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A Cytochrome P450 Serves as an Unexpected Terpene Cyclase during Fungal Meroterpenoid Biosynthesis

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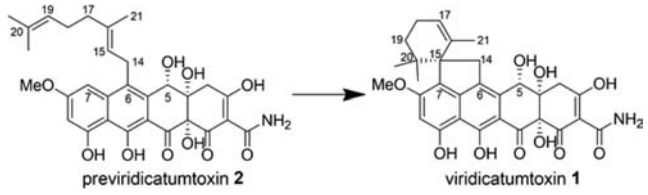
Supporting Information

ABSTRACT: Viridicatumtoxin (**1**) is a tetracycline-like fungal meroterpenoid with a unique, fused spirobicyclic ring system. Puzzlingly, no dedicated terpene cyclase is found in the gene cluster identified in *Penicillium aethiopicum*. Cytochrome P450 enzymes VrtE and VrtK in the *vrt* gene cluster were shown to catalyze C5-hydroxylation and spirobicyclic ring formation, respectively. Feeding acyclic previridicatumtoxin to *Saccharomyces cerevisiae* expressing VrtK confirmed that VrtK is the sole enzyme required for cyclizing the geranyl moiety. Thus, VrtK is the first example of a P450 that can catalyze terpene cyclization, most likely via initial oxidation of C17 to an allylic carbocation. Quantum chemical modeling revealed a possible new tertiary carbocation intermediate E that forms after allylic carbocation formation. Intermediate E can readily undergo concerted 1,2-alkyl shift/1,3-hydride shift, either spontaneously or further aided by VrtK, followed by C7 Friedel–Crafts alkylation to afford **1**. The most likely stereochemical course of the reaction was proposed on the basis of the results of our computations.

Viridicatumtoxin (**1**) is a fungal meroterpenoid consisting of an unusual monoterpene-derived spirobicyclic ring fused to an anhydrotetracycline-like, 2-carboxamide naphthacenedione scaffold.[1] Compound **1** and its derivative, viridicatumtoxin B, exhibit anti-MRSA activity.[2] Compound **1** and a C2 acetyl analogue, spirohexaline, have been demonstrated to inhibit bacterial undecaprenyl diphosphate synthase,[3] a potential new target for antibiotic development. The *vrt* gene cluster for the biosynthesis of **1** was identified in *Penicillium aethiopicum* using genome sequencing.[4] More recently, the prenyltransferase VrtC responsible for Friedel–Crafts alkylation at C6 of the naphthacenedione with geranyl diphosphate (GPP) to afford previridicatumtoxin (**2**) was characterized in detail.[5] However, the mechanism for forming the spirobicyclic ring (Scheme 1) that is further fused to C7 of the naphthacene core, especially in the absence of a dedicated terpene cyclase encoded in the gene cluster, remains intriguing. The spirobicyclic system with a hindered C15 quaternary carbon center is a striking structural feature that sparked a recent total synthesis effort and led to structure reassignment of viridicatumtoxin B as a quinone derivative of **1**.[6]

Scheme 1. Spirocyclization of the Geranyl Moiety of 2 To Afford 1



Our initial hypothesis proposed that cyclization of the geranyl substituent of **2** could begin with adding a hydroxyl at the allylic C17 position, followed by protonating the hydroxyl and forming an allylic cation accompanied by loss of water.[4] The carbocation can then initiate the cascade of cyclization reaction. We reasoned that the same enzyme that performs the C17 hydroxylation step may also orient the geranyl chain in a configuration that leads to regiospecific C15–C20 and C7–C15 cyclization events, thereby serving an additional function as a cryptic terpene cyclase. Cyclization of terpenes catalyzed by oxygenases is exceptionally rare, one example being oxidative cyclization of the monoterpene group of cannabigerolic acid by a berberine bridge enzyme-like flavin-dependent monooxygenase.[7]

As cytochrome P450 monooxygenases are likely candidates to catalyze the proposed C17 allylic oxidation, we began the search by genetically deleting each of the two genes encoding P450 monooxygenase (*vrtE* and *vrtK*) in the *vrt* gene cluster in *P. aethiopicum*. Using a double homologous gene-targeting approach described previously,[4] *vrtE* was deleted in the *P. aethiopicum* Δ*gsfA* strain. The Δ*gsfA* mutant was used as a background strain due to abolishment of griseofulvin biosynthesis, which provides a cleaner chemotype for metabolite analysis. The Δ*vrtE* strain lost the production of **1** but accumulated a new naphthacenedione intermediate **3** (*m*/*z* 402 $[M+H]^+$) (Figures 1 and S7). The compound was subsequently isolated and characterized by 1D and 2D NMR, with spectra comparable to those reported previously for anthrotainin[9] and 5-hydroxyanthrotainin,[5] except for the absence of the 8-*O*-methyl signal. A 1,3-keto–enol tautomer can also be detected in DMSO-d_6 (Table S2). Hence, **3** was assigned as the 8-*O*-desmethyl derivative of anthrotainin[9] as shown in Figure 1D.

