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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 X1) 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 PK genes appears to be produced under laboratory conditions (Pel et al., 2007; Andersen et al., 2013). In agreement with this view, genome sequences 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
predicted PKS and PKS-like genes in (Zabala et al., 2012). It is interesting to note that among the 33 putative new insights into the biosynthesis of this class of compounds azaphilone compounds, and further studies uncovered substantial clustering 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 mevalonic 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/ASPIDRAFT_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 (UHPLC)-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
Biosynthesis of Yanuthone D in Aspergillus niger

Figure 2. Extracted Ion Chromatograms
(A) Extracted ion chromatogram (EIC, m/z 153.0546 ± 0.005) of an A. niger reference strain (IBT 29539) and a 6-MSA producing strain (IST-44965/yanA).
(B) Base peak chromatograms (BPC) m/z 100-1,000 of the A. niger reference (KB1001), yanAΔ, and yanRa strains.
(C) EICs of yanuthone D (1) 503.2640 ± 0.005 (red) and yanuthone E (2) 505.2791 ± 0.005 (black) for KB1001, yanAΔ, and yanRaΔ. All chromatograms are to scale.

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 X₁) 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 13C₈-6-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 13C₈-6-MSA to KB1001 and the PKS48Δ strain at different time points during growth (24, 48, and 72 hr; see Experimental Procedures). The addition of 13C₈-6-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 13C₈-6-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, 13C₈-6-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 13C₈-6-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 (yanuthone) and the corresponding gene yanA. On the other hand, no labeled yanuthone X₁ was observed in KB1001 as well as in the PKS48 deletion strain after addition of 13C₈-6-MSA (mass spectra are shown in Figure S3), confirming our finding that yanuthone X₁ is formed in the absence of PKS48. Hence, we conclude that 6-MSA is not the precursor of yanuthone X₁.

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 detailed analyses, we propose that yanA is part of a larger gene cluster that includes ten genes (Table S3 and S4) encoding activities that are typical of PKs. These activities include thioesterase, dehydratase, and other enzymes that are involved in the modification of PKs. This indicates that yanuthones are produced through a complex pathway that involves the modification of a primary amino acid. The proposed gene cluster for yanuthones is shown in Figure 3A. The presence of this yanuthone, it was still present. This observation strongly suggested that some yanuthones are produced independently of PKS48.
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 44961 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 13C6-6-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 yanFΔ 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 yanFΔ 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 in 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 yanAΔ 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 yanAΔ 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 *ist1-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 yanBΔ-ΔΔ and yanG3 strains, new products were observed in the yanHΔ and yanAΔ 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...
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 X1 Structure
As mentioned above, yanuthone X1 (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 X1 (12) does not have the same C7 core scaffold but instead has a C6 core with a methoxy group directly attached to the six-membered ring at the expense of a methyl group (Figure 4). Despite the fact that yanuthone X1 (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 X1 (12) was absent in the yanC, yanD, yanE, and yanG deletion strains (Table S1). In contrast, yanuthone X1 (12) is produced in larger amounts in the yanA strain, which cannot produce 6-MSA.

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, IC50 values were 5- to 10-fold higher than the IC50 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
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; Schachtzabel 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 Zn2Cys6 transcription factors with this, YanR is homologous to Zn2Cys6 transcription factors. YanR 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 2-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-deacetoxyanuthone A (6), accumulates in the absence of YanH.

Unfortunately we did not detect any intermediates leading from 6-MSA to 7-deacetoxyanuthone 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 flocosus (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 gentisalcohol 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 C9-based 6-MSA can serve as the building block for the C7-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 strain. The identity of the gene product(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.
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 yanBΔ, yanCΔ, yanDΔ, and yanEΔ 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.

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.
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-deacetylxyanuthone A (6). In addition to yanuthone E (2), yanuthone J (9) accumulates in the yanFHΔ strain. Similarly, yanuthone F (4) accumulates in addition to 7-deacetoxyyanuthone A (3) in the yanFHΔ strain, and yanuthone H (7) accumulates in addition to 22-deacetylxyanuthone A (6) in the yanFHΔ 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-deacetylxyanuthone 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-deacetylxyanuthone, is toxic to C. albicans.

