# Structures of New Metabolites of Diacetoxyscirpenol in the Excreta of Orally Administered Rats

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Rats orally administered multiple doses of diacetoxyscirpenol (DAS, 2.8 mg/kg of body weight) eliminated 15-monoacetoxyscirpenol (15-MAS), scirpentriol (SCP), and two unknown metabolites named DRM-1 and DRM-2 in urine and feces. The parent compound was detected in neither urine nor feces. The metabolites 15-MAS, SCP, DRM-1, and DRM-2 were detected in urine at 3.5%, 4.9%, 9.5%, and 7.2% of the administered dose, and only the latter two metabolites were detected in feces at 9.5% and 18.9%, respectively. The new metabolites DRM-1 and DRM-2 were identified as 15-acetoxy-3α,4β-dihydroxytrichothec-9,12-diene and 3α,4β,15-trihydroxytrichothec-9,12-diene, respectively, on the basis of mass and nuclear magnetic resonance spectroscopy.

## INTRODUCTION

Diacetoxyscirpenol (DAS, anguidine, 4β,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene) is one of the trichothecene mycotoxins produced by the species of *Fusarium*, frequently isolated from various agricultural commodities (Gilgan et al., 1966; Mirocha et al., 1976; Siegfried, 1977; Scott et al., 1980; Bhat et al., 1983; Hintikka et al., 1983; Patterson, 1983). This toxin has been found in barley, wheat, rice, oats, corn, safflower, and mixed feeds throughout the world (Mirocha et al., 1976; Ghosal et al., 1977, 1978; Siegfried et al., 1977; Cirilli, 1983; Szathmáry, 1983) and the consumption of naturally contaminated feed has been associated with hemorrhagic bowel lesions in swine (Mirocha et al., 1976). Experimentally, DAS has been shown to have various biological activities including antifungal activity against *Candida albicans* (Bamburg and Strong, 1971), phytotoxicity (Brian et al., 1961), high acute mammalian toxicity accompanied by radiomimetic injury of the tissues (Sato and Ueno, 1977; Weaver et al., 1978; Hoerr et al., 1981), teratogenicity to chicken embryos (Veselý et al., 1982), and mutagenicity in onion cells (Reiss, 1974), but not in the Ames test (Wehner et al., 1978). Similarly, DAS was efficacious against colonic adenocarcinomas in mice (Corbett et al., 1977; Loeffler et al., 1965). However, no remission of colonic adenocarcinomas was observed in human clinical trials (Diggs et al., 1978).

To more fully understand the mode of action of DAS, it is important to clarify its metabolic fate in vivo. Currently, little information is available on the metabolism of DAS. In vitro metabolism studies utilizing liver microsomes of rat and rabbit demonstrated that 15-monoacetoxyscirpenol (15-MAS) was the only metabolite of DAS (Ohta et al., 1978). In addition, 15-MAS was also reported as the sole metabolite when DAS was incubated with bovine rumen microorganisms (Kiessling et al., 1984). On the other hand, DAS, 15-MAS, and scirpentriol (SCP) were detected in the serum of pigs orally administered DAS (Bauer et al., 1985). In this paper, we describe the structural elucidation of the metabolites detected in the excreta of rats orally administered DAS.

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## EXPERIMENTAL SECTION

**Mycotoxin.** Diacetoxyscirpenol was purchased from Myco-Lab Co. (Chesterfield, MO), 15-MAS, SCP, and T-2 tetraol were prepared by alkaline hydrolysis of DAS and T-2 toxin, respectively, and 4-monoacetoxyscirpenol (4-MAS) was prepared by acid hydrolysis of DAS (Sigg et al., 1965). The purity of these toxins exceeded 96% as determined by gas–liquid chromatography (GLC) with a hydrogen flame ionization detector (FID), depending upon an assumption that all the heights under GC peaks sum to 100%.

**Animal Treatment.** A total of 20 male Wistar rats weighing 200–270 g was purchased from Holtzman Co. (Madison, WI) and individually housed in metabolic cages (Nalgen Co., Rochester, NY) for 7 days prior to toxin administration. Water and feed (Wayne rodent blox, Continental Grain Co., Chicago, IL) were available ad libitum. Feed was analyzed and determined to be free from DAS, T-2 toxin, deoxynivalenol (DON), and aflatoxin.

