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Frenolicins C−G, Pyranonaphthoquinones from *Streptomyces* sp. RM-4-15

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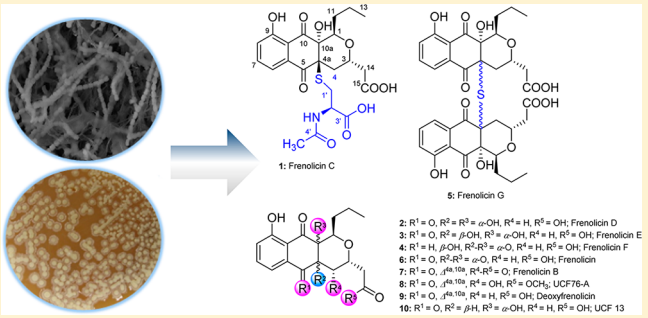
Supporting Information

ABSTRACT: Appalachian active coal fire sites were selected for the isolation of bacterial strains belonging to the class actinobacteria. A comparison of high-resolution electrospray ionization mass spectrometry (HRESIMS) and ultraviolet (UV) absorption profiles from isolate extracts to natural product databases suggested *Streptomyces* sp. RM-4-15 to produce unique metabolites. Four new pyranonaphthoquinones, frenolicins C−F (**1**−**4**), along with three known analogues, frenolicin (**6**), frenolicin B (**7**), and UCF76-A (**8**), were isolated from the fermentation of this strain. An additional new analogue, frenolicin G (**5**), along with two known compounds, deoxyfrenolicin (**9**) and UCF 13 (**10**), were isolated from the fermentation supplied with 18 mg/L of scandium chloride, the first example, to the best of our knowledge, wherein scandium chloride supplementation led to the confirmed production of new bacterial secondary metabolites. Structures **1**−**5** were elucidated on the basis of spectral analysis and chemical modification. While frenolicins are best known for their anticoccidial activity, the current study revealed compounds **6**−**9** to exhibit moderate cytotoxicity against the human lung carcinoma cell line (A549) and thereby extends the anticancer SAR for this privileged scaffold.



The Appalachian Mountains of eastern Kentucky are known for both their rich natural biodiversity and the production of coal.[1−5] Fires within abandoned underground mines throughout the region are a common occurrence and contribute to the emission of greenhouse gases and the dispersion of volatile organic and inorganic species through surface vents.[6−9] The Ruth Mullins underground coal mine fire, which unofficially dates back to 1960, is located in the high volatile A bituminous Pennsylvanian-age Hazard No.7 coal bed, Perry County, Kentucky. Recent studies of the Ruth Mullins site highlight a notable alteration of the soil composition in and around the fire's thermal vents with adjacent surface temperature variation ranging from 26.0 to 64.8 °C.[10] In the context of microbial diversity, the convergence of natural biodiversity and man-made environmental alteration upon the Ruth Mullins coal fire site offers an unprecedented environment for exploration. As part of a broad natural product discovery initiative, we examined soil samples collected from this site with a focus upon actinomycetes capable of producing novel secondary metabolites.

More than two dozen different actinomycetes were isolated from a single soil sample collected from the Ruth Mullins coal fire site. HPLC−high-resolution mass spectrometry (HPLC-HRMS) profiling of the corresponding culture extracts and metabolite database comparison revealed the potential presence of unique metabolites in the extract produced by *Streptomyces* sp. RM-4-15. In this report, we describe the comparative fermentation of *Streptomyces* sp. RM-4-15 in the absence and presence of scandium chloride and the corresponding isolation, structure elucidation, and biological activity of five new frenolicin analogues (**1**−**5**) and five previously reported analogues—frenolicin (**6**), frenolicin B (**7**), UCF76-A (**8**), deoxyfrenolicin (**9**), and UCF 13 (**10**)—produced by *Streptomyces* sp. RM-4-15. Consistent with previous studies that revealed notable enhancement of known metabolite production in the presence of scandium chloride,[11] the presence of scandium chloride in the current study led to marked improvements in production of frenolicin (**6**) and frenolicin B (**7**) by *Streptomyces* sp. RM-4-15. Interestingly, unlike prior studies, the production of frenolicin G (**5**), deoxyfrenolicin (**9**), and UCF 13 (**10**) by *Streptomyces* sp. RM-4-15 was notably only observed in the presence of the heavy

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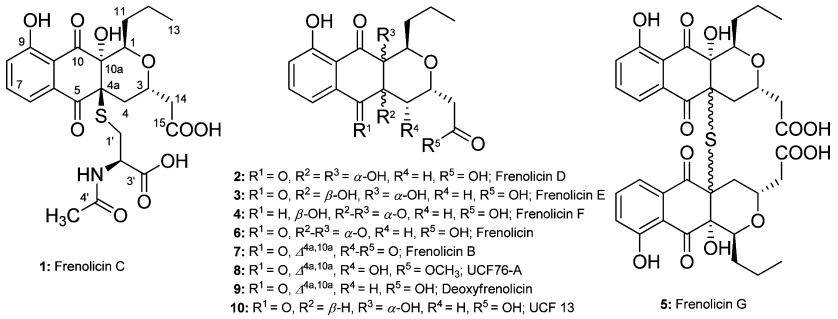

Figure 1. Structures of compounds **1**−**10**.

