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Geopyxins A–E, *ent*-Kaurane Diterpenoids from Endolichenic Fungal Strains *Geopyxis* aff. *majalis* and *Geopyxis* sp. AZ0066: Structure–Activity Relationships of Geopyxins and Their Analogues[1]

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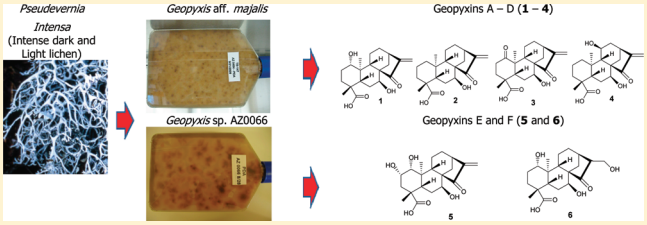
Supporting Information

ABSTRACT: Four new *ent*-kaurane diterpenoids, geopyxins A–D (**1**–**4**), were isolated from *Geopyxis* aff. *majalis*, a fungus occurring in the lichen *Pseudevernia intensa*, whereas *Geopyxis* sp. AZ0066 inhabiting the same host afforded two new *ent*-kaurane diterpenoids, geopyxins E and F (**5** and **6**), together with **1** and **3**. The structures of **1**–**6** were established on the basis of their spectroscopic data, while the absolute configurations were assigned using modified Mosher's ester method. Methylation of **1**–**3**, **5**, and **6** gave their corresponding methyl esters **7**–**11**. On acetylation, **1** and **7** yielded their corresponding monoacetates **12** and **14** and diacetates **13** and **15**. All compounds were evaluated for their cytotoxic and heat-shock induction activities. Compounds **2**, **7**–**10**, **12**, **14**, and **15** showed cytotoxic activity in the low micromolar range against all five cancer cell lines tested, but only compounds **7**–**9**, **14**, and **15** were found to activate the heat-shock response at similar concentrations. From a preliminary structure–activity perspective, the electrophilic α,β-unsaturated ketone carbonyl motif present in all compounds except **6** and **11** was found to be necessary but not sufficient for both cytotoxicity and heat-shock activation.



Recent studies have demonstrated that endolichenic fungi are rich sources of structurally diverse small-molecule natural products, some with interesting biological activities.[2] In our continuing search for bioactive and/or novel metabolites of plant- and lichen-associated fungi of the Sonoran Desert bioregion,[3] we have been particularly interested in the identification of compounds that activate the heat-shock response as a novel mode of anticancer activity.[4] Unlike most normal tissues, cancers must endure high levels of stress from the profound alterations in protein homeostasis caused by malignant transformation. Powerful innate adaptive mechanisms, especially the ancient, highly conserved transcriptional heat-shock response, are activated in cancers to enable survival under these conditions.[5] Natural products that further tax the heat-shock response may overwhelm the ability to cope and could provide leads for the development of new, broadly effective anticancer drugs.[6,7]

Here we report the investigation of two endolichenic fungal strains, *Geopyxis* aff. *majalis* and *Geopyxis* sp. AZ0066, inhabiting the live thallus of the intense dark and light lichen *Pseudevernia intensa* (Nyl.) Hale & W. L. Culb. (Parmeliaceae). This work led to the isolation and characterization of the new *ent*-kaurane diterpenes geopyxins A–D (**1**–**4**) from *G.* aff. *majalis* and geopyxins E (**5**) and F (**6**) together with geopyxins A (**1**) and C (**3**) from *Geopyxis* sp. AZ0066. This constitutes the first report of metabolites from a fungal strain of the genus *Geopyxis*. The cytotoxic and heat-shock-inducing activities of geopyxin B (**2**) prompted us to prepare several analogues of the geopyxins (**1**–**3**, **5**, and **6**), which were available to us in sufficient quantities, and evaluate these in a limited structure–activity relationship study. The outcome of this study supports our previous findings that diverse species from plants to fungi elaborate structurally complex secondary metabolites capable of acting on the stress responses of heterologous organisms.[6,7] From a practical drug discovery perspective, it further validates our heat-shock-guided approach to identifying natural products and their analogues that can target the malignant phenotype by altering protein homeostasis.

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Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectroscopic Data of Compounds 1−6 in $CDCl_3$

	1		2		3		4		5		6	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	81.8 (CH)	3.50, dd (11.4, 4.9) (β-H)	39.8 (CH_2)	1.85, m (α-H) 0.93, td (13.2, 4.8) (β-H)	213.6 (C)		39.7 (CH_2)	1.90, m (α-H) 1.18, m (β-H)	86.3 (CH)	3.06, d (9.1) (β-H)	81.8 (CH)	3.37, dd (11.3, 4.8) (β-H)
2	30.2 (CH_2)	1.60, m 1.93, m	18.2 (CH_2)	1.45, m 1.68, m	35.6 (CH_2)	3.19, dt (14.0, 6.4) 2.15, ddd (14.0, 4.6, 2.4)	18.8 (CH_2)	1.49, m 1.85, m	68.7 (CH)	3.79, m (β-H)	29.6 (CH_2)	1.85−1.91, m 1.54, m
3	35.4 (CH_2)	1.24, td (14.3, 4.4) (α-H) 2.14, dt (14.3, 3.5) (β-H)	37.4 (CH_2)	1.10, td (13.2, 4.0) (α-H) 2.16, brd (13.2) (β-H)	38.1 (CH_2)	1.53, td (13.8, 4.6) (α-H) 2.51, ddd (13.8, 6.4, 2.4) (β-H)	37.4 (CH_2)	1.14, dd (13.4, 4.5) (α-H) 2.15, dt (13.4, 4.8) (β-H)	43.4 (CH_2)	1.15, t (12.4) (α-H) 2.34, dd (12.4, 4.8) (β-H)	35.6 (CH_2)	1.12, m (α-H) 2.05, dd (13.4, 3.1) (β-H)
4	42.8 (C)		43.2 (C)		43.2 (C)		43.2 (C)		43.4 (C)		42.4 (C)	
5	45.0 (CH)	2.10, d (8.0) (β-H)	46.4 (CH)	2.02,m (β-H)	46.9 (CH)	2.38, dd (11.2, 3.6) (β-H)	46.0 (CH)	2.10, dd (12.9, 2.1) (β-H)	44.4 (CH)	2.01, m (β-H)	44.2 (CH)	1.80, m (β-H)
6	27.1 (CH_2)	1.94−1.98, m 1.94−1.98, m	27.3 (CH_2)	1.90, m 2.02, m	27.6 (CH_2)	2.03, m 2.03, m	27.2 (CH_2)	1.89, m 2.04, ddd (14.4, 3.4, 2.4)	26.9 (CH_2)	1.90−1.97, m 1.90−1.97, m	26.9 (CH_2)	1.85−1.91, m 1.85−1.91, m
7	72.1 (CH)	3.84, d (2.8) (α-H)	72.3 (CH)	3.91, dd (3.4, 2.0) (α-H)	71.6 (CH)	3.88, brs (α-H)	72.2 (CH)	3.94, dd (3.4, 2.0) (α-H)	72.2 (CH)	3.79, brs (α-H)	71.8 (CH)	3.76, brs (α-H)
8	53.5 (C)		53.1 (C)		52.3 (C)		51.1 (C)		53.3 (C)		54.4 (C)	
9	47.3 (CH)	2.09, m (β-H)	46.2 (CH)	1.82, d (7.9) (β-H)	37.9 (CH)	2.56, d (6.4) (β-H)	58.3 (CH)	1.95, s (β-H)	47.1 (CH)	2.02, d (7.8) (β-H)	47.1 (CH)	1.85−1.91, m (β-H)
10	44.5 (C)		40.0 (C)		54.3 (C)		38.5 (C)		44.9 (C)		44.6 (C)	
11	20.7 (CH_2)	1.48, m (α-H) 2.93, m (β-H)	18.8 (CH_2)	1.45, m (α-H) 1.83, m (β-H)	21.5 (CH_2)	1.65, m (α-H) 1.75, m (β-H)	66.1 (CH)	4.10, d (4.2) (α-H)	20.5 (CH_2)	1.45, m (α-H) 2.95, dd (15.7, 5.2) (β-H)	20.8 (CH_2)	1.15, m (α-H) 2.89, dd (16.1, 5.7) (β-H)
12	32.9 (CH_2)	1.63, m 1.95, m	32.6 (CH_2)	1.69, td (15.6, 6.0) 1.90, m	32.7 (CH_2)	1.71, m 1.81, m	41.2 (CH_2)	1.94, m 2.15, m	32.8 (CH_2)	1.59, brd (14.6) 1.90−1.97, m	25.5 (CH_2)	1.54, m 1.78, m
13	38.3 (CH)	3.06, d (2.3) (α-H)	38.0 (CH)	3.06, brd (2.4) (α-H)	37.8 (CH)	3.06, brs (α-H)	36.9 (CH)	3.05, d (3.4) (α-H)	38.2 (CH)	3.01, brs (α-H)	32.6 (CH)	2.57, d (3.8) (α-H)
14	35.3 (CH_2)	2.25, d (11.8) (α-H) 1.46, m (β-H)	34.8 (CH_2)	2.28, d (12.0) (α-H) 1.45, m (β-H)	35.1 (CH_2)	2.12, d (12.0) (α-H) 1.50, m (β-H)	34.8 (CH_2)	2.27, d (12.0) (α-H) 1.47, m (β-H)	35.3 (CH_2)	2.19, d (11.7) (α-H) 1.45, m (β-H)	36.1 (CH_2)	2.28, d (11.7) (α-H) 1.40, d (11.7) (β-H)
15	214.9 (C)		214.3 (C)		213.9 (C)		213.3 (C)		215.0 (C)		229.1 (C)	
16	149.5 (C)		149.1 (C)		148.7 (C)		150.0 (C)		149.4 (C)		56.0 (CH)	2.55, dd (6.8, 5.8)
17	115.6 (CH_2)	5.30, s 5.96, s	116.0 (CH_2)	5.31, s 5.98, s	116.6 (CH_2)	5.34, s 6.01, s	113.8 (CH_2)	5.30, s 5.88, s	115.8 (CH_2)	5.27, s 5. 92, s	59.1 (CH_2)	3.56, dd (11.1, 6.8) 3.89, dd (11.1, 5.8)
18	28.5 (CH_3)	1.22, s	28.7 (CH_3)	1.25, s	27.5 (CH_3)	1.30, s	28.7 (CH_3)	1.26, s	28.4 (CH_3)	1.20, s	28.3 (CH_3)	1.11, s
19	183.6 (C)		184.2 (C)		183.2 (C)		182.8 (C)		180.4 (C)		180.1 (C)	
20	11.5 (CH_3)	1.12, s	15.7 (CH_3)	1.02, s	15.5 (CH_3)	1.41, s	15.8 (CH_3)	0.96, s	12.7 (CH_3)	1.08, s	11.3 (CH_3)	1.04, s

