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BIOSYNTHESIS OF TRICHOTHECENE MYCOTOXINS: IDENTIFICATION OF ISOTRICHODIOL AS A POST-TRICHODIENE INTERMEDIATE

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Key Word Index—*Fusarium culmorum*; mycotoxin; trichothecene; biosynthesis; isotrichodiol.

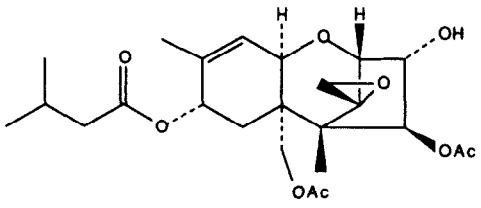
Abstract—When cultures of *Fusarium culmorum* CMI 14764 were treated with the furanocoumarin xanthotoxin, trichothecene biosynthesis was suppressed, and large amounts of trichodiene accumulated, along with smaller amounts of 12,13-epoxytrichothec-9-ene (EPT) and isotrichodermin. Trichodiene was shown to be a precursor of the trichothecene mycotoxins in *F. culmorum*, and when large amounts of trichodiene were supplied, a new trichodiene metabolite isotrichodiol was found to accumulate. This compound, 12,13-epxoy-2α,11α-dihydroxytrichodiene, was shown to be a biosynthetic precursor of the trichothecenes, and represents the first demonstrated post-trichodiene intermediate in the pathway to trichothecenes. Slow, acid-catalysed cyclization of isotrichodiol to EPT was demonstrated, but the rapid *in vivo* incorporation into EPT points to an enzyme-catalysed process in the fungus. The biosynthesis of oxygenated trichothecenes such as 3-acetyldeoxynivalenol in *F. culmorum* appears to require further 3-hydroxylation to isotrichotriol prior to cyclization.

INTRODUCTION

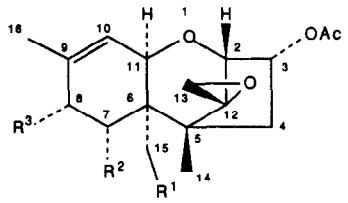
The trichothecenes are a group of sesquiterpene mycotoxins [1] produced by several genera of the Fungi Imperfecti, e.g. *Fusarium*, *Myrothecium* and *Trichothecium*, and are known to be responsible for the toxicity associated with a number of fungal-contaminated foodstuffs [2]. Their biosynthetic origin has been studied in some detail over the years [3, 4] and a sequence from mevalonate via farnesyl diphosphate [5], nerolidyl diphosphate [6], and trichodiene (TDN, **1**) [7, 8] is known to be operative. The parent trichothecene 12,13-epoxytrichothec-9-ene (EPT, **2**) has been isolated from *Trichothecium roseum* [9] and more recently from *Fusarium culmorum* [10] and this led to the hypothesis that EPT could be a common intermediate in the biosynthesis of all natural trichothecenes, which in turn are derived from it by a series of hydroxylation, esterification and other reactions. Thus, *F. culmorum* [10, 11] and other *Fusarium* speices [12] have been shown to accumulate a wide range of trichothecene structures with varying substitution patterns representing products from a stepwise sequence of oxygenation processes. The oxygen atoms in the epoxide and hydroxyl groups of T-2 toxin (**11**) are known to be derived from molecular oxygen [13], and mono-oxygenase enzymes are thus believed to be responsible for the hydroxylation reactions. By supplying specific mono-oxygenase inhibitors to cultures of *F. sambucinum* [14] or *F. sporotrichioides* [15], these oxygenation reactions can be markedly suppressed, and this results in accumulation of significant levels of TDN, which is presumably the last hydrocarbon intermediate in the pathway. Trichodiol (**6**), first isolated from *T. roseum* [16], is widely assumed to be a post-trichodiene intermediate, but there is no direct evidence to support its involvement, and the sequence between TDN and trichothecenes is completely unknown. We report the isolation of a new metabolite from cultures of *F. culmorum* and its characterization as **5**. This is shown to be derived from TDN, and is subsequently transformed by the fungus into trichothecene mycotoxins, and therefore appears to be the first demonstrated intermediate on this part of the biosynthetic pathway. A preliminary communication of part of this work has been published [17].

RESULTS AND DISCUSSION

Treatment of *F. sporotrichioides* cultures with the furanocoumarin xanthotoxin has been shown to result in inhibition of trichothecene biosynthesis, and accumulation of high levels of trichodiene [15]. Liquid cultures of *F. culmorum* CMI 14764 (2 days after subculture) were similarly treated with varying amounts of xanthotoxin (concentrations 0–1 mM) and then analysed for toxin production via GC-MS after a further six days. Under normal culture conditions, *F. culmorum* produces 3-acetyldeoxynivalenol (3-AcDON, **10**) and dihydroxycalonectrin (DHC, **13**) as major trichothecene metabolites [10, 18], but xanthotoxin treatment inhibited their production to minimal levels, and significant amounts of TDN accumulated. In addition, the trichothecenes EPT and isotrichodermin (ITD, **12**) were detected, with EPT at significantly higher levels than in the normal culture. Maximum yields of TDN were obtained with a xanthotoxin concentration of 0.1 mM (Fig. 1), whilst optimum levels of EPT were produced using higher xanthotoxin concentrations (0.2–0.8 mM). ITD levels remained reas-



