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Cycloheximide and Actiphenol Production in *Streptomyces* sp. YIM56141 Governed by Single Biosynthetic Machinery Featuring an Acyltransferase-less Type I Polyketide Synthase

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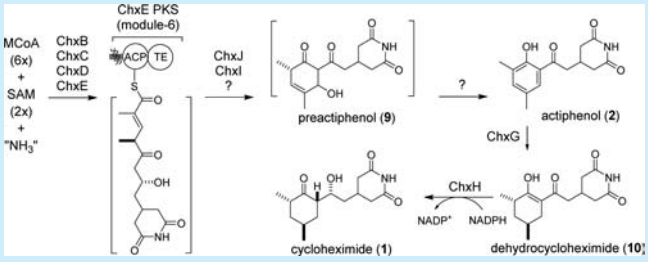
Supporting Information

ABSTRACT: Cycloheximide (**1**) and actiphenol (**2**) have been isolated from numerous *Streptomyces* species. Cloning, sequencing, and characterization of a gene cluster from *Streptomyces* sp. YIM65141 now establish that **1** and **2** production is governed by single biosynthetic machinery. Biosynthesis of **1** features an acyltransferase-less type I polyketide synthase to construct its carbon backbone but may proceed via **2** as a key intermediate, invoking a provocative reduction of a phenol to a cyclohexanone moiety in natural product biosynthesis.



Cycloheximide (**1**) is one of the most well-known members of the glutarimide-containing polyketide family of natural products and has been used for decades as an inhibitor of eukaryotic translation.[1] Actiphenol (**2**), sharing the same carbon skeleton as **1** but having a phenol in place of a cyclohexanone moiety, exhibits weak translation inhibiton activity.[1c,2] Other members of this family include streptimidone (**3**), 9-methylstreptimidone (**4**), iso-migrastatin (**5**), migrastatin, and lactimidomycin (**6**) (Figure 1A). Whereas **1** inhibits translation globally, **6** inhibits preferentially translation initiation but not elongation, a property that has been exploited recently in the development of the global translation initiation sequencing (GTI-seq) technology that enables high-resolution mapping of translation initiation sites across the entire transcriptome.[3] Members of this family have also been pursued as promising anti-metastatic drug leads for their potent cell migration inhibiton activity and cytotoxicity.[4]

During our recent efforts toward discovering inhibitors of eukaryotic translation, we rediscovered **1** from two *Streptomyces* species, YIM56141 and YIM56132.[1c] Interestingly, both species also produced **2**, along with other congeners. Upon delving into the literature, we found that many of the strains reported to produce **1** also produced **2**, including *Streptomyces griseus*,[2a] *Streptomyces albulus*,[2b,c] and *Streptomyces noursei*.[2d] Co-production of **1** and **2** raises an interesting question if they are biosynthetically related, and if true, the biosynthetic relationship between the phenol moiety of **2** and cyclohexanone moiety of **1** are fascinating and cannot be readily predicted a priori according to current knowledge of natural product biosynthesis.

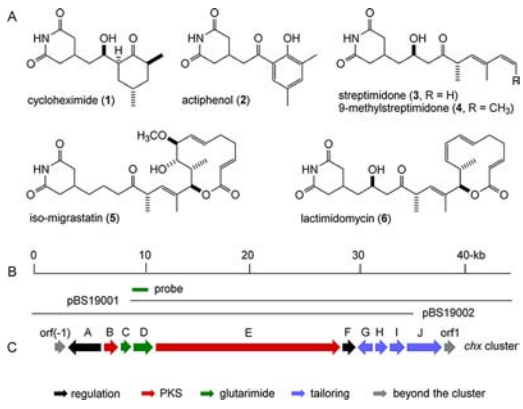


Figure 1. Cloning of the *chx* biosynthetic gene cluster from *Streptomyces* sp. YIM56141. (A) Structures of **1**, **2**, and selected glutarimide-containing polyketides (**3**–**6**). (B) Cloning of the *chx* cluster from *Streptomyces* sp. YIM56141 using a probe encoding glutarimide moiety biosynthesis as represented by two overlapping cosmids. (C) The *chx* cluster spanning ∼35 kb and consisting of 10 genes with their predicted functions color-coded.

