



Induction of clovamide by jasmonic acid in red clover

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Abstract

The effect of jasmonic acid (JA) on the secondary metabolism of 5-day-old red clover seedlings was investigated. Induction of the formation of four compounds was found in roots after treatment with 50 μ M JA for 48 h, while no induction was observed in the shoots. These compounds, whose formation was induced by JA addition, were isolated and identified as caffeoyl DOPA (clovamide), caffeoyltyrosine, *p*-coumaroyl DOPA and *p*-coumaroyltyrosine, by ion-spray MS and ¹H NMR analyses, and by chemical synthesis. Among them, clovamide was the most abundant, while the other amides represented only a minor portion. Clovamide started to increase in amount 24–36 h after treatment and reached a maximum after 96 h (2.81 nmol/mg fr. wt.). The induction of their formation was observed even with 5 μ M of JA, and the amount increased with concentrations up to 100 μ M. Treatment with 1 mM CuCl₂, which elicits accumulation of the phytoalexin maackiain in red clover, caused a decrease in clovamide amount. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Jasmonic acid (JA) and its methyl ester (MeJA) are widely distributed in the plant kingdom. It has been suggested that JA acts as a signal molecule in response to various environmental stimuli including wounding and infection by pathogens, on the basis of accumulation of JA under stress conditions, and activation of stress-related genes and enzymes after exogenous JA/MeJA treatments (Sembdner and Parthier, 1993; Creelman and Mullet, 1997). Treatments with JA/MeJA also affect secondary metabolism in plants. Gundlach et al. (1992) described induction of formation of secondary metabolites by treatment with MeJA in cell suspension cultures of 36 species of mono- and dicotyledonous plants. JA strongly elicits the production of

rice phytoalexins, oryzalexins, momilactones, and sakuranetin, in leaves and cell cultures of the plant (Nojiri et al., 1996; Kodama, 1996). More recently, accumulation of a hydroxycinnamic acid amide, *p*-coumaroylagmatine, a precursor of antifungal hordatine compounds, was reported in barley leaves following MeJA treatment (Lee et al., 1997).

In red clover (*Trifolium pratense* L.), a number of secondary metabolites have been identified. Among them, maackiain has been characterized as a pterocarpoid phytoalexin (Higgins and Smith, 1972), and its glucoside and malonyl glucoside occur as constitutive components. Isoflavonoids such as formononetin and biochanin A have been detected in both free and conjugated forms, and formononetin has been regarded as a precursor of maackiain (Dewick, 1975, 1977; Dewick and Ward, 1978). Another class of characteristic secondary metabolites in red clover include the hydroxycinnamic acid amides conjugated to aromatic amino acids. For example, caffeoyl DOPA (clovamide) has been isolated (Yoshihara et al., 1977), although no bio-

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logical function was elucidated. In this study, we investigated the effect of JA on secondary metabolism in red clover, including biosynthesis of pterocarpanoids, isoflavonoids and hydroxycinnamic acid amides, in order to better understand the role of JA in the defensive systems of red clover.

2. Results

Effects of JA on the composition of secondary metabolites in red clover (*Trifolium pratense* L.) were examined using 5-day-old seedlings. Shoots and roots were separately extracted with MeOH containing 2% HOAc, and the extracts were analyzed by reversed phase HPLC (Fig. 1). In the extracts from roots treated with 50 μ M JA for 48 h after wounding, increases in the size of three peaks at R_t 3.95 min (**1**), R_t 5.20 (**2**+**3**) and R_t 7.35 (**4**) were observed. Neither wounding alone nor JA treatment on intact roots caused any changes. In intact shoots, these peaks were found in a

