PEPTIDES OF *AMANITA PHALLOIDES*

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In central European forests two species of tuber leaf mushrooms are found from August until October: the yellow *Amanita mappa* and the greenish *Amanita phalloides*. The former contains no poisonous substance, whereas the latter is responsible for about one hundred victims annually all over the world. It contains several fatal poisonous cyclicpeptides, the chemistry of which will be discussed in this paper.

Serious investigation of these toxins was begun early this century by Ford and his colleagues in the U.S.A., who, however, did not succeed in isolating pure substances[1]. In the early thirties work was resumed in Heinrich Wieland's laboratory in Munich. In 1937 Lynen and U. Wieland[2] obtained a rapidly acting posion in crystalline form and designated it Phalloidin. Four years later an even more toxic component was crystallized and called Amanitin by H. Wieland and Hallermayer[3]. In 1959, together with O. Wieland, I reviewed the state of research[4]. At that time five toxins were known and identified; the paper chromatogram, shown schematically in *Figure 1*, of a purified extract of *A. phalloides* indicates the situation today.

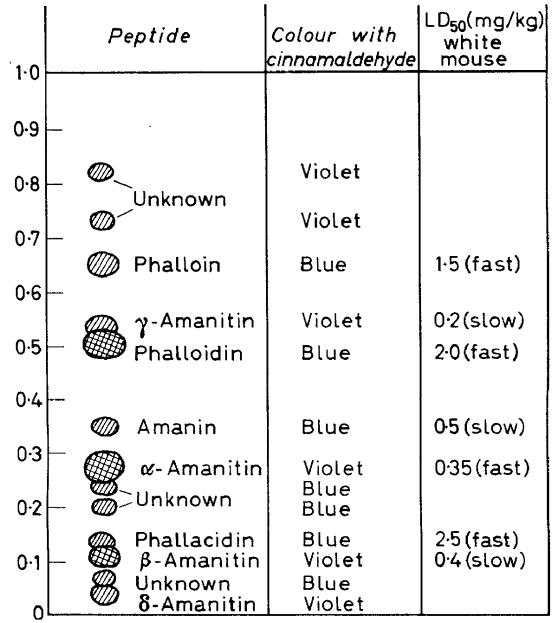


Figure 1. Components of *A. phalloides*

The diameters of the spots, and the closeness of hatching, represent the relative concentrations.

The number of defined toxic components has now increased to eight, which can be divided into two groups: (*i*) The more poisonous, slowly acting amanitines, which give a very sensitive violet colour reaction with cinnamaldehyde and hydrochloric acid and (*ii*) the less toxic, but more rapidly acting phalloidines, whose blue colour reaction with the same reagent is ten times weaker. Amanin, a very rare cyclopeptide which was characterized only a few months ago by Boehringer[5] in my laboratory occupies an intermediate position. It shows a blue colour reaction like the phalloidines and acts slowly like the amanitines. All these cyclopeptides are indole derivatives but they differ in their ultraviolet light absorption spectra (*Figure 2*).

