Trichothecene Mycotoxins from *Fusarium culmorum* Cultures

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Chemical analysis of the culture filtrates of *Fusarium culmorum* CMI 14764 has demonstrated the presence of seven trichothecene mycotoxins. Major metabolites are 3-acetyldeoxynivalenol and 7α,8α-dihydroxycalonectrin, with 3,15-diacetyldeoxynivalenol, deoxynivalenol, calonectrin, isotrichodermin and 12,13-epoxytrichothec-9-ene (EPT) as minor products. The occurrence of the rarely encountered unsubstituted trichothecene EPT is significant in that this compound may function as a common intermediate in the biosynthetic pathways to all natural trichothecenes. The structures of the known trichothecenes isolated from *F. culmorum* suggest a route in which EPT is sequentially oxygenated to the more complex deoxynivalenol derivatives.

Introduction

The trichothecenes are a major group of fungal toxins of particular importance because of their frequent occurrence in fungal contaminated foodstuffs, especially cereals. Several genera of the Fungi Imperfecti, *e.g. Fusarium, Myrothecium,* and *Trichothecium* are known to produce representatives of this group of sesquiterpene mycotoxins [1–3]. The known natural trichothecenes [1–3] are based on a parent 12,13-epoxytrichothec-9-ene (**1**) skeleton, but a considerable number of examples is encountered due to modification of this skeleton by oxygen substituents. Oxygen functions, mainly alcohols and simple esters, may occur at positions 3, 4, 7, 8 and 15, in a wide variety of combinations, but typically with well-defined stereochemistry at any particular carbon.

One such derivative, found with increasing frequency in crops such as wheat and corn, is deoxynivalenol (DON, vomitoxin) (**5**). In order to assist routine toxin analyses, and to extend toxicological evaluation of DON, we have developed a high-yielding method for the production of 3-acetyldeoxynivalenol (3-AcDON) (**6**), and hence DON by chemical hydrolysis, using cultures of *Fusarium culmorum* CMI 14764 [4]. By including labelled sodium acetate in the cultures, this procedure provided an easy and reliable method for obtaining ^{14}C-labelled 3-AcDON and DON of suitable activity for further metabolic studies in animals [5]. Biotransformation studies with other *Fusarium* species [6] extends the range of trichothecene structures available for detailed toxicological evaluation.

A second trichothecene metabolite isolated from cultures of *F. culmorum* was identified as 7α,8α-dihydroxycalonectrin (DHC) (**4**) [4], and other trichothecenes were detected as minor constituents. Concurrently with our own studies, Greenhalgh and coworkers at Agriculture Canada were also investigating the trichothecene and biogenetically-related constituents from cultures of the very same strain of *F. culmorum*, and they have reported the presence of twelve trichothecenes in this fungus [7–9]. Although we have not attempted to carry out an exhaustive investigation of the minor constituents from *F. culmorum,* we have identified five trichothecenes in addition to 3-AcDON and DHC, and we report their structures here. Two of these were also isolated by Greenhalgh *et al.* Of the three compounds not reported by this group, 12,13-epoxytrichothec-9-ene (EPT) (**1**) is the rarely encountered unsubstituted trichothecene, and its presence is of major significance to our knowledge of trichothecene biosynthesis.

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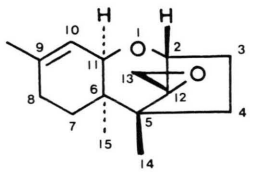
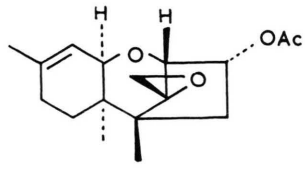
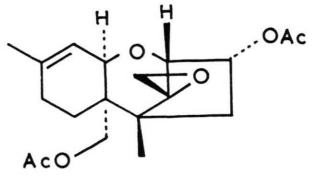


