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Trichothecenes Accumulated in Liquid Culture of a Mutant of *Fusarium sporotrichioides* NRRL 3299

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A UV-generated mutant of *Fusarium sporotrichioides* NRRL 3299 was altered in its ability to biosynthesize T-2 toxin, as shown by a rapid screen with monoclonal antibodies to T-2. This stable mutant accumulated two trichothecenes that were not observed in liquid cultures of the parent strain. The two compounds were identified as 3,15-diol 12,13-epoxytrichothec-9-ene and 3,15-diol 12,13-epoxytrichothec-9-ene 3-acetate on the basis of their nuclear magnetic resonance and mass spectra. This is the first report of either of these two compounds as secondary metabolites of *F. sporotrichioides* and of a trichothecene acetylated at C-3 by this species.

Trichothecenes are a class of chemically related secondary metabolites produced by various species of fungi. They are sesquiterpene alcohols or esters generally containing a 12,13-epoxide group and derived from a common tricyclic skeleton. Knowledge concerning the biosynthesis of trichothecenes has been reviewed by Tamm and Breitenstein (14). Farnesyl PP_i is enzymatically converted into the cyclic sesquiterpene trichodiene, compound 1 (Fig. 1), via a terpene cyclase. Details of the conversion, which involves a double 1,2 methyl migration and a 1,4 hydride shift, have been worked out by feeding specifically labeled mevalonate molecules to cultures of *Trichothecium roseum*, *Trichoderma* species, or *Myrothecium* species.

Trichothecenes are synthesized from trichodiene after a series of oxygenations and esterifications and rearrangement to the trichothecene nucleus. The biosynthetic intermediates in many of these steps, and the order in which they occur, are not known. Trichodiol, compound 2 (Fig. 1), was isolated by Nozoe and Machida (12) from mycelia of *T. roseum* and has been suggested as an intermediate in biosynthesis of trichothecenes. Recently, Corley et al. (2) isolated small quantities of trichotriol, compound 3 (Fig. 1), from solid cultures of *Fusarium sporotrichioides*. These authors reported the fortuitous decomposition of compound 3 to 3-hydroxytrichothecene (2) in $CDCl_3$ solution (with a trace of water present) in a nuclear magnetic resonance (NMR) tube. Whether this nonenzymatic conversion is a part of the normal trichothecene biosynthetic pathway in *F. sporotrichioides* to compounds which have oxygenation at C-3 is not known.

T-2 (3,4,8,15-tetraol 12,13-epoxytrichothecene 4,15-diacetate 8-isovalerate) toxin is the major trichothecene produced by *F. sporotrichioides* NRRL 3299 (9). Recent work with NRRL 3299 provided some additional information about the biosynthesis of T-2 by *F. sporotrichioides*. When NRRL 3299 is grown in liquid culture in the presence of ancymidol, trichothecene production is inhibited and large amounts of trichodiene accumulate (5). The remaining steps in the biosynthesis of T-2 by *F. sporotrichioides* include six oxygenations. Labeling studies demonstrated that these oxygenations all utilize molecular oxygen-dependent enzymes (6). Analysis of a leucine auxotroph of *F. sporotrichioides* revealed that leucine is the precursor to the isovalerate moiety at C-8. A large number of trichothecenes with hydroxyl groups or esters at combinations of C-3, C-4, C-15, C-7, and C-8 are produced by various *Fusarium* species. It is not known whether these reactions must occur in any required order.

To provide a tool to study these aspects of trichothecene biosynthesis, Beremand (1) reported a procedure to generate mutants of *F. sporotrichioides* NRRL 3299 in which T-2 synthesis is blocked. Using this procedure, we have identified a UV-induced mutant, MB2972, that produces only small amounts of T-2 (M. N. Beremand, P. J. Black, and R. D. Plattner, J. Cell. Biochem. Suppl. **12c**:261, 1988). The lower T-2 production of this mutant is accompanied by production of similar amounts of two trichothecenes that are less oxygenated. Here, we report the isolation and characterization of these compounds and discuss their relationship to the T-2 biosynthetic pathway.