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Here we report the cloning, sequencing, and characterization of a gene cluster from *Streptomyces* sp. YIM56141. In vivo and in vitro studies establish that the production of **1** and **2** is governed by single biosynthetic machinery, featuring an acyltransferase (AT)-less type I polyketide synthase (PKS) to construct their carbon backbones and a provocative phenol-to-cyclohexanone reduction in **1** biosynthesis.

We first cloned the gene cluster from *Streptomyces* sp. YIM56141 taking advantage of the biosynthetic knowledge of other glutarimide-containing polyketides. Thus, using degenerate primers designed according to the conserved genes encoding biosynthesis of the glutarimide moiety in **5** and **6** (Table S1 in Supporting Information (SI)), we amplified a fragment containing the amidotransferase gene (Supporting Information and Table S2). Using this fragment as a probe, we screened the genomic library of *Streptomyces* sp. YIM56141 and identified two overlapping cosmids, pBS19001 and pBS19002, that covered the *chx* cluster (Figure 1B). DNA sequencing of the two cosmids revealed 18 open reading frames (Figure 1C), and this sequence has been deposited in GenBank with the accession number JX014302. To determine the *chx* cluster boundaries, *orf(-1)* and *orf1* were inactivated, affording mutant strains SB19001 [Δ*orf(-1)*] and SB19002 (Δ*orf1*), the genotypes of which were confirmed by Southern analysis (Figures S1 and S2 in SI). HPLC analysis of SB19001 and SB19002 fermentations confirmed that both strains still produced **1** and **2** (Figure 2, panels II and III), hence establishing boundaries of the *chx* cluster that spans ∼35 kb and consists of 10 genes (Figure 1C).

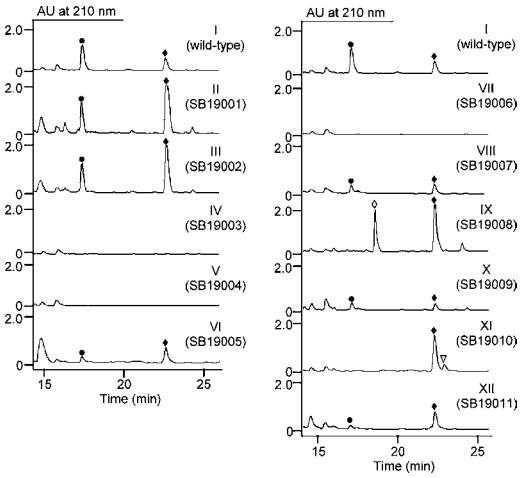

Figure 2. Inactivation of selected genes within the *chx* cluster supporting the proposed pathway for **1** and **2** biosynthesis. HPLC analysis of fermentations from *Streptomyces* sp. YIM56141 wild-type and recombinant strains: (I) wild-type, (II) SB19001 [Δ*orf(-1)*], (III) SB19002 (Δ*orf1*), (IV) SB19003 (Δ*chxE*), (V) SB19004 (Δ*chxJ*), (VI) SB19005 (Δ*chxJ*/*chxJ*), (VII) SB19006 (Δ*chxI*), (VIII) SB19007 (Δ*chxI*/*chxI*), (IX) SB19008 (Δ*chxG*), (X) SB19009 (Δ*chxG*/*chxG*), (XI) SB19010 (Δ*chxH*), and (XII) SB19011 (Δ*chxH*/*chxH*). Highlighted metabolites are **1** (●), **2** (♦), **10** (▽), and **11** (◊).

