Metabolism of Zearalenone by *Fusarium roseum* Graminearum

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Twenty products, associated with the metabolism of zearalenone by *Fusarium roseum* Graminearum, were analyzed with respect to time and cultural conditions. Statistical methods of analysis were used to select a set of six metabolites possibly related to zearalenone. The products of metabolism were analyzed by combination gas chromatography–mass spectroscopy with additional characterization by infrared and ultraviolet spectrophotometry. The components identified included the two isomers of 8'-hydroxyzearalenone, 6',8'-dihydroxyzearalene, 7'-zearaldienone, and 6-(carboxypentyl)-β-resorcylic acid (component 11). Three other derivatives were tentatively identified as the aldehyde of component 11, trihydroxyphenylacetic acid and 3,5-dihydroxyphenylacetic acid. Evidence for their genesis from zearalenone is presented. A suggested scheme of metabolism of zearalenone by *F. roseum* includes the sequential formation of the two isomers of 8'-hydroxyzearalenone, 6',8'-dihydroxyzearalene, and 6-(carboxypentyl)-β-resorcylic acid, followed by derivatives of phenylacetic acid.

Zearalenone [2,4-dihydroxy-6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)benzoic acid μ-lactone], Chart I, compound I, is a secondary metabolite of some strains of *Fusarium roseum*, *F. oxysporum*, *F. tricinctum*, and *F. moniliforme*. It has been implicated as a causal agent of hyperestrogenism in swine (Mirocha et al., 1971), and plays a regulatory role in the sexual processes of *F. roseum* (Wolf and Mirocha, 1973). These and other biological activities have been reviewed by Mirocha et al. (1971).

The biosynthesis of zearalenone has been shown to be typical of the polyketides (Steele et al., 1974), i.e. acetate malonyl-CoA. Several materials present in cultures of *F. roseum* have been reported to have properties similar to zearalenone (Mirocha et al., 1971). Bolliger and Tamm (1972) reported that 8'-hydroxyzearalenone (Chart I, compound II), 8'-*epi*-hydroxyzearalenone, 5-formylzearalenone, and 7'-dehydrozearalenone are present in cultures of *F. roseum*. Jackson et al. (1974) have determined that the materials described by Mirocha et al. (1971) as F-5-3 and F-5-4 are the 8'-hydroxyzearalenones. No investigations regarding the metabolic relations among these seemingly related metabolites have been reported, nor has the degradation of zearalenone by *F. roseum* been investigated.

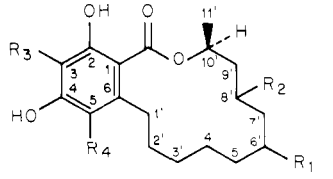
Several problems have impeded studies of the degradation of zearalenone even though data concerning the degradation products would be of some potential use to those concerned with the presence of zearalenone in foods and feeds. Some of the difficulties encountered are that, generally, zearalenone is produced by the fermentation of moist solid substances such as rice or corn. These contain large proportions of carbohydrates and lipids and thus limit the specific activity of zearalenone which can be economically obtained by the addition of a radiolabeled precursor. This precludes its use in determining which of the many metabolites found in cultures of *F. roseum* on solid substrates might arise from its metabolism. An alternative approach is to individually isolate and characterize each component of the complex mixture of metabolites obtained after culturing and attempt to infer the metabolic relationships among them on the basis of their chemical structures; this is both time consuming and expensive. An alternative is to correlate the concentrations of the various metabolites which appear upon culturing with the concentration of zearalenone over a relatively wide range of environmental conditions and cultural ages. Such an approach requires an analytical system capable of estimating many components in a sample without relying on specific features of the components in question. For zearalenone and many similar compounds temperature programmed gas chromatography (GC) of the trimethylsilyl ether (Me_3Si) derivatives of the various metabolites on relatively nonpolar liquid phases offers a fairly nonselective, sensitive, and quantitative method. Gas chromatography offers an additional advantage in that it can be effectively coupled with a mass spectrometer to obtain a considerable amount of qualitative data concerning the identity of the components being analyzed. In addition a great degree of selectivity can be introduced into the analysis by applying the techniques of multiple ion detection made available through the use of a mass spectrometer. Moreover, selective detection of nanogram quantities of one material in the presence of microgram quantities of interfering materials can be achieved using this means of analysis.

The apparent advantages of these approaches, as well as the availability of mass spectral studies of zearalenone and related compounds (Jackson et al., 1974) encouraged us to apply them to the problem of determining the fate of zearalenone in cultures of *F. roseum*.

MATERIALS AND METHODS

Source and Maintenance of *F. roseum*. The organism used in these studies, unless otherwise indicated, was a subculture of a mass isolate of *F. roseum* (Lk.) Sny. and Hans. from moldy corn collected near Mapleton, Minn. in

Chart I. Various Derivatives of Zearalenone



	$C_{1'-2'}$	R_1	R_2	R_3	R_4
I, zearalenone	CH=CH	=O	H_2	H	H
II, 8'-hydroxyzearalenone	CH=CH	=O	OH	H	H
III, 6',8'-dihydroxyzearalene	CH=CH	OH	OH	H	H
IV, zearalanone	CH_2—CH_2	=O	H_2	H	H
V, zearalanol	CH_2—CH_2	OH	H_2	H	H
VI, 3,5-dibromozearalenone	CH=CH	=O	H_2	Br	Br

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1963. This isolate was maintained on moist, autoclaved soil at 4°C, and designated as JRL (MAP 10).

Preparation of Shredded Wheat Cultures. Six shredded wheat biscuits (Nabisco; weight of each biscuit, 1.1–1.2 g) were placed in a petri plate, autoclaved for 1 hr at 121°C on each of two successive days, and then seeded with *F. roseum* from soil cultures. Each biscuit was then moistened with 1 ml of sterile 10% sucrose solution. After 5 days of incubation at 25°C and 95–100% relative humidity, two of these biscuits were crushed and suspended in 100 ml of sterile 10% sucrose solution and used as inoculum. Shredded wheat biscuits used in various experiments were sterilized as described and inoculated with 1 ml of this suspension. After inoculation, biscuits were examined periodically for bacterial and fungal contaminants.

In experiments which required the application of various derivatives of zearalenone, the substances were applied in 0.2 ml of ethanol per biscuit. The ethanol was removed under reduced pressure in a sterile chamber and the biscuits were then inoculated.

Sources of Experimental Compounds. Zearalenone was obtained from cultures of *F. roseum* on moist rice prepared as previously described (Steele et al., 1974). The material obtained from these cultures was purified by repeated crystallization from mixtures of ethyl acetate and isooctane until pure. Purity was determined by gas chromatography (GC) and thin-layer chromatography (TLC). The melting point of this product was 158–159°C.

The zearalanol isomer (Chart I, compound V), having a melting point of 180–181°C, was a gift from Commercial Solvents Corp., Terre Haute, Ind.