MATERIALS AND METHODS

Cultures and culture conditions. A culture of the T-2 toxin-producing isolate *F. sporotrichioides* NRRL 3299 (ATCC 24043) was derived from a single spore (6). This strain was originally isolated from corn (*Zea mays*) in France (3, 9). Strain MB2972 is a UV-induced mutant of NRRL 3299 which is greatly reduced in its ability to produce T-2 toxin (Beremand et al., J. Cell. Biochem. Suppl. **12c**:261, 1986). Media and culture conditions were as reported previously (1).

Extraction and analysis procedures. The culture filtrate or whole culture was extracted twice with an equal volume of ethyl acetate. The extracts were combined and evaporated to dryness on a rotary evaporator. The residue was suspended in 1 ml of ethyl acetate.

For gas chromatography (GC), a sample of the underivatized extract equivalent to about 5 to 10 μl of the original culture was injected directly to the GC-mass spectrometer (MS). Alternatively, a sample equivalent to 1 or 2 ml of the original culture was evaporated to dryness under nitrogen and reacted with 100 to 200 μl of silylating reagent (Tri-Sil/TBT; Pierce Chemical Co., Rockford, Ill.) for 1 h at 80°C. The reaction mixture was diluted to 1 ml with hexane, and 1

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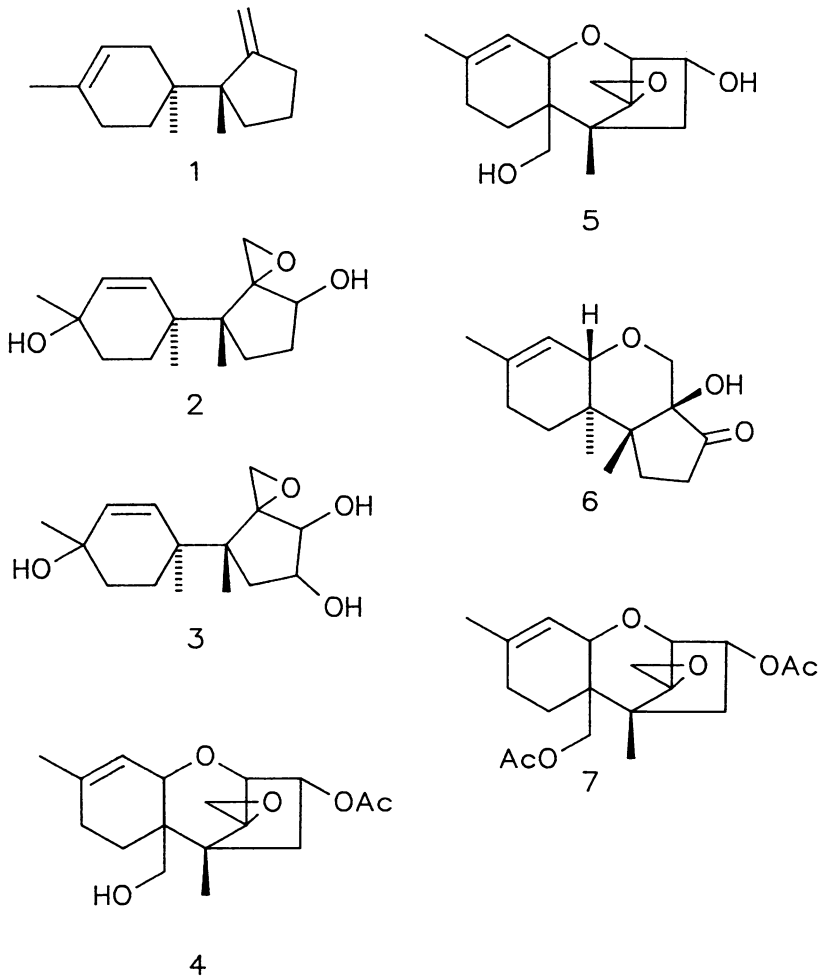

FIG. 1. Structures of compounds 1 through 7.

