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THREE NEW AFLAVININES FROM THE SCLEROTIA OF ASPERGILLUS TUBINGENSIS

Mark R. TePaske and James B. Gloer*

Department of Chemistry, University of Iowa, Iowa City, Iowa, 52242

Donald T. Wicklow and Patrick F. Dowd

Agricultural Research Service, Northern Regional Research Center, United States Department of Agriculture, Peoria, IL, 61604

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Abstract: Three new metabolites with the aflavinine ring system (2-4) have been isolated from the sclerotia (key survival structures) of the fungus Aspergillus tubingensis (NRRL 4700), and their structures were assigned primarily through NMR studies. Carbon and proton NMR assignments for these compounds are presented, and the antiinsectan activity of one of the compounds (4) is described.

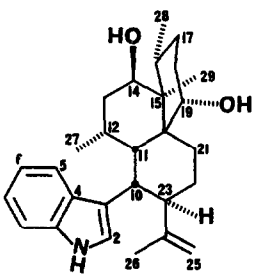
Many species of higher fungi produce specially-adapted propagules called sclerotia as a means of surviving harsh climates or nutrient-poor conditions.[1] The production of these durable resting bodies is an important part of the survival strategy of the producing organisms. Sclerotia lie dormant in soil, and survive for long periods during which they are exposed to large numbers of fungivorous insects. This observation led us to undertake chemical studies of sclerotia as potential sources of insect antifeedant or entomotoxic natural products. We have previously reported the isolation of four antiinsectan aflavinine derivatives (e.g., 1) from the sclerotia of Aspergillus flavus Link:Fr.[2-4] and a fifth, related insecticidal metabolite from the sclerotia of Aspergillus nomius Kurtzman, et al.[5] Our studies of the sclerotial metabolites of Aspergillus tubingensis (Schober) Mosseray (NRRL 4700), a member of the Aspergillus niger group,[6] have led to the isolation of three more new aflavinine derivatives. One of these compounds exhibits significant antiinsectan activity. We wish to report here the isolation, structure determination, and biological activity of these new metabolites, and to provide details of the NMR spectral characteristics of this class of compounds.

Results and Discussion

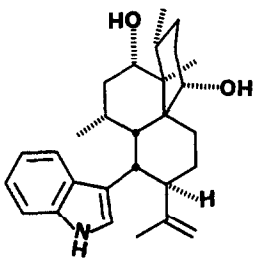
The hexane extract of the sclerotia of A. tubingensis exhibited potent activity against the fungivorous beetle Carpophilus hemipterus L. (Nitidulidae:coleoptera) and the widespread crop pest Heliothis zea (Brodie). The corresponding extract from A. flavus showed much weaker activity, suggesting the presence of different metabolites in the hexane extract of A. tubingensis. HPLC analysis of this extract indicated the presence of several indole

diterpenes not previously detected. Reversed-phase HPLC separation afforded three major components that showed close spectral resemblance to aflavinine derivatives isolated from the sclerotia of A. flavus.[3] The structures of the new metabolites were elucidated primarily by NMR decoupling and heteronuclear shift correlation experiments, and by comparison of their ^{1}H and ^{13}C NMR data with those of other aflavinine derivatives.[3,5]

Analysis of the most polar component by HREIMS indicated a molecular formula of $C_{28}H_{39}NO_2$ (M^+ 421.2962; Δ = -1.8 mmu). ^{1}H NMR, ^{13}C NMR, and mass spectral fragmentation data were virtually identical to those of 14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine (1) reported earlier,[3] with the exception of a significant downfield shift and absence of a large coupling constant for the signal corresponding to H-14. This change is consistent with the presence of an axial proton at position 14, rather than an equatorial proton, as in 1. A series of proton NMR decoupling experiments (Table I) verified the connectivities of the spin systems, and a heteronuclear shift correlation experiment provided carbon assignments for these spin systems (Table II). On the basis of these results, and by comparison of the data with those obtained for 1, the structure of this component was established as 14-epi-14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine (2).



