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Discovery of Cryptic Polyketide Metabolites from Dermatophytes Using Heterologous Expression in *Aspergillus nidulans*

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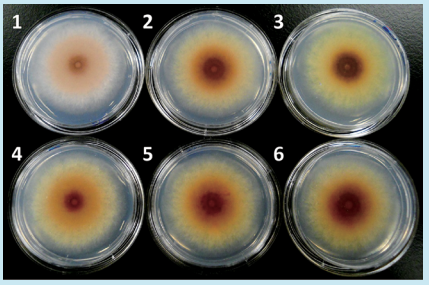
Supporting Information

ABSTRACT: Dermatophytes belonging to the *Trichophyton* and *Arthroderma* genera cause skin infections in humans and animals. From genome sequencing data, we mined a conserved gene cluster among dermatophytes that are homologous to one that produces an immunosuppressive polyketide in *Aspergillus fumigatus*. Using a recombination-based cloning strategy in yeast, we constructed fungal heterologous expression vectors that encode the cryptic clusters. When integrated into the model *Aspergillus nidulans* host, a structurally related compound neosartoricin B was formed, suggesting a possible role of this compound in the pathogenesis of these strains.



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Secondary metabolites (SMs) play important roles in the pathogenesis of microorganisms. In pathogenic fungi, polyketide and nonribosomal peptides have been shown to be potential virulence factors and immunosuppressants.[1−5] Identification of these factors is therefore highly important toward understanding the molecular basis of host−pathogen interactions.[6,7] Genome sequencing of numerous dermatophytes belonging to the *Trichophyton* and *Arthroderma* genera revealed that each genome encodes numerous SM clusters.[1,8] Unfortunately, under laboratory conditions many of the gene clusters in fungi are silent and hence mask the products encoded in them.[9] The difficulties involved in manipulating these fungi, such as long doubling times and lack of genetic tools, further impedes the establishment of metabolite-cluster correlations.

When analyzing the polyketide gene clusters from the sequenced dermatophyte genomes,[1,8] we found an orthologous gene cluster that is also conserved in the pathogenic fungi *Aspergillus fumigatus* and *Neosartorya fischeri*[10,11] (Figure 1). In each genome, the cluster contains a set of four genes that includes polyketide synthase (PKS), a β-lactamase like thioesterase (TE), a flavin-dependent monooxygenase (FMO), and a polycyclic prenyltransferase (pcPT).[10,11] In addition to these four well-conserved "core PKS genes", the neighboring regions encode additional genes, some of which are highly syntenic and conserved between genomes (Figure 1). We recently showed that when activated these core PKS genes from *A. fumigatus* and *N. fischeri* can synthesize a prenylated, tricyclic polyketide neosartoricin.[11] Neosartoricin was shown to inhibit T-cell proliferation with an IC_{50} of 3 μM and was proposed to possibly play a role in suppressing host adaptive immune response during infection.[11] The conservation of the core PKS genes in the dermatophytes (>90% identity between them) (Supplementary Table S3, Supporting Information) and the lower sequence homology to those in *A. fumigatus* and *N. fischeri* (~70% identity, Supplementary Table S3) raise an interesting question as to whether these fungi produce similar compounds and whether the presence of additional conserved genes may lead to further tailoring of the neosartoricin scaffold.

To examine the activities of the conserved core PKS genes from dermatophytes, we cloned the clusters using yeast recombination[12] followed by expression in *Aspergillus nidulans* (Supplementary Figure S1, Supporting Information). We initiated the cloning strategy by creating an *E. coli*-yeast-*Aspergillus* shuttle vector, pYH-wA-pyrG, which consists of a ColE1 origin of replication from SuperCos1, a yeast centromere sequence (CEN), and an autonomously replicating sequence (ARS)[13,14] (Supplementary Table S1; Methods). The choice of the low copy number in *Saccharomyces cerevisiae* allows stable maintenance of the subsequent large (>20 kb) plasmids containing cloned gene clusters. For fungal heterologous expression, we used the well-studied *A. nidulans* containing the Δ*nkuA* deletion (Supplementary Table S1), which minimizes nonhomologous recombination events.[15,16] The *wA* locus in *A. nidulans*, which encodes a pigment-encoding and nonessential PKS,[17] was chosen as the site for homologous integration of the gene clusters (Figure 2 and Supplementary Figure S1B). This allowed the facile, initial screening of correct

