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Sambutoxin, a New Mycotoxin Produced by Toxic *Fusarium* Isolates Obtained from Rotted Potato Tubers

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Ninety-nine isolates of *Fusarium* species were obtained from rotted potato tubers from various parts of Korea. Of these isolates, 80 were identified as *Fusarium oxysporum*, *F. solani*, or *F. sambucinum*. The isolates of these species were grown on autoclaved wheat grains and examined for toxicity in a rat-feeding test. A total of 8 of 57 *F. oxysporum* isolates, 3 of 14 *F. solani* isolates, and 5 of 9 *F. sambucinum* isolates caused the death of the rats. Of the 16 toxic isolates, 1 isolate of *F. oxysporum* produced a substantial amount of moniliformin, which could account for its toxicity. None of the other 15 isolates produced trichothecenes, moniliformin, fusarochromanone, fumonisin B_1, or wortmannin. *F. sambucinum* PZF-4 produced an unknown toxin in wheat culture. This new toxin, given the trivial name sambutoxin, caused toxic effects in rats, including body weight loss, feed refusal, hemorrhage in the stomach and intestines, and, finally, death when rats were fed diets supplemented with 0.05 and 0.1% sambutoxin. The toxin was also toxic to chicken embryos, and the 50% lethal concentration was 29.6 μg per egg. Sambutoxin formed as white crystals that turned purple when combined with reagents such as sulfuric acid and *p*-anisaldehyde. It exhibited a green color immediately after treatment with potassium ferricyanide-ferric chloride. Its UV spectrum had absorption maxima at 213, 233, and 254 nm, and its infrared spectrum showed an amide group at 1,650 and 1,560 cm^{-1} and a hydroxy group at 3,185 cm^{-1}. Mass spectrometry showed that the molecular weight of the toxin was 453 and the molecular formula was $C_{28}H_{39}NO_4$. Sambutoxin was produced by seven of the toxic *F. oxysporum* isolates and by five of the toxic *F. sambucinum* isolates but not by any of the toxic *F. solani* isolates.

Fusarium species are distributed worldwide as saprophytes, soil inhabitants, and parasites of many plants. Some isolates of certain species are capable of producing mycotoxins. During the past few decades, interest in *Fusarium* species has increased because they produce numerous mycotoxins involved in various mycotoxicoses of humans and animals (18).

Potato is a staple human food in Europe, North America, and Asia. After harvest, several *Fusarium* species invade potato tubers, and most strains can grow in rotted potato tubers as saprophytes. *Fusarium* dry rot is an important disease that can cause significant economic losses as a result of destruction of stored potatoes in the northern hemisphere (12). *Fusarium solani* var. *coeruleum* and *F. sambucinum* (also known as *F. sulphureum* with a sexual stage known as *Gibberella pulicaris*) are common causes of dry rot of potatoes in Europe and North America (4). Occasionally, other species, including *F. oxysporum* and *F. avenaceum*, are involved.

The production of trichothecenes is a common trait of *F. sambucinum* isolates from potato tubers with dry rot. Desjardins and Plattner (6) reported that most strains of *F. sambucinum* isolated from potato tubers with dry rot produced diacetoxyscirpenol (DAS) and monoacetoxyscirpenol, as well as other trichothecenes. Lafont et al. (15) also found several trichothecenes in potato tubers naturally and experimentally infected with *F. sambucinum* in France. Steyn et al. (23) found that *F. sambucinum* isolates from potatoes in an area of Iran with a high incidence of esophageal cancer were toxic to ducklings and produced DAS as well as other trichothecenes. El-Banna et al. (7) reported that trichothecenes such as deoxynivalenol (DON), acetyl-DON, and HT-2 toxin were produced in potato tubers inoculated with *F. solani* var. *coeruleum*. Trichothecenes were also detected in a few cultures of *F. sambucinum* in potato tubers. Siegried and Langerfeld (21) reported that potato extracts obtained after inoculation of potatoes with *F. sambucinum*, *F. solani*, and *F. oxysporum* were toxic to brine shrimp. The chemical nature of the mycotoxins produced in inoculated potato tubers by these isolates has not been described.

Although potato is not a staple food in Korea, it is an important crop and is cultivated in all provinces. Potatoes are usually planted during April and harvested during July in Korea. A high incidence of tuber rot is common during storage through the warm and humid summer. Han et al. (11) reported that the incidence of rotted potatoes ranged from 3 to 6% during the 5-month period from July to November. They also found that some potato samples were 40 to 45% decayed when they were purchased at markets. Lee (16) reported that *F. solani*, *F. sambucinum*, and *F. oxysporum* were major causes of rotted potato tubers during storage.

