

## Isoverrucarol Production by *Fusarium oxysporum* CJS-12 Isolated from Corn

KOOK-HYUNG KIM,<sup>1</sup> YIN-WON LEE,<sup>1\*</sup> CHESTER J. MIROCHA,<sup>2</sup> AND ROBERT J. PAWLOSKY<sup>2</sup>

*Department of Agricultural Biology, College of Agriculture, Seoul National University, Suwon 440-744, Korea,<sup>1</sup> and Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota 55108<sup>2</sup>*

Received 9 June 1989/Accepted 22 October 1989

**Isoverrucarol (3,15-dihydroxy-12,13-epoxy-trichothec-9-ene) was isolated and purified from wheat cultures of a toxic strain of *Fusarium oxysporum* CJS-12. The toxin was characterized by thin-layer chromatography, gas chromatography-mass spectrometry, mass spectrometry, and <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance spectrometry. Isoverrucarol caused toxic effects in rats, including loss of appetite, bodily weakness, severe mucosae of the stomach, and death, when administered orally at 10 and 20 mg/kg of body weight. The toxin also caused a definite dermatitic reaction of epidermis and an edematous-necrotic response of the dermis.**

*Fusarium* species are a widespread, cosmopolitan group of fungi, and they commonly colonize aerial and subterranean plant parts. They are capable of being pathogenic to many plants, causing symptoms such as wilt, scab, and rot in the field (12). Interest in the *Fusarium* species has increased as a result of the observed cause and effect relation of various mycotoxicoses in humans and animals (4-7, 18, 21). Within the past several decades, a group of chemically related compounds called trichothecenes have been isolated from several different species of toxigenic fusaria (10). This group of compounds is characterized by a tetracyclic sesquiterpene structure with a 6-membered oxane ring, a 12,13-epoxide group, and a 9,10-olefinic bond (15).

In a previous paper (9), we reported on the incidence of *Fusarium* species in corn seeds obtained from the corn-producing area in Korea and on their toxicity to laboratory animals. During chemical analyses we found one isolate of *Fusarium oxysporum* CJS-12 that produce a substantial amount of isoverrucarol (IVE). Production of IVE (also known as 3,15-dideacetylcalonecitrin) has been reported in liquid cultures of *Fusarium culmorum* and *Fusarium sporotrichioides* (3, 14). This paper describes the isolation, purification, and toxicity of IVE produced by a toxic strain of *F. oxysporum* CJS-12 grown on wheat.

### MATERIALS AND METHODS

**Fungus isolation.** The fungal isolate of *F. oxysporum* CJS-12 was obtained from corn collected from the Jeongsun district in Kangwon province. The strain was identified by mycological studies as described by Nelson et al. (12). Inocula of *F. oxysporum* CJS-12 were produced by the fungus on potato glucose agar incubated for 5 days at 25°C.

**Culture of the fungus.** Twenty-five Erlenmeyer flasks (1 liter), each containing 200 g of wheat and 120 ml of distilled water, were autoclaved for 1 h at 121°C on two consecutive days. The wheat was inoculated with mycelium plugs from 5-day-old potato glucose agar plugs of fungus. The cultures were incubated for 2 weeks at 22 to 26°C and then for 2 weeks at 10°C. Harvested wheat cultures were dried in a ventilated hood, milled in a Wiley mill, and stored at -15°C until used.

**Extraction and purification.** Dried wheat cultures (2.9 kg) were moistened to 30% with distilled water, extracted with

methanol (3 liters, three times) and vacuum filtered through Whatman no. 1 filter paper. The filtrate was concentrated in vacuo to about 500 ml. The aqueous phase was defatted twice with 500 ml of *n*-hexane and then partitioned three times with 500 ml of ethyl acetate. Evaporation of the ethyl acetate layer yielded a reddish yellow oily residue (9.5 g) which was biologically active in the rat skin test.