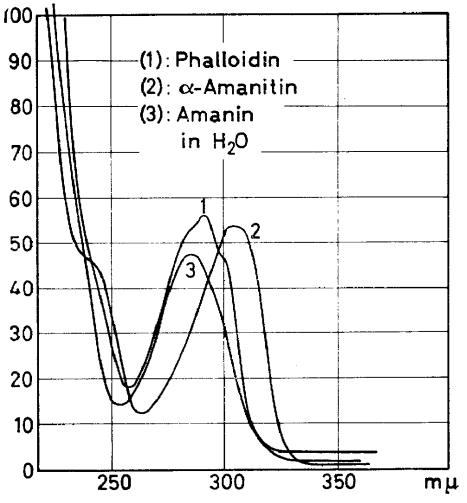
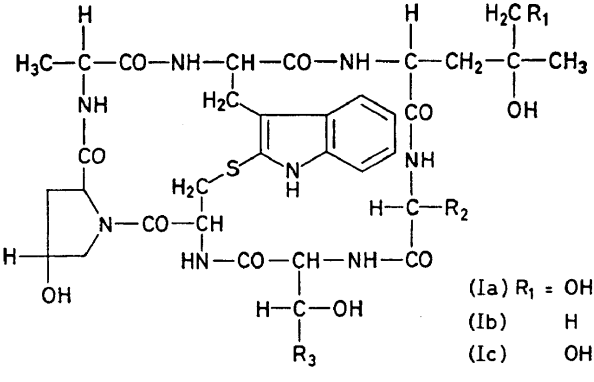
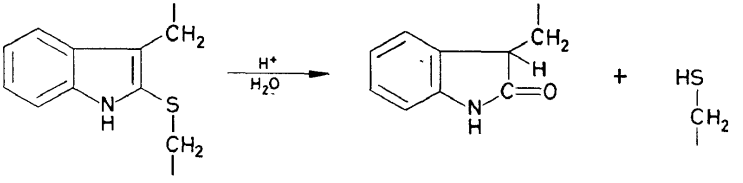


Figure 2. Ultraviolet spectra of phalloidin (1), α-amanitin (2) and amanin (3)

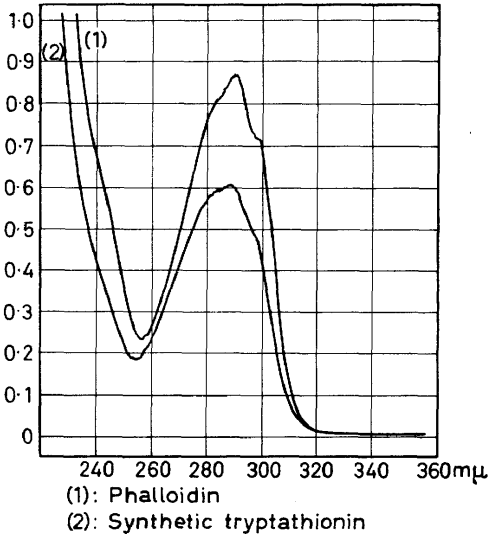
In this paper I shall concentrate mainly on the hitherto unknown elucidation of the structures of the amanitines and of amanin. The work resembles that used to determine the structures of phalloidin[6] (Ia), phalloin (Ib), and phallacidin (Ic).



Of the constituents, which are set free by hydrolysis of these latter compounds with 6N hydrochloric acid, only L-alanine is a normally occurring amino-acid. Hydroxy-proline belongs to the allo-series and threonine occurs in the D-form. The sulphur bridge, which probably results from oxidative coupling of cysteine with tryptophan, is split to L-cysteine and L-β-oxindolylalanine by acid hydrolysis.



The characteristic ultraviolet spectrum ($\lambda_{max.} = 292$ mμ) is due to this α-thioether of tryptophan. Compounds of this type can be synthesized by coupling β-substituted indoles with several sulphenyl chlorides and they exhibit light absorption properties very similar to those of phalloidin (*Figure 3*).



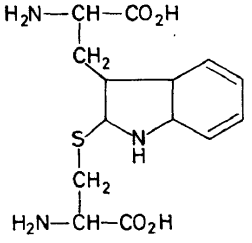
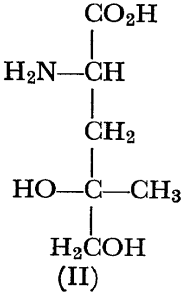
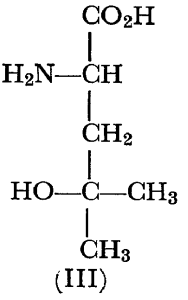


Figure 3. Ultraviolet spectra of phalloidin and synthetic tryptathionin

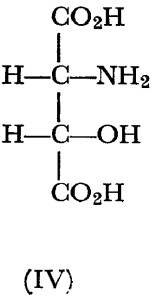
Among the products of hydrolysis of phalloidin and phallacidin a previously unknown amino-acid was found, L-erythro-γ,δ-dihydroxyleucin (II) which easily forms a γ-lactone.