Diacetoxyscirpenol was dissolved in physiological saline containing 5% ethanol at a concentration of 1.4 mg/mL. Rats were orally administered DAS (2.8 mg/kg of body weight) three times at 7-day intervals. Either urine or feces were collected daily for 21 days and combined for the first 3 days after each toxin administration. The samples were well mixed and divided into two parts. One part was saved for the quantitative analysis, and the other was for the isolation of the metabolites. Those samples were stored at −20 °C prior to analysis.

**Extraction and Purification of DAS and Its Metabolites in Excreta.** After addition of 330 g of sodium chloride, 1 L of the urine was extracted with acetonitrile (3 × 0.5 L) and acetonitrile–acetone (1:1, v/v, 3 × 0.5 L) followed by centrifugation. The organic extracts were combined and concentrated followed by addition of 10 mL of water. The water-soluble materials were loaded onto five SEP-PAK $C_{18}$ cartridges (Waters Associates Inc., Milford, MA) which were rinsed with 6 mL of water per cartridge. The water-insoluble residue was dissolved in 20 mL of 75% aqueous acetonitrile, loaded onto the above five cartridges, and eluted with 4 mL of 75% aqueous acetonitrile per cartridge. The eluates were combined, evaporated to dryness, and redissolved in 12 mL of methylene chloride–methanol (5:1, v/v). Anhydrous sodium sulfate (5 g) and cupric carbonate (2 g) were added, followed by filtration through Whatman No. 1 filter paper. The residue was rinsed twice with 5 mL of methanol. The filtrate was evaporated, redissolved in 10 mL of methylene chloride–methanol (9:1, v/v), added to a Florisil column (2 cm i.d., 60–100 mesh, 20 g, Fisher Scientific. Co., Fair

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Lawn, NJ). The metabolites were eluted with 200 mL of the same solvent.

Feces (1 kg) were extracted three times with 50% aqueous acetonitrile (1 × 2 L, 2 × 1 L) followed by centrifugation. The aqueous acetonitrile was defatted with *n*-hexane (2 × 1 L), and the hexane was discarded. Sodium chloride (1.2 kg) was added to the extracts to induce phase separation. The acetonitrile layer was collected, and the remaining aqueous layer was successively extracted with acetonitrile (2 × 1 L) and acetonitrile–acetone (1:1 v/v, 3 × 1 L) followed by centrifugation. The acetonitrile extracts were combined and purified as described above for urine. Additional purification of metabolites for spectroscopic analysis was accomplished on silica gel TLC plates. Bands 5 mm wide with $R_f$ values corresponding to 15-MAS and SCP were scraped and eluted with 5 mL of acetone.

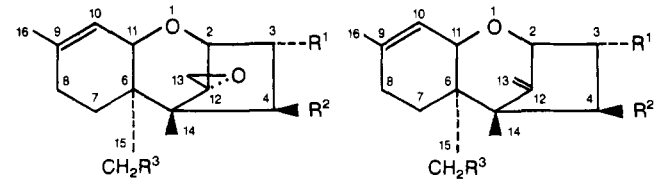
Quantification of the metabolites was performed by extracting aliquots of each sample for three dosing replications (135–165 mL of urine or 30–40 g of feces). Extraction and purification was accomplished as described above on a reduced scale. Florisil column eluates were concentrated and redissolved in a 2 mL of ethanol, and aliquots were removed for GLC analysis. For the recovery study, DAS, 15-MAS, and SCP were added to the control samples (10 mL of urine or 10 g of feces) at 10, 2.5, and 1.0 ppm levels, respectively.

**Gas–Liquid Chromatography (GLC).** Gas chromatography was performed on a Hewlett-Packard Model 5790A gas chromatograph equipped with an electron capture detector ($^{63}$Ni-ECD) and a 12 m × 0.2 mm i.d., 0.2-μm film OV-1 bonded phase fused silica capillary column. The operating conditions were as follows: column temperature programmed from 100[1] to 150 °C at 25 °C/min, from 150[4] to 230 °C at 4 °C/min and from 230 to 270 °C at 30 °C/min (hold time in minutes is shown in brackets); injector temperature 250 °C; detector temperature 325 °C; helium carrier gas 35 cm/s; makeup gas (argon–methane, 95:5) flow rate 45 mL/min. Quantification was accomplished using T-2 tetraol as a GLC internal standard and 15-MAS and SCP as standards for quantification of DRM-1 and DRM-2, respectively.

