

Dedicated to Prof. Kurt Mothes on the occasion of his seventieth birthday

p*-COUMAROYLPUTRESCINE, CAFFELOYLPUTRESCINE AND FERULOYLPUTRESCINE FROM CALLUS TISSUE CULTURE OF *NICOTIANA TABACUM*

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Abstract—*p*-Coumaroyl-, caffeoyl- and feruloylputrescine have been isolated from callus tissue culture of *Nicotiana tabacum*. Proof of the structures was obtained by hydrolysis, color reactions, and comparison of i r., u v and NMR spectra with those of synthetic compounds *p*-Coumaroylputrescine and caffeoylputrescine are new natural products

INTRODUCTION

DURING tracer studies of nictotine biosynthesis in callus tissue cultures of *Nicotiana tabacum* L., it was observed that considerable amounts of the radioactivity from administered ¹⁴C-labelled putrescine or ornithine were incorporated into at least four ninhydrin positive basic compounds. Intense absorption in u.v. light and positive reactions with diazotized sulfanilic acid and ferric chloride indicated the phenolic character of the compounds. It was clearly of interest to identify these compounds.

The occurrence of phenolic compounds linked to putrescine has already been observed in higher plants. Feruloylputrescine has been isolated and identified from citrus leaves and fruit by Wheaton and Stewart¹ and *p*-coumaroylagmatine by Stoessl.² Our investigations have now resulted in the isolation and identification of *p*-coumaroylputrescine(I), caffeoylputrescine(II) and feruloylputrescine(III). A part of this investigation has already been reported.³

RESULTS

Caffeoylputrescine

Fractionation of the tobacco callus extract on Amberlite CG-50 resin with an acetic acid concentration gradient resulted in the separation of caffeoylputrescine from the other two aromatic amides. On further fractionation on cellulose column chromatography, crystalline caffeoylputrescine was obtained. Since recrystallization of the free base was found to be difficult, the compound was converted to its hydrochloride. It decomposed at 245–250° with a change in form at about 96°, due to a loss of water of crystallization. Elementary analysis indicated an empirical formula of C₁₃H₁₈N₂O₃.

The compound gave a violet color with ninhydrin and was strongly fluorescent. The LeRosen test for aromatic compounds⁴ and the reaction with diazotized sulfanilic acid and

¹ T. A. WHEATON and I. STEWART, *Nature* **206**, 620 (1965).

² A. STOESSL, *Phytochem.* **4**, 973 (1965).

³ S. MIZUSAKI, Y. TANABE and M. NOGUCHI, *Agri Biol Chem.* **34**, 972 (1970).

⁴ A. L. LERSEN, R. T. MORAVEK and J. K. CARLTON, *Anal Chem.* **24**, 1335 (1952).

with ferric chloride were all positive. The u.v. absorption of the compound in water (219, 235, 295 and 320 nm) was similar to that of caffeic acid. Hydrolysis of the compound by refluxing in 2 N NaOH for 4 hr in N_2 gave caffeic acid and putrescine, which were identified by paper chromatography and i.r. spectroscopy. The i.r. spectrum of the compound in KBr disk shows an amide band A (carbonyl) at 1598 cm^{-1} and an amide band B at 1536 cm^{-1} . Absorption at 1655 cm^{-1} is ascribed to the $C=C$ stretching vibration in conjugation with the carbonyl. An absorption band at 980 cm^{-1} is considered to be due to the unsaturated $C-H$ deformation vibration at the *trans*-double bond.⁵

These results indicate that the compound is 1-(*trans*-3,4-dihydroxycinnamoylamino)-4-aminobutane. This is further confirmed by its NMR spectrum. The NMR spectrum of the compound in deuterium oxide showed the presence of three aromatic protons (δ 6.88, singlet and δ 6.76, triplet formed by partial overlapping of two doublets) and two protons (δ 7.10, doublet and δ 6.11, doublet, J value 15.0 c/s) corresponding to vinyl protons. The signal of the vinyl proton which is adjacent to the carbonyl group showed a chemical shift at a lower field than the other proton because of the effects of the conjugated carbonyl group and the aromatic ring current. The coupling constant between the vinyl protons of 15.0 c/s confirmed the *trans* arrangement about the double bond.⁶ The spectrum also revealed four protons (δ 3.05) attributable to two methylene groups adjacent to the amino group and four additional protons (δ 1.61) corresponding to the other methylene groups of 1,4-diaminobutane.

Final confirmation of the structure was obtained by i.r., u.v. and NMR spectral comparison with a synthetic specimen.

