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In Vitro Formation of 3′-Hydroxy T-2 and 3′-Hydroxy HT-2 Toxins from T-2 Toxin by Liver Homogenates from Mice and Monkeys

TAKUMI YOSHIZAWA,* TAE SAKAMOTO, AND KAZUMI OKAMOTO

Department of Food Science, Faculty of Agriculture, Kagawa University, Kagawa 761-07, Japan

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In vitro metabolism of T-2 toxin was studied in homogenates of mouse and monkey livers. In addition to several hydrolyzed products, including HT-2 toxin, neosolaniol, 4-deacetylneosolaniol, 15-deacetylneosolaniol, and T-2 tetraol, two metabolic products were isolated from the incubation mixture. Their structures were confirmed as 3′-hydroxy T-2 toxin and 3′-hydroxy HT-2 toxin on the basis of mass and nuclear magnetic resonance spectroscopy. The formation of these hydroxylated metabolites was found in the microsomes in the presence of NADPH, and the hydroxylation reaction was enhanced by treating mice with phenobarbital. The results suggest that a cytochrome P-450 is catalyzing the hydroxylation at the C-3′ position of T-2 and HT-2 toxins. An in vitro metabolic pathway of T-2 toxin in the hepatic homogenates containing the NADPH-generating system is proposed.

T-2 toxin, 4β,15-diacetoxy-8α-(3-methylbutyryloxy)-3α-hydroxy-12,13-epoxytrichothec-9-ene, is a toxic metabolite produced by various species of *Fusarium* which can cause feed refusal, emesis, dermal necrosis, hemorrhaging, leukopenia, immunological disorders, infertility, abortion, and inhibition of protein synthesis in various animal and biological systems (12). Furthermore, it is one of the most important trichothecene mycotoxins occurring naturally in various agricultural products (1, 3, 4, 7–10). As a result of consumption of contaminated products, humans and farm animals suffer from sublethal and lethal toxicoses (1, 5, 7, 8, 10, 11). In addition, residues of the toxin and its metabolites in animal products appear to be an important human health problem.

T-2 toxin, when orally administered to rodents, chickens, and a lactating cow, was rapidly metabolized into various products and eliminated into the excreta without specific accumulation in any organ (6, 13, 17). Recently, two major metabolites of T-2 toxin were isolated from the urine sample of a lactating cow and identified as 3′-hydroxy T-2 toxin (TC-1) and 3′-hydroxy HT-2 toxin (TC-3) (15, 16). These metabolites were found in plasma and milk in addition to bovine excreta (13) and may correspond to unidentified metabolites found in the excreta of chickens and rats (6, 17).

On the other hand, in liver homogenates and intestinal strips of rats, T-2 toxin was rapidly metabolized to HT-2 toxin, which was then converted into T-2 tetraol via 4-deacetylneosolaniol (18). This hydrolytic pathway was also observed in the hepatic homogenates of rabbits, swines, and cows, but HT-2 was the sole metabolite in homogenates of chickens, indicating species differences in the metabolic pathway of T-2 toxin (14). Despite the formation of these hydrolytic metabolites, no information on the in vitro metabolic modification of the toxin other than hydrolysis is available. In this study, attempts were made to elucidate the formation of the hydroxylated products of T-2 and HT-2 toxins in the homogenates of mouse and monkey livers.

MATERIALS AND METHODS

Chemicals. All trichothecenes used in this study were prepared in our laboratory. $NADP^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd., Tokyo, Japan; phenobarbital was from Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan; and sodium pentobarbital was from Pitman-Moore, Inc., Washington Crossing, N.J. All other reagents used were of analytical grade.

Animals and preparation of hepatic subcellular fractions. Female *ddys* mice weighing 40 to 50 g were used and treated in some experiments with phenobarbital or sodium pentobarbital in 0.8% NaCl (75 mg/kg of body weight by intraperitoneal injection for 4 consecutive days). Livers of Japanese monkeys (*Macaca fuscata*) were obtained from the Livestock Hygiene Service Center, Kagawa, Japan.