(II)



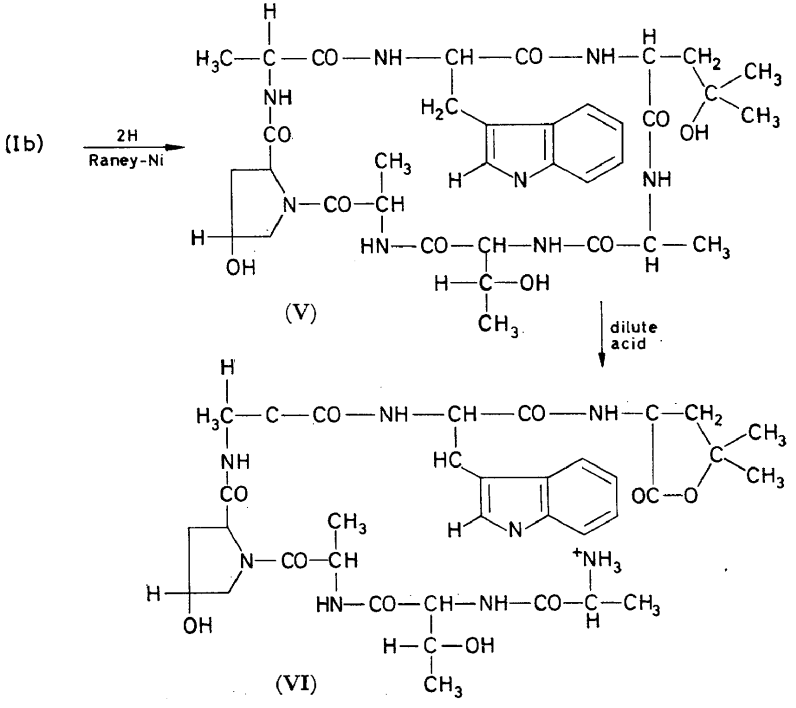
(III)



(IV)

γ-Hydroxyleucin (III), an analogous constituent of phalloin (Ib), was already known but had not previously been detected in Nature. It forms a γ-lactone equally easily. As mentioned later, lactonizing amino-acids are also structural elements of the other toxins of *A. phalloides*. They cause selective splitting of the peptide bond due to the neighbouring group effect of the γ-hydroxyl.

Phallacidin (Ic) has the properties of an acid. From its hydrolysate with hydrochloric acid—(not with sulphuric acid, which gives elimination of water) another unusual amino-acid, D-erythro-β-hydroxyaspartic acid



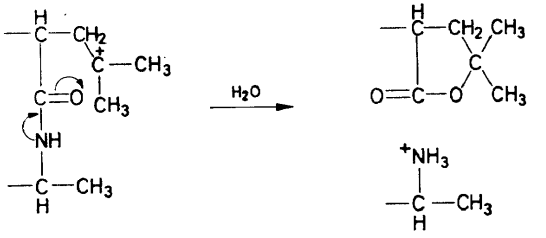
(IV), was isolated. The acid corresponds to D-threonine of the other toxic peptides, presumably also in biogenetic relation even though it has the erythro configuration. One more difference between phallacidin and phalloin is the occurrence of L-valine instead of L-alanine.

The elucidation of structure will now be summarized using phalloin as an example.

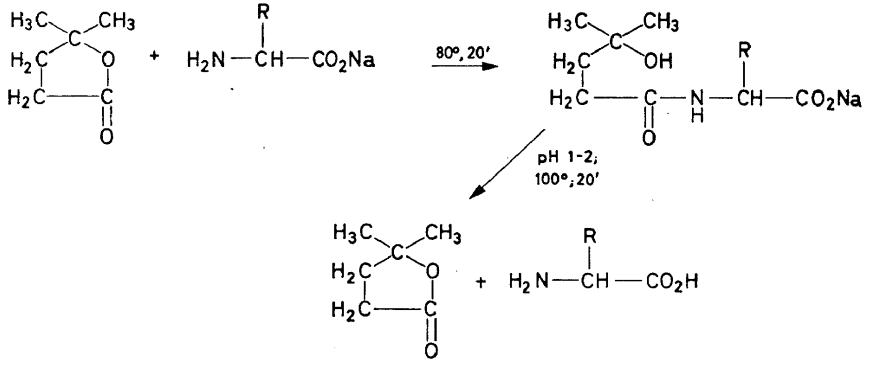
(*i*) The first step was the hydrogenolytic substitution with Raney-nickel of sulphur by two hydrogen atoms, yielding the non-toxic dethiophalloin (V).

