Published in final edited form as:

J Nat Prod. 2012 March 23; 75(3): 361–369. doi:10.1021/np200769q.

Geopyxins A – E, *ent*-Kaurane Diterpenoids from Endolichenic Fungal Strains, *Geopyxis* aff. *majalis* and *Geopyxis* sp. AZ0066: Structure-Activity Relationships of Geopyxins and their Analogues¹

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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

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DEDICATION

Dedicated to Dr. Gordon M. Cragg for his pioneering work on the development of natural product anticancer agents.

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 panel of five cancer cell lines. Majority-rule consensus tree indicating placement of strains AZ0484 and AZ0066 within Pyronemataceae (Pezizomycetes). Tables giving ¹H and ¹³C NMR spectroscopic data for compounds **7** – **15**. ¹H and ¹³C NMR, ¹H – ¹H COSY, HSQC, and HMBC spectra of compounds **1**, **4** and **5**; ¹H and ¹³C NMR, ¹H – ¹H COSY and HMBC spectra of compound **2**; ¹H and ¹³C NMR spectra of compounds **3**, **7** – **15**. ¹H and ¹³C NMR, HSQC and HMBC spectra of compound **6**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

fungi of the Sonoran Desert bioregion,³ we have been particularly interested in the identification of compounds which 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 (SAR) 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.

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- κ B activation inhibitory,²² and apoptosis-inducing²³ activities.

RESULTS AND DISCUSSION

Geopyxin A (**1**) obtained as a white amorphous solid, analyzed for C₂₀H₂₈O₅ by a combination of HRESIMS and ¹³C 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 ¹³C 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)CH₂CH₂–, –CHCH₂CH(O)–, and –CHCH₂CH₂CHCH₂– in **1** was evident from its ¹H–¹H COSY spectrum (Figure 1). The connectivity of the above three spin systems,

tertiary methyls, and non-protonated 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 CH₃-18²⁵ 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 with the carbonyl carbon C-15 (δ 214.9) placing it at C-7. Methylation (CH₃I/K₂CO₃/acetone) of **1** gave its methyl ester (**7**) further confirming the presence of a carboxylic acid group, while acetylation (Ac₂O/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 up-field shift to δ 206.5 as a result of acetylation suggested that the OH-7 has 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 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 CH₃ group on C-4 was inferred from its ¹³C chemical shift (δ 28.5)²⁹ which suggested that the carboxylic acid group is α -oriented; the ¹³C chemical shift value for the α -CH₃ group at C-4 has been reported to be at δ 16.7.³⁰ The NOE data for **7** (Figure 2) confirmed that both CH₃-20 and H-14 at δ 2.22 have 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**).

Geopyxin B (**2**), obtained as a white amorphous solid, analyzed for C₂₀H₂₈O₄ by a combination of HRESIMS and NMR data. The ¹H and ¹³C 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 presence of a methylene group (δ_C 39.8) which exhibited an HMBC correlation to CH₃-20 (δ 1.02). This was further confirmed by the up-field 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) confirmed the *S* absolute stereochemistry for C-7. Based on 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 C₂₀H₂₆O₅ by a combination of HRESIMS and NMR data. The ¹H and ¹³C 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 mono-keto compound as the only product which was identical with **3**. The ¹H NMR coupling pattern (brs) of the oxymethine proton at

δ 3.88 suggested the presence of 7 β -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 C₂₀H₂₈O₅ by a combination of HRESIMS and ¹³C NMR spectroscopy. The ¹H and ¹³C 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 formulae between these compounds confirmed that **4** contain 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 CH₃-20 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**. Based on 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, C₂₀H₂₈O₆, 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 ¹H and ¹³C 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)CH₂- identified from its ¹H-¹H 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 method³¹ 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 $\Delta\delta_{\text{S-R}}$ 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 C₂₀H₃₀O₆ by a combination of HRESIMS and ¹³C NMR data. The ¹H and ¹³C 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 -CHCH₂OH group instead of a -C=CH₂ group in **1**. In the HMBC spectrum, oxygenated methylene protons of **6** showed correlations with the ketone carbonyl at δ 229.1 (C-15), methine carbons at δ 32.6 (C-13), and 56.0 (C-16), confirming the above observation (Supporting Information, S3). The treatment of **6** with Ac₂O/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 up-field 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 12 β -H and 16-CH₂OH as observed before for related *ent*-kauranes bearing a 16-CH₂OH³³ suggested 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 work up, the absence of C-16 epimer and the previous reports^{33,34} of the natural occurrence of

