

Genome-Based Characterization of Two Prenylation Steps in the Assembly of the Stephacidin and Notoamide Anticancer Agents in a Marine-Derived *Aspergillus* sp.

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Abstract: Stephacidin and notoamide natural products belong to a group of prenylated indole alkaloids containing a core bicyclo[2.2.2]diazaoctane ring system. These bioactive fungal secondary metabolites have a range of unusual structural and stereochemical features but their biosynthesis has remained uncharacterized. Herein, we report the first biosynthetic gene cluster for this class of fungal alkaloids based on whole genome sequencing of a marine-derived *Aspergillus* sp. Two central pathway enzymes catalyzing both normal and reverse prenyltransfer reactions were characterized in detail. Our results establish the early steps for creation of the prenylated indole alkaloid structure and suggest a scheme for the biosynthesis of stephacidin and notoamide metabolites. The work provides the first genetic and biochemical insights for understanding the structural diversity of this important family of fungal alkaloids.

Introduction

Structurally complex fungal-derived natural products account for a significant number of clinical therapeutics for treatment of human and animal diseases.¹ Due to emerging appreciation for the high level of biodiversity within this group of eukaryotes, an increasing number of natural products have been isolated from fungal sources and screened for bioactive secondary metabolites.² Recently, a family of fungal-derived prenylated alkaloids has attracted increasing interest for its remarkably diverse bioactivities including insecticidal, antitumor, anthelmintic, calmodulin inhibitory, and antibacterial properties, and intriguing structural features. These natural products are comprised of L-tryptophan, a second cyclic amino acid residue, and one or two isoprene units (Scheme 1).³ The isolation and characterization of two key biosynthetic intermediates, prepara-herquamide (**1**) and premalbrancheamide (**2**), in the biosynthesis

of the paraherquamides (**3**) and malbrancheamides (**4**),^{4,5} respectively, suggest that two amino acid residues are condensed to generate the *cyclo*-L-tryptophan-L-proline analog **5** or **6**. The tryptophanyl subunit of the dipeptide is subsequently prenylated in a reverse manner to generate compound **7** or **8** (Scheme 1). The bicyclo[2.2.2]diazaoctane core in **1** and **2** possibly arises from an intramolecular Diels–Alder (IMDA) reaction after oxidizing **7** or **8** to form a putative pyrazine-derived azadienophile. However, the detailed understanding of assembly and modification of these biosynthetic building blocks remain highly obscure.

Recently, a group of new prenylated indole alkaloids, the notoamides (A–E, **9–13**), were isolated from a marine-derived *Aspergillus* sp. (Figure 1a).^{6,7} Interestingly, stephacidin A (**14**) and deoxybrevianamide E (**15**) were purified from the same fungal strain, indicating the possible role of **15** as a common biosynthetic intermediate.⁷ In 2006, a bimodular nonribosomal peptide synthetase (NRPS) gene (*ftmA*) was mined from an *A. fumigatus* genome sequence, and its heterologous expression led to accumulation of the *cyclo*-L-tryptophan-L-proline product

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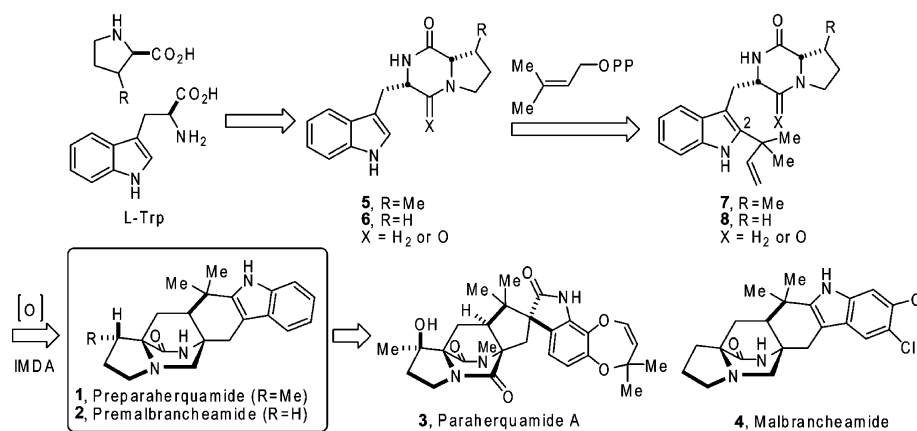
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Scheme 1. Biosynthetic Subunits and Putative Route to Paraherquamide (3) and Malbrancheamide (4)^a

^a Molecules in the boxes have been validated as the building blocks or biosynthetic intermediates based on precursor incorporation studies.