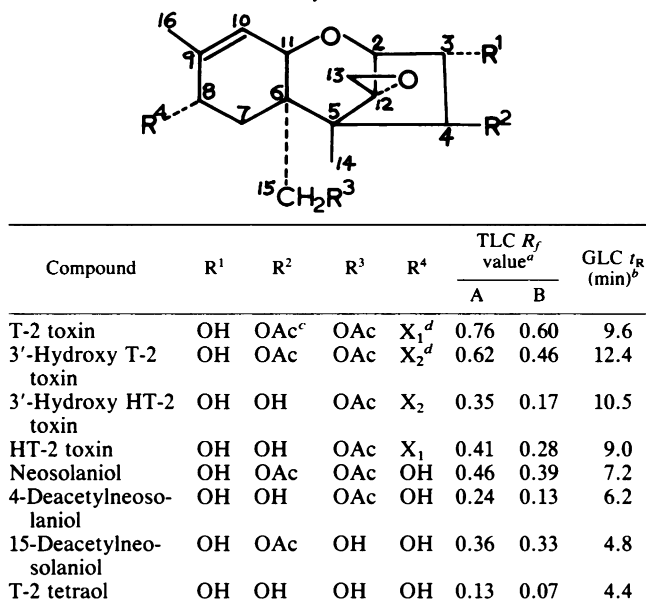
Individual livers were rinsed with cold 0.25 M sodium phosphate buffer (pH 7.4) and homogenized in 4 volumes of the same buffer with cooling in an ice bath. The homogenate was centrifuged at 9,000 × *g* for 20 min at 0°C, and the supernatant was filtered through glass wool to give the filtrate, referred to as the liver S-9 fraction. The S-9 fraction of mice was further centrifuged at 100,000 × *g* for 60 min at 0°C, and the resulting supernatant was used as cytosol. The precipitate, referred to as microsomes, was resuspended in the same buffer to give a microsomal enzyme solution of an equal volume to S-9. Protein levels in hepatic preparations were as follows: 26.2, 7.0, and 16.6 mg of protein per ml in S-9, microsomes, and cytosol, respectively.

Incubation of T-2 and HT-2 toxins with mouse liver fractions. The substrate was incubated for 60 min at 37°C with each hepatic subcellular fraction in 1-ml reaction mixtures (pH 7.4), which contained 0.5 ml of the hepatic preparation, 0.4 ml of phosphate buffer, 0.54 μmol of T-2 toxin or HT-2 toxin, 0.5 μmol of $NADP^+$, 5 μmol of glucose 6-phosphate, 8 μmol of $MgCl_2$, and 33 μmol of KCl. In addition, 0.75 U of glucose-6-phosphate dehydrogenase was added to the hepatic microsomal fraction.

Extraction and analysis of metabolites. After incubation, the reaction mixture was applied to a preconditioned column (14 cm by 1-cm inner diameter; packed in acetone and rinsed with water) of Amberlite XAD-2 resin (Rhom and Haas Co., Spring House, Pa.). The column was eluted successively with 50 ml each of water and acetone. The latter eluate was concentrated and redissolved in 2 ml of ethanol. A portion of this solution was transferred to a small test tube containing deoxynivalenol as an internal standard. After evaporation of the solvent, the residue was reacted

* Corresponding author.

TABLE 1. Chemical structures and resolution of T-2 toxin and its metabolites by TLC and GLC



Compound	R^1	R^2	R^3	R^4	TLC R_f value[a]		GLC t_R (min)[b]
					A	B	
T-2 toxin	OH	OAc[c]	OAc	X_1[d]	0.76	0.60	9.6
3'-Hydroxy T-2 toxin	OH	OAc	OAc	X_2[d]	0.62	0.46	12.4
3'-Hydroxy HT-2 toxin	OH	OH	OAc	X_2	0.35	0.17	10.5
HT-2 toxin	OH	OH	OAc	X_1	0.41	0.28	9.0
Neosolaniol	OH	OAc	OAc	OH	0.46	0.39	7.2
4-Deacetylneosolaniol	OH	OH	OAc	OH	0.24	0.13	6.2
15-Deacetylneosolaniol	OH	OAc	OH	OH	0.36	0.33	4.8
T-2 tetraol	OH	OH	OH	OH	0.13	0.07	4.4

[a] On silica gel TLC plates developed in solvent systems A and B (see text).

