Natural Occurrence of Fusarin C, a Mutagen Produced by *Fusarium moniliforme*, in Corn

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Fusarin C is a highly mutagenic compound produced by strains of *Fusarium moniliforme* and exhibits a mutagenic activity comparable with that of aflatoxin B_1 and sterigmatocystin. All the isolates of *F. moniliforme* and *Fusarium graminearum* tested were shown to produce fusarin C in culture, while none of the isolates of *F. moniliforme* var. *subglutinans* produced fusarin C. Chemical analyses of a sample of moldy home-grown corn from Butterworth, Transkei, indicated that fusarin C occurs naturally in both hand-selected visibly *Fusarium* infected and "healthy" corn kernels.

Extracts of several strains of *Fusarium moniliforme* have been shown to be mutagenic in the Ames *Salmonella*/microsome mutagenicity assay (Bjeldanes et al., 1979). A mutagenic compound, fusarin C, has recently been isolated from a culture of *F. moniliforme* strain MRC 826 (Gelderblom et al., 1983). The structures of fusarin C as well as of two related nonmutagenic metabolites, fusarins A and D (Gelderblom et al., 1984), have been elucidated (Figure 1).

Interest in the fungal production of this potent mutagen under laboratory conditions as well as under natural conditions on corn during cultivation is due to the ubiquitous nature of *F. moniliforme* on cereals and the fact that *F. moniliforme* has been shown to cause diverse lesions in experimental animals as well as the implication of *F. moniliforme* in the etiology of esophageal cancer.

Several isolates of *F. moniliforme* from Transkeian corn have been shown to cause equine leukoencephalomalacia in horses and to be highly toxic to experimental animals (Kriek et al., 1981a,b). Life-long exposure of rats to culture material of one of these isolates, *F. moniliforme* MRC 826, resulted in a high incidence of hepatocellular carcinoma and a high overall tumor incidence (Kriek et al., 1983). Culture material of *F. moniliforme* on corn has been demonstrated to cause a significant enhancement of nitrosamine-induced esophageal carcinogenesis in rats (Van Rensburg et al., 1982). A significantly higher incidence of *F. moniliforme* has been reported in home-grown corn produced in an area with a high human esophageal cancer rate than in a low rate area in Transkei, southern Africa (Marasas, 1982; Marasas et al., 1979, 1981). *F. moniliforme* has also been reported to be one of the most prevalent fungi associated with staple foodstuffs in Linxian County in the Henan Province, which is a high risk area for esophageal cancer in China (Li et al., 1980; Yang, 1980, 1982).

None of the active compounds responsible for the toxic or carcinogenic effects mentioned above have as yet been isolated or identified. It has not been resolved whether the fusarins are in any way associated with the known toxicity or carcinogenicity of *F. moniliforme* isolates. It is also not known whether the fusarins occur naturally in corn or not.

The mycotoxins moniliformin, deoxynivalenol, and zearalenone have been shown to occur naturally in a sample of moldy home-grown corn from Butterworth, the district with the highest esophageal cancer rate in Transkei (Thiel et al., 1982). In addition to *F. moniliforme*, this corn was also infected by *F. moniliforme* Sheldon var. *subglutinans* Wr. & Rk. [=*Fusarium sacchari* (Butl.) Gams var. *subglutinans* (Wr. & Rk.) Nirenberg] and *Fusarium graminearum* Schwabe (Thiel et al., 1982). In cultures on corn, moniliformin was only produced by isolates of *F. moniliforme* var. *subglutinans*, deoxynivalenol and zearalenone were produced by isolates of *F. graminearum*, and isolates of *F. moniliforme* did not produce any of these three mycotoxins (Thiel et al., 1982).

The same sample of moldy corn from Butterworth, Transkei, that was shown to contain moniliformin, deoxynivalenol, and zearalenone was used to determine whether fusarin C occurs naturally in corn or not. The production of fusarin C in culture by isolates of *F. moniliforme*, *F. moniliforme* var. *subglutinans*, and *F. graminearum* from Transkeian corn was also investigated. A comparative study where the mutagenic activity of fusarin C and the mycotoxins aflatoxin B_1 and sterigmatocystin were compared is also presented.

EXPERIMENTAL SECTION

Culture of Fungi. Preparation of the corn cultures of the different fusaria species was carried out as previously described (Kriek et al., 1981b). The cultures were incubated for 2 weeks at 25 °C, followed by 2 weeks at 15 °C, and stored at 4 °C until analyzed.

