Genome-Based Deletion Analysis Reveals the Prenyl Xanthone Biosynthesis Pathway in *Aspergillus nidulans*

James F. Sanchez,[†] Ruth Entwistle,[‡] Jui-Hsiang Hung,[§] Junko Yaegashi,[†] Sofina Jain,[†] Yi-Ming Chiang,[†,‖] Clay C. C. Wang,*,[†,⊥] and Berl R. Oakley*,[‡]

[†]Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, School of Pharmacy, 1985 Zonal Avenue, Los Angeles, California 90089, United States

[‡]Department of Molecular Biosciences, University of Kansas, 1200 Sunnyside Avenue, Lawrence, Kansas 66045, United States

[§]Department of Biotechnology, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan

[‖]Graduate Institute of Pharmaceutical Science, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan

[⊥]Department of Chemistry, University of Southern California, College of Letters, Arts, and Sciences, Los Angeles, California 90089, United States

S *Supporting Information*

ABSTRACT: Xanthones are a class of molecules that bind to a number of drug targets and possess a myriad of biological properties. An understanding of xanthone biosynthesis at the genetic level should facilitate engineering of second-generation molecules and increasing production of first-generation compounds. The filamentous fungus *Aspergillus nidulans* has been found to produce two prenylated xanthones, shamixanthone and emericellin, and we report the discovery of two more, variecoxanthone A and epishamixanthone. Using targeted deletions that we created, we determined that a cluster of 10 genes including a polyketide synthase gene, *mdpG*, is required for prenyl xanthone biosynthesis. *mdpG* was shown to be required for the synthesis of the anthraquinone emodin, monodictyphenone, and related compounds, and our data indicate that emodin and monodictyphenone are precursors of prenyl xanthones. Isolation of intermediate compounds from the deletion strains provided valuable clues as to the biosynthetic pathway, but no genes accounting for the prenylations were located within the cluster. To find the genes responsible for prenylation, we identified and deleted seven putative prenyltransferases in the *A. nidulans* genome. We found that two prenyltransferase genes, distant from the cluster, were necessary for prenyl xanthone synthesis. These genes belong to the fungal indole prenyltransferase family that had previously been shown to be responsible for the prenylation of amino acid derivatives. In addition, another prenyl xanthone biosynthesis gene is proximal to one of the prenyltransferase genes. Our data, in aggregate, allow us to propose a complete biosynthetic pathway for the *A. nidulans* xanthones.
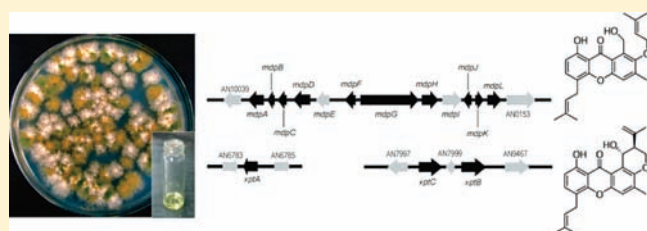
■ INTRODUCTION

Xanthones are polyphenolic compounds produced by higher plants, lichens, and fungi. In common they share a 9*H*-xanthen-9-one scaffold, but the class is highly diverse owing to functionalization with a range of substituents at various positions.[1] As a consequence, the xanthone core has been described as a "privileged structure,"[2] with members of this group exhibiting the potential to bind to a variety of targets. Indeed, xanthones have proven to be an important class of secondary metabolites. Over 250 of them have been shown to possess biological activities, including antimicrobial, antioxidant, cytotoxic, and neuropharmacological activities.[1]

In many instances xanthones are functionalized with prenyl groups, commonly the five carbon dimethylallyl moieties. Examples of prenylated xanthones include mangostanin and α-mangostin, which are strongly inhibitory to both sensitive and methicillin-resistant strains of *Staphylococcus aureus*.[3] γ-Mangostin

also displays potent inhibitory activity against mediators of prostaglandin release, COX-1 and COX-2.[4] Other prenylated xanthones have been found to be strongly active against *Bacillus subtilis*, *Staphylococcus faecalis*, *Staphylococcus typhi*, and *Candida glabrata*.[5] By themselves, prenyl groups are important contributors to the outstanding structural diversity of natural products.[6] Prenylated secondary metabolites isolated from plants, bacteria, and fungi display a large variety of medicinal properties, including antitumor, antiretroviral, and psychotrophic activities, that are often distinct from their nonprenylated precursors.[7]

