Genomics-driven Discovery of Phytotoxic Cytochalasans involved in the Virulence of the Wheat Pathogen *Parastagonospora nodorum*

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ABSTRACT

The aetiology of fungal pathogenesis of grains is critical to global food security. The large number of orphan biosynthetic gene clusters uncovered in fungal plant pathogen genome sequencing projects suggests that we have a significant knowledge gap about the secondary metabolite repertoires of these pathogens and their roles in plant pathogenesis. Cytochalasans are a family of natural products of significant interest due to their ability to bind to actin and interfere with cellular processes that involved actin polymerization, however, our understanding of their biosynthesis and biological roles remains incomplete. Here, we identified a putative polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) gene cluster (*phm*) that was upregulated in the pathogen *Parastagonospora nodorum* during its infection on wheat. Activation of the *phm* gene cluster by overexpression of the transcription factor *phmR* resulted in the production of two leucine-derived cytochalasans, phomacins D-E (**1-2**), and an acetonyl adduct phomacin F (**3**). Heterologous expression of the PKS-NRPS gene *phmA* and the *trans*-enoyl reductase (ER) gene *phmE* in *Aspergillus nidulans* resulted in the production of a novel 2-pyrrolidone precursor prephomacin (**4**). Reverse genetics and wheat seedling infection assays showed that Δ *phmA* mutants exhibited significantly reduced virulence compared to the wild type. We further demonstrated that both **1** and **2** showed potent actin polymerization inhibitory activities and exhibited potentially monocot-specific antigerminative activities. The findings from this study have advanced our knowledge base on the biosynthesis and biological roles of cytochalasans, the latter of which could have significant implications for our understanding of the molecular mechanisms of fungal-plant interactions.

Fungi, being a major source of natural products for drug discovery, also include major plant pathogens that threaten global food security. An increasing number of natural metabolites (also known as secondary metabolites) produced by plant-associating fungi are now known to play important roles in plant pathogenesis and other fungal-plant interactions.^{1, 2} Thus the discovery that the chemical diversity observed in laboratory cultures only consists of a small fraction of the biosynthetic potential encoded in fungal genomes represents both an immense opportunity for bioactive molecule discovery and a significant gap in our knowledge on the roles of the cryptic metabolites in plant pathogenesis.³

Cytochalasans are one such group of natural products that is of significant interest to both drug discovery and fungal-host interactions. They are polyketide-amino acid hybrid compounds structurally characterized by a macrocycle fused to an isoindolone core and are most well-known for their ability to bind to the barbed end of actin, thus interfering with various cellular processes that involved actin polymerization.⁴⁻⁶ In addition, cytochalasans have been shown to exhibit potent anti-angiogenic activity⁷ (cytochalasin E) while cytochalasin B has been observed to have a synergistic antitumor effect when co-used with the well-known microtubule-directed agent vincristine, signifying the potential applications of these compounds in cancer therapy.⁸ Cytochalasans have also been reported to exhibit other biological activities, including inhibition of glucose transport by cytochalasin B,^{9, 10} and HIV-1 protease by 18-dehydroxy cytochalasin H.¹¹

Many cytochalasans have been isolated from plant-associated fungi, including both pathogenic and endophytic,^{12, 13} however, their biological roles are unclear. In fungal plant pathogens, a number of phytotoxic cytochalasins have been found, such as the cytochalasins B, F, Z₂ and Z₃.¹⁴ Interestingly, cytochalasans have been shown to suppress cytoplasmic aggregation

involved in non-host defence via inhibition of actin polymerization. For example, cytochalasin A treatment allowed non-pathogens to penetrate into the non-host barley cells.¹⁵ However, no cytochalasan has yet been shown to play a role in the virulence of plant pathogens. Curiously, *ACE1*, the avirulence signaling gene of the rice blast fungus *Magnaporthe oryzae* has been proposed to produce a metabolite recognized by rice resistance gene *Pi33*.¹⁶ Recently, it was shown that *ACE1* encodes a hybrid polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) likely involved in the biosynthesis of a tyrosine-derived cytochalasan compound that is yet to be identified.¹⁷ All of these observations suggest that cytochalasans could play an important role in fungal-plant interactions.

