Bioactive Natural Products from a Sclerotium-Colonizing Isolate of *Humicola fuscoatra*

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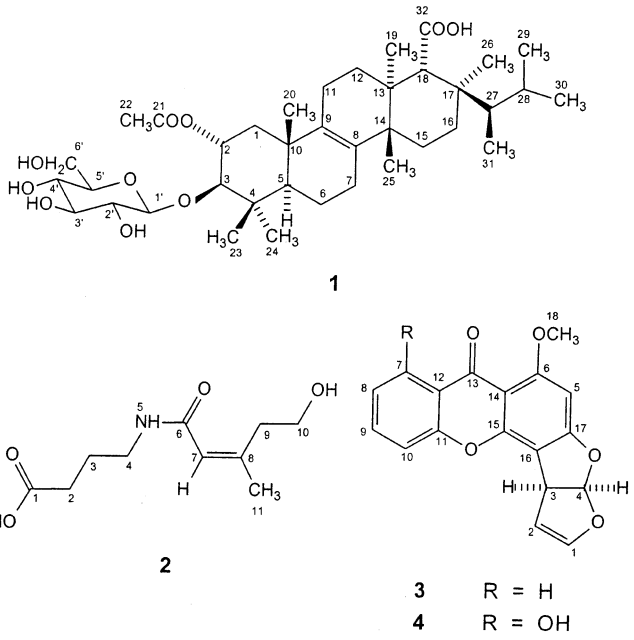
Chemical studies of an organic extract from solid-substrate fermentations of the mycoparasitic fungus *Humicola fuscoatra* NRRL 22980, originally isolated as a colonist of *Aspergillus flavus* sclerotia, afforded two new unrelated compounds that we have named fuscoatroside (**1**) and fuscoatramide (**2**). The structures of these metabolites were elucidated by analysis of NMR and MS data. Fuscoatroside (**1**) is a triterpenoid glycoside that exhibited activity in antifungal assays against *A. flavus*. This extract also contained the known metabolites 7-deoxysterigmatocystin, sterigmatocystin, isosclerone, and decarestrictines A_1 and I.

During long-term field studies of the viability of sclerotia produced by *Aspergillus* spp., we encountered a variety of mycoparasites that colonize these sclerotia, sometimes rendering them inviable. Mycoparasites are fungi that invade and colonize the hyphae or survival structures of other fungal species.[1] The EtOAc extract of solid-substrate fermentations of the mycoparasitic species *Humicola fuscoatra* (NRRL 22980; mitosporic fungi) was active against *Aspergillus flavus* in our bioassays, and this extract was examined as a potential source of anti-*Aspergillus* agents. Initial investigations led to the isolation of four known compounds (cerebrosides C and D, monorden, and monocillin IV) and a new monorden analogue.[1] Further studies of this extract have afforded several additional metabolites, including two new unrelated compounds, which we have named fuscoatroside (**1**) and fuscoatramide (**2**). Compound **1** shows significant activity against *A. flavus*. These studies also yielded the known metabolites 7-deoxysterigmatocystin (**3**), sterigmatocystin, isosclerone, and decarestrictines A_1 and I. This report focuses on the isolation and structure determination of the new compounds **1** and **2** and also includes spectral data for **3**, which have not been previously reported in the primary literature.



1

2

3 R = H

4 R = OH

The EtOAc extract of solid-substrate fermentation cultures of *H. fuscoatra* was fractionated by silica gel column chromatography. Additional silica gel chromatography or reversed-phase HPLC treatment of the resulting fractions afforded **1**−**3**, which were identified by analysis of NMR and MS data. The known fungal metabolites sterigmatocystin,[3] decarestrictines A_1 and I,[4,5] and isosclerone[6] were also isolated from the EtOAc extract and identified by comparison of their NMR data with literature values.

