JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Differences in PLP-Dependent Cysteinyl Processing Lead to Diverse *S*-Functionalization of Lincosamide Antibiotics

Min Wang,[†] Qunfei Zhao,[*,†,‡] Qinglin Zhang,[§] and Wen Liu[*,†,‡,§]

[†]State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, University of Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, China

[‡]State Key Laboratory of Microbial Metabolism, School of Life Science & Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

[§]Huzhou Center of Bio-Synthetic Innovation, 1366 Hongfeng Road, Huzhou 313000, China

Supporting Information

ABSTRACT: Pyridoxal-5′-phosphate (PLP)-dependent proteins constitute one of the largest and most important families of enzymes in living organisms. These proteins participate in numerous biochemical processes, many of which have not been characterized, and transform substrates containing an amino group through various reactions that share aldimine as a common intermediate. Herein, we report that the PLP-dependent enzymes CcbF and LmbF, which are highly related in phylogenesis, process cysteine *S*-conjugated intermediates in different ways and associate with individual downstream enzyme(s) toward distinct *S*-functionalization of the lincosamide antibiotics celesticetin and lincomycin A. CcbF catalyzes an unusual conversion that involves decarboxylation-coupled oxidative deamination of the cysteinyl group during the formation of a two-carbon alcohol linker, whereas LmbF is responsible for *β*-elimination, followed by *S*-methylation to produce a methylmercapto group. The two tailoring routes are variable and exchangeable with each other, allowing for *in vitro* combinatorial biosynthesis of a number of hybrid lincosamide antibiotics, including the natural product Bu-2545. These findings demonstrate the wide diversity of PLP chemistry in enzymatic catalysis and its promising applicability in creation of new molecules.

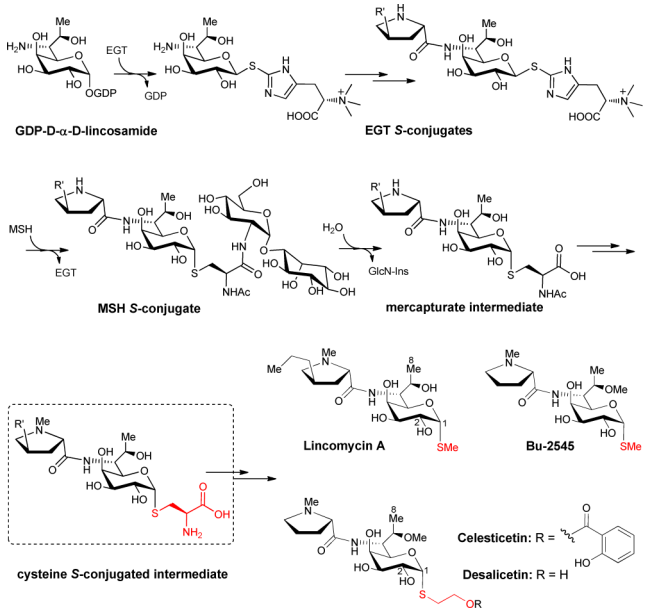

Figure 1. LMW thiols-programmed biosynthesis of lincosamide antibiotics, including lincomycin A, celesticetin and desalicetin, and their hybrid Bu-2545. The common cysteine *S*-conjugated intermediate is shown in dashed rectangle. The exposed cysteinyl and related downstream functional groups are colored red.

Lincosamide antibiotics (Figure 1), characterized by an eight-carbon aminosugar central to an amino acid residue and a sulfur appendage, include the therapeutic anti-infective agent lincomycin A and its naturally occurring analogues celesticetin and Bu-2545.[1] These antibiotics act on the 50S large subunit of bacterial ribosome, where their octose-based amide structures mimic the 3′-end of (de)acetyl-tRNA, and are therefore able to block protein synthesis at the initial stage of the elongation cycle.[2]

