Organic LETTERS

Letter

pubs.acs.org/OrgLett

Polyketide Production of Pestaloficiols and Macrodiolide Ficiolides Revealed by Manipulations of Epigenetic Regulators in an Endophytic Fungus

Guangwei Wu,[†] Haichuan Zhou,[†] Peng Zhang,[†] Xiuna Wang,[†] Wei Li,[†] Weiwei Zhang,[†] Xingzhong Liu,[†] Hong-Wei Liu,[†] Nancy P. Keller,[‡] Zhiqiang An,[§] and Wen-Bing Yin*[,†]

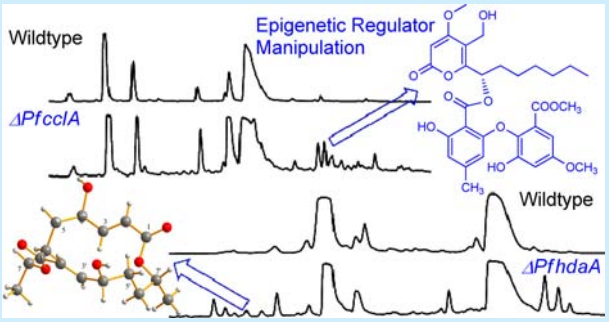
[†]State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, China

[‡]Department of Medical Microbiology and Immunology, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States

[§]Texas Therapeutics Institute, the Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center at Houston, Houston, Texas 77030, United States

Supporting Information

ABSTRACT: Regarding targeted disruption of epigenetic regulators, histone methyltransferase and deacetylase in a plant endophytic fungus *Pestalotiopsis fici* have been uncovered as an unexplored chemical repertoire. Manipulation of epigenetic regulators led to the isolation of 15 new polyketides, including pestaloficiols T−W (**1**−**3** and **5**), as well as 11 macrodiolide ficiolides A−K (**6**−**16**). Ficiolide K (**16**) was found to contain a very rare 1,6-anhydro-pyranose moiety. Finally, the biosynthetic origin of macrodiolide was characterized by isotope-labeling experiments.



Fungal secondary metabolites (SMs), or natural products, are known to possess potent pharmacological properties and have long been utilized for human medicines such as the well-known antibiotics penicillin and anidulafungin and the immunosuppressant cyclosporine.[1] With the availability of an ever-increasing number of genome sequences of natural product producing fungi, it is clear that the capacity of producing SMs is far more than we anticipated for most microbes, suggesting that most SM gene clusters are silent or expressed at low levels under standard cultivation conditions.[1] It has been reported that alleviating epigenetic repression, either by molecular genetic manipulation or by treatment with chemical inhibitors, results in global activation of silent fungal SM biosynthetic pathways.[2] For example, by deletion of the histone H3 lysine 4 methyltransferase encoding gene *cclA* in *Aspergillus nidulans*,[3] Bok et al. activated two silent gene clusters revealing monodictyphenone, emodins, and another antiosteoporosis polyketide, F9775A B. Deletion of the homologue of *cclA* gene in *A. oryzae* increased the production of astellolides.[4] This approach established a bridge connecting traditional natural chemists with microbiologists to address the common goal of drug discovery. Deletion of histone methyltransferase in the pathogenic fungi *A. fumigatus* and *Fusarium graminearum* regulates not only the biosynthesis of SMs such as gliotoxin, deoxynivalenol, and aurofusarin but also virulence.[5] By disruption of histone H3 deacetylase HdaA in an endophytic fungus *Calcarisporium arbuscular*, Mao et al. activated 75% of SM biosynthetic genes and found four new structures.[6] Taken together, this evidence shows that modifying the chromatin structure either genetically or chemically is an excellent approach to accessing new chemical diversity in fungi.

