Fungal Metabolites Which Contain Substituted 7,8-Dihydrofuro-[2,3-*b*]furans (DHFF) and 2,3,7,8-Tetrahydrofuro[2,3-*b*]furans (THFF)

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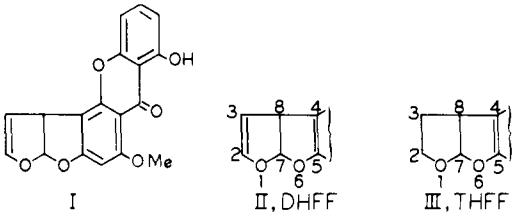
Since 1962 there have been reports of 16 mold metabolites which contain the unusual heterocyclic ring systems named in the title. These compounds are discussed with particular emphasis on the non-aflatoxin members of this group. Details of the NMR characteristics of the DHFF and THFF ring systems are elaborated, and it is suggested that NMR spectroscopy will provide the best clues to the possible presence of these structures in newly isolated compounds.

A new series of naturally-occurring oxygen heterocycles has been described in the chemical literature of the past six years. Since the structure of the first compound of this series, sterigmatocystin (I), was published (Bullock *et al.*, 1962) 15 more compounds, all mold metabolites, have been isolated and identified. The distinctive feature of this series is that all compounds contain either the unusual 7,8-dihydrofuro[2,3-*b*]furan (II, DHFF) or the more fully reduced 2,3,7,8-tetrahydrofuro[2,3-*b*]furan (III, THFF). The group of furofuran mold metabolites can be divided into three subgroups: the aflatoxin group in which a substituted coumarin is fused to the 4,5-ring positions of DHFF or THFF (Figure 1); the sterigmatocystin group in which a substituted xanthone is fused to the 4,5-ring positions of DHFF (Figure 2); and the group of mold pigments in which a substituted anthraquinone is fused to the 4,5-ring positions of DHFF or THFF (Figure 3). The fungal sources of these metabolites are listed in Table I.



A recent report (Wilson *et al.*, 1968) in which 121 fungal isolates representing 29 species were screened for aflatoxin production showed that only *Aspergillus flavus* and *Aspergillus parasiticus* produced aflatoxin. These authors specifically question the validity of earlier work (Hodges *et al.*, 1964; Kulik and Holaday, 1966; Scott *et al.*, 1967; Van Walbeek *et al.*, 1968) in which aflatoxins were reported to be produced by other species of *Aspergillus*, by some species of *Penicillia*, and an unknown species of *Rhizopus* (Table I). Their doubts about aflatoxin production by fungi other than *A. flavus* and *A. parasiticus* have been fortified by more negative findings (Mislivec *et al.*, 1968). Adding fuel to the controversy, a recent report describes the production of aflatoxin by a species of *Streptomyces* (Mishra and Murthy, 1968); this is the first report of aflatoxin production by a nonfungal species.

The first evidence of the extreme toxicity and carcinogenicity of aflatoxin B_1 stimulated a vast amount of research on the compounds of this group; however, the earlier chemical investigations on sterigmatocystin (Bullock *et al.*, 1962), now almost forgotten in the avalanche of papers on the aflatoxins, provided the key to structure for the whole group of naturally-occurring furofuran compounds.

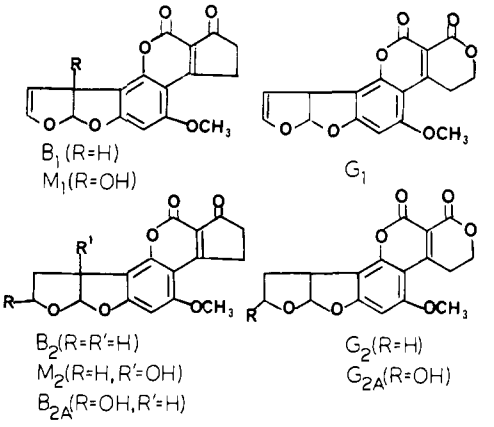


Figure 1. Aflatoxin group of DHFF and THFF compounds

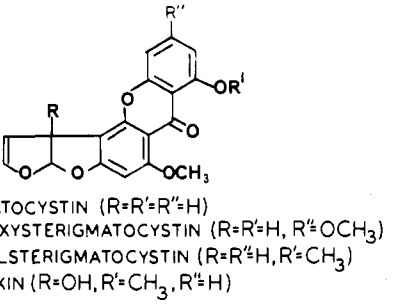


Figure 2. Sterigmatocystin group of DHFF compounds

It is becoming apparent that sterigmatocystin, and perhaps other compounds of the furofuran group beside the aflatoxins, are important toxic agents. The presently available evidence indicates that the presence of the THFF, or especially the DHFF moieties, have a great deal to do with the biological activities of the aflatoxins. We have prepared compounds of the type shown in Figure 4 (Rodricks, 1968a); these compounds contain the fused coumarin-cyclopentenone systems of aflatoxins B_1, B_2, M_1, M_2, and B_{2a}, but not the DHFF or THFF ring systems. In

