Metabolic Conversions of Trichothecene Mycotoxins: Biotransformation of 3-Acetyldeoxynivalenol into Fusarenon-X

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The trichothecene mycotoxin 3-acetyldeoxynivalenol was transformed by cultures of the diacetoxyscirpenol producer Fusarium sp. strain C37410-90 into four compounds, identified as deoxynivalenol, 15-acetyldeoxynivalenol, 3,15-diacetyldeoxynivalenol and fusarenon-X. The major transformations are the result of specific esterification and de-esterification processes, but the production of fusarenon-X involves in addition a novel 4ß-hydroxylation of the trichothecene ring system itself.

Introduction
The trichothecenes are a group of mycotoxins displaying a wide range of biological activities, including antibacterial, antiviral, antifungal and cytostatic activity, but they are perhaps most significant because of their toxicity and their frequent occurrence in fungal contaminated foodstuffs [1]. Several genera of the Fungi Imperfecti, e.g. Fusarium, Myrothecium, and Trichothecium are known to be producers of this group of sesquiterpene mycotoxins. The known natural trichothecenes [1] show considerable variations in structure, but are based on a parent 12,13-epoxytrichothec-9-ene (1) skeleton, and can conveniently be subdivided into two broad groups. These are the alcohols and simple esters, e.g. deoxynivalenol (DON, vomitoxin) (6) and the more complex macrocyclic esters such as the verrucarins and roridins. Most of the structures encountered in nature have a polyhydroxylated skeleton with some ester functions, usually acetate, though other esterifying acids may occur, particularly in the case of the macrocyclic esters. Oxygen functions 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. As in DON (6), an 8-oxygen function is frequently present as a carbonyl group.

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2: R = Ac, DAS
3: R = H, scirpentriol

The biosynthetic pathways to the trichothecenes have been studied in some detail over the years [2], and the origins of the basic skeleton from acetate and mevalonate via farnesyl pyrophosphate and subse-
quent rearrangement reactions are well-documented. From studies in *Trichothecium roseum* [3, 4], the parent structure (1) is suggested to arise via cyclization of the demonstrated intermediate trichodiene [5], and the toxin metabolites of this fungus are then produced by specific oxygenation and esterification processes. Although evidence for other organisms is generally lacking, it is assumed that the wide range of trichothecene structures known similarly arise from 12,13-epoxytrichothec-9-ene, by reactions that gradually build up the oxygenation pattern and ester functionality. Such transformations also tend to increase the toxicity of the trichothecene.

In view of the variety of natural trichothecenes known, and the frequently complex mixture of structures produced by a single organism, it is probable that hydroxylase and esterase enzymes employed by these fungi have relatively low specificities with respect to substrate. If so, it may be possible to exploit such enzymes to carry out specific biotransformations on a variety of different trichothecene substrates, thus extending access to mycotoxin materials and allowing more detailed toxicological evaluations.

We report here the feasibility of this approach by demonstrating the microbiological transformation of 3-acetyldeoxynivalenol (3-AcDON) (4) into other DON esters and also into the 4-oxygenated derivative fusarenon-X (9).

**Results and Discussion**

In an earlier communication [6], we described a high yielding method for the production of 3-AcDON using cultures of *Fusarium culmorum*. This method was easily modified to allow the isolation of 14C-labelled 3-AcDON suitable for further metabolic studies [6]. 3-AcDON contains the basic 12,13-epoxytrichothec-9-ene skeleton with the additional functionalities of an 8-keto, hydroxyls at C-7α and C-15, and an acetoxy C-3α, i.e. a 3α, 7α, 8, 15 oxygenation pattern. We also had available a culture of an unidentified *Fusarium* sp. (Bristol-Myers Company, strain C37410-90 [7]) which produces high levels (ca. 150—200 mg/l) of 4,15-diacetoxyscirpenol (DAS) (2) in liquid cultures. This molecule differs from 3-AcDON by possessing a 3α, 4β, 15 oxygenation pattern. By supplying *Fusarium* sp. with 3-AcDON as substrate, we anticipated observing the removal or addition of acetyl ester functions as demonstrated in other trichothecene metabolism studies [7–10], but we particularly wished to observe some 4-oxygenation process if the 4-hydroxylating enzyme showed broader substrate specificity, and would accept a substrate with the 7α-hydroxy-8-keto functionality.

