

A Novel Benzoic Acid Derivative from Yeast-Elicited *Ephedra distachya* Cultures

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Plants accumulate low molecular weight antibiotic substances against phytopathogen as a result of plant-defense mechanisms (Müller et al., 1940). They are called as phytoalexins and the substances which stimulate the accumulation of phytoalexins are termed as elicitors (Brooks et al., 1984). Phytoalexins have been reported in many plant families and most of them are concentrated on Angiosperms but rarely on Gymnosperms (Dixon et al., 1986). The model study using suspension cultures of *E. distachya*, one of a medicinal Gymnospermous plants, had been carried out in order to investigate the plant pathogen interaction mechanisms of this plant. It was found that *E. distachya* cultures produced a series of *p*-coumaroylamino acids by the elicitation with yeast extract (Song et al., 1992, 1994d). The produced *p*-coumaroylamino acids were supposed to be phytoalexins of the *Ephedra* (Song et al., 1992, 1994a, b, c). Besides the *p*-coumaroylamino acid peaks, an unidentified peak was detected on HPLC from the yeast-elicited *E. distachya* cultures. Thus, the isolation and structure determination of the compound were carried out as one of our serial works on 'plant-pathogen interaction mechanisms in *E. distachya*' (Song et al., 1992, 1994a, b, c, d).

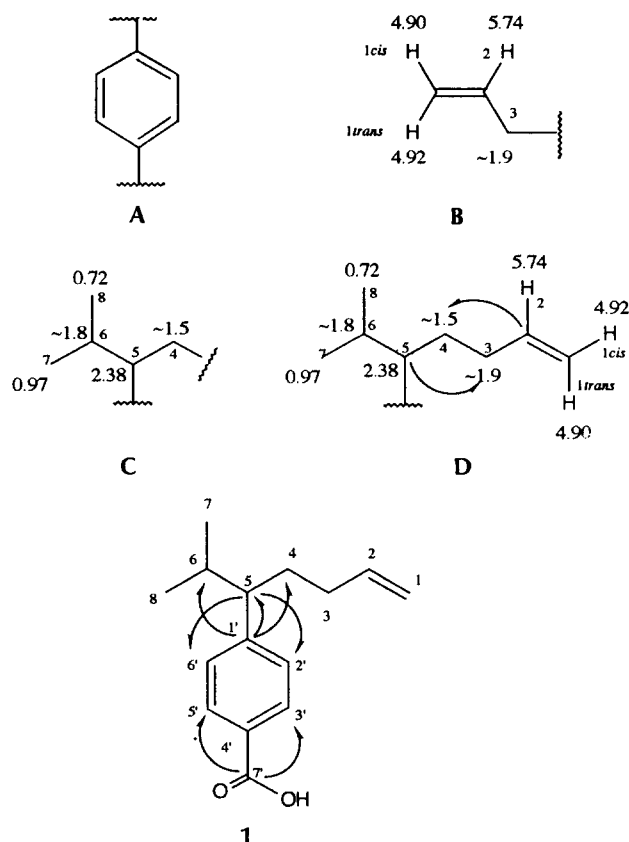
The suspension culture of *E. distachya* was performed according to the method described before (Song et al., 1992). 500 mg yeast ext was added to the 27-28 day old cultures and subsequently cultured for 48 hr. 60 g fr. wt. yeast-elicited *E. distachya* cultures were

harvested and extracted with 4 L CH₃OH in water bath. The CH₃OH extract was suspended in 1 N NaHCO₃ and the solution was extracted with ethylacetate (EtOAc). After removal of the organic layer, the aq. solution was acidified with 0.1 N HCl and extracted with EtOAc. The EtOAc soluble acidic fr (1.5 g) was applied on XAD-2 column (40 mm×340 mm) and eluted with H₂O and acetone with gradual increase of acetone concentration up to 100%. Seven frs (Fr I-VII) were obtained and re-chromatography of Fr VI on silicagel [Merck Art. 9385, 25 mm×300 mm, gradient elution with CHCl₃ and CH₃OH. Elution was started with CHCl₃ and finally washed with CHCl₃-CH₃OH (10:1)] gave six frs (Fr VI-1 to VI-6). Fr VI-4 (14 mg) was applied to silicagel again [Merck Art. 9385, 10 mm×180 mm), C₆H₆-EtOAc-CH₃COOH (4:1:0.1)] to obtain three frs (Fr. VI-4-a to 4-c). Purification of Fr. VI-4-c (7 mg) using HPLC equipped with TOSOH ODS 80Tm (column size; 7.3 mm×300 mm, mobile phase; 2% CH₃COOH in 85% CH₃OH, flow rate; 2 ml min⁻¹, detection; UV 254 nm) afforded 1 (4 mg). HPLC was carried out by TOSOH SC8010 HPLC system.

