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J. Nat. Prod., **1992**, 55 (8), 1080-1086• DOI:
10.1021/np50086a008 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 55, No. 8, pp. 1080-1086, August 1992

AFLAVARIN AND β-AFLATREM: NEW ANTI-INSECTAN METABOLITES FROM THE SCLEROTIA OF *ASPERGILLUS FLAVUS*

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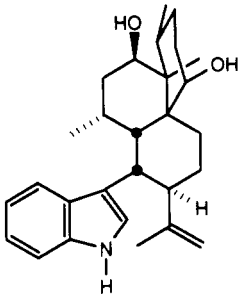
ABSTRACT.—Aflavarin [**3**], a new bicoumarin, and β-aflatrem [**11**], an isomer of the tremorgen aflatrem, were isolated from the sclerotia of *Aspergillus flavus*. The structures were determined through a series of 1D and 2D nmr experiments, assisted by spectral comparisons with known compounds. Aflavarin exhibits potent antifeedant activity against the fungivorous beetle *Carpophilus hemipterus*. β-Aflatrem causes a significant reduction in the growth rate of the corn earworm *Helicoverpa zea*. The presence of nominine [**13**] as a minor metabolite of *A. flavus* is reported for the first time.

Extracts of the sclerotia of *Aspergillus flavus* (Eurotiaceae) exhibit anti-insectan activity against the fungivorous beetle *Carpophilus hemipterus* (Nitidulidae), regardless of whether aflatoxins are present (1,2). We have previously reported the isolation of four aflavinine derivatives (e.g., **1**) (3) and a related metabolite, aflavazole [**2**] (4) from extracts of *A. flavus* sclerotia. All of these compounds deter feeding by *Car. hemipterus* and may be important in helping to protect the sclerotia from consumption or damage caused by fungivorous insects. Further studies of anti-insectan fractions from these extracts have provided another new, structurally unrelated, anti-insectan metabolite that we have called aflavarin [**3**]. Aflavarin is a bicoumarin related to the kotanins **4–6** (5,6) and the desertorins **7–9** (7), which have been isolated from *Aspergillus* and *Emericella* spp., respectively. The structure of aflavarin [**3**] was determined primarily on the basis of spectroscopic analysis and through spectral comparisons with data for related compounds. These studies also afforded a new isomer of the known tremorgen, aflatrem, which we have called β-aflatrem [**11**]. Details of the isolation, structure determination, and biological activity of these compounds are the subjects of this report.

Sclerotia of *A. flavus* (NRRL 6541) were produced by solid substrate fermentation on corn kernels (2). The CH_2Cl_2 extract of the sclerotia exhibited potent anti-insectan activity and was examined chemically. This extract was fractionated by reversed-phase flash chromatography followed by reversed-phase hplc to afford several indole diterpenoids described earlier (3,4), along with small quantities of three other unrelated components. Analysis of sclerotial extracts from several other strains of *A. flavus* revealed that these three other compounds were present at much higher levels in some cases (e.g., in NRRL 13462). Therefore, sclerotia of *A. flavus* NRRL 13462 were produced, extracted, and processed in the same manner to afford larger quantities of these three metabolites for chemical study. The most abundant of these was a new compound which we named aflavarin [**3**]. The other two metabolites were determined to be the known compounds kotanin [**4**] and demethylkotanin [**5**] through spectral comparisons with literature data (6,7). The molecular formula for **3** was established as $C_{24}H_{22}O_9$ (14 unsaturations) on the basis of hrfabms and ^{13}C-nmr data (Table 1). The presence of hydroxyl and carboxyl functionalities was indicated by ir and ^{13}C-nmr data. Analysis of ^{13}C-nmr and DEPT spectra indicated that aflavarin contained four MeO groups, one aromatic or vinylic Me group, one oxygenated methylene unit, eighteen sp^2-hybridized carbons, and only one proton not bound to carbon. Six of the ^{13}C signals appeared be-



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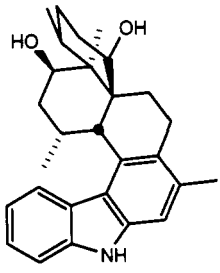

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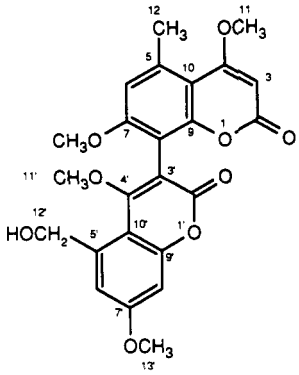

