Chemical and Biological Investigation of the Fungus Pulveroboletus r avenelii

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · FEBRUARY 2003

Impact Factor: 3.8 · DOI: 10.1021/np0203990 · Source: PubMed

Chemical and Biological Investigation of the Fungus *Pulveroboletus ravenelii*

Christine J. G. Duncan,[†] Muriel Cuendet,[‡] Frank R. Fronczek,[§] John M. Pezzuto,[‡] Rajendra G. Mehta,[‡,⊥] Mark T. Hamann,[†,∥] and Samir A. Ross*[,†,∥]

Department of Pharmacognosy, Thad Cochran National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, University, Mississippi 38677, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612, and Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803-1804

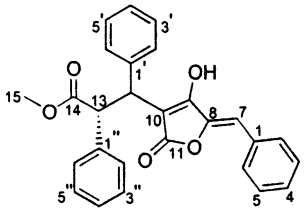
Received September 11, 2002

Two new compounds, pulveraven A (**1**) and pulveraven B (**2**), as well as vulpinic acid (**3**) and its previously unreported polymorph were isolated from the fruiting body of *Pulveroboletus ravenelii*. The structures were determined using a combination of NMR, MS, IR, optical rotation, molecular modeling, and X-ray analysis. The isolates were evaluated for antimicrobial activity as well as their potential to inhibit cyclooxygenase (COX) activity and carcinogen-induced preneoplastic lesion formation with mouse mammary organ culture (MMOC).

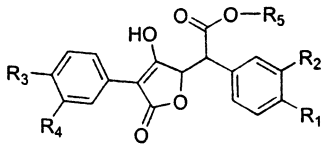
The Boletaceae is characterized by the presence of vertically arranged tubes, rather than gills, beneath the pileus.[1] Most members of this family are known as edibles, although a few are poisonous.[2,3] *Pulveroboletus* is a genus in the Boletaceae with a powdery to cobweb-like universal veil that covers the cap and stipe of young fruiting bodies.[3,4] Compounds isolated from *Pulveroboletus* include variegatic acid (**4**) and variegatorubin (**5**) from *P. lignicola*;[5] xercomic acid (**6**) from the cultured mycelium of *P. lignicola*;[6] vulpinic acid (**3**) from *P. ravenelii* and *P. cramesinus*; and compounds **7**−**10** from *P. auriflammeus*.[7]

Pulveroboletus ravenelii (Berk. & Curt.) Murr. is widely distributed in Eastern North America, but is most abundant in the Southeast, where it fruits during the summer and fall.[2,8] It has also been reported from California, Japan, China, Malaysia, Singapore, and Borneo.[3,8] Fruiting bodies appear on the ground, under conifers and hardwoods.[2,4,8] These materials are reportedly edible, having a mild taste and odor,[2,4,8] and are used in traditional Chinese medicine to cure lumbago and painful legs, numbed limbs, and tetany and to stop bleeding.[9] In addition, an 80% EtOH extract of *Pulveroboletus* (PS-7) demonstrated antibacterial activity against *Bacillus subtilis* (KCTC 1028), *Micrococcus luteus* (KCTC 1915), and *Staphylococcus aureus* (KCTC1621).[10]

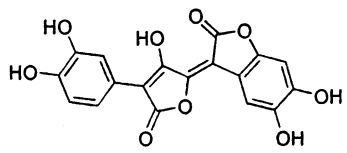
In our search for biologically active natural products from fungi, we discovered that a crude extract from *P. ravenelii* displayed strong activity against methicillin-resistant and -susceptible *S. aureus*. Analysis of this extract has resulted in the isolation of two novel compounds, **1** and **2**, as well as the isolation of vulpinic acid (**3**) and its previously unreported polymorph. Select biological activities of the isolated compounds are presented.



