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# Genetic Manipulation of the Pneumocandin Biosynthetic Pathway for Generation of Analogues and Evaluation of Their Antifungal Activity

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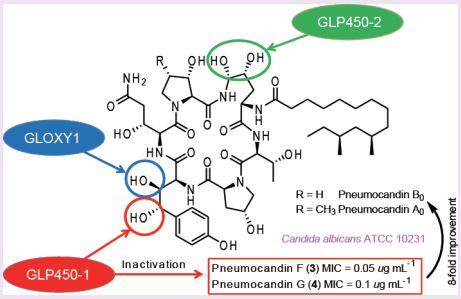
Supporting Information

**ABSTRACT:** Pneumocandins are lipohexapeptides of the echinocandin family that potently interrupt fungal cell wall biogenesis by noncompetitive inhibition of 1,3-$\beta$-glucan synthase. The pneumocandin biosynthetic gene cluster was previously elucidated by whole genome sequencing. In addition to the core nonribosomal peptide synthetase and polyketide synthase (*GLNRPS4* and *GLPKS4*), the pneumocandin biosynthetic cluster includes two P450-type hemeprotein monooxygenase genes (*GLP450-1* and *GLP450-2*) and four nonheme mononuclear iron oxygenase genes (*GLOXY1*, *GLOXY2*, *GLOXY3*, and *GLOXY4*), which function to biosynthesize and create the unusual sequence of hydroxylated amino acids of the mature pneumocandin peptide. Insertional inactivation of three of these genes (*GLP450-1*, *GLP450-2*, and *GLOXY1*) generated 13 different pneumocandin analogues that lack one, two, three, or four hydroxyl groups on 4*R*,5*R*-dihydroxy-ornithine and 3*S*,4*S*-dihydroxy-homotyrosine of the parent hexapeptide. Among them, seven analogues are previously unreported genetically engineered pneumocandins whose structures were established by NMR experiments. These new pneumocandins afforded a unique opportunity for side-by-side exploration of the effects of hydroxylation on pneumocandin antifungal activity. All of these cyclic lipopeptides showed potent antifungal activities, and two new metabolites pneumocandins F (**3**) and G (**4**) were more potent *in vitro* against *Candida* species and *Aspergillus fumigatus* than the principal fermentation products, pneumocandins $A_0$ and $B_0$.




Deliberate altering of the genetic makeup of an organism has become a powerful tool for the generation of novel analogues of bioactive natural products. Gene inactivation in the microbial producer of medicinally relevant secondary metabolites can significantly change the organism's metabolic profile. For example, gene deletions or insertions can result in the inactivation of biosynthetic genes for a given natural product and omission of the corresponding catalytic step from the biosynthetic pathway, often leading to new natural products. Therefore, the rapidly expanding toolkit for biosynthetic components and metabolic engineering offer powerful methods to improve natural product titers or produce novel natural products derivatives.[1,2]

Pneumocandins are lipohexapeptides of the echinocandin family and potently interrupt fungal cell wall formation by noncompetitive inhibition of 1,3-$\beta$-glucan synthase.[3−5] The most prevalent pneumocandin in fermentations of wild-type (wt) *Glarea lozoyensis* is pneumocandin $A_0$ (**2**; Figure 1A), and it was the first member pneumocandin to be isolated and structurally elucidated.[6,7] Subsequently other members of the family were isolated, including pneumocandin $B_0$ (**1**; Figure 1A), which was chosen as the starting point for semisynthesis of the first echinocandin-type antifungal drug, caspofungin acetate (Cancidas).[8,9] Structurally, pneumocandins are composed of a 10*R*,12*S*-dimethylmyristoyl side chain and a hexapeptide core which are cooperatively assembled by a polyketide synthase (PKS) and a nonribosomal peptide synthetase (NRPS). To date, several semisynthetic and total synthetic studies have explored structure−activity relationships (SAR) in pneumocandin derivatives,[10−15] but few studies have exploited biosynthetic methods to produce new analogues with the objective of improving the antifungal spectrum and potency.

In a previous study,[16] we identified the pneumocandin biosynthetic gene cluster in *Glarea lozoyensis* ATCC 20868 by whole genome bioinformatic analysis and by gene homology comparison to the recently characterized echinocandin B gene cluster from *Aspergillus rugulosus* (Figure 1B).[17,18] In addition to the core genes *GLPKS4* and *GLNRPS4*, the pneumocandin biosynthetic gene cluster also includes two P450-type hemeprotein monooxygenase genes (*GLP450-1* and *GLP450-2*) and four nonheme mononuclear iron oxygenase genes

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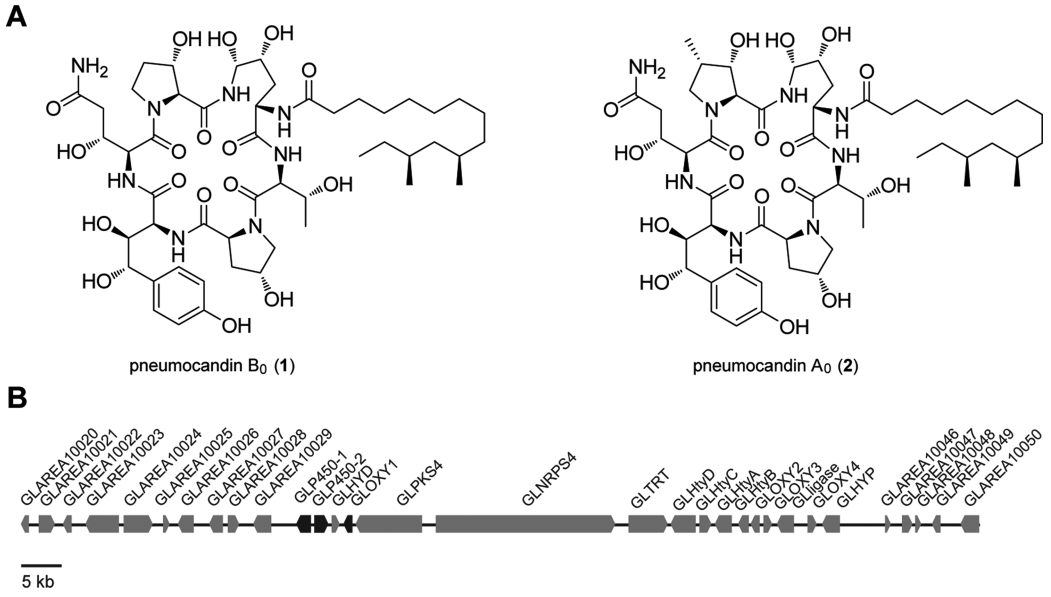


**Figure 1.** Structures of pneumocandins $B_0$ (**1**) and $A_0$ (**2**) and their biosynthetic gene cluster. (A) Pneumocandins $B_0$ (**1**) and $A_0$ (**2**). (B) Genetic organization of the pneumocandin gene cluster. Gene replacement mutants were generated in the genes indicated in black.