Corn Sample. A sample of moldy corn ears of the 1978 crop was obtained from a farm in the Butterworth district, Transkei, during July 1978 (Thiel et al., 1982). Ears visibly infected by *Fusarium* (pink, red, or purple discolored ears) were selected and shelled in a hand sheller. The kernels (a mixture of *Fusarium*-infected kernels, healthy kernels, and kernels infected with other fungi) were retained as sample M-84 (Thiel et al., 1982). For the purpose of this investigation, healthy kernels (sample M-84/C) and *Fusarium*-infected kernels (sample M-84/F) were selected from sample M-84 and ground in a coffee grinder, while kernels infected by other fungi were discarded.

Mutagenicity Assay. Mutagenicity was assayed as previously described (Gelderblom et al., 1983). A rat liver microsomal fraction (S-9) was prepared from phenobarbitone-induced BD IX male (200–250 g) rats (Marshall and McLean, 1969). The S-9 preparation (41.2 mg of protein/mL) was incorporated into the S-9 mixture at a ratio of 0.025 mL/mL of S-9 mixture.

Chloroform–2-propanol (1:1 v/v) ($CHCl_3$–IPA) extracts of samples M-84/C and M-84/F were prepared by extracting 50-g samples with 300 mL of $CHCl_3$–IPA by blending for 2 min in a Sorvall homogenizer and filtering through Whatman No. 1 filter paper. Extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness at 40 °C. The residues were dissolved in 3 mL of Me_2SO, and

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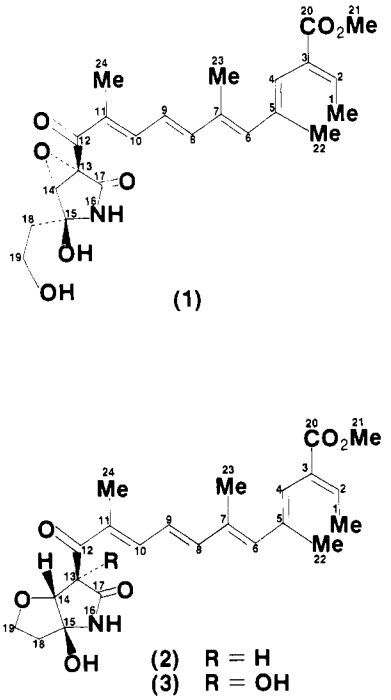

Figure 1. Chemical structure of fusarins A (1), C (2), and D (3).

different concentrations were titrated in the *Salmonella* test using tester strain TA 100.

The mutagenic activity of fusarin C (0.5, 1.16, and 2.32 nmol/plate) was compared with the activities of the potent mutagens aflatoxin B_1 (0.16, 0.32, and 0.64 nmol/plate) and sterigmatocystin (0.15, 0.31, and 0.62 nmol/plate). *Salmonella* strain TA 100 was used in this comparison employing a 100 mM phosphate buffer in the S-9 mixture for aflatoxin B_1 and sterigmatocystin and a 50 mM phosphate buffer for fusarin C. Plate counts were corrected for spontaneous back-mutations (approximately 100 revertants/plate) and each determination was done in triplicate.

Fusarin C Analyses. Samples M-84/C and M-84/F (50 g) were extracted by blending (2 min) and filtering successively with 150 mL of CH_2Cl_2–IPA (1:1 v/v) and 100 mL of H_2O and 100 mL of CH_2Cl_2–IPA (1:1 v/v). Filtrates were dried over anhydrous Na_2SO_4 and evaporated to dryness (at 40 °C). The wet cultures of the different fungal isolates were extracted in the same manner except that water was omitted in the first extraction and that 100 g of culture material was used.

The dry residues were extracted successively with 50 mL of petroleum ether (60–80°) and 2 × 50 mL of $CHCl_3$. Insoluble material during each extraction was removed by centrifugation (3000 rpm for 10 min). The petroleum ether extracts were reextracted with 2 × 50 mL of acetonitrile, and these acetonitrile phases were combined with the appropriate $CHCl_3$ extracts. The combined extracts (chloroform plus acetonitrile) were evaporated in vacuo at 40 °C and dissolved in 1 mL of MeOH–CH_2Cl_2 (1:19). These solutions were fractionated on a silica gel column (2.5 × 16 cm; 23 g of Kieselgel 60, 0.063–0.200 mm) with MeOH–CH_2Cl_2 (1:19) as the eluent. The first 100 mL of the eluate was discarded. The second 100 mL was collected and in the case of samples M-84/C and M-84/F concentrated to 50 mL. HPLC analyses were preformed on a Waters Associates liquid chromatograph equipped with a Laboratory Data Control fixed-wavelength detector (360 nm). Separations were carried out on a Ultrasphere (Beckman) silica column (4.6 mm × 25 cm; 5 μm) with MeOH–$CHCl_3$ (1:19) as the mobile phase at a flow rate of 1.0 mL/min.

