Metabolic Conversions of Trichothecene Mycotoxins: De-esterification Reactions Using Cell-Free Extracts of *Fusarium*

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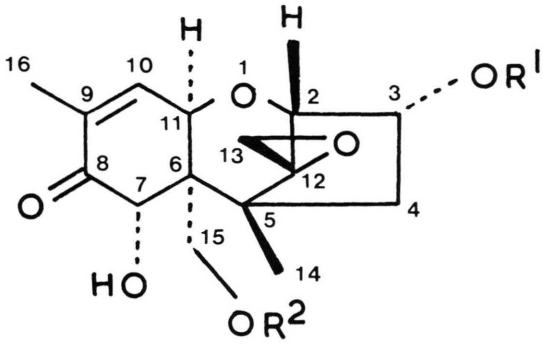
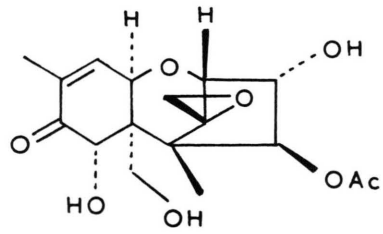
A crude cell-free extract from cultures of *Fusarium* sp. strain C37410-90 possessed significant esterase activity and hydrolyzed the trichothecene mycotoxin 3-acetyldeoxynivalenol (3-AcDON) to deoxynivalenol (DON) in high yield. Smaller amounts of 15-acetyl- and 3,15-diacetyl-esters of DON were also formed. The extract was capable of hydrolyzing a range of natural and semi-synthetic trichothecene esters, and showed a high degree of regioselectivity towards position 3. 3,4,15-Triacetylscirpentriol (TAS) was efficiently hydrolyzed to 4,15-diacetoxyscirpenol (DAS), and no further transformation was observed. The enzyme activity was partially purified.

Introduction

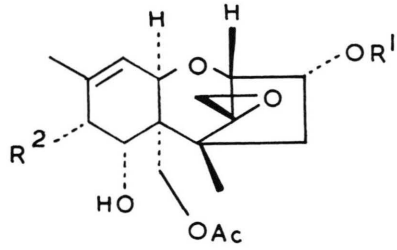
The trichothecenes are a group of sesquiterpene mycotoxins produced by several genera of the Fungi Imperfecti, and are known to be responsible for the toxicity associated with a variety of fungal-contaminated foodstuffs [1, 2]. To assist routine toxin analyses and extend toxicological evaluation studies, we have explored the production of trichothecene derivatives in fungal cultures [3, 4], and the possibility of using fungal enzyme systems to carry out specific biotransformations [5]. We recently reported [5] that the mycotoxin 3-acetyldeoxynivalenol (3-AcDON)



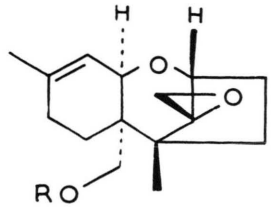
(**1**) $R^1 = R^2 = H$, DON
(**2**) $R^1 = Ac$, $R^2 = H$, 3-AcDON
(**3**) $R^1 = H$, $R^2 = Ac$, 15-AcDON
(**4**) $R^1 = R^2 = Ac$, 3,15-diAcDON
(**5**) $R^1 = Pr$, $R^2 = H$, 3-PrDON
(**6**) $R^1 = H$, $R^2 = Pr$, 15-PrDON
(**7**) $R^1 = R^2 = Pr$, 3,15-diPrDON



(**8**) fusarenon-X



(**9**) $R^1 = R^2 = H$, 3-deacetyl-7-hydroxycalonectrin
(**10**) $R^1 = Ac$, $R^2 = H$, 7-hydroxycalonectrin
(**11**) $R^1 = Ac$, $R^2 = OH$, DHC
(**12**) $R^1 = H$, $R^2 = OH$, 3-deacetylDHC



(**13**) 15-acetoxyEPT
(**14**) 15-hydroxyEPT

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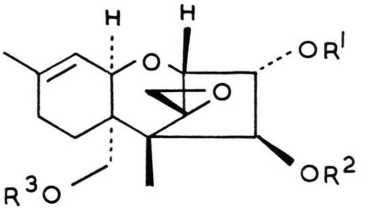

(**15**) $R^1 = R^2 = H$, $R^3 = Ac$, 15-MAS
(**16**) $R^1 = H$, $R^2 = R^3 = Ac$, DAS
(**17**) $R^1 = R^2 = R^3 = Ac$, TAS

