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TWO NEW TRICHOTHECENES FROM *FUSARIUM SPOROTRICHIOIDES*

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ABSTRACT.—Two new trichothecenes, 8-*n*-pentanoylneosolaniol [**8**] and 8-*n*-hexanoylneosolaniol [**9**], were isolated from corn grits cultured with *Fusarium sporotrichioides*. The structures of these compounds were elucidated using gc-ms, nmr, X-ray crystallography, and other spectroscopic techniques. Seven known trichothecenes were also isolated, and the relative cytotoxicity of these nine trichothecenes in cultured baby hamster kidney (BHK-21) cells was determined.

Trichothecene mycotoxins have been reported to be produced by the toxigenic fungus *Fusarium sporotrichioides* Sherbakoff (Moniliales) (1–9). The trichothecenes are known to be responsible for alimentary toxic aleukia (ATA), weight loss, vomiting, skin inflammation, and death in humans as well as livestock (10). The major trichothecenes produced by this fungus are T-2 toxin and/or neosolaniol (4,5,7), and HT-2 toxin (4,5,7) as well as a large number of minor products (1–5, 7, 9). We now report a strain of *F. sporotrichioides* that produces neosolaniol as the major metabolite as well as a number of C-8 substituted derivatives including two new trichothecenes, 8-*n*-pentanoylneosolaniol [**8**] and 8-*n*-hexanoylneosolaniol [**9**], which are present in lesser amounts while T-2 toxin is only found as a minor product. The cytotoxicity of all the isolated trichothecenes was measured, and the results are shown in Table 1.

F. sporotrichioides, isolated from Ethiopian wheat, was cultured (11) on ground corn grits at 10° for 28 days. The fermented corn grits were blended with $CHCl_3$-Me_2CO (85:15) (300 ml/jar) and the mixture was allowed to stand overnight followed by filtration. The solid residue was reextracted with Me_2CO (300 ml/jar), autoclaved, and discarded. The Me_2CO extract was combined with the $CHCl_3$-Me_2CO (85:15) extract and concentrated under reduced pressure. The remaining dark-green oil (ca. 300 ml/100 jars) obtained by this procedure was subjected to Florisil cc. The 300 ml of oil was dissolved in 700 ml C_6H_6–n-C_6H_{14} (2:1), and 50 ml aliquots were applied to the Florisil column. The column was eluted successively with 400 ml of C_6H_6–n-C_6H_{14} (2:1), 200 ml of CH_2Cl_2, 300 ml of $CHCl_3$-Me_2CO (9:1), and 250 ml of Me_2CO (a total of 20 Florisil cc were performed, and similar fractions were combined and concentrated under reduced pressure). Tlc indicated that the $CHCl_3$/Me_2CO (50 ml) and Me_2CO (60 ml) fractions contain trichothecenes. The C_6H_6/n-C_6H_{14} (250 ml) and CH_2Cl_2 (20 ml) fractions contained mainly corn oil and β-sitosterol. The Me_2CO fraction was highly enriched with neosolaniol [**1**] (4 g) as determined by tlc. Successive recrystallization of the $CHCl_3$/Me_2CO residue in Me_2CO/n-C_6H_{14} yielded a mixture of co-crystallized trichothecenes. Reversed-phase flash chromatography of this trichothecene mixture using MeOH-H_2O-HOAc (35:20:1) yielded neosolaniol [**1**] (61 mg/100 jars), NT-1 [**2**] (16 mg/100 jars), 8-acetylneosolaniol [**3**] (74 mg/100 jars), 8-propionylneosolaniol

