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Elucidation of Pyranonigrin Biosynthetic Pathway Reveals a Mode of Tetramic Acid, Fused γ-Pyrone, and *exo*-Methylene Formation

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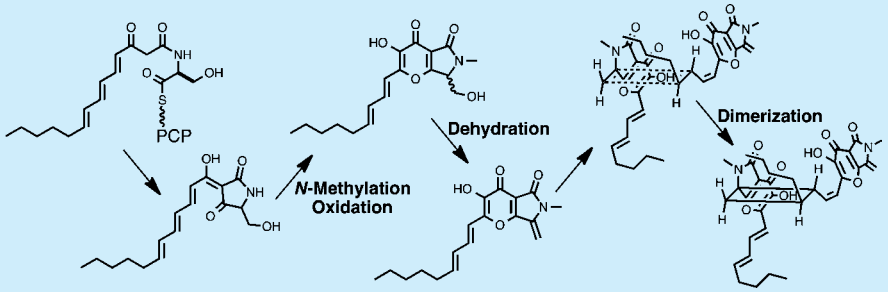
Supporting Information



ABSTRACT: Successful activation of the pyranonigrin biosynthetic gene cluster and gene knockout in *Aspergillus niger* plus in vivo and in vitro assays led to isolation of six new products, including a spiro cyclobutane-containing dimeric compound, which served as the basis for the proposed comprehensive pyranonigrin biosynthetic pathway. Two redox enzymes are key to forming the characteristic fused γ-pyrone core, and a protease homologue performs the *exo*-methylene formation.

Pyranonigrins are a family of antioxidative compounds isolated from *Aspergillus niger*. Previously, activation of the pyranonigrin biosynthetic gene cluster (see Figure S1 and Table S1, Supporting Information) was attempted by expressing the pathway-specific transcriptional regulator *pynR*. This achieved a modest induction of the production of pyranonigrin E[1] (**3**) (not to be confused with pyranonigrin E reported slightly afterward[2]) at a yield of 1.2 mg/L in *A. niger* ATCC 1015. To effect greater activation of the *pyn* gene cluster, we took advantage of the *kusA*- and *pyrG*-deficient *A. niger* A1179 strain that allows efficient targeted chromosome modification.[3] Using this strain, we replaced the original promoter for *pynR* with a strong *glaA* promoter[4] to generate AnKW2 (Figure S2). AnKW2 was able to produce **3** at a yield of 1 g/L. Furthermore, we were also able to isolate a new compound that we named pyranonigrin F (**1**), an unusual spiral cyclobutane-containing dimer of **3** (Figure 1, i). This high-level activation of the gene cluster provided an opportunity to conduct a detailed study for determining the complete pathway for the biosynthesis of pyranonigrins. For instance, the previous study[1] proposed that the γ-pyrone core of pyranonigrins formed spontaneously upon being released from PynA, the polyketide synthase−non-ribosomal peptide synthetase (PKS−NRPS) hybrid enzyme. However, details of the γ-pyrone formation or the formation of the *exo*-methylene group at C-7, which is absent in pyranonigrins A−D,[5] still remained ill defined. We conducted in vivo and in vitro characterization of the PKS−NRPS PynA and modification enzymes, PynB−E plus previously unrecognized PynG, PynH, and PynI, to determine which enzymes are responsible for which biosynthetic steps in the formation of the pyranonigrin family of natural products.

First, deletion of *pynA*, which encodes the PKS−NRPS hybrid enzyme (Table S1), or *pynI*, a predicted thioesterase (Table S1), abolished production of all pyranonigrins (Figure 1 i vs ii and viii). Although PynA has a terminal reductase domain, it appears to require PynI for the release of the straight-chain intermediate from PynA via the formation of a tetramic acid. Thioesterase-mediated tetramic acid formation and chain release have been observed before, but no report of a stand-alone thioesterase being responsible for tetramic acid formation and chain release from PKS−NRPS in a fungal biosynthetic system has been made before (Scheme 1).[6]

Next, deletion of *pynG* (Figure 1, i vs iii) and *pynD* (Figure 1, i vs iv) led to accumulation of intermediates devoid of the γ-pyrone ring **7** (Table S14 and Figures S35−S38) and **8** (Table S15 and Figures S39−S42), respectively, suggesting the involvement of PynG and PynD in the γ-pyrone formation. To examine in detail the biochemical functions of PynG and PynD, we cloned *pynG* and *pynD* from *A. niger* A1179.