(**2**) obtained from *Fusarium culmorum* was transformed by cultures of the 4,15-diacetoxyscirpenol (DAS) (**16**) producer *Fusarium* sp. strain C37410-90 into four compounds, deoxynivalenol (DON, vomitoxin) (**1**), 15-acetyldeoxynivalenol (15-AcDON) (**3**), 3,15-diacetyldeoxynivalenol (3,15-diAcDON) (**4**) and fusarenon-X (**8**). The main transformations were the result of specific esterification and de-esterification processes, but the production of fusarenon-X involved in addition a novel 4β-hydroxylation of the trichothecene ring system. In this paper, we report the isolation of a cell-free system from *Fusarium* sp. possessing a high degree of esterase activity and capable of hydrolyzing a range of natural and semisynthetic trichothecene ester derivatives. The extract showed a high degree of regioselectivity towards trichothecenes esterified at position 3, and the enzyme activity has been partially purified.

Results and Discussion

Preparation of cell-free extract

Cultures of *Fusarium* sp. (Bristol-Myers Company, strain C37410-90) [6] were grown in a two-stage fermentation process using conditions which produced a high yield (ca. 150–200 mg/l) of 4,15-diacetoxyscirpenol (DAS) in addition to smaller amounts of other trichothecene derivatives. The vast bulk of the trichothecenes are recovered from the culture medium and only minimal levels can be extracted from the mycelium. A cell-free extract from the mycelium was obtained by a freezing-grinding procedure, extracting into phosphate buffer (0.2 M, pH 7.2), then removing cellular debris by centrifugation at 5000 rpm. Enzymic activity was examined by adding a small amount of 3-acetyldeoxynivalenol (3-AcDON) dissolved in the minimum volume of DMSO to a portion of the extract. Trichothecenes present after incubation for 18 h at 4 °C were analyzed by TLC against standard materials, and significant deacetylation of 3-AcDON to deoxynivalenol (DON) was observed. In due course, after development of a quantitative HPLC assay for 3-AcDON, the pH optimum for the esterase activity was determined to be approx. 6.2, and the extracting buffer was modified accordingly. The ability of the cell-free extract to transform 3-AcDON was rapidly lost on storage at 4 °C, but enzyme activity could be maintained satisfactorily at this temperature for more than 11 days if phenylmethylsulphonyl fluoride (PMSF) and 2-mercaptoethanol were included in the preparation.

Biotransformation of trichothecene mycotoxins

In earlier studies using whole-cell cultures of *Fusarium* sp., 3-AcDON was observed to be biotransformed into DON, 15-acetyldeoxynivalenol (15-AcDON), 3,15-diacetyldeoxynivalenol (3,15-diAcDON) and fusarenon-X, in yields of 5%, 3.6%, 2.6% and 1.2% respectively [5]. DON and its acetate esters are produced as a result of de-esterification and/or esterification reactions, whilst fusarenon-X must have arisen *via* a 4β-hydroxylation reaction followed by 4-acetylation. To investigate the range of reactions catalyzed by the cell-free extract, a large-scale incubation was carried out using 3-AcDON (140 mg in 1 ml DMSO) and cell-free extract (19 ml) at 4 °C over 16 h. The mixture was worked up by extraction with ethyl acetate, then the component trichothecenes were separated by a combination of silica gel column and thin layer chromatography. They were characterized by ^1H NMR spectroscopy and analytical TLC against standard materials. Low levels of endogenous trichothecenes from the cell-free preparation were excluded by comparison with an ethyl acetate extract from the cell-free system.

The major transformation products were thus identified as DON (71% yield), 15-AcDON (5%) and 3,15-diAcDON (2%) (Table I), demonstrating a similarity with the whole-cell biotransformation products, though much higher overall yields and markedly different proportions were obtained. That these compounds were indeed biotransformation products from the added 3-AcDON was confirmed in a separate small-scale experiment using [^{14}C]-3-AcDON [3]. Autoradiography of the thin layer

Table I. Biotransformation products of trichothecenes using cell-free extract of *Fusarium* sp. C37410-90.

