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CHARACTERIZATION OF FUSARIN F, A NEW FUSARIN FROM *FUSARIUM MONILIFORME*[1]

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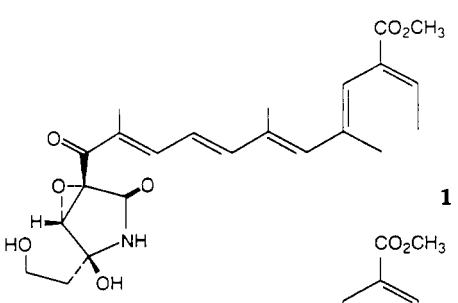
ABSTRACT.—The fusarins produced by seven *Fusarium moniliforme* strains grown in liquid culture were analyzed and characterized. Three fusarins were observed and identified, fusarin A [**2**], fusarin C [**1**], and a fusarin which had not been previously described, fusarin F [**4**]. No significant amounts of fusarin B or fusarin D [**3**] were detected. The time course of production of fusarins A, C, and F in liquid culture fermentations is described.

The genus *Fusarium* contains 24 toxigenic species perhaps best known for the production of trichothecenes and zearalenone. However, these species produce many other mycotoxins, including butenolide, moniliformin, fumonisins, and fusarins (1). One species that does not produce trichothecenes is *Fusarium moniliforme* Sheldon (Moniliaceae) (2). *F. moniliforme* has been shown to produce highly toxic and mutagenic compounds. Among these are the fumonisins, which have been directly associated with equine leukoencephalomalacia (LEM) (3) and have induced liver cancer in rats (4). Another compound, fusarin C [**1**], has also been associated with the mutagenic activity of *F. moniliforme* and with macrophage inhibition (5–7). Three other fusarins have been reported: fusarins A, B, and D (8). Of these, fusarins A and D have been characterized and shown to have structures **2** and **3**, respectively and to be non-mutagenic (9). There have been no further reports of fusarin B, and because fusarins A and D are non-mutagenic, most studies have centered on fusarin C. Crude fusarin extracts have been shown to be more mutagenic than pure fusarin C (10), suggesting that one or more other mutagens must be present. Our investigation of the fusarins produced in liquid culture by various strains of *F. moniliforme* resulted in the isolation of three fusarins, A, C, and an apparently new one with structure **4**, which we called fusarin F. The production profile of all three fusarins in liquid culture and a full characterization of fusarin E are reported here.



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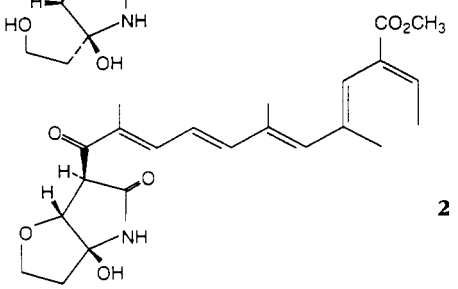

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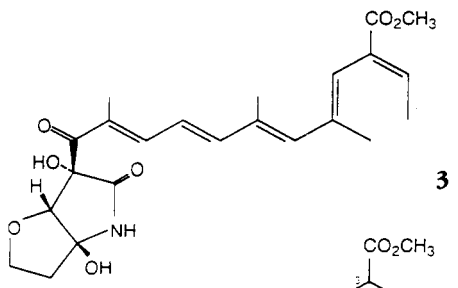

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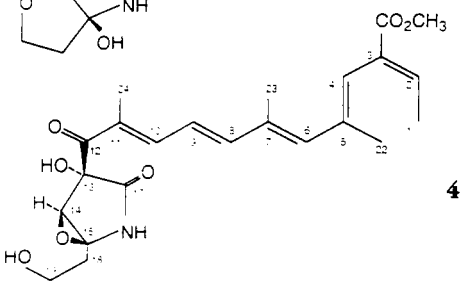

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[1]PRC Publication No. 1339.