Bioinformatics analysis of the 10 genes within the *chx* cluster revealed (i) two genes encoding an acyl carrier protein (ACP) (ChxC) and an amidotransferase (AMT) (ChxD) for glutarimide moiety biosynthesis, (ii) two genes encoding a discrete AT (ChxB) and a five-module AT-less type I PKS (ChxE) for biosynthesis of the glutarimide-containing polyketide backbone of both **1** and **2**, (iii) four genes encoding an enoylreductase (ChxG), a ketoreductase (ChxH), a cytochrome P450 oxidoreductase (ChxI), and a three-domain carboxylic acid reductase (ChxJ) for converting the nascent glutarimide-containing polyketide intermediate to **1** or **2**, respectively, and (iv) two genes encoding regulator proteins (ChxA and ChxF) for pathway regulation (Figure 1C and Table S3 in SI). The genetic organization of the *chx* cluster, as well as the deduced functions thereof, shows high similarity to gene clusters known for biosynthesis of other glutarimide-containing polyketides, including **4**,[5] **5**,[6] and **6**,[7] but also features several distinct features (Figure S3 in SI). While the similarities among the different pathways support the biosynthesis of a common glutarimide-containing polyketide intermediate, the variations among the tailoring enzymes account for channeling of the common intermediate into the various end products (Figure 3).

We next carried out in vivo experiments establishing the cloned *chx* gene cluster encoding the biosynthesis of both **1** and **2**. Central to the *chx* cluster is *chxE*, which encodes a five-module AT-less type I PKS that is highly homologous to the AT-less type I PKSs for **4**,[5] **5**,[6] and **6**[7] biosynthesis. ChxE and SmdI share an identical architecture with the exception of SmdI lacking the C-terminal thioesterase (TE) domain. ChxE and SmdI appear to be the result of a fusion between MgsE/LtmE module-3 and MgsF/LtmF module-4, which terminates after MgsF/LtmF module-6 with the TE domain from MgsG/LtmG (Figure S3 in SI). With the clear homology among ChxE, SmdI, MgsEFG, and LtmEFG (Table S3 in SI), we propose that they produce a common intermediate at module-6 (Figures 3 and Figure S3 in SI). Thus, in a biosynthetic analogy to **4**, **5**, and **6**, ChxC, ChxD, and ChxE consist of a six-module AT-less type I PKS, with ChxB loading the extender unit of malonyl-CoA to each of the six modules in trans, to biosynthesize the nascent glutarimide-containing polyketide intermediate (**7**) from six molecules of malonyl-CoA, two molecules of *S*-adenosylmethionine (SAM) (for the two CH_3 groups at C-11 and C-13), and an amino acid (as a donor for the "NH" group in the glutarimide moiety). The fact that there is only one methyltransferase (MT) domain in ChxE module-6 would suggest that this MT most likely acts twice to introduce the CH_3 groups at both C-11 and C-13 of **1** and **2** (Figure 3). While the latter prediction deviates from the collinear model for type I PKS, the identical domain and module architecture among the ChxE, SmdI, MgsF, and LtmF AT-less type I PKSs would suggest a similar biosynthetic logic for the installation of the analogous two CH_3 groups in **4**, **5**, and **6** (Figure S3 in SI).

The *chxE* gene was subsequently inactivated (SI), and the genotype (i.e., Δ*chxE*) of the resultant mutant strain SB19003 was confirmed by Southern analysis (Figure S4 and Table S2 in SI). HPLC analysis of SB19003 fermentation showed the abolishment of production of both **1** and **2** (Figure 2, panel IV), confirming the essential role ChxE plays in **1** and **2** biosynthesis and establishing **1** and **2** production is governed by single biosynthetic machinery (Figure 3).

