Sesquiterpene Quinones and Related Metabolites from *Phyllosticta spinarum*, a Fungal Strain Endophytic in *Platycladus orientalis* of the Sonoran Desert[1]

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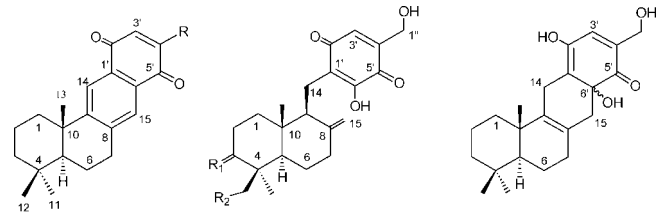
Five new metabolites, (+)-(5*S*,10*S*)-4′-hydroxymethylcyclozonarone (**1**), 3-ketotauranin (**3**), 3α-hydroxytauranin (**4**), 12-hydroxytauranin (**5**), and phyllospinarone (**6**), together with tauranin (**2**), were isolated from *Phyllosticta spinarum*, a fungal strain endophytic in *Platycladus orientalis*. The structures of the new compounds were determined on the basis of their 1D and 2D NMR spectroscopic data and chemical interconversions. All compounds were evaluated for inhibition of cell proliferation in a panel of five cancer cell lines, and only tauranin (**2**) showed activity. When tested in a flow cytometry-based assay, tauranin induced apoptosis in PC-3M and NIH 3T3 cell lines.

Plant-associated fungal strains are a rich source of structurally diverse and biologically active natural products.[2] In a continuation of our studies on plant-associated microorganisms of the Sonoran Desert for potential anticancer agents,[1] we have investigated an antiproliferative EtOAc extract of *Phyllosticta spinarum* (Botryosphaeriaceae), a fungal strain endophytic in the leaf tissue of oriental arbor-vitae (*Platycladus orientalis*; Cupressaceae), which is cultivated as an ornamental in southeastern Arizona. In this paper we report the isolation and structure elucidation of five new metabolites, (+)-(5*S*,10*S*)-4′-hydroxymethylcyclozonarone (**1**), 3-ketotauranin (**3**), 3α-hydroxytauranin (**4**), 12-hydroxytauranin (**5**), and phyllospinarone (**6**), along with tauranin (**2**). We also report the antiproliferative and apoptotic activity of tauranin (**2**) toward several cancer cell lines. Previous investigations of fungal strains of the genus *Phyllosticta* have resulted in the isolation of phycarone,[3,4] elsinochromes A−C,[5] phyllosinol,[6] phyllostine,[7] phyllostine II,[8] phyllosticta III,[8] several derivatives of cyclohex-2-en-1-one,[9,10] brefeldin A,[11] PM-toxins B and C,[12] and cholesterol.[13] Tauranin (**2**), a sesquiterpene quinone previously encountered in the mycelium of *Oospora aurantia*, a mold that grows on seeds of Japanese tea (*Thea japonica*),[14] has been reported to inhibit cholesterol biosynthesis.[15] It is noteworthy that a number of marine-derived sesquiterpene quinones have been reported to display inhibitory properties toward tyrosine kinases involved in cell signaling and proliferation.[16] This is the first report of metabolites from *P. spinarum* and the potential anticancer activity of tauranin (**2**).

Results and Discussion

Bioassay-guided fractionation of an antiproliferative EtOAc extract of *P. spinarum* involving solvent−solvent partitioning, size-exclusion chromatography, and preparative TLC furnished **1**−**6**. Compound **1** was obtained as a red crystalline solid that analyzed for $C_{22}H_{26}O_3$ by a combination of HRFABMS, ^{13}C NMR, and DEPT spectra and indicated 10 degrees of unsaturation. Its IR spectrum had absorption bands at 3425, 1690, and 1657 cm^{-1}, suggesting the presence of hydroxyl and carbonyl groups. Absorption bands at 272, 362, and 432 nm in its UV spectrum were