(*ii*) Selective opening of the peptide ring took place, when (V) was heated with dilute acids for a short time. The peptide bond between the carboxyl of a γ-hydroxyaminoacid and nitrogen was split with formation of the lactone (VI) of dethio-seco-phalloin.

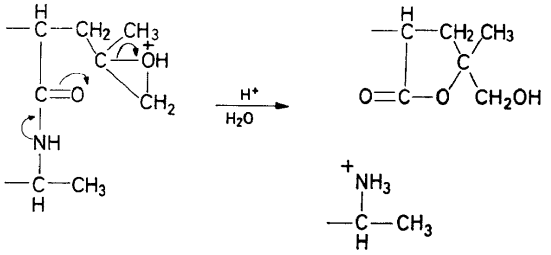
The reaction presumably occurs *via* a carbonium ion intermediate:



The principle of this reaction has been used by us[7] to develop a protecting group in peptide chemistry: the γ-hydroxy isocaproyl residue is introduced by heating a sodium salt of an amino-acid with isocaprolactone in imidazole. It is removed at pH 1–2 and 100° in 20 minutes.



An even milder regeneration namely by incubation with 0·5N acetic acid at 20° for 10 minutes, is possible if the side chain bears a γ,δ-epoxide ring[8]:

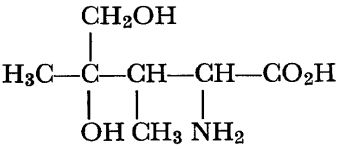


(*iii*) To establish the sequence of amino-acids in the heptapeptide (VI) the Edman degradation method was applied. It yielded successively the phenylthiohydantoin derivatives (PTH) of alanine, D-threonine, alanine (from cysteine), allo-hydroxyproline, alanine and tryptophan, thus disclosing the structures of phalloin, phalloidin and, with the appropriate mutations, of phallacidin[9].

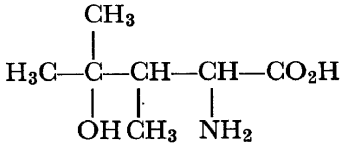
THE STRUCTURE OF THE AMANITINES

The determination of the structures of the amanitines proved more difficult. Annemarie Höfer[10] and, recently, Pfaender[11], Gebert[12], and Wehrt[13] contributed to the solution of the problem. It was proved many years ago by Boehringer[14], that α-amanitin is the amide of β-amanitin and that β-amanitin contains a lactonizing amino-acid which differs from that of α-amanitin[15].

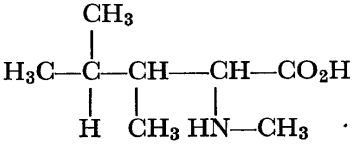
α-Amanitin yields on acid hydrolysis the lactone of β-methyl-γ,δ-dihydroxyleucine (VII). Most likely the corresponding building stone of γ-amanitin is (VIII), the relation to the γ-hydroxy compound (VII) being the same as that between the lactonizing amino-acids of phalloin and phalloidin. The carbon skeleton of this new amino-acid also occurs in *N*-methyl-β-methylleucine (IX), an amino-acid isolated from the antibiotic Etamycin by Sheehan *et al*[16].