(*GLOXY1*, *GLOXY2*, *GLOXY3*, and *GLOXY4*). Cytochrome P450 enzymes are involved in diverse oxidizing reactions, resulting in hydroxylation, epoxidation, dealkylation, and sulfoxidation in the fungi.[19] These genes may function to generate a complex of tailoring enzymes that are responsible for multiple oxidation steps leading to pneumocandin's hydroxylated amino acids. Previous SAR studies have observed that the absence of some hydroxyl groups on the peptide core significantly affected antifungal potency of the pneumocandins.[10,20−22] The genes of the echinocandin and pneumocandin pathways appear to have evolved from a common ancestor, yet some of the pathways' gene content and specificity of individual enzymes can vary significantly, leading to different amino acid compositions, side chain lengths and branching, and variations in relative abundances of major biosynthetic products.[23] Although some of the hydroxylation steps for the pneumocandin core amino acids can be inferred by their homology to the enzymes of echinocandin B biosynthesis (Supporting Information Table S1),[18] their function and impact on product profile need experimental confirmation.

Given the availability of the pneumocandin gene cluster sequence,[16,24] we wanted to understand how pneumocandin biosynthesis takes place. We also asked whether genetic manipulation methods could generate new pneumocandin analogues with improved antifungal potency. Here, we report on the identification and functional characterization of hydroxylation steps in pneumocandin biosynthesis in *G. lozoyensis* by insertional inactivation of three different genes, cultivation of insertional mutants blocked at those genes, and subsequent chemical analysis of their newly occurring products. The analysis also provides an explanation for the incomplete biosynthetic reactions that were likely responsible for the origin of some of the minor pneumocandin analogues previously obtained from pilot plant fermentations of mutant and wt strains during the development of Cancidas at Merck.[8,9,25−27] In total, 13 des-hydroxylated forms of pneumocandin analogues, including seven new congeners, were isolated, and their structures were elucidated by 1D and 2D NMR experiments. These newly biosynthesized cyclic lipopeptides exhibited a range of potent antifungal activities, and two new metabolites pneumocandins F (**3**) and G (**4**) were more potent *in vitro* against *Candida* species and *Aspergillus fumigatus* than pneumocandins $A_0$ (**2**) and $B_0$ (**1**).

## RESULTS AND DISCUSSION

**Inactivation of Two Cytochrome P450 Genes Generates Analogues with 3*S*-Hydroxy-homoTyr and Non-hydroxylated Orn.** Two cytochrome P450 enzyme encoding genes, *GLP450-1* and *GLP450-2*, are located upstream of *GLPKS4* and *GLNRPS4* in the pneumocandin biosynthetic gene cluster (Figure 1B). Because five of the six amino acid residues in the pneumocandin hexapeptide are hydroxylated, we predicted that the two P450 enzymes were responsible for amino acid hydroxylations. We therefore constructed two insertional mutant strains for *GLP450-1* and *GLP450-2* by an *Agrobacterium tumefaciens*-mediated transformation (AMT) method previously developed for *G. lozoyensis*.[16] Genes *GLP450-1* and *GLP450-2* were inactivated by inserting a hygromycin resistance gene into their coding regions. The hygromycin-resistant clones were rescued from the medium, and mutants with the desired disruption of the target gene were verified by PCR analysis (Supporting Information Figures S1 and S2). Fermentation extracts from the wt and two positive mutants were subsequently analyzed by LC−MS (Figure 2A and B).

LC−MS analysis of the *GLP450-1* inactivation mutant showed that production of pneumocandins $A_0$ (**2**, *m*/*z* calcd for $C_{51}H_{82}N_8O_{17}$ $[M + H]^+$ 1079.5871, found 1079.5863) and $B_0$ (**1**, *m*/*z* calcd for $C_{50}H_{80}N_8O_{17}$ $[M + H]^+$ 1065.5714, found 1065.5698) were abolished. Instead, these fermentations yielded two new derivatives, pneumocandin F (**3**) and G (**4**) (Figure 2A and B). Compounds **3** and **4** (Figure 2C) were purified from the scaled-up fermentation (4 L) of the *GLP450-1* mutant, and their structures were subsequently elucidated by 1D and 2D NMR experiments. Pneumocandin F (**3**) was assigned the elemental composition of $C_{50}H_{80}N_8O_{16}$ by HRESIMS analysis (*m*/*z* = 1049.5765 $[M + H]^+$, found 1049.5761), which was 16 mass units less than **1**, corresponding