An actively-growing culture of *Fusarium* sp. was filtered from its growth medium, resuspended in distilled water and incubated in shake culture in the presence of 14C-labelled 3-AcDON (5 mg/50 ml culture). The culture was monitored daily for toxin content by TLC. After five days, five distinct toxin bands were observed, autoradiography showing that all five were radioactive. The culture was then worked up and the metabolites isolated from the filtrate, separated and purified by TLC. Approximately half of each isolated toxin was hydrolyzed using NaOH/MeOH at room temperature, and the trichothecene hydrolysis products were compared chromatographically with authentic standards, to confirm the nature of the alcohol and establish that they too were radioactive and thus derived from 3-AcDON*.

The identity of the products was established by repeating the metabolism on a larger scale using the same methods with five 400 ml cultures each supplied with 40 mg unlabelled 3-AcDON, and analysing the purified components by MS and 250 MHz 1H NMR. NMR data for the products are given in Table I.

**Band 1.** This band, at lowest *R* <sub>t</sub>, cochromatographed with deoxynivalenol (DON), and was unchanged on base treatment. It had MS (M<sup>+</sup> at *m/z* 296) and NMR data identical to those of authentic DON. This product is therefore the result of enzymic hydrolysis of 3-AcDON.

**Band 3.** This zone on hydrolysis gave two spots corresponding to DON and scirpenetriol (3), and was therefore obviously a mixture. NMR analysis of the mixture indicated the presence of diacetoxyscirpenol (DAS), the major normal metabolite of *Fusarium* sp. The other principal component of the mixture was a monoacetoate derivative of DON. Both DAS (M<sup>+</sup> at *m/z* 366) and a monoacetyl DON (M<sup>+</sup> at *m/z* 338) were visible in the mass spectrum. Further TLC of this band separated DAS and this monoacetyl DON, and two very minor, as yet unidentified, components were also noted. The NMR spectrum confirmed the nature of the DON ester as 15-

* The 14C-labelled 3-AcDON contained approximately 7% of its activity in the acetyl group [6]. Transesterification processes might thus label at a low level a *Fusarium* sp. metabolite in its ester group.
Table I. ¹H NMR Chemical shift assignments and coupling constants for 3-AcDON and metabolites.

<table>
<thead>
<tr>
<th></th>
<th>3-AcDON (4)</th>
<th>15-AcDON (7)</th>
<th>3,15-diAcDON (5)</th>
<th>Fusarenon-X (9)</th>
<th>DON (6)</th>
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<tr>
<td>H-2</td>
<td>3.92 (d, J = 4.4)</td>
<td>3.67 (d, J = 4.4)</td>
<td>3.93 (d, J = 4.4)</td>
<td>3.83 (d, J = 4.7)</td>
<td>3.65 (d, J = 4.5)</td>
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<td>H-3</td>
<td>5.23 (dd, J = 11.2)</td>
<td>4.56 (dd, J = 10.6)</td>
<td>5.24 (dd, J = 10.9)</td>
<td>4.35 (dd, J = 4.7)</td>
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<td>H-4α</td>
<td>2.38 (dd, J = 15.0)</td>
<td>2.25 (dd, J = 14.9)</td>
<td>2.33 (dd, J = 15.1)</td>
<td>5.55 (d, J = 3.4)</td>
<td>2.24 (dd, J = 14.8)</td>
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<tr>
<td>H-4β</td>
<td>2.17 (dd, J = 15.2)</td>
<td>2.12 (dd, J = 14.8)</td>
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<td>H-7</td>
<td>4.84 (d, J = 1.9)</td>
<td>4.86 (d, J = 1.9)</td>
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<td>H-7O</td>
<td>3.83 (d, J = 1.9)</td>
<td>3.79 (d, J = 1.9)</td>
<td>3.79 (d, J = 1.9)</td>
<td>3.73 (d, J = 1.7)</td>
<td>3.87 (d, J = 1.8)</td>
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<td>H-10</td>
<td>6.61 (dq, J = 5.8)</td>
<td>6.63 (dq, J = 5.8)</td>
<td>6.59 (dq, J = 5.8)</td>
<td>6.68 (dq, J = 6.0)</td>
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<td>4.91 (d, J = 5.8)</td>
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<td>3.11 (d, J = 4.2)</td>
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<td>1.91 (dd, J = 1.4)</td>
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<td>1.90</td>
<td>2.16</td>
<td>2.18</td>
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</table>

Chemical shifts are in ppm from Me₄Si; coupling constants are in Hz; spectra recorded in CDCl₃ solution at 250 MHz.
* d, J = 12.6 in CDCl₃/D₂O.
** d, J = 11.8 in CDCl₃/D₂O.