Zearalanone (Chart I, compound IV) was prepared by the oxidation of zearalanol with CrO_3–H_2SO_4 in acetone (Fieser and Fieser, 1967). The product was extracted with diethyl ether, purified by TLC, and recrystallized from methylene chloride–petroleum ether. Its yield was ca. 86% and had a melting point of 189–190°C. Anal. Calcd for $C_{18}H_{24}O_5$: 320.1622. Found: 320.1612.

Dibromozearalenone (Chart I, compound VI) was prepared by the bromination of zearalenone using KBr–$KBrO_3$ in concentrated HCl as the brominating agent. The product obtained was purified by repeated crystallization from ethanol–water until no impurity was detectable by GC or TLC. The NMR spectrum of this derivative showed the absence of aromatic protons and the presence of olefinic protons (7.00, d, 1, J = 16 Hz). The molecular ion was found at m/e^+ 474, 476, and 478 as one would expect from the isotopes of bromine.

Demethylation of 2,4-dimethoxy-6-(carboxypentyl)-benzoic acid (1 mg/ml of CH_2Cl_2) was accomplished by adding a solution of BBr_3 (100 μl) in CH_2Cl_2 (1 ml) to the former at 0°C. The reaction mixture was stirred at room temperature for 24 hr and then hydrolyzed with water; the product was extracted with CH_2Cl_2 and was separated into four components by TLC on silica gel G. In addition to the unreacted starting material, two monomethylated products were detected.

Analytical Gas Chromatography. Trimethylsilyl ether (Me_3Si) derivatives used in gas chromatography were prepared by adding 25 μl of a mixture of *N,N*-bis(trimethylsilyl)acetamide and trichloromethylsilane (5:1, v/v) to a dry sample (0.1–1 mg) in a 0.5-dram vial with a polyethylene lined screw cap. The reaction was allowed to proceed for at least 30 min before analysis was carried out. Analysis of the sample was achieved by injecting 1 μl of the reaction mixture onto a 3 ft × 1/8 in. stainless steel column packed with 3% OV-1 methyl silicone on a solid support of 100–120 mesh Gas-Chrom Q. The carrier gas was high purity nitrogen with a flow rate of 20 ml/min. All separations were performed using a custom built Varian 1500 series dual column gas chromatograph with flame ionization detectors. The analytical work reported here was carried out using a linear temperature program of 4 to 6°C/min from 150 to 275°C followed by a 5-min isothermal period at 275°C.

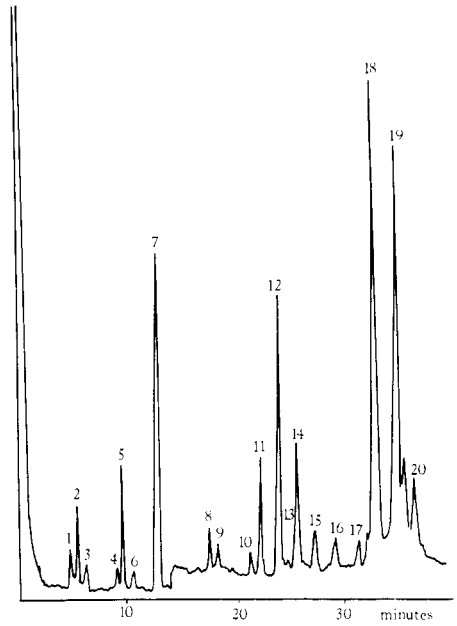


Figure 1. A gas chromatogram of the Me_3Si derivatives of components extracted from cultures of *Fusarium roseum* growing on shredded wheat. The numbers correspond to the component numbers used in the text.

Combined Gas Chromatography and Mass Spectrometry. An LKB-9000 combined gas chromatograph–mass spectrometer was used to obtain mass spectra of various gas chromatographic components as well as mass spectra of underivatized materials. Gas chromatographic conditions were similar to those above, but helium was used as the carrier gas. See Figure 1 for the separation of components.

Spectra were obtained at 70 eV, and were examined over a 0–750 mass range. Nominal masses were assigned to each fragment in the spectra, and up to 120 selected ions were measured. Appropriate background spectra were taken to correct for background contributions. These data were reduced to usable form with a FORTRAN program which was developed for this application (Steele, 1974). This program also calculated arrays of possible losses which proved useful in interpreting the spectra.

Thin-Layer Chromatography. TLC separations were carried out on 0.250-mm layers of silica gel G (Brinkmann). Preparative separations were carried out on 2-mm layers of silica gel Pf 254 (Brinkmann). Various solvents were used to develop the chromatograms, and these are specified in the appropriate sections.

Measurement of Gas Chromatographic Components from Cultures of *F. roseum*. Three groups of shredded wheat biscuits were inoculated with *F. roseum*. Treatment A consisted of cultures maintained at 25°C during the 1113-hr duration of the experiment. Treatment B consisted of biscuits maintained at 25°C for 110 hr and then transferred to 12°C for the remaining 1003 hr. Treatment C was as B but was returned to 25°C for the final 394 hr of incubation.

After each treatment reached its final incubation temperature, biscuits were removed at intervals of 3–5 days

for analysis. Each sample was ground and extracted with 10 ml of water-saturated ethyl acetate for at least 6 hr. A portion of this extract (0.5 ml) was transferred to a 0.5-dram vial and reduced to dryness under nitrogen, and the Me_3Si derivatives were analyzed by gas chromatography. A standard containing 0.5 μg of zearalenone as its bis(trimethylsilyl) ether was analyzed before and after each group of three samples to ensure proper quantitation. The relative quantities of each component were then estimated by comparison with the analytical standard. Individual components were identified by their relative retention times and by their position in the chromatographic pattern. Each metabolite was assigned a number which was used in identification of components throughout the experiment. Upon completion of the experiment, the collected data were analyzed using programs maintained by the University of Minnesota Computer Center (Frisch, 1973).

A typical sample containing all of the measurable components was selected, and mass spectra of the Me_3Si derivatives of each component were obtained using combined gas chromatograph–mass spectrometry. Separation of the components of this sample is presented in Figure 1 and the peaks are identified by number.

Isolation of Component 16 (6',8'-Dihydroxyzearalene). Fifty shredded wheat biscuits were prepared as previously described. After 5 days at 25°C, the cultures were transferred to a 12°C incubator for an additional 25 days. After this period they were extracted with ethyl acetate. The extract was concentrated to an oil and dissolved in 2 ml of chloroform. This was applied to a 1 cm × 15 cm silica gel (Matheson no. 923) column packed in chloroform. Six fractions were obtained by elution with 25 ml each of mixtures of chloroform and acetone containing 10, 25, 50, 75, and 100% acetone (v/v). The Me_3Si ether derivatives of these fractions were analyzed by GC and component 16 detected in the last two fractions. The two fractions were combined, concentrated, and applied to a preparative silica plate which was developed with chloroform–acetone (7:3, v/v). After recovery of the substances present in the various bands on the plate with acetone, analysis by GC indicated that component 16 corresponded to a blue fluorescent band (254 nm) at R_f 0.6. This fraction was chromatographed once more on a silica gel plate developed in chloroform–ethanol (9:1, v/v) and resulted in the isolation of material which was pure as determined by gas chromatography.