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Although *ent*-kauranes of fungal origin are rare and reported mainly from *Gibberella fujikuroi* and *Phaeosphaeria* sp. L487,[8] this class of diterpenoids has frequently been encountered in several plant families,[9a] and a recent report has suggested that in important crop plants such as rice and maize some *ent*-kauranes function as phytoalexins with potent antifungal and antifeedant activities.[9b] In addition, *ent*-kauranes are of considerable interest because of their cytotoxic,[10−13] anti-inflammatory,[14−16] antiangiogenic,[17] anti-HIV,[18] antioxidant,[19] anticholinesterase,[19] SIRT1 (silent information regulator two ortholog 1) inhibitory,[20] antibacterial,[21] NF-κB activation inhibitory,[22] and apoptosis-inducing[23] activities.

RESULTS AND DISCUSSION

Geopyxin A (**1**), obtained as a white, amorphous solid, analyzed for $C_{20}H_{28}O_5$ by a combination of HRESIMS and ^{13}C NMR spectroscopy. The UV λ_{max} at 238 nm indicated the presence of an α,β-unsaturated ketone carbonyl chromophore.[24] Its IR spectrum had absorption bands at 3489, 1706, and 1641 cm^{-1}, suggesting the presence of OH, carboxylic acid carbonyl, and α,β-unsaturated ketone carbonyl functionalities. The ^1H and ^{13}C NMR spectra together with HSQC data of **1** (Table 1) showed the presence of two tertiary methyls (δ_H 1.22 s, 1.12 s and δ_C 28.5, 11.5), seven methylenes, of which one is olefinic (δ_H 5.96 s, 5.30 s; δ_C 115.6), five methines, of which two are oxygenated [δ_H 3.84 d (J = 2.8 Hz, H-7), 3.50 dd (J = 11.4, 4.9 Hz, H-1); δ_C 72.1, 81.8] and one is allylic [δ_H 3.06 d (J = 2.3 Hz); δ_C 38.3], six quaternary carbons, of which one is olefinic (δ_C 149.5) and two are carbonyls (δ_C 214.9 and 183.6), and a set of aliphatic methylene and methine protons. Because an exocyclic methylene and two carbonyls accounted for three degrees of unsaturation, **1** should possess a tetracyclic framework. The presence of three spin systems, −CH(O)-CH_2CH_2−, −$CHCH_2$CH(O)−, and −$CHCH_2CH_2CHCH_2$−, in **1** was evident from its $^1H-^1H$ COSY spectrum (Figure 1). The connectivity of the above three spin systems, tertiary methyls, and nonprotonated carbons was established by the analysis of the HMBC correlations (Figure 1) to constitute an *ent*-kaurane skeleton. The tertiary methyl group at δ 1.12 showed HMBC correlations with the oxygenated carbon at δ 81.8 (C-1) and methine carbons at δ 45.0 (C-5) and 47.3 (C-9), placing it at C-10. The remaining tertiary methyl group at δ 1.22 assigned to CH_3-18[25] showed HMBC correlations with C-3 (δ 35.4), C-5 (δ 45.0), and the carboxylic acid carbonyl carbon (δ 183.6), placing methyl and carboxylic acid groups at C-4. The oxymethine proton at δ 3.50 showed HMBC correlations with C-5, C-9 (δ 47.3), and C-20 (δ 11.5), placing it at C-1. The remaining oxymethine proton at δ 3.84 showed HMBC correlations with C-5, C-9, C-14 (δ 35.3), and the carbonyl carbon C-15 (δ 214.9), placing it at C-7. Methylation (CH_3I/K_2CO_3/acetone) of **1** gave its methyl ester (**7**), further confirming the presence of a carboxylic acid group, while acetylation (Ac_2O/pyridine) of **1** yielded its diacetate (**13**), suggesting the presence of two OH groups. The chemical shift of the carbonyl carbon (C-15; δ 214.9)[26] and its upfield shift to δ 206.5 as a result of acetylation suggested that the OH-7 has a β-orientation.[27] This was further confirmed by the application of modified Mosher's ester method as described below. The relative stereochemistry of **1** was determined with the help of ^1H NMR and NOEDIFF data (Figure 2) of its methyl ester (**7**). The coupling pattern of the H-1 (dd, J = 11.4, 5.3 Hz) suggested it to have an axial orientation.[28] Irradiation of this

