Deepoxy T-2 Tetraol: A Metabolite of T-2 Toxin Found in Cow Urine

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T-2 toxin, when orally administered to a lactating cow, is metabolized, and its metabolic derivatives are excreted in the urine, blood, and feces. One of the major metabolites found in the urine was characterized by mass spectrometry as deepoxy T-2 tetraol. The structure of this new metabolite was confirmed by spectroscopic comparison with the synthetic compound obtained by reduction of T-2 toxin to its pentaol (with lithium aluminum hydride) and subsequent dehydration at C12–C13 with trifluoroacetic acid anhydride. This metabolite could be detected in the blood up to 24 h and in the urine up to 48 h after dosage.

T-2 toxin (3-hydroxy-4,15-diacetoxy-8-isovaleroxy-12,13-epoxytrichothec-9-ene) is a trichothecene produced by various species of *Fusarium* and has been implicated in various animal intoxications (Cole et al., 1981).

T-2 toxin, when orally administered to a lactating cow is rapidly transformed to several unidentified metabolites that do not accumulate in any specific organ (Yoshizawa et al., 1981). Recently two of these major metabolites, TC-3 and Iso-TC-1, have been characterized (Yoshizawa et al., 1982; Visconti et al., 1985). Moreover, Yoshizawa et al. (1985) described the deepoxy derivative of 3′-OH HT-2, 3′-OH T-2 triol, and T-2 tetraol in rat excreta. This paper describes a new metabolite found in the urine of a Holstein cow orally dosed with T-2 toxin. The metabolite was found in the urine as long as 48 h after administration of T-2. It could be detected in the whole blood samples collected from the same cow 24 h after administration when no other T-2 metabolites were detected. This paper reports the identification and the structural confirmation of this new metabolite and confirms the original observation of Yoshizawa et al. (1985) in which they reported the occurrence of the deepoxy derivative of T-2 tetraol in rat excreta.

Reagents. T-2 toxin was extracted and purified in our laboratory from *Fusarium sporotrichioides* by using the published procedure of Chi et al. (1977). Purity was checked by thin-layer chromatography and by gas chromatography/mass spectrometry and found to be 99% pure.

Apparatus. (a) Gas chromatograph–mass spectrometer (GC–MS): Hewlett-Packard Model 5978B with a DB5 capillary column (30 m × 0.25 mm) with covalently bonded liquid phase. (b) NMR instrument: Nicolet NT-300 WB FTNMR.

Procedure. A Holstein cow (365 kg) was administered a single dose of T-2 toxin (200 mg) orally in form of a capsule, with a balling gun. Blood was collected from the jugular vein with a heparinized catheter, and urine samples were collected by catheter at regular time intervals for analysis. After 48 h, the same cow was dosed with another 200 mg of T-2 toxin and blood and urine samples were collected.

Extraction and Cleanup. *Extraction of Blood Sample*: A blood sample (5 mL) was added to an equal volume of 30% methanol in water and mixed well with a vortex. The sample was loaded onto a Clean-elute column (#1020) (Analytichem, Harbor City, CA) and was eluted with ethyl acetate (50 mL). Prior to loading, the column was rinsed with 200 mL of ethyl acetate. The eluate was concentrated in rotary evaporator, transferred to a $^1/_2$-dram vial, and derivatized for GC–MS analysis as described in another section of the paper.

Extraction of Urine Sample: Urine samples (10 mL) were loaded onto a Clean-elute column (#1010) (Analytichem, Harbor City, CA). The column was eluted with ethyl acetate (50 mL); the ethyl acetate eluate was concentrated to dryness and dissolved in methanol (1 mL), and 5 mL of water was added. The solution was concentrated to 2 mL and was loaded onto a disposable XAD-2 column (5 × 1 cm). It was rinsed with water (10 mL) and eluted with 90% methanol in water. The methanol eluate was concentrated and derivatized with trifluoroacetic acid anhydride (TFAA) for GC–MS analysis.

Extraction and Isolation of New Metabolites: Urine (500 mL) collected 14 h after the second oral dose of T-2 toxin was loaded directly onto a glass column (40 × 10 cm i.d.) packed with CHEM TUBE hydromatrix (Analytichem, Harbor City, CA). It was eluted with 2 L of ethyl acetate. The ethyl acetate eluate was partitioned with water (200 mL), and the water layer was concentrated to 20 mL in a rotary evaporator. The concentrate (10 mL) was loaded onto a glass C 18 (Water Associates, Milford, MA) column (5 × 1.5 cm i.d.), rinsed with water (20 mL), and then eluted with 30% methanol in water (50 mL). The methanol eluate was concentrated and further purified on TLC plates with chloroform–methanol (80:20) as developing solvent. The purified product was found to contain traces of T-2 tetraol when analyzed by GC–MS.