To confirm the identity of the isolated compound, a mass spectrum of the Me_3Si derivative was obtained. In addition a mass spectrum of the underivatized material (direct probe) was obtained. Ultraviolet spectra of the isolated substance were taken in 95% ethanol and 0.05 *N* ethanolic NaOH. An infrared spectrum of 0.5 mg of the isolated substance in a KBr pellet was also taken. In addition, the gas chromatographic and mass spectral behavior of the isolated material was compared with an authentic sample of 2,4-dihydroxy-6-(6,8,10-trihydroxy-*trans*-1'-undecenyl)benzoic acid μ-lactone (Jackson et al., 1974).

The *n*-butylboronate–Me_3Si derivative of component 16 was prepared by treating 50 μg of the isolated substance with 50 μl of a 1% (w/v) solution of *n*-butylboronic acid in dry pyridine for 2 hr. The pyridine was removed under nitrogen, and the products were converted to the Me_3Si derivatives in the usual manner and examined by mass spectrometry and gas chromatography.

Isolation of Component 11 [6-(Carboxypentyl)-β-resorcylic Acid]. A procedure of purification similar to that used with component 16 was followed, but because of increased polarity of this product, an additional fraction consisting of 50 ml of acetone–H_2O (9:1) was used for final elution off the silica gel column. This fraction was concentrated and applied to an unactivated preparative silica gel plate which was then developed successively with chloroform–acetone (7:3) and chloroform–acetone (9:1). The various bands were removed, and the substances present eluted with acetone–water (9:1). Gas chromatographic analysis located component 11 in a broad band centered about R_f 0.5 of the TLC plate and revealed that the fraction also contained other components.

The fraction containing component 11 was dried under nitrogen and Me_3Si derivatives were formed in the usual manner. The solutions of the Me_3Si derivatives were injected in 7-μl portions onto a 0.5 × 7 mm stainless steel gas chromatographic column packed with 10% SE 30 on 60–80 mesh Chromosorb W. Separation was carried out isothermally at 250°C with a nitrogen flow rate of 70 ml/min. The column was connected to a stream splitter which diverted 90% of the flow to a collection system and the remainder of the effluent was directed to a flame ionization detector. The peak representing component 11 was collected and the condensed materials were dissolved in acetone containing 10% water to hydrolyze the Me_3Si derivatives. This solution was thoroughly dried under nitrogen and used in other characterization procedures.

An infrared spectrum was obtained of 250 μg of component 11 in a KBr disk. Ultraviolet spectra were obtained as described for component 16. Mass spectra were obtained of the Me_3Si derivatives of the component as well as the underivatized material (direct probe). For purposes of comparison, an infrared spectrum of 2,4-dihydroxybenzoic acid in KBr was also obtained.

Preparation of a *F. roseum* Isolate Capable of Growing at High Zearalenone Concentrations. *F. roseum* JRL (MAP 10) was grown on shredded wheat biscuits containing 1.0 mg of zearalenone/biscuit. After 5 days at 25°C those biscuits on which growth was noticeable were used to inoculate biscuits containing 2.0 mg of zearalenone/biscuit. Successive transfers to biscuits containing 5 and 10 mg of zearalenone were carried out until a strain capable of apparently normal growth on biscuits containing 10 mg of zearalenone/biscuit was obtained. This isolate was maintained on biscuits containing 10 mg of zearalenone/biscuit at 4°C, and designated as JRL (MAP 10)-B.

Catabolism of Zearalenone by *F. roseum*. Forty-two biscuits were prepared and 1, 2, 5, and 10 mg of zearalenone, respectively, was applied to each biscuit of a group of 7. In addition, four groups of two biscuits each were amended with the same amounts of zearalenone, but were not inoculated. The four groups of seven biscuits were inoculated with JRL (MAP 10)-B. The control groups were moistened with 1 ml of sterile 10% sucrose. All treatments were then incubated at 25°C for 30 days. A biscuit from each group was used to check for contamination, and the remainder were separately extracted and analyzed by gas chromatography of the Me_3Si derivatives.

Catabolism of Zearalanone by *F. roseum*. Ten shredded wheat biscuits were sterilized, and 0.5 mg of zearalanone (Chart I, compound IV) was added to each as described above. The biscuits were inoculated with JRL (MAP 10). An additional five biscuits were prepared in the same manner, but were moistened with sterile sucrose solution (10%) instead of being inoculated with the fungus (control). After 30 days of incubation at 25°C, five of the inoculated biscuits and five control biscuits were extracted with 50 ml of water-saturated ethyl acetate. A portion of

each extract was taken to dryness, and the Me_3Si ether derivatives were analyzed by gas chromatography and mass spectrometry. The multiple ion detector facility of the LKB 9000 was used to record the intensities at m/e 333, 335, 305, and 307. An identical procedure was carried out with the control sample. In addition the inoculated samples were examined at m/e 462, 521, and 536 in an attempt to detect zearalenol.

The crude extract from the inoculated biscuits was fractionated on a silica column as in the isolation of component 11. Those fractions enriched with respect to components 15 and 16 were examined by gas chromatography and mass spectrometry for the presence of hydrogenated analogues of the 8'-hydroxyzearalenones and component 16.

Catabolism of Dibromozearalenone by *F. roseum.* In an attempt to determine if component 11 was derived from zearalenone, an experiment similar to that described for zearalanone was performed, but dibromozearalenone was substituted for zearalanone. After fractionation of the extracts using a silica gel column as described for component 11, the acetone–water fraction was dried and examined using the multiple ion detector at m/e 714. The mass spectra of selected components were taken on the basis of this examination.

Catabolism of Zearalanol by *F. roseum.* Zearalanol (0.5 mg) was administered to cultures of *F. roseum* on shredded wheat biscuits. Incubation at 25°C for 14 days was followed by extraction and analysis by gas chromatography and mass spectrometry.

Isolation of 7'-Dehydrozearalenone. A culture of *F. roseum* on rice was dried and extracted with ethyl acetate, and the concentrated extract was partitioned between equal volumes of chloroform and 2 *N* Na_2CO_3 in aqueous solution. The aqueous layer was acidified with HCl and extracted with chloroform and the chloroform fraction was examined by gas chromatography and mass spectrometry. Prior to analysis by GC, the chloroform fraction was purified by chromatography on preparative silica gel plates which were developed in chloroform–ethanol (97:3, v/v). The upper edge of the band corresponding to zearalenone was removed from the plate and the substances present were recovered with acetone. These materials were then examined by mass spectrometry and gas chromatography.

These procedures were repeated using other cultures of *F. roseum*, but the use of carbonate was avoided.

RESULTS AND DISCUSSION

Statistical Relations among 20 Components from Cultures of *F. roseum.* A typical gas chromatogram of the 20 components is presented in Figure 1. The component numbers used throughout the text are those as listed in the chromatogram. Of the 46 samples examined in this fashion, the average deviation of duplicate samples about their mean value was less than 12% of the mean value. No microbial contamination was detected.