Pestalotiopsis is a well-studied genus of endophytic fungi which produces a wide array of natural products, notably including taxol, with pronounced biological activities.[7] Previous works with two related species—*P. crassiuscula* treated with the DNA methyltransferase inhibitor 5-azacytidine and *hid1* in *P. microspora* NK17 with deletion of the histone deacetylase—both resulted in increased production of SMs.[8] *P. fici* in this genus is a well isolated species and has provided over 80 novel chemical structures including unique skeletons chloropupukeananin, chloropupukeanone A, and chloropestolide A, identified by Che and co-workers.[9] Due to its SM producing capacity and unique ecological role, the genome of the fungus was sequenced.[10] Bioinformatic analysis of *P. fici* genome sequence indicated 76 biosynthetic gene clusters (BGCs) encoding potential pathways for SMs, including 30 polyketide synthases (PKSs), 14 nonribosomal peptide synthases (NRPSs), 16 NRPS-like enzymes, 12 terpene synthases (TSs), and 4 NRPS-PKS hybrids (Tables S1−S3, Supporting Information). Except for the PKS biosynthetic gene cluster of pestheic acid,[11] no

Received: February 28, 2016
Published: March 25, 2016

DOI: 10.1021/acs.orglett.6b00562
Org. Lett. 2016, 18, 1832−1835

others have been characterized. Therefore, *P. fici* is a prime source for genome mining of SMs via epigenetic manipulation.

In this study, we first identified two putative epigenetic related genes by a BLAST search of the *P. fici* genome for targeted gene disruption. PfCclA (PFICI_05127) is a homologue of *A. nidulans* CclA (38% identity, 53% similarity), and PfHdaA (PFICI_08988) is a homologue of *A. fumigatus* HdaA (49% identity, 65% similarity).[12] The previously described *Agrobacterium tumefaciens* mediated transformation (ATMT) method was used to create the *PfcclA* deletion strain (Figure S1, Supporting Information).[13] But the protocol involves multiple complicated manipulation steps and is time-consuming. Therefore, we optimized a protoplast transformation method[14] and then created *PfhdaA* deletion mutants (Figure S1, Methods, Supporting Information). In comparison to the ATMT method, which requires 14−17 days to complete a round of transformation, the improved protocol takes only 7−10 days. Analysis of PfCclA and PfHdaA deletion mutants yielded significantly enhanced polyketide productions of pestaloficiols T−W (**1**−**3** and **5**) and ficipyrone C (**4**) as well as 11 macrodiolide ficiolides A−K (**6**−**16**) (Figure 1) and led to the isolation of 15 novel polyketides (Figures 1 and 2). Ficiolide K (**16**) was found to contain a very rare 1,6-anhydropyranose moiety.

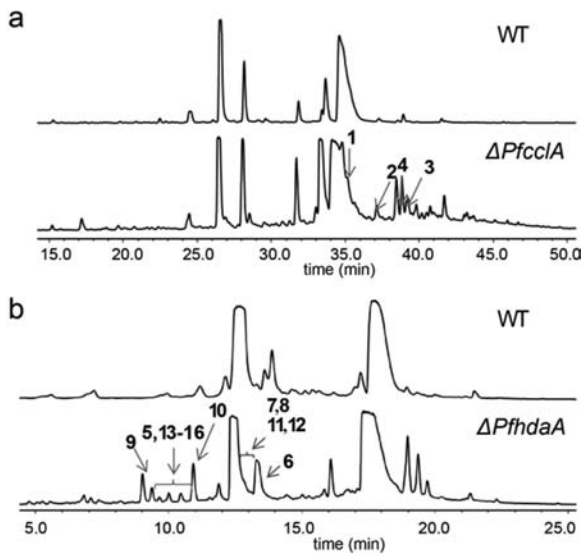


Figure 1. HPLC profiles of crude extracts from Δ*PfcclA* strains (a) and Δ*PfhdaA* strains (b) in comparison to wildtype (WT). Detection was carried out at 260 nm. The numbering of the peaks corresponds to the new natural products shown in Figure 2.

Here, we present the isolation and structural elucidation of **1**−**16**. In addition, we show the action of epigenetic regulators PfCclA and PfHdaA on the known compounds (Figure 3). Finally, we establish the biogenetic pathway for macrodiolide **6** on the basis of isotope-labeling experiments.

