Biosynthesis of Trichothecene Mycotoxins in *Fusarium culmorum* Cultures

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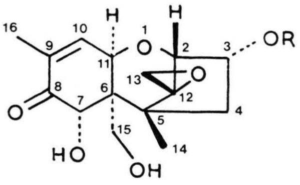
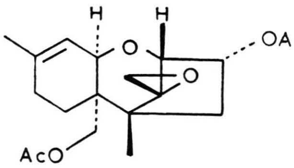
A high yielding production of the trichothecene mycotoxin 3-acetyldeoxynivalenol (3-AcDON) in cultures of Fusarium culmorum is described. By supplying [^{14}C]acetate, ^{14}C-labelled 3-AcDON suitable for further metabolic studies has been obtained. The pattern of labelling has been ascertained by using ^{13}C-labelled acetate precursors, and is in line with established biosynthetic data. A second trichothecene produced in significant amounts by F. culmorum has been identified as 3α, 15-diacetoxy-7α, 8α-dihydroxy-12, 13-epoxytrichothec-9-ene (7α, 8α-dihydroxycalonectrin).

Introduction

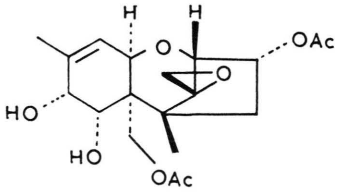
The trichothecenes are a group of sesquiterpene mycotoxins produced by several genera of the Fungi Imperfecti, eg. *Fusarium, Myrothecium,* and *Trichothecium* [1]. They are known to be responsible for the toxic properties of a variety of fungal-contaminated foodstuffs, especially cereals. One such derivative, found with increasing frequency in crops such as wheat and corn, is deoxynivalenol (DON, vomitoxin) (**1**). In order to assist routine toxin analyses, and to extend toxicological evaluation of DON, we have explored the production of this and related trichothecene derivatives in fungal cultures. We report here the high yield production of 3-acetyldeoxynivalenol (3-AcDON) (**2**) in cultures of *Fusarium culmorum,* and the use of these cultures to produce ^{14}C-labelled 3-AcDON (and hence DON) for toxicological and immunological studies.



1 R=H DON, vomitoxin
2 R=Ac 3-AcDON



3 Calonectrin



4 DHC

Results and Discussion

Cultures of *Fusarium culmorum* (*Calonectria nivalis;* CMI 14764) have been reported [2] to produce the trichothecene calonectrin (**3**) in yields of about 50 mg/l. When cultured in a chemically defined production medium, this organism yielded high levels (50 mg/l) of 3-AcDON instead of calonectrin, however. The material was readily isolated from the culture medium and purified by column chromatography giving a fraction from which 3-AcDON could easily be crystallised. The identity of the product was confirmed by MS (M^+338), ^{1}H NMR (Table I) and

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Table 1. ^{1}H Chemical shift assignments and coupling constants for 3-AcDON (**2**) and DHC (**4**).

	3-AcDON (**2**)		DHC (**4**)	
H-2	3.89	(d, J = 4.5)	3.80	(d, J = 4.4)
H-3	5.20	(ddd, J = 11.3, 4.4, 4.4)	5.19	(ddd, J = 11.1, 4.4, 4.4)
H-4α	2.37	(dd, J = 15.0, 4.4)	2.39	(dd, J = 15.1, 4.4)
H-4β	2.13	(dd, J = 15.0, 11.3)	2.16	(dd, J = 15.1, 11.1)
H-7	4.80	(d, J = 1.3)	4.02	(br dd, J ca. 7.3, 5.5)*
7-OH	3.82		2.74	(br d, J = 8.1)
H-8			4.54	(dd, J = 9.7, 5.4)**
8-OH			3.03	(d, J = 9.8)
H-10	6.58	(dd, J = 5.8, 1.5)	5.62	(dd, J = 5.9, 1.4)
H-11	4.67	(d, J = 5.8)	4.35	(d, J = 5.8)
H-13	3.09	(d, J = 4.3)	3.12	(d, J = 4.2)
	3.16	(d, J = 4.3)	3.23	(d, J = 4.2)
H-14	1.12		1.15	
H-15	3.73	(d, J = 11.7)	4.17	(d, J = 12.3)
	3.84	(d, J = 11.7)	4.48	(d, J = 12.3)
H-16	1.88		1.89	
H-18	2.12		2.13	
H-20			2.05	

