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Article

Molecular and Chemical Characterization of the Biosynthesis of the 6-MSA-Derived Meroterpenoid Yanuthone D in *Aspergillus niger*

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SUMMARY

Secondary metabolites in filamentous fungi constitute a rich source of bioactive molecules. We have deduced the genetic and biosynthetic pathway of the antibiotic yanuthone D from *Aspergillus niger*. Our analyses show that yanuthone D is a meroterpenoid derived from the polyketide 6-methylsalicylic acid (6-MSA). Yanuthone D formation depends on a cluster composed of ten genes including *yanA* and *yanI*, which encode a 6-MSA polyketide synthase and a previously undescribed O-mevalon transferase, respectively. In addition, several branching points in the pathway were discovered, revealing five yanuthones (F, G, H, I, and J). Furthermore, we have identified another compound (yanuthone X_1) that defines a class of yanuthones that depend on several enzymatic activities encoded by genes in the *yan* cluster but that are not derived from 6-MSA.

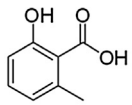
INTRODUCTION

Fungal polyketides (PKs) comprise a large and complex group of metabolites with a wide range of bioactivities. Hence, the group includes compounds that are used by fungi as pigments for UV-light protection, in intra- and interspecies signaling, and in chemical warfare against competitors (Williams et al., 1989). Many PKs are mycotoxins that are harmful to human health, e.g., patulin and the highly carcinogenic aflatoxins (Olsen et al., 1988). On the other hand, several PKs have a great medical potential, e.g., cholesterol-lowering statins (Endo et al., 1976), the antimicrobial and immunosuppressive mycophenolic acid (Bentley, 2000), the acetyl-coenzyme A acetyltransferase-inhibiting pyripyropenes (Frisvad et al., 2009), and the farnesyltransferase inhibiting andrastins (Rho et al., 1998). Although more than 6,000 different PKs have been isolated and characterized (AntiBase 2012), these compounds are likely only the tip of the iceberg. For example, for each fungus analyzed, only a small part of its full repertoire of PKs genes appears to be produced under laboratory conditions (Pel et al., 2007; Andersen et al., 2013). In agreement with this view, genome sequencing of several fungal species have uncovered far more genes for PKs production than can be accounted for by the number of compounds that they are actually known to produce. Hence, the chemical space of PKs is far from fully known, and many new drugs and mycotoxins await discovery.

The fungal genome sequencing projects have demonstrated that genes necessary for production of individual PKs often cluster around the gene encoding the polyketide synthase (PKS), which delivers the first intermediate in a given PK pathway. Although this is helpful for pathway elucidation, compounds produced by orphan gene clusters (Gross, 2007) can still not be easily predicted by bioinformatic tools (for review, see Cox, 2007 and Hertweck, 2009). This is because most fungal PKs are produced by type I iterative PKSs whose products are notoriously difficult to predict. Moreover, the specificities and the order of actions of the tailoring enzymes that modify the PK released from the PKS further complicate prediction of the end products. To elucidate the biochemical pathway of an orphan gene cluster, it is therefore necessary to create gene cluster mutations and/or to genetically reconstitute the pathway in a heterologous host. Subsequent analytical and structural chemistry analyses of the compounds that are present in the reference strain but not in the mutant strains and of compounds that accumulate in the mutant strains but are absent or present in minute amounts in the reference strain may deliver insights that can be used for pathway elucidation.

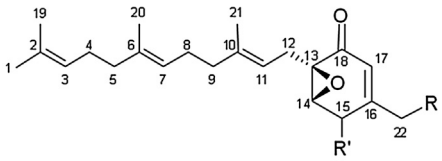
Aspergillus niger is an industrially important filamentous fungus, which has obtained GRAS status for use in several industrial processes and is used for production of organic acids and enzymes. Importantly, when the full genome sequence of *A. niger* was examined, a gene cluster resembling the fumonisin gene

A



6-MSA

B



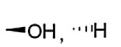
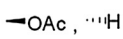
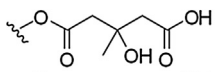
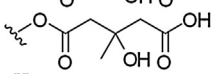
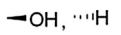
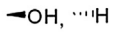
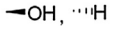
Compound	R	R'
Yanuthone A	OAc	
Yanuthone B	OAc	=O
Yanuthone C	OH	
Yanuthone D (1)		=O
Yanuthone E (2)		
7-deacetoxyyanuthone A (3)	H	
22-deacetylyanuthone A (6)	OH	

Figure 1. Chemical Structures of 6-MSA and Previously Described Yanuthones

(A) Chemical structure of 6-MSA.

(B) Chemical structures of previously described yanuthones: yanuthones A–E, 7-deacetoxyyanuthone A, and 22-deacetylyanuthone A (Bugni et al., 2000; Li et al., 2003).

cluster from *Gibberella moniliformis* was surprisingly identified, suggesting that this well-characterized fungus has the genetic potential to produce the carcinogenic fumonisins (Baker, 2006). This possibility was later confirmed by genetic and chemical analyses (Pel et al., 2007; Frisvad et al., 2007). The fact that the *A. niger* genome contains several orphan gene clusters for production of secondary metabolites (Fisch et al., 2009) raises the question of whether it can produce other bioactive PKs that could be harmful, or perhaps beneficial, to human health. To this end, one silent cluster in *A. niger* was recently activated by expression of a transcription factor-encoding gene, which was embedded in the cluster. The resulting strain produced six azaphilone compounds, and further studies uncovered substantial new insights into the biosynthesis of this class of compounds (Zabala et al., 2012). It is interesting to note that among the 33 predicted PKS and PKS-like genes in *A. niger*, one encodes a putative PKS, which is phylogenetically close to fungal 6-methylsalicylic acid (6-MSA) synthases (Fisch et al., 2009). Importantly, the model PK 6-MSA (Wattanachaisaereekul et al., 2008) (Figure 1A) is known to be the precursor to, for example, the mycotoxin patulin (Beck et al., 1990) produced by many *Aspergillus* and *Penicillium* species, substantiating the possibility that this gene could be the source of yet another unknown bioactive PK in *A. niger*. Importantly, none of these 6-MSA-derived compounds have been observed in *A. niger* (Nielsen et al., 2009). We therefore investigated whether *A. niger* has the potential to produce 6-MSA or 6-MSA-derived compounds.

Known yanuthones constitute a group of compounds that are derived from a six-membered methylated ring (the C_7 core scaffold) with three side chains: one sesquiterpene and two varying side chains (-R and R') (Figure 1B). In this study we demonstrate that in *A. niger*, 6-MSA is the precursor for formation of yanuthone D, which is an antibiotic against *Candida albicans*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus* (Bugni et al., 2000). We also show that yanuthone D is in fact a complex meroterpenoid synthesized by a pathway where 6-MSA is decarboxylated, heavily oxidized, and fused to a sesquiterpene and a mevalon moiety (the di-acid of mevalonic acid). This is surprising, because yanuthones have been hypothesized to originate from the shikimate pathway (Bugni et al., 2000).

RESULTS

A. niger PKS48 Encodes a 6-MSA Synthase

To investigate the possibility that the *A. niger* gene PKS48/ASPNIDRAFT_44965 encodes a 6-MSA synthase, we transferred the gene to *A. nidulans*, which has not been shown to produce 6-MSA and which does not contain a close homolog to known 6-MSA PKSs. To ensure a high expression level on a defined medium, the PKS48 gene was integrated into a well characterized integration site, *IS1* (Hansen et al., 2011), under control of the strong constitutive promoter *PgpdA*. As expected, the metabolite profile obtained with an *Aspergillus nidulans* reference strain (IBT 29539) did not show any indications of 6-MSA when analyzed by ultra-high-performance liquid chromatography (UPHLC)-UV-visible diode array detector (DAD)-high-resolution time-of-flight mass spectrometry (TOFMS) (Figure 2A). In contrast, the metabolite profile of the strain expressing PKS48 showed the presence of a prominent new peak, which had the same retention time as an authentic 6-MSA standard and displayed the same adducts and monoisotopic mass for the pseudomolecular ion. We therefore conclude that PKS48 encodes a 6-MSA synthase.