Table 1. NMR Data for Frenolicins C (1) and G (5)

	frenolicin C (**1**)			frenolicin G (**5**)		
position	δ_C,[a] type	δ_H[b] (*J* in Hz)	HMBC	δ_C,[c] type	δ_H[d] (*J* in Hz)	HMBC
1	77.0, CH	3.94, d (11.1)	3, 4a, 10a	80.1, CH	3.81, dd (9.8, 1.4)	3, 4a, 10a, 11, 12
3	62.8, CH	4.45, m		63.1, CH	3.89, m	14, 15
4α	29.4, CH_2	2.13, t (13.7, 11.9)	4a, 5, 10a	29.1, CH_2	2.11, d (13.7)	3, 5, 10a
4β		1.89, d (14.2)	3, 4a, 5		2.25, dd (15.0, 11.8)	3, 4a, 5, 14
4a	58.3, C			64.6, C		
5	189.5, C			191.5, C		
5a	132.8, C			135.0, C		
6	118.5, CH	7.42, d (7.4)	5, 8, 9a	121.8, CH	7.77, d (6.3)	5, 8, 9a
7	137.1, CH	7.73, t (7.5, 8.3)	5a, 9	138.7, CH	7.73, t (7.4, 8.2)	5a, 9
8	123.8, CH	7.28, d (8.4)	6, 9a	124.9, CH	7.26, d (6.9)	6, 9, 9a
9	160.5, C			163.1, C		
9a	114.4, C			115.8, C		
10	198.6, C			199.4, C		
10a	75.8, C			77.4, C		
11	29.2, CH_2	1.69, 2.32, m	12	31.1, CH_2	1.40, 1.51, m	12, 13
12	20.0, CH_2	1.31, 1.59, m	11, 13	21.8, CH_2	1.06, 1.31, m	11, 13
13	13.8, CH_3	0.90, t (7.3)	11, 12	14.3, CH_3	0.63, t (7.3)	11, 12
14	40.1, CH_2	2.38, 2.58,[e] m	3, 15	41.4, CH_2	2.40, 2.52, m	3, 4, 15
15	172.3, C			174.6, C		
9-OH		11.21, s		11.1,[b] s		
10a-OH		7.15, s	1, 4a, 10	7.32[b]		
1′	31.5, CH_2	2.44, 2.58,[c] m	4a, 2′, 3′			
2′	51.2, CH	4.12, m	1′, 3′, 4′			
3′	171.4, C					
4′	169.4, C					
5′	22.2, CH_3	1.74, s	4′			
N−H		8.16, d (6.7)	2′, 4′			

[a]Measured in DMSO-d_6, 100 MHz. [b]Measured in DMSO-d_6, 500 MHz. [c]Measured in CD_3OD, 100 MHz. [d]Measured in CD_3OD, 500 MHz. [e]Overlap.

metal additive. While frenolicins are best known for their anticoccidial activity,[12] the corresponding bioactivity assessment highlighted within also extends the anticancer structure−activity relationship (SAR) of this structural family.

RESULTS AND DISCUSSION

A four-day grown seed culture (300 mL) of *Streptomyces* sp. RM-4-15 was used to inoculate 4 L of production medium. Fermentation of the strain for six days followed by extraction, fractionation, and resolution of components within the crude extract mixture led to the isolation of four new compounds, frenolicins C (**1**, yield: 4.25 mg/L), D (**2**, yield: 2.5 mg/L), E (**3**, yield: 3.0 mg/L), and F (**4**, yield: 1.25 mg/L), in addition to the known analogues frenolicin (**6**, yield: 2.25 mg/L), frenolicin B (**7**, yield: 2.75 mg/L), and UCF76-A (**8**, yield: 0.5 mg/L). From a separate 10 L culture, which was supplemented with 18 mg/L of scandium chloride, compounds **6** and **7** were isolated with higher yield. An additional new analogue, frenolicin G (**5**, yield: 1.2 mg/L), along with two known compounds, deoxyfrenolicin (**9**, yield: 1.5 mg/L) and UCF 13 (**10**, yield: 0.8 mg/L), were also isolated and characterized.

Compound **1** was isolated as a yellow, amorphous powder. The HRESIMS of **1** displayed masses of m/z = 510.1380 $[M + H]^+$ and m/z = 508.1340 $[M - H]^-$ in positive and negative modes, respectively, suggesting a molecular formula of $C_{23}H_{27}O_{10}NS$ with 11 degrees of unsaturation. The analysis of the $^1H/^{13}C$ and gHSQC NMR data suggested the presence of two methyl, five methylene, and six methine groups and 10 quaternary carbons (Table 1). Comprehensive analysis of the COSY spectrum of **1** established four structural fragments as

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illustrated in Figure 2. The presence of three aromatic proton signals at δ_H 7.73 (1H, t, J = 7.5, 8.3 Hz, H-7), 7.42 (1H, d, J = 7.4 Hz, H-6), and 7.28 (1H, d, J = 8.4 Hz, H-8) indicated the presence of a trisubstituted benzene ring, which was further supported by the observation of COSY cross-peaks for H-6/H-7 and H-7/H-8 and HMBC correlations for H-6/C-9a, H-7/C-9, and H-8/C-9a (Figure 2). The designation of the *n*-propyl group at C-1 was supported by the key HMBC correlations H-1/C-11 and H-1/C-12. The singlet proton signal at δ_H 11.21 (s, OH-9) revealed HMBC correlations to C-8, C-9, and C-9a in acetone-d_6 (see Supporting Information S5), consistent with a hydroxyl group at C-9 position chelated with the C-10 carbonyl (δ_C 198.6) through an intramolecular hydrogen bond. Comparison of the NMR data of **1** with the known analogue frenolicin (**6**) indicated structural conservation with divergent C-4a and C-10a substitution. The hydroxyl proton at δ_H 7.15 (s) demonstrated HMBC correlations with C-1 (δ_C 77.0), C-4a (δ_C 58.3), and C-10 (δ_C 198.6), consistent with C-10a regiospecificity. Consistent with the presence of an amide bond, the NH proton at δ_H 8.16 (d, J = 6.7 Hz) lacked HSQC correlation but displayed a COSY methine (δ_H 4.12, H-2′) correlation and HMBC correlations with C-2′ (δ_C 51.2) and the *N*-acetyl carbonyl C-4′ (δ_C 169.4). The observation of the key HMBC cross-peaks [H_2-1′ (δ_H 2.44, 2.58) and C-2′ (δ_C 51.2), C-3′ (δ_C 171.4); H-2′ (δ_H 4.12) and C-1′ (δ_C 31.5), C-3′ (δ_C 171.4), C-4′ (δ_C 169.4); and the acetate methyl H_3-5′ (s, δ_H 1.74) and C-4′ (δ_C 169.4)] as well as the presence of a sulfur atom in the molecular formula of **1** suggested the presence of an *N*-acetyl-cysteine moiety connected at the C-4a position. This was further confirmed by the presence of a strong cross-peak of H_2-1′ and C-4a (δ_C 58.3) in the HMBC spectrum.