In this study we compared the toxicity of *Fusarium* isolates belonging to three species obtained from rotted potato tubers and attempted to account for their toxicity in terms of toxins known to be produced by *Fusarium* species. Later, one isolate of *F. sambucinum* PZF-4 was selected because it was the most toxic isolate and produced a mycotoxin that was named sambutoxin. This report also describes the production, the purification, some chemical and physical properties, and the toxicity of sambutoxin.

MATERIALS AND METHODS

Potato samples. Thirteen samples of rotted potato tubers, consisting of five to seven potatoes each, were collected from 11 different farmers' stocks in six provinces of Korea during

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January 1991. The potatoes were harvested in July 1990 and had been stored for 7 months.

Isolation and culture of *Fusarium* species. For each potato sample, 50 pieces (5 mm^3) were removed from the rotted parts with a razor, soaked in 2% NaOCl for 1 min, rinsed in sterile distilled water, transferred to potato dextrose agar (Bacto Potato Dextrose Agar Dehydrated; Difco Laboratories, Detroit, Mich.), and incubated at 25°C for 4 to 7 days. *Fusarium* species were transferred from the potato pieces to noncommercial potato dextrose agar or carnation leaf agar (8), or both, incubated under fluorescent lamps (5,000 lux) at 25°C, and identified to species with the criteria described by Nelson et al. (19). A total of 99 isolates were obtained from the rotted potato tubers. Of these, 80 belonging to *F. oxysporum*, *F. solani*, and *F. sambucinum* were selected and screened for toxicity by feeding the cultures to rats. Stock cultures of *Fusarium* isolates were single spored and then maintained on moist autoclaved soils and stored at −15°C.

Erlenmeyer flasks (1 liter), each containing 200 g of wheat and 120 ml of distilled water, were autoclaved for 1 h at 121°C twice with a 24-h interval. The wheat was inoculated with mycelium plugs from a 5-day-old potato dextrose agar plate of the fungus. The flasks were incubated for 2 weeks at 25°C and 2 weeks at 10°C. The mycelial mass and substrate were dispersed onto a screen-bottom tray and allowed to air dry in a ventilated hood. When dry, this inoculated substrate was ground to the consistency of flour and stored at −15°C until used.

Rat-feeding test. Female Sprague-Dawley rats, 21 days old and weighing approximately 50 g each, were obtained from the Experimental Animal Farm, Seoul National University. The rats were housed in individual cages and fed a 1:1 mixture of ground moldy wheat and complete rat diet. The animals and feeds were weighed at the beginning and end of the experiment. Three rats were used for each *Fusarium* isolate. Control rats received a 1:1 mixture of ground noninoculated wheat and complete rat diet. The rats were observed for 7 days, and major symptoms and death were recorded. Surviving rats were sacrificed by cervical distortion and examined for pathological changes in the tissues.

Mycotoxin standards. T-2 toxin, HT-2 toxin, T-2 tetraol, DAS, moniliformin (MON), and wortmannin were kindly supplied by C. J. Mirocha, Department of Plant Pathology, University of Minnesota. Ketotrichothecenes, including DON, nivalenol, 15-acetyl-DON, 3-acetyl-DON, and 4-acetylnivalenol, were kindly supplied by T. Yoshizawa, Department of Bioresource Science, Kagawa University, Japan. Zearalenone and fumonisin B_1 were purchased from Sigma Chemical Co., St. Louis, Mo.

Detection of known mycotoxins in *Fusarium* extracts. To detect trichothecenes and zearalenone in the toxic cultures, each ground culture (20 g) was extracted with 160 ml of acetonitrile-water (3:1, vol/vol) for 30 min and the extract was filtered through Whatman no. 1 filter paper. The filtrate was defatted with the same volume of *n*-hexane and concentrated to dryness. The residue was dissolved in 2 ml of methanol and applied to a Florisil column (2 cm [inside diameter] by 15 cm) containing 10 g of Florisil (60/100 mesh; Fisher Scientific Co., Pittsburgh, Pa.). The column was washed with 100 ml of *n*-hexane and then eluted with 100 ml of chloroform-methanol (9:1, vol/vol). The eluate was concentrated to dryness, and the residue was redissolved in 2 ml of methanol. The extracts were chromatographed on thin-layer chromatography (TLC) plates (Kiesel gel 60, 20 by 20 cm, 0.25 mm thick; E. Merck, Darmstadt, Germany) with the toxin standards. The trichothecenes were made visible by spraying the plates with *p*-anisaldehyde and heating at 110°C for 10 min.

MON, fumonisin B_1, fusarochromanone, and wortmannin were extracted and detected by methods described by Scott et al. (20), Gelderblom et al. (9), Lee et al. (17), and Abbas and Mirocha (1), respectively.

