nature chemical biology

Identification of the toxic trigger in mushroom poisoning

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We have isolated the small, highly strained carboxylic acid cycloprop-2-ene carboxylic acid from the Asian toxic mushroom *Russula subnigricans*. This compound is responsible for fatal rhabdomyolysis, a new type of mushroom poisoning that is indicated by an increase in serum creatine phosphokinase activity in mice. We found that polymerization of the compound at high concentrations via ene reaction abolishes its toxicity.

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A new type of mushroom poisoning, rhabdomyolysis, caused by repeated ingestion of *Tricholoma equestre*, was reported recently in France[1] and Poland[2]. Further, some other mushrooms that have been believed to be edible and that have even been cultivated have a similar effect[3–5]. In Japan, this type of acute poisoning, caused by *Russula subnigricans*, has been known since 1954 (ref. 6). Owing to its strong toxicity, from 2005 to 2007, consumption of this mushroom in Japan led to several cases of fatal poisoning (deaths/cases: 2/2 in 2005, 1/1 in 2006 and 1/3 in 2007). Thus far the country has experienced seven fatal accidents. Typical symptoms include nausea and diarrhea, which begin 30 min after ingestion, followed by speech impairment, convulsions, pupil contractions, stiff shoulders, backaches and the presence of myoglobin in urine. This myoglobinuria, the most distinctive symptom of the poisoning, comes from rhabdomyolysis[7]. In severe cases, additional symptoms include loss of consciousness, weakening of the heart and death[8]. Biochemical tests reveal a marked increase in plasma creatine phosphokinase (CPK) to greater than 90,000 U l^{-1} (normal values are about 200 U l^{-1}). This fungus is distributed widely in Asia, in countries such as Japan, Korea, China and Nepal. Similar poisonings due to this toadstool also occurred in Taiwan in 1998 (ref. 9). Despite the highly toxic nature of this mushroom, identification of the responsible toxin has been slow because of incorrect classification of several similar *Russula* species.

Previous studies on the constituents of an *R. subnigricans* candidate distributed in the Miyagi prefecture (northeast Japan) reported isolation of characteristic compounds[10–13], including cytotoxic phenolic compounds called russuphelins[10,11]. However, one report questioned whether the species was truly *R. subnigricans*[14]. Indeed, all of the accidental poisonings due to this mushroom occurred in western (not northeastern) Japan. Therefore, we compared two candidates, one collected in Miyagi and one from Kyoto (located in the western area where the first poisoning occurred). The Miyagi species closely resembles the Kyoto species, and both are toxic to mice when water extracts are injected intraperitoneally. However, when water extracts were administered orally, only the Kyoto species was toxic. Thus, the genuine *R. subnigricans* with lethal toxicity is the Kyoto species.

To isolate the toxin responsible, extracts were separated, guided by lethal toxicity in mice through oral injection (all animal experiments were performed with the approval of the Keio University School of Medicine Laboratory Animal Care and Use Committee). We cut the fruiting bodies of *R. subnigricans* collected in Kyoto into pieces and soaked them in water overnight at 4 °C (**Supplementary Methods** online). After filtration, the filtrate was concentrated under reduced

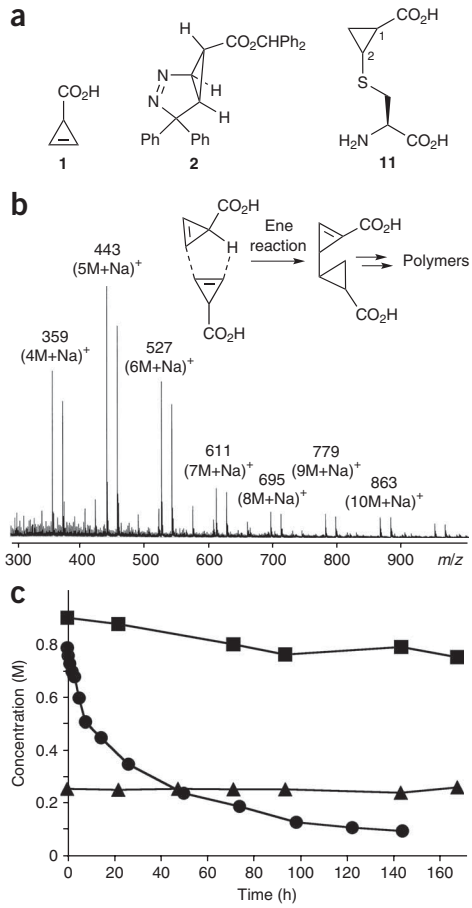

Figure 1 Structures and properties. (**a**) Structures of toxin **1** and its derivatives **2** and **11** (a 1:1 mixture of (1*S*,2*R*) and (1*R*,2*S*) diastereomers). (**b**) ESI-MS of the polymeric products. (**c**) Stability of **1** (●), **4** (■) and **5** (▲) in D_2O at ambient temperature, as determined by ^1H NMR spectroscopy.

