mushroom. The average value of HBA found in the mushroom samples analyzed was 10.7 ± 2.0 μg of HBA/g of mushroom. This value is surprisingly low if the sole or principal biosynthetic pathway leading to agaritine involved HBA. As mentioned earlier, agaritine has been found in these mushrooms in levels up to 0.07%. The level of agaritine in these mushrooms therefore is approximately 70-fold higher than that of HBA. It appears that either the biosynthesis of agaritine proceeds through alternate pathways not involving HBA or HBA is rapidly converted to other compounds involved in agaritine biosynthesis. HBA is presently under study for carcinogenic action in mice.

Registry No. HBA, 619-67-0.

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Paspalitrem C, a New Metabolite from Sclerotia of *Claviceps paspali*

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A new metabolite was purified from chloroform extracts of *Claviceps paspali* sclerotia by using column chromatography and preparative centrifugally accelerated, radial, thin-layer chromatography. The chemical structure of the metabolite was determined by ^1H and ^{13}C NMR spectroscopy to be 4-(3-methyl-2-butenyl)paspalinine (paspalitrem C). Paspalitrem C differed from the previously identified tremorgen, paspalitrem A, only by the position of attachment of the 3-methyl-2-butenyl unit to the indole ring.

Several tremorgenic as well as nontremorgenic, chemically related metabolites have been isolated from sclerotia of *Claviceps paspali* Stevens et Hall (Figure 1). This fungus infects *Paspalum* spp. and produces a staggers syndrome in cattle that graze the infected grass. The nontremorgens, paspaline (**1**) and paspalicine (**2**), were first reported from the dried mycelium of *C. paspali* (Fehr and Acklin, 1966), and their absolute stereochemical structures were subsequently determined by Springer and Clardy (1980). The tremorgens include paspalinine (**3**), 5-(3-methyl-2-butenyl)paspalinine (paspalitrem A) (**4**), and 5-(3-hydroxy-3-methyl-1-butenyl)paspalinine (paspalitrem B) (**5**) (Cole et al., 1977; Gallagher et al., 1980b). Another chemically related tremorgen, aflatrem (**6**), a metabolite of *Aspergillus flavus* (Wilson and Wilson, 1964; Gallagher et al., 1980a) and a logical distal product of *C. paspali* metabolism, has not been shown to be produced by *C. paspali*. In separate analyses of the mycelium-culture medium and sclerotia of several sclerotium-producing strains of *A. flavus*, Wicklow and Cole (1982) reported that aflatrem was detectable only in the sclerotia of *A. flavus*.

In the process of developing a high-performance liquid chromatographic system for the quantitation of these metabolites, a major unidentified component consistently appeared in *C. paspali* sclerotial extracts. We now report the purification and chemical identification of this previously unknown member of the paspalitrem group.

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EXPERIMENTAL SECTION

Extraction and Purification of Metabolites. Sclerotia of *C. paspali* were hand collected from infected *Paspalum* spp. in Georgia. Seven hundred grams of sclerotia was extracted 3 times with 2 L of chloroform per extraction by grinding with an Ultra Turrax homogenizer. The combined extracts were reduced in volume to an oily residue that was chromatographed on a silica gel column (4.5 cm i.d. × 40 cm) packed in benzene and eluted with a linear gradient of benzene to ethyl ether. One hundred eighty 17-mL fractions were collected, and every tenth fraction was subjected to thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Paspalitrem A (**4**), the unidentified metabolite, and related compounds (**1–3** and **5**) were contained in fractions 38–63. These fractions were combined, concentrated, and partitioned twice between 100 mL of hexane and 100 mL of 80% acetonitrile in water. The aqueous phase was reduced in volume and applied to a C_{18} reverse-phase column (3.5 cm i.d. × 27 cm) and eluted with a gradient of 50–90% acetonitrile in water. One hundred sixty fractions were collected and the following fractions were combined on the basis of TLC and HPLC analyses: 58–67, **5**; 79–90, **2**; 112–116, unknown metabolite; 117–120, unknown plus **4**; 121–124, **4**; 128–134, **1**.

High-performance liquid chromatographic analysis of the combined fractions 112–116 indicated the actual presence of two compounds eluting as a negatively skewed peak. A Model 7924 Chromatotron (Harrison Research,

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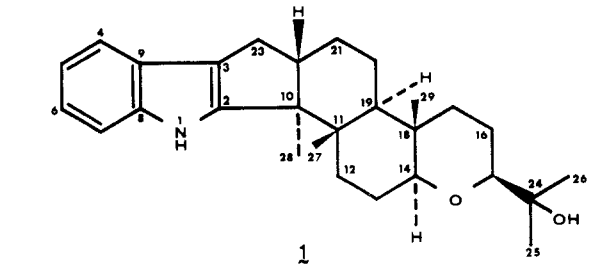
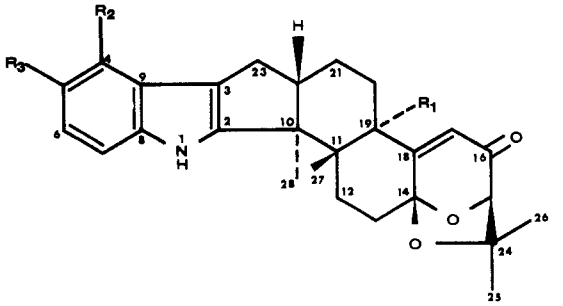


1



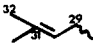
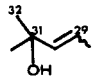
	R_1	R_2	R_3
2	H	H	H
3	OH	H	H
4	OH	H	
5	OH	H	
6	OH		H
7	OH		H

Figure 1. Structures of *C. paspali* tremorgens and related compounds.

