Deepoxynivalenol: A New Metabolite of Nivalenol Found in the Excreta of Orally Administered Rats

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The presence of a new metabolite of nivalenol in the excreta of orally administered rats was confirmed. The new metabolite, purified from rat feces, was identified as 3,4,7,15-tetrahydroxytrichothec-9,12-dien-8-one or deepoxynivalenol on the basis of mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. In multiple administration, 80% and 1% of the total dose were excreted as the deepoxy metabolite into feces and urine and the parent compound was detected at 7% and 1% in feces and urine, respectively.

Nivalenol (12,13-epoxy-3,4,7,15-tetrahydroxytrichothec-9-en-8-one) is one of the trichothecene mycotoxins produced by the species of *Fusarium* and has been found in barley harvested in Japan (Yoshizawa and Hosokawa, 1983; Dohi et al., 1984) and in various crops in the world (Visconti et al., 1984; Lee et al., 1985). Moreover, nivalenol together with deoxynivalenol has usually been found in commercial foods as low concentration level contamination (Onji et al., 1987). This toxin has been shown to have various toxicities (Ueno, 1983) including skin toxicity, hematological disorder, vomitting, feed refusing, and immunotoxicity.

Isolates of *Fusarium graminearum* from cereal kernels have been divided into two groups based on trichothecene production: one producing nivalenol and fusarenon X and the other deoxynivalenol and 3-acetyldeoxynivalenol (Ichinoe et al., 1983). In the culture of nivalenol-producing strains, nivalenol is considered to be an end product converted from fusarenon X. However, it has not been determined whether nivalenol might be metabolized further in animals.

Recently deepoxy metabolites of deoxynivalenol (Yoshizawa et al., 1983b), T-2 toxin (Yoshizawa et al., 1985a; Chatterjee et al., 1986), and diacetoxyscirpenol (Sakamoto et al., 1986) in the feces and urine of orally administered animals have been reported. However, the metabolic fate of nivalenol in vivo is still unknown. To fully understand the toxic influence of nivalenol on human health, it is important to clarify its metabolic fate in vivo. This paper describes the chemical structure of a new metabolite of nivalenol in urine and feces of orally administered rats.

EXPERIMENTAL SECTION

Reagents. Nivalenol was extracted from polished rice culture of *F. graminearum* and purified by centrifugal countercurrent partition chromatography in our laboratory (Onji et al., in press). The purity of nivalenol exceeded 91% in comparison with the authentic standard as determined by gas chromatography (GC) with electron capture detector (ECD), and the toxin was free from deepoxynivalenol and fusarenon X.

Apparatus: mass spectrometer (MS), JOEL JMS-DX 300; nuclear magnetic resonance spectrometer (NMR), JOEL JNM-GX 400; high-performance liquid chromatograph (HPLC), Shimazdu LC 6A-SPD 6A (UV detector) with LiChrospher RP-18 column (25 cm × 4.6 mm (i.d.), 10-μm particle size; Cica-Merck); gas chromatograph–mass spectrometer (GC–MS), Shimadzu GC/MS 6020 with glass column (2 m × 3 mm (i.d.)) packed with 2% OV-1 on Gaschrom Q; gas chromatograph (GC), Hewlett-Packard GC 5890A-ECD (^{63}Ni) with HP Ultra-2 capillary column (25 m × 0.2 mm (i.d.)).

Animal Treatment. Nivalenol was dissolved in water at a concentration of 0.6 mg/mL. Each of five male Wistar rats weighing 190–445 g was orally administered nivalenol (5 mg/kg of body weight) 12 times at 2- or 3-day intervals. A total of 100 mg of nivalenol was administered to five rats. These rats were individually housed in metabolic cages during toxin administration, and water and feed (Oriental Yeast Co., Ltd.) were available ad libitum. Feed was analyzed to be free from nivalenol. Either urine or feces were collected daily for 39 days and stored at −20 °C prior to analysis and purification.