To characterize the newly produced compounds from the Δ*PfcclA* and Δ*PfhdaA* strains, large scale fermentation was carried out. The organic extracts were fractionated by ODS and Sephadex LH-20 column chromatography (Methods, Supporting Information). The subfractions containing the targeted metabolites were then selected for further purification. After the semipreparative reversed-phase HPLC separation step, four new compounds were isolated from Δ*PfcclA* strains and named

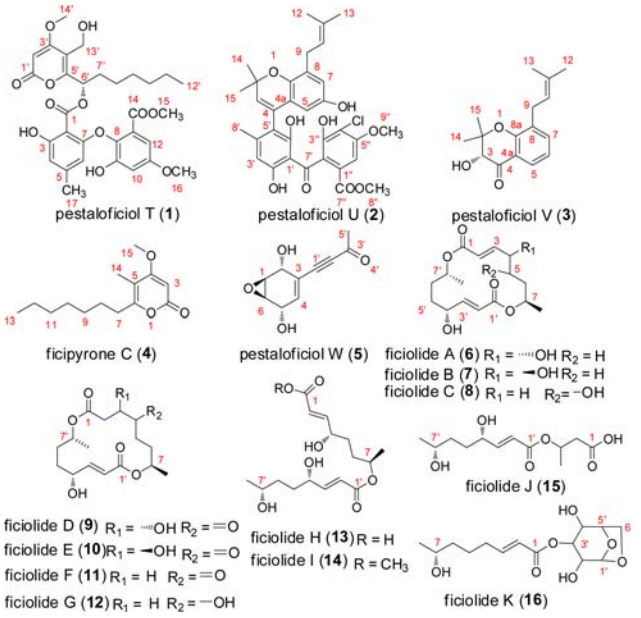


Figure 2. Compounds of **1**−**16** isolated from Δ*PfcclA* and Δ*PfhdaA* mutants.

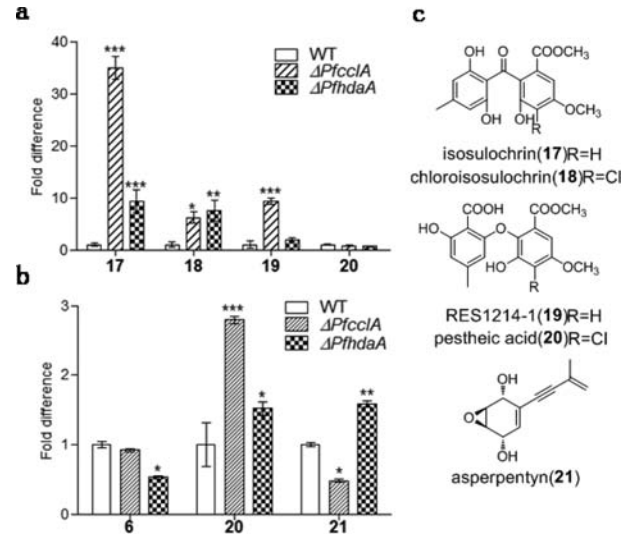
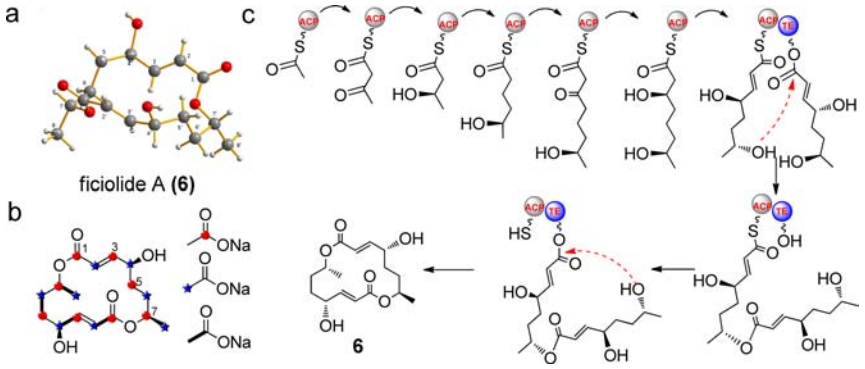


Figure 3. Known compound production regulated by PfCclA and PfHdaA under rice (a) and PDA (b) culture. (c) Compounds **17**−**21**.

