Organic LETTERS

Letter

pubs.acs.org/OrgLett

Pyrazolofluostatins A–C, Pyrazole-Fused Benzo[*a*]fluorenes from South China Sea-Derived *Micromonospora rosaria* SCSIO N160

Wenjun Zhang,[†,∥,§] Chunfang Yang,[†,§] Chunshuai Huang,[†,‡] Liping Zhang,[†] Haibo Zhang,[†] Qingbo Zhang,[†] Cheng-shan Yuan,[†] Yiguang Zhu,[†] and Changsheng Zhang*[,†]

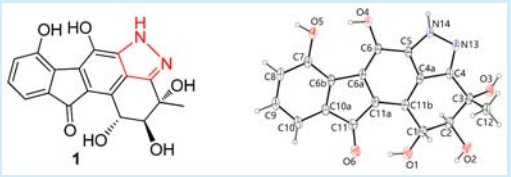
[†]Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China

[‡]University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China

[∥]State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, P. R. China

Supporting Information

ABSTRACT: Pyrazolofluostatins A–C (**1**–**3**), three new benzo[*a*]-fluorenes with an unprecedented carbon skeleton, were obtained from the South China Sea-derived *Micromonospora rosaria* SCSIO N160. Their structures were elucidated by extensive spectroscopic analyses. The structure of pyrazolofluostatin A (**1**) was confirmed by X-ray crystallographic analysis. Notably, **1**–**3** possessed a benzo[*cd*]indeno[2,1-*f*]indazol skeleton with a pyrazole-fused 6/5/6/6/5 pentacyclic ring system. Pyrazolofluostatin A (**1**) showed moderate antioxidation activity (EC_{50} 48.6 μM).



Fluostatins are a class of atypical angucyclines containing a distinctive tetracyclic benzo[*a*]fluorene skeleton.[1] The fluostatin family of natural products was reported to have diverse bioactivities including dipeptidyl peptidases inhibition and antibacterial and antitumor activities.[2] To date, 13 fluostatin analogues (fluostatins A–L and difluostatin A) have been discovered by various strategies, such as traditional isolation methods,[2a,3] environmental DNA-based metagenomic approach,[2b] and heterologous expression of the fluostatin gene cluster.[2c] We have reported the isolation of fluostatins C–F and I–K from a South China Sea-derived *Micromonospora rosaria* SCSIO N160.[3b] Recently, the identification of the fluostatin gene cluster from *M. rosaria* SCSIO N160 and the heterologous expression in *Streptomyces coelicolor* YF11,[4] led to the discovery of fluostatin L and a heterodimer difluostatin A.[2c] A careful investigation of the metabolite profile of *M. rosaria* SCSIO N160 revealed the presence of several minor components with characteristic UV spectra of benzo[*a*]fluorene. A large scale (40 L) culture and repeated separation of the crude extracts led to the discovery of pyrazolofluostatins A–C (**1**–**3**, Figure 1), containing an unusual pyrazole-fused 6/5/6/6/5 pentacyclic ring. Herein, we report the isolation, structure elucidation, and bioactivities of **1**–**3**, as well as a plausible biosynthetic pathway.

Figure 1. Structures of pyrazolofluostatins A–C (**1**–**3**).

The 40 L of fermentation cultures of *M. rosaria* SCSIO N160 were subjected to acetone extraction and resin (Amberlite XAD-16) absorption. Several chromatographic separation steps afforded **1** (7.8 mg), **2** (6.2 mg), and **3** (4.3 mg).

Pyrazolofluostatin A (**1**) was obtained as a dark-red crystal. Its molecular formula $C_{18}H_{14}N_2O_6$ was determined by high-resolution electrospray ionization mass spectrometry (HRESIMS) data (*m*/*z* 353.0784, $[M - H]^-$, calcd for 353.0779), corresponding to 13 degrees of unsaturation. The ^{1}H NMR spectrum of **1** (Table 1) showed the presence of a singlet methyl (δ_H 1.42, 3H, s), a pair of oxygenated and intercoupled methine groups (δ_H 5.03, 1H, d, *J* = 4.0 Hz; 3.80, 1H, d, *J* = 4.0 Hz), one 1,2,3-trisubstituted phenyl (δ_H 6.71, 1H, d, *J* = 7.5 Hz; 6.90, 1H, dd, *J* = 7.0, 7.5 Hz; 6.82, d, *J* = 7.0 Hz), and three exchangeable protons (δ_H 5.92, 1H, s; 5.21, 1H, s; 5.25, 1H, s). The ^{13}C and 2D NMR spectroscopic data of **1** (Table 1, Figure S1) displayed resonances for 18 carbons, which were ascribed to one methyl, two sp^3 methines, three sp^2 methines, and 12 quaternary carbons. Further 2D NMR analyses (Figure 2) indicated the presence of a typical fluorenone ring (A, B, and C) and a cyclohexene ring (D) in **1** and suggested that **1** belongs to the fluostatin family of natural products.[2,3b,c]