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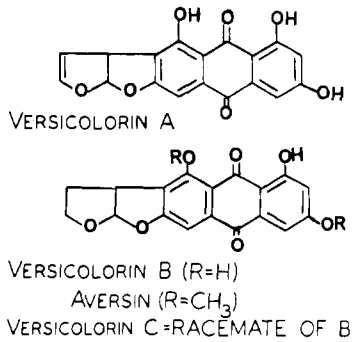


Figure 3. Anthraquinone group of DHFF and THFF compounds

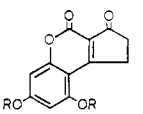


Figure 4. Aflatoxin analogs

either the chicken embryo or tissue culture systems in which aflatoxins B_1, B_2, and M_1 show severe toxic effects, the aflatoxin analogs which were prepared were completely inactive at levels 100 times those of aflatoxin B_1 (Rodricks, 1968a). Also, minor alterations in the furofuran portions of the various aflatoxins—e.g., hydration of the DHFF double bond in aflatoxin B_1—have a pronounced effect on the toxicity of these compounds (Pohland *et al.*, 1968; Schoental, 1967).

On the basis of this evidence it is reasonable to assume that other compounds which contain the DHFF or THFF ring systems could have biological activity similar to that of the aflatoxin group. This assumption has been demonstrated to be correct in the cases of sterigmatocystin and aspertoxin, although the data on these compounds do not begin to equal the mass accumulated on aflatoxin B_1.

Sterigmatocystin has been shown to be carcinogenic when administered subcutaneously to rats, and on the basis of a single experiment it was estimated that sterigmatocystin exhibits only about $^1/_{250}$ of the carcinogenic activity of an aflatoxin B_1-G_1 mixture (Dickens *et al.*, 1966). Some strains of *Aspergillus nidulans* and a *Bipolaris* species which were isolated from feedstuffs in South Africa proved to be very toxic to ducklings fed rations inoculated with these molds (Holzapfel *et al.*, 1966a). Sterigmatocystin was the major toxin isolated from these rations. The toxicity of sterigmatocystin to zebra fish larvae has been reported to be about equal to that of aflatoxin B_1 (Abedi, 1968).

The only other furofuran compounds on which toxicity data are available are aspertoxin and *O*-methylsterigmatocystin. The former compound has shown severe toxic effects on developing chick embryos (Rodricks *et al.*, 1968a) and has proved to be about $^1/_{15}$ as toxic as aflatoxin B_1 to zebra fish larvae (Abedi, 1968). The *A. flavus* metabolite, *O*-methylsterigmatocystin, has been reported to show very low toxicity to mice and ducklings (Burkhardt and Forgacs, 1968).

The toxicological study of compounds of the furofuran group has not been extensive other than in the case of the aflatoxins; the reports cited exhaust the literature references to toxicity studies on the nonaflatoxin compounds of this group. However, in the light of these reports, the nonaflatoxin furofurans warrant more attention.

A brief history of the chemical work on the xanthone (Figure 2) and anthraquinone (Figure 3) groups of furofuran compounds will be followed by a discussion of the means by which new furofuran compounds might be most easily identified, and it seems realistic to believe that new DHFF and THFF compounds will be found.

The studies of the metabolites of *Aspergillus versicolor* which resulted in the isolation of sterigmatocystin and related compounds (Figure 2) was not prompted by any recognition of a health problem, but rather as part of a systematic survey of fungal products. This is unlike the case of the aflatoxins which were isolated from feed implicated in animal mortality and related to the contamination of the feed with *A. flavus*.

