

## Peptide toxin components of *Amanita exitialis* basidiocarps

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**Abstract:** Eight peptide toxins were isolated and purified from basidiocarps of *Amanita exitialis* with high performance liquid chromatography and were subjected to ultraviolet, nuclear magnetic resonance and mass spectrometry. We identified seven peptide toxins,  $\alpha$ -amanitin,  $\beta$ -amanitin, amaninamide, phallacin, phallacidin, phallisacin and desoxoviroidin. The molecular weight (729.5 Da) of the eighth compound did not match that of any reported *Amanita* toxins and, although the UV absorption spectrum indicated it to be a phallotoxin, further studies are required to identify this component. This is the first report of amaninamide, phallacin, phallisacin and desoxoviroidin in this lethal mushroom species.

**Key words:** amanitins, HPLC, lethal mushroom, NMR

### INTRODUCTION

*Amanita exitialis* Zhu L. Yang & T.H. Li is a white mushroom of genus *Amanita*, section *Phalloideae* (Yang 2001) and, in common with *Amanita phalloides* (Fr.) Link, *Amanita verna* (Bull.: Fr.) Lam., *Amanita virosa* (Fr.) Bertillon and *Amanita fuliginea* Hongo, is highly poisonous. The mushroom has been reported only from Guangdong Province, China, (Yang 2005) where in 2000–2010 52 cases of *A. exitialis* poisoning, including 20 fatalities, were reported.

Peptide toxins are considered the major deadly toxins of poisonous *Amanita* and are classified into three groups, amatoxins (bicyclic octapeptides), phallotoxins (bicyclic heptapeptides) and virotoxins (monocyclic heptapeptides) (Chen and Oberlies 2005). Nine amatoxins, seven phallotoxins and six virotoxins have been identified. Amatoxins are 10–20 times more toxic than phallotoxins and virotoxins and are responsible for fatal human poisonings (Wieland and Faulstich 1978). Amatoxins and phallotoxins first were isolated from *A. phalloides* (Lynen and Wieland 1938, Wieland and Hallermayer 1941) and are common among lethal *Amanita* mushrooms, whereas virotoxins were discovered more recently and have been reported only in *A. virosa* (Faulstich et al. 1980).

*A. exitialis* was described formally in 2001 (Yang and Li 2001), and there are relatively few reports describing its toxins. Hu et al. (2003) used high-performance liquid chromatography (HPLC) to compare the distribution of the main amatoxins ( $\alpha$ -amanitin,  $\beta$ -amanitin) and phallotoxins (phallacidin, phallisin, phalloin) in cap, stipe and volva tissue.  $\alpha$ -Amanitin levels were observed by HPLC to vary at different stages of basidiocarp development (Deng et al. 2006). Amatoxins produced by mycelial cultures of *A. exitialis* were analyzed and characterized by HPLC and mass spectrometry (MS) (Zhang et al. 2005).

Previous work has focused on detecting and quantifying amatoxins and phallotoxins in *A. exitialis* using toxin standards, and the diversity of toxins in the species has not been investigated. Therefore we have isolated and purified the actual peptide toxin components in *A. exitialis* basidiocarps and determined their chemical properties.

### MATERIALS AND METHODS

*Collection of fungi.*—We collected fresh *A. exitialis* basidiocarps in Guangzhou, Guangdong, in spring 2008. Samples were dried at 60 °C in an electric dryer 4 h.

