Ovine Metabolism of Zearalenone to α-Zearalanol (Zeranol)

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This is the first report of a metabolic conversion in animals of zearalenone to zearalanols. Zearalenone was labeled with deuterium by base-catalyzed isotope exchange. The labeled zearalenone, in the form of its β-cyclodextrin inclusion complex, was administered orally and intravenously to sheep. In addition to deuteriozearalenone glucuronide, analysis of the urine revealed the presence of deuteriated α- and β-zearalenol and α- and β-zearalanol glucuronides. No zearalanone was detected in the urine. The problem of cis−trans isomerization of zearalenone in the presence of sunlight is also discussed.

Keywords: *Zeranol; zearalanol; zearalenone; zearalenol; metabolism; residue; Fusarium; mycotoxin*

INTRODUCTION

α-Zearalanol (**4**) (zeranol) is a growth promotant made commercially by reduction of zearalenone (**1**), an estrogenic β-resorcylic acid lactone produced by *Fusarium* species. In addition to zearalenone, these *Fusarium* species can also produce an array of related metabolites: α-zearalenol (**2**), β-zearalenol (**3**), α-zearalanol (**4**), β-zearalanol (taleranol) (**5**), and zearalanone (**6**) (Richardson et al., 1985).

Although a normal product of *Fusarium* metabolism, zeranol is deemed an industrial anabolic by European Union (EU) legislation, and its use was finally banned in the European Union in 1989 (European Union, 1988). The use of zeranol as a growth promotant is, however, permitted in many countries, including the United States and New Zealand. In New Zealand, the use of zeranol is rare and treated animals must be specially tagged to prevent export of their carcasses or offal to the EU. As part of the Ministry of Agriculture (MAF) program to monitor compliance with EU market access requirements, urine taken at random from animals sent to slaughter in New Zealand is analyzed. The detection of zeranol, mainly present as the glucuronide, in 51 of 698 urine samples analyzed between October 1, 1991, and September 30, 1992, initially caused concern that some animals treated with zeranol in New Zealand were not being tagged (W. T. Jolly, MAF Regulatory Authority, personal communication). Research undertaken by MAF, and subsequently published by Erasmuson et al. (1994), provided compelling evidence that urinary zeranol from pasture-fed cattle, sheep, deer, goats, and horses in New Zealand originated from a dietary source. Furthermore, Kennedy et al. (1995) reported several instances in which zeranol residues were present in cattle, swine, and sheep from EU member states, even though no evidence of zeranol treatment could be found.

It has been known for some time that at certain times of the year the levels of zearalenone and analogs present in New Zealand pastures are sufficient to depress ewe fertility (Smith et al., 1990, 1995; Towers, 1992; Towers and Sprosen, 1992) and that correspondingly high levels of zearalenone and zearalenol glucuronides are present in the urine of sheep in affected flocks (Towers and Sprosen, 1992; Sprosen et al., 1995). It therefore seemed likely that the zearalenone analogs in the pasture that were responsible for ewe infertility were also the source of the zeranol in the urine of those pasture-fed New Zealand livestock that had not been treated with zeranol implants.

di Menna et al. (1991) detected zearalenone but not α- or β-zearalenol in New Zealand pasture samples. However, Lauren et al. (1988) have shown that *Fusarium* isolates obtained from New Zealand pastures are capable of producing not only zearalenone but also α- and β-zearalenols, zeranol, taleranol, and possibly also zearalanone. It was therefore not possible to be certain whether the source of the zeranol in the urine of the New Zealand animals was primarily extrinsic (i.e., zeranol was produced by the *Fusarium* spp. present in the fodder) or intrinsic (i.e., zeranol was generated in the animal by metabolism of ingested zearalenone or zearalenols, produced by the *Fusarium* spp. present in the fodder) (Erasmuson et al., 1994).

Here we report transformation of zearalenone by ovine metabolism resulting in the presence of glucuronides of zeranol (as well as of zearalenone, α- and β-zearalenol, and taleranol) in the urine. Passage through the rumen was not necessary in order for these transformations to occur.

MATERIALS AND METHODS

General. Zearalenone (**1**), zearalanone (**6**), zeralane (1′,2′-dihydro-6′-desoxyzearalenone), α-zearalanol (**4**), and β-zearalanol (**5**) were from International Minerals & Chemical Corp., Terre Haute, IN, and β-cyclodextrin was from American Maize-Products Co., Hammond, IN. 3,3′,5,5′-Tetramethylbenzidine (TMB), α-zearalenol (**2**), β-zearalenol (**3**), and ovalbumin were from Sigma Chemical Co., and horseradish peroxidase (HRP) conjugated to anti-sheep immunoglobulin G (IgG) was from Silenus Laboratories Pty., Victoria, Australia. Unless stated otherwise, chemical manipulations of **1** were performed with protection from sunlight to prevent isomerization to *cis*-**1** (**7**).

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TLC was performed on silica gel plates (0.2 mm, Merck Art. 5554) with petroleum spirit−ethyl acetate (3:1) as eluent (**1**, R_f 0.13; **7**, R_f 0.18). Flash chromatography (Still et al., 1978) was performed on silica gel (Merck Art. 9385). HPLC analysis was performed with a silica gel column (Zorbax, 250 × 4.6 mm, 5 μm) and petroleum spirit−ethyl acetate−acetic acid (924:75:1) (1.0 mL min^{-1}) as eluent. Eluting compounds were detected with a Shimadzu RF-530 Fluorescence Spectromonitor (excitation at 285 nm, emission detection at 460 nm) and a Hewlett-Packard 1040M diode array UV detector connected in series (**1**, t_R 6.46 min; **7**, t_R 7.35 min).