Despite there being over 300 reported cytochalasans,¹⁸ only a handful of cytochalasan biosynthetic gene clusters have been characterized. Among them, the biosynthetic gene clusters of phenylalanine-derived cytochalasin E/K,¹⁹ tryptophan-derived chaetoglobosin A,²⁰ and *O*-methyltyrosine-derived pyrichalasin H²¹ have been described. A previous comparative analysis using the *ccs* cluster encoding cytochalasin E/K from *Aspergillus clavatus* indicated a putative cytochalasan biosynthetic gene cluster (*SNOG_00306-00314*, herein named *phm* cluster) in the genome of the damaging wheat pathogenic fungus *Parastagonospora nodorum* SN15.^{19, 22} Mining of the previous transcriptomics data of *P. nodorum*²³ uncovered that the genes within the *phm* cluster increased significantly during infection on wheat suggesting that encoded metabolite(s) could play important roles in wheat disease development.³ As part of our ongoing efforts to discover new bioactive metabolites and small molecule virulence mediators from fungi using chemical ecogenomics approach,²⁴⁻²⁶ we focused on the *phm* gene cluster as a candidate of interest. In this study, we used complementary homologous and heterologous expression approaches to characterize the *phm* cluster and showed that encoded for the biosynthesis of

leucine-derived cytochalasans, metabolites which have not been isolated from the well-characterized wheat pathogen previously. The phytotoxicity of the cytochalasans, their actin polymerization-inhibiting properties, and their roles in the virulence of *P. nodorum* against wheat were also investigated.

RESULTS AND DISCUSSION

A comprehensive study has previously characterized *P. nodorum* gene expression during infection and also axenic growth.²³ Detailed analysis of the expression of biosynthetic genes within this transcriptomics dataset revealed that the PKS-NRPS hybrid gene *SNOG_00308* (*phmA*) exhibited a distinct transcriptional profile whereby transcription increased over time *in planta* on wheat (*Avena sativum*) and peaked at 10-days post-inoculation (dpi) when the plant tissue was heavily necrotic and *P. nodorum* was undergoing asexual sporulation. The *in-planta* expression of *phmA* has been previously validated by reverse transcription qPCR.²⁷ The lack of coordinated expression of the flanking genes (*SNOG_00305* and *SNOG_00315*) and the genes further up- and downstream provided an indication as to the tentative boundaries of this biosynthetic gene cluster (named *phm* cluster, ~31kb).

The *phm* cluster was previously used as one of the example orphan gene clusters that share multiple homologs with the *ccs* cluster that encodes the biosynthesis for cytochalasin E/K.¹⁹ We performed a more detailed comparative analysis and revealed that PhmA shares significant protein identity with *A. clavatus* NRL1 CcsA (46%),¹⁹ *C. globosum* CHGG_01239 (41%)²⁰ and *M. grisea* N1980 PyiS (44%),²¹ which are the PKS-NRPSs characterized to be involved in the biosynthesis of different cytochalasans (Figure 1 and Table 1). PhmA also shares 43% protein identity with the PKS-NRPS encoded by the *M. oryzae* avirulence gene *ACE1*, which has been proposed to be involved in the biosynthesis of a tyrosine-derived cytochalasan.¹⁷ Importantly,

analysis of the enzymes encoded by the genes within the *phm* cluster revealed several homologs in the *ccs*, *CHGG_01239* and *pyi* gene clusters that have been implicated in cytochalasan biosynthesis,¹⁸ namely a *trans*-enoyl reductase (ER) PhmE, an α/β hydrolase PhmG and a putative Diels-Alderase PhmD (Figure 1 and Table 1). The extensive overlap between the *phm* cluster and the previously characterized cytochalasan gene clusters suggests that the *P. nodorum* *phm* cluster also likely encodes for the production of cytochalasan-like compound(s).

Figure 1. (A) The *phm* gene cluster in comparison to *ccsA*, *CHGG_01239*, *pyi* and *ACE1* clusters (homologues are in same colours, see Table 1). (B) The structures of cytochalasin E (encoded by *ccs* cluster), chaetoglobosin A (encoded by *CHGG* cluster) and pyrichalasin H (encoded by *pyi* cluster).

