Biosynthesis of 3-Acetyldeoxynivalenol and Sambucinol

IDENTIFICATION OF THE TWO OXYGENATION STEPS AFTER TRICHOIDEINE*

(Received for publication, October 16, 1998, and in revised form, January 29, 1999)

Lolita O. Zamir‡§, Anastasia Nikolakakis‡, Liren Huang§, Patrick St-Pierre§, Françoise Sauriol‡, Salvatore Sparace, and Orval Mamer**

From the §Centre de Recherche en Microbiologie Appliquée, Université du Québec, Institut Armand-Frappier, Laval, Quebec H7T 4Z3, Canada, the ‡Department of Chemistry, McGill University, Montreal, Quebec H3Z 2K6, Canada, the **Plant Science Department, McGill University, McConnell Campus, Ste-Anne de Bellevue, Quebec H9X 3V9, Canada, and the "Biomedical Mass Spectrometry Unit, McGill University, Montreal, Quebec H3A 1A3, Canada

The first two oxygenation steps post-trichodiene in the biosyntheses of the trichothecenes 3-acetyldeoxynivalenol and sambucinol were investigated. The plausible intermediates 2-hydroxytrichodiene (2α- and 2β-) and 12,13-epoxytrichodiene and the dioxygenated compounds 12,13-epoxy-9,10-trichoene-2-ol (2α- and 2β-) were prepared specifically labeled with stable isotopes. They were then fed separately and/or together to Fusarium culmorum cultures, and the derived trichothecenes were isolated, purified, and analyzed. The stable isotopes enable easy localization of the labels in the products by 1H NMR, 13C NMR, and mass spectrometry. We found that 2α-hydroxytrichodiene is the first oxygenated step in the biosynthesis of both 3-acetyldeoxynivalenol and sambucinol. The stereoisomer 2β-hydroxytrichodiene and 12,13-epoxytrichidiene are not biosynthetic intermediates and have not been isolated as metabolites. We also demonstrated that the dioxygenated 12,13-epoxy-9,10-trichoene-2α-ol is a biosynthetic precursor to trichothecenes as had been suggested in a preliminary work. Its stereoisomer was not found in the pathway. A further confirmation of our results was the isolation of both oxygenated trichodiene derivatives 2α-hydroxytrichodiene and 12,13-epoxy-9,10-trichoene-2α-ol as natural metabolites in F. culmorum cultures.

Trichothecenes are toxic secondary metabolites produced by fungi, in particular by Fusarium spp. They infect mostly wheat, grains, and corn and therefore affect human and animal health (1–4). Fusarium culmorum (HLX-1503) produces two major trichothecene metabolites (5): 3-acetyldeoxynivalenol (3-ADN)1 and sambucinol (SOL) (Fig. 1). These mycotoxins have been known for a long time (6); however, there is not yet an efficient detoxification procedure. Since trichodiene (TDN; 1, Fig. 1), the biosynthetic precursor to trichothecenes, is not toxic, knowledge of its first oxidized metabolite will enable the design of a potent inhibitor to trichothecenes. We therefore decided to focus on the identification of the oxygenation steps after the hydrocarbon trichodiene. The first oxygenated trichothecene derivatives isolated from F. culmorum were 9,10-trichoene-12,13-diol (2d) and 12,13-epoxy-9,10-trichoene-2α-ol (3a) (Fig. 1) (8). These compounds had been detected by the kinetic pulse-labeling method (7) and could accumulate when the inhibitor ancinymidol was used (8). In addition, they had been isolated with a radiolabel after feeding (3RS)-[2,14C]mevalonate to F. culmorum cultures and isolating and purifying the derived radiolabeled 2d and 3a. These compounds were separately fed to F. culmorum cultures, and the derived trichothecenes 3-ADN and SOL were recovered and analyzed. Metabolite 3a was found to be incorporated in both 3-ADN and SOL, whereas the trichothecenes obtained after the feeding of 2d were unlabeled. This preliminary result suggests that 3a might be a biosynthetic precursor to 3-ADN and SOL, whereas 2d is a dead end metabolite (8). This result is not extremely rigorous, since no degradation could be done on the radiolabeled trichothecenes obtained from the feeding of compound 3a to prove the labeled site. Indeed, there is a possibility that the metabolite 3a was degraded and then resynthesized into the trichothecenes. Since we wanted to determine with certainty the sequence of oxygenation post-trichodiene, our first goal was to determine unambiguously if 3a is the dioxygenated metabolite and then what is the first oxygenated precursor: 2a, 2b, or 2c. The isolated 2d was shown to be a dead end product and probably derives from opening of the epoxide 2c (8).

In this paper, we have rigorously proven, for the first time, the two oxygenation steps of trichodiene and their sequence. A biosynthetic scheme for all of the oxidations leading to 3-ADN and SOL is proposed.

