Accumulation of Trichothecenes in Liquid Cultures of a *Fusarium sporotrichioides* Mutant Lacking a Functional Trichothecene C-15 Hydroxylase

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A mutant strain of *Fusarium sporotrichioides* NRRL 3299 produced by disruption of *Tri11*, a gene encoding a cytochrome P-450 monooxygenase, was shown to be altered in its ability to biosynthesize T-2 toxin. This mutant strain produced four trichothecenes that were not observed in cultures of the parent strain. The compounds were identified as isotrichodermin, 8-hydroxyisotrichodermin, 8-hydroxyisotrichodermol, and 3,4,8-trihydroxytrichothecene on the basis of their nuclear magnetic resonance and mass spectra. This is the first report of these 8-hydroxytrichothecenes as metabolites of *F. sporotrichioides*. The accumulation of isotrichodermin and the results of whole-cell feeding experiments with a *Tri11*[−] strain confirm that oxygenation of C-15 is blocked.

Trichothecenes are sesquiterpene epoxides that are produced by several *Fusarium* species. Trichothecenes are derived from the parent hydrocarbon trichodiene. The biosynthesis of T-2 toxin in *Fusarium sporotrichioides* has been studied with the aid of mutant strains blocked at specific steps in the trichothecene pathway (7–9, 11–13). Analysis of the trichothecenes produced by mutant strains, together with whole-cell feeding experiments with suspected pathway intermediates, suggests that there is an ordered sequence of cyclizations, oxygenations, and acetylations in the conversion of trichodiene to T-2 toxin.

Labeling studies have demonstrated that at least six oxygenations of T-2 toxin utilize molecular oxygen-dependent enzymes (3). One of the genes (*Tri4*) encoding a cytochrome P-450 monooxygenase has been previously reported, and transformants lacking a functional *Tri4* gene accumulate the parent hydrocarbon trichodiene (9). A second cytochrome P-450 monooxygenase gene, *Tri11*, has recently been identified in the trichothecene gene cluster (1). We report here the isolation and chemical characterization of the trichothecene metabolites accumulated by a *Tri11*[−] mutant strain.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type strain used in this study is a T-2 toxin-producing strain, *F. sporotrichioides* NRRL 3299. Strain A11b is a mutant produced by disruption of the *Tri11* gene, as described elsewhere (1). Strain F15 is a mutant produced by disruption of *Tri4* (9). Mutant strains were maintained on slants of V-8 juice agar with hygromycin B. Conidial suspensions were prepared from strains grown on V-8 juice agar plates for 4 to 7 days and used to inoculate glucose-yeast extract-peptone (GYEP) liquid medium as described previously (11, 17). Rice cultures were prepared by inoculating rice in 2.8-liter Fernbach flasks with 2-day-old GYEP liquid cultures (11 ml/333 g of rice). The inoculated rice was incubated in the dark for 7 days at 28°C.

Whole-cell feeding experiments. Liquid cultures were inoculated with conidia washed from the V-8 plates, at a starting density of 5×10^4 conidia/ml in 10 ml of GYEP medium in a 50-ml Erlenmeyer flask, and incubated on a gyratory shaker (200 rpm) at 28°C. After 24 h, a 25 mM stock solution of the trichothecene in acetone was added to the culture to a final concentration of 250 μ M (1% acetone). Control cultures had acetone added to reach a final concentration of 1% acetone. Cultures were incubated on a rotary shaker at 28°C for an additional

5 days and were then extracted with ethyl acetate and analyzed by gas-liquid chromatography (GLC).

Extraction and isolation of compounds. Liquid cultures (eight, each 100 ml) were combined and extracted twice with equal volumes of ethyl acetate. The combined extracts were concentrated under reduced pressure. The syrup was separated on a silica gel column eluted with dichloromethane-methanol (19:1). Twelve 40-ml fractions were collected and separation was monitored by thin-layer chromatography (TLC) and GLC. Fractions 2 and 3 contained isotrichodermin (compound 1) and were combined and further purified on TLC plates developed in hexane-ethyl acetate (2:1). Fractions 4 and 5 contained 8-hydroxyisotrichodermin (compound 2) and T-2 toxin and were further separated on TLC plates developed in ether-hexane (4:1). Fractions 7 to 12 contained 8-hydroxyisotrichodermol (compound 3) and apotrichodiol and were further separated on a small silica gel column eluted with hexane-2-propanol (3:1).

