Sclerotiamide: A New Member of the Paraherquamide Class with Potent Antiinsectan Activity from the Sclerotia of *Aspergillus sclerotiorum*

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Sclerotiamide (**1**), a new fungal natural product related to the paraherquamides, was isolated by bioassay-guided fractionation of antiinsectan organic extracts from the sclerotia of *Aspergillus sclerotiorum* (NRRL 5167). The structure was determined primarily through ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC experiments. Sclerotiamide causes significant mortality and unusual physiological effects in dietary assays against the corn earworm *Helicoverpa zea*.

Our continuing interest in fungal sclerotia as sources of new antiinsectan metabolites[1−3] prompted us to investigate the chemistry of the sclerotia of *Aspergillus sclerotiorum* Huber (NRRL 5167). Bioassay-guided fractionation of a sclerotial extract of *A. sclerotiorum* afforded a new compound with potent antiinsectan activity. Details of the isolation, structure determination, and biological activity of this metabolite, sclerotiamide (**1**), are described here.

Sclerotiamide (**1**) has the molecular formula $C_{26}H_{29}N_3O_5$ (14 degrees of unsaturation), as deduced from ^{13}C-NMR (Table 1), DEPT, and HRFABMS data [$(M + H)^+$ at 464.2185, Δ 0.0 mmu]. The ^{1}H-NMR, ^{13}C-NMR, and DEPT spectra indicated the presence of four methyl groups, four methylene units, three exchangeable protons, four protonated sp^2 carbons, five quaternary sp^3 carbons, and two sp^3 methine units (one oxygenated). These data, together with a COSY experiment, defined isolated $CH_2CH_2CH_2N$ and $CHCH_2$ subunits, as well as an oxygen-substituted 1,2,3,4-tetrasubstituted benzene ring and a 1,2-disubstituted olefin. Two of the exchangeable proton signals were attributed to amide NH groups (broad singlets at 7.16 ppm and 9.69 ppm), and the third signal, a broad singlet at 4.68 ppm, was suggestive of an OH group. Resonances at 169.8, 173.7, and 179.4 ppm in the ^{13}C-NMR spectrum indicated the presence of three carboxyl carbons, at least two of which must be amide carbonyl groups.

Further evaluation of these spectra, along with HMQC and HMBC results (Table 1), revealed that sclerotiamide (**1**) is closely related to the paraherquamide group of fungal metabolites. Analysis of these data led to recognition of a nitrogen-substituted dimethylbenzopyran subunit, as is found in the known compounds marcfortine C[4] and paraherquamides F and G.[5] Although unambiguous NMR assignments for these compounds have not been reported, the relevant ^{1}H- and ^{13}C-NMR data for this subunit of **1** are consistent with data published for marcfortine C in $CDCl_3$, despite the minimal solubility of **1** in this solvent. Because this subunit accounted for the remaining oxygen atom, all three carboxyl carbons must be present as amide groups.

Table 1. NMR Data for Sclerotiamide (**1**)

position	δ_H (multiplicity, J_{HH})	δ_c	HMBC correlations (C-#)
1	9.69 (s)		
2		179.4	
3		70.1	
4	7.02 (d, 8.2)	126.8	C-3, 6, 7, 8
5	6.43 (d, 8.2)	109.7	C-6, 7, 9
6		153.8	
7		105.7	
8		139.8	
9		122.2	
10	5.38 (br s)	73.9	C-2, 3, 9, 11, 12, 19
10-OH	4.68 (br s)		
11		66.7	
12		169.8	
13			
14	3.45 (ddd, 13, 7.2, 6.6)	44.2	C-12, 15, 16, 17
	3.49 (ddd, 13, 7.2, 5.2)		
15	1.91 (m)	25.4	C-14, 16, 17
	2.06 (m)		
16	1.87 (m)	29.7[a]	C-14, 15, 17, 18, 20
	2.66 (ddd, 13, 6.9, 5.2)		
17		69.8	
18	1.82 (dd, 13, 9.0)	31.1	C-16, 17, 19, 20, 22
	2.03 (m)		
19	3.81 (br t, 9.4)	56.2	C-11, 12, 18, 22, 23, 24
20		173.7	
21	7.16 (s)		C-11, 17
22		44.5	
23	0.79 (s)	23.4	C-3, 19, 22, 24
24	0.87 (s)	19.8	C-3, 19, 22, 23
25	6.66 (d, 9.9)	117.5	C-6, 7, 8, 27
26	5.76 (d, 9.9)	131.2	C-7, 27, 28, 29
27		76.6	
28	1.41 (s)	28.0	C-26, 27, 29
29	1.41 (s)	28.1	C-26, 27, 28