In treatment A (25°C) zearalenone did not accumulate, but rather decreased from an initial value of 20–30 μg/biscuit to 6–10 μg/biscuit after 721 hr of incubation. In this treatment components 11 [2,4-dihydroxy-6-(carboxypentyl)benzoic acid], 15 (8'-hydroxyzearalenone), and 9 (dihydroxyphenylacetic acid) are significantly correlated with zearalenone. The correlation coefficients are, respectively, 0.76, 0.61, and 0.53.

In treatment B (25°C, 110 hr; 12°C, 1003 hr) the zearalenone content increased from about 30 μg/biscuit to 275 μg/biscuit after 1113 hr of incubation. In this treatment components 15, 11, 8, 9, 16, and 10 have respective correlation coefficients of 0.90, 0.88, 0.77, 0.76, 0.63, and 0.66 with zearalenone and are the only components significantly correlated with zearalenone at the 5% level. See Scheme IV for structures of components 8, 16, and 10.

Table I. Correlations among Selected Components from Cultures of *Fusarium roseum* Grown on Moist Shredded Wheat at 25°C for 110 hr and at 12°C for 903 hr (Each Entry Is the Correlation Coefficient Multiplied by 100)

Independent variable	Dependent variable						
	8	9	10	11	14	15	16
8	100[a]						
9	75	100					
10	76	61	100				
11	92	76	82	100			
14	77	76	66	88	100		
15	85	70	66	89	90	100	
16	62	55	57	69	63	72	100

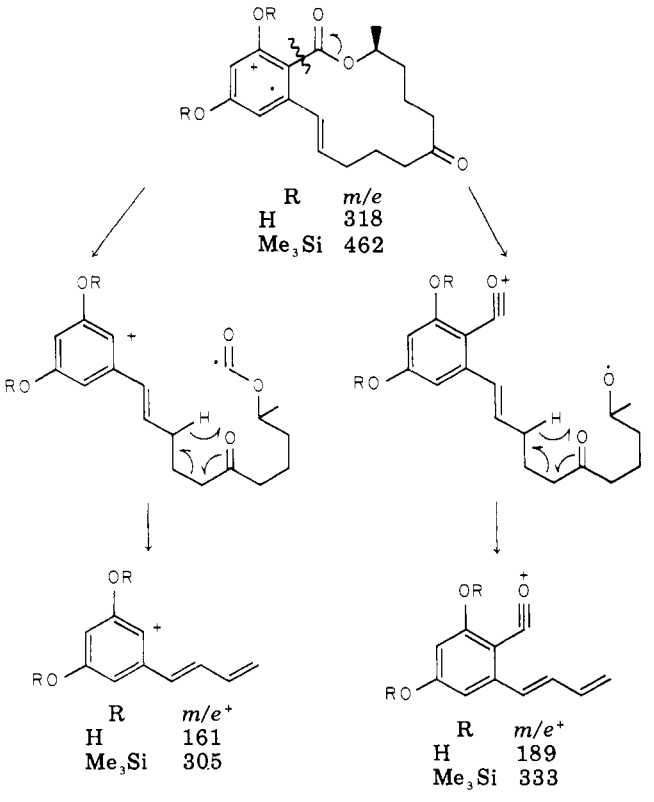
[a] Correlation coefficients greater than 0.56 are significant at the 0.01 level: 8 = 2,3,5-trihydroxyphenylacetic acid; 9 = 3,5-dihydroxyphenylacetic acid; 10 = 2-(5-carboxypentyl)-4,6-dihydroxybenzaldehyde; 11 = 2-(5-carboxypentyl)-4,6-dihydroxybenzoic acid; 14 = zearalenone; 15 = 8'-hydroxyzearalenone; 16 = 6',8'-dihydroxyzearalene.

In treatment C (25°C, 110 hr; 12°C, 609 hr; 25°C, 394 hr) the zearalenone content remained relatively constant at 175–200 μg/biscuit. Only component 15 (8'-hydroxyzearalenone) was significantly correlated with zearalenone. The coefficient in this instance was 0.70. When the data from all three treatments were pooled, components 15, 11, 9, 10, and 16 were significantly correlated with zearalenone. The respective correlation coefficients were 0.90, 0.89, 0.74, 0.62, and 0.60.

An initial set of components possibly related to zearalenone would thus consist of components 9, 10, 11, 15, and 16. An examination of the correlations of components 4, 6, 7, and 8 with members of this set reveals that component 8 is highly correlated with component 11 in treatment B. Similar correlations of components 4, 6, and 7 with components 9, 10, 11, 15, and 16 were less than 0.70 in all cases. Since zearalenone accumulated only in treatment B, and since component 8 is so highly correlated with component 11 in this instance, component 8 was included among the set of metabolites possibly related to zearalenone.

The correlations among the selected set of components in treatment B are presented in Table I. This treatment has been singled out for presentation because it represents conditions under which zearalenone production is optimum (Mirocha et al., 1971). As shown in this table, not only are the selected components correlated with zearalenone (component 14), but they form a strongly correlated group. This is particularly true in treatment B but the same tendency is apparent in the other treatments. In this treatment zearalenone is most highly correlated with the 8'-hydroxyzearalenone (component 15) and component 11. In turn, component 16 is most highly correlated with component 15 and component 11 is most highly correlated with component 8. An important observation is that the correlations between selected components correlated with zearalenone are frequently greater than the correlation of these components with zearalenone; for instance the correlation of component 8 with zearalenone is 0.77 while the correlation between component 8 and component 11 is 0.92. This leads to the concept that these correlation coefficients may reflect an underlying series of metabolic relationships in which one substance is the most immediate

Scheme I



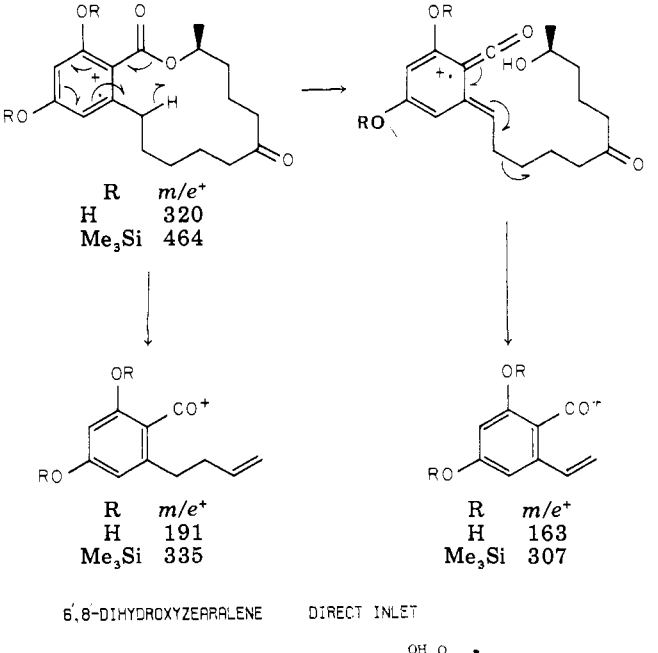
precursor of some compounds and those compounds in turn are the most immediate precursors (or products) of the compounds with which they are most highly correlated. Analytical methods for determining possible chains of causation such as this have been developed and applied in areas other than biology (Blalock, 1964). Such analytical methods depend on the fact that a cause and its effects are logically never simultaneous, and that the delay between cause and effect reduces the correlation between a cause and its effects. Thus, correlations among sets of variables may reflect the causal relations among the variables.