Table 1. Comparative analysis of the *phm*, *ccs*, *CHGG_01239*, *pyi* and *ACE1* gene clusters.

<i>phm</i> cluster proteins	<i>ccs</i> cluster homologues (% id*)	<i>CHGG_01239</i> cluster homologues (% id*)	<i>pyi</i> cluster homologues (% id*)	<i>ACE1</i> cluster homologues (% id*)	Deduced function
PhmB (SNOG_00306)	CcsD (42%)	CHGG_01243 (48%)	PyiD (37%)	CYP1 (38%)	P450
PhmR (SNOG_00307)	CcsR (29%)	CHGG_01237 (53%)	PyiR (56%)	n/a	Transcription factor
PhmA (SNOG_00308)	CcsA (46%)	CHGG_01239 (41%)	PyiA (44%)	ACE1 (43%)	PKS-NRPS hybrid

PhmC (SNOG_00309)	n/a	CHGG_01242_2 (37%)	n/a	OXR2 (38%)	FAD oxidase
PhmD (SNOG_00310)	CcsF (52%)	CHGG_01241 (52%)	PyiF (45%)	ORF3 (42%)	Putative Diels-Alderase
PhmE (SNOG_00311)	CcsC (50%)	CHGG_01240 (44%)	PyiC (47%)	RAP1 (47%)	Enoyl reductase
PhmF (SNOG_00312)	n/a	CHGG_01245 (40%)	n/a	OXR1 (39%)	Dehydrogenase
PhmG (SNOG_00313)	CcsE (52%)	CHGG_01246 (51%)	PyiE (46%)	ORFZ (49%)	α/β Hydrolase
PhmH (SNOG_00314)	n/a	n/a	PyiT (45%)	MFS1 (38%)	MFS transporter

Note: n/a means no corresponding homologues. % id* indicates protein identity in comparison to homologues.

Despite a number of previous studies on *P. nodorum* secondary metabolites²⁸⁻³⁰ and more recent studies, including metabolite profiling of a *sch1* mutant,³¹ the use of epigenetic modifiers,³² and growth on rice substrates,³³ which resulted in new compounds discovered from *P. nodorum*, no cytochalasan-like compound has been reported.³⁴ Our previous work on elsinochrome C²⁴ and stemphyloxin II²⁶ have shown that overexpression of pathway-specific transcription factor can be an effective mean to increase the targeted metabolite production and link the targeted gene cluster to its metabolite product(s) in *P. nodorum*. An analysis of the *phm* cluster identified a Zn₂Cys₆-type transcription factor gene (*SNOG_00307*, herein named as *phmR*). Thus, we introduced multiple ectopic copies of *phmR* under the regulation of the strong *gpdA* promoter into the *P. nodorum* genome.^{35,36} LC-DAD-MS analysis of the acetone extracts from the resulting mutants T2, T6 and T7 grown in minimal media liquid culture revealed the accumulation of two compounds, **1** (m/z [M+H]⁺ 416) and **2** (m/z [M+H]⁺ 430) that were absent in the wide-type (WT) strain and the production of compound **1** was more abundant in all transformants (Figure 2). To reproduce **1** and **2** on a larger scale for isolation of the compounds, the T2 mutant was grown in 6 L of stationary liquid culture under prolonged incubation at room temperature in the dark. The culture was harvested for extraction after three weeks when the production was detected to be at its peak, based on LC-MS sampling. In addition to **1** and **2**, a

third compound (**3**, m/z $[M+H]^+$ 472) (Figure S2) were purified using a combination of flash chromatography, Sephadex LH-20 and reverse-phase semi-prep HPLC. NMR analyses revealed that the structure of **1** was identical to the recently described cytochalasan, 18-*oxo*-19,20-dihydrophomacin C (Table S3), isolated from a marine sediment-derived fungus *Westerdykella dispersa*.³⁷ Here we renamed **1** as phomacin D for the ease of describing the compound and subsequent analogues, and their structural relationship to previously identified phomacins (all leucine-derived).³⁸ The molecular formula of **2** was determined as $C_{25}H_{35}NO_5$ based on the HRESIMS experiment (observed m/z 430.2673 $[M+H]^+$). Its NMR spectroscopic data (Table S4) closely resembled those of **1**, except for the presence of a carbonyl carbon (C-19) in **2** which replaced a methylene carbon in **1**, which was further confirmed by the key HMBC correlations from H₂-20 to C-19. Compound **2** is a novel leucine-derived cytochalasan and here named phomacin E.

