of crust of whole meal bread show the barely discernible traces of isoDON at m/z 512, but it is clearly evident although not completely resolved at m/z 497 (Figure 6c).

Both natural wheat and flour were analyzed for DON and in no cases was isoDON detected; it occurred only in samples that had been heat treated. Identical analytical procedures were used in an extensive survey of barley samples for DON (Gilbert et al., 1983) and for confirmation of positive DON results in wheat and corn samples from different countries (Osborne and Willis, 1984). In neither case was any evidence found for isoDON, although only 35 samples out of a total of 360 contained DON levels >0.1 mg/kg.

Samples of wheat-based breakfast cereal made from three different batches of naturally DON contaminated wheat at levels ranging from 0.35 to 0.75 mg/kg were analyzed. The manufacturing process involved pressure cooking at 45 psi, partial drying, milling, flaking, and finally toasting of the product at 175–180 °C. Analyses of this breakfast cereal showed that significant amounts of DON survived processing. As with the bread samples, there is an indication from the MID traces of the presence of isoDON, this being most evident in the trace for m/z 497 (Figure 6d).

In summary, DON undergoes isomerization, which can be induced either chemically or thermally to form an isomer, isoDON. Chemically, an enol intermediate is proposed, followed by acyl migration from C-8 to C-7 and double bond migration to the C-8, C-9 position. Thermally, a similar rearrangement can be induced as has been demonstrated synthetically and by its presence in bread, especially the crust, and wheat-based cereal.

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Registry No. I, 92397-71-2; II, 92397-72-3; DON, 51481-10-8.

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Structure of a Metabolic Derivative of T-2 Toxin (TC-6) Based on Mass Spectrometry

Robert J. Pawlosky and Chester J. Mirocha*

A new metabolite of T-2 toxin in cows named TC-6 has been tentatively identified as 3′-hydroxy-7-hydroxy-HT-2 toxin. The new metabolite is related to TC-3 (3′-hydroxy-HT-2 toxin) and like TC-3 forms two isomers when reacted with trifluoroacetic acid anhydride. Its only difference from TC-3 is a hydroxyl group tentatively assigned to the C-7 position. This metabolite is a product of T-2 metabolism in the cow and can be found in feces and urine.

T-2 toxin [4β,15-diacetoxy-8-[(3-methylbutyryl)oxy]-3-hydroxy-12,13-epoxytrichothec-9-ene] is a trichothecene mycotoxin produced by various species of *Fusarium* but predominantly by *Fusarium tricinctum* or *Fusarium sporotrichioides*. These two species are thought to be the same by some taxonomists but classified separately by others. The distribution of this toxin in animals has been studied by Chi et al. (1978) in chicks, Robison et al. (1979a,b) in bovine and porcine milk, Robison et al. (1979a,b) in swine, Yoshizawa et all. (1980) in chickens, and Yoshizawa et al. (1981) in a lactating cow.

The products of metabolism of T-2 in the cow have been reported as HT-2, neosolaniol, 4-deacetylneosolaniol, and unkown derivatives called TC-1, TC-3, TC-5, TC-6, TC-7, and TC-8 (Yoshizawa et al., 1981). Similarly, in the chicken, the metabolic products were described as HT-2, neosolaniol, TB-1, TB-3, TB-4, TB-5, TB-6, TB-7, and TB-8 (Yoshizawa et al., 1980). Presently we are not certain which of the unknown chicken metabolites (TB series) correspond to the bovine metabolites (TC series) although we feel they are identical or closely related. Recently, TC-1 and TC-3, found in the cow, were identified as 3′-hydroxy-T-2 toxin and 3′-hydroxy-HT-2 toxin, respectively (Yoshizawa et al., 1982). We have obtained mass spectral evidence for the identification of TC-6, and the presentation of this data is the subject of this paper.