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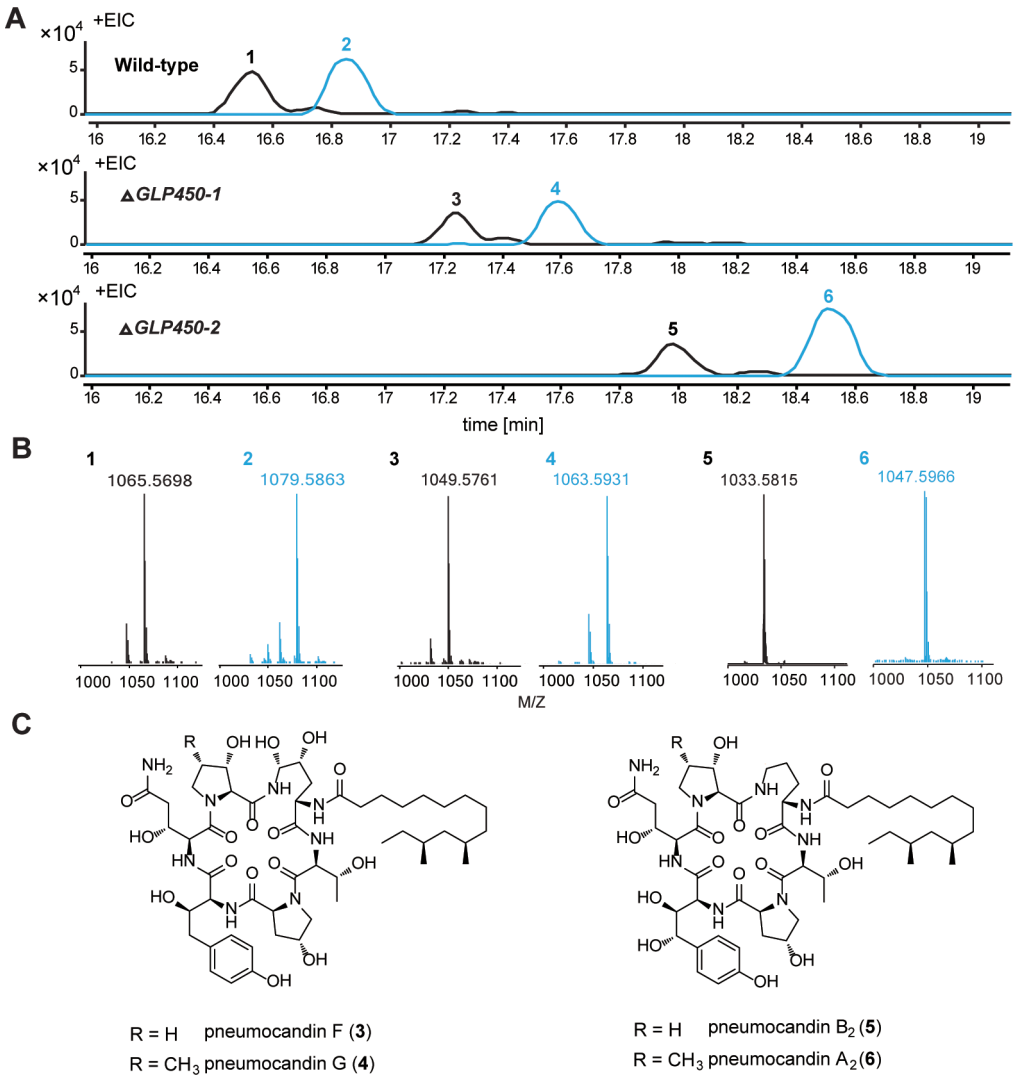


**Figure 2.** Insertional inactivation of two P450-type hemeprotein monooxygenase genes (Δ*GLP450-1* and Δ*GLP450-2*). (A) HPLC–MS analysis of crude extracts of Δ*GLP450-1* and Δ*GLP450-2* strains compared to the wild-type strain (extracted ion chromatogram). The *GLP450-1* inactivated mutant does not produce pneumocandins $B_0$ (**1**) and $A_0$ (**2**) but instead produces two new metabolites, pneumocandins F (**3**) and G (**4**). However, inactivation of *GLP450-2* abolishes production of **1** and **2**, and two later eluting compounds, pneumocandins $B_2$ (**5**) and $A_2$ (**6**), are observed instead. (B) HRESIMS of parent ions from pneumocandins produced by wild-type (**1** and **2**), Δ*GLP450-1* (**3** and **4**), and Δ*GLP450-2* (**5** and **6**). (C) Structures of **3**–**6** were determined by NMR, and metabolites **3** and **4** are two new genetically engineered pneumocandins.

to the monodeoxy form of **1**. The $^{1}H$ and $^{13}C$ NMR spectra (Supporting Information Figure S4A and B) of **3** displayed signals for structural features similar to those found in **1**, except that the oxymethine (C-4; $\delta_H/\delta_C$ = 4.28/75.8 ppm) of the L-homoTyr in **1** was replaced by a methylene ($\delta_H/\delta_C$ = 2.09/24.8 ppm) in the spectra of **3** (Supporting Information Table S3). These observations were also confirmed by $^{1}H-^{1}H$ COSY experiments that established an isolated proton spin-system corresponding to the C-2–C-4 (Supporting Information Figure S4C) and by HMBC correlations from H-2′ and H-6′ to the methylene carbon C-4 and from $H_2$-4 to C-1′, C-2′, and C-6′ (Supporting Information Figure S4D). Therefore, **3** was assigned as **1** lacking a hydroxyl group at the 4-position of the homoTyr residue. Similarly, comparison of the 1D NMR spectroscopic data (Supporting Information Table S3) of **4** and **2** and analysis of the 2D NMR data (Supporting Information Figure S5) confirmed that **4** was **2** missing one OH group at 4-homoTyr. These results indicated that GLP450-1 catalyzes the hydroxylation at C-4 of the L-homoTyr, the fourth residue of the pneumocandin hexapeptide core. Additionally, we implemented a chemical complementation experiment by feeding **4** to the *GLNRPS4* deletion mutant.[16] Extraction and analysis of the fed culture indicated that about 70% of **4** was converted to **2** (Supporting Information Figure S18), thus indicating that the hydroxylation reaction on the C-4 of L-homoTyr is one of the last steps during pneumocandin biosynthesis.

Similarly, fermentation extracts of the *GLP450-2* insertional mutant failed to produce **1** and **2** but yielded two dideoxy derivatives, **5** (*m*/*z* calcd for $C_{50}H_{80}N_8O_{15}$ $[M + H]^+$ 1033.5816, found 1033.5815) and **6** (*m*/*z* calcd for $C_{51}H_{82}N_8O_{15}$ $[M + H]^+$ 1047.5972, found 1047.5966; Figure 2A and B). An enzyme orthologue of GLP450-2, EcdH (68% similarity), was responsible for dihydroxylation of L-Orn in the echinocandin B hexapeptide from *A. rugulosus*.[18] Therefore, we predicted that GLP450-2 dihydroxylates L-Orn, the first residue in the pneumocandin hexapeptide core. The two target compounds **5** and **6** (Figure 2C) were purified from the