The process through which nature produces lincosamide antibiotics remained poorly understood until recent insights were gained through investigations into the biosynthetic pathway of lincomycin A. Eight-carbon sugar formation proceeds via a transaldol reaction, using D-fructose 6-phosphate or D-sedoheptulose 7-phosphate as the three-carbon donor and D-ribose 5-phosphate as the five-carbon acceptor.[3a] The resulting octose is then activated by GDP, to initiate a modification process that yields GDP-D-*α*-D-lincosamide[3b] (Figure 1). During the incorporation with a proline-like residue and *S*-functionalization at C-1, metabolic coupling of two bacterial low-molecular-weight (LMW) thiols, mycothiol (MSH) and ergothioneine (EGT), plays a constructive role that is potentially common for all related antibiotics.[4] These thiols function through two unusual S_N2 *S*-glycosylations to program lincosamide transfer, activation and modification. Specifically, EGT is a cryptic carrier to mediate molecular assembly, in contrast to MSH, which acts as a sulfur donor after thiol exchange (Figure 1). Mechanistically analogous to the thiol-mediated detoxification process,[5] hydrolysis of the MSH *S*-conjugate produces an *N*-acetyl-cysteinyl mercapturate intermediate, thereby indicating that the downstream route

Received: February 17, 2016
Published: May 12, 2016

DOI: 10.1021/jacs.6b01751
J. Am. Chem. Soc. 2016, 138, 6348−6351

toward lincomycin maturation would center on processing the remaining cysteinyl group following *N*-deacetylation (Figure 1).

The further conversion of mercapturate derivatives to excretive thiomethyl products has previously been established as an extended detoxification pathway in thiol-mediated metabolism.[6] This pathway relies on the PLP-dependent activity of cysteine *S*-conjugate β-lyases to cleave the C−S bond and expose the sulfhydryl group. In fact, several sulfur-containing natural products, e.g., gliotoxin and leinamycin, share cysteinyl-based β-elimination chemistry during their biosyntheses,[7] and lincomycin A is no exception. In its pathway, LmbF has recently been demonstrated to be a protein that possesses PLP-associated β-lyase activity;[8] however, the candidate for *S*-methylation of the thiol intermediate remains to be determined. Here, we targeted CcbF, which is a LmbF homologue (40% identity) involved in the biosynthesis of celesticetin[9] (Figures S1 and S2), a lincosamide antibiotic different from lincomycin A primarily in *S*-substitution.[1] Our efforts focused on elucidating whether a cysteine *S*-conjugate exists in the pathway and how CcbF, in association of its downstream enzymes, acts on this intermediate and initiates completely distinct tailoring to furnish a salicylated two-carbon appendage of sulfur. Relevant studies included a comparative analysis with lincomycin maturation upon completing this process by characterization of the *S*-methylation step.

To validate the necessity of CcbF for celesticetin biosynthesis in *Streptomyces caelestis*, we inactivated the encoding gene *ccbF*. Under applied culture conditions, the wild-type strain produced celesticetin and its non-salicylic precursor, desalicetin[10] (for both compounds, this study provides the complete 1D and 2D NMR spectral data for the first time) (Figure S4A and Supplementary Results). The Δ*ccbF* mutant strain failed to produce celesticetin and desalicetin; instead, it accumulated a new product (**1**), a trace of which was also examined in the wild-type strain (Figures 2 and S4A). A sufficient quantity of **1** was prepared through fermentation on a large scale. Subsequent 1D and 2D NMR analyses revealed that this compound is a cysteine *S*-conjugate intermediate (Supplementary Results), supporting the notion that both celesticetin and lincomycin A share a common LMW thiol-programmed biosynthetic route toward cysteinyl processing.

We expressed and purified CcbF from *Escherichia coli*. The resulting recombinant proteins appeared light yellow in color and exhibited an absorbance spectrum characteristic of PLP binding (Figure S5). CcbF alone was capable of transforming intermediate **1** *in vitro*; however, the rate was largely improved by supplementing exogenous PLP (Figure 2A). HPLC-MS analysis of the product profile revealed a new compound, **2** (Figure 2), which readily underwent hydration in solution (Figure S18). This compound was accordingly predicted to be a highly reactive aldehyde (the structure was further demonstrated below through enzymatic transformations to desalicetin) that is derived from **1** as a result of the activity of CcbF to remove the terminal carboxylate and α-amino groups of the cysteinyl.

