# Transformation of Deoxynivalenol (Vomitoxin) by Rumen Microorganisms

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The mycotoxin deoxynivalenol (vomitoxin) was incubated in vitro with rumen fluid and sampled at regular intervals. At feed contamination levels (up to 10 ppm) deoxynivalenol was virtually all transformed within a 24-h period. The single transformation product was identified as 3α,7α,15-trihydroxytrichothec-9,12-dien-8-one by IR, UV, MS, and NMR. A similar treatment of 3-acetoxydeoxynivalenol caused deacetylation prior to transformation.

Deoxynivalenol (3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one or vomitoxin) is a 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, food refusal, and growth depression in certain animals (Pathre and Mirocha, 1979).

Although the "no effect" level of deoxynivalenol in wheat or corn varies widely among animal species, ruminants appear to be relatively unaffected (Rohmer, 1983). Since rumen fermentation can act as a first line of defence against harmful or toxic substances in the diet (Church, 1980), we undertook to determine the effect on deoxynivalenol when incubated in vitro with rumen fluid. Our subsequent investigations revealed that at feed contamination levels (up to 10 ppm) deoxynivalenol was readily transformed by rumen microoganisms in vitro. This paper describes the conditions associated with the transformation and the structural determination of the end product.

## EXPERIMENTAL SECTION

**Chemicals.** Deoxynivalenol (mp 151–153 °C) and 3-acetoxynivalenol (mp 184–186 °C) were obtained from a liquid culture of *Fusarium graminearum* (Miller and Greenhalgh, 1983).

**Equipment.** 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 MHz. The mass spectra (MS) were determined with a Finnigan 4021 GC/MS coupled to an Incos 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 (GLC) equipped with a $^{63}Ni$ electron capture 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 215 °C, column 190 °C, detector 300 °C, and helium carrier gas flow rate 60 mL/min. Table I shows retention times and characteristic mass ions for the trifluoroacetylated deoxynivalenols analyzed under the above conditions. The lower limit of detection was 0.05 ppm.

**High-Performance Liquid Chromatography.** A Perkin-Elmer series 4 liquid chromatograph (HPLC) 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) at 220 nm and a Sigma 15 data station was used.

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**Table I. GC and MS Data of Trifluoroacetylated Deoxynivalenols**

| parent compd | retention time, min | characteristic ions, *m/e* |
|---|---|---|
| I | 4.02 | 374, 488 |
| II | 2.79 | 358, 472 |
| III | 7.13 | 343, 374 |

**Table II. Composition of Rumen in Vitro Solutions**

| | |
|---|---|
| solution A | |
| $NH_4HCO_3$, g/L | 4.0 |
| Na $HCO_3$, g/L | 35.0 |
| solution B | |
| $Na_2HPO_4$, g/L | 5.7 |
| $KH_2PO_4$, g/L | 6.2 |
| $MgSO_4 \cdot 7H_2O$, g/L | 0.6 |
| solution C | |
| $CaCl_2 \cdot 2H_2O$, g/L | 13.2 |
| $MnCl_2 \cdot 4H_2O$, g/L | 10.0 |
| $CoCl_2 \cdot 6H_2O$, g/L | 1.0 |
| $FeCl_3 \cdot 6H_2O$, g/L | 8.0 |
| solution D[a] | |
| cysteine hydrochloride·$H_2O$, g/100 mL | 0.625 |
| 1 N NaOH, mL/100 g | 4.0 |
| $Na_2S \cdot 9H_2O$, g/100 mL | 0.625 |

[a] Prepared immediately before use by dissolving the cysteine hydrochloride in 95 mL of water and 4 mL of 1 N NaOH, then adding the $Na_2S \cdot 9H_2O$, and dissolving it to give 100 mL.

The mobile phase (10% acetonitrile, 10% methanol in water) had a flow rate of 2 mL/min. Under these conditions deoxynivalenol (I) had a retention time of 3.26 min, 3-acetoxydeoxynivalenol (IV) 4.50 min, and the diene (II) 4.94 min at 40 °C. Mixtures of deoxynivalenol and the diene (II) were separated and purified accordingly.

