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THE ROLE OF ^{13}C-LABELED TRICHODIENE AND BAZZANENE IN THE SECONDARY METABOLISM OF *FUSARIUM CULMORUM*[1]

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ABSTRACT.—Trichodiene [3] has been reported as a precursor of the trichothecenes. To prove this relationship, [7′-^{13}C]-trichodiene was synthesized and fed to *Fusarium culmorum* cultures, resulting in specific incorporation at position 13 of both trichothecenes and trichothecene-related compounds. The use of the trichodiene epimer, ^{13}C-bazzanene [7], as an alternate substrate did not produce the expected labeled trichodiol or trichothecene analogues but rather an apotrichothecene analogue.

Farnesyl pyrophosphate (FPP) is recognized as the precursor of most sesquiterpenes (1). Cane and Ha (2) have also shown the involvement of nerolidyl pyrophosphate in the biosynthesis of trichothecenes (Figure 1). Labeling studies have linked FPP to both trichodiene [3] and the trichothecenes (2–9). However, the conversion of trichodiene to trichothecenes still remains to be clarified.

Two studies, one with ^{3}H-labeled (10) and the other with ^{14}C-labeled (8) trichodiene have been reported, but in neither case was the specificity of the labeling of trichothecenes verified. In other experiments, treatment of *Fusarium* cultures with mono-oxygenase inhibitors reduced the production of trichothecenes, resulting in an increase in trichodiene concentration (11,12). The isolation of trichodiol [4] (13) and

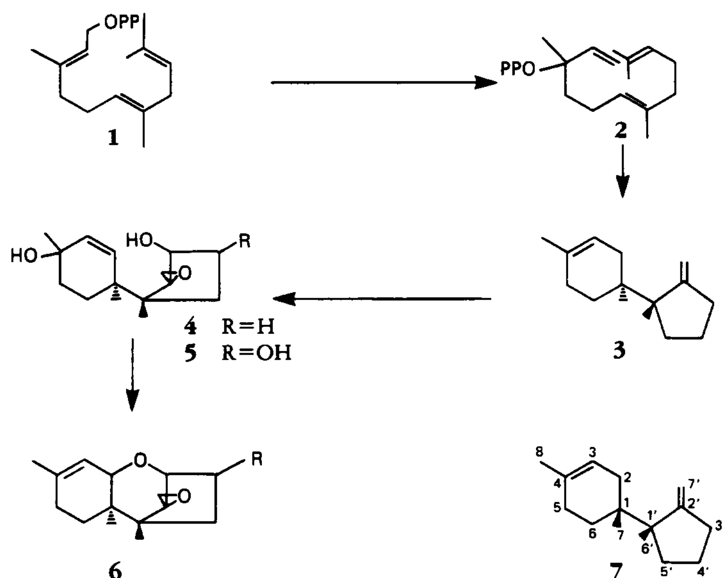

FIGURE 1. Biosynthetic pathway of trichothecenes: farnesyl pyrophosphate (FPP) [1], nerolidyl pyrophosphate [2], trichodiene [3], trichodiol [4], trichotriol [5], 12,13-epoxytrichothecene [6]. Also shown is the trichodiene epimer bazzanene [7].

[1]PRC Contribution #1209.

trichotriol [**5**] (14) indicates a possible oxidation pathway for the conversion of trichodiene to trichothecenes. The cyclization of trichotriol to 3-hydroxytrichothecene in $CHCl_3$ gave further credence to this hypothesis (14).

To further define the biosynthetic pathway of trichothecenes, specifically labeled [7′-^{13}C]-trichodiene [**3**] and its epimer, bazzanene [**7**], were synthesized and added to cultures of *Fusarium culmorum* HLX 1503 (W.G. Smith) Sacc. [W & R, G, B, J]. It was postulated that steric interaction between the vicinal methyl groups of bazzanene could prevent cyclization to a trichothecene-like structure, thus causing the accumulation of an oxidized intermediate. Bazzanene has been isolated as a secondary metabolite of *Bazzania pompeana* (15).

EXPERIMENTAL

INSTRUMENTS.—^{1}H and ^{13}C nmr spectra were obtained using either a Bruker AM250 or AM500 spectrometer. At 250 MHz, the ^{1}H spectra were acquired with 16K data points, a 2.2 KHz spectral window, and an 8 sec repetition rate. At 500 MHz, a 4.5 KHz spectral window was used. ^{13}C spectra were run either at 62.9 MHz with 16K data points, a 15 KHz spectral window, and a 3 sec repetition rate, or at 125.8 MHz with 32K data points, a 30 KHz spectral window, and a 3 sec repetition rate. Chemical shifts were referenced to residual $CHCl_3$ at 7.24 ppm and $CDCl_3$ at 77.0 ppm for ^{1}H and ^{13}C spectra, respectively, and reported relative to TMS. NOe's were determined by nOe difference spectra. ^{1}H assignments were determined by homonuclear correlation spectroscopy at 500 MHz. Using the COSY-45 pulse sequence (90-t_1-45), 512 × 1K FIDs of 16 scans each were acquired. Final digital resolution was 2.3 Hz/pt. ^{13}C assignments were determined using heteronuclear correlation spectroscopy. FIDs (256 × 4K) of 96 scans each were acquired with a sweep width of 3500 Hz in the ^{13}C dimension and 1000 Hz in the ^{1}H dimension. Mass spectra were obtained on a Finnigan MAT 4500 GC/MS system operating in the electron impact mode. A DB-5 fused silica column [20 m × 0.32 mm (i.d.), 0.25 μm film] was used with He as a carrier gas at 10 psi. The gc was programmed from 80° to 250° at 15°/min. The ^{13}C content was determined by gc-ms isotopic ratio analysis, modifying the procedure of Barrie *et al.* (16) for the Finnigan system. Measurements of the m/z 44 and m/z 45 peak intensities provided the ratio of ^{12}C to ^{13}C in each compound.