We then examined the associated chemicals in CcbF-mediated conversion of **1** to **2**. The omission of pyruvate (or α-ketoglutarate) from the mixture had no effect on the reaction (Figures 2A and S6), indicating that α-keto acid, which often serves as an ammonia (NH_3) acceptor in various transamination reactions to recycle PLP from the resultant pyridoxamine-5′-phosphate (PMP), is unnecessary. No PMP was detected during a 1-h examination period (Figure S7); consistently, the derivatization of the reaction mixture with *O*-phthaldialdehyde (OPA)/mercaptopropionic acid (MPA) revealed the production

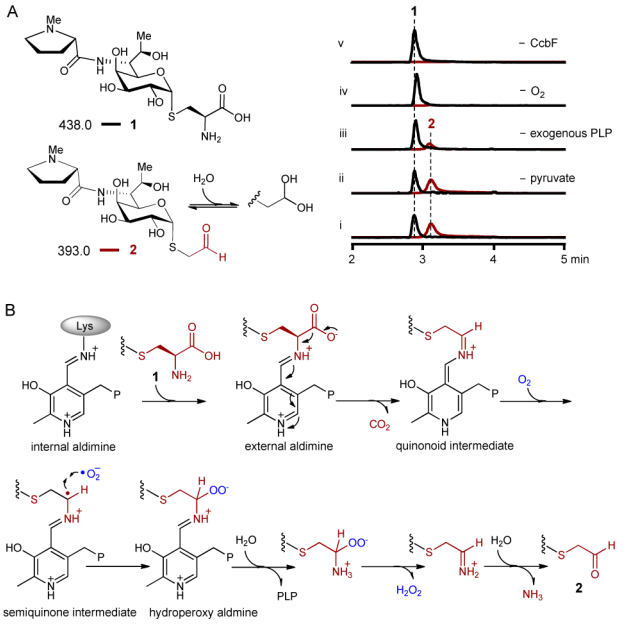

Figure 2. CcbF-catalyzed decarboxylation-coupled oxidative deamination. (A) Conversion of substrate **1** (black) to product **2** (ocher) *in vitro*. The ESI m/z $[M+H]^+$ modes of target compounds are indicated (left) and colored in the HPLC-MS traces (right). The transformations proceeding here derived from the reaction in which CcbF, **1**, exogenous PLP, and pyruvate were incubated under aerobic conditions during a 20-min incubation period (i), and included those in which pyruvate (ii), PLP (iii), O_2 (iv), or CcbF (v, control) was selectively omitted. (B) Proposed catalytic mechanism of CcbF. The cysteinyl and related downstream groups are highlighted (ocher). Oxygen and associated products are indicated (blue).

of free NH_3 (Figure S8). These results excluded the alternative that PLP is a co-substrate to accept NH_3 from the cysteinyl group of **1** and supported that PLP acts as a cofactor in the process of **2** production. Intriguingly, oxygen (O_2) was indispensable for CcbF-catalyzed conversion, which failed to occur under a strictly anaerobic condition (Figure 2A). Inspired by this finding, we further analyzed hydrogen peroxide (H_2O_2) in the reaction mixture. H_2O_2 was detected as anticipated, and over time, its production was completely in line with both the consumption of substrate **1** and the generation of product **2** (Figure S9). Consequently, CcbF catalyzes a PLP-dependent biotransformation of a cysteine *S*-conjugate to an aldehyde along with the co-production of carbon dioxide (CO_2), H_2O_2, and NH_3 under an aerobic condition (Figure 2).

A comparable PLP-dependent biotransformation is the well-established decarboxylation−transamination process that involves the activity of dialkylglycine decarboxylase (DGD).[11] This bifunctional enzyme catalyzes the conversion of dialkylglycine to the ketone product, in which PLP is also a cofactor but mediates the transfer of NH_3 onto pyruvate to generate alanine in an O_2-independent manner. Mechanistically analogous to typical PLP-dependent decarboxylases, both CcbF and DGD could share the early catalytic steps, as the conserved lysine residue (e.g., Lys260 for CcbF) at the active site that covalently binds PLP through internal aldimine formation could be exchanged with a substrate to provide a common external aldimine and trigger decarboxylation. Rather than the transamination occurring in DGD-catalyzed conversion that requires no O_2, the subsequent action of CcbF presumably involves an O_2-originated radical chemistry for oxidative deamination: (1) In the presence of O_2, single

