Biosynthesis of Trichothecenes and Apotrichothecenes

Lolita O. Zamir,*[,†,‡] Anastasia Nikolakakis,[†] Françoise Sauriol,[‡] and Orval Mamer[§]

Centre de Recherche en Microbiologie Appliquée, Université du Québec, Institut Armand-Frappier, 531 Boulevard des Prairies, Laval, Québec, Canada H7N 4Z3, Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, Quebec, Canada H3Z 2K6, and Biomedical Mass Spectrometry Unit, McGill University, 1130 Pine Avenue West, Montreal, Quebec, Canada H3A 1A3

Fusarium culmorum produces two major trichothecenes, 3-acetyldeoxynivalenol and sambucinol, and some minor apotrichothecenes. It was desired to investigate if during their biosynthesis a C-11-keto intermediate was involved. To verify this postulate, trichodiene, a known precursor to trichothecenes, was synthesized with two deuteriums at C-11 and one at C-15. It was then fed to *F. culmorum* cultures, and the derived metabolites were purified and analyzed. The results ruled out the involvement of an 11-keto intermediate but revealed two novel apotrichothecenes. The characterization of their structures suggested that one of the 2-hydroxy-11α-apotrichothecene stereoisomers (2α or 2*β*) could be converted to sambucinol. These apotrichothecenes were therefore synthesized labeled specifically with two deuteriums at C-4 and C-15 and fed to *F. culmorum* cultures. Indeed, the result established for the first time that 2α-hydroxy-11α-apotrichothecene was a precursor to sambucinol. A biosynthetic scheme for the production of trichothecenes and apotrichothecenes is described.

Keywords: *Fusarium culmorum; trichodiene; apotrichothecenes; 3-acetyldeoxynivalenol; sambucinol; 3-deoxysambucinol*

INTRODUCTION

Trichothecenes are toxic secondary metabolites produced by fungi and in particular by the ubiquitous *Fusarium* spp. (O'Donnell, 1993). They infect grains in the fields and are therefore of major concern in agriculture. The toxicity has been linked with trichothecenes, in particular with deoxynivalenol (DON; Figure 1) (Foster et al., 1986). The acetyl derivative, 3-acetyldeoxynivalenol (3-ADN), and sambucinol (SOL) (Figure 1) are the two major metabolites produced by *Fusarium culmorum* strain HLX 1503 (Greenhalgh et al., 1984; Mohr et al., 1984), which are investigated in this study. To rationally control the production of these mycotoxins, a thorough investigation of their biosynthesis is necessary. We have partially succeeded in designing inhibitors based on the late stages of trichothecene biosynthesis (Zamir et al., 1992a,b). We are now focusing on understanding earlier metabolic steps. The biosynthesis of the trichothecene mycotoxins can be divided into three stages: the conversion of mevalonate to the bicyclic hydrocarbon trichodiene, then to tricyclic metabolites, and finally to the trichothecenes. The steps involved in the first stage have been completely elucidated: trichodiene synthetase has been cloned and the gene isolated. We owe most of these elegant results to Hohn's and Cane's groups (Hohn and VanMiddlesworth, 1986; Hohn and Desjardins, 1992; Cane et al., 1995a,b, 1996). Numerous intermediates have been demonstrated in the next two stages, but there are still many unresolved problems. Trichodiene (TDN; Figure 1) and not its stereoisomer at C-15 [bazzanene (BZN)] was incorporated to trichothecenes (Zamir, 1989; Zamir et al., 1989; Savard et al., 1989). The sequence of oxidative steps post-trichodiene have not yet been resolved (Hesketh et al., 1991, 1993; Zamir et al., 1991). The first tricyclic metabolite that was proven to be a biosynthetic precursor to 3-ADN was shown to be isotrichodermin (ITD) (Zamir et al., 1990, 1996a,b) (Figure 1). On the other hand, 12,13-epoxytrichothec-9-ene (EPT) was incorporated into SOL via an intermediate 3-deoxysambucinol (preSOL). ITD was not a precursor to preSOL or SOL, 3-ADN was not derived from EPT, and there were no interconversions between EPT and ITD (Zamir et al., 1990) (Figure 1). The conversion of EPT to preSOL and SOL implies an initial oxidation with inversion of configuration, which is unknown in nature. Indeed, in the same organism we have previously shown that the stereochemistry of the oxygenation at C-3 of 3-ADN was shown to occur with retention of configuration (Zamir et al., 1987b; Zamir, 1989). Apotrichothecenes with a trans junction between cycles A and B were also reported as natural metabolites of *F. culmorum* and other *Fusarium* spp. (Zamir and Devor, 1987; Zamir et al., 1992c; Greenhalgh et al., 1989, 1990). Their biosynthesis is yet unknown. One of those apotrichothecenes, apotrichodiol (ATD) (Figure 1) (with a 3-α-hydroxyl), was not incorporated into the trichothecenes 3-ADN and SOL (Zamir et al., 1987a).

Two attractive biosynthetic postulates could explain the formation of apotrichothecenes and SOL: (1) the involvement of an 11-keto group in ring A of one of the precursors and (2) that 2-hydroxyapotrichothecenes with a cis junction between cycles A and B could be converted to SOL (Figure 1). To verify these hypotheses, we prepared the following compounds labeled specifi-

* Author to whom correspondence should be addressed [telephone (450) 687-5010, ext. 4260; fax (514) 481-2797; e-mail Lolita_Zamir@iaf.uquebec.ca].

† Centre de Recherche en Microbiologie Appliquée.

‡ Department of Chemistry.

§ Biomedical Mass Spectrometry Unit.

10.1021/jf980808r CCC: $18.00
Published on Web 04/03/1999

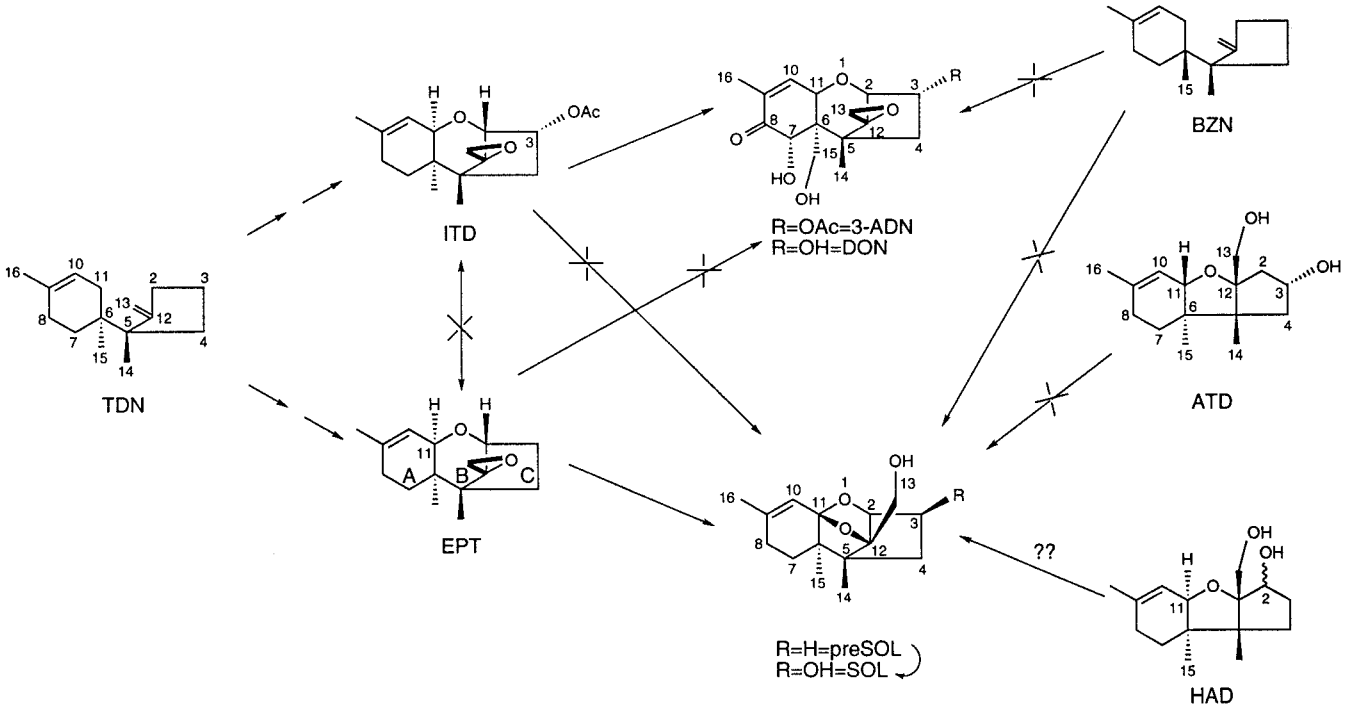

Figure 1. Biosynthetic interconversions between bicyclic metabolites and trichothecenes: TDN is a precursor to 3-ADN via ITD and to SOL via EPT and preSOL. The C-15-stereoisomer of trichodiene, BZN, and the apotrichothecene, ATD, are not precursors to the trichothecenes 3-ADN and SOL. The hypotheses investigated are (1) is there an 11-keto intermediate in the biosynthesis of trichothecenes and apotrichothecenes and (2) would HAD be a precursor to SOL?

cally with stable isotopes at two different sites: [11-dideutero-15-deutero]trichodiene, 11α-[4,15-tetradeutero]-2β,13β-apotrichodiol, and 11α-[4,15-tetradeutero]-2α,13β-apotrichodiol. The feedings of these postulated intermediates enabled us to understand the biosynthesis of SOL and apotrichothecenes.

MATERIALS AND METHODS

Instrumentation. All of the nuclear magnetic resonance (NMR) spectra were obtained at room temperature with a UNITY-500 spectrometer operating at 499.843 MHz for ^{1}H, at 125.697 MHz for ^{13}C, and at 76.735 MHz for ^{2}H. Positive ion fast atom bombardment mass spectra (FAB-MS) were obtained with a VG.ZAB-HS double-focusing instrument using a xenon beam having 8 kV of energy at 1 mA equivalent neutral current. Infrared spectra were measured in chloroform with a Perkin-Elmer model 683 infrared spectrophotometer. Flash chromatography was performed on silica gel 60, 230−400 mesh (EM Science). Thin-layer chromatography was conducted on silica gel 60 F-254 precoated TLC plates, 0.25 mm (EM Science). High-performance thin-layer chromatography (HPTLC) was run with LHP-KF, 0.2 mm plates (Whatman). Analytical high-performance liquid chromatography (HPLC) was performed on a Perkin-Elmer series 3B instrument coupled to an LC-75 spectrophotometric detector (Perkin-Elmer, Montreal, PQ) set at 204 nm. Preparative and semi-preparative HPLC were carried out on a Waters Delta Prep 3000 instrument coupled to a Lamda-Max model 481 LC spectrophotometric detector set at 204 nm (Waters, Montreal, PQ). *F. culmorum* slant cultures were homogenized using a Polytron homogenizer (Brinkman Instruments, Rexdale, ON).

