 Biosynthesis of the Trichothecene 3-Acetyldeoxynivalenol

IS ISOTRICHODERMIN A BIOSYNTHETIC PRECURSOR?*

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3-Acetyldeoxynivalenol is the major trichothecene produced by the fungus Fusarium culmorum. The first proven tricyclic intermediate in the biosynthesis of 3-acetyldeoxynivalenol has been shown by in vivo studies to be isotrichodermin, a natural metabolite of F. culmorum. Indeed, the feeding of ring-deuterated isotrichodermin resulted in ring-deuterated 3-acetyldeoxynivalenol as shown by NMR studies. In this work, we have shown that the 3-acetyl group of isotrichodermin is mostly lost in its metabolism to 3-acetyldeoxynivalenol. We have shown by two different approaches that the deacetylation occurs at an early step after the first oxygenation step at C-15. Derivatives of isotrichodermin lacking the 3-acetyl such as 3-deacetyl isotrichodermin or 3-oxo-12,13-epoxytrichothec-9-ene are not precursors to 3-acetyldeoxynivalenol. The role of this acetyl exchange mechanism is not clear presently.

Fusarium culmorum has been shown to produce two main secondary metabolites (Fig. 1): 3-acetyldeoxynivalenol (3-ADN)1 and sambucinol (SOL) (1, 2). The biosynthesis of these two trichothecenes has been shown to differ at the tricyclic stage (3). Isotrichodermin (ITD), a natural metabolite (4), has been shown to be the major biosynthetic precursor of 3-ADN, whereas 12,13-epoxytrichothec-9-ene (EPT) is converted only to SOL (Fig. 1). There seems to be no metabolic interconversion between EPT and ITD (3). In addition, many of the oxidative metabolic products of isotrichodermin were elucidated (5). Preliminary data on cell-free experiments have been reported (6–8).

We have recently investigated the fate of the 3-acetyl group in isotrichodermin conversion to 3-acetyldeoxynivalenol. It has always been assumed without any proof that the 3-acetyl in 3-acetyldeoxynivalenol originates from the 3-acetyl in isotrichodermin. This was based on the seemingly textbook definition of a biosynthetic precursor: it was produced by F. culmorum cultures (4), it was 27% incorporated into 3-acetyldeoxynivalenol, and the incorporation site was rigorously determined by 2H NMR of ring-deuterated isotrichodermin feedings (3). In order to ensure that we could use the easily synthesized [1-14C]acetyl isotrichodermin as a marker to isolate enzymes, we decided to confirm that indeed the 3-acetyl in isotrichodermin is retained in its conversion to 3-acetyldeoxynivalenol. We obtained surprising results, which will be discussed here.

EXPERIMENTAL PROCEDURES

Instrumentation—High performance liquid chromatography (HPLC) was performed on a Perkin-Elmer series 3B instrument coupled to an LC-75 variable wavelength detector (Perkin-Elmer) set at 224 nm and a Berthold LB 505 HPLC radioactivity monitor (Labserco, Oakville, Ontario). Thin layer chromatography separations using LHP-KF thin layer chromatography plates (Whatman) were analyzed with a Bioscan Imaging Scanner System 200 (Bioscan, Inc., Washington, D.C.). A Tracor Analytic Delta 300 instrument was used for liquid scintillation counting. Homogenization of F. culmorum cells was accomplished with a Bead-Beater (Biospec Products, Bartlesville, OK). Centrifugation was done using a Dupont-Sorvall SC centrifuge with an SS-34 rotor and a Beckman Instruments L5-75 ultracentrifuge with a 60 Ti rotor. A Perkin-Elmer model 559A uv-visible spectrophotometer equipped with a digital temperature controller was used for all spectrophotometric measurements. Proton NMR spectra were run on JEOL CPF-270 (270 MHz), while the deuterium NMR spectra were run on XL-300 operating at 64 MHz. The samples (2–5 mg) were dissolved in CDCl3 (in CHCl3 for deuterium NMR), and their spectra were recorded at ambient temperature (22 °C). For routine 1H NMR, a 45° pulse was used with an acquisition time of 4 s (no relaxation delay). The data were zero-filled, and resolution enhancement techniques were used to help resolve small couplings. The two-dimensional matrix was 1024 × 1024 data points after processing. The data were pseudoecho-shaped and symmetrized prior to plotting. The 1H NMR spectra were acquired using 80° pulse and 1-s acquisition times. The data were zero-filled and processed with 1-Hz line broadening. The solvent (CHCl3), used as internal reference, was set at 7.26 ppm.

