Antifungal and Antibacterial Metabolites from a Sclerotium-Colonizing Isolate of *Mortierella vinacea*

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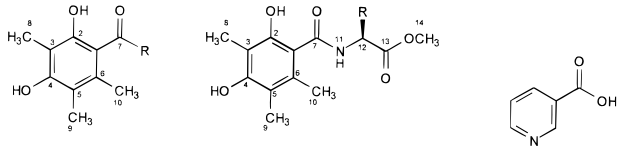
The known compound methyl 2,4-dihydroxy-3,5,6-trimethylbenzoate (**1**) and three new related metabolites, which we have named mortivinacins A (**2**), B (**3**), and C (**4**), were identified as metabolites of the fungus *Mortierella vinacea*. Nicotinic acid (**5**) was also encountered. This isolate of *M. vinacea* was obtained from an *Aspergillus flavus* sclerotium during field studies of sclerotium longevity in soil. Compounds **1**−**5** were isolated by chromatographic fractionation of organic extracts from *M. vinacea* solid−substrate fermentation cultures, and the structures were assigned by analysis of NMR and MS data. Compounds **1**, **2**, and **5** were responsible for the antibacterial and antifungal activities of the extract.

Our ongoing studies of fungi that exert antagonistic effects on competitors have resulted in the discovery of a variety of new antifungal agents.[1] Similar ecological considerations have recently led us to consider mycoparasites (fungi that parasitize other fungal species)[2] as potential sources of antifungal metabolites.[3] Prior chemical investigations of mycoparasitic fungi have provided a number of precedents demonstrating production by mycoparasites of agents with activity against the host species.[4,5] Our interest in mycoparasites arose from our work with fungal sclerotia.[1,6] Sclerotia are reproductive structures produced by certain fungi as a survival mechanism. These reproductive bodies may lie dormant in the soil for long periods of time. During field studies of the survival of *Aspergillus* sclerotia in soil, a variety of fungi were encountered as colonists (possibly mycoparasites) of the sclerotia. To our knowledge, chemical studies of fungi that colonize or parasitize *Aspergillus* sclerotia have not been previously reported. We view such colonists as rational sources to explore in search of antifungal agents, especially those active against *Aspergillus* spp. Anti-*Aspergillus* agents could be significant because of the importance of *Aspergillus* spp. as opportunistic pathogens, but compounds with activity against other fungal pathogens of humans or plants might also be encountered. One such sclerotium-colonizing species is the subject of this report. An isolate of *Mortierella vinacea* NRRL 22986 (Mortierellaceae) was obtained from a sclerotium of *Aspergillus flavus* that had been buried in soil in an Illinois cornfield for three years. Chemical investigation of extracts from *M. vinacea* cultures afforded a group of four related metabolites (**1**−**4**), three of which are new, as well as nicotinic acid (**5**). Details of this work are presented here.

M. vinacea was grown in solid−substrate fermentation on rice, and the mature cultures were extracted with EtOAc. The resulting extract showed antibacterial and antifungal activity. Bioassay-guided fractionation of this extract by Sephadex LH-20 column chromatography, followed by semipreparative reversed-phase HPLC, led to the isolation of compounds **1**−**5**.

A molecular formula of $C_{11}H_{14}O_4$ (five unsaturations) was established for compound **1** by HREIMS analysis. The ^{13}C NMR and DEPT spectral data (Table 1) were consistent with the presence of a hexasubstituted benzenoid moiety, and the ^1H NMR spectrum demonstrated the presence of hydrogen-bonded and nonchelated phenolic OH groups. The NMR data also indicated the presence of three aryl methyl groups, one methoxy group, and an ester carbonyl. The presence of a methyl ester was revealed by HMBC correlation of the methoxy proton signal at δ 3.91 to the ester carbonyl (C-7, δ 172.7). The hydrogen-bonded phenolic OH was placed ortho to the methyl ester substituent on the basis of its ^1H NMR chemical shift. Additional HMBC correlations of one aryl methyl signal (H_3-8) to two oxygen-bearing carbons (C-2, δ 156.7 and C-4, δ 159.6), as well as the carbon to which the methyl is attached (C-3, δ 106.2) established the location of this methyl group between the two oxygenated aromatic carbons. The two remaining methyl groups must occupy the two remaining sites on the aromatic ring, thereby permitting assignment of structure **1**. Interestingly, this relatively simple tetraketide has been described only once before as a natural product, from another isolate of *M. vinacea*.[7] It has also been reported as a synthetic product.[8]