ChxJ consists of three domains, an acyl-CoA ligase (AL), an ACP, and a reductase (R) that specifically reduces a carboxylic acid to an aldehyde via the intermediacy of an acyl-*S*-ACP and shares 59% identity with CAR from *Nocardia iowensis*, which reduces several carboxylic acids to their corresponding aldehydes (Table S3 in SI).[8] Inspired by the chemistry of CAR, we propose that ChxJ catalyzes the reduction of the carboxylic acid group in **7** to afford the aldehyde intermediate (**8**). Thus, the AL domain of ChxJ activates **7** and loads it to the ACP domain, and the resultant acyl-*S*-ACP intermediate is

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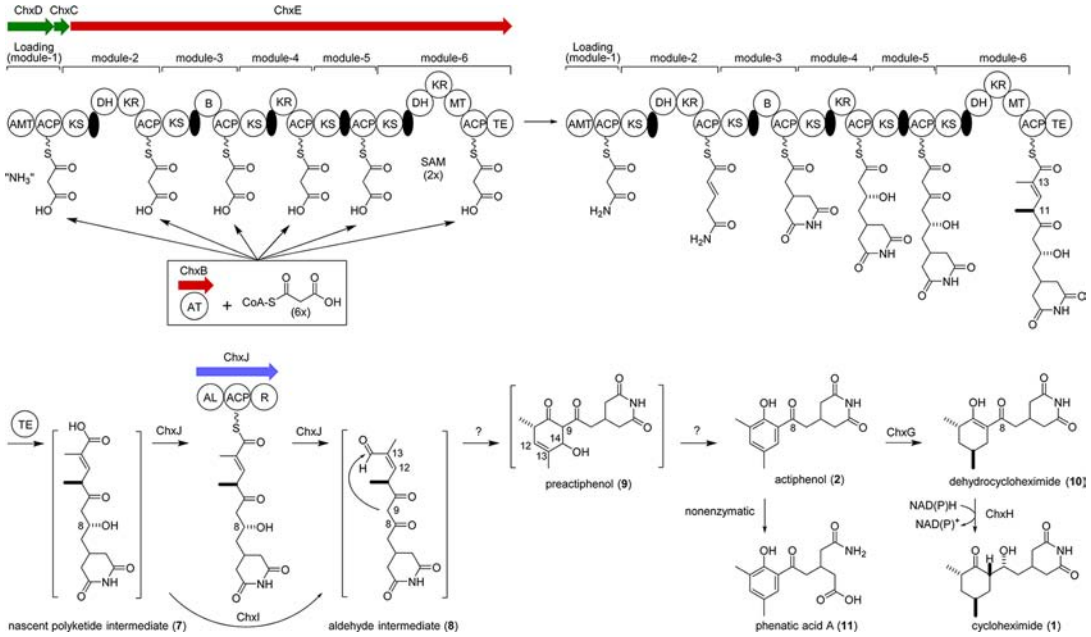

Figure 3. Proposed pathway for **1** biosynthesis featuring an AT-less type I PKS and proceeding via **2** as an intermediate. Abbreviations are ACP, acyl carrier protein; AL, acyl-CoA ligase; AMT, amidotransferase; B, *β*-branching; DH, dehydratase; KR, ketoreductase; KS, ketosynthase; MT, methyltransferase; R, acyl thioester reductase; SAM, *S*-adenosylmethionine; TE, thioesterase; and ?, unknown or nonenzymatic.

subsequently reduced by the R domain of ChxJ to afford **8**. ChxI belongs to the cytochrome P450 superfamily of oxidoreductases (Table S3 in SI), serving as the candidate to catalyze C-8 oxidation during the conversion from **7** to **8**. Oxidation at C-8 would set the stage for **8** to undergo an intramolecular aldol condensation between C-9 and C-14 to yield an intermediate such as preactiphenol (**9**), which features the carbon scaffold of both **1** and **2** (Figure 3). While a cis-double bond at C-12 and C-13 of **8** would be necessary to facilitate C-9 and C-14 cyclization, it is not known when and how this isomerization occurs from **7** to **8**. We also failed to identify any candidate responsible for the necessary aldol condensation from **8** to **9**, as well as the subsequent dehydration of **9** to **4** within the *chx* cluster. While it is tempting to speculate that both steps could be spontaneous, we cannot exclude the possibility that they are catalyzed by enzyme activities residing outside of the cloned *chx* cluster (Figure 3).

