# Some Minor Secondary Metabolites of *Fusarium sporotrichioides* DAOM 165006†

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More secondary metabolites were isolated in trace amounts from large-scale fermentations of *Fusarium sporotrichioides* DAOM 165006. They included two trichothecenes, 4-deacetoxy T-2 toxin and 4,8-isoneosolaniol, and five new 11-epiapotrichothecenes. 4-Deacetoxy T-2 toxin is unique in that trichothecenes from this species usually have an oxygen function at C-4. All the 11-epiapotrichothecene compounds isolated had an acetoxy moiety at C-2, and in addition some had oxygen functions at C-8 and C-15. The structures are interpreted in terms of the activity of the hydroxylases present in this fungal species. The absolute configuration of 13-hydroxy-3α,11-epoxyapotrichothec-9-ene was also determined by X-ray crystallographic analysis.

## INTRODUCTION

Secondary metabolites of the genus *Fusarium* are of interest mainly because of their toxigenic potential and occurrence in the diet of both animals and humans. In Ontario, *Fusarium sporotrichioides*, *F. avenaceum*, *F. moniliforme*, *F. graminearum*, *F. equiseti*, and *F. poae* were the most common species associated with infestation of winter wheat seeds in 1984 (Duthie et al., 1986). *F. sporotrichioides* is a problem particularly with cereals that have been overwintered in the field.

*Fusarium* species are known to produce a wide variety of mycotoxins including enniatins, fusarins, fumonisins, trichothecenes, and zearalenones, as well as individual compounds such as butenolide and moniliformin (Marasas et al., 1984). Each species appears to produce a characteristic mixture of toxins. In contrast, the trichothecene-related compounds such as the 11-epiapotrichothecenes together with sambucinol and sambucoin are thought to be produced by all *Fusarium* species (Greenhalgh et al., 1989b).

A chemotype of *F. sporotrichioides* produces T-2 toxin, HT-2, and neosolaniol (NEO). Characterization of the secondary metabolites produced by isolates of this species from various countries, e.g., Brazil (Mirocha et al., 1989), Canada (Greenhalgh et al., 1988), Hungary (Corley et al., 1986a,b, 1987), Japan (Ishii and Ueno, 1981), and Poland (Visconti et al., 1985), shows this chemotype to be common.

In addition to these major trichothecene metabolites, *F. sporotrichioides* DAOM 165006 produced a number of minor secondary metabolites, some of which have been reported previously (Greenhalgh et al., 1988). This paper describes the isolation and characterization of several new minor metabolites obtained from a large-scale liquid culture of this isolate, as well as configuration of the structure of 13-hydroxy-3α,11-epoxyapotrichothec-9-ene by X-ray crystallography.

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## MATERIALS AND METHODS

**Gas Chromatography/Mass Spectrometry (GC/MS).** Mass spectra were obtained on a Finnigan GC/MS system, Model 4500, with a DS 400 data system. Underivatized crude fungal extracts were injected on-column and chromatographed on a DB-5 fused silica capillary column (20 m × 0.32 mm i.d.; 0.25-μm film). The column was temperature programmed from 140 to 260 °C at 15 °C $min^{-1}$ with helium as the carrier gas at 10 psi.

**Nuclear Magnetic Resonance (NMR).** $^1H$ and $^{13}C$ NMR spectra were run on Bruker AM250 and AM500 NMR spectrometers. Chemical shifts were referenced to deuterochloroform at 7.24 and 77.0 ppm for $^1H$ and $^{13}C$, respectively, and reported relative to tetramethylsilane ($Me_4Si$). $^{13}C$ chemical shift assignments were made by using DEPT pulse sequence and $^1H/^{13}C$ heteronuclear correlation spectra (HETCOR) and by comparison with known compounds (Greenhalgh et al., 1989a). Confirmation of $^1H$ chemical shifts assignments was made by the use of $^1H/^1H$ homonuclear correlation spectra (COSY). The $^1H$ and $^{13}C$ NMR data for the trichothecene-related compounds are given in Tables I and II, respectively.

**X-ray Crystallography.** 3α,11-Epoxyapotrichothec-9-ene (VIII) crystallized as a dimer; a single orthorhombic crystal was analyzed by using an automated Picker four-circle diffractometer. Accurate cell parameters and intensity data were obtained at room temperature by using graphite-monochromatized Cu Kα radiation. The data were corrected for the Lorentz effect and measured for direct-beam polarization (Le Page et al., 1979) but not for absorption. The cell parameters were obtained by least-squares refinement of the setting angles of 64 reflections ($100 < 2\theta < 120$) [$L(Cu\ K\alpha_1) = 1.54056 Å$]. Other details are given in Table III.

The structure of VIII was solved by direct methods using MULTAN (Germain et al., 1971). The determination of absolute configuration was performed in two ways. First, the intensities of selected pairs of Friedel reflections and their symmetry-related reflections were measured, which showed the highest difference between calculated (+) and (−) structure factors. The intensity measurement data were in good agreement with the configuration used in the least-squares refinement. The chirality parameter ε was also included as a refinable in the least-squares cycles. Theoretical values of +1 and −1 would correspond to the correct and opposite hand conformations. The ε parameter was refined and converged to +0.8, thus assigning the absolute configuration of VIII. All computations were performed with the NRCVAX system of programs (Gabe et al., 1985). Neutral-atom scattering curves were from standard sources (Birmingham, 1974).