μl of this derivatized solution was analyzed by GC-MS. The column was a non-polar-bonded, fused silica capillary column (15 m by 0.2 mm; DB-1; J & W Scientific, Rancho Cordova, Calif.). Samples were injected in the splitless mode at 120°C. After 2 min, the column temperature was programmed to 200°C (40°C/min) and then to 270°C (10°C/min).

For thin-layer chromatography (TLC), 5 to 10 μl of the ethyl acetate extract was spotted on silica plates and the plates were developed in toluene-acetone (1:1). Spots were visualized by charring with sulfuric acid or by using nitrobenzyl pyridine spray reagent (13), which gives a bright blue spot for compounds containing the epoxytrichothecene nucleus.

Mass spectra were obtained on a Finnigan TSQ-46 tandem quadrupole mass spectrometer equipped with an Incos data system. Chemical ionization (CI) spectra were recorded with isobutane as the reagent gas at 0.3 torr (39.9 Pa) and 100°C. NMR experiments were performed on a Bruker WM-300 instrument. All ^1H NMR shifts are referenced to internal tetramethylsilane (TMS) (0.0 ppm). Reference standards of T-2 toxin, diacetoxyscirpenol (DAS), neosolaniol, and verrucarol were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Isolation procedures. A 4-liter culture of the mutant was extracted twice with equal volumes of ethyl acetate. The combined extracts were evaporated to dryness and resuspended in 20 ml of ethyl acetate. This extract was then separated on a preparative silica cartridge with a Waters Prepmaster liquid chromatograph. The column was eluted with CH_2Cl_2-methanol (98:2, vol/vol). Eighteen 500-ml fractions were collected, and the column was then washed with 500 ml of methanol. Separation was monitored by TLC, and fractions 7 and 8, which contained compound 4 (Fig. 1), were combined for additional purification. Compound 5 (Fig. 1) was found by TLC to be in the methanol wash (fraction 19). This fraction was reapplied to the silica cartridge and eluted with CH_2Cl_2-methanol (95:5). Fourteen 500-ml fractions were collected, and the column then was washed with 500 ml of methanol. Compound 5 was shown to be in fractions 4 and 5, which were combined for further purification.

Combined fractions containing compound 4 were purified further by high-pressure liquid chromatography on a Whatman PAC column. Separation of compound 4 was accomplished on an analytical column (20 cm by 4.6 mm) with detection of eluting components by differential refractometry. With hexane-ethyl acetate (60:40, vol/vol) as the solvent, compound 4 eluted as a peak with a retention of about 3 column volumes. Compound 6 (Fig. 1) eluted at ca. 1.5 column volumes. Preparative separations were made with a Magnum-9 column (50 cm by 9.4 mm), and 37 mg of compound 4 and 1 mg of compound 6 were isolated by repeated injections of portions of combined fractions 7 and

TABLE 1. EI and CI mass spectra of compounds 4 and 5 and their TMS derivatives

Compound	*m/z* (percent ionization)
EI	
4	43 (100), 91 (30), 105 (26), 109 (29), 122 (22), 140 (35), 159 (20), 171 (11), 175 (12), 189 (18), 199 (17), 217 (17), 235 (12), 262 (4), 265 (3), 278 (4), 290 (2)
5	43 (100), 91 (85), 109 (65), 123 (40), 140 (41), 159 (20), 171 (21), 175 (23), 189 (25), 199 (21), 221 (22), 236 (19), 248 (10), 266 (8)
4-TMS	43 (85), 73 (100), 91 (28), 103 (27), 105 (25), 187 (20), 199 (15), 262 (55), 290 (80)
5-TMS	73 (100), 91 (15), 103 (14), 106 (26), 181 (22), 197 (13), 212 (18), 217 (11), 277 (1), 292 (11), 305 (3), 307 (1), 320 (10)
Verrucarol-TMS	73 (100), 91 (11), 103 (17), 106 (15), 147 (15), 159 (21), 173 (21), 187 (10), 189 (11), 212 (4), 217 (9), 234 (3), 277 (7), 292 (1), 307 (8), 320 (1)
CI	
4	201 (50), 219 (30), 231 (27), 249 (45), 261 (20), 279 (77), 291 (89), 309 (100)-MH^+
5	201 (20), 219 (24), 231 (21), 237 (42), 249 (100), 267 (60)-MH^+
4-TMS	213 (11), 231 (17), 262 (8), 273 (12), 291 (30), 303 (11), 321 (13), 363 (17), 381 (100)-MH^+
5-TMS	199 (5), 213 (11), 231 (12), 273 (18), 303 (35), 321 (55), 339 (11), 393 (22), 411 (100)-MH^+
Verrucarol-TMS	199 (15), 213 (14), 231 (20), 277 (11), 285 (5), 295 (47), 303 (44), 321 (84), 339 (5), 393 (52), 411 (100)-MH^+