1 R = OCH_3 **3** R = H

2 R = SCH_3 **4** R = CH_3 **5**

The ^1H and ^{13}C NMR spectral data for mortivinacin A (**2**, Table 1) were also consistent with a hexasubstituted benzenoid. Other features observed in the ^1H NMR spectrum were almost identical with those of **1**, except for the presence of an upfield-shifted methyl signal (δ 2.46, H_3-11) in place of the methoxy signal. Once again, there were 11 individual resonances in the ^{13}C NMR spectrum. Interpretation of these data indicated the presence of six sp^2-hybridized carbons, four methyl groups, and one apparent ketone carbonyl (δ 198.3, C-7). A long-range HMBC correlation of H_3-11 to C-7 initially suggested the presence of a methyl ketone. However, since the EIMS of mortivinacin A shows a molecular ion peak at *m*/*z* 226, which is 16 mass units higher than that of **1**, and because no additional oxygen atoms could be accounted for by the units

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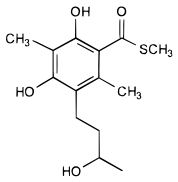
Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1**−**4**

	1[a]		mortivinacin A (**2**)[a]		mortivinacin B (**3**)[b]		mortivinacin C (**4**)[b]	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		107.2		117.5		113.1		117.5
2		156.7		155.0		155.9		155.7
3		106.2		107.6		109.1		109.1
4		159.6		155.9		156.5		156.4
5		114.8		115.2		115.7		115.7
6		137.5		134.7		133.2		133.3
7		172.7		198.3		171.9		174.3
8	2.117 (s)	8.0	2.107 (s)	8.1	2.08 (s)	8.7	2.077 (s)	8.7
9	2.124 (s)	11.8	2.114 (s)	11.7	2.09 (s)	12.0	2.083 (s)	12.0
10	2.41 (s)	18.8	2.51 (s)	19.3	2.41 (s)	18.1	2.38 (s)	18.1
11	3.91 (s)	51.8	2.46 (s)	13.1	7.59 (br m)		7.48 (br d)	
12					4.17 (d, 6)	41.9	4.67 (dq; 7, 7)	49.4
13						171.3		171.3
14					3.72 (s)	52.4	3.73 (s)	52.5
15							1.48 (d, 7)	17.4
OH-2	11.42 (s)		10.05 (s)		10.07 (s)		9.83 (s)	
OH-4	5.12 (br s)		5.08 (br s)		7.60 (br s)		7.47 (br s)	

[a] These data were recorded using $CDCl_3$. [b] These data were recorded using Me_2CO-d_6. Numerical values in parentheses indicate J values in Hz. All carbon assignments are consistent with DEPT results.

present, the presence of a methyl thiol ester, rather than a methyl ketone, was proposed. HREIMS data confirmed the presence of a sulfur atom. Moreover, the base peak in the mass spectrum corresponds to loss of 47 mass units, which is consistent with the expected $[M - SCH_3]^+$ fragmentation peak, and the ^{1}H and ^{13}C NMR chemical shifts for **2** are characteristic of a methyl thiol ester group.[9] These data led to the assignment of the structure of mortivinacin A as shown in **2**. The presence of a methyl thiol ester group is quite rare among secondary metabolites. An example resembling compound **2** is resorthiomycin (**6**), an antitumor antibiotic from *Streptomyces collinus*.[10]



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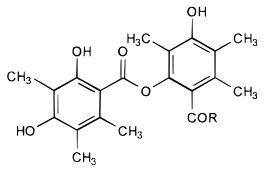
HREIMS data for mortivinacin B (**3**) indicated a molecular formula of $C_{13}H_{17}O_5N$ (six unsaturations). The ^{1}H NMR spectrum was nearly identical to that of **1**, except for the presence of an additional downfield-shifted methylene proton doublet coupled to a broad signal at δ 7.59 characteristic of a secondary amide proton. Assignment of an amide functionality was supported by the nitrogen atom in the molecular formula, and by the appearance of a second resonance in the carboxyl region of the ^{13}C NMR spectrum. ^{13}C NMR, DEPT, and selective INEPT data clearly indicated that this compound contains the same hexasubstituted benzoyl unit as **1** and **2**. Thus, the only difference must be in the identity of the acylated moiety. Selective INEPT irradiation of the methoxy proton signal at δ 3.72 (H_3-14) resulted in polarization transfer to one of the carbonyl signals (δ 171.3), indicating the presence of a methyl ester group. Irradiation of the methylene signal (δ 4.17) resulted in polarization transfer to both the ester (δ 171.3) and amide (δ 171.9) carbonyls, revealing the presence of an acylated glycine subunit. On the basis of these data, the structure of mortivinacin B was assigned as **3**.