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**Table I. 250-MHz $^1$H NMR Chemical Shifts and Coupling Constants for Trichothecene-Related Compounds**

| proton | I | III | IV | VI | VIII | IX |
|---|---|---|---|---|---|---|
| 2α | | | | | 2.13; 11.5 (AB) | |
| 2β | 5.18 m | 5.07; 12.1 (2β, 3α), 6.3 (2β, 3β) | 5.0; 12.0 (2β, 3α), 6.1 (2β, 3β), 3.8 (2β, 3) | 3.53; 2.1 (2, 3) | 1.44; 2.0 (2α, 4β) | 5.16 m |
| 3α | 1.95 m | 1.85 m | 1.96 m | | | 1.95 m |
| 3β | 2.20 m | 2.07 m | 2.10 m | 4.16; 2.1 (3β, 2β), 2.1 (3β, 4α), 1.0 (3β, 4β) | 4.51; 1.8 (3, 2α), 3.4 (3, 4α), 2.0 (3, 4β) | 2.05 m |
| 4α | 2.20 m | 2.17; 13.1 (AB) | 2.12; 10.3 (4α, 3α) | 2.33; 13.3 (AB) | 2.46; 13.5 (AB) | 2.24; 13.3 (AB) |
| 4β | 1.50 m | 1.24; 6.8 (4α, 3β), 6.8 (4β, 3β) | 1.22; 7.6 (4α, 3β), 11.1 (AB) | 1.07; 2.1 (4α, 3), 1.0 (4β, 3) | 1.16; 2.0 (4α, 2α), 3.4 (4α, 3β), 6.8 (4β, 3β) | 1.22; 8.2 (4α, 3α), 5.3 (4β, 3α), 7.7 (4β, 3β) |
| 7α | 1.17 m | 1.63; 14.2 (AB) | 1.60; 14.8 (AB) | 1.90; 13.2 (AB) | 1.34; 12.9 (AB) | 1.63; 14.1 (AB) |
| 7β | 2.90 m | 1.94; 7.5 (7β, 8β) | 2.10 m | 1.38; 6.0 (7β, 8), 0.8 (7β, 15) | 1.86; 1.6 (7α, 8α), 6.6 (7α, 8β), 5.6 (7β, 8α), 4.4 (7β, 8β), 0.8 (7β, 15) | 1.91 m |
| 8α | 1.80 m | | | 2.02 m | 1.80; 7.2 (AB) | |
| 8β | 2.20 m | 4.08; 7.5 (8β, 7β) | 5.21; 7.3 (8β, 7β) | 2.10 m | 2.03 | 4.10 m |
| 10 | 5.53 (br s) | 5.68; 1.1 (10, 16) | 5.82; 1.5 (10, 15) | 5.39; 1.4 (10, 16) | 5.35; 1.6 (10, 16) | 5.66; 1.5 (10,16), 3.3 (10, 8) |
| 11 | 4.29 | 4.05 (br s) | 4.10 (br s) | | | 4.09 (br s) |
| 13 | 3.48, 3.79; 12.2 (AB) | 3.82, 4.39; 11.8 (AB) | 3.98, 4.28; 11.8 (AB) | 3.74, 3.86; 12.2 (AB) | 3.66, 3.79; 12.1 (AB) | 3.31, 3.76; 11.6 (AB) |
| 15 | 3.97, 4.15; 11.8 (AB) | 1.06 (s) | 1.03 (s) | 0.91; 0.8 (15, 7β) | 0.90; 0.8 (15, 7β) | 1.08; 0.7 (15, 7β) |
| 16 | 1.63 (br s) | 1.77 (br s) | 1.67; 1.2 (16, 8β) | 1.77; 1.4 (16, 10) | 1.67; 1.6 (16, 10) | 1.77; 1.6 (16, 10) |

**Table II. 62.8-MHz $^{13}$C NMR Chemical Shift Assignments of Trichothecene-Related Compounds[a]**

| | I | III | IV | VI | VIII | IX |
|---|---|---|---|---|---|---|
| C-2 | 76.0 | 72.1 | 75.7 | 73.5 | 42.2 | 75.7 |
| C-3 | 29.3 | 31.7 | 31.7 | 78.7 | 76.3 | 31.8 |
| C-4 | 32.1 | 32.6 | 32.6 | 34.8 | 42.7 | 32.6 |
| C-5 | 58.4 | 55.3 | 55.7 | 49.9 | 50.0 | 55.7 |
| C-6 | 45.4 | 43.5 | 43.8 | 46.5 | 47.1 | 43.9 |
| C-7 | 28.1 | 38.5 | 36.1 | 28.2 | 28.3 | 38.5 |
| C-8 | 27.8 | 68.8 | 70.1 | 28.0 | 28.2 | 68.8 |
| C-9 | 135.3 | 136.2 | 133.3 | 142.2 | 141.7 | 136.1 |
| C-10 | 120.8 | 123.1 | 125.5 | 118.6 | 119.5 | 122.7 |
| C-11 | 82.6 | 82.4 | 81.9 | 105.6 | 105.5 | 82.3 |
| C-12 | 95.0 | 95.0 | 92.7 | 86.5 | 88.7 | 92.4 |
| C-13 | 62.4 | 62.8 | 64.3 | 62.5 | 65.3 | 64.4 |
| C-14 | 15.0 | 19.4 | 19.7 | 14.4 | 14.7 | 19.9 |
| C-15 | 65.5 | 16.7 | 15.9 | 14.1 | 13.9 | 16.5 |
| C-16 | 22.3 | 19.0 | 19.2 | 23.0 | 22.9 | 19.5 |
| C=O | 170.4[b] | 170.4[b] | 171.1[b] | | | 171.1 |
| $CH_3CO$ | 20.9 | 21.0 | 21.0 | | | 21.0 |
| | 21.0 | 21.1 | 21.1 | | | |

[a] I, 2,15-diacetoxy-13-hydroxy-11-epiapotrichothec-9-ene; III, 2,13-diacetoxy-8-hydroxy-11-epiapotrichothec-9-ene; IV, 2,8,13-triacetoxy-11-epiapotrichothec-9-ene; VI, 2,13-dihydroxy-3,11-epoxyapotrichothec-9-ene; VIII, 13-hydroxy-3,11-epoxyapotrichothec-9-ene; IX, 2-acetoxy-8,13-dihydroxy-11-epiapotrichothec-9-ene. [b] Two carbonyl carbon resonances.