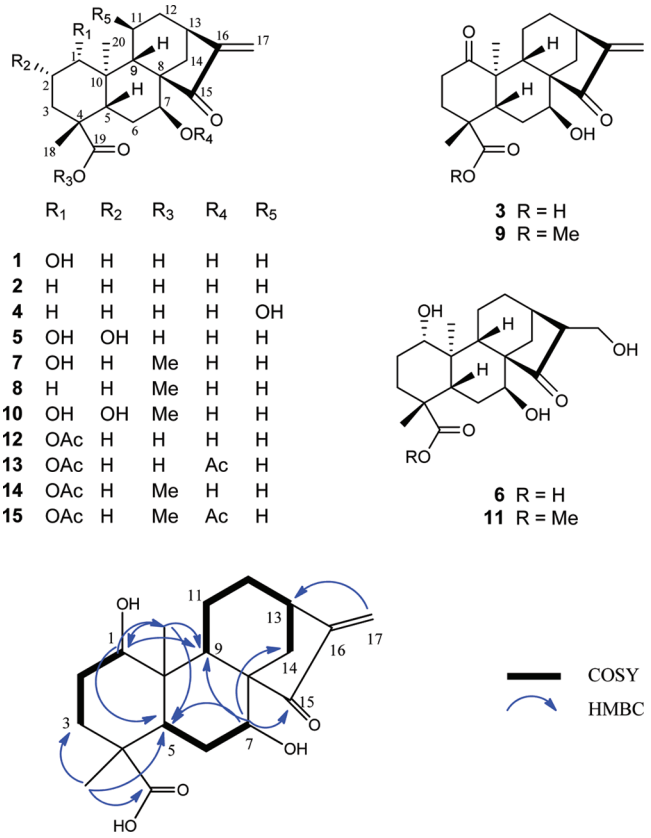

Figure 1. COSY and selected HMBC correlations for **1**.

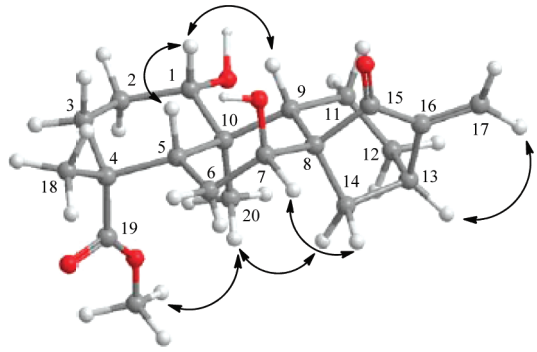

Figure 2. Selected NOEs observed in the NOEDIFF experiment of **7**.

proton led to significant enhancements of H-5 and H-9 signals, suggesting that these were also on the same side of the molecule and hence axially oriented. The β-orientation of the CH_3 group on C-4 was inferred from its ^{13}C chemical shift (δ 28.5),[29] which suggested that the carboxylic acid group is α-oriented; the ^{13}C chemical shift value for the α-CH_3 group at C-4 has been reported to be at δ 16.7.[30] The NOE data for **7** (Figure 2) confirmed that both CH_3-20 and H-14 at δ 2.22 have an α-orientation. The absolute stereochemistry of positions C-1 and C-7 of geopyxin A (**1**) were determined by the application of modified Mosher's ester method.[31] Reaction of **1** with (*R*)- and (*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl) afforded (*S*)- and (*R*)-bis-MTPA esters **1a** and **1b**, respectively (Figure 3). Analysis of $\Delta\delta$ ($\delta_S - \delta_R$) values confirmed the *S* absolute stereochemistry for both C-1 and C-7. Thus, the structure of geopyxin A was established as (1*S*,7*S*)-1,7-dihydroxy-15-oxo-*ent*-kaur-16-en-19-oic acid (**1**).

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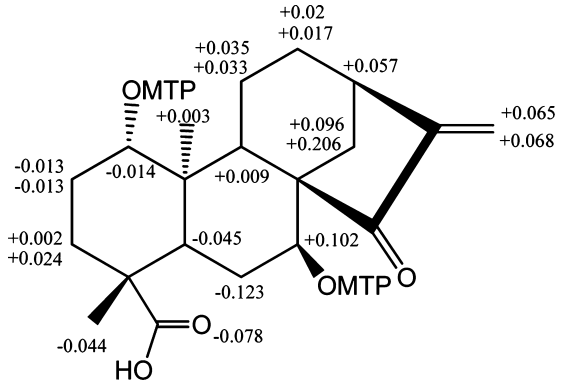

Figure 3. $\Delta\delta$ values [($\Delta\delta$ in ppm)= $\delta_S - \delta_R$] obtained for (*S*)- and (*R*)-bis MTPA esters (**1a** and **1b**, respectively) of geopyxin A (**1**).

Geopyxin B (**2**), obtained as a white, amorphous solid, analyzed for $C_{20}H_{28}O_4$ by a combination of HRESIMS and NMR data. The ^{1}H and ^{13}C NMR data of **2** (Table 1) were similar to those of **1**, and the molecular weight difference of 16 Da between these suggested that **1** was an oxygenated analogue of **2**. The major difference in NMR data was found to be the absence of the oxymethine group at δ_C 81.8, which was assigned to C-1 in **1**; instead **2** showed the presence of a methylene group (δ_C 39.8), which exhibited an HMBC correlation to CH_3-20 (δ 1.02). This was further confirmed by the upfield chemical shift of the two carbons (C-2 and C-10) adjacent to C-1 in **2** when compared with those of **1** (Table 1). The planar structure of **2** was thus determined to be 7-hydroxy-15-oxo-*ent*-kaur-16-en-19-oic acid. The absolute stereochemistry of C-7 was determined by the application of modified Mosher's ester method.[31] Reaction of geopyxin B with (*R*)- and (*S*)-MTPA-Cl afforded (*S*)- and (*R*)-MTPA esters **2a** and **2b**, respectively. Analysis of $\Delta\delta$ ($\delta_S - \delta_R$) values (Figure 4)

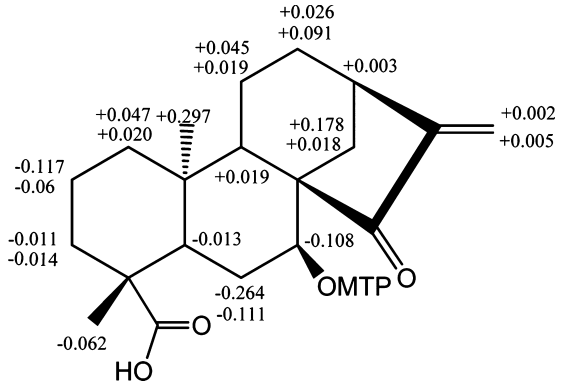

Figure 4. $\Delta\delta$ values [($\Delta\delta$ in ppm) = δ_S–δ_R] obtained for (*S*)- and (*R*)-MTPA esters (**2a** and **2b**, respectively) of geopyxin B (**2**).

confirmed the *S* absolute stereochemistry for C-7. On the basis of the above data, the structure of geopyxin B was established as (7*S*)-7-hydroxy-15-oxo-*ent*-kaur-16-en-19-oic acid (**2**).

Geopyxin C (**3**), obtained as a white, amorphous powder, analyzed for $C_{20}H_{26}O_5$ by a combination of HRESIMS and NMR data. The ^{1}H and ^{13}C NMR data of **3** (Table 1) were very similar to those of **1** except for the presence of an additional ketone carbonyl (δ_C 213.6) in **3** instead of an oxymethine group [δ_H 3.50 dd (J = 11.4, 4.9 Hz); δ_C 81.8] in **1**. Oxidation of **1** with pyridinium chlorochromate afforded a monoketo compound as the only product, which was identical with **3**. The ^{1}H NMR coupling pattern (brs) of the oxymethine proton at δ 3.88 suggested the presence of a 7β-OH group (see above) in **3**. Thus, geopyxin C was identified as (7*S*)-7-hydroxy-1,15-dioxo-*ent*-kaur-16-en-19-oic acid (**3**).

