Isolation of Vomiting Principles from the Mushroom *Rhodophyllus rhodopolius*

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The toxicity of the extracts of basidiocarp of *Rhodophyllus rhodopolius* was estimated by the effective dose inducing vomit in frogs. The ethanolic extract of the mushroom was submitted to dialysis, and the effective outer portion was chromatographed subsequently on cellulose, alumina, and reversed-phase ODS columns. The toxic vomiting principles that gave a positive Dragendorff reaction on a thin-layer chromatogram were isolated and identified with (2-hydroxyethyl)trimethylammonium salt (choline), tetrahydro-4-hydroxy-*N*,*N*,*N*,5-tetramethyl-2-furanmethanaminium salt (muscarine), and (4,5-dihydroxyhexyl)trimethylammonium salt (muscaridine), respectively, by comparison of ^1H NMR, ^{13}C NMR, and mass spectral data with those of authentic samples.

Rhodophyllus rhodopolius (Fr.) Quel. has been known in Japan as a poisonous mushroom. According to the annual statistical data from Japanese Ministry of Health and Welfare, *R. rhodopolius* came in the second to cause poisoning by mushrooms. Ingestion of the basidiocarp of this mushroom in different ways (soup, cooked, fried) causes nausea, vomiting, diarrhea, and sometimes stomachache (Imazeki, 1978).

The *Rhodophyllus* species occurring in Japan are regarded generally as harmless, and the toxic *R. rhodopolius* is often mistaken by amateur mushroom hunters for the edible mushroom *Rhodophyllus crassipes* (Imazeki and Toki; Imazeki and Hongo), since both species are morphologically very similar.

The occurrences of muscarine in *Amanita muscaria* (Eugster and Waser, 1954), *Clitocybe dealbata* (Hughes et al., 1966), and *Inocybe praeterisa* (Malone et al., 1962), choline in *Naemetoloma fasciculare* (Diak, 1977) and *Corprinus comatus* (List, 1960), muscaridine in *A. muscaria* (Kögl et al., 1960), and vinylglycine in *Rhodophyllus nidorous* (Dardenne et al., 1974) were reported. But neither the presence of any toxic principle in *R. rhodopolius* nor the occurrence of the above toxic principles in any other *Rhodophyllus* species has been reported. This paper is concerned with biological tests and vomiting toxic principles occurring in the ethanolic extract of the basidiocarp of *R. rhodopolius* that was studied by chromatographic separation and comparative NMR and mass spectroscopic analyses.

EXPERIMENTAL SECTION

Materials. *R. rhodopolius* and *R. crassipes* were collected in natural habitats in Yamanashi prefecture in the autumn of 1981–1983. The samples were dried in a freezer at −40 °C for 3 days, showing the moisture content 94% in each basidiocarp. The pulverized basidiocarps were stored in a desiccator prior to use.

Reagents. Analytical grade reagents were used for detection.

Chromatography. Thin-layer chromatography (TLC) utilyzed cellulose (Avicel) with a developing solvent of isopropyl alcohol *i*-PrOH/H_2O (3:1): cellulose column (4 × 36 cm), mobile solvent *i*-PrOH/H_2O (3:0.5); alumina E-type column (4 × 30 cm), mobile solvent methyl alcohol/acetone (1:4); reversed-phase (C_{18}) column (3 × 20 cm), mobile solvent 1% HOAc in CH_3CN/H_2O (6:4) and CH_3CN/H_2O (1:99).

Apparatus. NMR spectra were measured on a JEOL FX-270 spectrometer, in D_2O with sodium 3-(trimethylsilyl)propanesulfonate as an internal standard. Mass spectra were measured on a JEOL D-300 mass spectrometer with a source temperature from 50 to 270 °C.

Animals. The ddY strain male mice weighting 18–22 g and leopard (*Rana nigromaculata*) and ranid (*Rana japonica*) frogs, weighing 10–15 g were used for experiments. The animals were fed with a basal diet and water ad libitum before the experiments.

Bioassay. The dried basidiocarps (15 g) were extracted subsequently with petroleum ether, ethyl ether, chloroform, acetone, ethanol, and water. The residue was dialyzed to obtain the outer and inner solutions. Each extract (20 mg), except the aqueous extracts, was suspended in 1 mL of 1% Tween 60 solution, and the aqueous extracts were dissolved in 1 mL of distilled water. The test solution was administered orally to mice and frogs at approximately 5 mg in 0.2 mL per 10 g of body weight, respectively. The controls were pretreated by oral administration of 0.5 mL of 1% Tween 60 solution and 0.2 mL of distilled water, respectively. The poisoning symptoms of mice and frogs were observed at intervals of 5 min for 30 min after oral administration of the extracts to find chromodacryorrhea, salivation, and vomit, and occasionally respiratory paralysis to death.