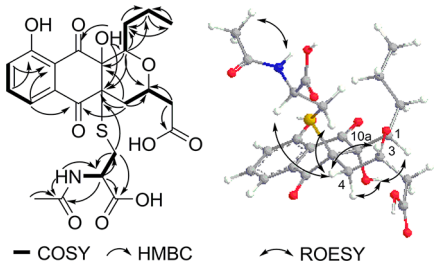

Figure 2. COSY, select HMBC, and ROESY correlations of frenolicin C (**1**).

The relative configuration of **1** was established by ROESY experiments (Figure 2). Two cross-peaks of H-1/OH-10a and OH-10a/H-4α observed in the ROESY indicated that they were α-oriented. Similarly, the ROESY correlations of H-3/H-4β and H-4β/H_2-1′ suggested their β-orientations. To determine the absolute configuration of the amino acid moiety, compound **1** was subjected to acid hydrolysis. The hydrolytically released amino acid was derivatized following Marfey's method, and the product was subsequently compared to amino acid standards (derivatized in an identical manner) via HPLC.[13] This analysis revealed the identity of the hydrolytic product as L-cysteine. On the basis of this cumulative analysis, the structure of **1** was established as depicted in Figure 1 and, as a new member of the frenolicin family, given the name frenolicin C.

HRESIMS of compounds **2** and **3** suggested both to be isomers of the molecular formula $C_{18}H_{20}O_8$. ^{1}H and ^{13}C NMR of both compounds were similar to those of **1** (Table 2); both notably lacked the *N*-acetyl-L-cysteine spectroscopic signatures but displayed a new hydroxy proton signal at δ_H 6.36 and 6.53 in **2** and **3**, respectively. Both hydroxyl protons displayed HMBC correlations with the corresponding C-4, C-4a, and C-

Table 2. NMR Data for Frenolicins D−F (2−4)

	frenolicin D (**2**)		frenolicin E (**3**)		frenolicin F (**4**)	
position	δ_C,[a] type	δ_H[b] (J in Hz)	δ_C,[a] type	δ_H[b] (J in Hz)	δ_C,[c] type	δ_H[b] (J in Hz)
1	79.4, CH	3.80, dd (2.7, 12.0)	78.8, CH	3.99, d (9.8)	68.2, CH	4.26, dd (6.3, 8.3)
3	65.4, CH	4.00, m	62.6, CH	4.32, m	61.4, CH	3.95, m
4α	34.0, CH_2	1.73, t (11.5)	32.8, CH_2	2.00, m	27.7, CH_2	1.76, dd (11.2, 14.3)
4β		2.27, dd (3.1, 12.5)		1.76, t (12.9)		2.60, dd (4.7, 14.5)
4a	75.7, C		75.0, C		63.7, C	
5	191.8, C		194.7, C		67.1, CH	4.78,d (8.6)
5a	134.1, C		132.7, C		141.8, C	
6	119.8, CH	7.59, d (7.5)	119.6, CH	7.45, d (7.5)	121.4, CH	6.95, d (7.7)
7	138.0, CH	7.82, t (7.6, 8.2)	137.1, CH	7.75, t (7.6, 8.3)	137.2, CH	7.58, t (7.6, 8.2)
8	124.4, CH	7.38, d (8.4)	124.7, CH	7.31, d (8.4)	117.3, CH	6.93, d (8.7)
9	161.6, C		162.5, C		160.7, C	
9a	116.1, C		115.8, C		112.8, C	
10	202.4, C		199.1, C		198.0, C	
10a	79.7, C		78.8, C		59.3, C	
11	30.9, CH_2	0.69, 1.22, m	28.1, CH_2	1.66, 2.35, m	28.9, CH_2	1.85, m
12	18.8, CH_2	1.05, 1.28, m	20.1, CH_2	1.26, 1.58, m	18.1, CH_2	1.38, 1.57, m
13	13.1, CH_3	0.64, t (7.4)	13.8, CH_3	0.90, t (7.4)	13.8, CH_3	0.92, t (7.4)
14	39.6, CH_2	2.42, 2.58, m	40.3, CH_2	2.42, 2.58, m	40.9, CH_2	2.29, 2.46, m
15	175.7, C		175.7, C		172.0, C	
4a-OH		6.36, s		6.53, s		
5-OH						6.09, d (8.6)
9-OH		11.96, s		11.58, s		11.28, s
10a-OH		6.09, s		6.76, s		

[a]Measured in $CDCl_3$, 100 MHz. [b]Measured in DMSO-d_6, 500 MHz. [c]Measured in DMSO-d_6, 100 MHz.

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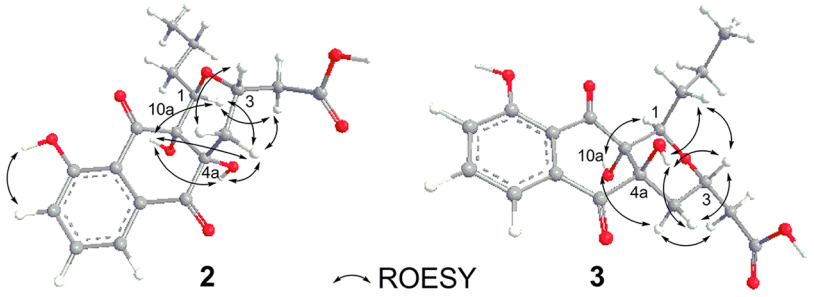

Figure 3. Key ROESY correlations of frenolicins D (**2**) and E (**3**).