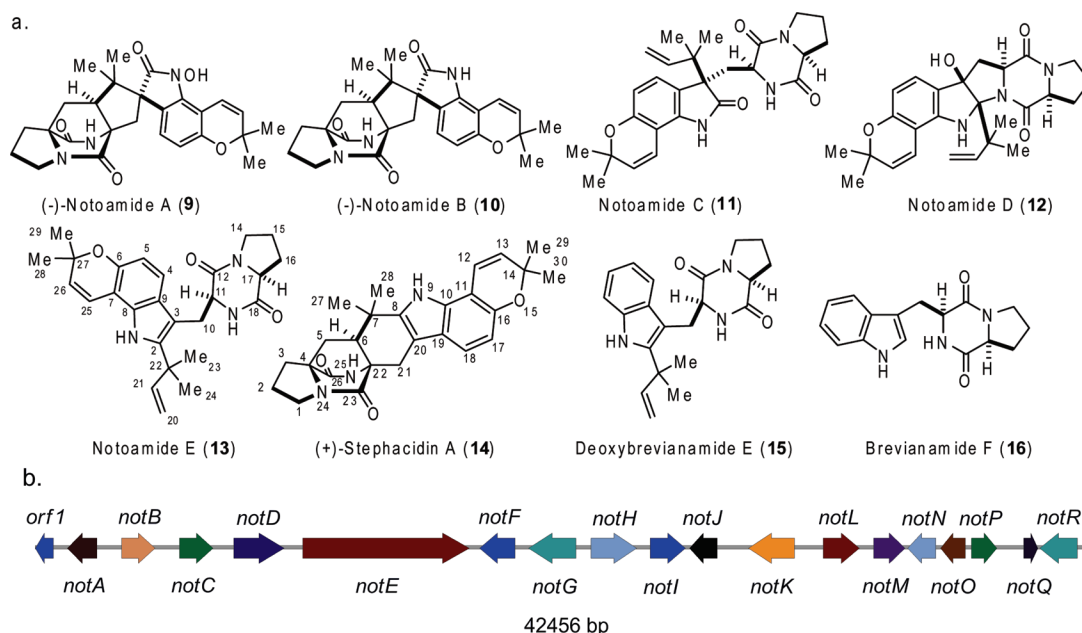


Figure 1. Genetic studies of fungal alkaloids produced in the marine-derived fungus *Aspergillus* sp. (a) Selected fungal alkaloids isolated from the marine-derived *Aspergillus* sp. Compound **16** was not reported in the fungal strain but was expected as the direct precursor of compound **15**. (b) The notoamide (*not*) biosynthetic gene cluster derived from complete sequencing and bioinformatic mining of *Aspergillus* sp. MF297-2 genome.

brevianamide F (**16**).⁸ We reasoned that an NRPS with a function coincident with FtmA would be expected for the notoamide biosynthetic pathway, where **16** in this marine-derived *Aspergillus* sp. is elaborated in an alternative manner compared to *A. fumigatus*⁹ that mediates biosynthesis of one ergot alkaloid, fumitremorgin. Herein, we report the identification of the first gene cluster for the biosynthesis of the stephacidin and notoamide family of prenylated alkaloids based on genome mining and biochemical analysis. These studies include a detailed characterization of the elusive deoxybrevianamide E synthase (e.g., reverse prenyl-transferase) as well as a second normal prenyltransferase that provide new insights into the assembly of the structurally and biologically diverse class of bicyclo[2.2.2]diazaoctane-derived natural products.

Results

Localization and Analysis of the Notoamide (*Not*) Gene Cluster from a Marine-Derived *Aspergillus* sp. through Genome Mining. The genome of the stephacidin- and notoamide-producing marine-derived *Aspergillus* sp. MF297-2 was sequenced to ~15 times coverage of the average published *Aspergillus* genome size (32.5 Mb) using Roche 454FLX technology (unpublished data). An open reading frame (*orf*) named *notE* (Figure 1b) was identified from the genome sequence using *fma* to probe for homologous genes.⁸ NotE is a presumed bimodular NRPS (Table 1) with adenylation (A)-thiolation (T)-condensation (C)-A-T-C domain organization and shares 47% amino acid sequence identity with FtmA (Table 1). In addition to *notE*, eighteen other genes were identified in a 42456-bp region of the chromosome encompassed by four overlapping genome assembly nodes (Figure 1b). At the left end of the gene cluster, the product of *orf1* was predicted to be the N-terminus of a capsule polysaccharide biosynthesis protein

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Table 1. Features of the *Not* Gene Products