11 T-2 toxin



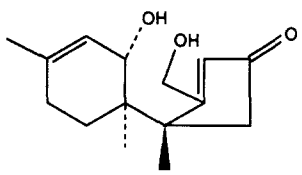
12 $R^1 = R^2 = R^3 = H$, ITD

13 $R^1 = OAc$, $R^2 = R^3 = OH$, DHC

14 $R^1 = OAc$, $R^2 = R^3 = H$, CAL

15 $R^1 = OH$, $R^2 = R^3 = H$, 15-deacetylCAL

16 $R^1 = OAc$, $R^2 = OH$, $R^3 = H$, 7-hydroxyCAL



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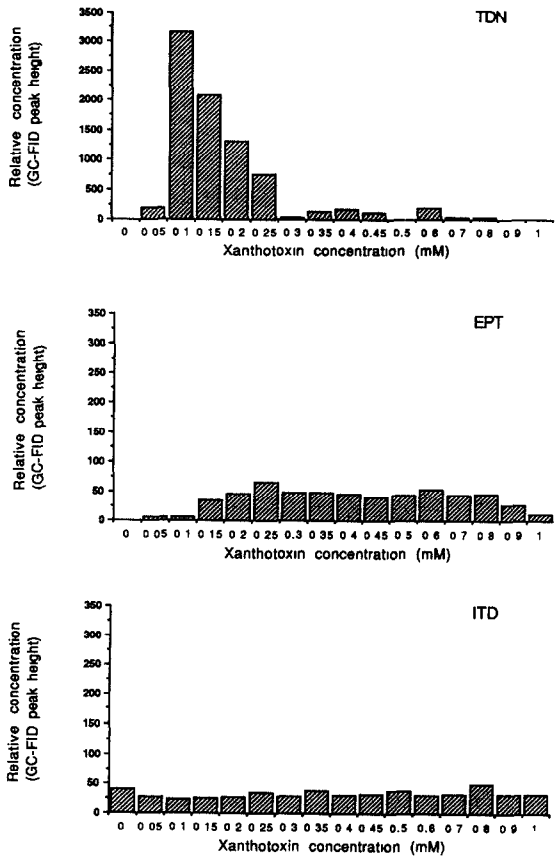


Fig. 1. Accumulation of trichodiene (TDN), 12,13-epoxytrichothec-9-ene (EPT) and isotrichodermin (ITD) in xanthotoxin-treated cultures of *F. culmorum*.

onably constant. Larger scale fermentations (0.1 mM inhibitor) gave TDN (500 mg l^{-1}), EPT (1 mg l^{-1}) and ITD (3 ml l^{-1}).

Further amounts of EPT were obtained by semi-synthesis from ITD, employing chemical deoxygenation via Barton reduction of the phenylthionocarbonate ester with tributyltin hydride [19]. A similar sequence has been reported independently by McCormick *et al.* [20], and EPT has also been obtained from verrucarol [21]. An alternative sequence using diacetoxyscirpenol as substrate gave good yields of 15-hydroxyEPT [19], but we were unable to transform this into EPT. A successful execution of this last step has since been reported by Zamir *et al.* [21].

By feeding sodium [2-^{14}C]acetate to a xanthotoxin-inhibited culture of *F. culmorum*, [^{14}C]TDN was produced with a specific activity suitable for further metabolic studies. Incorporation of [^{14}C]TDN (0.5 mg) into trichothecene toxins was demonstrated by feeding the material over 6 hr to a 30 ml culture of *F. culmorum* suspended in distilled water. TLC-autoradiography of extracts (Fig. 2, lane 1) indicated good incorporation of label into the major toxins 3-AcDON and DHC, with activity also present in EPT, ITD, calonectrin (CAL, **14**), 15-deacetylcalonectrin (**15**), and 7-hydroxycalonectrin (**16**). This 'normal' radiolabelled toxin profile was then compared with that from a similar experiment in which unlabelled TDN (5 mg) was fed 30 min before the labelled TDN (0.5 mg). All of the major toxins were radiolabelled, but their intensities were diluted due to the presence of the unlabelled precursor (Fig. 2, lane 2). In this experiment, a highly active material was also present, which in the normal profile appeared at only trace levels. This new metabolite (23 mg) was isolated from a larger scale feeding experiment in which unlabelled TDN (600 mg) was fed to mycelium from a 1.8 l culture. Small amounts of trichothecenes **2**, **10** and **12**–**16** were also isolated, along with recovered TDN (233 mg). The complete recovery of TDN from culture media appeared hampered by adsorption to mycelia.

The new metabolite was identified as **5** by a combination of mass, ^{1}H and ^{13}C NMR spectral methods. On TLC, it reacted with *p*-nitrobenzylpyridine spray reagent to give a bright sky blue colour. This reagent gives dark blue or violet colours with trichothecenes via the epoxide