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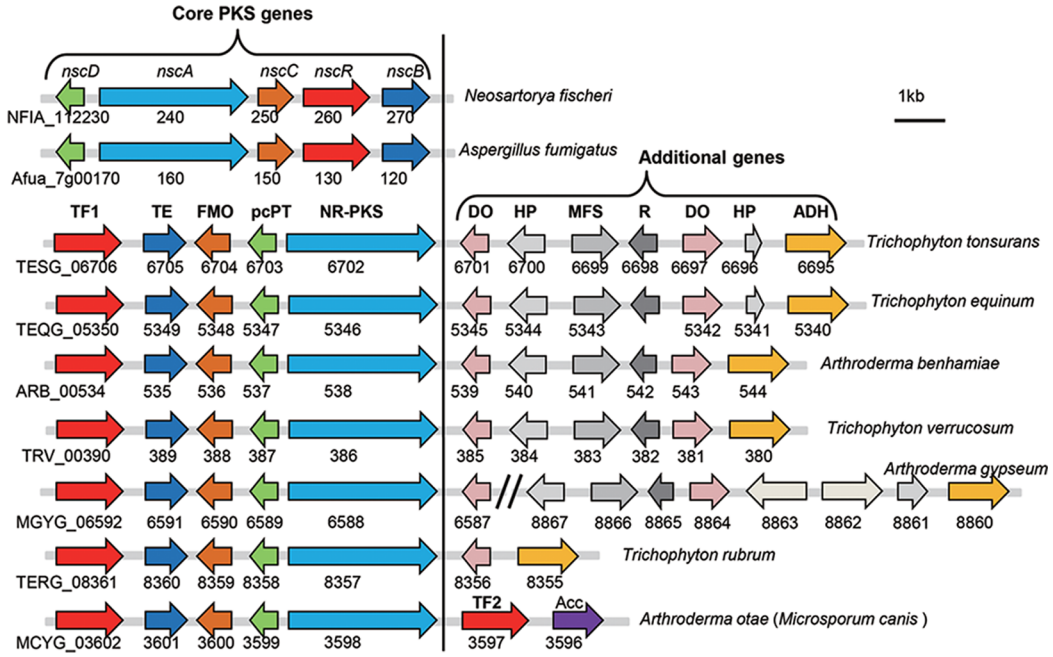

Figure 1. Comparison of conserved gene clusters across different dermatophyte and pathogenic fungi. All of the fungi shown contain a conserved core PKS gene cassette that consists of a nonreducing polyketide synthase (NR-PKS), β-lactamase thioesterase (TE), flavin-dependent monooxygenase (FMO), and polycyclic prenyltransferase (pcPT). A fungal transcription factor (TF) is also found immediately adjacent to the core PKS genes. In dermatophytes, additional genes are also found adjacent to the core PKS gene cassette, among which many are conserved across different fungi. Some of the genes include dioxygenase (DO), hypothetical protein (HP), amino acid racemase (R), major facilitator superfamily transporters (MFS), and alcohol dehydrogenase (ADH). In *A. otae,* an additional C6 transcriptional factor (TF2) is adjacent to the core PKS gene cassette.

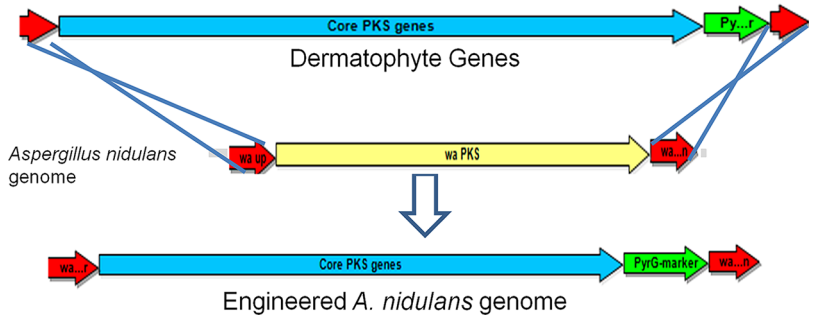

Figure 2. Strategy for integrating dermatophyte gene cluster into *A. nidulans.* The dermatophyte genes were cloned between *A. nidulans wA* flanking regions and then integrated into *wA* locus by replacing the *wAPKS* gene.

recombination through visual evaluation of the resulting white conidia.