The application of such techniques to the data from treatment B results in postulating that zearalenone gives rise to the 8'-hydroxyzearalenones, which in turn give rise to component 16, and that either the 8'-hydroxyzearalenones or zearalenone give rise to component 11 which in turn serves as a precursor to components 8, 9, and 10. Similar results are obtained for the pooled data and treatment A. Unfortunately, the confidence limits on the correlation coefficients are too broad to justify the unreserved acceptance of this result; however, the results of this analysis may have considerable diagnostic value.

The correlations discussed simply show the utility of such a statistical approach to solutions of metabolic relationships. Chemical proof of this relationship is shown in the following sections.

Characterization of Metabolites Related to Zearalenone. The mass spectra of the Me_3Si derivatives of components 8, 9, 10, 11, 14, 15, and 16 were obtained from the sample illustrated in Figure 1 by combined gas chromatography–mass spectrometry. The mass spectra were compared and analyzed for characteristic fragments of zearalenone and zearalanone derivatives (Schemes I and II) (Jackson, 1973; Jackson et al., 1974; Pathre and Mirocha, 1976). Particularly important are the intense ions at m/e^+ 333 and 305 for zearalenone derivatives and m/e^+ 335 and 307 for zearalanone derivatives.

Scheme II



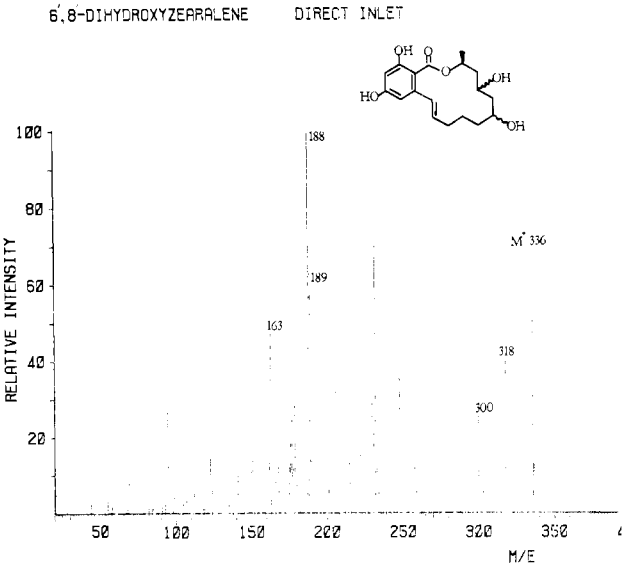


Figure 2. Mass spectrum of 6',8'-dihydroxyzearalene (component 16).

Component 14 (Zearalenone) and Component 15 (8'-Hydroxyzearalenone). The mass spectra of the trimethylsilyl ether derivatives of components 14 and 15 were found to have identical fragmentation patterns as that of authentic trimethylsilyl ether of zearalenone and the two isomers of 8'-hydroxyzearalenone, respectively.

Component 16 (6',8'-Dihydroxyzearalene). The high-resolution mass spectrum of component 16 (Figure 2) indicated a strong molecular ion at m/e^+ 336 (measured 336.1566) with the elemental composition of $C_{18}H_{24}O_6$. The spectrum showed two successive losses of H_2O from the molecular ion to yield intense ions at m/e^+ 318 and 300. In addition, two successive losses of $(CH_3)_3SiOH$ (90 mass units each) from the molecular ion (m/e^+ 624) of the trimethylsilyl ether derivative of component 16 are indicative of the presence of two aliphatic hydroxyl groups.

The ultraviolet spectrum of component 16 was identical with that of zearalenone and, upon treatment with base, it displayed a bathochromic shift identical with zearalenone. The infrared spectrum of component 16 is also similar to that of zearalenone except that the carbonyl absorption near 1700 cm^{-1} was lacking indicating the absence of the 6'-ketone.

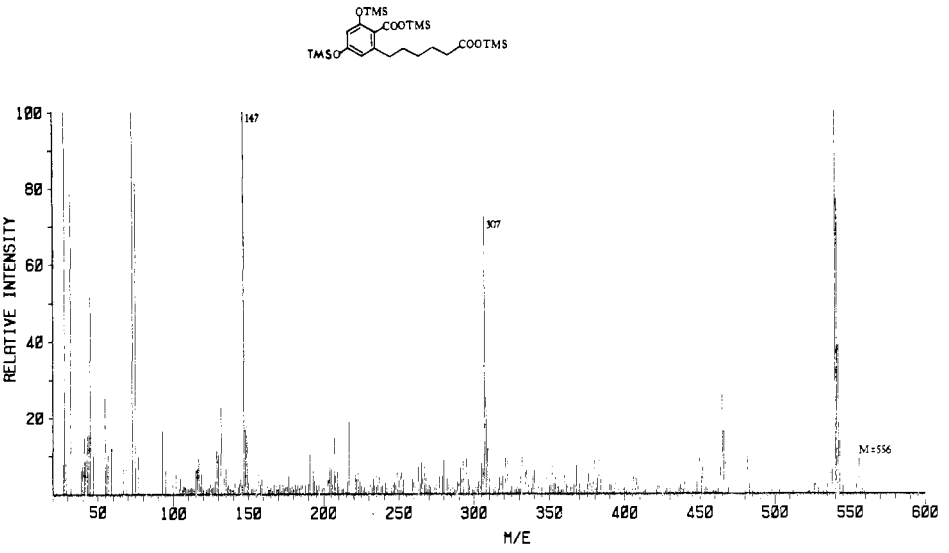
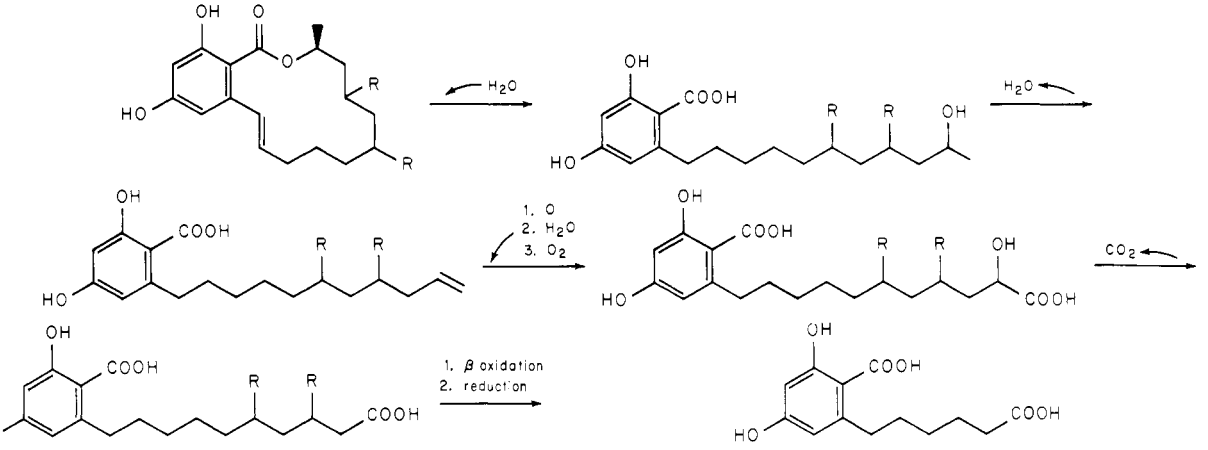


Figure 3. Mass spectrum of Me_3Si derivative of component 11 normalized to m/e^+ 147.