Protein	Size bp/aa	Exon	Function	Relative (identity/similarity [%])	Accession number
Orf1	731/224	1–113, 173–731	partial polysaccharide synthase	capsule polysaccharide biosynthesis protein from <i>Aspergillus fumigatus</i> (43/63)	XP_748327
NotA	1199/339	1293–1568, 1643–1971, 2023–2179, 2234–2491	negative regulator	NmrA family protein from <i>Ajellomyces capsulatus</i> (45/64)	EEH03447
NotB	1344/401	3486–4079, 4141–4487, 4568–4829	FAD binding domain protein	FAD binding domain protein from <i>A. clavatus</i> (44/63)	XP_001268514
NotC	1350/427	5819–6996, 7066–7168	prenyl- transferase	FtmH from <i>A. fumigatus</i> (50/66)	BAH24002
NotD	2025/621	8012–8294, 8389–8927, 8996–10036	oxidoreductase	oxidoreductase from <i>Microsporium canis</i> (40/59)	EEQ33235
NotE	6723/2241	10787–17509	NRPS	FtmA from <i>A. fumigatus</i> (47/67)	XP_747187
NotF	1431/453	17924–18053, 18126– 19354	prenyl- transferase	tryptophan dimethylallyltransferase from <i>Coccidioides posadasii</i> (40/62)	EER24759
NotG	1901/544	19899–20086, 20171– 20272, 20337–20635, 20689–20810, 20879– 21799	P450	cytochrome P450 from <i>A. fumigatus</i> (62/75)	XP_747185
NotH	1836/502	22422–22668, 22734– 22822, 22897–22996, 23060–23128, 23187– 23765, 23836–24257	P450	cytochrome P450 from <i>Neosartorya fischeri</i> (47/65)	XP_001261652
NotI	1423/434	24803–24962, 25021– 25939, 26003–26225	FAD binding domain protein	FAD binding domain protein from <i>A. clavatus</i> (44/63)	XP_001268514
NotJ	1113/371	26390–27502	unknown	hypothetical protein from <i>Salinispora arenicola</i> (52/65)	YP_001537335
NotK	1851/564	28771–29141, 29196– 29569, 29620–30389, 30445–30621	efflux pump	MFS transporter from <i>N. fischeri</i> (87/93)	XP_001265322
NotL	1455/484	31789–33243	transcriptional activator	C6 zinc finger domain protein from <i>N. fischeri</i> (53/62)	XP_001265321
NotM	1266/402	33816–34597 34654–35080	unknown	hypothetical protein from <i>Talaromyces stipitatus</i> (74/82)	XP_002482929
NotN	1126/340	35192–35244, 35299– 35895, 35948–36317	dehydrogenase	alcohol dehydrogenase from <i>Penicillium marneffeii</i> (60/76)	XP_002147947
NotO	993/331	36520–37512	short-chain dehydrogenases/ reductase	hypothetical protein from <i>Nectria hematococca</i> (66/80)	EEU36425
NotP	1020/322	37770–37930, 37985–38789	unknown	metallo- β -lactamase domain protein from <i>T. stipitatus</i> (80/88)	XP_002482927
NotQ	569/152	39871–40059, 40120– 40316, 40370–40439	unknown	hypothetical protein from <i>T. stipitatus</i> (88/94)	XP_002482928
NotR	1517/461	40514–41140, 41212– 41727, 41791–42030	transcriptional coactivator	hypothetical protein from <i>P. marneffeii</i> (45/61)	XP_002144868

involved in a primary metabolic pathway. At the right end of the gene cluster, a protein encoded by *notR* showed 38% sequence identity to the AflJ aflatoxin pathway transcriptional coactivator.¹⁰ Bioinformatic analysis indicated that NotB and NotI show high similarity to FAD-dependent monooxygenases while NotD is a presumed flavin-dependent oxidoreductase. NotG and NotH show high sequence similarity to fungal CYP450s, both of which might be involved in the formation of the isoprene-derived pyran ring (Scheme 2). Furthermore, NotN and NotO are predicted to function as a dehydrogenase and a short-chain dehydrogenase/reductase, respectively. The *notK* gene encodes a putative efflux pump, which might specify excretion of alkaloid products from the cell. As with NotR, NotL shares high protein sequence similarity to AflR while NotA is a predicted biosynthetic pathway transcriptional repressor.¹¹ These regulators offer opportunities to understand the notoamide pathway gene expression, and the potential to manipulate fungal

alkaloid production in this unique marine-derived *Aspergillus* sp.¹² NotC and NotF, two predicted aromatic prenyltransferases, presumably catalyze the two key prenylation reactions including a first reverse prenyltransfer step leading to **15**. NotC shows a 50% sequence identity to FtmH (also called FtmPT2) in *A. fumigatus* while NotF shows the highest identity (40%) to a putative dimethylallyl tryptophan synthase (EER24759) in *Coccidioides posadasii*.¹³ However, the putative functions of products encoded by *notJ*, *notM*, *notP*, and *notQ* remain unknown based on bioinformatics analysis.

