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Advances in *Aspergillus* secondary metabolite research in the post-genomic era

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Abstract

This review studies the impact of whole genome sequencing on *Aspergillus* secondary metabolite research. There has been a proliferation of many new, intriguing discoveries since sequencing data became widely available. What is more, the genomes disclosed the surprising finding that there are many more secondary metabolite biosynthetic pathways than laboratory research had suggested. Activating these pathways has been met with some success, but many more dormant genes remain to be awakened.

1 Introduction

Aspergillus, a genus of filamentous fungi, is renowned for its medical and commercial importance. Species in *Aspergillus* have been sources of lifesaving drugs, devastating toxins, or mass-produced industrial enzymes. Some species are pathogenic and pose a danger to immunocompromised patients. Laboratory research on *Aspergillus* has also contributed much knowledge about fundamental cell biology and biochemistry.

The significance of *Aspergillus* was cause for the sequencing of the genomes of some of the most well-known members of this genus. *A. fumigatus* is a common airborne pathogen, threatening susceptible patients with infection and life-threatening illness. *Neosartorya fischeri* (anamorph *A. fischerianus*) is genetically closely related to *A. fumigatus*, but it is rarely pathogenic. A chief motivation for its sequencing was thus to learn more about *A. fumigatus* pathogenicity. In parallel, *A. oryzae* is a close genetic cousin to *A. flavus*. Remarkably, whereas *A. flavus* is a contaminant of food stocks and a generator of the potent toxic and carcinogenic aflatoxins, *A. oryzae* has been safely used in East Asian cuisine for centuries.

A. terreus, like *A. fumigatus*, is a significant cause of aspergillosis, but it is also the main source of the anticholesterol drug lovastatin, with worldwide sales topping $10 billion

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annually. *A. nidulans* is a model organism that has been used for the past 60 years to study genetics and cell biology. Unlike many other Aspergilli, it has a well-characterized sexual cycle. *A. niger* has also served as an important model organism; it is also a major producer of enzymes and metabolites, including citric acid. The genomes of these species, and in addition *A. clavatus* and *A. carbonarius*, are now publicly available.

One of the predictions coming from the genome sequencing projects was that our understanding of *Aspergillus* secondary metabolism would profit from the provided data. Sequence information greatly facilitates the identification of natural product genes, the function of which can be demonstrated by molecular biological and biochemical approaches. When a set of genes involved in the formation of the same secondary metabolite are recognized, a biosynthesis can be proposed. Down the road, such advances should be useful for enhanced production of secondary metabolites of interest and the development of second-generation compounds with improved pharmacodynamic and pharmacokinetic characteristics.

This review examines the benefits genomic sequencing has brought to *Aspergillus* secondary metabolite research. As will be detailed below, one of the major findings from the data is that, given the number of putative secondary metabolite genes that have been found, many corresponding natural products have yet to be discovered. This untapped potential of *Aspergillus* has inspired researchers to undertake various strategies to induce the generation of previously unknown natural products. And for compounds that had already been reported, the genomic data has been instrumental in identifying genes and biosynthetic pathways at a rapid pace.

Unquestionably, much important work on *Aspergillus* secondary metabolism was completed or in progress before genome sequencing information became available. The study of the aforementioned aflatoxins[1] and lovastatin[2] were among the many works that not only taught us much about secondary metabolite regulation and organization but were part of the inspiration to sequence genomes in order to learn more. The interested reader is well-advised to turn to these achievements, too,[3] but this review is devoted to projects that were largely a product of the post-genomic era.

2 The status of Aspergillus genomic sequencing efforts

The task of fully sequencing the first *Aspergillus* genomes was a community effort, involving private companies, academic laboratories, and research institutes and funded by private and public entities. For example, *A. nidulans* FGSC A4 was initially sequenced by Cereon Genomics (Monsanto) in 1998, the threefold coverage becoming publicly available in 2003. Shortly thereafter the Whitehead Institute/MIT Center for Genome Research rereleased the sequence with additional coverage sequence to afford 13-fold coverage. A seminal article on the genome sequence was published in 2005, with authors representing The Broad Institute of MIT and Harvard, The Institute for Genomic Research (TIGR), and over 20 universities around the world.[4]

The work, and the teamwork, do not stop with the sequence data. Many times the initial gene annotations are incomplete or contain inaccuracies, such as merged genes and missed

exon calls. On the subject of *A. nidulans*, a goal spearheaded by the Euro-fungbase in collaboration with TIGR/J. Craig Venter Institute and university laboratories is to raise the number of functionally assigned proteins. Since then, over 2500 genes have been edited, and the percent of gene products with an informative name has increased from about 3% to 19%.[5] Table 1 lists several features of sequenced *Aspergillus* genomes.

The future will likely bring additional *Aspergillus* sequencing and annotation data that is the result of collaborations among institutions around the world. But there may also be a trend in which individual laboratories alone may sequence an *Aspergillus* genome (over 180 species left). Because fungal genomes tend to be compact, with few repetitive sequences, they may be amenable to assembly from the short sequence reads that come from low-cost, next-generation sequencing techniques, as demonstrated by the *de novo* assembly of raw sequence data from *Sordaria macrospora*.[6] The bioactive secondary metabolite profile of *Penicillium aethiopicum* motivated researchers to undertake 454 shotgun sequencing of a *P. aethiopicum* strain, covering approximately 90% of the genome.[7]

3 Technological progress in genome mining endeavors

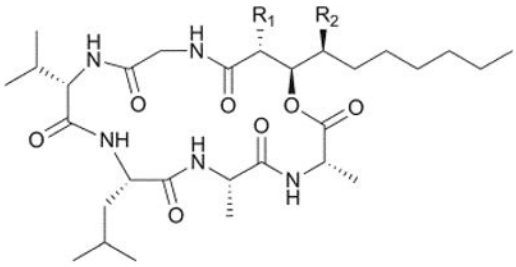
3.1 Methods to study existing secondary metabolites

Published sequenced genomes allow investigators to pinpoint putative secondary metabolite genes based on sequence similarity to established genes from other species. BLAST analysis allows assignment of putative polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), and hybrid PKS–NRPS genes, coding for the core, backbone structures of many fungal natural products. What is more, these genes together with tailoring and regulatory genes tend to exist in clusters, which is not the case for fungal primary metabolic genes or for secondary metabolism genes from other kingdoms, such as plants. Alternative explanations for this phenomenon have been proposed,[8–10] and although no one explanation has been universally accepted, it is unmistakable that this feature is fortuitous for researchers, as the identification of one secondary metabolite gene automatically implicates neighboring genes as suspects in the formation of the same metabolite. Web-based tools assist in systematically locating secondary metabolite clusters. One of them, Secondary Metabolite Unknown Regions Finder (SMURF; www.jcvi.org/smurf), is exclusively tailored to fungi.[11] Another, antibiotics and Secondary Metabolite Analysis Shell (antiSMASH), locates biosynthetic loci involved with the entire range of secondary metabolite classes.[12] Guided by SMURF, Tables 2–5 lists backbone genes in *A. nidulans*, *A. fumigatus*, *A. terreus* and *A. niger*, and actual or predicted products. When candidate genes are identified, several approaches can be taken to study their function in relation to a natural compound.

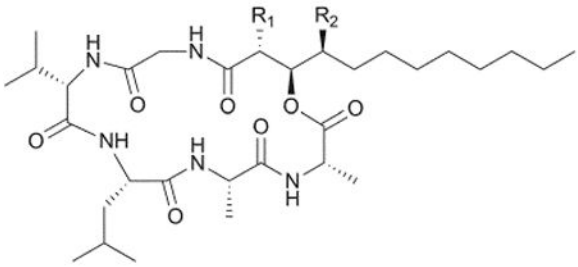
3.1.1 The development of efficient genetic deletion systems—Gene targeting is a useful technique to study fungal secondary metabolism. Deletion of the gene, or the replacement of its promoter with a regulatable promoter, should indicate its significance (if any) in the formation of a particular product. Knocking out genes has the additional advantage in that intermediates in a biosynthesis may accumulate in a deletant strain and offer clues to how a natural product is pieced together. However, the susceptibility of some intermediates to spontaneous rearrangements, degradation, or catalysis by endogenous enzymes can complicate analysis.

High rates of correct gene targeting will hasten progress in studying gene function. However, in *Aspergillus* species, rates hover as low as 0–20%.[13] Human Ku protein is a heterodimer of two polypeptides, Ku70 and Ku80. It plays a chief role in nonhomologous endjoining repair. In *Neurospora crassa*, correct homologous integration occurs only 3–5% of the time, but when the homologs to Ku70 and Ku80 were separately disrupted, each strain was able to integrate exogenous DNA into homologous sequences at a frequency of 100%.[14] Based on this finding, Ku homologs in *Aspergillus* have been deleted with the effect of substantially improving homologous integration efficiency. This approach has been demonstrated in *A. nidulans* (*nkuA*Δ),[15] *A. fumigatus (akuA*Δ and *akuB*^{KU80}Δ),[16,17] *A. niger (kusA*Δ),[18] *A. terreus (akuB*Δ),[19] and *A. oryzae (ku70*Δ and *ku80*Δ).[20,21] Because correct gene targeting frequencies are somewhat lower in *A. oryzae* compared with other *Aspergilli*, an alternative method was developed in which a homolog of human DNA ligase IV (*ligD*) was disrupted.[22,23] As a further aid to experiments, fusion PCR allows transforming sequences to be developed without ligation, and, if the genomic sequence is known, the targeted gene does not have to be cloned.[13,24,25]

Efficient gene knockout was used in the study of the emericellamides (**1–5**) in *A. nidulans*.[26] The emericellamides are molecules of mixed polyketide/peptide origin that were shown to have antibiotic activity, first found from a marine *Emericella* species.[27] Emericellamide A and four other related molecules (thus labeled C–F) were soon after isolated from *A. nidulans*. As expected, both a polyketide and a nonribosomal peptide synthetase gene are responsible for the formation of the emericellamides, as deletion of these genes eliminated the compounds' production. Two additional crucial genes (EasC and EasD) were identified within the cluster.



emericellamide A (**1**): $R_1 = R_2 = Me$
emericellamide C (**2**): $R_1 = Me$, $R_2 = H$
emericellamide D (**3**): $R_1 = H$, $R_2 = Me$



emericellamide E (**4**): $R_1 = Me$, $R_2 = H$
emericellamide F (**5**): $R_1 = H$, $R_2 = Me$

Efficient genetic targeting also allowed investigators to delete 32 known and putative PKS genes and search for any link to observed metabolites.[28] The study revealed that inactivating the PKS gene AN8383 eliminated production of two compounds of mixed polyketide-terpene origin, austinol (**6**) and dehydroaustinol (**7**). AN8383 under the control of an inducible promoter generated 3,5-dimethylorsellinic acid (**8**), a likely precursor in the synthesis of the meroterpenoids (Scheme 1).

