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Biosynthetic Pathway of the Reduced Polyketide Product Citreoviridin in *Aspergillus terreus* var. *aureus* Revealed by Heterologous Expression in *Aspergillus nidulans*

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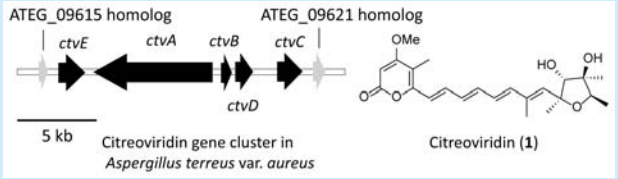
Supporting Information

ABSTRACT: Citreoviridin (**1**) belongs to a class of F1-ATPase β-subunit inhibitors that are synthesized by highly reducing polyketide synthases. These potent mycotoxins share an α-pyrone polyene structure, and they include aurovertin, verrucosidin, and asteltoxin. The identification of the citreoviridin biosynthetic gene cluster in *Aspergillus terreus* var. *aureus* and its reconstitution using heterologous expression in *Aspergillus nidulans* are reported. Two intermediates were isolated that allowed the proposal of the biosynthetic pathway of citreoviridin.



Recent genome sequencing efforts of filamentous fungi have clearly demonstrated that the number of secondary metabolites (SMs) that have been isolated and identified after decades of intensive efforts is very much smaller than the number of SM biosynthetic gene clusters within the genome.[1] The gene clusters that are not connected with any known compounds are called orphan gene clusters, and they are a promising source of novel therapeutics from the pharmacological point of view.[2] With the advent of new DNA sequencing technologies such as next-generation sequencing, we are witnessing a rapid accumulation of fungal genomes in public databases. Thus, natural product researchers are seizing this opportunity by intensely mining these fungal genomes for orphan gene clusters that produce medicinally valuable compounds.

Citreoviridin (**1**) is a highly reduced polyketide product that belongs to a class of fungal secondary metabolites that act as inhibitors of mitochondrial oxidative phosphorylation. Members of this class of ATP synthase inhibitors contain a methylated α-pyrone, a polyene linker, and either a tetrahydrofuran ring, as in the case of citreoviridin and verrucosidin, or a dioxabicyclooctane moiety, as in the case of aurovertin and asteltoxin (Figure 1).[3,4] Citreoviridin binds to the β-subunit of F1-ATPase, thereby uncompetitively inhibiting ATP hydrolysis and noncompetitively inhibiting ATP synthesis.[5,6] Recently, Chang et al.[7] used citreoviridin to target ectopic ATP synthase in breast cancer. Other members of this class of ATP synthase inhibitors have similarly been investigated as potential therapeutics against cancer.[8]

Because of the potent biological activity of citreoviridin, investigators have been interested in the biosynthesis of this mycotoxin since the 1980s. From ^{13}C-labeled precursor studies, Steyn et al.[9] found that the polyketide backbone of citreoviridin is derived from one acetyl-CoA starter unit, eight malonyl-CoA extender units, and five methyl groups from *S*-adenosylmethionine (SAM). The same group later proposed that tetrahydrofuran ring formation involved a bisepoxidation step followed by epoxide hydrolysis.[10] Asai et al.[11] proposed that citreomontanin (**2**) is likely the biogenic precursor of citreoviridin. Here we report our efforts to identify the biosynthetic gene cluster for citreoviridin using an *Aspergillus nidulans* heterologous expression system.[12] While our study was being completed, the biosynthesis gene cluster of a similar

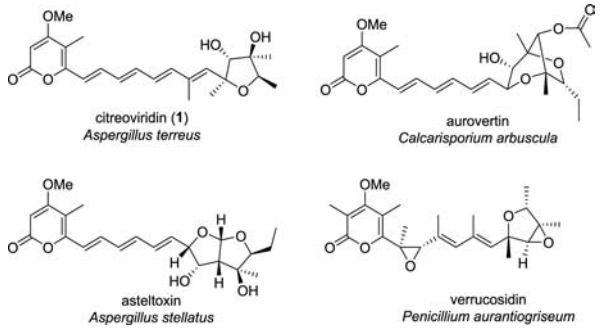


Figure 1. Citreoviridin and other inhibitors of mitochondrial oxidative phosphorylation produced by different fungal species.

