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Microbial Transformation of Zearalenone to a Zearalenone Sulfate

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The conversion of zearalenone by various microorganisms was studied. A new polar metabolite was formed in addition to α- and β-zearalenols. The structure of the new metabolite was determined as zearalenone-4-*O*-sulfate conjugate on the basis of enzymatic and acid hydrolysis, followed by mass spectrometry, nuclear magnetic resonance, and infrared spectroscopic analysis. The results obtained demonstrate that *Rhizopus arrhizus* catalyzes sulfation of zearalenone at the C-4 hydroxyl group.

The mycotoxin zearalenone I (Fig. 1) and some of its derivatives are produced by several *Fusarium* species that invade corn, wheat, and other cereals (13). This compound has been implicated in serious reproductive and toxicological problems to farm animals (12, 19) fed contaminated mouldy corn, causing hyperestrogenic syndrome (17) and consequently a large economic loss. Zearalenone and some of its metabolites exhibit anabolic and growth-promoting activities in cattle and lambs (22). This led many investigators (16, 18, 26) to conduct a wide range of in vivo and in vitro metabolic studies.

Limited information is currently available on the microbial metabolism of mycotoxins (25). In view of the fact that microorganisms can simulate metabolic patterns observed in mammalian systems (2, 5, 23) and predict metabolic pathways and toxicological and pharmacological properties, we initiated studies on the microbial metabolism of zearalenone. In this report we describe the isolation, identification, and spectroscopic characterization of zearalenone-4-*O*-sulfate from zearalenone as a major conjugate by *Rhizopus arrhizus* IFO-6155.

MATERIALS AND METHODS

Equipment. Melting points were determined on a Thomas Hoover Unimelt capillary melting point apparatus and are uncorrected. Infrared spectra were recorded on a Nicolet 5 DXB, FT-IR spectrophotometer. Mass spectra were determined on a VG 7070 E-HF mass spectrometer. ^1H (300 MHz) and ^{13}C (75 MHz) nuclear magnetic resonance (NMR) spectra were recorded in deuterated dimethyl sulfoxide solution with a Nicolet NT-300-W3 spectrophotometer using trimethylsilane ($\delta = 0$) as an internal reference standard. High-pressure liquid chromatography analysis was performed with a Beckman 110A pump and an ISCO variable-wavelength absorbance detector operated at 254 nm. An ultrasphere 5μ ODS column (4.6 mm by 25 cm, Altex Scientific Inc., Berkeley, Calif.) was used to identify the metabolites with an isocratic solvent system of water-saturated dichloromethane containing 1% absolute ethanol and 1% glacial acetic acid at a flow rate of 1 ml/min. The external standard method (24) was used for determining the percentage yield of α- and β-zearalenols.

Microorganisms. All cultures were grown on Sabouraud dextrose agar (Difco) slants and stored in a refrigerator at 4°C in sealed screw-cap tubes until needed. Microorganisms were either purchased from the American Type Culture Collection (ATCC strains) or obtained from the U.S. Department of Agriculture, Northern Regional Research Laboratories (Peoria, Ill.) (NRRL strains). Seven genera (23 species) of microorganisms were used for potential metabolism of zearalenone, including *Aspergillus* (4 species), *Curvularia* (2 species), *Cunninghamella* (3 species), *Fusarium* (3 species), *Penicillium* (2 species), *Rhizopus* (5 species), and *Streptomyces* (4 species).

Chromatographic conditions. Thin-layer chromatographic analyses were carried out on precoated silica gel G-25-UV_{254} glass plates (Macherey-Nagel Düren). They were developed in $CHCl_3$-CH_3OH (8:2, vol/vol), made visible under UV light, and sprayed with 1% ceric sulfate in 3 N H_2SO_4. Column chromatography was performed for the isolation of the metabolites with silica gel (40-140 mesh; Baker analyzed 3404) activated at 110°C for 30 min before use.

