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Identification of the Naturally Occurring Isomer of Zearalenol Produced by *Fusarium roseum* 'Gibbosum' in Rice Culture†

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One diastereomer of *trans*-zearalenol [2,4-dihydroxy-6-(6,10-dihydroxy-*trans*-1-undecenyl)-benzoic acid-μ-lactone] was isolated from cultures of *Fusarium roseum* 'Gibbosum.' This strongly estrogenic metabolite was identified by analysis of its mass spectrum and its behavior in thin-layer, high-pressure liquid and gas-liquid chromatographic systems. The concentration of zearalenol in cultures was 563 μg/g, or 7% of the 8,000-μg/g zearalenone content, while the two diastereomers of 8'-hydroxyzearalenone each occurred at 3% of the zearalenone level. Of the two possible diastereomers of zearalenol, the one occurring in cultures was identical to the low-melting-point (171°C) isomer (alpha) obtained by synthesis. In the rat uterus bioassay, the alpha zearalenol isomer was three times more estrogenic than zearalenone while the beta isomer was equal in activity in zearalenone. The two diastereomers of zearalenol can be distinguished from each other by the intensity of the m/e^+ 302 fragment of the mass spectrum of the pure underivatized compound.

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone] is an estrogenic compound synthesized by certain isolates of *Fusarium roseum, F. tricinctum, F. oxysporum,* and *F. moniliforme*; it causes hyperestrogenism in animals (4). The metabolite was characterized chemically by Urry et al. (10).

Six derivatives (Fig. 1) of zearalenone have been isolated from cultures of *F. roseum* 'Gibbosum' and *F. roseum* 'Graminearum.' They are: zearalenol (9), diastereomers of 8'-hydroxyzearalenone (2, 9, 11), diastereomers of 3'-hydroxyzearalenone (5), 6'8'-dihydroxyzearalene (8), 5-formylzearalenone, and 7'-dehydrozearalenone (11). Among the derivatives isolated from *Fusarium,* so far only zearalenol has been shown to be estrogenic. Zearalenol obtained by sodium borohydride reduction of zearalenone is a diastereomeric mixture. Peters (6) presented data which, although fragmentary, indicated that one diastereomer of zearalenol was inactive uterotropically while the other was about three times more estrogenic than zearalenone. From Peters' (6) report, it was not possible to ascertain the identity of the active diastereomer.

Shipchandler (7) and Hidy et al. (1) described two diasteromers of zearalenol, one with a melting point of 168 to 169°C and the other of 174 to 176°C. Stipanovic and Schroeder (9) reported the isolation of zearalenol from cultures of *F. roseum* 'Gibbosum' and *F. roseum* 'Semitectum' grown on moist autoclaved grain sorghum or cracked corn. However, they did not establish whether or not zearalenol isolated from *Fusarium* was a diastereomeric mixture. We decided to reinvestigate the presence of zearalenol in the isolates of *F. roseum* used in our laboratory and to characterize the naturally occurring diastereomer.

In this paper, the naturally occurring diastereomer of zearalenol produced in culture is referred to as zearalenol (low melting point), or alpha zearalenol, whereas the non-naturally occurring diastereomer is designated as *epi*-zearalenol (high melting point), or beta zearalenol. Since the termination of this work, zearalenol was identified as alpha, or R, in configuration (unpublished data) by X-ray crystallography and is the subject of a separate report.

The major toxic, non-estrogenic metabolite produced by this isolate of *F. roseum* is monoacetoxyscirpenol. In addition, the fungus produced large quantities of zearalenone (8,000 μg/g) as well as lesser amounts of non-estrogenic 8'-hydroxyzearalenone (F-5-4) and 8'-*epi*-hydroxyzearalenone (F-5-3).