larger amount than in the roots, although the over all magnitude was little affected by treatment with JA. Compounds **1** and **4** were isolated from 5-day-old seedlings by three chromatographic steps, and **2** and **3** were purified as a mixture in the same manner. The structures of the compounds were identified by use of ion spray-MS and ^1H NMR spectroscopy. Pseudomolecular ions, $[\text{M}+\text{H}]^+$, in positive ion spray-MS spectra, revealed the molecular weights of compounds **1**–**4** to be m/z 359, 343, 343 and 327, respectively. Compounds **1** and **2** gave a diagnostic ion at m/z 163, whereas **3** and **4** gave an ion at m/z 147, suggesting that **1** and **2** have a caffeoyl moiety, and that **3** and **4** have a *p*-coumaroyl moiety, respectively. The presence of a caffeoyl moiety in **1** and **2**, and a *p*-coumaroyl moiety in **3** and **4**, was confirmed by ^1H NMR spectroscopy. In the ^1H NMR spectra of **1** and **2**, the presence of a *trans* double bond and a 1,3,4-trisubstituted benzene ring was assigned to the caffeoyl moiety, whereas, in **3** and **4**, the *trans* double bond and a 1,4-disubstituted benzene ring were assigned to the *p*-coumaroyl moiety. The rest of the signals in the ^1H NMR spectra of **1** and **3**, including methine, methylene and 1,3,4-trisubstituted benzene ring resonances, indicated a 3,4-dihydroxyphenylalanine (DOPA) structure; in an analogous manner, methine, methylene and 1,4-disubstituted benzene ring resonances in the ^1H NMR spectra of **2** and **4** revealed the presence of a tyrosine moiety. The chemical shift of the proton connected to the nitrogen atom established an amide linkage of the hydroxycinnamic acids and amino acids in each case. From these data, **1**–**4** were identified as hydroxycinnamic acid amides, caffeoyl DOPA (clovamide), caffeoyltyrosine, *p*-coumaroyl DOPA and *p*-coumaroyltyrosine, respectively. Finally, the identity of each amide was established by chemical synthesis from

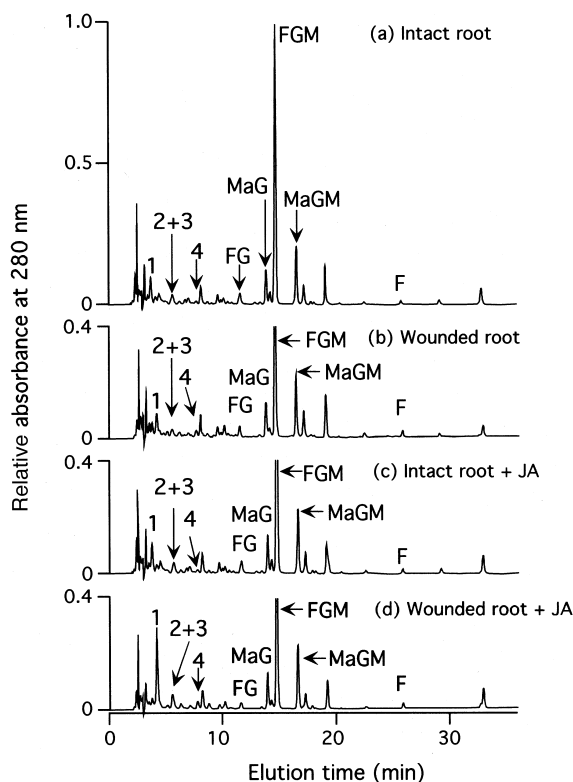


Fig. 1. HPLC chromatograms of the extracts from intact roots (a), wounded roots (b), intact roots treated with 50 μ M JA (c) and wounded roots treated with 50 μ M JA (d). **1**: caffeoyl DOPA (clovamide), **2**: caffeoyltyrosine, **3**: *p*-coumaroyl DOPA, **4**: *p*-coumaroyltyrosine, MaG: maackiain 7-*O*-glucoside, MaGM: maackiain 7-*O*-glucoside-6''-malonate, F: formononetin, FG: formononetin 7-*O*-glucoside, FGM: formononetin 7-*O*-glucoside-6''-malonate.

