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A gene cluster containing two fungal polyketide synthases encodes the biosynthetic pathway for a polyketide, asperfuranone, in *Aspergillus nidulans*

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Abstract

The genome sequencing of *Aspergillus* species including *A. nidulans* reveals that the products of many of the secondary metabolism pathways in these fungi have not been elucidated. Our examination of the 27 polyketide synthases (PKS) in *A. nidulans* revealed that one highly reduced PKS (HR-PKS, AN1034.3) and one non-reduced PKS (NR-PKS, AN1036.3) are located next to each other in the genome. Since no known *A. nidulans* secondary metabolites could be produced by two PKS enzymes, we hypothesized that this cryptic gene cluster produces an unknown natural product. Indeed after numerous attempts we found that the products from this cluster could not be detected under normal laboratory culture conditions in wild type strains. Closer examination of the gene cluster revealed a gene with high homology to a citrinin biosynthesis transcriptional activator (CtnR, 32% identity/47% similarity), a fungal transcription activator located next to the two PKSs. We replaced the promoter of the transcription activator with the inducible *alcA* promoter, which enabled the production of a novel polyketide that we have named asperfuranone. A series of gene deletions has allowed us to confirm that the two PKSs together with five additional genes comprise the asperfuranone biosynthetic pathway and leads us to propose a biosynthetic pathway for asperfuranone. Our results confirm and substantiate the potential to discover novel compounds even from a well-studied fungus by using a genomic mining approach.

Introduction

Aspergillus species are well known producers of medicinally important natural products such as lovastatin. As more and more *Aspergillus* species genomes are sequenced, it is becoming

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clear that there are far more secondary metabolism biosynthesis pathways than there are known secondary metabolites.[1–5] The large number of secondary metabolism gene clusters with unknown products has prompted a renewed search for secondary metabolites from *Aspergillus* species, even species such as *A. nidulans* that have been well studied in the lab.[6–8] By taking advantage of genomic sequencing and the development of a facile method for gene targeting in *A. nidulans*, we recently identified and characterized the emericellamide biosynthesis pathway, which involves one nonribosomal peptide synthetase (NRPS) and one polyketide synthase (PKS).[9,10] The search for new compounds in fungi fortunately has also been accompanied by advancements in the understanding of fungal secondary metabolite regulation. For example, the terrequinone A gene cluster was identified by the utilization of global regulator of secondary metabolism, *laeA*, followed by targeted gene deletions.[7] Another approach has been to upregulate the transcription activators that are often within silent metabolic clusters, and this has lead to the identification of the novel hybrid polyketide and nonribosomal peptide metabolites, aspyridones.[6]

Fungal polyketides are produced by multidomain type I PKSs which are iterative in nature. Fungal PKSs can be further grouped into non-reduced (NR), partially reduced (PR) and highly reduced (HR) PKS by examining the domains encoded in the gene.[11] NR-PKSs usually contain starter unit ACP transacylase (SAT), β-ketoacyl synthase (KS), acyl transferase (AT), product template (PT), acyl carrier protein (ACP), and Claisen-cyclase/thiolesterase (CLC/TE) domains. In some cases the NR-PKS also contains methyl transferase (CM) and reductase (R) domains. PR-PKSs contain KS, AT, dehydratase (DH), ketoreductase (KR), and terminate with ACP. HR-PKSs, on the other hand, contain KS, AT, and DH domains. In many cases DH is followed by a CM, enoyl reductase (ER), KR and a terminating ACP domain. The iterative nature of fungal PKSs means that in the majority of cases there is only one PKS involved in the biosynthesis of a particular fungal polyketide. However polyketides such as citrinin[12,13] and zearalenone[14] contain structures that require high levels of reduction early during biosynthesis and no reduction during the later steps, arguing that in certain cases two PKS are necessary where one PKS makes an advanced starter unit, which is passed on to a second PKS for further extension.[11] In the *A. nidulans* genome, there are at least three clusters where two PKS genes are located next to each other on the chromosome (AN1034.3 and AN1036.3, AN2032.3 and AN2035.3, and AN3612.3 and AN10430.3 using the Broad Institute gene designation system), two of which, AN1034.3 and AN2032.3, are NR-PKS. Here we report the induction of the cryptic AN1034.3 and AN1036.3 pathway in *A. nidulans* and the isolation of a novel polyketide, asperfuranone. A series of gene deletion experiments has allowed us to determine the boundaries of the asperfuranone gene cluster and the genes involved in the pathway.