Palo Alto, CA), which is a preparative centrifugally accelerated, radial, thin-layer chromatograph, was used for the final separation of these two metabolites. The rotor, or circular plate, was coated with a 1-mm layer of silica gel PF and eluted with hexane–ethyl acetate (8:2 v/v) at a flow rate of 4 mL/min. The sample was dissolved in 1 mL of ethyl acetate for application to the rotor. After the sample was applied, 25 mL of eluate was collected and then the Chromatotron was placed in the recycle mode for 7 min (28 mL). When taken out of the recycle mode, 4-mL fractions were collected and monitored by TLC and HPLC. Fractions 2 and 3 (collected after recycle) contained the major metabolite, and approximately 35 mg was subsequently precipitated from benzene–hexane solution. The minor component eluted in fractions 5 and 6.

Physical and Chemical Analyses. Thin-layer chromatographic analyses were performed on precoated silica gel 60 F-254 plates (5 by 10 cm; EM Laboratories, Inc., Elmsford, NY) developed in chloroform–acetone, 93:7 (v/v). Developed plates were sprayed first with 1% (*p*-dimethylamino)benzaldehyde in ethanol and then with 50% ethanolic sulfuric acid followed by heating for 2 min at 100 °C to visualize the metabolites.

High-performance liquid chromatographic (HPLC) analyses were carried out on a Waters Associates HPLC system consisting of an M6000A pump, a Model 720 system controller, a Model 730 data module, a Model 450 variable-wavelength UV detector at 231 nm, and a Z module with a 5 mm × 10 cm Radial-PAK C_{18} cartridge. A mobile phase of 67% acetonitrile in water and a variable flow rate from 2 to 4 mL/min gave separation of the paspalitrem metabolites in 15 min. Details of this procedure will be reported in a separate publication.

Ultraviolet (UV) spectra were run in methanol solution by using a Perkin-Elmer Model 552A spectrophotometer. Infrared (IR) spectra of samples prepared as KBr pellets were recorded with a Perkin-Elmer Model 1310 recording spectrophotometer. Low-resolution mass spectra were obtained with a Hewlett-Packard Model 5985 spectrometer. Samples were introduced by the direct probe method. Proton and ^{13}C NMR spectra were obtained on a Varian Associates XL-300 spectrometer at 300 and 75 MHz, respectively, in chloroform-*d* solutions. Carbon multiplicities were determined from the results of a DEPT (Doddrell et al., 1982) experiment. Homonuclear-correlated (Aue et al., 1976), homonuclear-*J* (Aue et al., 1976), and heteronuclear-correlated (Maudsley et al., 1977; Bodenhausen and Freeman, 1977) two-dimensional spectra were run to aid in the assignment of the spectra.

RESULTS AND DISCUSSION

Compounds 1–3 and 5 were identified in the eluate of the reverse-phase column by TLC and HPLC comparison with authentic standards. Paspalitrem A (4) and the unknown metabolite (7) could not be resolved by TLC, but HPLC analysis gave base-line resolution of the two compounds. The elution times of 7 and 4 were 7.4 and 8.5 min, respectively. A minor compound, which eluted in tubes 5 and 6 from the Chromatotron, had an HPLC retention time of 7 min, but an insufficient amount of this metabolite was available to elucidate its chemical structure.

The UV spectrum of 7 showed γ_{max}^{MEOH} 234 nm (log ϵ 4.55) and γ_{max}^{MeOH} 278 nm (log ϵ 4.02). The IR spectrum of 7 was essentially identical with that previously reported for 4 (Cole et al., 1977). In addition, the low-resolution mass spectral analysis of 7 showed a molecular ion at m/e 501, which was identical with that found for 4.

Compound 4 was identified as paspalitrem A by comparison of its NMR spectral properties with those previously reported (Cole et al., 1977). Several features of the NMR spectra of 7 were similar to those reported previously for 4 and for 6. The ^{13}C NMR spectrum of 7 exhibited 32 peaks similar to those of 4 and 6. Spectral editing techniques (Doddrell et al., 1982) showed that these peaks correspond to six methyl carbons, six methylene carbons, seven methine carbons, and thirteen quaternary carbons, which was also true for 4. Furthermore, it was clear from the ^{13}C NMR spectrum that 7 contained the basic paspalinine (Gallagher et al., 1980b) ring structure with an isoprene group attached to the six-membered ring of the indole portion of the molecule. The ^{1}H NMR spectrum of 7 clearly showed that there were three aromatic protons with coupling constants such that the three protons must be on adjacent carbons. Thus, 7 has the paspalinine ring system with an isoprene (3-methyl-2-butenyl) group attached at either carbon 4 or carbon 7. The presence of a methine peak at 109 ppm in the ^{13}C NMR spectrum of 7, characteristic of C-7 nonsubstituted indoles and similar to that observed in 6, confirmed that the indole ring is substituted on carbon 4 similar to that for 6. Thus, the structure of 7 was established as 4-(3-methyl-2-butenyl)-paspalinine for which we propose the trivial name paspalitrem C. NMR data for 7 are given in Table I.

