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Preparation and Characterization of the Deepoxy Trichothecenes: Deepoxy HT-2, Deepoxy T-2 Triol, Deepoxy T-2 Tetraol, Deepoxy 15-Monoacetoxyscirpenol, and Deepoxy Scirpentriol

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The production of deepoxy metabolites of the trichothecene mycotoxins T-2 toxin and diacetoxyscirpenol, including deepoxy HT-2 (DE HT-2), deepoxy T-2 triol, deepoxy T-2 tetraol, deepoxy 15-monoacetoxyscirpenol, and deepoxy scirpentriol is described. The metabolites were prepared by in vitro fermentation with bovine rumen microorganisms under anaerobic conditions and purified by normal and reverse-phase high-pressure liquid chromatography. Capillary gas chromatographic retention times and mass spectra of the derivatized metabolites were obtained. The deepoxy metabolites were significantly less toxic to brine shrimp than were the corresponding epoxy analogs. Polyclonal and monoclonal T-2 antibodies were examined for cross-reactivity to several T-2 metabolites. Both HT-2 and DE HT-2 cross-reacted with mouse immunoglobulin monoclonal antibody 15H6 to a greater extent than did T-2 toxin. Rabbit polyclonal T-2 antibodies displayed greater specificity to T-2 toxin compared with the monoclonal antibody, with relative cross-reactivities of only 17.4, 14.6, and 9.2% for HT-2, DE HT-2, and deepoxy T-2 triol, respectively. Cross-reactivity of both antibodies was weak for T-2 triol, T-2 tetraol, 3'OH T-2, and 3'OH HT-2.

The trichothecene mycotoxins are a group of toxic sesquiterpenoids produced by several genera of fungi and, most notably, species of the genus *Fusarium* (1). The basic ring structures of trichothecenes and their corresponding deepoxy analogs are shown in Fig. 1. T-2 toxin, diacetoxyscirpenol (DAS), nivalenol, and deoxynivalenol (DON, vomitoxin) are four of the more important members of the trichothecene mycotoxins. All four of these trichothecenes have been detected as naturally occurring contaminants of agricultural commodities. Consumption of trichothecene-contaminated feeds by livestock and poultry has been associated with adverse health effects including feed refusal, reduced feed efficiency, reduced weight gains, diarrhea, intestinal irritation, emesis, decreased immune response, and death (1, 9, 12, 17, 18, 25).

Pharmacokinetic studies have demonstrated that T-2 rapidly disappears from the plasma of animals which were administered T-2 intravascularly, with mean plasma depletion half-lives of 13.8, 17.4, and 5.3 min for swine (2), cattle (2), and dogs (22), respectively. DAS also has a short plasma half-life in animals, with a half-life of 11.6 min in swine and 6.4 min in cattle (7).

T-2, DAS, and DON are all extensively biotransformed in vivo to a variety of metabolites. Pathways of biotransformation include glucuronide conjugation (8, 10), ester hydrolysis (8, 22), oxidation of the C-8 isovaleryl group (8, 31), and epoxide reduction (4, 10, 21, 29, 30, 32, 33). Removal of oxygen from the epoxide group of a trichothecene mycotoxin to yield a carbon-carbon double bond was originally reported by Yoshizawa et al. (33). They found that rats receiving orally administered DON excreted a deepoxy metabolite which the authors labeled DOM-1 (deepoxy deoxynivalenol). Subsequently, deepoxy metabolites have been detected as in vitro biotransformation products of trichothecenes (11, 15, 23, 30), as well as in vivo metabolites of T-2 toxin (4, 8, 30, 32; R. L. Pfeiffer, S. P. Swanson, and W. B. Buck, submitted for publication), DAS (21), and DON (10, 20, 29, 33). Since the deepoxidation of trichothecene mycotoxins is an important pathway of metabolism in animals, quantities of pure metabolites are needed for toxicological evaluation and for analytical standards. Presently, standards for deepoxy trichothecenes remain limited. Côté et al. (11) previously reported a method for the production of deepoxy deoxynivalenol. Recently, we reported that rumen microflora biotransform T-2 and DAS under anaerobic conditions to give deepoxy products, including deepoxy HT-2 (DE HT-2), deepoxy T-2 triol (DE TRIOL), deepoxy 15-monoacetoxyscirpenol (DE MAS), and deepoxy scirpentriol (DE SCP) (23). In the present study, we describe a method for production and purification of the deepoxy metabolites of T-2 and DAS and results of the toxicity and anti-T-2 antibody cross-reactivity studies with the purified products.