**Table III. X-ray Crystallographic Data for 13-Hydroxy-3,11-epoxyapotrichothec-9-ene**

| | |
|---|---|
| formula | $C_{15}H_{22}O_3$ |
| fw | 250.3 |
| cryst syst | orthorhombic |
| space group | $P2_12_12_1$ |
| $a$, Å | 6.9175 (8) |
| $b$, Å | 17.326 (2) |
| $c$, Å | 23.047 (3) |
| $V$, Å$^3$ | 2762.2 |
| $Z$ | 8 |
| $p_{calc}$, g/cm$^3$ | 1.204 |
| crystal size, mm | 0.1 × 0.15 × 0.40 |
| radiation | Cu Kα (1.54056 Å) |
| octant measd | $hkl$ |
| max 2θ, deg | 120 |
| no. of unique reflns | 2927 |
| no. of observed reflns $[I_{net}/*(I_{net}) > 2.0]$ | 3400 |
| av std fluctuations, % | |
| abs coeff, mm$^{-1}$ | 0.62 |
| $R_f$ | 0.063 |
| $R_w$ | 0.025 |
| $S$ (Gof) | 1.8 |
| sec ext coeff, ×10$^{-6}$ m | 0.43 |

The space group and cell dimensions of VIII were $P2_12_12_1$ and $a$ = 6.9175(8), $b$ = 17.326(2), and $c$ = 23.047(3) Å, respectively. Further details of the crystallographic data, such as final atomic coordinates, bond lengths and angles, thermal parameters of atoms, and observed and calculated structure factors, are deposited as supplementary material.

**High-Pressure Liquid Chromatography (HPLC).** Preparative HPLC was conducted by using a Varian Vista 5500 series chromatograph equipped with a UV 200 detector operating at 205 nm. A 5-μm CN column (0.3 × 25 cm) CSC-S was used with gradient elution from 1% to 12% 2-propanol/hexane at a flow rate of 4 mL min$^{-1}$.

**Chemicals.** Merck Lichroprep Si-60 silica gel (25–40 μm) was used for preparative columns and Merck TLC grade silica gel 60 for vacuum liquid chromatography (VLC). All solvents used were HPLC grade glass distilled purchased from Caledon.

**Standards.** T-2 toxin, HT-2, DAS, NEO, 3′-hydroxy T-2, 3α,13- and 3β,13-dihydroxy-11-epiapotrichothec-9-ene (3α-APO and 3β-APO, respectively), 13-hydroxy-3α,11-epoxyapotrichothec-9-ene, sambucinol (SOL) and sambucoin (SAM) have been isolated previously (Greenhalgh et al., 1988, 1989a). The butyryl (BuNEO), isobutyryl (iBuNEO), and propionyl (PrNEO) analogues of NEO have also been isolated and synthesized (Savard and Greenhalgh, 1987).

**Fermentation.** The large-scale fermentation (250 L) of *F. sporotrichioides* DAOM 165006 in liquid culture was carried out at the Atlantic Research Laboratories, NRC, Halifax. Moss medium was used for the production fermentation, sucrose being replaced by table sugar. The fermentation conditions determined to be optimal for T-2 production were as follows: temperature, 27 °C; stirring rate, 285 rpm; air flow, 1.6 L min$^{-1}$ (Grenhalgh et al., 1988). The pH of the inoculum initially was 5.4 but rapidly dropped to pH 4, where it was maintained. After 7 days, the aqueous broth was adjusted to pH 8.3 and filtered through Celite (300 g) and Hyflo (3 kg). The mycelium was washed, and the combined filtrates (140 L) were treated with NaCl (10 kg) and then extracted with methylene chloride (3 × 30 L). The combined extracts were concentrated to ca. 2.5 L in a cyclone evaporator (5 °C, 100 Torr) and taken to dryness in vacuo, giving a viscous syrup.

**Extraction and Purification.** The crude fungal extract (117 g) was triturated with $CH_2Cl_2$ (500 mL), and the insoluble inorganic salts (3.8 g) were removed before the solution was concentrated. An aliquot containing about 20 g was applied to a column (15 cm × 1 m) containing Lichroprep Si-60 silica gel (1.5 kg), which was eluted with $CH_2Cl_2$ (2 L) followed by 1.5% MeOH/$CH_2Cl_2$ (2 L), 2.0% MeOH/$CH_2Cl_2$ (2 L), 3.0% MeOH/$CH_2Cl_2$ (2 L), 4.0% MeOH/$CH_2Cl_2$ (2 L), 4.5% MeOH/$CH_2Cl_2$ (4 L), 8.0% MeOH/$CH_2Cl_2$ (4 L), 10.0% MeOH/$CH_2Cl_2$ (2 L), 12.0% MeOH/$CH_2Cl_2$ (2 L), 15.0% MeOH/$CH_2Cl_2$ (2 L), 20.0% MeOH/$CH_2Cl_2$ (2 L), and finally MeOH (2 L). Some 155 fractions were collected, and each was analyzed by GC/MS by comparison with the PRC Mycotoxin MS data bank. Appropriate fractions were combined to give 24 fractions labeled F1–24.

*Fraction F1* (554 mg) contained mainly phthalates and some T-2 toxin and was not processed further.

*Fraction F3* (5.22 g) contained only T-2 toxin, which was recrystallized from isopropyl ether/hexane, mp 148–149 °C.