Scheme III. Tentative Scheme Explaining the Origin of Component 11 [6-(Carboxypentyl)-β-resorcylic Acid] from Zearalenone



component 11 [2,4-dihydroxy-6-(carboxypentyl)benzoic acid]

Component 16 reacted with *n*-butylboronic acid to yield an ester as determined by mass spectrometry. Further, component 16 gave a negative periodate test. These observations are consistent with the presence of 1,3-diol. Finally, the sodium borohydride reduction product of one of the isomers of 8'-hydroxyzearalenone was found to be identical with component 16 with respect to its mass spectrum, GLC retention time, and TLC R_f value. Hence, component 16 was identified as 6',8'-dihydroxyzearalene.

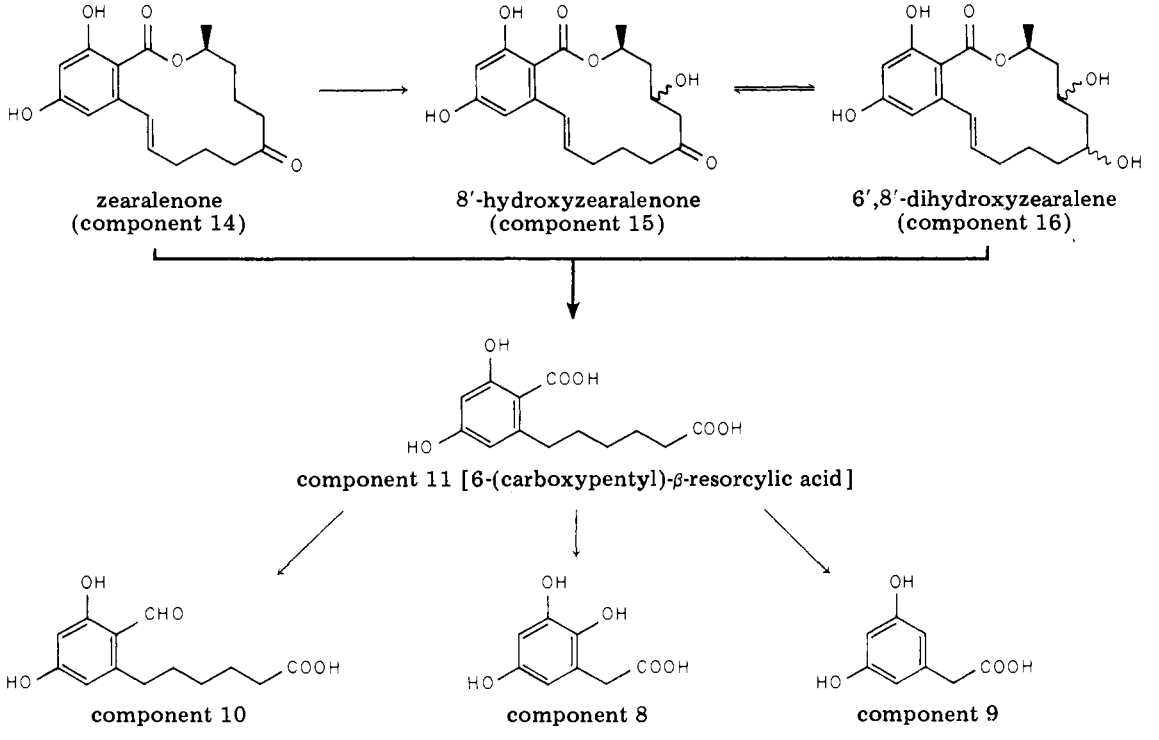
Component 11 (2,4-Dihydroxy-6-(carboxypentyl)-benzoic Acid). The mass spectrum of the Me_3Si derivative of component 11 had the highest mass at m/e^+ 556 with fragment losses of CH_3, -O-Me_3Si, and HO-Me_3Si. The spectrum also showed significant fragments at m/e^+ 335 and 307 which are characteristic fragments of zearalanone derivatives (see Figure 3).

The infrared spectrum of component 11 indicated a carboxyl group (3000, 1680, and 1275 cm^{-1}) and a di-meta-substituted aromatic ring (1600 and 840 cm^{-1}); the spectrum is similar to that of 2,4-dihydroxybenzoic acid. The ultraviolet spectrum of component 11 (maximum at 265 nm) is consistent with the 2,4-dihydroxybenzoic acid chromophore.

When 2,4-dimethoxy-6-(carboxypentyl)benzoic acid was demethylated with boron tribromide, one of the demethylated products had an R_f value identical with that of component 11. The demethylated product was isolated and identified as 2,4-dihydroxy-6-(carboxypentyl)benzoic acid. The comparison of the mass spectra and GLC retention time of the Me_3Si derivative of the demethylated product with that of the Me_3Si derivative of component 11 indicated that the latter is 2,4-dihydroxy-6-(carboxypentyl)benzoic acid (Scheme III).

Components 8, 9, and 10. The Me_3Si derivatives of components 8 and 9 displayed similar mass spectral fragmentation patterns. Component 8 (Me_3Si derivative) exhibited a molecular ion m/e^+ 472 and an intense fragment at m/e^+ 384 attributable to the loss of -O-$(CH_3)_2Si{=}CH_2$ ($M^+ - 88$); the remainder of the spectrum was similar to that of component 9. The mass spectrum Me_3Si derivative of component 9 yielded an ion at m/e^+ 384 (molecular ion). It is highly probable that component 8 is a hydroxylated derivative of 9. The Me_3Si derivative of component 10 yielded a molecular ion at m/e^+ 468 and a major fragment at m/e^+ 438 ($M^+ - CHO$); the remainder of the spectrum was similar to that of component 11. Component 10 then appears to be an aldehyde of component 11. Components 8, 9, and 10 could not be isolated in quantity for extensive characterization due to a limited quantity of extract. However, based on the mass spectral fragmentation pattern which is closely related to component 11 and 2,4-dihydroxybenzoic acid, structures of

Scheme IV. Tentative Scheme for the Metabolism of Zearalenone by *Fusarium roseum*



components 8, 9, and 10 are tentatively assigned as trihydroxyphenylacetic acid, dihydroxyphenylacetic acid, and 2,4-dihydroxy-6-(carboxypentyl)benzaldehyde.