1 (12*R*, 13*R*) and **2** (12*S*, 13*R*)



	R_1	R_2	R_3	R_4	R_5
3	H	H	H	H	CH_3
4	OH	OH	OH	OH	H
6	OH	H	OH	OH	H
7	OCH_3	H	OCH_3	OCH_3	CH_3
8	OCH_3	Cl	OCH_3	OCH_3	CH_3
9	OCH_3	H	OCH_3	H	CH_3
10	OCH_3	Cl	OCH_3	H	CH_3



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Results and Discussion

Compounds **1**, **2**, and **3** were isolated from the MeOH-soluble portion of the crude EtOH extract from *P. ravenelii* by various chromatographic techniques including vacuum liquid chromatography (VLC), centrifugal liquid chromatography (CLC), and HPLC. The molecular formula of **1** was established by HRESIMS as $C_{27}H_{22}O_5$, indicating 17 degrees of unsaturation. The IR spectrum revealed absorption bands for carbonyl (1735 and1704 cm^{-1}) and monosubstituted aromatic ring moieties (C−H stretching, 3086 and 3029 cm^{-1}; C−C stretching, 1495 and 1453 cm^{-1}; in-plane C−H bending, 1065 and 1164 cm^{-1}; 697 cm^{-1} out-of-plane C−H bending). The ^1H NMR spectrum (Table 1)

* To whom correspondence should be addressed. E-mail: sross@olemiss.edu.
† Department of Pharmacognosy, The University of Mississippi.
‡ Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago.
§ Department of Chemistry, Louisiana State University.
⊥ Department of Surgical Oncology, University of Illinois at Chicago.
∥ Thad Cochran National Center for Natural Products Research, The University of Mississippi.

Table 1. NMR Spectral Data for Compounds **1** and **2** in d_6-Acetone[a]

	1		**2**	
position	δ_C	δ_H	δ_C	δ_H
1	134.7		136.2	
2	131.3	7.78 d (7.4)	128.9	7.70 d (7.2)
3	129.9	7.42 m	127.9	7.30 m
4	129.5	7.33 m	125.4	7.11 m
5	129.9	7.42 m	127.9	7.30 m
6	131.3	7.78 d (7.4)	128.9	7.70 d (7.2)
7	107.0	6.49 s	96.9	5.95 s
8	144.7		150.7	
9	166[b]		178[b]	
10	105[b]		90[b]	
11	170.1		173.2	
12	44.2	4.85 d (12.0)	44.1	4.46 d (12.2)
13	54.5	5.00 d (12.0)	53.8	5.20 d (12.2)
14	174.4		173.9	
15	52.6	3.58 s	50.6	3.50 s
1′	141.6		144.9	
2′	129.9	7.42 m	128.6	7.45 m
3′	129.2	7.12 m	126.9	6.95 m
4′	127.6	7.04 m	124.6	6.85 m
5′	129.2	7.12 m	126.9	6.95 m
6′	129.9	7.42 m	128.6	7.45 m
1″	138.7		139.1	
2″	129.9	7.33 m	128.7	7.30 m
3″	129.5	7.18 m	127.6	7.13 m
4″	128.3	7.12 m	126.1	7.04 m
5″	129.5	7.18 m	127.6	7.13 m
6″	129.9	7.33 m	128.7	7.30 m

[a] Assignments were based on DEPT, COSY, HMQC, and HMBC experiments. TMS was used as the internal standard; chemical shifts are shown as δ, multiplicity, J (Hz). [b] Value determined from HMBC data.

contained one signal for a methoxy group at δ 3.58 (3H, s) and several signals (δ 7.78−7.03) in the aromatic region, which integrated for 15 protons and verified the presence of three monosubstituted phenyl rings. Additional ^{1}H NMR signals at δ 6.49 (1H, s, H-7), 4.85 (1H, d, J = 12.0 Hz, H-12), and 5.00 (1H, d, J = 12.0 Hz, H-13) were observed. The ^{13}C NMR spectrum (Table 1) showed the presence of two ester carbonyls (δ 174.4 and 170.1), one methoxy (δ 52.6), and three nonaromatic, methine carbon signals at δ 107.0 (C-7), 44.2 (C-12), and 54.5 (C-13). Analysis of the COSY spectra revealed spin-systems A (H-4, H-5, H-6), B (H-4′, H-5′, H-6′), C (H-12, H-13), and D (H-5″, H-6″). The chemical shifts of spin-systems A, B, and D indicated aromaticity.

The carbon skeleton of **1** was deduced from the results of an HMBC experiment (Figure 1). Signals in the HMBC experiment clearly connect spin-systems B, C, and D, which consists of two phenyl groups connected by two adjacent methine carbons. HMBC signals between H-7 and C-8, C-9, and C-2 suggest that spin-system A is an isolated benzylidene group. The ^{1}H and ^{13}C NMR assignments (Table 1) of compound **1**, a new natural product named pulveraven A, were unambiguously made using HMQC and HMBC NMR correlations.