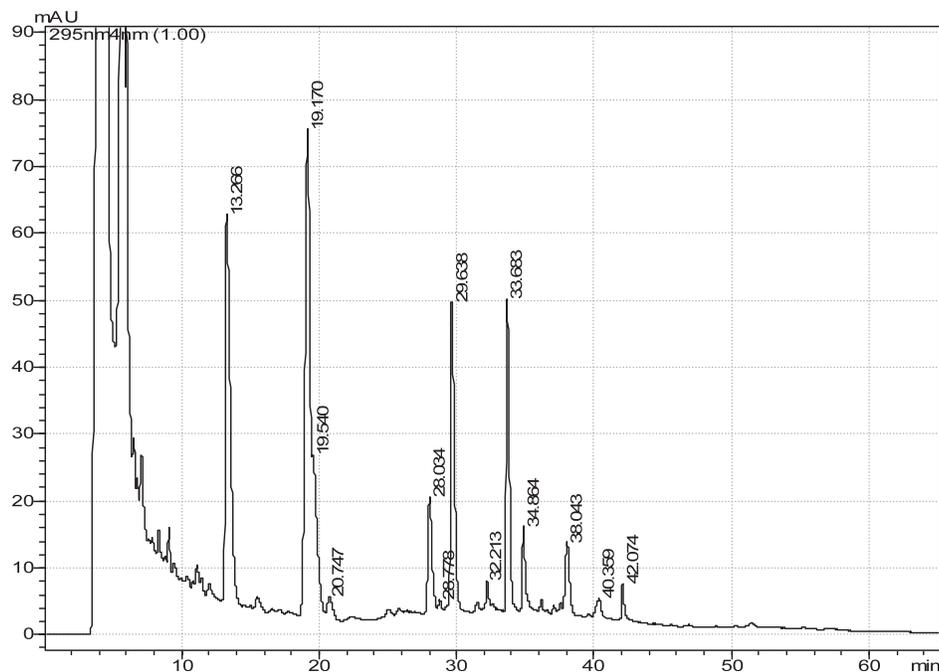


FIG. 1. HPLC profile of peptide toxins extracted from *Amanita exitialis*.

*Standard solutions and chemicals.*— $\alpha$ -Amanitin was obtained from Sigma-Aldrich and HPLC-grade acetonitrile was from Shanghai ANPEL Scientific Instrument Co. Ltd. All other solvents and chemicals were of analytical grade and from Guangdong Huankai Microbial Science and Technology Co. Ltd.

*Extraction of peptide toxins.*—Dried basidiocarps were ground to a powder with a SS280-D electric multifunction mixer (Guangzhou Huineng Industry and Trade Co. Ltd., Guangzhou), and a 50 g sample was extracted with 1000 mL methanol:water (1:1, v/v) at 25 C for 12 h on a rotary shaker (130 rpm). After centrifugation at 4000 *g* for 10 min the supernatant was collected and the pellet was extracted repeatedly as before. Supernatant fractions were combined, extracted twice with the same volume of petroleum ether (b.r. 30–60), and the extract was dried at 50 C in a RE-2000 rotary evaporator (Shanghai Yarong Biochemical Instrument Factory, Shanghai). The residue was dissolved in 100 mL double-distilled H<sub>2</sub>O before isolation and purification of the toxin components.

*Isolation and purification of peptide toxins.*—Peptide toxins were isolated and purified by reversed-phase HPLC with a Shimadzu LC-20A HPLC system (Shimadzu Inc., Japan) fitted with a Shimadzu SPD-M20A variable UV detector set at 295 nm. Separations were carried out with a YWG C-18 reverse phase HPLC column (250 × 10 mm I.D., particle size 5  $\mu$ m) (Dalian Elite Analytical Instruments Co. Ltd., China) at 40 C with the conditions described by Zhang et al. (2005) and Enjalbert et al. (1992). The mobile phases were (A) 0.02 M aqueous ammonium acetate-acetonitrile (90:10, v/v) and (B) 0.02 M aqueous ammonium acetate-acetonitrile (76:24, v/v), prepared with double-distilled water and adjusted to pH 5.0 in both cases with glacial acetic acid. All

solutions were degassed by sound before use. The elution profile consisted of four isocratic steps of 60 min total duration: (1) 0 → 15 min, A 100% → 95%, B 0 → 5%; (2) 15 → 40 min, A 95% → 20%, B 5 → 80%; (3) 40 → 50 min, A 20% → 0, B 80 → 100%; (4) 50 → 60 min, A 0 → 100%, B 100% → 0. The flow of the mobile phases was 3.0 mL/min.

*LC-MS spectrometry.*—Separation of *A. exitialis* peptide toxins and molecular weight determinations were performed with an Agilent 1100 LC/MSD trap system fitted with a Zorbax SB-C18 reverse phase HPLC column (150 × 2.1 mm I.D., particle size 3.5  $\mu$ m) (Agilent Inc., USA). The mobile phases and gradient elution modes were identical to those used for the isolation and purification of peptide toxins with HPLC, except that the flow of the mobile phase was 0.3 mL/min. We analyzed the sample with electrospray ionization (ESI) in the positive mode.

*NMR spectrometry.*—<sup>1</sup>H (1D) and <sup>13</sup>C (1D) NMR spectra of the peptide toxins were recorded at 300 K with a Bruker DMX-600 NMR spectrometer (Bruker Biospin GmbH, Karlsruhe, Germany) locked to the major deuterium signal of the CD<sub>3</sub>OD/CDCl<sub>3</sub> solvent (30:70). Reported chemical shift values (expressed in ppm) are relative to the internal standard, tetramethylsilane (TMS), and we denoted coupling constants in Hz frequency units.