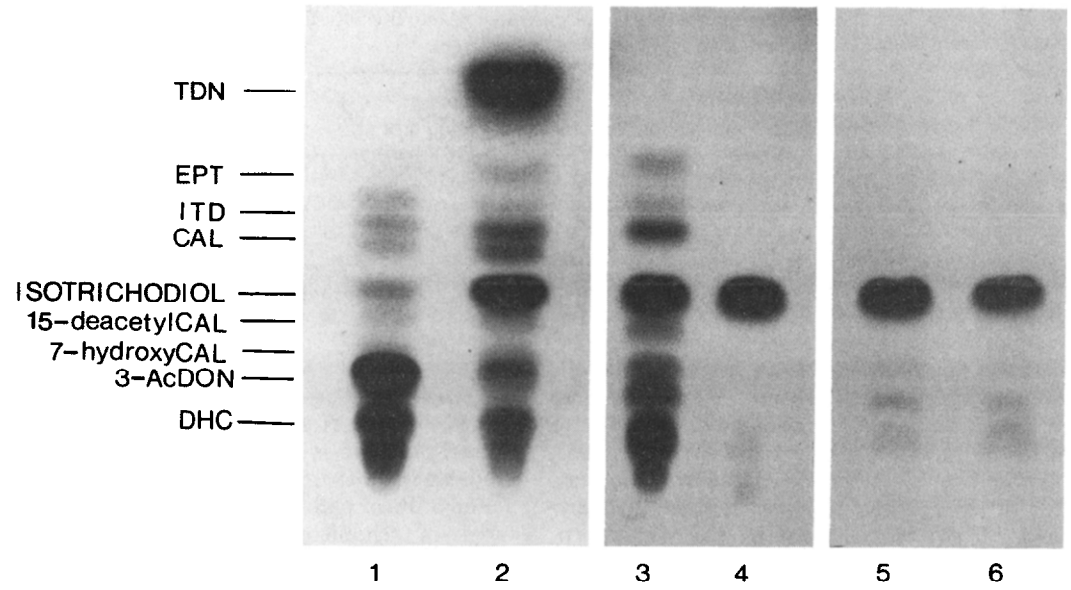


Fig. 2. TLC-autoradiograms from feeding experiments. Lane 1—fed [^{14}C]trichodiene. Lane 2—fed [^{14}C]trichodiene + unlabelled trichodiene. Lane 3—fed [^{14}C]isotrichodiol. Lane 4—fed [^{14}C]isotrichodiol, boiled mycelia. Lane 5—fed [^{14}C]isotrichodiol, mycelia in production medium in presence of N_2. Lane 6—fed [^{14}C]isotrichodiol, mycelia in production medium in presence of xanthotoxin (0.1 mM).

group [22], but this sky blue colour is typical of non-cyclized trichothecene-like materials such as trichodiol (**6**) and trichotriol (**8**) [23]. EIMS ([M]$^+$ at *m/z* 252.1733) indicated a molecular formula $C_{15}H_{24}O_3$ (requires 252.1741), with a base peak at *m/z* 84 (C_5H_8O), consistent with a retro-Diels–Alder fragmentation in ring A as seen for **4** and **7** and indicating monohydroxylation of this ring [23]. The GC-MS of the product after trimethylsilylation indicated formation of two derivatives, a di-TMS ether ([M − Me]$^+$ at *m/z* 381) and a mono-TMS ether ([M]$^+$ at *m/z* 324). RDA cleavage of ring A in the di-ether resulted in the base peak shifting to *m/z* 156, but this peak was insignificant in the spectrum of the mono-ether, which still retained *m/z* 84. This indicated the presence of a second hydroxyl group on ring B, and that the ring B hydroxyl is alkylated more readily than that on ring A.

The compound was clearly a 12,13-epoxide derivative (trichothecene numbering) as indicated by the doublets (J = 4.2 Hz) at δ3.06 and 3.32 in the ^{1}H NMR spectrum. The additional hydroxyl substituents incorporated into the basic trichodiene structure were readily assigned to positions 11α and 2α, by comparison with the spectrum for trichodiene itself, and those of trichothecene derivatives, especially EPT [10]. The stereochemistries at positions 11 and 2 were defined by characteristic J values [23]. Thus, H-10 was a broad singlet at δ5.14, and H-11β a broad singlet at δ4.60, i.e. $J_{10,11} < 1$ Hz, whereas in EPT, $J_{10,11\alpha} = 5.5$ Hz [10]. In ring B, H-2β appeared as a doublet (J = 4.1 Hz) at δ3.60, very similar to that found in EPT (δ3.72, *d*, J = 4.6 Hz), and indicates similar stereochemistries, with $J_{2\beta,3\alpha} \sim 0$ in both molecules as expected because molecular models predict a dihedral angle of about 90° for these two protons. The proton spectrum of **5** shared many features of those published for compounds **4**, **7** and **17** [23, 24], and allowed confirmation of the identity of the A-ring. A full assignment of the ^{1}H NMR spectrum was aided by COSY and NOE difference (DNOES) spectra. A significant NOE aiding the structural analysis was that between H-2β (δ3.60) and the C-13 proton at δ3.06, confirming the stereochemistry at C-2. NOE between the other C-13 proton (δ3.32) and δ1.25 helped to assign the latter signal to H-7β. Several coupling constants are effectively zero due to dihedral angles, including $J_{2\beta,3\alpha}$, $J_{7\alpha,8\beta}$ and $J_{3\alpha,4\beta}$.

The structure was further supported by ^{13}C NMR data, where again, signals corresponding to those in the spectra of **4**, **7** and **17** [23, 24] were encountered. The relationship of **5** to TDN was evident from the appearance of two new oxygenated methine signals at δ72.0 and 81.7, and the signals characteristic of 12,13-epoxytrichothecenes at δ69.4 and 49.5, coupled with corresponding signal losses. The structure **5** differs from that of trichodiol (**6**) in that the allylic alcohol function is at position 11α rather than 9α, and so the trivial name isotrichodiol is proposed for the new metabolite.

