Clovamides; L-Dopa Conjugated with
trans- and *cis*-Caffeic Acids in
Red Clover (*Trifolium pratense*)

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Received February 20, 1974

The polyphenol compounds have been shown to be correlated with a resistance to the plant disease. Sakuma¹⁾ found that in red clover (*Trifolium pratense*) the quantity of the polyphenols increased during the process of the disease and the spore germination of *Kabatiella caulivora* was inhibited completely by the active peroxidase-polyphenols system in the clover extract.

Therefore, attempts to isolate the polyphenols from the red clover led to confirmation of novel polyphenols named *trans*- and *cis*-clovamide (I and II) and known phasic acid²⁾ (III).

I. *trans*-Clovamide

II. *cis*-Clovamide

III. Phasic acid

Fresh leaves and stems of the plants (9.3 kg) were chipped and boiled in water. The filtrates were treated with neutral lead acetate

and the resulting precipitates were treated with hydrogen sulfide. The filtrates were concentrated and extracted with ethyl acetate. The extract was concentrated *in vacuo* and fractionated by the method of Sondheimer.³⁾ Fractions collected were monitored by UV absorption at 280 nm and separated mainly into two fractions (F₁ and F₂).

The F₁-compound (III) was obtained as a pure oily substance, 6015 mg (0.065% yield), $[\alpha]_D^{15} + 21.2^\circ$ ($c=4.2$, H₂O), PC; R_f 0.63 in 2% acetic acid. The IR spectrum revealed ab-

sorption band at $1700\sim 1720\text{ cm}^{-1}$ ($-\overset{\text{O}}{\parallel}\text{C}-\text{O}-$). Hydrolysis of the compound by allowing to stand in 2 N KOH for 20 hr under a nitrogen atmosphere gave caffeic acid, which was identified by paper chromatography, UV, NMR and IR. Methylated products (IV) with dimethylsulfate were fractionated on a silicic acid column and yielded oily substance, UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm (ϵ): 218 (12200), 235 (10500), 300 (sh) (11900), 327 (15800). The mass spectrum of this compound showed the molecular ion peak at m/e 352 and base peak m/e 191

($\text{MeO}-\text{C}_6\text{H}_3(\text{MeO})-\text{CH}=\text{CH}-\text{C}\equiv\text{O}^+$). The NMR spectrum in CDCl₃ suggested that *trans*-caffeic acid and malic acid constituted the compound III: 2.93 (2H, d, $J=6$ Hz, $-\text{CH}_2-$), 3.71 (3H, s, $-\text{COOMe}$), 3.77 (3H, s, $-\text{COOMe}$), 3.89 (6H, s, Ar-OMe), 5.61 (1H, t, $J=6$ Hz, $-\dot{\text{C}}\text{H}-$),

6.35 (1H, d, $J=16$ Hz, $\text{H}-\text{C}=\text{C}'$), 6.75~7.20 (3H, m, Ar-H), 7.69 (1H, d, $J=16$ Hz, $\text{H}-\text{C}=\text{C}'$). With these results described

above, F₁-compound (III) was identified as phasic acid²⁾ (*trans*-caffeoyl-L-malic acid).

Subsequently, by the paper chromatographic analysis in 2% acetic acid, the F₁₁-compound was shown to be a mixture of two components R_f 0.30 (I) and 0.52 (II), so that a further isolation was carried out over a cellulose powder column. As the result, I and II were obtained as pure oil in yield 920 mg (0.010% yield) and

1078 mg (0.012% yield) respectively. I showed $[\alpha]_D^{18} -30.9^\circ$ ($c=0.5$, H_2O) and UV λ_{max}^{EtOH} nm (ϵ): 218 (sh) (19100), 232 (sh) (15500), 292 (13500), 326 (14700). When the solution of I in 6N HCl was heated and then shaken with ether, a phenol reagent⁴¹-positive and ninhydrin-positive substance was obtained from the water soluble portion, and identified as 3-(3,4-dihydroxyphenyl)-L-alanine (L-dopa) by its mp $278\sim 280^\circ C$, specific rotation $[\alpha]_D^{20} -9.75^\circ$ ($c=1.0$, 1N HCl) and behaviors on paper-chromatography. Besides, I was methylated with dimethylsulfate and chromatographed over silicic acid. Elution with 10% ethylacetate in chloroform yielded a crystal (V), mp $152.0\sim 153.0^\circ C$, $[\alpha]_D^{25} +137^\circ$ ($c=1$, $CHCl_3$). Doragendorff's reaction was positive. This result indicates the presence of the amine group. *Anal.* Found: C, 64.36; H, 6.59; N, 3.16, Calcd. for $C_{23}H_{27}NO_7$: C, 64.49; H, 6.31; N, 3.04%. In the mass spectrum of V, M^+ ion peak was observed at m/e 429. Furthermore fragment ions corresponding to