EXPERIMENTAL PROCEDURES

Instrumentation—Analytical high performance liquid chromatography (HPLC) was performed on the same instrument used previously (9) or on a Perkin-Elmer Binary LC pump 230 coupled to a Waters 990 photodiode array detector or on a Waters 600 pump coupled to a Waters 990 photodiode array detector. Preparative and semipreparative HPLC were performed on a Waters Delta Prep 3000 instrument coupled to a Lambda-Max model 481 LC spectrophotometric detector. All ultraviolet detectors were set at 204 nm. Infrared spectra were measured in chloroform with a Perkin-Elmer model 693 infrared spectrophotometer. Mass chromatography was performed on silica gel 60, 230—400 mesh (EM Science). Thin layer chromatography was conducted on Silica Gel 60 F254 precoated TLC plates, 0.25 mm (EM Science). High performance TLC was run with LHP-KF, 0.2-mm plates (Whatman). Radiolabeled compounds were analyzed with a Bioscan Imaging Scanner System 200.
Synthesis of Compound 7

A solution of compound 7 (0.21 g; 0.51 mmol) in 2 ml of tetrahydrofuran was added dropwise. After stirring at room temperature and was treated with enone (0.036 g; 0.60 mmol) as a colorless oil. A solution of compound 7 (0.24 g; 0.51 mmol) in 2 ml of tetrahydrofuran was added dropwise. After stirring at room temperature and was treated with enone (0.036 g; 0.60 mmol) as a colorless oil. A solution of compound 7 (0.24 g; 0.51 mmol) in 2 ml of tetrahydrofuran was added dropwise. After stirring at room temperature and was treated with enone (0.036 g; 0.60 mmol) as a colorless oil.
of absolute ethanol was treated with pyridine (0.41 mL; 5.07 mmol) and freshly activated zinc (0.125 g; 1.91 mmol). The solution was heated at 79 °C for 2 h. Upon cooling to room temperature, the solution was filtered and diluted with ethyl acetate. The organic layer was washed with a saturated solution of NaHCO3 and brine to neutrality. The ethyl acetate layer was dried (MgSO4) and filtered. The residue was chromatographed on silica gel using hexane/ethyl acetate (95:5) to yield 0.1 mg (69%) of the desired deuterated 12,13-epoxytrichodione 2e as a colorless oil. 1H NMR (400-Hz acetonitrile-d6) δ (ppm) 2.75 (1H, br, s), 10.10 (1H, d, J = 4.9 Hz, H-13a), 3.16 (0.6H, d, J = 4.9 Hz, H-13a), 2.73 (0.6H, d, J = 4.4 Hz, H-13b), 2.70 (0.4H, d, J = 4.4 Hz, H-14b), 2.64–2.68 (2H, br, s, H-6a), 1.89 (1.2H, s, 1.8H, s, H-14b), 0.85 (1.2H, m, H-15), 0.82 (0.8H, t, J = 2.0 Hz, H-15), 1H NMR δ (ppm) 0.87 (0.6H, s, H-15), 0.84 (0.4H, s, H-15).

Synthesis of Compound 16 (Fig. 16)—Compound 16 was prepared from compound 6 employing the same procedure as for the preparation of compound 8 from compound 7. Compound 16 was isolated as white crystals, melting point 66–68 °C. (IR (CHCl3), vmax 3090, 3030(w), 2970, 1730, 1440, 1370, 1245, 970 cm−1; 1H NMR δ ppm) 6.84 (1H, d, J = 10.6, 2.0 Hz, H-11), 5.93 (1H, d, J = 10.6 Hz, H-10), 5.21 (1H, d, J = 2.6 Hz, H-13a), 5.01 (1H, d, J = 2.6 Hz, H-13b), 4.25 (1H, m, H-2), 2.46–3.16 (8H, m, H-3, H-4, H-7, H-8), 1.78 (1H, H-13a), 1.15 (3H, s, H-14). 9.39 (3H, s, t-Bu), 0.11, 0.08 (6H, s, SiMe3).

Synthesis of Compound 17 (Fig. 17) — Compound 17 (a colorless oil) was prepared from compound 6. The procedure for the preparation of compound 9 from compound 8. (IR (CHCl3), vmax 3090, 3060(w), 2960(s), 2860(s), 1650(w), 1625(w), 1580(w) cm−1; 1H NMR δ ppm) 6.8 (1H, d, J = 10.6, 4.0 Hz, H-11), 5.81 (1H, d, J = 10.6 Hz, H-10), 5.13 (1H, d, J = 2.0 Hz, H-13a), 4.96 (1H, d, J = 2.0 Hz, H-13b), 4.66 (1H, d, J = 13.1 Hz, H-14b), 3.57 (1H, H-13b), 2.16–2.18 (3H, m, H-3, H-4, H-7, H-8), 1.82 (1H, H-13a), 1.15 (3H, s, H-14), 0.93 (9H, t-Bu), 0.11, 0.08 (6H, s, SiMe3); 13C NMR δ ppm 153.6, 142.9, 136.5, 128.3, 110.0 (C-16 enriched), 106.7, 47.7, 40.3, 32.2, 31.6, 30.1, 27.1, 26.0, 25.1, 22.7, 20.7, 14.1, 4.8.

Synthesis of Compound 18 (Fig. 18) — Compound 18 (a colorless oil) was prepared from compound 17 similarly to the preparation of compound 10 from compound 9. (IR (CHCl3), vmax 3090(w), 3060(w), 2960(s), 1645(s), 1645(s) cm−1; 1H NMR δ ppm) 5.25 (1H, br, t, J = 5.4 Hz, H-10), 5.62 (1H, d, J = 1.3 Hz, H-13b), 5.17 (1H, d, J = 1.3 Hz, H-13a), 5.06 (1H, d, J = 2.6 Hz, H-13b), 4.25 (1H, br, t, J = 4.9 Hz, H-14b), 1.88 (1.2H, s, 1.8H, s, H-14b), 0.82 (1.1H, m, H-15), 0.79 (0.9H, t-Bu), 0.11, 0.09 (6H, s, SiMe3); 13C NMR δ ppm 159.9, 132.4, 120.3, 106.7, 48.7, 36.5, 32.7, 32.1, 31.6, 27.9, 26.0, 24.6, 23.3 (s, C-16 highly enriched), 22.2, 18.4, 17.7, 4.8.

