1 **Characterization of the Product of a Nonribosomal Peptide Synthetase-like**
2 **(NRPS-like) Gene Using the Doxycycline Dependent Tet-ON System in**
3 *Aspergillus terreus*

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5 Wei-Wen Sun^{a,†}, Chun-Jun Guo^{a,†}, Clay C. C. Wang^{a,b,*}

6 ^a Department of Pharmacology and Pharmaceutical Sciences, University of Southern
7 California, School of Pharmacy, 1985 Zonal Avenue, Los Angeles, California 90089,
8 USA.

9 ^b Department of Chemistry, University of Southern California, College of Letters,
10 Arts, and Sciences, Los Angeles, California 90089, USA.

11

12 * Correspondence should be addressed to C.C.C.W. (clayw@usc.edu)

13 [†] These authors contributed equally to this project.

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ABSTRACT

Genome sequencing of the fungus *Aspergillus terreus* uncovered a number of silent core structural biosynthetic genes encoding enzymes presumed to be involved in the production of cryptic secondary metabolites. There are five nonribosomal peptide synthetase (NRPS)-like genes with the predicted A-T-TE domain architecture within the *A. terreus* genome. Among the five genes, only the product of *pgnA* remains unknown. The Tet-on system is an inducible, tunable and metabolism-independent expression system originally developed for *Aspergillus niger*. Here we report the adoption of the Tet-on system as an effective gene activation tool in *A. terreus*. Application of this system in *A. terreus* allowed us to uncover the product of the cryptic NRPS-like gene, *pgnA*. Furthermore expression of *pgnA* in the heterologous *A. nidulans* host suggested that the *pgnA* gene alone is necessary for phenguignardic acid (**1**) biosynthesis.

Keywords: Tet-on system; *Aspergillus terreus*; Nonribosomal peptide synthetase-like; Natural Products; phenguignardic acid; Biosynthesis

32 INTRODUCTION

33 Filamentous fungi are important sources of secondary metabolites (SMs) that
34 have potential for pharmaceutical and agricultural purposes. Recent whole
35 genome-sequencing projects have revealed that the number of core structural
36 biosynthetic genes for putative SMs far exceeds the number of isolated natural
37 products, indicating that most fungal SM biosynthetic pathways are silent under
38 normal laboratory culture conditions (Chiang et al., 2009a). Several approaches, such
39 as overexpressing cluster-specific regulatory activators (Bergmann et al., 2007;
40 Chiang et al., 2009b), manipulation of the global regulator *laeA* expression (Bok et al.,
41 2006), and epigenetic regulation (Shwab et al., 2007), have been developed to activate
42 these silent biosynthetic pathways using primarily the model organism *Aspergillus*
43 *nidulans*. Many of these approaches have hinged on the use of the inducible *alcA*
44 promoter system that encodes the alcohol dehydrogenase I (ADHI) gene which is
45 positively regulated by the *alcR* gene (Ahuja et al., 2012; Bergmann et al., 2007; Bok
46 et al., 2009; Chiang et al., 2010; Chiang et al., 2009b; Waring et al., 1989; Yeh et al.,
47 2012).

48 Among the filamentous fungi, *Aspergillus terreus*, the main producer of
49 lovastatin, represents a potential reservoir of cryptic SMs. Several labs, including our
50 own, have been interested in mining the *A. terreus* genome for novel SMs (Awakawa
51 et al., 2009; Boruta and Bizukojc, 2014; Chiang et al., 2013; Gressler et al., 2011;
52 Guo et al., 2015; Guo et al., 2012; Guo et al., 2013a; Guo et al., 2014; Guo et al.,
53 2013b; Qiao et al., 2011; Wang et al., 2014; Xu et al., 2013; Zaehle et al., 2014).
54 Several attempts using the constitutive *gpdA* promoter (Gressler et al., 2011) and/or
55 the inducible *alcA* promoter to drive gene expression, have been evaluated in the
56 effort to turn on silent biosynthetic pathways present in *A. terreus*. However, these

57 attempts have not succeeded as envisioned, either because *gpdA* activation is
58 insufficient to yield a significant amount of the product although the target genes were
59 overexpressed (Gressler et al., 2011), or due to the absence of the ethanol catabolism
60 regulatory gene *alcR* in *A. terreus* as predicted by bioinformatic analyses (Flippi et
61 al., 2009).

