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Trichothecene Biosynthesis in *Fusarium sporotrichioides*: Origin of the Oxygen Atoms of T-2 Toxin

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Mass spectral analysis of T-2 toxin formed during the growth of *Fusarium sporotrichioides* (ATCC 24043) in the presence of $H_2{}^{18}O$ showed incorporation of up to three ^{18}O atoms per toxin molecule. The carbonyl oxygens of the acetates at C-4 and C-15 and of the isovalerate at C-8 were derived from H_2O. Toxin formed in the presence of ^{18}O molecular oxygen incorporated up to six ^{18}O atoms per toxin molecule. The overall incorporation was 78 and 92% of toxin molecules labeled for $H_2{}^{18}O$ and $^{18}O_2$ labeled samples, respectively. The oxygens of position 1, the 12,13-epoxide, and the hydroxyl groups at C-3, C-4, C-8, and C-15 were all derived from molecular oxygen.

The trichothecenes are a family of bicyclic sesquiterpenoid toxins which are produced by fusaria and related fungi and which have been implicated in mycotoxicoses of man and animals (12). Precursor feeding experiments in *Trichothecium roseum* have shown that the trichothecene carbon skeleton is formed by cyclization of farnesyl pyrophosphate via the intermediate trichodiene (4). The origin of the oxygen atoms, especially the mechanisms of formation of the pyran ring and of the 12,13-epoxide of the trichothecene nucleus, has not been determined. However, it is known that during hydroxylation of trichodiene by *T. roseum* and *Fusarium culmorum*, the oxygen atoms at C-3 and C-4 are introduced with retention of configuration (1, 3). We have been investigating the biosynthesis of 4β,15-diacetoxy-8α(3-methylbutyryloxy)-3-α-hydroxy-12,13-epoxytrichothec-9-ene (T-2 toxin) (Fig. 1) by *Fusarium sporotrichioides*. We report that the carbonyl oxygens of the acetates at C-4 and C-15 and of the isovalerate at C-8 were derived from water. The oxygens at position 1, the 12,13-epoxide, and the hydroxyl groups at C-3, C-4, C-8, and C-15 were all derived from molecular oxygen.

MATERIALS AND METHODS

Cultures and growth conditions. The fungal isolate used in these experiments was a single conidial isolate selected from a lyophilized stock culture of *F. sporotrichioides* NRRL 3299 (ATCC 24043) (9). The results of three experiments are reported here. In the first, $H_2{}^{16}O$ and $^{16}O_2$ were used; in the second, $H_2{}^{18}O$ and $^{16}O_2$ were used; and in the third, $H_2{}^{16}O$ and $^{18}O_2$ were used. For each experiment, a 300-ml Erlenmeyer flask containing 150 ml of 5% glucose–0.1% yeast extract–0.1% peptone was inoculated with conidia to a final concentration of 10^5 conidia per ml. Cultures were incubated for 48 h at 28°C in the dark at 200 rpm on a gyratory shaker. Mycelium was then collected on a sterile cloth filter and suspended in 50 ml of culture filtrate. One milliliter of this mycelial suspension was placed in a 10-ml Erlenmeyer flask, and 1 ml of labeled (98% atom enrichment; MSD Isotopes, Merck Chemical Division, St. Louis, Mo.) or unlabeled water was added. The flask was sealed with a rubber septum and then connected to a nitrogen-flushed reservoir and nitrogen flushed again. A 100-ml reservoir of labeled (98% atom enrichment) or unlabeled oxygen gas was then connected to the system. Successful toxin production depended on aerating the cultures; therefore, a peristaltic pump was used to slowly circulate gas through a sterile cotton filter and directly into the culture medium. A simple manostat served to maintain constant pressure throughout the incubation period. After 3 days of incubation at 28°C in the dark at 50 rpm on a reciprocal shaker, the culture was diluted with 5 ml of water and extracted five times with ethyl acetate to a final volume of 50 ml. The extract was evaporated to dryness and redissolved in 200 μl of methylene chloride.

