Secondary Metabolites Produced by *Fusarium sporotrichioides* DAOM 165006 in Liquid Culture

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A Canadian strain of *Fusarium sporotrichioides*, DAOM 16005, grown in liquid culture produced a variety of trichothecenes. The yields obtained in two different fermentation media, MYRO and Moss and Franks, were approximately the same; however, in the later medium, T-2 formed more rapidly and then was degraded to HT-2. All the trichothecenes isolated were 3,4-oxygenated compounds; T-2 was the major metabolite together with neosolaniol, HT-2, and DAS. Of some 16 minor metabolites detected, four were characterized by GC and ^{1}H NMR spectral data as being T-2 analogues. These were the C-8 propionate, isobutyrate, and butyrate esters of neosolaniol, which are new compounds, and the 3′-hydroxy derivative of T-2. The biosynthetic implication of the formation of these analogues is discussed.

Trichothecenes are a family of closely related sesquiterpenoid secondary metabolites produced by *Fusarium*, *Trichothecium*, *Myrothecium*, and *Stachybotyris*. The first member of this group, trichothecin, was isolated from *Trichothecium roseum* and was found to exhibit some antifungal activity (Freeman and Morrison, 1948). Subsequent observations have implicated various trichothecene-producing fungi in a number of cases of animal and human toxicoses due to the ingestion of moldy cereal grains. Further studies have led to the isolation and characterization of over 60 compounds differing principally in the degree of oxygenation of the trichothecene ring and the extent of acylation.

The preponderance of the pathogen *Fusarium graminearum* over other fungal species, such as *Fusarium sporotrichioides*, in grain at harvest time can be partially attributed to the more rapid growth of the former species at higher temperatures (~28 °C) that occur in summer. The optimum temperature for growth of *F. sporotrichioides* is lower, and infection usually occurs when grains are left in the fields in autumn under wet conditions or are over-wintered there. T-2 and related toxins subsequently are found in the crop.

Isolates of *F. sporotrichioides* are known to produce the 3,4-oxygenated trichothecenes. Visconti et al. (1985) isolated some 11 compounds including T-2 toxin as the major metabolite when the strain P-11 was cultured on rice. More recently, several novel trichothecenes were characterized from the isolate *F. sporotrichioides* MC-72083 cultured on a corn medium at 10 °C (Corley et al., 1986a,b). The present study examines the secondary metabolites produced by a Canadian isolate, *F. sporotrichioides* DAOM 165006, in liquid culture. This isolate was originally obtained from a sample of hay that induced severe mycotoxicosis in horses (Davis et al., 1982).

MATERIALS AND METHODS

Standards. T-2, HT-2, and DAS toxins were purchased from Sigma Chemical Co., St. Louis, MO. 3-Hydroxyapotrichothecene (APO) and sambucinol had been previously isolated from *Fusarium culmorum* (Greenhalgh et al., 1986b). The propionyl, butyryl, and isobutyryl analogues of T-2 toxin were synthesized from neosolaniol (Savard and Greenhalgh, 1987).

Apparatus. Mass spectra (MS) were obtained on a Finnigan GC/MS system, Model 4500, operating in the electron impact (EI) mode. Underivatized crude fungal extracts and trichothecene standards were separated on a DB-5 fused silica capillary column (20 m × 0.32 mm (i.d.), 0.25-μm film). All samples were injected on-column. The GC was temperature programmed from 140 to 260 °C at 15 °C/min with helium as the carrier gas at 10 psi. High-performance liquid chromatography (HPLC) was performed on a Varian Model 5500 system with a UV-200 detector operating at 205 nm and using a 5-μm Spherisorb CN column (25 cm × 0.94 cm (i.d.)) with a solvent flow of 4 mL/min.

^{1}H and ^{13}C NMR spectra were run on a Bruker WM 250-MHz spectrometer. The ^{1}H spectra were acquired with 16K data points, a 2200-Hz spectral window, 60° pulses, and 10-s repetition time. Chemical shifts are referenced to deuteriochloroform at 7.24 ppm and are reported relative to tetramethylsilane.

