

Molecular Genetic Characterization of the Biosynthesis Cluster of a Prenylated Isoindolinone Alkaloid Aspernidine A in *Aspergillus nidulans*

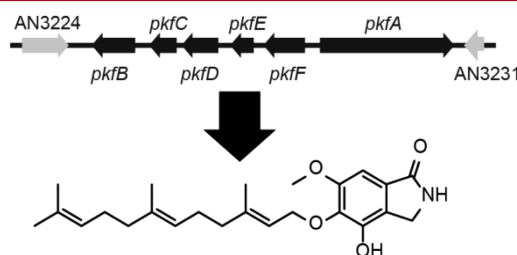
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ABSTRACT



Aspernidine A is a prenylated isoindolinone alkaloid isolated from the model fungus *Aspergillus nidulans*. A genome-wide kinase knockout library of *A. nidulans* was examined, and it was found that a mitogen-activated protein kinase gene, *mpkA*, deletion strain produces aspernidine A. Targeted gene deletions were performed in the kinase deletion background to identify the gene cluster for aspernidine A biosynthesis. Intermediates were isolated from mutant strains which provided information about the aspernidine A biosynthesis pathway.

Secondary metabolites (SMs) that filamentous fungi produce have served as a valuable source of low molecular weight molecules with a variety of biological activities. Many of the bioactive SMs that are easily accessible under conventional laboratory conditions have already been isolated and patented for drug development. However, advances in

genome sequencing revealed that there is an abundance of potential SM gene clusters that have yet to be associated with their final metabolites.^{1–3}

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One major group of SMs is polyketides (PKs) whose core structure is furnished by polyketide synthases (PKSs). Using a variety of genome mining methods, we and others have successfully identified the immediate products of all 14 nonreducing (NR)-PKSs in the model fungus *A. nidulans*.^{4–9} However, for several of these NR-PKSs in *A. nidulans*, the final, downstream metabolites remain unknown. We are interested in using this information to comprehensively link metabolites to genes and to elucidate their biosynthetic pathways.

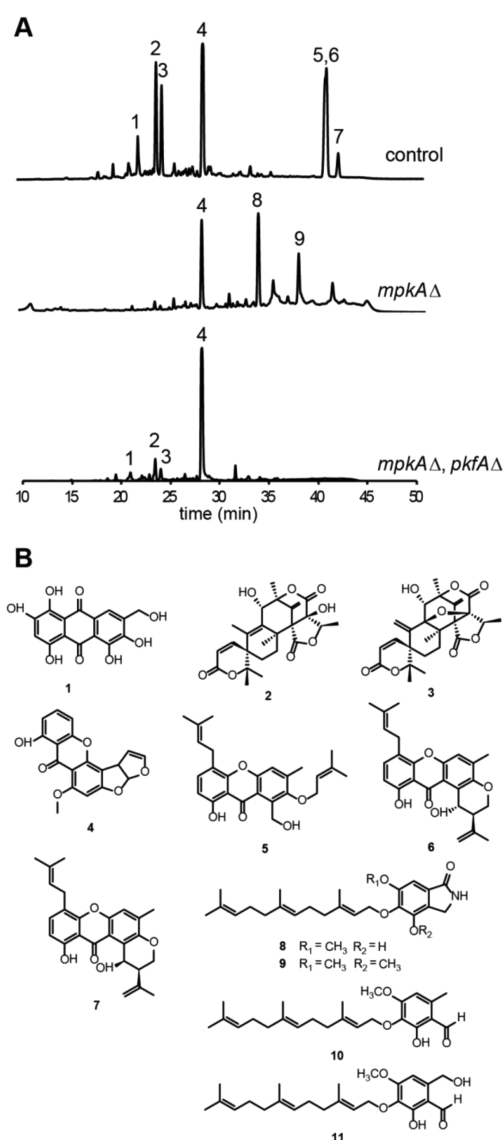


Figure 1. LC-DAD-MS analysis of a control strain and strains carrying *mpkAΔ* and both *mpkAΔ* and *pkfAΔ*. (A) HPLC profiles of extracts of strains as detected by UV absorbance at 254 nm. (B) Structures of compounds elucidated throughout this study. The compounds are as follows: asperthecin, **1**; austinol, **2**; dehydroaustinol, **3**; sterigmatocystin, **4**; emericellin, **5**; shamixanthone, **6**; epishamixanthone, **7**; aspernidine A, **8**; aspernidine C, **9**; aspernidine D, **10**; aspernidine E, **11**.