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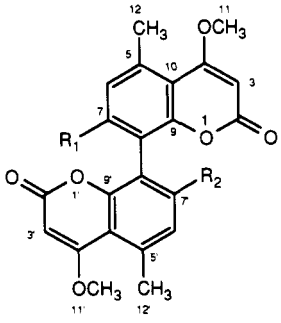
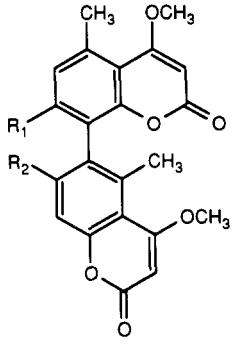


4 $R_1=R_2=OMe$
5 $R_1=OH$, $R_2=OMe$
6 $R_1=R_2=OH$



7 $R_1=R_2=OH$
8 $R_1=OH$, $R_2=OMe$
9 $R_1=R_2=OMe$

tween 159 and 170 ppm, implying that each was oxygenated in some way. The even number of sp^2-hybridized carbons implied that aflavarin must also contain an even number of carboxyl groups. The only way to rationalize these data and the large number of oxygenated sp^3 and sp^2 carbons with a total of only nine oxygen atoms was for aflavarin to contain two carboxyl groups and eight olefinic or aromatic double bonds. The remaining unsaturations must be accounted for by four rings. Acetylation of aflavarin induced a 0.55 ppm downfield chemical shift for the methylene proton AB pattern centered at 4.89 ppm, indicating the presence of a hydroxymethylene group in the natural product. Because of the absence of other exchangeable hydrogen atoms, both carboxyl groups in aflavarin must be ester functionalities.

The uv spectrum of aflavarin [224 (ϵ 31770), 236 (20300), 293 (20000), 309 (27500), 318 (27000)] was very similar to that of **4** [235 (ϵ 23400), 250 (12180), 290 (25100), 307 (28800), 316 (24300)]. The nmr data for kotanin [**4**] and those reported for the desertorins **7–9** (7) also bore a close resemblance to those of aflavarin. These similarities, in conjunction with the nmr spectral analysis above, strongly suggested that aflavarin is also a bicoumarin.

^1H- and ^{13}C-nmr data for aflavarin are presented in Table 1. One-bond ^1H-^{13}C connectivities were determined by analysis of single frequency heteronuclear decoupling experiments, and quaternary ^{13}C-nmr assignments were based on analysis of selective INEPT and COLOC data. The resulting assignments were supported by comparisons

TABLE 1. ^{1}H- and ^{13}C-nmr Data for Aflavarin [**3**].[a]

Position	^{1}H nmr	^{13}C nmr	Selective INEPT[b] Correlations	NOESY Correlations
2	—	161.43		
3	5.66 (s)	87.55	2[c],4,5[d],10	11
4	—	169.34		
5	—	139.14		
6	6.99 (br s)	111.33	3′[c],4[d],5[d],7,8,9[d],10,12	12,13
7	—	159.58		
8	—	107.75		
9	—	153.42		
10	—	107.70		
11	3.94 (s)	56.82	4	3
12	2.68 (br s)	23.55	4[d],5,6,8[d],9[d],10	6
13	3.84 (s)	56.40	7	6
2′	—	161.06		
3′	—	97.91		
4′	—	166.68		
5′	—	144.00		
6′	7.25 (d, 2.2)	110.64	4′[d],5′[c],7′,8′,9′[d],10′,12′	12′,13′
7′	—	162.22		
8′	6.90 (d, 2.2)	98.75	4′[d],5′[d],6′,7′,9′,10′	13′
9′	—	155.11		
10′	—	107.13		
11′	3.44 (s)	59.29	4′	12′
12′	4.84 (s)	61.47	5′,6′,7′[c],9′[c],10′	11′
13′	3.86 (s)	55.88	7′	6′,8′

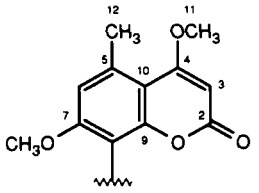
[a]Data were obtained in DMSO-d_6 at 360 and 90.7 MHz, respectively. Carbon multiplicities were determined by a DEPT experiment and are consistent with the assignments.

[b]Most of the 2- or 3-bond CH correlations were also observed in a COLOC experiment.

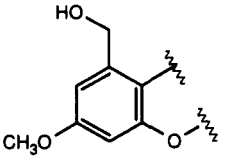
[c]These correlations were observed only in experiments optimized for 4 Hz or 2 Hz.

