Two New Modified Trichothecenes from Fusarium sporotrichioides

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TWO NEW MODIFIED TRICHOTHECENES FROM *FUSARIUM SPOROTRICHIOIDES*

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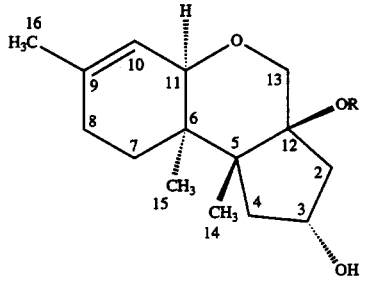
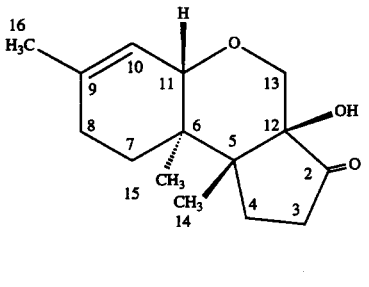
ABSTRACT.—Two new modified trichothecenes, 2-deoxy-11-*epi*-3α-hydroxysambucoin [**1**] and 2-deoxy-11-*epi*-12-acetyl-3α-hydroxysambucoin [**2**], were isolated from *Fusarium sporotrichioides* culture. This is the first report of modified trichothecenes where the two six-membered rings are cis-fused. Structures were elucidated using gc-ms, nmr, X-ray crystallography, and other spectroscopic techniques. Compounds **1** and **2** were screened for relative cytotoxicity in cultured baby hamster kidney (BHK-21) cells and found to be non-toxic.

The toxigenic fungus *Fusarium sporotrichioides* produces substantial amounts of T-2 toxin, HT-2, and neosolaniol and also produces a number of other trichothecenes in smaller amounts (1–9). Trichothecenes are responsible for alimentary toxic aleukia (ATA), weight loss, vomiting, skin inflammation, and death in humans as well as livestock (10). Previously, we reported a wild strain of *F. sporotrichioides* (isolated from 1987 Ethiopian wheat) to produce two new neosolaniol-derived trichothecene mycotoxins (11). From this same *Fusarium* strain we isolated and characterized two new modified trichothecenes, 2-deoxy-11-*epi*-3α-hydroxysambucoin [**1**] and 2-deoxy-11-*epi*-12-acetyl-3α-hydroxysambucoin [**2**]. This is the first report of modified trichothecenes where the two six-membered rings are cis-fused. Figure 1 shows the proposed biosynthesis of **1** and **2**.

F. sporotrichioides (wild strain isolated from Ethiopian wheat) was cultured (12) on ground corn grits contained in jars at 10° for 28 days, in three batches of 100 jars each. The fermented corn grits were blended with $CHCl_3$-Me_2CO (85:15) (300 ml/jar) and the mixture allowed to stand overnight. After filtration the remaining solid residue was blended with Me_2CO (300 ml/jar). The mixture was filtered and the solid autoclaved and



1 R=H
2 R=Ac



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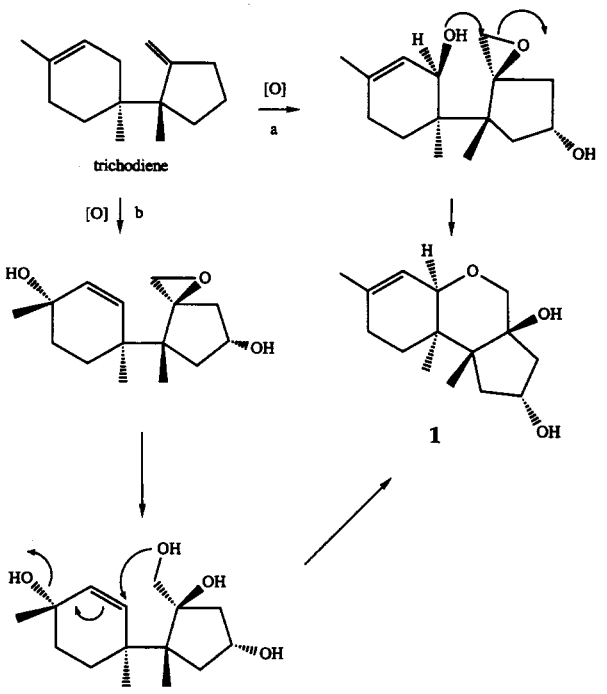