Results and Discussion

Examination of the *A. nidulans* genome reveals that one NR-PKS (AN1034.3) and one HR-PKS (AN1036.3) are located adjacent to each other in the chromosome. These genes are not sub-telomeric and are located in contig 15 (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html) on the right arm of chromosome VIII (http://www.gla.ac.uk/ibls/molgen/aspergillus/viiicontigs.html). We were curious as to whether the two PKSs could be responsible for the biosynthesis of a novel polyketide in *A. nidulans*. Attempts to culture *A. nidulans* under different culture conditions, however, did not result in the isolation of polyketides that would be biosynthesized by both the NR-PKS and HR-PKS. In addition, no difference in the secondary metabolite profile was observed when we compared an *A. nidulans* strain containing a deletion of AN1034.3 (AN1034.3Δ) against wild type strains (data not shown). These results suggested to us that the biosynthesis cluster in question is silent under normal laboratory growth conditions. Closer examination of the genes

surrounding the two PKSs revealed a gene, AN1029.3, with homology to *CtnR*, a citrinin biosynthesis transcriptional activator.[13] The N-terminus of AN1029.3 contains a fungal Zn_2Cys_6 binuclear cluster DNA-binding domain (Cys^{16}-Xaa_2-Cys^{19}-Xaa_6-Cys^{26}-Xaa_6-Cys^{33}-Xaa_2-Cys^{36}-Xaa_6-Cys^{43}). We attempted to turn on the expression of the entire gene cluster by selectively inducing expression of this transcription factor. Although at this point it wasn't clear if the two PKSs were from one cluster or from two adjacent secondary metabolite clusters, we replaced the native promoter of the C6 transcription factor AN1029.3 with the inducible alcohol dehydrogenase promoter *alcA*(p).[15] The replacement was accomplished using our previously reported strategy involving a *nkuA*Δ strain and fusion PCR.[10] The selective replacement of the promoter was verified using diagnostic PCR (data not shown). The promoter replacement was carried out in an *A. nidulans* strain carrying *stcJ*Δ which prevents production of the major polyketide sterigmatocystin.[16] The rationale for the use of a *stcJ*Δ strain is that the elimination of sterigmatocystin frees up the common polyketide precursor malonyl-CoA and facilitates detection and isolation of other metabolites. We compared the metabolite profile of the promoter replaced mutant under normal and inducing conditions (Figure 1). The resulting HPLC profile showed that one major and one minor new UV-active peaks could be detected in the induced strain, providing strong evidence that selective activation of the pathway-specific transcription factor AN1029.3 is capable of activating the entire gene cluster.

The new compounds were purified from large scale cultures by flash chromatography followed by preparative HPLC. We found that the minor compound was unstable and decomposed prior to the completion of the isolation process. The major compound however could be isolated and its structure was determined by a series of one- and two-dimensional NMR analyses. The absolute configurations of C-12 and C-13 were determined to be 12*R* and 13*S*, respectively, by a modified Mosher's method (for details of structure elucidation, see Supplemental data). C-3 was assigned as 3*S* since asperfuraone (**1**) is biosynthesized from compound **2** for which the C-3 stereochemistry has been determined previously (see below). This compound (**1**) has a completely novel polyketide structure consisting of a conjugated alkene chain and a furan subunit (Figure 3). We provide the name asperfuranone for this molecule. Asperfuranone (**1**) shares structural similarities with the azaphilone class of natural products.[17] Azaphilones are mostly fungal in origin and other members include sclerotiorin,[18] chlorofusin,[19–22] and isochromophilones.[23] To our knowledge, the asperfuranone biosynthesis pathway identified in this study is the first known biosynthesis pathway described for this class of natural products. Its structure, including a highly reduced side chain and an aromatic furan, provides evidence that two different types of PKSs are necessary for its biosynthesis.