Synthesis of Compound 20 (Fig. 2b) — 2p-Butyldimethylsilyloxy-16β-13H-trichodione 18 (56 mg; 0.17 mmol), in a 1 mL solution of tetraethylammonium fluoride (0.50 mL; 0.50 mmol) was treated with acetic acid (29 µl; 0.50 mmol) at room temperature for 36 h. The reaction was quenched with 0.5 mL of water, extracted with ether, dried on MgSO4, filtered, and evaporated to dryness. Flash chromatography on silica gel yielded the desired product as a colorless oil: 2β-hydroxy-16β-13H-trichodione. (IR (CHCl3), vmax 3600(m), 3450(br), 3080(w), 2960(s), 1645(w) cm−1; 1H NMR δ ppm) 5.28 (1H, br, t, J = 4.3 Hz, H-10), 5.15 (1H, d, J = 2.6 Hz, H-13a), 4.93 (1H, d, J = 2.6 Hz, H-13b), 1.25 (1.5H, br, s, H-6b), 2.18–1.78 (5H, m, H-3, H-4, H-7, H-8, H-11), 1.09 (3H, s, H-14), 0.93 (9H, t-Bu), 0.83 (3H, H-15), 0.11 (0.86 (6H, s, SiMe3); 13C NMR δ ppm 159.9, 132.4, 120.3, 106.7, 48.7, 36.5, 32.7, 32.1, 31.6, 27.9, 26.0, 24.6, 23.3 (s, C-16 highly enriched), 22.2, 18.4, 17.7, 4.8.

Feeding of 12,13-Epoxide-16β,17β-dibromo-28-ol 3b and Purification and Characterization of the Derived 3-ADN and SOL—The 12,13-epoxide-16β,17β-dibromo-28-ol 3b, 45 mg in total, was dissolved in methanol and was equally distributed among nine sterile 125-mL Erlemeyer flasks. The methanol was allowed to evaporate from the stoppered flasks overnight. To each of the nine flasks was added 0.1 mL of a 5% Brj 35 solution (15) and one 48-h production culture (previously prepared from a 3-day-old seed culture). Three controls were prepared by adding 0.1 mL of a 5% Brj 35 solution directly to the broth containing the production culture. The cultures were incubated for 7 days at 25 °C and 220 rpm. After 7 days, the three controls and the nine samples were filtered separately. The filtrates were saturated with NaCl and extracted with ethyl acetate. The organic extracts were dried over MgSO4, filtered, and concentrated in vacuo. The sample crude extract was fractionated by preparative HPLC using gradient A at 18 mL/min. Fraction 1 and fraction 3, corresponding to 3-ADN (tR = 36 min) and SOL (tR = 48...
min), respectively, were further purified by HPLC. The pure 3-ADN (t<sub>R</sub> = 37 min) that was isolated contained no 13C incorporation. SOL was acetylated by incubation at 25 °C for 18 h with 90 μl of deuterated acetic anhydride (Ac<sub>2</sub>O(D<sup>2</sup>)<sub>H</sub>) and 60 μl of pyridine, purified by HPLC, and the pure diacyl-SOL (t<sub>R</sub> = 49 min) also contained no 13C incorporation; therefore, no enriched carbon visible.

