Organic LETTERS

Letter

pubs.acs.org/OrgLett

Biosynthetic Study on Antihypercholesterolemic Agent Phomoidride: General Biogenesis of Fungal Dimeric Anhydrides

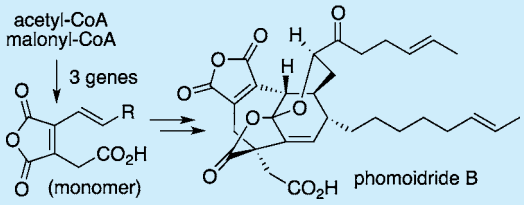
Ryuya Fujii,[†] Yusuke Matsu,[†] Atsushi Minami,[†] Shota Nagamine,[†] Ichiro Takeuchi,[†] Katsuya Gomi,[‡] and Hideaki Oikawa*[,†]

[†]Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

[‡]Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan

Supporting Information

ABSTRACT: To elucidate the general biosynthetic pathway of fungal dimeric anhydrides, a gene cluster for the biosynthesis of the antihypercholesterolemic agent phomoidride was identified by heterologous expression of candidate genes encoding the highly reducing polyketide synthase, alkylcitrate synthase (ACS), and alkylcitrate dehydratase (ACDH). An *in vitro* analysis of ACS and ACDH revealed that they give rise to anhydride monomers. Based on the established monomer biosynthesis, we propose a general biogenesis of dimeric anhydrides involving a single donor unit and four acceptor units.



Fungal metabolites with common maleic anhydride moieties represented by **1** in Figure 1 have been found in a wide range of fungi and lichens.[1] Among these, particularly unique members are dimeric anhydrides including phomoidride (**2**)[2] and glauconic acid (**3**)[3] (Figure 1). Dimeric anhydrides usually possess a nine-membered ring, which are often called "nonadride",[3] and also include the 8-membered ring dimer zopfielin[4] and the linear dimer cordyanhydride A (**4**).[5] Phomoidrides are potent inhibitors of Ras farnesyltransferase and squalene synthase.[2] Their biological activities and fascinating molecular framework consisting of a bicyclo[4.3.1]-deca-1,6-diene core with bridgehead olefin and maleic anhydride moieties have stimulated a number of synthetic studies that have included four total syntheses.[6]

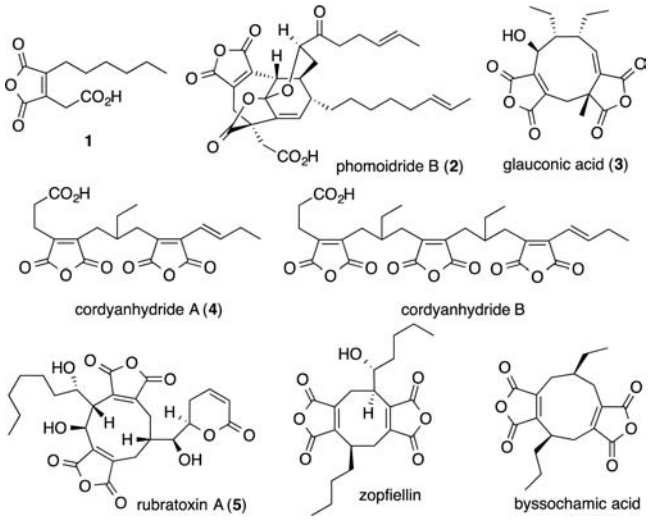


Figure 1. Representative fungal anhydrides.

Biosynthetic pathways of fungal dimeric anhydrides such as **3** and rubratoxin (**5**) have been studied via feeding experiments with isotopically labeled precursors.[7,8] After isolation of **2**, Sulikowski and co-workers studied phomoidride biosynthesis using a similar approach.[9] Additionally, biomimetic dimerizations of putative anhydride monomers or their tethered forms were studied by the Sutherland[7b] as well as Baldwin and Sulikowski groups.[10] Based on the successful formation of nonadride core-containing isoglaucanic acid with alternative cyclization products, Baldwin supported stepwise anionic condensations[10a] rather than [6 + 4]cycloaddition.[7b] However, to date, no genetic or biochemical studies have been performed.