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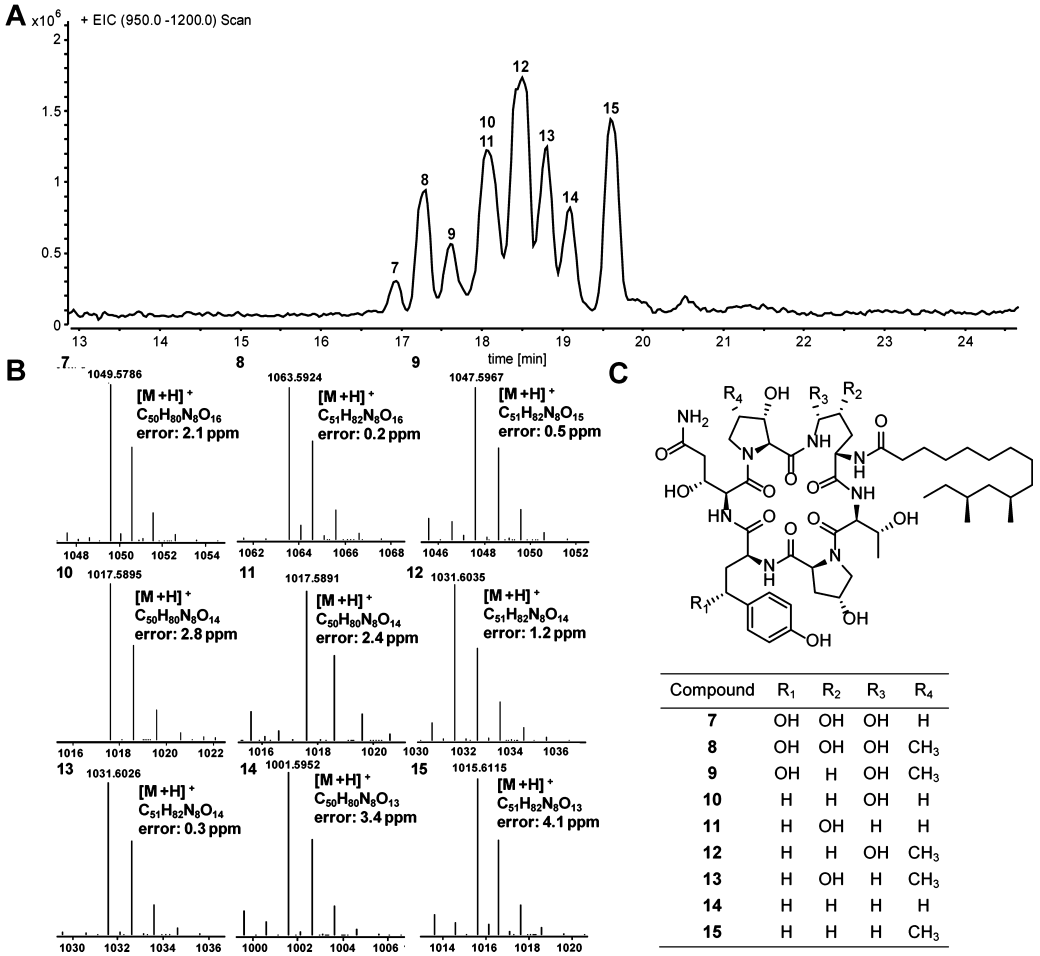


| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **7** | OH | OH | OH | H |
| **8** | OH | OH | OH | $CH_3$ |
| **9** | OH | H | OH | $CH_3$ |
| **10** | H | H | OH | H |
| **11** | H | OH | H | H |
| **12** | H | H | OH | $CH_3$ |
| **13** | H | OH | H | $CH_3$ |
| **14** | H | H | H | H |
| **15** | H | H | H | $CH_3$ |

**Figure 3.** Insertional inactivation of the nonheme iron oxygenase gene (*GLOXY1*). (A) HPLC−MS analysis of a crude extract of the Δ*GLOXY1* strain. Full-scan + mode spectrum was acquired over a scan range of *m*/*z* 950−1200. The inactivated mutant does not produce pneumocandins $B_0$ (**1**) and $A_0$ (**2**) but produces nine pneumocandins analogues (**7**−**15**) instead. (B) HRESIMS of parent ions from pneumocandin mutants produced by Δ*GLOXY1* (**7**−**15**). (C) Structures of compounds **7**−**15** were determined by NMR; **9**−**11**, **13**, and **14** are new pneumocandins. **7**, pneumocandin $B_1$; **8**, pneumocandin $A_1$; **12**, pneumocandin $A_3$; **15**, pneumocandin $A_4$.

scaled-up fermentation (1 L) of the *GLP450-2* mutant. NMR structural characterization of **5** and **6** (Supporting Information Figures S6 and S7) confirmed the two missing hydroxyl groups on the L-Orn residue. Compounds **5** and **6** are pneumocandin $B_2$ and pneumocandin $A_2$ (Figure 2B), respectively, which were previously reported as pneumocandin derivatives from a *G. lozoyensis* mutant strain ATCC 20958.[8,26] Although not confirmed experimentally, it is reasonable to speculate that chemical mutagenesis had inactivated the *GLP450-2* gene in ATCC 20958. Deletion of *GLP450-2* affected pneumocandin biosynthesis and generated **5** and **6**, suggesting that the dihydroxylation occurs after the release of the macrocyclic peptide. In order to verify this hypothesis, we fed compound **6** to the Δ*GLNRPS4* mutant[16] and recovered **2** at a conversion of about 35% (Supporting Information Figure S18), which is also consistent with previous experiments on echinocandin B biosynthesis.[18]

**Functional Inactivation of *GLOXY1* and Generation of Analogues with Nonhydroxylated Variations of Orn and homoTyr.** EcdG in the echinocandin biosynthetic gene cluster has been identified as catalyzing the C-3 hydroxylation on L-homoTyr; therefore we predicted that because *GLOXY1* is an orthologue of EcdG (similarity 68%), it likely hydroxylates the C-3 of L-homoTyr in the pneumocandin scaffold.[18] In a previous report,[18] the Δ*ecdG* strain generated an array of echinocandin analogues with modifications in the Orn and homoTyr moieties, so we presumed the inactivation of *GLOXY1* would result in a similar outcome. To confirm our hypothesis and to generate new pneumocandin analogues with potentially improved antifungal properties, AMT was used to inactivate *GLOXY1* (Supporting Information Figure S3). LC−MS analysis of the crude extract showed that the *GLOXY1* insertional mutant failed to produce pneumocandin $B_0$ (**1**) and $A_0$ (**2**) but exhibited a more complex pneumocandin metabolite profile (Figure 3A), producing a mixture of mono- (**7** and **8**), di- (**9**), tri- (**10**−**13**), and tetra-deoxy (**14** and **15**) analogues. All compounds visible in the UV profile were purified by HPLC from a scaled-up fermentation (6 L) of the Δ*GLOXY1* strain and then characterized by HRESIMS (Figure 3B) and NMR (Supporting Information Figures S8−S16). After analysis of their MS and NMR data, compounds **9**−**11**, **13**, and **14** were identified as new pneumocandins, while compounds **7**, **8**, **12**, and **15** were identified as pneumocandin $B_1$, pneumocandin $A_1$, pneumocandin $A_3$, and pneumocandin $A_4$, respectively, which were previously isolated from a *G. lozoyensis* mutant strain ATCC 20958.[8,26] Because the only difference between all the new analogues and pneumocandins $B_0$ (**1**) and $A_0$ (**2**) was the absence of different −OH groups, we established their