62 The Tet-on system is an inducible, tunable and metabolism-independent
63 expression system functional in filamentous fungi of the genus *Aspergillus*, including
64 *A. niger*, *A. fumigatus* and *A. nidulans* (Dümig and Krappmann, 2015; Kalb et al.,
65 2015; Kwon et al., 2014; Macheleidt et al., 2015; Meyer et al., 2011; Ouedraogo et
66 al., 2011; Richter et al., 2014; Samantaray et al., 2013; Wartenberg et al., 2012). It
67 was established by constitutively expressing a doxycycline-dependent transcriptional
68 activator rtTA under the *gpdA* promoter and fusing the target gene behind a minimal
69 promoter sequence (Pmin) preceded by several operator (*tetO7*) sites that rtTA would
70 bind to (Meyer et al., 2011) (Fig. 1). We were interested in adopting and employing
71 the Tet-on system in *A. terreus* as an important tool to mine its secondary
72 metabolome. There are five nonribosomal peptide synthetase (NRPS)-like genes with
73 predicted A-T-TE domain architecture within *A. terreus* genome, including *pgnA*,
74 *atmela*, *atqA*, *apvA* and *btyA* (Guo and Wang, 2014). The difference between NRPS
75 and NRPS-like genes is shown in Figure S3A. Among the five genes, only the product
76 of *pgnA* is unknown at the start of this study, while the other four genes have been
77 linked to their secondary metabolites (Guo et al., 2015; Guo et al., 2013a). Here we
78 report our efforts on activating the silent NRPS-like gene ATEG_08899.1 (*pgnA*) via
79 the Tet-on system. Application of this system to activate *pgnA* allowed us to discover
80 its product, phenguignardic acid (**1**, Fig. 1), a phytotoxic secondary metabolite
81 produced by *Guignardia bidwellii* the causal agent of black rot in grapes (Molitor et

al., 2012). Additional heterologous expression of *pgnA* in *A. nidulans* confirmed that the single gene *pgnA* is necessary to produce phenguignardic acid.

MATERIALS AND METHODS

Strains and molecular genetic manipulations.

The plasmid pVG4.1 containing the Tet-on system was kindly provided by Dr. Vera Meyer. The Tet-on system (*Ptet-on*) was amplified from pVG4.1 by PCR with primers F4 and R5 (Meyer et al., 2011). Replacement of endogenous promoters with *Ptet-on* was carried out as shown in Figure S1, Supporting Information. Primers used in this study are listed in Table S1. The fungal strains used in this study are listed in Table S2. The scheme for heterologous expression of the *pgnA* gene of *A. terreus* in *A. nidulans* strain LO4389 is shown in Figure S1. The construction of fusion PCR products, protoplast generation, and transformation were carried out as previously described (Chiang et al., 2013; Guo et al., 2013a). The scheme of diagnostic PCR is shown in Figure S2A, and all transformants were verified by diagnostic PCR.

Fermentation and LC-MS analysis.

A. terreus NIH2624 wild type strain and *Ptet-on_pgnA* mutant strains were cultivated at 37 °C in 25 ml LCMM liquid medium (6 g/l NaNO₃, 0.52 g/l KCl, 0.52 g/l MgSO₄·7H₂O, 1.52 g/l KH₂PO₄, 20 g/l lactose, 10 g/l D-glucose supplemented with 1 ml/l of trace element solution (EDTA disodium salt 50 g/l, ZnSO₄ · 7H₂O 22 g/l, H₃BO₃ 11.4 g/l, MnCl₂ · 4H₂O 5.06 g/l, CoCl₂·6H₂O 1.61 g/l, CuSO₄·5H₂O 1.57 g/l, (NH₄)₆Mo₇O₂₄·4H₂O 1.1 g/l, FeSO₄·7H₂O 4.99 g/l)) at 1 × 10⁶ spore/ml per 125

