Table I. Germination (Percent ±SD) of Uredospores Floated on Water Containing Tween 20 (0.005%) and Coumarin[a]

coumarin, μg/mL (ppm)	% germin	coumarin, μg/mL (ppm)	% germin
0	0.2 ± 0.1	25.0	74.3 ± 9.0
3.1	0.9 ± 0.2	50.0	49.7 ± 2.1
6.2	6.7 ± 1.5	100.0	11.0 ± 2.1
12.5	41.7 ± 7.0	200.0	0.4 ± 0.2

[a] Reported values are means of three replications.

present in the leaf and pod tissue at concentrations too low to markedly effect snap bean rust uredosphore germination. Knowledge of the presence of coumarin in snap bean pods may be important because coumarin has been reported to be a hepatotoxic compound in test animals and is listed as a carcinogenic compound (Dickens and Jones, 1965; Cohen, 1979; Evans et al., 1979).

In summary, coumarin, a biologically active compound, was isolated for the first time from snap bean leaves and pods at concentrations no greater than 3.0 μg/g fresh weight. The concentration of coumarin in the snap bean leaves and pods is not sufficiently high to counteract the uredospore inhibitor and to initiate germination of the snap bean rust uredospores. However, leachate from snap bean leaves was capable of counteracting the uredospore germination inhibitor, indicating the presence of an unknown factor or factors causing germination of the bean rust spores. Additional studies are being conducted to determine the identity of the other chemical constituent or constituents in snap bean tissue capable of counteracting the germination inhibitor.

Registry No. Coumarin, 91-64-5.

LITERATURE CITED

Allen, J. P. In "Encyclopedia of Plant Physiology New Series"; Springer-Verlag: Berlin, 1976; Vol 4, Chapter 2.
Allen, J. P. *Science (Washington, D.C.)* **1972**, *169*, 3497–3500.
Allen, J. P.; Dunkle, L. D. In "Morphological and Biochemical Events in Plant-Parasite Interactions"; The Phytopathological Society of Japan: Tokyo, 1971; pp 23–58.
Allen, J. P. *Phytopathology* **1955**, 45, 259–266.
Cohen, A. J. *Food Cosmet. Toxicol.* **1979**, *17*, 277–289.
Dickens, F.; Jones, H. E. H. *Br. J. Cancer.* **1965**, *19*, 392–403.
Evans, J. G.; Gaunt, I. F.; Lake, B. G. *Food Cosmet. Toxicol.* **1979**, *17*, 187–193.
French, R. C.; Graham, C. L.; Gale, A. W.; Long, R. K. *J. Agric. Food Chem.* **1977**, *25*, 84–88.
Heller, S. R.; Milne, G. W. A. "EPA/NIH Mass Spectral Data Base"; U.S. Department of Commerce: Washington, DC, 1978; Vol. 1, p 419, Reference Spectra 91-64-5.
Macko, V.; Staples, R. C.; Yaniv, Z.; Granados, R. R. In "The Fungal Spore: Form and Function"; Wiley: New York, 1976; pp 73–100.
Macko, V.; Staples, R. C.; Renwick, J. A. A. *Science (Washington, D.C.)* **1970**, *170*, 539–540.
Staples, R. C.; Yaniv, Z. In "Encyclopedia of Plant Physiology New Series"; Springer-Verlag: Berlin, 1976; Vol. 4, pp 88–103.
Stavley, J. R. *Plant Dis.* **1984**, *68*, 95–99.
Thomas, C. A.; Meiners, J. P. *Proc. Am. Phytopath. Soc.* **1977**, *4*, 219–220.
Thomas, C. A. unpublished data.
van Sumere, C. F.; van Sumere-de Preter, C.; Vining, L. C.; Ledingham, G. A. *Can. J. Microbiol.* **1957**, *3*, 847–862.