(VII)



(VIII)

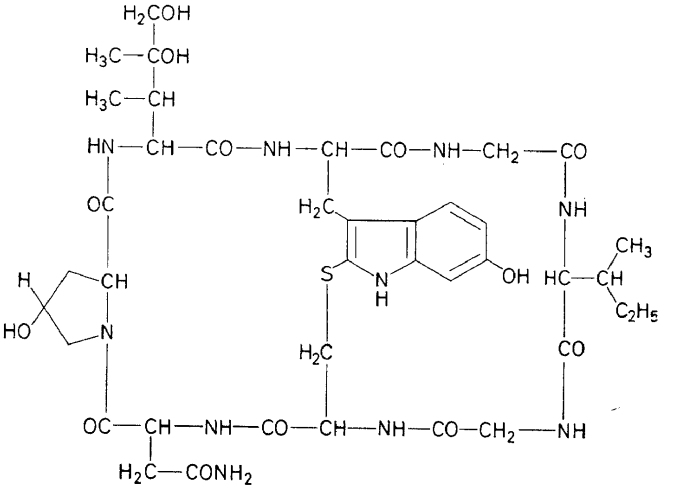


(IX)

The amanitins also contain sulphur, which is incorporated between an indole nucleus and the periphery. They reduce silver ions in ammoniacal solution. The ultraviolet light absorption maximum (302 mμ) is shifted

to higher wavelengths by alkali, furthermore they give azo dyes with diazonium salts (Pauly reaction), thus showing the presence of a phenolic hydroxyl group. The indole system of the amanitins is extremely unstable towards hydrolysis by strong acids. In this case the cysteine moiety is partially converted into cysteic acid, $HO_3S \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$.

As with phalloin, the different steps in the elucidation of the structure of α-amanitin, (X) are shown using the finally established formula. The first substantial progress was made by treating the toxin with Raney-nickel. This gave a non-toxic sulphur-free compound, dethio-α-amanitin, which exhibits ultraviolet light absorption very similar to that of 6-hydroxy-tryptophan (*Figure 4*).



(X) α—Amanitin
(XI) β—Amanitin: —COOH instead of $CONH_2$

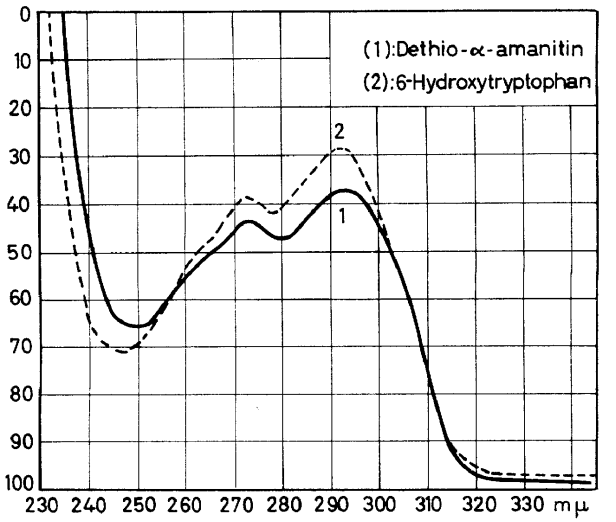
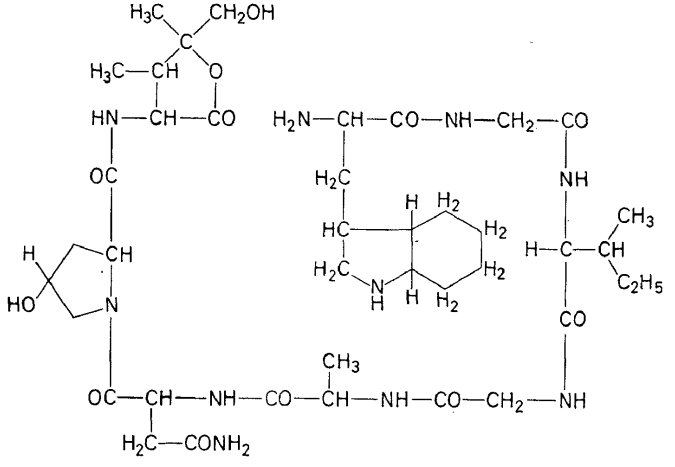


