Oxidative Transformation of Deoxynivalenol (Vomitoxin) for Quantitative and Chemical Confirmatory Purposes

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A simple and rapid method for the confirmation of deoxynivalenol residues in cereal crops was developed. The reaction involves a one-step treatment with aqueous periodate or freshly precipitated lead dioxide. Deoxynivalenol is quantitatively converted into a unique seven-membered ring lactone analogue. The trifluoroacetylated conversion product could be quantitated at levels equivalent to 0.05 ppm by electron capture gas–liquid chromatography. 3-Acetoxydeoxynivalenol was shown to undergo a similar oxidative transformation.

Deoxynivalenol (3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one or vomitoxin) is contamination trichothecene toxin produced by certain Fusarium species (Morooka et al., 1972; Yoshizawa and Morooka, 1973; Vesonder et al., 1973). It is a causative agent of emesis, feed refusal, and growth depression in animals (Pathre and Mirocha, 1979). The recent widespread occurrence of contamination by deoxynivalenol (Stoloff, 1983) has initiated development of a number of methods for its detection in cereal crops. Most of these methods (Szathmary et al., 1980; Scott et al., 1981; Cohen and Lapointe, 1982) utilize gas–liquid chromatography (GLC) as the determinative step with prior conversion of the compound to either Me_3Si ethers or heptafluorobutyrate esters. Subsequent detection by electron capture while extremely sensitive has the disadvantage of possible incorrect identification because closely related trichothecenes or coextractives, etc., may have misleadingly similar retention times. Such an example has recently been reported by Visconti and Palmisano (1982). In the absence of confirmatory mass spectral data a far-reaching purification of the initial extracts may be required. Alternatively, differentiation by selective chemical reactions may also be utilized.

This paper describes a unqiue chemical confirmatory test for deoxynivalenol and its 3-acetate analogue that is both rapid and quantitative.

EXPERIMENTAL SECTION

Chemicals. Deoxynivalenol was purchased from the Myco-Lab Co., Chesterfield, MO 63017. 3-Acetoxydeoxynivalenol was isolated from a liquid culture of *Fusarium graminearum* (Miller and Greenhalgh, 1983). Lead tetraacetate and trifluoroacetic anhydride were purchased from BDH Chemicals, Ltd., Poole, England. Sodium metaperiodate was purchased from the Fisher Scientific Co., Fair Lawn, NJ. Extrelut columns (Merck No. 11737) were purchased from BDH Chemicals, Ltd., Halifax, Nova Scotia, Canada.

Equipment. Melting points were determined on a Kofler hot-stage microscope and are uncorrected. Infrared (IR) spectra were determined by using a Beckman IR-20A spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained in $CDCl_3$ solution with Me_4Si as an internal standard on a Bruker W.M. 250 NMR spectrophotometer at 250 and 62.8 MHz. The mass spectra (MS) were determined with a Finnigan 3000 GC/MS coupled to a D6000 data acquisition system. Ultraviolet (UV) spectra were determined on a Perkin-Elmer Model LC-75 spectrophotometric detector.

Gas–Liquid Chromatography. A Tracor 222 gas chromatograph equipped with electron-capture ^{63}Ni detector (with linearizer) and a 1.8 m × 4 mm i.d. glass column packed with 2% OV-275 on 80–100-mesh H.P. Chromosorb W was used. Operating parameters were injection port 220 °C, column 190 °C, detector 300 °C, and helium carrier gas flow rate 60 mL/min. Under these conditions the 3-trifluoroacetoxy lactone (V) had a retention time of 8.94 min. A Hewlett-Packard 3380 A integrator was used to quantitate the peaks.

High-Performance Liquid Chromatograph. A Perkin-Elmer series 4 liquid chromatograph system (equipped with a Perkin-Elmer RP-18 octadecyl, 10-μm particle size, 4.6 mm × 25 cm column) in conjunction with a Model LC-75 spectrophotometric detector (8-μL flow cell) and a Sigma 15 data station was used. The mobile phase (10% acetonitrile–10% methanol in water) had a flow rate of 2 mL/min. Under these conditions deoxynivalenol had a retention time of 3.29 min at 40 °C.