Feruloylputrescine

Later fractions eluted from the Amberlite CG-50 resin contained two other aromatic amides. Treatment with Dowex 50 \times 4 resin and recrystallization from water gave yellow needles of feruloylputrescine, which did not have a sharp m.p. but changed form at about 96°, as observed by Wheaton and Stewart.¹ The hydrochloride of the compound had m.p. 150.5–151.5° with an empirical formula of $C_{14}H_{20}N_2O_3$. All its properties were similar to those of feruloylputrescine previously obtained from grape fruit.¹ Its identification as 1-(*trans*-3-methoxy-4-hydroxycinnamoylamino)-4-aminobutane was then confirmed by direct comparison with a synthetic specimen.

p-Coumaroylputrescine

p-Coumaroylputrescine was recovered from the mother liquor obtained by recrystallization of feruloylputrescine. Purification was achieved by preparative paper chromatography and recrystallization from water, yielding faint yellow needles of *p*-coumaroylputrescine. It had m.p. 182–183.5° with an empirical formula of $C_{13}H_{18}N_2O_2$.

This compound gave positive reactions with ninhydrin and diazotized sulfanilic acid, but not with ferric chloride. It exhibited the u.v. spectrum characteristic for derivatives of *trans-p*-coumaric acid.⁷ *trans-p*-Coumaric acid and putrescine were obtained on alkaline hydrolysis. Its structure as 1-(*trans*-4-hydroxycinnamoylamino)-4-aminobutane was confirmed by mixed m.p. and i.r. comparison with a synthetic sample.

⁵ L. CROMBIE, *J. Chem. Soc.* 2997 (1952).

⁶ J. A. POLE, W. G. SCHNEIDER and H. J. BERNSTEIN, *High-Resolution Nuclear Magnetic Resonance*, McGraw-Hill, New York (1956).

⁷ C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* 235, 2418 (1960).

DISCUSSION

Feruloylputrescine has been isolated and identified from citrus by Wheaton and Stewart,¹ and some related compounds such as aegelin,⁸ heraclavin,⁹ cinnamoylhistamine,¹⁰ and *p*-coumaroylputrescine² have been found in plants; however, caffeoylputrescine and *p*-coumaroylputrescine have not previously been reported. A predominance of aromatic amides of this type in the callus tissue of *N. tabacum* is notable. The caffeoylputrescine content of the tissue samples varied from 0.2 to 1 mg/fr. wt. and the other aromatic amides were found in lesser amounts. On the other hand, none of these compounds could be detected in normal tobacco tissue, indicating a difference in secondary metabolism between the two types of tissue.¹¹⁻¹³ Hydroxycinnamic acids are known to occur almost always as esters of quinic acid or glucose and glucosides in higher plants.^{7,14} However, the presence of substantial quantities of putrescine derivatives in growing cells suggest that these compounds play a role in the metabolism of cinnamic acid; the presence of bound putrescine is also of interest in relation to the biosynthesis of nicotine in tobacco plants.

EXPERIMENTAL

Callus Tissue Culture

Tobacco cells growing in suspension culture, derived from *N. tabacum* 'Bright Yellow', were used for this work. The stock cultures were routinely subcultured on Linsmaier and Skoog revised medium¹⁵ once a week for about 2 yr. Cells were grown in 500 ml Erlenmeyer flasks containing 100 ml of medium in darkness at 28° on a reciprocal shaker. The cells were harvested by suction filtration, washed with water, and stored in deep-freeze until extracted.

Isolation

Washed tissue (1.5 kg) was repeatedly extracted with 75% aq. methanol containing 0.5% acetic acid. The extract was concentrated *in vacuo* to about 200 ml. The water-insoluble materials formed during evaporation were removed by centrifugation. The supernatant was passed through a column (2 × 30 cm) of Amberlite CG-50 resin (H⁺ form) with water and basic materials were eluted with 2 N HOAc. The eluate was concentrated *in vacuo*, taken up in 100 ml of water, and placed on a column (2 × 30 cm) of Amberlite CG-50 resin (H⁺ form). The column was then treated with 4 l. of a linear gradient of HOAc from 0 to 5 N. The effluent was collected in 50 ml fractions. Caffeoylputrescine was found in fraction 10-20 and both *p*-coumaroylputrescine and feruloylputrescine in fraction 22-35. The caffeoylputrescine was purified on a cellulose column (2 × 100 cm), eluted with *n*-BuOH-HOAc-H₂O (13:2:5) and then converted to the hydrochloride. Repeated recrystallization from water afforded 220 mg of fine needles of caffeoylputrescine hydrochloride, decomp. 245-250°, changing form at about 96°. (Found C, 48.21; H, 7.10; N, 8.65. C₁₃H₁₈N₂O₃ · HCl · 2H₂O required: C, 48.28; H, 7.18; N, 8.68 %)

The fractions containing both *p*-coumaroylputrescine and feruloylputrescine were placed on a small column of Dowex 50 × 4 (NH₄⁺ form) with methanol, and the basic compounds were eluted with 2 N NH₄OH in methanol. The eluate was taken to dryness. The residue was dissolved in a small volume of water and on being left overnight at 0° it deposited feruloylputrescine, repeated recrystallization from water afforded 75 mg of yellow fine needles. The hydrochloride of the compound had m.p. 150-151.5° (Found C, 52.35; H, 7.22; N, 8.98. C₁₄H₂₀N₂O₃ · HCl · H₂O required: C, 52.75; H, 7.27; N, 8.79 %).