In order to substantiate the evidence used to elucidate the structure of TC-6 (Figure 1), it is necessary to review the reactivity of TC-1 and TC-3 in a chromatographic system (Pawlosky et al., 1984). Both TC-1 and TC-3 react with silylating reagents to form the fully silylated derivatives that resolve on a SE-54 (DB-5) bonded phase capillary column. In methane chemical ionization mass spectrometry, the $M^+ + 1$ ions 657 for TC-3 and 627 for TC_1 are formed. However, when TC-1 and TC-3 are reacted with trifluoroacetic acid anhydride, two isomers are

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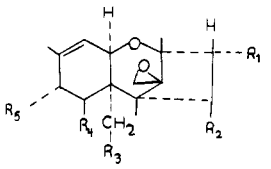

	R_1	R_2	R_3	R_4	R_5
I. underivatized TC-6	OH	OH	OAc	OH	$OCOCH_3COH(CH_3)_2$
II. TC-6 TFA isomer I.	OTFA	OTFA	OAc	OTFA	$OCOCH{=}C(CH_3)_2$
isomer II.	OTFA	OTFA	OAc	OTFA	$OCOCH_2(CH_3)C{=}CH_2$
III. TC-6 TMS 3-OTMS	OTMS	OTMS	OAc	OH	$OCOCH_2COTMS(CH_3)_2$
IV. TC-6 TMS 4-OTMS	OTMS	OTMS	OAc	OTMS	$OCOCH_2COTMS(CH_3)_2$

Figure 1. Tentative structure of TC-6 with its various trifluoroacetate and trimethylsilyl ether derivatives.

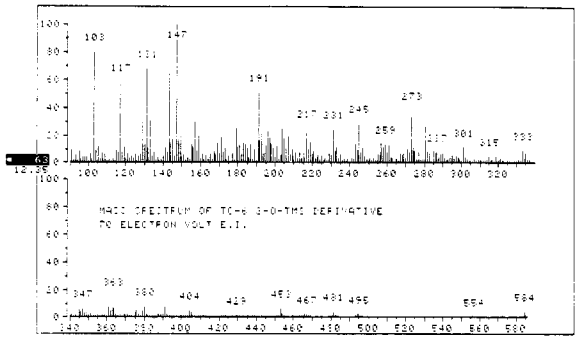

Figure 2. Mass spectrum of TC-6 Me_3Si derivative, 70 eV. Three hydroxyls are silated; one remains underivatized.

formed of each metabolite corresponding to two dehydration products of the C-3′ hydroxyl as described (Pawlosky et al., 1984). Thus any C-3′ hydroxylated product of T-2 toxin metabolism will form two isomers when reacted with trifluoroacetic acid anhydride (TFAA) resolvable on a 30-m capillary column. The diagnostic fragments identifying these derivatives are best found in positive chemical ionization (CI).

Positive CI with methane is well suited to determine the molecular weight of the trichothecenes derivatized with TFAA. The reactions with the methane plasma yield adduct ions of $M^+ + 1$, $M^+ + 29$ ($M^+ + C_2H_5$), and $M^+ + 41$ ($M^+ + C_3H_5$). These adducts, the dimer and trimer of methane, are used in establishing the identity of the molecular ion. Thus, the TFAA derivative of TC-1 positive CI (methane) will yield a base peak of 401, a $M^+ + 1$ of 561, and methane adducts of 589 and 601. Similarly, TC-3 yields a base peak of 455, a $M^+ + 1$ of 615, and methane adducts of 643 and 655. Trimethylsilyl ether derivatives are less susceptibile to the adduct formation but do form the protonated species.

