

Molecular Genetic Mining of the *Aspergillus* Secondary Metabolome: Discovery of the Emericellamide Biosynthetic Pathway

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DOI 10.1016/j.chembiol.2008.05.010

SUMMARY

The recently sequenced genomes of several *Aspergillus* species have revealed that these organisms have the potential to produce a surprisingly large range of natural products, many of which are currently unknown. We have found that *A. nidulans* produces emericellamide A, an antibiotic compound of mixed origins with polyketide and amino acid building blocks. Additionally, we describe the discovery of four previously unidentified, related compounds that we designate emericellamide C–F. Using recently developed gene targeting techniques, we have identified the genes involved in emericellamide biosynthesis. The emericellamide gene cluster contains one polyketide synthase and one nonribosomal peptide synthetase. From the sequences of the genes, we are able to deduce a biosynthetic pathway for the emericellamides. The identification of this biosynthetic pathway opens the door to engineering novel analogs of this structurally complex metabolite.

INTRODUCTION

Secondary metabolites are a rich source of medically useful compounds. The recent sequencing of a number of fungal genomes reveals, surprisingly, that common fungi may be a rich and underexploited source of secondary metabolites. The sequencing of the genomes of four species of the genus *Aspergillus*, for example (Galagan et al., 2005; Machida et al., 2005; Nierman et al., 2005; Pel et al., 2007), revealed that they have many more secondary metabolite pathways than was suspected from decades of natural product chemistry. *A. nidulans* has 27 polyketide synthases (PKSs) and 14 nonribosomal peptide synthetases (NRPSs), but the products of fewer than 10 biosynthesis gene clusters had been identified at the time the genome was completed (Bergmann et al., 2007; Bok et al., 2006; Haas, 2003; Hoffmeister and Keller, 2007; Marquez-Fernandez et al.,

2007; Scherlach and Hertweck, 2006). Moreover, the genes of individual secondary metabolite pathways are almost always clustered together in the genome, which potentially facilitates study and molecular genetic manipulation (Brown et al., 1996).

The realization that common laboratory fungi are a likely source of many more natural products has been accompanied by important advances in the molecular genetic manipulation of some of these fungi. One was the development of fusion polymerase chain reaction (PCR) techniques that have greatly facilitated the production of linear molecules for gene replacements, promoter replacements, and gene tagging (Kuwayama et al., 2002; Nayak et al., 2006; Szewczyk et al., 2006; Yang et al., 2004; Zarrin et al., 2005). A second was the discovery that deletion of KU genes, the products of which are required for nonhomologous end joining, greatly improves gene-targeting frequencies (da Silva Ferreira et al., 2006; Krappmann et al., 2006; Nayak et al., 2006; Ninomiya et al., 2004; Takahashi and Smithies, 1999). We have applied these approaches to natural product discovery in *A. nidulans* and, in particular, have used them to identify and characterize the emericellamide biosynthesis pathway.

RESULTS AND DISCUSSION

With the expectation that deleting each biosynthetic gene will result in the inability to synthesize one or more secondary metabolites in the same biosynthetic pathway, we randomly selected six NRPS genes, using the Broad Institute designation (http://www.broad.mit.edu/annotation/genome/Aspergillus_group/MultiHome.html)—that is, AN0607.3, AN1242.3, AN2545.3, AN2621.3, AN8412.3, and AN9244.3 (see Tables S1 and S2 available online)—and deleted each of them. One of the NRPS genes, AN8412.3, has recently been shown to be involved in aspyridone biosynthesis (Bergmann et al., 2007). The selective disruption of each gene was verified by diagnostic PCR (data not shown). The results of HPLC profiles show that only the AN2545.3 deletant exhibited a different profile from the control, whereas the other five NRPS deletants were the same as the wild-type (data not shown). These results highlight the fact that most biosynthetic gene clusters are cryptic, at least in common laboratory culture conditions. Though both wild-type and the AN2545.3 deletant still produce