HPLC CONDITIONS.—A 5-μm semi-preparative Si gel column [300 × 3 mm (i.d.)] was used with a Varian 5500 HPLC system. Separation of the epimers pairs **8**, **9**, and **11** was achieved using EtOAc-hexane (0.5:99.5) and that of the enol ethers **10** with hexane.

FLASH CHROMATOGRAPHY OF THE METHYL ESTERS **9**.—The size of the column used was twice that recommended by Still *et al.* (17). Typically, 230–400 mesh Si gel (250 g) was packed as a slurry in a 5-cm-diameter column to a height of 30 cm. The crude mixture of esters **9** (1.0 g) was chromatographed using EtOAc-hexane (2.5:97.5). Despite identical R_f's on tlc [EtOAc-hexane (10:90)], the two esters were resolved as determined by hplc analysis.

SYNTHESIS OF [7′-^{13}C]-BAZZANENE [**7**].—The Wittig reaction conditions employed were essentially the same as those used by Schlessinger and Schultz (18) for trichodiene [**3**], except that the Wittig reagent was made using ^{13}C-MeI instead of MeBr. NaH (65% in oil, 120 mg) was washed with hexane (3 × 2 ml) under N_2 in a sealable tube. DMSO (0.5 ml, triple-distilled under vacuum from CaH_2) was added, and the resulting suspension was heated to 80° under N_2 for 30 min. The solution was cooled, and ^{13}C-methyltriphenylphosphonium iodide (1.2 g) in DMSO (3 ml) was added. This solution was heated to 80° for 1 h and cooled, and bazzanenone (56 mg) in DMSO (0.6 ml) was added. The solution was then frozen in liquid N_2 and degassed through five freeze-thaw cycles, and the tube was sealed under vacuum. The tube was heated to 80° for 60 h, then cooled and the solution partitioned between H_2O (25 ml) and Et_2O (3 × 10 ml). The organic phase was washed with H_2O and brine, dried over Na_2SO_4, concentrated, and chromatographed on Si gel (2 g) with hexane to yield [7′-^{13}C]-bazzanene (16 mg). The ^{1}H-nmr spectrum is shown in Figure 2 and the nmr assignments are given in Table 1.

[7′-^{13}C]-TRICHODIENE [**3**].—The above procedure was used with trichoenone. The nmr assignments are included in Table 1.

FERMENTATION.—*F. culmorum* HLX 1503 (CMI 14764, Commonwealth Mycological Institute) was cultured at 28° in 250-ml flasks containing 50 ml of MYRO medium (pH 6.3) according to the procedure of Miller and Blackwell (19). [7′-^{13}C]-Bazzanene [**7**], [7′-^{13}C]-trichodiene [**3**], or a mixture of the two was dissolved in absolute EtOH (35–40 mg/ml) and added to the cultures in 5 aliquots (25 μl per flask) at 6-h intervals between 72 to 96 h after inoculation, for a total addition of 0.10–0.15 mg/ml culture with