**Derivatization of DAS and Its Metabolites.** An aliquot of the sample extract was evaporated to dryness, redissolved in 500 μL of toluene–acetonitrile (95:5, v/v), 75 μL of *N*-methylbis(trifluoroacetamide) (MBTFA; Pierce Chemical Co., Rockford, IL) was added, and the mixture was heated for 1 h at 60 °C. After cooling, the excess MBTFA was removed by partitioning the organic phase with 1 mL of 5% aqueous sodium bicarbonate. The organic layer was diluted to 4 mL with *n*-hexane and the trifluoroacetyl (TFA) ester derivatives were analyzed by GLC–ECD. In addition, metabolites were derivatized to trimethylsilyl ($Me_3Si$) ethers with Tri-Sil/TBT (Pierce Chemical Co.) for GC–MS analysis. Aliquots (ca. 0.5 μg) of the isolated metabolites were hydrolyzed by reacting with 200 μL of 1 N KOH in 80% methanol for 30 min at room temperature to check for modification of the 12,13-epoxy ring according to the procedure described previously (Yoshizawa et al., 1985b). After evaporation of the solvent, the hydrolyzed metabolites were analyzed by GLC–ECD as the TFA derivatives.

**Thin-Layer Chromatography (TLC).** Thin-layer chromatography was carried out on precoated silica gel plates (250-μm gel thickness 20 × 20 cm, J. T. Baker Chemical Co., Phillipsburg, NJ). Two TLC solvent systems were used: solvent A, chloroform–methanol (9:1, v/v) and solvent B, chloroform–acetone (3:2, v/v). Compounds were made visible under long-wave (354-nm) ultraviolet light by heating the plates at 130 °C for 5 min after treating with 30% $H_2SO_4$ in methanol. Additional TLC visualisation was accomplished by treatment with 4-(*p*-nitrobenzyl)pyridine (Takitani et al., 1979). Trichothecenes possessing a 12,13-epoxy ring yield blue spots with this reagent.

**Table I. Chemical Structures and Resolution of DAS and Its Metabolites by TLC and GLC**




skeleton A skeleton B

| compd[a] | skeleton | $R^1$ | $R^2$ | $R^3$ | TLC $R_f$[b] A | TLC $R_f$[b] B | GLC $t_R$,[c] min |
|---|---|---|---|---|---|---|---|
| DAS | A | OH | OAc[d] | OAC | 0.67 | 0.47 | 18.9 |
| 15-MAS | A | OH | OH | OAc | 0.40 | 0.13 | 14.3 |
| DRM-1 | B | OH | OH | OAc | 0.37 | 0.14 | 11.4 |
| SCP | A | OH | OH | OH | 0.17 | 0.03 | 10.4 |
| DRM-2 | B | OH | OH | OH | 0.17 | 0.03 | 7.7 |

[a] Abbreviations: DAS, diacetoxyscirpenol; 15-MAS, 15-monoacetoxyscirpenol; DRM-1, 15-acetoxydeepoxyscirpenol; SCP, scirpentriol; DRM-2, deepoxyscirpentriol. [b] TLC on silica gel plates developed in solvent systems A and B (see text). [c] Retention time of trifluoroacetate derivatives. [d] OAc = acetate.

**Spectroscopy.** Gas chromatography–mass spectroscopy (GC–MS) was performed on a JEOL QH-100 mass spectrometer at 20 and 70 eV using both TFA and $Me_3Si$ derivatives. Proton and $^{13}$C nuclear magnetic resonance (NMR) spectra were measured on a Hitachi R-90H Fourier transform NMR spectrometer (90 MHz) with tetramethylsilane as an internal standard.

## RESULTS AND DISCUSSION

**Quantification of DAS and Its Metabolites in Rat Excreta.** The recoveries of DAS, 15-MAS, and SCP added at 10, 2.5, and 1.0 ppm levels, respectively, were 111.8%, 100.4%, and 75.0% in urine and 63.9%, 100.2%, and 92.5% in feces, respectively (each percent was mean value of two replications).

Resolution of DAS and its metabolites by TLC and GLC is shown in Table I. Unknown metabolites DRM-1 and DRM-2 had $R_f$ values of 0.37 and 0.17 in solvent system A, respectively, on TLC, which were close to those of the corresponding epoxy compounds, i.e. 0.40 and 0.17 for 15-MAS and SCP, respectively. The similar tendency was also observed in solvent system B. On the other hand, DRM-1 and DRM-2 had retention times of 11.4 and 7.7 min (as TFA esters) on GLC, respectively, and showed good separation from 15-MAS (14.3 min) and SCP (10.4 min), respectively.