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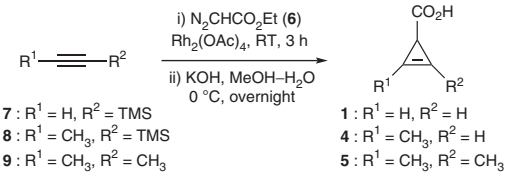
Received 5 December 2008; accepted 1 April 2009; published online 24 May 2009; doi:10.1038/nchembio.179



Scheme 1 Synthesis of **1** and its analogs **4** and **5**. TMS, trimethylsilyl group; RT, room temperature (20 °C).

pressure. Concentration to dryness substantially decreased the toxicity. Therefore, we avoided concentration to dryness during all the separation steps. The concentrated solutions were dialyzed against water. The dialyzate was concentrated and then successively separated by chromatography on octadecyl silica gel (ODS) and anion exchange resin. We subjected the solution obtained to gel filtration, and we combined the toxin-containing fractions and concentrated the solution to 1 ml under reduced pressure. The final solution contained approximately 15 mg (from 125 g of fruiting bodies) of toxin **1**, as determined by ^{1}H NMR analysis using an internal standard.

The ^{1}H NMR spectrum (300 MHz in D_2O/H_2O (1:5), TSP (trimethysilyl-2,2,3,3-tetradeuteropropionic acid sodium salt) = 0.00 p.p.m.) of toxin **1** was simple: δ 2.16 (t, J = 1.5 Hz, 1H) and 7.09 (d, J = 1.5 Hz, 2H) (**Supplementary Fig. 1** online). Its ^{13}C NMR spectrum (75 MHz in D_2O/H_2O (1:5), TSP = –2.00 p.p.m.) indicated three types of carbon atoms at δ 17.03, 103.89 and 183.16 p.p.m. (**Supplementary Fig. 2** online). Its molecular mass $[M]^+$ by HREI-MS (m/z) was 84.0211, which corresponds to $C_4H_4O_2$ (84.0211). These data indicate that the toxin is cycloprop-2-ene carboxylic acid (**1**) (**Fig. 1a**, **Supplementary Methods** and **Supplementary Figs. 3** and **4** online), which is a known synthetic compound[15] that has never been isolated from a natural source. Cycloprop-2-ene carboxylic acid appears to be the smallest carboxylic acid with strong lethal toxicity. Cyclopropenes are versatile components for synthetic organic chemistry[16]. In addition, some bioactive cyclopropenes have been reported[17].

To investigate the chemical functionality of the toxin, we converted compound **1** to stable and nonvolatile derivative **2** by reaction with diphenyldiazomethane (**3**) (**Fig. 1a**, **Supplementary Methods** and **Supplementary Figs. 5** and **6** online). We established the structure of **2** by ^{1}H NMR, ^{13}C NMR, MS and IR spectra, which also confirmed the structure of toxin **1**.

Next, to investigate the toxicity of some congeners, we synthesized compounds **1**, **4** and **5** (**Scheme 1**, **Supplementary Methods** and **Supplementary Figs. 7–10** online) according to a reported procedure[15], with a slight modification: ethyl diazoacetate (**6**) was added to the corresponding acetylene derivatives, **7**, **8** and **9**, in the presence of $Rh_2(OAc)_4$. The products were hydrolyzed under basic conditions to afford the corresponding carboxylic acids **1**, **4** and **5**.

