily identified as 5′-*S*-methyl-5′-thioadenosine and stigmast-4-ene-3-one by comparison of their UV, ^{1}H and ^{13}C NMR, and EIMS data with those reported in the literature [10, 11] and with standard material. The third compound (**1**) was Ehrlich's reagent [12] positive and its UV spectrum showed a typical indole absorption at 270–290 nm. The presence of the tryptophan moiety was verified by ^{1}H NMR. In the ^{13}C NMR spectrum, three additional resonances appeared at δ169.2 (*s*), 42.2 (*t*), 165.7 (*s*), indicating the presence of a malonyl residue in its structure. The amide stretching band at 1695 cm^{-1} in IR and coupling (J = 8.0 Hz) between α-NH (δ8.26) and α-CH (δ4.53) established its structure to be *Nα*-malonyltryptophan (MT). The D-isomer of MT has been isolated from more than 12 species [13, 14], however, its absolute configuration was proved by an indirect method [15, 16] and so far no optical property has been described. Thus L-MT was synthesized starting from L-Trp by a reported method [17]. All spectral data of MT (**1**) isolated from cell cultures were identical to those of synthetic L-MT, including specific rotation value. Malonyl conjugates of D-amino acids have been suggested as possible detoxication metabolites of exogenously added D-amino acids [18, 19]. Some reports suggested that the D-MT is a precursor of IAA biosynthesis or a possible storage form of IAA [20, 21]. Although L-MT has never been found in plants, synthetic L-MT was reported to be converted into indole-3-acetaldoxime, a possible precursor of IAA, in seedlings of Chinese cabbage [16]. It is of interest that L-MT was isolated for the first time from gymnospermous plant cell culture at 30–40 μg g^{-1} fr. wt.

To examine the effect of yeast extract on *E. distachya* cell cultures, commercial yeast extract up to 500 mg per 100 ml medium was added to the early stationary phase of cultures and further cultured for 48 hr. Harvested cells were extracted with MeOH and analysed by HPLC. Compared with the control, no significant increase of ephedrine nor pseudoephedrine production was noted for basic fraction of yeast extract added cells. Medium was also analysed by HPLC but no large changes were observed (data not shown). However, four new peaks (2–5) appeared on the chromatogram of non-basic fraction of elicited cells (Fig. 1). Production of three compounds (**2–4**) increased linearly to the amount of yeast extracted up to 500 mg per flask. Subsequent chromatographic separation afforded compounds **2–4**. Compound **5** which was found in yeast extract itself was

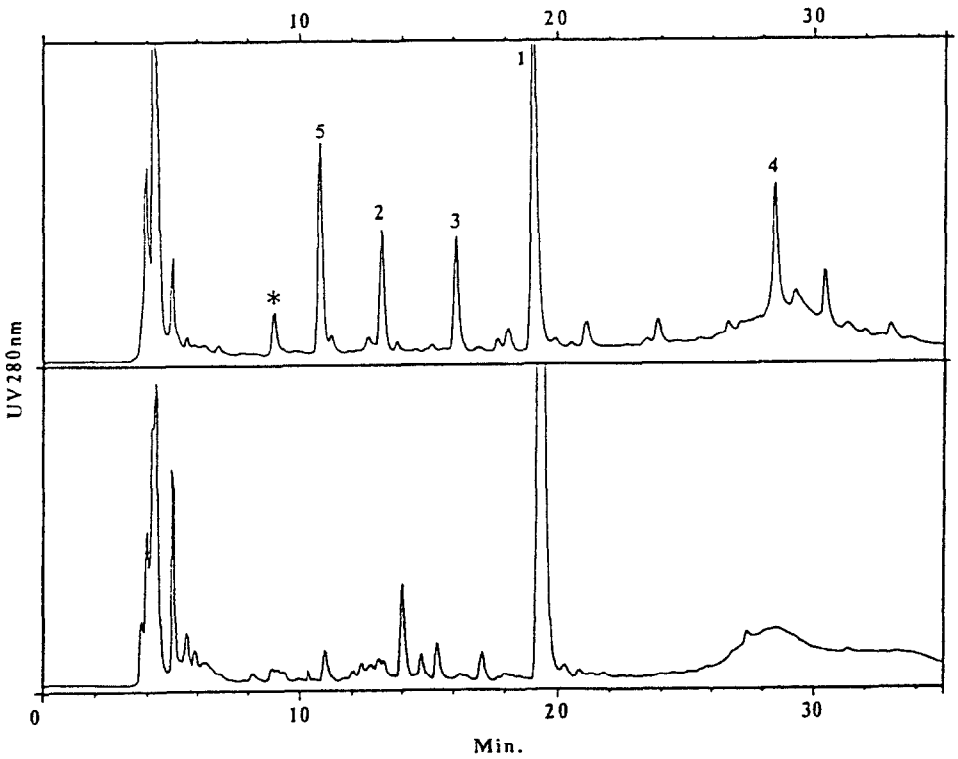
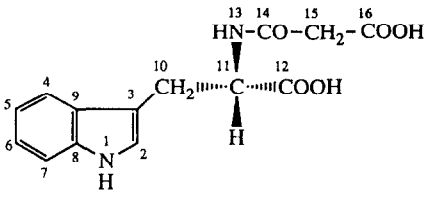