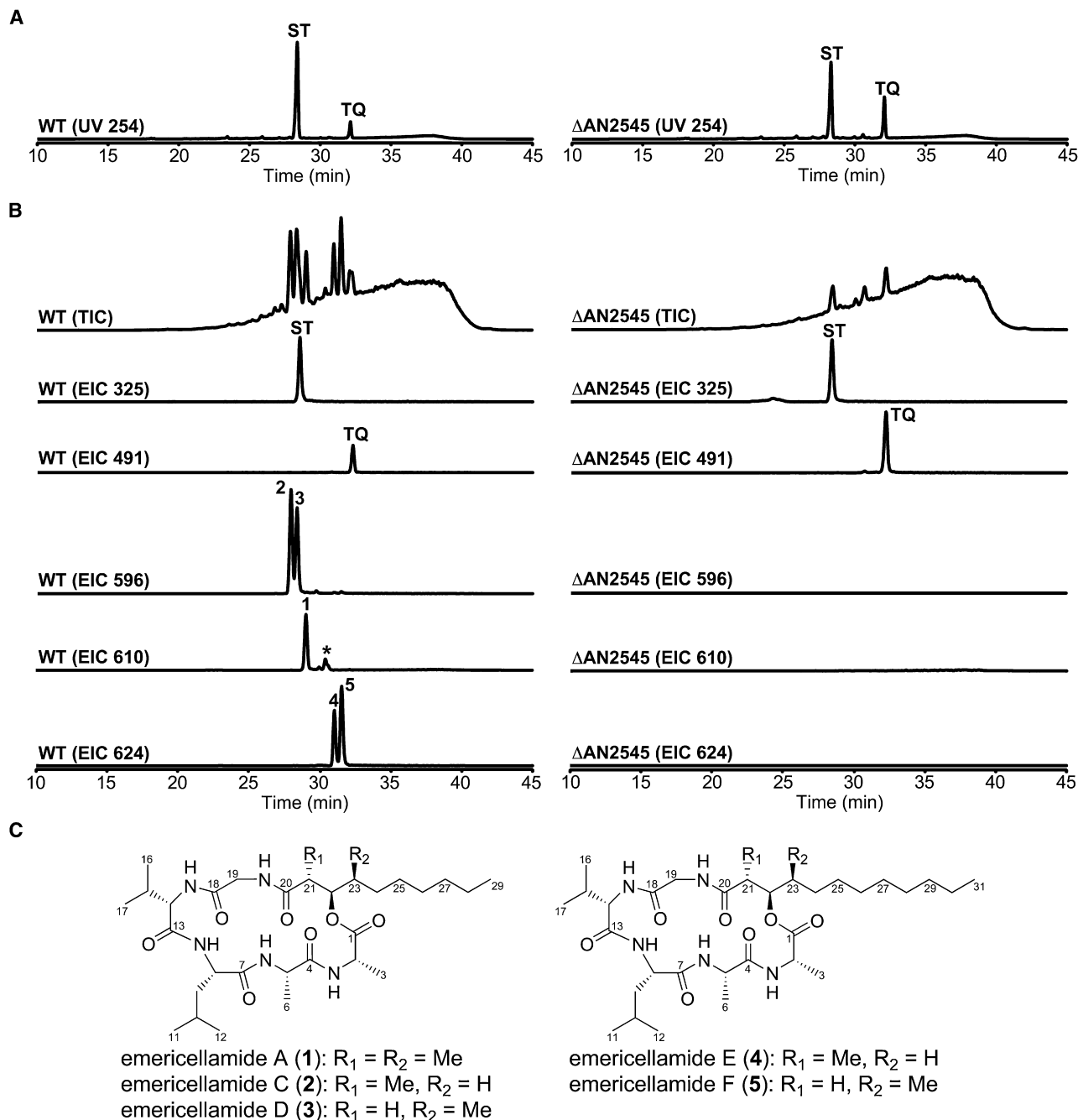


Figure 1. LC-DAD-MS Analysis of Wild-Type *A. nidulans* and Δ AN2545 Mutant Metabolites

(A) UV trace at 254 nm.