The physico-chemical properties of compound 1 were as follows: UV λ_{max}^{MeOH} nm (log ε): 206 (3.79), 238 (3.82); IR ν_{max}^{KBr} cm⁻¹: 2800-3400 (OH), 1691 (a,b unsaturated C=O); EI-MS m/z (rel. int.): 232 (6.7) [M]⁺, 149 (87.0) [M-83]⁺, 135 (100) [M-97]⁺; FAB-MS (glycerol as matrix, positive): 233 (11.2) [M+1]⁺; ¹H NMR: δ 8.01 (2H, d, J=8.4 Hz, H-3', H-5'), 7.25 (2H, d, J=8.4 Hz, H-2', H-6'), 5.74 (1H, dddd, J_{1,2,trans}=16.4 Hz, J_{1,2,cis}=10.4 Hz, J_{2,3a}=7.2 Hz, J_{2,3b}=6.0 Hz, H-2), 4.92 (1H, d, J=10.4 Hz, H-1, cis to H-2), 4.90 (1H, d, J=16.4, H-1, trans to H-2), 2.38 (1H, ddd, J=11.0, 8.0, 3.2 Hz, H-5), ca 1.9 (2H, m, H-3), ca 1.8 (1H, m, H-6) ca 1.5 (2H, m, H-4), 0.97 (3H, d, J=6.4 Hz, H-7), 0.72 (3H, d, J=6.4 Hz, H-8); ¹³C NMR (125 MHz, CDCl₃): δ 171.95 (s, C-7'), 151.21 (s, C-1'), 138.54 (d, C-2), 130.09 (d x 2, C-3', C-5'), 128.72 (d x 2, C-2', C-6'), 127.10 (s, C-4'), 114.62 (t, C-1), 52.72 (d, C-5), 33.41 (d, C-6), 32.07 (t, C-3), 31.86 (t, C-4), 20.96 (q, C-7), 20.73 (q, C-8).

Compound 1 showed typical ¹H resonances of 4-substituted benzene at δ 8.01 ppm (2H, d, J=8.4 Hz) and 7.25 (2H, d, J=8.4 Hz) as presented in partial structure A. The proton signal at δ 5.74 ppm showed very complicated coupling pattern which was caused by *trans* coupling with δ 4.90 ppm proton (1H, d, J=16.4 Hz), *cis* coupling with δ 4.92 ppm proton (1H, d, J=10.4 Hz) and vicinal couplings with methylene protons about δ 1.9 ppm (2H, m). By the irradiation of δ 1.90 ppm, the spin-spin coupling pattern of δ 5.74 ppm proton (dddd) was changed to doublets of doublet

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($J=16.4, 10.4$ Hz). From these observations, the partial structure B was supposed. HMQC (heteronuclear multiple quantum coherence) and ^1H - ^1H COSY spectrum showed that two doublet methyl protons at δ 0.97 and 0.72 ppm were coupled with a ca δ 1.8 ppm multiplet methine proton, indicating the presence of iso-propyl group. In addition, the methine proton at ca δ 1.8 ppm showed correlation spot with the methine proton at δ 2.38 ppm (H-5), and H-5 with methylene protons about 1.5 ppm (H-4) in ^1H - ^1H COSY. Partial structure C was suggested from the above data. The H-4 methylene protons presented correlation spot with methylene proton about δ 1.9 ppm (H-3) in ^1H - ^1H COSY. HMBC (heteronuclear multiple bond connectivity) study showed that δ 52.72 ppm C-5 correlated with H-3 and δ 138.54 ppm C-2 with H-4. As a result, partial structure B and C could be connected as partial structure D, namely 5-substituted 6-methyl-1-heptene moiety.

^{13}C NMR and DEPT (distortionless enhancement by polarization transfer) spectrum indicated the presence of three singlet carbons including a carbonyl carbon, three methylene carbons, six methine carbons and two

methyl carbons. In the combination of ^{13}C NMR data with EI-MS m/z 232 $[\text{M}]^+$ and FAB-MS m/z 233 $[\text{M}+1]^+$, the molecular formula was decided to $\text{C}_{15}\text{H}_{20}\text{O}_2$. The molecular formula and the chemical shift in ^{13}C NMR (δ 171.95 ppm, s) indicated that the carbonyl carbon should be assigned to be that of carboxylic acid. Since partial structure D has only one methine carbon which should be bonded to another moiety, structure 1 was deduced. To confirm this structure, HMBC study was carried out. The singlet carbon at δ 151.21 ppm (C-1') exhibited correlation spots with H-5, H-6 and H-4. Moreover, the C-5 correlated with the δ 7.25 ppm proton (H-2', H-6'). The carbonyl carbon at δ 171.95 ppm also showed correlation spot with the δ 8.01 ppm protons (H-3', H-5'). From these spectral data, the chemical structure of this compound was elucidated as 6-methyl-1-heptene-5-yl-4'-benzoic acid (1). 1 was isolated and identified for the first time in this work. We named it as distachic acid. The biological activity of compound 1 is under investigating.

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