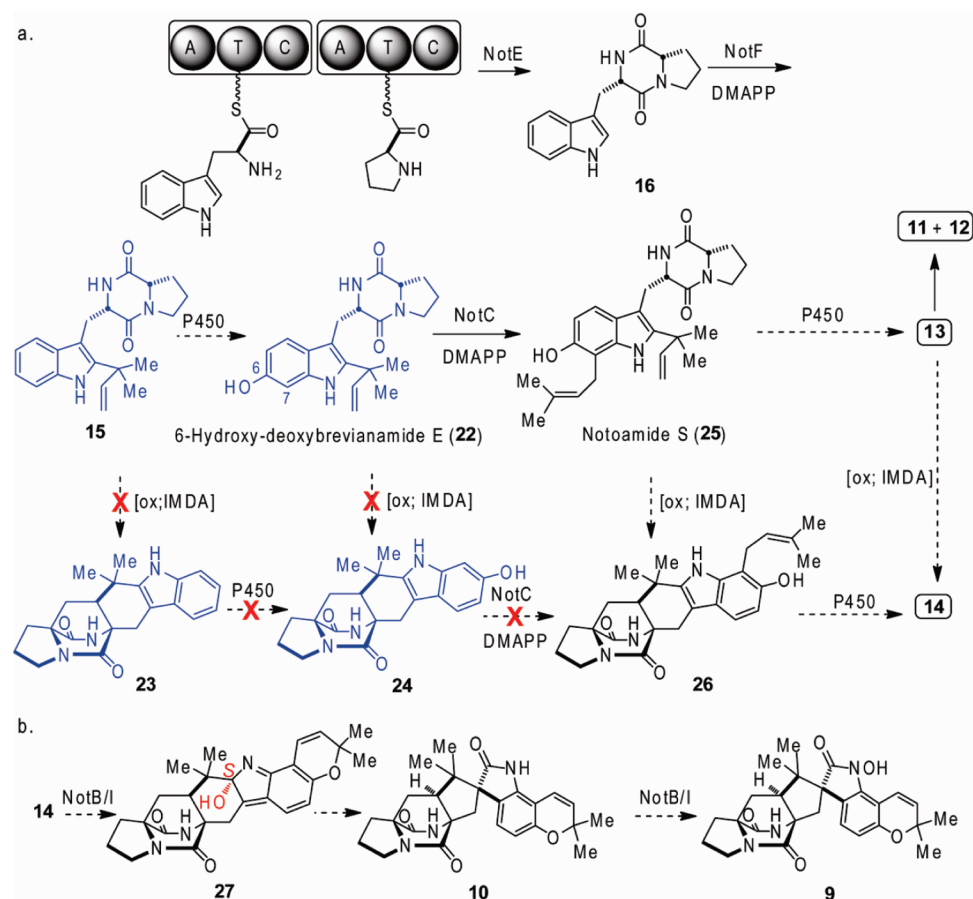
Determination of NotF as the Deoxybrevisanamide E Synthase. We first examined the role of NotF in notoamide biosynthesis. Its cDNA was prepared by removing the 72-bp intron using an overlapping PCR strategy (Supplementary Table 1). The recombinant enzyme was purified with Ni-NTA resin to about 90% purity, and its native protein status was determined as an oligomer with an observed molecular weight of 292 kDa

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Scheme 2. The Putative Biosynthetic Pathway for Stephacidin A (**14**) and the Notoamide Natural Products^a

^a (a) The early stages in the stephacidin and notoamide biosynthesis. **25** serves as the common precursor to **13** and **14**. **13** is then converted into **11** and **12**. Substrates used in NotC studies were labeled in blue. (b) Notoamide A (**9**) and B (**10**) are possibly derived from **14**. The solid arrows represent reactions that have been confirmed with bioinformatic analysis, biochemical analysis, or precursor incorporation experiments, while the dashed arrows indicate proposed biosynthetic steps. The red symbol X indicates the reaction is not supported by the current study.

(53.6 kDa as the calculated monomeric size) by gel filtration (Supplementary Figure 1).

Next, the function of NotF was tested with doubly ¹³C-labeled brevianamide F (**17**, Supplementary Figure 2). The product **18** exhibited the same retention time (17.38 min) as authentic **15** but its [M+H]⁺ ion was 354.19, bearing the expected 2.00-Da shift from [15+H]⁺ (352.19) (Figure 2a). In MS² analysis, **18** was fragmented in the same manner as **15** and the *m/z* differences (1 or 2 Da) of three major fragments (*m/z* values at 199.14, 286.17, and 298.14 for **18**) in two MS² spectra originated from the two ¹³C atoms in **17** (Supplementary Figure 3). These results demonstrate that NotF is the deoxybrevianamide E (**15**) synthase and catalyzes the key reverse prenylation at C-2 of the indole ring leading to the bicyclo[2.2.2]diazaoctane core during biosynthesis of many fungal alkaloids within this family. In contrast, **16** in *A. fumigatus* is ultimately converted to fumitremorgin following normal prenylation at C-2 by FtmB.⁹

As a next step, the substrate selectivity of NotF was investigated with L-Trp, **17**, cyclo-(L-Phe-L-Pro) **19**, cyclo-(L-Trp-L-Trp) **20**, and cyclo-(L-Trp-L-Tyr) **21** (Supplementary Figure 2). No prenylated products for any of these unnatural substrates were detected by LC-MS analysis. This result provides strong evidence for the early timing and high selectivity of the NotF-catalyzed reaction in the alkaloid biosynthetic pathway (Scheme 2a). Moreover, the structural similarities among **16**, **19**, **20**, and **21** suggested that both amino acid residues in **16**

are critical for selective interactions between the substrate and the NotF reverse prenyltransferase.

Determination of NotC as the 6-Hydroxy-7-prenyl-deoxybrevianamide E Synthase. The role of NotC, the second predicted prenyltransferase from the marine-derived *Aspergillus* sp. MF297–2, was also investigated. Its cDNA was similarly generated by an overlapping PCR strategy and was expressed in *E. coli* (Supplementary Table 1). The recombinant protein was purified with a single Ni-NTA affinity column, and its native protein status was determined as a monomer with an observed molecular weight of 61 kDa (51.1 kDa as the calculated monomeric size) by gel filtration (Supplementary Figure 1).

