Analysis of the Toxins of Amanitin-Containing Mushrooms

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We have recently developed a method for the analysis of toxins in single specimens of the mushroom *Amanita phalloides* (Fr.) Secr.[1]. Separation of the different toxins was achieved by column chromatography, on Sephadex LH$_{20}$ in water, of a methanolic extract of fresh or dried mushrooms, followed by further purification on thinlayers of silica gel, spectrophotometric evaluation, and, finally, identification of the toxins by amino acid analyses of their γ-hydroxylated leucines or isoleucines as lactones. This procedure afforded the quantitative determination of up to 10 toxins in 2 samples of *A. phalloides* from different sources.

In the present study the treatment outlined above was extended successfully to all the amanitin-containing species of mushrooms known in Europe so far. We examined two more samples of *A. phalloides* collected near Hirschhorn (Neckar, W.-Germany) in 1971 (sample III) and 1972 (sample IV), so that the analytical results from the 4 different samples of this species should shed light on the question as to what extent the toxic composition may differ, depending on location and season. A single small sample of the white species *Amanita verna*, of only 1.47 g of dry weight, was found near Würzburg (W.-Germany) in 1971[2]. Two samples of withe *Amanita virosa* were collected near Trento, Italy, 1972 (sample I)[3] and in Hägnan, Femsjö (Smoland, Sweden) in 1972[4] (sample II). We also investigated a sample of *Galerina marginata* (Fr.) Kühn, collected near Einsiedeln (Switzerland) in 1972[4]; these small, brown mushrooms, which grow on coniferous trunks, are, together with the stirpes *G. automnalis* and *G. venenata*, the only amanitin-containing mushrooms outside the *Amanita* group known to date. This had already been reported in 1966 by Tyler *et al.*[5] by chromatographic and pharmacologic evaluation.

The results are summarized in the table. For most of the samples of *A. phalloides* the sum of the toxins is about 0.7% of the dry weight, 0.1% being α-amanitin. Sample (III) shows, however, that in spite of a normal concentration of phallotoxins, the amount of α-amanitin may be only one twentieth of the value normally found. In all samples from *A. phalloides* the acidic phallotoxins are present in larger amount than the neutral ones.

Hydroxylation of the leucine side chain, which is the cause of the large variety of phallotoxins in *A. phalloides*, is unusually high in the mushrooms of sample (I), in which high amounts of trihydroxylated toxins (phallisin and phallisacin) have been formed at the expense of dihydroxylated toxins (phalloidin and phallacidin). However, hydroxylation was less extensive in sample (III), where, besides a high amount of the monohydroxylated phalloin, we detected traces of its acidic analog phallacin, which was recently discovered by Walch[6]. In addition to the well known series of phallotoxins and amatoxins, sample (I) contained 3 acidic amanitins, none of which was identical with ε-amanitin[7] and which were not found in other *A. phalloides* mushrooms.

The content of toxins was, as a whole, lower in *A. verna* than in *A. phalloides*. This white mushroom had only 70% of the acidic phallotoxins, 50% of the neutral phallotoxins and only 30% of the amanitins in comparison with *A. phalloides*. Besides the known peptides, however, 0.2 μmoles each of two neutral phallotoxins were detected, which could not be characterized because of their low concentrations.

The two samples of *A. virosa*, though of quite different origin, corresponded well to each other in the composition and concentration of toxins. α-Amanitin was 120 – 130%, and phalloidin up to 200% of the values for *A. phalloides*. These mushrooms, however, were free from β- and γ-amanitin, and, generally lacked the variety of toxins produced by hydroxylation of leucine and isoleucine side chains in *A. phalloides*. In sample (I) trace amounts of trihydroxylated phallotoxins were detected, the acidic one in the form of its sulfoxide[8].

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Abbreviations[9]: D-(OH)-abu, D-β (*threo*) hydroxy-α-aminobutyric acid (= D-threonine); ala, alanine; asn, Asparagine; asp, aspartic acid; ile, isoleucine; (OH) ile, γ-hydroxyisoleucine; (OH)$_2$ile, γ,δ-dihydroxyisoleucine; D-(OH) asp, D-β (*erythro*) hydroxyaspartic acid; gly, glycine; (OH) leu, γ-hydroxyleucine; (OH)$_2$leu, γ,δ-(*erythro*) dihydroxyleucine; (OH$_3$)leu, γ,δ,δ'-trihydroxyleucine; (OH)pro, γ-hydroxyproline; a-(OH) pro, allo-γ-hydroxyproline; ser, serine; thr, threonine; val, valine; cy (S) trp, β-alanyl-2-tryptophylsulfide; cy (SO) trp (OH), β-alanyl-2 (6-hydroxy) tryptophylsulfoxide; R (SO$_2$) trp, R-2-tryptophylsulfone.

Table I. mg-Amounts of toxins found in various amanitin-containing mushrooms, related on 2.0 g dry weight each.