However, only *pynG* expressed adequately in *Escherichia coli* (see the SI, Table S7 and Figures S11, S16, S17). In vitro assay of PynG was performed with **7** that was obtained from the culture of Δ*pynG*/AnKW2 strain (see the SI for details). The recombinant PynG-containing fraction from nickel-affinity chromatography (SI, Figures S16 and S17) was able to catalyze epoxidation of **7** to form **6** and **4** as confirmed by HRESIMS

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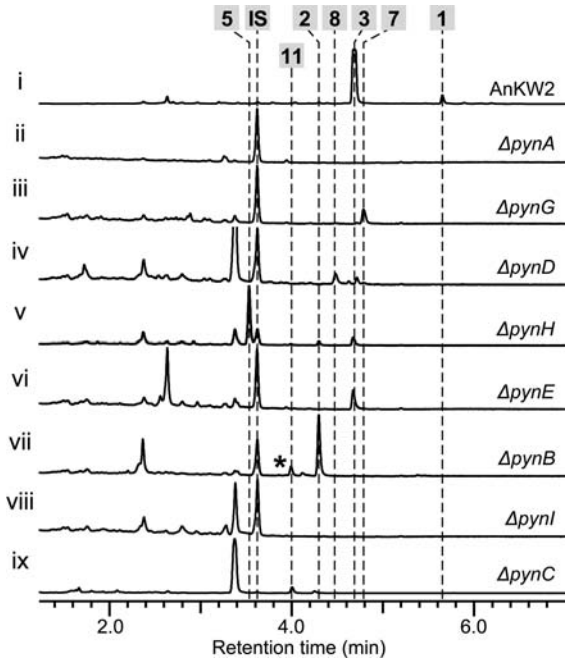
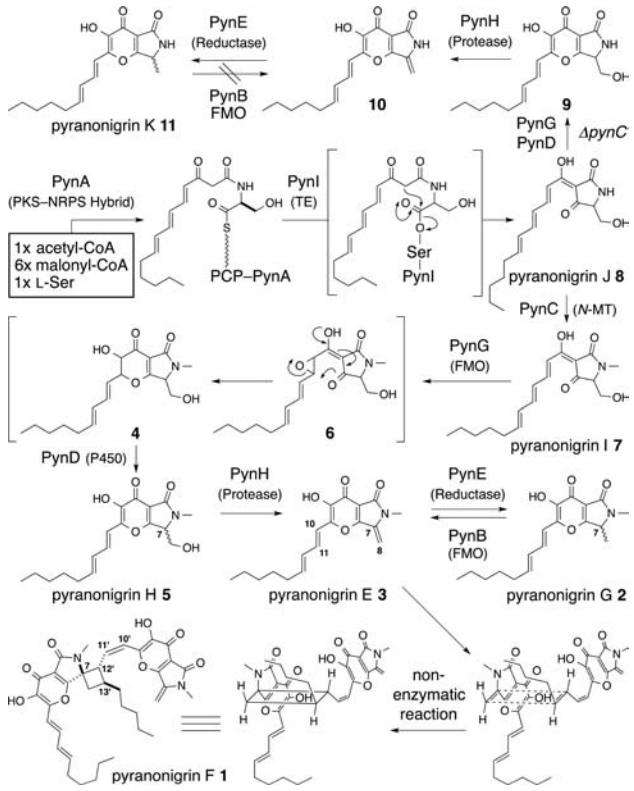


Figure 1. HPLC traces of metabolic extracts from the cultures of various AnKW2-based deletion strains prepared to identify the genes involved in different steps of the pyranonigrin biosynthesis. See the Supporting Information for details. All HPLC traces were monitored at 280 nm. *N*-Boc-L-tryptophan methyl ester was used as an internal standard (IS) throughout the study. Extract of the culture of (i) AnKW2 harboring pKW20088[7] for the expression of *pyrG* as a wild-type control, (ii) Δ*pynA* strain, (iii) Δ*pynG* strain, (iv) Δ*pynD* strain, (v) Δ*pynH* strain, (vi) Δ*pynE* strain, (vii) Δ*pynB* strain, (viii) Δ*pynI* strain, and (ix) Δ*pynC* strain. Note: peaks that elute earlier than 3.5 min in all panels and the small peak denoted by * that elutes at the same time as **11** in panel vii are all unrelated compounds.