Sterigmatocystin was first isolated in 1954 (Hatsuda and Kuyama, 1954) and the structure proof was completed eight years later (Bullock *et al.*, 1962), although preliminary reports (Birkinshaw and Hammady, 1957; Davies *et al.*, 1960) on the structure of this compound were published in the intervening years. A simple methoxylated derivative of sterigmatocystin, 6-methoxysterigmatocystin (Figure 2), has also been reported (Bullock *et al.*, 1963) as a metabolite of *A. versicolor*. The two other sterigmatocystin derivatives shown in Figure 2, *O*-methylsterigmatocystin (Burkhardt and Forgacs, 1968) and aspertoxin (Rodricks *et al.*, 1968), were isolated from aflatoxin-producing cultures of *A. flavus*. Aspertoxin, the most recent DHFF compound to be identified, was isolated and identified in our laboratory and concurrently and independently in the USDA Western Regional Laboratory (Rodricks *et al.*, 1968a, b; Waiss *et al.*, 1968). This latter compound carries an OH function on the DHFF ring system and is, in this respect, identical to aflatoxin M_1 (Figure 1). The isolation of these two sterigmatocystin relatives from cultures of *A. flavus* provides a considerable measure of support to the conjecture that the sterigmatocystins and the aflatoxins share a biogenetic pathway or that aflatoxin B_1 may be derived from a DHFF-xanthone related to sterigmatocystin (Holker

Table I. Reported Fungal Sources of Metabolites Containing the DHFF or THFF Ring Systems

Genus	Species	Reference
Aflatoxin Group (−FF Fused to a Substituted Coumarin)		
Aspergillus	*flavus*	Asao *et al.*, 1965
	ochraceus	Van Walbeek *et al.*, 1968
	parasiticus	Kulik and Holaday, 1966; Parrish *et al.*, 1966
	niger	Kulik and Holaday, 1966
	ruber	Kulik and Holaday, 1966
	ostianus	Scott *et al.*, 1967
Penicillium	*variable*	Kulik and Holaday, 1966
	citrinum	Kulik and Holaday, 1966
	frequentans	Kulik and Holaday, 1966
	puberulum	Hodges *et al.*, 1964; Kulik and Holaday, 1966
Rhizopus	sp.	VanWalbeek *et al.*, 1968
Streptomyces	sp.	Mishra and Murthy, 1968
Sterigmatocystin Group (−FF Fused to a Substituted Xanthone)		
Aspergillus	*flavus*	Burkhardt and Forgacs, 1968; Rodricks *et al.*, 1968a; Waiss *et al.*, 1968
	versicolor	Bullock *et al.*, 1962, 1963
Penicillium	*luteum*	Dean, 1963
Bipolaris	sp.	Holzapfel *et al.*, 1966a
Anthraquinone Group (−FF Fused to a Substituted Anthraquinone)		
Aspergillus	*versicolor*	Bullock *et al.*, 1963; Hamasaki *et al.*, 1965

and Underwood, 1964). Recent communications (Biollaz *et al.*, 1968; Donkersloot *et al.*, 1968) in which the biosynthesis of aflatoxin B_1 was examined with the aid of radioactive precursors suggest that aflatoxin B_1 may be derived from a precursor closely related to but not identical with the precursor of sterigmatocystin.

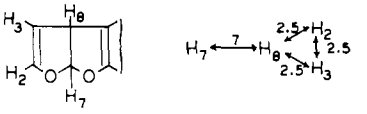
The third group of DHFF- and THFF-containing metabolites (Figure 3) are all substituted anthraquinones. They are all products of *A. versicolor*, and are all colored. A number of other metabolites of *A. versicolor* are anthraquinones, but do not possess a DHFF or THFF moiety. The three compounds known as versicolorins were identified by Japanese workers (Hamasaki *et al.*, 1965) who were engaged in studies of the metabolic products of molds. Aversin was identified by the same English group responsible for the work on sterigmatocystins, although the orientation of the THFF ring system on the anthraquinone ring was not definitely established (Bullock *et al.*, 1963). The structure shown in Figure 3 was proposed, along with a second structure in which the THFF ring system was fused to the 1,2-ring positions of the anthraquinone ($-OCH_3$ attached at C-3). The linear structure is shown in Figure 3, since it is the more likely one in view of the fact that the other compounds in this group have a linear furofuran-anthraquinone structure.

The task of identifying new DHFF and THFF compounds has been greatly simplified by the ground work laid during the chemical investigations of the 16 metabolites described. The early work on sterigmatocystin has proved to be crucial to the subsequent structure determinations. Because yields of sterigmatocystin from *A. versicolor* cultures are high—e.g., 1.3 grams per 100 grams of dried mycelium—extensive chemical degradation studies were possible. Until the recent degradation studies (Biollaz *et al.*, 1968) of radiolabeled aflatoxin B_1, this was the only thorough chemical degradation work which had been done on DHFF or THFF compounds, and provided convincing evidence for the DHFF and THFF ring systems. The total synthesis of aflatoxin B_1 (Büchi *et al.*, 1966, 1967) including the synthesis of the DHFF ring system of this compound has provided unequivocal proof of the furofuran ring structure.