[b] Retention times of trimethylsilyl ether derivatives.

[c] OAc, Acetate.

[d] $X_1 = OCOCH_2CH(CH_3)_2$; $X_2 = OCOCH_2C(OH)(CH_3)_2$.

with a mixture of *N*-trimethylsilylimidazole-*N,O*-bis(trimethylsilyl)acetamide-trimethylchlorosilane (3:3:2 [vol/vol/vol]; Wako Pure Chemical Industries, Ltd., Osaka, Japan) to convert into trimethylsilyl ethers and then quantified directly by gas chromatography. Silylation of T-2 metabolites was readily accomplished with this reagent.

Chromatography. Gas-liquid chromatography (GLC) was performed on a JEOL model JGC-20K gas chromatograph equipped with a hydrogen flame ionization detector. The operation conditions were: a glass column (1 m by 2 mm, inner diameter) packed with 2% OV-17 on Gas-Chrom Q of 80 to 100 mesh; column temperature programmed for 200 to 270°C at 8°C/min; and a flow rate of nitrogen of 55 ml/min.

Thin-layer chromatography (TLC) was carried out on preparative silica gel plates (0.25 mm, gel thickness) for analysis of the metabolites. The developing solvent systems used consisted of (A) chloroform-methanol (9:1, [vol/vol]) and (B) chloroform-acetone (3:2 [vol/vol]). Trichothecene metabolites were made visible under long-wave (354 nm) UV light by charring with 20% H_2SO_4 in methanol. Resolution of T-2 toxin and its metabolites by TLC and GLC was tabulated (Table 1).

Spectroscopy. Gas chromatography-mass spectroscopy was carried out in a Hitachi M-80 mass spectrometer at 20 and 70 eV. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured in a $CDCl_3$ solution with Me_4Si as an internal standard on a Hitachi R-90H Fourier transform NMR spectrometer (90 MHz).

Isolation of metabolites. (i) 3'-Hydroxy T-2 toxin. T-2 toxin (50 mg; 0.11 mmol) dissolved in 1 ml of ethanol was added to 100 ml of the hepatic S-9 fraction of Japanese monkeys, which contained 0.1 mmol of $NADP^+$, 1 mmol of glucose 6-phosphate, 1.6 mmol of $MgCl_2$, and 6.6 mmol of KCl, and incubated for 2 h at 37°C with aeration. The reaction mixture was applied directly to an Amberlite XAD-2 resin column (15 by 2 cm, inner diameter), rinsed with 100 ml of water, and eluted with 150 ml of acetone. After evaporation of the solvent, the residue was chromatographed on a column (13 by 1 cm, inner diameter; packed in chloroform) of Florisil (60 to 100 mesh; 5 g; Wako Pure Chemical Industries), which was eluted successively with 20 ml of chloroform and 20 ml each of the following concentrations of methanol in chloroform: 1, 2, 3, 5, and 10%. The eluate with 2% methanol in chloroform was further purified on silica gel TLC plates with chloroform-methanol (97:3).

(ii) 3'-Hydroxy HT-2 toxin. HT-2 toxin (20 mg; 0.047 mmol) dissolved in 0.4 ml of ethanol was added to 40 ml of the mouse hepatic S-9 fraction, which contained 0.04 mmol of $NADP^+$, 0.4 mmol of glucose 6-phosphate, 0.64 mmol of $MgCl_2$, and 2.64 mmol of KCl. After incubation at 37°C for 3

TABLE 2. In vitro metabolism of T-2 toxin and HT-2 toxin by the hepatic fractions of untreated mice and mice treated with phenobarbital

Subcellular fraction	NADPH-generating system[a]	Substrate	Product[b] (molar % of added substrate)							
			T-2	3'-OH-T-2	3'-OH-HT-2	HT-2	NEOS	4-DANS	15-DANS	TOL
S-9	−	T-2	2.3[c]			53.6	tr[d]	34.6	2.2	7.4
	+	T-2	7.3[c]	3.9	6.4	64.8	2.0	15.8		tr
	−	HT-2				50.9[c]		44.2		44.9
	+	HT-2			5.2	76.9[c]		18.1		tr
S-9[e]	+	T-2			52.5	17.9		10.2		19.5
	+	HT-2			21.5	52.6[c]		14.7		11.2
Microsome[e]	+	T-2		5.6	27.5	61.2		5.6	tr	tr
	+	HT-2			20.4	65.8[c]		6.7		7.0
Cytosol[e]	+	T-2	13.5[c]	tr	tr	71.3	5.0	10.2		tr

