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Isolation and Characterization of Zearalenone Sulfate Produced by *Fusarium* spp.†

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A water-soluble compound related to zearalenone was isolated from a culture of *Fusarium graminearum* 30 grown in rice. The structure of the novel metabolite was determined to be zearalenone-4-sulfate on the basis of fast-atom-bombardment mass spectrometry, proton nuclear magnetic resonance, UV spectroscopy, and by chemical and enzymatic reactions. Strains representing *Fusarium equiseti*, *Fusarium sambucinum*, and *Fusarium roseum* produced the sulfate conjugate as well. In the rat uterus enlargement bioassay, the metabolite or its hydrolysis product was found to retain the estrogenic activity characteristic of zearalenone. Natural occurrence of this novel metabolite might be significant because analytical methods devised for zearalenone in grain cannot detect the conjugate but the conjugate retains the biological properties of the mycotoxin when ingested by animals.

Zearalenone, a mycotoxin produced by several *Fusarium* spp., is frequently found to contaminate various cereal grains (3) and other commodities. A recent worldwide survey (24) reports zearalenone occurring in 58% (n = 45) of the corn samples collected in 19 different countries. The estrogenic syndrome triggered by zearalenone when it is fed to swine (7) includes diminished or nonfertility, prolonged estrus, reduced litter size, malformations in offspring, and juvenile hyperestrogenism. Zearalenone has also been implicated in decreased fertility cases in cows fed moldy hay (15).

Some strains of *Fusarium* spp. produce, in addition to zearalenone, other related metabolites such as alpha-zearalenol, which has also been found in feeds (18). The diastereomers of 8′-hydroxyzearalenone (13) and of 3′-hydroxyzearalenone (20) have been isolated and characterized from cultures of *Fusarium roseum*.

Another source for the study of zearalenone derivatives is the fluids of animals that have been dosed with the mycotoxin. Zearalenone is found as its glucuronide adduct in the urine of cows, rats, rabbits, and swine. The sulfate conjugate is also reported to occur in cow urine as determined indirectly by enzymatic hydrolysis and then analysis of zearalenone (17). However, the identity of the conjugate was not confirmed.

In the present study we report the isolation of the zearalenone sulfate conjugate from a rice culture of *Fusarium graminearum*. The chemical, spectral, chromatographic, and biological properties of this novel derivative of zearalenone are described.

MATERIALS AND METHODS

Isolation of the metabolite. *F. graminearum* 30 was originally isolated from a corn stalk in Minnesota (26) and when grown in autoclaved rice produced large amounts (1,500 mg/kg) of zearalenone (27). The ground moldy rice (100 g) was placed into a 1-liter Erlenmeyer flask and moistened with enough water to form a paste. A 200-ml volume of methanol-water (3:1, vol/vol) was added, and the flask was shaken for 1 h in a wrist-action shaker. The extract was filtered through Whatman no. 4 filter paper into a 1-liter separatory funnel. The residue was reextracted twice with 200 ml of methanol-water, and the filtrates were pooled in the funnel. Saturated sodium chloride solution (20 ml) was added to the funnel, and the extract was defatted by partitioning it twice with 300 ml of petroleum ether. The aqueous methanolic extract was poured into a round-bottomed flask, and about 80% of the methanol was evaporated in a rotary evaporator at 45 to 50°C. The remaining solution was diluted volume to volume with distilled water and partitioned with ethyl acetate (three times), which was collected and evaporated to dryness in a rotary evaporator. The residue was redissolved in 2 ml of chloroform-methanol (9:1, vol/vol) and loaded into a glass column (20 cm by 1.5 cm [inside diameter]) packed with 20 g of silica gel (100 to 200 mesh). The silica gel column was previously equilibrated with chloroform-methanol (98:2, vol/vol). The column was eluted successively with 50 ml of each one of the following mixtures of chloroform-methanol: 9:1, 8:2, and 6:4 (vol/vol). Fractions of 5 to 7 ml were collected and screened on silica gel–thin-layer-chromatography (TLC) plates which were developed with chloroform-methanol (8:2, vol/vol), and the zearalenone derivative was made visible under short-wavelength UV light. Fractions containing an intense fluorescent spot with an R_f value of 0.25 were pooled in a round-bottomed flask and concentrated to dryness in a rotary evaporator. The residue was redissolved in 0.35 ml of methanol and applied on silica gel-TLC plates (20 by 20 cm, 0.25-mm thickness; Merck) which were developed in chloroform-methanol (8:2, vol/vol). The fluorescent band with an R_f of 0.25 was scraped off the plates and eluted with chloroform-methanol (6:4, vol/vol). The solvent was evaporated, and the residue was crystallized with benzene and then dried with vacuum at room temperature overnight. About 20 mg of a yellow powder was obtained and then used for further chemical and biological tests.