Compound **3** was shown to be the C-20 acetyl derivative of **1**, which was supported by the ¹H-¹H correlation of H-20 (δ_H 3.31)/H₂-27 (δ_H 3.91 and 2.22) and the HMBC correlation from H-20 (δ_H 3.31) to C-28 (δ_C 207.1) in **3**, here named phomacin F. The structure of **3** and the absolute configuration were further confirmed by the Cu K α X-ray single-crystal diffraction with a Flack parameter³⁹ of -0.05 (5) (Figure 2). Overall, the acetyl group of **3** is unusual for cytochalasans. A thorough literature search uncovered previously reported leucine-derived cytochalasans, aspochalasins N and O,⁴⁰ with similar acetyl side chain on the macrocycle. It was suggested that the acetyl adducts are the product of reaction with acetone during the extraction step.⁴⁰ Similarly, this postulation was supported by treatment of **1** with acetone, MeOH and H₂O at room temperature, which successfully converted **1** to **3** (Figure S3). However, the possibility of **3** as a result of the reaction of **1** with endogenous methyl ketone metabolites cannot be completely

ruled out, and the observation suggests that the C-20 of **1** is highly reactive and could have biological significance (e.g. forming covalent bond with cellular macromolecules).

Figure 2. The *Phm* gene cluster encodes the production of **1-2**. (A) Metabolite profile analysis of *phmR*-OE strains in comparison to WT. (B) Structures of compounds **1-3** and the X-ray structure of **3**.

To further corroborate the relationship between the *phm* cluster and the biosynthesis of compounds **1-2**, the backbone PKS-NRPS gene *phmA* and its *trans*-ER partner *phmE* were heterologously expressed in *A. nidulans* LO7890,⁴¹ under the regulation of the *A. nidulans* *alcA* promoter on a hybrid yeast–fungal artificial chromosome (pYFAC) expression system.^{25, 42} Upon cyclopentanone induction, the *A. nidulans* transformants expressing *phmA* and *phmE* showed clear accumulation of a new peak (λ_{max} 276 nm, m/z 384 $[M+H]^+$) (Figure 3). Subsequent scale-up culture (4.0 L) and semi-prep HPLC purification yielded purified **4**. Detailed NMR structural characterization revealed that **4** is an acyclic polyolefinic compound containing a 4-pyrrolin-2-one moiety that is conjugated with the C-2 enol via C-3, here named prephomacin (Figure 3).

Previous work has suggested a putative biosynthetic pathway for cytochalasans.^{4, 18} Briefly, the aldehyde intermediate released by the C-terminal reductase domain undergoes a Knoevenagel condensation to form a 2-pyrrolidone. The pyrrolidone functions as a dienophile, and could undergo an intramolecular Diels–Alder cyclization with the polyketide terminal diene to generate the characteristic isoindolone-fused macrocycle core, which can be further modified by post-assembly enzymes to form structurally diverse cytochalasans. Prephomacin (**4**) is likely the enolized form of the proposed 2-pyrrolidone intermediate **5**, which contains eight differentially α and β -functionalized ketide units aminoacylated with leucine and has undergone Knoevenagel condensation to form the 2-pyrrolidone (Figure 3). However, when we expressed other *phm* biosynthetic genes (*phmB*, *C*, *D*, *F*, *G* and *H*) together with *phmAE*, the resulting *A. nidulans* still produced **4** without any detectable cyclized isoindolone (Figure S4). We propose that the incorrect tautomeric state in **4** formed in the *A. nidulans* host hindered the isoindolone core formation as the desirable dienophile required for the intramolecular Diels–Alder reaction is not present in **4**. These data are consistent with the previous reports whereby no complete cytochalasan biosynthetic pathway has been reconstructed heterologously.^{14,34,35} Previous efforts have only demonstrated the production of linear shunt precursors at the alcohol oxidation state via heterologous expression of the PKS-NRPS and *trans*-ER in the cytochalasan pathways.^{17, 43,}
⁴⁴ Interestingly, the pyrrolidone **4** is remarkably similar to the pyrrolidone intermediate obtained in the heterologous reconstruction of pathway to the decalin compound myceliothermophin, which was also in the incorrect tautomeric form.⁴⁵ In the case of myceliothermophin however, spontaneous oxidation of the intermediate resulted in an oxidized product with the correct tautomeric form that presents the diene and dienophile for the regio- and stereo-selective Diels–Alder cycloaddition catalyzed by the Diels–Alderase MycB to form the decalin system.⁴⁵