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MATERIALS AND METHODS

F. roseum 'Gibbosum' was grown in three 1-liter Erlenmeyer flasks on 300 g of moist autoclaved Uncle

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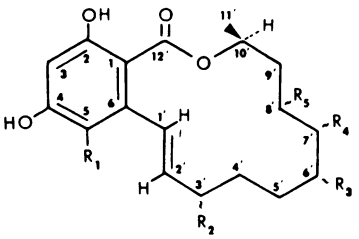

	R_1	R_2	R_3	R_4	R_5
Zearalenol	H	H_2	OH	H_2	H_2
Zearalenone	H	H_2	O	H_2	H_2
8'-hydroxyzearalenone	H	H_2	O	H_2	OH
6',8'-dihydroxyzearalene	H	H_2	OH	H_2	OH
3'-hydroxyzearalenone	H	OH	O	H_2	H_2
5-formylzearalenone	CHO	H_2	O	H_2	H_2
7'-dehydrozearalenone	H	H_2	O	H	H

FIG. 1. *Naturally occurring derivatives of zearalenone. The 8'-hydroxyzearalenones exist in culture as α and β isomers, while zearalenol exists in culture as the α isomer; the 8'-hydroxyzearalenones are not active uterotropically in the rat uterus bioassay, while the zearalenols and zearalenone are.*

Ben's rice (about 40% moisture content after autoclaving) for 7 days at 25°C and 30 days at 10 to 12°C. The cultures were extracted with acetonitrile, concentrated in vacuo and partitioned with petroleum ether (bp, 60 to 70°C). The petroleum ether containing the fats was discarded, the acetonitrile fractions were combined and concentrated to dryness in vacuo, and the residue was dissolved in 20 ml of acetone. Small portions (10 μl) of the extract were reacted with 100 μl of Tri-Sil BT (Pierce Chemical Co., Rockford, Ill.), and zearalenone, zearalenol, and 8'-hydroxyzearalenone were quantitated by gas-liquid chromatography (GLC). The identity of the metabolites was confirmed by mass spectrometry.

Other portions (0.5 ml) of the acetone solution were applied to 2-mm-thick Silica Gel G plates (E. Merck, Darmstadt) and repeatedly developed (10 to 15 times) in petroleum ether (bp, 30 to 50°C)–diethyl ether–glacial acetic acid (70:30:2, vol/vol) until the zearalenol reached an R_f value of about 0.4. The bands of silica gel containing zearalenol were scraped, combined, and eluted with acetone; the acetone concentrate was subjected to further purification by thin-layer chromatography (TLC) and then analyzed by gas-liquid chromatography (GLC), high-pressure liquid chromatography (HPLC), and combined gas chromatography-mass spectroscopy (GC-MS) (Fig. 2). The epimers of zearalenol were analyzed by analytical and preparatory means by using HPLC and a C_{18} Bondapak reverse-phase column (Waters Associates Inc., Milford, Mass.). The elution solvents were methanol–water (65:35, vol/vol). The diastereomers were repeatedly crystallized from methanol–water until no further increase in the melting point occurred.

Zearalenol was prepared from pure zearalenone by reduction of the 6'-ketone of zearalenone with sodium borohydride in ethanol. The resulting diastereomeric mixture was resolved and purified as described above by HPLC. Melting points were taken on a Fisher-Johns melting point apparatus (Fisher Chemical Co., Chicago, Ill.) and are uncorrected.

High resolution mass spectra were obtained on an AEI MS-30.

The diasteromers of synthetic zearalenol were tested for estrogenicity in the rat uterus bioassay. Compounds were applied to the shaved backs of weanling female rats at the rate of 2 mg each in one application of 100 μl of acetone. After 4 days the rats were sacrificed, and uteri were excised and immediately weighed.

Gas chromatographic analyses were made with Varian Series 1400 and Hewlett-Packard 1710A instruments equipped with a flame ionization detector under the following conditions: stainless steel column (1 m by 3 mm) packed with 3% QF-1 on 100 to 120-mesh Chromosorb-Q solid support (Applied Science Laboratories, Inc., State College, Pa.); N_2 carrier gas was 30 ml/min; injector and detector temperatures were 250°C; temperature program was from 150 to 250°C at 6°C per min.