(B) Positive ion mode: total ion current (TIC); extracted ion current (EIC) at m/z 325, sterigmatocystin $[M+H]^+$; EIC at m/z 491, terrequinone $[M+H]^+$; EIC at m/z 596, emicellamides C and D $[M+H]^+$; EIC at m/z 610, emicellamide A $[M+H]^+$; EIC at m/z 624, emicellamides E and F $[M+H]^+$. *A minor compound that has same MS/MS fragmentation with emicellamide A.

(C) Chemical structures of emericellamides.

sterigmatocystin (ST) and terrequinone (TQ) (Figure 1A), five compounds with $m/z = 609$ ($m/z = 610$ $[M+H]^+$, compound **1**), $m/z = 595$ ($m/z = 596$ $[M+H]^+$, compounds **2** and **3**), and $m/z = 623$ ($m/z = 624$ $[M+H]^+$, compounds **4** and **5**) were missing in the AN2545.3 deletant (Figure 1B). A search of known *A. nidulans* secondary metabolites from CrossFire Beilstein database re-

vealed that these five compounds had not been previously described in *A. nidulans*.

All five compounds were isolated from large-scale cultivation of wild-type *A. nidulans* by purification, initially by flash chromatography and then by preparative HPLC. Full one- and two-dimensional NMR analysis of compound **1** identified the product