Sample preparation and hydrolysis. Culture media extracts were analyzed for T-2 by using a quadrupole tandem mass spectrometer. Trimethylsilyl (TMS) derivatives of each extract were made from 20-μl portions of the above samples by evaporation of the methylene chloride under nitrogen and addition of 20 μl of Tri-Sil/TBT (Pierce Chemical Co., Rockford, Ill.). The samples were allowed to stand at room temperature for at least 1 h before analysis by gas chromatography-mass spectrometry (GC-MS). Injection of 1 μl of the derivative (equivalent to 20 μl of the 2-ml liquid culture) provided adequate GC-MS results.

A portion of extract equivalent to 200 μl of each 2-ml culture was hydrolyzed by adding 100 μl of 0.1% sodium methoxide in methanol for 1 h at room temperature. This mixture was then washed through a silica microcolumn (ca. 5 cm by 0.5 mm) with 1 ml of methanol to remove sodium methoxide and evaporated to dryness. The dry residue was derivatized with 20 μl of Tri-Sil TBT, and 1 μl was analyzed for TMS-derivatized T-2 tetraol by GC-MS.

Instrumentation. The crude underivatized extract was analyzed by using the solids probe, and the TMS derivative of the sample was analyzed by GC-MS on a Finnegan 4535 ITSQ instrument. The mass spectrometer source was operated in the chemical ionization mode with isobutane (0.3 torr [ca. 40.0 Pa]) or ammonia (0.2 torr [ca. 26.7 Pa]) as the chemical ionization gas. Normal mass spectra were obtained by operating the first two quandrupoles in the all pass (radiofrequency only) mode and scanning the third quadrupole. In tandem mass spectrometry experiments (10),

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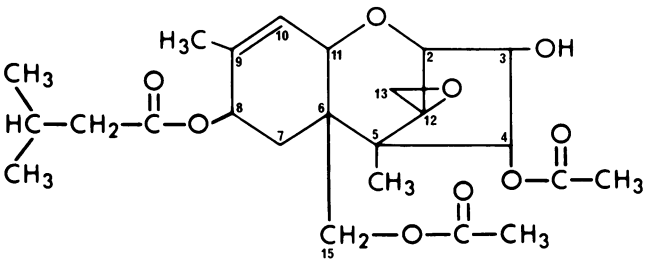

FIG. 1. Structure of T-2 toxin.

the protonated molecule (MH^+) was selected by the first quadrupole, and daughter fragments were formed in a collision cell (quadrupole 2) by collision with argon (1 mtorr [ca. 0.13 Pa] and 20 V). The resulting daughter ions were detected and mass analyzed by the third quadrupole.

GC-MS analyses were made with a fused silica capillary DB-1 column (30 m by 0.25 mm; J & W Scientific). The linear flow rate of helium in the column was 50 cm/s. The injection port temperature was held at 250°C. Samples were injected in the split mode with a split ratio of approximately 60:1. The outlet of the capillary column was directly coupled into the source of a quadrupole tandem mass spectrometer. The gas chromatograph temperature was held at 120°C for 2 min and then programmed to increase to 270°C at 5°C per min and held at that temperature.

RESULTS AND DISCUSSION

The amounts of T-2 formed in the control and in the $^{18}O_2$-labeled and $H_2{}^{18}O$-labeled cultures of *F. sporo-*

TABLE 2. Relative abundance of ^{18}O-labeled T-2 tetraol TMS derivative[a]

No. of ^{18}O atoms incorporated	*m/e*	Relative abundance of:		
		Standard T-2 tetraol	Hydrolyzed $H_2{}^{18}O$-labeled T-2 toxin	Hydrolyzed $^{18}O_2$-labeled T-2 toxin
0	585	1.0	1.0	
	586			
	587	100	100	14
	588	38	41	6
1	589	20	22	5
	590	7	6	4
2	591	2	2	29
	592			13
3	593			68
	594			32
4	595			100
	596			46
5	597			94
	598			40
6	599			44
	600			8
	601			11

[a] The empirical formula and molecular weight of the TMS derivative of T-2 tetraol are $C_{27}H_{54}O_6Si_4$ and 586, respectively.