## RESULTS

*Isolation and purification of peptide toxins.*—HPLC of crude toxin preparations extracted from *A. exitialis* revealed eight clearly distinguishable peaks with R<sub>t</sub> values of 13.266, 19.170, 19.540, 28.034, 29.638, 33.683, 34.864 and 42.074 min (FIG. 1). The eight

TABLE I. Peptide toxins isolated and purified from *Amanita exitialis* basidiocarps

Compound	Retention time (min)	Wavelength for maximum UV absorbance (nm)	Molecular weight (Da)	Peptide toxin
1	13.266	303	919.4	$\beta$ -Amanitin
2	19.170	303	918.4	$\alpha$ -Amanitin
3	19.540	283	848.8	Desoxoviroidin
4	28.034	303	902.4	Amaninamide
5	29.638	290	862.8	Phallisacin
6	33.683	290	846.4	Phallacidin
7	34.864	290	729.5	Not identified
8	42.074	290	830.5	Phallacin

compounds were collected and purified repeatedly by HPLC until  $\sim 90\%$  purity was achieved. The wavelengths at which individual compounds exhibited maximum UV absorbance values are provided (TABLE I). Maximum absorbance values for compounds 1, 2 and 4 were recorded at 303 nm, which is characteristic of amatoxins, whereas the maximum absorbance value for compound 3 was observed at 283 nm, in accordance with that reported for virotoxins. Maximum absorbance values of the other compounds were recorded at 290 nm, which is a feature of phallotoxins.

*Mass spectrometry analysis.*—The eight purified compounds and standard  $\alpha$ -amanitin were analyzed with MS, and the corresponding molecular weights were calculated according to their molecular ion peaks (TABLE 1). The molecular weight of compound 2 was identical to that of standard  $\alpha$ -amanitin reported by Wieland and Hallermayer (1941), and the molecular weights of the other compounds (with the exception of compound 7) were also in accordance with documented data (Wieland 1949; Faulstich et al. 1975, 1980; Buku et al. 1980; Wieland and Schnabel 1962). Our data support the presence of seven peptide toxins in *A. exitialis*:  $\alpha$ -amanitin,  $\beta$ -amanitin, amaninamide, phallacin, phallacidin, phallisacin and desoxoviroidin. The first three compounds are assigned to the amatoxin group, desoxoviroidin is a virotoxin, and the remaining three compounds are phallotoxins.

*NMR analysis.*—The molecular structures of the two main toxins, compounds (1 and 2), were analyzed by NMR (SUPPLEMENTARY TABLE 1). The  $^1\text{H}$  NMR spectrum of compound 1 contained signals at  $\delta$  8.9–8.0, corresponding to the NH moiety of seven amino acids, and at  $\delta$  6.0–3.5, corresponding to  $\alpha$ -H peaks of eight amino acids. The  $^{13}\text{C}$  NMR spectrum of compound 1 contained signals at  $\delta$  173.4–168.8, corresponding to the CO moieties of eight amino acids. When we compared NMR data for compound 1

and compound 2 the latter contained one signal for an additional N but lacked the signal for one O, in accord with the presence of an asparagine residue in compound 2 in place of aspartic acid. The NMR data for compound 1 and 2 were identical to those reported respectively for  $\beta$ -amanitin and  $\alpha$ -amanitin by Yang et al. (2003).

#### DISCUSSION

Toxins produced by different *Amanita* species have been studied extensively, and to date 22 *Amanita* peptide toxins have been described. Our data showed that eight peptide toxins are present in the basidiocarps of *A. exitialis*, four of which were not reported previously in this species (Hu et al. 2003, Chen et al. 2003). We identified compounds 1 and 2 as  $\beta$ -amanitin and  $\alpha$ -amanitin respectively based on NMR spectrometry and MS data. As far as we are aware, this is the first report of amaninamide, phallacin, phallisacin and desoxoviroidin in *A. exitialis*, which now represents a new source of these toxins. Phallisin and phalloin, reported by Hu et al. (2003), were not detected in *A. exitialis* under the conditions adopted in our study.

The molecular weight (729.5 Da) of compound 7 did not match that of any reported *Amanita* toxins and, although the UV absorption spectrum suggested it to be a phallotoxin, further studies are required to identify this component.

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