1



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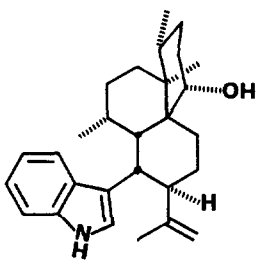

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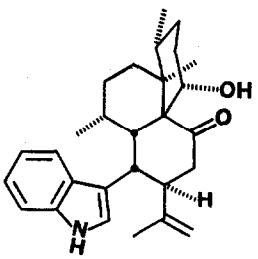

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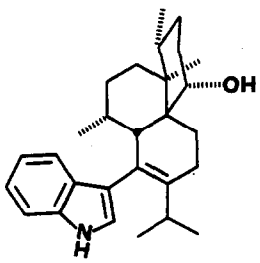

5

HREIMS analysis of the second new compound was consistent with the elemental composition $C_{28}H_{39}NO$ (M^+ 405.3044; Δ = 1.2 mmu). NMR data were very similar to those of 1 and 2, except for the absence of downfield-shifted proton and carbon signals associated with position 14, and the presence of an additional aliphatic triplet in the carbon NMR spectrum.

Based on this observation, and the presence of one less oxygen in the molecular formula, the structure of this metabolite was assigned as 10,23-dihydro-24,25-dehydroaflavinine (3). This structure is consistent with the results of homonuclear decoupling and selective INEPT[7] experiments, which permitted assignment of proton and carbon NMR signals (Tables I and II).

Table I. Proton NMR Data for Compounds 2-5*

#	2	3	4	5
1	8.04 (br s)	7.89 (br s)	8.00 (br s)	8.02 (br s)
2	7.80 (br s)	7.02 (d; 2.2)	7.03 (br s)	6.88 (d; 2.2)
3	--	--	--	--
4	--	--	--	--
5	7.52 (br d; 7.6)	7.52 (br d; 7.8)	7.55 (d; 7.8)	7.42 (br d; 7.8)
6	7.11 (dd; 7.6,6.8)	7.09 (dd; 6.8,7.8)	7.13 (dd; 7.8,6.8)	7.08 (dd; 7.1,7.8)
7	7.16 (dd; 8.1,6.8)	7.16 (dd; 6.8,8.1)	7.19 (dd; 7.6,6.8)	7.17 (dd; 7.1,8.1)
8	7.32 (d; 8.1)	7.32 (br d; 8.1)	7.36 (d; 7.6)	7.36 (br d; 8.1)
9	--	--	--	--
10	3.68 (dd; 5.9,12.9)	3.68 (dd; 5.1,12.9)	4.05 (dd; 5.4,13.2)	--
11	2.73 (dd; 5.9,5.8)	2.62 (m)	3.00 (dd; 5.4,4.6)	2.43 (br d; 5.1)
12	1.42 (m)	1.45 (m)	1.47 (m)	1.96 (m)
13	0.85 (m), 1.68 (m)	1.16 (m), 1.54 (m)	1.54 (m), 2.15 (m)	1.14 (m), 1.64 (m)
14	4.74 (br s)	0.86 (m), 1.63 (m)	0.93 (m), 1.21 (m)	1.18 (m), 1.55 (m)
15	--	--	--	--
16	2.18 (m)	2.18 (m)	2.08 (m)	2.03 (m)
17	1.19 (m), 1.68 (m)	1.36 (m), 1.75 (m)	1.73 (m), 1.96 (m)	1.23 (m), 1.69 (m)
18	1.38 (m), 1.78 (m)	1.86 (m), 2.21 (m)	1.41 (m), 2.32 (m)	1.75 (m), 2.06 (m)
19	4.73 (br s)	4.84 (br s)	5.19 (br s)	4.46 (br s)
20	--	--	--	--
21	1.74 (m), 2.15 (m)	1.88 (m), 2.04 (m)	--	1.73 (m), 1.85 (m)
22	1.62 (m), 2.31 (m)	1.75 (m), 1.84 (m)	2.42 (dd; 6.8,14.4) 3.08 (dd; 12.5,14.4)	2.10 (m), 2.22 (m)
23	3.38 (ddd, 4.6,13,13)	3.19 (m)	3.67 (ddd; 13,12.5,6.8)	--
24	--	--	--	2.57 (septet; 7)
25	4.77, 4.86 (br s)	4.64, 4.78 (br s)	4.68, 4.81 (br s)	0.82 (d; 7.1)
26	1.44 (s)	1.51 (s)	1.60 (br s)	0.96 (d; 6.8)
27	1.29 (d, 5.8)	1.24 (d; 7.1)	1.03 (d; 6.1)	1.08 (d; 7.1)
28	0.78 (d, 6.8)	0.77 (d; 6.8)	0.75 (d; 6.8)	0.75 (d; 6.8)
29	1.02 (s)	0.97 (s)	1.38 (s)	0.98 (s)

*Data recorded in $CDCl_3$ at 360 MHz.