The oily residue was suspended in 30 ml of chloroform-methanol (97:3, vol/vol) and introduced onto a silica gel column (5 by 150 cm) containing 500 g of silica gel (Kiesel gel 60, 70 to 230 mesh; E. Merck, Darmstadt, Federal Republic of Germany). The column was successively eluted with chloroform-methanol in ratios of 97:3 (1 liter), 95:5 (1 liter), and 90:10 (1 liter). Fractions (50 ml each) were collected and monitored by thin-layer chromatography (TLC). Fractions (12 to 36) were combined and evaporated to dryness in vacuo. The residue (2.9 g) was dissolved in chloroform-methanol (97:3, vol/vol) and applied directly to a Florisil column (150 g, 3.5 by 60 cm, 60 to 100 mesh; Fisher Scientific Co., Pittsburgh, Pa.) topped with 30 g of anhydrous sodium sulfate. Chloroform-methanol (99:1, 97:3, 95:5, and 90:10 [vol/vol], 200 ml of each) was added to the column and fractions (5 ml each) were collected. The fractions collected (1 through 95) were reduced to three samples, called F1 (43 to 49), F2 (50 to 70), and F3 (71 to 80), by combining the fractions indicated in parentheses. The purified compound was obtained from fraction F2, which displayed only a single spot on TLC developed in chloroform-methanol (9:1, vol/vol). The fractions F1 and F3, which contained impurities, were purified by TLC on a silica gel plate (0.25 mm thickness; E. Merck AG, Darmstadt, Federal Republic of Germany) by using chloroform-methanol (9:1, vol/vol) as the developing solvent. The compound was scraped off the plate and eluted from the silica gel.

**Crystallization.** Fraction F2 and the toxin purified from F1 and F3 were pooled and dried in vacuo. The residue, dissolved in a minimal volume of ethyl acetate and *n*-hexane, was added, and the mixture was kept at 10°C for a few hours until crystals formed. White crystals of the toxin (760 mg) were obtained by filtering the ethyl acetate-hexane layer with water suction and rinsing twice with *n*-hexane.

**TLC.** Purity of the isolated toxin was confirmed by TLC analysis. The toxin was spotted on silica gel TLC plates (0.25 mm thickness; E. Merck AG, Darmstadt, Federal Republic of Germany) and developed in seven different solvent sys-

\* Corresponding author.

TABLE 1. TLC resolution of IVE in various solvent systems<sup>a</sup>

Solvent system	Ratio by volume	R <sub>f</sub>
Chloroform-methanol	95:5	0.29
Chloroform-methanol	9:1	0.51
Chloroform-acetone	3:2	0.55
Benzene-acetone	3:2	0.41
Benzene-acetone	1:1	0.59
Ethyl acetate-toluene	3:1	0.28
Ethyl acetate-hexane	3:1	0.41

<sup>a</sup> The toxin was visualized with 4-(*p*-nitrobenzyl)pyridine and tetraethylenepentamine. Only one blue spot was seen in all the solvent systems.

tems (Table 1). It was visualized with 4-(*p*-nitrobenzyl)pyridine and tetraethylenepentamine as described by Takitani et al. (16). The R<sub>f</sub> values of the toxin in seven different solvent systems were recorded. Various physical methods were used to determine the reactions of various chemical reagents with the toxin. The plates were sprayed with 20% sulfuric acid in methanol, a solution of freshly prepared *p*-anisaldehyde as described by Pathre and Mirocha (13), and 0.1% ninhydrin in methanol. The following methods were used to detect the toxin on TLC plates after spraying them with chemical reagents: naked-eye observation for color changes at room temperature, color changes after charring (110°C, 10 min) the plates, and fluorescence under long- and shortwave UV light irradiation before and after charring.

**Spectroscopy.** Electron impact mass spectrometry of the toxin was performed on a VG 7070 EQ mass spectrometer. The sample was introduced via a direct-insertion probe.

Gas chromatography-mass spectrometry analysis was carried out on a gas chromatograph-mass spectrometer (model 5987B; Hewlett-Packard Co., Palo Alto, Calif.) equipped with an updated computerized data system. The toxin was reacted at room temperature with Tri-Sil-BT (Pierce Chemical Co., Rockford, Ill.) to form a trimethylsilyl (TMS) derivative. The reaction was allowed to proceed for 20 min prior to injection of the toxin into the gas chromatograph. Injection (1 μl each) was made into a DB-5 covalent bonded capillary column (30 m by 0.25 mm). A temperature program from 140 to 260°C (10°C/min) was used, with an injector temperature at 300°C and the detector set at 200°C, and helium (10 lb/in<sup>2</sup>) was used as the carrier gas.

<sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) spectra were recorded at 250 and 62.8 MHz, respectively, on an NMR spectrometer (model WM-300; Bruker Analytische Messtechnik GmbH, Rheinstetten, Federal Republic of Germany). Chemical shifts are referenced to deuteriochloroform at 7.24 ppm for <sup>1</sup>H and 77.0 ppm for <sup>13</sup>C and reported to tetramethylsilane.