*Fraction F5* (2.72 g) was an orange oil, which contained predominantly T-2 with trace amounts of BuNEO, PrNEO, DAS, and 3′OH T-2. The oil was subjected to VLC on silica gel, yielding 12 fractions. (a) Fraction 5-5 (112 mg) on HPLC cleanup (5% 2-propanol/hexane) gave a pale oil, I (3 mg): GC Rt 8.42 min; MS calcd for $C_{19}H_{28}O_6$ 352.189, found 352.187; *m/z* 43 (base), 55 (15), 69 (11), 81 (14), 95 (15), 107 (27), 124 (44), 155 (9), 219 (21), 337 (12), 352 ($M^+$). The $^1H$ and $^{13}C$ NMR spectra (Tables I and II) were consistent with the structure 2α,15-diacetoxy-13-hydroxy-11-epiapotrichothec-9-ene. (b) Fraction 5-10 (35 mg) was purified by HPLC (6% 2-propanol/hexane) to afford a colorless oil, II (4 mg): GC Rt 12.01 min; MS calcd for $C_{22}H_{32}O_7$ 408.215, found 408.209; MS CI *m/z* 43 (43), 199 (31), 247 (base), 307 (59), 409 (23), 408 ($M^+$); $^1H$ NMR δ 0.74 (3 H, H-14), 0.89, 0.91 (6 H, H-4′,5′), 1.69 (3 H, H-16), 1.90–2.30 (2 H, H-7, *J* mult), 2.05–2.25 (2 H, H-4, *J* mult), 2.00–2.20 (2 H, H-3′, *J* mult), 2.21 (2 H, H-2′), 2.81, 3.02 (2 H, H-13, $J_{AB}$ = 3.9 Hz), 3.43 (1 H, H-2), 3.91, 4.15 (2 H, H-15, $J_{AB}$ = 12.2), 4.24 (1 H, H-11, $J_{11,10}$ = 5.8 Hz), 4.34 (1 H, H-3, $J_{3,2}$ = 4.7 Hz), 5.70 (1 H, H-8, $J_{8,7}$ = 5.7 Hz), 5.73 (1 H, H-10, $J_{10,11}$ = 5.8 Hz); $^{13}C$ NMR δ 12.0 (C-14), 20.2 (C-16), 22.3 (C-4′,5′), 25.6 (C-3′), 27.1 (C-7), 41.7 (C-6), 42.0 (C-4), 43.4 (C-2′), 45.5 (C-5), 48.4 (C-13), 64.6 (C-15), 65.3 (C-12), 67.6 (C-11), 68.5 (C-8), 68.9 (C-3), 79.6 (C-2), 124.3 (C-10), 136.0 (C-9), 172.8 (C-1′). II was identified as 15-acetoxy-3-hydroxy-8-isovaleryl-12,13-epoxytrichothecen-9-ene (4-deacetoxy T-2).

*Fraction F14* (3.28 g) was a mixture of crystals and oil and contained NEO and a number of unknowns. The fraction F-14 was rechromatographed on silica gel. (a) Fraction 14-2 (170 mg) on further purification by preparative HPLC afforded a clear colorless oil, III (66 mg): GC Rt 9.09 min; MS calcd for $C_{19}H_{28}O_6$ 352.189, found 352.188; *m/z* 43 (base), 55 (48), 69 (27), 81 (25), 93 (52), 109 (49), 123 (61), 140 (48), 149 (28), 308 (27), 352 ($M^+$); $^1H$ and $^{13}C$ NMR (Tables I and II). On acetylation, III gave triacetyl derivative IV: calcd for $C_{21}H_{30}O_7$ 394.199, found 394.121; MS *m/z* 43 (base), 93 (38), 109 (29), 122 (48), 121 (47), 133 (24), 215 (19), 233 (24), 355 (18), 394 ($M^+$). The triacetyl derivative IV was characterized as 2,8,13-triacetyl-11-epiapotrichothec-9-ene from its NMR and MS spectra. This corroborates the structure, 2,13-diacetoxy-8-hydroxy-11-epiapotrichothec-9-ene assigned to III. (b) Fraction 14-3 was further purified by HPLC (6% 2-propanol/hexane) to give a crystalline product, V (3 mg): mp 104–105 °C; GC Rt 5.38 min; MS *m/z* 350. This compound had NMR and MS spectra identical with those of synthetic diacetylsambucinol (mp 105–106 °C). (c) Fraction 14-4 cleanup by HPLC (6% 2-propanol/hexane) gave an oil, VI (9 mg): GC Rt 7.47 min; MS *m/z* 55 (22), 82 (24), 109 (49), 124 (base), 161 (27), 251 (9), 266 (4, $M^+$). From the NMR data VI was assigned the structure 2,13-dihydroxy-3,11-epoxyapotrichothec-9-ene.

*Fraction F15* (1.69 g) GC/MS analysis revealed that the fraction contained SOL, NEO, and HT-2 together with an unknown. VLC on silica gel with 1.5–10% methanol in chloroform resulted in 24 fractions. Fraction 15-14 (352 mg) on HPLC (6% 2-propanol/hexane) gave an oil, VII (4 mg): GC Rt 8.23 min; MS *m/z* 43 (base), 232 (30), 107 (17), 91 (15), 121 (14), 55 (12), 145 (12), 173 (12), 185 (11), 382 ($M^+$). VII was identified as 4,8-diacetylisoneosolaniol (4,8-diacetyl T-2 tetraol).