An additional variant of yanuthone F (4) was identified in the yanFHΔ 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 yanFHΔ, 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-deacetylxyanuthone A (6) revealed a novel compound yanuthone I (8), which is identical to 22-deacetylxyanuthone 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 X1 Defines a Novel Class of Yanuthones

Because yanuthones are based on a C7 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 C9 polyketide precursor 6-MSA, which is decarboxylated to form the C7 core of the yanuthone structure. In contrast, the novel yanuthone X1 (12) has a C9 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 X1 (12), class II. The two classes of yanuthones share several enzymatic steps. First we note that the sesquiterpene side chain in yanuthone X1 (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 X1 (12) are the groups attached to C-15. In the case of yanuthone X1 (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 X1 (12) cannot be mevalonated by Yanl.

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 X1, although similar in structure, is not derived from 6-MSA, but the yet unknown precursor to yanuthone X1, does employ several enzymes encoded by the yan cluster. An important finding in the elucidation of the biosynthesis is the identification of yanl encoding an O-methyl 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 13C-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. niger, 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. niger was made as described by Cove (1968), but with 1% glucose, 10 mM NaNO₃, 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-13C₆ (99 atom % 13C, Sigma-Aldrich) or D-glucose, 7.3 g/l (NH₄)₂SO₄, 1.5 g/l KH₂PO₄, 1.0 g/l MgSO₄·7 H₂O, 1.0 g/l NaCl, 0.1 g/l CaCl₂, 0.1 ml of Antifoam 204
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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-Lyzer LT (QIAGEN) by treatment samples for 1 min at 45 MHz. The A. niger histone 3-encoding gene, hntA (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 \( \Delta \Delta Ct = \Delta Ct_{\text{target}} - \Delta Ct_{\text{reference}} \), where \( \Delta Ct_{\text{target}} = Ct_{\text{target}} - Ct_{\text{reference}} \). The calibrator \( Ct \) 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 UPLC-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 was a reverse-phase Ketex C18, 0.1 mm × 2.1 mm (Phenomenex), and the column temperature was maintained at 40°C. A linear water-acetonitrile (liquid chromatography-mass spectrometry gradient) valve 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 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) (Kitgaard 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); H2O, and 1:1 heptane was added, resulting in two phases. To the MeOH/H2O phase H2O was added to a ratio of 1:1, and metabolites were extracted with dichloromethane (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-ETDAc-MeOH. Solvents of HPLC grade, and H2O 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 semi-preparative 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 C18 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 MilliQ 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 1H, 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-\text{d}_3, and signals were referenced by solvent signals for aceto- 
nitrile-\text{d}_3 at \delta_N = 1.94 ppm and \delta_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 (\delta) 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 measured 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$_2$-6-MSA was not commercially available, it was 
produced in-house from the 6-MSA-producing strain by batch cultivation. Spores 
sporulated 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 2.10$^8$ spores/l. A Sartorius 1 l bioreactor (Sartorius) with a working volume of 0.8 l equipped 
with two Rushton six-blade disc turbines was used. The pH electrode (Mettrier) 
was calibrated according to manufacturer standard procedures. The biore-
actor was sparged with sterile atmospheric air, and off-gas concentrations of 
oxxygen 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$_2$SO$_4$. Start 
conditions were pH 3.0, stir rate: 100 rpm, and air flow: 0.1 volume of air 
per hour 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 quantitative paper filter followed 
by centrifugation at 8,000 \( \times \) 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 re-
dissolved in 20 ml of ethyl acetate and dried under vacuum onto 3 g of Sepra ZT C18 
(Phenomenex) resin prior to packing into a 25 g SNAP column (Biogel) with 
22 g of pure resin in the base. The crude extract was fractionated on an Isolera 
stationary phase as monitored by off-gas CO$_2$ concentration.

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 \( \mu \)l of spore suspension was added to the well, and 
plates were incubated at 30°C for 5 days. 100 \( \mu \)g of $^{13}$C$_2$-6-MSA, m-cresol, 
and tolualquinol (toluquinol) was added to the plates after 24, 48, and 72 hr, 
respectively. Agar plugs were taken both as reported previously (Smadsgaard, 
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 
fractionation on an Isolera 
concentration of approximately 2.5 \( \times \) 10$^5$ cells/ml. Inoculated media 
were seeded into 96-well microtiter plates in aliquots of 200 \( \mu \)l 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$_2$SO and applied at 100 to 5 \( \mu \)M (1% Me$_2$SO per well). 
The plates were incubated at 35°C at 1.200 rpm shaking with a 2 mm ampli-
tude. Optical density was recorded every hour for 20 hr. Endpoint optical den-
sities 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 extrap-
olated 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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