

Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*

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In the postgenomic era it has become increasingly apparent that the vast number of predicted biosynthesis genes of microorganisms is not reflected by the metabolic profile observed under standard fermentation conditions. In the absence of a particular (in most cases unknown) trigger these gene loci remain silent. Because these cryptic gene clusters may code for the biosynthesis of important virulence factors, toxins, or even drug candidates, new strategies for their activation are urgently needed to make use of this largely untapped reservoir of potentially bioactive compounds^{1,2}. The discovery of new microbial metabolites through genome mining has proven to be a very promising approach^{3–15}. Even so, the investigation of silent gene clusters is still a substantial challenge, particularly in fungi¹⁶. Here we report a new strategy for the successful induction of a silent metabolic pathway in the important model organism *Aspergillus nidulans*, which led to the discovery of novel PKS-NRPS hybrid metabolites.

Mining the recently sequenced *A. nidulans* genome¹⁷ for cryptic (or orphan) gene clusters that might code for the biosynthesis of polyketides or polypeptides, we noted the presence of a single putative hybrid polyketide synthase–nonribosomal peptide synthetase (PKS-NRPS)-encoding gene (*apdA*; *A. nidulans* Database entry AN8412.3). The deduced gene product shows typical motifs of PKS and NRPS domains, as well as an additional C-terminal reductase domain. Obviously, ApdA is a rare fungal PKS-NRPS hybrid. The 11.9-kilobase (kb) gene is flanked upstream by genes coding for several putative oxidoreductases and downstream by exporter and activator genes (Fig. 1 and Supplementary Table 1 online). Despite the large amount of metabolic data available for *A. nidulans*, the role of this gene cluster has not been clarified. So far only two related fungal PKS-NRPS hybrids have been attributed to metabolic functions. Gene inactivation experiments revealed that they are involved in the biosynthesis of the tetramic acid derivatives equisetin (10)¹⁸ and fusarin (11)¹⁹ in *Fusarium* spp. Although an orthologous hybrid synthetase gene has been detected in the genome of *Magnaporthe grisea*²⁰, the encoded pathway has not yet been elucidated²¹.

In seeking to determine the function of the cryptic biosynthesis gene cluster, several strategies are conceivable. In a recent *A. nidulans* genome mining approach, investigators used targeted gene inactivation⁸. However, this technique is only a viable option when the metabolite is constitutively (or at least under certain conditions) produced by the strain²². However, no predicted PKS-NRPS hybrid metabolites were detectable by HPLC-MS screening of a panel of 40 extracts prepared under different culture conditions⁷, which indicates that the gene cluster is silent under standard fermentation conditions. Thus, gene expression needs to be induced, for example by heterologous expression using defined promoters or by promoter exchange within the genome (homologous recombination). For eukaryotes, however, promoter exchange can be cumbersome and is normally used for individual genes only. Furthermore, many fungi only show a very low frequency of homologous recombination, which hampers promoter exchange. Another drawback is that overexpression of a single gene of a gene cluster often leads to limitation of another gene product of the same cluster. Finally, gene expression and (as in the lovastatin (12) pathway) the functioning of enzymes may be context dependent²³. A solution to this problem lies in the expression of pathway-specific regulatory genes, which are present in many secondary-metabolite gene clusters. The advantages of such an approach are that (i) only a small gene needs to be handled and (ii) an ectopic integration is sufficient, thus bypassing all limitations of homologous recombination. Most conveniently, this strategy allows for the concerted expression of all pathway genes.

We noted that the cryptic *A. nidulans* PKS-NRPS gene cluster contains a putative activator gene designated *apdR* (*A. nidulans* Database entry AN8414.3). The deduced gene product is related to