Fermentation. Conditions were optimized for the production of T-2 by *F. sporotrichioides* DAOM 165006 with a New Brunswick Scientific Magnaferm fermentor equipped with oxygen and pH controls and a CO_2 analyzer (ADC), interfaced with a Hewlett-Packard data acquisition system (Brewer et al., 1987).

A malt extract agar slant of the isolate was macerated in sterile distilled water (50 mL). An aliquot (2.5 mL) was used to inoculate 10 250-mL Erlenmeyer flasks, each containing inoculation medium (50 mL). The flasks were inoculated at 28 °C for 48 h on a rotary shaker (3.81-cm throw) at 220 rpm. The resulting mycelium was macerated and used to inoculate 10 L of MYRO production medium. Both the inoculation and production media were prepared as previously described (Miller and Blackwell, 1986). The fermentation conditions were as follows: temperature, 28 °C; stirring rate, 375 rpm; air flow, 1.25 h min^{-1}; the pH was allowed to fall to 4.0 and then maintained by addition of sterile 0.8 N NaOH. Alternatively, the production medium of Moss and Frank (1985) was used under the same fermentation conditions. After 7 days, the culture filtrate was adjusted to pH 8.3 and extracted on Clinelut columns with ethyl acetate as solvent. The procedure was scaled up to 180 L and worked up as previously described to give the crude fungal extract (Greenhalgh et al., 1986b).

Extraction and Purification. The ether-soluble portion (7.2 g) of the crude fungal material was chromatographed in two equal portions in a glass column (5 × 29 cm) containing activated LiChroprep Si-60 (225 g). The eluting solvents consisted of CH_2Cl_2 (500 mL) followed by 79:1 CH_2Cl_2/MeOH (500 mL), 59:11 CH_2Cl_2/MeOH (1000 mL), 39:1 CH_2Cl_2/MeOH (500 mL), 24:1 CH_2Cl_2/MeOH (500 mL), 4:1 CH_2Cl_2/MeOH (500 mL), and finally MeOH (500 mL). The eluate was collected in 74 fractions, each 50 mL, which were monitored by thin-layer chromatography, and appropriate fractions from the columns were

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0021-8561/88/1436-0216$01.50/0 Published 1988 by the American Chemical Society

Table I. 250-Mz ^{1}H Chemical Shift and Coupling Constants for Trichothecenes from *F. sporotrichioides*