It is clear from the foregoing data that the set of components selected by the statistical approach as possibly related to zearalenone was found to be structurally related to zearalenone.

Catabolism of Exogenously Applied Zearalenone. When an isolate of *F. roseum*, selected to grow at high concentrations of zearalenone, was grown on shredded wheat containing 0–10000 ppm of zearalenone, it was found that the consumption of zearalenone at 1000 ppm was relatively low (23%) while increasing concentrations gave steadily increasing percentages of consumption. At 2000, 5000, and 10000 ppm, the percent of the zearalenone metabolized was 30, 44, and 65%, respectively. As far as we can determine, this represents the first analytical data indicating that zearalenone is metabolized by *F. roseum*.

Analyses of the amounts of the 8'-hydroxyzearalenones and component 16 (6',8'-dihydroxyzearalene) showed that as the amount of zearalenone recovered increased from 800 to 2800 ppm (5000 ppm was the initial concentration), the amount of 8'-hydroxyzearalenones and component 16 increased by a factor of 2.5. An initial administration of 10000 ppm of zearalenone resulted in a decrease of the quantities of these two components to values below that obtained at 5000 ppm of added zearalenone but still greater than that obtained at 1000 ppm. These results are largely consistent with the original statistical findings that the 8'-hydroxyzearalenones and component 16 are correlated with zearalenone.

In this experiment the concentration of component 11 [6-(carboxypentyl)-β-resorcylic acid] was greatly affected by the exogenous addition of zearalenone.

Catabolism of Exogenously Applied Zearalanone. When extracts of *F. roseum* cultures grown with added zearalanone were examined by multiple ion detection (ions were monitored at *m/e* 305, 307, 335, and 333), the results analogous to those shown in Scheme IV were obtained. Zearalanone was metabolized to the 8'-hydroxyzearalanones and to 6',8'-dihydroxyzearalane (dihydro analogue of component 16), i.e., c and e of Figure 4. The 8'-hydroxyzearalanones and 6',8'-dihydroxyzearalane were not found in zearalanone as contaminants nor were they found in cultures of *F. roseum*

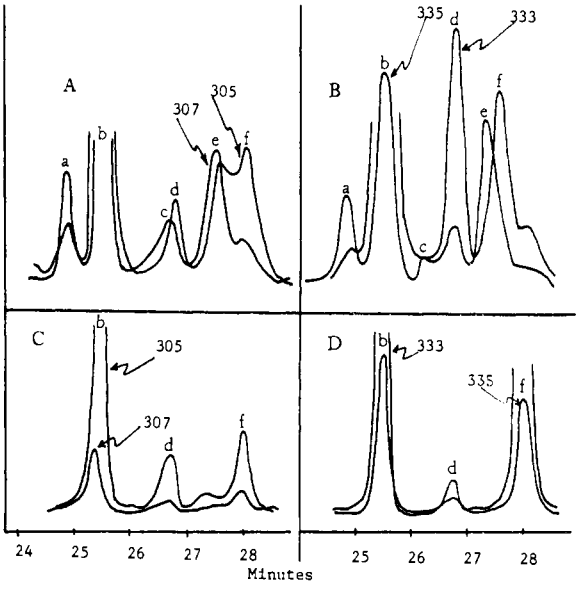


Figure 4. Mass fragmentogram of various derivatives of zearalenone obtained by multiple ion detection: (A) simultaneous measurement of m/e^+ 307 and 305 of an extract of a culture of *Fusarium roseum* amended with zearalanone after 30 days of growth; (B) same as A except that fragments having m/e^+ values of 335 and 333 were monitored; (C) same as A except that no zearalanone was added, (D) same as C except that fragments m/e^+ 335 and 333 were monitored. Fragments m/e^+ 307 and 335 were used to monitor zearalanone derivatives and m/e^+ 305 and 333 for derivatives of zearalenone. Refer to Schemes I and II for significance of these masses. The lower case letters represent: (a) zearalanone; (b) zearalenone; (c) 8'-hydroxyzearalanone; (d) 8'-hydroxyzearalenone; (e) 6',8'-dihydroxyzearalane; (f) 6',8'-dihydroxyzearalene.

to which zearalanone was not administered. The presence of these analogues was confirmed by combination gas chromatography–mass spectrometry following purification.

When purified fractions were examined by mass spectrometry (trimethylsilyl ethers), intense fragments were found at m/e 626, 335, and 307 confirming the presence of 6',8'-dihydroxyzearalane. Mass fragments of m/e 552, 335, and 307 were also found in the component suspected of being 8'-hydroxyzearalanone, thereby confirming its identity. This evidence supports the hypothesis that naturally occurring zearalenone may be metabolized to both isomers of 8'-hydroxyzearalenone and 6',8'-hydroxyzearalene (component 16).

The formation of component 11 from zearalenone cannot be examined by this method; accordingly dibromozearalenone was used in a similar experiment as described above. Initial attempts to detect the molecular ion of the Me_3Si derivative of dibromo component 11 in crude extracts of cultures fed dibromozearalenone failed. Upon fractionation of the crude extract on silica gel columns, a fragment at m/e^+ 714 was detected by multiple ion detection. The gas chromatographic component responsible for this had a retention time of 1.2 times that of the peak identified as the Me_3Si derivative of component 11. The multiple ion detector maximum was contained in but was not coincident with this larger peak. A mass spectrum was obtained of the materials in the leading edge of the mixed gas chromatographic component. This mass spectrum had ions at m/e 712, 714, and 716 with an approximate intensity ratio of 1:2:1 as would be expected of a dibromo compound. Fragments with this same intensity ratio were found at m/e 697, 699, and 701 ($M^+ - CH_3$). Due to considerable interference no other ions attributable to the dibromo analogue could be located.

These results support the hypothesis that component 11 is derived from zearalenone. In view of the structural relationship of component 11 to zearalenone, and the relatively good correlation between the two substances over a range of cultural ages and environmental conditions, it is logical to maintain this hypothesis until tests using radiolabelled zearalenone can be carried out.

Catabolism of Zearalanol. When zearalanol (Chart I, compound V) was incubated with *F. roseum* as described, a material having a mass spectrum identical with that of the Me_3Si derivative of zearalanone (Chart I, compound IV) was detected by gas chromatography–mass spectrometry. The new material accounted for 70% of the added zearalanol of which 10% was recovered. Zearalanone was not detected under identical conditions in the absence of *F. roseum*.

These results indicated that *F. roseum* can readily oxidize at least one 6'-hydroxy form of zearalenone. It seems likely that the 6'-hydroxyl group is readily oxidized in general. This may account for the failure to detect zearalenol in culture. In addition these results suggest that the 8'-hydroxyzearalenones and component 16 are readily interconverted.