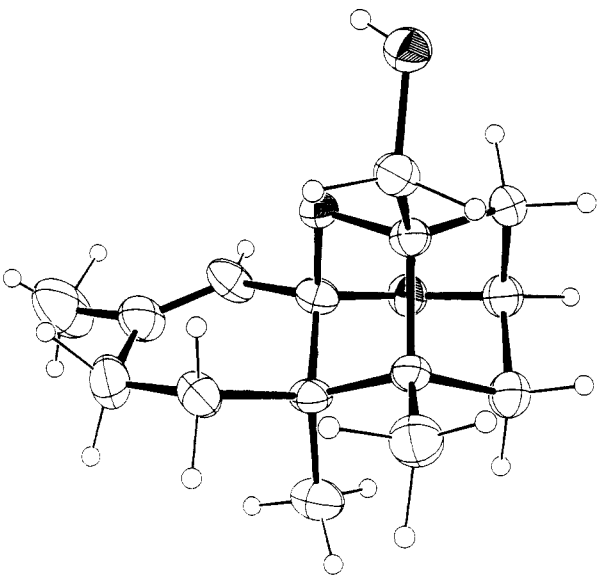


**Figure 1.** ORTEP II drawing of 13-hydroxy-3α,11-epoxyapotrichothecene.

*Fraction F16* (3.34 g) was a mixture of crystals and yellow oil. The crystals (mp 234–235 °C) were identified as SOL (190 mg) by GC/MS. The yellow oil was chromatographed on silica gel to yield eight fractions which were further purified by preparative HPLC (6% 2-propanol/hexane). (a) Fraction 16-2 (76 mg) contained two compounds, 3α-APO (28 mg), which was recrystallized from 2-propanol/hexane (mp 139–141 °C), and 3β-APO (10 mg), which was an oil. (b) Fraction 16-3 (190 mg) contained a number of compounds including VIII (16 mg), which was isolated by HPLC (4% 2-propanol/hexane) and crystallized from acetone/hexane (mp 90–92 °C). The structure and stereochemistry of VIII were confirmed by X-ray crystallography (Figure 1). (c) Fraction 16-4 (400 mg) on HPLC cleanup gave a pale yellow oil, which showed a single peak on GC analysis, IX (11 mg): GC Rt 8.41 min: $[\alpha]_D$: 43.2° ($CHCl_3$; MS calcd for $C_{17}H_{26}O_5$ 310.178, found 310.178; *m/z* 43 (base), 55 (30), 81 (26), 95 (34), 97 (60), 109 (33), 123 (95), 140 (36), 149 (18), 266 (38), 310 ($M^+$). This compound, which possessed one acetyl moiety, gave the triacetyl derivative IV on acetylation. On the basis of its MS and NMR spectral data, IX was assigned the structure 2α-acetoxy-8,13-dihydroxy-11-epiapotrichothec-9-ene.

## RESULTS AND DISCUSSION

Some 83 nonmacrocyclic trichothecenes have been isolated from natural sources (Grove, 1988). Of these, 21 have been reported as being produced by *F. sporotrichioides*. In vitro studies with *F. sporotrichioides* isolates from different locations and in various media indicate that T-2 toxin, HT-2, and NEO are the major metabolites produced by this species.

*F. sporotrichioides* produces mainly type A trichothecenes (Table IV), one exception being 8-oxo DAS (Corley et al., 1987; cf. 4,15-diacetyl-7-deoxynivalenol; Greenhalgh et al., 1988). Two other trichothecenes were isolated in this study, 4-deacetoxy T-2 and 4,8-isoneosolaniol (4,8-diacetyl T-2 tetraol). 4-Deacetoxy T-2 is the 15-acetyl derivative of sporotrichiol previously reported by Corley et al. (1986a). It is known that in liquid cultures the degree of acetylation of the trichothecenes is greater than that found with solid cultures. 4-Deacetoxy T-2, sporotrichiol, and 8-hydroxytrichothecene (Corley et al., 1986a) are unique among the trichothecene metabolites produced by *F. sporotrichioides* in that they lack an oxygen function at C-4. 4,8-Diacetyl T-2 tetraol was first reported to be produced by what was purported to be *F. tricinctum* (Ilus et al., 1977) and later by *F. solani* M-1-1 (Ishii and Ueno,

**Table IV. Trichothecenes Produced by Four Isolates of *F. sporotrichioides***

| compd | substitution[a] R1 | R2 | R3 | R4 | isolate[b] |
|---|---|---|---|---|---|
| T-2 | OH | OAc | OAc | OiVa | 1-4 |
| acetyl | OAc | OAc | OAc | OiVa | 3 |
| 3[1]-hydroxy | OH | OAc | OAc | OiVa | 2-4 |
| T-2 triol | OH | OH | OH | OiVa | 2, 3 |
| 4-deoxy | OH | H | OH | OiVa | 3 |
| T-2 tetraol | OH | OH | OH | OH | 1-3 |
| 4-acetyl | OH | OAc | OH | OH | 2 |
| 8-acetyl | OH | OH | OH | OAc | 2, 3 |
| 15-acetyl | OH | OH | OAc | OH | 1, 2 |
| 4,8-diacetyl | OH | OAc | OH | OAc | 1, 2 |
| HT-2 | OH | OH | OAc | OiVa | 1-4 |
| 3[1]-hydroxy | OH | OH | OAc | OiVa | 2 |
| 4-propionyl | OH | OPr | OAc | OiVa | 2 |
| NEO | OH | OAc | OAc | OH | 1-4 |
| 8-acetyl | OH | OAc | OAc | OAc | 3, 4 |
| 8-butryl | OH | OAc | OAc | OBu | 4 |
| 8-isobutryl | OH | OAc | OAc | OiBu | 4 |
| 8-propionyl | OH | OAc | OAc | OPr | 4 |
| DAS | OH | OAc | OAc | H | 3, 4 |
| 8-oxo | OH | OAc | OAc | O= | 3, 4 |
| TCC, 8-hydroxy | H | H | H | OH | 3 |

[a] OAc = $OCOCH_3$; OBu = $OCOCH_2CH_2CH_3$; OiBu = $COCH(CH_3)_2$; OPr = $COCH_2CH_3$; OiVa = $COCH_2CH_2CHC(CH_3)_2$. [b] 1, Japanese; Czapek, 25 °C, 20 days (Ishii and Ueno, 1981); 2, Polish; rice, 27 °C, 14 days/12 °C, 14 days (Visconti et al., 1985); 3, Hungarian; corn, 10 °C, 21 days (Corley et al., 1986, 1987a,b); 4, Canadian; Myro, 25 °C, 20 days (Greenhalgh et al., 1988).