**Feeding of 12,13-Epoxy-[16-13C]9,10-trichone-2α-ol 3a and Purification and Characterization of the Derived 3-ADN, SOL, pre-SOL, and 11α,2β,13β-Apotrichodiol—**The 12,13-epoxy-[16-13C]9,10-trichone-2α-ol, 35 mg in total, was dissolved in ether and equally distributed among eight sterile 125-ml Erlenmeyer flasks. The ether was allowed to evaporate. The eight flasks were then stored for 3 days at 25 °C overnight. The acetic anhydride and pyridine were evaporated. FAB-MS: (M<sup>+</sup> + Na<sup>+</sup>): 273.1468; C<sub>13</sub>H<sub>18</sub>O<sub>3</sub>Na<sup>+</sup> requires 273.1467. The 13C NMR spectra of SOL show incorporation of 13C at 23.0 ppm corresponding to C-16 at 15.3 ppm. The percentage of 13C incorporation calculated by low resolution mass spectrometry was 44.1%. From NMR we obtained the ratio of 32:68 between 13C-enriched and 12C-nonenriched metabolites by comparing the integration peaks of the doublet derived from the coupling of 13C-H-16 and the singlet 12C-H-16 at 1.92 ppm (J<sub>13C-12C</sub> = 129.0 Hz). Fraction 3, corresponding to SOL (t<sub>R</sub> = 55 min) was isolated and evaporated. FAB-MS: (M<sup>+</sup> + H<sup>+</sup>): 267.1597; C<sub>13</sub>H<sub>18</sub>O<sub>4</sub>Na<sup>+</sup> requires 267.1596. The 13C NMR spectra of SOL show incorporation of 13C at 23.0 ppm, corresponding to C-16. The percentage of 13C incorporation calculated by low resolution mass spectrometry was 49.9%. From NMR we obtained the ratio of 57:43 between 13C-enriched and 12C-nonenriched metabolites by comparing the integration peaks of the doublet derived from the coupling of 13C-H-16 and the singlet 12C-H-16 at 1.76 ppm (J<sub>13C-12C</sub> = 126.5 Hz). Fraction 5, corresponding to pre-SOL (t<sub>R</sub> = 62–64 min) was further purified by analytical HPLC using 55% methanol, 45% water at 1 ml/min (t<sub>R</sub> = 64 min). After evaporation, the structure was confirmed by high resolution mass spectrometry. FAB-MS: (M<sup>+</sup> + Na<sup>+</sup>): 273.1466; C<sub>13</sub>H<sub>18</sub>O<sub>4</sub>HNa<sup>+</sup> requires 273.1467. The 13C NMR spectra of pre-SOL show incorporation of 13C at 23.0 ppm, corresponding to C-16. The percentage of 13C incorporation calculated by low resolution mass spectrometry was 51.7%. From NMR, we obtained the ratio of 44:56 between 13C-enriched and 12C-nonenriched metabolites by comparing the integration peaks of the doublet derived from the coupling of 13C-H-16 and the singlet 12C-H-16 at 1.84 ppm (J<sub>13C-12C</sub> = 126.0 Hz). Fraction 6 (t<sub>R</sub> = 64–69 min), from HPLC gradient A, was acetylated with 145 μl of deuterated acetic anhydride and 135 μl of pyridine at 25 °C overnight. The acetic anhydride and pyridine were evaporated under a stream of nitrogen, and the acetylated fraction was purified by analytical HPLC using 65% methanol, 35% water at 1 ml/min (t<sub>R</sub> = 56 min). NMR characterization identified this compound as 11α,2β,13β-apotrichodiol, which has previously been isolated from cultures of *F. culmorum* (14). The 13C NMR of 11α,2β,13β-apotrichodiol shows incorporation of 13C at 23.4 ppm corresponding to C-16. The 1H NMR spectra of the decacylated compound (11α,2β,13β-apotrichodiacetate) is as follows: δ(ppm) 5.55 (1H, br, m-H, H-10), 5.16 (1H, t, J<sub>13C-12C</sub> = 5.1 Hz, H-2), 4.38 (1H, d, J<sub>13C-12C</sub> = 11.7 Hz, H-13A), 4.12 (1H, d, J<sub>13C-12C</sub> = 11.7 Hz, H-13B), 3.77 (1H, br, J<sub>13C-12C</sub> = 5.3 Hz, H-11), 1.94 (1H, br, J<sub>13C-12C</sub> = 1.7 Hz, H-3A, 1.71 (3H, d, J = 126.0 Hz, H-18), 1.64 (1H, om, H-3B), 1.04 (3H, s, H-14), 0.82 (3H, s, H-15); H NMR (δ ppm) 2.01, 1.97 (6H, s, OAc); 13C NMR (δ ppm) 118.4 (J<sub>13C-C</sub> = 1.8 Hz, C-10), 118.6 (J<sub>13C-C</sub> = 1.8 Hz, C-10), 81.6 (s, C-2), 79.0 (s, C-11), 66.0 (s, C-13), 34.4 (s, C-4), 30.2 (s, C-3), 23.4 (s, C-16 enriched), 17.9 (s, C-14), 15.1 (s, C-15). From this 1H NMR, we obtained a ratio of 90:10 between 13C-enriched and 12C-nonenriched metabolites by comparing the integration peaks of the doublet derived from the coupling of 13C-H-16 and the singlet 12C-H-16 at 1.71 ppm (J<sub>13C-12C</sub> = 126.0 Hz).

**Feeding of 2α-Hydroxy-[16-13C]trichodiene 2a and/or [15-2H]12,13-Epoxytrichodiene 2c and Purification and Characterization of the Derived 3-ADN and SOL—**2α-Hydroxy-[16-13C]trichodiene 2a (10 mg) and total dissolved in acetone, was equally distributed to six 125-ml Erlenmeyer flasks. A mixture of 2α-hydroxy-[16-13C]trichodiene 2a (10 mg) and [15-2H]12,13-epoxytrichodiene 2c (10 mg) dissolved in acetone was equally distributed to each of five 125-ml Erlenmeyer flasks. The ace-
of the filtrate (5 liters in total) was extracted with ethyl acetate (3 liters) to remove the mycelia. Each liter of the filtrate was filtered through miracloth (Calbiochem) to remove the mycelia. Each liter of the filtrate was saturated with MgSO₄, filtered, and concentrated in vacuo. The crude extract was dissolved in methanol to be repurified by fractionation on semipreparative HPLC using gradient A, at 3 ml/min. The peak observed at 23.0 ppm. When a mixture of 2a and 2e was fed there was not enough sambucinol to isolate.

