Isotrichodiol: A Post-trichodiene Intermediate in the Biosynthesis of Trichothecene Mycotoxins

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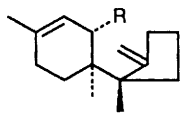
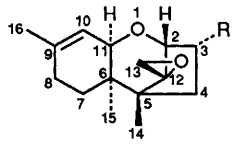
Feeding experiments with labelled trichodiene (**1**) in *Fusarium culmorum* cultures have resulted in the isolation of a new metabolite, isotrichodiol (**9**), that is subsequently transformed into trichothecene toxins, and thus represents the first demonstrated post-trichodiene intermediate in the biosynthetic pathway to trichothecenes.

The biosynthetic origin of the trichothecene mycotoxins[1] has been studied in some detail over the years,[2,3] and a sequence from mevalonate *via* farnesyl diphosphate,[4] nerolidyl diphosphate,[5] and trichodiene [TDN, (**1**)][6,7] is known to be operative. Isolation of the parent trichothecene 12,13-epoxytrichothec-9-ene [EPT, (**3**)], initially from *Trichothecium roseum*[8] and more recently from *Fusarium culmorum*[9] supports the hypothesis that EPT is 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. Feeding experiments[3] have confirmed that isotrichodermin [ITD, (**5**)] is incorporated into 3-acetyldeoxynivalenol [3-AcDON, (6)], the major trichothecene metabolite of *F. culmorum*, and this intermediate may arise by hydroxylation and acetylation from EPT. However, based on the observation[10] of non-enzymic cyclisation of trichotriol (**8**) to isotrichodermol (**4**), an alternative sequence involving introduction of the 3-hydroxy before cyclisation has also been proposed.

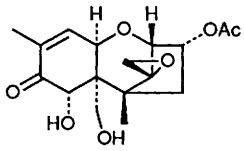
Mono-oxygenase enzymes are believed to be responsible for the hydroxylation and epoxidation reactions,[11] and by supplying specific mono-oxygenase inhibitors to cultures of *F. sambucinum*[12] or *F. sporotrichioides*,[13] these oxygenation processes can be markedly suppressed, resulting in accumulation of significant levels of TDN, which is presumably the last hydrocarbon intermediate in the pathway. Although trichodiol (**7**), first isolated from *T. roseum*,[14] is widely assumed to be a post-trichodiene intermediate, there is no direct evidence to support its involvement, and the sequence between TDN and trichothecenes is completely unknown. This communica-



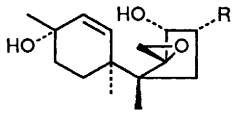
TDN (1) R = H
(2) R = OH



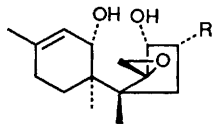
EPT (3) R = H
(4) R = OH
ITD (5) R = OAc



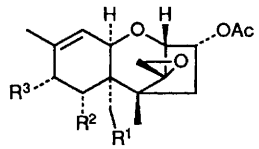
3-AcDON (6)



(7) R = H
(8) R = OH



(9) R = H
(10) R = OH



DHC (11) R^1 = OAc, R^2 = R^3 = OH
CAL (12) R^1 = OAc, R^2 = R^3 = H
(13) R^1 = OH, R^2 = R^3 = H
(14) R^1 = OAc, R^2 = OH, R^3 = H

tion describes the isolation from cultures of *F. culmorum* of a new metabolite isotrichodiol (**9**). This is 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.

Cultures of *F. culmorum* (2 days after subculture) were treated with varying amounts of xanthotoxin[13] and then analysed for toxin production *via* GCMS after a further 6 days. This treatment inhibited production of the major trichothecene metabolites [3-AcDON and dihydroxycalonectrin (DHC, **11**)], and resulted in the accumulation of significant amounts of TDN, together with increased amounts of EPT and ITD. Maximum yields of TDN were obtained with a xanthotoxin concentration of 0.1 mM, whilst optimum levels of EPT and ITD were produced using 0.7 mM xanthotoxin. Larger scale fermentations (0.1 mM inhibitor) gave TDN (500 mg/l), EPT (1 mg/l), and ITD (3 mg/l) and by feeding sodium [2-^{14}C]acetate, [^{14}C]TDN was produced with a specific activity suitable for further metabolic studies.