1981). Both these isolates, however, have now been reclassified as *F. sporotrichioides* (Marasas et al., 1984). 4,8-Diacetyl T-2 tetraol is also reported to be produced by other *Fusarium* species, e.g., *F. acuminatum* (Visconti et al., 1989) and *F. compactum* (Cole et al., 1988). In the latter case, the chemical shift data are at variance with that reported by Ilus et al. (1977) and would agree better for 8,15-diacetyl T-2 tetraol.

Although trichothecene-related compounds, e.g., APOs, SAM, and SOL, are only minor secondary metabolites, they are of interest because of their potential synergistic action with trichothecenes. A discrepancy between toxicoses induced in animals on mycotoxin-contaminated feed and the amount of mycotoxin present has been reported (Trenholm et al., 1984). In general, the trichothecene-related compounds are derived from trichodiene like the trichothecenes, but their biosynthesis appears to involve a different oxidized intermediate. They possess a trans-A/B ring fusion as opposed to the cis-A/B ring fusion found in the trichothecenes. Although the first trichothecene-related compounds reported were sambucinol and sambucoin (Mohr et al., 1984), the 11-epiapotrichothecenes now are the most numerous of the compounds in this group. 3$\alpha$-APO is a precursor of VIII, the absolute conformation of which is shown in Figure 1, as determined by X-ray crystallography and which confirms the structure based previously on NMR data (Greenhalgh et al., 1989a). Like sambucinol, VIII is a ketal but with a 3,11 oxygen bridge instead of the 2,11 oxygen bridge in sambucinol. It is possible that this compound is a precursor of Sporol (3,11,9,15-diepoxy-13-hydroxyapotrichothecane) isolated by Corley et al. (1986a) and the structure later corrected (Ziegler et al., 1988).

The EI/MS of VI ($M^+$, $m/z$ 266) showed a fragmentation pattern like that of sambucinol, suggesting that it could be an isomer. Its $^1H$ NMR, however, was more similar to that of the ketal VIII, but with a $CH_2$ group replaced by a CHOH function to account for a methine resonance at 3.5 ppm. The second CHOH methine proton had moved upfield from 4.5 to 4.16 ppm. The presence of a $CHOHCHOHCH_2$ unit in the COSY spectrum indicated hydroxylation of the C-ring. Acetylation of VI gave a diacetyl derivative in which the H-13 AB system and the CHOH resonance at 3.5 ppm were shifted downfield. This would suggest that VI has a structure similar to that of VIII rather than SOL. NOE difference spectra indicated that the hydroxyl function was located at C-2 with an $\alpha$-configuration. This was corroborated by the absence of any W coupling between H-2$\alpha$ and H-4$\alpha$ as found in VI. A strong NOE was observed between the $CH_3$-14 protons (0.91 ppm) and H-4 (2.3 ppm) and between the H-13 methylene protons and H-2$\beta$ (3.5 ppm), signifying that these protons are all on the $\beta$-face of the molecule. Further confirmation of the proposed structure came from the $^{13}C$ NMR spectrum, which compared more closely to that of VIII (Table II). Specifically, the $^{13}C$ chemical shifts of C-12 and C-13 at 86.1 and 62.5 ppm, respectively, indicate that C-12 is located at the junction of two five-membered rings as in VIII (88.7 and 65.3 ppm). In both SOL and its diacetyl derivative, the C-12 and C-13 resonances occur at 93–94 and 59–59.5 ppm, respectively. Thus, VI was assigned the structure 2$\alpha$,13-dihydroxy-3$\alpha$,11-epoxyapotrichothec-9-ene.

The structures of compounds III and IX were obviously closely related since they gave the same triacetyl derivative, IV. In the case of III, a single resonance at 2.01 ppm (6 H) indicated the presence of two coincidental acetoxy groups. On acetylation, the only $^1H$ resonance of III affected was the one at 4.08 ppm (1 H), which had moved downfield to 5.28 ppm. Coupling of this proton with H-7$\beta$ (1.94 ppm, $J$ = 7.5 Hz) suggested that the hydroxyl moiety was at C-8 and has an $\alpha$-configuration since $J_{7\beta,8\beta}$ is similar to those of other trichothecenes having an oxygen function at C-8. The AB system at C-13 was not affected by acetylation, showing that one of the acetoxy groups in III must be at C-13.

The methine resonance at 5.07 ppm, associated with the remaining acetoxy group in III, must be located at C-2 or C-4 because of the coupling pattern. The NOE difference spectra obtained on irradiation of $CH_3$-15 revealed a pentet at 2.17 ppm and doublet of quartets at 1.85 ppm, which were assigned to H-4 and H-3, respectively. Hence, the second acetoxy group is located at C-2. Irradiation of $CH_3$-14 enhanced the H-11 resonance, confirming the trans-A/B ring fusion of the 11-epiapotrichothecenes. In addition, some enhancement was observed of the resonances at 1.24 and 1.95 ppm, which are attributed to H-4 and H-7, respectively. Irradiation of the triplet at 5.10 ppm produced only a small enhancement to half of the C-13 AB system, indicating the acetoxy group to be pseudoaxial on the $\beta$-face of the molecule. A positive enhancement was observed also to both protons of the AB system at 2.07 and 1.85 ppm, associated with the H-3 methylene protons. With a knowledge of $^1H$ NMR assignments, those of the $^{13}C$ spectrum were determined by a $^1H/^{13}C$ heteronuclear correlation spectra (Figure 2). The data are consistent with the proposed structure of 2$\alpha$,13-diacetoxy-8$\alpha$-hydroxy-11-epiapotrichothec-9-ene for III.

