Natural Products

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Evidence for the Natural Toxins from the Mushroom *Trogia venenata* as a Cause of Sudden Unexpected Death in Yunnan Province, China**

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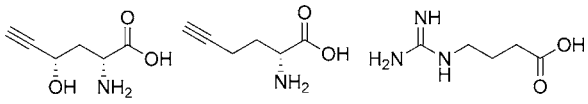
Over the past 30 years, more than 260 apparently healthy villagers in Yunnan Province, southwest China, have suddenly died of an unknown cause.[1] Yunnan Sudden Unexplained Death (SUD) often strikes in time-space clusters during the rainy season, from June to August, in villages between 1800 and 2400 m above sea level.[1,2] Epidemiologic studies in 2005 implicated mushroom picking as a risk factor, and studies from 2006–2009 further implicated eating a previously undescribed species of mushroom, which we have named *Trogia venenata* Zhu L. Yang (Figure 1, see also the descriptions in the Supporting Information). We have isolated and characterized three toxic compounds from the fruiting bodies of this mushroom to further substantiate the hypothesis that *T. venenata* is responsible for SUD.

Herein, we describe two unusual and toxic amino acids (**1** and **2**, Scheme 1) as well as the known toxin γ-guanidinobutyric acid (**3**) that were isolated from the mushroom *T. venenata*. The isolation of these active constituents was guided by oral toxicity tests in mice (all animal experiments were performed with the approval of the Kunming Institute of Botany Ethical Committee for Experimental Use of Animals). Amino acid toxins **1** and **2** killed mice with an LD_{50} value of 71 and 84 mg kg^{-1} respectively. *T. venenata* has been epidemiologically implicated as a contributing cause of Yunnan SUD, which is an important local health concern of hereto undefined etiology.

Figure 1. Toxic mushroom *Trogia venenata* Zhu L. Yang.



1 **2** **3**

Scheme 1. Structures of two toxic amino acids 2*R*-amino-4*S*-hydroxy-5-hexynoic acid (**1**) and 2*R*-amino-5-hexynoic acid (**2**) and the known toxin γ-guanidinobutyric acid (**3**) isolated from fruiting bodies of *Trogia venenata*.

In July 2009, samples of *T. venenata* were collected from Tengchong County in Yunnan Province, near to a village where several *T. venenata*-associated Yunnan SUD clusters had occurred. We subjected fresh fruiting bodies of *T. venenata* (9.7 kg) to three successive extractions with ethanol at room temperature. Concentration of the combined ethanol soltions under reduced pressure gave crude extract (60.2 g), which was suspended in water and partitioned with ethyl acetate. Animal testing confirmed that the toxic factor was present in the aqueous phase. The water extract (40.5 g) was subjected to preparative medium-pressure liquid chromatography (MPLC) on a reverse-phase C_{18} column (RP-C_{18}, MeOH/H_2O, 0–100%) to yield 16 fractions. Fraction A was

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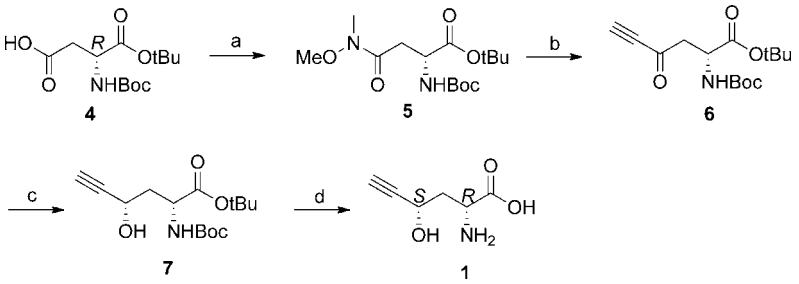
eluted with pure water and further separated by using an Amberlite 732 cation-exchange resin column (H_2O/NH_4OH (1.0 M), 0–100 %). Fraction A3 was obtained at a concentration of 0.3 M of NH_4OH, then repeatedly purified by chromatography on a microcrystalline cellulose column (*n*BuOH/EtOH/HOAc/H_2O, 4:1:1:2), a Sephadex LH-20 column (MeOH/H_2O, 7:3), and an RP-C_{18} silica gel column (pure H_2O) to give compounds **1** (905 mg), **2** (436 mg), and **3** (44.5 mg).



Scheme 2. Total synthesis of compound **1**. Reagents and conditions: a) Et_3N, $CH_3ONHCH_3{\cdot}HCl$, BOP·PF_6 (BOP = benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium), CH_2Cl_2 (97 % yield); b) HC≡CMgBr (5 equiv), Et_2O, −78 °C (78 % yield); c) (*S*)-B-methyl Corey-Bakshi-Shibata (CBS) catalyst (2 equiv), BH_3–SMe_2 (2 equiv), toluene (61 % yield); d) CF_3CO_2H (99 % yield).

