ACS chemical biology

Letters

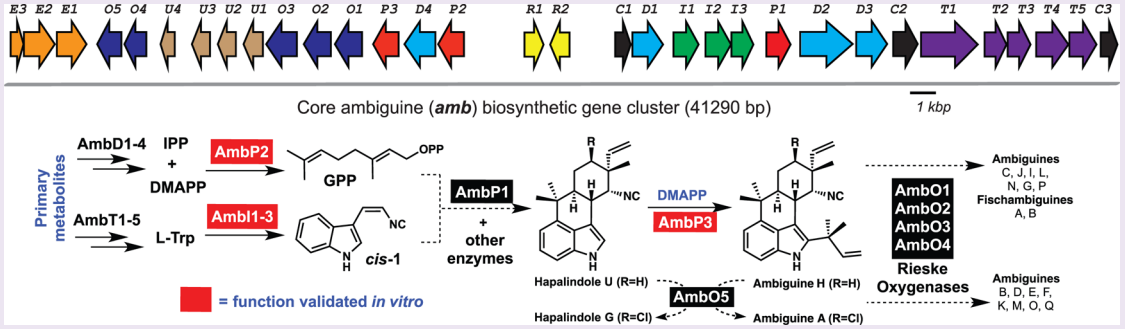
pubs.acs.org/acschemicalbiology

Biosynthesis of Ambiguine Indole Alkaloids in Cyanobacterium *Fischerella ambigua*

Matthew L. Hillwig, Qin Zhu, and Xinyu Liu*

Department of Chemistry, University of Pittsburgh, 219 Parkman Avenue, Pittsburgh, Pennsylvania 15260, United States

Supporting Information



ABSTRACT: Ambiguines belong to a family of hapalindole-type indole alkaloid natural products, with many of the members possessing up to eight consecutive carbon stereocenters in a fused pentacyclic 6-6-6-5-7 ring scaffold. Here, we report the identification of a 42 kbp ambiguine (*amb*) biosynthetic gene cluster that harbors 32 protein-coding genes in its native producer *Fischerella ambigua* UTEX1903. Association of the *amb* cluster with ambiguine biosynthesis was confirmed by both bioinformatic analysis and *in vitro* characterizations of enzymes responsible for 3-((*Z*)-2′-isocyanoethenyl) indole and geranyl pyrophosphate biosynthesis and a C-2 indole dimethylallyltransferase that regiospecifically tailors hapalindole G to ambiguine A. The presence of five nonheme iron-dependent oxygenase coding genes (including four Rieske-type oxygenases) within the *amb* cluster suggests late-stage C−H activations are likely responsible for the structural diversities of ambiguines by regio- and stereospecific chlorination, hydroxylation, epoxidation, and sp^2−sp^3 C−C bond formation.

Hapalindole-type natural products are a class of structurally diverse terpenoid indole alkaloids that are produced by true-branching heterocystous filamentous cyanobacteria *Fischerella* sp. and *Hapalosiphon* sp. in the order Stigonematales. Since the initial isolation of hapalindole A from *Hapalosiphon fontinalis* (hence, the coinage of hapalindole) by Moore and co-workers in 1984,[1] more than 70 members of hapalindole-type natural products have been isolated from a diverse set of geological locations worldwide.[2−9] These natural products possess a broad range of biological activities, including insecticidal activities against *Chironomus* larvae,[9] antibacterial and antifungal against human pathogens *Mycobacterium tuberculosis* and *Candida albicans*,[3,7,8] and anticancer activities against multidrug-resistant ovarian cancer cell lines.[2,10] The major classes of hapalindole-type molecules include hapalindoles,[1,2] fischerindoles,[4] welwitindolinones,[5] and ambiguines[3,6−8] that share a common molecular feature where an indole and an isonitrile group are connected by a carbon−carbon motif (C10−C11) that is appended with a monoterpene unit (Figure 1A). Fusion of the exomethylene carbon C-16 with indole backbones in the tricyclic hapalindoles provides tetracyclic hapalindoles and fischerindoles that, upon rearrangement, can also lead to the bridged tetracyclic welwitindolinones. Decoration of tetracyclic hapalindoles with a *tert*-prenyl group at C-2 of the indole ring results in ambiguines, of which many have a fused pentacyclic 6-6-6-5-7 or 6-6-6-5-6 ring system (Figure 1B).