**Thin-Layer Chromatography.** Mixtures of deoxynivalenol and the diene (II) were separated from other in vitro fermentation extracted material on preparative thin-layer plates (coated with silica gel GF) by development in ethyl acetate. The compounds were detected by UV and eluted from the silica gel with acetone.

**In Vitro Rumen Fermentation Procedure.** Rumen contents were collected from a rumen-fistulated cow that was maintained on timothy hay. The sample of rumen contents was taken before the morning feeding and was put into a prewarmed thermos bottle. The bottle was completely filled to eliminate any air space. The inoculum was prepared by blending rumen contents in a Waring blender for 2 min under carbon dioxide, by squeezing them through four layers of cheesecloth, and then filtering them through a 5-cm pad of glass wool (also under carbon dioxide) to remove feed particles.

To 250-mL Erlenmeyer flasks containing ground climax timothy hay (0.5 g) was added 39 mL of media [1 g of trypticase, 200 mL of water, 100 mL of solution A (see Table II), 100 mL of solution B, 1.0 mL of solution C, and 2 mL of solution D]. Except for controls 1 mL of the

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**Table III. Rate of Transformation of Deoxynivalenol (I) and 3-Acetoxydeoxynivalenol (IV) by Rumen Microorganisms**

| trichothecene | amount, ppm | incubation time, h | trichothecenes recovered, % I | II | IV |
|---|---|---|---|---|---|
| I | 5 | 6 | 64.9 | 26.1 | |
| | | 12 | | 79.4 | |
| | | 24 | | 81.4 | |
| | | 48 | | 87.1 | |
| I | 10 | 6 | 65.2 | 23.7 | |
| | | 12 | 4.0 | 81.2 | |
| | | 24 | | 84.0 | |
| | | 48 | | 89.4 | |
| I | 50 | 6 | 73.0 | 18.1 | |
| | | 12 | 63.6 | 32.7 | |
| | | 24 | 52.4 | 39.7 | |
| | | 48 | 46.2 | 48.8 | |
| I | 100 | 6 | 79.9 | 13.1 | |
| | | 12 | 72.5 | 18.9 | |
| | | 24 | 68.7 | 20.1 | |
| | | 48 | 57.4 | 37.8 | |
| IV | 100 | 6 | 19.8 | 5.2 | 68.9 |
| | | 12 | 22.3 | 13.5 | 43.1 |
| | | 24 | 38.0 | 29.8 | 16.4 |
| | | 48 | 58.5 | 33.6 | |

appropriate aqueous solution of deoxynivalenol was added (i.e., amounts equivalent to 5, 10, 50, and 100 ppm in the feed). The flasks and contents were then saturated with and kept under carbon dioxide to a pressure of 10–20 cm of water. Saturation was judged complete when a control flask containing media and 0.05 mL of a resazurin solution (1 g of resazurin/L) remained pink. Two milliliters of solution D was added to lower the redox potential as indicated by the media containing resazurin turning colorless.

After 30 min, 10 mL of freshly prepared rumen fluid inoculum was added to each flask, and the samples were incubated at 40 °C under a positive pressure of 10 cm of water (provided by carbon dioxide). This procedure is similar to that described by Goering and Van Soest (1970). Incubations covered periods of 6, 12, 24, and 48 h, respectively, for each different concentration of deoxynivalenol. Flasks containing (1) no rumen fluid inoculum and (2) rumen fluid inoculum to which 0.1 mL of toluene had been added acted as fermentation controls.

