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Bolevenine, a toxic protein from the Japanese toadstool *Boletus venenatus*

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Abstract

A toxic protein, called bolevenine, was isolated from the toxic mushroom *Boletus venenatus* based on its lethal effects on mice. On SDS–PAGE, in either the presence or absence of 2-mercaptoethanol, this protein showed a single band of ~12 kDa. In contrast, based on gel filtration and MALDI-TOFMS, its relative molecular mass was estimated to be ~30 kDa and ~33 kDa, respectively, indicating that the protein consists of three identical subunits. This toxin exhibited its lethal activity following injection at 10 mg/kg into mice. The N-terminal amino acid sequence was determined up to 18, and found to be similar to the previously reported bolesatine, a toxic compound isolated from *Boletus satanas*.

Keywords: *Boletus venenatus*; Boletaceae; Mushroom poisoning; Toxic protein; Bolevenine; Trimeric protein

1. Introduction

Most of the Boletaceae mushrooms have long been thought to be edible; however, recently, a few species, such as *Boletus satanas* (distributed in Europe and America), *Xanthoconium affine* (synonym, *Boletus affinis*; Japanese name, utsuroi-iguchi; distributed in Japan, North America, and Madagascar), and *Tylopilus* sp. (Japanese name, mikawakuroamiashi-iguchi), were found to be exceptions. Of these, *B. satanas* induces gastroenteritis in humans and its responsible proteinaceous toxin, bolesatine, has been isolated by a French group (Kretz et al., 1989). *X. affine* can also result in death of cows after ingestion, and the responsible toxin has been found to be the protein bolaffinine (Razanamparany et al., 1986). The *Tylopilus* sp. had also been toadstool of some health concern, and recently a Japanese group demonstrated that the responsible toxin for acute toxicity on mice was the 2-butyl-1-azacyclohexene iminium salt (Watanabe et al., 2002).

In Japan, in addition to the *X. affine* and *Tylopilus* sp., *Boletus venenatus* (Japanese name, dokuyamadori or tahei-iguchi) is known to be toxic. Ingestion of the mushroom causes a severe gastrointestinal syndrome, such as nausea, repetitive vomiting, diarrhea, and stomachache. Severe poisoning brings about dehydration; fortunately, most of the patients, however, usually recover in a few days. Regarding *B. venenatus*, there has been confusion between similar species, but recent taxonomic studies have made it possible to identify this species (Nagasawa, 1995). This situation prompted us to attempt to identify the toxic component of the Japanese Boletaceae mushroom. Guided

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by its lethal effect on mice, we isolated a toxic protein and have called it bolevenine.

2. Results and discussion

2.1. Purification

The lethal effect on mice was used as the toxicity index for the isolation of the toxic component. Fruiting bodies were extracted with water and MeOH, and each extract was injected into mice. Only the aqueous extract exhibited toxicity. Next, the aqueous extract underwent ultrafiltration, and the resulting filtrate and retentate were tested for toxicity. Only the retentate showed toxic activity, which after heating at 70 °C for 20 min, disappeared. The toxicity of the retentate under various pH conditions was also tested, and was maintained the pH 4–10 range. These properties suggested that the toxic compound was a protein.

The aqueous extract was dialyzed against water, with the retentate subjected to cation-exchange chromatography on CM-52 resin using 20 mM citrate–NaOH buffer (pH 4.5). Stepwise elution with the citrate buffer containing NaCl was effective for concentrating the toxin. Only the C3 fraction eluted during 423–477 ml (Fig. 1) showed toxic activity. Lyophilization of this fraction with the citrate buffer produced a colorless solid with less solubility and less bioactivity; therefore, this fraction was used for the next purification step without lyophilization. The C3 fraction was thus dialyzed against 20 mM Tris–HCl buffer (pH 8.0), and then the retentate was subjected to anion-exchange chromatography on DE-52 resin equilibrated in 20 mM Tris–HCl buffer (pH 8.0). Stepwise elution with the buffer containing NaCl gave three fractions (D1–D3, Fig. 2). Among them, the lethal toxic effect was observed in both D1 and D2 fractions, with the lethal doses for both on mice being almost equivalent. Although both samples exhibited a broad band of about ~12 kDa by SDS–PAGE analysis, an isoelectric focusing analysis also indicated that