Gas Chromatography and Mass Spectrometry. *Gas Chromatographic Conditions*: injector temperature, 275 °C; detector temperature, 275 °C; temperature programming, 80–280 °C at 20 °C/min; carrier gas flow, 1 mL/min helium.

Positive Chemical Ionization Conditions: source temperature, 150 °C; electron energy, 200 eV; methane source pressure, 0.6 torr; derivatization, (i) trifluoroacetate (TFA) derivatives prepared by reacting with trifluoroacetic acid anhydride at 60 °C for 30 min and (ii) Me_3Si derivatives prepared by reacting with a mixture of chloroform–TBT (1:1) for 30 min at room temperature.

Synthesis of Deepoxy T-2 Tetraol. *Synthesis of Trichothec-9-ene-3,4,8,12,15-pentaol*: synthesis was performed according to the method of Grove et al. (1984) by reacting T-2 toxin (30 mg) with lithium aluminum hydride (35 mg) in tetrahydrofuran (10 mL) under reflux conditions for 12 h. Its identity was confirmed by using trimethylsily (Me_3Si) ether and trifluoroacetate derivatives by GC–MS and also by proton NMR.

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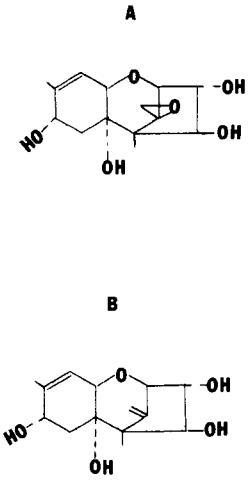

Figure 1. Structure of T-2 tetraol (A) and deepoxy T-2 tetraol (B).

Reaction of T-2 Pentaol with Trifluoroacetic Acid Anhydride (TFAA). A mixture of T-2 pentaol (5 mg) and trifluoroacetic acid anhydride (2 mL) was allowed to react at 60 °C for 12 h. Excess TFAA was removed under a gentle stream of nitrogen. It was hydrolyzed with 2 N ammonium hydroxide solution in methanol. GC–MS analysis of the TFA derivative of the hydrolyzed product showed it to be a mixture of three major compounds. (1) Deepoxy T-2 tetraol TFA derivative: yield 30%; retention time 8.26 min; m/z 667 (MH^+). (2) T-2 pentaol TFA derivative (completely derivatized): yield 40%; retention time 8.48 min; (MH^+) – TFA m/z 667. (3) T-2 pentaol TFA derivative with one underivatized hydroxyl group (presumably tertiary): yield 20%; retention time 9.53 min; ($MH^+ - H_2O$) m/z 571.

This mixture was concentrated and purified on TLC plate by using chloroform–methanol (80:20) as developing solvent.

NMR Spectroscopy. ^1H NMR of purified deepoxy T-2 tetraol was taken in deuterated chloroform.

RESULTS AND DISCUSSION

The blood and urine samples collected after the first and second dose were analyzed by GC–MS. A new metabolite, whose TFA derivative showed a protonated molecular ion at m/z 667, 16 mass units less than T-2 tetraol TFA, was detected. A 16-mass-unit shift was also observed in all diagnostic fragments as compared with corresponding ions in T-2 tetraol TFA derivative. Its retention time was 0.6 min earlier than T-2 tetraol. This new metabolite could be detected in the blood after 24 h and in the urine up to 48 h, but is maximum production in the urine occurred 13 h after the second administration of T-2 toxin to the cow. Urine collected at that particular time was used for extraction and purification of this compound. This metabolite has an R_f value of 0.6 in chloroform–methanol (80:20), identical with that of T-2 tetraol. Because of the difficulty in resolving this compound from T-2 tetraol on a TLC plate using different solvent systems, it was necessary to synthesize it chemically for its structural confirmation. The synthetic product when sprayed with a 20% methanolic sulfuric acid solution charred; it showed a brown spot that fluoresced under wavelength irradiation (365 nm). Its color reaction after treatment with Katos reagent (Takitani et al., 1979) was negative, suggesting the absence of the 12,13-epoxy function in the new metabolite.

TFA derivatives of both T-2 tetraol (Figure 1a) and the new metabolite (Figure 1b) were analyzed by GC–MS in methane chemical ionization (CI), and the results are compared in Table I.