One such metabolite of interest is aspernidine A. Aspernidine A was discovered previously by the Hertweck group by screening 45 different culture conditions using the *A. nidulans* AXB4A2 strain (see Table S2, Supporting Information, for genotype) and was isolated from 14 L of malt medium cultured for 7 days.¹⁰ The aromatic group in this compound suggested that its biosynthesis is initiated by an NR-PKS. Prior to this study, our group had not been able to detect the production of aspernidine A in the genome-sequenced strain *A. nidulans* FGSC A4 and all other FGSC A4 background mutant strains. A chief requirement for the elucidation of the genetic biosynthetic basis of SMs is the availability of a strain that expresses the required gene cluster to yield enough material for chemical and biological characterization. We have recently obtained and screened a genome-wide kinase knockout (KO) library, a resource provided by the Fungal Genetics Stock Center (FGSC) to the *Aspergillus* research community,¹¹ with the expectation that manipulating the expression of kinases, which are key players in many aspects of regulation and signal transduction, would be a novel approach for activating cryptic gene clusters. Through screening of this library (a total of 98 kinase KO strains), we found one strain (*mpkAΔ*) that consistently produced compounds **8** and **9** which were distinctly different from the compounds produced by the control strain (**1–7**) (Figure 1A). Compound **8** was isolated from this strain from yeast agar glucose (YAG) medium at a titer of almost 15 mg/L and identified as aspernidine A. *MpkA* is one of four mitogen-activated protein kinase (MAPK) genes that the *A. nidulans* genome harbors.^{12–17} The kinase has been shown to play a pivotal role in cell wall integrity signaling as well as in regulation of germination

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of conidial spores and polarized growth.^{18,19} The discovery of a strain of *A. nidulans* that produces aspernidine A (**8**) in high titer allowed us to identify and analyze the gene cluster involved in its biosynthesis. Herein we report the identification of the biosynthetic gene cluster for aspernidine A biosynthesis through a series of targeted gene deletions in the *mpkAΔ* background. We isolated and characterized 3 novel related compounds, aspernidine C-E (**9–11**), allowing us to propose a biosynthesis pathway for aspernidine A.

For full characterization of compound **8**, the *mpkAΔ* strain was cultivated at a larger scale and the target compound was purified using flash chromatography and preparative HPLC. ¹H and ¹³C NMR analysis identified **8** as aspernidine A.¹⁰ Additionally, a potentially new metabolite **9** was isolated, and the similarity of UV spectra and MS fragmentation patterns (Figure S1, Supporting Information) suggested it was structurally related to **8**. Compound **9** had a molecular formula of C₂₅H₃₅NO₄ (deduced from HRESI-MS), suggesting the presence of an additional methyl group. This was confirmed by one- and two-dimensional NMR spectroscopy (Figures S2 and S3, Supporting Information). Though several isoindoline derivatives have been identified from fungi, compound **9** appears to be a new compound, which we named aspernidine C.

The chemical structure of aspernidine A led us to hypothesize that it might be derived from orsellinaldehyde, which was shown previously to be the product of the NR-PKS, PkfA.⁴ To test this hypothesis, we generated a *pkfA* deletion in the *mpkAΔ* background, replacing *pkfA* with the *A. fumigatus pyroA* gene, a nutritional selection marker required for pyridoxine biosynthesis. The deletion was verified by diagnostic PCR. We cultured this *mpkAΔ*, *pkfAΔ* double mutant and the *mpkAΔ* strain under the same conditions that yielded aspernidine A. In the double mutant, aspernidine A production was eliminated (Figure 1A). This confirmed that PkfA, the PKS encoded by *pkfA*, is required for the biosynthesis of aspernidine A.