FIGURE 1. Proposed biosynthesis of the *cis*-fused modified trichothecene 2-deoxy-11-*epi*-3α-hydroxysambucoin [**1**].

discarded. The Me_2CO extract and the $CHCl_3$-Me_2CO (85:15) extract were combined and concentrated under reduced pressure, which yielded a dark green oil. The oil (ca. 1000 ml/300 jars) was dissolved in 2 liters C_6H_6–*n*-C_6H_{14} (2:1), and 50-ml aliquots were applied to Florisil columns. Columns were eluted successively with 400 ml C_6H_6–*n*-C_6H_{14} (2:1), 200 ml CH_2Cl_2, 300 ml $CHCl_3$-Me_2CO (9:1), and 250 ml Me_2CO. A total of 60 Florisil cc were performed, and similar fractions were combined and concentrated under reduced pressure. Tlc indicated that the C_6H_6–*n*-C_6H_{14} (750 ml) and CH_2Cl_2 (60 ml) fractions contained primarily corn oil and β-sitosterol. The $CHCl_3$-Me_2CO (150 ml) and Me_2CO (200 ml) fractions contained a mixture of trichothecenes. The residue from the $CHCl_3$/Me_2CO fraction was recrystallized from Me_2CO–*n*-C_6H_{14} (1:1) to yield a mixture of trichothecenes. Reversed-phase flash cc of the solid trichothecene mixture using MeOH-H_2O-HOAc (35:20:1) (14 ml×100 fractions) yielded, per 300 jars, neosolaniol (180 mg, fractions 7–10), NT-1 (48 mg, fractions 8–13), 8-acetylneosolaniol (222 mg, fractions 14–16), 8-propionylneosolaniol (66 mg, fractions 22–23), 8-isobutyrylneosolaniol (25 mg, fractions 29–37), 8-*n*-butyrylneosolaniol (225 mg, fractions 29–37), T-2 toxin (90 mg, fractions 54–65), 8-*n*-pentanoylneosolaniol (180 mg, fractions 69–74), and 8-*n*-hexanoylneosolaniol (45 mg, fractions 78–84). The Me_2CO fraction was dissolved in 500 ml C_6H_6-Me_2CO (2:1) and subjected to Florisil cc. Aliquots of 25 ml were applied to a Florisil column. The column was eluted successively with 200 ml C_6H_6-Me_2CO (2:1), 250 ml $CHCl_3$-Me_2CO (4:1), and 250 ml Me_2CO. A total of 28 Florisil cc were performed, and similar fractions were combined and concentrated under reduced pressure. The residue from the Me_2CO fraction was chromatographed using flash cc eluting with solvent mixtures of increasing polarity: 650 ml C_6H_6-Me_2CO (65:35), 400 ml C_6H_6-Me_2CO (57:63), 200 ml Me_2CO, 400 ml