[a] This signal was obscured by the solvent signal in the ^{13}C-NMR spectrum and was assigned based on HMQC and HMBC data.

Additional HMBC correlations confirmed the fusion of a pyrrolidone ring to the benzopyran subunit. The oxygenated methine proton at 5.38 ppm (H-10) and the aryl proton H-4 (7.02 ppm) each correlated to the quaternary carbon C-3 (70.1 ppm). The remaining aryl proton (H-5; 6.43 ppm) and H-10 both showed correlations to C-9 (122.2 ppm), indicating that C-3 is attached to both C-9 and C-10. The signal for H-10 also showed correlations to C-11 (66.7 ppm), C-19 (56.2 ppm), and to two amide carbonyl carbons (C-2 and C-12). Because

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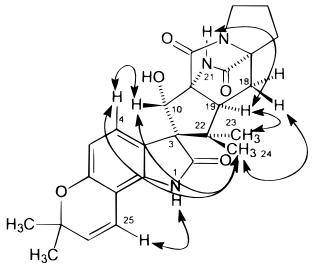


Figure 1. Key NOESY Interactions For Sclerotiamide (**1**).

protons elsewhere in the molecule showed correlations to C-12 (169.8 ppm) and to the third amide carbonyl (C-20; 173.7 ppm), but not to the amide carbonyl at 179.4 ppm, this carbonyl carbon (C-2) must be linked to the aryl nitrogen N-1 to complete the pyrrolidone ring. The downfield ^{13}C-NMR chemical shift observed for C-3 (70.1 ppm) is consistent with substituent effects and corresponding shifts in other paraherquamide-type structures.[4,5]

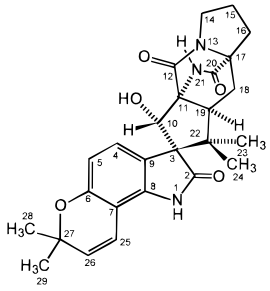
The bridged tetracyclic portion (C-10 through C-24) of **1** was established based on the results of 1D-NMR, COSY, and HMBC experiments. Although there were some similarities between the ^{1}H- and ^{13}C-NMR data for this subunit of **1** and the published assignments for the corresponding portion of the known compound paraherquamide (**2**),[5] significant shift differences at many positions required independent analysis of the data to establish unambiguously the remainder of the structure.

The signals for NH-21 and the methine proton H-19 of the $CHCH_2$ unit (3.81 ppm) each showed HMBC correlations to C-11. Because H-21 is an amide NH, and H-10 is not coupled to either H-19 or NH-21, the quaternary carbon C-11 must be linked to C-10 to permit these correlations. Based on these results and on data presented above, N-21, C-12, and C-19 must also be linked to C-11. Correlations of H_3-23 and H_3-24 with C-3, C-22, and C-19 permitted connection of C-22 to both C-3 and C-19 and established the presence of a cyclopentanoid ring.