Chemical shifts are in ppm from Me_4Si; coupling constants are in Hz; spectra recorded in $CDCl_3$ solution.
* d, J = 5.3 in $CDCl_3/D_2O$
** d, J = 5.4 in $CDCl_3/D_2O$

^{13}C NMR (Table II) data. NMR assignments were made using COSY and fully coupled 2 D techniques, and the data presented in Tables I and II complement those reported for 3-AcDON in a recent paper by Blackwell *et al.* [3]. However, the assignments for the ^{13}C chemical shifts of C-7 and C-11 have been reversed, agreeing with an earlier assignment [4] for these carbons in DON, and now confirmed beyond doubt by coupling data from the [$^{13}C_2$]acetate feeding experiment described below.

Table 2. ^{13}C Chemical shift assignments and coupling constants for 3-AcDON (**2**) and DHC (**4**) derived from sodium [$^{13}C_2$]acetate.

	3-AcDON	(**2**)	DHC (**4**)	
	δ	J	δ	J
C-2	79.0	37	78.6	37
C-3	71.3	36	71.2	36
C-4	40.4	(34)	41.4	(34)
C-5	45.8	(35)	46.2	
C-6	52.0	38	46.4	37
C-7	74.4	38	69.8	37
C-8	199.9	(50, 39)	70.6	
C-9	135.8	45	139.3	44
C-10	138.4	47	122.5	48
C-11	70.2	46	70.3	
C-12	65.2	32	64.7	33
C-13	47.4	33	47.9	33
C-14	14.1	(38)	15.0	(38)
C-15	62.1	(39)	63.3	(39)
C-16	15.2	45	20.4	43
C-17	170.4	59	170.4	56
C-18	20.9	59	20.9	59
C-19			170.0	59
C-20			21.1	59

Chemical shifts are in ppm from Me_4Si; coupling constants are in Hz; spectra recorded in $CDCl_3$ solution. Coupling constants in brackets were obtained from low intensity satellite signals.

Although fermentation conditions for the production of 3-AcDON were not systematically altered to achieve optimum yields, under the conditions used, maximum production occurred about 10 days after inoculation of the growth medium. In longer term cultures (ca. 4–6 weeks), the presence of DON was also observed, probably as the result of hydrolysis of 3-AcDON by esterases in the culture [5]. Small amounts of calonectrin (**3**) were also isolated from the cultures, but 3-AcDON is the major metabolite, a feature also noted by Greenhalgh and coworkers [6]. 3-AcDON has also been reported as a major metabolite from another strain of *F. culmorum* [7].

The biosynthesis of the trichothecene skeleton from acetate and mevalonate *via cis-trans* farnesyl pyrophosphate and subsequent rearrangement reactions (Fig. 1) has been well-documented for some years [8]. In principle, therefore, it is possible to produce labelled trichothecene mycotoxins *via* biosynthetic methods using inexpensive labelled acetate precursors, to give multiple-labelled products, though having a defined labelling pattern. In practice, this approach has not provided material of sufficient enrichment for further metabolic studies [9, 10]. Encouraged by the excellent yields of 3-AcDON produced by the *F. culmorum* cultures, we carried