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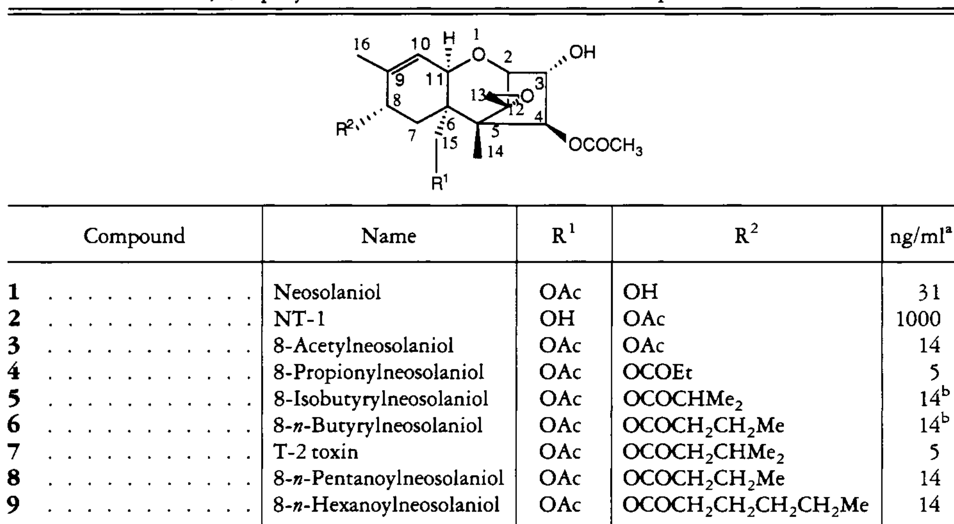
TABLE 1. Baby Hamster Kidney Cell (BHK-21) Cytotoxicity Bioassay and Structure of 12,13-Epoxytrichothecenes Isolated from *Fusarium sporotrichioides*.



Compound	Name	R^1	R^2	ng/ml[a]
1	Neosolaniol	OAc	OH	31
2	NT-1	OH	OAc	1000
3	8-Acetylneosolaniol	OAc	OAc	14
4	8-Propionylneosolaniol	OAc	OCOEt	5
5	8-Isobutyrylneosolaniol	OAc	$OCOCHMe_2$	14[b]
6	8-*n*-Butyrylneosolaniol	OAc	$OCOCH_2CH_2Me$	14[b]
7	T-2 toxin	OAc	$OCOCH_2CHMe_2$	5
8	8-*n*-Pentanoylneosolaniol	OAc	$OCOCH_2CH_2Me$	14
9	8-*n*-Hexanoylneosolaniol	OAc	$OCOCH_2CH_2CH_2CH_2Me$	14

[a]Approximate LC_{100}; each value represents the average of 2 × 12 assays.
[b]Cytotoxicity of trichothecenes **5** and **6** was determined as a 1:9 mixture.

[**4**] (22 mg/100 jars), 8-isobutyrylneosolaniol [**5**], 8-*n*-butyrylneosolaniol [**6**] [**5** and **6** were obtained as an inseparable mixture (50 mg/100 jars) in an approximate 1:9 ratio (isobutyryl/*n*-butyryl) determined by ^{1}H-nmr], T-2 toxin [**7**] (29 mg/100 jars), and two previously unreported trichothecenes, 8-*n*-pentanoylneosolaniol [**8**] (60 mg/100 jars) and 8-*n*-hexanoylneosolaniol [**9**] (15 mg/100 jars) (Table 1). The two new trichothecenes were purified using preparative reversed-phase hplc with MeOH-H_2O (3:1) as the mobile phase.

8-*n*-Pentanoylneosolaniol [**8**], $C_{24}H_{34}O_9$, isolated as colorless crystals from ACN/H_2O, mp 152–153°, has bands in its ir spectrum indicative of the presence of hydroxyl

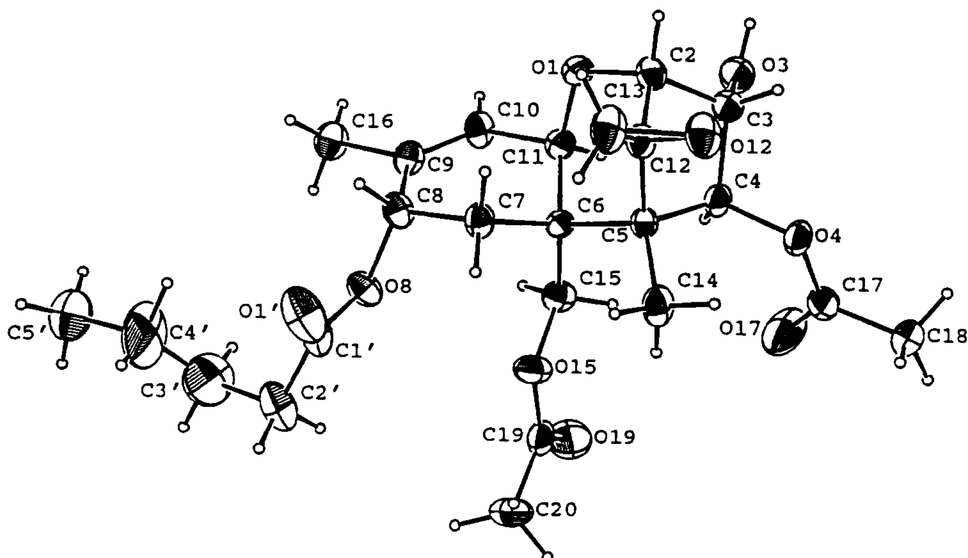


FIGURE 1. ORTEP drawing of 8-*n*-pentanoylneosolaniol [**8**].