The incorporation of [^{14}C]TDN into isotrichodiol was measured in a further experiment, and a specific incorporation of 67% was recorded. When ^{14}C-labelled isotrichodiol was refed to a culture of *F. culmorum* suspended in distilled water, the radiolabelled toxin profile after 6 hr was very similar to that obtained with labelled TDN (Fig. 2, lane 3), and demonstrated its incorporation into EPT, ITD, CAL, 7-hydroxycalonectrin, 15-deacetylcalonectrin, 3-AcDON and DHC. Specific incorporations in the range 31–79% were recorded for 3-AcDON, DHC, and 7-hydroxycalonectrin (Table 1). A control experiment using the boiled mycelium showed no chemical modification of isotrichodiol under these incubation conditions (Fig. 2, lane 4). In further experiments, it was demonstrated that incorporation of isotrichodiol into the trichothecenes was almost completely inhibited when the culture had previously been exposed to xanthotoxin, or if the fungus was cultured under a nitrogen atmosphere (Fig. 2, lanes 5 and 6). In the latter case, the presence of additional ATP in the culture medium did not restore trichothecene biosynthesis. Isotrichodiol thus appears to

Table 1. Incorporation of [^{14}C]isotrichodiol* into trichothecene toxins in *Fusarium culmorum* cultures

Trichothecene	Mg isolated	Sp. act. (kBq mM^{-1})	Sp. incorpn (%)
3-AcDON (**10**)	1.4	41.6	79
DHC (**13**)	2.6	31.3	59
7-HydroxyCAL (**16**)	0.6	19.4	31

*Isotrichodiol (18 mg, 52.9 kBq mM^{-1}) fed to 400 ml culture over 6 hr.

be an intermediate between TDN and EPT and the other trichothecene structures. Cyclization of isotrichodiol to EPT might be expected to occur readily, and indeed, under appropriate acid-catalysed conditions, this transformation can be detected. However, this *in vitro* cyclization is slow (35% yield over 3 days) and other byproducts are formed. The very rapid incorporation of isotrichodiol into trichothecenes *in vivo* as demonstrated in these 6 hr experiments suggests that if cyclization to EPT is part of the pathway, then this process is likely to be controlled in the fungus by an appropriate cyclase enzyme. The involvement of a phosphorylated derivative would provide a better leaving group for the process, but there is no evidence to suggest that this is necessary. A similar slow chemical transformation under acidic conditions of **7** ('isotrichotriol' is now proposed as trivial name for this compound) to trichotriol (**8**) and then isotrichodermol (**9**) has been reported [20], but again, this is not rapid enough to feature in a biosynthetic pathway without enzymic intervention.

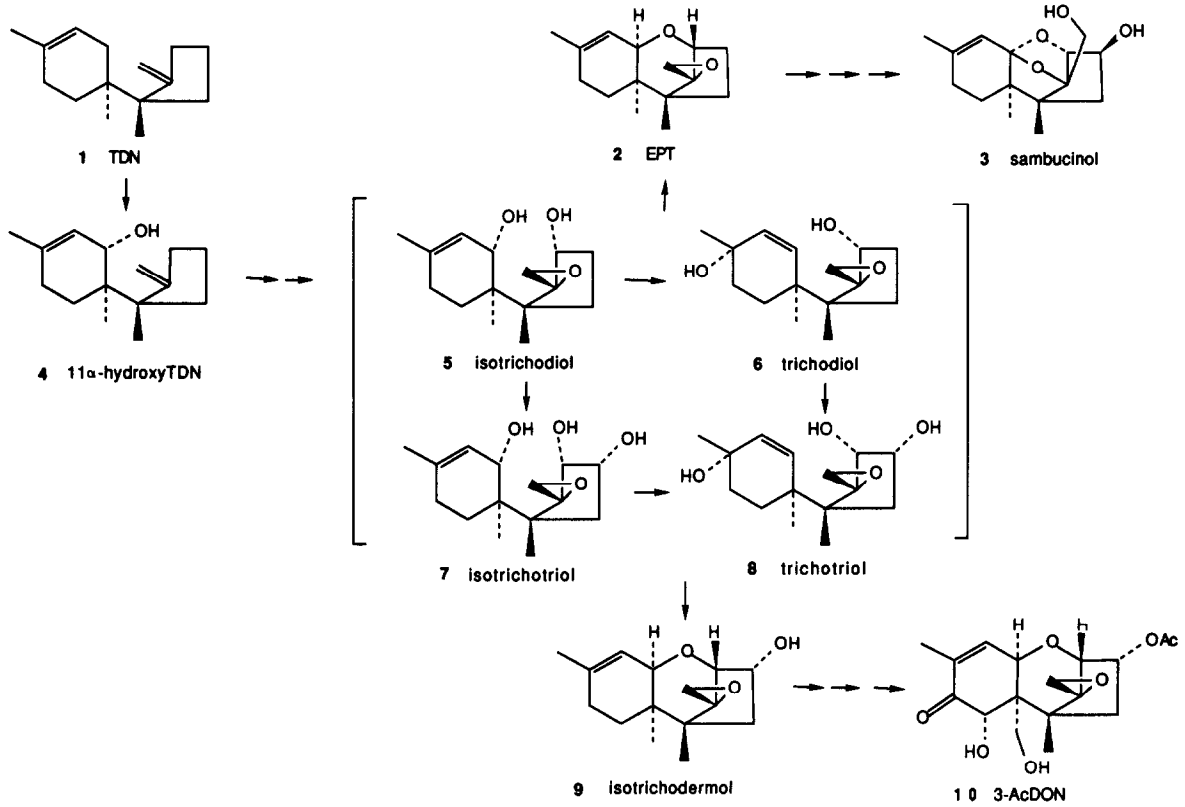
Isotrichodiol can be envisaged as the product of probably three oxygenation reactions on TDN, hydroxylations at positions 11α and 2α, and epoxidation at C-12, C-13. Isotrichotriol (**7**) along with 11α-hydroxytrichodiene (**4**) have been isolated from mutant strains of *F. sporotrichioides* [23], and isotrichodiol may thus be considered part of the sequence from **4** to **7**. Feeding experiments using unlabelled precursors [20] showed a *F. sporotrichiodes* mutant was able to use the non-cyclized trichothecene-like compounds isotrichotriol (**7**), and trichotriol (**8**) and its 9β-epimer, as well as the trichothecenes isotrichodermol (**9**) and isotrichodermin (**12**), to resume biosynthesis of T-2 toxin (**11**). Most significantly, addition of trichodiol (**6**) or EPT did not prompt synthesis of T-2 toxin. In other studies [21], radiolabelled isotrichodermin was shown to be a precursor of 3-AcDON (**10**) in *F. culmorum*. EPT was found to be involved in the biosynthesis of the trichothecene-related metabolite sambucinol (**3**) in *F. culmorum*, but was not incorporated into 3-AcDON. These experiments, coupled with the demonstrated *in vitro* cyclization of trichotriol (**8**) to isotrichodermol (**9**) [25], have pointed to a sequence in which introduction of the 3-hydroxyl occurs *before* cyclization to the trichothecene. Accordingly, trichodiene is suggested to be transformed to isotrichodermol via intermediates trichodiol (**6**) and trichotriol (**8**) [20, 21]. The role, if any, of trichodiol in trichothecene biosynthesis has yet to be demonstrated. Allylic hydroxylation of TDN at position 11 giving 11α-hydroxytrichodiene (**4**) and then further modification to isotrichodiol seems more plausible than introduction of oxygen at position 9. Further hydroxylation would lead to isotrichotriol (**7**) (Scheme 1). Trichodiol and trichotriol may be formed from **5** and **7** respectively by allylic isomerization. It is not certain from the information available whether trichodiol, trichotriol or isotrichotriol are on a direct pathway, or arise as side reactions. The variety of trichothecene and pre-trichothecene structures so far identified in *Fusarium* species point to the operation of metabolic grids rather than unique pathways, but such aspects remain to be clarified.