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Table 1. *In Vitro* Antifungal Activities of Compounds 1−15 (MIC, $\mu$g mL$^{-1}$)[a]

| | fungal pathogens | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| compounds | *Candida albicans* ATCC 10231 | *Candida albicans* ATCC 90028 | *Candida albicans* MDACC1[b] | *Candida tropicalis* ATCC 750 | *Candida glabrata* ATCC 2001 | *Candida glabrata* MDACC1[b] | *Candida parapsilosis* ATCC 90018 | *Aspergillus. fumigatus* FGSC A1240 |
| **1** | 0.4 | 0.8 | 12.5 | 0.1 | 0.8 | 12.5 | 0.4 | 3.2 |
| **2** | 0.8 | 0.8 | 25 | 0.2 | 1.6 | 25 | 0.4 | 3.2 |
| **3** | 0.05 | 0.2 | 6.4 | 0.04 | 0.2 | 3.2 | 0.4 | 0.8 |
| **4** | 0.1 | 0.1 | 12.5 | 0.04 | 0.2 | 12.5 | 0.4 | 3.2 |
| **5** | 1.6 | 3.2 | 100 | 3.2 | 3.2 | 100 | 0.4 | 3.2 |
| **6** | 1.6 | 1.6 | 50 | 0.8 | 3.2 | 50 | 0.4 | 3.2 |
| **7** | 6.4 | 3.2 | 50 | 3.2 | 3.2 | 50 | 3.2 | 3.2 |
| **8** | 3.2 | 3.2 | 50 | 3.2 | 3.2 | 50 | 1.6 | 3.2 |
| **9** | 3.2 | 3.2 | 50 | 6.4 | 3.2 | 50 | 1.6 | 3.2 |
| **10** | 3.2 | 6.4 | 100 | 3.2 | 6.4 | 100 | 1.6 | 3.2 |
| **11** | 3.2 | 3.2 | 50 | 3.2 | 3.2 | 50 | 1.6 | 3.2 |
| **12** | 1.6 | 1.6 | 50 | 0.8 | 3.2 | 50 | 1.6 | 3.2 |
| **13** | 3.2 | 3.2 | 50 | 3.2 | 6.4 | 50 | 1.6 | 3.2 |
| **14** | 3.2 | 3.2 | 100 | 1.6 | 3.2 | 100 | 1.6 | 3.2 |
| **15** | 0.8 | 0.8 | 50 | 0.2 | 1.6 | 50 | 1.6 | 3.2 |
| amphotericin B | 1.6 | 1.6 | 1.6 | 3.2 | 1.6 | 1.6 | 0.8 | 0.8 |
| caspofungin | <0.025 | <0.025 | 6.4 | <0.025 | 0.025 | 6.4 | 0.4 | 0.01 |

[a]Compounds **1** and **2** are pneumocandins $B_0$ and $A_0$. [b]Spontaneous caspofungin-resistant strain isolated at the M. D. Anderson Cancer Center.

structures by comparison of their $^1$H NMR data with those of pneumocandins $B_0$ (**1**) and $A_0$ (**2**)[26] and by analysis of their $^1$H−$^1$H COSY correlations observed for relevant protons (Supporting Information Figures S8−S16). The dideoxy form **9** (*m*/*z* calcd for $C_{51}H_{82}N_8O_{15}$ [M + H]$^+$ 1047.5967, found 1047.5972) is pneumocandin $A_0$ (**2**) lacking two hydroxyl groups on 3-homoTyr and 4-Orn because the $^1$H−$^1$H COSY NMR data (Supporting Information Figure S10) of **9** showed two isolated spin-systems in L-homoTyr (CH-2−$CH_2$−3-CHOH-4) and Orn (CH-2−$CH_2$−3-$CH_2$−4-CHOH-5). In a similar fashion, trideoxy derivatives **10** and **11** were pneumocandin $B_0$ (**1**) missing both hydroxyl groups on L-homoTyr, while also lacking one of either of the hydroxyl group at C-4 of L-Orn (compound **10**) and C-5 of L-Orn (compound **11**). Another trideoxy form **13** was determined to be a pneumocandin $A_0$ (**2**) derivative analogous to compound **11**; the new compound **14** was pneumocandin $B_0$ (**1**) lacking all four hydroxyl groups on both L-homoTyr and L-Orn. Therefore, the structures of **9**−**15** were proposed as shown in Figure 3C. Because two monodeoxy forms **7** and **8** corresponded to an absence of a hydroxyl group at the 3-position of the L-homoTyr residue, *GLOXY1* was therefore confirmed to mediate hydroxylation of the C-3 of L-homoTyr. Moreover, the presence of all these variants of C-4 of L-homoTyr moieties and the C-4 and C-5 of L-Orn (dideoxy in **9**, trideoxy in **10**−**13**, and tetradeoxy in **14** and **15**) suggested that inactivation of *GLOXY1* significantly decreased the hydroxylation efficiency on the C-4 position of L-homoTyr by GLP450-1 and the C-4 and C-5 positions of L-Orn by GLP450-2. As previously hypothesized in the case of echinocandin B, an altered conformation of the hexapeptide macrocycle may result when specific −OH groups are missing.[18] This result also indicated that the unhydroxylated L-Orn and L-homoTyr can be accepted and incorporated into the growing peptide core by GLNRPS4 and further supported that the sequence of hydroxylation reactions on the C-4 of L-homoTyr by GLP450-1 and on the C-4 and C-5 on L-Orn by GLP450-2 occur during the last steps of pneumocandin biosynthesis.