8. Similarly, 26 mg of compound 5 was isolated from combined fractions 4 and 5 above by using hexane-ethyl acetate (25:75, vol/vol) for elution.

RESULTS

F. sporotrichioides NRRL 3299 secretes T-2 toxin into the medium when grown in liquid shake cultures. Seven-day-old liquid cultures of this fungal strain produced T-2 toxin at a level of 100 to 200 μg/ml as determined by both GC analysis and by a competitive inhibition enzyme-linked immunoassay (1). In addition to accumulating 100 to 200 μg/ml of T-2, 7-day-old liquid cultures of NRRL 3299 also accumulated lower levels of neosolaniol and DAS (about 1 to 10 μg/ml). GC-MS of TMS derivatives from 7-day-old liquid cultures of the mutant MB2972 showed only a small amount of T-2 (about 1 to 2 mg/ml). No neosolaniol or DAS was detected. However, two components that eluted earlier in the analysis, at 6.0 and 6.5 min, were present at high levels (about 60 mg/ml each). Neither of the components accumulated by the mutant were observed in GC-MS of the TMS derivatives from NRRL 3299.

TLC of culture filtrate extracts from the mutant showed no intense spots for T-2 toxin, neosolaniol, or DAS. There were two components, not observed in TLC of the wild type, that developed bright blue spots when the plate was sprayed with the nitrobenzyl pyridine spray reagent (13). Small quantities of these two components, compound 4, R_f 0.75, and compound 5, R_f 0.5, were isolated by preparative TLC from a 7-day-old 25-ml liquid shake culture of the mutant. The TMS derivative of compound 4 eluted from the GC at 6.5 min. The TMS derivative of compound 5 eluted at 6.0 min.

The CI mass spectra (Table 1) of compound 4 had an intense protonated molecule observed at *m/z* 309, indicating a molecular weight of 308. In the electron ionization (EI) spectrum, the base peak was *m/z* 43, suggesting the presence of an acetate group. The CI spectrum also had an intense ion at *m/z* 249, indicating the loss of an acetate (60 daltons). The CI spectrum of compound 5 had an abundant protonated molecule at *m/z* 267, indicating a molecular size of 266; that was 42 daltons less than compound 4. These observations, along with the TLC R_fs, suggested that compound 4 was a monoacetate derivative of compound 5.

Base peaks in the CI spectra of TMS derivatives of the two components were protonated molecules at *m/z* 381 (compound 4) and 411 (compound 5), indicating that there were two derivatizable hydrogens on compound 5 and one on compound 4. These data were consistant with the proposal that these compounds had the 12,13-epoxytrichothec-9-ene nucleus, that compound 4 had a hydroxyl group and an acetate, and that compound 5 had two hydroxyl groups.

The epoxytrichothec-9-ene nucleus of T-2 toxin has undergone four additional oxygenations. They are the free hydroxyl at C-3, acetates at C-4 and C-15, and an isovalerate ester at C-8. The two trichothecenes, compounds 4 and 5, produced by the mutant appeared to be lacking two of these oxygens. The TMS derivative of compound 5, the dihydroxy trichothecene from the mutant, had a GC retention time ca. 1 min less than the retention time of the TMS derivative of verrucarol (4,15-diol 12,13-epoxytrichothec-9-ene), and it had a different mass spectrum in both EI and CI. If trichothecenes accumulated by the mutant are intermediates of the T-2 biosynthetic pathway, the likely possibilities for the dihydroxy trichothecene are a 3,15-, 3,8-, or 8,15-dihydroxy isomer.