[a] The hepatic fraction was incubated with (+) or without (−) the NADPH-generating system, which consisted of $NADP^+$, glucose 6-phosphate, $MgCl_2$, and KCl. The microsomal fraction was incubated with this system containing glucose-6-phosphate dehydrogenase.

[b] Abbreviations: T-2, T-2 toxin; 3'-OH-T-2, 3'-hydroxy T-2 toxin; 3'-OH-HT-2, 3'-hydroxy HT-2 toxin; HT-2, HT-2 toxin; NEOS, neosolaniol; 4-DANS, 4-deacetylneosolaniol; 15-DANS, 15-deacetylneosolaniol; TOL, T-2 tetraol. Values are means of triplicate experiments.

[c] Unmetabolized substrate after incubation for 60 min.

[d] tr, Trace amount.

[e] The subcellular fraction was prepared from livers of mice treated with phenobarbital (75 mg/kg per day intraperitoneally) for 4 consecutive days.

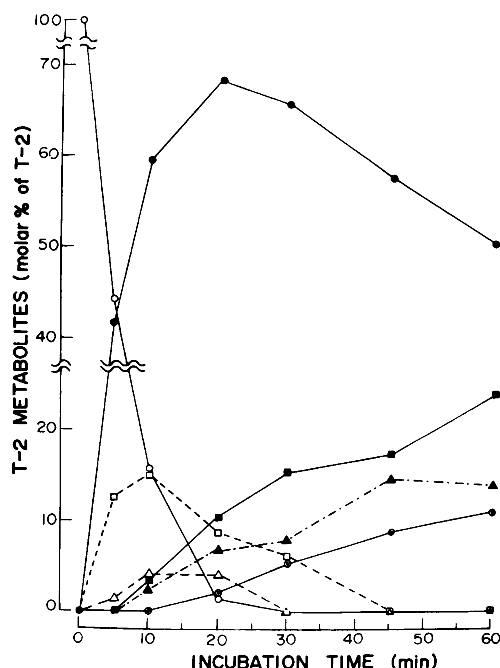


FIG. 1. Time course of the in vitro metabolism of T-2 toxin in the NADP$^+$-supplemented hepatic S-9 fraction from phenobarbital-treated mice. Symbols: ○, T-2 toxin (substrate); ●, HT-2 toxin; □, 3'-hydroxy T-2 toxin; ■, 3'-hydroxy HT-2 toxin; △, neosolaniol; ▲, 4-deacetylneosolaniol; ⊙, T-2 tetraol. A trace amount of 15-deacetylneosolaniol was also detected by TLC and GLC.

h with aeration, the mixture was applied to an Amberlite XAD-2 resin column (10 by 2 cm, inner diameter), rinsed with 50 ml of water, and eluted with 130 ml of acetone. The eluate was purified on silica gel TLC plates with chloroform-methanol (9:1 [vol/vol]).

RESULTS AND DISCUSSION

Metabolism of T-2 toxin in hepatic fractions of mice. T-2 and HT-2 toxins were metabolized to several hydrolyzed derivatives in the hepatic S-9 fraction from normal (phenobarbital-untreated) mice (Table 2). When NADP$^+$ supplemented the S-9 fraction, such hydroxylated metabolites as 3'-hydroxy T-2 and 3'-hydroxy HT-2 toxins were produced. The structural elucidation of these metabolites is described in the following section. The hydroxylation reaction in the S-9 fraction from phenobarbital-treated mice was four to five times higher as compared with that in the untreated mice, i.e., the yield of the metabolites was 52% from T-2 toxin and 21% from HT-2 toxin after incubation for 60 min. The rate of hydroxylation at the C-3' position was also dependent on the dosage of phenobarbital (data not shown). Pentobarbital was a weak inducer of the hydroxylation reaction, which was increased approximately 20% by treating mice with 75 mg of sodium pentobarbital per kg of body weight by intraperitoneal injection for 4 consecutive days. Furthermore, these hydroxylated metabolites were not formed in the cytosol, but formed in the microsomes in the presence of the NADPH-generating system. These results suggest that a cytochrome P-450 is catalyzing the hydroxylation reaction.