In *Aspergillus*, identification of genes responsible for the biosynthesis of a specific polyketide is facilitated by the fact that these biosynthesis genes are usually clustered. We deleted twelve genes surrounding the two PKSs to identify genes involved in asperfuranone biosynthesis, from AN1027.3 to AN11288.3 (Figure 2A). Because the *A. nidulans* genome annotation has been revised three times the gene numberings are no longer strictly sequential. AN1029.3 was not deleted because the induction of this gene was necessary for asperfuranone (**1**) production. The twelve deletants were cultivated under inducing conditions and their metabolite profiles examined by LC/MS. Analysis of the deletants shows that AN1033.3Δ, AN1034.3Δ, AN1035.3Δ and AN1036.3Δ fully eliminated asperfuranone (1) production while AN1031.3Δ and AN1032.3Δ strongly diminished production (Figure 2B). We believe we have established the border of the gene cluster using our deletion studies since AN1027.3Δ, AN1028.3Δ, AN1030.3Δ, AN11287.3Δ, AN1037.3Δ, and AN11288.3Δ_strains all produced asperfuranone (1) in substantial, albeit varying, amounts. We designate this cluster as the *afo* gene cluster. The genes involved in asperfuranone (**1**) production are, AN1029.3 (which we now designated *afoA* using the standard *A. nidulans* gene format) the transcription factor that regulates expression of the cluster, AN1031.3 (*afoB*) a gene with high homology to efflux

pumps, AN1032.3 (*afoC*) a gene with high homology to citrinin biosynthesis oxidoreductase, AN1033.3 (*afoD*) a gene with high homology to salicylate hydroxylase, AN1034.3 (*afoE*) a NR-PKS, AN1035.3 (*afoF*) a gene with high homology to FAD/FMN-dependent oxygenase, and AN1036.3 (*afoG*) a HR-PKS (Table 1). Asperfuranone (**1**) is thus biosynthesized by two polyketide synthases (AN1034.3 and AN1036.3) and three additional genes (AN1032.3, AN1033.3, and AN1035.3). Interestingly, deletion of AN1031.3 a gene with high homology to efflux pumps also strongly diminished asperfuranone (**1**) production, indicating that this efflux pump is likely responsible for the transport of this polyketide out of the cell.

Among the genes involving in asperfuranone (1) production, AN1033.3Δ produced a large amount of intermediates that were UV 254 nm active. We were able to purify three of the compounds (**2**, **3**, and **4**, Figure 2C) from large scale culture by flash chromatography followed by preparative HPLC. The major compound **2** was identified as 2,4-dihydroxy-6-(5,7-dimethyl-2-oxo-*trans*-3-*trans*-5-nonadienyl)-3-methylbenzaldehyde using both one- and two-dimensional NMR analyses in acetone-d_6. (for details of structure elucidation, see Supplemental data) as well as by comparison to the NMR spectra according to the literature[17] in chloroform-*d*. Interestingly, when the NMR spectra of compounds **3** and **4** were taken in acetone-d_6, both compounds showed superimposeable ^{1}H and ^{13}C spectra with compound **2**, suggesting that they were compounds which were either tautomers or in equilibrium (Figure 2D). When dissolved in MeOH for LC/MS analysis, compound **2** equilibrated to compounds **3** and **4**, and these compounds could be resolved in HPLC. When dissolved in acetone-d_6 for NMR analysis, all the isomers favored the equilibrium state of compound **2**. From the evidence above, compounds **3** and **4** were proposed to be six-member-ring α and βhemiacetals (Figure 2D).

By analyzing the genes involved in asperfuranone (**1**) production and the intermediate (**2**) isolated from the AN1033.3 deletant, we were able to propose a biosynthetic pathway for asperfuranone (**1**) (Figure 3). AN1036.3 (*afoG*), a HR-PKS is responsible for producing the 3,5-dimethyloctadienone moiety from acetyl-CoA, three malonyl-CoA, and two SAM (S-adenosyl methionine). The 3,5-dimethyloctadienone moiety is then loaded onto the SAT domain of AN1034.3 (*afoE*) and extended with four malonyl-CoA and one SAM. Interestingly, the C terminal region of AN1034.3 (*afoE*) contains a male sterility protein homolog which probably functions as a fatty acyl reductase.[24] This domain also has high homology to the R domain (thiolester reductase) of 3-methylorcinaldehyde synthase (MOS), recently described by Cox and Simpson et al.[25] Consistent with the reductive releasing activity of the R domain found in AN1034.3 (*afoE*), compound **2** could be synthesized by AN1034.3 (*afoE*) after reductive release and aldol condensation. Compound **2** has been isolated from other azaphilone producing organisms and is likely a common intermediate in the biosynthesis of this entire class of natural products.[17] This finding also suggests that the diversity of azaphilones structures is due to additional downstream modifying enzymes.