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Table 1. Citreoviridin (1) Biosynthesis Genes in *A. terreus* var. *aureus*, Their Homologues in Other Species, and Gene Function Predictions

gene	putative function	*A. terreus* NIH2624 homologue aa (% identity, % similarity) gene (% identity)	*C. arbuscula* homologue aa (% identity, % similarity)	*M. anisopliae* homologue aa (% identity, % similarity)
ctvA	polyketide synthase	XP_001218239.1 (89, 93) ATEG_09617 (88)	AurA (46, 60)	XP_007824472.2 (47, 63)
ctvB	SAM-dependent methyltransferase	XP_001218240.1 (94, 98) ATEG_09618 (91)	AurB (53, 67)	XP_007824471.1 (52, 67)
ctvC	flavin-dependent monooxygenase (FMO)	XP_001218242.1 (92, 96) ATEG_09620 (87)	AurC (47, 67)	XP_007824468.2 (45, 64)
ctvD	hydrolase	XP_001218241.1 (84, 88) ATEG_09619 (85)	AurD (31, 47)	XP_007824469.1 (33, 48)
ctvE	ATP synthase β-chain	XP_001218238.1 (96, 97) ATEG_09616 (87)	none	XP_007824466.2 (83, 89)

highly reduced polyketide, aurovertin from *Calcarisporium arbuscula*, was reported.[13]

Organisms sometimes achieve self-resistance against the metabolites they produce by harboring duplicate or resistant targets within the metabolite biosynthetic gene cluster.[14] For instance, *Aspergillus terreus* is thought to protect itself from the antifungal effects of lovastatin by encoding a copy of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the target of lovastatin, in the lovastatin biosynthetic locus. In fact, resistance-gene-driven genome mining has led to the identification of the mycophenolic acid and thiotetronic acid gene clusters.[15,16]

Bioinformatic analysis of *A. terreus* strain NIH2624 revealed that the organism contains an extra copy of the F1-ATPase β-subunit of the known target of citreoviridin (ATEG_09616 and ATEG_07609).[5] One gene, ATEG_09616, is located adjacent to a highly reducing poyketide synthase (HR-PKS), ATEG_09617, and other putative tailoring enzymes required for the synthesis of the tetrahydrofuran ring and methylated α-pyrone in citreoviridin. Specifically, a methyltransferase (ATEG_09618), flavin-dependenent mono-oxygenase (ATEG_09620), and hydrolase (ATEG_09619) were found in the locus. Moreover, genome database searches showed that *Metarhizium anisopliae*, a known producer of the structurally similar toxin aurovertin,[17] also harbors a cluster containing a β-subunit of ATP synthase, and genes in the cluster have homology to the locus we identified (Table 1).

Initial bioinformatics analysis was conducted on NIH2624 because it was the only *A. terreus* strain with a publicly available genome at the time of the analysis. However, despite extensive studies conducted on its secondary metabolites, NIH2624 has not been reported to produce citreoviridin.[18] On the other hand, the *A. terreus* var. *aureus* strain CBS503.65 is a known producer of the toxin.[19] When we cultured both strains, we detected the production of citreoviridin only in *A. terreus* var. *aureus* CBS503.65 (Figure S1). Furthermore, initial attempts at the heterologous expression of ATEG_09617 were unsuccessful in obtaining the production of the expected polyketide (data not shown). These results led us to investigate the citreoviridin biosynthetic gene cluster in the *A. terreus* var. *aureus* CBS503.65 strain.