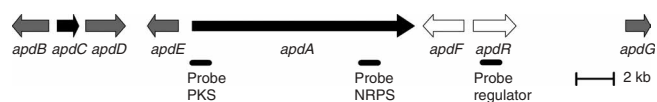
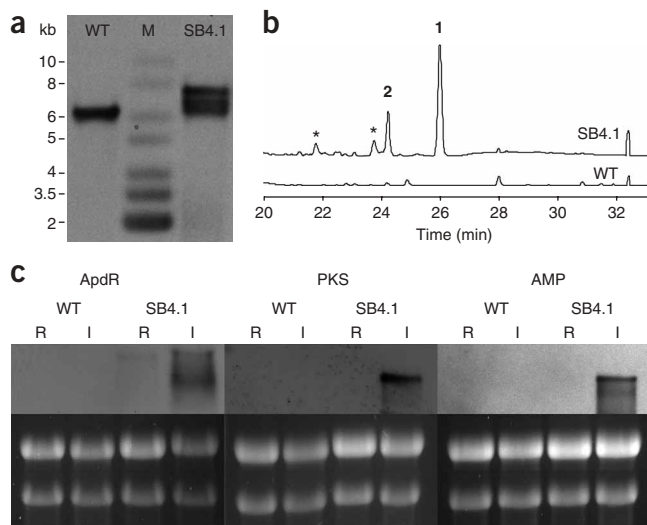


Figure 1 Organization of the *apd* gene locus in *A. nidulans*. Black open reading frame (ORF), gene encoding PKS-NRPS; gray ORFs, genes encoding polyketide tailoring enzymes; white ORFs, regulator and exporter genes. Bars indicate regions for Northern hybridization (selected).

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the C6 transcription factor of *Aspergillus fumigatus* (XP_731535) and to a putative regulator with a GAL4-type Zn₂Cys₆ binuclear cluster DNA-binding domain from *Aspergillus flavus*. To prove the concept that the homologous overexpression of a regulatory gene can lead to activation of a silent gene cluster, we amplified the putative activator gene *apdR* from genomic DNA and cloned it into expression vector pAL4 (ref. 24). Transformation of *A. nidulans* AXB4A2 with the plasmid harboring *apdR* under the control of the inducible alcohol dehydrogenase promoter *alcAp* of *A. nidulans* resulted in several mutant strains that are chemically inducible (Supplementary Methods online). By Southern blot analysis, we identified transformants with multiple ectopic integrations of the construct. The transcription of the regulatory gene was unequivocally proven by northern blot. A substantial amount of *apdR* steady state mRNA was detectable in a selected transformant under inducing conditions. This mRNA was completely absent under noninducing conditions (Fig. 2).

Notably, under inducing conditions the *apdA* gene encoding the PKS-NRPS hybrid synthetase was substantially transcribed. The presence of the full transcript was proven by two different probes targeting both 5' and 3' regions of the *apdA* mRNA. Conversely, no transcript was detected under noninducing conditions. This observation further confirms that under the conditions applied, this gene cluster is silent without induction. Further northern blot analyses revealed that five other predicted pathway genes (*apdB*, *apdC*, *apdD*, *apdE* and *apdG*) are only expressed upon induction, whereas flanking genes (*A. nidulans* Database entries AN8404.3, AN8405.3, AN8406.3, AN8407.3 and AN8416.3) are not induced or are silent (Supplementary Fig. 1 online). Taken together, these molecular data not only delineate the boundaries of the *apd* locus but also give strong evidence that the entire biosynthesis gene cluster can be chemically induced in the transformant. As a result, we sought to monitor the production of new metabolites in the mutants.

In fact, HPLC coupled to diode array and mass detectors revealed that the induced strains produce two main products and two minor compounds (Fig. 2b). Large-scale cultivation of the best producer strain (*A. nidulans* SB4.1) under inducing conditions provided sufficient amounts for full structure elucidation.

The main metabolites were isolated from the crude extract by flash chromatography on silica gel and further purified by size-exclusion chromatography on Sephadex LH-20 and preparative HPLC. Their structures were elucidated by extensive one- and two-dimensional

Figure 2 Monitoring the induction of aspyridone biosynthesis. (a) Southern blot analysis. Chromosomal DNA of *A. nidulans* wild type and transformant strain SB4.1 was digested with *EcoRI*. The blot was hybridized with a 2.3-kb DNA fragment obtained by PCR from *A. nidulans* chromosomal DNA with the oligonucleotides *apdRfor* and *apdRrev* (Supplementary Table 3). (b) HPLC profiles of extracts from *A. nidulans* wild type and chemically inducible transformant strain SB4.1 producing novel compounds **1** and **2** and two minor compounds (*). Both strains were incubated in *Aspergillus* minimal medium (AMM) under *alcAp*-inducing conditions. (c) Northern blot analysis. Total RNA (10 µg) from the *A. nidulans* wild type and transformant strain was analyzed. Both strains were incubated in AMM under *alcAp*-repressing and *alcAp*-inducing conditions. *ApdR*, *PKS* and *AMP* are probes used for hybridization; WT, *A. nidulans* wild-type strain AXB4A; SB4.1, transformant strain; M, marker; I, inducing conditions; R, repressing conditions.