EXPERIMENTAL

ORGANISMS.—Seven strains of *F. moniliforme* were studied: MRC 826, a South African strain isolated and identified by W.F.O. Marasas, Tygerberg, RSA; B33A, DAOM 212259, and DAOM 212260, three strains isolated by George Bean of the University of Maryland from Maryland corn fed to horses that died of LEM and identified by Paul Nelson of Pennsylvania State University; DAOM 183571, isolated from Thailand (11); DAOM 195767, isolated from corn from Ontario, Canada and identified by Gordon Neish of Agriculture Canada; and DAOM 212261 (M3783) from western Canada, isolated and identified by R.M. Clear of the Canadian Grain Commission, Winnipeg and provided by Jeffrey Farber of Health and Welfare Canada, Ottawa. Culture strains are deposited as follows: MRC, Medical Research Council, P.O. Box 70, Tygerberg; B, Department of Botany, University of Maryland, College Park; DAUM, Biosystematics Research Center, Agriculture Canada, Ottawa, Ontario.

GROWTH CONDITIONS.—Organisms were initially grown on 2% malt extract agar slants for 7–10 days. A slant was macerated in 27 ml of sterile H_2O. Aliquots (2.5 ml) of the resulting suspension were added to 250-ml Erlenmeyer flasks containing 50 ml of inoculation medium made up of ultrapure H_2O (1 liter), NH_4Cl (3 g), $FeSO_4 \cdot 7H_2O$ (0.2 g), $MgSO_4 \cdot 7H_2O$ (2 g), KH_2PO_4 (2 g), peptone (2 g), yeast extract (2 g), malt extract (2 g), and glucose (20 g). After 48 h of incubation in the dark at 28° on a rotary shaker (220 rpm, 3.81 cm throw), the suspension was macerated, and 2.5 ml aliquots were added to 250-ml Erlenmeyer flasks containing 50 ml of production medium consisting of ultrapure H_2O (1 liter), $(NH_4)_2HPO_4$ (1 g), KH_2PO_4 (3 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (5 g), sucrose (40 g), and glycerol (10 g). The cultures were covered with aluminum foil and incubated as described earlier for up to 12 days.

EXTRACTION.—Cultures (3 flasks, 150 ml) were harvested on each sampling day, vacuum-filtered together through Whatman #1 filter paper, and extracted with EtOAc (3 × 100 ml). The extract was dried over anhydrous Na_2SO_4 and concentrated to yield 10–20 mg of crude extract. During these operations, all glassware was covered with aluminum foil to block light. Originally, samples were then flash-chromatographed on Si gel (230–400 mesh) with $CHCl_3$ followed by MeOH-$CHCl_3$ (1:9), the latter solvent eluting a yellow band which contained all the fusarins. This pre-hplc purification step was later abandoned when it was shown to cause extensive decomposition of fusarin F.

HPLC ANALYSIS.—A Varian 5000 hplc instrument was used in conjunction with a Varian 9060 Polychrom diode array detector set at 362 nm for the chromatographic profile. Analysis of fusarin production was done with either a Lichrosorb 5 μm RP-18 250 × 4.6 mm column and an H_2O-MeOH (40:60) solvent system at 1 ml/min, or a Partisil 5 μm ODS-3 250 × 4.6 mm column and an H_2O-MeOH (30:70) solvent system at 1 ml/min. Evaluation of the concentration of fusarins was based on the absorption of a fusarin C standard (30 μg for a 1.0 AU peak). Isolation of fusarins was performed using either a Partisil 10 μm ODS-2 300 × 10 mm column and an H_2O-MeOH (60:40) solvent system at 2 ml/min or a CSC 10 μm 300 × 10 mm CN column with an MeOH-CH_2Cl_2 (2.5:97.5) solvent system at 2 ml/min.

CHARACTERIZATION.—Mass spectra were obtained on a Finnigan MAT 4500 MS system operating in the ei mode. Ir spectra were recorded with a Perkin-Elmer Infracord spectrophotometer. Uv spectra were obtained on a Varian DMS 200 uv-vis spectrophotometer. ^1H- and ^{13}C-nmr spectra were obtained on either a Bruker AM250 or AM500 spectrometer. Chemical shifts are referenced to residual $CHCl_3$, at 7.24 ppm for ^1H spectra and $CDCl_3$ at 77.0 ppm for ^{13}C spectra, and reported relative to TMS. Homonuclear correlation spectra were obtained using the 90-t_1-45 FID pulse sequence (COSY 45). Heteronuclear correlation spectra were also obtained to confirm the assignments of the ^{13}C resonances.