Extraction and Purification of New Metabolites. Feces (about 1 kg) were twice extracted with 3 L of acetonitrile followed by filtration. The concentrated extract (20 mL) was partitioned with ethyl acetate–water (3:7, v/v, 500 mL). After the water layer was concentrated to 50 mL, the water-soluble materials were loaded on Amberlite XAD-2 resin packed in glass column (25 cm × 2 cm (i.d.)). It was rinsed with 380 mL of water and eluted with 240 mL of methanol. The methanol eluate was concentrated to 5 mL in a rotary evaporator and further purified on DEAE-Sephadex A-25 column (15 cm × 1.2 cm (i.d.)) by elution with methanol–water (1:6, v/v). Additional purification of the new metabolite was accomplished on HPLC. The new metabolite fractions were combined, evaporated to dryness, and recrystallized from a small volume of water in a refrigerator.

High-Performance Liquid Chromatography. The partially purified materials were repeatedly fractionated with LiChrosphere RP-18 column by monitoring the absorption at 254 nm. Mobile phase: programmed 15% methanol for 5 min and 15–50% methanol linear ramp for 20 min; flow rate, 1.5 mL/min.

Thin-Layer Chromatography (TLC). TLC was carried out on precoated silica gel plates (HPTLC plate, 250-μm gel thickness, 10 × 10 cm, E. Merck, Darmstadt) with a chloroform–methanol (7:1, v/v) solvent system. The visualization was accomplished by treatments with aluminum chloride (Kamimura et al., 1981), 4-(*p*-nitrobenzyl)pyridine (Takitani et al., 1979), or chromotropic acid (Baxter et al., 1983).

Gas Chromatography–Mass Spectrometry. GC conditions: injector temperature, 240 °C; column oven temperature, 190 °C; He carrier gas flow rate, 30 mL/min.

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Table I. ^{1}H NMR Chemical Shift Assignments and Coupling Constants for Nivalenol and Its New Metabolite Deepoxynivalenol

proton posn	^{1}H chem shift,[a] δ (coupling const, Hz)	
	nivalenol	deepoxynivalenol
2	3.53 ($J_{2,3}$ = 5.0)	4.16 ($J_{2,3}$ = 4.8)
3	4.16 ($J_{3,2}$ = 5.0, $J_{3,4}$ = 5.0, $J_{3,OH}$ = 4.0)	3.85 ($J_{3,2}$ = 4.8, $J_{3,4}$ = 3.2)
3-OH	4.48	
4	4.55 ($J_{4,3}$ = 5.0, $J_{4,OH}$ = 3.5)	4.54 ($J_{4,3}$ = 3.2)
4-OH	3.85	
7	4.82 ($J_{7,OH}$ = 2.7)	4.59[b]
7-OH	4.03	3.92
10	6.57 ($J_{10,11}$ = 6.0, $J_{10,16}$ = 1.5)	6.54 ($J_{10,11}$= 6.0, $J_{10,16}$ = 1.5)
11	4.72 ($J_{11,10}$ = 6.0)	4.75 ($J_{11,10}$ = 6.0)
13	2.93 (J_{AB} = 4.5)	5.17 (J_{AB} = 1.0)
	2.90	4.92
14	1.08 (s)[c]	1.78 ($J_{14,2}$ = 1.5, $J_{14,4}$ = 1.0)
15	3.84 (J_{AB} = 11.5, $J_{15,OH}$ = 5.0)	3.85 (J_{AB} = 11.5)
	3.74	3.76
15-OH	3.76	
16	1.79 ($J_{16,10}$ = 1.5, $J_{16,11}$= 1.0)	1.36[b]

[a] From tetramethylsilane. [b] Coupling could not be detected because of the contamination of trace salt. [c] Singlet signal.

MS conditions: ion source temperature, 190 °C; ionization voltage, 70 eV for electron impact ionization, 100 eV for chemical ionization; reagent gas, isobutane.

Gas Chromatography. GC conditions: injector temperature, 240 °C; detector temperature (^{63}Ni-ECD), 300 °C; carrier gas linear velocity, 30 cm/s; split ratio, 1:60; column oven temperature, hold at 150 °C (1 min), programmed 150–270 °C at 40 °C/min and 270–300 °C at 4 °C/min; derivatization, trimethylsilyl (TMS) derivatives prepared by reacting with 0.5 mL of trimethylsilyl chloride–(trimethylsilyl)imidazole–ethyl acetate (0.2:1:9, v/v) at room temperature for 15 min.

Spectroscopy. Mass spectra were measured at 30-eV ionization voltage by a direct-insertion method. ^{1}H and ^{13}C NMR spectra were measured in acetone-d_6 with tetramethylsilane as an internal standard at 400 and 100 MHz, respectively.