Extraction and purification of sambutoxin. (i) Extraction. A 2-kg sample of a ground-wheat culture of *F. sambucinum* PZF-4 was divided into five subsamples of 400 g. Each subsample was placed in a 3-liter flask and successively extracted three times with chloroform (totaling 7,500 ml) and methanol (totaling 7,500 ml) in a reciprocating shaker at room temperature (23 to 26°C). The extracts were filtered through Whatman no. 2 filter paper and concentrated to dryness. The chloroform and methanol extracts and the remaining residue were bioassayed in a rat-feeding test. Each extract was dissolved in 200 ml of acetone, and a portion (20 ml) equivalent to 200 g of fungal culture was mixed with 200 g of complete rat diet. Control feed was prepared by mixing 20 ml of acetone alone with 200 g of complete rat diet. After drying at 40°C overnight, each diet was fed to three experimental rats (21-day-old female Sprague-Dawley rats) for 7 days.

(ii) Silica gel column chromatography. The chloroform extract was dissolved in a minimal volume of chloroform and loaded onto a silica gel column (4.5 cm [inside diameter] by 50 cm) containing 200 g of silica gel (Kiesel gel 60, 70/230 mesh; E. Merck). The column was eluted with a stepwise solvent system of neat chloroform to chloroform-methanol (9:1, vol/vol). The eluate was collected in 10-ml fractions with a fraction collector. The fractions were monitored by TLC and reduced to three fractions called F1, F2, and F3, which were bioassayed in a rat-feeding test. Both the F1 and F2 caused death of rats, but since F2 was the more toxic, it was purified further. A 10-g sample of F2 was suspended in 7 ml of ethyl acetate–*n*-hexane (3:1, vol/vol) and loaded onto a silica gel column (3.6 cm [inside diameter] by 60 cm) containing 150 g of silica gel (Kiesel gel 60, 230/400 mesh; E. Merck). The column was eluted with ethyl acetate–*n*-hexane (3:1, vol/vol), and the eluate was reduced to three fractions called F21, F22, and F23, which were bioassayed in a rat-feeding test.

(iii) Purification by preparative HPLC. The toxic F22 fraction (1.4 g) was finally purified by preparative high-performance liquid chromatography (HPLC) (Waters Delta Prep 4000; Millipore Co., Milford, Mass.). The following equipment and conditions were used for purification: column, μBondapak C_{18} (19 mm [inside diameter] by 300 mm; particle size, 15 to 20 μm; Millipore Co.); mobile phase, 80% methanol in water; flow rate, 10 ml/min; UV detector, 254 nm. The preparative HPLC yielded sambutoxin as a colorless solid.

(iv) Crystallization. The fraction from preparative HPLC was dissolved in 3 ml of methanol and held at 4°C for 5 h. White crystals of sambutoxin were collected by filtration and recrystallized twice from methanol as described above.

(v) Bulk purification of sambutoxin. Approximately 2 kg of culture material was extracted with ethyl acetate instead of chloroform. The extract was concentrated to 500 ml and back-extracted with 400 ml of distilled water three times. The aqueous layer was discarded, and the organic layer was concentrated to dryness. The residue was fractionated as described above.

(vi) Purity of sambutoxin. The isolated sambutoxin was analyzed on TLC plates (0.25 mm thick) with 10 different developing systems (Table 1). Sambutoxin was visualized by spraying the plates with *p*-anisaldehyde and heating them at 110°C for 10 min; the R_f values were recorded.

Color reactions of sambutoxin. Sambutoxin was chromato-

TABLE 1. TLC resolution of sambutoxin in various solvent systems[a]

Solvent system	Ratio by vol	R_f
Chloroform-methanol	9:1	0.45
Chloroform-methanol	95:5	0.33
Chloroform-acetone	3:2	0.33
Ethyl acetate-hexane	3:1	0.08
Ethyl acetate-toluene	3:1	0.15
Benzene-acetone	1:1	0.33
Toluene-methanol	7:3	0.54
Chloroform-methanol-water	6:3:1	0.90
Acetonitrile-water-benzene	90:6:4	0.64
Butanol-methanol-water	8:1:2	0.77

[a] The developed TLC plates were sprayed with *p*-anisaldehyde and heated at 110°C for 10 min. Only one spot was seen in all solvent systems.

graphed on TLC plates (0.25 mm thick) and developed in chloroform-methanol (9:1, vol/vol). Eight reagents were used to determine their reaction with sambutoxin on TLC plates (Table 2). The following methods were used to detect sambutoxin on TLC after its treatment with various sprayed reagents: naked-eye observation for color changes at room temperature immediately after its treatment with the spraying reagent; long- and short-wave UV light for color fluorescence; and heating at 110°C for 10 min for color change reactions or color fluorescence reactions.