Using *in vivo* yeast recombination cloning (Supplementary Figure S1A), the core PKS genes (TESG_6702−6705), along with a putative pathway specific fungal transcription factor (TF, TESG_6706) (Figure 1) from *Trichophyton tonsurans* was cloned into the pYH-wA-pyrG vector adjacent to the *A. fumigatus pyrG* (AfpyrG) selection marker. The entire cassette was flanked by upstream and downstream sequences of the *wA* gene (Supplementary Figure S1A). The native promoter of the TF (TESG_6706) gene was replaced with the *A. nidulans gpdA* promoter. The use of the constitutive promoter is to ensure expression of the foreign gene cluster in *A. nidulans*, since neosartoricin-like compounds are not isolated from the dermatophytes under a variety of culture conditions (data not shown). The resultant plasmid containing the four core PKS genes was then linearized and introduced into *A. nidulans*. A number of the nutrition-complemented transformants displayed the loss of the green spore pigments associated to the *wA* mutant phenotype. In addition, a yellow pigmentation was observed for these mutants, which is similar to the *N. fischeri* strain overexpressing neosartoricin in our previous study[11] (Supplementary Figure S2).

Following PCR verification of the integration of all four genes, the strain TWY1.1 (TESG_06702−6706) was cultivated in GMM media with 0.5 μM pyridoxine HCl, and the metabolites were extracted and analyzed with LC−MS (Figure 3). Compared to the isogenic control TWY2.2 (Δ*wAPKS*) in which the *wA* locus was disrupted with the *pyrG* cassette alone, a new metabolite (yield 10 mg/L) was observed with UV absorption identical to that of neosartoricin. Mass analysis showed that the compound has m/z = 443 $[M + H]^+$ (Supplementary Figure S3C), which is 42 mass units (mu) lower than that of neosartoricin. The new metabolite (hereafter named neosartoricin B) was purified from the crude extract and fully characterized by one- and two-dimensional NMR analyses

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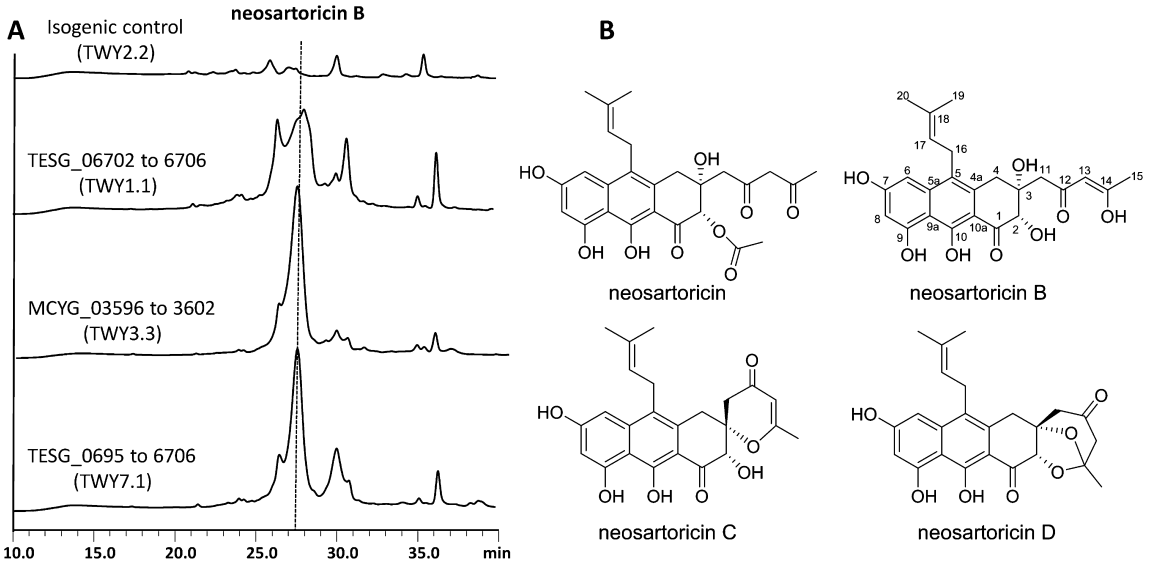

Figure 3. Production of neosartoricins from synthetic mutants in *A. nidulans*. (A) HPLC traces (400 nm) of organic extracts from different strains. Trace 1: isogenic control TWY2.2. Trace 2: TWY1.1 (TESG_06702 to 6706). Trace 3: TWY3.3 (MCYG_03596 to 3602). Trace 4: TWY7.1 (TESG_0695 to 6706). (B) Structures of neosartoricin, neosartoricins B, C, and D.