**Sample Analysis.** At the end of each incubation period, ethyl acetate (150 mL) was added to the Erlenmayer flask and the solution was mechanically shaken for 15 min. Next, a 60-mL portion of ethyl acetate layer was taken from the flask and the solvent removed on a rotary evaporator (water bath at 35 °C). The residue was dissolved in appropriate amounts of methanol prior to quantitation of the trichothecenes present by HPLC. For confirmatory purposes, samples containing low levels (i.e., 5 and 10 ppm) of trichothecenes were purified by HPLC and treated with trifluoroacetic anhydride (TFAA) (100 $\mu$L) in benzene (2 mL) for 10 min at room temperature. The benzene and excess trifluoroacetic anhydride were removed on a rotary evaporator (water bath at 35 °C), and the derivatized material was dissolved in benzene (1 mL) prior to GLC analysis.

In order to obtain sufficient quantities of the deoxynivalenol transformation product for physical characterization, amounts of deoxynivalenol equivalent to 1000 ppm in the feed were incubated with rumen microoganisms for 7 days. After extraction with and removal of ethyl acetate, the residue was submitted to a preliminary cleanup by preparative thin-layer chromatography. Preparative HPLC was then utilized to separate unreacted deoxynivalenol from the transformed product.

**Table IV. Proton NMR Spectra of Compounds I and II (Chemical Shifts, $\delta$)**

| hydrogen no. | I | II |
|---|---|---|
| 2 | 3.62 (d, 1 H, $J$ = 4.5 Hz) | 4.25 (d, 1 H, $J$ = 2.5 Hz) |
| 3 | 4.53 (m, 1 H) | 4.30 (m, 1 H) |
| 4 | 2.14 (ABX, 2 H) | 2.18 (ABX, 2 H) |
| 7 | 4.83 (d, 1 H, $J$ = 1.6 Hz) | 4.73 (d, 1 H, $J$ = 1.5 Hz) |
| 10 | 6.61 (dd, 1 H, $J$ = 5.9 Hz) | 6.63 (dd, 1 H, $J$ = 6.0 Hz) |
| 11 | 4.80 (d, 1 H, $J$ = 5.9 Hz) | 4.75 (d, 1 H, $J$ = 6.0 Hz) |
| 13 | 3.10 (AB, 2 H) | 5.11 (s, 1 H), 5.24 (s, 1 H) |
| 14 | 1.13 (s, 3 H) | 1.46 (s, 3 H) |
| 15 | 3.80 (AB, 2 H, $J$ = 11.7 Hz) | 3.85 (AB, 2 H, $J$ = 11.8 Hz) |
| 16 | 1.86 (s, 3 H) | 1.88 (s, 3 H) |

## RESULTS AND DISCUSSION

Analysis by HPLC and GLC of ethyl acetate extracts taken from the in vitro rumen samples (incubated over four different time periods) revealed a steady decrease in the quantity of deoxynivalenol present (Table III). The corresponding appearance of equivalent amounts of a less polar compound was confirmed by comparison with control samples. As shown in Table III, transformation of deoxynivalenol was essentially complete within 24 h at levels up to 10 ppm (in the feed). The total recovered trichothecenes for all samples averaged approximately 89% (of the trichothecenes added) after 48 h. As expected (Kallela and Vasenius, 1982), the quantity of toxin added was important. For samples in which deoxynivalenol was added in high doses (>10 ppm), the percentage loss of toxin eventually became less than in samples where the amount of toxin added was relatively small (<10 ppm).

In order to obtain sufficient quantities of the transformation product for physical characterization, several large-scale incubations with rumen fluid were carried out. These incubations (7-days duration and activity had abated) averaged approximately 50% conversion of deoxynivalenol at concentrations equivalent to 1000 ppm in the feed.