observed at m/e 206, 191 and 151 (base peak). The IR spectrum revealed absorption bands

at 3250, 1595, 1540, 1265 ($>NH$), $1730(-\overset{\text{O}}{\underset{\text{O}}{\text{C}}}-)$, 1645, $970 \left(\begin{array}{c} \text{H} \backslash \\ \text{C}=\text{C} / \\ \text{H} \end{array} \right)$, 840 and 800

$\left(\begin{array}{c} \text{H} \\ | \\ \text{C}_6\text{H}_4 \\ | \\ \text{H} \end{array} \right) \text{cm}^{-1}$. The UV spectrum in EtOH

showed an absorption maximum at 220, 233, 290 and 320 nm ($\epsilon=22200$, 24200, 19100 and 22200). The NMR spectrum ($CDCl_3$) of V indicated four Ar-OMe (3.77, 3H; 3.81, 3H; 3.88, 6H), one- COOMe (3.71, 3H), six Ar-H ($6.5\sim 7.2$, 6H, m), $\begin{array}{c} \text{H} \backslash \\ \text{C}=\text{C} / \\ \text{H} \end{array}$ (6.28, 1H, d, $J=16$ Hz and 7.56, 1H, d, $J=16$ Hz), $>NH$ (6.13, 1H, d, $J=8$ Hz), $-\overset{|}{\text{CH}}$ (4.99, 1H, dt, $J=8$ and 5 Hz) and $-\text{CH}_2-$ (3.10, 2H, d, $J=5$ Hz). Hydro-

genation of I over palladium-black, followed by methylation with dimethylsulfate, furnished pentamethyl dihydrocompound (VI), mp $103.0\sim 103.5^\circ C$, $[\alpha]_D^{18} +56^\circ$ ($c=1.0$, $CHCl_3$) and its structure was determined by MS, NMR and IR spectrum. When refluxed in 6N HCl, V afforded O,O'-dimethylhydrocaffeic acid, mp $95\sim 96^\circ C$. An oxidation product of V, using potassium permanganate in NaOH, was identified as veratric acid. From these data, the structure of I was elucidated to be N-*trans*-caffeoyl-3-(3,4-dihydroxyphenyl)-L-alanine.

On the other hand, oily substance II, exhibited $[\alpha]_D^{19} +28.7^\circ$ ($c=0.57$, H_2O) and UV λ_{max}^{EtOH} nm (ϵ): 288 (6700), 320 (5500), which quite differed from that of I. Methylation of II with dimethylsulfate afforded pentamethyl compound (VII), $[\alpha]_D^{18} +69^\circ$ ($c=2$, $CHCl_3$). The mass spectrum revealed m/e 429 (M^+) with a fragmentation pattern similar to that of V. Additional fact suggesting the absence of a *trans*-double bond was obtained from no signal at 980 cm^{-1} in the IR spectrum, and these results led us to postulate that VII might be a geometrical isomer (*cis*-form) of V. This validity was given by the NMR spectrum;⁵⁾ the signals at 5.89 (1H, d, $J=15$ Hz) and 6.61 (1H, d, $J=15$ Hz), which were assigned to *cis*-double bond by comparing with the spectra of *trans*- and *cis*-*p*-methoxy-cinnamic acids. Finally, a hydrogenated product of VII on palladium-black was compatible with dihydro compound, mp $103.5\sim 104.0^\circ C$, $[\alpha]_D^{18} +60.0^\circ$ ($c=1$, $CHCl_3$), *Anal.* Calcd. for $C_{23}H_{29}NO_7$: C, 64.04; H, 6.73; N, 3.25. Found: C, 64.04; H, 6.73; N, 3.25%. The spectral data of its MS, IR, UV and NMR were completely identical with those of VI. Thus, the structure of II was established to be N-*cis*-caffeoyl-3-(3,4-dihydroxyphenyl)-L-alanine.

Although several N-acylamino acids have been found in nature,^{6,7)} L-dopa conjugates such as clovamides are the first findings, and a interest is to assume that L-dopa, as a drug against Perkinsonism,⁸⁾ may be released from them by a biological system.

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