NMR and MS Measurements. The NMR spectra were obtained on 1−2 mg (natural product) and 5−10 mg (synthetic compounds) dissolved in 0.3−0.4 mL of deuteriochloroform. The solvent was used as an external reference (77.0 ppm for ^{13}C and 7.25 ppm for ^{1}H and ^{2}H). For the ^{1}H spectra, eight transients were recorded with an 8000 Hz spectral window, a 45° pulse, 30K data points, and 2 s of acquisition time. The data were zero filled to 65K points during the processing. The ^{13}C NMR spectra were obtained with a spectral window of 30007.5 Hz using a 40° pulse and an acquisition time of 1.092 s. For synthetic compounds, 500 to 4000 transients were needed. For the natural products, 40000−70000 transients were acquired. The ^{2}H NMR spectra were obtained in chloroform using a spectral window of 2290 Hz, an 80° pulse, 4K data points, 0.894 s for the acquisition time, and 200−1000 transients. The correlation spectroscopy (COSY) experiments were performed in absolute value mode using 4 transients on 512 increments. The relaxation delay was set to 1 s. The data were processed with pseudo-echo shaping, using zero filling on the evolution domain (final matrix size was 2K × 2K). The spectral window was reduced to 3312 Hz to provide excellent resolution. The nuclear Overhauser effect 2D (NOESY) experiment (hypercomplex phase mode) was obtained using a mixing time of 0.3 s and a relaxation delay of 1 s. The acquisition was repeated for 32 transients, and 256 complex increments were acquired. The data were processed using a Gaussian apodization function. The final data matrix has 1K by 1K points (zero filling was used in the evolution domain). For the compounds studied, when the diagonal signals were phased negative, the cross-peaks resulting from dipolar relaxation nuclear Overhauser effect (NOE) were phased positive. The heteronuclear multiple quantum coherence (HMQC) experiment with a preceding bilinear rotational decoupling (BIRD) nulling period was used with hypercomplex phased mode. The recycling delay was set to 1 s while the nulling period (following the BIRD pulse) was set to 0.4 s. The acquisition was repeated 32 times, and 256 free induction decays (FIDS) were acquired. During acquisition of the proton spectra, ^{13}C broadband waltz decoupling was applied. The data were processed with Gaussian function and with zero filling in the evolution domain (^{13}C). The final matrix size was 1K by 1K. The spectral window in the carbon domain was 140 ppm while it was of ∼6.5 ppm in the proton domain. The heteromultiple bound correlation (HMBC) experiment was acquired using similar conditions but without the null period and without ^{13}C decoupling during acquisition. The *tau* delay to emphasize long-range coupling was set to 100 ms. Also, the carbon window was set larger to accommodate carbonyl carbons (180 ppm) in the carbon domain. The HMBC data were processed using shifted Gaussian function and zero filling on both the

detection and the evolution domain (matrix size 2K by 2K). Data were displayed and plotted in an absolute value mode.

Low-resolution FAB mass spectra ($R = 2000$) were obtained in a matrix composed of 3-nitrobenzyl alcohol initially containing ~15% dimethyl sulfoxide and 5% 1 M aqueous NaCl. This mixture provided protonated and sodiated ions that were usually very prominent. High-resolution spectra ($R = 10000$) were obtained in NaCl-saturated glycerol by peak matching the protonated or sodiated quasimolecular ions against appropriate glycerol cluster ions of known composition.

The site of deuterium incorporation was determined by NMR analyses, whereas the extent of incorporation was established by mass spectrometry. The extent of deuterium labeling in metabolites, obtained from feedings of labeled substrate, was determined by correction of the appropriate protonated or sodiated quasimolecular ion clusters present in a computer average of 10 scans in low-resolution FAB. The correction applied was the conventional reduction of the intensity of each member of the cluster by an amount calculated to be the contribution of that member by natural abundance heavy isotope substitution in all lighter ions of the cluster. The remaining intensities then represent the relative abundances of each labeled species. Interfering isobaric ions could be detected by comparing the relative intensities of the members of the protonated cluster with those of the sodiated cluster. As sodium is monoisotopic, any relative intensity differences between the two clusters are immediately apparent and indicate the presence of unrelated isobaric ions (possibly from minor contaminants) in the protonated or sodiated ion clusters. In this event, the cluster yielding apparently the lower enrichment was always found under high-resolution conditions to have no unrelated isobaric ions, and this was the cluster used for the correction described above.

HPLC. Analytical HPLC was performed with two Whatman Partisil 10 ODS-2 analytical columns in series (4.6 × 250 mm). Unless otherwise specified, semipreparative HPLC was performed with two Whatman Partisil 10 ODS-2 Mag 9 semipreparative columns in series (9.4 × 250 mm). Preparative HPLC was performed with one Partisil 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 15:85 methanol/water and a final concentration of 75:25 methanol/water, which was then held for 40 min. Program 2 consisted of a linear gradient lasting 50 min with an initial concentration of 15:85 methanol/water and a final concentration of 75:25 methanol/water, which was held for 30 min. This was then increased linearly over 10 min to 100% methanol and held for 20 min before re-equilibration to 15:85 methanol/water.

Strain and Cultivation Conditions. A 2% potato dextrose agar slant of *F. culmorum* strain (HLX-1503) was grown as previously described (Zamir and Devor, 1987). Seed cultures were grown for 3 days. Unless otherwise specified, production cultures were incubated for 48 h and consisted of 25 mL of production medium in 125 mL Erlenmeyer flasks that had previously been inoculated with 1.25 mL of seed culture homogenate.

Synthesis of [11-Dideutero-15-deutero]trichodiene and [11-Dideutero-15-deutero]bazzanene. A combination of the methods of Gilbert and Kelly (1986) and VanMiddlesworth (1986) was used with some minor modifications. The major differences derived from the introduction of two deuteriums at C-11, which had not been done previously. All of the intermediates synthesized were identified by comparison of their NMR spectra with the reported values of the unlabeled compounds.

4-Methyl-3-cyclohexene-[2-dideutero]-1-carbonyl chloride was prepared using acryloyl chloride and [1,1-dideutero]isoprene (Gajewski et al., 1989) following the reported method for the unlabeled compound (Gilbert and Kelly, 1986). 1-(Hydroxymethyl)-2-methyl-1-cyclopentene was synthesized according to the method of VanMiddlesworth (1986) except that dry tetrahydrofuran was used instead of ether and the reaction was conducted at ambient temperature for 18 h (as compared to 1 h), resulting in an improved yield of 85% instead of 40%.

(2-Methyl-1-cyclopentenyl)methyl 4-methyl-3-cyclohexene-[2-dideutero]-1-carboxylate was prepared using 4-methyl-3-cyclohexene-[2-dideutero]-1-carbonyl chloride and 1-(hydroxymethyl)-2-methyl-1-cyclopentene following the reported method for the unlabeled compound (Gilbert and Kelly, 1986). The only modification was the use of dichloromethane (instead of pyridine), which was easier to evaporate and improved the yield slightly from 81 to 91%. The ^{1}H NMR spectrum of (2-methyl-1-cyclopentenyl)methyl 4-methyl-3-cyclohexene-[2-dideutero]-1-carboxylate was identical to that of the unlabeled compound.

4-Methyl-1-(1-methyl-2-methylenecyclopentyl)-3-cyclohexene-[2-dideutero]-1-carboxylic acids were prepared according to the reported procedure for the unlabeled compounds (Gilbert and Kelly, 1986). We found it unnecessary to isolate the intermediate trimethylsilyl ester of the carboxylic acids, which we hydrolyzed during workup with a saturated ammonium chloride solution. Integration of the C-14 methyl protons that have different chemical shifts shows that this mixture is 60% (α^{15}COOH) and 40% (β^{15}COOH). Esterification of these acids with diazomethane followed by reduction with a large excess of $LiAlH_4$ gave the diastereomeric mixtures of [11-dideutero]-trichodienol and [11-dideutero]bazzanenol following the procedure for unlabeled compounds. These diastereomers were separated on preparative HPLC using 75% methanol/25% water at 20 mL/min to obtain 95% pure [11-dideutero]trichodienol ($t_R = 71.2$ min) and 82% [11-dideutero]bazzanenol ($t_R = 75.2$ min). Each diastereomer was oxidized at C-15 according to the procedure of VanMiddlesworth (1986) to yield an aldehyde in order to enable the introduction of label. Indeed, further reaction with sodium borodeuteride (NaB^2H_4) and one more oxidation with pyridinium chlorochromate (PCC) gave [11-dideutero-15-deutero]trichodien-15-al and [11-dideutero-15-deutero]bazzanen-15-al. These labeled aldehydes were reduced by the Wolff−Kishner reduction (VanMiddlesworth, 1986) to give the desired [11-dideutero-15-deutero]trichodiene and [11-dideutero-15-deutero]bazzanene, R_f 0.46, hexane: ^{1}H NMR δ 5.27 (s, 1H, H-10), 4.94 (od, 0.6H, H-13A), 4.93 (od, 0.4H, H-13A), 4.77 (d, $J = 2.0$ Hz, 0.4H, H-13B), 4.71 (d, $J = 2.6$ Hz, 0.6H, H-13B), 1.02 (s, 1.2 H, H-14), 1.09 (s, 1.8H, H-14), 0.81 (t, $J_{H,D} = 4.0$ Hz, 2H, H-15), 1.62 (s, 3H, H-16); ^{2}H NMR δ 1.95, 1.68 (1.2^{2}H, ^{2}H-11), 2.12, 1.54 (0.8^{2}H, ^{2}H-11), 0.83 (s, 1^{2}H, H-15).

Feeding of [11-Dideutero-15-deutero]TDN. The synthetic 60:40 mixture of [11-dideutero-15-deutero]TDN/[11-dideutero-15-deutero]BZN was fed to production cultures of *F. culmorum* that had been previously inoculated from 3-day-old seed cultures. Ten milligrams of the deuterated mixture dissolved in 120 μL of methanol was added to each of six sterile 125 mL Erlenmeyer flasks. Sterile 5% Brij 35 solution (0.1 mL) and a 48-h-old production culture of *F. culmorum* were added to each flask. Two identical controls were prepared with the Brij 35 solution but without deuterated precursor. The cultures were incubated for 96 h at 25 °C and 220 rpm. After filtration, the culture mediums were pooled and extracted with ethyl acetate, which was then dried and evaporated. The extract was dissolved in methanol and fractionated by semipreparative HPLC using program 2 at a flow rate of 3 mL/min. Thirteen fractions were collected as follows: fraction 1, 36.0−43.0 min; fraction 2, 43.0−45.7 min; fraction 3, 45.7−49.9 min; fraction 4, 49.9−52.9 min; fraction 5, 52.9−56.0 min; fraction 6, 56.0−60.8 min; fraction 7, 60.8−66.5 min; fraction 8, 66.5−73.4 min; fraction 9, 73.4−78.0 min; fraction 10, 78.0−84.6 min; fraction 11, 84.6−95.0 min; fraction 12, 95.0−102.0 min; and fraction 13, 102−112 min.

Purification of 3-ADN Derived from [11-Dideutero-15-deutero]TDN (Figure 2 and Table 1). Fraction 1, corresponding to 3-ADN, was purified on semipreparative HPLC using 35:65 methanol/water at 3 mL/min ($t_R = 34.0$ min). The NMR data of this pure compound showed that it was 3-ADN with deuteriums at C-11 and C-15 (Table 1). The high-resolution mass spectrum confirmed that indeed the metabolite isolated is 3-ADN, whereas the low-resolution mass spectrum was used to determine the deuterium enrichment (under Results and Discussion).