Oxidation Procedure. A reference sample was prepared by stirring deoxynivalenol (20 mg) in chloroform (50 mL) with a 1.0% aqueous solution of sodium metaperiodate (20 mL) at room temperature. Alternately, a suspension of freshly precipitated lead dioxide (prepared by the addition of 100 mg of lead tetraacetate to 20 mL of water) could be substituted for the periodate. After 30 min, thin-layer chromatography (silica gel GF) by development in ethyl acetate–hexane (60:40) indicated complete conversion of deoxynivalenol. The reaction mixture was neutralized with sodium bicarbonate, transferred to a 125-mL separatory funnel, and shaken, and the chloroform layer was removed. The aqueous layer was reextracted with chloroform and the combined extracts were taken to dryness on a rotary evaporator (water bath at 35 °C). Crystallization of the residue (18.9 mg) from ethyl acetate gave the lactone II, mp 192–194 °C. In a similar manner the 3-acetoxy lactone IV, mp 157–158 °C, was prepared from 3-acetoxydeoxynivalenol (III). The 3-acetoxylactone (IV) had identical melting point, mixed IR, NMR, MS, and GLC retention time with that of the product from acetylation (acetic anhydride–pyridine, overnight) of the lactone II.

Analytical Procedures. The wheat subsample (20 g) (pulverized in a hammer mill) was homogenized with 40% aqueous methanol (100 mL) in a Waring blender for 3 min and the homogenate filtered through Whatman No. 1 filter paper. The blender was rinsed with water (50 mL) and the washings were filtered. The combined filtrate was

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Table I. Proton NMR Spectra of Compounds I-IV (Chemical Shifts, δ)

hydrogen no.	I	II	III	IV
2	3.62 (d, 1 H, J = 4.5 Hz)	3.77 (d, 1 H, J = 4.5 Hz)	3.87 (d, 1 H, J = 4.5 Hz)	4.02 (d, 1 H, J = 4.5 Hz)
3	4.53 (m, 1 H)	4.64 (m, 1 H)	5.18 (m, 1 H)	5.31 (m, 1 H)
4	2.14 (ABX, 2 H)	2.16 (ABX, 2 H)	2.20 (ABX, 2 H)	2.25 (ABX, 2 H)
7	4.83 (d, 1 H, J = 1.6 Hz)	10.02 (s, 1 H)	4.79 (d, 1 H, J = 2.1 Hz)	9.98 (s, 1 H)
10	6.61 (dd, 1 H, J = 5.9 Hz)	6.42 (dd, 1 H, J = 4.5, 1.6 Hz)	6.57 (dd, 1 H, J = 5.9, 1.5 Hz)	6.40 (dd, 1 H, J = 4.6, 1.2 Hz)
11	4.80 (d, 1 H, J = 5.9 Hz)	5.54 (d, 1 H, J = 4.8 Hz)	4.66 (d, 1 H, J = 5.9 Hz)	5.27 (d, 1 H, J = 4.8 Hz)
13	3.10 (AB, 2 H)	2.83 (AB, 2 H)	3.11 (AB, 2 H)	2.87 (AB, 2 H)
14	1.13 (s, 3 H)	0.75 (s, 3 H)	1.12 (s, 3 H)	0.76 (s, 3 H)
15	3.80 (AB, 2 H, J = 11.7 Hz)	4.35 (AB, 2 H, J = 13.7 Hz)	3.77 (AB, 2 H, J = 11.7 Hz)	4.13 (AB, 2 H, J = 13.8 Hz)
16	1.86 (s, 3 H)	1.92 (s, 3 H)	1.85 (s, 3 H)	1.92 (s, 3 H)
Ac			2.10 (s, 3 H)	2.10 (s, 3 H)