NMR measurements and by IR spectroscopy and MS experiments (Supplementary Fig. 2 online). The molecular formula (C₁₉H₂₃NO₄) of compound **1** was deduced from high-resolution ESI-MS measurements. The ¹³C NMR spectrum confirmed the presence of 19 carbon atoms, which we assigned to seven quaternary, seven methine, two methylene and three methyl groups through distortionless enhancement by polarization transfer (DEPT) experiments. The H,H correlated spectroscopy (COSY) spectrum revealed the spin system of **1**, and the conclusion was supported by the heteronuclear multiple bond correlation (HMBC) couplings (Fig. 3). The ¹H NMR spectrum showed three broad signals of exchangeable protons that were assigned to two hydroxyl groups and one amide group (δ_H 16.74, 9.47 and 11.60 p.p.m.). Furthermore, the proton spectrum revealed the presence of a typical AA'BB' spin system (attributable to H2' and H6', and to H3' and H5'), clearly establishing the substitution pattern of the *p*-hydroxyphenyl moiety. The HMBC indicated a coupling of H6 to C4, C2, C1' and C5 and elucidated the partial structure of the pyridone ring (Fig. 3). Comparison of the ¹³C NMR shifts of **1** with experimental and calculated NMR shifts of *syn* and *anti* isomers of similar partial structures suggests a *syn* configuration at C8 and C10 (refs. 25,26). Metabolite **2** was identified as a derivative of **1** with a similar chromophoric system and a molecular formula of C₁₉H₂₃NO₅. The one-dimensional NMR data confirmed this assumption. The difference between the two compounds was exclusively due to the hydroxylated phenyl substituent. Instead of the characteristic set of methine carbon signals in **1**, only three aromatic methine carbon resonances were detected in **2**. An additional quaternary carbon that resonates at δ_C 146.2 p.p.m. (C3') indicates the substitution with a second hydroxyl group. HMBC cross peaks between H5' and C3' and between H6' and C2', and between H6' and C5, established the structure of the 3',4'-dihydroxyphenyl substituent (Fig. 3).

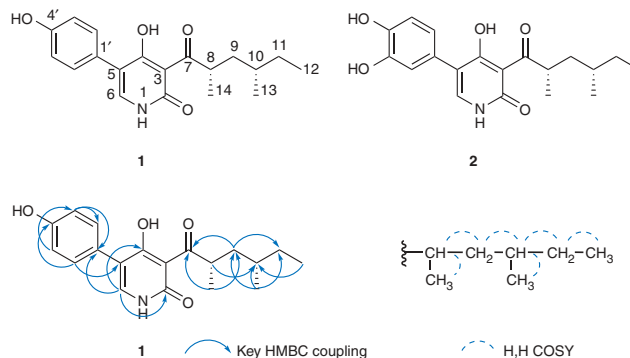
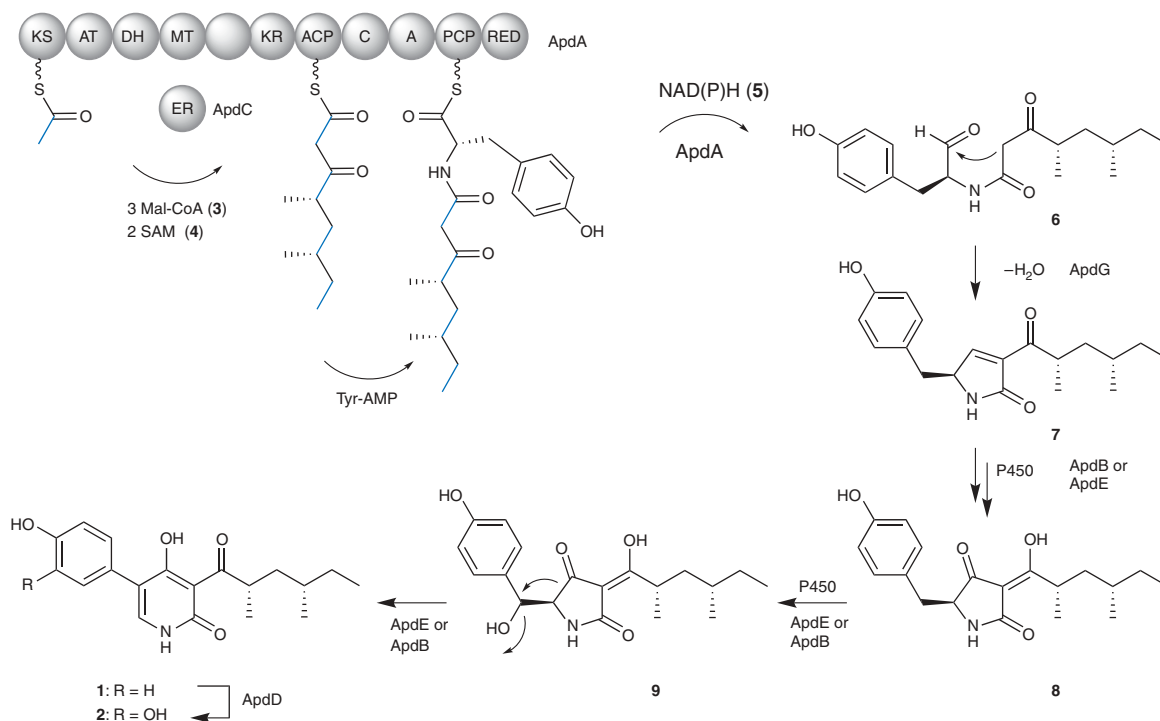