Spectral measurements. The melting point was determined with a Yanaco micro-melting point apparatus. The UV spectrum was recorded in a methanol solution on a Hitachi 340 double-beam spectrophotometer. The infrared spectrum was obtained by a JASCO A-302 IR spectrophotometer with the sample encased in a KBr pellet. Low-resolution (LR) mass spectra were recorded on a double-focusing high-resolution (HR) mass spectrometer (JMS-AX505; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV. The sample was introduced via a direct-insertion probe. The LR electron impact (EI) mass spectrum was recorded at 70 eV and a source temperature of 200°C. The LR-chemical ionization (CI) analysis was carried out with methane as the reagent gas, at a source pressure of about 61 Pa. The filament electron energy was 200 eV, and the source temperature was 200°C. The direct-insertion probe was heated to 100°C in the EI and CI analyses. LR fast atom bombardment (FAB) analysis was carried out with a glycerol matrix and Ar as the bombarding gas. The HR FAB mass spectrum was measured by a JEOL JMS-SX102A mass spectrometer equipped with a JMA-DA7000 data system, operating at a resolution of 1,500. The analysis was carried out at an accelerating voltage of 10 kV with an *m*-nitrobenzyl alcohol matrix and Xe as the bombarding gas. The mass was determined accurately by using software peak matching and a dual-stage alternating FAB probe at a resolution of 8,000. Polyethylene glycol was used as a reference material.

TABLE 2. Color reaction of sambutoxin

Chemical reagent(s)	Color reaction at:	
	27°C	110°C
Aluminum chloride[a]		
p-Anisaldehyde[b]		Purple
2,4-Dinitrophenylhydrazine[c]		
Ferric chloride[d]		
Hydrochloric acid[e]		
20% Sulfuric acid in methanol		Pale purple
4-(*p*-Nitrobenzyl)pyridine–tetraethylene-pentamine[f]		
Ninhydrin solution[g]		
2% Potassium ferricyanide–2% ferric chloride[h]	Green	Green

[a] Aluminum chloride solution (1%) in ethanol.
[b] *p*-Anisaldehyde, 0.5 ml, was added to the mixture of 85 ml of methanol, 10 ml of glacial acetic acid, and 5 ml of concentrated sulfuric acid.
[c] A 10-ml volume of hydrochloric acid (36%) was added to a solution of 1 g of 2,4-dinitrophenylhydrazine in 100 ml of ethanol.
[d] Solution of ferric chloride (1 to 5%) in 0.5 N hydrochloric acid.
[e] Hydrochloric acid (36%) and ethanol were mixed in the volume ratio of 1:4.
[f] The plate was sprayed with a 3% (wt/vol) solution of 4-(*p*-nitrobenzyl)pyridine in chloroform-carbon tetrachloride (2:3, vol/vol), dried, heated for 30 min at 150°C, cooled, and sprayed with 10% (vol/vol) tetraethylene-pentamine in chloroform-carbon tetrachloride (2:3).
[g] Ninhydrin (0.1%) in methanol.
[h] A mixture of 2% potassium ferricyanide in H_2O and 2% ferric chloride in H_2O.

Toxicity test of sambutoxin. (i) Rat-feeding test. Sambutoxin was incorporated into complete rat diets at concentrations of 0.05 and 0.1% and fed to 21-day-old female Sprague-Dawley rats. Each treatment group consisted of three rats. After a 1-week feeding period, all surviving rats were sacrificed and examined for pathological changes in tissues.

(ii) Chicken embryo test. Fertile eggs were obtained from inbred Single-Comb White Leghorn females crossed with Rhode Island Red males and were set in a forced-air incubator at 38°C and 60% relative humidity. The eggs were candled on day 4 of incubation, when the blood vessels were visible. Visible shell membranes of eggs were removed with forceps, and 20 μl of sambutoxin dissolved in ethanol was dispensed onto the egg membrane at 12.5, 25, 50, 100, and 200 μg per egg to determine the toxicity to chicken embryos. The holes in the shell were sealed with paraffin immediately after injection. The control eggs received 20 μl of ethanol alone, and each treatment group consisted of 40 eggs. The eggs were candled again on days 4, 8, and 15 after injection, and the mortality of the embryos was recorded. The 50% lethal dose of sambutoxin was calculated by probit analysis (22).