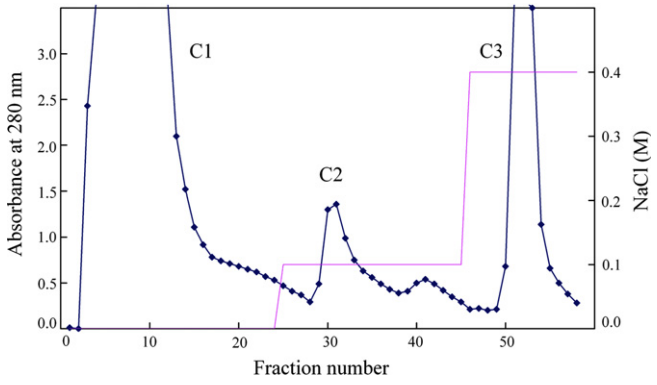

Fig. 1. Cation exchange chromatography of the crude extract of *B. venenatus* on a CM-52 column; stepwise elution with 20 mM citrate buffer (pH 4.5) containing NaCl (0, 100, 400 mM, each 180 ml) was carried out with 9 ml fractions collected.

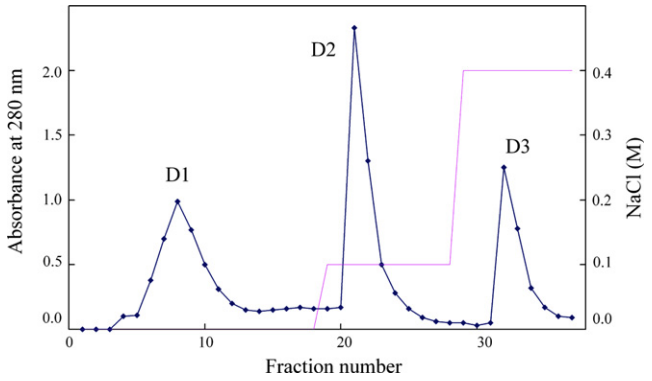

Fig. 2. Anion exchange chromatography of the C3 fraction on a DE-52 column; After the resin was washed with 20 mM Tris–HCl buffer (pH 8.0, 150 ml), stepwise elution with the buffer containing NaCl (100, 400 mM, each 90 ml) was carried out with 9 ml fractions collected.

both fractions consisted of a mixture of proteins. The D1 fraction consisted of one major band along with three minor bands; on the other hand, the D2 fraction consisted of the same major band along with six minor bands. Therefore, further purification of the D1 fraction was carried out. The D1 fraction underwent gel filtration chromatography on Sephacryl S-100HR. Elution with 20 mM Tris–HCl buffer (pH 8.0) afforded a single fraction (S1, Fig. 3); however, isoelectric focusing analysis indicated that the S1 fraction still contained a small amount of impurities. Finally, the S1 fraction was purified by anion-exchange chromatography on Q Sepharose to give one main fraction (Q1, Fig. 4) and one minor fraction (Q2). The Q1 fraction showed a single band by both SDS–PAGE and isoelectric focusing analyses. We called this toxin bolevenine. Bolevenine exhibited hair erection and decreased mobility on mice by injection of 10 mg/kg through i.p. route. After developing these initial symptoms, the mice died within 18–36 h.

2.2. The M_r, pI, and partial structure of bolevenine

The M_r of bolevenine was estimated to be by gel filtration using FPLC about 30,000 (Fig. 5). On the other hand,

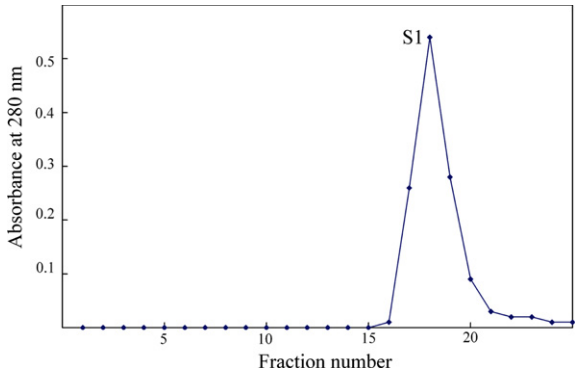

Fig. 3. Gel filtration of D1 fraction using a Sephacryl S-100 HR column; 20 mM Tris–HCl buffer (pH 8.0) was used as eluate, with 2 ml fractions collected.