Fusarin F [**4**].—Ir 3300, 3000, 2350, 1710, 1580, 1400, 1250, 1200, 1030, 895, 840 cm^{-1}; uv ($CHCl_3$) λ max 370 nm (ε = 40200) with shoulder at 293 nm (ε = 9700); hrms calcd for $C_{23}H_{29}NO_7$, 431.1944, found 431.1933. Eims and nmr data for all three fusarins may be found in Tables 1, 2, and 3. Tlc on Si gel (Whatman LK6DF linear-K 250 μm, 5 × 20 cm channelled plates), solvent *i*PrOH-$CHCl_3$ (1:9): R_f fusarin A 0.56, R_f fusarin C 0.38, R_f fusarin F 0.30.

RESULTS AND DISCUSSION

CHARACTERIZATION.—Hplc analysis of EtOAc extracts of *F. moniliforme* cultures showed three main peaks with fusarin-type uv spectra. Nmr and ms analysis of the three peaks isolated showed that fusarin A [**2**] was eluted first from a CN column and last from a reversed-phase column while fusarin C [**1**] was the middle peak in both cases. The other peak was originally thought to be fusarin D [**3**]. However, its ^{13}C-nmr spectrum did not fit the structure proposed (9). Tlc analysis of the fusarins also showed that the new fusarin, fusarin F [**4**], was not fusarin B since its R_f on Si gel did not match the

TABLE 1. Eims Data for Compounds **1**, **2**, and **4**.

Compound		
1	**2**	**4**
431 $[M]^+$ (5%)	415 $[M]^+$ (6%)	431 $[M]^+$ (9%)
		399 (4%)
	368 (36%)	
314 (15%)		314 (15%)
245 (30%)	245 (38%)	245 (34%)
241 (35%)		241 (38%)
240 (36%)		240 (23%)
213 (82%)	213 (64%)	213 (83%)
	212 (53%)	
197 (56%)	197 (50%)	197 (40%)
185 (100%)	185 (100%)	185 (100%)

expected value determined from the work of Wiebe and Bjeldanes (8). Taking into account R_f's of 0.56 and 0.36 for **2** and **1**, respectively, fusarin B should have an R_f of 0.42 and **3**, 0.26. The R_f of **4** was 0.30.

Compound **4** was shown to be rather unstable. Purification of the fusarin fraction from an *F. moniliforme* extract by flash chromatography on Si gel caused a great reduction of the concentration of **4** as measured by hplc. It was also noticed that separation of the fusarin peaks on a reversed-phase column with an MeOH/H_2O solvent system (Figure 1) induced a rearrangement of **4** to **1** as verified by nmr and hplc. While **4** was found to be isomerized to **1** rather readily, small amounts of **4** were also found to be produced in samples of **1** upon standing. Considering the structures **4** and **3** of fusarins F and D, respectively, it is surprising that **4** rearranges so readily to **1** and not to the apparently more stable **3**. This could be explained by strong H-bonding of the 19-OH of **4** with

TABLE 2. ^{1}H-nmr Data for Compounds **1**, **2**, and **4** (in $CDCl_3$).

Proton	Compound		
	1	**2**	**4**
H-1	1.76 dd (7.3, 1.3)	1.77 dd (7.2, 1.3)	1.77 dd (7.2, 1.4)
H-2	6.97 q (7.3)	6.97 q (7.2)	6.96 q (7.2)
H-4	6.05 bs	6.06 bs	6.06 bs
H-6	6.25 bs	6.28 bs	6.27 bs
H-8	6.74 d (15)	6.83 d (15)	6.76 d (15)
H-9	6.61 dd (15, 11)	6.61 dd (15, 11)	6.62 dd (15, 11.5)
H-10	7.44 d (11)	7.44 d (11)	7.47 d (11.7)
H-13		4.37 s	
H-14	3.99 d (2.5)[a]	4.23 d (1.2)	4.11 d (2.4)
H-18	2.10 m	2.35 m	2.22 m
	2.10 m	2.23 m	2.09 m
H-19	4.10 m	4.08 m	3.99 m
	3.96 m	3.91 m	3.92 m
H-21	3.73 s	3.73 s	3.73 s
H-22	1.71 d (1.4)	1.72 d (1.4)	1.72 d (1.4)
H-23	2.06 s	2.07 s	2.07 s
H-24	1.99 s	1.96 s	1.97 s

[a]Coupling is to 16-NH proton as observed in COSY spectra and as reported by Gelderblom *et al.* (9).