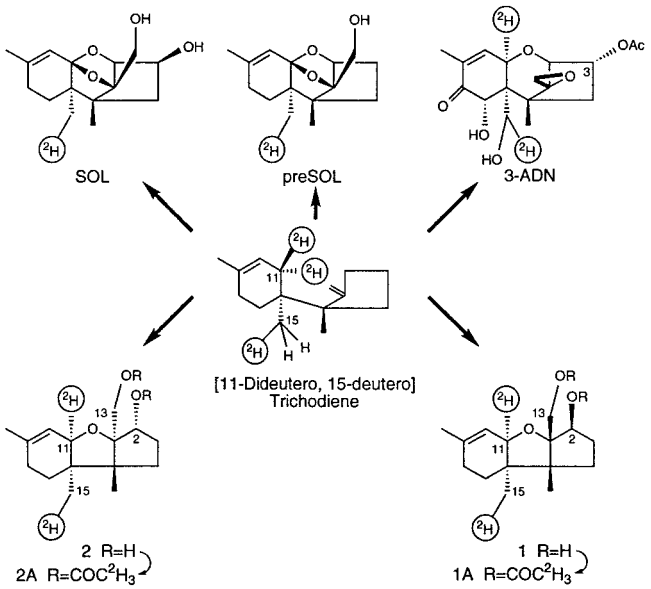

Figure 2. Results of the feeding of [11-dideutero-15-deutero]-trichodiene to *F. culmorum*: it was incorporated into 3-ADN, preSOL, and SOL, and two new apotrichothecenes (**1**, **2**) were also isolated. Metabolite **1** incorporated the deuteriums at C-11 and C-15 (between 11 and 17% enrichment at each position), whereas metabolite **2** almost quantitatively (H-11 cannot be observed in the ^{1}H NMR). These apotrichothecenes were perdeuteroacetylated (**1A** and **2A**) to facilitate their characterization.

Table 1. Proton and Deuterium Analysis of 3-ADN Derived from [11-Dideutero-15-deutero]trichodiene Feeding to *F. culmorum* Cultures

position	δ^1H[a]	δ^2H
2	3.91 (d), J = 4.4 Hz	
3	5.23 (dt), J = 4.4, 4.4, 11.7 Hz	
4a	2.36 (br m)	
4b	2.17 (dd), J = 11.2, 15.1 Hz	
7	4.83 (br)	
10	6.60 (br m)	
11	4.69 (d), J = 4.9 Hz	4.69
13a	3.18 (d), J = 4.4 Hz	
13b	3.12 (d), J = 4.4 Hz	
14	1.16 (s)	
15a	3.88 (d), J = 11.7 Hz	3.88
15b	3.78 (d), J = 11.7 Hz	3.82
16	1.90 (br s)	
OAc	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.

Purification of SOL Derived from [11-Dideutero-15-deutero]TDN (Figure 2 and Table 2). The fraction corresponding to SOL (fraction 4) was purified on analytical HPLC using 50:50 methanol/water at 1 mL/min (t_R = 41.0 min). The NMR data of the SOL derived from this feeding are shown in Table 2. The high-resolution mass spectrum confirmed that indeed the metabolite isolated is SOL, whereas the low-resolution mass spectrum was used to determine the deuterium enrichment (see Results and Discussion).

Purification of PreSOL Derived from [11-Dideutero-15-deutero]TDN (Figure 2 and Table 3). Fraction 6 (t_R = 56.0−60.8 min) was rotary evaporated to eliminate the methanol, and the aqueous residue was extracted with ethyl acetate (6 × 6 mL) through a Chem Elut 1003 tube. To facilitate the purification, the residue was acetylated with perdeuteroacetic anhydride (0.25 mL) and pyridine (0.15 mL) at 25 °C for 18 h. Upon evaporation, the residue was fractionated by analytical HPLC using 60:40 methanol/water for 50 min, and then a linear gradient was used to 90:10 methanol/water in 30 min and then held at 90:10 for 10 min, all at 1 mL/min. The peak

Table 2. Proton and Deuterium Analysis of SOL Derived from [11-Dideutero-15-deutero]trichodiene Feeding to *F. culmorum* Cultures

position	δ^1H[a]	δ^2H[a]
2	3.92 (s)	
3	4.24 (dd), J = 4.4, 8.3 Hz	
4a	2.59 (dd), J = 8.3, 15.1 Hz	
4b	1.50 (dd), J = 4.4, 15.1 Hz	
7a	1.82 (m), J = 5.9, 12.2, 12.2 Hz	
7b	1.36 (dd), J = 5.4, 12.2 Hz	
8a	2.10 (br m)	
8b	1.97 (dd), J = 5.8, 18.6 Hz	
10	5.44 (br s)	
13a	4.11 (d), J = 11.2 Hz	
13b	4.06 (d), J = 11.2 Hz	
14	1.07 (s)	
15	0.82 (s)	0.84
16	1.75 (br 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.

Table 3. Proton, Deuterium, and Carbon-13 NMR Data for Perdeuteroacetyl-preSOL[a]

position	δ^1H[b]	δ^2H	δ^{13}C[c]	δ^{13}C[d]
2	4.11 (dd), J = 2.9, 2.2 Hz		82.35	82.31
3a/b	1.87 (mo)		27.55	27.79
4a	1.98 (mo)		33.06	33.59
4b	1.55 (m)			
5			**50.65**	50.65
6			**47.95**	47.95
7a	1.85 (mo)		29.43	30.33
7b	1.3 (ddd), J = 13.0, 5.6, 1.5 Hz			
8a/8b	2.11 (br m)/1.95 (mo)		29.12	29.21
9			**145.51**	145.51
10	5.49 (m, 7 lines), J = 1.2 Hz		116.48	116.47
11			**107.18**	107.18
12			**93.28**	93.28
13a	4.39 (d), J = 12.4 Hz		60.12	60.20
13b	4.30 (d), J = 12.4 Hz			
14	0.99		16.28	16.48
15	0.88	0.90	14.09	14.48
16	1.76 (br s)		22.86	23.01
OAc-d3		2.07		

[a] The preSOL derived from the [11-dideutero-15-deutero]trichodiene feeding to *F. culmorum* cultures was perdeuteroacetylated to facilitate its purification. [b] 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. [c] The ^{13}C chemical shifts have been extracted from the HMQC experiment (±0.2 ppm). The numbers in boldface characters represent quaternary carbons; their shifts have been obtained from the HMBC experiment (±0.2 ppm). [d] The ^{13}C chemical shifts of standard unlabeled acetylated-preSOL are added for comparison.

corresponding to perdeuteroacetyl-preSOL (t_R = 73 min) was further purified on HPTLC plates using 15% ethyl acetate/85% hexane as the eluting solvent. The band corresponding to perdeuteroacetyl-preSOL was isolated, R_f 0.24, hexane/ethyl acetate (85:15). It was rigorously identified by NMR spectra (Table 3) and mass spectrometry (see Results and Discussion).

Purification of 11α-[11-Deutero-15-deutero]-2*β*,13*β*-apotrichodiol (1) and 11α-[11-Deutero-15-deutero]-2α, 13α-apotrichodiol (2) Derived from [11-Dideutero-15-deutero]TDN (Figures 2 and 3; Tables 4 and 5). Fractions 7 and 8 were pooled, evaporated, and extracted with ethyl acetate (7 × 15 mL) through a Chem Elut 1020 tube. The residue was acetylated with 0.25 mL of perdeuteroacetic anhydride and 0.15 mL of pyridine at 25 °C for 18 h. The product was fractionated by semipreparative HPLC using 65:35 methanol/water for 45 min, changing linearly to 90:10 methanol/water in 30 min and then held at 90:10 for 10 min, all at 3 mL/min. The fraction at t_R = 59 min was purified on one HPTLC plate eluting twice with 20% ethyl acetate/80%

Table 4. Proton, Deuterium, and Carbon-13 NMR Data for 1A and Synthetic 3A[a]

	1A				**3A**		
position	δ^1H[b]	δ^2H	δ^{13}C[c]		δ^1H[b]	δ^2H	δ^{13}C[c]
2	5.16 (t), J = 5.1 Hz		81.59		5.16 (t), J = 5.1 Hz		81.56
3a	1.95		30.16		1.93 (dd), J = 13.2, 5.1 Hz		29.96
3b	1.66				1.64 (dd), J = 13.2, 5.2 Hz		
4a	2.08 (dt), J = 13.7, 7.1, 7.1 Hz		34.44		2.08 (m)	*2.05*	
4b	1.48 (dt), J = 13.6, 7.8, 7.8 Hz				*1.47 (m)*	*1.46*	
5/6			**56.29**				**56.07**
6/5			**43.46**				**43.27**
7a	1.63		26.53		1.63		26.45
7b	1.30				1.30		
8a/8b	2.10/1.92		28.14		2.10/1.90		28.08
9			**139.81**				**139.79**
10	5.56 (br s)		118.39		5.55 (br d)		118.34
11	3.77 (br d), J = 5.1 Hz	*3.78*	**79.02**		3.76 (d), J = 5.1 Hz		**78.99**
12			**90.93**				**90.92**
13a	4.38 (d), J = 11.6 Hz		65.98		4.38 (d), J = 11.5 Hz		65.99
13b	4.12 (d), J = 11.6 Hz				4.12 (d), J = 11.5 Hz		
14	1.05		17.90		1.04		17.81
15	0.82	*0.83*	15.09		0.82	*0.81 (2^2 H)*	14.5
16	1.71 (br s)		23.41		1.70		23.40
OAc-d3		*2.02*	169.88	Ac	2.03		169.76
		1.80	171.10		1.99		171.00

[a] The NMR values for **1A** derived from the [11-dideutero-15-deutero]trichodiene feeding to *F. culmorum* cultures followed by perdeuteroacetylation are identical to those for the synthetic **3A** except for the location of the deuterium labels. [b] The letters s, d, t, q, and m following the signals stand for singlet, doublet, triplet, quartet, and multiplet signals; br and o show overlapping lines. [c] The ^{13}C chemical shifts have been extracted from the HMQC experiment (±0.2 ppm). The numbers in boldface characters represent quaternary carbons; their shifts have been obtained from the HMBC experiment (±0.2 ppm).

Table 5. Proton, Deuterium, and Carbon-13 NMR Data for Perdeuterodiacetylated Metabolite 2A and Synthetic 4A[a]

	2A			**4A**		
position	δ^1H[b]	δ^2H	δ^{13}C[c]	δ^1H[b]	δ^2H	δ^{13}C[c]
2	5.01 (dd), J = 5.9, 9.7 Hz		75.62	5.12 (dd), J = 2.7, 4.4 Hz		78.71
3a/b	1.98/1.75		29.86	1.77 (m)/1.76 (m)		29.18
4a	2.10		32.06		*2.05*	
4b	1.35				*1.27*	
5/6			**54.90**			**56.13**
6/5			**43.28**			**42.84**
7a	1.75		26.85	1.68 (o td), J = 12.7, 12.7, 5.8 Hz		25.77
7b	1.35			1.29 (dd), J = 12.7, 5.6 Hz		
8a/8b	2.02/1.91		28.40	2.02 (m)/1.92 (dd), J = 18.6, 5.6 Hz		28.06
9			**139.53**			**140.45**
10	5.56 (br s)		118.36	5.55 (br)		118.08
11		*3.85*	**79.71**	3.83 (d), J = 4.4 Hz		**79.16**
12			**89.38**			**89.43**
13a	4.35 (d), J = 11.7 Hz		64.11	4.27 (d), J = 11.2 Hz		66.79
13b	4.10 (d), J = 11.7 Hz			3.99 (d), J = 11.2 Hz		
14	1.05		21.64	0.94 (s)		15.78
15-DH_2	0.80 (br s)	*0.82*	17.26		*0.78*	
-H_3	0.82 (s)					
16	1.72 (br s)		23.29	1.70		23.41
OAc-d3		*2.04*	171.03		*2.05*	

[a] Perdeuterodiacetylated metabolite **2A** is very different from perdeuterodiacetylated metabolite **1A** (which is identical to synthetic **3A**, Table 4) and from compound **4A**. [b] The letters s, d, t, q, and m following the signals stand for singlet, doublet, triplet, quartet, and multiplet signals; br and o show overlapping lines. [c] The ^{13}C chemical shifts have been extracted from the HMQC experiment (±0.2 ppm). The numbers in boldface characters represent quaternary carbons; their shifts have been obtained from the HMBC experiment (±0.2 ppm).

hexane. Two compounds were isolated: 11α-[11-deutero-15-deutero]-2*β*,13*β*-apotrichodiperdeuteroacetate (**1A**) (R_f 0.37) and 11α-[11-deutero-15-deutero]-2α,13α-apotrichodiperdeuteroacetate (**2A**) (R_f 0.29). The NMR spectral data of the diperdeuteroacetylated metabolites **1** and **2**, **1A** and **2A**, are shown in Tables 4 and 5, respectively. The mass spectra of **1A** and **2A** differ by the extent of deuteration (discussed under Results and Discussion).