Production of sambutoxin by the toxic isolates. The presence of sambutoxin was investigated with the 16 cultures of toxic *Fusarium* isolates. A 20-g portion of culture was moistened with 10 ml of distilled water and extracted with 100 ml of ethyl acetate for 30 min in a wrist action shaker. After filtration through Whatman no. 2 filter paper, the filtrate was back-extracted with 80 ml of distilled water and then concentrated to dryness. The residue was redissolved in 2 ml of chloroform and applied to a Florisil column (2 cm [inside diameter] by 20 cm). The column was packed with 10 g of Florisil (60/100 mesh) and topped with 5 g of anhydrous sodium sulfate. After being washed with 100 ml of *n*-hexane, the column was eluted with 100 ml of chloroform-methanol (9:1, vol/vol). The eluate was concentrated to dryness and redissolved in 2 ml of methanol. The extract was analyzed by TLC and HPLC. For the HPLC analysis, the following equipment and conditions were used: instrument, Shimadzu SCL-10A; Zorbax C_{18} column (4.6 mm [inside diameter] by 150 mm; particle size, 5 μm; Dupont Co., Kyoto, Japan); mobile phase, a linear-gradient solvent system of CH_3OH-H_2O (76:24 [vol/vol] to 96:4 [vol/vol]); flow rate, 1 ml/min; UV detector, 254 nm. The retention time of sambutoxin was 21.58 min.

RESULTS

Toxicity of *Fusarium* isolates. *Fusarium* spp. (99 isolates) were recovered from 13 samples of rotted potatoes collected at 11 sites. Representatives of 11 species were identified: *F.*

TABLE 3. Toxicity of *Fusarium* isolates obtained from rotted potato tubers and lethal to rats

Fusarium sp.	Strain	No. of rats which died[a]	Toxic sign(s)[b]	Mean wt change (g)[c]
None (control)		0	—	15
F. oxysporum	POF-23	1	—	−11
	PKF-12	1	IH	−18
	PBF-16	1	I&SH	−10
	PDF-3	1	IH	−13
	PJF-5	1	I&SH, Ht	ND[d]
	PJF-18	2	IH&M	ND
	PPF-9	3	I&SH	ND
	PKF-8	3	IH&M	ND
F. solani	PCF-16	1	SH	−13
	PPF-1	1	IH	−7
	PBF-2	3	I&SH	ND
F. sambucinum	PZF-5	1	IH	−16
	PKF-1	1	I&SH	−10
	PZF-6	2	IH&M	ND
	PZF-3	3	I&SH	ND
	PZF-4	3	I&SH, IM, Ht	ND

[a] Rats fed ground moldy wheat-complete diet (1:1) and control rats fed an autoclaved wheat-complete diet (1:1). Three rats were used for each treatment.
[b] —, no detectable toxic effect; I, intestine; S, stomach; H, hemorrhage; M, mucosa; H, hematuria.
[c] Mean weight change of surviving rats (minus sign signifies weight loss).
[d] ND, not determined because rats died.

oxysporum (57 isolates), *F. solani* (14 isolates), *F. sambucinum* (9 isolates), *F. reticulatum* (4 isolates), *F. equiseti* (3 isolates), *F. lateritium* (2 isolates), *F. subglutinans* (2 isolates), *F. chlamydosporum* (2 isolates), *F. proliferatum* (1 isolate), *F. acuminatum* (1 isolate), and *F. tricinctum* (1 isolate); 3 isolates could not be identified. Of the 99 isolates, 80 belonging to three abundant species were screened for the toxicity in rats. Of these 80 isolates, 16 (20%) caused death, 52 (65%) caused a loss in body weight of 0.5 to 19.5 g, and 28 (35%) caused a gain in body weight of 1.0 to 18.0 g. A total of 8 of 57 *F. oxysporum* isolates, 3 of 14 *F. solani* isolates, and 5 of 9 *F. sambucinum* isolates caused death. The toxicity of the 16 *Fusarium* isolates which caused death during the test period is summarized in Table 3. Of the 16 cultures, 15 were highly toxic by each of the following criteria: marked feed refusal, weight loss, death (at least one of the three rats in each treatment group), hemorrhage and severe mucosa of stomach or intestines or both, and reduced fat bodies among the tissues. One culture of *F. oxysporum* POF-23 caused death, but no visible toxic signs were observed in the tissues.

Assay for mycotoxins. The cultures of the 16 toxic isolates were tested for T-2, HT-2, T-2 tetraol, DAS, DON, 15-acetyl-DON, 3-acetyl-DON, nivalenol, 4-acetylnivalenol, zearalenone, MON, fumonisin B_1, fusarochromanone, and wortmannin. All isolates except one were negative for the toxins. One isolate of *F. oxysporum* PKF-8 produced over 1,000 ppm of MON when the culture extract was analyzed by TLC; the substantial amount of MON would account for its toxicity.