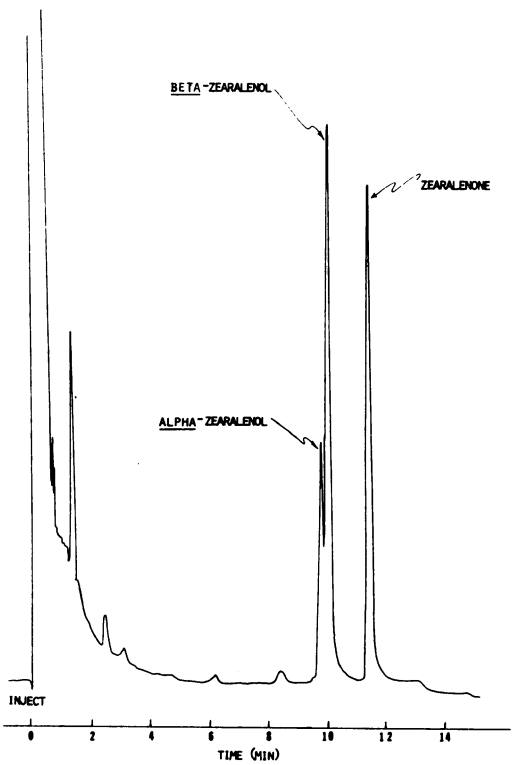

FIG. 2. *Separation of the alpha and beta isomers of trans-zearalenol and zearalenone by GLC. The metabolites were resolved on a Hewlett-Packard 5710A gas chromatograph under the following conditions: column, 3% QF-1 on 100 to 120-mesh Gas Chrom Q, 1 m-by-3 mm stainless steel; N_2 and H_2 flow rate was 30 ml/min; air flow was 240 ml/min and a temperature program of 150 to 250°C at 6°C/min.*

Low-resolution mass spectra were taken at 70 eV on an LKB 9000 combination GC-MS. Masses were calculated and tabulated by an on-line computer. Multiple scans of background signals were taken before and after the zearalenol peak and subtracted from the zearalenol spectrum by the computer. The zearalenol peak was scanned repeatedly to verify the presence of a single component. Direct inlet spectra of underivatized compounds were taken in the same fashion.

RESULTS

The low-resolution mass spectrum (direct probe) of the naturally occurring zearalenol is shown in Fig. 3, and the mass spectrum of its trimethylsilyl ether derivative is shown in Fig. 4. The naturally occurring isomer has the identical fragmentation pattern as the low-melting-point isomer obtained after resolution of the synthetically derived diastereomeric mixture.

Diastereomeric zearalenol—separation and properties. The high-pressure liquid chromatography of the diastereomeric mixture of zearalenol, which was obtained by sodium borohydride reduction, on a reverse phase column provided an excellent separation. The more polar diastereomer (faster moving) had a melting point of 176.0 to 177.5°C. This diastereomer was designated *epi*-zearalenol. The other diastereomer (less polar) melted at 171.0 to 171.5°C.

No separation of these diastereomers was observed when the mixture was analyzed by GLC on columns packed with 3% OV-1 or 3% OV-17 on Gas Chrom Q. However, satisfactory resolution was obtained on 3% QF-1 phase as shown in Table 1 and Fig. 2.

Separation of the two diastereomers of zearalenol was also attempted by TLC (Table 2). Two solvent systems: (i) chloroform–ethanol (97:3) and (ii) petroleum ether (60 to 70°C)–diethyl ether–glacial acetic acid (70:30:2), provided sufficient resolution of the mixture. *epi*-Zearalenol had a lower R_f value than the other diastereomer. There was a considerable difference in the fluorescent intensities between these two isomers. The diastereomer with the higher R_f exhibited a significantly more intense (about four