Feeding of 2α-hydroxy-[16,13C]trichodiene 2b and Purification and Characterization of the Derived 3-ADN and SOL—The 2α-hydroxy-[16,13C]trichodiene, 50 mg in total, was dissolved in methanol and equally distributed among 10 sterile 125-ml Erlenmeyer flasks. The methanol was allowed to evaporate from the stoppered flasks overnight. To each of the 10 flasks was added 0.1 ml of a 5% Brij 35 solution and one 48-h production culture. The cultures were incubated for 5 days at 25 °C and 220 rpm. After 5 days, the two controls and the 10 samples were filtered separately. The filtrates were saturated with NaCl and extracted with ethyl acetate. The organic extracts were dried over MgSO₄, filtered, and concentrated in vacuo. For the feeding of 3a, we acetylated it with radiolabeled acetic anhydride. The compound was dissolved in a mixture of 120 μl of pyridine and 25 μl (12.5 μCi) of [1-13C]acetic anhydride (100 mCi/mmol). The mixture was incubated for 24 h at 25 °C and then 145 μl of unlabeled acetic anhydride was added, and the mixture was incubated for an additional 24 h at 25 °C. Following the removal of acetic anhydride and pyridine, methanol was added, and the solution was fractionated by analytical HPLC using 80% methanol and 20% water at a flow rate of 1 ml/min. The peak observed at tₑ = 56 min was collected and extracted using a ChemElut tube (Extube®, Varian) with 4 × 10 ml of pentane and evaporated under a stream of nitrogen to 20 μl and spotted on a high performance TLC plate. The plate was developed with ethyl acetate/hexane (1:4) and then analyzed by the Bio scan imaging scanner system. One symmetrical radioactive peak was detected with an RFₚ of 0.81. In order to ensure that the compound was pure and was identical to synthesized 2α-acetyl-trichodienol, it was purified on HPLC using three different conditions of elution at 1 ml/min, and its retention time coincided with the standard. The conditions were as follows: 80% methanol, 20% water, tₑ = 56 min; 85% methanol, 15% water, tₑ = 32 min; and HPLC program B, tₑ = 61 min.

Identification of the Two Oxygenation Steps after Trichodiene

**Fig. 2.** Syntheses of 2α-hydroxy-[16,13C]trichodiene 2a; 12,13-epoxy-[16,13C]9,10-trichoene-2α-ol 3a; and 12,13-epoxy-[16,13C]9,10-trichoene-2β-ol 3b. TBDMs, t-butyldimethylsilyloxy.

**Fig. 3.** A, synthesis of [15⁻³H]12,13-epoxytrichodiene 2c; B, synthesis of 2β-hydroxy-[16-13C] trichodiene 2b. OTBDMS, t-butyldimethylsilyloxy.
RESULTS AND DISCUSSION

Putative Post-trichodiene Oxygenated Trichothecene Precursors (Fig. 1)—Putative post-trichodiene oxygenated precursors were synthesized with a stable isotope label (13C or 2H) at a nonlabile position and fed to F. culmorum cultures. The derived metabolites were analyzed by 13C or 2H NMR and mass spectrometry to determine if the precursor was incorporated and the site and extent of incorporation. In order to confirm the preliminary data (8) suggesting that 12,13-epoxy-9,10-trichene-2β-ol (3a, Fig. 1) is a post-trichodiene biosynthetic precursor to trichothecenes, we synthesized it with a 13C label at C-16 (Fig. 2). Its stereoisomer 12,13-epoxy-[16-13C]9,10-trichene-2β-ol (3b, Fig. 2) was also prepared with a 13C label at C-16. We wanted to determine if both stereoisomers were precursors to trichothecenes, implying a 2-keto-12,13-epoxy-9,10-trichene intermediacy. The syntheses of two of the three plausible monooxygenated trichodiene intermediates (2a and 2b, Figs. 2 and 3) are closely related to that of 3a and 3b. We therefore decided to synthesize the different stereoisomers of the putative monooxygenated precursors (2a–2c, Figs. 2 and 3) as well as 3a and 3b, specifically labeled with stable isotopes (13C or 2H). The dioxygenated trichodiene intermediates 3a and 3b (Fig. 2) and the monooxygenated stereoisomers of 2-hydroxytrichodiene (2a- and 2β-) were labeled with a 13C label at C-16 (2a and 2b, Figs. 2 and 3), while 12,13-epoxytrichodiene was labeled with a deuterium at C-15 (2c, Fig. 3A). We have prepared only the β-epoxide (2c), since all natural trichothecenes have this stereochemistry. The rationale for using a different isotope for 2a, 2b, and 2c is to enable the simultaneous feeding of two plausible monooxygenated precursors (2a and 2c; 2b) under the exact same conditions. The analysis of the derived products will therefore determine the relative incorporations. We confirmed these results by separate feedings of each compound. Below, the synthetic methods for specifically labeling 3a and 3b and 2a, 2b, and 2c will be described.