**Oral and skin toxicity of purified toxin to rats.** Twenty-one-day-old female Sprague-Dawley rats weighing approximately 50 g each were obtained from the Experimental Animal Farm, Seoul National University. All rats were housed in individual cages and given feed and water ad libitum after treatment. Purified crystals of the toxin were dissolved in 40% aqueous ethanol and administered in a single oral dose by gastric intubation by using a hooked cannula needle attached to a syringe. Ten rats were used, with 10 and 20 mg of the toxin per kg of body weight. Control rats were intubated with 0.1 ml of 40% aqueous ethanol carrier solvent. The rats were observed frequently for 5 days after dosing, and the major symptoms and death were recorded. Skin toxicity was measured by application of the toxin to the backs of the rats. An area (3 by 3 cm) on the backs of the rats was shaved with an electric clipper. The

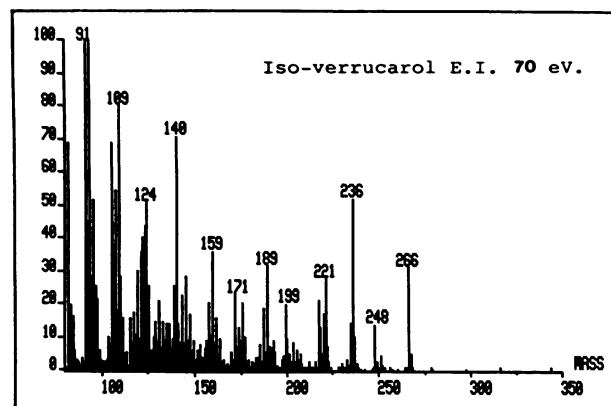


FIG. 1. Full electron impact (E.I.) mass spectrum of isoverrucarol by direct inlet. The molecular ion in the E.I. spectrum appears at  $m/e^+$  266.

toxin was dissolved in acetone and applied to the shaved skin of the rats with a capillary pipette. Only one test solution, in a volume of 25 μl or 50 μl, was applied to each rat. Three rats were used for each toxin level, with doses of 0.5 and 1.0 mg per rat. Control rats received only acetone. After 72 h, all rats were sacrificed and examined within 5 min after death for gross cutaneous and subcutaneous toxicity signs.

## RESULTS AND DISCUSSION

Culturing of *F. oxysporum* CJS-12 on sterile wheat produced 262 mg of pure crystals of the toxin per kilogram of moldy wheat. The purified toxin was subjected to TLC with seven different solvent systems to verify the purity and R<sub>f</sub> value. It was made visible by spraying the developed chromatograms with 4-(*p*-nitrobenzyl)pyridine-tetraethylenepentamine (16). Only one spot, having various R<sub>f</sub> values depending on the solvent systems, was visualized by using the described procedures (Table 1). The toxin appeared as a blue spot when sprayed with 4-(*p*-nitrobenzyl)pyridine-tetraethylenepentamine and exhibited a brownish violet color with *p*-anisaldehyde. The toxin exhibited a brown color with sulfuric acid after being heated for 10 min at 110°C. The toxin did not show any color or fluorescence properties with ninhydrin solution. The color reaction with these spraying agents suggested that the toxin is a trichothecene lacking an α, β-enone system, and according to Ueno et al. (19), such trichothecenes exhibit a blue fluorescence under long-wavelength (365 nm) UV irradiation after being sprayed with sulfuric acid and heated.

A mass spectrum of underivatized toxin displayed molecular ion (M<sup>+</sup>) at 266 and major fragment ions at  $m/e^+$  236, 221, 199, 189, 171, 159, 140, and 124 (Fig. 1). This trichothecene has the same molecular weight as verrucarol but different fragmentation patterns when the mass spectrum of this trichothecene was compared with that of verrucarol (2). A mass spectrum of the TMS derivative of the trichothecene is shown in Fig. 2A. Prominent fragments were at  $m/e^+$  410, 320, 292, 277, 212, 196, 181, 159, and 106; the molecular ion (M<sup>+</sup>) is at  $m/e^+$  410. Compared with the spectrum of TMS derivative of verrucarol shown in Fig. 2B, both compounds have the same molecular ion and the same fragments at  $m/e^+$  320, 277, 196, 106, 103, and 91. However, the relative intensities of major fragments are different. The increment of