Strain and Cultivation Conditions—F. culmorum strain HLX 1503 was grown as described previously (9). The seed cultures (50 ml of seed medium in a 250-ml Erlemeyer flask) were incubated for 3 days. The production cultures (25 ml of production medium in a 125-ml Erlemeyer flask) were incubated for 2 days.

High Performance Liquid Chromatography—Analytical HPLC was performed with two Whatman partisl 10 ODS-2 analytical columns in series (4.6 × 500 mm). Semipreparative HPLC was performed with two Whatman partisl 10 ODS-2 Mag 9 semipreparative columns in series (9.4 × 500 mm). Preparative HPLC was performed with one partisl 10 ODS-2 MAG-20 preparative column (22 × 500 mm). Program 1 consisted of a linear gradient that lasted for 50 min with an initial concentration of 15% methanol, 85% water and a final concentration of 75% methanol, 25% water, which was then held for 40 min. All ultraviolet detectors were set at 204 nm. Program 2 consisted of a linear gradient lasting 50 min with an initial concentration of 15% methanol, 85% water and a final concentration of 75% methanol, 25% water, which was held for 30 min and then increased linearly for 10 min to 100% methanol. The concentration was held for 20 min at 100% methanol before equilibrating to 15% methanol, 85% water. Program 3 consisted of a linear gradient from 15% methanol, 85% water to 75% methanol, 25% water over 25 min. This concentration of methanol was held for 20 min and then increased linearly for 5 min to 100% methanol and maintained for 20 min.

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‡ The abbreviations used are: 3-ADN, 3-acetyldeoxynivalenol; SOL, sambucinol; EPT, 12,13-epoxytrichothec-9-ene; HPLC, high performance liquid chromatography; ITD, isotrichodermin; DON, deoxynivalenol; tR, retention time.
Is Isotrichodermin a Biosynthetic Precursor to 3-ADN?

![Diagram of 3-ADN and Fusarium culmorum cells](image)

**Fig. 1.** Tricyclic biosynthetic precursors to 3-acetyldeoxynivalenol and to sambucinol, the major metabolites produced by *F. culmorum.*

**Fig. 2.** Radiolabeled *in vivo* experiments. The location on the rings of the $^{14}$C label is indicated by $\bullet$ and on the 1'-acetate by $\star$. The relative size of the structures emphasizes the ratios obtained.

**[11,14-C]Acetyl Isotrichodermin (Fig. 2)—[11,14-C]Acetyl isotrichodermin was prepared and purified according to Ref. 6. The radioactivity of the pure [11,14-C]acetyl isotrichodermin was $7.2 \times 10^4$ dpm, and the specific activity was $9.1 \times 10^3$ dpm/mg or $2.53 \times 10^3$ dpm/μmol.