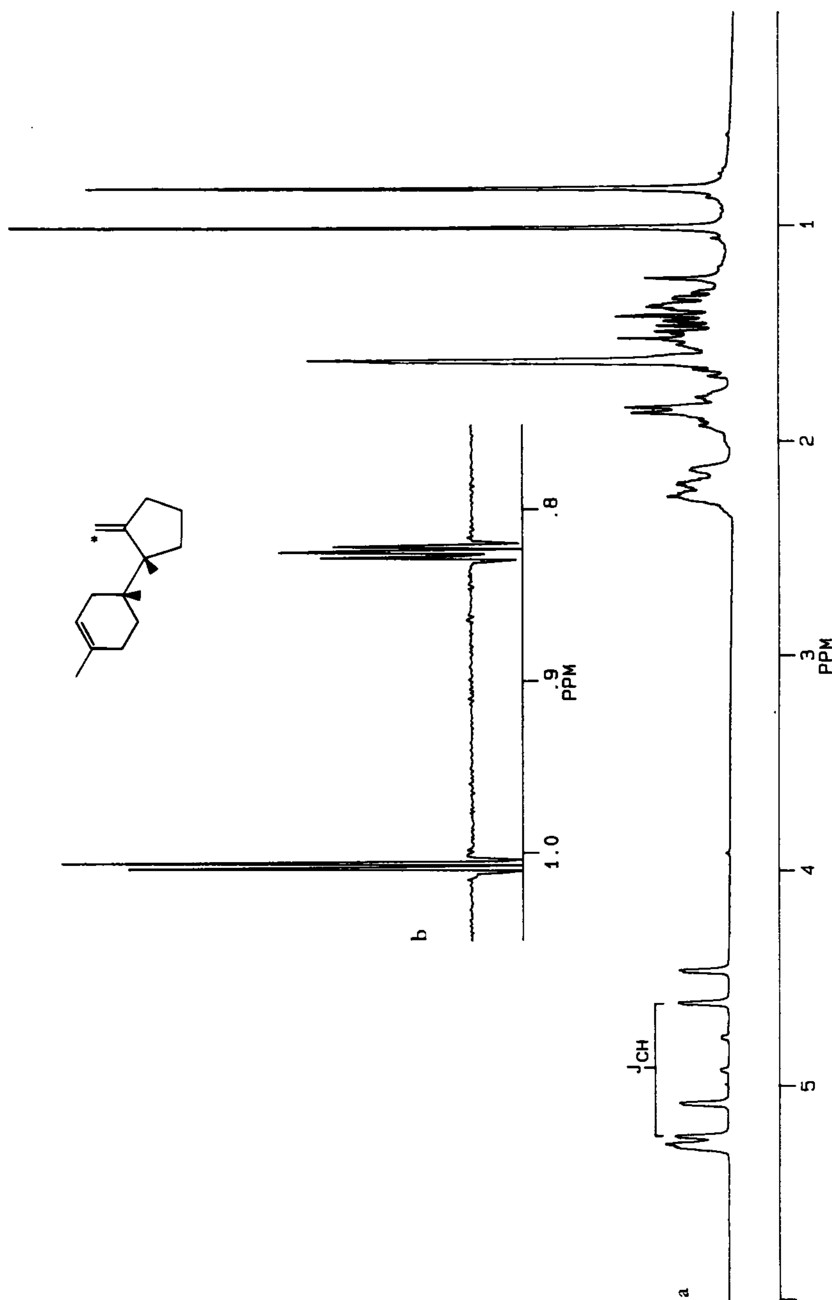

FIGURE 2. (a) ^{1}H-nmr spectrum of [7′-^{13}C]-bazzanene, showing the ^{1}H-^{13}C coupling. (b) Long-range coupling of the vicinal methyl groups to ring protons as seen by resolution enhancement.

TABLE 1. Nmr Assignments for Bazzanene [7] and Trichodiene [3].

Position	Compound			
	Bazzanene [7]		Trichodiene [3]	
	^{13}C	^{1}H	^{13}C	^{1}H
1	36.7		36.8	
2	32.3	α: 2.15 β: 1.63	33.0	α: 1.73 β: 2.02
3	120.6	5.27	120.5	5.27
4	132.6		132.4	
5	37.0	1.8–1.9	37.3	1.8–1.9
6	23.4	α: 1.40 β: 1.52	23.3	α: 1.36 β: 1.62
7	17.6	0.82 (s)	17.9	0.84 (s)
8	23.2	1.62 (bs)	23.2	1.62 (bs)
1′	50.2		50.6	
2′	160.0		160.0	
3′	38.9	A: 2.28 B: 2.18	38.8	A: 2.30 B: 2.20
4′	28.0	A: 1.3–1.4 B: 1.3–1.4	28.2	A: 1.3–1.4 B: 1.3–1.4
5′	27.8	α: 1.84 β: 1.65	27.8	α: 1.90 β: 1.6–1.65
6′	23.6	1.00 (s)	24.0	1.02 (s)
7′	106.3	A: 4.77 (bd) B: 4.93 (bd)	106.9	A: 4.72 (bd) B: 4.95 (bd)

a total volume of EtOH of 125 μl per flask. Three replicate flasks per experiment were used, eight for the mixture of substrates. Previous studies[2] have shown that the production of 3-acetyl-4-deoxynivalenol (3-ADON) in liquid cultures is significantly reduced by the presence of EtOH; however, amounts of absolute EtOH lower than 0.5 ml per flask cause no significant change in 3-ADON production. Two controls were also cultured, one to which no additions were made and the other to which EtOH was added at the same rate as to the other flasks.

The cultures were harvested after 7 days, each set of replicates was pooled, the hyphae were filtered off, and the medium was adsorbed on CHEM-TUBE columns (100 or 300 ml) and extracted with EtOAc (×5 column volumes). After drying over Na_2SO_4, the organic solution was concentrated to dryness and analyzed by gc-ms and nmr.