NMR Spectroscopy of DHFF and THFF Compounds. Having unequivocal proof of the structure of this ring system, identification of new members of the furofuran group is simplified. The unique NMR spectral characteristics which can now be assigned to the DHFF and THFF portions of these compounds probably provide the best means for identifying new compounds of this group. As will be shown, the character of the NMR spectra is independent of the ring system to which the DHFF or THFF moiety is fused, and the values for the chemical shifts and coupling constants fall within a narrow range for each furofuran structure. These data are presented in Tables II, III, IV, and V; they have been gathered from the literature and from work in this laboratory. In almost every case the solvent was $CDCl_3$, although in three cases the low solubility of a compound forced use of DMSO-d_6 or pyridine-d_5. The solvent difference had little effect on the spectra.

There are major difficulties with an NMR spectroscopic technique for identifying compounds, the most serious of which are the requirements that the compounds to be investigated be of rather high purity and that substantial amounts be available for analysis. Unfortunately, these

Table II. NMR Spectra of DHFF Ring Systems



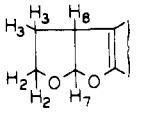
Numbers alongside arrows represent coupling constants (J's in Hz) (Asao *et al.*, 1965)

Range of Chemical Shifts (δ^a)	Proton	Multiplets (J's in Hz)
6.45–6.52	H_2	Triplet, 2.5
5.46–5.53	H_3	Triplet, 2.5
6.84–7.05	H_7	Doublet, *ca.* 7
4.81–4.86	H_8	Triplets of doublet, 2.5 and 7.0

Compounds Represented	Reference
Sterigmatocystin	Bullock *et al.*, 1962
O-Methylsterigmatocystin	Burkhardt and Forgacs, 1968
6-Methoxysterigmatocystin acetate	Bullock *et al.*, 1963
Versicolorin A, trimethyl ether	Hamasaki *et al.*, 1967
Aflatoxin B_1	Asao *et al.*, 1965

[a] Downfield from TMS.

Table III. NMR Spectra of THFF Ring Systems

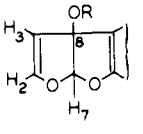


Range of Chemical Shifts (δ^a)	Proton	Multiplets (J's in Hz)
3.60–3.80	H_2	Complex
2.15–2.25	H_3	Complex
6.22–6.48	H_7	Doublet, 4.8–6.0
4.00–4.24	H_8	Complex

Compounds Represented	Reference
Dihydrosterigmatocystin	Knight *et al.*, 1965
O-Methylaversin	Bullock *et al.*, 1963
Dihydroversicolorin A	Hamasaki *et al.*, 1967
Aflatoxins B_2 and G_2	Hartley *et al.*, 1963
(±) Tetrahydro-4,6-dimethoxyfuro-[2,3,-*b*]benzofuran	Knight *et al.*, 1966

[a] Downfield from TMS.

Table IV. NMR Spectra of 8-Hydroxy and 8-Acetoxy DHFF Compounds

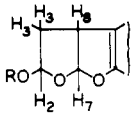


Range of Chemical Shifts (δ^a)	Proton	Multiplets (J's in Hz)
6.64–6.9	H_2	Doublet, 2–3
5.66–5.8	H_3	Doublet, 2–3
6.38–6.83	H_7	Singlet

Compounds Represented	Reference
Aflatoxin M_1	Holzapfel *et al.*, 1966b; Masri *et al.*, 1967
Aspertoxin	Waiss *et al.*, 1968
Aspertoxin acetate	Rodricks *et al.*, 1968b

[a] Downfield from TMS.