The NMR spectroscopic data of **1** were similar to those of fluostatin C.[3b] The downfield shifts of the C-2 (δ_C 78.9, Δ 14.9 ppm) and C-3 (δ_C 71.1, Δ 21.6 ppm) in **1** suggested the

Received: December 16, 2016
Published: January 11, 2017

DOI: 10.1021/acs.orglett.6b03745
Org. Lett. 2017, 19, 592–595

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Assignments of Compounds 1−3 (J in Hz within Parentheses)

	1[a]		2[a]		3[a]	
no.	δ_H multi (J in Hz)	δ_C multi	δ_H multi (J in Hz)	δ_C multi	δ_H multi (J in Hz)	δ_C multi
1	5.03, d (4.0)	69.3, CH	5.01, d (7.3)	69.9, CH	5.21, br s	66.3, CH
2	3.80, d (4.0)	78.9, CH	3.52, d (7.3)	80.7, CH	3.97, br d (2.0)	75.6, CH
3		71.1, C		69.9, C	3.33, dq (3.0, 7.0)	30.9, CH
4		152.0, C		151.9, C		150.4, C
4a		121.3, C		122.1, C		123.7, C
5		137.5, C		135.5, C		135.7, C
6		144.1, C		143.1, C		138.9, C
6a		125.3, C		121.6, C		121.5, C
6b		133.0, C		133.1, C		130.9, C
7		153.4, C		153.2, C		150.8, C
8	6.71, d (7.5)	122.4, CH	6.78, d (7.0)	123.3, CH	6.98, d (7.5)	122.8, CH
9	6.90, dd (7.0, 7.5)	127.5, CH	6.95, dd (7.0, 7.5)	128.6, CH	7.12, dd (7.5, 7.0)	129.4, CH
10	6.82, d (7.0)	113.1, CH	6.88, d (7.0)	114.5, CH	7.05, d (7.0)	115.5, CH
10a		135.1, C		137.5, C		137.1, C
11		194.3, C		195.3, C		192.8, C
11a		124.7, C		125.5, C		125.5, C
11b		124.8, C		128.0, C		127.8, C
12	1.42, s	22.6, CH_3	1.58, s	23.8, CH_3	1.36, d (7.0)	13.3, CH_3
1-OH	5.92, s		6.02, s		5.37, br s	
2-OH	5.21, s		5.07, br s		4.88, br s	
3-OH	5.25, s		4.85, br s			

[a]Recorded at 500 MHz in DMSO-d_6; assignments were based on DEPT, HSQC, COSY, HMBC, and NOESY experiments.

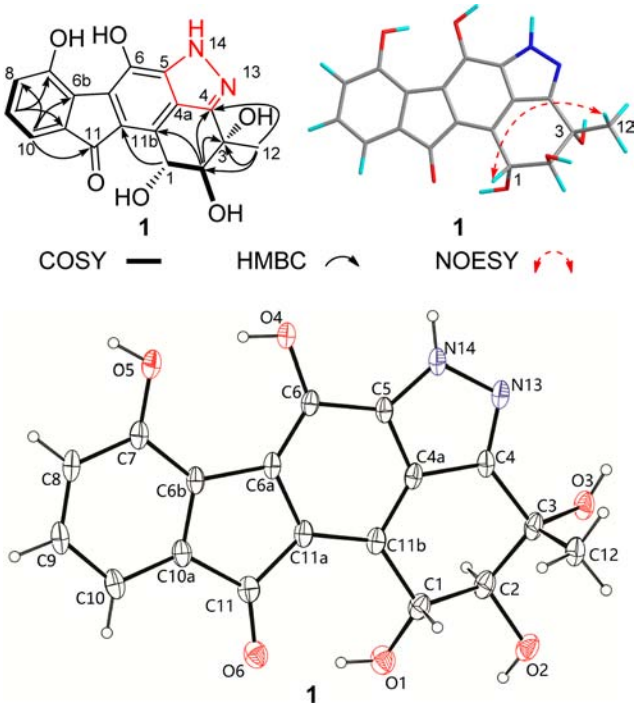


Figure 2. Selected COSY, HMBC, and NOESY correlations of **1** and the ORTEP representation of **1**.