related *ent*-kauranes bearing 16 α -CH₂OH suggested that **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 *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 non-small cell lung cancer), SF-268 (human CNS glioma), MCF-7 (human breast cancer), PC-3M (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 μ M, 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 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 are 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 which 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-QP8000a and JEOL HX110A spectrometers, respectively. Analytical and preparative thin layer chromatography (TLC) were performed on pre-coated 0.25 mm thick plates of silica gel 60 F₂₅₄; 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 Waters 996 Photodiode Array Detector and Waters Prep LC Controller utilizing Empower pro software and using a RP column (Phenomenex Luna 5 μm , C₁₈, 100 $^\circ\text{A}$, 250 $\times$ 10 mm) with a flow rate 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 $^\circ$ 53'00" N, 109 $^\circ$ 12'18" W).⁴² The thallus was cut into 2 $\times$ 2 cm segments, washed in tap water, surface-sterilized by agitating sequentially in 95% EtOH for 30 sec, 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 $\times$ 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 condition at room temperature (ca. 21.5 $^\circ\text{C}$). 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 strain⁴² 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 Mesquite⁴³ with a core alignment containing 148 closely related endophytic, endolichenic, and saprotrophic fungi from the same site⁴² and 19 described species of Pyronemataceae (Ascomycota) chosen based on 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 7 strains of *Geopyxis* spp. corresponding to all available sequences in GenBank for the relevant loci (partial LSU rDNA), as well as 9 representative genera within the family. Phylogenetic analyses using maximum likelihood were conducted in GARLI⁴⁶ 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 endpoint. 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 (EGFP) 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 two-fold dilutions of pure compounds, or a single concentration of extracts and fractions were added to triplicate wells. DMSO vehicle alone (volume not to exceed 0.1%) served as a negative control. Cells were incubated overnight, the medium removed and wells 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 and 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 CuSO₄ and incubated at 160 rpm and 28 °C. The glucose level in the medium was monitored using glucose strips (URISCAN glucose strip). 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 H₂O, dried over anhydrous Na₂SO₄ 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 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 with 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 sub-fractions ($F_1 - F_{12}$). 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 7 sub-fractions ($F_{2A} - F_{2G}$). Sub-fractions ($F_{2B} - F_{2D}$) 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). Sub-fraction F_4 on evaporation gave an additional quantity of **1** (29.9 mg). Further separation of the sub-fraction 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), 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 sub-fractions ($F_1 - F_{11}$). 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; $[\alpha]_D^{25} -79.9$ (c 0.11, CH_3OH); UV (EtOH) $\lambda_{\max}$ (log ϵ) 238 (3.52) nm; IR (KBr) $\nu_{\max}$ 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 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{20}\text{H}_{27}\text{O}_5$, 347.1864).

Geopyxin B (2): white amorphous solid; $[\alpha]_D^{25} -118.8$ (c 0.11, CH_3OH); UV (EtOH) $\lambda_{\max}$ (log ϵ) 237 (3.07) nm; IR (KBr) $\nu_{\max}$ 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 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{20}\text{H}_{27}\text{O}_4$, 331.1915).

Geopyxin C (3): white amorphous solid; $[\alpha]_D^{25} -112.7$ (c 0.18, CH_3OH); UV (EtOH) $\lambda_{\max}$ (log ϵ) 238 (2.94) nm; IR (KBr) $\nu_{\max}$ 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 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{20}\text{H}_{27}\text{O}_5$, 347.1853).

Geopyxin D (4): white amorphous solid; $[\alpha]_D^{25} -49.2$ (c 0.31, CH_3OH); UV (EtOH) $\lambda_{\max}$ (log ϵ) 236 (3.13) nm; IR (KBr) $\nu_{\max}$ 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 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{20}\text{H}_{27}\text{O}_5$, 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 and 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 1L of the medium (PBD) and incubated at 160 rpm and 28 °C. The glucose level in the medium was monitored using glucose strips (URISCAN glucose strip), 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 H₂O, dried over anhydrous Na₂SO₄ and evaporated under reduced pressure to give 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 followed by EtOAc/MeOH (99:1) to afford 30 (150 mL) fractions (F₁ – F₃₀). Fractions F₆ – F₁₇ (1.30 g) were found to contain geopyxin A (**1**) as the major compound (>95%). Chromatographic separation of fraction F₅ gave geopyxin C (**3**, 12.0 mg). Fraction, F₂₃ (220.0 mg) was further fractionated by column chromatography over silica gel (10.0 g) made up in CH₂Cl₂ and eluted with CH₂Cl₂ containing increasing amounts of MeOH to give 120 (8 mL) fractions and those having similar TLC patterns were combined to give 14 sub-fractions (A – N). The sub-fraction E (30.0 mg) was purified by column chromatography over silica gel (1.0 g) using CH₂Cl₂/MeOH (98:2) as eluant to give **5** (25.9 mg). Fraction, F₂₄ (195.0 mg) was subjected to column chromatography over silica gel (4.0 g) made up in CH₂Cl₂ and eluted with CH₂Cl₂ containing increasing amounts of MeOH to give **6** (161.9 mg).

Geopyxin E (5): white amorphous solid; $[\alpha]_D^{25} -93.5$ (c 0.13, CH₃OH); UV (EtOH) $\lambda_{\max}$ (log ϵ) 239 (2.84) nm; IR (KBr) $\nu_{\max}$ 3323, 2933, 2868, 1703, 1641, 1473, 1444, 1406, 1259, 1201, 1076, 1049, 983 945, 905, 733 cm⁻¹; for ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 363.1812 [M – H]⁻ (calcd for C₂₀H₂₇O₆, 363.1813).

