CHEMICAL AND TOXICOLOGICAL STUDIES WITH CYCLOPEPTIDES OF *AMANITA PHALLOIDES*

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INTRODUCTION

Cyclic peptides have attracted much interest during the past few years for several reasons. Chemists studying problems of protein conformation can profit from knowledge about the stereochemistry of the peptide bond. Investigators in the field of natural products soon took charge of the new class of compounds after the antibiotic properties of Gramicidin S had been recognized. This cyclic peptide was followed by a great number of other homodetic and heterodetic cyclopeptides, which display not only bacteriostatic but also toxic action[1]. On account of their different effects, cyclopeptides are also a source of great interest to the physiologist, pharmacologist and biochemist. One of the main tasks of biological and chemical research is to get information about the mechanism of these different actions. Although we are still a long way from understanding them, I should like now to consider the relationships between constitution and activity in the toxic substances of the poisonous mushroom *Amanita phalloides*.

CHEMISTRY OF THE TOXIC SUBSTANCES[2]

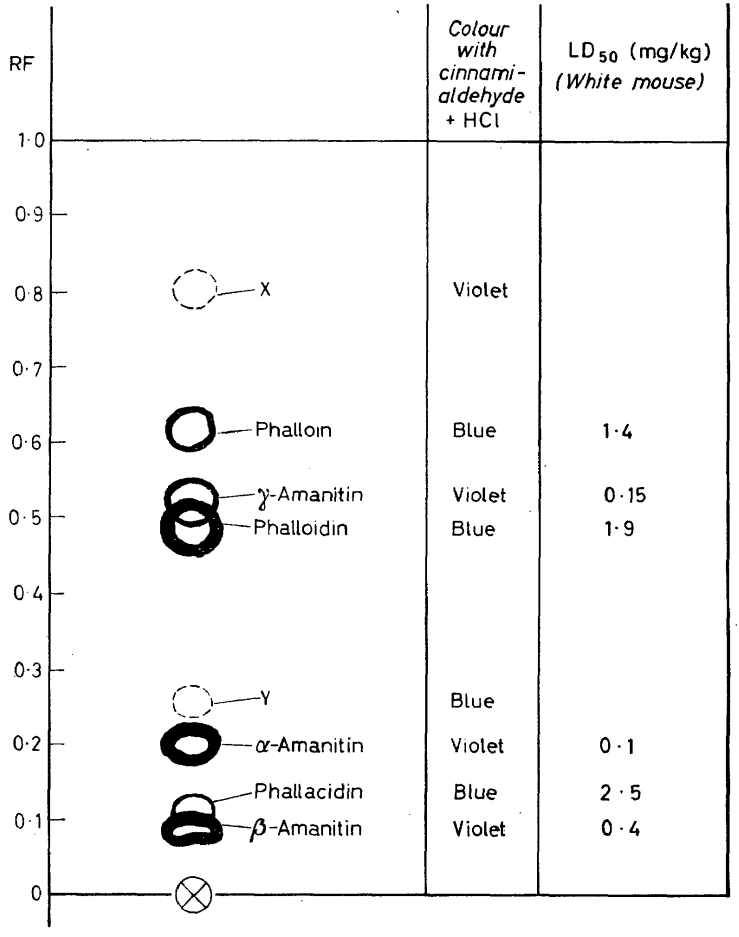
The methanolic axtracts of *A. phalloides* contain, besides many ballast substances, the phytotoxins summarized schematically in *Table 1* according to their RF-values and the colours produced on reaction with cinnamaldehyde and hydrochloric acid. The thickness of the outlines of the different spots corresponds to the relative concentrations of toxins. Two groups of substances can be recognized, the more toxic *amanitins*, which give a very sensitive violet colour reaction, and the less toxic but more rapidly acting *phalloidines*, which become blue in a much less sensitive colour reaction with the above mentioned reagent. Both of these classes are derivatives of indole, but they differ in their ultra-violet light absorption spectra (*Figure 1*).