Table V. NMR Spectra of 2-Hydroxy and 2-Acetoxy THFF Compounds



	Range of Chemical Shifts (δ^a)	Proton	Multiplets (J's in Hz)
R=H	5.16–5.95	H_2	Complex
	2.13–2.50	H_3	Complex
	6.45–6.64	H_7	Doublet, 6
	4.05–4.22	H_5	Complex
R=CH$_3$C(=O)–	6.38–6.47	H_2[b]	Complex

Compounds Represented	Reference
Aflatoxin B_{2a}	Büchi *et al.*, 1967; Pohland *et al.*, 1968
Aflatoxin G_{2a}	Dutton and Heathcote, 1968
Aflatoxin B_{2a}, acetate	Büchi *et al.*, 1967; Dutton and Heathcote, 1968
Aflatoxin G_{2a}, acetate	Dutton and Heathcote, 1968

[a] Downfield from TMS.
[b] Only proton absorption significantly affected by acetylation.

requirements are usually difficult to meet for many natural products, and the furofuran compounds are, in general, no exception. Nevertheless, the NMR spectra of the furofurans still provide the best means yet available to demonstrate the presence of this unusual ring system.

The characteristics of the NMR spectrum of the DHFF ring system, which were first interpreted during the structural work on aflatoxin B_1 (Asao *et al.*, 1965), are given in Table II. The observed chemical shifts for the four protons fall into a very narrow range for the five DHFF compounds named. To explain the pattern observed it is necessary to assume that the coupling constants for the H_2-H_3, H_3-H_8, and H_2-H_8 protons are all about 2.5 Hz. This fortuitous circumstance greatly simplifies the NMR spectra of DHFF compounds, and since the constitution of the DHFF ring system has been definitely established by chemical means (and by synthesis in the case of aflatoxin B_1), there is little doubt about this interpretation of the spectra. The NMR spectral characteristics of THFF compounds are presented in Table III. The spectra are not nearly so simple as those of the DHFF compounds, although a pattern is still distinguishable. The bands observed for protons H_2, H_3, and H_8 were multiplets and were not analyzed further. Since the DHFF moiety can be easily converted by catalytic hydrogenation to the THFF ring system, the demonstration of a change in the NMR pattern from that shown in Table II to that in Table III will provide very convincing evidence for a DHFF system.

In Table IV are presented NMR data for the two compounds, one in the aflatoxin group and one in the sterigmatocystin group, which possess an OH function in the 8-position of the DHFF moiety. A third compound, aspertoxin acetate, prepared to circumvent solubility problems, carries an acetoxy group on the 8-position; these three can be classified together for NMR purposes. The general pattern here is simpler than the two previous ones mentioned. As with DHFF, the ranges of chemical shifts for the three protons are quite narrow, and the doublet-doublet-singlet pattern is very simple.

The NMR data presented in Table V are for 2-hydroxy THFF compounds and 2-acetoxy THFF compounds. The 2-hydroxy compounds are known as aflatoxins B_{2a} and G_{2a} (Figure 1). Aflatoxin B_{2a}, also known as aflatoxin B_1 hemiacetal, has been prepared by acid-catalyzed hydration of aflatoxin B_1 (Pohland *et al.*, 1968) and also by total synthesis (Büchi *et al.*, 1966, 1967). The NMR data from spectra of both the naturally-occurring and synthetic compounds are included in Table V. The narrow range of chemical shifts observed demonstrates the existence of a specific NMR pattern for this type of THFF compound. Note that acetylation of the OH group significantly affects the position of absorption of H_2 but not the other proton absorptions.

Finally, the complete spectra of two furofuran compounds are presented in Figures 5 and 6. The most simple DHFF spectrum (Figure 5), that of aspertoxin acetate, is one example. The signals for the three protons in the DHFF ring system of aspertoxin acetate at δ5.66 (doublet, J = 2 H), δ6.64 (doublet, J = 2 H), and δ6.83 (singlet) may be attributed to protons H_c, H_b, and H_a, respectively. The coupling between protons H_b and H_c can be demonstrated by double irradiation. The signal for the proton H_a, which appears at δ6.83, is superimposed on the complex multiplet which is attributable to the aromatic proton absorptions. This latter situation is encountered in the case of the xanthone and anthraquinone groups of furofurans, but the spectra are still interpretable.

The spectrum shown in Figure 6 is that of aflatoxin B_{2a} and is the most complex spectrum of the furofuran groups. The strong absorptions near δ7.0 are due to the aromatic protons of the solvent, pyridine. The absorption due to the proton H_2 appears as the multiplet at δ5.95. The very complex absorptions in the range δ2.0 to 3.0 represent the protons H_3 and also the group of four aliphatic protons on the cyclopentenone ring. This superposition of proton absorptions renders the spectrum very complex, but interpretation is still possible.