Substrate	Product	Yield [%]
3-AcDON (**2**)	DON (**1**)	71
	15-AcDON (**3**)	5
	3,15-diAcDON (**4**)	2
	3-deacetyl-7-hydroxycalonectrin (**9**)	trace
15-AcDON (**3**)	DON (**1**)	25
3,15-diAcDON (**4**)	15-AcDON (**3**)	20
	DON (**1**)	unrecorded
DON (**1**)	none	
3-PrDON (**5**)	DON (**1**)	22
	15-acylDON ?	trace
15-PrDON (**6**)	DON (**1**)	15
DAS (**16**)	none	
15-MAS (**15**)	none	
TAS (**17**)	DAS (**16**)	67
DHC (**11**)	3-deacetylDHC (**12**)	14
15-acetoxyEPT (**13**)	15-hydroxyEPT (**14**)	23

chromatogram showed almost all of the recovered radioactivity to reside in DON, with very low levels in spots corresponding to 15-AcDON and 3,15-diAcDON. A further trace product, detectable by autoradiography, ran just above DON on TLC, and was subsequently isolated in low yield from the large-scale non-radioactive experiment. It was tentatively identified from NMR data (Table II) as 3-deacetyl-7-hydroxycalonectrin (**9**). This material was absent from the control *Fusarium* sp. extract, and thus appears to be derived by biotransformation from 3-AcDON. In this case, therefore, 3-deacetylation and 15-acetylation are accompanied by reduction of the 8-keto group, a novel *Fusarium*-mediated biotransformation. The possible derivation of (**9**) by 3-deacetylation of 7-hydroxycalonectrin (**10**), a known *Fusarium* metabolite reported in cultures of *F. roseum* [7], must be considered, however, though contamination of the 3-AcDON substrate with 7-hydroxycalonectrin is unlikely from the analytical data. In neither experiment could the hydroxylation product fusarenon-X be detected, and the predominant enzyme activity observed was thus for de-esterification. No transformations of 3-AcDON were detected when the cell-free extract was heat denatured at 60 °C for 15 min.

A range of natural and semi-synthetic trichothecene derivatives was subsequently tested as substrates for the cell-free extract to identify a structure-activity profile for esterase/acyltransferase en-

Table II. ^{1}H NMR Chemical shift assignments and coupling constants for trichothecene substrates and products.

	3-PrDON (**5**)	15-PrDON (**6**)	3,15-diPrDON (**7**)	(**9**)
H-2	3.93 (d, J = 4.5)	3.67 (d, J = 4.5)	3.93 (d, J = 4.4)	3.55 (d, J = 4.5)
H-3	5.24 (ddd, J = 11.2, 4.5, 4.5)	4.54 m	5.20 (ddd, J = 10.9, 4.5, 4.5)	4.5 m
H-4α	2.38 (dd, J = 15.1, 4.4)	2.26 (dd, J = 14.8, 4.4)	2.2 m	2.0–2.2 m
H-4β	2.17 (dd, J = 15.1, 11.3)	2.13 (dd, J = 14.8, 10.6)	2.2 m	2.0–2.2 m
H-7	4.84 (d, J = 1.9)	4.86 (d, J = 2.0)	4.84 (d, J = 1.9)	4.6 m
H-8α				2.0–2.2 m
H-8β				2.43 (dd, J = 17.3, 5.9)
H-10	6.59 (dq, J = 5.9, 1.5)	6.63 (dq, J = 5.8, 1.4)	6.58 (dq, J = 5.9, 1.6)	5.42 (br d)
H-11	4.69 (d, J = 5.9)	4.92 (d, J = 5.8)	4.73 (d, J = 5.8)	4.26 (d, J = 5.1)
H-13	3.13 (d, J = 4.3)	3.11 (d, J = 4.2)	3.13 (d, J = 4.2)	3.10 (d, J = 4.3)
	3.19 (d, J = 4.3)	3.17 (d, J = 4.2)	3.18 (d, J = 4.2)	3.25 (d, J = 4.3)
H-14	1.16	1.10	1.12	1.14
H-15	3.78 (d, J = 11.7)	4.27	4.24 (d, J = 12.0)	4.06 (d, J = 12.3)
	3.88 (d, J = 12.0)		4.31 (d, J = 12.0)	4.33 (d, J = 11.8)
H-16	1.90 (dd, J = 1.3, 0.8)	1.90 (dd, J = 1.5, 0.8)	1.90	1.75
Ac				2.10
Pr	1.20 (t, J = 7.6)	1.07 (t, J = 8.6)	1.07 (t, J = 7.6)	
	2.42 (q, J = 7.6)	2.15 (q, J = 8.6)	1.20 (t, J = 7.5)	
			2.16 (q, J = 7.5)	
			2.44 (q, J = 7.6)	
3-OH				
4-OH				
7-OH	3.82 (d, J = ca. 2)	3.79 (d, J = 2.0)	3.78 (d, J = 1.8)	
8-OH				

Table II. Continued.