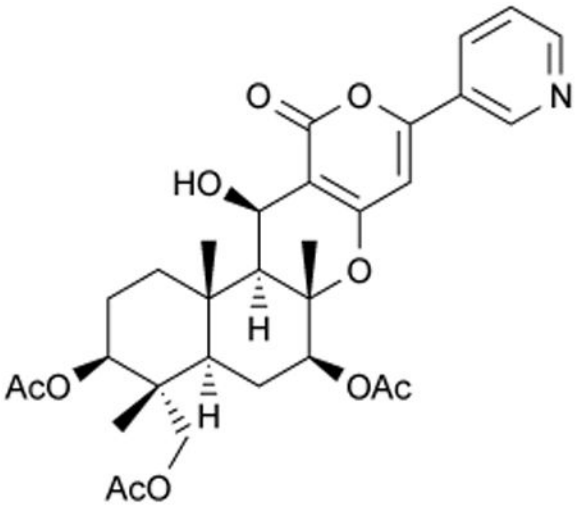
3.1.2 Gene amplification and overexpression—A common method to study a secondary metabolite gene is to amplify the gene of interest, overexpress it in a heterologous host, purify the enzyme, and then examine the enzyme's function, often by incubating it with a supposed secondary metabolite intermediate and any cofactors that are predicted to be necessary. Examples provided below illustrate that the approach has filled in many gaps in our knowledge of various biosynthetic pathways. Given the substrate promiscuity of many secondary metabolite enzymes, however, it may be difficult to assign specific function when the encoding gene is not clustered with other secondary metabolite genes that give context to the overall biosynthesis.

As one example, a consideration of the structure of the mycotoxin acetylaszonalenin (**9**), a dipeptide derivative of tryptophan and anthranilic acid that has been prenylated and acetylated, prompted experimenters to mine the *N. fischeri* genome for a cluster containing NRPS, prenylation, and acetylation genes.[29] The prenyltransferase gene *AnaPT* in such a cluster was cloned and overexpressed. To serve as the starting molecule, the predicted first intermediate, (*R*)-benzodiazepinedione (**10**) was acquired through synthesis. Recombinant AnaPT catalyzed reverse prenylation at the C-3 position, and at this stage ring formation

between C-2 of the indole ring and N-12 of the diketopiperazine seems to occur as well (Scheme 2). Isolated product aszonalenin (**11**) and acetyl coenzyme A were then incubated with the acetylating protein in the cluster, AnaAT. The enzymatic product was confirmed to be acetylaszonalenin.

3.1.3 Heterologous production of natural products—A whole set of genes from one organism may be transferred to a host organism that does not naturally contain these genes. This is a well-established method to induce the expression of genes from a strain that is less-than-optimal in generating the associated natural product.[30] Ideally, heterologous expression can be used to assess the function of these transferred genes. However, the approach has some limitations, including the difficulty of handling large genes and gene clusters, as well as finding a suitable host.

Despite these challenges, researchers succeeded in reconstituting up to five steps of the biosynthesis of the meroterpenoid pyripyropene A (**12**) from *A. fumigatus*, a potent inhibitor of acyl-coenzyme A:cholesterol acyltransferase, using *A. oryzae* M-2-3 as a host.[31] In the search for a terpene cyclase they discovered a protein with unusual sequence and primary structure. The protein bears homology to an efflux pump, raising the possibility that it in fact serves a dual purpose: catalyzing a late-stage cyclization step and, perhaps as a self-resistance move, exporting the metabolite from the cell. Further, the experimenters fed benzoic acid, instead of the natural precursor nicotinic acid, to a transformant and ultimately obtained a close analog to a compound that is an inhibitor of inducible nitric-oxide synthase in human cells. Their approach points to the flexibility of secondary metabolite biosynthetic enzymes, allowing the formation of novel metabolites with distinct biological properties.



pyripyropene A (**12**)

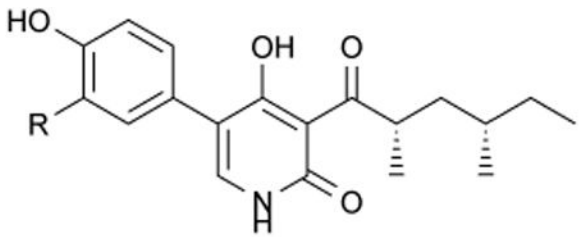
3.2 Uncovering hidden biosynthetic pathways

It came as a surprise to many researchers that *Aspergillus* genomes were indicating that the number of putative biosynthetic genes far exceed the number of secondary metabolites that had been acquired from those species. Because of the extensive investigation of some of

these fungi, it is unlikely that many secondary metabolites have simply escaped notice. Rather, it is plausible to conclude that most of the genes that have not yet been associated with a secondary metabolite are not expressed or expressed in very low amounts in a laboratory culture setting. The discovery of the untapped metabolic potential of *Aspergillus* both inspires excitement and poses a challenge: How can researchers unearth the hidden secondary metabolites of *Aspergillus*, especially with an incomplete knowledge of their regulation?

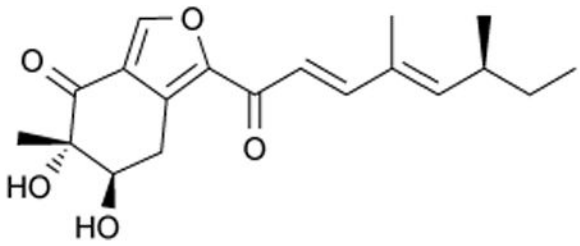
Remarkably, various strategies have been successful to this end, many of which take advantage of some interesting characteristics of fungal secondary metabolism. Besides the aforementioned clustering phenomenon, many (but not all) secondary metabolite gene clusters contain a class of zinc binuclear ($Zn(_{II})_2Cys_6$) transcription factor genes, which are unique to fungi.[32]

3.2.1 Controlling expression of cluster-specific transcriptional activators—In *A. nidulans* a plasmid carrying the transcriptional gene *apdR* under the control of the inducible *alcA* promoter was transformed, leading to the upregulation of the putative entire cluster to which the gene belongs.[33] As a result, two new related polyketide–nonribosomal peptide hybrids, aspyridones A and B (**13–14**), were acquired.



aspyridone A (**13**) R = H
aspyridone B (**14**) R = OH

In another approach that also took note of the fact that many secondary metabolite gene clusters contain their own regulatory gene, the native promoter of one of these genes, *afoA*, was replaced by an inducible promoter, and as a result the novel polyketide asperfuranone (**15**), with structural similarities to the azaphilone class of natural products, was generated.[34] Under *alcA*-inducing conditions, the nearby genes could be deleted and analyzed for their effect on asperfuranone formation. Asperfuranone was found to come from the product of a highly reduced PKS, which was then loaded onto a nonreduced PKS and then further tailored to lead to the final product.



asperfuranone (**15**)

3.2.2 LaeA, a global regulator of secondary metabolism—LaeA (Loss of aflR expression A), a nuclear protein, was first found in *A. nidulans*,[8,35] and orthologs have since been identified in *A.fumigatus, A. terreus, A. flavus*,[36] as well as in fungi beyond *Aspergillus*.[37–40] Its deletion was found to cause the decrease of sterigmatocystin and penicillin production in *A. nidulans* and of gliotoxin production in *A. fumigatus*,[41] and lovastatin in *A. terreus*.[8] Deletion also leads to reduced virulence in *A. fumigatus*[41,42] and *A. flavus*.[43] Over-expression of LaeA, on the other hand, boosted the generation of the above products. The data suggest that the protein has a broad, global role in secondary metabolite regulation. Indeed, microarray data from *A. fumigatus* revealed that up to 13 of 22 studied gene clusters were affected by LaeA.[44] Comparing the deletion and overexpression mutants of LaeA in *A. nidulans* was instrumental in defining the terrequinone A (**16**) gene cluster.[45,46]

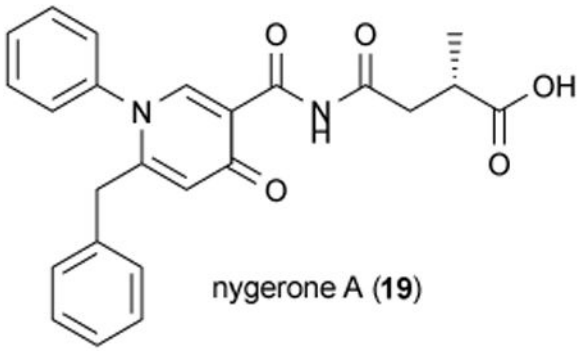
Each of the five terrequinone A genes was characterized by another group through heterologous overexpression.[47] TdiD was established as a pyridoxal-5′-phosphate-dependent L-tryptophan aminotransferase that converted tryptophan to indole pyruvic acid (**17**). Next, the tridomain NRPS TdiA couples two tethered units together to yield dimethylasterriquinone D (**18**). The combination of TdiB, TdiC, and TdiE was reported to be necessary to proceed substantially to terrequinone A, and that TdiC is an NADH-dependent quinone reductase, generating a hydroquinone that is primed for two prenylations catalyzed by TdiB.[47] TdiE is necessary to direct the pathway away from a shunt metabolite (Scheme 3). Concurrent independent research was in agreement about the roles of TdiA and TdiD[48] and characterizes TdiB as a catalyst for reverse prenylation of the indole moiety.[49]

Recent methodology, including the employment of suppressor screens that can remediate loss of *laeA*, may uncover new global regulators of secondary metabolism. For example, such a screen identified a bZIP protein called RsmA (Restorer of secondary metabolism A), which was independently able to enhance secondary metabolite production in *A. nidulans*.[50]

Based on its homology to histone and arginine methyltransferases, LaeA is believed to function by influencing chromatin. As chromatin can exist in an “open” (euchromatin) or “closed” (heterochromatin) state, experimenters considered the possibility that the manipulation of the interplay between these two states may open up previously closed sections of the genome to the transcriptional machinery. LaeA, in fact, was shown to reverse the establishment of heterochromatic marks.[51] Subsequent research has confirmed that

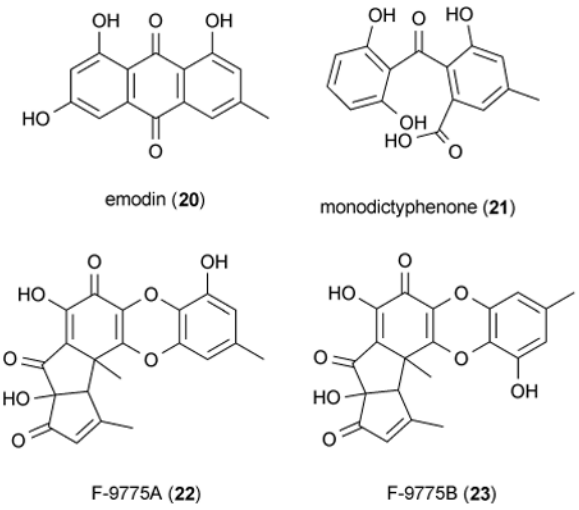
control of chromatin remodeling in *Aspergillus* and elsewhere may affect its metabolic profile.