Me_2CO-MeOH (1:1), and 50 ml MeOH. Fractions (14 ml) were collected and analyzed by tlc, and similar fractions were combined. The trichothecenes 15-acetyl-T-2 tetraol (1.5 mg, fractions 22–26) and 8-acetyl-T-2-tetraol (6 mg, fractions 32–35) were isolated and identified by gc-ms after conversion to their TMSi- and TFA-derivatives, and compared with authentic samples. The polar fractions of the Si gel cc were combined, concentrated at reduced pressure, and subjected to reversed-phase cc eluting with MeOH-H_2O-HOAc (35:20:1) and collecting 40×10-ml, 8×50-ml, and 1×350-ml fractions which were analyzed by tlc. Similar fractions were combined. The known trichothecenes, per 300 jars, acuminatin (20 mg, fractions 27–31), FS-1 (7 mg, fractions 22–26, fraction 31), scirpenol (6 mg, fractions 26–31), T-2 tetraol (5 mg, fractions 18–19), T-2 triol (3 mg, fractions 18–21), 15-acetylscirpenol (2 mg, fractions 35–39), DON (deoxynivalenol) (1 mg, fractions 12–15), 8α-hydroxytrichothecolone (1 mg, fractions 19–22), and verrucarol (1 mg, fractions 19–22) were isolated and identified by gc-ms of their TMSi- and TFA-derivatives, and compared with authentic samples. 2-Deoxy-11-*epi*-3α-hydroxysambucoin (70 mg, fractions 46–47) [**1**] and 2-deoxy-11-*epi*-12-acetyl-3α-hydroxysambucoin (20 mg, fraction 49) [**2**] were also isolated, and their structures were elucidated using gc-ms, nmr, ir, and X-ray crystallography. Compounds **1** and **2** were screened for relative cytotoxicity in cultured baby hamster kidney (BHK-21) cells and found to be non-toxic (LC_{100}=1000 ng/ml). The cytotoxicity of the known trichothecenes was reported previously (11).

2-Deoxy-11-*epi*-3α-hydroxysambucoin [**1**], $C_{15}H_{24}O_3$, was crystallized from MeOH/H_2O. The colorless crystals had a melting point of 177–178°, and its ir spectrum indicated the presence of hydroxyl group (film, 3412 cm^{-1}). The eims (TMSi derivative) (745 s) *m/z* (rel. int.) $[M]^+$ 396 (1), 378 (4), 258 (100), 108 (63), 73 (60); cims (TMSi derivative) (745 s) *m/z* (rel. int.) 397 (18) $[M+1]^+$, 381 (15), 307 (22), 291 (100), 263 (42), 217 (53), 199 (32), 73 (21); and cims (TFA derivative) (481 s) *m/z* (rel. int.) $[M+1]^+$ 445 (45), 331 (100), 217 (13), 115 (67), 93 (80) indicate the presence of two hydroxyl groups. The mol wt was determined to be 252. ^{1}H-nmr ($CDCl_3$, 400 MHz) and ^{13}C-nmr ($CDCl_3$, 100 MHz) spectral data (Table 1) suggest this compound is related to the modified trichothecene sambucoin [**3**]. Information about carbon multiplicities was obtained by DEPT. One- and long-bond heteronuclear correlations were determined with HMQC (Figure 2) and HMBC 2D nmr experiments. COSY (Figure 2) gave information on through-bond interactions between protons present in the molecule. Comparing these data with those reported for sambucoin [**3**] (13), **1** is not oxidized at C-2, but has a hydroxyl group at C-3. The coupling pattern of H-11 with H-10 suggests the configuration at C-11 is inverted with respect to the configuration of sambucoin [**3**]. NOe experiments were not conclusive regarding the orientation of the hydroxyl group at C-3. To confirm the orientation of the hydroxyl group at C-3 and the stereochemistry of C-11, a single crystal X-ray analysis was performed, and the resultant ORTEP drawing is shown in Figure 3. Positional parameters are reported in Table 2.

2-Deoxy-11-*epi*-12-acetyl-3α-hydroxysambucoin [**2**], $C_{17}H_{26}O_4$, was isolated as an oil. Its ir spectrum indicates the presence of hydroxyl and ester functionalities (film, 3408 and 1736 cm^{-1}). The cims (TFA derivative) (776 s) *m/z* (rel. int.) $[M+1]^+$ 391 (10), 331 (17), 301 (38), 277 (22), 217 (55), 111 (100) shows an *m/z* 60 (from 391 to 331), which suggests the presence of an acetyl group. Loss of *m/z* 114 (from 217 to 111) corresponds to the loss of trifluoroacetic acid, which confirms the presence of a hydroxyl group. This information suggests a mol wt of 294, which agrees with the mol wt expected for the acetyl derivative of **1**. ^{1}H-nmr (MeOD, 400 MHz) and ^{13}C-nmr (MeOD, 100 MHz) spectral data (Table 1) of **2** confirm that this compound is related to **1**. By comparison to the ^{13}C-nmr data from **1**, the acetyl group is placed at position 12, as the only observed