Syntheses of Compounds 3 and 4 (Figure 4). *Synthesis of 11α-[4,15-Tetradeutero]-2β,13β-apotrichodiol (**3**, Figure 4).* A solution of [4,15-tetradeutero]-12,13-epoxytrichothecene (Zamir et al., 1990) (0.53 g; 0.0022 mol) in 11 mL of methanol was treated at 25 °C with 22 mL of a 0.25 M sulfuric acid solution for 18 h following the conditions reported for unlabeled EPT (Grove, 1988). The solution was neutralized with a saturated solution of sodium bicarbonate and extracted with chloroform. The organic layer was washed with brine, dried ($MgSO_4$), filtered, and evaporated in vacuo. The residue was chromatographed using hexane/ethyl acetate (25:75) to give 0.433 g (76%) of the desired compound as a viscous syrup: R_f 0.39, hexane/ethyl acetate (30:70); IR (cm^{-1}) 3700−3200 broad band, 3005, 2965, 2940, 2880, 1675, 1450, 1385, 1250, 1045, 1000; ^{1}H NMR δ 5.54 (dq, $J_{10,11}$ = 5.0 Hz, $J_{10,Me}$ = 1.4 Hz, 1H, H-10), 4.18 (dd, $J_{2,3B}$ = 9.8 Hz, $J_{2,3A}$ = 6.5 Hz, 1H, H-2), 3.81 (br s, 2H, H-13A,B), 3.79 (br d, $J_{10,11}$ = 5.0 Hz, 1H, H-11), 3.38 (CH_2OH), 2.70 (C-2-OH), 1.99 (dd, $J_{A,B}$ = 12.3, $J_{3A,2}$ = 6.3 Hz, 1H, H-3A), 1.72 (br s, 3H, H-16), 1.61 (br t, 1H, H-3B), 0.97 (s, 3H, H-14), 0.74 (br s, 1H, H-15); ^{2}H NMR δ 1.77, 1.29 (s, 2^2H, ^{2}H-4), 0.75 (d, $J_{H,D}$ = 1.8 Hz, 2^2H, ^{2}H-15). The high-resolution

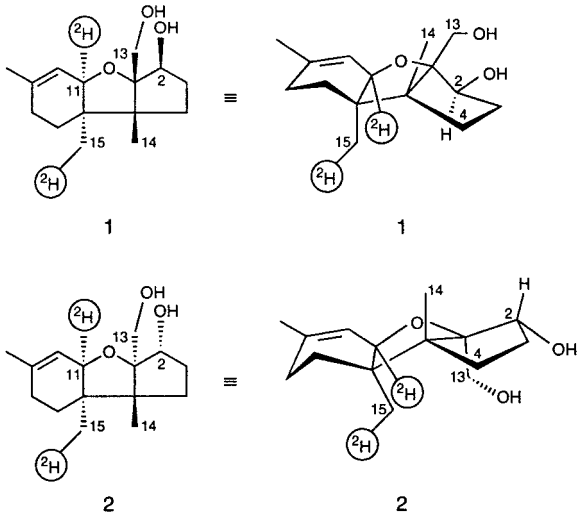

Figure 3. Tridimensional structures of metabolites **1** and **2**.

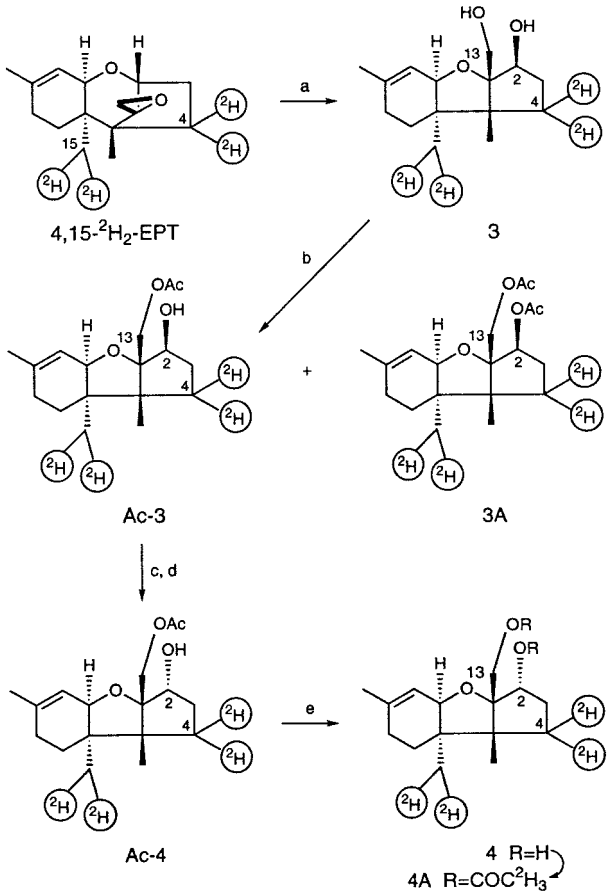

Figure 4. Semisyntheses of apotrichothecenes **3** and **4** and their diacetates **3A** and **4A**. The starting material, [4,15-tetradeutero]EPT, was prepared according to the literature (Zamir et al., 1990). The reactions used are (a) H_2SO_4 (0.25 M), MeOH; (b) Ac_2O, pyridine, CH_2Cl_2; (c) PCC, CH_2Cl_2; (d) $NaBH_4$, MeOH, 0 °C; and (e) K_2CO_3, MeOH.

mass spectra of compound **3** confirmed the structure shown in Figure 4 (see Results and Discussion).

*Synthesis of 11α-[4,15-Tetradeutero]-13β-acetoxy-2β-apotricho-ol (**Ac-3**, Figure 4).* A solution of 11α-[4,15-tetradeutero]-2β,13β-apotrichodiol (**3**, Figure 4) (0.433 g; 0.00169 mol) in 20 mL of dichloromethane was treated with pyridine (0.8 mL; 0.01 mol) and then with acetic anhydride (0.185 mL; 0.00196 mol) at 25 °C for 40 h. At this time more acetic anhydride (0.054 mL; 0.57 mmol) was added and stirring was resumed for an additional 24 h. The solution was diluted with dichloromethane and then washed successively with 10% HCl, saturated $NaHCO_3$ solution, and brine. The combined aqueous layers were extracted with ethyl acetate. The organic layers were combined, dried ($MgSO_4$), filtered, and evaporated in vacuo. Flash chromatography using hexane/ethyl acetate (60:40) yielded 0.227 g (54% yield) of the desired crystalline compound (**Ac-3**, Figure 4): R_f 0.30, hexane/ethyl acetate (50:50); mp 91−93 °C from petroleum ether; IR (cm^{-1}) 3620, 3580−3300 broad band, 3005, 2960, 2935, 2875, 1735, 1675, 1465, 1455, 1385, 1250, 990; ^1H NMR δ 5.55 (dq, $J_{10,11} = 5.2$ Hz, $J_{10,Me} = 1.4$ Hz, 1H, H-10), 4.49 (d, $J_{A,B} = 11.7$ Hz, 1H, H-13A), 4.20 (d, $J_{A,B} = 11.7$ Hz, 1H, H-13B), 4.12 (t, $J_{2,3} = 5.5$ Hz, 1H, H-2), 3.75 (br d, $J_{10,11} = 5.3$ Hz, 1H, H-11), 2.43 (br s, OH), 2.11 (s, 3H, C-13-OAc), 1.89 (dd, $J_{A,B} = 12.8$ Hz, $J_{A,2} = 5.0$ Hz, 1H, H-3A), 1.71 (s, 3H, H-16), 1.65 (dd, $J_{A,B} = 12.8$ Hz, $J_{B,2} = 6.0$ Hz, 1H, H-3B), 1.01 (s, 3H, H-14), 0.77 (br s, 1H, H-15); ^2H NMR δ 2.04, 1.44 (s, 2^2H, ^2H-4), 0.80 (s, 2^2H, ^2H-15). A diacetyl derivative (**3A**, Figure 4) (0.074g; 15% yield) was also obtained: R_f 0.60, hexane/ethyl acetate (50:50); IR (cm^{-1}) 3010, 2965, 2940, 2880, 1735, 1675, 1450, 1385, 1375, 1255, 990; ^1H NMR δ 5.55 (br d, 1H, H-10), 5.16 (t, $J_{2,3} = 5.1$ Hz, 1H, H-2), 4.38 (d, $J_{A,B} = 11.5$ Hz, 1H, H-13A), 4.12 (d, $J_{A,B} = 11.5$ Hz, 1H, H-13B), 3.76 (d, $J_{10,11} = 5.1$ Hz, 1H, H-11), 2.03 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.93 (dd, $J_{A,B} = 13.2$ Hz, $J_{A,2} = 5.1$ Hz, 1H, H-3A), 1.70 (s, 3H, H-16), 1.64 (dd, $J_{A,B} = 13.2$ Hz, $J_{B,2} = 5.2$ Hz, 1H, H-3B), 1.04 (s, 3H, H-14), 0.82 (s, 1H, H-15); ^2H NMR δ 2.05, 1.46 (s, 2^2H, ^2H-4), 0.81 (s, 2^2H, ^2H-15); ^{13}C NMR δ 171.00, 169.76, 139.79, 118.34, 90.92, 81.56, 78.99, 65.99, 56.07, 43.27, 29.96, 28.08, 26.45, 23.40, 21.11, 21.02, 17.81, 14.50.