Because of the intriguing structural complexities and diversities within hapalindole-type natural products, they have been subjected to extensive synthetic studies by many laboratories worldwide since 1984, which culminated in more than a dozen total syntheses within all four major classes of hapalindole-type molecules.[11] In sharp contrast to the synthetic efforts associated with these molecules, their biosynthesis at the molecular level remains unknown because neither genes nor enzymes associated with hapalindole-type molecule biogenesis have been identified. The existing biosynthetic hypothesis on the generation of hapalindoles was initially formulated by Moore and more recently by others[4−6,12] and suggests a key enzymatic step involving a chloronium ion or a proton-catalyzed polycyclization of 3-(2′-isocyanoethenyl) indole **1** (*cis* or *trans*) with a monoterpene *β*-ocimene **2** (E or Z) to furnish tricyclic hapalindoles (Figure 1C), which can be further enzymatically elaborated to other classes of hapalindole-type molecules. This hypothesis calls for an unprecedented enzyme, which remains to be identified, that can accommodate

Received: September 4, 2013
Accepted: November 1, 2013

   dx.doi.org/10.1021/cb400681n | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

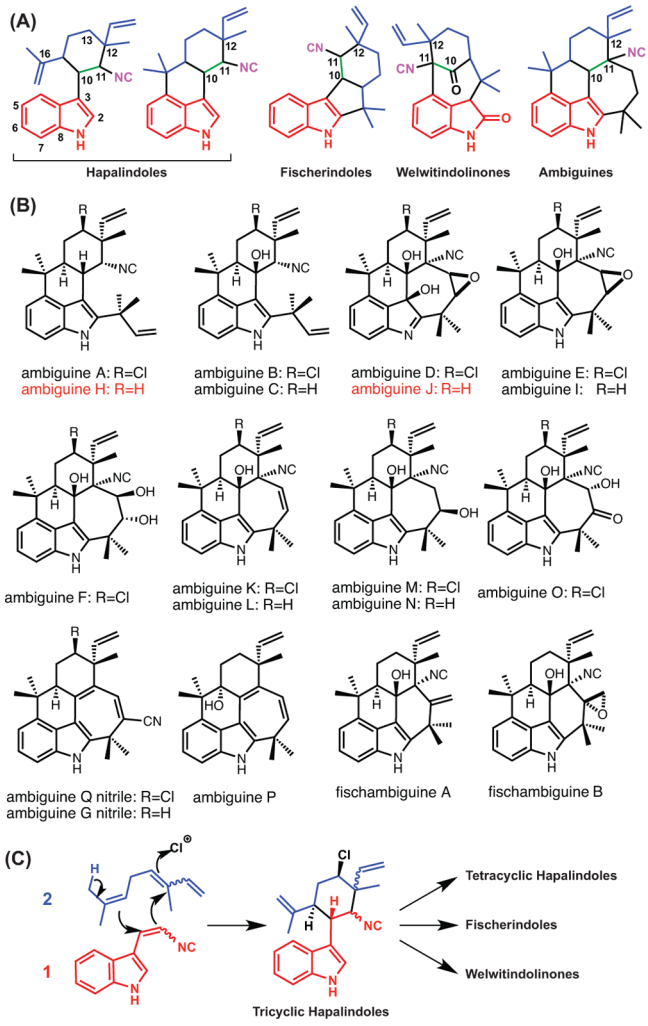


Figure 1. (A) Highlights of shared indole monoterpenoid structural elements of hapalindole-type natural products (backbone stereochemistries and additional functionalities are removed for simplicity). (B) Structural diversities of ambiguines produced by cyanobacteria *Fischerella ambigua*. Except for ambiguines H and J (named in red),[6] all other 19 structures have been isolated from a single strain, *F. ambigua* UTEX1903.[3,7,8] Seven pairs of ambiguines (A/H, B/C, D/J, E/I, K/L, M/N, and Q/G) are structurally identical to each other except for a single substitution at C-13, which is either chlorine or hydrogen. (C) Existing hapalindole biosynthetic hypothesis proposed by Moore and others.[4−6,12]

both chloronium ion and proton at the active site and can catalyze a carbocation-based cyclization cascade between two molecular components.

In view of the scarce molecular information associated with hapalindole-type molecule's biosynthesis, we set out to use a genomics-based approach to identify their biosynthetic genes. Among four major families of hapalindole-type natural products, we chose ambiguines produced by *Fischerella ambigua* UTEX1903 as the target to initiate our program, which was based on the following considerations. First, ambiguines are the only member of hapalindole-type molecules that are modified with a *tert*-prenyl group at C-2 of the indole ring, suggesting a dedicated indole prenyltransferase will be required for this tailoring event,[13] providing us with a unique bioinformatic lead to mine the target gene cluster. Second, the relative stereochemistry of indole ring and isonitrile group across the C10−C11 bond in all ambiguines isolated to date (Figure 1B) is strictly *cis*, suggesting they are likely all derived from a single biosynthetic precursor, *cis*-**1**, of which biosynthesis may be linked to isonitrile synthase superfamily,[14−18] thus providing us with a second unique bioinformatic clue. Third, to the best of our knowledge, *F. ambigua* UTEX1903 is the only axenic cyanobacterial hapalindole-type-molecule producer that was publicly available at the time we initiated this program.[19] We anticipated the axenic status of this strain would facilitate our efforts in assembling raw genomic data derived from next-generation sequencing.