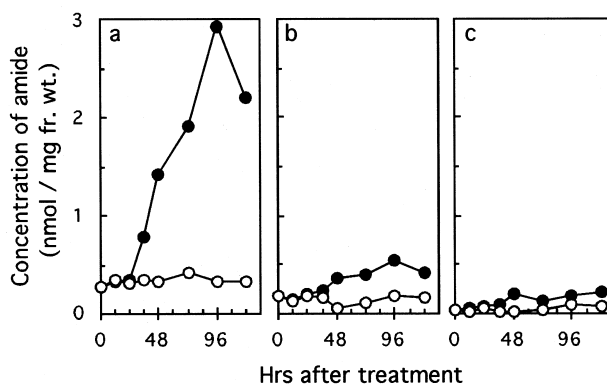


Fig. 2. Time course of the accumulation of amide compounds in wounded roots treated with 50 μ M JA (●) and untreated (○). (a): caffeoyl DOPA (clovamide) (**1**), (b): caffeoyltyrosine (**2**) plus *p*-coumaroyl DOPA (**3**), (c): *p*-coumaroyltyrosine (**4**). Each data point expresses the mean of four experiments.

the corresponding hydroxycinnamic acids and amino acids using dicyclohexylcarbodiimide.

The effects of JA were also investigated as regards effects on the amounts of maackiain, maackiain 7-*O*-glucoside, maackiain 7-*O*-glucoside-6"-malonate, formononetin, formononetin 7-*O*-glucoside and formononetin 7-*O*-glucoside-6"-malonate (Fig. 1). Maackiain was not detected in either JA-treated or untreated seedlings. Although other compounds were found in varying amounts, the changes in relative amounts were minor as an effect of JA treatment.

Changes in the amounts of amides following treatment with 50 μM JA were next investigated (Fig. 2). The amount of **1** started to increase after a 24-h lag period and reached a maximum 96 h post-treatment (2.81 nmol/mg fr. wt.), being ca. 10 times greater than in control roots. Thereafter, compound **1** decreased to 80% of the maximum after 120 h. Compounds **2** and **3** were quantified as a mixture, where their over all accumulation varied in a fashion similar to **1**, although the amount (0.54 nmol/mg fr. wt. as mixture) was approximately one-fifth that of **1** at 96 h. The level of compound **4** also increased slightly after JA-treatment (0.20 nmol/mg fr. wt. at 48 h), and remained at a constant level thereafter up to 120 h. On the other hand, the amounts of **1**–**4** in roots wounded, but not treated with JA, did not change throughout the time period examined.

The dependency of the induced formation of hydroxycinnamic acid amides was next investigated, as a

function of various JA concentrations (Fig. 3). The amount of each compound was determined 48 h after treatment. The induction of **1** was observed even with 5 μM JA, and the amount increased with JA concentration up to 100 μM (1.20 nmol/mg fr. wt.). At 500 μM , the amount of **1** decreased to a level as low as that in the control roots. The dose-response curves for other amides were similar to that for **1**, though the amounts were small.

Various substances were finally tested for induction of accumulation of clovamide and maackiain. In this regard, JA addition resulted in the increased accumulation of **1** but not maackiain, whereas hexa-*N*-acetylchitohexaose, chitosan, laminarin, and salicylic acid were all ineffective on increasing the levels of both **1** and maackiain. Copper chloride, on the other hand, caused the decrease of **1** to an undetectable level at 1 mM, although it elicited the increased production of maackiain (0.57 nmol/mg fr. wt.).