ml flask with shaking at 180 rpm. After 18 h of incubation, 25 μ l sterile doxycycline (50 μ g/ μ l) was added into the culture of the *Ptet-on_pgnA* mutant strains, making the final concentration of doxycycline at 50 μ g/ml. The culture of *A. terreus* NIH2624 wild type strain and *Ptet-on_pgnA* mutant strains without adding doxycycline were used as controls. Then the incubator temperature was lowered to 30 °C and the culture medium was collected after 72 h. The medium was filtered and extracted with equal volume of ethyl acetate (EtOAc). Then the pH of the medium was adjusted to around 2 by addition of 6M HCl and extracted with equal volume of EtOAc again. The EtOAc extracts were combined and evaporated *in vacuo*, and dissolved in 1 ml 20% DMSO in MeOH and a portion (10 μ l) was examined by high performance liquid chromatography-photodiode array detection-mass spectrometry (HPLC-DAD-MS) analysis. The condition for the MS analysis was carried out as previously described (Guo et al., 2013a).

A. nidulans strain LO4389 and the *pgnA* heterologous expression strains were cultivated at 37 °C in 50 ml LMM liquid medium (6 g/l NaNO₃, 0.52 g/l KCl, 0.52 g/l MgSO₄·7H₂O, 1.52 g/l KH₂PO₄, 20 g/l lactose supplemented with 1 ml/l of trace element solution) at 1×10^6 spore/ml per 125 ml flask with shaking at 180 rpm. The nutrients pyridoxine and riboflavin were supplemented. After 18 h of incubation, 44 μ l cyclopentanone was added into the medium to make final concentration at 10 mM for *alcA* induction as previously reported (Chiang et al., 2013). Then the incubator temperature was lowered to 30 °C and the culture medium was collected 72 h after cyclopentanone induction. The medium was filtrated and extracted with equal volume of EtOAc. Preparation of the HPLC-MS samples and the condition for MS analysis were as previously described (Guo et al., 2013a).

130 **Isolation of secondary metabolites.**

131 For structure elucidation, the *Ptet-on_pgnA* mutant strain was cultivated at 37
132 °C in a total of 1.5 liter LCMM liquid medium (50 ml per 125 ml flask) at 1×10^6
133 spore/ml with shaking at 180 rpm. To induce expression of the *pgnA* gene, sterile
134 doxycycline at a final concentration of 50 µg/µl was added into each flask after 18 h
135 of incubation. Then the incubator temperature was lowered to 30 °C and the culture
136 medium was collected after 72 h induction. The medium was filtered and extracted
137 with equal volume of ethyl acetate (EtOAc). Then the pH of the medium was adjusted
138 to around 2 by addition of 6M HCl and extracted with equal volume of EtOAc again.
139 The EtOAc layers were combined and evaporated to a crude extract. Further
140 purification was carried on by gradient HPLC on a C18 reverse phase column
141 [Phenomenex Luna 5µm C18, 250 × 10 mm] with a flow rate of 5.0 ml/min and
142 measured by a UV detector at 304 nm. The gradient system was MeCN (solvent B)
143 and 5% MeCN/H₂O (solvent A) both containing 0.05% TFA. The gradient condition
144 for HPLC analysis of crude extract from the *Ptet-on_pgnA* mutant strain was 0-5 min
145 100-80% A, 5-35 min 80-40% A, 35-38 min 40-0% A, 38-40 min 0-0% A, 40-42 min
146 0-100% A. Compound **1** (2.1 mg/L) was eluted at about 32.5 min.

147

148 **Compound identification.**

149 NMR spectra were collected on a Varian Mercury Plus 400 spectrometer. High
150 resolution electrospray ionization mass spectrum (HRESIMS) of compound **1** was
151 obtained on Agilent 6210 time of flight LC-MS. Optical rotation was measured with a
152 Jasco P-2000 polarimeter at a wavelength of 589 nm.

The compound phenguignardic acid (**1**) was isolated as an amorphous colorless solid. The molecular formula was established to be C₁₈H₁₄O₅ by its ¹³C NMR, DEPT and HRESIMS spectral data (HRESIMS, *m/z*=309.0757, expected 309.0763 [M-H]⁻). For UV-Vis and ESIMS spectra, see Figure 3; For NMR data and spectra (chloroform-*d*), see Table 1 and Figure S5.

RESULTS

Activation of the *pgnA* gene using an inducible *Ptet-on* stimulates the production of phenguignardic acid.