RESULTS AND DISCUSSION

Nivalenol and its new metabolite extracted from feces and urine were detected by GC-ECD as trimethylsilyl ether after partial purification. Trimethylsilyl ether of nivalenol and an unknown metabolite had strong recorder response for ECD at retention times of 8.20 and 9.24 min, respectively, on capillary GC.

The trimethylsilyl ether of unknown metabolite detected at 9.24 min on capillary GC was analyzed by GC–MS. The molecular ion was found at m/z 584 (calcd for $C_{27}H_{52}O_6Si_4$ 584.28) as a small peak in the EI mass spectrum and at m/z 585 (calcd for $C_{27}H_{53}O_6Si_4$ 585.29) as a strong peak in the CI mass spectrum, which were 16 mass units less than the values for nivalenol.

Since the separation of nivalenol from an unknown metabolite could not be accomplished by normal-phase HPLC or TLC, reversed-phase HPLC was employed to purify an unknown metabolite. Our purification method by HPLC of an unknown metabolite from feces gave a quantity (20 mg) of the crystalline compound at 95.6% purity, enough to determine the chemical structure spectroscopically. The new metabolite was eluted at retention time of 6.32 min on HPLC, later than nivalenol. Nivalenol and its new metabolite had R_f values of 0.27 and 0.26 on TLC, respectively. When sprayed with aluminum chloride or chromotropic acid reagents followed by heating, the new metabolite showed a weak fluorescent spot under 365-nm irradiation or a positive purple spot, respectively. But its color reaction after treatment with 4-(*p*-nitrobenzyl)-pyridine reagent was negative. These results of thin-layer chromatography suggest that the new metabolite has a carbonyl group at the C-8 position, a β-oxyethyl group at the C-6 position, but no epoxy ring at the C-12,13 position.

Table II. ^{13}C NMR Chemical Shift Assignments for Nivalenol and Its New Metabolite Deepoxynivalenol

carbon no.	^{13}C chem shift,[a] δ		carbon no.	^{13}C chem shift,[a] δ	
	nivalenol	deepoxy-nivalenol		nivalenol	deepoxy-nivalenol
2	81.19	81.44	10	139.65	140.09
3	81.13	81.51	11	70.25	70.04
4	81.03	80.96	12	65.65	154.10
5	45.14	53.92	13	45.70	107.92
6	50.20	54.48	14	8.24	12.77
7	74.93	74.86	15	61.63	61.46
8	200.65	201.27	16	15.23	15.30
9	135.79	135.53			

[a] From tetramethylsilane.

Mass spectra of the new metabolite of nivalenol, compared with that of nivalenol, are shown in Figure 1.

Molecular ions at m/z 296 for the new metabolite (calcd for $C_{15}H_{20}O_6$ 296.1260) and at m/z 312 for nivalenol (calcd $C_{15}H_{20}O_7$ 312.1209) were observed. And comparative fragmentation for the new metabolite to nivalenol was observed (Figure 1). Precise masses were obtained at 296.1265 for the former and 312.1212 for the latter by peak matching with ions in the spectrum of perfluorokerosene as a standard.

The ^{1}H and ^{13}C NMR spectral assignments for the new metabolite and nivalenol were summarized in Tables I and II, based on the H–H and C–H shift 2D-correlated spectroscopy, respectively.

In ^{1}H NMR spectra, signals at δ 5.17 and 4.92 assigned to vinylic protons at C-13 of the new metabolite were observed, instead of a signal at δ 2.92 due to methylene protons at C-13 position of nivalenol. In addition, shifts to the low magnetic field of signal at C-2 proton and C-14 protons in the new metabolite were also observed. In ^{13}C NMR spectra, signals at δ 65.65 and 45.70 due to the C-12 and C-13 carbons of nivalenol were shifted to δ 154.10 and 107.92 in the new metabolite, respectively. These characteristic chemical shifts in ^{1}H and ^{13}C NMR spectra at C-12 and C-13 carbons were comparable to those of deepoxy metabolites of other trichothecene mycotoxins (Blackwell et al., 1984) including DOM-I (Yoshizawa et al., 1983b), deepoxy T-2 (Chatterjee et al., 1986), and DRM-1 and DRM-2 (Sakamoto et al., 1986) with a trichothec-9,12-diene skeleton.