The accumulation of isotrichodiol when *F. culmorum* cultures are challenged with a large excess of the natural precursor TDN could be due to exhaustion of cofactors necessary for trichothecene biosynthesis. The mycelium was transferred from its culture medium to distilled water, and despite the short duration of the experiments, this would deprive the organism of essential nutrients, as well as inducing stress. A similar accumulation of isotrichodiol was observed when a number of unnatural semi-synthetic TDN analogues were fed in the same way. Thus, 9β,10β-epoxytrichodiene and 9β,10β;12,13-diepoxytrichodiene both inhibited incorporation of labelled TDN into trichothecenes and initiated isotrichodiol production. In these cases, endogenous TDN is presumably used to synthesize isotrichodiol, and the initiators could be acting as enzyme inhibitors, perhaps blocking an essential cyclase system. These possibilities are being investigated further.

EXPERIMENTAL

General. NMR: 400 MHz (^{1}H) or 100 MHz (^{13}C), $CDCl_3$, TMS int. standard; MS: probe, 70 eV; TLC: 0.2 mm silica gel (Merck Kieselgel 60 F_{254}). Toxins were visualized using spray reagents 20% aq. H_2SO_4 with heating at 150° for 2–5 min [26] or 1% 4-(*p*-nitrobenzyl)pyridine in $CHCl_3$–CCl_4 with heating at 150° for 5 min, followed by spraying with 10% tetraethylenepentamine in $CHCl_3$–CCl_4 [22]. Autoradiograms were obtained by placing TLC plates in contact with Hyperfilm-βmax (Amersham) for 1–4 days prior to development (Kodak D-19 developer). Centrifugal TLC (CTLC) was performed on a Chromatotron (Harrison Research) using Merck Kieselgel 60. CC: Merck Kieselgel 60 (70–230 mesh); GC: capillary column (J&W, DB-5; 30 m × 0.25 mm), H_2 2 ml min^{-1}, injection temp. 300°, temp. prog. 1 min at 100°, then 10° min^{-1} to 270°. Detection was by FID or EIMS (70 eV). Peak heights were used to assess relative amounts of metabolites in GC analyses.

Culture of fungus. Fusarium culmorum CMI 14764 was maintained on Czapek Dox (CD) agar (Oxoid) slopes at 25° in the dark. CD agar plates (8 cm diam.) were inoculated with mycelia/agar portions (*ca* 5 mm cubes) cut from mature CD slope cultures (3–4 weeks) and incubated at 27° for 6 days in the dark.



Scheme 1. Proposed biosynthetic pathway to trichothecene mycotoxins in *F. culmorum*.

Mature growths were cut from excess peripheral agar and old inoculum source, combined, and liquidized for 1 min in sterile dist. H_2O (15 ml per plate). Aliquots (20 ml) of this homogenate were used to inoculate 1 l flasks, each containing 200 ml of CD production medium [$(NH_4)H_2PO_4$, 1 g; K_2HPO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 5 g; sucrose, 40 g; glycerol, 10 g; H_2O to 1 l)]. Cultures were incubated in the dark at 27° on an orbital shaker (250 rpm) for 8 days. The cultures were filtered through muslin and the filtrate extracted with EtOAc. (× 3). The combined extracts were dried over $MgSO_4$ and evapd to an oil.

Effects of xanthotoxin inhibition. Fusarium culmorum cultures (32 × 50 ml; 250 ml flasks) were incubated (200 rpm) for 2 days at 27° in the dark. Pairs of cultures were treated with varying amounts of xanthotoxin (10.8 mg ml^{-1} in DMSO) to give a concentration series of 0–0.5 mM in 0.05 mM increments, and 0.60–1.00 mM in 0.10 mM increments. Additional DMSO was added so that each culture received 1 ml. After a further 6 days incubation, each 50 ml culture was filtered (Whatman No 4 paper) under red. pres. and extracted with EtOAc (3 × 50 ml). The extracts were dried ($MgSO_4$), filtered, and evapd to dryness under red. pres. Extracts were dissolved in $CHCl_3$–MeOH (9:1; 1 ml per 15 mg extract) and analysed by GC. A further study on selected samples using GC-MS was performed to identify TDN, 3-AcDON, EPT and ITD.