Previous data have shown *A. terreus* lacks the *alcR* gene, therefore an alternative to the *alcA* induction system needed to be developed (Flipphi et al., 2009). For this study, we adopted the Tet-on system developed for *A. niger* to *A. terreus* for promoter replacement. Our analysis identified five NRPS-like genes with A-T-TE domain architecture in *A. terreus*. Since the products of the other four NRPS-like genes in *A. terreus* were known, we focused on replacing the promoter of the *pgnA* gene (ATEG_08899.1), a gene whose product was still unknown. We replaced the native promoter of *pgnA* with the *Ptet-on* promoter cloned from an *A. niger* plasmid (Meyer et al., 2011). The replacement was accomplished by using a *kusA*-, *pyrG*-mutant strain of *A. terreus* and fusion PCR. All strains were verified by diagnostic PCR (Fig. S2). We cultivated two separate promoter exchange strains and a control strain in LCMM media and used doxycycline (Dox) as an inducer. Metabolite profiles from each strain were analyzed by LC-DAD-MS, and the *Ptet-on_pgnA* strains with Dox induction were able to produce a new metabolite with *m/z* of 309 [M-H]⁻ (compound **1**, Fig. 2A and Fig. 3). The compound was isolated from scaled-up

cultivation of the *Ptet-on_pgnA* strain by semi-preparative HPLC purification. The molecular formula was established to be C₁₈H₁₄O₅ by its ¹³C NMR, DEPT and HRESIMS spectral data, and by comparison to the published NMR spectra of phenguignardic acid (Molitor et al., 2012). The compound also exhibited an optical rotation of $[\alpha]_D^{21} = -53$ (c = 0.4, CH₂Cl₂), indicating it is (-)-phenguignardic acid ((R)-**1**, Fig. 1) (Stoye et al., 2013).

Heterologous expression of *pgnA* in the heterologous host *A. nidulans* LO4389 demonstrates that only one gene is necessary for biosynthesis of compound **1.**

Our data so far suggested that turning on the expression of a single gene *pgnA* in *A. terreus* was necessary to elicit the compound **1** production. To confirm that indeed overexpression of only one single gene *pgnA* without any other accessory genes in *A. terreus* is sufficient for compound **1** biosynthesis, we expressed *pgnA* in a heterologous host, *A. nidulans* LO4389 (Ahuja et al., 2012). We have deleted the biosynthesis clusters of the major UV active metabolites sterigmatocystin from the selected strain of *A. nidulans*. The *pgnA* gene was fused with the *alcA* promoter by fusion PCR and transformed into *A. nidulans* using our standard procedure (Fig. S1, Chiang et al., 2013). We cultivated the *OE:pgnA A. nidulans* strain in lactose minimal media (LMM) and as a control we also cultivated the parental *A. nidulans* strain. We induced the *alcA* promoter using 50 µg/ml cyclopentanone as inducer. Using HPLC-DAD-MS we detected compound **1** in the *OE:pgnA A. nidulans* and not the *A. nidulans* parental control strain demonstrating that only *pgnA* is necessary for compound **1** biosynthesis (Fig. 2B).

201 DISCUSSION

202 The *A. terreus* genome contains fourteen NRPS-like genes and the SM products
203 for the majority of them are unknown (Khaldi et al., 2010). Five among the fourteen
204 NRPS-like genes have the predicted A-T-TE domain architectures, missing the
205 condensation (C) domain compared to typical NRPSs (Fig. S3) (Fischbach and
206 Walsh, 2006; Guo and Wang, 2014). The adenylation (A) domain recognizes and
207 activates the aryl acid substrates, and loads them onto the thiolation (T) domain. The
208 thioesterase (TE) domain shares the missing condensation (C) domain function, and is
209 responsible for condensation and final product release (Balibar et al., 2007). The
210 alignment of PgnA amino acid sequence with other NRPS-like proteins is shown in
211 Figure S4. BLASTP analysis of the protein sequence of *pgnA* shows 56% similarity
212 with TdiA, a characterized NRPS-like protein involved in terrequinone biosynthesis.
213 In the biosynthesis of the terrequinone core, the A domain of TdiA is shown to
214 activate and load indole-pyruvic acid as its substrate (Balibar et al., 2007). Based on
215 the function of TdiA, we propose a speculative biosynthetic pathway for **1** in Figure 4.
216 It involves the activation of phenylpyruvic acid (PPA), a precursor available in both
217 *A. terreus* and *A. nidulans*, by the PgnA A domain to AMP-PPA followed by loading
218 the PPA unit to the T domain and eventually transferring to the TE domain. Another
219 PPA unit is loaded onto the T domain. The TE domain likely promotes the enolate
220 formation on the attached unit, followed by a nucleophilic attack on the carbonyl to
221 yield an ether linkage between the two units. Then the TE domain likely catalyzes a
222 similar reaction to give the cyclized dioxolanone core and releases compound **1** (Fig.
223 4).