Nonetheless, for **4**, this is the first time that a 2-pyrrolidone intermediate that has been proposed for cytochalasan biosynthetic pathway has been successfully observed, albeit in the incorrect tautomeric form. Based on this and the published PKS-NRPS biosynthetic paradigm,⁴⁶ the biosynthetic pathway of **1-3** was proposed (Figure S8).

Figure 3. Heterologous production of prephoamcin (**4**) and the biogenesis of phoamcin D (**1**). (A) Metabolite profile analysis of *A. nidulans* expressing *phmA* and *phmE* comparing with control strain carrying empty plasmid and the structure of compound **4**. (B) The proposed biosynthetic pathway of phoamcin D (**1**) from prephoamcin (**4**).

A number of cytochalasans from fungal plant pathogens have been shown to exhibit phytotoxicity.¹² Consequently, we investigated whether phomacins **1-3** could show similar phytotoxic activity against wheat. Leaf infiltration assays showed no necrosis on wheat leaves treated with up to 200 ppm (1 ppm = 1 µg/ml) of **1-3**. The capacity of phomacins to affect wheat seed germination was also assessed. Complete inhibition of wheat seed germination was observed for **1** and **2** at 100 ppm (Figure 4 and S9), while **3** exhibited only moderate antigerminative activity even at 200 ppm (Figure S9). Furthermore, we found that **1** at 100 ppm

was also able to inhibit the seed germination of another monocot *Eragrostis tef* (commonly known as teff or William's lovegrass). Interestingly, **1** did not show any inhibition of seed germination against the dicots *Arabidopsis thaliana* and *Lepidium sativum* (garden cress) (Figure 4). This suggests that the antigerminative activity of **1** could be specific to monocots. By comparison, and as a control, cytochalasin D inhibited the germination of the monocot wheat and the dicot *Arabidopsis thaliana* tested above (Figure S10).

Figure 4. Seed germination results of phomacin D (**1**) at 100 ppm and 5 days against different plants.

Cytochalasans are also renowned for their capability to bind to actin and inhibit its polymerization. As such, we sought to determine if **1-3** also impaired the ability of actin to form polymers. Using a commercial actin polymerization assay kit (Cytoskeleton Inc, Colorado), **1** and **2** inhibited actin polymerization at a level exceeding the positive control cytochalasin D (Figure 5). Interestingly, phomacin F (**3**) was not significantly different from the negative control pyrene inferring that it was unable to inhibit actin polymerization. Notably, the actin polymerization-inhibitory activities of phomacins **1** and **2** (and the positive control cytochalasin D) correlated with their ability to inhibit monocot seed germination, while **3** failed to inhibit actin polymerization exhibited no antigerminative activity. This suggests that the macrocycle portion of **1** and **2** is important for both actin-binding and antigerminative activity and that acetylation at C-20 inactivated the bioactivities. It also suggests that the actin polymerization-inhibitory properties of the phomacins tested are potentially co-related to the antigerminative

activity. However, the monocot-specific nature of **1** remains an enigma and warrants further in-depth investigation.

Figure 5. The effect of the phomacins on actin polymerization. Error bars shown represent standard errors.