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Production of Deepoxydeoxynivalenol (DOM-1), a Metabolite of Deoxynivalenol, by in Vitro Rumen Incubation

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A method for the production and purification of deepoxydeoxynivalenol (DOM-1), the only known metabolite of deoxynivalenol (DON), is presented. An in vitro incubation technique with rumen microorgansms was used either with an extract from highly DON-contaminated corn or with DON-contamination corn as a substrate. The incubation of 1500 mg of DON from corn extract yielded, after extraction and purification, 340 mg of DOM-1. The incubation of ground DON-contaminated corn was advantageous over the incubation of the extract from the corn because it was more time and cost effective.

Deoxynivalenol (DON, vomitoxin) is a mycotoxin produced primarily by the fungus *Fusarium graminearum*. Infection of corn and cereal grains by *F. graminearum* and concomitant DON production occur most frequently during those years when the maturation and harvest season is wet and cool. The deleterious effects of DON in swine and laboratory animals have been described (Vesonder et al., 1976; Friend et al., 1982; Chavez, 1984; Khera et al., 1984; Tryphonas et al., 1984; Trenholm et al., 1985). These effects included feed refusal, decreased feed efficiency, reduced weight gain, emesis, depletion of hepatic glycogen, and hypoglycemia. However, very few metabolism studies have been conducted to determine the fate of this toxin in animals. Yoshizawa et al. (1983) were the first to characterize a metabolite of DON, namely DOM-1, in rat urine and feces. In our laboratory we have recently found DOM-1 in milk of cows fed a diet containing DON-contaminated corn. (Cote et al., submitted for publication). Since DON is a frequent contaminant of livestock feed

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grains infected by *Fusarium* species (Vesonder et al., 1977; Trenholm et al., 1981; Côté et al., 1984; Scott, 1984) and DOM-1 might be a contaminant of foods of animal origin, gram quantities of this metabolite are needed for toxicologic testing.

The present study reports a method of producing DOM-1 from *Fusarium* field innoculated corn using an in vitro rumen fermentation technique.

EXPERIMENTAL METHOD

Apparatus and Reagents. Extraction column: 300-mL Chem Tube (Analytichem International, Harbor City, CA). Column: 5-cm i.d., slurry packed with 80 g of silica (70–230 mesh) in chloroform. Column: flash chromatography (Still et al., 1978) 5-cm i.d., slurry packed with 250 g of Florisil (100–200 U.S. mesh) in methylene chloride. TLC plates: silica gel 60 precoated 10 × 20 cm, developed with methylene chloride–methanol (90:10). Spray reagent: sulfuric acid (30% in methanol). HPLC system: Perkin-Elmer Series 4 with a RSIL silica column (ALLTECH 25 cm × 10 mm, 10-μm particle size). HPLC system: Perkin-Elmer Series 10 with a 10 mm × 25 cm C_{18} column (ALLTECH, 10-μm particle size). Detector: UV detector, Perkin-Elmer LC 85. Gas–liquid chromatograph: Hewlett-Packard 5840 A equipped with an electron capture detector. Derivatization agent: trimethylsilyl derivatizing reagent mixed in a ratio of 5:1 (trimethylsilyl)imidazole and trimethylchlorosilane (Sigma Chemical Co., St. Louis, MO).

DON Corn Extraction. The extraction was done according to the procedure of Scott et al. (1984), with the following modifications. A 200-g ground sample was extracted three times with a total volume of 1500 mL of acetonitrile–water (84:16) in a 1-L Erlenmeyer flask in three steps: (1) extraction with 600 mL for 2 h; (2) extraction with 500 mL overnight; and (3) extraction with 400 mL for 2 h. The combined extracts were filtered through a bed of Celite and partitioned against petroleum ether. The petroleum ether layer was discarded, and the aqueous–acetonitrile layer was concentrated to 250 mL prior to its addition to a 300-mL Chem Tube. DON was eluted with ethyl acetate (6 × 150 mL) and concentrated.

DON Column Chromatography. The residue was dissolved in 25 mL of methylene chloride and the resultant mixture added to a flash chromatography column containing a Florisil (250 g) slurry packed in methylene chloride. The column was rinsed with 200 mL of methylene chloride–acetone (95:5) and DON was eluted with 800 mL of methylene chloride–methanol (95:5) at a flow rate of 40 mL/min under low pressure. A total of 20 fractions (40 mL) was collected from which 20-μL aliquots were spotted on TLC plates that were subsequently developed with methylene chloride–methanol (90:10). DON was made visible by heating the plates in an oven at 110 °C for 5 min after spraying them with sulfuric acid. DON was detected in fractions 7–15.