The HRESIMS, IR, ^{1}H, ^{13}C, COSY, HMQC, and HMBC data acquired for compound **2** were comparable to the data acquired for pulveraven A (**1**). A comparison of the ^{13}C NMR data, however, revealed different chemical shifts for carbons C-7 through C-11 (Table 1), indicating a variation near the lactone ring.

The absolute configuration of **1** and compound **2** at positions C-12 and C-13 was deduced from molar rotation angle values. The molar rotation angle $[M]_D$ is directly proportional to the specific rotation $[\alpha]_D$; $[M]_D = [\alpha]_D \times MW/100$. The computed molar rotation angle values were −442 for the 12*R*,13*R* configuration and −243 for the 12*S*,13*R* configuration.[11] Molar rotation angle values obtained from the measured specific rotation of **1** and **2** were −394.3 and −152.9, respectively. Thus, the absolute configuration of compounds **1** and **2** at positions 12 and 13 may be assigned on the basis of the magnitude and sign of the modeled and measured molar rotation angle values as 12*R*,13*R* and 12*S*,13*R*, respectively. Discrepancies observed between the modeled and calculated molar rotation angle values may be due to the low solution concentrations used in measuring optical rotation as well as the relatively small basis set used for optical rotation computations.[11] On the basis of the above evidence, the structure of compound **2**, a new natural product named pulveraven B, was established as the 12-*S* diastereomer of pulveraven A (**1**).

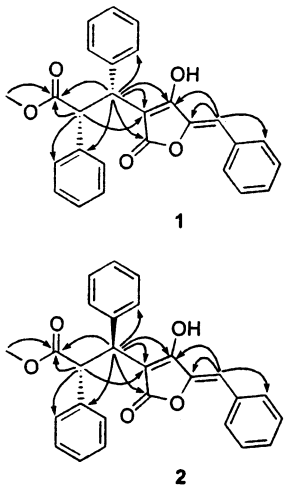

Figure 1. Selected HMBC correlations for compounds **1** (12*R*,13*R*) and **2** (12*S*,13*R*).

The molecular formula of compound **3** was established by HRESIMS as $C_{19}H_{14}O_5$, which indicated 13 degrees of unsaturation. The IR spectrum contained absorption bands at 2952, 2923, and 2854 cm^{-1} (asymmetrical and symmetrical C−H stretching), 1770 cm^{-1} (α,β-unsaturated-γ-lactone), 1712 and 1679 cm^{-1} (conjugated ester with intramolecular H-bond), 1573 cm^{-1} (C=O stretching), 1494 cm^{-1} (C−C stretching), 1303, 1274, and 1062 cm^{-1} (aromatic in-plane C−H bending), and 898, 781, and 692 cm^{-1} (out-of-plane C−H bending) and was in agreement with data reported for vulpinic acid by Harper and Letcher.[12] The aromatic region of the ^{1}H NMR spectra showed signals at δ 7.38−7.49, integrating for 10 protons, and a signal at δ 3.91, integrating for three protons, thus indicating the presence of a methoxy group. The proton spectrum (Table 2) is in close agreement with the literature.[13,14]

The structure of compound **3**, vulpinic acid, was confirmed by X-ray crystallography. Crystallization of **3** was attempted using several different solvents, but MeOH yielded a yellow crystal fragment suitable for X-ray analysis. Low-temperature crystallographic results obtained in the current study were in agreement with the less precise room-temperature structure reported in the literature (Table 3, Figure 2).[15]

Similar to compound **3**, crystallization of another isolate was attempted using several solvents. Final crystallization of the isolate from MeOH yielded yellow needles suitable for X-ray analysis. The results indicate that this isolate is a previously unreported polymorph of **3**, with a torsional angle difference of 7.29° at $C_{17}-C_{12}-C_5-C_6$ and a difference in the rotation of the phenyl ring at $C_{12}-C_5-C_6-C_7$ by 44.76° (Table 3, Figure 3).