Geopyxin D (**4**), obtained as a white, amorphous powder, analyzed for $C_{20}H_{28}O_5$ by a combination of HRESIMS and ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR spectroscopic data of **4** (Table 1) closely resembled those of **2**. The only difference was the presence of an oxygenated methine group [δ_H 4.10 d (J = 4.2 Hz); δ_C 66.1] in **4** instead of a methylene group in **2**, suggesting that **4** is an oxygenated derivative of **2**. The difference in molecular formulas between these compounds confirmed that **4** contains an additional oxygen atom. The oxymethine proton at δ 4.12 showed HMBC correlations with C-13 (δ_C 36.9) and C-8 (δ_C 51.1) (Supporting Information, S1a) placing this additional OH group at C-11. Irradiation of CH_3-20 of **4** during the NOEDIFF experiment caused enhancement of H-11 (δ 4.10), H-14 (δ 2.27), and H-6 (δ 2.04), suggesting that they were α-oriented and hence 11-OH should be β-oriented (Supporting Information, S1b). Because compounds **1** and **4** presumably share a common biosynthetic origin, it is proposed that **4** should have the same configuration at C-7 as **1**. On the basis of this and the above observed NOE correlations the absolute configuration at C-11 of **4** was determined as *S*. Thus, geopyxin D was identified as (7*S*,11*S*)-7,11-dihydroxy-15-oxo-*ent*-kaur-16-en-19-oic acid (**4**).

Repeated chromatographic separation of the EtOAc extract of *Geopyxis* sp. AZ0066 over silica gel resulted in the isolation of two new compounds, geopyxins E and F (**5** and **6**), together with geopyxins A (**1**) and C (**3**). The molecular formula, $C_{20}H_{28}O_6$, was assigned to geopyxin E (**5**) on the basis of its HRESIMS and NMR data and indicated the presence of one more oxygen atom than geopyxin A (**1**). Comparison of ^{1}H and ^{13}C NMR data of **5** (Table 1) with those of **1** showed them to be similar with some minor differences. The significant difference was the presence of an oxygenated methine group (δ_C 68.7) in **5** replacing a methylene (δ_C 30.2) group in **1**. This was placed at C-2, due to the presence of the spin system $-CH(O)CH(O)CH_2-$ identified from its ^{1}H–^{1}H COSY spectrum. In the NOEDIFF experiment (Supporting Information, S2), irradiation of H-1 (δ 3.06) exhibited enhancement of H-2, indicating both protons were β-oriented and hence the OH group at C-2 should be α. Application of modified Mosher's ester method[31] for the determination of absolute stereochemistry at C-1 and C-2 of geopyxin E was not attempted, as related 1,2-bis-MTPA esters are reported to show interactions in $\Delta\delta_{S-R}$ effects among the modification sites due to their close proximity.[32] However, biosynthetically it could share a common origin with geopyxin A (**1**). This together with the NOE correlations observed above led to the identification of geopyxin E as (1*R*,2*S*,7*S*)-1,2,7-trihydroxy-15-oxo-*ent*-kaur-16-en-19-oic acid (**5**).

Geopyxin F (**6**), obtained as a white, amorphous powder, analyzed for $C_{20}H_{30}O_6$ by a combination of HRESIMS and ^{13}C NMR data. The ^{1}H and ^{13}C NMR data of **6** (Table 1) were similar to those of **1**, except for the absence of the *exo*-cyclic double bond in **6**. It showed the presence of an oxygenated methylene group, suggesting that it contained a $-CHCH_2OH$ group instead of a $-C{=}CH_2$ group in **1**. In the HMBC spectrum, oxygenated methylene protons of **6** showed correlations with the ketone carbonyl at δ 229.1 (C–15) and methine carbons at δ 32.6 (C-13) and 56.0 (C-16), confirming the above observation (Supporting Information, S3). The

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Table 2. Cytotoxic Activity of Compounds 2, 7−10, 12, 14, and 15 against a Panel of Five Cancer Cell Lines[a]

	cell line[b]				
compound	NCI-H460	SF-268	MCF-7	PC-3M	MDA-MB-231
2	2.25 ± 0.15	2.35 ± 0.31	4.32 ± 1.55	5.41 ± 0.91	3.31 ± 0.44
7	1.08 ± 0.15	0.72 ± 0.00	0.79 ± 0.04	2.27 ± 0.07	0.73 ± 0.14
8	0.92 ± 0.01	0.69 ± 0.06	0.63 ± 0.12	1.48 ± 0.11	0.91 ± 0.10
9	2.28 ± 0.10	1.56 ± 0.16	1.67 ± 0.45	3.63 ± 0.09	3.38 ± 0.37
10	2.27 ± 0.17	1.02 ± 0.03	2.08 ± 0.20	3.99 ± 0.34	3.81 ± 0.48
12	3.52 ± 0.25	2.37 ± 0.14	3.48 ± 0.20	3.88 ± 0.21	4.11 ± 0.15
14	0.82 ± 0.05	0.66 ± 0.02	0.53 ± 0.14	1.54 ± 0.04	1.15 ± 0.07
15	1.26 ± 0.05	1.18 ± 0.07	1.18 ± 0.07	2.31 ± 0.37	1.93 ± 0.10
doxorubicin	0.42 ± 0.30	0.62 ± 0.07	0.42 ± 0.13	0.24 ± 0.09	0.58 ± 0.23

[a]Results are expressed as IC_{50} values ± standard deviation in μM. Doxorubicin and DMSO were used as positive and negative controls. Compounds **1**, **3**−**6**, **11**, and **13** were found to be inactive at 5.0 μM. [b]Key: NCI-H460 = human non-small-cell lung cancer; SF-268 = human CNS cancer (glioma); MCF-7 = human breast cancer; PC-3M = metastatic human prostate adenocarcinoma; MDA-MB-231= human breast adenocarcinoma.

treatment of **6** with Ac_2O/pyridine gave the diacetate **13**, which was previously obtained by the acetylation of **1** (see above), suggesting that it was formed as a result of dehydration and acetylation of **6**. The upfield shift of both C-12 (δ_C 25.5) and the oxygenated C-17 (δ_C 59.1) due to a γ-steric compression effect between 12β-H and 16-CH_2OH as observed before for related *ent*-kauranes bearing a 16-CH_2OH[33] suggested the β-orientation of the hydroxymethylene group at C-16 of **6**. Although it is possible that **6** was formed from **1** as a result of the addition of a molecule of water during the workup, the absence of the C-16 epimer and the previous reports[33,34] of the natural occurrence of related *ent*-kauranes bearing 16α-CH_2OH suggested **6** to be a genuine natural product. Since acetylation of both geopyxin A (**1**) and geopyxin F (**6**) afforded the same product and **1** was determined to have an *S* configuration at C-1 and C-7, geopyxin F should also have the same absolute configuration at these two positions. Thus, geopyxin F was identified as (1*S*,7*S*)-1,7,17-trihydroxy-15-oxo-*ent*-kauran-19-oic acid (**6**).