Scheme 1. Biosynthetic Pathway Involving Methylation, Dehydration, and Multiple Oxidation Steps for the Formation of Pyranonigrins from a Polyketide−Peptide Hybrid Straight Chain[a]



[a]Key: TE, thioesterase; *N*-MT, *N*-methyltransferase; FMO, FAD-containing monooxygenase; P450, cytochrome P450.

analysis (Figure 2, i vs ii). Bioconversion of **7** with *the pynG*-expressing *Saccharomyces cerevisiae* SCKW12, which was derived from *S. cerevisiae* BY4705,[8] provided a clearer observation for the formation of **6** and **4** (Figure 2, iii vs iv, and Figure S15). However, those compounds proved to be too unstable for more detailed characterizations. To obtain a stable conversion product of **7**, we attempted coexpression of *pynG* and *pynD* in SCKW12 for bioconversion of **7** (see the SI for details). This in vivo assay resulted in the formation of **5** (Figure 2, iii vs v), which contained the complete γ-pyrone moiety as fully characterized by high-resolution electrospray ionization LC−MS (HRESIMS) and 1D and 2D NMR (Table S13 and Figures S31−S34). As expected from the lack of an epimerase domain in the NRPS portion of PynA, **7** and **8** retained the L-configuration at C-7 as indicated by the rotation data (Tables S14 and S15). Surprisingly, analysis of the rotation data for **5** revealed that it was a racemic mixture. Related compounds **2** and **11** (discussed below) were also racemic mixtures. This can be explained by the previous observation[9] that the acidic nature of H-7 of those compounds allowed epimerization at these positions under mildly basic conditions. The enzymes that act on those racemic compounds, especially FMO (PynB) and P450 (PynD), may have relaxed substrate specificity to tolerate the stereochemical flexibility at the C-7 position, especially since the position carries a relatively small substituent, namely a methyl group in **2** and **11** and a hydroxymethyl group in **5**. Amino acid sequence analysis assigned PynG and PynD to be an FAD-containing monooxygenase (FMO) and a cytochrome P450, respectively (Table S1 and Figure S1). Based on the observation outlined above, we propose a mechanism in which PynG catalyzes an epoxidation-mediated cyclization[10] to form the dihydro-γ-pyrone moiety in **4** (Scheme 1, bracketed steps), followed by PynD-catalyzed oxidation of the alcohol to the ketone and enolization to yield the characteristic tetramic acid-fused γ-pyrone core of pyranonigrins (Scheme 1).

Deletion of *pynH* resulted in an accumulation of **5** (Figure 1, i vs v). Thus, we attempted in vivo bioconversion experiments using **5** as a fed substrate to determine the exact function of this predicted aspartyl protease (Table S1). For this study, we generated a strain derived from *A. nidulans* A1145[11] that expresses *pynH* from an expression plasmid (see the SI for details). Those in vivo assays revealed that **3** was formed from **5** in the presence of PynH (Figure 3, i vs ii, Table S12, and Figure S30). These results suggest that dehydration of a serine side chain can give rise to the *exo*-methylene structure. To confirm whether L-serine is incorporated into **3** by PynA, we fed [1-^{13}C]-L-serine to AnKW2 in an in vivo incorporation assay. Analysis of the isolated product **3** from the culture by HRESIMS showed an increase of one atomic mass unit, indicating that L-serine is incorporated into **3** as an extender unit (Figure S20). Recently, a dehydratase NisB has been shown to catalyze dehydration of a serine side chain to form an *exo*-methylene group in the ribosomally synthesized nisin.[12] However, a similar mechanism applied toward tailoring a

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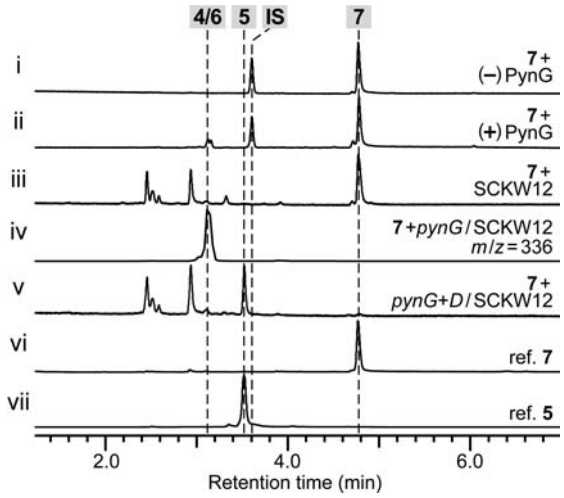