TABLE 1. Relative abundance of ^{18}O-labeled T-2 toxin[a]

No. of ^{18}O atoms incorporated	Underivatized T-2				T-2 TMS derivative			
	m/e	Relative abundance containing ^{18}O from:			*m/e*	Relative abundance containing ^{18}O from:		
		Natural abundance	$H_2{}^{18}O$	$^{18}O_2$		Natural abundance	$H_2{}^{18}O$	$^{18}O_2$
0	465	5	12	21	537	8	7	
	466	1	2	9	538	3	3	
	467	100	61 (60)	33 (11)	539	100	62	9
	468	20	13	10	540	32	27	3
1	469	4	100 (100)	17 (10)	541	9	100	10
	470	0.5	17	5	542	1	42	2
2	471		90 (94)	39 (41)	543		93	30
	472		28	8	544		37	5
3	473		28 (31)	73 (85)	545		32	61
	474		7	12	546		10	9
4	475			100 (100)	547		2	100
	476			21	548			19
5	477			94 (63)	549			88
	478			21	550			19
6	479			45 (16)	551			31
	480			10	552			6

[a] A small signal appeared in the isobutane chemical ionization spectrum of the TMS derivative of T-2 toxin at $(M-H)^+$ due to proton abstraction. The abundance of this ion was approximately equal to the level of the $[MH + 2]^+$ ion attributed to naturally occurring isotopes (^{13}C). Therefore, the relative abundances of ions at the expected masses were used directly as a measure of the abundance of the ^{18}O atoms without correction for natural isotope abundances or proton abstraction. The predicted distribution of labeled atoms in underivatized T-2 (shown in parentheses) was calculated from the binomial distribution, assuming three atoms to be labelable from H_2O and six to be labelable from O_2 and with average isotope enrichments of 43 and 61% for underivatized H_2O- and O_2-labeled samples, respectively. The empirical formula and molecular weight for each compound are as follows: underivatized T-2, $C_{24}H_{34}O_9$, 466; T-2 TMS derivative, $C_{27}H_{42}O_9Si$, 538.