With the initial confirmation of ambiguine production in axenic *F. ambigua* UTEX1903 (Figure SI-1), its genomic DNA was isolated and subjected to next-generation sequencing using a Roche 454 GS FLX+ system (see the Supporting Information for methods). An approximately 350 Mbp read was generated, and this raw data was assembled into 250 contigs that gives a draft genome size of *F. ambigua* UTEX1903 at 6.93 Mbp with ca. 50× coverage and GC content at 40.1%. Both of the genome size of UTEX1903 and its GC content are in agreement with other recently sequenced cyanobacteria in the order Stigonematales.[20] With high-quality draft genome data in hand, we used genes in the family of bacterial aromatic (indole) prenyltransferase and isonitrile synthase as bioinformatic leads to search for contig(s) that may coharbor these genes (see the Supporting Information for methods). This effort led to the identification of a 94 kbp contig (Figure 2). Annotation of the protein-coding genes within the contig revealed the putative ambiguine (*amb*) biosynthetic gene cluster that spans ca. 41 kbp with 32 protein-coding genes. Upstream of the *amb* cluster resides a 47 kbp nonribosomal peptide synthetase (NPRS)-containing gene cluster, of which the core NRPS genes are highly homologous to those in the nostocyclopeptide (*nos*) biosynthetic gene cluster.[21] The *nos* and *amb* clusters are further flanked with transposable elements (*orf*2, 3, and −3) as well as

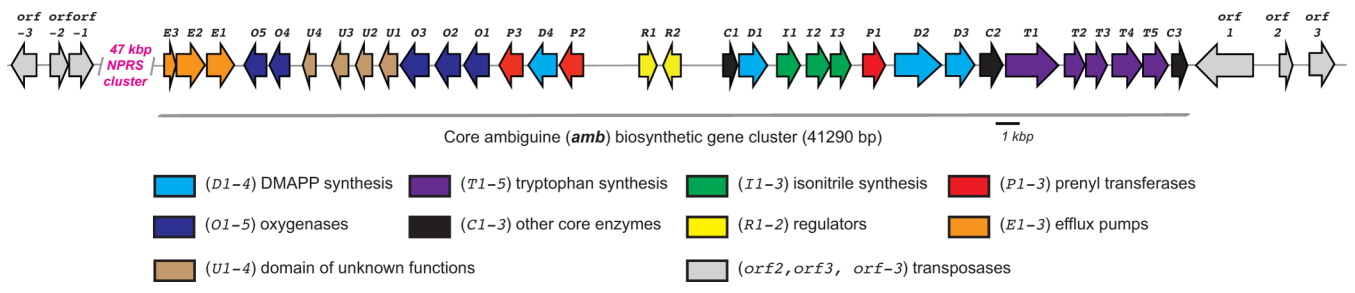


Figure 2. Illustration of the ambiguine biosynthetic gene cluster in *F. ambigua* UTEX1903. Gene functions are grouped on the basis of their putative roles associated with ambiguine biosynthesis. Full annotation of protein-coding genes in the *amb* cluster can be found in the Supporting Information, Table SI-1.

dx.doi.org/10.1021/cb400681n | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