Molecular genetic analysis of the biosynthesis of prenylated and nonprenylated xanthones, as well as prenylations in general, would greatly advance our understanding of fungal secondary metabolite biosynthesis and, by relating gene sequence to function, facilitate
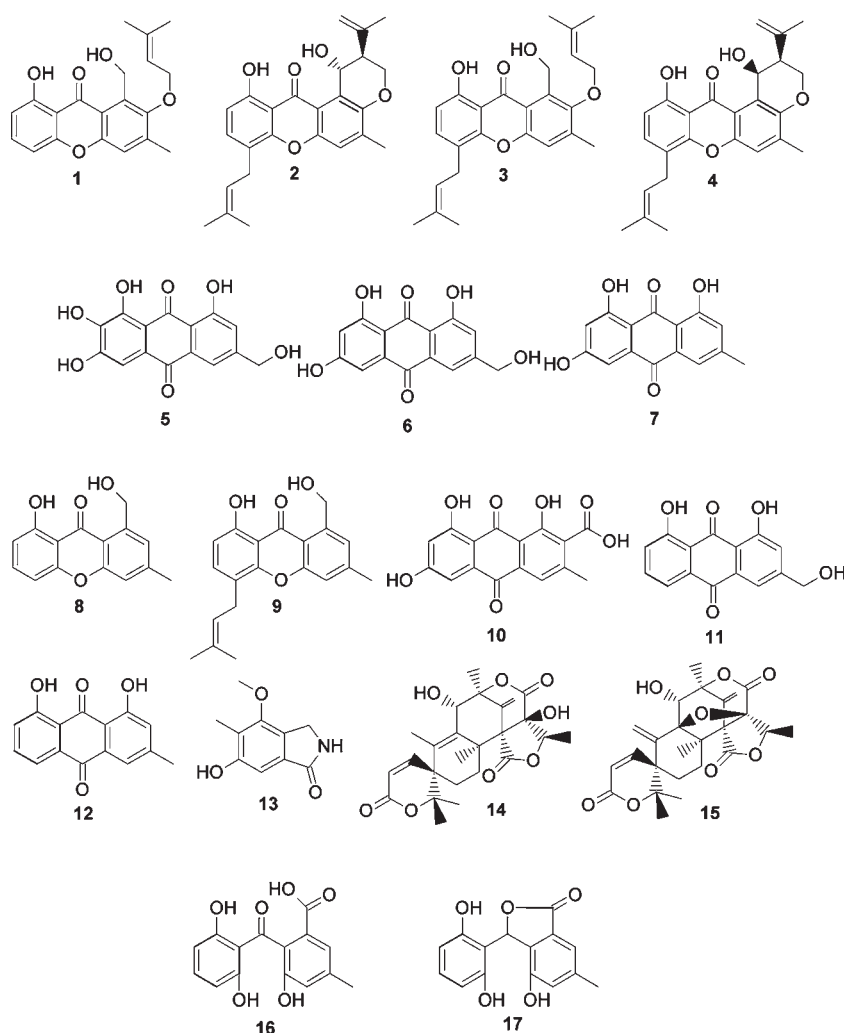
Figure 1. Prenyl xanthones and compounds that emerged from the study of targeted gene deletions. The compounds are as follows: variecoxanthone A, **1**; shamixanthone, **2**; emericellin, **3**; epishamixanthone, **4**; 2,ω-dihydroxyemodin, **5**; ω-hydroxyemodin, **6**; emodin, **7**; 9*H*-xanthen-9-one, 8-hydroxy-1-(hydroxymethyl)-3-methyl-, **8**; paeciloxanthone, **9**; endocrocin, **10**; aloe-emodin, **11**; chrysophanol, **12**; cichorine, **13**; austinol, **14**; dehydroaustinol, **15**; monodictyphenone, **16**; and 1(3*H*)-isobenzofuranone, 3-(2,6-dihydroxyphenyl)-4-hydroxy-6-methyl-, **17**.

prediction of the function of homologous genes. Moreover, identification of the genes involved in the production of particular secondary metabolites offers the possibility of enhanced production through replacement of their native promoters with strong or inducible promoters. In addition, semisynthesis, mutasynthesis, and chemoenzymatic synthesis that lead to second-generation compounds with improved pharmacodynamic and pharmacokinetic properties should greatly benefit from identification and manipulation of the genes involved in secondary metabolite production.

The filamentous fungus *Aspergillus nidulans* is, in principle, an excellent organism in which to study the biosynthesis of prenylated xanthones. It is known to produce two prenylated xanthones, shamixanthone (**2**) and emericellin (**3**) (Figure 1).[8,9] Its genome has been sequenced and is reasonably well annotated,[10,11] and development of an efficient gene targeting system[12,13] has greatly facilitated the targeted deletion of secondary metabolite genes.[14,15] We have taken advantage of these attributes to identify and characterize the prenyl xanthone biosynthesis pathway in *A. nidulans*.

We previously identified, using a set of deletions of the aromatic, nonreducing PKS genes in a chromatin remodeling mutant background, a PKS gene, *mdpG*, that is responsible for synthesis of the

related polyketides monodictyphenone and emodin.[16,17] Here, we employed the same set of deletions in the investigation of prenyl xanthone synthesis and found that, again, *mdpG* is responsible. Deletion of genes flanking *mdpG* revealed that the prenylated xanthone biosynthetic pathway is complex, involving the products of nine genes that cluster with *mdpG*. Identification of intermediate compounds that accumulate in the deletion strains provides important clues as to the steps in the xanthone biosynthesis pathway. Many of these intermediates have not previously been identified from *A. nidulans* and are of potential medical interest. Surprisingly and interestingly, however, the prenyltransferase genes required for prenyl xanthone biosynthesis are not clustered with the PKS gene. Bioinformatic analysis of the *A. nidulans* genome allowed us to identify putative prenyltransferase genes elsewhere in the genome whose products might be required for prenylation of the xanthones. By deleting each of these candidate genes, we found two genes that are required for prenylation of xanthones. They are distant from the *mdpG* cluster, and it is unlikely that they could have been identified by traditional approaches. Interestingly, the products are members of a family of prenyltransferases that are recognized to prenylate amino acids and their derivatives, as

opposed to polyketides. Lastly, we discovered a gene proximal to one of the prenyltransferase genes that, upon deletion, leads to the loss of two of the four prenyl xanthones that we identified. Our data, in aggregate, allowed us to unravel the complex xanthone biosynthesis pathway in *A. nidulans*, and they highlight the utility of genomics coupled with powerful molecular genetic methods for determining biosynthetic pathways, especially when the cluster is complex and critical genes are distant from the primary cluster.