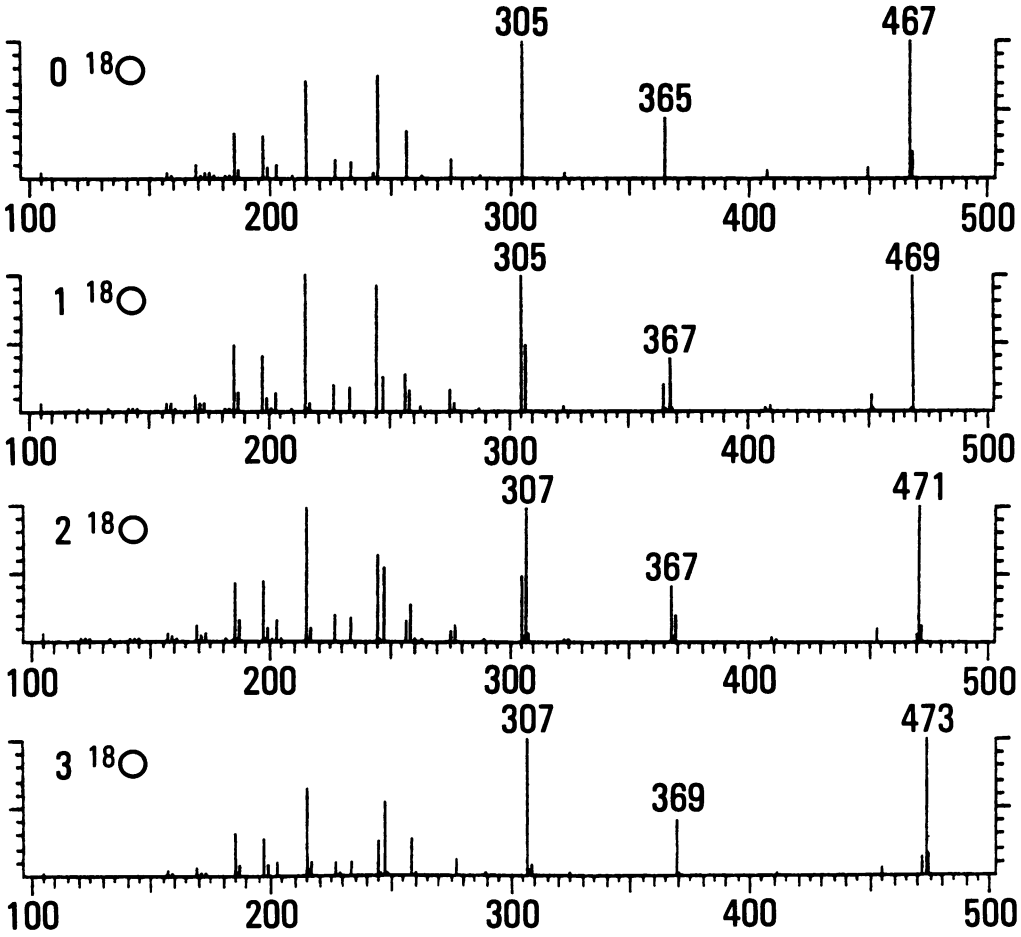

FIG. 2. Daughter spectra of MH^+ ions from $H_2{}^{18}O$-labeled T-2 toxin with incorporation of zero to three ^{18}O atoms.

trichioides were estimated by GC-MS of the TMS derivative of the ethyl acetate extract. The yield of T-2 in all three samples was approximately 250 μg/ml. Both sources of ^{18}O gave rise to labeled T-2 toxin molecules as well as unlabeled toxin. The degree of ^{18}O incorporation was determined by the ratios of the MH^+ isotope cluster (Table 1) for the TMS derivative at *m/z* 539 to 552. These values were confirmed by analyzing the MH^+ region of the spectrum of the underivatized sample at *m/z* 467 to 480. The overall incorporation was 78 and 92% of toxin molecules labeled for $H_2{}^{18}O$ and $^{18}O_2$, respectively. The analysis of underivatized T-2 was made directly from the crude ethyl acetate extract and thus was more subject to chemical noise from other components in the sample matrix. Nevertheless, in all three samples, the data in the MH^+ region for underivatized T-2 toxin were in excellent agreement with the data obtained by GC-MS of the TMS derivative.

In the presence of $H_2{}^{18}O$, toxin was a mixture of molecules containing zero to three labeled atoms (*m/z* 539, 541, 543, and 545 in the TMS derivative and *m/z* 467, 469, 471, and 473 in the underivatized toxin). The observed distribution of the TMS-derivatized and underivatized toxin molecules was in good agreement with the prediction based on three labelable atoms (Table 1).

In the presence of $^{18}O_2$, up to six ^{18}O atoms were incorporated per molecule of toxin. Again, there was good agreement between the observed distribution and the prediction based on six labelable atoms (Table 1). Both $^{18}O_2$- and $H_2{}^{18}O$-labeled samples contained fewer labeled oxygen atoms than predicted. This was probably due to the presence of some T-2 toxin or partially oxygenated intermediates in the cultures before exposure to labeled oxygen.