Metabolic pathway of T-2 toxin in hepatic homogenates of mice. Figure 1 shows the time course of T-2 metabolism in the NADP$^+$-supplemented hepatic S-9 fraction from phenobarbital-treated mice. As the concentration of added T-2 toxin was rapidly decreased in the reaction mixture, its metabolites, including HT-2 toxin, 3'-hydroxy T-2 toxin, neosolaniol, and 4-deacetylneosolaniol, exhibited maximal levels within 20, 10, 15, and 45 min, respectively, followed by gradual decrease, indicating that these metabolites are intermediate products in the metabolism of T-2 toxin. On the other hand, the remaining metabolites, 3'-hydroxy HT-2 toxin and T-2 tetraol, were resistant to further metabolism in the S-9 fraction. From these results, the pathway shown in Fig. 2 is proposed for the in vitro metabolism of T-2 toxin, in which two enzymatic reactions, hydrolysis and hydroxylation, are included. By enzymatic hydrolysis, T-2 toxin is initially converted into HT-2 toxin and neosolaniol. Thereafter, the former metabolite is converted to T-2 tetraol via 4-deacetylneosolaniol, and the latter is metabolized to T-2 tetraol via either 4-deacetylneosolaniol or 15-deacetylneosolaniol. The subcellular localization of the enzymes participating in the hydrolysis of T-2 metabolites was reported in a previous paper (14). By enzymatic hydroxylation, on the other hand, T-2 and HT-2 toxins are oxidized at the C-3' position to afford 3'-hydroxy T-2 and 3'-hydroxy HT-2 toxins, respectively. 3'-Hydroxy HT-2 toxin is also produced from 3'-hydroxy T-2 toxin by hydrolysis at the C-4 ester linkage. The metabolic conversion of these hydroxylated products into neosolaniol, 4-deacetylneosolaniol, or 15-deacetylneosolaniol did not occur under the conditions adopted in this study.

Chemical structures of hydroxylated metabolites. T-2 toxin was metabolized efficiently by the S-9 fraction of monkey liver in the presence of the NADPH-generating system to afford a new metabolite (yield, 24.9%). The electron impact mass spectrum of the trimethylsilylated metabolite was identical to that of synthetic compound: *m/z* 626 (M$^+$) (15). In ^{13}C NMR spectroscopy (Table 3), the signal at δ 25.64 (d)

TABLE 3. ^{13}C chemical shifts of T-2 toxin and its metabolites in $CDCl_3$

Carbon or compound	Chemical shift (ppm downfield from Me_3Si)		
	T-2 toxin	3'-Hydroxy T-2 toxin	3'-Hydroxy HT-2 toxin
2	78.66 d (78.5)[a]	78.72 d	78.69 d
3	78.01 d (76.8)	78.39 d	80.36 d
4	84.06 d (84.2)	84.40 d	81.39 d
5	48.37 s (48.3)	48.45 s	48.92 s
6	42.90 s (42.9)	42.96 s	42.50 s
7	27.78 t (27.8)	27.90 t	27.55 t
8	67.90 d (67.8)	68.51 d	68.62 d
9	135.95 s (135.8)	135.59 s	135.60 s
10	123.59 d (123.4)	124.15 d	124.19 d
11	67.18 d (67.2)	67.20 d	67.17 d
12	64.20 s (64.4)	64.21 s	64.59 s
13	47.03 t (47.1)	47.11 t	46.65 t
14	6.72 q (6.8)	6.83 q	6.92 q
15	64.49 t (64.1)	64.58 t	64.49 t
16	20.18 q (20.2)	20.28 q	20.28 q
1'	172.31 s (172.0)	172.33 s	172.10 s
2'	43.46 t (43.4)	46.65 t	46.79 t
3'	25.64 d (25.7)	69.06 s	69.07 s
4'	22.27 q (22.4)	29.06 q	29.01 q
5'	22.35 q (22.4)	29.51 q	29.47 q
CH_3*CO*	169.78 s (169.5)	169.98 s	170.41 s
	171.96 s (172.0)	172.15 s	
*CH_3*CO	20.86 q (20.2)	20.95 q	21.16 q
	20.92 q (21.0)	21.04 q	

[a] From Cole and Cox (2).