pestaloficiols T−V (**1**−**3**) and ficipyrone C (**4**). **1** is a heterodimer composed of compounds RES-1214-1 and ficipyrone A[15] via ester bond linkage. NMR spectroscopy data and HMBC correlation from H-6′ to C-1 provided evidence to establish the complete planar structure of **1** (Figures 2 and S2, Table S6, Supporting Information). To determine the absolute configuration of C-6′, **1** was subjected to a basic hydrolysis reaction to yield ficipyrone A which exhibited the same electronic circular dichroism (ECD) Cotton effects as previously reported (Figure S3, Supporting Information),[15] suggesting the 6′*S* absolute configuration. The molecular formula $C_{31}H_{36}O_{12}$ of **1** was deduced by HRESIMS at *m*/*z* 601.2288 $[M + H]^+$ (calcd for $C_{31}H_{37}O_{12}$, 601.2207). To the best of our knowledge, **1** has not been discovered in nature before. Compound **2** is a chlorinated product of pestaloficiol L[16] with HRESIMS at *m*/*z* 609.1893 $[M + H]^+$ (calcd for $C_{33}H_{34}O_9Cl$, 609.1886), representing a

DOI: 10.1021/acs.orglett.6b00562
Org. Lett. 2016, 18, 1832−1835

Scheme 1. Crystal Structure of Ficioilde A (6) (a) and Proposed Biogenetic Pathway for 6 Established on the Basis of Stable Isotope Incorporation Patterns (b,c)



new feature of a heterodimer containing a chloroisosulochrin moiety. NMR spectroscopy showed that a proton signal ($\delta_{C/H}$ 106.4/6.65) of isosulochrin unit in pestaloficiol L was replaced by an aromatic nonprotonated carbon (δ_C 113.8) of the chloroisosulochrin unit in **2**, indicating the chlorine atom was located at the C-4″ position (Figures 2 and S2, Table S7, Supporting Information). Compound **3** is a similar to pestaloficiol J[16] with the difference that a hydroxyl group at C-6 of pestaloficiol J appears instead at C-3 in compound **3**. The correlations of the gem-dimethyl proton from H_3-14 and H_3-15 to C-3 provide evidence for assigning the hydroxyl group at C-3 (Figure S2, Table S8, Supporting Information). The absolute configuration of **3** was established by ECD spectra for the respective lowest-energy conformers of (3*R*)-3,[17] suggesting 3*R* absolute configuration (Figures S4 and S5, Supporting Information). The structure of **4** as a key precursor of ficipyrone A was determined by NMR spectroscopy (Figure S2, Table S9, Supporting Information). A relatively rare family of 16-membered ring macrodiolides and related derivatives (**6**−**16**, namely ficiolide A−K), **5** (namely pestaloficiol W), and the known compound **21** (asperpentyn[18]) were isolated from Δ*PfhdaA* mutants (Figures 2 and 3). Structures of the macrodiolides were determined by HRESIMS, 1D and 2D NMR spectra (Figures S2, S6, and S7, Tables S10−S17, Supporting Information). Symmetric macrodiolide **6** was isolated as a major component possessing the same planar structure as pyrenophorol from several other fungi[19] and a synthetic product.[20] The small differences in chemical shifts between **6** and pyrenophorol revealed different stereochemistry. The mono-MTPA ester, di-MTPA esters and X-ray crystal diffraction (Cu Kα, Flack parameter = 0.00, CCDC number: 1414142) confirmed **6** was a diastereoisomer of pyrenophorol at C-4/4′. Thus, (4*R*,7*R*,4′*R*,7′*R*)-**6** was finally established, which is consistent with the absolute configuration of the synthetic product based on the specific rotation (no reported NMR data).[20] Ficiolide A (**6**) was isolated from nature for the first time. The stereochemistry of other macrodiolides **7**−**16** was determined according to the hydrolysis reaction, based on the stereochemistry of **6** and biogenic origin (Methods, Supporting Information). Unexpectedly, ficiolide K (**16**) was found to contain a 1,6-anhydropyranose moiety, a very rare fragment in natural product chemistry. **5** and **21** are epoxyquinoids, a subclass of naturally occurring cyclohexane epoxides.[21] In contrast to **21**, the double bond at C-3′═C-4′ was oxidized to a ketone moiety in structure **5**. The absolute configuration of **5** was assigned by comparing ECD data between **5** and **21** (Figures 2, 3, S2, and S8−S10, Supporting Information).