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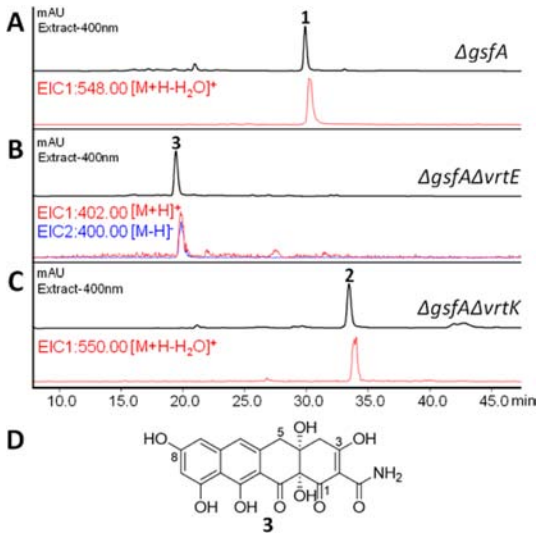


Figure 1. Deletion of individual P450 genes in the *vrt* gene cluster in *P. aethiopicum*. LCMS analyses of extracts from (A) Δ*gsfA*, (B) Δ*gsfA*/Δ*vrtE*, and (C) Δ*gsfA*/Δ*vrtK*. (D) Structure of **3**.

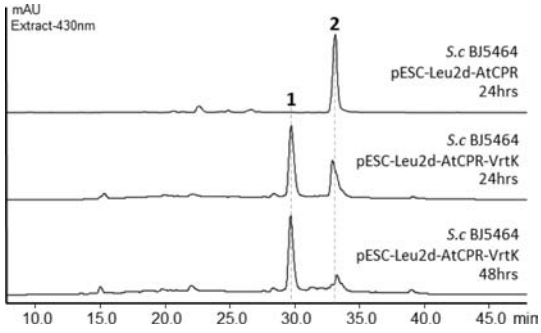


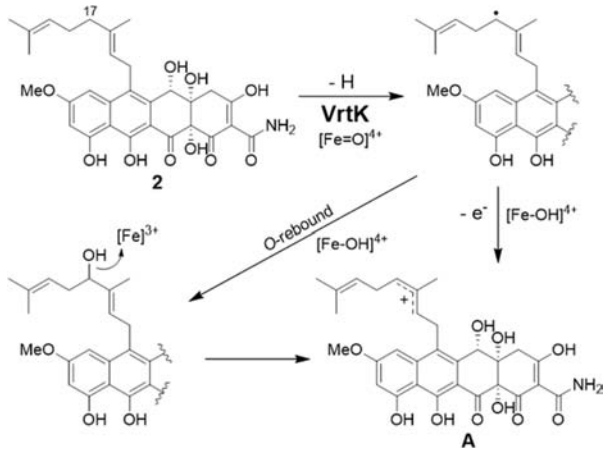
Figure 2. Biotransformation of **2** to **1** by *S. cerevisiae* BJ5464 coexpressing AtCPR and VrtK. The control (top) yeast harbors the pESC-leu2d-AtCPR plasmid without *vrtK*.

Elucidating the structure of **3** implies that the function of VrtE is to perform a single hydroxylation at C5 of **3**. **3** is the expected product of the nonreducing PKS VrtA, which functions in collaboration with auxiliary enzymes VrtG (dimanganese-containing Claisen cyclase) and VrtH (C12a monooxygenase).[8] Accumulation of **3** suggests that downstream enzymes, including 8-*O*-methyltransferase (VrtD) and prenyltransferase (VrtC), require the installation of C5-OH by VrtE for substrate recognition.

On the other hand, *vrtK* deletion in the *P. aethiopicum* Δ*gsfA* strain abolished production of **1** but accumulated a new intermediate **2** with *m*/*z* 550 $[M+H-H_2O]^+$ and *m*/*z* 568 $[M+H]^+$ in the culture extract (Figures 1 and S7). Comparing **2** with a standard shows that it is the uncyclized meroterpenoid **2**, which is the product of the VrtC-catalyzed geranylation as shown previously (Table S3).[5] This confirms our hypothesis that VrtK is the P450 enzyme that initiates cyclization of the spirobicyclic ring that eventually transforms **2** into **1**. However, the genetic result alone does not exclude the possibility that other enzymes in *P. aethiopicum* may be involved in the cyclization step following oxidation of the terpene moiety in **2**.