■ RESULTS

Characterization of Prenylated Xanthones from *A. nidulans*. LO2026, an *A. nidulans* strain carrying a deletion of the *stcJ* gene (*stcJ*Δ), which is required for the biosynthesis of the carcinogenic secondary metabolite sterigmatocystin,[18] was cultivated on Yeast Extract Sucrose (YES) agar for 5 days at 37 °C. The rationale for the use of an *stcJ*Δ strain is that elimination of sterigmatocystin frees up the common polyketide precursor malonyl-CoA and facilitates detection and isolation of other metabolites. The strain also carries *nkuA*Δ to facilitate gene targeting.[12] Reverse-phase LC/MS analysis of the crude organic extract revealed the presence of several late-eluting, nonpolar metabolites (Figure 2). For full characterization, we cultivated the strain on YES plates at a larger scale and purified the compounds using flash chromatography, HPLC, and preparatory TLC when necessary. NMR characterization revealed the molecules to be shamixanthone (**2**) and emericellin (**3**), as well as, in lesser amounts, two prenylated xanthones not previously observed from this species, variecoxanthone A (**1**)[19] and epishamixanthone (**4**).[20] (Figure 1; NMR data available in the Supporting Information, Structural Characterization and Figures S3−S15). We also found that other solid media including Yeast Agar Glucose (YAG) and shredded wheat are conductive to xanthone production.

Analysis of Prenyl Xanthone Biogenesis through Targeted Deletions. The structural similarity of the prenyl xanthones (Figure 1) suggests a common biosynthetic origin, and the presence of aromatic rings is indicative of formation catalyzed by a nonreduced polyketide synthase (NR-PKS).[21] Analysis of the sequenced genome of *A. nidulans* suggests there are 12 NR-PKS genes in this species. The products of two of the genes, *stcA* encoding the sterigmatocystin PKS and *wA*, a spore pigment PKS, were already determined,[22,23] leading us to focus on the 10 remaining NR-PKSs. In a previous project, we discovered that deletion of *cclA*, a *bre2* homologue orchestrating histone H3 lysine 4 methylation, resulted in the synthesis of related aromatic compounds including monodictyphenone and emodin under conditions in which these molecules were normally not observed.[16] For that project we deleted the 10 annotated NR-PKS genes in a *cclA*Δ background and discovered that deletion of one NR-PKS gene, AN0150.4 (using the nomenclature of the Central Aspergillus Data Repository, CADRE, http://www.cadre-genomes.org.uk/, and the *Aspergillus* genome database, http://www.aspgd.org/) in a *cclA*Δ background, resulted in the elimination of these compounds.[17] We designated AN0150.4 *mdpG* and the PKS it encodes MdpG. (See Table 1 for our gene designations and the corresponding annotations from CADRE/AspGD and the Broad Institute Aspergillus Comparative Database.) For the current project we utilized the same 10 NR-PKS deletant strains to learn whether any of them were required for the synthesis of the prenyl xanthones in *A. nidulans*. We were surprised to discover that, in addition to monodictyphenone and its related molecules,
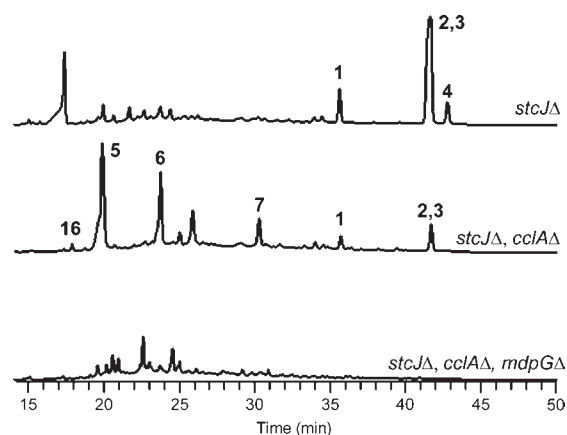

Figure 2. HPLC profiles of extracts from *stcJ*Δ; *stcJ*Δ, *cclA*Δ; and *stcJ*Δ, *cclA*Δ, *mdpG*Δ strains, as detected by UV absorbance at 254 nm. Numbering of peaks correspond to the compounds in Figure 1. Shamixanthone **2** and emericellin **3** elute at the same retention time.

the xanthones disappeared in the *mdpG*Δ strain (Figure 2) and not in the other deletant strains (data not shown). This reveals that MdpG is the PKS responsible for the synthesis of not only emodin, monodictyphenone, and related compounds but also the xanthones.

Next, we tried to identify additional genes involved in *A. nidulans* prenyl xanthone biosynthesis. Facilitating the search, secondary metabolism genes in *A. nidulans* are usually clustered, prompting us to focus on the genes surrounding *mdpG* (AN10039 to AN0153) (Figure 3). To study the synthesis of the xanthones as it would occur naturally, without the influence of a potentially powerful chromatin remodeling mutation, we deleted these genes in the LO2026 background carrying the wild-type *cclA* gene (*cclA*+). All deletions were verified by diagnostic PCR (using three different primer sets for each gene) (see Materials and Methods).

Attempted deletion of AN0153, homologous to a DNA-binding protein, failed to yield any viable transformants, in agreement with previous observations.[17] This gene appears to be essential for viability and unrelated to secondary metabolism and was thus excluded from further study here. Deletion of the other genes significantly reduced or eliminated prenyl xanthone formation except for AN10039, *mdpE*Δ, and *mdpI*Δ (Figures 3 and 4). Two differences were observed in relation to our investigation using *cclA*Δ: In the *cclA*Δ background, deletion of the transcriptional regulator gene *mdpE* eliminates the synthesis of monodictyphenone and related compounds; however, for the prenyl xanthones this gene does not appear consequential (Figure 3). Additionally, the monooxygenase gene *mdpD* was not found to be important for the production of monodictyphenone and analogous products in the *cclA*Δ background, whereas it plays a crucial role in prenyl xanthone formation.