A previous study indicated that SM production in *P. fici* is dependent on culture conditions.[10] Epigenetic regulation of SM yields would help improve production for bioactive molecules. Therefore, we assessed the SM production of PfCclA and PfHdaA deletion mutants on select rice and PDA media. Except for new peaks (Figure 1) characterized in this study, the purified known compounds **17**−**21** (Figure 3c) were used as standards.[9] The production of six *P. fici* metabolites **6**, **17**−**21** were greatly altered in both mutants. In rice culture, mutants of Δ*PfcclA* and Δ*PfhdaA* both greatly increased the synthesis of compounds **17**, **18**, and **19** (35.0-, 6.2-, and 9.3-fold for the Δ*PfcclA* mutant, 9.3-, 7.6-, and 2.0-fold for the Δ*PfhdaA* mutant, respectively) (Figure 3a) over WT. In PDA medium, the production of compounds **20** and **21** were slightly increased (1.5-fold) and the production of compound **6** slightly decreased (0.5-fold) in the Δ*PfhdaA* strain compared to WT (Figure 3b). The production of compounds **6** and **21** were slightly decreased and the production of compound **20** increased (2.8-fold) in the Δ*PfcclA* strain compared to WT (Figure 3b).

The biosynthesis of macrodiolides, rarely reported in fungi,[22] was unique, and the mechanism of diolide formation was reported in actinomycetes.[23] To better understand the biosynthetic origin of fungal macrodiolide, we performed stable isotope labeling experiments with [1-^{13}C] sodium acetate, [2-^{13}C] sodium acetate, and [1,2-$^{13}C_2$] sodium acetate (Scheme 1). After culturing, the labeled **6** was purified and further analyzed by ^{13}C NMR. The abundance of each carbon atom was determined from the ^{13}C-signal intensities in the one-dimensional spectrum (Table S21, Supporting Information). When sodium [1-^{13}C] acetate was added, increments of eight ^{13}C resonances, C-1/1′, C-3/3′, C-5/5′, and C-7/7′, were observed to be at similar levels whereas these carbon atoms were not labeled by sodium [2-^{13}C] acetate (Scheme 1). Noticeable enrichments of C-2/2′, C-4/4′, C-6/6′, and C-8/8′ from [2-^{13}C] sodium acetate were observed. Thus, results indicate that all 16 carbons in **6** were derived from acetate building blocks. In the spectrum of ficiolide A labeled from [1,2-$^{13}C_2$] sodium acetate, all the carbon atoms appeared as distinct triplets flanked by two strong satellite signals and the similar J_{C-C} values of C-1(1′)/C-2(2′), C-3(3′)/C-4(4′), C-5(5′)/C-6(6′), and C-7(7′)/C-8(8′) unequivocally indicated the incorporation of 8 intact acetate-derived C2 units in **6** (Scheme 1, Table S21, Supporting Information). The above-mentioned results confirmed the polyketide origin of **6**.

DOI: 10.1021/acs.orglett.6b00562
Org. Lett. 2016, 18, 1832−1835

In summary, we studied the global impact of chromatin remodeling on the natural products production profile in *P. fici*. By improving the genetic transformation system to enable a quicker process to obtain mutants, we found 15 new structures (Figure 2). Surprisingly, 11 macrodiolide compound ficiolides A to K were highly produced in the PfHdaA deletion strain (Figures 1b and 2). Our study also elucidated the biosynthetic origin of compound **6** by isotope-labeling experiments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00562.