Phalloidin group

The structural formulae of phalloidin (Ia), phalloin (Ib) and phallacidin (Ic)[3], which were established in our laboratory[4], are shown on page 341.

Of the building units only the alanine molecules are normally occurring amino-acids (L-forms). Hydroxyproline belongs to the allo-series and threonine (in Ia and Ib) occurs in the D-form. The sulphur bridge coming from an oxidative coupling of L-tryptophane with L-cysteine is hydrolysed giving L-cysteine and oxindolylalanine by boiling with strong acids. The characteristic ultra-violet spectrum is due to this α-thioether bridge, which can also

Table 1. Schematic representation of a paper chromatogram of the toxic ingredients of *A. phalloides*



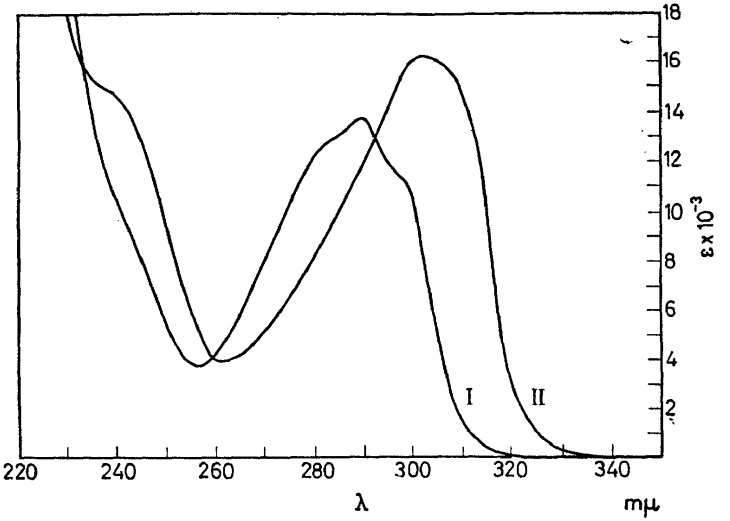
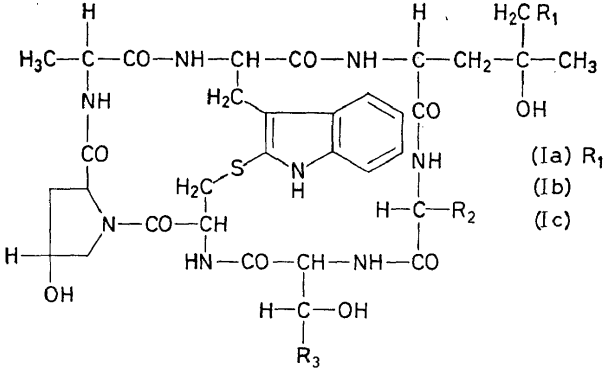
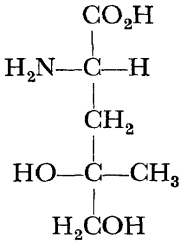


Figure 1. Ultra-violet light absorption spectra in water of phalloidin (I) and amanitin (II)

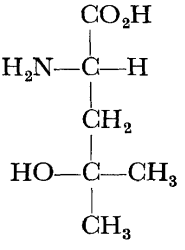


(Ia) R_1 = OH R_2 = CH_3 R_3 = CH_3
(Ib) H CH_3 CH_3
(Ic) OH $CH(CH_3)_2$ CO_2H

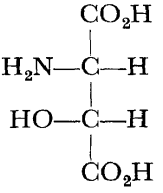
be formed synthetically. A new natural substance, the γ-lactone of γ,δ-dihydroxyleucine, was discovered in the hydrolysate of phalloidin (Ia) and phallacidin (Ic). Of the four possible diastereomeric compounds, the amino-acid was identified as the L-erythro compound II.