GC−MS analysis of reactions involving **1** were performed with an HP-1 (Hewlett-Packard) methyl silicone capillary column (20 m × 0.25 mm, 0.25 μm) installed on an HP5980 GC instrument interfaced to an HP5970B mass-selective detector. The GC column was temperature programmed from 200 (0.5 min hold) to 250 °C at 35 °C min^{-1} and then to 285 °C at 2 °C min^{-1} (10 min hold) (**1**, t_R 18.20 min; **7**, t_R 17.06 min).

One- and two-dimensional ^1H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were determined at 300 K from $CDCl_3$ solutions with a Bruker AC-300 instrument fitted with a standard 5 mm probe head. Chemical shifts are reported relative to internal $CHCl_3$ (δ ^{13}C 77.06, δ ^1H 7.27). ^{13}C NMR signal multiplicities (d, t, or q) were determined with the DEPT135 sequence. NOE-difference experiments were performed with an irradiation power of 40 L. NOE-difference spectra were obtained by subtraction of an off-resonance control fid from the irradiated fid and Fourier transformation of the resulting difference fid. Two-dimensional COSY, ^{13}C−^1H correlated, and inverse-mode heteronuclear multiple-bond correlation (HMBC) spectra were determined in absolute value mode. All animal manipulations were approved by animal ethics committees established under the Animal Protection (code of ethical conduct) Regulations Act, 1987.

ELISA Analysis of Urine. "Zearalenone" (a term which we use to cover cross-reacting metabolites as well as **1**) was analyzed by ELISA as described by Towers and Sprosen (1992). Briefly, free "zearalenone" was determined by assay of ethyl acetate extracts of urine. Total "zearalenone" was determined in the same manner after treatment of the urine with β-glucuronidase as follows. Urine (0.5 mL) was diluted with NaOAc buffer (0.2 M, pH 5.5, 0.5 mL) containing β-glucuronidase (3000 IU) and incubated for 16 h at 37 °C before being extracted twice with ethyl acetate (0.5 mL). The extracts were pooled, solvent was removed under a stream of dry N_2, and the residue was dissolved in methanol−H_2O (2:3, 0.5 mL) for ELISA.

The sample (50 μL) and the antiserum (50 μL) were incubated in microtiter plate wells precoated with poly(lysine)−zearalenone *O*-(carboxymethyl)oxime (2 μg mL^{-1}) for 1 h and blocked with ovalbumin (1%, w/v) for 0.5 h at 20 °C. The plates were washed before and after incubation with HRP-labeled anti-sheep antibody for 1 h at 20 °C. Plates were developed by incubation with TMB substrate solution (prepared by addition of TMB in DMSO (100 μL, 10 mg mL^{-1}) to NaOAc buffer (10 mL, 0.1 M, pH 5.5) containing H_2O_2 (0.005%)) for 15 min, after which the reaction was stopped by addition of H_2SO_4 (50 μL, 2 M) and the absorbance at 450 nm was determined with a microplate spectrophotometer.

The content of "zearalenone" in samples was determined relative to standards of **1** and is expressed in zearalenone equivalents. Urinary creatinine concentrations were determined by standard methods at MAF Animal Health Laboratory, Ruakura, and total "zearalenone"−creatinine ratios were calculated to partially correct for variations in urine concentration. Results of these analyses are presented in Table 2.

GC−MS Analysis of Urine. Briefly, a minor adaptation of a general purpose anabolic assay (Erasmuson et al., 1994) was used. After hydrolysis with glucuronidase, the urine was extracted twice with *tert*-butyl methyl ether (*t*-BME)−hexane (3:7); the extracts were combined, evaporated, and then further purified by normal phase HPLC with hexane−*t*-BME−methanol−acetic acid (139:50:10:1) as eluent on a Whatman PAC column. Two fractions were collected, one containing the ketones **1** and **6** and a second fraction containing the alcohols **2**−**5**. These fractions were combined, added to 20 ng of zearalane, and evaporated under a stream of nitrogen. The product was then derivatized with MSTFA−hexane (1:3) for 10 min at 60 °C to form the TMS ethers, the solvent was evaporated in a stream of nitrogen, and the residue was taken up in toluene (25 μL). The toluene contained epiandrosterone butyrate (1 μg mL^{-1}) as an internal retention time standard, and the zearalane was included as a recovery standard.

The toluene solution was analyzed for **1**−**6**, at various deuterium enrichments, by GC−MS on an HP Ultra2 column (25 m × 0.2 mm) fitted with an HP 5972 MSD and an autoinjector, with splitless injection of the sample (1 μL). GC column temperature was ramped from 100 to 290 °C in stages and held at 290 °C from 14.5 to 21.0 min before cooling.

Under the conditions used, the major ion for deuterio-**1** bisTMS ether was at *m/z* 466 (M + 4). Given that the molecular weights of the underivatized metabolites of **1** are either 2 or 4 mass units higher than that of **1**, the monitoring pattern chosen followed an even-number pattern above the natural (undeuteriated) isotopic ion. Ions monitored were *m/z* 435 (16.5−19.0 min) and then from 19.0 to 21.0 min, *m/z* 270, 433, 437, 462, 464, 466, 468, 536, 538, 540, and 542. The recovery standard (zearalane, as its bisTMS ether), monitored at *m/z* 435, eluted near 17.17 min. The retention time standard (epiandrosterone butyrate) eluted near 20.57 min. The natural isotopic abundance ions of the compounds were monitored at *m/z* 462 (**1**, 20.00 min), 464 (**6**, 19.35 min), 536 (**2**, 20.31 min, and **3**, 20.60 min), 433 and 538 (for both **4**, 19.45 min, and **5**, 19.64 min). The deuteriated compounds eluted ca. 0.04 min before their undeuteriated analogs.

Undeuteriated standards were spiked into blank urine samples at 0−20 ng mL^{-1} for **4**−**6** and at 0−200 ng mL^{-1} for **1**−**3**. The response of the GC−MS to each compound, after minor corrections for recovery estimated from the zearalane response, was regressed against the concentration of spike, and the resulting linear concentration−response correlation was used to estimate the concentrations of both undeuteriated and deuteriated analytes.