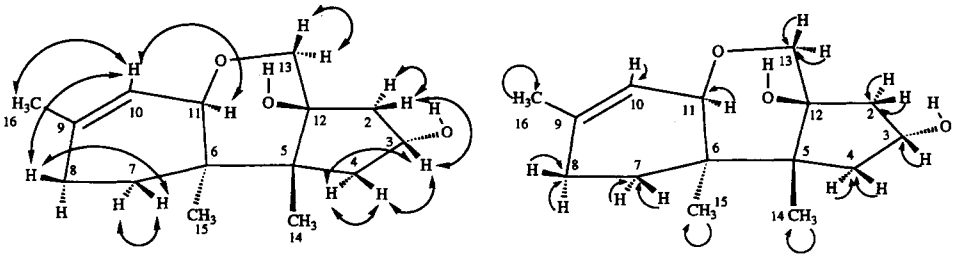
TABLE 1. Nmr Data on 2-Deoxy-11-*epi*-3α-hydroxysambucoin [**1**] (in $CDCl_3$) and 2-Deoxy-11-*epi*-12-acetyl-3α-hydroxysambucoin [**2**] (in MeOD). (^{1}H-nmr at 400 MHz and ^{13}C-nmr at 100 MHz, coupling constants in Hz).

Position	Compound			
	1		**2**	
	^{1}H	^{13}C	^{1}H	^{13}C
2_a	1.46 dd (15.0, 2.4)	44.1 t	1.40 dd (14.9, 2.0)	45.9 t
2_b	2.28 dd (15.0, 9.4)	—	2.32 dd (14.9, 8.4)	—
3	4.44 ddd (15.6, 7.8, 2.4)	67.0 d	4.4 m	67.5 d
4_a	1.85 m	44.2 t	1.91 m	44.8 t
4_b	2.02 m	—	2.12 m	—
5	—	48.6 s	—	obscured by solvent
6	—	35.0 s	—	35.2 s
7_a	1.53 dd (13.3, 2.0)	24.3 t	1.68 dd (5.5, 2.0)	24.5 t
7_b	1.91 dd (8.0, 2.0)	—	1.95 dd (9.0, 2.0)	—
8	1.95–2.0 m	28.2 t	1.95–2.0 m	29.2 t
9	—	139.5 s	—	140.4 s
10	5.52 dd (5.6, 0.8)	119.5 d	5.45 dd (6.0, 1.0)	120.8 d
11	3.69 bs	75.1 d	3.6 bs	75.9 d
12	—	77.6 s	—	86.9 s
13_a	3.66 d (12.6)	74.0 t	3.58 d (15.1)	74.5 t
13_b	3.95 d (12.6)	—	3.75 d (15.1)	—
14	0.97 s	16.6 q	0.96 s	16.8 q
15	0.71 s	16.1 q	0.73 s	16.3 q
16	1.70 s	23.0 q	1.68 s	22.3 q
CO*Me*			2.05 s	22.4 q
*C*OMe				not observed

significant chemical shift difference occurs at C-12: δ 77.6 (s) in **1** vs. 86.9 (s) in **2** (R_3C-OH vs. R_3C-OAc).