Incorporation of TDN into trichothecene toxins was demonstrated by feeding [^{14}C]TDN (0.5 mg) to cultures of *F. culmorum* over 6 h.† TLC-autoradiography of extracts indicated good incorporation of label into the toxins 3-AcDON and DHC, with activity also present in EPT, ITD, calonectrin [CAL, (**12**)], 15-deacetylcalonectrin (**13**), and 7-hydroxycalonectrin (**14**). This 'normal' radiolabelled toxin profile was 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 owing to the presence of the unlabelled precursor. However, an additional, highly active material was present. This new metabolite was isolated from a larger scale feeding experiment using unlabelled TDN,‡ and was identified as (**9**).§ This structure differs from trichodiol (**7**) in that the allylic alcohol function is at position 11α (trichothecene numbering) rather than 9α, and the trivial name isotrichodiol is proposed. [^{14}C]TDN was incorporated into isotrichodiol with a specific incorporation of 67%.‡

When ^{14}C-labelled isotrichodiol was refed to a culture of *F. culmorum*, the radiolabelled toxin profile was essentially the same as that obtained with labelled TDN, and demonstrated its incorporation into EPT, ITD, CAL, 7-hydroxycalonectrin, 15-deacetylcalonectrin, 3-AcDON, and DHC. Specific incorporations into 3-AcDON, DHC, and 7-hydroxycalonectrin in the range 31—79% were recorded.¶ A control experiment with boiled mycelium showed no chemical modification of isotrichodiol under these incubation conditions. Isotrichodiol thus appears to be an intermediate between TDN and EPT and the other trichothecene structures. Although cyclisation to EPT might be expected to occur readily, there is no evidence to indicate this is achieved chemically under acid-catalysed conditions, and it is presumably controlled in the fungus by an appropriate cyclase enzyme, perhaps *via* a phosphorylated derivative.

Isotrichodiol can be envisaged as the product of probably three oxygenation reactions on TDN, hydroxylations at positions 11α and 2α, and epoxidation at C(12,13). The related structure (**10**) along with 11α-hydroxytrichodiene (**2**) have been isolated from mutant strains of *F. sporotrichioides*,[15] and isotrichodiol may thus be considered part of the sequence from (**2**) to (**10**). Indeed, the isotrichodiol structure has been postulated as an intermediate in the pathway to sambucinol and sambucoin, trichothecene-related metabolites from *F. sambucinum*.[16] The role, if any, of trichodiol (**7**) itself in trichothecene biosynthesis has yet to be demonstrated. Allylic hydroxylation of TDN at position 11 giving 11α-hydroxytrichodiene (**2**) and then further modification to

† [^{14}C]TDN [0.5 mg (7.55 MBq/mM) in 50 μl acetone] was fed to cultures of *F. culmorum* CMI 14764 [30 ml, 42 h after subculture, filtered and resuspended in H_2O (6 ml)]. After 6 h incubation, the cultures were worked up by filtration and EtOAc extraction. Toxins were separated by TLC (silica; hexane–EtOAc, 1:1), visualised by autoradiography, and identified by comparison with authentic standards.

‡ 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). Complete recovery of TDN from culture media is hampered by adsorption to mycelia. Feeding of [^{14}C]TDN (280 mg; 124 kBq/mM) gave [^{14}C]isotrichodiol (8 mg; 83.7 kBq/mM), purified to constant specific activity by circular TLC, then TLC. Purity was further checked by TLC, NMR and capillary GC of the TMS ether.

§ Isotrichodiol was identified by a combination of MS, ^{1}H NMR, ^{13}C NMR, COSY and DNOES. The compound (M^+ 252.1733; $C_{15}H_{24}O_3$ requires 252.1741) was clearly a 12,13-epoxide derivative (trichothecene numbering) as indicated by the doublets (*J*, 4.2 Hz) at δ 3.06 and 3.32 in the ^{1}H NMR spectrum. Two additional hydroxy substituents had been incorporated, and these were readily assigned to positions 11α and 2α from ^{1}H NMR data, in particular from characteristic *J* values.[15] Thus, H(10) was a broad singlet at δ 5.14, H(11β) a broad singlet at 4.60, and H(2β) a doublet (*J*, 4.1 Hz) at 3.60. All other spectral data were fully in accord with the structure (**9**).

¶ [^{14}C]Isotrichodiol (18 mg; 52.9 kBq/mM) gave a range of ^{14}C-labelled trichothecenes as indicated by autoradiography. 3-AcDON (1.4 mg; 41.6 kBq/mM), DHC (2.6 mg; 31.3 kBq/mM) and 7-hydroxycalonectrin (0.6 mg; 19.4 kBq/mM) were isolated and purified to constant specific activity by repeated TLC and/or recrystallisation.

isotrichodiol seems more plausible than introduction of oxygen at position 9. However, trichodiol may be formed from isotrichodiol by an allylic isomerisation, either as the next intermediate on the pathway, or as a side reaction. If isotrichodiol cyclises to EPT, then isotrichodermol (**4**) may arise by similar cyclisation of (**10**), rather than by hydroxylation of EPT. The variety of trichothecene and pretrichothecene 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.

We thank the Ministry of Agriculture, Fisheries and Food, Food Science Division for financial support.

Received, 23rd March 1990; Com. 0/01279C

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