(Supplementary Table S4 and Supplementary NMR spectra). Neosartoricin B is structurally similar to neosartoricin, with the loss of acetyl group at the C2 hydroxyl. All other structural features, including the polyhydroxylated aromatic scaffold, C5 prenylation, and 1,3-diketo substituent at C3 were all verified to be identical. The C2 acetyl group in neosartoricin was attributed to the action of an unidentified and likely endogenous acetyltransferase in *A. fumigatus*. The absence of this group in neosartoricin B may be attributed to the lack of this activity in *A. nidulans*.

During purification of neosartoricin B, we observed that under slightly acidic conditions, neosartoricin B can be converted into two additional compounds neosartoricins C and D (Figure 3B), both of which have the molecular weight of 424 (Supplementary Figure S4). Full structural characterizations were performed to reveal their structures as shown in Figure 3B. Interestingly, neosartoricin C is a spirocyclic compound that is cyclized through the attack of C3 hydroxyl on C14, followed by dehydration. On the other hand, neosartoricin D is a further cyclized compound in which attack of C2 on C14 in neosartoricin C resulted in the formation of the acetal-containing dioxabicyclo-octanone ring. Both of these compounds are novel and possibly represent related metabolites of the gene cluster. Indeed, analysis of the organic extract of TWY1.1 also revealed small amounts of neosartoricins C and D, although it is not known whether these are formed during culturing or during the extraction process.

Other highly identical and syntenic genes were found immediately adjacent to most of the core PKS gene ensembles in some of the dermatophytes, including *T. tonsurans*, *T. equinum*, *T. verrucosum*, and *A. benhamiae* (Figure 1). Among them are included enzymes that one may anticipate to associate with the modification of secondary metabolites, including putative dioxygenase (DO), amino acid racemase (R), and alcohol dehydrogenase (ADH). Hence it appears tantalizingly possible that among these organisms neosartoricin B may be further morphed into a different product that may have additional biological properties. To assess the possible roles of these enzymes in tailoring of neosartoricin B, we used a similar strategy to insert different combinations of these genes from *T. tonsurans* into the neosartoricin B-producing host TWY1.1 (Supplementary Figure S1B). Specifically, we added TESG06695−6701, TESG06695−6701 (without 6696), and TESG_06701 to yield TWY7.1, TWY8.1, and TWY9.1, respectively. However, follow-up analysis of the extracts from these strains yielded metabolic profiles identical to that of TWY1.1 (Figure 3 and Supplementary Figure S3A). This suggests that although highly conserved, the genes either may not express or do not cotranscribe with the core PKS genes. Indeed, RT-PCR analysis of the genes TESG_06698 and TESG_06701 in TWY7.1 showed that the genes included in this cassette are not cotranscribed with the PKS-associated genes with the constitutively expressed TF (Supplementary Figure S5) and hence are likely not involved in the neosartoricin B pathway. Lastly, six contiguous genes in *Arthroderma otae* (*Microsporum canis*) (MCYG_03596−3602) including the four core PKS genes, a TF gene and a putative aminocyclopropane carboxylic acid synthase (ACCS) gene were also introduced into *A. nidulans* to create TWY3.3, which was confirmed to produce neosartoricin B as well (Figure 3 and Supplementary Figure S2).

Our discovery of neosartoricin B demonstrates that the highly conserved core PKS genes (PKS, TE, FMO, and pcPT) in dermatophytes can synthesize nearly the same prenylated aromatic polyketide as in *A. fumigatus* and *N. fischeri*, which we showed have notable immunosuppressive activities. Neosartoricin B may therefore mediate immunomodulatory interactions with the host during infection and colonization of the pathogenic fungi. The biosynthetic mechanism of neosartoricin B is expected to follow that proposed for neosartoricin.[11] Hence the neosartoricin compounds may represent conserved SM shared by pathogenic fungal strains. Our approach shows the potential to use the model fungus to analyze the products of biosynthetic pathways from difficult to handle organisms.