indicative of a 1,4-naphthoquinone moiety.[17] The presence of a 2(or 3),6,7-trisubstituted 1,4-naphthoquinone moiety in **1** was supported by its ^1H NMR signals at δ 7.97 (1H, s), 7.32 (1H, s), and 6.96 (1H, s) and the ^{13}C NMR signals for two carbonyl carbons (δ 181.7 and 178.8) and eight aromatic carbons (δ 153.2, 145.2, 141.4, 137.3, 131.4, 130.9, 128.6, and 127.5). A detailed analysis of the ^{13}C NMR spectrum of **1** with the help of HSQC data revealed the presence of three methyl carbons (δ 33.2, 24.4, and 21.6), six methylene carbons (δ 60.4, 41.4, 38.4, 30.9, 18.9, and 18.5) of which one is oxygenated, one methine carbon (δ 49.9), and two quaternary carbons (δ 38.2 and 33.5), in addition to the carbons assignable to a 1,4-naphthoquinone moiety (see above). The $^1H-^1H$ correlations observed in the DQF-COSY spectrum suggested the presence of $CH_2CH_2CH_2$ and CH_2CH_2CH spin systems. The ^1H and ^{13}C NMR spectroscopic data of **1** resembled those of (+)-cyclozonarone (**7**),[18] except that in **1** a CH_2OH group was found in place of the aromatic proton at δ 6.90 (d, J = 10.3 Hz), suggesting that **1** is a derivative of **7**. In the HMBC spectrum (Figure 1), the aromatic proton at δ 7.97 showed correlations with the quaternary carbon at δ_C 38.2, two quaternary aromatic carbons at δ_C 145.2 and 131.4, and the carbonyl carbon at δ_C 178.8. Thus, the remaining carbonyl carbon signal at δ 181.7 in the ^{13}C NMR spectrum of **1** was assigned to C-5′. The proton at δ 6.96 showed HMBC correlations with C-7 (δ 30.9), C-9 (δ 153.2), and C-1′ (δ 128.6), placing this proton at C-15. The presence of HMBC correlations from H_2-1″ (δ_H 4.49) to the carbonyl carbon (C-5′, δ_C 181.7), and the protonated aromatic carbon (C-3′, δ_C 141.4), unambiguously placed the CH_2OH group at C-4′. The absolute configuration of the naturally occurring (−)-cyclozonarone[19] has been established as 5*R*,10*R* by comparison of its spectroscopic data and optical rotation (−89.1) with those of (+)-cyclozonarone.[20] The optical rotation of **1** (+87.2) suggested that it is a derivative of (+)-cyclozonarone. The structure of **1** was thus elucidated as

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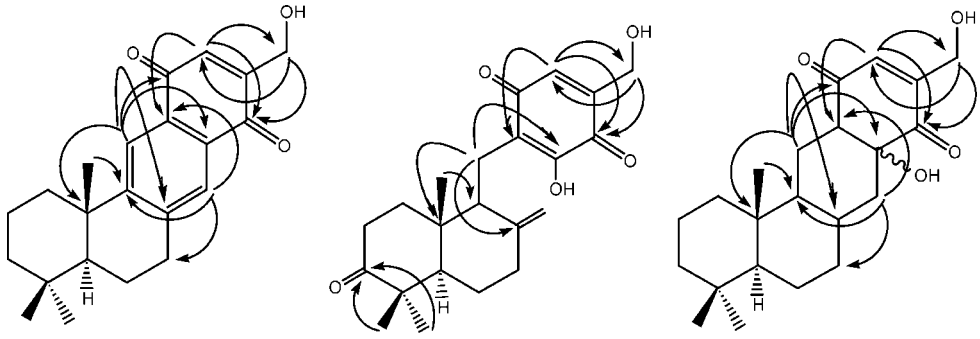

1 **3** **6**

Figure 1. Selected HMBC correlations for **1**, **3**, and **6**.

Table 1. ^{1}H NMR Data (500 MHz, $CDCl_3$) for Compounds **2**−**5**

	δ_H multiplicity (*J* in Hz)			
position	**2**	**3**	**4**	**5**
1	1.91 dt (12.8, 4.8) 1.39 m	2.33 m 1.93 dt (12.8, 6.5)	1.81 dt (12.6, 4.3) 1.52 dt (12.6, 4.3)	1.25–1.65 m
2	1.51 m 1.48 m	2.63 ddd (15.2, 12.8, 6.5) 2.44 m	1.36 dd (12.9, 4.3) 1.39 dd (12.9, 4.3)	1.25–1.65 m
3	1.51 m 1.21 dt (13.3, 3.9)		3.29 dd (11.3, 4.3)	1.25–1.65 m
5	1.16 dd (12.5, 2.0)	1.65 m	2.34 br d (10.4)	1.80 br d (14.2)
6	1.78 m 1.29 dd (12.5, 4.7)	1.66 m 1.48 dd (13.2, 4.3)	1.72 m 1.41 dd (12.9, 4.8)	1.62 m
7	2.27 br d (12.8) 1.90 dt (12.8, 4.7)	2.07 ddd (12.8, 6.6, 3.8) 1.93 dt (12.8, 4.3)	2.31 ddd (12.6, 4.6, 2.4) 1.90 dt (12.6, 4.8)	2.27 ddd (12.5, 4.2, 3.1) 1.94 dt (12.5, 5.0)
9	2.38 m	2.42 m	1.15 m	2.45 m
11	0.81 s	1.02 s	0.77 s	0.80 s
12	0.85 s	1.08 s	0.98 s	3.39 d (10.9) 3.10 d (10.9)
13	0.75 s	0.93 s	0.76 s	0.76 s
14	2.64 dd (13.8, 11.2) 2.52 dd (13.8, 2.7)	2.73 dd (13.9, 10.9) 2.49 dd (13.9, 2.8)	2.66 dd (13.9, 11.0) 2.50 dd (13.9, 2.9)	2.65 dd (13.9, 11.0) 2.53 dd (13.9, 3.1)
15	4.65 s	4.77 d (0.8) 4.74 d (0.8)	4.69 d (1.2) 4.67 d (1.2)	4.66 d (1.2)
3′	6.66 s	6.66 t (1.9)	6.65 t (1.7)	6.65 t (1.9)
1″	4.52 s	4.52 d (1.9)	4.52 d (1.7)	4.52 d (1.9)
OH-3			1.25 (brs)	
OH-6′	6.96 s	6.95 s	6.94 s	6.92 s

(+)-(5*S*,10*S*)-4′-hydroxymethylcyclozonarone. This is the first report of isolation of a (+)-cyclozonarone derivative from a natural source.