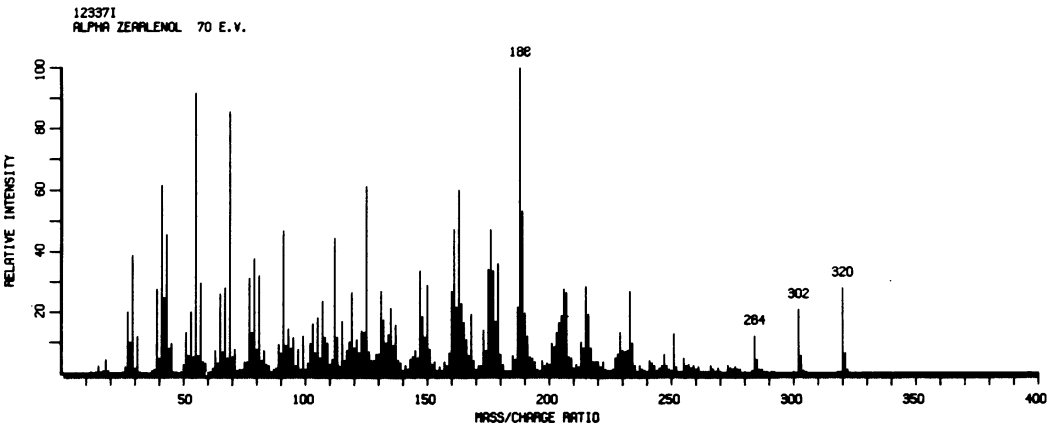

FIG. 3. *The mass spectrum of zearalenol (naturally occurring and alpha [R] in configuration) obtained by direct probe analysis (70 eV) on an LKB-9000 low-resolution mass spectrometer. The intensity of the m/e⁺ 302 fragment is always less than its corresponding beta isomer.*

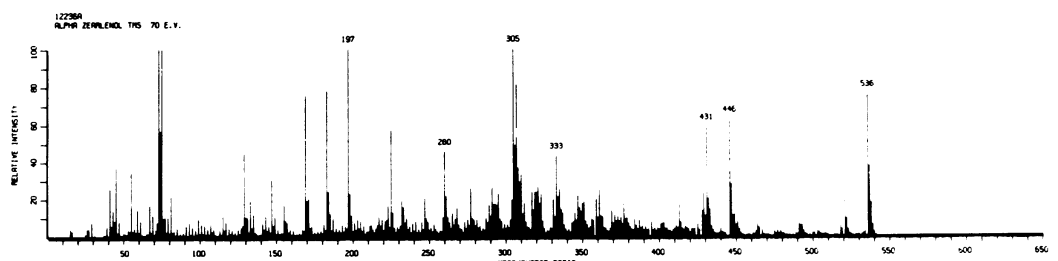

FIG. 4. *Mass spectrum of the trimethylsilyl ether of naturally occurring trans isomer of zearalenol normalized to m/e⁺ 305. The molecular ion is 536, and the base peak is m/e⁺ 305; the spectrum was obtained on a combination GC-MS (LKB-9000) at 70 eV with a gas chromatographic column and conditions as described in the text.*

to five times) fluorescence than that of *epi*-zearalenol.

The synthetic diastereomeric mixture of zearalenol had a differential solubility in chloroform or methylene chloride. The more soluble diastereomer in those solvents was found to be the isomer with the lower melting point (alpha). This differential solubility was useful in obtaining *epi*-zearalenol in a pure form by fractional crystallization.

The mass spectra of the two diastereomers of zearalenol were identical with respect to the fragmentation pattern and intensities of major ions except that of m/e^+ 302, which is formed by the removal of a water molecule from the molecular ion. The intensity of m/e^+ 302 of *epi*-zearalenol in a normalized spectrum obtained by resolution mass spectrometry (base peak at m/e^+ 188) was about equal to the intensity of the molecular ion. However, a sizable reduction (ca. 30%) was observed in the intensity of m/e^+ 302 in the spectrum of lower melting diastereomer (Fig. 5).

Identity of naturally occurring zearalenol. The naturally occurring zearalenol, when analyzed by HPLC (reverse phase), GLC (3% QF-1), and by TLC indicated a single component and not a diastereomeric mixture. Further, it was found to conform to the identity of the diastereomer of zearalenol which had the lower melting point. Examination of the culture extract by GLC, GC-MS, and HPLC did not reveal the presence of *epi*-zearalenol.