Production of Yanuthones D and E Is Eliminated by Deletion of PKS48

The fact that 6-MSA has not previously been reported from *A. niger* prompted us to investigate whether this compound could be a precursor to a known secondary metabolite produced by this fungus. We therefore cultivated an *A. niger* reference strain (KB1001) and an *A. niger* PKS48Δ strain on four different solid media (minimal medium [MM], CYA, YES, and MEA) that are known to trigger the production of a wide range of metabolites (Nielsen et al., 2011). The resulting UHPLC-DAD-TOFMS metabolite profiles were almost identical (Figure S1 available online), showing that the PKS48Δ mutation did not induce a global response on the secondary metabolism. However, on YES and MM media, we identified two compounds that were produced by KB1001, but not by the PKS48Δ strain (Figures 2B and 2C; Table S1). UHPLC separation with UV-visible and

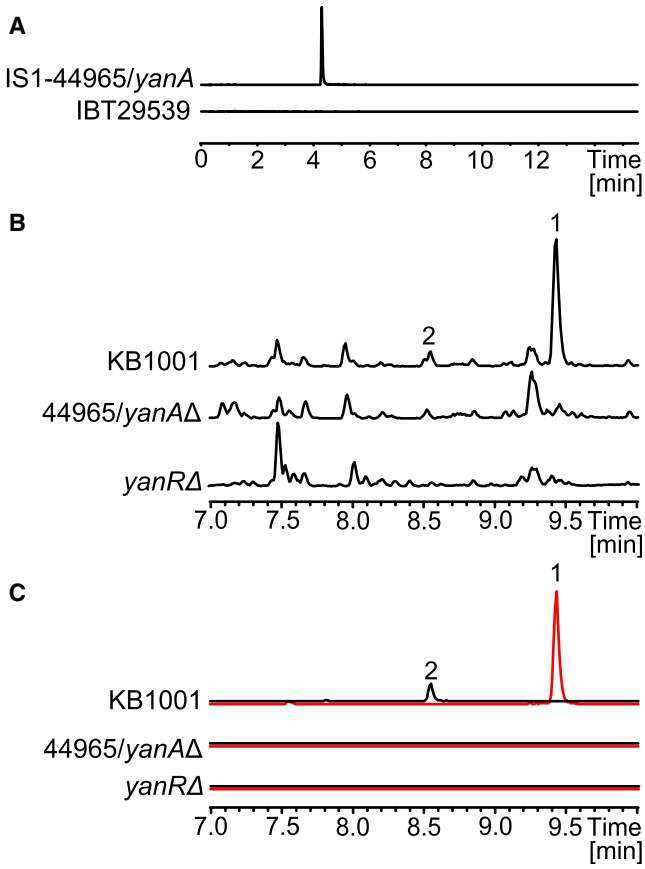


Figure 2. Extracted Ion Chromatograms

(A) Extracted ion chromatogram (EIC, *m/z* 153.0546 ± 0.005) of an *A. nidulans* reference strain (IBT 29539) and a 6-MSA producing strain (IS*1*-44965/*yanA*).

(B) Base peak chromatograms (BPC) *m/z* 100-1,000 of the *A. niger* reference (KB1001), *yanA*Δ, and *yanR*Δ strains.

(C) EICs of yanuthone D (**1**) 503.2640 ± 0.005 (red) and yanuthone E (**2**) 505.2791 ± 0.005 (black) for KB1001, *yanA*Δ, and *yanR*Δ.

All chromatograms are to scale.

high-resolution MS detection as well as MS/MS suggested that the two compounds were yanuthones D and E. This was confirmed by isolation of the compounds, nuclear magnetic resonance (NMR) spectroscopy, and circular dichroism (CD) (Tables S3 and S4). Hence, production of yanuthones D and E appears to be based on the use of 6-MSA as a key precursor. In this scenario, one carbon must be eliminated from C_8-based 6-MSA to form the C_7 core scaffold of yanuthones D and E.

Yanuthones Constitute a Complex Group of Compounds That Appear to Originate from Different Precursors

In addition to yanuthones D and E, *A. niger* has previously been reported to produce yanuthones A, B, and C, 1-hydroxyyanuthone A, 1-hydroxyyanuthone C, and 22-deacetylyanuthone A (Bugni et al., 2000), and 7-deacetoxyyanuthone A has been reported from the genus *Penicillium* (Li et al., 2003) (Figure 1B). We thus examined the extracted ion chromatograms from the UHPLC-DAD-TOFMS profiles obtained by KB1001 for the presence of these metabolites. In extracts obtained after cultivation on MM, YES, and CYA media, this analysis identified trace amounts of a compound (yanuthone X_1) with a mass and elemental composition corresponding to the yanuthone isomers A and C. The nature of this compound was further investigated by MS/MS, and its fragmentation pattern was similar to the pattern of other yanuthones, showing characteristics such as loss of a sesquiterpene chain. Moreover, the UV-visible spectrum of the compound was similar to spectra obtained for yanuthones D and E, substantiating that this compound was a yanuthone. Surprisingly, when the UHPLC-DAD-TOFMS metabolite profiles obtained with the PKS48Δ strain were examined for the presence of this yanuthone, it was still present. This observation strongly suggested that some yanuthones are produced independently of PKS48.

Fully Labeled $^{13}C_8$-6-MSA Is Incorporated into Yanuthones D and E In Vivo

The fact that some yanuthones could be produced independently of PKS48, combined with the fact that yanuthones have been proposed to originate from the shikimate pathway, raised the possibility that the absence of yanuthones D and E in the PKS48 deletion strain potentially could be the result of an indirect effect. To investigate this possibility, we fed fully labeled $^{13}C_8$-6-MSA to KB1001 and the PKS48Δ strain at different time points during growth (24, 48, and 72 hr; see Experimental Procedures). The addition of $^{13}C_8$-6-MSA did not seem to adversely affect the growth rate, and the morphologies of the colonies of the two strains were identical (Figure S2). This indicates that the amounts of $^{13}C_8$-6-MSA added (2-10 μg/ml) did not significantly influence strain fitness. Metabolites were then extracted from the plates and analyzed by UHPLC-DAD-TOFMS. For both strains, $^{13}C_8$-6-MSA was incorporated into yanuthones D and E, resulting in a mass shift of 7.023 Da. This is in agreement with the scenario described above, where one carbon atom must be eliminated from 6-MSA in the biosynthetic processing toward yanuthones D and E. Moreover, the MS-based metabolite profiles also showed that $^{13}C_8$-6-MSA was exclusively incorporated into compounds related to yanuthones. These compounds are only present in tiny amounts and are likely intermediates or analogs of yanuthone D or E, because they share the same UV chromophore and because their masses corresponded to water loss(es) or gain from yanuthone D or E. Based on these results, we named the 6-MSA synthase (encoded by PKS48/ASPNIDRAFT_44965) YanA (*yan*uthone) and the corresponding gene *yanA.* On the other hand, no labeled yanuthone X_1 was observed in KB1001 as well as in the PKS48 deletion strain after addition of $^{13}C_8$-6-MSA (mass spectra are shown in Figure S3), confirming our finding that yanuthone X_1 is formed in the absence of PKS48. Hence, we conclude that 6-MSA is not the precursor of yanuthone X_1.