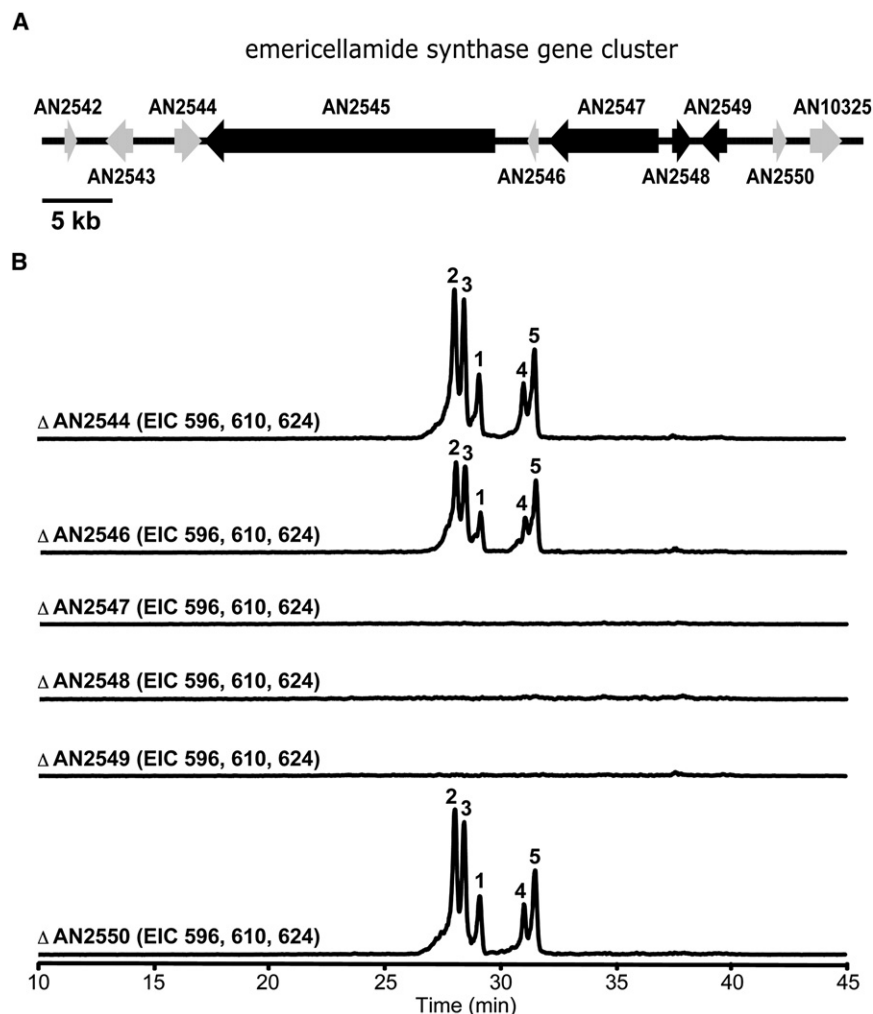


Figure 2. Boundary of Emericellamide Synthesis eas Cluster

(A) Organization of the emericellamide synthase gene cluster in *A. nidulans*. Black open reading frame (ORF), genes involved in emericellamide biosynthesis; gray ORF, genes not involved in the pathway.

(B) LC-MS analysis of *A. nidulans* mutant metabolites in positive ion mode, EIC at m/z 596, emericellamides C and D $[M+H]^+$; EIC at m/z 610, emericellamide A $[M+H]^+$; EIC at m/z 624, emericellamides E and F $[M+H]^+$.

EIC of 610 (Figure 1B). Although not present in sufficient quantity to allow full NMR characterization, this compound could be another long-chained emericellamide that did not undergo methyltransfer.

In fungi, biosynthesis genes of secondary metabolites are generally clustered. To identify genes in the emericellamide biosynthesis pathway, additional genes surrounding the NRPS (from AN2542.3 to AN10325.3; Figure 2A) were deleted and the mutant strains analyzed (Figure 2B and data not shown). The data show that, using the BLAST NCBI protein database, AN2547.3 (a type I PKS homolog), AN2548.3 (an acyltransferase homolog) and AN2549.3 (an AMP-dependent CoA-ligase homolog) are involved in the emericellamide biosynthetic pathway. Though the structures of emericellamides clearly suggest the involvement of a PKS, the involvement of the two additional

as emericellamide A (Figure 1C and Tables S3 and S4), a mixed cyclic polyketide nonribosomal peptide (active against methicillin-resistant *Staphylococcus aureus*) produced by a marine *Emericella* species (Oh et al., 2007). (*Emericella* is the genus designation generally used for species of *Aspergillus* with a demonstrated sexual cycle.) The four other compounds were, to our knowledge, completely new emericellamides (Figure 1C), and we solved their structures by a combination of one- and two-dimensional NMR analyses and by comparison to emericellamide A (1). (For detailed structural elucidation, see the Supplemental Data.) The MS fragmentation also supports the assigned structures (Figures S2 and S3). The intensities of dehydration fragments in compounds 3 and 5 are stronger than compounds 2 and 4 ($m/z = 295, 323, 408, 436,$ and 507 for 2 and 3; $m/z = 323, 351, 436, 464,$ and 535 for 4 and 5). These fragmentation data also support the assigned structures of the aliphatic side chains, because compounds 3 and 5 have two carbonyl α -protons that increase the probability of H_2O elimination to generate α,β -unsaturated carbonyl fragments. Similarity in structures and the single biosynthetic origin leads us to name compounds 2–5 emericellamides C–F (Figure 1C). In addition to emericellamide A (1), a minor peak having the same MS/MS fragments (data not shown) with emericellamide A (1) was also observed in the

genes provides evidence that the transfer of the polyketide intermediates (i.e., the acyl side chains) from the PKS to the first thiolation domain of the NRPS is mediated by additional enzymes. Our data show that deletants of AN2544.3 (a sugar transporter homolog) and AN2546.3 (a hypothetical protein) continue to produce emericellamides.