Recently, we have successfully elucidated the biosynthetic pathways of various fungal metabolites such as terpenes,[11] indole diterpenes,[12] and polyketides[13] with a heterologous expression system in *Aspergillus oryzae*. We applied this highly reliable expression system to solve unknown biosynthetic mechanisms of dimeric anhydrides. Herein, we report the identification of gene clusters for the biosynthesis of **2** and related metabolites and functional analysis of monomer biosynthetic genes and propose a unified model of fungal dimeric anhydride biosynthesis.

Feeding experiments with isotopically labeled simple precursors such as acetate and succinate showed that the monomer backbones of these metabolites were constructed of fatty acyl chains and a C4-diacid, which was most likely oxaloacetate.[7,8] Studies on the identification of monomer units in dimeric anhydrides will be described later. Based on the phomoidride structure and biochemical studies on alkyl citrate

Received: October 12, 2015
Published: November 11, 2015

DOI: 10.1021/acs.orglett.5b02934
Org. Lett. 2015, 17, 5658−5661

formation,[14] we speculated that fatty acid synthase (FAS) or highly reducing iterative polyketide synthase (HR-PKS) would provide the corresponding fatty acyl chain and that the citrate synthase homologue is a key enzyme for backbone construction.

To identify the phomoidride biosynthetic gene cluster, a genomic analysis of the unidentified fungus ATCC 74256 was performed. Using gene cluster searching tools (local Blast searches and the 2ndFind program[15]), we found more than 10 clusters containing HR-PKS genes. Among them, we identified a single cluster (*phi*) that possessed homologous genes to those of fungal methylcitrate dehydratase[16] (*phiI*, identity 58.7%) and methylcitrate synthase[16] (*phiJ*, identity 17.3%) adjacent to the gene *phiA* encoding HR-PKS (Figure 2, Table S1). Domain

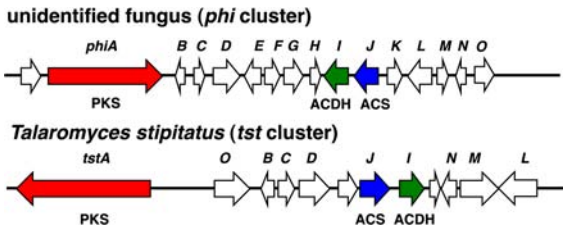


Figure 2. Two putative gene clusters for fungal anhydrides **2** and its related metabolite.

analysis of HR-PKS *phiA* showed that it possesses typical *β*-ketoacylsynthase (KS), acyl transferase (AT), dehydratase (DH), *C*-methyl transferase (MT), enoylreductase (ER), ketoreductase (KR), and acyl carrier protein (ACP) domains consistent with those required for the monomer biosynthesis of **2**, except for the MT domain. Involvement of a nonmethylated polyketide intermediate in the biosynthesis of **2** suggests that the MT domain in PhiA may be inactive similar to the MT domains of Pks1 (T-toxin), MlcA (compactin), and SorbA (sorbicillol).[17]

This putative biosynthetic gene cluster tentatively consisted of 15 genes, of which three of them are likely MFS transporters (*phiDL*) and transcription factor (*phiO*) as shown in Figure 2 and Table S1. In this cluster, we could not detect any genes encoding hydrolytic enzymes for hydrolysis of ACP-bound polyketide chains or acyl-CoA ligase for the generation of citrate synthase substrates. Thus, the PKS product acyl-ACP is most likely the substrate of PhiJ. Based on these data, we speculated that three *phiAIJ* genes are responsible for monomeric anhydride biosynthesis. Direct conversion of the ACP bound polyketide chain is known for several iterative PKS products.[18] The amino acid sequence of putative alkylcitrate synthase (ACS) *phiJ* differed significantly from standard citrate synthases and methylcitrate synthases involving propionic acid metabolism. Thus, by using the *phiJ* gene as a query sequence, homologous gene clusters were found in the database (Figure S4). Among them, one cluster (*tst*) in *Talaromyces stipitatus* has *phiAIJ* homologues *tstAIJ*, which showed significant similarity (55−65%) to *phiAIJ* (Figure 2, Table S1), and thus we also examined their heterologous expression although there has been no report of isolation of dimeric anhydrides from this fungus.