***In Vitro* Antifungal Activities.** The variations of *in vitro* antifungal activities of these lipopeptide variants are compared in Table 1. The antifungal activity of each compound was determined as the minimal inhibitory concentration (MIC) that achieved 100% inhibition of the eight tested pathogenic fungi (*Candida albicans* ATCC 10231, *C. albicans* ATCC 90028, *C. albicans* MDACC1, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 2001, *C. glabrata* MDACC1, *C. parapsilosis* ATCC 90018, and *Aspergillus. fumigatus* FGSC A1240) with amphotericin B (AMB) and caspofungin used as reference drugs. As shown in Table 1, most of the analogues showed good inhibitory activities against all the tested fungal pathogens, except for elevated MICs for the caspofungin-resistant strains of *C. albicans* MDACC1 and *C. glabrata* MDACC1. The assays revealed strong activity against wt *C. albicans* with their MIC values in the range of 0.05−6.4 $\mu$g mL$^{-1}$. In particular, pneumocandins F (**3**) and G (**4**), two compounds lacking the 4-hydroxyl moiety on the homoTyr, were found to be more active than AMB and pneumocandins $B_0$ (**1**) and $A_0$ (**2**). Their MIC values were 0.05 $\mu$g mL$^{-1}$ and 0.1 $\mu$g mL$^{-1}$, respectively, against *Candida albicans* ATCC 10231, which were 8-fold improvements in antifungal potency compared to the corresponding wt fermentation products pneumocandins $B_0$ (**1**) and $A_0$ (**2**). Compounds **3** and **4** were also the most effective inhibitors of the caspofungin-resistant strains, albeit the MICs were much higher than against the wt strains (Table 1). Several compounds (such as **1**−**4** and **15**) were more potent against wt *Candida* species than the positive control AMB. Most of the compounds also showed good potency against *A. fumigatus* (MIC range from 0.8 $\mu$g mL$^{-1}$ to 3.2 $\mu$g mL$^{-1}$) but, as expected, were less inhibitory than against *Candida* spp.

**The Complete Pneumocandin Biosynthetic Pathway.** Our previous studies identified the pneumocandin biosynthetic gene cluster in *G. lozoyensis* and confirmed that GLNRPS4 and GLPKS4 were the essential core megasynthases for the biosynthesis of the hexapeptide and 10*R*,12*S*-dimethylmyristoyl side chain, respectively.[16] In addition to GLNRPS4 and


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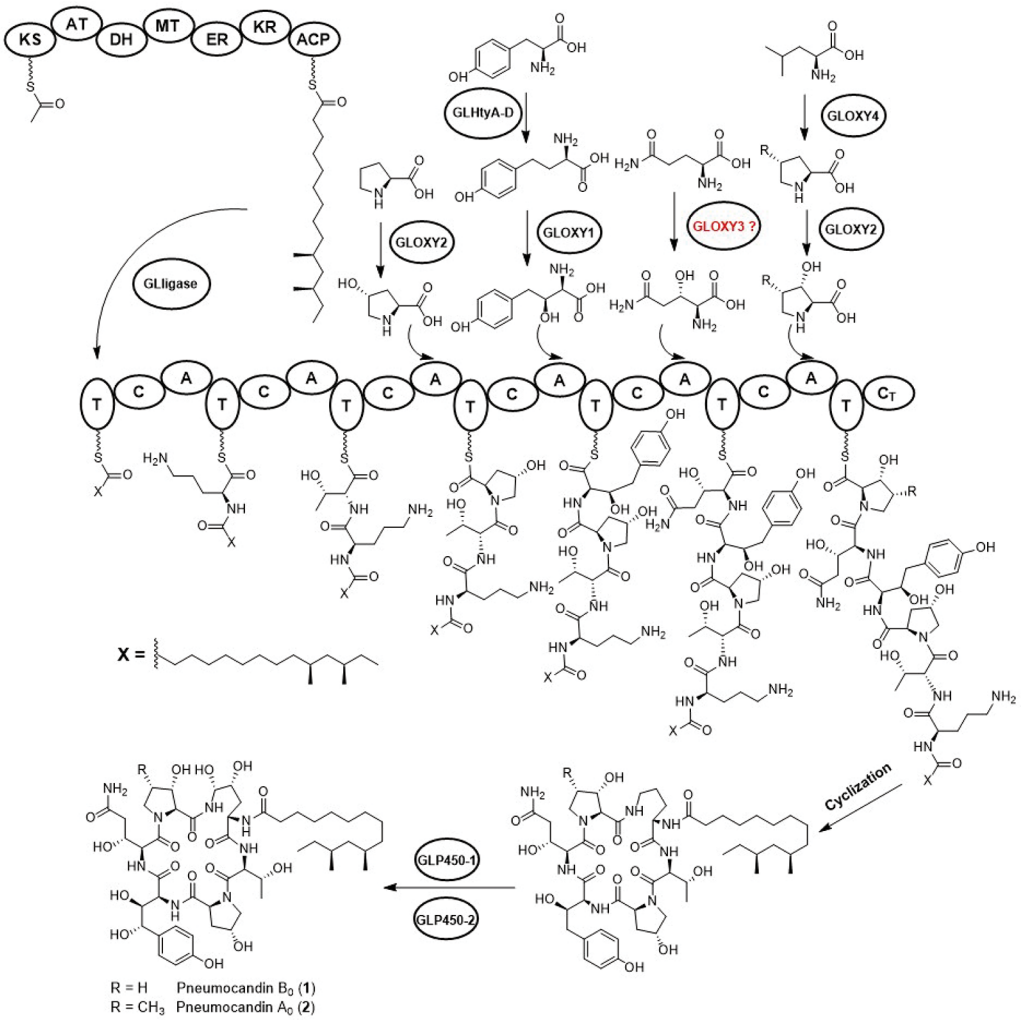
**Scheme 1. Proposed Pneumocandin Biosynthetic Pathway**[a]




[a]KS, ketosynthase domain; AT, acyltransferase domain; DH, dehydratase domain; MT, methyltransferase; ER, enoylreductase domain; KR, ketoreductase domain; ACP, acyl carrier protein; A, adenylation domain; T, thiolation domain; C, condensation domain; $C_T$, terminal condensation domain. The function of GLOXY3 (red) has been inferred because it is only present in pathways with hydroxyl-Gln in their peptide core (Supporting Information Table S1).[23].

GLPKS4, the pneumocandin biosynthetic cluster encodes a complex array of tailoring enzymes which spans about 66 kb (from *GLP450-1* to *GLHYP*). Most of these genes have an orthologue in the echinocandin pathway (Supporting Information Table S1), which indicates that the corresponding enzymes have functions equivalent to those of the echinocandin B pathway.[18] Based on the previous studies[16,18,28] and our current findings, we propose the complete pneumocandin biosynthetic pathway as outlined in Scheme 1. The 10,12-dimethylmyristoyl side chain is synthesized by GLPKS4, and its migration to the first thiolation domain of GLNRPS4 is mediated by the acyl-CoA ligase (GLligase), followed by its acylation to L-Orn to trigger elongation of the cyclic hexapeptide. Thr, 4-hydroxy-Pro (generated by GLOXY2),[22,28] 3-hydroxy-homoTyr (generated by GLHtyA–D and GLOXY1),[17,18] 3-hydroxy-Gln and 3-hydroxy-Pro (generated by GLOXY2),[22,28] or 3-hydroxy-4-methyl-Pro (generated by GLOXY4 and GLOXY2)[22,28] are sequentially added to the growing chain in the same sequence as in EcdA.[17] Like EcdA, the last C domain of GLNRPS4 is proposed to be responsible for cyclization by condensation to form the peptide bond between L-Orn and 3-hydroxy-4-methyl-Pro or 3-hydroxy-Pro. Finally, the C-4 of L-homoTyr and C-4 and C-5 on L-Orn are hydroxylated by GLP450-1 and GLP450-2, respectively.