224 With the elucidation of the product of *pgnA* by utilizing the Tet-on system,
225 product characterization of all five NRPS-like genes with A-T-TE domain

architecture in *A. terreus* has been completed. Both activation of the single *pgnA* gene in native *A. terreus* and the heterologous expression in *A. nidulans* led to the identical product, indicating that indeed the single *pgnA* gene is necessary for compound **1** production. The identification and structural characterization of phenguignardic acid (**1**) has demonstrated the effectiveness of the Tet-on system for activating silent genes to discover natural products in *A. terreus*. Once the genome information is accessible for *Guignardia bidwellii*, the sequence of PgnA could facilitate the mining of the gene responsible for phenguignardic acid biosynthesis in *Guignardia bidwellii*.

In conclusion, we report here the adoption of the Tet-on system as an effective gene activation tool in *A. terreus*. Application of the Tet-on system in *A. terreus* allowed us to discover the product of the NRPS-like gene, *pgnA*. With the heterologous expression of *pgnA* in *A. nidulans*, we have demonstrated that *pgnA* gene alone is necessary for phenguignardic acid (**1**) biosynthesis in *A. terreus*.

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347

348

FIGURE LEGENDS

Table 1. NMR data for compound **1** (400 and 100 MHz in CDCl₃). The left two lanes are experimental data, and the right two lanes are published data.

Figure 1. Schematic overview of the doxycycline-regulated gene expression system (*Ptet-on*, gray part) used in this study. The constitutive promoter *PgpdA* drives the expression of a doxycycline (Dox)-dependent transcriptional activator rtTA (*TcgrA* as a terminator). In the absence of Dox, rtTA does not bind to the rtTA dependent *tetO7*-Pmin promoter, so there is no transcription activation of the target gene. In contrast, in the presence of Dox, rtTA binds to *tetO7*-Pmin and initiates the transcription of the target gene. *A. fumigatus pyrG* is used as a selection marker for transformation.

Figure 2. (A) HPLC profiles of extracts from *A. terreus* wild type, *Ptet-on_pgnA* mutant with and without doxycycline induction as detected by UV (200-600 nm). The maximum uAU value for compound **1** peak is around 110000. (B) HPLC profiles of extracts from *A. nidulans* LO4389 (control), and *pgnA* heterologous expression strain (*alcA_pgnA*).

Figure 3. UV-Vis and ESIMS spectra of compound **1**.

Figure 4. Proposed biosynthetic pathway for compound **1**.

373 **FIGURES AND TABLES**

374 **Table 1.**

Position	δH (J in Hz)	δC	δH (J in Hz)	δC
2		106.1, C		105.9, C
4		135.7, C		135.5, C
5		163.1, C		162.9, C
6		168.6, C		168.7, C
7	3.51, d (16.0) 3.44, d (16.0)	40.9, CH ₂	3.51, d (14.7) 3.44, d (14.7)	41.0, CH ₂
8	6.22, s	109.3, CH	6.20, s	109.7, CH
1'		132.4, C		132.3, C
2'/6'	7.60, d (8.0)	130.0, C	7.59, m	130.0, CH
3'/5'	7.34, t (8.0)	128.8, CH	7.34, m	128.9, CH
4'	7.31, m	129.1 CH	7.31, m	129.2 CH
1''		131.0, CH		130.7, CH
2''/6''	7.22, m	131.2, CH	7.22, m	131.2, CH
3''/5''	7.16, m	128.5, CH	7.16, m	128.6, CH
4''	7.16, m	127.8, CH	7.16, m	127.9, CH

375

376 **Figure 1.**

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379 **Figure 2.**