TABLE 3. ^{13}C-nmr Data for Compounds **1**, **2**, and **4** (in $CDCl_3$).

Carbon	Compound		
	1	**2**	**4**
C-1	15.9	15.9	15.9
C-2	140.1	140.1	140.1
C-3	130.4	130.4	130.4
C-4	126.2	126.2	126.2
C-5	137.4	137.4	137.4
C-6	140.9	140.9	140.9
C-7	134.8	134.8	134.8
C-8	149.3	149.2	149.2
C-9	123.4	123.5	123.4
C-10	146.3	146.2	146.5
C-11	133.4	133.8	133.5
C-12	190.2	192.5	189.5
C-13	61.9	56.6	64.2
C-14	63.7	85.7	62.4
C-15	85.4	94.4	84.8
C-17	170.1	175.4	168.2
C-18	35.9	37.5	39.2
C-19	58.2	68.5	57.6
C-20	167.6	166.4	167.6
C-21	51.9	51.9	51.9
C-22	18.7	18.8	18.7
C-23	14.1	14.1	14.1
C-24	11.4	11.6	11.3

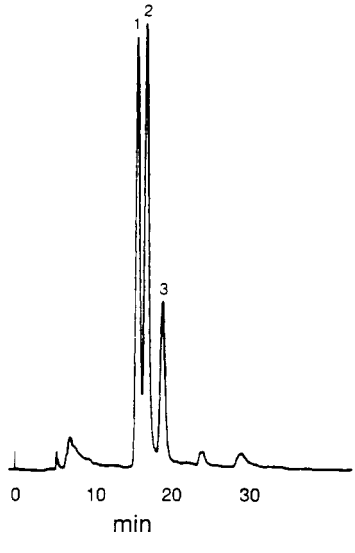

FIGURE 1. Hplc chromatogram of an EtOAc extract of *Fusarium moniliforme* B33A. Column: CSC ODS-2, 10 μm, 25 × 0.94 cm. Solvent: 70% MeOH, 30% H_2O. Detector: uv, 340 nm. Peak 1 fusarin E [**4**]; peak 2 fusarin C [**1**]; peak 3 fusarin A [**2**].

the 17-carbonyl and the 16-NH, thus slowing down its reaction with the epoxide in relation to the reaction between the 13-OH and the epoxide.

Separation of the fusarins from a crude *F. moniliforme* EtOAc extract on a CN column with 2.5% MeOH in CH_2Cl_2 as eluent prevented the rearrangement of **4** to **1** and allowed the isolation of pure **4**. Its ^1H- and ^{13}C-nmr spectral data are reported in Tables 2 and 3, respectively, along with those of the other two fusarins isolated. The data reported here were obtained in $CDCl_3$, while literature data are usually reported in CD_2Cl_2. Nmr data were also obtained in CD_2Cl_2 for more direct comparison with literature values.

Structure **4** was assigned to fusarin F based on the following analysis. Ms data (Table 1) showed that **4** had a mol wt of 431 amu, the same as that of fusarin C [**1**]. Hrms confirmed that **4** had the same molecular formula as **1**. Nmr data showed that **4** and **1** had very similar structures, the only differences occurring in the chemical shifts associated with the pyrrolidone ring and the ethanolic side chain. Little more information could be obtained from the ^1H spectrum of **4**. The resonance for H-14 had moved slightly downfield, while those for the H-19's had come closer to one another and those for the H-18's had moved apart.

The ^{13}C spectra were more informative. The C-13 of **4** had a chemical shift typical of an oxygen-bearing carbon, more likely as a hydroxyl group than as an epoxide since it was at a lower field than the C-13 of **1**. The carbonyl resonances for C-12 and C-17 of **4** were both upfield of those of **1**. This is due to the reduction in the polarization of the carbonyl groups by hydrogen-bonding to the hydroxyl group on C-13. The similarity of the chemical shifts of the C-14's suggested that this carbon still carried an epoxide. If this carbon was part of a five-membered ring as in **2** or **3**, one would expect its chemical shift to be 85–90 ppm, instead of 63.0 ppm, where it is found. If C-14 bore a free hydroxyl group, it would be expected at 70–80 ppm. Similarly, the chemical shift of C-19 would be much different if it was part of a ring. At 57.6 ppm, it is a lot closer to that of the open-form **1** at 58.2 ppm than to that of the closed **2** at 68.5 ppm. The same effect can be seen in the chemical shift of C-15, which is more characteristic of the strained epoxide form that brings chemical shifts upfield than of a five-membered ring as in either **2** or **3**. Analysis of the ^{13}C-nmr data for **3** (R. Vleggaar, personal communication) confirmed our conclusions, as the signals of the pyrrolidone ring carbons correlated well with the expected values and were very different from those of **4**. The spontaneous isomerization of **4** to **1** also suggested a close relationship between the two structures, as would be expected from isomeric α-hydroxy epoxides.