The biosynthetic pathway of emericellamides can be deduced by analyzing the four genes in the gene cluster, which we designate the eas (emicellamide synthesis) cluster. The biosynthesis of emericellamide initiates from the highly reducing iterative type I polyketide synthase EasB (AN2547.3; Figure 3). The enzyme contains the ketosynthase (KS), acyl transferase (AT), methyltransferase (MT), dehydrogenase (DH), enoylreductase (ER), ketoreductase (KR), and acyl carrier protein (ACP) domains. The single-module fungal polyketide iteratively catalyzes the formation of the linear polyketide chain. Isolation and identification of emericellamide derivatives differing in the polyketide chain length and degree of methylation provide evidence that EasB produces several polyketides that can be further processed by the downstream enzymes. The polyketide intermediate is not transferred directly to the NRPS EasA (AN2545.3) but instead is mediated by two additional enzymes as supported by our genetic data. The polyketide is released from EasB as a linear

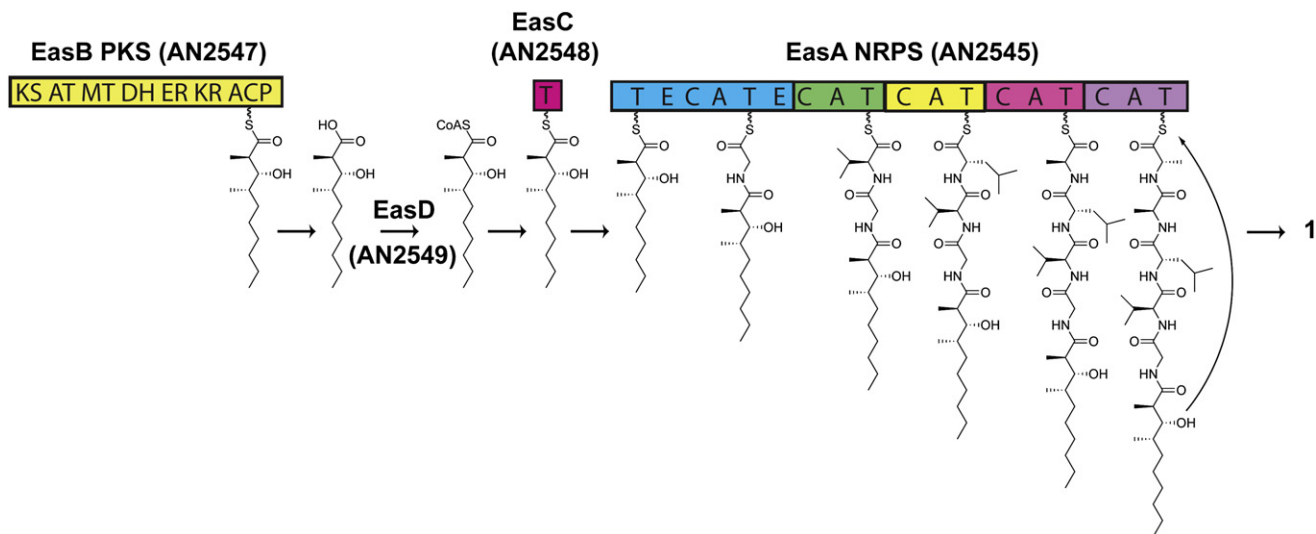


Figure 3. Model for Emericellamide A (1) Biosynthesis

polyketide carboxylic acid, which is converted to a CoA thioester by the acyl-CoA ligase EasD (AN2549.3). The substrate is then loaded onto the acyltransferase EasC (AN2548.3), which shuttles the polyketide intermediate to the first thiolation (T) domain of the NRPS EasA (AN2545.3). Examples of transferase-mediated transfer between carrier protein domains have recently been characterized in the coronamic acid biosynthesis pathway (Strieter et al., 2007). The NRPS EasA is a multimodular enzymatic assembly that contains 18 domains grouped into five modules corresponding to each of the five amino acid monomers incorporated. The second to the fifth modules contain the three core domains, the condensation (C) domain responsible for catalyzing peptide bond formation, adenylation (A) domain responsible for selecting the amino acid monomer substrate, and the thiolation (T) domain. The first module in the NRPS contains a unique T-E-C-A-T-E structure where (E) domains are epimerization domains. We propose that the first of the two thiolation domains is responsible for accepting the incoming polyketide from the acyltransferase EasC. The second thiolation domain is responsible for accepting the glycine amino acid adenylylated by the A domain in the module. The condensation domain then catalyzes the C–N bond formation between the upstream acyl thioester and the downstream aminoacyl thioester. The roles of the two epimerization domains located in the first module of the NRPS are unclear as glycine is the amino acid activated by the module. The NRPS does not contain a TE domain at the end of module 5, presumably suggesting that TE is not necessary for the cyclization of the emericellamides. Of the NRPS in the *A. nidulans* genome, only ACV NRPS (AN2621.3), terrequinone NRPS (AN8513.3), and the unannotated NRPS AN3396.3 contain a TE domain as part of the enzyme. The nine residues that define the signature sequence of A domain selectivity were identified and analyzed using two publicly available bioinformatic software programs (Table 1; Rausch et al., 2005; Yadav et al., 2003). Both software tools, Search NRPS/PKS and NRPSpredictor, correctly predicted that the first module codes for glycine. However, the specificity of the A domain in the second module was only cor-

