# THE ISOLATION AND IDENTIFICATION OF SEVERAL TRICHOTHECENE MYCOTOXINS FROM *FUSARIUM HETEROSPORUM*

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ABSTRACT.—An isolate of *Fusarium heterosporum* Nees ex Fr. that parasitizes the honeydew and immature sclerotia of *Claviceps paspali* was shown to be highly toxigenic to day-old chickens. The major toxicity of culture extracts of the fungus was due to the presence of several trichothecene mycotoxins. Six trichothecenes were isolated and chemically identified by spectroscopic methods as T-2 toxin (**1**), HT-2 toxin (**2**), T-2 tetraol (**6**), 3α,4β,dihydroxy-15-acetoxy-8α-(3-hydroxy-3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene (**3**), 3α,4β,15-trihydroxy-8α-(3-hydroxy-3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene (**5**), and 3α,4β,8α-trihydroxy-15-acetoxy-12,13-epoxytrichothec-9-ene (**4**). The acute toxicities of the trichothecenes were compared to the effects of T-2 toxin in day-old chickens and on the back skins of rabbits. The comparative inhibitory effects of the trichothecene mycotoxins in the *Triticum* coleoptile bioassays were also determined.

*Fusarium heterosporum* Nees ex Fr. is an ubiquitous saprobic fungus that colonizes the honeydew and immature sclerotia of *Claviceps paspali*, *C. purpurea*, and probably most other ergot fungi (1,2,3). As *F. heterosporum* colonizes and overgrows the honeydew, it prevents the maturation of the sclerotium and thus acts as a natural biological control. Matured sclerotia of *C. purpurea* that were partially colonized by *F. heterosporum* rotted rapidly and did not germinate when moistened (Cunfer and Mathre, unpublished).

Colonization by *F. heterosporum* is easily identified because it produces a bright pink to orange mycelial stroma over most of the immature sclerotium. The stroma is composed of dense masses of multiseptate conidia. These conidia are disseminated in the same manner as ergot conidia. When *F. heterosporum* and ergot conidia reach a susceptible flower at the same time, *F. heterosporum* does not prevent infection by the ergot fungus. However, as soon as honeydew appears, the *F. heterosporum* conidia germinate and colonize the honeydew (1).

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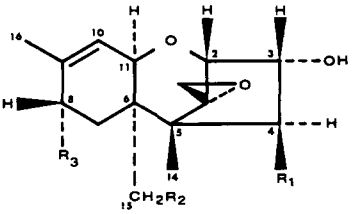
By the end of each summer in Georgia and throughout most of the southern United States, 30 to 50% of *C. paspali* stromata infecting dallisgrass (*Paspalum dilatatum* Poir.) flowers are colonized by *F. heterosporum*. *F. heterosporum* produces abundant numbers of conidia, both *in vitro* and *in vivo*, and chlamydospores that persist for long periods until they reach a favorable substrate.

Several taxonomic systems are used for the Fusaria. *F. heterosporum* is the species name according to the scheme of Booth (4). In the Snyder and Hansen (5) system, it is *F. roseum* Lk. ex Fr.; according to the Woolenweber (6) system, it is called *F. sambucinum* Fuckel.

The potential for mycotoxin production by *F. heterosporum* on ergot honeydew has been previously studied. Mower *et al.* (3) were unable to detect psychotrophic compounds from cultures of *F. heterosporum* isolated from *C. purpurea* or from cultures of *F. heterosporum* grown on ergotamine tartrate.



1 $R_1$ = OAc, $R_2$ = OAc, $R_3$ = $OCOCH_2CH(CH_3)_2$

2 $R_1$ = OH, $R_2$ = OAc, $R_3$ = $OCOCH_2CH(CH_3)_2$

3 $R_1$ = OH, $R_2$ = OAc, $R_3$ = $OCOCH_2COH(CH_3)_2$

4 $R_1$ = OH, $R_2$ = OAc, $R_3$ = OH

5 $R_1$ = OH, $R_2$ = OH, $R_3$ = $OCOCH_2COH(CH_3)_2$

6 $R_1$ = OH, $R_2$ = OH, $R_3$ = OH

FIG. 1. Chemical structures of some trichothecene mycotoxins.