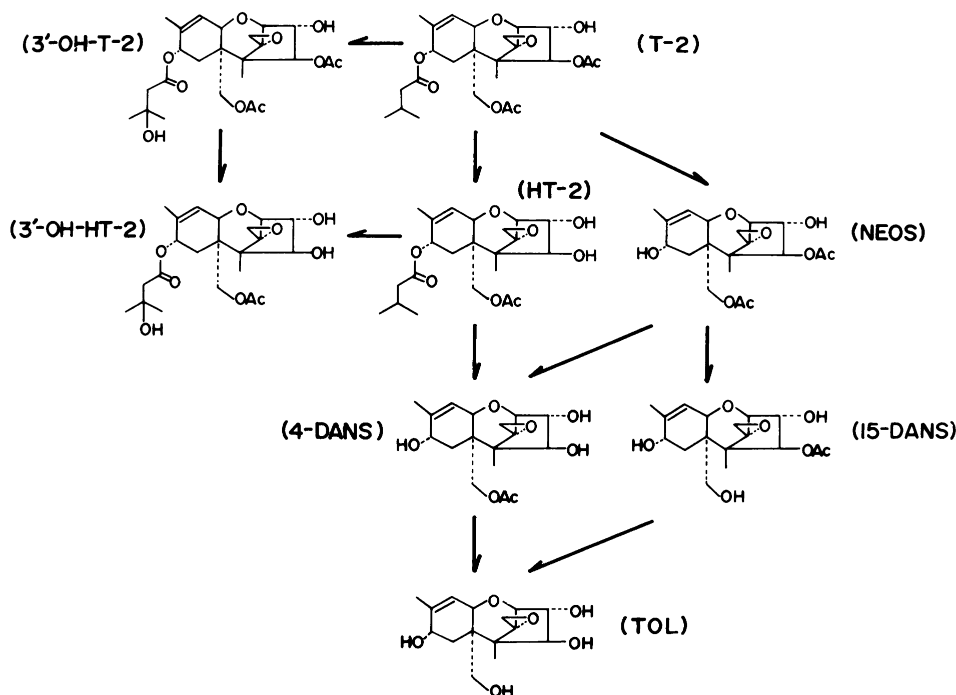


FIG. 2. Proposed pathways for the in vitro metabolism of T-2 toxin in the hepatic homogenates of animals in the presence of the NADPH-generating system. DANS, deacetylneosolaniol; NEOS, neosolaniol; TOL, T-2 tetraol; 3'-OH-T-2, 3'-hydroxy T-2 toxin; 3'-OH-HT-2, 3'-hydroxy HT-2 toxin.

due to the C-3' position of T-2 toxin was shifted downfield to δ 69.06 (s) in the metabolite, and signals of C-2', C-4', and C-5' were also shifted downfield by 3 to 7 ppm. Remaining chemical shifts of the metabolite corresponded well with those observed in the spectrum of T-2 toxin (Table 3). Moreover, the ^{1}H NMR spectrum of the new metabolite