**Purification of [4,8,14-$^{14}$C]Isotrichodermin (Fig. 2)—Eight 54-h-old 25-ml production cultures of *F. culmorum* were each fed $2.7 \times 10^9$ dpm of [3R]-[2-$^{14}$C]mevalonate (2.3 GBq/mmol) and then were incubated for another 20 h (9). The [4,8,14-$^{14}$C]isotrichodermin was isolated as described previously (3). The purified [4,8,14-$^{14}$C]isotrichodermin and standard isotrichodermin were both subjected to HPLC with 65% methanol, 35% water as eluting solvent. The $R_f$ of standard isotrichodermin was 57.6 min, and the $R_f$ of [4,8,14-$^{14}$C]isotrichodermin was 57.4 min. Finally, both standard isotrichodermin and [4,8,14-$^{14}$C]isotrichodermin (20 μl/1000 μl) were spotted on a LHP-KF plate. The plate was developed with ethyl acetate/methanol (98:2) and then analyzed with the Bioscan Imaging Scanner System. One symmetrical radioactive peak was detected, which had an $R_f$ of 0.67. The standard isotrichodermin was detected with iodine vapor and had an $R_f$ of 0.66. The yield of [4,8,14-$^{14}$C]isotrichodermin was $2.6 \times 10^4$ dpm.

**Feeding of [11-$^{14}$C]Acetyl Isotrichodermin and [4,8,14-$^{14}$C]Isotrichodermin to a F. culmorum Production Culture (Fig. 2)—Both [11-$^{14}$C]acetyl ITD (6.5 $\times 10^5$ dpm) and [4,8,14-$^{14}$C]ITD (1.9 $\times 10^5$ dpm) in methanol were added to a sterile 125-ml Erlenmeyer flask. The methanol was evaporated under nitrogen, and 0.1 ml 5% Brij 35 was added. Next, a 55-h-old 25-ml production culture of *F. culmorum* was added, and the culture was shaken at 220 rpm in the dark at 25°C for an additional 70 h. The culture medium was extracted on two 1020 Cheme-lut tubes with 8 $\times$ 12 ml of ethyl acetate for each tube.

**Purification of 3-ADN from the [11-$^{14}$C]Acetyl ITD and [4,8,14-$^{14}$C]ITD Feeding (Fig. 2)—The extract was fractionated by analytical HPLC on program 1 at 1 ml/min, and the peak corresponding to 3-ADN was detected, which had an $R_f$ of 0.67. The standard isotrichodermin with 8 $\times$ 12 ml of ethyl acetate for each tube.

**Hydrolysis of a Mixture of [11-$^{14}$C]Acetyl ITD and [4,8,14-$^{14}$C]ITD—**Two aliquots of the mixture were injected individually onto the HPLC, and the radioactivity collected under the acetyl ITD region was 1941 dpm. NMR analysis confirmed the structure to be that of 3-ADN.

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**TABLE I**

Proton and deuterium NMR analyses of the deuterated isotrichodermin derivatives lacking an acetyl at C-3:

| Deuterated isotrichodermin derivative | $\delta^H$ | $\delta^D$ |
|--------------------------------------|----------|-----------|
| $[4,8,15-2H3]$-deacetyl isotrichodermin (1) | 3.84 (s) | 3.25 (s) |
| $[4,8,15-2H3]$-deacetyl 3-oxo-12,13-epoxytrichothec-9-ene (3) | 3.42 (d) | 3.05 (d) |

(See Table 1 for more details.)

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The letters s, t, q, and m following the signals stand for singlet, doublet, triplet, and multiplet signals. br and o show broad or overlapping lines.

**Two deuterium signals appear in the deuterium spectrum, one at 1.99 ppm or 2.51 ppm for D-4 (integrating for one deuterium) and one signal at 0.74 ppm or 0.78 ppm for D-15A and -B protons (integrating for one deuterium together) for 1 and 3, respectively.

**Deuterated isotrichodermin derivatives synthesized.** 1. 
[4,8,15-2H3]-deacetyl isotrichodermin; 2. [2-$^{14}$C]-[4,8,15-2H3]-isotrichodermin; 3. [4,8,15-2H3]-oxo-12,13-epoxytrichothec-9-ene. The deuteriums at C-4 and C-15 and the deuterated acetyl are indicated by a shaded circle.

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$^{10}$ The letters s, d, t, q, and m following the signals stand for singlet, doublet, triplet, quartet, and multiplet signals. br and o show broad or overlapping lines.