diluted with water to exactly 200 mL and a 10-mL aliquot (equivalent to a 1.0-g sample) was poured onto an Extrelut column. The measuring cylinder was rinsed with 10 mL of water and this was also added to the column. After 10 min the column was eluted with methylene chloride (40 mL) and the eluate discarded. The column was then eluted with ethyl acetate (40 mL), the eluted solvent removed under vacuum, and the residue taken up in methanol (0.5 mL) for HPLC analysis. For confirmation of positive HPLC determinations, chloroform (15 mL) was added and the mixture stirred with a solution of 0.1% periodate (5 mL) for 10 min. The reaction mixture was neutralized with sodium bicarbonate (important) and decanted into a 60- or 125-mL separatory funnel and the chloroform layer removed. Extraction was repeated with another 10 mL of chloroform and the chloroform extracts were reduced to dryness on a rotary evaporator. The residue was taken up in benzene (ca. 2 mL) and treated with trifluoroacetic anhydride (25 μL) for 10 min at room temperature or 30 s in a water bath (40 °C). (If the trifluoroacetic anhydride is not fresh, this step should be repeated to ensure complete trifluoroacetylation). The solution was reduced to dryness under vacuum, benzene (1 mL) was added, and 10-μL aliquots were injected into the GLC for analysis. The TFA derivative is quite stable (less than 5% hydrolysis over a week at room temperature).

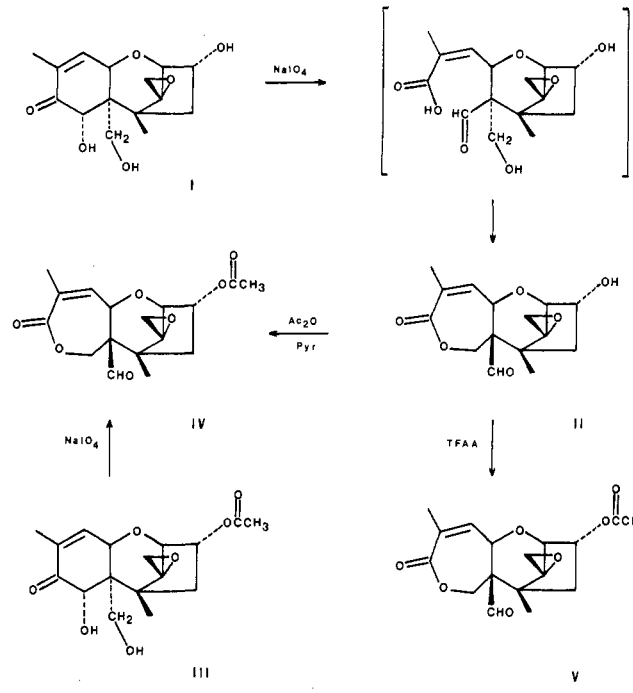

Figure 1. Reaction scheme for the oxidative transformation of deoxynivalenol (I) and 3-acetoxydeoxynivalenol (III).

RESULTS AND DISCUSSION

All naturally occurring trichothecenes contain a trichothecane ring system, unsaturation at C-9 and -10, and an epoxy group at C-12 and -13. They are classified into four groups according to their chemical characteristics. Deoxynivalenol (I) because it possesses a carbonyl group at C-8 is designated as belonging to the B group of trichothecenes. Nivalenol-type trichothecenes are also unique in possessing a hydroxyl group at the C-7 position. Since α-hydroxy ketone systems are normally prone to selective oxidation, a chemical confirmatory test based on this feature of deoxynivalenol was pursued.