3.2.3 Chromatin restructuring through enzyme inhibitors or by deletion of a chromatin remodeling protein—Various enzymes catalyze the addition or removal of small functional groups, such as acetyl, methyl, and phosphoryl, onto the tails of histone proteins. These groups appear to influence the architecture of the surrounding chromatin; for instance, acetylated histone tails are generally associated with the open state, whereas deacetylated histones are related with closed chromatin. It was postulated that blocking the enzymes that catalyze deacetylation could potentially open up previously inaccessible secondary metabolite genetic loci and in turn bring about its associated natural product. Indeed, treatment of *Alternaria alternata* and *Penicilium expansum* with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) induced the production of a number of undefined metabolites.[52] In *A. niger*, the addition of the HDAC inhibitor suberoylanilidehydroxamic acid (SAHA) generated the production of nygerone A (**19**), containing a unique 1-phenyl-pyridin-4(1H)-one core.[53]



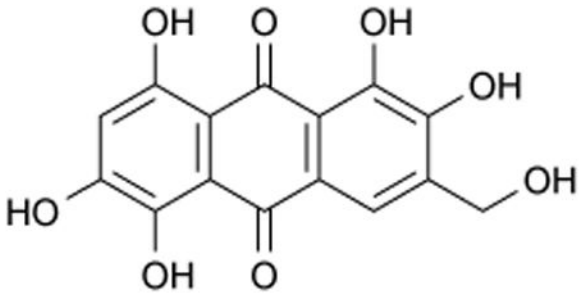
nygerone A (**19**)

Investigators also considered the effects of removing critical members of chromatin regulatory proteins through targeted gene deletion. *A. nidulans* contains CclA, a putative ortholog of the *Saccharomyces cerivisiae* Bre2 protein, belonging to a complex (COMPASS), which both activates and represses chromatin-related processes through lysine 4 histone H3 methylation. Two classes of secondary metabolites emerged when the *cclA* gene was knocked out: emodin (**20**), monodictyphenone (**21**), and related compounds, which all depend on the PKS gene AN0150; and F9775 A/B (**22–23**), which stems from the gene AN7909.[54]



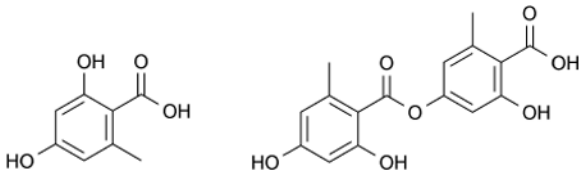
The research so far on the epigenetic link to secondary metabolites suggests that alterations in the chromatin landscape can have a dramatic effect on a fungus's metabolic profile, although no work to date has reported that one single approach yields the entire array of hidden natural products from one fungus. Nevertheless, epigenetic strategies, if they cannot access an entire genome, are still useful in opening up at least part of the secondary metabolome that had previously been closed off to researchers.

In addition to histone proteins, other targets may touch upon secondary metabolite regulation. The single sumoylation gene of *A. nidulans*, *sumO*, was shown to affect the production of several metabolites.[55] The gene's removal led to decreased amounts of austinol (**6**) and dehydroaustinol (**7**) production but also a significant increase in the generation of the polyketide asperthecin (**24**). The substantial amount of asperthecin permitted researchers to identify a cluster of three genes containing the responsible polyketide synthase gene, a gene thought to be needed for the release of the polyketide from its enzyme, and a monooxygenase gene. It is not yet clear how the deletion of *sumO* affects *A. nidulans* secondary metabolism, but as the small product SUMO is added post-translationally to a variety of proteins, it may play a role in the regulation of at least some secondary metabolites.



asperthecin (**24**)

3.2.4 Mimicking ecological systems through co-incubation with microorganisms—Another successful strategy was based on the realization that, in nature, fungi often co-inhabit ecosystems with other species, and indeed the purpose of many secondary metabolites apparently is to assist the producing organism secure its niche, whether the interaction is antagonistic or symbiotic. This has indeed translated to the induction of secondary metabolites when a fungus is co-incubated with another organism.[27,56–58] Again in *A. nidulans*, it was demonstrated that cultivation with a particular actinomycete, *Streptomyces rapa-mycinicus*, prompted the upregulation of 248 genes including a PKS and NRPS gene.[59] It was demonstrated that, rather than arising from a chemical signal, the induction was triggered by the physical interaction of the two organisms. Metabolic profiling revealed the emergence of the polyketide orsellinic acid (**25**) as well as lecanoric acid (**26**) and F9775 A and B (**22–23**), which may be derived from the simple tetraketide, found to be encoded by *orsA* (AN7909).[59,60]



orsellinic acid (**25**) lecanoric acid (**26**)

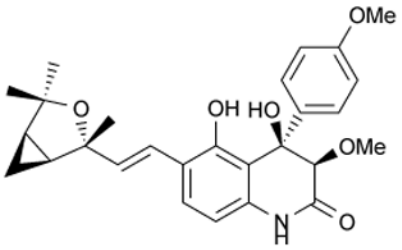
3.2.5 One strain many compounds (OSMAC)—Despite the unmistakable value of sophisticated molecular biological research in uncovering previously undetected secondary metabolites, a potentially equally powerful method involves little more than a change in recipe. Articulated as OSMAC (One Strain – Many Compounds), the strategy endeavors to expose the metabolic diversity of fungi (among other organisms) by subjecting the same strain to a number of different culture conditions.[61] A frequent approach is to alter the broth composition in which the organism is cultivated. Other parameters may be adjusted, such as temperature, shaking speed, and even flask shape and size.

Our understanding of the mechanisms that make OSMAC effective can only be as complete as our knowledge of the complex regulatory networks that influence secondary metabolism. It is realized that heightened glucose, ammonium, or phosphate concentrations often (but not necessarily) curb secondary metabolism formation.[62,63] However, in many cases it is not clear why a certain environment results in the desired effect of a new metabolite. Considering the facts that altering culture conditions is usually not labor intensive, but that many attempts will not lead to a different metabolic profile, a sensible course of action is to test out many different conditions at once.

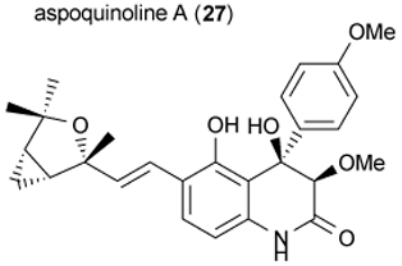
OSMAC inspired the discovery that simple culture conditions, namely *A. nidulans* in a sucrose-based media without shaking, led to the formation of orsellinic acid (**25**) and F-9775 A and B (**22–23**).[60] Interestingly, F-9775 A and B has now been demonstrated to be a result of three separate approaches: chromatin remodeling,[54] co-incubation with an actinomycete,[59] and alteration of culture conditions. The straightforward nature of changing conditions facilitated the discovery of a three-gene cluster through genetic deletion analysis

and the acquisition of two bioactive compounds, gerfelin and diorcinol, from two of the knockout strains.

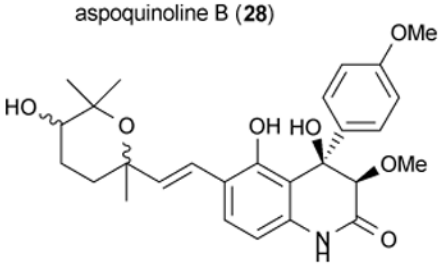
As other examples, one group prepared 40 different conditions and found that one (and not the 39 others) yielded four related quinoline-2-one alkaloids named aspoquinolones (**27–30**).[64] An additional round of investigation was rewarded with the discovery of the prenylated isoindolinone alkaloids, aspernidine A and B (**31–32**).[65]



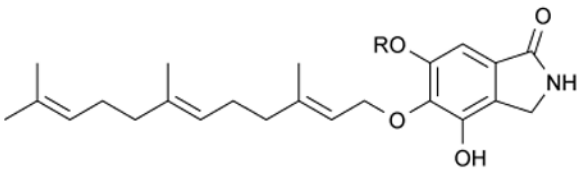
aspoquinoline A (**27**)



aspoquinoline B (**28**)



aspoquinolines C (**29**) and D (**30**)



aspernidine A (**31**) R = Me
asperinidine B (**32**) R = H

4 Genetic characterization of other *Aspergillus* secondary metabolites

4.1 Prenylated xanthones from *A. nidulans*

Investigation into the biosynthesis of the naturally occurring prenyl xanthones of *A. nidulans* (**33–36**) revealed that the identified genes are separated into three distinct genomic loci.[66]

The two necessary prenyltransferase genes are distant from a main cluster, and another gene is proximal to one of the prenyl-transferase genes (Scheme 4). This and other examples of "non-clustering" in the literature[67–69] suggest that fungal secondary metabolite genetic clustering should be thought of as a guiding principle rather than an unbreakable rule. The study also detailed that the main cluster of genes was the same as the one responsible for emodin (**17**), monodictyphenone (**18**), and related compounds, which were only detected in a chromatin remodeling mutant strain. The genes outside the cluster complete a biosynthesis in which emodin and monodictyphenone may be intermediates.

These findings suggest that applications of strategies that aim to upregulate a genetic cluster may not equally influence genes that pertain to the biosynthesis but reside outside the cluster. Consequently, the "real" natural product may not be formed, but rather one or more molecules representing a portion of the biosynthesis. However, since many intermediates on their own possess potent biological activity, an original aim of these aforementioned approaches, the generation of bioactive secondary metabolites, may be satisfied after all.

4.2 *A. fumigatus* secondary metabolites

4.2.1 Gliotoxin—Of all the metabolites generated by *A. fumigatus*, gliotoxin (**37**) may be the most scrutinized of them all. Gliotoxin is an epipolythiodioxopiperazine containing a transannular disulfide bridge. This structure is believed to a major factor in the metabolite's – and the organisms's – harmful effects, *via* the generation of reactive oxygen species from cycling between the oxidized and reduced forms, and/or the formation of mixed disulfides with proteins in the host cell.[70,71] The metabolite has been associated with the virulence of the producing species.[72,73]

The gliotoxin cluster (*gli*) was identified promptly after the sequence information for *A. fumigatus* strain Af293 became available.[74] The 12-membered cluster includes a gene for an NRPS (*gliP*),[75,76] a transcription factor (*gliZ*),[77] and a protein with a putative pyridoxal 5′-phosphate binding domain (*gliI*),[78] and a cytochrome P450 monooxygenase (*gliC*)[79] each of which was determined to be essential for gliotoxin formation (Scheme 5).

Work with recombinant GliP showed that it assembles an L-Phe-L-Ser-thioester intermediate, tethered to one of the thiolation domains of the three-module NRPS (**38**).[80]

At the other end of the biosynthesis, *gliT* encodes a flavin-dependent oxidoreductase which mediates oxidation of the dithiol precursor (**39**) to gliotoxin.[81,82] This protein has been shown to confer resistance from exogenous gliotoxin, suggesting a protective role for this gene product.

Investigators are attempting to unfold the secrets of gliotoxin biosynthesis between beginning and end stages. The deletion of the glutathione *S*-transferase (GST) gene homolog *gliG* led to a putative shunt metabolite lacking sulfur, suggesting that GliG may not be a usual GST but somehow provides the sulfur groups.[79,83] In another experiment, differential analysis by 2D NMR spectroscopy (DANS) was utilized to observe differences in the metabolic profile between wild type and *gliZ* knockout strains.[84] Remarkably, nineteen metabolites, nine of them novel, were detected in the wild type strain alone, allowing

researchers to outline a biosynthesis in which many of the intermediates remain tethered to the NRPS.