Figure 4. Ultraviolet spectra of dethio-α-amanitin and 6-hydroxytryptophan

Hence it follows that the phenolic hydroxyl-group is attached to the 6-position. The suspected 6-hydroxytryptophan of dethioamanitin, however, is not stable enough to survive the partial hydrolysis now necessary as the next step. To stabilize the side chain a hydrogenation over platinum was carried out. In this reaction not only saturation of the aromatic system takes place but also elimination of the phenolic hydroxyl-group. A peptide of octahydrotryptophan is obtained, perhydro-dethioamanitin, which migrates electropheretically to the cathode on account of its basicity. Now the selective opening of the peptide ring with weak acids gave the octapeptide secoperhydro-dethioamanitin (XII).



(XII)

Total hydrolysis of (XII) gave the following amino-acids: glycine, isoleucine, alanine (formed from cysteine by treatment with Raney-nickel), hydroxyproline (the normal, not the allo-isomer), aspartic acid, octahydrotryptophan and β-methyl-γ,δ-dihydroxyleucine. None of these amino-acids disappeared on incubation with D-amino acid oxidase, thus suggesting that all of them belong to the L-series.

The Edman degradation method with a similar octapeptide, whose indole nucleus was only partially hydrogenated, gave the PTH-derivatives of the amino-acids which, beginning from the amino-end of the chain, were obtained in the sequence shown in *Figure 5*†.

The sole structural element not directly proved by the reactions described above is the 2-position of the thioether bridge. We tried to furnish evidence by comparison of the ultraviolet spectra of amanitine and synthetic model substances. Grimm[17] obtained, *e.g.*, the thioether (XIII) by reacting 2-methyl-6-methoxyindole with ethylsulphenylchloride.

† *Note added in proof:* On re-examination it was found that the positions of isoleucine and 6-hydroxytryptophan have to be exchanged, leading to a structural formula which differs from that reported earlier.

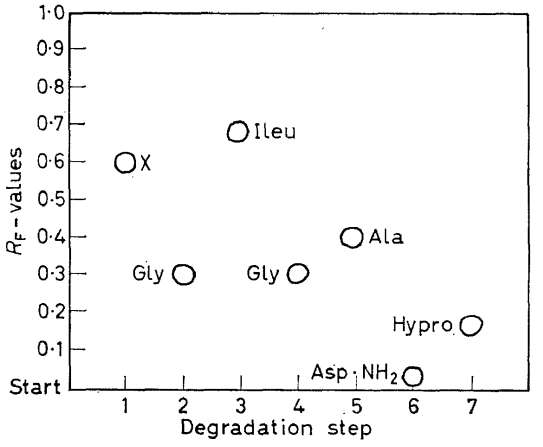
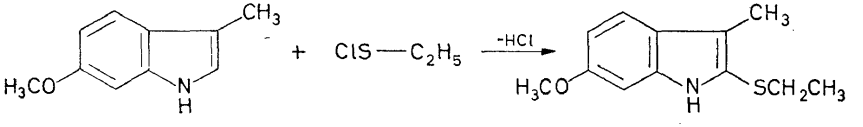


Figure 5. Thin layer chromatogram of PTH-derivatives in Edman degradation of (XII)

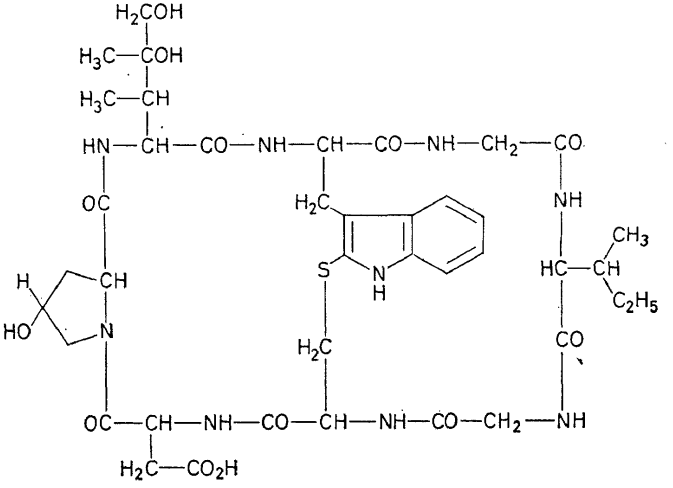


(XIII)