and ester functionalities (film, 3407, 1732 cm^{-1}). The uv spectrum supported the presence of an isolated double bond (MeCN, λ max 198 nm, ε max 6600). The eims (TMS derivative), 436 (10) $[M - C_5H_9O_2]^+$, 350 (18), 290 (15), 185 (23), 157 (26), 122 (74), 73 (100), and cims (TFA derivative) spectral data 563 (6) $[M + 1]^+$, 461 (6), 401 (100), 341 (4), 227 (3), are consistent with the trichothecene nucleus fragmentations reported previously (12,13). The ^{1}H-nmr ($CDCl_3$, 300 MHz) and the ^{13}C-nmr ($CDCl_3$, 75 MHz) spectral data (Table 2) suggest that **8** is closely related to the co-occurring T-2 toxin [**7**] (14,15). Differences between **8** and **7** are found in the aliphatic region of the nmr spectra assigned to the ester sidechain at C-8 [i.e., ^{13}C nmr **8** see Table 2; **7** C-1′ (172.0, s), C-2′ (43.4, t), C-3′ (25.7, d), C-4′ (22.4, q)]. Information about carbon multiplicities was obtained by DEPT. From the above data, it was clear that **8** contained an *n*-pentanoyl ester group. To confirm the placement of the pentanoyl ester at C-8, a single crystal X-ray analysis was performed on **8**, and the resulting ORTEP drawing is shown in Figure 1. The final atomic coordinates are given in Table 3.

8-*n*-Hexanoylneosolaniol [**9**], $C_{25}H_{36}O_9$, a colorless glass, has bands in its ir spectrum indicative of the presence of hydroxyl and ester functionalities (film, 3418, 1733

TABLE 2. Nmr Data on 8-*n*-Pentanoylneosolaniol [**8**] and 8-*n*-Hexanoylneosolaniol [**9**] in $CDCl_3$ (^{1}H-nmr at 300 MHz and ^{13}C-nmr at 75 MHz, coupling constants in Hz).

Position	Compound			
	8		**9**	
	^{1}H	^{13}C	^{1}H	^{13}C
2	3.70 d (4.8)	78.7 d	3.70 d (4.8)	78.7 d
3	4.16 bp	78.4 d	4.16 bp	78.4 d
4	5.28 d (3.0)	84.6 d	5.28 d (3.0)	84.6 d
5	—	48.5 s	—	48.4 s
6	—	43.0 s	—	43.0 s
7_a	1.93 d (15.3)	27.7 t	1.93 d (15.3)	27.7 t
7_b	2.40 dd (15.3, 5.9)		2.39 dd (15.3, 3.0)	
8	5.28 d	68.1 d	5.28 d	68.1 d
9	—	136.3 s	—	136.3 s
10	5.81 d (5.5)	123.6 d	5.81 d (5.5)	123.6 d
11	4.34 d (5.5)	67.3 d	4.34 d (5.5)	67.3 d
12	—	64.3 s	—	64.3 s
13_a	2.80 d (3.6)	47.2 t	2.80 d (3.6)	47.2 t
13_b	3.07 d (3.6)		3.06 d (3.6)	
14	0.82 s	6.9 q	0.81 s	6.9 q
15_a	4.06 d (12.6)	64.6 t	4.06 d (12.6)	64.6 t
15_b	4.29 d (12.6)		4.29 d (12.6)	
16	1.75 s	20.3 q	1.75 s	20.3 q
1′	—	173.4 s	—	173.4 s
2′	2.27 t (7.5)	34.2 t	2.26 t (7.4)	34.5 t
3′	1.63 q (7.5)	27.0 t	1.61 m (7.4)	31.3 t
4′	1.35 m (7.5)	22.3 t	1.31 m	24.7 t
5′	0.92 t (7.5)	13.6 q	1.31 m	22.3 t
6′			0.89 t (7.4)	13.9 q
*Me*CO	2.03 s	21.0 q	2.03 s	21.0 q
	2.15 s	21.0 q	2.15 s	21.0 q
Me*C*O	—	172.7 s	—	170.1 s
	—	172.7 s	—	172.7 s
3-OH	3.20 bs	—	3.21 bs	—