Table 2. ^{1}H NMR Spectral Data for Compound **3** and Its Polymorph in d_6-Acetone[a]

position	**3** δ_H	polymorph δ_H
1		
2	7.49[b] d (7.0)	7.67 d (7.4)
3	7.38−7.48[c] m	7.39 t (7.7)
4	7.38−7.48[d] m	7.28 t (7.4)
5	7.38−7.48[c] m	7.39 t (7.7)
6	7.49[b] d (7.0)	7.67 d (7.4)
7		
8		
9		
10		
11		
12		
13	3.91 s	3.86 s
1′		
2′	8.18[b] d (7.7)	8.36 d (7.4)
3′	7.38−7.48[c] m	7.21 t (7.7)
4′	7.28[d] t (7.2)	6.96 t (7.2)
5′	7.38−7.48[c] m	7.21 t (7.7)
6′	8.18[b] d (7.7)	8.36 d (7.4)

[a] TMS was used as the internal standard; chemical shifts are shown as δ, multiplicity, J (Hz). [b] Assignment inferred from polymorph data. [c,d] Assignments carrying the same superscript in each column may be interchanged.

Table 3. Crystallographic Data for Polymorphs of Vulpinic Acid

	3	polymorph
formula	$C_{19}H_{14}O_5$	$C_{19}H_{14}O_5$
mol wt	322.30	322.30
space group	monoclinic $P2_1/n$	monoclinic $P2_1/c$
a (Å)	7.6780(2)	11.4643(8)
b (Å)	7.8440(3)	7.5023(6)
c (Å)	25.9950(9)	18.5725(4)
β (°)	94.580(2)	103.655(4)
V (Å^3)	1560.58(9)	1552.2(2)
D_x (Mg m^{-3})	1.372	1.379
Z	4	4
μ(Mo Kα) (mm^{-1})	0.100	0.100
temperature (K)	150	120
cryst	yellow, fragment	yellow, needle
cryst dimens (mm)	0.32 × 0.15 × 0.10	0.45 × 0.15 × 0.10
θ range (deg)	2.5−27.5	2.5−30.0
no. of unique data	3546	4326
no. of obsd data	1976	1538
criterion for obsd	$I > 2\sigma(I)$	$I > 2\sigma(I)$
no. of refined variables	222	223
intensity decay (%)	none	none
R	0.030	0.042
R_w	0.099	0.087
max. resid density (e Å^{-3})	0.19	0.30
min. resid density (e Å^{-3})	−0.19	−0.19

The ^{1}H NMR spectra of **3** and the polymorph differed, when recorded in d_6-acetone (Table 2). The reason for these observed differences is unclear, but similar phenomena are demonstrated with kahalalide F.[16] The crystallographic data, however, clearly demonstrate that the difference between **3** and its polymorph is a variation in the rotation of the aromatic ring.

Vulpinic acid (**3**) possesses diverse biological activities, and lichens containing **3** have a strong history of medicinal use. For example, Eskimos[17] and people of Northern Europe have used lichens containing **3** to poison the wolf[17,18] and fox.[18,20] Lichens containing **3** are used as fodder for reindeer and emergency food by Arctic and Subarctic peoples.[21] In central Europe, members of the genus *Cetraria*, which is known to produce **3**, have been used as laxatives and have been taken for coughing, including that associated with tuberculosis.[20,22]

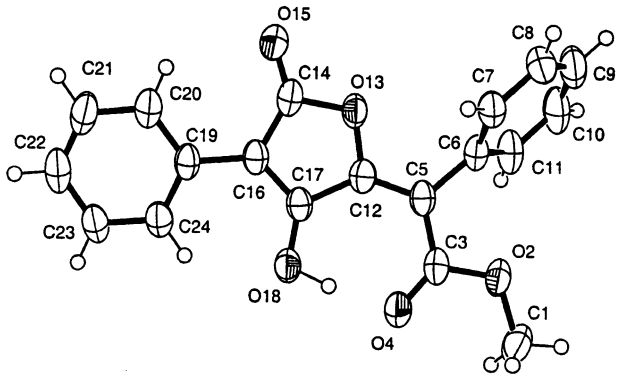

Figure 2. ORTEP drawing of compound **3**.