3. Discussion

In the present study, it was demonstrated that accumulation of clovamide congeners (**1**–**4**) is increased by JA treatment in roots of red clover. Compound **1** has been identified as a constitutive component (Yoshihara et al., 1977), but for **2**–**4** this is the first report of their occurrence in this plant. Hydroxycinnamic acids linked to an amine function are common types of hydroxycinnamic acid conjugates (Strack, 1997), but amides with aromatic amino acids have been found in a relatively few plants (Van Heerden et al., 1980; Clifford et al., 1988). Hydroxycinnamic acid amides have been implicated in inducible defense systems of plants. Avenanthramides, a series of substituted *N*-cinnamoylanthranilates, have been characterized as oat phytoalexins (Mayama et al., 1981; Miyagawa et al., 1995). In Solanaceous plants, such as potato (Clarke, 1982), tobacco (Negrel and Martin, 1984) and tomato (Pearce et al., 1998), the production of *N*-hydroxycinnamoyltyramine is activated in response to various environmental stimuli. Moreover, increased accumulation of *p*-coumaroyl- and feruloylagmatines, precursors of the antifungal compounds known as hordatines, was demonstrated after fungal infection in barley (Peipp et al., 1997). Interestingly, their increased accumulation was reproduced by treatments with JA (Lee et al., 1997), suggesting the involvement of MeJA in their inducible defense systems. This situation is very similar to our findings of the induced levels of clovamide by JA-treatment, regarding the chemical structure of the induced compounds and the activity of JA. It is thus presumed that the induction of clovamide accumulation is a part of the root defense system of red clo-

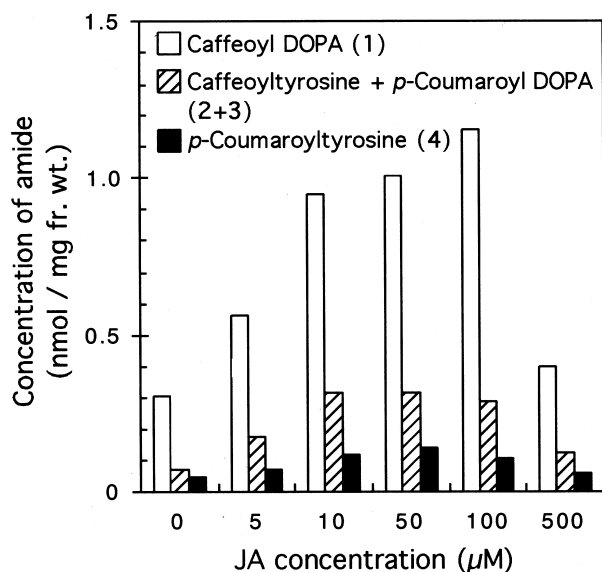
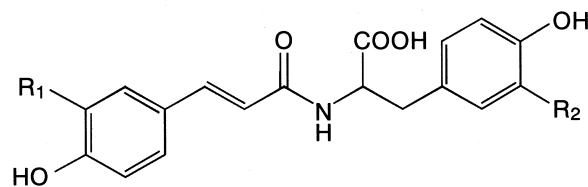


Fig. 3. Effects of the concentration of JA on the accumulation of amides in red clover roots. Seedlings were treated with JA at various concentrations for 48 h. Each data point expresses the mean of five experiments.

ver, in a manner analogous to hydroxycinnamic acid amides in other plants.

Diverse stimuli are known to induce formation of hydroxycinnamic acid amides in various plants; these include wounding (Negrel et al., 1993), chitoooligosaccharides (Bordin et al., 1991), laminarin (Miyagawa et al., 1998), and heavy metal ions (Fink et al., 1990). In red clover, wounding alone did not increase the levels of **1**, although wounding seemed to be necessary for induced formation of **1** by JA. Furthermore, the levels of **1** were not increased by the above elicitors, indicating that the stimulus which triggers the production of amide compounds is species-dependent. It is of particular interest that CuCl_2 , which elicited accumulation of maackiain, caused a decrease in amounts of **1**, and no increased levels of maackiain occurred following JA treatment. A signaling role for JA in phytoalexin induction has been proposed in opium poppy, lettuce and rice (Gundlach et al., 1992; Nojiri et al., 1996; Kodama, 1996). However, our findings suggest that the stimulation of amide production by JA is independent from the induction of formation of the pterocarpoid phytoalexins in red clover. The mechanism leading to the decrease of **1** after CuCl_2 treatment is unclear. The oxidative polymerization of **1** seems to be a possible explanation, because **1** could be in contact with oxidative enzymes, which were localized to different compartments, under conditions where the integrity of the membranes was lost by the toxic effects of CuCl_2 (Doncheva, 1998).

