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INVESTIGATION OF THE ALKALOIDS OF *AMANITA* SPECIES[1]
II. *AMANITA CITRINA* AND *AMANITA PORPHYRIA*

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Modern authorities (Hennig 1958) consider *Amanita citrina* to be a weakly toxic mushroom. Its earlier reputation as a deadly poisonous species no doubt stemmed from confusion with *Amanita phalloides*. In 1953, Wieland and Motzel isolated bufotenine from *A. citrina* collected in Europe, and Tyler (1961) subsequently confirmed the presence of that alkaloid in American specimens of the fungus. Catalfomo and Tyler (1961) also detected bufotenine in the closely related *Amanita porphyria*. No additional analytical studies of these species have been reported.

Preliminary chromatographic examination of ethanolic extracts of sporocarps of both species which had been collected in Germany in the fall of 1963 revealed the presence of a number of Ehrlich-positive compounds in addition to bufotenine. Consequently, it was decided to examine authentic sporocarps of both fungi in an attempt to identify these minor constituents. *A. citrina* was also cultivated on sterile liquid media to determine the biosynthetic capabilities of its mycelium under these conditions.

Experimental

The dates and collection sites of the specimens of *Amanita citrina* S. F. Gray and *Amanita porphyria* (Fr.) Secr. utilized in this study are reported in table 1. When preliminary analyses revealed no appreciable differences in the composition of the bases found in the different collections, all were utilized indiscriminately.

After drying in a forced-air oven at 55° C, the samples were finely powdered and defatted by exhaustive extraction with petroleum ether in a Soxhlet apparatus. A 10-g sample was then extracted by shaking with three consecutive 50-ml portions of 70% ethanol for thirty-minute periods. After filtering, the combined extract was reduced to a small volume *in vacuo*, the resulting concentrated solution adjusted to pH 10 with sodium hydroxide and saturated with sodium chloride. It was then extracted by shaking with three consecutive portions of water-saturated *n*-butanol, the butanol extracts combined, an equal volume of petroleum ether added, and this solution extracted with three consecutive portions of 2% tartaric acid solution. The acidic solution was rendered alkaline, saturated with sodium chloride, and extracted as before. After combining, the butanol extract was evaporated to dryness *in vacuo* and the remaining residue dissolved in 10 ml of 70% ethanol. Extracts thus prepared were utilized for thin-layer and paper chromatography.

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Table 1

Time and place of *Amanita citrina* and *Amanita porphyria* collections

Sample No.	Collection Site	Date (1963)
	A. citrina	
1.	Gransee	Sept. 10
2.	Berlin/Grunewald	Sept. 7, 8
3.	Halle/Dölauer Heide	Sept. 16
4.	Brettenberge Forest/Harz	Sept. 11
5.	Ramberg Forest/Harz	Sept. 11
6.	Blankenhaim by Sangerhausen	Sept. 15
7.	Bad Schmiedeberg	Sept. 17
	A. porphyria	
1.	Berlin/Grunewald	Sept. 8
2.	Quedlinburg	Sept. 10

Numerous chromatographic systems were tested to find one which would give effective separation of the Ehrlich-positive compounds present in the extracts. Thin-layer plates prepared with Merck silica gel G (acc. to Stahl) developed with a solvent mixture composed of chloroform containing 5% methanol, saturated with 25% ammonium hydroxide (Legler and Tschesche, 1963) proved to be most satisfactory. Quantities (< 600 µl) of the extract were applied as streaks (2–6 cm) and the plates developed twice with this solvent system. Tryptamine derivatives were detected by spraying with Ehrlich's reagent (2% p-dimethylaminobenzaldehyde in 15% hydrochloric acid) followed by exposure to the fumes of aqua regia and hydroxylated compounds with Pauly's reagent prepared according to Kutáček (Hais and Macek,