The AN1033.3 (*afoD*) deletant accumulated a large amount of compound **2** suggesting that AN1033.3 is the next enzyme in the biosynthesis sequence. AN1033.3 hydroxylates the side chain at the benzylic position of compound **2**. After benzylic hydroxylation, a furan ring is formed after five-member ring hemiacetal formation and water elimination (Figure 3). AN1035.3 (*afoF*) and AN1032.3 (*afoC*) are proposed to oxidize the α-diketone proton and to reduce the unconjugated carbonyl group, respectively, to generate asperfuranone (**1**). Since no intermediates could be isolated form AN1035.3 (*afoF*) and AN1032.3 (*afoC*) deletants, the sequence of these two enzymes is not fully understood. Since AN1032.3 (*afoC*) deletant still produces a small amount of asperfuranone (**1**), other endogenous oxydoreductase might catalyze the same reaction with much less efficiency.

The asperfuranone gene cluster contains two transcription factors. By replacing the native promoter of AN1029.3 with an *alcA* inducible promoter we showed that the entire asperfuranone gene cluster can be induced. However, when the second transcription factor AN1028.3 in the cluster was deleted, the pathway was still induced. These results indicate that induction of AN1029.3 alone is sufficient to turn on the whole asperfuranone biosynthetic pathway and AN1028.3 is probably not involved in the regulation of the pathway.

Genomic sequencing has revealed that the fungal secondary metabolome is far richer than once thought. In this study we report that by inducing a transcription activator located next to two PKSs genes, we have been able to identify and activate an otherwise cryptic pathway. The pathway produces a novel polyketide, asperfuranone (**1**) with structural similarities to the azaphilone class of natural products. We showed by a series of gene deletion experiments that biosynthesis of asperfuranone (**1**) requires, at a minimum, two PKS and three additional enzymes. The identification of the asperfuranone biosynthesis gene cluster in the model organism *A. nidulans* is significant because the vast number of genetic tools available for *A. nidulans* will facilitate the rational biosynthesis pathway engineering of asperfuranone and possibly other azaphilones.

Materials and Methods

Strains and molecular genetic manipulations

A. nidulans strains used in this study are listed in Table S1. Construction of fusion PCR products, protoplast production and transformation were carried out as described.[10] Primers used in this study are listed in Table S2. Diagnostic PCR of the deletant and WT strains was performed using the external primers used in the first round of PCR. The difference in the size between the gene replaced by the selective marker and the native gene allowed us to determine if transformants carried correct gene replacements. In cases in which the sizes of both the WT and deletant products were similar, the diagnostic PCR was performed using one of the external primers and the primer located inside the marker gene. In those cases, the deletants gave the PCR product of the expected size while no product was present in non-deletants.

Fermentation and LC/MS analysis

A. nidulans alcA(p)-AN1029.3 and asperfuranone cluster gene deletant strains (Table S1) were grown in 50 ml liquid LMM medium (15g/L lactose, instead of 10g/L glucose in GMM medium)[9] supplemented when necessary with pyridoxine (0.5 mg/L) at 37°C with shaking at 200 rpm. For AN1029.3 induction, cyclopentanone at a final concentration of 30 mM was added to the medium after 18 h of incubation. Culture medium was collected 48 h after cyclopentanone induction by filtration and extracted twice with a volume of EtOAc equal to the culture volume. The combined EtOAc layers were evaporated *in vacuo*, re-dissolved in 1 mL of MeOH and 10 μL was injected for HPLC-DAD-MS analysis. LC/MS was carried out in positive mode using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer with an RP C_{18} column (Alltech Prevail C18 3 μm 2.1 × 100mm) at a flow rate of 125 μL/min. The solvent gradient for HPLC was 95% MeCN/H_2O (solvent B) in 5% MeCN/H_2O (solvent A), both containing 0.05% formic acid: 0% B from 0 to 5 min, 0 to 100% B from 5 to 35 min, maintained at 100% B from 35 to 40 min, 100 to 0% B from 40 to 45 min, and re-equilibration with 0% B from 45 to 50 min. Conditions for MS included a capillary voltage 5.0 kV, a sheath gas flow rate at 60 arbitrary units, an auxiliary gas flow rate at 10 arbitrary units, and the ion transfer capillary temperature at 350°C.

Compound spectral data

Optical rotations were measured on a JASCO P-1010 digital polarimeter, IR spectra were recorded on a Perkin–Elmer 983G spectrophotometer. NMR spectra, detailed in supplementary

data, were run on a Varian Mercury Plus 400 spectrometer, and HRESIMS spectrum was obtained on Waters LCT Premier XE electrospray time-of-flight mass spectrometer.