	(**12**)	15-acetoxyEPT (**13**)	15-hydroxyEPT (**14**)	15-MAS (**15**)
H-2	3.55 (d, J=4.5)	3.73 (d, J=4.7)	3.71 (d, J=5.0)	3.64 (d, J=4.8)
H-3	4.5 m			4.25 m
H-4α	2.30 (dd, J=14.9, 4.3)			4.25 m
H-4β	2.10 (dd, J=14.9, 10.8)	1.8–2.2 m	1.8–2.2 m	
H-7	4.04 (br dd, J=ca. 8, 6)			1.7–2.1 m
H-8α				
H-8β	4.56 (dd, J=9.9, 5.4)			
H-10	5.66 (dq, J=5.9, 1.4)	5.43 (br d, J=ca. 5.8)	5.43 (br d)	5.49 (br d, J=5.5)
H-11	4.49 (d, J=5.8)	3.73 (d, J=ca. 4.7)	3.67 (d, J=ca. 5)	3.95 (d, J=5.3)
H-13	3.11 (d, J=4.2)	2.89 (d, J=4.1)	2.90 (d, J=4.1)	2.77 (d, J=3.9)
	3.22 (d, J=4.2)	3.17 (d, J=4.1)	3.17 (d, J=4.1)	3.05 (d, J=3.9)
H-14	1.13	0.84	0.93	0.85
H-15	4.17 (d, J=12.4)	3.85 (d, J=12.1)	3.50 (br dd, J=ca. 12, 5)	3.90 (d, J=12.3)
	4.49 (d, J=12.4)	4.11 (d, J=12.1)	3.7 m	4.20 (d, J=12.3)
H-16	1.90	1.72	1.73	1.73
Ac	2.04	2.05		2.07
Pr				
3-OH				2.83
4-OH				3.33
7-OH	2.61 (br d, J=ca. 8)			
8-OH	3.02 (d, J=9.3)			

	DAS (**16**)	TAS (**17**)
H-2	3.71 (d, J=4.9)	3.87 (d, J=4.9)
H-3	4.17 (dd, J=5.0, 2.8)	5.20 (dd, J=4.9, 3.4)
H-4α	5.14 (d, J=3.0)	5.75 (d, J=3.3)
H-4β		
H-7	1.7–2.1 m	1.8–2.1 m
H-8α		
H-8β		
H-10	5.54 (br d, J=5.4)	5.49 (br d, J=5.5)
H-11	4.11 (br d, J=5.3)	3.99 (br d, J=5.8)
H-13	2.79 (d, J=4.0)	2.81 (d, J=4.0)
	3.08 (d, J=4.0)	3.09 (d, J=4.0)
H-14	0.83	0.77
H-15	3.97 (d, J=12.3)	4.06 (d, J=12.3)
	4.18 (d, J=12.3)	4.27 (d, J=12.3)
H-16	1.73	1.73
Ac	2.06	2.07
	2.15	2.12
		2.16
Pr		
3-OH	3.27 (d, J=2.9)	
4-OH		
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. NMR spectral data for DON (**1**), 3-AcDON (**2**), 15-AcDON (**3**), 3,15-diAcDON (**4**) and DHC (**11**) are given in ref. [4] and [5].

zymes present. The mycotoxins (5–10 mg) were dissolved in minimum amounts of acetone and incubated with the cell-free extract at 4 °C for 18 h. Transformation products were isolated and purified by TLC, and yields determined by weighing. The results of these experiments are also summarized in Table I, and NMR data confirming the nature of the resultant products are given in Table II.

The only detectable product from 15-AcDON was DON, demonstrating effective deacetylation of this ester, and thus the ability of the cell-free extract to deacetylate at position 15 as well as position 3. When the diester 3,15-diAcDON was employed, the major product was 15-AcDON and smaller amounts of DON were present. No 3-AcDON was detected. This indicates that deacetylation at position 3 predominates over 15-deacetylation.

The enzyme or enzymes responsible were also able to hydrolyze propionyl esters of DON, since the semi-synthetic 3-propionyldeoxynivalenol (3-PrDON) (**5**) and 15-propionyldeoxynivalenol (15-PrDON) (**6**) were both transformed to DON, though in lower yields than found for the corresponding acetyl esters. TLC analysis showed a very small proportion of 3-PrDON had probably been reacylated. The compound was chromatographically analogous to 3,15-dipropionyldeoxynivalenol (3,15-diPrDON) (**7**), but the amount isolated was insufficient for further structural analysis, and whether a propionyl or acetyl ester was produced is unknown. Some acetylation of 3-AcDON to 3,15-diAcDON had been observed with both the whole-cell and cell-free systems. In contrast, incubation of DON with the cell-free extract yielded no esterified transformation products, and indeed, no transformations at all were observed.