The *yan* Gene Cluster Comprises Ten Genes

To determine whether *yanA* defines a gene cluster for a biosynthetic pathway toward yanuthones D and E, ten genes up- and downstream of *yanA* were annotated using FGeneSH (Softberry) and AUGUSTUS software (Stanke and Morgenstern, 2005). Subsequently, these twenty putative genes were examined using the NCBI Conserved Domain Database (Marchler-Bauer et al., 2011) for open reading frames (ORFs) encoding activities that are typically employed for the modification of PKs. Based on these

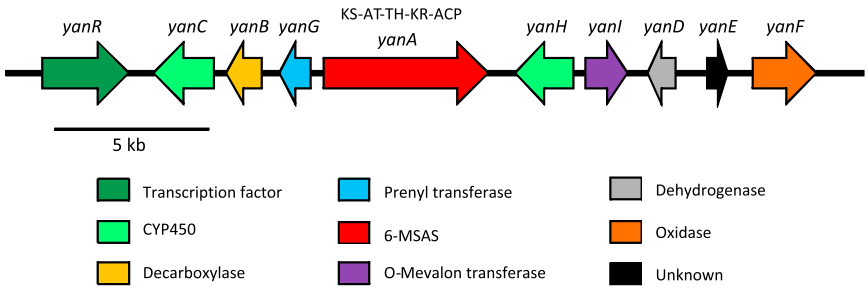


Figure 3. The Proposed *yan* Cluster
The *yanA* 6-MSA synthase-encoding gene is flanked by nine cluster genes (*yanB*, *yanC yanD*, *yanE*, *yanF*, *yanG*, *yanH*, *yanI*, and *yanR*) whose products contain all necessary activities for conversion of 6-MSA into yanuthone D.

analyses, eight additional genes could potentially belong to the *yanA* cluster, including genes encoding a transcription factor (TF), a prenyl transferase, an O-acyltransferase, a decarboxylase, two oxidases, two cytochrome P450s (CYP450s), and a dehydrogenase (Figure 3; Table S2). Together with *yanA* and 192604 (a gene with no known homologs), these eight genes form a cluster of ten genes that are not interrupted by any of the remaining eleven genes included in the analysis. The fact that one of the ten genes in this cluster (44961) putatively encodes a TF raised the possibility that expression of the genes involved in yanuthones D and E production is controlled by this TF. In agreement with this view, deletion of 44961 resulted in a strain that did not produce these two yanuthones (Figures 2B and 2C). To further delineate the *yanA* gene cluster, we determined the expression levels of the ten cluster genes as well as of four flanking genes by RT-quantitative PCR (qPCR) in a 44961Δ strain and KB1001. When the two data sets were compared, we found, as expected, that expression from 44961 is eliminated in the 44961Δ strain where the entire gene is deleted (Figure S4). More importantly, the analysis demonstrated that expression from the other nine genes in the cluster was significantly downregulated in the 44961Δ strain as compared to KB1001 (p value < 0.05). Specifically, the expression was reduced more than 10-fold for seven of the genes, including *yanA*. Expression of the remaining two genes, 54844 and 44964, was expressed at a level corresponding to 20% and 11%, respectively, of the level obtained with KB1001. In contrast, expression levels from the four flanking genes were not significantly different from KB1001 (Figure S4). Next, we individually deleted the remaining eight genes in the proposed *yan* gene cluster, which encode putative activities for PK modification. None of the resulting strains, including 192604Δ, produced yanuthone D, indicating that all genes belong to the *yan* cluster (Table S1). As a control, the four additional genes flanking this cluster were also individually deleted, but all these four strains produced yanuthone D. Based on these analyses and the results from the RT-qPCR, we propose that the *yan* gene cluster is composed by 10 genes, *yanA*, *yanB*, *yanC*, *yanD*, *yanE*, *yanF*, *yanG*, *yanH*, *yanI*, and *yanR*, where *yanR* encodes a TF that regulates the gene cluster (Figure 3; Table S2). Finally, all ten genes were simultaneously deleted in one strain. When $^{13}C_8$-6-MSA was fed to this strain, no labeled metabolites were detected, showing that all 6-MSA-derived yanuthones depend on this gene cluster (see above).

YanF Converts Yanuthone E into Yanuthone D

As the first step toward elucidating the order of reaction steps in the pathway toward yanuthones D and E, we asked whether yanuthones D and E are two different end products or whether one is an intermediate in the pathway toward production of the other. To this end, we note that individual deletion of genes in the *yan* gene cluster generally resulted in loss of production of both yanuthones D and E on YES medium. The only exception is the *yanF*Δ strain, which produced substantial amounts of yanuthone E (**2**), but no yanuthone D (**1**) (Figure 4). These findings suggest that YanF converts yanuthone E into yanuthone D, which is the true end product of the pathway. Interestingly, the *yanF*Δ strain produced a new and unknown compound, which was not detected in KB1001. Elucidation of its structure revealed a yanuthone E analog with a hydroxylation at C-2 at the expense of the first double bond (between C-2 and C-3) in the sesquiterpene moiety (Table S4). This compound was named yanuthone J (**9**).

m-Cresol and Toluquinol Are Intermediates of the Yanuthone D Biosynthesis

Deletion of *yanB*, *yanC*, *yanD*, *yanE*, and *yanG* did not produce any detectable intermediates, and the phenotype of these mutations therefore does not link any of the genes to specific reaction steps in the pathway toward formation of yanuthone D. However, one of the five putative enzymes, YanC, has a defined homolog, PatI, in the *Aspergillus clavatus* patulin biosynthesis pathway (Artigot et al., 2009) where it catalyzes the oxidation of *m*-cresol into toluquinol, suggesting that toluquinol and *m*-cresol are also likely intermediates in the yanuthone biosynthesis. To test this hypothesis, we fed *m*-cresol and toluquinol to the *yanA*Δ strain. Analysis of the metabolite profiles of the two strains indeed showed that addition of *m*-cresol or toluquinol restored production of yanuthones D and E in the *yanA*Δ strain (Figure 5).

In an attempt to further elucidate the role of the five enzymes, the corresponding genes were inserted into plasmid pDHX2 (Figure S5) and individually expressed in the *A. nidulans* strain harboring the *yanA* gene. No new compounds were produced in these *IS1-yanA* strains expressing *yanC*, *yanD*, *yanE*, and *yanG*, despite the fact that 6-MSA was produced in high amounts (Figure S6). Similarly, in the strain expressing *yanB*, no new product was observed, but in this case 6-MSA was absent, indicating that 6-MSA is a substrate for YanB.

Deletion of *yanI* and *yanH* Reveals Key Intermediates in the Biosynthesis of Yanuthone D

In contrast to the *yanB*Δ-*E*Δ and *yanG*Δ strains, new products were observed in the *yanH*Δ and *yanI*Δ strains. Deletion of *yanH* resulted in a strain where the most prominent compound accumulating is 7-deacetoxyyanuthone A (**3**) (NMR data in Table S4). Interestingly, we also identified two compounds in this strain (Figure 4). Isolation and structure elucidation revealed two C-1 oxidized yanuthone derivatives, which we named

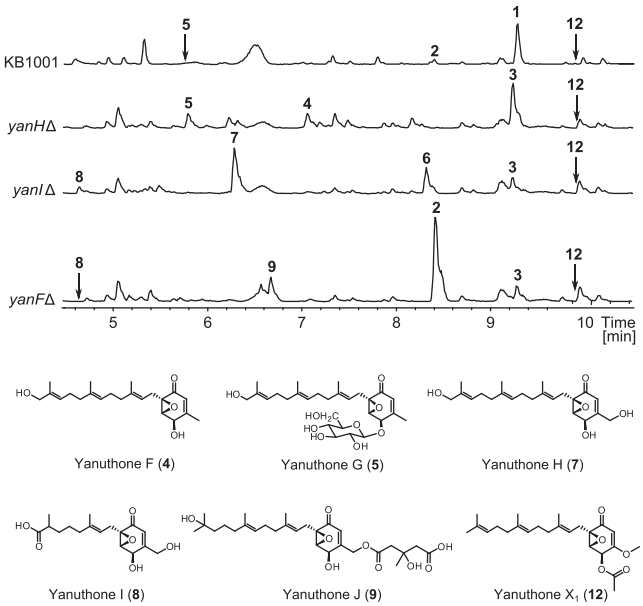


Figure 4. BPC *m/z* 100–1,000 of Reference Strain KB1001, *yanH*Δ, *yanI*Δ, and *yanF*Δ