proton	T-2	8-propionyl-NEO	8-isobutyryl-NEO	8-butyryl-NEO	3′-OH-T-2	4,15-DA-7-DON
2	3.68 ($J_{2,3}$ = 4.9)	3.68 ($J_{2,3}$ = 4.9)	3.69 ($J_{2,3}$ = 4.9)	3.68 ($J_{2,3}$ = 4.9)	3.67 ($J_{2,3}$ = 4.9)	3.77 ($J_{2,3}$ = 4.9)
3	4.14 ($J_{3,2}$ = 4.9, $J_{3,4}$ = 2.9)	4.12 ($J_{3,2}$ = 4.9, $J_{3,4}$ = 2.8)	4.14 ($J_{3,2}$ = 4.9, $J_{3,4}$ = 2.9)	4.13 ($J_{3,2}$ = 4.9, $J_{3,4}$ = 2.9)	4.14 ($J_{3,2}$ = 4.9, $J_{3,4}$ = 2.9)	4.21 ($J_{3,2}$ = 4.9, $J_{3,4}$ = 2.9)
4	5.28 ($J_{4,3}$ = 2.8)	5.27 ($J_{4,3}$ = 2.8)	5.35 ($J_{4,3}$ = 2.9)	5.27 ($J_{4,3}$ = 2.9)	5.27 ($J_{4,3}$ = 2.9)	5.06 ($J_{4,3}$ = 2.9)
7	1.89 (J_{AB} = 15.3)	1.93 (J_{AB} = 15.1)	1.83 (J_{AB} = 15.0)	1.91 (J_{AB} = 15.9)	1.90 (J_{AB} = 15.0)	2.46 (J_{AB} = 15.9)
	2.38 ($J_{7,8}$ = 5.7)	2.38 ($J_{7,8}$ = 5.8)	2.40	2.39 ($J_{7,8}$ = 5.3)	2.40 ($J_{7,8}$ = 5.2)	2.92 ($J_{7,15}$ = 1.6)
8	5.26 ($J_{8,7}$ = 5.7)	5.27 ($J_{8,7}$ = 5.8)	5.26 ($J_{8,7}$ = 5.6)	5.27 ($J_{8,7}$ = 5.5)	5.26 ($J_{8,7}$ = 5.3)	
10	5.78 ($J_{10,11}$ = 5.9)	5.80 ($J_{10,11}$ = 6.0, $J_{10,16}$ = 1.2)	5.80 ($J_{10,11}$ = 5.4)	5.79 ($J_{10,11}$ = 4.9)	5.79 ($J_{10,11}$ = 5.9)	6.59 ($J_{10,11}$ = 5.9, $J_{10,16}$ = 1.5)
11	4.32 ($J_{11,10}$ = 5.9)	4.33 ($J_{11,10}$ = 6.0)	4.38 ($J_{11,10}$ = 5.4)	4.30 ($J_{11,10}$ = 4.9)	4.35 ($J_{11,10}$ = 5.9)	4.51 ($J_{11,10}$ = 5.9)
13	2.78 (J_{AB} = 3.9)	2.78 (J_{AB} = 3.9)	2.78 (J_{AB} = 3.9)	2.78 (J_{AB} = 3.9)	2.78 (J_{AB} = 3.9)	2.79 (J_{AB} = 3.9)
	3.04	3.04	3.05	3.04	3.04	3.07
14	0.79	0.79	0.79	0.79	0.78	0.80
15	4.03 (J_{AB} = 12.6)	4.03 (J_{AB} = 12.6)	4.02 (J_{AB} = 12.6)	4.03 (J_{AB} = 12.5)	4.04 (J_{AB} = 12.6)	4.10 (J_{AB} = 12.5)
	4.27	4.27	4.31	4.27	4.26	4.17
16	1.73	1.72 ($J_{16,10}$ = 1.2)	1.73	1.72	1.73	1.82
2′	2.12	2.28 ($J_{2',3'}$ = 7.5)	2.45 ($J_{2',3'}$ = $J_{2',4'}$ = 7.0)	2.33 ($J_{2',3'}$ = 7.4)	2.42	
3′	2.00	1.13 ($J_{3',2'}$ = 7.5)	1.16 ($J_{3',2'}$ = 7.0)	1.64 ($J_{2',3'}$ = $J_{3',4'}$ = 7.4)		
4′	0.94 ($J_{3',4'}$ = 7.0)		1.16 ($J_{2',4'}$ = 7.0)	0.93 ($J_{4',3'}$ = 7.4)	1.27	
5′	0.95 ($J_{3',5'}$ = 7.0)					1.27
CH_3 Ac (5)	2.01	2.01	2.01	2.01	2.02	1.97
(4)	2.12	2.13	2.13	2.13	2.12	2.14

then combined to give seven fractions (F1–F7). Further purification of these fractions was accomplished by HPLC with an isocratic solvent system of 2-propanol and hexane (*i*-PrOH/hexane), in the proportions indicated.

Fraction F1 was comprised of 23 fractions, which gave an oil (39.4 mg), and consisted mainly of phthalates. It was discarded.

Fraction F2. Fractions 24–32 gave an oil (147.4 mg) comprised mainly of phthalates, together with isotrichodermin, calonectrin, and sambucoin. These compounds were characterized by GC/MS but not purified.