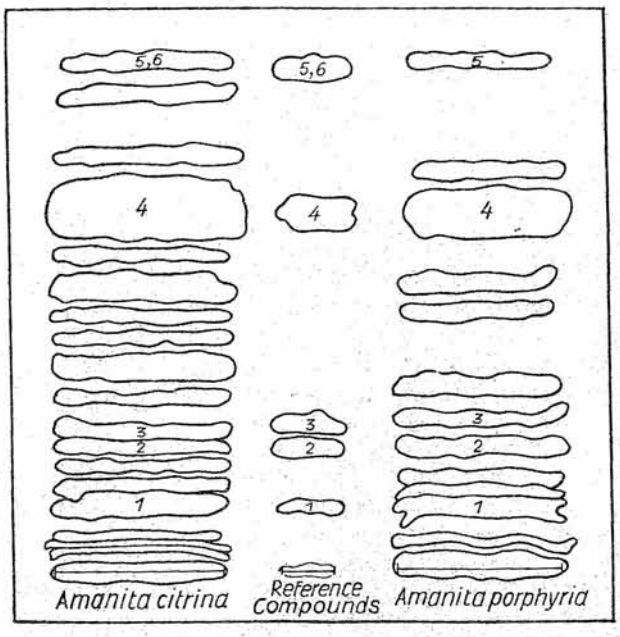

Fig. 1: Thin-layer chromatogram of purified extracts of *A. citrina* and *A. porphyria* together with reference compounds 1 = bufotenine-N-oxide; 2 = serotonin; 3 = N-methylserotonin; 4 = bufotenine; 5 = 5-methoxy-N,N- dimethyltryptamine; 6 = N,N-dimethyltryptamine. Spots are visualized with Ehrlich's reagent

1958). Identification of the bases was effected by co-chromatography with reference compounds including bufotenine, bufotenine-N-oxide, dehydrobufotenine, N,N-dimethyltryptamine, N-methylserotonin, 5-methoxy-N-methyltryptamine, 5-methoxy-N,N-dimethyltryptamine, 5-methoxytryptamine, psilocin, psilocybin, serotonin, and tryptamine.

Nearly 20 different Ehrlich-positive spots could be detected by this procedure in the *A. citrina* extracts and approximately 12 in those of *A. porphyria*. Of these, five spots in both extracts had mobilities coinciding with bufotenine-N-oxide, serotonin, N-methylserotonin, bufotenine, and 5-methoxy-N,N-dimethyltryptamine and N,N-dimethyltryptamine (the latter two compounds moved together to form a single spot). A typical chromatogram is illustrated in figure 1.

To substantiate further the identity of these compounds, additional chromatograms were prepared, and the areas equivalent to the five spots were scraped from the plates. The compounds were eluted from the silica gel with warm water or 70% ethanol, according to their respective solubilities, and the extracts again spotted, together with reference compounds, on thin-layer plates and on S. and S. 2043 bm filter paper. The thin-layer plates were developed as previously described; the paper chromatograms were subjected to circular development in two solvent systems, *n*-butanol : acetic acid : water (4 : 1 : 1) (BAcW) and *n*-propanol : 1 N ammonium hydroxide (5 : 1) (PrAm). Average R_F values and estimated relative concentrations of the tryptamine derivatives detected in both *A. citrina* and *A. porphyria* are recorded in table 2. In each case these compounds failed to separate from the respective reference compounds when chromatographed singly and in admixture. This, rather than the R_F values which may vary appreciably in the circular chromatographic technique, was utilized as the criterion of identity. As may be seen in figure 1, a number of spots in both extracts remain unidentified.

Table 2
R_F values and estimated relative concentrations
of tryptamine derivatives detected in *Amanita* species

Compound	R_F Values (ave.)		Relative Concentration[a]	
	BAcW	PrAm	*A. citrina*	*A. porphyria*
Bufotenine-N-oxide	0,45	0,50	4	2
Serotonin	0,58	0,66	2	1
N-Methylserotonin	0,66	0,73	2	1
Bufotenine	0,69	0,85	10	8
5-Methoxy-N,N-dimethyltryptamine	0,78	0,92	0,25	0,1
N,N-Dimethyltryptamine	0,85	0,97	0,25	0

[a] Expressed as 10 = maximum, 0 = none.

To determine the biosynthetic capabilities of mycelial cultures of *A. citrina*, a nutrient solution of the following composition was utilized: malt extract, 25 ml; glucose, 10 g; peptone, 1 g; thiamine, 150 mcg; distilled water *ad* 1,000 ml. After autoclaving, the pH of this medium was 5,7. Erlenmeyer flasks (100 ml), each containing 25 ml of this nutrient solution, were inoculated with *A. citrina* and incubated at 25° C in the absence of light. The organism grew extremely slowly but eventually produced a thick white mycelial pad on the surface of the medium (figure 2). After 85 days, six such cultures were harvested, yielding a total of 55,5 ml of medium and 3,01 g fresh weight (0,54 g dry weight) of mycelium.