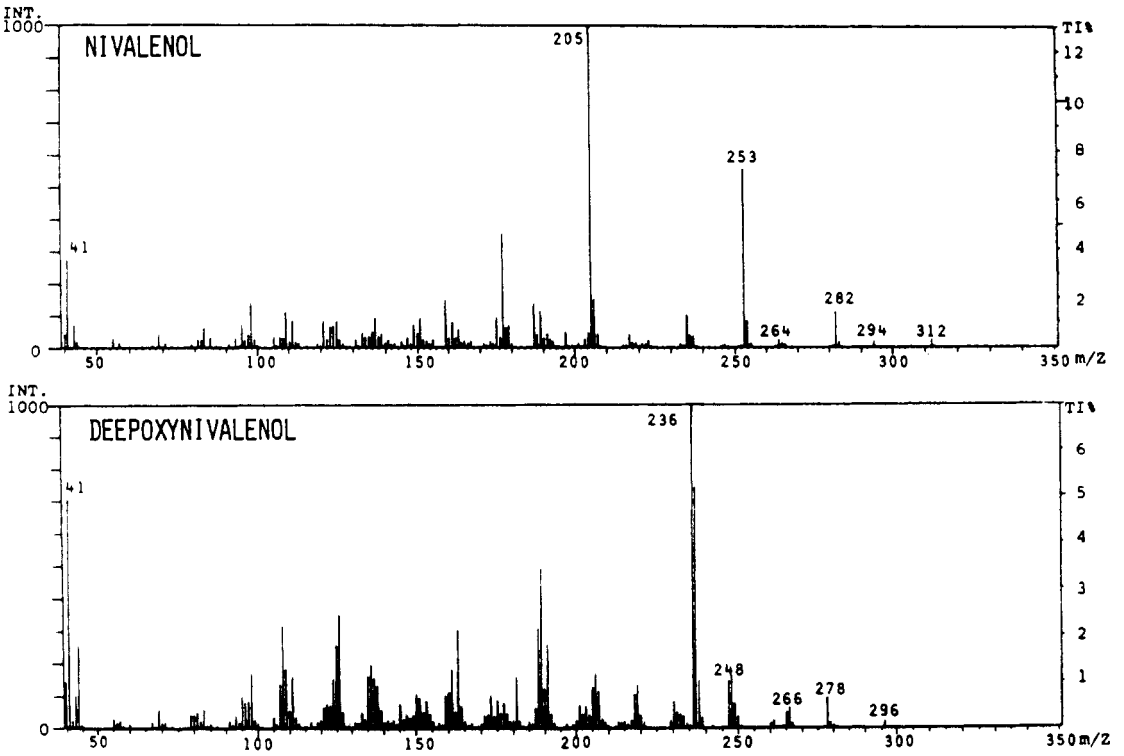

Figure 1. Mass spectra of nivalenol and its new metabolite deepoxynivalenol (30 eV).

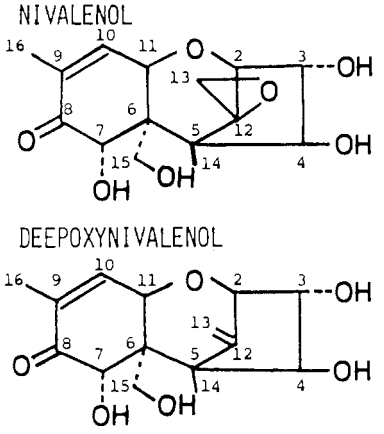

Figure 2. Structures of nivalenol and deepoxynivalenol.

On the basis of these data, the chemical structure of the new metabolite of nivalenol was identified as 3,4,7,15-tetrahydroxytrichothec-9,12-dien-8-one, namely deepoxynivalenol (Figure 2).

Yoshizawa et al. (1983b) have reported that a metabolite of deoxynivalenol excreted in urine and feces of orally administered rats was identified as deepoxydeoxynivalenol named DOM-I by GLC–MS and proton NMR spectrometry. Transformation of deoxynivalenol to DOM-1 by rumen microorganism (King et al., 1984) and T-2 toxin to deepoxy T-2 tetraol in the mixed culture of mice intestinal flora under anaerobic conditions (Yoshizawa et al., 1985a) have been reported. Recently, deepoxy T-2 tetraol in cow urine (Chatterjee et al., 1986) and two deepoxy metabolites of diacetoxyscirpenol, DRM-1 and DRM-2, in rat excreta (Sakamoto et al., 1986) from orally administered toxins have been confirmed. However, the metabolic fate and deepoxy metabolite of nivalenol have not been reported yet.