A previous report showed that the production of **2** was induced under low pH conditions.[19] Therefore, we initially examined the expression of proposed *phi* genes under both nonproducing and producing conditions. We confirmed that the three genes [iterative type-I PKS *phiA* and ACS *phiJ*] were specifically expressed under conditions that allowed the fungus to produce **2** (Figures S1 and S2). To examine the putative functions of the *phiAIJ* genes, they were introduced to *A. oryzae* NSAR1 with plasmids pTAex3-*phiA* and pUSA2-*phiJI*. The resultant transformant AO-*phiAIJ* produced the new metabolite **6** having a characteristic UV absorption (λ_{max} 312 nm) for maleic anhydride conjugated with olefin (Figure 3, Figure S3).[6b]

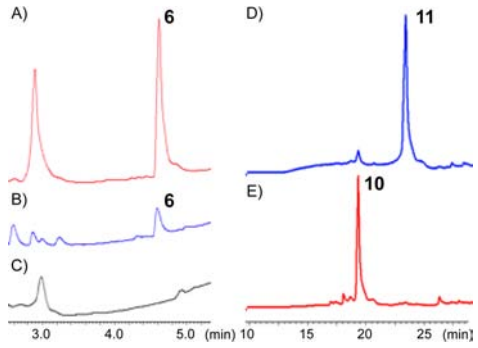
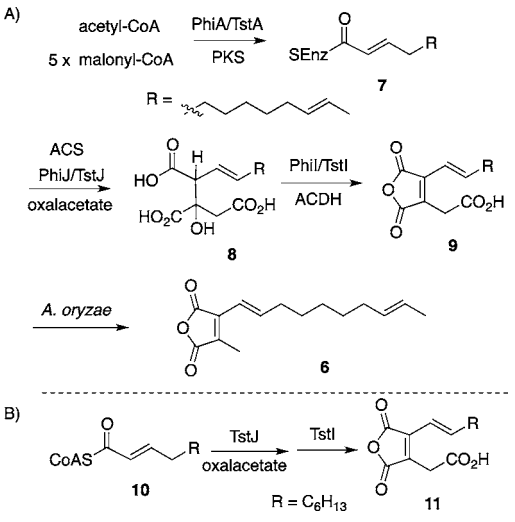


Figure 3. HPLC analytical data. (A) Metabolites produced by transformants AO-*tstAJI*, (B) AO-*phiAJI*, and (C) *A. oryzae* NSAR1 (wild type). (D) Reaction products of 1-decenoyl-CoA and oxalacetate with recombinant TstJ and TstI; (E) without enzymes.

Although we could not obtain a sufficient amount of **6** for structure determination from AO-*phiAIJ*, the corresponding transformant AO-*tstAIJ* prepared in a similar manner gave the same product (Figure 3). Its molecular formula $C_{15}H_{21}O_3$ was determined by HR-MS (m/z 249.1484 $[MH]^+$), and the ^{13}C NMR spectrum supported the presence of the maleic anhydride moiety (2 × C═O: 166.4, 164.7 ppm; C═C: 135.0, 137.3 ppm). Extensive NMR data analysis enabled us to determine its structure as shown in Scheme 1A. The structure of **6** possessed a C10 side chain with a propenyl group which is characteristic for putative biosynthetic intermediates **8** and **9**. Therefore, the product is proposed to be derived by decarboxylation of the putative monomer intermediate **9**, which is constructed by

Scheme 1. Proposed Anhydride Monomer Biosynthesis: (A) Heterologous Expressions of *phiAIJ*/*tstAIJ* in *A. oryzae*; (B) in Vitro Analysis of TstJ/TstI



DOI: 10.1021/acs.orglett.5b02934
Org. Lett. 2015, 17, 5658−5661

coupling of the C12-fatty acyl-ACP **7** and oxalacetic acid (Scheme 1A).