11α-[4,15-Tetradeutero]-13β-acetoxy-2-ketoapotrichothecene. A solution of 11α-[4,15-tetradeutero]-13β-acetoxy-2β-apotricho-ol (**Ac-3**, Figure 4) (0.340 g; 0.0011 mol) in 45 mL of dichloromethane was cooled to 0 °C and was treated with pyridinium chlorochromate (0.98 g; 0.0045 mol). The reaction mixture was stirred at 0 °C for 10 min, at which time it was warmed to 25 °C and stirred for 2.5 h. The mixture was filtered through a column of dry silica gel, rinsed with dichloromethane, and eluted with hexane/ethyl acetate (75:25). This gave 0.28 g (83%) of the desired compound: R_f 0.24, hexane/ethyl acetate (75:25); IR (cm^{-1}) 3020, 2970, 2940, 2920, 2880, 1750, 1675, 1465, 1450, 1375, 1250, 1045, 990; ^1H NMR δ 5.54 (dm, $J_{10,11} = 5.1$ Hz, 1H, H-10), 4.37 (d, $J_{A,B} = 11.3$ Hz, 1H, H-13A), 4.14 (d, $J_{A,B} = 11.3$ Hz, 1H, H-13B), 3.80 (br d, $J_{10,11} = 5.3$ Hz, 1H, H-11), 2.40 (d, $J_{A,B} = 18.8$ Hz, 1H, H-3B), 2.00 (s, 3H, C-13-OAc), 1.72 (br s, 3H, H-16), 1.13 (s, 3H, H-14), 0.82 (br s, $J_{H,D} = 1.7$ Hz, 1H, H-15); ^2H NMR δ 1.91, 1.59 (s, 2^2H, ^2H-4), 0.83 (s, 2^2H, ^2H-15).

*11α-[4,15-Tetradeutero]-13β-acetoxy-2α-apotricho-ol (**Ac-4**, Figure 4).* A solution of the previously prepared ketone (0.28 g; 0.94 mmol) in 31 mL of methanol was cooled to 0 °C and was treated with $NaBH_4$ (0.11 g; 0.0029 mol) for 15 min. The reaction mixture was diluted with $CHCl_3$ to 200 mL and washed to neutrality with a saturated solution of NaCl. The volatiles were evaporated in vacuo. Flash chromatography using hexane/ethyl acetate (65:35) afforded 0.238 g (84%) of the desired product: R_f 0.24, hexane/ethyl acetate (65:35); IR(cm^{-1}) 3620−3400 broad band, 3010, 2960, 2940, 2880, 1735, 1675, 1450, 1385, 1250, 1045, 990; ^1H NMR δ 5.55 (dq, 1H, H-10), 4.09 (d, $J_{A,B} = 11.7$ Hz, 1H, H-13A), 4.03 (d, $J_{A,B} = 11.7$ Hz, 1H, H-13B), 3.96 (q, $J_{2,3A} = J_{2,OH} = 2.3$ Hz, $J_{2,3B} = 4.6$ Hz, 1H, H-2), 3.92 (br d, $J_{10,11} = 5.3$ Hz, 1H, H-11), 2.87 (s, OH), 2.06 (s, 3H, C-13-OAc), 1.73 (br s, 3H, H-16), 0.93 (s, 3H, H-14), 0.76 (br s, 1H, H-15); ^2H NMR δ 2.09, 1.19 (s, 2^2H, ^2H-4), 0.78 (s, 2^2H, ^2H-15).

*11α-[4,15-Tetradeutero]-2α,13β-apotrichodiol (**4**, Figure 4).* A solution of 11α-[4,15-tetradeutero]-13β-acetoxy-2α-apotricho-ol (**Ac-4**) (0.253 g; 0.848 mmol) in 28 mL of dry methanol was treated with anhydrous K_2CO_3 (0.67 g; 0.0048 mol) at 25 °C for 16 h. The methanol was evaporated, 30 mL of a saturated NaCl solution was added to the residue, and the mixture was extracted with $CHCl_3$. The organic layer was washed with brine. The volatiles were dried ($MgSO_4$), filtered, and evaporated. The residue was chromatographed using hexane/ethyl acetate (35:65) providing 0.205 g (94%) of a viscous syrup: R_f 0.33, hexane/ethyl acetate (30:70); IR (cm^{-1}) 3660−3300 broad

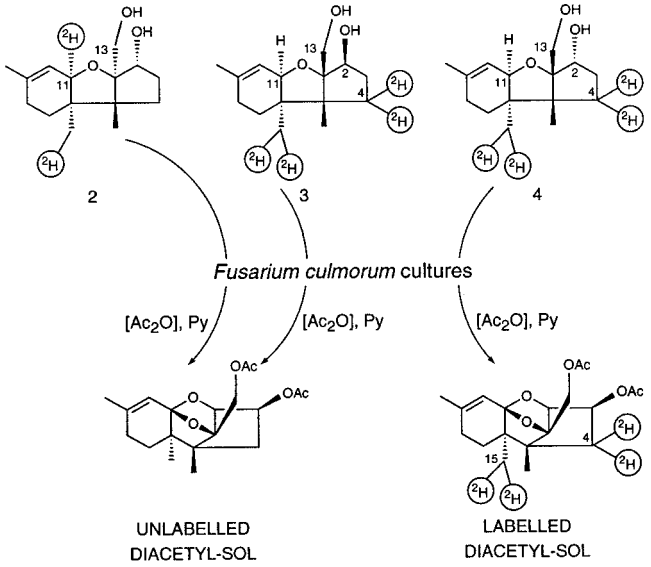

Figure 5. Results of the separate feedings of metabolite **2** and semisynthetic apotrichothecenes **3** (which is identical to metabolite **1** except for the location of the deuteriums) and **4** to *F. culmorum* cultures. To purify the derived SOL, it was acetylated. The only apotrichothecene that was found to be a biosynthetic precursor of SOL is **4**.

band, 3010, 2965, 2940, 2885, 1675, 1450, 1385, 1250, 1000; ^{1}H NMR δ 5.58 (dm, $J_{10,11}$ = 5.2 Hz, 1H, H-10), 4.02 (t, $J_{2,3B}$ = 7.2, $J_{2,3A}$ = $J_{2,OH}$ = 2.3 Hz, 1H, H-2), 3.93 (br d, $J_{10,11}$ = 5.4 Hz, 1H, H-11), 3.70 (dd, $J_{A,B}$ = 12.2, $J_{A,OH}$ = 4.4 Hz, 1H, H-13A), 3.40 (dd, $J_{A,B}$ = 11.7, $J_{B,OH}$ = 8.1 Hz, 1H, H-13B), 2.81 (d, $J_{H2,OH2}$ = 2.5 Hz, C-2-OH), 1.98, 1.71 (d, 2H, H-3), 1.75 (br s, 3H, H-16), 1.71 (dd, CH_2OH), 0.92 (s, 3H, H-14), 0.77 (br s, 1H, H-15); ^{2}H NMR δ 2.09, 1.16 (s, 2^2H, ^{2}H-4), 0.78 (s, 2^2H, ^{2}H-15). The high-resolution mass spectra of compound **4** (see Results and Discussion) confirmed the structure shown in Figure 4. The perdeuterodiacyl derivative was also prepared for further characterization: 11α-[4,15-tetradeutero]-2α,13*β*-apotrichodiol (**4**, Figure 4) (4 mg) was incubated with 145 μL of perdeuteroacetic anhydride and 135 μL of pyridine for 18 h at 25 °C. The reagents were coevaporated with heptane in vacuo, and the residue was chromatographed on a short column of silica gel with hexane/ethyl acetate (75:25) to give pure **4A**. ^{1}H NMR δ 5.55 (br, 1H, H-10), 5.12 (dd, $J_{2,3A}$ = 2.7 Hz, $J_{2,3B}$ = 4.4 Hz, 1H, H-2), 4.27 (d, $J_{A,B}$ = 11.2 Hz, 1H, H-13A), 3.99 (d, $J_{A,B}$ = 11.2 Hz, 1H, H-13B), 3.83 (d, $J_{10,11}$ = 4.4 Hz, 1H, H-11), 1.77 (m, 1H, H-3A), 1.76 (m, 1H, H-3B), 1.70 (s, 3H, H-16), 0.94 (s, 3H, H-14); ^{2}H NMR δ 2.05, 1.27 (s, 2^2H, ^{2}H-4), 0.78 (s, 2^2H, ^{2}H-15), 2.05 (s, 6^2H, OAc); ^{13}C NMR δ 140.45, 118.08, 89.43, 79.16, 78.71, 66.79, 56.13, 42.84, 29.18, 28.06, 25.77, 23.41, 15.78.

Feedings of 11α-[11-Deutero-15-deutero]-2α,13α-apotrichodiol (2); 11α-[4,15-Tetradeutero]-2*β*,13*β*-apotrichodiol (3); and 11α-[4,15-Tetradeutero]-2α,13*β*-apotrichodiol (4) (Figure 5) to *F. culmorum* Cultures. Each apotrichodiol was fed separately to 48 h production cultures of *F. culmorum* (previously prepared from 3-day-old seed cultures). 11α-[11-Deutero-15-deutero]-2α,13α-apotrichoperdeuterodiacetate (**2A**, Figure 2) was hydrolyzed to **2** by dissolving it in 140 μL of methanol and adding 100 μL of 0.1 N NaOH for 18 h at 25 °C. The solution was neutralized with 40 μL of 0.1 N HCl and was placed in a sterile 125 mL Erlenmeyer flask. Twenty milligrams of each apotrichodiol **3** and **4** was distributed evenly (2.0 mg) in each of 10 125 mL Erlenmeyer flasks. To each Erlenmeyer flask was added 0.5 mL of a 5% Brij 35 solution to enhance the solubility of the hydrocarbons. Next, a 48-h-old 25 mL production culture was transferred to each Erlenmeyer containing the apotrichodiols, and the cultures were shaken at 220 rpm in the dark at 25 °C for an additional 5 days. After filtration, the culture media were saturated with NaCl and extracted with ethyl acetate. The crude extracts were partitioned by preparative HPLC using program 2 at a flow rate of 18 mL/min.

Purification and Characterization of the SOL Derived from Separate Feedings of Apotrichodiols 2, 3, and 4 (Figure 5). Each peak corresponding to SOL (t_R = 48.0 min) was further purified on semipreparative HPLC using 50:50 methanol/water at 3 mL/min (t_R = 59.0 min). The peaks corresponding to SOL were each acetylated by incubating for 18 h at 25 °C with 300 μL of acetic anhydride and 190 μL of pyridine. Further purification by flash chromatography on silica gel using hexane/ethyl acetate (65:35) provided pure samples of diacetyl-SOL for NMR. The diacetyl-SOL metabolites obtained from the feedings of 11α-[11-deutero-15-deutero]-2α,13α-apotrichodiol (**2**, Figure 5) and 11α-[4,15-tetradeutero]-2*β*,13*β*-apotrichodiol (**3**, Figure 5) were identical to standard unlabeled diacetyl-SOL and showed no deuterium enrichment. The diacetyl-SOL derived from the feeding of 11α-[4,15-tetradeutero]-2α,13*β*-apotrichodiol (**4**, Figure 5) showed two deuterium substitutions at C-4 and two at C-15: ^{2}H NMR δ 2.61 (H-4a), 1.41 (H-4b), 0.85 (H-15). The mass spectral data confirmed these results.