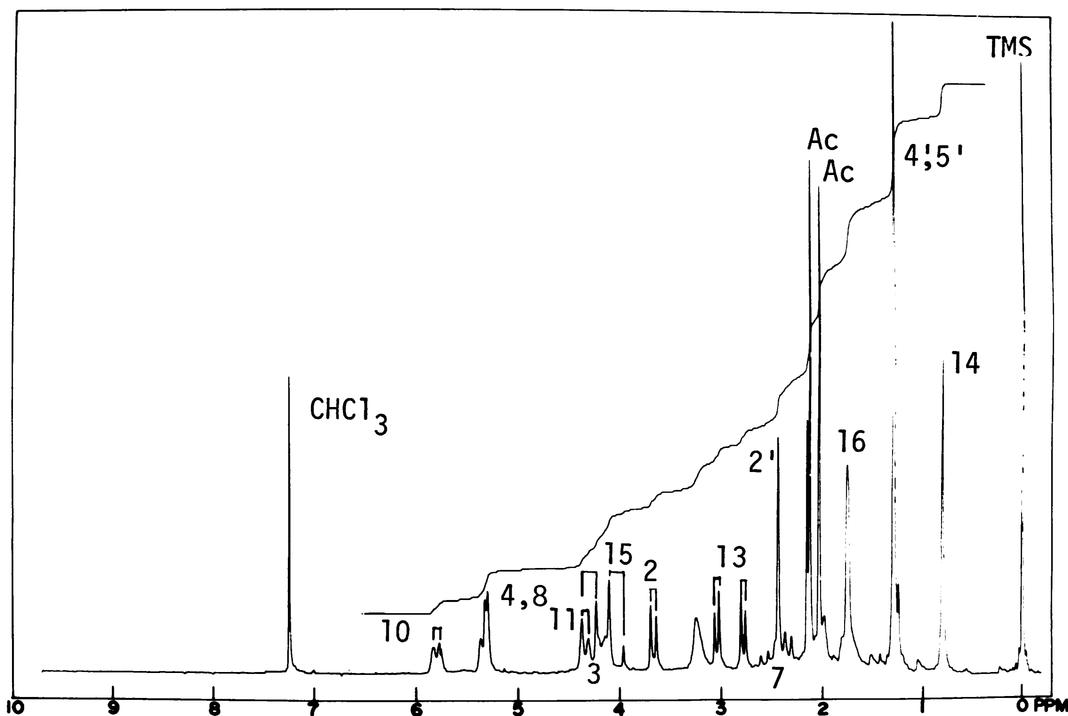


FIG. 3. ^{1}H NMR spectrum (90 MHz; in $CDCl_3$) of 3'-hydroxy T-2 toxin metabolized from T-2 toxin by monkey liver homogenates in the presence of the NADPH-generating system. TMS, Tetramethylsilane; Ac, acetyl.

(Fig. 3) was identical to that of synthetic 3′-hydroxy T-2 toxin, 4β,15-diacetoxy-3α-hydroxy-8α-(3-hydroxy-3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene (refer to the literature [16] for the chemical shifts and the assignment). A diagnostic resonance due to *gem*-dimethyl protons of the C-8 substituent was observed at δ 1.27 as a singlet rather than a doublet resonance at δ 0.96 (J = 6 Hz) in T-2 toxin.

With regard to the other hydroxylated metabolite, it was formed from HT-2 toxin in a yield of 3.2% after incubation with the mouse hepatic S-9 fraction containing the NADPH-generating system. ^{1}H NMR spectrum and mass spectrum (as a trimethylsilyl ether, $M^+ = m/z$ 656) of the purified product were identical with those of synthetic 3′-hydroxy HT-2 toxin, 15-acetoxy-3α,4β-dihydroxy-8α-(3-hydroxy-3-methyl butyryloxy)-12,13-epoxytrichothec-9-ene (15, 16). As shown in the ^{13}C NMR spectrum of the metabolite, a singlet signal due to C-3′ was observed at δ 69.07, indicating the introduction of a hydroxy group in place of the hydrogen in the C-3′ position.

This is the first report on the in vitro formation of the hydroxylated metabolites of T-2 toxin in hepatic homogenates of animals. These metabolites were found in the plasma, milk, and excreta of a lactating cow (13) and may correspond to the metabolites found in the excreta of chickens and rats (6, 17). This indicates that many animals, including primates, are capable of hydroxylating T-2 and HT-2 toxins at the C-3′ position. As described in a previous paper (16), the acute toxicity of 3′-hydroxy T-2 toxin was slightly higher than that of the parent toxin, whereas the toxicity of 3′-hydroxy HT-2 toxin was approximately 30% in comparison with that of HT-2 toxin, suggesting that the susceptibility of animals to T-2 toxin is dependent on the rate of hydrolysis and hydroxylation in the metabolic interconversion of T-2 metabolites. The interspecies differences in the pharmacokinetics of T-2 metabolites in animals remain to be established.

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