Labeling of Zearalenone. To K_2CO_3 (125 mg) in D_2O (1.0 mL) was added **1** (16.5 mg) in THF (200 μL). The resulting solution was kept in the dark at ambient temperature for 24 h. The reaction mixture was added to H_2SO_4 (0.2 M, 60 mL) and extracted with CH_2Cl_2 (2 × 20 mL). No *cis*-zearalenone (**7**) could be detected by TLC, HPLC, or GC−MS. The product was dried ($MgSO_4$), concentrated in vacuo, and purified by flash chromatography with CH_2Cl_2−CH_3OH (19:1) as eluent. The resultant product was identical with authentic zearalenone by TLC and HPLC, but the GC−MS and NMR analyses were, however, consistent with deuterium-substituted zearalenone.

Deuterio-**1** (average deuterium content 3.4 atoms/molecule): ^1H NMR δ ($CDCl_3$) 1.38 (3H, d, J = 6.5 Hz, H-11′), 1.49 (1H, m, H_A-4′), 1.65 (2H, m, H_A-9′, H_B-9′), 1.80 (2H, m, H_A-8′, H_B-8′), 2.05−2.28 (total 2.9H, m, H_A-3′, H_B-4′, H_A-5′, H_A-7′), 2.40 (1H, m, H_B-3′), 2.61 (0.5H, br d, H_B-7′), 2.86 (0.4H, br d, H_B-5′), 5.00 (1H, m, H-10′), 5.68 (1H, ddd, J = 3.7, 10.4, 15.2 Hz, H-2′), 5.81 (1H, br s, 3-OH), 6.35 (0.6H, s, H-3), 6.41 (0.04H, br s, H-5), 7.02 (1H, dd, J = 1.8, 15.2 Hz, H-1′), 12.05 (1H, s, 2-OH); ^{13}C NMR δ ($CDCl_3$) 20.9 (C-11′), 21.0 (C-4′), 22.3 (C-8′), 31.0 (C-3′), 34.8 (C-9′), 36.4 (*C*DH-5′, 3 lines, 1:1:1, J = 19.8 Hz), 42.7 (*C*DH-7′, 3 lines, 1:1:1, J = 19.0 Hz), 43.0 (CH_2-7′), 73.5 (C-10′), 102.5 (C-3), 103.9 (C-1), 108.2 (*C*D-5, 3 lines, 1:1:1, J = 19.8 Hz), 132.5 (C-2′), 133.2 (C-1′), 144.0 (C-6), 160.6 (C-4), 165.5 (C-2), 171.4 (C-12′), 211.7 (C-6′); EI-MS (GC−MS, selected ion mode, 70 eV) *m/z* 325 (1.2%), 324 (7.8), 323 (31.9), 322 (74.6), 321 (100), 320 (65.9), 319 (19.3), 318 (M^+, 1.9).

Isolation of *cis*-Zearalenone (7). Zearalenone was isomerized by modification of the method of Peters (1972). Zearalenone (**1**) (50 mg) in CH_3OH (20 mL) was placed outdoors in a sealed quartz tube and analyzed periodically by HPLC for the presence of **7**. After 4 weeks, the methanol was removed in vacuo and the residue purified by flash chromatography with petroleum spirit−ethyl acetate (3:1) as the eluent to give **7** (15 mg): ^1H NMR δ ($CDCl_3$) 1.34 (3H, d, J = 6.4 Hz, H-11′), 1.45−2.6 (12H, m, 6 × CH_2), 5.14 (1H, m, H-10′), 5.40 (1H, m,

Table 1. ^{1}H and ^{13}C NMR Assignments for Zearalenone (1) and Its Cis-Isomer (7)

	1		7	
	^{13}C	^{1}H	^{13}C	^{1}H
1	103.9		104.4	
2	165.5		165.5	
3	102.5	6.36	102.3	6.38
4	160.7		160.7	
5	108.5	6.42	111.6	6.20
6	144.0		142.2	
1′	133.2	7.02	130.1	6.62
2′	132.4	5.68	132.0	5.40
3′	31.1	2.18, 2.41	29.0	2.24 (2H)
4′	21.1	1.54, 2.18	21.4	1.50, 2.14
5′	36.8	2.23, 2.86	40.8	2.24 (2H)
6′	211.9		212.0	
7′	43.0	2.21, 2.61	41.7	2.24, 2.50
8′	22.3	1.81 (2H)	21.4	1.50, 1.90
9′	34.8	1.66 (2H)	33.1	1.50, 1.90
10′	73.5	5.00	73.8	5.14
11′	20.9	1.37	21.4	1.34
12′	171.4		171.0	
3-OH		6.03		*a*
5-OH		12.07		11.97

[a] Not detected.

H-2′), 6.20 (1H, d, J = 2.1 Hz, H-5), 6.38 (1H, d, J = 2.1 Hz, H-3), 6.62 (1H, d, J = 11.6 Hz, H-1′). The ^{1}H and ^{13}C NMR assignments of **7** are given in Table 1.

Administration of Zearalenone. Deuterio-**1** (6.0 mg) was dissolved in ethanol (5 mL) and added dropwise with stirring to a solution of β-cyclodextrin (300 mg) in water (15 mL). The solution was concentrated in vacuo to a white powder. This powder was dissolved in sterile isotonic saline solution (18 mL) for administration to sheep.

In late October 1995, two adult Romney ewes (30−35 kg), neither of which had ever been treated with zeranol, were held in individual metabolism crates and offered fresh water and a freshly cut mixture of ryegrass (*Lolium perenne*) and prairie grass (*Bromus unioloides*) (ad libitum). Each sheep had a 12 G Foley catheter inserted via the urethra into the urinary bladder, connected by gravity feed to a plastic collection bag. The day before the experiment, one sheep had a 16 G indwelling intravenous catheter inserted into the jugular vein. This catheter was tested for patency and position and left filled with heparinized saline.