As no genome sequence of the *A. terreus* var. *aureus* CBS503.65 strain is publicly available, we designed primers based on the sequence of the NIH2624 strain in an attempt to clone homologues of the HR-PKS ATEG_09617 and its surrounding genes. At the onset we were unsure whether the strategy would be successful. Gratifyingly, the putative citreoviridin biosynthetic genes were cloned with relatively little difficulty. In total, five genes were cloned: *ctvA*, *ctvB*, *ctvC*, *ctvD*, and *ctvE* (Figure 2A). Sequence analysis of the homologues revealed that the two share 80−90% identity at the nucleotide level and 90−95% identity at the amino acid level. On the basis of function prediction (Table 1), we hypothesized that *ctvA* to *ctvD* constitute the core biosynthetic gene cluster while *ctvE* is the resistance gene.

Each gene in the putative citreoviridin gene cluster was fused to the alcohol-inducible *alcA*(p) by fusion PCR and transformed into the host *A. nidulans* LO8030 according to the procedure developed by us.[12] The major known metabolites of the heterologous host *A. nidulans* LO8030 were deleted to facilitate the detection of new compounds and to free up metabolite precursors such as acetyl-CoA and malonyl-CoA.[12,20] Correct integration of the gene into the chromosome was verified by diagnostic PCR (Figure S3).

We first overexpressed the HR-PKS CtvA in an attempt to identify the highly reduced polyketide backbone of **1**. In the *ctvA* overexpression strain we detected the production of a new metabolite, compound **3**, compared with the LO8030 host control (Figure 2B, i and ii). Compound **3** is bright yellow and has an ESI-MS peak at m/z 339 $[M + H]^+$. It has a strong UV−vis absorption around 420 nm (Figure S2), consistent with the polyene chromophore of an ER-less HR-PKS.[21]

Scale-up and NMR characterization of the purified compound revealed the structure of compound **3**. The ^{1}H and ^{13}C NMR spectra of **3** were found to be almost identical to those of **2** except for the disappearance of the methoxy resonances (δ_H = 3.82 (3H, s), δ_C = 56.3; Table S3). Therefore, **3** is a demethylated form of the proposed citreoviridin biogenic intermediate **2** (Figure 3).

Since we did not know whether the methyltransferase or the monooxygenase would act next in the biosynthetic pathway, we made two distinct mutant strains, *ctvAB* and *ctvAC*. We cultured both strains and analyzed their metabolite profiles. The *ctvAC* strain had a metabolic profile similar to that of the *ctvA*-only strain (Figure 2B, iv), while in the *ctvAB* strain we observed the disappearance of compound **3** and the appearance of a new peak (Figure 2B, iii). The corresponding ESI-MS peak was at m/z 353 $[M + H]^+$, suggesting the addition of a methyl group. Indeed, by comparison with published ^{1}H and ^{13}C NMR spectra (Figures S4 and S5) we confirmed the identity of the new compound as citreomontanin (**2**).

The formation of the tetrahydrofuran ring has been proposed to involve two steps: bisepoxidation by a monooxygenase and epoxide ring opening by a regioselective hydrolase.[10] Since