Compounds **1**−**15** were evaluated for in vitro inhibition of cell proliferation/survival using a panel of five cancer cell lines, NCI-H460 (human nonsmall cell lung cancer), SF-268 (human CNS glioma), MCF-7 (human breast cancer), PC-3 M (human metastatic prostate adenocarcinoma), and MDA-MB-231 (human metastatic breast adenocarcinoma). Cells were exposed to serial dilutions of test compounds for 72 h in RPMI-1640 media supplemented with 10% fetal bovine serum, and relative viable cell number was measured by standard dye reduction assay using resazurin (AlamarBlue).[35] Among the natural *ent*-kauranes, only geopyxin B (**2**) was found to be cytotoxic (Table 2 and Supporting Information S4). In contrast, all methyl ester analogues except **11** showed cytotoxic activity at low micromolar concentrations, and among these 1-*O*-acetylmethylgeopyxin A (**14**) was the most active. However, no evidence for significant cell-type selectivity was observed. Compounds **1**−**15** were also evaluated for heat-shock-inducing activity over a range of concentrations using a heat-shock reporter cell line.[4,6] As demonstrated for a concentration of 2.5 μM, the natural *ent*-kaurane geopyxin B (**2**) showed weak activity, whereas its methyl ester **8** and 1-*O*-acetylmethylgeopyxin A (**14**) induced the greatest response (Figure 5). Heat-shock activation occurred in the same concentration range at which these compounds demonstrated cytotoxic activity against human cancer lines, but not all cytotoxic compounds induced a heat-shock response. This structure−activity relationship is consistent with our previous finding that heat-shock activation is

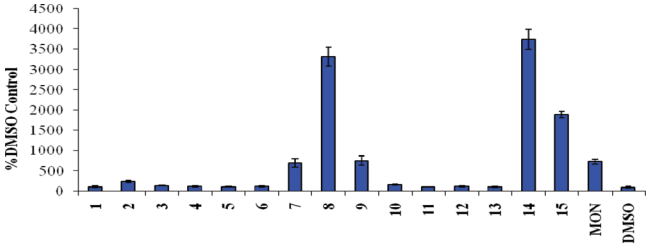

Figure 5. Cell-based heat-shock induction assay. The materials tested were DMSO (negative control), monocillin I (MON, positive control), and compounds **1**−**15**. All samples were tested at 2.5 μM except for MON, which was tested at 0.5 μM. The mean and standard deviation of triplicate determinations are presented, expressed as a percentage of the negative control; results are representative of three independent experiments.

not a general consequence of toxicity.[6] For example, conventional cytotoxic chemotherapeutics such as doxorubicin and cisplatin do not induce the response.[36] Instead, activation of the heat-shock response represents a specific response to disturbances of protein homeostasis within the cell. Consistent with this concept, we found that the α,β-unsaturated ketone carbonyl motif present in all compounds except **6** and **11** was necessary but not sufficient for both tumor cell growth inhibition and heat-shock activation. This electrophilic moiety possesses strong potential for Michael addition of the reactive cysteine residues exposed on many key regulatory proteins in cells. Rather than forming adducts with proteins indiscriminately, however, it is likely that these compounds selectively add nucleophilic residues, which are poised to react by virtue of local electronic and structural constraints as well as the particular pH maintained in specific intracellular compartments.[37] Some of these proteins are perhaps the fundamental electrophile sensors in cells that are among the first-line defenses for launching adaptive transcriptional and post-transcriptional responses.[38,39]

CONCLUSIONS

Here and in our previous studies we have found that the α,β-unsaturated ketone carbonyl moiety in these *ent*-kauranes and other small-molecule natural products is necessary, but not sufficient, for their biological activities. A number of compounds with this moiety were completely inactive in our assays. A plausible explanation may be that these molecules contain a structurally complex targeting moiety that confers

target selectivity and a more generic effector motif that confers thiol reactivity via Michael addition chemistry. We have seen similar structure−activity scenarios for several other heat-shock-active natural products encountered during our screening efforts.[6] The chemical reactivity of such heat-shock-inducing compounds makes elucidating their mechanisms of action challenging. While targets for some thiol-reactive molecules have been proposed, it is still unclear if these are the most biologically meaningful interactions. At high concentrations of compound, targeting may be swamped, and more promiscuous adduct formation can occur, leading to less relevant effects.[40,41] These natural products have been honed by nature over an evolutionary time scale to modulate the biology of diverse species by engaging the heat-shock response and perhaps other transcriptional responses that act system-wide to impact their survival. In this regard, they could prove very useful in probing the mechanisms responsible for maintaining protein homeostasis in the cell and perhaps serve as leads for the development of broadly effective anticancer drugs with novel modes of action.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in $CHCl_3$ with a JASCO Dip-370 digital polarimeter. UV spectra were recorded on a Shimadzu UV-1601 UV−vis spectrophotometer. IR spectra were recorded on a Shimadzu FTIR-8300 spectrometer using KBr disks. 1D and 2D NMR spectra were recorded in $CDCl_3$ with a Bruker Avance III 400 spectrometer at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR using residual $CHCl_3$ as an internal reference. Low-resolution and high-resolution MS were recorded on Shimadzu LCMS-QP8000α and JEOL HX110A spectrometers, respectively. Analytical and preparative thin layer chromatography (TLC) were performed on precoated 0.25 mm thick plates of silica gel 60 F_{254}; spraying with a solution of anisaldehyde in EtOH followed by heating was used to visualize compounds on analytical TLC. Preparative HPLC was performed on a Waters Delta Prep 4000 preparative chromatography system equipped with a Waters 996 photodiode array detector and a Waters Prep LC controller utilizing Empower Pro software and using a RP column (Phenomenex Luna 5 μm, C_{18}, 100 Å, 250 × 10 mm) with a flow rate of 3.0 mL/min; chromatograms were acquired at 254 and 270 nm.

Fungal Isolation and Identification. In June 2007, a healthy thallus of *Pseudevernia intensa* was collected from the lower branches of a mature individual of *Pseudotsuga menziesii* in a mixed coniferous forest ca. 9.5 km northwest of the Southwestern Research Station in the eastern Chiricahua Mountains of southeastern Arizona, USA (2100 m.a.s.l.; 31°53′00″ N, 109°12′18″ W).[42] The thallus was cut into 2 × 2 cm segments, washed in tap water, surface-sterilized by agitating sequentially in 95% EtOH for 30 s, 0.5% NaOCl for 2 min, and 70% EtOH for 2 min following previous procedures,[42] and then surface-dried under sterile conditions before cutting into 2 × 2 mm pieces. Pieces were plated on 2% malt extract agar (MEA) in a Petri plate. The plate was sealed with Parafilm and incubated under ambient light/dark conditions at room temperature (ca. 21.5 °C). Two of the emergent fungi were isolated into pure culture on 2% MEA, vouchered in sterile water, and deposited as living vouchers at the Robert L. Gilbertson Mycological Herbarium at the University of Arizona under accession nos. ARIZ0484 and ARIZ0066 (AZ0484, AZ0066). Total genomic DNA was isolated from fresh mycelium of each strain,[42] and the nuclear ribosomal internal transcribed spacers and 5.8s gene (ITS rDNA; ca. 600 bp) and an adjacent portion of the nuclear ribosomal large subunit (LSU rDNA; ca. 500 bp) were amplified as a single fragment by PCR. Positive amplicons were sequenced bidirectionally as described previously.[42]

Sequence data for these strains were aligned manually in Mesquite[43] with a core alignment containing 148 closely related endophytic, endolichenic, and saprotrophic fungi from the same site[42] and 19 described species of Pyronemataceae (Ascomycota) chosen on the basis of highest-affinity BLASTn matches to the GenBank non-redundant (nr) database in NCBI and recent phylogenetic analyses within the family.[44,45] Taxon sampling included seven strains of *Geopyxis* spp. corresponding to all available sequences in GenBank for the relevant loci (partial LSU rDNA), as well as nine representative genera within the family. Phylogenetic analyses using maximum likelihood were conducted in GARLI[46] using the GTR+I+G model of evolution as determined by ModelTest,[47] followed by 1000 bootstrap replicates to assess support for the resulting topology. The analysis placed AZ0484 and AZ0066 with strong support within *Geopyxis* (data not shown). The alignment then was trimmed to exclude (1) all redundant sequences of *Geopyxis* and (2) all terminal taxa placed outside of *Geopyxis,* except for three strains of *Trichophaea* and one of *Pulvinula,* used as outgroups or to clarify the boundaries of *Geopyxis* in a second analysis. Analysis of 38 strains as described above placed AZ0484 with strong support as sister to *Geopyxis majalis*; thus it was determined here as *Geopyxis* aff. *majalis* AZ0484. AZ0066 was placed with strong support within *Geopyxis,* albeit with unknown affinity at the species level. Thus it was determined here as *Geopyxis* sp. AZ0066 (Supporting Information, S5).