DOI: 10.1021/jacs.6b01751
J. Am. Chem. Soc. 2016, 138, 6348−6351

electron transfer may occur on external aldimine to produce a radical semiquinone intermediate and superoxide anion, combination of which at Cα would form hydroperoxy aldimine. (2) After PLP release, the resulting intermediate might undergo H_2O_2 elimination and imine hydrolysis to produce aldehyde (Figure 2B). This mechanism of O_2 activation and incorporation has previously been proposed for certain cofactor-free oxidases, such as urate oxidase;[12] however, its coupling with the PLP chemistry that is common for carbanion stabilization is extremely rare. The only precedents include a plant phenylacetaldehyde synthase and a DOPA decarboxylase mutant, which have activity similar to that of CcbF for decarboxylation-coupled oxidative deamination, and Ind4, an enzyme that has been identified very recently for oxidation of an unactivated carbon−carbon bond in the biosynthesis of indomycin.[13]

We next established the biosynthetic route downstream of aldehyde **2** *in vitro* through *in situ* enzymatic transformations producing desalicetin. The search for potential candidates was based on a comparative analysis of the biogenesis of celesticetin and lincomycin A, which led to the identification of the genes *ccb4* and *ccb5* from the *ccb* cluster (Figure S1). *Ccb4* and *ccb5*, which are functionally unassigned and lack the counterparts in the *lmb* cluster, encode proteins resembling various *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferases and NAD(P)H-dependent oxidoreductases, respectively. The two proteins were expressed, purified, and then simultaneously incorporated into the above reaction mixture involving CcbF. Desalicetin was effectively produced, thus confirming the function of Ccb4 in the *O*-methylation of lincosamide at C-7 and Ccb5 in the reduction of the terminal aldehyde group (Figure 3). The addition of Ccb4 or Ccb5 alone resulted in the *O*-methylated product **3** or alcohol **4**, both of which were further converted to desalicetin in the presence of Ccb5 or Ccb4, indicating a high promiscuity of their catalytic sequences (Figures 3, S19, and S20). *S. caelestis* appears to have surrogates encoded outside of the *ccb* cluster: mutating *ccb4* or *ccb5* alone only partially abolished the production of celesticetin and desalicetin (Figure S4A).

For a comparison in cysteinyl processing, we completed the maturation process of lincomycin A in *Streptomyces lincolnensis*. The inactivation of *lmbF* resulted in intermediate **5** (Figures 4 and S4B, and Supplementary Results). Incubating this cysteine *S*-conjugate with the purified β-lyase LmbF led to effective cleavage

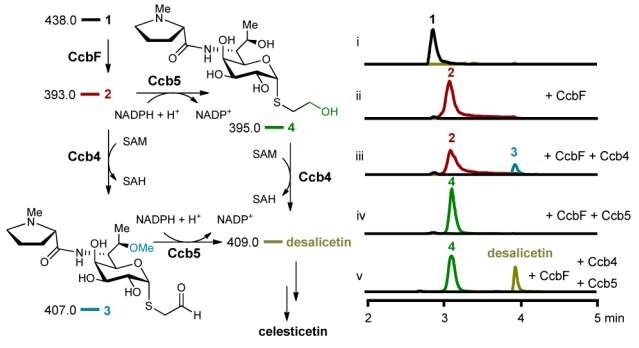

Figure 3. *In vitro* enzymatic transformations of **2** to desalicetin involving the activities of Ccb4 and Ccb5. The ESI m/z $[M+H]^+$ modes of target compounds are indicated in the biosynthetic routes (left) and colored in the HPLC-MS traces (right). The transformations derived from the control reaction in which **1** and exogenous PLP were incubated under aerobic conditions during a 2-h incubation period (i), in the presence of CcbF alone (ii), CcbF and Ccb4 (iii), CcbF and Ccb5 (iv), or CcbF, Ccb4, and Ccb5 (v).