All NMR-elucidated compounds are shown for comparison of intensity and relative retention times. Below are structures of the yanuthones identified in this study. The structures of yanuthone D (**1**), yanuthone E (**2**), 7-deacetoxyyanuthone A (**3**), and 22-deacetylyanuthone A (**6**) are shown in Figure 1.

yanuthone F (**4**) and yanuthone G (**5**) (NMR data in Table S4). Yanuthone G (**5**) is a glycosylated version of yanuthone F (**4**), which can also be detected in trace amounts in KB1001 (Table S1). Deletion of *yanI* resulted in a strain producing the known compounds 7-deacetoxyyanuthone A (**3**) and 22-deacetylyanuthone A (**6**) (NMR data in Table S4; Figure 1B). Importantly, the latter compound corresponds to yanuthone E (**2**) without the mevalon moiety. In addition, two compounds were produced. The structures were elucidated by NMR spectroscopy, revealing that one, which we named yanuthone H (**7**), is very similar to 22-deacetylyanuthone A (**6**), but with a hydroxyl group at C-1 (Figure 4; Table S4). The other compound, which we named yanuthone I (**8**), is a modification of 22-deacetylyanuthone A (**6**) with a shorter and oxidized terpene (NMR data in Table S4). We note that yanuthone I (**8**) was also detected in trace amounts in KB1001 (Table S1).

Determination of the Yanuthone X_1 Structure

As mentioned above, yanuthone X_1 (**12**) has an elemental composition corresponding to yanuthone A and C but was biosynthesized from another precursor than yanuthone D and E. We therefore isolated and elucidated the structure (Figure 4; Table S4). This analysis confirmed that yanuthone X_1 (**12**) does not have the same C_7 core scaffold but instead has a C_6 core with a methoxygroup directly attached to the six-membered ring at the expense of a methyl group (Figure 4). Despite the fact that yanuthone X_1 (**12**) and yanuthones D and E employ different precursors, they share common features like the epoxide and the sesquiterpene side chain, and we therefore hypothesized that they share common enzymatic steps during their biosynthesis. In agreement with this, examination of the metabolite profiles obtained with the *yan* gene deletion strains revealed that yanuthone X_1 (**12**) was absent in the *yanC*, *yanD*, *yanE*, and *yanG* deletion strains (Table S1). In contrast, yanuthone X_1 (**12**) is produced in larger amounts in the *yanA*Δ strain, which cannot produce 6-MSA.

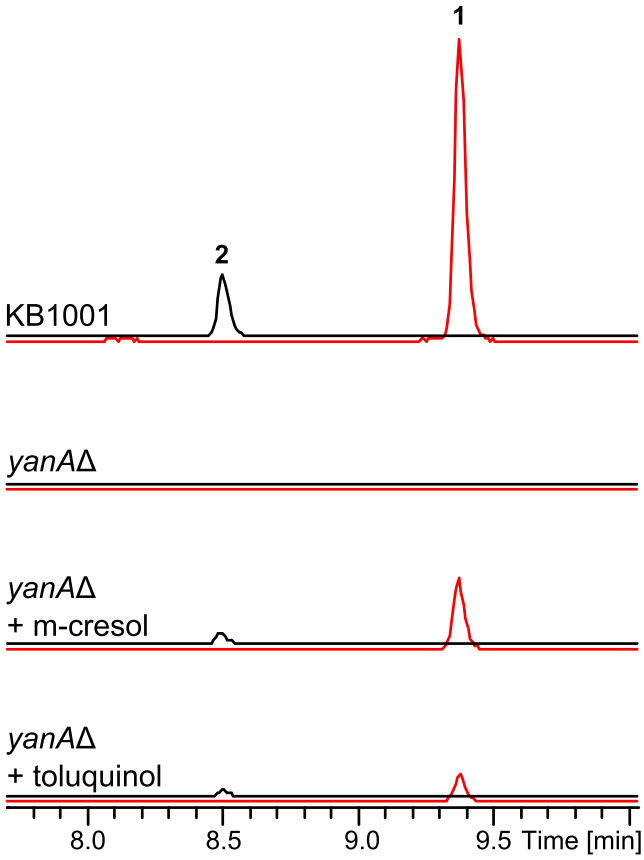


Figure 5. Feeding with Unlabeled *m*-cresol and Toluquinol

Shown are EICs of yanuthone D (**1**) 503.2640 ± 0.005 (red) and yanuthone E (**2**) 505.2791 ± 0.005 (black) for KB1001 and the *yanA*Δ strain with and without feeding. Chromatograms are to scale.

Antifungal Activity of Yanuthones

Yanuthones have earlier been reported to display antimicrobial activity (Bugni et al., 2000), and we therefore tested all ten yanuthones presented in this study for antifungal activity toward *C. albicans* (Table 1). Among these compounds, our analysis identified yanuthone D as the most toxic species in agreement with the fact that it represents the most likely end point of the pathway. Among the remaining yanuthones, three other species, yanuthone G, yanuthone H, and 22-deacetylyanuthone A, exhibited antimicrobial activity. In these cases, IC_{50} values were ~5- to 10-fold higher than the IC_{50} value determined for yanuthone D.

DISCUSSION

Elucidation of the Biosynthetic Route from 6-MSA toward Yanuthone D

We have used a combination of bioinformatics, genetic tools, chemical analyses, and feeding experiments to investigate

Table 1. The Half-Maximal Inhibitory Concentration for *C. albicans* Treated with a Small Library of Yanuthones

Compound	Origin	Isolate	IC_{50} (μM)
Yanuthone D	*A. niger*	KB1001	3.3 ± 0.5
Yanuthone E	*A. niger*	KB1001	>100
Yanuthone F	*A. niger*	*yanHΔ*	>100
Yanuthone G	*A. niger*	*yanHΔ*	38.8 ± 5.1
Yanuthone H	*A. niger*	*yanIΔ*	24.5 ± 1.1
Yanuthone I	*A. niger*	*yanIΔ*	>100
Yanuthone J	*A. niger*	*yanFΔ*	>100
7-deacetoxyyanuthone A	*A. niger*	KB1001	>100
22-deacetylyanuthone A	*A. niger*	KB1001	19.4 ± 1.8
Yanuthone X_1	*A. niger*	KB1001	>100

The IC_{50} values were calculated based on duplicate experiments carried out in three independent trials and annotated with their respective SD.

whether 6-MSA is produced and whether it is used for production of toxic secondary metabolites in *A. niger*. Our work demonstrates that 6-MSA is synthesized by the YanA PKS and then subsequently modified into the antimicrobial end product yanuthone D. This is intriguing because yanuthones have previously been suggested to originate from shikimic acid (Bugni et al., 2000). Yanuthones have previously been observed on YES agar (Klitgaard et al., 2014; Nielsen et al., 2009) and a mixture of yeast, beef, and casein extract (Bugni et al., 2000). In this study yanuthones were detected on solid YES and MM medium, but not on solid CYA or MEA medium, and yanuthone synthesis is therefore conditionally induced. To this end, we find that yanuthone D is not produced in liquid YES and MM medium, in agreement with the fact that secondary metabolism is generally turned off in submerged cultures (González, 2012; Schachtschabel et al., 2013).