In Vitro Rumen Fermentation Procedure I. Rumen fluid was obtained from a fistulated steer fed alfalfa hay ad libitum. The in vitro procedure was similar to that described by Leedle and Hespell (1980) with the following modifications. Clarified rumen fluid as prepared by centrifuging at 6555*g* for 20 min at 21–25 °C. The supernatant solution was retained, pooled, and distributed in glass bottles and then autoclaved at 15 psi for 20 min. The resulting clarified sterilized rumen fluid constituted 15% of the fermentation medium (Table I). The inoculum for the fermentation was also 15% of the total volume and was obtained fresh from the same fistulated steer mentioned above. Each 1-L Erlenmeyer flask contained 700 mL of

Table I. Composition of the in Vitro Fermentation Incubation Medium

ingredients	%	ingredients	%
clarified rumen fluid	19	cellulose[c]	0.1
mineral soln 1[a]	8	$NaHCO_3$	0.05
mineral soln 2[b]	8	water	54
trypticase	0.1	inoculum	15
starch	0.1	toxin	0.043

[a] Mineral solution 1: K_2HPO_4 (0.6%). [b] Mineral solution 2: KH_2PO_4 (0.6%), $(NH_4)_2SO_4$ (0.6%), NaCl (1.2%), $MgSO_4 \cdot 7H_2O$ (0.2%), $CaCl_2 \cdot 2H_2O$ (0.2%). [c] Chopped unscented tissue paper.

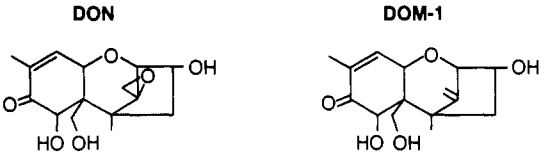

Figure 1. Structure of deoxynivalenol (DON) and its metabolite DOM-1.

total incubate to which 300 mg of DON dissolved in water (1 mL) was added 24 h after the beginning of the fermentation process. The fermentation system was incubated at 37 °C for an additional 5 days during which the pH was monitored and maintained at 6.8.

Extraction of DOM-1. The incubation medium was filtered through a Buchner funnel containing Celite. The filtrate was saturated with NaCl and partitioned three times with ethyl acetate. The combined ethyl acetate extracts (2.1 L) were dried over anhydrous sodium sulfate (ca. 100 g) and filtered. The sodium sulfate was then rinsed with an additional 200 mL of ethyl acetate, and the combined ethyl acetate solution was concentrated with a rotary evaporator.

DOM-1 Column Chromatography. The residue was redissolved in 20 mL of chloroform and added to a column (5 × 50 cm) containing a silica slurry packed in chloroform. The column was rinsed with 200 mL of chloroform–acetone (95:5), and DOM-1 was eluted with 250 mL of chloroform–methanol (90:10). Fractions of 10 mL were collected and monitored on TLC plates. Fluorescent spots were detected in fractions 4–13.

Semipreparative HPLC. The residue was dissolved in ca. 1 mL of methylene chloride–acetone (97:3) and the resultant mixture subjected to a normal-phase HPLC. Fractions of 2 mL were collected and monitored on TLC plates. Fluorescent spots at the R_f of DON were detected in fractions 40–66. Fractions with similar profiles were combined and redissolved in water for separation of DON and DOM-1 by reversed-phase HPLC.

In Vitro Rumen Fermentation and Cleanup Procedure II. A rumen incubation was processed as previously described except that 15 g of DON-contaminated ground corn (1300 ppm), instead of the corn extract, was added directly to 225 mL of medium (four replicates). The extraction of DOM-1 from the incubate was accomplished as described previously. The silica column cleanup was performed as described above except the elution solvent was changed to 1000 mL of chloroform–acetone (70:30). Fractions (ca. 40 mL) were collected, and DOM-1 was eluted in fractions 9–14. Final purification of DOM-1 was accomplished by reversed-phase HPLC using 35% methanol.