To obtain further evidence of the actual enzymatic reaction product, we overexpressed both *tstJ* and *tstI* in *E. coli*. Large-scale incubation of substrate mimic 2-decenoyl-CoA (**10**) and oxalacetate with the recombinant TstJ and TstI gave a new product (Figure 3, D, E). Subsequent HR-MS data ($C_{14}H_{19}O_5$, m/z 267.1261 $[MH]^+$) suggested that the enzymatic reaction product is 4-octyl-*cis* aconitic anhydride **11**. NMR analysis enabled us to confirm the structure of **11** (Scheme 1B). Together with the availability of anhydride related to **11** by synthesis,[21] the reasonable stability of **11** from the TstJ/TstI reaction supports the involvement of the adventitious decarboxylase for the production of **6** in the host *A. oryzae*. Although homo-**A** and dihydro analogs of **A** such as **1** are found in various fungi, **A** has rarely been isolated as an intact form (Figure 4).[1,5b] To our knowledge, however, the decarboxylated

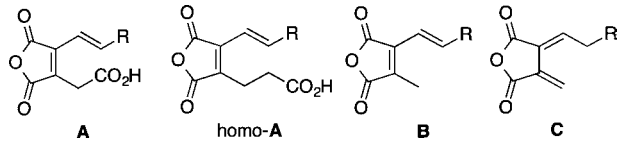


Figure 4. Various anhydride monomer units **A**–**B** isolated from fungi and putaitive unit **C**.

form **B** of the corresponding anhydride **A** is isolated as in the case of producers of zopfiellin.[1,20] This suggests that fungi including producers of dimeric anhydrides have an adventitious decarboxylase specific for monomer anhydride **A**.

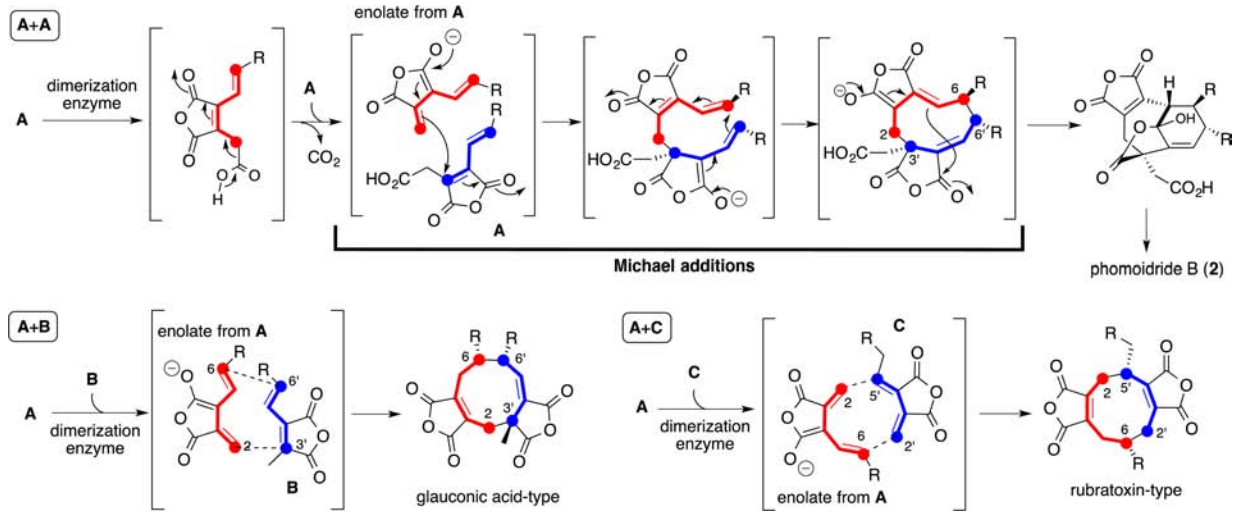
Our in vivo and in vitro analyses of PhiAIJ/TstAIJ indicated that dialkylmaleic anhydrides **A** and homo-**A**[5b] are constructed by ACS-catalyzed condensation of keto diacids (oxalacetate or α-ketoglutarate) and fatty acyl-ACPs (varying chain lengths of C6–C12), which are supplied by HR-PKS (Scheme 1A). In this condensation, deprotonation occurs at the γ-position and the C–C formation takes place at the α-position.[22] Co-isolation of *cis*-aconitic anhydride **1** and hexylitaconic acid in the fermentation of *Aspregillus niger* 5-no.22[23] suggests that similar decarboxylation and specific protonation of **A** results in the formation of **B** and its isomer fulgenic anhydride **C**[4] (Scheme S1). Involvement of **B** and **C** in nonadride biosynthesis was proposed by Sutherland.[4] Sulikowski noted decarboxylative condensation but did not specify its timing.[15] Now, we update the original Sutherland biogenesis incorporating Sulikowski's findings[7a,15] with our work described herein (Scheme 2).