Compound **2** was identified as tauranin by comparison of its spectroscopic (UV, IR, ^{1}H NMR) and physical data with those reported in the literature.[14,21] However, the ^{1}H NMR spectrum of **2** was only partially assigned by these workers,[14,21] and its ^{13}C NMR data have not been reported previously. Since several metabolites encountered in this study appeared to be related to tauranin, it was necessary to completely assign its ^{1}H and ^{13}C NMR data. This was done with the help of HMBC correlations, and these data are presented in Tables 1 and 2, respectively. The molecular formula of compound **3** was determined as $C_{22}H_{28}O_5$ from its HRFABMS and ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C spectroscopic data of this compound (Tables 1 and 2, respectively) showed very close resemblances to those of tauranin (**2**), suggesting that they had the same carbon skeleton. The major difference was the absence of one of the CH_2 groups in **3**; instead, it showed the presence of a carbonyl group. Since the ^{1}H−^{1}H COSY spectrum of this compound indicated the presence of two moieties of CH_2CH_2, the carbonyl group must be located at either C-1 or C-3. Both methyl groups (δ_H 1.08 and 1.02) at C-4 showed HMBC correlations with the carbonyl carbon at δ 216.7, locating this group at C-3 (Figure 1). Thus, this compound was identified as 3-ketotauranin (**3**).

Compound **4** analyzed for $C_{22}H_{30}O_5$ by a combination of HRFABMS and ^{13}C NMR spectroscopy and indicated eight degrees of unsaturation. The ^{1}H and ^{13}C NMR spectroscopic data of **4** (Tables 1 and 2, respectively) were found to be similar to those of **3**, indicating that they contained the same carbon skeleton. The only difference observed in the ^{1}H NMR spectrum of **4** was the presence of two additional proton signals assignable to a CH(OH) group, suggesting that **4** may be derived from 3-ketotauranin (**3**) by reduction of the carbonyl group at C-3 to a secondary alcohol moiety. This was further confirmed by its ^{13}C NMR spectrum, which

Table 2. ^{13}C NMR Data (125 MHz, $CDCl_3$) for Compounds **2**−**5**

	δ_C			
position	**2**[a]	**3**[a]	**4**[a]	**5**
1	38.8 CH_2	37.8 CH_2	36.8 CH_2	38.1
2	19.5 CH_2	34.8 CH_2	28.0 CH_2	18.8
3	42.0 CH_2	216.7 C	78.7 CH	38.0
4	33.6 C	39.7 C	39.2 C	35.4
5	55.4 CH	55.0 CH	54.6 CH	48.6
6	24.5 CH_2	25.1 CH_2	24.0 CH_2	24.2
7	38.3 CH_2	37.3 CH_2	38.1 CH_2	38.3
8	148.9 C	147.3 C	148.1 C	148.5
9	54.4 CH	53.4 CH	54.1 CH	54.3
10	40.1 C	47.8 C	39.8 C	40.1
11	21.7 CH_3	21.7 CH_3	15.4 CH_3	17.6
12	33.6 CH_3	26.1 CH_3	28.3 CH_3	72.2
13	14.1 CH_3	13.8 CH_3	14.1 CH_3	14.6
14	19.1 CH_2	19.4 CH_2	19.2 CH_2	19.1
15	106.6 CH_2	107.9 CH_2	107.5 CH_2	106.8
1′	122.3 C	121.4 C	121.9 C	122.2
2′	187.6 C	187.4 C	187.5 C	187.5
3′	133.9 CH	133.9 CH	133.9 CH	133.9
4′	142.3 C	142.5 C	142.3 C	142.3
5′	183.1 C	183.0 C	183.2 C	183.1
6′	151.0 C	151.0 C	150.9 C	151.0
1″	58.8 CH_2	58.4 CH_2	58.8 CH_2	58.9

[a] Multiplicities from HSQC and DEPT.

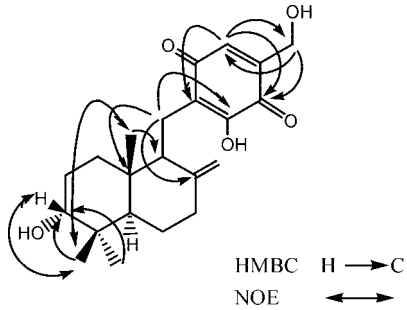

Figure 2. Selected HMBC and NOE correlations for **4**.

lacked the signal due to the carbonyl carbon at δ 216.7; instead, it showed the presence of an oxygenated methine carbon at δ 78.7. In the HMBC spectrum of **4** (Figure 2), both methyl groups (δ_H 0.77 and 0.98) at C-4 showed correlations with this oxygenated methine carbon, confirming the placement of the OH group at C-3. Irradiation of the methyl signal at δ_H 0.98 (CH_3-12) showed enhancement of the signals at δ 0.76 (CH_3-13) and 3.29 (H-3), indicating that both these methyl groups and the proton attached to the oxygenated carbon (H-3) must have the same relative configuration. Since CH_3-13 has a β-orientation, the proton at C-3 also should have the same β-configuration, leading to the identification of this compound as 3α-hydroxytauranin (**4**).