Table II. Carbon-13 NMR Data for Compounds 2-5*

Position	2	3	4	5
1	--	--	--	--
2	123.4	122.8	122.5	121.2
3	115.9	116.7	114.9	118.6
4	127.5	127.4	127.2	127.6
5	118.3	118.4	118.0	119.6
6	119.0	119.0	119.4	118.6
7	121.6	121.6	122.0	121.8
8	111.1	111.1	112.4	111.0
9	135.7	136.0	136.1	135.9
10	34.8	34.6	34.0	125.5
11	38.0	43.6	43.9	43.7
12	29.3	29.8[b]	29.6	31.3
13	29.9	27.9[c]	29.6	25.7
14	71.7	25.3	28.3[d]	27.6
15	39.3	39.3	38.4	38.5
16	32.2	31.4[b]	31.3	29.1
17	28.5	28.2[c]	24.7	25.4
18	31.4[a]	28.9[c]	30.2[d]	21.8
19	68.8	69.0	72.7	71.1
20	46.9	44.0	58.1	42.4
21	24.9	24.6	214.2	30.1
22	30.9[a]	30.1[c]	44.9	20.5
23	39.5	38.7	47.3	141.0
24	150.0	150.3	146.8	31.0
25	111.4	111.0	111.4	21.9
26	18.1	18.4	18.2	20.8
27	18.1	21.9	18.0	18.1
28	15.9	15.8	14.7	15.7
29	17.9	18.4	18.1	18.1

*Data recorded in $CDCl_3$ at 90.7 MHz. All carbon multiplicities are in agreement with the assignments.

[a-d]Carbon assignments denoted by identical superscript letters are interchangeable

The third new metabolite has the molecular formula $C_{28}H_{37}NO_2$ (M+ 419.2839; Δ = 1.5 mmu), and contains a ketone group as indicated by IR and ^{13}C NMR data. This metabolite exhibited the same proton spin systems as compound 3, except that the $-CH_2CH_2CH-$ fragment corresponding to carbons 21-23 of structure 3 was shortened by one CH_2 unit, and the terminal CH_2 of the new system was shifted downfield. This information permitted location of the ketone group at C-14, and led to proposal of the structure as 10,23-dihydro-24,25-dehydro-21-oxo-aflavinine (4). Confirmation of this structure was provided by selective INEPT correlations, including an experiment that showed H-11 to be long-range coupled to the ketone carbonyl carbon. Compound 4 is the first reported member of the aflavinine class to be functionalized at position 21. The selective INEPT data afforded correlations accounting for most of the carbon signals of this molecule (Table II), and other carbon assignments were made by analogy to the data for 2.

The relative stereochemistry shown for compounds 2-4 was proposed by analogy to that of aflavinine (5), which was established prior to our studies by x-ray crystallography,[8] and these assignments are supported by NMR similarities. The relative stereochemistry shown for positions 10 and 23 in structures 2-4 is proposed on the basis of structural and NMR spectral analogy to compound 1.[3]

One of the new metabolites (4) exhibits activity against H. zea and C. hemipterus in controlled feeding trials. Compound 4 caused a 68% weight reduction in H. zea relative to controls after one week when incorporated into a standard test diet at 125 ppm. The same concentration also causes a 38% reduction in feeding rate by C. hemipterus. Despite their close structural relationship to 4, compounds 2 and 3 did not show antiinsectan effects in either assay when tested at the same level. Compounds 2 and 4 exhibit mild antibacterial activity, affording respective zone sizes of 15 and 19 mm in standard disk assays against Bacillus subtilis when tested at 100 μg/disk.

Comparative examination of the hexane extract of A. tubingensis with those of A. flavus (NRRL 6541) and Aspergillus parasiticus Speare (NRRL 13539) by HPLC showed that compounds 2 and 3 are present in the sclerotia of both A. flavus and A. parasiticus, albeit in smaller quantities than those found in A. tubingensis. During these studies, aflavinine itself (5) was isolated as the major hexane-soluble component of the sclerotia of both A. flavus and A. parasiticus, but it is inactive in our antiinsectan assays. Compound 5 had been encountered before only as a product of A. flavus,[8] and had not been reported as a sclerotial component. Surprisingly, neither aflavinine nor the aflavinine derivatives we reported earlier from A. flavus[3] were detected in the sclerotia of A. tubingensis. Complete NMR assignments for 5 were made by analysis of homonuclear decoupling and heteronuclear shift correlation experiments. These assignments are included here (Tables I and II) to permit spectral comparison to compounds 2-4. Prior to this report, carbon assignments for members of the aflavinine group had not been published.