ACETYLATION OF EXTRACT AND ISOLATION OF **19**.—Half of the concentrated extract from the ^{13}C-bazzanene-fed culture (17 mg) was dissolved in CH_2Cl_2 (2 ml) and acetylated with acetyl chloride (0.1 ml) and pyridine (0.1 ml) at room temperature. After 20 h, the solution was washed with a saturated $NaHCO_3$ solution followed by brine, dried over anhydrous Na_2SO_4, and chromatographed on Si gel (3 g) with EtOAc-hexane (1:9) (25 ml) followed by EtOAc-hexane (1:3), which eluted the labeled diacetate **19** as determined by ^{13}C nmr. Ms *m/z* $[M]^+$ 337 (4), 322 (28), 295 (8), 277 (7), 220 (15), 135 (13), 124 (79), 108 (68), 107 (53), 97 (68), 93 (34), 42 (base); ^{1}H nmr δ 0.81 (3H, Me-15), 1.03 (3H, Me-14), 1.71 (3H, Me-16), 2.05 (3H, Ac), 2.06 (3H, Ac), 3.84 (1H, H-11, $J_{11,10}=4.1$ Hz), 4.09 (1H, H-13, $J_{AB}=11.9$, $J_{H,C}=147$), 4.33 (1H, H-13, $J_{AB}=11.9$, $J_{H,C}=147$), 5.00 (1H, H-2, $J_{2,3}=9.3$, $J_{2,3}=6.4$, $J_{2,4}=3$), 5.54 (1H, H-10, $J_{10,11}=4.1$); ^{13}C nmr δ 17.7 (C-15), 21.0 (Ac), 21.3 (Ac), 21.9 (C-14), 23.4 (C-16), 27.0 (C-7), 28.6 (C-8), 29.9 (C-4), 32.2 (C-3), 37.5 (C-6), 43.6 (C-5), 64.4 (C-13), 75.9 (C-2), 80.3 (C-11), 118.7 (C-10), 139.4 (C-9), 170.9 (Ac), 172.6 (Ac).

RESULTS AND DISCUSSION

Some modifications were made to the synthesis of trichoenone and bazzanenone as described by Kraus and Thomas (20). Failure to separate the enol ethers **10** by flash

[2]Blackwell and Miller, unpublished data.

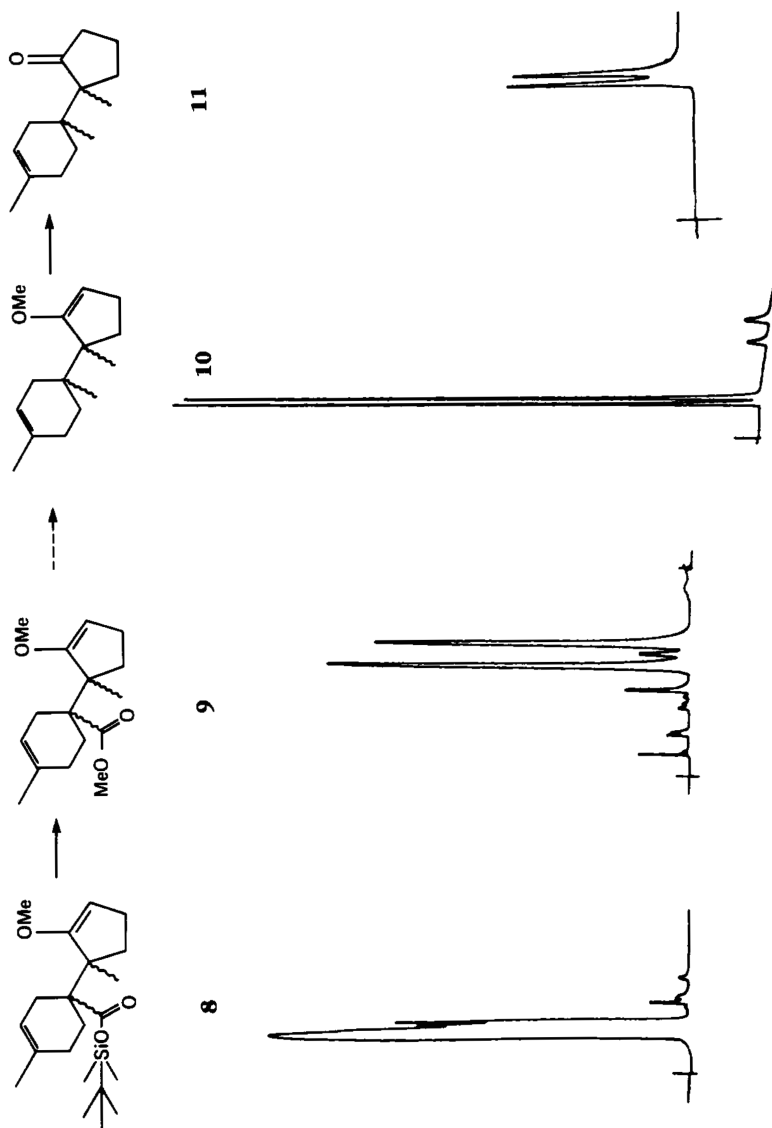

FIGURE 3. Hplc analysis of trichodiene [3] and bazzanene [7] synthetic precursors.

chromatography led to an investigation of the separation of the other precursors **8**, **9**, and **11** (Figure 3). The best hplc separation was that of the methyl esters **9** on a Si gel column with EtOAc-hexane (0.5:99.5). Flash chromatography of these compounds was subsequently found to separate the two epimers.