Our initial investigations centered on the utilization of reagents known to specifically oxidize α-hydroxy ketones to α-diketones, i.e., bismuth trioxide, cupric acetate, cupric carbonate, cupric sulfate, ferric chloride, etc. When these reagents proved unacceptable, other less selective oxidants were appraised. Lead dioxide [which was recently demonstrated as more effective than lead tetraacetate for conversion of maleic hydrazide into a diazoquinone (King, 1983)] was included among the latter reagents. Results from the eventual treatment of deoxynivalenol (I) with lead dioxide (freshly precipitated) proved intriguing. Monitoring of the reaction by thin-layer chromatography indicated a rapid, quantitative transformtion of deoxynivalenol into a substantially less polar compound. MS data indicated a parent ion at m/e 294 for the new compound. Its IR spectra had carbonyl absorptions at 1728 and 1716 cm^{-1} but the UV spectrum exhibited a maxima at 218 nm, indicating that the α,β-enone system was still intact. A proton NMR spectra (Table I) confirmed loss of the C-7 hydrogen and the appearance of an aldehydic proton. The spectrum was consistent with the C-2, C-3, and C-4 hydrogens being in a similar environment to that of deoxynivalenol (I) but the downfield shift of H-11 suggested greater polarization of the conjugated α,β-enone system. A ^{13}C NMR spectrum (Table II) contains a resonance at 203.7 ppm suggestive of a nonconjugated aldehyde and a resonance at 171 ppm consistent with an α,β-unsaturated ester. These spectral properties were considered supportive of the seven-membered ring lactone structure II. The formation of II may be rationalized by assuming initial oxidative cleavage at C-7–C-8 to yield a C-7 aldehyde and a C-8 carboxylic acid, respectively (Figure 1). Subsequent or concerted esterification of the C-15 hydroxyl with the C-8 carboxylic acid would lead to the lactone isolated. Good evidence for the postulated sequence of events (unique for PbO_2) was obtained when treatment of deoxynivalenol (I) with periodate yielded a similar compound; i.e., periodate is known (Sklarz, 1967)

Table II. ^{13}C NMR Spectra of Compounds I-IV (Chemical Shifts, δ)

carbon no.	I	II	III	IV
2	80.6 (1)[a]	80.8 (1)	79.1 (1)	79.2 (1)
3	68.6 (1)	67.7 (1)	71.3 (1)	70.2 (1)
4	43.0 (2)	42.0 (2)	40.5 (2)	39.4 (2)
5	46.0 (0)	46.1 (0)	45.9 (0)	45.6 (0)
6	52.1 (0)	64.0 (0)	52.0 (0)	63.6 (0)
7	70.2 (1)	203.6 (1)	70.2 (1)	202.5 (1)
8	202.3 (0)	170.1 (0)	199.8 (0)	170.2 (0)
9	135.7 (0)	129.0 (0)	135.8 (0)	129.7 (0)
10	138.5 (1)	140.0 (1)	138.4 (1)	139.2 (1)
11	74.4 (1)	71.5 (1)	74.5 (1)	71.6 (1)
12	65.7 (0)	64.1 (0)	65.1 (0)	63.9 (0)
13	47.2 (2)	47.4 (2)	47.5 (2)	47.8 (2)
14	13.9 (3)	11.0 (3)	14.1 (3)	11.4 (3)
15	61.4 (2)	66.0 (2)	62.2 (2)	66.0 (2)
16	14.9 (3)	17.9 (3)	15.2 (3)	18.5 (3)
CH_3 (Ac)			20.9 (3)	20.9 (3)
C=O (Ac)			170.3 (0)	170.3 (0)

[a] Multiplicity (number of directly attached protons); cf. Blackwell et al. (1983).

Table III. Recovery of Deoxynivalenol from Fortified Wheat Samples

deoxynivalenol added, ppm	average recovery,[a] %
0.5	87.1
1.0	90.3
2.0	88.0
5.0	91.6
mean recovery	89.3

CV = 2.4%

[a] Quantitated by conversion to the trifluoroacetylated lactone (V) for electron capture GLC analysis (duplicate samples).

to routinely cleave α-hydroxy ketones into their respective aldehydic and carboxylic acid components.

It was subsequently demonstrated that 3-acetoxydeoxynivalenol (III) also underwent an oxidative conversion with lead dioxide or periodate. Spectroscopic data (Tables I and II) for the conversion product was consistent with the expected 3-acetoxy lactone structure (IV). Appropriately, acetylation of the deoxynivalenol-derived lactone (II) furnished an identical product.

Although the deoxynivalenol lactone (II) could be gas chromatographed directly, its trifluoroacetoxy derivative (V) (readily formed by reaction with trifluoroacetic anhydride at room temperature) exhibited superior GLC characteristics and was extremely sensitive to electron capture detection.