TABLE 3. Positional and Thermal Parameters for 8-*n*-Pentanoylneosolaniol [**8**].[a]

Atom	x	y	z	B (Å^2)[b]
O-1	0.0547(4)	0.4295(2)	0.2260(3)	3.72(9)
O-1′	0.0478(5)	0.1178(s)	0.1407(4)	6.7(1)
O-3	0.2321(5)	0.5313(2)	0.3295(3)	4.8(1)
O-4	0.3081(4)	0.4151(2)	0.4926(3)	3.83(9)
O-8	0.2202(4)	0.1994(2)	0.1001(3)	3.88(9)
O-12	−0.0566(4)	0.3746(2)	0.4630(3)	5.0(1)
O-15	0.3889(4)	0.2257(2)	0.2780(3)	4.26(9)
O-17	0.5459(5)	0.3863(3)	0.4612(4)	8.5(2)
O-19	0.6408(5)	0.2310(3)	0.2733(4)	7.0(1)
C-1′	0.1712(7)	0.1324(3)	0.1104(5)	4.8(2)
C-2	0.0238(7)	0.4407(3)	0.3240(4)	3.5(1)
C-2′	0.2878(8)	0.0809(3)	0.0753(5)	6.1(2)
C-3	0.1568(6)	0.4738(3)	0.3765(5)	3.6(1)
C-3′	0.288 (1)	0.0772(6)	−0.0277(7)	10.7(3)
C-4	0.2680(6)	0.4131(3)	0.3943(4)	3.2(1)
C-4′	0.159 (1)	0.0665(7)	−0.0761(7)	13.3(3)
C-5	0.1779(6)	0.3435(3)	0.3694(4)	3.0(1)
C-5′	0.159 (1)	0.0558(5)	−0.1807(6)	7.7(2)
C-6	0.2144(6)	0.3239(3)	0.2631(4)	2.7(1)
C-7	0.0975(7)	0.2702(3)	0.2261(4)	3.5(1)
C-8	0.1085(7)	0.2547(3)	0.1218(4)	3.5(1)
C-9	0.1557(7)	0.3177(3)	0.0640(4)	3.7(1)
C-10	0.1901(6)	0.3787(3)	0.1020(4)	3.8(1)
C-11	0.1957(6)	0.3930(3)	0.2050(4)	3.4(1)
C-12	0.0157(6)	0.3711(3)	0.3715(4)	3.3(1)
C-13	−0.1255(7)	0.3313(4)	0.3906(5)	5.0(2)
C-14	0.2094(7)	0.2822(3)	0.4353(4)	4.3(1)
C-15	0.3791(7)	0.2985(3)	0.2512(4)	3.7(1)
C-16	0.1512(8)	0.3058(4)	−0.0410(4)	5.0(2)
C-17	0.4503(7)	0.4011(3)	0.5156(5)	4.5(1)
C-18	0.4740(8)	0.4035(4)	0.6189(5)	6.1(2)
C-19	0.5261(7)	0.1977(3)	0.2863(4)	4.3(1)
C-20	0.5226(8)	0.1223(4)	0.3151(5)	6.2(2)
3-OH	0.160 (7)	0.564 (3)	0.332 (5)	4.0[c]

[a]Figures in parentheses are ESD.

[b]Anisotropically refined atoms are given in the form of the isotropic equivalent displacement parameter defined as: $(4/3)*[a^2*\beta(1,1)+b^2*\beta(2,2)+c^2*\beta(3,3)+ab(\cos\gamma)*\beta(1,2)+ac(\cos\beta)*\beta(1,3)+bc(\cos\alpha)*\beta(2,3)]$.