[d]These correlations were observed only in experiments optimized for 2 Hz.

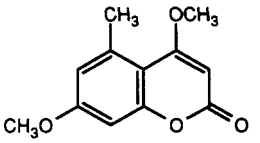
with relevant ^{13}C nmr assignments reported for desertorin C [**9**] in the same solvent (DMSO-d_6) (7). Firm evidence for the partial structures **a** and **b** was provided by the ^{1}H-nmr spectrum, homonuclear decoupling experiments, ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, selective INEPT, and COLOC data (Table 1). Placement of an MeO group at C-4 rather than C-3 in partial structure **a** was based on ^{13}C-nmr chemical shifts, a NOESY correlation between H_3-12 and H_3-11, and the observation of four-bond correlations of both H-6 and H_3-12 with C-4. Although couplings between protons and carbons separated by more than three bonds are typically small (ca. 0–1 Hz), it is not uncommon to observe such correlations in aromatic systems when the experiments are optimized for small J values (8). The observation of only one selective INEPT correlation between H-3 and a member of the aromatic ring (C-10) in experiments optimized for large J values (7, 10, or 12 Hz) is also consistent with this assignment. The partial structure **a** resembles the siderin-like moiety that has been proposed to dimerize biogenetically to form kotanin (9). Upon establishment of partial structure **a** as a coumaryl unit, and upon consideration of the atoms remaining, it became evident that partial structure **b** must also be part of a coumaryl moiety. Carbons 3′ and 4′ of this second coumaryl subunit must be linked to an MeO group and partial structure **a** to give one of two possible structures. Aflavarin was assigned structure **3** through analysis of additional long-range correlations observed in selective INEPT and NOESY experiments. Four-bond correlations of both H-6′ and H-8′ with the methoxylated carbon (C-4′) were observed. Furthermore, a NOESY interaction was observed between the C-



a



b



10

12′ methylene proton signal and the H_3-11′ MeO group signal. Thus, since the MeO group must be located at C-4′, the two subunits could be linked as shown in structure **3** by default. Positive confirming evidence for this linkage was provided by detection of a four-bond coupling between H-6 and C-3′ in a selective INEPT experiment optimized for a 2-Hz coupling. All other NOESY and selective INEPT data (Table 1) are consistent with the assignment of structure **3** for aflavarin. As expected for a compound of this type, aflavarin exhibited an optical rotation due to restricted rotation about the single bond linking the two coumaryl subunits. The numbering system shown for aflavarin is analogous to that employed for the desertorins **7–9** (7).

It was originally postulated that compounds **4–9** might be shikimate-derived. However, it has been shown in isotope labelling studies with *Aspergillus niger* and *Aspergillus variecolor* that both kotanin [**4**] and the presumed siderin precursor **10** (9) are formed via the polyketide pathway (10,11). Thus, it seems likely that aflavarin is also a polyketide metabolite of analogous origin, arising via dimerization of a siderin precursor with connectivity at a different site. Unlike the desertorins and kotanins, the linkage between the two coumaryl subunits of aflavarin involves one of the pyrone rings, and one of the sideryl subunit Me groups (C-12′) is oxygenated. To our knowledge, aflavarin is the first bicoumarin reported to be linked in a 3′,8 (or 3,8′) fashion.

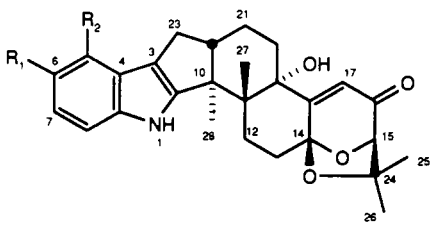
Aflavarin exhibits anti-insectan activity against both adults and larvae of *Car. hemipterus*, causing respective feeding reductions of 66% and 53% relative to controls when incorporated into a standard test diet at a concentration of 100 ppm (dry wt). Interestingly, demethylkotanin [**5**] exhibits no activity against *Car. hemipterus* at the same concentration in this assay. Although our studies of *Aspergillus* sclerotia have afforded a variety of compounds with activity against the corn earworm, *Helicoverpa zea*, aflavarin is the first non-aflavinine derivative we have isolated that shows significant activity against the more ecologically relevant fungivorous insect *Car. hemipterus*. However, it does not exert significant effects on *H. zea*. In other tests, aflavarin exhibits no antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus*, or *Candida albicans* in standard disk assays at 100 μg/disk. Aflavarin does show some cytotoxicity toward human solid tumor cell lines (12), affording ED_{50} values of 7.5, 55.0, and 5.8 μg/ml against non-small lung carcinoma A-549, breast adenocarcinoma MCF-7, and colon adenocarcinoma HT-29 cells, respectively.