Figure 2. In vitro analysis of the activity of PynG against **7** and yeast in vivo characterization of PynG and PynD for the formation of **5**. See the SI for details. The HPLC traces were monitored at 280 nm (i and ii) and 350 nm (iii, v–vii). (i) Negative control with **7** and heat-inactivated PynG (denoted by a negative sign). (ii) In vitro reaction with **7** and PynG. (iii) Negative control for yeast bioconversion of **7**. SCKW12 harboring the expression vectors without *pynG* and *pynD* (pKW1250 and pKW5050, respectively[7]) were used. (iv) In vivo yeast conversion of **7** to **4**/**6** by PynG. Trace corresponding to the *m*/*z* of **4** and **6** (*m*/*z* = 336) extracted from (iv) is shown. (v) In vivo conversion of **7** to **5** by SCKW12 expressing *pynG* and *pynD*. The authentic reference of (vi) **7** and (vii) **5** are also shown.

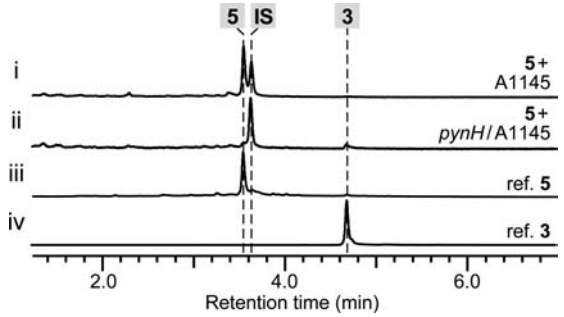

Figure 3. In vivo conversion of **5** to **3** by the protease PynH in *A. nidulans* A1145. See the SI for details. All traces were monitored at 280 nm. HPLC profiles of the culture of (i) the wild type strain with **5** as a negative control and (ii) *A. nidulans* A1145 carrying the *pynH* expression vector pKW10007 with **5**. The authentic references of (iii) **5** and (iv) **3** are also shown.

nonribosomal peptide-containing compound as seen here by PynH has not been reported previously.

Deletion of *pynC* led to a small accumulation of **8** and the formation of a desmethylated product **11** (Figure 1, i vs ix, Tables S15–S16 and Figures S39–S46). Furthermore, the production of desmethylated forms of **9** and **10** was also confirmed by HRESIMS (Figure S19). To address the timing of *N*-methylation in the biosynthetic pathway, we performed an in vitro assay (Figure S18) using purified *E. coli*-produced PynC (Figure S16). This analysis showed that PynC performs *N*-methylation of **8**, but not **11**, suggesting that γ-pyrone-containing late intermediates like **9**, **10**, and **11** are not recognized by PynC.

Lastly, deletion of *pynB* caused an accumulation of **2** (Figure 1, i vs vii), while deletion of *pynE* led to a reduced accumulation of **3** (Figure 1, i vs vi). PynE, a predicted reductase having an NADPH binding domain (Table S1), was produced in *E. coli* successfully and purified to homogeneity (SI, Table S7 and Figures S10 and S16). In vitro reaction with the recombinant PynE showed that PynE reduced the *exo*-methylene of **3** into a pendant methyl to form **2** (Figure 4). Interestingly, we also

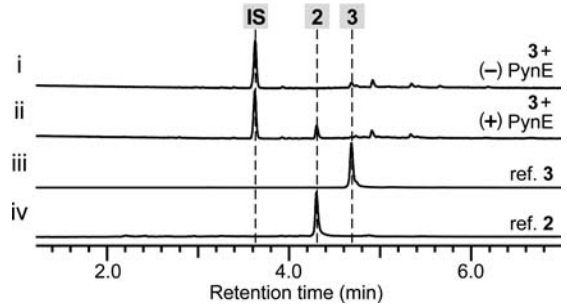

Figure 4. In vitro analysis of the activity of the reductase PynE against **3** for the formation of **2**. See the SI for details. All of the HPLC traces were monitored at 280 nm. (i) Negative control with **3** and heat-inactivated PynE. (ii) In vitro reaction with **3** and PynE for the formation of **2**. The authentic references of (iii) **3** and (iv) **2** are also shown.

observed a complete depletion of **2** and the formation of **3** in an in vivo assay using PynB, an FMO, produced in SCKW12 (Figure 5, i vs ii and iii). While a clear peak was not observed