We became interested in the toxin-producing potential of *F. heterosporum* because of its widespread occurrence in nature. We now report the isolation and identification of several trichothecene mycotoxins from *F. heterosporum* isolated from *C. paspali* sclerotia in middle Georgia in the summer of 1977. Comparative acute toxicities of the trichothecenes are presented.

## EXPERIMENTAL

ISOLATION AND CULTURE OF FUNGUS.—The strain of *Fusarium heterosporum* Nees ex Fr. [= *F. roseum* Lk. ex Fr.] was isolated from *Claviceps paspali* honeydew on *Paspalum distichum* L. at Experiment, Ga., in the summer of 1977. The isolate was grown on fresh potato-dextrose agar at 20 to 25° in light supplied by fluorescent tubes. Under these conditions, *F. hetero-*

*sporum* sporulated profusely in 2 to 3 days. This material was used to inoculate subsequent cultures.

The toxigenic nature of the fungus was ascertained by culturing it in 500-ml Erlenmeyer flasks containing 25 g of shredded wheat supplemented with 50 ml of Difco mycological broth (pH 4.8) plus YES medium (7). The cultures were incubated at 25 to 28° for 2 weeks and extracted once with an equal volume of chloroform. The total chloroform extract from one flask was given to 5 one-day-old chickens to determine the toxigenicity of the fungus (8).

*F. heterosporum* was mass cultured in forty 2.8-liter Fernbach flasks containing 200 g of shredded wheat supplemented with 200 ml of Difco mycological broth plus YES medium. The cultures were incubated at 27 to 30° for 3 weeks. The cultures were extracted 2 times with chloroform to extract the toxins.

PURIFICATION OF TOXINS.—The crude chloroform extract was fractionated on a silica gel column (9.5 x 17 cm) eluted with 3 liters each of toluene, ethyl ether, ethyl acetate, acetone, and methanol. All fractions were tested for toxicity on day-old chickens (8). All gross fractions except toluene were highly toxic.

The ethyl ether and ethyl acetate fractions were combined and chromatographed on a