[c]This atom was refined isotropically.

cm^{-1}). The uv spectrum supported the presence of an isolated double bond (MeCN, λ max 204 nm, ε max 5400). The eims (TMS derivative), 436 (9) $[M-C_6H_{11}O_2]^+$, 350 (18), 290 (17), 185 (25), 157 (27), 122 (82), 73 (100), and cims (TFA derivative) spectral data, 577 (8) $[M+1]^+$, 461 (5), 401 (100), 341 (6), 227 (4), are also consistent with the trichothecene nucleus fragmentations seen in **8**. The ^{1}H-nmr ($CDCl_3$, 300 MHz) and the ^{13}C-nmr ($CDCl_3$, 75 MHz) (Table 2) spectral data suggest that **9** is closely related to **8** and **7**. Differences between **9** and **7** are again found in the aliphatic region of the nmr spectra assigned to the ester side chain at C-8 [i.e., ^{13}C nmr **9** see Table 2; **7** C-1′ (172.0, s), C-2′ (43.4, t), C-3′ (25.7, d), C-4′ (22.4, q)]. Information about carbon multiplicities was obtained by DEPT. From the above data, it was clear that **9** contained an *n*-hexanoyl ester group (placed at C-8 by biosynthetic analogy with **8**). The absolute stereochemistry of **8** and **9** is assumed to be the same as all previously isolated trichothecenes (16).

MATERIALS AND METHODS

PHYSICAL ANALYSES.—Uv spectra were obtained on a Perkin-Elmer Lambda 3B uv/vis spectrophotometer. Ir spectra were obtained on a Fourier transform Nicolet 20DBX spectrometer. Samples were cast as a film on an NaCl plate and run neat. Mass spectra of the samples were obtained on a Finnigan INCOS 50 system quadrapole mass spectrometer interfaced with a Hewlett Packard 5840 gas chromatograph. Chromatographic separations were made on a 37 meter × 0.32 mm (i.d.) fused silica capillary column Ultra I (0.5 μm) using He as the carrier gas at 25 psi. Injections were on a 2 m × 0.53 mm guard column at 70°. The column oven was programmed from 70° to 170° at 25°/min, then ramped from 170° to 300° at 5°/min and held for 4 min. Mass spectrometer conditions: ion source temperature 175°, transfer line 300°, scan rate 50–700 amu/sec, ionizing voltage 70 eV. Ei (electron ionization) and pci (positive chemical ionization) used CH_4 with ion source at 120°. For the TMS derivative, each toxin was dissolved in MeCN, and a 10 to 50 μg aliquot of each toxin was evaporated to dryness with N_2 and then treated with 200 μl TMSi (*N*-trimethylsilylimidazole, Pierce Chemical Co.) in a reaction vial for 1 h at 65°. H_2O (1 ml) and isooctane (1 ml) were added and the mixture was vortexed and centrifuged. The isooctane layer was analyzed by gc-ms. For the TFA derivative, each toxin was dissolved in MeCN and a 10 to 50 μg aliquot of each toxin was evaporated to dryness with N_2 and reacted with 50 μl of TFAA (trifluoroacetic anhydride, Pierce Chemical Co.) in a reaction vial at 65° for 30 min. After excess reagent was evaporated with a stream of N_2, the residue was dissolved in 100 μl of toluene, and 1 μl was injected in the gc-ms. All nmr experiments were performed on a Nicolet NT-300 WB spectrometer equipped with 5 mm ^1H and ^{13}C probes operating at 300.06 and 75.45 MHz, respectively. All ^1H-nmr chemical shifts were referenced to internal TMS (0.0 ppm), and all ^{13}C-nmr chemical shifts were referenced against the deuterated solvent used ($CDCl_3 = \delta$ 77.0).

FUNGAL MATERIAL.—*F. sporotrichioides* was isolated from a sample of Ethiopian wheat harvested in 1987, and the identity confirmed by Dr. Oscar Calvert of the University of Missouri. The strain was destroyed as per USDA instructions; it was a wild strain.

CULTURE CONDITIONS.—*F. sporotrichioides* was grown on yeast malt agar plates for 14 days at 25°. Sterilized H_2O was added to the agar plates, and the conidia and spores were loosened with a sterilized wire loop and transferred into a larger volume of sterilized distilled H_2O (100 ml/agar plate). One quart Ball® canning jars (100) containing 100 g of Quaker Oats® white corn grits were autoclaved for 30 min. Aliquots (2 ml) of the mycelium/H_2O mixture and 33 ml of sterile H_2O were pipetted into each jar of corn grits. The jars were shaken and the lids loosened to allow for respiration. After 24 h of incubation at 10° in darkness, the jars were shaken again to ensure complete dispersal of the mycelium. The jars were incubated for a total of 28 days at 10° in darkness.