These substances show ultraviolet absorption spectra, which resemble that of amanitin in the position of $\gamma_{max.}$ but not in the shape of the peak. All other positions of the sulphur bond, however, are unlikely for reasons which cannot be explained in detail here. In this situation the investigation of amanin brought clarification.

Amanin

Amanin (XIV), the poison with the amanitin-like toxic action and with



(XIV) Amanin

the colour reaction of phalloidin, gives on hydrolysis nearly the same amino-acids as α-amanitin, the sole difference being the formation of cysteine (instead of cysteic acid) and of β-oxindolyltryptophan—as with phalloidin. Dethioamanin, produced by treatment of amanin with Raney-nickel exhibits—like the phalloidin product—the indole-spectrum of tryptophan. According to these observations amanin must be an amanitin with the indole moiety of phalloidin—but unfortunately its ultraviolet spectrum does not correspond to that of phalloidin (*Figure 2*). This

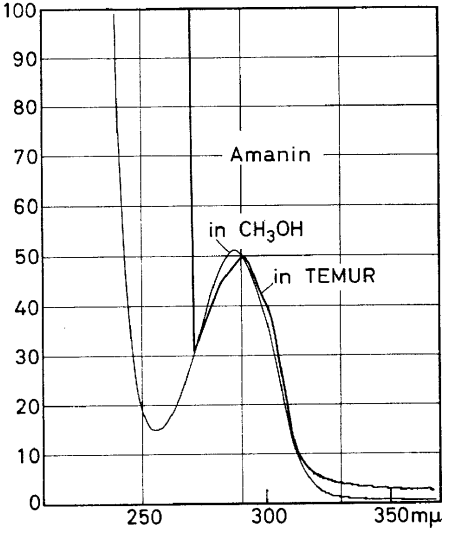


Figure 6a. Ultraviolet spectra of amanin in CH_3OH and in TEMUR

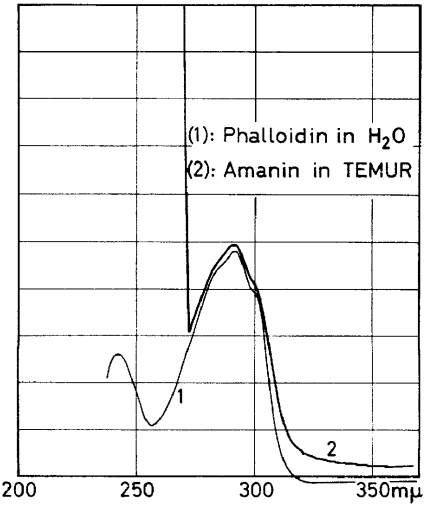


Figure 6b. Ultraviolet spectra of phalloidin in water (1) and of amanin in TEMUR (2)

discrepancy might arise from differences in the molecular environment of the chromophoric systems.

In the latter the imino-group of the indole nucleus be may situated next to the oxygen of a peptide linkage making possible a hydrogen bond. In accordance with this suggestion the shape of the ultraviolet peak is clearly different in methanol and in a hydrogen bond breaking solvent such as tetramethylurea (*Figure 6a*).