rectly predicted by NRPSpredictor to be valine. The A domain selectivity of the last three modules was predicted incorrectly by both programs. Many characterized NRPS used by these bioinformatic software tools are bacterial in origin, and understanding of fungal NRPS is still in its infancy. Identification of the products of fungal NRPS from genome sequencing could help in the development of a better understanding of the fungal NRPS code.

SIGNIFICANCE

The rapid completion of fungal genome sequencing provides an unprecedented opportunity to study fungal secondary metabolism pathways. In this communication, we report the application of gene-targeting approaches to functionally characterize a pathway containing both a PKS and an NRPS working to produce the cyclic polyketide peptide emericellamides. Previously emericellamide A was only discovered in a marine *Emericella* and produced in very small amounts when the fungus was cultured alone. When the marine fungus was stimulated by coculturing with a bacterium, production of emericellamide A was increased 100-fold (Oh et al., 2007). By comparison, *A. nidulans* is a well-studied, established laboratory model fungus, and the emericellamides are produced by the *A. nidulans* cultured alone in levels comparable to the bacterium stimulated marine fungus.

Table 1. Signature Sequences of the Adenylation (A) Domains

Amino Acid	Module	Signature Sequence Position								
		235	236	239	278	299	301	322	330	331
Gly	1	D	I	Q	G	V	L	A	M	Q
Val	2	D	A	S	Q	I	G	G	I	Y
Leu	3	D	I	H	F	V	G	A	I	A
Ala	4	D	L	L	V	V	A	G	I	L
Ala	5	D	I	A	I	L	V	A	I	L

Our efficient gene deletion approach allows rapid annotation of the genes in *A. nidulans* and can be applied to other secondary metabolite clusters. Replacement of the native promoter with either inducible or strong constitutive promoters is feasible and can provide a rational route to increase the production of emericellamides. The identification of the emericellamide pathway opens the door to the engineering of the PKS and NRPS for the production of novel analogs. Finally, additional functional annotation in *A. nidulans* and other fungi will improve both our understanding of fungal secondary metabolite biosynthesis and bioinformatic analysis of fungal NRPS and PKS.

EXPERIMENTAL PROCEDURES

Molecular Genetic Procedures

Construction of fusion PCR products, protoplast production, and transformation were carried out as described (Szewczyk et al., 2006). For construction of the fusion PCR fragments, two ~1000 base-pair fragments upstream and downstream of the targeted gene were amplified from genomic *A. nidulans* DNA by PCR. Fusion PCR was set up with the two amplified flanking sequences and the *A. fumigatus* *pyrG* selectable marker cassette. The three fragments were fused into a single molecule and amplified with two nested primers (Figure S1 and Table S2). The *ΔnkuA* strain TN02A3 (Nayak et al., 2006) was used as a recipient strain. Diagnostic PCR of deletant strains was performed using the external primers used in the first round of the PCR. The difference in the size between the gene replaced by the selective marker and the native gene allowed the determination of correct gene replacement. In cases when the sizes of both the wild-type and deletant products were similar, diagnostic PCR was performed using one of the external primers and a primer located inside the marker gene. In those cases, the deletant gave the PCR product of the expected size versus no product in the wild-type strain.