Stephacidin A (**14**) is a central advanced intermediate featuring a pyran ring but lacking a spiroindole implicating the mode of assembly and timing of the bicyclo[2.2.2]diazaoctane family of fungal alkaloids.^{7,14} We propose that **14** is produced from deoxybrevianamide E (**15**) in a series of reactions, including hydroxylation at C-6 (following **13** numbering system), followed by normal prenylation at C-7, oxidation of the dioxopiperazine ring, IMDA and ring closure to the pyran (Scheme 2a). This hypothesis and the order of these reactions were examined by the determination of NotC activity with four

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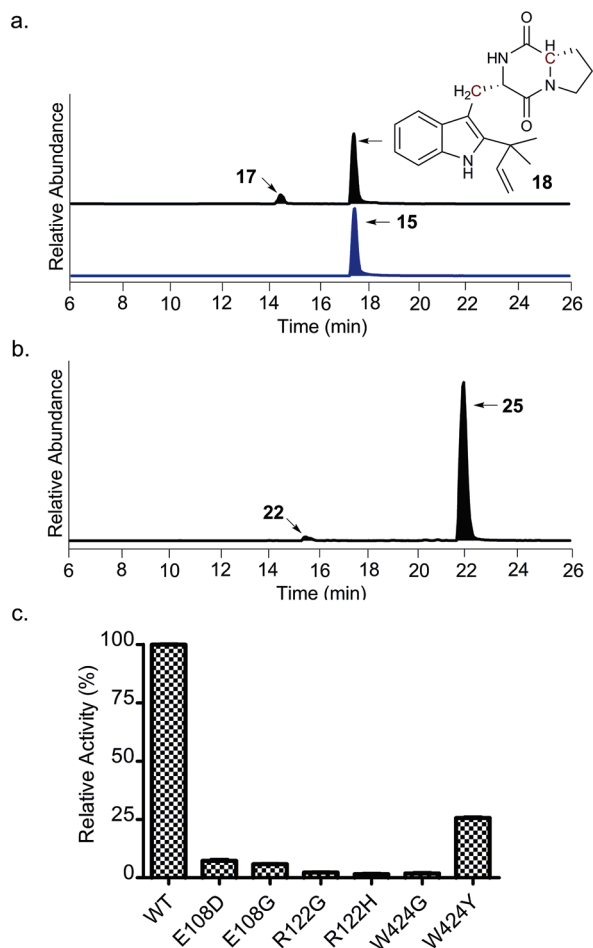


Figure 2. Determination of NotF and NotC prenyltransferase activities. (a) Identification of NotF product (**18**) by LC-MS/MS analysis as described in Methods. (b) Identification of NotC reaction product (**25**) by LC-MS/MS analysis as described in Methods. The product (**25**) was further characterized by ^1H and ^{13}C NMR analysis. (c) Investigation of key residues in the reaction of the reverse prenyltransferase NotF by site-directed mutagenesis. Data shown are means $\pm$ s.d. from two independent experiments.

structurally related putative substrates, **15**, **22**, **23**, and **24** (Scheme 2a). Compounds **22** and **24** were synthesized according to Supplementary Schemes 1 and Scheme 2, respectively. NotC showed high selectivity toward 6-hydroxy-deoxybrevianamide E (**22**) with three additional substrates failing to be converted to products by the enzyme (Figure 2b). The m/z value of the product was 436.17, the same as that of the singly prenylated **22** (calculated MW of 435.25), in MS analysis. Also, three major fragment ions in the MS² spectrum of the enzyme product exhibited m/z values at 280.20, 368.17, and 380.22 (Supplementary Figure 4). The difference between these and the major ions in MS² spectrum of **15** is 84-Da, which is consistent with the MW sum of one oxygen atom and one isoprene unit linked to the indole ring of **15**. We also chemically synthesized notoamide S (6-hydroxy-7-prenyl-deoxybrevianamide E, **25**)¹⁵ as the authentic standard and compared the authentic substance with the NotC product by LC and ^1H NMR analyses. Both compounds exhibit the same LC retention time (Supplementary Figure 5) and displayed identical ^1H NMR spectra (Supplementary Figure 6). The NotC product was further confirmed to

be notoamide S (**25**)¹⁵ by comparison with **13** (Supplementary Table 2). Chemical shifts of ^1H and ^{13}C NMR spectra between the product and **13** were essentially identical with the exception of C-25 and C-27.⁶ The significant differences at these two positions reflect the double bond position in the attached isoprene unit and also indicate that the pyran is not formed in **25**. These results demonstrated that NotC catalyzes a normal prenyltransfer reaction at C-7 of the indole aromatic ring system in **22**. Moreover, analysis of NotC substrate selectivity suggests that the biosynthetic pathway of **13** and **14** might not proceed through substrates **23** or **24** (Scheme 2a). Instead, **15** is first hydroxylated at C-6 of the indole ring, and the product **22** is subsequently prenylated at C-7 by NotC to generate **25**, a biosynthetic precursor of both **13** and **14**. Accordingly, notoamide J was isolated from the culture of this marine-derive fungus, and contains only one C-6 (following **13** numbering system) hydroxy group.¹⁶ The precise path from **25** to **14** requires further investigation, although we have demonstrated that stephacidin A (**14**) does not arise directly from notoamide E (**13**), suggesting that generation of the pyran follows formation of the bicyclo[2.2.2]diazaoctane core.⁶