Figure 3 Structures of aspyridones A (**1**) and B (**2**).



Scheme 1 Model for aspyridone biosynthesis. KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; MT, methyltransferase; ACP, acyl carrier protein; A, adenylation domain; C, condensation domain; PCP, peptidyl carrier protein; RED, reductase domain; SAM, *S*-adenosylmethionine; Mal-CoA, malonyl coenzyme A.

We named the new metabolites produced by the induced strain aspyridones A and B because of their pyridone moieties. They are similar but not identical to a variety of other pyridones isolated from fungi, such as militarinone D (**13**)²⁷, tenellin (**14**)²⁸ and farinosone A (**15**)²⁹.

Notably, these metabolites are mainly produced by insect pathogens. In a broad bioactivity screening, aspyridones A and B showed moderate cytotoxicity.

The hypothetical biosynthesis pathway for the aspyridones can be rationalized by the deduced gene functions in the *apd* locus. The polyketide–amino acid backbone is first assembled by the PKS-NRPS hybrid ApdA (3,931 amino acids; **Scheme 1**), which is similar to the equisetin and fusarin pyrrolinone synthases^{18,19}. By analogy, ApdA would catalyze three Claisen condensations, as well as β -keto processing and methylation. Because ApdA lacks a designated enoylreductase (ER) domain, the required activity is likely provided by a free-standing ER *in trans*. The best candidate for this role is ApdC, which is most similar to the *trans* ER LovC involved in lovastatin biosynthesis²³. Upon formation of the polyketide backbone on the thioester template, the triketide is transferred to the NRPS module and linked to tyrosine. The presence of a C-terminal reductase domain suggests that the hybrid molecule is down-loaded by reductive cleavage. The resulting aldehyde would readily undergo an intramolecular Knoevenagel condensation. A number of oxidative steps would be required for the transformation of the resulting pyrrolinone via a tetramic acid intermediate (**Scheme 1**). We identified several candidate genes for these oxidative steps in the expressed *apd* gene locus. ApdB shows similarity to GliC (Entrez Protein code AAW03306) and other cytochrome P450 oxygenases of *A. fumigatus* (Entrez Protein code XP_754325) and may have a role in the oxidation of the tetramic acid. ApdE has substantial similarity to a cytochrome P450 alkane hydroxylase of *A. fumigatus* (Entrez Protein

code XP_749919) and is thus implicated in the formation of the proposed hydroxylated intermediate **9**. ApdB and ApdE might also be involved in the cryptic pyridone rearrangement. Finally, the transformation of aspyridone A into aspyridone B is likely catalyzed by the predicted FAD-dependent monooxygenase ApdD, which is related to ring hydroxylases such as 2,4-dichlorophenol 6-monooxygenase from *Bradyrhizobium* sp. (**Supplementary Table 1**). The exact biosynthetic mechanism of pyridone formation has long been a matter of intensive studies and controversy^{30–32}. The discovery of the aspyridone biosynthesis gene cluster now creates the basis for a detailed biochemical investigation of this rearrangement.