MATERIALS AND METHODS

Chemicals. T-2 toxin was prepared in our laboratory from cultures of *Fusarium sporotrichioides*. DON and DAS were purchased from Myco Lab Co., Chesterfield, Mo. Deepoxy deoxynivalenol was prepared from DON as previously described (11). HT-2, T-2 triol (TRIOL), T-2 tetraol, 15-monoacetoxyscirpenol (MAS), and scirpentriol (SCP) were prepared by alkaline hydrolysis of T-2 and DAS, respectively (28). 3'OH T-2 and 3'OH HT-2 were prepared by incubating T-2 toxin with S-9 liver fractions (centrifuged [9,000 × *g*] supernatant fraction of liver homogenate) prepared from rats treated with phenobarbital (16). All solvents were glass distilled (Fisher Scientific Co., Itasca, Ill.).

Culture conditions. Fresh ruminal fluid was obtained from a fistulated dairy cow maintained on a timothy hay diet. Inoculum was prepared by filtering rumen contents through cheesecloth and blending the filtrate for 1 min under a carbon dioxide atmosphere. A total of 567 ml of clarified rumen

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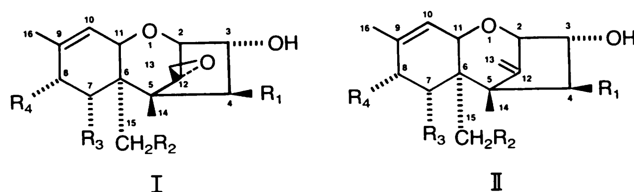

FIG. 1. Structures of trichothecenes (I) and their corresponding deepoxy analogs (II). (See Table 1 for composition of R groups.)

fluid-mineral medium (11) was added to 1-liter Erlenmeyer flasks containing 100 mg of T-2 toxin or 50 mg of DAS. The flasks were bubbled with carbon dioxide for 5 min, and then 133 ml of inoculum was added, and the pH was adjusted to 6.8 with sodium carbonate. The flasks were stoppered with fermentation locks and incubated for 5 days at 37°C.

Extraction. The incubation mixtures were filtered through a bed (2 by 15 cm) of Celite 545 (Johns-Manville Products Corp., Manville, N.J.) under vacuum, and the bed was washed with 200 ml of water. To the aqueous filtrates, 500 g of Amberlite XAD-4 (resin; Rohm & Haas Co., Philadelphia, Pa.) was added, and the solutions were shaken for 12 h. The mixture was transferred to a column (2.5 by 12.5 cm), the aqueous layer was drained, and the column was washed with an additional 500 ml of water. The toxins were eluted with 0.5 liters of acetone, and the eluate was concentrated to dryness on a rotary evaporator.

Column chromatography. The crude toxin extracts were redissolved in a minimum volume of dichloromethane-methanol (9:1, vol/vol) and chromatographed on a column (2.5 by 12.5 cm) of Florisil (60/100 mesh) (Fisher Scientific Co., Itaska, Ill.). The column was rinsed with 100 ml of dichloromethane-acetone (97:3, vol/vol), and the toxins were eluted with 400 ml of dichloromethane-methanol (9:1, vol/vol). Fractions were monitored by silica thin-layer chromatography with chloroform-methanol (9:1, vol/vol) as the solvent system. Metabolites were visualized by charring with sulfuric acid. DE MAS and MAS coeluted in the first 200 ml, whereas SCP and DE SCP coeluted in the next volume (300 to 500 ml). Separation of HT-2 and DE HT-2 from TRIOL and DE TRIOL was not achieved with this column. Appropriate fractions were combined and concentrated to dryness for further high-pressure liquid chromatography (HPLC) purification.