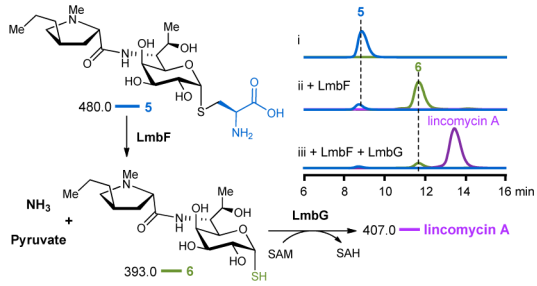

Figure 4. *In vitro* enzymatic transformations of **5** to lincomycin A involving the activities of LmbF and LmbG. The ESI m/z $[M+H]^+$ modes of target compounds are indicated in the biosynthetic routes (left) and colored in the HPLC-MS traces (right). The transformations derived from the control reaction in which **5** and exogenous PLP were incubated during a 2-h incubation period (i), in the presence of LmbF alone (ii) or LmbF and LmbG (iii).

of the C−S bond *in vitro*, generating the highly reactive thiol intermediate **6** along with the coproducts, NH_3 and pyruvate, the latter of which was characterized here by chemical derivatization with 2,4-dinitrophenylhydrazine (Figures 4, S10, and S22). The *lmb* cluster contains three genes that encode SAM-dependent methyltransferases, among which LmbW and LmbJ have previously been confirmed to perform *C*- and *N*-methylation, respectively, during the formation of the proline-derived residue.[14] We thus expressed and purified the remaining protein LmbG from *E. coli*, and assayed its activity after LmbF-catalyzed reaction to prepare substrate **6** *in situ*. As anticipated, LmbG efficiently catalyzed the conversion of **6** to the mature molecule lincomycin A, demonstrating the *S*-methylation activity of LmbG (Figures 4 and S23).

The availability of two *S*-functionalization strategies for lincosamide antibiotic maturation allowed us to reconstitute the biosynthetic pathway of Bu-2545 *in vitro*. Bu-2545, isolated from *Streptomyces* sp. H230-5,[1e] is a naturally occurring chimera between celesticetin and lincomycin A: it shares the 7-*O*-methyl-6-*N*-prolinyl-lincosamide unit with the former molecule but bears a methylmercapto group like the latter. According to the common logic of thiol-programmed biosynthesis of lincosamide antibiotics, the reconstitution began with the branching point for cysteinyl processing of the celesticetin intermediate **1**, in combination with the tailoring enzymes from the two biosynthetic pathways of lincomycin A and celesticetin (Figure 5A). Incubation of **1** with the β-lyase LmbF led to a new thiol, **7**, which was then transformed through LmbG-catalyzed *S*-methylation to the methylmercapto product **8**, demonstrating the effectiveness and flexibility of the thiomethyl appendage route (Figures 5A, S24, and S25). Furthermore, the incorporation of the *O*-methyltransferase Ccb4, derived from the celesticetin pathway, into the above reaction mixture and into a mixture lacking LmbG produced the target Bu-2545 and its *S*-demethylated thiol **9**, respectively (Figures 5A, S26, and S27).

We therefore extended the idea of *in vitro* combinatorial biosynthesis to process the cysteinyl group of the lincomycin intermediate **5** to generate a two-carbon *S*-linker characteristic of celesticetin (Figure 5B). Incubation with CcbF for 2-h resulted in the partial conversion of **5** into aldehyde **10**; however, the further transformation of **10** to alcohol **11** by the NADPH-dependent oxidoreductase Ccb5 was complete (Figures 5B, S28, and S29). Compared to its counterpart LmbF, CcbF appeared to be more stringent in substrate tolerance and its associated decarbox-