To further investigate the role of VrtK, we expressed VrtK heterologously in *Saccharomyces cerevisiae* BJ5464. VrtK was cloned into pESC-leu2d-AtCPR vector containing the *A. terreus* cytochrome P450 reductase (AtCPR) gene under the regulation

Scheme 2. Proposed C17 Carbocation Formation by VrtK



of Gal10 promoter. AtCPR has been demonstrated to perform coupled reduction of fungal P450 in *S. cerevisiae* for biotransformations.[10] The activity of VrtK was examined by feeding 1 mg of substrate **2** to 100 mL of galactose-induced yeast culture expressing AtCPR and VrtK. A slight conversion of **2** to **1** can be observed after 24 h of incubation, while no conversion was observed in the control yeast culture expressing only AtCPR. Nearly complete biotransformation of **2** to **1** was obtained when **2** was fed to a culture with higher density yeast cells prepared by concentrating 500 mL of culture to 100 mL (Figure 2). No C17-hydroxylated derivative or other intermediate derived from **2** can be detected under any tested culturing conditions. The relatively slower conversion rate and requirement of concentrated yeast cells for biotransformation could be attributed to the poor expression level of the VrtK in *S. cerevisiae*, the poor solubility of the substrate **2**, and/or the poor entry of **2** into the yeast cells. To test if VrtK could cyclize analogues of **2** with a dimethylallyl moiety, the yeast culture expressing VrtK was fed with the dimethylallyl analogue of TAN1612,[5] but the substrate could not be converted by VrtK under the same conditions (Figure S8).

Successful biotransformation of **2** to **1** supports the proposal that VrtK is the sole enzyme required for cyclization of the geranyl moiety of **2** to the spirobicyclic ring in **1**. Cyclization could be initiated by C17 hydroxylation via the typical hydrogen abstraction/oxygen rebound mechanism followed by ionization to the allylic cation intermediate **A** in the presence of Lewis acidic heme iron (Scheme 2). A similar mechanism is observed for the P450 enzyme LovA involved in lovastatin biosynthesis, whereby an allylic alcohol is first formed and then dehydrates, presumably via an allylic cation intermediate, to give monacolin L by proton loss.[10a] However, the absence of a C17-hydroxylated intermediate in the biotransformation assay suggests that VrtK may alternatively initiate the cyclization cascade via dehydrogenation of allylic C17 to give a carbon radical followed by electron transfer to the iron-heme center to form the corresponding carbocation A (Scheme 2). Such a mechanism has been proposed for P450-catalyzed desaturation reactions,[11] e.g., alkyl dehydrogenation/hydroxylation of capsaicinoids.[12] Indeed, a homology search of VrtK indicates that the enzyme shares significant protein sequence similarity (42% identity) to versicolorin desaturase VerB (StcL) in the aflatoxin pathway, which catalyzes desaturation of the bisfuran ring system of versicolorin B to afford versicolorin A.[13]

The mechanism of the geranyl cyclization was previously probed by feeding of [^{13}C,^{2}H]-labeled mevalonates.[14] Observa-

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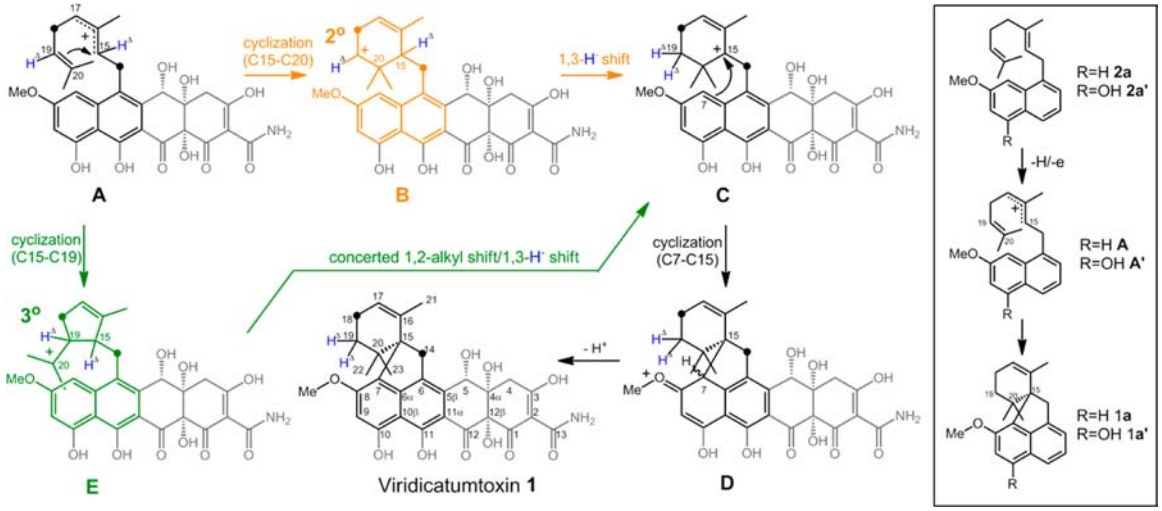