HPLC purification. Normal phase purification of metabolites was accomplished by using a 10-μm silica column (25 cm by 10 mm; Alltech Associates, Inc., Applied Science Div., Deerfield, Ill.) at a flow rate of 2 ml/min. Appropriate fractions from the Florisil columns were combined and chromatographed with the following mobile phases: DE MAS, chloroform-acetone (95:5, vol/vol); DE SCP, chloroform-acetone (9:1, vol/vol); DE HT-2 and DE TRIOL, chloroform-acetone gradient from 96:4 (vol/vol) to 9:1 (vol/vol) over 60 min. Fractions (1 ml) were collected and monitored by both thin-layer chromatography and gas-liquid chromatography. Appropriate fractions were combined and further purified by reverse-phase HPLC on a 10 μm C-18 column (10 mm by 25 cm; Alltech) at a flow rate of 2 ml/min. Mobile phases and elution volumes were as follows: DE SCP, 40% methanol (50 to 62 ml); DE MAS, 50 to 100% methanol (70 to 94 ml); DE HT-2, 60 to 100% methanol (58 to 76 ml); and DE TRIOL, 50 to 100% methanol (79 to 92 ml). Crystallization of the metabolites was effected by dissolving the purified metabolites in a minimal volume of ethyl acetate and slowly dripping the solution into hexane. Crystals were collected and dried overnight in a vacuum desiccator.

Preparation of deepoxy tetraol. DE HT-2 (1.0 mg) was hydrolyzed with 0.1 N NaOH in 95% methanol (0.5 ml) for 45 min at room temperature. After removal of the solvent, the mixture was redissolved in chloroform-acetone (1:3, vol/vol) and chromatographed on a 1.0-g column of silica (Analytichem, Harbor City, Calif.) in the same solvent.

Toxicity testing. Brine shrimp toxicity bioassays were performed as described by Bergers et al. (3).

T-2 antibody cross-reactivity determinations. Rabbit polyclonal antibodies were prepared with T-2 hemisuccinate–bovine serum albumin conjugate as described by Chu et al. (5). Mouse immunoglobulin G monoclonal antibody 15H6 (14), purified from mouse ascites fluid, was obtained from the U.S. Department of Defense. The specificity of the antibody for the various epoxy and deepoxy T-2 metabolites was determined in competitive binding analyses by varying the concentrations of unlabeled metabolites in the presence of a constant quantity of antiserum to give 50% binding inhibition with tritium-labeled T-2 toxin (5). Tritium-labeled T-2 toxin was synthesized (1 Ci/mM) with the tritium label in the C-3 position by the method of Wallace et al. (26). Radiochemical purity was >99% as determined by thin-layer chromatography.

Derivatization and GC. The corresponding trimethylsilyl (TMS) derivatives of the metabolites were formed before gas chromatography (GC) analysis by dissolving 25 to 50 μg of the compounds in 50 μl of TMS reagent (11:2:3, vol/vol/vol; bis[TMS]trifluoroacetamide, trimethylchlorosilane, trimethyl silylimidazole) and heating for 10 min at 60°C. After cooling to room temperature, the mixture was diluted with 50 μl of ethyl acetate containing triacontane ($C_{30}H_{62}$, 0.5 mg/ml) as an internal standard. Trifluoroacyl (TFA) derivatives were prepared as previously described (16). Capillary GC was performed on a model 5790 chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame ionization detector and a DB1701 capillary column (30 m by 0.25 mm [inside diameter] by 0.25 μm film; J & W Scientific, Folsom, Calif.). The column oven was programmed from 250°C to 275°C (hold, 5 min) at 5°C/min for analysis of TMS derivatives and from 225°C to 275°C (hold, 5 min) at 5°C/min for analysis of the TFA derivatives. Other conditions included detector, 300°C; injector, 275°C; and a hydrogen carrier gas linear velocity of 45 cm/s. A split-injection mode was used with a split ratio of 40/1.

GC-mass spectrometry (MS) of the TMS and TFA trichothecene derivatives was accomplished on a model 300 series mass spectrometer (Extranuclear, Pittsburgh, Pa.) with positive and negative methane chemical ionization. Ionization conditions were as follows: source temperature, 150°C; electron energy, 200 eV.