Asperfuraonone (1)—light yellow gum; $[\alpha]_D^{22}$ +8.4° (MeOH, c 0.4); IR ν_{max}^{ZnSe} 3385, 1682, 1659, 1580, 1418, 1184, 1140, 1059, 979 cm^{-1}; For UV-Vis and ESIMS spectra, see Figure S1; For ^1H and ^{13}C NMR data (acetone-d_6), see Table S3. HRESIMS, $[M+H]^+$ *m*/*z* found 333.1696; calc. for $C_{19}H_{25}O_5$: 333.1702.

2,4-Dihydroxy-6-(5,7-dimethyl-2-oxo-*trans*-3-*trans*-5-nonadienyl)-3-methylbenzaldehyde (2)—colorless needle; $[\alpha]_D^{21}$ +47.3° ($CHCl_3$, c 0.3); IR ν_{max}^{KBr} 3235, 2962, 1669, 1615, 1589, 1426, 1303, 1255, 1188, 1125, 1093, 982 cm^{-1}; For UV-Vis and ESIMS spectra, see Figure S1; For ^1H and ^{13}C NMR data (acetone-d_6), see Table S3; ^1H and ^{13}C NMR data ($CDCl_3$), in good agreement with the published data.[17]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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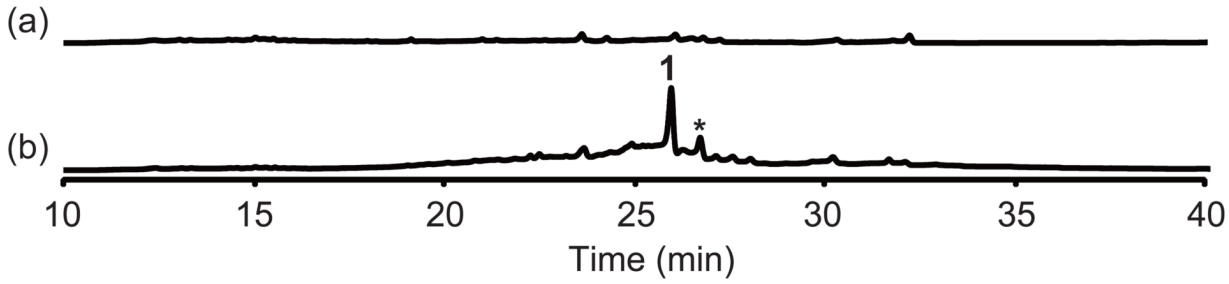
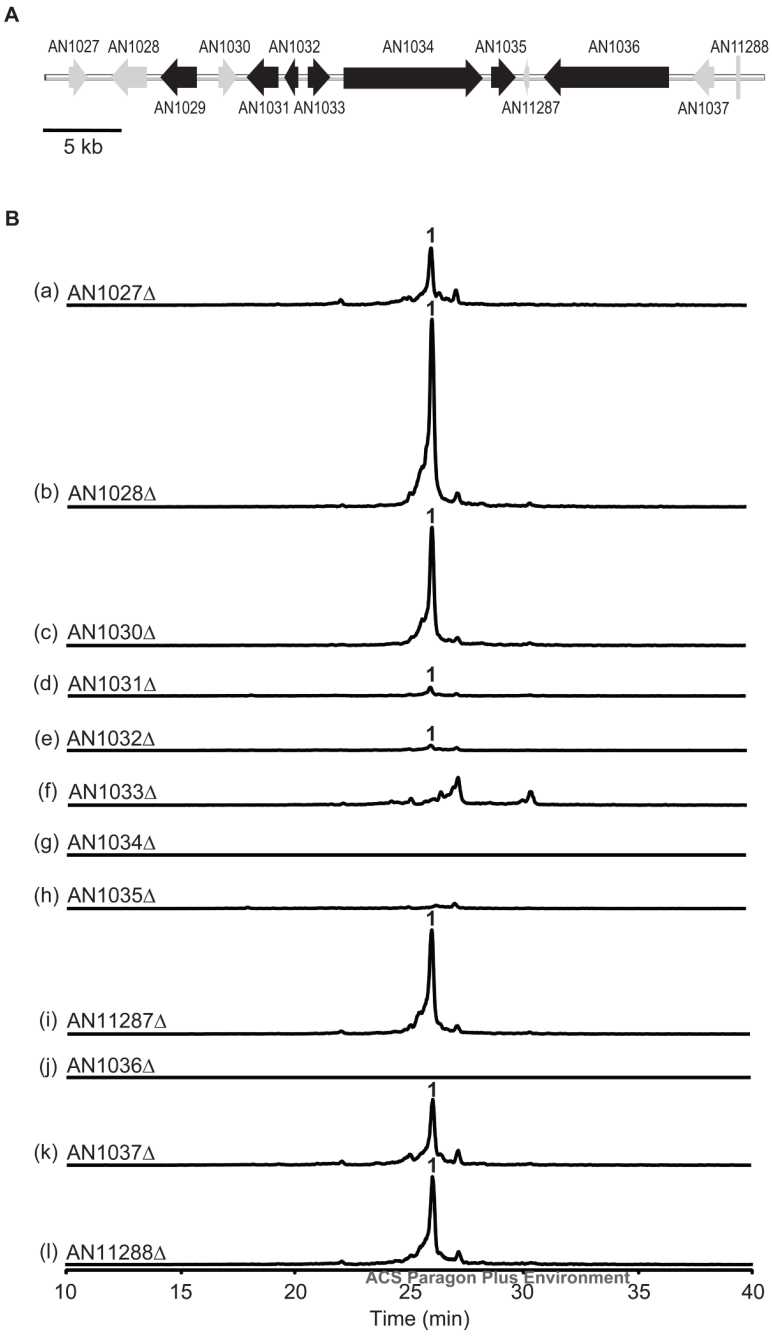
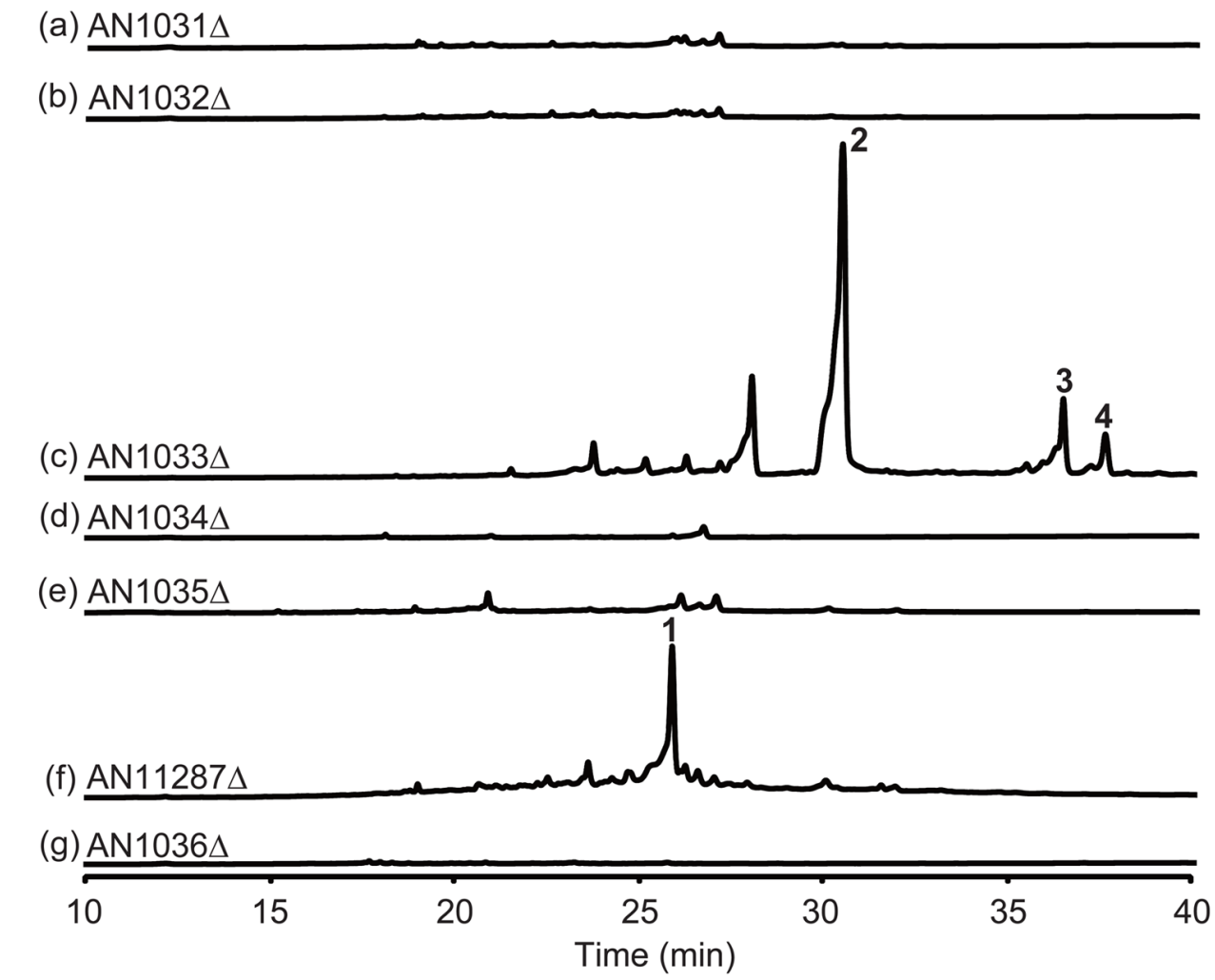