presence of two hydroxy groups at C-2/C-3 in **1**, instead of the epoxy ring at C-2/C-3 in fluostatin C. This assignment was supported by the COSY correlation between OH-2/H-2 (Figure 2). Since the established fluostatin-type skeleton (rings ABCD) in **1** has accounted for 18 carbons and six oxygens, only two nitrogen atoms remained unassigned. The upfield shift of 41.3 ppm at C-4 (δ_C 152.0) in **1**, compared to that of fluostatin C, suggests that the carbonyl (C═O) in fluostatin C was substituted by a C═N in **1**. The aromatic singlet signal at δ_H 7.43 (H-5) in fluostatin C was absent in **1**. Taking into account these facts, a pyrazole ring (E) fused to rings C and D was assumed to satisfy the 13 degrees of unsaturation. Thus, the planar structure of **1** was proposed as shown in Figure 1. The *trans* configuration between H-1 and H-2 in **1** was indicated by the J value of H-1/H-2 ($^{3}J_{H1-H2}$ 4.0 Hz).[3b] The NOE correlations between H-1 and H_3-12 in **1** indicated that both hydroxy groups at C-1 and C-3 were on the same side (Figure 2). Finally, **1** was crystallized in a mixture of MeOH/H_2O (v/v, 17/1). An X-ray analysis using Cu Kα radiation (CCDC 1481191[5]) confirms the structure of **1** (Figure 2). On the basis of a Flack parameter value of 0.05(3), the absolute configuration of **1** was assigned as 1*R*, 2*S*, and 3*S*.

Pyrazolofluostatin B (**2**) was isolated as a red powder. The molecular formula of **2** was assigned as $C_{18}H_{14}N_2O_6$ ([M − H]$^-$, m/z 353.0788, calcd for 353.0779) by HRESIMS, the same as that of **1**. The 1D and 2D NMR spectroscopic data of **2** (Table 1, Figure S2) were highly similar to those of **1**, suggesting that compound **2** might be a stereoisomer of **1**. The planar structure of **2** was confirmed by detailed 2D NMR data analysis (Figure 3) and comparison with that of **1**. On the basis of the larger $^{3}J_{H1-H2}$ (7.3 Hz) coupling constant in **2** than that in **1** ($^{3}J_{H1-H2}$ 4.0 Hz), a *cis* configuration between H-1 and H-2 in **2** was suggested. The observed NOE correlations of OH-1/OH-3 and H-2/H_3-12 in **2** (Figure 3, Figure S2) indicate that both hydroxy groups at C-1 and C-3 are on the same side. Cumulatively, the absolute configuration of **2** was tentatively assigned as 1*R*, 2*R*, and 3*S* upon comparing with **1**.

Pyrazolofluostatin C (**3**) was obtained as a red solid. The molecular formula of **3** was assigned as $C_{18}H_{14}N_2O_5$ (m/z 337.0838, [M − H]$^-$, calcd for 337.0830) by HRESIMS. The ^{1}H and ^{13}C NMR spectroscopic data of **3** and **1** are also highly similar (Table 1, Figure S3). The major difference was that the oxygenated quaternary carbon (δ_C 71.1) in **1** was not found in **3**, while one more methine carbon (δ_C 30.9) was present in **3**.