Cleanup procedures described in the literature for deoxynivalenol-contaminated samples (Cohen and Lapointe, 1982; Scott et al., 1981) though relatively complex can satisfactorily be utilized prior to confirmatory testing with the oxidation step outlined. We have found that the use of Extrelut columns (i.e., the aqueous phase remains on the support and lipophilic substances are extracted with organic solvents) gave satisfactory recoveries (Table III) and a cleanup acceptable for detection of deoxynivalenol at levels equivalent to 0.1 ppm using HPLC with an ultraviolet detector. Subsequent periodate oxidation and pertrifluoroacetylation of the samples allowed confirmation and quantitation to 0.05 ppm by electron capture GLC. Figure 2 illustrates an application of the reaction for the chemical confirmation of deoxynivalenol in a naturally infected sample of wheat. GLC–mass spectrometry was used to confirm that the peak occurring on GLC was due

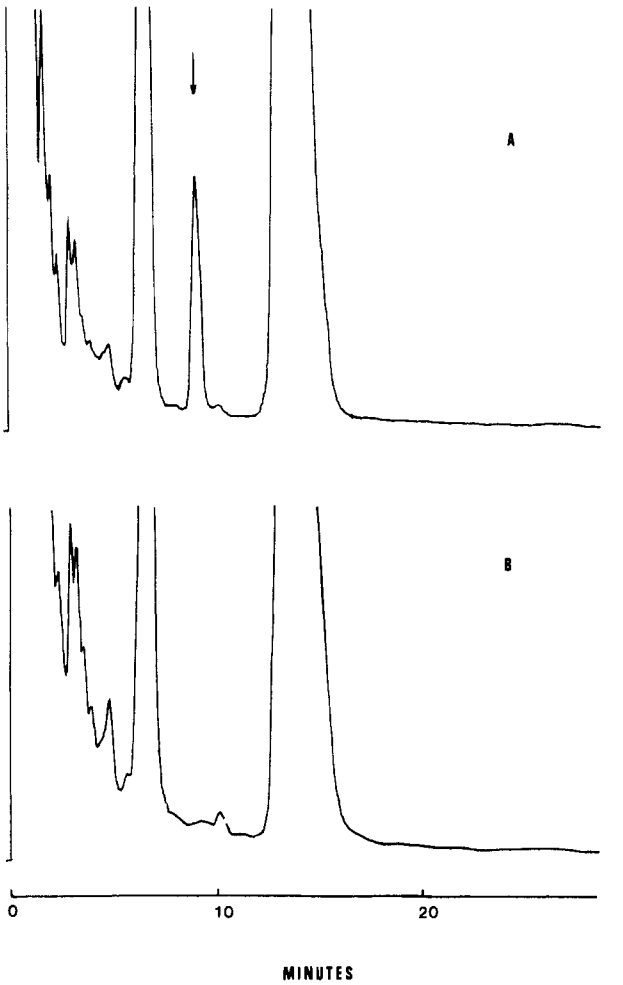

Figure 2. Gas chromatograms of (A) an extract of naturally contaminated Ontario winter whet after cleanup, oxidation with periodate, and TFAA derivatization, 10 μL of 1.0 g equiv of wheat/mL corresponding to 0.8 ppm of deoxynivalenol, and (B) an extract of uninfected wheat following similar treatment.

to the pertrifluoroacetylated oxidation product (V). The mass spectral fragmentation pattern exhibited prominent ions at *m/e* 390 (27.9%), 294 (41.7%), 265 (14.4%), and 97 (100%).

The oxidative transformation outlined provides a rapid and reliable method for chemically confirming the presence of suspected deoxynivalenol (I) and 3-acetoxydeoxynivalenol (III) residues. A meaningful application of the reaction to other trichothecenes with the appropriate C-7, C-8, and C-15 substituents should also prove effective.