The deletant strains that reduced prenyl xanthone production were examined for the presence of any new metabolites that might represent intermediates in the biosynthetic pathway of the xanthones. Extracts from strains carrying deletions of *mdpG*, *mdpA*, and *mdpF* contained no obvious intermediates from the prenyl xanthone biosynthesis pathways. A strain carrying a deletion of *mdpH* did not display new metabolites in significant amounts, but a compound with the mass and retention time of endocrocin (**10**), recently identified from an *mdpH*/*cclA* double-deletant strain,[17] was observed.

Table 1. Gene Designations of the Text and the Corresponding Annotations from Two Websites[a]

gene designation	putative function	CADRE/AspGD annotation	Broad annotation
AN10039	histidine acid phosphatase	AN10039.4	unannotated
mdpA	regulatory gene	AN10021.4	ANID_10021.1
mdpB	dehydratase	AN10049.4	ANID_10049.1
mdpC	ketoreductase	AN0146.4	ANID_00146.1
mdpD	monooxygenase	AN0147.4	ANID_00147.1
mdpE	regulatory gene	AN0148.4	ANID_00148.1
mdpF	Zn-dependent hydrolase	AN0149.4	ANID_00149.1
mdpG	polyketide synthase	AN0150.4	ANID_00150.1
mdpH	hypothetical protein	AN10022.4	ANID_11847.1 + ANID_11848.1
mdpI	acyl-CoA synthase	AN10035.4	ANID_10035.1
mdpJ	glutathione S transferase	AN10038.4	ANID_10038.1
mdpK	oxidoreductase	AN10044.4	ANID_10044.1
mdpL	Baeyer–Villiger oxidase	AN10023.4	ANID_10023.1
AN0153	MYB DNA binding protein	AN0153.4	ANID_00153.1
xptA	prenyltransferase	AN6784.4	ANID_06784.1
xptB	prenyltransferase	unannotated	ANID_12402.1 + ANID_12430.1
xptC	GMC oxidoreductase	AN7998.4	ANID_07998.1

[a] CADRE (http://www.cadre-genomes.org.uk) which uses the same designations as the *Aspergillus* genome database (AspGD, http://www.aspgd.org) and the Broad Institute Aspergillus Comparative Database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). Putative functions are from BLAST searches performed previously[16] or for this study.
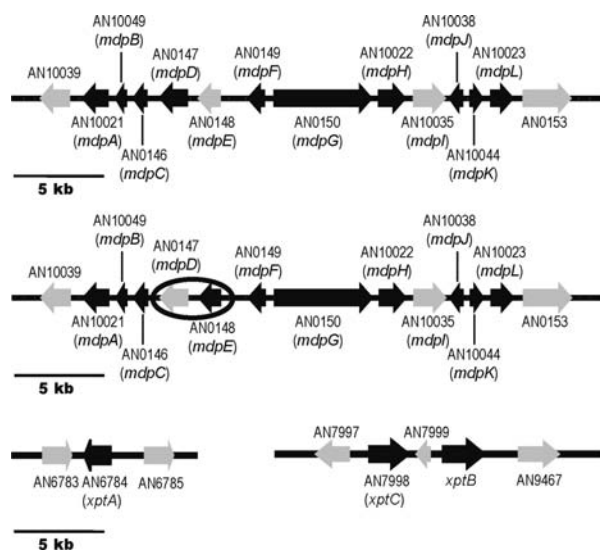

Figure 3. (Top) Organization of the genes surrounding the PKS *mdpG* involved in prenyl xanthone biosynthesis. Each arrow indicates gene size and direction of transcription. On the basis of a set of deletions we created and analyzed, genes shown in black are involved in prenyl xanthone biosynthesis while those in gray are not. (Middle) Organization of the genes for monodictyphenone biosynthesis in a *cclAΔ* background (based on ref 17). AN0147 and AN0148 are circled to emphasize that AN0147 is unnecessary for monodictyphenone generation but required for xanthone synthesis, whereas AN0148 is necessary for monodictyphenone generation but not required for xanthone synthesis. (Bottom) Organization of the genes outside of the *mdpG* cluster that are involved in prenyl xanthone synthesis. The genes that were successfully deleted were AN10039 through *mdpL*, *xptA*, *xptC*, AN7999, and *xptB*.

The *mdpLΔ* strain, the extract of which displayed the greatest number of significant peaks in the chromatogram, was grown in large scale on YES plates, and the major metabolites were isolated by flash chromatography and HPLC and characterized primarily using NMR. The compounds were determined to be 2,ω-dihydroxyemodin (**5**), ω-hydroxyemodin (**6**), emodin (**7**), aloe-emodin (**11**), and chrysophanol (**12**) (Figure 4; NMR data shown in the Supporting Information). Also, *mdpCΔ*, *mdpJΔ*, and *mdpKΔ* extracts each exhibited **5**, **6**, and **7**, as determined by their masses and retention times in comparison to the *mdpLΔ* extract, but little or no **11** or **12**. Chromatograms of *mdpBΔ* extracts similarly displayed the three metabolites, along with a number of small, indistinct peaks.

The *mdpDΔ* extract also yielded **5**, **6**, and **7** but with addition of monodictyphenone (**16**), a core xanthone structure (**8**), and the C-prenylated analog paeciloxanthone (**9**). Finally, the polyketide cichorine (**13**) and the meroterpenoids austinol (**14**) and dehydroaustinol (**15**) could be detected in all strains tested, indicating they are unrelated to prenyl xanthone biosynthesis.