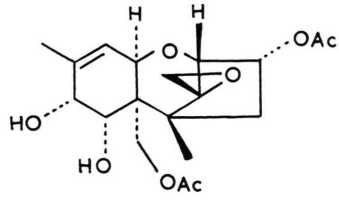
1 EPT



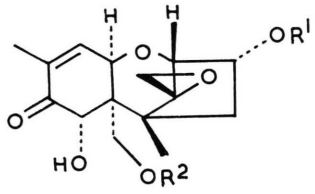
2 ITD



3 CAL



4 DHC



5 $R^1 = R^2 = H$, DON

6 $R^1 = Ac$, $R^2 = H$, 3-AcDON

7 $R^1 = R^2 = Ac$, 3,15-diAcDON

Results and Discussion

Cultures of *Fusarium culmorum* CMI 14764 were grown in a chemically-defined production medium as described previously [4]. After 9 days, the culture medium was separated from the mycelium and extracted with ethyl acetate. Fractionation of this extract on a silica gel column gave the major toxins 3-AcDON (**6**) and DHC (**4**) in yields of approximately 75 mg/l and 15 mg/l respectively [4]. Other fractions were assayed for trichothecene content by TLC and resolved further by column chromatography or TLC as appropriate. If possible, the compounds were obtained in crystalline form before MS and ^{1}H NMR analysis.

Proton NMR data (250 MHz) for the isolated compounds are presented in Table I. The NMR spectral data for 3-AcDON were assigned [4] completely using COSY and fully-coupled 2-D techniques [10], and complemented those reported by the Agriculture Canada group [11]. The structures of the other trichothecene metabolites were readily deduced by comparison of their ^{1}H NMR spectra with that for 3-AcDON, so the previously reported spectra for 3-AcDON and DHC are also included in Table I.

Column fractions yielding the major metabolite 3-AcDON also contained small amounts (ca. 1.5 mg/l of culture) of 3,15-diacetyldeoxynivalenol (3,15-

Table I. ^{1}H NMR Chemical shift assignments and coupling constants for trichothecene mycotoxins from *Fusarium culmorum* CMI 14764.

	DON (**5**)	3-AcDON (**6**)	3,15-diAcDON (**7**)	DHC (**4**)
H-2	3.65 (d, J=4.5)	3.92 (d, J=4.4)	3.93 (d, J=4.4)	3.80 (d, J=4.4)
H-3	4.54 (ddd, J=10.6, 4.5, 4.5)	5.23 (ddd, J=11.2, 4.5, 4.5)	5.24 (ddd, J=10.9, 4.5, 4.5)	5.19 (ddd, J=11.1, 4.4, 4.4)
H-4α	2.24 (dd, J=14.8, 4.4)	2.38 (dd, J=15.0, 4.5)	2.33 (dd, J=15.1, 4.7)	2.39 (dd, J=15.1, 4.4)
H-4β	2.09 (dd, J=14.8, 10.8)	2.17 (dd, J=15.2, 11.1)	2.19 (dd, J=15.0, 11.0)	2.16 (dd, J=15.1, 11.1)
H-7	4.85 (d, J=1.8)	4.84 (d, J=1.9)	4.84 (d, J=1.9)	4.02 (brdd, J ca. 7.3, 5.5)
H-8				4.54 (dd, J=9.7, 5.4)
H-10	6.63 (dq, J=5.8, 1.5)	6.61 (dq, J=5.8, 1.5)	6.59 (dq, J=5.8, 1.5)	5.62 (dd, J=5.9, 1.4)
H-11	4.83 (d, J ca. 6)	4.70 (d, J=5.8)	4.72 (d, J=5.8)	4.35 (d, J=5.8)
H-13	3.10 (d, J=4.3)	3.12 (d, J=4.3)	3.13 (d, J=4.2)	3.12 (d, J=4.2)
	3.17 (d, J=4.3)	3.19 (d, J=4.3)	3.17 (d, J=4.2)	3.23 (d, J=4.2)
H-14	1.15	1.16	1.12	1.15
H-15	3.75 (brd, J=11.8)	3.77 (dd, J=11.7, 3.1)	4.25 (d, J=12.0)	4.17 (d, J=12.3)
	3.90 (brd, J=11.8)	ca. 3.86 (m)	4.30 (d, J=12.0)	4.48 (d, J=12.3)
H-16	1.90 (dd, J=1.4, 0.7)	1.90 (dd, J=1.3, 0.8)	1.91	1.89
Ac		2.15	2.16	2.13
			1.90	2.05
7-OH	3.87 (d, J=1.8)	3.83 (d, J=1.9)	3.79 (d, J=1.9)	2.74 (brd, J=8.1)
8-OH				3.03 (d, J=9.8)