(II)



(III)



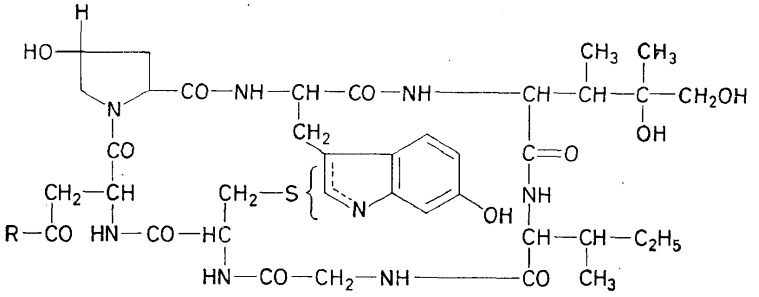
(IV)

The only difference in phalloin (Ib) compared with phalloidin is the presence of a hydrogen atom instead of the hydroxy group in the δ-position of the branched amino-acid. γ-Hydroxyleucine (III) was already known, but had not previously been found in nature. It also forms a lactone easily.

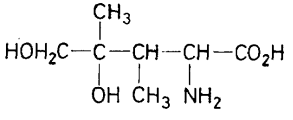
Phallacidin (Ic), recently isolated in a pure state, shows the same ultraviolet spectrum as the former compounds, but has acidic properties. From the hydrolysate a hydroxyaminodicarboxylic acid was obtained which turned out to be D-erythro-β-hydroxyaspartic acid (IV). It certainly corresponds to D-threonine in phalloidin although it possesses the erythro-configuration. Another difference between phallacidin and phalloidin (phalloin) is the occurrence of L-valine instead of alanine.

AMANITIN GROUP

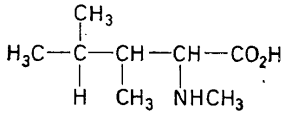
Our knowledge of the chemistry of the more toxic amanitins is less complete on account of the lability of the chromophoric group. Here also there is a bicyclic structure with a sulphur bridge. The bridge, however, is not the same as in phalloidin, because after acidic hydrolysis the bound cysteine appears as cysteic acid, whereas the aromatic bridge head changes its ultra-violet spectrum in a characteristic manner. The same holds for alkaline hydrolysis which also takes place very easily. Formula (V), a hypothetical one, contains the amino-acids that have been detected in a probable sequence. These are hydroxyproline (not the allo-form), L-aspartic acid, glycine, isoleucine, and a further lactonizing, branched amino-acid (VI), which contains one more methyl group than compound (II). Its structure was established as β-methyl-γ,δ-dihydroxyleucine, a structure which has the same carbon skeleton as the side chain of ergosterol. The same is true for *N*-methyl-β-methyl-leucine (VII) which has been discovered as a building block of the antibiotic Etamycin by Sheehan *et al*[5].



(Va) α-Amanitin R = NH_2
(Vb) β-Amanitin R = OH



(VI)



(VII)

The relation of α- to β-amanitin (Vb) is that of an acid amide to the corresponding carboxylic acid. α-Amanitin was obtained from β-amanitin with ammonia *via* the mixed anhydride with propionic acid. In γ-amanitin, recently obtained in an analytically pure state[6], there is once again an amino-acid capable of lactonization; it certainly differs from (VI), perhaps by the absence of the γ-hydroxyl group.

Pharmacology and biochemistry of amanita poisoning[7]

The actual intoxication by the crystalline phytotoxins manifests itself by morphologic changes of the parenchymatous organs, notably of the liver which is attacked first. The action of phalloidines and amanitines seems to be essentially similar, but there are great differences in their toxicities and in the onset of their effects: α-amanitin is about 20 times more toxic than phalloidin, but with a lethal dose of the latter death in mice occurs within 1 hour, whereas α-amanitin even in large amounts shows its effect only after 20 hours. The central organ to be affected is the liver, which 1 hour after intoxication with phalloidin has already increased in weight by 30–60 per cent due to haemorrhagic bleeding. After a short period the ability to form glycogen from added glucose is lost. The blood glucose rises initially and then drops to subnormal levels shortly before death.