Some of the hydroxycinnamic acid amides, such as avenanthramides (Mayama et al., 1981) and *p*-coumaroylagmatine (Stoessl and Unwin, 1970), display toxic effects against pathogens. However, in our preliminary experiments, **1–4** exhibited little antifungal activity against *Curvularia trifolii*, *Biopolaris oryzae*, and *Colletotrichum lagenarium* up to 0.5 mM (data not shown). Accordingly, compounds **1–4** may serve as substrates for reinforcement of cell walls via formation of phenolic polymers. Except for the minor component **4**, they have catechol groups that are easily oxidized by either enzymatic or chemical reactions. Peroxidases are putative enzymes responsible for the incorporation of amides into cell walls, whose activities are reportedly induced by JA treatment (Moons et al., 1997; Curtis et al., 1997). Incorporation of hydroxycinnamic acid amides into cell walls has also been reported in tobacco (Negrel and Lherminier, 1987), potato (Keller et al., 1996) and opium poppy (Facchini, 1998). In light of these facts, the incorporation of clovamide and related compounds into cell wall components is under investigation, as well as the spatial and temporal correlation between oxidative enzyme activity and amide production.



- 1: $R_1 = \text{OH}, R_2 = \text{OH}$ (Caffeoyl DOPA = clovamide)
- 2: $R_1 = \text{OH}, R_2 = \text{H}$ (Caffeoyltyrosine)
- 3: $R_1 = \text{H}, R_2 = \text{OH}$ (*p*-Coumaroyl DOPA)
- 4: $R_1 = \text{H}, R_2 = \text{H}$ (*p*-Coumaroyltyrosine)

4. Experimental

4.1. General

^1H NMR spectra were recorded on a Bruker AC-300 spectrometer in $\text{DMSO}-d_6$ solution using TMS as an internal standard. Positive ion-spray ionization mass spectra were obtained using Perkin-Elmer-Sciex API-165 instrument (ion-spray voltage: 5 kV, orifice voltage: 30 V, nebulizer gas: air, curtain gas: nitrogen) combined with a Shimadzu 10A HPLC system equipped with an ODS column (Wakosil-II 5C18 HG, 150 mm $\times$ 4.6 mm). The following HPLC conditions were used: linear gradient: 0–65% B/A for 35 min (solvent A: H_2O –TFA (999:1, v/v), solvent B: MeCN); flow rate: 0.8 ml/min.

4.2. Plant material and analysis of secondary metabolites

Red clover seeds (*Trifolium pratense* L. cv. Kenland, Yukijirushi Shubyo, Japan) were sown on moistened germination paper and maintained at 25° with a 12-h period of illumination from fluorescent lamps (60 W m^{-2}) in growth chambers. After 120-h incubation, the roots of red clover seedlings were wounded using carborundum. The seedlings were immediately placed on a 24-well tissue culture plate (10 mm $\times$ 16 mm) containing 0.5 ml of aqueous JA (Wako, Japan) solution per well. After incubation at 25° for 48 h, the seedlings were separated into roots and shoots, and extracted with 0.5 ml of HOAc–MeOH (2:98, v/v) under ultrasonication. The extracts were subjected to reversed phase HPLC analysis (column: Wakosil-II 5C18 HG, 150 mm $\times$ 4.6 mm, Wako, Japan). The following HPLC conditions were used: linear gradient: 20–65% B/A for 35 min (solvent A: HOAc– H_2O (3:97, v/v), solvent B: MeCN); flow rate: 0.8 ml/min; detection: 280 nm.