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METHODS

Strain, Media, and Growth Conditions. The fungal strains used in this study are listed in Supplementary Table S1. All strains were grown at 37 °C on glucose minimum medium (GMM)[18] and when appropriate were supplemented with 0.56 g uracil/L, 1.26 g uridine/L, and 0.5 μM pyridoxine HCl and maintained as glycerol stocks at −80 °C. *Escherichia coli* strains XL-1 Blue (Stratagene) was used for DNA manipulation.

Gene Cloning, Plasmid Construction, and Genetic Manipulation. The plasmids utilized in this work are listed in Supplementary Table S1. The oligonucleotide sequences for PCR primers are given in Supplementary Table S2. PCR reactions were performed with Phusion high-fidelity DNA polymerase (New England Biolabs). PCR screening for transformants and mutants were carried out with Quick-Load Taq 2X Master Mix (New England Biolabs).

The basic strategy for assembling of large PCR fragments is splicing by overlapping extension (SOE)-PCR and yeast homologous recombination as described in Supplementary Figure S1. *Saccharomyces cerevisiae* strain BJ5464-NpgA (*MATα ura3-52 his3-Δ200 leu2-Δ1 trp1 pep4::HIS3 prb1Δ1.6R can1 GAL*) was used as the host.[12] The vector pYH-WA-pyrG is constructed by in vivo yeast recombination of three linear DNA fragments consisting of (1) the ColE1 origin of replication and *ampR* from SuperCos1 vector (Stratagene), (2) a yeast centromere sequence (*CEN*), autonomously replicating sequence (*ARS*), and a URA3 marker from pXP742,[19] and (3) a 5′wA-AfpyrG-3′wA cassette synthesized by fusion PCR. The *pyrG* auxotrophy marker was amplified from *A. fumigatus* genomic DNA, while the 5′ and 3′ regions of the *wA* gene were amplified from *A. nidulans* genomic DNA. All three DNA fragments for in vivo yeast recombination contained a minimum of 35 bp overlapping bases with the flanking fragments. Primers used for construction of the plasmid are listed in Supplementary Table S2.

Using the same strategy, all plasmids pWY13.2, 14.1, 15, 16, and 17.1 (Supplementary Table S1) were constructed in this study. Overlapping regions between two flanking segments of TESG_06695−6706 and MCYG_03596−3602 ranged from 60 to 150 bp and overlapping regions between the segments and the vector pYH-WA-pyrG were 40 bp. Briefly, four PCR fragments (ca. 4 kb/each fragment) of TESG_06706−6702 were amplified with *T. tonsurans* genomic DNA by using designated primers (Supplementary Table S2), respectively. The *gpdA* promoter was amplified by using *A. nidulans* genomic DNA and fused with transcription factor (TF) TESG_06706 using designated primers (Supplementary Table S2). Then, all fragments, *Nhe*I digested vector were gel purified and transformed into *S. cerevisiae* BJ5464-NpgA by using a *S. c.* EasyComp Transformation Kit (Invitrogen). The obtained yeast colonies were characterized by PCR. Yeast plasmids were isolated by using a Zymoprep (D2001) Kit (Zymo Research) and transformed into *E. coli* XL1 Blue. The fragments for MCYG_03596−3602 from *A. otae* were assembled to create plasmid pWY14.2. For the construction of plasmids pWY15, 16, and 17.1, the selectable marker AfpyroA was amplified from *A. fumigatus* assembled with *Not*I digested pYH-WA-pyrG and amplified genomic DNA segments from the fungal hosts (Figure 1). All plasmids were confirmed by restriction enzyme digestion and sequencing.

Transformation of *A. nidulans*. *A. nidulans* strain RJMP1.49 was used as the recipient host. Fungal protoplast preparation and transformation were modified according to the description from Bok and Keller.[20] The modifications are the following: (1) Culture medium for conidia germination was reduced to 20−50 mL, and germination time was shortened. A total of 20−50 mL of sterile liquid Minimal Medium (LMM, containing the appropriate supplements) was innoculated with about 5×10^8 fresh spores (1×10^7 conidia/mL) and shaken at 37 °C and 280 rpm for approximately 5−6 h for spore germination. The germination time can be shortened 1−2 h if yeast extract is added to medium. (2) Digestion temperature with lysing enzyme was adjusted to 37 °C, which can reduce digestion time 1−2 h. Optionally, digestion can also be performed at room temperature overnight to obtain better protoplasts. A 5−15 μg sample of plasmids were linearized and transformed into RJMP1.49. Transformation with linearized pYH-WA-pyrG was used as control. Transformants were verified by using diagnostic PCR with appropriate primers (Supplementary Table S2).