	CAL (**3**)	ITD (**2**)	EPT (**1**)
H-2	3.76 (d, J=4.6)	3.75 (d, J=4.6)	3.72 (d, J=4.6)
H-3	5.17 (ddd, J=10.0, 5.2, 4.8)	5.18 (ddd, J=10.0, 5.1, 5.0)	
H-4α			
H-4β	1.8–2.2 m	1.8–2.2 m	1.8–2.2 m
H-7			
H-8			
H-10	5.48 (dq, J=5.4, 1.3)	5.47 (dq, J=5.5, 1.4)	5.42 (dq, J=5.5, 1.5)
H-11	4.02 (d, J=5.6)	3.98 (d, J=5.7)	3.70 (d, J=5.5)
H-13	2.87 (d, J=4.0)	2.86 (d, J=4.0)	2.89 (d, J=4.3)
	3.11 (d, J=4.0)	3.10 (d, J=4.0)	3.16 (d, J=4.2)
H-14	0.84	0.82	0.81
H-15	3.84 (d, J=12.2)	0.76	0.76
	4.08 (d, J=12.2)		
H-16	1.72	1.72	1.71
Ac	2.12	2.14	
	2.05		
7 OH			
8-OH			

Chemical shifts are in ppm from Me_4Si; coupling constants are in Hz; spectra recorded in $CDCl_3$ solution at 250 MHz.

diAcDON) (**7**). This was isolated by TLC from the mother liquors remaining after crystallization of 3-AcDON, and readily identified by spectral data. The compound had M^+ at m/z 380, and its NMR spectrum showed only minor differences from that of 3-AcDON. The appearance of a second acetate methyl signal at δ 1.90, and a downfield shift for the H-15 methylene doublets from δ ca. 3.8 to δ ca. 4.3 were consistent with the introduction of a 15-acetyl group. In addition, the compound was in all respects identical to material isolated earlier during biotransformation studies with 3-AcDON [6], and to semisynthetic 3,15-diAcDON derived by acetylation of 3-AcDON.

The parent alcohol DON was isolated in yields of about 4 mg/l from more polar fractions. This was identified by comparison with authentic standards (including material derived by hydrolysis of 3-AcDON), MS data (M^+ at m/z 296) and its NMR spectrum [6]. The latter, compared with that for 3-AcDON, showed no acetyl signals, and the expected upfield shifts for H-2, H-3 and H-4. In our earlier communication [4], we had noted the presence of

DON in *F. culmorum* cultures, particularly after fermentation had progressed for longer periods of time (ca. 4–6 weeks). This may result from the presence of esterase enzymes in the culture acting on 3-AcDON, rather than implying that DON is the biosynthetic precursor of 3-AcDON. Biotransformation studies [6, 12–15] have demonstrated the frequent occurrence of such esterase reactions in *Fusarium* species.

Several metabolites were observed in less-polar fast-eluting fractions from the column. The amounts isolated were quite small, and fractions from several 2 l fermentations were combined to provide sufficient material for full identification. One of these was characterized as calonectrin (CAL) (**3**). Cultures of *F. culmorum* CMI 14764 had earlier been reported to produce calonectrin in quite high yields of about 50 mg/l [16]. Our own studies [4] have shown 3-AcDON as the major metabolite, and are confirmed by the independent studies of Greenhalgh and coworkers [7]. The presence of calonectrin as a minor metabolite has since been reported by the latter group [8]. The identity of our minor metabolite as calonectrin was deduced mainly from the NMR data. Comparison with the spectrum for 3-AcDON showed several significant changes. Thus, loss of the 8-carbonyl was reflected in the upfield shifts for H-10 (δ 6.61 → δ 5.48), H-11 (δ 4.70 → δ 4.02) and H-16 (δ 1.90 → δ 1.72) The change for H-14 from δ 1.16 to δ 0.84 is a consequence of the loss of the 7-hydroxyl, which also affects the separation of the characteristic doublets for H-13. The signals for protons H-8 and H-7 now overlap those for H-4, and give an unresolved multiplet in the δ 1.8–2.2 region. The spectrum for CAL is similar to that for 7α,8α-dihydroxycalonectrin (DHC) (**4**), except for the changes consequent on the loss of substitution at C-7 and C-8, and agrees with literature data [17]. The EIMS for CAL gave no molecular ion, but a major fragment at *m/z* 290 (M^+-acetic acid). The molecular mass was, however, confirmed by CIMS (MH^+ at *m/z* 351).