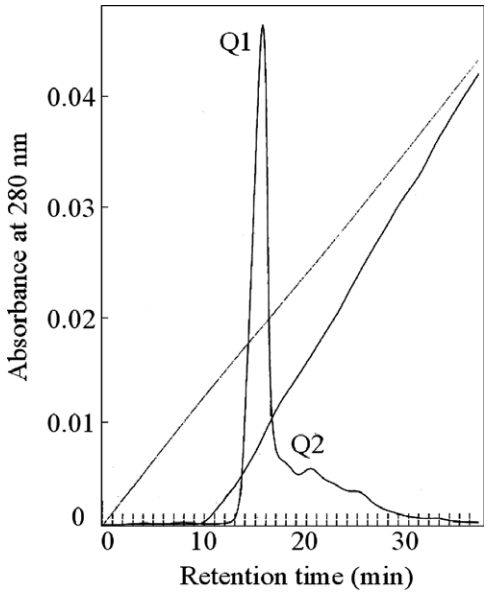

Fig. 4. Anion exchange chromatography of the S1 fraction on a HiLoad 26/10 Q Sepharose HP column connected to an AKTA prime system; For the elution of the sample, a linear gradient of NaCl from 0 to 250 mM was applied for 40 min at a flow rate of 2 ml/min with monitoring at 280 nm. 2 ml fractions were collected.

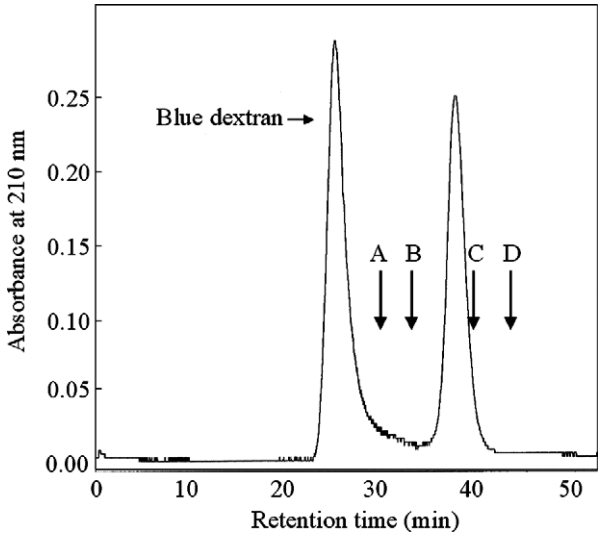

Fig. 5. Chromatogram using Superdex 75 10/300 GL connected to a FPLC system; bolevenine was dissolved in 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, and applied to the column. Elution of the sample was carried out at a flow rate of 1 ml/min with monitoring at 210 nm. The markers of the relative molecular mass were BSA (A; 67,000), egg albumin (B; 45,000), pectate lyase (C; 23,800), and cytochrome C (D; 12,400).

the MALDI-TOFMS analysis showed peaks at 11,000 and 33,000 (Fig. 6). Whereas SDS–PAGE gave a single band of ~12 kDa regardless of presence or absence of 2-mercaptoethanol (Fig. 7). Considering these data, bolevenine is a trimer complex which consists of three identical subunits of 11,000. Isoelectric focusing showed that the p*I* of bolevenine was about 6.5 (Fig. 8).