DOI: 10.1021/acs.orglett.6b03745
Org. Lett. 2017, 19, 592−595

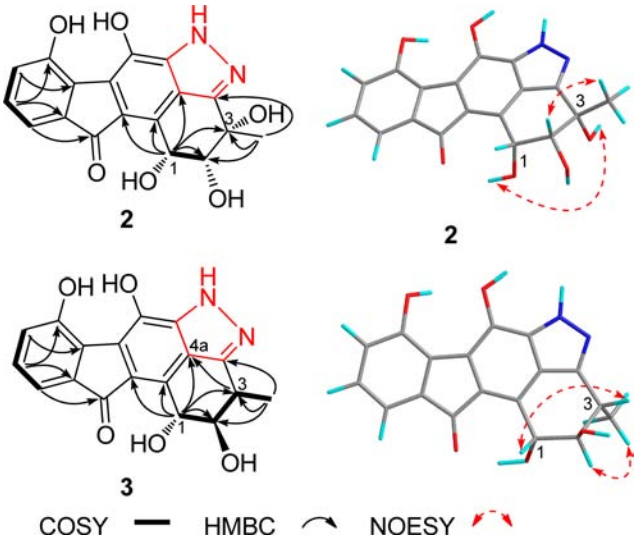


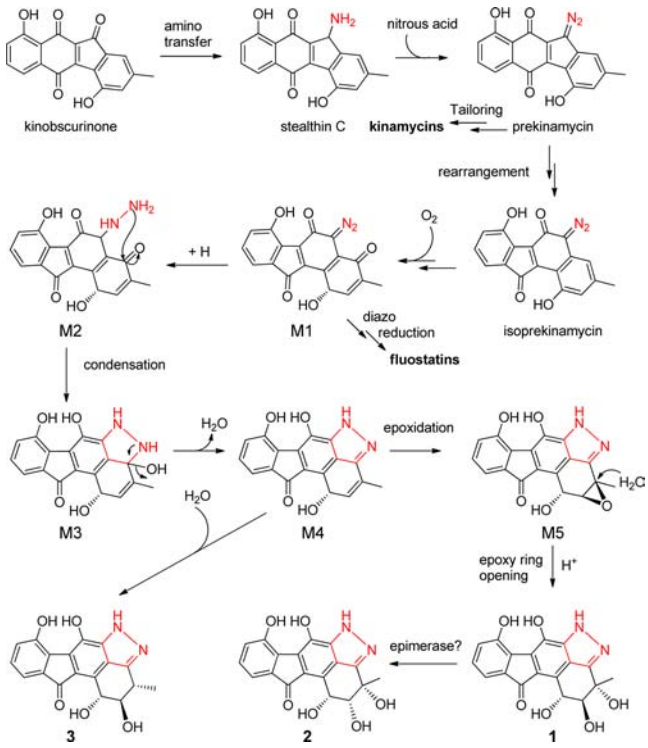
Figure 3. Selected COSY, HMBC, and NOESY correlations of **2** and **3**.

Therefore, pyrazolofluostatin C (**3**) was deduced to be different from **1** by the absence of the OH group at C-3. This assignment was confirmed by the COSY correlation of H-3/H_3-12, and the HMBC correlations from H_3-12 to C-2/C-3/C-4 (Figure 3). Thus, the planar structure of **3** was determined as shown in Figure 1. The *trans* configurations of H-1/H-2 and H-2/H-3 in **3** were supported by the small *J* values ($J_{H\text{-}1/H\text{-}2}$ = 2.0 Hz and $J_{H\text{-}2/H\text{-}3}$ = 3.0 Hz) and the observed NOE correlations of H-1/H-3 and H-2/H_3-12 in **3** (Figure 3, Figure S3).[3b] Considering a similar biosynthetic origin as **1**, the absolute configuration of **3** was tentatively assigned as 1*R*, 2*R*, and 3*S*.

Thus, pyrazolofluostatins A–C (**1**–**3**) were structurally elucidated to contain a 6/5/6/6/5 pentacyclic ring system with an unusual pyrazole-fused benzo[*cd*]indeno[2,1-*f*]indazole skeleton. Pyrazole-containing natural products are a rare class of compounds but are of great pharmaceutical significance with a variety of biological activities.[6] Since the first report of a naturally occurring pyrazole-containing natural product, *β*-pyrazol-1-ylalanine from watermelon (*Citrullus vulgaris* var. Tom Watson) seeds in 1959,[7] about 40 pyrazole derivatives have been isolated from natural sources,[8] with the most recent occurrence of pyrazole alkaloids from watermelon (*Citrullus lanatus*) seeds.[9] Synthetic efforts have brought more than 200 pyrazole derivatives for developing therapeutic agents with antimicrobial, anticancer, antianxiety, and anti-inflammatory activities.[6] Pyrazolofluostatins A–C (**1**–**3**) showed weak antimicrobial activities against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Bacillus thuringensis* SCSIO BT01, *Bacillus subtilis* SCSIO BS01, and *Candida albicans* ATCC 10231. Pyrazolofluostatins A–C (**1**–**3**) displayed no cytotoxicities against four human cancer cell lines SF-268, MCF-7, NCI-H460, and HepG2. Interestingly, pyrazolofluostatin A (**1**) exhibited moderate antioxidation activity (EC_{50} 48.6 *μ*M, Table S2).

