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STIZOLOBIC AND STIZOLOBINIC ACIDS IN *AMANITA PANTHERINA*

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Abstract—Stizolobic acid, β-(6-carboxy-α-pyron-4-yl)alanine and stizolobinic acid, β-(6-carboxy-α-pyron-3-yl)-alanine have been isolated from the toxic mushroom *Amanita pantherina*.

Amanita pantherina is the most frequent cause of non-fatal mushroom poisoning in the Pacific Northwest. Twenty persons were hospitalized by *A. pantherina* poisoning in the Seattle area in spring 1973. Symptoms consist of hallucination and muscular spasms followed by deep sleep. Complete recovery generally occurs in 24–48 hr, but deaths have occurred. Drug-use fads of the past decade contributed to re-publication of 18th century accounts of uses of *Amanita muscaria* for hallucinogenesis in Siberia.[1] *A. muscaria* and *A. pantherina* are chemically and morphologically very similar. However *A. pantherina* is more abundant in the Pacific Northwest and more frequently involved in poisonings.

Previous investigations of the chemistry of *A. muscaria* and *A. pantherina* in Europe and Japan led to the identification of several amino acids and related compounds of significance to this problem.[2] Ibotenic acid and its decarboxylation product, muscimol, are responsible, at least in part, for the hallucinogenic effect. Muscazone and 4-hydroxy-2-pyrrolidinone are probably related to the biosynthesis of ibotenic acid. Muscarine, though toxic, is present at too low a level to play a role in muscaria-pantherina toxicity.

Quantitation of these metabolites in local *A. pantherina* disclosed the presence of an additional non-protein amino acid giving an orange ninhydrin colour characteristic of lathyrine,[3] and other aromatic heterocyclic alanines possessing an activated methylene.[4] The electrophoretic behavior of the new amino acid indicated a strong acid with $pK_a < 2$. As expected for a heterocyclic aromatic amino acid, it was effectively absorbed from aqueous ethanol on to activated charcoal and eluted by ethanolic HCl. The new amino acid was enriched by ion exchange techniques and further purified by either gradient elution from an anion exchanger, gradient elution from a cation exchanger, or cellulose chromatography. After these steps what appeared to be a single substance could now be resolved on analytical chromatography into two isomers, one giving an

[1] GOLDSMITH, O. (1762) *Letters from a Citizen of the World to his Friends in the East*, Letter xxxii, A. Mushroom Feast among the Tartars, London.

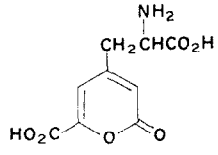
[2] EUGSTER, C. H. (1969) *Prog. Chem. Nat. Prod.* **27**, 261.

[3] BELL, E. A. and FOSTER R. G. (1962) *Nature* **194**, 91.

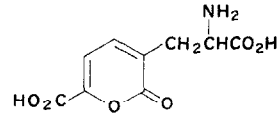
[4] SPRINGER, R. H., HAGGERTY, JR., W. J. and CHENG, C. C. (1965) *J. Heterocycl. Chem.* **2**, 49.

orange (stizolobic acid) and the other a yellow brown color (stizolobinic acid) with ninhydrin. Final separation of the isomers was achieved by prep. PC and electrophoresis.

The single long wavelength UV maximum of the orange ninhydrin-reactor, λ_{max}^{EtOH} 301 nm (ε 3100), matches that of an α-pyrone[5] and no other simple heterocycle. The slow spectral shift in NaOH to 338 nm (complete after 3 hr) with an isobestic point at 314 nm is particularly characteristic of an α-pyrone.[6]



Stizolobic acid



Stizolobinic acid

On a longer time scale the UV curve cross-over point for the orange ninhydrin-reactor begins to diverge from a true isosbestic point, and the long wavelength absorption decays, indicating slow formation of a third, and final compound, having λ_{max} 263, 272, 279 nm. Although insufficient material was available to identify this compound, it is known that stizolobic acid is converted to 4-methyl-pyridine-2-carboxylic acid, λ_{max} 257, 264, 270 nm, by prolonged acid treatment.[7] Its IR spectrum contains characteristic α-pyrone carbonyl absorption at 1715 cm^{-1} and pyrone C=C stretch at 1630 cm^{-1} as well as bands ascribable to NH_3^+ and $-CO_2^-$. Two isolated aromatic proton singlets are observable in the aqueous NMR spectrum at 6·81 and 7·63 ppm. Corresponding aromatic proton singlets occur at 6·43 and 7·02 ppm in 4-bromomethyl-6-carbethoxy-α-pyrone.[8]

The UV spectrum of the yellow-brown ninhydrin-reactor is similar to that of the orange ninhydrin-reactor but distinguishable in that the pyrone maximum and the ring-opened enone anion maximum are closer and no isosbestic point is observable due to rapid formation (1 min) of a third product. The qualitative difference in the acid base spectra of the two metabolites is identical to the difference previously observed for the isomeric alanyl-α-pyrone-6-carboxylic acids, stizolobic acid and stizolobinic acid.[6]

The identity of the isomeric pair of amino acids with stizolobic and stizolobinic acids was confirmed by comparison of ninhydrin colors and by electrophoresis and chromatography in several solvents with synthetic samples of the DL acids.