On the day after catheterization, deuterio-**1**−β-cyclodextrin solution (3.0 mL; 1 mg of deuterio-**1**) was administered via the jugular catheter, which was then washed with heparinized saline (2 mL). The other sheep was dosed orally with the remainder of the solution (15 mL; 5 mg of deuterio-**1**) by intraruminal intubation. Urine from both sheep was collected 0−2, 2−4, 4−8, and 8−24 h after administration, via the urinary catheters. Samples were frozen immediately after collection and stored at −18 °C until analysis. Creatinine concentrations and ELISA analyses for "zearalenone" were performed within 3 days, and GC−MS analysis within 1 week, of sample collection.

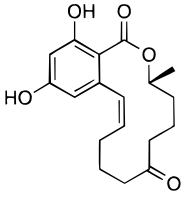
RESULTS AND DISCUSSION

We wished to determine whether zearalenone (**1**), a known contaminant of New Zealand pastures, could be metabolized to zearalanols (**4** and **5**) by sheep (either by the animal itself or by the action of the microbial flora of its gastrointestinal tract and bladder). Although administration of **1** to sheep with monitoring of their urine for **4** and **5** might answer this question, results from such an experiment would be confounded by the possible presence of **1**−**6** in the animal's feed because New Zealand pastures contain detectable levels of zearalenone analogs throughout the year (Towers and Sprosen, 1992; Sprosen et al., 1995). To circumvent this problem, we labeled **1** with deuterium prior to administration and then monitored the resulting deuterium-labeled metabolites in the urine by GC−MS. This approach should provide reliable information about zearalenone metabolism, even in the presence of a significant background exposure of the experimental animals to natural (i.e., unlabeled) zearalenone analogs.

The β-cyclodextrin complex of zearalenone was formed by adapting standard procedures (Frömming and Szejtl, 1994) and was water soluble. This permitted the administration of deuterio-**1**, in the form of its β-cyclodextrin complex, in isotonic saline solution without the use of cosolvents. Oral administration of such complexes generally results in uptake of the guest molecule, with very little absorption of the host molecule (β-cyclodextrin) from the gastrointestinal tract, whereas intravenous administration of cyclodextrin complexes generally results in rapid delivery of the guest to the tissues, with rapid elimination of the cyclodextrin (Frömming and Szejtl, 1994).

Deuterium Labeling. Shipchandler (1975) reported that base-catalyzed hydrolysis of **1** in D_2O resulted in products with deuterium at the 3-, 5-, 5′-, and 7′-positions. We hoped that use of milder conditions would result in adequate levels of deuterium incorporation without hydrolysis of **1**.

In a preliminary exchange reaction, TLC (with CH_2Cl_2−CH_3OH, 19:1, a system which does not resolve **1** and **7**, as eluent) indicated that no significant degradation of **1** had occurred after 16 h. Subsequent GC−MS analysis of this material, however, revealed the presence of a second component in addition to **1**. The mass spectra of both components were identical, with molecular ion envelopes indicating that substantial incorporation of deuterium had occurred. The identity of the second component was established by NMR as deuterio-*cis*-zearalenone (deuterio-**7**). An authentic specimen of **7** was prepared by modification of the method of Peters (1972), and a complete assignment of its ^{1}H and ^{13}C NMR signals (Table 1) was obtained with the aid of two-dimensional NMR experiments. Notably, in an inverse-mode HMBC experiment, the 2-OH signal correlated with the C-1, C-2, and C-3 resonances (104.4, 165.5, and 102.3 ppm, respectively), and H-11′ (1.34 ppm) correlated with C-10′ and C-9′ (73.8 and 33.1 ppm, respectively). Irradiation of H-1′ in an NOE-difference experiment enhanced H-2′ (and vice-versa), thereby verifying the cis-disposition of the olefinic protons, but no NOE enhancement was observed between H-5 and either olefinic proton (H-1′ or H-2′). The assignments presented in Table 1 for **7** are in agreement with the partial assignment advanced by Muñoz et al. (1989), other than for the reversal of the aryl C-3 and C-5 resonances, and the assignment of the proton resonance at 2.50 ppm to H-7′ rather than to H-5′.



cis-zearalenone (**7**)

Peters (1972) has shown that **1** is isomerized to **7** by irradiation with a mercury lamp, but our deuteriation reaction had only been exposed to sunlight (through 5 mm of glass). Nevertheless, we found that protection

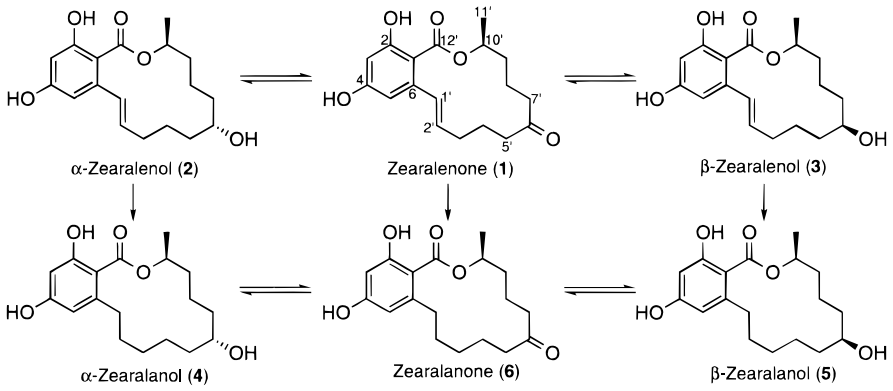


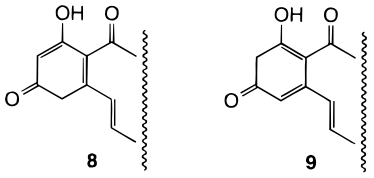
Figure 1. Proposed ovine metabolism of orally or intravenously administered zearalenone.

of exchange reactions from light (with aluminum foil) completely prevented isomerization. Furthermore, exposure of **1** (in CH_3OH, kept in glass vials on the bench) to diffuse indirect sunlight slowly generated **7**, whereas identical samples protected from light did not undergo detectable isomerization. For this reason, all manipulations of **1** were performed with protection from light, and products were checked for the presence of **7** by appropriate TLC, HPLC, or GC−MS analyses. The above observations indicate that precautions should be taken to prevent unnecessary exposure of samples containing zearalenes to UV−visible light and that reports of the occurrence of cis-analogs of **1** as natural products (e.g., Richardson et al., 1985; Lauren et al., 1988; Muñoz et al., 1989) may be due to exposure of the trans-isomers to light.