RESULTS AND DISCUSSION

The deepoxy metabolites DE HT-2, DE TRIOL, DE MAS, and DE SCP were prepared by preparative incubation of T-2 and DAS with rumen microflora under anaerobic conditions. Crystals of DE HT-2 were not obtained, but rather an amorphous white solid was produced upon drying in a vacuum desiccator. Final yields of purified metabolites were as follows: DE HT-2, 20 mg; DE TRIOL, 7 mg; DE MAS, 7 mg; DE SCP, 5 mg. Deepoxy tetraol was not detected in any incubation mixture.

Alkaline hydrolysis of DE HT-2 yielded a more polar compound which remained near the origin of the silica gel

TABLE 1. Capillary GC retention times of trichothecenes and their corresponding deepoxy analogs[a]

Compound (compound no.)	RT[b]	RT[c]	R_1	R_2	R_3	R_4	Ring
T-2 (1)	8.256	10.683	OAc	OAc	H	ISV	I
HT-2 (2)	7.528	7.573	OH	OAc	H	ISV	I
DE HT-2 (3)	5.675	5.772	OH	OAc	H	ISV	II
TRIOL (4)	5.945	6.165	OH	OH	H	ISV	I
DE TRIOL (5)	4.455	4.572	OH	OH	H	ISV	II
TETRAOL (6)	3.702	3.735	OH	OH	H	OH	I
DE TETRAOL (7)	2.629	2.581	OH	OH	H	OH	II
DAS (8)	4.459	6.478	OAc	OAc	H	H	I
MAS (9)	3.865	4.235	OH	OAc	H	H	I
DE MAS (10)	2.848	2.972	OH	OAc	H	H	II
SCP (11)	3.109	3.035	OH	OH	H	H	I
DE SCP (12)	2.310	2.212	OH	OH	H	H	II
DON (13)	3.023	3.345	H	OH	OH	=O	I
DOM-1 (14)	2.430	3.080	H	OH	OH	=O	II
ISTD	7.046[d]	9.075[e]					

[a] See Fig. 1 for the structures of the trichothecene skeleton (ring I) and their corresponding deepoxy analogs (ring II) and locations of R groups 1 to 4. Abbreviations: RT, retention time; Ac, acetyl; ISV, $OCOCH_2CH(CH_3)_2$; DE TETRAOL, deepoxy T-2 tetraol; TETRAOL, T-2 tetraol; DOM-1, deepoxy deoxynivalenol; ISTD, internal standard; =O, double-bonded oxygen.

[b] GC retention times (minutes) of corresponding TMS ether derivatives. See Materials and Methods for chromatographic conditions.

[c] GC retention times (minutes) of TFA ester derivatives.

[d] Triacontane internal standard ($C_{30}H_{62}$).

[e] *N*-Octacosane internal standard ($C_{28}H_{58}$).

thin-layer chromatography plate, and cochromatographed with T-2 tetraol. GC analyses of the TMS ether and TFA ester derivatives of the resulting hydrolysis products revealed a single peak identified as deepoxy T-2 tetraol by GC-MS, with M + H at *m/z* 667 (positive chemical ionization) and M^+ at *m/z* 666 (negative chemical ionization) as the TFA derivatives and M + H at *m/z* 571 (positive chemical ionization) as the TMS derivative.

Deepoxy metabolites of T-2 and DAS separated poorly by thin-layer chromatography from their epoxy analogs (21, 32). In the present study, normal phase column chromatography also did not completely separate the deepoxy trichothecenes from their epoxy analogs. Although normal phase HPLC was more efficient in resolving the metabolites, the separation was not adequate to eliminate all epoxy trichothecenes from the deepoxy fractions and additional purification by reverse-phase HPLC was required. Deepoxy trichothecenes eluted later than their epoxy analogs by reverse-phase HPLC. Attempts to use only reverse-phase HPLC for purification were unsuccessful because colored substances contaminated the deepoxy fractions. With the combination of both normal and reverse-phase HPLC, the metabolites DE HT-2, DE TRIOL, deepoxy tetraol, DE MAS, and DE SCP were obtained with purities of ≥99.5%, as determined by capillary GC of the derivatized products.

Both the TFA and TMS derivatives of the deepoxy trichothecenes eluted earlier than did their epoxy analogs by GC. The TMS derivatives were more stable (16) and were preferred for routine analysis; however, TFA derivatives were utilized for mass spectral analysis because of their greater sensitivity. GC retention times of the epoxy and deepoxy trichothecenes are given in Table 1.