Fraction F3. Fractions 33–49 yielded an oil (3.07 g). It contained T-2 (1.95 g), which was crystallized from isopropyl ether (mp 148–149 °C). The mother liquor (800 mg) was analyzed by GC/MS, and in addition to T-2 and DAS four unknown compounds were detected. Three of these unknown compounds had mass spectra that bore a strong resemblance to that of T-2. These compounds were purified by preparative HPLC (elution with 5% *i*-PrOH/hexane) followed by rechromatography in 3% *i*-PrOH/hexane. Three compounds were obtained. Oil I: 2 mg; R_t (3% *i*-PrOH/hexane) 19 min; MS, *m/z* 121 (base), 71 (80), 105 (48), 180 (45), 185 (26). Crystalline material II: 2.28 mg; R_t 21 min; mp 182–183 °C; MS, *m/z* 57 (base), 121 (89), 180 (61), 105 (48), 185 (35). Oil III: 8.20 mg; R_t 32 min; MS, *m/z* 380 (M^+), 109 (base), 121 (97), 100 (64), 173 (52), 189 (45), 278 (21). Preparative TLC (hexane/acetone/formic acid, 65:33:2) with double development afforded compound IV (2 mg): R_f 0.42; MS *m/z* 121 (base), 71 (98), 191 (62), 180 (60), 105 (57), 195 (38).

Fraction F4. Fractions 50–55 were a mixture of crystals and an oil (455.5 mg). It consisted mainly of T-2.

Fraction F5. Fractions 56–61 gave an oil (2.09 g). Analysis by GC/MS indicated the presence of neosolaniol, sambucinol, and HT-2, together with a fifth unknown compound (V). The mass spectrum of the latter had many similarities to that of T-2, but on GC analysis it eluted later than T-2. The entire fraction was first chromatographed on LiChroprep Si-60 (100 g) in a glass column (30 × 3.5 cm), using CH_2Cl_2 containing increasing amounts of EtOAc as the eluting solvent. Some 21 fractions (100 mL each) were collected and monitored by TLC and GC/MS. Those fractions containing the unknown V were pooled and further purified by HPLC (9% *i*-PrOH/hexane) to yield an oil: 12.8 mg; R_t 30.5 min; MS, *m/z* 121 (base), 59 (92), 105 (57), 185 (48), 180 (45). Analysis of the ^{1}H NMR

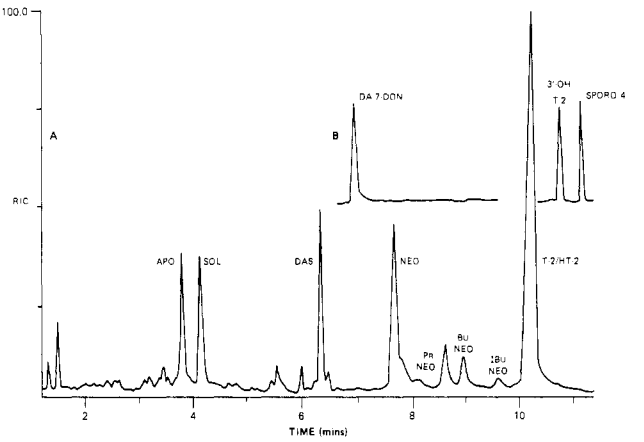

Figure 1. Reconstituted total ion chromatogram of crude fungal extract of *F. sporotrichioides* DAOM 165006 in liquid culture. GC conditions: DB-5 capillary column; 20 m long, i.d. 0.32 mm; film thickness, 0.25 μm; oven programmed from 140 to 260 °C at 15 °C/min; helium carrier gas at 10 psi. (A) Key: APO = 3-hydroxyapotrichothecene; SOL = sambucinol; DAS = diacetoxyscirpenol; NEO = neosolaniol; Pr-NEO = propionylneosolaniol; Bu-NEO = butyrylneosolaniol; *i*-Bu-NEO = isobutyrylneosolaniol. (B) Key: DA-7-DON = 4,15-diacetoxy-7-deoxynivalenol; 3′-OH-T-2 = 3′-hydroxy-T-2; SPORO 4 = unknown.

spectral data (Table I; Figure 2) indicates the compound to be 3′-hydroxy-T-2 toxin. This compound has been isolated previously from *F. sporotrichioides* strain P-11 (Visconti et al. 1985).