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Captan-Treated Seed Corn in Alcohol Production

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Utilization of surplus captan-treated corn has long been of concern to seed producers. Results of this study show that it can be used effectively as a substrate for ethanol fermentation. When captan-treated seed corn and untreated corn were used in 10-L fermentations with recycled distillers' solubles, higher ($P < 0.10$) alcohol production resulted from captan-treated corn (91.8%) than from untreated corn (84.0%). Chicks fed diets containing 10% and 20% distillers' dried grains (DDG) from the treated corn fermentations had higher gain:feed ratios at day 7 than did chicks fed diets containing DDG from control fermentations. No significant differences in any performance measurement occurred at day 14, but chicks fed DDG from the treated corn fermentations had the highest rates of gain and gain:feed ratios. Practical use of this procedure will probably require approval by appropriate regulatory agencies.

The controversy over the method for disposal of surplus captan-treated seed corn has been ongoing for a number of years. Captan [*N*-[(trichloromethyl)thio]tetrahydrophthalimide] is used on all seed corn produced (45.5 g of captan/45.5 kg of seed corn) and acts to protect the seed from fungal infestation. An estimated 1.97 billion lb of seed is treated with captan every year, with a carry-over of unused seed corn (55–110 million lb) resulting. Captan is believed to have toxic properties; this potential toxicity, when incorporated directly into animal diets, has been studied (Ackerson and Mussehl, 1955; Dowe et al., 1957; Theuninck et al., 1981) with varying results. Evidence of harmful effects on humans has also been inconsistent (Environmental Protection Agency, 1980). Until recently, the approved method of disposal was by burial. However, the Environmental Protection Agency has changed the method of disposal of the treated corn; it now allows feeding to cattle or hogs if the captan level is below 100 ppm after either roasting or washing (*Fed. Regist.*, 1981). Washing the seed corn with water degrades captan to sulfur, chloride, and 4-cyclohexene-1,2-dicarboximide (Wolfe et al., 1976). If a strong base is used as a wash, the primary breakdown product is also 4-cyclohexene-1,2-dicarboximide (Coats and Dahm, 1980).

One use for surplus captan-treated corn could be as a fermentation substrate for alcohol production, with recovery of the solid portion (distillers' dried grain, DDG) as an animal feed. To reduce the cost of concentrating solids after a fermentation, the spirits industry recycles part of the distillers' solubles (up to 20%) back into the makeup water of the next fermentation. Recycling also seems to accelerate yeast growth and fermentation. Extensive recycling has not won widespread approval, but studies (Ronkainen et al., 1978; Wall et al., 1983) have shown alcohol yields did not diminish even when 70% or more of the distillers' solubles were recycled. Also, researchers have used ethyl alcohol as a wash to remove the captan, fermented the washed corn to produce alcohol, and then used this alcohol for the next wash (Steinberg et al., 1982).

This report describes a study of (1) the fermentability of captan-treated seed corn while recycling the distillers' solubles in increasing amounts (0, 25, 50, 75, and 100%) and (2) the performance of chicks fed a diet containing the spent grains from the fermentations.

MATERIALS AND METHODS

Captan-treated seed corn (obtained commercially) and yellow-dent field corn were ground through a 0.063-in. screen with a Fitzpatrick Homoloid Model J. T. mill. Each type was mixed thoroughly before use.

Fermentations of both captan-treated seed corn and untreated field corn were run in tandem using 20-L stainless steel fermentors equipped with stirrers and temperature control jackets. Ground corn (2945 g of captan-treated or field corn) was added to 6.25 L of distilled H_2O (some or all of this water would be substituted with distillers' solubles when recycling). The pH was adjusted to 6.2, and 7.5 mL of a bacterial α-amylase (Taka-therm, Miles Laboratories) was added for liquefaction. The fermentors were then heated to 90 °C with stirring and held for 1 h. At the end of this period, 1950 mL of distilled H_2O (again, some or all might be recycled distillers' solubles) was added and the temperature of the fermentors was dropped to 60 °C. The pH was adjusted to 4.0 and 22.5 mL of a fungal glucoamylase (Diazyme L-100, Miles Laboratories) was added for conversion of dextrins to glucose. The fermentors were held at 60 °C for 2 h, after which they were cooled to 32 °C and the pH was adjusted to 5.0. The pH adjustments were achieved with either dilute NaOH or dilute HCl. A yeast inoculum was added (5% v/v) and

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