Compound **1** was obtained as colorless prism-like crystals and reacted with ninhydrin on a TLC plate. The molecular formula of **1** is $C_6H_9NO_3$ as determined by HRMS ((ESI): *m/z* calcd for $C_6H_9NO_3Na^+$: 166.0480 [M+Na^+]; found: 166.0478,). The HRMS data agree with the ^{13}C NMR and DEPT spectral data, which suggest the presence of a linear carbon chain that is composed of a carbonyl group ($\delta = 173.9$ ppm, C1), a methine connected to an amino group ($\delta = 53.5$ ppm, C2), a terminal triple bond ($\delta = 85.0$ ppm, s, C5; 76.6, d, C6), an oxymethine group ($\delta = 60.1$ ppm, C4), and a central methylene unit ($\delta = 38.4$ ppm, C3). The HSQC data revealed a connection between the bonded CH pairs, and the ^1H NMR and COSY spectra of **1** indicated the existence of the spin system from C2 to C4. Furthermore, HMBC correlations from H2 to the carbonyl carbon ($\delta =$ 173.9 ppm) and C3 established the linkage of the α-amino acid moiety to C3, whereas the connection between C4 and the acetylenic bond was deduced from HMBC correlations between H4, C5, and C6. From this evidence, structure **1** was proposed for the toxin. The downfield position of the resonance at $\delta = 4.46$ ppm in the ^1H NMR spectrum, as well as the upfield shift of the signal at $\delta = 60.1$ ppm in the ^{13}C NMR spectrum from the C4 methine carbon adjacent to the acetylene unit were in accordance with the chemical shifts reported in the literature.[3]

The absolute configuration of amino acid **1** was determined as 2*R*,4*S* by both matrix-mode and optical rotation computations based on DFT methods.[4] Therefore, compound **1** is 2*R*-amino-4*S*-hydroxy-5-hexynoic acid (Scheme 1). The structure and absolute configuration of the compound were confirmed by a total synthesis that commenced from *N*-*tert*-butyloxycarbonyl(Boc)-D-aspartic acid-1-*tert*-butyl ester (**4**, Scheme 2, see also the Supporting Information).

Compound **2** was identified as 2-amino-5-hexynoic acid by comparison of its spectroscopic data with literature values.[5,6] The optical rotation (−2.7°, see the Supporting Information) was the opposite sign to that of 2*S*-amino-5-hexynoic acid reported in the literature (+3.8°).[7] This indicates that **2** has the 2*R* configuration (Scheme 1).

Both amino acids **1** and **2** were lethal and have estimated oral LD_{50} values in ICR mice of 71 and 84 mg kg^{-1}, respectively (see the Supporting Information). We also isolated γ-guanidinobutyric acid (**3**) which was reported to lead to seizures in rabbits,[8,9] but we did not evaluate its toxicity in mice. The total content of **1** and **2** in the fruiting bodies was 2000 mg per 1000 g of dried fruiting body after extraction and isolation by an optimized method that uses water instead of ethanol. If these LD_{50} values were applied to humans (60 kg), it would correspond to the ingestion of about 400 g of dried *T. venenata*.

We obtained post mortem blood from the heart of a 27-year-old man in Xiangyun County, Yunnan province, who died suddenly on August 18, 2009. We compared this blood sample with an authentic sample of **1** (see Supporting Information, Figure 1–3). Similar fragment ions were present in the LC-MS/MS of the blood sample and of authentic **1**, which suggests that **1** was present in the blood sample. This village had experienced repeated clusters of Yunnan SUD in the past and investigators had found *T. venenata* in the environs. This finding served as direct evidence that the man had eaten the mushroom before he died.

Observations from cases of Yunnan SUD suggest that a toxin that affects cardiac muscle might be responsible. A decade ago, a new type of mushroom poisoning, rhabdomyolysis, which was caused by repeated ingestion of *Tricholoma equestre*, was reported in France.[10] A recent report from Japan has attributed deaths and rhabdomyolysis to cycloprop-2-ene carboxylic acid, a toxin from *Russula subnigricans*.[11] Accordingly, we determined the creatine kinase (CK) levels in the serum of mice as a marker for cardiac and skeletal muscle toxicity.

Mice that were treated with **1** and **2** from had a 1.1–1.6-fold increase in serum CK levels relative to control mice that received water (Table 1). This effect included a dose response. The magnitude of this increase was not consistent with the fivefold or greater increase above the upper limit of normal CK level that has been proposed for a diagnosis of rhabdomyolysis, nor with the 14-fold increase in CK level that was reported in the case of the *R. subnigrans* toxin.[11] An elevated CK level can result from a more widespread death of cells, which includes brain cells, and could indicate general metabolic toxicity.[12]

An extract of *T. venenata* caused profound hypoglycemia (median 0.66 mmol L^{-1} glucose) in mice within 2 h of oral exposure (data not shown). This level of serum glucose will rapidly lead to depletion of ATP and neural cell death, and thus may explain the fatal outcome in humans and experimental animals.[13] One other botanical toxin, hypoglycin from the ackee fruit, is known to reduce blood glucose to this extent.[14] Hypoglycin is a specific inhibitor of isovaleryl CoA

Table 1: Serum creatine kinase (CK) levels in mice after three consecutive daily oral exposures to **1**, **2**, or *p*-phenylenediamine.