Culture and fermentation procedures. Biotransformation experiments were performed by shake culture techniques, with the two-stage fermentation procedures and the media and culture conditions described previously (7). After 24, 48, 72, and 120 h, 3-ml samples of the complete incubation mixtures were withdrawn and extracted with 1.5 ml $CHCl_3$-CH_3OH (9:1, vol/vol), and 30 μl of the extracted layer was spotted on plates for analysis. Most transformations were continued until no further increases in metabolites were observed.

Microbial metabolism of zearalenone by *R. arrhizus* IFO-6155. A total of 400 mg of zearalenone I was dissolved in 2 ml of dimethyl formamide and distributed equally among 20 1-liter culture Erlenmeyer flasks, each containing 200 ml of 24-h-old *R. arrhizus* stage II culture. The cultures were incubated on a rotary shaker (27°C, 200 rpm) for 11 days. The entire incubation mixtures were combined and filtered. The cells (230 g, wet weight) were extracted with methanol (three times with 250 ml) and concentrated under vacuum to a yellowish white suspension. The suspension was filtered and washed with acetone to yield 105 mg of a white solid (metabolite II), which was recrystallized from methanol-acetone. The purity of this solid was determined by thin-layer chromatography with GF_{25}-UV_{254} silica plates developed in ethyl acetate-ether-formic acid (90:9:1, vol/vol) and observed as one spot with an R_f of 0.32 by viewing under UV light (254 nm) and spraying with ceric sulfate reagent. The aqueous filtrate was extracted three times with an equal

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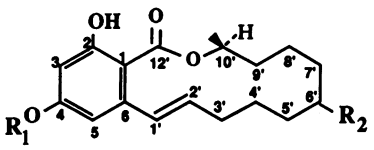

		R_1	R_2
I	Zearalenone	H	= O
II	Zearalenone-Sulfate	SO_3^-	= O
III	α-Zearalenol	H	H, OH
IV	β-Zearalenol	H	OH, H

FIG. 1. Structure of zearalenone and its metabolites.

volume of $CHCl_3$-CH_3OH (9:1, vol/vol) to allow maximum accumulation of the metabolite. The combined extract was washed with water, dried over anhydrous Na_2SO_4, and concentrated to a brown viscous residue (100 mg). The residue was purified by loading on the top of a silica gel column chromatography with a gradient of $CHCl_3$ (100%) to $CHCl_3$-CH_3OH (70%). Similar fractions (R_f, 0.61) were pooled, concentrated, and recrystallized from $CHCl_3$-CH_3OH (96:4, vol/vol) to yield 75 mg of white needles of metabolite III (retention time, 11 min; melting point, 169 to 170°C). Compound III was identified as α-zearalenol as reported previously (8). β-Zearalenol was also obtained from *Fusarium oxysporum* S.F3 and characterized as reported previously (8).

The isolated metabolite II (zearalenone-4-sulfate) had a melting point at >300°C and infrared (KBr) absorption maxima at 3,290, 1,690, 1,645, 1,612, 1,460, 1,050 (sulfuric acid), 960, and 800 cm^{-1} (S-O stretching). Anion fast-atom bombardment mass spectrometry showed fragments at *m/z* 398 (M^+, calculated for $C_{18}H_{22}O_8S$), 318 (100%, M^+-SO_3^-, 300, 188, 151, 125, 112, 69, and 51. ^1H and ^{13}C NMR (deuterated dimethyl sulfoxide) data are shown in Table 1.

Hydrolysis of zearalenone-4-sulfate. (i) Acid hydrolysis. Metabolite II (10 mg) was dissolved in 25 ml of 0.1 N HCl and refluxed in a round-bottomed flask for 2 h (2). After cooling to room temperature, the hydrolysate was extracted with chloroform. The recrystallized material (from $CHCl_3$) showed melting point, infrared, and ^1H and ^{13}C NMR data that were identical to those of zearalenone (8). The aqueous solution of the hydrolysate showed a white precipitate upon addition of a few drops of a mixture of 10% $BaCl_2$ diluted with HCl solution.