We have also shown that *yanA* defines a gene cluster of ten members: *yanA*, *yanB*, *yanC*, *yanD*, *yanE*, *yanF*, *yanG*, *yanH*, *yanI*, and *yanR*, which is regulated by YanR. In agreement with this, YanR is homologous to Zn_2Cys_6 transcription factors that are commonly involved in regulation of secondary metabolite production. The fact that deletion of *yanR* completely abolished production of yanuthone D suggests that YanR acts as an activator of the *yan* cluster. Additionally, analyses of strains where the remaining genes in the *yan* cluster were individually deleted have allowed us to isolate and characterize the full structures of three intermediates. Based on these compounds, we propose the entire pathway for yanuthone D formation including addition of a sesquiterpene and a mevalon to the core polyketide moiety at different stages of the biosynthesis (Figure 6).

In our model, the last intermediate in the pathway is yanuthone E (**2**), which is converted into the end product yanuthone D (**1**) by oxidation of the hydroxyl group at C-15 in a process catalyzed by YanF. The fact that yanuthone E (**2**) is present in KB1001 indicates that it may act as a reservoir for rapid conversion into the more potent antibiotic compound yanuthone D. Yanuthone E (**2**) is likely formed from 22-deacetylyanuthone A (**6**) by attachment of mevalon to the hydroxyl group at C-22. Because 22-deacetylyanuthone A (**6**), but not yanuthone E (**2**), accumulates in the *yanI*Δ strain, we propose that YanI, a putative O-acyltransferase, catalyzes this step. Intriguingly, YanI therefore appears to be an O-mevalon transferase, an activity, which, to the best of our knowledge, has not previously been described in the literature. Next, we propose that 22-deacetylyanuthone A (**6**) is formed by hydroxylation of C-22 of 7-deacetylyanuthone A (**3**). In agreement with this view, 7-deacetylyanuthone A (**3**), but not 22-deacetoxyyanuthone A (**6**), accumulates in the absence of YanH.

Unfortunately we did not detect any intermediates leading from 6-MSA to 7-deacetoxyyanuthone A (**3**) in any of the deletion strains in *A. niger*. The remaining tentative steps in the pathway were therefore deduced from bioinformatics and feeding experiments. First, analyses of patulin formation in *Aspergillus flocco-sus* (previously identified as *Aspergillus terreus*; Jens C. Frisvad, personal communication) and in *A. clavatus* have shown that it requires decarboxylation of 6-MSA into *m*-cresol (Artigot et al., 2009; Puel et al., 2010). This step is catalyzed by 6-MSA decarboxylase (Light, 1969), which has been proposed to be encoded by *patG* (Puel et al., 2010). *m*-Cresol is then converted into gentisyl alcohol in two consecutive hydroxylation steps catalyzed by the two cytochrome P450s CYP619C3 (PatH) and CYP619C2 (PatI). However, CYP619C2 may also act directly on *m*-cresol to form the co-metabolite toluquinol, which is not an intermediate toward patulin. When we inspected the *yan* gene cluster for similar activities, we found a putative 6-MSA decarboxylase (YanB) and CYP619C2 (YanC), but not CYP619C3. These observations suggest that *m*-cresol and toluquinol are intermediates in yanuthone D formation. We present two lines of evidence in support of this view. First, our feeding experiments demonstrate that both compounds can be converted into yanuthone D. Second, heterologous expression of *yanA* in *A. nidulans* leads to production of 6-MSA. This compound disappears if the strain also expresses *yanB*, indicating that 6-MSA is a substrate for the putative 6-MSA decarboxylase YanB. Together these results strongly suggest that *m*-cresol is formed directly from 6-MSA by a decarboxylation reaction, which is most likely catalyzed by YanB. This reaction explains how C_8-based 6-MSA can serve as the building block for the C_7-based core unit of yanuthones. Moreover, the analyses show that toluquinol is an intermediate in the production of yanuthone D and that it is formed from *m*-cresol in a process most likely catalyzed by the putative cytochrome P450 encoded by *yanC*. Conversion of toluquinol into 7-deacetylyanuthone A (**3**) requires epoxidation and prenylation. Based on the fact that prenylated toluquinol is never observed in KB1001 or mutant strains, we propose that epoxidation precedes prenylation. In this scenario, toluquinol is epoxidated into (**10**), which is in equilibrium with the tautomer (**11**). This compound (**11**) is then prenylated to form 7-deacetylyanuthone A (**3**) as a sesquiterpene moiety is attached to C-13 of (**11**). The latter reaction is likely catalyzed by YanG, a putative prenyltransferase. This is supported by the observation that yanuthone D (**1**) and all detectable intermediates, including 7-deacetoxyyanuthone A (**3**), were absent in the *yanG*Δ strain. The identity of the gene products(s) responsible for epoxidation of toluquinol is less clear. Among the putative activities encoded by the genes in the *yan* cluster, which have not been assigned to any reaction step during the analyses above, we note the presence of a putative dehydrogenase (YanD) and one with an unknown activity and with no obvious homologs (YanE) as judged by BLAST

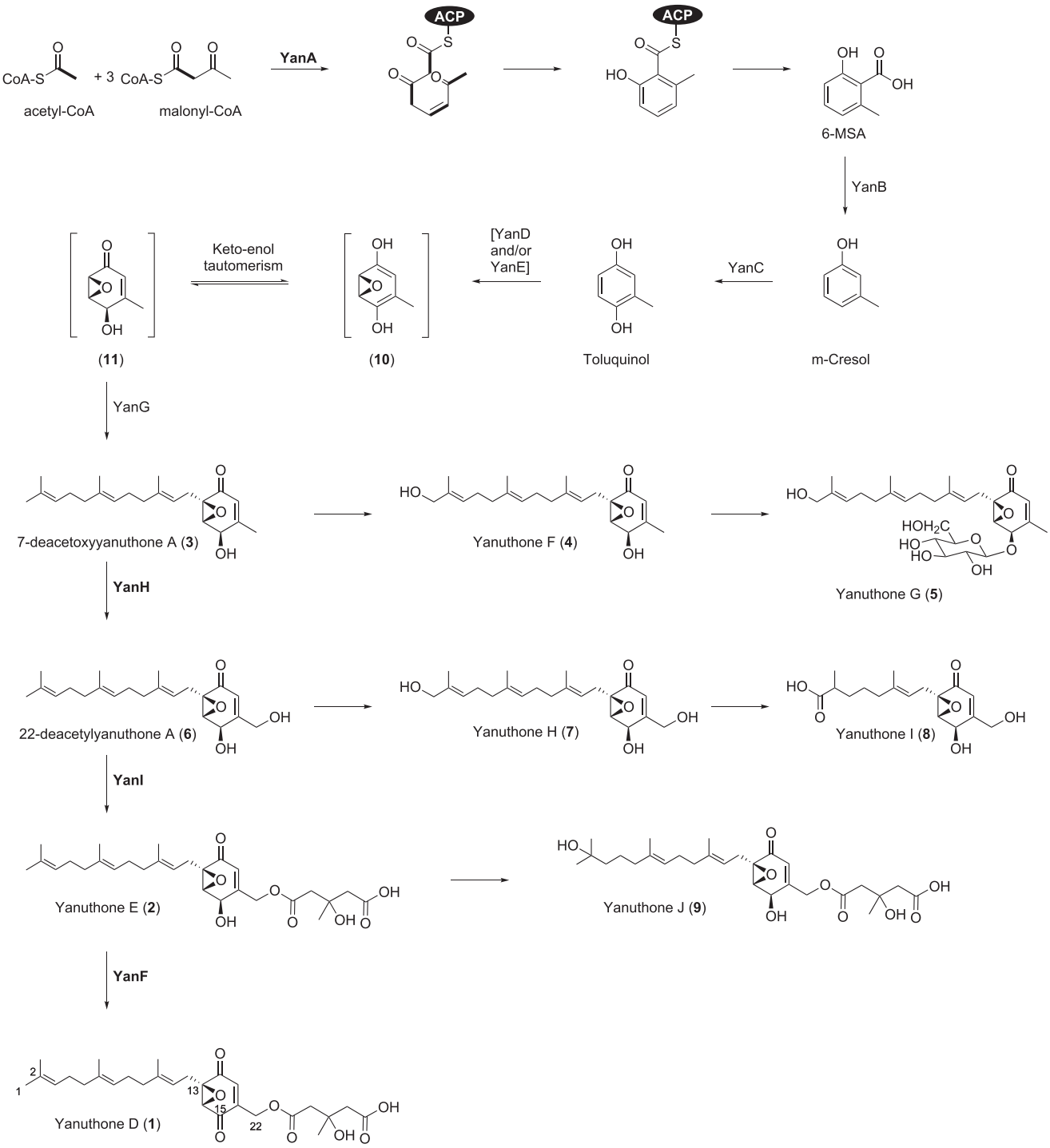


Figure 6. Proposed Biosynthesis of yanuthone D

Structures and enzymatic activities in brackets are hypothesized, activities in plain text have been proposed from bioinformatics, and activities in bold have been experimentally verified.