The UV and IR spectra of the new compound were virtually identical with those of deoxynivalenol. Its mass spectra, however, indicated a parent ion of $m/e$ 280, which is $m/e$ 16 less than that observed for deoxynivalenol. Moreover, a key feature of its $^1$H NMR spectra (Table IV) was the absence of absorptions ascribable to a 12,13-epoxide group and the appearance of two distinct proton singlets at $\delta$ 5.11 and 5.24. These new absorptions are compatible with the presence of vinylic protons and are comparable to the values obtained (i.e., $\delta$ 4.81 and 5.24) for a verrucarol analogue (i.e., 4$\beta$,15-diacetoxytrichothec-9,12-diene) prepared by $LiAlH_4$ reduction of verrucarol's 12,13-epoxide function and then dehydration of the resultant 12-hydroxy group (Gutzwiller et al., 1964). Predictably, the $^1$H NMR spectra also indicated that the C-2 proton had shifted downfield from $\delta$ 3.62 to 4.25. This can be explained by its proximity to the 12,13 double bond. On the basis of its aforementioned spectral characteristics, the new compound was tentatively assigned the structure 3$\alpha$,7$\alpha$,15-trihydroxytrichothec-9,12-dien-8-one (II) (see Figure 1).

Further evidence for the structural assignment was established by chemical means. For example, reaction of II with TFAA yielded a ditrifluoroacetate derivative (as did deoxynivalenol under similar conditions) and treatment

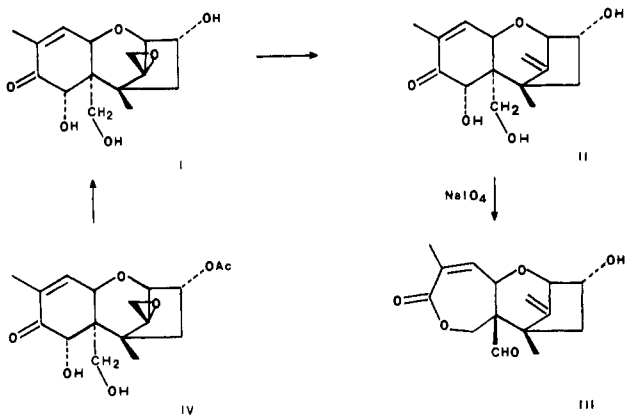


**Figure 1.** Reaction scheme for the transformation of deoxynivalenol (I) and 3-acetoxydeoxynivalenol (IV) by rumen microganisms.

with periodate produced a compound having spectral properties consistent with those of the 3-hydroxy 8-lactone compound III (see Table I). The latter reaction was recently reported for deoxynivalenol (I) and 3-acetoxydeoxynivalenol (IV) (King et al., 1984) and is a unique means of identifying trichothecenes with A-ring functionalities similar to that of deoxynivalenol (I).

The biochemical details of the pathway leading from deoxynivalenol (I) to the diene (II) are uncertain. Previous studies (Ueno et al., 1971, 1976) have demonstrated that fusarenon-X (4$\beta$-acetoxy-3$\alpha$,7$\alpha$,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one), when administred to mice and rats, underwent deacetylation only. Similarly, Kiessling et al. (1982) incubated diacetoxyscirpenol (4$\beta$,15-diacetoxy-3$\alpha$,7$\alpha$-dihydroxy-12,13-epoxytrichothec-9-ene) with rumen fluid and noted only removal of the C-4 acetate group. Our investigations involving 3-acetoxydeoxynivalenol (IV) incubated with rumen fluid also yielded a deacetylated product, namely, deoxynivalenol (I). Although substantial amounts of the deoxynivalenol were subsequently modified (Table III), no evidence for the presence of an analogous 3-acetoxydiene was detected.

Generally, epoxides are reactive toward SH groups, and Ueno and Matsumoto (1975) have demonstrated the reactivity of the epoxide group in trichothecenes toward some thiol enzymes. It is therefore likely that a specific epoxide reductase may be involved in the transformation of deoxynivalenol by rumen microorganisms. Although a hydroxy vitamin K byproduct in the enzymatic reduction of vitamin K epoxide to vitamin K quinone has been isolated (Fasco et al., 1983), our results with deoxynivalenol are akin to the benzopyrene epoxide reductase examples where neither hydroxy byproduct nor intermediates have been detected (Booth et al., 1975).