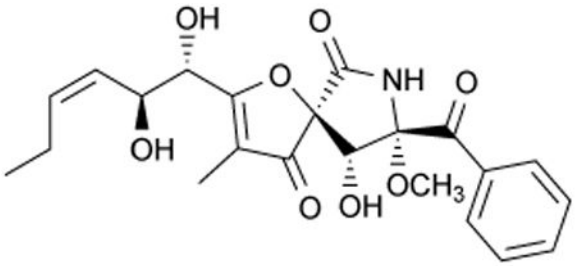
4.2.2 Fumigaclavine C—Fumigaclavine C (**40**) belongs to the ergot alkaloid class of natural products, a diverse group with many potent pharmacological activities. A putative gene cluster for fumigaclavine C was detected in *A. fumigatus* through bioinformatic investigations.[85,86] From this cluster the gene (*dmaW/fgaPT2*) was both deleted[87] and cloned and overexpressed.[85] Examination revealed that it functions as a dimethylallyltryptophan synthase (DMATS), appending a dimethylallyl group on the 4-position of tryptophan to give **41** (Scheme 6). The X-ray structure of FgaPT2 was determined with a resolution of 1.76 Å.[88] Interestingly, it showed significant structural similarity to the ABBA family of bacterial prenyltransferases without having any significant similarity at the amino acid level.

FgaMT was also cloned and overexpressed and was found to catalyze the *N*-methylation of 4-dimethylallyltryptophan (**41**) with the addition of *S*-adenosylmethionine.[89] This methylated product (**42**) was accumulated upon deletion of either the putative catalase gene *easC* or the putative oxidoreductase *easE*.[90] Their specific contributions, and whether they work in tandem or sequentially, await discovery. The possible product, chanoclavine I (**43**), is oxidized to yield chanoclavine-1-aldehyde (**44**), as evidenced by the *in vitro* reaction with cloned and overexpressed *FgaDH*.[91]

A homolog of the Old Yellow Enzyme gene in yeast, *easA*, was amplified and overexpressed.[92] By incubating with chanoclavine I-aldehyde (**44**) and subsequently reducing with sodium cyanoborohydride, researchers obtained festuclavine (**45**). Reducing the alkene of chanoclavine-1 aldehyde allows the new single bond to rotate and orient the aldehyde group for iminium formation. Another team, using purified recombinant proteins, showed that EasA (named FgaOx3 here) and FgaFS, of unknown function, were crucial for the transformation of chanoclavine-1 aldehyde to festuclavine.[93] Festuclavine formation could only be observed if the enzymes were incubated together or in a sequence with FgaOx3 preceding FgaFS.

An undetermined step hydroxylates festuclavine to fumigaclavine B (**46**), then the putative acetyltransferase FgaAT catalyzes the conversion of fumigaclavine B to fumigaclavine A (**47**), as elaborated by a similar overexpression study.[94] A second prenyltransferase gene product (FgaPT1) was shown to catalyze the addition of a prenyl group to the late-stage intermediate fumigaclavine A to fumigaclavine C (**40**).[95]

4.2.3 Pseurotin A—The structure of pseurotin A (**48**), together with previous feeding experiments, suggested that the molecule is a product of a hybrid PKS/NRPS, and there is only one obvious PKS/NRPS gene in the *A. fumigatus* genome. Researchers both deleted and overexpressed this gene, *psoA*, to find that pseurotin A was eliminated or accumulated, respectively.[96] Pseurotin A was recently found to be induced in laboratory hypoxic conditions, an environment designed to mimic the conditions *A. fumigatus* may encounter in the human lung.[97]



pseurotin A (**48**)

4.2.4 Fumiquinazolines—Researchers validated that an adenylation domain of the acetylaszonalenin NRPS (see above) activates anthranilic acid as anthranilyl-AMP,[98] which is then loaded onto the corresponding thiolation domain. Because adenylation domains feature ten-residue long sequence "codes" that correspond to the amino acid they are specific for, the experimenters used the presumed code for anthranilic acid to search for similar sequences in other fungi. This information was used to confirm that an NRPS module from *A. fumigatus* also activates anthranilate.[98]

Next, the group identified two adjacent genes, coding for a monomodular NRPS and a flavin adenine dinucleotide-dependent monooxygenase. Starting with chemically synthesized fumiquinazoline F (**49**), the group showed that the recombinant monooxygenase oxidizes the 2′–3′ double bond in the indole ring, and the NRPS presents L-alanine to acylate the oxidized indole (Scheme 7). Intramolecular cyclization completes the synthesis of fumiquinazoline A (**50**).[99]

Subsequent work has shown that another gene codes for an FAD-dependent amide oxidase that generates a nascent imine in fumiquinazoline A that is captured by the –OH group of the oxidized indole ring to form the remarkably complex, seven-ring spirohemiaminal fumiquinazoline C (**51**). This molecule undergoes slow spontaneous conversion to fumiquinazoline D (**52**).[100]

4.2.5 Fumitremorgins—The diverse family of fumitremorgins includes brevianamide F (**53**), fumitremorgins A (**54**), B (**55**), and C (**56**), and verruculogen (**57**).[101] As research validated, the structural similarities suggest a common biosynthetic origin. Interestingly, different members of the family have different biological properties. Fumitremorgin C, for one, is a potent and specific inhibitor of breast cancer resistance protein.[102,103] Verruculogen, on the other hand, is a tremorgenic mycotoxin.[104]

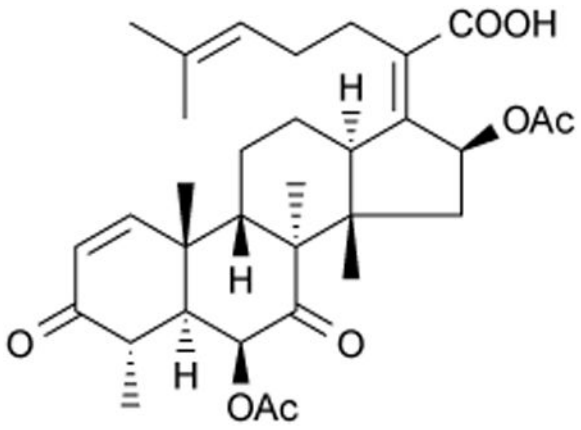
Experimenters noticed that two prenyltransferases are expected to be involved in the biosynthesis of fumitremorgin B (**55**) and related compounds.[105] The identification of two putative prenyltransferase genes in proximity with each other prompted the overexpression of *ftmPT1* and its recognition as a catalyst for the prenylation of synthetic brevianamide F (**53**) (Scheme 8). As expected, the bimodular NRPS gene in the cluster, upon overexpression in both *A. fumigatus* and *A. nidulans*, was shown to be the generator of brevianamide F.[106]

Disruption of cytochrome P450 gene *ftmC* led to a metabolite profile in which trypostatin B (**58**), the prenylated derivative of brevianamide F (**53**), was most prominent.[107] A strain with

a deletion of another cyctochrome P450 gene, *ftmE*, revealed trypostatin A (**59**), and to a smaller extent, **60**, suggesting that the biosynthetic steps after trypostatin B are hydroxylation of C-6 followed by methylation of the new hydroxyl group, then C–N bond closure to form a five-ring system.[107] *FtmG* was also disrupted, resulting in fumitremorgin C (**56**) and suggesting the corresponding enzyme dihydroxylates the intermediate.[107] Reconsitution experiments confirmed roles for FtmC, FtmE, and FtmG.

The second prenyltransferase gene, *ftmPT2*, was overex-pressed and shown to be responsible for appending a prenyl group to the indole nitrogen of **61**.[108] Purified FtmOx1, a non-heme Fe(II) and α-ketoglutarate-dependent dioxygenase catalyzes endoperoxide formation to yield verruculogen (**57**).[109] Incubation with an $^{18}O_2$-enriched atmosphere reveals that both oxygen atoms come from one molecule of O_2. Gene knockout results of the same gene agreed with its role as verruculogen synthase.[110] An unidentified prenyltransferase is expected to convert verruculogen to fumitremorgin A (**54**).

4.2.6 Helvolic acid—Plants are the major producers of triterpenes with biological activity, but the large number of candidate tailoring genes makes it difficult to assign function to them. *A. fumigatus*, however, is a producer of the terpene-derived antibiotic helvolic acid (**62**). A putative oxidosqualene cyclase gene with some sequence identity to fungal lanosterol synthase genes was identified in the *A. fumigatus* genome, as well as tailoring genes and a transporter gene that all cluster together and could plausibly contribute to helvolic acid biosynthesis.[111] The OSC gene, *AfuOSC3*, was PCR amplified and expressed in yeast, the transformant bearing a known precursor to helvolic acid. Coexpression with the cluster's dehydrogenase/reductase gene or a P450 gene yielded oxidized backbone products that are in agreement with the idea the cluster is responsible for forming a tetracyclic ring system which is then tailored to helvolic acid.

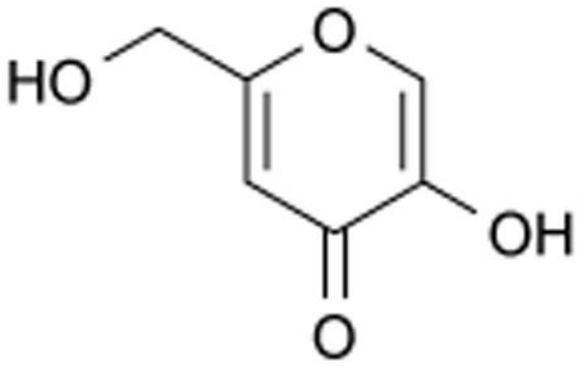


helvolic acid (**62**)

4.3 *A. oryzae* secondary metabolites

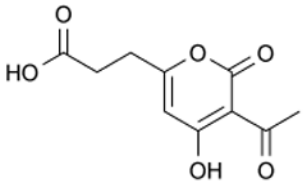
4.3.1 Kojic acid: Kojic acid (**63**) has been found to have a number of properties, including antibacterial and antifungal, and it is used in cosmetics to lighten skin. Experimenters scrutinized gene transcription profiles from DNA microarray data.[112] Based on observations

that kojic acid production increased with time until day 7 and that it was inhibited by sodium nitrate, the laboratory compared gene expression levels in a pair of conditions (7-day culture *vs*. 4-day culture, 4-day culture *vs*. 2-day culture, and without nitrate *vs*. with nitrate) and identified genes that were highly expressed in one condition relative to the other condition. No gene was commonly more than 2-fold enhanced from all three experiments, but the two most strongly induced genes in the 7 day *vs*. 4 day experiment were validated, upon gene disruption, to be responsible for kojic acid synthesis. The genes are separated by a third gene, which, too, was deleted and found to be behind the formation of the metabolite.[113] The genes encode for an enzyme with an oxidoreductase motif, a transcription factor, and a transporter protein. Glucose, rather than a backbone polyketide, peptide, or terpene, is believed to serve as the starting material.