It is interesting to note that in the biosynthesis of 4 and 7 by *C. paspali* sclerotia the 3-methyl-2-butenyl group occurs at both the 4- and 5-position of the indole ring of paspalinine. On the other hand, in the production of 6 by

Table I. ^{13}C NMR Data[a,b] for Paspalitrem C and Related Compounds

position	paspalitrem C	paspalitrem A	aflatrem
1	– (7.70, s)	–	–
2	151.2	151.2	151.8
3	116.7	116.7	115.1
4	131.8	117.6	140.6
5	118.9 (6.86, dd, J = 7.3, 1.5)	127.9	115.8
6	120.9 (7.01, dd, J = 7.3, 7.3)	120.9	118.9
7	109.3 (7.13, dd, J = 7.3, 1.5)	109.4	110.6
8	139.8	139.8	139.3
9	124.5	124.6	123.3
10	51.1	51.2	50.5
11	39.8	39.9	34.9
12	21.1 (1.81, m)	21.1	21.0
13	26.9 (2.0, m)	27.0	26.4
14	104.3	104.4	104.5
15	87.9 (4.31, s)	88.0	87.6
16	197.2	197.3	197.0
17	117.6 (5.82, s)	117.6	116.9
18	169.8	169.8	170.0
19	77.6	77.6	77.6
20	28.2 (2.73, 2.04, m)	28.3	28.2
21	31.9 (2.78, 2.04, m)	29.4	32.6
22	48.6 (2.86, m)	48.7	48.0
23	33.8 (1.91, m)	33.8	33.8
24	78.7	78.7	78.0
25	23.0 (1.18, s)	23.6	22.9
26	28.8 (1.44, s)	28.9	28.8
27	23.6 (1.24, s)	23.1	22.9
28	16.2 (1.38, s)	16.3	16.0
29	29.3 (3.62, d, J = 7.0)	32.0	111.1
30	123.6 (5.41, t, J = 7.0)	123.7	140.6
31	133.0	133.0	41.3
32	17.9 (1.75, s)	18.0	29.3
33	25.8 (1.76, s)	25.8	29.5

[a] Chemical shifts in ppm downfield from Me_4Si. [b] ^1H NMR data in parentheses; J in hertz.

A. flavus sclerotia, an α,α-dimethylallyl group is attached to the 4-position of paspalinine. No member of this group of metabolites has been reported from *A. flavus* with a functionality attached at the 5-position.

Although an insufficient quantity of 7 was obtained to determine its tremor-inducing potential through biological assays, the probability that it is tremorgenic is high based on its structural features. The compound is identical with the tremorgen paspalitrem A except for the positioning of the 3-methyl-2-butenyl group. As such, it has the key structural feature necessary for tremor induction, i.e., the tertiary hydroxyl group on carbon 19. Cole (1981) reported that all members of the paspalitrem group possessing that hydroxyl were tremorgenic, whereas those lacking it (1 and 2) were not. Therefore, paspalitrem C, in all likelihood, represents another in a growing list of fungal metabolites capable of inducing tremors in vertebrate animals and potentially involved in naturally occurring tremorgenic syndromes.

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Registry No. 1, 11024-56-9; 2, 11024-55-8; 3, 63722-91-8; 4, 63722-90-7; 5, 63764-58-9; 7, 90866-61-8.

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Rapid Extraction and Detection of Aflatoxins B_1 and M_1 in Beef Liver

Geng-Sun Qian[1] and George C. Yang*

A method for the determination of aflatoxins B_1 and M_1 in small samples (1 g) of beef liver, using a simple and rapid extraction and cleanup procedure, has been developed. C_{18} disposable columns are used in conjunction with normal-phase high-performance liquid chromatography with a packed-cell fluorescence detector. The method is particularly suitable for the analysis of small autopsy and biopsy specimens, which are not amenable to analysis by published methods due to sample size limitations. Recoveries from liver samples fortified at the 0.5-ppb level were 96% for aflatoxin B_1 and 58% for aflatoxin M_1.

Associations between the ingestion of aflatoxin B_1 (B_1), a fungal metabolite found in many agricultural products, and both acute and chronic toxicoses have been the subject of considerable research (Stoloff, 1977). More recently, a relationship between Reye's Syndrome and aflatoxin exposure has been implied (Ryan et al., 1979). The limited available sample for the extraction of B_1 and its major metabolite, aflatoxin M_1 (M_1), from animal tissues by using existing methods (Stubblefield and Shotwell, 1981; Trucksess et al., 1982) prompted the development of a

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