In conclusion, we have identified a silent PKS-NRPS hybrid gene cluster by genome mining, and we have successfully applied a new strategy for the concerted activation of the cryptic gene locus. Ectopic expression of the pathway-specific regulator under the control of an inducible promoter allowed us to chemically switch on a silent fungal pathway, which so far has not been possible. The expression of the regulatory component, its PKS-NRPS target gene, and five other predicted pathway genes was unequivocally proven by Northern hybridization. In addition, as a result of the induction we were able to isolate and identify two new cytotoxic metabolites, aspyridones A and B, that belong to the pyridone class of metabolites. We have provided the proof of principle for a strategy that may be generally applicable to cryptic gene clusters, particularly in eukaryotes.

Note added in proof: The tenellin biosynthesis gene cluster has recently been identified³³.

METHODS

Generation of recombinant plasmids and probes for Southern blot analyses.

For construction of the expression plasmid pAL4apdR, the *apdR*-encoding sequence was amplified by PCR from genomic DNA using Vent_r DNA

polymerase (New England Biolabs). The sequence-specific oligonucleotides apdRfor and apdRrev were synthesized by biomers.net, both introducing an *Xma*I site at each end of the fragment (Supplementary Table 2 online). The DNA fragment produced was purified using the Invisorb Spin DNA Extraction Kit (Invitex) and cloned into the pCR4-TOPO vector (Invitrogen). After restriction digestion with *Xma*I, the purified *apdR*-encoding DNA fragment was cloned into the *Xma*I-digested pAL4 vector²⁴, which codes for the *alcA* promoter of *A. nidulans* and the *pyr-4* gene of *Neurospora crassa* as a selectable marker. The resulting plasmid pAL4pdR was sequenced by MWG Biotech and contained the 2.3-kb fragment of the *apdR* sequence under the control of the *alcA* promoter. The probe for Southern blot analysis was generated by PCR amplification of the same 2.3-kb fragment using the same oligonucleotides (Supplementary Table 3 online).

Isolation of RNA and northern blot analysis. Mycelia were harvested and broken using liquid nitrogen as previously described³⁴. Total RNA was prepared using the RNeasy Plant Minikit (Qiagen) according to the manufacturer's instructions. The amount of RNA was determined spectrophotometrically. Northern blot analysis was performed with 10 µg of total RNA according to the manufacturer's instructions (Roche). To generate probes, chromosomal DNA of the *A. nidulans* strain AXB4A2 was used as the template in a PCR reaction. apdRfor and apdRrev (Supplementary Table 3) were used to produce the *apdR*-specific probe primers, and this gave an 800-base-pair (bp) DNA fragment. The *apdA* gene transcript was analyzed using two different probes, which bound to either the 5' or 3' end of the transcript. For detection of the 5' end of the transcript a DNA fragment of 799 bp was generated with oligonucleotides PKSporefor and PKSporerev. The 3' end of the transcript was detected using an 802-bp DNA fragment obtained with primers NRPSporefor and NRPSporerev (Supplementary Table 3).

Isolation and structure elucidation of aspyridones. The crude extract from the 13-liter fermentation was separated by flash chromatography on silica gel (eluents: CHCl₃ (100%) 500 ml, CHCl₃/MeOH (95:5) 700 ml, CHCl₃/MeOH (90:10) 500 ml, CHCl₃/MeOH (80:20) 400 ml, flow rate 30 ml min⁻¹). Metabolite-containing fractions were further purified by size-exclusion chromatography on Sephadex LH-20 (eluent MeOH) and preparative HPLC (gradient mode with MeCN/H₂O: 0.5% MeCN to 83% MeCN in 30 min, then 83% MeCN for 10 min, UV detection 245 nm).