Rice cultures were extracted by soaking the rice overnight with ethyl acetate. The concentrated extracts were dissolved in a small volume of dichloromethane and filtered through a pad of silica gel 60 (70/230 mesh; E. Merck AG, Darmstadt, Germany) on a Büchner funnel with a fritted disk and washed successively with dichloromethane and methanol. The methanol wash was concentrated under reduced pressure and separated initially on a silica gel column eluted with dichloromethane-methanol (9:1). Fractions were monitored by TLC and GLC and further separated by open-column chromatography and preparative TLC as described above. Compound 3,4,8-trihydroxytrichothecene was purified on TLC plates developed in hexane-ethyl acetate-methanol (11:11:3).

Trichothecene toxin assays. For time course and feeding experiments, liquid cultures were analyzed for trichothecene toxins by GLC. A 5-ml sample from 25 ml of liquid shake culture in GYEP medium was transferred to a test tube and mixed with 2 ml of ethyl acetate by vortexing (60 s). The layers were separated by centrifugation. The organic layer was removed, and a 1- or 2- μ l sample was analyzed by GLC. Concentrations of trichothecenes were determined with appropriate standard curves. Compound identifications were confirmed by GC-mass spectrometry (MS).

General physical and chemical methods. Filtration and open-column chromatography were done on silica gel 60, and TLC was done on silica gel 60-F254 plates (5 by 10 cm or 20 by 20 cm, 0.25 mm thick; Merck). Fractions were visualized under a shortwave UV lamp. Trichothecenes were detected with the 4-(*p*-nitrobenzyl)pyridine-tetraethylenepentamine detection system as described previously (16) but with methanol solutions of the two reagents instead of carbon tetrachloride-chloroform solutions. Acetate derivatives were prepared with pyridine and acetic anhydride.

GLC measurements were made by flame ionization detection with a Hewlett-Packard 5890 gas chromatograph fitted with a 30-m fused silica capillary column (DB-1; diameter, 0.25 μ m; J&W Scientific Co., Palo Alto, Calif.). For routine screening of a trichothecene toxin phenotype, the column was held at 120°C at injection, then heated to 210°C at 15°C/min and held for 1 min, and then heated to 260°C at 5°C/min and held for 8 min. Low-resolution mass spectra were obtained by GC-MS with a Hewlett-Packard 5891 mass-selective detector fitted with a DB-5 MS column (15-m length, 0.25-mm film thickness).

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FIG. 1. Chromatogram of *F. sporotrichioides* liquid culture extracts analyzed by GLC. Cultures were grown on GYEP medium for 7 days. (A) NRRL 3299 (*Tri11⁺*). The locations of T-2 toxin, diacetoxyscirpenol (DAS), and apotrichodiol are indicated. (B) Strain A11b (*Tri11⁻*). The locations of isotrichodermin, 8-hydroxyisotrichodermin, 8-hydroxyisotrichodermol, 3,4,8-trihydroxytrichothecene, T-2 toxin, and apotrichodiol are indicated.

RESULTS AND DISCUSSION

Trichothecene profile. *F. sporotrichioides* NRRL 3299 GYEP cultures produce a number of closely related trichothecenes in liquid culture: T-2 toxin, neosolaniol, propylneosolaniol, butylneosolaniol, and diacetoxyscirpenol as well as the modified trichothecene apotrichodiol (Fig. 1A). T-2 toxin typically constitutes 60 to 80% of the total trichothecenes (100 to 250 $\mu\text{g/ml}$) produced by 7-day-old liquid shake cultures of this strain. Seven-day-old GYEP cultures of *F. sporotrichioides* A11b produced only a small amount of T-2 toxin (less than 10 $\mu\text{g/ml}$), and the other trichothecenes normally produced by the parent strain were not detected. However, three additional peaks were present in GLC traces of A11b liquid culture extracts (Fig. 1B). TLC analysis of extracts of seven-day-old cultures showed the presence of three components that gave a blue reaction with 4-(*p*-nitrobenzyl)pyridine-tetraethylenepentamine spray reagent, characteristic of epoxide-containing compounds.