Fig. 1. Chromatogram of normally cultured *E. distachya* cell (lower trace) and yeast extract elicited cell (upper trace); see Experimental. Each peak number corresponds to its compound number. * 5′-*S*-methyl-5′-thioadenosine.

identified as Trp from its R_f on the chromatogram. Compound **4** was readily identified as cinnamic acid from its spectral data. The ^1H and ^{13}C NMR spectra of **2** and **3** indicated the presence of *p*-coumaroyl moiety in both of these compounds. Amide stretching band at 1695 cm^{-1} in IR and carbon resonances at δ171.2 (*s*) and 40.7 (*t*) of **2** together with its molecular ion peak at *m/z* 221 in the EI mass spectrum suggested it to be *p*-coumaroylglycine. The IR, UV, and ^1H NMR spectra of **3** were similar to those of **2** except for the presence of an additional doublet methyl signal (δ1.31, $J = 7.2$ Hz) in the ^1H NMR which corresponded to the carbon resonance at δ17.3 (*q*). All the above data and a molecular ion peak at *m/z* 235 in the EI mass spectrum established its structure as *p*-coumaroylalanine. Structures of **2** and **3** were verified by chemical synthesis of authentic compounds [17]. The amount of natural **3** was too small to measure the specific rotation; however, its CD spectrum was an exact mirror image of that of synthetic **3** prepared from L-Ala. HPLC analysis using an enantiomer-discriminating column showed that natural **3** is the optically pure D-isomer. The glycine conjugate of *p*-coumaric acid has been identified in human urine [22] and the synthetic product was once tested for its inhibitory effect against melanin biosynthesis in human and guinea-pig [23]. This is the first time that the production of **2** and **3** has been confirmed in a plant. *N*-Acylamino acids such as *N*-benzoyl-L-leucine and *N*-phenylacetyl-L-leucine were reported to inhibit the growth of several phytopathogens [24]. These facts and the induction of both **2** and **3** (3–4 $\mu g\ g^{-1}$ fr. wt) by yeast extract addition to the culture under optimal conditions



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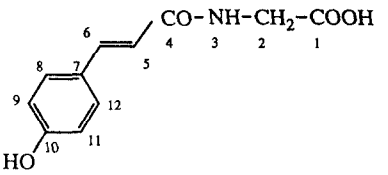

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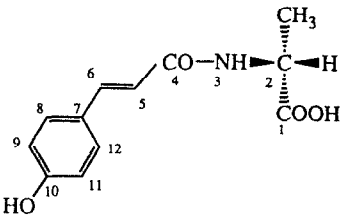

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suggest that they might be produced as phytoalexins in intact *E. distachya* plants.

EXPERIMENTAL

General. Mps: uncorr. IR were measured in KBr disks. EIMS at 70 eV. ^1H and ^{13}C NMR were recorded in DMSO-d_6, 40° on 400 MHz and 100 MHz, respectively. Chemical shifts are given in δ (ppm) from TMS.

Tissue culture. The callus was induced by placing sterile explant (5–10 mm long) derived from the aerial part of *E. distachya* on the solid Murashige–Skoog's medium, containing 30 g sucrose, 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg kinetin l^{-1}. The callus was transferred to MS liquid medium (100 ml per 500 ml flask) containing the same elements as solid medium, and successively cultured at 28–29°, 100 rpm in the dark.