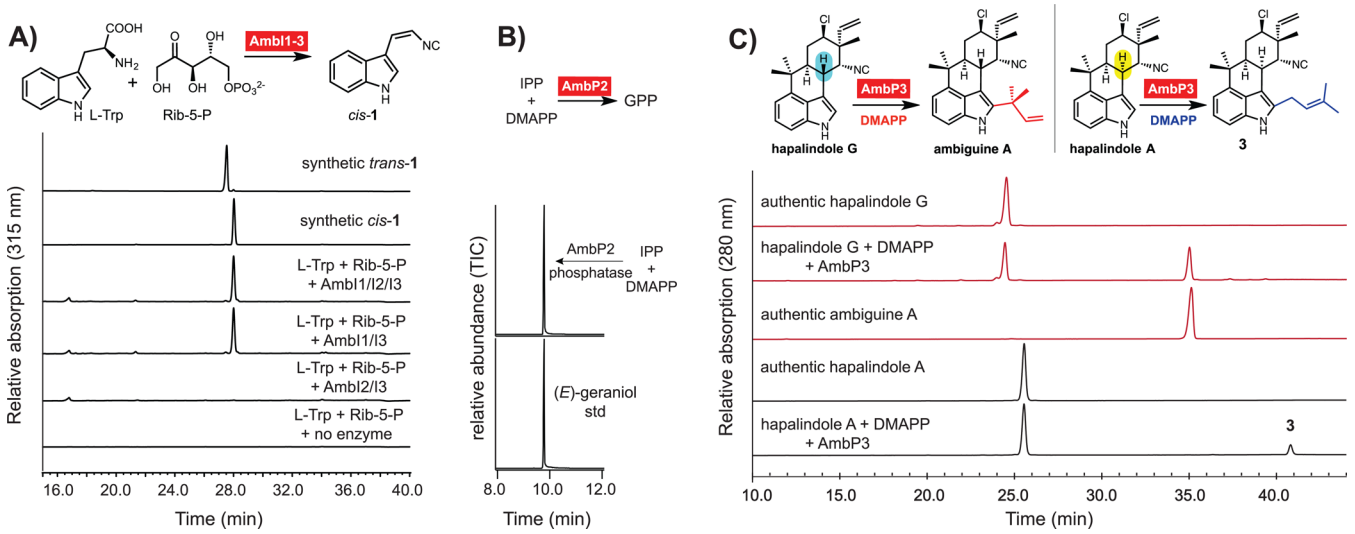


Figure 3. *In vitro* characterization of five key enzymes (AmbI1−3, AmbP2, and AmbP3) in ambiguine biosynthesis. (A) HPLC chromatograph with a UV detector showing AmbI1−3 is responsible for the stereoselective generation of *cis*-**1** from L-tryptophan and ribulose-5-phosphate. (B) GC chromatograph with an MS detector showing that AmbP2 is a dedicated GPP synthase. (C) HPLC chromatograph with a UV detector showing that AmbP3 is an exquisite dimethylallyltransferase that can regioselectively tailor hapalindole G to ambiguine A. AmbP3 can also take hapalindole A (epimer of hapalindole G) as a substrate with a decreased catalytic efficiency and install a forward dimethylallyl group at its C-2 indole ring to furnish **3**.

with plasmid replication and partitioning genes (*orf*−1/−2, *par*A/*par*B homologue), implying boundaries of the gene clusters as well as their potential mobile natures and the possibility of an extrachromosomal source.

Detailed bioinformatic analysis of the *amb* cluster (Table SI-1) allowed for the functional annotation of 28 genes based on their homologies with others, which can be further categorized into eight groups according to their roles in ambiguine biosynthesis. Besides two regulatory genes (*amb*R1/R2), three transporter genes (*amb*E1−3), four sequence-redundant domain of unknown function genes (*adm*U1−4) (Figure 2), the *amb* cluster contains five dedicated genes for tryptophan biosynthesis (*amb*T1−5) that correspond to *trp*A−E in the classical *trp* pathway as well as four dedicated genes for isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) biosynthesis (*amb*D1−4) that correspond to *dxs*, *dxr*, *isp*G, and *isp*H in the nonmevalonate pathway. The presence of a large set of dedicated genes for tryptophan and isoprenoid biosynthesis is consistent with the structural origin of ambiguines as terpenoid indole alkaloids that require L-Trp, DMAPP, and IPP as essential building blocks for their assembly (*vide infra*).

To further study the functions of the remaining 12 core *amb* biosynthetic proteins calls for their *in vitro* reconstitution because there is no validated procedure currently available for gene disruptions in Stigonematales. To this end, we cloned and overexpressed AmbI1−3 and AmbP1−3 (see the Supporting Information for methods) and validated the functions of AmbI1−3, AmbP2, and AmbP3 as the enzymes responsible for the assemblies of indole-isonitrile *cis*-**1**, geranyl pyrophosphate (GPP), and ambiguine A from hapalindole G. The *in vitro* study of AmbP1-3 also allowed us to conclude that ambiguine biosynthesis does not involve the chloronium-ion-mediated polycyclization event with *β*-ocimene **2**, as previously proposed (Figure 1C).[4−6,12]

AmbI1−3 are homologues of isonitrile synthase IsnA/IsnB, where both AmbI1 and AmbI2 share homology to IsnA and AmbI3, to IsnB. Because all previous studies on IsnA/IsnB superfamily genes are related to *trans*-**1**-type isonitrile production,[14−18] we proceeded to examine if AmbI1−3 is responsible for the stereoselective synthesis of *cis*-**1**, a biosynthetic precursor to all ambiguines. To address this question unequivocally, AmbI1/I2/I3 were overexpressed and purified individually as an N-His-tagged proteins in *Escherichia coli*. We also chemically synthesized *cis*-**1** and *trans*-**1** as standards for the enzymatic assays (see the Supporting Information for methods). Upon treatment of equal molar amounts of L-tryptophan (L-Trp) and ribulose-5-phosphate (Rib-5-P) with AmbI1/I2/I3 in the presence of *α*-ketoglutarate and Fe(II),[15] we observed the robust production of *cis*-**1** but not *trans*-**1** according to comparative HPLC analyses with synthetic standards (Figure 3A). In addition, AmbI1 and AmbI3 alone were sufficient to generate *cis*-**1** from L-Trp and Rib-5-P, but AmbI2 and AmbI3 were not, suggesting that AmbI2 is functionally redundant *in vitro*.

