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Ruthmycin, a New Tetracyclic Polyketide from *Streptomyces* sp. RM-4-15

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Abstract

ruthmycin (1)

The isolation and structural elucidation of a new tetracyclic polyketide (ruthmycin) from *Streptomyces* sp. RM-4-15, a bacteria isolated near thermal vents from the Ruth Mullins underground coal mine fire in eastern Kentucky, is reported. In comparison to the well-established frenolicin core scaffold, ruthmycin possesses an unprecedented signature C₃ bridge and corresponding fused six member ring. Preliminary in vitro antibacterial, anticancer and antifungal assays revealed ruthmycin to display moderate antifungal activity.

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Supporting Information Available: Experimental procedures, HRESIMS, CD, ¹H-, ¹³C-NMR, HSQC, HMBC and ROESY spectra of ruthmycin (1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Pyranonaphthoquinones are polyketides produced by various bacteria and fungi, the majority of which have been isolated from actinomycetes.¹ The frenolicins are a predominate group of pyranonaphthoquinones known for their antibacterial, antifungal and antiprotozoal activities that typically contain a C-1 propyl side chain and a C-3 acetate, acetate *O*-methyl ester or a corresponding C-3/C-4 fused cyclic lactone, as exemplified by deoxyfrenolicin, UCF76-A and frenolicin B, respectively (Figure 1).² As part of our ongoing natural product initiative to characterize new metabolites produced by actinomycete isolates from unique environments within Appalachia (including underground and surface coal mines, thermal vents from underground mine fires and mine reclamation sites), we have adapted a HRMS-based metabolomics strategy to rapidly delineate unique metabolite signatures as a primary strain prioritization strategy. This process has facilitated the recent discovery of a range of structurally diverse new bacterial metabolites including isopterocarpone and representative herbimycins, venturicidins and frenolicins.³ With respect the latter, several frenolicins from the *Streptomyces* sp. RM-4-15 (an isolate from the soil collected near a thermal vent of the Ruth Mullins coal fire), exhibited notable anticancer *in vitro* cytotoxicity,^{3d} prompting an interest to extend the SAR of this privileged scaffold via medicinal chemistry⁴ and/or expanding fermentation-based strategies.

In the course of fermentation-based scale up (30 L) to produce select frenolicins for animal studies, a new metabolite was detected that displayed a distinct LC-MS signature from known frenolicins of *Streptomyces* sp. RM-4-15 origin or related compounds in Antibase⁵ but with UV characteristics reminiscent of frenolicin F (Figure 1).^{3d} As described herein, subsequent isolation, purification, and structure elucidation of the newly identified metabolite revealed a new tetracyclic skeletal framework, henceforth referred to as ruthmycin (**1**) in reference to the producing strain's point of origin. Preliminary activity assays revealed ruthmycin to display moderate antifungal activity.

A seed culture of *Streptomyces* sp. RM-4-15 cultivated in medium A for 3 days was used to inoculate 30 L of production medium (4% W/V) and fermentation was continued for 7 days at 28 °C and 200 rpm. The culture broth was centrifuged and the supernatant and mycelial cake separately extracted with EtOAc. Based on the corresponding HPLC and TLC profiles, both extracts were combined and subsequent removal of solvent afforded 7.5 g reddish-brown crude extract. The obtained crude extract was fractionated by preparative C₁₈ column chromatography. The fraction eluted with 40% CH₃CN/H₂O was subsequently subjected to Sephadex LH-20 column chromatography followed by semi-preparative C₁₈ HPLC to yield compound **1** in pure form (5 mg, yield: 0.17 mg/L, see Supporting Information).