Analysis of ^{1}H NMR, ^{13}C NMR, and DEPT data indicated the molecular formula $C_{14}H_{19}O_5N$ for mortivinacin C (**4**), which differs from that of **3** by a methylene unit. This formula was confirmed by observation of a molecular ion peak at m/z 281 in the EIMS. The ^{13}C NMR spectrum contained 14 carbon signals, with the methylene carbon signal in the spectrum of compound **3** being replaced by methine (δ 49.4) and methyl carbon resonances (δ 17.4). The ^{1}H NMR spectrum differed from that of **3** by the appearance of a methine multiplet (δ 4.67) coupled to a methyl doublet (δ 1.48). These observations, together with other data closely paralleling those for mortivinacin B (**3**) led to the assignment of structure **4** for mortivinacin C. Acid hydrolysis of mortivinacin C (**4**), followed by chiral TLC analysis, confirmed the presence of the alanine unit and enabled its identification as the L-isomer.

The known compound 3-pyridinecarboxylic acid (**5**; nicotinic acid) was also isolated as a minor constituent of the *M. vinacea* extract. Nicotinic acid is well known as a common metabolite of plants and microorganisms.[11]

Only a few compounds have been previously described from the genus *Mortierella*.[7,11−13] Interestingly, compound **1** (= KS-506f) and several dimeric and tetrameric structures containing this aromatic subunit (e.g., KS-506h, **7**; KS-506m, **8**) have been reported from an isolate of *M. vinacea* as inhibitors of histamine release and/or calmodulin-dependent phosphodiesterase.[7,11,13]



7 R = OCH_3

8 R = $SCH_2CH_2COCH_3$

Compound **1** and mortivinacin A (**2**) both produced ca. 30-mm zones of inhibition in 48-h disk assays[3] against *Fusarium verticillioides* (NRRL 25457) at 250 μg/disk. Nicotinic acid (**5**) caused a similar-sized zone of reduced growth at this level, while mortivinacins B (**3**) and C (**4**) were inactive. A nystatin standard afforded a 19-mm zone at 100 μg/disk in this assay. Compounds **1** and **2** also showed antibacterial activity (10- to 15-mm zones of inhibition) in standard disk assays against *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 14053) at 100 μg/disk. A gentamicin sulfate standard caused 24- to 26-mm zones of inhibition in these assays at 50 μg/disk.

Only mortivinacin A (**2**) showed significant activity against *C. albicans* (ATCC 10453), affording a 15-mm zone of inhibition upon testing at 100 μg/disk. A zone of the same size was obtained with a filipin standard at 50 μg/disk. All five compounds were inactive in assays against *A. flavus* (NRRL 6541) at 250 μg/disk. Thus, although metabolites with antifungal effects were encountered in these cultures of *M. vinacea* and our prior studies have shown that anti-*Aspergillus* metabolites may be obtained from mycoparasites of *A. flavus* sclerotia,[3] no anti-*Aspergillus* metabolites were encountered in this instance.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded using $CDCl_3$ or CD_3COCD_3 solutions with a Bruker AC-300 spectrometer at 300 and 75 MHz, respectively. Residual solvent signals (δ 7.24/77.0 and δ 2.04/29.8, respectively) were used as internal references. UV spectra were recorded using a Beckman model 168 photodiode array detector. HREIMS data were obtained with a VG ZAB−HF double-focusing mass spectrometer. The specific rotation was measured on a JASCO model DIP-1000 digital polarimeter. Samples of nystatin, gentamicin sulfate, filipin, and nicotinic acid were obtained from Sigma Chemical Co., St. Louis, MO. Details of other general experimental procedures have been provided previously.[14]

Fungal Material. *Mortierella vinacea* Dixon-Stewart NRRL 22986 was isolated by D. T. Wicklow from an individual fungal sclerotium of *Aspergillus flavus* (Link:Fr.) that had been buried in soil for three years (1989−1992) in the Illinois River Valley Sand Field, Kilbourne, IL. Although *M. vinacea* is not routinely listed with fungal taxa recognized as mycoparasites,[2,15] the fungus was repeatedly isolated from carpophores of *Armillaria matsutkae* and is reported to inhibit the development of the soil-borne plant pathogenic fungi *Cochliobolus sativus* and *Rhizoctonia solani*.[16]