Table 1. Putative Function of Genes within the Aspernidine A Cluster

gene designation	putative function	AspGD annotation	Broad annotation
<i>pkfB</i>	cytochrome p450	AN3225	ANID_03225.1
<i>pkfC</i>	short-chain dehydrogenase	AN3226	ANID_03226.1
<i>pkfD</i>	hypothetical protein	AN3227	ANID_03227.1
<i>pkfE</i>	prenyltransferase	AN3228	ANID_03228.1
<i>pkfF</i>	choline dehydrogenase	AN3229	ANID_03229.1
<i>pkfA</i>	polyketide synthase	AN3230	ANID_03230.1

We next set out to identify additional genes involved in aspernidine A biosynthesis. Taking advantage of the fact that

secondary metabolism genes in *A. nidulans* are usually clustered, we focused on the genes surrounding *pkfA* (Table 1, Figure 2A). We performed gene deletions in the *mpkAΔ* background, and all deletions were verified by diagnostic PCR. Again, all strains were cultivated in the same culture conditions that yielded aspernidine A. LC/MS analysis of the extracts from the gene deletion strains showed elimination of aspernidine A in deletants of genes from AN3225 through AN3230 (Figure 2B). The putative functions of the genes within the cluster along with the genes immediately outside are shown in Table 1. Deletants of AN3224 and AN3231 continued to produce aspernidine A, indicating that we have identified the boundaries of the gene cluster. We now designate the genes surrounding *pkfA* as *pkfB–pkfF*.

In silico analysis was carried out using the Aspergillus 16-way comparative database asp2_v7 provided through the Aspergillus Genome Database (AspGD, <http://www.aspgd.org/>) to compare the surrounding genes of this proposed gene cluster with those of other fungal species. Interestingly, this analysis shows that our proposed aspernidine A gene cluster is present as an insertion of ~20kb in *A. nidulans* within a highly conserved region of the *Aspergillus* genome (Figure S8, Supporting Information). Furthermore,

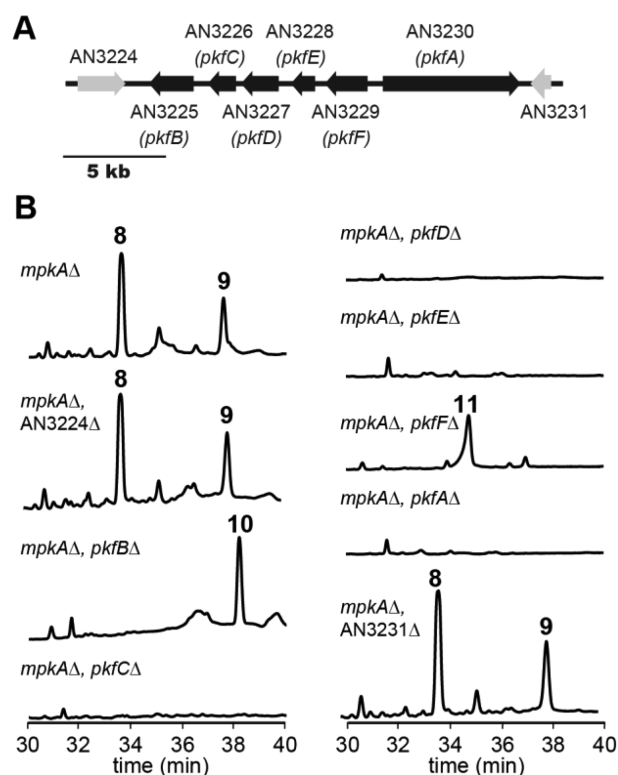
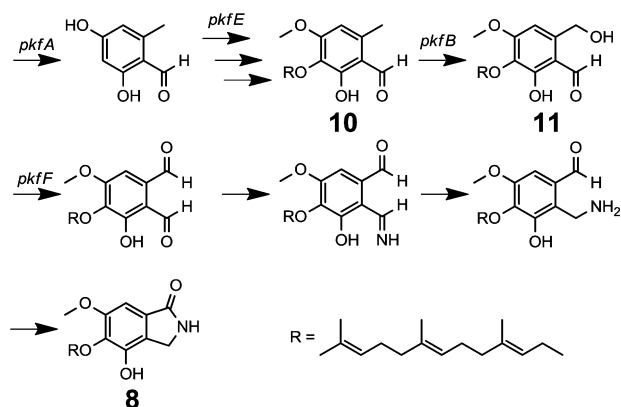