Larger amounts of compounds 4 and 5 were purified from the filtrate of a 4-liter shake liquid culture of MB2972 to obtain sufficient material for NMR analysis and to establish their structures. ^1H and ^{13}C NMR spectra of compounds 4 and 5 in comparison with literature data (11, 12) clearly established the structures as 3,15-diol 12,13-epoxytrichothec-9-ene, 3-acetate and 3,15-diol 12,13-epoxytrichothec-9-ene (Table 2).

The structure of the minor compound 6, isolated during purification of compound 4, was revealed by its mass and ^1H NMR spectra, which matched those reported by Greenhalgh et al. (8) (Table 3).

DISCUSSION

Both compounds 4 and 5 have been previously reported as fungal secondary metabolites. Compound 4 was isolated and characterized along with calonectrin, compound 7 (Fig. 1), by Gardner et al. (7) in *Calonectria nivalis*. Greenhalgh et al. (8) reported the isolation of minor quantities of compound 5 along with the 15-acetoxy, 3-hydroxy isomer of compound 4 in the mother liquor from an extract of a liquid culture of *Fusarium culmorum* after crystallization of 3-acetyl-deoxynivalenol (3-acetyl-DON). This is the first report of compounds 4 and 5 as the major trichothecenes produced by a fungus and the first report of either as secondary metabolites of *F. sporotrichioides*.

TABLE 2. ^{1}H NMR and ^{13}C NMR data for compounds 4 and 5

NMR	ppm[a]	
	Compound 4	Compound 5
^{1}H proton		
H-2	3.73 d, J = 4.4	3.48 d, J = 4.4
H-3	5.15 td, J = 4.4, 10.5, 4.6	4.42 td, J = 4.5, 5.0, 10
H-4	2.28 dd, J = 4.6, 14.4	2.15 m
	2.18 dd, J = 10.5, 14.4	
H-7	1.93 td, J = 6.2, 6.0, 12.6	1.79 m
	1.90 td, J = 6.2, 2.1, 12.6	
H-8	2.04–2.18 cm	2.06 m
H-10	5.48 d, J = 5.4	5.50 d, J = 4.8
H-11	3.98 d, J = 5.4	4.10 d, J = 4.8
H-13	3.08 d, J = 4.0	3.07 d, J = 4.0
	2.87 d, J = 4.0	2.85 d, J = 4.0
H-14	0.92 s	0.90 s
H-15	3.67 bd, J = 11.4	3.68 d, J = 11.7
	3.48 bd, J = 11.4	3.45 d, J = 11.7
H-16	1.73 s	1.74 s
OH	1.59 bs	2.78 bs
Acetate	2.12 s	
^{13}C carbon		
C-2	77.99 d	79.8 d
C-3	71.4 d	69.1 d
C-4	39.23 t	42.1 t
C-5	45.16 s	45.6 s
C-6	44.09 s	44.1 s
C-7	20.77 t	20.8 t
C-8	28.38 t	28.5 t
C-9	140.36 s	140.6 s
C-10	119.15 d	119.1 d
C-11	68.25 d	68.6 d
C-12	65.20 s	65.7 s
C-13	48.51 d	48.5 d
C-14	12.36 q	12.6 q
C-15	62.52 t	62.6 t
C-16	23.18 q	23.3 q
Acetate	170.46 s; 20.93 q	

[a] Analyzed in $CDCl_3$ solution. Chemical shifts are reported as ppm relative to TMS, and coupling constants (J) are in hertz.