The T-2 metabolite (TC-6) found in cow urine and feces was extracted and resolved by thin-layer chromatography as described (Yoshizawa et al., 1981), reacted with TFAA and TBT separately, resolved on a SE-54 capillary column, and analyzed by mass spectrometry, positive chemical ionization (PCI), and electron impact (EI). The trimethylsilyl ether derivative eluted with a retention time of 12.4 min, and the electron impact spectrum matched the TC-6 EI spectrum from our last metabolic study (Yoshizawa et al., 1981) (Figure 2). The TFAA derivative of TC-6, like TC-3 and TC-1, resolved into two individual components (isomers) with identical mass spectra (Figure

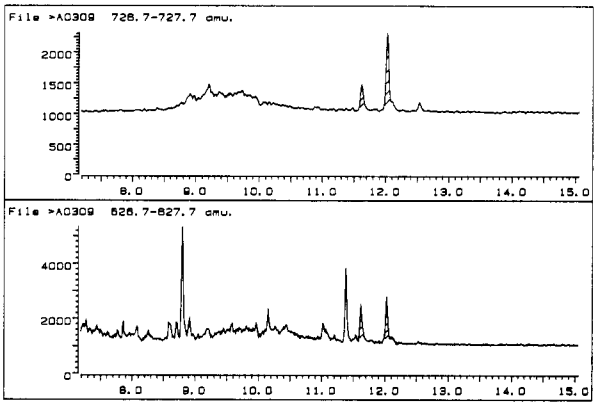

Figure 3. Selected ion chromatogram of the TFA derivative of TC-6. The $M^+ + 1$ (727) and the base peak (627) are coincident and can be found at retention times of 11.6 and 12.0 min.

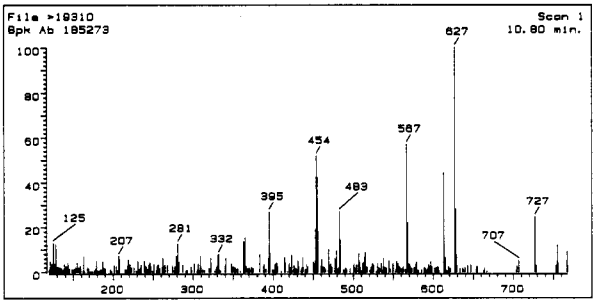

Figure 4. Mass spectrum of TC-6 TFA when reacted with TFAA methane chemical ionization. The dehydrated product's $M^+ + 1$ is 727, $M^+ + 29$ is 755, and $M^+ + 41$ is 767.

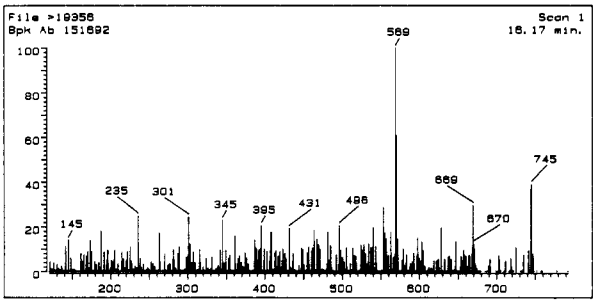

Figure 5. Mass spectrum of the TC-6 Me_3Si derivative. Methane chemical ionization. All hydroxyl groups are silated; $M^+ + 1$ is 745.

3). The TC-6 TFAA chemical ionization spectrum displayed a base peak at 627, a second intense peak at 454, and a $M^+ + 1$ ion of 727 (Figure 4). The $M^+ + 29$ ($M + C_2H_5$) ion is found at 755 and $M^+ + 41$ ($M^+ + C_3H_5$) is found at 767. The dimer and trimer of methane identified the $M^+ + 1$ ion as 727.

The TFA spectra of TC-6 can be compared directly to those of TC-3 or HT-2 TFA derivatives. Mass 727 is 110 mass units greater than that of HT-2, $M^+ + 1$ (617), and represents the presence of another *O*-TFA group (+112 amu) and a double bond (−2 amu). The pseudomolecular ion for TC-6 is 112 mass units greater than TC-3 $M^+ + 1$ and represents an additional hydroxyl group on the TC-3 molecule.

In order to establish the number of hydroxyl groups, TC-6 was reacted with TBT (Pierce Chemical Co.) to form the trimethylsilyl ether derivative. Two products formed, a minor one in which four hydroxyl groups had reacted yielding a $M^+ + 1$ of 745 (Figure 5) and the major component (90% of the total; retention time 12.4 min) with three hydroxyl groups and one unreacted (Figure 6).