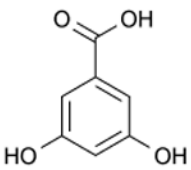


kojic acid (**63**)

4.3.2 Csypyrone B1 and DHBA: The formation of most fungal polyketides is catalyzed by iterative type I PKSs. However, it was noted that in *A. oryzae* several genes, *csyA*, *csyB*, *csyC*, and *csyD*, encode type III PKSs, which have a simple ketosynthase architecture with an active site for multistep reactions.[114] They are independent of acyl carrier proteins. Interestingly, no ortholog of *csyB* is present in *A. flavus*, despite the genetic similarity with *A. oryzae*. A plasmid carrying *csyB* was transformed into *A. oryzae* M-2-3 strain. A new product was isolated and characterized as csypyrone B1 (**64**).[115] In a similar fashion, the transformation of *csyA* led to the tetraketide 3,5-dihydroxybenzoic acid (DHBA) (**65**).[116]



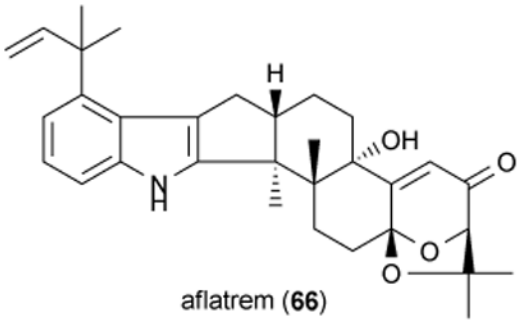
csypyrone B1 (**64**)



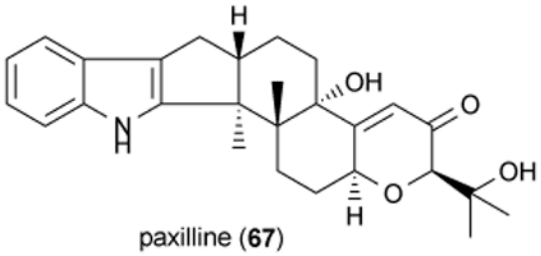
DHBA (**65**)

4.4 *A. flavus* secondary metabolites

4.4.1 Aflatrem—Prior to the availability of *A. flavus* genome sequence data, researchers studying the tremorgenic indole-diterpene aflatrem (**66**) had used degenerate primers for conserved domains of geranylgeranyl synthases to clone a GGPP synthase gene (*atmG*) and used chromosome walking to identify a cluster containing two additional secondary metabolite genes (*atmC and atmM*).[117] *Penicillium paxilli* generates a structurally similar indole-diterpene, paxilline (**67**). A plasmid containing a copy of *atmM* was introduced into a strain of *P. paxilli* missing the ortholog *paxM*, rescuing paxilline production and implicating *atmM* (and the clustered genes) in aflatrem biosynthesis.



aflatrem (**66**)



paxilline (**67**)

Following the whole genome sequencing of *A. flavus*, four additional candidate aflatrem genes were located, on another chromosome, based on their homology to paxilline genes.[67] This time, the monooxygenase gene *atmP* was introduced into a *P. paxilli paxP* mutant, which resulted predominantly in the synthesis of paxilline.

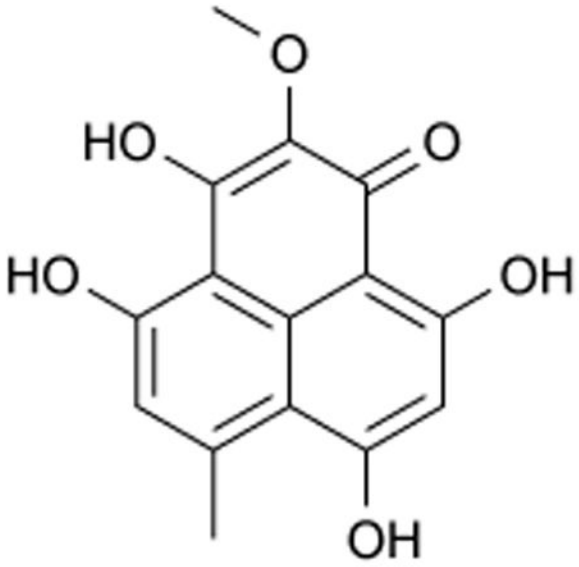
4.4.2 α-cyclopiazonic acid—The mycotoxin α-cyclopiazonic acid (CPA) (**68**) is a nanomolar inhibitor of Ca^{2+}-ATPase. It is produced by, among others, *A. flavus* and a strain of *A. oryzae*, and it is noted to be co-expressed with aflatoxin. Experimenters located the putative CPA prenyltransferase gene in the region between the aflatoxin gene cluster and the next telomere in CPA-nonproducing *A. oryzae* RIB40.[118] Sequence comparison of this region with CPA-producing *A. oryzae* NBRC4177 showed that NBRC4177 has an extension of 17–18 kb, and that RIB40 contains only a portion of the putative PKS–NRPS gene *cpaA*. Deletion of *cpaA* in NBRC4177 and resulting removal of CPA confirmed its role. Heterologous expression of *cpaA* leads to *cyclo*-acetoacetyl-L-tryptophan (**69**) (Scheme 9).[119] An analysis of the subtelomeric region of *A. flavus* flanking aflatoxin genes also identified putative CPA genes.[120] Gene deletion identified a crucial PKS–NRPS (*pks-nrps*), monoamine oxidase (*maoA*), and prenyltransferase gene (*dmaT*).

The study of the CPA PKS–NRPS afforded intriguing information that may pertain to many reductase R domains belonging to this class of protein. Using a recombinant PTP-R didomain and PTP and R monodomains, a team established that the R domain of CpdA actually does not function as a reductase, because Leu is in place of the Ser-Tyr-Lys catalytic triad for reduction, providing evidence that the R domain instead functions as a Dieckmann cyclase.[121]

4.5 Naptho-γ-pyrones and melanin from *A. niger*

A. niger generates a number of structurally similar, dimeric naphtho-γ-pyrones. Its genome features a homolog to a gene, *alb1*, in *A. fumigatus* that was demonstrated to encode a naphtho-γ-pyrone synthase upon heterologous expression. The *A. niger albA* gene was disrupted, resulting both in the elimination of all the naptho-γ-pyrones and in a white/colorless conidial phenotype.[122] The deletant of *aygA*, homologous to *A. fumigatus ayg1* whose protein converts a polyketide product to the tetrahydroxynapthalene precursor of melanin had little effect on naphtho-γ-pyrone formation but resulted in orange pigmented conidia, prompting researchers to propose that AlbAp produces a polyketide that is a branch point in both naphtho-γ-pyrone and melanin synthesis and that AygAp directs some of the polyketide to melanin formation.

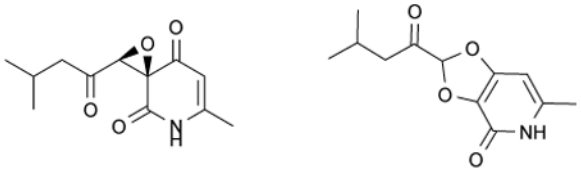
Another team obtained three different strains with lighter pigmentation compared to wild type.[123] Candidate genes behind the phenotypes were identified, based on homologues in *A. fumigatus*. Complementation with the *fwnA* mutant with the same gene labeled *alb1* in the other study, restored pigmentation. The other mutants, *olvA* and *brnA*, were complemented with the *ayg1* homolog and a multicopper oxidase homolog, respectively, to restore color. In agreement with the other work, the *fwnA*Δ strain cannot accumulate naphtho-γ-pyrones, but it was also noted that funalenone (**70**) is dependent on *fwnA*, *olvA*, and *brnA*, suggesting that it may be a product of the pigmentation pathway.



funalenone (**70**)

4.6 *A. terreus* secondary metabolites

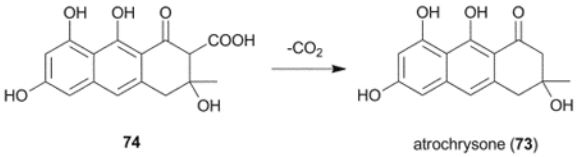
Experimenters located a PKS–NRPS gene in the *A. terreus* genome, but initial attempts to acquire the corresponding compound from culture were unsuccessful.[19] A reporter strain was then engineered, with the gene's promoter fused with the *E. coli lacZ* gene. Investigations with pH and media composition revealed that transcription was repressed when various sugars were utilized but activated when casamino acids (CAs) were introduced instead. Transcription was also stimulated by alkaline pH. The metabolite profile agreed, as two compounds of apparent polyketide and peptide origin, flavipucine (**71**), and isoflavipucine (**72**), were obtained from CA-containing media. The NRPS portion of the PKS-NRPS was reconstituted, and by incubating the NRPS with various amino acids and thiols, researchers obtained over 60 different thiopyrazine compounds in good yields, demonstrating the substrate promiscuity of this part of the megasynthase.[124]



flavipucine (**71**) isoflavipucine (**72**)

Experimenters noted a candidate emodin anthrone synthase PKS gene, *ACAS*, in *A. terreus* NIH2624 that was highly homologous to one in *A. terreus* RED1.[125] They found that the recombinant protein could catalyze loading and condensation of malonyl-CoA units, but, lacking a TE/CYC domain, it was unable to release its product. However, coincubation with the enzyme coded by the gene upstream by *ACAS*, *ACTE*, which is homologous to metallo-β-lactamases, resulted in the release of several products, chiefly atrochrysone (**73**).

Heterologous expression of the two genes in *A. oryzae* led to a number of metabolites that supported the notion that atrochrysone carboxylic acid (**74**) was the product of the PKS gene, and that the metallo-β-lactamase was responsible for hydrolyzing the thioester bond between the PKS and the octaketide.



74 atrochrysone (**73**)

5 Conclusion

A few years' hindsight has validated predictions that genomic sequencing of *Aspergillus* members would remarkably catalyze our advances in fungal secondary metabolite research. The above examples have illustrated that the data provided by sequencing projects have facilitated the identification of secondary metabolite genes and accelerated their characterization. What is more, the information has revealed to us that many more compounds await discovery, and the same information has helped to enable ways to bring these compounds to light.

In the course of this research as a whole, much has also been learned about the kinds of chemical transformations related to these pathways and their mechanistic detail. Because this large body of knowledge could constitute another comprehensive review, it has been left out here but is elaborated in many of the cited articles.

So what lies in the future? It may become clear that much of the “low-hanging fruit” of the well-studied *A. nidulans* and *A. fumigatus* species may have been depleted, and it will be up to scientists to persist in developing creative ways to unfold the secrets of the pathways that have been more resistant to discovery. In these organisms still, the majority of putative secondary metabolite genes have not been assigned to a known compound. It is not clear how many of them will be ultimately expressed, but as a comprehension of secondary metabolite regulation grows, it is likely that this knowledge will have a positive impact on efforts to give a voice to silent genes.