Semi-synthetic 15-acetoxy-12,13-epoxytrichothec-9-ene (15-acetoxyEPT) (**13**) was de-esterified to 15-hydroxy-12,13-epoxytrichothec-9-ene (15-hydroxyEPT) (**14**), demonstrating the ability of the extract to de-esterify a trichothecene system which lacks the 8-keto function, and which also lacks the oxygenation at positions 3 and/or 4 typically present in most natural derivatives. The natural *F. culmorum* metabolite 7,8-dihydroxycalonectrin (DHC) (**11**) differs from 3,15-diAcDON in having an 8-hydroxyl instead of the 8-keto function. This compound was deacetylated at position 3 giving 3-deacetyl-7,8-dihydroxycalonectrin (**12**), but no 15-deacetylation was detected.

Fusarium sp. C37410-90 naturally produces a range of trichothecene esters based on scirpentriol, with 4,15-diacetoxyscirpenol (DAS) (**16**) forming the major constituent. To investigate biotransformations in the scirpentriol group of toxins, which lack oxygenation at positions 7 and 8, it was necessary to ensure removal of trace levels of such compounds from the cell-free extract. A partially-purified preparation was obtained by removing particulate matter by ultracentrifugation followed by ammonium sulphate precipitation and micropore filtering (see Experimental). When the three scirpentriol esters DAS, 15-acetylscirpentriol (15-monoacetylscirpentriol, 15-MAS) (**15**), and 3,4,15-triacetylscirpentriol (TAS) (**17**) were incubated with this preparation, 15-MAS and DAS were not transformed, whereas a very high proportion of TAS was deacetylated to DAS. Thus, only position 3 on the scirpentriol skeleton appeared susceptible to hydrolysis by the enzyme preparation, and position 15 was unaffected. If separate enzymes are responsible for site-specific de-esterifications, the partial purification procedure may have eliminated the enzyme which deacetylates position 15. It is unlikely however that the procedure used would separate two apparently closely-related enzymes, but incubations with the crude cell-free extract were also carried out for comparison. With the crude cell-free extract, traces of scirpentriol esters are already present, and consequently only the major transformations can confidently be assessed without using radiolabelled materials. However, the results reinforced those observed with the partially purified system: only TAS was deacetylated giving DAS. However, minor transformations involving deacetylation from positions 4/15 would probably not be detected in this system and cannot be excluded. The 15-deacetylation of **13** demonstrates the ability of the enzyme to deacetylate at position 15 when the cyclohexene ring is not oxygenated.

These experiments demonstrate the presence of at least one enzyme in the cell-free extract from *Fusarium* sp. C37410-90 which will de-esterify trichothecene mycotoxins. However, it is more likely that two such enzymes are present. One enzyme shows specificity for position 3 and is apparently unaffected by the oxygenation/esterification pattern of the remainder of the trichothecene skeleton. The second enzyme de-esterifies at position 15. This enzyme seems more specific for its substrate and attacks preferentially substrates with the DON oxygen-

ation pattern. One of these enzymes, or perhaps a third enzyme, acetylates at position 15, so that 3-AcDON substrate is eventually transformed to 15-AcDON and 3,15-diAcDON. Acetylation does not require the addition of acetyl-CoA to the extract, and removal of endogenous acetyl-CoA *via* dialysis did not result in loss of acetylating activity. It is possible that acetate is obtained by the de-esterification of the substrate provided, *i.e.* 3-AcDON, and this may explain the lack of acetylation when DON was used as substrate.

Yoshizawa and Luangpitsuksa recently reported [8] that two different types of *Fusarium graminearum* strains could be identified, based on their abilities to de-esterify 3-AcDON and 15-AcDON. One type, which normally biosynthesized 3-AcDON, would deacetylate 3,15-diAcDON and 15-AcDON at position 15, whereas the second type, 15-AcDON producers, de-esterified 3,15-diAcDON and 3-AcDON at position 3. These activities were also demonstrated in cell-free extracts. The authors suggested that DON-producing strains of *F. graminearum* differed according to the presence of deacetylating enzymes which were considered to be position specific. In the light of these data, it is highly likely that *Fusarium* sp. contains two regiospecific de-esterifying enzymes. The present results demonstrate that with the cell-free extract de-esterification at position 3 predominates over de-esterification at position 15, perhaps because of higher levels of the first enzyme, or because it has higher specific activity.