5, with a C-4a hydroxy. ROESY experiments to establish C-4a relative stereochemistry revealed the presence of cross-peaks for H-1/H-4α, H-1/H_2-14, H-1/OH-10a, H-4α/OH-4a, and OH-4a/OH-10a indicating all as cofacial and the C-4a and C-10a hydroxys of compound **2** to adopt an α-orientation. In a similar manner, ROESY cross-peaks for H-1/OH-10a, H-4α/H-10a, and H-4α/H_2-14 in compound **3** indicated a similar cofacial α-orientation, while the ROESY correlations of H-3/H-4β, H-3/OH-4a, H-4β/H-4a, and OH-4a/H_2-11 supported the β-orientation of OH-4a (Figure 3). On the basis of this cumulative analysis, the structures of **2** and **3** were established as depicted in Figures 1 and 3 and, as new members of the frenolicin family, named frenolicins D and E, respectively.

Compound **4** was obtained as a yellow, amorphous powder. The presence of a molecular ion at m/z = 371.1107 $[M + Na]^+$ in the HRESIMS spectrum suggested a molecular formula of $C_{18}H_{20}O_7$, which, when compared to frenolicin (**6**), contained two additional protons. The ^{13}C NMR data of **4** was similar to those of frenolicin (**6**), with both displaying similar C-4a–C-10a epoxy signatures (Table 2). In contrast to **6**, the ^{13}C spectrum suggested compound **4** to lack a C-5 carbonyl, and the detection of two additional coupled proton signals at δ_H 6.09 (d, J = 8.6) and 4.78 (d, J = 8.6) also suggested the presence of a secondary alcohol. Of these latter resonances, the proton at δ_H 4.78 (d, J = 8.6) displayed HMBC correlations with C-4a, C-5, C-4, C-5a, C-6, C-9a, and C-10a, consistent with C-5 regiospecificity. The subsequent ROESY correlations of OH-5/H-4β and H-4β/H-3 in compound **4** implicated a β-orientation of the C-5 OH group. To determine the relative configuration of the oxirane ring, compound **4** and the standard **6** were both treated with Dess-Martin periodinane at room temperature following previously reported methods.[14] The ^1H NMR data of the oxidized products of **4** and **6** were indistinguishable, supporting an identical α-orientation of oxirane in both compounds. On the basis of this cumulative analysis, the structure of **4** was established as depicted in Figure 1 and, as a new member of the frenolicin family, named frenolicin F.

Three previously reported compounds—frenolicin (**6**),[15−17] frenolicin B (**7**),[17] and UCF76-A (**8**)[18]—were also identified as fermentation products of *Streptomyces* sp. RM-4-15 via comparison to previously reported spectroscopic data. In light of a recent report that indicated the positive effect of scandium on the expression level of pathway-specific positive regulatory genes,[11] we tested the effect of this rare earth element on the production of secondary metabolites of *Streptomyces* sp. RM-4-15. Addition of 18 mg/L scandium chloride in a parallel fermentation of the same strain revealed a new compound (**5**) along with two additional known compounds, deoxyfrenolicin (**9**)[15−17] and UCF 13 (**10**).[19] Interestingly, compounds **8** and **9** both eluted at the identical retention time and possessed identical UV characteristics (Figure S46).

Compound **5** was obtained as a yellow, amorphous powder, and its molecular formula was established as $C_{36}H_{38}O_{14}S$ by HRESIMS at m/z 727.2107 $[M + H]^+$ and m/z 725.1947 $[M - H]^-$. Only 19 hydrogen and 18 carbon atoms were observed in the ^1H NMR and ^{13}C NMR, respectively. The tentative molecular weight of the compound deduced from NMR analysis appeared to be half of that observed in the HRESIMS data, suggesting compound **5** to be a symmetrical dimer. The NMR data of **5** were similar to those of frenolicin C (**1**). The close chemical shift of C-4a of **5** to that of **1** suggested that compound **5** contains a sulfur atom at the C-4a position. Analysis of 2D NMR data including COSY and HMBC (Figure 4) led to the elucidation of the structure of **5**. Compound **5**

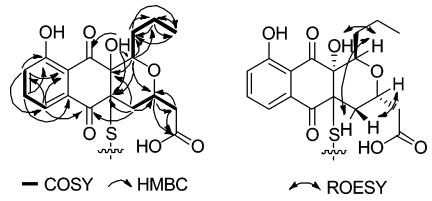

Figure 4. COSY, select HMBC, and ROESY correlations of frenolicin G (**5**).

contained the **1** frenolicin core wherein a thioether bridge links two symmetrical units at the 4a position. The relative configuration of **5** was established through the analyses of ROESY data. While the NOE correlations observed between H-1 and OH-10a, H-1 and H-4α, and H-3 and H-4β helped establish the configuration of most chiral centers, we were unable to assign the relative configuration at the 4a position via NMR experiments. A number of experiments to produce single crystals for the X-ray diffraction analysis for compound **5** were not successful. Hence, the structure of **5** is reported as depicted in Figure 1 and, as a new family member, subsequently named frenolicin G.

Frenolicins are typically characterized by the presence of a pyranonaphthoquinone moiety with propyl substitution at the C-1 position, and they are known for their antibacterial, antifungal, and antiprotozoal activities.[15,17,20−22] Frenolicin B (**7**) was recently identified as a putative AKT-selective kinase inhibitor, while UCF76-A (**8**) was reported to inhibit Ras p21 farnesylation.[18,23−26] Inspired by the putative anticancer mechanisms reflected within these recent studies, we compared the new analogues (**1**–**5**) and the known frenolicins (**6**–**10**) in a standard cancer cell line cytotoxicity assay using the human non-small-cell lung carcinoma A549. While compounds **6**–**9** displayed moderate cytotoxicity in this assay (with IC_{50}'s

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ranging from 0.28 to 5.77 μM), the new isolates **1**–**5** were inactive (IC_{50}'s > 80 μM) in the parallel assays (Table 3).