Fraction F6. Fractions 62–67 were an oil (1.25 g) and contained neosolaniol and hydroxyapotrichothecene. Neosolaniol (640 mg) was readily crystallized from this fraction (mp 170–171 °C). HPLC purification of the mother liquor (10% *i*-PrOH/hexane at 4 mL/min) gave another compound VI (0.5 mg; R_t 24 min), which remains an unknown and has been given the name SPORO 4.

Fraction F7. Fractions 68–74 gave an oil (215.5 mg). It contained the most polar material present in the crude mixture and was not amenable to analysis by GC/MS.

RESULTS AND DISCUSSION

Culturing *F. sporotrichioides* DAOM 165006 on sterile rye (24 days at 20 °C) produced some T-2 (Davis et al., 1982). The conditions for the optimization of the production of T-2 by this isolate in liquid culture were investigated. The three-stage procedure developed for 3-

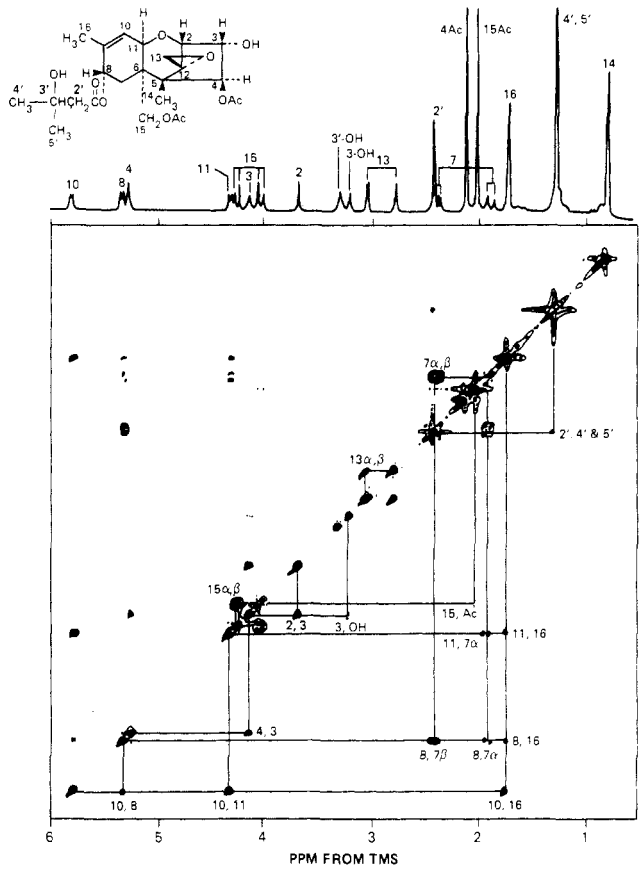

Figure 2. $^1H/^1H$ correlation spectrum for 3′-OH-T-2 toxin. A 90–t_1–45 (COSY-45) pulse sequence was employed to give a data matrix of 512 × 1K fid's of 16 scans; final data resolution was 1 Hz/pt. The spectrum was symmetrized about the diagonal to delete noise from the high-intensity acetyl methyl resonances. The single-pulse spectrum is shown at the top of the figure.

acetoxydeoxynivalenol (Greenhalgh et al., 1986b) gave yields of 30 mg/L (for 8-L batch) of T-2 toxin as determined by HPLC analysis. Replacement of the medium (glucose, malt extract, yeast extract, peptone) used in the second stage of this procedure by that used for producing the inoculum resulted in an increase of at least 10-fold in the yield. Under the fermentation conditions (7 days at 28 °C), the MYRO medium gave typical yields of T-2 (850 mg/L), HT-2 (30 mg/L), and neosolaniol (400 mg/L). Moss and Frank's medium (1985) gave comparable yields of the various mycotoxins; however, the maximum yields occurred at 96-120 h. After this time T-2 was rapidly degraded, principally to HT-2.