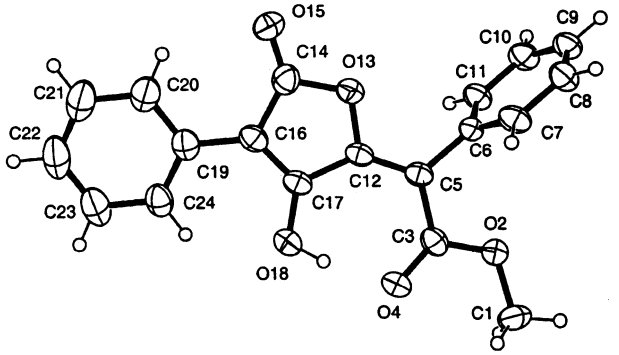

Figure 3. ORTEP drawing of vulpinic acid polymorph.

Vulpinic acid (**3**) and its polymorph showed strong *in vitro* activity against *S. aureus*, having IC_{50} values of <2 μg/mL. The polymorph showed greater in vitro activity against methicillin-resistant *S. aureus*, with an IC_{50} value of 9 μg/mL, compared to the IC_{50} value of 20 μg/mL demonstrated by **3**. Compound **1** was not active against methicillin-resistant and -susceptible *S. aureus* (IC_{50} values ≥ 15 μg/mL), and insufficient quantities of compound **2** did not permit biological evaluation in these systems.

Compounds **1**, **2**, and **3** were evaluated for their potential to inhibit cyclooxygenase-1 and -2 (COX-1 and COX-2), and carcinogen-induced preneoplastic lesion formation in mouse mammary organ culture (MMOC) assays, according to established protocols.[23,24] Pulveraven B (**2**) was inactive (IC_{50} values > 100 μg/mL) in both the COX-1 and -2 inhibition assays. Accordingly, pulveraven A (**1**) was not anticipated to function as an active inhibitior, but this was not tested due to insufficient material. Compound **3** and its polymorph showed weak activity against COX-1 with IC_{50} values of 218 and 192 μM, respectively, and were inactive as COX-2 inhibitors. In addition, compound **3** was inactive (IC_{50} values > 10 μg/mL) in the mouse mammary organ culture (MMOC) assay, although its polymorph showed weak activity (52% of inhibition at 10 μg/mL). On the other hand, pulveraven A (**1**) and pulveraven B (**2**) showed good activities, with IC_{50} values of 6.0 and 0.8 μM, respectively, in the MMOC assay. This is of interest since **1** and **2** are diastereoisomers, but stereoselectivity leads to ∼10-fold greater activity with **2**. The magnitude of this response warrants further investigation.

Experimental Section

General Experimental Procedures. 1D and 2D NMR spectra of compounds **1**, **3**, and the vulpinic acid polymorph were recorded in acetone-d_6 on a Bruker Avance DPX-500 spectrometer. NMR spectra of pulveraven B (**2**) were recorded in acetone-d_6 on a Bruker 600 cryo-probe spectrometer. IR

spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer. UV spectra were recorded on a HP 8453 UV/Vis diode array spectrophotometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. High-resolution mass spectra were measured on a Bruker-Magnex BioAPEX spectrometer with electrospray ionization. HPLC was carried out on a Waters 600 model system with a 2487 Dual λ absorbance detector, except for pulveraven A (**1**), in which a Waters 510 model system was used. X-ray crystallographic data were recorded on a KappaCCD (with Oxford Cryostream) diffractometer using M*o* Kα radiation (λ = 0.71073 Å).

Fungal Material. Specimens were collected on the Black Creek Trail in the De Soto National Forest, Mississippi, on July 10, 1999, by the Gulf States Mycological Society (GSMS). Mycologists present at the GSMS Spring 1999 foray identified the specimens. A voucher specimen (62920) is housed in the Pullen Herbarium of the University of Mississippi (MISS).

Extraction and Isolation. Fresh fungal material, consisting of two mushrooms weighing 8.96 g, was exhaustively extracted at room temperature with 95% EtOH and dried under vacuum to yield 823 mg of crude extract, a bright yellow solid. The crude extract was divided into MeOH-soluble and -insoluble fractions. A portion (266 mg) of the MeOH-soluble fraction was purified by HPLC using a NH_2 column [5 μm, 250 × 10 mm, EtOAc/MeOH, 80:20, 10 mL/min, detection at 254 nm]. The fraction (3.8 mg) eluting at 9.2 min was further purified by C_{18} HPLC [4 μm, 3.9 × 150 mm, MeOH/H_2O, 70:30, with 0.1% acetic acid, 1.5 mL/min, detection at 256 nm] and provided pulveraven A (**1**) (1.7 mg), which eluted at 3.39 min.