Figure 3. Proposed mechanisms for formation of **1**. Previously proposed mechanism via secondary carbocation **B** (in black and orange path). Newly proposed mechanism based on the results of quantum chemical calculations (black and green path). Box: model systems for computational modeling. H^{Δ} and ● indicate ^{2}H and ^{13}C labels on the geranyl moiety from (3*RS*)-[5-^{13}C,4-$^{2}H_2$]-mevalonolactone.[14]

tion of two ^{2}H atoms at C19 of **1**, derived from feeding of (3*RS*)-[5-^{13}C,4-$^{2}H_2$]mevalonolactone, provided evidence for a 1,3-hydride shift from C15 to C19 (corresponding to C2 and C6 of GPP) during the conversion of **2** to **1** (Figure 3). It was proposed that formation of the spirobicyclic ring is preceded by attack of C20 on C15 (Figure 3, **A**→**B**) followed by a 1,3-hydride shift to give intermediate **C**. With the new biosynthetic insight, we sought to further our understanding of the cyclization reaction mechanism using computational quantum chemistry (MPW1PW91/6-31+G(d,p)//B3LYP/6-31+G(d,p), MPWB1K/6-31+G(d,p)//B3LYP/6-31+G(d,p) M06-2X/6-31+G(d,p)//B3LYP/6-31+G(d,p), and B3LYP/6-31+G(d,p)/6-31+G(d,p)//B3LYP/6-31+G(d,p) calculations; see Supporting Information).[15] The model system shown in Figure 3 (box) was utilized to examine the inherent reactivity of putative carbocations (i.e., in the absence of the enzyme).

Based on our calculations, we propose that the mechanism postulated by Horak et al. (Figure 3, orange path) be revised to a mechanism involving tertiary carbocation intermediate **E** (Figure 3, green path) instead of secondary carbocation **B**,[14] which was found not to be a minimum on the potential energy surface. Cation **E** is predicted to undergo ring expansion (1,2-alkyl shift) and 1,3-hydride shift through a single-step reaction in which these two events are combined asynchronously.[16]

We examined variations on this pathway, differing in conformations/configurations of the intermediates involved, but not in the configuration of **1** produced. The transition-state structures involved in each of these four pathways are shown in Figure 4. We predict that all four paths are energetically accessible (see Supporting Information for all energies), but path 1 (Figure 5) does not require any conformational changes of intermediates. Thus, if the reactant is preorganized by VrtK into the conformation shown in Figure 5 for path 1, rearrangement of the active-site structure to accommodate conformational changes of the substrate is not required.[17] The overall barrier for path 1 is predicted to be <10 kcal/mol. For the rearrangement to follow path 2, 3, or 4, formation of additional intermediates and/or substantial changes to substrate conformation during the cyclization/rearrangement cascade are predicted to be necessary. Thus, it is likely that the active site of VrtK provides a structural template that chaperones reactive carbocation **A** to **D** via path 1

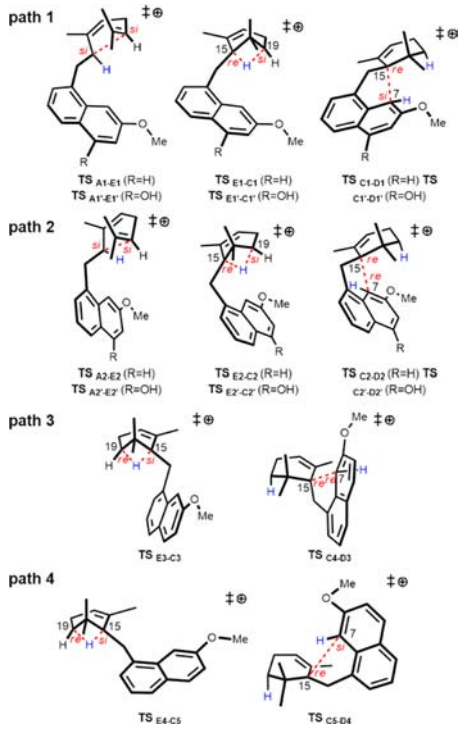

Figure 4. Different conformations/configurations of transition-state structures for reaction steps along the black/green pathway shown in Figure 3.