Detection of 1',7'-Zearaldienone. A derivative having a mass spectrum and TLC and GLC properties indicative of 1',7'-zearaldienone (Jackson et al., 1974) was detected in cultures of *F. roseum* when extracted as previously described. When the purification procedure was altered so as to eliminate the use of Na_2CO_3, this component was no longer detected. Bolliger and Tamm (1972), using Na_2CO_3 in their extraction and purification procedure, isolated this same derivative from cultures of *F. roseum* and reported it as a natural product. The question of whether this material is a natural metabolite or whether it arises as an artifact due to dehydration of the 8'-hydroxyzearalenones upon exposure to base remains to be answered.

Tentative Scheme for the Metabolism of Zearalenone. It has been shown that the 8'-hydroxyzearalenones and 6',8'-dihydroxyzearalene (component 16) probably arise from zearalenone. In addition, a substituted resorcylic acid, 6-(carboxypentyl)-β-resorcylic acid (component 11), has been shown to be structurally related to zearalenone. This substance might arise from zearalenone and some evidence obtained using dibromozearalenone suggests that it does. Additional support is provided by the statistical relation of this material to zearalenone in cultures of *F. roseum*.

Three other components (8, 9, and 10) appear to be structurally and statistically related to zearalenone. These materials have not been fully characterized because they occur in trace amounts and were not isolated in sufficient quantity from the limited numbers of cultures available. Tentative structures have, however, been assigned on the basis of the mass spectra of their Me_3Si derivatives and are found in Scheme IV.

An overall scheme for the degradation of zearalenone by *F. roseum* is presented in Scheme IV. Parts of this scheme must be considered tentative until components 8, 9, and 10 are examined in detail. This scheme is, however, consistent with much of what is known concerning the metabolism of aromatic compounds. Presumably the phenylacetic acid derivatives (components 8 and 9) would be capable of eventually entering the tricarboxylic acid cycle through the ring fission pathways described by Dagley (1971).

The findings of these studies that zearalenone is converted to at least three and possibly six identifiable metabolites has implications bearing on the analysis of feed samples suspected to contain zearalenone. Component 11, in particular, might serve as an indicator of the potential presence of zearalenone. In addition, continuing studies concerning the role of zearalenone in regulation of the sexual stage of *F. roseum* (Wolf and Mirocha, 1973) must consider the various interconversions when studying structure–activity relationships of dose–response relationships.

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Monoacetoxyscirpenol. A New Mycotoxin Produced by *Fusarium roseum* Gibbosum

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Fusarium roseum Gibbosum was isolated from corn suspected of causing illness and death in several dairy cattle. When this isolate was grown on corn or rice and consumed by rats, turkey poults, swine, or young chickens, it caused illness and death. Two toxic metabolites were isolated from corn on which this isolate was grown. Chemical and spectral evidence indicate that both toxins are 12,13-epoxytrichothecenes. Monoacetoxyscirpenol, a major toxic component, has been shown to be 15-acetoxy,-3α,4β-dihydroxy-12,13-epoxytrichothec-9-ene and is the only toxic component in the culture when grown on rice. The other toxic component is scirpentriol (3α,4β,15-trihydroxy-12,13-epoxytrichothec-9-ene), found when grown on corn. Monoacetoxyscirpenol is the principal toxic metabolite produced by this isolate of *Fusarium*.

In Minnesota, a number of undiagnosed cases of animal toxicosis pass through the Veterinary Diagnostic Laboratory which have no explanation except that perhaps the feed might be implicated in explaining signs of intoxication such as abortion, diarrhea, emesis, loss of weight gain, hemorrhagia, and death. Routinely, these feed samples are analyzed for toxic fungi and toxic components. One such case involved abortion and death in dairy cattle and swine. An isolate of *Fusarium roseum* Gibbosum, among other fungi, was isolated from the feed, grown on autoclaved corn, and analyzed for toxicity in rats. One major toxic metabolite was isolated from this isolate and its identification is the subject of this paper.

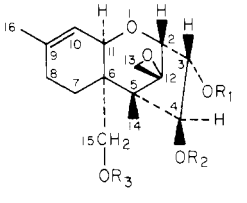
RESULTS AND DISCUSSION

The cultures of *Fusarium roseum* Gibbosum grown on rice were harvested, dried, moistened, and extracted with ethyl acetate (see Scheme I for extraction and cleanup procedure). Toxicities of the various fractions were determined by feeding to white rats.

The extensive chromatographic separations of the partially purified extract resulted in the isolation of a pure crystalline toxin. Its molecular weight was determined by high-resolution mass spectroscopy and found to be 324, with an elemental analysis of $C_{17}H_{24}O_6$. Preliminary interpretation of the mass spectral data showed an m/e^+ of 264 (measured 264.1373) as the highest significant mass in the spectrum with an elemental composition of $C_{15}H_{20}O_4$. However, the mass spectrum of the trimethylsilyl (Me_3Si) ether showed an apparent molecular ion at m/e^+ 468 (measured 468.2387 and elemental composition of $C_{23}H_{40}O_6Si_2$), instead of the expected molecular ion at m/e^+ 408 ($C_{21}H_{36}O_4Si_2$) which was due to the loss of acetic acid from the parent ion (m/e^+ 468). Therefore, the ion at m/e^+ 264 in the underivatized toxin was not the molecular ion, but rather an ion arising from the rapid loss of CH_3COOH from the parent ion (Figure 1).

The infrared spectrum (Figure 2) of the toxin in a KBr disk showed the presence of a free hydroxyl (3400 cm^{-1}), an ester group (1715, 1250 cm^{-1}), and an olefinic linkage (3010, 1680, 835 cm^{-1}). The ultraviolet spectrum did not show any significant absorption except end absorption. Deuterium exchange, trimethylsilylation, and acetylation indicated the presence of two hydroxyl groups.

The toxin yielded a triol **3** when hydrolyzed in a solution of 0.6 *N* K_2CO_3 which when acetylated by *N*-acetylimidazole in refluxing methylene chloride was quantitatively converted into its triacetate **4**. This reaction proceeded smoothly and was much simpler than the classical acetylation by acetic anhydride; see structures **1**–**8** of various derivatives of the trichothecene toxins.



	R_1	R_2	R_3
1	H	H	$COCH_3$
2	H	$COCH_3$	$COCH_3$
3	H	H	H
4	$COCH_3$	$COCH_3$	$COCH_3$
5	Me_3Si	Me_3Si	$COCH_3$
6	$COCF_3$	$COCF_3$	$COCH_3$
7	D	D	$COCH_3$
8	H	$COCH_3$	H

The triacetate **4** formed in the above reaction was identical with the acetylation product of the toxin. Hydrolysis of **4** in 0.2 *N* NH_4OH–MeOH yielded a mixture consisting of the triol **3**, the toxin, and a monohydroxy compound. The latter was identified as diacetoxyscirpenol **2** (3α-hydroxy,4β,15-diacetoxy-12,13-epoxytrichothec-9-ene), a known mycotoxin (Sigg et al., 1965; Dawkins, 1966;

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