Comparative assays of A. flavus sclerotia, mycelia, and conidia have previously shown that aflavinine derivatives found in the sclerotia are not found in the conidia or mycelia, and neither sclerotia nor aflavinines are formed in liquid cultures of A. flavus.[3] A. tubingensis is unusual because it is the only Aspergillus species we have encountered thus far that forms sclerotia in liquid culture. Interestingly, it is also the only species we have studied that forms significant quantities of aflavinine derivatives in liquid culture. This observation is consistent with the proposed correlation of aflavinine production with sclerotium formation.[2,3,5] Further studies of sclerotial metabolites produced by Aspergillus spp. and other fungi are underway in our laboratory.

Experimental

General. Cultures of A. tubingensis (NRRL 4700), A. flavus (NRRL 6541), and A. parasiticus (NRRL 13539) were obtained from the ARS culture collection at the USDA Northern Regional Research Center in Peoria, IL. Sclerotia were prepared by solid substrate fermentation on corn as described[2] and stored at 4°C until used. HPLC separations employed a Beckman model 332 binary gradient system with an Altex reversed-phase semipreparative column (5-μ C_{18} particles; 250 X 10 mm; UV detection at 215 nm; 2.0 mL/min; 90:10 MeOH-H_2O) unless otherwise stated. Retention times reported below were measured under these conditions. NMR experiments were performed on a Bruker WM-360 instrument, and chemical shifts were measured using the solvent signal as a reference ($CDCl_3/CHCl_3$; 7.24 ppm for ^1H NMR, 77.0 ppm for ^{13}C NMR). Carbon NMR multiplicities were established by DEPT experiments. Other general procedures, insect bioassays, NMR experiments, and instrumentation employed in this work have been described previously.[2,3,5,9]

Isolation and Characterization of Sclerotial Metabolites. Sclerotia produced by A. tubingensis (98.7 g) were ground to a powder with a mortar and pestle, and extracted with hexane (10 x 250 mL). Filtration and evaporation of the solvent afforded 474 mg of hexane extract as a yellow oil. Reversed-phase HPLC of the hexane extract as described above afforded pure compound 2 (86.7 mg), along with fractions containing compounds 3 and 4. Component 3 (7.3 mg) was isolated by HPLC on the same phase using a different solvent composition (85:15 MeOH-H_2O; 2.5 ml/min), and component 4 (30.4 mg) was isolated by further chromatography on a 10-μ Hamilton PRP-1 column (250 x 4.6 mm, 100% MeOH, 1.0 mL/min).

14-Epi-14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine (2) has: mp 79-82°C; $[\alpha]_D$ -5.6° (c 1.06, $CHCl_3$); HPLC retention time 10.6 min; UV (MeOH) 292 (ϵ 2100), 284 (2290), 226 nm (16400); ^1H NMR, Table I; ^{13}C NMR, Table II; EIMS (70 eV) 421 (M^+; rel. int. 40%), 388 (0.3), 334 (7.2), 306 (6.9), 243 (3.3), 226 (5.1), 196 (14), 182 (45), 168 (43), 159 (16), 146 (18), 130 (100), 117 (14); HREIMS, obs. 421.2962; calcd. for $C_{28}H_{39}NO_2$, 421.2980.

10,23-Dihydro-24,25-dehydroaflavinine (3) has: mp 192-194oC; $[\alpha]_D$ -1.2o (c 0.5, $CHCl_3$); HPLC retention time 18.3 min; UV (MeOH) 291 (ε 4100), 284 (4400), 226 nm (31600); ^1H NMR, Table I; ^{13}C NMR, Table II; EIMS (70 eV) 405 (M^+; rel. int. 22%), 390 (1.3), 372 (2.6), 330 (2.4), 236 (1.8), 210 (7.8), 196 (20), 184 (14), 182 (14), 168 (15), 156 (27), 143 (17), 130 (100), 115 (11); HREIMS, obs. 405.3044; calcd. for $C_{28}H_{39}NO$, 405.3032.