The spectrum of amanin in TEMUR is almost identical with that of

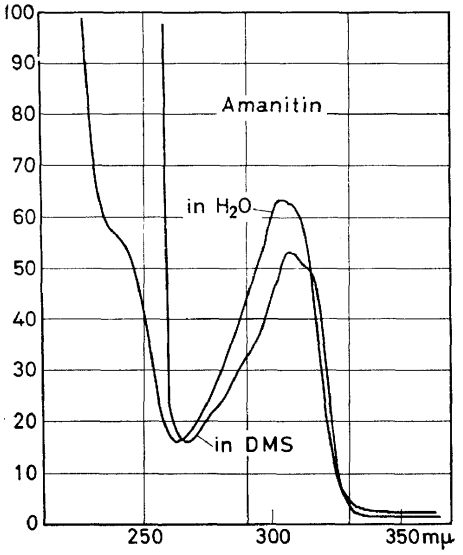


Figure 7a

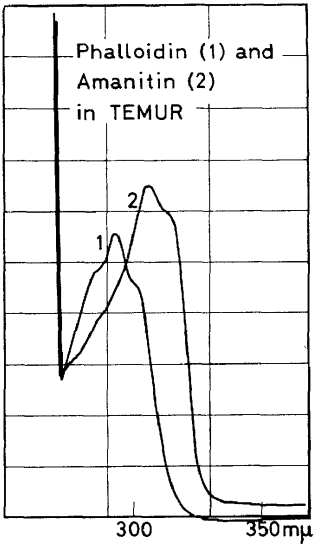


Figure 7b

phalloidin (in water) (*Figure 6b*) and the true character of the compound is now evident.

The amanitines also show more articulate ultraviolet spectra in solvents like DMS, DMF or TEMUR than in water or methanol (*Figure 7a*). With its shoulder, especially in the descending part, the curve of amanitin nearly fits that of phalloidin—apart from the shift to higher wavelengths due to the phenolic hydroxyl group (*Figure 7b*). One can also recognize distinct similarities between amanitin and the model thioether (XIII) (*Figure 8*).

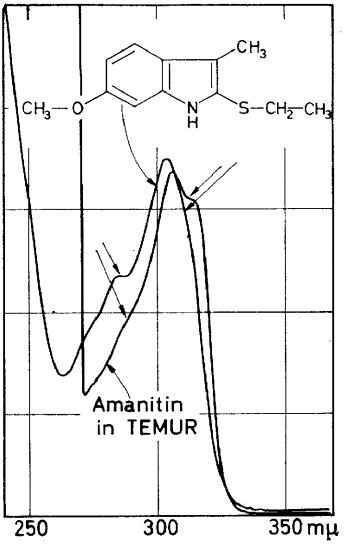
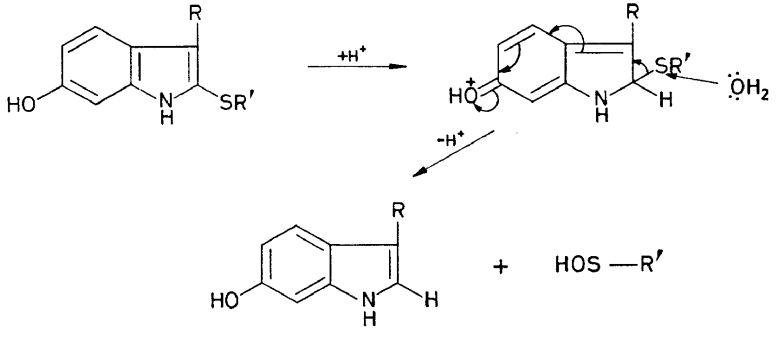


Figure 8

The shoulders in the ascending and in the descending branch are situated at the same wavelengths; the remaining difference could be sought due to an extra interaction of the 6-hydroxy group of amanitin with other parts of the peptide ring. We are therefore able to formulate the structure of α- and β-amanitin as (X) and (XI). As mentioned earlier, the sulphur bridge of the amanitines on hydrolysis gives cysteic acid (besides cysteine). An explanation for this unusual "electrophilic" hydrolysis, which differs from the "nucleophilic" hydrolysis of phalloidin and amanin is indicated.



Finally, I should like to record my appreciation of the invaluable help provided by my various co-workers in bringing this investigation of the toxic constituents of A. phalloides to a satisfactory conclusion.

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[1] For a complete collection of literature concerning the history of research on *A. phalloides* see in H. Raab. *Z. Physiol. Chem.* **207,** 157 (1932).
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