The EI mass spectrum of compound 1 exhibited a molecular ion at m/z 292 consistent with a trichothecene with one acetoxy moiety. Compound 1 was identified as isotrichodermin (Fig. 2a) by comparison with a mass spectral library and published nuclear magnetic resonance (NMR) and mass spectral data. Production of isotrichodermin has been previously reported for a number of *Fusarium* species (5, 6, 15), and this compound was detected as a minor component in liquid cultures of *F. sporotrichioides* O2 (a *Tri3⁻* mutant that accumulates decalonectrin and didcalonectrin in liquid culture) (13). Isotrichodermin has been shown to be a key intermediate in *Fusarium culmorum* biosynthesis of 3-acetyldeoxynivalenol (18).

The EI mass spectrum of compound 2 exhibited a molecular ion at m/z 308 consistent with a trichothecene with one hydroxyl and one acetoxy substituent but differed significantly

FIG. 2. Structures of isotrichodermin (a), 8-hydroxyisotrichodermin (b), 8-hydroxyisotrichodermol (c), and 3,4,8-trihydroxytrichothecene (d).

from the mass spectrum of 15-decalonectrin (14), which has a characteristic fragment at m/z 140. The mass spectrum of compound 2 had prominent ions at m/z 123 and 109. The proton NMR spectrum displayed characteristic epoxide doublets at δ 2.87 and 3.06, an acetyl methyl signal, and three additional methyl signals, which confirmed that compound 2 was a trichothecene with one acetyl group and that no oxygen substituent was attached to C-15. The chemical shift of the double doublet at δ 5.16, which was assigned to the C-3 proton, suggested that the acetyl group was at C-3. The chemical shift of the A-ring proton doublet (δ 4.10) indicated that the second hydroxy group was at the C-8 position. Compound 2 was identified as 8-hydroxyisotrichodermin (Fig. 2b). Production of this trichothecene has been reported previously for other *Fusarium* species, including *Fusarium crookwellense* and *F. roseum* (5, 15).

The EI mass spectrum of compound 3 exhibited a molecular ion at m/z 266. The fragmentation pattern of compound 3 was similar to that of 8-hydroxyisotrichodermin, with prominent peaks at m/z 109 and 123. The proton NMR spectrum also corresponded to that of 8-hydroxyisotrichodermin, differing in the chemical shift of the C-3 proton and the absence of an acetyl methyl signal. Acetylation of compound 3 with acetic anhydride and pyridine resulted in two products, 8-hydroxyisotrichodermin and 8-acetoxyisotrichodermin. Compound 3 was therefore identified as 8-hydroxyisotrichodermol (Fig. 2c). This trichothecene has been reported as a very minor constituent of *F. crookwellense* (10), but full spectral data have not yet been reported.

Solid media, such as rice or corn grits, have been used to produce large quantities of fungal metabolites. GC analysis of extracts from A11b rice cultures indicated that the same three trichothecenes found in liquid cultures, isotrichodermin, 8-hydroxyisotrichodermin, and 8-hydroxyisotrichodermol, were produced, although 8-hydroxyisotrichodermol was the major constituent (70% trichothecene fraction). An additional trichothecene that was detected as a minor component in liquid cultures was isolated from extracts of rice cultures and characterized.

The migration of compound 4 during TLC indicated that it was a more polar compound than the three trichothecenes

isolated from liquid cultures. The EI mass spectrum of compound 4 did not exhibit an apparent molecular ion but had a fragmentation pattern similar to that of 8-hydroxyisotrichodermin and 8-hydroxyisotrichodermol, with prominent fragments at m/z 109 and 123. The mass spectrum of the acetylated derivative had an ion at m/z 348 (M-60) consistent with a trichothecene with three acetoxy groups. The proton NMR of compound 4 displayed characteristic epoxide doublets at δ 2.9 and 2.76 and three methyl signals but no acetyl methyl signals, suggesting that compound 4 was a trichothecene with three hydroxyl groups. The appearance of a doublet at δ 4.03, assigned to C-8, and a doublet and double doublet at δ 2.18 and 1.69, assigned to the C-7 protons, confirmed that there was one A-ring hydroxyl group at C-8. The appearance of the H-3 double doublet at δ 5.19 and the H-4 doublet at δ 5.57 in the proton NMR of the triacetate confirmed the presence of oxygen substituents at C-3 and C-4. Compound 4 was therefore identified as a new trichothecene, 3,4,8-trihydroxytrichothecene (Fig. 2d).