(ii) Enzyme hydrolysis. A sample of 5 mg of the metabolite II was incubated with sulfatase from *Helix pomatia* (Sigma Chemical Co., St. Louis, Mo.) in 5 ml of 0.1 M acetate buffer (pH 5) at 37°C (9). Control experiments were performed concurrently in the absence of enzyme. Complete hydrolysis was observed after 5 h of incubation. The mixture was then concentrated and used for thin-layer chromatographic analysis, which showed a compound with the melting point and R_f values similar to those of zearalenone. Again, the addition of a few drops of a mixture of 10% $BaCl_2$ diluted with HCl solution to the aqueous layer resulted in the formation of white precipitate of $BaSO_4$.

RESULTS AND DISCUSSION

Screening-scale studies of zearalenone have shown that different fungal species are capable of metabolizing this mycotoxin to a number of metabolites. Of the 23 microorganisms screened, *Streptomyces griseus* (ATCC 13273), *Streptomyces rutgersensus* (NRRL-B 1256), and *Rhizopus arrhizus* (IFO-6155) metabolized zearalenone to α-zearalenol with yields of 40, 25, and 18%, respectively. Meanwhile, *Aspergillus niger* (ATCC 111394), *S. rutgersensus* (NRRL-B 1256), and *F. oxysporum* S-F3 yielded β-zearalenol at 10, 25, and 15%, respectively. It is interesting to note the formation of α- and β-zearalenols by *S. rutgersensus*. This demonstrates the flexibility of the lactone ring; it is possible that two separate reductases are involved in these stereospecific

TABLE 1. ^1H and ^{13}C NMR chemical shifts of zearalenone and metabolite II[a]

Carbon no.	δH		δC	
	Metabolite II	Zearalenone	Metabolite II	Zearalenone
1	–[b]	–	105.20 (0), s	104.79
2	12.10 (OH)	11.60 (OH)	164.95 (0), s	165.11
3	7.15 (1 H, d, J = 2.6)	6.49	103.55 (1), d	102.79
4	–	9.90 (OH)	162.51 (0), s	160.11
5	6.87 (1 H, d, J = 2.6)	6.42	108.75 (1), d	109.11
6	–	–	144.21 (0), d	143.85
1′	7.11 (1 H, d, J = 15.2)	7.00	133.12 (1), d	134.71
2′	5.82 (2 H, m)	5.80	132.45 (1), d	131.95
3′	2.27 (2 H, m)	2.10	31.81 (2), t	30.90
4′	1.80 (2 H, m)	1.81	22.31 (2), t	22.50
5′	2.80	2.90	43.30 (2), t	43.05
6′	–	–	210.21 (0), s	211.82
7′	2.65 (2 H, m)	2.62	36.85 (2), t	36.76
8′	1.69 (2 H, m)	1.65	21.50 (2), t	21.09
9′	2.38 (2 H, m)	2.21	35.02 (2), t	34.79
10′	4.86 (1 H, m)	5.01	74.34 (1), d	74.52
11′	1.40 (3 H, d, J = 6.20)	1.36	20.50 (3), q	21.07
12′	–	–	171.05 (0), s	171.37

[a] The numbers within parentheses for the ^{13}C-NMR data indicate the numbers of hydrogens attached to the corresponding carbon and were determined from distortionless enhanced polarization transfer experiments.

[b] –, No protons at this position.

enzymatic reactions. In a cell-free system in the presence of 4-pro-S[^{3}H]NADPH and 4-pro-R[^{3}H]NADPH (1), *R. arrhizus* IFO 6155 metabolized zearalenone to α-zearalenol (18%) and zearalenone sulfate (26%).