All seven strains of *F. moniliforme* studied produced similar ratios of the three fusarins but different overall amounts ranging from 7 to 225 mg/liter of fusarins. Our isolation of **4** and not **3**, while the South Africans found **3** and no **4**, could be due to the culture conditions, especially the substrate used (autoclaved corn vs. liquid culture). The lability of **4** may also have prevented its isolation.

PRODUCTION PROFILE.—Further evidence that **4** could be a precursor of **1** was obtained when the production of the three fusarins by strain B33A was studied by hplc over a period of 12 days (Figure 2). Fusarins were found in cultures of this strain 1 day after inoculation. Production generally increased until day 10, when their concentrations started declining. Strains MRC 826 and B33A produced similar proportions of the three fusarins (Table 4). In contrast, when MRC 826 was grown on autoclaved corn, fusarin A was the major compound produced (12). In liquid culture, MRC 826 produced fusarin A in the least amount of the three fusarins determined. All strains tested produced fusarins A, C, and F (Table 4). Fusarins have been reported from Canadian, US, and South African isolates of *F. moniliforme* (4,8,13). This is the first report of a

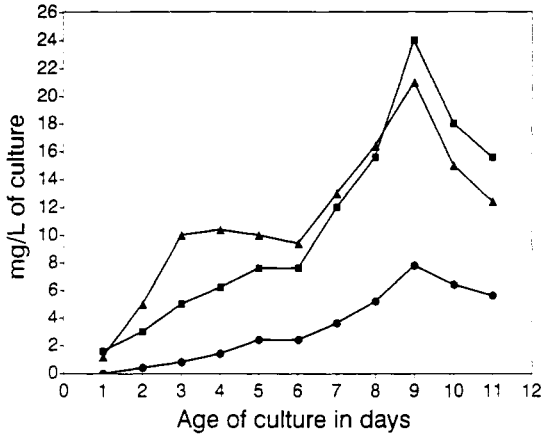

FIGURE 2. Concentrations of fusarins A [**2**], C [**1**], and F [**4**] in *Fusarium moniliforme* B33A cultures over a period of 12 days. ▲ fusarin F [**4**]; ■ fusarin C [**1**]; ● fusarin A [**2**].

fusarin-producing strain from Southeast Asia. Culture extracts of the Thai strain (DAOM 183571) had previously been shown to be toxic to mice (11). Under these conditions, the strain DAOM 212260 produced the most fusarin C and total fusarins. A previous study of fermentations of strain DAOM 212261 (M3783) in the same medium found a higher yield of fusarin C than reported here (13). This could have been due to the rearrangement of fusarin F to C upon workup.

TABLE 4. Distribution of Fusarins at the Time of Maximum Production in Liquid Culture.

Strain of *Fusarium moniliforme*	Source	Compound		
		1 (mg/liter)	**2** (mg/liter)	**4** (mg/liter)
MRC 826	South Africa	30	12	25
B33A	USA	24	8	21
DAOM 212260	Canada	75	30	120
DAOM 212259	Canada	66	24	90
DAOM 183571	Thailand	35	15	30
DAOM 195767	Canada	2.8	1.7	2.2
DAOM 212261 (M3783)	Canada	22	9	16

A plot of the ratios of the concentrations of fusarins A and F to that of fusarin C (Figure 3) shows the proportion of fusarin F increasing rapidly and then decreasing as the concentration of fusarin C is still increasing. While not all strains showed the same profile, they all showed a faster decrease of fusarin F concentration than of fusarin C. This is another indication that fusarin F is a precursor of fusarin C while fusarin A is a co-metabolite.

To summarize, fusarin F has been isolated from *F. moniliforme* cultures for the first time and characterized. Its production does not parallel that of any other fusarin, suggesting that it is not an artifact due to a rearrangement during the analytical process.