Fermentation and Purification

Spores of *A. nidulans* wild-type R153 and deletion strains were inoculated (1×10^6 /ml) into 100 ml glucose minimal medium (6 g/l NaNO₃, 0.52 g/l KCl, 0.52 g/l MgSO₄ · 7H₂O, 1.52 g/l KH₂PO₄, 10 g/l D-glucose, 1 ml/l trace element solution [Cove, 1966]) supplemented with pyridoxine (0.5 μg/ml) and cultivated at 37°C with shaking at 250 rpm. After 4 days, culture medium was collected by filtration and extracted with the same volume of EtOAc two times. The combined EtOAc layer was evaporated in vacuo and redissolved in MeOH (1 mg/ml) for HPLC-DAD-MS analysis. For structure elucidation, a 10-liter fermentation volume was extracted with EtOAc to yield a gray powder (350 mg), which was applied to a Si gel column (Merck 230–400 mesh, ASTM, 10 × 100 mm) and eluted with CHCl₃-MeOH mixtures of increasing polarity (fraction A, 1:0, 300 ml; fraction B, 19:1, 300 ml; fraction C, 9:1, 300 ml; fraction D, 7:3, 300 ml). The solvent of fraction B, containing emericellamides, was evaporated in vacuo and resuspended in CHCl₃. The emericellamides, which do not dissolve in CHCl₃, were collected by filtration. Enriched emericellamides were separated by preparative HPLC (Phenomenex Luna 5 μm C18 [2], 250 × 21.2 mm) with a flow rate of 10.0 ml/min and measured by a UV detector at 200 nm. The gradient system was MeCN (solvent B) in 5% MeCN/H₂O (solvent A): 60% to 100% B from 0 to 15 min, 100% B from 15 to 20 min, and re-equilibration with 60% B from 20 to 22 min. A mixture of emericellamides C and D (6.6 mg), emericellamide A (1.2 mg), and a mixture of emericellamides E and F (5.6 mg) were eluted at 11.4, 12.5, and 14.8 min, respectively. Emericellamides C (2.8 mg, $t_R = 36.2$ min) and D (2.5 mg, $t_R = 37.8$ min) were further purified by the same HPLC using isocratic 50% B solvent. Emericellamides E (1.4 mg, $t_R = 47.0$ min) and F (2.6 mg, $t_R = 49.1$ min) were further purified by the same HPLC using isocratic 55% B solvent.

SUPPLEMENTAL DATA

Supplemental Data include detailed structural characterization of emericellamides, *A. nidulans* strains and primers used in this study, fusion PCR for gene disruption, ¹H and ¹³C NMR spectral data, and MS/MS fragmentation

of emericellamides, and are available with this article online at <http://www.chembiol.com/cgi/content/full/15/6/527/DC1/>.

ACKNOWLEDGMENTS

This work was supported by NIH grants GM 075857 (C.C.C.W.), GM 031837 (B.R.O.), and GM 084077 (C.C.C.W. and B.R.O.). H.S. is a recipient of the USC School of Pharmacy Undergraduate Summer Fellowship. E.K. is a recipient of the Rose Hill Undergraduate Fellowship. K.W. is a recipient of fellowships from the Japan Antibiotics Research Association, Pfizer Infectious Disease Foundation, and the Agricultural Chemical Research Foundation. High-resolution mass spectrometry analyses were performed in the Mass Spectrometry Laboratory of the Division of Chemistry and Chemical Engineering at the California Institute of Technology, supported in part by the National Science Foundation Materials and Research Science and Engineering Program. We also thank Brian Stoltz for access to a digital polarimeter and IR spectrophotometer.

Received: December 13, 2007

Revised: April 28, 2008

Accepted: May 6, 2008

Published: June 20, 2008

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