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Structures of New Metabolites from Fusarium Species : an Apotrichothecene and Oxygenated Trichodienes.

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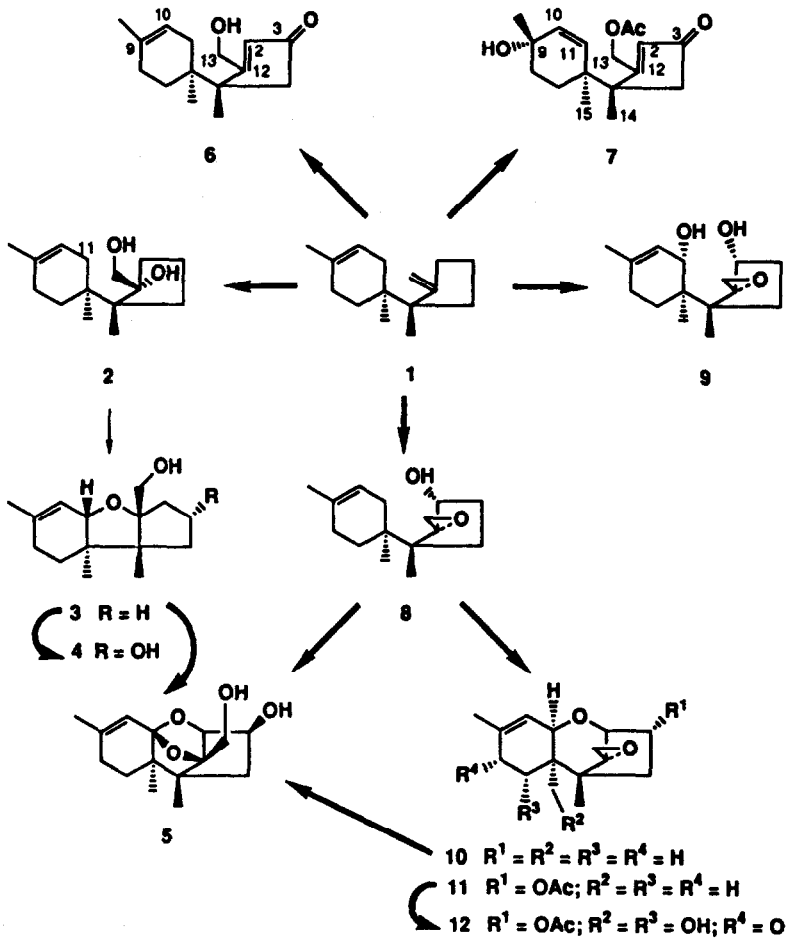
Abstract : Three new natural products were isolated from *Fusarium spp*, one apotrichothecene and two oxygenated derivatives of trichodiene. The apotrichothecene was a precursor to apotrichodiol and sambucinol.

Apotrichothecenes were for a long time considered to be the products of the rearrangements of trichothecenes under acidic conditions.[1,2] In all the acid catalyzed rearrangements of the trichothecenes to the apotrichothecenes the *cis* stereochemistry between the rings A and B is always retained.[1,2] The first natural product found with an apotrichothecene structure was apotrichothec-9-ene-3α,13-diol[3] (trivially named apotrichodiol **4**), and was shown by NOE experiments [3] and later by X-ray crystallography[4] to have a *trans* junction between rings A and B. Apotrichodiol was shown to be a dead-end metabolite[3] which is not a precursor to trichothecenes. Trichodiene **1**, is the first bicyclic compound which was shown[5,6] to be a biosynthetic precursor to trichothecenes. A dioxygenated trichodiene derivative[7] **8**, and trioxygenated trichodiene metabolite[8] **9**, have been shown to be incorporated into trichothecenes. The discovery of new apotrichothecenes or oxygenated trichodienes in similar fungal species would help understand the metabolic events.

In this publication, we report the detection and characterisation of a new apotrichothecene and a new dioxygenated derivative of trichodiene in *Fusarium culmorum*. Their structures were found to be 13-hydroxy-apotrichothec-9-ene **3**, and 13-hydroxy-tricho-2(12),9(10)-diene-3-one **6**. In *Fusarium sambucinum*, we have isolated and determined the structure of a new trioxygenated derivative of trichodiene : 9α-hydroxy-13-acetyltricho-2(12),10(11)-diene-3-one **7**. The new apotrichothecene **3**, was shown to be a transient intermediate and was proven to be a precursor to apotrichodiol **4**, and to the trichothecene sambucinol **5**.[9]

The kinetic pulse-labelling method[10] was used extensively[3,7,11,12] to detect plausible intermediates to trichothecenes. We fed (3RS)[2-^{14}C]mevalonate to *F. culmorum* cultures and followed the radiolabelled metabolites formed with time and an HPLC peak with a retention time in the vicinity of 12,13-epoxytrichothecene[13] **10** (R_t: 72.6min ± 0.4; R_t of **10**; 71.4min ± 0.3) behaved like a transient intermediate. It started to be formed ten minutes after the feeding of (3RS)[2-^{14}C] mevalonate and decreased in amounts as the end-products **4,5,12**, accumulated[13] (metabolite **4**: R_t 52.4 min ± 0.2; metabolite **5**: R_t 49.1 ± 0.5; metabolite **12** R_t: 36.2 min). When the level of this plausible transient intermediate is at a minimum (24 h) the end-products (**4,5,12**) have almost reached their maximum.