Fusarin F is distinct from fusarins A, B, and C by tlc. While its retention time is close to that of fusarin D, its nmr characteristics are different from those of fusarin D as well as those of fusarins A and C. Fusarin B has never been isolated.

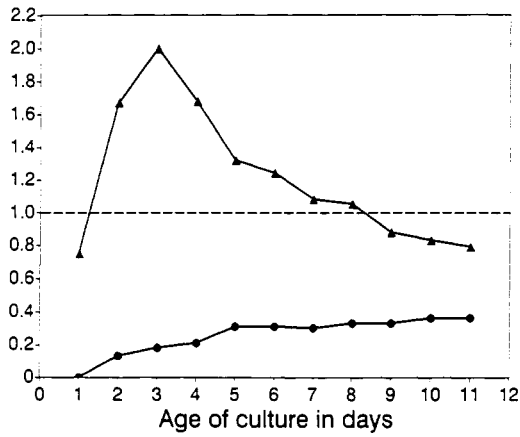

FIGURE 3. Ratios of the concentrations of fusarin F to fusarin C (▲) and fusarin A to fusarin C (●), produced by *Fusarium moniliforme* B33A over a period of 12 days.

Fusarin F appears to be less stable than fusarin C, as the equilibrium between the two structures favors fusarin C. The higher reactivity of the fusarin F epoxide could make it a more potent mutagen than fusarin C. The presence of fusarin F in crude samples of fusarin C may account for the higher mutagenicity of crude fusarin C compared to pure fusarin C as observed by Lu *et al.* (10).

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LITERATURE CITED

1. M.E. Savard, R. Greenhalgh, and J.W. ApSimon, in: "Studies in Natural Products Chemistry." Ed. by Atta-ur-Rahman, Elsevier, Amsterdam, 1990, Vol. 6, Part D, pp. 213–259.
2. C.J. Mirocha, K.H. Abbas, and R.F. Vesonder, *Appl. Environ. Microbiol.*, **56**, 520 (1990).
3. W.F.O. Marasas, T.S. Kellerman, W.C.A. Gelderblom, J.A.W. Coetzer, P.G. Thiel, and J.J. van der Lugt, *Onderstepoort J. Vet. Res.*, **55**, 197 (1988).
4. W.C.A. Gelderblom, K. Jaskiewicz, W.F.O. Marasas, P.G. Thiel, R.M. Horak, R. Vleggaar, and N.P.J. Kriek, *Appl. Environ. Microbiol.*, **54**, 1806 (1988).
5. W.C.A. Gelderblom, P.G. Thiel, K.J. van der Merwe, W.F.O. Marasas, and H.S.C. Spies, *Toxicon*, **21**, 467 (1983).
6. Z. Dong and Y. Zhang, *J. Exp. Clin. Cancer Res.*, **6**, 31 (1987).
7. J.M. Farber and P.M. Scott, in: "*Fusarium* Mycotoxins, Taxonomy and Pathogenicity." Ed. by J. Chelkowski, Elsevier, Amsterdam, 1989, pp. 41–52.
8. L.A. Wiebe and L.F. Bjeldanes, *J. Food Sci.*, **46**, 1424 (1981).
9. W.C.A. Gelderblom, W.F.O. Marasas, P.S. Steyn, P.G. Thiel, K.J. van der Merwe, P.H. van Rooyen, R. Vleggaar, and P.L. Wessels, *J. Chem. Soc., Chem. Commun.*, 122 (1984).
10. S.-J. Lu, Z.A. Ronai, M.H. Li, and A.M. Jeffrey, *Carcinogenesis*, **9**, 1523 (1988).
11. R. Greenhalgh, J.D. Miller, G.A. Neish, and H.B. Schiefer, *Appl. Environ. Microbiol.*, **50**, 550 (1985).
12. P.S. Steyn and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, 1189 (1985).
13. J.M. Farber and G.W. Sanders, *Appl. Environ. Microbiol.*, **51**, 381 (1986).

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NOTE ADDED IN PROOF: Another compound of different structure has recently been reported as fusarin E (D.R. Sanson, Ph.D. diss., University of Missouri). Although this has not yet been published in a reviewed journal, it was decided to refer to compound **4** as fusarin F to avoid confusion.