Both *chxJ* and *chxI* were subsequently inactivated (SI), and the genotypes of the resultant mutant strains SB19004 (i.e., Δ*chxJ*) and SB19006 (i.e., Δ*chxI*) were confirmed by Southern analysis (Figures S5, S6, and Table S2 in SI). Two additional recombinant strains were also constructed (Table S2 in SI), in which the Δ*chxJ* and Δ*chxI* mutations in SB19004 and SB19006 were complemented by expressing a functional copy of *chxJ* (SB19005) or *chxI* (SB19007) in trans. HPLC analysis of their fermentations confirmed that **1** and **2** production was completely abolished in SB19004 and SB19006 (Figure 2, panels V and VII) and **1** and **2** production was restored, albeit only partially, in SB19005 and SB19007 (Figure 2, panels V, VI, VII, VIII), consistent with the essential roles proposed for ChxI and ChxJ in **1** and **2** biosynthesis. Failure to accumulate any discrete intermediate by SB19004 and SB19006, however, prevented us from providing direct evidence supporting the intermediacy of **7** or **8** in **1** and **2** biosynthesis or shedding light into the timing of C-8 oxidation, which could occur before, during, or after ChxJ catalysis (Figure 3).

We finally carried out in vivo and in vitro experiments on *chxG* and *chxH* to delineate the biosynthetic relationship between **1** and **2**, unveiling **2** as a key intermediate to **1**. ChxG belongs to the Old Yellow Enzyme (OYE) family of flavoprotein oxidoreductases that are capable of C═C bond reduction of a wide range of substrates,[9] and ChxH is a member of the short-chain dehydrogenase/reductase superfamily consisting of a large number of NAD(P)H oxidoreductases that provide varying enzymatic activities and act on a broad spectrum of substrates[10] (Table S3 in SI). Both *chxG* and *chxH* were inactivated (SI), the genotypes of the resultant mutant strains SB19008 (i.e., Δ*chxG*) and SB19010 (i.e., Δ*chxH*) were confirmed by Southern analysis (Figures S7 and S8 in SI), and the Δ*chxG* and Δ*chxH* mutations were also complemented by expressing functional copies of *chxG* (SB19009) and *chxH* (SB19011) in trans, respectively (Table S2 in SI). Remarkably, HPLC analysis of their fermentations showed complete abolishment of production of **1** but not **2** in both SB19008 and SB19010 (Figure 3, panels IX and XI) and partial restoration of **1** production in SB19009 and SB19011 (Figure 3, panels X and XII). These findings unambiguously established **2** as an intermediate for **1** biosynthesis, the transformation of which to **1** requires minimally ChxG and ChxH (Figure 3).

Close examination of the HPLC profiles further revealed a significantly increased production of **2**, accompanied by the accumulation of phenatic acid (**11**), a known metabolite of nonenzymatic hydrolysis of **2**,[1c] in SB19008 and accumulation of dehydrocycloheximide (**10**), in addition to **2**, in SB19010 (Figure 2, panels IX and XI). The identity of **10** was unambiguously established by ^{1}H and ^{13}C NMR analysis (Table S4 and Figure S9 in SI), which has been isolated previously from **1** and **2** producers such as *S. noursei*.[2d] Taken together, these results suggest that ChxG catalyzes reduction of **2** to **10**, a provocative proposal for an enzymatic reduction of a phenol to a cyclohexanone moiety in natural product biosynthesis, and that ChxH catalyzes the final step of **1** biosynthesis, reducing **10** to **1** (Figure 3). Controlled reduction

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of a benzene ring has been difficult in both laboratories and biological systems due to its high resonance energy. Although members of the OYE family have been implicated in the reduction of trinitrotoluene,[9] structural characterization of the partially reduced cyclic products remains elusive to date. ChxG, which shows high sequence homology to known members of OYE family (Figure S10 in SI), therefore could serve as an excellent model to study how flavoproteins modulate redox potential to catalyze the reduction of a benzene ring.