The discovery of pyrazolofluostatins provides evidence to support the previous hypothesis that the biosynthesis of fluostatins, kinamycins, and lomaiviticins shares similar diazo-containing intermediates.[2c,10] Given that kinobscurinone,[11] stealthin C,[12] and prekinamycin[13] have been confirmed to be precursors in the kinamyicn biosynthetic pathway, we propose that the formation of pyrazolofluostatins involves the following key steps (Scheme 1): (i) a transamination reaction converts kinobscurinone to stealthin C; (ii) the conversion of stealthin C

Scheme 1. Plausible Biosynthetic Pathway of 1–3



to prekinamycin involves an unusual *N*–*N* bond formation to afford the diazo group, in which the distal nitrogen was putatively derived from nitrous acid, similar to the proposed biosynthesis of pyridazine unit in azamerone,[14] and the formation of the diazo group confirmed in cremeomycin biosynthesis;[15] (iii) tailoring modifications of prekinamycins produce diverse kinamycin structures, alternatively, a water-mediated rearrangement of prekinamycin scaffold yields isoprekinamycin,[10a,16] a metabolite isolated from the kinamycin producer; (iv) a subsequent oxidation of isoprekinamycin leads to a proposed intermediate **M1**, which is a precursor for biosynthesizing fluostatins after removing the diazo group, or undergoes a reduction to produce **M2**; (v) a condensation between the amino group and the C-5 keto group in **M2** yields **M3**, a dehydration of which leads to the formation of the pyrazole ring in **M4**; (vi) a hydration of the $\Delta^{2,3}$ bond converts **M4** to **3**; (vii) alternatively, the epoxidation of $\Delta^{2,3}$ in **M4** leads to **M5**, and finally, opening of the epoxy ring in **M5** completes the biosynthesis of pyrazolofluostatins A (**1**) and B (**2**). It is well known that opening of epoxides generally provides products with *trans*-configurations (e.g. **1**), a previous study has proposed the generation of a *cis*-configured product daldinone E through enzymatic epimerization of its *trans*-configured precursor (a product of epoxide opening),[17] indicating that **2** could be formed in a similar way.

In conclusion, pyrazolofluostatins A–C (**1**–**3**) were isolated from marine-derived *M. rosaria* SCSIO N160 and represented the first examples of pyrazole-containing fluostatins featuring an unprecedented 6/5/6/6/5 pentacyclic skeleton. The unusual molecular architecture of **1**–**3**, especially the pyrazole moiety, implies an intriguing biosynthetic mechanism awaiting for further genetic and biochemical investigations.

DOI: 10.1021/acs.orglett.6b03745
Org. Lett. 2017, 19, 592–595

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03745.

Experimental procedures and characterization data for compounds (PDF)

X-ray crystallization of **1** (CIF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: czhang@scsio.ac.cn or czhang2006@gmail.com.

ORCID

Haibo Zhang: 0000-0003-3796-5095

Changsheng Zhang: 0000-0003-2349-3138

Author Contributions

§W.Z. and C.Y. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support was provided by NSFC (41676165, 31630004, 31290233, 31125001), Guangdong Province (2015A030308013, GD2012-D01-002), Chinese Academy of Sciences (XDA11030403, QYZDJ-SSW-DQC004), and the State Key Laboratory of Bioactive Substance and Function of Natural Medicines (GTZK201402). We appreciate Dr. Yuchan Chen and Prof. Weimin Zhang of Guangdong Institute of Microbiology for their assistance in cytotoxicity assays. We are grateful to the analytical facilities in SCSIO.

■ REFERENCES

(1) (a) Wang, B.; Guo, F.; Ren, J. W.; Ai, G. M.; Aigle, B.; Fan, K. Q.; Yang, K. Q. *Nat. Commun.* **2015**, *6*, 7674. (b) Yu, M.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2008**, *130*, 2783−2785.

(2) (a) Akiyama, T.; Nakamura, K. T.; Takahashi, Y.; Naganawa, H.; Muraoka, Y.; Aoyagi, T.; Takeuchi, T. *J. Antibiot.* **1998**, *51*, 586−588. (b) Feng, Z.; Kim, J. H.; Brady, S. F. *J. Am. Chem. Soc.* **2010**, *132*, 11902−11903. (c) Yang, C.; Huang, C.; Zhang, W.; Zhu, Y.; Zhang, C. *Org. Lett.* **2015**, *17*, 5324−5327.