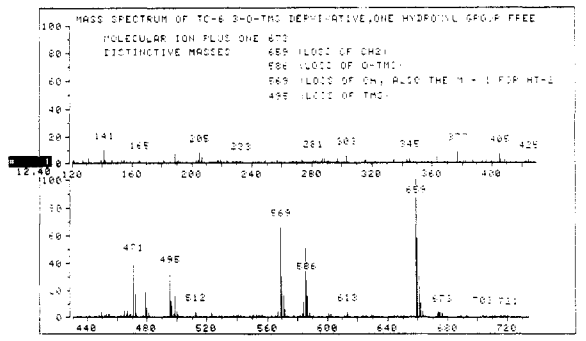

Figure 6. Mass spectrum of the TC-6 Me_3Si derivative. Methane chemical ionization. Three hydroxyl groups were silated; one remains underivatized. $M^+ + 1$ is 673.

Thus, the molecule TC-6 has four hydroxyl groups and one of them is resistant to derivatization presumably owing to steric hindrance.

Interpretation of Mass Spectra. The CI mass spectra of the TFA derivatives of HT-2, TC-1, TC-3, and TC-6 as well as the Me_3Si (TMS in the Figures) derivative of TC-6 form the basis for the elucidation of structure. The TFA derivatives of TC-1, TC-3, and TC-6 all initially lose the dehydrated isovaleroxy group to form the $M^+ - 100$ (Figure 4). In the case of TC-6, the $M^+ - 100$ fragment is the base peak (627). Then, as the acetate (60 amu) and a trifluoroacetate (113 amu) groups are lost, 455 and 454 become the major ions. In the case of TC-1 and TC-3, the base peaks are formed similarly through the loss of the isovaleroxy (100 amu) and acetate groups to form 401 and 455, respectively. Since the intense ions of TC-6-TFA are similar to those of TC-3- and HT-2-TFA, its structure must be more similar to HT-2 than to T-2. Furthermore, the isovaleroxy group has been hydroxylated in the 3-position since (1) the TFAA derivative gives two resolvable isomers that characteristically lose 100 mass units from the molecular ion and (2) the Me_3Si derivatives show the presence of four hydroxyl groups.

In attempts to form the Me_3Si ether of TC-6, the largest resolvable component represents three silylated groups and one free hydroxyl. Only 10% of TC-6 has all four hydroxyls silylated. The former species has a $M^+ + 1$ of 673. Fragment 659 is formed by a loss of 14 mass units (CH_2), fragment 586 is formed by a loss of an *O*-TMSMe_3Si (90), and fragment 596 (base peak) is the protonated HT-2-$(Me_3Si)_2$ derivative and is formed by the loss of a free hydroxyl group (17 mass units). Fragment 495 is formed by the loss of an additional Me_3Si group (73) and occurs in TC-3 as well.

DISCUSSION AND CONCLUSION

Mass spectral data from the chemical ionization spectra of TC-6-TFA and TC-6-Me_3Si provide information concerning its molecular structure. TC-6 is a dihydroxylated derivative of HT-2 toxin. The molecular weight of TC-6 was determined based on the trimethylsilyl ether derivative of TC-6. The fully silylated material represents four hydroxyl groups and is present in a lesser amount than the species with three Me_3Si reacted hydroxyls. The molecular weight of TC-6 in the underivatized form is calculated to be 456.

One hydroxyl group is present at the C-3′ position; like TC-3 and TC-1, it dehydrates in the presence of TFAA to produce two isomers. The isomers are resolvable by chromatography and have identical mass spectra. The other hydroxyl group is present on the main skeletal portion of the molecule and appears to be the hydroxyl that is resistant to derivatization with silylating reagents. It is tentatively assigned to the C-7 position of TC-6. The remaining two hydroxyls are identical with those found in HT-2 and TC-3 in the C-3 and C-4 positions.