Compound **5** was obtained as a yellow, amorphous solid that analyzed for $C_{22}H_{30}O_5$ by a combination of HRFABMS and ^{13}C NMR data. The ^1H and ^{13}C NMR data of **5** (Tables 1 and 2, respectively) showed very close resemblances to those of tauranin (**2**), the major difference being the absence of the signals (δ_H, 0.85 s; δ_C, 33.6) due to CH_3-12 and the presence of signals [δ_H, 3.39 and 3.10 (each 1H, d, J = 10.9); δ_C, 72.2] due to a CH_2OH group. Thus, this metabolite was identified as 12-hydroxytauranin (**5**).

The molecular formula of phyllospinarone (**6**) was determined as $C_{22}H_{30}O_4$ from its HRFABMS and ^{13}C NMR data and indicated eight degrees of unsaturation. Its IR spectrum had absorption bands at 3420, 1658, and 1632 cm^{-1}, indicating the presence of hydroxyl, conjugated carbonyl, and olefinic groups. The ^1H NMR spectrum of **6** revealed the presence of an olefinic proton [δ 7.04 (t, J = 1.5 Hz)], three methyl groups on tertiary carbons (δ 1.05, 0.93, and 0.88), an oxygenated CH_2 group attached to an olefinic carbon (δ 4.44, dd, J = 5.3, 1.5 Hz), a CH_2 in a cyclic system sandwiched by two double bonds [δ 3.37 and 2.87 (d each, J = 16.4 Hz)], an allylic CH_2 in a cyclic system [δ 2.31 and 2.01 (d each, J = 17.2 Hz)], 11 protons assignable to $CH_2CH_2CH_2$ and CH_2CH_2CH spin systems, and 3 OH protons (δ 7.18, 4.22, and 4.03). The ^{13}C NMR spectrum, while confirming the above structural moieties in **6**, suggested the presence of a conjugated carbonyl carbon (δ 182.2), six sp^2 carbons [δ 149.8 (CH), 142.1 (C), 136.9 (C), 135.6 (C), 131.5 (C), and 123.8 (C)], and three quaternary sp^3 carbons (δ 68.2, 39.2, and 34.5), of which one is oxygenated (δ 68.2). These data suggested that **6** contains a tetracyclic structure similar to that of 4′-hydroxymethylcyclozonarone (**1**). In the HMBC spectrum (Figure 1), the proton at δ_H 2.31 showed correlations with C-7 (δ_C 33.0), C-9 (δ_C 136.9), and C-1′ (δ_C 131.5), suggesting that it should be attached to C-15 (δ_C 46.2). HMBC correlations of the signal at δ_H 3.37 with C-10 (δ_C 39.2), C-8 (δ_C 123.8), and C-6′ (δ_C 68.2) and with the oxygenated carbon at δ_C 142.1 allowed the assignment of the proton at δ_H 3.37 to H-14 and the oxygenated carbon at δ_C 142.1 to C-2′. The olefinic proton at δ_H 7.04 showed HMBC correlations with C-1′ (δ_C 131.5), C-1″ (δ_C 59.6), and the carbonyl carbon at δ_C 182.2, assigning it to H-3′ and the carbonyl carbon to C-5′. The CH_2OH group was placed at C-4′ with the help of the HMBC correlations of the signal at δ_H 4.44 to C-3′ (δ_C 149.8) and C-5′ (δ_C 182.2). Finally, prolonged exposure of phyllospinarone (**6**) to air gave 4′-hydroxymethylcyclozonarone (**1**), suggesting that **6** should have the same carbon skeleton as **1**. The structure of phyllospinarone was thus established as 7a,11-dihydroxy-9-hydroxymethyl-1,2,3,4,4a,5,6,7,7a,12,12b-undecahydro-4,4,12b-trimethylbenz[*a*]anthracen-8-one (**6**). The co-occurrence of 4′-hydroxymethylcyclozonarone (**1**), tauranin (**2**), and phyllospinarone (**6**) in *P. spinarum* is significant, as **6** may be considered an intermediate in the biosynthesis of naturally occurring tetracyclic benz[*a*]anthraquinones related to 4′-hydroxymethylcyclozonarone (**1**) from sesquiterpene quinones.