Cytotoxicity Assay. Relative cell growth and survival were measured in 96-well microplate format using the fluorescent detection of resazurin (AlamarBlue) dye reduction as an end point. Compounds were tested against human non-small-cell lung cancer (NCI-H460), CNS glioma (SF-268), breast cancer (MCF-7), metastatic prostate adenocarcinoma (PC-3M), and human metastatic breast adenocarcinoma (MDA-MB-231). Serial dilutions of test compounds or vehicle control (DMSO) were added to triplicate wells. After 72 h incubation, dye solution was added to each well (1:10 dilution). After brief agitation, incubation was continued for 4 h at 37 °C before data acquisition using a microplate fluorometer (Ex/Em: 560/590). Mean fluorescence intensity per well as a measure of relative viable cell number in compound-treated wells was compared to that of DMSO-treated wells. The conventional chemotherapeutic drug doxorubicin served as a positive control.

Heat-Shock Induction Assay (HSIA). Mouse fibroblasts stably transduced with a reporter construct encoding enhanced green fluorescent protein under the transcriptional control of a minimal consensus heat-shock element were used to measure the heat-shock-inducing activity of extracts and pure compounds as previously described.[25] Reporter cells were seeded into flat-bottom 96-well plates (Falcon; Becton Dickinson, Lincoln Park, NJ) at a density of 20 000 cells/well and allowed to adhere overnight (one column of wells was left empty to serve as a blank control). The following day, serial 2-fold dilutions of pure compounds or a single concentration of extracts and fractions was added to triplicate wells. DMSO vehicle alone (volume not to exceed 0.1%) served as a negative control. Cells were incubated overnight, the medium was removed, and wells were rinsed once with PBS, followed by addition of 150 μL of PBS to each well. Fluorescence was determined on an Analyst AD (LJL Biosystems) plate reader equipped with filter sets for excitation at 485 nm and emission at 525 nm.

Cultivation and Isolation of Metabolites of *Geopyxis* aff. *majalis*. A seed culture of *G.* aff. *majalis* (AZ0484) grown on PDA for two weeks was used for inoculation. Mycelia were scraped out, mixed with sterile water, and filtered through a 100 μm filter to separate spores from the mycelia. Absorbance of the spore solution was measured (at 600 nm) and adjusted to between 0.3 and 0.5. This spore solution was used to inoculate 4 × 2.0 L Erlenmeyer flasks, each holding 1.0 L of the medium (PDB) containing 0.25 mM $CuSO_4$ and incubated at 160 rpm and 28 °C. The glucose level in the medium was monitored using glucose strips (URISCAN glucose strips). On day 66, the strip gave a green color for the glucose test, indicating the absence of glucose in the medium. Mycelia were then separated by filtration, and the filtrate was extracted with EtOAc (3 × 2 L). The combined EtOAc layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give crude EtOAc extract (1.05 g), which showed activity in HSIA. A portion (938.0 mg) of this extract was partitioned between hexane and 80% aqueous MeOH. The

HSIA-active 80% aqueous MeOH fraction was diluted to 50% aqueous MeOH by addition of water and extracted with $CHCl_3$. The $CHCl_3$ fraction (795.0 mg) was then subjected to gel permeation chromatography over a column of Sephadex LH-20 (30 g) made up in hexanes/CH_2Cl_2 (1:4) and eluted with hexanes/CH_2Cl_2 (1:4, 500 mL), CH_2Cl_2/acetone (3:2, 400 mL), CH_2Cl_2/acetone (1:4, 400 mL), CH_2Cl_2/MeOH (1:1, 100 mL), and finally MeOH (100 mL). Of the 78 fractions (20 mL each) collected, those having similar TLC patterns were combined to give 18 major fractions [A (20.4 mg), B (28.6 mg), C (16.3 mg), D (12.4 mg), E (14.7 mg), F (15.4 mg), G (10.7 mg), H (47.5 mg), I (23.5 mg), J (20.9 mg), K (20.9 mg), L (135.7 mg), M (217.6 mg), N (24.9 mg), O (56.6 mg), P (21.1 mg), Q (15.9 mg), R (96.1 mg), and S (42.1 mg)]. Fraction M (100.0 mg) was chromatographed over a column of silica gel (4.0 g) made up in hexanes/EtOAc (4:1) and eluted with hexanes containing increasing amounts of EtOAc followed by EtOAc. The fractions eluted with hexanes/EtOAc (7:3) were combined to give **1** (55.2 mg). Fraction L (135.0 mg) was further fractionated on a column of silica gel (5.0 g) by elution with hexanes/Et_2O (1:1), hexanes/Et_2O (3:7), and Et_2O to give 55 fractions (6 mL each), and those having similar TLC patterns were combined to give 12 subfractions (F_1–F_{12}). Fraction F_2 (35.9 mg) was rechromatographed over a column of silica gel (1.0 g) made up in CH_2Cl_2/MeOH (100:2) and eluted with CH_2Cl_2/MeOH (100:2) followed by CH_2Cl_2/MeOH (100:4) to give seven subfractions (F_{2A}–F_{2G}). Subfractions F_{2B}–F_{2D} were combined (20.5 mg) and purified by RP-HPLC using 60% aqueous CH_3CN containing 0.25% HCOOH to give **2** (13.2 mg, t_R = 24 min). Subfraction F_4 on evaporation gave an additional quantity of **1** (29.9 mg). Further separation of subfraction F_5, (9.5 mg) by RP-HPLC using 65% aqueous CH_3CN containing 0.25% HCOOH gave **1** (1.5 mg, t_R = 9 min) and **4** (1.7 mg, t_R = 14 min). Fraction O was chromatographed over a column of silica gel (3.5 g) made up in CH_2Cl_2/MeOH (100:2) and eluted with CH_2Cl_2/MeOH (100:2) and CH_2Cl_2/MeOH (100:4) followed by MeOH to afford 40 (6 mL) fractions, and those having similar TLC patterns were combined to give 11 subfractions (F_1–F_{11}). Of these, F_2 and F_3 were combined (36.7 mg) and subjected to RP-HPLC using 50% aqueous CH_3CN containing 0.25% HCOOH to give **3** (21.2 mg, t_R = 17.5 min).

*Geopyxin A (**1**):* white, amorphous solid; $[\alpha]^{25}_D$ −79.9 (*c* 0.11, CH_3OH); UV (EtOH) λ_{max} (log ε) 238 (3.52) nm; IR (KBr) ν_{max} 3489, 3157, 2939, 1755, 1706, 1641, 1473, 1442, 1409, 1263, 1244, 1199, 1157, 1078, 1037, 987, 941 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 347.1862 $[M - H]^-$ (calcd for $C_{20}H_{27}O_5$, 347.1864).

*Geopyxin B (**2**):* white, amorphous solid; $[\alpha]^{25}_D$ −118.8 (*c* 0.11, CH_3OH); UV (EtOH) λ_{max} (log ε) 237 (3.07) nm; IR (KBr) ν_{max} 3467, 2931, 2864, 1708, 1641, 1462, 1441, 1263, 1238, 1164, 1078, 1045, 980, 941 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 331.1913 $[M - H]^-$ (calcd for $C_{20}H_{27}O_4$, 331.1915).

*Geopyxin C (**3**):* white, amorphous solid; $[\alpha]^{25}_D$ −112.7 (*c* 0.18, CH_3OH); UV (EtOH) λ_{max} (log ε) 238 (2.94) nm; IR (KBr) ν_{max} 3363, 2929, 2866, 1726, 1703, 1639, 1471, 1443, 1410, 1280, 1281, 1045, 982 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 347.1857 $[M + H]^+$ (calcd for $C_{20}H_{27}O_5$, 347.1853).