A further toxin isolated from the less-polar fraction had an NMR spectrum very similar to that of calonectrin. The only major differences were the loss of one acetyl signal, the loss of the H-15 methylene doublets at δ 3.84 and 4.08, together with the appearance of a new methyl singlet at δ 0.76. This indicated loss of the 15-acetoxy substituent; the remaining acetyl was at position 3 as shown by the signal for H-3 at δ 3.75. Mass spectral analysis gave M^+ at *m/z* 292, with a major fragment at *m/z* 249 (M^+-acetyl). Thus, structure (**2**), 3α-acetoxy-12,13-epoxytrichothec-9-ene, may be assigned to this compound, a structure also reported by Greenhalgh [8] as a minor constituent of *F. culmorum*, and given the trivial name isotrichodermin (ITD). Our spectral data are in complete agreement with those published [18].

The final compound to be characterized from this fraction was the least polar, and was identified as 12,13-epoxytrichothec-9-ene (EPT) (**1**). The NMR spectrum had signals for H-2, H-10, H-13, H-14, H-15 and H-16 almost identical with those present in the spectrum of ITD. Differences were the loss of the acetyl methyl from δ 2.14, loss of H-3 from δ 5.18, a more complex multiplet in the δ 1.8–2.2 region, and a small upfield shift from δ 3.98 to 3.70 for H-11. EI mass spectral data gave M^+ at *m/z* 234, with a major fragment at *m/z* 219 (M^+–Me). No M-18, M-30 or M-60 fragments were present, indicating a lack of oxygen substituents. All these data are consistent with the assignment of the EPT structure to this compound, and they correspond well with literature values quoted for this material [19]. The three compounds CAL, ITD and EPT were isolated in yields of approximately 0.25, 0.9 and 0.7 mg/l respectively.

The isolation of EPT is of some considerable significance. This rarely detected unsubstituted trichothecene was first isolated by Nozoe and Machida from cultures of *Trichothecium roseum* [19] and it has been suggested as a key link between trichodiene and the more complex trichothecene structures [20]. EPT has been demonstrated to be produced from labelled trichodiene in *T. roseum* [21], but as yet there is little direct evidence for its further metabolism. However, it is likely that EPT could be a common intermediate in the biosynthesis of all natural trichothecenes, which can be derived from it by a series of hydroxylation and other sequences. Recent evidence has demonstrated the origin of oxygen atoms in the epoxide and hydroxyl groups of T-2 toxin is from molecular oxygen [22]. In addition, oxygen substituents at C-3 and C-7 in 3-AcDON are introduced with retention of configuration [23]. These observations point towards oxygenations catalysed by hydroxylase enzymes, and that hydroxylation most probably occurs after the trichothecene skeleton has been assembled.

The array of trichothecene structures now identified in *F. culmorum* gives further support for this hypothesis. The 15 compounds isolated are listed in

Table II. Trichothecene mycotoxins reported in *Fusarium culmorum* CMI 14764.