The three atoms derived from $H_2{}^{18}O$ were located by hydrolysis followed by GC-MS analysis of the TMS derivative of the T-2 tetraol formed. After the carbonyl oxygens of the isovalerate at C-8 and the acetates at C-4 and C-15 were removed by hydrolysis, the resulting T-2 tetraol mass spectrum showed no labeled ^{18}O atoms and was identical to that of unlabeled T-2 tetraol (data not shown). Therefore, the acetate and isovalerate carbonyl oxygens contained all of the water-derived ^{18}O atoms. Although the mechanism of incorporation of labeled water has not been determined, it is possible that the labeled oxygen of isovalerate may originate from leucine via transamination (8), and that of acetate may originate from β-oxidation of fungal fatty acids (8). Incorporation via exchange with the oxygen of water molecules cannot be disproved by our data (2). However, there was no loss of ^{18}O label from toxin incubated in water at neutral pH.

The mass spectrum of T-2 tetraol, obtained upon hydrolysis of molecular $^{18}O_2$-labeled toxin, had the same isotope distribution as labeled T-2, showing that none of the label was removed when the isovalerate and acetate carbonyl oxygens were hydrolyzed (Table 2). These results can be explained by assuming that the oxygens of all four

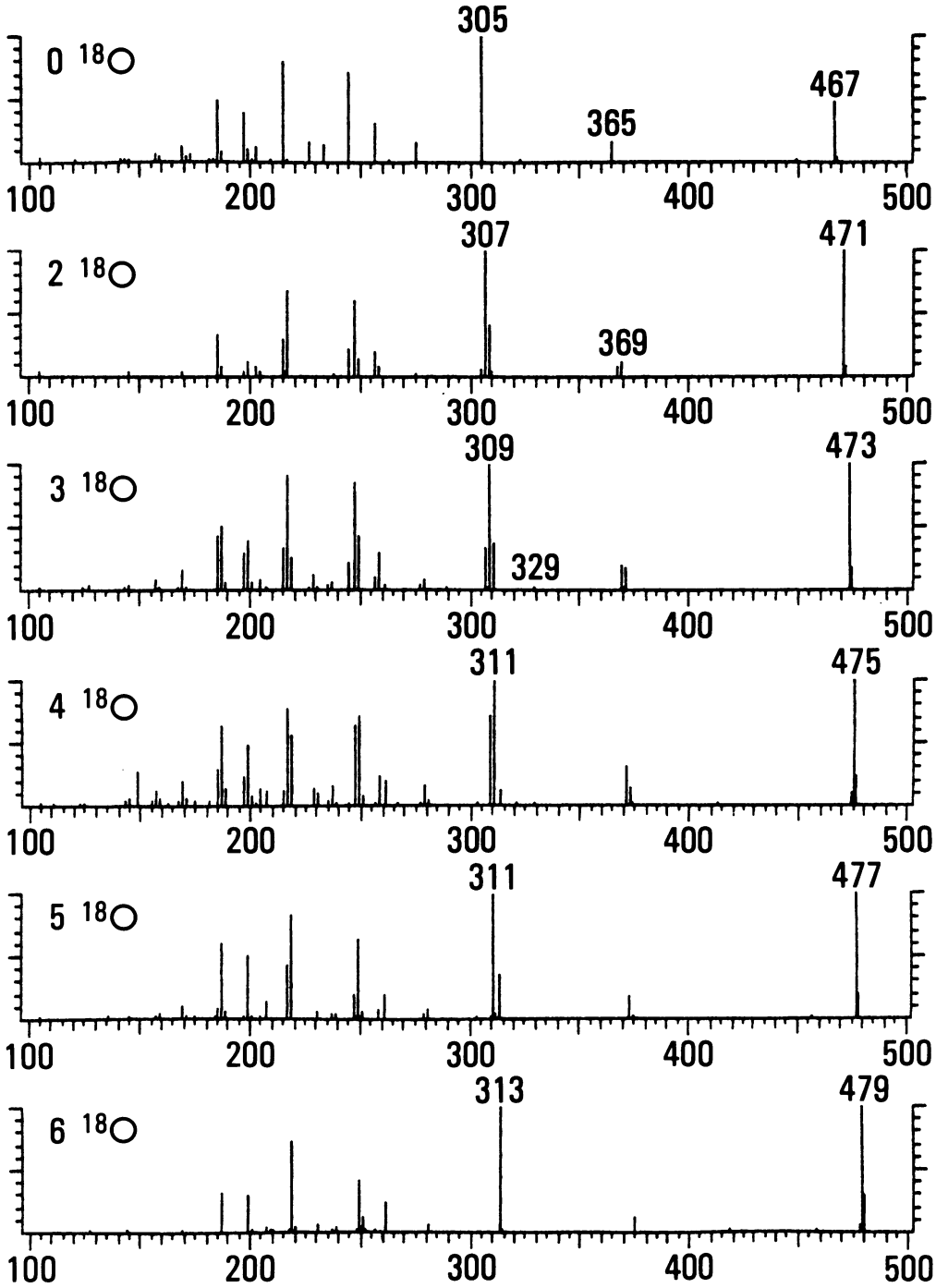

FIG. 3. Daughter spectra of MH^+ ions from $^{18}O_2$-labeled T-2 toxin with incorporation of zero to six ^{18}O atoms.

hydroxyls, as well as those of the pyran ring and the 12,13-epoxide, are derived exclusively from molecular oxygen and not from water.

To obtain additional data on the labeling of acetate and isovalerate oxygens, toxin samples were examined by tandem mass spectroscopy. Daughter ions from MH^+ toxin molecules with zero, one, two, and three ^{18}O atoms in the $H_2{}^{18}O$-labeled sample and with zero, two, three, four, five, and six ^{18}O atoms in the $^{18}O_2$-labeled sample were individually recorded (Fig. 2 and 3).