Scheme Trichodiene metabolites: the heavy arrows represent proven or common biosynthetic steps.

In order to determine that this plausible transient intermediate is indeed a precursor to one or more of the end-products it was purified[13] to constant specific activity and re-fed to *F. culmorum* cultures. The 3-acetyldeoxynivalenol **12**, derived from this feeding was unlabelled. On the other hand, the derived sambucinol **5**, and apotrichodiol **4**, remained radiolabelled with constant specific activity after repeated purifications[3,11] by : i) repetitive HPLC's aided by a radioactivity detector ii) TLC and Bioscan radioactivity scanner analysis iii) acetylation in the presence of standard sambucinol or apotrichodiol which caused the change of the retention times of both compounds (diacetyl-sambucinol R_t[13] : 60.7 min; diacetyl-apotrichodiol R_t: 33.8 min (70% MeOH, 30% H_2O)) and transfer of all the radioactivity to the new peaks iv) deacetylation and regeneration of the original natural products, with the original retention times (sambucinol R_t: 48.9 min[13]; apotrichodiol R_t: 53.4 min (50% MeOH, 50% H_2O)) and transfer of the radioactivity to the original peaks. The percent incorporation[7] of this unknown intermediate was 7,5% into apotrichodiol and 1% into

sambucinol. In order to identify this transient intermediate 2.3g of crude extract obtained from the media of *F. culmorum* production cultures[10] grown for 10 hours (to maximize the amount of intermediate) was separated by preparative HPLC. The unknown was purified with ^{14}C-labelled intermediate (obtained from the kinetic pulse-labelling experiment) as a marker. Following repeated purifications by HPLC with the ^{14}C-labelled marker the pure compound was analyzed by NMR[14] and characterized as 13-hydroxy-apotrichothec-9-ene **3**. A trichodiene derivative previously isolated from *F. culmorum*, metabolite **2** (could easily be a biosynthetic precursor to 13-hydroxy-apotrichothec-9-ene. Indeed, it would only require an allylic hydroxylation at C-11 and dehydration. A new minor metabolite was also extracted from the large scale fermentation of *F. culmorum*. Acetylation, deacetylation and repetitive HPLC enabled us to purify this new compound (R_t[15]: 54.5 min) and NMR analysis[16] enabled us to assign its structure as 13-hydroxy-tricho-2(12),9(10)-diene-3-one **6**. Analysis of the extracts of *F. sambucinum*, led us to isolate a new major metabolite with a related structure. The first separation by flash chromatography from fifty culture flasks containing 40ml media led to 1.46g of the end-product 4,15-diacetoxyscirpenol and 0.42g of unknown which was further purified by HPLC to yield 0.2g of pure compound identified by spectroscopic techniques [17] as 9α-hydroxy-13-acetyltricho-2(12),10(11)-diene-3-one **7**. Related structures to metabolites **6** and **7** had previously been found in *Fusarium spp.* Indeed compound **6** however with an additional 11α-hydroxyl and compound **7** without an acetyl group at C-13 were isolated from *F. sambucinum*[18].

The biosynthesis of the trichothecenes 3-acetyldeoxynivalenol **12**, and sambucinol **5** derived from different biosynthetic branchpoints. A hydroxyl group at C-3 seems to be the decisive factor. Indeed, 3-acetyldeoxynivalenol **12** is derived from isotrichodermin **11**, and sambucinol **5** from metabolite **10** lacking an oxygenated group at C-3. There are no biosynthetic interconversions between metabolites **10** and **11**. We could therefore predict that the branchpoint between the biosynthesis of 3-acetyldeoxynivalenol and sambucinol is at the bicyclic level. The bicyclic precursors (trichodiene **1** and metabolites **8**, **9**) to both trichothecenes (3-acetyldeoxynivalenol **12** and sambucinol **5**) lack an oxygen function at C-3. Therefore an oxygenated derivative of trichodiene with an oxygen function at C-3 would only be converted to isotrichodermin **11** and 3-acetyldeoxynivalenol **12**. The discovery of such derivatives (**6** and **7**), in the present work, supports this hypothesis.