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dpm for one injection and 597 dpm for the second injection. Two aliquots of the mixture were hydrolyzed. The radioactivity collected under the deacyl ITD region was 567 dpm for the first injection and 164 dpm for the second injection. From these data the average ratio of 3.5:1 for radioactivity in [14C]acetyl ITD to radioactivity in [4,8,14-14C]ITD was calculated.

Synthesis of [4\(\beta\),15-2H]3-Deacetyl ITD (Fig. 3)—The [4\(\beta\),15-2H]3-deacetyl ITD (1) was prepared as described previously (3) and was subsequently used for the synthesis of [2\(^-\)\(^2\)H\(_3\), 4\(\beta\),15-2H] ITD (2) and of [4\(\beta\),15-2H]3-oxo-EPT (3, Fig. 3). The \(^1\)H and \(^2\)H NMR of 1 is shown in Table I.

Synthesis of [2\(^-\)\(^2\)H\(_3\), 4\(\beta\),15-2H]ITD (Fig. 3)—A solution of [4\(\beta\),15-2H]3-deacetyl ITD (1) (0.061 g; 0.24 mmol) in 3.6 ml of pyridine was treated with 4-dimethylaminopyridine (0.008 g; 0.065 mmol) and [\(^2\)H\(_6\)]acetic anhydride (0.064 ml; 0.64 mmol). The acetylation was completed after stirring at 25 °C for 90 min. The pyridine was evaporated with heptane. The residue was flash-chromatographed on silica (10) using hexane/ethyl acetate (80:20) as the eluting solvent. [2\(^-\)\(^2\)H\(_3\), 4\(\beta\),15-2H]ITD (2) was obtained (0.070 g; 97%) as a white crystalline powder (m.p.: 145.5–147°C) (Fig. 3, Table II).

Synthesis of [4\(\beta\),15-2H]3-Oxo-EPT (Fig. 3)—A solution of [4\(\beta\),15-2H]3-deacetyl ITD (1) (0.014 g; 0.055 mmol) in 2.2 ml of dry dichloromethane was cooled to 0°C and was treated with pyridinium chlorochromate (0.050 g; 0.23 mmol). The oxidation was completed after stirring at 0 °C for 30 min and at 25 °C for 60 min. The heterogeneous mixture was filtered through a column of dry silica gel, and the desired products were eluted with dichloromethane.

**Table II**

Proton and deuterium NMR analyses of doubly deuterated isotrichodermin, [2\(^-\)\(^2\)H\(_3\), 4\(\beta\),15-2H]ITD (2) and its unlabeled counterpart, ITD

| Position | \(\delta \) \(^1\)H | \(\delta \) \(^2\)H |
|----------|-----------------|-----------------|
| 2        | 3.75 (d, J=4.6) | 2.11            |
| 3        | 5.18 (m, J=16.0)|                 |
| 4        | 2.15 (dd, J=3.4, 14.7)| 2.08 (d, J=14.7) |
| 5        | 1.32 (m)         |                 |
| 6        | 1.83 (d)         |                 |
| 7        | 5.87 (m, J=3.3)  |                 |
| 8        | 3.96 (m, J=1.5)  |                 |
| 9        | 2.09 (d, J=4.0)  |                 |
| 10       | 2.86 (d, J=6.0)  | 0.85            |
| 11       |                 | 0.82 (s)        |
| 12       |                 | 1.71 (br s)     |
| 13a      |                 |                 |
| 13b      |                 |                 |
| 14       |                 | 0.72 (s)        |
| 15       |                 | 0.82 (s)        |
| 16       |                 | 1.71 (br s)     |
| 17Ac     |                 | 2.11 (s)        |

The shaded areas represent the deuterated positions. The open circles indicate unlabeled acetate.