Crystallization and Spectroscopy. Chromatography fractions containing DOM-1 from procedure I were evaporated in vacuo, and the residue was subjected to vacuum (0.5 torr) to remove trace amounts of solvent. Proton magnetic resonance were obtained on a Varian 200-MHz spectrometer. Mass spectra were determined at 70 eV by

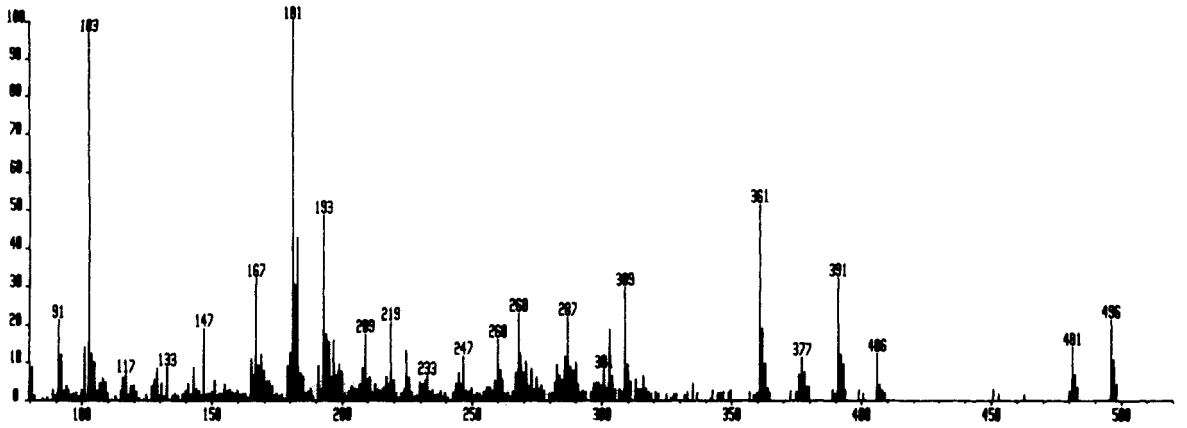

Figure 2. Mass spectrum of DOM-1 trimethylsilyl ether obtained from a VG 7070E, electron impact at 70 eV. DOM-1 retention time was 3.5 min on a 3% OV-17, 3-ft packed column with a flow rate of 35 mL/min at 190 °C.

electron impact with a VG 7070 E GC/MS.

RESULTS AND DISCUSSION

The transformation of DON into DOM-1 (Figure 1) was previously described by King et al. (1984). The method presented herein for the production of DOM-1 was developed with use of field-inoculated corn, highly contaminated with DON (1300 ppm). The concentration of DON in the corn was determined by gas–liquid chromatography (GC) with electron-capture detection. From 3 kg of corn extracted was obtained 3 g crude DON (77% yield).

With rumen incubation procedure I, in which a semipurified corn extract was used, five incubations (300 mg of DON each) yielded 75–90% transformation of DON to DOM-1. Therefore, it was necessary to use a C_{18} reversed-phase HPLC or C_{18} flash chromatography (not described) to separate DON from DOM-1 because this could not be accomplished by normal-phase HPLC or TLC. A total of 340 mg (24% yield) of crystalline DOM-1 was obtained. This amounts does not include several HPLC fractions containing DOM-1 with impurities that were not further purified and crystallized.

The ^{1}H NMR spectrum of DOM-1 taken in acetonitrile-d_3 was as follows (200 MHz): δ 6.54 (dd, J = 6.08, 1.59 Hz, 1 H, C10), 5.12 and 4.94 (2 s, 2 H, C13), 4.81 (d, J = 5.71 Hz, 1 H, C11), 4.56 (d, J = 2.86 Hz, 1 H, C7), 4.12–4.08 (m, 2 H, C3 and C4), 3.80 (d, J = 2.86 Hz, 1 H, C2), 3.68 (m, 2 H, C15), 3.48 (d, J = 3.49 Hz, 1 H), 2.88 (m, 1 H), 2.28 (dd, J = 4.13, 14.6 Hz, 1 H), 1.79 (s, 3 H, C14). The electron-impact mass spectrum of DOM-1 trimethylsilyl ether is given in Figure 2. Molecular ion at m/e 496 was 16 mass units less than DON. The spectrum was consistant with the loss of oxygen and was identical with the spectrum obtained in rat urine (Yoshizawa, 1983).