The mass spectra of compounds 1 through 12 were determined by methane-positive chemical ionization of the TFA and TMS derivatives. The mass spectra of compounds 3, 5, 7, 10, and 12 (TMS ether derivatives) are given in Fig. 2. In general, the major fragments of the deepoxy trichothecenes were each 16 mass units less than that of the corresponding epoxy congener. The chemical ionization mass spectra of TFA derivatives displayed less fragmentation than did those of the corresponding TMS derivatives. Although the TFA ester derivatives gave greater sensitivity by GC-MS compared with the TMS derivatives, the stability of the TMS ether derivatives was much greater. Degradation was detected within 24 h with the TFA derivatives, whereas TMS derivatives were stable for at least 1 week (data not shown).

As in previous studies (23), direct deepoxidation products of T-2 or DAS (deepoxy T-2 or deepoxy DAS) were not detected; only their deacylation products were. Because of the relatively slow rate of epoxide reduction observed with this in vitro system, coupled with the more rapid rate of C-4 ester hydrolysis, it is unlikely that direct epoxide reduction occurs with trichothecenes containing a C-4 ester.

The comparative toxicities of T-2, DAS, and their metabolites were established by a brine shrimp bioassay (Table 2). The 50% lethal concentration of T-2 in this bioassay system, 111 ng/ml, was similar to the previously reported value of 86 ng/ml by Bergers et al. (3). The deacylated products of T-2 displayed a general decrease in toxicity corresponding to a loss of esterified side groups. In contrast to T-2 toxin, MAS, the initial C-4 hydrolysis product of DAS, was more toxic to brine shrimp than the parent compound was. The relative toxicities of DAS and MAS apparently are dependent upon the test system employed. DAS is a more potent protein synthesis inhibitor in Vero cells, whereas MAS is more toxic than DAS to mice administered these mycotoxins intraperitoneally or subcutaneously (24). Although the C-4 hydrolysis product MAS was slightly more toxic than DAS in the brine shrimp bioassay, hydrolysis of all esters to yield the parent alcohol SCP resulted in a significant decrease in toxicity. In all cases, the deepoxy trichothecene analogs displayed little or no toxicity to the brine shrimp under these bioassay conditions. The highest concentration of the deepoxy metabolites examined, 6,000 ng/ml, resulted in the death of less than 50% of the brine shrimp. Reduction of the epoxide group produced at least a 50-fold reduction in toxicity compared with T-2 toxin. Taking into consideration the finding that the deepoxy metabolite preparations were 99.5% pure and the major contaminants were the corresponding epoxy congeners, epoxide reduction may be viewed as a significant single-step detoxification reaction.

To date, little information pertaining to the toxicity of deepoxy trichothecenes has been reported. Yoshizawa et al. (30) found that the acute toxicity of deepoxy 3′OH HT-2 to mice was extremely low compared with its epoxy analog 3′OH HT-2. In the present study, the deepoxy trichothecenes exhibited little or no toxicity to brine shrimp, thus demonstrating that the reduction of the epoxide group in trichothecene mycotoxins by anaerobic microflora is an effective means of detoxification. The role that epoxide reduction plays as a pathway for trichothecene detoxification in situ and possible species variations in toxicity remains to be established.

The binding specificities of two different types of anti-T-2 antibodies were assessed by a competitive radioimmunoassay. The cross-reactivity of compounds 2 through 6 to the antibodies relative to T-2 toxin is given in Table 3. The results demonstrate that the rabbit polyclonal antibodies were more specific for T-2 toxin than were the mouse monoclonal antibodies. HT-2 and DE HT-2 cross-reacted less than 20% of the time compared with T-2 toxin. The polyclonal antibodies displayed little capacity to bind TRIOL, DE TRIOL, T-2 tetraol, 3′OH T-2, or 3′OH HT-2.