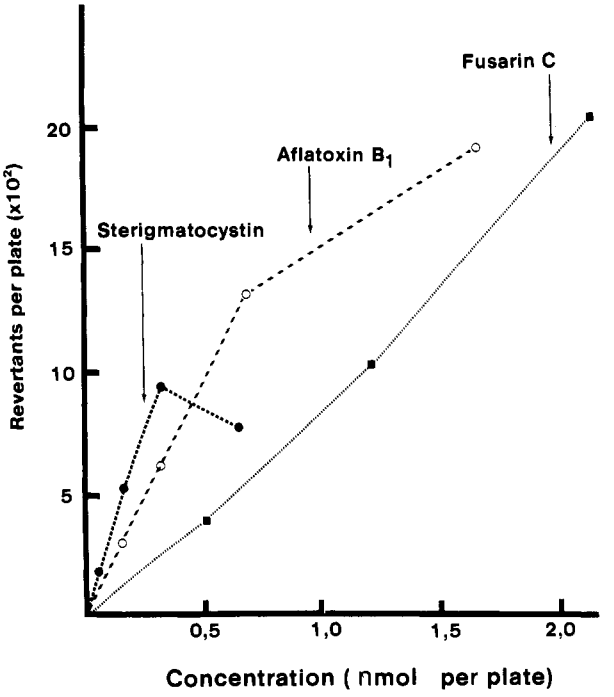

Figure 2. Evaluation of the mutagenic potential of fusarin C against aflatoxin B_1 and sterigmatocystin. Different concentrations of each mutagen were titrated against *Salmonella* strain TA 100 in the presence of S-9 (0.5 mg of protein/mL of S-9 mixture). Values represent means of triplicate determinations.

Samples were "spiked" in two different ways with standard fusarin C solutions (0.028 mg/mL) to confirm the identity of the peak eluting in the position of fusarin C. First, in the determination of the percentage of recovery of fusarin C, sample M-84/C was spiked by adding fusarin C (0.84 mg) to the dry sample prior to extraction. Second, the purified column eluates of M-84/C and M-84/F were spiked with standard fusarin C solutions (0.54 μg/mL and 0.028 mg/mL, respectively) before HPLC analyses. The "fusarin C" in the silica gel column eluate of sample M-84/F was further purified on Sephadex LH-20 columns with MeOH–CH_2Cl_2 (1:19) and CH_2Cl_2 as the eluting solvents as previously described (Gelderblom, 1982).

The effect of UV light on the isolated "fusarin C" was compared with its effect on authentic fusarin C standard by exposing solutions in CH_2Cl_2 to long-wave UV light (±30 s) and monitoring the effect by HPLC analysis under the conditions described above.

RESULTS AND DISCUSSION

The relative mutagenic activities of fusarin C and the potent mutagens aflatoxin B_1 and sterigmatocystin at varying concentrations are illustrated in Figure 2. Each of these dose–response curves represents results obtained under optimized conditions for the relevant mutagen. Preliminary experiments indicated that optimal mutagenic activity could be obtained for all three compounds when using an S-9 preparation from phenobarbital-induced rats at a concentration of 0.5 mg of protein/plate (Gelderblom, 1982). Under these conditions sterigmatocystin was the most potent mutagen followed by aflatoxin B_1 and fusarin C while sterigmatocystin became toxic at concentrations above 0.3 nmol/plate (Figure 2). When using the concentration of the mutagen that gives rise to a revertant colony count of 500 as a criterion for the mutagenic potency, sterigmatocystin was 4 times and aflatoxin B_1 2 times as potent as fusarin C.

All 20 *F. moniliforme* isolates tested produced fusarin C at concentrations varying from 700 mg/kg for strain