The experiments described below have been carried out in the laboratory of Otto Wieland in Munich, using the rapidly acting phalloidin. In order to obtain results which were not influenced by regulations of the whole organism, an isolated perfused rat liver preparation was used[8]. Among other results, it appeared that the carbohydrate metabolism is not affected directly by phalloidin, although this seemed to be the case in experiments with whole animals. Without poison, a glucose level of 120–150mg per cent is set up after 60 minutes perfusion, and then remains constant. In the presence of phalloidin, blood sugar slowly rises, but only after one hour. After addition of 2,4-dinitrophenol or glucagon, however, a sudden rise is observed, indicating the readiness of the isolated liver for glycogenolysis. Accordingly, the carbohydrate metabolism is only gradually affected by the toxin. More immediate is its effect on the secretory performance. In the presence of 10^{-4} M phalloidin, bile secretion stops within 15 minutes. Thus, the elimination of bromosulphthalein and bilirubin is blocked immediately after administration of phalloidin, as shown in *Figure 2*. The slower acting α-amanitin produces this effect only after 4 hours. Simultaneously, a strong excretion of different liver enzymes, notably of the glutamic-oxalacetic-transaminase (GOT) takes place (*Figure 3*). The features first observable biochemically point to a reaction of phalloidin with cell and tissue structures of the liver. The microsomes show an especially marked affinity. This has been shown in experiments with a radioactive labelled toxic derivative of phalloidin, which was used in the perfused liver preparation instead of the original toxin[9]. After subsequent homogenization of the liver and differential centrifugation, radioactivity was found in cell nuclei and débris, mitochondria and microsomes. The activity can be washed out with more-or-less difficulty by re-suspending the materials in fresh buffer and centrifugation. As can be seen in *Figure 4* only the microsome fraction retains a constant amount of activity after the second washing. This

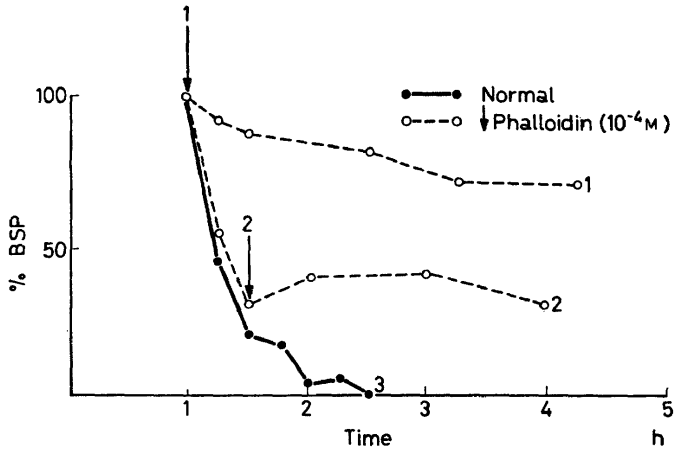


Figure 2. Excretion of bromosulphthalein by isolated rat liver with and without phalloidin (10^{-4} M)

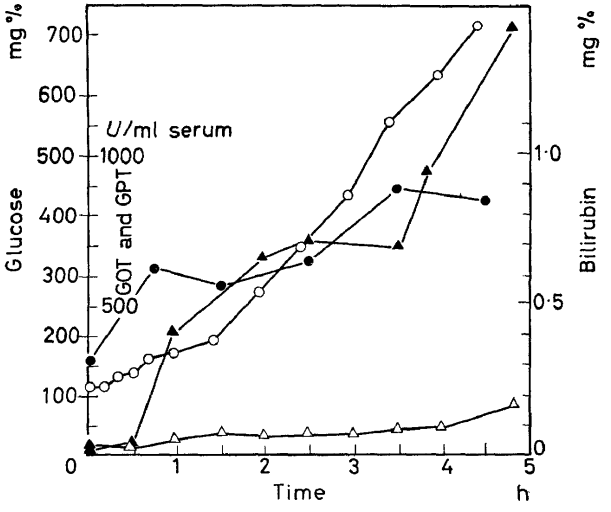


Figure 3. SGOT (▲—▲), SGPT (△—△), Glucose (○—○) and bilirubin (●—●) in serum of a rabbit after poisoning with phalloidin

corresponds well to the results of Hultin *et al.*[10], who found that incorporation of amino-acids into the protein of the liver microsome fraction is inhibited after poisoning the animals with phalloidin 1–2 hours before.