For the other *Aspergillus* species with sequenced genomes, the next several years are likely to continue to see new secondary metabolite developments as experimenters continue to devote effort to these organisms. Beyond, literally dozens of other *Aspergillus* members may be replete with compounds with remarkable biological properties. Expect again that future sequence data will translate into an abundance of discoveries of new secondary metabolites, and an understanding about how they came to be.

Acknowledgments

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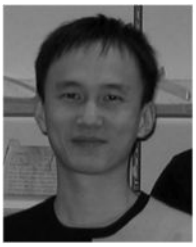
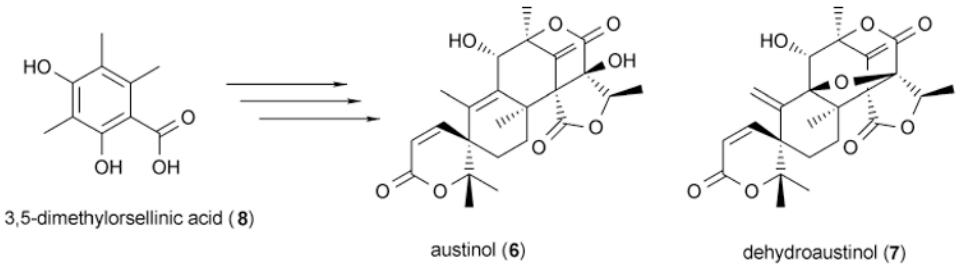
James F. Sanchez: James Sanchez has recently earned his doctorate in pharmaceutical sciences at the University of Southern California, previously receiving a B.S. in chemistry at the University of California, Irvine. His research has focused on the molecular genetic analysis of secondary metabolites from Aspergillus nidulans.

Amber D. Somoza: Amber Somoza is completing her doctoral research in chemistry at the University of Southern California after having received her B.S. from California State University of Long Beach in chemistry. Her research focuses on applying synthetic chemistry and metabolic engineering to generate structurally diverse natural products analogs using Aspergillus nidulans.

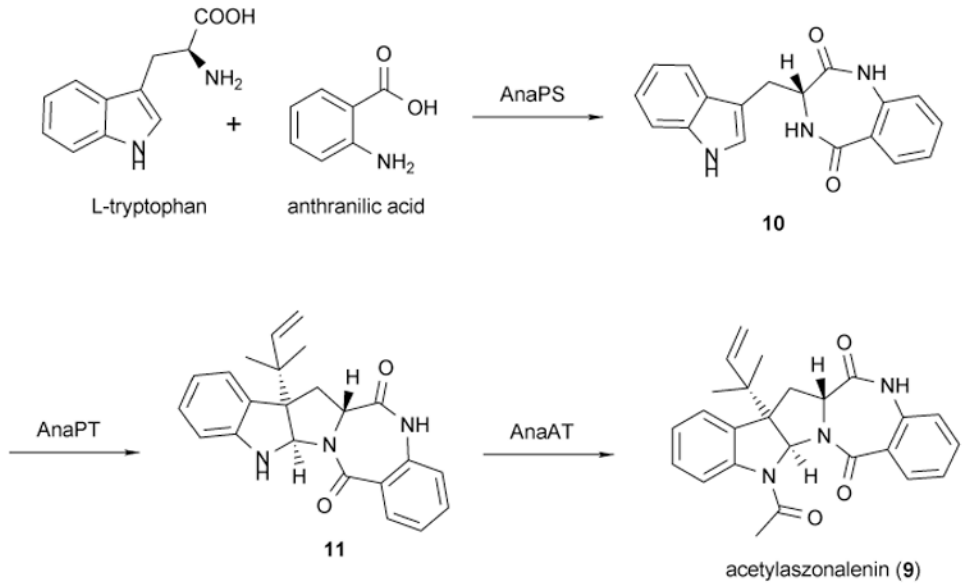
Nancy P. Keller: Nancy Keller is a Professor in the Departments of Medical Microbiology and Bacteriology at the University of Wisconsin, Madison, Wisconsin, USA. Her interest in fungal secondary metabolism dates back to her days in the Peace Corps in Lesotho, Africa from first hand knowledge of moulded and toxic food supplies. Her interests range from basic science studies of genetic regulation of fungal secondary metabolism to the role of these metabolites in pathogenicity of plant (e.g. A. flavus) and human (e.g. A. fumigatus) pathogenic fungi.



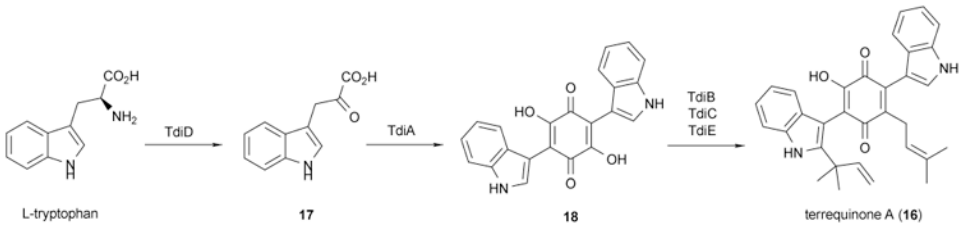
Clay C. C. Wang: Clay Wang is currently Associate Professor in the Departments of Pharmacology and Pharmaceutical Sciences and Chemistry at the University of Southern California. While as an undergrad at Harvard he attended a seminar given by Professor Chaitan Khosla, which sparked his lifelong interest in natural product biosynthesis. He obtained his Ph.D. in chemistry at Caltech with Professor Peter Dervan and completed a NIH post-doctoral fellowship with Professor Chaitan Khosla at Stanford University. The current focus of his laboratory is in fungal natural product biosynthesis, regulation, and engineering.



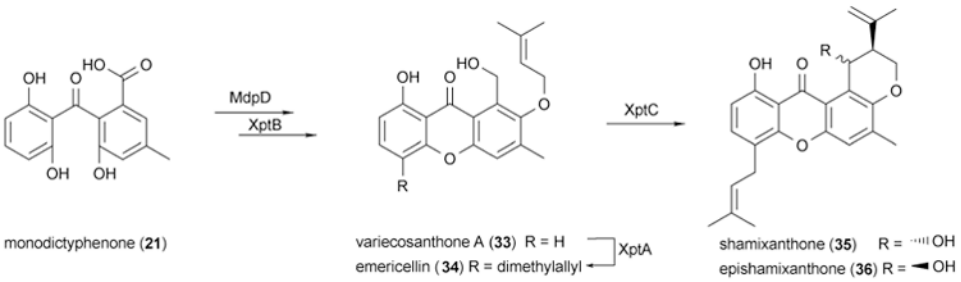
Scheme 1.
The biosynthetic pathway for austinol and dehydroaustinol.



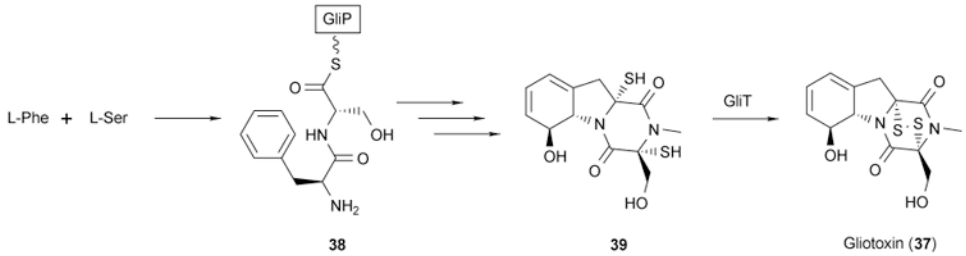
Scheme 2.
The biosynthetic pathway for acetylaszonalenin.



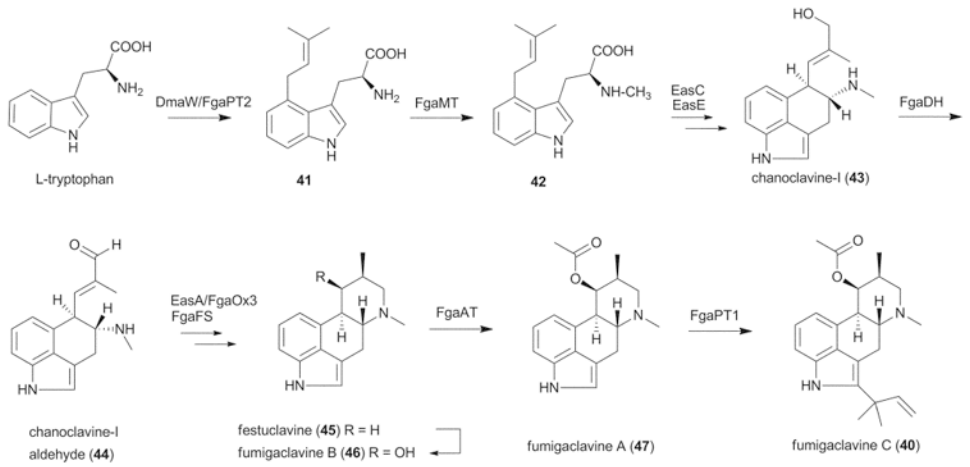
Scheme 3.
The biosynthetic pathway for terrequinone A.



Scheme 4.
The biosynthetic pathway for prenyl xanthones in *A. nidulans*.

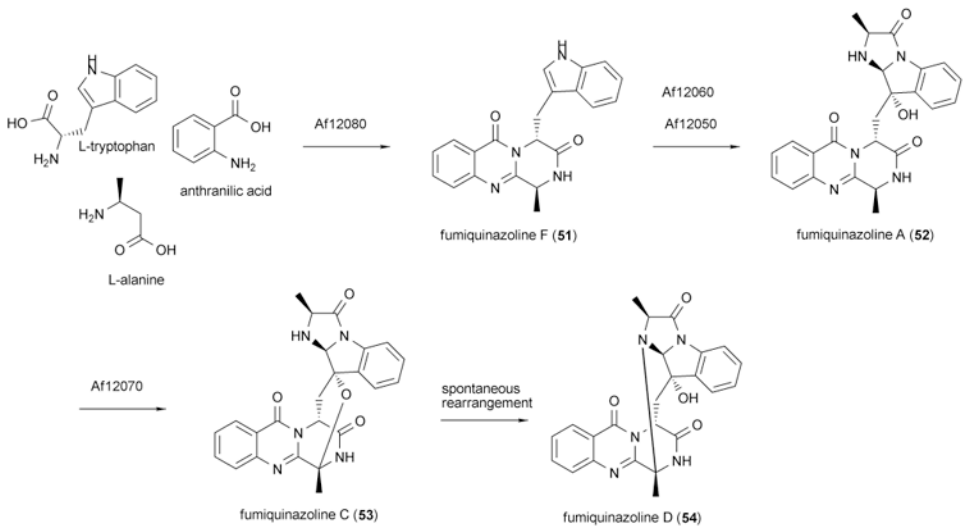


Scheme 5.
The biosynthetic pathway for gliotoxin.