M. vinacea was cultured on slants of potato dextrose agar (PDA) for 14 days (25 °C). Spore inoculum obtained from these cultures was suspended in sterile distilled H_2O to give a final spore/cell suspension of 1×10^6/mL. Fermentations were carried out in three Erlenmeyer flasks each containing 200 g of rice. Distilled H_2O (200 mL) was added to each flask and the contents soaked overnight before autoclaving for 30 min. After the flasks had cooled to room temperature, they were inoculated with 3.0 mL of the spore inoculum and incubated for 61 days at 25 °C.

Extraction and Isolation of 1−5. After incubation, the flask contents were combined, mechanically fragmented, and extracted with EtOAc (3 × 500 mL). The combined EtOAc extracts were filtered and evaporated to give a yellow oil (4.6 g). The extract was partitioned between hexane and 10% H_2O in MeOH. The antifungal H_2O−MeOH fraction (1.8 g) was then subjected to Sephadex LH-20 column chromatography. The antifungal fraction (600 mg) eluting with 1:4 hexane−CH_2Cl_2 was rechromatographed on a Si gel column eluting with a $CHCl_3$−MeOH gradient. An antifungal fraction eluted with 2% $CHCl_3$−MeOH (25 mg) was further purified by reversed-phase HPLC (Rainin Microsorb C_{18} column, 1 × 25 cm, 5-μm particles, 2.0 mL/min, 40−100% CH_3CN in 0.1% HCOOH−H_2O over 40 min, monitored by UV absorption at 215 nm) to afford compound **1** (3.2 mg, t_R 26.6 min) and mortivinacin A (**2**, 5.2 mg, t_R 22.5 min). A fraction eluted with 4% $CHCl_3$−MeOH (55 mg) was also further purified by HPLC using the same column and a gradient of 10−50% CH_3CN in 0.1% HCOOH−H_2O over 40 min to yield mortivinacin B (**3**, 16.1 mg, t_R 32.5 min) and mortivinacin C (**4**, 0.8 mg, t_R 38 min). Another antifungal fraction (45 mg, eluted with 60% MeOH−$CHCl_3$), when subjected to reversed-phase HPLC with a gradient of 0−30% CH_3CN in 0.1% HCOOH−H_2O over 30 min, afforded nicotinic acid (**5**, 7.8 mg, t_R 9.1 min), which was identified by comparison of its mp, MS, ^{1}H NMR, and ^{13}C NMR data to those of a commercial sample.

Methyl 2,4-dihydroxy-3,5,6-trimethylbenzoate (1): properties for **1** (^{1}H NMR, ^{13}C NMR, HMBC, IR, UV, MS) matched those previously reported.[7] NMR data are included in Table 1 for comparison with **2**−**4**.

Mortivinacin A (2): pale yellow glass; UV (MeOH) λ_{max} 212 (ϵ 4400), 264 (1200), 304 (790); IR ($CDCl_3$) 3600, 2930, 1653, 1606, 1275, 1222 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HMBC correlations (H# → C#) H_3-8 → C-2, -3, -4; H_3-9 → C-4, -5, -6; H_3-10 → C-1, -5, -6; H_3-11 → C-7; OH-2 → C-1, -2, -3; OH-4 → C-3, -5; EIMS (70 eV) *m/z* 226 ($M^{\bullet+}$; rel int 25), 179 (100), 150 (39), 77 (38); HREIMS obsd, M^+ at *m/z* 226.0666, calcd for $C_{11}H_{14}O_3S$, 226.0664.

Mortivinacin B (3): pale yellow glass; UV (MeOH) λ_{max} 212 (ϵ 5200), 258 (1200), 304 (400); IR ($CDCl_3$) 3603, 2956, 1746, 1672, 1626, 1524, 1439, 1370, 1215 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; selective INEPT correlations (H# → C#) H_3-8 → C-2, -3, -4; H_3-9 → C-5, -6; H_3-10 → C-1, -5, -6; H-12 → C-7, -13; H_3-14 → C-13; OH-2 → C-1, -2, -3; OH-4 → C-3, -4, -5; EIMS (70 eV) *m/z* 267 ($M^{\bullet+}$; rel int 54), 178 (83), 150 (100), 77 (31); HREIMS obsd, M^+ at *m/z* 267.1090, calcd for $C_{13}H_{17}O_5N$, 267.1106.