	Toxin	Amino acid composition variable	 constant	*A. phall.* I [1]	*A. phall.* II [1]	*A. phall.* III	*A. phall.* IV	*A. verna*	*A. virosa* I	*A. virosa* II	*Galerina marginata*
Phallotoxins	Phallisacin	(OH)$_3$ leu val D-(OH)asp		6.0	2.6	1.7	2.3	<0.1	0.1 *	–	–
	Phallacidin	(OH)$_2$ leu val D-(OH)asp	ala a-(OH)pro	2.4	3.7	3.4	3.4	3.8	3.0	1.4	–
	Phallacin	(OH)leu val D-(OH)asp	cy(S)trp	–	–	<0.1	–	–	–	–	–
	Phallisin	(OH)$_3$ leu ala D-(OH)abu		0.8	0.2	0.2	0.3	1.0	<0.1	–	–
	Phalloidin	(OH)$_2$ leu ala D-(OH)abu		0.9	2.0	2.2	2.2		4.1	4.2	–
	Phalloin	(OH)leu ala D-(OH)abu		<0.1	–	0.3	0.3	–	–	–	–
Amatoxins	Amanin	(OH)$_2$ ile asp cy(SO)trp		<0.1	0.6	–	–	–	–	–	–
	β-Amanitin	(OH)$_2$ ile asp cy(SO)trp(OH)	gly$_2$ ile	2.3	1.2	<0.1	1.3	0.4	–	–	<0.1 **
	α-Amanitin	(OH)$_2$ ile asn cy(SO)trp(OH)	(OH)pro	2.0	1.8	0.1	2.2	0.5	2.3	2.7	0.8
	γ-Amanitin	(OH)ile asn cy(SO)trp(OH)		0.2	0.2	–	0.4	<0.1	–	–	–
	Amanullin	ile asn cy(SO)trp(OH)		–	<0.1	–	0.2	–	–	–	–
Virosa-toxins	Viroidin	thr, ala, val, ser, (OH)$_2$ leu, R(SO$_2$)trp		–	–	–	–	–	2.2 μmoles	2.0 μmoles	–
Peptides not yet elucidated	* As sulfoxide. ** Not identified by amino acid analysis.			3 species of acidic amatoxins				2 neutral phallotoxins 0.2 μmoles each	0.4 μmoles of a sulfone, closely related to viroidin 2 phallo-toxines	0.5 μmoles	

A. virosa contained two toxins not previously found in *A. phalloides*. Their UV absorption spectra indicated that they were α-tryptophyl sulfones[8]. Their toxicity was 2.0 mg/kg on intraperitonal injection in white mice; the dose and progress of intoxication were very similar to that of phalloidin. The major compound, about 2 μmoles per 2 g of dried material, for which we propose the name *viroidin*, on hydrolysis yielded alanine, valine, threonine, serine, dihydroxyleucine and contained tryptophylsulfone (trpSO_2R). It could not be proved that the sulfur originated from a cystein residue, because in the acidic hydrolysate not even traces of cysteic acid were found, as was the case for the corresponding hydrolysate of phalloidin sulfone[8]. Likewise, the configuration and the sequence of the amino acids still remain to be elucidated. For the minor compound, ca. 0.5 μmoles/2 g of dried material, we were able to show the presence of the same amino acids except for dihydroxylated leucine, which remains uncertain, because of the small amount of material. In addition to these sulfones, sample (I) contained 2 phallotoxins not further characterized.

Finally, the sample of *Galerina marginata* was devoid of any phallotoxins. It contained about 40% of the amount of α-amanitin in *A. phalloides*, and perhaps traces of an acidic amanitin, which, however, was only detected on thin layer plates by its violet colour reaction with cinnamic aldehyde/hydrochloric acid.

The results show that these analyses of toxins may be used for chemical taxonomy. So *A. virosa* clearly proves to be an individual species also from its toxin pattern. Some doubts, however, remain for *A. verna*, for which only one small mushroom was available. The low concentrations of toxins in this case could likewise be explained by the fact that the sample investigated was a dwarf form.

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[1] H. Faulstich, D. Georgopoulos, and M. Bloching, J. Chromatogr. [Amsterdam] **79**, 257 [1973].

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[4] A gift from Prof. M. Moser, Innsbruck, whom we wish to thank sincerely, also for the sample of *Galerina marginata*.

[5] V. E. Tyler, L. R. Brady, R. G. Benedict, J. M. Khanna, and M. H. Malone, Lloydia **26**, 154 [1963].

[6] St. Walch, diploma work, University of Heidelberg 1973.

[7] Th. Wieland and A. Buku, Liebigs Ann. Chem. **717**, 215 [1968].

[8] H. Faulstich, Th. Wieland, and C. Jochum, Liebigs Ann. Chem. **713**, 186 [1968].

[9] For formulas see the review of: Th. Wieland and O. Wieland, Microbial Toxins, Vol. **8** (S. J. Ajl, S. Kadis, and A. Giegler, ed.). Academic Press, New York, London 1972.