The procedure for the synthesis of [7′-^{13}C]-trichodiene [**3**] from trichoenone (18,21) was also modified slightly by use of methyltriphenylphosphonium iodide instead of the bromide. The lower volatility of MeI as compared to MeBr facilitated the synthesis of this salt. [7′-^{13}C]-Bazzanene [**7**] was similarly synthesized from bazzanenone, despite reports to the contrary (21). To verify that the success of the reaction was not due to the use of the iodide salt, it was repeated with the bromide salt with identical results.

Nmr analysis of trichodiene and bazzanene confirmed the chemical shifts previously reported (20–25). Because no assignment of the various methylene resonances had been reported, a more extensive study was performed using resolution enhancement, nOe difference and 2D-nmr methods (Table 1).

Resolution enhancement of the trichodiene and bazzanene ^{1}H spectra showed some long-range coupling of the Me-7 and Me-6′. In the spectrum of [7′-^{13}C]-bazzanene (Figure 2), the resonance at 0.82 ppm appeared as a triplet and that at 1.00 ppm as a doublet (Figure 2b). Long-range "W"-couplings are possible between the Me-7 and H-2α and H-6α methylene protons of bazzanene, and for the Me-6′ group only with H-5′α (Figure 4a). The triplet was thus assigned to the Me-7 and the doublet to the Me-6′. ^{1}H/^{1}H correlation spectra (COSY) then permitted assignment of the 2α, 6α, and 5′α protons and their geminal partners. The resonances at 5.27, 4.93, and 4.77 ppm were assigned to the vinylic 3-CH and 7′-CH_2. Assignment of H-2, H-5, and H-3′ could then be made from COSY spectra. Assignment of the ^{13}C spectra was then made possible with ^{1}H/^{13}C heterocorrelation experiments.

The W couplings between Me-7 and H-2α and H-6α of bazzanene are only possible if these protons are in pseudo-axial conformations. It thus follows that the preferred conformation of bazzanene is that shown in Figure 4a. This conformation is also in accord with the W coupling observed in the bazzanene COSY spectrum between H-2β and H-6β and the nOe's observed. Not only is there the predictable nOe between the 6′ and 7 methyl groups but also between Me-6′ and the H-2′s. A strong nOe is also visible between both Me-6′ and Me-7 and the vinylic proton at 4.77 ppm, which can thus be assigned the "endo" position. Other nOe's are illustrated in Figure 4.

A similar analysis of the trichodiene spectra yielded the results shown in Table 1 and Figure 4b. The α configuration of the Me-7 of trichodiene is the only feature in which it differs from bazzanene. It follows that W coupling is seen between the Me-7 and the H-2β and H-6β instead of the α protons as in bazzanene.

A metabolic study was carrried out with *F. culmorum* HLX 1503 cultures to which the ^{13}C-labeled bazzanene and trichodiene were added in absolute EtOH. The timing of the additions was chosen in order to enhance incorporation into 3-ADON. Previous studies (26) have shown that 3-ADON production begins approximately 48 h after inoculation, and that the maximum rate of production occurs between 48 and 100 h.

^{13}C-nmr analysis of the crude extracts showed significant incorporation of ^{13}C (Figure 5). The major resonances in the control spectrum (Figure 5a) could be assigned to 3-ADON. Most of the remaining resonances were assigned to butenolide, dihydroxycalonectrin(DHCAL), 3,13-dihydroxy-11-*epi*-apotrichothecene (APO), and a small amount of sambucinol. The spectrum of the extract from the ^{13}C-trichodiene-treated cultures (Figure 5b) showed complete disappearance of ^{13}C-trichodiene (as evidenced by lack of a significant peak at 106.9 ppm) and incorporation of ^{13}C into both trichothecenes and trichothecene-related compounds. The resonances that were en-

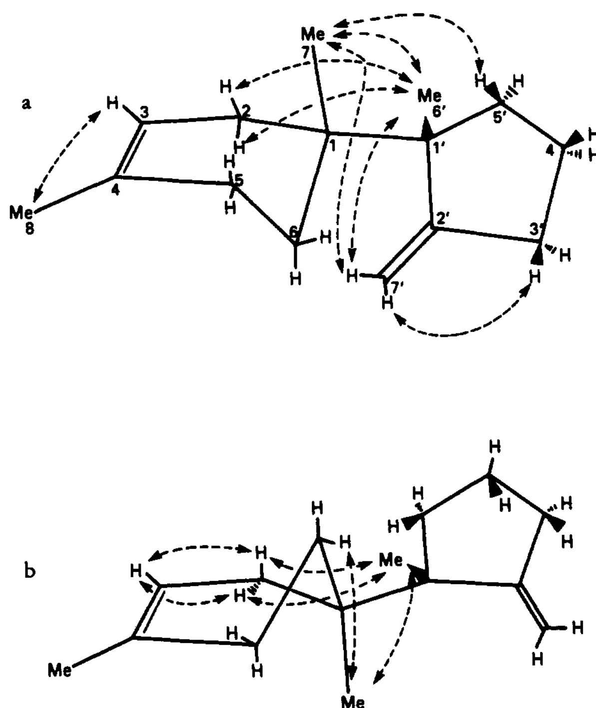

FIGURE 4. NOe's observed for (a) bazzanene [**7**] and (b) trichodiene [**3**].