Isolation and Purification of the Toxic Principles. By the above biological assay the ethanolic extract showed a poisonous effect to mice and frogs. Accordingly the mushroom was treated by the following process to isolate and purify the poisonous principle. The dried and powdered mushroom (60 g) was extracted with Et_2O for 16 h. The residue obtained on evaporation was refluxed twice with EtOH for 48 h. The combined EtOH extracts (I) were concentrated in vacuo and submitted to dialysis using cellophane membrane with a cutoff of molecular weight (mol wt) 1000. The outside portion of the dialysis (II) was concentrated under reduced pressure. The residue was chromatographed on a column of Avicel using *i*-PrOH/H_2O (3:1) as the solvent to afford a crude toxic fraction (III). From this fraction a toxic principle was collected from Avicel TLC developed with *i*-PrOH/H_2O (3:1) with a positive spot at R_f 0.4–0.7 detected by spraying with Dragendorff's reagent. Furthermore, the toxic fraction (III) was chromatographed on a column of Avicel using *i*-PrOH/H_2O (3:0.5) as a mobile phase to afford a partly purified toxic principles (IV). The principle (IV) was purified on a column of alumina using a mobile phase of

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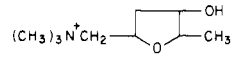
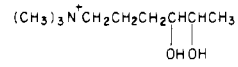
0021-8561/85/1433-1204$01.50/0

Table I. Mass Spectral Analysis of the Extracted Toxic Principles[a]

m/z	fragment	
	Choline	
104 (M^+)	$C_5H_{14}NO$	$(CH_3)_3N^+CH_2CH_2OH$
89 ($M^+ - 15$)	$C_4H_{11}NO$	
58 ($M^+ - 15 - 31$)	C_3H_8N	
	Muscarine	
174 (M^+)	$C_9H_{20}NO_2$	
159 ($M^+ - 15$)	$C_8H_{17}NO_2$	
116 ($M^+ - 58$)	$C_6H_{12}O_2$	
58 ($M^+ - 116$)	C_3H_8N	
	Muscaridine	
176 (M^+)	$C_9H_{22}NO_2$	
161 ($M^+ - 15$)	$C_8H_{19}NO_2$	
87 ($M^+ - 15 - 74$)	$C_5H_{13}N$	
58 ($M^+ - 15 - 29$)	C_3H_8N	

[a] m/z 58 $[(CH_3)_2{}^+N{=}CH_2]$ (Newton et al., 1983); EI-MS, temp 50–270 °C.

MeOH/$(CH_3)_2CO$ (1:4), and the eluate was divided into two portions. One portion was purified on an ODS reversed-phase column with CH_3CN/H_2O (6:4) added with 1% HOAc to afford muscarine and muscaridine. The other portion was purified on a column of ODS with CH_3CN/H_2O (1:99) to give choline.

RESULTS AND DISCUSSION

The ethanolic extracts of *R. rhodopolius* have been shown to contain principles toxic for mice and frogs. Oral administration of 5 mg of I in 0.2 mL of distilled water showed the toxic symptoms and resulted in deep breathing, salivation, blinking of the eyes, curled backs, wide open mouth with jumping, trembling, and frequently death in mice and in vomiting or death in frogs. These toxic symptoms were very similar to those produced by quaternary ammonium salts (Hodge, 1944; Gyermek and Unna, 1958; 1960; Tyler, 1958; Witkop et al., 1959). However, no toxic symptoms were associated with any other solvent extracts of *R. crassipes*. The outside portion of dialysis, the molecular weight of which was less than 1000, induced vomiting and occasional death in mice. Vomiting and diarrhea were also observed in cats and dogs with fraction II. Fraction II gave a red to orange yellow spot on Avicel TLC at R_f 0.4–0.7 on spraying with Dragendorff's reagent. Alumina column chromatography of the toxic principles using MeOH/acetone as the solvent gave three spots with Dragendorff's reagent on Avicel TLC. These were isolated and identified as choline giving the red–purple spot and muscarine and muscaridine giving yellow–orange spots, respectively. Final purification of the toxic principles of *R. rhodopolius* were obtained by reversed-phase column chromatography and subjected to EI–MS to give mass spectra in Table I. Recently, Unger et al. (1981) reported that the presence of choline and muscarine in *Inocybe napipes*, which was confirmed by means of EI–MS and gave results similar to our data. The mass spectra of choline, muscarine, and muscaridine were measured to confirm the molecular weight of the proposed molecular formula of compound: $C_5H_{14}NO$, $C_9H_{20}NO_2$, and $C_9H_{22}NO_2$, respectively. The fragment m/z 58, C_3H_8N, was used for monitoring trimethylammonium groups as reported by Newton et al. (1983). The ^1H and ^{13}C NMR spectra of the toxic principles were taken up in D_2O. The ^1H NMR signal at δ 3.22 showed the presence of a $(CH_3)_3N^+$ group in the molecules of choline, muscarine, and muscaridine (Pochet and Huynh-Dinh, 1982). The signal at δ 3.05 was assigned to $-CH_2O-$ and δ 4.08 to $-CH_2-$. The ^{13}C NMR signal at δ 56.6 was assigned to CH_3N corresponding to the trimethylammonium group (Breitmaier et al., 1970; Toda et al., 1981). The triplet signals at δ 56.6 and 70.1 were assigned to $-CH_2O-$, and $-CH_2-$, respectively. The mass and NMR spectra data of these toxic principles were found to be identical with those of authentic samples of choline, muscarine, and muscaridine, respectively. The contents of choline (0.14%), muscarine (0.05%), and muscaridine (0.05%) in the dried mushroom were measured by densitometric scanning TLC. In conclusion, the vomiting principles in *R. rhodopolius* were large amounts of choline, muscarine, and muscaridine, which were identified by means of chemical technique coupled with two bioassays.

ACKNOWLEDGMENT

The authors thank Dr. S. Suzuki for his kindness in measuring NMR, S. Horii for his help with the mass spectra and Dr. Y. Okada for his advice to perform this study.

Registry No. Choline, 62-49-7; muscarine, 300-54-9; muscaridine, 6801-43-0.

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Received for review March 28, 1985. Accepted August 5, 1985.