The nutrient solution was adjusted to pH 10 with 10% sodium hydroxide, saturated with sodium chloride, and extracted by shaking with three consecutive portions of *n*-butanol. This

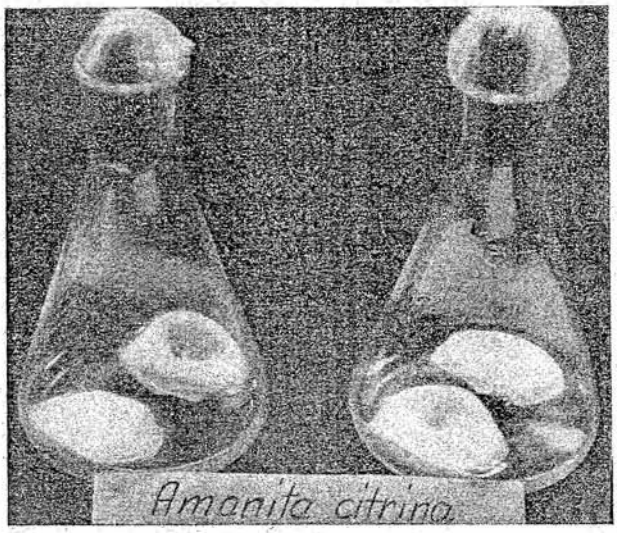

Fig. 2: Mycelial cultures of *Amanita citrina*

extract was purified as previously described and the final ethanol solution adjusted to a volume of 2 ml. The mycelium was finely powdered, extracted with three successive portions of 70% ethanol and the combined solution also purified by partition as outlined for the sporocarp extracts. Final volume of this solution was also adjusted to 2 ml.

Quantities (100–500 µl) of both extracts were chromatographed as before in thin-layer and paper chromatographic systems. Bufotenine, which was present in both extracts, was accompanied by traces of other Ehrlich-positive compounds, but the latter were not unequivocally identified.

In order to determine the approximate concentrations of bufotine in the nutrient solution and the mycelium, the size and color intensity of spots on the thin-layer plates were compared visually with a series of reference spots produced by chromatographing different concentrations of bufotenine and spraying with Ehrlich's reagent followed by exposure to aqua regia vapor. From the data thus obtained, it was calculated that the nutrient solution contained a concentration of bufotenine approximating 0,00003% and the mycelium 0,03% (dry-weight basis).

Discussion

Five tryptamine derivatives, in addition to the previously detected bufotenine, were identified in extracts of sporocarps of *A. citrina*. These included bufotenine-N-oxide, serotonin, N-methylserotonin, 5-methoxy-N,N-dimethyltryptamine, and N,N-dimethyltryptamine. With the exception of N,N-dimethyltryptamine, these same compounds were also identified in sporocarp extracts of *A. porphyria*.

Based on the relative amounts of these compounds shown to be present, it may be concluded that both of these fungi possess active methylation systems, since the amount of bufotenine accumulated far exceeds the concentration of

its demethylated precursor serotonin or the partially methylated N-methyl-serotonin. The relatively large number of tryptamine derivatives which remain unidentified in both species, some of which are present in relatively large concentrations, preclude definite conclusions regarding the details of the metabolic relationships of these compounds.

Although N,N-dimethyltryptamine is known to function as a psychotomimetic agent in human beings following ingestion, it is doubtful if the extremely small amount present in *A. citrina* would be sufficient to induce these symptoms after consumption of normal quantities of the mushroom. The absence of any report classifying *A. citrina* as a hallucinogenic mushroom tends to confirm this idea. However, until the identity of the other tryptamine derivatives of both *A. citrina* and *A. porphyria* is established, or until modern pharmacological studies clarify their physiological activities, they should continue to be classified as potentially dangerous species.

The ability of mycelial cultures of *A. citrina* to biosynthesize even small amounts of bufotenine is of considerable interest. Metabolic capabilities of mycelial cultures of basidiomycetes are often quite different from those of sporocarps, as revealed by the frequent inability of the former to accumulate certain secondary metabolic products. For example, sporocarps of numerous *Panaeolus* species contain appreciable quantities of serotonin and its precursor, 5-hydroxytryptophan. Efforts to detect these compounds in mycelial cultures of these species have been uniformly unsuccessful (Benedict and Tyler 1962). *A. citrina* cultures offer a suitable system for studies of the biosynthesis of bufotenine and related 5-hydroxytryptamine derivatives.

Summary

Bufotenine, bufotenine-N-oxide, serotonin, N-methyl-serotonin, 5-methoxy-N,N-dimethyltryptamine, and N,N-dimethyltryptamine were identified chromatographically in sporocarp extracts of *A. citrina* and, with the exception of N,N-dimethyltryptamine, in similar extracts of *A. porphyria*. Surface cultures of *A. citrina* were found to be capable of biosynthesizing bufotenine, small quantities of the alkaloid being detected in the mycelium and the culture medium.

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