Mycotoxins Produced by *Fusarium tricinctum* as Possible Causes of Cattle Disease

Fusarium tricinctum, which frequently occurs on tall fescue grass and on corn, produces 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone and 4β,15-diacetoxy - 8α - (3 - methylbutyryloxy) - 12,13-epoxytrichothec-9-en-3α-ol (T-2 toxin) when grown on laboratory media. The acetamido lactone, prepared synthetically from 4-ethoxy-4-hydroxy-2-butenoic acid γ-lactone, was injected intramuscularly at 3.8 mg/kg into a heifer for 90 days and produced dry gangrene at the end of the tail. This lesion is one of the two most characteristic signs sometimes observed in cattle grazing on tall fescue grass. T-2 toxin, isolated from cultures of *F. tricinctum* grown on Sabouraud's agar, was injected at 0.1 mg/kg into a steer for 65 days and caused death from internal hemorrhaging similar to that occasionally found in cattle after ingestion of moldy corn.

Cattle grazing on pastures of tall fescue grass (*Festuca arundinacea* Schreb.) occasionally develop a syndrome called "fescue foot," a noninfectious disease characterized by signs that include loss of weight, arched back, rough hair coat, lameness in the hind quarters, and dry gangrene of the tail and feet (Jacobson *et al.*, 1963; Yates, 1962). Clinical signs of this disease have been produced experimentally in cattle by intraruminal administration of extracts prepared from toxic fescue hay (Jacobson *et al.*, 1963). The sporadic and seasonal nature of fescue toxicity suggested that toxic fungal metabolites were present on areas of fescue pasture (Yates *et al.*, 1969). Extracts of several fungi isolated from tall fescue grass caused death when injected intraperitoneally into mice. Nearly all the toxigenic fungi isolated belong to the genus *Fusarium* (Yates *et al.*, 1969).

One of the major problems of toxicosis in farm animals associated with ingested moldy corn is general internal hemorrhaging sometimes resulting in death (Albright *et al.*, 1964). Preparations from cultures of fungi isolated from corn were tested in laboratory animals, and *Fusarium tricinctum* proved a potent toxin producer (Bamburg *et al.*, 1968a, 1969).

One strain of *F. tricinctum* (NRRL 3249) isolated from tall fescue hay produced the toxins 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone (I, Figure 1), and 4β,15-diacetoxy-8α - (3 - methylbutyryloxy) - 12,13 - epoxytrichothec - 9 - en-3α-ol, trivially called T-2 toxin (III, Figure 1), when grown on laboratory media (Yates *et al.*, 1968). This fungal strain, earlier thought to be *F. nivale*, is now considered an atypical strain of *F. tricinctum*. Compound I had no optical rotation and, presumably, was racemized during isolation. Compound III is also produced by the T-2 strain of *F. tricinctum* isolated from moldy corn (Bamburg *et al.*, 1968b).

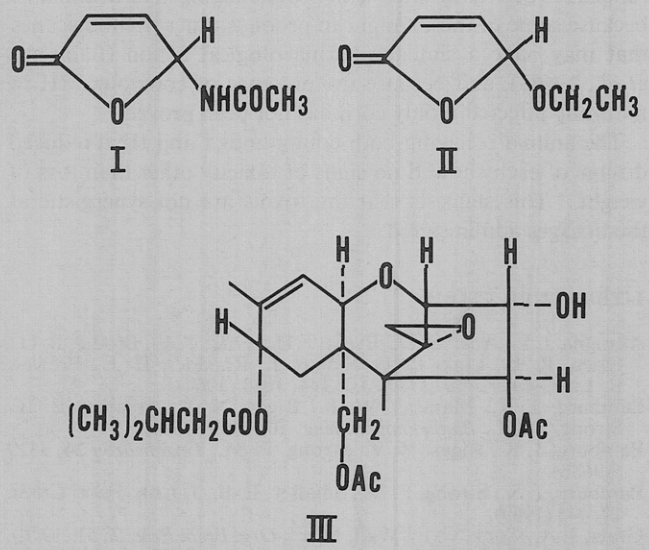
This paper describes the results of intramuscular injection of compounds I and III into cattle and the procedures employed in the preparation of these compounds.