The molecular formula for fuscoatroside (**1**) was determined to be $C_{38}H_{62}O_{10}$ (eight unsaturations) by HRFABMS ($[M - H + 2Na]^+$ at *m*/*z* 723.4077). The FABMS spectrum contained an ion at *m*/*z* 516 consistent with the loss of a six-carbon sugar unit. The ^1H and ^{13}C NMR data (Table 1) and DEPT results indicated the presence of 10 methyl groups (including one acetate methyl), eight methylene units (one oxygenated), five aliphatic quaternary carbons, a tetrasubstituted olefin unit, two carboxy groups, an anomeric carbon, and 10 additional methine carbons, six of which are oxygenated. These spectral data were suggestive of a triterpenoid glycoside structure containing five exchangeable protons.

The COSY spectrum for **1** indicated the presence of six isolated proton spin systems corresponding to subunits C1−C3, C5−C7, C11−C12, and C15−C16, in addition to a 2,3-dimethylpropyl group (C31−C27−C28−C29/30) and a sugar subunit (C1′−C6′). Construction of the triterpenoid system, the placement of its substituents, and assignment of chemical shifts were accomplished in a straightforward fashion through the analysis of HMBC correlations (Table 1), particularly those associated with the numerous, relatively well-resolved methyl groups. These correlations alone enabled near completion of the aglycone structure. The only remaining connections not directly established by these COSY-derived spin systems and the methyl-group HMBC correlations were the linkages of C-7 to C-8, C-9 to C-11, and C-18 to C-32. These connections were demonstrated

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Table 1. NMR Data for Fuscoatroside (**1**) in DMSO-d_6

C#	δ_H (mult., J_{HH})	δ_C	HMBC (H→C#)	C#	δ_H (mult., J_{HH})	δ_C	HMBC (H→C#)
1	1.15 (m)	41.0	2, 3, 5, 10, 20	22	1.99 (s)	21.0	21
	1.90 (m)		2, 3, 5, 9, 10, 20				
2	4.96 (m)	69.3	3	23	0.83 (s)	17.4	3, 4, 5, 24
3	3.20 (d, 10.2)	87.8	2, 4, 23, 24, 1′	24	1.06 (s)	27.5	3, 4, 5, 23
4		40.5		25	0.93 (s)	21.7	8, 13, 14, 15
5	1.12 (m)	49.4	3, 10, 20	26	1.19 (s)	20.5	16, 17, 18, 27
6	1.36 (m)	18.9	5, 7, 10	27	1.15 (m)	47.6	16, 29, 31
	1.68 (m)		5, 7, 8, 10				
7	1.95 (m)	26.3	5, 8	28	2.15 (m)	25.9	27, 31
	2.12 (m)		6				
8		133.4		29	0.84 (d, 7.2)	18.2	27, 28, 30
9		133.6		30	0.79 (d, 6.6)	24.7	27, 28, 29
10		37.8		31	0.71 (d, 7.2)	8.0	17, 27, 28
11	1.11 (m)	18.2	12	32	11.8 (OH; br s)	175.2	
	1.96 (m)		12				
12	1.15 (m)	30.8	19	1′	4.18 (d, 7.8)	104.8	3
	1.55 (m)		9				
13		37.1		2′	2.90 (dt, 7.8, 5.4)	74.2	1′, 3′
14		40.0		3′	3.08 (m)	76.9	4′
15	1.36 (m)	25.1	14	4′	3.05 (m)	69.9	2′, 3′
	1.64 (m)		13				
16	1.20 (m)	28.8	26	5′	3.04 (m)	76.5	4′
	1.58 (m)		18				
17		39.8		6′	3.64 (br dd, 10, 5.4)	61.1	4′
					3.47 (dt, 10, 5.4)		5′
18	2.59 (s)	52.3	13, 14, 19, 26, 27, 32	2′-OH	4.99 (d, 5.4)		1′, 2′, 3′
19	1.01 (s)	16.4	12, 13, 14, 18	3′-OH	4.86 (d, 5.2)		2′, 3′, 4′
20	0.98 (s)	20.7	9, 10, 1, 5	4′-OH	4.81 (d, 5.4)		4′, 5′
21		170.0		6′-OH	4.24 (t, 5.4)		5′