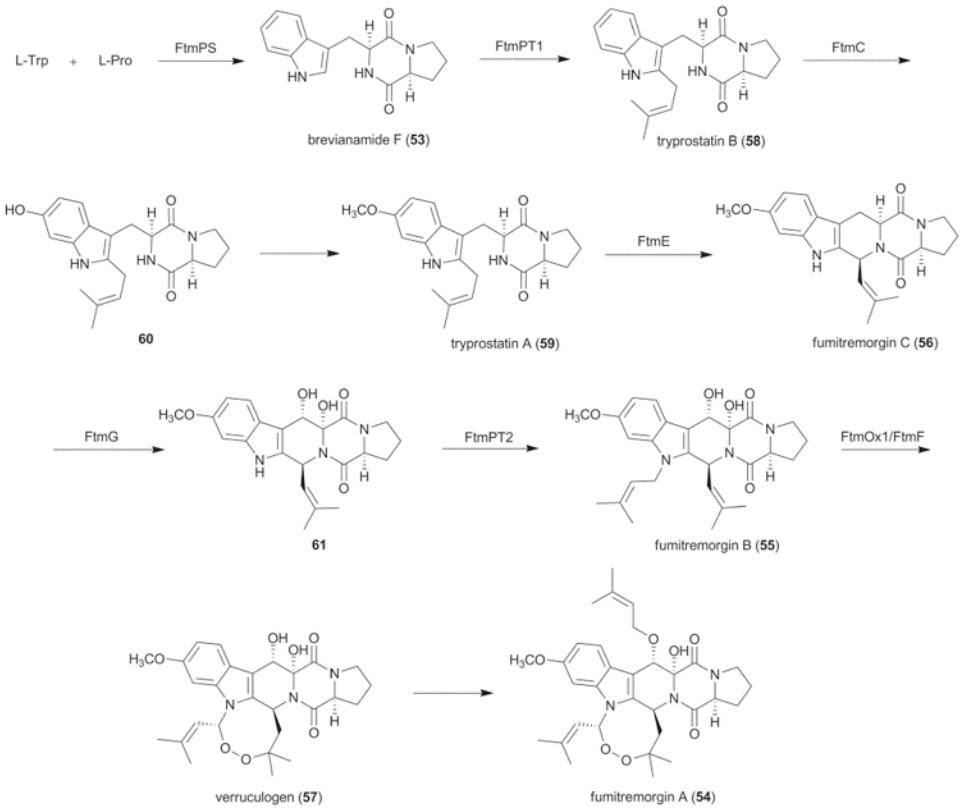


Scheme 6.

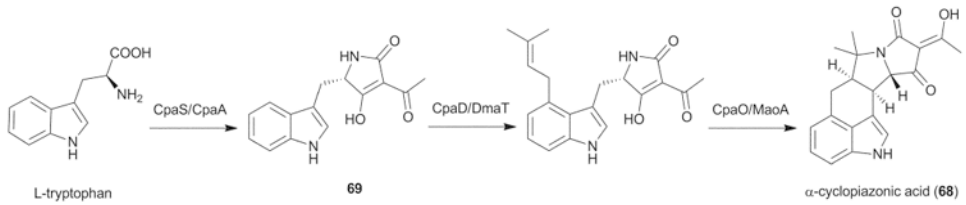
The biosynthetic pathway for fumigaclavine C.



Scheme 7.
The biosynthetic pathway for fumiquinazoline D.



Scheme 8.
The biosynthetic pathway for fumitremorgin A.



Scheme 9.
The proposed biosynthetic pathway for CPA.

Table 1

Features of *Aspergillus genomes*

Species	Strain	Size (Mb)	Chromosomes	Protein-coding Genes	Putative SM Core Genes[a]	PKS	PKS-like[b]	NRPS	NRPS-like[b]	Hybrid
A. carbonarius[126]	IMI 388653	36.30[126]	8	11,624[126]	61	28	—	33	—	—
A. clavatus[127]	NRRL 1	27.86[128]	8	9,121[128]	39	16	1	12	6	4
N.fischeri[127]	NRRL 181	32.55[128]		10,406[128]	46	17	1	19	9	0
A. flavus[129]	NRRL 3357	36.79[128]	8	12,604[128]	62	25	3	18	14	2
A. fumigatus[130]	Af293	29.38[128]	8	9,887[128]	34	13	2	13	5	1
A. fumigatus[127]	A1163	29.20[128]	8	9,906[128]	33	13	1	13	5	1
A. nidulans[4]	FGSC A4	30.07[128]	8	11,214[131]	52	24	3	12	12	1
A. niger[132]	CBS 513.88	37.20[128]	8	14,071[131]	79	35	4	17	16	7
A. niger[c] [133,134]	ATCC 1015	34.85[135]	8	11,197[135]	57	33	—	15	—	9
A. oryzae[136]	RIB40	37.12[128]	8	12,063[128]	61	27	0	16	16	2
A. terreus	NIH2624	29.33[128]	8	10,406[128]	65	28	2	20	14	1

[a] genes identified by SMURF,[11] except for A. niger ATCC 1015[135] and A. carbonarius[126]

[b] non-canonical domain structure.

[c] *A. niger* strain ATCC 9029 (NRRL 3) has also been sequenced.

abbreviations: SM = secondary metabolite, Hybrid = PKS–NRPS.

Table 2
Secondary metabolism gene clusters in *A. nidulans*

No.	Broad designation	Gene name	Gene type[a]	Actual or predicted product[b]
1	AN0016.3	*pes1*	NRPS	
2	AN0150.3	*mdpG*	NR-PKS	**monodictyphenone (18), emodin (17),**[54] **xanthones (30–33)**[66]
3	AN0523.3		NR-PKS	
4	AN0607.3	*sidC*	NRPS	**ferricrocin**[137]
5	AN1034.3	*afoE*	NR-PKS	**asperfuranone (15)**[34]
6	AN1036.3	*afoG*	PR-PKS	**asperfuranone (15)**[34]
7	AN1242.3		NRPS	cyclotripeptide
8	AN1680.3		NRPS-like	
9	AN1784.3		PR-PKS	
10	AN2032.3		NR-PKS	
11	AN2035.3		PR-PKS	
12	AN2064.3		NRPS-like	
13	AN2545.3	*easA*	NRPS	**emericellamides (1–5)**[26]
14	AN2547.3	*easB*	PR-PKS	**emericellamides (1–5)**[26]
15	AN2621.3	*acvA*	NRPS	**penicillin**[138]
16	AN2924.3		NRPS-like	
17	AN3230.3		NR-PKS	
18	AN3386.3		NR-PKS	
19	AN3396.3		NRPS-like	
20	AN3495.3		NRPS-like	
21	AN3496.3		NRPS	
22	AN3612.3		PR-PKS	
23	AN4827.3		NRPS-like	
24	AN5318.3		NRPS-like	
25	AN6000.3	*aptA*	NR-PKS	**asperthecin (21)**[55]
26	AN6236.3	*sidD*	NRPS	siderophore
27	AN6431.3		PR-PKS	
28	AN6444.3		NRPS-like	
29	AN6448.3		NR-PKS	
30	AN6791.3		PR-PKS	
31	AN7071.3		NR-PKS	
32	AN7084.3		PKS-like	
33	AN7489.3		PKS-like	
34	AN7815.3	*stcJ*	FAS subunit alpha	**sterigmatocystin**[139]
35	AN7825.3	*stcA (pksST)*	NR-PKS	**sterigmatocystin**[139]
36	AN7838.3		PKS-like	
37	AN7884.3		NRPS	
38	AN7909.3	*orsA*	NR-PKS	**orsellinic acid (22),**[59,60] **F-9775A (19), B (20)**[54,59]

No.	Broad designation	Gene name	Gene type[a]	Actual or predicted product[b]
39	AN8105.3		NRPS-like	
40	AN8209.3	*wA*	NR-PKS	**naphthopyrone (YWA1)**[140]
41	AN8383.3		NR-PKS	
42	AN8412.3	*apdA*	Hybrid	**aspyridone A (13), B (14)**[33]
43	AN8513.3	*tdiA*	NRPS	**terrequinone A (9)**[46]
44	AN8910.3		PR-PKS	
45	AN9005.3		PR-PKS	
46	AN9226.3		NRPS	
47	AN9244.3		NRPS	
48	AN9291.3		NRPS-like	
49	AN10297.3		NRPS-like	
50	AN10430.3		NR-PKS	
51	AN10486.3		NRPS-like	
52	AN10576.3		NRPS	
53	AN11191.3		PR-PKS	

[a] abbreviations: polyketide synthase (PKS), non ribosomal peptide synthetase (NRPS), PKS–NRPS (Hybrid), fatty acid synthase (FAS), nonreduced polyketide synthase (NR-PKS), partially reduced polyketide synthase (PR-PKS), highly reduced polyketide synthase (HR-PKS)

[b] Actual products are highlighted in bold.

Table 3

Secondary metabolism gene clusters in *A. fumigatus*

No.	Af293 gene (Broad designation)	A1163 gene (Cadre designation)	Gene name	Gene type[a]	Actual or predicted product[b]
1	Afu1g01010	no homolog		PR-PKS	
2	Afu1g10380	AFUB_009800	*pesB (pes1)*	NRPS	
3	Afu1g17200	AFUB_016590	*sidC*	NRPS	**ferricrocin, hydroxyferricrocin**[141,142]
4	Afu1g17740	AFUB_045790		PR-PKS	
5	Afu2g01290	AFUB_018370		PR-PKS	
6	Afu2g05760	AFUB_022790		PKS-like	
7	Afu2g17600	AFUB_033290	*alb1(pksP)*	NR-PKS	**YWA1**[143]
8	Afu3g01410	AFUB_046990		PR-PKS	
9	Afu3g02530	no homolog		PKS-like	
10	Afu3g02570	no homolog		NR-PKS	
11	Afu3g02670	AFUB_045610		NRPS-like	
12	Afu3g03350	AFUB_044900	*sidE*	NRPS	
13	Afu3g03420	AFUB_044830	*sidD*	NRPS	**fusarinine C, triacetylfusarinine C**[141,142]
14	Afu3g12920	AFUB_036270	*pesF*	NRPS	
15	Afu3g13730	AFUB_035460	*pesG*	NRPS	
16	Afu3g14700	AFUB_034520		PR-PKS	
17	Afu3g15270	AFUB_033950	*pesH*	NRPS	
18	Afu4g00210	AFUB_100730		NR-PKS	
19	Afu4g14560	AFUB_071800		NR-PKS	
20	Afu5g10120	AFUB_057720		NRPS-like	
21	Afu5g12730	AFUB_060400	*pesI*[144]	NRPS	
22	Afu6g03480	AFUB_094810		NRPS-like	
23	Afu6g08560	AFUB_074520		NRPS-like	
24	Afu6g09610	AFUB_075660	*pesJ*	NRPS	
25	Afu6g09660	AFUB_075710	*gliP*	NRPS	**gliotoxin (37)**[74–76]
26	Afu6g12050	AFUB_078040		NRPS	**fumiquinazolines (49–52)**[99]
27	Afu6g12080	AFUB_078070		NRPS	**fumiquinazolines (49–52)**[98]
28	Afu6g13930	AFUB_000820	*pyr2*	PR-PKS	**pyripyropene A (12)**[31]

No.	Af293 gene (Broad designation)	A1163 gene (Cadre designation)	Gene name	Gene type[a]	Actual or predicted product[b]
29	Afu7g00160	AFUB_086700		NR-PKS	
30	Afu8g00170	AFUB_086360	*ftmA*	NRPS	**fumitremorgins (53–59)**[106]
31	Afu8g00370	AFUB_086200		PR-PKS	
32	Afu8g00540	AFUB 086030	*psoA*	hybrid	**pseurotin A (48)**[96]
33	Afu8g01640	AFUB_084950		NRPS-like	
34	Afu8g02350	AFUB_084240		NR-PKS	
35	no homolog	AFUB 079710		PKS	
36	no homolog	AFUB_045640		PKS	

[a] abbreviations: polyketide synthase (PKS), non ribosomal peptide synthetase (NRPS), PKS–NRPS (Hybrid), non reduced polyketide synthase (NR-PKS), partially reduced polyketide synthase (PR-PKS), highly reduced polyketide synthase (HR-PKS).