A preparative-scale fermentation was performed with *R. arrhizus* with zearalenone I as a substrate, and metabolite II was isolated and purified as a major metabolite conjugate. This metabolite was found to be present mainly in the cells and not in the fermentation broth. Extraction of metabolite II with methanol resulted in a pure product, which recrystallized from CH_3OH-CH_3COCH_3. The fast-atom bombardment mass spectrum of metabolite II showed a molecular ion *m/z* of 398 (M^+), which coincided with the addition of 80 atomic mass units of the SO_3 moiety to zearalenone. The loss of SO_3 from the M^+ ion gives an *m/z* of 318, which is indicative of an oxygen-sulfate conjugate. Based upon this information, the metabolite was tentatively assigned structure II: zearalenone sulfate. To confirm the presence of the sulfate group, metabolite II was hydrolyzed by acid to yield aglycone, which was identified as zearalenone. The aqueous solution from the acid hydrolysis was treated with 10% $BaCl_2$-HCl solution, resulting in a white precipitate presumed to be $BaSO_4$. Further support for the presence of a sulfate conjugate was obtained after treatment of II with *Helix pomatia* sulfatase. The formation of the aglycone identified as zearalenone as well as the formation of a white precipitate after the addition of $BaCl_2$-HCl to the aqueous layer strongly support the presence of a sulfate group in metabolite II. Infrared spectroscopy of the metabolite showed a peak at 1,050 cm^{-1}, due to the presence of a sulfate moiety, and an absorption maximum at 3,290 cm^{-1}, indicating the presence of an intramolecular hydrogen bonding between the C-2 hydroxyl group and the lactone carbonyl, which in turn indicates C-4 substitution. Also, the frequency at 1,690 cm^{-1} of the lactone carbonyl supports C-4 substitution, rather than the reported C-2 substitution at 1725 cm^{-1} (8). ^{1}H NMR of the metabolite II showed a chemical shift pattern similar to that of zearalenone except for the aromatic protons in the metabolite, which were observed at δ 7.15 and 6.87 as compared with δ 6.49 (H-3), and 6.42 (H-5) in zearalenone (Table 1). The presence of the signal at δ 12.10 in metabolite II and the absence of the chemical shift at δ 9.90 of the C-4 phenolic hydroxyl group of zearalenone is indicative of C-4 substitution. The ^{13}C NMR spectrum of metabolite II showed all the resonances of the 18 carbon signals of zearalenone (Table 1). The chemical shifts of the aliphatic carbons in metabolite II did not change significantly. The chemical shift of the quaternary carbons at δ 105.20, 164.95, 162.51, and 144.2 of the metabolites, which correspond to δ 104.79 (C-1), 165.11 (C-2), 160.11 (C-4), and 143.85 (C-6) of zearalenone, are quite similar except for the C-4 signal, which is deshielded by 2.40 ppm, indicating substitution at the C-4 phenolic hydroxyl group. Also the C-1′ signal was shielded by 1.59 ppm from that of zearalenone. The effect of aromatic substitution of zearalenone was discussed in detail previously (6).

The results obtained from mass, infrared, and ^{1}H and ^{13}C NMR spectrometry and acid and sulfatase hydrolyses clearly support the identification of the sulfate conjugate at the C-4 position of zearalenone.

Sulfation and glucuronidation are well-documented metabolic pathways in the mammalian system (10, 11). However, glycosylation by microorganisms is not a common pathway (15). Sulfation by a microbial system is extremely rare. Phenolic hydroxyl groups are good substrates for sulfate conjugation (3), and sulfate conjugates of phenolic hydrocarbons have been reported (4, 14). Zearalenone metabolic studies on turkeys indicated the formation of zearalenone sulfate, but no further structural elucidation was reported (20). These reactions are poorly understood in microbial systems, and further studies are needed to understand the role of microbial transformation of mycotoxins in nature. This work is the first report of zearalenone sulfation by microbial transformation. It should provide new insights for the mechanism of formation of similar mycotoxin conjugates in nature.

ADDENDUM

Plasencia and Mirocha (21) have recently shown that zearalenone 4-sulfate is also produced by *Fusarium graminearum* grown on rice.

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