Enzyme assay and partial purification of enzyme

To follow the enzymic 3-deacetylation of 3-AcDON to DON, 3-AcDON in DMSO (to give a final concentration of 0.5 mg/ml) was incubated with enzyme extract at 22 °C, and samples were removed at regular intervals. To assess the amount of 3-AcDON remaining at any one time, an HPLC assay was devised. This involved ethyl acetate extraction of the trichothecenes from the aqueous incubation, followed by chromatography on a reversed-phase Spherisorb ODS2 column using acetonitrile-H_2O, 4:6 containing HCl (0.1 ml/l) and UV detection (214 nm). The addition of acid to the mobile phase considerably sharpened the peaks produced, at approximately 2 min (DON) and 4 min (3-AcDON), using a flow rate of 1.1 ml/min. The 3-AcDON concentration was assessed from the peak area (*via* integrator) and a calibration curve. The efficiency of EtOAc extraction from the incubation mixture had been found to be satisfactory (ca. 90%) and reproducible provided levels of 3-AcDON used did not exceed 0.5 mg/ml. A standard curve using concentrations within the range 0.03–0.5 mg/ml was thus employed for these assay studies. From the sequential analyses, the concentration of 3-AcDON remaining after 240 min was determined, and the enzyme activity (see Table III for definition of unit of enzyme activity) calculated. Protein concentrations were established by the micro-Lowry method.

Partial purification of the enzyme was achieved by ultracentrifugation at 100000 g, ammonium sulphate precipitation to 60–80% saturation, micropore filtration (MW cutoff 100000) and ion-exchange chromatography (Trisacryl M). Yields of protein and enzyme purification factors are shown in Table III. Overall, this purification procedure led to an improvement of only 22-fold, and thus considerable further effort is required to purify the enzyme to homogeneity.

The availability of an enzyme catalyzing the hydrolysis of ester groups at position 3 of a broad range of trichothecene mycotoxins has potential application for specific modification of trichothecene esters. Chemical hydrolysis of trichothecene acetates using basic reagents is typically nonspecific [9], although with mild bases, some selective deacetylation may be achieved, mainly a consequence of neighbouring group participation [10]. Thus, in nivalenol and deoxynivalenol derivatives, neighbouring group participation by the 7α-hydroxyl group enhances the rate of hydrolysis of 15-acetate substituents, and hydrolysis of 4β-esters is facilitated in scirpentriol derivatives by the presence of neighbouring 3α-hydroxyl groups [9]. By using this esterase activity, selective hydrolysis at position 3 is now possible. Although a purified enzyme would be preferred for such transformations, the use of even the crude cell-free extract may achieve quite high overall yields of 3-deacetylated trichothecene derivatives from a range of substrates. Selective 3-deacetylation of trichothecene esters has also been observed using several other whole-cell cultures, *e.g. Fusarium graminearum* [8, 11], *F. nivale* [11, 12], *F. roseum* [13], *F. solani* [12, 13] and *Acinetobacter calcoaceticus* [14], thus indicating that similar enzyme preparations could be obtained from other microbial sources.

Table III. Partial purification of esterase from *Fusarium* sp. C37410-90.

Step	Protein Volume of Fraction [ml]	Concn. [mg/ml]	Total Amount [mg]	Enzyme Concn. [units/ml]	Spec. Act. [units/mg protein]	Total Amount [units]	Yield [%]	Purificn. Factor
Crude cell-free extract	380	3.99	1516	5.6	1.4	2072	100	1
Supernatant from 100000 × *g* centrifugation	355	3.48	1235	3.9	1.1	1385	67	0.8
Ammonium sulphate precipitation	20	2.48	49.6	23.5	9.5	470	23	6.8
Micropore filtration	8	1.60	12.8	22.5	14.1	180	9	10.1
Ion exchange + micropore filtration	2	0.64	1.3	19.5	30.5	39	2	21.8

1 unit of enzyme activity is defined as that amount which catalyses de-esterification of 0.1 mg 3-acetyldeoxynivalenol (3-AcDON) in 2 h.

Experimental

Culture of fungus

Fusarium sp. strain C37410-90 (Bristol-Myers Company) was maintained on Sabouraud agar (Oxoid) slants at 4 °C in the dark. A six day old culture was macerated with sterile distilled H_2O (10 ml) in a homogenizer, and the homogenate used to inoculate a seed medium of composition: glucose, 20 g; malt extract, 2 g; yeast extract, 2 g; peptone, 2 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 2 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; NH_4Cl, 3 g; H_2O, 1 l (200 ml per 1 l Erlenmeyer flask, 5% inoculum). The seed medium was incubated in the dark on a rotary shaker (200 rpm) at 26 °C for 48 h, and was then used to inoculate a production medium of composition: $NH_4H_2PO_4$, 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, 1 l (400 ml per 2 l Erlenmeyer flask, 10% inoculum). The fermentation was continued at 22 °C, 100 rpm for a further 48 h, then the mycelia were harvested by filtering through two layers of muslin, and washing with water. Yields were typically 40 g wet weight (2.5 g dry weight) per litre of production medium.