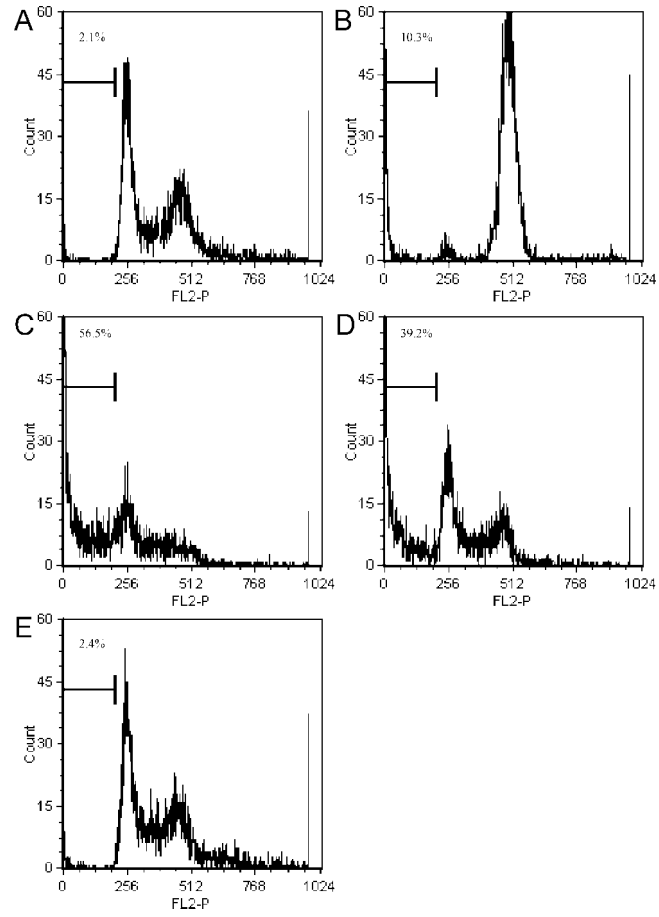

Figure 3. Induction of apoptosis by toxic concentrations of tauranin (**2**) after 24 h treatment in PC-3M: (A) DMSO control, (B) nocodazole (0.5 μg/mL), (C) **2** (10.0 μM), (D) **2** (5.0 μM), (E) **2** (2.5 μM). Marker indicates percentage of sub-G_1 apoptotic cells.

Compounds **1**−**6** were evaluated for *in vitro* antiproliferative activity against a panel of five sentinel cancer cell lines, NCI-H460 (non small cell lung), MCF-7 (breast), SF-268 (CNS glioma), PC-3 M (prostate), and MIA Pa Ca-2 (pancreatic). Cells were exposed to serial dilutions of test compounds for 72 h in RPMI 1640 media supplemented with 10% fetal bovine serum and glutamax, and cell viability was evaluated by the MTT assay.[22] Only tauranin (**2**) showed antiproliferative activity against the cancer cell lines tested (Table 3); surprisingly, all other compounds were found to be inactive at concentrations up to 5 μM, suggesting that subtle structural changes may result in significant differences in the level of antiproliferative activity displayed by these sesquiterpene quinones. In an attempt to elucidate the mechanism(s) responsible for the antiproliferative activity of tauranin, drug-sensitive human prostate cancer (PC-3M) and mouse fibroblast (NIH 3T3) cells were subjected to the cell cycle analysis and apoptosis induction analysis using flow cytometry. Apoptosis is a common cellular response to cytotoxic agents, and the accumulation of sub-G_1 cells is considered a hallmark in apoptotic cell death.[23] Treatment with tauranin (**2**) at cytotoxic concentrations (2.5−10.0 μM) resulted in a significant and dose-dependent accumulation of PC-3M cells in the sub-G_1

Table 3. Antiproliferative Activity of Tauranin (**2**) against a Panel of Human Solid Tumor Cell Lines[a]

	cell line[b]				
compound	NCI-H460	MCF-7	SF-268	PC-3M	MIA Pa Ca-2
2	4.3	1.5	1.8	3.5	2.8
Dox	0.01	0.07	0.04	1.11	ND

[a] Results are expressed as IC_{50} values in μM: ND, not determined. [b] Key: NCI-H460, non small cell lung cancer; MCF-7, breast cancer; SF-268, CNS cancer (glioma); PC-3M, metastatic prostate cancer; MIA Pa Ca-2, pancreatic carcinoma. Doxorubicin (Dox) was used as positive control.

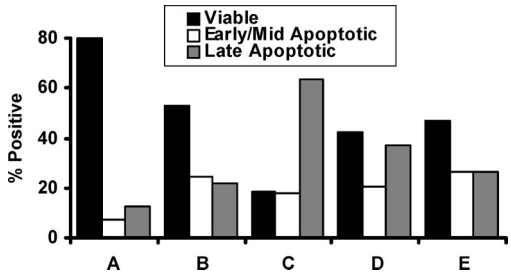

Figure 4. Toxic concentrations of tauranin (**2**) lead to apoptosis in NIH 3T3 after a 24 h exposure. NIH 3T3 cells were stained with Guava Multi-Caspase kit according to manufacturer's instructions. (A) DMSO, (B) nocodazole (0.50 μg/mL), (C) **2** at 5.00 μM, (D) **2** at 2.50 μM, (E) **2** at 1.25 μM.

phase (Figure 3); similar behavior was also observed toward the NIH 3T3 cells (data not shown). NIH 3T3 cells, which showed an increased sensitivity to **2**, were then subjected to a cytometry-based caspase activation assay to determine the relative amount of cells committed to apoptosis. In this assay, the cell viability was determined by exclusion of 7-AAD (7-amino-actinomycin D), a stain unable to permeate healthy and early to midapoptotic cells, but which readily enters late-stage apoptotic and dead cells; a cell-permeable fluorochrome-conjugated inhibitor of caspases, sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK), binds covalently to caspases that have been activated in cells committed to the apoptotic pathway, resulting in a fluorescent signal proportional to the number of active caspases within the cell.[24] Tauranin (**2**) treatment resulted in an increase of NIH 3T3 cells undergoing apoptosis in a dose-dependent manner, indicating cytotoxicity and progression toward cell death (Figure 4). Although the toxicity of tauranin is not specific to cancer cells, its ability to induce apoptosis within 24 h at low μM concentrations warrants its further evaluation as a potential anticancer agent.