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13. The HPLC instrument was a Varian 5000 connected to a variable wavelength detector and to a Berthold (B504) radioactivity monitor. HPLC conditions for the purification of the transient intermediate as well as for coumponds **4,5,10,12,** diacetyl sambucinol and sambucinol: 2xODS-2 analytical columns (4.6 x 500mm) in series eluted at 1ml/min using 0-50 min 15-75% methanol, 50-90 min 75% methanol. The fraction between 72.3-74.6 min was collected and repetitively purified on HPLC using : i) 72% methanol R_t: 34.2 min ii) 0-30 min 60-80% methanol, 30-50 min 80% methanol R_t=39.0 min iii) 0-50 min 15-75% methanol, 50-90 min 75% methanol R_t= 73.5 min. The purity of this radioactive peak was also examined using an LHP-KF high performance thin layer chromatography using ethyl acetate : methanol (95:5 V/V) as solvent. R_t: 0.66 as detected by a Bioscan Imaging Scanner System 200.
14. The ^{1}H nmr of metabolite **3** shows three methyls : one of them on a double bond (1.652 ppm) the others on quaternary (0.913; 0.931 ppm), H_{10}:5.532 ppm (septet J = 1.7 Hz) coupled (from COSY) to 1.652 ppm and to H_{11} (4.153 ppm), CH_2OH on a quaternary (3.265 broad doublet and 3.760 doublet of doublets J_{AB}:11.0 ppm). On acetylation CH_2OAc shifted to 4.195 and 3.980 AB quartet J_{AB} = 11.6 Hz. Other protons: 2.3-1.2 ppm. The ^{13}C NMR of **3** and DEPT experiments and comparison with the ^{13}C nmr of acetylated **3** and **4** rigorously prove the structure of **3**. Indeed **4** possessing a hydroxyl group at C-3 present a ß-OH and γ-OH effect in the ^{13}C nmr. In **3** C-2/C-4 are more shielded and C-5 and C-12 appear at lower field (2.5-4 ppm) than **4**. ^{13}C nmr of **3** in ppm C-2/C-4: 36.66 (t); C-3: 25.92 (t); C-4/C-2: 36.41 (t); C-5/C-6: 55.08 (s); C-6/C-5: 45.27 (s); C-7: 27.65 (t); C-8: 29.27 (t); C-9: 135.30 (s); C-10: 121.23 (d); C-11: 81.20 (d); C-12: 96.45 (s); C-13: 64.47 (t); C-14: 19.24 (q); C-15: 15.79 (q); C-16: 22.60 (q).
15. HPLC conditions for the isolation of the unknown trichodiene derivative : ODS-2 MAG20 column (22 x 500mm) eluted using 20ml/min: 0-50 min, 15-75% methanol; 50-90 min 75% methanol. Then on 2 x ODS-2 analytical columns (4.6 x 500mm) 1ml/min 60% methanol , R_t = 41.6 min, 59% methanol, R_t = 51.7 min. Acetylation with d_6 acetic anhydride and elution on HPLC with 59% methanol for 44 min follow up by a gradient of 59-90% methanol for 50 min, R_t = 82.8 min.
16. Comparison of the nmr of **6** with the known derivative with an additional OH at C-11[18] rigorously establish the structure of **6**. ^{1}H nmr of acetylated **6** in ppm : H-2: 6.104 (t) $J_{2,13}$=1.8Hz; H-4a: 2.684 (d) J_{4a-4b}= 18.5Hz; H-4b, 2.070 (d); H-7a,b/H-8a,b/H-11: 1.9 - 1.2 (om); H-10: 5.23 (brm); H-13a: 4.939 (dd) J_{AB}=17.1Hz, J_{13a-2}:= 2.0Hz; H-13b: 4.829 (dd) J_{AB}=17.1Hz, J_{13b-2}= 1.5 Hz; H-14: 0.880 (s); H-15: 1.230 (s); H-16: 1.634 (brs).
17. The ^{1}H nmr of **7** shows three methyls on saturated quaternary carbons, three olefinic protons, two of which are coupled with a *cis* coupling (10.2Hz) and further coupled with small couplings (1.7; 1.0 Hz) to two more shielded protons (1.2 - 1.7 ppm). The most deshielded olefinic proton H-2 (6.2 ppm) is a triplet with small long range coupling to H-13a,b (4.6; 4.4 ppm). No other coupling is observed, therefore this olefinic proton is isolated. The presence of a keto group is confirmed by an isolated AB spin at position 4 (2.6; 2.1 ppm) coupled with a very large geminal coupling (18.8 Hz) typical for a CH_2-located α to CO. The COSY nmr allows us to distinguish Me-14 from Me-15 since Me-14 shows a cross peak with the most deshielded H-4. ^{13}C nmr of **7** in ppm: C-2: 129.6 (d); C-3: 207.1 (s); C-4: 49.0 (t); C-5: 51.9 (s); C-6: 39.4 (s); C-7: 33.9 (t); C-8: 27.6 (t); C-9: 66.2 (s); C-10/C-11: 134.7/132.5 (d); C-12: 186.7 (s); C-13: 66.1 (t); C-14/C-15: 22.2/21.6 (q); C-16: 30.4 (q); Ac (CO): 183.7 (s).
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