riched occurred at 47.4 ppm (assigned to the epoxide carbon C-13 of 3-ADON), 47.8 ppm (assigned to C-13 of DHCAL), and 58.8, 59.7, 63.8, and 68.3 ppm (assigned to the C-13 positions of deoxysambucinol, sambucinol, APO, and sambucoin, respectively). None of the other carbon resonances of these compounds appeared to be enriched, indicating that trichodiene was incorporated intact into the above species. Several additional minor resonances appearing at 106–110 ppm may be due to the trichodiene's having undergone a variety of oxidations without changing the C-7′ alkene group.

Purification of the main metabolite, 3-ADON, confirmed this specificity of labeling, as its ^{13}C-nmr spectrum showed 18% incorporation of ^{13}C at position 13 only. Enrichment of the C-13 position in DHCAL was 9%, reflecting the previous observation that this metabolite is biosynthesized before 3-ADON and does not increase during the time of 3-ADON synthesis (19). In addition, no enrichment was observed in culmorin, a metabolite also derived from FPP, but via a different cyclization pathway than trichodiene. These results confirm that trichodiene is the direct precursor of both the trichothecenes and the modified trichothecenes (27).

The ^{13}C-nmr spectrum of the extract of bazzanene-treated cultures (Figure 5c) showed no incorporation into 3-ADON, indicating that the trichothecene ring structure cannot be formed from this compound. The two main resonances which appeared highly enriched occurred at 63.8 ppm and 62.7 ppm and were assigned to unknown compounds A and B, respectively. Several other resonances appeared somewhat enriched (21.8, 66.5, 66.9, 68.3, and 78.1 ppm), indicating either some modification of the labeled vinyl group of bazzanene or possibly some degradation of bazzanene leading