The alternative assignment for the hydroxyl placed at C-7 is C-15 with a concomitant placement of an acetate group at C-7. Such an assignment does not conform with the present data because (i) a hydroxyl at C-15 is very reactive with Me_3Si whereas the C-7 is hindered and (ii) there is no precedent for an acetyl group at C-7 in any of the trichothecene derivatives reported so far. Thus, assignment of a hydroxyl at C-7 and an acetate at C-15 is reasonable and awaits confirmation by NMR analysis.

The above data are our evidence to support the structure of TC-6 as shown in Figure 3. In the absence of sufficient material for analysis by NMR spectroscopy, we feel confident that the mass spectral data support the structure as shown. Moreover, the analysis of the data contained herein supports the identification of two other derivatives (TC-8 and iso-TC-1) found in our studies of T-2 metabolism.

EXPERIMENTAL SECTION

Two kilograms of bovine feces taken from a T-2-treated cow was extracted with 4 L of acetonitrile (2 L × 2). The acetonitrile was filtered through Whatman filter paper No. 4 and concentrated to 500 mL. The acetonitrile was dried over 20 g of anhydrous sodium sulfate and filtered through a Whatman filter paper No. 2. The sulfate was rinsed with 125 mL of acetonitrile, and the solvent was partitioned with 200 mL of petroleum ether (60:40) twice. The acetonitrile layer was taken and evaporated to dryness and brought up in 20 mL with methanol–water (9:1). The solution was added to an Amberlite XAD-2 column, 30 cm, and rinsed with 100 mL of water. The column was eluted with 150 mL of methanol–water (9:1). The eluant was concentrated to dryness and brought up to 20 mL of chloroform. The mixture was chromatographed on four high-performance (200) silica gel TLC plates (20 × 20 cm) with chloroform–methanol (9:1) as the developing solvent. A 3 mm wide band with an R_f value of 0.35 corresponding to TC-6 was scraped and eluted with 100 mL of chloroform (50 mL × 2). The eluant was filtered through Whatman filter paper No. 1, and the filtrate was rinsed with 25 mL of acetone. The solvent was evaporated to dryness and brought up in 10 mL of chloroform. The sample was divided in half and transfered to two $^1/_2$-dram vials with Teflon screw-capped lids. The solvent was evaporated, and one sample was reacted with 200 μL of trifluoroacetic acid anhydride (TFAA) at 60 °C for 20 min. The excess TFAA was evaporated, and 20 μL of chloroform was used as the carrier solvent for the gas chromatographic run. One microliter was injected for analysis. The other half of the sample was reacted with 10 μL of the silylating reagent Tri/Sil TBT (Pierce Chemical Co., Rockford, IL) and 10 μL of chloroform. One microliter was taken for sample analysis.

Chromatographic Conditions. Samples were delivered on to a 30-m fused silica, SE-54 (DB-5) coated column with a splitless injection technique. The oven temperature program was 80–300 °C at 30 °C/min. The injection port temperature was 250 °C. The transport line temperature was 270 °C. A helium flow rate of 60 mL/min inlet and 1.5 mL/min exit was used.

Mass Spectrometer Conditions. The mass spectrometer was tuned with perfluorotributylamine in positive methane chemical ionization. The source temperature was set at 150 °C and source pressure at 0.6 torr.

Registry No. T-2, 21259-20-1; TC-6, 91860-55-8; TC-6 (isomer I), 91860-56-9; TC-6 (isomer II), 91860-57-0.

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Reaction Products (Isomers) of Two Metabolic Derivatives of T-2 Toxin (TC-1 and TC-3) When Reacted with Trifluoroacetic Acid Anhydride

Robert J. Pawlosky, Chester J. Mirocha,* and Takumi Yoshizawa

T-2 metabolites TC-1 and TC-3 (3′-hydroxy-T-2 toxin and 3′-hydroxy-HT-2 toxin) when reacted with trifluoroacetic acid anhydride each forms two isomers. The isomers resolve on SE-54 (DB-5) capillary columns and are unsaturated in either the C-3′ or C-2′ position. This characteristic reaction serves as an aid in locating other unknown metabolites of T-2 that contain a hydroxyl group on the C-3′ position.