Isolation of sambutoxin. The toxicity of culture material of *F. sambucinum* PZF-4 and culture extracts to rats is shown in Table 4. Rats fed a diet containing a 1:1 mixture of crude culture and complete rat diet died of hemorrhaging in the stomach and intestines within 4 days after treatment. No toxicity was observed in the control group fed complete rat diet with 20 ml of acetone. After extraction, most of the toxicity was recovered in the $CHCl_3$ extract, while the methanol extract and residue caused only feed refusal and loss in body weight but not death. After the first silica gel column chromatography, most of the toxicity was recovered in the F2 fraction, although a portion of the toxicity was found in the F1 fraction (Table 5). Further purification of the F2 fraction on the second silica gel column indicated that a compound with an R_f value of 0.08 (F22 fraction) on a TLC plate with ethyl acetate–*n*-hexane (3:1, vol/vol) as the developing solvent system was responsible for the bulk of the toxicity applied to the column (Table 5). Subsequent fractionation of this fraction (F22 fraction) on an HPLC column (C_{18}) yielded one pure-white solid compound. During the large-scale extraction, 250 mg of purified sambutoxin was obtained from 2 kg of crude culture.

TABLE 4. Toxicity to rats of *F. sambucinum* PZF-4 culture material and fractions obtained from the extraction step

Fraction[a]	No. of rats which died[b]	Mean wt change (g)[c]
Control	0	20.0
Culture material	3	ND[d]
Chloroform extract	3	ND
Methanol extract	0	−10.0
Residue	0	−11.0

[a] The control was prepared by mixing 20 ml of acetone alone with 200 g of complete rat diet and drying the mixture at 40°C overnight. The extract obtained from 2 kg of culture was dissolved in 200 ml of acetone, and 20 ml of the solution was mixed with 200 g of complete rat diet and dried at 40°C overnight. Residue obtained after successive extraction of 2 kg of culture material was dried at 40°C for 1 day to remove solvent completely, and 200 g of the residue was mixed with 200 g of complete rat diet.
[b] Three rats were used for each treatment.
[c] Mean weight change of surviving rats (minus sign signifies weight loss).
[d] ND, not determined because rats died.

Physical properties of sambutoxin. Sambutoxin was subjected to TLC with 10 different solvent systems to verify its purity and R_f value. It was made visible by spraying the developed chromatograms with *p*-anisaldehyde. Only one spot, with various R_f values depending on the solvent system, was detected (Table 1). The purity of sambutoxin was greater than 98% in the HPLC analysis.

TABLE 5. Toxicity to rats of fractions obtained from two silica gel column fractionations of the chloroform extract of *F. sambucinum* PZF-4 culture material

Fraction[a]	No. of rats which died[b]	Mean wt change (g)[c]
Control	0	18.5
F1	1	−10.5
F2	3	ND[d]
F3	0	17.5
F21	0	−5.5
F22	2	ND
F23	0	15.0

[a] Control was prepared by mixing 20 ml of acetone alone with 200 g of complete rat diet and drying the mixture at 40°C overnight. The first silica gel column containing the chloroform extract was eluted with a stepwise solvent system of neat chloroform to chloroform-methanol (9:1, vol/vol) to give F1, F2, and F3 fractions. The F2 fraction was then subjected to the second silica gel column, which was eluted with ethyl acetate–*n*-hexane (3:1, vol/vol) to give F21, F22, and F23 fractions. Each fraction was dissolved in acetone, and a portion of the solution equivalent to 200 g of fungal culture was mixed with 200 g of complete rat diet and dried at 40°C overnight.
[b] Three rats were used for each treatment.
[c] Mean weight change of surviving rats (minus sign signifies weight loss).
[d] ND, not determined because rats died.

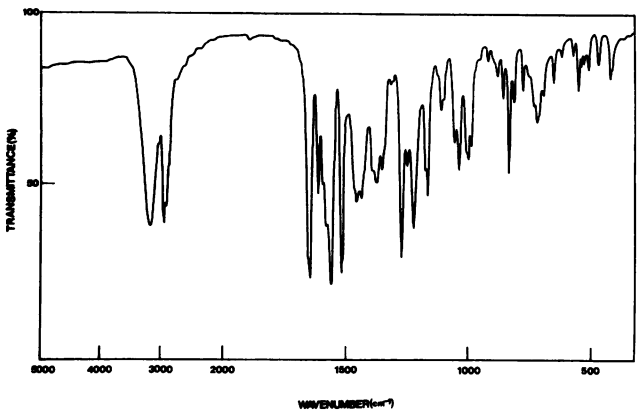

FIG. 1. Infrared spectrum of sambutoxin with the sample encased in a KBr pellet.

Sambutoxin was spotted on silica gel TLC plates, which were developed with chloroform-methanol (9:1, vol/vol). Various spraying reagents were used to determine the color reactions of sambutoxin (Table 2). To the naked eye, the toxin did not exhibit a color or fluorescence properties without additional preparation. Sambutoxin did not show any fluorescence properties with any chemical reagents after reaction or after reaction followed by heating at 110°C for 10 min. It appeared as a purple spot when sprayed with *p*-anisaldehyde and 20% sulfuric acid in methanol after being heated at 110°C for 10 min. It exhibited a green color reaction with potassium ferricyanide-ferric chloride, and the color did not change after treatment at 110°C for 10 min. Sambutoxin did not exhibit any color properties with aluminum chloride, 2,4-dinitrophenylhydrazine, ferric chloride, hydrochloric acid, 4-(*p*-nitrobenzyl)pyridine, or ninhydrin before or after being heated at 110°C for 10 min (Table 2).