Total ion chromatogram and selected ion-mode GC−MS analyses established that the deuteriation procedure introduced 2−6 deuterium atoms, with an average incorporation level of 3.4 deuterium atoms/molecule of **1**. The sites at which the deuterium atoms had been incorporated were determined by analysis of ^1H and ^{13}C NMR data. A complete assignment of the ^1H and ^{13}C NMR resonances of zearalenone, supported by one- and two-dimensional $^1H-^1H$ (COSY) and $^{13}C-^1H$ correlation data (optimized for the detection of one-bond couplings), has been reported by Blackwell et al. (1984). Subsequently, Pathre et al. (1990) ultilized long range (two- and three-bond) $^{13}C-^1H$ correlated COLOC NMR data to show that the C-2 and C-4 (quaternary), and also the C-3 and C-5 (methine) (and their corresponding H-3 and H-5), assignments, advanced by Blackwell et al. (1984), should be reversed. The ^1H and ^{13}C NMR assignments established in the present investigation are given Table 1 and are in accord with those of Blackwell et al. (1984) as revised by Pathre et al. (1990). The chemical shifts of the aliphatic protons (C-1′−C-11′) were verified in a series of one- and two-dimensional NMR experiments analogous to those described by Blackwell et al. (1984) and Smith and Watson (1987). The chemical shifts of the aliphatic protons are similar to those of Smith and Watson (1987) and typically 0.03−0.10 ppm higher than those reported by Blackwell et al. (1984). This discrepancy is partly accounted for by their assignment of chemical shifts relative to the $CHCl_3$ resonance, which was given a value of 7.24 ppm (compared to 7.27 ppm in the present study).

NOE-difference results supported the H-3, H-5, H-1′, and H-2′ assignments given for **1** in Table 1. For example, irradiation of H-2′ (5.68 ppm), but not of H-1′ (7.02 ppm), enhanced H-5 (6.42 ppm). It follows from this observation that the preferred orientation of the olefinic C-1′−C-2′ bond is transoid with respect to the aryl C-1−C-6 bond of **1**, as depicted in Figure 1. This observation is in accord with the work of Smith and Watson (1987), based on the NMR data for, and crystal structures of, **1** and **2**.

^1H and ^{13}C NMR data established that competitive deuteriation (exchange) of the protons attached to C-3, C-5, C-5′, and C-7′ had occurred, with rates (assuming that washing of the deuteriated material with MeOH to remove freely exchangeable aryl-OD contributions does not result in back-exchange of the introduced aryl-methine or aliphatic methylene deuterium atoms) for C-5 > C-5′ > C-3 ≈ C-7′. Deuterium incorporation at C-3 (ca. 50%) and C-5 (96%) can be envisaged as proceeding via conjugated enone structures such as **8** and **9**.



Proton-decoupled ^{13}C NMR analysis demonstrated that in deuterio-**1**, C-5′ was deuteriated to a greater extent than was C-7′ because the C-5′ resonance of the deuteriated material was comprised of CH_2 (singlet-like signal) and CHD (triplet-like signal) contributions, whereas the C-7′ was represented only by a CHD (triplet-like) signal (and presumably also by a CD_2 contribution that was not detected because of a combination of its reduced intensity due to (i) suppression of NOE by replacement of ^1H with ^2H and (ii) more extensive coupling (^2H has $I = 1$)). The $^{13}C-^2H$ coupling constants determined for the monodeuteriated component of the C-5, C-5′, and C-7′ signals of deuterio-**1** are given under Materials and Methods.

ELISA Analysis of Urine. The ELISA recognizes zearalenone (**1**) (100%) but also has appreciable cross-reactivities with **2** (220%), **3** (60%), **4** (110%), **5** (35%), and **6** (45%). For this reason, results obtained with the ELISA are reported in terms of "zearalenone" equivalents.

Although ELISA analysis of the urine from the dosed sheep revealed that only low levels of free "zearalenone" were present, treatment of the urine with β-glucuronidase resulted in the detection of much higher levels (Table 2). This is due to hydrolysis of conjugates, which are not recognized by the antibodies (and so are not detected by the ELISA), by the β-glucuronidase. Although this suggests that the urinary conjugates are principally glucuronides, the presence of low levels of sulfatase activity in the β-glucuronidase means that the

Table 2. Zearalenone Metabolite (As Measured by ELISA) and Creatinine Concentrations in Sheep Dosed with Deuterio-1

sample (h)	zearalenone equivalents (ng mL^{-1}) free	total	creatinine (μM)	"zearalenone"/creatinine (μmol/mol)
		Oral Dose		
pre-dose	nd[a]	0.59	4790	0.39
0−2 h	0.33	7.95	3435	7.3
2−4 h	0.38	>50[b]	4896	>32[b]
4−8 h	0.61	>50[b]	10155	>15[b]
8−24	0.42	2.00	8354	0.75
		Intravenous Dose		
predose	nd	0.56	5750	0.31
0−2 h	0.32	2.41	4444	1.7
2−4 h	0.25	1.43	4007	1.1
4−8 h	0.22	2.20	5170	1.3
8−24 h	0.09	1.95	4491	1.4

[a] Not detected. [b] Outside the accurate analytical range of the ELISA.

possibility that some sulfate conjugates are also present in the urine cannot be discounted.