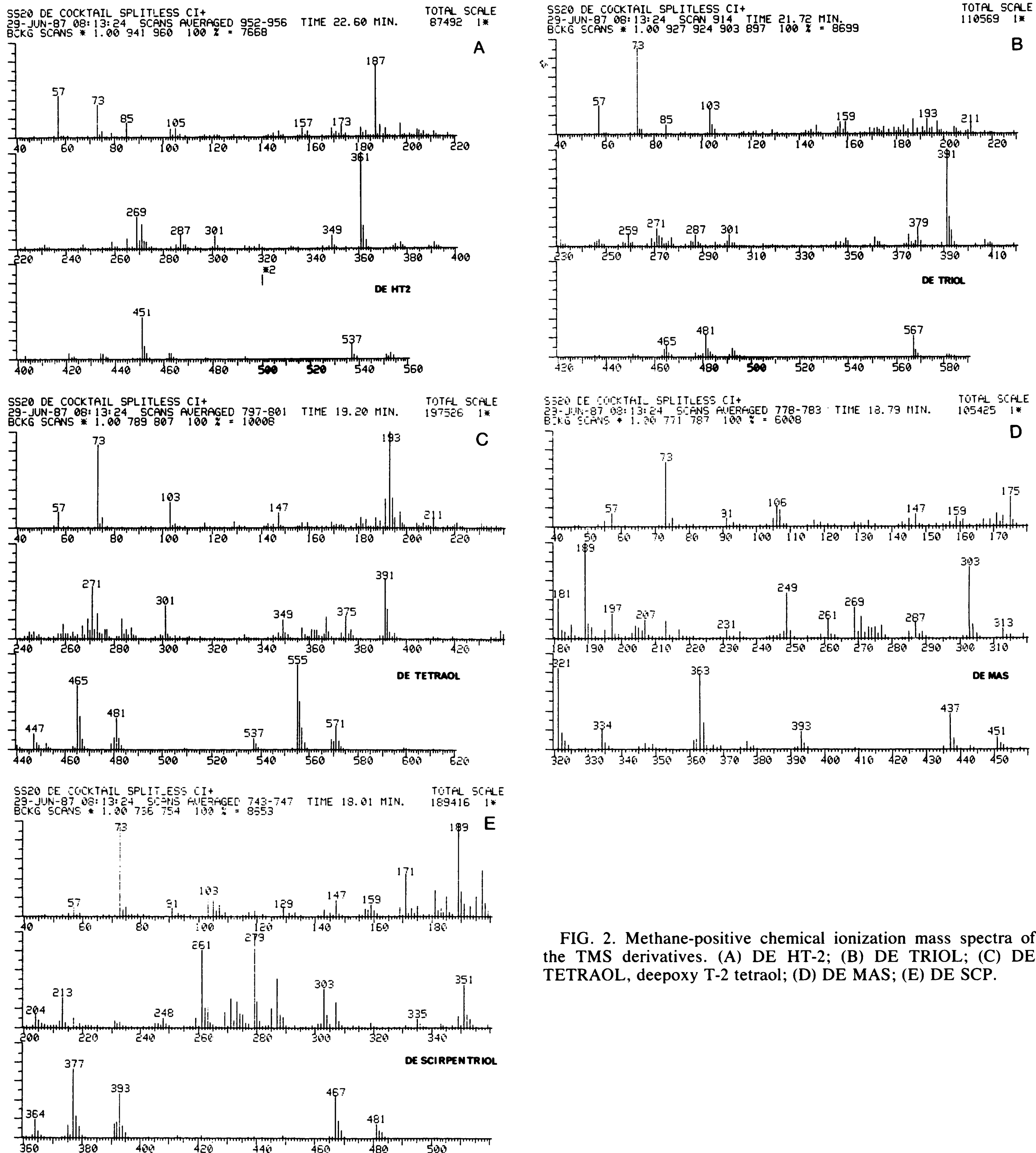

FIG. 2. Methane-positive chemical ionization mass spectra of the TMS derivatives. (A) DE HT-2; (B) DE TRIOL; (C) DE TETRAOL, deepoxy T-2 tetraol; (D) DE MAS; (E) DE SCP.