EXPERIMENTAL

PHYSICAL ANALYSES.—Ir spectra were obtained on a Fourier transform Nicolet 20 DBX spectrometer. Samples were cast as a film on a NaCl plate, air dried, placed in a desiccator which was pumped for 3–5 h under vacuum, and run neat. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Mass spectra were obtained on a Finningan INCOS 50 system quadrapole mass spectrometer interfaced with a Hewlett Packard 5840 gas chromatograph. Chromatographic separations were made on a 37 mm×0.32 mm (i.d.) fused silica capillary column Ultra I (0.5 μm). Helium was used as the carrier gas at 25 psi. 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°,



Couplings One-bond carbon-hydrogen correlations (HMQC)

FIGURE 2. Couplings observed by COSY and one-bond carbon-hydrogen correlations (HMQC) on 2-deoxy-11-*epi*-3α-hydroxysambucoin [**1**].

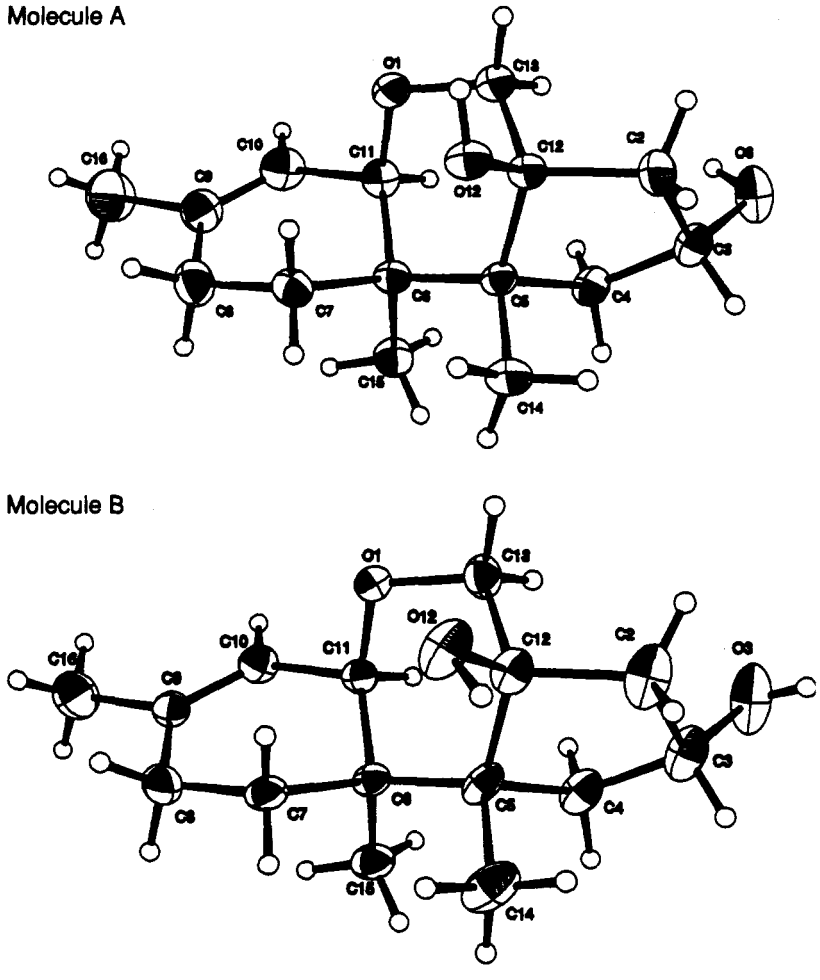


FIGURE 3. ORTEP drawing of the two independent molecules of 2-deoxy-11-*epi*-3α-hydroxysambucoin [**1**].

transfer line 300°, scan rate 50–700 amu/sec, ionizing voltage 70 eV. Eims and PCI (positive chemical ionization) used methane with ion source at 120°. TMSi derivative: The sample was dissolved in MeCN, and a 10 to 50 μg aliquot was evaporated to dryness with N_2 and then treated with 200 μl *N*-trimethylsilylimidazole, Pierce Chemical Co. in a reaction vial for 1 h at 65°. H_2O (1 ml) and 1 ml isooctane were added, vortexed, and centrifuged. The isooctane layer was analyzed by gc-ms. TFA derivative: The sample was dissolved in MeCN, and a 10 to 50 μg aliquot was evaporated to dryness with N_2 and reacted with 50 μl of 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 toluene and 1 μl was injected in the gc-ms. All nmr experiments were performed on a Varian Unity 400 spectrometer equipped with 5 mm ^1H and ^{13}C probes operating at 399.9 and 100.6 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 ($CDCl_3$=77.0 ppm and MeOD=40.0 ppm). Single crystal X-ray analysis was performed on an Enraf-Nonius CAD4 automated κ axis diffractometer.