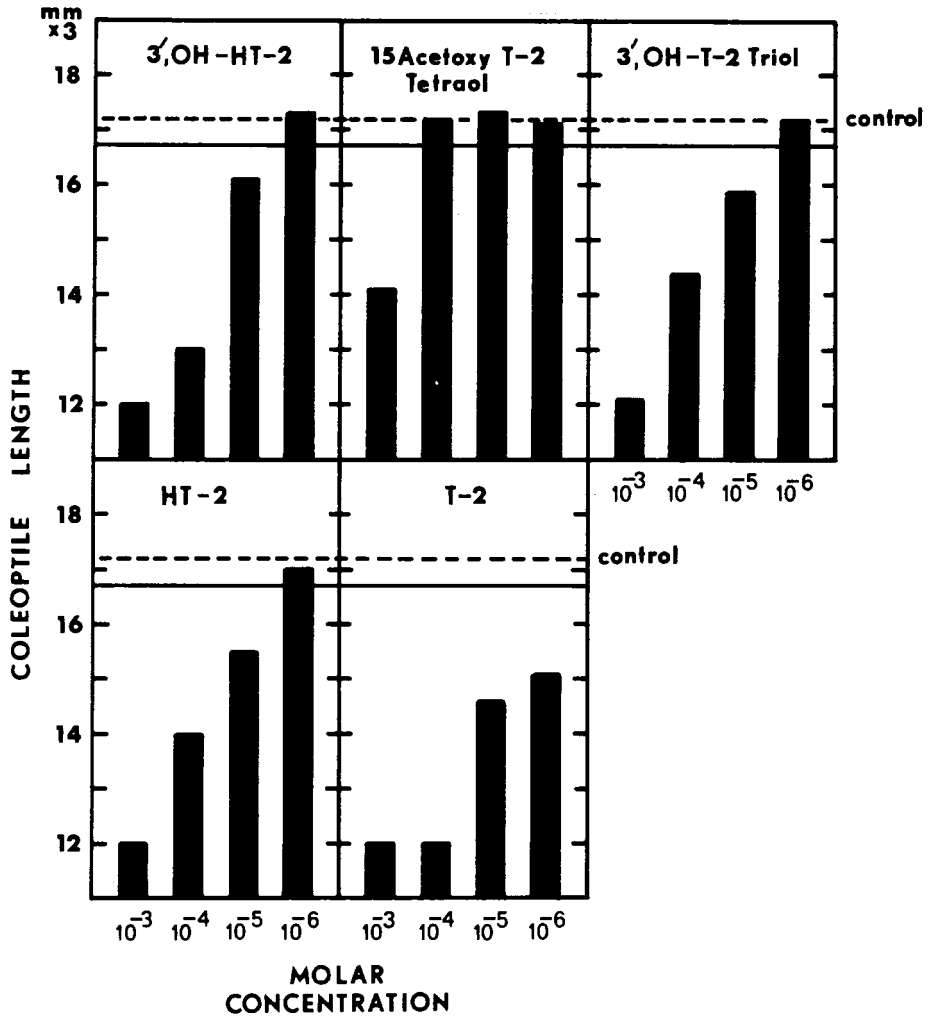



FIG. 2. Inhibitory effects on the growth of wheat coleoptiles (*Triticum aestivum* L. cv. Wakeland) by some trichothecene mycotoxins. Control, dotted line. Significant inhibition below solid line ($P<0.01$).

$C_{18}$ reverse-phase column (3.5 x 10 cm) with a gradient elution from 30 to 80% acetonitrile-water. A total of 107 17-ml fractions were collected. Toxicity was associated with tubes 31 to 65. These tubes were combined and reduced in volume, and toxin **1** was crystallized at 5° (mp 150 to 152°).

The gross acetone fraction was fractionated on a silica gel column (3.5x 40 cm) with a linear gradient from ethyl acetate to acetone (211 17-ml fractions collected). The eluant was combined into four groups (A, B, C, and D) based on tlc analysis.

Fraction A was applied to a $C_{18}$ reverse-phase column (3.5 x 10 cm) and eluted with a linear gradient from 10 to 80% acetonitrile-water. Toxicity was associated with fractions 51 to 96 (139 17-ml fractions collected). These tubes were combined and concentrated, and toxin **2** was crystallized from solutions at 5°.

Fraction B was chromatographed on a $C_{18}$ reverse-phase column (3.5 x 10 cm) with a linear gradient from 5 to 50% acetonitrile-water. A total of 112 17-ml fractions were collected. Fractions 46 to 58 were combined and reduced in volume, and toxin **3** was precipiated from solution at 5°.

Fraction C was chromatographed on a $C_{18}$ reverse-phase column packed in water. The sample was applied in water, and the column was eluted with a linear gradient from water to 50% acetonitrile-water. A total of 70 17-ml fractions were collected. Fractions 12 to 18, when combined and concentrated, yielded toxin **4**.

Fraction D was applied to a $C_{18}$ reverse-phase column (3.5 x 10 cm) packed in 10% acetonitrile-water. The sample was applied in methanol-water, and the column was eluted with a linear gradient from 10 to 50% acetonitrile-water (110 17-ml fractions collected). Fractions 35 to 44 were combined and yielded toxin **5**.

The crude gross methanol fraction was applied to a silica gel column (3.5 x 40 cm) packed in ethyl acetate and eluted with a gradient from ethyl acetate to methanol (112 17-ml fractions collected). Fractions 77 to 90 were combined and yielded toxin **6**.

SPECTRAL ANALYSES.—Proton and $^{13}C$-nmr spectra were obtained on a Varian Associates XL-100-12 nmr spectrometer equipped with the 620-L disk data system. The $^{1}H$ spectra were run in the continuous wave mode, whereas the $^{13}C$ spectra were run in the Fourier transform (FT) mode. Typical operating conditions for the $^{13}C$ spectra were spectral width, 5 kHz; pulse angle, 30°; repetition rate, 2.8 sec.; square-wave modulated proton decoupling, 100 Hz; data points, 8,000; and exponential line broadening, −1.0. Single-frequency, off-resonance proton decoupled (sford) $^{13}C$ spectra were obtained to aid in the assignment of the carbon resonances. The sample for nmr analysis was prepared by dissolving 25 mg of the compound in 0.5 ml of $CDCl_3$ to which a small amount of tetramethylsilane (TMS) was added as an internal reference. Both the $^{1}H$ and $^{13}C$ spectra were run on this solution in a 5-mm sample tube.