Both the MS and NMR data of IX were similar to those of III, except for the absence of an acetoxy group. On acetylation, IX gave the triacetyl derivative IV, in which the H-13 AB system resonance had shifted from 3.5 to 4.0 ppm. This demonstrates the presence of a free hydroxyl group at C-13. Acetylation of IX also gave a $^{13}C$ resonance at 62.8 ppm, upfield from 64.4 ppm in III. An $\alpha$-effect was observed for the C-12 resonance with a 3 ppm downfield shift to 95.0 ppm. These data are in agreement with the

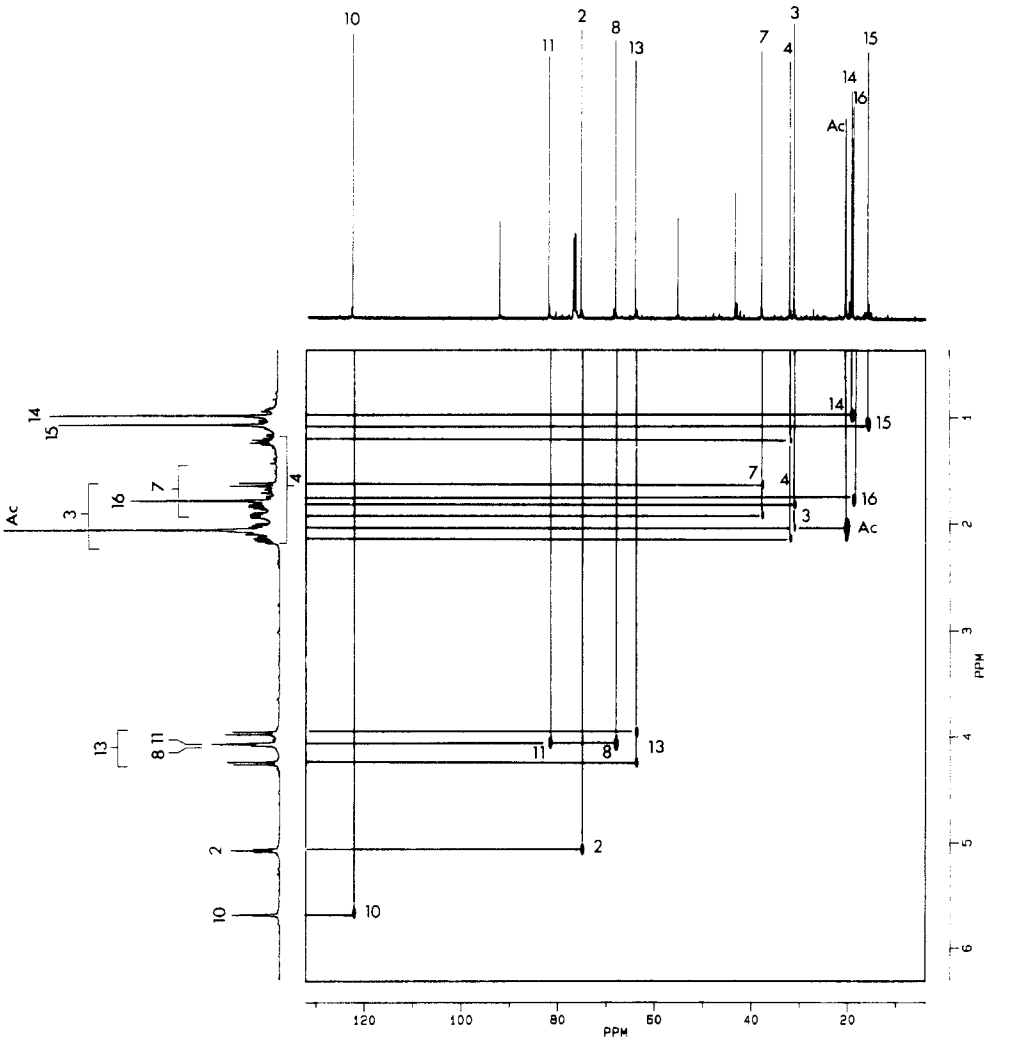
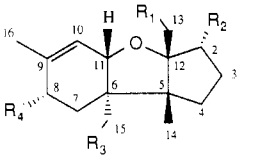



**Figure 2.** $^{1}H/^{13}C$ correlation spectrum for 2,13-dihydroxy-8-hydroxy-11-epiapotrichothecene.

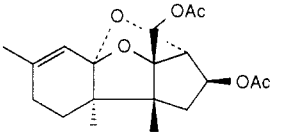
structure 2$\alpha$-acetoxy-8$\alpha$,13-dihydroxy-11-epiapotrichothec-9-ene for IX.