The white crystals of sambutoxin had a melting range of 196.5 to 197.5°C. They were soluble in methanol, ethanol, butanol, ethyl acetate, chloroform, benzene, toluene, and acetone and insoluble in *n*-hexane and water.

Spectroscopic analyses of sambutoxin. The UV absorption of sambutoxin in methanol took place at wavelengths of 213 nm (ε, 38,000), 233 nm (ε, 17,000), and 254 nm (ε, 29,000). The infrared spectrum (Fig. 1) showed an amide group at 1,650 and 1,560 cm^{-1} and a hydroxyl group at 3,185 cm^{-1}.

The LR-EI mass spectrum of sambutoxin displayed a strong molecular ion at *m/z* 453 and fragment ions at *m/z* 396, 354, 286, 272, 258, 243, 230, and 217 (Fig. 2A). The cleavage from *m/z* 453 to 437 indicated the presence of a hydroxyl group somewhere in the structure of sambutoxin. The LR-CI mass spectrum of sambutoxin had strong $(M+1)^+$, $(M+15)^+$, and $(M+29)^+$ ion peaks at *m/z* 454, 468, and 482, respectively (Fig. 2B). The FAB mass spectrum of sambutoxin displayed a protonated molecular ion at *m/z* 454 (Fig. 2C).

HR-FAB mass spectrometry gave the molecular formula $C_{28}H_{40}NO_4$ (MH^+, *m/z* 454.2959; found, *m/z* 454.2957, Δ −0.8 mmu).

Toxicity of sambutoxin. In the rat-feeding test, a 0.05% level of sambutoxin in rat diet caused death at 4 to 7 days after treatment and a 0.1% level caused death within 4 days of the initial treatment. Rats that died following either treatment had hemorrhaging in the stomach and intestines accompanied by tissue degeneration.

In a chicken embryo test, 200 μg of sambutoxin per egg killed 100% of the chick embryos, half of which died within the first 4 days of incubation after the injection of sambutoxin. Sambutoxin elicited dose-related mortality responses over the range of 12.5 to 100 μg per egg. The calculated 50% lethal dose in this system for sambutoxin was 29.6 μg per egg.

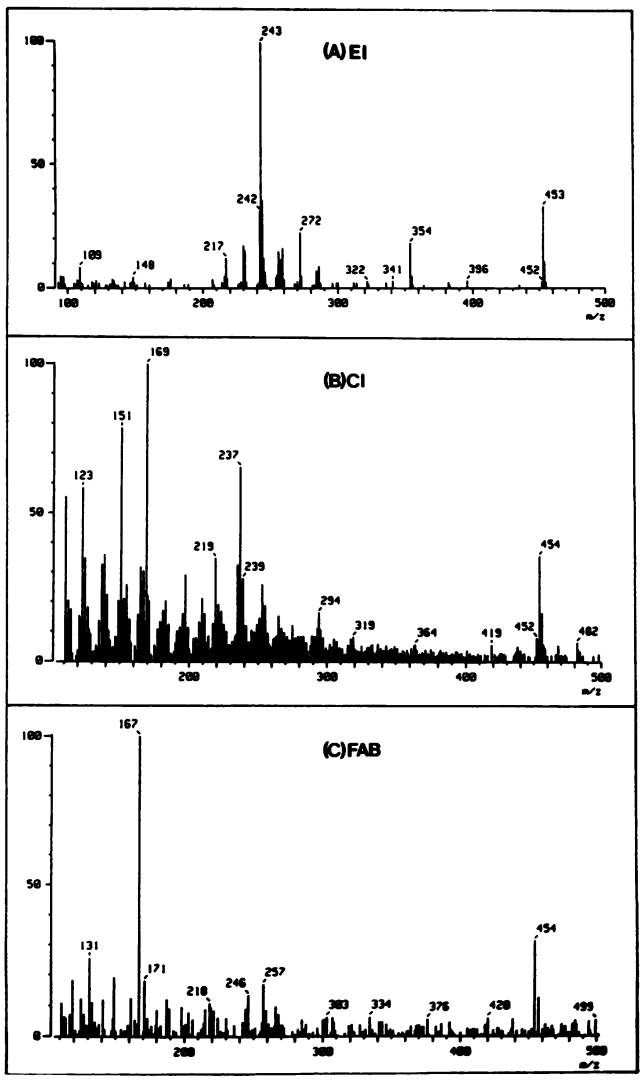

FIG. 2. LR mass spectrum of sambutoxin obtained by EI at 70 eV (the molecular ion is *m/z* 453) (A), by CI at 200 eV ($[M+1]^+$ peak is at *m/z* 454) (B), and by FAB ($[M+1]^+$ peak is at *m/z* 454) (C).