**Fig. 4.** Results of in vivo feedings of deuterated isotrichodermin derivatives. The relative size of the structures emphasizes the amounts fed and produced. The shaded areas represent the deuterated positions. The open circles indicate unlabeled acetate.
Table III

Proton and deuterium NMR analyses of dideuterated 3-acetyldeoxyxynovalenol 4 obtained from the feedings of the following labeled substrates: [4β, 15-2H3]-deacetyl isotrichodermin (1), [2β, 15-2H3]-iso- 

tsambucinol (3). The letters s, d, t, q, and m following the signals stand for singlet, doublet, triplet, quartet, and multiplet signals. br and o show broad or overlapping lines. The shaded areas represent the deuterated positions.

| Positon | δ 1H | δ 1H' | δ 2H |
|---------|------|-------|------|
| 2       | 3.90 (d, 4.5) | 3.90 (d, 4.5) | 0.00 |
| 3       | 5.22 (d, 4.5, 11.3) | 5.21 (d, 4.6, 4.8, 11.2) | 0.00 |
| 4       | 2.37 (d, 4.4) | - | - |
| 5       | 4.82 (s) | 4.82 (s) | 4.82 (s) |
| 6       | 6.98 (q) | 6.98 (q) | 6.98 (q) |
| 7       | 4.38 (d, 6.5) | 4.38 (d, 6.5) | 4.38 (d, 6.5) |
| 9       | 3.17 (d, 2.4, 1) | 3.17 (d, 2.4, 1) | 3.17 (d, 2.4, 1) |
| 10      | 5.15 (s) | 5.15 (s) | 5.15 (s) |
| 11      | 3.87 (m) | 3.87 (m) | 3.87 (m) |
| 12      | 3.10 (d, 2.7, 1) | 3.10 (d, 2.7, 1) | 3.10 (d, 2.7, 1) |
| 13      | 1.90 (s) | 1.90 (s) | 1.90 (s) |
| 14      | 2.14 (s) | 2.14 (s) | 2.14 (s) |

a The letters s, d, t, q, and m following the signals stand for singlet, doublet, triplet, quartet, and multiplet signals. br and o show broad or overlapping lines. The shaded areas represent the deuterated positions.
Isotrichodermin a Biosynthetic Precursor to 3-ADN?

Table IV

Proton and deuterium NMR analyses of deuterated calonectrin (5) and of recovered isotrichodermin (6 and 7) obtained from the feeding of excess [2'-2H3, 4β,15-2H]isotrichodermin (2) and [4β,15-2H]deacetyl isotrichodermin (1), respectively

The shaded areas represent the deuterated positions.

| Position | δ^1H | δ^2H |
|----------|------|------|
| 2        | 3.76 (d) J=4.6 |  |
| 3        | 5.17 (t) J=4.3 |  |
| 4α       | 2.14 (dd) | 2.10 |
| 4b       |       |      |
| 7        | 2.05-1.78 (m) |  |
| 8        | 2.05-1.78 (m) |  |
| 10       | 5.48 (br,dn) |  |
| 11       | 4.02 (br,d) J=5.4 |  |
| 13α      | 3.11 (d) J=4.0 |  |
| 13β      | 2.87 (d) J=4.0 |  |
| 14       | 0.84 (s) |  |
| 15α      | 4.07 (s)/3.83 (s) | 3.84 |
| 15β      | 4.09 (d)/3.85 (d) J=14.3 |  |
| 16       | 1.73 (br s) | 2.05, 2.13 (2s) |
| OAc      | 2.05 | 2.08

a The letters s, d, t, q, and m following the signals stand for singlet, doublet, triplet, quartet, and multiplet signals. br and o show broad or overlapping lines.

b In the deuterium NMR of both calonectrin (5) and isotrichodermin (6) the acetyl (CD3-CO) (2.08 ppm or 2.10 ppm) and D-4 (2.10 ppm) overlap. However, the integration of the peaks reveals that some acetyl is still partially deuterated. Indeed, this is obtained from the relative height of D-4/CD3 with respect to the Me-15.

FIG. 5. In vitro feeding of doubly deuterated isotrichodermin. The 15-deacetylcalonectrin derived from that feeding has retained all the deuteriums. The shaded areas represent the deuterated positions.