Identification of Two Genes Required for Prenylation of the Xanthone Scaffold. Although our results revealed a gene cluster responsible for synthesis of *A. nidulans* prenyl xanthones, the lack of a prenyltransferase gene in this cluster made it clear that the required prenyltransferase gene(s) must be located elsewhere in the genome. On the basis of homology to known genes of this class,[24] we were able to identify several putative prenyltransferase genes in the *A. nidulans* genome: AN6784.4, AN8514.4 [*tdiB*[25]], AN10289.4, AN11080.4, AN11194.4, and AN11202.4. We deleted each of these genes, and the deletant strains were cultured on YES plates. LC/MS analysis revealed that deletion of AN6784.4, but not the other prenyltransferase genes, resulted in the elimination of **2**–**4** (Figure 5a) and accumulation of a compound which upon large-scale isolation and characterization was confirmed to be variecoxanthone A (**1**). Because AN6784.4 is a prenyltransferase gene required for synthesis of the xanthones, we hereby designate it *xptA* (xanthone prenyltransferase A).

Since the *A. nidulans* xanthones contain two prenyl groups (except for variecoxanthone A, which only has one), it was
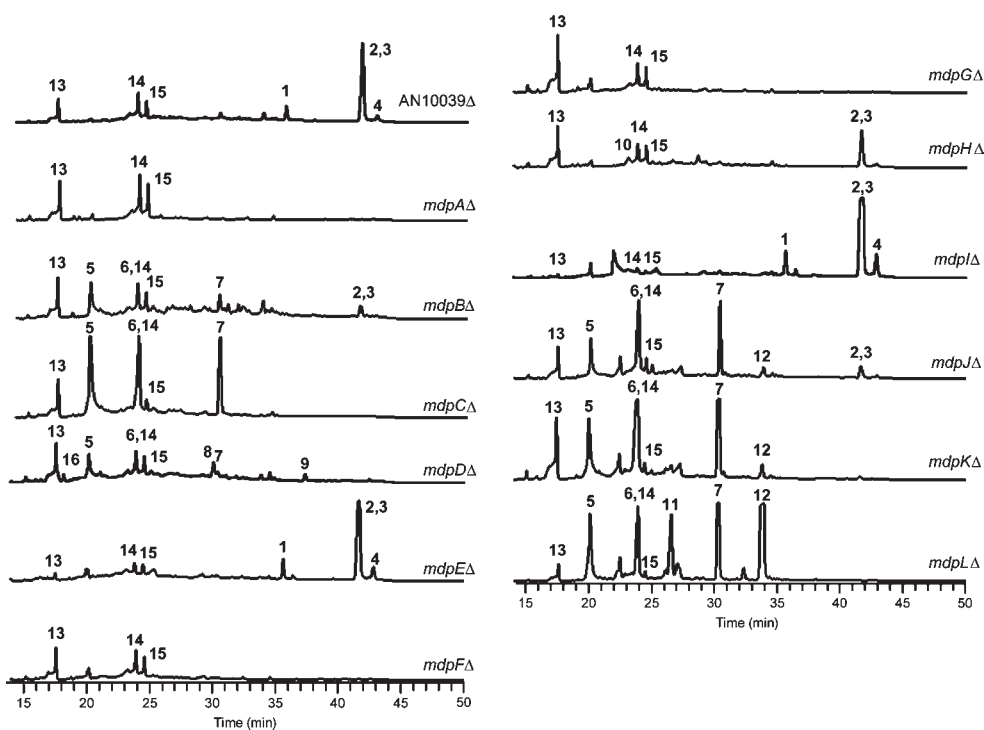
Figure 4. HPLC extracts of strains in the cluster as detected by UV absorbance at 254 nm. ω-Hydroxyemodin **6** and the unrelated metabolite austinol **14** elute at the same retention time.

apparent that an additional prenyltransferase gene was required. We hypothesized that the genome must contain an additional, unannotated prenyltransferase gene. We performed a local BLAST search of the Broad Institute Aspergillus Comparative Database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) using AN6784.4 as a query sequence. We identified two annotated partial gene fragments designated ANID_12402.1 and ANID_12430.1. Inspection of the two fragments revealed that the 3′-end of ANID_12402.1 overlaps with the 5′-end of ANID_12430.1 by ∼600 bp. Further, the Web site predicted that the sequence of ANID_12402 is prematurely truncated at its 3′-end. For these reasons we concluded that the nonoverlapping portion of ANID_12430 was in fact the continuation of ANID_12402 and that the 5′-end of ANID_12402 and the 3′-end of ANID_12430 are the boundaries of a single gene. Indeed, the sequence of this putative gene is found intact in CADRE/AspGD but without annotation. Deletion of this gene led to the elimination of the xanthones and accumulation of several metabolites, including **7**, **12**, **16**, and a putative monodictyphenone derivative (**17**) (Figure 5a). We designate this new gene *xptB* (xanthone prenyltransferase B).

Identification of an Additional Prenyl Xanthone Biosynthesis Gene. The data were suggestive of a biosynthesis in which emericellin (**3**) is a precursor to shamixanthone (**2**) and epi-shamixanthone (**4**) (see Discussion). Even after identification of *xptA* and *xptB* it was clear that a gene encoding the enzyme responsible for the transformation from emericellin to shamixanthone and epishamixanthone was required but not yet identified. Given the tendency of fungal secondary metabolite genes to exist in clusters, we reasoned that the missing gene might reside proximate to *xptA* or *xptB*. An analysis of the putative function of the genes neighboring *xptA* revealed none that were likely involved in secondary metabolism; however, AN7998.4 and

AN7999.4 upstream of *xptB* were possible candidates. Deletion of AN7998.4 yielded a metabolite profile that continued to display variecoxanthone A (**1**) and **3** but lacked **2** and **4** (Figure 5b and 5c), revealing that AN7998.4 is the missing gene in the biosynthesis of the two prenyl xanthones. We designate AN7998.4 as *xptC*. The amino acid sequence indicates that it is a member of the glucose–methanol–choline (GMC) oxidoreductase superfamily, a broad class that includes cellobiose dehydrogenase, choline dehydrogenase, and methanol oxidase.[26] Deletion of AN7999.4 had no obvious effect on prenyl xanthone biosynthesis.