Finally, we overexpressed *chxH* in *E. coli* (SI and Table S2) and purified ChxH to near homogeneity (Figure S11 in SI). ChxH catalyzed efficient reduction of **10** to **1**, requiring NADPH or NADH (Figure S12A in SI) and exhibiting an optimal pH at 7.2 in 100 mM sodium phosphate (Figure S12B in SI), and showed no activity toward **11** as an alternative substrate. Pseudo-first-order kinetic studies under steady state conditions (SI) showed that plots of initial velocity versus the concentration of substrates or cofactors all displayed Michaelis–Menten kinetics, allowing the determination of the corresponding K_M's, and k_{cat}'s (Figure S13 in SI). Thus, as summarized in Table S5 in SI, ChxH exhibited apparent K_M's for **10** at 44 ± 4 μM and 139 ± 23 μM upon saturation of NADPH and NADH, apparent K_M's for NADPH and NADH at 34 ± 4 μM and 75 ± 8 μM upon saturation of **10**, and apparent k_{cat}'s at 599 ± 69 min^{-1} and 20 ± 3 min^{-1} with NADPH and NADH, respectively. ChxH was also competent to catalyze the reverse reaction from **1** to **10**, exhibiting apparent K_M's for **1** at 99 ± 7 μM and 162 ± 32 μM upon saturation of $NADP^+$ and NAD^+, apparent K_M's for $NADP^+$ and NAD^+ at 76 ± 5 μM and 233 ± 37 μM upon saturation of **1**, and apparent k_{cat}'s at 387 ± 23 min^{-1} and 13 ± 3 min^{-1} with $NADP^+$ and NAD^+, respectively. ChxH therefore prefers NADPH (K_M = 34 ± 4 μM) to NADH (K_M = 75 ± 8 μM) and **10**-to-**1** (K_M = 44 ± 4 μM and k_{cat}/K_M = 14 μM^{-1} min^{-1}) to **1**-to-**10** conversion (K_M = 76 ± 5 and k_{cat}/K_M = 5.1 μM^{-1} min^{-1}). These findings provided direct evidence, further supporting the intermediacy of **2** in **1** biosynthesis with ChxG and ChxH catalyzing the last two steps of the pathway (Figure 3).

In summary, in vivo and in vitro characterizations of the *chx* gene cluster have now revealed that **1** and **2** biosynthesis is governed by single biosynthetic machinery, which explains why **1**, **2**, and congeners are often isolated together.[1,2] The glutarimide-containing polyketide backbone of **1** is assembled similarly to that of other members of this family of natural products such as **4**, **5**, and **6**, featuring an AT-less type I PKS.[5−7] Comparative studies among these machineries provide an outstanding opportunity to study glutarimide biosynthesis and many of the common features unique to AT-less type I PKSs.[11] Our findings also support that ChxG and ChxH are necessary and sufficient to catalyze the conversion of **2** to **1** as the last two steps for **1** biosynthesis, invoking a provocative phenol-to-cyclohexanone reduction that to our knowledge is unprecedented in natural product biosynthesis.[9]

■ ASSOCIATED CONTENT

Supporting Information

Complete description of materials and methods, supporting tables (S1−S5), and supporting figures (S1−S13). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Leach, B. E.; Ford, J. H.; Whiffen, A. J. *J. Am. Chem. Soc.* **1947**, *69*, 474. (b) Kornfeld, E.; Jones, R. G.; Parke, T. V. *J. Am. Chem. Soc.* **1949**, *71*, 150−158. (c) Huang, S.-X.; Yu, Z.; Robert, F.; Zhao, L.-X.; Jiang, Y.; Duan, Y.; Pelletier, J.; Shen, B. *J. Antibiot.* **2011**, *64*, 163−166.