analysis of the GenBank database (Altschul et al., 1990). We hypothesize that one or both of these enzymes catalyze epoxidation. The fact that neither 6-MSA, *m*-cresol, toluquinol, nor any other intermediates were detected in the *yanB*Δ, *yanC*Δ, *yanD*Δ, and *yanE*Δ strains suggests that these small, aromatic compounds must be rapidly degraded or converted into other compound(s), or they may be incorporated into insoluble material, e.g., the cell wall.

Accumulation of Intermediates in the Yanuthone D Pathway Triggers Formation of Novel Yanuthones

Disruption of the biosynthetic pathway toward yanuthone D results in formation of three branch points in the pathway toward yanuthone D: at yanuthone E (**2**), at 7-deacetoxyyanuthone A (**3**), and at 22-deacetylyanuthone A (**6**). In addition to yanuthone E (**2**), yanuthone J (**9**) accumulates in the *yanF*Δ strain. Similarly, yanuthone F (**4**) accumulates in addition to 7-deacetoxyyanuthone A (**3**) in the *yanH*Δ strain, and yanuthone H (**7**) accumulates in addition to 22-deacetylyanuthone A (**6**) in the *yanI*Δ strain. In all cases, the sesquiterpenes of the accumulated intermediates in the main pathway are oxidized at C-1 or C-2. Because hydroxylation is a known detoxification mode, we speculate that the abnormally high amount of potentially toxic intermediates 7-deacetoxyyanuthone A (**3**), 22-deacetylyanuthone A (**6**), and yanuthone E (**2**) triggers the cell to initiate phase I type of detoxification processes in which the toxic intermediates are hydroxylated. This hypothesis is supported by the fact that there is no obvious assignment of an enzyme with this activity, encoded by the *yan* gene cluster, and by the fact that one of the intermediates, 22-deacetylyanuthone, is toxic to *C. albicans*.

An additional variant of yanuthone F (**4**) was identified in the *yanH*Δ strain, in which yanuthone F (**4**) is glycosylated at the hydroxyl group at C-15 to form yanuthone G (**5**). The glucose moiety of yanuthone G (**5**) is intriguing because sugar moieties are rare in fungal secondary metabolites, and the fact that yanuthone G (**5**) is detected in KB1001 shows that it is a naturally occurring compound (Figure 4; Table S1). Because yanuthone G (**5**) production is upregulated in *yanH*Δ, we suggest that glycosylation poses a second (phase II conjugation) type of mechanism for further detoxification of possible toxic intermediates.

The branch point at 22-deacetylyanuthone A (**6**) revealed a novel compound yanuthone I (**8**), which is identical to 22-deacetylyanuthone A (**6**) and yanuthone H (**7**) but with a shorter and oxidized sesquiterpene chain. A similar modification has been observed in the biosynthetic pathway for production of mycophenolic acid (Regueira et al., 2011). Here it was proposed to occur by oxidative cleavage between C-4 and C-5 of the sesquiterpene chain. Alternatively, it could occur by terminal oxidation of a geranyl side chain.

Yanuthone X_1 Defines a Novel Class of Yanuthones

Because yanuthones are based on a C_7 scaffold, they were previously proposed to originate from shikimic acid (Bugni et al., 2000). However, in our study we demonstrate that yanuthones D and E originate from the C_8 polyketide precursor 6-MSA, which is decarboxylated to form the C_7 core of the yanuthone structure. In contrast, the novel yanuthone X_1 (**12**) has a C_6 core scaffold that does not originate from 6-MSA and does not require decarboxylation by YanB. Based on this we define two classes of yanuthones: those that are based on the polyketide 6-MSA, class I, and those that are based on the yet unknown precursor leading to the formation of yanuthone X_1 (**12**), class II. The two classes of yanuthones share several enzymatic steps. First we note that the sesquiterpene side chain in yanuthone X_1 (**12**) is likely attached by YanG, as is the case for yanuthone D. Second, it depends on enzyme activities of YanC, YanD, and YanE, but not of YanB. Together this suggests that the precursor is a small aromatic compound similar to 6-MSA but lacking the carboxylic acid. Importantly, the main difference between yanuthone D and yanuthone X_1 (**12**) are the groups attached to C-16. In the case of yanuthone X_1 (**12**), this position is oxidized, whereas in yanuthones D and E there is a carbon-carbon bond that originates from the methyl group of 6-MSA. Consequently, yanuthone X_1 (**12**) cannot be mevalonated by YanI.

SIGNIFICANCE

This study has identified a cluster of 10 genes, which is responsible for production of antimicrobial yanuthone D in *A. niger*. We show that yanuthone D is based on the polyketide 6-MSA and not on shikimic acid as previously suggested, and we have proposed a detailed genetic and biochemical pathway for converting 6-MSA into yanuthone D. Interestingly, we have revealed that yanuthone X_1, although similar in structure, is not derived from 6-MSA, but the yet unknown precursor to yanuthone X_1 does employ several enzymes encoded by the *yan* cluster. An important finding in the elucidation of the biosynthesis is the identification of *yanI* encoding an O-mevalon transferase, which represents a different enzymatic activity. We have discovered that the pathway toward yanuthone D branches when intermediates accumulate, because three intermediates are hydroxylated. Two of the hydroxylated compounds are further modified by oxidative cleavage of the sesquiterpene and glycosylation, respectively, resulting in five yanuthones. The discovery of a glycosylated compound, yanuthone G, is intriguing because glycosylated compounds are very rare in fungal secondary metabolism. We successfully employed an interdisciplinary approach for solving the biosynthetic pathway: applying gene deletions, heterologous gene expression, UHPLC-DAD-MS, MS/MS, structural elucidation by NMR spectroscopy and CD, and feeding experiments with ^{13}C-labeled and unlabeled metabolites. Together, our analyses have cast insights into understanding the complexity of fungal secondary metabolism.

EXPERIMENTAL PROCEDURES

Strains and Media

The strain IBT 29539 was used for strain constructions in *A. nidulans*. ATCC1015-derived strain KB1001 was used for strain constructions in *A. niger*. All fungal strains prepared in the present work (Table S5) have been deposited in the IBT Culture Collection at the Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark. *Escherichia coli* strain DH5α was used for propagating plasmids, except *E. coli* ccdB survival2 cells (Invitrogen), which were used for plasmids carrying the *ccdB* gene.

MM for *A. nidulans* was made as described by Cove (1966), but with 1% glucose, 10 mM $NaNO_3$, and 2% agar. MM for *A. niger* was prepared as described by Chiang et al. (2011). YES, MEA, and CYA were prepared as described by Frisvad and Samson (Samson et al., 2010). When necessary, media were supplemented with 4 mM L-arginine, 10 mM uridine, 10 mM uracil, and/or 100 μg/ml hygromycin B (InvivoGen). Luria-Bertani (LB) medium was used for cultivation of *E. coli* strains and consisted of 10 g/l tryptone (Bacto), 5 g/l yeast extract (Bacto), and 10 g/l NaCl (pH 7.0). When necessary, LB was supplemented with 100 μg/ml ampicillin.