■ DISCUSSION

We used a combination of genomics, efficient gene targeting, and natural products chemistry to elucidate the complex prenyl xanthone biosynthesis pathway in *A. nidulans*, the first such pathway to be deciphered in a fungus. This pathway is complex, involving the products of 10 clustered genes and three genes located apart from the main cluster. The genes *mdpA* through *mdpL* are located on the right arm of Chromosome VIII, whereas *xptA* is located on the right arm of Chromosome I and *xptB* and *xptC* are found on the left arm of Chromosome II. In all cases the genes are ∼0.5 Mb from a telomere. Other uncommon examples of separated fungal secondary metabolism genes include the genes for T-toxin biosynthesis in *Cochliobolus heterostrophus*, with 9 genes in two unlinked loci, and the nonclustered acetyltransferase gene *Tri101* involved in trichothecene biosynthesis in *Fusarium* species, having a different evolutionary history from other trichothecene genes.[27,28] Without genomics and efficient gene targeting it would have been difficult, if not impossible, to identify all of the genes in the pathway. In particular, it would have probably been extremely difficult to identify the three genes that are separated in the genome from the bulk of the xanthone

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biosynthesis genes in the absence of the ability to identify candidate genes and to delete them en masse.

The two prenyltransferases, encoded by the *xptA* and *xptB* genes, are particularly interesting. They are both homologous to members of a class of enzymes known as fungal indole prenyltransferases.[29] For example, XptA is 27% identical at the amino acid level to SirD from *Leptosphaeria maculans*[30] and 24% identical to FgaPT2 from *Aspergillus fumigatus*.[31] XptB is 29%

and 27% identical to these enzymes, respectively, and XptA and XptB are 37% identical to each other. Unlike many other prenyltransferase enzymes, the fungal indole prenyltransferases are soluble, lack (N/D)DxxD motifs, and continue to be active in the absence of Mg^{2+}.[29] The fungal indole prenyltransferases are capable of regular and reverse C-prenylations as well as N- and O-prenylations. Their solubility may be useful for chemoenzymatic synthesis in terms of ease of manipulation and efficiency of catalysis. All members of this class studied in detail to date prenylate compounds of amino acid origin, whereas our data reveal that XptA and XptB clearly prenylate polyketides. There were hints from the literature that this class might have a range of substrates extending to polyketides. For example, the X-ray structure of dimethylallyl tryptophan synthase (DMATS) from *A. fumigatus* revealed a common architecture (but no significant primary amino acid similarity) with the bacterial enzyme NphB that catalyzes addition of a 10-carbon geranyl moiety to a polyketide-based aromatic scaffold.[32] In addition, a gene in *Penicillium aethiopicum* that is homologous to indole prenyltransferases is proximal to a polyketide gene necessary for the formation of viridicatumtoxin and was proposed to contribute to addition of a geranyl group to that metabolite.[33] Our findings provide the first direct evidence, however, that this class of prenyltransferases can prenylate polyketides, and the possibility must be considered that XptA and XptB may prenylate polyketide substrates beyond those reported in this study.

Our deletions reveal the genes required for prenyl xanthone synthesis, but correlation of the deleted genes with the intermediates that accumulate in the deletion strains allows us to propose a biosynthetic pathway for the *A. nidulans* prenyl xanthones (Figure 1). We previously detailed the synthesis of monodictyphenone (**16**) in a *cclA*Δ background, which was dependent on many of the same secondary metabolite genes also responsible for the prenyl xanthones.[17] We propose that **16** is an intermediate en route to prenyl xanthones (Figure 6). One new observation, however, is that from the *mdpL*Δ strain we have now isolated chrysophanol (**12**).

Earlier work by others used isotopic precursors to propose the biosynthesis of **2** and a similar metabolite, tajixanthone, in *Aspergillus variecolor*.[34] The labeling patterns from [1-^{13}C]- and [2-^{13}C]-acetate incorporation suggested an octaketide precursor,
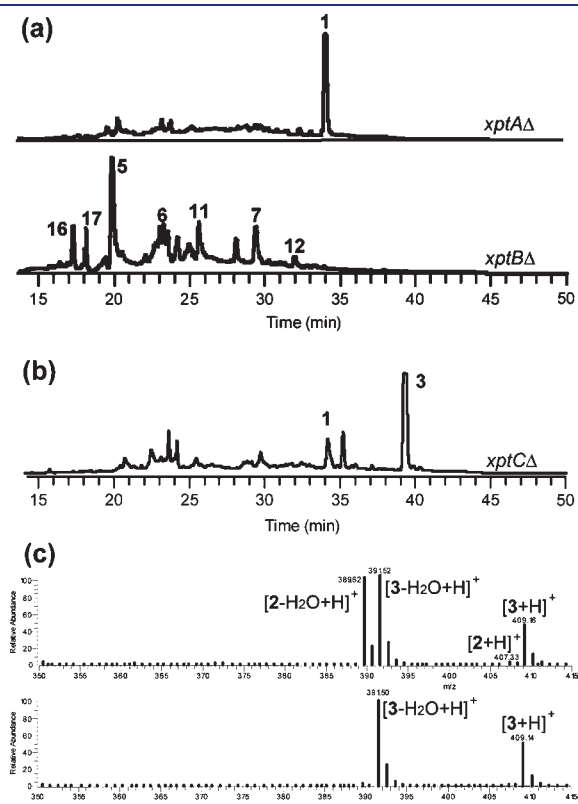