Syntheses of 12,13-Epopy-[16-13C]9,10-trichoene-2β-ol 3b, 12,13-Epopy-[16-13C]9,10-trichoene-2α-ol 3a, 2α-Hydroxyl-[16-13C]trichodiene 2a, 2β-Hydroxyl-[16-13C]trichodiene 2b, [15-2H]12,13-Epopytrichodiene 2c (Figs. 2 and 3)—This is the first time these labeled compounds were prepared. The 13C label was introduced via a Wittig reaction of a ketone with Ph3P13CH3I. No unlabeled reagent was added in order to ensure 100% incorporation of 13C. The 2H label was derived from reduction of an alkyl group at C-15 with NaBH4, thereby introducing one deuterium in the resulting primary alcohol in the two prochiral hydrogens, prior to reducing it to a methyl group. The reactions involved in the syntheses of 3a, 3b, and 2a are the following. The tricarbonyl [4-methoxy-1-methyl-1-(1-methyl-2-methylenecyclopentyl)-(2-5-η)-cyclohexa-2,4-dienyl] iron (4) was prepared according to Pearson’s method (11, 25) and was oxidized using selenium dioxide and a 90% solution of tert-butyl hydroperoxide (16). Compound 5 was obtained with an allylic alcohol at C-2 with a β-orientation as confirmed by NOESY experiments. Indeed, the NOE correlations of 5 show a strong interaction between H-2 and Me-15, thereby confirming the existence of a β-OH on C-2. Decomplexation of compound 5, using CuCl2 in ethanol (17) produced enone 6. Subsequent epoxidation of enone 6 using vanadyl acetylacetonate with a 90% solution of tert-butyl hydroperoxide (16) and silylation of the epoxide 7 with t-butyldimethylsilyl chloride produced compound 8. Wittig olefination of compound 8 with Ph3P13CH3I (12) enabled the introduction of the 13C label at C-16 (trichothecene numbering) to afford compound 9, which was subsequently reduced with sodium in liquid ammonia to give compound 10 (11, 25). Desilylation of compound 10 with tetrabutylammonium fluoride produced 12,13-epoxy-[16-13C]9,10-trichoene-2β-ol (3b). This compound was acetylated (Ac-3b), and its NOE correlations determined by the NOESY experiment show a strong interaction between H-2 and Me-15, thereby confirming the existence of a β-OH on C-2.

In order to synthesize compound 3a containing the C-2 hydroxy with an α configuration, the allylic alcohol of compound 3b had to be inverted. Alcohol 3b was inverted via its para-toluenesulfonyloxy intermediate 1 with cesium acetate in dimethylformamide (18) to produce acetate 12, which was then hydrolyzed to obtain 12,13-epoxy-[16-13C]9,10-trichoene-2α-ol 3a. The NOE correlations of 12 determined by the NOESY experiment show a strong interaction between H-2 and H-13b and between Me-14 and H-3a, thereby confirming the existence of an α-OAc on C-2. After hydrolysis, compound 3a has therefore an α-OH on C-2. The proton NMR spectra of 3a show C-16 as a doublet (J = 125.2 Hz, Fig. 4A) derived by the coupling
\(^{13}\text{C}-\text{H}-16\) and no singlet that would arise from \(^{12}\text{C}-\text{H}-16\). This constitutes a proof that we succeeded in synthesizing practic-ally 100\% with \(^{13}\text{C}\) at position 16.

When acetate 12 was treated with WCl\(_6\) and \(n\)-butyllithium (19), the olefin 13 was produced, and upon hydrolysis, \(2a\)-hydroxy-\([16-^{13}\text{C}]\)trichodiene 2a was isolated. 2\(b\)-Hydroxy-\([16-^{13}\text{C}]\)trichodiene 2b was also prepared from enone 6 using the same procedure as for the preparation of compound 3b except that 6 was not epoxidized. The NOE correlations of 2b determined by the NOESY experiment show a strong interaction between H-2 and Me-15, thereby confirming the existence of a \(\beta\)-OH on C-2.

\([15-^{2}\text{H}]12,13\)-Epoxytrichodiene (2c) was prepared from \([15-^{2}\text{H}]\)trichodiene (14) by bromination of the deuterated trichodiene 14 to give the 9,10-dibromide, which was epoxidized, using \(m\)-chloroperoxybenzoic acid, to give the 12,13-epoxy-9,10-dibromide 15 (13). Compound 15 was debrominated using zinc dust in ethanol (20) to yield the desired deuterated epoxytrichodiene 2c.

\textit{Biosynthesis of Trichothecenes: Dioxygenated Precursor Post-trichodiene (Fig. 4)—} Radiolabeling experiments suggested that 12,13-epoxy-9,10-trichoene-2\(a\)-ol was a precursor to trichothecenes (8). The site of incorporation could not be obtained, since no degradation was done on the derived radiolabeled trichothecenes. We therefore decided to confirm it by synthesizing this potential precursor with \(^{13}\text{C}\) at position 16: 12,13-epoxy-\([16-^{13}\text{C}]9,10\)-trichoene-2\(a\)-ol 3a. After the feeding, no degradations are necessary, since we can locate the label by \(^{13}\text{C}\) NMR spectroscopy. In order to ensure that only the 2\(a\)-stereoisomer is the precursor, we also synthesized 12,13-epoxy-\([16-^{13}\text{C}]9,10\)-trichoene-2\(b\)-ol (3b, Figs. 1 and 2). When 3b was fed to \(\textit{F. culmorum}\) cultures, the 3-ADN isolated showed no enrichment in \(^{13}\text{C}\), and after 927 scans, all carbons were of equal intensity. Similarly, the isolated SOL, which was acetylated with deuterated acetic anhydride for purification reasons, also showed no enrichment in \(^{13}\text{C}\).