Isolation of trichodiene (*TDN*, **1**). The crude extract from 3 × 200 ml CD production medium cultures exposed to 0.1 mM xanthotoxin as above was dissolved in EtOAc (3 ml) and loaded on to a silica gel column (10 cm × 6 cm diam.) packed in redistilled hexane. The column was eluted first with hexane (500 ml), then EtOAc (200 ml) and MeCN (400 ml), collecting frs of *ca* 70 ml. Frs 4–6 were pooled and evapd to dryness under red. pres. at 30° to yield TDN as an oil (300 mg). TDN gives a brown colour with the H_2SO_4 spray, which on exposure to air over *ca* 30 min at room temp. becomes a characteristic purple colour. ^{1}H NMR ($CDCl_3$); δ5.29 (1H, *dq*, *J* = 5.4, 1.3 Hz, H-10), 4.96 (1H, *dd*, *J* = 1.3, 1.1 Hz, H-13), 4.73 (1H, *dd*, *J* = 1.3, 1.3, H-13), 2.4–1.3 (12H, *m*, H-2, H-3, H-4, H-7, H-8, H-11), 1.64 (3H, *ddd*, *J* = 1.4, 0.7, 0.6 Hz, H-16), 1.04 (3H, *d*, *J* = 0.5 Hz, H-15), 0.85 (3H, *s*, H-14); ^{13}C NMR ($CDCl_3$): δ160.0 (C-12), 132.3 (C-9), 120.6 (C-10), 106.7 (C-13), 50.8 (C-5), 38.9 (C-2), 37.4 (C-4), 37.0 (C-6), 33.2 (C-11), 28.3 (C-7), 27.9 (C-8), 24.1 (C-14), 23.4 (C-3), 23.2 (C-16), 18.0 (C-15); EIMS *m/z* (rel. int.): 204 $[M]^+$ (5), 189 (3), 122 (3), 121 (8), 119 (4), 110 (18), 109 (100), 108 (83), 107 (18), 96 (29), 95 (21), 93 (22), 91 (11).

[^{14}C]*Trichodiene*. A 2-day-old CD production medium culture (200 ml) was exposed to 0.1 mM xanthotoxin as above. Portions (1 ml) of a soln of Na[2-^{14}C]acetate (37 MBq; 1.9 GBq mM^{-1}, 5 ml) were added at times 0, 8, 31, 77, and 100 hr after addition of xanthotoxin, and the culture was then worked-up at time 144 hr. Extraction and purification gave [^{14}C]trichodiene (164 mg; sp. act. 8.45 MBq mM^{-1}). Purity was checked by TLC, GC and NMR.

12,13-*Epoxytrichothec-9-ene* (*EPT*, **2**) *and isotrichodermin* (*ITD*, **12**). Crude extract (3 g) from 15 × 260 ml 2-day-old CD production medium cultures exposed to 0.7 mM xanthotoxin for 6 days was fractionated by CTLC (4 mm plate) eluting with hexane (100 ml) then hexane–EtOAc mixts (17:3, 100 ml; 33:17, 100 ml) and collecting 25 ml frs: frs 1–4 gave TDN (600 mg); frs 6–10 were combined and further purified by CTLC [1 mm plate; hexane–EtOAc, 4:1, 1.5 ml frs, giving EPT (4 mg) in frs 28–31]; frs 11–13 were combined and further purified by CTLC [1 mm plate; toluene–EtOAc, 3:1; 1.5 ml frs, collecting frs 21–26. Fur-

ther CTLC of these frs (1 mm plate; hexane–EtOAc, 1:1; 1 ml frs, collecting frs 24–28) gave ITD (11 mg)]. Spectral data for EPT and ITD were as previously reported [10].

Feeding experiments with [^{14}C]*TDN*. [^{14}C]TDN (0.5 mg, 37 kBq mg^{-1}) in 50 μl Me_2CO was fed to a culture (30 ml, 42 hr after subculture, filtered and resuspended in 6 ml H_2O) for 6 hr. The culture was worked-up by filtration and EtOAc extraction, the toxins separated by TLC (hexane–EtOAc, 1:1), visualized by autoradiography, and identified by comparison with authentic standards [10, 18].