Mass spectra were run on a VG-Micromass AZB-2F spectrometer, and samples were introduced by the direct probe technique. Electron-impact spectra were run using 70 eV, and chemical-ionization spectra were run with isobutane as the reagent gas.

VERTEBRATE BIOASSAY.—The acute vertebrate toxicity studies were conducted with day-old chickens according to the method of Kirksey and Cole (8). The $LD_{50}$ determinations were made with single oral doses of 25, 50, 100, 200, or 400 μg per chicken (7 replications of each dosage level). Samples were prepared with corn oil as the inert carrier and dosed at 1 cc/chicken via crop intubation (8). Mortality counts were taken up to 6 days after dosing.

The dermal toxicities of the toxins were compared to that of T-2 on the shaved back skin of 4 rabbits. The method used was a modification of the method proposed by Chung *et al.* (9). Each toxin dissolved in acetone was applied topically to the back skins of rabbits in 10-μl aliquots at levels of 0.16, 0.32, 0.64, and 1.28 μg per spot. Two replications of each concentration of each toxin were performed on the back skins of 4 rabbits.

PLANT BIOASSAY.—Four-millimeter-long coleoptiles were cut from etiolated wheat seedlings (*Triticum aestivum* L. cv. Wakeland) that had been grown in the dark at 22±1° for 4 days on moist sand in trays (10). Working under a green safelight (11), we removed the seedlings from the sand, and stems were individually separated from the roots and caryopses. The tips of the seedlings were fed into a Van der Weij guillotine and the terminal 2 mm were cut off and discarded. The next 4 mm were retained for bioassay, and ten of the coleoptile pieces were added to each test tube, which contained 2 ml of phosphate-citrate buffer solution at pH 5.6 plus 2% sucrose (11) and a predetermined amount of toxin. Each toxin was tested at $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$M, and controls were included. To facilitate solubility of the toxins, 5 μl of acetone (12) was added to each test tube before the buffer solution was added. Coleoptiles were incubated in the dark in a Coller tube apparatus at 0.25 rpm for 24 hours, then measured by projecting their images magnified 3 times by a photographic enlarger (13). Data were statistically analyzed (14).

## RESULTS AND DISCUSSION

Compounds **1**, **2**, and **6** were identified as T-2 toxin (**1**), HT-2 toxin (**2**), and T-2 tetraol (**6**) by comparison of their $^{1}H$- and $^{13}C$-nmr spectra and mass spectra

with those of authentic standards, and with data reported previously in the literature for those compounds (15). The electron-impact mass spectrum of **3** showed prominent peaks at *m/e* 263, 203, and 121, with a small peak at 381 for the highest mass. The chemical-ionization mass spectrum showed a peak at *m/e* 441 ($M^+$+1), with additional peaks at 423 ($M^+$+1–$H_2O$), 405 ($M^+$+1–2$H_2O$), 381 ($M^+$+1–AcOH), and 363 ($M^+$+1–AcOH–$H_2O$), with other major peaks at 263, 203, and 121. Thus, the molecular weight of **3** was established as 440. The $^1$H-nmr spectrum of **3** (table 1) was very similar to that of the trichothecene HT–2 toxin with the exception that the doublet reported for the two methyl groups on the isovaleryl side-chain is not split, but appears as a singlet shifted slightly downfield (16). The $^{13}$C-nmr spectrum (table 2) contained peaks for 22 carbons. Again, with the exception of the isovaleryl side-chain, the $^{13}$C-nmr spectrum of **3** was similar to that reported for HT–2 toxin (15). The nmr data along with the mass spectrum suggested the molecular formula $C_{22}H_{32}O_9$ for **3**, i.e., **3** contained one additional oxygen as compared with HT–2 toxin. Closer inspection of the $^1$H- and $^{13}$C-nmr data for **3** and comparison with data for HT–2 toxin (**2**) (15,16) suggested that **3** contained a hydroxy group in place of the hydrogen on the methine carbon of the isovaleryl side-chain. This conclusion was supported by the following observations: (a) the protons of the two methyl groups appeared as a singlet rather than a doublet; (b) the $^1$H-nmr spectrum contained a singlet for the methylene protons of the side-chain rather than the splitting observed for these protons in HT–2 toxin (16); (c) the doublet and triplet (sford spectrum) observed for C–3′ and C–2′ at 25.0 and 42.6 ppm, respectively, in HT–2 toxin were replaced by a singlet and triplet (sford) at 68.05 and 47.86 ppm, respectively, for C–3′ and C–2′ of **3**; and (d) the $^{13}$C peaks observed for the methyl carbons of HT–2 toxin at 21.9 ppm were shifted downfield to 28.9 and 29.5 ppm in **3**, consistent with a hydrogen on an adjacent carbon being replaced with a hydroxy group. Thus, the structure of **3** was established as 3α,4β,dihydroxy-15-acetoxy-8α-(3-hydroxy-3-methylbutyryloxy)-12,13-epoxy-trichothec-9-ene.