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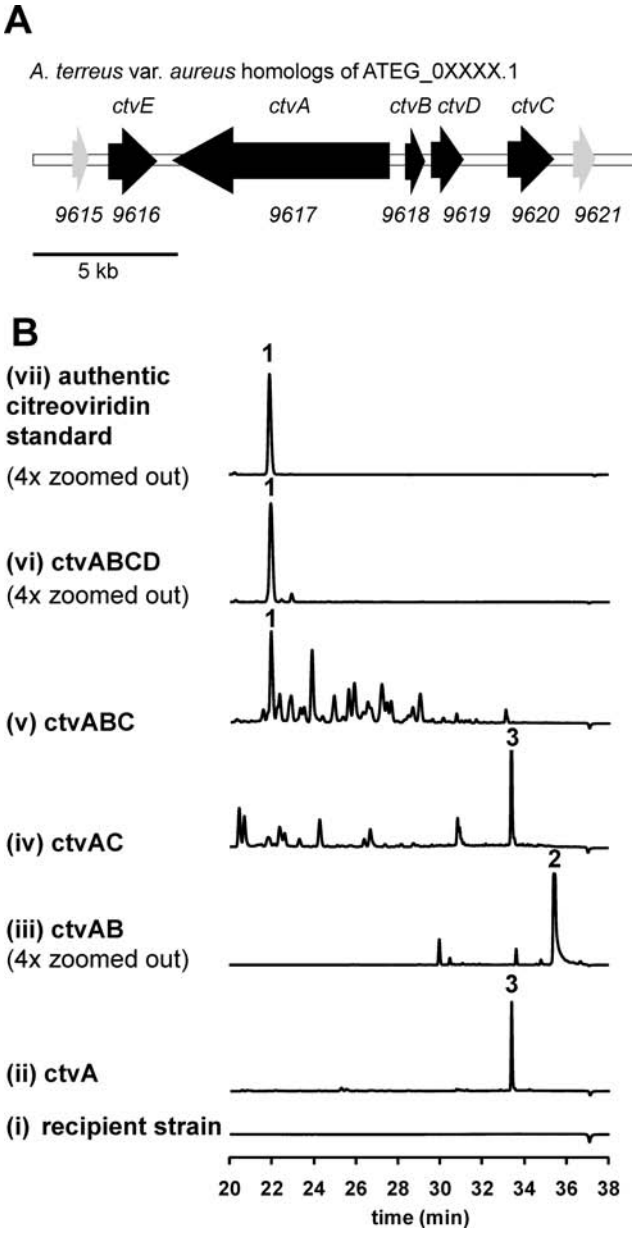

Figure 2. (A) The citreoviridin biosynthesis gene cluster. Black open reading frames are involved in the biosynthesis while gray ones are not. (B) HPLC profiles of metabolites extracted from the culture medium of *A. nidulans* strains expressing citreoviridin cluster genes under the control of *alcA*(p). HPLC analysis was carried out using a C18 reversed-phase column. Detection was at 403 nm. "4× zoomed out" indicates that the *y*-axis scale is 4 times larger.

CtvC is the only monooxygenase in the cluster, we first generated a triple overexpression *ctvABC*. We observed the emergence of many peaks, including that of **1** (Figure 2B, v). Some of these peaks correspond to species with molecular weight of 402 and similar UV−vis absorbance patterns, suggesting that they might be stereoisomers of **1**. Since bisepoxides are unstable and reactive, spontaneous hydrolysis and degradation might be responsible for these peaks. Citreoviridin **1** seems to be one of the major products, and it might be due to the greater stability of the allylic carbocation intermediate compared with the other carbocation intermediates that could form from epoxide opening (Figure 3). When *ctvD* was added to generate the *ctvABCD* overexpression strain, the other peaks disappeared and only the peak for compound **1** remained (Figure 2B, vi and vii). These results indicate that the four genes *ctvA*, *ctvB*, *ctvC*, and *ctvD* are sufficient for citreoviridin biosynthesis.

On the basis of our results, we propose the biosynthetic pathway for citreoviridin shown in Figure 3. The HR-PKS CtvA has a domain structure of KS-AT-DH-CMet-KR-ACP. It accepts acetyl-CoA as the starter unit and catalyzes eight iterations of malonyl-CoA extension and four iterations of SAM-dependent methylation at C4, C12, C14, and C16. The KR and DH domains selectively act on the first six iterations to generate the hexaene chain. In the last three iterations, the KR and DH domains terminate their functions to yield a β,δ-diketo ester moiety, which then undergoes intramolecular cyclization to yield the α-pyrone in compound **3**. Subsequently, CtvB methylates the α-pyrone hydroxyl group of **3** to generate **2**. In order to form the tetrahydrofuran ring with the correct stereochemistry, the terminal alkenes of **2** need to undergo isomerization to yield a (17*Z*)-hexaene,[10,11] a step that could be catalyzed by CtvC. The (17*Z*)-hexaene then undergoes bisepoxidation by CtvC to form a (17*R*,16*R*,15*S*,14*R*)-bisepoxide moiety. Lastly, CtvD acts as a regioselective hydrolase to form the tetrahydrofuran ring with the substituents in the correct absolute configuration, completing the biosynthesis of **1**.