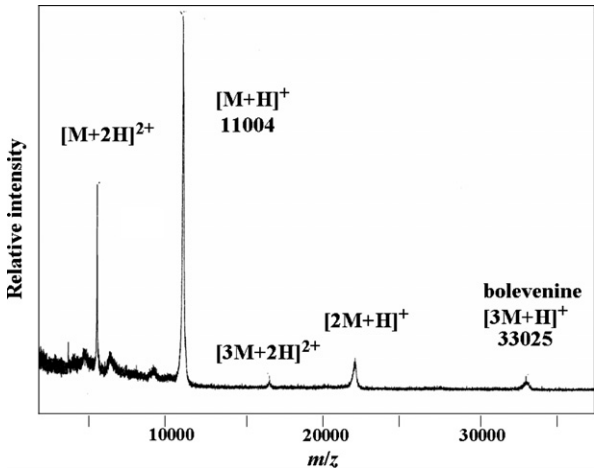

Fig. 6. MALDI-TOFMS spectrum of bolevenine. "M" means the monomer (subunit) of bolevenine. CHCA (α-cyano-4-hydroxycinnamic acid) was used as the matrix.

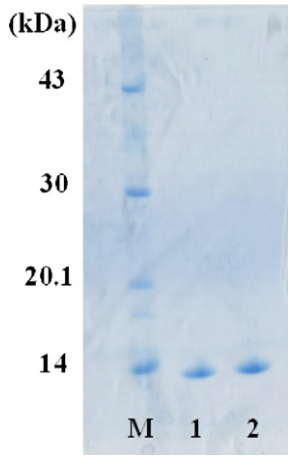

Fig. 7. SDS–PAGE of bolevenine; samples were heated for 10 min at 100 °C in the presence (lane 1) and absence (lane 2) of 2-mercaptoethanol and applied to a 15% gel. Protein bands were stained with Coomassie Brilliant Blue G-250.

The N-terminal amino acid sequence was analyzed up to 18 (Fig. 9) by a protein sequencer and the sequence was searched using the NCBI-Blast database. A homolog, bolesatine, which is a toxic protein isolated from *B. satanas* (Kretz et al., 1992b), was found during this search.

2.3. Discussion

B. venenatus contains at least two toxins with different chromatographic behavior on the DE-52 column. Only the toxin contained in the D1 fraction was isolated, and it is called bolevenine. Although heating (at 70 °C for 20 min) inactivated bolevenine, changing the pH (4–10) had no effect on the activity; moreover, this protein also resisted proteolysis (data not shown). The properties of being tolerant to both pH and proteolysis might be one of the reasons why poisoning can occur. This toxin was

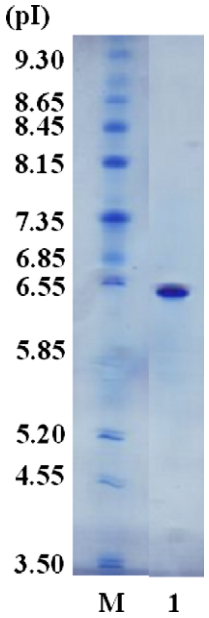

Fig. 8. Isoelectric focusing of bolevenine (lane 1); Sample (10 μg) was applied to a gel plate (Ampholine PAG plate, pH 3.5–9.5). Protein bands were stained with Coomassie Brilliant Blue G-250.

also shown to consist of three identical subunits. Comparing the SDS–PAGE bands of the D1 and D2 fractions, both fractions showed a single band of ~12 kDa, and when comparing their isoelecric focusing bands, an identical band was observed along with some other bands. Furthermore, using MALDI-TOFMS, both fractions showed about 11,000 and 33,000 as their average M_rs. Taking these data into account, the other toxins (the minor components of the D1 fraction and the components of the D2 fraction) might be a similar trimeric protein consisting of similar subunits.

The proteinaceous toxins already isolated from the Boletaceae mushrooms are bolesatine and bolaffinine. The M_rs of these toxins and bolevenine are quite different: bolesatine, 63 ± 3 kDa, single-chain (Kretz et al., 1989); bolaffinine, 22 kDa, single-chain (Razanamparany et al., 1986); bolevenine, 33 kDa, trimer of single-chain protein of 11 kDa. The p*I* values are different from each other (bolesatine, 8.3 ± 0.1 (Kretz et al., 1991b); bolaffinine, 9–10 (Razanamparany et al., 1986); bolevenine, 6.5). Provided that the M_r of the bolesatine is 66 kDa, all three M_rs are a multiple (*n*: 6 for bolesatine, 2 for bolaffinine, and 1 for bolevenine) of 11 kDa. This may indicate that these three proteins are homologous proteins, although their entire sequences are still unknown.