Isotrichodiol (**5**). 3 × 200 ml CD production medium cultures (42-hr-old) were filtered under red. pres. (Whatman No 4 paper) and the mycelia washed with 3 × 50 ml sterile dist. H_2O, then resuspended in H_2O (160 ml) in a 1 l flask. Trichodiene (156 mg) in Me_2CO (0.6 ml) was added, and the culture incubated at 27°, 250 rpm, for 30 min. A further portion of TDN (44 mg in 0.2 ml Me_2CO) was added and the incubation continued for 5.5 hr. The mycelia was filtered and washed with EtOAc (3 × 20 ml), and the combined filtrate and washings extracted with EtOAc (3 × 50 ml). The combined extracts were dried over $MgSO_4$ and evapd to an oil (120 mg). This was chromatographed by CTLC (1 mm plate) in hexane–EtOAc (1:1) collecting 1 ml frs. Isotrichodiol (11.8 mg) was obtained as crystals, mp 138–140° from frs 33–36. ^{1}H NMR ($CDCl_3$): δ5.14 (1H, *br s*, H-10), 4.60 (1H, *br s*, H-11β), 3.60 (1H, *d*, J = 4.1 Hz, H-2β), 3.32 (1H, *d*, J = 4.2 Hz, H-13), 3.06 (1H, *d*, J = 4.2 Hz, H-13), 2.82 (1H, *td*, J = 13.3, 6.9 Hz, H-4α), 2.00 (1H, *m*, H-8α), 1.91 (1H, *tdd*, J = 13.3, 7.6, 4.3 Hz, H-3β), 1.79 (1H, *br dd*, J = 18.1, 5.8 Hz, H-8β), 1.69 (1H, *dd*, J = 13.6, 6.8 Hz, H-3α), 1.66 (3H, *br s*, H-16), 1.55 (2H, *m*, H-4β, 7α), 1.25 (1H, *ddd*, J = 14.1, 13.6, 6.2 Hz, H-7β), 0.94 (3H, *s*, H-14), 0.93 (3H, *s*, H-15); ^{13}C NMR ($CDCl_3$): δ135.1 (C-9), 125.6 (C-10), 81.7 (C-2), 72.0 (C-11), 69.4 (C-12), 49.5 (C-13), 46.9 (C-5), 41.2 (C-6), 37.3 (C-4), 30.4 (C-3), 29.2 (C-7), 27.9 (C-8), 22.4 (C-16), 20.4 (C-14), 12.6 (C-15); EIMS *m/z* (rel. int.): 252 [M]$^+$ (4), 237 (11), 234 (5), 224 (5), 219 (10), 177 (10), 151 (10), 133 (9), 127 (3), 125 (13), 124 (42), 121 (28), 107 (43), 93 (47), 84 (100); CIMS (CH_4, 100 eV): 253 ([M + 1]$^+$, 10%), 235 (23), 217 (37), 199 (30), 137 (18), 127 (25), 125 (35), 109 (100), 97 (58), 93 (43), 81 (73); EIMS di-TMS ether: 381 ([M − Me]$^+$, 0.3%), 339 (0.9), 313 (0.3), 306 (1), 291 (1), 169 (20), 156 (100), 73 (89); EIMS mono-TMS ether: 324 ([M]$^+$, 1), 309 (2), 306 (0.7), 296 (1), 219 (4), 211 (6), 169 (38), 107 (96), 84 (32), 73 (100).

A feeding experiment using 600 mg unlabelled TDN gave isotrichodiol (23 mg), EPT (1.5 mg), ITD (1.0 mg), CAL (2.5 mg), 15-deacetylcalonectrin (3.5 mg), 7-hydroxycalonectrin (4.0 mg), 3-AcDON (17 mg), DHC (21 mg), and recovered TDN (233 mg). Spectral data for CAL, 3-AcDON, and DHC were as previously reported [10, 18]. ^{1}H NMR ($CDCl_3$) 15-deacetylcalonectrin (**15**): δ5.48 (1H, *br d*, J = *ca* 5 Hz, H-10), 5.17 (1H, *ddd*, J = 9.7, 5.0, 4.7 Hz, H-3), 3.98 (1H, *br d*, J = 4.9 Hz, H-11), 3.75 (1H, *d*, J = 4.7 Hz, H-2), 3.69 (1H, *d*, J = 11.8 Hz, H-15), 3.50 (1H, *d*, J = 11.7 Hz, H-15), 3.10 (1H, *d*, J = 4.0 Hz, H-13), 2.87 (1H, *d*, J = 4.0 Hz, H-13), 2.2–1.5 (6H, *m*, H-4, H-7, H-8), 2.12 (3H, *s*, Ac), 1.74 (3H, *br s*, H-16), 0.93 (3H, *s*, H-14), agreeing with ref. [27]; 7-hydroxycalonectrin (**16**): δ5.41 (1H, *br d*, J = 5.7 Hz, H-10), 5.19 (1H, *ddd*, J = 8.3, 7.1, 4.6 Hz, H-3), 4.58 (1H, *br dd*, J = *ca* 11, 6 Hz, H-7β), 4.32 (1H, *d*, J = 12.3 Hz, H-15), 4.12 (1H, *d*, J = 5.7 Hz, H-11), 4.07 (1H, *d*, J = 12.3 Hz, H-15), 3.26 (1H, *d*, J = 4.4 Hz, H-13), 3.12 (1H, *d*, J = 4.4 Hz, H-13), 3.80 (1H, *d*, J = 4.4 Hz, H-2), 2.43 (1H, *dd*, J = 17.7, 5.6 Hz, H-8β), 2.2–1.5 (4H, *m*, H-4, H-7α, H-8α), 2.11 and 2.10 (each 3H, *s*, Ac), 1.74 (3H, *br s*, H-16), 1.16 (3H, *s*, H-14), agreeing with ref. [28].

[^{14}C]*Isotrichodiol*. Feeding of [^{14}C]TDN (280 mg, 124 kBq mM^{-1}) gave [^{14}C]isotrichodiol (8 mg, 83.7 kBq mM^{-1}), purified to constant sp. act. by CTLC and TLC. Purity was further checked by TLC, NMR, and GC of the TMS ether.

Feeding experiments with [^{14}C]*isotrichodiol*. (*a*) *Normal conditions*: a filtered and washed mycelial mat from one 200 ml CD production medium culture (42 hr) was divided into 4 portions. Two 25 ml flasks containing 7.5 ml H_2O each received one portion, and to these cultures were added [^{14}C]isotrichodiol (2.1 mg, 0.67 kBq, in 0.1 ml Me_2CO) or [^{14}C]TDN (2.1 mg, 0.67 kBq, in 0.1 ml Me_2CO). Mycelium in a third flask was boiled for 10 min, cooled, then [^{14}C]isotrichodiol as above was added. The 3 cultures were incubated at 27°, 250 rpm for 6 hr, and worked-up by extracting the filtrates with EtOAc. The extracts were analysed by TLC and autoradiography.