MATERIALS AND METHODS

Preparation of 4-Acetamido-4-hydroxy-2-butenoic Acid γ-Lactone (I). This compound was synthesized in approximately 30-g batches using a modification of the procedure of Gratz *et al.* (1970). The key intermediate, 4-ethoxy-4-hydroxy-2-butenoic acid γ-lactone (II, Figure 1), was prepared by a modified procedure of Schenck (1953) in which oxygen was passed into an irradiated mixture of furfural (4.2 moles), eosin (5.4 mmoles), and vanadium pentoxide (0.55 mmole) in absolute ethanol (1500 ml). The light was provided by a circular arrangement of 26 20-w fluorescent lamps. After 4 days, the solvent was removed *in vacuo*, and the residue was dissolved in carbon tetrachloride and passed through a column of silica gel (Brinkmann 70 to 325 mesh) to remove some polar byproducts. The concentrated carbon tetrachloride eluate was distilled through a spinning band column to give the desired intermediate, bp 42° to 44° C (0.3 mm). The nuclear magnetic resonance (nmr) spectrum was consistent with structure II.

In a typical conversion of the intermediate to compound I, 0.66 mole of compound II was dissolved in concentrated hydrochloric acid (60 ml) and allowed to stand for 15 min. Acetamide (1.4 moles) was added and the mixture heated on a steam bath for 25 min. The reaction mixture was cooled, diluted with tetrahydrofuran to dissolve compound I, and filtered. After removal of the solvent *in vacuo*, the residue was taken up in ethyl acetate, treated with sodium sulfate and Darco G-60 activated carbon, and filtered. The filtrate was concentrated *in vacuo* and the residue crystallized from acetone. The crystals were removed by filtration and washed thoroughly with acetone cooled in dry ice-ethanol to give 20 to 25 g of compound I, mp 115° to 116.5° C, homogeneous on Silica Gel G (Brinkmann) thin-layer chromatography (tlc) (acetone-carbon tetrachloride 1 to 2 v/v; detection with iodine vapor; R_f 0.2). An additional 7 to 15 g of compound I could be obtained from the mother liquor by chromatography on silica gel with ethyl acetate as the eluting solvent. Synthetic 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone was found by mixed mp, tlc, infrared (ir) spectroscopy, and nmr spectroscopy to be identical to compound I isolated from cultures of *F. tricinctum* (Yates *et al.*, 1968).

Production of T-2 Toxin (III). Compound III was isolated from cultures of the T-2 strain of *F. tricinctum* (NRRL 3299, furnished to us by E. B. Smalley, University of Wisconsin). Spore inoculum, prepared by growing the fungus on yeast extract agar (Haynes *et al.*, 1955) at 25° C for 7 days, was streaked over the surface of Sabouraud's agar (Yates *et al.*, 1968) in 40 petri plates. The cultures were grown on a window sill at room temperature for 5 to 10 days. The agar was diced and extracted with ethyl acetate in a Soxhlet extractor for 24 hr. After concentration of the extract *in vacuo*, the residue was covered with carbon tetrachloride and allowed to stand for at least 2 hr. The carbon tetrachloride extract



I II III

Figure 1. Structures of 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone (I), 4-ethoxy-4-hydroxy-2-butenoic acid γ-lactone (II), and T-2 toxin (III)

was filtered through a pad of Celite. After three such extractions, the combined, concentrated filtrates were chromatographed on silica gel (75 g per g of concentrate). Nonpolar material was eluted with chloroform. The solvent was changed to acetone in chloroform (1 to 9 v/v) whereupon compound III was rapidly eluted. Fractions containing compound III according to silica gel G tlc (toluene-ethyl acetate 1 to 3 v/v; spray with concentrated sulfuric acid and heat to reveal a green spot; R_f 0.45) were combined, concentrated, and crystallized from benzene-hexane to give compound III, mp 146° to 147.5° C. Comparison of the compound obtained in this manner with an authentic sample (Bamburg *et al.*, 1968b) (tlc, ir spectroscopy, and nmr spectroscopy) showed them to be identical. Yields of crystalline, homogenous compound III averaged 280 mg per 40 agar plates.

Administration of 4-Acetamido-4-hydroxy-2-butenoic Acid γ-Lactone (I) and T-2 Toxin (III) to Cattle. Beginning Dec 13, 1968, compounds I and III were injected intramuscularly into the shoulders or thighs of cattle so that each quarter received an injection every fourth day (Table I). The cattle were turned out during the day and at night kept in an unheated barn where the temperature was above freezing. Average daily maximum temperatures during the experiment were: Dec 27°, Jan 22°, Feb 31°, and March 33° F. The cattle were fed a diet of tall fescue hay from a source with no previous history of toxicity. Hair was clipped from the tail of each animal, including the control, to aid observations.