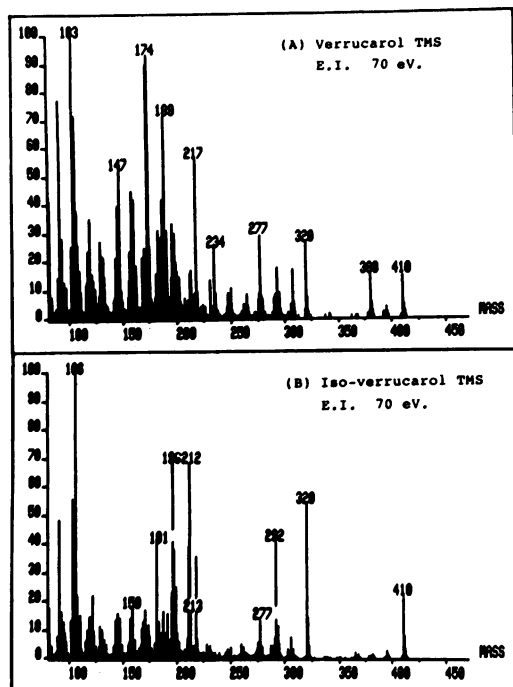


FIG. 2. Full mass spectra of the TMS derivative of verrucarol (A) and IVE (B). Both metabolites have the same molecular ion ( $M^+$ ) at  $m/e^+$  410.

144 mass units when the toxin was derivatized with TMS ether suggests that the toxin has two hydroxyl groups in its structure.

Further evidence supporting the structure of the trichothecene was obtained from the  $^1\text{H}$  and  $^{13}\text{C}$  NMR analysis. The  $^1\text{H}$  NMR spectrum of the trichothecene gave the following signals: 0.84 (s, 3H, C-14), 1.68 (s, 3H, C-16), 1.79 (m, 2H, C-7), 2.0 (m, 2H, C-8), 2.15 (m, 2H, C-4), 2.87 and 3.01 (each, d, 2H, C-13,  $J = 4.0$  Hz), 3.40 (d, 1H, C-2,  $J = 4.4$  Hz), 3.38 and 3.61 (each, d, 2H, C-15,  $J = 11.7$  Hz), 4.07 (d, 1H, C-11,  $J = 4.8$  Hz), 4.34 (td, 1H, C-3,  $J = 4.5, 5.0, \text{ and } 10.0$  Hz), and 5.43 (d, 1H, C-10,  $J = 4.8$  Hz). The  $^{13}\text{C}$  NMR of the trichothecene taken in the Bruker WM-300 NMR spectrometer is shown in Table 2. The signals at  $\delta 39.5$  due to

TABLE 2.  $^{13}\text{C}$  chemical shifts ( $\delta$  value) of IVE and verrucarol

Carbon no.	IVE	VER <sup>a</sup>
C-2	79.7 d	78.5
C-3	68.9 d	39.5
C-4	41.9 t	74.0
C-5	45.5 s	48.7
C-6	43.9 s	43.7
C-7	20.7 t	20.9
C-8	28.4 t	28.0
C-9	140.4 s	140.4
C-10	119.1 d	118.7
C-11	68.4 d	66.4
C-12	65.7 s	65.6
C-13	48.5 d	47.4
C-14	12.5 q	6.8
C-15	62.4 t	62.1
C-16	23.3 q	22.9

<sup>a</sup> VER, Verrucarol, 4,15-dihydroxy-12,13-epoxy-trichothec-9-ene (taken from Cole and Cox [2]).

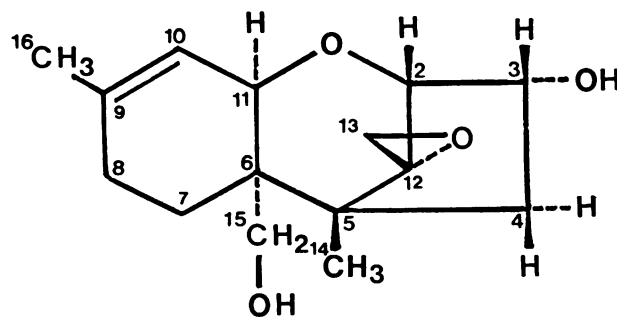


FIG. 3. Chemical structure of IVE.

the C-3 position of verrucarol was shifted upfield to  $\delta 68.9$  (d) in the trichothecene, whereas the signal of C-4 was shifted downfield from 74.0 to 41.9 (t). Remaining chemical shifts of the trichothecene correspond well with those listed in the spectrum of verrucarol (Table 2). From these results, the spectrum of the trichothecene was unambiguously established as 3,15-dihydroxy-12,13-epoxy-trichothec-9-ene (IVE). The structure of IVE is shown in Fig. 3.