For batch cultivation the medium contained 20 g/l D-glucose-$^{13}C_6$ (99 atom % ^{13}C; Sigma-Aldrich) or D-glucose, 7.3 g/l $(NH_4)_2SO_4$, 1.5 g/l KH_2PO_4, 1.0 g/l $MgSO_4 \cdot 7\ H_2O$, 1.0 g/l NaCl, 0.1 g/l $CaCl_2$, 0.1 ml of Antifoam 204

(Sigma), and 1 ml/l trace element solution. Trace element solution contained 0.4 g/l $CuSO_4 \cdot 5\ H_2O$, 0.04 g/l $Na_2B_2O_7 \cdot 10\ H_2O$, 0.8 g/l $FeSO_4 \cdot 7\ H_2O$, 0.8 g/l $MnSO_4 \cdot H_2O$, 0.8 g/l $Na_2MoO_4 \cdot 2\ H_2O$, and 8.0 g/l $ZnSO_4 \cdot 7\ H_2O$.

Construction of Basic Vectors for Strain Construction

All primers are listed in Table S6. The PfuX7 polymerase (Nørholm, 2010) was used in all PCRs. Fragments were assembled via uracil-excision fusion (Geu-Flores et al., 2007) into a compatible vector.

pDH56 and pDH57 are designed for integration of novel genes into the *IS1* site of *A. nidulans*. In pDH56, the AsiSI/Nb.BtsI uracil-excision cassette of CMBU1111 (Hansen et al., 2011) is modified into a *ccdB-cm*^{R} AsiSI/Nb.BtsI uracil-excision cassette. Unlike with CMBU1111, new fragments can be introduced into this cassette and cloned in *ccdA*-deficient *E. coli* strains like DH5α without generating background because false positives resulting from incomplete digestion of the USER cassette are eliminated (Bernard and Couturier, 1992). Specifically, the suicide gene *ccdB* and the chloramphenicol resistance gene *cm*^{R} (*ccdB-cm*^{R}) construct was PCR amplified (using primers 84 and 85) from pDONR (Invitrogen) and inserted into CMBU1111 by uracil-excision cloning in a manner that reconstituted the original uracil excision cassette on either side of the insert. pDH57 was constructed from pDH56 by removing an undesirable Nb.BtsI nicking site located in the *amp*^{R} gene. pDH56 was PCR amplified in two pieces (81 + 76 and 75 + 80). 75 and 76 were designed to introduce a silent mutation into the Nb.BtsI recognition site. Fragments were assembled via uracil-excision cloning, and correct clones were verified by sequencing. The gene targeting substrate for insertion of the 6-MSA synthase gene *yanA* was made by amplifying the synthase gene *yanA* (PKS48/ASPNIDRAFT_44965) from IBT 29539 genomic DNA (primers 1 and 2) and inserted into pDH57, yielding pDH57-*yanA*.

The pDHX2 vector is AMA1-based and designed for episomal gene expression. pDHX2 was constructed by USER fusion of five fragments: (1) *E. coli* origin of replication (*ori*^{R}) and the *E. coli* ampicillin resistance gene (*amp*^{R}); (2) the 5′ half of AMA1; (3) the 3′ half of AMA1; (4) 0.5 kb of the P*gpdA* promoter, an AsiSI/Nb.BtsI USER cassette containing *ccdB* and *cm*^{R}, and the TtrpC terminator; and (5) the *A. fumigatus pyrG* selection marker (Figure S5). Fragment 1 was amplified from pDH57 (primers 77 + 78); fragments 2, 3, and 5 were amplified from pDEL2 (primers 86 + 89, 87 + 88, and 82 + 83) (Nielsen et al., 2008); and fragment 4 was amplified from pDH57 (primers 79 + 80). Fragments were assembled as described by Geu-Flores et al. (2007) using equal molar amounts of purified PCR product, and correct clones were verified by restriction digestion. Plasmids for episomal heterologous expression of cluster genes were constructed by PCR amplification of ORFs using primers 3–12 pairwise. Genes were inserted into AsiSI/Nb.BtsI-digested pDHX2 as described by Nour-Eldin et al. (2006), resulting in pDHX2-*yanB*, pDHX2-*yanC*, pDHX2-*yanD*, and pDHX2-*yanE*. Plasmids were verified by sequencing.

Plasmids carrying gene targeting substrates for gene deletion in *A. niger* were constructed by PCR amplification of upstream (US) and downstream (DS) targeting sequences along with the *hph* marker, conferring resistance to hygromycin B. US and DS targeting sequences were generated using the primers 17–72, and *hph* was amplified from pCB1003 (McCluskey et al., 2010) using primers 13 + 14. The three fragments were assembled into the CMBU0020 vector (Hansen et al., 2011).

Strain Construction

Protoplasting and gene-targeting procedures were performed as described previously for *A. nidulans* (Johnstone et al., 1985; Nielsen et al., 2006) and *A. niger* (Chiang et al., 2011). NotI-linearized pDH57-*yanA* was transformed into IBT 29539. Transformants were verified by diagnostic PCR as described by Hansen et al. (2011).

Strains for episomal expression of cluster genes were constructed by transforming the IS1-yanA strain with circular plasmids (pDHX2-*yanB*, pDHX2-*yanC*, pDHX2-*yanD*, and pDHX2-*yanE*) using *pyrG* as a selectable marker.

A. niger deletion strains were constructed by transforming KB1001 with bipartite gene targeting substrates. The substrates were generated by PCR amplification of the US::*hph*::DS cassettes of the CMBU0020-based plasmids using primers GENE_US-FW+73 and 74+GENE_DS-RV. Deletion strains were selected on 100 μg/ml hygromycin B and verified by diagnostic PCR.

RNA Extraction and RT-qPCR

RNA isolation from the *A. niger* strains and subsequent quantitative RT-PCRs were done as previously described by Hansen et al. (2011) except that biomass for RNA isolation was prepared with a Tissue-Lyser LT (QIAGEN) by treating samples for 1 min at 45 MHz. The *A. niger* histone 3-encoding gene, *hhtA* (ASPNIDRAFT_52637) and gamma-actin-encoding gene *actA* (ASPNIDRAFT_200483) were used as internal standards for normalization of expression levels. All primers used for quantitative RT-PCR are shown in Table S6 (primers 90–121). The relative expression levels were approximated based on $2^{\Delta\Delta c(t)}$, with $\Delta\Delta c(t) = \Delta c(t)_{(normalized)} - \Delta c(t)_{(calibrator)}$, where $\Delta c(t)_{(normalized)} = \Delta c(t)_{(target\ gene)} - \Delta c(t)_{(actA\ or\ hhtA)}$.The calibrator c(t) values are those from the *A. niger* reference strain KB1001. Statistical analysis of RT-qPCR results was performed as a Student's t test, and the error bars indicate the SD.

Chemical Analysis of Strains

Unless otherwise stated, strains were cultivated on solid MM media and incubated at 37°C for 5 days. Extraction of metabolites was performed as described by Smedsgaard (1997). 6-MSA was purchased from (Apin Chemicals). Analysis was performed using UPHLC-DAD-TOFMS on a maXis 3G orthogonal acceleration quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an electrospray ionization (ESI) source and connected to an Ultimate 3000 UHPLC system (Dionex). The column used was a reverse-phase Kinetex 2.6 μm C_{18}, 100 mm × 2.1 mm (Phenomenex), and the column temperature was maintained at 40°C. A linear water-acetonitrile (liquid chromatography-mass spectrometry grade) gradient was used (both solvents were buffered with 20 mM formic acid) starting from 10% (v/v) acetonitrile and increased to 100% in 10 min, maintaining this rate for 3 min before returning to the starting conditions in 0.1 min and staying there for 2.4 min before the following run. A flow rate of 0.4 ml•min^{-1} was used. TOFMS was performed in ESI+ with a data acquisition range of 10 scans per second at *m/z* 100–1,000. The TOFMS was calibrated using Bruker Daltonics high precision calibration algorithm by means of the use of the internal standard sodium formate, which was automatically infused before each run. This provided a mass accuracy of better than 1.5 ppm in MS mode. UV-visible spectra were collected at wavelengths from 200 to 700 nm. Data processing was performed using DataAnalysis 4.0 and Target Analysis 1.2 software (Bruker Daltonics) (Klitgaard et al., 2014). Tandem MS was performed with fragmentation energies from 18 to 55 eV.