Acetyldioxyvalenol (15-AcDON) (7). Significant shifts for H-3 (upfield) and H-15 (downfield) relative to 3-AcDON immediately indicated the change in position of esterification. The 7-hydroxyl was still unesterified as demonstrated by the observed coupling of this hydroxyl proton to H-7. Literature data [11] supported the identification. 15-AcDON presumably arises via a 2-stage process involving hydrolysis and esterification of 3-AcDON.

Band 4. Band 4 chromatographed with 3-AcDON, was hydrolysed to DON, and was confirmed by MS (M⁺ at m/z 354) and NMR (Table I) to be unchanged 3-AcDON.

Band 5. This band, at highest Rₜ, was also hydrolyzed to DON, had M⁺ at m/z 380 and was a diacetate of DON. NMR analysis showed the acetyl groups to be attached at the 3- and 15-hydroxyls. The 7-hydroxyl was again coupled to H-7, the signal for H-3 was at the same chemical shift as that in 3-AcDON, and those for the two H-15's were at similar shifts as in 15-AcDON. In 15-AcDON, the two protons were observed as a singlet (δ 4.26), but in 3,15-diacetyldioxyvalenol, 3,15-diAcDON (5) they had become magnetically nonequivalent and were observed as doublets at 4.25 and 4.30 (J = 11.9 Hz), in agreement with literature data [12]. 3,15-DiAcDON is therefore the result of specific acetylation of 3-AcDON by the fungus.
of enzymic oxygenation at position 4 of a DON derivative.

Compounds (4), (5), (6), (7) and (9) were obtained in yields of 9.0 mg, 5.2 mg, 10.0 mg, 7.2 mg and 2.3 mg respectively from a 5×400 ml culture supplied with a total of 200 mg 3-AcDON (4). The recovery of metabolites was thus rather poor (overall ca. 17%) but at this stage no optimization of conditions or media has been attempted. It would appear that most of the added trichothecene has been catabolized by the fungus, probably as a consequence of carrying out the incubation in a nutrient-deficient medium. However, this did have the advantage that production of the normal pattern of trichothecenes by the fungus was suppressed to minimum levels, and only relatively small amounts of DAS were present in the product mixture, thus simplifying purification procedures.

The acetylation and deacetylation transformations observed are unexceptional and parallel a number of earlier reports on fungal metabolism of similar substrates [8–10]. Since all four compounds DON, 3-AcDON, 15-AcDON and 3,15-diAcDON were recovered from the culture filtrate, the relative importance of acetylation or deacetylation to the organism, or the order in which the reactions are carried out cannot be assessed. Indeed, the four compounds could well be interrelated via a metabolic grid as shown in Fig. 1. However, if the esterase activities are site-specific, they would be of significant value in

![Fig. 1. Biotransformations of 3-AcDON in Fusarium sp.](image URL)
selectively modifying the esterification pattern of trichothecene substrates, and enzymatic transformations could supplement less-specific chemical methods. Varying levels of specificity have already been reported for Fusarium esterases [7–10].

The transformation of 3-AcDON into fusarenon-X is of much greater significance than the esterification reactions however. Fusarenon-X presumably arises via 4ß-hydroxylation of DON to nivalenol (8) followed by 4-acetylation (Fig. 1). Fungal hydroxylation of the trichothecene skeleton at a nonactivated position is potentially a most valuable transformation to extend accessibility to polyhydroxylated trichothecene structures for study of biological activities and assessment of structure-activity profiles. To date, the only successful demonstration of skeletal hydroxylation of a trichothecene is the production of 16-hydroxyverrucarins A and 16-hydroxyverrucarin B from the corresponding verrucarins using Rhizopus arrhizus [13]. However, this represents hydroxylation in an activated position, and such a transformation is attainable chemically, though in poorer yields [13]. 16-Hydroxytrichothecenes are not known as natural toxins whereas the present transformation gives low yields of a natural 4-hydroxy derivative. The hydroxylating enzyme in Fusarium sp. is normally involved in the production of DAS and hydroxylates some yet to be identified trichothecene substrate which lacks oxygenation at C-7 and C-8. However, it will also hydroxylate DON which does have oxygen substituents at C-7 and C-8, showing a somewhat lower substrate specificity. If substrate specificity is sufficiently low, then this fungus under optimized conditions, or the enzyme itself, could be used to produce a range of 4ß-hydroxylated trichothecenes. A 4ß-hydroxylase acting on the unsubstituted trichothecene (1) is implicated in the biosynthesis of trichothecin and trichothecolone via trichodermol [3].