Compound **1**⁶ was obtained as a yellow amorphous powder. HRESIMS data established the molecular formula of **1** as C₂₁H₂₃O₇ (*m/z* 387.1437 [M+H]⁺) indicating eleven degrees of unsaturation. The IR spectrum of **1** displayed characteristic absorptions for OH (3374 cm⁻¹) and C=O (1717 cm⁻¹) functionality while the proton NMR and COSY spectra indicated the presence of a trisubstituted benzene ring at δ_H 7.65 (dd, *J* = 7.6, 8.4, H-7), 7.35 (d, *J* = 7.6, H-6) and 6.88 (d, *J* = 8.4, H-8), a four carbon fragment and two three carbon fragments (highlighted in bold, Figure 2) – all signatures consistent with frenolicin-type architecture. A key proton signal observed at δ_H 6.46 (s, 5-OH) in DMSO-*d*₆, with no detectable HSQC carbon atom correlations and HMBC correlations with C-4a (δ_C 140.3), C-5 (δ_C 73.0), C-5a (δ_C 149.8) and C-16 (δ_C 60.0), supported the presence of an adjacent C-5-OH. This was further supported by HMBC correlations from H-6 to C-5, and from H₂-16 to C-5 and C-5a. A distinguishing allylic proton signal at δ_H 6.33 (s, H-4), which displayed HSQC correlation with C-4 (δ_C 121.8) and HMBC correlations with C-3 (δ_C 66.5), C-4a (δ_C 140.3), C-5 (δ_C 73.0) and C-14 (δ_C 39.6), supported the presence of an unprecedented C-4/C-4a double bond in the context of known frenolicin-type scaffolds. By comparing the **1** ¹³C-NMR and MS data to that of frenolicin B, three extra carbons must be accounted for in **1** – a ketone (δ_C

204.7, C-17) and two methylenes (δ_C 60.0 (C-16) and δ_C 54.5 (C-18), respectively). While the $^1\text{H-NMR}$ of **1** acquired in CDCl_3 failed to resolve the corresponding CH_2 proton signals of interest, the use of acetone- d_6 enabled unambiguous assignment of the target protons (see Supporting Information). The subsequent HMBC correlations from H_2 -16 to C-4a, C-5 and C-17, and from H_2 -18 to C-1, C-10 and C-16 provided further support for the new cyclohexanone ring D deriving from a unique C-5/C-10a C_3 bridge insertion of the frenolicin core skeleton (Figure 1).

The relative configuration of **1** was assigned based on ROESY spectral analyses (Figure 2). NOE correlations between H-3 and H_2 -11 indicated a cofacial relationship, consistent with the β -orientation of frenolicin B.^{2b} H-1/H-18 and H-16/H-18 cross peaks provided key support for the assignment of their α -orientation and the corresponding β -orientation of the 5-OH. Thus, the relative configuration of **1** was proposed as illustrated in Figure 1.

Despite extensive effort, attempts to produce suitable crystals of **1** for X-ray diffraction were unsuccessful. Alternatively, electronic circular dichroism (ECD) analysis was utilized as an indirect determination of absolute configuration. Specifically, time-dependent density functional theory (TDDFT) was used to calculate the ECD spectra of the two possible enantiomers of **1**.⁷ Using GaussSum 2.2,⁸ the theoretical ECD spectra were then compared to the actual **1** ECD spectra (experimentally determined in MeOH, see Supporting Information, Figure S14). This comparison revealed a best experimental/actual ECD spectral match for the 1*R*, 3*R*, 5*S*, 10*aR* isomer (Figure 3). Thus, the absolute configuration of **1** is put forth as 1*R*, 3*R*, 5*S*, 10*aR*.

As a preliminary assessment of biological activity, ruthmycin (**1**) was tested in simple antibacterial, anticancer and antifungal assays. In an antifungal disc diffusion assay, **1** outperformed amphotericin B and cycloheximide against *Aspergillus nidulans* (ATCC 38163), a fungal strain known to be resistant to polyene antifungals.⁹ In microdilution assays, **1** moderately inhibited both *Saccharomyces cerevisiae* (ATCC 204508, MIC 30 μM) and *Staphylococcus aureus* (ATCC 6538, MIC 60 μM) but had no effect on *Salmonella enterica* (ATCC 10708, at concentrations $\geq 120 \mu\text{M}$). Compound **1** displayed no apparent cytotoxicity against model human cancer cell lines (human non-small cell lung A549; human prostate PC3) at concentrations $\leq 10 \mu\text{M}$.