Three genes in the prenyltransferase superfamily were identified in the *amb* cluster: *amb*P2 belongs to the superfamily of *trans*-isoprenyl diphosphate synthase and thus is a candidate geranyl pyrophosphate (GPP) synthase that provides the monoterpene backbone for ambiguines and *amb*P1 and *amb*P3 are homologues to bacterial aromatic prenyltransferases that can transfer C5 or C10 isoprenoids to aromatic backbones including phenolics, tryptophans and phenazines[13] and thus constitutes a valid candidate for decorating hapalindole U or G with a *tert*-prenyl group from DMAPP to generate ambiguine H or A, an essential step for the subsequent maturation of ambiguines. Because the *amb* pathway lacks the apparent terpene synthase homologue to generate **2**, a previously hypothesized common precursor for tricyclic hapalindole biosynthesis,[4−6,12] we set out to verify the *in vitro* roles of AmbP1−3 in ambiguine biosynthesis and to determine if they are related to the production of **2**.

Following the cloning, overexpression, and purification of His-tagged AmbP1−3 from *E. coli*, we first tested if AmbP2 is a dedicated GPP synthase. Although GPP is an essential component for bacterial cell wall, cofactor, and small molecular terpenoid biosynthesis, dedicated GPP synthase of bacterial origin has been rarely identified and characterized *in vitro*.[22] Upon incubation of IPP and DMAPP with AmbP2 in the presence of Mg(II) followed by enzymatic dephosphorylation,

dx.doi.org/10.1021/cb400681n | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

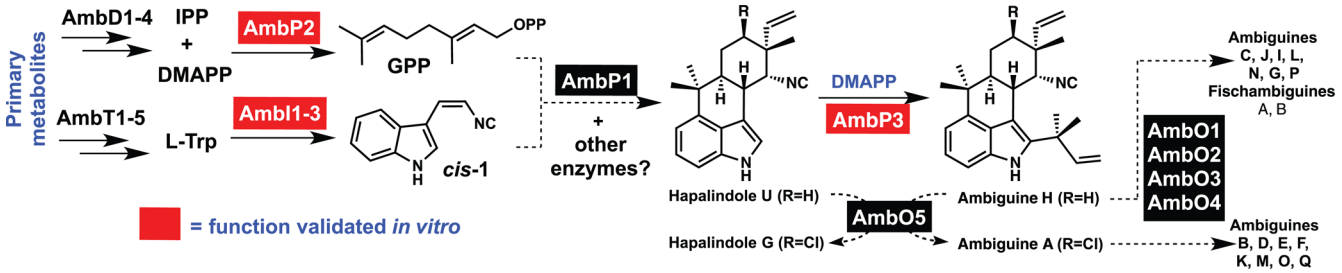


Figure 4. Current view of ambiguine biosynthesis at the molecular level on the basis of the *in vitro* characterization of AmbI1−3, P2, and P3 (highlighted in red) and bioinformatic correlations of key maturation enzymes (highlighted in black) in the *amb* cluster with the structural features of ambiguines.

we observed the formation of (*E*)-genraniol by GC−MS analysis (Figure 3B), which matched the authentic standard, whereas no isoprenoid derived from farnesyl/geranylgeranyl pyrophosphate (FPP/GGPP) or **2** was observed, demonstrating AmbP2 is an exquisite GPP synthase.

We subsequently examined whether AmbP1 or AmbP3 can tailor hapalindole G to ambiguine A. Upon treatment of purified hapalindole G with DMAPP and AmbP3, the generation of a new molecular entity was observed that matched the retention time of authentic ambiguine A by HPLC analysis (Figure 3C), whereas AmbP1 showed no effect under identical assay conditions. Full characterization of the enzymatic product derived from AmbP3 and hapalindole G was achieved using 1D/2D NMR spectroscopy, confirming its structural identity to be ambiguine A (Figures SI-2, -3, and -4). Both the apparent k_{cat} of AmbP3 (5.3 ± 0.5 min^{-1}) with hapalindole G and DMAPP as substrates and its activity independent of divalent metals (e.g., Mg^{2+}) (Figure SI-5) are consistent with the enzymatic profiles of other bacterial aromatic prenyltransferases,[23−25] collectively proving AmbP2 is a dedicated dimethylallyl transferase for ambiguine A biosynthesis. We also examined the substrate promiscuity of AmbP3 toward other indole derivatives. AmbP3 does not accept L/D-Trp, *trans*/*cis*-**1**, or Trp-containing linear or cyclic dipeptide as substrates, suggesting that the terpenoid component in hapalindole G is a crucial recognition element by AmbP3. Consistent with this hypothesis, when we treated purified hapalindole A, a C-10 epimer of hapalindole G, with AdmP3 and DMAPP, new molecule **3** was generated with an observed k_{cat} = 2.2 ± 0.3 min^{-1} (Figure 3C). NMR spectroscopic analysis of **3** unambiguously showed that it is a forward dimethylallyl modified hapalindole A (Figure SI-6) and that it is distinct from the native reverse prenylation in ambiguine A. This result indicates that in addition to the overall indole terpenoid scaffold, the 3D conformation of hapalindoles are equally crucial for AmbP3 to achieve exquisite selectivity. It also shows that AmbP3 can be used as a versatile prenylation catalyst for the structural diversification of hapalindoles.