The average concentration of the naturally occurring zearalenol was 563 μg/g, while that of zearalenone was 8,000 μg/g; 8′-hydroxyzearalenones were present in the concentration of 240 μg/g. The production of zearalenol and 8′-hydroxyzearalenone in culture was 7 and 6%, respectively, relative to zearalenone.

Biological activity. The rat uterotropic assay showed that the naturally occurring diaster-

TABLE 1. *Separation of trimethylsilyl derivatives of various derivatives of zearalenone by GLC on 3% QF-1*[a]

Compound	Retention time (min)	Relative retention index
Zearalenone (F-2)	11.80	1.00
8′-Hydroxyzearalenone (F-5-4)	11.70	0.99
3′-Hydroxyzearalenone (F-5-1)	11.55	0.98
8′-*epi*-Hydroxyzearalenone (F-5-3)	11.40	0.97
trans-epi-Zearalenol (beta)	10.50	0.89
trans-Zearalenol (alpha)	10.40	0.88
cis-epi-Zearalenol (beta)	10.25	0.87
cis-Zearalenol (alpha)	10.20	0.86

[a] Column was 1 m by 3 mm OD stainless steel; QF-1 was on 100 to 120 mesh Chromosorb Q; temperature was 150 to 250°C at 6°C/min; flame ionization detector was with N_2 at 30 ml/min, H_2 at 20 ml/min, and air at 300 ml/min.

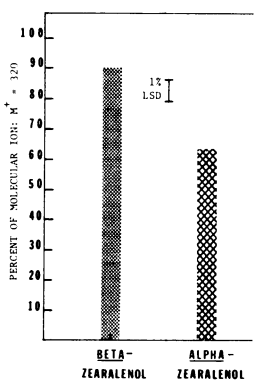

FIG. 5. *Analysis of the difference in intensity of m/e^+ 302 between underivatized alpha and beta isomers of zearalenol. The naturally occurring alpha isomer has an intensity 63% of the molecular ion (M^+) whereas the non-naturally occurring beta isomer has an intensity of 91% of the M^+ (Tukey's test, $P < 0.01$).*

TABLE 2. *Summary of the characteristic properties of alpha zearalenol and beta zearalenol*

Diastereomer	MP (°C)[a]	Mol wt	Fluorescence	Estrogenicity	Occurrence in culture	Mass spectra (underivatized)	R_f value[b]	HPLC retention[c] (min)
Alpha zearalenol	171–171.5	320 ($C_{18}H_{24}O_5$)	Intense blue	More	Yes	*m/e* 302, 63% of M^+ = 320	0.38	12.80
Beta zearalenol	176–177.5	320 ($C_{18}H_{24}O_5$)	Less intense blue	Less	No	*m/e* 302, 91% of M^+ = 320	0.25	10.00

[a] After crystallization from H_2O:CH_3OH.

[b] In either 70:30:2 (petroleum ether–ethyl ether–glacial acetic acid) or 97:3 ($CHCl_3$:CH_3OH) on Silica Gel G; plate developed five times.

[c] Column, micro-Bondapak C_{18} (30 cm by 4 mm); mobile phase, methanol–water (65:35); flow rate, 1 ml/min; detector, ultraviolet light (254 nm).

TABLE 3. *Uterotropic activity of alpha zearalenol (naturally occurring isomer), beta zearalenol, and zearalenone*

Treatment	Dose (mg)	Uterine wt[a, b] (mg)	Feed consumed (g)	Wt gain[a] (g)
Acetone control	0	38.5 ± 1.50 a	28.0 ± 2.00	18.0 ± 1.00
Zearalenone	2	67.5 ± 8.50 b	14.0 ± 7.00	7.5 ± 7.50
Beta zearalenol	2	65.0 ± 25.00 b	25.0 ± 3.00	19.0 ± 1.00
Alpha zearalenol	2	204.0 ± 64.00 c	23.0 ± 1.00	16.0 ± 1.00

[a] Weights are the mean of two rats. Means followed by the same letter are not significantly different ($P < 0.05$).