Preparation of cell-free extract

Mycelia (160 g wet weight) were suspended in distilled water (80 ml), frozen with liquid nitrogen, then allowed to partially thaw for 10 min at room temperature. Whilst semi-firm, the sample was thoroughly ground in a mortar, then PPM buffer (0.2 M sodium phosphate buffer pH 6.2, 4 mM PMSF, 5 mM 2-mercaptoethanol; 60 ml) was added, and the resultant mixture was centrifuged at 5000 rpm for 5 min. The pellet was re-extracted with two further 60 ml portions of buffer, and the supernatants were combined. The final volume of cell-free extract was typically about 400 ml, and the pH was adjusted to 6.2 with dilute NaOH as necessary.

Cell-free transformation of 3-AcDON

Cell-free extract from *Fusarium* sp. (19 ml) was added to a solution of 3-AcDON (140 mg) in DMSO (1 ml). The solution was incubated with stirring at 4 °C for 16 h, then extracted with ethyl acetate (6 × 20 ml). The combined extracts were dried over $MgSO_4$, and evaporated. A control experiment using DMSO (0.25 ml) and cell-free extract (5 ml) was incubated and extracted similarly, and used to indicate compounds endogenous to the extract.

The transformation extract was dissolved in acetone (2 ml) and applied to a column (50 × 3 cm) of silica gel (Merck Kiesel gel 60, 70–230 mesh), eluting with ether-acetone, 9:1, and collecting 5 ml fractions. Fractions were monitored for trichothecene derivatives by TLC (Merck Kiesel gel

GF254 plates, 0.2 mm thick), developed with ether-acetone, 9:1, hexane-acetone, 2:1, or chloroform-methanol, 9:1. Spray reagents 4-(*p*-nitrobenzyl)pyridine [15], 20% H_2SO_4 [16], or 10% $AlCl_3$ [17] were used to visualize the trichothecenes. Appropriate fractions were combined, and individual trichothecenes were isolated and purified by preparative TLC using the above solvent systems. Products were dried under vacuum, weighed, then fully characterized by NMR. Yields were DON (99 mg), 15-AcDON (7 mg), 3,15-diAcDON (3 mg), 3-deacetyl-7-hydroxycalonectrin (< 1 mg).

A small-scale experiment was performed using [^{14}C]-3-AcDON (3.59 MBq/mM, 1.2 mg) [3] and unlabelled 3-AcDON (3.8 mg) in DMSO (0.5 ml) and cell-free extract (5 ml). The resultant products were analyzed by TLC (ether-acetone, 9:1) with authentic standards of 3-AcDON, 15-AcDON, 3,15-diAcDON and DON, and this plate was then used for autoradiography.

Cell-free transformations of trichothecenes

Cell-free extract (5–10 ml) was added to a solution of the trichothecene mycotoxin (5–10 mg) in acetone (0.3–0.5 ml), and the mixture was incubated with stirring at 4 °C for 18 h. The mixture was extracted with ethyl acetate (3 × 10 ml), the combined extracts evaporated and separated by TLC using solvent systems ether-acetone, 9:1, hexane-acetone, 2:1, chloroform-methanol, 9:1, or chloroform-methanol, 4:1 as appropriate. Products were dried under vacuum, weighed, then fully characterized by NMR. In each case, control incubations of toxin + acetone + buffer, and acetone + cell-free extract were carried out simultaneously.

The mycotoxins 15-MAS, DAS, and TAS were incubated with a partially purified esterase preparation. This was obtained (see below) by centrifugation of the cell-free extract at 100000 × *g*, then precipitation by the addition of ammonium sulphate. The enzyme was redissolved in PPM buffer, then subjected to micropore filtration. The toxin (10 mg) in acetone (0.3 ml) was diluted with PPM buffer (2.6 ml), enzyme (0.4 ml) was added, and the mixture was incubated as above.