by further HMBC data, including correlations of H-18 with the carboxyl carbon C-32, H-12b with olefin carbon C-9, and H-6b and H7a with the other olefin carbon (C-8). This assignment was supported by comparison to the related known compound WF 11605,[7] which differs from **1** in that it contains a ketone carbonyl at C-16 in place of the methylene unit. Antibiotic WF 11605 was originally reported from an unidentified fungal isolate as a specific antagonist of leukotriene B_4 and identified by X-ray crystallography of a derivative of the aglycone. The triterpenoid-derived tetracyclic skeleton found in **1** and in WF 11605 is unusual. To our knowledge, fuscoatroside is only the second fungal metabolite of this type to be reported and the first to be described from an identified source.

Acid hydrolysis of fuscoatroside (**1**) afforded a monosaccharide that was identified as D-glucose on the basis of its ^{1}H NMR and optical rotation data. The coupling constant (J = 7.8 Hz) between the anomeric proton H-1′ and the adjacent axial methine proton H-2′ in the spectrum of the intact glycoside **1** was indicative of a β-linkage. Location of the sugar substituent at C-3 of the triterpenoid skeleton was confirmed by an HMBC correlation between H-3 and anomeric carbon C-1′. Although no relevant HMBC correlation was observed for the acetate carbonyl carbon, this substituent was placed at the only remaining oxygenated site on the triterpenoid subunit (C-2) on the basis of chemical shift considerations and by comparison with the data for WF 11605. The relative stereochemistry of **1** was proposed as shown by analogy to that of WF 11605, and this assignment was fully consistent with coupling constant information and NOESY data obtained for **1**. In disk assays against *A. flavus*, fuscoatroside (**1**) afforded an 18 mm zone of inhibition at 200 μg/disk.

The molecular formula for the second new metabolite, fuscoatramide (**2**), was determined to be $C_{10}H_{17}O_4N$ (three unsaturations) by HRFABMS analysis [$(M + H)^+$ at m/z 216.1228]. The NMR data (Table 2) contained resonances for a vinyl methyl group, five methylene units (one oxygenated), two olefinic carbons (one protonated), a secondary amide unit, and one ester or acid carbon. These data accounted for all three unsaturations and revealed that fuscoatramide is acyclic. Three exchangeable protons were required to accommodate the molecular formula and DEPT data, thereby requiring the presence of alcohol and acid groups in addition to the amide unit.

Table 2. NMR Data for Fuscoatramide (**2**) in Acetone-d_6

position	δ_H (mult., J_{HH})	δ_C	selective INEPT correlations (H→C#)
1		174.3	
2	2.33 (t, 7.2)	31.6	1, 3, 4
3	1.78 (tt, 7.2, 7.2)	25.8	1, 2, 4
4	3.25 (m)	38.9	2, 3, 6
5	7.39 (br m)		
6		168.0	
7	5.82 (q, 1.2)	122.1	6, 9, 11
8		150.9	
9	2.72 (t, 6.0)	37.1	7, 8, 10, 11
10	3.66 (t, 6.0)	60.8	8, 9
11	1.85 (d, 1.2)	24.9	7, 8, 9