Quantification of the new metabolite was accomplished using the purified deepoxynivalenol. The ratio of ECD-GC response of deepoxynivalenol to nivalenol was 0.9. In feces, nivalenol and deepoxynivalenol were detected at 7% and 80% of administered nivalenol, respectively. On the other hand, in urine, either nivalenol or deepoxynivalenol was found only at 1%. When nivalenol was orally administered to rats with single dose, deepoxynivalenol was excreted predominantly in feces rather than in urine, and the excretion was about 24 h later than that of nivalenol (manuscript in preparation). The metabolite predominantly detected in feces in the present study was the deepoxy compound, suggesting that gastrointestinal microorganisms participate into deepoxidation of nivalenol in rats as described in other 12,13-epoxytrichothecenes.

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Uptake, Translocation, and Metabolism of [^{14}C]Thuringiensin (β-Exotoxin) in Corn

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The absorption, translocation, and metabolism of [^{14}C]thuringiensin (β-exotoxin), an insecticide, derived from *Bacillus thuringiensis* was investigated in corn. Corn was harvested 3 and 7 days after its roots or leaves were exposed to thuringiensin. Corn absorbed more thuringiensin at 7 than 3 days of root exposure. Less than 10% of the applied thuringiensin was absorbed after 7 days of exposure. Only 12% of the foliar-applied thuringiensin was detected in the whole plant, and amounts absorbed at 3 and 7 days were similar. About 80% of the applied radioactivity was found in the leaf wash at both times of harvest, and only 20% of the absorbed was translocated out of the treated leaf. More than 95% of the absorbed radioactivity remained in the root. Time did not affect the distribution pattern of root- or foliar-applied thuringiensin in different parts of corn. In this study, thuringiensin was not readily absorbed by root or leaves of corn and had limited mobility in the plant. The insecticide was also not metabolized by corn shoot after 3 and 7 days of exposures. The implications of these results are discussed.

Thuringiensin (β-exotoxin) is an insecticide produced by the entomopathogen bacteria *Bacillus thuringiensis*. The insecticide is produced commercially by fermentation and can be formulated as stabilized emulsion, wettable powder, or dust (Burges, 1982). It is a nucleotidic ATP analogue (Benz,1966; Bond et al., 1969) that inhibits the production of DNA-dependent RNA polymerase and consequently the production of ribosomal RNA (Lecadet and De Barjac, 1981). The insecticide has shown potential for the control of insects on field crops, trees, ornamentals, vegetables, and stored grain and grain products (Miller et al., 1983). It is most effective to immature Lepidoptera, Diptera, Coleoptera, Hymenoptera, Isoptera, and Orthoptera (Burgerjon and Martouret, 1971).

In corn, it has given effective control of the larvae as well as the adults of *Heliothis zea* (Herbert and Harper, 1985; Ignoffo and Gregory, 1972). Although its effect on *Heliothis* and other insects has been documented extensively (Burgerjon and Martouret, 1971; Herbert and Harper, 1985; Sebasta et al., 1981), there are no studies on its behavior in plant species. In order to maximize the efficacy of thuringiensin aimed at insects, it is important to understand how much of the insecticide is absorbed and the extent of its translocation out of the site of application in plants. The objectives of this research were to investigate thuringiensin absorption, translocation, and metabolism when applied to roots or leaves of corn.

METHODS AND MATERIALS

Plant Culture. Corn (*Zea mays* L. cv. Sunbelt 1860) was grown from seed in a greenhouse (24/20 °C day/night) in planter flats (Styrofoam flats of 66 × 33 × 12.7 cm) containing sand. Plants were watered and fertilized with half-strength Hoagland and Arnon (1950) nutrient solution as needed.

[^{14}C]Thuringiensin Application. At the three-leaf stage of corn, uniform plants were selected and transferred to 1-qt darkened jars with 900 mL of half-strenth Hoagland and Arnon solution, pH 7–7.5. The jars were then trans-

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