*Geopyxin D (**4**):* white, amorphous solid; $[\alpha]^{25}_D$ −49.2 (*c* 0.31, CH_3OH); UV (EtOH) λ_{max} (log ε) 236 (3.13) nm; IR (KBr) ν_{max} 3462, 2938, 1756, 1705, 1639, 1474, 1443, 1410, 1283, 1281, 1045, 982 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 347.1864 $[M - H]^-$ (calcd for $C_{20}H_{27}O_5$, 347.1864).

Cultivation and Isolation of Metabolites of *Geopyxis* sp. AZ0066. A seed culture of *Geopyxis* sp. AZ0066 grown on PDA for two weeks was used for inoculation. Mycelia were scraped out, mixed with sterile water, and filtered through a 100 μm filter to separate spores from the mycelia. Absorbance of the spore solution was measured (at 600 nm) and adjusted to between 0.3 and 0.5. This spore solution was used to inoculate 2 × 2.0 L Erlenmeyer flasks, each holding 1 L of the medium (PBD) and incubated at 160 rpm and 28 °C. The glucose level in the medium was monitored using glucose strips (URISCAN), and after 7 months, the strip gave a green color for the glucose test, indicating the absence of glucose in the medium. Mycelia were then separated by filtration, and the filtrate was extracted with EtOAc (3 × 1.2 L). The combined EtOAc layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give a crude EtOAc extract (2.42 g), which showed activity in HSIA. A portion (2.10 g) of this extract was chromatographed over a column of silica gel (50.0 g) made up in hexanes/EtOAc (7:3) and eluted with hexanes/EtOAc (7:3), hexanes/EtOAc (6:4), EtOAc, and EtOAc/MeOH (99:1) to afford 30 (150 mL) fractions (F_1–F_{30}). Fractions F_6–F_{17} (1.30 g) were found to contain geopyxin A (**1**) as the major compound (>95%). Chromatographic separation of fraction F_5 gave geopyxin C (**3**, 12.0 mg). Fraction F_{23} (220.0 mg) was further fractionated by column chromatography over silica gel (10.0 g) made up in CH_2Cl_2 and eluted with CH_2Cl_2 containing increasing amounts of MeOH to give 120 (8 mL) fractions, and those having similar TLC patterns were combined to give 14 subfractions (A–N). Subfraction E (30.0 mg) was purified by column chromatography over silica gel (1.0 g) using CH_2Cl_2/MeOH (98:2) as eluant to give **5** (25.9 mg). Fraction, F_{24} (195.0 mg) was subjected to column chromatography over silica gel (4.0 g) made up in CH_2Cl_2 and eluted with CH_2Cl_2 containing increasing amounts of MeOH to give **6** (161.9 mg).

*Geopyxin E (**5**):* white, amorphous solid; $[\alpha]^{25}_D$ − 93.5 (*c* 0.13, CH_3OH); UV (EtOH) λ_{max} (log ε) 239 (2.84) nm; IR (KBr) ν_{max} 3323, 2933, 2868, 1703, 1641, 1473, 1444, 1406, 1259, 1201, 1076, 1049, 983 945, 905, 733 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 363.1812 $[M - H]^-$ (calcd for $C_{20}H_{27}O_6$, 363.1813).

*Geopyxin F (**6**):* white, amorphous solid; $[\alpha]^{25}_D$ −63.3(*c* 0.10, CH_3OH); IR (KBr) ν_{max} 3423, 2941, 2872, 1709, 1641, 1445, 1412, 1250, 1213, 1161, 1070, 1022, 984, 735 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 365.1967 $[M - H]^-$ (calcd for $C_{20}H_{29}O_6$, 365.1970).

Preparation of Methyl Ester Derivatives. To a solution of **1** (10.0 mg) in acetone (0.5 mL) were added K_2CO_3 (20.0 mg) and CH_3I (0.2 mL), and the mixture was stirred at 25 °C. After 1 h the reaction mixture was filtered, and the filtrate was evaporated under reduced pressure to give methylgeopyxin A (**7**, 10.3 mg). Four other methyl ester derivatives, methylgeopyxin B (**8**), methylgeopyxin C (**9**), methylgeopyxin E (**10**), and methylgeopyxin F (**11**), were prepared from **2**, **3**, **5**, and **6**, respectively, in the same way as described above for the preparation of **7**.

*Methylgeopyxin A (**7**):* white, amorphous solid; IR (KBr) ν_{max} 3489, 3442, 2945, 2874, 1726, 1711, 1641, 1465, 1423, 1257, 1238, 1190, 1149, 1080, 1033, 975 cm^{-1}; ^1H NMR data, see Supporting Information S6; ^{13}C NMR data, see Supporting Information S7; APCIMS (−) mode *m*/*z* 361 $[M - H]^-$.

*Methylgeopyxin B (**8**):* white, amorphous solid; ^1H NMR data, see Supporting Information S6; ^{13}C NMR data, see Supporting Information S7; APCIMS (−) mode *m*/*z* 345 $[M - H]^-$.

*Methylgeopyxin C (**9**):* white, amorphous solid; ^1H NMR data, see Supporting Information S6; ^{13}C NMR data, see Supporting Information S7; APCIMS (+) mode *m*/*z* 343 $[M + H - H_2O]^+$.

*Methylgeopyxin E (**10**):* white, amorphous solid; ^1H NMR data, see Supporting Information S6; ^{13}C NMR data, see Supporting Information S7; APCIMS (+) mode *m*/*z* 379 $[M + H]^+$, 361 $[M + H - H_2O]^+$, 343 $[M + H - 2H_2O]^+$, 325 $[M + H - 3H_2O]^+$.

*Methylgeopyxin F (**11**):* white, amorphous solid; ^1H NMR data, see Supporting Information S6; ^{13}C NMR data, see Supporting Information S7; APCIMS (+) mode *m*/*z* 363 $[M + H - H_2O]^+$, 345 $[M + H - 2H_2O]^+$, 327 $[M + H - 3H_2O]^+$.

Acetylation of Geopyxin A. To a solution of **1** (30.0 mg) in pyridine (0.5 mL) was added Ac_2O (0.5 mL), and the mixture was stirred at 25 °C. After 48 h pyridine and excess Ac_2O were evaporated under reduced pressure and by adding EtOH, and the residue was chromatographed over a column of silica gel (1.0 g) made up in CH_2Cl_2 and eluted with CH_2Cl_2 containing increasing amounts of MeOH to give **12** (22.3 mg) and **13** (7.8 mg).

*1-O-Acetylgeopyxin A (**12**):* white, amorphous solid; IR (KBr) ν_{max} 3467, 2955, 2878, 1726, 1701, 1643, 1445, 1416, 1247, 1240, 1215, 1151, 1149, 1088, 1024, 984 cm^{-1}; ^1H NMR data, see Supporting Information S8; ^{13}C NMR data, see Supporting Information S9; APCIMS (−) mode *m*/*z* 389 $[M - H]^-$.

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*1,7-O-Diacetylgeopyxin A (**13**):* white, amorphous solid; ^{1}H NMR data, see Supporting Information S8; ^{13}C NMR data, see Supporting Information S9; APCIMS (−) mode *m/z* 431 $[M - H]^-$.

Oxidation of Geopyxin A. To a solution of **1** (10.0 mg) in dry CH_2Cl_2 (0.5 mL) at 0 °C was added pyridinium chlorochromate (30.0 mg), and the mixture was stirred at 0 °C. After 3 h the reaction mixture was chromatographed over a column of silica gel (200 mg) made up in CH_2Cl_2 and eluted with 2% MeOH in CH_2Cl_2 to give **3** (6.2 mg).

Acetylation of Methylgeopyxin A. To a solution of **7** (10.0 mg) in pyridine (0.5 mL) was added Ac_2O (0.2 mL), and the mixture was stirred at 25 °C. After 48 h pyridine and excess Ac_2O were evaporated under reduced pressure and by adding EtOH, and the residue was separated by preparative TLC (silica gel) using 8% MeOH in CH_2Cl_2 as eluant to give **14** (6.1 mg) and **15** (3.8 mg).