Figure 5. (a) HPLC extracts of the prenyltransferase deletant strains *xptA*Δ and *xptB*Δ as detected by UV absorption at 254 nm. (b) HPLC extract of *xptC*Δ. (c) Mass spectra of the major LC/MS peak from (top) *stcJ*Δ control extract and (bottom) *xptC*Δ extract.
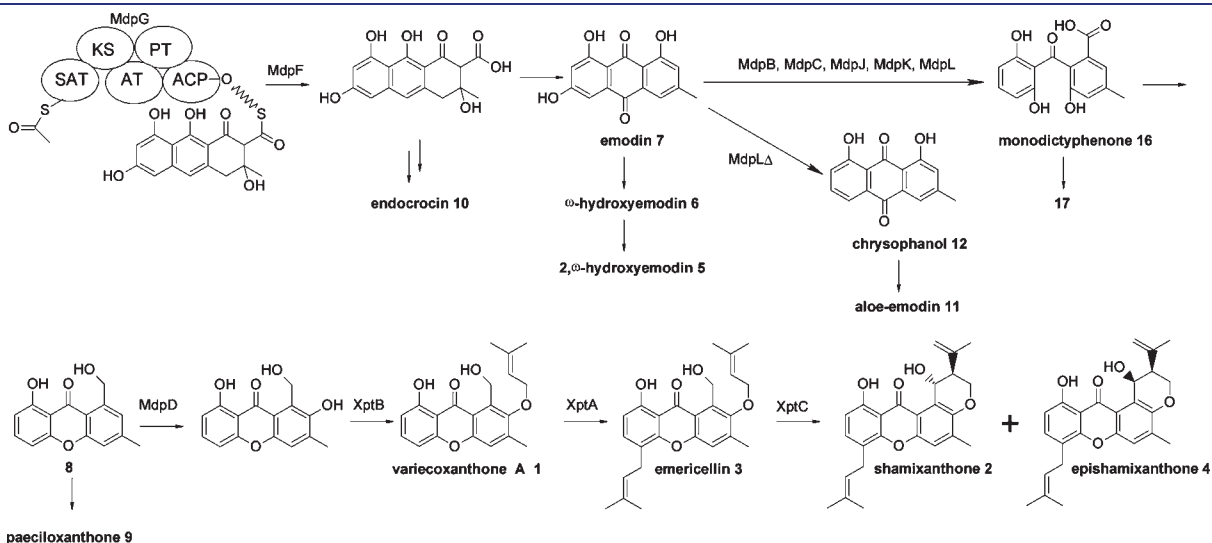

Figure 6. Proposed biosynthetic pathway of prenyl xanthones.

which could condense into an anthrone and possibly autoxidize to an anthraquinone. Interestingly, the earlier work also found that isotopically labeled **12** was incorporated into tajixanthone; it was proposed that xanthone formation proceeds from an octaketide to **7** and further to **12**. Because our earlier analysis[17] indicates that **12** is not in the direct biosynthetic pathway from **7** to **16**, we propose that it is a shunt metabolite; more specifically, the *mdpL* knockout may yield an unstable intermediate that transforms to chrysophanol. **7** might not be converted to **12** when the biosynthesis is uninterrupted. An alternative explanation for incorporation of **12** into tajixanthone is that **12** is metabolized to **7** at a low rate through an endogenous mechanism. Aloe-emodin (**11**), the ω-hydroxylated analog of **12**, may be a side product arising from oxidation by an endogenous cytochrome P450 enzyme.[35]

Elucidating the enzymatic conversion of **7** to **16** is complicated by the fact that the process appears to be dependent on five of the clustered genes, *mdpB*, *mdpC*, *mdpJ*, *mdpK*, and *mdpL*, and that the metabolite profiles from each of the corresponding deletion strains are similar. As a further complication, the presence of several metabolites in a deletant strain (for example, five major compounds from *mdpL*Δ) makes it difficult to ascribe their roles in the biosynthesis and their relation to each other. We refer the reader to ref 17 for a discussion of the possible functions of *mdpB*, *mdpC*, *mdpJ*, *mdpK*, and *mdpL* in monodictyphenone biosynthesis. Our data allow us, however, to deduce the remainder of the prenyl xanthone biosynthesis pathway with considerable confidence.

In this study we show conclusively that four genes, *mdpD*, *xptA*, *xptB*, and *xptC* are, at a minimum, responsible for conversion of monodictyphenone to the prenyl xanthones. It is easiest to deduce the steps of the biosynthetic pathway by working back from the final products, shamixanthone (**2**) and epishamixanthone (**4**). The *xptC* deletion does not accumulate **2** or **4** but does accumulate emericellin (**3**) and variecoxanthone A (**1**). This allows us to deduce that XptC is required for conversion of **3** or **1** to **2** and **4**. The *xptA* deletion strains accumulate **1** but not **3**, **2**, or **4**. This result allows us to deduce that XptA is required for the C-prenylation of **1** to form **3** and also allows us to deduce that **3** is the compound that is converted by XptC to **2** and **4**. Given the similarity of XptC to oxidative enzymes, we suggest that oxidation of the primary alcohol of **3** to an aldehyde, followed by ene cyclization,[19] may yield **2** and its epimer **4**. From the facts that XptA catalyzes the C-prenylation of **1** and no prenylated xanthones are found in *xptB* deletion strains, we deduce that XptB catalyzes the O-prenylation required for formation of **1** and that the O-prenylation occurs before the C-prenylation and is a prerequisite for the C-prenylation. From the *mdpD* deletant we isolated compound **8,** which contains the xanthone core but does not bear functionality at the 2 position, at which the final products feature the O-prenyl group. Oxidation of the xanthone core at the C-2 position is necessary for O-prenylation, so we postulate that MdpD, homologous to known monooxygenases, catalyzes oxidation prior to O-prenylation by XptB. In the absence of the C-2 oxygen, XptA is able to yield the C-prenylated product **9** in small amounts. In the *xptB* deletant strain, we were able to detect compound **17**, which we suspect to be a shunt metabolite arising from reduction of the carbonyl group of monodictyphenone (**16**) and condensation with the carboxylic acid.