Table I clearly shows that the molecular weight of the TFA derivative of the new metabolite is 666, i.e. 16 mass units less than the molecular weight of T-2 tetraol TFA derivative (682). This compound undergoes fragmentation like T-2 tetraol (TFA), losing a TFA group sequentially to form the major diagnostic ions. The molecular ion was

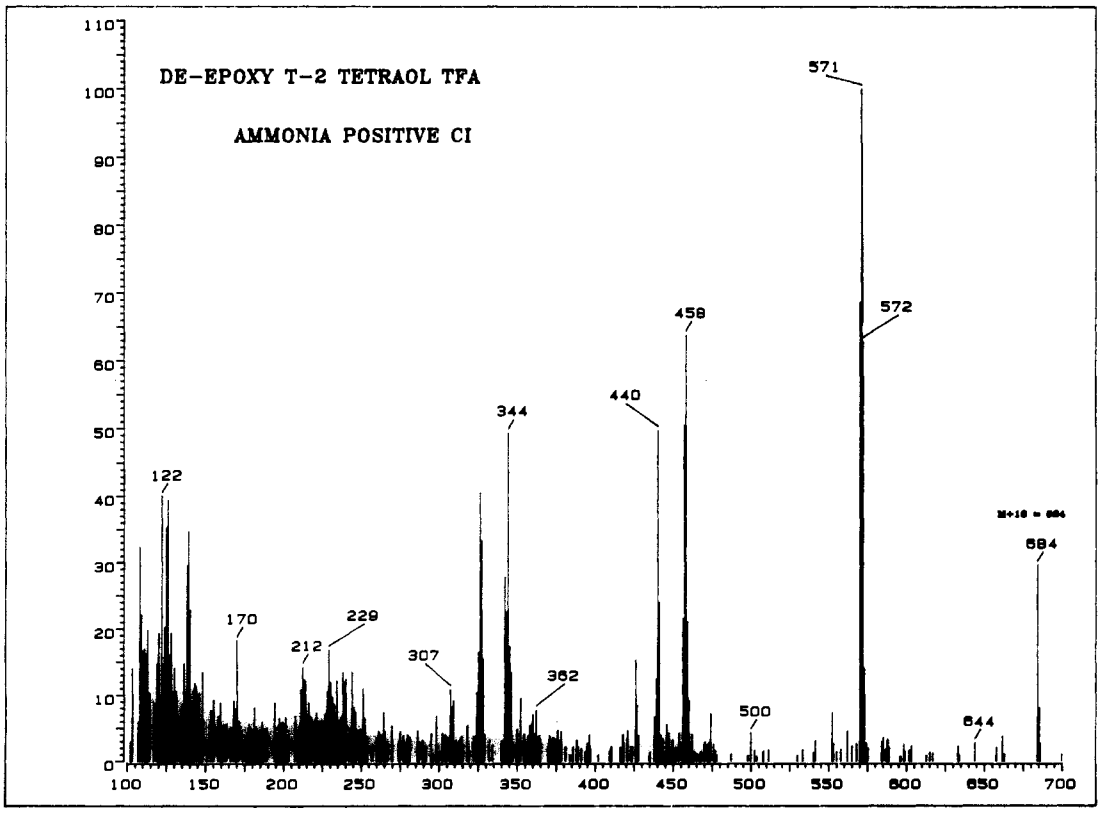

Figure 2. Positive chemical ionization mass spectrum of deepoxy T-2 tetraol in ammonia. (M + 18) is at m/z 684.