The results from ELISA (Table 2) generally parallel those obtained by GC−MS for the orally dosed (Table 3), but not for the intravenously dosed (Table 4), sheep. According to Frömming and Szejtli (1994), a significant proportion of intravenously administered β-cyclodextrin is usually rapidly excreted in the urine, so the interference with the ELISA may be due to the formation of inclusion complexes between the urinary zearalenone metabolites and β-cyclodextrin present in the urine of the intravenously dosed ewe.

GC−MS Analysis of Urine. Zearalenone and its metabolites (**1**−**6**) were quantitated by GC−MS as their TMS ethers (Tables 3 and 4). Because of the likelihood of partial loss of label during fragmentation in the mass spectrometer, the concentrations of unlabeled **1**−**6** were determined from the M^+ ions of their TMS ethers, and the concentrations of deuterio-**1**−**6** were estimated from the corresponding $(M + 4)^+$ ions. A sample of deuterio-**1** was analyzed in the same manner, and no deuterio-**2**−**6** could be detected; therefore, the deuterio-**2**−**5** found in the urine of the dosed animals—often at concentrations exceeding that of deuterio-**1** (Tables 3 and 4)—are due to metabolism of deuterio-**1**, and not to the presence of contaminating deuterio-**2**−**6** in the administered deuterio-**1**. The presence of an interference affected the values obtained for zearalenone at *m*/*z* 462 but not at *m*/*z* 466, so that apparently elevated values for the levels of undeuteriated **1** (but not of its metabolites **2**−**5**) were obtained for the predose urine samples from both ewes (Tables 3 and 4).

No loss of deuterium was detected by GC−MS when a specimen of deuterio-**1** was stored in the dark in ethanol−water (1:4) at 20 °C for 11 days. Nevertheless, the variation with time of the relative intensities of the M + 2, M + 4, and M + 6 ions of the TMS derivatives of each metabolite in the urine was monitored during GC−MS analysis to assess whether significant loss of the deuterium label occurred during metabolism of **1**. The changes found for **3** were typical, with (M + 2)/(M + 4) increasing by 15% and (M + 6)/(M + 4) decreasing by 4% over the course of the experiment, indicating that slow exchange of deuterium was occurring in vivo. The loss by exchange of deuterium from (M + 4)-**3** appeared to be partially offset by loss of deuterium from more heavily deuteriated molecules (e.g., **3**-d_5) because ((M + 2) + (M + 6))/(M + 4) decreased by only 4% during the 24 h of the experiment. As a result of the deuterium exchange, the concentrations given in Tables 3 and 4 give only an approximate measurement of the degree to which zearalenone (**1**) is converted to metabolites **2**−**5** with time.

That deuterio-**4** and -**5** were found in the urine of sheep dosed with deuterio-**1** demonstrates that ovine metabolism of **1** can produce **4** and **5**, previously regarded as industrial anabolics.

Implications. Sprosen et al. (1995) have established the urinary total "zearalenone" (as measured by ELISA)−creatinine ratio as a marker for zearalenone intoxication of sheep, and as an indicator for the resulting fertility reduction. Levels in excess of 12.5 μmol of "zearalenone"/mol of creatinine are associated with significant reductions in lambing percentages, and such levels were found in 32−68% of New Zealand flocks sampled during a nationwide survey from 1991 to 1994. The "zearalenone"−creatinine ratios found for the dosed animals in the present experiment were within the range normally found in pasture-fed sheep during autumn and would be typical of flocks moderately, but not severely, affected by a natural pasture-sourced zearalenone intoxication (Sprosen et al., 1995). It therefore appears that the qualitative aspects of zearalenone metabolism observed in the present study do not result from administration of unrealistically high doses of **1**.

Table 3. Concentrations of Metabolites of 1 (ng mL^{-1}), Detected as Their TMS Ethers by GC−MS in Selected-Ion Mode at the Indicated Values of *m*/*z*, in Urine Collected from a Ewe at Various Times after an Oral Dose of Deuterio-1 (5 mg)

	zearalenone (**1**)		α-zearalenol (**2**)		β-zearalenol (**3**)		α-zearalanol (**4**)		β-zearalanol (**5**)	
time (h)	*m*/*z* 462	*m*/*z* 466	*m*/*z* 536	*m*/*z* 540	*m*/*z* 536	*m*/*z* 540	*m*/*z* 538	*m*/*z* 542	*m*/*z* 538	*m*/*z* 542
predose	13.5	0.47	0.00	0.00	1.02	0.30	0.00	0.00	0.00	0.00
0−2	9.22	8.90	0.25	11.88	0.52	13.94	0.00	0.80	0.00	0.54
2−4	2.80	2.62	0.00	5.60	0.17	5.76	0.06	0.32	0.00	0.29
4−8	2.92	2.83	0.08	9.31	0.50	12.31	0.00	0.42	0.00	0.50
8−24	7.61	5.20	0.36	17.11	0.98	26.29	0.10	0.73	0.00	0.97

Table 4. Concentrations of Metabolites of 1 (ng mL^{-1}), Detected as Their TMS Ethers by GC−MS in Selected-Ion Mode at the Indicated Values of *m*/*z*, in Urine Collected from a Ewe at Various Times after an Intravenous Dose of Deuterio-1 (1 mg)

	zearalenone (**1**)		α-zearalenol (**2**)		β-zearalenol (**3**)		α-zearalanol (**4**)		β-zearalanol (**5**)	
time (h)	*m*/*z* 462	*m*/*z* 466	*m*/*z* 536	*m*/*z* 540	*m*/*z* 536	*m*/*z* 540	*m*/*z* 538	*m*/*z* 542	*m*/*z* 538	*m*/*z* 542
predose	5.67	0.80	0.60	0.68	1.68	1.19	0.14	0.00	0.00	0.00
0−2	5.57	5.53	0.75	19.50	1.72	32.58	0.07	0.39	0.00	0.55
2−4	2.54	2.25	0.77	6.35	1.62	12.00	0.15	0.35	0.00	0.42
4−8	1.89	2.12	0.41	5.79	0.70	8.75	0.00	0.25	0.00	0.33
8−24	0.94	1.26	0.21	2.48	0.41	3.47	0.09	0.13	0.00	0.27