Aflavarin is a major metabolite of the sclerotia of this strain of *A. flavus* (isolated yield 0.06% of the dry wt of the sclerotia, 4.2% of the CH_2Cl_2 extract). Considering its sclerotial concentration and the potency of its effects on *Car. hemipterus*, aflavarin could be significant in helping to defend sclerotia from feeding by fungivorous insects. Analytical hplc analysis of sclerotial extracts from eleven other strains of *A. flavus* and *Aspergillus parasiticus* showed that at least seven of them contained aflavarin. The presence of aflavarin as a trace constituent of the other four strains could not be readily ruled out due to the complexity of the extracts.

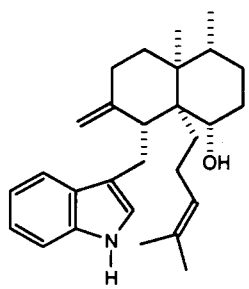
Studies of the hexane extract of this *A. flavus* isolate afforded a second new anti-in-

sectan metabolite. Fractionation by reversed-phase hplc afforded several known compounds, including aflatrem [**12**] (13), aflavinine (14), nominine [**13**] (15), paspalinine (16), and paspaline (17), along with small amounts of the aflavinine derivatives (e.g., **1**) and aflavazole [**2**] cited earlier. Of these, only nominine had not been previously reported as a metabolite of *A. flavus*. The most abundant component **11** of this mixture did not correspond with any known compounds, although its spectral data indicated a close relationship to aflatrem [**12**]. The compound was established as an isomer of aflatrem on the basis of hreims and ^{13}C-nmr data. Analysis of ^{1}H-nmr data and decoupling experiments suggested that **11** differed from aflatrem only in the location of the 1,1-dimethyl-2-propenyl substituent on the indole nucleus (i.e., at C-6 rather than C-5). Selective INEPT experiments were used to verify this conclusion and to assist in making carbon assignments. The key experiment involved irradiation of the aromatic proton doublet at 7.43 (J = 1.5 Hz), which resulted in strong polarization transfer to C-3 and to the quaternary carbon of the side chain (C-29), placing the proton at the 5 position of the indole moiety and locating the alkenyl substituent at the 6 position. All other data were consistent with these assignments. Compound **11** is even more closely related to paspalitrem C [**14**], a metabolite of *Claviceps paspali* (18) differing only in the arrangement of the side-chain, but because it was isolated from *A. flavus*, the name β-aflatrem is proposed for **11**.

^{1}H- and ^{13}C-nmr data for β-aflatrem [**11**] are presented in Table 2. The relative stereochemistry is proposed by analogy to aflatrem. Proton spin systems were determined by analysis of ^{1}H-^{1}H COSY data and homonuclear decoupling experiments. One-bond ^{1}H-^{13}C connectivity was determined by analysis of HMQC (19) data, and quaternary ^{13}C-nmr assignments were based upon ^{13}C-nmr δ values and selective INEPT results. The ^{13}C-nmr assignments for C-12 and C-13 in β-aflatrem are inverted relative to those reported for paspalitrem C [**14**] (18), but this is the only point of disagreement in these assignments.



11 R_1=$C(Me)_2CH{=}CH_2$, R_2=H
12 R_1=H, R_2=$C(Me)_2CH{=}CH_2$
14 R_1=$CH_2CH{=}CMe_2$, R_2=H



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β-Aflatrem is the first paspalinine-type metabolite from *A. flavus* to contain a substituent at the 6 position of the indole ring. β-Aflatrem exhibits significant activity against *H. zea* (20), causing a 57% reduction in weight gain relative to controls when incorporated into a standard diet at a concentration of 100 ppm. Compound **11** does not show activity against *Car. hemipterus* at this concentration.

Examination of sclerotial extracts from two strains of *A. parasiticus* (NRRL 6433, aflatoxigenic; and NRRL 13539, non-aflatoxigenic) and a strain of *Aspergillus subolivaceus* (NRRL 4998) indicated that β-aflatrem was present in each case, albeit as a minor constituent.