Homonuclear decoupling experiments allowed the assignment of isolated proton spin-systems corresponding to the fragments C-2−NH-5, C-7/C-8/C-11, and C-9−C-10. These fragments were linked together on the basis of selective INEPT experiments (Table 2). The connectivity of the oxygenated C-9−C-10 subunit to the methyl-substituted olefin at C-8 was established by correlations of methylene protons H_2-9 with C-7, C-8, and C-11. The chemical shift value for C-4 (δ 38.9), together with selective INEPT correlations of H_2-4 and H-7 with carbonyl carbon C-6, suggested that the secondary amide group linked positions C-4 and C-7. A correlation between H_2-3 and the remaining carbonyl carbon (C-1) indicated attachment of this carboxyl group to C-2. The double bond was assigned the *Z*-configuration by comparison of ^{13}C NMR chemical shifts of the allylic methylene carbon (C-9, δ 37.1) and methyl carbon (C-11, δ 24.9) to the shifts of analogous signals for 3,7-dimethyl-3-(*Z*)-6-octadien-5-one-1-*O*-β-D-glucoside.[8] Thus, fuscoatramide was assigned structure **2**. This

metabolite bears resemblance to fusarinine,[9] a known fungal metabolite possessing an *N*-hydroxyornithine unit in place of the γ-aminobutyryl moiety found in **2**.

The structure of 7-deoxysterigmatocystin (**3**) was determined by comparison of its ^{1}H NMR, ^{13}C NMR, IR, and MS data to those of the closely related known compound sterigmatocystin (**4**), which was also present. The mass spectrum of **3** showed a difference of 16 mass units relative to **4**, the IR spectrum lacked an OH absorption, and the ^{1}H NMR spectrum lacked a proton resonance at δ 13.7 that was assigned to an intramolecularly hydrogen-bonded phenolic OH proton in sterigmatocystin. In addition, the ^{13}C NMR spectrum indicated the presence of an extra sp^2 methine carbon (δ 127.0) in place of an oxygenated sp^2 carbon (δ 164.9) found in **4**, and the ^{1}H NMR spectrum revealed the presence of an ortho-disubstituted benzenoid ring in place of the 1,2,3-trisubstituted ring found in **4**. These data indicated that **3** lacked the 7-OH substituent present in sterigmatocystin. Compound **3** has been described previously in the patent literature from a *Chaetomium* sp. as insecticidal antibiotic PF 1093.[10] However, to our knowledge, neither this analogue nor its spectral data have been reported in the primary literature.

Fuscoatroside (**1**) and two known compounds reported earlier (monorden and monocillin IV)[1] appear to be responsible for the *A. flavus* activity of the crude extract. Decarestrictines A_1 and I were previously reported from an *Aspergillus* sp. as inhibitors of cholesterol biosynthesis.[4,5] Isolation of anti-*Aspergillus* agents from this colonist of *A. flavus* sclerotia provides support for the concept that fungi that parasitize sclerotia can serve as sources of agents with antifungal activity against the host.

Experimental Section

General Experimental Procedures. The ^{1}H NMR spectrum of **1**, as well as all HMQC, HMBC, COSY, and NOESY spectra, were recorded with a Bruker AMX-600 spectrometer. ^{1}H NMR spectra of **2** and **3**, and all ^{13}C NMR spectra, were recorded with a Bruker AC-300 spectrometer. HMQC and HMBC experiments were optimized for $^1J_{CH} = 152$ Hz and $^nJ_{CH} = 8$ Hz, respectively. Chemical shifts were assigned using solvent signals for DMSO-d_6 (δ_H 2.49, δ_C 39.5), acetone-d_6 (δ_H 2.04, δ_C 29.8), or $CDCl_3$ (δ_H 7.24, δ_C 77.0). Other general experimental details have been reported elsewhere.[1]

Fungal Material. *Humicola fuscoatra* Traaen was isolated by DTW from a fungal sclerotium of *Aspergillus flavus* Link: Fr. that had been buried in soil for three years (1989−1991) in a Georgia cornfield (Coastal Plains Research Station, Tifton, GA). A culture of this fungus has been deposited with the ARS Culture Collection, Peoria, IL, and assigned the accession number NRRL 22980. *H. fuscoatra* was grown on slants of potato dextrose agar (PDA) for 14 days (25 °C). A cell and spore suspension prepared from the PDA slants represented the inoculum. Fermentations were carried out in duplicate 3 L Fernbach flasks, each containing 200 g of rice. Distilled water (200 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in.^{2} for 30 min. After the flasks had cooled to room temperature, they were inoculated with 3.0 mL of the spore/cell suspension and incubated for 40 days at 25 °C.