FAB mass spectrometry. Negative-ionization fast-atom-bombardment (FAB) mass spectrometry was carried out in a VG7070EQ mass spectrometer. The compound was dis-

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solved in methanol, and 5 μl of the solution was mixed with the thioglycerol matrix on the probe.

A resolution of 2,500 was used in obtaining an accurate mass measurement. Reference masses were *m/z* 413 and 369 from polyethylene glycol.

Chemical and acid hydrolysis. One milligram of the isolated compound was treated separately with 1% hydrochloric acid and sulfatase from limpets (Sigma Chemical Co., St. Louis, Mo.). Acid hydrolysis was carried out at 65°C for 4 h, while enzymatic hydrolysis was performed in a water bath at 37°C for 4 h. When the reaction was completed, the reaction mixtures were extracted with methylene chloride and both the organic and aqueous fractions were analyzed; the aqueous-phase residue was redissolved in 0.1 ml of water and treated with 3 drops of a 30% solution of barium chloride. The organic-layer residue was analyzed for zearalenone by TLC and compared with authentic zearalenone standard. For further confirmation, the organic layer was evaporated to dryness and reacted with the derivatization reagent BT (Pierce Chemical Co., Rockford, Ill.) for the formation of the trimethylsilyl ether. The derivative was analyzed by gas chromatography-mass spectrometry and compared with authentic zearalenone-trimethylsilyl.

UV spectroscopy. Zearalenone and the isolated compound were dissolved in methanol, and their UV absorption spectra were recorded on a Beckman DB-GT grating spectrophotometer.

NMR spectroscopy. The proton-nuclear magnetic resonance (NMR) spectrum of the compound was recorded in a 300-MHz Nicolet NT-300-W3 spectrometer by using tetramethylsilane as the internal standard and CD_3OD as solvent.

Biological activity. The estrogenic activity of the compound was compared to that of zearalenone in the rat uterus enlargement bioassay. Eighteen Sprague-Dawley 20-day-old virgin rats (Bio-Lab Corp., St. Paul, Minn.) were divided randomly into three groups of six rats each and housed in the Research Animal Resources facility, University of Minnesota, St. Paul, Minn. Equivalent molar amounts (630 nmol) of zearalenone or zearalenone sulfate (as calculated by the molecular weight of the anion, i.e., 397) were administered intragastrically to each rat in 0.5 ml of the carrier solvent (20% ethanol in sterile water). A control group was dosed with the carrier solvent. Twenty-four hours after gastric intubation, the animals were weighed and killed by cervical dislocation. The animals were dissected, and the uteri were removed and weighed in an analytical balance. The ratio of the uterus weight to total body weight was recorded for each animal. The mean and standard deviation were calculated for each group, and data were analyzed by using the Student's *t* test.

Production of zearalenone sulfate by *Fusarium* spp. Zearalenone sulfate production was determined by TLC analysis of the extract of rice in which isolates representing various species known to produce zearalenone were grown. For this analysis, 20 g of rice was extracted with 120 ml of methanol-water (3:1, vol/vol) and defatted as described in the isolation procedure. The water-methanol fraction was concentrated to evaporate most of the methanol, and the aqueous phase was then partitioned with ethyl acetate (50 ml, three times). The organic layer was dried, redissolved in 1 ml of methanol, and spotted on the TLC plate which was cospotted with purified zearalenone sulfate. Quantitative analysis of the compound was done by high-pressure liquid chromatography by using a C_{18} reverse-phase column with water-acetonitrile-methanol (2:1.6:1, vol/vol/vol) as mobile phase and fluorescence detection (excitation wavelength, 274 nm; emission wavelength, 418 nm).