Naturally-occurring frenolicin analogues reported to date mainly differ via subtle C-4, C-4a and C-10a alterations with the recently reported frenolicin F (also from *Streptomyces* sp. RM-4-15) as the first example of a C-5-hydroxy bearing analog.^{2,3d} The unusual C-5/C-10a three carbon bridge and resulting fused D ring system clearly distinguishes ruthmycin (**1**) as a new tetracyclic structural scaffold. Herbarone (Figure 4), a tricyclic polyketide isolated from the sea hare associated fungus *Torula herbarum*, shares structural similarities with the A-B-D ring system of **1**.¹⁰ Biosynthetically, the fused C ring of herbarone has been proposed to derive from a rearrangement of the polyketide core culminating in an intramolecular aldol reaction to provide the key C5/C6 C ring connection. While metabolic labeling and/or shunt metabolites in support of the proposed herbarone biosynthetic pathway are lacking, invoking an analogous putative rearrangement en route to **1** is implausible. In contrast, the intact frenolicin core of **1** suggests installation of the 3-carbon unit (and the culminating key D ring fusion event) to directly diverge from a late stage frenolicin biosynthetic intermediate.¹¹

In conclusion, the isolation and characterization of ruthmycin (**1**) highlights the characterization of a new tetracyclic polyketide produced by an actinomycete isolated from the soil associated with underground coal fires in eastern Kentucky. As such, this study highlights the potential for the continued study of under/unexplored terrestrial environments for the discovery of novel natural product scaffolds. In addition, the structure of ruthmycin

reflects a notable adaptation of the naturally-occurring frenolicin core via the potential participation of unique biosynthetic machinery. Thus, studies are underway to elucidate the identity of the signature 3-carbon precursor and the corresponding mechanism of late stage D ring formation en route to **1**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Brimble MA, Duncalf LJ, Nairn MR. *Nat Prod Rep*. 1999; 16:267–281. [PubMed: 10399361]
2. (a) Ellestad GA, Kunstmann MP, Whaley HA, Patterson EL. *J Am Chem Soc*. 1968; 90:1325–1332. [PubMed: 5636537] (b) Iwai Y, Kora A, Takahashi Y, Hayashi T, Awaya J, Masuma R, Oiwa R, Omura S. *J Antibiot*. 1978; 31:959–965. [PubMed: 711620] (c) Hara M, Soga S, Shono K, Eishima J, Mizukami T. *J Antibiot*. 2001; 54:182–186. [PubMed: 11302493] (d) Salaski EJ, Krishnamurthy G, Ding WD, Yu K, Insaf SS, Eid C, Shim J, Levin JI, Tabei K, Toral-Barza L, Zhang WG, McDonald LA, Honores E, Hanna C, Yamashita A, Johnson B, Li Z, Laakso L, Powell D, Mansour TS. *J Med Chem*. 2009; 52:2181–2184. [PubMed: 19309081]
3. (a) Shaaban KA, Wang X, Elshahawi SI, Ponomareva LV, Sunkara M, Copley GC, Hower JC, Morris AJ, Kharel MK, Thorson JS. *J Nat Prod*. 2013; 76:1619–1626. [PubMed: 23947794] (b) Shaaban KA, Singh S, Elshahawi SI, Wang X, Ponomareva LV, Sunkara M, Copley GC, Hower JC, Morris AJ, Kharel MK, Thorson JS. *J Nat Prod*. 2013; 76:1441–1447. [PubMed: 23944931] (c) Shaaban KA, Singh S, Elshahawi SI, Wang X, Ponomareva LV, Sunkara M, Copley GC, Hower JC, Morris AJ, Kharel MK, Thorson JS. *Nat Prod Res*. 2013; 27:1080–1088. [PubMed: 23944931] (d) Wang X, Shaaban KA, Elshahawi SI, Ponomareva LV, Sunkara M, Zhang Y, Copley GC, Hower JC, Morris AJ, Kharel MK, Thorson JS. *J Nat Prod*. 2013; 76:1441–1447. [PubMed: 23944931] (e) YouTube, Web. University of Kentucky Research Communications; Nov 6. 2013 <http://www.youtube.com/watch?v=VglEEjMviVA>
4. Zhang Y, Wang X, Sunkara M, Ye Q, Ponomareva LV, She QB, Morris AJ, Thorson JS. *Org Lett*. 2013; 15:5566–5569. [PubMed: 24151973]
5. Laatsch, H. *AntiBase*. Wiley-VCH; Weinheim, Germany: 2012.
6. Ruthmycin (1): $C_{21}H_{22}O_7$; yellow amorphous powder; $[\alpha]_D^{25} + 13.3^\circ$ (c 0.7, MeOH); UV (MeOH) λ_{max} (log ϵ) 342 nm (2.47); IR (KBr) ν_{max} 3374, 3177, 1717, 1680, 1607, 1334, 1121, 1068 cm^{-1} ; ^{13}C and 1H NMR data, see Table 1; APCI-MS: m/z 387.2 $[M + H]^+$, m/z 385.2 $[M - H]^-$, and m/z 771.6 $[2M - H]^-$; (+)-HR-ESI-MS: m/z 387.1437 (calcd for $C_{21}H_{23}O_7$, 387.1438).
7. (a) Menna M, Aiello A, D'Aniello F, Imperatore C, Luciano P, Vitalone R, Irace C, Santamaria R. *Eur J Org Chem*. 2013; 16:3241–3246. (b) Piao SJ, Song YL, Jiao WH, Yang F, Liu XF, Chen WS, Han BN, Lin HW. *Org Lett*. 2013; 15:3526–3529. [PubMed: 23829334]
8. O'Boyle NM, Tenderholt AL, Langner KM. *J Comput Chem*. 2008; 29:839–845. [PubMed: 17849392]
9. (a) Moore CB, Sayers N, Mosquera J, Slaven J, Denning DW. *J Infection*. 2000; 41:203–220. (b) Kontoyiannis DP, Lewis RE, May GS, Osherov N, Rinaldi MG. *Mycoses*. 2002; 45:406–407. [PubMed: 12421291]
10. Geng WL, Wang XY, Kurtán T, Mándi A, Tnag H, Schulz B, Sun P, Zhang W. *J Nat Prod*. 2012; 75:1828–1832. [PubMed: 23043498]