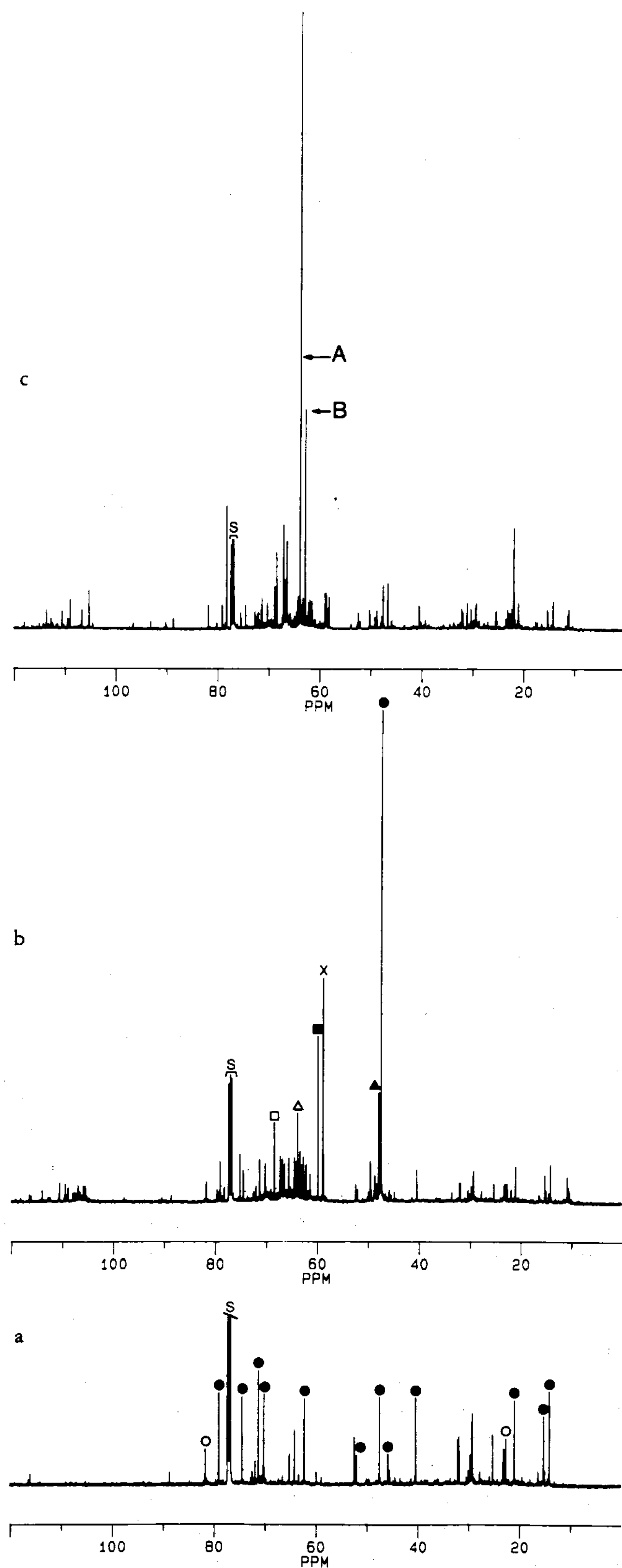

FIGURE 5. A portion of the ^{13}C-nmr spectra of crude extracts: (a) control; (b) enriched from [7′-^{13}C]-trichodiene; (c) enriched from [7′-^{13}C]-bazzanene. Symbols used: ● = 3-ADON, ○ = butenolide, × = deoxysambucinol, ■ = sambucinol, ▲ = dihydroxycalonectrin, □ = sambucoin, Δ = APO, s = solvent.

to scrambling of the label. Several other resonances between 105 and 110 ppm could be due to traces of oxidized metabolites of bazzanene with the labeled vinyl group preserved.

Incorporation of the label was confirmed by analysis of the crude extract by isotope ratio monitoring gc-ms (irm-gcms). This method, using $^{12}CO_2/^{13}CO_2$ ratios for each gc peak, yielded the results shown in Table 2. Data for the control sample provide a measure of the accuracy of the method since each peak analyzed yielded a ^{13}C-level of $1.1\pm0.1\%$. The enriched samples showed a slightly elevated background, possibly due to some scrambling of the label. The butenolide peak, which is well separated and non-mevalonate-derived, showed no labeling. The 1.3% level for culmorin in the ^{13}C-trichodiene-fed cultures was considered to be within background levels due to the errors associated with the low amounts of material and the absence of enriched culmorin resonances in the ^{13}C spectrum. The trichothecenes and related compounds showed definite enrichment.

TABLE 2. ^{13}C Abundance in *Fusarium culmorum* Metabolites.

Compounds	Control	+ ^{13}C-Trichodiene	+ ^{13}C-Bazzanene
Butenolide	1.2%	1.1%	1.1%
Culmorin	1.1	1.3	1.8
Unknown A	—	—	8.4
Sambucoin	1.1	2.8	1.9
Apotrichothecene	1.0	2.5	2.0
Unknown B	—	—	6.2
Sambucinol	1.1	3.3	2.0
3-ADON	1.0	2.3	1.2

The ^{13}C-bazzanene-fed cultures showed a background elevation for most peaks to 1.8–2.0%. This may have been due to the oxidation of a small amount of bazzanene to a variety of compounds with retention times similar to those of the known compounds or to the presence of a small amount of trichodiene in the bazzanene sample. The presence of a number of small unassigned labeled peaks in the ^{13}C-nmr spectrum of the crude extract supports the former hypothesis. Despite this elevated background, two peaks were indicated as having a very high level of labeling and were assigned to the unknowns A and B.

The gc-ms analysis of the [7′-^{13}C]-bazzanene-fed culture showed two major new compounds (A and B) with retention times close to that of APO. The larger of the two peaks, A, had a molecular ion of *m/z* 253, thus indicating the addition of three oxygen atoms. Compound B had a molecular ion of *m/z* 295, suggesting it could be an acetylated derivative of compound A. The similarity of the two fragmentation patterns is in accord with such a relationship. Both A and B showed $[M-15]^+$ and $[M-32]^+$ ions and had very similar patterns below *m/z* 220, with major ions at *m/z* 220, 135, 124, 108, 107, 97, and 93.

Trichodiol [4] (mol wt 252) is generally considered to be an intermediate in the conversion of trichodiene to the trichothecenes. The mass spectrum of A was significantly different from that of trichodiol (28), whereas the mass spectra of trichodiene and bazzanene are almost identical. Furthermore, the epoxide group of trichodiol leads to a ^{13}C chemical shift of 50.7 ppm for C-7′ (14). In compound A, the enriched C-7′ chemical shift was 63.8 ppm, which is characteristic for a primary alcohol as found in APO-type compounds. Compound A was found to be unstable, and on acetylation yielded compound C with a mol wt of 337, suggesting it to be a diacetate. The change in the

chemical shift of the ^{13}C-labeled resonance from 63.8 ppm to 64.4 ppm confirmed it to be an acetylated primary alcohol. The formation of a diacetate suggested that the third oxygen of A belonged to either a tertiary alcohol or an ether. Because compound C contained only one double bond (from its ^{13}C-nmr spectrum) and its molecular formula did not allow for the presence of a third hydroxyl group without further unsaturation, the third oxygen had to be part of an ether linkage. Comparison of ^{13}C chemical shifts of C with those of bazzanene and 8-hydroxy-2,13-diacetoxy-11-*epi*-apotrichothecene [**20**] (29) suggested it to be a cyclized product, not a trichothecene analogue but an apotri-