The collective results from the *in vitro* characterization of AmbP2/P3, in addition to the fact that AmbP1/P2/P3 lack the ability to convert GPP to *β*-ocimine **2**, indicates that the biosynthesis of the core hapalindole tri- or tetracyclic scaffold is clearly different from previously proposed[4−6,12] and suggests that AmbP1 likely plays a key role in the formation of the hapalindole tetracyclic scaffold by directly utilizing *cis*-**1** and GPP (Figure 4). Our hypothesis that the direct enzymatic conversion of *cis*-**1** and GPP to hapalindole U constitutes a key step in ambiguine biosynthesis is also consistent with the fact that there is no obvious chloroperoxidase-like enzyme encoded in the pathway or in the UTEX1903 genome that can provide chloronium ion, as previously proposed.[4−6,12] On the contrary, the *amb* pathway encodes five nonheme iron (NHI)-dependent oxygenases, among which *amb*O5 belongs to the Fe(II)/*α*-ketoglutarate-dependent oxygenase family. Because NHI-dependent hydroxylase and halogenase share strong mechanistic homology,[26,27] it is plausible that *amb*O5 is a valid halogenase that mediates the transformation of nonchlorinated ambiguines/hapalindoles to chlorinated ones by regio- and stereoselective installation of a C−Cl bond at the C-13 of the ambiguine backbone. This proposal is also in agreement with the observation that the vast majority of ambiguines are isolated as chlorinated and nonchlorinated pairs (Figure 1B), suggesting that nonchlorinated ambiguine is likely a biosynthetic intermediate that can be tailored at a late stage of biosynthesis (Figure 4).

The remaining four NHI oxygenases belong to the Rieske oxygenase superfamily that are likely the key catalysts in the structural diversification of ambiguines from ambiguine A or H (Figure 4). This hypothesis is consistent with the structural features of ambiguines, such as ambiguine D, which is the most oxidatively decorated ambiguine in the family and is four oxidation states above ambiguine A. Achieving this set of individually unique oxidative transformations would call for four distinct 2e oxidation events through a sequence of enzymatic transformations mediated by AmbO1−4 (Figure SI-7). Although the involvement of a single Rieske oxygenase in the oxidative biosynthetic maturation of pyrrolnitrin and streptorubin B have been demonstrated,[28,29] it is striking to observe four highly homologous Rieske oxygenases that share nearly 80% sequence identity (Figure SI-8) are likely responsible for the regio- and stereospecific sp^2−sp^3 C−C bond formation, hydroxylation, and epoxidations across two different C−C double bonds in the ambiguine maturation process. Oxidative diversifications in the related natural products from plants, bacteria, and fungi have been traditionally related to FAD-dependent and cytochrome P450 oxygenase families.[30−32] These Rieske-type enzymes will therefore warrant future mechanistic studies to provide additional insights on enzyme evolution.

Our identification of the *amb* cluster provides the first genetic and biochemical insights on the biosynthesis of ambiguines and related hapalindole-type molecules at a detailed molecular level. The *in vitro* characterization of five key enzymes in the *amb* pathway, including unusual isonitrile synthases (AmbI1−3) for stereoselective assembly of *cis*-**1**, a rare dedicated bacterial GPP synthase, AmbP2, and a unique hapalindole dimethylallyl transferase, AmbP3, that is crucial for the initial assembly of ambiguines, collectively solidified the linkage between the *amb* pathway and ambiguine biosynthesis

dx.doi.org/10.1021/cb400681n | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

and provides the basis for future *in vitro* pathway engineering related to the combinatorial generation of hapalindole-like indole alkaloid natural products. The study of enzymatic properties of AmbP1−3 conclusively demonstrates that core hapalindole scaffold biogenesis does not involve the chloronium ion or a proton-catalyzed polycyclization of indole-isonitrile **1** with *β*-ocimene **2**. This work thus sets the stage for further exploration of novel enzymatic transformations in the biosynthesis of ambiguines, including the formation of core terpenoid indole scaffold from *cis*-**1** and GPP and oxidative diversification of ambiguines by Rieske-type oxygenases, which will be the subjects of future studies. In addition, it provides the foundation for comparative biosynthetic studies of other hapalindole-type natural products produced by terrestrial cyanobacteria in the order Stigonematales.