DOI: 10.1021/jacs.6b01751
J. Am. Chem. Soc. 2016, 138, 6348−6351

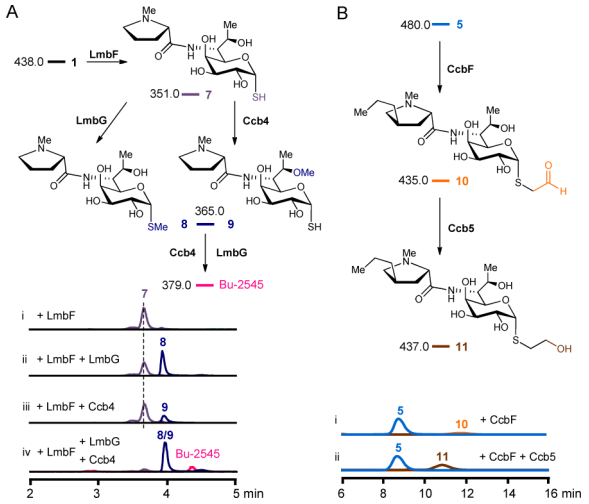

Figure 5. *In vitro* combinatorial biosynthesis of lincosamide antibiotics. The ESI m/z $[M+H]^+$ modes of target compounds are indicated in the biosynthetic routes (top) and colored in the HPLC-MS traces (bottom). (A) Generation of Bu2545 and related products bearing a lincomycin-like sulfur appendage. The transformations were conducted by incubating the celesticetin intermediate **1** with LmbF (i), LmbF and LmbG (ii), LmbF and Ccb4 (iii), or LmbF, Ccb4, and Ccb5 (iv). (B) Generation of the products with a celesticetin-like sulfur appendage. The transformations were conducted by incubating the lincomycin intermediate **5** with CcbF (i) or CcbF and Ccb5 (ii).

ylation-coupled oxidative deamination reaction could be a limiting step in processing cysteine *S*-conjugated surrogates.

In conclusion, we have established two paradigms for distinct *S*-functionalization of the lincosamide antibiotics celesticetin and lincomycin A, whose biosynthesis share the thiol-programmed pathway of molecular assembly and sulfur incorporation but branch at cysteinyl processing to build the specific sulfur appendages. The celesticetin route involves a rare PLP-dependent activity for decarboxylation-coupled oxidative deamination that releases the two-carbon skeleton (after reduction) as a linker to append salicylate, and during this process, *O*-methylation occurs at C-7 of the lincosamide unit. In contrast, the lincomycin route involves β-elimination to reserve the sulfhydryl group of cysteinyl only, followed by *S*-methylation to furnish the methylmercapto functionality. *In vitro* permutation of these two maturation routes produced lincosamide hybrids of celesticetin and lincomycin A, including the natural member Bu-2545, for which a similar hybrid pathway can be foreseen in the native producer *Streptomyces* sp. H230-5.[1e] The tool box of synthetic biology is thus enriched, as the ability to design variable sulfur appendage for the production of sulfur-containing active molecules has been established. CcbF and LmbF are functionally distinct but highly homologous to each other, implying that phylogenetically they evolved from a common PLP-dependent prototype protein. A genome sequence-based survey revealed a number of homologues of CcbF and LmbF that remain functionally unassigned.Characterization of their potentially diverse activities, arising from the malleable nature of PLP chemistry, would allow access to unpredictable new biological processes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b01751.

Supplementary Methods, Results, Figures S1−S29, and Tables S1−S6 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*massfly@sioc.ac.cn

*wliu@mail.sioc.ac.cn

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Wei Deng at NCPSS for technical support. This work was supported in part by grants from NSFC (81302674 for Q. Zhao; 21520102004, 31430005, and 21472231 for W.L.), STCSM (14JC1407700 and 15JC1400400 for W.L.), and MST (2012AA02A706 for W.L.) of China.

■ REFERENCES

(1) (a) Mason, D. J.; Dietz, A.; DeBoer, C. *Antimicrob. Agents Chemother.* **1962**, 554. (b) Herr, R. R.; Bergy, M. E. *Antimicrob. Agents Chemother.* **1962**, 560. (c) Hoehsema, H.; Crum, G. F.; DeVries, W. H. *Antibiot. Annu.* **1955**, *2*, 837. (d) Hoeksema, H. *J. Am. Chem. Soc.* **1968**, *90*, 755. (e) Hanada, M.; Tsunakawa, M.; Tomita, K.; Tsukiura, H.; Kawaguchi, H. *J. Antibiot.* **1980**, *33*, 751.