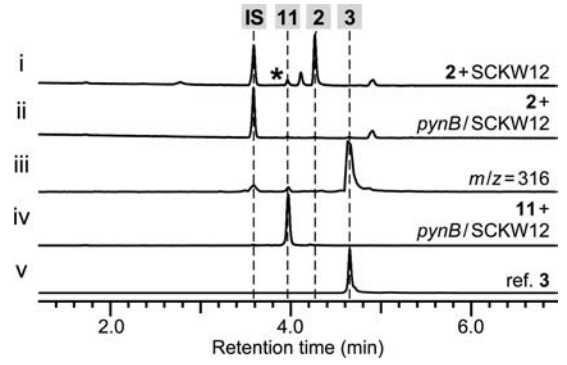

Figure 5. In vivo characterization of the monooxygenase PynB. See the SI for details. All of the HPLC traces except (iii) were monitored at 280 nm. (i) Negative control with **2** and SCKW12 without *pynB*. What appears to be a small peak for **11** (denoted by *) is an unrelated compound. (ii) SCKW12 harboring the *pynB* expression vector pKW20221 with **2** for the formation of **3**. (iii) Trace corresponding to the *m*/*z* of **3** (*m*/*z* = 316) extracted from (ii). (iv) SCKW12 harboring pKW20221 with **11**, showing the lack of formation of **10**. (v) The authentic reference of **3** is also shown.

for **3** in the in vivo assay (Figure 5, ii) due to its instability, its presence was confirmed by LC–MS (Figure 5, iii). However, **11** was not transformed into **10** in the same reaction (Figure 5, iv), indicating the requirement of the *N*-methyl group in this PynB-catalyzed oxidation reaction (Scheme1 and Figure 5).

A dimeric pyranonigrin **1** was isolated in this study (Table S10 and Figures S21–S25), and we elucidated the relative configuration of its cyclobutane moiety to be 7*R**,12′*S**,13′*S** (Scheme 1). Rotation data of **1** (Table S10) showed that it is a racemic mixture containing an enantiomer having 7*S**,12′*R**,13′*R** stereochemistry. The chemical structure of **1** suggested that **1** is a dimer of **3**, and we were able to observe that **3** dimerized via a nonenzymatic intermolecular [2 + 2]

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cycloaddition reaction to form **1** over time. The low production level of **3** is likely the reason **1** was not isolated before. We also observed an increasing accumulation of compounds having the same m/z as **1** in AnKW2 (Figure S47). The peaks might be composed of diastereomers of **1** and geometric isomers originating from epimerization of the 10,11 olefin. We propose that an intermolecular [2 + 2] cycloaddition between the *exo*-methylene of **3** and 10,11 olefin of another molecule of **3** leads to the formation of **1** (Scheme 1).

In this study, we combined targeted gene deletion, heterologous in vivo bioconversion, and in vitro assays to identify the main pathway for the formation of pyranonigrins. Through the study, we identified six of the intermediates formed and the enzymes responsible for the methylation and oxidation steps leading to the conversion of a polyketide−peptide hybrid intermediate into γ-pyrone-containing natural products. The most notable was the discovery of PynH, a predicted protease that performs dehydration of a serine side chain to form an *exo*-methylene in **3**. A similar reaction is catalyzed by NisB during the biosynthesis of ribosomal peptide-derived lantibiotics,[12] but it is highly unusual to find an *exo*-methylene-forming enzyme that is involved in the modification of a fungal polyketide−nonribosomal peptide hybrid compound. The significant difference in the amino acid sequence between PynH and NisB suggests that there is a significant mechanistic difference in how those two enzymes achieve dehydration of an amino acid side chain. Through *glaA* promoter-based targeted activation of a transcription regulator, comprehensive gene knockout, and detailed in vivo/in vitro analyses, we were able to draw a complete picture of the complex pathways for the biosynthesis of the pyranonigrin family compounds. Those studies allowed us to identify unique enzymes, such as the protease-like protein PynH that performs an *exo*-methylene formation and the stand-alone thioesterase PynI that is capable of concomitant tetramic acid formation and chain release from the PKS−NRPS hybrid enzyme PynA. We also identified the formation of **1**, a dimerization product of **3** derived via a nonenzymatic [2 + 2] cycloaddition reaction. Findings from this study reiterate the notion that fungal natural product biosynthesis relies heavily on redox enzymes[13] and employs enzymatic processes found elsewhere, such as the dehydration-mediated *exo*-methylene formation described in this report, to generate complex chemical structures that can give rise to unique bioactivities. Such knowledge will be valuable in further characterization and engineering of natural product biosynthetic systems for bioproduction of novel compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02435.

Data from the NMR and MS determination of the compounds and additional experimental information (PDF)

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Notes

The authors declare no competing financial interest.

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