Table 3. Cytotoxicity of Compounds 1–10 against the A549 Cell Line

compound	IC_{50} (μM)	95% confidence interval
1	>80	
2	>80	
3	>80	
4	>80	
5	>80	
6	5.77	5.144–6.479
7	0.28	0.248–0.330
8	0.35	0.318–0.394
9	1.07	0.867–1.327
10	>80	

Although the molecular target that contributes to the cancer cell cytotoxicity of the frenolicin family remains to be determined, these results suggest an intact quinone as indispensable for the cytotoxic activity. The similar potencies of frenolicin B and UCF-76A suggest the exocyclic lactone to have minimal influence on bioactivity, while the free C-14 carboxylate and/or the C-4 hydroxyl contribute to the differing activities of deoxyfrenolicin and UCF76-A. This general trend is consistent with the previous observation that frenolicin B was both a more potent AKT inhibitor in vitro (~2-fold) and a more potent antiproliferative agent against the PTEN-deleted breast tumor cell line MDA468 (~5-fold) than deoxyfrenolicin.[24] Mechanistically, pyranonaphthoquinones are postulated to inhibit AKT via irreversible alkylation of the allosteric T-loop site.[23] This mechanism is unique to classical AKT kinase inhibitors, which act via competitive inhibition of the ATP-binding site in a relatively nonselective manner.

The reported identification and characterization of five new frenolicins support the contention that unique man-made ecological environments may serve as reservoirs for new/novel microbial natural products. Among a total of 138 reported natural products containing a pyranonaphthoquinone core known to date, only 21—kalafungin,[27] the nanomycins,[28] the lactoquinomycins,[29] and arizonin[30]—are considered to be closely related structurally to the frenolicins. Therefore, the addition of five new structures to this subset is a notable expansion. In addition, amino acid conjugated naphthopyrones, exemplified by frenolicin C, have not been previously identified, and the thioether linkage sulfur source within the homodimeric frenolicin G is intriguing from a biosynthetic perspective. This study also highlights the first example, to the best of our knowledge, wherein scandium chloride supplementation led to the confirmed production of new bacterial secondary metabolites. Given the intense focus upon the development of technologies to induce metabolite production from cryptic biosynthetic pathways within the past decade,[31−33] this observation might have a broader potential in the discovery of previously inaccessible bacterial metabolites.

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were recorded on a GE Ultraspec 8000 spectrophotometer. IR spectra were measured on a Nicolet FTIR 6700 spectrophotometer. All NMR data were recorded at 500 MHz for ^{1}H and at 100 MHz for ^{13}C with Varian Inova NMR spectrometers. HRESIMS experiments were carried out using an AB SCIEX TripleTOF 5600 System. HPLC analyses were performed using an Agilent 1260 system equipped with a DAD detector and a Phenomenex C_{18} column (4.6 × 150 mm, 0.5 μm). Semipreparative HPLC separation was performed using a Varian Prostar 210 HPLC system equipped with a PDA detector 330 using a DiscoveryBio wide pore C_{18} column (25 × 21.2 mm, 10 μm; flow rate, 8 mL/min). All solvents used were of ACS grade and purchased from Pharmco-AAPER (Brookfield, CT, USA). Sephadex LH-20 (25–100 μm) was purchased from GE Healthcare (Little Chalfont, UK). C_{18}-functionalized silica gel (40–63 μm) was purchased from Material Harvest Ltd. (Cambridge, UK). Silica gel (230–400 mesh) for column chromatography was purchased from Silicycle (Quebec City, Canada). TLC silica gel plates (60 F_{254}) were purchased from EMD Chemicals Inc. (Darmstadt, Germany).

Isolation of *Streptomyces* sp. RM-4-15 and Preliminary Screening of the Metabolites. The soil sample was collected from the Ruth Mullins underground mine fire, Perry County, KY (coordinates: N 37°18.725′ and W 83°10.3335′). *Streptomyces* sp. RM-4-15 was isolated from soil following minor modifications of prior precedent.[34] Specifically, a soil sample (0.5 g) was suspended in 1.0 mL of sterile H_2O, and the suspension was heated to 75 °C for 10 min to eliminate nonsporulating bacteria. Following serial dilution (10^{-1}, 10^{-2}, 10^{-3}) of the suspension with sterile water, a 100 μL aliquot was spread on oatmeal agar and on ISP4 agar plates supplemented with nalidixic acid (75 μg/mL) and cycloheximide (50 μg/mL). A number of sporulating bacterial colonies were observed after a week of incubation at 28 °C, and each colony was subsequently streaked on an M2 agar plate (glucose, 4.0 g/L; malt extract, 10.0 g/L; yeast extract, 4.0 g/L; and agar, 15.0 g/L; pH of the medium was adjusted to 7.2 with 2 M NaOH prior to sterilization). Individual bacterial colonies were isolated from the second-generation plate and fermented in 50 mL of soytone–glucose medium (glucose, 20 g/L; soytone, 10 g/L; $CaCO_3$, 2 g/L; and $CoCl_2$, 1 mg/L; pH 7.2) using 250 mL baffled Erlenmeyer flasks. Cultures were allowed to grow for six days at 28 °C with agitation (250 rpm). Amberlite XAD-16 (0.5 g, Sigma, St. Louis, MO, USA) resin was added to the culture 24 h prior to harvesting. Each individual culture was transferred to a 50 mL falcon tube, and the mixture centrifuged at 300*g* for 15 min. The supernatant was discarded, and the mycelia-XAD resin portion was washed twice with 80 mL of distilled water prior to the addition of 15 mL of methanol to generate the crude extract. Methanol extract was separated from the resin using Whatman filter paper (150 mm diameter, 11 μm pore size). Each extract was then subjected to HPLC/HRESIMS analysis. Parental mass values were determined based on consideration of all relevant adducts (e.g., $[M + Na]^+$, $[M + NH_4]^+$, and/or $[M - H_2O]^+$), and the corrected molecular masses used as a batch query against the Natural Products Identifier AntiBase 2012[35] to ascertain the potential for chemical novelty. On the basis of this analysis, *Streptomyces* sp. RM-4-15 was identified as among the strains capable of producing novel metabolites.