Preparative Isolation of Selected Metabolites

The fungal strains were cultivated on 10-200 YES plates at 30°C for 5 days. For details about each extraction, see Table S3. Extracts were filtered and concentrated in vacuo. The combined extract was dissolved in 9:1 methanol (MeOH):H_2O, and 1:1 heptane was added, resulting in two phases. To the MeOH/H_2O phase H_2O was added to a ratio of 1:1, and metabolites were extracted with dichlormethane (DCM). The phases were concentrated separately in vacuo. The DCM phase was adsorbed onto diol column material and dried before packing into a SNAP column (Biotage) with diol material. The extract was fractionated on an Isolera flash purification system (Biotage) using seven steps of heptane-DCM-EtOAc-MeOH. Solvents were of HPLC grade, and H_2O was purified and deionized by a Millipore system through a 0.22 μm membrane filter.

The Isolera fractions were subjected to further purification on a semipreparative high-performance liquid chromatography (HPLC), which was either a Waters 600 controller with a 996 photodiode array detector (Waters) or a Gilson 322 controller connected to a 215 Liquid Handler, 819 Injection Module, and a 172 DAD (Gilson). This was achieved using a Luna II C_{18} column (250 × 10 mm, 5 μm; Phenomenex) or a Gemini C6-Phenyl 110A column (250 × 10.00 mm, 5 μm; Phenomenex). 50 ppm TFA was added to acetonitrile of HPLC grade and Milli-Q water. For choice of system, flow rate, column, gradients, and yields, see Table S3.

NMR and Structural Elucidation

The 1D and 2D spectra were recorded on a Unity Inova-500 MHz spectrometer (Varian). Spectra were acquired using standard pulse sequences, and ^{1}H, double quantum filtered-correlated spectroscopy, nuclear Overhauser effect spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple bond correlation spectra were acquired. The deuterated solvent

was acetonitrile-d_3, and signals were referenced by solvent signals for acetonitrile-d_3 at δ_H = 1.94 ppm and δ_C = 1.32/118.26 ppm. The NMR data was processed in Bruker Topspin 3.1 or ACD NMR Workbook. Chemical shifts are reported in ppm (δ) and scalar couplings in hertz. The sizes of the *J* coupling constants reported in the tables are experimentally measured values from the spectra. There are minor variations in the measurements that may be explained by the uncertainty of *J*. Descriptions of the structural elucidations are shown in Table S4.

CD spectra were obtained from a J-710 spectropolarimeter (Jasco). The methanol dissolved samples (1 mg/3 ml) were analyzed in 0.2 cm optical path length cells at 20°C, and the spectra were recorded from 200 to 500 nm. Optical rotation was measured on a PerkinElmer 321 Polarimeter.

Production and Purification of Fully Labeled ^{13}C-6-MSA

Because fully labeled $^{13}C_8$-6-MSA was not commercially available, it was produced in-house from the 6-MSA-producing strain by batch cultivation. Spores propagated on CYA media plates for 7 days at 30°C were harvested with 10 ml of 0.9% NaCl through Mira cloth. The spores were washed twice with 0.9% NaCl. The batch fermentation was initiated by inoculation of $2 \cdot 10^9$ spores/l. A Sartorious 1 l bioreactor (Satorious) with a working volume of 0.8 l equipped with two Rushton six-blade disc turbines was used. The pH electrode (Mettler) was calibrated according to manufacturer standard procedures. The bioreactor was sparged with sterile atmospheric air, and off-gas concentrations of oxygen and carbon dioxide were measured with a Prima Pro Process Mass Spectrometer (Thermo-Fischer Scientific). Temperature was maintained at 30°C, and pH was controlled by addition of 2 M NaOH and H_2SO_4. Start conditions were pH: 3.0, stir rate: 100 rpm, and air flow: 0.1 volume of air per volume of liquid per minute (vvm). These conditions were changed linearly in 720 min to pH: 5.0, stir rate: 800 rpm, and air flow: 1 vvm. The strain was cultivated until glucose was depleted, as measured by glucose test strips (Machery-Nagel), and the culture had entered stationary phase as monitored by off-gas CO_2 concentration.

The entire volume of the reactor was harvested, and the biomass was removed by filtration through a Whatman 1 qualitative paper filter followed by centrifugation at 8,000 × *g* for 20 min to remove fine sediments. The 6-MSA was then recovered from the supernatant by liquid-liquid extraction using ethyl acetate with 0.5% formic acid.

The organic extract then dried in vacuo to give a crude extract that was redissolved in 20 ml of ethyl acetate and dry loaded onto 3 g of Sepra ZT C18 (Phenomenex) resin prior to packing into a 25 g SNAP column (Biotage) with 22 g of pure resin in the base. The crude extract was fractionated on an Isolera flask purification system (Biotage) using an water-acetonitrile gradient starting at 15:85 going to 100% acetonitrile in 23 min at a flow rate of 25 ml min^{-1} and kept at that level for 4 min. Fractions were collected using UV detection at 210 and 254 nm, resulting in a total of 15 fractions, of which 3 were pooled and analyzed. 6-MSA concentration was assessed using a Dionex Ultimate 3000 UHPLC coupled with a ultimate 3000 RS diode array detector (Dionex) equipped with a Poroshell 120 phenyl hexyl 2.1 × 100 mm, 2.7 μm (Agilent) column. Finally, purity (98.7%) was analyzed by UHPLC-TOFMS (Figure S3A).

Feeding Experiments

Solid YES plates were prepared using a 6 mm plug drill to make a well in the middle of the agar. 25-100 μl of spore suspension was added to the well, and plates were incubated at 30°C for 5 days. 100 μg of ^{13}C-6-MSA, *m*-cresol, and toluquinol (ortoluquinol) was added to the plates after 24, 48, and 72 hr, respectively. Agar plugs were taken both as reported previously (Smedsgaard, 1997) and also separately from the center, the middle, and the rim of the colony, respectively, to verify diffusion and absorption of the 6-MSA and the location of yanuthone production. Samples were analyzed as described in "Chemical Analysis of Strains."

Antifungal Susceptibility Testing

All compounds were screened for antifungal activity toward *C. albicans* in accordance with the CLSI standards using RPMI 1640 medium adjusted to pH 7 with 0.165 M MOPS buffer (CLSI, 2012). Inoculum was prepared to a final concentration of approximately 2.5×10^3 cells per ml. Inoculated media were seeded into 96-well microtiter plates in aliquots of 200 μl using a Hamilton STAR liquid handling workstation with an integrated Thermo Cytomat shaking incubator and Biotek Synergy Mx microplate reader. The pure compounds were dissolved in Me_2SO and applied at 100 to 5 μM (1% Me_2SO per well). The plates were incubated at 35°C at 1,200 rpm shaking with a 2 mm amplitude. Optical density was recorded every hour for 20 hr. Endpoint optical densities from compound screens were normalized to the negative controls, and susceptibility was evaluated as the percentage of reduction in optical density. All bioactive compounds were tested in duplicate in three independent trials to ensure reproducibility and to evaluate potency of the compound toward the target organism. The half-maximal inhibitory concentration (IC_{50}) was extrapolated from compound specific dilution sequences and annotated as the average concentration for which 50% inhibition plus minus the SD was observed.

SUPPLEMENTAL INFORMATION

Supplemental information includes six figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.01.013.

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