■ ASSOCIATED CONTENT

Supporting Information

Materials and Methods; Figures SI-1 to SI-12; Tables SI-1 & SI-2. This material is available free of charge via the Internet at http://pubs.acs.org. The nucleotide sequence of the *amb* cluster was deposited in GenBank under accession number KF664586.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: xinyuliu@pitt.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by Department of Chemistry startup fund and a central research development fund (2011-2013), both through the University of Pittsburgh.

■ REFERENCES

(1) Moore, R. E., Cheuk, C., and Patterson, G. M. L. (1984) Hapalindoles: New alkaloids from the blue-green alga *Hapalosiphon fontinalis*. *J. Am. Chem. Soc. 106*, 6456−6457.

(2) Moore, R. E., Cheuk, C., Yang, X. Q. G., Patterson, G. M. L., Bonjouklian, R., Smitka, T. A., Mynderse, J. S., Foster, R. S., Jones, N. D., Swartzendruber, J. K., and Deeter, J. B. (1987) Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. *J. Org. Chem. 52*, 1036−1043.

(3) Smitka, T. A., Bonjouklian, R., Doolin, L., Jones, N. D., Deeter, J. B., Yoshida, W. Y., Prinsep, M. R., Moore, R. E., and Patterson, G. M. L. (1992) Ambiguine isonitriles, fungicidal hapalindole-type alkaloids from three genera of blue-green algae belonging to the Stigonemataceae. *J. Org. Chem. 57*, 857−861.

(4) Park, A., Moore, R. E., and Patterson, G. M. L. (1992) Fischerindole L, a new isonitrile from the terrestrial blue-green alga *Fischerella muscicola*. *Tetrahedron Lett. 33*, 3257−3260.

(5) Stratmann, K., Moore, R. E., Bonjouklian, R., Deeter, J. B., Patterson, G. M. L., Shaffer, S., Smith, C. D., and Smitka, T. A. (1994) Welwitindolinones, unusual alkaloids from the blue-green algae *Hapalosiphon welwitschii* and *Westiella intricata*. Relationship to fischerindoles and hapalinodoles. *J. Am. Chem. Soc. 116*, 9935−9942.

(6) Raveh, A., and Carmeli, S. (2007) Antimicrobial ambiguines from the cyanobacterium *Fischerella* sp. collected in Israel. *J. Nat. Prod. 70*, 196−201.

(7) Mo, S. Y., Krunic, A., Chlipala, G., and Orjala, J. (2009) Antimicrobial ambiguine isonitriles from the cyanobacterium *Fischerella ambigua*. *J. Nat. Prod. 72*, 894−899.

(8) Mo, S. Y., Krunic, A., Santarsiero, B. D., Franzblau, S. G., and Orjala, J. (2010) Hapalindole-related alkaloids from the cultured cyanobacterium *Fischerella ambigua*. *Phytochemistry 71*, 2116−2123.

(9) Becher, P. G., Keller, S., Jung, G., Roderich, D. S., and Juttner, F. (2007) Insecticidal activity of 12-epi-hapalindole J isonitrile. *Phytochemistry 68*, 2493−2497.

(10) Smith, C. D., Zilfou, J. T., Stratmann, K., Patterson, G. M. L., and Moore, R. E. (1995) Welwitindolinone analogues that reverse P-glycoprotein-mediated multiple drug resistance. *Mol. Pharmacol. 47*, 241−247.

(11) A complete list of references associated with the accomplished total chemical syntheses of hapalindole-type natural products is included in the Supporting Information.

(12) Richter, J. M., Ishihara, Y., Masuda, T., Whitefield, B. W., Llamas, T., Pohjakallio, A., and Baran, P. S. (2008) Enantiospecific total synthesis of the hapalindoles, fischerindoles, and welwitindolinones via a redox economic approach. *J. Am. Chem. Soc. 130*, 17938−17954.

(13) Bonitz, T., Alva, V., Saleh, O., Lupas, A. N., and Heide, L. (2011) Evolutionary relationships of microbial aromatic prenyltransferases. *PLoS One 6*, e27336-1−e27336-8.

(14) Brady, S. F., and Clardy, J. (2005) Systematic investigation of the *Escherichia coli* metabolome for the biosynthetic origin of an isocyanide carbon atom. *Angew. Chem., Int. Ed. 44*, 7045−7048.

(15) Brady, S. F., and Clardy, J. (2005) Cloning and heterologous expression of isocyanide biosynthetic genes from environmental DNA. *Angew. Chem., Int. Ed. 44*, 7063−7065.