The MH^+ parent ion of unlabeled T-2 toxin (*m/z* 467) had a weak daughter ion (*m/z* 449), probably due to the loss of water at C-3. A second weak daughter ion (*m/z* 407) was equivalent to loss of acetic acid (−60) from C-4 or C-15. A more intense daughter ion (*m/z* 365) corresponded to the loss of isovaleric acid (−102) from C-8, and a second (*m/z* 305) corresponded to the combined loss of isovaleric and acetic acids (−162). The ion at *m/z* 305 and other daughter ions at lower *m/z* values undoubtedly arose from the parent through multiple collisions in quadrupole 2.

The MH^+ parent ions of ^{18}O-labeled T-2 toxin had weak daughter ions equivalent to losses of singly ^{18}O-labeled acetic acid (−62), isovaleric acid (−104), and combined isovaleric and acetic acid (−166). No loss of doubly ^{18}O-labeled acetic acid (−64) or isovaleric acid (−106) was observed in either the water-labeled sample or the O_2-labeled sample. Therefore, a maximum of one oxygen per ester was labeled. However, our isotope technique does not conclusively disprove the role of O_2 in biosynthesis of the carbonyl oxygens of T-2 toxin, because oxygen atoms of carbonyl groups can exchange under some conditions with the oxygen of water molecules (2).

A great deal of interest has been directed toward identifying trichothecenes and determining their biosynthetic relationships (13). A variety of minor metabolities of oxygenated trichothecenes have been isolated from fungal cultures (5, 7, 11). However, their intermediacy in the biosynthetic pathway has seldom been established by precursor feeding experiments. The results of this study demonstrate that molecular oxygen is the biosynthetic precursor for all six noncarbonyl oxygens of T-2 toxin, including those of the pyran ring and the 12,13-epoxide. It may be concluded that these six oxygenations are catalyzed by aliphatic hydroxylases (14). Enzymatic hydroxylation of a variety of substrates proceeds with retention of steric configuration and can be catalyzed by either dioxygenases or monooxygenases (6). These enzyme mechanisms can be distinguished by their sensitivity to a variety of inhibitors. Studies of the effects of monooxygenase inhibitors on T-2 toxin biosynthesis in intact cells of *F. sporotrichioides* are now in progress.

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