RESULTS AND DISCUSSION

Role of Specifically Labeled Trichodiene at C-11 and C-15. We wanted to investigate if any precursor to trichothecenes (in particular to SOL) or to apotrichothecenes would involve a keto group at C-11. Because trichodiene is a known precursor to trichothecenes (Zamir et al., 1989; Savard et al., 1989), we thought that by introducing two deuteriums at C-11 we could (1) check this postulate and (2) enhance the possibility of trapping interesting putative intermediates. On the other hand, we needed to have another deuterium in another site of the molecule as a control in case both deuteriums at C-11 were eliminated. We chose as a second site C-15, and we therefore needed to synthesize 11-dideutero-15-deuterotrichodiene, which had never been prepared previously. There are, however, many syntheses of trichodiene in the literature including [16-^{14}C]trichodiene by Zamir and Huang (1992), who utilized the elegant synthesis of Pearson and O'Brien (Pearson and O'Brien, 1989; O'Brien et al., 1989). This procedure does not allow easy introduction of a deuterium or tritium label. [15-^{3}H]Trichodiene with the respective [15-^{3}H]bazzanene diastereomer differing in the stereochemistry of the methyl group at C-15 was prepared by VanMiddlesworth (1986) using an adaptation of the procedure of Gilbert and Kelly (1986). BZN (Figure 1), which has been isolated from *Bazzania pompeana* (Ohta et al., 1977), is a diastereomer of TDN differing in the stereochemistry at C-15 and is not a metabolite of *Fusarium* species. We have previously shown with radiolabeling experiments that BZN is not incorporated into trichothecenes and has no influence on the growth of *F. culmorum* (Zamir et al., 1989). We have therefore used a combination of the methods of Gilbert and Kelly (1986) and VanMiddlesworth (1986) for the synthesis of our deuterated substrate. Some changes were necessary to allow for the introduction of two deuteriums at C-11, and some slight modifications to the procedure are described in detail under Materials and Methods.

2-Hydroxyapotrichothecenes and Syntheses of 11α-[4,15-Tetradeutero]-2*β*,13*β*-apotrichodiol (3) and 11α-[4,15-Tetradeutero]-2α,13*β*-apotrichodiol (4) (Figure 4). We have previously shown that apotrichodiol (ATD) (Figure 1), which has a trans junction between rings A and B (11*β*) and a hydroxyl at C-3, was not a precursor to trichothecenes (Zamir et al., 1987a).

We wanted to determine if one of the apotrichothecenes with 11α and C-2-OH (α or β) was a precursor to SOL. We therefore had to synthesize these two diastereomers (11α, C-2α-OH; 11α, C-2β-OH) with stable isotopes. We chose to synthesize 11α-[4,15-tetradeutero]-2β,13β-apotrichodiol (**3**) and 11α-[4,15-tetradeutero]-2α,13β-apotrichodiol (**4**) (Figure 4), which had not been prepared before. [4,15-Tetradeutero]-12,13-epoxytrichothecene was prepared according to the literature (Zamir et al., 1990). When this compound was exposed to a 0.25 M H_2SO_4 solution in methanol (Grove, 1988), the appropriate rearranged product, 11α-[4,15-tetradeutero]-2β,13β-apotrichodiol, was obtained in 76% yield (**3**, Figure 4). The structure of **3** was confirmed by mass spectrometry and by the NMR spectra of the diacetylated derivative **3A** (Table 4). The low-resolution mass spectrum of **3** showed ions at 279, 257, and 239 corresponding to MNa^+, MH^+, and $[MH - H_2O]^+$, respectively. Under high resolution, the first two had exact masses of 279.18746 and 257.20538, respectively. $C_{15}H_{20}{}^2H_4O_3Na^+$ and $C_{15}H_{20}{}^2H_4O_3H^+$ require 279.18742 and 257.20548, respectively. Correction of the low-resolution spectrum for natural abundance heavy isotope inclusion showed that the product was 73.16% labeled with four deuteriums, 16.18% with three, 6.40% with two, 2.50% with one, and 1.75% unlabeled.

The stereoisomer 11α-[4,15-tetradeutero]-2α,13β-apotrichodiol (**4**, Figure 4) cannot be obtained directly from [4,15-tetradeutero]-12,13-epoxytrichothecene. It was synthesized from the apotrichothecene **3** in three steps. The first reaction was a selective acetylation of the hydroxyl group at C-13 (Schuda et al., 1984) followed by oxidation of the 2β-hydroxyl (Corey and Suggs, 1975; Müller and Tamm, 1975) to a ketone. Reduction with sodium borohydride in methanol at 0 °C led to the C-13 monoacetylated 2α-hydroxyapotrichothecene (**Ac-4**, Figure 4). This last reaction occurs stereoselectively because it allows the molecule to assume a less hindered conformation. Indeed, the end product is less sterically hindered because the C-13-CH_2OAc group is trans to the 2α-hydroxyl group, whereas 11α-[4,15-tetradeutero]-13β-acetoxy-2β-apotricho-ol is more hindered with the C-13-CH_2OAc being cis to the 2β-hydroxyl group. Removal of the acetyl group at C-13 with anhydrous K_2CO_3 in methanol at room temperature (Schuda et al., 1984) afforded the desired 11α-[4,15-tetradeutero]-2α,13β-apotrichodiol (**4**, Figure 4). The structure was confirmed by mass spectra. The high-resolution mass spectra showed an exact mass at 257.20538. $C_{15}H_{20}{}^2H_4O_3H^+$ requires 257.20548. Compound **4** was then diperdeuteroacetylated (**4A**) for NMR identification (Table 5).

Purification of the Metabolites Derived from the Feeding of [11-Dideutero-15-deutero]trichodiene. The results obtained from the radiolabeled experiment ([15-^{3}H]trichodiene) showed conclusively that the BZN diastereomer is not incorporated and does not inhibit the production of trichothecenes (Zamir et al., 1989). Therefore, we did not have to separate the 60:40 mixture of diastereomers by extensive HPLC separations. The 60:40 [11-dideutero-15-deutero]trichodiene mixture was fed to cultures of *F. culmorum* to which a sterile Brij 35 solution was added to solubilize the hydrocarbons. The HPLC peaks corresponding to 3-ADN and SOL were collected, pooled, and purified. In addition, the HPLC peak corresponding to preSOL was isolated, acetylated with perdeuteroacetic anhydride, and further purified. The use of labeled acetic anhydride distinguishes each $-COC^2H_3$ introduced from others that may be biosynthetically acetylated. In addition, two other peaks that do not correspond to any of the known *Fusarium* metabolites were collected and purified. To ensure their purity, they were also acetylated with perdeuteroacetic anhydride and purified.

Label Distribution of 3-ADN and SOL Derived from [11-Dideutero-15-deutero]trichodiene (Tables 1 and 2). The 3-ADN derived from this feeding was enriched with deuteriums as will be seen from the analysis of its ^{1}H and ^{2}H NMR spectra (Table 1) as well as the mass spectrometry analyses. The ^{1}H NMR spectrum is similar to the one described in the literature (Zamir and Devor, 1987). The mass determined by high-resolution mass spectrometry of the 361 Da sodiated quasimolecular ion is 361.1264; $C_{17}H_{22}O_7 + Na^+$ requires 361.1263, confirming rigorously that the metabolite isolated is indeed 3-ADN. The quasimolecular ion region of the low-resolution FAB spectrum of this sample shows prominent and significant ions at 279, 301, 339, and 361 Da corresponding to $MH^+ - HOAc$, $MNa^+ - HOAc$, MH^+, and MNa^+. respectively. When we subtract from the measured intensities of the sodiated 361 Da ion cluster, the calculated intensities due to natural abundance heavy isotope substitution, we find that the sample is composed of 84.7% unlabeled 3-ADN, 4.8% monodeuterated 3-ADN, and 10.6% dideuterated 3-ADN. The 10.6% value determines therefore the extent of incorporation of trideuterotrichodiene and is in accord with previous values obtained from radiolabeled experiments (Zamir, 1989; Zamir et al., 1989). The locations of these deuteriums were determined by the deuterium NMR spectrum (Table 1) obtained for that compound, which showed the presence of three signals appearing at 3.82, 3.88, and 4.69 ppm and integrating in a ratio of 0.5:0.5:1, respectively. These results demonstrate that one of the deuteriums at C-11 (4.69 ppm) and the deuterium at C-15 (3.82 and 3.88 ppm) were retained from the trideuterotrichodiene feeding. This proves conclusively that the biosynthesis of 3-ADN does not involve an 11-keto intermediate. In addition, this result gives us some information on the enzyme hydroxylating the $-^{15}CH_2{}^2H-$ group of trichodiene. This enzyme is not specific; indeed, it has equally hydroxylated both prochiral hydrogens. We had seen the same nonspecific hydroxylase at $-^{15}CH_2{}^2H-$ group in isotrichodermin (Zamir et al., 1990).

The NMR spectrum of the SOL derived from the feeding was similar to the one described in the literature (Zamir and Devor, 1987). In addition, unambiguous confirmation that it is indeed SOL was obtained by high-resolution mass spectrometry: the mass measured for the 267 Da protonated quasimolecular ion is 267.1597; $C_{15}H_{22}O_4 + H^+$ requires 267.1596. The quasimolecular ion region of the low-resolution FAB spectrum of SOL shows prominent and significant ions at 267 and 289 Da, corresponding to protonated and sodiated SOL, respectively. Correcting again in the manner described above for natural abundance heavy isotope substitution in the protonated ion demonstrates that the sample is composed of 91.0% unlabeled and 9.0% monodeuterated SOL: only one deuterium remained. To produce SOL, trichodiene must lose the two protons at C-11, and as a result the SOL isolated from the feeding of [11-dideutero-15-deutero]trichodiene to *F. culmorum* was labeled only with the deuterium at C-15 ($\delta = 0.84$) (Table 2).

Detection and Structure Characterization of PreSOL, an Intermediate between TDN and SOL (Table 3). In this work we have employed two techniques in conjunction to try to trap or accumulate biosynthetic intermediates or shunt metabolites: (1) the large amount of trideuterotrichodiene fed (10 mg per flask) and (2) the kinetic isotope effect with a ^{2}H-labeled precursor where one or two C−^{2}H bonds have to be broken to yield the product. C−^{2}H bond cleavage requires more energy and is therefore slower than cleavage of a C−H bond and may slow the mechanism enough to enhance intermediate accumulation. To facilitate the purification of the compounds obtained from the feeding, they were acetylated with perdeuteroacetic anhydride. One of the compounds isolated from the feeding had a retention time on HPLC close to that of standard acetyl preSOL, and it was further purified by HPTLC. PreSOL was previously reported only when an excess of the precursor 12,13-epoxytrichothec-9-ene was fed to *F. culmorum* cultures (Zamir et al., 1990). Indeed, the NMR spectra of the compound obtained from the trideuterotrichodiene fed was identical to that of a sample of unlabeled standard acetyl-preSOL (Table 3) except for the presence of deuteriums at C-15 and in the acetate. Additional confirmation was obtained from mass spectrometry. The mass of the 296 Da quasimolecular ion measured by high-resolution mass spectrometry is 296.1942; C_{17} $H_{21}O_4{}^2H_3 + H^+$ requires 296.1941. The quasimolecular ion region of the low-resolution FAB spectrum of perdeuteroacetyl-preSOL shows prominent and significant ions at 296 and 318 Da, corresponding to protonated and sodiated perdeuteroacetyl-preSOL, respectively. When we subtract from the measured intensities of the 296 Da ion cluster the calculated intensities due to natural abundance heavy isotope substitution, we find that the sample of perdeuteroacetyl-preSOL is 93.2% substituted with three deuterium atoms and 6.8% with four. The extent of labeling with three deuteriums is high because they were obtained from synthesis (by acetylation of the primary alcohol at C-13 with perdeuteroacetic anhydride), and the fourth deuterium is derived biosynthetically from labeled trichodiene. ^{2}H NMR analysis (Table 3) revealed the sites of deuterium substitution: a deuterium at C-15 (0.90 ppm) and three deuteriums at C-13 (2.07 ppm) introduced by acetylation with perdeuteroacetic anhydride. Therefore, the feeding of the trichodiene with two deuteriums at C-11 enabled the isolation of preSOL, a biosynthetic precursor of SOL (Zamir et al., 1990) not usually found in the culture media.