Identification of *Streptomyces* sp. RM-4-15. Genomic DNA was isolated from streaked colonies and purified using Qiaquick PCR purification kit (Qiagen). Universal primers (27F, 5′-AGAGTTTGATCMTGGCTCAG-3′; 1492R, 5′-GGTTACCTTGTTACGACTT-3′) and HF Taq (New England Biolabs) were used for the amplification of the 16S rRNA gene following prior precedent.[36] The amplified product was purified using Qiaquick gel extraction kit (Qiagen) and sequenced. BLAST search of the amplified 1244 bp fragment revealed 99% identity to the 16S rRNA gene of *Streptomyces coeruleorubidus* strain ISP 5145. The sequence has been deposited in GenBank under the accession number KC297232.

Fermentation. *Streptomyces* sp. RM-4-15 was cultivated in three 250 mL Erlenmeyer flasks, each containing 50 mL of medium A (soluble starch, 20.0 g/L; glucose, 10.0 g/L; peptone, 5.0 g/L; yeast extract, 5.0 g/L; NaCl, 4.0 g/L; K_2HPO_4, 0.5 g/L; $MgSO_4{\cdot}7H_2O$, 0.5 g/L; $CaCO_3$, 2.0 g/L). After three days of incubation at 28 °C with 200 rpm agitation, the cultures were used to inoculate 40 flasks (250 mL), each containing 100 mL of medium A. The fermentation was continued for seven days at 28 °C with 200 rpm agitation. An identical procedure was used to culture *Streptomyces* sp. RM-4-15 in 10 L of medium A containing 18 mg/L $ScCl_3$.

Extraction, Isolation, and Purification. All 40 culture flasks were combined, and 200 g of Celite was added into the culture to facilitate filtration. The mycelial cake−Celite portion was extracted with acetone (2 × 500 mL) and subjected to filtration. Evaporation of the effluent afforded 0.5 g of a reddish-brown crude extract. The culture broth was subjected to an XAD-16 resin column (800 g), washed with water until the effluent became colorless, and then eluted with 5 L of methanol. The MeOH extract was concentrated under reduced pressure to obtain a crude extract (4.0 g). HPLC and TLC analysis indicated an identical set of metabolites in both mycelial cake and culture extracts. Therefore, the extracts were combined (4.5 g) for further processing. Components of the crude extract (4.5 g) were separated by silica gel column chromatography using a gradient of $CHCl_3$−MeOH (100:0−0:100) to yield nine fractions, I−IX. Fraction II (95 mg) was subjected to a Sephadex LH-20 column, and methanol was used to elute compounds at a flow rate of 2 mL/min. The major fraction obtained was dried and then further chromatographed using a C_{18} column (10 × 1 cm) eluted with 60% MeOH−water to afford frenolicin B (**7**, 11 mg). Fraction III (80 mg) was first subjected to a Sephadex LH-20 (MeOH; flow rate, 2 mL/min), followed by preparative TLC ($CHCl_3$−10% MeOH) to yield UCF76-A (**8**, 2 mg). Similarly, fraction IV (105 mg) was loaded onto a C_{18} column (10 × 1 cm) and was separated with 50% aqueous MeOH to afford frenolicin (**6**, 9 mg). Fraction VI (53 mg) was first subjected to a Sephadex LH-20 column (MeOH), and the major fraction was further purified by using a semipreparative HPLC to yield compound **4** (5 mg) following an identical procedure described earlier in the General Experimental Procedures section. Similarly, a gradient of acetonitrile and water was used in HPLC to isolate 17, 10, and 12 mg of compounds **1**−**3**, respectively.

A separate fermentation batch (10 L), accomplished in medium A containing scandium chloride (18 mg/L $ScCl_3$), was centrifuged and filtered over Celite. The supernatant was extracted with EtOAc (4 × 800 mL), and the EtOAc extract subsequently evaporated in vacuo at 38 °C to afford 3.4 g of a yellow crude extract. The biomass (mycelium) was extracted with EtOAc (3 × 600 mL), and then the organic fraction was evaporated to yield 1.7 g of crude extract. Both extracts were combined and separated by an HP-20 resin column (100 g) with a gradient of aqueous CH_3CN (20%, 40%, 60%, 80%, and 100%) to yield five fractions. Five compounds were separated from 60% aqueous CH_3CN fraction III (250 mg) by preparative HPLC, frenolicin (**6**, 50 mg), frenolicin B (7, 42 mg), frenolicin G (**5**, 12 mg), deoxyfrenolicin (**9**, 15 mg), and UCF 13 (**10**, 8 mg).

*Frenolicin C (**1**):* yellow, amorphous powder; UV (MeOH) λ_{max} (log ε) 354 nm (3.60); IR (KBr) ν_{max} 2930, 1717, 1690, 1653, 1603, 1453, 1257, 1220, 1002 cm^{-1}; ^{13}C and ^1H NMR data, see Table 1; HRESIMS *m*/*z* 510.1380 (calcd for $C_{23}H_{28}O_{10}NS$, 510.1434), *m*/*z* 508.1340 (calcd for $C_{23}H_{26}O_{10}NS$, *m*/*z* 508.1277).

*Frenolicin D (**2**):* yellow, amorphous powder; UV (MeOH) λ_{max} (log ε) 351 nm (4.80); IR (KBr) ν_{max} 2935, 1702, 1644, 1602, 1454, 1164, 1265, 1220, 1123, 1047 cm^{-1}; ^{13}C and ^1H NMR data, see Table 2; HRESIMS *m*/*z* 387.1016 (calcd for $C_{18}H_{20}O_8Na$, *m*/*z* 387.1056).

*Frenolicin E (**3**):* yellow, amorphous powder; UV (MeOH) λ_{max} (log ε) 348 nm (5.21); IR (KBr) ν_{max} 2959, 1705, 1651, 1604, 1454, 1261, 1223, 1127, 1020 cm^{-1}; ^{13}C and ^1H NMR data, see Table 2; HRESIMS *m*/*z* 365.1212 (calcd for $C_{18}H_{21}O_8$, *m*/*z* 365.1236).

*Frenolicin F (**4**):* yellow, amorphous powder; UV (MeOH) λ_{max} (log ε) 341 nm (3.34); IR (KBr) ν_{max} 3400, 2962, 1709, 1615, 1455, 1203, 1113, 1027 cm^{-1}; ^{13}C and ^1H NMR data, see Table 2; HRESIMS *m*/*z* 371.1078 (calcd for $C_{18}H_{20}O_7Na$, *m*/*z* 371.1107).