Instability during concentration and volatility were the most troublesome properties of toxin **1** during both isolation and toxicity assessment. For example, when a solution of **1** (8 mg) in water (0.4 ml) was lyophilized, a small amount (0.2 mg) of **1** was recovered in the trap (liquid N_2, –196 °C), while most was polymerized and remained as colorless powder. The ESI-MS analysis indicated that the nonvolatile powder consisted of a mixture of polymers; observed mass numbers included 359 $[4M+Na]^+$, 443 $[5M+Na]^+$, 527 $[6M+Na]^+$ and 611 $[7M+Na]^+$ (**Fig. 1b** and **Supplementary Methods**). The half-life of **1** (0.8 M in D_2O) was about 20 h at ambient temperature (20 °C). These results can be explained by the easily polymerizable nature of cyclopropenes via ene reaction[18]. In contrast, the derivatives **4** and **5** did not undergo polymerization at ambient temperature (**Fig. 1c**). The stability of cyclopropenes depends on their substituents. All natural cyclopropenes that have been isolated (that is, sterculic acids[19] and calysterols[20]) are 1,2-disubstituted or 1,2,3-trisubstituted cyclopropenes, and are much more stable than **1**. Furthermore, introduction of substituents on the cyclopropene framework also affects the reactivity toward nucleophiles. For example, we added L-cysteine (**10**) to a solution of **1** in water and concentrated the product *in vacuo* to afford the adduct **11** as a diastereomeric mixture (**Fig. 1a**; **Supplementary Methods** and **Supplementary Figs. 11** and **12** online). In contrast, both **4** and **5** remained unchanged upon addition of **10**, even at elevated temperature (60 °C).

We tested the toxicities of the samples synthesized by oral administration in mice (**Table 1** and **Supplementary Methods**). The toxicities of **1**, **4** and **5** are summarized in **Table 1**. For toxin **1**, we observed tremor, hair erection and decreased mobility within 3 h of ingestion. In the worst cases, the mice died through collapse and tonic extension. Introduction of a methyl group into the skeleton of **1** considerably reduced toxicity; addition of two methyl groups eliminated lethal toxicity. Myoglobinuria could not be reproduced in mice by administration of toxin **1**. However, mice given **1** exhibited a significant ($P < 0.01$) increase in serum CPK activity (3441 ± 1012 U l^{-1}, 5 mg kg^{-1})[7] compared to controls—which received either water (240 ± 34 U l^{-1}), a methanol extract that contained almost no toxin (171 ± 15 U l^{-1}, 1 g kg^{-1}) and phenylenediamine (positive control, 397 ± 21 U l^{-1}, 70 mg kg^{-1})—which indicates progressive rhabdomyolysis (**Supplementary Methods** and **Supplementary Table 1** online)[21]. Preliminary biological experiments indicated that toxin **1** showed no antibacterial activity against many strains and no cytotoxicity against several cell types, which implies that toxin **1** does not directly attack myocytes, but triggers the rhabdomyolysis and subsequent lethal poisoning (**Supplementary Methods**).

We estimated the amount of **1** contained in fruiting bodies from the ^{1}H NMR spectrum of the crude extract. The content was very high: 360 mg per 500 g of fruiting body. The LD_{100} value (the dose lethal to 100% of animals tested) by oral injection in mice was 2.5 mg kg^{-1}. If this LD_{100} value was applied to humans, it would correspond to about two or three pieces (diameter of the cap: 6–7 cm) of the mushroom.

We have identified the genuine toxic *R. subnigricans* and isolated the responsible toxin, cycloprop-2-ene carboxylic acid. This acid causes severe rhabdomyolysis, probably not by direct interaction with myocytes but by a trigger for some other biochemical reactions. This study should facilitate investigations of similar effects of other mushrooms (including *T. equestre*) and clarification of the mechanisms of rhabdomyolysis.

Table 1 Lethal toxicities

Compound	LD_{100} (p.o.) (mg kg^{-1})	LD_{100} (i.p.) (mg kg^{-1})
1	2.5	2.5
4	50	–
5	>250	–

Lethal toxicities of cycloprop-2-ene carboxylic acids (**1**, **4** and **5**) in mice were tested by oral (p.o.) and intraperitoneal (i.p.) injection.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

ACKNOWLEDGMENTS
We thank T. Suda, S. Morimoto, T. Ueda and K. Yokoyama (Shiga University) for their help in collecting and identifying the mushrooms. We are grateful to F. Hashizaki (Ueno Zoological Gardens) for the help in the animal experiments.

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AUTHOR CONTRIBUTIONS
M.M. performed all experiments and all data analysis, except for histopathological, antibacterial activity and cytotoxicity analyses; Y.S. performed data analysis and helped write the manuscript; K.I. performed histopathological analyses; K.N. performed antibacterial activity and cytotoxicity analyses; M.I. performed antibacterial activity and cytotoxicity analyses; K.H. was the principal investigator of the project and contributed to data analysis and manuscript writing; M.N. performed data analysis, helped write the manuscript and provided funding.

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