Diacetoxyscirpenol and its metabolites were quantified by GLC–ECD as TFA derivatives (Table II). The parent compound was detected in neither urine nor feces eliminated within the first 3 days. In urine, 15-MAS, SCP, and two unknown metabolites named DRM-1 and DRM-2 were detected at 3.5%, 4.9%, 9.5%, and 7.2% of the administered dose, respectively. On the other hand, only the metabolites DRM-1 and DRM-2 were found in feces at 9.5% and 18.9% of the dose, respectively.

**Chemical Structures of the Metabolites.** The metabolites 15-MAS and SCP detected in urine were analyzed by GC–MS as the corresponding $Me_3Si$ derivatives. The molecular ion of $Me_3Si$–15-MAS was found at $m/z$ 468 (calcd for $C_{23}H_{40}O_6Si_2$ 468.2365). Other predominant

**Table II. Quantification of DAS and Its Metabolites in Excreta of Rats Orally Administered DAS**

| excreta[a] | rec,[b] % of dose | | | | | |
|---|---|---|---|---|---|---|
| | DAS | 15-MAS | DRM-1 | SCP | DRM-2 | total |
| urine | ND[c] | 3.5 ± 0.8 | 9.5 ± 1.8 | 4.9 ± 0.5 | 7.2 ± 0.3 | 25.0 ± 0.9 |
| feces | ND | ND | 9.5 ± 1.0 | ND | 18.9 ± 1.2 | 28.5 ± 1.8 |
| total | 0.0 ± 0.0 | 3.5 ± 0.8 | 19.1 ± 2.0 | 4.9 ± 0.5 | 26.1 ± 1.2 | 53.5 ± 1.4 |

[a] Urine or feces eliminated within the first 3 days after each dose were pooled and analyzed as TFA esters by capillary GLC-ECD. [b] Molecular percent of the administered DAS. Values are the mean of three dosing replications ± standard deviation. [c] Nondetected.

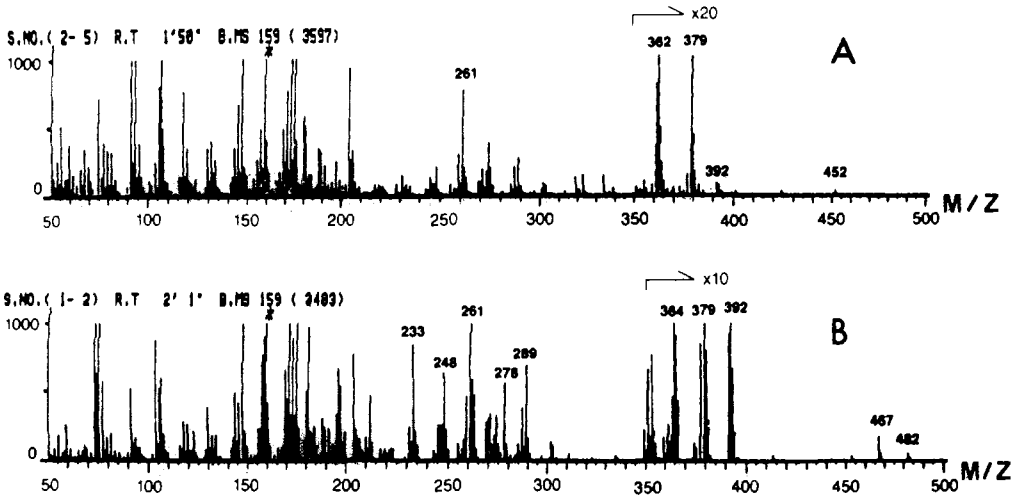



**Figure 1.** Mass spectra of $Me_3Si$ ethers of DRM-1 (A) and DRM-2 (B) (70 eV).

fragment ions at $m/z$ 378 [$M^+ - (CH_3)_3SiOH$] and 159 (base peak) were also found in the authentic $Me_3Si$-15-MAS. The mass spectrum of $Me_3Si$-SCP was identical with authentic SCP, displaying a molecular ion at $m/z$ 498 (calcd for $C_{24}H_{46}O_5Si_3$ 498.2655) and other fragment ions at $m/z$ 483 ($M^+ - CH_3$), 408 [$M^+ - (CH_3)_3SiOH$], and 277 (base peak).