On the other hand, the 3-ADN derived from the feeding of 3a under the exact same conditions showed a peak very clearly in the \(^{13}\text{C}\) NMR spectra at C-16 (15.3 ppm) (Fig. 4). Also, the average percentage of \(^{13}\text{C}\) incorporation (ratio between \(^{13}\text{C}\)-enriched and nonenriched 3-ADN) as calculated by mass spectrometry and \(^1\text{H}\) NMR data is 38\%. Three more compounds isolated from the feeding of 3a showed in the \(^{13}\text{C}\) NMR spectra a large peak at C-16 (Fig. 4): SOL, pre-SOL (a precursor to SOL (21)), and a dead end metabolite, 11\(a\)-2\(b\),13\(b\)-apotrichodiol, previously isolated from \(\textit{F. culmorum}\) (14). This last metabolite was diacetylated after isolation to facilitate its purification (Fig. 4). The structure of this apotrichothecene was rigorously proven by independent synthesis (14) (Fig. 4). No mass spectral data are available for 11\(a\)-2\(b\),13\(b\)-apotrichodiol, because it decomposed prior to analysis. We have, however, the detailed \(^1\text{H}\) and \(^{13}\text{C}\) NMR characterization, and they are identical to the ones reported except for the enrichment in \(^{13}\text{C}\). It is interesting to note that the conversion of 3a to these three metabolites
Identification of the Two Oxygenation Steps after Trichodiene

Fig. 6. Biosynthesis of 3-ADN and SOL. The metabolite in brackets has not been isolated in *F. culmorum* but has been found in other fungi (24). The thick arrows emphasize metabolic steps that have been rigorously proven. The other arrows are postulated based on the present work.

seems even more efficient than to 3-ADN. Indeed, the percentage of incorporation of 3a into SOL is ~54%; into pre-SOL ~48%, and into 11α-2β,13β-apotrichodiol ~90% (experimental). This very large incorporation of 3a into this apotrichothecene will be discussed below.

We also succeeded in isolating 12,13-epoxy-9,10-trichoene-2α-ol from a large amount of crude extract prepared from *F. culmorum*. There was enough metabolite to characterize by NMR spectroscopy, and the data were identical to those of the unlabeled synthesized compound.

Biosynthesis of Trichotheccenes: Sequence of the Oxygenations Post-trichodiene (Figs. 5 and 6)—The results shown in Fig. 5 prove conclusively for the first time that the sequence of the two oxygenation steps of the hydrocarbon trichodiene are as follows: first hydroxylation at position 2α, leading to 2α-hydroxytrichodiene, followed by the epoxidation at C-12-C-13 to give 12,13-epoxy-9,10-trichoene-2α-ol, which has been successfully incorporated to both 3-ADN and SOL (see above). The feeding of 2α-hydroxy-[16,13C]trichodiene 2a to *F. culmorum* cultures gave 3-ADN and SOL (which was diacetoylated in order to purify it) with a clearly enriched C-16 in the 13C NMR spectra at 15.2 and 23.0 ppm, respectively (Fig. 5). In addition, the incorporation of 2a into 3-ADN and SOL was significant as calculated by mass spectrometry and 1H NMR data (experimental): ~28% into 3-ADN and ~37% into SOL. When the two possible monooxygenated trichodiene precursors, 2α-hydroxy-[16-13C]trichodiene 2a and [15-2H]12,13-epoxytrichodiene 2c, were simultaneously fed to *F. culmorum* cultures, the production of 3-ADN and SOL was inhibited to the extent that no SOL could be isolated for characterization. The only trichothecene that was produced, 3-ADN, was highly enriched with 13C at position 16 (Fig. 5). There was no deuterated 3-ADN produced. In order to ensure that the 2α-hydroxyl system is absolutely required, we also synthesized the stereoisomer: 2β-hydroxy-[16,13C]trichodiene 2b and fed it to *F. culmorum* cultures. It seems to inhibit considerably the production of trichothecenes. SOL could not be isolated, and the 3-ADN obtained was unlabeled. We are therefore very confident that the first oxygenation step post-trichodiene is the formation of 2α-hydroxytrichodiene. In addition, we succeeded in finding 2α-hydroxytrichodiene as a metabolite by radioactive dilution. A large amount of crude extract was prepared from production cultures of *F. culmorum*. After successive purifications on HPLC, a very small peak was found with the same retention time as unlabeled 2α-hydroxytrichodiene. The amount was too small to isolate and characterize by NMR. We therefore decided to acetylate that minute quantity with [1-13C]acetic anhydride and obtained a single peak on HPLC that had all of the radioactivity transferred to the new peak at a retention time identical to that of standard 2α-acetyltrichodiene. In one experiment, the compound was purified to constant specific activity under three different analytical HPLC conditions that gave peaks with retention times identical to that of the synthetic acetylated standard. A Bioscan tracing of 2α-[1-13C]trichodiene acetate demonstrated the symmetrical distribution.