FUNGAL CULTURES.—*F. sporotrichioides* was isolated from a sample of Ethiopian wheat harvested in 1987, and the identity was 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®

TABLE 2. Positional and Thermal Parameters of 2-Deoxy-11-*epi*-3α-hydroxysambucoin [**1**].[a]

Atom	*x*	*y*	*z*	B(Å^2)[b]
O-1A	0.2391 (4)	0.2744 (3)	0.80790 (12)	3.17 (12)
C-13A	0.2564 (5)	0.3141 (4)	0.74340 (19)	3.02 (19)
C-12A	0.1338 (6)	0.4279 (4)	0.72941 (17)	2.50 (17)
O-12A	−0.0991 (4)	0.40600	0.72971 (13)	2.90 (12)
C-5A	0.1970 (6)	0.5266 (4)	0.77978 (18)	2.57 (17)
C-14A	0.0348 (6)	0.6320 (4)	0.77367 (19)	3.46 (20)
C-6A	0.2167 (5)	0.4799 (4)	0.85061 (17)	2.75 (17)
C-15A	0.3466 (6)	0.5698 (5)	0.89230 (18)	3.86 (20)
C-7A	−0.0088 (6)	0.4572 (4)	0.87981 (18)	3.34 (18)
C-8A	0.0071 (7)	0.4084 (5)	0.94862 (21)	4.48 (23)
C-9A	0.1840 (8)	0.3192 (5)	0.96015 (21)	4.51 (24)
C-16A	0.1825 (10)	0.2536 (6)	1.02424 (23)	7.4 (3)
C-10A	0.3336 (7)	0.2992 (5)	0.91737 (22)	4.38 (22)
C-11A	0.3380 (6)	0.3588 (4)	0.85241 (20)	3.35 (19)
C-2A	0.2065 (6)	0.4788 (5)	0.66421 (17)	3.36 (19)
C-3A	0.3969 (6)	0.5652 (5)	0.67915 (18)	3.26 (18)
O-3A	0.5950 (5)	0.5327 (4)	0.64962 (13)	4.76 (17)
C-4A	0.4176 (6)	0.5700 (4)	0.75295 (18)	3.18 (17)
O-1B	0.7625 (4)	0.1962 (3)	0.67254 (12)	3.01 (12)
C-13B	0.7249 (6)	0.1994 (5)	0.60459 (19)	3.45 (20)
C-12B	0.8460 (6)	0.1015 (5)	0.57036 (18)	3.17 (19)
O-12B	1.0725 (4)	0.1343 (4)	0.57511 (14)	4.28 (15)
C-5B	0.7931 (6)	−0.0239 (5)	0.60039 (18)	3.14 (18)
C-14B	0.9492 (7)	−0.1202 (5)	0.57416 (23)	4.91 (22)
C-6B	0.7933 (5)	−0.0241 (4)	0.67570 (18)	2.68 (17)
C-15B	0.6684 (6)	−0.1339 (4)	0.70094 (22)	3.95 (21)
C-7B	1.0279 (6)	−0.0223 (4)	0.70481 (20)	3.55 (19)
C-8B	1.0379 (7)	−0.0159 (5)	0.77817 (22)	4.32 (22)
C-9B	0.8630 (7)	0.0587 (4)	0.80748 (19)	3.54 (20)
C-16B	0.8841 (8)	0.0752 (6)	0.87870 (22)	5.9 (3)
C-10B	0.6986 (6)	0.1038 (5)	0.77227 (19)	3.42 (19)
C-11B	0.6787 (6)	0.0880 (4)	0.70064 (18)	2.67 (18)
C-2B	0.7583 (7)	0.0920 (6)	0.50002 (21)	4.81 (23)
C-3B	0.5732 (6)	−0.0013 (5)	0.50067 (20)	4.23 (23)
O-3B	0.3651 (5)	0.0478 (5)	0.48251 (14)	5.87 (20)
C-4B	0.5654 (6)	−0.0494 (5)	0.56931 (20)	3.70 (20)
HO-12A	0.119 (7)	0.341 (4)	0.7165 (20)	4.7[c]
HO-3A	0.660 (7)	0.491 (4)	0.6735 (21)	4.7[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]These atoms were refined isotropically.