(16) Brady, S. F., Bauer, J. D., Clarke-Pearson, M. F., and Daniels, R. (2007) Natural products from isnA-containing biosynthetic gene clusters recovered from the genomes of cultured and uncultured bacteria. *J. Am. Chem. Soc. 129*, 12102−12103.

(17) Clarke-Pearson, M. F., and Brady, S. F. (2008) Paerucumarin, a new metabolite produced by the pvc gene cluster from *Pseudomonas aeruginosa*. *J. Bacteriol. 190*, 6927−6930.

(18) Crawford, J. M., Portmann, C., Zhang, X., Roeffaers, M. B., and Clardy, J. (2012) Small molecule perimeter defense in entomopathogenic bacteria. *Proc. Natl. Acad. Sci. U.S.A. 109*, 10821−10826.

(19) *F. ambigua* UTEX1903 was obtained from the culture collection of algae at the University of Texas at Austin and was rendered axenic by Dr. David R. Nobles, Jr. on Jan 23, 2009 (http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=4360).

(20) Dagan, T., Roettger, M., Stucken, K., Landan, G., Koch, R., Major, P., Gould, S. B., Goremykin, V. V., Rippka, R., Tandeau de Marsac, N., Gugger, M., Lockhart, P. J., Allen, J. F., Brune, I., Maus, I., Puhler, A., and Martin, W. F. (2013) Genomes of Stigonematalean cyanobacteria (subsection V) and the evolution of oxygenic photosynthesis from prokaryotes to plastids. *Genome Biol. Evol. 5*, 31−44.

(21) Becker, J. E., Moore, R. E., and Moore, B. S. (2004) Cloning, sequencing, and biochemical characterization of the nostocyclopeptide biosynthetic gene cluster: Molecular basis for imine macrocyclization. *Gene 325*, 35−42.

(22) Mann, F. M., Thomas, J. A., and Peters, R. J. (2011) Rv0989c encodes a novel (E)-geranyl diphosphate synthase facilitating decaprenyl diphosphate biosynthesis in *Mycobacterium tuberculosis*. *FEBS Lett. 585*, 549−554.

(23) Pojer, F., Wemakor, E., Kammerer, B., Chen, H., Walsh, C. T., Li, S. M., and Heide, L. (2003) CloQ, a prenyltransferase involved in clorobiocin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A. 100*, 2316−2321.

(24) Saleh, O., Gust, B., Boll, B., Fiedler, H. P., and Heide, L. (2009) Aromatic prenylation in phenazine biosynthesis: Dihydrophenazine-1-carboxylate dimethylallyltransferase from *Streptomyces anulatus*. *J. Biol. Chem. 284*, 14439−14447.

(25) Schultz, A. W., Lewis, C. A., Luzung, M. R., Baran, P. S., and Moore, B. S. (2010) Functional characterization of the cyclomarin/cyclomarazine prenyltransferase CymD directs the biosynthesis of unnatural cyclic peptides. *J. Nat. Prod. 73*, 373−377.

(26) Matthews, M. L., Neumann, C. S., Miles, L. A., Grove, T. L., Booker, S. J., Krebs, C., Walsh, C. T., and Bollinger, J. M., Jr. (2009) Substrate positioning controls the partition between halogenation and

hydroxylation in the aliphatic halogenase, SyrB2. *Proc. Natl. Acad. Sci. U.S.A. 106*, 17723−17728.
(27) Kulik, H. J., and Drennan, C. L. (2013) Substrate placement influences reactivity in non-heme Fe(II) halogenases and hydroxylases. *J. Biol. Chem. 288*, 11233−11241.
(28) Lee, J., Simurdiak, M., and Zhao, H. (2005) Reconstitution and characterization of aminopyrrolnitrin oxygenase, a Rieske N-oxygenase that catalyzes unusual arylamine oxidation. *J. Biol. Chem. 280*, 36719−36727.
(29) Sydor, P. K., Barry, S. M., Odulate, O. M., Barona-Gomez, F., Haynes, S. W., Corre, C., Song, L., and Challis, G. L. (2011) Regio- and stereodivergent antibiotic oxidative carbocyclizations catalysed by Rieske oxygenase-like enzymes. *Nat. Chem. 3*, 388−392.
(30) Wang, P., Gao, X., and Tang, Y. (2012) Complexity generation during natural product biosynthesis using redox enzymes. *Curr. Opin. Chem. Biol. 16*, 362−369.
(31) Walsh, C. T., and Wencewicz, T. A. (2013) Flavoenzymes: Versatile catalysts in biosynthetic pathways. *Nat. Prod. Rep. 30*, 175−200.
(32) Podust, L. M., and Sherman, D. H. (2012) Diversity of P450 enzymes in the biosynthesis of natural products. *Nat. Prod. Rep. 29*, 1251−1266.