Compound I has a molecular formula of $C_{19}H_{28}O_6$ by MS. Its $^{1}H$ NMR spectrum showed no $CH_3$-15 resonance, but instead it had an additional AB system between 3 and 4 ppm, suggesting an oxygen function at C-15. The presence of a hydroxy group at C-13 was confirmed by comparison of the $^{13}C$ spectrum with that of III. An $\alpha$-effect is observed at C-13, shifting the resonance upfield from 64.3 to 62.8 ppm, while a $\beta$-effect is observed on the resonance assigned to C-12, which moved downfield by 2 ppm to 95.0 ppm. The resonances at 62.4 and 65.5 ppm were assigned to C-13 and C-15, respectively, by comparison with the known spectra of 11-epiapotrichothecenes. The COSY spectrum revealed a $CHOAcCH_2CH_2$ system to that in III, implying that a second acetoxy group is situated at C-2. The structure assigned to I was 2$\alpha$,15-diacetoxy-3-hydroxy-11-epiapotrichothec-9-ene. The structures of the trichothecene-related compounds isolated in this study are shown in Figure 3.

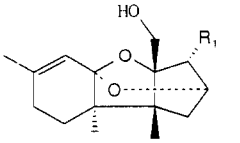
It is known that in T-2 produced by *F. sporotrichioides* the six oxygen atoms in the trichothecene ring are derived from molecular oxygen (Desjardins et al., 1986). These oxidations, which are stereospecific, could be attributed to mono- or dihydroxylases. Each *Fusarium* species appears to be associated with specific hydroxylases, judging by the different levels of oxygenated trichothecenes produced by different species. Baldwin et al. (1986) biotransformed 3-acetyldeoxynivalenol (ADON) into fusarenon X using an unidentified DAS producing *Fusarium* sp. Thus, the hydroxylase in this species responsible for oxidation at C-4 in the trichothecene ring responds to the configuration in the C-ring of ADON, regardless of that in the A-ring. The oxygen function at C-4 in the trichothecenes always has an 4$\beta$-configuration.




| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| I | OH | OAc | OAc | H |
| III | OAc | OAc | H | OH |
| IX | OH | OAc | H | OH |
| IV | OAc | OAc | H | OAc |




V




| | R1 |
|---|---|
| VIII | H |
| VI | OH |

**Figure 3.** Some trichothecene-related secondary metabolites isolated from *F. sporotrichioides*.

Biooxidation at C-8 in the trichothecene ring normally results in an oxygen function with an 8$\alpha$-configuration. Two exceptions have been reported, 8$\beta$-hydroxytrichoth-

ecene (Corley et al., 1986b) and 8β-hydroxysambucoin (Corley et al., 1987); both are minor metabolites produced by *F. sporotrichioides* MC72083. Oxidation in the A-ring appears independent of the substitution in the C-ring as demonstrated by the 8α-oxygen function in III, IV, and IX. Beremand et al. (1988) have identified a genetic locus responsible for oxidation at C-8 and indicated the presence of a separate gene responsible for subsequent acetylation of the C-8 oxygenated moiety.

Although the biosynthesis of trichodiene has been elaborated (Cane et al., 1985), the intermediate steps involved in the bioconversion of trichodiene to trichothecenes and 11-epiapotrichothecenes still remain to be clarified. That trichodiene is a precursor to both classes of compounds has been demonstrated (Savard et al., 1989). The isolation of trichodiol (Nozoe and Machida, 1972) and trichotriol (Corley et al., 1987) and their conversion to trichothecene and 3-hydroxytrichothecene, respectively, indicated that biooxidation of the trichodiene intermediate at C-2 and C-3 preceded cyclization. Recently, McCormich et al. (1990) showed that oxidation of the intermediate at both C-2 and C-3 was essential for the formation of T-2 by the mutant strain *F. sporotrichioides* MB5493. Unfortunately, the latter experiment does not provide a definitive answer regarding the oxidative state of C-3 prior to cyclization in other strains, since oxidation at C-3 by this mutant strain could be blocked. Oxidation at C-4, C-8, and C-15 obviously occurs postcyclization.

In the case of the 11-epiapotrichothecenes, biooxidation could follow a similar sequence as for the trichothecenes, albeit from a different trichodiene-derived intermediate. Since the 11-epiapotrichothecenes have the same conformation in the A-ring, it would be expected that oxidation would occur at C-8 if C-8 hydroxylated trichothecenes are present. The difference in the configuration of the C-rings of trichothecenes and 11-epiapotrichothecenes could account for the lack of oxidation at the C-4 position in the latter. Oxidation at C-2 is of interest in that it could provide a possible biosynthetic route to sambucinol.

In summary, a trichothecene and four 11-epiapotrichothecenes not previously reported are described together with the natural occurrence of diacetylsambucinol. These 11-epiapotrichothecenes serve to epitomize the specificity of the C-8 hydroxylase system present in *F. sporotrichioides*. Molecular models suggest that the absence of oxidation at C-4 in the 11-epiapotrichothecenes can be attributed to the different configuration of the C-ring from that in the trichothecenes.

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**Supplementary Material Available:** X-ray data for compound VIII, consisting of the final atomic coordinates and bond lengths and angles (6 pages). Ordering information is given on any current masthead page.

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**Registry No.** T–2, 21259-20-1; T-2 acetyl, 21259-21-2; T-2 3′-hydroxy, 84474-35-1; T-2 triol, 34114-98-2; T-2 triol 4-deoxy, 101401-89-2; T-2 tetraol, 34114-99-3; T-2 tetraol 4-acetyl, 111827-62-4; T-2 tetraol 8-acetyl, 94099-58-8; T-2 tetraol 15-acetyl, 74833-39-9; T-2 tetraol 4,8-diacetyl, 65180-29-2; HT-2, 26934-87-2; HT-2 3′-hydroxy, 78368-54-4; HT-2 4-propionyl, 104903-80-2; NEO, 36519-25-2; NEO 8-acetyl, 65041-92-1; NEO 8-butryl, 98813-18-4; NEO 8-isobutryl, 111112-48-2; NEO 8-propionyl, 111112-47-1; DAS, 2270-40-8; DAS 8-oxo, 77620-47-4; TCC 8-hydroxy, 104903-78-8.