T-2 toxin [4β,15-diacetoxy-8-[(3-methylbutyryl)oxy]-3-hydroxy-12,13-epoxytrichothec-9-ene] is a potent mycotoxin produced by some isolates of various species of *Fusarium* that can cause hemorrhaging, abortion, dermal necrosis, emesis, leukopenia, immunodepression, hindquarter paresis, and inhibition of protein synthesis in various animals and biological systems (Mirocha, 1983). When intubated into dairy cows, various metabolic derivatives were discovered, some of which were called TC-1 and TC-3 (Yoshizawa et al., 1981). The latter were identified as the C-3′ hydroxylation products of T-2 and HT-2, respectively (Yoshizawa et al., 1982). Their structures are shown in Figure 1. When reacted with silylating reagents such as TBT (Pierce Chemical Co.), one major trimethylsilyl ether derivative is formed of each metabolite that is resolvable by capillary gas chromatography. When TC-1 and TC-3 are reacted with trifluoroacetic acid anhydride, two isomers of each derivative are formed and are the subject of this paper. Trifluoroacetyl (TFA) derivatives were chosen because their detection sensitivity is much greater than that of Me_3Si derivatives in chemical ionization mass spectroscopy.

EXPERIMENTAL SECTION

Derivative Formation (Trimethylsilyl Ethers) of T-2, HT-2, TC-1, and TC-3 Toxins. Standards of T-2 and HT-2 toxin were obtained from *Fusarium* cultures that were extracted and purified in our laboratory. TC-1 and TC-3 were supplied by T.Y. and were obtained from a synthetic preparation. The purity of the toxins approximated 98%. Stock solutions (20 μL each) of 100 ng/μL were pipetted into a $^1/_2$-dram vial with a Teflon-lined screw cap. After the solvent was evaporated, 10 μL of the silylating reagent (Tri-Sil/TBT, Pierce Co., Rockford, IL) and 10 μL of chloroform were added. The samples were left at room temperature for 5 min to equilibrate, and then 1 μL was injected for analysis.

Derivative Formation (Trifluoroacetate) of T-2, HT-2, TC-1, and TC-3. Standards were transferred to $^1/_2$-dram vials as described in the silylation procedure above. The solvent was evaporated, and 200 μL of trifluoroacetic acid anhydride (Pierce Chemical Co., Rockford, IL) was added. The samples were heated at 60 °C for 20 min. The TFAA was then evaporated under nitrogen, and chloroform was added as the carrier solvent. One microliter was injected into the gas chromatograph.

Gas Chromatographic and Mass Spectral Conditions. A 30-m, 0.25-i.d. fused silica capillary column coated with dimethyl silicone gum (SE-54) was used. The samples were introduced by splitless injection with a delay time of 0.5 min. Flow rates were 60 mL/min, inlet, and 1.5 mL/min, exit. The injection port temperature was 250 °C. The GC oven was programmed from 80 to 300 °C at 30 °C/min.

The mass spectrometer was tuned with perfluorotributylamine with methane as the reagent gas. The source temperature was set at 150 °C, and pressure was measured at 0.6 torr for methane and 0.2 torr for ammonia. The electron energy was set at 200 eV.

RESULTS AND DISCUSSION

TC-1 (III), when reacted with a Me_3Si-donating reagent, will produce the predominant species consisting of the reaction product of two sylilated hydroxyl groups, one at C-3 and the other at C-3′, resulting in a molecular ion of 626. On the other hand, the TFA derivative of TC-1 forms two predominant isomeric species resolvable by capillary gas chromatography, neither of which represents reaction with two hydroxyls but rather only one of the pair. The two isomers formed have a molecular ion of 560. The molecular ion of the TFA derivative of the parent compound (T-2 toxin) is 562, 2 mass units more than that of

Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota 55108 (R.J.P. and C.J.M.), and Department of Food Science, Faculty of Agriculture, Kagawa University, Kagawa, Japan (T.Y.).

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