Table I. Fusarin C Production by Isolates of Different *Fusarium* Species

Fusarium species	isolate no.	area of origin	concn, mg/kg of dry weight[a]
F. moniliforme	MRC 1069	Transkei	655
	MRC 828	Transkei	247
	MRC 826	Transkei	83
	MRC 980	Transkei	258
	MRC 1054	Transkei	77
	MRC 1070	Transkei	298
	MRC 1896	Transkei	57
	MRC 820	Transkei	447
	MRC 1058	Transkei	177
	MRC 1064	Transkei	352
	MRC 614	South Africa	0.63
	MRC 712	South Africa	724
	MRC 716	South Africa	179
	MRC 809	South Africa	160
	MRC 958	South Africa	294
	MRC 1471	KwaZulu	172
	MRC 2595	KwaZulu	206
	MRC 1439	KwaZulu	58
	MRC 1784	United States	7
	MRC 2079	Egypt	42
F. moniliforme var. *subglutinans*	MRC 115	Transkei	0
	MRC 602	Transkei	0
	MRC 1899	Transkei	0
	MRC 822	Transkei	0
	MRC 1092	Transkei	0
F. graminearum	MRC 1113	Transkei	1
	MRC 1125	Transkei	39
	MRC 2052	Transkei	2
	MRC 1117	Transkei	2
	MRC 1123	Transkei	1

[a] Values are means of duplicate determinations.

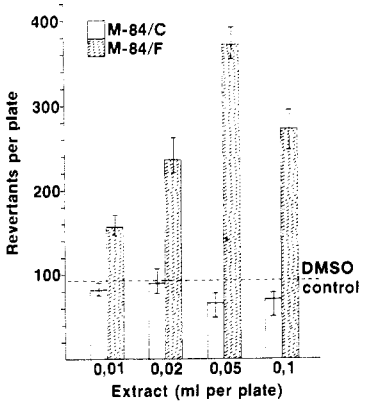

Figure 3. Mutagenicity of $CHCl_3$–IPA extracts of samples M-84/C and M-84/F in the presence of S-9 (1 mg of protein/mL of S-9 mixture). Values are means of triplicate determinations and vertical bars represent the range.

MRC 712 to 0.6 mg/kg for strain MRC 614 (Table I). Fusarin C was also produced by all five *F. graminearum* strains tested but not by any of the five strains of *F. moniliforme* var. *subglutinans*.

The presence of fusarin C in the two naturally contaminated samples M-84/C and M-84/F was demonstrated in several different ways. First, $CHCl_3$–IPA extracts of sample M-84/F were shown to contain mutagenic activity (Figure 3). A maximum mutagenic response of 4 times the background revertant counts was registered for the extract prepared from sample M-84/F while no mutagenic activity could be detected for sample M-84/C. Second, HPLC analyses of purified extracts of M-84/F and M-84/C showed a peak eluting in the same position as fusarin C (Figures 4 and 5). Upon spiking these extracts with authentic fusarin C and analyzing the "spiked" extracts

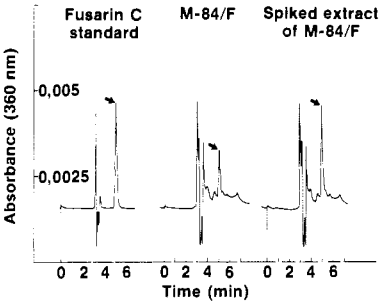

Figure 4. Separation of fusarin C standard (0.028 mg/mL), purified, and "spiked" purified column extracts of sample M-84/F, by silica gel HPLC.

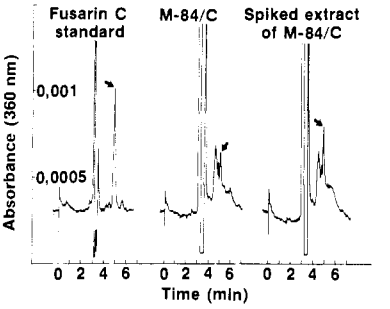

Figure 5. Separation of fusarin C standard (0.54 μg/mL), purified, and "spiked" purified column extracts of sample M-84/C by silica gel HPLC.

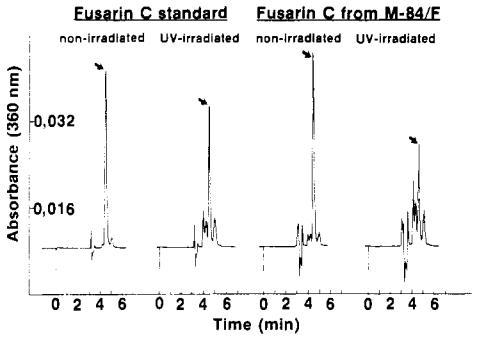

Figure 6. Comparison of the HPLC-eluting chromatograms of UV-irradiated fusarin C standard (0.028 mg/mL) and purified fusarin C from sample M-84/F. Double the amount of each sample was injected during UV irradiation.