Biochemical Characterization of NotF and NotC. Both NotF and NotC tolerated a broad range of temperature (4 to 42 °C for NotF while 16 to 42 °C for NotC) and pH (6.0 to 9.0) (Supplementary Figure 7). Enzyme activity was independent of divalent cation, although addition of 5 mM Mg^{2+} , Ca^{2+} or Mn^{2+} slightly enhanced catalysis (about 100–120%) (Supplementary Figure 8). Significant reduction of enzyme activity (2%–35%) was observed with Cu^{2+} , Zn^{2+} , Fe^{2+} , or Sn^{2+} (5 mM). Unlike previous observations with a normal aromatic prenyltransferase (MaPT)¹⁷ and CloQ,¹⁸ EDTA caused only minor effects on NotF and NotC activity (remaining 90–95%), possibly indicating that the active-site pocket of both prenyltransferases might be less exposed to solvent. However, the exact mechanism for nonchelating inhibition of this group of metal-independent enzymes by EDTA remains unclear.

The reactions of both NotF and NotC followed Michaelis–Menten kinetics (Supplementary Figure 9). The K_m and $V_{\max}$ values for doubly ^{13}C -labeled brevianamide F (**17**) in the NotF reactions were $4.33 \pm 0.43 \mu\text{M}$ and $0.89 \pm 0.02 \mu\text{M}/\text{min}$, respectively, giving a maximal turnover value of $19.1 \pm 0.4 \text{ min}^{-1}$. Similarly, the enzyme K_m and $V_{\max}$ values for dimethylallyl diphosphate (DMAPP) were $1.31 \pm 0.22 \mu\text{M}$ and $1.18 \pm 0.03 \mu\text{M}/\text{min}$, respectively. Its maximal turnover value ($25.3 \pm 0.6 \text{ min}^{-1}$) and enzyme catalytic efficiency value ($19.31 \mu\text{M}^{-1} \cdot \text{min}^{-1}$) were slightly higher than those of **17** ($k_{\text{cat}}/K_m = 4.41 \mu\text{M}^{-1} \cdot \text{min}^{-1}$). Compared to FtmB using **16** as a substrate in a normal prenylation step ($K_m = 55 \mu\text{M}$, $k_{\text{cat}}/K_m = 6.08 \mu\text{M}^{-1} \cdot \text{min}^{-1}$), NotF showed more restricted substrate selectivity and higher substrate binding affinity with a similar enzyme catalytic efficiency.¹⁹ In addition, we also determined the kinetic parameters for the NotC normal prenyltransferase. The K_m and $V_{\max}$ values for **22** in the NotC reactions were $2.64 \pm 0.33 \mu\text{M}$ and $1.30 \pm 0.04 \mu\text{M}/\text{min}$, respectively, while for DMAPP these values were determined to be $1.89 \pm 0.20 \mu\text{M}$ and $1.45 \pm 0.03 \mu\text{M}/\text{min}$, respectively. Similar to NotF, the maximal turnover

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value ($67.4 \pm 1.4 \text{ min}^{-1}$) and enzyme catalytic efficiency value ($35.66 \mu\text{M}^{-1} \cdot \text{min}^{-1}$) of NotC were also slightly higher than those of **22** ($60.5 \pm 1.9 \text{ min}^{-1}$, $22.92 \mu\text{M}^{-1} \cdot \text{min}^{-1}$, respectively).

In this study, three key amino acid residues located in the predicted NotF reaction pocket were mutated to probe the reaction mechanism of this reverse aromatic prenyltransferase (Supplementary Figure 10). R108 was predicted to be a substitute for the divalent metal ion and to interact with the DMAPP pyrophosphate group. Generation of the R108H and R108G mutants (Supplementary Figure 1a) resulted in proteins with less than 2% catalytic activity, confirming the vital role of R108 for effective catalytic function (Figure 2c). Another highly conserved key residue for substrate binding in NotF is E108, which may form a H-bond with N–H in the **16** indole ring system.²⁰ Both E108D and E108G mutants lost at least 92% of their activity (Figure 2c). This result suggests that a specific side chain length in this acidic amino acid residue is important for H-bond formation. Recently, the crystal structure of FgaPT2, a normal fungal aromatic prenyltransferase, revealed the presence of a defined network consisting of five Tyr residues to prevent the DMAPP-derived carbocation from reacting adventitiously with nucleophiles.²⁰ All of these residues are conserved in NotF except Y413, which is replaced with W424 (Supplementary Figure 10). Although W424Y still retained about 25% of its activity for production of **18**, the corresponding W424G lesion resulted in >98% loss of catalytic activity, validating the importance of this analogous aromatic network in the notoamide reverse prenyltransferase reaction (Figure 2c).