Experimental methods, figures, tables, full spectroscopic data, and NMR spectra of new compounds (NMR, MS, and CD) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yinwb@im.ac.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Drs. Jinwei Ren and Wenzhao Wang (Institute of Microbiology, CAS) for MS and NMR data collection. We thank Dr. Georgina Salazar at the University of Texas Health Science Center at Houston for her careful and critical reading of the manuscript. This work was supported by the National Natural Science Foundation of China (Grant 21502220 to G.W.). W.B.Y. is a scholar of "the 100 Talents Project" of CAS.

■ REFERENCES

(1) (a) Hoffmeister, D.; Keller, N. P. *Nat. Prod. Rep.* **2007**, *24*, 393−416. (b) Bills, G.; Li, Y.; Chen, L.; Yue, Q.; Niu, X. M.; An, Z. *Nat. Prod. Rep.* **2014**, *31*, 1348−1375. (c) Rutledge, P. J.; Challis, G. L. *Nat. Rev. Microbiol.* **2015**, *13*, 509−523.

(2) (a) Cichewicz, R. H. *Nat. Prod. Rep.* **2010**, *27*, 11−22. (b) Albright, J. C.; Henke, M. T.; Soukup, A. A.; McClure, R. A.; Thomson, R. J.; Keller, N. P.; Kelleher, N. L. *ACS Chem. Biol.* **2015**, *10*, 1535−1541. (c) Strauss, J.; Reyes-Dominguez, Y. *Fungal Genet. Biol.* **2011**, *48*, 62−69. (d) Tang, H. Y.; Zhang, Q.; Gao, Y. Q.; Zhang, A. L.; Gao, J. M. *RSC Adv.* **2015**, *5*, 2185−2190. (e) Asai, T.; Morita, S.; Shirata, N.; Taniguchi, T.; Monde, K.; Sakurai, H.; Ozeki, T.; Oshima, Y. *Org. Lett.* **2012**, *14*, 5456−5459. (f) Asai, T.; Morita, S.; Taniguchi, T.; Monde, K.; Oshima, Y. *Org. Biomol. Chem.* **2016**, *14*, 646−651.

(3) Bok, J. W.; Chiang, Y.-M.; Szewczyk, E.; Reyes-Dominguez, Y.; Davidson, A. D.; Sanchez, J. F.; Lo, H.-C.; Watanabe, K.; Strauss, J.; Oakley, B. R. *Nat. Chem. Biol.* **2009**, *5*, 462−464.

(4) Shinohara, Y.; Kawatani, M.; Futamura, Y.; Osada, H.; Koyama, Y. *J. Antibiot.* **2016**, *69*, 4−8.

(5) (a) Palmer, J. M.; Bok, J. W.; Lee, S.; Dagenais, T. R.; Andes, D. R.; Kontoyiannis, D. P.; Keller, N. P. *PeerJ* **2013**, *1*, e4. (b) Liu, Y.; Liu, N.; Yin, Y.; Chen, Y.; Jiang, J.; Ma, Z. *Environ. Microbiol.* **2015**, *17*, 4615−4630.

(6) Mao, X. M.; Xu, W.; Li, D.; Yin, W. B.; Chooi, Y. H.; Li, Y. Q.; Tang, Y.; Hu, Y. *Angew. Chem., Int. Ed.* **2015**, *54*, 7592−7596.

(7) Yang, X.-L.; Zhang, J.-Z.; Luo, D.-Q. *Nat. Prod. Rep.* **2012**, *29*, 622−641.

(8) (a) Yang, X. L.; Huang, L.; Ruan, X. L. *J. Asian Nat. Prod. Res.* **2014**, *16*, 412−417. (b) Niu, X.; Hao, X.; Hong, Z.; Chen, L.; Yu, X.; Zhu, X. *J. Microbiol. Biotechnol.* **2015**, *25*, 579−588.

(9) (a) Liu, L. *Mycology* **2011**, *2*, 37−45. (b) Liu, L.; Li, Y.; Li, L.; Cao, Y.; Guo, L.; Liu, G.; Che, Y. *J. Org. Chem.* **2013**, *78*, 2992−3000. (c) Liu, L.; Li, Y.; Liu, S. C.; Zheng, Z. H.; Chen, X. L.; Zhang, H.; Guo, L. D.; Che, Y. S. *Org. Lett.* **2009**, *11*, 2836−2839. (d) Liu, L.; Liu, S. C.; Jiang, L. H.; Chen, X. L.; Guo, L. D.; Che, Y. S. *Org. Lett.* **2008**, *10*, 1397−1400.