The electron-impact mass spectrum of **4** gave a *m/e* at 340 for $M^+$ with additional peaks at 322 ($M^+-H_2O$), 203, 175, and 121 amu (basepeak). In the isobutane chemical-ionization mass spectrum, peaks were observed at *m/e* 341 ($M^+$+1), 323 ($M^+1-H_2O$), 305 ($M^+$+1−2$H_2O$), 281, 263 (basepeak), 245, 233, 215, and 121 amu. These data suggested a molecular weight of 240 for **4**. The $^1$H-nmr spectrum of **4** (table 1) was typical of that of trichothecenes and indicated the presence of one acetate group. This was confirmed in the $^{13}$C-nmr spectrum of **4** (table 2). The $^{13}$C-nmr spectrum of **4** suggested the presence of the basic T–2 tetraol skeleton with one additional acetate group.

Comparison of the $^{13}$C-nmr data reported for acetyl T–2 toxin, T–2 toxin, HT–2 toxin, T–2 triol, and T–2 tetraol (15) indicated that C–9 was shifted downfield and C–10 was shifted upfield when the isovaleryl group was not present. The similarity of the chemical shifts for C–9 and C–10 in **4** with those in T–2 tetraol indicated that the acetate group in **4** was not located on C–8. Further comparison of the above compounds showed that the absence of an acetate ester on C–15 resulted in an upfield shift for C–15 of ∼2 pp,. Because the shift for C–15 of **4** was similar to that of T–2 toxin and HT–2 toxin, and the remaining hydroxyl-bearing carbons (C–3, C–4, and C–8) were similar to their corresponding shifts in T–2 tetraol, the acetate group must be located on C–15. Thus, the structure of **4** was established as 3α,4β,8α-trihydroxy-15-acetoxy-12, 13-epoxy-trichothec-9-ene.

TABLE 1. $^1$H-Nmr chemical shifts for some trichothecenes.

| Proton/compound | 3 | 4 | 5 |
|---|---|---|---|
| 2 | 3.28 ($J$=4.6) | 3.24 ($J$=4.9) | 3.21 ($J$=4.8) |
| 3 | 4.02 | 3.89 | 3.84 ($J$=4.8, 2.9) |
| 4 | 4.02 | 4.06 | 4.42 ($J$=2.9) |
| 7 | 2.09 | 2.06 | 2.05 |
| 8 | 5.18 | 5.19 | 5.15 ($J$=5.8) |
| 10 | 5.65 ($J$=5.2) | 5.40 | 5.63 ($J$=5.4) |
| 11 | 4.10 | 4.03 | 4.05 ($J$=5.4) |
| 13 | 2.66, 2.88 ($J$=4.0) | 2.62, 2.84 ($J$=4.1) | 2.61, 2.83 ($J$=4.2) |
| 14 | 0.65 | 0.67 | 0.68 |
| 15 | 3.84, 4.10 ($J$=12.0) | 3.89, 4.03 | 3.24, 3.64 ($J$=12.0) |
| 16 | 1.67 | 1.72 | 1.66 |
| $CH_3CO$ | 1.99 | 1.97 | — |
| 2' | 2.31 | — | 2.40 |
| 4' | 1.19 | — | 1.20 |
| 5' | 1.19 | — | 1.20 |

Values are chemical shifts in ppm downfield from TMS. Coupling constants in Hz are given in parenthesis.