Figure 1.
HPLC profiles of extracts as detected by UV absorption at 254 nm in non-induction (a) and induction (b) conditions. The Y-axis of both profiles was at the same order of magnitude. *: a minor metabolite that decomposed during purification.





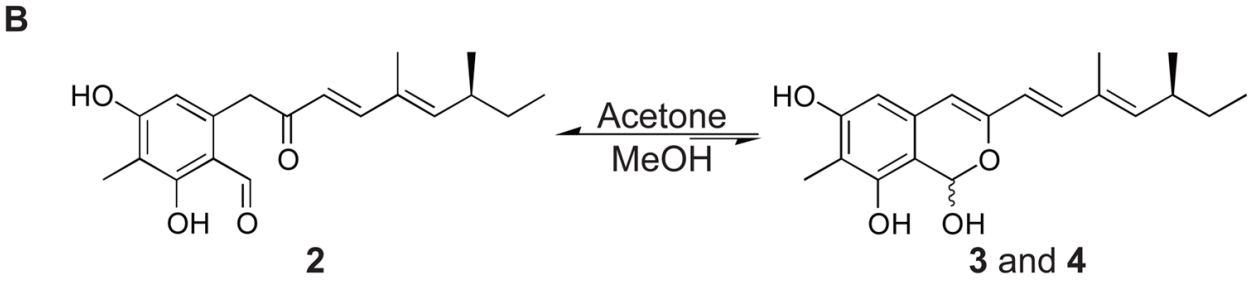


Figure 2.

(A) Organization of the asperfuranone synthase gene cluster in *A. nidulans*. Each arrow indicates the direction of transcription and relative sizes of the ORFs deduced from analysis of the nucleotide sequences. Black ORF's, AN1029.3 (transcriptional activator), AN1031.3 (efflux pump), AN1032.3 (oxydoreductase), AN1033.3 (salicylate hydroxylase), AN1034.3 (NR-PKS), AN1035.3 (oxygenase/oxidase), AN1036.3 (HR-PKS) are required for asperfuranone biosynthesis. Grey ORF's are genes not involved in asperthecin biosynthesis according to our deletion analysis. (**B**) Analysis of the effects on asperfuranone production of deletions of genes surrounding the asperfuranone PKSs. Positive mode extracted ion chromatograms (EIC) at *m*/*z* 333 of AN1027.3Δ (a), AN1028.3Δ (b), AN1030.3Δ (c),

AN1031.3Δ (d), AN1032.3Δ (e), AN1033.3Δ (f), AN1034.3Δ (g), AN1035.3Δ (h), AN11287.3Δ (i), AN1036.3Δ (j), AN1037.3Δ (k), AN11288.3Δ (l). The Y-axis of each profile was at the same order of magnitude. (**C**) Detailed analysis of the effects on asperfuranone production of deletions of genes surrounding the asperfuranone PKSs. UV at 254 nm of AN1031.3Δ (a), AN1032.3Δ (b), AN1033.3Δ (**c**), AN1034.3Δ (d), AN1035.3Δ (e), AN11287.3Δ (f), AN1036.3Δ (g). The Y-axis of each profile was at the same order of magnitude. (**D**) Hemiacetal formation of **3** and **4** from compound **2**.