The functional significance of the epoxide reduction in deoxynivalenol by rumen microganisms in vitro is unclear, but evidence has been presented (Sato and Ueno, 1977) that the epoxide group on the trichothecene nucleus is important not only for cytotoxicity but also for the induction of vomiting. Thus, pending the outcome of further research, one might speculate that the transformation described acts as a means of detoxification. If so, it may be a significant contributing factor to the relatively high "no effect" levels observed on feeding ruminants deoxynivalenol contaminated feed.

Since submission of this paper, Yoshizawa et al. (1983) have reported the isolation of a similar deoxynivalenol transformation product from in vivo experiments with rats.

ACKNOWLEDGMENT

We thank B. A. Blackwell, Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario, Canada, for the NMR detrminations, P. J. Silk, New Brunswick Research and Productivity Council, Fredericton, New Brunswick, Canada, for the GC/MS data, and J. Keough, Agriculture Canada, Fredericton, New Brunswick, Canada for technical assistance.

**Registry No.** I, 51481-10-8; II, 88054-24-4; IV, 50722-38-8.

LITERATURE CITED

Booth, J.; Hewer, A.; Keysell, G. R.; Sims, P. *Xenobiotica* **1975**, *5*, 197.

Church, D. E. "Digestive Physiology and Nutrition of Ruminants. Volume 3. Practical Nutrition, 2nd ed.; O & B Books, Inc.: Corvallis, OR, 1980, p 351.

Fasco, M. J.; Preusch, P. C.; Hildebrand, E.; Suttie, J. W. *J. Biol. Chem.* **1983**, *258*, 4372.

Goering, H. L.; Van Soest, P. J. *U.S., Agric. Res. Serv., ARS* **1970**, *No. 379*.

Gutzwiller, J.; Mauli, R.; Sigg, H. P.; Tamm, Ch. *Helv. Chim. Acta* **1964**, *47*, 2234.

Kallela, K.; Vasenius, L. *Nord. Vetinaermed.* **1982**, *34*, 336.

Kiessling, K.-H.; Pettersson, H.; Olsen, M.; Tideman, K. *Proc. IUPAC Symp. Mycotoxins Phycotoxins, 5th* **1982**, 301.

King, R. R.; Greenhalgh, R.; Blackwell, B. A. *J. Agric. Food Chem.* **1984**, *32*, 72.

Miller, D.; Greenhalgh, R., Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario, Canada, unpublished results, 1983.

Morooka, N.; Uratsiji, N.; Yoshizawa, T.; Yamamoto, H. *J. Food Hyg. Soc. Jpn.* **1972**, *13*, 368.

Pathre, S. V.; Mirocha, C. J. *J. Am. Oil Chem. Soc.* **1979**, *56*, 820.

Rohmer, T. R. *Feedstuffs* **1983**, *April 11*, 30.

Sato, N.; Ueno, Y. "Mycotoxins in Human and Animal Health"; Pathotox; Park Forest South, IL, **1977**; p 304.

Ueno, Y.; Matsumoto, H. *Chem. Pharm. Bull.* **1975**, *23*, 2439.

Ueno, Y.; Matsumoto, Y.; Ito, I.; Ohta, M. *Proc. Annu. Meet. Jpn. Pharm. Soc., 96th* **1976**, *3*, 240.

Ueno, Y.; Ueno, I.; Iitoi, Y.; Tsunoda, H.; Enomoto, M.; Ohtsubo, K. *Jpn. J. Exp. Med.* **1971**, *41*, 521.

Vesonder, R. F.; Ciegler, A.; Jensen, A. H. *Appl. Microbiol.* **1973**, *26*, 1008.

Yoshizawa, T.; Morooka, N. *Agric. Biol. Chem.* **1973**, *37*, 2933.

Yoshizawa, T.; Takeda, H.; Ohi, T. *Agric. Biol. Chem.* **1983**, *47*, 2133.

Received for review October 5, 1983. Revised manuscript received February 6, 1984. Accepted June 21, 1984.