TABLE 3. Mass and ^{1}H NMR spectra of compound 6

Analysis	Spectrum
^{1}H NMR	
H-3	2.59 ddd, J = 3.0, 11.9, 20, 1H
	2.29 ddd, J = 8.0, 10.5, 20, 1H
H-4[a]	1.9–2.06 m
	1.76 ddd, J = 8, 13.5, 11.1
H-7	1.45 ddd, J = 2.0, 5.6, 13.3
H-8[a]	1.9–2.06 m
H-10	5.21 bs, 1H
H-11	3.96 bs, 1H
H-13	3.44 d, J = 11.3, 1H
	4.21 d, J = 11.2, 1H
H-14	0.66 s, 3H
H-15	1.15 s, 3H
H-15	1.65 bs, 3H
EI[b]	43 (100), 55 (62), 69 (95), 81 (70), 93 (94), 109 (62), 124 (53), 235 (50), 250 (70)
CI	109 (10), 125 (5), 127 (6), 187 (3), 215 (15), 233 (31), 251 (100, MH^+)

[a] Signals overlap.
[b] m/z (percent ionization).

NRRL 3299 normally accumulates small amounts of DAS which lacks oxygenation at C-8. Two mutants, MB1370 and MB1716, which are apparently blocked in their ability to oxygenate C-8, were identified previously (1). The mutant MB2972 accumulates only a small amount of T-2 and large amounts of compounds lacking oxygenation at both C-8 and C-4. This suggests that the block is at an earlier step in trichothecene biosynthesis than in the previously identified mutants. Comparison of the amounts of compounds 4 and 5 produced by MB2972 (about 60 μg/ml each) and the amount of T-2 produced by the wild-type parent NRRL 3299 (about 100 μg/ml) suggests that compounds 4 and 5 accumulate at the expense of T-2. Therefore, MB2972 may be defective in an enzymatic step in the T-2 biosynthetic pathway, and the small amount of T-2 produced by the mutant (about 1 to 5 μg/ml) may mean that the enzyme is impaired and only slightly functional or that more than one enzyme can perform the affected function.

One interpretation is the MB2972 is blocked or impaired in its ability to oxygenate C-4. It is curious that the acetate in compound 4 is on C-3, not C-15, since the parent strain accumulates only trichothecenes acetylated at C-15. Another possibility for the block in MB2972 is that the enzyme that normally acetylates C-15 is impaired and that acetylation of C-15 must precede oxygenation of C-4. If this is the block in the mutant, it is not obvious whether acetylation of C-3 is a necessary step in the biosynthesis of T-2 in *F. sporotrichioides*, with the acetate being removed later, or whether C-3 is acetylated only as a result of the mutation in MB2972. No production of a trichothecene with acetylation at C-3 by a strain of *F. sporotrichioides* has been reported previously.

Several pieces of circumstantial evidence suggest that, in *F. sporotrichioides*, acetylation of C-3 may normally be involved in biosynthesis of those trichothecenes that have a hydroxyl group on C-3. First, biosynthesis of other trichothecenes that have a hydroxyl group at C-3 proceed in this fashion. DON biosynthesis involves the production of 3-acetyl-DON, followed by a late deacetylation at C-3. Strains of *Fusarium graminearum* and *Fusarium roseum* grown on corn or rice produce both 3-acetyl-DON and DON (10). Miller et al. (11) reported that 3-acetyl-DON was detected first, followed by a later increase in the level of DON and a drop in the level of 3-acetyl-DON. The strain of *F. culmorum* used by the Canadian group to produce large amounts of DON only produced 3-acetylated DON in liquid culture (10). Second, research on microbial biotransformations by Yoshizawa and co-workers (15, 16) has shown the presence of enzymes in cultures of *Fusarium nivale*, *F. roseum*, and *Fusarium solani* that deacetylate C-3 of 3-acetyl-DON. In microsomal preparations (4) of *F. sporotrichioides* NRRL 3299, an enzymatic activity has been found that removes an acetate from C-3 of calonectrin and triacetoxyscirpenol (A. E. Desjardins and R. D. Plattner, unpublished data).

Experiments involving feeding various trichothecenes and precursors to MB2972 and the other mutants to clarify the site of the blockage in MB2972, determine whether there is any required order of the oxygenations in the biosynthetic pathway to T-2, and establish the role of acetylation of C-3 in the biosynthesis of trichothecenes by *F. sporotrichioides* are presently under way and will be reported separately.

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