Isotrichodermin a Biosynthetic Precursor to 3-ADN? (Fig. 2)

The result obtained with the doubly radiolabeled isotrichodermin was unexpected. We therefore decided to confirm it with isotrichodermin labeled with stable isotopes in the ring as well as the acetate in the 3-position. The method is less sensitive than radiolabeled experiments, but the locus of incorporation can be easily and accurately determined by 2H NMR. In addition, if indeed the 3-acetyl in isotrichodermin is not retained in its conversion to 3-ADN, then two other isotrichodermin derivatives, 3-deacetyl ITD and 3-oxo-EPT, could be substrates. We therefore had to prepare them with deuterium labeling. Their syntheses were accomplished in the following manner: the [4β,15-2H]3-deacetyl ITD (1) was prepared as described previously (3) and was subsequently used for the synthesis of [2'-2H3, 4β,15-2H]ITD (2) and of [4β,15-2H]3-oxo-EPT (3, Fig. 3). In the first case, this necessitated an acetylation with [2H6]acetic anhydride and in the second case an oxidation of the 3-hydroxyl to a keto-group. After extensive purification and analyses by 1H and 2H NMR, the three deuterated compounds were ready to be fed to F. culmorum cultures (Tables I and II).

Feedings of [2'-2H3, 4β,15-2H]ITD (2, Fig. 4)—We have previously shown that feeding an excess of substrate enables us to...
accumulate biosynthetic intermediates that are otherwise undetected or present in minute quantities (5). We therefore fed in duplicate three different amounts of [2-2H3, 4β,15-2H]ITD (2) to F. culmorum production cultures. The results are shown in Fig. 4. The size of the structures in Fig. 4 emphasizes the amounts fed. When small amounts of [2-2H3, 4β,15-2H]ITD (2) were fed, 3-ADN was obtained with deuteriums only in the ring-positions at C-4 and C-15 and absolutely no deuteriums in the 3-acetyl group (Fig. 4 and Table III). This confirms our preliminary results with the radiolabeled compounds. The discrepancy between 100% loss of the 3-acetyl in the deuterium study versus 89% loss in the radiolabeled investigation emphasizes the relative sensitivity of the methods. The feeding with radiolabeled substrates uses trace amounts, and the detection via radioactivity is so sensitive that very small incorporations (11%) can be detected. On the other hand, the inferior sensitivity of deuterium labeling is compensated with definite determination of the site of incorporation, with no danger of easy contamination.

When larger amounts of [2-2H3, 4β,15-2H]ITD (2) were fed, the production of the end product 3-ADN was inhibited, and one of its known (5) biosynthetic intermediates, calonectrin (5, Fig. 4 and Table IV), accumulated. It is interesting to note that in this case two-thirds of the calonectrin obtained from the feeding had no deuteriums in the 3-acetyl group, whereas one-third retained the three deuteriums (Fig. 4 and Table IV). In addition, the isotrichodermin recovered from the feedings of excess substrate had the same distribution as the calonectrin; two-thirds had lost the deuteriums at C-3 (6, Fig. 4 and Table IV). This last result suggests that the loss of deuteriums in the 3-acetyl of isotrichodermin occurs at the initial stages of its metabolism to 3-acetyldeoxynivalenol (the first oxidation step at C-15) (Fig. 4 and Table IV).