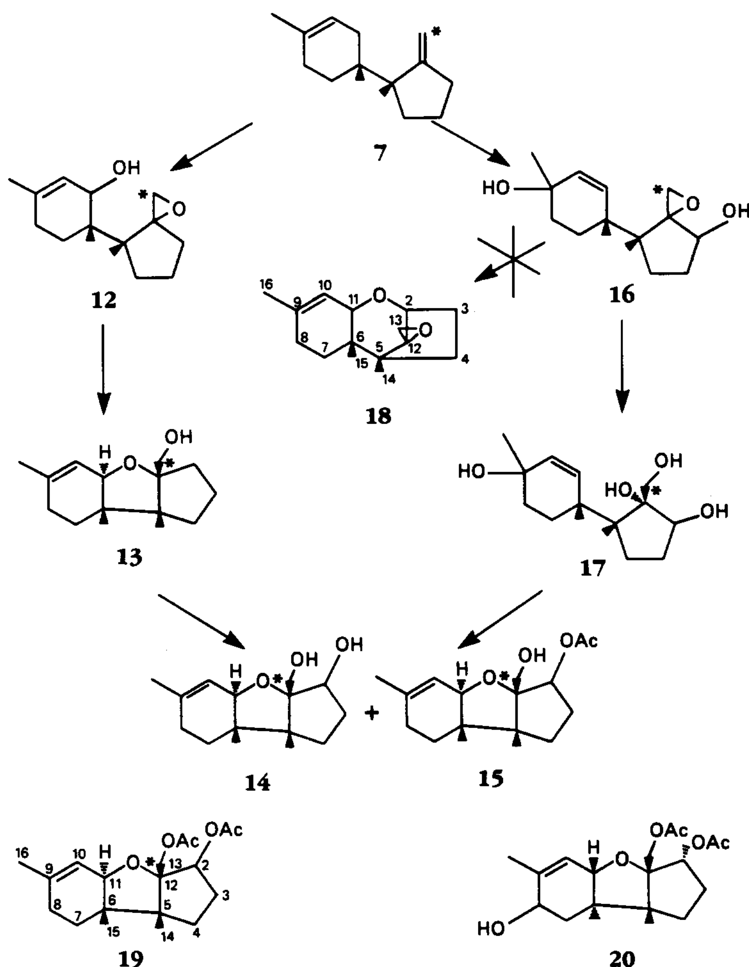

FIGURE 6. Proposed oxidation pathways for bazzanene [7].

chothecene analogue. Computer simulations (using MMP2 minimization) have shown that the molecular mechanics energy for a C-3′/C-2 cyclized bazzanene derivative would be much too high (156.8 kcal·mol^{-1}) to yield a stable compound due to massive distortion of normal geometry (30). Analysis of three-dimensional models showed this C-2′/C-2 cyclization to impose less strain on the molecule than a trichothecene-like cyclization. Further analysis of the nmr spectra of compound C yielded structure **19**. The configuration of the bridgehead proton (H-11, using the trichothecene numbering system, Figure 6) was determined to be α because of the size of $J_{10,11}$ (4.1 Hz) and the

gauche conformation of the vicinal Me-14 and Me-15 groups. The H-11β configuration would force these two methyl groups into a highly strained eclipsed conformation. The 13-CH_2 was assigned a β configuration because of the strain imposed on ring B if the 13-CH_2 was in an α configuration. The second acetate group of C had to be at C-2 because the resonance for the corresponding ring proton showed an extra coupling, which could only be attributed to long-range ^{1}H-^{13}C coupling with the labeled C-13. Only H-2 is close enough to C-13 to show this coupling. The configuration of the 2-OAc could not be determined.

Compound B, shown to be the monoacetate of A by ms, was assigned the structure **15**. The chemical shift of the labeled C-13 (62.7 ppm) compared to that in compound A [**14**] (63.8 ppm) indicated that the acetate group was not at C-13. That the oxidized bazzanene cyclizes as it does to give an epiapotrichothecene analogue suggests two possible oxidation pathways: the APO mechanism suggested previously (31) (Figure 6), going through the epiapotrichothecene analogue **13**, or the trichodiol-type oxidation pattern (through **16**) in which hydration of the epoxide takes place before the difficult 2-11 cyclization to a trichothecene-like structure, leading to the easier 12-11 cyclization. More work is required to determine which route is preferred.

The fact that A differs from trichodiol or a trichodiol epimer does not refute the possibility of trichodiol being an intermediate between trichodiene and the trichothecenes. The different stereochemistry of bazzanene and trichodiene could result in different oxygenation patterns.

In conclusion, the specificity of the incorporation of the label of [7'-^{13}C]-trichodiene at C-13 of the trichothecenes and trichothecene-related compounds proves that trichodiene is indeed an intermediate in the biosynthesis of the trichothecenes. The lack of incorporation of the label from [7'-^{13}C]-bazzanene into any trichothecene-like compounds shows the importance of the stereochemistry required for cyclization. The oxygenation of bazzanene implies that the oxygenases involved in the oxidation of trichodiene are not specific. The production of apotrichothecene-like compounds suggests that under conditions which inhibit a trichothecene-like cyclization, the apotrichothecene cyclization dominates.

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