A GC/MS of the underivatized crude fungal extract from the large batch is shown in Figure 1. The yields were lower than obtained on a smaller scale. The major product was T-2 (306 μg/g), which was not resolved from HT-2 (24 μg/g) on this column. Other major metabolites included neosolaniol (89 μg/g), diacetoxyscirpenol (DAS; 156 μg/g), and sambucinol, all of which have been previously reported as secondary metabolites of *F. sporotrichioides* (Ishii and Ueno, 1981; Corley et al., 1986a,b; Visconti et al., 1985). Hydroxyapotrichothecene (APO) was also present in a significant quantity. It is of interest to note that both APO and the modified trichothecene sambucinol appear to be produced by all the *Fusarium* species we have studied to date in liquid culture (Greenhalgh et al., 1986a,b; Lauren et al., 1987). In addition to these six major compounds, some 14 other metabolites were detected in trace amounts.

Characterization of four of these minor metabolites (I, II, IV, V) proved to be relatively simple. Their MS exhibited EI fragmentation patterns similar to that of T-2, indicating them to be analogues. All lacked a significant (M^+) ion. The ^1H NMR spectral data of these four T-2 analogues also reflect the similarities of their structures. Thus, the chemical shifts of the protons associated with the epoxide (C-13), methylene (C-15), methyl (C-14, C-16) moieties, and the methine (C-2, C-3, C-4) protons in ring C are virtually identical (Table I).

From the mass spectral and NMR data obtained, compounds I, II, and IV have been identified as 3-hydroxy-4,15-diacetoxy-8-(butyryloxy)-12,13-epoxy trichothec-9-ene (Bu-NEO), 3-hydroxy-4,15-diacetoxy-8-(propionyloxy)-12,13-epoxytrichothec-9-ene (Pr-NEO), and 3-hydroxy-4,15-diacetoxy-8-[(2-methylpropionyl)oxy]-12,13-epoxytrichothec-9-ene (*i*-Bu-NEO), respectively. These structures were confirmed by synthesis from neosolaniol (Savard and Greenhalgh, 1987).

The $^1H/^1H$ correlation spectrum (Cosy-45) for compound V (identified as 3′-OH-T-2) provides data on the extent of coupling between the various protons (Figure 2). Assignments of this spectrum are consistent with the trends observed for other trichothecenes with varying substitution patterns at C-7 and C-8 (Greenhalgh et al., 1986a; Lauren et al., 1987). In the H-7 AB system (J_{AB} = 15 Hz), the lower field multiplet which couples strongly with H-8 can be assigned to H-7β ($J_{7\beta,8\beta}$ = 5.2 Hz). The $J_{7\alpha,8\beta}$ coupling is too small to be resolved (J < 0.5 Hz) in the 1D spectrum but appears in the COSY plot.

With the B ring of 3′-OH-T-2 in the chair configuration, there are two conformations that the 8α-OH can assume equatorial when the A ring is in the boat configuration, or axial when it is in the half-chair. The calculated coupling constants for the axial conformation derived from the Karplus relationship gives the values $J_{7\alpha,8\beta} \sim 0$ and $J_{7\beta,8\beta} \sim 5$ Hz, which agree with those found. Most naturally occurring trichothecenes characterized thus far and containing an 8-OH group have the α-configuration. However, Corley et al. (1986b) recently isolated 8-hydroxytrichothecene, which was assigned the β-configuration based on NMR data (J = 5.5, 9.3 Hz). These assignments are supported by observed values of $J_{7,8}$ = 5 and 8 Hz, respectively, in synthetic 8-hydroxy epimers (α and β) of neosolaniol (Kaneko et al., 1982).

The broad resonance at 3.18 ppm is attributed to the 3-hydroxy proton, since it is coupled to H-3, and the second broad resonance (3.31 ppm) not coupled is assigned to the 3′-hydroxy proton. The two methyl groups at C-4′ and C-5′ are equivalent and appear as a slightly broadened singlet (1.25 ppm, 6 H), as do the C-2′ methylene protons (2.43 ppm). Some long-range coupling is apparent in the COSY plot between the C-2′ methylene and the terminal methyl groups (4′ and 5′), which accounts for this broadening. In addition, long-range coupling is observed between H-8 and both H-10 and H-16. Weak *W* coupling is observed between H-11 and H-7α due to the pseudochair configuration of the A ring.