11. (a) Fitzgerald JT, Ridley CP, Khosla C. J Antibiot. 2011; 64:759–762. [PubMed: 21934692] (b) Mikko MK, Terhi O, Takaaki T, Susumu O, Koji I. Curr Opin Chem Biol. 2013; 17:562–570. [PubMed: 23886982]

Figure 1.
Ruthmycin (1) and representative naturally-occurring frenolicin analogs.

Figure 2.
COSY, key HMBC and ROESY correlations of ruthmycin (**1**).

Figure 3.
Experimental and theoretical ECD spectra of ruthmycin (**1**).

Figure 4.
The structure of hebarone, a metabolite of the sea hare-associated fungus *Torula herbarum*.

Table 1NMR Data for ruthmycin (**1**) in acetone- d_6 (δ in ppm, J in Hz)

no.	δ_C , type	δ_H (J in Hz)	HMBC
1	76.3, CH	4.09, d (10.3)	
3	66.5, CH	4.68, t (5.5, 5.8)	4, 4a, 14, 15
4	121.8, CH	6.33, d (1.6)	3, 5, 4a, 10a, 14
4a	140.3, C		
5	73.0, C		
5a	149.8, C		
6	115.9, CH	7.35, d (7.6)	5, 8, 9a
7	139.3, CH	7.65, dd (7.6, 8.4)	5a, 9
8	117.6, CH	6.88, d (8.4)	6, 9a
9	163.0, C		
9a	115.1, C		
10	205.3, C		
10a	52.8, C		
11	29.2, CH ₂	1.55, 1.75, m	
12	20.0, CH ₂	1.42, 1.64, m	13
13	14.1, CH ₃	0.92, t (7.0)	11, 12
14	39.6, CH ₂	2.72, m	3, 4, 15
15	171.9, C		
16	60.0, CH ₂	2.61, 3.02, m	4a, 5, 5a, 17
17	204.7, C		
18	54.5, CH ₂	2.58, 3.33, m	1, 10, 16
5-OH		6.46 ^a , s	4a, 5, 5a, 16 ^a
9-OH		12.15, s	8, 9, 9a

^a measured in DMSO- d_6 , 500MHz.