IVE caused toxic effects, including loss of appetite, bodily weakness, severe mucosae of the stomach and intestines, and death, when administered orally at 10 and 20 mg/kg of body weight. No deaths occurred in the control group intubated with the ethanol carrier alone. Death occurred within 4 to 48 h after dosing. All surviving rats administered IVE at 10 mg/kg of body weight exhibited decreased feed consumption and displayed negative weight gains after 5 days (Table 3). All surviving rats started bleeding from the nose and eyes 24 h after dosing. All rats dosed with IVE at 20 mg/kg of body weight died within 24 h. The 50% lethal dose value of IVE at 24 h was approximately 10 mg/kg of body weight. The 50% lethal dose value of IVE compared with several 50% lethal dose values of 12, 13-epoxytrichothecenes for rats previously calculated (8, 17, 20, 22) is larger than T-2 toxin, diacetoxyscirpenol, fusarenone-X, roridin A, and verrucarol A; however, it is more toxic than deoxynivalenol, 3-acetyl-deoxynivalenol, and T-2 tetraol. Rats treated with IVE at levels of 0.5 and 1.0 mg per rat developed a definite dermatitic reaction, degeneration of epidermis, edema, and necrosis of the dermis. The intensity of skin reaction increased with concentration. These skin reactions confirmed that IVE applied on the skin of the rats resulted in reactions similar to those of most trichothecenes and characterized by necrosis of the dermis and epidermis (1, 11, 15).

IVE was first reported by Greenhalgh et al. (3). They isolated trace amounts of IVE along with trace amounts of

TABLE 3. Mortality, weight gain, and feed consumption of rats administered a single oral dose of isoverrucarol

Dose (mg/kg)	Mortality <sup>a</sup>	Weight gain <sup>b</sup> (%)	Feed consumption <sup>c</sup> (%)
0	0/10	100.0	100.0
10	7/10	-38.3	54.6
20	10/10		

<sup>a</sup> Number of rats which died during 5-day period after administration of the IVE/total number in the sample.

<sup>b</sup> Mean weight gain for surviving rats 5 days after dosing (percentage of controls).

<sup>c</sup> Mean feed consumption for surviving rats 5 days after dosing (percentage of controls).

calonectrin and 3-deacetylcalonectrin in a liquid culture of *F. culmorum*. Recently, IVE was produced at the level of 6.5 mg/liter of liquid culture by a mutant of *F. sporotrichioides* NRRL 3299 (14). Our results suggest that the use of solid substrates and selected isolates would offer a more facile method for large-scale production of IVE. Attempts were made to find the production of the other trichothecenes, but the presence of the other trichothecenes was not found. This is the first report of the production of IVE on a solid substrate by an *F. oxysporum* isolate.

#### ACKNOWLEDGMENT

This research was supported by the Sunkyong Agricultural Research Fund, College of Agriculture, Seoul National University, Suwon, Korea.