**Conclusion.** The pneumocandin peptide undergoes a series of hydroxylation reactions during its biosynthesis. In this study, we identified two P450s and one oxygenase that are involved in the hydroxylation of pneumocandin amino acid residues and applied insertional inactivation to generate three mutant strains that collectively produced 13 pneumocandin analogues, including seven new compounds (**3**, **4**, **9**–**11**, **13**, and **14**), some with potentially improved antifungal potency (i.e., **3** and **4**).

GLP450-1 is responsible for the hydroxylation on the C-4 of L-homoTyr, and this hydroxyl group is regarded as an important moiety for the antifungal activity of pneumocandins.[12,21] The improved activity of **3** and **4** closely parallels the increased antifungal activity reported for semisynthetically prepared deoxymulundocandin that also lacked the C-4 hydroxyl of homoTyr.[29,30] Previous SAR studies showed manipulation of the stereochemistry of C-4 hydroxyl group greatly impacted antifungal activity.[12,21] Inversion of the C-4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hydroxyl group caused about a 70-fold decrease in potency,[21] while our antifungal studies indicated a loss of this hydroxyl yielded a more potent inhibitor (8-fold improvements in the antifungal potency). To the best of our knowledge, this is the first experimental inactivation of this echinocandin pathway gene.[18]

GLP450-2, the orthologue of EcdH in the echinocandin B biosynthetic cluster,[18] has been proven to hydroxylate both C-4 and C-5 on L-Orn, suggesting that it could work iteratively on the C-4 and C-5 of L-Orn. Several P450 enzymes that iteratively hydroxylate their substrates have been described.[18,31,32] Our antifungal bioassay studies indicated that 4,5-dihydroxyl-L-Orn was a critical moiety for the antifungal activity of pneumocandins, because removal of both hydroxyl groups decreased potency about 4-fold.

*GLOXY1*, an orthologue of EcdG (similarity 68%) in the echinocandin biosynthetic gene cluster,[18] is responsible for the hydroxylation of the C-3of L-homoTyr in the pneumocandin scaffold. When *GLOXY1* was deleted, the loss of the C-3 hydroxyl group on the homoTyr residue led to incomplete hydroxylations by GLP450-1 and GLP450-2, likely due to conformational changes to the hexapeptide core. The erratic hydroxylation pattern resulted in nine variants at positions on the Orn and homoTyr residues (compounds **7**−**15**). None of the Δ*GLOXY1* mutant-produced compounds showed a clear improvement in anti-*Candida* activity compared to pneumocandins $A_0$ (**2**) and $B_0$ (**1**), although all of them were equally potent as **1** and **2** against *A. fumigatus*.

In summary, insertional inactivation of key pathway genes provided a means to explore structural diversity and timing of biosynthetic steps in the pneumocandin family of natural products. Modifications in the pneumocandins $A_0$ (**2**) and $B_0$ (**1**) molecules contributed to discovery of two new analogues, pneumocandins F (**3**) and G (**4**), that were more potent antifungals than the parent molecules. Caspofungin-resistant strains of *C. albicans* and *C. glabrata*, predictably and unfortunately, showed cross resistance to these new pneumocandins. As the patent cycles for the echinocandin antifungal drugs reach their expiration, interest is increasing in development of improved echinocandin drugs for their replacement[33] and more efficient manufacturing processes for generic versions.[34] Genetic manipulations of the biosynthetic pathway, such as those reported here, along with other manipulations can modify product spectrum and improve yields[35] and may contribute to developing improved pneumocandins from higher yielding strains.

## METHODS

**General Experimental Procedures.** All $^1H$, $^{13}C$, and 2D NMR data were collected on a Bruker 600 or a 500 MHz NMRs equipped with a 5 mm triple resonance cryoprobe at 298 K. Residual solvent signals were used as a reference ($CD_3OD$: $\delta_H$ 3.31/$\delta_C$ 49.2). The high resolution mass spectra for each compound were acquired with an Agilent 6520 Q-TOF system in the positive ionization mode. For Q-TOF/MS conditions, voltages of the fragmentor and capillary were kept at 130 and 3500 V, respectively. Nitrogen was provided as the nebulizing and drying gas. Temperature of the drying gas was kept at 300 °C. The flow rate of the drying gas and the pressure of the nebulizer were 10 L $min^{-1}$ and 25 psi, respectively. Full-scan spectra were acquired over a scan range of $m/z$ 80−1500 at 1.03 spectra $s^{-1}$.

**Strains and Plasmids.** The original pneumocandin producing strain of *G. lozoyensis* ATCC 20868 was obtained from American Type Culture Collection (ATCC) and was used as the wt recipient in AMT experiments. *Escherichia coli* DH5α was routinely used for plasmid propagation. The plasmid pAg1-H3 described previously was used for the gene inactivation vector construction.[36] Reference wt fungal pathogen strains were purchased from the ATCC and the Fungal Genetics Stock Center (FGSC). Caspofungin-resistant strains of *C. albicans* (MDACC 1) and *C. glabrata* (MDACC 1) were clinical isolates from the laboratory of Prof. Dimitrios Kontoyiannis, the M. D. Anderson Cancer Center, Houston, Texas.

**Gene Inactivation in *G. lozoyensis*.** To construct the gene inactivation mutants of *G. lozoyensis*, the DNA fragments of the upstream and downstream regions of target genes were amplified from the genomic DNA by using the primers listed in Supporting Information Table S2. Amplifications were carried out by using Phusion high-fidelity DNA polymerase following the manufacturer's instruction (NEB, USA). Homologous fragments with restriction sites were inserted into the multiple cloning site on pAg1-H3 to generate inactivation vectors. The protocol for gene inactivation in *G. lozoyensis* was described previously with modifications.[36] The conidia of *G. lozoyensis* were washed with 0.05% Tween-20, followed by 15 min of vortexing, and rinsed twice with distilled water. Conidia were resuspended in 750 μL of distilled water. The cultivation of *A. tumefaciens* and the subsequent transformation method were described by Chen et al.[16] The conidial suspension was mixed with an equal volume (750 μL) of *A. tumefaciens* culture, vortexed for 2 min, spread on IMAS agar, and cocultivated at 28 °C for 2 days. The coculture was then covered with M-100 supplemented with 300 μg $mL^{-1}$ cefotaxime and 200 μg $mL^{-1}$ hygromycin B and incubated at 25 °C for 2 to 3 weeks before isolating hygromycin B resistant transformants. Fungal genomic DNA from the wt strain and the transformants were extracted as previously described.[36] Primers used for PCR screening are listed in Supporting Information Table S2.