Experimental Section

General Experimental Procedures. Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO Dip-370 digital polarimeter using $CHCl_3$ as solvent. UV spectra were recorded on a Shimadzu UV-1601 UV–vis spectrophotometer. IR spectra for KBr disks were recorded on a Shimadzu FTIR-8300 spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ or acetone-d_6 with a Bruker DRX-500 instrument at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR using residual $CHCl_3$ or acetone as internal standard. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. Low-resolution and high-resolution MS were recorded respectively on Shimadzu LCMS- QP8000α and JEOL HX110A mass spectrometers. Whatman LRP-2 was used for reversed-phase column chromatography, and reversed-phase preparative TLC was performed on Merck RP-18 F_{254S} precoated aluminum sheets.

Isolation and Identification of the Fungal Strain. Healthy, asymptomatic foliage of one individual of *Platycladus orientalis* was harvested at the Campus Arboreum at the University of Arizona (32.231° N, 110.952° W, elevation 787 m; mean annual temperature = 20.2 °C) in April 2005.[25] This site, which is located within the Sonoran Desert bioregion, is relatively xeric, receiving an average of 30.5 cm of precipitation annually. Plant tissue was washed in tap water and then surface-sterilized by sequential immersion in 95% ethanol (10 s), 10% Clorox (0.5% NaOCl, 2 min), and 70% ethanol (2 min). Following treatment, the foliage was cut into small pieces (ca. 2 mm in length) and transferred under sterile conditions to 2% malt extract agar (MEA), which encourages growth of diverse endophytic fungi.[25] Plates were sealed with Parafilm and incubated up to two months at room temperature under ambient light. One of the emergent fungi was subcultured on 2% MEA, photographed, and deposited as a living voucher in the Robert L. Gilbertson Mycological Herbarium at the University of Arizona (ARIZ) under the accession number BA 9149b-4. Because endophytic fungi frequently lack reproductive structures in culture, this isolate could not be identified beyond the level of phylum on the basis of morphology alone. We therefore isolated total genomic DNA from the mycelium following our previously described procedure[26] and used primers ITS1F and LR3 to amplify and sequence the internal transcribed spacer regions and 5.8s gene (ITS) and the first 600 bp of the nuclear ribosomal large subunit (LSU). LSU data were integrated into existing alignments for the Dothideomycetes (Ascomycota) based on highest-affinity matches obtained through BLAST searches of the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) and FASTA searches implemented through the Fungal Metagenomics Project (http://biotech.inbre.alaska.edu/fungal-metagenomics). Subsequent phylogenetic analyses indicated the taxonomic placement of BA 9149b-4 within *Phyllosticta spinarum,* a finding confirmed by ITS-based matches using GenBank and FASTA.[25] The mycelial growth of this isolate is consistent with *Phyllosticta,* but reproductive structures could not be obtained in culture.

Extraction and Isolation. A culture of *P. spinarum* (BA 9149b-4) grown in PDB (2 L) was filtered, and the filtrate was extracted with EtOAc (5 × 500 mL). Evaporation under reduced pressure afforded the EtOAc extract (315.2 mg), which exhibited antiproliferative activity toward a panel of five cancer cell lines. A portion (300 mg) of the EtOAc extract was partitioned between hexane and 80% aqueous MeOH and the bioactive aqueous MeOH fraction was diluted to 50% aqueous MeOH by the addition of water and extracted with $CHCl_3$. Evaporation of solvents under reduced pressure yielded the hexane (25.2 mg), $CHCl_3$ (245 mg), and 50% aqueous MeOH (25.8 mg) fractions. A portion (240 mg) of the bioactive $CHCl_3$ fraction was subjected to gel-permeation chromatography over a column of Sephadex LH-20 (20 g) made up in hexane−CH_2Cl_2 (1:4) and eluted with hexane−CH_2Cl_2 (1:4) (400 mL), CH_2Cl_2−acetone (3:2) (150 mL), CH_2Cl_2−MeOH (1:1) (100 mL), and finally with MeOH (250 mL). Sixteen fractions (F_1−F_{16}) of 25 mL of each were collected with hexane−CH_2Cl_2 (1:4). The remaining fractions were found to be similar by TLC and therefore combined to give one major fraction (F_{17}). Preparative TLC (silica gel, CH_2Cl_2) of fraction F_4 (4.7 mg) yielded **1** (2.3 mg; R_f, 0.6). Column chromatography of the combined fractions F_9−F_{11} (89.4 mg) on silica gel (3.0 g) and elution with CH_2Cl_2 followed by CH_2Cl_2 containing increasing amounts of MeOH afforded **2** (29.2 mg). Preparative TLC (silica gel, CH_2Cl_2−MeOH, 93:7) of F_5 (4.5 mg) gave **3** (1.6 mg; R_f, 0.5) as a yellow solid. Fraction F_{14} (35.7 mg) was separated on preparative TLC (silica gel, CH_2Cl_2−MeOH, 93:7) to give an orange solid (7.3 mg), which was further separated by preparative TLC (RP-18, MeOH−H_2O, 80:20) to give **4** (2.3 mg; R_f, 0.6) and **5** (1.4 mg; R_f, 0.5). Repeated preparative TLC purification of the fraction F_{13} (24.9 mg) furnished **6** [2.3 mg; R_f, 0.5 (RP-18, MeOH-H_2O, 18:15)].