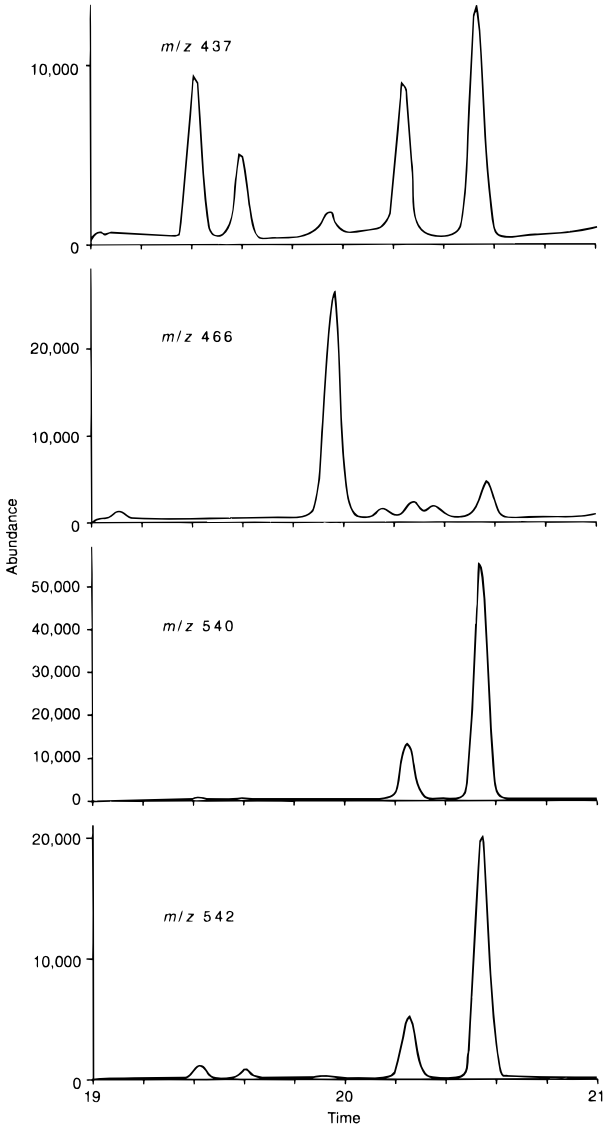


Figure 2. Selected ion chromatograms from GC−MS analysis of a sample of ovine urine collected 0−2 h after the oral dose of deuterio-**1**. The quantitation for this sample appears in Table 3, and retention times for **1**−**6** are given under Materials and Methods.

Furthermore, Erasmuson et al. (1994) found levels of zearalanols (**4** + **5**) of up to 3.3 ng mL^{-1}, and of up to 140 ng mL^{-1} zearalenols (**2** + **3**), in the urine of a control group of rams that were not treated with zeranol. They also found an approximately linear correlation between the concentration of zearalanols and the square root of the concentration of zearalenols in the urine. When the data for the deuteriated metabolites from the dosed animals (Tables 3 and 4) are plotted alongside those from the trial of Erasmuson et al. (1994) (Figure 3), it can be seen that values for the dosed animals fall within the normal range for pasture-fed animals. This suggests that metabolic processing of deuterio-**1** observed in the present study was similar to that which takes place during natural exposure to **1** and was not greatly perturbed by our experimental methodology.

In view of the ability of the ruminal microflora to reduce olefinic and carbonyl bonds in steroids (Miles et al., 1994), oral administration of **1** might be expected to result in significant conversion by ruminal metabolism to zeranol (**4**) and taleranol (**5**). Although ruminal metabolism was not directly investigated in the present experiment, the fact that deuterio-**4** and -**5** were detected in the urine of a sheep dosed intravenously with deuterio-**1** indicates that ruminal metabolism is not a prerequisite for this conversion. Indeed, zearalenone-contaminated feed has been implicated in several recent reports of zeranol residues in nonruminants (Erasmuson et al., 1994; Kennedy et al., 1995).

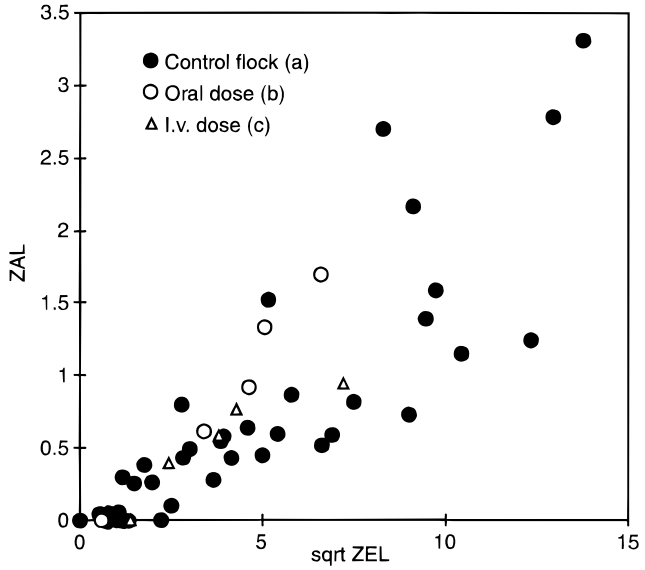


Figure 3. Plot of zearalanol (**4** + **5**) concentration (ng mL^{-1}) versus the square root of zearalenol (**2** + **3**) concentration (ng mL^{-1}) in the urine of (a) a control flock (data from Erasmuson et al., 1994), (b) the ewe dosed orally with deuterio-**1**, and (c) the ewe dosed iv with deuterio-**1**. Data for b and c are taken from Tables 3 and 4, respectively.