Next, we sought to determine whether the biosynthesis of **1** and **2** facilitated the infection of wheat by *P. nodorum*. To this end, *phmA* in *P. nodorum* was targeted for gene disruption and the ability of $\Delta phmA$ strains to infect wheat were compared with WT. Two of these $\Delta phmA$ disrupted mutants, $\Delta phmA-1$ and $\Delta phmA-2$, were inoculated onto the leaves of the wheat cultivar Emu Rock. The WT and an ectopic mutant (Ec), where the knockout construct had randomly inserted into the *P. nodorum* genome, were inoculated as positive controls. After seven days, the leaves were collected, imaged and scored for disease (Figure 6). Leaves infected with both *P. nodorum* SN15 WT and Ec showed significant necrosis and tissue damage as expected at 7 dpi. The symptoms of disease though were significantly reduced for both of the $\Delta phmA$ mutants (50% lower than WT) implying that *phmA*, and therefore the phomacins, are required by *P. nodorum* to successfully colonise and infect wheat.

Figure 6. (A) Representative images of wheat leaves infected with each of the four strains at 7 dpi. (B) Disease scores of the infected leaves in (A) (0 being uninfected and 5 completely necrotic). Leaves were scored at 7 dpi and at least 10 leaves infected by each strain were scored. The disease scores are an average from the scoring of each leaf. Standard error bars are shown.

In conclusion, we transcriptionally activated an *in-planta* expressed PKS-NRPS gene cluster *phm* in *P. nodorum* and this resulted in the isolation of three leucine-derived cytochalasans **1-3**, which had never been previously reported from this wheat pathogen. Compound **2** and the acetonide derivative **3** are novel metabolites. This study thus expands the series of cryptic phytotoxic metabolites that we have uncovered for this important wheat pathogen *P. nodorum*, including elsinochrome C,²⁴ novel alternapyrones²⁵ and stemphyloxin II²⁶, and further advanced our understanding of the pathogenicity mechanism of *P. nodorum*. It also once again highlights chemical ecogenomics as a powerful approach for unravelling the hidden chemical ecology of fungal-host interactions.³ Reports to date for cytochalasans have shown that the amino acids

incorporated in the compounds are mainly tryptophan, tyrosine, phenylalanine, leucine and alanine.^{4, 18} The *phm* gene cluster here represents the first example encoding a leucine-derived cytochalasan biosynthetic pathway and expand our knowledge on the diversity of cytochalasan biosynthetic gene clusters, which is currently limited to the few that encode aromatic amino acid-derived cytochalasans (Figure 1).¹⁹⁻²¹ Heterologous expression of *phm* backbone genes in *A. nidulans* led to the production of a novel pathway 2-pyrrolidone precursor **4**, which provided new insights into the biosynthesis of cytochalasans.

Importantly, our reverse genetics and wheat seedling assays demonstrated that the *phm* cluster is important for the *P. nodorum* virulence against wheat. Congruently, both **1** and **2** exhibited antigerminative phytotoxicity and were shown to inhibit actin polymerization. It has been established that cytoskeletal rearrangement is an important mechanism of plant defense against fungal penetration by driving the formation of cytoplasmic aggregation around the penetration site.⁴⁷ Therefore, it is logical to speculate that *P. nodorum* produces phomacins to disrupt the cytoskeletal rearrangements via binding to actin and leading to an inhibition of cytoplasmic aggregation. However, we cannot rule out the involvement of secondary mechanisms in the phytotoxicity observed for **1-2**. For example, cytochalasin B was demonstrated to inhibit glucose transport by binding to human glucose transporter GLUT1.¹⁰ Similar secondary mechanisms could be involved, especially given that there were significant differences in the inhibition of germination of monocot versus dicot plants that we tested.

To our knowledge, this is the first demonstration of the involvement of cytochalasans in the virulence of fungal plant pathogens. Further in-depth studies, including high-resolution imaging of the cytoskeleton dynamics in the plants during the interactions with *P. nodorum* in comparison with the non-phomacin-producing mutant, would be required to corroborate the

hypothesis. Given the wide-spread nature of cytochalasans among plant-associated fungi observed both in chemical isolation studies and in searching the fungal genome sequence databases (Figure S11), it is highly likely that production of cytochalasans to target actin polymerization in plants is a commonly used strategy by fungi to facilitate plant-host infection. The monocot-specific nature of **1** and **2** is highly interesting and warrants further investigation to untangle the structure-activity relationship of cytochalasan-induced phytotoxicity as a strategy towards development of monocot-specific herbicides.