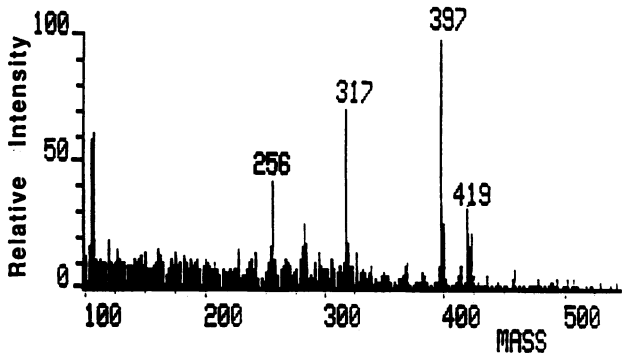

FIG. 1. Negative-ionization FAB mass spectrum of the isolated metabolite in thioglycerol matrix. Molecular anion $(M\text{-}H)^-$, *m/z* 397, loses the sulfonic group to yield *m/z* 317 which corresponds to zearalenone molecular weight minus a proton. Peak at *m/z* 419 corresponds to the sodium adduct of the molecular ion.

RESULTS

Treatment of the compound with 1% hydrochloric acid yielded zearalenone which was identified in the methylene chloride layer by normal-phase TLC (R_f = 0.77 in chloroform-methanol [9:1, vol/vol]) and by the electron-impact mass spectrum of the trimethylsilyl ether derivative. The diagnostic ions of zearalenone-trimethylsilyl ether were found as described by Mirocha et al. (16): M^+ 462, 447, 429, 333, 305, 260, and 151. The water-soluble fraction produced a white precipitate of barium sulfate when it was reacted with a 30% aqueous solution of barium chloride. Sulfatase treatment of the compound also gave zearalenone as the aglycone and sulfate ion which precipitated when treated with barium chloride.

The negative-ionization FAB mass spectrum of zearalenone sulfate (Fig. 1) showed three major peaks: the molecular anion $(M\text{-}H)^-$ at *m/z* 397 loses the sulfonic group (SO_3^-) to yield *m/z* of 317 $(M\text{-}SO_3\text{-}H)^-$. The peak with *m/z* 419 corresponds to $(M-H+Na)^-$. The empirical formula proposed for the compound was $(C_{18}H_{21}O_8S)^-$ corresponding to a calculated mass of 397.0957 and was confirmed by exact mass measurement with negative-ionization FAB mass spectrometry. The spectrum revealed the ion with an observed mass of 397.0979 which differs from the calculated empirical formula by only 2.2 millimass units. The UV spectrum (Fig. 2) of the zearalenone conjugate in methanol revealed an appreciable shift in two bands. The maximum absorption band of 236 nm in zearalenone is shifted to 228 nm in the conjugate, whereas the band at 274 in the parent compound is observed at 260 nm in the conjugate. These observed shifts are attributed to the electron-withdrawing effect of the sulfate group (2).

The ^1H-NMR spectrum (Table 1) shows that the phenolic hydroxyl group at C-4 observed at 9.30 ppm in zearalenone is not present in zearalenone sulfate, supporting the assignment of the position of the sulfate ester at C-4 in zearalenone.

The novel compound isolated from the *F. graminearum* culture was identified as zearalenone-4-sulfate (Fig. 3) on the basis of mass spectrometry, chemical and enzymatic reac-