In the unified biogenetic model (**A** + **A**, **A** + **B**, **A** + **C**), only enolate derived from **A** acts as a nucleophile while four units **A**–**C** and homo-**A** act as electrophiles in dimerization (Scheme 2, Scheme S2). In the dimerizations (9-membered ring formation) from these units, decarboxylation of **A** (C5-unit) results in an enolate, which undergoes initial Micheal addition with electrophiles (**A**–**C** and homo-**A**, C4-unit), and then the second intramolecular Micheal addition produces various nonadrides. All feeding experiments in previous studies were based on the consideration of a single monomer [**A** in **2**; **B** in glauconic acid (**3**) and rubratoxin (**5**)], which may have caused discrepancies in prior experimental results. Applying our unified model to the feeding studies, we can reasonably explain these discrepancies. A previous study on the biosynthesis of **2** with a specifically ^{2}H-labeled monomer **A** showed incorporation of ^{2}H at two positions, indicating that this dimerization requires two **A** units.[7a] However, this dimerization mode (**A** + **A**) cannot apply to that of structurally related **3**. In this case (**A**+**B**), monomer **B** acts as an electrophile and this proposal is well correlated with an efficient incorporation of ^{3}H-labeled **B** into **3**.[6] Failure to incorporate ^{14}C-labeled **B** in the biosynthesis of **5** is also explained by the involvement of the mode (**A** + **C**), thus indicating that **B** is not a precursor.[6c]

Using the same set of monomers **A** and **C**, two alternative couplings yield 1,5-dialkyladduct byssochlamic acid[24] and 1,2-dialkyladduct heveadride,[25] respectively (Scheme S2). Furthermore, this model can apply for the formation of linear oligomeric adducts cordyanhydrides.[5] In this oligomerization, the enolate from **A** attacks electrophile homo-**A** at the C6′-position of the side chain to yield the linear coupling product **4**, which may accept another monomer **A** to give a trimer (Scheme S2).

In our unified model, the enolate, which is susceptible to water, is essential for the dimerization. This suggests that a dimerization enzyme for the homocoupling (**A** + **A**) should catalyze both enolate formation and sequential Micheal additions in the same active site. This type of dimerization has been reported for the biosynthesis of ditryptophenaline.[26]

Scheme 2. Unified Model of Fungal Dimeric Anhydride Biosynthesis



DOI: 10.1021/acs.orglett.5b02934
Org. Lett. 2015, 17, 5658–5661

On the other hand, substrates **B** and **C**[7b] for heterocoupling (**A** + **B** or **A** + **C**) can be obtained by decarboxylation catalyzed by an individual enzyme. Thus, two enzymes may be involved in the heterocoupling. To elucidate the most intriguing dimerization step, inactivation or heterologous expression of genes found in the *phi/tst* gene clusters are required.