Concerning their mode of action only that of bolesatine has been reported, i.e. these include inhibition of protein synthesis (Kretz et al., 1989, 1992a,b; Basset et al., 1991, 1995), agglutination effects (Gachet et al., 1996; Ennamany et al., 1998), lipid peroxidation (Ennamany et al., 1995), as well as resistance to proteolysis (Kretz et al., 1991a,b).

Establishing the mechanism of poisoning caused by bolevenine is the subject of further investigation.

3. Experimental

3.1. General

All separation procedures were carried out at 4 °C. Each fraction was monitored by UV spectra (U-2001, Hitachi) at 280 nm otherwise stated.

3.2. Materials

The fruiting bodies of *Boletus venenatus* were collected during 2002–2003 in the Nagano and Gifu Prefectures, Japan, and stored at −30 °C until use.

3.3. Purification

Fruiting bodies of *B. venenatus* (250 g) were cut into pieces, soaked in water (500 ml), and extracted overnight. The mixture was filtered through filter paper (No. 5A, Kiriyama) under suction and the filtrate was concentrated in vacuo to 1/10 volume. The solution was then dialyzed (M_r 12,000–14,000) against H_2O ($3\,l \times 2$) overnight. The retentate was lyophilized to give a crude extract (1.3 g). A second similar extraction gave the second crop (0.6 g). The lethal effect was observed in the crude extract by injection of 5 mg/capita.

The combined extracts (460 mg) were dissolved in 20 mM citrate–NaOH buffer (pH 4.5, 20 ml) and applied to a cation-exchange column (CM-52, 2.8×10 cm, Whatman) equilibrated with the same buffer. Stepwise elution with 20 mM citrate buffer (pH 4.5) containing NaCl (0, 100, 400 mM, each 180 ml) was carried out, with 9 ml fractions collected. The C3 fraction eluted between 423 and 477 ml was concentrated to 1/10 volume and dialyzed against 20 mM Tris–HCl buffer (pH 8.0, $1\,l \times 3$).

	1				5					10					15			
bolevenine	T	W	S	A	F	L	N	N	Q	S	V	K	L	A	M	L	L	P
bolesatine[a]	T	W	R	I	Y	L	N	N	Q	T	V	K	L	A	L	L	L	P

a: Data from Kretz et al (1992b)

Fig. 9. N-terminal amino acid sequences of bolevenine and bolesatine; amino acids identical in both toxins are underlined.

The retentate was loaded onto an anion-exchange column (DE-52, 2.6 × 7 cm, Whatman) previously equilibrated with 20 mM Tris–HCl buffer (pH 8.0). After the resin was washed with buffer (150 ml), stepwise elution with 20 mM Tris–HCl buffer (pH 8.0) containing NaCl (100, 400 mM, each 90 ml) was carried out. With 9 ml fractions collected as above. The D1 fraction that eluted between 45 and 117 ml was concentrated in vacuo to 1/10 volume, and then dialyzed against 5 mM Tris–HCl buffer (pH 8.0, 2 l). The retentate was next lyophilized. The amount of protein in the D1 fraction was about 1.7 mg as estimated from the Bradford method (1976) using BSA as standard. A lethal effect was observed in the D1 fraction by injection of 150 μg/capita.

The D1 fraction (3.4 mg) was dissolved in 20 mM Tris–HCl buffer (pH 8.0, 2 ml), and then subjected to gel filtration on a Sephacryl S-100HR column (1.2 × 40 cm, GE Healthcare Bio-Sciences) pre-equilibrated with the same buffer. Using the same buffer as the eluate, 2 ml fractions were collected. The fractions that eluted between 20 and 40 ml were combined and evaporated in vacuo to 1/10 volume. This fraction (S1) was then dialyzed against 5 mM Tris–HCl buffer (pH 8.0, 2 l) with the retentate lyophilized. The amount of the protein in the S1 fraction was about 3.2 mg as estimated by the Bradford method (1976).

