Mechanism Proposal for a Fluorescent Amanitin Derivative Formation

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A quantitative fluorimetric detection of α-amanitin treated by H_3PO_4 is proposed. Spectra for both amanitin and tryptophan suggest an extended aromatic structure clearly confirmed by ¹H NMR spectrography.

The bicyclooctapeptide product isolated from *Amanita phalloides* α-amanitin has been found to be responsible for the intoxication by this mushroom [1]. It inhibits RNA polymerase II (EC 2.7.7.6) in higher eukaryotes [2–5].

The covalent structure of α-amanitin has been elucidated as a cyclo-(L-α-asparagyl-4-hydroxy-L-prolyl-4,5-dihydroxy-L-isoleucyl-6-hydroxy-2-mercapto-L-tryptophyl-glycyl-L-isoleucyl-glycyl-L-cysteinyl) cyclo (4 → 8) S-oxide [6]. The spatial arrangement closely related to the toxicity [7–9] was recently determined by NMR spectroscopy [10] and found to be similar to the structure of α-amanitin in the crystalline state [11] with a backbone conformation stabilized by intramolecular bonds [12] giving the molecule a pseudo-symmetry axis along which lies the indole moiety (see Fig. 1). Thus it may be reasoned that this symmetry is recognized by RNA polymerase as suggested for other regulatory proteins [13, 14].

We worked out the procedure described by Palyza [15] for separating toxic products from *Amanita phalloides* by preparative T. L. C. α-amanitin gave a characteristic blue spot after the only spraying of the silica gel thin layer chromatogram with phosphoric acid and heating at 85 °C. In our hands, this method gave not only colored but fluorescent spots under the UV light of a mercury lamp. The observed fluorescence excitation and emission wavelengths were found to be quite different from the initial ones attributed to the tryptophan residue.

The tryptophyl initial fluorescence was no more found. It seemed obvious that only the tryptophan indole ring may present electronic perturbations leading to such changes in the fluorescence spectrum. This prompted us to study the tryptophan behaviour in the above conditions. We found that the new fluorimetric detection may be quantitative in the fluorimeter cuvette. Fig. 2 shows a range of linear sensitivity. Dioxan, which is known to give a reduced dielectric constant, minimizing the interactions of charged peripheral groups, was used as the solvent. Water, ethanol quenched fluorescence and so did toluene.

With both tryptophan and α-amanitin, information available from fluorescence changes were consistent with a chemical modification of the initial

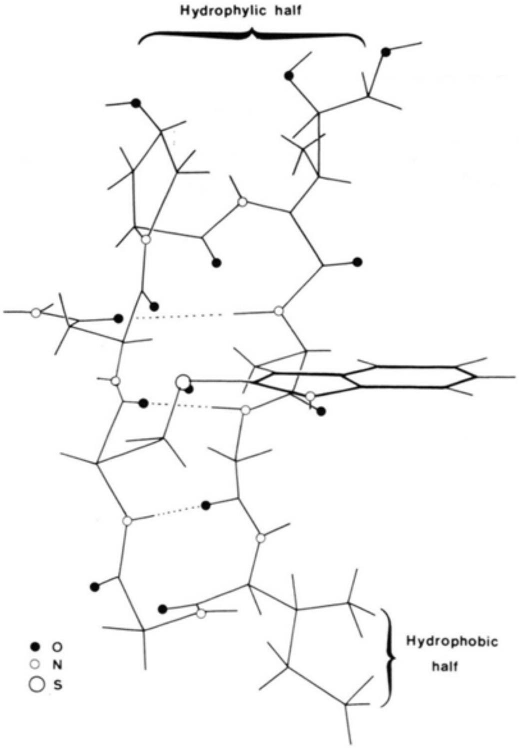

Fig. 1. A view of a steric model of α-amanitin emphasizing the analogy of the molecule with a DNA sequence: the indole ring may be looked upon structurally as adjacent base pairs of the DNA helix and the polar asparagyl-hydroxy prolyl-dihydroxyisoleucyl moiety as the hydrophylic osidic region.