#### LITERATURE CITED

- Bamburg, J. R., and F. M. Strong. 1971. 12,13-Epoxytrichothecenes, p. 207–292. In S. Kadis, A. Ciegler, and S. Ajl (ed.), *Microbial toxins*, vol. 7. Academic Press, Inc., New York.
- Cole, R. J., and R. H. Cox. 1981. Verrucarol, p. 157–159. In *Handbook of toxic fungal metabolites*. Academic Press, Inc., New York.
- Greenhalgh, R., D. Levandier, W. Adams, J. D. Miller, B. A. Blackwell, A. J. McAlees, and A. Taylor. 1986. Production and characterization of deoxynivalenol and other secondary metabolites of *Fusarium culmorum* (CMI 14764, HLX 1503). *J. Agric. Food Chem.* **34**:98–102.
- Greenway, J. A., and R. Puls. 1976. Fusariotoxicoses from barley in British Columbia. I. Natural occurrence and diagnosis. *Can. J. Comp. Med.* **40**:12–16.
- Hsu, I. C., E. B. Smalley, F. M. Strong, and W. E. Ribelin. 1972. Identification of T-2 toxin in moldy corn associated with a lethal toxicosis in dairy cattle. *Appl. Microbiol.* **24**:684–690.
- Joffe, A. Z. 1971. Alimentary toxic aleukia, p. 139–189. In S. Kadis, A. Ciegler, and S. Ajl (ed.), *Microbial toxins*, vol. 7. Academic Press, Inc., New York.
- Joffe, A. Z. 1962. Biological properties of some toxic fungi isolated from overwintered cereals. *Mycopathol. Mycol. Appl.* **16**:201–221.
- Kosuri, N. R., E. B. Smalley, and R. E. Nichols. 1971. Toxicologic studies of *Fusarium tricinctum* (Corda) Snyder and Hansen from moldy corn. *Amer. J. Vet. Res.* **32**:1843–1850.
- Lee, Y. W., K. H. Kim, and H. S. Chung. 1988. Toxicity of *Fusarium* isolates obtained from corn-producing area in Korea. *Korean J. Plant Pathol.* **4**:40–48.
- Marasas, W. F. O., P. E. Nelson, and T. A. Tousson. 1984. *Toxigenic Fusarium species*. Pennsylvania State University Press, University Park, Pa.
- Mirocha, C. J. 1983. Effect of trichothecene mycotoxins on farm animals, p. 177–194. In Y. Ueno (ed.), *Trichothecenes—chemistry, biology and toxicological aspects*. Kodansha, Ltd., Tokyo.
- Nelson, P. E., T. A. Tousson, and W. F. O. Marasas. 1986. Identification of *Fusarium* species. Pennsylvania State University Press, University Park, Pa.
- Pathre, S. V., and C. J. Mirocha. 1977. Assay methods for trichothecenes and review of natural occurrence, p. 229–253. In J. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman (ed.), *Mycotoxins in human and animal health*. Pathotox Publishers, Inc., Park Forest South, Ill.
- Plattner, R. D., L. W. Tjarks, and M. N. Beremand. 1989. Trichothecenes accumulated in liquid culture of a mutant of *Fusarium sporotrichioides* NRRL 3299. *Appl. Environ. Microbiol.* **55**:2190–2194.
- Smalley, E. B., and F. M. Strong. 1974. Trichothecenes, p. 199–228. In I. H. F. Purchase (ed.), *Mycotoxins*. Elsevier Biomedical Press, Amsterdam.
- Takitani, S., Y. Asabe, T. Kato, M. Suzuki, and Y. Ueno. 1979. Spectrodensitometric determination of trichothecene mycotoxins with 4-(p-nitrobenzyl)pyridine on silica gel thin-layer chromatograms. *J. Chromatogr.* **172**:335–339.
- Ueno, Y., and K. Ishii. 1985. Chemical and biological properties of trichothecenes from *Fusarium sporotrichioides*, p. 307–316. In J. Lacey (ed.), *Trichothecenes and other mycotoxins*. John Wiley & Sons, Inc., New York.
- Ueno, Y., K. Ishii, K. Sakai, S. Kanaeda, H. Tsunoda, T. Tanaka, and M. Enomoto. 1972. Toxicological approaches to the metabolites of *Fusaria*. IV. Microbial survey on “Bean-hulls poisoning of horses” with the isolation of toxic trichothecenes, neosolaniol and T-2 toxin of *Fusarium solani* M-1-1. *Jpn. J. Exp. Med.* **42**:187–203.
- Ueno, Y., N. Sato, K. Ishii, K. Sakai, H. Tsunoda, and M. Enomoto. 1973. Biological and chemical detection of trichothecene mycotoxins of *Fusarium* species. *Appl. Microbiol.* **25**:699–704.
- Ueno, Y., and I. Ueno. 1978. Toxicology and biochemistry of mycotoxins, p. 107–188. In K. Uruguchi and M. Yamazaki (ed.), *Toxicology-biochemistry and pathology of mycotoxins*. Kodansha, Ltd., Tokyo.
- Yoshizawa, T., Y. Matsuura, Y. Tsuchiya, N. Morooka, K. Kitani, M. Ishinoe, and H. Kurata. 1979. On the toxigenic *Fusaria* invading barley and wheat in southern Japan. *Jpn. J. Food Hyg. Soc.* **20**:21–26.
- Yoshizawa, T., and N. Morooka. 1973. Deoxynivalenol and its monoacetate: new mycotoxins from *Fusarium roseum* and moldy barley. *Agric. Biol. Chem.* **37**:2993–2934.