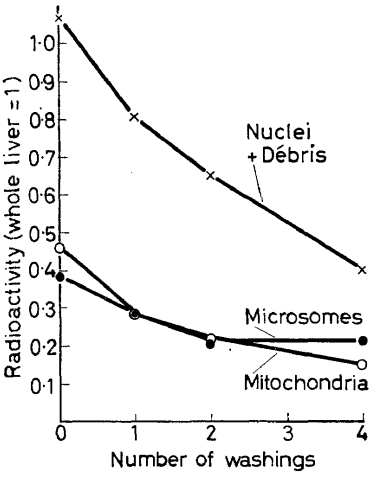


Figure 4. Retention of radioactivity in particles of rat liver cells intoxicated with ^{35}S-mercaptophalloidin (XIII) *in vitro* after several washings

Chemical variations of phalloidin

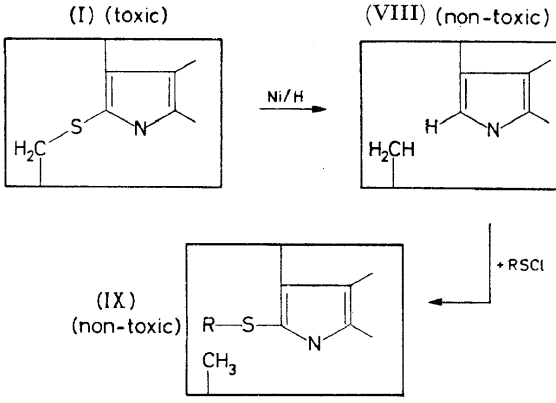
To help in understanding the very complicated action, and possibly the mode of fixation of the toxic substance, the phalloidin molecule was systematically altered and then tested for toxicity. Common to all amanita toxins are

(*i*) a bicyclic molecular structure
(*ii*) the presence of a γ-hydroxy amino-acid and, presumably,
(*iii*) the occurrence of a D-amino-acid.

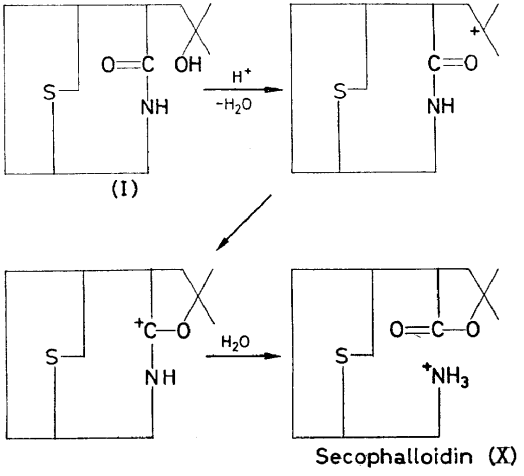
These features will now be considered separately.

(*i*) The sulphur bridge can be removed by heating the toxins with Raney nickel in a hydrogenolytic reaction, yielding a monocyclic desthiopeptide (VIII, a, b or c as in I). These desthio compounds are not toxic, even in concentrations a hundred times greater than those used with the toxins. However, it is not merely a thioether group, attached to the α-position of the indole nucleus, which is responsible for the toxic properties: the monocyclic thioether (IX), prepared by subsequent introduction of RS into desthiophalloidin (VIIIa)[11], also showed no toxicity.