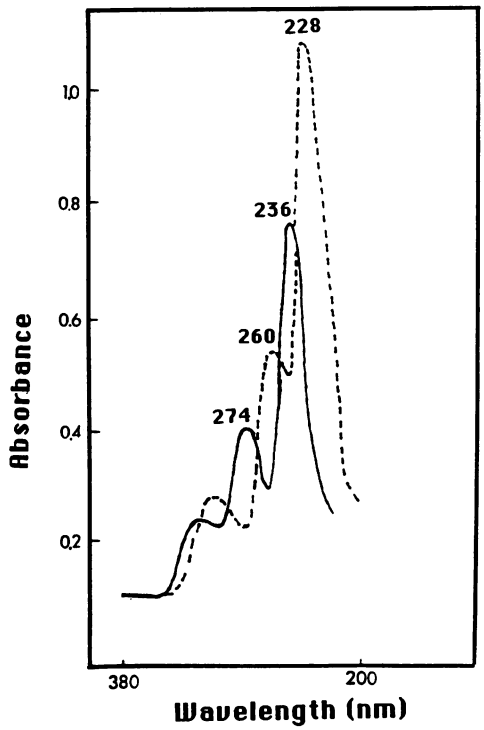

FIG. 2. UV absorption spectrum of zearalenone (——) and of the isolated metabolite (– – – –) in methanol.

tions, and UV and NMR spectroscopies. The metabolite is soluble in water, methanol, ethanol, and ethyl acetate, slightly soluble in chloroform, and insoluble in hexane, benzene, and petroleum ether. In normal-phase TLC, the metabolite has an R_f value of 0.25 (chloroform-methanol, 8:2, vol/vol). In reverse-phase high-pressure liquid chromatography, by using the mobile phase indicated for zearalenone analysis, it elutes at 1.42 min (Fig. 4) from a 25-cm-long C_{18} column. Although the retention time is close to that of the solvent front, adequate quantification of its concentration in cultures was possible by using the purified metabolite as standard. Because conjugation adds 79 mass units to the molecular weight of zearalenone, the molar concentration was used for comparison. The production of zearalenone and zearalenone sulfate by *F. graminearum* 30 over a 30-day period follows a similar pattern, maintaining a constant molar ratio (Fig. 5).

TABLE 1. ^{1}H-NMR chemical shifts of zearalenone and its metabolite in CD_3OD

Position	Chemical shift of:			
	Zearalenone		Zearalenone sulfate	
	ppm	(J=Hz)	ppm	(J=Hz)
2-OH	12.06		12.10	
H-3	6.93	(1.4)	6.90	(1.4)
4-OH	9.30			
H-5	6.36	(1.3)	6.80	(1.3)
H-1′	7.01	(1.4)	6.92	(1.4)
H-2′	6.21	(1.2)	6.77	(1.3)
H-3′	2.12		2.24	
H-4′	1.74		1.72	
H-5′	2.84		2.70	
H-6′				
H-7′	2.62		2.65	
H-8′	1.65		1.67	
H-9′	2.30		2.43	
H-10′	5.03	(1.0)	5.04	(1.0)
11′-CH_3	1.37		1.36	

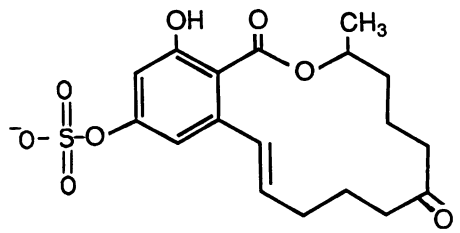

FIG. 3. Chemical structure of zearalenone-4-sulfate.

Four different *Fusarium* species were found to produce the metabolite (Table 2) when grown in rice substrate. Concentrations ranging from 300 nmol/g of rice (123 ppm) to 580 nmol/g of rice (230 ppm) were found in the five cultures tested. Molar ratios of zearalenone to zearalenone sulfate varied from 12:1 to 2:1.

The rat uterus enlargement bioassay (Fig. 6) showed that when zearalenone sulfate is ingested by rats, it partially retains the estrogenic properties of the parent compound.

DISCUSSION

The identity of the water-soluble metabolite isolated from *F. graminearum* 30 was established as zearalenone-4-sulfate by using the criteria set by natural products chemists in the systematic identification of flavonoid sulfates (2).