The molecular ion of DRM-1 $Me_3Si$ ether was found at $m/z$ 452 [calcd for $C_{23}H_{40}O_5Si_2$ 452.2416, $M^+$($Me_3Si$-15-MAS) - oxygen atom] with other fragment ions at $m/z$ 437 [$M^+$($Me_3Si$-DRM-1) - $CH_3$], 362 [$M^+$($Me_3Si$-DRM-1) - $(CH_3)_3SiOH$], 289, and 261 (Figure 1). All of these fragment ions were shifted down by 16 mass units compared with corresponding fragments in the $Me_3Si$-15-MAS. Other predominant ions at $m/z$ 175 and 159 (base peak) were observed in both $Me_3Si$-DRM-1 and $Me_3Si$-15-MAS.

The molecular ion of DRM-2 $Me_3Si$ ether was found at $m/z$ 482 [calcd for $C_{24}H_{46}O_4Si_3$, 482.2706, $M^+$($Me_3Si$-SCP) - oxygen atom] (Figure 1). Other fragment ions at $m/z$ 467 [$M^+$($Me_3Si$-DRM-2) - $CH_3$], 392 [$M^+$($Me_3Si$-DRM-2) - $(CH_3)_3SiOH$], 379, and 261 were also shifted down by 16 mass units compared with corresponding fragments in the $Me_3Si$-SCP. Other predominant ions including $m/z$ 159 (base peak) and 147 were observed in both $Me_3Si$-DRM-2 and $Me_3Si$-SCP.

The proton NMR of DRM-2 dissolved in chloroform-$d$ and dimethyl-$d_6$ sulfoxide (3:2, v/v, plus $D_2O$) was as follows: $\delta$ 1.04 (s, 3 H, $CH_3C$), 1.67 (s, 3 H, $CH_3C$), 5.30 (d, 1 H, $J$ = 5.0 Hz, C=CH). Instead of a doublet resonance at $\delta$ 2.59 and 2.80 (each 1 H, $J$ = 4.2 Hz) due to methylene protons of the epoxy ring in SCP, singlet resonances were observed at $\delta$ 4.58 and 4.94 in DRM-2, which were assigned to terminal methylene protons at the C-13 position. The $^{13}C$ NMR of DRM-2 dissolved in chloroform-$d$ and dimethyl-$d_6$ sulfoxide (3:2, v/v) was as follows: $\delta$ 11.31 (C-14), 20.12 (C-7), 23.11 (C-16), 27.90 (C-8), 44.12 (C-6), 52.32 (C-5), 60.95 (C-15), 67.35 (C-11), 78.63, 79.21, 79.88 (C-2, 3, 4), 119.61 (C-10), 138.30 (C-9). Signals at $\delta$ 64.79 (C-12) and 45.91 (C-13) in SCP were shifted to $\delta$ 152.91 and 105.25 in DRM-2, respectively. Chemical shifts of both carbons and protons at the C-12 and C-13 positions of DRM-2 were similar to those of verrucarin K (Breitenstein and Tamm, 1977), deepoxydeoxynivalenol (DOM-1) (Yoshizawa et al., 1983), and T-2 deepoxy metabolites (Yoshizawa et al., 1985b) with a trichothec-9,12-diene skeleton.

In addition, DRM-1 did not give SCP, but rather DRM-2 as a parent alcohol after alkaline hydrolysis. DRM-2 remained unchanged, indicating that the trichothecene nucleus of these compounds was modified. Furthermore, DRM-1 and DRM-2 were negative to the color reaction on a TLC plate after treating with 4-(*p*-nitrobenzyl)pyridine, suggesting the absence of the 12,13-epoxy function in the metabolites. On the basis of these data, chemical structures of DRM-1 and DRM-2 were identified as 15-acetoxy-3$\alpha$,4$\beta$-dihydroxytrichothec-9,12-diene, i.e. 15-acetoxy-deepoxyscirpenol, and 3$\alpha$,4$\beta$,15-trihydroxytrichothec-9,12-diene, i.e. deepoxyscirpentriol (Table I), respectively.