(*ii*) The γ-hydroxy group of the branched amino acid, present in all of the toxins, is the reason for a preferential splitting of the peptide bond derived from its carboxylic group. The peptide ring of phalloidin is opened giving secophalloidin (X) even on standing at room temperature for 1/2 hour in 50 per cent trifluoroacetic acid. This splitting also occurs in the presence of weak bases. Accordingly, acyl groups with similar side chains can be used as *N*-protecting groups in peptide chemistry[12].



Though the seco-compounds likewise exhibit no toxic action, a fixation mechanism could be suggested which consists in a similar reaction of the appropriate peptide bond with a specific nucleophilic group of the cell structure. However, this is unlikely to be true, since keto-phalloidin (XI),



H^+-catalysed ring opening of phalloidin

which is obtained from phalloidin by oxidation of the branched glycol side chain by periodate, retains the whole activity although there is no γ-t OH group.

We come now to derivatives of phalloidin, in which toxicity remains more or less unaltered. These are molecules, whose bicyclic structure is intact and whose periphery has been changed in different ways[13]. Nature itself provides three examples of this class of compounds with her three variants of phalloin (Ia, b and c). Artificially, modifications can be brought about at the branched side chain of phalloidin or phallacidin and at the carboxylic group of the latter compound. In both cases derivatives were obtained which still showed toxicity. *Table 2* represents a series of transformations.

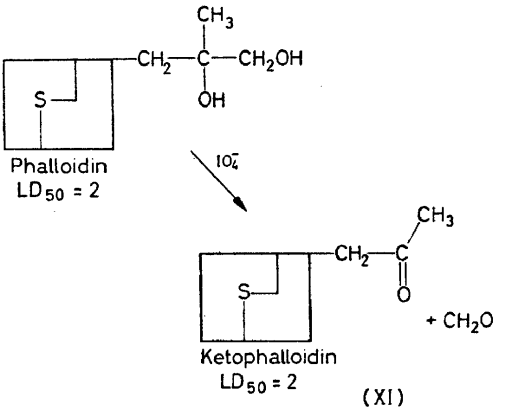
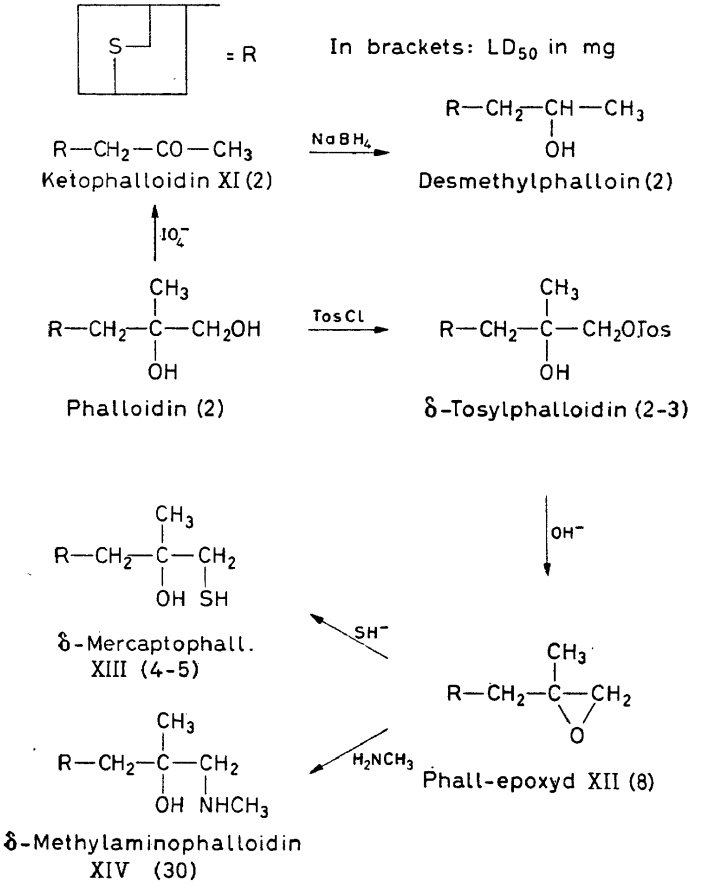