Isolation of Metabolites. The fermented rice was mechanically fragmented and extracted repeatedly with EtOAc (3 × 500 mL). A portion of the EtOAc extract (7.0 g) was purified by silica gel vacuum liquid chromatography (VLC) using a 6 cm × 5 cm column. The material was eluted with a step gradient beginning with 10% hexane in CH_2Cl_2 (1500 mL), followed by 1, 2, 3, 4, 8, 15, and 30% MeOH in CH_2Cl_2 (2000, 1000, and 750 mL for 1, 2, and 3% MeOH in CH_2Cl_2, respectively, and 500 mL each for the remaining eluents). The fraction eluting with 15% MeOH−CH_2Cl_2 (367 mg) was further purified on a silica gel column (38 cm × 2.2 cm) using successive gradients of 1% MeOH in CH_2Cl_2 to 50% MeOH in CH_2Cl_2. The fraction eluting with 15% MeOH in CH_2Cl_2 (85 mg) was triturated with acetone, yielding 38 mg of insoluble material. A 30 mg portion of this material was purified by semipreparative reversed-phase HPLC (Hamilton 10 μm particle size PRP-1 column; 50→100% CH_3CN in 0.1% HCOOH−H_2O in 20 min) to afford fuscoatroside (**1**; 6.5 mg, t_R 24.4 min).

A 36 mg fraction that eluted with 12% MeOH in CH_2Cl_2 from the silica gel column was purified by semipreparative reversed-phase HPLC (Dynamax C_{18} column, 5 μm particle size, 60 Å pore size, 10 × 250 mm; 10→60% CH_3CN in 0.1% HCOOH−H_2O in 30 min, at 3.0 mL/min) to afford fuscoatramide (**2**; 2.2 mg, t_R 13.2 min).

The fractions that eluted with 1% MeOH in CH_2Cl_2 from the original VLC column were combined on the basis of their TLC behavior. The combined fractions (1.8 g) were further fractionated on a silica gel column (36 cm × 2.2 cm) using step gradients of 5−80% EtOAc in CH_2Cl_2, followed by 1−10% MeOH in CH_2Cl_2. A fraction eluting with 10% EtOAc (137 mg) consisted of crude sterigmatocystin (**4**). The fractions that eluted with 20 and 40% EtOAc in CH_2Cl_2 were combined (1.04 g, wet), and 70 mg of this material was purified by semipreparative reversed-phase HPLC (Dynamax column as above; 40 → 80% CH_3CN in 0.1% HCOOH in 20 min) to afford isosclerone (1 mg; t_R 7.2 min) and 7-deoxysterigmatocystin (**3**; 6.2 mg, t_R15.8 min).

A smaller sample of crude EtOAc extract (1.0 g) was subjected to silica gel VLC using a step gradient from CH_2Cl_2 to MeOH. The fractions eluting with 3−5% MeOH were combined (359 mg) and chromatographed on a column of Sephadex LH-20 eluting sequentially with 4:1 CH_2Cl_2−hexane, 4:1 CH_2Cl_2−acetone, and 3:2 CH_2Cl_2−acetone. The fraction eluting with 4:1 CH_2Cl_2−acetone was subjected to semipreparative reversed-phase HPLC (Dynamax column as above; 20 → 100% CH_3CN in 0.1% HCOOH in 20 min) to afford decarestrictine I (6.7 mg; t_R 9.5 min) and decarestrictine A_1 (7.6 mg, t_R 10.5 min).