In contrast, the monoclonal antibody displayed approximately twice the binding activity to HT-2 and DE HT-2 as it did to T-2 toxin. The greater affinity of the monoclonal antibody 15H6 for HT-2 compared with its affinity for T-2 toxin was previously described (14); however, the affinities of anti-T-2 antibodies to deepoxy metabolites have not been reported. Although the data presented support the conclusion that the 12,13-epoxide group is essential for toxicity, the strong cross-reactivity of monoclonal antibodies to DE HT-2 demonstrates that the epoxide moiety is not an essential epitope for antibody recognition. Of additional interest was the observation that the monoclonal antibody displayed limited (10%) cross-reactivity to DE TRIOL but not to TRIOL, its epoxy congener. Recently, Gendloff et al. (13) reported the production of mouse monoclonal T-2 antibodies which displayed strong cross-reactivity to the metabolites

TABLE 2. Comparative toxicities of trichothecene mycotoxins and their deepoxy analogs to brine shrimp[a]

Compound	LC_{50} (ng/ml)[b]	Deepoxy analog	LC_{50} (ng/ml)
T-2	111		
HT-2	258	DE HT-2	>6,000[c]
TRIOL	1,377	DE TRIOL	>5,000[d]
TETRAOL	964	DE TETRAOL	≫6,000[e]
DAS	178		
MAS	128	DE MAS	≫6,000[e]
SCP	6,000	DE SCP	≫6,000[e]
DON	2,788	DOM-1	≫6,000[e]

[a] Abbreviations: LC_{50}, 50% lethal concentration; TETRAOL, T-2 tetraol; DE TETRAOL, deepoxy T-2 tetraol; DOM-1, deepoxy deoxynivalenol.
[b] Concentration which caused 50% lethality to brine shrimp in 24 h at 25°C. Each value is the mean of duplicate assays.
[c] Less than 50% mortality observed at 6,000 ng/ml, the highest concentration examined.
[d] Less than 20% mortality at 5,000 ng/ml, the highest concentration examined.
[e] Less than 20% mortality at 6,000 ng/ml, the highest concentration examined.

3'OH T-2 (100%, relative to T-2 toxin) and 3'OH HT-2 (20%, relative to T-2 toxin) but only weak cross-reactivity to HT-2 (2%, relative to T-2 toxin).

These data demonstrate that antibodies produced against trichothecenes can vary greatly in their sensitivity and must be evaluated for cross-reactivity with a variety of metabolites, including deepoxy compounds, for accurate results to be obtained. However, in certain circumstances, strong cross-reactivity to trichothecene metabolites may be advantageous. Since deepoxidation and 3'-hydroxylation (with respect to T-2 toxin) are major pathways of biotransformation, antibodies which cross-react with these metabolites would be useful for screening biological fluids.

Recently, antibodies and immunoassay tests have been developed for the analysis of T-2 toxin (5, 13, 14, 19, 27, 34), DAS (6, 34), and DON triacetate (35). Immunoassays should provide rapid and inexpensive means for screening feed or biological fluid samples for trichothecenes. Immunologically based screening assays offer several advantages over chromatographic and spectroscopic methods of analysis including ease of analysis, high sensitivity, decreased cost, and high specificity. However, immunoassays are screening techniques requiring confirmation of positive samples, generally accomplished by GC or GC-MS techniques. Since both HT-2 and DE HT-2 strongly cross-react with mouse monoclonal antibody to T-2 toxin, confirmation of positive immunoassay samples by GC-MS must take into account not only T-2, but also metabolites of T-2. The present report describes a method for production of deepoxy trichothecene standards which are essential for confirmation of trichothecene exposure.

TABLE 3. Cross-reactivity comparison of epoxy and deepoxy T-2 metabolites to polyclonal and monoclonal T-2 antibodies

Compound[a]	% Cross-reactivity[b] of:	
	Polyclonal T-2[c]	Monoclonal T-2[d]
T-2	100	100
HT-2	17.4	225
DE HT-2	14.6	189
TRIOL	0.5	0.2
DE TRIOL	1.8	9.2
TETRAOL	≪1	≪1
3'OH T-2	0.4	0.4
3'OH HT-2	≪1	2.6

[a] TETRAOL, T-2 tetraol.
[b] Relative binding cross-reactivity of trichothecene metabolites to the antibodies compared with T-2 toxin (nanograms of T-2 required to displace tritium-labeled T-2 by 50%/nanograms of metabolite required to displace tritium-labeled T-2 by 50%) × 100.
[c] Rabbit polyclonal T-2 antibodies.
[d] Mice monoclonal T-2 antibody strain 15H6.

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