Although there have been no equivalent 2-hydroxy THFF compounds in the xanthone or anthraquinone groups, this superimposition of absorptions should not be a problem in the event of their isolation since there are no aliphatic protons present in these two groups of compounds to interfere with the absorptions from protons on the THFF ring system. The acetal proton H_7 absorbs at δ6.64 (doublet, J = 6 Hz) and can be easily discerned. The absorption due to H_3 is the complex multiplet centered at δ4.10. This is the most complex of the furofuran spectra, but some of the THFF ring protons do absorb in a fairly narrow and characteristic range. The complex absorptions exhibited by some of the ring protons in the THFF compounds are of little positive value in identification of

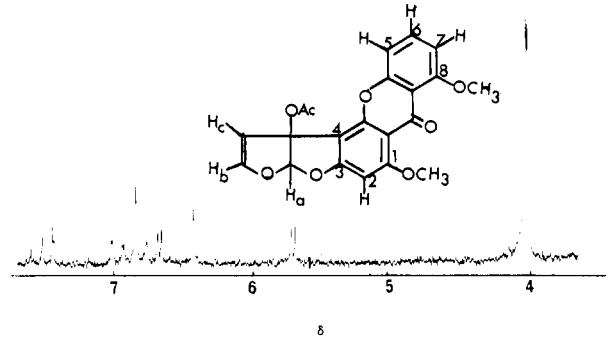


Figure 5. NMR spectrum of aspertoxin acetate (solvent = $CDCl_3$)

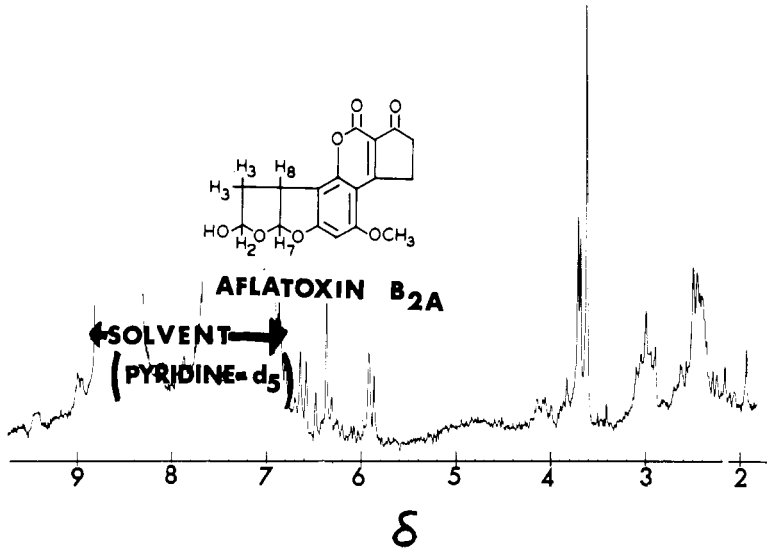


Figure 6. NMR spectrum of aflatoxin B_{2a}

these ring systems, and this is a major shortcoming of the NMR spectroscopic approach to the identification of furofuran compounds.

Although the NMR spectra of furofuran compounds can become as complex as the last one shown, the NMR patterns provide the best clue yet available for identifying these compounds. New compounds in the furofuran series should be most easily identified by their NMR spectra, although these spectra will be entirely different for furofuran compounds which have substitution patterns different from those mentioned above, or which are substituted by groups different from those mentioned above. A further limitation on the method is that it will fail to identify stereoisomers.

NOTE ADDED IN PROOF

Since this paper was first presented two new furofuran-containing mold metabolites have been reported. A mutant strain of *A. versicolor* has produced 5-methoxysterigmatocystin (Holker, J. S. E., Kagal, S. A., *Chem. Commun.* **(1969)**, p. 1574 and a strain of *A. parasiticus* has yielded a compound containing the furofuran system fused to a coumarin ring (Stubblefield, R. D., Shotwell, O. L., Shannon, G. M., Abstracts, 60th Annual Meeting, American Oil Chemists' Society, San Francisco, Calif., April 1969).

In addition to the above there has been a report on the carcinogenicity of sterigmatocystin in rats [Purchase, I. F. H., Van der Watt, J. J., *Food Cosmet. Toxicol.* **6,** 555 (1968)] and on the toxicity of sterigmatocystin toward mice and ducklings [Lillehoj, E. B., Ciegler, A., *Mycopathol. Mycolog. Appl.* **35,** 373 (1968)]. Methods for the analysis of sterigmatocystin have been published [Vorster, L. J., Purchase, I. F. H., *Analyst* **93,** 694 (1968); Vorster, L. J., *Analyst* **94,** 136 (1969)].

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