*Frenolicin G (**5**):* yellow, amorphous powder; UV (MeOH) λ_{max} (log ε) 359 nm (9.49); ^{13}C and ^1H NMR data, see Table 1; HRESIMS *m*/*z* 727.2107 (calcd for $C_{36}H_{39}O_{14}S$, *m*/*z* 727.2061), *m*/*z* 725.1947 (calcd for $C_{36}H_{37}O_{14}S$, *m*/*z* 725.1904).

Determination of Amino Acid Configuration. The absolute configuration of the cysteine residue was determined following Marfey's method.[13] Specifically, compound **1** (1.0 mg) was hydrolyzed in 6 N HCl (1 mL) at 110 °C for 14 h. After drying under nitrogen, the residue was dissolved in 2 mL of EtOAc−H_2O (1:1). The aqueous layer was dried in vacuo, to which a solution of 1% Marfey's reagent in acetone (200 μL) was added, followed by 1 M $NaHCO_3$ (50 μL). The reaction was heated to 40 °C for 1 h, cooled to room temperature, and acidified with 2 N HCl (25 μL). The reaction mixture was diluted with MeOH (0.5 mL) and analyzed by HPLC using the following gradient: 0−40 min, linear gradient from 5% to 50% CH_3CN in 50 mM TBDHS buffer (a mixture of 40 mL of 0.5 M KH_2PO_4, 10 mL of 0.5 M K_2HPO_4, 1.7 g of tetrabutylammonium bisulfate, and 20 mL of acetonitrile, pH was adjusted to 6.0 with 0.5 M K_2HPO_4, then adjusted with water to 1 L). Derivatized standards were prepared from authentic D- and L-cysteine (50 μL of a 50 mM stock) following an identical procedure. The retention times for Marfey's derivatives were as follows: derivative prepared from authentic L-cysteine, 38.6 min; from authentic D-cysteine, 40.3 min; and from compound **1**, 38.6 min (Supporting Information Figure S9).

Oxidation of Frenolicin F (4). Oxidation of frenolicin F was accomplished following prior precedent.[14] Specifically, 1.0 mg of compound **4** was dissolved in 0.5 mL of $CDCl_3$, and 1.5 mg of Dess-Martin periodinane was added in the solution. The mixture was stirred for 10 min at room temperature and then subjected to NMR analyses. ^1H NMR (500 MHz) data of the product was compared with the data of the standard frenolicin (**6**). ^1H NMR (500Mz, $CDCl_3$): δ_H 4.62 (H-1), 4.11 (H-3), 7.59 (H-6), 7.64 (H-7), 7.28 (H-8), 11.53 (OH-9), 0.98 (H_3-13), 2.60, 2.92 (H_2-14).

Cancer Cell Line Cytotoxicity. A resazurin-based cytotoxicity assay, also known as the AlamarBlue assay, was used to assess the cytotoxicity of agents against the human lung non-small-cell carcinoma cell line A549 where the degree of cytotoxicity was based upon residual metabolic activity as assessed via reduction of resazurin (7-hydroxy-10-oxido-phenoxazin-10-ium-3-one) to its fluorescent product resorufin. A549 (ATCC, Manassas, VA, USA) was grown in DMEM/F-12 Kaighn's modification and MEM/EBSS media, respectively (Thermoscientific, Rockford, IL, USA), with 10% heat-inactivated fetal bovine serum, 100 μg/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. Cells were seeded at a density of 2×10^3 cells per well onto 96-well culture plates with a clear bottom (Corning, NY, USA), incubated 24 h at 37 °C in a humidified atmosphere containing 5% CO_2, and exposed to standard toxin (positive controls: 1.5 mM hydrogen peroxide, 10 mg/mL actinomycin D) and test compounds for two days. To assess residual metabolic activity, 150 mM resazurin (Sigma, St. Louis, MO, USA) was added to each well and the plate was shaken gently for 10 s and incubated for another 3 h (A549 cells) in a 37 °C incubator to allow viable cells to convert resazurin into resorufin. The fluorescence intensity for resorufin was detected on a FLUOstar Omega scanning microplate spectrofluorometer (BMG Labtech, Cary, NC, USA) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The assay was repeated in three independent experiment replications. In each replication, the emission of fluorescence of resorufin values in treated cells were normalized to, and expressed as a percent of, the mean resorufin emission values of positive control (untreated, metabolically active cells; 100%, all cells are viable).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H NMR, ^{13}C NMR, HSQC, HMBC, ROESY, and HRESIMS spectra of frenolicins C−G (**1**−**5**) and ^1H and ^{13}C NMR spectra of compounds **6**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): J.S.T. is a co-founder of Centrose (Madison, WI, USA).