Sulfated metabolites have been widely studied in mammals and other animal species as end products of the metabolism of xenobiotics (5, 22). In the plant kingdom, sulfated products, mainly flavonoids, have been isolated from more than 250 species, including dicotyledons and monocotyledons (2). In fungi, however, few studies of sulfate conjugates appear in the literature. Choline sulfate is produced by *Aspergillus nidulans* presumably as a sulfur reserve (12). Studies by Cerniglia et al. (6) report the sulfate conjugation of aromatic hydrocarbons in liquid fermentation by *Cunninghamella elegans*.

Although only a limited survey among our isolates was done, we were able to detect zearalenone sulfate in four species of *Fusarium*, all of them known to produce the parent mycotoxin. The ratio of zearalenone to zearalenone sulfate is high compared with the production of other zearalenone derivatives (21).

In the mammalian systems, the condensation with sulfate occurs via the formation of an activated sulfur donor, 3′-phosphoadenosine-5′-phosphosulfate, which is conjugated with an alcohol group by a sulfotransferase (5). Although the reaction is primarily used for phenols, other hydroxyl groups can be sulfated as well. The ability to conjugate phenols and alkyl alcohols in *Fusarium* spp. has been demonstrated in this and one other study, although the mechanism of conjugation is unknown. Sulfate conjugation in *Fusarium* spp. has been reported by Vesonder et al. (25), who found a sterol sulfate in corn cultures of *F. graminearum*. In addition to zearalenone and sterols, *Fusarium* spp. produce other mycotoxins, such as trichothecenes and

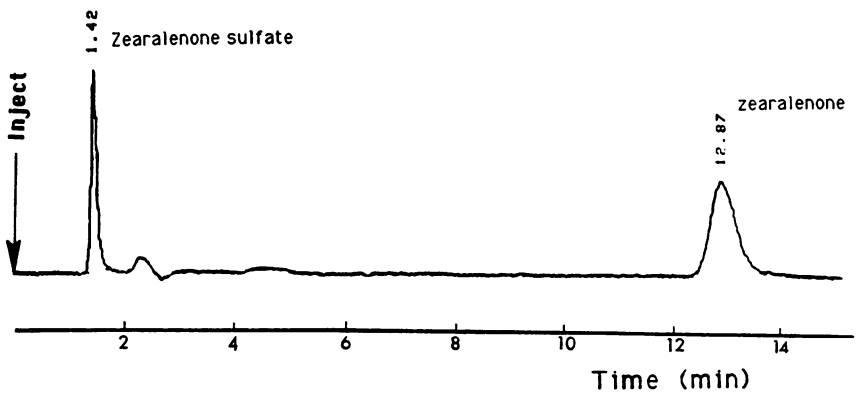

FIG. 4. Elution profile of zearalenone and zearalenone sulfate separated on a μBondapak C_{18} column by using methanol-acetonitrile-water (1:1.6:2, vol/vol/vol) as mobile phase and a fluorescence monitor for detection. Retention time of each metabolite is indicated at the top of the peak.

fumonisins, which also contain hydroxyl groups that can be conjugated with sulfate esters.

Other organisms, when incubated in vitro with zearalenone, are able to conjugate the mycotoxin with D-glucose; *Rhizopus* sp. (14), *Mucor bainieri* (9), and corn cells in tissue culture (10) form the 4-O-beta-glucoside conjugate, whereas *Thamnidium elegans* produces the 2,4-O-beta-diglucoside (8). Gareis et al. (11) report the natural occurrence of zearalenone glucoside in barley and wheat, suggesting that the mycotoxin is being conjugated by the plant cells. Although this might occur, conjugation of zearalenone by *Fusarium* spp. is possible. This is the first report on the production of a zearalenone conjugate by the same organism responsible for zearalenone synthesis.

A noteworthy difference between glucose and sulfate conjugates is their relative stability to acid hydrolysis. While glucosides are very resistant to acid, which might explain their lack of activity in swine (11), aryl sulfate esters are readily hydrolyzed by acid (23). Zearalenone sulfate is also susceptible to acid hydrolysis; therefore, zearalenone is released when the conjugate is ingested by animals. Intestinal absorption into the bloodstream might explain how, in the rat uterus enlargement bioassay (Fig. 6), the conjugated zearalenone triggered an estrogenic response characteristic of the mycotoxin.