Feeding of [4β,15-2H]Deacetyl ITD (1) and of [4β,15-2H]3-Oxo-EPT (3) to F. culmorum Cells (Fig. 4)—The almost complete loss of the 3-acetyl group in isotrichodermin in its in vivo conversion to 3-acetyldeoxynivalenol led us to consider two other possible substrates: 3-deacetyl isotrichodermin (with a hydroxyl at C-3) or the isotrichodermin derivative with a ketone at C-3 (3-oxo-EPT). They were synthesized with deuteriums at positions 4β and 15 (1 and 3, Fig. 3) and fed to F. culmorum. The first observation on these two feedings was the compounds inhibited considerably the production of the end products: 3-acetyldeoxynivalenol and sambucinol. For example, when 5 mg of [4β,15-2H]deacetyl isotrichodermin (1, Fig. 3) were fed, the only metabolites detected were the recuperated starting material and [4β,15-2H]isotrichodermin (7, Table IV). However, some 3-ADN and SOL were isolated from the 1- and 2.5-mg feedings. As expected in the feeding of both substrates, the sambucinol isolated contained no deuteriums. On the other hand, these deuterated isotrichodermin derivatives were incorporated into 3-ADN (4, Fig. 4), as could be easily observed from the deuterium NMR (Table III).

In Vitro Metabolism of [2-2H3, 4β,15-2H]ITD (2, Fig. 5)—In order to determine the fate of the 3-acetyl group of isotrichodermin in its cell-free metabolism, doubly deuterated isotrichodermin (in the ring and in the 3-acetyl group), [2-2H3, 4β,15-2H]ITD (2) was fed to a microsomal fraction of F. culmorum. After the incubation period, the extract was fractionated by HPLC (using program 3), and a major single peak was obtained (ts = 26.0 min). This new peak was purified by analytical HPLC. Proton and deuterium NMR established rigorously this compound to be 15-deacetylcalonectrin deuterated at positions 4 and 15 and at the C-3-O-acetyl position (8, Fig. 5 and Table V). Therefore, in the microsomes where isotrichodermin is only metabolized to 15-deacetylcalonectrin (8), the acetyl moiety is retained.

**DISCUSSION**

### Is Isotrichodermin a Precursor to 3-Acetyldeoxynivalenol?

Isotrichodermin was first detected as a metabolite of various *Fusarium* species including *F. culmorum* (4). Investigating the sequence of appearance of metabolites in *F. culmorum* by the kinetic pulse-labeling method (9) revealed that the disappearance of isotrichodermin with time coincided with the formation of 3-acetyldeoxynivalenol and had no effect on the biosynthesis of sambucinol (3). In addition, radiolabeled and deuterated isotrichodermin (ring-deuterated) were very good precursors (27–50% total incorporations) (3). The nonincorporation of the 3-acetyl group of isotrichodermin into the 3-acetyl of the product 3-acetyldeoxynivalenol (Figs. 2 and 4) might suggest that 3-deacetyl isotrichodermin is in fact the real biosynthetic intermediate. This compound was therefore synthesized with two deuteriums at C-4 and C-15 (1, Fig. 3). Our feeding experiments in this work showed that 1 inhibited the growth of the fungi and was incorporated into the product 3-acetyldeoxynivalenol but at a much lower extent than isotrichodermin. In addition, 3-deacetyl isotrichodermin was never isolated as a

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**TABLE V**

Proton and deuterium NMR analyses of deuterated 15-deacetylcalonectrin (8) derived from in vitro feeding of doubly deuterated isotrichodermin, [2-2H3, 4β,15-2H]ITD (2)

The shaded areas represent the deuterated positions. This table emphasizes the retention of all of the deuteriums that were present in the starting material.

| Position | δ1H | δ2H  |
|----------|-----|-----|
| 2        | 3.76 (d) J = 4.4 | 5.17 (t) J = 4.2 |
| 3        | 2.20 (om)         | 2.20             |
| 4a       | 5.50 (br. d) J = 5.4 | 3.99 (br. d) J = 5.3 |
| 4b       | 3.11 (d) J = 4.0 | 2.88 (d) J = 4.0 |
| 7        | 0.93 (s)         |                  |
| 8        | 3.68 (s)         | 3.69             |
| 10       | 3.50 (s)         | 3.50             |
| 11       | 1.75 (br. s)     | 2.10             |

*The symbols s, d, t, and m following the signals stand for singlet, doublet, triplet, and multiplet signals. br and o show broad or overlapping lines.