TABLE 2. ^{1}H- and ^{13}C-nmr Data for β-Aflatrem [**11**].[a]

Position	^{1}H nmr	^{13}C nmr	Position	^{1}H nmr	^{13}C nmr
1	7.73 (s)	—	18	—	169.84
2	—	152.45	19	—	77.61
3	—	117.22	20	2.73 (m), 2.83 (m)	28.25
4	—	124.94	21	2.45 (dd, 12.5, 10.3)	27.58
5	7.43 (d, 1.5)	115.32		2.73 (m)	
6	—	140.11	22	2.83 (m)	48.55
7	7.13 (dd, 1.5, 8.8)	119.54	23	1.92 (m), 1.97 (m)	33.82
8	7.27 (d, 8.8)	111.04	24	—	78.71
9	—	138.14	25	1.14 (s)	23.07
10	—	51.45	26	1.42 (s)	28.76
11	—	39.86	27	1.19 (s)	23.53
12	2.26 (m), 2.69 (m)	26.93	28	1.23 (s)	16.27
13	1.75 (m), 1.82 (m)	21.14	29	—	41.05
14	—	104.36	30	6.12 (dd, 10.5, 17.3)	149.01
15	4.32 (s)	87.96	31	5.05 (br d, 10.5)	109.87
16	—	197.28		5.09 (br d, 17.3)	
17	5.86 (s)	117.58	32/33	1.48 (br s)	28.83, 28.76

[a]Data were obtained in $CDCl_3$ at 360 and 90 MHz, respectively. Carbon assignments are based upon multiplicities, HMQC, and selective INEPT results.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Strains of *A. flavus* and *A. parasiticus* were obtained from the Agricultural Research Service (ARS) collection at the USDA National Center for Agricultural Utilization Research in Peoria, IL. Sclerotia were produced by solid substrate fermentation on autoclaved corn kernels using general procedures that have been previously described (2) and were stored at 4° until extraction. ^{1}H- and ^{13}C-nmr data were obtained on Bruker AC-300 and WM-360 spectrometers, and chemical shifts were recorded by using the corresponding solvent signals (e.g., 2.49 or 39.5 ppm for DMSO-d_6) as references. One-bond C-H correlations were established using single frequency decoupling or HMQC experiments. Long-range C-H correlations were obtained from COLOC data and/or selective INEPT experiments optimized for 2, 4, 7, 10, or 12 Hz. Details of other experimental procedures and insect bioassays have been described elsewhere (2–4, 20).

ISOLATION OF COMPOUNDS **3–5**.—Ground *A. flavus* sclerotia (NRRL 13462, 437.80 g) underwent Soxhlet extraction successively with pentane (450 ml for 48 h) and CH_2Cl_2 (450 ml for 72 h). The CH_2Cl_2 extract (6.42 g of 9.15 g total) was divided into 300-mg portions, and each portion was subjected to a rapid preliminary purification by chromatography on a reversed-phase flash column (1 × 10 cm; C_{18}; 40–63 μm particles) using a step gradient from 50 to 60% MeOH/H_2O in 5% increments. Compounds **3–5** were contained in fractions eluting at 50% and 55% MeOH. Separation of these fractions by semipreparative reversed-phase hplc [Beckman Ultrasphere 5-μ C_{18} column, 250 × 10 mm, MeOH-H_2O (70:30) at 2.0 ml/min] afforded aflavarin [**3**] (retention time 6.9 min, total isolated yield from 6.42 g of CH_2Cl_2 extract 271.0 mg), demethylkotanin [**5**] (9.3 min, 12.9 mg), and kotanin [**4**] (13.1 min, 5.8 mg). Aflavazole [**2**] and four aflavinine derivatives (including **1**) were also present in these flash column fractions; the isolation and properties of these compounds have been reported previously (3,4).

Aflavarin [**3**] was isolated as light brown needles with the following properties: mp 178–180° (dec); $[\alpha]_D$ −48.5° (c = 0.34 g/dl, MeOH); uv (MeOH) 224 (ε 31770), 236 (20300), 293 (20000), 309 (27500), 318 (27000); ^{1}H nmr ($CDCl_3$) 7.03 (1H, d, J = 2.2 Hz), 6.75 (1H, d, 2.2), 6.68 (1H, s), 5.53 (1H, s), 4.90 (1H, d, 12.6), 4.88 (1H, d, 12.6), 3.95 (3H, s), 3.86 (6H, s), 3.55 (3H, s), 2.70 (3H, s); ^{1}H nmr (DMSO-d_6), ^{13}C nmr, NOESY, and selective INEPT data see Table 1; ir (neat) 3611, 3035, 2981, 2944, 1708, 1602, 1456, 1363, 1330, 1258 cm^{-1}; eims (70 eV) $[M]^+$ 454 (rel. int. 41%), 437 (12), 420 (73), 407 (12), 394 (21), 368 (54), 353 (20), 337 (20), 313 (31), 264 (36), 251 (31), 236 (58), 212 (36), 182 (36), 162 (100), 129 (51), 109 (76); hrfabms found $[M+H]^+$ 455.1361, calcd for $C_{24}H_{22}O_9$ + H, 455.1342.