In addition to assisting in elucidating the biosynthetic pathway, many of the shunt products and intermediate molecules are bioactive. For instance, **11** is cytotoxic to neuroblastoma, Ewing

sarcoma, and pPNET cell lines and inhibits neuroblastoma tumors in vivo but is not toxic to normal cells nor animal models.[36] **9** displays antimicrobial and antiacetylcholineesterase activity and is also cytotoxic against hepG2 cells.[37] Compound **8** has antifungal and antibacterial activities.[38] These findings indicate that a benefit of targeted deletions of genes is the accumulation of useful quantities of biosynthetic intermediates that may themselves possess noteworthy biological properties.

■ CONCLUSION

Previous work demonstrated that a cluster of 10 genes was responsible for several related compounds that emerged in a chromatin deletant strain of *A. nidulans*, including monodictyphenone.[17] The current work reveals that four prenyl xanthones rely on most of these genes for their synthesis but in addition require a monooxygenase gene within the cluster and three genes outside of the cluster, including two belonging to a family of indole prenyltransferase genes and one homologous to GMC oxidoreductase genes. The combined data allow us to propose that these latter genes are involved in the later stages of prenyl xanthone formation to complete a biosynthesis in which monodictyphenone is a precursor.

■ MATERIALS AND METHODS

Generation of Fusion PCR Fragments, *A. nidulans* Protoplasting, and Transformation. All gene deletions were carried out according to the gene targeting procedures of Szewczyk et al.[13] Two ~1000 base pair fragments upstream and downstream of every targeted gene were amplified from *A. nidulans* genomic DNA using PCR. Primers used in this study are listed in Table S1, Supporting Information. The two amplified flanking sequences and an *A. fumigatus pyrG* selectable marker cassette were fused together by PCR using nested primers. *A. nidulans* strains in this study are displayed in Table S2, Supporting Information. Protoplast generation and transformation were performed as described.[13] The strain LO2026 carrying a deletion of the *stcJ* gene that eliminates sterigmatocystin production was used as the recipient strain. Diagnostic PCR of the deletant strains was carried out using the external primers from the first round of PCR. The difference in the size between the gene replaced by the selective marker and the native gene allowed us to establish if the transformants carried correct gene replacements. For further verification, diagnostic PCR was performed two more times, using one of the external primers and a primer located inside the marker gene, then the other external primer and an internal primer. In these instances, the deletants yielded the PCR product of the expected size whereas no product was present in nondeletants.

Media and Cultivation of Strains. YES media was prepared by combining 20 g of yeast extract, 120 g of sucrose, 20 g of agar, and 2 mL of trace element solution[15] in 1 L H_2O. For LC/MS screening, spores of LO2026 (the control strain) and three strains of each gene deletant were individually cultivated (1×10^7 spores) on 10×150 mm Petri dishes containing YES agar and cultivated at 37 °C for 5 days. The agar was chopped into ~2 cm^2 pieces, and the material was extracted using sonication with methanol, followed by 1:1 methanol:dichloromethane. The organic solvents were removed in vacuo, and the remaining material was partitioned between H_2O (25 mL) and ethyl acetate (25 mL $\times$ 2). The combined ethyl acetate layers were evaporated, and the crude material was redissolved at a concentration of 20 mg/mL in DMSO and then diluted 5-fold in methanol.

LC/MS Analysis. LC/MS was carried out using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer with an RP C18 column (Alltech Prevail; 2.1×100 mm with a 3 μm particle size) at a flow rate of 125 μL/min and monitored by a UV detector at 254 nm. The solvent

gradient was 95% MeCN−H$_2$O (solvent B) in 5% MeCN−H$_2$O (solvent A) both containing 0.05% formic acid:0% B from 0 to 5 min, 0 to 100% B from 5 to 35 min, 100% B from 35 to 40 min, 100% B to 0% B from 40 to 45 min, and reequilibration with 0% B from 45 to 50 min.

Isolation of Metabolites. The LO2026 (*stcJ*Δ), LO3387 (*mdpL*Δ), LO3337 (*mdpD*Δ), LO3896 (*xptA*Δ), and LO4178 (*xptB*Δ) strains were each cultivated in 25 × 150 mm Petri dishes containing 2 L YES media and extracted in the same manner as above. The crude material was subjected to silica gel column chromatography, using ethyl acetate and hexanes as the eluent. The materials were further separated by preparative HPLC [Phenomenex Luna 5 μm C18 (2), 250 × 21.2 mm] with a flow rate of 5.0 mL/min and measured by a UV detector at 250 nm. Shamixanthone (**2**), emericellin (**3**), epishamixanthone (**4**), and paecilo-xanthone (**9**) required further purification using preparative TLC. See Supporting Information for more details about isolation.

Structural Characterization. See the Supporting Information under Structural Characterization and Figures S3−S15.

■ ASSOCIATED CONTENT

S **Supporting Information.** *A nidulans* strains and primers used in this study, detailed structural characterization, purification methods, and complete refs 10 and 11. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
boakley@ku.edu; clayw@usc.edu

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