Aspyridone A (1) characterization. White amorphous powder (10 mg); ¹H NMR (500 MHz, d₆-DMSO): δ 7.54 (s, 1H; H6), 7.23 (d, *J* = 8.6 Hz, 2H; H2'/H6'), 6.76 (d, *J* = 8.6 Hz, 2H; H3'/5'), 4.35 (m, 1H; H8), 1.53 (m, 1H; H9α), 1.36 (m, 1H; H10), 1.31–1.21 (m, 2H; H11α/H9β), 1.11 (m, 1H; H11β), 1.04 (d, *J* = 6.7 Hz, 3H; H14), 0.84 (d, *J* = 6.5 Hz, 3H; H13), 0.80 (m, 3H; H12); ¹³C NMR (125 MHz, d₆-DMSO): δ 212.3 (C7), 176.0 (C4), 161.3 (C2), 156.7 (C4'), 140.7 (C6), 130.1 (C2'/C6'), 123.4 (C1'), 115.0 (C3'/C5'), 112.4 (C5), 105.3 (C3), 39.7 (C9), 39.5 (C8), 31.8 (C10), 29.4 (C11), 18.8 (C13), 16.9 (C14), 11.2 (C12). IR (ATR, solid film): ν̄ = 2959, 2925, 1644, 1604, 1542, 1514, 1452, 1428, 1377, 1263, 1212, 1174, 991, 832, 698 cm⁻¹; UV (MeOH): λ_{max} (lgε) = 203 (4.39), 247 (4.37), 344 (3.74) nm. (+)-ESI-MS *m/z* 352 [M+Na]⁺, *m/z* 330 [M+H]⁺; high-resolution ESI-MS (*m/z*): [M+Na]⁺ calcd. for C₁₉H₂₃NO₄Na, 352.1519; found, 352.1520.

Aspyridone B (2) characterization. White amorphous powder (5 mg); ¹H NMR (500 MHz, CD₃OD): δ 7.45 (s, 1H; H6), 6.91 (d, *J* = 1.9 Hz, 1H; H2'), 6.79 (d, *J* = 8.2 Hz, 1H; H5'), 6.74 (dd, *J* = 8.2/1.8 Hz, 1H; H6'), 4.35 (m, 1H; H8), 1.63 (m, 1H; H9α), 1.38 (m, 1H; H10), 1.38–1.26 (m, 2H; H11α/H9β), 1.14 (m, 1H; H11β), 1.12 (d, *J* = 6.8 Hz, 3H; H14), 0.90–0.84 (m, 6H; H13/H12); ¹³C NMR (125 MHz, CD₃OD): δ 214.5 (C7), 177.5 (C4), 163.9 (C2), 146.2 (C3'), 146.0 (C4'), 140.6 (C6), 125.8 (C1'), 121.8 (C6'), 117.5 (C2'), 116.3 (C5'), 116.0 (C5), 107.1 (C3), 41.9 (C8), 41.2 (C9), 33.6 (C10), 30.9 (C11), 19.4 (C13), 17.6 (C14), 11.7 (C12). IR (ATR, solid film): ν̄ = 2958, 2926, 1644, 1600, 1540, 1520, 1454, 1377, 1306, 1259, 1200, 1118, 1005, 814, 783, 697 cm⁻¹; UV (MeOH): λ_{max} (lgε) = 204 (4.40), 214 (4.41), 251 (4.21), 344 (3.61) nm. (+)-ESI-MS *m/z* 368 [M+Na]⁺, *m/z* 346 [M+H]⁺; high-resolution ESI-MS (*m/z*): [M+Na]⁺ calcd. for C₁₉H₂₃NO₅Na, 368.1474; found, 368.1476.

Accession codes. *A. nidulans* Database: ApdB (AN8408.3), ApdC (AN8409.3), ApdD (AN8410.3), ApdE (AN8411.3), ApdA (AN8412.3), ApdF (AN8413.3), ApdR (AN8414.3) and ApdG (AN8415.3) (see also Supplementary Table 1). Also, flanking genes are listed as entries AN8404.3, AN8405.3, AN8406.3, AN8407.3 and AN8416.3. Entrez Protein: *A. fumigatus* cytochrome P450 (XP_754325), *A. fumigatus* cytochrome P450 alkane hydroxylase (XP_749919) and *A. fumigatus* GliC (AAW03306). All accession codes were deposited as part of previous studies.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

S.B. and J.S. carried out the molecular studies; K.S. and C.L. performed structural elucidation; A.B. and C.H. designed the experiments and wrote the article.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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