ISOLATION.—All solvents used for extraction and chromatography were ACS grade purchased from Fisher. All solvents used for hplc were glass-distilled solvents purchased from Burdick & Jackson. The Florisil (Fisher 60–100 mesh) was packed by pouring into a 5 × 20 cm gravity column [C_6H_6-*n*-C_6H_{14} (2:1)] to a depth of 10 cm. A 2-cm layer of anhydrous Na_2SO_4 (Fisher) was added on top of the Florisil. Rp flash cc was performed with J.T. Baker Octadecyl (C_{18}) reagent. Si gel tlc plates were 10 cm × 20 cm HFL uniplates®, 250 μm thick, purchased from Analtech. After development, compounds were identified by their quenching behavior at 254 nm or by spray visualization using a chromogenic reagent. The first *p*-anisaldehyde spray reagent for tlc analysis was prepared with MeOH–HOAc–H_2SO_4–*p*-anisaldehyde (85:15:5:0.5). The second blue spray reagent was administered in two parts (17). The tlc plate was first sprayed with 1% 4-(*p*-nitrobenzyl)pyridine dissolved in CCl_4-$CHCl_3$ (3:2) and oven-heated at 150° for 30 min. The plate was cooled and sprayed with 10% tetraethylenepentamine in CCl_4-$CHCl_3$ (3:2). The epoxide-containing trichothecenes give a blue color. Preparative hplc was conducted on a Perkin-Elmer Series 10 liquid chromatograph with a Perkin-Elmer LC-235 diode array detector operating at 195 nm using MeOH-H_2O (3:1) as the mobile phase. The column was a Microsorb 5 μm Module–C-18 semiprep column (10 mm i.d. × 25 cm), purchased from Rainin Instrument Co.

SINGLE-CRYSTAL X-RAY ANALYSIS OF 8-*n*-PENTANOYLNEOSOLANIOL [**8**].[2]—Crystal data for 8-*n*-pentanoylneosolaniol [**8**], $C_{24}H_{34}O_9$: colorless plate from ACN/H_2O, 0.15 × 0.25 × 0.30 mm, orthorhombic space group $P2_12_12_1$, a = 8.723 (10) Å, b = 18.820 (12) Å, c = 14.365 (7) Å; V = 2358 Å^3; and D_c = 1.314 g/cm^3 for Z = 4. Diffraction data: Enraf-Nonius CAD4 automated κ-axis diffractometer, graphite-monochromated Mo radiation [λ (Kα) = 0.71073 Å], θ–2θ mode, 2θ max = 48°, 2473 reflec-

[2]Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK.

tions [2445 unique, $R_i = 0.059$ for 1611 unique reflections with $I > 2.0\ \sigma(I)$; corrected for anomalous dispersion, but absorption ignored ($\mu = 0.9\ cm^{-1}$). Solution: direct methods (SHELXS-86) (18). Refinement: Full matrix least squares, SDP (Enraf-Nonius), anisotropic thermal parameters for non-H atoms; H atoms calculated and included as riding atoms; 3-OH located on difference Fourier and refined with fixed isotropic temperature factors.

MYCOTOXIN BIOASSAY PROCEDURE.—Baby hamster kidney (BHK-21) cells were obtained from the American Type Culture Collection. Cells were grown at 37° in 5% CO_2 as monolayers in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Company) containing 10% serum Plus (JRH Biosciences), 2 mM L-glutamine, and 10 mM HEPES. The trichothecenes were dissolved in MeOH at concentrations of 0.1–10 mg/ml, and 1:100 dilutions were made with culture medium. Serial 3-fold dilutions of the trichothecenes were made in 96-well microtiter plates containing nonconfluent BHK-21 cells (ca. 20,000 cells/well). Each serial dilution of trichothecenes was added to 4 microtiter wells. Each well contained a total of 200 µl DMEM. The MeOH concentration never exceeded 0.1% in the individual wells, which resulted in no significant toxic effects to the BHK-21 cells. The microtiter plates were incubated at 37° in 5% CO_2 for 24 h and the wells examined under the microscope for the absence of cells, shrinkage of cells, or large quantities of cellular debris compared to cells treated with 0.1% of MeOH. The LC_{100}, the concentration of toxin that caused cell death in 100% of the wells, was determined (19), and is summarized in Table 1.

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