HPLC assay for 3-AcDON

A solution of 3-AcDON (2 mg/ml) in DMSO was serially diluted with PPM buffer to produce concentrations of 0.5, 0.25, 0.125, 0.063, and 0.031 mg/ml. Duplicate portions (0.5 ml) of each sample were extracted into ethyl acetate (1 ml) in a stoppered tube by vigorous shaking for 1 min. The resulting emulsions were broken by centrifugation at 3000 rpm for 5 min. The sample tubes were cooled in ice, unstoppered, and 0.8 ml samples of the organic phases were carefully removed. Solvent was removed under a gentle stream of nitrogen, and the residues were dissolved in 0.2 ml portions of the HPLC mobile phase (acetonitrile-water, 40:60, containing 0.1 ml/l 12 M HCl). The solution was analyzed by HPLC using a Spherisorb ODS2 column (250 × 4.6 mm) with a flow rate of 1.1 ml/min, a UV detector (214 nm) and integrator. A standard curve was obtained by plotting the concentration of 3-AcDON against peak area.

Assay of esterase activity

A mixture of 3-AcDON solution (0.75 mg/ml in DMSO; 2 ml), heat denatured (60 °C for 15 min) cell-free extract (0 or 0.8 ml) and enzyme preparation (1 or 0.2 ml depending on activity; to give a final volume of 3 ml, containing 0.5 mg/ml 3-AcDON) was incubated at 22 °C with stirring. Samples (0.5 ml) were removed after periods of 1, 3, 6, and 10 h, extracted with ethyl acetate (1 ml), and assayed for 3-AcDON content by HPLC as described above. From these data, the amount of 3-AcDON remaining after 2 h was deduced. A unit of enzyme activity was defined as that amount which catalyzed removal of 0.1 mg of 3-AcDON in 2 h.

Partial purification of protein

Protein concentrations were estimated by a modified micro-Lowry procedure [18]. All purification steps were conducted at 4 °C.

Crude cell-free extract (370 ml) was centrifuged (40000 rpm, 100000 × *g*) for 60 min. The resulting mixture comprised a thin surface layer and a particulate pellet, neither of which possessed any significant esterase activity, together with the supernatant solution. To the supernatant, solid ammonium sulphate (138 g, giving 60% saturation) was slowly added over 1.5 h. The pH was adjusted to 6.2 with dilute NaOH, and stirring continued for a further 1 h. After standing for 24 h, the mixture was centrifuged at 18000 rpm for 20 min, and the precipitate discarded.

Further ammonium sulphate (51 g, giving 80% saturation) was added to the supernatant over 1 h, the pH was again adjusted to 6.2, and stirring continued for 1 h. After standing for 24 h, the mixture was centrifuged at 18000 rpm for 20 min, and the supernatant decanted off and discarded. The pellet was dissolved in PPM buffer (50 ml), and the solution filtered through a micropore membrane (Amicon Diaflo XM100A Ultracentrifugation Membrane) under nitrogen gas until 5 ml remained unfiltered. This was diluted with distilled water (45 ml) and the filtration continued until approx. 1 ml of solution remained. This was diluted with distilled water (7 ml).

The enzyme solution was diluted further with distilled water to 50 ml, and applied to a column (11 × 2.5 cm) of CM-Trisacryl M cation exchange resin pre-equilibrated with Tris buffer (0.05 M, pH 7, 200 ml). The column was then eluted with water, monitoring the eluate at 280 nm, and collecting 10 ml fractions. Fractions were assayed for esterase activity by incubating portions (0.5 ml) with 3-AcDON (10 mg/ml in DMSO, 0.05 ml) at 4 °C with stirring for 18 h. These mixtures were extracted with ethyl acetate (1 ml) and the extent of de-esterification assayed by TLC (ether-acetone, 9:1). Esterase activity was found to be concentrated in fractions 6, 7 and 8. These fractions were combined and their volume reduced to 2 ml by further micropore filtration.

Trichothecene substrates

Trichothecene mycotoxins were obtained from cultures of *Fusarium culmorum* (CMI 14764) and *Fusarium* sp. (Bristol-Myers strain C37410-90) as described previously [3–5]. 15-AcetoxyEPT was synthesized from DAS *via* a deoxygenation sequence [19].

3-Propionyldeoxynivalenol (3-PrDON) and 15-propionyldeoxynivalenol (15-PrDON) were synthesized by dissolving DON (112 mg) in dry pyridine (14 ml), and heating under reflux at 120 °C with propionic anhydride (0.15 ml) for 80 min. The reaction mixture was cooled in ice, neutralized with 6 M HCl, then extracted with ethyl acetate (3 × 30 ml). The combined extracts were evaporated and the products isolated by TLC using hexane-acetone, 2:1. The esters 3-PrDON (49 mg, 37%) and 15-PrDON (30 mg, 22%), together with some 3,15-diPrDON, were eluted and dried under vacuum. NMR data are presented in Table II.

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