There are no published reports of zeranol and taleranol as metabolites of ruminant, or nonruminant, animals dosed with zearalenone. This could, however, be due to the use in many of these studies of HPLC—a method with much lower sensitivity for **4** and **5** than for fluorescent analogs **1**−**3**—for metabolite analysis.

CONCLUSION

Although based on data from only two animals, our results show that sheep (and possibly other livestock, including nonruminants) are capable of metabolizing zearalenone, a common contaminant of both pasture- and grain-based feeds, to zearalenols and on to zearalanols. Our results are consistent with ovine metabolism of **1** taking place as depicted in Figure 1.

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LITERATURE CITED

Blackwell, B. A.; Greenhalgh, R.; Bain, A. D. Carbon-13 and proton nuclear magnetic resonance spectral assignments of deoxynivalenol and other mycotoxins from *Fusarium graminearum*. *J. Agric. Food Chem.* **1984**, *32*, 1078−1083.

Erasmuson, A. F.; Scahill, B. G.; West, D. M. Natural zeranol (α-zearalanol) in the urine of pasture-fed animals. *J. Agric. Food Chem.* **1994**, *42*, 2721−2725.

European Union. Council directive of 7 March 1988 prohibiting the use in livestock farming of certain substances having a

hormonal action (88/146/EEC). *Off. J. Eur. Communities* **1988**, L 70/16.

Frömming, K.-H.; Szejtli, J. Cyclodextrins in Pharmacy. *Topics in Inclusion Science*; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1994; Vol. 5.

Kennedy, D. G.; McEvoy, J. D. G.; Blanchflower, W. J.; Hewitt, S. A.; Cannavan, A.; McCaughey, W. J.; Elliott, C. T. Possible naturally occurring zeranol in bovine bile in Northern Ireland. *J. Vet. Med. B* **1995**, *42*, 509−512.

Lauren, D. R.; di Menna, M. E.; Greenhalgh, R.; Miller, J. D.; Neish, G. A.; Burgess, L. W. Toxin-producing potential of some *Fusarium* species from a New Zealand pasture. *N. Z. J. Agric. Res.* **1988**, *31*, 219−225.

di Menna, M. E.; Lauren, D. R.; Sprosen, J. M.; MacLean, K. S. *Fusarium* and zearalenone on herbage fractions from short and long pasture. *N. Z. J. Agric. Res.* **1991**, *34*, 445−452.

Miles, C. O.; Wilkins, A. L.; Erasmus, G. L.; Kellerman, T. S. Photosensitivity in South Africa. VIII Ovine metabolism of *Tribulus terrestris* saponins during experimentally induced geeldikkop. *Onderstepoort J. Vet. Res.* **1994**, *61*, 351−359.

Muñoz, L.; Castro, J. L.; Cardelle, L.; Castedo, L.; Riguera, R. Acetylated mycotoxins from *Fusarium graminearum*. *Phytochemistry* **1989**, *28*, 83−85.

Pathre, S. V.; Khadikar, P. V.; Mirocha, C. J. Assignment of C-2 and C-4 of zearalenones. *Magn. Reson. Chem.* **1990**, *28*, 370−371.

Peters, C. A. Photochemistry of zearalenone and its derivatives. *J. Med. Chem.* **1972**, *15*, 867−868.

Richardson, K. E.; Hagler, W. M., Jr.; Mirocha, C. J. Production of zearalenone, α- and β-zearalenol, and α- and β-zearalanol by *Fusarium* spp. in rice culture. *J. Agric. Food Chem.* **1985**, *33*, 862−866.

Shipchandler, M. T. Chemistry of zearalenone and some of its derivatives. *Heterocycles* **1975**, *3*, 471−520.

Smith, W. B.; Watson, W. H. A conformational investigation of zearalenone and the 6′-zearalenols by carbon and proton NMR spectroscopy. *Magn. Reson. Chem.* **1987**, *25*, 975−980.

Smith, J. F.; di Menna, M. E.; McGowan, L. T. Reproductive performance of Coopworth ewes following oral doses of zearalenone before and after mating. *J. Reprod. Fert.* **1990**, *89*, 99−106.

Smith, J. F.; Wesselink, C.; Parr, J.; Sprosen, J. M.; Fowke, E. A.; Towers, N. R.; Laboyrie, D. Effect of zearalenone on ewe pregnancy rates. In *Toxinology and Food Safety Research Report 1992−95*; Garthwaite, L., Ed.; AgResearch: Hamilton, New Zealand, 1995; pp 41−43 (ISBN 0-478-04859-9)

Sprosen, J. M.; Armstrong, J. A.; Garthwaite, I.; Towers, N. R. National "zearalenone" exposure survey. In *Toxinology and Food Safety Research Report 1992−95*; Garthwaite, L., Ed.; AgResearch: Hamilton, New Zealand, 1995; pp 40−41 (ISBN 0-478-04859-9)

Still, W. C.; Kahn, M.; Mitra, A. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **1978**, *43*, 2923−2925.

Towers, N. R. Zearalenone induced infertility in sheep. *Proc. 22nd Sheep and Beef Cattle Seminar*; Publication 145 Veterinary Continuing Education; Massey University: Palmerston North, New Zealand, 1992; pp 159−178.

Towers, N. R.; Sprosen, J. M. *Fusarium* mycotoxins in pastoral farming: Zearalenone induced infertility in sheep. In *Recent Advances in Toxinology Research, Vol. 3*; Gopalakrishnakone, P., Tan, C. K., Eds.; Proceedings of the 10th World Congress on Animal, Plant and Microbial Toxins; National University of Singapore: Singapore, 1992. pp 272−284.

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