In a larger scale experiment, [^{14}C]isotrichodiol (18 mg, 52.9 kBq mM^{-1}) was fed to mycelium from a 400 ml culture as above. 3-AcDON (1.4 mg, 41.6 kBq mM^{-1}), DHC (2.6 mg, 31.3 kBq mM^{-1}) and 7-hydroxycalonectrin (0.6 mg, 19.4 kBq mM^{-1}) were isolated and purified to constant sp. act. by repeated TLC and/or recrystallization.

(*b*) *In presence of xanthotoxin*: aliquots (5 ml) of CD production medium culture (30-hr-old) were placed in three 25 ml flasks. Two flasks were treated with xanthotoxin (in 0.1 ml DMSO) to give 0.1 and 0.4 mM concentrations respectively. The 3 flasks were incubated at 27°, 250 rpm for 18 hr, after which time [^{14}C]isotrichodiol (0.5 mg, 83.7 kBq mM^{-1} in 50 μl Me_2CO) was added to each. The cultures were incubated for a further 6 hr, then worked-up by filtration and EtOAc extraction.

(*c*) *In presence of nitrogen*: aliquots (5 ml) of CD production medium culture (48 hr old) were placed in three 25 ml flasks. Two flasks were purged with N_2 gas for 30 min, after which time they received [^{14}C]isotrichodiol (0.5 mg, 83.7 kBq mM^{-1} in 50 μl Me_2CO), and in one case ATP (16.7 mg in 0.2 ml H_2O) in addition. The third flask received [^{14}C]isotrichodiol but was not purged with N_2. The cultures were incubated at 27°, 250 rpm for 6 hr, then worked-up by filtration and EtOAc extraction.

Cyclization of isotrichodiol. Isotrichodiol (7.7 mg) in Me_2CO (0.5 ml) was added to 200 ml sterile YEPD-5G medium [20], previously adjusted to pH 4.5 with dil HCl, and incubated at 28°, 250 rpm, for 3 days. The products were extracted with EtOAc, and sepd by TLC (hexane–EtOAc, 1:1). All isotrichodiol had been consumed, and 4 compounds were detected. The major product was identified as EPT (2.5 mg), and shown to be spectrally identical to natural material [10]. Similar results were obtained when isotrichodiol was incubated in 0.1 M K-Pi buffer (pH 5.9) at 28° for 5 days.

3-Hydroxy-12,13-epoxytrichothec-9-ene (isotrichodermol, **9**). A soln of 3-acetoxy-12,13-epoxytrichothec-9-ene (isotrichodermin, ITD; 30 mg) in MeOH (1.7 ml) was treated with NH_4OH (2 M, 1.2 ml), stirring at room temp. for 24 hr. The reaction was terminated by carefully adjusting the pH to 7.0 with M HCl, and the MeOH was removed under red. pres. The aq. residue was extracted with CH_2Cl_2 (3 × 10 ml), the organic layers combined, dried over $MgSO_4$, and evapd to an oil (23 mg). Purification by CTLC (1 mm plate; hexane-EtOAc, 2:3) gave isotrichodermol as crystals (9 mg, 35%). ^{1}H NMR as ref. [25].

12,13-Epoxytrichothec-9-ene 3-phenylthionocarbonate. To a soln of isotrichodermol (9 mg) in dry CH_2Cl_2 (1 ml) containing 4-dimethylaminopyridine (4.7 mg) and pyridine (18 μl) was added phenylchlorothionocarbonate (25 μl). The reaction was stirred at room temp. under a N_2 atmosphere for 20 hr, during which time the colour changed from orange to green. Addition of CH_2Cl_2 (40 ml), washing with M HCl (2 × 25 ml) and satd NaCl soln (30 ml) followed by drying over $MgSO_4$ and evapn gave a green oil (70 mg). This was purified by CTLC [1 mm plate; petrol (40–60°)–EtOAc, 17:3] to give the 3-phenylthionocarbonate ester as a pale yellow oil (6 mg, 48%). ^{1}H NMR ($CDCl_3$): δ7.46–7.13 (5H, *m*, Ph), 5.66 (1H, *ddd*, J = 9.2, 6.1, 4.7 Hz, H-3), 5.50 (1H, *br d*, J = 5.4 Hz, H-10), 4.01 (1H, *d*, J = 5.4 Hz, H-11),

3.97 (1H, *d*, $J = 4.7$ Hz, H-2), 3.13 (1H, *d*, $J = 3.9$ Hz, H-13), 2.90 (1H, *d*, $J = 3.9$ Hz, H-13), 2.4–1.4 (6H, *m*, H-4, 7, 8), 1.73 (3H, *br s*, H-16), 0.84 and 0.80 (each 3H, *s*, H-14, H-15).

12,13-*Epoxytrichothec-9-ene* (*EPT*, **2**). To a soln of 12,13-epoxytrichothec-9-ene 3-phenylthionocarbonate (6 mg) in dry C_6H_6 (0.5 ml) containing 2,2'-azo-bis(2-methylpropionitrile) (AIBN; 1 mg) was added tri-*n*-butyltin hydride (6.5 μl). The reaction was stirred under a N_2 atmosphere at 80° for 75 min, then evapd under red. pres. to give an oil (14 mg). This was purified by CTLC (1 mm plate; hexane–EtOAc, 1:1) and TLC (hexane–EtOAc, 1:1) to give EPT (1.2 mg, 50%) as an oil, spectrally identical to the natural product [10].

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