(+)-(5*S*,10*S*)-4′-Hydroxymethylcyclozonarone (1): red crystalline solid; mp 163 °C (dec); $[\alpha]^{25}_D$ +87.2 (*c* 0.1, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 432 (3.52), 362 (3.83), 272 (4.69) nm; IR (KBr) ν_{max} 3425, 2926, 1690, 1657, 1595, 1456, 1418, 1389, 1269, 1089, 924 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.97 (1H, s, H-14), 7.32 (1H, s, H-15), 6.96 (1H, s, H-3′), 4.49 (2H, d, J = 1.2 Hz, H-1″), 2.97 (1H, dd, J = 18.5 and 6.1 Hz, H-7a), 2.87 (1H, ddd, J = 18.5, 10.8, and 7.7 Hz, H-7b), 2.35 (1H, brd, J = 13.0 Hz, H-1a), 1.92 (1H, brdd, J = 13.5 and 7.8 Hz, H-6a), 1.77−1.63 (3H, m, H-2a, 2b, and 6b), 1.49 (1H, brd, J = 13.3 Hz, H-3a), 1.36 (1H, td, J = 13.0 and 3.9 Hz, H-1b), 1.27 (1H, dd, J = 12.6 and 2.4 Hz, H-5), 1.22 (1H, dd, J = 13.3 and 4.0 Hz, H-3b), 1.21 (3H, s, H-13), 0.98 (3H, s, H-12), 0.97 (3H, s, H-11); ^{13}C NMR ($CDCl_3$) δ 181.7 (C, C-5′), 178.8 (C, C-2′), 153.2 (C, C-9), 145.2 (C, C-8), 141.4 (CH, C-3′), 137.3 (C, C-4′), 131.4 (C, C-6′), 130.9 (CH, C-15), 128.6 (C, C-1′), 127.5 (CH, C-14), 60.4 (CH_2, C-1″), 49.9 (CH, C-5), 41.4 (CH_2, C-2), 38.4 (CH_2, C-1), 38.2 (C, C-10), 33.5 (C, C-4), 33.2 (CH_3, C-12), 30.9 (CH_2, C-7), 24.4 (CH_3, C-13), 21.6 (CH_3, C-11),

18.9 (CH_2, C-3), 18.5 (CH_2, C-6); HRFABMS *m/z* 339.1959 [M + 1]$^+$(calcd for $C_{22}H_{27}O_3$, 339.1960).

Tauranin (2): yellow solid; mp 153 °C (dec); $[\alpha]^{25}_D$ −139.5 (*c* 0.13, $CHCl_3$); $[\alpha]^{25}_D$ −149.1 (*c* 0.1, MeOH); lit[14] $[\alpha]^{21}_D$ −148 (*c* 0.10, MeOH); UV (EtOH) λ_{max} (log ϵ) 416 (3.93), 268 (4.95) nm; IR (KBr) ν_{max} 3314, 2928, 1653, 1638, 1618, 1460, 1344, 1280, 1192, 1086, 901 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; APCIMS (+) ve mode *m/z* 359 [M + 1]$^+$; APCIMS (−) ve mode *m/z* 358 [M]$^+$.

3-Ketotauranin (3): yellow, amorphous solid; $[\alpha]^{25}_D$ −130.2 (*c* 0.12, MeOH); UV (EtOH) λ_{max} (log ϵ) 410 (4.11), 269 (5.12) nm; IR (KBr) ν_{max} 3324, 2926, 1650, 1634, 1620, 1465, 1346, 1285, 1187, 809 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HRFABMS *m/z* 371.1872 [M − 1]$^-$ (calcd for $C_{22}H_{27}O_5$, 371.1859).

3α-Hydroxytauranin (4): yellow, amorphous solid; $[\alpha]^{25}_D$ +139.5 (*c* 0.13, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 416 (3.91), 269 (4.95) nm; IR (KBr) ν_{max} 3410, 2930, 1654, 1636, 1622, 1465, 1330, 1270, 1180, 1083, 807 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HRFABMS *m/z* 373.2022 [M − 1]$^-$ (calcd for $C_{22}H_{29}O_5$, 373.2015).