(10) Wang, X.; Zhang, X.; Liu, L.; Xiang, M.; Wang, W.; Sun, X.; Che, Y.; Guo, L.; Liu, G.; Guo, L. *BMC Genomics* **2015**, *16*, 28.

(11) Xu, X.; Liu, L.; Zhang, F.; Wang, W.; Li, J.; Guo, L.; Che, Y.; Liu, G. *ChemBioChem* **2014**, *15*, 284−292.

(12) Lee, I.; Oh, J.-H.; Shwab, E. K.; Dagenais, T. R.; Andes, D.; Keller, N. P. *Fungal Genet. Biol.* **2009**, *46*, 782−790.

(13) (a) Wang, X.; Wu, F.; Liu, L.; Liu, X.; Che, Y.; Keller, N. P.; Guo, L.; Yin, W.-B. *Fungal Genet. Biol.* **2015**, *81*, 221−228. (b) Chen, L.; Yue, Q.; Zhang, X.; Xiang, M.; Wang, C.; Li, S.; Che, Y.; Ortiz-Lopez, F. J.; Bills, G. F.; Liu, X.; An, Z. *BMC Genomics* **2013**, *14*, 339.

(14) (a) Yin, W. B.; Amaike, S.; Wohlbach, D. J.; Gasch, A. P.; Chiang, Y. M.; Wang, C. C.; Bok, J. W.; Rohlfs, M.; Keller, N. P. *Mol. Microbiol.* **2012**, *83*, 1024−1034. (b) Yin, W.-B.; Chooi, Y. H.; Smith, A. R.; Cacho, R. A.; Hu, Y.; White, T. C.; Tang, Y. *ACS Synth. Biol.* **2013**, *2*, 629−634.

(15) Liu, S.; Liu, X.; Guo, L.; Che, Y.; Liu, L. *Chem. Biodiversity* **2013**, *10*, 2007−2013.

(16) Liu, L.; Liu, S.; Niu, S.; Guo, L.; Chen, X.; Che, Y. *J. Nat. Prod.* **2009**, *72*, 1482−1486.

(17) Peng, J.; Gao, H.; Li, J.; Ai, J.; Geng, M.; Zhang, G.; Zhu, T.; Gu, Q.; Li, D. *J. Org. Chem.* **2014**, *79*, 7895−7904.

(18) Mühlenfeld, A.; Achenbacht, H. *Phytochemistry* **1988**, *27*, 3853−3855.

(19) (a) Krohn, K.; Farooq, U.; Flörke, U.; Schulz, B.; Draeger, S.; Pescitelli, G.; Salvadori, P.; Antus, S.; Kurtán, T. *Eur. J. Org. Chem.* **2007**, *2007*, 3206−3211. (b) Kind, R.; Zeeck, A.; Grabley, S.; Thiericke, R.; Zerlin, M. *J. Nat. Prod.* **1996**, *59*, 539−540.

(20) Dommerholt, F.; Thijs, L.; Zwanenburg, B. *Tetrahedron Lett.* **1991**, *32*, 1499−1502.

(21) Marco-Contelles, J.; Molina, M. T.; Anjum, S. *Chem. Rev.* **2004**, *104*, 2857−2900.

(22) Kastanias, M. A.; Chrysayi-Tokousbalides, M. *J. Agric. Food Chem.* **2005**, *53*, 5943−5947.

(23) (a) Zhou, Y.; Murphy, A. C.; Samborskyy, M.; Prediger, P.; Dias, L. C.; Leadlay, P. F. *Chem. Biol.* **2015**, *22*, 745−754. (b) Zhou, Y.; Prediger, P.; Dias, L. C.; Murphy, A. C.; Leadlay, P. F. *Angew. Chem., Int. Ed.* **2015**, *54*, 5232−5235.

DOI: 10.1021/acs.orglett.6b00562
Org. Lett. 2016, 18, 1832−1835