Addition of yeast extract and HPLC analysis. Yeast extract (Bacto Difcolab) was suspended in H_2O (100 mg ml^{-1}) and autoclaved. Autoclaved yeast extract soln (5 ml per100 ml medium) was added to 28-day-old cell cultures which were subsequently cultured for 48 hr. A control was made by adding sterile H_2O (5 ml per100 ml medium) instead of yeast extract to the cell cultures. After filtration, 10 g each fresh cells was suspended in 40 ml MeOH and extracted by sonication for 20 min. An aliquot (10 ml) of supernatant was taken out and evapd under red. pres. To this extract, 2 ml MeOH was added, followed by ODS-Pak filtration, and 10 μl of each filtrate was injected for HPLC analysis. Analysis conditions were as follows: column; TOSOH ODS-120T (6.0 mm i.d. × 150 mm), mobile phase; MeCN and 2% HOAc, programmed gradient elution (0 min, 10%; 20 min, 30%; 30 min, 80%; 35 min, 10% MeCN), flow rate; 0.8 ml min^{-1}, detection; UV 280 nm. EtOAc soluble basic fr. (20 mg) was suspended in 40 ml 0.1 M HCl and filtered through ODS-Pak, then 10–50 μl filtrate was injected. HPLC analysis of the basic fr. was carried out on the same column and flow rate as above. The other conditions were as follows: mobile phase; 0.2 M NaH_2PO_4 (pH 3)–MeOH (9:1), detection; UV 210 nm. R_t: ephedrine (23.43 min), pseudoephedrine (26.30 min). TSKgel Enantio L1 (4.6 mm i.d. × 250 mm) column was used for the analysis of enantiomeric purity of natural *p*-coumaroyl-alanine. Mobile phase, 0.25 mM $CuSO_4$; column temp., 50°; flow rate, 0.5 ml min^{-1}; detection, UV 280 nm. R_t: 14.27 min (L-isomer, R_t: 14.70 min).

Extraction and purification. Normally cultured cells (1230 g fr. wt) were extracted with 70% Me_2CO and separated to EtOAc soluble non-basic (1470 mg) and basic fr. (170 mg) according to routine method. A portion of EtOAc soluble non-basic fr. (340 mg) was applied on silica gel [Wakogel C-200, $CHCl_3$–MeOH (50:1 to 3:1)]. Further purification by prep. HPLC (RP-18, Merck Linchroprep., 95% MeOH or 50% MeOH) gave stigmast-4-ene-3-one and 5′-*S*-methyl-5′- thioadenosine, respectively (less than 5 mg each). The rest of EtOAc soluble non-basic fr. (1130 mg) was applied on silica gel [Merck Art 9385, $CHCl_3$–MeOH–HCO_2H–H_2O (60:7:3:1)]. Ten frs (I–X) were collected and cinnamic acid was isolated by rechromatography [Wakogel C-200, C_6H_6–EtOAc–HCO_2H (50:8:4)] of fr. I. Prep. HPLC [RP-18, Merck Linchroprep., MeCN–H_2O–HOAc (50:147:3)] of fr. X afforded compound **1**. Compounds **2** and **3** were purified from the yeast extract elicited cells by exactly the same procedures as those of **1**.

Synthesis of compounds **1–3**. *N,N*-Dicyclohexylcarbodiimide (16 mmol) was added to a cooled solution of *p*-coumaric acid or malonic acid (15 mmol each) and *N*-hydroxysuccinimide (16 mmol) in freshly dist. dioxane (22.5 ml). The reaction mixture was stirred for 5 hr at room temp. The insoluble material was filtered off and washed with cold dioxane. The filtrate was added to a soln of amino acids (17 mmol each) and $NaHCO_3$ (17 mmol) in H_2O (24 ml) and the reaction mixture stirred at room temp. for 24 hr. Dioxane was evapd and the aq. layer extracted with EtOAc (30 ml, × 3), the residual aq. layer was acidified with conc. HCl (pH 2) and the mixture allowed to cool. The ppt. formed was filtered and the products were purified by recrystallization from appropriate solvents.