Mortivinacin C (4): pale yellow glass; $[\alpha]_D$ −16° (*c* 1.9 mg/mL, $CHCl_3$); UV (MeOH) λ_{max} 210 (ϵ 3300), 258 (1300), 304 (400); ^{1}H and ^{13}C NMR data, see Table 1; EIMS (70 eV) *m/z* 281 ($M^{\bullet+}$; rel int 76), 249 (10), 220 (7), 192 (4), 178 (100), 150 (97), 77 (49); HREIMS obsd, M^+ at *m/z* 281.1261, calcd for $C_{14}H_{19}O_5N$, 281.1263.

Acid Hydrolysis of Mortivinacin C (4). A sample of mortivinacin C (0.3 mg) was placed in a vacuum hydrolysis tube along with 1 mL of 6N HCl. The mixture was heated to 110°C for 24 h. Upon cooling to room temperature, the solution was extracted with Et_2O, and the aqueous phase was collected, evaporated, and analyzed by chiral TLC (Machery−Nagel Chiralplate; 4:1:1 *n*-BuOH−HOAc−H_2O). Comparison with authentic DL- and L- standards indicated that the alanine obtained from hydrolysis of mortivinacin C was the L-isomer (R_f of the product obtained from mortivinacin C = 0.38; $R_{f,D}$ = 0.21, $R_{f,L}$ = 0.38).

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References and Notes

(1) Gloer, J. B. In *The Mycota IV*; Wicklow, D. T., Söderström, B., Eds.; Springer: Heidelberg, 1997; pp 249−268.
(2) Jeffries, P. In *The Mycota IV*; Wicklow, D. T., Söderström, B., Eds.; Springer: Heidelberg, 1997; pp 149−164.
(3) Wicklow, D. T.; Joshi, B. J.; Gamble, W. R.; Gloer, J. B.; Dowd, P. F. *Appl. Environ. Microbiol.* **1998**, *64*, 4482−4484.
(4) Huang, Q.; Tezuka, Y.; Hotanaka, Y.; Kikuchi, T.; Nishi, A.; Tubaki, K. *Chem. Pharm. Bull.* **1994**, *42*, 2612−2617.
(5) Morris, R. A. C.; Ewing, D. F.; Whipps, J. M.; Coley-Smith, J. R. *Phytochemistry* **1995**, *39*, 1043−1048.
(6) Gloer, J. B. *Acc. Chem. Res.* **1995**, *28*, 343−350.
(7) Yasuzawa, T.; Saitoh, Y.; Sano, H. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1989**, *31*, 617−624; *Chem. Abstr.* **1990**, *112*, 175314p.
(8) Hamilton, R. J.; Sargent, M. V. *J. Chem. Soc., Perkin Trans. 1* **1976**, 943−944.
(9) Suzuki, H. *J. Antibiot.* **1990**, *48*, 129−138.
(10) Tahara, M. *J. Antibiot.* **1991**, *44*, 255.
(11) *Dictionary of Natural Products* on CD-ROM, Chapman and Hall Electronic Publishing Division: New York, version 7.1, 1998.
(12) Kamiyama, T.; Umino, T.; Nakayama, N.; Yoshiko, I.; Tomoko, S.; Yamashita, Y.; Yamaguchi, A.; Yokose, K. *J. Antibiot.* **1992**, *45*, 424−427.
(13) Kuroda, K.; Kase, H.; Ando, K.; Kawamoto, I.; Yasuzawa, T.; Sano, H.; Goto, J.; Yamada, K. Eur. Pat. Appl. EP 335,386, 1988; *Chem. Abstr.* **1990**, *113*, 22205z.
(14) Alfatafta, A. A.; Gloer, J. B.; Scott, J. A.; Malloch, D. *J. Nat. Prod.* **1994**, *57*, 1696−1702.
(15) Lumsden, R. D. In *The Fungal Community*, 2nd ed.; Carroll G. C., Wicklow, D. T., Eds., Marcel Dekker: New York, 1992; pp 275−293.
(16) Domsch, K. H.; Gams, W.; Anderson, T.-H. *Compendium of Soil Fungi*; Academic Press: London, 1980: Vol. 1; 859 pp.

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