The consequences of one Fusarium species metabolizing a trichothecene toxin produced by another Fusarium species are of vital significance to the analysis of food materials likely to be contaminated by mycotoxins. Foodstuffs are routinely screened for known trichothecene contaminants such as DON, T-2 toxin, HT-2 toxin, etc., but the range of toxins encountered could be increased if mixed populations of Fusaria infect the plant source and fungal transformations occur. Since literature data [1] suggest fusarenon-X is considerably more toxic than 3-AcDON, DON or DAS, its presence in a mixed culture of Fusarium sp. and F. culmorum might significantly affect the overall toxicity relative to the individual cultures.

**Experimental**

3-AcDON and 14C-labelled 3-AcDON (97 μCi/mM) were obtained from cultures of Fusarium culmorum (CMI 14764) as described previously [6].

**Culture of fungus**

Fusarium sp. strain C37410-90 (Bristol-Myers Company) was maintained on Sabouraud agar (Oxoid) slants at 25 °C in the dark. Mycelium for the transformation experiments was cultured in a Czapek-Dox seed medium: 20 g glucose, 2 g malt extract, 2 g yeast extract, 2 g peptone, 2 g KH₂PO₄, 2 g MgSO₄·7H₂O, 0.2 g FeSO₄, 3 g NH₄Cl and H₂O (1 l), sterilized at 121 °C for 30 min. The fungus was grown for 2 days in the dark at 25 °C on a rotary shaker (125 rpm), before being filtered, washed and resuspended in an equivalent volume of sterile distilled H₂O.

**Biotransformation experiments**

Initial experiments were carried out using 3 × 50 ml cultures adding 14C-labelled 3-AcDON (5 mg dissolved in 0.5 ml EtOH) per flask. Cultures were then incubated at 25 °C/125 rpm as before and monitored daily by TLC (Merck Silica gel 60 F254 plates, developed with diethyl ether–acetone, 9:1, visualized using spray reagents 20% H₂SO₄ [14] or 4-(p-nitrobenzyl)pyridine [15]). After 5 days, the cultures were filtered through muslin, and the filtrate extracted with ethyl acetate (4 × 20 ml). The combined extracts were dried over anhydrous MgSO₄ and evaporated to yield an oil (6 mg).

Preparative TLC using one 200 × 200 × 0.5 mm silica plate (Et₂O–Me₂CO, 9:1; visualization UV₂₅₄) yielded five main bands which were eluted with acetone. Approximately half of each band was dissolved in MeOH (0.1 ml) and stirred at room temperature with aq. NaOH (0.4%, 0.1 ml) in a Reacti-vial. Samples were analyzed at intervals over 30 min using TLC in two solvent systems (Et₂O–Me₂CO, 9:1 and CHCl₃–MeOH, 4:1). Radioactivity was checked by autoradiography of the TLC plates.
A larger scale transformation employed $5 \times 400 \text{ ml}$ cultures adding 40 mg unlabelled 3-AcDON in 0.8 ml EtOH per flask. Subsequent work up gave an oil (84 mg) which was separated by preparative TLC (five $200 \times 200 \times 0.5 \text{ mm}$ plates) yielding 3-AcDON (9.0 mg), DON (10.0 mg), fusarenon-X (2.3 mg) and 3,15-diAcDON (5.2 mg). The remaining band was purified further by TLC (CHCl$_3$–MeOH, 9:1) giving 15-AcDON (7.2 mg). $^1$H NMR data for the products are given in Table I. EI and CI mass spectra were identical to those of authentic standards, or analogous to published data [1].

Acknowledgements
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