Figure 2. Gangrenous tail of heifer receiving 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone (I)

RESULTS AND DISCUSSION

The cattle experiments were performed during the winter because fescue foot occurs more frequently and with greater severity in cold weather than in warm weather. Reduction in blood flow to the extremities in a cold environment might facilitate development of clinical signs of fescue foot (Yates *et al.*, 1969).

The control animal did not exhibit any adverse effects other than failure to gain weight, probably because of the poor nutritional quality of the fescue. The other three animals lost weight during the experiment (Table I).

Table I. Dosage Schedule for Intramuscular Injection of Compounds I and III into Cattle

Compound	Daily Dose		Vehicle	Days	Animal	Weights	
	Mg	Mg/kg				Initial, lb	Final, lb
None	...	...	0.5 ml EtOH and 2.5 ml PG[a]	90	Heifer	450	455
I	1100	3.8	7 ml saline[b]	90	Heifer	630	507
III	30[c]	0.1	0.5 ml EtOH and 2.5 ml PG	65	Steer	650	540
I and III	265 and 18[d]	0.9 and 0.06	0.5 ml EtOH and 5 ml saline	42	Heifer	655	550

[a] PG is propylene glycol. [b] Saline solutions were adjusted to pH 6.5 to 6.8. [c] This dose was selected after adjustments from initial 72-mg dose (3 days) which made the animal anorexic. [d] Received 530 mg of I and 36 mg of III for 6 days; then 36 doses at the lower level.

The end of the tail of the animal receiving compound I had a reddish tint and was scaly on day 15. By day 54 the tip had darkened and there was a distinct white ring around the tail about 4 cm from the tip. By day 61 necrosis at the ring had progressed to the stage that the end of the tail was connected only by a thin strip of bony tissue (Figure 2), which broke on day 89. In the later stages of the experiment, the back of this animal arched; this sign was not observed in any of the other cattle. Postmortem examination, which included inspection of sagittal sections of all eight digits of the feet, revealed no gross abnormalities other than local abscesses which developed at the injection sites. Thus, compound I produced two signs often seen in tall fescue toxicosis—namely, gangrene of the tail and an arched back. However, involvement of this compound in fescue foot remains circumstantial for two reasons. First, there was no hoof damage in these initial experiments with cattle. Second, even though it is produced by a fungus found on toxic fescue, the presence of the compound on fescue grass has not been demonstrated. Once compound I is found on the grass, it must be shown that signs of fescue foot can be produced upon oral administration of the compound at a dose comparable to the amount ingested by cattle in the pasture.

The extent to which tall fescue grass is involved in this disease has not been determined. The disease may be associated with tall fescue simply because the grass is commonly used as a winter pasture. Alternatively, tall fescue may contain factors which predispose the cattle to the effects of compound I.

The animal receiving compound III had a dark red tail tip on day 15; however, the tail did not undergo further change. On day 64 the animal had a thin bloody discharge from both nostrils. By day 65 it experienced difficulty in breathing, had bloody feces, and succumbed that evening. A clotting time taken 12 hr before death was 6 to 7 times normal. Postmortem lesions included: Local abscesses at the injection sites, petechial-ecchymotic hemorrhages of the epi- and endocardium, congestion of cervical lymph nodes, possible bleeding from turbinates, hypostatic congestion of the lungs, scattered ecchymotic hemorrhages in the small intestine, massive hemorrhage into the lumen of the large intestine, and a few petechial hemorrhages on the abomasum. There was a general loss of body fat, including that on the heart as well as the subcutaneous and mesenteric fat. The general internal hemorrhage observed in this animal resembled that seen in cattle affected with the hemorrhagic syndrome resulting from consumption of moldy corn. Here, too, the evidence is still circumstantial because other toxin-producing fungi have been associated with hemorrhaging in farm animals, because some of these fungi can produce other trichothecanes that may have a similar pharmacological action (Bamburg *et al.*, 1968a), and because the presence of compound III in naturally infected moldy corn has not been proved.

The animal receiving both compounds I and III at reduced dosage of each showed no signs of toxicity other than loss of weight. This suggests that the toxins are not synergistic at the dosages administered.

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Michael D. Grove[1]
Shelly G. Yates[1]
William H. Tallent[1]
John J. Ellis[1]
Ivan A. Wolff[1]
Narayanarao R. Kosuri[2]
Roy E. Nichols[2]

[1]Northern Regional Research Laboratory
Agricultural Research Service
U.S. Department of Agriculture
Peoria, Ill. 61604
[2]Department of Veterinary Science
University of Wisconsin
Madison, Wis. 53706

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