4.3. Purification of hydroxycinnamic acid amides

Red clover seedlings (83.6 g, 5-day-old) were extracted three times with 300 ml of HOAc–MeOH

(2:98, v/v) under ultrasonication. The combined extract, after concentration to ca. 100 ml, was washed with hexane and fractionated with an ODS column (Cosmosil 75C₁₈-OPN, 50 g, 81 mm × 40 mm, Nacalai, Japan) by eluting with H₂O and MeOH (500 ml each). The MeOH fraction which contained hydroxycinnamic acid amides was fractionated on a polyamide column (Polyamide C-200, 30 g, 91 mm × 40 mm, Wako, Japan) by eluting with 500 ml each of H₂O, H₂O–MeOH (1:1, v/v), MeOH and HOAc–MeOH (3:17), respectively. Finally, amides were purified from the HOAc–MeOH (3:17) fraction by reversed phase preparative HPLC (column: Wakosil-II 5C₁₈ HG, 250 mm × 20 mm). The following HPLC conditions were used: linear gradient: 0–40% B/A for 15 min (solvent A: HOAc–H₂O (3:97, v/v), solvent B: MeCN); flow rate: 10 ml/min; detection: 280 nm. Because compounds **2** and **3** were eluted together, they were analyzed as a mixture.

Compound **1** (11.2 mg): ion-spray MS, m/z (rel. int.): 360 [M+H]⁺ (49), 163 (100); ¹H NMR spectral data (300 MHz, DMSO-*d*₆): δ 2.73 (1H, *dd*, J = 13.9, 9.2 Hz), 2.92 (1H, *dd*, J = 13.9, 4.8 Hz), 4.44 (1H, *ddd*, J = 9.2, 8.0, 4.8 Hz), 6.41 (1H, *d*, J = 15.7 Hz), 6.50 (1H, *dd*, J = 8.1, 1.8 Hz), 6.62 (1H, *d*, J = 8.20 Hz), 6.63 (1H, *s*), 6.76 (1H, *d*, J = 8.1 Hz), 6.86 (1H, *dd*, J = 8.2, 1.8 Hz), 6.96 (1H, *d*, J = 1.8 Hz), 7.21 (1H, *d*, J = 15.7 Hz), 8.21 (1H, *d*, J = 7.2 Hz), 8.7–9.4 (4H, *br m*).

Compounds **2–4** were isolated and identified by comparison with authentic standards and comparison with literature data (Yoshihara et al., 1977; Van Heerden et al., 1980; Clifford et al., 1988).

4.4. Preparation of synthetic **1–4**

Synthesis of caffeoyl DOPA (**1**) was carried out by the method of Villegas and Brodelius (1990) with a slight modification. Caffeic acid (3.0 mmol), L-3,4-dihydroxyphenylalanine methyl ester hydrochloride (1.5 mmol) and dicyclohexylcarbodiimide (3.3 mmol) were dissolved in dry pyridine (10 ml) and the mixture was stirred at room temperature for 24 h. After evaporation of pyridine in vacuo, the residue was dissolved in MeOH (20 ml). The solution was cooled in an ice bath after which 2 M KOH (200 ml) was added. The mixture was stirred under N₂ at room temperature for 4 h and then neutralized with HOAc. The solvent was evaporated in vacuo and the residue was dissolved in MeOH (50 ml).

Dicyclohexylurea and salts were removed by filtration. After evaporation of solvent, the filtrate was fractionated using a polyamide column (Polyamide C-200) by eluting with H₂O–MeOH (3:1, v/v). Finally, caffeoyl DOPA was purified by reversed phase preparative HPLC (column: Wakosil-II 5C₁₈ HG, 250

mm × 20 mm) using H₂O–MeCN (77:23, v/v) as solvent (yield 33.4%).