The S1 fraction (100 μg) was dissolved in 20 mM Tris–HCl buffer (pH 8.0, 400 μl) and loaded onto a HiLoad 26/10 Q Sepharose column (GE Healthcare Bio-Sciences) connected to an AKTA prime system (GE Healthcare Bio-Sciences). The column had been equilibrated with 20 mM Tris–HCl buffer (pH 8.0), and for sample elution, a linear gradient of NaCl from 0 to 250 mM was applied for 40 min at a flow rate of 2 ml/min with monitoring at 280 nm; 2 ml fractions were then collected. The fractions eluting between 28 and 32 ml, corresponding to the major peak in the chromatogram, were combined and evaporated in vacuo to 1/10 volume. This fraction (Q1) was next dialyzed against H_2O (100 ml) and the retentate lyophilized. These manipulations were repeated 32 times and the total amount of the combined product was 2.1 mg, as estimated by the Bradford method (1976). A lethal effect was observed in the Q1 fraction by injection of 100 μg/capita.

3.4. SDS–PAGE and isoelectric focusing

SDS–PAGE was performed as described by Laemmli (1970) using a 15% acrylamide gel. Samples (each 2 μg) were heated at 100 °C for 10 min in the presence or absence of 2-mercaptoethanol before its application. A calibration kit (LMW Marker Kit, GE Healthcare Bio-Sciences) was used as the standard molecular mass markers.

Isoelectric focusing was performed using a prepared gel, Ampholine PAG plate (5 × 11 cm) at pH 3.5–9.5 (GE Healthcare Bio-Sciences). A sample (10 μg) in 20 mM Tris–HCl buffer (pH 8.0) was applied to the gel plate (3 cm from anode), and run on a Multiphor II (horizontal electrophoresis apparatus, GE Healthcare Bio-Sciences) according to the manufacturer's instructions. The p*I* value was determined using the Broad p*I* Kit (pH 3.5–9.3, GE Healthcare Bio-Sciences) as the p*I* markers.

Protein bands were stained with Coomassie Brilliant Blue G-250 (Fluka).

3.5. Determination of relative molecular mass

3.5.1. MALDI-TOFMS

MALDI-TOFMS was measured using a Voyager DE-RP or Voyager DE-STR (Applied Biosystems) in the linear mode. Calibration was performed using ACTH (18–39) (adrenocorticoptropic hormone fragment 18–39) and BSA (bovine serum albumin) as the relative molecular mass standards. CHCA (α-cyano-4-hydroxycinnamic acid), SA (sinapinic acid), and FA (ferulic acid) were used as the matrix.

3.5.2. Gel filtration by FPLC

Purified bolevenine (100 μg) was dissolved in 20 mM phosphate buffer (pH 7.0), and applied to a Superdex 75 column connected to a FPLC system (GE Healthcare Bio-Sciences). The column had been pre-equilibrated with the buffer containing 150 mM NaCl with elution of the sample performed at a flow rate of 1 ml/min with monitoring at 210 nm. The markers of the relative molecular mass, BSA (67,000), egg albumin (45,000), and cytochrome C (12,400), were purchased from Sigma–Aldrich. Pectate lyase (23,800) was obtained by the method of Miyairi et al. (2004).

3.6. Amino acid sequence analysis

The N-terminal amino acid sequence of bolevenine was analyzed using a PPSQ-10 protien sequencer (Shimadzu).

3.7. pH Stability and thermal stability

The crude aqueous extract was filtered using filter paper (No. 5A, Kiriyama), and the filtrate concentrated in vacuo. The residue (40 mg) was dissolved in the following buffers and the mixture was allowed stand at 4 °C overnight: 50 mM citrate–NaOH buffer (pH 4, 5, 6), 50 mM Tris–HCl buffer (pH 7, 8), and 50 mM NH_3–CO_2 buffer (pH 9, 10). Each solution was next dialyzed against H_2O, with the retentates lyophilized. Each residue was used for biological assays. Activities were observed for all samples.

The concentrated filtrate (120 mg) was dissolved in H_2O and then heated at 70 °C for 20 min, which led to a white precipitate. After filtration, the resulting solution was concentrated in vacuo. This sample was injected into three mice and no lethal activities were observed.