(3) (a) Baur, S.; Niehaus, J.; Karagouni, A. D.; Katsifas, E. A.; Chalkou, K.; Meintanis, C.; Jones, A. L.; Goodfellow, M.; Ward, A. C.; Beil, W.; Schneider, K.; Sussmuth, R. D.; Fiedler, H. P. *J. Antibiot.* **2006**, *59*, 293−297. (b) Zhang, W.; Liu, Z.; Li, S.; Lu, Y.; Chen, Y.; Zhang, H.; Zhang, G.; Zhu, Y.; Liu, J.; Zhang, C. *J. Nat. Prod.* **2012**, *75*, 1937−1943. (c) Schneider, K.; Nicholson, G.; Strobele, M.; Baur, S.; Niehaus, J.; Fiedler, H. P.; Sussmuth, R. D. *J. Antibiot.* **2006**, *59*, 105−109.

(4) Zhou, H.; Wang, Y.; Yu, Y.; Bai, T.; Chen, L.; Liu, P.; Guo, H.; Zhu, C.; Tao, M.; Deng, Z. *Curr. Microbiol.* **2012**, *64*, 185−190.

(5) Crystallographic data have been deposited in the Cambridge Crystallographic Data Center with the deposition number CCDC 1481191. A copy of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB21EZ, U.K. (fax, + 44(0)-1233-336033; e-mail, deposit@ccdc.cam.ac.uk).

(6) (a) Khan, M. F.; Alam, M. M.; Verma, G.; Akhtar, W.; Akhter, M.; Shaquiquzzaman, M. *Eur. J. Med. Chem.* **2016**, *120*, 170−201. (b) Kumar, V.; Kaur, K.; Gupta, G. K.; Sharma, A. K. *Eur. J. Med. Chem.* **2013**, *69*, 735−753.

(7) Fowden, L.; Foe, F. F.; Ridd, J. H.; White, R. F. M. *Proc. Chem. Soc.* **1959**, 131−132.

(8) Blair, L. M.; Sperry, J. *J. Nat. Prod.* **2013**, *76*, 794−812.

(9) Kikuchi, T.; Ikedaya, A.; Toda, A.; Ikushima, K.; Yamakawa, T.; Okada, R.; Yamada, T.; Tanaka, R. *Phytochem. Lett.* **2015**, *12*, 94−97.

(10) (a) Janso, J. E.; Haltli, B. A.; Eustaquio, A. S.; Kulowski, K.; Waldman, A. J.; Zha, L.; Nakamura, H.; Bernan, V. S.; He, H.; Carter, G. T.; Koehn, F. E.; Balskus, E. P. *Tetrahedron* **2014**, *70*, 4156−4164. (b) Wang, B.; Ren, J. W.; Li, L. Y.; Guo, F.; Pan, G. H.; Ai, G. M.; Aigle, B.; Fan, K. Q.; Yang, K. Q. *Chem. Commun.* **2015**, *51*, 8845−8848.

(11) Gould, S. J.; Melville, C. R. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 51−54.

(12) Gould, S. J.; Melville, C. R.; Cone, M. C.; Chen, J.; Carney, J. R. *J. Org. Chem.* **1997**, *62*, 320−324.

(13) Gould, S. J.; Chen, J.; Cone, M. C.; Gore, M. P.; Melville, C. R.; Tamayo, N. *J. Org. Chem.* **1996**, *61*, 5720−5721.

(14) (a) Cho, J. Y.; Kwon, H. C.; Williams, P. G.; Jensen, P. R.; Fenical, W. *Org. Lett.* **2006**, *8*, 2471−2474. (b) Winter, J. M.; Jansma, A. L.; Handel, T. M.; Moore, B. S. *Angew. Chem., Int. Ed.* **2009**, *48*, 767−770.

(15) Sugai, Y.; Katsuyama, Y.; Ohnishi, Y. *Nat. Chem. Biol.* **2016**, *12*, 73−75.

(16) Proteau, P. J.; Li, Y. F.; Chen, J.; Williamson, R. T.; Gould, S. J.; Laufer, R. S.; Dmitrienko, G. I. *J. Am. Chem. Soc.* **2000**, *122*, 8325−8326.

(17) Du, L.; King, J. B.; Cichewicz, R. H. *J. Nat. Prod.* **2014**, *77*, 2454−2458.

DOI: 10.1021/acs.orglett.6b03745
Org. Lett. 2017, 19, 592−595