(2) (a) Highet, R. J.; Prelog, V. *Helv. Chim. Acta* **1959**, *42*, 1523−1526. (b) Rao, K. V.; Cullen, W. P. *J. Am. Chem. Soc.* **1960**, *82*, 1127−1128. (c) Rao, K. *J. Org. Chem.* **1960**, *25*, 661−662. (d) Vondracek, M.; Vanek, Z. *Chem. Ind.* **1964**, 1686−1687.

(3) (a) Obrig, T. G.; Culp, W. J.; McKeehan, W. L.; Hardesty, B. *J. Biol. Chem.* **1971**, *246*, 174−181. (b) Schneider-Poetsch, T.; Ju, J.; Eyler, D. E.; Dang, Y.; Bhat, S.; Merrick, W. C.; Green, R.; Shen, B.; Liu, J. O. *Nat. Chem. Biol.* **2010**, *6*, 209−217. (c) Lee, S.; Liu, B.; Lee, S.; Huang, S.-X.; Shen, B.; Qian, S.-B. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, E2424−2432. (d) Stern-Ginossar, N.; Weisburd, B.; Michalski, A.; Vu, T. K. L.; Hein, M. Y.; Huang, S.-X.; Ma, M.; Shen, B.; Qian, S. B.; Hengel, H.; Mann, M.; Ingolia, N. T.; Weissman, J. S. *Science* **2012**, *338*, 1088−1093.

(4) (a) Shan, D.; Chen, L.; Njardarson, J. T.; Gaul, C.; Ma, X.; Danishefsky, S. J.; Huang, X.-Y. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3772−3776. (b) Ju, J.; Rajski, S. R.; Lim, S.-K.; Seo, J.-W.; Peters, N. R.; Hoffmann, F. M.; Shen, B. *J. Am. Chem. Soc.* **2009**, *131*, 1370−1371. (c) Chen, L.; Yang, S.; Jakoncic, J.; Zhang, J.; Huang, X.-Y. *Nature* **2010**, *464*, 1062−1066. (d) Rajski, S. R.; Shen, B. *ChemBioChem.* **2010**, *11*, 1951−1954.

(5) Wang, B.; Song, Y.; Luo, M.; Chen, Q.; Ma, J.; Huang, H.; Ju, J. *Org. Lett.* **2013**, *15*, 1278−1281.

(6) Lim, S.-K.; Ju, J.; Zazopoulos, E.; Jiang, H.; Seo, J.-W.; Chen, Y.; Feng, Z.; Rajski, S. R.; Farnet, C. M.; Shen, B. *J. Biol. Chem.* **2009**, *284*, 29746−29756.

(7) The lactimidomycin biosynthetic gene cluster can be accessed at GenBank under acession number GQ274954.

(8) (a) He, A.; Li, T.; Daniels, L.; Fotheringham, I.; Rosazza, J. P. N. *Appl. Environ. Microbiol.* **2004**, *70*, 1874−1881. (b) Venkitasubramanian, P.; Daniels, L.; Rosazza, J. P. N. *J. Biol. Chem.* **2007**, *282*, 478−485.

(9) (a) Williams, R. E.; Bruce, N. C. *Microbiology* **2002**, *148*, 1607−1614. (b) Toogood, H. S.; Gardiner, J. M.; Scrutton, N. S. *ChemCatChem* **2010**, *2*, 892−914.

(10) (a) Kavanagh, K. L.; Jörnvall, H.; Persson, B.; Oppermann, U. *Cell. Mol. Life Sci.* **2008**, *65*, 3895−3906. (b) Kallberg, Y.; Oppermann, U.; Persson, B. *FEBS J.* **2010**, *277*, 2375−2386.

(11) (a) Cheng, Y.-Q.; Tang, G.-L.; Shen, B. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3149−3154. (b) Cheng, Y.-Q.; Coughlin, J. M.; Lim, S.-K.; Shen, B. *Methods Enzymol.* **2009**, *459*, 165−186. (c) Piel, J. *Nat. Prod. Rep.* **2010**, *27*, 996−1047. (d) Musiol, E. M.; Weber, T. *MedChemComm* **2012**, *3*, 871−889.