A trioxgenative derivative of trichodiene (4a, Fig. 6) has been isolated and converted to 3-ADN (22, 23). The conversion of 4a to sambucinol has never been shown, but it seems that the fungal strain utilized did not produce sambucinol, since it was not reported. We can therefore postulate that 4a is probably the more plausible trioxgenated intermediate of 3-ADN and SOL. The biosynthetic pathway of 3-ADN and SOL seems to bifurcate at an early stage (21). An attractive metabolite that would be converted to both trichotheccenes could be 4a. A fourth hydroxylation could give compound 5a, which is a natural product and has been isolated from *Fusarium sporotrichiodes* (24). Metabolite 5a has a hydroxyl at position 3 with the correct stereochemistry and would be converted to the tricyclic metabolite isotrichodermin (Fig. 6), a proven biosynthetic precursor to 3-ADN (9, 21). The conversion of isotrichodermin to 3-ADN involves four oxidations and six metabolic conversions from 3a. 12,13-Epoxytrichothec-9-ene (Fig. 6, EPT) has been converted to pre-SOL and SOL with no connection to the isotrichodermin metabolic pathway (21). Therefore, we can see (Fig. 6) that more steps are involved from 3a to 3-ADN than from 3a to pre-SOL and SOL, which accounts for the relative incorporations. One conversion that seems particularly efficient is the feeding of 12,13-epoxy-[16,13C]9,10-trichoene-2a-ol 3a to *F. culmorum* cultures, which leads to 90% of the 13C being incorporated into the apotrichothecene 11α-[16,13C]2β,13β-apotrichodiol (Fig. 6). One possibility could be that the SN2-type water attack on C-2, the cleavage of the ether bond, and opening of the epoxide of 12,13-epoxytrichothec-9-ene could happen simultaneously on the enzyme surface. On the other hand, the conversion of 12,13-epoxytrichothec-9-ene to pre-SOL and SOL is probably more involved. Knowledge of the sequence of the different oxygenations will be very helpful in understanding these ubiquitous enzymes.

REFERENCES

1. Rotter, B. A., and Prelusky, D. B. (1996) *Toxicol. Environ. Health* 48, 1–34.
2. Denijs, M., Van Egmond, H. P., Rombouts, F. M., and Notermans, S. H. W. (1997) *J. Food Safety* 17, 161–191.
3. Müller, H. M., Reimann, J., Schumacher, U., and Schwadorf, K. (1997) *Myco-pathologia* 137, 185–192.
4. Liu, W. Z., Langseth, W., Skinner, H., Elen, O. N., and Sundheim, L. (1997) *Eur. J. Plant Pathol.* 103, 589–595.
Identification of the Two Oxygenation Steps after Trichodiene

5. Marasas, W. F. D., Nelson, P. E., and Toussoun, T. A. (1984) *Toxigenic Fusarium Species: Identity and Mycotoxicology*, pp. 147–151, Pennsylvania State University Press, University Park, PA.

6. Greenhalgh, R., Hanson, A. W., Miller, J. D., and Taylor, A. (1984) *J. Agric. Food Chem.* 32, 945–948.

7. Zamir, L. O., and Devor, K. A. (1987) *J. Biol. Chem.* 262, 15348–15353.

8. Zamir, L. O., Devor, K. A., Morin, N., and Sauriol, F. (1991) *J. Chem. Soc. Chem. Commun.* 1033–1035.

9. Zamir, L. O., Nikolakakis, A., Devor, K. A., and Sauriol, F. (1996) *J. Biol. Chem.* 271, 27353–27359.

10. Zamir, L. O., Devor, K. A., and Sauriol, F. (1991) *J. Biol. Chem.* 266, 14992–15000.

11. Pearson, A. J., and O'Brien, M. K. (1989)*J. Org. Chem.* 54, 4663–4673.

12. Colvin, E. W., Malchenko, S., Raphael, R. A., and Roberts, J. S. (1973) *J. Chem. Soc. Perkin Trans. 18*, 1989–1997.

13. Hesketh, A. R., Gledhill, L., Bycroft, B. W., Dewick, P. M., and Gilbert, J. (1995) *Phytochemistry* 32, 93–104.

14. Zamir, L. O., Nikolakakis, A., Sauriol, F., and Maner, O. (1999) *J. Agric. Food Chem.* (1999), in press.

15. Zamir, L. O., Gauthier, M. J., Dever, K. A., Nadeau, Y., and Sauriol, F. (1989) *J. Chem. Soc. Chem. Commun.* 598–600.

16. Sharpless, K. B., and Verhoeven, T. R. (1979) *Aldrichimica Acta* 12, 63–74.

17. Thompson, D. J. (1976) *J. Organomet. Chem.* 108, 381–383.

18. Huffman, J. W., and Desai, R. C. (1983) *Synth. Commun.* 13, 553–557.

19. Sharpless, K. B., Umbreit, M. A., Nieh, M. T., and Flood, T. C. (1972) *J. Am. Chem. Soc.* 94, 6538–6540.

20. Kazlauskas, R. J., Weissfich, A. N. E., Rappaport, A. T., and Cucia, L. A. (1991) *J. Org. Chem.* 56, 2656–2665.

21. Zamir, L. O., Devor, K. A., Nikolakakis, A., and Sauriol, F. (1999) *J. Biol. Chem.* 265, 6713–6725.

22. Hesketh, A. R., Gledhill, L., Marsh, D. C., Bycroft, B. W., Dewick, P. M., and Gilbert, J. (1991) *Phytochemistry* 30, 2237–2243.

23. McCormick, S. P., Taylor, S. L., Plattner, R. D., and Beremand, M. N. (1990) *Appl. Environ. Microbiol.* 56, 702–706.

24. Zamir, L. O., and Huang, L. (1992) *J. Labelled Compd. Radiopharm.* 31, 915–924.