The mother liquor obtained by filtration of feruloylputrescine was chromatographed in *n*-BuOH-HOAc-H₂O (13:2:5) and the bands of *p*-coumaroylputrescine were eluted with water. The extract was purified on a small column of Dowex 50 × 4 resin (NH₄⁺ form) and repeated recrystallization of the product from water afforded 15 mg of faint yellow needles of *p*-coumaroylputrescine. It had m.p. 182-183.5°. (Found C, 61.05; H, 7.70; N, 10.83. C₁₃H₁₈N₂O₂ · H₂O required: C, 61.88; H, 7.98; N, 11.10 %)

⁸ R. N. CHAKRAVARTI and B. DASGUPTA, *J. Chem. Soc.* 1580 (1958).

⁹ L. CROMBIE, *J. Chem. Soc.* 995 (1955).

¹⁰ J. S. FITZGERALD, *Australian J. Chem.* 17, 375 (1964).

¹¹ L. M. BLAKELY and F. C. STEWARD, *Am. J. Botany* 51, 809 (1964).

¹² B. L. WILLIAMS and T. W. GOODWIN, *Phytochem.* 4, 81 (1965).

¹³ T. W. GOODWIN, *Chemistry and Biochemistry of Plant Pigments*, Academic Press, New York (1965).

¹⁴ J. B. HARBORNE and J. J. CORNER, *Biochem. J.* 81, 242 (1961).

¹⁵ E. M. LINSMAIER and F. SKOOG, *Physiol. Plantarum* 18, 100 (1965).

1-(Trans-3,4-dihydroxycinnamoylamino)-4-aminobutane

The acid chloride of diacetyl-*trans*-caffeic acid (prepd. from 530 mg of the acid¹⁶ via SOCl_2) in 20 ml CHCl_3 was added to a cooled solution of putrescine (220 mg) in CHCl_3 . The solid formed was filtered off, washed with CHCl_3 and extracted with methanol. The methanolic extract was concentrated to dryness and the residue was treated with 10 ml of 2 N NaOH for 2 hr at room temperature. After acidification with conc. HCl, the solution was concentrated to dryness and the residue was extracted with methanol. The extract, after treatment on a column of Dowex 50 \times 4 resin (NH_4^+), afforded the desired product. Recrystallization from water gave colorless fine needles of 1-(*trans*-3,4-dihydroxycinnamoylamino)-4-aminobutane (180 mg); decomp. 247–250°. (Found C, 48.30, H, 7.12, N, 8.63. Calc. for $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_3 \cdot \text{HCl} \cdot 2\text{H}_2\text{O}$ C, 48.28, H, 7.18, N, 8.68%.)

1-(Trans-3-methoxy-4-hydroxycinnamoylamino)-4-aminobutane

To a cooled solution of putrescine (220 mg) in CHCl_3 (20 ml) was added a CHCl_3 solution of acetyl-*trans*-ferulic acid chloride (500 mg).¹⁷ The product was hydrolyzed with 2 N NaOH under the same conditions as mentioned above. After acidification with conc. HCl, the solution was evaporated to dryness and the residue was treated on a column of Dowex 50 \times 4 resin (H^+ form). Repeated recrystallization of the product from water gave yellow needles of 1-(*trans*-3-methoxy-4-hydroxycinnamoylamino)-4-aminobutane (250 mg). The hydrochloride of the compound had m.p. 151–152°. (Found: C, 52.45, H, 7.20, N, 8.89. Calc. for $\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_3 \cdot \text{HCl} \cdot \text{H}_2\text{O}$ C, 52.75, H, 7.27, N, 8.79%.)

1-(Trans-4-hydroxycinnamoylamino)-4-aminobutane

Acetyl-*trans*-*p*-coumaroylputrescine was prepared by adding the acid chloride of acetyl-*trans*-coumaric acid (410 mg) to a solution of putrescine (220 mg) in CHCl_3 (20 ml). The desired product was obtained by hydrolyzing the acetyl derivative and by successive treatment with Dowex 50 \times 4 resin. Repeated recrystallization from water afforded faint yellow needles of 1-(*trans*-4-hydroxycinnamoylamino)-4-aminobutane (210 mg), m.p. 182–183°. (Found C, 61.52, H, 7.80, N, 11.0. Calc. for $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_2 \cdot \text{H}_2\text{O}$ C, 61.88; H, 7.98, N, 11.10%.)

¹⁶ E. PACSU and C. STIEBER, *Ber* **62**, 2976 (1929)

¹⁷ L. S. FOSDICK and A. C. STRAKE, JR., *J. Am. Chem. Soc.* **62**, 3352 (1940)