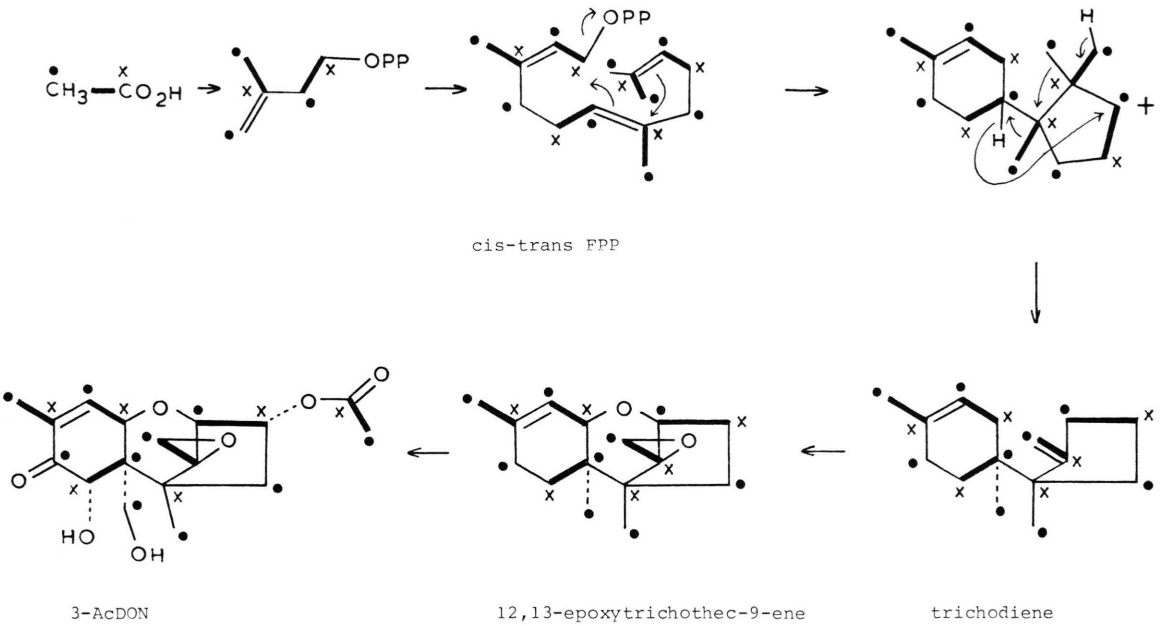

Fig. 1. Biosynthesis of 3-AcDON (**2**) from ^{13}C-labelled acetate.

out preliminary experiments in which sodium [1-^{14}C]acetate (50 μCi/l) was added to the growing culture, two days after inoculation. After 7 days metabolism, the culture was worked up to yield [^{14}C]-3-AcDON (80 mg/l; 1.08 μCi/mM). Base hydrolysis of the ester function gave DON, retaining 93% of the activity, indicating very substantial labelling in the trichothecene skeleton. This feeding experiment was then repeated using sodium [2-^{14}C]acetate (2 mCi/l), which after workup and purification yielded [^{14}C]-3-AcDON (70 mg/l; 97 μCi/mM), corresponding to an incorporation of 1.0%. This material is sufficiently active for toxicological studies and has been passed on to the British Industrial Biological Research Association, Carshalton, where *in vivo* metabolism studies are under way.

The labelling pattern produced in 3-AcDON from [2-^{14}C]acetate was checked by a similar feeding using sodium [2-^{13}C]acetate. The proton-noise decoupled ^{13}C NMR spectrum of the enriched sample of 3-AcDON differed from that of the natural abundance spectrum in that signals from C-2, C-4, C-6, C-8, C-10, C-13, C-14, C-15, C-16 were substantially enhanced (ca. 1.7–3.1% enrichment), in accord with the proposed pathway (Fig. 1). The acetyl methyl was not significantly enhanced. Additional evidence for the formation of the C-6:C-15 bond by a rearrangement involving migration of C-15 is provided by the observation of satellite signals (J = 38.8 Hz) flanking both of these resonances. That this spin-spin interaction is visible is a consequence of the high level of ^{13}C-enrichment resulting in multiple-labelled isoprene units.

3-Acetyldeoxynivalenol derived from sodium [$^{13}C_2$]acetate gave data consistent with those from similar feeding experiments leading to trichothecene mycotoxins [11]. Pairs of coupled carbon atoms were visible in the ^{13}C NMR spectrum, confirming the intact incorporation of acetate units for carbons 2 and 3, 6 and 7, 9 and 16, 10 and 11, 12 and 13 in the trichothecene skeleton, and at a much lower enrichment level in the acetyl side-chain C-17/18. The remaining signals, C-4, 5, 8, 14 and 15 were enriched in accord with Figure 1. Again, the high level of ^{13}C enrichment in 3-AcDON gave rise to multiple labelling and to additional couplings. Thus, virtually all the ^{13}C-^{13}C coupling constants (Fig. 2) may be assigned from this spectrum. Most significant of these are the observed couplings between C-3/4, C-7/8, C-5/14 and C-6/15 indicating multiple labelling between isoprene units, the latter two examples arising through methyl migrations.

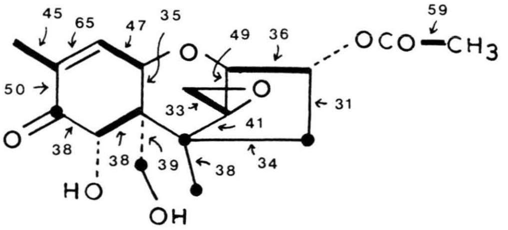

Fig. 2. Labelling pattern and ^{13}C-^{13}C coupling constants (Hz) in 3-AcDON (**2**) derived from sodium [$^{13}C_2$]acetate.