Figure 2. Boundary of the aspernidine A biosynthesis gene cluster. (A) Organization of genes surrounding the PKS *pkfA* involved in aspernidine A biosynthesis in *A. nidulans*. Black open reading frames (ORFs) are genes involved in aspernidine A biosynthesis, while gray ORFs are genes not involved in aspernidine A biosynthesis. (B) HPLC profile of extracts of strains in the cluster as detected by UV absorption at 254 nm. Numbers on peaks correspond to the compounds shown in Figure 1B.

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Scheme 1. Proposed Biosynthetic Pathway of Aspernidine A



Andersen et al. developed an algorithm to accurately predict SM gene clusters based on an annotated genome sequence and a catalog of gene expression.²⁰ Using this algorithm, they predicted the number of genes in the cluster for *pkfA* to be six, which matches our findings from targeted gene deletions. These data further suggest that we have correctly determined the extent of the gene cluster responsible for the biosynthesis of aspernidine A.

The deletant strains in which aspernidine A production was eliminated were examined for intermediates or shunt products that are part of the biosynthetic process for aspernidine A (Figure 2B). Extracts from strains carrying deletions of *pkfC*, *pkfD*, *pkfE*, and *pkfA* showed no obvious intermediates. Strains carrying deletions of *pkfB* and *pkfF* each displayed a significant new peak in the chromatogram. Both strains were cultured at a larger scale and the metabolites were isolated by flash chromatography and preparative HPLC. The structures of compounds **10** and **11**, which we have named aspernidine D and E, respectively (Figure 1B), were determined using both one- and two-dimensional NMR spectroscopy (see the Supporting Information for detailed structural characterization, Supplementary Figures S4–S7).

Our gene deletion data and the intermediates we isolated and structurally characterized by NMR allow us to

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propose a biosynthetic pathway for aspernidine A (Scheme 1). The starting point is the production of orsellinaldehyde by the NR-PKS PkfA as shown previously.⁴ Although we were unable to identify the immediate products after polyketide biosynthesis, isolation of compound **10** from the *mpkAΔ*, *pkfBΔ* strain led us to propose that hydroxylation, methylation of one of the phenol groups, and prenylation, presumably catalyzed by the gene product of *pkfE*, a prenyltransferase gene, would be needed to yield aspernidine D (**10**). Subsequently, the gene product of the cytochrome P450 monooxygenase gene *pkfB* is responsible for hydroxylation of aspernidine D (**10**) to yield aspernidine E (**11**). An aromatic dialdehyde, asperugin A, was found as a metabolic product of *A. rugulosus*, a species closely related to *A. nidulans*.²¹ This compound shows structural relation to the aspernidines,¹⁰ leading us to hypothesize that the choline dehydrogenase gene *pkfF* may be responsible for further oxidation of aspernidine E (**11**), to form this dialdehyde intermediate. Furthermore, this intermediate will need to be transformed in a series of steps, some of which are enzyme-mediated, to generate aspernidine A (**8**). Although fungal SM biosynthetic genes are often clustered in one region of the chromosome, we have identified two examples in *A. nidulans* where the genes are located in at least two distinct genomic loci and therefore we cannot exclude the possibility that additional genes in the genome are involved in aspernidine A biosynthesis.^{22,23}

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Supporting Information Available. Experimental methods, primers and *Aspergillus nidulans* strains used in this study, synteny alignment of surrounding genes, compound characterization, and spectral data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The authors declare no competing financial interest.