Phalloidin
LD50 = 2
IO4-
Ketophalloidin
LD50 = 2
(XI)
+ CH2O

Table 2


= R
In brackets: LD50 in mg
Ketophalloidin XI (2)
NaBH4
Desmethylphalloin (2)
IO4-
Phalloidin (2)
TosCl
δ-Tosylphalloidin (2-3)
OH-
SH-
δ-Mercaptophall.
XIII (4-5)
Phall-epoxyd XII (8)
H2NCH3
δ-Methylaminophalloidin
XIV (30)

δ-Mercaptophalloidin (XIII) with ^{35}S has been used in the above mentioned experiments to follow the absorption of the toxic substance by different cell particles. The lower toxicity of phall-epoxyd (XII) is probably due to hydrolytic ring opening with formation of the non-toxic secophalloidin (X), which occurs here with particular ease. δ-Methylaminophalloidin (XIV) perhaps exhibits, on account of its basicity, different permeation properties and may therefore be less toxic. Phallacidin (Ic) is a carboxylic acid which can be transformed into its amide by reaction of a mixed anhydride with ammonia[3]. The amide of phallacidin is a little less toxic (LD_{50} = 4–5) than phallacidin, in contrast to the corresponding situation in the amanitin field. There the amide (α-aminitin Va) is four times more toxic than the carboxylic acid β-amanitin (Vb).

By means of the mixed anhydride method several other derivatives were prepared from β-amanitin[14]. RF-values and toxicities are summarized in *Table 3*. Here one dependence is visible: the more lipophilic a substance is, the less is its toxicity.

Table 3. Toxicity and RF of some derivatives of β-amanitin

Substance	LD_{50} (μg/kg, mouse)	R (*ref. to amanitin*)
α-Amanitin	39	1·00
β-Amanitin	97	0·37
β-Amanitinmethyl-ester	760	1·30
β-Amanitin-thiophenyl-ester	600	1·72
β-Amanitinanilide	4450	1·65
β-Amanitin-dodecyl-amide	>4000	2·63

Up to now it appears that only the bicyclic structure can produce toxicity. It might be concluded that to obtain a toxic substance the synthesis of some similar bicyclic molecule would suffice. However, a stereochemical complication intervenes here which will cause enormous difficulties. Models show that bridged cyclic compounds can exist in two atropisomeric forms if the bridge is big enough to prevent it swinging across the ring (*Figure 5*). Isomers A and

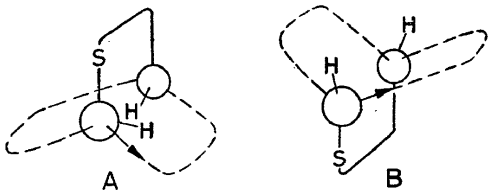


Figure 5. Atropisomers of bicyclic peptides

B can be markedly different in energy and entropy, and in a thermodynamically controlled synthesis only one of them would be expected to arise. This idea is supported by attempts to close the ring of secophalloidin (X) in order to regain the toxic phalloidin (Ia). After many attempts to carry out

this simple reaction, we succeeded[15] in obtaining a ninhydrin-negative neutral substance by heating the seco compound (X) for several hours in molten imidazole. Imidazole, a catalyst for nucleophilic substitutions at the carboxyl group, enables the amino group of (X) to form the peptide bond. The bicyclic substance formed, was not, however, identical with phalloidin, as was demonstrated by its non-toxic properties even at a dose level 100 mg per kg. We can assume that the intramolecular peptide synthesis gave the energetically more stable, non-active isomer, whilst in the mushroom the other compound is built up. The fact that only one form is toxic can be easily understood in view of the great specificity of living surfaces, but a general explanation of the reason why one isomer is toxic and the other is not, cannot be given at present. The chemistry of living surfaces, microstructures and membranes is only just developing and coming into an active phase. Perhaps experiments with the rather specific poisons of *Amanita phalloides* may help in exploring this complicated field of biology.

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