*1-O-Acetylmethylgeopyxin A (**14**):* white, amorphous solid; ^{1}H NMR data, see Supporting Information S8; ^{13}C NMR data, see Supporting Information S9; APCIMS (+) mode *m/z* 405 $[M + H]^+$.

*1,7-O-Diacetylmethylgeopyxin A (**15**):* white, amorphous solid; ^{1}H NMR data, see Supporting Information S8; ^{13}C NMR data, see Supporting Information S9; APCIMS (+) mode *m/z* 387 $[M + H - CH_3COOH]^+$.

Preparation of the (*S*)- and (*R*)-MTPA Ester Derivatives of Geopyxin A (1) by a Convenient Mosher Ester Procedure. Geopyxin A (**1**, 1.0 mg) was transferred into a clean NMR tube and was dried completely under the vacuum of an oil pump. Deuterated pyridine (0.5 mL) and (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl) (4.0 μL) were added into the NMR tube immediately under a stream of N_2, and then the NMR tube was shaken carefully to mix the sample and MTPA chloride evenly. The reaction in the NMR tube was monitored immediately by ^{1}H NMR and left at 25 °C for 14 h to give the bis-(*S*)-MTPA ester derivative (**1a**) of **1**. ^{1}H NMR data of **1a** (400 MHz, pyridine-d_5; data were assigned on the basis of the correlations of the ^{1}H−^{1}H COSY, HSQC, and HMBC spectra): δ 6.035 (1H, s, H-17a), 5.771 (1H, dd, *J* = 9.6, 5.2 Hz, H-7), 5.341 (1H, d, *J* = 0.8 Hz, H-17b), 4.407 (1H, t, *J* = 2.8 Hz, H-1), 2.852 (1H, dd, *J* = 8.8, 4.4 HZ, H-13), 2.442 (1H, dd, *J* = 9.6, 5.2 Hz, H-6a), 2.293 (1 h, dd, *J* = 12.8, 6.8 Hz, H-5), 2.126 (1H, m, H-6b), 2.080 (2H, *J* = 12.0, H-12a, H-14a), 1.928 (1H, dd, *J* = 13.6, 4.0 Hz, H-9), 1.866 (1H, dd, *J* = 12.0, 4.8 Hz, H-14b), 1.815 (2H, m, H_2-2), 1.584 (1H, m, H-11a), 1.528 (1H, m, H-3a), 1.479 (1H, m, H-3b), 1.358 (1H, m, H-12b), 1.321 (1H, m, H-11b), 1.030 (3H, s, H_3-18), 1.013 (3H, s, H_3-20). In the same manner described for **1a** another portion of **1** (1.0 mg) was reacted in a second NMR tube with (*S*)-(−) MTPA-Cl (4.0 μL) at 25 °C for 14 h using d_5-pyridine (0.5 mL) as solvent, to afford the bis-(*R*)-MTPA ester derivative (**1b**). ^{1}H NMR data of **1b** (400 MHz, pyridine-d_5): δ 5.970 (1H, s, H-17a), 5.273 (1H, s, H-17b), 5.669 (1H, dd, *J* = 10.0, 5.6 Hz, H-7), 4.421 (1H, t, *J* = 2.8 Hz, H-1), 2.778 (1H, dd, *J* = 9.2, 4.8 Hz, H-13), 2.520 (1H, dd, *J* = 12.8, 9.6 Hz, H-6a), 2.338 (1H, dd, *J* = 12.8, 6.8 Hz, H-5), 2.249 (1H, dd, *J* = 12.8, 5.6 Hz, H-6b), 2.060 (1H, m, H-12a), 2.000 (1H, d, *J* = 12.0 Hz, H-14a), 1.919 (1H, dd, *J* = 13.6, 4.0 Hz, H-9), 1.828 (2H, m, H_2-2), 1.660 (1H, dd, *J* = 12.0, 4.8 Hz, H-14b), 1.551 (1H, m, H-11a), 1.526 (1H, m, H-3a), 1.455 (1H, m, H-3b), 1.341 (1H, m, H-12b), 1.286 (1H, m, H-11b), 1.074 (3H, s, H_3-18), 1.010 (3H, s, H_3-20).

Preparation of the (*S*)- and (*R*)-MTPA Ester Derivatives of Geopyxin B (2) by a Convenient Mosher Ester Procedure. In the same manner described for **1a** and **1b**, portions (1.0 mg each) of geopyxin B were reacted in two NMR tubes with (*R*)-(+)-MTPA-Cl (4 μL) and (*S*)-(−)-MTPA-Cl (4.0 μL) at 25 °C for 14 h using d_5-pyridine (0.5 mL) as solvent, to afford (*S*)-MTPA and (*R*)-MTPA ester derivatives, **2a** and **2b**, respectively. ^{1}H NMR data of **2a** (400 MHz, pyridine-d_5): δ 6.085 (1H, s, H-17a), 5.317 (1H, s, H-17b), 3.706 (1H, dd, *J* = 3.6, 2.0 Hz, H-7), 2.959 (1H, m, H-13), 2.156 (1H, d, *J* = 12.8 Hz, H-3a), 2.154 (1H, dd, *J* = 12.8 Hz, H-5), 2.036 (1H, d, *J* = 12.0 Hz, H-14a), 1.809 (1H, d, *J* = 8.0 Hz, H-9), 1.741 (1H, m, H-12a), 1.714 (1H, m, H-2a), 1.670 (2H, m, H-1a), 1.526 (3H, m, H-6a, H-11a, and H-12b), 1.334 (3H, m, H-2b, H-11b, and H-14b), 1.258 (1H, dd, *J* = 11.6, 2.0 Hz, H-6b), 1.156 (3H, s, H_3-18), 1.044 (1H, m, H-3b), 0.976 (3H, s, H_3-20), 0.765 (1H, td, *J* = 13.6, 4.0 Hz, H-1b). ^{1}H NMR data of **2b** (400 MHz, pyridine-d_5): δ 6.083 (1H, s, H-17a), 5.322 (1H, s, H-17b), 3.814 (1H, dd, *J* = 3.6, 2.0 Hz, H-7), 2.956 (1H, m, H-13), 2.167 (2H, dd, *J* = 12.8, 1.8 Hz, H-3a), 1.858 (1H, d, *J* = 12.0 Hz, H-14a), 1.790 (3H, m, H-2a, H-6a, and H-9), 1.650 (1H, m, H-12a), 1.623 (1H, m, H-1a), 1.500 (1H, m, H-12b), 1.483 (1H, m, H-11a), 1.451 (1H, m, H-2b), 1.369 (1H, m, H-6b), 1.316 (1H, m, H-14b), 1.315 (1H, m, H-11b), 1.218 (3H, s, H_3-18), 1.058 (1H, m, H-3b), 0.745 (1H, td, 13.2, 4.0 Hz, 1b), 0.679 (3H, s, H_3-20).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Selected HMBC correlations of compounds **4** and **6**. Selected NOEs observed in the NOEDIFF experiments of compounds **4** and **5**. Cytotoxic activity of compounds **1**−**15** against a panel of five cancer cell lines. Majority-rule consensus tree indicating placement of strains AZ0484 and AZ0066 within Pyronemataceae (Pezizomycetes). Tables giving ^{1}H and ^{13}C NMR spectroscopic data for compounds **7**−**15**. ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HSQC, and HMBC spectra of compounds **1**, **4**, and **5**; ^{1}H and ^{13}C NMR, ^{1}H −^{1}H COSY, and HMBC spectra of compound **2**; ^{1}H and ^{13}C NMR spectra of compounds **3** and **7**−**15**. ^{1}H and ^{13}C NMR, HSQC, and HMBC spectra of compound **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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