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Biosynthesis of the Fungal Estrogen F-2 and a Naturally Occurring Derivative (F-3) by *Fusarium moniliforme*[1]

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The fungal metabolites, F-2 and F-3, associated with estrogenism in swine, are produced by some races of *Fusarium moniliforme* isolated from toxic feeds.

The estrogenic syndrome in swine as found in Minnesota is characterized by the development of a swollen, edematous vulva in females, shrunken testes in young males, enlarged mammary glands in the young of both sexes, and, possibly, abortion in pregnant gilts and sows. Young animals also come into standing heat prematurely. It is a fairly common and serious disease of swine and has been reported from various parts of the world (2, 5, 6, 12). The evidence now is that a major cause of this disease is an estrogenic metabolite of *Fusarium graminearum* (*Gibberella zeae*).

The estrogen, 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl) β-resorcylic acid lactone, has been called by various names, such as "F-2" by us, "RAL" by Commercial Solvents Corp., and more recently "Zearalenone." It has recently been found in hay associated with infertility in dairy cattle (8). It has also been shown to affect the development of the sexual stage of *G. zeae* (3) and various species of other ascomycetes and basidiomycetes (10). It may be an important sex-regulating hormone operative in many fungi.

Caldwell and Tuite (1) investigated the production of F-2 by various species of *Fusarium* and concluded that, of the species tested, only *F. roseum* and *G. zeae* produced the F-2 metabolite when grown on a corn substrate at 16 C for 3 weeks. We investigated the production of F-2 and F-3 by numerous isolates of *F. graminearum* and other species of *Fusarium*. Among these were isolates of *F. moniliforme* (asexual stage, some isolates of which produce the perfect stage called *G. fujikuroi* and which also produce gibberellic acid) that were found to produce in culture not only F-2, but also a chemically related derivative we call F-3. *F. moniliforme* is a common invader of corn plants grown in the field and may occur throughout the plant, including the embryo of the kernel. It is our objective to present evidence that certain isolates of *F. moniliforme* produce F-2.

Source of cultures. In our work with possibly toxicogenic fungi, *F. moniliforme* has been isolated frequently from corn stored on the cob in cribs, from shelled corn, and from samples of feed of which corn was the major ingredient. Of the two isolates under discussion here, one came from shelled corn, the other from a sample of feed. The isolates differed from one another in amount of aerial mycelium when growing on agar media, and in the amount of red pigment produced, but both produced conidia typical of *F. moniliforme*.

Extraction and characterization of F-2. To identify F-2 in cultures of *Fusarium*, four methods have been used to distinguish between naturally occurring F-2 and closely related derivatives found during the study of the biosynthesis of this compound. These compounds have been named F-3 and F-5-1 through F-5-7.

F. moniliforme was seeded onto autoclaved corn, which was incubated at 22 to 26 C for 2 weeks and then at 10 to 12 C for 6 weeks. The cultures were then dried, brought up to 15% moisture, and extracted with methylene chloride. The methylene chloride was concentrated on a flash evaporator to a syrup-like consistency; petroleum ether [boiling point (bp), 30 to 60 C] was added and then equilibrated with an equal amount of acetonitrile. The acetonitrile was concentrated and appropriate portions of it were spotted on thin-layer chromatograph plates of silica gel and developed with chloroform-ethyl alcohol (97/3). The F-2 from *Fusarium* was identified by comparing its mobility with that of an F-2 standard spotted alongside the unknown and by co-spotting the standard with the unknown. They were found to be identical. The

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F-2 constituent, separated on the thin-layer chromatograph, was eluted from the silica gel with ethyl alcohol and its absorption spectrum in the ultraviolet region was determined. An acceptable spectrum of F-2 was obtained, but it was not as good as desired because of an interfering substance which eluted from the silica gel with the F-2 and apparently had the same R_F value. The impurity was eliminated by rechromatography in a benzene-ethyl acetate (50/50) solvent system. After elution with ethyl alcohol, an ultraviolet absorption spectrum identical to that of F-2 was obtained. Alternatively, the extract was placed on a silica gel (Grace, 100 to 200 mesh, grade 923) column and developed in succession with petroleum ether (bp, 30 to 60 C), petroleum ether (bp, 60 to 70 C), benzene, and methylene chloride. The methylene chloride effluent was collected in two parts as it came off the column, a yellow and a clear fraction, and these fractions were kept separate. Crystals were obtained from the clear fraction and were analyzed by infrared spectrophotometry (Perkin-Elmer, model 257); their absorption maxima were found identical to those of F-2.

The compound was characterized further by gas-liquid chromotography [10-ft (305-cm) SE-30 (General Electric) column held at 265 C in a Varian-Aerograph chromatograph]. A trimethylsilyl ether derivative of the metabolite from *Fusarium* was made by reacting it with *N,O*-bis-(trimethylsilyl)-acetamide and chromatographing it with internal standards. The *F. moniliforme* metabolite had a retention time identical to that of authentic F-2.

The extracts of the *F. moniliforme* culture were also analyzed for the presence of F-3. The latter compound is a closely related derivative of F-2 and is also suspected of having estrogenic activity (7). As determined by gas-liquid chromatography, F-3 was present in all the cultures tested.

Studies involving the chemical isolation and characterization of F-3 show it to be a highly labile compound subject to oxidation. It is readily extractable from biological material by the same methods and solvents as with F-2. Its absorption spectrum is identical to that of F-2 except that F-3 lacks an absorption maximum at 314 nm.

F-3, like F-2, reacts with silating agents [*N,O*-bis-(trimethylsilyl)-acetamide] to form the trimethyl silyl ether. The latter compound can then be separated on the SE-30 column; it has a retention of about 2 min less than that of F-2 at about 260 C. Unlike F-2, F-3 does not react with Girard's reagent (trimethylaminoacetohydrazide chloride) and can be separated from F-2 by this method.

F-3, like F-2, can be separated from other biological constituents on a silica gel column. With solvents of the eluotropic series, it elutes off the column in the diethyl fraction.

The following species of *Fusarium* were also tested for their ability to produce F-2 when grown on a corn substrate for 2 weeks at 22 to 24 C, and 5 weeks at 10 to 12 C: *F. lateritium*, *F. tricinctum*, *F. nivale*, *F. episphaeria*, *F. rigidiusculum*, *F. oxysporum*, *F. roseum*, and *F. solani*. Of these, only *F. roseum* produced F-2 (3,600 μg/ml). The above cultures were provided and identified by R. R. Nelson of Pennsylvania State University.

The discovery that *F. moniliforme* can produce F-2 supports the contention that derivatives of the resorcylic acid lactone may be more widespread among fungi than originally thought. F-2 has already been shown to have striking effects on controlling the development of the sexual stage of fungi, and it or its derivatives may indeed be hormones that are functional in many different fungi. Compounds similar in chemical structure have previously been reported among the fungi, e.g., curvalarin, produced by *Curvularia* sp., *Penicillium steckii* Zaleski, and *P. expansum* Link (11); radicicol, produced by *Nectria radicicola* (9); and monorden, produced by *Monosproium* sp. (4).

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