Ion-spray MS, m/z (rel. int.): 360 [M+H]⁺ (52), 163 (100); ¹H NMR spectral data (300 MHz, DMSO-*d*₆): δ 2.72 (1H, *dd*, J = 13.9, 9.2 Hz), 2.90 (1H, *dd*, J = 13.9, 5.0 Hz), 4.44 (1H, *ddd*, J = 9.2, 8.0, 5.0 Hz), 6.41 (1H, *d*, J = 15.7 Hz), 6.48 (1H, *dd*, J = 8.0, 1.9 Hz), 6.61 (1H, *d*, J = 8.2), 6.62 (1H, *s*), 6.74 (1H, *d*, J = 8.1 Hz), 6.83 (1H, *dd*, J = 8.2, 1.8 Hz), 6.94 (1H, *d*, J = 1.8 Hz), 7.20 (1H, *d*, J = 15.7 Hz), 8.18 (1H, *d*, J = 8.0 Hz), 8.66 (1H, *br s*), 8.71 (1H, *br s*), 9.11 (1H, *br s*), 9.35 (1H, *br s*).

Caffeoyltyrosine (**2**), *p*-coumaroyl DOPA (**3**) and *p*-coumaroyltyrosine (**4**) were synthesized using the above protocol. Caffeoyltyrosine (**2**): ion-spray MS, m/z (rel. int.): 344 [M+H]⁺ (50), 163 (100); ¹H NMR spectral data (300 MHz, DMSO-*d*₆): δ 2.72 (1H, *dd*, J = 13.8, 9.3 Hz), 2.98 (1H, *dd*, J = 13.9, 4.8 Hz), 4.44 (1H, *ddd*, J = 9.3, 8.2, 4.8 Hz), 6.41 (1H, *d*, J = 15.7 Hz), 6.65 (2H, *d*, J = 8.2 Hz), 6.74 (1H, *d*, J = 8.2 Hz), 6.83 (1H, *d*, J = 8.3 Hz), 6.94 (1H, *s*), 7.03 (2H, *d*, J = 8.2 Hz), 7.19 (1H, *d*, J = 15.7 Hz), 8.21 (1H, *d*, J = 8.2 Hz), 9.06 (1H, *br s*), 9.19 (1H, *br s*), 9.36 (1H, *br s*).

p-Coumaroyl DOPA (**3**): ion-spray MS, m/z (rel. int.): 344 [M+H]⁺ (45), 147 (100); ¹H NMR spectral data (300 MHz, DMSO-*d*₆): δ 2.73 (1H, *dd*, J = 13.8, 9.2 Hz), 2.91 (1H, *dd*, J = 13.8, 4.7 Hz), 4.44 (1H, *ddd*, J = 9.2, 8.0, 4.7 Hz), 6.48 (1H, *d*, J = 8.1 Hz), 6.49 (1H, *d*, J = 15.4 Hz), 6.61 (1H, *d*, J = 8.0 Hz), 6.62 (1H, *s*), 6.79 (2H, *d*, J = 8.1 Hz), 7.28 (1H, *d*, J = 15.7 Hz), 7.38 (2H, *d*, J = 8.3 Hz), 8.16 (1H, *d*, J = 8.0 Hz), 8.68 (1H, *br s*), 8.73 (1H, *br s*), 9.83 (1H, *br s*).

p-Coumaroyltyrosine (**4**): ion-spray MS, m/z (rel. int.): 328 [M+H]⁺ (59), 147 (100); ¹H NMR spectral data (300 MHz, DMSO-*d*₆): δ 2.80 (1H, *dd*, J = 13.8, 9.2 Hz), 2.98 (1H, *dd*, J = 13.8, 4.8 Hz), 4.47 (1H, *ddd*, J = 9.2, 8.1, 4.8 Hz), 6.48 (1H, *d*, J = 15.7 Hz), 6.65 (2H, *d*, J = 8.4 Hz), 6.79 (2H, *d*, J = 8.6 Hz), 7.03 (2H, *d*, J = 8.4 Hz), 7.28 (1H, *d*, J = 15.7 Hz), 7.38 (2H, *d*, J = 8.6 Hz), 8.19 (1H, *d*, J = 8.1 Hz), 9.19 (1H, *br s*), 9.82 (1H, *br s*).

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