EXPERIMENTAL SECTION

Strain and culturing conditions. The wild-type *P. nodorum* strain SN15 was obtained from the Department of Agriculture and Food Western Australia (DAFWA). The wild-type and mutant strains generated in this study were maintained on V8-supplemented potato dextrose agar (PDA) plates or defined minimal medium (30 g sucrose, 2 g NaNO₃, 1 g K₂HPO₄, 0.5 g KCl, 0.5 g MgSO₄·7H₂O, 0.01 g ZnSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 2.5 mg CuSO₄·5H₂O in 1 L, adjusted to pH 6.0). The standard growth condition for *P. nodorum* strains was at 22°C under a 12h dark/near-UV light regime. *Saccharomyces cerevisiae* BJ5464 was used for *in vivo* homologous recombination to construct the heterologous expression plasmids. *A. nidulans* LO7890 was used for heterologous expression of *phm* genes.

Construction of *P. nodorum* overexpression and disruption mutants. The plasmid, pBAR-*phmR*, for overexpressing the *phmR* transcription factor gene in the *phm* cluster was constructed by cloning the gene (amplified from *P. nodorum* genomic DNA using LIC-*phmR*-F/R, Table S1) into the pBARGPE1-LIC expression plasmid by ligation-independent cloning as described previously.³⁶ The original plasmid pBARGPE1 was obtained from the Fungal Genetics Stock Centre (FGSC) and contains an *A. nidulans* *gpdA* promoter and *trpC* terminator for consecutive expression, and a *Streptomyces hygroscopicus* *bar* gene for glufosinate resistance. The disruption cassette for the PKS-NRPS gene *phmA* was constructed by using a fusion PCR approach as described.⁴⁸ Regions flanking the upstream and downstream of *phmA* were amplified

with the primer sets phmA-KO-P1/KO-P3 and phmA-KO-P4/KO-P6 (Table S1), and the hygromycin resistance (*hyg*) cassette was amplified with the primers *hyg*-F/R from the pAN7-1 plasmid.⁴⁸ Nested primer pairs phmA-KO-P2/P5 (Table S1) were used to generate the *phmA* disruption cassette containing the *hyg* marker by fusion PCR. Transformation of *P. nodorum* was achieved by polyethylene-glycol-mediated protoplast transformation as described previously.²⁴ For constructing the *phmR*-OE strains, *P. nodorum* SN15 was transformed with the plasmid pBAR-*phmR* and the transformants were selected on glufosinate ammonium-containing medium.⁴⁹ For *phmA* disruption, *P. nodorum* was transformed with *phmA* disruption cassette generated above and the transformants were selected on hygromycin.

Heterologous expression of *phmA* and *phmE* in *A. nidulans*. *Phm* genes were cloned into the pYFAC episomal vectors by yeast transformation-assisted recombination. Full length of *phmA* was amplified by PCR with two sets of primers of PalcA-*phmA*-F/*phmA*-mid-F and *phmA*-mid-R/*pyrG*-*phmA*-R (Table S1). The two overlapping DNA fragments and *PacI*-digested pYFAC-*pyrG* were introduced into yeast competent cells and generated pYFAC-CL25 by yeast homologous recombination. *PhmE* was amplified with the primers of PalcA-*phmE*-F/T1-*phmE*-R and ligated into linear expression vector *PacI*-digested pYFAC-*ribo* to get pYFAC-CL26. The resulting plasmids pYFAC-CL25 and pYFAC-CL26 were used to transform *A. nidulans* LO7890 protoplasts by polyethylene glycol protoplast transformation as described previously.

Metabolite profile LC-MS analysis. The mycelia of *P. nodorum* and *A. nidulans* from liquid culture were extracted with acetone. For liquid cultures, the metabolites were extracted with an organic solvent mixture containing ethyl acetate/methanol/acetic acid at 89.5:10:0.5 ratio. The crude extracts were dried down *in vacuo* and re-dissolved in methanol for LC-MS analysis. The analyses of the metabolite profiles were performed on an Agilent 1260 liquid chromatography (LC) system coupled to a diode array detector (DAD) and an Agilent 6130 Quadrupole mass spectrometer (MS) with an ESI source. Chromatographic separation was performed at 40°C using a Kinetex C18 column (2.6 μ m, 2.1 mm i.d. 3 100 mm; Phenomenex) with 5-95% MeCN-H₂O (0.1% (v/v) formic acid) gradient in 10 minutes followed by isocratic 95% MeCN for 3 minutes with a flow rate of 0.75 mL/min. The MS data were collected in the *m/z* range 100–1000 amu at positive and negative mode.