Table II. Concentration of Fusarin C in Hand-Selected *Fusarium*-Infected and "Healthy" Corn Kernels

concn, mg/kg	
healthy kernels (sample M-84/C)	*Fusarium*-infected kernels (sample M-84/F)
0.02	0.28

by HPLC, we again detected an increased single symmetrical peak in the position of fusarin C. Third, further purification of the extract from sample M-84/F by Sephadex LH-20 column chromatography and analysis by HPLC again confirmed the presence of a peak eluting in the same position as fusarin C. Fourth, exposure of a solution of this purified compound to long-wave UV light gave rise to the formation of exactly the same three breakdown products as formed from fusarin C under similar conditions as shown by HPLC analyses of the irradiated solutions (Figure 6).

The fusarin C content of samples was quantified by comparing the peak heights of fusarin C peaks in HPLC chromatograms with those of standard fusarin C solutions. In the analytical procedure, including the extraction and purification steps, 80% of the fusarin C could be recovered,

which can be considered to be satisfactory for this type of analysis.

The concentration of fusarin C in sample M-84/F was found to be 0.28 mg/kg (Table II) while a low amount (0.02 mg/kg) could also be detected in sample M-84/C. This latter concentration was apparently too low to be detected in a mutagenicity assay (Figure 2).

It can be concluded that fusarin C is a highly mutagenic metabolite with a mutagenic potency comparable to the potent mutagens aflatoxin B_1 and sterigmatocystin. Fusarin C was shown to be a secondary metabolite of several strains of both *F. moniliforme* and *F. graminearum* but not of those strains of *F. moniliforme* var. *subglutinans* tested. It was proved to occur naturally in both *F. moniliforme* infected and "healthy" corn samples as the "fusarin C" peak coincided precisely with that of authentic fusarin C. Even after exposure to long-wave UV light, the HPLC chromatograms of the breakdown products of fusarin C and the isolated compound corresponded perfectly. In practice, the cooccurrence of fusarin C with the other *Fusarium* mycotoxins, moniliformin, deoxynivalenol, and zearalenone, in corn merits further investigation to determine any potential danger to human health.

Registry No. Fusarin C, 79748-81-5; aflatoxin B_1, 1162-65-8; sterigmatocystin, 10048-13-2.

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Identification of *p*-Hydrazinobenzoic Acid in the Commercial Mushroom *Agaricus bisporus*

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p-Hydrazinobenzoic acid (HBA) was identified in the cultivated mushrooms of the western hemisphere *Agaricus bisporus*. The commercial mushroom purchased locally was found to contain 10.7 ± 2.0 μg of HBA/g of mushroom (wet weight). HBA was quantitated in the fungus by high-performance liquid chromatography. The structure of HBA was confirmed by mass spectrometry.

The commercial cultivated mushroom, *Agaricus bisporus*, contains relatively large amounts of the hydrazide N^{β}-[γ-L-(+)-glutamyl]-4-(hydroxymethyl)phenylhydrazine (agarithine) (Levenberg, 1960, 1961). Kelly et al. (1962) found 0.04% agaritine in these mushrooms (wet weight), while we have found levels up to 0.07% (Ross et al., 1982).

Although agaritine did not induce tumors in mice (Toth et al., 1981a,b), over 50 hydrazine derivatives have induced tumors in laboratory animals (Toth, 1975, 1980, 1984; Toth et al., 1978, 1981a,b). The aim of this present study is to identify biosynthetic hydrazine precursors to agaritine that are present in this commercial mushroom. Schutte et al. (1972) have examined the incorporation of isotopically labeled compounds into agaritine by *A. bisporus*. There was no significant incorporation from 3-^{14}C-labeled tyrosine or phenylalanine. However, incorporation of 0.14%, 0.68%, and 4.1% was obtained with [U-^{14}C]shikimic acid, [2-^{14}C]glutamic acid, and *p*-aminobenzoic acid, respectively. This finding indicates that *p*-aminobenzoic acid is a precursor, with agaritine being one of the few natural products known to be derived from it. The postulated intermediates between *p*-aminobenzoic acid and agaritine have not yet been isolated (LaRue, 1977).

LaRue (1977) has postulated *p*-hydrazinobenzoic acid (HBA) and N^{β}-[γ-L-(+)-glutamyl]-4-carboxyphenylhydrazine as possible biosynthetic precursors of agaritine. We report herein the successful isolation, identification, and quantitation of HBA in *A. bisporus*.

EXPERIMENTAL SECTION

Materials. HBA was purchased from Eastman Kodak Co. (Rochester, NY) and purified by recrystallization. All solvents used for solvent extraction and thin-layer chromatography (TLC) were glass distilled, and solvents used for high-performance liquid chromatography (HPLC) were UV spectroscopic grade.

High-Performance Liquid Chromatography (HPL-

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