**LC−MS Analysis.** The wt *G. lozoyensis* and mutant strains were cultured on malt-yeast extract agar at 28 °C for 5 days to produce conidia and mycelia. Agar cultures were cut into small pieces (0.5 $cm^3$), and 15 pieces were used to inoculate Erlenmeyer flasks (500 mL), each containing 100 mL of H medium.[13] The flasks of the inoculated media were incubated at 25 °C on a rotary shaker at 220 rpm for 14 days. The cultures were extracted with 100 mL of isobutanol, and the organic phase was evaporated to dryness and redissolved in MeOH at 30 mg $mL^{-1}$. Then, 10 μL of each dissolved extract was injected for HPLC−DAD−MS analysis on a linear gradient of 10−90% acetonitrile (MeCN) in water (with 0.1% formic acid) for 28 min at a flow rate of 1 mL $min^{-1}$ through an Agilent Zorbax Eclipse Plus $C_{18}$ reverse phase column (4.6 × 150 mm, 5 μm). LC−MS spectra were obtained on an Agilent 6120 single quadrupole LC−MS using positive electrospray ionization.

**Scaled-up Cultivation of Insertional Mutants and Compound Isolation for Structural Elucidation.** The procedures for fermentation were described by Schwartz et al.[6] Conidia from oat bran agar were inoculated into a 500 mL-Erlenmeyer flask containing 100 mL of seed medium (KF medium). The seed medium was incubated for 5 days with agitation at 220 rpm. A total of 4 mL of seed medium was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of production medium (H medium,[13] the total fermentation volumes were 4 L for Δ*GLP450-1*, 1 L for Δ*GLP450-2*, and 6 L for Δ*GLOXY1*) and cultivated at 25 °C with agitation at 220 rpm for 14 days.

The fermented culture of Δ*GLP450-1* was extracted with 4 L of isobutanol, and the organic solvent was evaporated to dryness under a vacuum to afford the crude extract (4.0 g), which was fractionated with a reversed-phase $C_{18}$ column (5−100% MeOH in $H_2O$ over 35 min; 40 mL $min^{-1}$) coupled to a Grace Reveleris X2 flash chromatography system. Fractions (125 mg) eluted with 70% and 80% MeOH were combined and further purified by semipreparative RP HPLC (Agilent Zorbax SB-$C_{18}$ column; 5 μm; 9.4 × 250 mm; 50% MeCN in $H_2O$ with 0.1% formic acid over 30 min; 2 mL $min^{-1}$) to afford **3** (4.5 mg, $t_R$ 18.75 min) and **4** (15.5 mg, $t_R$ 21.35 min).

The fermented culture of Δ*GLP450-2* was extracted with 1 L of isobutanol, and the organic solvent was evaporated to dryness under a vacuum to afford the crude extract (1.5 g), which was fractionated on a silica gel column (0−20% MeOH in $CH_2Cl_2$ over 30 min; 30 mL $min^{-1}$) by flash chromatography. Fractions (75 mg) eluted with 1%


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and 3% MeOH were combined and further purified by RP HPLC (the same gradient as in purification of **3**) to afford **5** (2.2 mg, $t_R$ 24.10 min) and **6** (3.9 mg, $t_R$ 29.05 min).

The fermented culture of Δ*GLOXY1* was extracted with 6 L of isobutanol, and the organic solvent was evaporated to dryness under a vacuum to afford the crude extract (8.0 g), which was fractionated with a reversed-phase $C_{18}$ column (10−100% MeOH in $H_2O$ over 35 min; 40 mL $min^{-1}$) coupled to a flash chromatography system. Fractions (220 mg) were eluted with 70% and 75% MeOH, combined, and further purified by RP HPLC (the same gradient as in purification of **3**) to afford **7** (3.5 mg, $t_R$ 16.20 min), **8** (5.0 mg, $t_R$ 17.45 min), and **9** (3.0 mg, $t_R$ 20.60 min). The fractions (0.8 g) were eluted with 80% and 90% MeOH and combined and separated by a Sephadex LH-20 CC, eluting with MeOH. The resulting subfractions were purified by RP HPLC (48% MeCN in $H_2O$ with 0.1% formic acid over 55 min; 2 mL $min^{-1}$) to afford **10** (2.5 mg, $t_R$ 32.24 min), **11** (3.0 mg, $t_R$ 34.05 min), **12** (8.0 mg, $t_R$ 37.91 min), **13** (3.3 mg, $t_R$ 42.82 min), **14** (2.5 mg, $t_R$ 46.85 min), and **15** (3.5 mg, $t_R$ 53.90 min).

Structural elucidation of the above 13 pneumocandin analogues was determined by MS and NMR spectroscopy (Figures 2B, 3B, and Supporting Information Figures S8−S16).[26]

**Antifungal Activity Assay.** *In vitro* antifungal activity was measured according to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations.[37] The minimum inhibitory concentration (MIC) was determined by means of the serial dilution method in 96-well plates with RPMI 1640 (Sigma-Aldrich) buffered with 0.165 M MOPS (Sigma-Aldrich) as the test medium. Amphotericin B and caspofungin were used as the reference drugs. Test compounds were dissolved in DMSO and serially diluted in a growth medium. The visual end point and the optical density readings of microplate wells were measured relative to positive and negative controls. The strains were incubated at 35 °C, and the MICs were determined at 24 h for *Candida* species, and at 48 h for *A. fumigatus*.[38] Viability was determined with the aid of a plate reader using PrestoBlue resazurin dye (Life Technologies) as the viability indicator. The spectrophotometric MIC value was defined as the lowest concentration of a test compound that resulted in a culture with a density equal to 100% inhibition when compared to the growth of the untreated control. Each experiment for the set of test compounds (e.g., Supporting Information Figure S17) was carried out in parallel at three different times to ensure accuracy.

## ■ ASSOCIATED CONTENT

### Supporting Information

Protein sequence comparisons, PCR primer list, PCR validation result of the deletion mutants, NMR data for compounds **3**−**15**, bioassay results, and feeding experiment. 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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