In conclusion, we have identified biosynthetic gene clusters for phomoidride and the related metabolite. Heterologous expression of three *phiAIJ/tstAIJ* genes gave the decarboxylated monomer **6**. Involvement of a type-**A** monomer was confirmed by in vitro reactions of unsaturated acyl-CoA and oxalacetate with TstJ and TstI to yield **11**. Elucidation of type-**A** monomer biosynthesis allowed us to propose a unified biogenesis of dimeric anhydrides. Currently, we are working on an intriguing dimerization reaction with several candidate genes found in several biosynthetic gene clusters for putative dimeric anhydrides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02934.

Experimental procedures, analytical and spectral data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: hoik@sci.hokudai.ac.jp.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by a MEXT research grant on Grant-in-Aid for Challenging Exploratory Research 25560398 and partly by Grant-in-Aid for Scientific Research (A) 15H01835 to H.O. We are grateful to Mr. Y. Miyashita for measurement of ESI-MS data.

■ REFERENCES

(1) (a) Turner, W. B.; Aldridge, D. C. *Fungal metabolites. Vol. II*; Academic Press: New York, 1983; pp 367−383. (b) Chen, X. L.; Zheng, Y. G.; Shen, Y. C. *Chem. Rev.* **2007**, *107*, 1777−1830.

(2) Dabrah, T. T.; Kaneko, T.; Massefski, W.; Whipple, E. B. *J. Am. Chem. Soc.* **1997**, *119*, 1594−1598.

(3) Barton, D. H. R.; Gondinho, L. D. S.; Sutherland, J. K. *J. Chem. Soc.* **1965**, 1779−1786 reference cited therein.

(4) Watanabe, T.; Yasumoto, K.; Murata, M.; Tagawa, M.; Narushima, H.; Furusato, T.; Kuwahara, M.; Hanaue, M.; Seki, T. *Jpn. Kokai Tokkyo Koho* **1994**, JP 06-184157, EP 582267, US 5346919.

(5) (a) Isaka, M.; Tanticharoen, M.; Thebtaranonth, Y. *Tetrahedron Lett.* **2000**, *41*, 1657−1660. (b) Yamanishi, R.; Okada, K.; Tamugi, N.; Iwashima, M.; Iguchi, K. *Bull. Chem. Soc. Jpn.* **2000**, *73*, 2087−2091.

(6) Spiegel, D. A.; Njardarson, J. T.; McDonald, I. M.; Wood, J. L. *Chem. Rev.* **2003**, *103*, 2691−2727.

(7) (a) Bloomer, J. L.; Moppett, C. E.; Sutherland, J. K. *J. Chem. Soc. C* **1968**, 588−591. (b) Huff, R. K.; Moppett, C. E.; Sutherland, J. K. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2584−2590.

(8) Nieminen, S.; Payne, T. G.; Senn, P.; Tamm, C. *Helv. Chim. Acta* **1981**, *64*, 2162−2174.

(9) (a) Spencer, P.; Agnelli, F.; Williams, H. J.; Keller, N. P.; Sulikowski, G. A. *J. Am. Chem. Soc.* **2000**, *122*, 420−421. (b) Sulikowski, G. A.; Agnelli, F.; Spencer, P.; Koomen, J. M.; Russell, D. H. *Org. Lett.* **2002**, *4*, 1447−1450.

(10) (a) Baldwin, J. E.; Beyeler, A.; Cox, R. J.; Keats, C.; Pritchard, G. J.; Adlington, R. M.; Watkin, D. J. *Tetrahedron* **1999**, *55*, 7363−7374. (b) Baldwin, J. E.; Adlington, R. M.; Roussi, F.; Bulger, P. G.; Marquez, R.; Mayweg, A. V. W. *Tetrahedron* **2001**, *57*, 7409−7416. (c) Sulikowski, G. A.; Agnelli, F.; Corbett, R. M. *J. Org. Chem.* **2000**, *65*, 337−342.