[b] Fresh weight.

eomer of zearalenol is about three times more estrogenic than zearalenone ($P < 0.05$; Table 3), while *epi*-zearalenol was about as active as zearalenone (i.e., the means are not significantly different by Tukey's test at 5%).

DISCUSSION

The diastereomer of zearalenol corresponding to the naturally occurring zearalenol has been analyzed by X-ray crystallography and has been shown to be α-zearalenol. The absolute configuration at C-6′ was determined to be R (unpublished information; to be published elsewhere).

Hidy et al. (1) reported that the *epi*-zearalenol isomer (higher melting point isomer), though estrogenically active, was less active than zearalenone, while the zearalenol (low melting point isomer) was described as simply estrogenic with no further description. Peters (6), on the other hand, reported that one diastereomer of zearalenol was inactive estrogenically while the other was observed to be about three times more active than zearalenone.

Steele et al. (8) anticipated the existence of zearalenol but did not find it probably because his chromatographic columns (GLC) would not resolve zearalenol from the 8′-hydroxyzearalenones.

Zearalenol obtained from cultures is very difficult to purify except by HPLC, and our evidence from TLC, GLC, HPLC, and mass spectrometry indicates the presence of only one isomer.

The presence of zearalenol in cultures of *F. roseum* is important because it suggests the possibility of occurrence of zearalenol in animal feeds. Moreover, zearalenol is about three times more estrogenic than zearalenone. There is also the possibility that some isolates of *F. roseum* may produce more zearalenol than found in this study and other isolates may produce zearalenol exclusively.

These reports indicate the need for development of an analytical method to detect zearalenol as well as zearalenone in feedstuffs.

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LITERATURE CITED

1. **Hidy, P. H., R. S. Baldwin, R. L. Greasham, C. L. Keith, and J. R. McMullen.** 1977. Zearalenone and some derivatives: production and biological activities. Adv. Appl. Microbiol. **22**:59–82.
2. **Jackson, R. A., S. W. Fenton, C. J. Mirocha, and G. Davis.** 1974. Characterization of two isomers of 8′-hydroxyzearalenone and other derivatives of zearalenone. Agric. Food Chem. **22**:1015–1019.
3. **Kuo, C. H., D. Taub, R. D. Hoffsommer, N. L. Wendler, W. H. Urry, and G. Mullenbach.** 1967. The resolution of (±) zearalenone. Determination of the absolute configuration of the natural enantiomorph. Chem. Commun., p. 761–762.
4. **Mirocha, C. J., and C. M. Christensen.** 1974. Oestrogenic mycotoxins synthesized by *Fusarium*, p. 129–148. *In* I.F.H. Purchase (ed.), Mycotoxins. Elsevier, Amsterdam.
5. **Pathre, S. V., C. J. Mirocha, and S. W. Fenton.** 1978. 3′-Hydroxyzearalenone: two new metabolites produced by *Fusarium roseum*. Proc. Am. Chem. Soc., Abstr. 32, Miami.
6. **Peters, C. A.** 1972. Photochemistry of zearalenone and its derivatives. J. Med. Chem. **15**:867.
7. **Shipchandler, M. T.** 1975. Chemistry of zearalenone and some of its derivatives. Heterocycles **3**:471–520.
8. **Steele, J. A., C. J. Mirocha, and S. V. Pathre.** 1976. Metabolism of zearalenone by *Fusarium roseum* 'Graminearum.' Agric. Food Chem. **24**:89–96.
9. **Stipanovic, R. D., and H. W. Schroeder.** 1975. Zearalenol and 8′-hydroxyzearalenone from *Fusarium roseum*. Mycopathology **57**:77–78.
10. **Urry, W. H., H. L. Wehrmeister, E. B. Hodge, and P. H. Hidy.** 1966. The structure of zearalenone. Tetrahedron Lett. **37**:3109–3114.
11. **von Bollinger, G., and C. Tamm.** 1972. 306. Vier neue Metabolite von *Gibberella zeae*: 5-Formyl-zearalenon, 7′-Dehydrozearalenon, 8′-Hydroxy- und 8′-*epi*-Hydroxyzearalenon. Helv. Chim. Acta **55**: 3030–3048.