(2) (a) Reusser, F. *Antimicrob. Agents Chemother.* **1975**, *7*, 32. (b) Wilson, D. N. *Nat. Rev. Microbiol.* **2014**, *12*, 35.

(3) (a) Sasaki, E.; Lin, C.-I.; Lin, K.-Y.; Liu, H.-w. *J. Am. Chem. Soc.* **2012**, *134*, 17432. (b) Lin, C.-I.; Sasaki, E.; Zhong, A.; Liu, H.-w. *J. Am. Chem. Soc.* **2014**, *136*, 906.

(4) (a) Zhao, Q.; Wang, M.; Xu, D.; Zhang, Q.; Liu, W. *Nature* **2015**, *518*, 115. (b) Wang, M.; Zhao, Q.; Liu, W. *BioEssays* **2015**, *37*, 1262.

(5) (a) Newton, G. L.; Buchmeier, N.; Fahey, R. C. *Microbiol. Mol. Biol. Rev.* **2008**, *72*, 471. (b) Jothivasan, V. K.; Hamilton, C. J. *Nat. Prod. Rep.* **2008**, *25*, 1091.

(6) Cooper, A. J. L.; Krasnikov, B. F.; Niatsetskaya, Z. V.; Pinto, J. T.; Callery, P. S.; Villar, M. T.; Artigues, A.; Bruschi, S. A. *Amino Acids* **2011**, *41*, 7.

(7) (a) Scharf, D. H.; Chankhamjon, P.; Scherlach, K.; Heinekamp, T.; Roth, M.; Brakhage, A. A.; Hertweck, C. *Angew. Chem.* **2012**, *124*, 10211. (b) Ma, M.; Lohman, J. R.; Liu, T.; Shen, B. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 10359.

(8) Kamenik, Z.; Kadlcik, S.; Radojevic, B.; Jiraskova, P.; Kuzma, M.; Gazak, R.; Najmanova, L.; Kopecky, J.; Janata, J. *Chem. Sci.* **2016**, *7*, 430.

(9) Čermák, L.; Novotná, J.; Ságová-Marečková, M.; Kopecký, J.; Najmanová, L.; Janata, J. *Folia Microbiol.* **2007**, *52*, 457.

(10) Argoudelis, A. D.; Brodasky, T. F. *J. Antibiot.* **1972**, *25*, 194.

(11) (a) Toney, M. D.; Hohenester, E.; Keller, J. W.; Jansonius, J. N. *J. Mol. Biol.* **1995**, *245*, 151. (b) Sun, S. X.; Zabinski, R. F.; Toney, M. D. *Biochemistry* **1998**, *37*, 3865.

(12) Fetzner, S.; Steiner, R. A. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 791.

(13) (a) Kaminaga, Y.; Schnepp, J.; Peel, G.; Kish, C. M.; Ben-Nissan, G.; Weiss, D.; Orlova, I.; Lavie, O.; Rhodes, D.; Wood, K.; Porterfield, D. M.; Cooper, A. J.; Schloss, J. V.; Pichersky, E.; Vainstein, A.; Dudareva, N. *J. Biol. Chem.* **2006**, *281*, 23357. (b) Bertoldi, M.; Cellini, B.; Montioli, R.; Borri Voltattorni, C. *Biochemistry* **2008**, *47*, 7187. (c) Du, Y.-L.; Singh, R.; Alkhalaf, L. M.; Kuatsjah, E.; He, H.-Y.; Eltis, L. D.; Ryan, K. S. *Nat. Chem. Biol.* **2016**, *12*, 194.

(14) (a) Pang, A.-P.; Du, L.; Lin, C.-Y.; Qiao, J.; Zhao, G.-R. *J. Appl. Microbiol.* **2015**, *119*, 1064. (b) Najmanová, L.; Kutejová, E.; Kadlec, J.; Polan, M.; Olšovská, J.; Benada, O.; Novotná, J.; Kameník, Z.; Halada, P.; Bauer, J.; Janata, J. *ChemBioChem* **2013**, *14*, 2259.

DOI: 10.1021/jacs.6b01751
J. Am. Chem. Soc. 2016, 138, 6348−6351