Stizolobic acid and stizolobinic acid have been isolated previously only from *Stizolobium* species (velvet bean) and detected in further *Mucuna* species of Leguminoseae.[9] *Mucuna* species have been suggested for commercial isolation of L-DOPA. Evidence that stizolobic and stizolobinic acids arise by metapyrocatechase cleavage of L-DOPA has been summarized.[7] If L-DOPA is present in *A. pantherina*, it must be at levels too low to be detected by $FeCl_3$, ninhydrin and Pauly tests on appropriate fractions. Nor were any other phenols detected in the aqueous alcoholic extracts. However, the white flesh of the

[5] FRIED, J. and ELDERFIELD, R. C. (1941) *J. Org. Chem.* **6**, 566; VAN DAM, M. J. D. and KOGL, F. (1964) *Rec. Trav. chim.* **83**, 39.
[6] SENOH, S., IMAMOTO, S., MAENO, Y., TOKUYAMA, T., SAKAN, T., KOMAMINE, A. and HATTORI, S. (1964) *Tetrahedron Letters* 3431.
[7] SENOH, S. (1965) *Nippon Kagaku Zasshi* **86**, 1087.
[8] IMAMOTO, S., MAENO, Y., SENOH, S., TOKUYAMA, T. and SAKAN, T. (1966) *Nippon Kagaku Zasshi* **87**, 1230.
[9] HATTORI, S. and KOMANINE, A. (1959) *Nature* **183**, 1116.

mushroom blackens rapidly on comminution in aqueous alcohol in the manner of *Cytisus scoparius* and other legumes known to contain appreciable amounts of L-DOPA and related catechols.

Stizolobic and stizolobinic acid are absent from local redcapped *A. muscaria*, but low levels were found in some *A. gemmata* varieties previously suggested[10] to be *A. pantherina–gemmata* hybrids on the basis of ibotenic acid content, and very low levels in at least one yellow-capped form of *A. muscaria*, also considered to be a possible *gemmata* hybrid.

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EXPERIMENTAL

All samples of *Amanita* were collected at Tenino, Washington and used either fresh or stored frozen for not more than two years. Stizolobic and stizolobinic acid do not survive air-drying of mushrooms and storage for several years at room temp.

4-l. batches of 70% ethanolic extracts of *A. pantherina* were loaded on a 3·5 × 60 cm column of Dowex AG 3 × 4A, 20–50 mesh, in the acetate form. Effluent was monitored by electrophoresis, and loading was continued until stizolobic–stizolobinic acid appeared in the effluent. The column was washed with 10% HOAc until the effluent was free of amino acids. The eluent was then changed to M HCl. Early fractions contained glutamic and aspartic acid. Stizolobic–stizolobinic acid was collected in a 300 ml fraction containing some glutamic and aspartic acid and additional highly acidic ninhydrin-positive substances.

The stizolobic–stizolobinic acid concentrate was chromatographed on Whatman 3MM paper in *n*-BuOH–HOAc–H_2O (12 : 3 : 5), developing twice by ascent. Compounds were located by use of ninhydrin and recovered from the paper in the usual manner. Relatively light loadings of about 5 mg/sheet (57 cm) were used to obtain partial resolution of stizolobic and stizolobinic acids.

Fractions containing mainly stizolobic acid and those containing mainly stizolobinic acid were electrophoresed separately on sheets of Whatman 3MM in a pH 2 formate-acetate buffer at 1000 V for 3 hr. Loading and resolution were optimized at 1–2 mg/sheet. Several 10–20 mg batches of electrophoretically and chromatographically pure amino acids were prepared in this manner.

The pH 2 electrophoretic order of acidic non-protein amino acids identified to date in *A. pantherina* or *A. muscaria* from anode-to-cathode is: stizolobic (15 mm from origin), stizolobinic (35 mm), ibotenic (58 mm), muscazone (75 mm), aspartic reference (165 mm), glutamic reference (180 mm). Typical apparent migration distances, sensitive to loading, impurities and electroosmotic flow, are given in parentheses for 1500 V, 2 hr. A related compound, tricholomic acid, migrates between muscazone and aspartic acid. Stizolobic and stizolobinic acids are readily resolved in PhOH–H_2O, 4 : 1 (R_f 0·24 and 0·33 respectively) but not in *n*-BuOH–HOAc–H_2O, 12:3:5 (R_f 0·12 and 0·15 on chromatograms in which ibotenic acid had R_f 0·25).

[10] BENEDICT, R. G. TYLER, V. E. and BRADY, L. R. (1966) *Lloydia* **29**, 333.