Samples of sclerotia produced by seven other *A. flavus* isolates (NRRL culture numbers 13461, 26638, 6556, 13892, 13048, 6541, and 27468) and four *A. parasiticus* isolates (NRRL culture numbe 6433, 13539, 13005, and 13006) were obtained by solid substrate fermentation on corn kernels. The

ples were individually powdered, extracted with CH_2Cl_2, and analyzed by reversed-phase hplc as reported elsewhere (2). Aflavarin was detected as a metabolite of four isolates of *A. flavus* (27468, 6541, 13461, and 13892) and three isolates of *A. parasiticus* (13539, 13005, and 13006).

ACETYLATION OF AFLAVARIN.—A sample of aflavarin (0.8 mg) was combined with 0.25 ml of pyridine and 0.10 ml of Ac_2O, and the solution was stirred at room temperature for 16 h. Distilled H_2O (0.25 ml) was added, and the solvents were evaporated to afford 0.9 mg of monoacetylaflavarin: ^{1}H nmr ($CDCl_3$) 6.93 (1H, d, 2.2), 6.78 (1H, d, 2.2), 6.68 (1H, s), 5.53 (1H, s), 5.46 (1H, d, 12.6), 5.42 (1H, d, 12.6), 3.95 (3H, s), 3.86 (6H, s), 3.53 (3H, s), 2.70 (3H, s), 2.15 (3H, s).

ISOLATION AND CHARACTERIZATION OF β-AFLATREM [**11**].—A sample of *A. flavus* NRRL 13462 sclerotia (52.1 g) was separately subjected to exhaustive extraction with hexane, and this extract (268.0 mg) was directly subjected to preparative reversed-phase hplc [8 μm C_{18} column, 2.14 × 25 cm, MeOH-H_2O (90:10) at 8.4 ml/min] to afford the following major components: paspalinine (Rt 17 min, 7.5 mg), aflatrem [**12**] (26 min, 7.8 mg), β-aflatrem [**11**] (32 min, 22.3 mg), nominine [**13**] (34 min, 2.2 mg), aflavinine (38.5 min, 12.2 mg), and paspaline (66 min, 1.4 mg). β-Aflatrem [**11**] was obtained as a yellow crystalline solid with the following properties: mp 188–190°; $[\alpha]_D$ +77.9° (c = 0.011 g/dl, $CHCl_3$); uv (MeOH) 233 (ε 18500), 264 (6300), 296 (2700); ^{1}H nmr and ^{13}C nmr see Table 2; selective INEPT results (^{1}H signal irradiated–^{13}C signals observed) H-5–C-3, -9, -29, H-7–C-5, -9, -29, H-8–C-4, -6, H-15–C-14, H-25–C-15, -24, -26, H-26–C-15, -24, H-27–C-12, -19, H-30–C-32, H-32/33–C-6, -29, -31, -32, -33; ir (CH_2Cl_2) 3572, 3468, 3060, 2971, 2937, 1690, 1614, 1455, 1275, 894 cm^{-1}; eims (70 eV) $[M]^+$ 501 (rel. int. 20), 4.86 (21), 483 (18), 468 (18), 443 (10), 428 (16), 425 (31), 410 (48), 387 (17), 372 (4), 344 (5), 250 (31), 237 (37), 222 (23), 198 (35), 182 (50), 168 (37), 130 (100); hreims found 501.2877, calcd for $C_{32}H_{39}NO_4$, 501.2881.

ACKNOWLEDGMENTS

This work was conducted under Cooperative Research Agreement No. 58-5114-M-010 between the USDA Agricultural Research Service and the University of Iowa. The University of Iowa nmr and ms Central Resource Facilities are gratefully acknowledged. We thank the Purdue University Cancer Center for cytotoxicity data. We also thank the National Science Foundation (CHE-8905894), Biotechnology Research and Development Corporation, and the Alfred P. Sloan Foundation for financial support.

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Received 23 December 1991