	Amino acid **1**			Amino acid **2**		
Exposure mg kg^{-1}	*n*[a]	Mean CK	Rel. inc.[b]	*n*[a]	Mean CK	Rel. inc.[b]
0[c]	4	680	–	4	410	–
10	0	–	–	4	460	1.1
20	4	740	1.1	4	460	1.1
40	4	1040	1.5	4	670[d]	1.6
60	4	1080[d]	1.6	0	–	–
Pos. cont.[e]	4	1250	1.8	4	600	1.5

[a] *n* is the number of mice tested. [b] Rel. inc. is the relative increase versus the control mice that were treated with water (value of exposure/value of water control). [c] Control mice treated with water. [d] Difference in mean value relative to the water control $p < 0.05$ (t-test). [e] Positive control mice treated with *p*-phenylenediamine (70 mg kg^{-1}).

dehydrogenase[15] and blocks β oxidation. Consequently, cells can no longer use lipids as an energy source, and a profound hypoglycemia results.[16,17] In the chemical structures of **1** and **2** there is a δ-unsaturated bond, which is similar to hypoglycin. Further investigations are needed to determine if **1** and **2** have this specific or a similar biochemical effect.

In summary, we have found that the fruiting bodies of the mushroom *T. venenata* contain two toxic amino acids, 2*R*-amino-4*S*-hydroxy-5-hexynoic acid (**1**) and 2*R*-amino-5-hexynoic acid (**2**). Furthermore, we have directly detected 2*R*-amino-4*S*-hydroxy-5-hexynoic acid in the blood of a victim of Yunnan SUD. These findings substantiate epidemiologic and initial toxicological findings. These toxins alone were sufficient to cause death in experimental mice without the presence of other potential toxins from this or other mushrooms, or from underlying disease. A campaign to warn Yunnan villagers against eating the mushroom has reduced reports of SUD in the affected area of Yunnan to zero in 2010 and 2011.

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[1] G. Q. Shi, J. Zhang, W. L. Huang, T. Yang, S. D. Ye, X. D. Sun, Z. X. Li, X. H. Xie, F. R. Li, Y. B. Wang, J. M. Ren, *Chin. J. Epidemiol.* **2006**, *27*, 96 – 101.
[2] R. Stone, *Science* **2010**, *329*, 132 – 134.
[3] Z. Y. Zhou, F. Wang, J. G. Tang, L. Z. Fang, Z. J. Dong, J. K. Liu, *J. Nat. Prod.* **2008**, *71*, 223 – 226.
[4] H. J. Zhu, J. Ren, Jr., C. Pittman, *Tetrahedron* **2007**, *63*, 2292 – 2314.
[5] Y. Aoyagi, T. Sugahara, *Phytochemistry* **1985**, *24*, 1835 – 1836.
[6] S. Collet, F. Carreaux, J. L. Boucher, S. Pethe, M. Lepoivre, R. Danion-Bougot, D. Danion, *J. Chem. Soc. Perkin Trans. 1* **2000**, 177 – 182.
[7] S. I. Hatanaka, J. Furukawa, T. Aoki, H. Akatsuka, E. Nagasawa, *Mycoscience* **1994**, *35*, 391 – 394.
[8] F. Irreverre, R. L. Evans, A. R. Hayden, R. Silber, *Nature* **1957**, *180*, 704 – 705.
[9] D. Jinnai, A. Sawai, A. Mori, *Nature* **1966**, *212*, 617 – 617.
[10] R. Bedry, I. Baudrimont, G. Defeieux, E. E. Creppy, J. P. Pomies, J. M. Ragnaud, M. Dupon, D. Neau, C. Gabinski, S. De Witte, J. C. Chapalain, P. Godeau, *N. Engl. J. Med.* **2001**, *345*, 798 – 802.
[11] M. Matsuura, Y. Saikawa, K. Inui, K. Nakae, M. Igarashi, K. Hashimoto, M. Nakata, *Nat. Chem. Biol.* **2009**, *5*, 465 – 467.
[12] W. H. Bagley, H. Yang, K. H. Shah, *Int. Emerg. Med.* **2007**, *2*, 210 – 218.
[13] P. E. Cryer, *J. Clin. Invest.* **2007**, *117*, 868 – 870.
[14] C. H. Hassall, K. Reyle, P. Feng, *Nature* **1954**, *173*, 356 – 357.
[15] K. Tanaka, E. M. Miller, K. J. Isselbacher, *Proc. Natl. Acad. Sci. USA* **1971**, *68*, 20 – 24.
[16] K. Melde, H. Buettner, W. Boschert, H. P. O. Wolf, S. Ghisla, *Biochem. J.* **1989**, *259*, 921 – 924.
[17] K. Melde, S. Jackson, K. Bartlett, H. S. A. Sherratt, S. Ghisla, *Biochem. J.* **1991**, *274*, 395 – 400.