Samples of 3-AcDON obtained by a single crystallisation of bulked column fractions were usually contaminated with another metabolite, though this could be removed by further recrystallisation. The contaminating metabolite was obtained in pure form (ca. 15 mg/l) from the crude extract by chromatography and identified as the new trichothecene 3α,15-diacetoxy-7α,8α-dihydroxy-12,13-epoxytrichothec-9-ene (7α,8α-dihydroxycalonetrin, DHC) (**4**) very recently reported also as a metabolite of *F. roseum* [12]. This compound had M^+ 382 and its ^1H NMR spectrum (Table I) resembled that of 3-AcDON except for the new acetyl on the 15-hydroxyl, shifting H-15 downfield, and H-10 and H-11 were shifted upfield due to the loss of the carbonyl. Protons at 7 and 8 were coupled to their respective hydroxyls, which couplings were lost *via* the addition of D_2O, giving $J_{7,8}$ = 5.4 Hz. The configurations at C-7 and C-8 may be assigned from the deshielding of H-14 (7α-OH as in 3-AcDON), and the 7,8 coupling constant which is analogous to that of other 8α-substituted compounds, eg. T-2 toxin and HT-2 toxin [1, 3, 13]. Interpretation of the ^{13}C NMR spectrum (Table II) was complicated by groups of signals around 70 ppm and 21 ppm. Assignments given in Table II differ from those reported [12] only in these regions and are backed by the added information available from ^{13}C-enriched metabolites available from the feeding experiments. Thus, since the labelling pattern will be identical to that in 3-AcDON, the analysis of couplings and enrichments may be used in the assignment of signals with similar δ values. Assignments of C-8 (enriched by [2-^{13}C]acetate), C-3 and C-7 (coupled to C-2 and C-6 respectively after [$^{13}C_2$]acetate feeding) then become straightforward.

The biosynthetic relationship existing between the three compounds so far isolated from *F. culmorum* has yet to be established. It is likely that calonectrin could be a precursor of both DHC and 3-AcDON. If 3-AcDON is the end product of metabolism, then deacetylation at position 15 is a necessary transformation. From a range of metabolites isolated from *F. roseum* [12], Greenhalgh has suggested sequences in which either DHC or 7-deoxy-3-AcDON could act as a precursor of 3-AcDON. The precise sequence in which the trichothecene skeleton is further substituted requires further investigation.

Experimental

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions at 250 MHz and 62.89 MHz respectively using a Bruker WM 250 spectrometer. 2D spectra were recorded on a Bruker WM 300 spectrometer operating at 300 MHz (^1H) and 75.47 MHz (^{13}C).

Culture of fungus

Fusarium culmorum (CMI 14764) was maintained on Czapek Dox agar (Oxoid) slants at 25 °C in the dark. The production medium [14] consisted of 1 g $NH_4.H_2PO_4$, 3 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 5 g NaCl, 40 g sucrose, 10 g glycerol, and H_2O (1 l), and was autoclaved at 121 °C for 30 min. Five 1 l Erlenmeyer flasks each containing 200 ml production medium were inoculated with the fungus, then incubated in the dark at 25 °C on a rotary shaker (100 rpm). After 48 hr, sodium [2-^{14}C]acetate (58 mCi/mM; 2 mCi) in 10 ml sterile H_2O was divided equally among the five flasks. The fermentations were monitored daily for trichothecene production by TLC analysis (Merck Silica gel 60 F_{254} plates, developed with diethyl ether-acetone, 9:1, visualised using spray reagents 20% H_2SO_4 [15] or 4-(p-nitrobenzyl)pyridine [16]. After 9 days, the cultures were filtered through muslin and the filtrate extracted with ethyl acetate (6 × 50 ml). The combined extracts were dried over anhydrous $MgSO_4$ and evaporated to give a viscous brown oil (2.13 g).

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. Fractions 20–22 on evaporation yielded a viscous oil which was taken up in the minimum amount of acetone, and crystallised by the addition of diethyl ether. It was recrystallised from the same solvent mixture to give *3-acetyldeoxynivalenol* (**2**) (70 mg); mp. 185 °C, lit. [6] 185 °C; sp. act. 97 μCi/mM; M^+ 338.

Fractions 25–27 were treated in the same manner to yield *7α,8α-dihydroxycalonectrin* (**4**) (15 mg); mp 189 °C, lit. [12] 190–2 °C, M^+ 382.

Acknowledgement

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