Fuscoatroside (1): white amorphous solid; mp 93−95 °C; $[\alpha]_D$ −26° (*c* 0.3 mg/mL, MeOH); UV (MeOH) λ_{max} 213 (ε 1400); IR (CH_2Cl_2) ν_{max} 3437, 2991, 2941, 1658, 1640, 1315, 1067, 1022, 950 cm^{-1}; ^{1}H NMR, ^{13}C NMR, and HMBC data, see Table 1; relevant NOESY correlations (H↔H #) H-2↔H-2′, H_3-20, H_3-23; H-3↔H-5, H-1′, H_3-24; H-18↔H-28, H_3-25, H_3-30, H_3-31; H_3-19↔H-12a, H_3-26; H_3-20↔H-2, H_3-23; H_3-23↔H-2, H_3-20; H_3-24↔H-3; H_3-25↔H-18; H_3-26↔H_3-19, H_3-31; H-1′↔H-3, H-3′; HRFABMS [M − H + 2 Na]$^+$ obsd at *m*/*z* 723.4077, calcd for [$C_{38}H_{62}O_{10}$ − H + 2 Na], 723.4060.

Hydrolysis of Fuscoatroside (1). A sample of fuscoatroside (2.6 mg) was dissolved in 300 μL of DMSO and combined with 700 μL of 6 M HCl in a hydrolysis tube. The hydrolysis tube was sealed and heated at 100 °C for 24 h. The reaction mixture was then combined with 2 mL of H_2O, and the resulting solution was extracted with 2 × 2 mL of EtOAc. The organic extract was dried ($MgSO_4$) and evaporated to afford the corresponding aglycone (1.8 mg; 90% yield). Upon evaporation, the aqueous extract afforded D-glucose (0.6 mg; 95% yield), which was identified on the basis of its ^{1}H NMR and optical rotation data in comparison with an authentic standard.

Fuscoatramide (2): colorless oil; UV (MeOH) λ_{max} 233 (ε 3900); IR (CH_2Cl_2) ν_{max} 3442, 3440, 2937, 1726, 1663, 1631, 1530 cm^{-1}; ^{1}H NMR, ^{13}C NMR, and selective INEPT data, see Table 2; HRESIMS [M + H]$^+$ at *m*/*z* 216.1228, calcd for $C_{10}H_{17}O_4N$ + H, 216.1236.

7-Deoxysterigmatocystin (3): pale yellow solid; $[\alpha]_D$ −184° (*c* 0.1 mg/mL, acetone); UV (MeOH) λ_{max} 248 (ε 10000), 304 (ε 6400), 336 (ε 3500); IR (CH_2Cl_2) ν_{max} 3544, 2942, 1700, 1636, 1456, 1440, 1238 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.28 (1H, dd, 1.6, 7.9, H-7), δ 7.72 (1H, ddd, 1.8, 6.8, 8.8, H-9), δ 7.37 (1H, d, 8.5, H-10), δ 7.32 (1H, ddd, 0.9, 6.8, 8.2, H-8), δ 6.78 (1H, d, 7.2, H-4), δ 6.46 (1H, dd, 2.2, 2.5, H-1), δ 6.38 (1H, s, H-5), δ 5.41 (1H, dd, 2.4, 2.7, H-2), δ 4.75 (1H, ddd, 2.2, 2.5, 7.1, H-3), δ 3.95 (1H, s, H-18); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 175.1 (C-13), 163.6 (C-17), 163.3 (C-6), 154.6 (C-11),

154.2 (C-15), 145.3 (C-1), 127.0 (C-7), 124.1 (C-9), 123.1 (C-12), 113.3 (C-4), 113.1 (C-10), 107.6 (C-14), 106.4 (C-18), 102.7 (C-2), 90.3 (C-5), 56.7 (C-18), 48.0 (C-3); EIMS (70 eV) *m*/*z* 308 (M^+; rel int 78), 291 (14), 279 (100), 262 (12), 249 (31), 221 (17), 205 (12), 165 (14), 152 (17).

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