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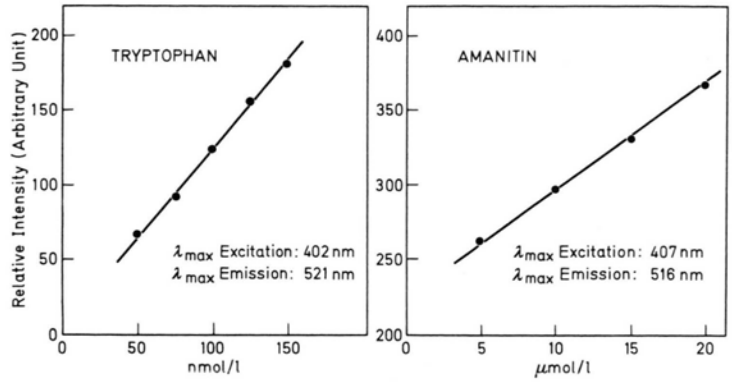
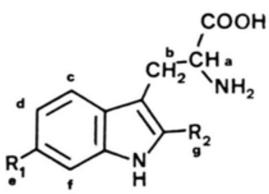


Fig. 2. Quantitative detection of fluorescent derivatives of tryptophan and amanitin.

structure. Thus, in the presence of phosphoric acid and at a temperature of about 90 °C, the excitation wavelength value (402 nm) observed corresponding to a weak transition energy, was related to the formation of a more extended aromatic structure. An intramolecular cyclization might be involved leading to previously described pyrrolo[2,3-b]indole [16] pyrido[4,3-b]indole or pyrido[2,3-b]indole [17] rings, when the 2-position of the indole moiety is attacked, or leading to the benz[c-d]indole heterocycle **2a** [18−20] when ring closure occurred at the 4-position.

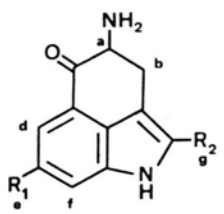
In similar experimental conditions, α-amanitin could be first degraded to a tryptophan derivative, the tryptathionin **1a** as stated by Palyza [15]: the easy accessibility of the indole ring is clearly shown on Fig. 1.

Then, under above Friedel-Crafts conditions, tryptathionin would be converted to a tricyclic compound **2b**, a derivative of the benz[c-d]indole ring whose excitation fluorescence wavelength value (407 nm) is close to that of the **2a** compound.



1a R_1 = H
R_2 = H
Tryptophan

1b R_1 = OH
R_2 = S(O)CH_2CH(NH_2)COOH
Tryptathionin



2a
b

The cyclization to a pyrrole or pyrido − indole involving the 2-position of the indole ring was obviously impeded by the presence of a bulky thione-substituent in the tryptathionin structure.

The formation of a benz[c-d]indole ring was substantiated by ^{1}H NMR spectroscopic study. The tryptophan model was submitted to heating up to 95 °C in $^2H_2O/^2H_3PO_4$ 83%, 1/1 (V/V) medium and the chemical transformations were followed by NMR spectroscopy. NMR spectrum of **1a** was not modified by heating from 25 °C to 75 °C: a: 4.25 (1 H, t); b: 3.31 (2 H, d); J_{a-b} = 6 Hz; c: 7.63 (1 H, d); d: 7.32 (1 H, dd); e: 7.25 (1 H, dd); f: 7.55 (1 H, d); J_{c-d} = 8.3 Hz; J_{e-f} = 8.2 Hz; J_{d-e} = 7.0 HZ; g: 7.28 (1 H, s).

Raising the temperature to 85 °C and moreover to 95 °C caused significant modifications in the aromatic region. **2a**: a: 4.28 (1 H, t); b: 3.36 (2 H, d); J_{a-b} = 6 Hz; d: 7.56 (1 H, d); e: 7.24 (1 H, dd); f: 7.51 (1 H, d); J_{d-e} = 7.0 Hz; J_{e-f} = 8.2 Hz; g: 7.26 (1 H, s).

Thus NMR data unambiguously established that cyclization occurs at the 4-position of the indole ring: signal attributed to *c*-proton disappears and the downfield shift of *d*-proton is a reflection of the greater deshielding effect of the neighboring carbonyl group.

Finally, our results strongly suggest that fluorescent compounds obtained from these tryptophan derivatives by phosphoric acid treatment and heating to 85 °C are relevant to a Uhle's ketone form [18].

Thus, reported here was a more accurate detection method for toxic products from *Amanita phalloides*. Moreover, this method may be extended to other tryptophan peptides.

Acknowledgements

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