Another portion (41 mg) of the MeOH-soluble material from the crude extract was fractionated into 39 200 mL fractions using a chromatotron coated with a 2 mm thick layer of silica gel GF_{254} [fractions 1−22 100% EtOAc, fractions 23−33 EtOAc/MeOH, 75:25, fractions 34−39 EtOAc/MeOH, detection at 254 nm]. Fractions 9−15 were combined (3.5 mg) and further fractionated using C_{18} HPLC [4 μm, 3.9 × 150 mm, 1.5 mL/min, detection at 228 nm] starting with 5 min isocratic 100% H_2O, followed by a gradient to obtain MeOHCH_3CN/H_2O with 0.1% acetic acid, 30:30:40, at 20 min. The fraction (1.1 mg) eluting at 14.6 min was purified by C_{18} HPLC [5 μm, 250 × 9.4 mm, MeOH/H_2O, 60:40, 2.5 mL/min, detection at 256 nm] and provided **2** (0.5 mg), which eluted at 31.63 min. Fraction 27 (5 mg) from the chromatotron was further purified using a C_{18} Bond Elute (500 mg). Fraction 1 (2.8 mg) eluted with 100% H_2O and was further purified using C_{18} HPLC [4 μm, 3.9 × 150 mm, 25% MeOH/CH_3CN/H_2O with 0.1% acetic acid, 25:30:45, 1.5 mL/min, detection at 228 nm], thus providing the vulpinic acid polymorph (0.05 mg), eluting at 5.09 min, and vulpinic acid (**3**) (0.2 mg), eluting at 8.08 min. This final separation was repeated several times. Fractions with comparable ^{1}H NMR spectra in d_6-acetone were combined to yield amounts suitable for additional NMR analysis (compound **3**, 2.0 mg; vulpinic acid polymorph, 1.6 mg).

Pulveraven A (1): yellowish-white solid, $[\alpha]_D$ −92.3° (*c* 0.11, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 225sh and 300 (4.30) nm; IR (NaCl disk) ν_{max} 3086, 3029, 2952, 1735, 1704, 1602, 1495, 1453, 1288, 1228, 1164, 1065, 697 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 427.1564 (calcd for $C_{27}H_{23}O_5$, 427.1545).

Pulveraven B (2): whitish orange solid, $[\alpha]_D$ −35.8° (*c* 0.05, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 220sh, 304 (3.75), and 361 (8.22) nm; IR (NaCl disk) ν_{max} 3061, 3028, 2953, 2942, 2853, 1722, 1563, 1494, 1452, 1433, 1274, 1168, 1128, 1073, 1032, 696 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 427.1564 (calcd for $C_{27}H_{23}O_5$, 427.1545).

Vulpinic acid (3): yellow fragment (MeOH) [lit.[14] mp 150−151 °C]; $[\alpha]_D$ −19.2° (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ϵ) 290 (9.21), 374 (3.73) nm [lit.[14] (EtOH)/nm 234 (ϵ 11986), 276 (ϵ 10318), 366 (ϵ 11106)]; IR (NaCl disk) ν_{max} 2952, 2923, 2854, 1770, 1712, 1679, 1573, 1494, 1436, 1303, 1274, 1062, 898, 781, 692 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; crystal data, see Tables 3, 4; HRESIMS *m*/*z* 323.0905 (calcd for $C_{19}H_{15}O_5$, 323.0919).

Vulpinic acid polymorph: yellow needle (MeOH); $[\alpha]_D$ +1.6° (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ) 290 (4.03), 374 (3.55) nm; IR (NaCl disk) ν_{max} 2923, 2854, 1704, 1556, 1494, 1425, 1299, 1230, 1143, 1031, 900, 782, 763, 694, 620 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; crystal data, see Tables 3, 5; HRESIMS *m*/*z* 323.0900 (calcd for $C_{19}H_{15}O_5$, 323.0919).