Production of sambutoxin by the toxic isolates. Most of the 16 toxic isolates were able to produce sambutoxin; it was produced by 7 toxic *F. oxysporum* isolates and 5 toxic *F. sambucinum* isolates but not by any of the *F. solani* isolates. The sambutoxin-producing isolates produced the toxin at levels ranging from 2.8 to 204 μg/g, and the average concentration of sambutoxin in wheat culture of *F. sambucinum* PZF-4 was 200 μg/g. One MON producer (*F. oxysporum* PKF-8) did not produce sambutoxin.

DISCUSSION

In stored potato tubers with rot symptoms, the predominant *Fusarium* species isolated was *F. oxysporum*, followed by *F. solani* and *F. sambucinum*. Lee (16) also reported these to be

the dominant species associated with rot of potato tubers in storage in Korea.

Most of the toxic isolates, when grown on wheat as the substrate, caused hemorrhage of the stomach and intestines in rats. Abbas et al. (2) reported that 52.3% of the *Fusarium* isolates obtained from Arctic areas of Norway caused congestion and hemorrhage of tissues in rats. The mycotoxins produced by toxic isolates including *F. oxysporum* and *F. sambucinum* were mainly nontrichothecenes such as MON, wortmannin, and HM-8. Recently, HM-8 toxin was characterized as chlamydosporol (3). The toxic isolates obtained from rotted potato tubers produced no known trichothecenes or other known mycotoxins, except MON, suggesting that the toxicity was due to an uncharacterized toxin(s). Therefore, we wanted to identify the toxic principles in the cultures of the toxic isolates. By using various chromatography and spectroscopy techniques, sambutoxin could be purified to an extent that rendered it suitable for studies of acute or chronic toxicity in animals and for physical and chemical investigations.

Chemical and spectroscopic evidence suggested that sambutoxin was a newly discovered mycotoxin. The absorptions at 1,650 and 1,560 cm^{-1} in the IR spectrum of sambutoxin are similar to those of equisetin and tenuazonic acid, suggesting that sambutoxin has a methyl pyridone ring (5, 26). Sambutoxin also might have a phenolic group, because a mixture of potassium ferricyanide-ferric chloride can be used to detect such a phenolic group. More recently, we have determined the chemical structure of sambutoxin from nuclear magnetic resonance spectroscopy (14).

Sambutoxin caused feed refusal, loss of body weight, hemorrhage in the stomach and intestines, and death when the rats were fed complete diets supplemented with 0.05 or 0.1% sambutoxin. The acute lethal toxicity of sambutoxin at the 0.05% level corresponds approximately to the toxicity of the mixture of crude culture and control diet. These results suggest that sambutoxin does not account for all of the toxicity associated with *F. sambucinum* PZF-4 and that an uncharacterized toxin(s) is also produced by the isolate. Fraction F1, containing unpurified toxins other than sambutoxin, also caused death accompanied by hemorrhage in the stomach and intestines in the feeding test. We briefly examined fraction F1 by TLC. Several compounds turned purple when sprayed with *p*-anisaldehyde and 20% sulfuric acid, suggesting that these components are structurally related to sambutoxin. These components, together with the sambutoxin in the culture, may be responsible for the hemorrhage and death in the feeding experiment. In addition, sambutoxin caused chicken embryo mortality. Compared with the 50% lethal doses of other mycotoxins for chicken embryos (10, 13, 24, 25), the 50% lethal dose of sambutoxin is higher than those of T-2 toxin and aflatoxin; however, it is lower than those of DON and tenuazonic acid.

The mycotoxins responsible for hemorrhage in cases of mycotoxicoses in farm animals have not all been identified, although trichothecenes, such as T-2 and DAS, may be responsible for some portion of the hemorrhagic disease syndrome observed under field conditions. Abbas and Mirocha (1) reported that wortmannin, a hemorrhagic factor, was produced by several *Fusarium* species; however, the natural occurrence of wortmannin has not been reported in agricultural products. Although sambutoxin caused hemorrhage and death at high doses in this study, more toxicological data are needed to account for a portion of frank hemorrhage found in the stomach and intestines of farm animals. Surveys on the occurrence of sambutoxin-producing isolates of *Fusarium* species and sambutoxin in agricultural products including rotted potatoes are expected to provide valuable information on risk assessment of sambutoxin. Also, the possibility that sambutoxin is produced by other *Fusarium* species or is responsible for animal and human syndromes of unknown etiology requires further investigation.

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