in a way that allows its inherent reactivity to be expressed while reducing the energy barrier for cyclization further.[15,16]

To our knowledge, this is the first report of a cytochrome P450 enzyme capable of catalyzing terpene cyclization. Interestingly, P450 albaflavenone monooxygenase from *Streptomyces coelicolor* A3 was recently discovered to contain a moonlighting class I terpene synthase active site and can convert farnesyl diphosphate to farnesenes in addition to its original function in oxidizing epi-isozizaene.[18] In contrast to class I terpene cyclases, which generate an carbocation by ionizing the allylic diphosphate ester, class II terpene cyclases promote cyclization by protonating a terminal double bond.[19] VrtK is similar to class II terpene

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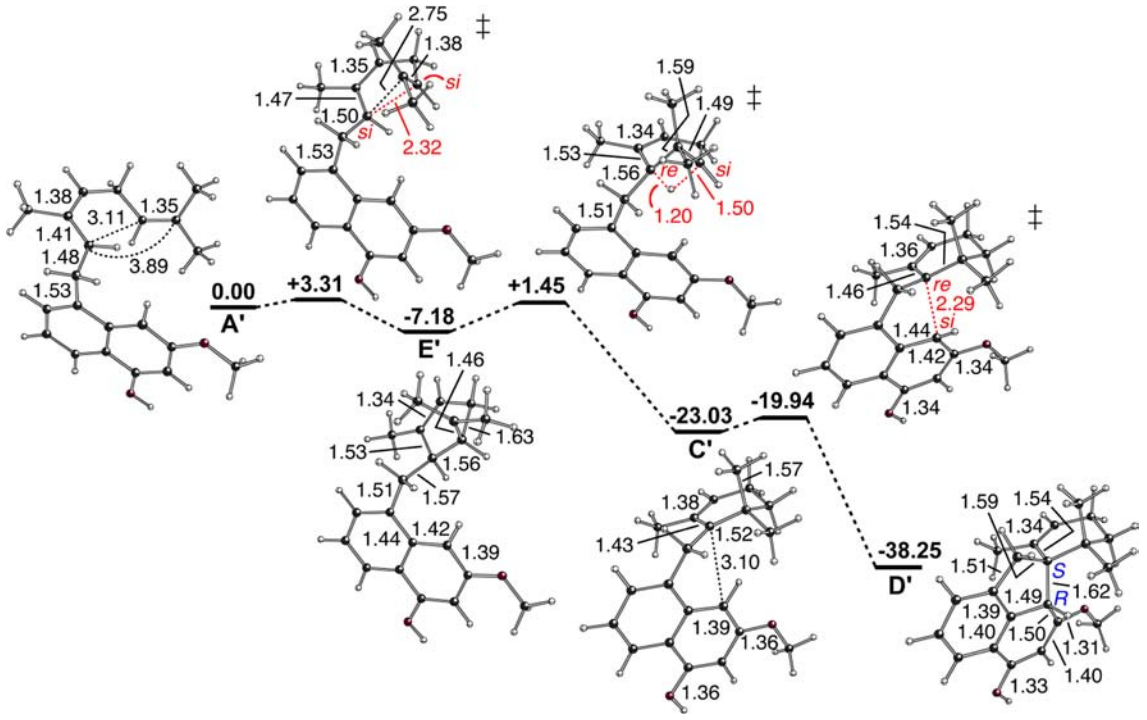

Figure 5. Reaction energy diagram (M06-2X/6-31+G(d,p)//B3LYP/6-31+G(d,p)) for path 1 (Figure 4, R = OH). Relative energies (kcal/mol) and selected distances (Å) are shown.

cyclases in that it does not act on a diphosphate terpene substrate; however, a carbocation is proposed to be generated by oxidation of an allylic carbon (C17), corresponding to C4 of geranyl moiety. This discovery expands our knowledge of the catalytic diversity of cytochrome P450 enzymes and provides new insights into meroterpenoid biosynthesis in fungi.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods, computational details, and NMR spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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