With rumen incubation procedure II, 15 g of ground DON-contaminated corn, instead of the corn extract and starch, was added directly to the medium. After 3 days of incubation, no DON was detected with GC electron-capture detection. Only two cleanup steps were necessary to obtain DOM-1 at 97.6% purity. The substitution of chloroform–methanol (90:10) with chloroform–acetone (70:30) used as the first cleanup step improved overall purification. The use of chloroform–acetone (70:30), however, required a 4-fold volume of solvent to elute most of the DOM-1.

Incubating the corn directly in the in vitro rumen system provided several advantages over that of the corn extract. These included (1) improved reproducibility among different incubation flasks, (2) less time and effort due to the shorter procedure, (3) less solvent required, and (4) overall reduced cost for production of DOM-1.

This method should facilitate the production of DOM-1 in quantities sufficient for metabolic and toxicologic studies.

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Registry No. DOM-1, 88054-24-4; DON, 51481-10-8.

LITERATURE CITED

Chavez, E. R. *Can. J. Anim. Sci.* **1984**, *64*, 717.
Côté, L.-M.; Reynolds, J. D.; Vesonder, R. F.; Buck, W. B.; Swanson, S. P.; Coffey, T.; Brown, D. C. *J. Am. Vet. Med. Assoc.* **1984**, *184*, 189.
Côté, L.-M.; Dahlem, A. M.; Yoshizawa, T.; Swanson, S. P.; Buck, W. B., sumitted for publication in *J. Dairy Sci.*
Friend, D. W.; Trenholm, H. L.; Elliot, J. I.; Hartin K. E. *Can. J.Anim. Sci.* **1982**, *62*, 1211.
Khera, K. S.; Arnold D. L.; Whalen C.; Angers G.; Scott, P. M. *Toxicol. Appl. Pharmacol.* **1984**, *74*, 345.
King, R. R.; McQueen, R. E.; Levesque D.; Greenhalgh R. *J. Agric. Food Chem.* **1984**, *32*, 1181.
Leedle, J. A. Z.; Hespell, R. B. *Appl. Environ. Microbiol.* **1980**, *39*, 709.
Scott, P. M. *Toxigenic Fungi: Their Toxins and health Hazard*; Kodansha Ltd.: Tokyo, 1984.
Scott, P. M.; Lawrence, G. A.; Telli, A.; Irengar, J. R. *J. Assoc.Off. Anal. Chem.* **1984**, *67*, 32.
Still, W. C.; Khan, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
Trenholm, H. L.; Cochran, W. P.; Cohen, H.; Elliot, J. I.; Farnwoth, E. R.; Freind, D. W.; Hamilton, R. M. G.; Neish, G. A.; Standish, J. F. *J. Assoc. Off. Anal. Chem.* **1981**, *58*, 992A.
Trenlolm, H. L.; Thompson, B. K.; Hartin, K. E.; Greenhalgh, R.; McAllister, A. J. *J. Dairy Sci.* **1985**, *68*, 1000.
Tryphonas, H; O'Grady, L.; Arnold, D. L.; McGuire, P. F.; Karpin-ski, K.; Vesonder, R. F. *Toxicol. Lett.* **1984**, *23*, 17.
Vesonder, R. F.; Ciegler, A.; Jensen, A. H.; Rohwedder, W. K.; Weislender, D. *Appl. Environ. Microbiol.* **1976**, *31*, 180.
Vesonder, R. F.; Ceigler, A.; Rogers, R. F.; Burbridge, K. A.; Bothast R. J.; Jensen, A. H. *Appl. Environ. Microbiol.* **1978**, *36*, 885.
Yoshizawa, T.; Takeda, H.; Ohi, T. *Agric. Biol. Chem.* **1983**, *47*, 2133.

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