This is the first report of 8-hydroxyisotrichodermin, 8-hydroxyisotrichodermin, and 3,4,8-trihydroxytrichothecene from *F. sporotrichioides*. Biosynthesis of T-2 toxin in *F. sporotrichioides* appears to proceed in a directed order with oxygenation at C-3 followed by C-15, then C-4, and then C-8 (4). As a result, most trichothecenes produced by these strains, if oxygenated at C-8, are also oxygenated at C-3, C-4, and C-15. However, production of some compounds not fitting this pattern has been reported for wild-type and mutant strains. Cultures of wild-type *F. sporotrichioides* grown on corn grits produced two compounds with 8-hydroxylation, sporotrichiol and 8-hydroxytrichothecene, as minor constituents (2, 14). Cultures of an *F. sporotrichioides* UV mutant, MB2972, grown on rice produce several uncyclized trichothecenes, including 2,3,11-isotrichotriol, 2,3,9-trichotriol, and 2,3,8,11-isotrichotetraol (13). It has been previously shown (11) that trichotriol and isotrichotriol will cyclize both enzymatically and nonenzymatically to isotrichodermin in acidic medium. Since the pH of GYEP cultures of A11b dropped from 5.3 at the time of inoculation to 4.0 after 7 days, cultures were examined for the transient production of isotrichotriols and isotrichotetraols, but none were detected. In addition, isotrichotetraol was added to acidified GYEP medium to determine if nonenzymatic conversion of isotrichotetraol to 8-hydroxyisotrichodermin was possible. No 8-hydroxyisotrichodermin was detected, nor has it been isolated from cultures which produced isotrichotetraols, suggesting that hydroxylation at C-8 may occur either before or after cyclization but that cyclization of isotrichotetraol does not occur.

To investigate whether additional trichothecene components were produced transiently in liquid cultures of A11b, the strain was grown on GYEP medium for 10 days and samples were removed periodically and analyzed by GLC. Figure 3 shows the production of isotrichodermin, 8-hydroxyisotrichodermin, 8-hydroxyisotrichodermol, apotrichodiol, and T-2 toxin over the 10-day time course. Only isotrichodermin and 8-hydroxyisotrichodermin were detected at 24 h, and the level of 8-hydroxyisotrichodermin peaked at 48 h. The results indicate that 8-hydroxyisotrichodermol may be the deacetylation product of 8-hydroxyisotrichodermin. No significant deacetylation of isotrichodermin was detected.

The accumulation of isotrichodermin in the *Tri11⁻* mutant suggested that oxygenation of C-15 was blocked and that *Tri11* controls conversion of isotrichodermin to 15-decalonecitrin. To test this hypothesis, cultures of A11b were amended with solutions of 15-decalonecitrin or 4,15-diacetoxyscirpenol. Both of

FIG. 3. Time course experiment. Cultures were grown on GYEP medium for 10 days and analyzed by GLC. ●, isotrichodermin; ■, 8-hydroxyisotrichodermin; ○, apotrichodiol; ×, T-2 toxin; ▲, 8-hydroxyisotrichodermol.

these compounds were efficiently converted to T-2 toxin by A11b within 3 days.

To determine if the 8-hydroxy trichothecenes produced by A11b could be converted to T-2 toxin, cultures of *F. sporotrichioides* F15 (a *Tri4⁻* mutant which accumulates only trichodiene) (9) were amended with solutions of either 8-hydroxyisotrichodermin or 8-hydroxyisotrichodermol. No T-2 toxin production was observed in either case, although 8-hydroxyisotrichodermol was completely converted to 8-hydroxyisotrichodermin.

The trichothecene profile of *F. sporotrichioides* A11b confirms that oxygenation at C-15 is blocked in the *Tri11⁻* mutant. The 8-hydroxylated trichothecenes that were isolated do not appear to be biosynthetic intermediates of T-2 toxin. The variety of compounds that have been reported with C-8 oxygenation suggests that there is not a strong substrate specificity for C-8 oxygenation. Oxygenation at C-4 appears to be more specific in *Fusarium* with calonectrin acting as the substrate. Although feeding experiments indicated that 8-hydroxyisotrichodermin and 8-hydroxyisotrichodermol were not on the T-2 toxin pathway, the appearance of low levels of T-2 toxin in A11b liquid cultures suggests that other oxygenases are able to complete the pathway through C-15 oxygenation.