Trichothecene		3α	7α	8α	15	Reference
(i)	*unsubstituted*					
	12,13-epoxytrichothec-9-ene (EPT)					*
(ii)	*3-mono-oxygenated*					
	3-deacetylisotrichodermin	OH				9
	isotrichodermin (ITD)	OAc				8, *
(iii)	*3,8-dioxygenated*					
	8-hydroxyisotrichodermin	OAc		OH		9
(iv)	*3,15-dioxygenated*					
	dideacetylcalonectrin	OH			OH	8
	3-deacetylcalonectrin	OH			OAc	8
	15-deacetylcalonectrin	OAc			OH	8, 16
	calonectrin (CAL)	OAc			OAc	4, 8, 16, *
(v)	*3,8,15-trioxygenated*					
	8-hydroxycalonectrin	OAc		OH	OAc	8
	8-ketocalonectrin	OAc		=O	OAc	8
	8-keto-15-deacetylcalonectrin	OAc		=O	OH	8
(vi)	*3,7,8,15-tetraoxygenated*					
	7,8-dihydroxycalonectrin (DHC)	OAc	OH	OH	OAc	4, 8, *
	deoxynivalenol (DON)	OH	OH	=O	OH	4, *
	3-acetyldeoxynivalenol (3-AcDON)	OAc	OH	=O	OH	4, 7, 8, *
	3,15-diacetyldeoxynivalenol (3,15-diAcDON)	OAc	OH	=O	OAc	*

* This work.

Table II, combining data from the present studies with those of Greenhalgh and coworkers. The structures can conveniently be grouped according to oxygenation pattern, with representatives of unsubstituted (1 compound), 3-mono-oxygenated (2 compounds), 3,8-dioxygenated (1 compound), 3,15-dioxygenated (4 compounds), 3,8,15-trioxygenated (3 compounds) and 3,7,8,15-tetraoxygenated (4 compounds) trichothecenes. If esterification/deesterification processes are regarded as secondary modifications of the structure, a biosynthetic sequence in which EPT is 3-oxygenated, then transformed further into 3,15-dioxygenated, 3,8,15-trioxygenated and 3,7,8,15-tetraoxygenated compounds may be postulated. The presence of 8-hydroxyisotrichodermin indicates an alternative sequence from 3-mono-oxygenated to 3,8,15-trioxygenated compounds, but in view of the greater number of examples of 3,15-dioxygenated structures isolated, it could represent a less important minor pathway. Analysis of *F. roseum* cultures [17] has shown the presence of 7-hydroxy and 8-hydroxy derivatives of both ITD and CAL, indicating that the oxidation reactions proceed simultaneously, but at different rates. The two species *F. culmorum* and *F. roseum* produce remarkably similar patterns of trichothecene metabolites [9], but the presence of 3,7-dioxygenated and 3,7,15-trioxygenated compounds has not yet been demonstrated in *F. culmorum*.

Experimental

Culture of fungus

Fusarium culmorum (CMI 14764) was maintained on Czapek Dox agar (Oxoid) slants at 25 °C in the dark. The production medium [24] consisted of 1 g $NH_4 \cdot H_2PO_4$, 3 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 g NaCl, 40 g sucrose, 10 g glycerol, and H_2O (1 l), and was autoclaved at 121 °C for 30 min. Ten 1 l Erlenmeyer flasks each containing 200 ml production medium were inoculated with the fungus, using a mycelial homogenate from an agar plate. The mycelium (5–7 day old) from one 8 cm diameter plate was homogenised in a blendor with sterile distilled H_2O (10 ml), and the contents were added to one 200 ml batch of production medium. The cultures were incubated in the dark at 25 °C on a rotary shaker (100 rpm) for 9 days. The cultures were fil-

tered through muslin and the filtrate extracted with ethyl acetate (5 × 50 ml). The combined extracts were dried over anhydrous $MgSO_4$ and evaporated to give a viscous brown oil.

Isolation of trichothecenes

The crude EtOAc extract above was fractionated on a silica gel column (Merck silica gel 60, 70–230 mesh; 40 cm × 3 cm) by eluting with diethyl ether-acetone, 9:1. Fractions (12 ml) were collected, analysed by TLC and combined as appropriate. TLC analysis used Merck silica gel 60 F_{254} plates, developed with diethyl ether-acetone, 9:1, visualised using spray reagents 20% H_2SO_4 [25] or 4-(*p*-nitrobenzyl)pyridine [26].