Characterization of the Structures and Label Distribution of Metabolites 1 and 2 Derived from [11-Dideutero-15-deutero]trichodiene (Figures 2 and 3). Two novel metabolites that have not been reported in *Fusarium* species were also obtained from the feeding of [11-dideutero-15-deutero]trichodiene. These metabolites (**1** and **2**) were acetylated with perdeuteroacetic anhydride to facilitate their purification (**1A** and **2A**, Figure 2) and to distinguish them from naturally acetylated metabolites. In addition, the synthetic 11α-[4,15-tetradeutero]-2β,13β-apotrichodiol (**3**) and 11α-[4,15-tetradeutero]-2α,13β-apotrichodiol (**4**) (Figure 4) and their diacetylated derivatives **3A** and **4A** will be used for comparison purposes and to secure the stereochemistry of the new apotrichothecenes **1** and **2**. The NMR spectrum of **3A** was identical to that of **1A** derived from the feeding of [11-dideutero-15-deutero]trichodiene except for the location of deuteriums (Table 4). The NMR data for the diacetylated **1A**, **3A**, and **2A**, **4A** are shown in Tables 4 and 5.

The assignments of the ^{13}C chemical shifts are based on the two-dimensional experiments HMQC and HMBC. The structures obtained fit the apotrichothecenes shown in Figure 2. We will first consider the structure of metabolite **1**. The coupling constants can be used initially to determine the stereochemistry: (i) H-2, which is a triplet, has a $J_{2-3} = 5.1$ Hz. This medium-large coupling with both H-3 protons might suggest that H-2 is α (Hesketh et al., 1991). (ii) H-11, which is a broad doublet, has a $J_{10-11} = 5.1$ Hz. This large coupling is also consistent with α stereochemistry for H-11. Additional confirmation was obtained by the NOESY correlations. The key NOEs that support the stereochemistry shown are the following: (a) H-13 is correlated with the methyl 14 and with H-4b (1.48 ppm), which shows the β orientation. (b) H-11 has an NOE with the methyl 15, H-2 and H-4a (2.08 ppm), confirming an α conformation. (c) The stereochemistry of the hydroxyl group in position 2 is based on H-2 having an NOE correlation with H-3a (1.95 ppm) and with H-11 in the α side. The deuteriums are localized on C-11 and C-15, which prove their origin from [11-$^2H_2$,15-^{2}H]-trichodiene. Unambiguous proof of this structure was given by the semisynthesis of this metabolite **3** (11α-[4,15-tetradeutero]2β,13β-apotrichodiol) as well as its diacetate **3A**. Indeed, the NMR data of **3A** are identical to those of **1A** except for the location of the deuterium labels (Table 4). The isomer with position 2 inverted **4** (11α-[4,15-tetradeutero]-2α,13β-apotrichodiol) and its perdeuterodiacetate **4A** were also prepared. Comparison of the COSY, HMQC, and HMBC of metabolites **1** and **2** perdeuterodiacetylated **1A**, **2A**) show that they have identical skeletons: therefore, compound **2A** is also an apotrichothecene. The major differences between the two compounds lie in their levels of deuterium enrichment. Both metabolites show incorporation of two deuteriums, one at C-11 and one at C-15 (apart from the six deuteriums introduced by synthetic perdeuteroacetylation). In the case of **1A**, the labeling can be detected in ^{2}H NMR (Table 4). Indeed, a signal is observed at 0.83 ppm for ^{2}H-15 and one at 3.78 ppm for ^{2}H-11, whereas the two labeled acetyls are found at 1.80 and 2.02 ppm. The incorporation of deuteriums has been confirmed by mass spectrometry, which could also give the extent of deuterium enrichment. The low-resolution mass spectrum of **1A** showed ions at 343 and 280 corresponding to MH^+ and $[M - HOCOC^2H_3]^+$, respectively. The 343 Da ion includes the six deuteriums introduced by acetylation with perdeuteroacetic anhydride. When we subtract from the measured intensities of the 343 Da ion cluster the calculated intensities due to natural abundance heavy isotope substitution, we find that **1A** is 70.58% substituted with six deuteriums, 11.27% with seven, and 17.46% with eight. Perdeuterodiacetylated **2A** was most unusual because it was almost 100% labeled. In fact, in the ^{1}H NMR, H-11 is difficult to measure. The ^{13}C signal of methyl 15 is much weaker than that for methyl 14 and appears broader due to deuterium coupling. These data were confirmed by mass spectrometry. The high-resolution mass spectrum for **2A** showed for the MH^+ ion an exact mass 345.25158 and $C_{19}H_{20}{}^2H_8O_5H^+$ requires 345.25171. Correction of the low-resolution spectrum for natural abundance heavy isotope inclusion showed that the

compound was 41.5% labeled with eight deuteriums, 16.4% with seven, 8.9% with six, 20.1% with five, 9.0% with four, and 4% with three. Measurement of the extent of deuterium incorporation in **2A** by deuterium NMR is likely to be more precise than by FAB-MS. Indeed, even though the ionization process produces ions of low internal energy, the quasimolecular ion cluster in FAB always shows evidence of fragmentation resulting in the loss of a number of hydrogen atoms, which may include some of the deuterium label. Although corrections for natural abundance heavy isotope inclusion (chiefly ^{13}C) are routinely made in these determinations, these are approximations (estimated to be correct to within 5% in relative terms) as it is difficult to evaluate the extent of deuterium loss in these fragmentations.

If we compare the ^{13}C chemical shifts of the carbons in ring A for metabolite **2** with that of **1** and compounds **3** and **4** where the stereochemistry is well established, we find that all the shifts are very similar. This analysis therefore suggests that in all of these compounds the H-11 configuration is the same and is therefore α. On the other hand, if we compare the ^{13}C chemical shifts of the carbons in ring A for metabolite **2** with that of apotrichodiol for which X-ray crystallography has unambiguously proved the 11*β*-stereochemistry as well as that of other 11*β*-natural apotrichothecenes (Savard et al., 1989; Zamir et al., 1987a, 1992c), we see that they are very different. We can therefore rigorously conclude that both metabolites **1** and **2** have an 11α-stereochemistry.

The difference in stereochemistry between **1** and **2** could be due to the orientation of the substituent at C-2. For the H-2 of **2**, we observe a doublet of doublet with $J = 9.7$ and 5.9 Hz. These couplings are very different from H-2 of compound **1** (t, $J = 5.1$ Hz). Metabolite **1** is identical to the synthetic compound **3** and differs from **4** (H-2 dd, $J = 4.4$ and 2.7 Hz) only in the stereochemistry at C-2. If we compare the NMR data (^1H and ^{13}C) of metabolite **2** with those of synthetic **4**, *they are very different*. We can therefore conclude that the main difference between **1** and **2** is not the stereochemistry at C-2 *but at the bond C-12−C-13*. Indeed, the carbon-13 NMR of perdeuterodiacetylated metabolite **2A** differs from that of **3A** or **4A** at C-2, C-13, and C-14 (Tables 4 and 5).

In metabolite **2**, for the H-2, we observe a doublet of doublet with $J = 9.7$ and 5.9 Hz. These couplings are very different from those of metabolite **1** (which is identical to the synthetic compound **3**) (triplet, $J = 5.1$ Hz). The synthetic stereoisomer **4**, which differs from **1** or **3** only by the stereochemistry at C-2, is also a doublet of doublet but with $J = 4.4$ and 2.7 Hz. Therefore, metabolite **2** must have a different stereochemistry at C12−C13.

In the ^{13}C NMR of compounds **3** and **4** where the hydroxyl group is 2*β* or 2α, respectively, the only shifts affected are C-2 (∼ −3 ppm) and Me-14 (∼ −2 ppm). Metabolite **2** differs from **1** not only at C-13 (∼ −2 ppm) but also at *C-2 by a large shift* (∼ −6 ppm). This big difference cannot be due *only* to the orientation of C-13. Therefore, we can conclude that the 2-hydroxyl group in **2** is probably α.

We feel confident about correctly characterizing metabolites **1** (11α-[11-deutero-15-deutero]-2*β*,13*β*-apotrichodiol) and **2** (11α-[11-deutero-15-deutero]-2α,13α-apotrichodiol), which are shown in Figure 3 with their 3D structures.

Feeding of 11α-[11-Deutero-15-deutero]-2α,13α-apotrichodiol (2), 11α-[4,15-Tetradeutero]-2*β*,13*β*-apotrichodiol (3), and 11α-[4,15-Tetradeutero]-2α, 13*β*-apotrichodiol (4) to *F. culmorum* Cultures. Metabolite **2** was highly deuterated (almost 100%); we could therefore refeed it to *F. culmorum* cultures and isolate the derived SOL. NMR analysis showed that no deuterium labels were found in the SOL obtained from that feeding. The same result was observed when the semisynthetic apotrichothecene **3** was separately fed to the fungi. On the other hand, when 11α-[4,15-tetradeutero]-2α,13*β*-apotrichodiol **4** was fed to *F. culmorum* cultures, the ^2H NMR of the SOL isolated showed a signal at 0.85 ppm corresponding to H-15 and at 1.41 and 2.61 ppm for H-4b and H-4a, respectively. This experimental result proves unambiguously that 11α-[4,15-tetradeutero]-2α,13*β*-apotrichodiol (**4**) is a biosynthetic precursor to SOL. This interesting result leads us to a possible explanation for a hitherto intriguing biotransformation: the conversion of the hydrocarbon EPT to SOL. The plausible biogenetic theories for this transformation are outlined in the next section.

Biogenetic Theories for the Conversion of EPT to PreSOL. The conversion of EPT to preSOL could be explained by various mechanisms:

(i) The most obvious one is based simply on a comparison of the two structures of EPT and preSOL (Figures 1 and 6): a hydroxylation step at C-11 with *inversion* of configuration followed by opening of the epoxide. The main drawback of this possible mechanism is that hydroxylations with *inversion* of configuration are unknown in nature to our knowledge. Furthermore, the stereochemistry of the oxygenation at position C-3 of 3-ADN in the same fungi *F. culmorum* has been rigorously shown to occur with *retention* of configuration (Zamir et al., 1987b; Zamir, 1989). It would be very unusual to have one oxygenation occurring with *retention* of configuration and another one with *inversion* both in the same microorganism: *F. culmorum*.

(ii) A more plausible pathway would be opening of the ether in ring B of EPT and formation of a possible oxygenated trichodiene intermediate that would have a keto group at C-11. This would not be unusual because it could arise by a simple oxidation of a hydroxyl group at C-11. This proposed intermediate could easily be converted by a variety of mechanisms to preSOL and then to SOL.

(iii) Another putative mechanism could be opening of the ether in ring B of EPT (induced by a backside OH^- attack at C-2) and formation of an apotrichothecene resulting from 11*β*-hydroxyl opening of the epoxide from the rear resulting in a *cis junction* between rings A and B, *a β-C12−C13 bond* and a *2α-hydroxyl group*. This apo derivative would then be hydroxylated with *retention* of configuration at C-11 and dehydrated to give preSOL. The only drawback of this alternative is that apotrichothecenes have only been recently isolated in nature and only with a trans junction between ring A and B (Zamir et al., 1987a; Greenhalgh et al., 1989). The only apotrichothecenes known with a cis junction between A and B prior to this work were obtained by treating trichothecenes with acids (Grove, 1988).