Geopyxin F (6): white amorphous solid; $[\alpha]_D^{25} -63.3$ (c 0.10, CH₃OH); IR (KBr) $\nu_{\max}$ 3423, 2941, 2872, 1709, 1641, 1445, 1412, 1250, 1213, 1161, 1070, 1022, 984, 735 cm⁻¹; for ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 365.1967 [M – H]⁻ (calcd for C₂₀H₂₉O₆, 365.1970).

Preparation of Methyl Ester Derivatives

To a solution of **1** (10.0 mg) in acetone (0.5 mL) were added K₂CO₃ (20.0 mg) and CH₃I (0.2 mL) and stirred at 25 °C. After 1h the reaction mixture was filtered and 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) $\nu_{\max}$ 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; ¹³C 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; ¹³C 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; ¹³C NMR data, see Supporting Information S7; APCIMS (+) mode m/z 343 [M + H – H₂O]⁺.

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 $[\text{M} + \text{H}]^+$, 361 $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$, 343 $[\text{M} + \text{H} - 2\text{H}_2\text{O}]^+$, 325 $[\text{M} + \text{H} - 3\text{H}_2\text{O}]^+$.

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 $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$, 345 $[\text{M} + \text{H} - 2\text{H}_2\text{O}]^+$, 327 $[\text{M} + \text{H} - 3\text{H}_2\text{O}]^+$.

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 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 $[\text{M} - \text{H}]^-$.

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

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 stirred at 0 °C. After 3 h 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 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; ^1H NMR data, see Supporting Information S8; ^{13}C NMR data, see Supporting Information S9; APCIMS (+) mode m/z 405 $[\text{M} + \text{H}]^+$.

1,7-O-Diacetylmethylgeopyxin A (15): white amorphous solid; ^1H NMR data, see Supporting Information S8; ^{13}C NMR data, see Supporting Information S9; APCIMS (+) mode m/z 387 $[\text{M} + \text{H} - \text{CH}_3\text{COOH}]^+$.

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 NMR tube was monitored immediately by ^1H NMR and left at 25 °C for 14 h to give bis (*S*)-MTPA ester derivative (**1a**) of **1**. ^1H

NMR data of **1a** (400 MHz, pyridine-*d*₅; data were assigned on the basis of the correlations of the ¹H–¹H 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 (1H, 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, H₂–2), 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, H₃–18), 1.013 (3H, s, H₃–20). 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**). ¹H 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, H₂–2), 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, H₃–18), 1.010 (3H, s, H₃–20).

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. ¹H 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, H₃–18), 1.044 (1H, m, H–3b), 0.976 (3H, s, H₃–20), 0.765 (1H, td, *J* = 13.6, 4.0 Hz, H–1b). ¹H 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, H₃–18), 1.058 (1H, m, H–3b), 0.745 (1H, td, 13.2, 4.0 Hz, 1b), 0.679 (3H, s, H₃–20).

Supplementary Material

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Acknowledgments

Financial support for this work from the National Cancer Institute (Grant R01 CA90265) and National Institute of General Medical Sciences (Grant P41 GM094060) are gratefully acknowledged. We also thank the National Science Foundation for supporting collection and identification of fungal strains (grant DEB-064099 to A.E.A.), the NSF IGERT Program in Genomics at The University of Arizona for a graduate fellowship (J.M.U.), and the College of Agriculture and Life Sciences at the University of Arizona for institutional support of E.M.K.W. and M.K.G., Timothy Bolton and Lauren West for their assistance in preparing large-scale cultures.

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

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

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

Figure 4.
 $\Delta\delta$ values [$(\Delta\delta \text{ in ppm}) = \delta_S - \delta_R$] obtained for (S) - and (R) -MTPA esters (2a and 2b, respectively) of geopyxin B (2).

Figure 5.

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

Table 1

¹H (400 MHz) and ¹³C (100 MHz) NMR Spectroscopic Data of Compounds 1 – 6 in CDCl₃

Position	1		2		3		4		5		6	
	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)	3.19, dt (14.0, 6.4) 2.15, ddd (14.0, 4.6, 2.4)	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)	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)

	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
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	116.0 (CH ₂)	5.31, s	116.6 (CH ₂)	5.34, s	113.8 (CH ₂)	5.30, s	115.8 (CH ₂)	5.27, s	59.1 (CH ₂)	3.56, dd (11.1, 6.8)
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

Table 2

Cytotoxic Activity of Compounds **2**, **7** – **10**, **12**, **14** and **15** Against a Panel of Five Cancer Cell Lines^{a,b}

Compound	Cell line ^b				
	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

^aResults are expressed as IC₅₀ values ± standard deviation in μM. Doxorubicin and DMSO were used as positive and negative controls.

^bCompounds **1**, **3** – **6**, **11**, and **13** were found to be inactive at 5.0 μM.

^cKey: 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.