Proposed Biosynthetic Pathway for the Stephacidin and Notoamide Biosynthesis in Marine-Derived *Aspergillus* sp. MF297-2. Based on the biochemical characterization of two prenyltransferases and the predicted biochemical function of related gene products in the isolated gene cluster, a putative notoamide biosynthetic pathway is proposed (Scheme 2). Briefly, we presume that **16** is produced from L-Trp and L-Pro by the NotE NRPS and is subsequently reverse prenylated at C-2 by NotF to produce **15**. In the next step, the indole ring is hydroxylated at C-6, which is likely catalyzed by one of the two *not* pathway-encoded P450s. NotC is then responsible for normal prenylation at C-7 position of **22** to produce **25**. From this intermediate, notoamide E (**13**) is generated following ring closure to the pyran, a process possibly controlled by the second P450, and then may be converted into notoamide C (**11**) and notoamide D (**12**).⁶ The direct connection from **13** to **14** was not observed in double ¹³C-labeled precursor incorporation experiments, whereby **13** was previously envisioned to be converted by an oxidase to the pyrazine-derived dienophile followed by IMDA to directly produce stephacidin A (**14**) (Scheme 2a).⁶ The enzyme(s) that promote bicyclo[2.2.2]diazoctane formation remain unknown. Recently, a flavin-dependent oxidase in solanapyrone biosynthesis was shown to catalyze both oxidation and subsequent Diels–Alder cycloaddition reactions,²¹ indicating that the predicted oxidoreductases (e.g., NotB, NotD, and NotI) are possible candidates for catalyzing the IMDA reaction in the notoamide biosynthesis. Alternative to the pathway through **13**, **14** may be produced from **25** after the stepwise oxidation, IMDA, and ring closure (Scheme 2a). We propose that **14** is

regiospecifically hydroxylated by a monooxygenase, possibly NotB or NotI, to give **27** for subsequent pinacol-like rearrangement to produce notoamide B (**10**). Such an intriguing rearrangement reaction has not been previously observed in a natural product biosynthetic pathway, and a putative mechanism is proposed (Supplementary Figure 11). A rare *N*-hydroxylation reaction is required to generate the final notoamide A product (**9**) (Scheme 2b). The biosynthetic scheme proposed here provides an initial understanding of the assembly and modification of biosynthetic building blocks for this important group of bioactive prenylated fungal alkaloids.

Discussion

The advent of next-generation sequencing has provided tremendous opportunities to identify novel natural products and their biosynthetic pathways through genome mining of bacterial, fungal,²² and plant²³ genomes. This approach has proved increasingly important in current natural product biosynthesis studies with the availability of increasing numbers of microbial genome sequences. For example, many orphan biosynthetic gene clusters were identified from the genome sequence of well-studied *Streptomyces coelicolor* A3(2), and novel natural products such as 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids were uncovered with the guidance of genome mining.^{24,25} In this study, we employed an allied approach to study the biosynthesis of prenylated indole alkaloids and also to initiate an understanding of fungal genetic evolution and adaptation to different environmental niches. The entire genome of the notoamide-producing marine-derived *Aspergillus* sp. was sequenced at ~15X coverage, enabling the *not* gene cluster identification through sequence file database comparison to *fmaA* (Figure 1). With this gene cluster it is now possible to pursue studies toward a complete understanding of the assembly, tailoring, and regulation of this family of bioactive fungal alkaloids and to further develop them as medicinal agents.

Fungal aromatic prenyltransferases have attracted increasing interest because of their important roles in the biosynthesis of natural products and potential applications in drug development. Currently, more than 100 putative indole prenyltransferases have been revealed by BLAST searches in the public genome database.²⁶ Biochemical investigations of over 10 recombinant enzymes in this group finds that catalytic functions are independent of divalent metal ions.^{26,27} In these reactions, an isoprene unit can be transferred onto different positions of the indole ring system.²⁷ Moreover, in a normal prenylation reaction, DMAPP alkylates an aromatic substrate through its C1' atom via an S_N2 displacement, while the C3' position is involved in the reverse prenyltransfer reaction via an S_N2' displacement. Remarkably, these enzymes can utilize a series of structurally similar analogs as their aromatic substrates.^{17,27,28} In contrast to other characterized enzymes in this group, NotF (reverse) and NotC (normal) prenyltransferases showed highly restricted substrate specificities. NotF specifically prenylates **16** (at C-2),

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confirming its role as the elusive deoxybrevianamide E synthase, while only **22** is utilized by NotC (C-7 alkylation) in the biosynthesis of the stephacidin and notoamides (Figure 2). The mechanism of the reverse prenyltransferase was also probed by site-directed mutagenesis to understand the reaction of this group of enzymes.^{17,20} Future structural studies of NotF and the comparison to FgaPT2²⁰ are expected to contribute further information about the regio- and stereospecificity of the reverse and normal prenyltransfer reactions. We expect this analysis will likely illuminate the lack of facial selectivity previously observed for the reverse prenylation step²⁹ and facilitate expansion of the enzyme substrate range and efficiency. More importantly, the combined studies of NotF and NotC provide direct evidence to establish a biosynthetic scheme for this family of bioactive prenylated fungal alkaloids. Finally, the high *in vitro* catalytic efficiencies of recombinant NotF and NotC suggest their potential value as biocatalysts for chemoenzymatic production of bioactive fungal alkaloid analogs in drug development.