5 Five deuterium signals appear in the deuterium spectrum, one at 2.20 ppm for D-4 (integrating for one deuterium), one signal at 3.69/3.50 ppm for D-15A and -B protons (integrating for one deuterium together), and one at 2.10 ppm for D-2 (integrating for three deuteriums).
natural product, whereas isotrichodermin is produced by *F. culmorum* (4). A second possibility for a biosynthetic intermediate close to isotrichodermin but with no acetyl at C-3 was 3-oxo-12,13-epoxytrichothe-9-ene. Similarly, the labeled 3-oxo-12,13-epoxytrichothe-9-ene (also not a natural metabolite) (3, Fig. 3), inhibited the growth of *F. culmorum* and was incorporated into 3-acetyldeoxynivalenol to a lower extent than isotrichodermin. Therefore, isotrichodermin must be the biosynthetic precursor, but on its metabolism to 3-acetyldeoxynivalenol it loses its acetate and is reacetylated *de novo* by an acetylase. The biosynthetic step where the initial deacetylation occurs has been determined by the feeding of excess doubly deuterated isotrichodermin (two deuteriums positioned on the ring and three on the 3-acetyl). As we have seen previously (5), when an excess of substrate is fed, the metabolic turnover is repressed, causing the enzymes to be saturated. This results in the accumulation of intermediates generally found in trace amounts. Therefore, when we fed an excess of doubly deuterated isotrichodermin (two deuteriums positioned on the 3-acetyl), the end product 3-acetyldeoxynivalenol was not detected. The starting material (6, Fig. 4) was recovered with two deuteriums on the ring and partly deuterated on the 3-acetyl. In addition, calonectrin, a known precursor of 3-acetyldeoxynivalenol (5), was also obtained with the same label distribution (5, Fig. 4): the deuteriums in the ring are retained, but two-thirds of the deuteriums on the 3-acetyl have been lost. We therefore may conclude that the deacetylation occurs at an early step, either just prior to the first oxygenation step at C-15 or thereafter. We therefore decided to reinvestigate this result with an *in vitro* system.

*In Vivo and in Vitro* Metabolism of ITD (Figs. 2, 4, and 5)—*In vivo* experiments have demonstrated that during metabolism the 3-acetyl of ITD is mostly lost in its metabolism to 3-acetyldeoxynivalenol (Figs. 2 and 4). Feeding with large amounts of precursor (Fig. 4) suggested that the deacetylation occurs after the first hydroxylation at C-15. Due to the importance of this result, we wanted to confirm it with a different experiment. We know that with microsomal preparations, the main product obtained is 15-deacetyl isotrichodermin (15-DAC) (6) with no traces of the end product, 3-acetyldeoxynivalenol. This is probably due to the extensive washing of the microsomes during their preparation, hence disappearance of the enzymes required for the subsequent steps between 15-DAC and 3-ADN. Since 15-DAC is the end product, we could investigate with this system the fate of the 3-acetyl in the metabolism of isotrichodermin. Feeding the doubly labeled isotrichodermin ([2,4,15-2H]ITD, indicated as 2 in Fig. 3) to a cell free preparation could demonstrate if the 3-deacetylation occurs prior to the 15-hydroxylation or after. The result shown in Fig. 5 demonstrates that in the 15-DAC derived from that feeding, the 3-acetyl of ITD is fully retained. Thus, this experiment with microsomal preparation confirms what was suggested by the *in vitro* experiments, namely that the loss of acetate occurs after the first oxygenation step of isotrichodermin. The loss of acetate is probably linked to the acetate turnover, i.e., it is hydrolyzed off and then is put back on again using ATP and coenzyme A to form acetyl-CoA, which can acetylate deacetylated intermediates in the cell. We have shown earlier that DON can be acetylated to 3-ADN by *F. culmorum* cells (9). We have seen that ITD is a better precursor than deacetylated ITD. Therefore, despite the existence of an acetyl exchange mechanism, the acetyl seems to have an essential role that is not clear presently.

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