The mass spectrum of **5** did not give a molecular ion peak in either the electron-impact or chemical-ionization spectrum. However, its structure was readily apparent from its $^1$H- and $^{13}$C-nmr spectra. The $^1$H-nmr spectrum of **5** (table 1) was similar to that of **3** in that a six-proton singlet was observed for what appeared to be the methyl protons of the isovaleryl side chain. In contrast to **3**, however, there were no acetate methyl groups in **5**. The $^{13}$C-nmr spectrum (table 2) of **5** contained 19 peaks. Intensity considerations suggested that one of the peaks was due to two carbon atoms. Thus, **5** contained 20 carbons. Furthermore, comparison of the $^{13}$C-nmr data of **5** with those of **3** established the presence of

TABLE 2. $^{13}$C-Nmr chemical shifts for some trichothecenes.

| Carbon/compound | 3 | 4 | 5 |
|---|---|---|---|
| 2 | 78.43 d | 78.48 d | 78.49 d |
| 3 | 78.87 d | 79.01 d | 79.18 d |
| 4 | 79.45 d | 79.49 d | 79.18 d |
| 5 | 47.95 s | 47.86 s | 48.06 s |
| 6 | 41.90 s | 42.57 s | 43.37 s |
| 7 | 26.40 t | 30.09 t | 26.66 t |
| 8 | 67.58 d | 64.82 d | 68.12 d |
| 9 | 134.43 s | 138.93 s | 133.93 s |
| 10 | 124.19 d | 120.38 d | 124.76 d |
| 11 | 66.22 d | 66.65 d | 66.45 d |
| 12 | 64.28 s | 64.61 s | 64.75 s |
| 13 | 45.44 t | 45.58 t | 45.58 t |
| 14 | 6.93 q | 6.78 q | 6.92 q |
| 15 | 63.85 t | 64.61 t | 61.37 t |
| 16 | 19.81 q | 20.34 q | 19.78 q |
| $CH_3CO$ | 20.83 q | 20.82 q | — |
| $CH_3CO$ | 169.41 s | 169.60 s | — |
| 1' | 169.89 s | — | 170.11 s |
| 2' | 47.86 t | — | 47.81 t |
| 3' | 68.05 s | — | 67.91 s |
| 4' | 29.17 q | — | 29.08 q |
| 5' | 29.37 q | — | 29.64 q |

Values are chemical shifts in ppm downfield from TMS.

the 3'-hydroxyisovaleryl side chain and the absence of acetate groups. The nmr data for **5** are therefore consistent with 3α,4β,15-trihydroxy-8α-(3-hydroxy-3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene.

The $LD_{50}$ values observed for day-old chicks of the various trichothecenes in order of decreasing toxicity were 1.75 mg/kg for T-2, 6.25 mg/kg for HT-2, 8.5 mg/kg for **3**, and >10 mg/kg for **4** and T-2 tetraol.

The $ED_{50}$ values for the dermal toxicity on back skin of rabbits of various trichothecenes were <0.16 μg for T-2, between 0.16 and 0.32 μg for HT-2, >1.28 μg for **3**, **4**, **5**, and T-2 tetraol. HT-2 was slightly less active than T-2, whereas no dermal toxicity was observed for the other four trichothecenes at the levels tested.

Of the five toxins assayed, T-2 toxin was the most potent and significantly inhibited (P 0.01) wheat coleoptiles at all concentrations tested ($10^{-3}$ to $10^{-6}$M). Conversely, toxin **4** inhibited coleoptiles 59% relative to controls at $10^{-3}$M and was relatively inactive. Toxins **3**, **5** and HT-2 induced approximately the same inhibitory effects at $10^{-3}$, $10^{-4}$, and $10^{-5}$M (figure 2) (17). Neosolaniol monoacetate was less active than T-2 toxin but was more active than the aforementioned toxins at $10^{-6}$M (18).

We have shown that *F. heterosporum* produced at least six 12,13-epoxytrichothec-9-ene mycotoxins. This is the first report of a strain of *F. heterosporum* parasitic on *Claviceps paspali* honeydew that is capable of producing trichothecenes in culture. Furthermore, three of the trichothecenes isolated—**3**, **4**, and **5**—represent new structures that have not been previously isolated from a fungal source.

The discovery of a trichothecene-producing isolate of *F. heterosporum* from *C. paspali* honeydew introduces the possibility of trichothecene exposure to cattle grazing in pastures infected with *C. paspali* and *F. heterosporum*.

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