APPENDIX

8-Hydroxyisotrichodermin (compound 3). EI mass spectrum (70 eV), m/z (relative intensity): 266 (0.1, M^+), 207 (0.6), 182 (2.8), 161 (5.6), 217 (16.8), 123 (89), 109 (100), 95 (41.7). EI mass spectrum of diacetate: 350 (0.1, M^+), 308 (0.9), 292 (0.7), 135 (18.1), 122 (100), 106 (52.4), 95 (25.5), 91 (47.7). 1H NMR (300 MHz, $CDCl_3$): δ 5.62 (1H, d, J = 5.8 Hz, H-10), 4.47 (1H, m, H-3), 4.19 (1H, d, J = 5.8 Hz, H-11), 4.12 (1H, d, J = 5.5 Hz, H-8), 3.48 (1H, d, J = 4.6 Hz, H-2), 3.06 (1H, d, J = 4.0 Hz, H-13a), 2.87 (1H, d, J = 4.0 Hz, H-13b), 2.22 (1H, dd, J = 14.3 Hz and 5.7 Hz, H-7a), 2.02 (2H, m, H-4a and H-4b), 1.87 (3H, s, 16-Me), 1.70 (1H, d, J = 14.3 Hz, H-7b), 0.98 (3H, s, 15-Me), 0.76 (3H, s, 14-Me). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 138.8 (C-9), 121.9 (C-10), 79.8 (C-2), 71.5 (C-3), 69.4 (C-11), 68.1 (C-8), 65.7 (C-12), 48.6 (C-13), 45.9 (C-5), 41.0 (C-4), 39.6 (C-6), 33.6 (C-7), 20.6 (C-16), 19.1 (C-15), 11.2 (C-14).

3,4,8-Trihydroxytrichothecene (compound 4). EI mass spectrum (70 eV), m/z (relative intensity): 205 (4.8), 187 (10.5), 123 (100), 109 (63), 95 (33). 1H NMR (300 MHz, CD_3OD): δ 5.51 (1H, d, J = 5.9 Hz, H-10), 4.08 (2H, m, H-3, H-4), 4.03 (1H, d, J = 5.4 Hz, H-8), 3.93 (1H, d, J = 5.9 Hz, H-11), 3.39 (1H, d,

$J = 4.3$ Hz, H-2), 2.90 (1H, d, $J = 4.1$ Hz, H-13a), 2.76 (1H, d, $J = 4.1$ Hz, H-13b), 2.18 (1H, dd, $J = 14.3$ Hz and 5.7 Hz, H-7a), 1.82 (3H, s, 16-Me), 1.69 (1H, d, $J = 14.3$ Hz, H-7b), 1.00 (3H, s, 14-Me), 0.75 (3H, s, 15-Me). ^{13}C NMR (75.5 MHz, CDCl_3): δ 140.6 (C-9), 122.4 (C-10), 81.2, 81.1, 80.8 (C-2, C-3, C-4), 72.6 (C-11), 68.1 (C-8), 47.3 (C-5), 41.4 (C-6), 34.9 (C-7), 20.9 (C-15), 19.1 (C-16), 6.35 (C-14).

Triacetate. EI mass spectrum: 366 (0.3), 348 (0.3), 185 (5.2), 173 (10.2), 122 (100), 95 (37.7), 91 (38.3). ^1H NMR (300 MHz, CD_3OD): δ 5.71 (1H, d, $J = 4.7$ Hz, H-10), 5.57 (1H, d, $J = 3.4$ Hz, H-4), 5.24 (1H, d, $J = 5.5$ Hz, H-8), 5.19 (1H, dd, $J = 4.9$ Hz and 3.4 Hz, H-3), 3.97 (1H, d, $J = 5.9$ Hz, H-11), 3.83 (1H, d, $J = 4.9$ Hz, H-2), 3.04 (1H, d, $J = 3.9$ Hz, H-13a), 3.81 (1H, d, $J = 3.9$ Hz, H-13b), 2.14 (3H, s, acetate methyl), 2.11 (3H, s, acetate methyl), 2.06 (3H, s, acetate methyl), 1.74 (3H, br-s, 16-Me), 1.06 (3H, s, 14-Me), 0.64 (3H, s, 15-Me).

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