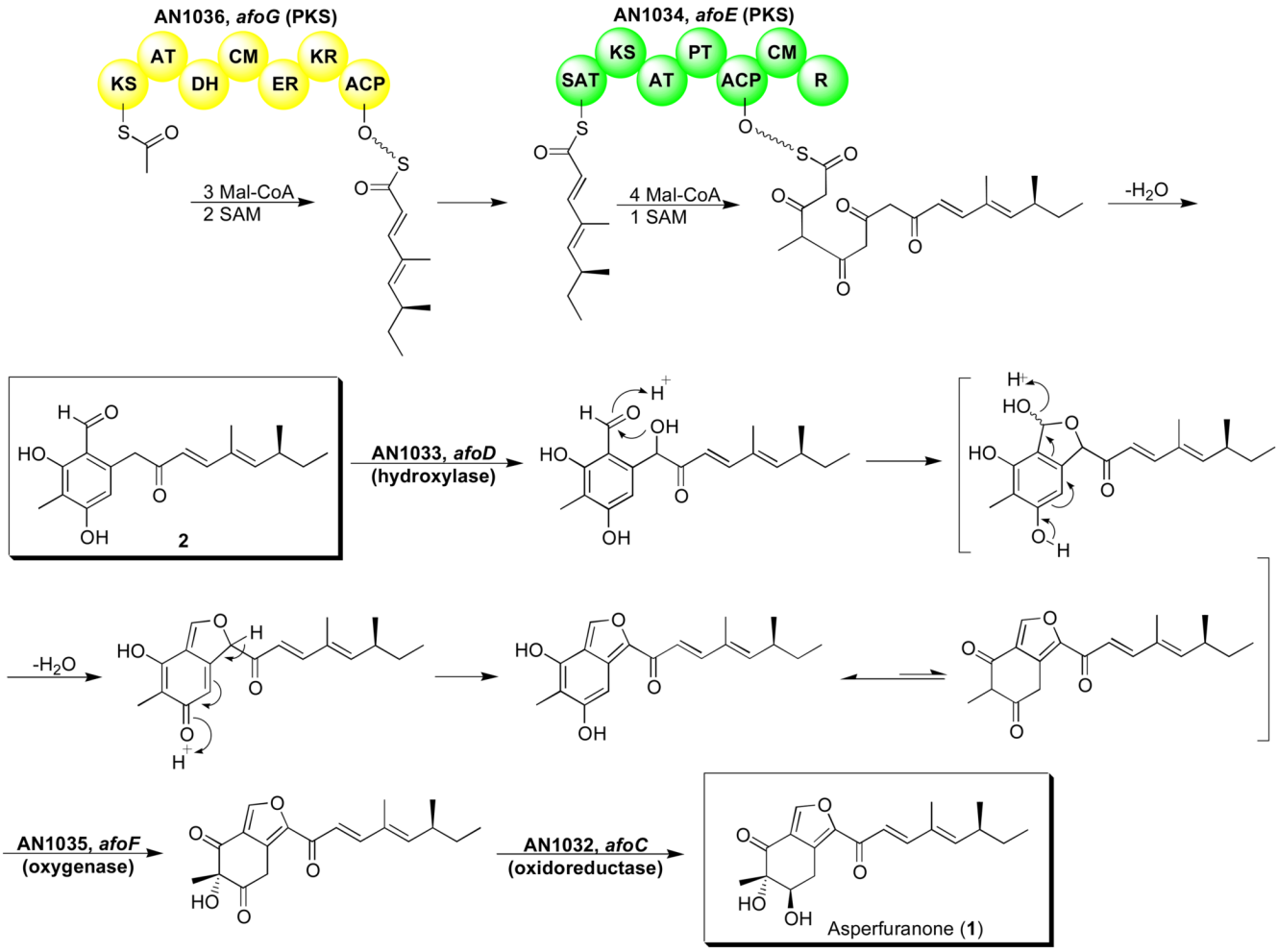


Figure 3.
Proposed biosynthesis pathway for asperfuranone (**1**) in *A. nidulans*. All intermediates are hypothetical except those boxed.

Table 1

Gene identities in asperfuranone clusters

ORFs		Homologues	
Gene	**Predicted size (kDa)**	**Match from BLAST search at NCBI (accession no.)[a]**	**Identity/similarity (%)**
AN1029.3 (*afoA*)	74	citrinin biosynthesis transcriptional activator CtnR (BAE95337)	32/47
AN1031.3 (*afoB*)	57	efflux pump (BAC20568)	37/55
AN1032.3 32 (*afoC*)		citrinin biosynthesis oxydoreductase CtnB (BAE95339)	44/57
AN1033.3 (*afoD*)	49	salicylate hydroxylase (BAA61829)	33/47
AN1034.3 (*afoE*)	304	citrinin polyketide synthase (BAD44749)	41/59
AN1035.3 (*afoF*)	52	FAD/FMN-dependent oxygenase/oxidase (BAD83683)	24/43
AN1036.3 (*afoG*)	273	polyketide synthase (BAC20566)	41/60

[a] Closest homologues with published functions found in a BLAST search of the NCBI non-redundant database.