The present work is a strong supporter of this last mechanism because we have proved that such an apotrichothecene (compound **4**) is a biosynthetic precur-

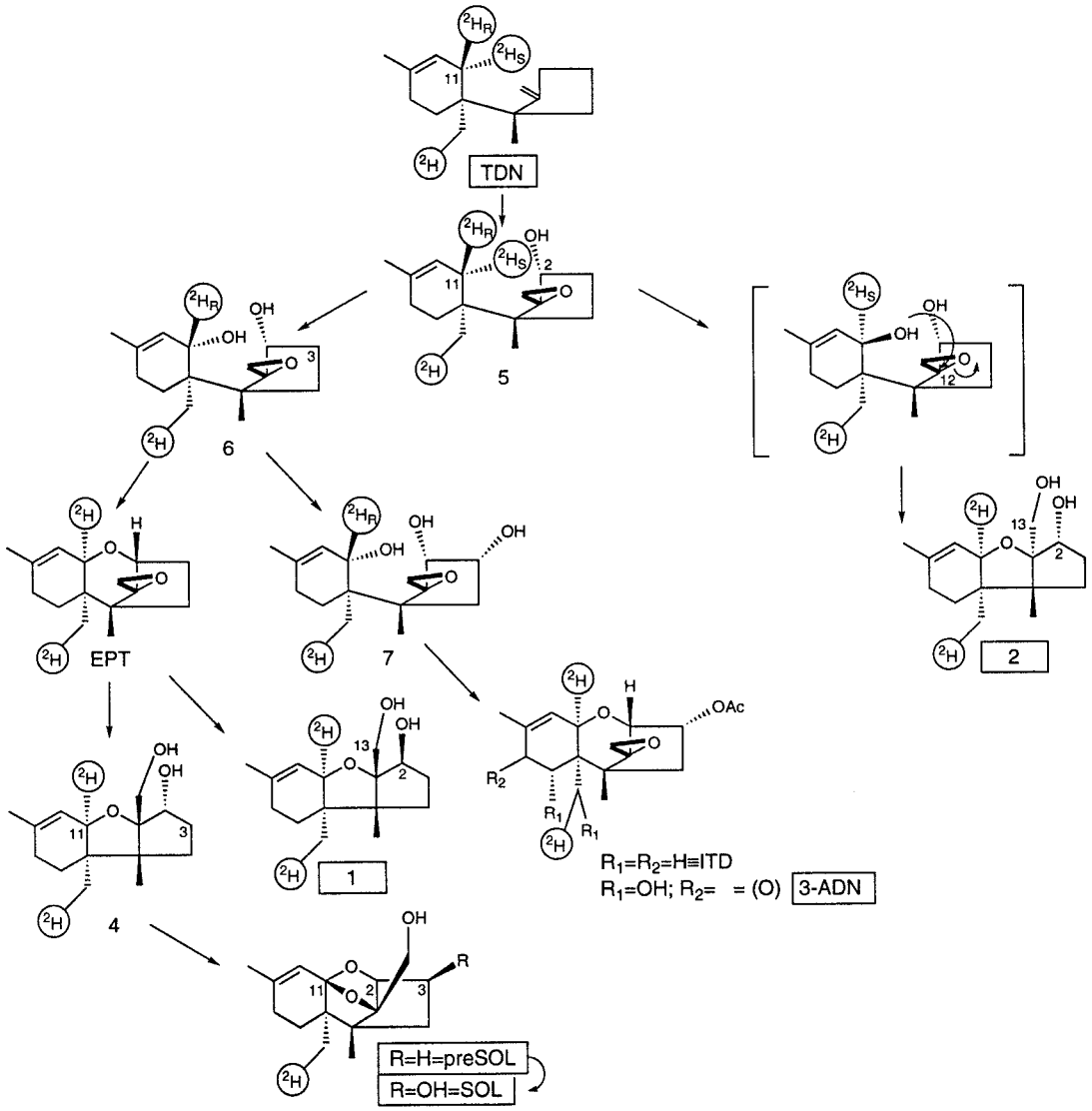

Figure 6. Postulated biosynthetic pathway from TDN to the apotrichothecenes (**4**, **1**, and **2**) and to the trichothecenes 3-ADN, preSOL, and SOL. Unlabeled oxygenated trichodiene species **5** and **6** have been isolated from *F. culmorum* (Zamir et al., 1991; Hesketh et al., 1991), whereas nondeuterated **7** has been found in a mutant of *F. sporotrichioides* (McCormick et al., 1990).

sor to SOL. In addition, the accumulation of the metabolites **1** and **2** with a cis junction between rings A and B supports the proposal that they could arise biosynthetically. Both compounds were shown in this work to be obtained from trichodiene. In addition, the very high level of labeling of metabolite **2** ensures that it derives from trichodiene with very few biosynthetic steps. A general biosynthetic scheme that could explain all of the experimental data obtained on trichothecenes and apotrichothecenes is described in the next section.

Biosynthesis of 3-Acetyldeoxynivalenol, Apotrichothecenes, and SOL and Intermediacy of EPT and ITD (Figure 6). The deuteriums at C-11 and C-15 were found in the 3-ADN obtained from the feeding of [11-dideutero-15-deutero]trichodiene (Table 1). This experimental result constitutes a proof that an 11-keto precursor was not involved in the biosynthesis of 3-ADN. In the case of SOL, both deuteriums at C-11 are lost during the biosynthesis and the deuterium at C-15 remained, which proved that, indeed, [11-dideutero-15-deutero]trichodiene was incorporated. The finding of preSOL as well as SOL with a deuterium at C-15 (Figure 2) is additional evidence that despite the two deuteriums at C-11, the biosynthesis of trichothecenes is not inhibited. Because in preSOL and in SOL both hydrogens at C-11 are lost, we cannot yet rule out an 11-keto precursor. In Figure 6, we postulate a biosynthetic scheme that can explain all of the reported experimental results: (i) the formation of apotrichothecenes **1** and **2**; (ii) the incorporation of semisynthetic apotrichothecene **4** into SOL; (iii) the conversion of EPT to preSOL and SOL; (iv) the nonincorporation of **1** (or **3**) and **2** into preSOL and SOL; and (v) the biosynthesis of 3-ADN via ITD.

The first dioxygenated trichodiene that has been shown to be incorporated to 3-ADN and to SOL is 2α-hydroxy-12,13β-epoxytrichodiene (Zamir et al., 1991) (**5**, Figure 6). The next step involves the intermediate 2α,-11α-dihydroxy-12,13β-epoxytrichodiene (**6**, Figure 6), which has been incorporated into 3-ADN (Hesketh et al., 1990, 1991, 1993). We have shown that the biosynthesis of 3-ADN goes through ITD, whereas that of SOL is via EPT and that the differentiation probably occurs at the trichodiene stage (Zamir et al., 1990). The probable precursor of ITD would therefore be 2α,3α,-11α-trihydroxy-12,13β-epoxytrichodiene (**7**, Figure 6), which has been isolated from a *Fusarium sporotrichioides* mutant (McCormick et al., 1990). The segment **5**

→ **6** → **7** → ITD and 3-ADN has been previously suggested by many authors and has been recently described in a review (Dewick, 1997, and references cited therein). The isolation of metabolite **2**, which was nearly 100% labeled from the feeding of [11-dideutero-15-deutero]trichodiene, demonstrates that it is not only derived from trichodiene but derived *in very few biosynthetic steps*. One plausible mechanism is shown in Figure 6. We have seen that the enzyme which hydroxylates the two prochiral hydrogens in C-15-deuterated trichodiene (this work) or C-15-deuterated isotrichodermin (Zamir et al., 1990) is not selective and replaces both hydrogens with equal probability. We could therefore postulate a similar situation with the two deuteriums at C-11 of compound **5** (Figure 6). If the ^2H_R of H-11 is replaced with hydroxyl, and this β-hydroxyl opens the epoxide from the top side of C-12, metabolite **2** will be obtained with an inverted C12−C13 side chain and a 2α-hydroxyl (Figure 6). This could explain the degree of deuterium labeling of metabolite **2** because after hydroxylation only one biosynthetic step is needed. If the ^2H_S of H-11 of compound **5** (Figure 6) is replaced with a hydroxyl, the reported intermediate 2α,11α-dihydroxy-12,13β-epoxytrichodiene (Hesketh et al., 1990, 1991, 1993) (**6**, Figure 6) is formed. At that stage the biosynthetic paths for 3-ADN or to SOL diverge. Compound **6** is either cyclized to EPT or oxygenated to a trihydroxylated trichodiene intermediate, 2α,3α,11α-trihydroxy-12,13β-epoxytrichodiene (**7**, Figure 6), which is then probably converted to ITD and 3-ADN.

The plausible conversion of EPT to metabolite **1** or to the apotrichothecene **4** can easily be explained by an attack of an OH^- on C-2 (front side for **1** or back side for **4**) followed by rear side opening of the epoxide. Metabolite **1** was not incorporated to preSOL, and this is understandable because the 2-OH does not have the required stereochemistry. It is a dead-end metabolite and therefore was accumulated after the feeding of [11-dideutero-15-deutero]trichodiene to *F. culmorum*. On the other hand, the apotrichothecene **4** was not isolated probably because of its easy conversion to preSOL, which involves a hydroxylation at C-11 with retention of configuration and dehydration. Biosynthesis of SOL would then just necessitate a hydroxylation at C-3. The discovery of 11α-deuterium-labeled apotrichothecenes (**1** and **2**, Figure 6) from the feeding of [11-dideutero-15-deutero]trichodiene and the finding of the conversion of 11α-[4,15-tetradeutero]-2α,13β-apotrichodiol (**4**) into labeled SOL can be considered as an indirect proof that an 11-keto intermediate is not involved in the biosynthesis of SOL.

The only experimental result that does not seem to fit with this mechanism is a preliminary finding (Zamir et al., 1992c) of a low conversion (1%) of radiolabeled apotricho-ol (with 11β-stereochemistry) to SOL. The radiolabeled apotricho-ol was obtained from (3*RS*)[2-^{14}C]-mevalonate feeding, and no degradation to determine the radiolabel position in the derived SOL was done. Therefore, these data probably result from complete degradation of apotricho-ol prior to resynthesis of SOL. This is in fact the disadvantage of using radiolabeled precursors when it is not easy to localize the radiolabel, and therefore a degradation to an early intermediate cannot be detected. On the other hand, this is the advantage of stable isotopes such as ^2H or ^{13}C when the position of the label is easily determined by NMR. We are therefore very confident in the results of this work because it was done with specific deuterium labeling, and at each biosynthetic step we have determined the position of the label.

ABBREVIATIONS USED

3-ADN, 3-acetyldeoxynivalenol; SOL, sambucinol; TDN, trichodiene; BZN, bazzanene; ITD, isotrichodermin; EPT, 12,13-epoxytrichothec-9-ene; PCC, pyridinium chlorochromate; preSOL, 3-deoxysambucinol; ATD, apotrichodiol; HAD, 2-hydroxy-*cis*-apotrichothecene; HPLC, high-performance liquid chromatography; HP-TLC, high-performance thin layer chromatography; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect 2D; HMBC, heteromultiple bound correlation; HMQC, heteronuclear multiple quantum coherence; BIRD, bilinear rotational decoupling; FAB, fast atom bombardment; MS, mass spectrometry; FIDS, free induction decays.

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Received for review July 24, 1998. Revised manuscript received January 12, 1999. Accepted March 5, 1999. We thank the Natural Sciences and Engineering Research Council of Canada and Canadian Breast Cancer Research Initiative for support of this work via operating grants to L.O.Z. and the Medical Research Council for grants to O.M.

JF980808R