Evaluation of COX-1 and COX-2 Activity by Quantitation of PGE_2. The effect of test compounds on COX activity was determined by measuring PGE_2 production. Reaction mixtures were prepared in 100 mM Tris-HCl buffer, pH 8.0, containing 1 μM heme, 500 μM phenol, 300 μM epinephrine, sufficient amounts of COX-1 or COX-2 to generate 150 ng of PGE_2/mL, and various concentrations of test samples. The reaction was initiated by the addition of arachidonic acid (final concentration, 10 μM) and incubated for 10 min at room temperature (final volume, 200 μL). Then, the reaction was terminated by adding 20 μL of the reaction mixture to 180 μL of 27.8 μM indomethacin, and PGE_2 was quantitated by an ELISA method. Samples were diluted to the desired concentration with 100 mM potassium phosphate buffer (pH 7.4) containing 2.34% NaCl, 0.1% bovine serum albumin, 0.01% sodium azide, and 0.9 mM Na_4EDTA. Following transfer to a 96-well plate (Nunc-Immuno Plate Maxisorp, Fisher) coated with a goat anti-mouse IgG (Jackson Immuno Research Laboratories), the tracer (PGE_2-acetylcholinesterase, Cayman Chemical, Ann Arbor, MI) and primary antibody (mouse anti-PGE_2, Monsanto, St. Louis, MO) were added. Plates were then incubated at room temperature overnight, reaction mixtures were removed, and wells were washed with a solution of 10 mM potassium phosphate buffer (pH 7.4) containing 0.01% sodium azide and 0.05% Tween 20. Ellman's reagent (200 μL) was added to each well, and the plate was incubated at 37 °C for 3−5 h, until the control wells yielded an optical density of 0.5−1.0 at 412 nm. A standard curve with PGE_2 (Cayman Chemical, Ann Arbor, MI) was generated on the same plate, which was used to quantify the PGE_2 levels produced in the presence of test samples. Results were expressed as a percentage, relative to control (solvent-treated) samples, and dose−response curves were constructed for the determination of IC_{50} values.

Mouse Mammary Organ Culture Assay. The inhibition of preneoplastic lesion formation in mouse mammary organ culture was performed as previously described.[24] BALB/c female mice (4 weeks old; Charles River, Wilmington, MA) were pretreated for 9 days with 1 μg of estradiol and 1 mg of progesterone. On the tenth day, the mice were sacrificed and the second thoracic mammary glands were dissected on silk and transferred to 60 mm culture dishes containing 5 mL of Waymouth's 752/1 MB medium supplemented with 100 units of streptomycin (100 μg/mL), penicillin (100 units/mL), and glutamin (35 μg/mL). The glands were incubated for 10 days (37 °C, 95% O_2 + 5% CO_2) in the presence of growth-promoting hormones (5 μg of insulin, 5 μg of prolactin, 1 μg of aldosterone, and 1 μg of hydrocortisone per mL of medium). Glands were exposed to 2 μg/mL 7,12-dimethylbenz[*a*]anthracene between 72 and 96 h. After exposure, glands were rinsed and transferred to new dishes with fresh medium. The fully differentiated glands were then permitted to regress by withdrawing all hormones except insulin for 14 additional days. For initial evaluation, test compounds were present in the medium during days 1−10 of culture (10 μg/mL); mammary glands were scored for incidence of lesions. For agents found to be active under these conditions, mammary glands (15 per treatment) were incubated with test agent concentrations ranging from 0.01 to 10 μg/mL. The glands were scored for the presence or absence of alveolar lesions.

Crystallographic data for the structure(s) reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

Acknowledgment. The authors would like to thank Dr. Nikolaus H. Fischer, The University of Mississippi, for helpful discussions and suggestions; Dr. D. Chuck Dunbar and Mr. Frank Wiggers, Thad Cochran National Center for Natural Products Research, for NMR and HRESIMS instrumental assistance; Dr. Helena Kovacs, Bruker Switzerland, for providing NMR spectra (600 MHz); Profs. Peter Wipf and David N. Beratan and Mr. Gustavo Moura, The University of Pittsburgh, for optical rotation computations; the mycologists and members of the Gulf States Mycological Society for providing *P. ravenelii*; and the Thad Cochran National Center for Natural Product Research, for the antimicrobial assay and a graduate student stipend. This work was supported in part by program project P01 CA48112 awarded by the NCI.

Supporting Information Available: ^{1}H, ^{13}C NMR and HRESIMS data for **1** and **2** material are available free of charge via the Internet at http://pubs.acs.org.

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