Correlations of the $CHCH_2$-unit methylene protons (H_2-18) with the terminal CH_2 of the $CHCH_2CH_2N$ unit (C-16; 29.7 ppm) and the N-substituted quaternary carbon C-17 (69.8 ppm), together with a correlation observed between the NH-21 amide proton and C-17, required linkage of both C-16 and the amide carbonyl C-20 to C-17. The CH_2N methylene proton signals at 3.45 and 3.49 ppm (H_2-14) each correlated with C-12, requiring connection of N-13 with C-12 to form the remaining amide bond, and completing the assignment of the gross structure of sclerotiamide as shown in **1**.

The relative stereochemistry assigned for sclerotiamide was deduced by analysis of NOESY data (Figure 1). X-ray crystallographic studies previously conducted on the related compound paraherquamide (**2**),[6] and examination of a Dreiding molecular model of sclerotiamide, suggested that **1** adopts a conformation in which the central five-membered ring is orthogonal to the plane of the indolinone subunit. NOESY correlations between H-4, H-10, and H_3-24 indicated that H-10 and H_3-24 are both on the face of the cyclopentanoid ring that orients them toward H-4, fixing the relative stereochemistry at C-3 as shown. NOESY interactions between H-19, NH-21, and H_3-23 placed the corresponding substituents together on the opposite face of the cyclopentanoid ring. The only other stereocenter is C-17, which must have the relative configuration shown to permit connection of the amide bridge to C-11. The relative stereochemistry proposed for sclerotiamide (**1**) is in agreement with the relevant features of the relative stereochemistry of paraherquamide[6] and with those of the absolute stereochemistry established by X-ray analysis of a semisynthetic paraherquamide analogue. The absolute stereochemistry shown for **1** is proposed by analogy to that of paraherquamide (**2**).

Although members of this structural class have been previously reported as fungal metabolites (e.g., marcfortines from *Penicillium roqueforti*,[4,8] and antiparasitic paraherquamides from *Penicillium charlesii*[5] and *Penicillium paraherquei*[6]), such compounds have not been reported before as metabolites of *Aspergillus* spp. Sclerotiamide is unique in that it is hydroxylated at position C-10, is oxidized at C-12, and lacks methyl groups at N-21 and C-16, relative to the closest known analogue, paraherquamide F (**3**).[5] Compound **1** retains an unreduced diketopiperazine unit, as expected for intermediates in the biosynthesis of the paraherquamides and marcfortines. Brevianamides A and B, which lack the OH group and the pyran ring and have a different connectivity pattern for the pyrrolidone ring relative to **1**, contain a similar intact bridged diketopiperazine unit.[9]



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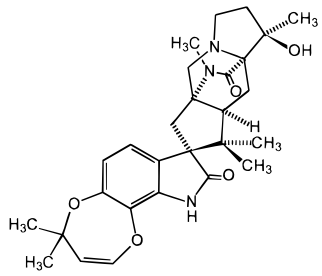

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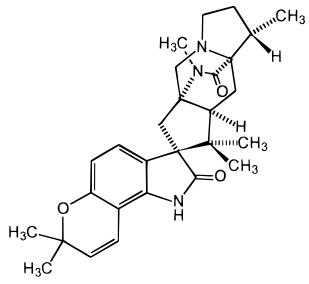

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Although the isolated yield of **1** was limited by the complexity of the *A. sclerotiorum* sclerotial extract, HPLC analysis indicated that it is a major component and is responsible for most of the observed antiinsectan activity. Sclerotiamide (**1**) exhibited potent activity in assays against first instar larvae of the corn earworm *Helicoverpa zea*, causing a 46% mortality rate against *H. zea* when incorporated into a standard test diet at 200 ppm. A 98% reduction in growth rate relative to controls was observed among survivors. Some unique physiological effects were also noted. Many of the dead larvae showed indications that the cuticle/exoskeleton

had ruptured. Larvae were laterally shriveled (suggesting liquid contents had leaked out), and blackened (an indication of the activity of the blood-clotting mechanism). Among the surviving larvae, the area just prior to the rear of the body was often bloated to as much as twice the diameter of the rest of the body. In a feeding assay against adults and larvae of the fungivorous beetle *Carpophilus hemipterus*, compound **1** induced reductions in feeding rates of 44% and 40%, respectively, at a 100-ppm dietary level. In contrast, compound **1** showed little cytotoxicity, displaying GI_{50} values > 25 μg/mL against nearly all of the 60 cell lines in the NCI tumor cell bioassay panel.