12-Hydroxytauranin (5): yellow, amorphous solid; $[\alpha]^{25}_D$ −91.5 (*c* 0.05, MeOH); UV (EtOH) λ_{max} (log ϵ) 416 (3.01), 268 (4.03) nm; IR (KBr) ν_{max} 3420, 2928, 1652, 1642, 1620, 1460, 1350, 1286, 1087, 900 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HRFABMS *m/z* 373.2024 [M − 1]$^-$ (calcd for $C_{22}H_{29}O_5$, 373.2015).

Phyllospinarone (6): white, amorphous solid; UV (EtOH) λ_{max} (log ϵ) 318 (4.38), 250 (4.77) nm; IR (KBr) ν_{max} 3420, 2926, 1658, 1632, 1480, 1372, 1287, 1087 cm^{-1}; ^{1}H NMR (acetone-d_6) δ 7.18 (1H, s, OH-2′), 7.04 (1H, t, J = 1.5 Hz, H-3′), 4.44 (2H, dd, J = 5.3 and 1.5 Hz, H-1″a and 1″b), 4.22 (1H, s, OH-6′), 4.03 (1H, t, J = 5.3 Hz, OH-1″), 3.37 (1H, brd, J = 16.4 Hz, H-14a), 2.87 (1H, d, J = 16.4 Hz, H-14b), 2.31 (1H, d, J = 17.2 Hz, H-15a), 2.01 (1H, d, J = 17.2 Hz, H-15b), 1.98–1.88 (3H, m, H-1a, H-7a, and H-7b), 1.73 (1H, m, H-6a), 1.69 (1H, dt, J = 13.8 and 3.5 Hz, H-3a), 1.51 (1H, dt, J = 13.8 and 3.4 Hz, H-3b), 1.47–1.42 (2H, m, H-2a and H-6b), 1.28 (1H, dd, J = 12.6 and 1.9 Hz, H-5), 1.22 (1H, dt, J = 13.2 and 4.0 Hz, H-2b), 1.20 (1H, dt, J = 13.7 and 3.7 Hz, H-1b), 1.05 (3H, s, CH_3-13), 0.93 (3H, s, CH_3-12), 0.88 (3H, s, CH_3-11); ^{13}C NMR (acetone-d_6) δ 182.2 (C, C-5′), 149.8 (CH, C-3′), 142.1 (C, C-2′), 136.9 (C, C-9), 135.6 (C, 4′), 131.5 (C, C-1′), 123.8 (C, C-8), 68.2 (C, C-6′), 59.6 (CH_2, C-1″), 53.0 (CH, C-5), 46.2 (CH_2, C-15), 43.1 (CH_2, C-2), 39.2 (C, C-10), 37.7 (CH_2, C-1), 34.5 (C, C-4), 34.3 (CH_3, C-12), 33.0 (CH_2, C-7), 23.9 (CH_2, C-14), 22.6 (CH_3, C-11), 20.5 (CH_3, C-13), 20.3 (CH_2, C-3), 20.2 (CH_2, C-6); HRFABMS *m/z* 359.2220 [M + 1]$^+$ (calcd for $C_{22}H_{31}O_4$, 359.2222).

Bioassay for Antiproliferative Activity. The tetrazolium-based colorimetric assay (MTT assay)[22] was used for the *in vitro* assay to measure inhibition of proliferation of non small cell lung cancer (NCI-H460), breast cancer (MCF-7), CNS glioma (SF-268), prostate cancer (PC-3M), and pancreatic carcinoma (MIA Pa Ca-2) cell lines as previously reported.[27] All samples for assays were dissolved in DMSO.

Apoptosis Assay. The ability of tauranine (**2**) to induce apoptosis in PC-3M and NIH 3T3 was assayed via flow cytometry. PC-3M cells were harvested from flasks at approximately 75% confluency, seeded into six-well plates at a density of 2.5 × 10^5 cells/well, and allowed to reattach overnight in a 5% CO_2 atmosphere incubator at 37 °C. NIH 3T3 cells were harvested from flasks at 100% confluency to achieve synchronization, seeded into six-well plates at a density of 3.0 × 10^5 cells/well, and allowed to reattach overnight in a 10% CO_2 atmosphere incubator at 37 °C. Treatment solutions were prepared in RPMI 1640 (PC-3M) or DMEM (NIH 3T3) containing 10% fetal bovine serum, penicillin (100 IU/mL), streptomycin (50 μg/mL), and Glutamax (2 mM). Test samples of tauranine (**2**) and nocodazole were prepared in DMSO. After cell adhesion, medium was changed to treatment solutions containing either 0.1% DMSO (negative control), 0.5 μg/mL nocodazole (positive control), or various concentrations of tauranine. Floating and adherent cells were harvested by trypsinization, resuspended in complete media, and washed twice with PBS. DNA content of PC-3M was measured with Guava cell cycle reagent according to manufacturer's specifications. Percentage of NIH 3T3 involved in the apoptotic pathway was determined with the Guava PCA-96 MultiCaspase detection kit according to the manufacturer's instructions. Fluorescently labeled PC-3M and NIH 3T3 cells were acquired on a Guava EasyCyte using CytoSoft software.

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