N-*Malonyl-L-tryptophan* (**1**). Obtained as a powder, mp 82°, $[\alpha]_D^{20}$ 47.4° (MeOH; *c* 1.0); UV λ_{max}^{MeOH} nm (log ε): 275.5 (3.76), 282 (3.79), 290 (3.72); IRν_{max} cm^{-1}: 3400, 1720, 1695; EIMS *m/z* (rel. int.): 246 (8.87) $[M-44]^+$, 180 (100) [quinolinium]$^+$; ^1H NMR δ10.77 (1H, *br s*, H-1), 8.26 (1H, *d*, J = 8.0 Hz, α-NH), 7.52 (1H, *br d*, J = 8.0 Hz, H-4), 7.33 (1H, *br d*, J = 8.0 Hz, H-7), 7.14 (1H, *d*, J = 2.4 Hz, H-2), 7.06 (1H, *ddd*, $J_{6,7}$ = 8.0 Hz, $J_{6,5}$ = 7.6 Hz, $J_{6,4}$ = 0.8 Hz, H-6), 6.97 (1H, *ddd*, $J_{4,5}$ = 8.0 Hz, $J_{5,6}$ = 7.6 Hz, $J_{5,7}$ = 0.8 Hz, H-5), 4.53 (1H, *ddd*, $J_{11,13}$ = 8.0 Hz, $J_{11,10a}$ = 5.2 Hz, $J_{11,10b}$ = 7.2 Hz, H-11), 3.19 (2H, *s*, H-15), 3.17 (1H, *dd*, $J_{10a,10b}$ = 14.8 Hz, $J_{10a,11}$ = 5.2 Hz, H-10a), 3.06 (1H, *dd*, $J_{10a,10b}$ = 14.8 Hz, $J_{10b,11}$ = 7.2 Hz, H-10b); ^{13}C NMR δ172.9 (*s*, C-12), 169.2 (*s*, C-16), 165.7 (*s*, C-14), 136.0 (*s*, C-8), 127.2 (*s*, C-9), 123.5 (*d*, C-2),120.8 (*d*, C-6), 118.2 (*d*, C-5), 118.1 (*d*, C-4), 111.2 (*d*, C-7), 109.5 (*s*, C-3), 53.0 (*d*, C-11), 42.2 (*t*, C-15), 27.1 (*t*, C-10).

p-*Coumaroylglycine* (**2**) *and* p-*coumaroyl-D-alanine* (**3**). Compound **2** was obtained as platelets from aq. EtOH, mp 232–233°; elemental analysis (Found: C, 59.44; H, 5.06; N, 6.14. $C_{11}H_{11}O_4N$ requires: C, 59.73, H, 5.01; N, 6.33%); UV λ_{max}^{MeOH} nm (log ε): 226 (4.10), 294 (4.33), 301 (4.33), 309 (4.34); IR ν_{max} cm^{-1}: 3400, 1730, 1695; EIMS *m/z* (rel. int.): 221 (7.21) $[M]^+$, 147 (100) $[M-74]^+$; ^1H NMR δ8.15 (1H, *t*, J = 5.6 Hz, H-3), 7.40 (2H, *d*, J = 8.4Hz, H-8, H-12), 7.34 (1H, *d*, J = 16.0Hz, H-6), 6.79 (2H, *d*, J = 8.4 Hz, H-9, H-11), 6.49 (1H, *d*, J = 16.0 Hz, H-5), 3.85 (2H, *d*, J = 5.6 Hz, H-2); ^{13}C NMR δ171.2 (*s*, C-1), 165.6 (*s*, C-4), 158.8 (*s*, C-10), 139.1 (*d*, C-6), 129.1 (*d*, C-8, C-12), 125.7 (*s*, C-7), 118.1 (*d*, C-5), 115.6 (*d*, C-9, C-11), 40.7 (*t*, C-2). Compound **3** was crystallized as platelets from C_6H_6–HOAc, mp 206–207°; elemental analysis (Found: C, 61.18; H, 5.39; N, 5.91. $C_{12}H_{13}O_4N$ requires: C, 61.27; H, 5.57; N, 5.95%); $[\alpha]_D^{20}$ −34.0° (MeOH; *c* 1.0, by synthetic one); UV λ_{max}^{MeOH} nm (log ε): 227 (3.91), 295 (4.20), 301 (4.20), 310 (4.20); IR ν_{max} cm^{-1}: 3400, 1720, 1695; EIMS *m/z* (rel. int.) 235 (6.4) $[M]^+$, 147 (100) $[M-88]^+$; ^1H NMR δ8.16 (1H, *d*, J = 7.2 Hz, H-4), 7.39 (2H, *d*, J = 8.4 Hz, H-8, H-12), 7.35 (1H, *d*, J = 16.0 Hz, H-7), 6.79 (2H, *d*, J = 8.4 Hz, H-9, H-11), 6.48 (1H, *d*, J = 16.0 Hz, H-6), 4.32 (1H, *quin*, J = 7.2 Hz, H-2), 1.31 (3H, *d*, J = 7.2 Hz, Me); ^{13}C NMR δ174.0 (*s*, C-1), 164.9 (*s*, C-4), 158.8 (*s*, C-10), 139.0 (*d*, C-6), 129.1 (*d*, C-8, C-12), 125.8 (*s*, C-7), 118.2 (*d*, C-5), 115.6 (*d*, C-9, C-11), 47.5 (*d*, C-2), 17.3 (*q*, Me).

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