Comparison of the citreoviridin biosynthetic gene cluster with the aurovertin gene clusters in *C. arbuscula* and *M. anisopliae* showed that there is 30−50% amino acid identity (Table 1). While the *C. arbuscula* gene cluster lacks the putative resistance gene, in *M. anisopliae* the ATP synthase β-subunit is highly conserved compared to that in *A. terreus* var. *aureus*.

CtvA selects acetyl-CoA as the starter unit, while AurA preferentially uses propionyl-CoA as the primer for biosynthesis. Currently, the only other HR-PKS or HR-PKS module known to use propionyl-CoA as a priming unit is the PKS-nonribosomal peptide synthase hybrid PsoA.[22] In nonreducing polyketide synthases (NR-PKSs), the starter unit acyltransferase (SAT) domain is responsible for starter unit selection,[23] yet the HR-PKSs do not contain SAT domains. These results suggest that the KS domain might possess innate starter unit selectivity. Our identification of CtvA, which has significant amino acid and domain architecture conservation with AurA (69% amino acid identity between the KS domains as predicted by antiSMASH), paves the way for future domain swapping experiments between the two closely related enzymes to verify the model in which the KS domain possesses a programming role.[24]

The findings of our study, in combination with the published studies done on the aurovertin biosynthetic cluster, will allow us to identify the biosynthetic gene clusters of asteltoxin in *A. stellatu* and verrucosidin and its derivatives in sponge-derived *Penicillium aurantiogriseum* once their genomes become publicly available.[25,26] The elucidation of the citreoviridin biosynthetic pathway strengthens the hypothesis that four genes are sufficient to generate the remarkable diversity among these ATP synthase inhibitors.

In conclusion, we have reported the elucidation of the citreoviridin biosynthetic pathway in *A. terreus* var. *aureus* by using *A. nidulans* as a heterologous expression host. We successfully reconstituted the pathway and demonstrated that four genes, *ctvA*, *ctvB*, *ctvC*, and *ctvD*, are sufficient for the production of the toxin.

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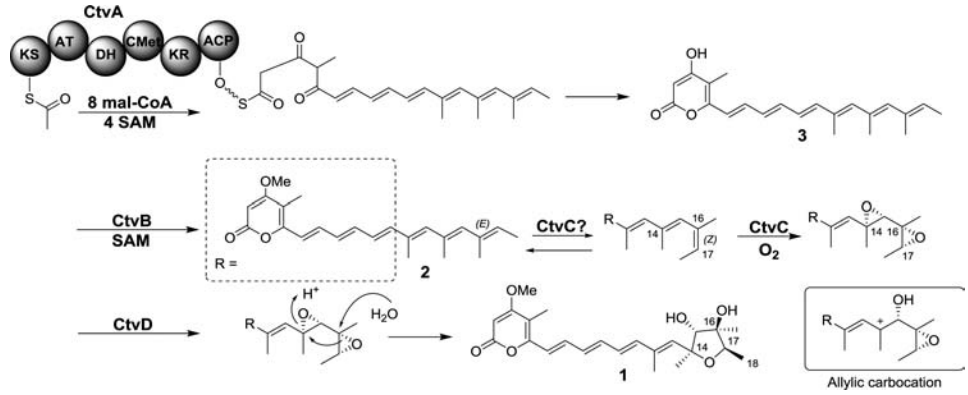

Figure 3. Proposed biosynthetic pathway for citreoviridin (**1**). The more stable allylic carbocation is shown in the solid-line box.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00299.

General methods, compound characterization and spectral data, diagnostic PCR, and sequence information (PDF)

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Notes

The authors declare no competing financial interest.

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