Another minor metabolite isolated, compound III, was identified as 4,15-diacetyl-7-deoxynivalenol (DA7-DON) by MS and ^1H NMR. This compound has been isolated previously from *Fusarium crookwellense* (Lauren et al., 1987). Its ^1H NMR spectrum shows the typical coupling constant for the H-7 AB system (J = 15.9 Hz), in addition to long-range coupling between H-7 and one of the H-15 protons (J = 1.6 Hz). This coupling is common to all compounds that have methylenic protons at C-7 and a carbonyl moiety at C-8. It may result from the more rigid structure of ring A incurred with this substitution pattern.

F. sporotrichioides DAOM 165006, in common with other isolates of this species, produced 3,4-oxygenated

trichothecenes, together with 3-hydroxyapotrichothecene and sambucinol, all of which are thought to be derived from the common intermediate trichodiene. In liquid culture, both *F. graminearum* and *F. culmorum* produced only 3-oxygenated trichothecenes. This could be interpreted as primary oxidation of the trichothecene ring occurring in the C ring at position 3 for the latter species and position 3,4 for *F. sporotrichioides* with secondary oxidation at the 7,8- and 15-positions. Although 8-hydroxytrichothecene was isolated from *F. sporotrichioides* grown on sterile grain, this compound has not been found in liquid culture of this strain. The isolation of several trichothecenes oxygenated at position 3 and 4 but not in the A ring, such as DAS, suggests that the C-8 position is not a primary oxidation site.

In both *F. graminearum* and *F. culmorum*, acylation of the hydroxylated trichothecene ring is limited to acetylation. By contrast, *F. sporotrichioides* produces a variety of acylated compounds, with variation at only the 8-OH group. Recent biosynthetic studies with *F. sporotrichioides* using ^{13}C-labeled acetate as a precursor show that the extent of ^{13}C incorporation in the C-8 acyl groups of *T*-2 and its analogues is much less than in the ring (Blackwell, 1986). This indicates that the acylating moiety involved in reaction with the 8-OH group is not derived by the mevalonate biosynthetic pathway as are the trichothecenes. One possible source of the acylating moiety would be via the amino acid pathway, where the propionate ester could arise from leucine. Experiments are in progress to test this hypothesis.

Chakrabarti and Ghosal (1986) reported the isolation of conjugates such as palmitoyltrithecolone and palmitoylscirpentriol from tropical fruit, as well as their production on culturing *Fusarium moniliforme* isolates. These compounds, which are esterified in the C-8 position, indicate the proclivity of the 8-OH group to react with a variety of acylating moieties.

In summary, the Canadian strain *F. sporotrichioides* DAOM 165006 in liquid culture produces the same major metabolites as other isolates of this species under various fermentation conditions. As a result of the more toxic nature of the 3,4-oxygenated trichothecenes as compared with that produced by *F. graminearum*, acceptable limits need to be established for animal feeds made from *F. sporotrichioides* infested grains.

ACKNOWLEDGMENT

We thank A. Ashley and P. Lafontaine for the mass spectral data and W. Adams and G. Montgomery for the analyses and technical assistance.

Registry No. T-2, 21259-20-1; HT-2, 26934-87-2; 3′-hydroxy-T-2, 84474-35-1; NEO, 36519-25-2; SOL, 90044-33-0; DAS, 2270-40-8; APO, 111085-77-9; DA-7-DON, 77620-47-4; Bu-NEO, 98813-18-4; Pr-NEO, 111112-47-1; *i*-Bu-NEO, 111112-48-2; isotrichodermin, 91423-90-4; calonectrin, 38818-51-8; sambucoin, 90044-34-1.

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Received for review January 2, 1987. Accepted July 7, 1987. PRC Contribution No. 1068.