Fractions 13–17 contained small amounts of toxins and these fractions from 8 l culture media were combined before further analysis. Column chromatography (silica gel 60, 70–230 mesh, 25 cm × 2.5 cm column; ethyl acetate-hexane, 1:1; 2 ml fractions) gave *12,13-epoxytrichothec-9-ene* (EPT) (**1**) (5.5 mg) in fractions 25–31. ^{1}H NMR: see Table I. EIMS: 234 (M^+, 44%), 219 (61), 191 (5), 177 (10), 163 (11), 149 (11), 135 (14), 123 (22), 107 (55), 93 (71). Fractions 41–46 yielded *3α-acetoxy-12,13-epoxytrichothec-9-ene* (*isotrichodermin*, ITD) (**2**) (7.4 mg). ^{1}H NMR: see Table I. EIMS: 292 (M^+, 7%), 277 (11), 249 (4), 202 (7), 189 (4), 173 (9), 159 (5), 149 (9), 133 (7), 124 (33), 108 (34), 93 (38).

Fractions 18–19 gave a small amount of *calonectrin* (CAL) (**3**) which was purified by TLC (1 silica plate, 200 × 200 × 0.5 mm; diethyl ether-acetone, 9:1). Yield 2 mg. ^{1}H NMR: see Table I. EIMS: 290 (25%), 262 (20), 247 (3), 202 (5), 199 (5), 187 (5), 171 (6), 159 (8), 123 (19); CIMS: 351 (MH^+, 100%), 291 (48), 290 (41), 273 (13), 262 (34), 249 (16), 231 (34).

Fractions 20–22 contained the major amount of toxin. The combined fractions yielded an oil which was crystallized from acetone-diethyl ether, giving *3-acetyldeoxynivalenol* (3-AcDON) (**6**) (150 mg), m.p. 185 °C, lit. [7] 185 °C. ^{1}H NMR: see Table I. EIMS: 338 (M^+, 4%), 320 (1), 308 (1), 290 (16), 278 (1), 265 (1), 248 (5), 241 (4), 231 (9), 223 (15), 203 (12), 181 (26), 163 (29), 151 (18), 135 (24), 125 (16), 107 (21), 98 (30). The mother liquors from the crystallization were purified further by TLC (2 silica plates, 200 × 200 × 0.5 mm; diethyl ether-acetone, 9:1) to give, in addition to 3-AcDON, *3,15-diacetyldeoxynivalenol* (3,15-diAcDON) (**7**). Yield 3 mg. ^{1}H NMR: see Table I. EIMS: 380 (M^+, 4%), 320 (3), 291 (11), 249 (3), 231 (12), 223 (7), 203 (10), 181 (23), 163 (40), 151 (12), 135 (18), 107 (18), 98 (29). This compound was identical with material synthesised (pyridine-Ac_2O) from 3-AcDON, crystallized ether, m.p. 109 °C, lit. [17] 117–118.5 °C.

Fractions 25–27 were combined, evaporated and crystallized from acetone-diethyl ether to give *7α,8α-dihydroxycalonectrin* (DHC) (**4**). Yield 30 mg, m.p. 189 °C, lit. [18] 190–2 °C. ^{1}H NMR, see Table I. EIMS: 382 (M^+, < 1%), 322 (3), 304 (8), 291 (2), 276 (4), 262 (2), 223 (4), 187 (4), 163 (5), 159 (5), 149 (6), 135 (8), 121 (12), CIMS: 383 (MH^+, 15%), 365 (34), 323 (18), 305 (35), 287 (7), 275 (12), 263 (23), 245 (27), 233 (12), 215 (30), 189 (29).

Fractions 35–38 yielded a yellow oil containing small amounts of *deoxynivalenol* (DON) (**5**). This was purified further by TLC (2 silica plates, 200 × 200 × 0.5 mm; $CHCl_3$–MeOH, 85:15) yielding DON as a colourless oil (8 mg). ^{1}H NMR: see Table I. EIMS: 296 (M^+, 5%), 278 (8), 265 (8), 248 (73), 231 (16), 217 (8), 203 (24), 181 (30), 175 (39), 163 (40), 151 (42), 135 (56), 125 (47), 107 (57), 98 (100). This compound was identical with material obtained by hydrolysis (NaOH/MeOH) [6] of 3-AcDON.

Acknowledgements

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