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■ REFERENCES

(1) Petranka, J. W. *Salamanders of the United States and Canada*; Smithonian Institute Press: Washington, DC, 1998, p 587.
(2) Bernardo, J.; Spotila, J. R. *Biol. Lett* **2006**, *2*, 135−139.
(3) Neves, R. J.; Bogan, A. E.; Williams, J. D.; Ahlstedt, S. A.; Hartfield, W. *Aquatic Fauna in Peril: The Southeastern Perspective*, Special Publication No. 1; Southeastern Research Institute; Lenz Design and Communications: Decatur, GA, 1997; pp 43−86.
(4) Stein, B. A.; Kutner, L. S.; Adams, J. S. *Precious Heritage: The Status of Biodiversity in the United States*; Oxford University Press: Oxford, UK, 2000.
(5) History of Coal in Kentucky. In *Kentucky Coal Facts*, 12th ed.; Division of Fossil Energy Development: Frankfort, KY, 2012; pp 1−101.
(6) O'Keefe, J. M. K.; Henke, K. R.; Hower, J. C.; Engle, M. A.; Stracher, G. B.; Stucker, J. D.; Drew, J. W.; Staggs, W. D.; Murray, T. M.; Hammond, M. L.; Adkins, K. D.; Mullins, B. J.; Lemley, E. W. *Sci. Total Environ.* **2010**, *408*, 1628−1633.
(7) Zhao, Y.; Zhang, J.; Chou, C.; Li, Y.; Wang, Z.; Ge, Y. *Int. J. Coal. Geol.* **2008**, *73*, 52−62.
(8) Carras, J. N.; Day, S. J.; Saghafi, A.; Williams, D. J. *Int. J. Coal. Geol.* **2009**, *78*, 161−168.
(9) Hower, J. C.; Henke, K.; O'Keefe, J. M. K.; Engle, M. A.; Blake, D. R.; Stracher, G. B. *Int. J. Coal Geol.* **2009**, *80*, 63−67.
(10) Silva, L. F. O.; Oliveira, M. L. S.; Philippi, V.; Serra, C.; Dai, S. F.; Xue, W. F.; Chen, W. M.; O'Keefe, J. M. K.; Romanek, C. S.; Hopps, S. G.; Hower, J. C. *Int. J. Coal Geol.* **2012**, *94*, 206−213.
(11) Kawai, K.; Wang, G.; Okamoto, S.; Ochi, K. *FEMS Microbiol. Lett.* **2007**, *274*, 311−315.
(12) Maestrone, G.; Schildknecht, H.; Untawale, G. G. U.S. Patent 4,839, 382, 1989.
(13) Marfey, P. *Carlsberg. Res. Commun.* **1984**, *49*, 591−596.
(14) Dess, D. B.; Martin, J. C. *J. Org. Chem.* **1983**, *48*, 4155−4156.
(15) Ellestad, G. A.; Kunstmann, M. P.; Whaley, H. A.; Patterson, E. L. *J. Am. Chem. Soc.* **1968**, *90*, 1325−1332.
(16) Ellestad, G. A.; Whaley, H. A.; Patterso, E. *J. Am. Chem. Soc.* **1966**, *88*, 4109−4110.
(17) Iwai, Y.; Kora, A.; Takahashi, Y.; Hayashi, T.; Awaya, J.; Masuma, R.; Oiwa, R.; Omura, S. *J. Antibiot.* **1978**, *31*, 959−965.
(18) Hara, M.; Soga, S.; Shono, K.; Eishima, J.; Mizukami, T. *J. Antibiot.* **2001**, *54*, 182−186.
(19) Nakano, H.; Hara, M.; Saito, Y.; Inata, Y.; Kawada, S.; Okabe, M. Japan Patent 04154778, 1992.
(20) Armer, R. E.; Dutton, C. J.; Fenner, B. R.; Greenwood, S. D.; Hall, K. T.; Rudge, A. J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 139−142.
(21) Fitzgerald, J. T.; Henrich, P. P.; O'Brien, C.; Krause, M.; Ekland, E. H.; Mattheis, C.; Sa, J. M.; Fidock, D.; Khosla, C. *J. Antibiot.* **2011**, *64*, 799−801.
(22) Fitzgerald, J. T.; Ridley, C. P.; Khosla, C. *J. Antibiot.* **2011**, *64*, 759−762.
(23) Toral-Barza, L.; Zhang, W. G.; Huang, X.; McDonald, L. A.; Salaski, E. J.; Barbieri, L. R.; Ding, W. D.; Krishnamurthy, G.; Hu, Y. B.; Lucas, J.; Bernan, V. S.; Cai, P.; Levin, J. I.; Mansour, T. S.; Gibbons, J. J.; Abraham, R. T.; Yu, K. *Mol. Cancer Ther.* **2007**, *6*, 3028−3038.
(24) Salaski, E. J.; Krishnamurthy, G.; Ding, W. D.; Yu, K.; Insaf, S. S.; Eid, C.; Shim, J.; Levin, J. I.; Tabei, K.; Toral-Barza, L.; Zhang, W. G.; McDonald, L. A.; Honores, E.; Hanna, C.; Yamashita, A.; Johnson, B.; Li, Z.; Laakso, L.; Powell, D.; Mansour, T. S. *J. Med. Chem.* **2009**, *52*, 2181−2184.
(25) Fernandes, R. A.; Chavan, V. P.; Mulay, S. V.; Manchoju, A. *J. Org. Chem.* **2012**, *77*, 10455−10460.
(26) Kraus, G. A.; Li, J.; Gordon, M. S.; Jensen, J. H. *J. Am. Chem. Soc.* **1993**, *115*, 5859−5860.
(27) Johnson, L. E.; Dietz, A. *Appl. Microbiol.* **1968**, *16*, 1815−1821.
(28) Tanaka, H.; Nagai, T.; Marumo, H.; Omura, S. *J. Antibiot.* **1975**, *XXVIII*, 868−875.
(29) Leo, P.-M.; Morin, C.; Philouze, C. *Org. Lett.* **2002**, *4*, 2711−2714.
(30) Hochloqski, J. E.; Brill, G. M.; Andres, W. W.; Spanton, S. G.; McAlpine, J. B. *J. Antibiot.* **1987**, *XL*, 401−407.
(31) Chiang, Y.-M.; Chang, S.-L.; Oakley, B. R.; Wang, C. C. C. *Curr. Opin. Chem. Biol.* **2011**, *15*, 137−143.
(32) Ochi, K.; Hosaka, T. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 87−98.
(33) Corre, C.; Challis, G. L. *Nat. Prod. Rep.* **2009**, *26*, 977−986.
(34) Abdelfattah, M. S.; Kharel, M. K.; Hitron, J. A.; Baig, I.; Rohr, J. *J. Nat. Prod.* **2008**, *71*, 1569−1573.
(35) Laatsch, H. *AntiBase 2012, The Natural Compound Identifier*; Wiley-VCH: Hoboken, NJ, 2012.
(36) Lane, D. J. In *Nucleic Acid Techniques in Bacterial Systematics*; Stackebrandt, E.; Goodfellow, M., Eds.; John Wiley and Sons Ltd.: New York, 1991; pp 115−147.