Identification and characterization of the notoamide gene cluster also provides the initial basis to understand the formation of three pairs of antipodal natural products derived from a marine-derived and a terrestrial *Aspergillus* sp.^{30,31} In the marine-derived fungal strain, (–)-notoamide B (**10**), (–)-versicolamide B (**38**), and (+)-stephacidin A (**14**) are produced,^{7,30} while their antipodal counterparts, **39**, **40**, and **41**, respectively, are isolated from the terrestrial *A. versicolor* NRRL 25660 strain (Supplementary Figure 12).³¹ Based on the putative notoamide biosynthetic pathway, we propose that formation of **14** and **41** might be controlled by the IMDA reaction. Subsequently, **10** and **39** are possibly derived from **14** and **41**, respectively, in these two distinct fungal strains. It remains unclear whether generation of (–)- and (+)-versicolamide B (**38** and **40**) occurs in the pathway through stephacidin A due to their opposite stereogenic centers at C-6 (Supplementary Figure 12).³⁰ Instead, **13** might be converted into **11**, subsequently producing **38** in the following IMDA reaction in the marine-derived fungus.³⁰ The detailed biochemical characterization of biosynthetic enzymes from both fungal strains is in progress and will shed more light on the biosynthesis of these unique antipodal natural products.

Identification of biocatalysts from fungal alkaloid biosynthetic pathways may also enable production of natural products and their analogs through heterologous expression and metabolic engineering.^{32–34} It is estimated that >99% of microorganisms in the environment fail to grow in the laboratory, and the potential to find pharmaceutically important natural products from fungal sources remains vastly underexplored. Introducing natural product gene clusters into more technically and industrially amenable microorganisms such as *E. coli* and yeast represents an attractive way to obtain suitable quantities of natural products and to identify novel leads in drug discovery and development programs.³⁴ Moreover, a microorganism can

be further optimized for the efficient production of a target metabolite using traditional mutation and selection methods, as well as new tools from systems biology and synthetic biology.³⁵ Identification of the notoamide gene cluster provides such an opportunity to produce bioactive fungal alkaloids and analogs thereof through pathway engineering and heterologous expression.

Methods

Materials and Strains. Authentic deoxybrevianamide E (**15**), doubly ¹³C-labeled brevianamide F (**17**), and keto-premalbrancheamide (**23**) were synthesized following previously published procedures.^{4,36} Standard methods for DNA isolation and manipulation were performed as described by Sambrook et al.³⁷ Genomic DNA from *Aspergillus* MF297-2 was isolated with a MasterPure Yeast DNA Purification kit (Epicenter Biotechnologies) as described in the manual. The GenBank accession numbers for *notC*, *notF*, and the complete assembled *not* gene cluster are GU564534, GU564535, and HM622670, respectively. *E. coli* DH5 α was used for cloning and plasmid harvesting while *E. coli* BL21 CodonPlus-(DE3)-RIPL was used for protein overexpression.

Expression and Purification of NotC and NotF. Details about the preparation of *notC* and *notF* cDNAs and of *notF* mutant DNAs are included in the Supporting Information and Supplementary Table 1. The expressed enzymes were purified with a single NNTA column (Supporting Information). As determined by SDS-PAGE analysis, the purity of proteins was more than 90%. The native status of proteins was determined by gel filtration (Supplementary Figure 1).

Determination of Enzyme Activities. Compounds **22** and **24** were chemically synthesized to examine NotC activity (Supporting Information). The 100- μ L reaction mixture contained 0.5 μ g of NotF, its mutants, or NotC; 5 mM MgCl₂; 0.1 mM **17** (NotF or its mutants) or **22** (NotC); and 0.15 mM DMAPP in the reaction buffer (50 mM Tris-Cl, pH 7.5, 10% glycerol, and 3 mM β -mercaptoethanol). The reaction was initiated by adding enzyme after prewarming the other components at room temperature for 1 min. After mixing well and briefly centrifuging, the reactions were further incubated at room temperature for 45–60 min and stopped with 10 μ L of 1.5 M trichloroacetic acid. The mixtures were mixed and centrifuged at 13 000g for 5 min. An aliquot of the 100- μ L solution was subjected to HPLC coupled with an XBridge C18 column (5 μ m, 4.6 mm $\times$ 250 mm), at a wavelength of 222 nm. Solvent B (acetonitrile in 0.1% TFA) was increased from 30% to 40% for 5 min and then increased to 80% over 20 min for the detection of products. LC-MS² analysis was performed by using a ThermoFinnigan LTQ linear ion-trap instrument equipped with an electrospray source and a Surveyor HPLC system at room temperature. Separations were performed with an XBridge C18 (3.5 μ m, 2.1 mm $\times$ 150 mm) column at a flow rate of 200 μ L/min with solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). Solvent B was kept at 2% in solvent A for 4 min and then was gradually increased to 90% over 16 min. After being washed with 90% solvent B for 2 min, the column was further re-equilibrated with 2% solvent B for 10 min. The spectra were recorded in positive ion mode. Product **25** was further characterized with ¹H and ¹³C NMR analysis (Supporting Information).

Kinetics Analysis. The 100- μ L reaction mixture contained 0.25 μ g of NotF or 0.11 μ g of NotC and 5 mM MgCl₂ in the reaction buffer. Details about the experiment procedures were included in the Supporting Information. All experiments were performed in duplicate. The data were fit to the Michaelis–Menten equation in Prism 4.0 (GraphPad Software).

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Supporting Information Available: Remaining methods, including genome sequencing and assembly, assay for enzyme

metal independence, detailed synthetic procedures of **22** and **24**, determination of enzyme optimal conditions, characterization of **25**, antipodal fungal prenylated alkaloids, and the complete list of authors in ref 25. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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