(11) (a) Fujii, R.; Minami, A.; Tsukagoshi, T.; Sato, N.; Sahara, T.; Ohgiya, S.; Gomi, K.; Oikawa, H. *Biosci., Biotechnol., Biochem.* **2011**, *75*, 1813−1817. (b) Ye, Y.; Minami, A.; Mandi, A.; Liu, C.; Taniguchi, T.; Kuzuyama, T.; Monde, K.; Gomi, K.; Oikawa, H. *J. Am. Chem. Soc.* **2015**, *137*, 11846−11853.

(12) Liu, C.; Tagami, K.; Minami, A.; Matsumoto, T.; Frisvad, J. C.; Suzuki, H.; Ishikawa, J.; Gomi, K.; Oikawa, H. *Angew. Chem., Int. Ed.* **2015**, *54*, 5748−5752.

(13) Ugai, T.; Minami, A.; Fujii, R.; Tanaka, M.; Gomi, K.; Oikawa, H. *Chem. Commun.* **2015**, *51*, 1878−1881.

(14) Mahlen, A. *Eur. J. Biochem.* **1971**, *22*, 104−114.

(15) 2ndFind: http://biosyn.nih.go.jp/2ndfind/.

(16) (a) Horswill, A. R.; Escalante-Semerena, J. C. *J. Bacteriol.* **1999**, *181*, 5615−5623. (b) Ibrahim-Granet, O.; Dubourdeau, M.; Latgé, J. P.; Ave, P.; Huerre, M.; Brakhage, A. A.; Brock, M. *Cell. Microbiol.* **2008**, *10*, 134−148.

(17) (a) Fujii, I.; Yoshida, N.; Shimomaki, S.; Oikawa, H.; Ebizuka, Y. *Chem. Biol.* **2005**, *12*, 1301−1309. (b) Fahad, A. a.; Abood, A.; Fisch, K. M.; Osipow, A.; Davison, J.; Avramovic, M.; Butts, C. P.; Piel, J.; Simpson, T. J.; Cox, R. J. *Chem. Sci.* **2014**, *5*, 523−527.

(18) (a) Du, L.; Lou, L. *Nat. Prod. Rep.* **2010**, *27*, 255−278. (b) Hiratsuka, T.; Suzuki, H.; Kariya, R.; Seo, T.; Minami, A.; Oikawa, H. *Angew. Chem., Int. Ed.* **2014**, *53*, 5423−5426. (c) Winter, J. M.; Cascio, D.; Dietrich, D.; Sato, M.; Watanabe, K.; Sawaya, M. R.; Vederas, J. C.; Tang, Y. *J. Am. Chem. Soc.* **2015**, *137*, 9885−9893.

(19) Sulikowski, G. A.; Pongdee, R. *Synlett* **2006**, 354−363.

(20) Futagawa, M.; Adachi, M.; Hirai, Y. *Jpn. Kokai Tokkyo Koho* **1998**, JP 10-316510.

(21) Adlington, R. M.; Baldwin, J. E.; Cox, R. J.; Pritchard, G. J. *Synlett* **2002**, *2002*, 820−822.

(22) Chittori, S.; Savithri, H. S.; Murthy, M. R. N. *J. Struct. Biol.* **2011**, *174*, 58−68.

(23) (a) Weidenmueller, H. L.; Cavagna, F.; Fehlhaber, H. W.; Prave, P. *Tetrahedron Lett.* **1972**, *13*, 3519−3522. (b) Almassi, F.; Ghisalberti, E. L.; Rowland, C. Y. *J. Nat. Prod.* **1994**, *57*, 833−836.

(24) Baldwin, J. E.; Barton, D. H. R.; Sutherland, J. K. *J. Chem. Soc.* **1965**, 1787−1797.

(25) Crane, R. I.; Hedden, P.; Macmillan, J.; Turner, W. B. *J. Chem. Soc., Perkin Trans. 1* **1973**, 194−200.

(26) Saruwatari, T.; Yagishita, F.; Mino, T.; Noguchi, H.; Hotta, K.; Watanabe, K. *ChemBioChem* **2014**, *15*, 656−659.

DOI: 10.1021/acs.orglett.5b02934
Org. Lett. 2015, 17, 5658−5661