canning jars (300) containing 100 g of Quaker Oats® white corn grits were autoclaved for 30 min. Aliquots (2 ml) of the mycelium/H_2O mixture were pipetted into each jar of corn grits, and 33 ml of sterile H_2O was added. 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 in the dark for a total of 28 days at 10°.

ISOLATION.—All solvents used for extraction and Florisil, tlc, and flash chromatography were ACS grade purchased from Fisher. All solvents used for hplc were glass-distilled solvents purchased from Burdick & Jackson. Florisil, 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 was added on top of Florisil. Reversed-phase flash column chromatography was performed with J.T. Baker Octadecyl (C_{18}) (40 μm). Normal phase flash cc used EM® reagent 40–63 micron Kieselgel 60 Si gel. Normal phase tlc plates were Si gel HFL uniplates®, 250 μm thick, purchased from Analtech; reversed-phase tlc used Whatman $KC_{18}F$® plates,

0.20 mm thick. After development, compounds were identified by their quenching behavior at 254 nm or by spray visualization using a chromogenic spray reagent. *p*-Anisaldehyde spray reagent for tlc analysis was prepared with MeOH–HOAc–H_2SO_4–*p*-anisaldehyde (Eastman Organic Chemicals) (85:15:5:0.5). The blue spray reagent was administered in two parts (14). The tlc plate was sprayed with 1% 4-(*p*-nitrobenzyl)pyridine (Aldrich) in CCl_4-$CHCl_3$ (3:2) and oven-heated at 150° for 30 min. The plate was cooled and sprayed with 10% tetraethylenepentamine (Aldrich) in CCl_4-$CHCl_3$ (3:2). 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 semipreparative column (10 mm i.d.×25 cm long), purchased from Rainin Instrument Co.

SINGLE CRYSTAL X-RAY ANALYSIS OF 2-DEOXY-11-*epi*-3α-HYDROXYSAMBUCOIN [**1**].[2]—Crystal data for **1**, $C_{15}H_{24}O_3$: colorless plate from MeOH/H_2O; 0.06×0.30×0.35 mm; monoclinic space group $P2_1$, a=6.0990 (20) Å, b=11.1460 (20) Å, c=20.704 (8) Å, β=91.32 (3); V=1407.1 (8) Å^3, and D_{calcd}=1.191 g/cm^3 for Z=4. Diffraction data: graphite monochromated Mo radiation [λ (Kα)=0.71073 Å], θ–2θ mode, 2θ max=45.9°, 3014 reflexions (2588 unique, R_i=0.036 for 2170 unique reflexions with I>2.0 σ (I)); no correction was made for absorption. Solution: direct methods (SHELXS-86) (15). Refinement: full matrix least squares, SDP (Enraf-Nonius), anisotropic thermal parameters for non-H atoms; H atoms calculated and included as riding atoms. HO3 and HO12 located on difference Fourier and refined with fixed isotropic temperature factors.

CYTOTOXICITY BIOASSAY.—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. 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. MeOH concentration never exceded 0.1% in the individual wells, which resulted in no significant toxic effects to the BHK-21 cells. 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 wth 0.1% MeOH. The LC_{100}, the concentration of toxin that caused cell death in 100% of the wells, was determined (16).

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[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, 12 Union Road, Cambridge CB2 1EZ, UK.

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