Experimental Section

General Experimental Procedures. The culture of *A. sclerotiorum* (NRRL 5167) was obtained from the Agricultural Research Service (ARS) Collection at the National Center for Agricultural Utilization Research in Peoria, Illinois. *A. sclerotiorum* NRRL 5167 was received by the ARS culture collection in 1945 from W. H. Weston, Harvard University, as UP093 (= QM 4999). The culture was isolated from a binocular reticle, Panama Canal Zone, and identified by K. B. Raper as *A. sulphureus* (Fres.) Thom and Church. In 1969, the culture was re-examined and identified as *A. sclerotiorum* Huber by D. I. Fennell.[10] This strain is known to produce ochratoxin A when grown on pearled wheat.[11] For the present study, sclerotia were produced by solid substrate fermentation on autoclaved corn kernels and were harvested using procedures previously described.[12] Protocols for insect bioassays have been reported elsewhere.[13]

^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker AC-300 instrument. HMQC and HMBC experiments were performed on a Bruker AMX-600 spectrometer and were optimized for $^1J_{CH}$ = 152 Hz and $^nJ_{CH}$ = 8 Hz, respectively. All NMR spectra were recorded using Me_2-CO-d_6, and chemical shift values were referenced to the corresponding solvent signals. LRFABMS and HRFABMS data were recorded on a VG ZAB-HF mass spectrometer. HPLC separations were accomplished using a Rainin Dynamax-60A C_{18} column (8-μm particles, 21.4 mm × 25 cm) at a flow rate of 10 mL/min with UV detection at 215 nm.

Isolation of Sclerotiamide (1). A sample of ground sclerotia (353 g) was extracted with 2 × 1 L of $CHCl_3$. Evaporation of the solvent yielded 4.0 g of $CHCl_3$ extract. A portion (1.6 g) was subjected to Sephadex LH-20 column chromatography, eluting with CH_2Cl_2−MeOH (1:1). Five fractions were collected and bioassayed. The first fraction (1.5 g) contained the activity and was further purified by chromatography on Sephadex LH-20 using hexane−toluene−MeOH (3:1:1) as the eluting solvent. Based on TLC, the resulting fractions were pooled to generate 15 subfractions. The active subfraction (subfraction 6; 69 mg) was subsequently purified by reversed-phase HPLC using a CH_3CN−H_2O (30:70) solvent system to yield 15.6 mg of sclerotiamide (**1**).

Sclerotiamide (1): white solid; mp 239−242 °C dec; $[\alpha]_D$ −55.1° (*c* 0.001 g/mL, MeOH); HPLC t_R 16.2 min (30:70 CH_3CN-H_2O); UV λ_{max} (MeOH) 215 (ϵ 5100), 250 (ϵ 8700), 280 (ϵ 3700); IR (neat) ν_{max} 3410, 2930, 1700, 1646 cm^{-1}; EIMS (70 eV) *m/z* 463 (M^+; 21), 377 (100), 362 (13), 256 (27), 240 (19), 181 (35), 165 (45), 151 (49), 83 (41), 69 (56), 55 (58), 43 (96); LRFABMS (DTT−DTE matrix) *m/z* 464 ($[M + H]^+$, 100), 436 (47), 401 (19), 377 (27), 256 (17); ^{1}H-NMR, ^{13}C-NMR, and HMBC data, see Table 1; HRFABMS found 464.2185 ($[M + H]^+$), calcd for $C_{26}H_{29}N_3O_5$ + H, 464.2185.

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Supporting Information Available: ^{1}H-NMR, ^{13}C-NMR, and DEPT spectra for sclerotiamide (**1**) (3 pages). Ordering information is given on any current masthead page.

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