Yoshizawa et al. (1982a, 1982b) and Pawlosky and Mirocha (1984) identified hydroxylated T-2 toxin metabolites (3′-OH-T-2, 3′-OH-HT-2, 3′-OH-7-OH-HT-2) in the urine of a cow dosed orally with T-2 toxin. In contrast, hydroxylated metabolites of DAS were not detected in the present study. Ohta et al. (1978) reported that the microsomal nonspecific carboxyesterase from rat and rabbit livers hydrolyzed DAS to 15-MAS. These data indicate that the liver microsomal enzymes are involved in the in vivo hydrolysis pathway, i.e. DAS was hydrolyzed to SCP via 15-MAS. The only metabolites detected in feces in the present study were the deepoxy compounds, suggesting that the gastrointestinal microorganisms participate in the deepoxidation reaction of DAS in rats. King et al. (1984) reported that the deepoxidation of DON was observed in the incubation mixture with bovine rumen microorganisms. In addition, Yoshizawa et al. (1985a) found that T-2 toxin and its derivatives were converted to the deepoxy metabolites in the mixed culture of mice intestinal flora under anerobic conditions. These data suggest that the deepoxidation performed by gastrointestinal or rumen organ-

isms is one of the common reactions that participate in the in vivo metabolism of 12,13-epoxytrichothecenes.

Diacetoxyscirpenol was highly toxic to animals, i.e. the $LD_{50}$ value in rats was 0.75 mg/kg, ip; however, this toxin was found neither urine nor feces, implying total biotransformation of the orally administered DAS. The toxicity of the hydrolyzed metabolites 15-MAS ($LD_{50}$ in rats 0.752 mg/kg, sc) and SCP ($LD_{50}$ in rats 0.81 mg/kg, ip) were comparable to that of DAS (Brian et al., 1961; Cole and Cox, 1981). But those minor metabolites accounted for 3.5% (15-MAS) and 4.9% (SCP) of the dose (Table II). With regard to the toxicity of the deepoxy trichothecenes, deepoxidation of 3′-OH HT-2 toxin ($LD_{50}$ = 22.8 mg/kg) resulted in extremely less toxic products (3′-OH deepoxy HT-2 toxin) (Yoshizawa et al., 1985a), and removal of the epoxide from DAS by reduction or rearrangement produced nontoxic compounds (10,13-cyclotrichothecene or apotrichothec-9-ene) (Grove and Mortimer, 1969). On the basis of those reports, DRM-1 and DRM-2 lacking the epoxide function are assumed to be less toxic than the corresponding epoxy compounds, although the toxicity of those metabolites is unknown at this moment. In addition, the detectable level of either deepoxy metabolite in rat urine or feces was quantitatively greater than that of either DAS, 15-MAS, or SCP (Table II), implying that the in vivo mechanisms responsible for the formation of the deepoxy metabolites may play an important role in the toxic mode of action for DAS. Further studies on the toxicity and the metabolic fate of deepoxy compounds remain to be established.

**Abbreviations Used**: diacetoxyscirpenol (DAS, 4β,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene; 15-monoacetoxyscirpenol (15-MAS), 15-acetoxy-3α,4β-dihydroxy-12,13-epoxytrichothec-9-ene; scirpentriol (SCP), 3α,4β,15-trihydroxy-12,13-epoxytrichothec-9-ene; 15-acetoxydeepoxyscirpenol (DRM-1), 15-acetoxy-3α,4β-dihydroxytrichothec-9,12-diene; deepoxyscirpentriol (DRM-2), 3α,4β,15-trihydroxytrichothec-9,12-diene; deoxynivalenol (DON), 3α,7α,15-trihydroxytrichothec-12,13-epoxytrichothec-9-en-8-one; deepoxydeoxynivalenol (DOM-1), 3α,7α,15-trihydroxytrichothec-9,12-dien-8-one; 3′-hydroxy T-2 toxin (3′-OH-T-2), 4β,15-diacetoxy-3α-hydroxy-8α-[(3-hydroxy-3-methylbutyryl)oxy]-12,13-epoxytrichothec-9-ene; 3′-hydroxy HT-2 toxin (3′-OH-HT-2), 15-acetoxy-3α,4β-dihydroxy-8α-[(3-hydroxy-3-methylbutyryl)oxy]-12,13-epoxytrichothec-9-ene; 3′-hydroxydeepoxy HT-2 toxin (3′-OH deepoxy HT-2), 15-acetoxy-3α,4β-dihydroxy-8α-[(3-hydroxy-3-methylbutyryl)oxy]-trichothec-9,12-diene; FID, hydrogen flame ionization detector; ECD, electron-capture detector; GLC, gas–liquid chromatography.

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**Registry No.** DAS, 2270-40-8; 15-MAS, 2623-22-5; SCP, 2270-41-9; 15-acetoxy-3α,4β-dihydroxytrichothec-9,12-diene, 101199-14-8; 3α,4β,15-trihydroxytrichothec-9,12-diene, 101199-15-9.

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