RNA Extraction and Reverse Transcriptase PCR (RT-PCR). Spores (1×10^6) from TWY2.1 (Isogenic control), TWY1.1, TWY3.3, and TWY7.1 were inoculated into 10 mL of LMM with pyridoxine (0.5 μM) and cultivated at 37 °C for 2 days under dark conditions with two replicates each. Then, the mycelia were harvested and total RNA was extracted using the Ambion RiboPure-Yeast Kit according to the instructions (Invitrogen, Carlsbad, CA). For transcription assessment of dermatophyte clusters in *A. nidulans*, the single strand cDNAs from TWY2.1, TWY1.1, TWY3.3, and TWY7.1 were synthesized by using ImProm-II Reverse Transcription System (Promega, Madison, WI). Genes coding for putative TF1 (TESG_06706), NR-PKS (TESG_06702), dioxygenase (TESG_06701), and asp/glu racemase (TESG_06698) were used for the transcription assessment of TESG_06695−6706 from *T. tonsurans*. Genes coding for putative TF1 (MCYG_03602) and NR-PKS (MCYG_03598) were used for the assessment of MCYG_03596−3602 transcription from *A. otae*.

Fermentation and LC−MS analysis. *A. nidulans* strains were cultivated at 37 °C in liquid GMM (supplemented with 0.5 μM pyridoxine HCl) at 1.0×10^5 spores per 10 cm plate in the dark. After 2 days, 700-μL cultures were taken from each strain and transferred to 1.5-mL eppendorf tubes. The cultures were extracted with 700 μL of ethyl acetate (EtOAc)/methanol (MeOH)/acetic acid (AcOH) (89:10:1). The organic phase was evaporated to dryness and redissolved in 100 μL of MeOH. Then 10 μL of dissolved extract was injected for high performance liquid chromatography-photodiode array detection-mass spectrometry (HPLC-DAD-MS) analysis. LC−MS spectra were obtained on a Shimadzu 2010 EV liquid chromatography mass spectrometer using positive and negative electrospray ionization and a Phenomenex Luna 5 μm, 2.0 mm × 100 mm C18 reverse-phase column. Samples were separated on a linear gradient of 5−95% acetonitrile (CH_3CN) in water (0.1% formic acid) for 30 min at a flow rate of 0.1 mL/min followed by isocratic 95% CH_3CN in water (0.1% formic acid) for another 15 min.

Neosartoricin B Extraction and Purification. Neosartoricin B was purified from a 2-day TWY1.1 culture in stationary liquid GMM culture (100 mL/per plate, 10 plates) with 0.5 μM pyridoxine HCl. The compound was extracted from the cultures using equal volume of EtOAc/MeOH/AcOH (89:10:1) twice. After evaporation of the organic phase, the crude extracts were separated by a chromatographic step on a

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Sephadex LH-20 column using MeOH/chloroform ($CHCl_3$) (9:1) as the mobile phase. Further purification was carried out by reverse-phase HPLC using a Beckman Coulter System Gold LC with a Phenomenex Luna 250 × 10 mm, 5 μm C18 column. The compounds were separated using a solvent gradient of 50−80% solvent B (CH_3CN) at a flow rate of 2.5 mL/min over 30 min. Neosartoricins C and D were purified using the same procedures as described above except 0.1% trifluoroacetic acid was added to the HPLC solvents. The HPLC fractions containing pure compounds were pooled and dried completely under vacuum before NMR analysis.

NMR Characterization. All ^1H, ^{13}C, and 2D ($^1H-^{13}C$ HSQC and $^1H-^{13}C$ HMBC) NMR were performed on a Bruker DRX-500 spectrometer at the UCLA Department of Chemistry and Biochemistry NMR facility. $CDCl_3$ was used as the solvent for neosartoricin B, C, and D.

■ ASSOCIATED CONTENT

Supporting Information

Supporting figures, tables, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

W.B.Y. designed and carried out the experiments, created plasmids used, analyzed the data, and wrote the manuscript. R.A.C. and Y.H. analyzed NMR data. A.R.S. and T.C.W. prepared the dermatophyte cultures. Y.H.C. and Y.T. designed the experiments, analyzed the data, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

Note added during revision: During review of the manuscript, a study using a similar genetic approach to study *Aspergillus* metabolites was reported.[21]

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