Extraction and Isolation. For isolation of **1-3**, the *P. nodorum* *phmR*-OE2 strain was grown in 6 L liquid defined minimal medium at 22°C for 48 h with shaking followed by stationary incubation at 24°C for another

3 weeks in the dark. The mycelia were extracted with acetone and the extract was dried *in vacuo*. The crude extract was then partitioned with ethyl acetate/H₂O and the organic layer was then dried down and separated on a Reveleris flash chromatography system (Grace) using a CH₂Cl₂/CH₃OH gradient on a Reveleris HP silica flash cartridge for initial separation. Fractions verified by LC-MS to contain the target compounds were combined and then applied to a Sephadex LH-20 column chromatography (CC) followed by semi-prep HPLC with a C18 column (Grace, 5 μ m, 10 $\times$ 250 mm) (isocratic, MeCN–H₂O, 55:45, flow rate at 4.3 mL min^{–1}) to afford compounds **1** (8.5 mg), **2** (1.0 mg) and **3** (7.5 mg). For isolation of **4**, an *A. nidulans phmAE*-expressing strain was grown in 4 litres shake-flask culture in glucose minimal medium (10 g glucose, 50 ml 20 $\times$ nitrate salts, 1 ml trace elements in 1L) supplemented with pyridoxine at 37°C 180 rpm for 18 h. To induce the expression of *phmAE* under *PalcA* regulation, 2.5 mL of cyclopentanone was added per litre culture. The cultures were incubated for another 2 days after induction at 25°C 180 rpm before the cells were extracted using acetone and the extract was dried *in vacuo*. The crude extract was then fractionated on a Sephadex LH-20 CC. Fractions identified to contain the target compound by LC-MS were combined for further purification using a semi-prep HPLC with a C18 column (Agilent, 5 μ m, 21.2 $\times$ 150 mm) (isocratic, MeCN–H₂O, 5:95, flow rate at 6.0 mL min^{–1}) to obtain **4** (30 mg).

X-ray Crystallographic Analysis of 3. C₂₈H₄₁NO₅, $M = 471.62$, colorless crystal, $0.32 \times 0.24 \times 0.18$ mm³, monoclinic, space group $P2_1$ (No. 4), $a = 8.8287(1)$, $b = 11.0571(1)$, $c = 13.3265(1)$ Å, $\beta = 102.649(1)^\circ$, $V = 1269.36(2)$ Å³, $Z = 2$, $D_c = 1.234$ g cm^{–3}, $\mu = 0.668$ mm^{–1}. $F_{000} = 512$, Cu K α radiation, $\lambda = 1.54178$ Å, $T = 100(2)$ K, $2\theta_{\max} = 134.5^\circ$, 23588 reflections collected, 4513 unique ($R_{\text{int}} = 0.0237$). Final $GooF = 1.004$, $R1 = 0.0378$, $wR2 = 0.0990$, R indices based on 4458 reflections with $I > 2\sigma(I)$ (refinement on F^2), $|\Delta\rho|_{\max} = 0.53(4)$ e Å^{–3}, 319 parameters, 1 restraint. Lp and absorption corrections applied. The Flack parameter³⁹ value was $-0.05(5)$.

Phytotoxicity, virulence and actin polymerization inhibition assays. The phytotoxicity and antigermination were performed as previously described.²⁵ To determine the virulence of the *P. nodorum* mutants, the attached leaf pathogenicity assay was performed as previously described with minor modifications.⁴⁹ The effect on

actin polymerization was assessed using the Actin Polymerization Biochem assay (Cytoskeleton Inc, Denver, CO). Further experimental details of the biological assays are provided in Supporting Information.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge on the ACS Publications website.

All experimental and characterization details, NMR data and spectra of **1-4** (PDF)

CCDC contains 1942077 the X-ray crystallographic data of **3** (CIF)

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Notes

The authors declare no competing financial interest.

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