3.8. Bioassay on mice

The lethality was assayed by intraperitoneal injection of the sample into female ddY strain mice (9.5–10.5 g of weight, Japan SLC). Samples were dissolved in 0.9% aqueous NaCl solution (500 μl). When a lethal effect was observed within 36 h, the sample was regarded as toxic.

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References

Basset, L., Badoc, A., Deffieux, G., Counord, J., Andrieux, M., Creppy, E.E., 1991. Effets de la bolesatine, toxine proteique extraite du bolet satan sur des cellules en culture. Bull. Soc. Pharm. Bordeaux 130, 39–45.

Basset, L., Ennamany, R., Portail, J.P., Kretz, O., Deffieux, G., Badoc, A., Guillemain, B., Creppy, E.E., 1995. Effects of bolesatine on a cell line from the SP2/O thymic lymphosarcoma. Toxicology 103, 121–125.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Ennamany, R., Marzetto, S., Saboureau, D., Creppy, E.E., 1995. Lipid peroxidation induced by bolesatine, a toxin of *Boletus satanas*: implication in m^5dC variation in Vero cells related to inhibition of cell growth. Cell Biol. Toxicol. 11, 347–354.

Ennamany, R., Bingen, A., Creppy, E.E., Kretz, O., Gut, J.P., Dubuisson, L., Balabaud, C., Sage, P.B., Kirn, A., 1998. Aspirin and heparin prevent hepatic blood stasis and thrombosis induced by the toxic glycoprotein bolesatine in mice. Hum. Exp. Toxicol. 17, 620–624.

Gachet, C., Ennamany, R., Kretz, O., Ohlmann, P., Krause, C., Creppy, E.E., Dirheimer, G., Cazenave, J.P., 1996. Bolesatine induces agglutination of rat platelets and human erythrocytes and platelets in vitro. Hum. Exp. Toxicol. 15, 26–29.

Kretz, O., Creppy, E.E., Boulanger, Y., Dirheimer, G., 1989. Purification and some properties of bolesatine, a protein inhibiting in vitro protein synthesis, from the mushroom *Boletus satanas* Lenz (Boletaceae). Arch. Toxicol. (Suppl. 13), 422–427.

Kretz, O., Creppy, E.E., Dirheimer, G., 1991a. Disposition of the toxic protein, in rats: its resistance to proteolytic enzymes. Xenobiotica 21, 65–73.

Kretz, O., Creppy, E.E., Dirheimer, G., 1991b. Characterization of bolesatine, a toxic protein from the mushroom *Boletus satanas* Lenz and its effects on kidney cells. Toxicology 66, 213–224.

Kretz, O., Barbieri, L., Creppy, E.E., Dirheimer, G., 1992a. Inhibition of protein synthesis in liver and kidney of mice by bolesatine: mechanistic approaches to the mode of action at the molecular level. Toxicology 73, 297–304.

Kretz, O., Reinbolt, J., Creppy, E.E., Dirheimer, G., 1992b. Properties of bolesatine, a translational inhibitor from *Boletus satanas* Lenz. Amino-terminal sequence determination and inhibition of rat mitochondrial protein synthesis. Toxicol. Lett. 64/65, 763–766.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Miyairi, K., Ogasawara, A., Tonouchi, A., Hosokawa, K., Kudou, M., Okuno, T., 2004. Low-molecular-weight pectate lyase from *Streptomyces thermocarboxydus*. J. Appl. Glycosci. 51, 1–7.

Nagasawa, E., 1995. A new poisonous species of Boletus from Japan. Rep. Tottori Mycol. Inst. 33, 1–6.

Razanamparany, J.L., Creppy, E.E., Perreau-Bertrand, J., Boulanger, Y., Dirheimer, G., 1986. Purification and characterization of bolaffinine, a toxic protein from *Boletus affinis* Peck (Boletaceae). Biochimie 68, 1217–1223.

Watanabe, R., Kita, M., Uemura, D., 2002. A novel dipeptide, *N*-γ-glutamyl boletine, and a cyclic iminium toxin from the mushroom *Tylopilus* sp. (Boletaceae). Tetrahedron Lett. 43, 6501–6504.