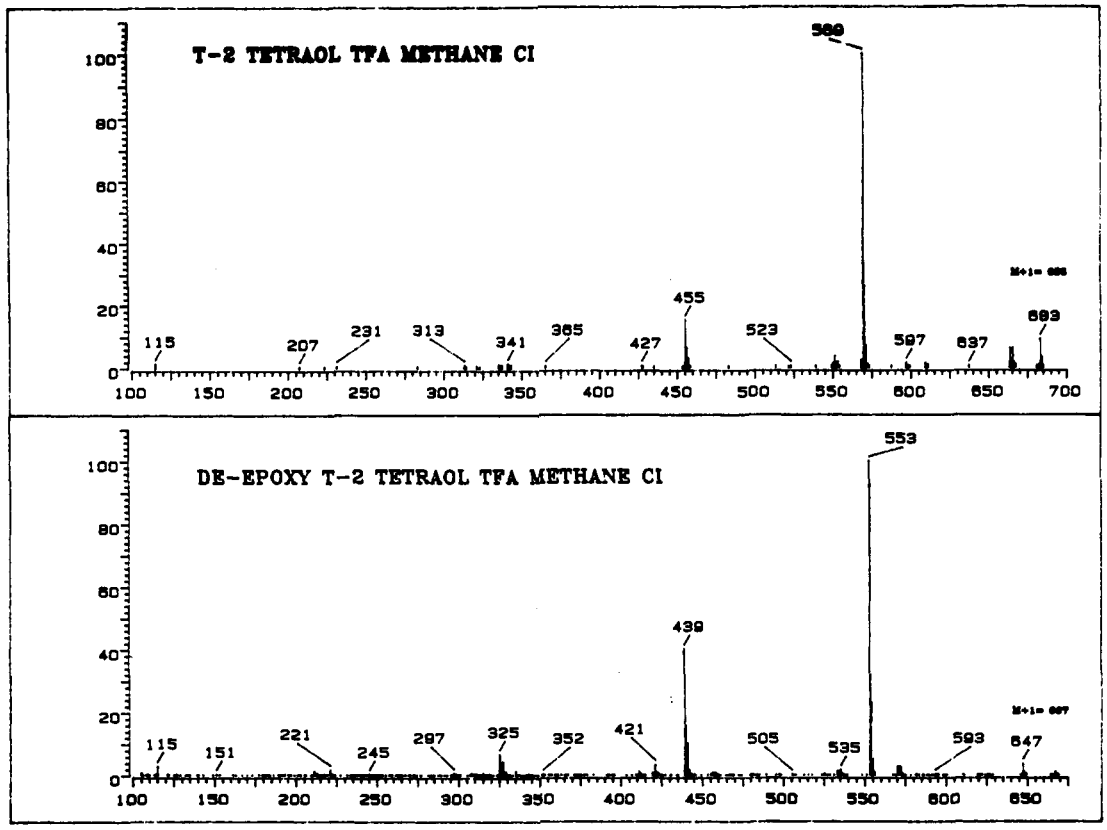

Figure 3. Mass spectra: (A) T-2 tetraol (trifluoroacetate derivative) in positive chemical ionization (methane). The base peak is at m/z 569, and the MH^+ is at m/z 683. Characteristic fragments are at m/z 455, 569, and 683. (B) deepoxy T-2 tetraol (trifluoroacetate derivative) in positive chemical ionization (methane). Fragments at m/z 439, 553, and 667 ($M^+ + 1$) correspond to m/z 455, 569, and 683 of T-2 tetraol except for the loss of an oxygen atom from the oxirane ring.

Table I. Comparison of Diagnositc Masses (m/z) of T-2 Tetraol and Deepoxy T-2 Tetraol in Positive Chemical Ionization (Methane)

	T-2 tetraol TFA	deepoxy T-2 tetraol of TFA
ret time on 30-m capillary col, min	8.88	8.26
MH^+	683	667
MH^+ - TFA	569	553
MH^+ - 2 TFA	455	439
MH^+ - 3 TFA	341	325

further confirmed when the TFA derivative was analyzed by positive chemical ionization in ammonia; m/z 684 (666 + 18) was obtained (Figure 2). In order to show that the deepoxy compound was not produced from T-2 tetraol during the derivatization process with TFAA, its Me_3Si derivative was also compared with Me_3Si T-2 tetraol. The Me_3Si derivative of the new metabolite had MH^+ at m/z 571, which is 16 mass units less than the Me_3Si derivative of T-2 tetraol (MH^+ at m/z 587).

The proton magnetic resonance spectrum of T-2 tetraol analyzed in our laboratory showed a doublet at δ 2.79 and 3.01 that is due to methylene protons of the epoxide group at C-13 position. In contrast the spectrum of the new metabolite showed two singlets at δ 4.85 and the other at δ 5.18 that can be attributed to the terminal methylene group at C-13 position.

On the basis of these data, the chemical structure of the new metabolite is described as 3,4,8,15-tetrahydroxytrichothec-9,12-diene. The mass spectrum of the trifluoroacetate derivative of T-2 tetraol and the deepoxy T-2 tetraol is shown in Figure 3.

Recently, a deepoxy derivative of T-2 tetraol was identified in rat excreta by Yoshizawa et al. (1985). The presence of this metabolite in the blood and urine of animals suggests the possibility that it can be used as a means of monitoring T-2 intoxication of farm animals.

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Registry No. T-2 toxin, 21259-20-1; deepoxy T-2 tetraol, 99531-48-3.

LITERATURE CITED

Chi, M. S.; et al. *Poult. Sci.* **1977**, *56*, 103–116.
Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites*; Academic: New York, 1981.
Grove, M. D.; Burmeister, H. R.; Taylor, S. L.; Weisleder, D.; Plattner, R. D. *J. Agric. Food Chem.* **1984**, *32*, 541–544.
Takitani, S.; Asabe, Y.; Kato, T.; Suzuki, M.; Ueno, Y. *J. Chromatogr.* **1979**, *127*, 33.
Visconti, A.; Treeful, L.; Mirocha, C. J. *Biomed. Mass Spectrosc.*, in press.
Yoshizawa, T.; Mirocha, C. J.; Behrens, J. C.; Swanson, S. P. *Food Cosmet. Toxicol.* **1981**, *19*, 31.
Yoshizawa, T.; Sakamoto, T.; Kuwamura, K. *Appl. Environ. Microbiol.* **1985**, *50*, 676–679.
Yoshizawa, T.; Sakamoto, T.; Ayano, Y.; Mirocha, C. J. *Agric. Biol. Chem.* **1982**, *16*, 2613–2615.

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