The presence of zearalenone sulfate in feeds might explain the estrogenic syndrome in the field in which zearalenone is identified at low concentrations, but it is not enough to explain all of the symptoms observed in the animals.

The conjugation of an organic molecule with the sulfate ion results in important changes in the chemical and physical properties of the parent compound. Mainly, the solubility in water and acidity increases. As a detoxification mechanism in mammals, increased water solubility implies facilitated urinary and biliary excretion (5). From an analytical point of view, increased water solubility implies that the sulfate conjugate of zearalenone cannot be extracted and analyzed by analytical methods currently devised for zearalenone. Chloroform extraction, acid-base partition cleanup, and separation by reverse-phase high-pressure liquid chromatography as described in the current official method of the Association of Official Analytical Chemists for analysis of zearalenone and alpha-zearalenol (4) cannot detect the sulfate conjugate. Figure 4 shows that, by the retention time at which the conjugate elutes, the conjugate would be masked by components of the corn matrix in a routine analysis. Zearalenone sulfate has not yet been detected in feeds.

Current methodology for analyzing zearalenone conjugates in serum and urine (17, 19) uses indirect techniques for the detection of sulfate adducts; the sample is treated with hydrolytic enzymes and the free zearalenone is quantified. For demonstration of the natural occurrence of zearalenone conjugates, a similar approach can be followed in feed analyses. However, reliable techniques for the direct determination of zearalenone conjugates should be developed to assess the total amount of zearalenone to which humans and animals are exposed.

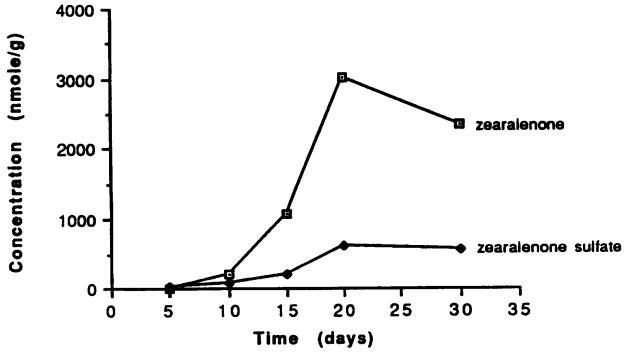

FIG. 5. Production of zearalenone and zearalenone sulfate by *F. graminearum* 30 grown in rice at 25°C over a 30-day period. Both metabolites were quantified by high-pressure liquid chromatography.

TABLE 2. Production of zearalenone and zearalenone sulfate in rice culture by various *Fusarium* spp.

Fusarium species and code	Reference	Amt produced (nmol/g of rice)		Molar ratio
		Zearalenone	Zearalenone sulfate	
F. graminearum 30	27	2,400	570	4:1
F. graminearum 1	1	3,770	370	10:1
F. equiseti 2	1	3,700	300	12:1
F. sambucinum N45B	1a	980	440	2:1
F. roseum "*gibbosum*"	20	5,000	580	10:1

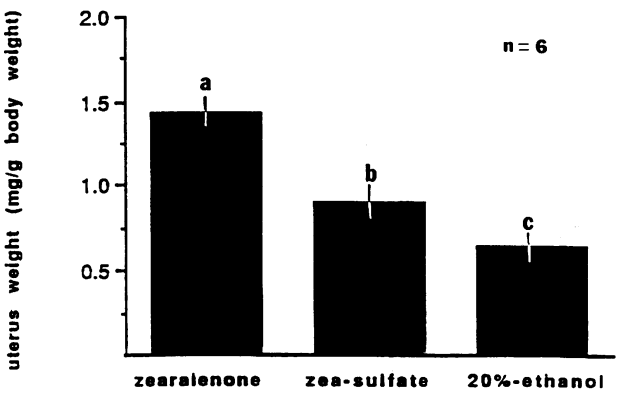

FIG. 6. Uterotropic activity of equimolar doses of zearalenone and zearalenone sulfate in the rat uterus enlargement bioassay. The third group was dosed with the carrier solvent (20% ethanol). Bars represent mean of six rats per group plus standard deviation. Different letters at the top indicate differences at $P < 0.05$.

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