10,23-Dihydro-24,25-dehydro-21-oxo-aflavinine (4) has: mp 138-141°C ; $[\alpha]_D$ -13.5°; (c 0.72, $CHCl_3$); HPLC retention time 12.2 min; UV (MeOH) 291 (ε 1750), 283 (2100), 273 (1400), 267 (1200), 224 nm (18200); IR ($CHCl_3$) 3415, 2929, 2870, 1695, 1457; ^1H NMR, Table I; ^{13}C NMR, Table II; EIMS (30 eV) 419 (M^+; rel. int. 15%), 401 (3.7), 322 (2.9), 302 (2.7), 265 (3.8), 248 (3.7), 242 (5.3), 232 (5.2), 225 (7.9), 217 (5.2), 208 (4.8), 196 (28), 182 (82), 168 (60), 157 (70), 146 (27), 130 (100), 117 (17); HREIMS, obs. 419.2839; calcd. for $C_{28}H_{37}NO_2$, 419.2824.

Aflavinine (5) has: mp 103-105°C; $[\alpha]_D$ +24.9° (c 0.98, $CHCl_3$); HPLC retention time 25.4 min; UV (MeOH) 291 (ε 2710), 283 (3010), 225 nm (18700); ^1H NMR, Table I; ^{13}C NMR, Table II; EIMS (30 eV) 405 (M^+; rel. int. 100%), 387 (19), 372 (27), 362 (24), 344 (21), 330 (5.4), 304 (6.9), 276 (12), 262 (18), 246 (11), 234 (14), 220 (17), 211 (15), 194 (17), 168 (21), 130 (30), 117 (6.8).

Liquid culture of A. tubingensis. Small volumes (50 ml each) of two different media suitable for production of sclerotia on agar in petri dishes (1.5% corn steep liquor/0.5% glucose/0.2% yeast extract, and 2.0% glucose/0.5% yeast extract) were placed in 250-ml Erlenmeyer flasks, autoclaved, and inoculated with A. tubingensis. The cultures were aerated by agitation on an orbital shaker at 200 rpm for 21 days. The media were individually vacuum-filtered and extracted exhaustively with hexane. Evaporation of the solvent afforded an extract that was dissolved in MeOH and analyzed by reversed-phase HPLC. In each case, compounds 2-4 were present in virtually the same ratio as in the sclerotial extract. The culture grown on corn steep liquor produced more mycelial growth, more sclerotia, and more of the compounds than the culture grown in glucose/yeast extract medium. The concentrations of 2-4 in this medium ranged from 0.5 to 7 μg/ml, as approximated by HPLC integration and comparison of peak areas to those afforded by analysis of standard solutions of 2-4. All three metabolites were also present in the mycelium, which was extracted and analyzed after manual separation from the 18 mg of sclerotia that were formed. The mass of the mycelium could not be determined because of the presence of some residual corn solids, but the total amounts of 2-4 detected in the mycelium were approximately twice the total amounts found in the medium.

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References and Notes

1. Willets, H. J. Biol. Rev. Cambridge Philos. Soc. 1971, 46, 387.
2. Wicklow, D. T.; Dowd, P. F.; TePaske, M. R.; Gloer, J.B. Trans. Br. Mycol. Soc. 1988, 91, 433.
3. Gloer, J. B.; TePaske, M. R.; Sima, J. S.; Wicklow, D. T.; Dowd, P. F. J. Org. Chem. 1988, 53, 5457.
4. The numbering system shown for the aflavinine ring system has been revised here and in reference 5 to better illustrate its presumed biogenetic origin.
5. Gloer, J. B.; Rinderknecht, B. L.; Wicklow, D. T.; Dowd, P. F. J. Org. Chem., in press
6. Raper, K. B.; Fennell, D. I. The Genus Aspergillus. Williams and Wilkins: Baltimore, 1965, p. 314.
7. Bax, A. J. J. Mag. Res. 1984, 57, 314.
8. Gallagher, R. T.; McCabe, T.; Hirotsu, K.; Clardy, J.; Nicholson, J.; Wilson, B. J. Tetrahedron Lett. 1980, 21, 243.
9. Dowd, P. F. Entomol. Exp. Appl. 1988, 47, 69.