[b] Actual products are highlighted in bold.

Table 4
Secondary metabolism gene cluster in *A. terreus*

No.	Broad designation	Gene name	Gene type[a]	Actual or predicted product[b]
1	ATEG_00145.1	*pes1*		
2	ATEG_00228.1		NRPS	
3	ATEG_00282.1			
4	ATEG_00325.1	*ftmA*	Hybrid	**flavipucine (71), isoflavipucine (72)**[19]
5	ATEG_00700.1		NRPS-like	
6	ATEG_00881.1		NRPS	
7	ATEG_00913.1		NR-PKS	
8	ATEG_01002.1		NRPS	
9	ATEG_01052.1		NRPS-like	
10	ATEG_01894.1		PR-PKS	
11	ATEG_02004.1		NRPS-like	
12	ATEG_02403.1		NRPS-like	
13	ATEG_02434.1		NR-PKS	
14	ATEG_02831.1		NRPS	
15	ATEG_02944.1		NRPS	
16	ATEG_03090.1		NRPS-like	
17	ATEG_03432.1		NR-PKS	
18	ATEG_03446.1		PR-PKS	
19	ATEG_03470.1		NRPS	
20	ATEG_03528.1		NRPS	
21	ATEG_03576.1		NRPS	
22	ATEG_03629.1		NR-PKS	
23	ATEG_03630.1		NRPS-like	
24	ATEG_04322.1		NRPS	
25	ATEG_04323.1		NRPS	
26	ATEG_04718.1		PR-PKS	
27	ATEG_04975.1		NRPS-like	
28	ATEG_05073.1	*sidC*	NRPS	siderophore
29	ATEG_05795.1		NRPS-like	
30	ATEG_06056.1		PR-PKS	
31	ATEG_06113.1		NRPS	
32	ATEG_06206.1		NR-PKS	
33	ATEG_06275.1	*atX*	PR-PKS	**6-methylsalicyclic acid (MSAS)**[145]
34	ATEG_06680.1		PR-PKS	
35	ATEG_06765.1		PKS-like	
36	ATEG_06998.1		NRPS-like	
37	ATEG_07067.1		PR-PKS	
38	ATEG_07279.1		PR-PKS	
39	ATEG_07282.1		PR-PKS	

No.	Broad designation	Gene name	Gene type[a]	Actual or predicted product[b]
40	ATEG_07358.1		NRPS	
41	ATEG_07379.1		NR-PKS	
42	ATEG_07380.1		NRPS-like	
43	ATEG_07488.1	*sidD*	NRPS	siderophore
44	ATEG_07500.1		NR-PKS	YWA1
45	ATEG_07659.1		PR-PKS	
46	ATEG_07661.1		NR-PKS	
47	ATEG_07894.1		NRPS-like	
48	ATEG_08172.1		PR-PKS	
49	ATEG_08427.1		NRPS	
50	ATEG_08448.1		NRPS	
51	ATEG_08451.1		NR-PKS	**atrochyrsone (73)**[125]
52	ATEG_08662.1		NR-PKS	
53	ATEG_08678.1		NRPS-like	
54	ATEG_09019.1		NRPS	
55	ATEG_09033.1		NRPS-like	
56	ATEG_09064.1		NRPS	
57	ATEG_09068.1		NRPS	
58	ATEG_09088.1		PR-PKS	
59	ATEG_09100.1		PKS	
60	ATEG_09142.1		NRPS-like	
61	ATEG_09617.1		PR-PKS	
62	ATEG_09961.1	*lovB*	HR-PKS	**lovastatin**[2]
63	ATEG_09968.1	*lovF*	HR-PKS	**lovastatin**[2]
64	ATEG_10080.1		PKS-like	
65	ATEG_10305.1		NRPS	

[a] abbreviations: polyketide synthase (PKS), non ribosomal peptide synthetase (NRPS), PKS–NRPS (Hybrid), nonreduced polyketide synthase (NR-PKS), partially reduced polyketide synthase (PR-PKS), highly reduced polyketide synthase (HR-PKS).

[b] Actual products are highlighted bold.

Table 5

Secondary metabolism gene clusters in *A. niger*

No.	CBS 513.88 gene (NCBI Designation)	ATCC 1015 gene[146] (JGI Designation)	Gene name	Gene type[a]	Actual or predicted product[b]
1	An01g00060[c]	Aspni1:55511		FAS subunit alpha	
2	An01g01130	no homolog		PKS	
3	An01g06930	Aspni1:225574	*fum1*	HR-PKS	fumonisins[147,148]
4	An01g06950	Aspni1:225587		PR-PKS	
5	An01g11770	und		NRPS-like	
6	An02g00210	und		NRPS-like	
7	An02g00450	Aspni1:118617		HR-PKS	
8	An02g00840	und		NRPS-like	
9	An02g05070	Aspni1:36929		NRPS	
10	An02g08290	Aspni1:118624		Hybrid	
11	An02g09430	Aspni1:37260		HR-PKS	
12	An02g10140	und		NRPS-like	
13	An02g14220	und		PKS-like	
14	An03g00650	Aspni1:128584		NRPS	
15	An03g01820	no homolog		PKS	
16	An03g03520	Aspni1:191228	*sidD*	NRPS	siderophore
17	An03g05140	Aspni1:118598		HR-PKS	
18	An03g05440	Aspni1:191422		NR-PKS	
19	An03g05680	und		NRPS-like	
20	An03g06010	Aspni1:44571		NRPS	
21	An03g06380	Aspni1:191702		HR-PKS	
22	An04g01150	und		NRPS-like	
23	An04g04340	Aspni1:44005		PR-PKS	
24	An04g04380	und		NRPS-like	
25	An04g06260	Aspni1:118635		NRPS	
26	An04g09530	Aspni1:51499		NR-PKS	
27	An04g10030	Aspni1:118662		HR-PKS	
28	An05g01060	Aspni1:118599		NRPS	

No.	CBS 513.88 gene (NCBI Designation)	ATCC 1015 gene[146] (JGI Designation)	Gene name	Gene type[a]	Actual or predicted product[b]
29	An06g00430[c]	Aspni1:175936		PKS	
30	An06g01300	Aspni1:207636	*sidC*	NRPS	siderophore
31	An07g01030	no homolog		PKS	
32	An08g02310	Aspni1:52774		NRPS	
33	An08g03790	Aspni1:176722		Hybrid	
34	An08g04820	und		NRPS-like	
35	An08g09220	und		NRPS-like	
36	An08g10930	und		PKS-like	
37	An09g00450	und		NRPS-like	
38	An09g00520	no homolog		NRPS	
39	An09g01290	Aspni1:43495		HR-PKS	
40	An09g01690	Aspni1:212679		NRPS	
41	An09g01860	Aspni1:56946		NR-PKS	
42	An09g01930	Aspni1:188817		HR-PKS	
43	An09g02100	und		PKS-like	
44	An09g05110	und		NRPS-like	
45	An09g05340	Aspni1:188697		HR-PKS	
46	An09g05730	Aspni1:56896	*fwnA or albA*	NR-PKS	**naphtho-γ-pyrones, melanin**[122,123]
47	An10g00140	Aspni1:44965		PR-PKS	
48	An10g00630	und		PKS-like	
49	An11g00050	Aspni1:118659		NRPS	
50	An11g00250	Aspni1:179585		Hybrid	
51	An11g03920	Aspni1:179079		HR-PKS	
52	An11g04280	Aspni1:39026		PR-PKS	
53	An11g05500	und		NRPS-like	
54	An11g05570	Aspni1:47991		HR-PKS	
55	An11g05940	no homolog		PKS	
56	An11g05960	no homolog		PKS	
57	An11g06460	Aspni1:118644		Hybrid	
58	An11g07310	no homolog		PKS	
59	An11g09720	Aspni1:118629		PR-PKS	

No.	CBS 513.88 gene (NCBI Designation)	ATCC 1015 gene[146] (JGI Designation)	Gene name	Gene type[a]	Actual or predicted product[b]
60	An12g02050[c]	Aspni1:190014		NR PKS	
61	An12g02670	Aspni1:189378		HR-PKS	
62	An12g02730	no homolog		PKS	
63	An12g02840	Aspni1:43807		NRPS	
64	An12g07070	Aspni1:118666		HR-PKS	
65	An12g07230	Aspni1:42205		NRPS	
66	An12g10090	und		NRPS-like	
67	An12g10860	und		NRPS-like	
68	An13g02430	Aspni1:128638		HR-PKS	
69	An13g02460	und		NRPS-like	
70	An13g02960	no homolog		PKS	
71	An13g03040	no homolog		NRPS	
72	An14g01910	Aspni1:41618		Hybrid	
73	An14g04850	Aspni1:41846		Hybrid	
74	An15g02130	Aspni1:181803		HR-PKS	
75	An15g04140	Aspni1:210217		HR-PKS	
76	An15g05090	Aspni1:118744		PR-PKS	
77	An15g07530	Aspni1:182031		NRPS	
78	An15g07910	no homolog		NRPS	ochratoxin[147]
79	An15g07920	no homolog		PKS	ochratoxin[147]
80	An16g00600	und		NRPS-like	
81	An16g06720	Aspni1:118601		NRPS	
82	An18g00520	Aspni1:187099		Hybrid	
83	no homolog	Aspni1:55153		NRPS	
84	no homolog	Aspni1:118581		Hybrid	
85	no homolog	Aspni1:128601		Hybrid	
86	no homolog	Aspni1:138585		HR PKS	
87	no homolog	Aspni1:171221		PR PKS	
88	no homolog	Aspni1:194381		NR-PKS	
89	no homolog	Aspni1:211885		PR-PKS	

[a] abbreviations: polyketide synthase (PKS), non ribosomal peptide synthetase (NRPS), PKS–NRPS (Hybrid), fatty acid synthase (FAS), nonreduced polyketide synthase (NR-PKS), partially reduced polyketide synthase (PR-PKS), highly reduced polyketide synthase (HR-PKS).

[b] Actual products are highlighted in bold.

[c] gene not identified by SMURF abbreviations: und = undetermined.